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Nottingham**

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**Changes in Transcription and Phospho-signalling in
Breast Tumour Hypoxia and Hypoxia-
reoxygenation**

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Abstract

Hypoxia (Low oxygen) is found in 50% of breast cancers. Clinically, hypoxia is associated with increased invasion, metastasis, therapy resistance, and worse patient survival. Hypoxia drives major hallmarks of cancer phenotypes, including invasion. Breast cancer adaptation to hypoxia is regulated by the HIF transcription factors and epigenetic modifications. In addition, hypoxia modulates signalling pathways. Hypoxic tumour cells can become reoxygenated as they invade away from hypoxic tumour regions or via hypoxia-induced tumour vascularisation. We investigated the impact of hypoxia-reoxygenation on gene expression, signalling, and cell phenotypes. Our study involved examining the changes in gene expression in hypoxia and hypoxia-reoxygenation in MDA-MB-231 and MCF7 cell lines using RNA sequencing. The analysis revealed significant changes in gene expression in hypoxia (1537 and 1965 genes, respectively) and in hypoxia-reoxygenation (2091 and 737 genes, respectively). A considerable number of gene expression changes induced by hypoxia were sustained upon reoxygenation, with 38% in MDA-MB-231 and 11.5% in MCF7. These genes were found to be enriched for metabolic processes and signalling pathways such as Wnt and TGF β signalling. We also examined the expression of these genes in breast tissues using transcriptomic and clinicopathological data. We discovered that several genes such as CST1, MUC1, CDH2, and EGLN3, which are known to be associated with metastasis and poor prognosis in breast cancer patients, were significantly expressed in TNBC and HER2(-ve) tissues. Furthermore, we explored the possible mechanism by which the changes in gene expression induced by hypoxia are sustained upon hypoxia-reoxygenation. We found that activation of growth factors under hypoxia might initiate a positive feedback loop responsible for this phenomenon.

In this study, we examined the effects of hypoxia and hypoxia-reoxygenation on signalling pathways. We used phospho-RTK and phospho-kinase arrays to screen for changes in phospho-signalling. The results showed that 24% of RTKs and 31% of intracellular kinases had increased phosphorylation levels under hypoxia. Additionally, 70% of RTKs and 67% of intracellular kinases had increased phosphorylation levels under hypoxia-reoxygenation. We conducted further analysis through Western blot and found a significant increase in phosphorylation levels of some targets, such as phospho-STAT3 (Y705), phospho-AKT(Thr308),

and ALK, under hypoxia and/or hypoxia-reoxygenation. Our study aimed to investigate the effects of hypoxia-reoxygenation on cell phenotype and response to chemotherapy drugs. Our findings revealed that hypoxia-reoxygenation led to an increase in cell invasion and resistance to chemotherapy drugs, cisplatin and doxorubicin when compared to normoxia. To address this issue, we employed kinase inhibitors, Crizotinib, WP1066, and MK-2206, to target phosphorylation, migration, and chemotherapy resistance. Our results showed that kinase inhibitors reduced phosphorylation, suppressed migration, and suggested a possible impact on reducing chemotherapy resistance towards cisplatin and doxorubicin in MDA-MB-231 and MCF7 cells.

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COVID Impact Statement

Unfortunately, the work submitted in this thesis was impacted significantly by the COVID-19 pandemic. The PhD project began in October 2019, and by March 2020 the research laboratories were closed for 5 months until August 2020. In addition, travel restrictions during that time delayed my return to the UK until October 2020. Following the return to the laboratory, further research time was lost due to the intensive logistical issues that arose from the pandemic, including capacity restrictions and reagent supply problems. While all efforts were made to mitigate the lost research time, this was not always possible due to the practical nature of the research. This work has been completed within the original deadline and an extension has not been taken, and therefore represents a project completed with an interrupted period of around 7 months.

List of Abbreviations

A2R	adeno- sine A2 receptor
ALCL	anaplastic large-cell lymphoma (ALCL)
ALK	Anaplastic Lymphoma Kinase
AML	acute myloid leukaemia
ATP	adenosine 5'-triphosphate
Bax	BCL2 Associated X, Apoptosis Regulator
Bcl-XL	B-cell lymphoma-extra large
c-myc	MYC Proto-Oncogene, BHLH Transcription Facto
CSRP2	cysteine-rich protein 2
DDR1	Discoidin domain receptor family, member 1
DEGs	differentially expressed genes
DUSP8	Dual Specificity Phosphatase 8
EGFR1	human epidermal growth factor receptor type 1
ERKs	Extracellular signal-regulated kinases
HER2	human epidermal growth factor type-2 receptors
ICAM1	Intercellular Adhesion Molecule 1
IGF1R	insulin-like growth factor 1 receptor
IHC	immunohistochemical analysis
IL-1 β	interleukin-1 β
JAK	receptor-associated Janus kinase
JAK2	Janus Kinase 2
JNKs	1-5, c-Jun amino-terminal kinases
Mcl-1	MCL1 Apoptosis Regulator, BCL2 Family Member
MMPs	matrix metalloproteinases
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MUC1	Mucin 1, Cell Surface Associated
NSCLC	non–small cell lung carcinoma
PGF	placental growth factor
PI3K	phosphoinositide 3-kinase
STAT	signal transducer and activator of transcription
TGFB	Transforming growth factor beta
TGFB2	Transforming growth factor-beta 2
TNF- α	Tumor necrosis factor alpha
VEGF-A.	vascular endothelial growth factor-A
VEGFA	Vascular Endothelial Growth Factor A
VEGFC	Vascular Endothelial Growth Factor C
VEGFR	vascular endothelial growth factor receptor
WNKs	With-no-lysine [K] kinases

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1.0.0 Introduction

1.1.0 Cancer

Cancer is a wide collection of diseases characterised by uncontrolled growth and the ability to spread to distant tissues. In addition, cancer pathogenesis consists of different stages indicating the involvement of several gene mutations which lead to abnormal cell proliferation (Hassanpour, S. 2017). Tumours develop into malignant cancer by acquiring the ability to invade the surrounding tissues and metastasise in the body, 90% of all cancer death is related to metastasis due to the damage in the tissues at the secondary site of the tumour formation (Chaffer CL. Et al. 2011)

1.1.1. Hallmarks of Cancer

The principle of “Hallmarks of cancer” was first described by Weinberg and Hanahan (2000), demonstrating six phenotypes that orchestrate the multiple processes required for normal cell transformation into malignant cells. These included self-sufficiency in growth signals, insensitivity to growth suppressive signals, ability to evade programmed cell death, enabling replicative immortality, sustained angiogenesis, and tissue invasion and metastasis (Hanahan D, Weinberg RA. 2000). This also included enabling characteristic, genome Instability which generates genetic diversity that accelerates hallmarks acquisition and inflammation to support multiple hallmarks functions (Hanahan D, Weinberg RA. 2000).

Weinberg and Hanahan updated six hallmarks of cancer and incorporated two emerging hallmarks: reprogramming energy metabolism and evading immune destruction (Hanahan D, Weinberg RA. 2011). This edition also included an enabling characteristic: tumor-promoting inflammation. It has been described that inflammation selects for several hallmarks capabilities by providing prognostic factors, survival, growth and releasing chemicals such as reactive oxygen species (ROS) (Hanahan D, Weinberg RA. 2011). ROS can cause further mutation in the nearby cancer cells. The authors also concentrated on the tumour microenvironment as a critical step in understanding cancer cells (Hanahan D, Weinberg RA. 2011).

The latest edition on the hallmark of cancer included the two emerging characteristics as core hallmarks and proposed additional emerging hallmarks: phenotypic plasticity and disrupted differentiation (Figure 1.1) (Hanahan D. 2022). This edition also included two new enabling characteristics: non-mutational epigenetic reprogramming and the microbiome (Hanahan D. 2022).

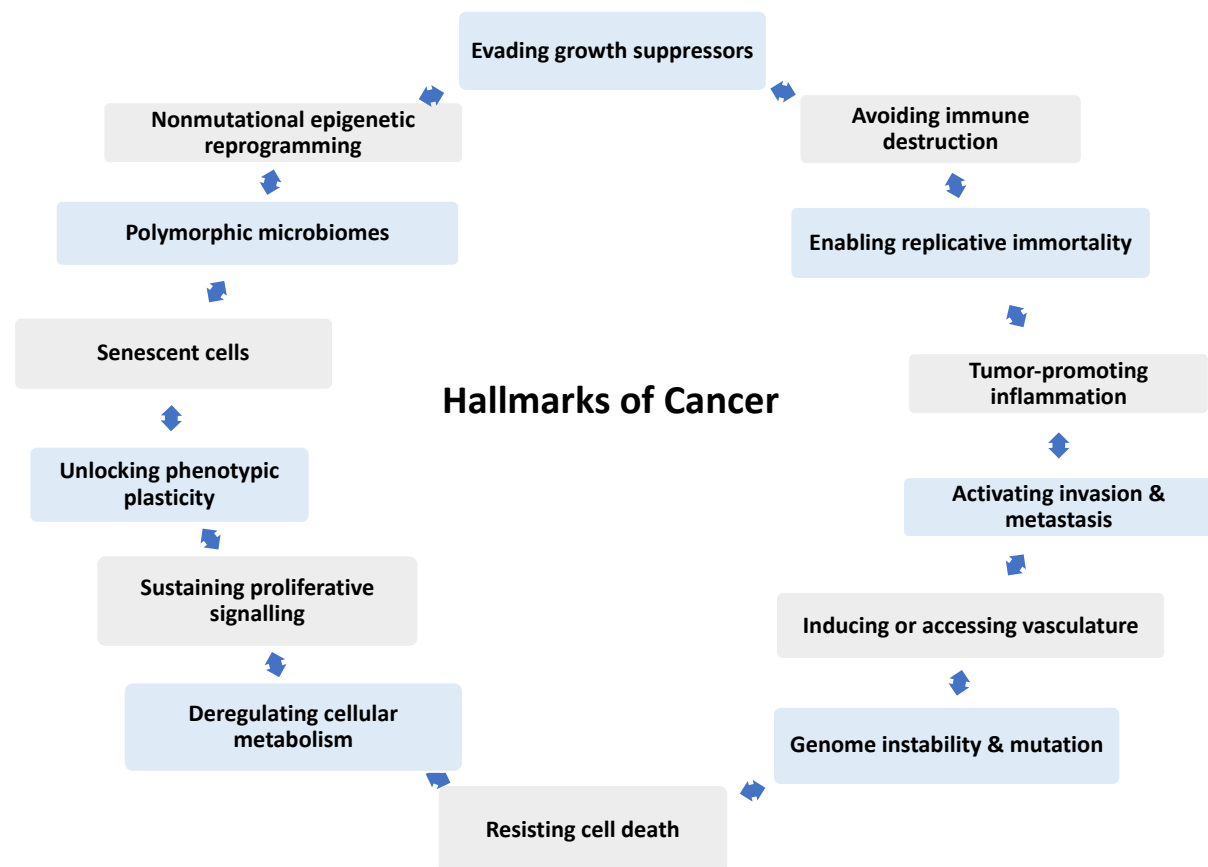


Figure 1.1: A schematic representation of the Hallmarks of Cancer. Adapted from (Hanahan D. 2022) (License Number: 5775230194819, License date: Apr 24, 2024)

1.2.0 Breast Cancer

Breast cancer is the most common type of cancer affecting women worldwide (Ferlay et al., 2013). In the early stages of non-metastatic disease, currently available therapies can cure 70-80% of patients (Harbeck et al., 2019). However, these therapies are less effective against advanced breast cancer and distant organ metastases, which tend to be incurable. At the molecular level, breast cancer is a heterogeneous disease characterised by several different features (McAnena et al., 2018). These include the presence of hormone receptors (HR), such as the estrogen receptor (ER) and progesterone receptor (PR), as well as the presence of

human epidermal growth factor receptor 2 (HER2, encoded by ERBB2) and/or BRCA mutations (McAnena et al., 2018). The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) recommend that ER/PR measurement should include the proportion and/or percentage of tumour cells that stain positive in the tissue section or cytology preparation, the overall intensity of staining (strong, moderate, weak), and the hormone receptor status. Estrogen receptor (ER) and progesterone receptor (PR) expression is considered negative when less than 1% of tumour cells stain positively and positive when 1% or more stain positively, while uninterpretable results occur when pre-analytic variables prevent accurate assessment (Hammond et al., 2010).

Estrogen receptor (ER) is a key diagnostic determinant; 70–75% of invasive breast carcinomas are characterised by high ER expression (Miah S. et al. 2019 and Zhang MH. Et al. 2013). 50% of ER-positive patients express the progesterone receptor (PR), and it's rarely expressed in ER-negative breast cancer. ER regulates PR expression; therefore, physiological PR values show the functional ER pathway. ER and PR are produced excessively in breast cancer and are considered critical diagnostic and prognostic biomarkers of breast cancer (Hicks DG, Lester SC. 2016 and Nicolini A. et al. 2017). Low levels of PR are associated with aggressiveness of the tumour, poorer recurrence and prognosis. In contrast, high levels are associated with overall survival, recurrence time, and treatment failure or progression time (Purdie CA. et al. 2013).

Human epidermal growth factor receptor 2 (HER2) overexpression occurs in the early stages of breast carcinogenesis and increases the detection rate of metastatic or recurrent breast cancer by 50%-80% (Iqbal N. et al. 2014). Moreover, the amplification of HER2 leads to increased activation of proto-oncogenic signalling pathways, which can result in uncontrolled cell growth and is associated with worse clinical outcomes of HER2+ cases. In addition, overexpression of HER2 is associated with shorter disease-free periods (Krishnamurti U. et al. 2009).

Ki-67, a nuclear proliferation marker expressed in all cell cycle phases except G0 (Gerdes and Li, 1991), is also a key marker of breast cancer. Ki67 determines proliferative activities and reflects the aggressiveness of the cancer with treatment response and recurrence time

(Haroon S. et al. 2013). A high expression level of Ki-67 in breast cancer is associated with worse outcomes (Trihia et al., 2003).

1.2.1. Classification of Breast Cancer Subtypes

Breast cancer can be classified using various approaches such as genetic, gene expression analysis, histology, and molecular pathology (Table 1.1). Recent advancements in miRNA profiling, multi-omics technologies, and principal component analysis-based iterative PAM50 subtyping have improved the accuracy of the subtypes of breast cancer (Tao et al., 2019, Søkilde et al., 2019, Raj-Kumar et al., 2019). However, there are inconsistencies in the classifications due to measurement noise, especially when the receptor levels in the tumour are at the edge between positive and negative (Raj-Kumar et al., 2019). Gene expression profiling has helped in the assessment of patients and treatments. Based on the immunohistochemical expression of hormone receptors, breast cancer can be classified into four main categories: estrogen receptor-positive (ER+), progesterone receptor-positive (PR+), human epidermal growth factor receptor-positive (HER2+), and triple-negative (TNBC) lacking the expression of the hormone receptors (Table 1.2) (Koboldt et al., 2012).

Classifier	Variables
Presentation	• Detection (Screen-detected versus symptomatic). • Stage (Early stage, locally advanced, or metastatic). • *Signs and symptoms (inflammatory BC, lump size, consistency, shape, and fixation, skin and nipple changes, axilla, laterality, and focality) • Menopausal status (premenopausal versus postmenopausal). • Others: gender, age, ethnic origin, family history.
Imaging	Mass shape, margin, depth, and site, breast composition, calcification, axillary findings, laterality
Pathological	
Morphological classification (mainly diagnostic and prognostic)	
Tumour differentiation	
Tumour type:	Several tumour types (currently at least 18 tumour types) are described and some types include multiple variants based on the combination of cytological, architecture features, and secretory activity and stromal features
Tumour grade	Three grades based on the degree of differentiation and similarity to terminal duct lobular units (TDLUs)
Disease extent	
Tumour stage	Invasive tumour size, infiltration of other tissues, lymph node status, and assessment of lesions at distant sites
Other factors	
	Lymphovascular invasion (present or absent), presence and extent of the <i>in situ</i> lesions (DCIS), stromal features such as TILs, Paget's disease, focality, bilaterality, and excision status
Molecular classification (mainly predictive but can provide diagnostic and prognostic value)	
Single gene classifier	• Oestrogen receptor and HER2 are the most important classifiers to guide treatment decision with the addition of PDL1. • Other markers include progesterone receptor (PR), KI67 as prognostic markers. • Familial predisposition genes such as BRCA1, BRCA2, and PALB2.
Multiple gene classifier	Multigene prognostic signatures are composed of multiple genes assessed together to assess risk in certain BC groups mainly the luminal class.
Global gene expression and genomic classification	• Intrinsic molecular subtypes including luminal, basal and HER2 enriched. • Mutation signatures • Integrated class classification based on a combination of transcriptomic and genomic (e.g. gene copy number) classification
Therapy classification	• Systemic therapy naïve versus treated patients. • Neoadjuvant therapy treated versus adjuvant treated patients. • Type of therapy (hormone, cytotoxic, targeted, or immunotherapy). • Line of therapy (first-line therapy versus second- or third-line)

Table 1.1: Main classification systems of breast cancer. Adapted from (Rakha, E.A., Tse, G.M. and Quinn, C.M. 2022) (Copyright licence: CC BY-NC 4.0).

	Luminal A	Luminal B	HER2	TNBC
Frequency (%)	50%	15%	20%	15%
ER	Yes	Yes	Some cases	No
PR	Yes	Some cases	Some cases	No
HER	No	No	Yes	No
miRNAs	<i>Let-7f, Let-7c, miR-10, miR-29a, miR-181a, miR-223 and miR-652</i>	<i>miR155, miR-93, miR-18a, miR-135b, miR-718, miR-4516, miR-210, and miR-125b-5p</i>	<i>miR-150 and miR-142-3p</i>	<i>miR-153, miR-10b, miR-26a, and miR146a</i>
Prognosis	Good	Middle	Middle/Bad	Bad
Therapy	Hormonal	Hormonal/Chemo	Hormonal/ Chemo/ Herceptin	Chemo/ Experimental

Table 1.2: Characteristics of breast cancer subtypes. Adapted from (Orrantia-Borunda E. et al. 2022). (Copyright licence: CC BY-NC 4.0)

ER, estrogen receptor; HER, human epidermal receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor; TNBC, triple-negative breast cancer

Luminal A

Luminal A breast cancer accounts for 40% of all breast tumours and is identified by being positive for ER and/or PR, negative for HER2, with low levels of Ki-67 (Fragomeni et al., 2018, Subik et al., 2018). Luminal A cancers have a better prognosis compared to Luminal B (91% 5-year recurrence-free (RFS) and 96.5% tumour-specific 5-year overall survival (OS) (Herr et al., 2019). Treatment of Luminal A patients begins with surgery to remove the tumour and may include chemotherapy and radiotherapy to remove any remaining tumour cells, followed by

hormone therapy (Goldhirsch et al., 2009 and Classes et al., 2020). Luminal A tumours respond best to endocrine therapy, such as antioestrogen or aromatase inhibitors (Wong, 2012).

Luminal B

Luminal B breast cancer accounts for 20% of all breast cancer cases and is associated with a more aggressive phenotype. Luminal B breast cancer is hormone receptor-positive, which has estrogen receptor (ER) and/or progesterone receptor (PR) and is either HER2 positive or negative (Fragomeni et al., 2018). This type of breast cancer is known to grow faster and has a worse prognosis compared to luminal A. Treatment for Luminal B includes surgery, chemotherapy, radiotherapy, and hormone therapy (Classes et al., 2020).

HER2 positive

HER2 breast cancers account for 10-15% of all breast cancers (Orrantia-Borunda E. et al. 2022). They are characterised by high HER2 expression and absence of ER and PR. Two subgroups can be observed: luminal HER2 (E+, PR+, HER2+ and Ki-67:15–30%) and HER2-enriched (HER2+, E-, PR-, Ki-67>30%) (Krishnamurti U, Silverman JF. 2014). Compared to luminal cancers, HER2 tumours grow faster and have a worse prognosis. The treatments for HER2 breast cancers include chemotherapy and HER2 protein targeted treatment such as Herceptin (trastuzumab), Enhertu (fam-trastuzumab- deruxtecan-nxki) and Tykerb (lapatinib) and tyrosine kinase inhibitors such as lapatinib and neratinib (Orrantia-Borunda E. et al. 2022).

Triple-Negative Breast Cancer (TNBC)

TNBCs account for 15-20% of all diagnosed breast cancers and have a rapid growth rate (Nakhjavani et al., 2019, Ho-Yen, 2012). TNBC tumours are identified as being ER, PR negative and HER2 negative (Onitilo et al., 2009, Nakhjavani et al., 2019). TNBC subtype is further classified into several additional subgroups, including basal-like (BL1 and BL2), claudin-low, mesenchymal (MES), luminal androgen receptor (LAR), and immunomodulatory (IM) (Kumar P, Aggarwal R. 2016). These subgroups have unique clinical outcomes, phenotypes, and pharmacological sensitivities. Studies have shown that TNBC patients often have germline BRCA1 mutations. Approximately 70% of breast tumours with BRCA1 mutation have low or absent expression of ER, PR and HER2 and have certain recurrence patterns, morphological features, and death rates (CARTER et al., 2012). It has also been suggested that BRCA1

mutation patients are more likely to develop TNBC, high nuclear grade and larger tumour burden than patients carrying BRCA2 mutation and noncarriers patients (Chen et al., 2018). Several characteristics distinguish TNBC, such as aggressiveness, early relapse and increased likelihood of advanced stage at presentation. TNBC presents alteration in DNA repair genes, increased proliferation rate and increased genomic instability. Histologically, it is a poorly differentiated, highly proliferative, heterogeneous neoplasm which involves subsets of variable prognosis. Immunohistochemically, TNBCs are subdivided into basal and non-basal TNBC; basal is characterised by expression EGFR1 and cytokeratins (CK)5/6. (CK)5/6 are not expressed by non-basal TNBC (Orrantia-Borunda E. et al. 2022).

TNBC patients do not benefit from the targeted therapy used for other breast cancer subtypes since they are deficient in the expression of ER, PR and HER2 (Nakhjavani et al., 2019). Moreover, TNBC tumours tend to be aggressive and less sensitive toward classical chemotherapies such as taxane and anthracycline. Therefore, fewer treatment options are available for TNBC patients, and they have the poorest prognosis among all types of breast cancers (Brenton et al., 2005). Consequently, researchers need to identify new strategies to target TNBC that could limit the progression and metastasis of this tumour group and improve patients' outcomes.

1.2.2. Breast Cancer Incidence and Survival rates

Distance metastases are common among breast cancer patients; the median survival time for patients with advanced breast cancer is 2-3 years (Bonotto M. et al. 2014). About 6-7% of newly diagnosed breast cancer patients are diagnosed with metastasis, and around 30% of stage-diagnosed patients have recurrent or metastatic disease (Berman et al., 2013). Studies have shown that distance metastasis can occur in various organs, such as the lung (1.52%), bone (3.28%), brain (0.35%), and liver (3.28%) (Xiao W. et al. 2018). The metastatic sites and subtypes are known to affect overall survival. The Luminal B subtype, and not the Luminal A subtype, had a higher incidence of bone metastasis (Xiao W. et al. 2018). Both Luminal subtypes had higher metastasis rates in the brain, lungs, and liver, with liver metastasis being the most common. On the other hand, TNBC showed higher rates of metastases in the liver and lungs and lower rates of bone metastasis compared to Luminal A (Xiao W. et al. 2018).

Breast cancer subtypes have different impacts on survival rates, according to the National Cancer Institute. The 5-year relative survival percentage' vary among breast cancer subtypes, with the luminal A subtype having the best survival pattern with a 94.4% survival rate which was followed by 90.7% luminal B subtype, 84.4% HER2 subtype and the worst survival in the TNBC subtype with 77.1% survival pattern (National Cancer Institute (NIH). 2022). The stage at diagnosis can also be a critical factor in determining survival outcomes.

Breast cancer subtypes have varying impacts on the relapse of breast cancer (Smid M. et al. 2008). TNBC and HER2 have the highest local and regional recurrence rates, with 7.6% and 3.3% for TNBC and 7.5% and 3.4% for HER2. On the other hand, the Luminal A subtype was recurrent in 1.5% and 0.7%, and the Luminal B subtype was recurrent in 2.9% and 1.5% of local and regional relapses, respectively (Ignatov A. et al. 2018). It is worth noting that even with a low recurrence rate in the Luminal and Luminal B subtypes, recurrence can still occur after ten years (Ignatov A. et al. 2018). Hence, factors such as pattern and recurrence time should be considered when making treatment decisions.

1.2.3. Breast Cancer Treatment and Therapies

Different approaches are currently in place for the treatment of breast cancer patients, including systemic therapy approaches and locoregional therapy (restricted to a localised region) such as surgery and radiational therapy. Systemic therapy includes immunotherapy and chemotherapy, targeted towards HR-positive disease (ER and PR).

Chemotherapy in Breast Cancer

Chemotherapy is a treatment method that involves using a combination of cytotoxic drugs to treat breast cancer patients (National Breast Cancer Foundation, 2020). Cytotoxic drugs are toxic to cells, and they work by targeting the cell cycle, interfering with cell proliferation, DNA, RNA, and metabolism (Schirmacher, V. 2018). These drugs are grouped by type, including alkaloids, alkylating agents, antimetabolites, and antibiotics. Alkaloids target mitosis and the cytoskeleton (β -tubulin), while antimetabolites target pyrimidine or purine metabolic enzymes (Seeber S. et al. 1993; Schirmacher, V. 2018). Chemotherapy is generally effective in the treatment of several types of lymphoma, but it may not be effective in other types of

cancer, such as carcinoma. However, it can extend overall survival rates up to 5% when treating head and, neck, colon, and breast cancers (Morgan G. 2004).

Chemotherapy is associated with multiple side effects, including immediate signs of toxicity and late signs of chronic toxicity (Schirrmacher. 2017 and Koeppen. et al., 2018). Immediate signs and effects can be observed on skin hair and extend to all organs such as the lungs, heart, and brain (Koeppen BM. et al. 2018). Chronic effects include drug resistance, infertility, and carcinogenicity (Schirrmacher, V. 2018). Patients undergoing chemotherapy may also experience poor appetite and diarrhoea, which can be attributed to changes in the balance of the cells lining the intestinal tract (National Breast Cancer Foundation, 2020).

Chemotherapy is commonly used for TNBC, mainly taxane and anthracycline-based. Platinum-based chemotherapy has also shown promising results in neoadjuvant and metastatic settings (Lebert, J.M. et al. 2018).

Radiotherapy in Breast Cancer

Radiotherapy, also known as radiation therapy, is a medical treatment that involves using high-energy rays to target cancer cells (Jereczek-Fossa BA. et al. 2003). It is one of the standard therapies used in breast cancer treatment to prevent local recurrence and reduce the frequency of distant metastasis and mortality rate. Studies have shown that the MDA-MB-231 cell line (TNBC subtype) is less sensitive to radiation than the MCF7 cell line (Luminal A subtype) (Chen X. et al. 2017, Zhou ZR. et al. 2017). However, it's important to note that the radiation dose delivered to the heart during radiotherapy can increase the risk of ischemic heart disease, which in turn affects overall survival rates (Darby et al., 2013). One form of radiotherapy is proton radiotherapy (PRT), which offers a better dose distribution (Levin et al., 2005). Studies suggest that protons can lower the mean heart dose compared to non-PRT, making it a promising treatment option for high-risk patients (Bradley et al., 2016, Patel et al., 2017, and Ranger et al., 2018). However, radiation therapy can have side effects, which can vary from patient to patient. Common side effects include breast heaviness, fatigue, and skin issues such as redness, dryness, and discolouration (Wanchai, A., Hoskin et al., 2006).

Molecular Targeted Therapies in Breast Cancer

Breast cancer diagnosis and treatment are based on molecular-level categorisation, which considers the hormone status and growth factor receptors. However, the development of resistance to treatment and recurrence of cancer has led researchers to focus on developing new therapies. Targeted therapies have shown promising results in overcoming this issue by directly targeting molecular abnormalities. Clinical trials of targeted therapies have shown positive outcomes, and their combination with existing treatments has been investigated (Nakhjavani et al., 2019). Various targeted therapies, including monoclonal antibodies, antibody-drug conjugates, tyrosine kinase inhibitors, cyclin-dependent kinase 4/6 inhibitors, PI3K/Akt/mTOR pathway inhibitors, PARP inhibitors, and anti-angiogenic drugs are used in TNBC treatment (Table 1.3).

Immune checkpoint modulators	Targets within the nucleus	Intracellular targets and signalling mediators	Cell surface targets
PD-L1	BRCA and platinum-based treatment	AR	VEGFR2
CTLA-4	PARP	HSP90	EGFR
LAG-3	HDAC	CDKs	FGFR
TIM-3	p53, ChK1 and ATR	Proteins involved in apoptosis	Trop-2
Hh and NRP-2 signalling pathway	DNA binding agents	PI3K/AKT/mTOR pathway	GPNMB
RAF-MEK-ERK pathway			
JAK			

Table 1.3: Summary of molecular targeted therapies for TNBC. (Adapted from Nakhjavani et al., 2019) (Copyright licence: CC BY-NC 4.0 DEED).

I. BRCA and Platinum-based Treatment

Mutations in BRCA1 (Breast Cancer Susceptibility Gene) and BRCA2 genes lead to DNA instability and increase the cells' sensitivity towards agents that interact with DNA, such as platinum salts. These genes are responsible for repairing double-stranded DNA breaks. About 80% of BRCA1 mutation tumours exhibit TNBC characteristics associated with higher tumor grade and aggressiveness (Andreopoulou E. et al., 2015). There has been a rise in the use of platinum-based drugs, including anthracyclines, antimetabolites, and microtubule inhibitors, in adjuvant and metastatic settings (Nakhjavani et al., 2019). Phase II clinical trials have shown that platinum monotherapy effectively treats patients with BRCA1/2 mutations (Isakoff et al., 2015).

II. Poly-ADP ribose-polymerases (PARP)

PARP inhibitors (PARPi) are responsible for repairing single-stranded DNA breaks. PARP can be inhibited at the active site of the enzyme using PARPi agents or, alternatively, using PARP trappers (Nakhjavani et al., 2019). The PARP/PARPi complex binds to the damaged area without requiring catalytic activity for PARP-dependent DNA damage repair. Upon the stalling of the PARP on the DNA, the double-strand break occurs. Usually, functioning BRCA can repair this damage in a healthy cell, resulting in the cell's survival. However, with BRCA mutations, the break in the double strand endures, leading to cell death. Therefore, platinum-based drugs, together with PARPi agents, can be used for patients carrying BRCA mutations. Currently, there are five different PARPi agents in clinical trials, phase II or III, such as olaparib. Olaparib has been used to treat patients with germline BRCA mutations and metastatic breast cancer and has shown promising activity (Kaufman et al., 2015). A clinical trial in phase I/Ib study of olaparib and carboplatin showed that a combination of these drugs is tolerable in TNBC (Lee et al., 2017). Veliparib, another drug in phase I clinical trial (NCT01104259), was combined with cisplatin and vinorelbine in TNBC and BRCA mutation breast cancer, and it was well-tolerated with promising response rates (Rodler et al., 2016).

Veliparib, is another drug that completed clinical trials phase I and II clinical trials. phase I clinical trial (NCT01104259) combined with Veliparib cisplatin and vinorelbine in BRCA

mutated TNBC patients and was well tolerated with promising response rates (Rodler et al., 2016).

III. Programmed cell death protein 1 (PD-1) and its ligand

Several targeted checkpoints are currently undergoing clinical trials in TNBC, including programmed cell death protein 1 (PD-1) and its ligand (PD-L) (Nakhjavani et al., 2019). PD1 is a surface receptor expressed on T cells and pro-B cells, and it has an immunosuppressive role when engaged with its ligand. The activation of this receptor can be inhibited by PD-1 inhibitors, thus improving immune function and allowing cancer cells to be attacked. PD-1 has two ligands: PD-L1 and PD-L2. Approximately 40.9% of TNBC tumours express PD- L1, which is associated with high levels of tumour infiltrating lymphocytes (TILs) (Schalper et al., 2014; Schmid et al., 2018). Several PD-1 and PD-L1 inhibitors are under clinical trials in TNBC such as pembrolizumab , nivolumab , spartalizumab, toripalimab and camrelizumab (Nakhjavani et al., 2019). These inhibitors have been combined with other treatments such as pembrolizumab and cisplatin (NCT03644589), pembrolizumab and doxorubicin (NCT02957968), nivolumab and doxorubicin (NCT03815890), camrelizumab and apatinib (NCT03394287) (Nakhjavani et al., 2019).

Additionally, several signalling pathways that have been modulated in TNBC can now be targeted with molecular targeted therapies such as PI3K/AKT/mTOR pathway, RAF-MEK-ERK pathway and JAK. This can affect multiple cellular processes, such as proliferation, migration, metabolism and cell cycle progression.

Targeted therapy for ER+ (Endocrine Therapy)

Endocrine therapy is a type of treatment used for patients with breast cancer who have been identified as Hormone Receptor (HR)-positive. This includes patients who are estrogen receptor (ER)-positive and/or progesterone receptor (PR)-positive. While endocrine therapy is used for early-stage HR+ patients, about 30% of patients may eventually relapse and develop metastatic disease (Reinert T, Barrios CH. 2016). Moreover, during treatment, patients commonly develop resistance to endocrine therapy. Multiple mechanisms are proposed to be responsible for endocrine therapy resistance, including different molecular pathways (Osborne CK, Schiff R. 2011, and Szostakowska M. et al. 2019). These molecular

pathways include 1) interaction between the estrogen pathway and the cyclin D-CDK4/6-retinoblastoma (Rb) pathway; 2) epigenetic abnormalities modified by histone deacetylase (HDACs); 3) mutation or amplification of growth factor receptor tyrosine kinase family; 4) ESR1 acquired mutation; and 5) abnormal cellular microenvironment and immune response (Szostakowska M. et al. 2019). These mechanisms have been studied, leading to the development of many targeted therapies to overcome or delay endocrine therapy resistance.

I. Cyclin-Dependent Kinase (CDK) 4/6 inhibitors

CDKs are crucial cell cycle regulators and belong to the serine/threonine protein kinase family. CDK4/6 forms a complex with cyclin D that phosphorylates Rb, promoting the release of the E2F transcription factor. This upregulates the transcription of genes related to the cell cycle and enables cells to enter the S phase (Bian L. et al. 2020). The progression of cancer cells from the G1 phase to the S phase can effectively be blocked with CDK4/6 inhibitors. In HR-positive breast cancer, the CDK4/6 and ER signal double suppression can restore control of the cell cycle and block breast cancer cell proliferation (Goel S. et al. 2017). CDK4/6 inhibitors such as palbociclib, ribociclib, and abemaciclib have been effectively used in multiple trials for HR-positive advanced breast cancer since 2015, including PALOMA, MONALEESA, and MONARCH trials (Finn RS. et al. 2015, Finn RS. et al. 2016, Rugo HS. et al. 2019, and Tripathy D. et al. 2018). Patients in these studies started with CDK4/6 inhibitors combined with aromatase inhibitors for first-line therapy and progressed to CDK4/6 inhibitors combined with fulvestrant (Slamon DJ. et al. 2018, Cristofanilli M. et al. 2016, and Sledge GW. et al. 2017).

II. Histone Deacetylase (HDAC) inhibitors

HDACs have vital roles in gene expression regulation and chromosome structure modification. Breast cancer progression and endocrine therapy resistance correlate with aberrant gene expression caused by epigenetic alterations modified by HDACs (Osborne CK, Schiff R. 2011, and Saxena NK, Sharma D. 2010). HDAC inhibitors can modulate or reverse epigenetic alteration, such as Tucidinostat (Falkenberg KJ, Johnstone RW. 2014, and Gao S. et al. 2017). Tucidinostat is an HDAC inhibitor that downregulates estrogen-independent growth factor signalling pathways and restores anti-estrogen agents sensitivity (Ning ZQ. et al. 2012, and Gao S. et al. 2017).

III. PI3K-AKT-mTOR target inhibitors

The PI3K gene mutation is associated with tumour proliferation and metastasis. 40% of HR-positive HER2-negative breast cancer patients have PIK3CA mutations (Mollon L. et al., 2018, and Goncalves, MD. et al., 2018). Data from pre-clinical studies suggested that inhibiting PI3K signalling may activate the ER pathway, which will lead to overcoming endocrine resistance (Bian L. et al. 2020). The currently available PI3K α inhibitors include pan-PI3K inhibitors such as buparlisib and pictilisib and selective PI3K α inhibitors such as taselisib and alpelisib. The Food and Drug Administration has approved Alpelisib as the first PI3K inhibitor for HR-positive ABC patients with endocrine resistance (André F. et al. 2019). AKT inhibitors are also targets for the activated PI3K/AKT signalling pathway, which is frequently activated in HR-positive breast cancer and associated with endocrine therapy resistance. Other PI3K/AKT signalling pathway inhibitors include mTOR inhibitors, as they are the downstream target for this signalling pathway (Dey N, De P and Leyland-Jones B. 2017). Both AKT inhibitors and mTOR inhibitors are under investigation (Schmid P. et al. 2019, Bachelot, T.D. *et al.* 2019, and Jones, R.H. et al. 2019).

1.3.0 Tumour Microenvironment

Three main processes influence cancer initiation and progression. Somatic mutations can cause oncoprotein gain-of-function and tumour suppressor loss-of-function. Epigenetic alterations can also change the gene expression pattern. Alterations in the tumour microenvironment can also lead to changes in gene expression (Semenza, 2016). The tumour microenvironment is divided into cellular and chemical microenvironments. The cellular microenvironment includes stromal cells, tumour cells, and the extracellular matrix (ECM). Stromal cells, such as macrophages and myeloid-derived suppressor cells (MDSC), promote primary tumour growth or metastasis (Semenza, 2016). The chemical microenvironment includes factors such as pH, Partial Pressure of Oxygen (PPO₂), and the concentration of molecules like NO and metabolites such as lactate, glucose, and glutamine.

1.4.0 Hypoxia

Hypoxia (low oxygen level) exerts a high impact on the cellular microenvironment by assisting cancer progression, thereby resulting in metastasis and invasiveness, leading to mortality

(Semenza, 2016). Hypoxic stimulation can drive alterations in gene expression, hence leading to cell signalling alterations, which can be observed in many cancers, including breast cancer (Semenza, 2016). Studies have shown that it is important to monitor the oxygen (O_2) content in tumour tissue as it can determine the occurrence of metastasis (Vaupel, 2009). Furthermore, hypoxia has been established as an adverse indicator of a patient's prognosis independent of the established prognostic parameters such as histological grade, clinical tumour stage, histology, and nodal status (Vaupel, 2009).

Solid tumours often experience hypoxia due to the high oxygen consumption of cancer cells that are constantly dividing. The formation of abnormal blood vessels within the tumour can cause a decrease in oxygen availability. Additionally, the distance of cells from the nearest blood vessel also affects the oxygen concentration, with cells closest to blood vessels having a higher concentration. As the distance from the blood vessels increases, oxygen availability decreases (Semenza, 2010). Investigations showed that in normal breast tissue, the partial pressure of oxygen (PO_2) accounts for 65 mm Hg, compared to breast tumour where the mean PO_2 ranges from 2.5 to 28 mm of mercury (Hg), with a 10 mm Hg median value (Vaupel, Hockel & Mayer, 2007). Tumours with a PO_2 less than 10 mm Hg are subjected to a high risk of metastasis and mortality (Vaupel, Mayer, Hockel, 2004).

1.4.1 Hypoxia-inducible factors (HIFs) Structure

Hypoxic stress in the tumour microenvironment triggers the expression of downstream genes through central transcription factors known as Hypoxia-inducible factors (HIFs) (Semenza, G. 2003). The HIF family includes two subunits, α and β . The α subunit includes three proteins, namely, HIF-1 α , HIF-2 α , and HIF-3 α . HIF-1 β , also known as aryl hydrocarbon nuclear translocator (ARNT), was discovered before HIF-1 α . It is a heterodimeric partner of aryl hydrocarbon receptor (AhR) (Semenza GL. 1998, Tian H, et al. 1997). When HIF-1 β binds with AhR, it facilitates HIF-1 β translocation to the nucleus (Wang GL et al., 1995). The structure of the two subunits is related to the nuclear proteins found in *Drosophila* (Per and Sim, PAS). These proteins have a basic-helix-loop-helix (bHLH) motif and belong to the bHLH-PAS protein family (Jiang BH et al., 1995, Wang GL, Semenza GL, 1995). The unique domains (b, HLH, PAS and TAD) of the bHLH proteins allow them to control both their own transcriptions and the expression of other family members. The bHLH-PAS motifs play a critical role in the

heterodimer formation between HIF-1 α and HIF-1 β subunits, as well as binding to the HRE-DNA sequence on the target genes (Masoud GN, Li W. 2015) (Figure 1.2).

Two transactivation domains (TAD) are responsible for HIF-1 α transcriptional activity: NH₂-terminal (N-TAD) and COOH-terminal (C-TAD), both have different responsibilities (Li H, Ko HP, Whitlock JP. 1996). C-TAD modulate gene transcription of HIF-1 α under hypoxia by interacting with co-activators such as CREB binding protein (CBP/p300), where N-TAD stabilises HIF-1 α against degradation. In addition, the structure of all HIF- α subunits differs from that of HIF-1 β in that an oxygen-dependent degradation domain (ODDD) overlaps with the N-TAD. The stability of O₂ regulation is mediated by this ODDD domain (Bruick RK, McKnight SL. 2001).

HIF- α isoforms are related (HIF-1 α , HIF-2 α , and HIF-3 α) (Li H, Ko HP, Whitlock JP. 1996 and Ema M. et al. 1997). 48% sequence similarity of HIF-1 α is shared with HIF-2 α , allowing them to hetero-dimerize with HIF-1 β and bind with HREs (Ema M. et al. 1997). While HIF-1 α is expressed in all body tissues, HIF-2 α and HIF-3 α are found in specifically selected tissues (Wiesener, M. S. et al. 2003 and Semenza, G. L. 2012). The overexpression of HIF-1 α and HIF-2 α has been observed in a variety of tumours which often indicates an aggressive tumour and poor prognosis (Keith B, Johnson RS, Simon MC. 2011). HIF-2 α has been detected in several organs, including the heart, lung, brain, liver, pancreas, intestine, and kidney (Wiesener MS. et al. 2003).

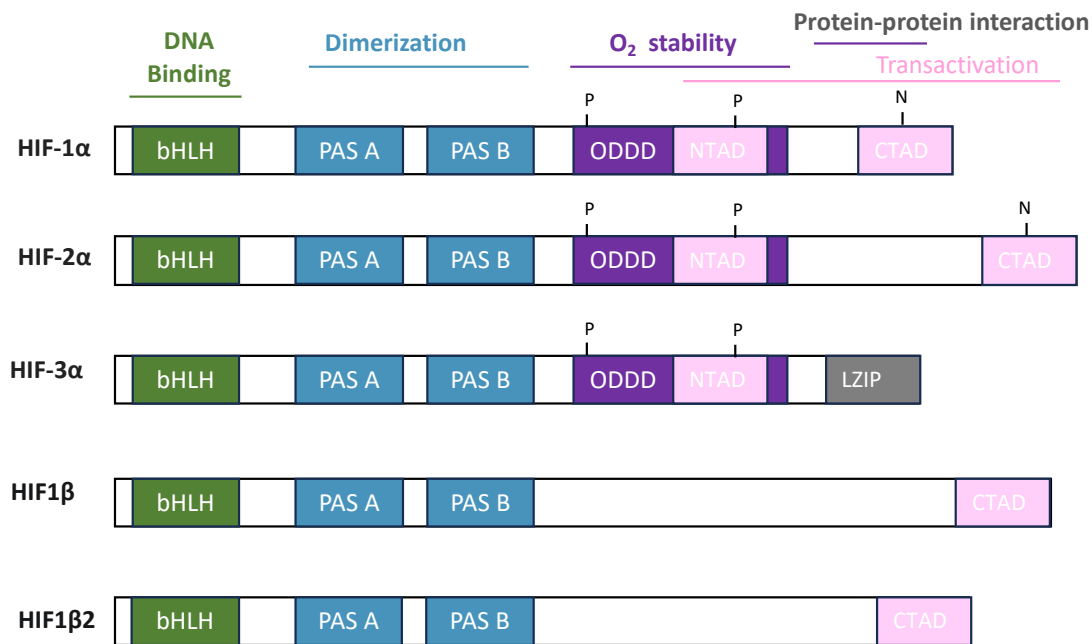


Figure 1.2: The functional domains of HIF isoforms. All isoforms possess a bHLH domain responsible for DNA binding highlighted in green, while PAS domain responsible for dimerization is marked in blue. The ODD domain, which overlaps with the NTAD domain, highlighted in purple and pink respectively, plays a crucial role in mediating oxygen regulation stability. However, this domain is not part of the HIF1 β isoform. The N-TAD domain is responsible for the transcriptional activities of the isoforms, while the N-TAD and C-TAD domains are responsible for the transactivation of the HIF α subunits. In the case of HIF-3 α , the C-TAD domain is replaced by the LZIP domain, highlighted in grey, which regulates protein-protein interaction.

Adapted from (Ravenna L, Salvatori L and Russo MA. 2016). (License Number: 5775870171564, License date: Apr 25, 2024)

1.4.2 Regulation of Hypoxia-inducible factors (HIFs)

The cellular availability of oxygen regulates the expression of the α -subunits, whereas the β subunit expression is independent of oxygen levels and is expressed constitutively (Kaelin WG Jr, Ratcliffe PJ. 2008 and Semenza, G. L. 2012). The abundance of HIFs is controlled by oxygen-dependent proline hydroxylase family (PHDs), namely PHD1, PHD2 and PHD3 (Ivan, M. et al 2001, Markolovic, S. et al. 2015). The enzymatic activity of these requires iron, oxygen and 2-oxoglutarate (Jaakkola, P. et al. 2001 and Markolovic, S. et al. 2015). Under hypoxic conditions, the lack of oxygen that is required as a co-factor for PHD2 activity results in the inactivation of PHD2, resulting in HIFs accumulation (Kaelin 2005 and Gao et al., 2007).

Under normoxic conditions, two specific proline residues (564 and/or 402) located within the ODDD of HIF- α proteins get hydroxylated by PHDs. Once hydroxylated, HIF- α binds to the β -domain of von Hippel–Lindau (pVHL), a component of the E3-ubiquitin ligase complex, which ultimately leads to the degradation of HIF-1 α / HIF-2 α by the 26S proteasome (Cockman, ME et al. 2000 and Kamura, T. 2000) (Figure 1.3).

Under hypoxic conditions, PHD enzymatic activity is inhibited, resulting in the prevention of HIF- α hydroxylation and ubiquitin-mediated proteasome degradation (Wu, D. et al. 2015 and Bao, X. et al. 2021). HIF- α subunits are translocated to the nucleus, where they dimerise with HIF-1 β , forming a complex associated with its cofactors CBP/p300 (Majmundar, A.J. et al. 2010). This binds to the DNA sequence 5'-RCGTG-3' on the hypoxia-responsive elements (HREs) region, thereby activating the transcription of HIF target genes. R denotes a purine residue (A/G) in the HRE core recognition sequence (Majmundar, A.J. et al. 2010). The HIF target genes encode proteins that are known to be involved in glycolysis, erythropoiesis, angiogenesis, resistance to chemotherapy and radiotherapy, cell survival, invasion, and metastasis (Semenza 2013). HIF-3 α has an opposite role in induced-hypoxia-gene expression; the high expression of HIF-3 α suppresses angiogenesis and reduces cell proliferation (Barth, D. A. et al. 2020) (Figure 1.3).

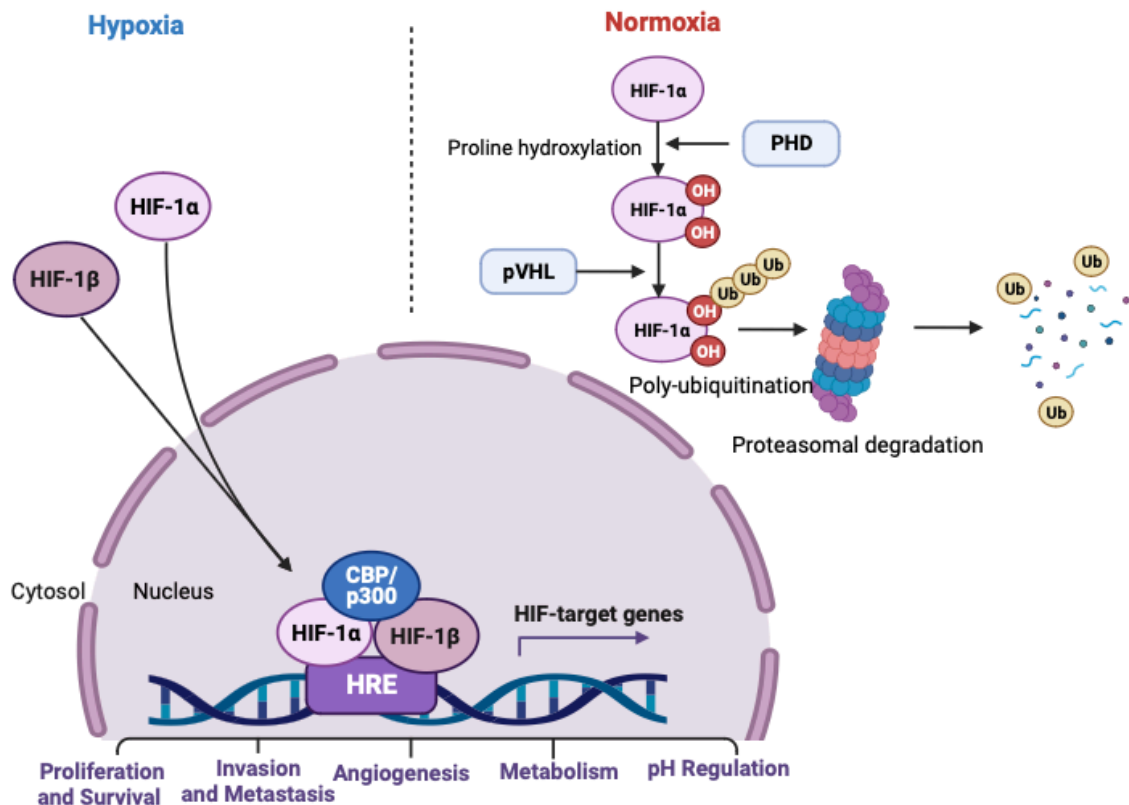


Figure 1.3: HIFs regulation under normoxic and hypoxic conditions. Under normoxic conditions, HIF- α is hydroxylated by PHDs ultimately leads to the degradation of HIF-1 α / HIF-2 α . Under hypoxic condition, PHD enzymatic activity is inhibited, HIF-1 α translocate to the nucleus where it dimerises with HIF-1 β to forming a complex associated with CBP/p300 and bind to the DNA sequence on HRE activating the transcription of HIF targeted genes. These genes involved in proliferation and survival, invasion and metastasis, angiogenesis, metabolism, pH regulation.

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1.4.3 Hypoxia and Angiogenesis

Angiogenesis is a process where new blood vessels grow. As the tumours grow, additional nutrients and oxygen are required; in addition, tumours induce new blood vessel formation. In normal physiology, new blood vessel formation is regulated by pro and anti-angiogenic signalling balance. When this balance is lost on the tumour, it results in unstructured vasculature. Growth factors and their receptors play a critical role in regulating angiogenesis, including FGF2 (FGFR1, 2, 3,4), VEGF, VEGFR-1,2, and 3, HGF (c-MET), ANG-1 and 2 (TIE2) (Welti J, et al. 2013). Growth factors are associated with signalling pathways, particularly the Notch pathway. Notch pathways regulate endothelial tip cell formation and function (Gridley T. 2013). Under hypoxic conditions, factors that regulate angiogenesis increase (Figure 1.6).

HIF increase angiogenesis genes in response to hypoxia, including VEGF, ANG1, 2, FLT-1, TIE2, MMP2, 9, FLK1 and PDGFC (Semenza GL. 2014). HIF-1 regulates proangiogenic chemokines and receptors and, as a result, aids in recruiting endothelial progenitor cells to hypoxic sites and enhances endothelial cell proliferation and division (Manalo et al., 2005 and Ceradini et al., 2004).

1.4.4 Different roles of HIF-1 α and HIF-2 α

HIF-1 α and HIF-2 α are two proteins with similar domain structures and regulatory mechanisms, and both bind to HRE-activating HRE-induced reporter genes (Loboda A. et al. 2010). Despite these similarities, HIF-1 α and HIF-2 α have different roles, which can be seen in their distinct functions and stability. In breast cancer cell lines, it takes 4-16 hours for HIF-1 α expression to stabilise, followed by a gradual decrease, whereas it takes 24 hours for HIF-2 α expression to stabilise (Cavadas MAS. et al. 2015, Stiehl DP. Et al. 2012). HIF-1 α is linked with metabolic reprogramming, angiogenesis, invasion, and metastasis, leading to different cellular phenotypes (Ratcliffe PJ. et al. 2007 and Harris AL. 2002). On the other hand, HIF-2 α plays a critical role in promoting the cancer stem cell phenotype, and its stabilisation occurs over a longer time (Kise K. et al. 2016 and Holmquist-Mengelbier L. et al. 2006). Moreover, glycolytic gene expression is induced by HIF-1 α and not HIF-2 α in various cell types (Hu, CJ. 2003). LDHA and PDK1, two critical enzymes that aid in the primarily glycolytic phenotype switch, are activated by HIF-1 α (Kierans SJ. Et al. 2021 and Kozlov AM. et al. 2020). Hence, the transcriptional cascade responsible for cellular responses to oxygen availability is independent of which HIF binds to the HRE. As a result, many cellular processes are controlled under hypoxia (Pouyssegur J, López-Barneo J. 2016).

1.4.5 Regulation of HIFs in Breast Cancer

Breast cancer has been associated with high levels of HIF, which are linked to more aggressive tumours (Bos R. et al. 2001). Overexpression of HIF-1 α in breast cancer patients can also be used as an additional prognostic factor and is usually associated with poor prognosis, disease-free (DFS) and overall survival (OS) (Shamis SAK. Et al. 2001, Dales JP. et al. 2010 and Marton I. et al. 2012). In breast cancer, HIF-1 α overexpression is regulated through different pathways: (i) hypoxia-induced, perinecrotic HIF-1 α overexpression associated with increased expression

of hypoxia-induced genes (CA IX and GLUT-1), resulting in poor prognosis and (ii) diffuse HIF-1 α overexpression without critical hypoxia-associated downstream effects, resulting in better prognosis (Vleugel MM. et al. 2005). Hypoxia is associated with breast cancer invasiveness in different studies. Under hypoxia, HIF-1 α upregulates invadopodial actin-bundling protein CSRP2 and MMP-9 expression, both affecting breast cancer invasion (Hoffmann C. et al., 2018, Choi JY. et al. 2011). Recent studies have revealed that extracellular ATP can activate HIF-1 α in breast cancer cells. The ATP-HIF-1 α signalling pathway is governed by STAT3-ALDOA and PI3K/AK, contributing to chemoresistance (Yang H. et al. 2022). Targeting HIF-1 α signalling may be a potential therapy for breast cancer.

Studies have shown that breast cancer cells' responses to normoxic and hypoxic microenvironments display diverse phenotypic characteristics. Under hypoxia, expression of hematopoietic pre-B cell leukaemia transcription factor interacting protein (HPIP) is induced by HIF-1 α , which facilitates cell survival and induces invasion and migration of cells, EMT and metastatic phenotype. HIF-1 α stabilises by accumulating HPIP to support cell growth (Khumukcham SS. et al. 2022). HIF-1 α also induces CLDN6 gene expression in breast cancer cells under hypoxia. CLDN6 act as a tumour suppressor gene, and increased expression of CLDN6 reduces HIF-1 α stability by decreasing SENP1 expression and preventing the deSUMOylation of HIF-1 α (Jia Y. et al. 2020). This negative feedback loop reduces hypoxia-induced metastatic phenotype in breast cancer.

HIF-2 α can serve as a biomarker for breast cancer progression and patient survival. The expression of HIF-2 α has a direct correlation with the size of the tumour, lymph node involvement, and metastasis. Furthermore, high levels of HIF-2 α expression are linked with poor overall survival in breast cancer patients (Wang HX. et al. 2014). HIF-2 α expression under hypoxic conditions can stimulate the Notch3 pathway, leading to increased migration and invasion of MCF7 breast cancer cells (Wang JG and Yuan L. 2016). Additionally, HIF-2 α regulates the expression of CD44, a marker for breast cancer stem cells, and activates cancer stem cells via the PI3K/AKT/mTOR signalling pathway in MDA-MB-231 cancer cells (Bai J. et al. 2020). The downregulation of HIF-2 α suppresses stemness and induces apoptosis in MDA-MB-231 breast cancer cells (Bai J. et al. 2020).

1.4.6 RTKs and hypoxia signalling in cancer

Independently from oxygen levels, HIF-1 α can be regulated through several signalling events, including multiple growth factors and signalling molecules (Kallio PJ, 1997 and Masoud GN, Li W. 2015). Under the normoxic condition, the half-life of HIF-1 α is very short (approximately 5 min), and it undergoes degradation (Salceda S, Caro J. 1997). Meanwhile, under hypoxia, the stability and transcriptional activity of HIF-1 α is controlled by multiple pathways through post-translational modifications, including ubiquitination, acetylation, hydroxylation, and phosphorylation reactions (Kallio PJ, 1997 and Masoud GN, Li W. 2015).

Targeting the HIF system and RTK system can be vital for current cancer therapy since deregulated signalling through RTK pathways correlates with therapy resistance, worst prognosis, and increased mortality, similar to the hypoxic response in cancer cells (Choura and Rebai, 2011; Casaletto and McClatchey, 2012). Several studies have demonstrated the crosstalk between RTKs and hypoxia-related signalling. Hypoxia can activate the expression of specific RTKs and/or their corresponding ligands, while some RTKs can trigger HIF machinery activation (Denko et al., 2000, Glück AA. et al. 2015). It has also been shown that under hypoxic conditions, the regulation of RTK signalling occurs in an HIF-dependent manner.

I. VEGF

The VEGF family consists of four homodimeric members: VEGF-A, VEGF-B, VEGF-C and VEGF-D. These bind and activate three corresponding VEGF receptors, VEGFR-1, VEGFR-2 and VEGFR-3, which are expressed by endothelial and various tumour cells (Abdel- Rahman, 2015; Hamerlik et al., 2012). Under hypoxic conditions, tumour cells increase the expression of VEGF family members in a paracrine fashion, which in turn promotes endothelial cell proliferation and leads to increased angiogenesis (Forsythe et al., 1996). It has been shown that the expression of VEGFA is induced by hypoxia in most cell types, both in vitro and in vivo (Harris, 2002; Simiantonaki et al., 2008) (Ferrara and Davis-Smyth, 1997). The transcriptional activation of VEGF can be triggered directly or indirectly by HIF-1. Several studies have reported that the stability of VEGF mRNA is enhanced under hypoxic conditions in addition to increased transcriptional activation (Levy et al., 1996; Ikeda et al., 1995; Stein et al., 1995). It has been shown that VEGF mRNA and protein levels were increased due to hypoxia-induced adenosine release resulting from decreased recycling by adenosine kinase. This in turn,

stimulated A2R and activated the cAMP-dependent protein kinase A (PKA) pathway, ultimately leading to increased VEGF expression (Bontemps et al., 1993; Takagi et al., 1996).

Deregulated PDGF production can also increase VEGF production, suggesting another indirect mechanism mediated by the HIF system (Abdel-Rahman, 2015; Chung et al., 2010). It has been shown that HIF-1 binds to a site in intron 3 of the PDGF-B gene and activates its transcription in breast cancer cells (Schito et al., 2012). This correlation between HIF-1 expression and PDGF-B has been observed in various studies on breast cancer and glioma. (Schito et al., 2012) (Yoshida et al., 2006). The PDGF family consist of disulfide-bonded homodimers of A-, B-, C- and D-polypeptide chains, in addition to the heterodimer PDGF-AB. The formation of new blood vessels is promoted via binding of PDGF isoforms to α and β tyrosine kinase receptors (PDGFR α and PDGFR β) (Schito et al., 2012).

Furthermore, VEGF signalling affects HIF protein expression and phosphorylation in multiple cell types. VEGF induced the activation of VEGFR-1 which has led to an increase in HIF-1 and HIF-2 protein levels in neuroblastoma cells (Nilsson et al., 2010). In addition, HIF-2 levels have been found to correlate with VEGF expression and are associated with poor patient outcomes in clinical neuroblastoma specimens (Holmquist-Mengelbier et al., 2006). The inhibition of VEGF/VEGFR-1 autocrine signalling decreased HIF-1 phosphorylation, ERK1/2 activated by VEGFR-1 signalling in newly arisen hypoxia. In turn, HIF-1 activated by ERK1/2, regulates VEGF and VEGFR-1, resulting in further stimulation of ERK1/2. Neuroblastoma cells with VEGFR-1 expression exhibit hypoxia-mediated resistance to chemotherapeutic agents, including doxorubicin (Das B. et al. 2005 and Glück AA. et al. 2015).

II. IGF1R and IR

Insulin-like growth factor 2 (IGF2) is an RTK ligand that plays a crucial role in hypoxia-induced tumorigenesis. It exerts its biological activity by binding to the IGF1R tyrosine kinase (Enguita-Germán and Fortes, 2014). In tumours, the activation of IGF1R is usually caused by increased ligand availability and not by point mutation or gene amplification (Fukuda et al., 2002). The activation of IGF1R occurs when IGF1 or IGF2 bind to it (Zhang et al., 1997). In glioblastoma cells, the activation of IGF1R is stimulated by either IGF1 or IGF2, which increases HIF-1 expression, HIF-1 DNA binding activity and activated transcription of

downstream target genes (Gariboldi et al., 2010). In colon cancer cells, IGF1 treatment resulted in increased HIF-1 expression, which was triggered by stimulation of HIF-1 synthesis (Fukuda et al., 2002). IGF2 lacks a defined HRE that contains the HIF-1 binding site. Therefore, HIF-1 possibly affects IGF2 expression indirectly (Feldser et al., 1999). In colon cancer, IGF2 is highly regulated and correlated with HIF-1 overexpression. It has been shown that hypoxia induces IGF2 expression in HepG2 hepatocellular carcinoma (HCC) cells (Kim et al., 1998). PI3K/AKT/mTOR is involved in IGF1R signalling; IGF1R signalling activates PI3K/AKT/mTOR via the recruitment of insulin receptor substrates (IRS) 1 and 2 to the activated receptor (Chitnis et al., 2008). Activation of PI3K/AKT/mTOR may have a self-inhibitory effect on HIF-1 synthesis, as mTOR is known to downregulate IRS1/2. Several pathways have been shown to mediate IGF-dependent signalling such as the JAK/STAT pathway. IGF1R can recruit STAT3 independently of IRS1/2 and ultimately modulate AKT expression (Xu et al., 2005). The inhibition of STAT3 or IGF1R can have therapeutic benefits on hypoxic tumours with autocrine regulation of the IGF-HIF-1 axis. Glioblastoma cells exposed to IGF1R inhibition (NVP-AEW541) revoked HIF-1 synthesis (Gariboldi et al., 2010). Several cellular responses and signalling effectors are common to IGF1/2, insulin, and insulin receptors (IR). Insulin regulates the metabolic pathway responsible for energy storage and utilisation. Hypoxia and insulin cause an increase in glucose transporter levels and glycolytic enzymes, thereby contributing to an increase in glycolysis (Glück AA. et al. 2015). The binding of insulin to IR in renal epithelial cells induces the translation of HIF-1 by activating the PI3K/AKT/mTOR pathway (Treins et al., 2002). It is suggested that insulin-induced HIF-1 translation may involve both AKT/mTOR-dependent and AKT-dependent/mTOR-independent pathways. Additionally, insulin stimulation for HIF-1 expression activates VEGF transcription (Treins et al., 2002).

III. EGFR

The ErbB family RTKs consist of EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. Hypoxia exerts a strong impact on this RTK family, aberrant activation by the EGF family of growth factors associated with invasion, metastasis, and tumorigenesis (Arteaga and Engelman. 2014, Momose et al. 2013). ErbB activity is deregulated due to gene amplification, mutation, and overexpression in several cancers and is associated with poor prognosis and therapy resistance (Glück AA. et al. 2015). It has been demonstrated that EGFR signalling has

increased HIF-1 α levels in multiple tumours, including breast cancer (Peng et al., 2006) HIF-1 α regulation by the ErbB family is cell-type and cancer-type specific. Some studies have reported that EGF increases HIF-1 α mRNA and protein levels, while others demonstrated that EGFR signalling leads to elevated protein translation and not mRNA levels (Cheng et al. 2013, Pore et al., 2006). The activation of EGFR can trigger multiple signal transduction pathways, including the PI3K/AKT pathway and RAS/RAF/MAPK pathway (Vivanco and Sawyers, 2002, Lewis et al., 1998) (Figure 1.4). HER2 also play a critical role in HIF-1 regulation. Increasing activity of HER2 has been observed in breast cancer and correlated with higher tumour grade, therapy resistance and worse patient survival (Pegram et al., 1998; Rilke et al., 1991). HER2 overexpression induced VEGF mRNA and protein expression (Bagheri-Yarmand et al., 2000)

1.4.7 Hypoxia and Intracellular Pathways Regulation

In addition to HIF's role in regulating the expression of genes participating in several cellular mechanisms, hypoxia signalling interacts with different cellular pathways such as PI3K/Akt/mTOR pathway, nuclear factor kappa-B (NF- κ B) pathway, Wnt/ β -catenin, ERK signalling and ER stress (Figure 1.4).

Several studies have shown that the PI3K-mTOR signalling pathway plays a crucial role in regulating the mRNA levels of HIF- α (Hudson CC. et al. 2002 and Düvel K. et al. 2010). In cancer cells, upregulation of PI3K-mTOR signalling can promote mRNA expression, facilitate HIF- α activity, and induce angiogenesis (Brugarolas JB. et al. 2003) (Figure 1.4). Additionally, under hypoxic conditions, mTORC1 phosphorylates STAT3, resulting in induced HIF-1 α RNA expression (Dodd KM. et al. 2015). It has been demonstrated that upregulation of the mTOR signal promotes HIF-1 α to induce metabolic reprogramming and enhance T cell survival (Chisolm DA, Weinmann AS. 2015). The growth of tumours and angiogenesis are also regulated by PI3K signalling through AKT activation and other targets by inducing the expression of HIF-1 and VEGF (Jiang BH. et al. 2001). Interleukin-6 (IL-6) activates downstream Janus kinase (JAK)-STAT3 signalling pathway therapy upregulates HIF- α expression (LaGory EL. et al. 2016). Toll-like receptor (TLR) signal activation drives the downstream NF- κ B pathway to increase HIF- α transcription (Rius J. et al. 2008). NF- κ B signalling also promotes HIF-1 β expression (van Uden P. et al. 2011).

The expression of HIF-1 α can be influenced by the ERK pathway, which affects the HIF signal. According to a study, AKT and ERK pathways regulate the expression of HIF-1 α in lung cancer cells after hyperthermia treatment (Wan J. et al. 2016). Additionally, photodynamic therapy (PDT) can increase HIF-1 α expression through the ROS-ERK axis, enhancing therapy resistance (Lamberti MJ. et al. 2017). The activation of MAPK signalling also contributes to HIF activation through p300/CBP (Sang N. et al. 2003).

The Wnt/ β -catenin signalling pathway also contributes to the HIF-1 α signal in cancer cells (Liu HL. et al. 2015). Wnt/ β -catenin can promote PI3K/Akt signalling, thereby regulating HIF-1 α function (Lau MT. et al. 2011). Additionally, HIF-1 α can regulate the Wnt/ β -catenin pathways, resulting in the promotion of breast tumorigenesis and progression by regulating the expression of calreticulin. Calreticulin is a multifunctional protein known to participate in various cellular processes, including cancer progression (Liu X. et al. 2021). Notch/HIF-1 α signalling pathway can regulate angiogenesis, liver regeneration and EMT. (Chen Y. et al. 2019, Li Y. 2020, and De Francesco EM. et al. 2018).

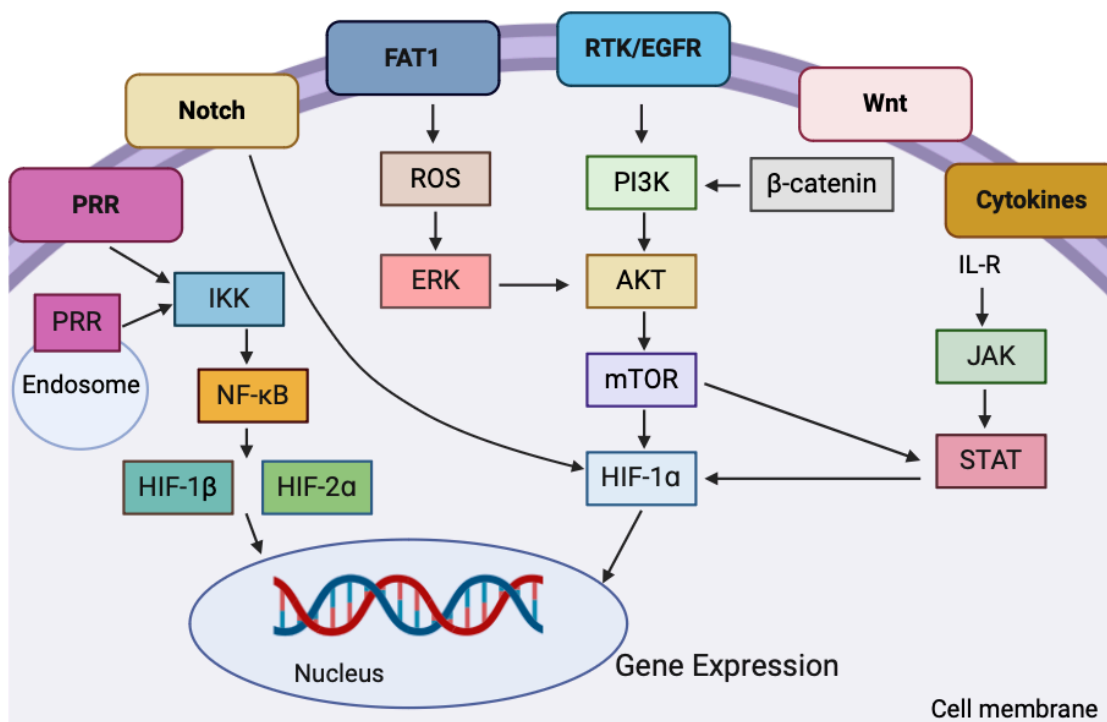


Figure 1.4: The interaction among HIF signal with multiple signalling pathways.

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1.4.8 Additional signalling regulating HIF-1 α pathway

The HIF-1 α pathway can also be regulated at different levels for example, HIF-1 α synthesis is regulated by growth factors-related pathways. HIF-1 α stability is regulated by pVHL-related pathways, Mdm2-p53 mediated ubiquitination and proteasomal degradation pathway and Hsp90. HIF-1 α transactivation is regulated by Hsp90 and factor inhibiting HIF-1 (FIH-1) (Masoud GN, Li W. 2015).

I. Growth factor-related pathways

Several studies have demonstrated that the translation of HIF-1 α protein can increase in response to activation of the PI3K pathway (Semenza G. 2002 and Jiang BH. et al. 2001 and Conrad PW. et al. 1999). PI3K regulates protein synthesis by targeting Akt and mTOR, which is a downstream component. mTOR carries out its function by phosphorylating the eIF-4E binding protein (4E-BP1), which disrupts the integrity of the two components and inhibits cap-dependent mRNA translation. This results in the enhanced translation of the HIF-1 α protein. Moreover, mTOR promotes the phosphorylation of its substrate, the ribosomal protein S6, through the phosphorylation of S6K, thereby inducing protein translation (Masoud GN, Li W. 2015). This pathway is counteracted by the tumour suppressor protein PTEN, which reverses the phosphorylation of PI3K products (Gingras A-C. et al. 2001).

Certain growth factors can activate RAS, which in turn stimulates the RAS/RAF/MEK/ERK kinase cascade. The activation of ERK can lead to the phosphorylation of 4E-BP1, S6K, and MAP kinase interacting kinase (MNK). MNK can directly phosphorylate eIF-4E, resulting in an increase in the mRNA translation rate into HIF-1 α protein (Masoud GN, Li W. 2015). ERK plays a role in regulating HIF-1 α synthesis and transcriptional activity. The co-activator CBP/p300 is phosphorylated by ERK, which increases the formation of the HIF-1 α /p300 complex, resulting in the stimulation of its transcriptional activation function (Sang N. et al. 2003).

II. Mdm2 pathway

Under normoxic conditions, the protein p53 promotes the degradation of HIF-1 α through its binding to HIF-1 α and facilitating its ubiquitination and proteasomal degradation by Mdm2. However, under hypoxic conditions, loss or mutation in the p53 tumour suppressor gene

prevents the HIF-1 α Mdm2-mediated degradation (Ravi R. et al. 2000). In certain types of tumours, the loss of the p53 tumour suppressor gene leads to increased levels of HIF-1 α (Bae MK. et al. 2002).

III. Heat shock protein 90 (Hsp90)

Hsp90, a molecular chaperone, is associated with HIF-1 α . Hsp90 is involved in the stabilisation and transactivation of HIF-1 α . HIF-1 α can be stabilised against non-VHL-dependent degradation via Hsp90. Additionally, the transactivation of HIF-1 α is initiated by direct binding of HIF-1 α and Hsp90. This binding induces changes in HIF-1 α structure to fit and couple with HIF-1 β to initiate transactivation (Gradin K. et al. 1996 and Masoud GN, Li W. 2015). Geldanamycin (GA), an Hsp90 antagonist, has been found to revoke HIF-1 α levels independent of oxygen availability (Isaacs JS et al. 2002).

1.4.9 Hypoxia and therapy resistance

Tumor hypoxia results in the development of resistance to treatment. Radiotherapy acts on cells by generating ROS which causes irreparable damage to cellular DNA and promotes apoptosis. This is due to the lower oxygen availability in hypoxic conditions, which makes cells three times more resistant to radiation than cells in normoxic conditions (Zhang Y. et al. 2021, Rockwell S. et al. 2009).

Immunotherapy is a well-developed therapy for cancer. However, the low response rate of patients to this treatment is influenced by various factors, including hypoxia (Fu Z. et al. 2021). Hypoxia results in an increase in metabolic shift by elevating glycolysis, which leads to an increase in the levels of adenosine (Hatfield SM, Sitkovsky M. 2016.). Adenosine is a potent suppressor of T cells, which reduces the effectiveness of immunotherapy. Hypoxia also increases suppressor T cells, and the action of HIF-1 α represses the adaptive immune system (Facciabene A. et al. 2011, Leone RD, et al. 2015). Additionally, hypoxia downregulates estrogen receptor- α expression and function, which makes the cancer cells resistant to hormonal therapy (Rani A. et al. 2019).

1.5.0 Oxygen gradient in breast cancer cells

The human breast tissue has physiological oxygen levels of approximately 8.5% O₂, whereas the hypoxic levels are around 1.5 % O₂ (Vaupel P, Höckel M, Mayer A. 2007). As breast cancer progresses, different stages of hypoxia may occur depending on the tumour microenvironment at the primary tumour site. Healthy and cancerous cells can experience oxygen level fluctuations, leading to intermittent hypoxia, which ultimately affects the molecular features of metastatic cells (Gutsche K. et al. 2016 and Cooper C, et al. 2004). The reoxygenation of circulating cancer cells occurs at around 5% O₂. Additionally, metastatic lesions of breast cancer have a poorer oxygen status than primary tumours. Furthermore, higher hypoxic fractions are observed in local recurrences when compared to the primary tumour. In breast cancer, hypoxia develops independently of tumour characteristics such as clinical size, histology, grade and stage (Vaupel P, Höckel M, Mayer A. 2007).

1.6.0 Hypoxia-Reoxygenation

Hypoxia induces metastasis in breast cancer (Gilkes, D. M., & Semenza, G. L. 2013 and Da Motta, L. L. *et al.* 2017), and studies have found that metastasis genes are enriched within well-oxygenated regions in the bloodstream (Boidot, R. *et al.* 2014) (Figure 1.5). These metastasis genes within the circulating tumours have maintained hypoxia gene signatures, which have been previously seen *in vitro* and in breast cancer patients (Boidot, R. *et al.* 2014). Under hypoxia, changes in chromatin accessibility are dependent on oxygen availability and reversed upon reoxygenation (Batie M, Frost J, Shakir D, Rocha S. 2022).

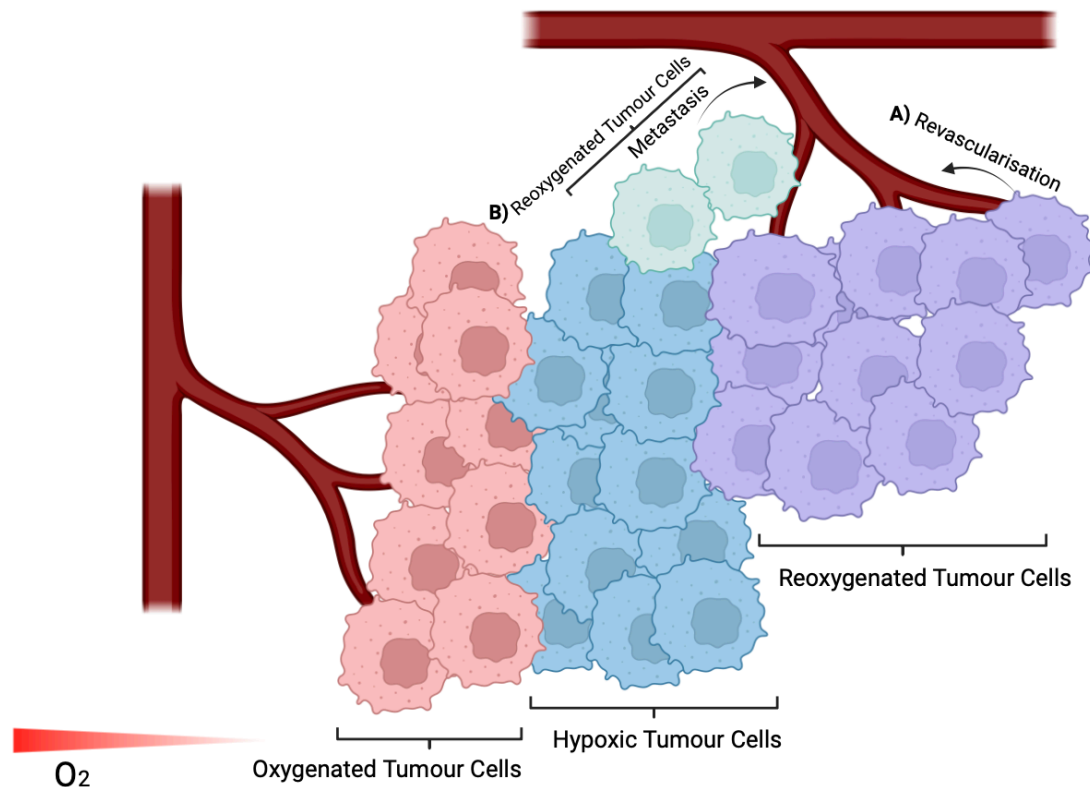


Figure 1.5: Schematic representation of the Mechanisms of Reoxygenation:

A) Revascularisation of the tissue. **B)** Hypoxia drives metastasis; invading from hypoxic tumour microenvironment into oxygenated tissues into blood vessels and become reoxygenated. Figure Created in BioRender.

1.6.1 Classification of Acute, chronic, and intermittent (cyclic) hypoxia

The duration of hypoxia, levels of oxygen and the presence of hypoxia-reoxygenation cycles have yet to be clearly defined. Various studies have discussed the necessary duration for cancer cells to induce specific phenotypes during acute or chronic hypoxia. It is worth noting that there are different classifications of hypoxia based on the duration of exposure to low oxygen levels. According to a previous study by Bayer C. et al. (2011), hypoxia lasting less than two hours is classified as acute, while hypoxia lasting longer than two hours is classified as chronic (Bayer C. et al. 2011). However, this classification is based solely on experimental observations and disregards the underlying pathophysiological and pathogenetic processes. Han et al. (2017) classified hypoxia lasting 24 hours as chronic, whereas Jarman et al. (2019) classified the same duration as acute hypoxia (Han et al. 2017 and Jarman et al. 2019).

Recently, Liu Q et al (2022) provided a clearer understanding of acute, chronic, and intermittent, relying on the literature on hypoxia within the in vitro breast cancer research

field. Accordingly, it was proposed that (i) Acute hypoxia (AH) occurs when cells are exposed to an environment with 1% O₂ or less for no longer than 24 hours, as this is when HIF-1 α is most active and stabilised. (ii) Chronic hypoxia (CH) is when cells are exposed to an environment with 1% O₂ or less for longer than 48 hours, as HIF-2 α activation occurs during this time. (iii) Intermittent hypoxia (IH) occurs when cells are subjected to a minimum of two phases of hypoxia (1% O₂ or less), followed by a minimum of one phase of reoxygenation through exposure to normoxia (8.5% O₂ or higher) (Liu Q. et al. 2022). It is essential to understand these classifications when studying the effects of hypoxia on tumour progression.

1.6.2 Cause of different forms of hypoxia

The proximity to the nearest oxygen source determines the hypoxic profile of cancer cells. The uncontrolled proliferation of cancer cells can cause hypoxia by distancing them from the closest oxygen source, leading to variation of hypoxia from acute to chronic (Bader SB. et al. 2020). Acute hypoxia occurs when small blood vessels are blocked due to irregular erythrocyte flow or increased cell mass and can be reversed by regaining blood flow. Chronic hypoxia occurs when cancer cells over-proliferate, increasing spatial cellular density and distance from nearby blood vessels. Cyclic hypoxia occurs when unevenly distributed, disorganised, and immature tumour vasculature is blocked, leading to hypoxia and reoxygenation periods. Cells exposed to hypoxia followed by reoxygenation can develop “reoxygenation injury” that involves tissue damage, oxidative stress, and free radical formation (Vaupel P, Mayer A, Höckel M 2004 and Saxena K, Jolly MK. 2019).

1.6.3 Evidence of Cyclic (Intermittent) hypoxia

In 1979, Brown introduced a new form of hypoxia called cyclic hypoxia. This was initially observed in tumour tissue and was caused by irregular oxygen perfusion due to the disordered and poorly structured network of blood vessels in the tumour (Brown, J., 1979). The fluctuations in perfusion resulted in periods of poor and better oxygenation, leading to cyclic exposure of cells to hypoxia and reoxygenation (Michiels, C., Tellier, C. and Feron, O., 2016)

The intermittent perfusion was also revealed by Trotter et al. in murine tumours by intravenously injecting two fluorescent stains, Hoechst 33342 (double-strand DNA fluorescent intercalating molecule) and DiOC7, with a time delay of 20 minutes between administration

of stains. This was to mark the cells directly adjacent to blood vessels by diffusing out of the perfused blood vessels. The two marks used had different fluorescence excitation and emission properties, which allowed recognition of the stains within the same tissue section. Intermittent perfusion was indicated by the unmatched staining in regions that happened in this 20-minute time-lapse (Trotter. M.J. et al. 1989a and 1989b).

1.6.4 Impact of Intermittent (cyclic) hypoxia

Hypoxia affects several cellular processes that are crucial for the growth, invasion, and metastasis of a tumour. Metastasis is a complex process that involves several steps, such as dissemination from the primary tumour, circulation entry, survival during circulation in matrix-deprived conditions, and forming new colonies in distant organs (Gupta GP, Massagué J. 2006). Metastatic cells adapt to stressful conditions during the metastatic journey, such as lack of oxygen or nutrient availability. In recent years, scientific attention has increasingly focused on studying hypoxia-reoxygenation and its impact on tumour progression. The Hallmarks of Cancer by Hanahan D and Weinberg RA have been instrumental in studying the role of hypoxia's different forms, including acute, chronic, or intermittent (cyclic hypoxia) (Hanahan D and Weinberg RA 2011, 2022). Saxena K and Jolly MK. 2019 reviewed the effect of cyclic hypoxia on various hallmarks of cancer (Saxena K and Jolly MK. 2019). In addition, Liu Q et al. 2022 demonstrated the role of acute, chronic, and intermittent hypoxia in the selection of hallmarks of cancer in breast cancer (Liu Q et al. 2022).

Intermittent hypoxia also called cyclic hypoxia, is when the cell is exposed to cycles of hypoxia and reoxygenation (H-R) cycles. Compared to acute and chronic hypoxia models, the intermittent hypoxia model imitates the oxygenation status in tumours as the oxygen gradient constantly changes between normoxia and hypoxia due to heterogeneous blood supply and dysfunctional tumour vascularisation (Matuszewska K, et al. 2021). In addition, studying the impact of intermittent hypoxia is crucial to understanding hypoxia's role in breast cancer progression due to the physiological relevance between intermittent hypoxia and breast microenvironment (Liu Q. et al., 2022).

Angiogenesis in hypoxia-reoxygenation

Angiogenesis, the process of forming new blood vessels, plays a crucial role in the growth and progression of tumours. Under hypoxic conditions, HIF-1 and HIF-2 stimulate essential factors involved in angiogenesis (Conway EM. et al. 2001 and Semenza GL and Wang GL. 1997). In Hep3B cells under acute hypoxia (1% O₂ for 2-4 hours), erythropoietin, a key hormone that promotes angiogenesis, is directly enhanced through HIF-1 (Semenza GL and Wang GL. 1997). In the tumour microenvironment, both tumour cells and endothelial cells have different roles in supporting tumour growth. Endothelial cells help form new blood vessels, supplying oxygen and nutrients for tumour growth. In contrast, tumour cells secrete crucial factors involved in angiogenesis, such as VEGF, to support the survival of endothelial cells (Toffoli S, Michiels C. 2008).

Intermittent hypoxia affects both endothelial and tumour cells, exhibiting a proangiogenic effect (Kochan-Jamrozky K. et al. 2019). Research has shown that HIF-1 α response is enhanced by intermittent hypoxia, driving tumour angiogenesis, as observed in studies by Martinive P, et al. (2006) and Toffoli S, et al. (2009). Endothelial cells showed increased survival under proapoptotic stimuli and enhanced migratory ability and lumen formation in a HIF-1 α -dependent manner when exposed to intermittent hypoxia (0.5–1% O₂, 1 h hypoxia; 30 min reoxygenation, 3–4 cycles) (Martinive P, et al. 2006 and Toffoli S, et al. 2009). Cyclic hypoxia (8% O₂, 10 min hypoxia; 10 min reoxygenation, 12 cycles) increased angiogenesis and tumour perfusion in a VEGF-A-dependent manner, accompanied by increased vessel density (Kochan-Jamrozky K. 2019 and Gaustad JV. et al. 2013). However, liver cells exposed to intermittent hypoxia (1% O₂, 24 h hypoxia, 24 h reoxygenation, 3 cycles) expressed lower VEGF-A when compared to acute hypoxia (1% O₂, 48 h). Furthermore, endothelial cells in a conditioned medium showed less proliferation and tube formation (Dong G. et al. 2019). Intermittent hypoxia-induced angiogenic factors have been found to improve tissue repair and recovery (Viscor G. et al., 2018). It can be concluded that the angiogenic response of cells under intermittent hypoxia depends on the hypoxia-reoxygenation time scale and/or cell type. Further research is needed to better understand these factors and address the angiogenic response of cells under intermittent hypoxia.

EMT, invasion migration and metastasis in hypoxia-reoxygenation

Epithelial-mesenchymal transition (EMT) is a biological process that occurs in cells and can increase the ability of cancer cells to metastasise. This process suppresses epithelial characteristics, such as apicobasal polarity and cell-cell adhesion, while inducing mesenchymal characteristics, like invasion and migration. Usually, cancer cells use EMT to spread from the primary tumour, invade surrounding tissues, and migrate to distant sites where they undergo a mesenchymal-epithelial transition (MET) to develop secondary tumours (Celià-Terrassa T, Kang Y. 2016). The loss of E-cadherin and increased Vimentin are markers of EMT in cells (Nieto MA. 2013).

Under hypoxic conditions, HIF-1 α levels are upregulated, and it has been shown to elevate EMT and metastasis by directly regulating the expression of TWIST, a crucial EMT transcription factor. It has been found that HIF-1, TWIST, and SNAIL are co-expressed in the primary tumours of head and neck cancer patients, correlating with increased metastasis and poor prognosis (Yang MH. Et al. 2008).

EMT, invasion and migration can be influenced by intermittent hypoxia. MDA-MB-231 cells exposed to intermittent hypoxia (1% O₂, 12 h hypoxia, 12 h reoxygenation, 10 cycles) showed accumulation of HIF-1 α , which led to upregulated of vimentin and decreased cell proliferation. In addition, intermittent hypoxia led to increased migration of MDA-MB-231, which was dependent on the hypoxia-reoxygenation cycle's numbers (Liu L. et al. 2017). Pancreatic cancer cells under intermittent hypoxia (1% O₂, 12 h hypoxia, 12 h reoxygenation, 5 cycles) showed enhanced invasiveness ability in addition to stem-like cell characteristics and increased expression of HIF-1 α , Vimentin, E-cadherin, N-cadherin and autophagy-related proteins LC3-II and Beclin (Zhu H. et al. 2014). Autophagy is induced by increased levels of HIF-1 α , pancreatic cancer stem cells enhanced metastatic ability, and EMT associated with HIF-1 α and autophagy. Autophagy is a process where cells are responsible for their own protein and organelle delivery to the lysosome for degradation, in addition, autophagy facilitates ROS-altered mitochondria removal and oxidative stress reduction (Yang Z. et al. 2009 and Levine B. 2005). Hypoxia upregulates autophagy, enhancing cell survival and growth (Zhu H. et al. 2014). Medulloblastoma cells under intermittent hypoxia (1% O₂, 48 h hypoxia, 48 h reoxygenation, 18–20 cycles) showed increased EMT, invasion and migration when compared to normoxia. In

addition, the expression of several mesenchymal markers was induced under intermittent hypoxia, such as SNAIL, Vimentin and N-cadherin) and reduced expression of epithelial markers such as Zo-1, E-cadherin in medulloblastoma cells (Gupta R. et al. 2010). Invasive breast cancer phenotype is strongly promoted by intermittent hypoxia and reoxygenation compared to chronic hypoxia (Liu L. et al. 2017).

Metastasis can be affected by intermittent hypoxia, as evidenced in various studies. For instance, in KHT murine fibrosarcoma, intermittent hypoxia (2-7% breathing O₂, 10 min hypoxia, 10 min reoxygenation, 12 cycles, 7 days per week) led to an increase in lung metastasis compared to hypoxia (2-7% breathing O₂, 120 min continuous hypoxia, 7 days per week) (Cairns RA, et al. 2001). Similar results were observed in an in vitro study with human cervical cancer cells under intermittent hypoxia, where increased lymph node metastasis and reduced tumour growth were reported (Cairns RA, Hill RP. 2004).

In a recent study on the human breast cancer xenograft model, it was found that intermittent hypoxia (1% O₂, 24 h hypoxia, 24 h reoxygenation, 9 days) led to increased pro-tumorigenic cytokine secretion and higher expression of pro-metastatic genes, resulting in higher lung metastasis when compared to hypoxia (1% O₂, 9 days) (Chen A. et al. 2018). These studies underscore the role of intermittent hypoxia in promoting cancer cell metastasis when compared to hypoxia, whether acute or chronic. However, further research is needed to determine the role of HIF-1 α and/or HIF-2 α in initiating these phenotypes.

Metabolic shift in intermittent (cyclic) hypoxia

Despite the varying oxygen availability, cells exposed to intermittent hypoxia preferentially undertake glycolysis. This suggests that these cells develop resilience to the microenvironmental stress that they may encounter during metastasis (Chen A, et al. 2018). Under hypoxia, HIF-1 α is stabilised and plays a critical role in influencing the metabolic shift in tumours. Certain genes, such as GLUT1 and PDK1, are known to promote glycolysis and reduce oxidative phosphorylation, which enables cancer cells to generate sufficient energy despite the lack of oxygen (Zeng W. et al. 2015). The cellular metabolism response to intermittent hypoxia has been studied in obstructive sleep apnea. Various metabolic alterations have been

reported, such as lipid and glycolysis alteration under intermittent hypoxia (Ryan S. 2017 and Drager LF. et al. 2010). Intermittent hypoxia can induce dyslipidemia, an imbalance of lipids, by upregulating lipid biosynthesis in the liver, resulting in increased lipolysis in adipose tissue, with subsequent free fatty acid flux to the liver and lipoprotein clearance inhibition (Drager LF. et al. 2010). Furthermore, intermittent hypoxia can affect glucose metabolism and cause sympathetic activation, leading to increased counter-regulatory hormones and fatty acids, systemic inflammation, and direct injury to pancreatic beta-cells (Drager LF. et al. 2010). In breast cancer cells (MCF7), intermittent hypoxia has been found to increase the survival of cells and enhance the expression of 15 genes involved in the glycolysis pathway (Jarrar Y. et al. 2020). The metabolic shift observed in hypoxia also persisted in normoxia for breast cancer cells (MCF10) cultured in cyclic hypoxia over long time periods. Increased expression of HIF-1 α and GLUT1 was observed in normoxia following the hypoxic periods (Verduzco D. et al. 2015).

1.6.5 Therapy resistance in intermittent (cyclic) hypoxia

It is known that tumours undergo a process of reoxygenation after being treated with radiotherapy and certain types of chemotherapy (Dewhirst MW, Cao Y, Moeller B 2008). Therefore, studying intermittent hypoxia can provide a better understanding of the effects of chemotherapy and radiotherapy on cancer cells. Cyclic hypoxia can induce chemoresistance and radio-resistance in tumour cells, as observed in several studies (Table 1.4).

I. Chemotherapy in intermittent hypoxia

Exposure to cyclic hypoxia can cause chemotherapy resistance in cancer cells. For example, in glioblastoma multiform cells, cyclic hypoxia induces resistance to carmustine (BCNU) and doxorubicin through the efflux transporter ATP Binding Cassette Subfamily B Member 1 (ABCB1), which is upregulated by HIF-1 α (Chou CW. Et al. 2012). Similarly, in xenograft models, the tumour area exposed to cyclic hypoxia shows increased expression of ABCB1 and chemoresistance. Cyclic hypoxia also induces temozolomide (TMZ) resistance in the same cells by increasing ROS formation activating HIF-1 α and NF- κ B (Chen WL. et al. 2015). These studies suggest that blocking ROS or HIF-1 can be a promising strategy to overcome cyclic hypoxia-induced chemoresistance in cancer cells.

Cancer cells exposed to cyclic hypoxia can also respond to chemotherapy by enhancing their ability to resist apoptosis. Cyclic hypoxia, compared to chronic hypoxia, induces upregulation of anti-apoptotic gene B-cell lymphoma extra-large (Bcl-xL) in glioblastoma and medulloblastoma cell lines in a HIF-1-dependent manner (Gupta R. et al. 2011 and Chen WL. et al. 2015). Exposure of epithelial cells to cyclic hypoxia (50 cycles for 50 days) has been shown to change the expression of tumour suppressor genes and oncogenes such as HIF-1 α , E-cadherin and loss of p53, leading to increased cell survival compared to cells treated under normoxia with chemotherapeutic etoposide (Verduzco D. et al. 2015). These findings suggest that cancer cells exposed to cyclic hypoxia in early stages can select for stable and specific phenotypic and genotypic properties, which can even be maintained when normoxic conditions are restored, leading to enhanced tumour progression and therapy resistance.

II. Radiotherapy in intermittent hypoxia

Intermittent hypoxia can lead to cells acquiring radioresistance through HIF-1 α and signalling-dependent mechanisms or HIF-1 α independent mechanisms. For example, lung cancer cell lines exposed to intermittent hypoxia (0.1% O₂, 24-hour hypoxia, 72-hour reoxygenation, 20 cycles) showed increased radiation resistance that was dependent on HIF-1 α (Liu Y. et al. 2010). Increased levels of reactive oxygen species (ROS) were also found to be associated with HIF-1-induced radioresistance in tumor cells under cyclic hypoxia. In glioma cells, intermittent hypoxia increased radioresistance via ROS-induced HIF-1 α protein levels (Hsieh CH. et al. 2010). Glioblastoma cells exposed to intermittent hypoxia (0.5-1% O₂, 1-hour hypoxia, 30-minute reoxygenation, 3 cycles) showed greater radioresistance than those exposed to chronic hypoxia (0.5-1% O₂, 4-hour hypoxia) (Hsieh CH. et al. 2010). Glioma xenograft models exposed to intermittent hypoxia (7% O₂, 1-hour hypoxia, 30-minute reoxygenation, 3 cycles) also showed higher radioresistance compared to those exposed to chronic hypoxia (7% O₂, 4-hour hypoxia) (Hsieh CH. et al. 2010). In addition, higher levels of NOX4 (NADPH oxidase subunit 4), ROS, and radioresistance were observed in cells under cyclic hypoxia (Hsieh CH. et al. 2012). NOX4 was found to be an important radioresistance mediator in glioblastoma cell lines and tumour models exposed to cyclic hypoxia (0.5-1% O₂, 10-minute hypoxia, 10-minute reoxygenation, 12 cycles) (Hsieh CH. et al. 2012). In endothelial cells, cyclic hypoxia resulted in the nuclear accumulation of HIF-1 in response to reactive oxygen, which increased HIF-1-regulated cytokines that enhanced radioresistance (Moeller BJ. et al. 2004) (Table 1.4).

Intermittent hypoxia-induced radioresistance can be affected by mechanisms independent of HIF-1 α . Cyclic hypoxia increases ROS production as a mechanism to prevent resultant oxidative stress. Failure to do so could lead to radio-sensitization of cancer cells. Cancer cells can adapt to increased ROS levels produced during intermittent hypoxia through glutamic-oxaloacetic transaminase (GOT1) and mitochondrial citrate carrier (SLC25A1). Cyclic hypoxia-induced upregulation of GOT1 in lung cancer cells exposed to cycling hypoxia (<0.1% O₂, 48 h hypoxia, 120 h reoxygenation, 16 or 25 cycles) resulted in NADPH synthesis rewiring and regeneration of glutathione using glutamine, as reported in a study by Matschke J. et al. in 2016. Inhibition of GOT1 or glutamine deprivation led to reduced glutathione levels, increased ROS levels, and restoring cell death when cells were exposed to radiation (Matschke J. et al. 2016). SLC25A1 was also found to be upregulated under cyclic hypoxia (<1% O₂, 48 h hypoxia, 120 h reoxygenation, 16 and 25 cycles) and protected cells against high levels of ROS. Inhibition of SLC25A1 resulted in enhancing radio-sensitivity of cancer cells (Hlouschek J. et al. in 2018). It was found that radioresistance under cyclic hypoxia was mediated by PERK/eIF2 α signalling, which protected cells against ROS produced during cycling hypoxia periods by inducing cysteine uptake and glutathione synthesis, as reported by Rouschop KM. et al. in 2013. Meanwhile, HIF-1 did not appear to be essential in this process.

Cell Line/Mouse Model	Intermittent Hypoxia	Chronic Hypoxia	Effect of IH	IH vs CH	Reference
U251 and U87 ^{\$}	0.5–1% O ₂ , 1 h H; 30 min R; 3 cycles	0.5–1% O ₂ , 3 h	Resistance to temozolomide treatment mediated by Bcl-xL	Greater chemoresistance under IH	Chen W.-L. et al. 2015
U87 and GBM8401 ^{\$}	0.5–1% O ₂ , 1 h H, 30 min R, 3 cycles	1% O ₂ for 4 h	Resistance to doxorubicin and BCNU treatment mediated by ABCB1	Greater chemoresistance under IH	Chou C. et al. 2012
TLT xenograft model [#]	7% O ₂ , 1 h H, 30 min R, 3 cycles	7% O ₂ , 3 h	Increased radioresistance and tumor regrowth	Greater tumor cell and vasculature Radioresistance under IH	Martinive P. et al. 2006
Fsall and B16-F10 ^{\$}	<1% O ₂ , 1 h H, 30 min R, 3 cycles	NA	Increased radioresistance	NA	Martinive P. et al. 2006
A549 and NCI-H446 ^{\$}	0.1% O ₂ , 24 h H, 72 h R, 20 cycles	0.1% O ₂ , 16 h	Radioresistance promoted by increased S phase proliferation	Greater radioresistance shown by IH conditioned cells	Liu Y. et al. 2006
U87 cells ^{\$}	0.5–1% O ₂ , 1 h H, 30 min R, 3 cycles	0.5–1% O ₂ for 4 h	Increased radioresistance	Greater radioresistance shown by IH conditioned cells	Hsieh C.-H., et al. 2010
U87 xenograft with regulatable HIF-1 [#]	7% O ₂ , 1 h H, 30 min R, 3 cycles	7% O ₂ , 4 h	Increased radioresistance and tumor regrowth	Greater surviving fraction and tumor regrowth in mice treated with IH	Hsieh C.-H., et al. 2010
GBM8401 and U251 ^{\$}	0.5–1% O ₂ , 10 min H, 10 min R, 12 cycles	0.5–1% O ₂ , 4 h	Radioresistance mediated by NOX4	Greater NOX4 induction and ROS production under IH	Hsieh C.-H., et al. 2012
U373-MG and HCT116 ^{\$}	<0.02% O ₂ , 1 h H, 1 h R, 2–5 cycles	NA	Radioresistance mediated by PERK/eIF2α	NA	Rouschop K.M. et al. 2013
NCI-H460, DU145 and T98G ^{\$}	<1% O ₂ , 48 h H, 120 h R, 16 and 25 cycles	NA	Radioresistance mediated by SLC25A1	NA	Hlouschek J., Hansel C., Jendrosseck V., Matschke J. 2018
NCI-H460, DU145 and T98G ^{\$}	<1% O ₂ , 48 h H, 120 h R, 16 and 25 cycles	NA	Radioresistance mediated by SLC25A10	NA	Hlouschek J., et al. 2018
NCH-H460, DU145 and T98G ^{\$}	<0.1% O ₂ , 48 h H, 120 h R, 16 or 25 cycles	NA	Increased radioresistance mediated by GOT1	NA	Matschke J. et al. 2016

Table 1.4: Effect of intermittent hypoxia on cancer therapies. Adapted from (Saxena and Jolly 2019) (Copyright licence: CC BY 4.0 DEED). h—hour, H—hypoxia, R—reoxygenation, NA—not available, #—in vivo, \$—in vitro.

1.7 Project aims:

This study aims to investigate hypoxia-reoxygenation phenomena in breast cancer. I hypothesise that hypoxia-reoxygenation has an impact on gene expression, signalling, and cell phenotypes. This study will specifically focus on three main aims:

Aim 1

Investigate the transcriptomic changes under hypoxia and hypoxia-reoxygenation by:

- 1- Preparing RNAseq libraries in breast cancer tumours TNBC and Luminal A cell lines.
- 2- Use bioinformatic analysis tools to understand the molecular and biological functions of differentially expressed genes under hypoxia and hypoxia-reoxygenation conditions uniquely and for those that undergo changes in hypoxia and maintain that in reoxygenation.
- 3- Validate mRNA expression levels using qPCRs.

Aim 2

Investigate the changes in signalling under hypoxia and hypoxia-reoxygenation by:

- 1- Using screen arrays to examine the changes in phosphorylation of Receptor Tyrosine Kinases (RTKs) and intracellular kinases.
- 2- Further investigate phosphorylation status by western blotting.

Aim 3

Investigate whether hypoxia-reoxygenation is also associated with hypoxia-induced cell phenotype changes such as invasion and chemotherapy resistance by:

- 1- Performing invasion assay and MTS assay.
- 2- Examine whether kinase inhibitors are valuable for targeted therapy in breast cancer by assessing their ability to reduce migration and overcome chemotherapy resistance in breast cancer cells.

2.0 Materials and Methods

2.1 Cell Culture

2.1.1 Maintenance of the cells

Cell culture was performed under sterile conditions in a Class II Microbiological Safety Cabinet (BioMAT2, Contained Air Solutions, Manchester, UK). The following breast cancer cell lines used in this project were originally purchased from the American Type Culture Collection (ATCC): MDA-MB-231 and MCF7. The cells were cultured in a complete medium consisting of Dulbecco's modified Eagle's medium (DMEM- high glucose, Sigma Aldrich, MO, USA) supplemented with 10% [v/v] foetal bovine serum (FBS, Sigma Aldrich, MO, USA) unless stated otherwise.

For experiments involving the study of phosphorylated proteins, cells were serum starved in 0.5% FBS 24 hours prior to beginning the experiment. Cells were maintained at 37°C in a humidified incubator at 5% CO₂. Cells were routinely tested for mycoplasma using the Plasmotest™ mycoplasma detection kit (InvivoGen, CA, USA). Cells were authenticated by STR and profiled at regular intervals by eurofins.

Cells were authenticated with Eurofins Genomics Europe Applied Genomics GmbH, Anzinger Str. 7 a, D-85560 Ebersberg. The genetic characteristics were determined by PCR-single-locus technology. 16 independent PCR-systems D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, AMEL, D5S818, FGA, D19S433, vWA, TPOX and D18S51 were investigated. ASN-0002 core markers were included, Thermo Fisher, AmpFISTR® Identifiler® Plus PCR Amplification Kit. In parallel, positive, and negative controls were carried out, yielding correct results.

2.1.2 Passaging of Cell Lines

Cell lines were routinely sub-cultured when they reached approximately 70% confluence in a T75cm² flask (Corning, Fisher Scientific, MA, USA). Cells were passaged three times a week, and cell passage numbers were limited to no more than 15-18 passages. Culture media was aspirated, and cells were washed once with sterile phosphate-buffered saline (PBS, Sigma Aldrich, MO, USA). Cells were detached from the tissue culture flask using 1X Trypsin

Ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, MO, USA) for 3-5 minutes at 37°C in a 5% CO₂ humidified incubator.

Dissociation of cells was confirmed by microscopy, and the trypsin solution was quenched using the appropriate volume (2x volume of trypsin) of fresh complete media pre-warmed to 37°C to neutralise trypsin activity. Cells were then pelleted by centrifugation at 12,000 x g for 5 min. Pellets were resuspended in 10ml of complete media and typically re-seeded 1:10 into a new tissue culture flask made up to the appropriate volume of fresh complete media.

2.1.3 Hypoxic Culture Conditions

Hypoxia was induced by incubating cells at 1% O₂ in an *invivo2* 400/500 hypoxic workstation (Baker Ruskinn, South Wales, UK). CO₂ levels were maintained at 5% and the temperature at 37°C. The length of hypoxic incubation varied by experiment.

Physoxia was induced by incubating cells at 8.5% O₂ in 5% CO₂ and at 37°C in an *invivo2* 400 hypoxic workstation (Baker Ruskinn, South Wales, UK).

2.1.4 Hypoxia-Reoxygenation Culture Conditions

Hypoxia-reoxygenation was induced following two steps. Step 1, incubating cells at hypoxic conditions (1% O₂, 5% CO₂, 37°C) in an *invivo2* 400/500 hypoxic workstation (Baker Ruskinn, South Wales, UK). Step 2, cells were then moved to normoxic conditions or physoxic conditions, depending on the experiment. Normoxic condition (21% O₂, 5% CO₂, 37°C) humidified incubator. Physoxic condition (8.5% O₂, 5% CO₂, 37°C) in an *invivo2* 400/500 hypoxic workstation (Baker Ruskinn, South Wales, UK). The length of hypoxic, normoxic and physoxic incubation varied by experiment.

2.1.5 Cell Counting

Cell lines were trypsinised, detached from the flasks, pelleted by centrifugation and resuspended in a complete medium as previously described. Cells were counted using TC10™ automated cell counter (Bio-Rad, CA, USA) using Dual-chamber cell counting slides TC10™/TC20™ (Bio-Rad, CA, USA) according to the manufacturer's instruction. Briefly, 10 µL of cell suspension was transferred into both chambers on the cell counting slide and inserted into

the machine. After counting, the cells/ml reading was used to calculate the dilutions of cells required for further experiments.

2.1.6 Cryopreservation of Cell Lines

Cells were grown in the flask to 70% confluency, trypsinised, and pelleted by centrifugation as described above (2.1.2). Cells were counted as described previously (2.1.5), resuspended at a concentration of 1×10^6 cells per mL in freezing media (90% [v/v] FBS, 10% [v/v] dimethyl sulfoxide [DMSO] (Sigma Aldrich, MO, USA)). 0.5 mL of the cell suspension was transferred into individual cryovials (Brooks Life Sciences, Manchester, UK). Cryovials were inserted into freezing containers (Mr Frosty; Thermo Scientific, MA, USA) containing 100% isopropanol (Sigma-Aldrich, MO, USA) filled to the recommended level and frozen overnight at -80°C . The following day, frozen cryovials were transferred into a cryogenic storage system (Brooks Biostore™ III Cryo; Azenta Life Sciences, MA, USA) for long-term storage.

For the thawing of frozen cells, cryovials were incubated in a 37°C water bath for 1 minute. Cell suspensions were then transferred immediately into 15 mL falcon tubes (Cellstar®, Greiner Bio-one, Austria) containing 5 mL pre-warmed complete medium and pelleted by centrifugation as described above (2.1.2). The pellet was resuspended in 5 mL pre-warmed complete medium, transferred into a T25cm² flask, and maintained at 37°C in a 5% CO₂ humidified incubator.

2.2 Western Blot

2.2.1 Protein Extraction

Protein was extracted from cells cultured to 70% confluency in 10cm Petri dishes (Corning®, Thermo Fisher Scientific, MA, USA) under appropriate conditions. Dishes were removed from the cell culture incubator and immediately placed onto ice. Cell culture media was aspirated; cells were washed once with 5 mL ice-cold PBS or TBS for experiments studying phosphorylated proteins and then lysed in 100 μL radioimmunoprecipitation assay (RIPA) buffer (Cell Signalling Technology, MA, USA) containing 1x protease inhibitor cocktail (10 $\mu\text{L}/\text{mL}$) (Sigma Aldrich, MO, USA) and phosphatase inhibitor cocktail 3 (10 $\mu\text{L}/\text{mL}$) (Sigma Aldrich, MO, USA). Cells were scraped from the dish using a cell scraper (Corning®, Thermo Fisher Scientific, MA, USA), and lysate was collected into a 1.5ml Eppendorf tube (Starlab,

Germany). Cell lysates were homogenised by 1ml syringe and needle (21-gauge needle (Terumo)). Lysates were then cleared by centrifugation at 12,000 x g for 10 minutes in a 4°C centrifuge. The supernatant was then transferred to a fresh 1.5ml Eppendorf and stored at -80°C.

For HIF protein immunoblotting, cells were lysed in urea buffer (Appendix A) containing SDS, DTT and 1x protease inhibitor cocktail. Cells were collected homogenised, and lysates were cleared by centrifugation as described above. The supernatant was transferred to a fresh 1.5ml Eppendorf and stored at -80°C. For hypoxic conditions, cells were lysed inside the hypoxia chamber to prevent reoxygenation.

2.2.2 Protein Quantification

Bradford assay was used to quantify protein extracts. Briefly, a stock solution of bovine serum albumin 50 mg/mL (BSA (Lee BioSolutions, MO, USA) was produced by dissolving 0.5g BSA powder in 10 mL distilled H₂O. 80 µL of BSA stock solution was then dissolved in 920 µL PBS to produce a top working concentration of 4000 µg/mL. Serial dilutions were then performed using the working BSA solution to generate a series of standard concentrations: 2000 µg/mL, 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.625 µg/mL. PBS was used as a negative control. Experimental samples were diluted 1:10 in PBS. 10 µL of standard concentrations, and experimental samples were plated in triplicate in a 96-well plate (Falcon®, Corning®, Thermo Fisher Scientific, MA, USA). The Bradford assay dye reagent concentrate (Bio-Rad, CA, USA) was prepared by diluting 1:5 in distilled H₂O immediately prior to use. 200 µL of the assay dye was added to each well, the plate was read using an Infinite® F50 absorbance microplate reader (Tecan, Switzerland) at 620 nm, and data was collected using Magellan™ data analysis software. Raw data was exported to Microsoft Excel for additional analysis.

2.2.3 Sample Preparation

Samples were prepared for western blotting, typically using 40 µg of protein sample as calculated by the Bradford assay (2.1.2). The calculated volume of cell lysate was mixed with a working solution of 4x Laemmli sample buffer (Bio-Rad, CA, USA) containing 900 µl 4x Laemmli and 100 µl β-mercaptoethanol (Sigma Aldrich, MO, USA). The final volume was

produced by adding distilled H₂O to give a final concentration of 1x Laemmli. Samples were boiled at 95°C for 5 min and then centrifuged briefly to collect excess from the lid of tubes.

2.2.4 Gel Electrophoresis

Prepared samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) based on their size using an appropriate percentage of polyacrylamide gel (Table 2.1). 1.0 mm polyacrylamide gels were prepared using the Mini-PROTEAN Tetra Handcast system (Appendix) (Bio-Rad, CA, USA). Typically, a 4% stacking gel was used. The gels were assembled in a running module in the gel tank, which was then filled with 1X running buffer (Appendix). 4 µL of Precision Plus Protein™ Dual colour standards (Bio-Rad, CA, USA) were used to allow the identification of protein molecular weights. Gels were run initially at 80 volts until the ladder began to separate, after which the voltage was increased to 120 volts until the dye front ran out of the gel (approx. 120 min).

Protein Size	Gel Acrylamide Percentage
4-40 kDa	20%
12-45 kDa	15%
10-70 kDa	12%
15-100 kDa	10%
5-200 kDa	8%
> 200 kDa	6%

Table 2.1: Percentage of acrylamide required to produce different percentage resolving gels for the separation of different-sized proteins.

2.2.5 Gel Transfer

Separated proteins were transferred from the gel onto a nitrocellulose membrane (0.45 µm pore size, Bio-Rad, CA, USA) using the Trans-Blot® Turbo™ Transfer machine (Bio-Rad, CA, USA). the tissue stacks and nitrocellulose membrane were soaked in 1L of 1X transfer buffer (600ml of dH₂O, 200ml ethanol and 200ml of 5X transfer buffer) prior to assembly in the transfer cassette. Proteins were transferred on either a high molecular weight (1.3 amps, 25 volts, 12 min) or mixed molecular weight (1.3 amps, 25 volts, 7 min) transfer programme depending on the size of the target proteins.

2.2.6 Immunoblotting

Following the transfer, membranes were blocked in 5% BSA or 3% milk [w/v] (according to antibody recommendations) in 1X Tris-buffered saline (TBS) containing 0.1% Tween20 (TBS-T) (Sigma Aldrich, MO, USA) for 1 hour. The membranes were incubated with the appropriate primary antibody diluted as described in Table 2.2 at 4°C overnight with gentle rocking.

The following day, membranes were washed three times in TBS-T (0.1% [v/v]) for 5 minutes each on a rocking platform. The membranes were then incubated with the appropriate fluorescent secondary antibody at room temperature for 1 hour, protected from light with foil on a rocking platform. IRDye® 680RD and 800CW anti-rabbit, anti-mouse, and anti-goat IgG (LI-COR Biosciences, NE, USA) secondary antibodies were used, diluted 1:10,000 in either 5% BSA or 3% milk [w/v]-TBS-T solution. The membranes were washed three with TBS-T on a rocking platform for 5 min each and stored in 1xTBS before imaging using the Odyssey Fc imaging system (LI-COR Biosciences, NE, USA). β -actin was used as a loading control. Band densitometry analysis was performed using the ImageStudio Lite software (LI-COR Biosciences, NE, USA), and data was exported to Microsoft Excel for further analysis.

Protein	Company	Catalogue #	Dilution
HIF1 α	BD Biosciences	610959	1:500
HIF2 α	Cell Signalling Technology	7096S	1:500
Human ALK/CD246	R&D Systems, Inc.	MAB4210-SP	1 μ g/mL
Stat3 (79D7)	Cell Signalling Technology	4904	1:2000
Phospho-Stat3 (Tyr705)	Cell Signalling Technology	9131	1:1000
Akt (pan) (40D4)	Cell Signalling Technology	2920	1:2000
Phospho-Akt (Thr308) (244F9)	Cell Signalling Technology	4056	1:1000
Phospho-CREB (Ser133)	Cell Signalling Technology	9198	1:1000
HSP60 (D307)	Cell Signalling Technology	4870	1:1000
β -Catenin (D10A8) XP®	Cell Signalling Technology	8480	1:1000
β -Actin	Santa Cruz Biotechnology	sc-47778	1:1000

Table 2.2: Summary of the primary antibodies used in this study along with dilution factor.

2.3 Proteome Profiler Human Phospho-RTK Array

Proteome Profiler Human Phospho-RTK Array Kit (R&D Systems, MN, USA) was used to assess the phosphorylation of 49 human receptor tyrosine kinases simultaneously according to the

manufacturer's instructions. Briefly, 2×10^6 MDA-MB-231 cells were seeded in 10cm Petri dishes (Corning®, Thermo Fisher Scientific, MA, USA) and incubated at 37°C in a 5% CO₂ humidified incubator overnight for cell attachment. The following day, dishes were incubated under appropriate conditions (2.1). At the end of the experiment time, dishes were washed with PBS and lysed with 250µl Lysis Buffer 17 supplemented with a protease inhibitor cocktail (10µl/ml). Samples were agitated for 30 minutes at 4°C and then centrifuged at 14,000 x g at 4°C for 5 min. The supernatant was transferred to a fresh 1.5ml Eppendorf tube and quantified immediately using the Bradford assay previously described (2.2.2). Array membrane (1 per sample) was blocked in 2ml Array Buffer 1 in the multi-well dish provided for 1 hour at room temperature. 300µg of protein lysate was diluted in 1.5ml Array Buffer 1, 1.5ml sample was added to the membrane after aspiration of the block solution. The sample was incubated with the membranes overnight at 4°C on a rocking platform.

The following day, membranes were removed from the multi-well dish, and the membrane was washed three times in 20ml 1X Wash Buffer at room temperature for 10 minutes. The membrane was returned to the multi-well dish and incubated for 2 hours at room temperature with Detection Antibody diluted in 2ml 1X Array Buffer 2. The membrane was washed 3 times with 20ml 1X wash buffer for 10 minutes each. The membrane was aligned on a sheet of Parafilm and covered in 1ml of prepared Chemi Reagent mix for 1 minute. The membrane was imaged using the Odyssey FC Imaging System. Densitometry analysis was performed using ImageStudio Lite software (LI-COR Biosciences, NE, USA), and data was exported to Microsoft Excel for further analysis.

2.4 Proteome Profiler Human Phospho-Kinase Array

The Proteome Profiler Human Phospho-Kinase Array kit (R&D Systems, MN, USA) was used to assess protein phosphorylation of 37 human kinases and total amounts of 2 related proteins simultaneously across different conditions, according to the manufacturer's instructions. Briefly, 2×10^6 MDA-MB-231 cells were seeded in 10cm Petri dishes (Corning®, Thermo Fisher Scientific, MA, USA) and incubated at 37°C in a 5% CO₂ humidified incubator overnight for cell attachment. The following day, dishes were incubated under appropriate conditions (2.1). At the end of the experiment time, dishes were washed with PBS and lysed with 250µl Array Buffer 6 supplemented with a protease inhibitor cocktail (10µl/ml). Samples were agitated for 30 minutes at 4°C and then centrifuged at 14,000 x g at 4°C for 5 min. The supernatant was

transferred to a fresh 1.5ml Eppendorf tube and quantified immediately using the Bradford assay previously described (2.2.2). Part A and Part B membranes (1 pair per sample) were blocked in 1ml Array Buffer 1 in the multi-well dish provided for 1 hour at room temperature. 600µg of protein lysate was diluted in 2ml Array Buffer 1, and 1ml of sample was added to each Part A and Part B membrane after aspiration of the block solution. The sample was incubated with the membranes overnight at 4°C on a rocking platform.

The following day, membranes were removed from the multi-well dish, and both Part A and Part B were washed together three times in 20ml 1X Wash Buffer at room temperature for 10 minutes. Membranes were returned to the multi-well dish and incubated for 2 hours at room temperature with either Detection Antibody Cocktail A or Detection Antibody Cocktail B diluted in 1ml 1X Array Buffer 2/3. Membranes were washed separately 3 times with 20ml 1X wash buffer for 10 minutes each. Streptavidin-HRP was diluted 1:2000 in 1X Array Buffer 2/3, and membranes were incubated in 1ml of the reagent at room temperature for 30 minutes. Part A and Part B membranes were washed a final 3 times together for 10 minutes each in 20ml 1X Wash Buffer. Membranes were aligned on a sheet of Parafilm and covered in 1ml of prepared Chemi Reagent mix per membrane pair for 1 minute. Membranes were imaged using the Odyssey FC Imaging System. Densitometry analysis was performed using ImageStudio Lite software (LI-COR Biosciences, NE, USA), and data was exported to Microsoft Excel for further analysis.

2.5 RNA Extraction and Quantitative PCR

2.4.1 RNA Extraction

I. TRIzol RNA Isolation Reagents

Cells were cultured to approximately 70% confluency in 10cm Petri dishes (Corning®, Thermo Fisher Scientific, MA, USA) under appropriate conditions. Dishes were removed from the cell culture incubator or hypoxic chamber and placed onto ice. Cell culture medium was aspirated, and 1ml TRI Reagent® (Sigma Aldrich, MO, USA) was added per 10cm² dish and incubated at room temperature for 3-5 minutes. All TRI Reagent® was removed from the dishes and placed in a clean 1.5 mL Eppendorf tube. 200 µL of chloroform was added, and samples were mixed by vigorous shaking by hand for 30 s. Samples were centrifuged at 12,000 x g for 20 min in a 4°C centrifuge. 400 µL of the aqueous phase was removed and placed into a fresh 1.5 mL

Eppendorf tube containing 500 µL isopropanol. Samples were mixed thoroughly by shaking by hand and then incubated at - 80°C for 2 hours or overnight. Samples were centrifuged at 12,000 x g for 20 min at 4°C. The supernatant was removed from the tube, and pellets were washed with 800µl of 75% ethanol and centrifuged at 12,000 x g for 5 min. Ethanol was aspirated from the RNA pellet, which was left to air dry for a maximum of 10 min. The pellet was dissolved in 20 µL RNase-free water, incubated on ice for 20 min and stored at -80°C until required.

II. Qiagen RNeasy column

Cells were cultured to approximately 70% confluency in 10cm Petri dishes (Corning®, Thermo Fisher Scientific, MA, USA) under appropriate conditions. Cell culture medium was aspirated, and the dishes were washed once with ice-cold PBS, which was subsequently removed. RNA extraction was carried out using the RNeasy Mini kit (Qiagen, Germany). Briefly, 600 µL RLT lysis buffer supplemented with 10µl/ml BME was added to each dish. Cells were scraped from the dish using a cell scraper (Corning®, Thermo Fisher Scientific, MA, USA). Lysate was transferred to a 1.5ml Eppendorf tube and homogenised by passing through a 20-gauge needle (0.9 mm diameter) and syringe 10 times. All centrifugation steps were carried out at 12,000 x g. One volume of 70% ethanol was then added to the lysate and mixed by pipetting. The samples were then added to the RNeasy spin column, placed within a 2 mL collection tube and centrifuged for 15 seconds. The flow-through was discarded. 350 µL of wash buffer RW1 was added to the spin column, which was then centrifuged for another 15 seconds and the flow-through was discarded. On-column DNase digestion was performed by adding 10µl DNase I stock to 70µl Buffer RDD and adding the 80µl DNase I incubation mix directly onto the centre of the membrane, incubated for 15 minutes at room temperature. 350µl Buffer RW1 was added to each spin column, followed by centrifugation for 15 seconds, and the flow-through was discarded. 500µl Buffer RPE was added to each spin column, followed by centrifugation for 15 seconds, and the flow-through was discarded. This step was repeated with a 2-minute centrifugation to dry the column. The spin column was then placed into a clean 1.5 mL Eppendorf tube, and 30 µL of RNase-free water was added directly to the spin column membrane. The column was then centrifuged for 1 min to elute the RNA, and the samples were stored at -80°C until required.

2.5.2 RNA Quantification

RNA quantification was performed using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA) by measuring absorbance at 260 nm. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the RNA, with a ratio of >2.0 being classified as pure.

2.5.3 cDNA Synthesis

cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific, MA, USA). 2 µg of total RNA was calculated and diluted to a total of 10µl with RNase-free water per reaction. 10µl of cDNA Mastermix was added per sample for a final 20µl volume. The Reverse transcription Mastermix prepared was multiplied by the number of reactions required.

Component	Volume (µl)
10 x RT Buffer	2.0
25 x dNTP Mix (100mM)	0.8
10 X RT Random Primers	2.0
Ultra-Pure Water	4.2
MultiScribe Reverse Transcriptase	1.0
Total Volume for 1 Reaction	10

Table 2.3: Reverse transcription Mastermix components per sample.

Samples were placed into a Veriti® 96-well Thermal Cycler (Applied Biosystems™, Thermo Fisher Scientific, MA, USA), and the cDNA cycling programme was selected. Upon completion of the programme, samples were removed and diluted 1:10 by adding 180µl RNase-free water and stored at -20°C.

Step	Temperature (°C)	Time (Minutes)
1	25	10
2	37	120
3	85	5
4	4	-

Table 2.4: Cycling programme for the cDNA synthesis reaction.

2.5.4 Quantitative PCR

Quantitative PCR (qPCR) was performed using the Viia7 Real-Time PCR System (Applied Biosystems, Fisher Scientific, MA, USA) and reactions were prepared using the LightCycler® 480 SYBR Green I Master mix (Roche, Switzerland). A qPCR master mix was prepared for each pair of targets (Table 2.5), with β -actin used as a control housekeeping gene on each plate run, and molecular grade H₂O was used as a negative control. qPCR primers were purchased from Sigma Aldrich, and sequences are listed in the Appendix. All samples were run in triplicate in a 96-well (Applied Biosystems, Fisher Scientific, MA, USA).

Component	Volume (μ L)
Forward Primer (10 μ M)	1 μ l
Reverse Primer (10 μ M)	1 μ l
Ultra-Pure Water	6 μ l
SYBR Green master mix	10 μ l
cDNA	2 μ l
Total Volume for 1 Reaction	20μl

Table 2.5: qRT-PCR master mix components for qPCR reaction.

Plates were spun down and run on the Viia7 Real-Time PCR System. Programme details are shown in Table 2.6. Upon completion of the programme, the Ct values generated were exported to an Excel file for analysis by the Δ Ct method.

Step	Temperature ($^{\circ}$ C)	Time (Seconds)
Initial denaturation	95	30
40 cycles	95	15
	58	15
	72	10
Final hold	4	-

Table 2.6: Cycling programme for qRT-PCR.

2.6 RNA Sequencing

2.6.1 Sample Preparation

Cells were cultured to 70% confluency in 10cm Petri dishes (Corning®, Thermo Fisher Scientific, MA, USA) under appropriate conditions. RNA-sequencing samples were prepared using the RNeasy Mini kit (Qiagen, Germany) described previously (2.4.1), as suggested by Novogene, due to the enhanced purity and integrity of the RNA.

RNA purity was assessed as described in section 2.4.2. RNA integrity was assessed and quantified using an Agilent TapeStation automated electrophoresis workflow. RNA samples were then subjected to eukaryotic mRNA sequencing (Illumina 150PE, 20 million reads) by Novogene (UK).

2.6.2 Eukaryotic RNA-Seq library preparation and sequencing

RNA prepared above (2.5.1) was diluted to 200ng/μl and shipped to Novogene UK for their sequencing service. Prior to undergoing library preparation, additional quality control steps were carried out. RNA integrity and contamination were assessed by agarose gel electrophoresis. Assessment of RNA integrity (RIN) and RNA quantification was also performed using an Agilent 2100 bioanalyser. RNA contamination analysis was performed using a Nanodrop 2000.

Novogen sequencing service consisted of 150 bp paired-end RNA sequencing carried out on an Illumina NovaSeq 6000 S4 platform with 20 million reads per sample. Bioinformatics analysis was included in the service, with the Homo Sapiens (GRCh38/hg38) reference genome used.

2.6.3 Bioinformatic analysis

Raw RNA-Sequencing reads in FASTQ format were initially processed to ensure the quality of the sequencing. The following reads were eliminated to achieve clean reads: 1) Remove reads containing adapters, 2) Remove reads containing N > 10% (N represents base that could not be determined), 3) Remove low-quality reads: The Qscore (Quality value) of over 50% bases of the read is ≤ 5. The foundation for all downstream analysis was based on high-quality reads and clean data. For mapping clean reads to the reference genome or the transcriptome,

the HISAT2 software was used to align for RNA-seq sequencing data analysis to avoid losing a lot of effective junction reads.

Quantification of gene expression from raw RNA-sequencing counts was carried out using the read summarization program featureCounts (Liao Y. et al. 2014). DESeq2 package in R (version 1.36) were used for the differential expression analysis of three biological replicates per condition. Briefly, a negative binomial distribution model was used by DESeq2 to analyse raw RNA-sequencing counts and to perform differential expression hypothesis testing between groups using the Wald test. Wald test generated p-values which was corrected for multiple comparisons to reduce the number of false positives using the Benjamini-Hochberg method, this is to control the false discovery rate (FDR). This approach ranked genes based on their p-value, the p-value was then multiplied by m/rank where m = total number of tests. Genes with an adjusted p-value of <0.05 identified in DESeq2 were assigned as differentially expressed (Love, M.I. et al. 2014).

Gene ontology (GO) and gene set enrichment analyses (GSEA) were performed with an over-representation analysis (ORA) using WebGestalt. Statistically significant terms were defined by an FDR <0.05 (Liao Y. et al. 2019). Bubble plots were generated using SRplo (Tang D. et al. 2023). KEGG pathway analysis was also performed using GSEA software (Subramanian A. et al. 2005). Additional analyses were performed using the Protein Analysis Through Evolutionary Relationships (PANTHER) software for comparison (Mi H, Thomas P. 2009). Venn diagrams were generated using Venny 2.1 (Oliveros, J.C. 2007-2015). Gene expression analysis was performed using Breast Cancer Gene-Expression Miner v5.0 (bc-GenExMiner v5.0). The portal includes sixty-two breast cancer cohorts and one healthy breast cohort with their corresponding clinicopathological information. The Analyses are based on microarray or RNAseq transcriptomic data (Jézéquel P. et al. 2021).

2.7 Functional Assays

2.7.1 Compounds

Doxorubicin hydrochloride was kindly provided as a 3.6 mM solution by the pharmacy at Nottingham University City Hospital (Nottingham, UK) and stored at 4°C. Cisplatin was kindly provided as a 3.3 mM solution by the pharmacy at Nottingham University City Hospital

(Nottingham, UK) and stored at room temperature. ALK inhibitor Crizotinib was purchased from Cell Signalling (4401, Cell Signalling Technology, MA, USA). STAT3 Inhibitor III, WP1066, was purchased from Sigma Aldrich (573097-10MG, Sigma Aldrich, MO, USA). AKT inhibitor MK-2206 2HCl was purchased from Selleck Chemicals (S1078, Selleck Chemicals, USA). The compounds were suspended in 100% v/v dimethyl sulphoxide (DMSO) (276855-250ML, Sigma Aldrich, UK) at 10 mM and stored at -80 °C.

2.7.2 Cell viability

MTS assay was used to assess the cell viability using an MTS reagent (CellTiter 96® AQueous One Solution, Promega, UK), according to the manufacturer's instructions. Typically, cells were first cultured in the appropriate conditions and then seeded in appropriate amounts in complete media into 96-well plates. The plate was incubated at 37°C in a humidified incubator at 5% CO₂ for 24 hours prior to allow cells to adhere. The following day, drugs were added at various concentrations in triplicate wells, and the cells were incubated at (37°C, 5% CO₂) for the required time. On the detection day, 20 µL of MTS reagent was added to each well, and the plate was incubated in the dark for 1 to 4 hours at 37 °C. A FLUOstar Optima plate reader was employed to measure absorbance at 490 nm due to formazan. Data was exported to Microsoft Excel for further analysis. The analysis normalised the average readings for treated cells and average readings of control cells. Survival curves were plotted, and the differences between samples were calculated using the Student's t-test with GraphPad Prism (version 7).

2.7.3 Invasion assay

Cells were cultured in a T75cm² flask (Corning, Fisher Scientific, MA, USA) at the appropriate conditions. On day 1 of the invasion assay, complete culture media was aspirated, and starvation media (supplemented with 2% [v/v] foetal bovine serum FBS) was added. Cells were starved for 24 hours incubated at a humidified incubator (37°C, 5% CO₂). On day 2, 24 Well ThinCert® Cell Culture Inserts (8.0µm pore diameter, 662638, Greiner Bio-One, Kremsmünster Austria) were prepared by placing them in their respective wells in the provided plate. Inserts were coated with Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (A1413201, Thermo Scientific, MA, USA) diluted in ice-cold free media to 2mg/ml, and 200µl were added per insert to create a uniformed layer. The plate was

then incubated at (37°C, 5% CO₂) for 2 hours. 150µl were aspirated, and inserts were washed gently with PBS by aspiration. 100µl of 2% FBS medium supplemented with Hydroxyurea (1mM final concentration) was added per insert. The starved cells from day one were then harvested and counted as described previously (2.1.2 and 2.1.5). The cells were spun down by centrifugation, and the pellets were then resuspended in 1mL 2% FBS medium plus Hydroxyurea. A 100µl from cell suspension was then added per insert. The inserts were then transferred to their respective well and topped with 600µl medium. This was incubated at 37°C, 5% CO₂) for 24 hours. On day 3, the medium was aspirated from the plate; inserts were washed with PBS. Inserts were then transferred to a new plate prepared with 4% FA for fixation and incubated for 15 minutes at room temperature. Following the incubation time, inserts were washed with PBS and transferred to a new plate prepared with 500µl DAPI (1mg/ml, 1:100 in PBS) and incubated, protected from light at room temperature. The inserts were washed with PBS, and a scalp was used to cut the membrane from the insert. The membranes were placed on Superfrost® microscope slides (Z692255, Sigma Aldrich, MO, USA) (basal facing up) and topped with 10µl Epredia™ Immu-Mount™ (9990402, Thermo Scientific, MA, USA) medium, which was then covered with a coverslip (19mm). This was imaged immediately with a fluorescent microscope to count the number of migrated cells. Images were exported to Fiji image processing software (Version 2.1.0/1.53) for further analysis (Schindelin J. et al. 2012). The images were then analysed, and data were exported to Microsoft Excel for further analysis. GraphPad Prism (version 7) was used for Statistical analysis using an Unpaired Student-test.

2.7.4 Migration assay

Migration assay were used to analyse cell migration under different conditions. Cells were first cultured in the appropriate conditions with a complete medium and then harvested and counted as described previously (2.1.2 and 2.1.5). Cells suspension containing an appropriate amount of the cells was then seeded into Culture-Insert purchased from ibidi (2 Well in µ-Dish 35 mm, 81176, IBIDI GMBH, Gräfelfing, Germany), according to the manufacturer's instructions. This was incubated at 37°C in a humidified incubator at 5% CO₂ for 24 hours to allow the cell to adhere. The following day, culture inserts were removed, and medium supplemented with 1% [v/v] foetal bovine serum and the desired concentration of the kinase

inhibitor or DMSO for the vehicle control were added. The wound closure was observed under a microscope (Nikon Eclipse Ti) using NIS-elements imaging software (Nikon, NY, USA) at different times (0, 16, 24, and 48 hours). Images were exported to Fiji image processing software (Version 2.1.0/1.53) for further analysis (Schindelin J. et al. 2012). The images were then analysed, and data were exported to Microsoft Excel for further analysis. GraphPad Prism (version 7) was used for Statistical analysis using a two-way analysis of Variance (ANOVA).

2.7.5 Statistical analysis

The results are presented as the mean $\pm$ standard error of the mean (SEM) for continuous variables. The statistical analysis was performed using GraphPad Prism (version 7) software (GraphPad Software, San Diego, CA, USA). Differences between groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Student's unpaired t-test was used to determine the significant differences between the two groups. A p-value < 0.05 was considered statistically significant.

3.0 Investigating the transcriptomic profile of breast cancer cells under Hypoxia and Hypoxia-Reoxygenation.

3.1 Introduction

RNAseq was invented over a decade ago and its application has enabled more accurate analysis of diseases that have limited approaches and analysis such as cancer. As medical professions are now leading toward higher effective treatments with fewer side effects and advanced targeted therapy, RNAseq has improved the treatment design as it can help scientists detect the resistant genes against currently available therapies. Therefore, studying the transcriptome is vital for understanding the function of the genome, identifying the molecular structure of the cell, and gaining insight into the cause and development of the disease (Wang B, Kumar V, Olson A, Ware D. 2019). Recent years have witnessed advancements in medicine, yet more research is still needed for cancer as it is considered one of the most life-threatening diseases. Development in the RNAseq has assisted in advancements in targeted therapies by detecting gene mutation in numerous types of cancer such as head and neck cancer (Qi Z, Barrett T, Parikh AS, Tirosh I, Puram SV. 2019), acute myeloid leukaemia (AML) (Yu J, et al. 2020) and oligodendroglioma (Puram SV, et al. 2017).

RNA sequencing has helped in shaping the hypoxic gene signature future in many tumours. In a study of the hypoxic transcriptional profile of breast cancer cells, a large-scale RNA sequencing of 31 breast cancer cell lines representing breast cancer subtypes and normal mammary epithelial was used to identify 42-gene expression signatures under hypoxia. Not only this, but the RNAseq analysis has also identified 4 novel hypoxia-regulated and HIF-1 responsive genes, including Fucosyltransferase 11 (FUT11), Dynein axonemal heavy chain 11 (DNAH11), TRPM8 channel-associated factor 2 (TCAF2), and Caspase-14 (CASP14) (Ye IC, Fertig EJ, DiGiacomo JW, Considine M, Godet I, Gilkes DM. 2018). Other examples of hypoxic gene signatures generated by RNAseq can be seen in, lung adenocarcinoma with a 29-hypoxic gene signature (Lane B, et al. 2022) and in Soft Tissue Sarcoma (STS) with a 24-hypoxia signature (Yang L, et al. 2019). These studies present robust hypoxic signatures that can be used as prognostication and can support the identification of patients for hypoxia-targeted therapies in clinical trials.

3.2 Hypothesis and Aims

Hypoxia induces changes in gene expression levels, affecting the transcription of genes involved in multiple processes such as signalling, metabolism, angiogenesis, metastasis, invasion, and therapy resistance (Semenza 2013). The effect of hypoxia-reoxygenation on gene expression levels and its mechanism in breast cancer is under investigation.

I hypothesise that hypoxia-induced gene expression changes are maintained upon hypoxia-reoxygenation in breast tumour cells, which can be reflected in the RNAseq data. In addition, I hypothesise that a possible mechanism for hypoxia-reoxygenation to maintain hypoxia-induced gene expression changes is through a positive feedback loop from growth factors signalling, which can be seen in gene expression levels of growth factors in the RNAseq data. The maintaining of hypoxia-induced gene expression in hypoxia-reoxygenation can be associated with poor prognosis in breast cancer patients and aggressiveness phenotypes.

To test this hypothesis, two RNAseq data, MDA-MB-231 and MCF7, will be analysed for the following aims:

- 1) Analyse the pathway enrichment of the differentially expressed genes (DEGs) in hypoxia and hypoxia-reoxygenation.
- 2) Identify the upregulated differentially expressed genes and pathways in hypoxia that are maintained upon hypoxia-reoxygenation (overlapped DEGs).

For this specific group (overlapped DEGs), additional analysis will be performed to:

- Investigate gene enrichment and leading genes in the data.
 - Pathway analysis to identify genes that are involved in signalling and transcription regulators.
 - Investigate and compare the expression of top-upregulated overlapped DEGs in healthy, tumour-adjacent, non-TNBC, TNBC, HER2(-ve) and HER2(+ve) tissue.
 - Investigate prognostic associations of top-upregulated overlapped DEGs in TNBC and Luminal A using METABRIC and RNA-sequencing data.
 - Validate the mRNA levels of top-upregulated overlapped DEGs via qPCR.
- 3) Identify the upregulated growth factors in hypoxia and hypoxia-reoxygenation.
 - 4) Validate the mRNA levels of growth factors via qPCR.

3.3 Results

3.3.1 Transcriptomic profile of TNBC and Luminal A cells under Hypoxia and Hypoxia-reoxygenation.

To investigate the TNBC and luminal A transcriptomic profile MDA-MB-231 cell line and MCF7 cell line were used. MDA-MB-231 cells were incubated in normoxia (72 hours 21% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 21% O₂) prior to RNA extraction as described in the Methods (Figure 3.1 A). MCF7 were incubated under physiological oxygen levels (8.5% O₂), representing the oxygen levels within human breast tissue (Vaupel P, Höckel M, Mayer A (2007)). Cells were incubated in physoxia (72 hours 8.5% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂).

From both cell lines, the total RNA extracts were prepared for RNA sequencing from three independent experimental repeats. Before sending for sequencing, the total RNA extracts were tested for purity and degradation in line with Novogene's sample requirements. The 260/280 ratio was obtained using Nanodrop, with a ratio of >1.8 indicating the absence of protein or DNA contamination; all RNA samples had a 260/280 ratio of > 2.0, reflecting the purity of the RNA samples for downstream applications (Table 3.2).

RNA integrity was assisted using the Agilent Bioanalyser and TapeStation System by providing RNA samples (200ng/μl) to the Deep-Seq facility (University of Nottingham). For each sample, an RNA Integrity Number (RIN) was obtained ranging from 1-10 with 1 representing the most degraded RNA profile and 10 representing the most intact RNA profile and a RIN value of >4 is considered acceptable for sequencing. All RNA samples had a RIN of >9 (Table 3.1 and Table 3.2).

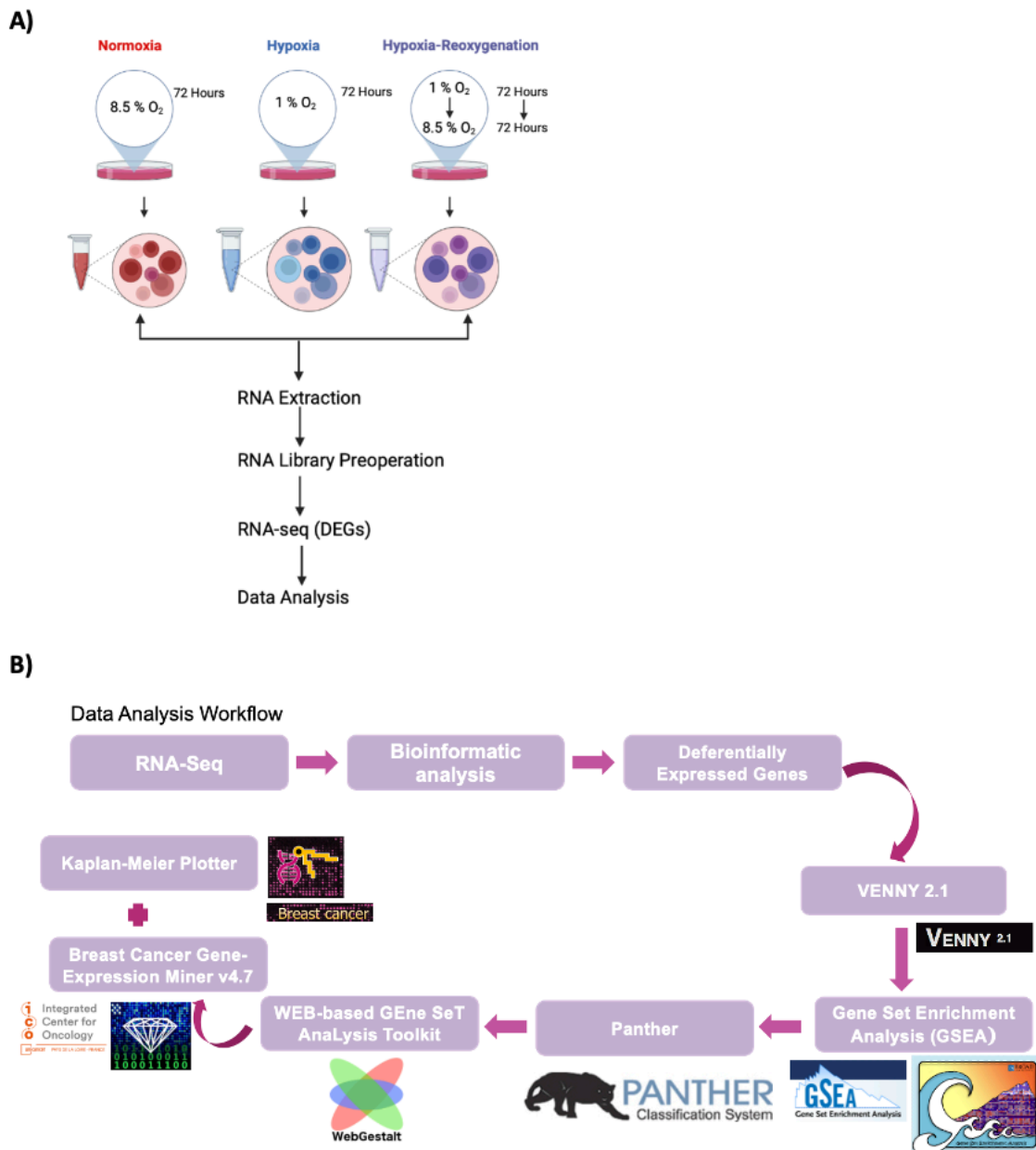


Figure 3.1 Schematic diagram illustrating the experimental approaches used to generate and analyse the RNAseq data. A) Sample preparation to Data analysis, **MDA-MB-231** cells were subjected to normoxia (72 hours 21% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 21% O₂). **MCF7** cells were subjected to physiological-normoxia (72 hours 8.5% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂). **B)** Bioinformatic workflow data analysis, The RNAseq data produced were analysed using accessible software tools. Figure created in BioRender.

Well	Sample Description	RIN
A1	Ladder	-
B1	Normoxia R1	10.0
C1	Hypoxia R1	9.9
D1	Reoxygenation R1	9.9
E1	Normoxia R2	10.0
F1	Hypoxia R2	10.0
G1	Reoxygenation R2	10.0
H1	Normoxia R3	10.0
A2	Hypoxia R3	9.8
B2	Reoxygenation R3	10.0

Table 3.1 RNA purity and integrity values of MDA-MB-231 samples submitted for RNA-sequencing.

Sample Description	260/280 Ratio	RIN
NOR2 Normoxia	2.09	9.7
NOR3 Normoxia	2.09	9.5
NOR4 Normoxia	2.08	9.6
HYP2 Hypoxia	2.07	10.0
HYP3 Hypoxia	2.1	9.8
HYP4 Hypoxia	2.08	9.9
REOX2 Hypoxia-Reoxygenation	2.1	9.9
REOX3 Hypoxia-Reoxygenation	2.06	9.7
REOX4 Hypoxia-Reoxygenation	2.09	9.5

Table 3.2: RNA purity and integrity values of the MCF7 samples submitted for RNA sequencing.

RNA sequencing checks

RNA samples were analysed at the Deep-Seq facility (University of Nottingham), and an RNA gel electrophoresis image was obtained as a pre-analysis step to check the quality of the RNA samples and to show there was no degradation within the samples before they were sent for sequencing. The RNA gel electrophoresis image showed two distinct bands corresponding to the 18S and 28S ribosomal RNA (rRNA) subunits (Figure 3.2A and Figure 3.3A). The RNA samples showed two complete, clear, and intense rRNA bands where the intensity of the 28S rRNA band (upper band) was stronger than the intensity of the 18S rRNA band (lower band). This result without smearing indicated a good quality of the RNA samples.

Another pre-analysis step was to check that the level of hypoxia was consistent among experimental repeats and that is by analysing the RNA samples by qPCR. Following cDNA synthesis (explained in the Methods), qPCR was performed using hypoxia-inducible genes CA9, BNIP3, HIG2 and VEGF along with glycolytic gene LDHA (Turner KJ, et al 2002) (Figure 3.2B and Figure 3.3B). Studies showed that under hypoxia, glycolysis levels are increased when HIF-1 α is stabilised by mitochondrial ROS or oxygen deprivation this can include an increase in glycolytic genes such as LDHA, which is a key checkpoint of anaerobic glycolysis (Lum JJ, et al 2007). Recent studies also showed that HIF1/2 α could activate LDHA expression in human pancreatic cancer (PC) cells, elevating the growth and migration of PC cells (Cui XG, et al 2017).

In the MDA-MB-231 cell line, CA9 was significantly upregulated by over 20-fold compared to normoxia ($p < 0.01$, $n = 3$) (Figure 3.2B). In the MCF7 cell line, all three targets had significantly high expression levels under hypoxia with CA9 showing the highest expression levels in hypoxia with 61.4 Log₂Fold change ($p < 0.01$, $n = 3$) followed by BNIP3 with 5.2 Log₂Fold change ($p < 0.0001$, $n = 3$) and finally HIG2 with 2.1 Log₂Fold change ($p < 0.0001$, $n = 3$) (Figure 3.3B-D).

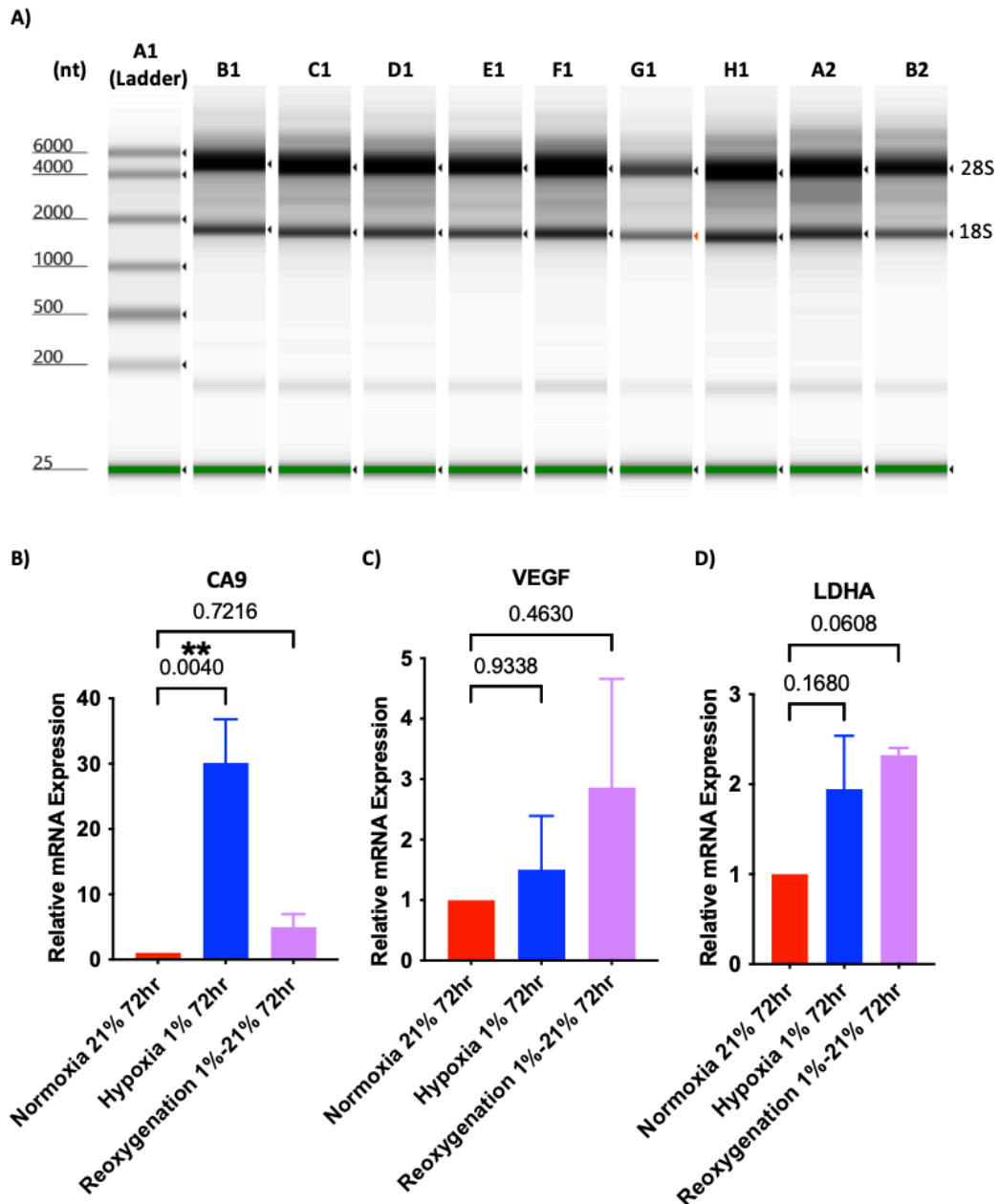


Figure 3.2: Validation of MDA-MB-231 RNA samples prior to RNA-sequencing. RNA samples were exposed to normoxia (72 hours 21% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 21% O₂). **A)** RNA gel electrophoresis of RNA samples representing two clear rRNA bands. **B)** CA9, VEGF and LDHA mRNA expression in the RNA samples under hypoxia and hypoxia-reoxygenation compared to normoxia. Error bars indicate $\pm$ SEM. Data analysed using a one-way ANOVA. ** $p < 0.01$. $n = 3$. (Christopher Paul Carroll of the McIntyre lab generated the MDA-MB-231 cell line RNAseq samples and performed the qPCR in this figure).

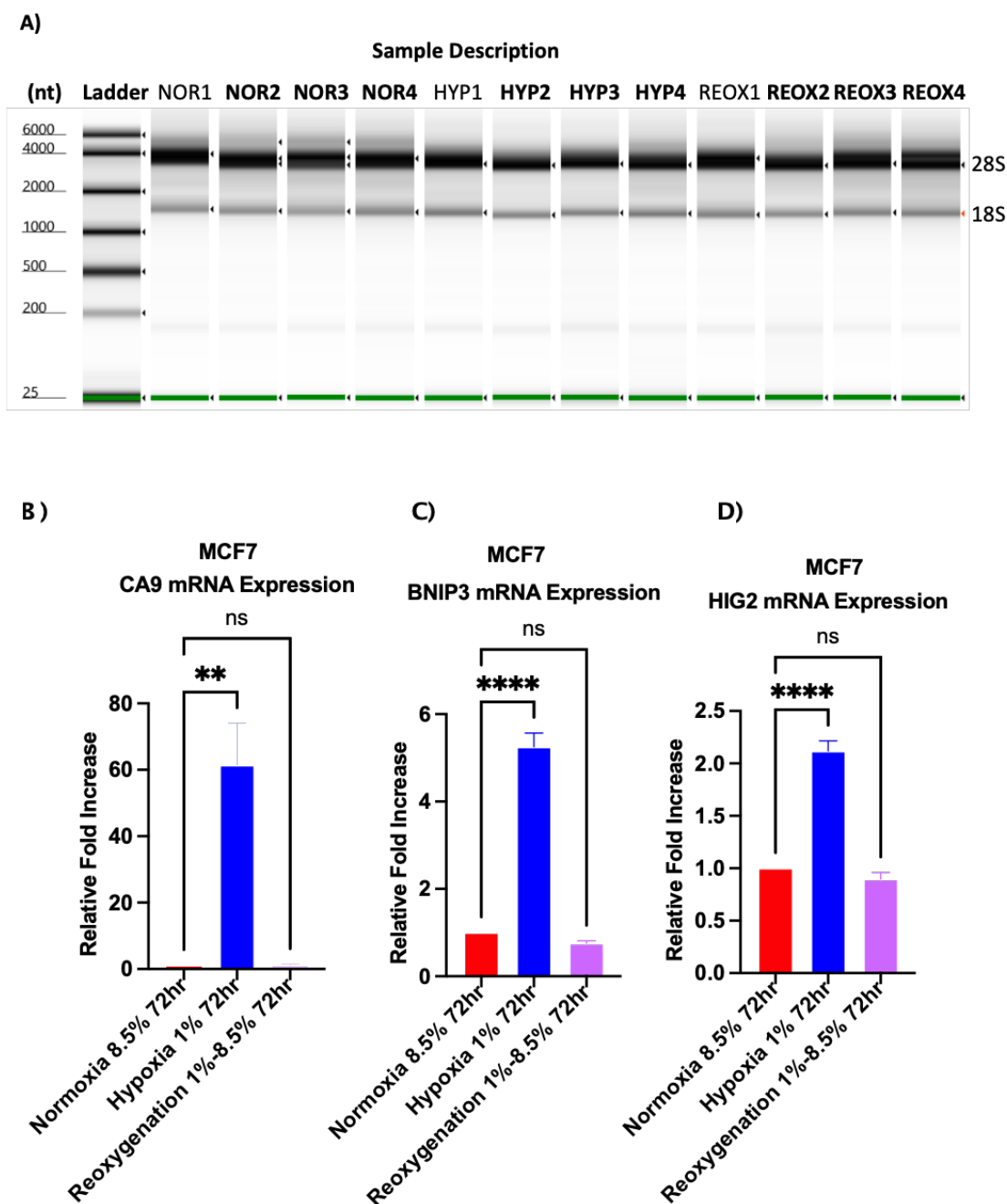


Figure 3.3 Validation of MCF7 RNA samples prior to RNA-sequencing. RNA samples were exposed to physoxia (72 hours 8.5% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂) **A)** RNA gel electrophoresis of RNA samples representing two clear rRNA bands. **B)** CA9, BNIP3 and HIG2 mRNA expression in RNA samples under hypoxia and hypoxia-reoxygenation compared to normoxia. Error bars indicate $\pm$ SEM. Statistical analysis using one-way ANOVA. ** $p < 0.01$, **** $p < 0.0001$. $n = 3$

RNA samples were sent for analysis at Novogene for sequencing, and it was essential to run quality control (QC) checks before downstream analysis. This is because any quality issues with the RNAseq data can significantly influence the analytical results and lead to an inaccurate conclusion. The QC included checks for sequencing error rate distribution, checks for A/G/C/T distribution and sequence data filtering. Gene expression level analysis (FPKM) and RNAseq correlation are illustrated in (Figure 3.4 and Figure 3.5)

Figure 3.4A and Figure 3.5A compared gene expression levels in different conditions (normoxia, hypoxia and hypoxia-reoxygenation) using the FPKM (short for the expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced) method. This method considers both sequencing depth and gene length in the counting of fragments. In the RNAseq experiment, the gene expression level is estimated by the abundance of transcripts (count of sequencing) that are mapped to the genome or exon. The gene expression levels were approximately the same across all MDA-MB-231 and MCF7 RNAseq samples, indicating that hypoxia and hypoxia-reoxygenation conditions did not influence the overall gene expression levels (Figure 3.4A and Figure 3.5A)

The Pearson correlation matrix between samples is demonstrated in (Figure 3.4B and Figure 3.5B). Correlation of the gene expression levels between samples is an important indicator to verify reliability and sample selection, it is used to demonstrate the experiment is repeatable and for the differential gene expression analysis. The correlation coefficient is closer to 1, the higher the similarity of the samples is. All RNAseq samples had a correlation coefficient above 0.94, indicating a strong positive correlation between samples and the absence of outliers (Figure 3.4B and Figure 3.5B).

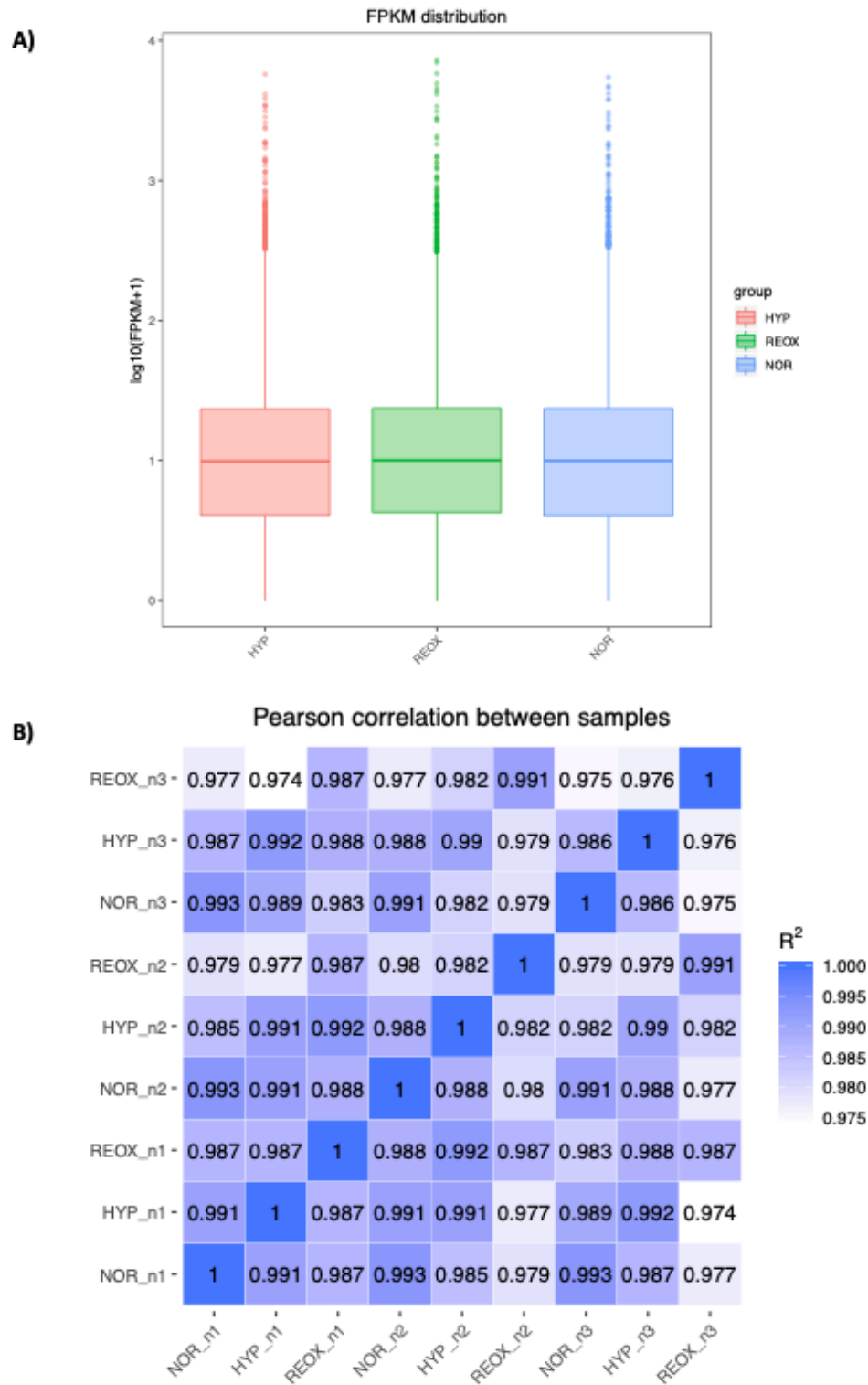


Figure 3.4 RNA-sequencing gene expression distributions and sample correlation in MDA-MB-231 RNAseq. A) Violin plot illustrating the gene expression levels in different conditions. The horizontal axis indicates the sample names, the vertical coordinate indicates the $\log_{10}(\text{FPKM}+1)$, and each violin has 5 statistical magnitudes (max value, upper quartile, median, lower quartile, and min value). **B)** Pearson correlation matrix displaying correlation coefficient values for each of the 9 samples. R^2 : The square of the Pearson coefficient.

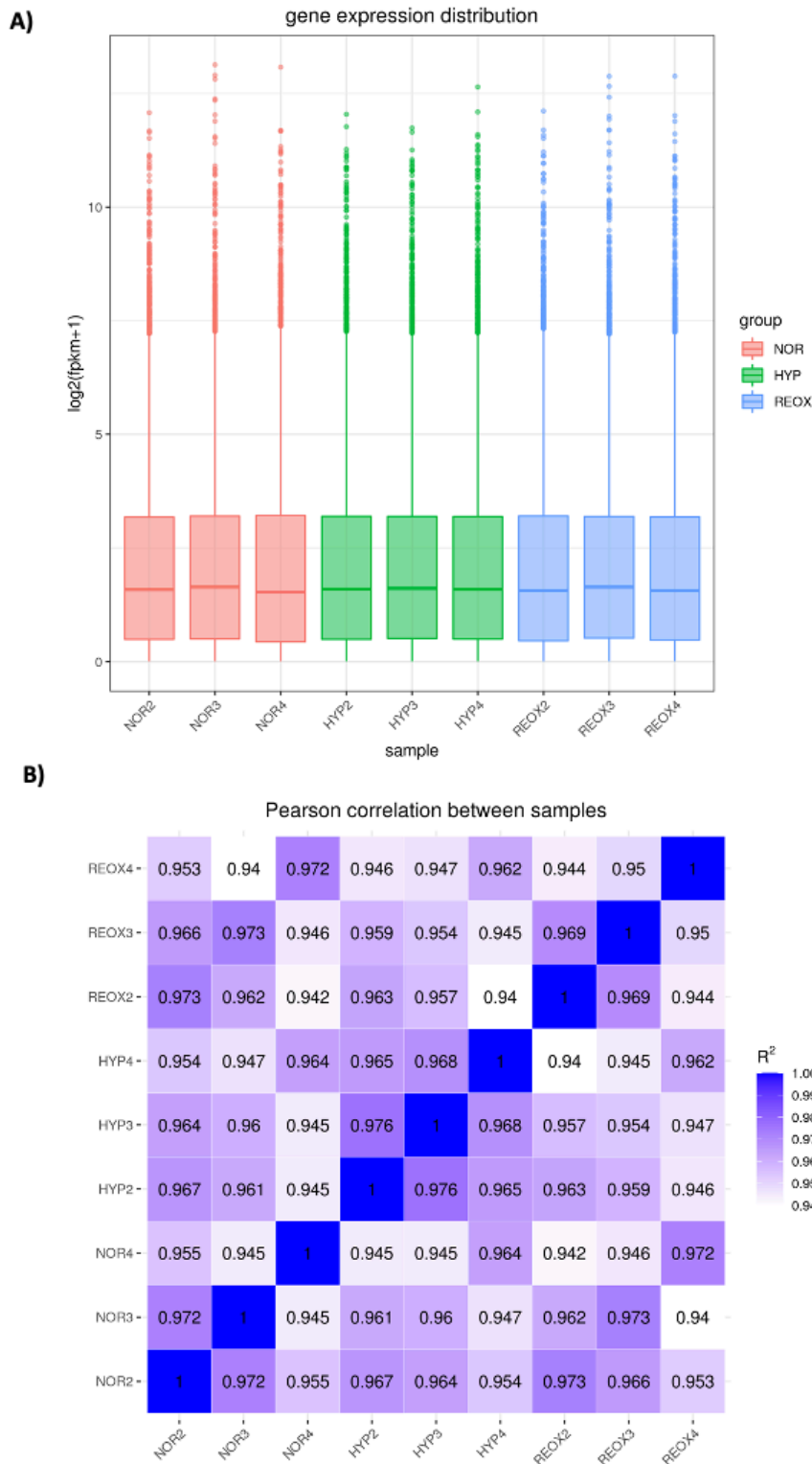


Figure 3.5 Displays MCF7 RNAseq's gene expression levels and sample correlation via RNA-sequencing. A) The violin plot shows gene expression under different conditions. The horizontal axis shows the sample names, the vertical axis shows the $\text{Log}_{10}(\text{FPKM}+1)$, (max value, upper quartile, median, lower quartile, and min value). **B)** The Pearson correlation matrix shows correlation coefficient values for each of the 9 samples. R^2 : The square of the Pearson coefficient.

Cluster Analysis of Differentially Expressed Genes

The cluster analysis (hierarchical clustering) of differential expression genes is shown in (Figure 3.6 and Figure 3.7). The cluster analysis of differential expression genes was used to evaluate the expressed pattern of differential expression genes under different experimental conditions based on their FPKM values. This method can recognise the unknown function of transcripts or unknown genes, showing that the same kind of transcripts have similar functions or participate in the same metabolic process or cellular pathways (Vidman et al, 2019). The horizontal axis indicates the experiment conditions while the vertical coordinate indicates the genes and the colours represent gene expression levels from blue (low read counts) to red (high read counts) in the MDA-MB-231 cell line (Figure 3.6) and from green (low read counts) to red (high read counts) in MCF7 cell line using $\log_{10}(\text{FPKM}+1)$ value.

The clustering included hypoxia with normoxia, hypoxia-reoxygenation with normoxia, and hypoxia-reoxygenation with hypoxia. In MDA-MB-231 RNAseq, the analysis of differentially expressed genes within hypoxia showed significant upregulation of the following hypoxia-inducible genes and adjusted p-values; CA9 ($p=0.00000000147$, $n=3$), LDHA ($p=4.38\text{E-}37$, $n=3$), BNIB3 ($p=1.03\text{E-}35$, $n=3$) and VEGFs: VEGFA ($p=0.00000898$, $n=3$), and VEGFC ($p=0.000000194$, $n=3$). VEGFs were also significantly upregulated in hypoxia-reoxygenation, VEGFA ($p=0.00000015043$, $n=3$), VEGFB ($p=0.000016522$, $n=3$), and VEGFC ($p=0.0000000000074835$, $n=3$). Interestingly, HIF1A was also significantly upregulated in hypoxia-reoxygenation ($p=0.0015004$, $n=3$) (Figure 3.6).

In MCF7 RNAseq, the analysis of DEGs within hypoxia showed significant upregulation of several hypoxia-inducible genes and the adjusted p-values; BNIP3P1 ($\text{Log}_2\text{Fold Chang}=2.7997682060125$, $p\text{-value}=0.008$, $n=3$) and CA9 ($\text{Log}_2\text{Fold Chang}=5.64892487019746$, $p=3.97144962641135\text{E-}17$, $n=3$). HIF1A-AS2 was also found to be upregulated under hypoxia ($\text{Log}_2\text{Fold Chang}=3.78033278108945$, $p=5.32945969938588\text{E-}08$). EGLN3 which is another hypoxia-inducible gene was found to be upregulated under hypoxia-reoxygenation ($\text{Log}_2\text{FoldChang}=1.23440997353089$, $p=0.0000337624923997256$, $n=3$) (Figure 3.7).

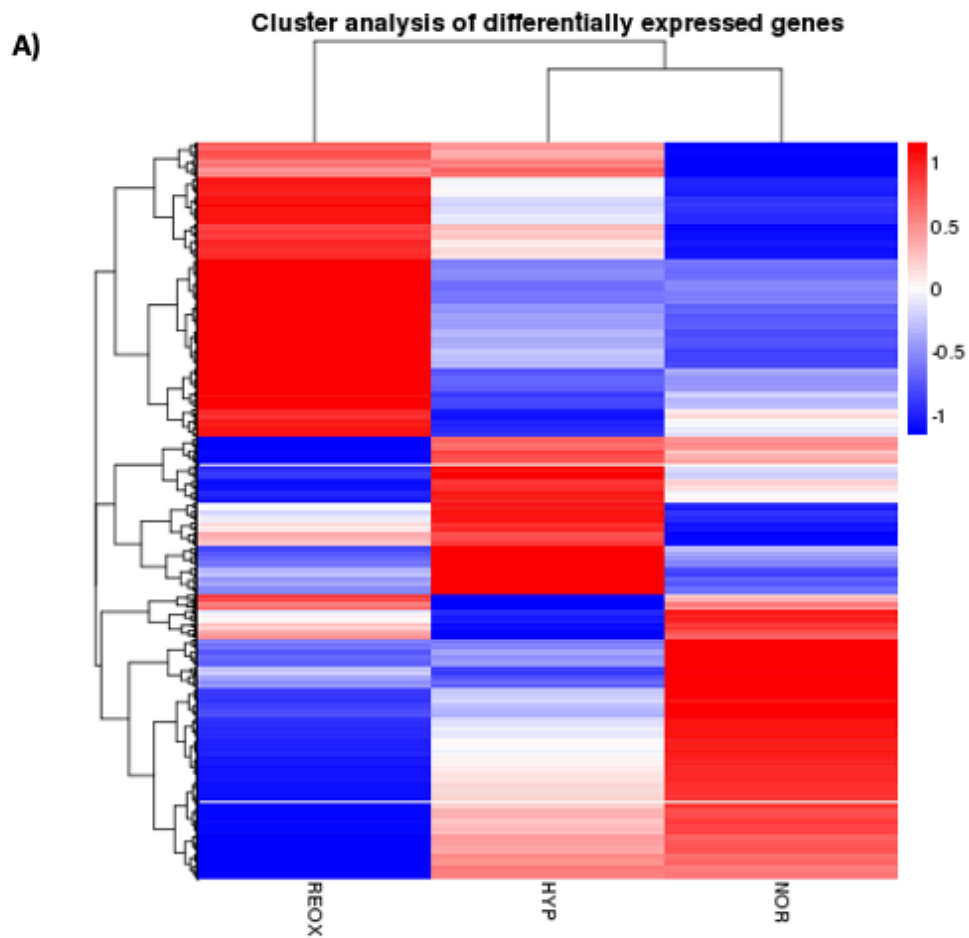


Figure 3.6 Shows the results of a Hierarchical Cluster analysis of differentially expressed genes in the MDA-MB-231 RNAseq conditions. The $\text{Log}_{10}(\text{FPKM}+1)$ value was used to cluster differential gene expression. The colours represent gene expression levels from blue (low read counts) to red (high read counts). The three conditions are NOR= normoxia (72 hours 21% O_2), HYP= hypoxia (72 hours 1% O_2) and REOX= hypoxia-reoxygenation (72 hours 1% O_2 -72 hours 21% O_2).

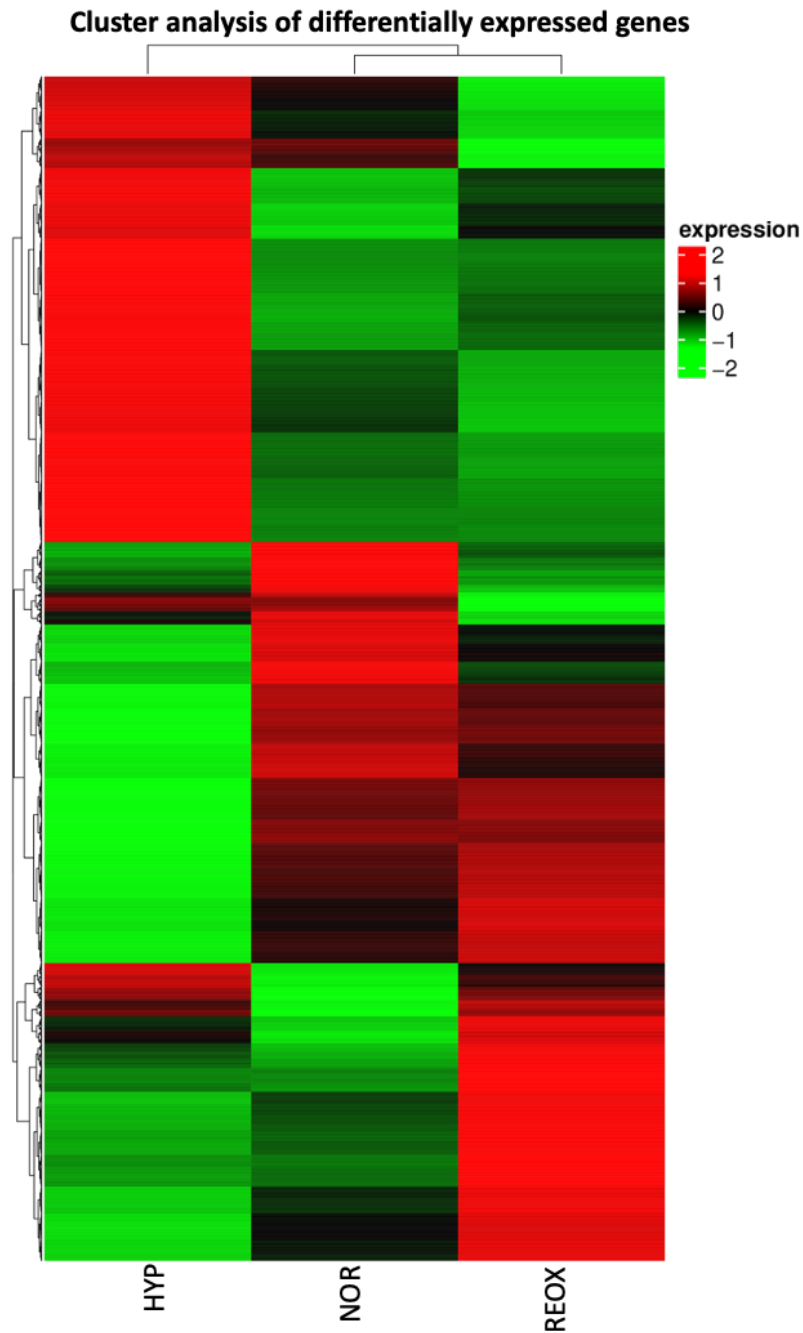


Figure 3.7 shows a hierarchical cluster analysis of differentially expressed genes in **MCF7 RNAseq conditions**. Gene expression levels are represented by colours ranging from green (low read counts) to red (high read counts) using the $\text{Log}_{10}(\text{FPKM}+1)$ value. NOR, HYP, and REOX are normoxia (72 hours 8.5% O_2), hypoxia (72 hours 1% O_2), and hypoxia-reoxygenation (72 hours 1% O_2 -72 hours 8.5% O_2), respectively.

Screening of Differentially Expressed Genes in MDA-MB-231 and MCF7 RNAseq

The first step of this analysis was to identify the overall distribution of differentially expressed genes among different experimental groups. Figure 3.8 and Figure 3.9 displays volcano plots for each RNAseq comparison between two experimental groups; the focus here was on hypoxia compared to normoxia and hypoxia-reoxygenation compared to normoxia showing several significant DEGs either upregulated or downregulated.

MDA-MB-231 RNAseq, hypoxia compared to normoxia showed 1537 significant DEGs in total, 952 significant DEGs upregulated, and 585 significant DEGs downregulated (Figure 3.8A). Hypoxia-reoxygenation, compared to normoxia, showed 3257 significant DEGs in total, 1653 significant DEGs upregulated, and 1604 significant DEGs downregulated in the MDA-MB-231 cell line (Figure 3.8B). Interestingly, there were more changes in DEGs in the hypoxia-reoxygenation profile compared to the hypoxic profile (Figure 3.8).

MCF7 RNAseq, hypoxia was compared to normoxia and as can be seen in the figure (Figure 3.9A), 1965 significantly DEGs in total with 1077 significantly upregulated DEGs and 888 significantly downregulated DEGs (Figure 3.9A). Hypoxia-reoxygenation was compared to normoxia, with 736 significantly DEGs in total, 472 significantly upregulated DEGs and 264 significantly downregulated DEGs (Figure 3.9B).

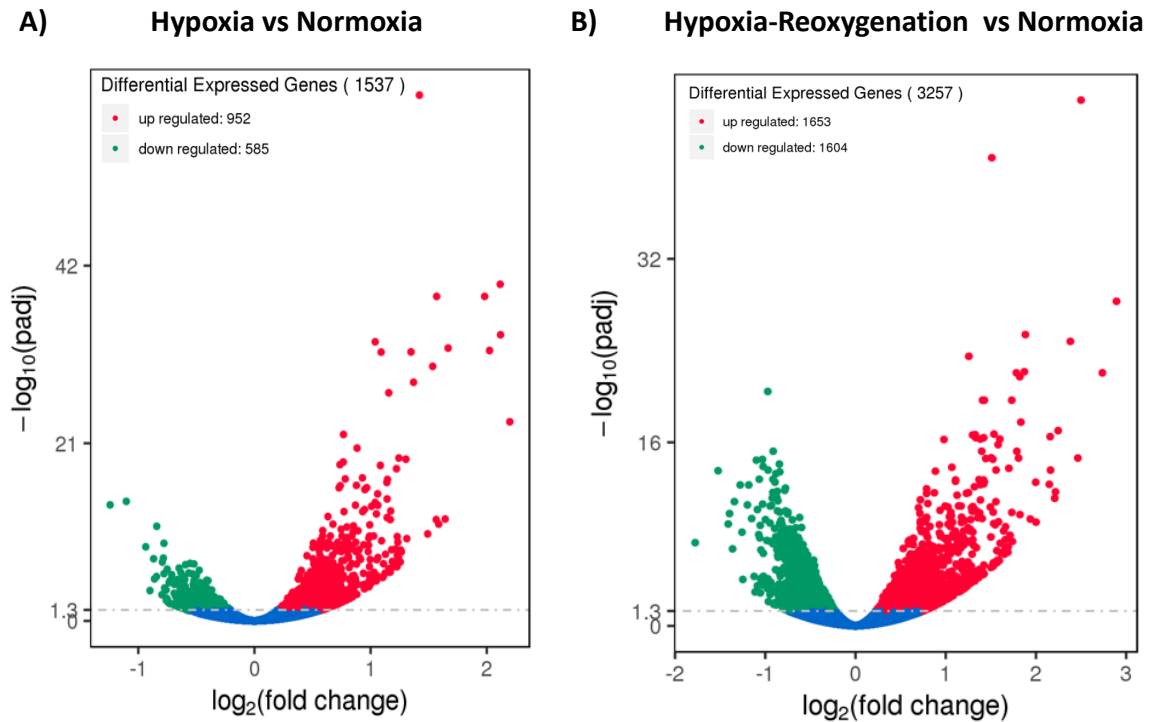


Figure 3.8 Volcano plots displaying DEG distribution from MDA-MB-231 RNAseq. **A)** hypoxia vs control normoxia **B)** hypoxia-reoxygenation vs control normoxia. Red (upregulated), green (downregulated) and blue dots (no differential expression between groups). X-axis: $\log_2$ Fold change in gene expression plotted against statistical significance in $\log_{10}$ (padj). n=3.

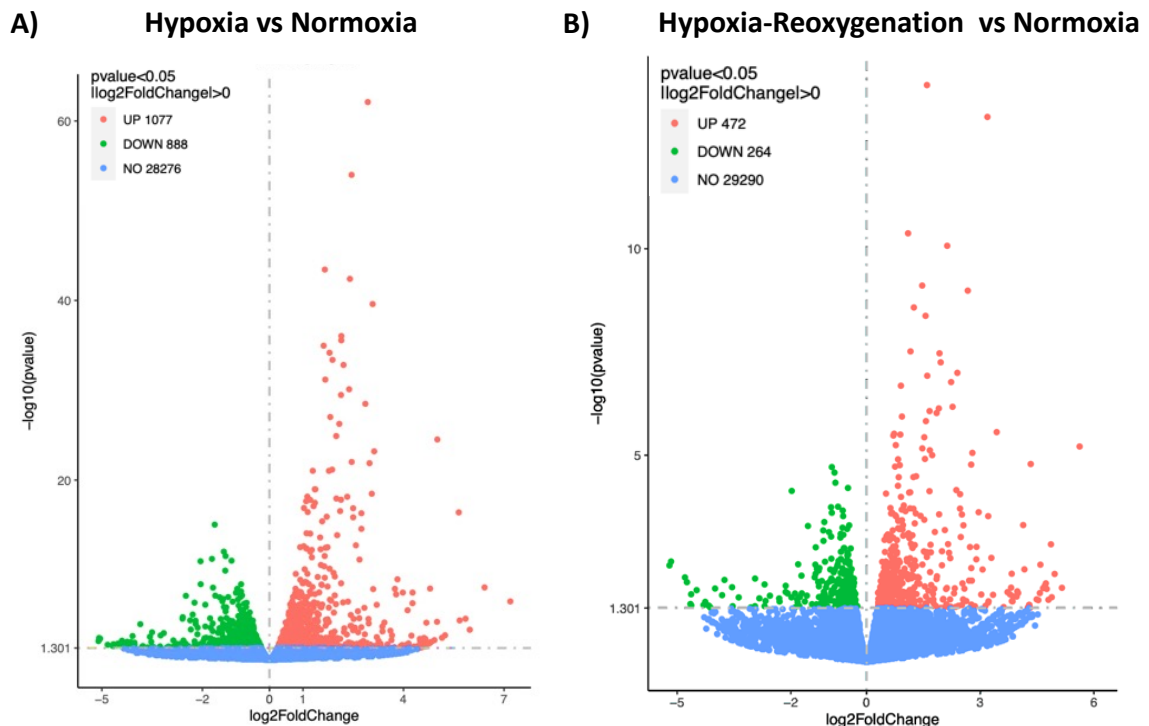


Figure 3.9 Volcano plots showing distribution of DEGs from MCF7 RNAseq: A) hypoxia vs control normoxia **B)** hypoxia-reoxygenation vs control normoxia. Red dots (upregulated DEGs), green dots (downregulated DEGs), blue dots (no differential expression). X-axis displays $\log_2$ Fold change in gene expression plotted against statistical significance in $\log_{10}(\text{padj})$. $n=3$.

KEGG pathway Analysis in hypoxia vs normoxia

KEGG (Kyoto Encyclopedia of Genes and Genomes) is an online database that links genomic information with functional information. The PATHWAY database contains information regarding cellular processes such as membrane transport, metabolism signal transduction and cell cycle (Kanehisa 2023). The KEGG pathway analysis was used here to identify the significantly enriched pathways associated with differentially expressed genes in hypoxia compared to normoxia and in hypoxia-reoxygenation compared to normoxia. Figure 3.10 and Figure 3.11 show KEGG dot-plot diagrams; the gene count enriched in the pathway is reflected by the size of the dots, while the colour of the dots reflects the significance of the pathway enrichment.

MDA-MB-231 RNAseq, five KEGG pathways were significantly upregulated in hypoxia compared to normoxia, highlighted by red boxes in (Figure 3.10). The most significant upregulated pathway is Glycolysis / Gluconeogenesis was the top upregulated pathway in the

MDA-MB-231 cell line with 16 DEGs upregulated in the pathway ($p=8.33506018346022E-08$). This was predictable as glycolysis increases in response to hypoxia, the pathway is enriched with hypoxia-inducible genes such as ALDOA, LDHA and ENO1 (Table 3.3). It's known that the metabolism of cancer cells shifts from oxidative phosphorylation to anaerobic glycolysis under hypoxic environments. A large number of these enzymes are controlled by HIF1 (Semenza GL. et al. 1994).

Other enriched pathways upregulated significantly in hypoxia compared to normoxia in MDA-MB-231 KEGG analysis include Fructose and mannose metabolism pathway ($p=7.39874105877286E-06$), Focal adhesion pathway ($p=0.000433246703640846$), Endocytosis pathway ($p=0.000464534647817796$) and Renal cell carcinoma pathway ($p=0.000686476982632548$) (Figure 3.10). More information about the KEGG pathway is presented in (Table 3.3) including unique KEGG ID for each significantly upregulated KEGG pathway, the gene ratio of differentially expressed genes to all genes which are correlated with each specific pathway, probability value (p-value) and the genes ID.

In MCF7 RNAseq, the analysis showed several enriched pathways significantly upregulated in hypoxia compared to normoxia. The top enriched pathway was Pathways in cancer with 42 DEGs ($p=0.00115106703543431$), followed by the PI3K-Akt signalling pathway with 34 DEGs ($p=0.0000224237608001951$). Other enriched upregulated pathways included Focal adhesion with 30 DEGs ($p=4.85580529148145E-08$) and the p53 signalling pathway with 12 DEGs ($p=0.000463811829440472$). As anticipated, HIF-1 signalling pathway with total of 18 genes ($p=1.90812509482466E-06$), Carbon metabolism pathway with 18 DEG ($p=0.0000313353634087219$), and Glycolysis with 17 DEGs ($p=1.23941507107256E-08$) were among the enriched upregulated pathways under hypoxia in MCF7 cell line. All mentioned pathways are highlighted by red boxes in (Figure 3.11).

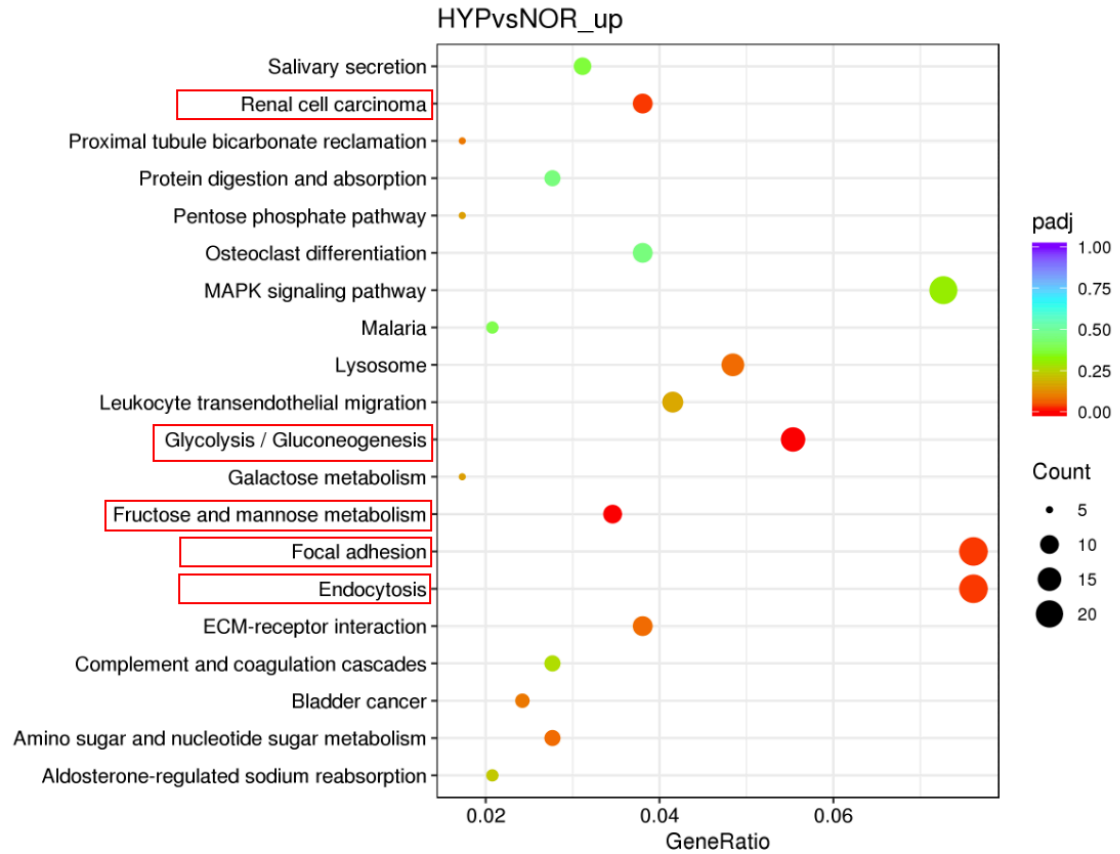


Figure 3.10 KEGG pathway analysis of significantly upregulated genes in hypoxia compared to normoxia in MDA-MB-231 RNAseq. The y-axis displays the name of the pathway, the x-axis shows the gene ratio of differentially expressed genes to all genes, which are found with this pathway. The size of the dot reflects the gene count, while the colour of the dots reflects pathway enrichment significance. Significantly upregulated pathways in hypoxia vs normoxia are highlighted in red boxes.

KEGG ID	Description	Number of Genes	Gene Ratio	p-value	Gene ID
hsa00010	Glycolysis / Gluconeogenesis	16	16/289	8.34E-08	PFKP/PKM/ENO1/PGM1/PGK1/GPI/GAPDH/TPI1/ENO2/LDHA/PFKL/ALDOA/HK1/HK2/PDHB/PGAM1
hsa00051	Fructose and mannose metabolism	10	10/289	7.40E-06	PFKP/TPI1/PFKFB4/PFKFB2/PFKL/ALDOA/HK1/HK2/PFKFB3/MPI
hsa04510	Focal adhesion	22	22/289	0.00043325	FYN/TNC/MYLK/MYL9/MYL12A/ITGB8/CAV2/CAV1/COL1A1/VEGFA/PGF/COL5A1/ITGB4/IGF1R/PDGFC/VEGFC/ITGA5/COL1A2/MAP2K1/RELN/LAMB3/PPP1CB
hsa04144	Endocytosis	22	22/289	0.00046453	EHD2/NEDD4L/ERBB3/PRKCZ/NEDD4/CAP1/EPS15/TGFB2/CAV2/CAV1/CXCR4/PARD6B/PSD4/RAB11FIP5/IGF1R/PIP5K1A/FGFR4/VPS28/PIP5KL1/HSPA1B/HSPA1A/HSPA1L
hsa05211	Renal cell carcinoma	11	11/289	0.00068648	TGFB2/VEGFA/SLC2A1/PGF/MAP2K2/EGLN3/ETS1/EGLN1/VEGFC/FLCN/MAP2K1

Table 3.3 Show significant KEGG pathways upregulated in hypoxia compared to normoxia in MDA-MB-231 RNAseq. Each pathway is identified by a unique KEGG ID, and the table includes the gene ratio of differentially expressed genes to all genes related to that pathway, the probability value (p-value), and the gene ID.

Hypoxia vs Normoxia Upregulated KEGG Pathways Analysis

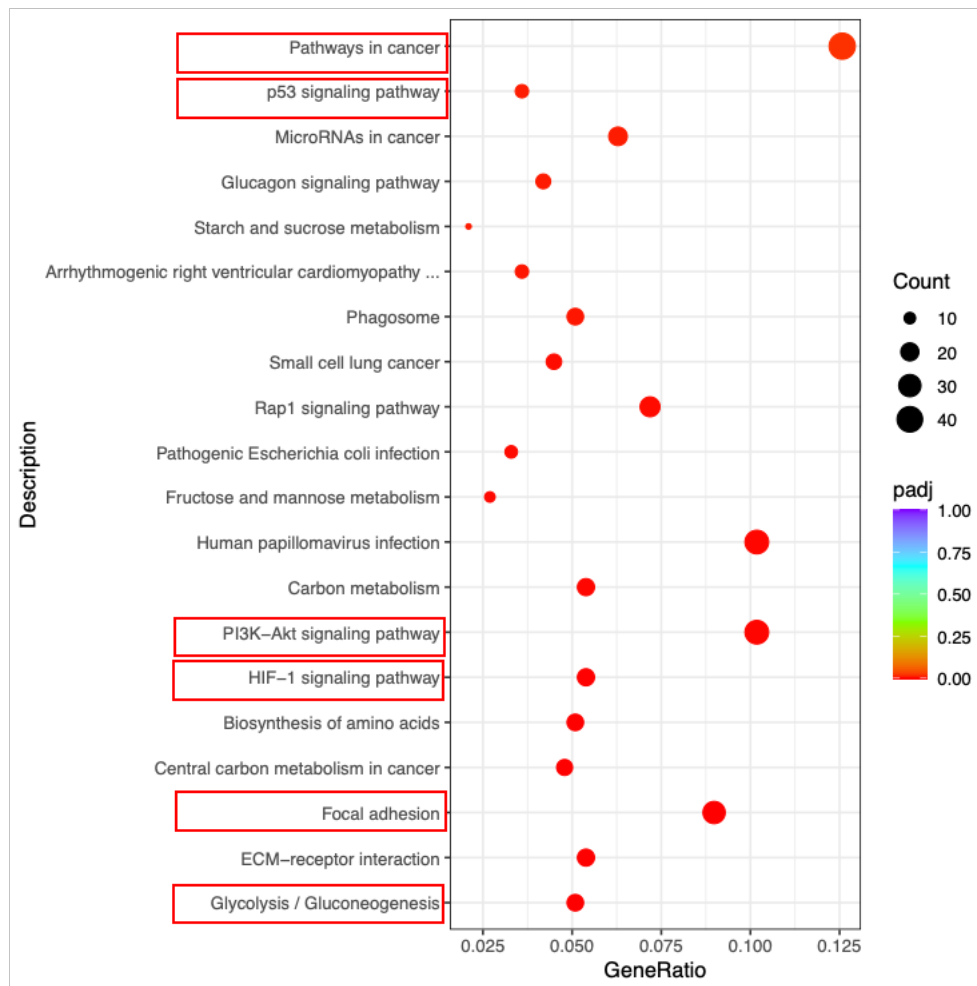


Figure 3.11 Displays KEGG pathway analysis of genes significantly upregulated in hypoxia compared to normoxia in MCF7 RNAseq. The y-axis shows pathway names, the x-axis shows the gene ratio of differentially expressed genes to all genes present in the pathway. The size of the dot represents the gene count, while the dot colour shows the significance of the pathway enrichment. Red boxes highlight significantly upregulated KEGG pathways in hypoxia vs normoxia.

KEGG pathway Analysis in hypoxia-reoxygenation vs normoxia

In MDA-MB-231 RNAseq, The KEGG pathway analysis revealed that 11 enriched pathways were significantly upregulated in hypoxia-reoxygenation when compared to normoxia, highlighted by red boxes in (Figure 3.12). To better understand the relationship between these pathways, they were classified into four main categories based on the KEGG pathway maps classification, which forms the basis of the KEGG Orthology system (KO).

The first category was transport and catabolism, which included the Lysosome pathway with 38 upregulated DEGs ($p=1.19E-11$), and the phagosome pathway with 29 upregulated DEGs ($p=0.00010048$). The second category was signalling, which included the Cell adhesion molecules (CAMs) pathway with 27 upregulated DEGs ($p=7.85E-05$) and the Cytokine-cytokine receptor interaction pathway with 43 upregulated DEGs ($p=0.00011726$). The third category was immune system/ immune disease, which included the Rheumatoid arthritis pathway with 28 upregulated DEGs ($p=1.93E-09$), Complement and coagulation cascade pathway with 18 upregulated DEGs ($p=5.82E-05$), and NOD-like receptor signalling pathway with 14 upregulated DEGs ($p=0.00061434$). The fourth and final category was genetic information processing, which included Protein processing in the endoplasmic reticulum pathway with 40 upregulated DEGs ($p=1.81E-08$).

Another interesting finding is Steroid biosynthesis pathway was among the significant enriched pathways in hypoxia-reoxygenation showing that metabolism is being upregulated under hypoxia-reoxygenation. The KEGG pathway database also provides a network database, showing KEGG networks that are part of these pathways. Cytokine-cytokine receptor interaction pathway was found to be networking with the TGFB signalling pathway. The rheumatoid arthritis pathway was found to be networking with the VEGF signalling pathway. Unique KEGG ID for each significantly upregulated KEGG pathway, gene ratio of differentially expressed genes and the p-value are shown in (Table 3.4).

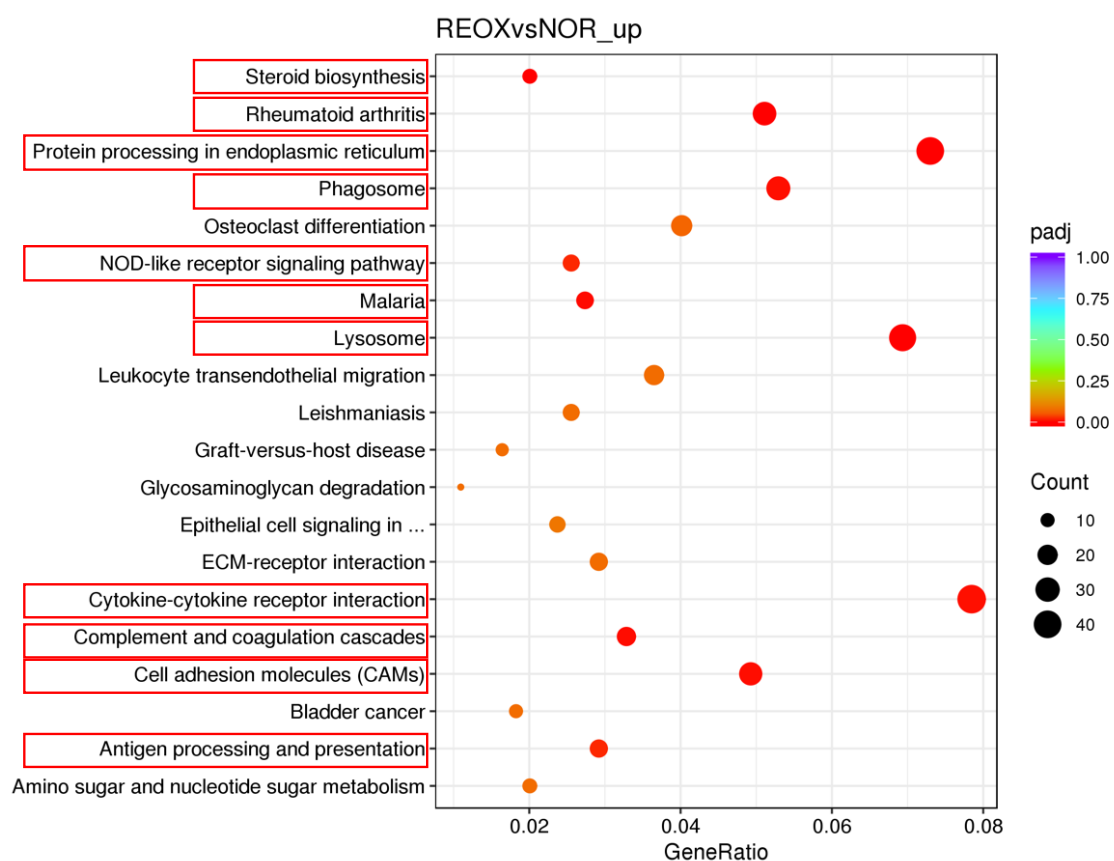


Figure 3.12 KEGG pathway analysis of significantly upregulated genes in hypoxia-reoxygenation compared to normoxia in MDA-MB-231 RNAseq. The y-axis shows the pathway name, x-axis shows the gene ratio of differentially expressed genes to all genes in the pathway. Dot size reflects gene count and colour reflects pathway significance. Red boxes highlight significantly upregulated KEGG pathway in hypoxia-reoxygenation.

KEGG ID	Description	Number of Genes	Gene Ratio	p-value
hsa04142	Lysosome	38	38/548	1.19E-11
hsa05323	Rheumatoid arthritis	28	28/548	1.93E-09
hsa04141	Protein processing in endoplasmic reticulum	40	40/548	1.81E-08
hsa00100	Steroid biosynthesis	11	11/548	2.13E-07
hsa05144	Malaria	15	15/548	4.14E-05
hsa04610	Complement and coagulation cascades	18	18/548	5.82E-05
hsa04514	Cell adhesion molecules (CAMs)	27	27/548	7.85E-05
hsa04145	Phagosome	29	29/548	0.00010048
hsa04060	Cytokine-cytokine receptor interaction	43	43/548	0.00011726
hsa04621	NOD-like receptor signaling pathway	14	14/548	0.00061434
hsa04612	Antigen processing and presentation	16	16/548	0.00063489

Table 3.4 Significant KEGG pathways upregulated in hypoxia compared to normoxia in MDA-MB-231 RNAseq. Displayed here are the unique KEGG IDs for each pathway, the genes ratio of differentially expressed genes to all genes for each pathway and the corresponding p-value.

In MCF7 RNAseq, the KEGG pathway showed that five enriched pathways were significantly upregulated in hypoxia-reoxygenation compared to normoxia, highlighted by red boxes in (Figure 3.13). Based on the KEGG pathway maps classification, four pathways were classified as infectious diseases, including influenza pathway with 18 DEGs ($p=6.27E-08$), Measles pathway with 15 DEGs ($p=3.86E-07$), Herpes simplex infection pathway with 13 DEGs ($p=0.00039882$), and Hepatitis C pathway with 12 DEGs ($p=0.00018861$). RIG-I-like receptor signalling pathway with 8 DEGs ($p=0.00024704$) was classified as the immune system.

The five enriched pathways that were significantly upregulated in hypoxia-reoxygenation compared to normoxia were found to be interacting with critical pathways. The KEGG network database showed that the PI3K signalling pathway was networking with influenza, measles, and Hepatitis C pathways and linked to the Herpes simplex infection pathway. The apoptosis pathway was networking with influenza, Hepatitis C, and Herpes simplex infection pathways; it's also related to measles and RIG-I-like receptor signalling pathways. ERK signalling pathway

was networking with influenza and Hepatitis C pathways. In addition, the JAK-STAT signalling pathway was found to be related to all mentioned KEGG pathways (Table 3.5). MAPK signalling pathway related to influenza, Hepatitis C pathway and RIG-I-like receptor signalling pathway. p53 signalling pathway related to measles and Herpes simplex infection pathways. Unique KEGG ID for each significantly upregulated KEGG pathway, gene ratio of differentially expressed genes and the p-value are shown in (Table 3.5).

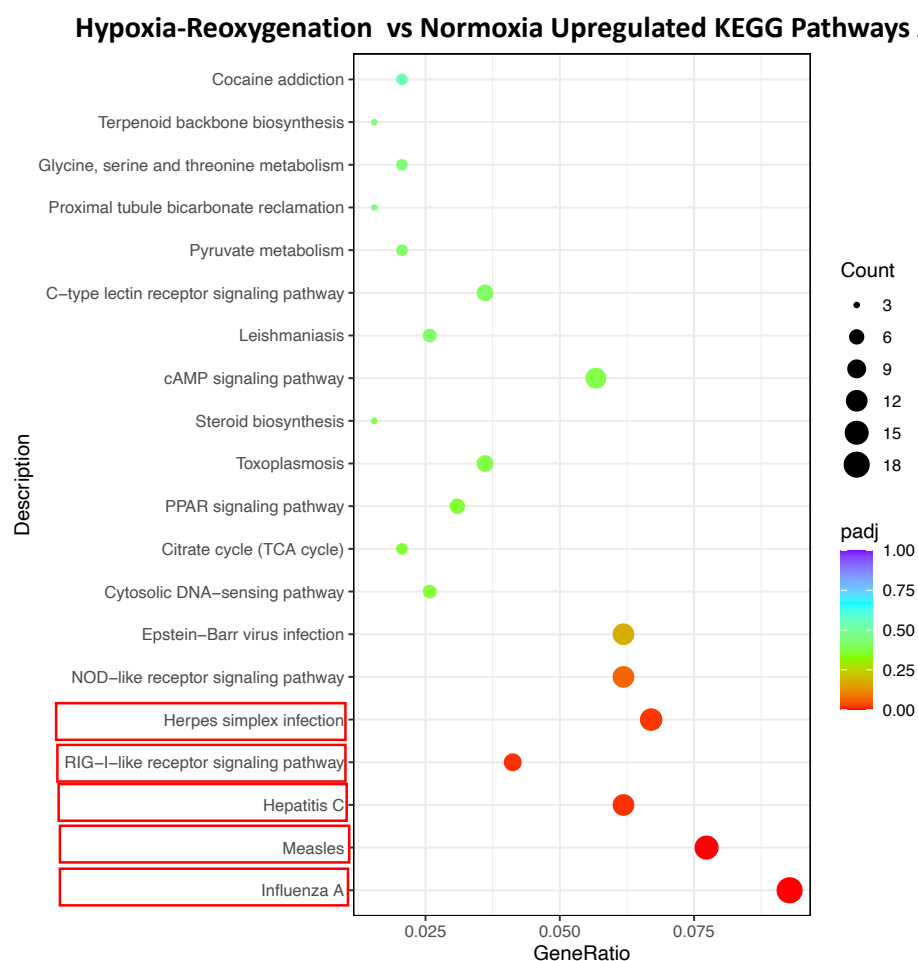


Figure 3.13 Displays the KEGG pathway analysis of significantly upregulated genes in hypoxia-reoxygenation compared to normoxia in MCF7 RNAseq. the graphs show the name of the pathway on the y-axis and the gene ratio of differentially expressed genes to all genes on the x-axis. The size of the dot represents the gene count while the colour of the dots shows the significance of the pathway enrichment. The red boxes highlight the significantly upregulated KEGG pathway.

KEGG ID	Description	Number of Genes	Gene Ratio	p-value
hsa05164	Influenza A	18	18/194	6.27E-08
hsa05162	Measles	15	15/194	3.86E-07
hsa05168	Herpes simplex infection	13	13/194	0.00039882
hsa05160	Hepatitis C	12	12/194	0.00018861
hsa04622	RIG-I-like receptor signaling pathway	8	8/194	0.00024704

Table 3.5 KEGG pathways significantly upregulated in MCF7 RNAseq during hypoxia-reoxygenation compared to normoxia. For each pathway, it shows the unique KEGG ID, gene ratio of differentially expressed genes to all genes involved with that pathway, and the p-value.

3.3.2 Maintaining the hypoxia-induced transcriptomic profile during hypoxia-reoxygenation in TNBC and MCF7 cells.

One of the main hypotheses of this study evolves around the maintaining of the hypoxia-induced transcriptomic profile during hypoxia-reoxygenation and its impact on breast cancer progression. RNAseq was performed in two breast cancer cell lines, MDA-MB-231 and MCF7, with the aim of addressing this hypothesis.

Co-expression of DEGs in hypoxia and hypoxia-reoxygenation in breast cancer cells

The analysis first looked at the total DEGs in each condition (hypoxia vs normoxia, hypoxia-reoxygenation vs normoxia and hypoxia-reoxygenation vs hypoxia) and then the co-expression of all DEGs to determine the number of genes that are expressed uniquely and shared between conditions (Figure 3.14 and Figure 3.15). The main aim was to determine the percentage of DEGs that are co-expressed in hypoxia and hypoxia-reoxygenation in both RNAseq. MDA-MB-231 RNAseq revealed that 42% of the hypoxic profile is shared with hypoxia-reoxygenation, and a total number of 651 significant DEGs (up and downregulated) co-expressed in hypoxia-reoxygenation (Figure 3.14B). The main interest was in the significantly upregulated DEGs; the analysis revealed that 358 significantly upregulated DEGs overlapped in hypoxia-reoxygenation (Figure 3.14C). MCF7 RNAseq showed that 11% of the hypoxic profile was found to be shared with hypoxia-reoxygenation in the MCF7 cell line. A total number of 228 DEGs (up and down downregulated) have co-expressed in hypoxia and hypoxia-reoxygenation. Similar to the MDA-MB-231 analysis, the main interest was in the significantly upregulated DEGs; 96 upregulated DEGs overlapped in hypoxia and hypoxia-reoxygenation (3.15C).

Further investigation was carried out on the identified lists of interest from MDA-MB-231 RNAseq (358 overlapped significantly upregulated DEGs) and MCF7 RNAseq list (96 overlapped significantly upregulated DEGs). The analysis investigated the molecular, and biological functions, pathway analysis, correlation with patient's outcome, and survival analysis of the specified DEGs. The workflow for this analysis is shown in (Figure 3.1B).

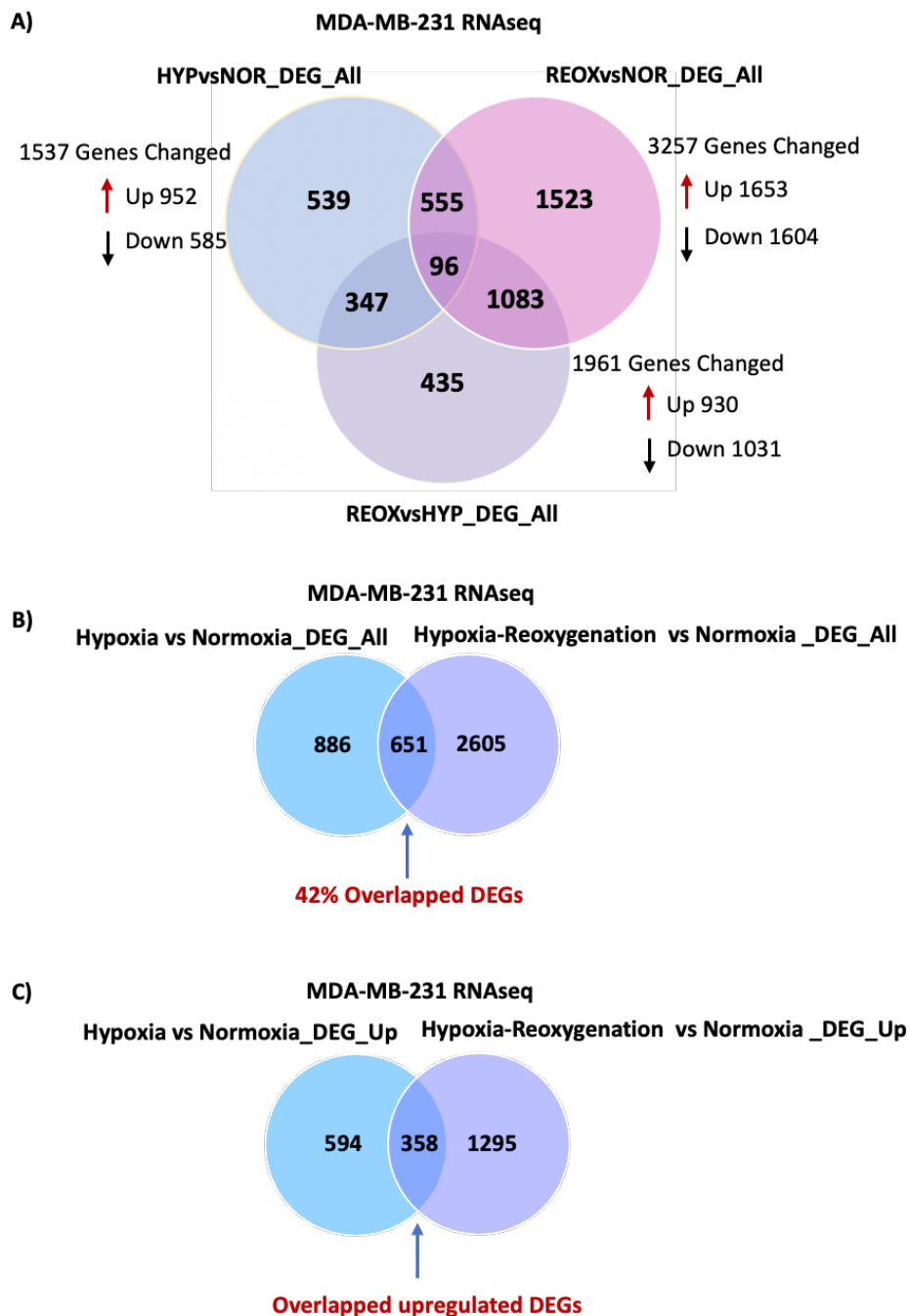


Figure 3.14 Venn diagrams showing co-expression of differentially expressed genes in MDA-MB-231 RNAseq. A) Co-expression of all DEGs (up and downregulated) in three groups, hypoxia vs normoxia, hypoxia-reoxygenation vs normoxia and hypoxia-reoxygenation vs hypoxia. **B)** Co-expression of all DEGs (up and downregulated) in hypoxia vs normoxia and hypoxia-reoxygenation vs normoxia. 42% overlapped genes between the two groups. **C)** Co-expression of only upregulated DEGs in hypoxia vs normoxia and hypoxia-reoxygenation vs normoxia with 358 overlapped genes. Red arrows (upregulated differentially expressed genes) black arrows (downregulated differentially expressed genes) and blue arrows (overlapped DEGs). Total DEG list for each condition represented in each circle. n=3.

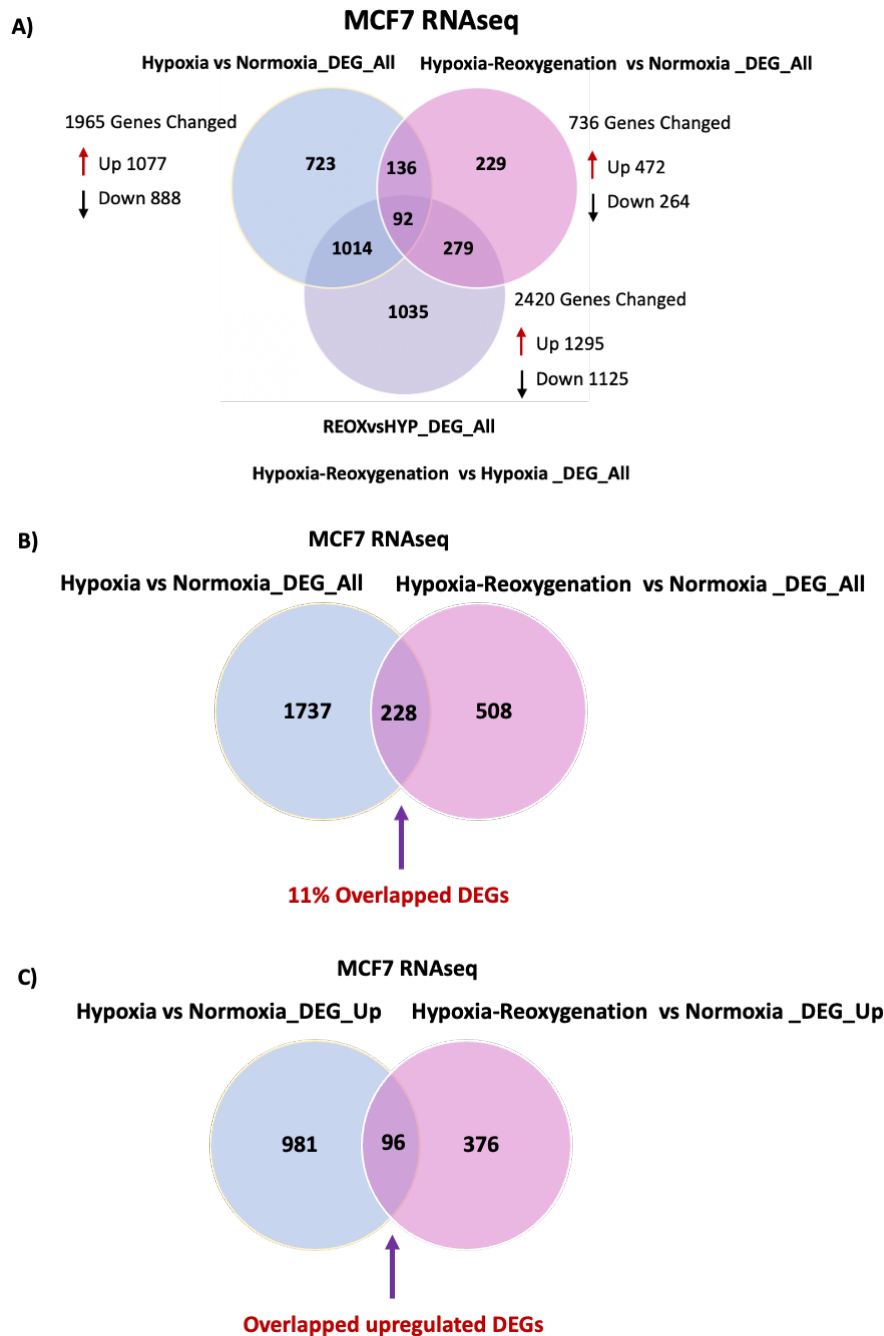


Figure 3.15 Shows Venn diagrams of co-expressed differentially expressed genes in MCF7 RNAseq. A) Shows co-expression of all DEGs (up and downregulated) in three groups: hypoxia vs normoxia, hypoxia-reoxygenation vs normoxia, and hypoxia-reoxygenation vs hypoxia. **B)** Shows co-expression of all DEGs (up and downregulated) in hypoxia vs normoxia and hypoxia-reoxygenation vs normoxia, with 11% overlapped genes between the two groups. **C)** Shows co-expression of only upregulated DEGs in hypoxia vs normoxia and hypoxia-reoxygenation vs normoxia, with 96 upregulated DEGs overlapped between the two groups. The Venn diagrams use red arrows (upregulated differentially expressed genes), black arrows (downregulated differentially expressed genes), and purple arrows (overlapped DEGs). The sum of the numbers in each circle represents the total DEG list for that condition. n=3.

PANTHER classification system

The PANTHER classification system is a powerful tool for understanding how protein functions and biological roles have evolved over time by analyzing protein sequence evolution (Mi H, Guo N, Kejariwal A, Thomas PD. 2007). The system uses computational algorithms to generate a vast collection of phylogenetically defined protein families and subfamilies, which are then classified by biologists using software that associates ontology terms with the protein families (Thomas PD, et al. 2003). In this study, we used the PANTHER classification system to investigate the molecular and biological functions of specific DEGs in MDA-MB-231 and MCF7 RNAseq data (as shown in Figure 3.14C and Figure 3.15C) and to explore the relevant pathways in these RNAseq datasets.

Molecular Functions of the overlapped DEGs

MDA-MB-231 list of 358 overlapped significantly upregulated DEGs were submitted to PANTHER for analysis. The molecular function analysis (Figure 3.16) revealed that the majority of the DEGs (more than 100 DEGs from 358 DEGs) in this list participated in binding functions (GO:0005488) followed by catalytic activity functions (GO: 0003824) (approximately 80 DEGs) (Figure 3.16A). The binding molecular function was explored further to determine the type of binding; the analysis followed the protein binding (GO: 0005515) and showed that 26% of submitted DEGs participated in the signalling receptor binding category (GO:0005102) (Figure 3.16B). Table 3.6 shows the list of these 18 DEGs and their PANTHER protein classification. Four growth factors, VEGFC, TGFB2, PGF, and VEGFA were part of the analysis (Table 3.6).



Figure 3.16 Shows PANTHER pathway analysis for molecular functions in MDA-MB-231 RNAseq. A) displays a bar graph of the different molecular function categories, with the x-axis showing the category and the y-axis showing the number of DEGs in each category. **B)** presents a pie chart that illustrates the different protein binding categories, with the signalling receptor binding highlighted in yellow Chart tooltips are read as: Category name (Accession): # genes; Percent of gene hit against total # genes; Percent of gene hit against total # Function hits. This figure is generated by the PANTHER classification system. Figure generated using PANTHER Classification system (Thomas PD. et al. 2022)

Gene Name	PANTHER Protein Class
SH2B2	Scaffold/adaptor protein
WNT10A	Intercellular signal molecule
ADM	Peptide hormone
VEGFC	Growth factor
TGFB2	Growth factor
PGF	Growth factor
VEGFA	Growth factor
SHH	-
SEMA3C	Membrane-bound signaling molecule
ITGA5	Integrin

Table 3.6 Features the significant upregulated DEGs in signalling receptor binding (GO:0005102) in MDA-MB-231 RNAseq. Adapted from PANTHER Classification system (Thomas PD. et al. 2022)

The molecular function analysis for the MCF7 list of 96 overlapped significantly upregulated DEGs showed that 30% (30 DEGs) participated in binding functions (GO:0005488) and 24% (24 DEGs) participated in catalytic activity functions (GO: 0003824) (Figure 3.17A). Four DEGs of the catalytic activity functions acted on the protein kinase activity (GO: 0004672), including PLK2, AC093012, SIK1B and BRSK2. The binding molecular function was explored further to determine the type of binding; 21 DEGs were involved in protein binding, and 8 DEGs were involved in signalling receptor binding (GO:0005102) (Figure 3.17B). Table 3.7 shows the list of the eight DEGs involved in signalling receptor binding and their PANTHER protein classification. Two growth factors were involved in this analysis: INHBA and INHA.

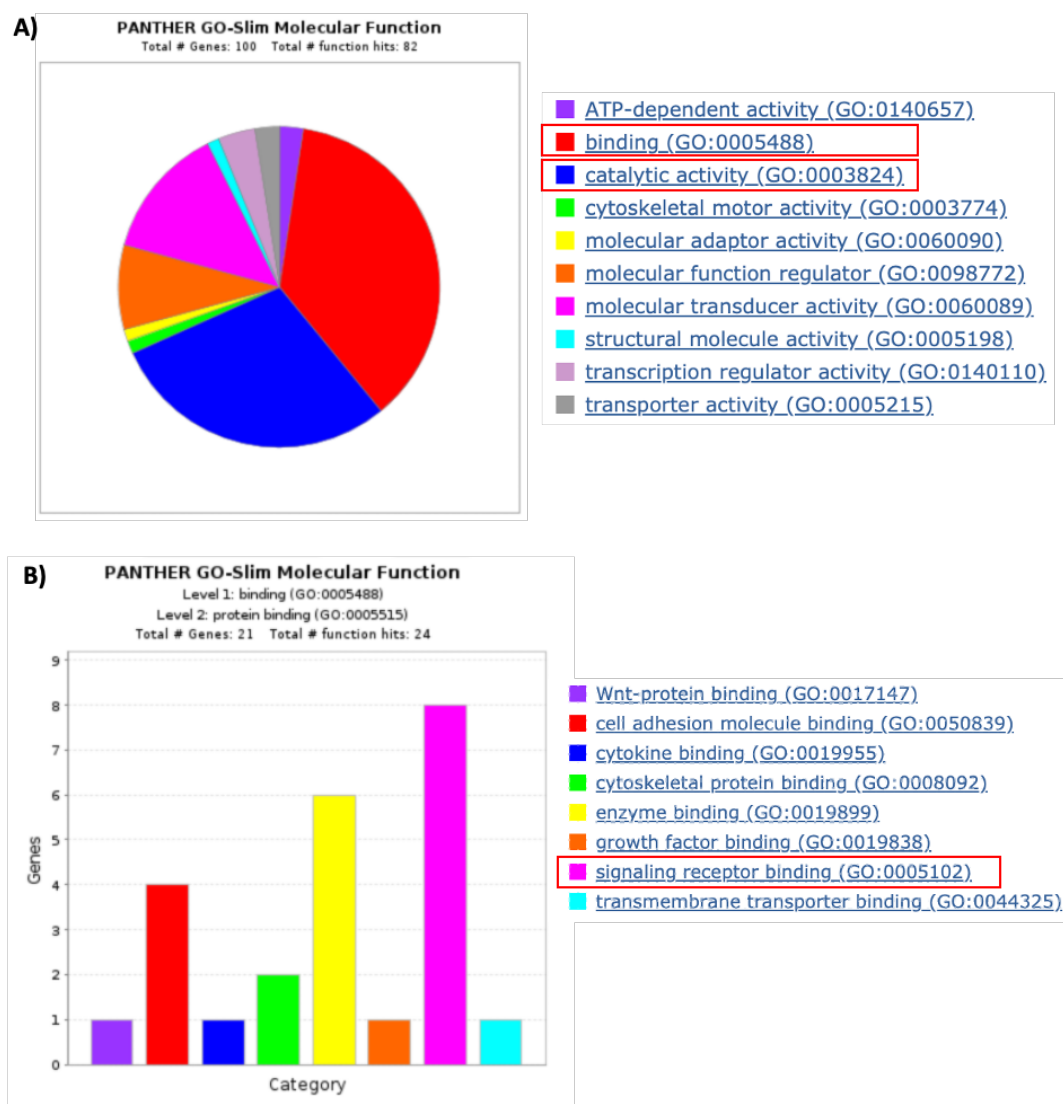


Figure 3.17 Shows the results of PANTHER pathway analysis for the molecular functions of the MCF7 RNAseq list (96 overlapped significantly upregulated DEGs in hypoxia and hypoxia-reoxygenation from MCF7 RNAseq). A) Pie chart illustrates the different molecular function categories. B) Bar graph showing the different protein binding categories. Top enriched categories highlighted in red boxes. Chart tooltips are read as: Category name (Accession): # genes; Percent of gene hit against total # genes; Percent of gene hit against total # Function hits. This figure is generated by the PANTHER classification system. Figure generated using PANTHER Classification system (Thomas PD. et al. 2022)

Gene Name	PANTHER Protein Class
APLP1	protease inhibitor
NMB	-
FN1	extracellular matrix protein
INHBA	growth factor
INHA	growth factor
NMB	membrane-bound signaling molecule
LRRK2	scaffold/adaptor protein
SEMA5B	membrane-bound signaling molecule

Table 3.7 Displays the list of the overlapped significantly upregulated DEGs featured in the signalling receptor binding (GO:0005102) from the MCF7 RNAseq. Adapted from PANTHER Classification system (Thomas PD. et al. 2022)

Biological process of the overlapped DEGs

The MDA-MB-231 biological process analysis for the overlapped 358 significantly upregulated DEGs in hypoxia and hypoxia-reoxygenation showed that 56% (193 DEGs) participated in cellular process (GO:0009987), with the majority being part of cell communication (GO:0007154, 62 DEGs). Other featured biological processes include 35% in biological regulation (GO:0065007, 120 DEGs), 30% in metabolic process (GO:0008152, 104 DEGs), 21 % in response to stimulus (GO:0050896, 72 DEGs) and 17 % in signalling (GO:0023052, 60 DEGs) (Figure 3.18A).

It is well known that cell signalling is modified under hypoxic conditions. To identify the type of signalling overlapped between hypoxia and hypoxia-reoxygenation in MDA-MB-231 RNAseq, further analyses were performed exploring the signal transduction for the 60 DEGs in signalling highlighted by the green box in (Figure 3.18A). The analysis showed that 27 DEGs were participating in the cell surface receptor signalling pathway (GO:0007166) and 19 DEGs were participating in intracellular signal transduction (GO:0035556) (Figure 3.18B). The list of these DEGs is explained in (Table 3.8).

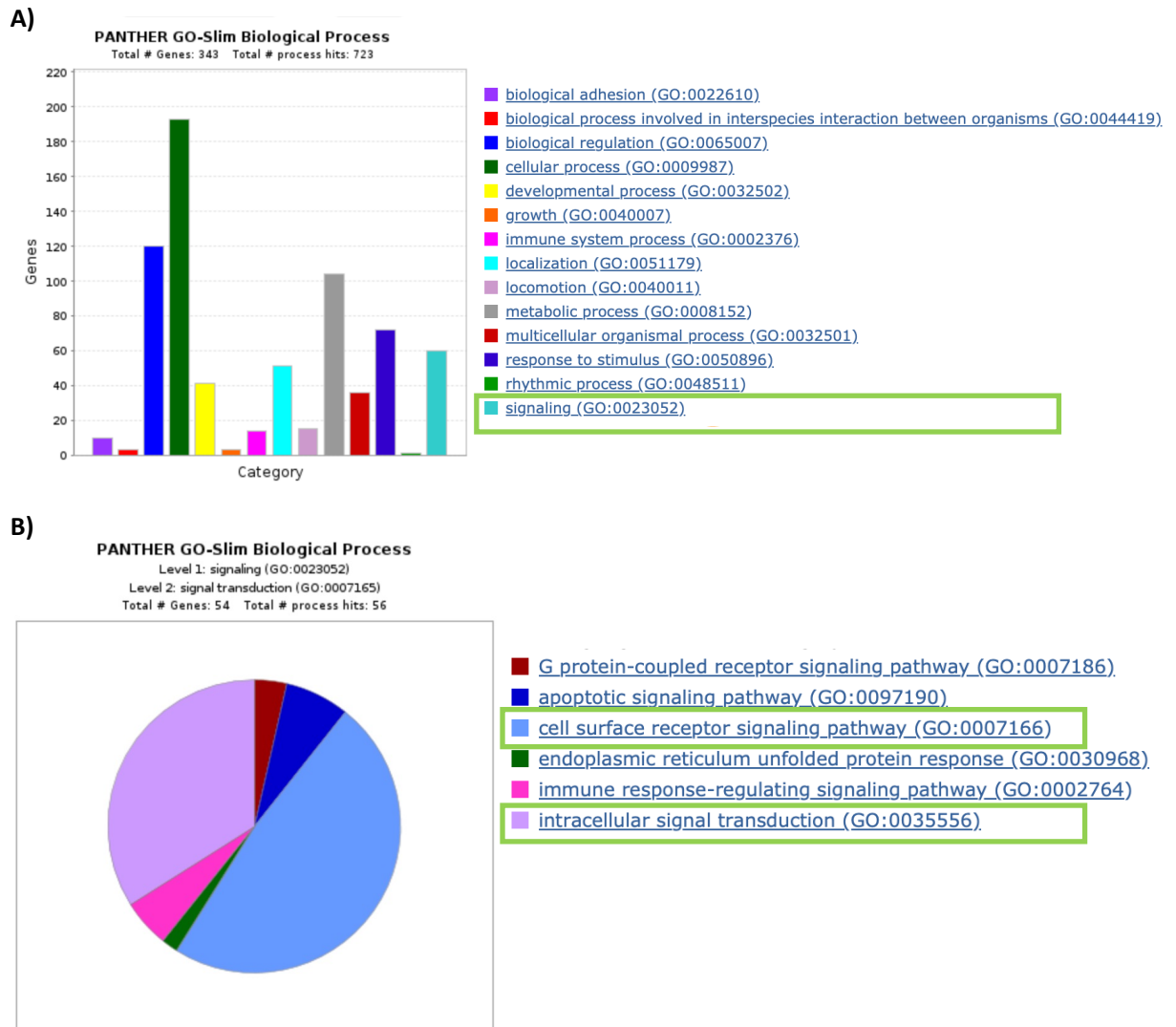


Figure 3.18 PANTHER pathway analysis for the biological process featured in the overlapped significantly upregulated DEGs in hypoxia and hypoxia-reoxygenation in MDA-MB-231 RNAseq. A) Bar graph showing the different biological process. The x-axis shows the category and the y-axis show number of DEGs in each category green box highlight the focus of the next analysis. **B)** Bar chart showing the signal transduction, green box highlights the focus of the analysis. Figure generated using PANTHER Classification system (Thomas PD. et al. 2022)

Cell surface receptor signaling pathway (GO:0007166)	Intracellular signal transduction (GO:0035556)
FSTL3	RALA
SH2B2	SH2B2
DEDD2	CYLD
CYLD	ADM
WNT10A	NABP1
TPBG	DUSP3
VEGFC	DAPK3
FST	TNIP1
TGFB2	DENND3
PGF	DUSP10
VEGFA	ARHGEF28
LEPROT	DUSP1
SHH	OPTN
SEMA3C	FLCN
LZTS2	MAP2K2
OSMR	NYAP1
NEDD9	DGKA
NOG	WWC1
ITGA5	DUSP8
IGFBP3	
ITGB8	
SEMA4B	
SDCBP	
FAM83A	
ERRFI1	
FYN	
DKK1	

Table 3.8 Displays DEGs significantly upregulated participating in cell surface receptor signalling pathway (GO:0007166) and intracellular signal transduction (GO:0035556) in MDA-MB-231 RNAseq. Adapted from PANTHER Classification system (Thomas PD. et al. 2022).

The MCF7 RNAseq biological process analysis for the 96 overlapped significantly upregulated DEGs in hypoxia and hypoxia-reoxygenation showed 55% (55 DEGs) participated in the cellular process (GO:0009987), 33% biological regulation (GO:0065007, 33 DEGs), 30% in metabolic process (GO:0008152, 30 DEGs), 23 % in response to stimulus (GO:0050896, 23 DEGs), and 20 % in signalling (GO:0023052, 20 DEGs) (Figure 3.19A).

The cellular process was further investigated, and the analysis showed that within this, 27% of DEGs participated in the cellular metabolic process (GO:0044237), and 20% of DEGs in cell communication (GO:0007154) (Figure 3.19B) (Table 3.9).

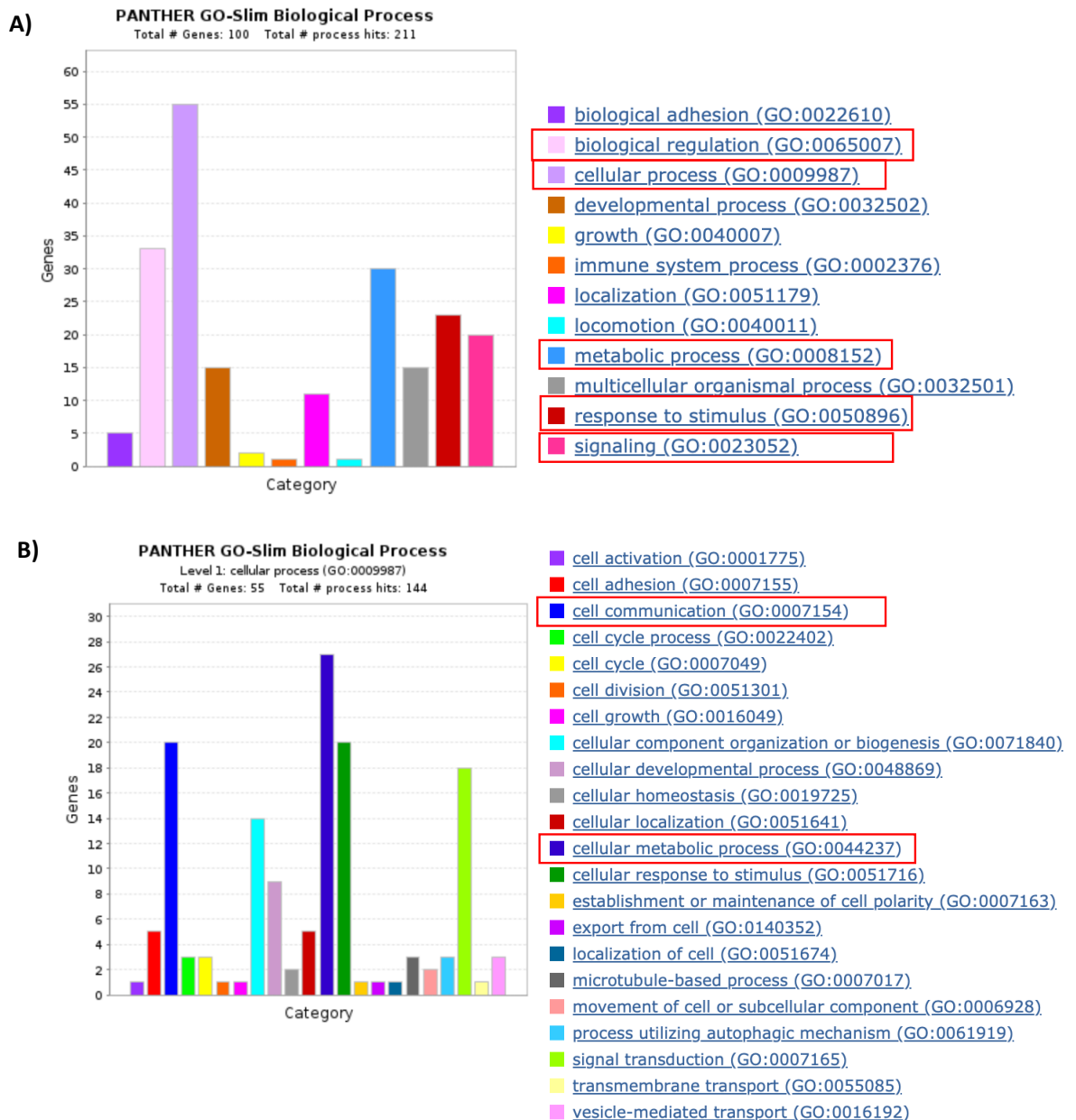


Figure 3.19 PANTHER pathway analysis for the biological Process of 96 overlapped significantly upregulated DEGs in hypoxia and hypoxia-reoxygenation in the MCF7 RNAseq. A) Bar graph illustrating the different biological process categories. B) Bar graph showing the different cellular processes. Red boxes highlight the top enriched categories. Chart tooltips are read as Category name (Accession): # genes; Percent of gene hit against total # genes; Percent of gene hit against total # Function hits. This figure was generated using the PANTHER Classification system (Thomas PD. et al. 2022).

Gene ID	PANTHER Protein Class
PPARA	C4 zinc finger nuclear receptor
AC093012	-
HACD1	dehydratase
PSPH	phosphatase
LIX1L	-
SESN3	peroxidase
PTGS1	oxygenase
AP003068	-
TRAF4	scaffold/adaptor protein
ALDOC	aldolase
C1GALT1	transferase
TP53INP2	-
PCK2	kinase
CRLF1	transmembrane signal receptor
BCAR3	-
COMT	methyltransferase
TIMP3	protease inhibitor
LHX2	homeodomain transcription factor
SOX9	-
SIK1B	non-receptor serine/threonine protein kinase
LOXL2	oxidase
CBS	lyase
PLAC8	-
INHBA	growth factor
INHA	growth factor
BRSK2	non-receptor serine/threonine protein kinase
LRRK2	scaffold/adaptor protein

Table 3.9 Displays the list of the overlapped significantly upregulated DEGs enriched in the cellular metabolic process (GO:0044237) from the MCF7 RNAseq. Adapted from PANTHER Classification system (Thomas PD. et al. 2022).

PANTHER Pathways in the overlapped DEGs

the PANTHER pathway analysis of MDA-MB-231 RNAseq showed enrichment for several signalling pathways including Gonadotropin-releasing hormone receptor pathway (P06664), integrin signalling pathway (P00034), Wnt signalling pathway (P00057), TGF-beta signalling pathway (P00052), VEGF signalling pathway (P00056), PDGF signalling pathway (P00047), Ras Pathway (P04393) and p53 pathway (P00059) (Figure 3.20). The analysis also showed enrichment for the oxidative stress response pathway (P00046), which is known to activate the MAPK signalling pathway (Son Y. et al. 2011). Studies found that kinases such as ERK, JNK and p38 get activated in response to oxidative stress via the production of reactive oxygen species (ROS), which can, in turn, affect apoptosis and survival of the cells (J. A. McCubrey, M. M. Lahair, and R. A. Franklin, 2006). Another interesting finding from the analysis is enrichment in the angiogenesis pathway (P00005), which is involved in the migration, growth, and differentiation of endothelial cells (Figure 3.20).

MCF7 PANTHER pathway analysis highlighted enrichment of the 96 overlapped significantly upregulated DEGs in multiple pathways such as the Cadherin signalling pathway (P00012), Gonadotropin-releasing hormone receptor pathway (P06664), Wnt signalling pathway (P00057) and TGF-beta signalling pathway (P00052), integrin signalling pathway (P00034), and Dopamine receptor-mediated signalling pathway (P05912) (Figure 3.21). A list of these top enriched pathways, along with the genes featured in each pathway, is presented in (Table 3.10).

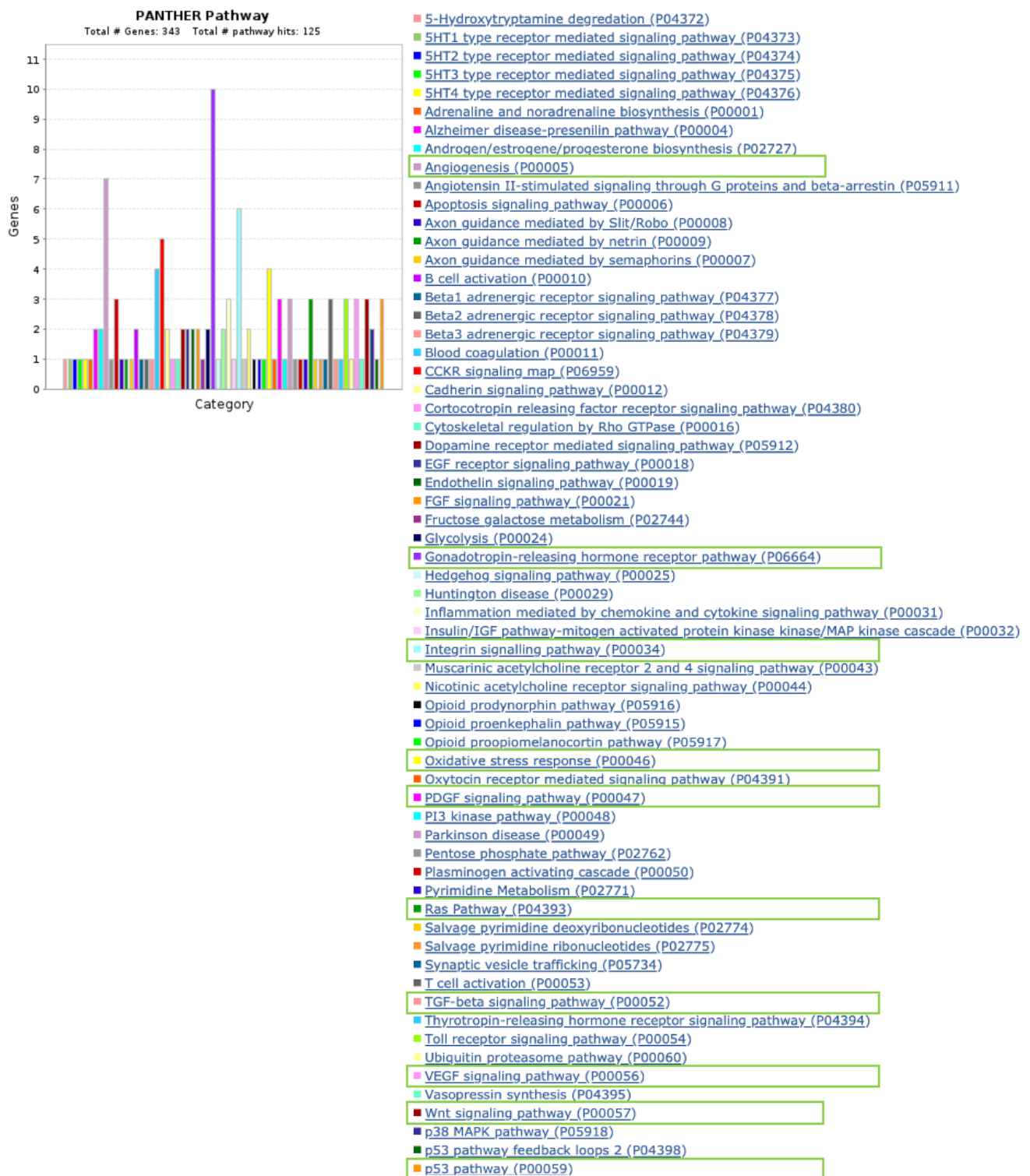


Figure 3.20 Shows a bar chart of PANTHER pathways in the analysis of upregulated DEGs in hypoxia and hypoxia-reoxygenation from MDA-MB-231 RNAseq. PANTHER recognized 343 DEGs and 125 pathway hits, with green boxes highlighting enriched pathways. Figure generated using PANTHER Classification system (Thomas PD. et al. 2022).

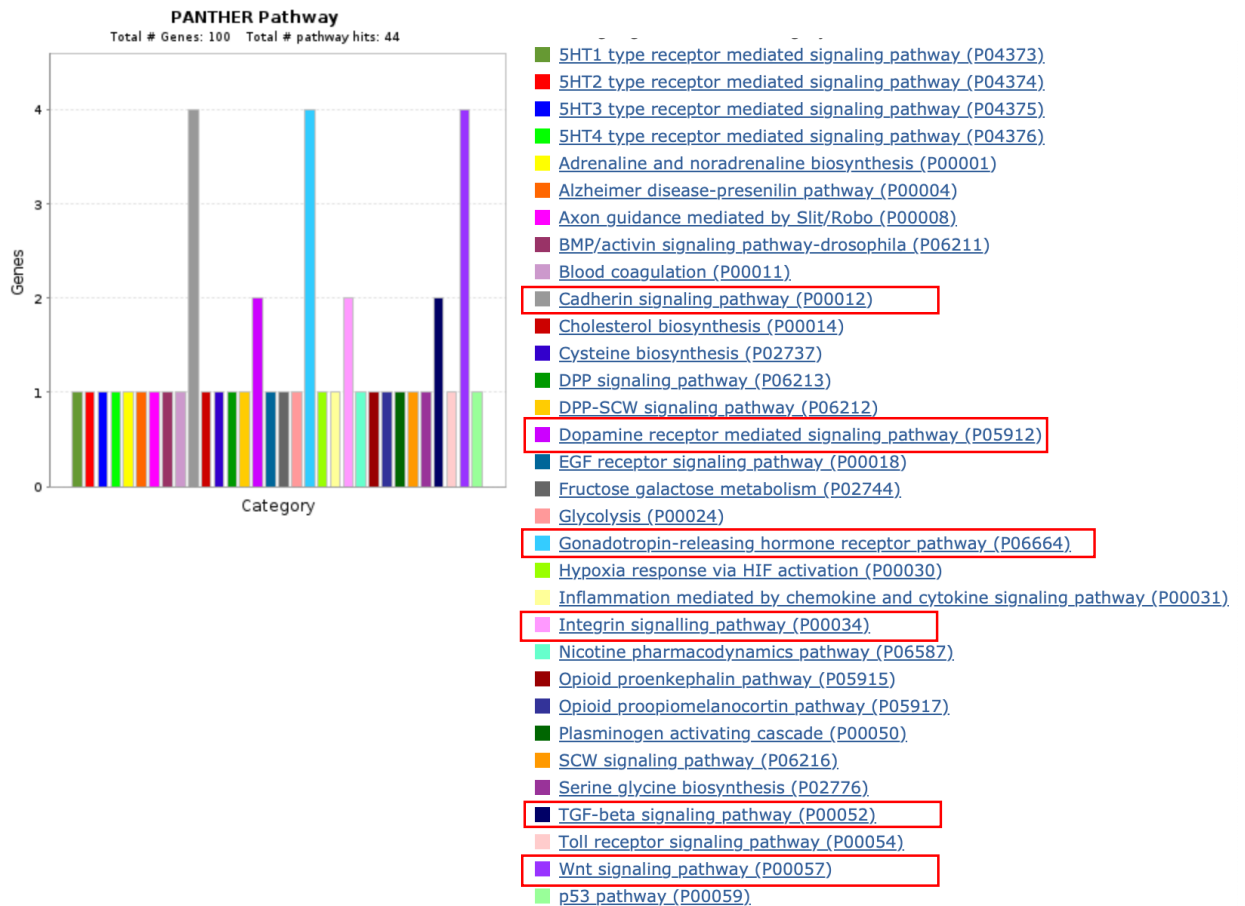


Figure 3.21 Bar chart displaying the PANTHER pathways analysed from the MCF7 RNAseq list (96 overlapped significantly upregulated DEGs in hypoxia and hypoxia-reoxygenation from MCF7RNAseq). Red boxes highlight the top enriched pathways. Chart tooltips are read as: Category name (Accession): # genes; Percent of gene hit against total # genes; Percent of gene hit against total # Function hits. This figure generated by PANTHER classification system. Figure generated using PANTHER Classification system (Thomas PD. et al. 2022).

PANTHER Pathway Category	Accession	Genes
Cadherin signalling pathway	(P00012)	CDH10 / CDH2 / FZD8 / PCDH7
Gonadotropin-releasing hormone receptor pathway	(P06664)	INHA / INHBA / LHX2 / PPARA
Wnt signaling pathway	(P00057)	CDH10 / CDH2 / FZD8 / PCDH7
TGF-beta signaling pathway	(P00052)	NHBA / CITED2
Dopamine receptor mediated signalling pathway	(P05912)	KCNK3 / COMT
Integrin signalling pathway	(P00034)	FN1 / LAMB3

Table 3.10 Shows the most enriched pathways from upregulated DEGs in MCF7 RNAseq during hypoxia and hypoxia-reoxygenation. Adapted from PANTHER Classification system (Thomas PD. et al. 2022).

Additional PANTHER protein classification analysis of the overlapped DEGs

The PANTHER database also provides entry to protein class ontology, which includes commonly used class of protein function; this was adapted from PANTHER molecular function ontology. Here, this feature was used to investigate protein class associated with the MDA-MB-231 RNAseq 358 significantly upregulated overlapped DEGs in hypoxia and hypoxia-reoxygenation (Figure 3.22A). gene-specific transcriptional regulator class consisted of several DNA-binding transcription factors (Figure 3.22B). The analysis revealed that 24 DEGs were found to be associated with the 5 different DNA-binding transcription factors, including HMG box transcription factor (PC00024, 1 DEG), Basic helix-loop-helix transcription factor (PC00055, 1 DEG), Basic leucine zipper transcription factor (PC00056, 5 DEGs), Helix-turn-helix transcription factor (PC00116, 6 DEGs), and Zinc finger transcription factor (PC00244, 11 DEGs). Table 3.11 shows the different DNA-binding transcription factors along with their unique ID, gene name and PANTHER protein classification (Table 3.11).

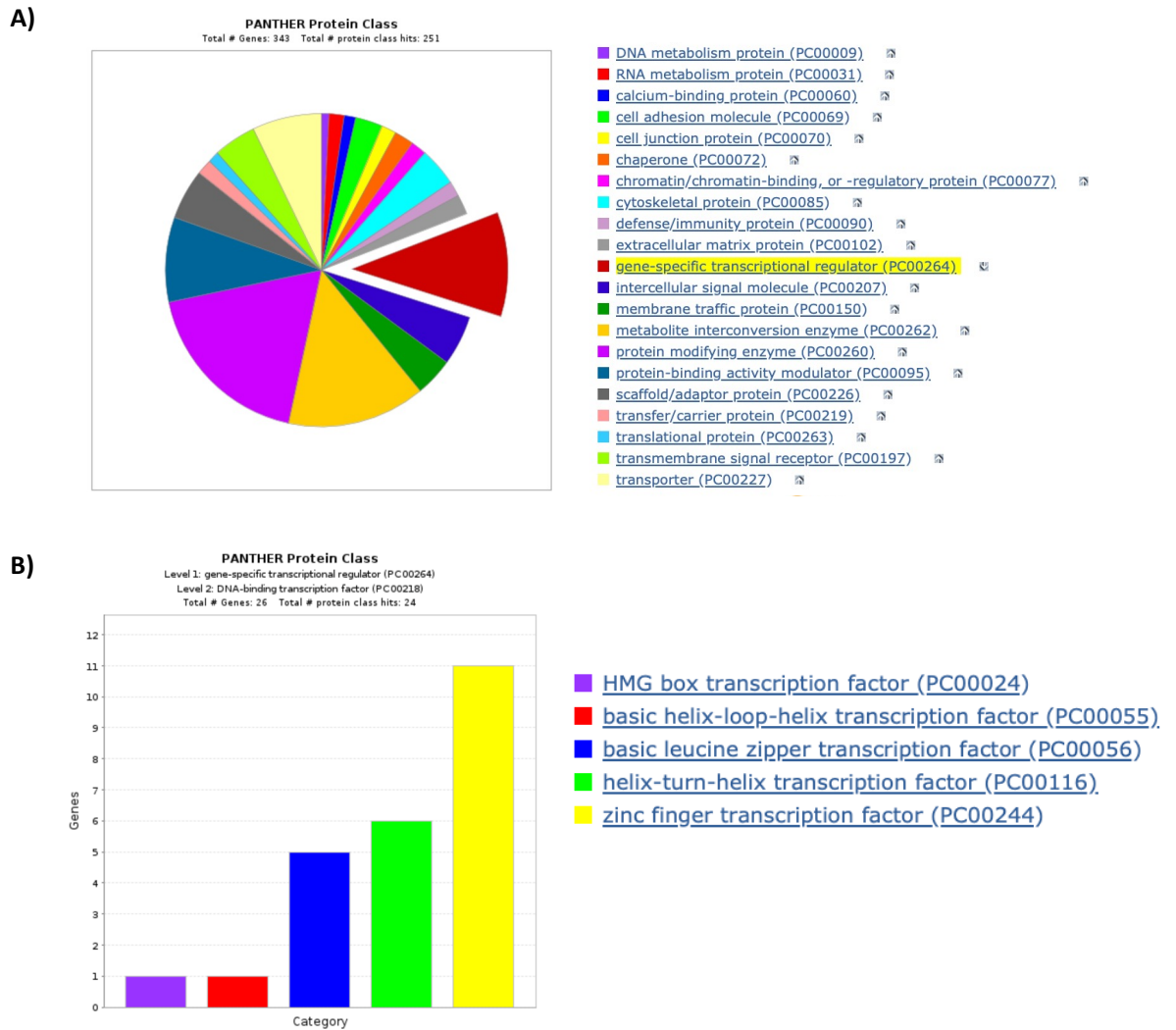


Figure 3.22: Bar chart displaying downstream protein class analysis in MDA-MB-231 RNAseq. A) Panther protein classes involved in the overlapped DEGs list in MDA-MB-231. **B)** DNA-binding transcription factors within the gene-specific transcription regulator class. Figure generated using PANTHER Classification system (Thomas PD. et al. 2022)

DNA-binding transcription factor (PC00218)	Gene Name	PANTHER Protein Class
HMG box transcription factor (PC00024)	SOX4	HMG box transcription factor
Basic helix-loop-helix transcription factor (PC00055)	MXI1	basic helix-loop-helix transcription factor
Basic leucine zipper transcription factor (PC00056)	LZTFL1	basic leucine zipper transcription factor
	NFIL3	basic leucine zipper transcription factor
	MAFF	basic leucine zipper transcription factor
	CEBPD	basic leucine zipper transcription factor
	MAFK	basic leucine zipper transcription factor
Helix-turn-helix transcription factor (PC00116)	FOXO4	winged helix/forkhead transcription factor
	HOXD1	homeodomain transcription factor
	PAX8 AC016683	homeodomain transcription factor
	TGIF1	homeodomain transcription factor
	ETS1	winged helix/forkhead transcription factor
	FLI1	winged helix/forkhead transcription factor
Zinc finger transcription factor (PC00244)	KLF3	C2H2 zinc finger transcription factor
	ZNF84	C2H2 zinc finger transcription factor
	ESR2	C4 zinc finger nuclear receptor
	KLF4	C2H2 zinc finger transcription factor
	ZNF160	C2H2 zinc finger transcription factor
	RORA	C4 zinc finger nuclear receptor
	KLF7	C2H2 zinc finger transcription factor
	ZBTB7B	C2H2 zinc finger transcription factor
	KLF5	C2H2 zinc finger transcription factor
	ZNF517	C2H2 zinc finger transcription factor
	ZNF165	C2H2 zinc finger transcription factor

Table 3.11 List of associated genes in the DNA-binding transcription factor class (PC00218). Adapted from PANTHER Classification system (Thomas PD. et al. 2022)

3.4 Analysis of the top significantly upregulated DEGs overlapped in hypoxia and hypoxia-reoxygenation

Both previously identified lists of overlapped MDA-MB-231 (358 DEGs) and MCF7 (96 DEGs) (Figure 14C and Figure 15C) have been adjusted to focus only on the top targets in each set based on the Log₂Fold change. The top 20 DEGs featured in MDA-MB-231 RNAseq and MCF7 RNAseq are presented in (Table 3.12) and (Table 3.13). Each dataset was arranged from the targets with the higher Log₂Fold changes to the targets with the smallest Log₂Fold changes, along with the read count and p-values for each target (Table 3.12) and (Table 3.13).

These top 20 genes of interest identified from each dataset were further investigated to explore their gene expression in breast cancer based on transcriptomic and clinicopathological data. In addition to exploring their association with clinical outcomes, including overall survival (OS) or disease-free survival (DFS). qPCR experiments were then performed to validate the RNAseq results.

Gene Name	Hypoxia-Reoxygenation	Normoxia	Log ₂ Fold Change	p-value
CST1	11453.4029	1708.602301	2.4992	9.56E-51
LURAP1L	284.328049	38.53106693	2.2459	9.98E-21
PTPRR	126.1369024	21.96560967	1.8224	1.49E-12
GPRC5B	746.8954148	170.7661215	1.8067	4.94E-18
SPINT1	440.6014353	104.4787746	1.7878	1.15E-18
CST4	2795.355641	717.1401482	1.7827	4.86E-26
ICAM1	2322.581773	340.2522489	1.7378	6.47E-10
DUSP8	103.2829453	22.58575203	1.6625	2.23E-11
PTPRB	126.8538164	24.3967492	1.6596	4.30E-10
CLDN1	415.6054007	52.90532231	1.651	1.15E-08
NYAP1	123.7927081	27.2953946	1.6415	5.74E-11
CDK18	87.19073699	18.51039699	1.6034	9.55E-10
DUSP1	1322.107207	385.4278032	1.5993	8.21E-20
TNFSF15	819.0471396	64.42902249	1.5939	4.84E-08
RRAD	675.2071734	199.4743153	1.5801	2.74E-19
RELN	93.39026283	13.68095704	1.573	4.66E-08
TM4SF19	146.5954737	39.89499704	1.5311	1.09E-11
MUC1	605.8776413	172.7782346	1.5259	8.37E-13
ZNF165	128.9462894	34.43901198	1.5003	2.20E-10
TNFSF9	240.0708628	71.51380051	1.4943	2.66E-13

Table 3.12: Top 20 significantly upregulated DEGs overlapped in hypoxia and hypoxia-reoxygenation in MDA-MB-231 RNAseq. The list is arranged from DEGs with higher Log₂Fold change to the smallest.

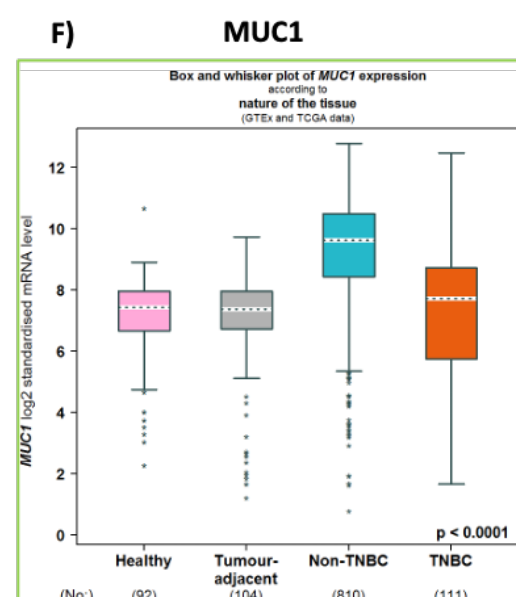
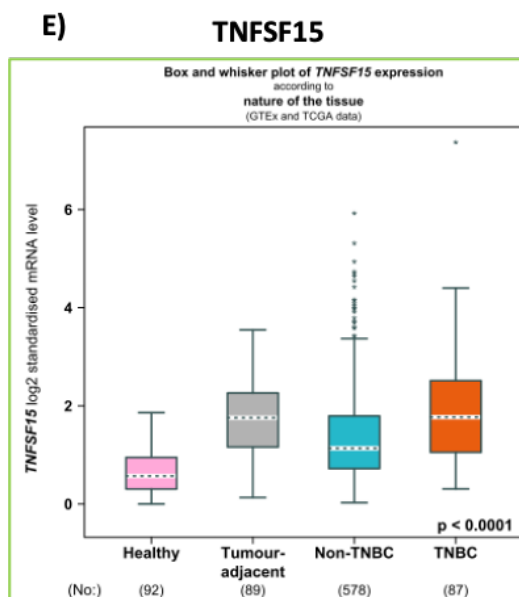
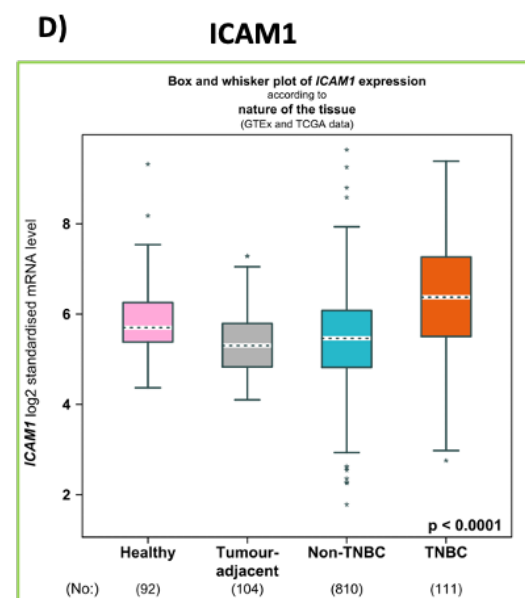
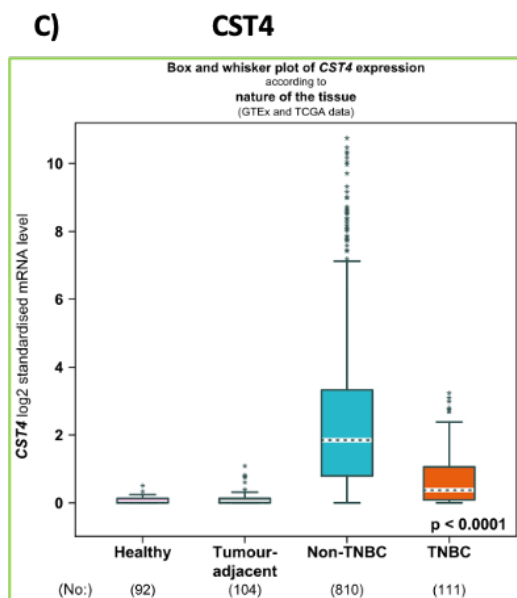
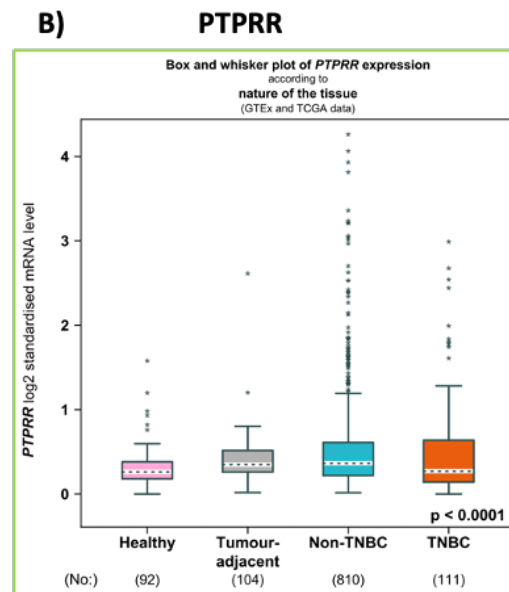
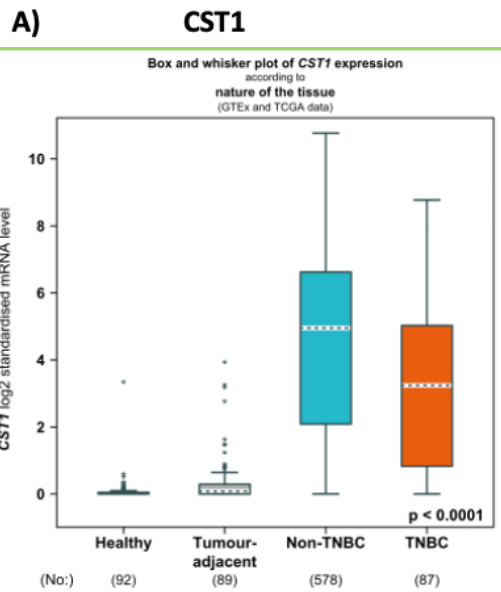
Gene Name	Hypoxia-Reoxygenation	Normoxia	Log ₂ Fold Change	p-value
KRT17P8	3.37443715	0	4.292902157	0.04221456
BPIFB2	8.924823958	0.60923158	3.831034169	0.0073889
FGB	17.32304981	1.84994161	3.21042627	0.00029819
PTGS1	533.4447328	58.571619	3.190139005	6.32E-14
CDH2	22.05290546	3.74457283	2.55594738	0.00049717
AC093012.1	7.24576915	1.22958659	2.548174478	0.03081869
KCNK3	61.53198072	12.7850957	2.270448761	6.72E-07
P3H2	27.7498105	5.94967539	2.20980137	0.003107
SEMA5B	49.49947164	10.7035263	2.203157261	0.03082025
CDH10	22.60193964	5.73990262	1.990796109	0.00210964
GLP2R	268.9626682	70.8817479	1.924420756	3.40E-08
CHRD	1552.11409	414.115298	1.906074528	7.35E-07
EDIL3	69.15500341	21.7944085	1.669355476	7.60E-06
DIO2	1281.576248	404.899151	1.662122341	8.52E-07
ABCA4	266.7592	84.2463104	1.659469579	7.41E-05
FZD8	837.2712549	303.21302	1.467260755	7.79E-10
MDGA2	44.05026392	16.8169136	1.393135347	0.01294204
PRR29	33.91673347	12.9384678	1.377126655	0.00932757
ANK2	148.0352543	58.5199303	1.336727374	0.01251446
ODAM	49.88152728	20.1427244	1.317183153	0.02291499

Table 3.13 Top 20 significantly upregulated DEGs overlapped in hypoxia and hypoxia-reoxygenation from MCF7 RNAseq. The list is arranged from DEGs with higher Log2Fold change to the smallest.

3.4.1 Expression of the overlapped DEGs in breast cancer tissues.

The expression of the top 20 significantly upregulated DEGs in hypoxia and hypoxia-reoxygenation from MDA-MB-231 and MCF7 RNAseq dataset in breast cancer tissues was investigated using the Breast Cancer Gene-Expression Miner (bc-GenExMiner). The online tool was used to compare the expression of these genes in healthy (non-diseased/normal tissue), tumour adjacent, and breast cancer patient's tumour tissues, which included TNBC, HER2 (-ve), and (+ve) tissues. This comparison was done using an RNAseq TCGA dataset (n=743).

From the MDA-MB-231 RNAseq dataset, the analysis showed that several genes were significantly overexpressed in TNBC tissues compared to healthy tissues ($p < 0.0001$), including CST1, PTPRR, CST4, ICAM1, TNFSF15, MUC1, ZNF165 and SPINT1 (Figure 3.23A-H). Using the same tool, the analysis also compared the expression of the MDA-MB-231 RNAseq dataset in HER2 (-ve) tissues and showed that CST1, PTPRR, CST4, TNFSF15, MUC1, ZNF165 and SPINT1 significantly overexpressed in HER2- tissue compared to healthy tissue ($p < 0.0001$) (Figure 3.24A-F). The analysis investigated all top 20 genes of interest in the dataset (Table 3.12), but they were not presented here as either their expression was not significant or they were not overexpressed in TNBC or HER2-ve tissue when compared to healthy tissue.



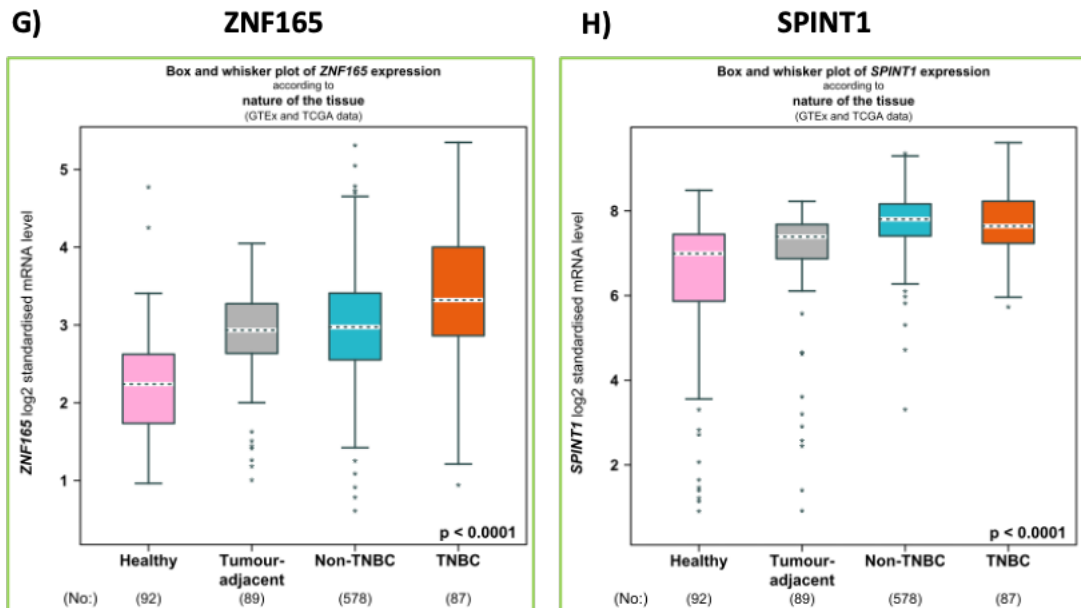
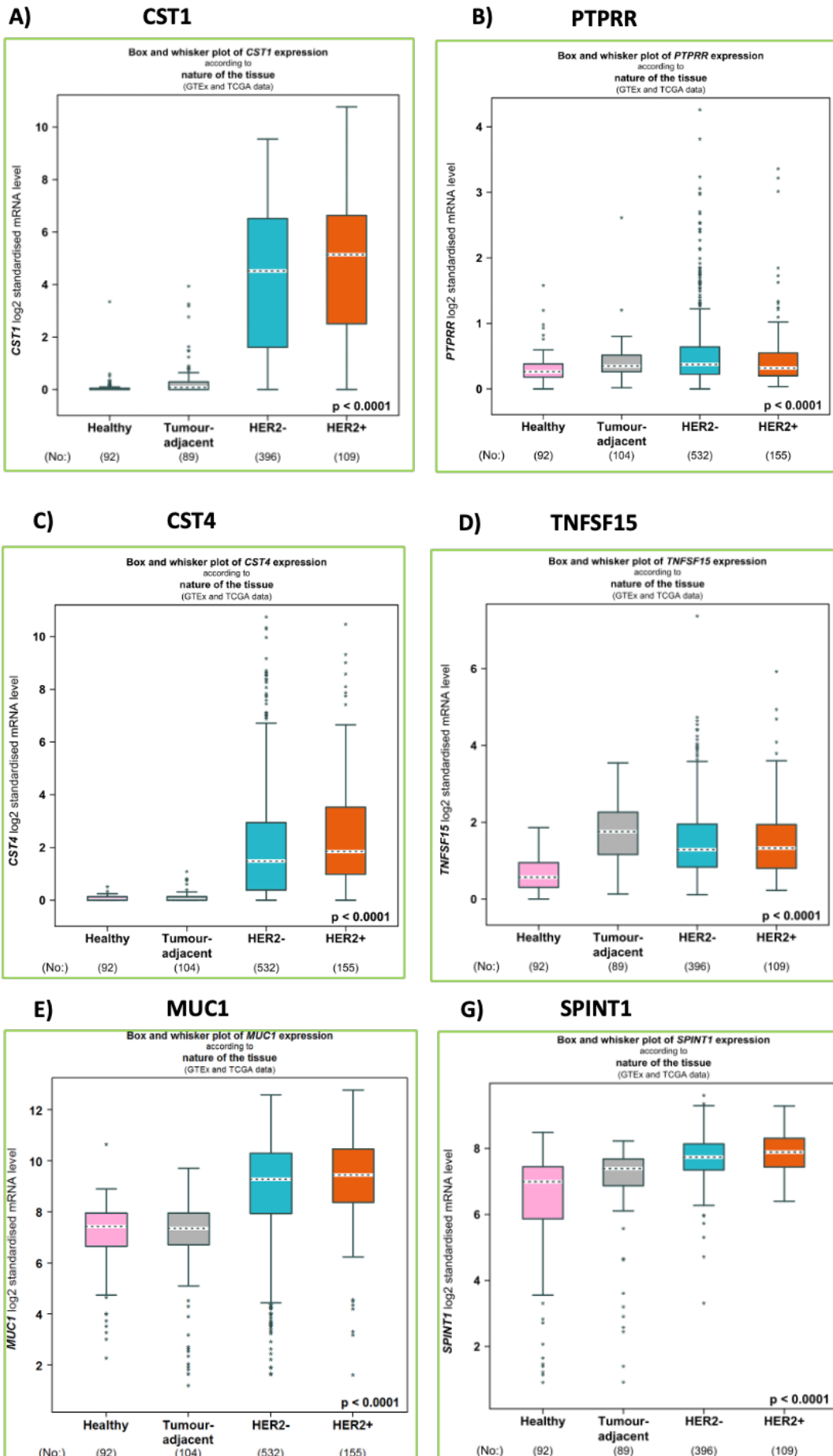


Figure 3.23 Displays genes expression in healthy, tumour adjacent, non-TNBC, and TNBC tissues using a box and whisker plot. Overlapped upregulated DEGs in hypoxia and hypoxia reoxygenation in MDA-MB-231 RNAseq. Figure generated using bc-GenExMiner (Jézéquel P. et al. 2021)



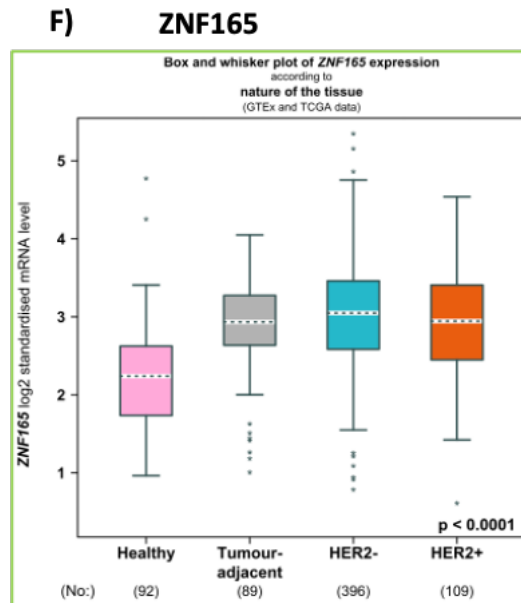
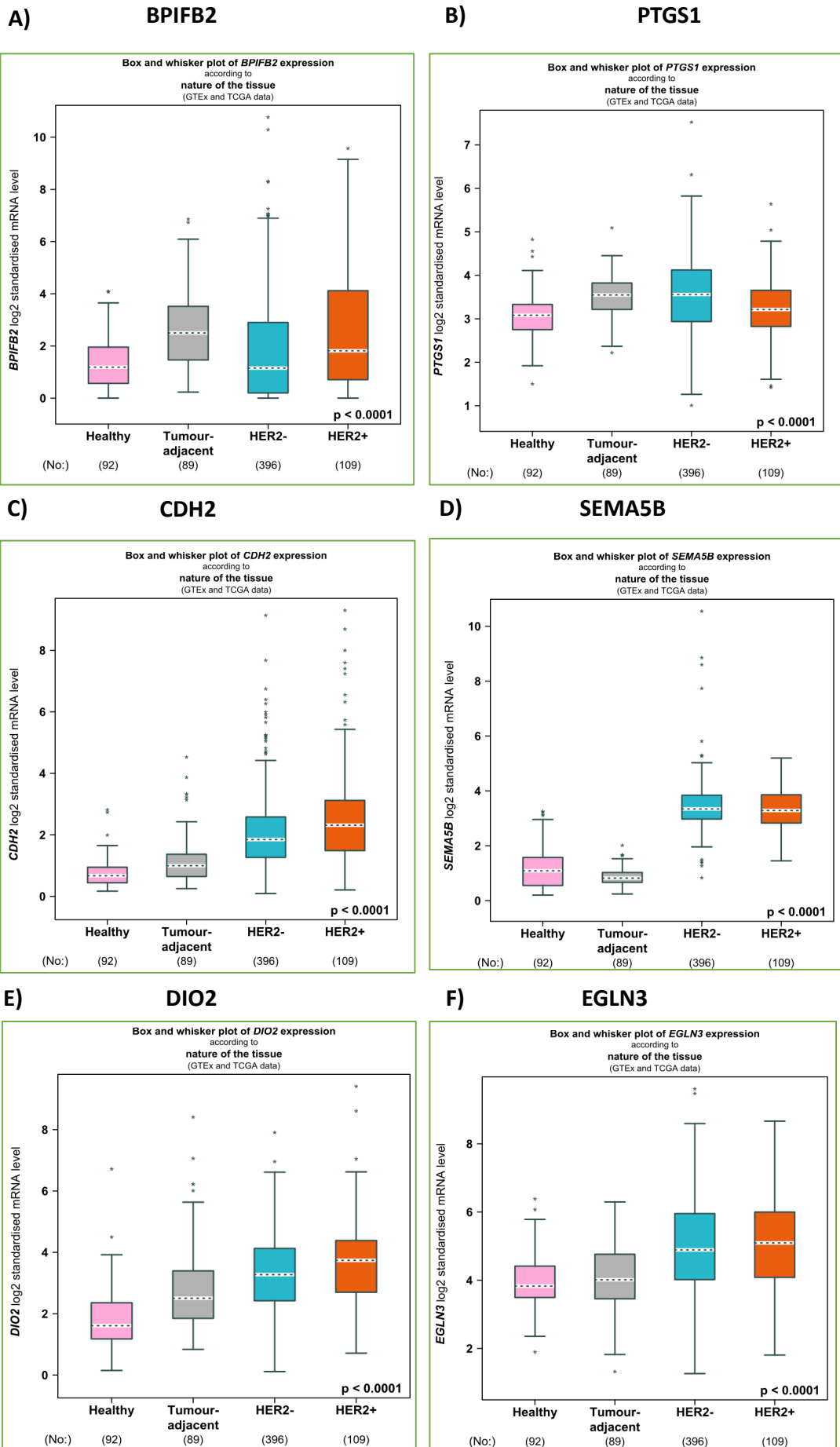


Figure 3.24 Shows the expression of genes in healthy, tumour-adjacent, HER2-ve, and HER+ve tissues, using a box and whisker plot. Overlapped upregulated DEGs in hypoxia and hypoxia reoxygenation in MDA-MB-231 RNAseq. Figure generated using bc-GenExMiner (Jézéquel P. et al. 2021)

Several significantly upregulated genes overlapped in hypoxia and hypoxia-reoxygenation in MCF7 cells were also overexpressed in HER2- tissues compared to healthy tissue ($p < 0.0001$), including BPIFB2, PTGS1, CDH2, SEMA5B, DIO2, EGLN3, EDIL3 and MDGA2 (Figure 3.25A-H). To compare the expression in different breast tissues, the same tool was used, and the analysis compared the expression of the top 20 overlapped genes in the MCF7 RNAseq dataset in TNBC tissues ($n=743$). CDH2, SEMA5B, DIO, EGLN3 and MDGA2 overexpressed in TNBC tissues compared to healthy tissue ($p < 0.0001$). ODAM significantly showed overexpression in TNBC tissues compared to healthy tissue ($p < 0.0001$) but not in HER2- tissues compared to healthy tissue (Figure 3.26F). Other targets from the DEGs featured in Table 3.13 have been explored, but they were significantly overexpressed more in healthy tissues than in HER2- and TNBC tissues ($p < 0.0001$), including PRR29, ANK2, GLP2R and CHRD (supplementary figure). Table 3.14 compares the top 20 overlapped DEGs in hypoxia and hypoxia-reoxygenation in MCF7 RNAseq and their expression in breast cancer patients' tissues.



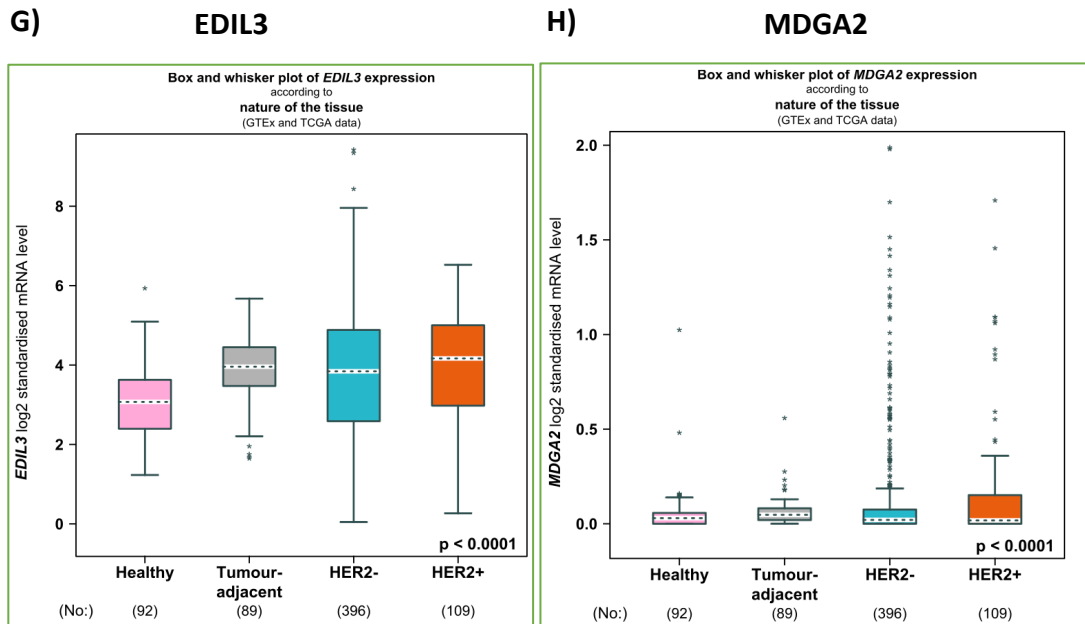


Figure 3.25 Shows a box and whisker plot of gene expression in healthy, tumor-adjacent, HER2-, and HER+ tissue. Overlapped upregulated DEGs in hypoxia and hypoxia reoxygenation in MCF7 RNAseq. Figure generated using bc-GenExMiner (Jézéquel P. et al. 2021)

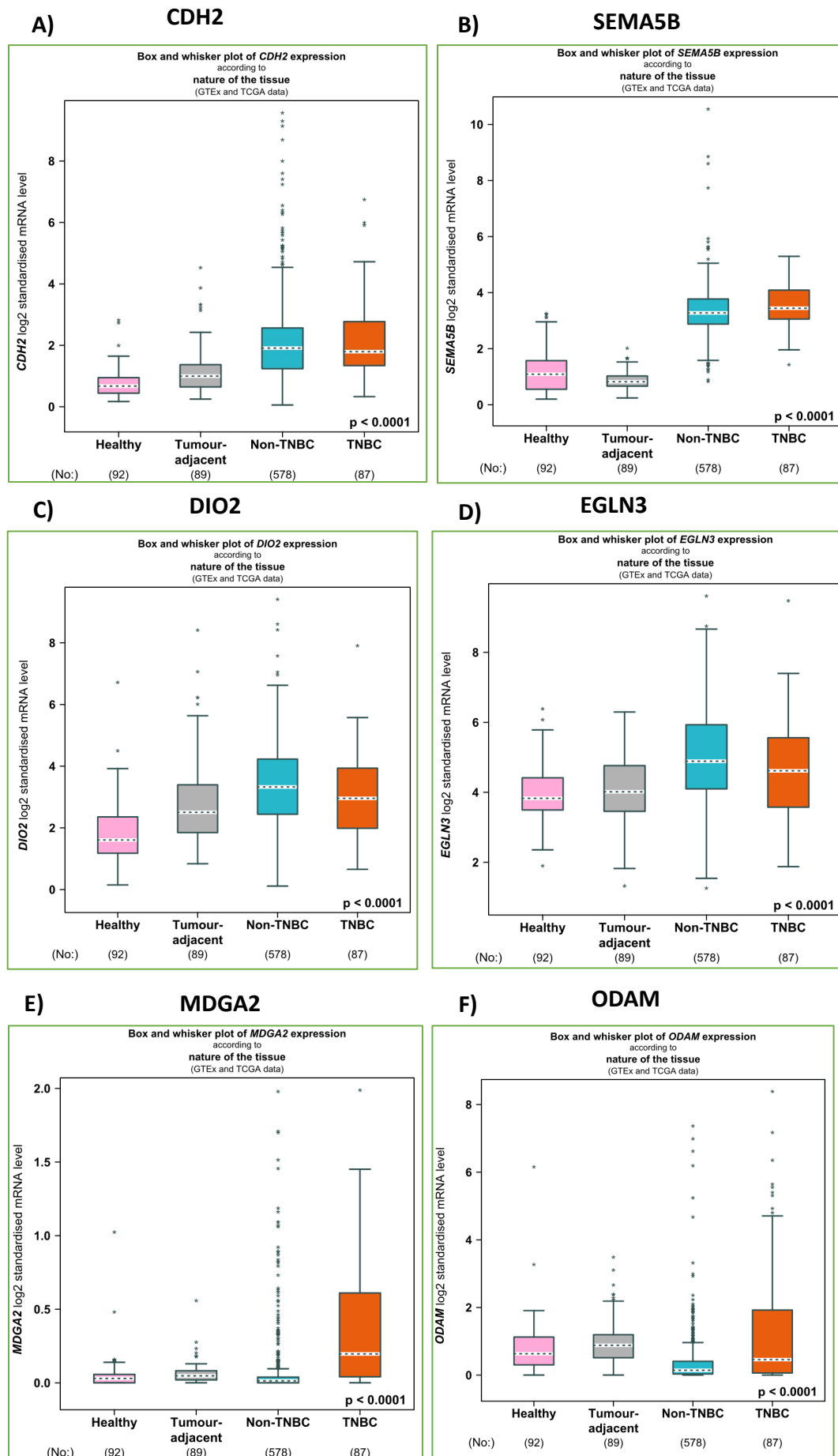


Figure 3.26 shows the expression of genes in healthy, tumor adjacent, non-TNBC, TNBC, and TNBC based on the nature of tissue using a box and whisker plot. Overlapped upregulated DEGs in in hypoxia and hypoxia reoxygenation in MCF7 RNAseq. Figure generated using bc-GenExMiner (Jézéquel P. et al. 2021)

Gene ID	HER2- vs Healthy	p-value	TNBC vs Healthy	p-value 2
ABCA4	HER2- < Healthy	p<0.01	TNBC = Health	p>0.01
FZD8	HER2- = Healthy	p>0.10	TNBC = Health	p>0.01
ODAM	HER2- = Healthy	p>0.10	TNBC > Healthy	p < 0.0001
PRR29	HER2- < Healthy	p< 0.0001	TNBC < Healthy	p < 0.0001
ANK2	HER2- < Healthy	p< 0.0001	TNBC < Healthy	p < 0.0001
CDH10	HER2- < Healthy	p<0.05	TNBC < Healthy	p>0.01
GLP2R	HER2- < Healthy	p< 0.0001	TNBC < Healthy	p< 0.0001
CHRD	HER2- < Healthy	p< 0.0001	TNBC < Healthy	p< 0.0001
EDIL3	HER2- > Healthy	p< 0.0001	TNBC = Health	p>0.01

Table 3.14 Comparing the expression of genes in different breast tumour tissues. Overlapped upregulated DEGs in hypoxia and hypoxia reoxygenation in MCF7 RNAseq. Adapted from bc-GenExMiner (Jézéquel P. et al. 2021)

3.4.2 qPCR validation of the overlapped upregulated DEGs

MDA-MB-231 and MCF7 cell lines were used to validate the mRNA expression of some of the genes that were upregulated in hypoxia and maintained in hypoxia-reoxygenation (genes featured in Table 3.12 and Table 3.13). Following the cDNA synthesis (explained in the Methods), MDA-MB-231 and MCF7 cells were exposed to physoxia (72 hours 8.5% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂). The MDA-MB-231 RNAseq experiment was conducted under normoxia (21% O₂). However, for the qPCR experiment, the cells were investigated under physoxia (8.5% O₂) for two reasons: 1) physoxia (8.5% O₂) represents the oxygen levels within human breast tissue (Vaupel P, Höckel M, Mayer A, 2007), and 2) provides a better interpretation of gene expression levels for genes of interest when compared to MCF7 RNAseq, which was conducted under physoxia (8.5% O₂). The mRNA expression was analysed using qPCRs.

MDA-MB-231 RNAseq qPCR validation

The qPCR validation for the targets identified from the MDA-MB-231 RNAseq is presented in Figures (Figure 3.27) and (Figure 3.28). The analysis showed that mRNA expression of MUC1 was significantly upregulated over 1.5-fold in hypoxia (Log₂fold change=1.788, p<0.05, n=3), the expression has doubled significantly in hypoxia-reoxygenation (Log₂fold change=2.8, p<0.001, n=3) showing that MUC1 was significantly upregulated in hypoxia and maintained in hypoxia-reoxygenation (Figure 3.27A).

ICAM1 (Log₂fold change=1.3, p= 0.281, n=3) and DUSP8 (Log₂fold change=1.45, p= 0.9, n=3) showed upregulation in hypoxia, however it wasn't significant. Both ICAM1 and DUSP8 have significantly upregulated in hypoxia-reoxygenation, ICAM1 (Log₂fold change=1.75, p<0.05, n=3) and DUSP8 (Log₂fold change=1.45, p<0.05, n=3) (Figure 3.27B and Figure 3.27C). DUSP1 has significantly upregulated in hypoxia (Log₂fold change=1.45, p<0.05, n=3), but the expression was not maintained upon hypoxia-reoxygenation (Figure 3.27D). CST1 expression has significantly downregulated in hypoxia and hypoxia-reoxygenation (Figure 3.27E).

The expression of some of these genes was also validated in the MCF7 cell line (Figure 3.29), including MUC1, ICAM and DUSP8 (Figure 3.89A-C). The analysis showed upregulation of

mRNA expression under hypoxia and significant upregulation under hypoxia-reoxygenation (p-value= p<0.05).

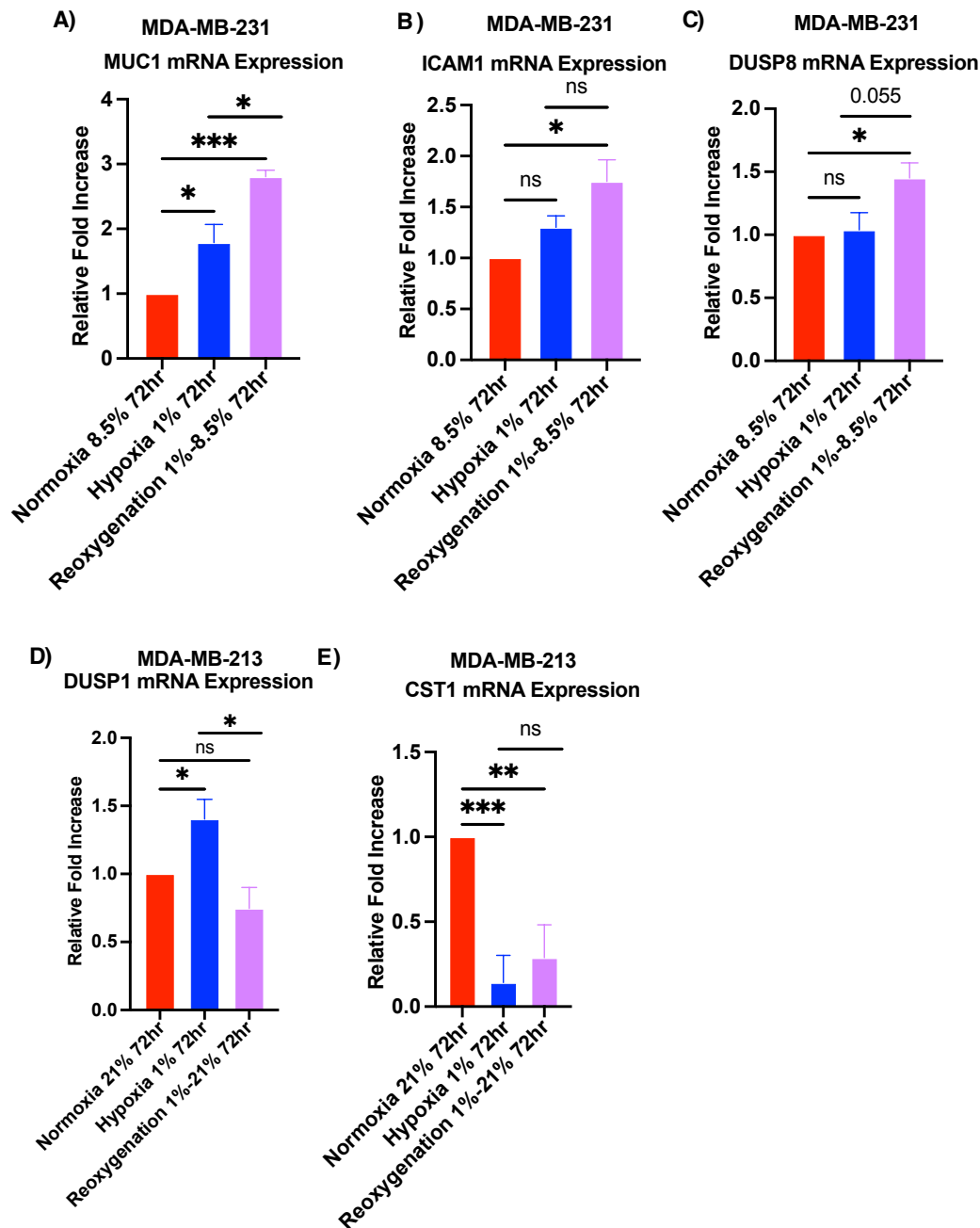


Figure 3.27 Validation of the overlapped upregulated DEGs in hypoxia and hypoxia-reoxygenation in MDA-MB-231 RNAseq in MDA-MB-231 cell line using qPCR. A) – C) MUC1, ICAM1 and DUSP8 mRNA expression, RNA samples were generated as described previously following exposure to physoxia (72 hours 8.5% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂). D-E) DUSP1 and CST1 mRNA expression, RNA samples were exposed to normoxia (72 hours 21% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 21% O₂). The expression levels in MDA-MB-231 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Statistical analysis using one-way ANOVA followed by Dunnett's multiple comparisons test. *p<0.05, **p<0.01, and ***p<0.001, respectively. n=3

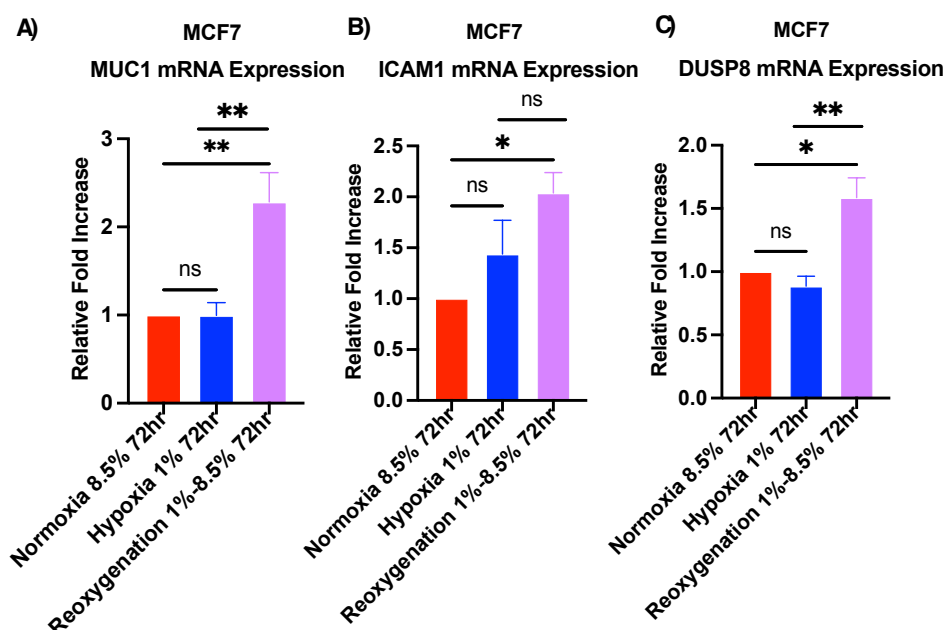


Figure 3.28 Validation of the overlapped upregulated DEGs in hypoxia and hypoxia-reoxygenation in MDA-MB-231 RNAseq in MCF7 cell line using qPCR. RNA samples were generated as described previously following exposure to physoxia (72 hours 8.5% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂). **A) – C)** MUC1, ICAM1 and DUSP8 mRNA expression in MCF7 cells under hypoxia and hypoxia-reoxygenation. The expression levels in MCF7 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Statistical analysis using one-way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$. $n = 3$

MCF7 RNAseq qPCR validation

The qPCR validation for the targets identified from the MCF7 RNAseq is presented in Figures (Figure 3.29) and (Figure 3.30). The analysis showed that the mRNA expression of BPIFB2 significantly upregulated in hypoxia (Log₂fold change=2.54, $p < 0.05$, $n = 3$); the expression was upregulated in hypoxia-reoxygenation, however, it was not as significant (Log₂fold change=1.49, $p = 0.5$, $n = 3$) (Figure 3.29A). PTGS1 upregulated in hypoxia (Log₂fold change=1.7, $p = 0.1$, $n = 3$) and significantly upregulated in hypoxia-reoxygenation (Log₂fold change=14, $p < 0.0001$) (Figure 3.29B). EDIL3 had significant upregulation (over 2-fold) in hypoxia (Log₂fold change=2.18, $p < 0.05$, $n = 3$) and maintained significantly in hypoxia-reoxygenation (Log₂fold change=2.58, $p < 0.01$, $n = 3$) (Figure 3.29D).

CDH2 (Log₂fold change=1.21, p=0.444, n=3) and SEMA5B (Log₂fold change=1.47, p=0.58, n=3) mRNA expression upregulated in hypoxia, and in hypoxia-reoxygenation CDH2 (Log₂fold change=1.5, p=0.054, n=3) and SEMA5B (Log₂fold change=1.5, p=0.537, n=3), however these were not statistically significant (Figure 3.30C and Figure 3.29E).

The qPCR validation has also included additional interesting targets from the significantly overlapped upregulated DEGs in hypoxia and hypoxia-reoxygenation from MCF7 RNAseq, such as LOXL2 and INHBA (Figure 3.29F-G). LOXL2 is a hypoxia-inducible gene; the MCF7 RNAseq showed that it is upregulated in hypoxia and maintained upon reoxygenation. The qPCR validation showed significant upregulation for LOXL2 under hypoxia (Log₂fold change=4.85, p<0.0001, n=3), the upregulation maintained in hypoxia but was not statistically significant (Log₂fold change= 1.588, p=0.251, n=3) (Figure 3.29F). INHBA is a growth factor and a member of the TGF- β superfamily of proteins, which has also overlapped in hypoxia and hypoxia-reoxygenation in MCF7 RNAseq. qPCR validation of INHBA showed that it was significantly upregulated in hypoxia (Log₂fold change=3.777, p<0.01, n=3) the expression was maintained in hypoxia-reoxygenation but was not statistically significant (Log₂fold change= 1.607, p-value=0.687, n=3) (Figure 3.29G).

MCF7 RNAseq results (Table 3.13) were also validated in MDA-MB-231 cell line (Figure 3.30). the analysis showed that some genes had upregulated in hypoxia and maintained in hypoxia-reoxygenation such as EDIL3, LOXL2 and INHBA while other genes had downregulation in hypoxia and upregulation in hypoxia-reoxygenation however none of these genes were statistically significant (Figure 3.30A-F).

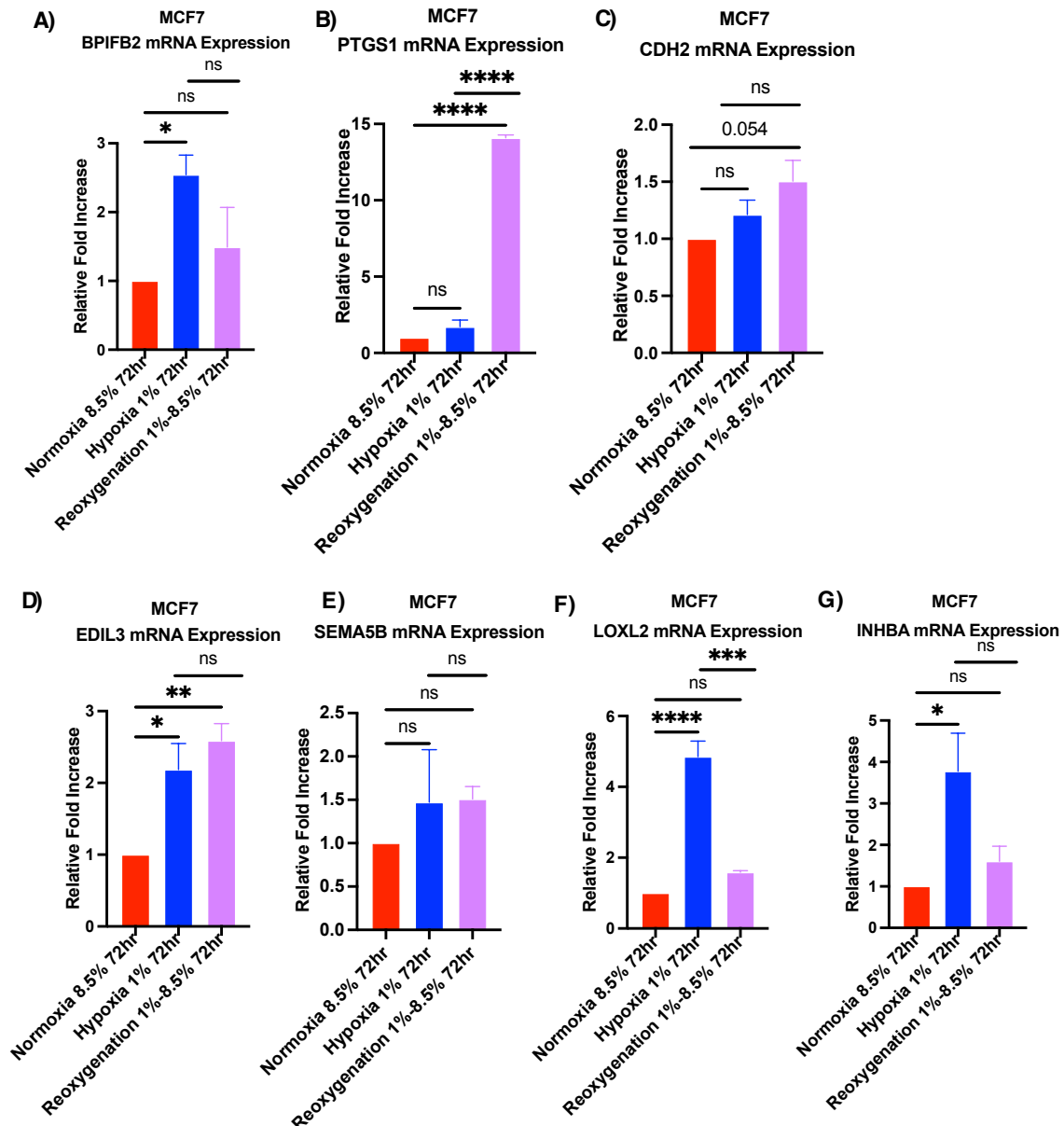


Figure 3.29 Shows validation of the overlapped upregulated DEGs in hypoxia and hypoxia-reoxygenation from MCF7 RNAseq in MCF7 cell line using qPCR. RNA samples were generated as described previously following exposure to physoxia (72 hours 8.5% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂). **A-E)** mRNA expression of BPIFB2, PTGS1, CDH2, EDIL3 and SEMA5B, from list of top 20 overlapped DEGs in hypoxia and hypoxia-reoxygenation from MCF7 RNAseq. **F-G)** mRNA expression of LOXL2 and INHBA, from list of 96 overlapped DEGs in hypoxia and hypoxia-reoxygenation from MCF7 RNAseq. The expression levels in MCF7 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Statistical analysis using one-way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ respectively. $n = 3$.

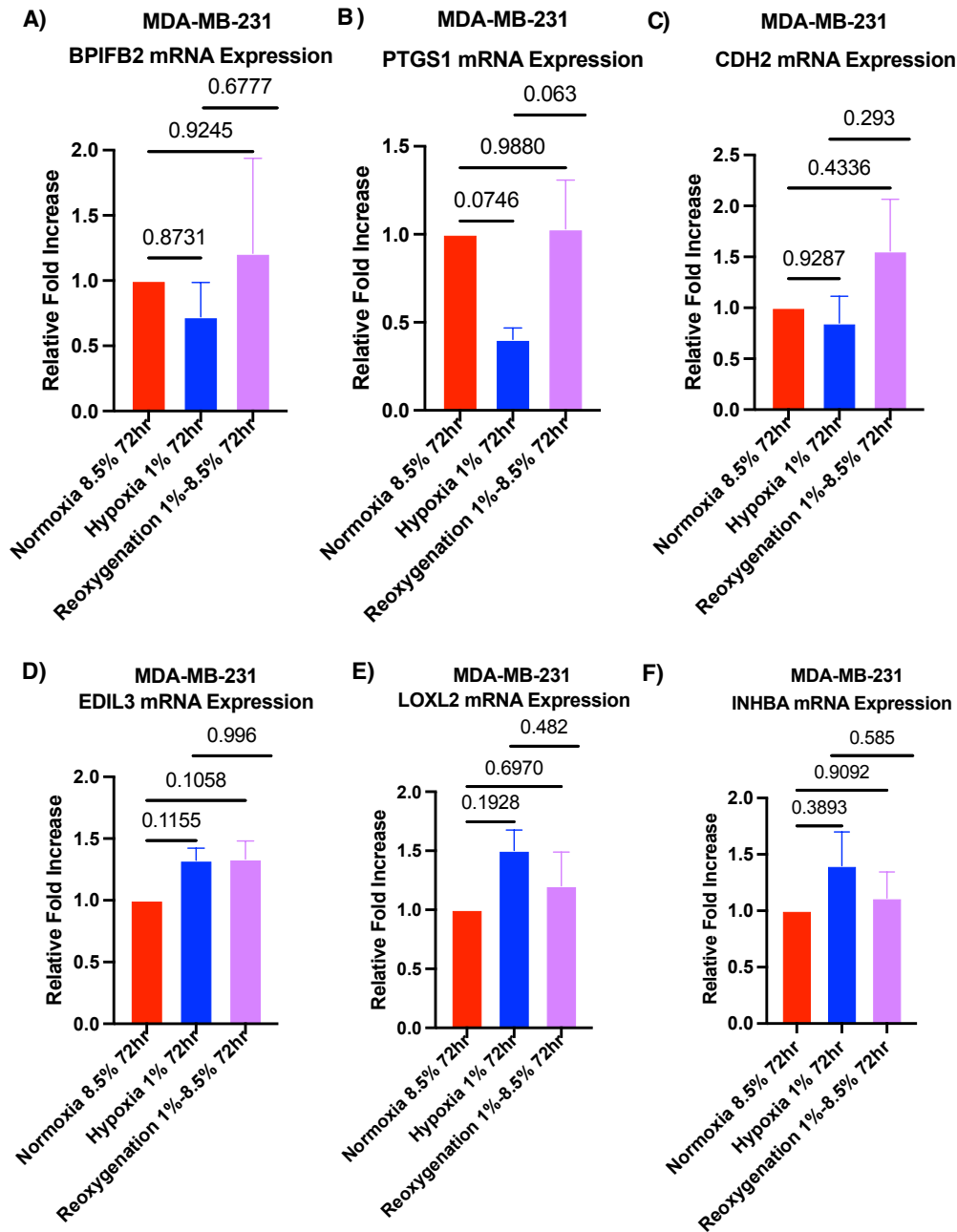


Figure 3.30 Shows validation of the overlapped upregulated DEGs in hypoxia and hypoxia-reoxygenation from MCF7 RNAseq in the MDA-MB-231 cell line using qPCR. RNA samples were generated as described previously following exposure to physoxia (72 hours 8.5% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂). **A-E)** mRNA expression of BPIFB2, PTGS1, CDH2, EDIL3 and SEMA5B from the list of top 20 overlapped DEGs in hypoxia and hypoxia-reoxygenation from MCF7 RNAseq. **F-G)** mRNA expression of LOXL2 and INHBA, from the list of 96 overlapped DEGs in hypoxia and hypoxia-reoxygenation from MCF7 RNAseq. The expression levels in MDA-MB-231 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Statistical analysis using one-way ANOVA followed by Dunnett's multiple comparisons test. n=3.

3.4.3 Hypoxia and Hypoxia-reoxygenation regulate growth factors in breast cancer.

After analysing the RNAseq libraries of two types of breast cancer cells, TNBC (MDA-MB-231) and Luminal A (MCF7), it was observed that several growth factors were upregulated under hypoxic conditions and in hypoxia-reoxygenation. To identify these growth factors, the PANTHER PATHWAY classification system was used, which provided a list of 95 known growth factors in humans (*Homo Sapiens*). The VENNY online tool was then used to compare the PANTHER growth factors list with the list of upregulated DEGs under hypoxia vs normoxia conditions (Figure 3.31A and Figure 3.31C), as well as under hypoxia-reoxygenation vs normoxia conditions (Figure 3.31B and Figure 3.31D) in both RNAseq libraries.

The analysis revealed that 9 different growth factors were expressed under hypoxia and another 9 expressed under hypoxia-reoxygenation in MDA-MB-231 RNAseq (Figure 3.31A-B). Interestingly, there were few similarities between hypoxia and hypoxia-reoxygenation as some growth factors expressed under both conditions including TGFB2, VEGFA, VEGFC and PGF (Table 3.15). MCF7 RNAseq analysis revealed that 11 growth factors expressed under hypoxia and 3 under hypoxia-reoxygenation (Figure 3.31C-D). A list of all expressed growth factors under hypoxia and hypoxia-reoxygenation in both RNAseq libraries is included in (Table 3.13).

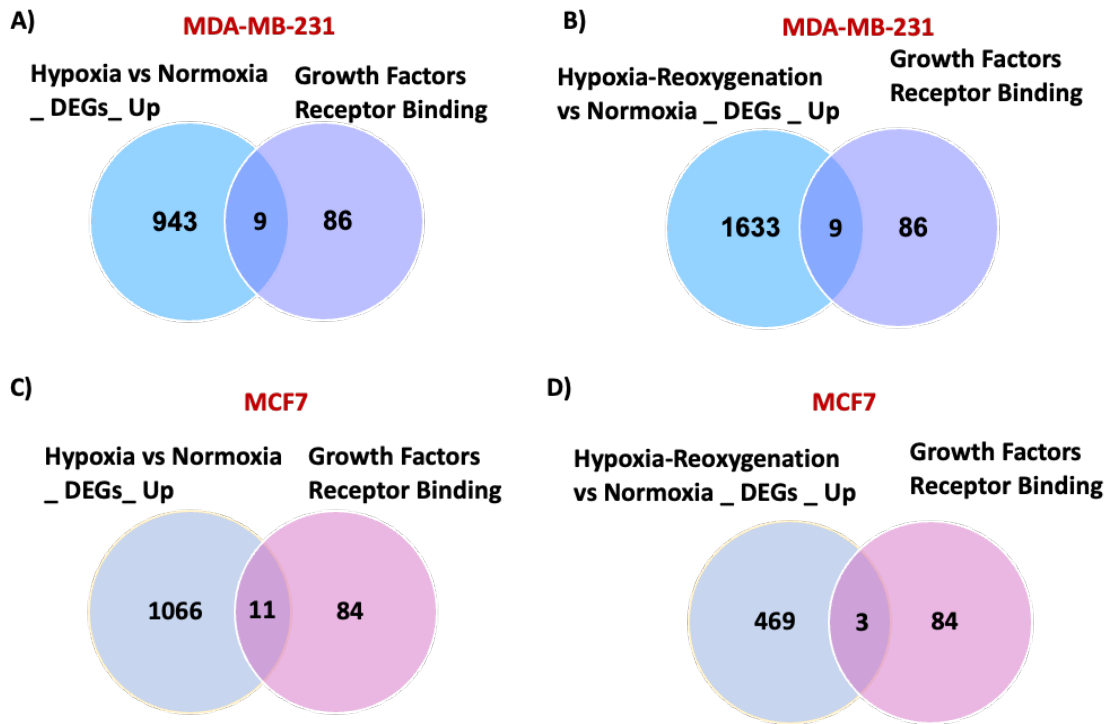


Figure 3.31 Displays Venn diagrams of growth factor co-expression in MDA-MB-231 and MCF7 RNAseq experiments. The list of growth factors was generated by PANTHER and compared to the DEGs upregulated in hypoxia vs normoxia and hypoxia-reoxygenation vs normoxia in both cell lines. **A)** Co-expression of growth factors in hypoxia vs normoxia upregulated DEGs in MDA-MB-231 RNAseq **B)** Co-expression of growth factors in hypoxia-reoxygenation vs normoxia upregulated DEGs in MDA-MB-231 RNAseq. **C)** Co-expression of growth factors in hypoxia vs normoxia upregulated DEGs in MCF7 RNAseq **D)** Co-expression of growth factors in hypoxia-reoxygenation vs normoxia upregulated DEGs in MCF7 RNAseq.

MDA-MB-231 RNAseq		MCF7 RNAseq	
Hypoxia vs Normoxia	Hypoxia-Reoxygenation vs Normoxia	Hypoxia vs Normoxia	Hypoxia-Reoxygenation vs Normoxia
INHBB	INHBA	INHBA	INHBA
TGFB2	TGFB2	INHA	INHA
NRG1	GDF15	INHBE	GDF15
BMP4	PDGFA	BMP4	
VEGFA	VEGFA	VEGFA	
VEGFC	VEGFC	VEGFC	
PGF	PGF	FGF13	
PDGFC	VEGFB	VEGFB	
ARTN	BMP2	PDGFB	
		MDK	
		INHBC	

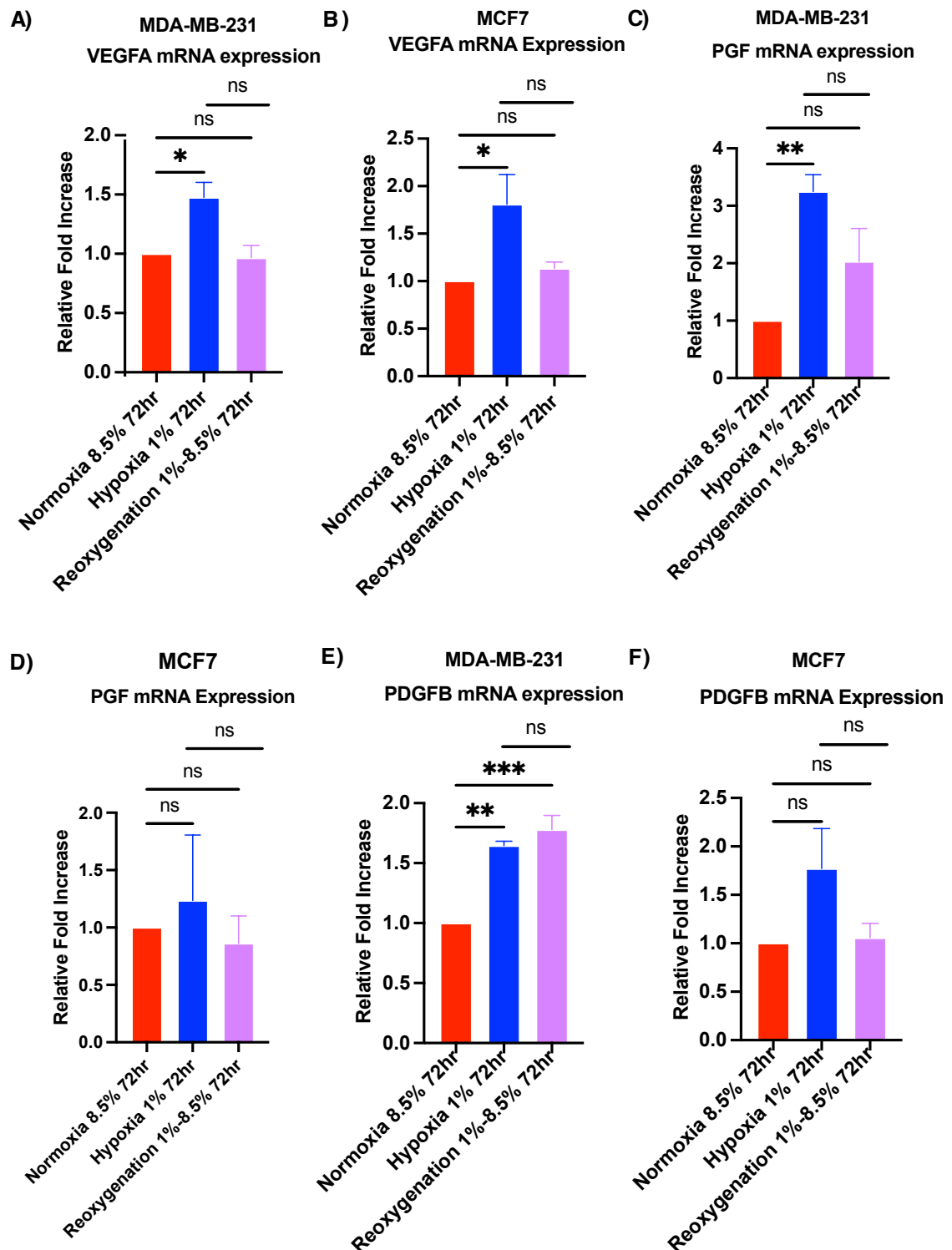
Table 3.15 Growth factors featured in the upregulated DEGs lists for MDA-MB-31 RNAseq and MCF7 RNAseq. The lists included upregulated DEGs in hypoxia vs normoxia and hypoxia-reoxygenation vs normoxia.

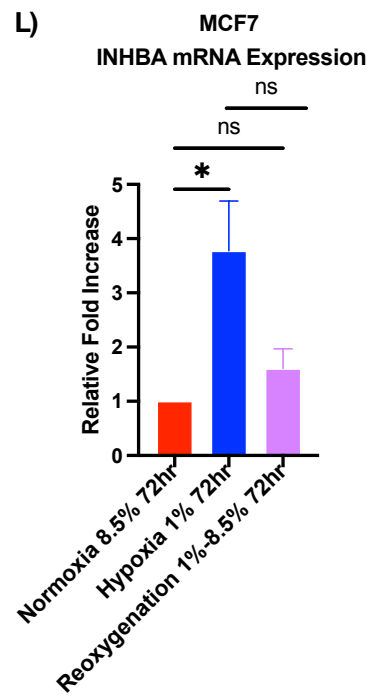
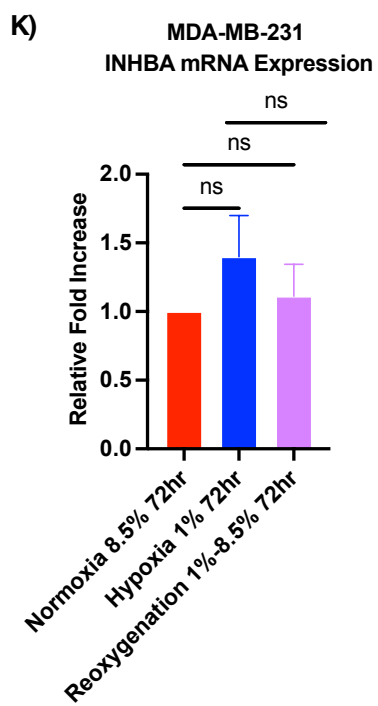
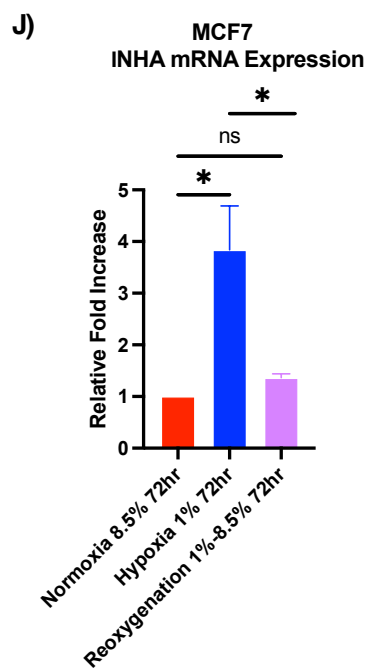
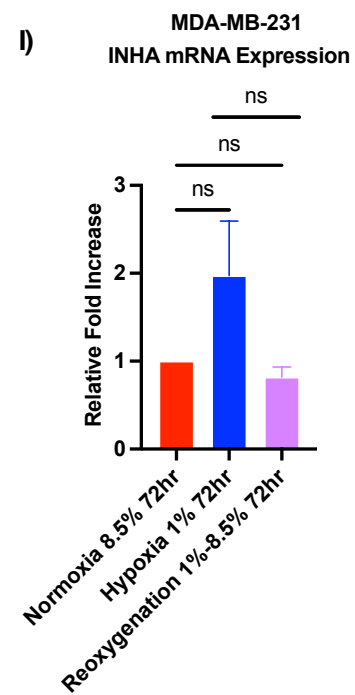
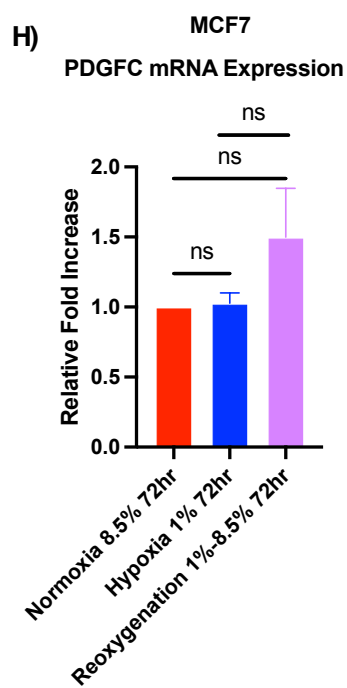
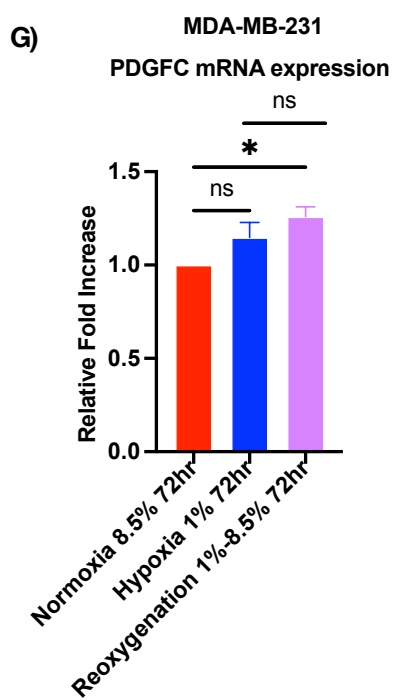
3.4.4 qPCR validation for the upregulated growth factors

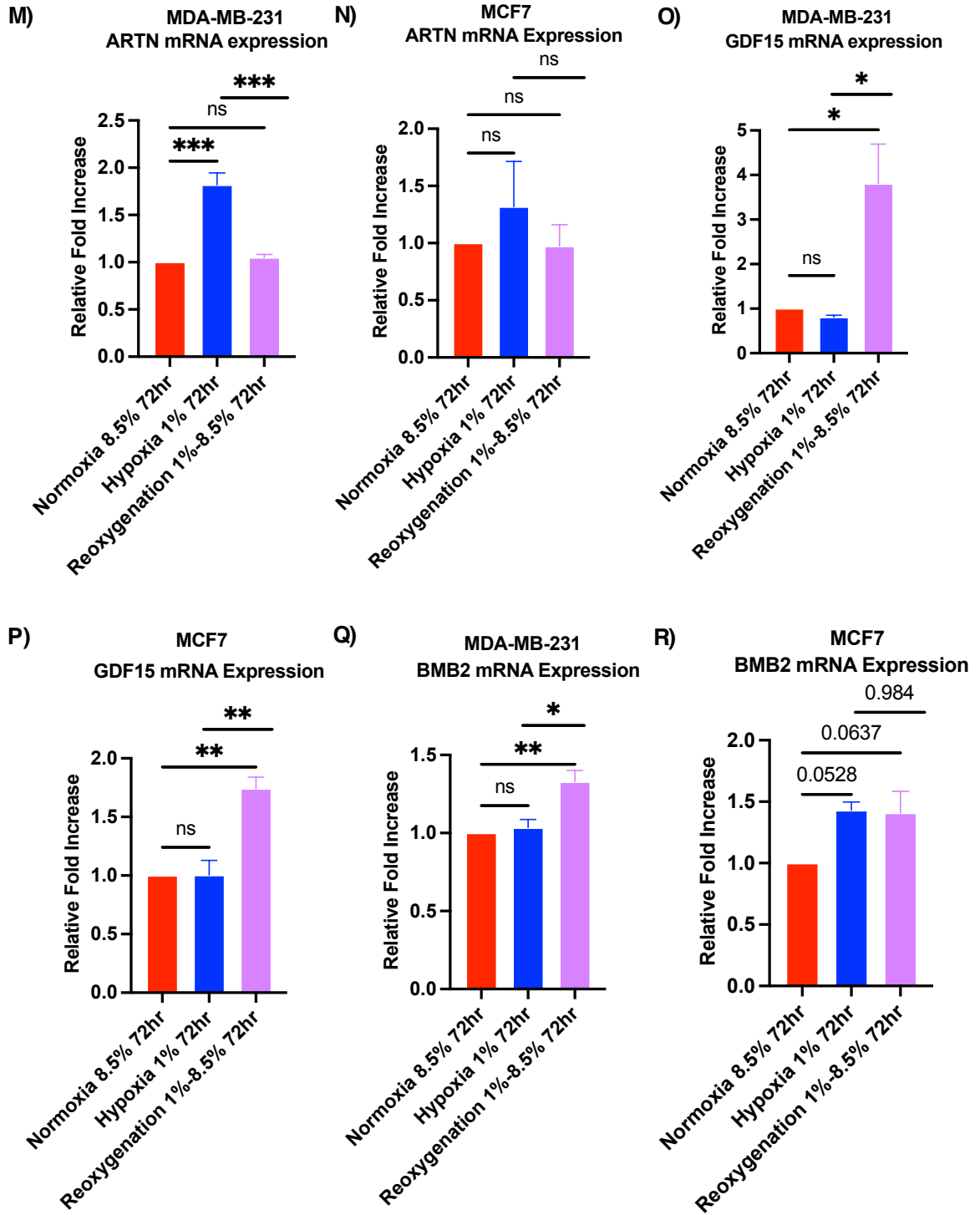
MDA-MB-231 and MCF7 lines were used to validate the mRNA expression of the featured growth factors in MDA-MB-231 and RNA seq libraries by qPCR (Table 3.15). The qPCR analysis showed that several growth factors had significant upregulation in the under hypoxia in MDA-MB-231 cells such as VEGFA (Log₂fold change=1.47, p<0.05, n=3), PGF (Log₂fold change=3.24, p<0.01, n=3), PDGFB (Log₂fold change=1.64, p<0.01, n=3) and ARTN (Log₂fold change=1.82, p<0.001, n=3) (Figure 3.33). In MCF7 cells, growth factors also had significant upregulation under hypoxia such as VEGFA (Log₂fold change=1.8, p<0.05, n=3), INHA (Log₂fold change=3.84, p<0.05, n=3), INHBA (Log₂fold change=3.77, p<0.05, n=3) and BMB2 (Log₂fold change=1.49, p<0.05, n=3) (Figure 3.32).

Additionally, multiple growth factors had significant upregulation under hypoxia-reoxygenation in MDA-MB-231 cells such as PDGFB (Log₂fold change=1.77, p<0.001, n=3),

PDGFC (Log₂fold change=1.25, p<0.05, n=3), GDF15 (Log₂fold change=3.8, p<0.05, n=3), BMP2 (Log₂fold change=1.32, p<0.01, n=3), and TGFB2 (Log₂fold change=2.32, p<0.05, n=3) and in MCF7 cells such as GDF15 (Log₂fold change=1.74, p<0.01, n=3) (Figure 3.32). Additional growth factors from Table 3.15 have been validated in MDA-MB-231 and MCF7 cell lines; however, these were not statistically significant.







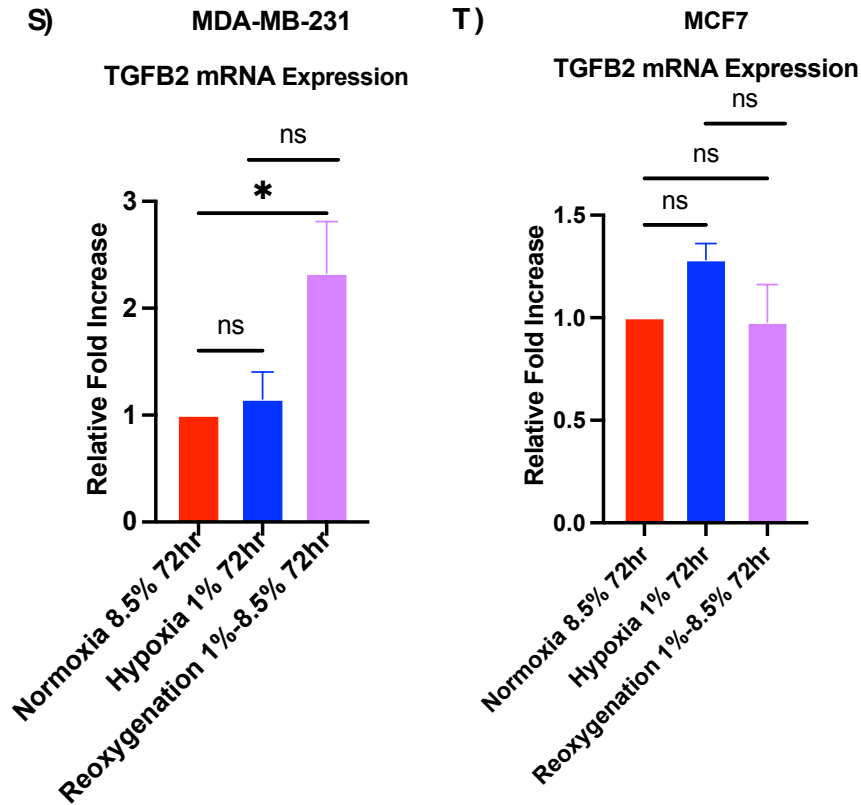


Figure 3.32 Shows validation of upregulated growth factors featured in hypoxia and hypoxia-reoxygenation from MCF7 RNAseq and MDA-MB-231 RNAseq using qPCR. RNA samples were generated as described previously following exposure to physoxia (72 hours 8.5% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂), cDNA synthesis protocol and finally qPCR **A-T**) mRNA expression of different growth factors in MDA-MB231 and MCF7 cell line. The expression levels in MCF7 and MDA-MB-231 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Statistical analysis using one-way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ respectively. $n = 3$

3.4.5 Hypoxia-reoxygenation gene signature

This section aims to identify specific hypoxia-reoxygenation genes that have been upregulated under hypoxia-reoxygenation in MDA-MB-231 and MCF7 cells. Surprisingly, the analysis revealed 63 DEGs that are expressed under hypoxia-reoxygenation in TNBC and Luminal A cells (Figure 3.33). The KEGG pathway analysis for these specific genes showed that the majority of DEGs enriched in the metabolic process (42 DEGs) (Figure 3.34A). The analysis has also highlighted that 37 DEGs are enriched in protein binding (Figure 3.34C).

The 63 genes identified are: DUSP8 , NFKBIA, EPAS1, LCP1, PTPRH, UCA1, RCAN1, MAP2, GDF15, TMPRSS2, TNFSF10, CEB2, SAMD12, LAMP3, ADAMTS6, SCARF1, FBXW10, SQLE, REC8, NOV, MSMO1, BRSK2, ALDOC, NTN4, SLC2A6, INHBA, TCN1, TSC22D3, IFI27, CPE, VGLL3, FZD8, XAF1, MVD, IGFBP3, AVPI1, AGFG2, SC5D, NAV2, ELF3, ANK2, FN1, BCL3, ATP1B1, PPP2R5B, STARD4, MIR22HG, PPARG, MRPS6, DAB2, TMEM173, ACSS2, IDH1, COMT, LRRC61, PCK2, PRRG4, LAMB3, DDX60L, MAFK, BCAR3, ACSL4, and LOXL2.

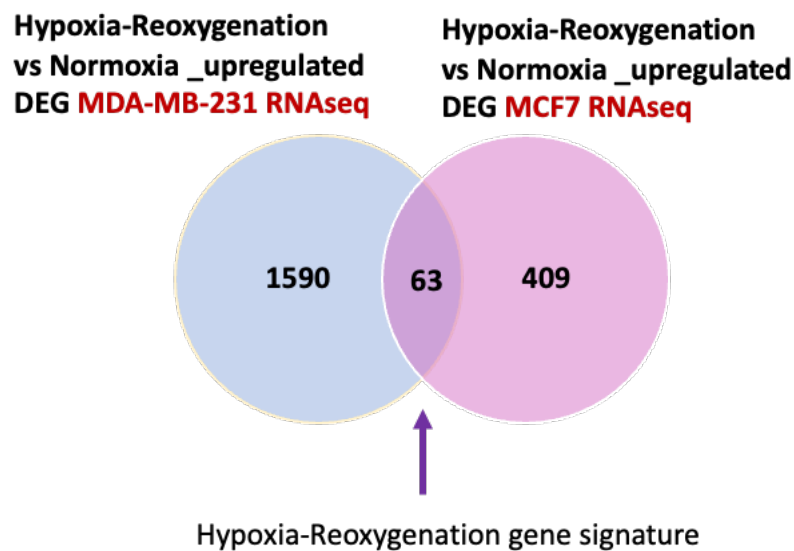
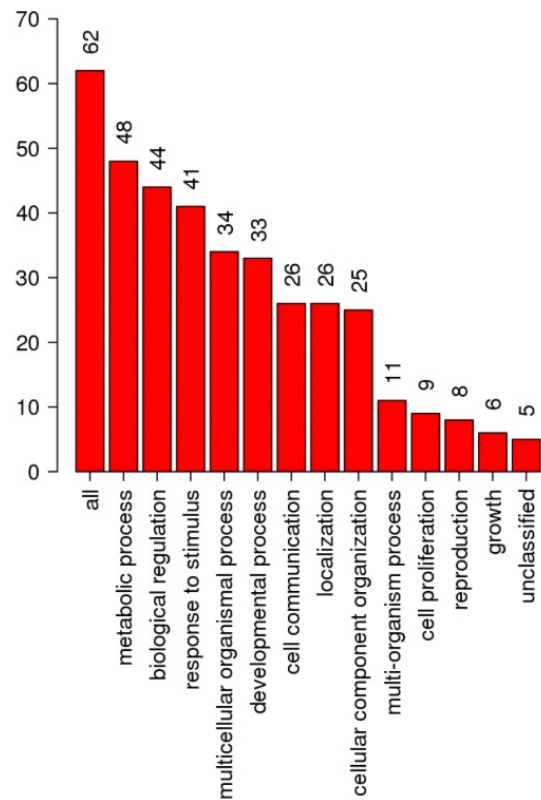


Figure 3.33 Shows the overlap between upregulated DEGs under hypoxia-reoxygenation from MDA-MB-231 and MCF7 RNA sequencing libraries. There are 63 DEGs expressed in both libraries.

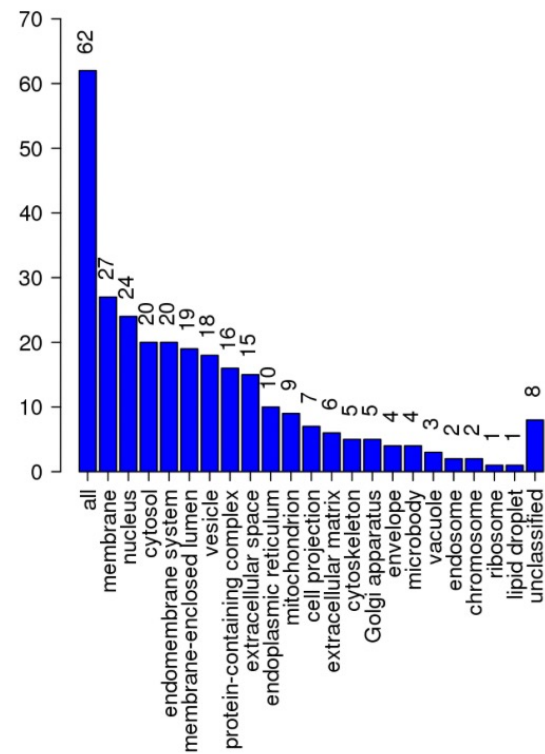
A)

Bar chart of Biological Process categories



B)

Bar chart of Cellular Component categories



C)

Bar chart of Molecular Function categories

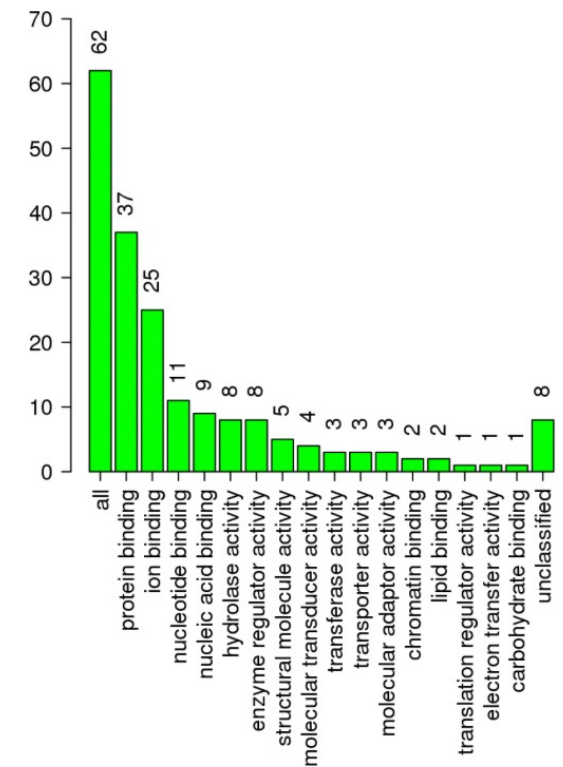


Figure 3.34 Displays the Gene ontology analysis for 63 differentially expressed gene signature in Hypoxia-reoxygenation. A) Bar chart of Biological Process categories, **B)** Bar chart of Cellular Components categories, **C)** Bar chart of Molecular Function categories. Figure generated using WebGestalt (Liao Y. et al. 2019).

3.5 Discussion

3.5.1 RNA sequencing application in cancer

In this chapter, we have explored the transcriptomic profile of two breast cancer subtypes that are known to be associated with hypoxia (Bianchini, G. et al. 2016, Podo, F. et al. 2010, Ye IC. et al. 2018, Bernardi R, Gianni L. 2014). MDA-MB-231 represents the TNBC subtype, and MCF7 represents the Luminal A were investigated using the RNA Sequencing technique. This technique has helped to have an in-depth study of the breast cancer transcriptome under hypoxia and hypoxia-reoxygenation. In this study, RNAseq libraries have been generated from cells incubated in physoxia (72 hours 8.5% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂). The RNA sequencing libraries generation was followed by bioinformatic analysis of the obtained results using several online available tools to gain more understanding of the gene expression changes under hypoxic and hypoxia-reoxygenation conditions. The study design and the bioinformatic tools used in this analysis are explained in (Figure3.1).

3.5.2 Regulation of gene expression in breast cancer under hypoxia

One of the aims of this chapter was to analyse the pathway enrichment of the differentially expressed genes (DEGs) of breast cancer cells under hypoxia and hypoxia-reoxygenation. As expected, MCF7 RNAseq showed enrichment for the HIF-1 signalling pathway under hypoxia and enrichment in the PI3K-Akt and p53 signalling pathway. Active HIF-1 α pathway has been linked previously to chemotherapy resistance in cancer cells (Comerford K.M., et al. 2004). Comparing the two RNAseq libraries, several KEGG pathways under hypoxia were enriched in glycolysis, focal adhesion and fructose and mannose metabolism. Recent cancer review has highlighted the role of fructose metabolism in cancer (Krause N, Wegner A. 2020). Fructose can metabolise in different ways; the fructose metabolic pathways can overlap with glucose degradation pathways hence playing a vital role in cell survival and growth. Similar to glucose, fructose can also affect cancer cells proliferation, growth, and survival (Boroughs L.K., et al. 2015). GLUT5 is a fructose transporter, a recent study has shown that upregulation of GLUT5 correlated with poor prognosis in lung adenocarcinoma patients, as overexpression of GLUT5 enhanced migration, invasion, and proliferation (Weng Y., et al. 2018). In addition, a recent study has shown that hypoxia triggers focal adhesion turnover in mesenchymal TNBC cells

(Nguyen TMH. 2023). Hypoxia can promote the migration of mesenchymal TNBC cells through activation of the Yes-associated protein YAP (YAP) transcription factor (Nguyen TMH. 2023).

3.5.3 Regulation of gene expression in breast cancer under hypoxia-reoxygenation

The investigation of transcriptomic profiles of breast cancer cells under hypoxia-reoxygenation showed enrichment of DEGs in the steroid biosynthesis pathway, showing that metabolism is being upregulated under hypoxia-reoxygenation in the MDA-MB-231 cell line. Cellular metabolism in cyclic hypoxia has been studied in Obstructive sleep apnea (OSA), where several metabolic effects have been identified, including glucose metabolism and lipid alteration (Drager LF, Jun JC, Polotsky VY. 2010 and Ryan S. 2017). However, more research is needed to fully understand cellular metabolism under hypoxia-reoxygenation or cyclic hypoxia in breast tumours. In addition, the analysis here showed that 63 significantly upregulated DEGs co-expressed under hypoxia-reoxygenation in MDA-MB-231 and MCF7 RNAseq, suggesting a possible hypoxia-reoxygenation gene signature. However, further investigation is required to confirm this. Interestingly, 43 genes from the identified hypoxia-reoxygenation gene signature here were enriched in the metabolic process. This strongly suggests that metabolism is increased under hypoxia-reoxygenation in breast cancer. Boidot et al, have previously generated cyclic hypoxia gene signatures using 20 tumour cell lines and clinical data sets from 2,150 breast cancer patients to be used as predictive of clinical outcomes in patients. However, the cells in this study were submitted to cyclic hypoxia as (24 cycles 30 min) incubation under normoxia (21% O₂) and 30 min incubation under hypoxic (1% O₂) conditions to reproduce tumour hypoxic fluctuations (Boidot R, Branders S, Helleputte T. et al. 2014).

Another interesting finding from the RNAseq analysis presented here is that it showed enrichment of significant upregulated DEGs in the apoptotic pathways under hypoxia-reoxygenation from both RNAseq libraries (MDA-MB-231 and MCF7). Previously, studies have shown that cyclic hypoxia induces the ability of cells to resist apoptosis in response to chemotherapy in glioblastoma (Chen WL, Wang CC, Lin YJ, Wu CP, Hsieh CH. 2015). The cyclic hypoxia used in the study was 3 cycles of (0.5–1 % O₂ 1 h) interrupted by (5 % CO₂) and air for 30 min for cycling hypoxic treatment to persistent (0.5–1 % O₂ 4 h) for non-interrupted hypoxic treatment (Chen WL, Wang CC, Lin YJ, Wu CP, Hsieh CH. 2015).

The analysis has also revealed that the NOD-like receptor signalling pathway is significantly enriched under hypoxia-reoxygenation in MDA-MB-231 and MCF7 cells. Cell adhesion molecules (CAMs) pathway and Cytokine-cytokine receptor interaction pathway have been significantly enriched in MDA-MB-231 cells. Another interesting finding is that several significant KEGG pathways enriched in hypoxia-reoxygenation are networking with vital signalling pathways, including the VEGF signalling pathway, TGFB signalling pathway, MAPK signalling pathway, PI3K signalling pathway, ERK signalling pathway and, mTOR signalling pathway (Kanehisa, M., et al. 2023).

3.5.4 The hypoxic transcriptomic profile is maintained under hypoxia-reoxygenation

Previous studies have found that circulating tumours (Metastatic genes) have maintained hypoxia gene signatures, which have been previously seen in vitro and in patients (Boidot, R. *et al.* 2014). The main aim of this study was to determine how much of the transcriptional profile of hypoxic TNBC and Luminal A cells overlapped upon hypoxia-reoxygenation. Surprisingly, in MDA-MB-231 cells, 42% of the hypoxic profile is shared with hypoxia-reoxygenation, and a total number of 651 significant DEGs (up and downregulated) have been maintained in hypoxia-reoxygenation. Interestingly, 11% of the hypoxic profile was found to be shared with hypoxia-reoxygenation in MCF7 cells, and a total number of 228 DEGs (up down downregulated) were maintained in hypoxia-reoxygenation in MCF7 cells. This suggests that the hypoxia transcriptomic profile is maintained under hypoxia-reoxygenation in breast cancer cells.

The downstream analysis of both RNAseq libraries has focused on the significant DEGs upregulated in hypoxia and maintained in hypoxia-reoxygenation. The analysis showed that MDA-MB-231 had 358 significant DEGs and MCF7 had 96 significant DEGs upregulated in hypoxia and maintained in hypoxia-reoxygenation. Enrichment in metabolism was strongly noticed in TNBC and Luminal A overlapped analysis, with over 30% of significantly DEGs upregulated in hypoxia and maintained in hypoxia-reoxygenation enriched in the metabolic process. This indicates that hypoxic-induced metabolism is maintained upon reoxygenation of breast cancer cells.

Interestingly, 17% of TNBC and 20% of Luminal A overlapped significantly upregulated DEGs enriched in signalling. Here I have shown that several pathways are significantly enriched in the maintained hypoxic profile in hypoxia-reoxygenation. These pathways include the Gonadotropin-releasing hormone receptor pathway, Wnt signalling pathway, TGF-beta signalling pathway and integrin signalling pathway. In addition, the VEGF signalling pathway, PDGF signalling pathway, Ras Pathway, oxidative stress response and p53 pathway was enriched uniquely in TNBC cells, while the Cadherin signalling pathway and Dopamine receptor-mediated signalling pathway were enriched uniquely in Luminal A.

Studies has shown that hypoxia signalling can interact with other cellular pathways such as ERK (Malekan, M., Ebrahimzadeh, M. A. & Sheida, F. 2021), TGFB (Mingyuan X, et al. 2017) and mTOR (Düvel K, et al. 2010). In TNBC, studies showed that deregulated Wnt/ β -catenin signalling correlated with aggressiveness of the tumour and poor outcomes, in addition, abnormal Wnt/ β -catenin signalling in TNBC patients can lead to developing distance metastasis, especially to the lung and brain (Dey, N. et al. 2013). TGF- β signalling is known to promote proliferation, EMT, angiogenesis, metastasis and chemotherapy resistance (Neuzillet C. et al. 2015). Studies showed that chemoresistance in TNBC associated with increase in TGF- β signalling (Bhola NE. et al. 2013)

In a comparison between the identified overlapped significantly upregulated DEGs in hypoxia and hypoxia-reoxygenation (from both RNAseq libraries) with the published breast cancer transcriptomic data (TCGA n=743), several DEGs were significantly overexpressed in TNBC and HER2- tissues compared to healthy tissues such as CST1, PTPRR, MUC1, CDH2, EGLN3, DIO and MDGA2 (Jézéquel P. et al. 2021). High expression of CST1 promoted clone formation cell proliferation and metastasis in breast cancer cells (Dai DN, et al. 2019). In addition, CST1, MUC1 and CDH2 are associated with poor prognosis in breast cancer patients (Dai DN, et al. 2019 and Jing X, et al 2019 and Xu M, et al. 2021). The analysis here showed that maintained hypoxic genes in hypoxia-reoxygenation are associated with the overexpression of genes linked to poor prognosis in breast cancer patients. The genes identified by the RNAseq analysis above can be used as potential prognostic markers and therapeutic targets.

3.5.5 Growth Factors upregulated under hypoxia and hypoxia-reoxygenation

The study here revealed that a hypoxic transcriptomic profile is maintained upon hypoxia-reoxygenation in breast cancer and aimed to investigate this mechanism. A recent study by Batie. M, Frost, J, Shakir, D and Roche, S has discussed this in the chromatin accessibility context (Batie M, Frost J, Shakir D, Rocha S. 2022). Their study investigated the sensitivity of hypoxia-induced changes in chromatin accessibility toward reoxygenation, in this case, reoxygenation condition as 24 h hypoxia (1% oxygen), followed by 1 h at normoxia (21% oxygen). Majority of differentially open chromatin regions (DORs) (up and down-regulated DOR) levels at 24 hr hypoxia returned to their normoxic levels when reoxygenation occurred. indicating that hypoxia-induced changes in chromatin accessibility are dependent on HIF, where chromatin accessibility is increased under hypoxia. In addition, changes in chromatin accessibility under hypoxia are dependent on the availability of oxygen and reversed upon reoxygenation (Batie M, Frost J, Shakir D, Rocha S. 2022).

The study here proposes a possible mechanism for such phenomena, it could be possible that changes induced by hypoxia are maintained in hypoxia-reoxygenation due to a positive feedback loop from the signalling (Figure 3.37). MDA-MB-231 and MCF7 RNAseq analysis identified several growth factors that were upregulated under hypoxia and hypoxia-reoxygenation (Table 3.15). This has been further investigated, and the expression of these growth factors under hypoxia and hypoxia-reoxygenation were examined via qPCR. The analysis revealed that several growth factors were significantly upregulated under hypoxia in MDA-MB-231 and MCF7 cells, including VEGFA, PDGFB, PGF, INBA, INHA, BMP2 and ARTN. Further investigation is required to conclude this mechanism.

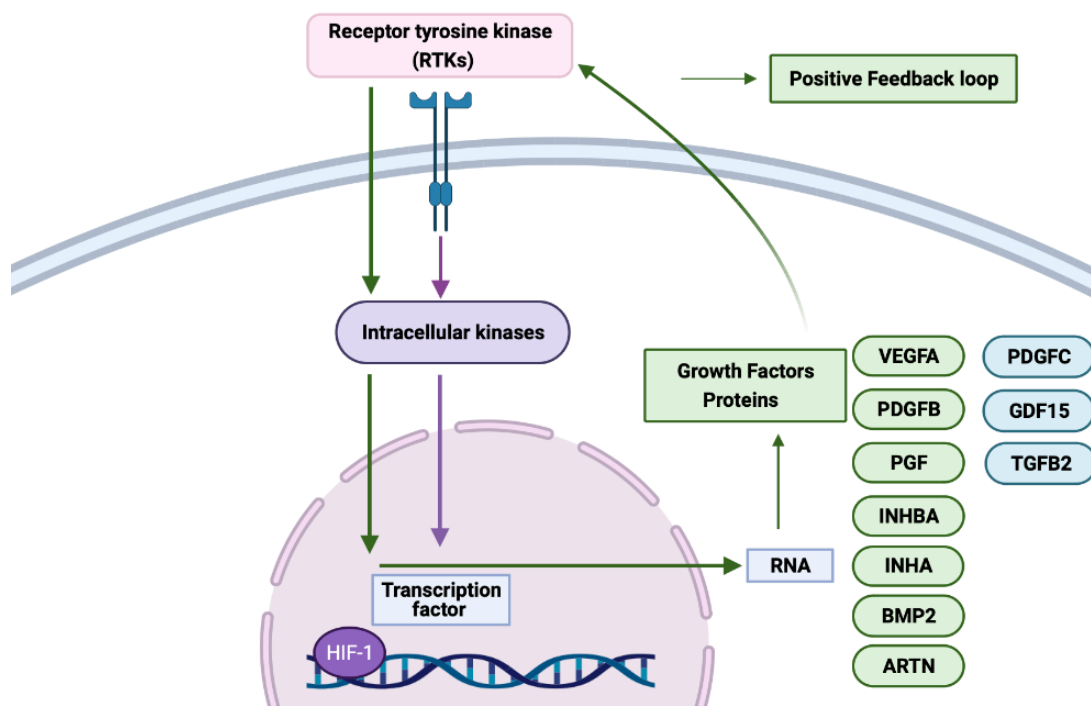


Figure 3.37 Schematic diagram explaining the positive feedback loop hypothesis.

Hypoxia upregulates growth factor expression under hypoxic conditions. Once upregulated, they activate unique Receptor Tyrosine Kinases (RTKs), which in turn activate downstream intracellular kinases affecting the transcription of hypoxic targeted responses. The response is maintained in hypoxia-reoxygenation as part of a positive feedback loop. The hypoxic targeted response involves gene transcription for metabolism, angiogenesis, proliferation, survival, and pH regulation. This can also increase tumour metastasis, invasion, and resistance to therapy.

Growth factors upregulated under hypoxia and hypoxia-reoxygenation, validated by qPCR, are indicated by green boxes (expression has been significantly upregulated under hypoxic conditions) and blue boxes (expression has been significantly upregulated under hypoxia-reoxygenation) respectively. Figure created in BioRender.

3.6 Conclusion

In conclusion, previously identified lists of overlapped upregulated DEGs in hypoxia and hypoxia-reoxygenation from MDA-MB-231 RNAseq (358 DEGs) and MCF7 RNAseq (96 DEGs) were further investigated. This investigation focused on the significant top targets with highest Log₂Fold change.

The first investigation explored the expression of the targeted genes in breast cancer tissues using transcriptomic and clinicopathological data. From the MDA-MB-231 RNAseq, CST1, PTPRR, CST4, ICAM1, TNFSF15, MUC1, ZNF165 and SPINT1 were significantly overexpressed

in TNBC tissues when compared to healthy tissues. These were also investigated in HER2(-ve) tissues, and CST1, PTPRR, CST4, TNFSF15, MUC1, ZNF165, and SPINT1 were significantly overexpressed in HER2(-ve) tissues when compared to healthy tissues. From the MCF7 RNAseq, BPIFB2, PTGS1, CDH2, SEMA5B, DIO2, EGLN3, EDIL3 and MDGA2 significantly overexpressed in HER2(-ve) tissues when compared to healthy tissues. These were also investigated in TNBC tissues, and CDH2, SEMA5B, DIO, EGLN3 and MDGA2 were overexpressed in TNBC tissues compared to healthy tissues.

The second investigation explored the mRNA expression of the targeted genes using qPCRs. From the MDA-MB-231 RNAseq, ICAM1, MUC1, DUSP8, DUSP1, CST1 were investigated in MDA-MB-231 cell line. ICAM1, MUC1 and DUSP8 showed a significant upregulation in hypoxia-reoxygenation when compared to normoxia. Hence, these genes were also investigated in the MCF7 cell line. From MCF7 RNAseq, BPIFB2, PTGS1, EDIL3, CDH2, SEMA5B, LOXL2, and INHBA were investigated in MCF7 cell line. PTGS1, CDH2, and EDIL3 showed a significant upregulation in hypoxia-reoxygenation when compared to normoxia, PTGS1 and LOXL2 showed a significant upregulation in hypoxia-reoxygenation when compared to hypoxia, and BPIFB2 and INHBA showed a significant upregulation in hypoxia when compared to normoxia. Hence, BPIFB2, PTGS1, CDH2, EDIL3, LOXL2, and INHBA were also investigated in the MDA-MB-231 cell line.

In addition to this, based on the analysis from both RNAseq libraries, several growth factors were identified for being upregulated under hypoxia and hypoxia-reoxygenation. The mRNA expressions of these genes were explored in MDA-MB-231 and MCF7 cell lines by qPCR, including VEGFA, PGF, PDGFB, PDGFC, INHA, INHBA, ARTN, GDF15, BMB2 and TGFB2.

4.0 Chapter 2: Investigating the role of kinase signalling in hypoxia and hypoxia-reoxygenation in breast cancer.

4.1 Introduction

Cell signal transduction plays a key role in the development and progression of cancer. Deregulation of cell signalling pathways can promote proliferation, progression and survival in tumour cells (Feitelson M. et al. 2015). In breast cancer cells, many alterations that affect cell signalling pathways have been described with great focus on those mediated by the ER and HER2/Neu or c-ErbB2, which are involved directly in breast cancer progression and development (Booy E. et al. 2011). The activation of HER2 receptors promotes signalling of other pathways, such as MAPKs PI3K/Akt/mTOR pathways. (Hermida M. et al. 2017, Feng Y. et al. 2018). Alterations in PI3K/Akt/mTOR have been found in several tumours, including breast cancer. Deregulation of PI3K/Akt/mTOR has been linked to multiple cancer hallmarks, including genomic instability, metabolic programming and uncontrolled proliferation in tumour cells (Hanahan D., Weinberg R. et al. 2011, Lien E. C. et al. 2017). Activation in signalling pathways has been linked to therapy resistance toward antitumour therapies (Martini M. et al. 2014). Therefore, it's important to investigate the activation of different signalling pathways in cancer in order to identify them and target them with available kinase inhibitors as an alternative way to overcome therapy resistance. Different approaches are now being developed to explore activation of cell signalling cancer. One of the useful tools is the use of a phospho-RTK array and a phospho-kinase array. These arrays have been used to profile phosphorylation in tumours such as in melanoma (Pietrobono S. et al. 2022). They have also been used in multiple studies as useful screen for kinase inhibitors (Neradil J. et al. 2019, Sariyar E. et al. 2023, Khayat MT. et al. 2022).

4.2 Hypothesis and Aims

Tumour cells have been shown to adapt to hypoxic environments by altering signalling pathways, resulting in increased proliferation, migration and resistance to chemotherapy, radiotherapy, and immune checkpoint blockade (Chen, Z., Han, F., Du, Y. et al., 2023). I hypothesise that hypoxia and hypoxia-reoxygenation modulate signalling pathways in breast cancer. I also hypothesise that hypoxia increases the phosphorylation levels of receptor tyrosine kinases (RTKs) and intracellular kinases, which are maintained upon hypoxia-reoxygenation.

To test these hypotheses, I will use the following aims:

- 1) Investigate the activation status of Receptor Tyrosine kinases (RTKs) in hypoxia and hypoxia-reoxygenation compared to normoxia in the MDA-MB-231 cell line using a Phospho-RTK array.
- 2) Investigate the activation status of intracellular kinases in hypoxia and hypoxia-reoxygenation compared to normoxia in the MDA-MB-231 cell line using a Phospho-Kinase array.
- 3) Investigated the activation status of specific RTKs, identified using Phospho-RTK arrays, in MDA-MB-231 and MCF7 cell lines by western blot.
- 4) Investigate the activation status of specific phosphokinases, identified using Phospho-kinase arrays, in MDA-MB-231 and MCF7 cell lines by western blot.

4.3 Results

4.3.1 Hypoxia-reoxygenation in Breast Cancer modulates phosphorylation levels of Receptor Tyrosine Kinases (RTKs).

To Investigate the activation status of Receptor Tyrosine kinases (RTKs) in hypoxia and hypoxia-reoxygenation compared to normoxia, the Proteome Profiler Human Phospho-RTK Array was used as described in the Methods. This array enabled the detection of tyrosine phosphorylation of 49 human receptor tyrosine kinases. Cells were incubated in normoxia (72 hours 21% O₂), hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 21% O₂) before protein extraction. Samples were applied to analysis using the arrays, followed by data analysis and western blot validation (Figure 4.1A). At the same time as generating experimental samples for the arrays experiment, additional lysates were generated to confirm that these samples were hypoxic in hypoxia before starting the experiment using HIF-1 α stabilisation as a biomarker for hypoxia (Figure 4.1B). Figure 4.1B shows HIF-1 α induction as expected in hypoxic conditions, and surprisingly, it also indicates that HIF-1 α is still expressed in hypoxia-reoxygenation conditions at a lower level than is detected in hypoxia. An example of the raw data images after the chemiluminescent detection in normoxia (control), hypoxia and hypoxia-reoxygenation is demonstrated in (Figure 4.2A). Figure 4.2A displays reference spots along with an example of spots for example RTKS (RYK, EGFR, Axl (Dtk), Eph R, Insulin R (ALK) and Insulin R (IGF-I R)) in duplicate spots (Figure 4.2A). The duplicate spots were then quantified using densitometry analysis in line with the manufacturer's instructions (Figure 4.2B).

The phospho RTK array was repeated in triplicate, and three independent biological replicates were used in the analysis. The analysis included averaged values of the two raw signals for each protein target; the values for the hypoxia and hypoxia-reoxygenation were normalised to normoxia, and the Log₂Fold change was calculated accordingly to investigate the alteration of phospho-RTK signalling in hypoxia and hypoxia-reoxygenation. 17 of the 49 human receptor tyrosine kinases produced a level of detection above the background level in the three conditions (normoxia, hypoxia and hypoxia-reoxygenation) over the three independent biological repeats; only these were included in the analysis. All RTKs are described here along with their receptor family as (RTK Receptor family (RTK name)) respectively.

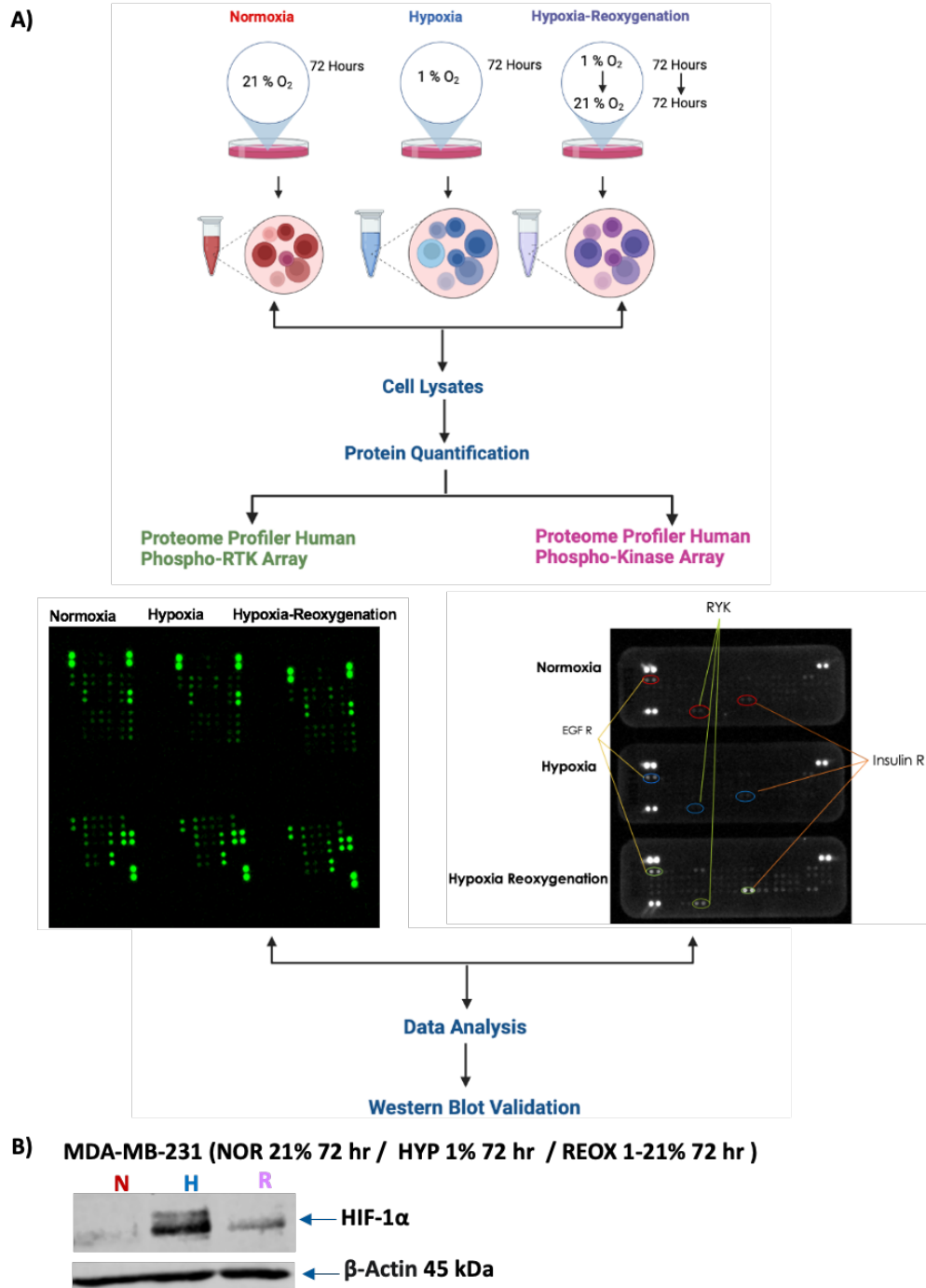


Figure 4.1. Experimental process for the phospho-RTK array and phospho-kinase array experiments. **A)** Schematic diagram illustrating the experimental approaches used to investigate the phosphorylation of RTKs and intracellular kinases in breast cancer cells; cells were incubated at specific conditions normoxia (72 hours 21% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 21% O₂), cell lysates were generated. Arrays were performed and analysed, followed by western blot validation for the promising targets. **B)** Representative western blots confirming HIF1α induction as a marker to validated hypoxic conditions during the generation of samples used in the arrays (Phospho-RTK and Phospho-Kinase arrays)

Phosphorylation of RTKs under hypoxia compared to normoxia

The analysis showed that 24% (5 out of 17) of the RTKs analysed displayed upregulated phosphorylation under hypoxia, with a value of 0.5 Log₂Fold change and above when compared to normoxia. These RTKs included Insulin R (IGF-I R) (Log₂Fold change=1.98, n=3), Insulin R (ALK) (Log₂Fold change=3.2, n=3), Eph R (EphB2) (Log₂Fold change=0.84, n=3), and DDR1 (Log₂Fold change=0.822, n=3) as compared to normoxia, although the adjusted p-value was not significant for any of these (Figure 4.2B). On the other hand, EGF R (ErbB3) was significantly downregulated in hypoxia when compared to normoxia (Log₂Fold change=-1.45, p<0.05, n=3). Furthermore, several other RTKs also displayed reduced phosphorylation in hypoxia compared to normoxia, with no significant statistical difference (Figure 4.2B).

Phosphorylation of RTKs under hypoxia-reoxygenation compared to normoxia

The analysis also revealed that 12 of 17 RTKs analysed displayed upregulated phosphorylation under hypoxia-reoxygenation with a value of 0.5 Log₂Fold change and above when compared to normoxia accounting for 70% of the total profiled RTKs (Figure 4.2B). Three of these RTKs were statistically significant, including Insulin R (ALK) (Log₂Fold change=3.2, p<0.01, n=3), ErbB2 (EGF R) (Log₂Fold change=1.5, p<0.01, n=3), EGF R (EGF R) (Log₂Fold change=0.77, p<0.05, n=3) (Figure 4.2B). Additional RTKs displayed an upregulation in hypoxia-reoxygenation compared to normoxia such as VEGF R (VEGF R1) (Log₂Fold change=1.69, n=3), FGFR (FGF R3) (Log₂Fold change=1.48, n=3), DDR1 (Log₂Fold change=1.35, n=3), and Insulin R (IGF-I R) (Log₂Fold change=1.3, n=3) (Figure 4.2B). Although these additional changes in phosphorylation were detectable, statistical analysis did not identify significance. This is likely due to variation between the experimental repeats.

Phosphorylation of RTKs under hypoxia and hypoxia-reoxygenation compared to normoxia

The analyses identified four RTKs that displayed increased phosphorylation in hypoxia and hypoxia-reoxygenation compared to normoxia: Insulin R (IGF-I R), Insulin R (ALK), VEGF R1 (VEGF R), and DDR1 (Figure 4.2B).

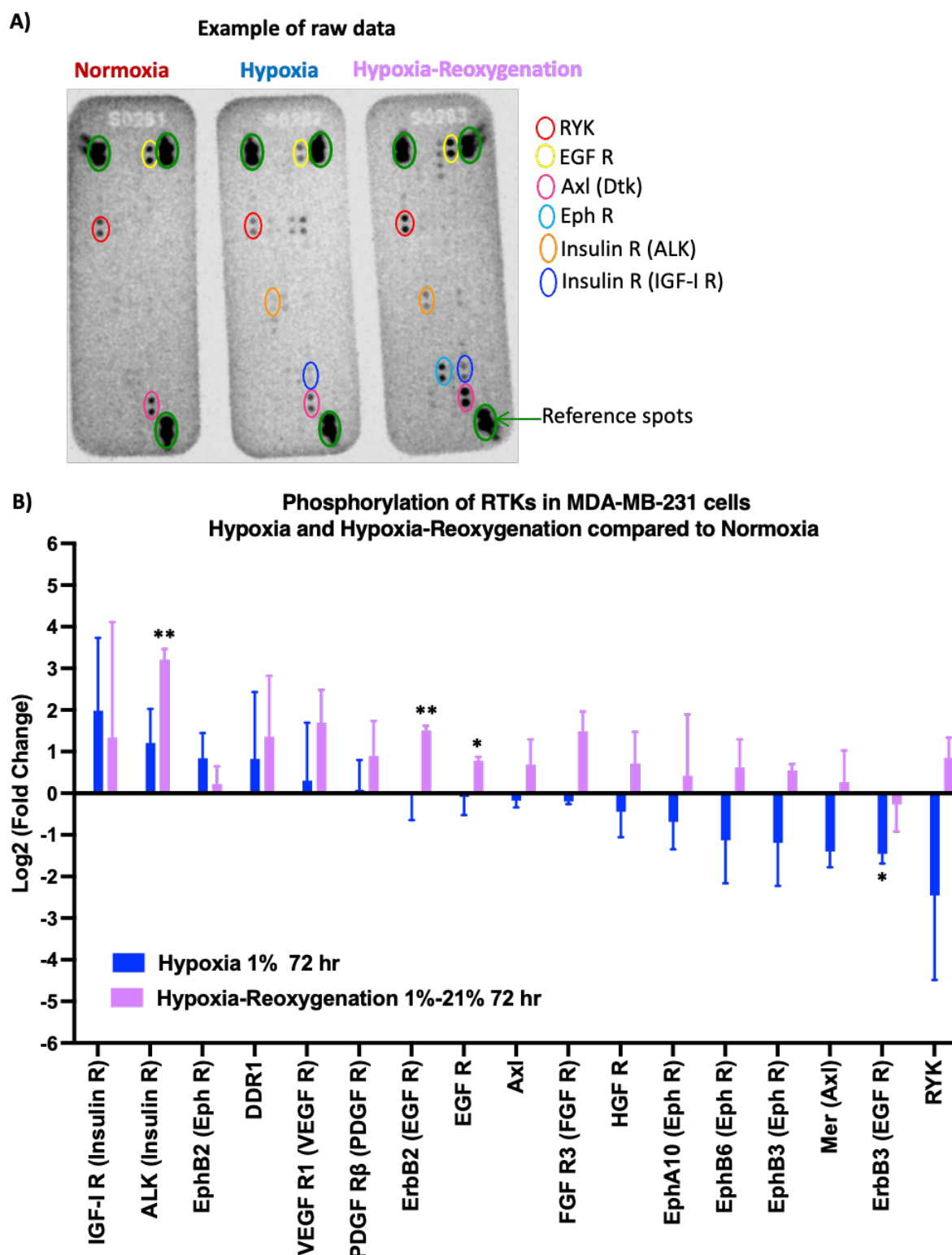


Figure 4.2. Phospho-RTK array, Representative images, and summary analysis of the phosphorylation of RTKs in MDA-MB-231 cells. **A)** Representative image of experimental raw data of the Phospho-RTK arrays for normoxia, hypoxia and hypoxia-reoxygenation; reference spots are highlighted along with an example of protein targets spotted in duplicate. **B)** Bar-graph showing the Log₂Fold changes in phosphorylation of RTKs targets normalising hypoxia and hypoxia-reoxygenation to normoxia (control). Values obtained from the raw images and densitometry analysis were performed as described above. Error bars indicate $\pm$ SEM. Data was analysed using a 2-way ANOVA. * $p < 0.05$, ** $p < 0.01$. $n = 3$.

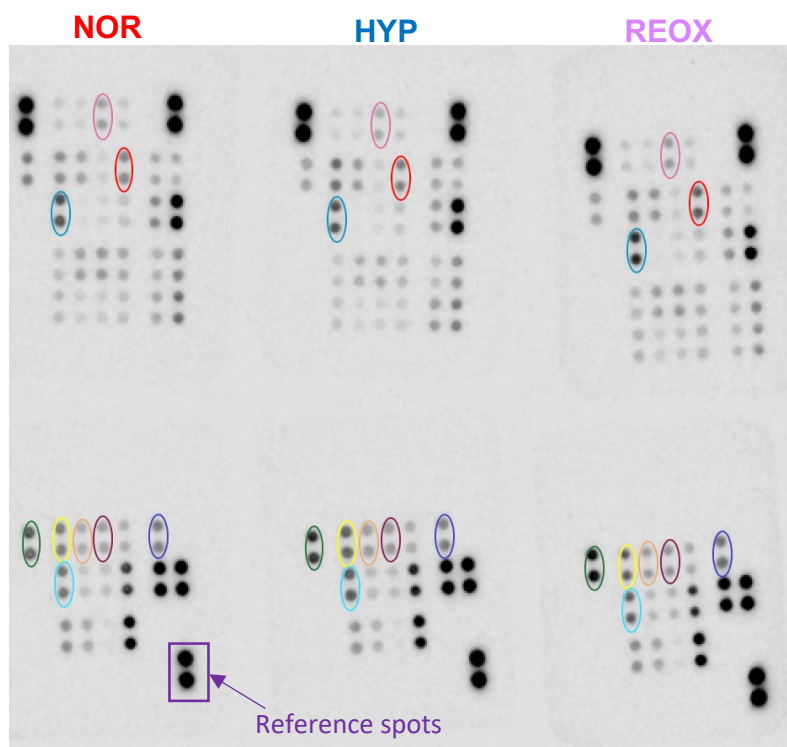
4.3.2 Hypoxia-Reoxygenation in Breast Cancer modulates phosphorylation levels of Intracellular kinases.

To Investigate the regulation of phospho-signalling pathways under hypoxia and hypoxia-reoxygenation in breast cancer, a phospho-kinase array experiment was conducted. The Proteome profiler human phospho-kinase array was used to detect the phosphorylation of 37 human kinases and total amounts of 2 total proteins simultaneously. TNBC cells (MDA-MB-231) were incubated in normoxia (72 hours 21% O₂), hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 21% O₂) before protein extraction and conducting the array, as described above (Figure 4.1). The phospho-kinase array experiment was repeated in triplicate, and the analysis was performed using three independent biological replicates. Unfortunately, the composition of the commercial kit has changed between the first experimental repeat and the second/third experimental repeats. This has resulted in the inter-replicate variation, which has affected the statistical significance of the analysis. However, despite this challenge, the study highlights changes that were the greatest, which will be further investigated using western blot to determine if they are biologically relevant.

Figure 4.3A demonstrates the raw images after the chemiluminescent detection in normoxia (control), hypoxia and hypoxia-reoxygenation, and examples of reference spots and examples of phospho-kinase targets in duplicate spots. Densitometry analysis was used to quantify the duplicate spots in line with the manufacturer's instructions. The phospho-kinase array coordinates used to identify the phospho-kinase targets are presented (Figure 4.3B). Data was normalised to normoxic values, and a comparison was made with the values obtained from hypoxia and hypoxia-reoxygenation (Figure 4.4). Figure 4.4 displays a heatmap using normalised values of hypoxia and hypoxia-reoxygenation, arranged from the targets having the greatest increase changes to the greatest decrease in hypoxia compared to normoxia (Figure 4.4A) and hypoxia-reoxygenation normalised to normoxia (Figure 4.4B). The Log₂Fold change was calculated to investigate the alteration of phospho-signalling in hypoxia and hypoxia-reoxygenation compared to normoxia (Figure 4.5).

A) Raw Image

PK N=1



PK1:

CREB (S133): C3, C4

FAK (Y397): F5,F6

Src (Y419): D1,D2:

HSP60: G11,G12

AKT123 (T308): B11,B12

STAT3 (Y727): F11,F12

WNK1 (T60): F13,F14

STAT3 (Y705): E11,E12

p70 S6 Kinase (T421/S424): D11,D12

B) Human Phospho-Kinase Array Coordinates

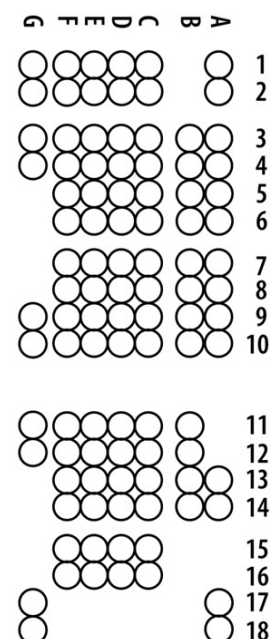
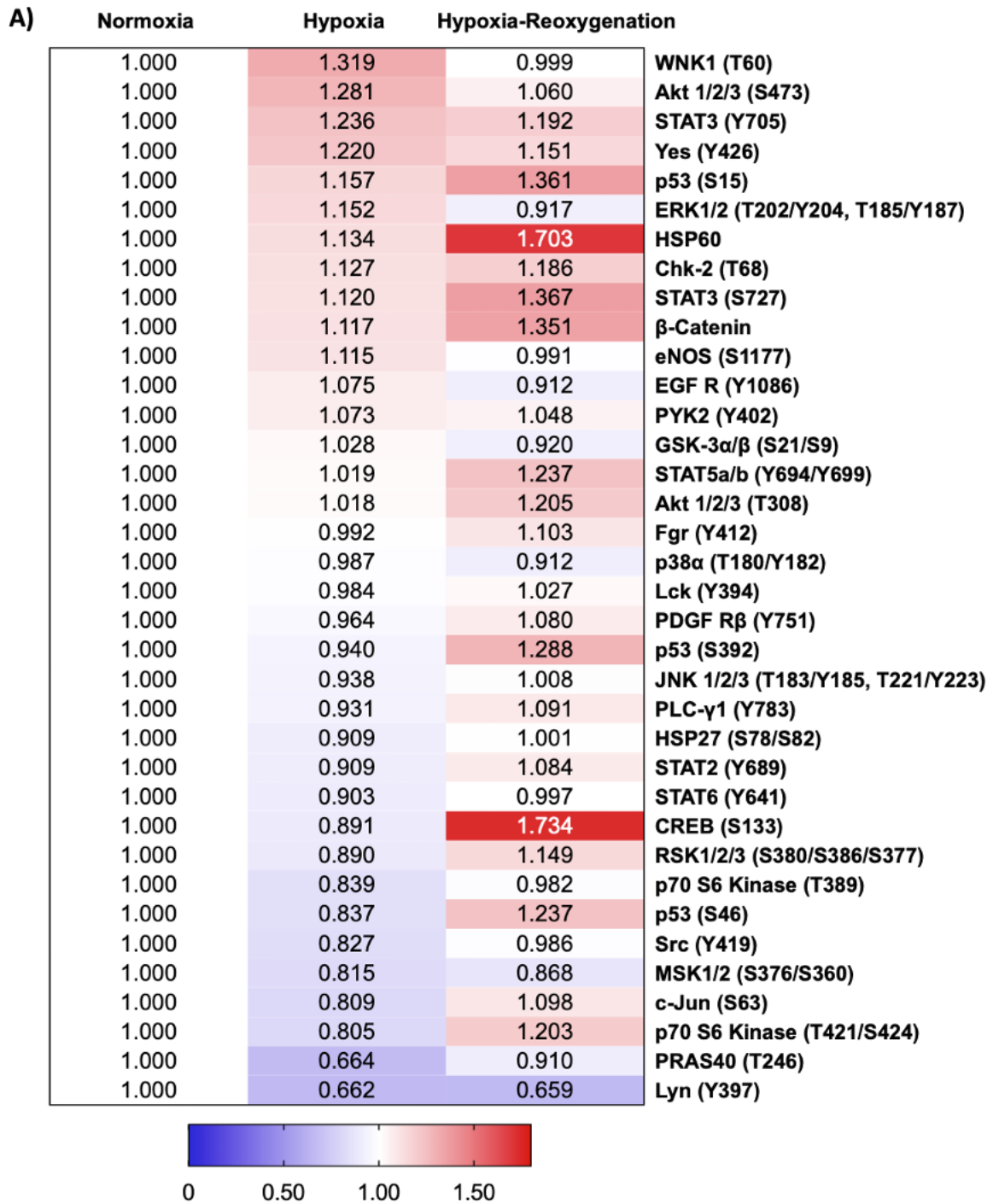


Figure 4.3. Proteome profiler phosphokinase array in MDA-MB-231 cell line. A) Representative image of experimental raw data of the Phospho-kinase array for normoxia, hypoxia and hypoxia-reoxygenation; reference spots are highlighted along with an example of protein targets spotted in duplicate. **B)** Image for the coordinate reference used for the analyte identification



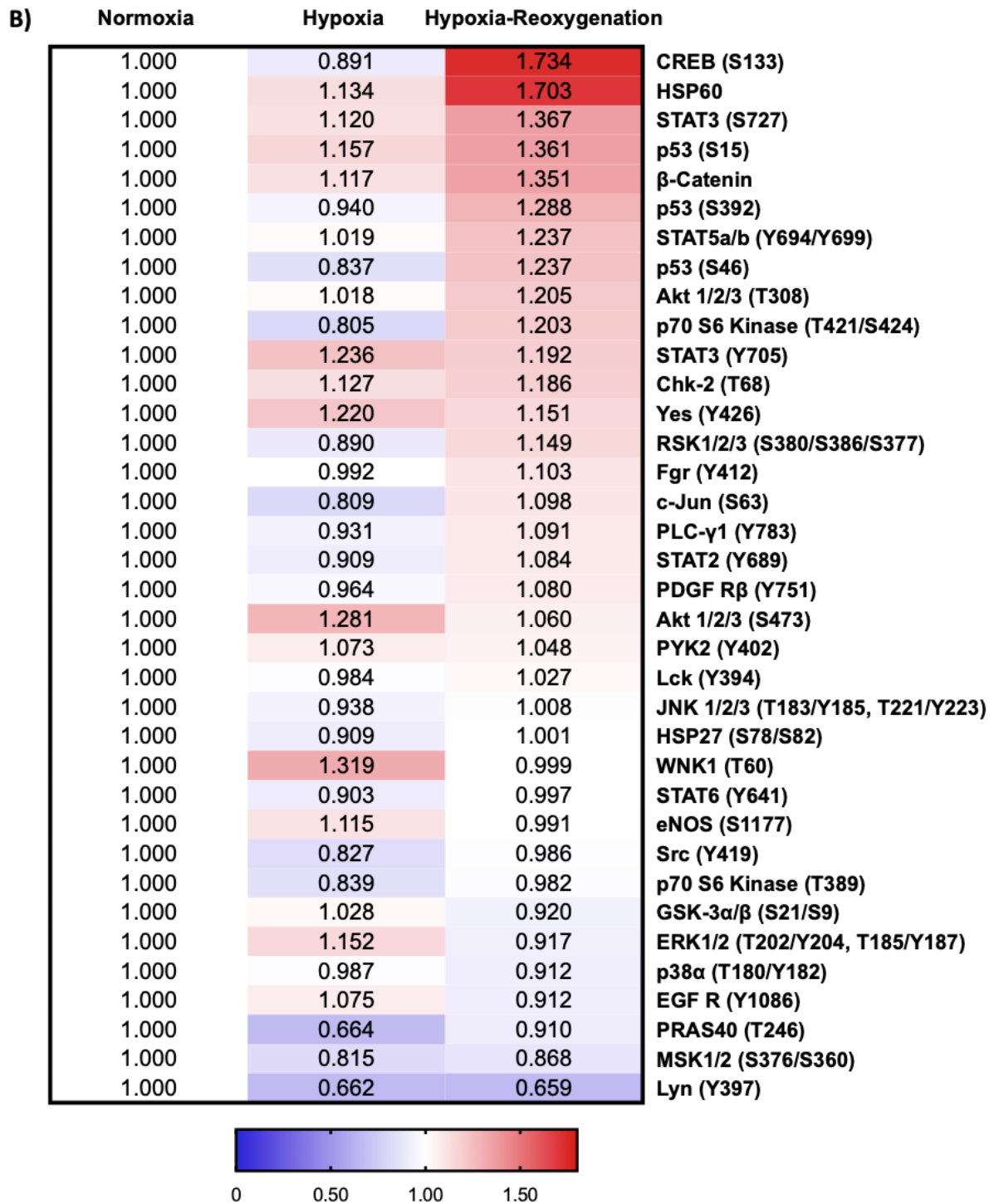


Figure 4.4. Heatmap showing the phospho-signalling changes in hypoxia and hypoxia-reoxygenation when normalised to normoxia. Densitometry analysis was performed on the raw data images as described above. Normalised values of hypoxia and hypoxia-reoxygenation **A)** Arranged from the targets having the greatest changes to the minor changes in hypoxia normalised to normoxia. **B)** Arranged from the targets having the greatest changes to the minor changes in hypoxia-reoxygenation normalised to normoxia. The values presented in the heatmap represent the average of the target for each condition in hypoxia normalised to normoxia and hypoxia-reoxygenation normalised to normoxia. Values of 1 are equivalent to control normoxia (white), values below 1 represent downregulations (blue), and values above 1 represent upregulations (red).

Phosphorylation of Intracellular kinases under hypoxia compared to normoxia

The analysis revealed that several kinase targets had increased phosphorylation under hypoxia when compared to normoxia. Out of 36 intracellular kinases profiled, 11 kinases (31%) increased in hypoxia including WNK1 (T60) (Log₂Fold change=0.43, n=3), Akt 1/2/3 (S473) (Log₂Fold change=0.389, n=3), ERK1/2 (T202/Y204, T185/Y187) (Log₂Fold change=0.33 n=3), Yes (Y426) (Log₂Fold change=0.31, n=3), p53 (S15) (Log₂Fold change=0.3, n=3), Chk-2 (T68) (Log₂Fold change=0.18, n=3), PYK2 (Y402) (Log₂Fold change=0.074, n=3), β-Catenin (Log₂Fold change=0.007, n=3), EGF R (Y1086) (Log₂Fold change=0.069, n=3) and STAT3 at two phosphorylation sites at (Y705) (Log₂Fold change=0.26, n=3) and at (S727) (Log₂Fold change=0.07, n=3) (Figure 4.5). Several intracellular kinases showed a decrease in hypoxia, including PRAS40 (T246) (Log₂Fold change=-0.59, p<0.01 n=3). The statistical analysis showed that only PRAS40 (T246) was significant (Figure 4.5).

Phosphorylation of Intracellular kinases under hypoxia-reoxygenation compared to normoxia

The analysis has also shown that multiple kinase targets had increased phosphorylation under hypoxia-reoxygenation when compared to normoxia (Figure 4.5). Out of the 36 intracellular kinases that were analysed, 24 kinases (67%) were observed to be upregulated during hypoxia-reoxygenation, including CREB (S133) (Log₂Fold change=0.866, n=3), Jun (S63) (Log₂Fold change=, n=3), (Log₂Fold change=, n=3), (Log₂Fold change=, n=3), (Log₂Fold change=, n=3), (Log₂Fold change=, n=3), Akt 1/2/3 (T308) (Log₂Fold change=0.294, n=3), p53 at two phosphorylation sites (S392) and (S46) (Log₂Fold change=0.54, n=3) and (Log₂Fold change=0.26, n=3) respectively (Figure 4.5). The array also investigated total phosphorylation of two targets β-Catenin (Log₂Fold change=-0.59, p<0.05 n=3) and HSP60. In term of statistical significance, only β-Catenin was statistically significant (Figure 4.5).

Phosphorylation of Intracellular kinases under hypoxia and hypoxia-reoxygenation compared to normoxia

One of the main aims of this study was to investigate the phosphorylation of intracellular kinases under hypoxia and hypoxia-reoxygenation. The analysis showed that multiple intracellular kinases upregulated in hypoxia and maintained in hypoxia-reoxygenation,

including Akt 1/2/3 (S473), Yes (Y426), p53 (S15), Chk-2 (T68), β -Catenin and STAT3 at two phosphorylation sites at (Y705) and (S727) (Figure 4.5).

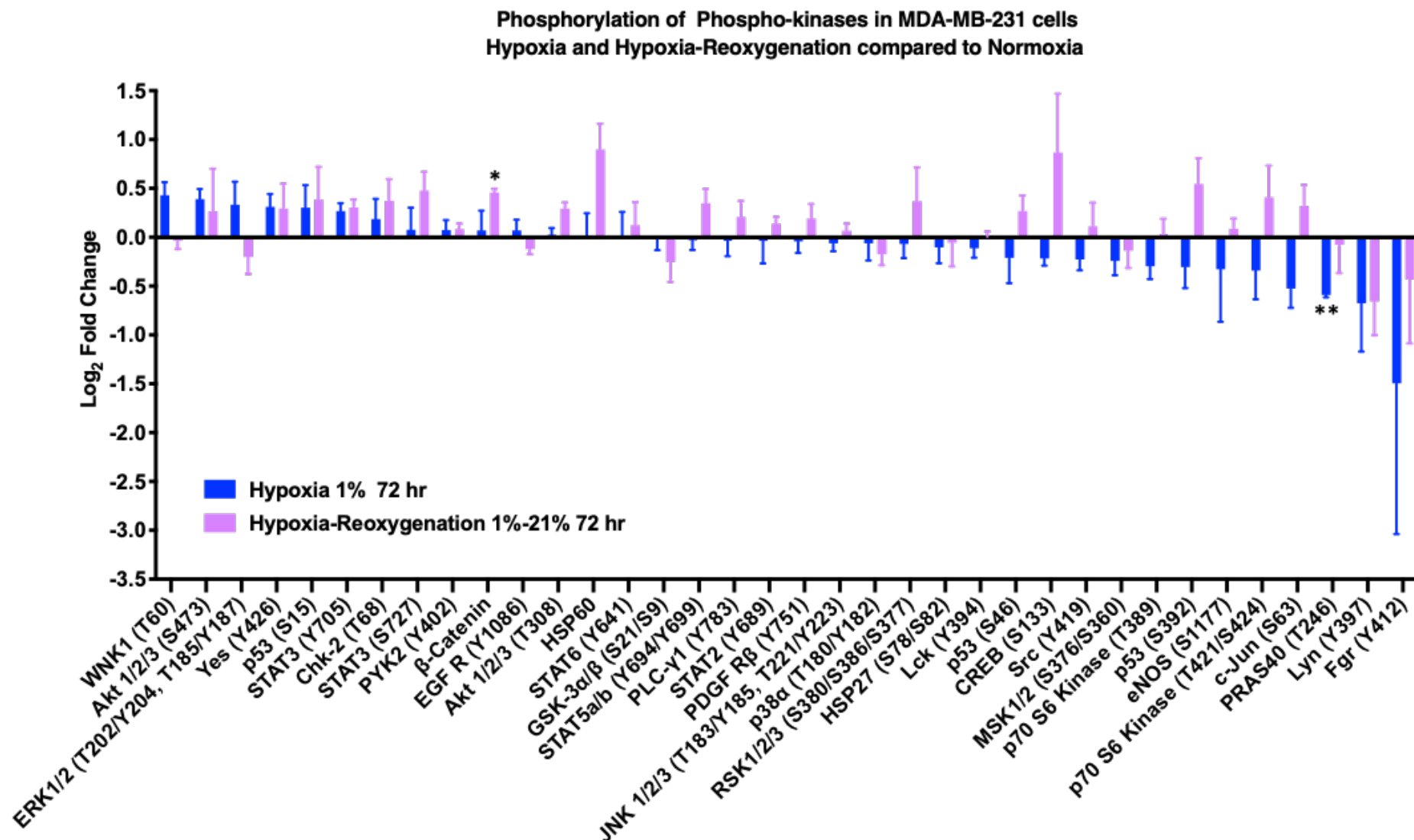


Figure 4.5. Phospho-kinase array results of the impact of hypoxia and hypoxia-reoxygenation on phosphokinase levels in MDA-MB-231 cells. Values obtained from the densitometry analysis of raw images. Phospho-proteins are arranged above from the most upregulated in hypoxia compared to normoxia (log₂ fold change) to most downregulated. Blue bars are Hypoxia (1% O₂ 72 hours), purple bars are hypoxia-reoxygenation (1%O₂ 72 hours followed by 21%O₂ 72 hours) Error bars indicate ± SEM. Data was analysed using a 2way ANOVA. *p<0.05, **p<0.01. n=3.

4.3.3 Western blot investigation

Following the phospho-RTK array and the intracellular kinase array analysis, several targets were selected for further investigation. From the phospho-RTK array, Insulin R (ALK) was identified to be upregulated under hypoxia and significantly under hypoxia-reoxygenation compared to normoxia. Therefore, it was selected for further investigation. However, selecting targets from the phospho-kinase array was challenging due to the inter-replicate variation mentioned earlier. Few proteins were statistically significant; the study here focused on those that were closest to significance to be investigated in further studies (Table 4.1). Finally, STAT3 (Y705), AKT 1/2/3 (T308), CREB (S133), and HSP60 were selected.

As this study focused on signal transduction to understand the phosphorylation state of proteins of interest, a western blot was used to further investigate the results from the arrays. Researchers commonly use western blotting to calculate the amount of phosphorylated to unphosphorylated protein in samples. Initially, the MDA-MB-231 cell line was used for this array; for further investigation of the results, the MCF7 cell line was used in addition to the MDA-MB-231 cell line.

For the western blot investigation, MDA-MB-231 cells and MCF7 cells were incubated in conditions and time points used in the array, that is, normoxia (72 hours 21% O₂) or hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 21% O₂) and seeded in 10% FBS [v/v] as per protocol. Additional samples were added to this investigation; normoxia, hypoxia and hypoxia-reoxygenation samples incubated in their different conditions and seeded in 10% FBS as mentioned previously, 24 hours before protein extraction 10% FBS medium were removed, and 0.5% FBS [v/v] medium was added to the cells. The 0.5% FBS conditions were key to investigating the phospho-signalling to investigate if the changes observed were impacted by the concentration and composition of FBS (Nonnis S. et al. 2016).

Kinase Target	Phosphorylation Site	Normoxia vs. Hypoxia		Normoxia vs. Hypoxia-reoxygenation	
		Log2 Fold change	p-value	Log2 Fold change	p-value
WNK1	T60	0.430	0.1266	0.039	0.8520
Akt 1/2/3	S473	0.389	0.1004	0.263	0.7958
STAT3	Y705	0.268	0.1209	0.304	0.1044
STAT3	S727	0.076	0.9247	0.476	0.1981
β -Catenin	N/A	0.070	0.9176	0.458	0.0111*
Akt 1/2/3	T308	0.026	0.9036	0.294	0.0600
HSP60	N/A	0.012	0.9980	0.896	0.1201
CREB	S133	-0.217	0.1460	0.866	0.4180
c-Jun	S63	-0.525	0.1748	0.317	0.4138
PRAS40	T246	-0.519	0.0026**	-0.077	0.9506

Table 4.1 Shows changes induced by hypoxia and hypoxia-reoxygenation after normalising to normoxia. Densitometry analysis was performed on the raw images as described above. Targets arranged above from the greatest changes to the minor changes in hypoxia compared to normoxia (Log₂Fold change). *p<0.05, **p<0.01. n=3.

Insulin R (ALK) phosphorylation under hypoxia and hypoxia-reoxygenation in breast cancer

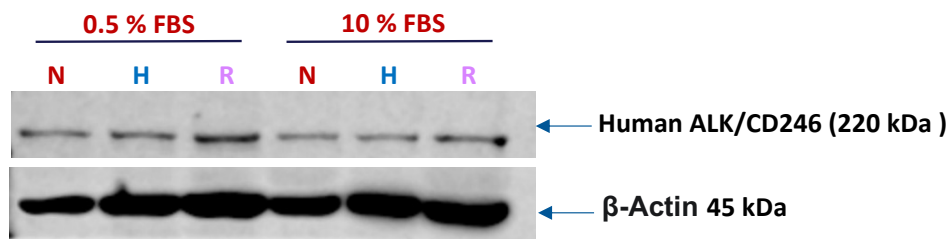
Figure 4.6 and Figure 4.7 shows a representative western blot image of the 3 completed replicates for ALK phosphorylation in MDA-MB-231 cells and MCF7 cells along the quantification (Figure 4.6 and Figure 4.7). ALK phosphorylation levels in each condition were first normalised to their respective β -Actin levels. Then hypoxia and hypoxia-reoxygenation conditions were normalised to normoxia condition (0.5% FBS hypoxia and hypoxia-reoxygenation normalised to 0.5% FBS normoxia and 10% FBS hypoxia and hypoxia-reoxygenation normalised to 10% FBS normoxia). This was to compare the changes in ALK phosphorylation under hypoxia and hypoxia-reoxygenation compared to normoxia. The human ALK/CD24 antibody used here matches the exact target in the phospho-RTK array experiment.

In MDA-MB-231 cells, ALK phosphorylation increased slightly under hypoxic conditions when normalised to β -Actin levels in 0.5% and 10% FBS conditions (Log₂Fold change=1.1, p=0.77 n=3) and (Log₂Fold change=1.17, p=0.71 n=3) respectively (Figure 4.6). As expected from the phospho-RTK array, in hypoxia-reoxygenation compared to normoxia, the ALK phosphorylation increased significantly in 0.5% FBS condition (Log₂Fold change=1.7, p<0.05,

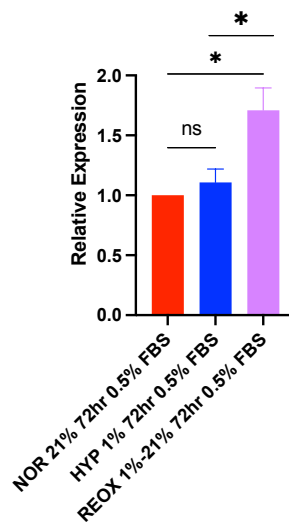
n=3) but the increase was not statistically significant in the 10% FBS condition (Log₂Fold change=1.35, p=0.3, n=3) when normalised to β -Actin levels (Figure 4.6).

In MCF7 cells, a slight induction of ALK phosphorylation was observed in hypoxia compared to normoxia when normalised to β -Actin levels in 0.5% FBS condition (Log₂Fold change=1.22, p=0.9, n=3) however slight decrease was observed in 10% FBS condition (Log₂Fold change=0.8, p=0.5, n=3) (Figure 4.6). Under hypoxia-reoxygenation, ALK phosphorylation increased in 0.5% FBS condition (Log₂Fold change=2.3, p=0.1, n=3) and in 10% FBS condition (Log₂Fold change=1.5, p<0.05, n=3) when compared to normoxia after normalising to β -Actin levels (Figure 4.6).

A) MDA-MB-231 (NOR 21% 72 hr / HYP 1% 72 hr / REOX 1-21% 72 hr)



**B) MDA-MB-231
Human ALK/CD246
Normalised to β-Actin**



**C) MDA-MB-231
Human ALK/CD246
Normalised to β-Actin**

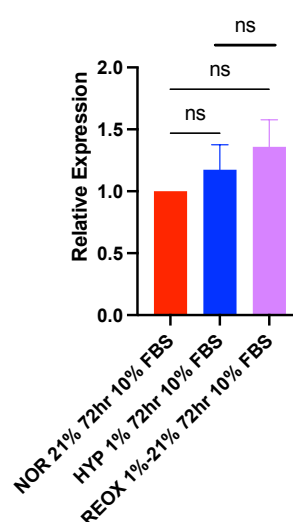


Figure 4.6. Phosphorylation of ALK in MDA-MB-231 cell. **A)** Representative Western blot image for ALK(CD246) phosphorylation in different conditions under 0.5% FBS and 10% FBS. phosphorylated ALK levels were normalised to the β-Actin levels first, then hypoxia and hypoxia-reoxygenation conditions were normalised to normoxia. **B)** Hypoxia 0.5% FBS and hypoxia-reoxygenation 0.5% FBS were normalised to normoxia 0.5% FBS. **C)** Hypoxia 10% FBS and hypoxia-reoxygenation 10% FBS were normalised to normoxia 10% FBS. The expression levels in MDA-MB-231 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia

Error bars indicate ± SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. *p<0.05. n=3

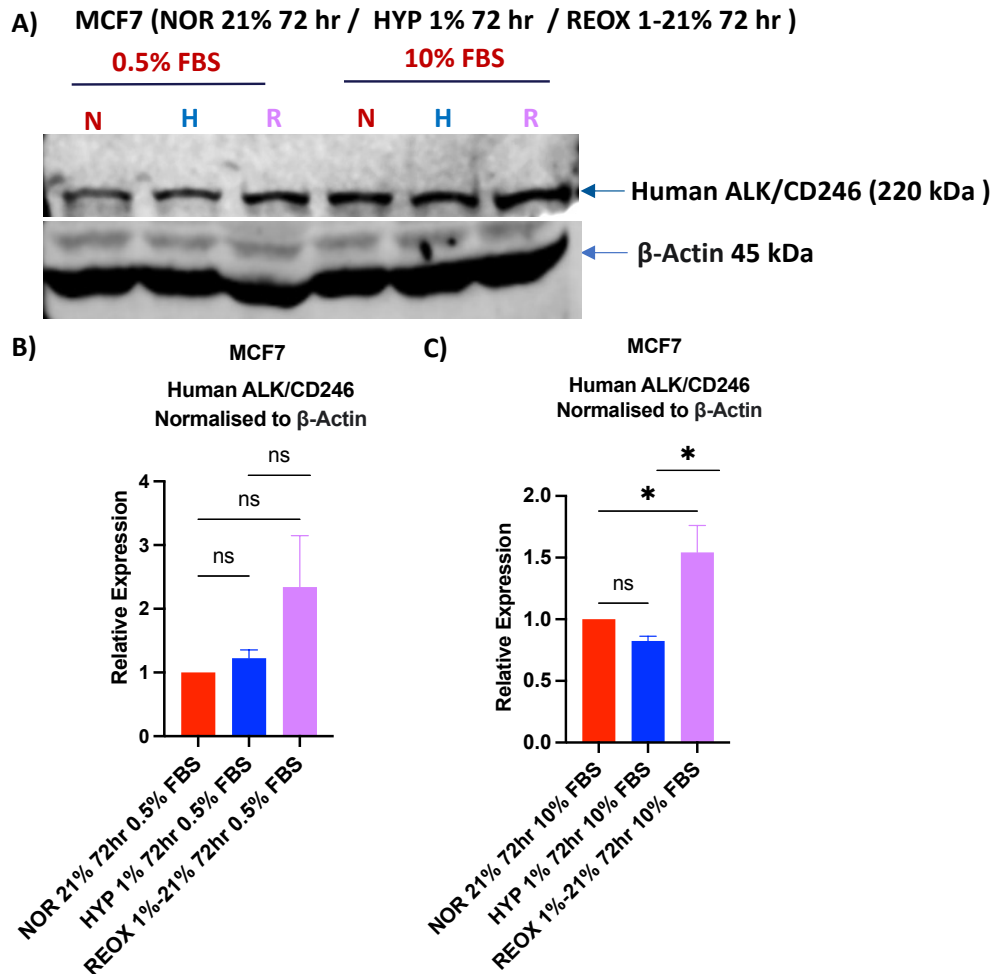


Figure 4.7. Phosphorylation of ALK in MCF7 cell. **A)** Representative Western blot image for ALK(CD246) phosphorylation in different conditions under 0.5% FBS and 10% FBS. phosphorylated ALK levels were normalised to the β-Actin levels first, then hypoxia and hypoxia-reoxygenation conditions were normalised to normoxia. **B)** Hypoxia 0.5% FBS and hypoxia-reoxygenation 0.5% FBS were normalised to normoxia 0.5% FBS. **C)** Hypoxia 10% FBS and hypoxia-reoxygenation 10% FBS were normalised to normoxia 10% FBS. The expression levels in MCF7 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$. $n = 3$

STAT3 phosphorylation under hypoxia and hypoxia-reoxygenation in breast cancer

Figure 4.8 and Figure 4.9 shows representative western blot images of phosphorylated STAT3 (Y705), total STAT3 and β -Actin in MDA-MB-231 and MCF7 cell lines with quantifications shown in (Figure 4.8B-G) and (Figure 4.9B-G). The first quantification displays the ratio of phospho-STAT3 (Y705) levels to β -Actin levels in each condition (phospho-STAT3 (Y705)/ β -Actin); in hypoxia and hypoxia-reoxygenation (Figure 4.8B and Figure 4.8BE) (Figure 4.9B and Figure 4.9E). The second quantification displays the ratio of phospho-STAT3 (Y705) levels to total STAT3 levels (phospho-STAT3 (Y705)/T-STAT3) (Figure 4.8C and Figure 4.8F) (Figure 4.9C and Figure 4.9 F). This quantification measured the changes in the phosphorylation status of STAT3 (Y705) compared to the total amount of STAT3 present in each sample. The third quantification analyses the ratio of the total STAT3 level to β -Actin levels (T-STAT3/ β -Actin) (Figure 4.8D and Figure 4.8G) (Figure 4.9D and Figure 4.9 G). This was to distinguish between changes in STAT3 expression vs. changes in the phosphorylation status STAT3 (Y705).

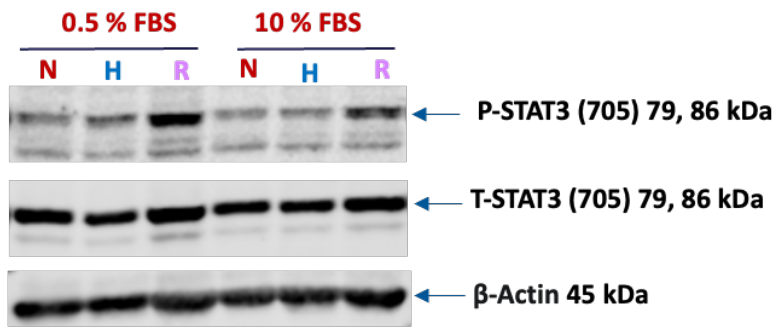
In MDA-MB-231, Phospho-STAT3 (Y705) increased in hypoxia compared to normoxia when normalised to β -Actin with a 2.76-fold increase in 0.5% FBS and a 1.46-fold increase in 10% FBS. Phospho-STAT3 (Y705) increased in hypoxia-reoxygenation compared to normoxia when normalised to β -Actin with a 3.11-fold increase in 0.5% FBS and a 2-fold increase in 10% FBS (Figure 4.8B and 4.8 E). However, these changes were not statistically significant.

Phospho-STAT3 (Y705) increased significantly in hypoxia and hypoxia-reoxygenation compared to normoxia when normalised to total STAT3 in 0.5% FBS and 10% FBS. In 0.5% FBS conditions, phospho-STAT3 (Y705) had a significant increase in hypoxia ($\text{Log}_2\text{Fold change}=3$, $p<0.01$, $n=3$) and hypoxia-reoxygenation ($\text{Log}_2\text{Fold change}=2$, $p<0.05$, $n=3$) compared to normoxia. Similarly, in 10% FBS conditions, phospho-STAT3 (Y705) had a significant increase in hypoxia ($\text{Log}_2\text{Fold change}=1.88$, $p<0.05$, $n=3$) and a significant 1.76-fold increase in hypoxia-reoxygenation ($\text{Log}_2\text{Fold change}=1.76$, $p<0.05$, $n=3$) compared to normoxia (Figure 4.8C and Figure 4.8F).

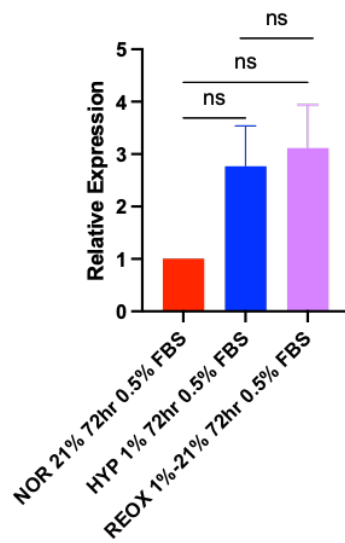
The expression of total STAT3 had slightly decreased in hypoxia compared to normoxia when normalised to β -Actin with a 0.89-fold decrease in 0.5% FBS and 0.76-fold decrease in 10% FBS conditions. In hypoxia-reoxygenation, the total STAT3 expression had increased by 1.45-

fold in 0.5% FBS and by 1.12-fold in 10% FBS compared to normoxia. Statistically, these were not significant (Figure 4.8D and Figure 4.8G).

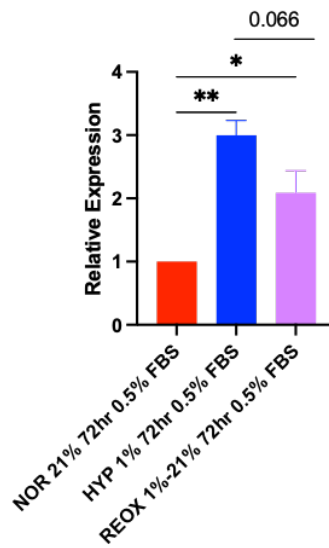
A) MDA-MB-231 (NOR 21% 72 hr / HYP 1% 72 hr / REOX 1-21% 72 hr)



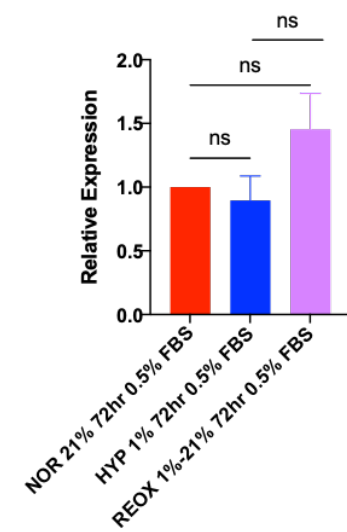
**B) MDA-MB-231
P-STAT3 (Y705)
Normalised to β-Actin**



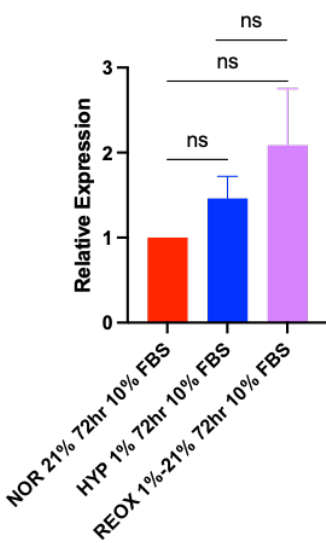
**C) MDA-MB-231
P-STAT3(Y705)
Normalised to Total STAT3**



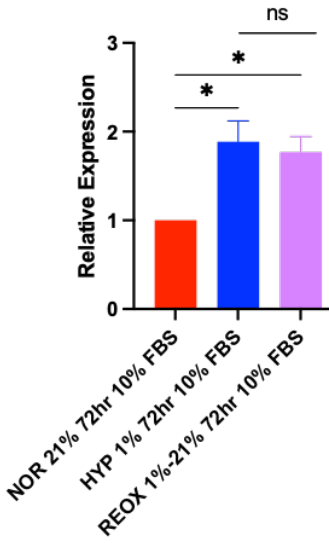
**D) MDA-MB-231
Total STAT3
Normalised to β-Actin**



**E) MDA-MB-231
P-STAT3 (Y705)
Normalised to β-Actin**



**F) MDA-MB-231
P-STAT3(Y705)
Normalised to Total STAT3**



**G) MDA-MB-231
Total STAT3
Normalised to β-Actin**

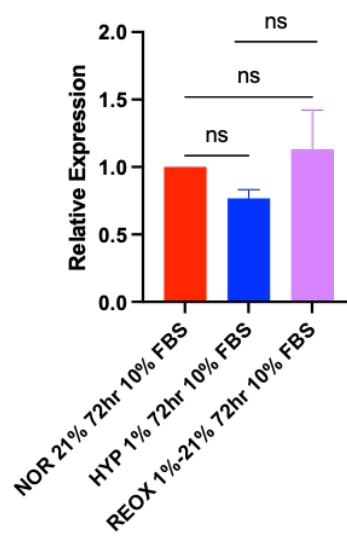


Figure 4.8. Phosphorylation of STAT3 (Y705) in MDA-MB-231 cell under hypoxia and hypoxia-reoxygenation compared to normoxia. A) Representative Western blot image for phospho-STAT3 (Y705), total STAT3 and loading control β -Actin. **B)-D)** Quantification for phospho-STAT (Y705) and total STAT3 in 0.5% FBS conditions. **E)-G)** Quantification for phospho-STAT (Y705) and total STAT3 in 10% FBS conditions. The expression levels in MDA-MB-231 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$. $n = 3$.

Figure 4.9 shows representative western blot images of phosphorylated STAT3 (Y705) in MCF7 cells. Phospho-STAT3 (Y705) significantly increased in hypoxia compared to normoxia when normalised to β -Actin ($\text{Log}_2\text{Fold change} = 1.5$, $p < 0.05$, $n = 3$) in 0.5% FBS condition. However, in 10% FBS conditions, phospho-STAT3 (Y705) decreased in 10% FBS conditions ($\text{Log}_2\text{Fold change} = 0.85$, $p = 0.9$, $n = 3$). Phospho-STAT3 (Y705) significantly increased in hypoxia-reoxygenation compared to normoxia when normalised to β -Actin in 0.5% FBS condition ($\text{Log}_2\text{Fold change} = 2$, $p < 0.01$, $n = 3$). It has also increased in 10% FBS condition ($\text{Log}_2\text{Fold change} = 1.72$, $p = 0.3$, $n = 3$) (Figure 4.9B, Figure 4.9E).

Phospho-STAT3 (Y705) increased in hypoxia and hypoxia-reoxygenation compared to normoxia when normalised to total STAT3 in both conditions, 0.5% FBS and 10% FBS. In 0.5% FBS conditions, phospho-STAT3 (Y705) had a significant 2.39-fold increase in hypoxia ($p < 0.05$, $n = 3$) and a 1.81-fold increase in hypoxia-reoxygenation compared to normoxia ($p = 0.4$, $n = 3$). In 10% FBS conditions, phospho-STAT3 (Y705) had a 1.55-fold increase in hypoxia and a 1.87-fold increase in hypoxia-reoxygenation when compared to normoxia however, statistically, this was not significant (Figure 4.9C and Figure 4.9F).

The expression of total STAT3 decreased in hypoxia compared to normoxia when normalised to β -Actin with a significant 0.56-fold decrease in 0.5% FBS ($p < 0.05$, $n = 3$) and 0.41-fold decrease in 10% FBS ($p = 0.09$, $n = 3$). On the other hand, expression of total STAT3 increased in hypoxia-reoxygenation compared to normoxia when normalised to β -Actin in 0.5% FBS condition with 1.32-fold ($p = 0.052$, $n = 3$) and remained unchanged in 10% FBS condition ($\text{Log}_2\text{Fold change} = 1.06$, $p = 0.9$, $n = 3$).

A) MCF7 (NOR 21% 72 hr / HYP 1% 72 hr / REOX 1-21% 72 hr)

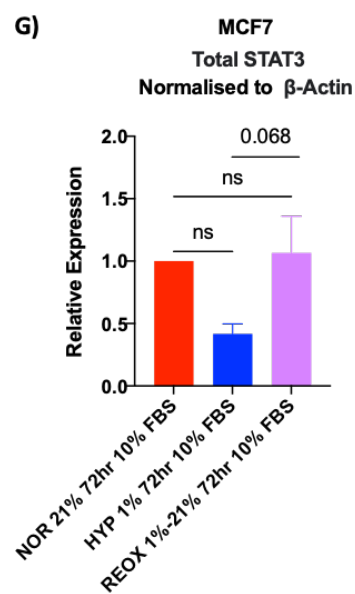
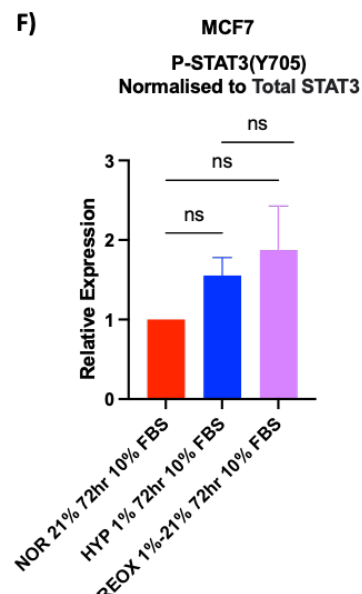
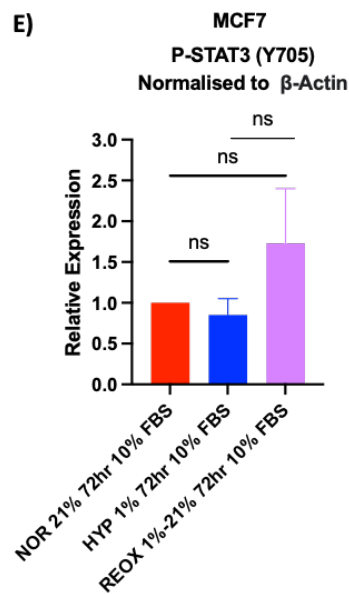
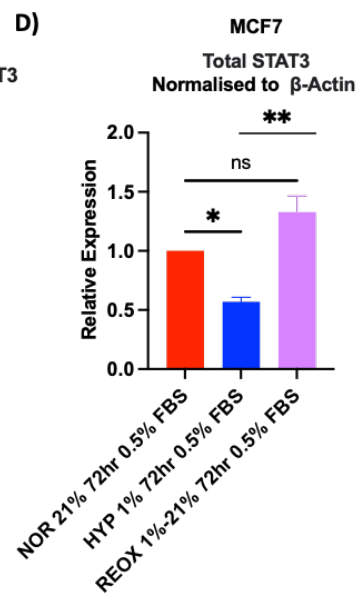
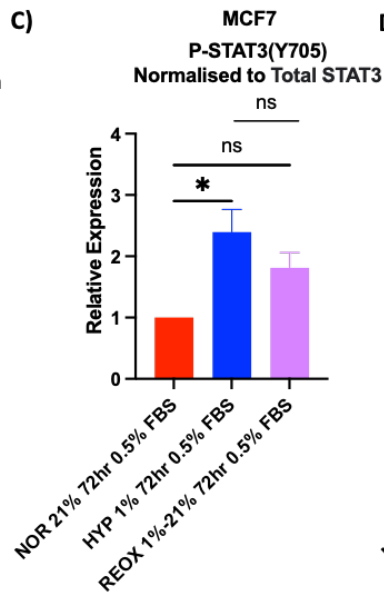
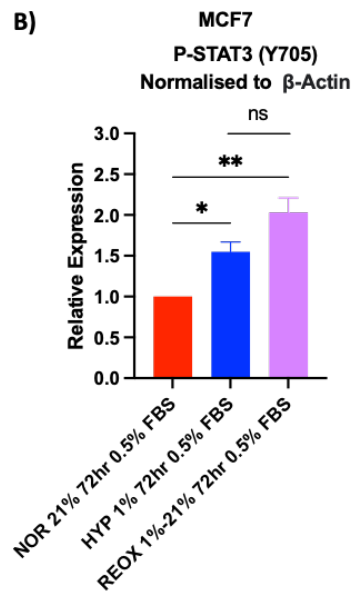
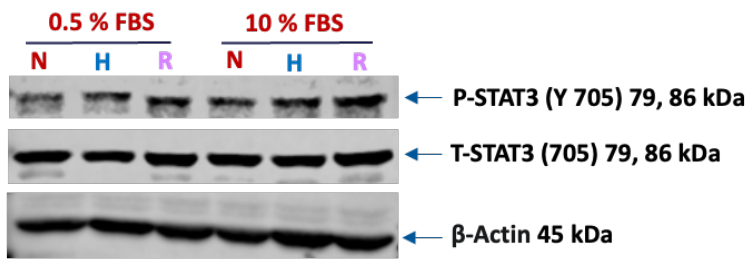


Figure 4.9. Phosphorylation of STAT3 (Y705) in MCF7 cells under hypoxia and hypoxia-reoxygenation compared to normoxia. A) Representative Western blot image for phospho-STAT3 (Y705), total STAT3 and loading control β -Actin. **B)-D)** Quantification for phospho-STAT (Y705) and total STAT3 in 0.5% FBS conditions. **E)-G)** Quantification for phospho-STAT (Y705) and total STAT3 in 10% FBS conditions. The expression levels in MCF7 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$. $n = 3$.

AKT 1/2/3 phosphorylation under hypoxia and hypoxia-reoxygenation in breast cancer

Figure 4.10 and Figure 4.11 shows representative western blot images of phosphorylated AKT (Thr308) and total AKT in MDA-MB-231 and MCF7 cell lines with quantification shown in (Figure 4.10B-G) and (Figure 4.11B-G). The first quantification displays the ratio of phospho-AKT 1/2/3 (Thr308) to β -Actin levels in each condition (AKT 1/2/3 (Thr308)/ β -Actin) (Figure 4.10B and Figure 4.10E) (Figure 4.11B and Figure 4.11E). The second quantification displays the ratio of phospho-AKT 1/2/3 (Thr308) levels of each condition total AKT levels (phospho-AKT 1/2/3 (Thr308)/ T-AKT) (Figure 4.10C and Figure 4.10F) (Figure 4.11C and Figure 4.11F). This quantification measured the changes in the phosphorylation status AKT 1/2/3 (Thr308) compared to the total AKT present in each sample. The third quantification interrogates the ratio of the total AKT to β -Actin levels (T-AKT/ β -Actin) (Figure 4.10D and Figure 4.10G) (Figure 4.11D and Figure 4.11G). This was to distinguish between changes in AKT expression vs. changes in the phosphorylation status STAT3 (Y705).

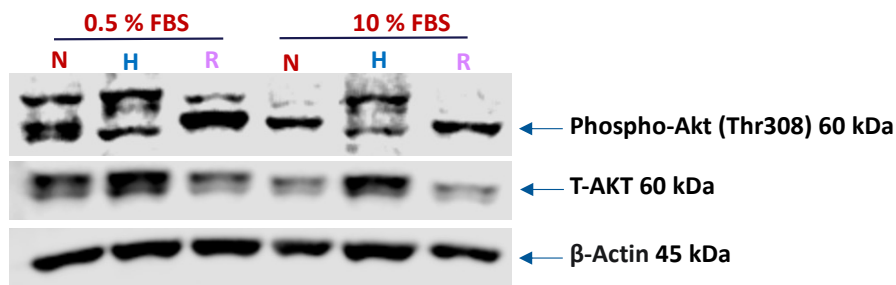
In MDA-MB-231 cells, the analysis identified that phospho-AKT 1/2/3 (Thr308) changed in hypoxia compared to normoxia when normalised to β -Actin with a 1.69-fold increase in 0.5% FBS condition and 0.76-fold decrease in 10% FBS condition. In hypoxia-reoxygenation compared to normoxia, phospho-AKT 1/2/3 (Thr308) increased significantly with a 2.7-fold increase in 0.5% FBS ($p < 0.05$, $n = 3$) and 1.65-fold increase in 10% FBS ($p < 0.05$, $n = 3$) (Figure 4.10B and Figure 4.10E).

Phospho-AKT 1/2/3 (Thr308) increased significantly in hypoxia and hypoxia-reoxygenation compared to normoxia when normalised to total AKT in 0.5% FBS conditions with 1.73-fold increase in hypoxia ($p < 0.05$, $n = 3$) and 1.81-fold increase in hypoxia-reoxygenation ($p < 0.05$,

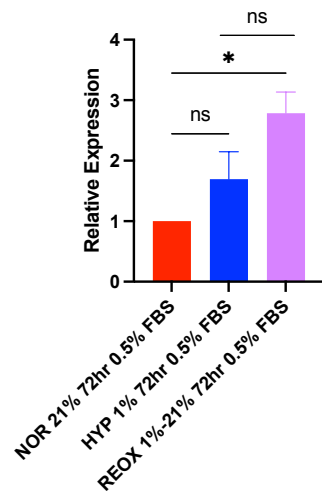
n=3). Similar results were found with the phospho-AKT 1/2/3 (Thr308) in hypoxia and hypoxia-reoxygenation compared to normoxia when normalised to total AKT in the 10% FBS conditions; hypoxia had a 1.16-fold increase and hypoxia-reoxygenation had a 1.33-fold increase, however, statistically, this was not significant (Figure 4.10C and Figure 4.10F).

The expression of total AKT remained constant in hypoxia compared to normoxia when normalised to β -Actin in both 0.5% FBS and 10% FBS conditions. In hypoxia-reoxygenation compared to normoxia, expression of total AKT remained constant when normalised to β -Actin in 0.5% FBS; however, this has increased significantly to 1.3-fold in 10% FBS condition ($p<0.05$, $n=3$) (Figure 4.10D and Figure 4.10G).

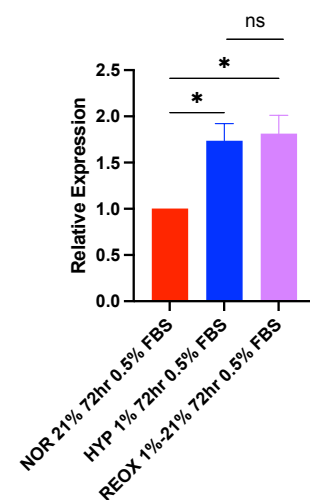
A) MDA-MB-231 (NOR 21% 72 hr / HYP 1% 72 hr / REOX 1-21% 72 hr)



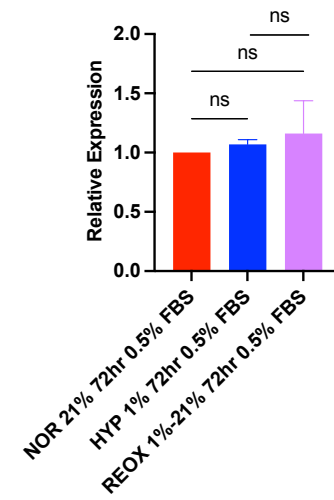
B) MDA-MB-231
P-AKT(Thr308)
Normalised to β-Actin



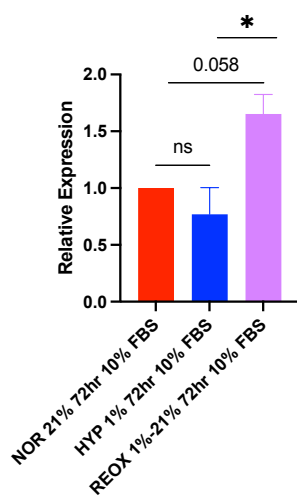
C) MDA-MB-231
P-AKT(Thr308)
Normalised to Total AKT



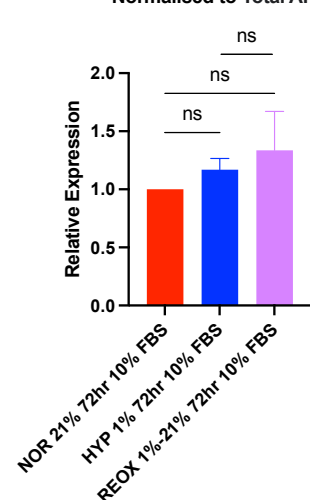
D) MDA-MB-231
Total AKT
Normalised to β-Actin



E) MDA-MB-231
P-AKT(Thr308)
Normalised to β-Actin



F) MDA-MB-231
P-AKT(Thr308)
Normalised to Total AKT



G) MDA-MB-231
Total AKT
Normalised to β-Actin

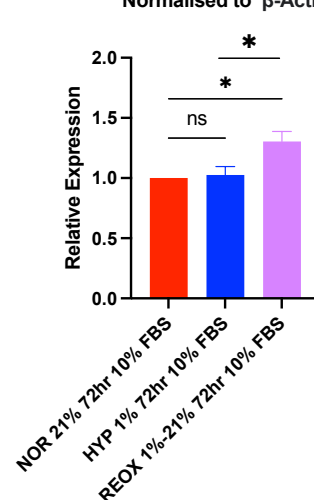


Figure 4.10 Phosphorylation of AKT (Thr 308) in MDA-MB-231 cells under hypoxia and hypoxia-reoxygenation compared to normoxia. A) Representative Western blot image for phospho-AKT (Thr 308), total AKT and loading control β -Actin. **B)-D)** Quantification for phospho-AKT (Thr 308) and total AKT in 0.5% FBS conditions. **E)-G)** Quantification for phospho-AKT (Thr 308) and total AKT in 10% FBS conditions. The expression levels in MDA-MB-231 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

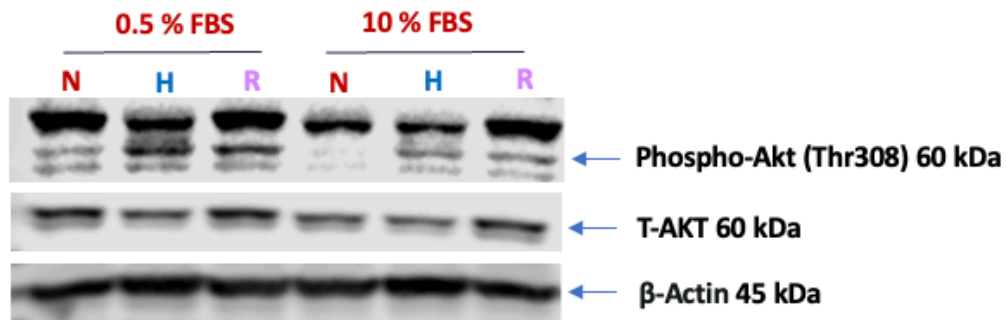
Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$. $n = 3$.

In MCF7 cells, the analysis revealed that phospho-AKT 1/2/3 (Thr308) has significantly increased in hypoxia and hypoxia-reoxygenation compared to normoxia when normalised to β -Actin. In 0.5% FBS conditions, phospho-AKT 1/2/3 (Thr308) increased substantially to 2.37-fold in hypoxia ($p < 0.01$, $n = 3$) and to 1.78-fold in hypoxia-reoxygenation ($p < 0.05$, $n = 3$). In 10% FBS conditions, phospho-AKT 1/2/3 (Thr308) increased to 2.48-fold in hypoxia and to 2-fold in hypoxia-reoxygenation; however, statistically, this was not significant (Figure 4.11B and Figure 4.11E).

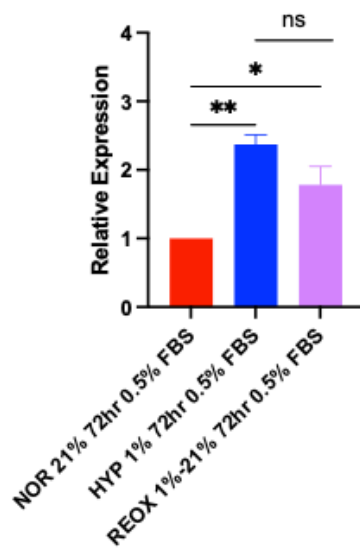
Phospho-AKT 1/2/3 (Thr308) increased significantly in hypoxia compared to normoxia when normalised to total AKT in 0.5% FBS conditions ($\text{Log}_2\text{Fold change} = 3.17$, $p < 0.001$, $n = 3$) and in 10% FBS ($\text{Log}_2\text{Fold change} = 2.7$, $p < 0.05$, $n = 3$). phospho-AKT 1/2/3 (Thr308) also increased in hypoxia-reoxygenation compared to normoxia when normalised to total AKT in 0.5% FBS conditions ($\text{Log}_2\text{Fold change} = 1.67$, $p = 0.1$, $n = 3$) and in 10% FBS conditions ($\text{Log}_2\text{Fold change} = 1.64$, $p = 0.4$, $n = 3$) (Figure 4.11C and Figure 4.11F).

The expression of total AKT has decreased in hypoxia compared to normoxia when normalised to β -Actin in both 0.5% FBS and 10% FBS conditions, significantly in 0.5% FBS conditions ($\text{Log}_2\text{Fold change} = 0.78$, $p < 0.001$, $n = 3$) and decreased in 10% FBS ($\text{Log}_2\text{Fold change} = 0.87$, $p = 0.2$, $n = 3$). In hypoxia-reoxygenation compared to normoxia, the expression of total AKT has significantly increased in both 0.5% FBS and 10% FBS conditions when normalised to β -Actin with 1.2-fold increase in 0.5% FBS conditions ($p < 0.001$, $n = 3$) and 1.3-fold increase in 10% FBS conditions ($p < 0.05$, $n = 3$) (Figure 4.11D and Figure 4.11G).

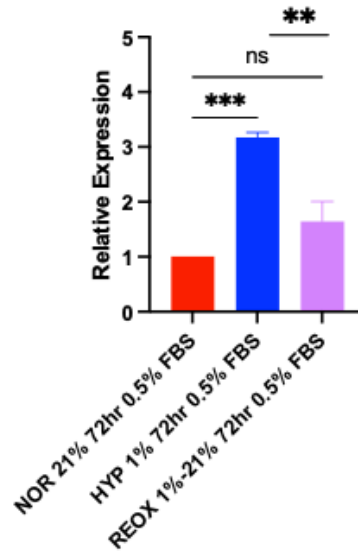
A) MCF7 (NOR 21% 72 hr / HYP 1% 72 hr / REOX 1-21% 72 hr)



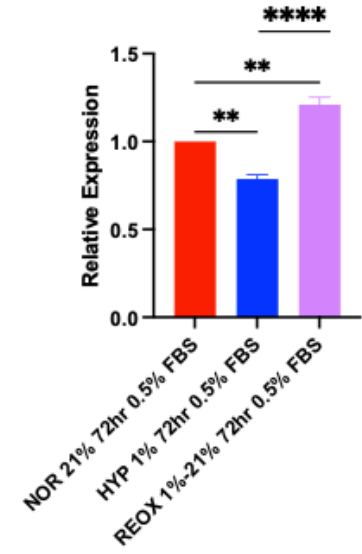
B) MCF7
P-AKT(Thr308)
Normalised to β-Actin



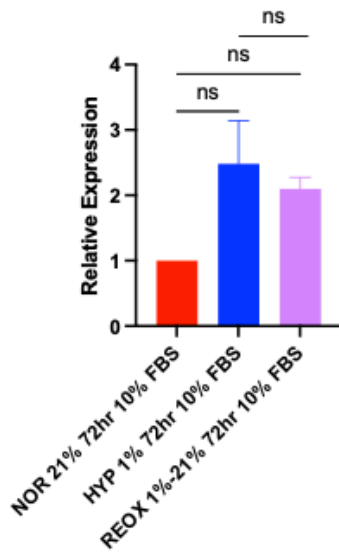
C) MCF7
P-AKT(Thr308)
Normalised to Total AKT



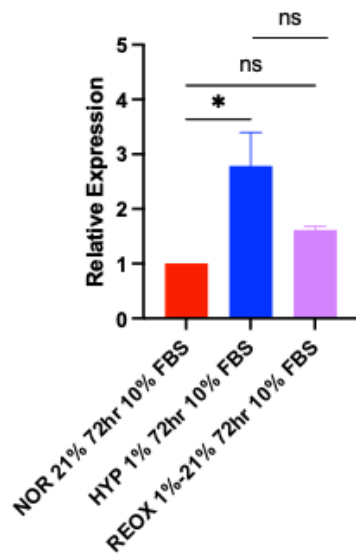
D) MCF7
Total AKT
Normalised to β-Actin



E) MCF7
P-AKT(Thr308)
Normalised to β-Actin



F) MCF7
P-AKT(Thr308)
Normalised to Total AKT



G) MCF7
Total AKT
Normalised to β-Actin

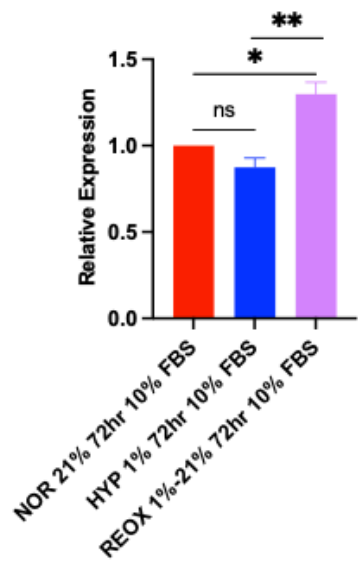


Figure 4.11. Phosphorylation of AKT (Thr 308) in MCF7 cells under hypoxia and hypoxia-reoxygenation compared to normoxia. **A)** Representative Western blot image for phospho-AKT (Thr 308), total AKT and loading control β -Actin. **B)-D)** Quantification for phospho-AKT (Thr 308) and total AKT in 0.5% FBS conditions. **E)-G)** Quantification for phospho-AKT (Thr 308) and total AKT in 10% FBS conditions. The expression levels in MCF7 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 3$.

CREB phosphorylation under hypoxia and hypoxia-reoxygenation in breast cancer

Figure 4.12. and Figure 4.13. shows a representative western blot for phosphorylation of CREB (Ser 133) in MDA-MB-231 cells and MCF7 cells. The quantification displays the ratio of phospho-CREB (Ser 133) levels to β -Actin levels in each condition (phospho-CREB (Ser 133)/ β -Actin); in hypoxia and hypoxia-reoxygenation (Figure 4.12B and Figure 4.12C) (Figure 4.13B and Figure 4.13C).

In MDA-MB-231 cells, surprisingly, the phosphorylation of CREB (Ser 133) increased dramatically in hypoxia and hypoxia-reoxygenation compared to normoxia when normalised to β -Actin levels with a 4-fold increase in 0.5% FBS conditions ($p < 0.01$, $n = 3$) and 2.7-fold increase in 10% FBS conditions ($p < 0.01$, $n = 3$) (Figure 4.12B and Figure 4.12C). Phosphorylation of CREB (Ser 133) also increased in hypoxia-reoxygenation compared to normoxia when normalised to β -Actin levels with a 1.4-fold increase in 0.5% FBS conditions; however, the phosphorylation levels remained constant in hypoxia-reoxygenation compared to normoxia in 10% FBS conditions (Figure 4.12B and Figure 4.12C). Statistically, this was not significant (Figure 4.12B and Figure 4.12C).

Surprisingly, in MCF7 cells, phospho-CREB (Ser 133) had a similar dramatic increase in hypoxia compared to normoxia when normalised to β -Actin in both 0.5% FBS and 10% FBS conditions. phospho-CREB (Ser 133) had significantly increased to 3.35-fold in 0.5% FBS condition ($p < 0.001$, $n = 3$) and to 3.1-fold increase in 10% FBS ($p < 0.0001$, $n = 3$). Phosphorylation of CREB (Ser 133) remained constant in hypoxia-reoxygenation compared to normoxia when

normalised to β -Actin levels in both conditions; this was not statistically significant (Figure 4.13B and Figure 4.13C).

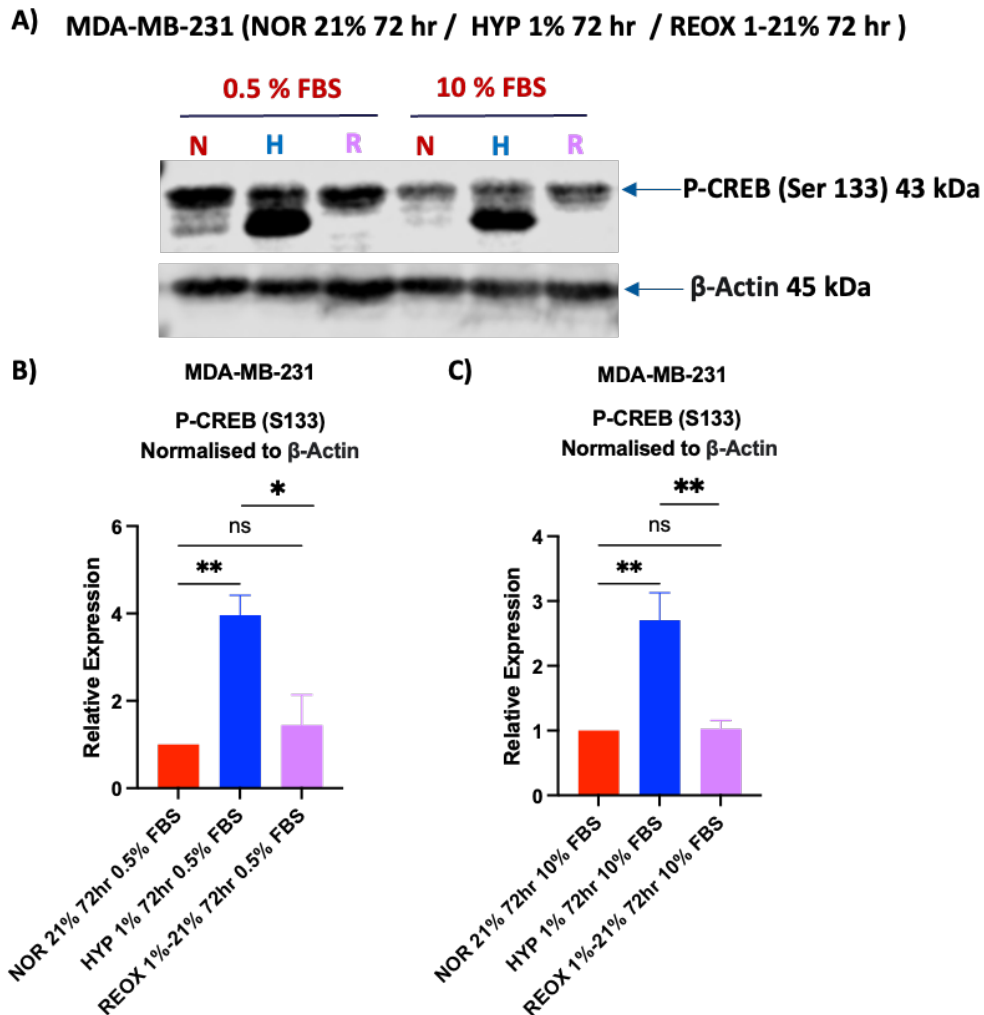


Figure 4.12 Phosphorylation of CREB (Ser133) in MDA-MB-231 cell under hypoxia and hypoxia-reoxygenation compared to normoxia. A) Representative Western blot image for phospho-CREB (Ser133), loading control β -Actin. **B)** Quantification for phospho-CREB (Ser133) in 0.5% FBS conditions. **C)** Quantification for phospho-CREB (Ser133) in 10% FBS conditions. The expression levels in MDA-MB-231 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. ** $p < 0.01$. $n = 3$.

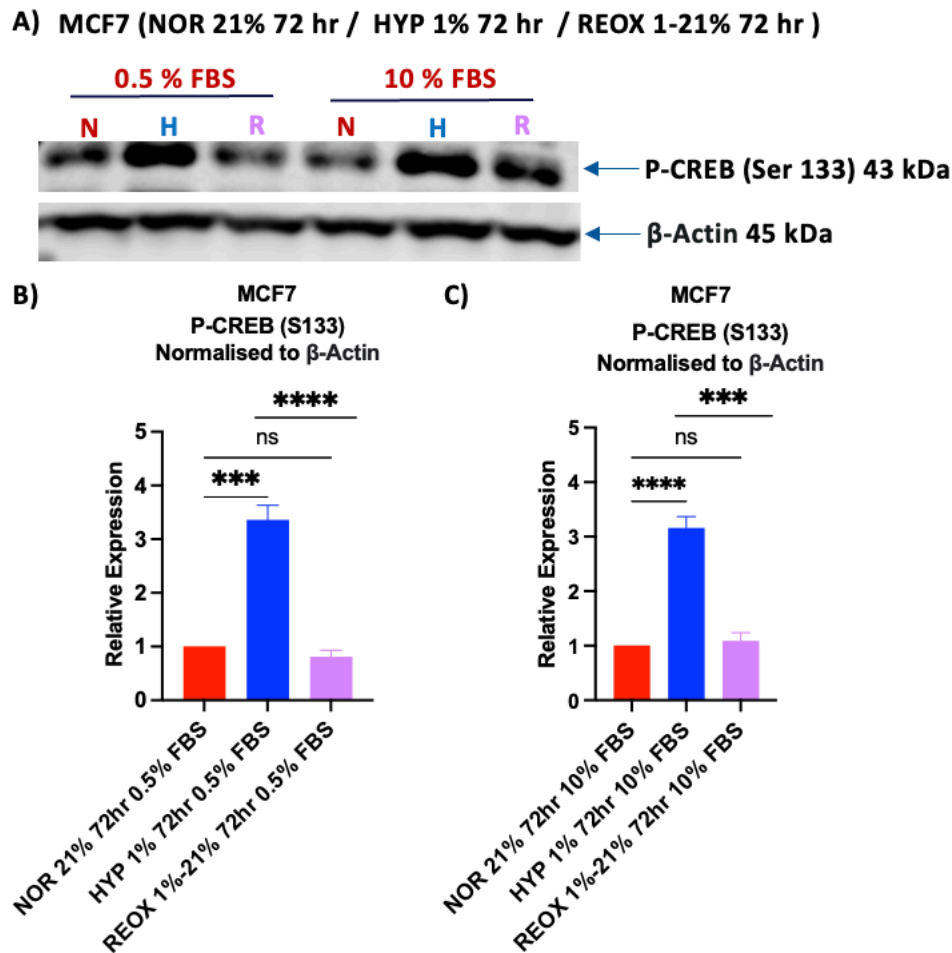


Figure 4.13. Phosphorylation of CREB (Ser133) in MCF7 cell under hypoxia and hypoxia-reoxygenation compared to normoxia. A) Representative Western blot image for phospho-CREB (Ser133), loading control β -Actin. **B)** Quantification for phospho-CREB (Ser133) in 0.5% FBS conditions. **C)** Quantification for phospho-CREB (Ser133) in 10% FBS conditions. The expression levels in MCF7 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. *** $p < 0.001$, **** $p < 0.0001$. $n = 3$.

HSP60 phosphorylation under hypoxia and hypoxia-reoxygenation in breast cancer

The phospho-kinase array conducted has also investigated 2 related total proteins, β -Catenin and HSP60. The analysis has further investigated the expression of total HSP60 by western blot in MDA-MB-231 and MCF7. Additional investigation of phospho-HSP60 was undertaken using phos-tag Acrylamide system, which is used to study the protein phosphorylation levels

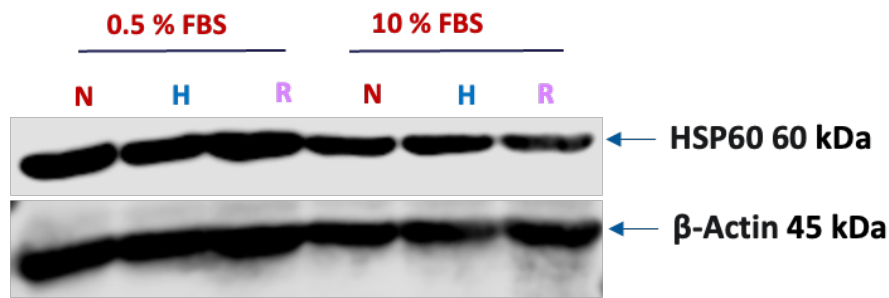
when there is no available phospho-specific antibodies. The phos-tag acrylamide system is combined with SDS-PAGE to separate phosphorylated from non-phosphorylated proteins. Multiple optimisations were undertaken to use this system to study the phosphorylation of HSP60. However, this study had to stop due to the time limitation at the end of the project.

Figure 4.14 and Figure 4.15 shows representative western blot images of HSP60 expression in MDA-MB-231 and MCF7 cell lines with quantification shown in (Figure 4.14B and Figure 4.14C) (Figure 4.15B and Figure 4.15C). The quantification displays the ratio of total HSP60 levels to β -Actin levels (HSP60/ β -Actin).

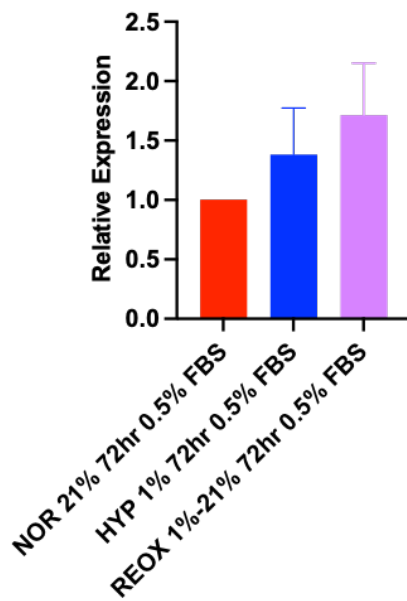
In MDA-MB-231, the expression of HSP60 increased in hypoxia compared to normoxia when normalised to β -Actin in 0.5% FBS condition ($\text{Log}_2\text{Fold change}=1.3$, $n=2$) and in 10% FBS condition ($\text{Log}_2\text{Fold change}=1.14$, $n=2$). This increase has also been observed in hypoxia-reoxygenation compared to normoxia when normalised to β -Actin in 0.5% FBS condition ($\text{Log}_2\text{Fold change}=1.7$, $n=2$) and in 10% FBS condition ($\text{Log}_2\text{Fold change}=1.3$, $n=2$) (Figure 4.15). Additional biological repeats are required to confirm these results.

In MCF7, the expression of HSP60 decreased in hypoxia compared to normoxia when normalised to β -Actin in 0.5% FBS condition ($\text{Log}_2\text{Fold change}=0.6$, $n=3$) and in 10% FBS condition ($\text{Log}_2\text{Fold change}=0.7$, $n=3$). In hypoxia-reoxygenation compared to normoxia, expression of HSP60 slightly increased in 0.5% FBS condition ($\text{Log}_2\text{Fold change}=1.15$, $n=3$) and decreased in 10% FBS condition ($\text{Log}_2\text{Fold change}=0.9$, $n=3$) (Figure 4.15). Statistically, this was not significant.

A) MDA-MB-231 (NOR 21% 72 hr / HYP 1% 72 hr / REOX 1-21% 72 hr)



B) MDA-MB-231
HSP60 Normalised to β-Actin



C) MDA-MB-231
HSP60 Normalised to β-Actin

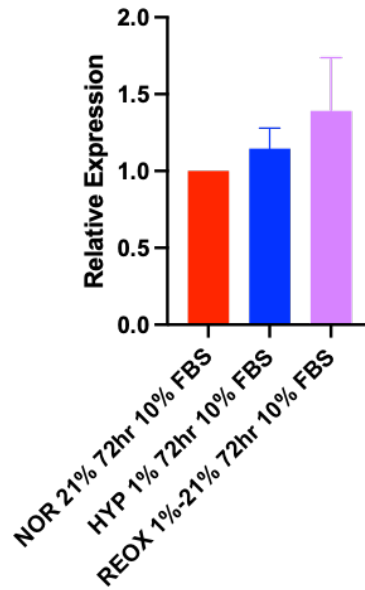


Figure 4.14. HSP60 expression in MD-MB-231 cell under hypoxia and hypoxia-reoxygenation compared to normoxia. A) Representative Western blot image for HSP60 and loading control β-Actin. **B)** Quantification for HSP60 in 0.5% FBS conditions. **C)** Quantification for HSP60 in 10% FBS conditions.

Error bars indicate $\pm$ SEM. n=2

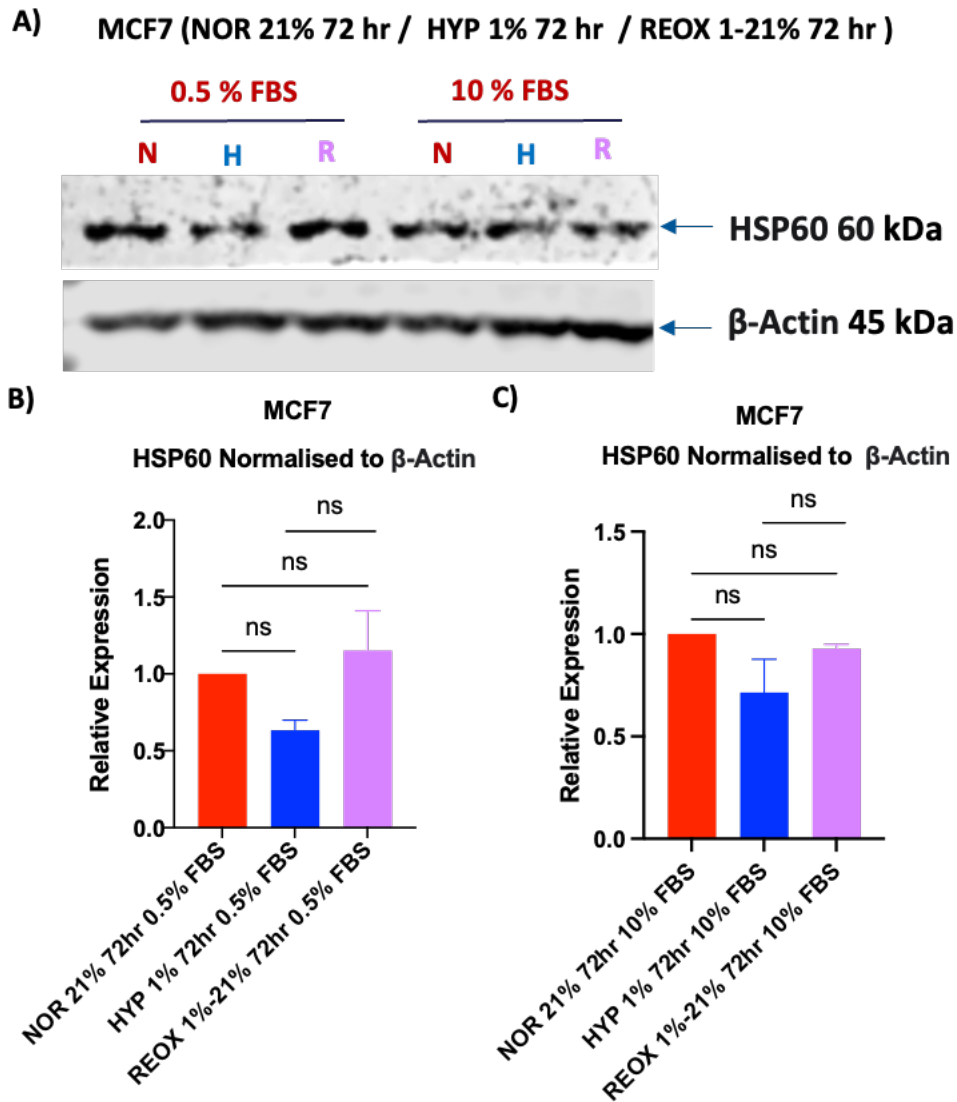


Figure 4.15. HSP60 expression in MCF7 cell under hypoxia and hypoxia-reoxygenation compared to normoxia. A) Representative Western blot image for HSP60 and loading control β -Actin. **B)** Quantification for HSP60 in 0.5% FBS conditions. **C)** Quantification for HSP60 in 10% FBS conditions. The expression levels in MCF7 cells compared hypoxia and hypoxia-reoxygenation to normoxia and compared hypoxia-reoxygenation to hypoxia.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. $n=3$

4.5 Discussion

4.5.1 Phospho-signalling changes in breast cancer

In this study we hypothesised that activation of receptor tyrosine kinases (RTKs) and intracellular kinases changes hypoxia and hypoxia-reoxygenation., some of the hypoxia-induced changes is maintained in hypoxia-reoxygenation. To test this, we investigated the phospho-signalling alteration using two arrays to explore receptor tyrosine kinases (RTKs) and intracellular kinases, followed by western blotting to further investigate the observed results. The screen was completed in the MDA-MB-231 cell line, which is representative of TNBC. Further investigations by western blot included MCF7, representative of Luminal A. Cells were incubated in normoxia (72 hours 21% O₂), hypoxia (72 hours 1% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 21% O₂).

The screen analysis in MDA-MB-231 cells presented here revealed that 24% of RTKs and 31% of intracellular kinases are upregulated under hypoxia such as WNK1 (T60), ERK1/2 (T202/Y204, T185/Y187) and Chk-2 (T68). Whereas 70% of the RTKs and 67% of intracellular kinases are upregulated under hypoxia-reoxygenation such as EGFR (ErbB2) and FGFR (FGF R3). These targets have been explored previously in cancer studies, and some of these are promising therapeutic targets due to their involvement with the tumour microenvironment.

ERKs, JNKs and p38 isoforms are part of the MAPKs family of proteins that regulate mitosis, differentiation, metabolism, apoptosis and survival, making them promising therapeutic targets in cancer (Roux PP. et al. 2004, Buscà R et al. 2016). Studies have shown that phosphorylation of ERK1/2 stabilises HIF-1 α , leading to chemoresistance in cancer, including breast cancer (Kopecka J. et al. 2016). In addition, overexpression of ERK1/2 and ERK5 has been linked to poor survival in breast cancer patients (Bhatt AB. et al. 2021). Inhibition of both PI3K/Akt and MEK5/ERK5 signalling showed an effective reduction in the proliferation and survival of TNBC compared to inhibiting either pathway alone, suggesting a crosstalk between ERK and AKT signalling pathway in TNBC (Wright TD. Et al. 2020). Another identified kinase from the arrays screen was Chk-2 (T68). It has been previously discovered that hypoxia induces the phosphorylation and activation of checkpoint kinase CHK2, consequently leading to the activation of the BRCA1 protein. BRCA1 protein plays a significant role in regulating the cell cycle checkpoints and repairing DNA in mammalian cells (Gibson SL. et al. 2006). In addition,

WNK1 has also been identified by the screen array in this study. WNK1 was found to be involved in the migration and invasion of breast cancer and contributed to the metastatic phenotype. The inhibition of WNK1 reduced the invasion and migration in breast cancer models (Jaykumar AB. Et al. 2021)

One of the main aims of this study was to identify kinases (RTKs and intracellular kinases) that have been upregulated under hypoxia and maintained upon hypoxia-reoxygenation in breast cancer. The analysis identified several RTKs such as Insulin R (IGF-I R and ALK), VEGF R1 (VEGF R) and DDR1, in addition to several intracellular kinases such as p53 (S15), Yes (Y426), Chk-2 (T68), Akt 1/2/3 (S473) and STAT3 at two phosphorylation sites at (Y705) and (S727) to be upregulated under hypoxia and maintained upon hypoxia-reoxygenation. Here we further investigated phosphorylation of ALK, STAT3 (Y705) and AKT 1/2/3 (Thr 308) by western blot.

4.5.2 Alteration of Insulin R signalling pathway under hypoxia and hypoxia-reoxygenation in breast cancer

The analysis here showed that the insulin R signalling pathway is upregulated under hypoxia and hypoxia-reoxygenation and that through the activity of receptor tyrosine kinase insulin R family members: ALK and IGF-I R. The western blot investigation showed that ALK phosphorylation increases slightly under hypoxia and dramatically under hypoxia-reoxygenation in both breast cancer cell lines.

ALK is a member of the insulin receptor superfamily of transmembrane tyrosine kinase receptors. ALK activation is involved in the tumorigenesis of anaplastic large-cell lymphoma (Morris SW. et al. 1994), lung cancer, and neuroblastoma (Della Corte CM. et al. 2018). Recent studies suggested that ALK expression correlates with poor prognosis in PDGFRA-mutated gastrointestinal stromal tumours and ovarian high-grade serous carcinoma (HGSC) in addition to its association with phenotypic aggressiveness in ovarian high-grade serous carcinoma (HGSC) and breast cancer (Wu, Y. et al. 2023, Matsumoto T. et al. 2021, Matsumoto T. et al. 2021, Siraj, A.K. et al. 2015). High expression of ALK was found to be due to increased copy number and gene amplification and translocation (Siraj, A.K. et al. 2015). The alterations in the ALK-RAS pathway were detected in neuroblastoma risk categories and associated with poor outcomes (Mlakar et al. 2021).

The role of ALK signalling in the development of breast cancer is currently being studied. ALK expression has been observed in different histological breast cancer subtypes (Perez-Pinera. et al. 2007). In addition, increased ALK copy number correlated with recurrence risk in inflammatory breast cancer (IBC) patients (Kim MH. et al. 2015). ALK pathway activity was positively associated with recurrence risk in breast cancer patients, and high ALK pathway activity indicated a significant risk factor for hormone receptor-negative and high-grade breast cancer (Liu D and Wu Y. 2020). ALK signalling pathway is known to activate multiple pathways, including JAK and STAT (Li Y. ET AL. 2021), PI3K-AKT (Slupianek A. et al. 2001), mTOR (Arvais EW. et al. 2006) and MAPK signalling cascades which eventually affect cell proliferation, invasion, migration, angiogenesis, and inhibition of apoptosis (Stoica GE. et al. 2002). The activation of some of these pathways, such as PI3K-AKT, JAK/STAT3 and sonic hedgehog, promoted tumour metastasis and correlated with cell-differentiation in breast cancer (Banerjee K, Resat H. 2016, Kenna MM. et al. 2018, Riobo-Del Galdo NA. et al 2019).

A recent study showed that increased activity of the ALK pathway is associated with high pathological grade and loss of PR and ER expression in breast cancer patients; in addition, it has been associated with breast cancer recurrence (Liu D. et al. 2020). A common downstream activation pathway is shared among ALK, PR/ER, and HER2, including Ras/MAPK and PI3K/Akt signalling pathways. These pathways lead to increased transcription, growth, cell proliferation and survival. Thus, these groups exhibit similarities in prognostic effects on patients (Hrustanovic G, Bivona TG. 2015, Moore NF. et al. 2014, Ciruelos Gil EM. 2014, Díaz-Serrano A. et al. 2018). Moreover, role of ALK in hypoxic response has been investigated, it was found that under hypoxic conditions, ALK regulates HIF1 α and HIF2 α expression in ALCL and NSCLC. ALK activity also controls hypoxic pathways in these tumours (Martinengo C. et al. 2014). Another study showed that hypoxia induced resistance to ALK inhibitors (crizotinib or alectinib) in NSCLC (Kogita A. et al. 2014). Therefore, based on these findings and results from this study, it can be concluded that activation in ALK signalling could have a functional role in breast cancer pathogenesis.

4.5.3 The phosphorylation status of STAT3 is increased under hypoxia and hypoxia-reoxygenation in breast cancer.

This study found that the STAT3 signalling pathway is upregulated during hypoxia and remains so during hypoxia-reoxygenation due to phosphorylation at two sites, Y705 and S727. STAT3 family was found to be regulated by hypoxia-reoxygenation; STAT3 Y705, STAT3 S727, and STAT5a/b (Y694/Y699) have all been upregulated under hypoxia-reoxygenation. Western blotting investigation of phospho-STAT3 Y705 in breast cancer cell lines showed that phosphorylation levels of STAT3 (Y705) significantly increased under hypoxia and hypoxia-reoxygenation when compared to normoxia in breast cancer cell lines (MDA-MB-231 and MCF7). However, the expression of total STAT3 significantly decreased under hypoxia in MCF7 cells and possibly in MDA-MB-231 cells. It is possible that total STAT3 expression increases under hypoxia-reoxygenation in both breast cancer cell lines.

STAT3 is a member of the STAT protein family, and it can be phosphorylated by JAK in response to growth factors and hemodynamic stress. It regulates several cellular processes, such as proliferation, differentiation, cell growth, migration, and apoptosis (Chakraborty D. et al., 2017; Wei L-H. et al., 2003, He J. et al., 2018). STAT3 also has oncogenic properties, controlling the expression of genes involved in invasion, migration, cell proliferation, immunosuppression, anti-apoptosis, autophagy, and stem cell regeneration (Ma J.-H. et al., 2020). Its activation promotes angiogenesis and the metastasis of cancer cells (Wu F. et al., 2021).

Dysregulation of STATs has been linked to various cancer types, including breast cancer (Pencik J. et al., 2016). Patients with high p-STAT3 positive tumors have shown shorter progression-free survival and worse overall survival (Fei Y. et al., 2020 and Lin GS et al., 2014). Moreover, p-STAT3 expression has been recognized as a distinct prognostic indicator of the prognosis of GBM patients (Lin GS et al., 2014). Breast cancer progression is also promoted by the phosphorylation of STAT3. Oncostatin M (OSM), a cytokine belonging to the IL-6 family, can induce upregulation of IL-6 and phosphorylation of STAT3 to activate STAT3 and HIF-1 α in ER (-) and ER (+) breast cancer cells, thereby promoting cancer progression (Tawara K. et al., 2019).

Hypoxia can induce STAT3 activation and promote breast cancer stemness and metastasis (Abyaneh HS. et al., 2018). A recent study has reported that the expression of a subset of hypoxia-dependent genes is regulated by STAT3 (Dinarello, A. et al., 2023). Additionally, VEGF, a key marker for angiogenesis, is activated by hypoxia and can be directly activated by STAT3 activation (Weis SM, Cheresch DA. 2011) or indirectly through the STAT3/HIF-1 α -dependent pathway (Tamura R. 2019).

4.5.4. The phosphorylation status of AKT is increased under hypoxia and hypoxia-reoxygenation in breast cancer.

In this study, AKT signalling pathway was found to be upregulated during hypoxia and hypoxia-reoxygenation via phosphorylation at two specific sites: Thr 308 and S473. Through western blotting investigation, phosphorylation of AKT Thr 308 has significantly increased in both breast cancer cell lines (MDA-MB-231 and MCF7) under hypoxia and hypoxia-reoxygenation. Interestingly, the total expression of AKT has remained constant in the MDA-MB-231 cell line under hypoxia, while it has significantly decreased in the MCF7 cell line. However, the total expression of AKT has significantly increased in both breast cancer cell lines under hypoxia-reoxygenation.

The intracellular signalling pathway PI3K/Akt plays a significant role in the cell cycle process. Once PI3K is activated, it phosphorylates and activates Akt, which localises in the cellular membrane (King D et al. 2015). The activated Akt version participates in many biological functions, including the activation of mTOR (Rafalski VA. Et al. 2011). Several biological molecules can enhance the PI3K/Akt pathway, including insulin-like growth factor (IGF)-1 (Peltier J. et al. 2007), insulin (Rafalski VA.2011), and EGF (Ojeda L. et al. 2011). RTKs activate the PI3K/Akt pathway, which enhances the expression of HIF-1 α . PI3K/Akt pathway regulates HIF-1 α , and several growth factors activate the PI3K/Akt pathway, including EGF, IGF, PDGF, TGF, TNF- α , IL-1 β (Niu G. et al. 2008). The glucose uptake in cancer cells is enhanced by AKT activation through GLUT1 regulation (Hoxhaj G and Manning BD. 2020).

The phosphorylation of threonine and serine aids the translocation of Akt from the cytoplasm to the nucleus. It mediates enzymatic biological effects, including inhibition of apoptosis, cell proliferation, migration, and carcinogenesis (Xie Y. et al. 2019). High phosphorylation levels

of Akt Thr 308 are associated with poor overall survival in AML patients (Gallay, N. et al. 2009). Overexpression of phospho-AKT is considered an adverse prognostic factor in breast cancer for overall survival (OS) and disease-free survival (DFS) (Yang ZY et al. 2015). Recent IHC analysis showed that 42.4% of breast invasive ductal carcinoma (BIDC) had high phospho-AKT expression, and that was significantly associated with increased expression of insulin receptor substrate 1 (IRS-1) (Luo J. et al. 2018). High phospho-AKT expression is significantly associated with HER2 overexpression (Park SS et al. 2007).

4.6 Conclusion

This study demonstrated changes in phospho-signalling under hypoxia and hypoxia-reoxygenation in MDA-MB-231 and MCF7. The identified kinases with upregulated phosphorylation under both hypoxia and hypoxia-reoxygenation and the activation of their pathways can suggest their involvement with breast cancer tumorigenesis. This can be used as promising therapeutic targets in breast cancer to fight the aggressiveness, metastatic, and therapy resistance characteristics acquired by cancer cells under the hypoxic environment, which can also maintain upon the reoxygenation of cancer cells.

5.0 Chapter 3: Investigating the impact of Hypoxia-reoxygenation on breast cancer cell phenotype and therapy resistance.

5.1 Introduction

TNBC tumours lack ER, PR and HER2, they cannot benefit from hormonal-targeted therapies (Nakhjavani et al., 2019). Chemotherapy is commonly used for TNBC to reduce the aggressiveness of this tumour (Marra A., Viale G., Curigliano G. 2019). However, multiple studies have reported that hypoxia increased therapy resistance in cancer cells, including chemotherapy resistance (Xiong H., et al. 2017 and Rebucci M, Michiels C. 2013). Hypoxia has been reported in breast cancer subtypes and was more evident in TNBC (Tutzauer, J. et al. 2022). Therefore, TNBC is associated with aggressiveness and worse prognosis in women (Yao H, et al. 2017). The lack of targeted therapies accounts for the poor prognosis in aggressive tumours. Hence, more understanding of the molecular basis of aggressive tumours such as TNBC is needed, along with more investigation of the clinical trials to facilitate the improvement of new and innovative drugs to overcome therapy resistance in TNBC and other aggressive tumours. In addition, recent studies have reported several molecular mechanisms and signalling pathways associated with TNBC proliferation, metabolism and survival leading to acquired therapy resistance (Qi F., Qin W.X., Zang Y.S. 2019, Monitor W.R., Salas A., Melo F.H.M. 2018). This indicates that kinase inhibitors can be employed in TNBC as potential targeted therapy.

The Luminal A subtype of breast cancer is characterised by the presence of estrogen receptor (ER+) and the absence of amplification of the human epidermal growth factor receptor (HER2-) (Orrantia-Borunda E. et al. 2022). These types of cancers typically depend on estrogen and can be treated by blocking the function of estrogen receptors, which is often effective. However, the tumour can become resistant over time through different mechanisms (Anderson, D.H. 2021). For recurrent breast cancer that no longer responds to estrogen, new targeted therapies are needed to address the evolving molecular characteristics initiated by the tumour (Anderson, D.H. 2021). Several emerging treatments are being considered for Luminal A breast cancer, including the use of PI3K/Akt/mTOR pathway inhibitors to target cell growth and survival pathways, inhibition of poly (ADP-ribose) polymerase (PARP) to target DNA repair pathways, and targeting cell cycle progression through cyclin-dependent kinase

4/6 (CDK4/6)/RB pathway (Miricescu D. et al. 2021, Anderson, D.H. 2021, and Piezzo M. et al.2020). Combining different therapeutic approaches is also being considered for Luminal A breast cancer to overcome mechanisms leading to therapy resistance (Anderson, D.H. 2021)

5.2 Hypothesis and Aims

Hypoxia induces breast cancer cells to invade, migrate and become chemo-resistant (Yong L. et al., 2022). I hypothesise that these characteristics such as increased invasiveness and chemoresistance can persist even after hypoxia in hypoxia-reoxygenation in breast cancer cells. As continuous work from this study (Results Chapter 2), I hypothesise that specific kinase inhibitors, some of which have already been approved for clinical use in alternative settings, can be used to target the protein kinases that had increased phosphorylation under hypoxia-reoxygenation in breast cancer cells. In addition, I hypothesise that these kinase inhibitors can target the migration and chemoresistance of breast cancer cells acquired under hypoxia-reoxygenation.

To test this hypothesis, the TNBC cell line (MDA-MB-231) and Luminal A cell line (MCF7) will be used for the following aims:

- 1) Investigate the breast cancer cells' invasiveness under hypoxia-reoxygenation compared to normoxia using a Transwell invasion assay.
- 2) Investigate whether kinase inhibitors, Crizotinib, WP1066, and MK-2206, can impact the phosphorylation of breast cancer cells under normoxia and hypoxia-reoxygenation using western blot.
- 3) Measure the cell viability in response to chemotherapy drugs cisplatin and doxorubicin on breast cancer cells under hypoxia-reoxygenation compared to normoxia using MTS assay.
- 4) Evaluate cell viability in response to the combination of chemotherapy drugs and kinase inhibitors on breast cancer cells under hypoxia-reoxygenation compared to normoxia using MTS assay.
- 5) Evaluate the migration of breast cancer cells when treated with kinase inhibitors under normoxia and hypoxia-reoxygenation using migration assay.

5.3 Results

5.3.1 Does Hypoxia-reoxygenation increase the capacity of breast tumour cells to invade?

Transwell invasion assay was used in this study to compare breast cancer cells' invasiveness under hypoxia-reoxygenation compared to physoxia. This was to address whether hypoxia-reoxygenation increases the capacity of breast cancer cells to invade. In this study, breast cancer cells were incubated under physiological oxygen levels (8.5% O₂), representing the oxygen levels within human breast tissue (Vaupel P, Höckel M, Mayer A (2007)). Cells were incubated in physoxia (72 hours 8.5% O₂) or hypoxia-reoxygenation (72 hours 1% O₂-72 hours 8.5% O₂) and were then serum starved in 2% FBS for 24 hours before trypsinisation for the Transwell cell invasion assay as described in the Methods (Methods). The cells were then allowed to invade through the Reduced Growth Factor basement membrane matrix for 24 hours, followed by formaldehyde fixation and DAPI staining. The cells were then imaged using a fluorescent microscope (Figure 5.1 and Figure 5.2).

The analysis has revealed that hypoxia-reoxygenation significantly increased the invasiveness of MDA-MB-231 cells by 57% compared to physoxia (32%) ($p < 0.01$, $n = 3$) (Figure 5.1). The analysis has also showed that hypoxia-reoxygenation significantly increases the invasiveness of MCF7 cells by 56% compared to physoxia (18%) ($p < 0.0001$, $n = 3$) (Figure 5.2).

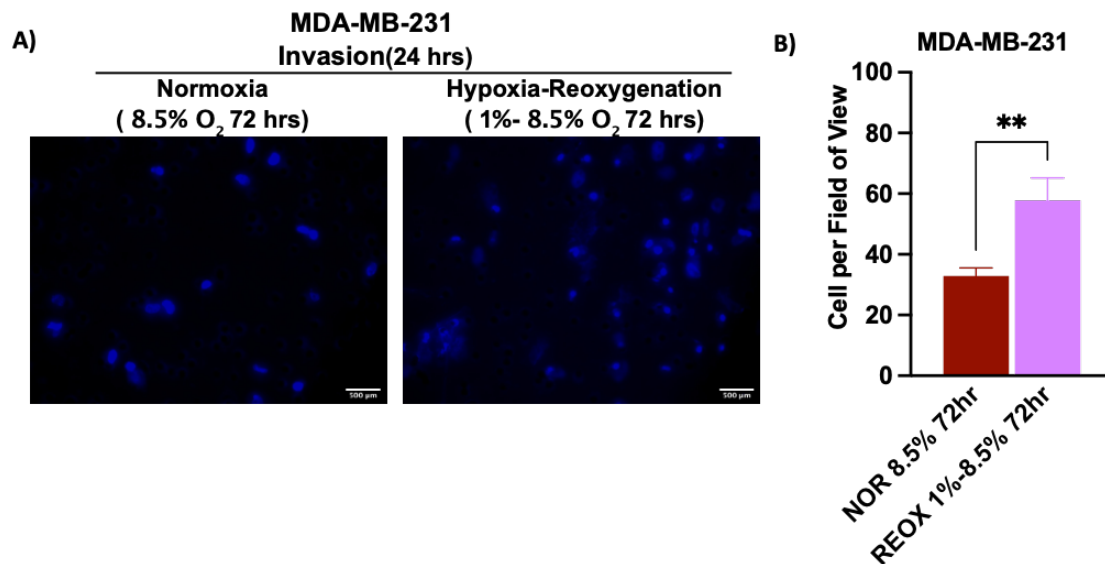


Figure 5.1 Hypoxia-Reoxygenation increase invasion in MD-MB-231 cells. **A)** Representative images of Transwell invasion in MDA-MB-231 cells under physoxia (8.5% O₂ 72) and Hypoxia-reoxygenation (1%-8.5% O₂ 72 hours conditions). **B)** Quantification of image analyses showing the percentage of invasion of cells following physoxia and hypoxia-Reoxygenation

Error bars indicate $\pm$ SEM. Data was analysed using an Unpaired t-test. **p<0.01, n=3.

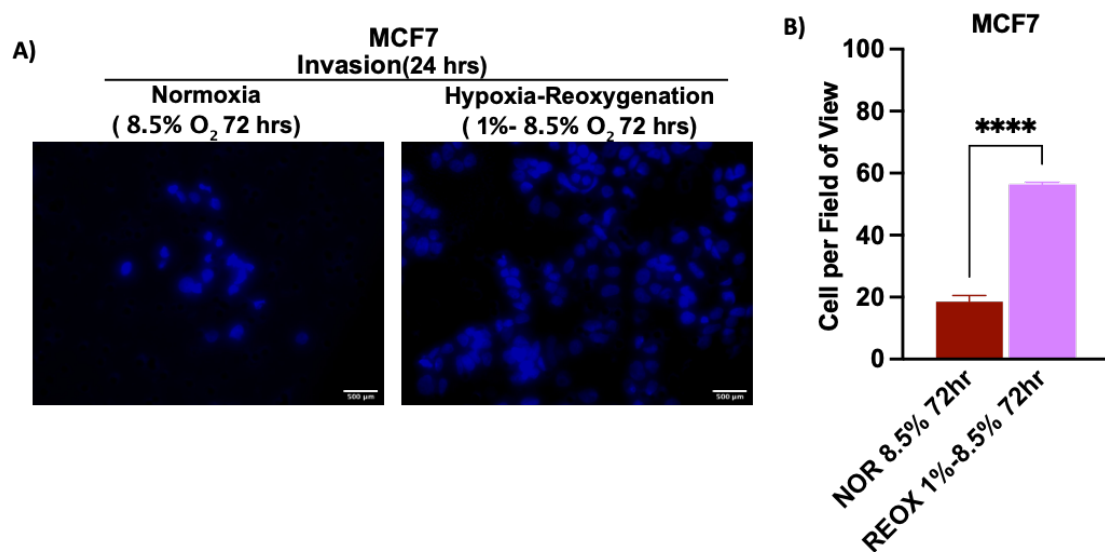


Figure 5.2 Hypoxia-Reoxygenation increase invasion in MCF7. **A)** Representative images of Transwell invasion in MCF7 cells under physoxia (8.5% O₂ 72) and Hypoxia-reoxygenation (1%-8.5% O₂ 72 hours conditions). **B)** Quantification of image analyses showing the percentage of invasion of cells following physoxia and hypoxia-Reoxygenation

Error bars indicate $\pm$ SEM. Data was analysed using an Unpaired t-test. ****p<0.0001, n=3.

5.3.2 Kinase inhibitors reduced phosphorylation under normoxia and hypoxia-reoxygenation

Previously, in this study (4.0 Chapter 2), the phospho-RTK array and phospho-kinase array were used as screens to identify changes in phosphorylation in breast cancer cells MDA-MB-231. The phospho-RTK array screen identified three significant RTKs which had increased phosphorylation under hypoxia-reoxygenation compared to normoxia: Insulin R (ALK) ($p < 0.01$, $n=3$), ErbB2 (EGF R) ($p < 0.01$, $n=3$), and EGFR ($p < 0.05$, $n=3$). In addition, the phospho-kinase array screen also identified increased phosphorylation in several targets under hypoxia and hypoxia-reoxygenation, such as STAT3 (Y705) and AKT 1/2/3 (T308); however, these were not statistically significant. Still, results were promising, and these targets were selected for further investigation by western blots. The western blot analysis showed that phosphorylation significantly increased under hypoxia and hypoxia-reoxygenation for these targets ALK, STAT3 (Y705), and AKT 1/2/3 (T308). Specific kinase inhibitors were selected for each target to assess their role in breast cancer cell development: Crizotinib for ALK, WP1066 inhibitor for STAT3, and MK-2206 for Akt 1/2/3.

Here, breast cancer cells were incubated under normoxia (72 hours at 21% O₂) and hypoxia-reoxygenation (72 hours at 1% O₂ followed by 72 hours at 21% O₂) and then treated for 24 hours with kinase inhibitor using inhibitor's IC₂₅ and IC₅₀ concentrations previously shown in the literature before collecting the cell lysate for further investigation. The control sample used for these experiments was the (normoxia, non-treated) sample. For future experiments, including an additional control sample (hypoxia-reoxygenation, non-treated) would be of great value for this investigation. The IC₂₅ is the drug concentration required to cause a 25% reduction in cell proliferation. In contrast, the IC₅₀ is the drug's minimum concentration needed to cause a 50% reduction in cell growth (Lozon L. et al. 2022). Western blot was then conducted, and phosphorylation levels were quantified relative to the housekeeping gene β -actin.

Crizotinib, ALK inhibitor

Crizotinib, an oral bioavailable tyrosine kinase inhibitor, acts against the ALK kinase domain and is effective against tumours that express ALK (Della Corte. Et al., 2018). Crizotinib was recently approved by the Food and Drug Administration (FDA) for clinical use for both adult

and paediatric patients with inflammatory anaplastic lymphoma kinase (ALK)-positive myofibroblastic tumours (IMT) who are unresectable, refractory, or recurrent starting at age one year (Center for Drug Evaluation and Research, F. 2022). The effect of crizotinib has been demonstrated in several studies, and the IC_{50} was identified using cell viability by (MTT) colorimetric assay as 5.16 μ M in MDA-MB-231 and 2.88 μ M for MCF7 (Ayoub NM. et al. 2017, Ayoub, N.M. et al. 2021)

In this study, Crizotinib was found to reduce the phosphorylation of ALK in the MDA-MB-231 and MCF7 cell lines compared to the control vehicle-only normoxia. In MDA-MB-231, phosphorylated ALK decreased significantly by 59% ($p < 0.05$, $n = 3$) and 72% ($p < 0.01$, $n = 3$) when treated with 2.5 μ M Crizotinib in normoxia or hypoxia-reoxygenation, respectively (Figure 5.3B). However, under 5 μ M Crizotinib in normoxia and hypoxia-reoxygenation, the phosphorylation of ALK decreased by 77% ($p < 0.01$, $n = 3$) and 61% ($p < 0.05$, $n = 3$), respectively (Figure 5.3B).

In MCF7, treatment with a 1.5 μ M concentration of Crizotinib significantly reduced the phosphorylation of ALK by 31% ($p < 0.0001$, $n = 3$) in normoxia and by 23% ($p < 0.0001$, $n = 3$) in hypoxia-reoxygenation ($p < 0.0001$) (Figure 5.4B). When treated with 3 μ M concentration, the effect of Crizotinib was even more substantial, and phosphorylation of ALK decreased by 26% ($p < 0.0001$, $n = 3$) in normoxia and by 14% in hypoxia-reoxygenation ($p < 0.0001$, $n = 3$) (Figure 5.4B).

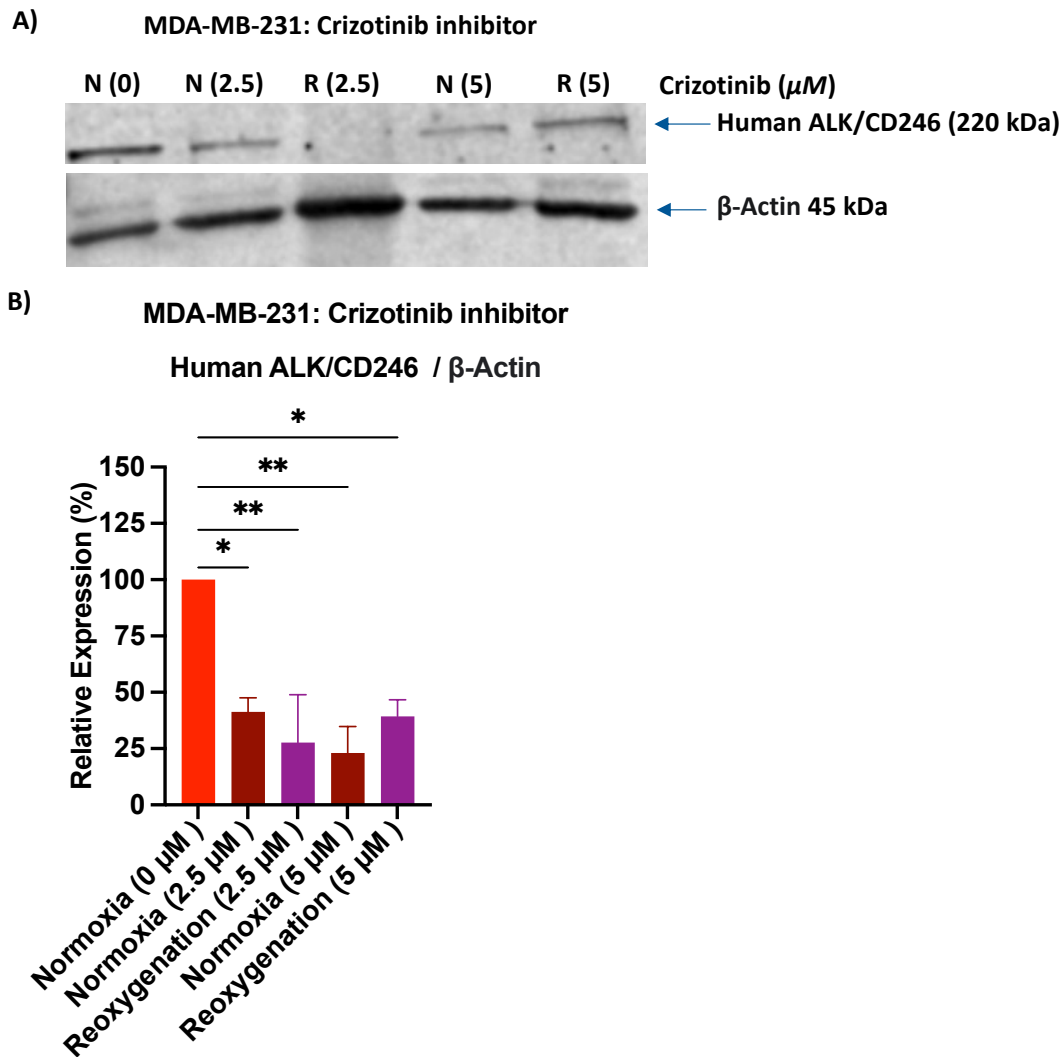


Figure 5.3. Crizotinib inhibitor inhibited ALK phosphorylation under normoxia and hypoxia-reoxygenation in MDA-MB-231 cells. Cells treated with Crizotinib inhibitor (2.5 μM and 5 μM) in normoxia and hypoxia-reoxygenation compared to control vehicle only normoxia. **A)** Representative Western blot image for ALK(CD246) phosphorylation and loading control β -Actin. **B)** Quantification for ALK(CD246) phosphorylation in control and treated MDA-MB-231 cells. N=Normoxia, R=hypoxia-reoxygenation.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$. $n = 3$.

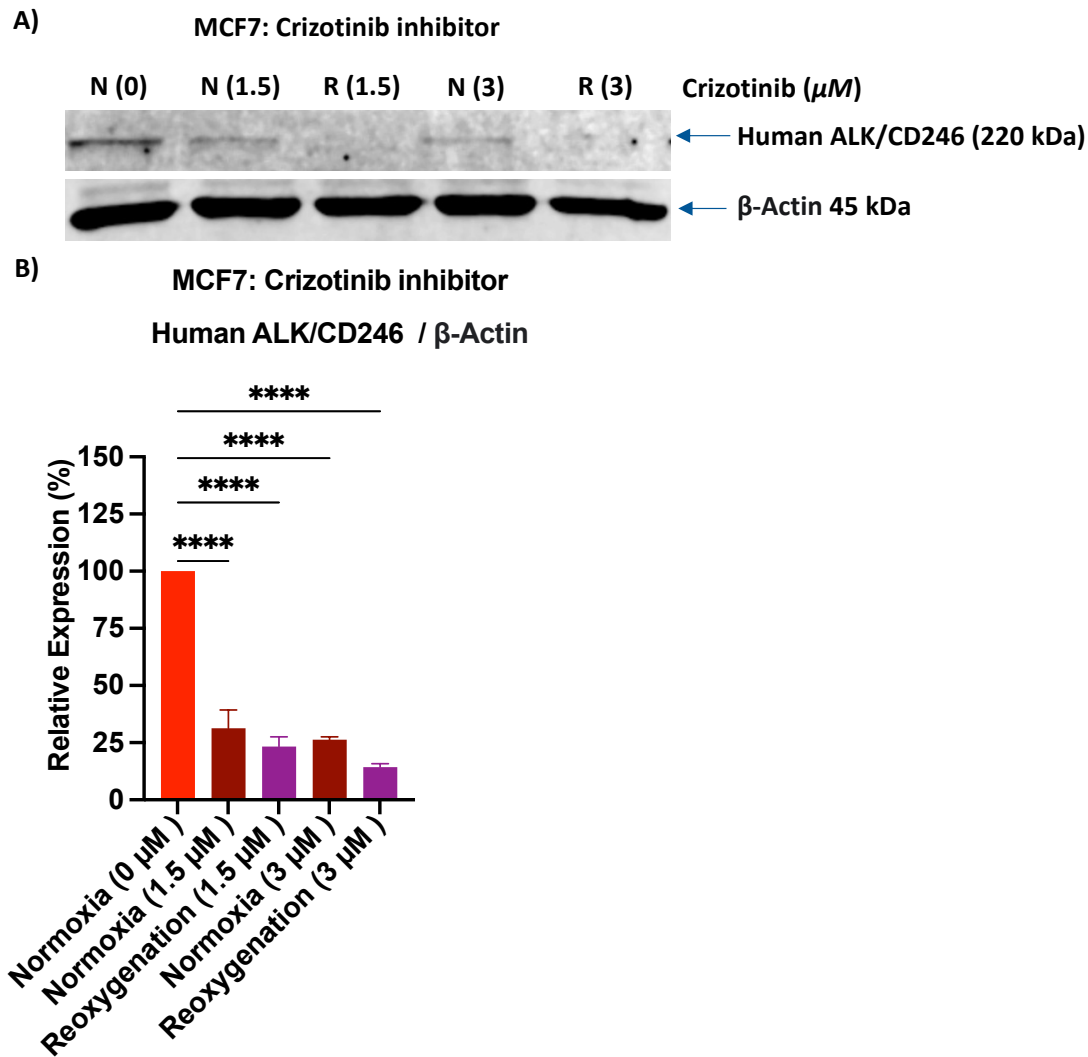


Figure 5.4. Crizotinib inhibitor inhibited ALK phosphorylation under normoxia and hypoxia-reoxygenation in MCF7 cells. Cells treated with Crizotinib inhibitor (1.5 μM and 3 μM) in normoxia and hypoxia-reoxygenation compared to control vehicle only normoxia. **A)** Representative Western blot image for ALK(CD246) phosphorylation and loading control β -Actin. **B)** Quantification for ALK(CD246) phosphorylation in control and treated MCF7 cells. N=Normoxia, R=hypoxia-reoxygenation.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. **** $p < 0.0001$. $n = 3$.

WP1066, STAT3 inhibitor

WP1066 inhibitor is a cell-permeable version of AG490 tyrphostin. It has been found to be a potent anti-tumour agent by inhibiting the STAT3 pathway. Several studies have shown that WP1066 has the ability to inhibit STAT3 phosphorylation (Xue ZJ. et al. 2014, Horiguchi A. et al. 2010, Ferrajoli A. et al. 2007). Moreover, WP1066 showed inhibition of JAK2 phosphorylation and degradation of JAK2 protein, which further blocked its downstream STAT3 and PI3K pathways (Ferrajoli A. et al. 2007). Both in vitro and in vivo studies have been conducted for WP1066. The in vitro studies have demonstrated selective cytotoxicity towards malignant glioma cells by inhibiting STAT3 activation (Iwamaru A. et al. 2007). Furthermore, WP1066 has been found to decrease brain metastases incidence and enhance survival in a preclinical model of breast cancer brain metastasis (Lee HT. et al. 2015).

WP1066 was found to be effective in inhibiting malignant glioma growth in viability assays. It demonstrated IC₅₀ concentrations of 5.6 μ M and 3.7 μ M in U87-MG and U373-MG, respectively (Iwamaru A. et al. 2007). Further studies have shown that WP1066 can prevent STAT3 phosphorylation in bladder cancer cells and renal cell carcinoma at a concentration of 5 μ M, at 2.5 μ M, WP1066 can reduce cell survival and proliferation in tumour cells (Tsujiya Y. et al. 2017, Horiguchi A. 2010). In addition, WP1066 inhibited the cell viability of breast cancer cells at a concentration of 3 μ M, (Lee HT. et al. 2015).

In this study WP1066 was tested to investigate whether it can reduce phosphorylation of STAT3 in breast cancer cells. MDA-MB-231 and MCF7 cells were incubated under normoxia (72 hours at 21% O₂) and hypoxia-reoxygenation (72 hours at 1% O₂ followed by 72 hours at 21% O₂) and then treated with 2.5 μ M and 5 μ M WP1066 for 24 hours and examined the phosphorylation of STAT3 at Y705. Treated normoxia and hypoxia-reoxygenation breast cancer cells with WP1066 2.5 μ M and 5 μ M were normalised to control vehicle-only normoxia (0 μ M) (Figure 5.5 and Figure 5.6).

The effects of WP1066 on the MDA-MB-231 cell line were examined, and it was found that WP1066 significantly inhibited the phosphorylation of STAT3 (Y705) at 2.5 μ M and 5 μ M concentrations compared to the control normoxia. In normoxia and hypoxia-reoxygenation, the relative expression of cells treated with 2.5 μ M decreased by 45% and by 66%, respectively

($p < 0.0001$, $n=3$). Moreover, a 5 μM concentration of WP1066 was found to have an even more substantial effect on phospho-STAT3 (Y705), with the relative expression dropping by 18% in both normoxia and hypoxia-reoxygenation conditions ($p < 0.0001$, $n=3$) (Figure 5.5B). While examining the effect of WP1066 on total STAT3 levels, it was observed that 2.5 μM WP1066 slightly inhibited the total STAT3 levels, with the relative expression dropping by 69% and by 87% in normoxia and hypoxia-reoxygenation conditions, respectively ($p < 0.05$, $n=3$). However, at 5 μM WP1066, the relative expression dropped by 13% and by 23%, respectively, in normoxia and hypoxia-reoxygenation conditions ($p < 0.0001$, $n=3$) (Figure 5.5C).

In MCF7 cells, WP1066 inhibited the phosphorylation of STAT3 noticeably under 2.5 μM and 5 μM concentrations apart from normoxia treated with 2.5 μM WP1066. The relative expression of phospho-STAT3 (Y705) dropped by 69% -statistically not significant- in normoxia and by 37% ($p < 0.05$, $n=3$) in hypoxia-reoxygenation with the 2.5 μM WP1066. Moreover, 5 μM WP1066 significantly inhibited phospho-STAT3 (Y705), where relative expression went down by 28% ($p < 0.01$, $n=3$) in normoxia and hypoxia-reoxygenation (Figure 5.6B). The relative expression levels of total STAT3 remained stable under all conditions in MCF7 cells treated with WP1066, ranging between 81% to 90%. Statistically, this was not significant (Figure 5.6C).

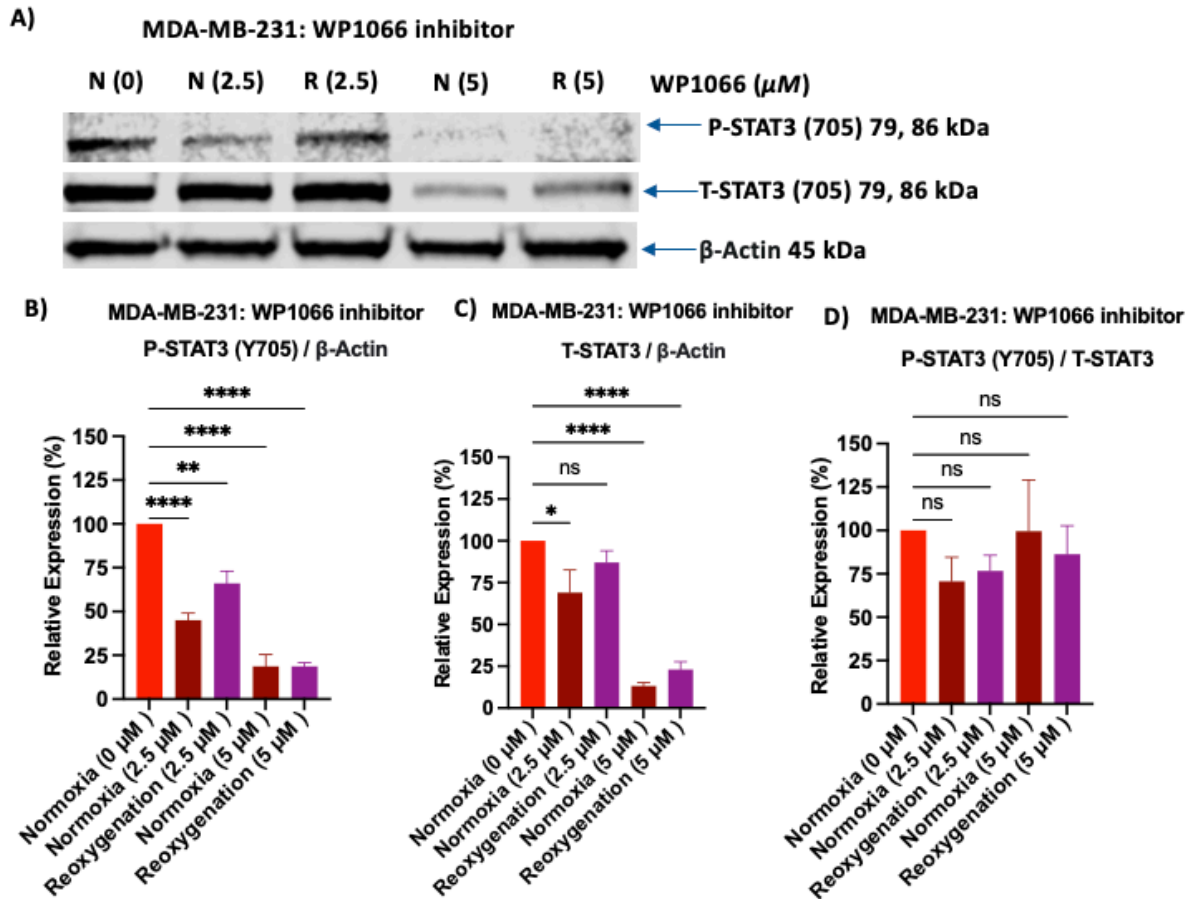


Figure 5.5. WP1066 inhibitor inhibited the phosphorylation of phospho-STAT3 (Y705) and reduced expression of total STAT3 under normoxia and hypoxia-reoxygenation in MDA-MB-231 cells. MDA-MB-231 cells treated with WP1066 inhibitor (2.5 and 5 μ M) under normoxia and hypoxia-reoxygenation compared to control vehicle only normoxia (0 μ M). **A)** Representative Western blot image for phospho-STAT3 (Y705), total STAT3 and loading control β -Actin. **B)** Quantification for phospho- STAT3 (Y705) in control and treated MDA-MB-231 cells. **C)** Quantification for total STAT3 in control and treated MDA-MB-231 cells. **D)** Quantification for phospho- STAT3 (Y705)/ total STAT3 in control and treated MDA-MB-231 cells. N=Normoxia, R=hypoxia-reoxygenation.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 3$

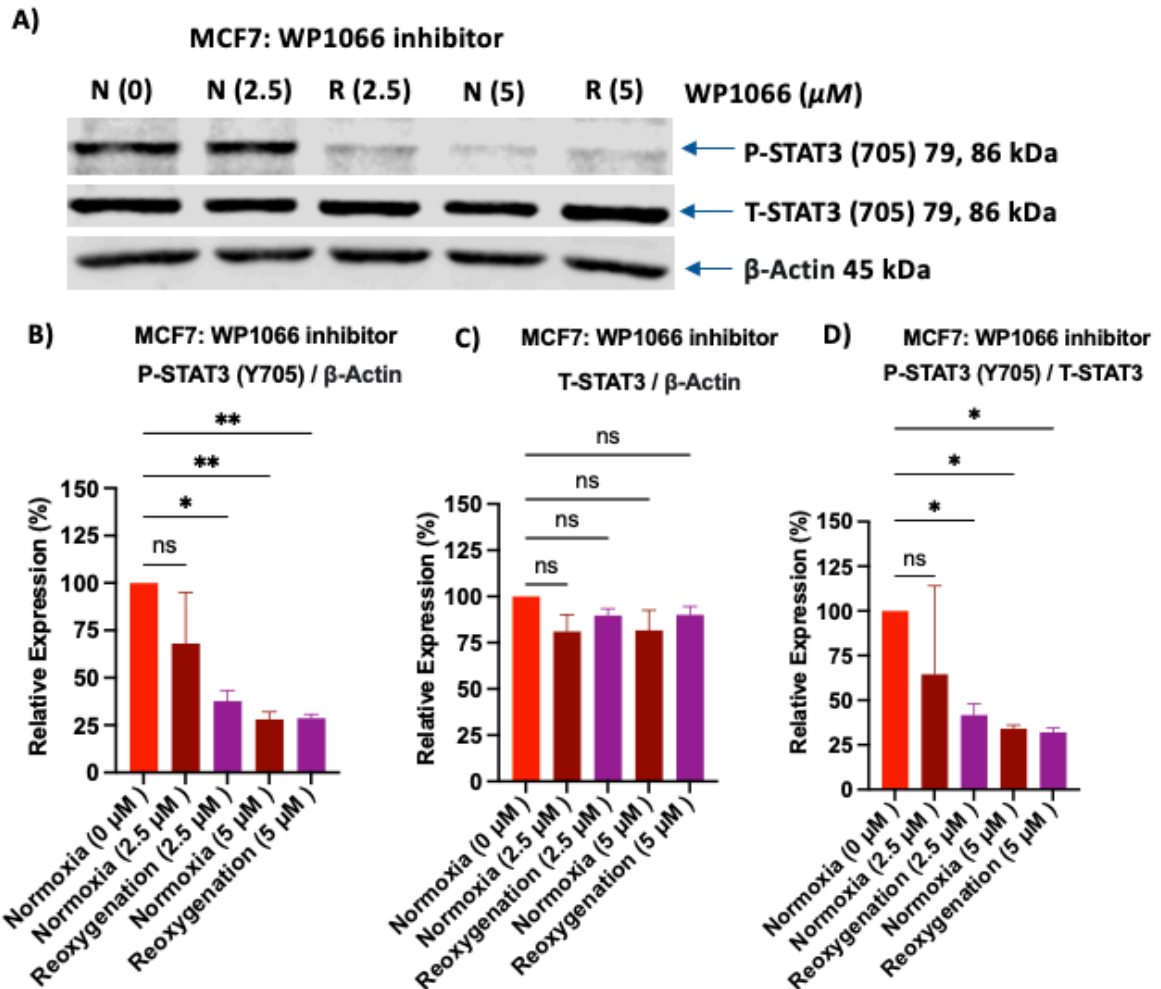


Figure 5.6. WP1066 inhibitor inhibited the phosphorylation of phospho-STAT3 (Y705) and reduced expression of total STAT3 under normoxia and hypoxia-reoxygenation in MCF7 cells. MCF7 cells treated with WP1066 inhibitor (2.5 and 5 μ M) under normoxia and hypoxia-reoxygenation compared to control vehicle only normoxia (0 μ M). **A)** Representative Western blot image for phospho-STAT3 (Y705), total STAT3 and loading control β -Actin. **B)** Quantification for phospho- STAT3 (Y705) in control and treated MCF7 cells. **C)** Quantification for total STAT3 in control and treated MCF7 cells. **D)** Quantification for phospho-STAT3 (Y705)/ total STAT3 in control and treated MCF7 cells. N=Normoxia, R=hypoxia-reoxygenation.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. * p <0.05, ** p <0.01. n =3

MK-2206, AKT inhibitor

MK-2206 is an inhibitor of Akt 1/2/3. Clinical trials have shown its safety in humans and antitumor activity (Yap TA. Et al. 2011, Molife LR. Eta la. 2014 and Brown, J. et al. 2017). Several studies in colorectal, prostate and lung cancer cells have shown that treatment with MK-2206 at concentrations of 5 μ M reduces AKT phosphorylation in cancer cells, while concentrations of 2.5 μ M inhibited multiple downstream targets of AKT (Iida M. et al. 2013, Al-Saffar. et al. 2018, Hirai H. et al. 2010 and Malkomes, P. et al. 2016).

MK-2206 was investigated to determine whether it reduced phosphorylation of the AKT in breast cancer cells. Thus, MDA-MB-231 and MCF7 cells were incubated under normoxia (72 hours at 21% O₂) and hypoxia-reoxygenation (72 hours at 1% O₂ followed by 72 hours at 21% O₂) and then treated with 2.5 μ M and 5 μ M MK-2206 for 24 hours and examined the phosphorylation of AKT at Thr308. Treated normoxia and hypoxia-reoxygenation breast cancer cells with 2.5 μ M and 5 μ M MK-2206 were normalised to control vehicle-only normoxia (Figure 5.7 and Figure 5.8).

In the MD-MB-231 cell line, MK-2206 significantly reduced phosphorylation of AKT (Thr308) in all conditions ($p < 0.0001$, $n = 3$) when compared to the control normoxia, with relative expressions ranging between 30%-10% (Figure 5.7B). However, MK2206 had less effect on the total AKT levels, as the total AKT levels remained constant in all conditions compared to the control normoxia, with relative expressions ranging between 69%-79% (Figure 5.7C).

In the MCF7 cell line, treatment with 2.5 μ M MK-2206 reduced phospho-AKT (Thr308) expression by 28% and by 22% in normoxia and hypoxia-reoxygenation, respectively. With 5 μ M MK-2206, the phospho-AKT (Thr308) expression reduction was even greater, dropping by 17% and by 12% in normoxia and hypoxia-reoxygenation, respectively (Figure 5.8B). Interestingly, treatment with 2.5 μ M MK-2206 reduced total AKT protein expression by 63% and by 60% in normoxia and hypoxia-reoxygenation, respectively. Treatment with 5 μ M MK-2206 had an even greater effect, dropping total AKT protein expression by 59% and by 50% in normoxia and hypoxia-reoxygenation, respectively (Figure 5.8C).

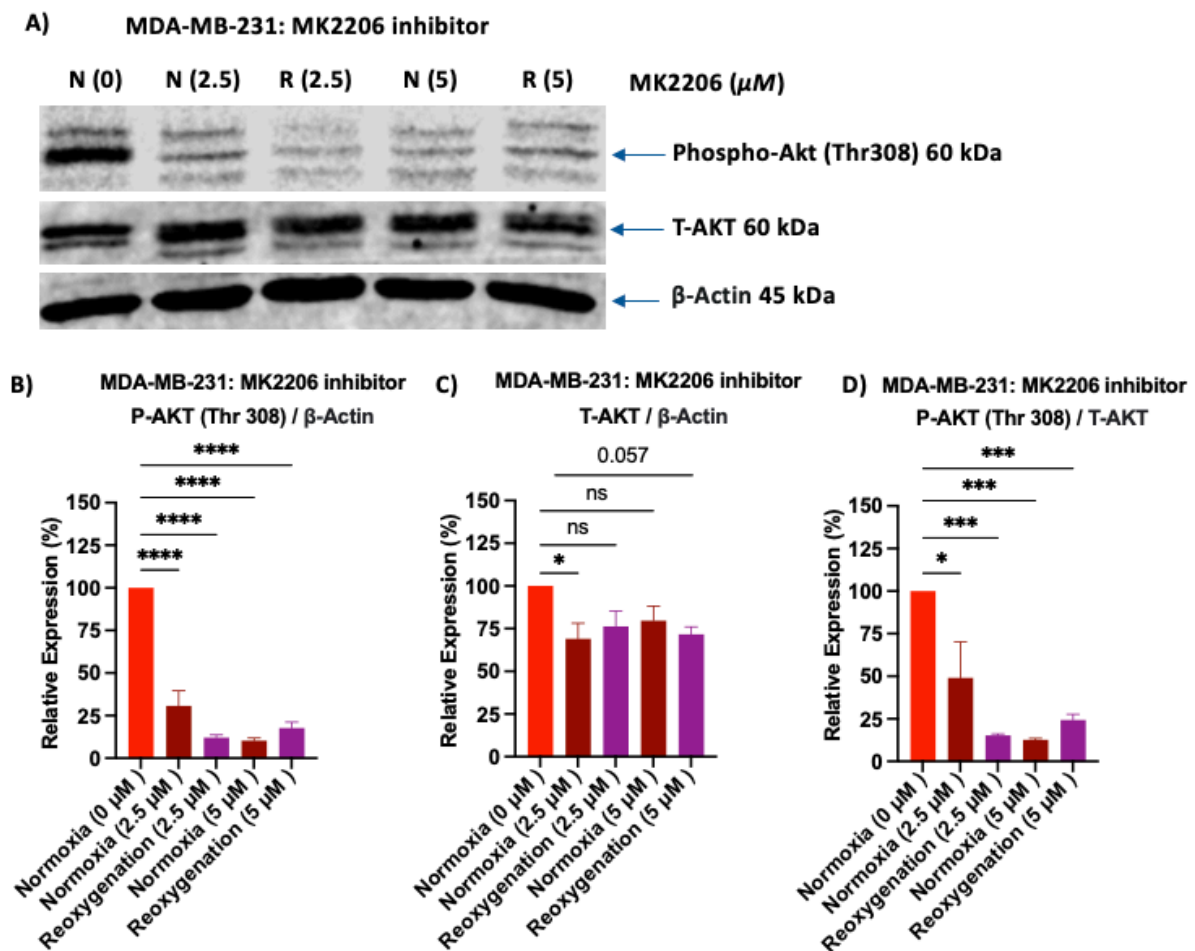


Figure 5.7. MK-2206 inhibitor reduced the phosphorylation of AKT (Thr 308) in normoxia and hypoxia-reoxygenation in MDA-MB-231 cells. MDA-MB-231 cells treated with MK2206 inhibitor (2.5 and 5 μ M) under normoxia and hypoxia-reoxygenation compared control vehicle only normoxia (0 μ M) **A)** Representative Western blot image for phospho-AKT (Thr 308) alongside total AKT and loading control β -Actin **B)** Quantification for phospho-AKT (Thr 308) in control and treated MDA-MB-231 cells. **C)** Quantification for total AKT in control and treated MDA-MB-231 cells. **D)** Quantification for phospho-AKT (Thr 308)/ total AKT in control and treated MDA-MB-231 cells. N=Normoxia, R=hypoxia-reoxygenation.

Error bars indicate $\pm$ SEM. Data was analysed using a One-Way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. $n = 3$.

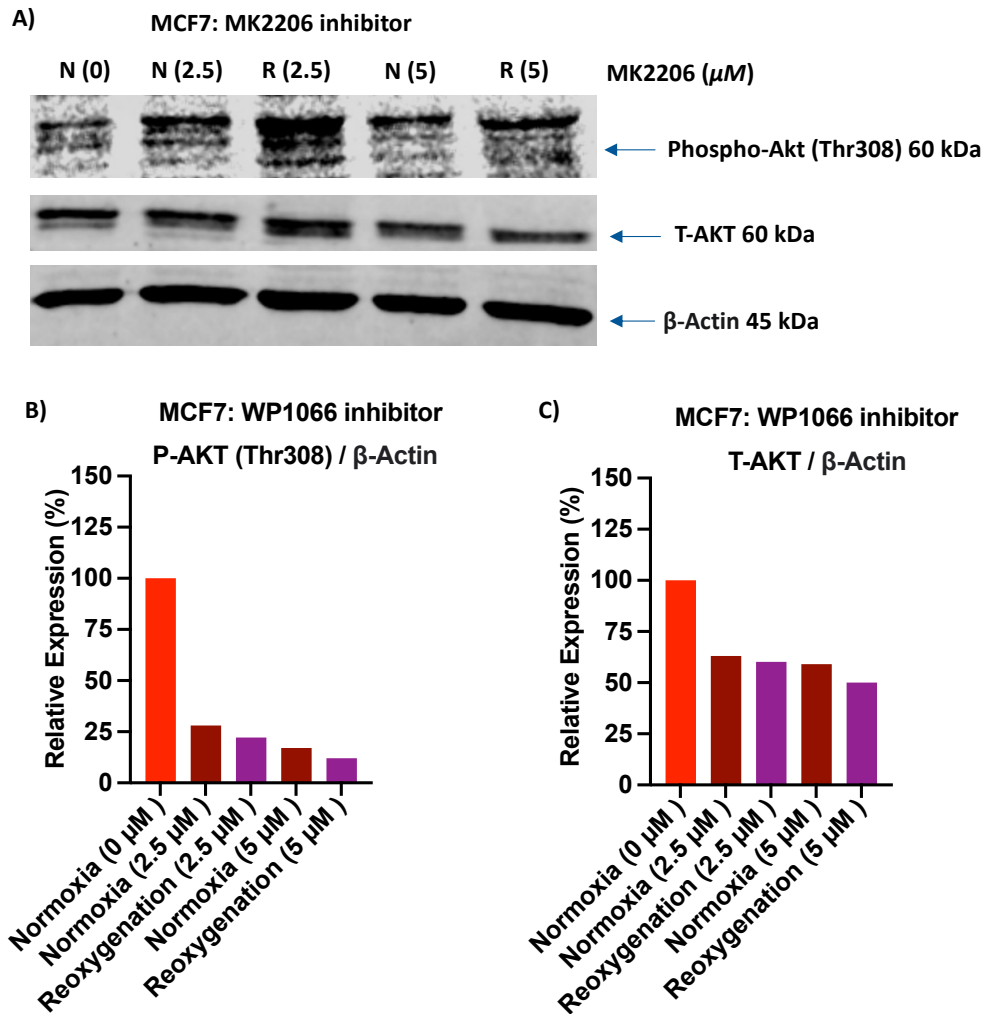


Figure 5.8. MK-2206 inhibitor reduced the phosphorylation of AKT (Thr 308) in normoxia and hypoxia-reoxygenation in MCF7 cells. MCF7 cells treated with MK2206 inhibitor (2.5 and 5 μ M) under normoxia and hypoxia-reoxygenation compared to control vehicle only normoxia (0 μ M). **A)** Representative Western blot image for phospho-AKT (Thr 308), total AKT and loading control β -Actin. **B)** Quantification for phospho-AKT (Thr 308) in control and treated MCF7 cells. **C)** Quantification for total AKT in control and treated MCF7 cells. N=Normoxia, R=hypoxia-reoxygenation. n=2.

5.3.3 Hypoxia-reoxygenation increases chemotherapy resistance in breast cancer cells

TNBC patients are deficient in the expression of ER, PR and HER2 hence, they do not benefit from these targeted therapies (Nakhjavani et al., 2019). Chemotherapy is a standard treatment for TNBC; however, TNBC patients frequently develop resistance. Hypoxia is also known to be implicated in chemotherapy resistance (Rohwer N. et al. 2011). Cyclic hypoxia can also induce chemotherapy resistance, which is seen in glioblastoma multiform cells (Chou CW. Et al. 2012). This study has two aims, first to address the impact of hypoxia-reoxygenation on breast cancer cells treated with chemotherapy drugs cisplatin and doxorubicin compared to normoxia. The second aim is to investigate the impact of the combination of chemotherapy drugs (cisplatin and doxorubicin) with kinase inhibitors (Crizotinib and WP1066) on cell viability of breast cancer cells under hypoxia-reoxygenation compared to normoxia.

Cisplatin impact cancer cells by modulating multiple process and pathways, including transcription, apoptosis, and p53 pathway signalling pathway (Pabla N. et al. 2008, Dasari S, Tchounwou PB. 2014). Cisplatin can also trigger cell death by elevating the oxidative stress. Resistance to cisplatin is a major concern in chemotherapy, which can be due to multiple factors such as defects in the apoptotic pathway, increased DNA damage repair, microRNA deregulation or transcription factor alteration (Fallahi H, Godini R. 2019). Doxorubicin, another chemotherapy drug, works in two main ways to act on cancer cells. Firstly, it intercalates into DNA and interferes with topoisomerase-II-mediated DNA repair; secondly, through the production of free radicals that result in damage to proteins, DNA, and cellular membranes (Thorn C. et al. 2011).

Breast cancer cell lines, MDA-MB-231 and MCF7 were first incubated under normoxia (72 hours at 21% O₂) and hypoxia-reoxygenation (1%-21% O₂ 72 hours). After the incubation, the cells were exposed to varying concentrations of cisplatin and doxorubicin, ranging from 0 µM to 5 µM, for an additional 48 hours (as shown in Figure 5.9A). cell viability was then measured by MTS assay as described previously (Methods).

The MTS survival data indicated that hypoxia-reoxygenation significantly increased the resistance toward cisplatin in both MDA-MB-231 ($p < 0.0001$, $n=3$) and MCF7 ($p < 0.0001$, $n=3$) breast cancer cell lines when compared to normoxia (Figure 5.7B-C).

Importantly, hypoxia-reoxygenation also increased the resistance of both breast cancer cell lines toward doxorubicin significantly in MDA-MB-231 ($p < 0.0001$, $n=3$) but non-significantly in MCF7 ($p=0.0619$, $n=3$). The results were deemed significant using the Unpaired Student T-test, as presented in Table 5.1. Additional statistical test was performed using 2way ANOVA followed by Tukey's multiple comparisons test as presented in Table 5.2.

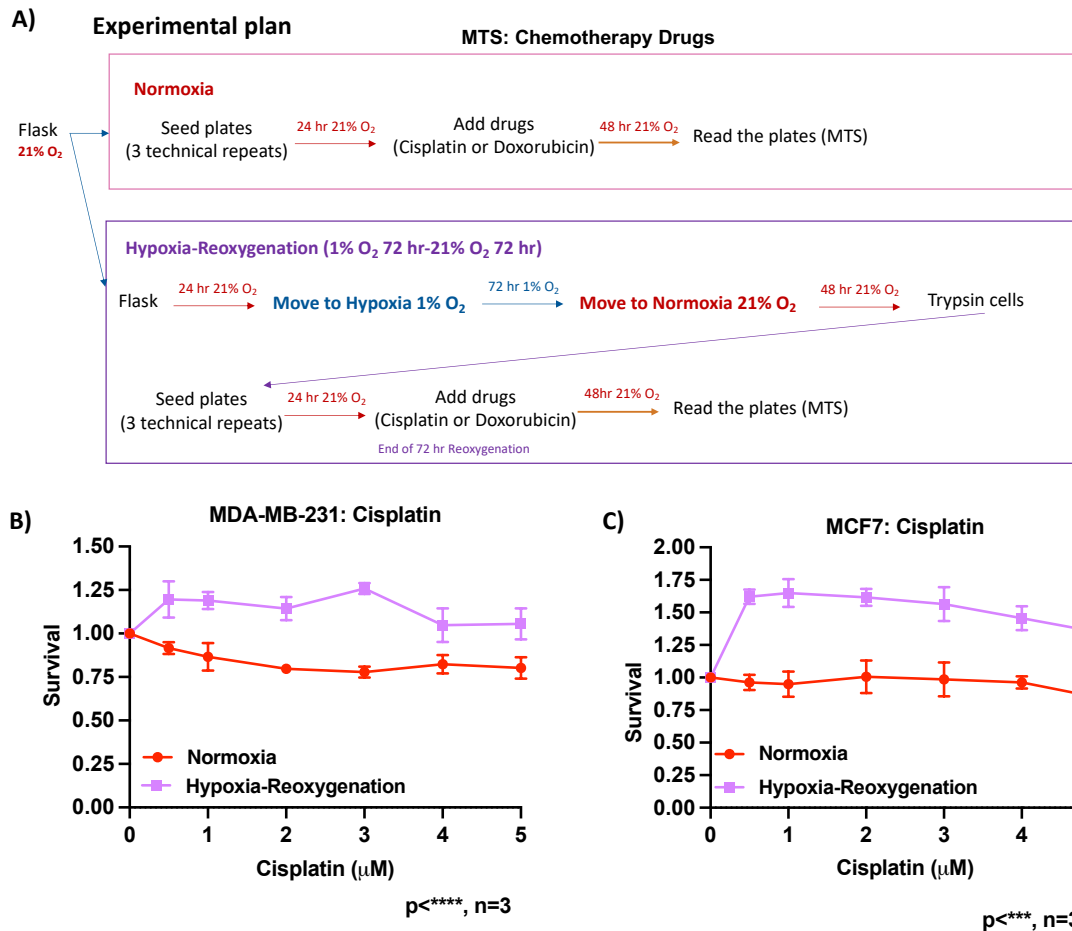


Figure 5.9 Survival data (MTS), breast cancer cells that undergo hypoxia-reoxygenation is more resistance to the chemotherapy drug Cisplatin. A) Experimental plan showing the steps involved in measuring the cell viability of breast cancer cells toward cisplatin and doxorubicin via MTS assay. **B)** Graph showing the survival fraction of MDA-MB-231 cells under hypoxia-reoxygenation and normoxia when treated with cisplatin. **C)** Graph showing the survival fraction of MCF7 cells under hypoxia-reoxygenation and normoxia when treated with cisplatin.

Error bars indicate $\pm$ SEM. Data was analysed using Two-tailed Unpaired t-test. *** $p < 0.001$, **** $p < 0.0001$, $n=3$

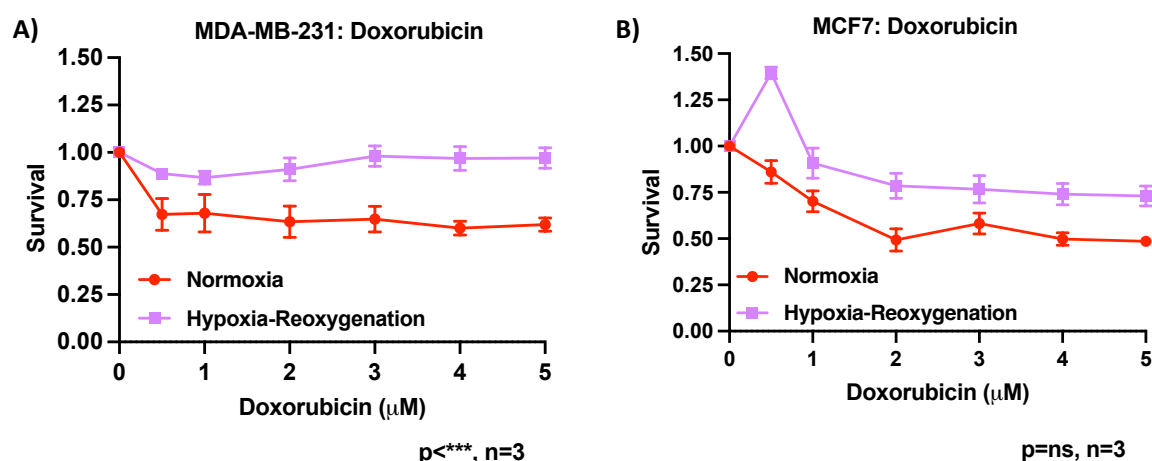


Figure 5.10 Survival data (MTS), breast cancer cells that undergo hypoxia-reoxygenation are more resistance to the chemotherapy drug Doxorubicin. A) Graph showing the survival fraction of MDA-MB-231 cells under hypoxia-reoxygenation and normoxia treated with doxorubicin. **B)** Graph showing the survival fraction of MCF7 cells under hypoxia-reoxygenation and normoxia treated with doxorubicin.

Error bars indicate $\pm$ SEM. Data was analysed using Two-tailed Unpaired t-test., *** $p < 0.001$, $n=3$.

Test (Unpaired T-test)	p-value summary	Significance summary (P < 0.05)?
MDA-MB-231: NOR vs REOX Cisplatin	<0.0001 Two-tailed	Yes
MDA-MB-231: NOR vs REOX Doxorubicin	0.0008 Two-tailed	Yes
MCF7: NOR vs REOX Cisplatin	0.0001 Two-tailed	Yes
MCF7: NOR vs REOX Doxorubicin	0.0619 Two-tailed	No

Table 5.1 Statistical analysis result of the survival data for breast cancer cells treated with different concentrations of chemotherapy drugs under hypoxia-reoxygenation and normoxia using Two-tailed Unpaired t-test. * $p < 0.001$, **** $p < 0.0001$. $n=3$**

Cell line: Chemotherapy drug	(μ M Concentration) Condition	Statistical test	Significance summary ($P < 0.05$)?	Adjusted P Value
MDA-MB-231: Cisplatin	(0 μ M) NOR vs (0 μ M) REOX	2way ANOVA	ns	>0.9999
	(0.5 μ M) NOR vs (0.5 μ M) REOX	2way ANOVA	ns	0.1203
	(1 μ M) NOR vs (1 μ M) REOX	2way ANOVA	*	0.0408
	(2 μ M) NOR vs (2 μ M) REOX	2way ANOVA	*	0.0216
	(3 μ M) NOR vs (3 μ M) REOX	2way ANOVA	***	0.0004
	(4 μ M) NOR vs (4 μ M) REOX	2way ANOVA	ns	0.3783
	(5 μ M) NOR vs (5 μ M) REOX	2way ANOVA	ns	0.217
MCF7: Cisplatin	(0 μ M) NOR vs (0 μ M) REOX	2way ANOVA	ns	>0.9999
	(0.5 μ M) NOR vs (0.5 μ M) REOX	2way ANOVA	***	0.0007
	(1 μ M) NOR vs (1 μ M) REOX	2way ANOVA	***	0.0003
	(2 μ M) NOR vs (2 μ M) REOX	2way ANOVA	**	0.0019
	(3 μ M) NOR vs (3 μ M) REOX	2way ANOVA	**	0.0037
	(4 μ M) NOR vs (4 μ M) REOX	2way ANOVA	*	0.0213
	(5 μ M) NOR vs (5 μ M) REOX	2way ANOVA	*	0.0177
MDA-MB-231: Doxorubicin	(0 μ M) NOR vs (0 μ M) REOX	2way ANOVA	ns	>0.9999
	(0.5 μ M) NOR vs (0.5 μ M) REOX	2way ANOVA	ns	0.3555
	(1 μ M) NOR vs (1 μ M) REOX	2way ANOVA	ns	0.556
	(2 μ M) NOR vs (2 μ M) REOX	2way ANOVA	ns	0.088
	(3 μ M) NOR vs (3 μ M) REOX	2way ANOVA	*	0.018
	(4 μ M) NOR vs (4 μ M) REOX	2way ANOVA	**	0.0061
	(5 μ M) NOR vs (5 μ M) REOX	2way ANOVA	*	0.0102
MCF7: Doxorubicin	(0 μ M) NOR vs (0 μ M) REOX	2way ANOVA	ns	>0.9999
	(0.5 μ M) NOR vs (0.5 μ M) REOX	2way ANOVA	****	<0.0001
	(1 μ M) NOR vs (1 μ M) REOX	2way ANOVA	ns	0.2957
	(2 μ M) NOR vs (2 μ M) REOX	2way ANOVA	*	0.0274
	(3 μ M) NOR vs (3 μ M) REOX	2way ANOVA	ns	0.4584
	(4 μ M) NOR vs (4 μ M) REOX	2way ANOVA	ns	0.1223
	(5 μ M) NOR vs (5 μ M) REOX	2way ANOVA	ns	0.1125

Table 5.2. Statistical analysis results of the survival data for breast cancer cells treated with different concentrations of chemotherapy drugs under hypoxia-reoxygenation and normoxia using 2way ANOVA test.

Data was analysed using 2way ANOVA, Followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 3$

5.3.4 Kinase inhibitors suppress resistance to chemotherapy drugs induced by hypoxia-reoxygenation

Previously (section 5.3.3), the study demonstrated that hypoxia-reoxygenation increased resistance to the chemotherapy drugs cisplatin and doxorubicin. Additionally, the results revealed that kinase inhibitors significantly decreased phosphorylation levels of their respective kinases (5.3.2). Based on the results of this research, we conducted further investigations to determine whether a combination of chemotherapy drugs and kinase inhibitors could overcome chemotherapy resistance in breast cancer cells under hypoxia-reoxygenation, as compared to normoxia.

Breast cancer cells were incubated in normoxia (72 hours at 21% O₂) and hypoxia-reoxygenation (1%-21% O₂ 72-72 hr) conditions followed by treatment with cisplatin (0 μ M to 10 μ M) or doxorubicin (0 μ M to 8 μ M) for 24 hours as explained in the methods and in (Figure 5.11A). After the chemotherapy treatment (24 hours), cells were treated with kinase inhibitor Crizotinib (5 μ M) in MDA-MB-231 and (1.5 μ M) in MCF7 for another 24 hours, and cell viability was then measured by MTS assay as explained in the methods (Methods).

According to the MTS survival data, the viability of cells under hypoxia-reoxygenation treated with cisplatin was reduced in a dose-dependent manner by 5 μ M Crizotinib in MDA-MB-231 ($p < 0.001$, $n = 3$) and 1.5 μ M Crizotinib in MCF7 ($n = 2$) as compared to cisplatin-treated cells under normoxia, (Figure 5.11B and Figure 5.11C). Additionally, in MDA-MB-231 ($p = 0.33$, $n = 3$) and MCF7 ($n = 2$), Crizotinib reduced the cell viability of doxorubicin-treated cells under hypoxia-reoxygenation when compared to doxorubicin-treated cells under normoxia (Figure 5.11D and Figure 5.11E). However, it is necessary to conduct further research to determine whether crizotinib will have a better impact at higher doses of cisplatin and doxorubicin.

The results were deemed significant using the Unpaired Student T-test. An additional statistical test was performed using 2way ANOVA followed by Tukey's multiple comparisons test as presented in Table 5.3.

A) Experimental plan

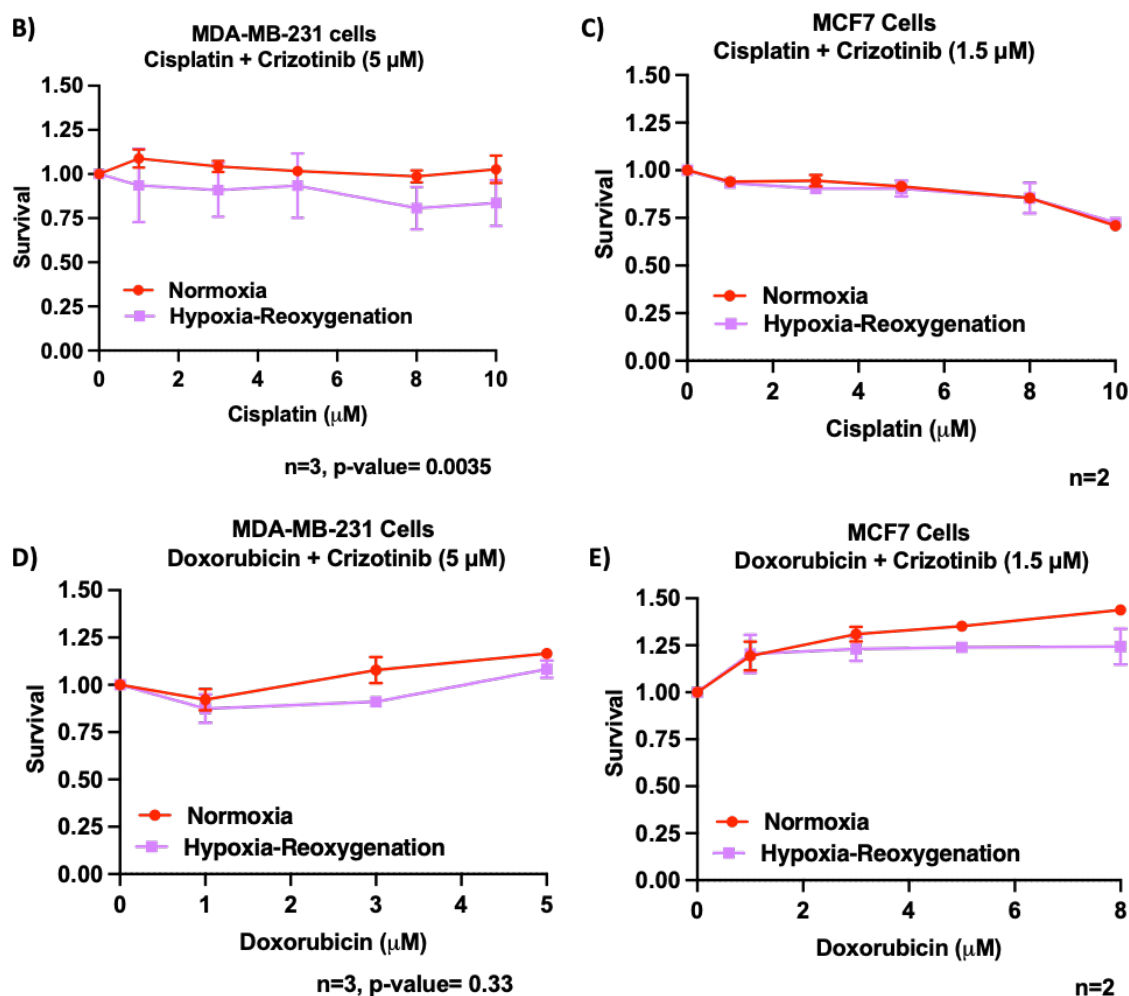
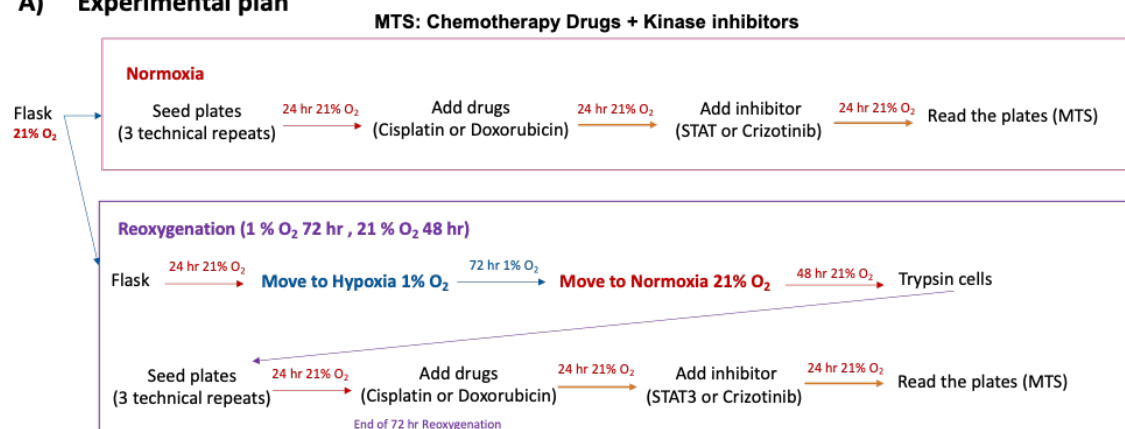


Figure 5.11 Survival data (MTS), breast cancer cells treated with chemotherapy drugs and Crizotinib under normoxia and hypoxia-reoxygenation. A) Experimental plan showing the steps involved in measuring the cell viability of breast cancer cells toward the combination of chemotherapy drugs and Crizotinib via MTS assay. B-C) Graph showing Cisplatin + Crizotinib treatment in MDA-MB-231 and MCF7 cells. D-E) Graph showing Doxorubicin + Crizotinib treatment in MDA-MB-231 and MCF7 cells. Error bars indicate $\pm$ SEM. Data was analysed using a Two-tailed Unpaired t-test.

Cell line: Chemotherapy drug + inhibitor (μM Concentration)	(μM Chemotherapy drug Concentration) Condition	Statistical test	Significance summary ($P < 0.05$)?	Adjusted P Value
MDA-MB-231: Cisplatin + Crizotinib (5 μM)	(0 μM) NOR vs (0 μM) REOX	2way ANOVA	ns	>0.9999
	(1 μM) NOR vs (1 μM) REOX	2way ANOVA	ns	0.8938
	(3 μM) NOR vs (3 μM) REOX	2way ANOVA	ns	0.9522
	(5 μM) NOR vs (5 μM) REOX	2way ANOVA	ns	0.999
	(8 μM) NOR vs (8 μM) REOX	2way ANOVA	ns	0.7566
	(10 μM) NOR vs (10 μM) REOX	2way ANOVA	ns	0.6886
MDA-MB-231: Doxorubicin+ Crizotinib (5 μM)	(0 μM) NOR vs (0 μM) REOX	2way ANOVA	ns	>0.9999
	(1 μM) NOR vs (1 μM) REOX	2way ANOVA	ns	0.9944
	(3 μM) NOR vs (3 μM) REOX	2way ANOVA	ns	0.2099
	(5 μM) NOR vs (5 μM) REOX	2way ANOVA	ns	0.8897

Table 5.3 Statistical analysis results of survival data (MTS), breast cancer cells treated with chemotherapy drugs and Crizotinib under normoxia and hypoxia-reoxygenation.

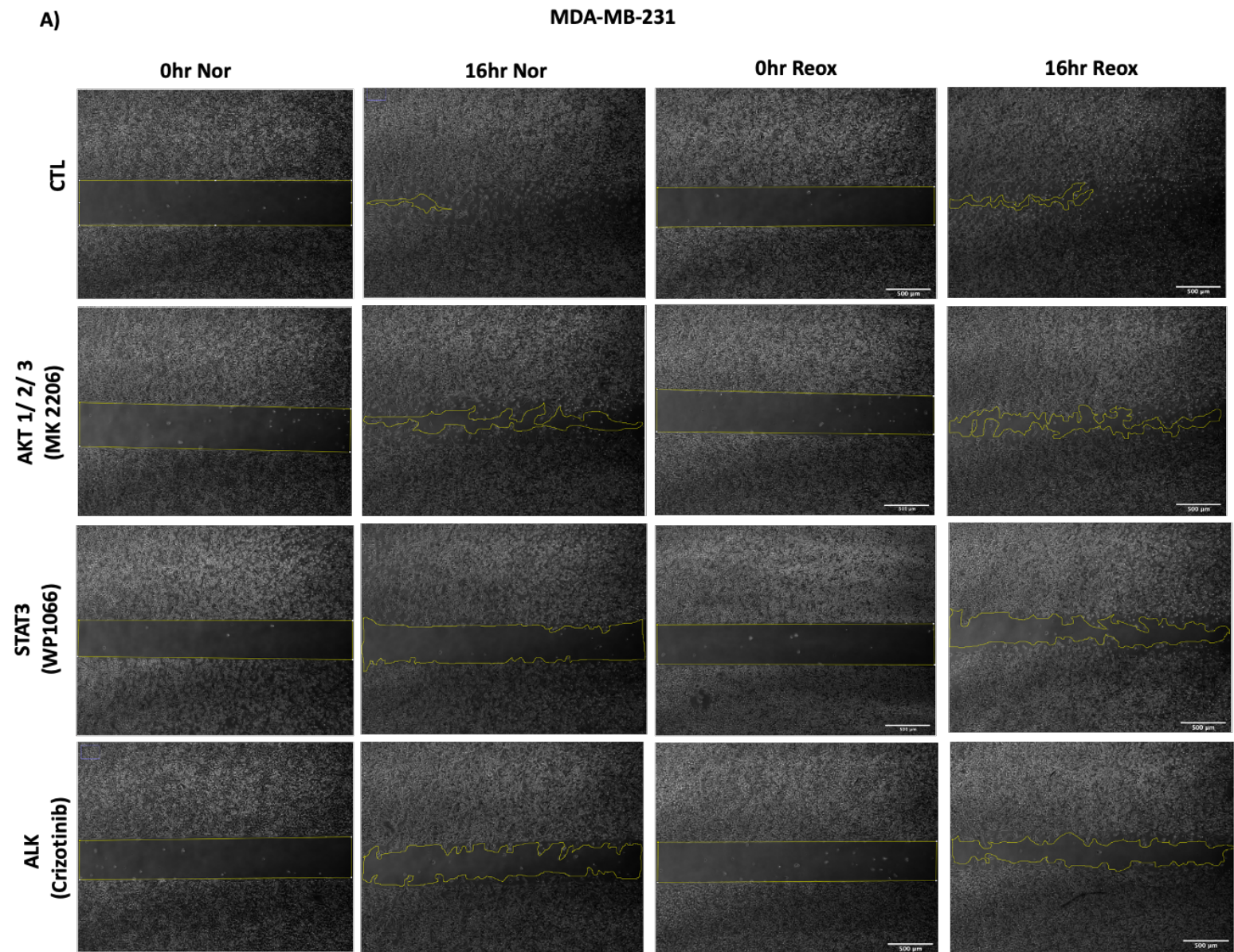
Data was analysed using 2way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n=3$

5.3.5 Migration

Previously, we screened for the changes in phosphorylation under hypoxia and hypoxia-reoxygenation compared to normoxia by performing two arrays investigating phospho-RTKs and phospho-kinases. We have further investigated the phosphorylation of selected targets from the array using a western blot. The results showed a significant increase in phosphorylation of STAT3 (Y705, AKT (Thr 308) and ALK under hypoxia and/or hypoxia-reoxygenation (4.0 Chapter 2). Following on these results, we have investigated the use of kinase inhibitors to investigate whether phosphorylation can be reduced under hypoxia-reoxygenation and normoxia. Results have shown a significant decrease in the phosphorylation of STAT3 (Y705) and ALK and a possible decrease in AKT (308) (5.3.2). Studies have shown that intermittent hypoxia increases the migration of breast cancer cells (MDA-MB-231) (Liu L. et al. 2017). Based on these findings, we hypothesised that kinase inhibitors can suppress the migration of breast cancer under hypoxia-reoxygenation and normoxia.

To test this, cells were incubated under normoxia (at 21% O₂ for 72 hours) and hypoxia-reoxygenation (at 1% O₂ for 72 hours followed by 21% O₂ for 72 hours. After incubation, the cells were seeded in the inserts and allowed to attach before adding the medium containing the appropriate dose concentration of kinase inhibitor or DMSO vehicle control as described in the methods (Methods). MDA-MB-231 cells were imaged at 0 and 16 hours, whereas MCF7 cells were imaged at 0, 24, and 48 hours. The analysis here compared normoxia and hypoxia-reoxygenation to DMSO vehicle controls at 0 hours.

In MDA-MB-231 cells, results showed that ALK inhibitor Crizotinib (5 µM) strongly inhibited the mobility of MDA-MB-231 cells. The percentage of the gap closure remained high at 72% (p<0.001, n=3) in normoxia and 70% (p<0.01, n=3) in hypoxia-reoxygenation (Figure 5.12). Similarly, the STAT3 inhibitor WP1066 (2.5 µM) also inhibited the mobility of MDA-MB-231 cells. After 16 hours, the percentage of the gap closure in normoxia was 66% (p<0.01, n=3) and 54% (p<0.05, n=3) in hypoxia-reoxygenation (Figure 5.12). Regarding the AKT inhibitor MK-2206 (2.5 µM), after 16 hours in both normoxia and hypoxia-reoxygenation, the percentage of the gap closure was 32% and 35%, respectively, in the MDA-MB-231 cells (Figure 5.12). However, this was not statistically significant.



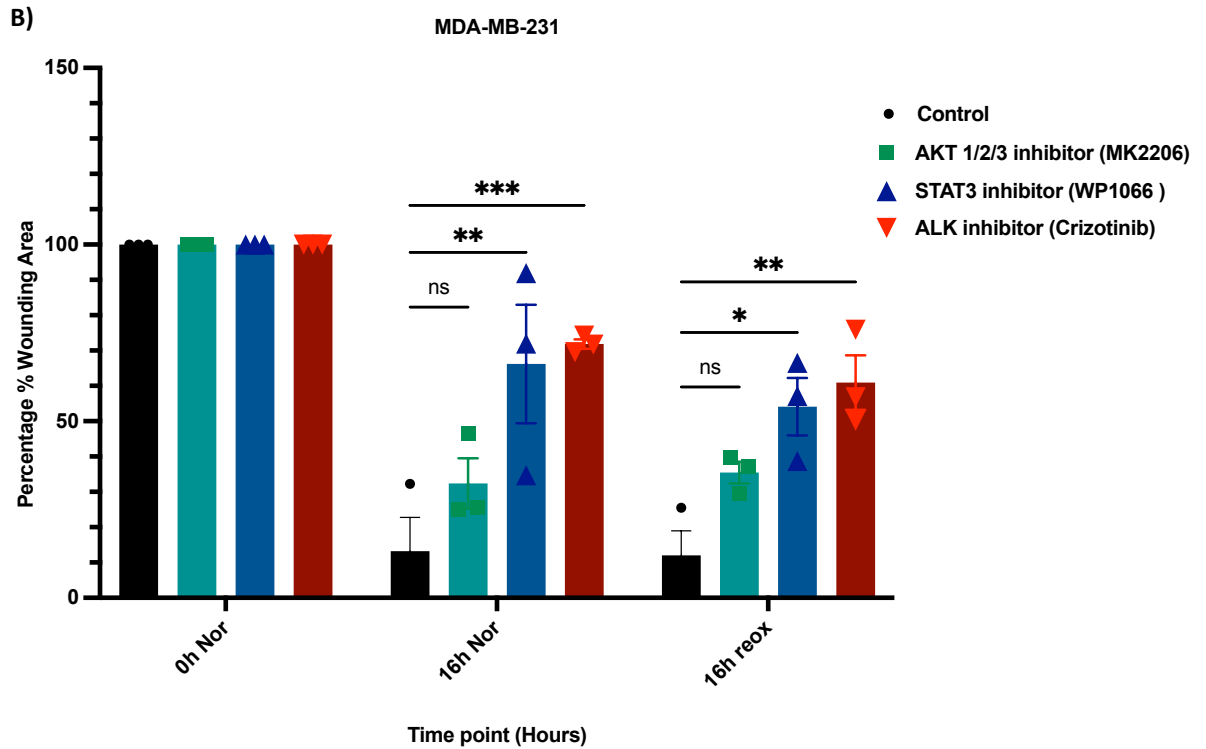
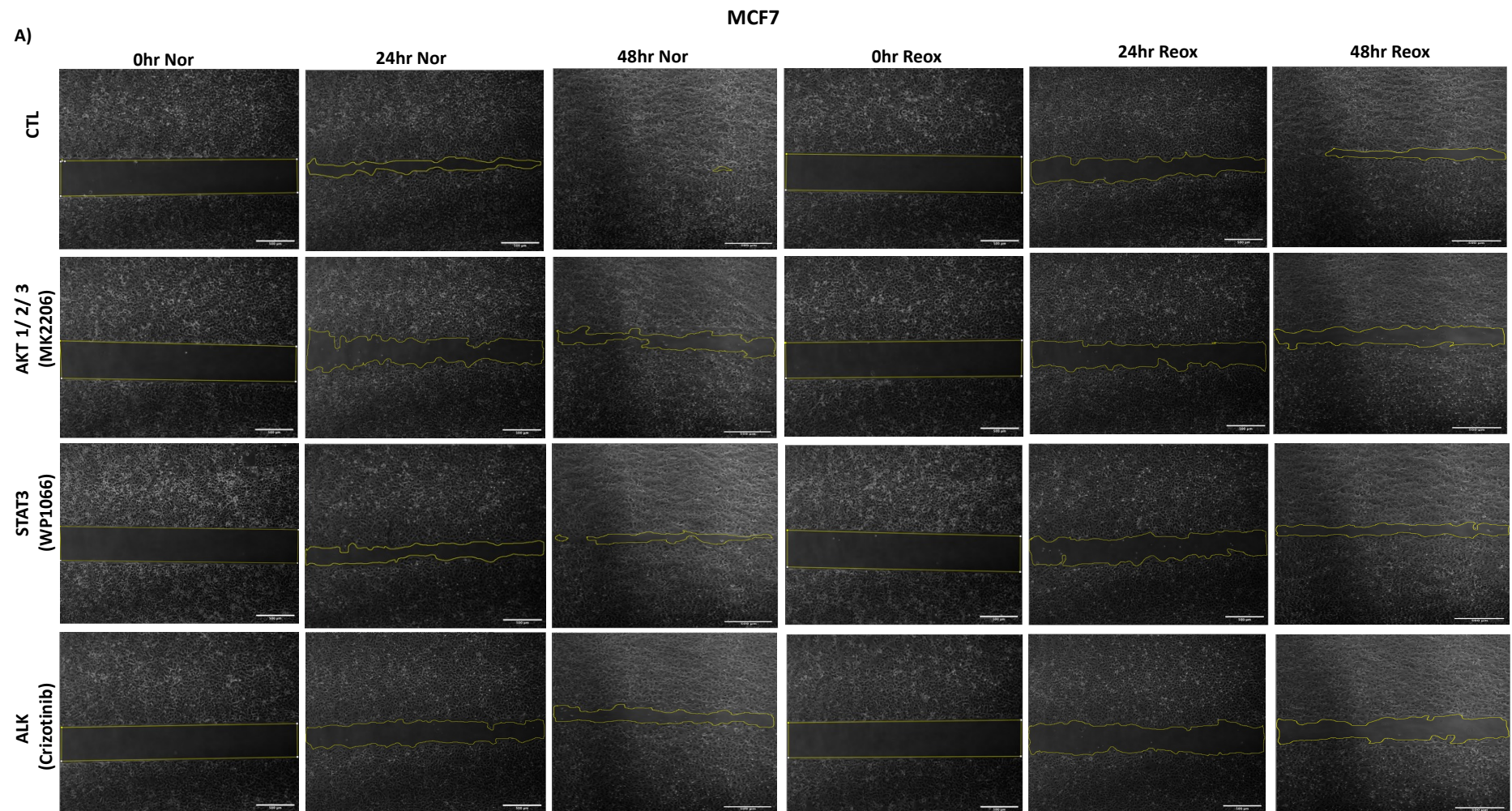


Figure 5.12 Migration of MDA-MB-231 cells treated with kinase inhibitors under normoxia and hypoxia-reoxygenation. A) Representative images of migration of the MDA-MB-231 cells under normoxia and Hypoxia-Reoxygenation conditions treated with kinase inhibitors for 16 hours. **B)** Bar graph showing the percentage of the gap closure in treated cells after 16 hours compared to 0 hour.

Error bars indicate $\pm$ SEM. Statistical analysis using A two-way-Analysis of Variance (ANOVA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 3$

In the MCF7 cells, image analysis was conducted after 24 hours of migration and after 48 hours of migration. After 24 hours of migration, crizotinib at a concentration of 1.5 μ M suppressed the migration of both normoxia and hypoxia-reoxygenation the percentage of the gap closure was 48.6% in normoxia ($p < 0.05$, $n = 3$) and 56.5% in hypoxia-reoxygenation ($p < 0.05$, $n = 3$). In 48 hours of migration, the percentage of the gap closure was 26.6% in normoxia and 32% in hypoxia-reoxygenation, both statistically not significant (Figure 5.13B).

With the 2.5 μ M WP1066 concentration, in 24 hours of migration, the percentage of the gap closure was 45.3% ($p < 0.05$, $n = 3$) and 47% ($p < 0.05$, $n = 3$) in normoxia and hypoxia-reoxygenation, respectively. In 48, the percentage of the gap closure was 15.6% in normoxia and 12% in hypoxia-reoxygenation. In addition, after 24 hours of migration, 2.5 μ M MK-2206 inhibitor suppressed the migration under normoxia and hypoxia-reoxygenation, with the percentage of the gap closure reduced by 50% ($p < 0.01$, $n = 3$) in normoxia and 56% in hypoxia-reoxygenation ($p < 0.05$, $n = 3$). In 48 hours of migration with MK-2206, the percentage of the gap closure was 35% and 32% in normoxia and hypoxia-reoxygenation cells, respectively (Figure 5.13B).



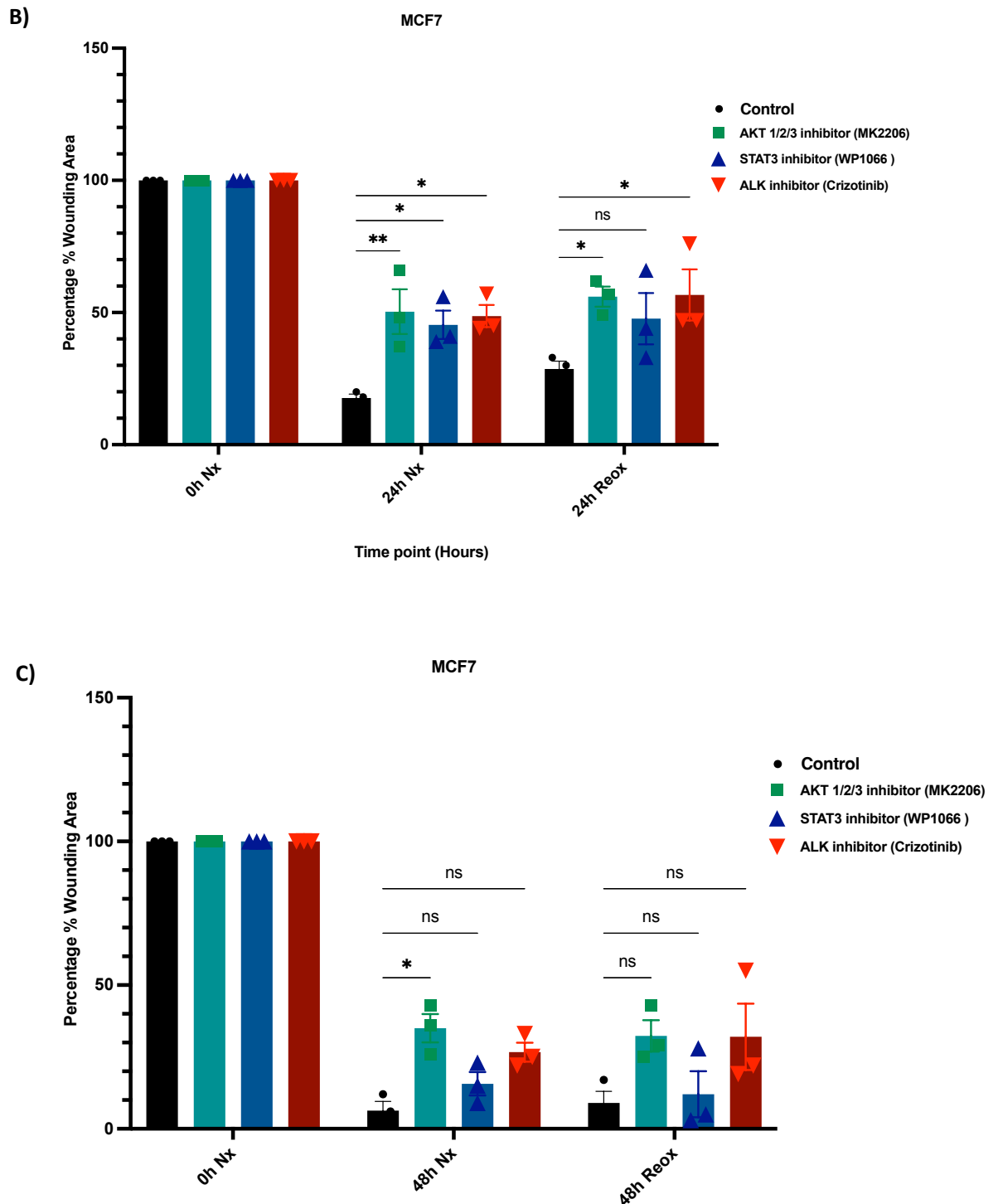


Figure 5.13 Migration of MCF7 cells treated with kinase inhibitors under normoxia and hypoxia-reoxygenation. A) Representative images of migration of the MCF7 cells under normoxia and hypoxia-reoxygenation conditions treated with kinase inhibitors for 24 and 48 hours. **B)** Bar graph showing the percentage of the gap closure in treated cells after 24 hours compared to 0 hours. **C)** Bar graph showing the percentage of the gap closure in treated cells after 48 hours compared to 0 hours.

Error bars indicate $\pm$ SEM. Statistical analysis using a two-way analysis of Variance (ANOVA). * $p < 0.05$, ** $p < 0.01$. $n = 3$

5.0. Discussion

In this chapter, the impact of hypoxia-reoxygenation on the phenotype of breast cancer cells has been explored. This aimed to address two critical hypotheses. The first hypothesis states that breast cancer cells can maintain their acquired characteristics under hypoxic conditions upon hypoxia-reoxygenation, such as enhanced invasion and metastasis and resistance to chemotherapy. The second hypothesis is that kinase inhibitors have the potential to inhibit some of the acquired characteristics of breast cancer cells under hypoxia and hypoxia-reoxygenation, such as suppressing migration, inhibiting altered phospho-signalling, and enhancing the response of breast cancer cells to chemotherapy.

5.4.1 Hypoxia-reoxygenation induced the invasion of breast cancer cells

Cancer cells are known to acquire the ability to invade and metastasise, which is a crucial characteristic of the hallmarks of cancer cells (Hanahan D. 2022). Moreover, hypoxia-inducible factor (HIF) is activated when exposed to low oxygen levels. It triggers gene transcription that regulates various cancer hallmarks, such as invasion and metastasis (Semenza, G.L 2003).

This study explores the effect of hypoxia-reoxygenation on the invasiveness of breast cancer cells. The analysis revealed that hypoxia-reoxygenation (1%-8.5% O₂ 72 hours) significantly increased the invasiveness of breast cancer cells MDA-MB-231 and MCF7 when compared to physoxia (8.5% O₂ 72 hours) following the 24-hour Transwell invasion assay through Matrigel.

Studies have shown that both cyclic and intermittent hypoxia can enhance the invasiveness and migration of cancer cells *in vitro*. For example, a previous study has shown that cyclic hypoxia enhanced the invasiveness and migration of gastric cancer cells *in vitro* (Miao, ZF. et al. 2014). Intermittent hypoxia (18-20 cycles of hypoxia and normoxia; each cycle consisted of 1% O₂ 48 h hypoxia followed by 20% O₂ 48 h normoxia) enhanced the invasiveness, migration, and angiogenesis of medulloblastoma (Gupta R, et al. 2011). In addition, Intermittent hypoxia (5 cycles of hypoxia and normoxia; each cycle consisted of 1% O₂ 12 h hypoxia followed by 20% O₂ 12 h normoxia) increased invasiveness of pancreatic cancer cell lines in Transwell assay (Zhu H. et al. 2014)

Another study found that the migration of MDA-MB-231 cells increased under intermittent hypoxia (1% O₂, 12 h hypoxia, 12 h reoxygenation, 10 cycles); however, the effect depended on the number of hypoxia-reoxygenation cycles (Liu L. et al. 2017).

Epithelial-mesenchymal transition (EMT) was also found to be related to HIF-1 α in inducing the invasiveness of tumour cells, as several studies have demonstrated that overexpression of HIF-1 (hypoxia) promotes EMT and the metastatic phenotype (Yang, MH. et al. 2008). Intermittent hypoxia was found to induce EMT, and an increased expression of HIF-1 α , E-cadherin, Vimentin and N-cadherin was detected in the migratory pancreatic cells under intermittent hypoxia (Zhu H. et al. 2014). Studies showed that EMT was induced, which resulted in the expression of mesenchymal markers such as N-cadherin, SNAIL and Vimentin. In contrast, the expression of epithelial markers such as E-cadherin and Zo-1 was reduced in medulloblastoma cells (Gupta R, et al. 2011). Furthermore, HIF-1 α co-expressed with TWIST and Snail in head and neck carcinoma patients, associated with metastasis and a poor prognosis (Yang, MH. Et al. 2008). Therefore, the role of HIF-1 α in inducing EMT and promoting tumour cell invasiveness has been well documented in various studies.

5.4.2 Role of kinase inhibitors in breast cancer

In this study, an investigation was carried out to explore the activation of phospho-RTKs and intracellular kinases in breast cancer cells (MDA-MB-231 and MCF7) under hypoxia, hypoxia-reoxygenation and normoxia conditions. Arrays were used to screen for the activation status of 49 RTKs and 37 kinases Western blot. The findings here revealed that hypoxia-reoxygenation significantly increased the phosphorylation of ALK in both MDA-MB-231 and MCF7 cells. Furthermore, upregulation of the STAT3 pathway under hypoxia-reoxygenation was observed; the levels of phosphorylated STAT3 (Y705) significantly increased in breast cancer cell lines MDA-MB-231 and MCF7 under hypoxia and hypoxia-reoxygenation when compared to normoxia. Additionally, the AKT signalling pathway was upregulated during hypoxia and hypoxia-reoxygenation, with a significant increase in phospho-AKT Thr 308 observed in both breast cancer cell lines.

Based on these results, the aim was to investigate the potential of kinase inhibitors in inhibiting the phosphorylation of kinases, suppressing migration, and overcoming

chemotherapy resistance in breast cancer cells under hypoxia-reoxygenation compared to normoxia. The following inhibitors were selected: Crizotinib (ALK inhibitor), WP1066 (STAT3 inhibitor), and MK-2206 (AKT inhibitor).

Crizotinib

This study found that Crizotinib inhibited total ALK expression in breast cancer cells MDA-MB-231 and MCF7. The concentration of 2.5 μ M-5 μ M crizotinib in MDA-MB-231 cells and 1.5 μ M-3 μ M in the MCF7 cells significantly inhibited total ALK expression in cells incubated under normoxia and hypoxia-reoxygenation when compared to control normoxia ($p<0.05$) and ($p<0.0001$) respectively. Following the 16 hours of incubation with 5 μ M Crizotinib in MDA-MB-231, the mobility of cells was significantly suppressed under both conditions of normoxia and hypoxia-reoxygenation when compared to vehicle-treated controls ($p<0.001$ and $p<0.01$). Similarly, in MCF7, 1.5 μ M crizotinib has also suppressed the migration of normoxia and hypoxia-reoxygenation cells following the 24-hour incubation time

The study here also showed that treating MDA-MB-231 cells with cisplatin in dose-dependent manners followed by 5 μ M Crizotinib led to a significant reduction in the cell proliferation rate of hypoxia-reoxygenation cells when compared to similar treated normoxia cells (p -value <0.001). It is also possible that Crizotinib has similar effects when combined with doxorubicin in MDA-MB-231 cells (p -value=ns). Additionally, combined treatment of cisplatin or doxorubicin with 1.5 μ M Crizotinib in MCF7 cells showed that crizotinib could potentially lower the previously observed hypoxia-reoxygenation-induced cytotoxicity when cells were treated with chemotherapy drugs alone. However, additional biological repeats are needed to confirm it ($n=2$).

Studies have shown that crizotinib treatment impacts the phosphorylation of cancer cells. It has been found to suppress TGF β signalling by preventing Smad phosphorylation, which is independent of ALK/MET/ROS1 in non-small cell lung cancer (NSCLC) cells (Park, S. et al., 2022). Additionally, crizotinib has been found to competitively inhibit TGF β receptor I kinase activity, thereby reducing the migration, invasion, and metastasis capacity of NSCLC (Park, S. et al., 2022). It has also been observed to reduce the downstream signalling phosphorylation AKT and ERK in ovarian cancer cells (Huang XX. et al., 2017). Treatment with crizotinib has

been shown to inhibit the growth of cancer cells. In breast cancer, MDA-MB-231 and MCF7 cells, growth was inhibited at 2-12 μM and 0.5-7 μM , respectively (Ayoub NM. et al. 2017). Moreover, in a dose-dependent manner, crizotinib treatment inhibited cell growth with IC50 values of 2.88 μM in MCF7 cells (Ayoub, N.M. et al. 2021). In ovarian cancer cells, crizotinib has been found to inhibit cell growth, arrest the G2/M phase of the cell cycle, and induce apoptosis (Huang XX et al. 2017).

WP1066

This study found that the phosphorylation of STAT3 Y705 in breast cancer cells (MDA-MB-231 and MCF7 cells) can be targeted by WP1066. The study showed that incubating these cells with 2.5-5 μM of WP1066 significantly inhibited phospho-STAT3 Y705 under both normoxia and hypoxia-reoxygenation conditions ($p < 0.0001$ and $p < 0.05$). The concentration of WP1066 did not show any significant effect on the total STAT3 expression levels in MCF7 cells ($p\text{-value} = \text{ns}$). However, in MDA-MB-231 cells, a higher concentration of WP1066 significantly inhibited the total STAT3 expression levels. Additionally, 16 hours of incubation with 2.5 μM of WP1066 significantly suppressed the mobility of MDA-MB-231 cells incubated under normoxia and hypoxia-reoxygenation ($p < 0.01$ and $p < 0.05$) compared to vehicle controls. A similar effect of WP1066 on the mobility of MCF7 cells was noticed following the 24-hour incubation.

It has been shown that in renal cancer cells (Caki-1 and 786-O) and bladder cancer cells (T24 and UMUC-3), the phosphorylation of STAT3 (Y705) inhibited with 5 μM WP1066 and 2.5 μM WP1066 inhibited cell survival and proliferation (Horiguchi A. et al. 2010 and Tsujita Y. et al. 2017). In renal cancer cells under both normoxic and hypoxic conditions, WP1066 downregulated the expression of HIF1a and HIF2a, which decreased the production of VEGF and inhibited in vitro angiogenesis (Horiguchi A. et al. 2010). A study on breast cancer cells found that inhibiting STAT3 with WP1066 reduced brain metastasis caused by breast cancer. WP1066 also inhibited STAT3 activation in endothelial cells associated with tumours, which reduced cell infiltration and angiogenesis (Lee HT. et al. 2015). Another study found that WP1066 inhibited STAT3 phosphorylation and induced apoptosis in retinoblastoma cells (Sun, F. et al. 2018). Another study in the MDA-MB-231 cell line found that WP1066 reduced levels of phospho-STAT3 and upregulated ROS production (Linher-Melville. Et al. 2015). ROS act as a

signal transducer at low levels to activate cell proliferation invasion, migration, and angiogenesis; in contrast, high levels of ROS cause damage to nucleic acids, proteins, lipids, organelles and membranes, leading to cell death (Nakamura H. et al. 2021)

MK-2206

Here in this study, it has been demonstrated that phosphorylation of AKT Thr308 can be inhibited with MK-2206 inhibitor. The 2.5- 5 μ M MK-2206 concentration inhibited the phosphorylation of cells incubated under normoxia and hypoxia-reoxygenation significantly in MDA-MB-231 cells and possibly in MCF7 compared to control normoxia cells. However, the total expression of AKT remained high in MDA-MB-231 under all conditions; it could have dropped to almost half in MCF7 cells treated with a higher concentration of MK-2206. In addition, cells incubated under normoxia and hypoxia-reoxygenation and treated with a lower concentration of MK-2206 (2.5 μ M) had slower mobility when compared to vehicle controls, both MDA-MB-231 cells after 16 hours of incubation and MCF7 cells after 24 hours.

In line with data presented here, previous studies have shown that MK-2206 inhibited the phosphorylation of AKT S473 with 5 μ M concentration and a decrease in phosphorylation of AKT Tr308 and rpS6 was observed in dose-dependent manners in non-small cell lung cancer cells with acquired resistance to cetuximab (Iida M, et al. 2013). Moreover, multiple downstream AKT targets could be inhibited with 2.5 μ M MK-2206, including eIF4E, c-jun, GSK3 β , IRS-1, Raf1, rpS6, and IKK α (Iida M, et al. 2013). In a study conducted by Al-Saffar et al., it was demonstrated that MK-2206 at a concentration of 5 μ M inhibited the AKT pathway by decreasing the phosphorylation of AKT Ser4237 and RPS6 (Ser240/244) in human prostate cancer cells treated with the drug, as compared to the control group. Furthermore, treatment with MK-2206 resulted in apoptosis induction after 12 and 24 hours, cell growth inhibition, and G1 cell cycle arrest after 24 hours of treatment (Al-Saffar et al. 2018). A study revealed that MK-2206 has reduced the growth rate of Colorectal cancer cells, leading to a significant decrease in their stemness capacity to form colonospheres in vitro and tumour formation in vivo. Additionally, the study observed a significant reduction in the growth of primary patient-derived tumorspheres. (Malkomes, P. et al. 2016). Further, MK-2206 has moved on to a number of Phase I/II clinical trials against malignancies, among which is HER2 positive or negative breast cancer (Ma CX. et al. 207).

5.4.3 Hypoxia-reoxygenation increased the resistance of breast cancer cells toward the chemotherapy drugs Cisplatin and Doxorubicin.

Stabilising the HIF-1 α protein under hypoxia contributes to pro-survival processes in cancer cells, such as abnormal proliferation, metabolic alteration, and angiogenesis (Kietzmann T, et al. 2016). These processes promote chemoresistance and radio-resistance in cancer cells (Senthebane DA, et al. 2017). This study aimed to investigate if hypoxia-reoxygenation contributes to the chemoresistance of TNBC and Luminal A breast cancer cells toward Cisplatin and Doxorubicin. The study here investigated this through cell viability; the result showed that cells under hypoxia-reoxygenation are more resistant to cisplatin and doxorubicin compared to cells under normoxia in MDA-MB-231 and MCF7 cell lines.

According to a recent study conducted by Godet I et al. in 2021, MDA-MB-231 cells that were exposed to intratumoral hypoxia in the primary tumour and then metastasised to the lung and liver were found to be less responsive to paclitaxel and doxorubicin, but not to 5-FU or cisplatin (Godet I. et al. 2023). It's important to note that the study only analysed the MDA-MB-231 cell line and did not include other cancer cell lines, cancer types or patients (Godet I. et al. 2023). Studies have shown that doxorubicin resistance can be due to the activation of a HIF-1 α dependent mechanism in MDA-MB-231 breast cancer cells (Sullivan, R. et al. 2006). Another factor contributing to this resistance is increased intracellular ROS levels, also stimulated by chemotherapy and radiotherapy (Perillo B. et al. 2020). This increase in ROS can trigger apoptosis via intrinsic or extrinsic pathways (Perillo B. et al. 2020). Doxorubicin can also accumulate ROS and stimulate the Fenton reaction, generating hydroxyl radicals (Kotamraju S. et al. 2002). This reaction has been used to treat various cancers, including acute lymphocytic leukaemia and breast cancer (Kotamraju S. et al. 2002). A research study examined the impact of cycling hypoxia on MCF7 cancer stem cells (CSCs) and their resistance to doxorubicin. The study found that CSCs that experienced intermittent hypoxia (INTR.10, INTR.20, INTR.30, INTR.40) showed higher levels of resistance to doxorubicin compared to the parental MCF7 cells and normoxic CSCs (Alhawarat FM, et al. 2019).

As for hypoxia-induced cisplatin resistance in cancer cells, a study has investigated how hypoxia causes resistance to cisplatin in lung cancer cells. The study found that when lung cancer was exposed to hypoxia, the levels of HIF-1 α and p53 increased, protecting the lung

cancer cells from the effects of cisplatin (Guo Q. et al. 2018). The upregulation of p53 led to activating its downstream target gene, P21, which blocked the cell cycle at the G1-G0 phase and inhibited cell proliferation. As a result, the effectiveness of cisplatin on lung cancer cells (A549) decreased (Guo Q. et al. 2018).

Kinase inhibitors can be potential therapeutic targets to overcome chemotherapy resistance in breast cancer cells.

Here we investigated the combination of chemotherapy drugs, cisplatin and doxorubicin, with the kinase inhibitor crizotinib. The result indicated that this combination reduced the cell viability of cells under hypoxia-reoxygenation compared to those under normoxia in MDA-MB-231 and MCF7 cell lines. However, further investigation, including higher doses of chemotherapy drugs, is required to confirm this.

Combined treatment of crizotinib with chemotherapy drugs can result in overcoming chemotherapy resistance. Crizotinib combined with cisplatin inhibited in vitro and in vivo ovarian cancer cell growth (Huang XX. Et al. 2017). Combined treatment of crizotinib and doxorubicin increased the doxorubicin accumulation in the nucleus and enhanced hepatocellular carcinoma cell death. This drug combination activates PERK-JNK- autophagic cell death pathways (Shao M et al. 2021).

Research has shown that the combination of MK-2006 and MLN4924 inhibitors can effectively inhibit the migration of breast cancer cells MDA-MB-231 and SK-BR3 (Chen X. et al. 2018). Additionally, this combination has been found to increase the induction of apoptosis compared to treatment with MLN4924 inhibitor alone (Chen X. et al. 2018). MLN4924 inhibitor is a first-in-class NEDD8-activating enzyme (NAE) inhibitor, which can deactivate cullin-RING ligases and accumulate their substrates to trigger apoptosis (Brownell JE, et al. 2010). Several studies have investigated its antitumor activity and found it effective in inducing apoptosis (Oladghaffari M. et al. 2017). Another recent study also showed that combining ruxolitinib - Janus kinase (JAK)1/JAK2 inhibitor- and MK-2206 in MDA-MB-231 cells decreased proliferation by the dual suppression of PI3K/AKT and JAK2/STAT5 signalling (Guvendir Celik E. et al. 2023). This was achieved by upregulating the expression of PTEN and PI3K and

downregulating the levels of AKT, JAK2, STAT5, Caspase-9, Caspase-7, PARP, c-Myc, and Bcl-2 (Guvenir Celik E. et al. 2023).

6.0 Conclusion and Future Direction

The aim of this project was to study the effects of hypoxia-reoxygenation in gene expression, signalling and cell phenotype on breast cancer cells. It is known that hypoxia induces metastasis in breast cancer cells (Gilkes, D. M., & Semenza, G. L. 2013), and that metastasis-related genes are more prevalent in well-oxygenated regions of the bloodstream (Boidot, R. et al. 2014). A study has also shown that circulating tumour cells maintain a metastasistic enriched transcriptome, suggesting that a hypoxic gene signature is maintained in these tumour cells (Boidot, R. et al. 2014). In this project, we investigated changes in gene expression, signalling, and cell phenotype in hypoxia and hypoxia-reoxygenation compared to normoxia/physoxia. In addition, we investigated whether the hypoxic profile is maintained during hypoxia-reoxygenation in breast cancer cells and its role in cancer progression and therapy resistance. The main findings are summarised below.

6.1 The impact of hypoxia-reoxygenation in gene expression in breast cancer

Here in this project, we generated two RNAseq libraries generated from TNBC cell line (MDA-MB-231) and = Luminal A cell line (MCF7) to quantify the changes in gene expression in hypoxia and hypoxia-reoxygenation compared to normoxia in the MDA-MB-231 cell line and compared to physoxia in the MCF7 cell line. We identified changes in gene expression in hypoxia (1537 and 1965 significantly changed genes, respectively) and in hypoxia-reoxygenation (2091 and 737 genes, respectively) in MDA-MB-231 and MCF7. VEGFs (VEGFA, VEGFB, and VEGFC) and EGLN3 were found to significantly upregulate under hypoxia-reoxygenation. Cyclic hypoxia has been found to increase tumour perfusion and angiogenesis in a VEGF-A-dependent manner (Kochan-Jamroz K. 2019, Gaustad JV. et al. 2013). KEGG pathway analysis also that hypoxia-reoxygnation RNAs were enriched for TGF β and Wnt signalling and also interacted with several pathways, including apoptosis, p53, PI3K, MAPK, ERK and JAK-STAT3 pathways. In addition, we found that 63 DEGs upregulated under hypoxia-reoxygenation were common to both cell lines, where the majority (42 DEGs) show enrichment for metabolic processes, suggesting a possible hypoxia-reoxygenation gene signature in breast cancer. We have also shown that gene expression changes induced by hypoxia were maintained upon reoxygenation, with 38% in MDA-MB-231 and 11.5% in MCF7. These maintained genes were enriched in metabolic processes such as ALDOC, PTGS and LOXL2 and in signalling pathways such as Wnt and TGF β signalling. We have also shown that

multiple genes were significantly expressed in TNBC and HER(-ve) breast cancer patients and associated with metastasis and poor prognosis in breast cancer patients such as CST1, MUC1, CDH2, and EGLN3. The impact of metabolic shift under intermittent hypoxia has been studied in obstructive sleep apnea (Ryan S. 2017, Drager LF. et al. 2010). Upregulation of genes involved in glycolysis pathways has also been observed in breast cancer cells under intermittent hypoxia (Jarrar Y. et al. 2020, Verduzco D. et al. 2015).

In addition, we observed a significant upregulation of several growth factors in hypoxia in both RNAseq datasets and validated their expression by qPCR, such as VEGFA, ARTN, INHA, PDGFB, and PGF. Here, we proposed a possible mechanism by which hypoxia-induced changes are maintained upon hypoxia-reoxygenation through the activation of growth factors in hypoxia, which can initiate a positive feedback loop responsible for this phenomenon.

6.2 The impact of hypoxia-reoxygenation signalling in breast cancer

In this project, we investigated the impact of hypoxia and hypoxia-reoxygenation in signalling pathways. We screened for changes in the phosphorylation of RTKS and intracellular kinases using phospho-RTK array and phosphokinase arrays in the MDA-MB-231 cell line. We found that during hypoxia, 24% of RTKs and 31% of intracellular kinases had increased phosphorylation levels. Additionally, following hypoxia-reoxygenation, 70% of RTKs and 67% of intracellular kinases had increased phosphorylation levels. We investigated this further using a western blot in MDA-MB-231 and MCF7 cell lines. We have shown that phosphorylation of STAT3 (Y705), AKT (Thr308), and ALK increases in these cell lines under hypoxia and/or hypoxia-reoxygenation.

6.3 The impact of hypoxia-reoxygenation cell phenotype in breast cancer

In addition to the observed changes in transcription and signalling induced by hypoxia-reoxygenation the phenotypic consequences of these changes. We showed that hypoxia-reoxygenation resulted in increased invasion by 58% in MDA-MB-231 and by 56% in MCF7 compared to physoxia. We also showed that hypoxia-reoxygenation increased resistance to chemotherapy drugs cisplatin and doxorubicin in MDA-MB-231 and MCF7. Following the phospho-RTK and phospho-kinase arrays screen and the western blot investigations, we identified several promising kinase targets, and we further investigated the use of kinase

inhibitors in breast cancer cells. MDA-MB-231 and MCF7 were treated with kinase inhibitors Crizotinib, WP1066, and MK-2206, and we observed a reduction in the phosphorylation under normoxia and hypoxia-reoxygenation compared to the untreated cells. We have also investigated the role of kinase inhibitors targeting STAT3, AKT and ALK pathways in breast cancer cells as potential targeted therapy; we showed that kinase inhibitors suppressed the migration under hypoxia-reoxygenation and physoxia compared to vehicle control. In addition, chemotherapy drugs cisplatin and doxorubicin were combined with Crizotinib in a dose-dependent manner, and we observed increased sensitivity of breast cancer cells toward chemotherapy drugs following this combination. However, more biological repeats are needed to confirm this.

6.4 Future Direction

RNAseq demonstrated that DEGs are upregulated under hypoxia and maintained upon hypoxia-reoxygenation. For future work, metabolic pathways under hypoxia-reoxygenation could be explored further to determine their impact on the invasiveness and metastatic behaviour of breast cancer cells. It would be necessary to conduct further investigation into the activation of growth factor signalling during hypoxia and their role in initiating a positive feedback loop that could potentially maintain the hypoxic profile upon hypoxia-reoxygenation. To comprehend this mechanism, it is recommended to perform a knockdown of these growth factors in breast cancer cells and evaluate transcriptional activation under hypoxia and hypoxia-reoxygenation. In this study, we explored whether the hypoxic profile is maintained during hypoxia-reoxygenation in MDA-MB-231 and MCF7 cell lines. For future research, it would be beneficial to include other breast cancer cells, such as additional TNBC and Luminal A cell lines, to investigate any similarities and differences between subtypes. While we have already looked into the clinical relevance of some genes maintained during hypoxia-reoxygenation in breast cancer tissues using transcriptomic and clinicopathological data, additional research can be conducted to confirm these findings. For instance, we could employ Tissue Microarray to identify potential diagnostic and prognostic markers.

In this project, MDA-MB-231 and MCF7 showed increased resistance toward chemotherapeutic drugs cisplatin and doxorubicin under hypoxia-reoxygenation. As for future work in this project, it is recommended to expand the investigation by screening for more

chemotherapeutic agents and kinase inhibitors. Additionally, the response of breast cancer cells under normoxia and hypoxia-reoxygenation should also be investigated at higher doses of cisplatin and doxorubicin. Combining chemotherapy drugs with kinase inhibitors could also help determine whether higher doses of chemotherapy drugs with kinase inhibitors can make breast cancer cells more sensitive to chemotherapy and lead to a better prognosis in TNBC. According to studies, using crizotinib in combination with chemotherapy drugs has shown promising results in overcoming chemotherapy resistance in ovarian cancer (Huang XX. et al. 2017). Studies have demonstrated that increased formation of reactive oxygen species (ROS) is linked with the resistance of cancer cells to chemotherapy and radiotherapy under cyclic hypoxia (Hsieh CH. et al. 2012, Matschke J. et al. 2016, Chen WL. et al. 2015, Hsieh CH. et al. 2010). In another study, it was observed that cells which had metastasised to the lung and liver and undergone intratumoral hypoxia in the primary tumour had a diminished response to doxorubicin (Godet I. et al. 2023, Alhawarat FM, et al. 2019). Several studies have also shown that ROS contributes to doxorubicin resistance in breast cancer cells (Perillo B. et al. 2020, Kotamraju S. et al. 2002). Therefore, future research for this project should include evaluating ROS levels under hypoxia-reoxygenation. Further investigation can include targeting ROS as a prospective strategy to overcome drug resistance in breast cancer cells induced by hypoxia-reoxygenation.

6.5 Limitation of Methodology

It is important to take into account the various limitations that were present during the course of this project. For instance, although some experiments were conducted using physiological oxygen levels in human breast tissues (8.5% O₂) for physoxia(normoxia), other experiments used the widely used oxygen concentration in the literature for normoxia (21% O₂). This limitation should be considered when interpreting the results. In addition, the definition of hypoxia-reoxygenation as (1% O₂ for 72 hours followed by 21% O₂ or 8.5% O₂ for 72 hours) was based on the project's own definition as there is currently no defined duration of hypoxia, levels of oxygen and the presence of hypoxia-reoxygenation cycles in the literature (Han et al. 2017, Jarman et al. 2019, Bayer C. et al. 2011). This makes it difficult to compare the results with other cyclic and intermittent hypoxia studies. Moreover, the lack of statistical significance in the phospho-RTK array and phospho-kinase array could be attributed to the inter-variation between biological replicates. However, to ensure robust results this project conducted three

biological replicates, while other studies only used one replicate when screening with these arrays (Neradil. et al. 2019)

In our study, we focused solely on breast cancer cell lines. However, while these cell lines are useful for assessing responses to drugs and tumour phenotypes, they have limited clinical relevance (Gillet JP, 2011). To obtain more clinically significant results, it would be beneficial to validate our findings on 3D cultures, such as organoids. Organoids are particularly useful as they replicate tumour heterogeneity and characteristics, making them more similar to tumours in patients (Luo L. et al., 2022). Additionally, they serve as a bridge between animal models and human patients, providing a more accurate representation of the effects of drugs on tumours.

7.0 Appendix

Supplementary Experimental Information

1- qPCR Primer Sequences

Target	Sequence
CA9	F: CTTGGAAGAAATCGCTGAGG
	R: TGGAAGTAGCGGCTGAAGTC
VEGFA	F: TCTTCAAGCCATCCTGTGTG
	R: TCTCTCCTATGTGCTGGCCT
LDHA	F: AACATGGCAGCCCTTTTATG
	R: ATTGGAGTTTGCAGTGACCA
B-ACTIN	F: ATTGGCAATGAGCGGTTT
	R: GGATGCCACAGGACTCCAT
MUC1	F: TTTCCAGCCCGGGATACCTA
	R: ACAAGTTGGCAGAAGTGGCT
DUSP8	F: GCTCCGTCAACATCTGCTGC
	R: CCGCGTGCTCTGGTCATAGA
DUSP1	F: GAGGCCATTGACTTCATAGACTC
	R: CATAAGGTAAGCAAGGCAGATGG
CST1	F: GGTACTAAGAGCCAGGCAACA
	R: AAGGCACAGGTGTCCAAGTT
BNIB3	F: TGCTGCTCTCTCATTTGCTG
	R: GACTCCAGTTCTTCATCAAAAGGT
HIG2	F: CCGACTTTCCTCCGGACT
	R: CCTTCTGAAAGGCCTCTGG
BPIFB2	F: GCTTGGGCAAGTAGGCTGG
	R: GCCTTGTTGAGTCGGACCAC
PTGS1	F: TAGTGCCACCAGGCATCAGA
	R: CCAGACGGTTAGAGGCTGGA
CDH2	F: TGGACGGTTCGCCATCCAGAC
	R: AGTCGATTGGTTTGACCACGGTG
EDIL3	F: GCGAATGGAACCTTCTGGCTGTG
	R: GAGC GTTCTGAAGATGCTGGAG
SEMA5B	F: TGTCCTGTGCGGAATGTGAC
	R: CCTGAGTTGTCCCATCCAA

LOXL2	F: CCAGTGTGGTCTGCAGAGAG
	R: CCTGTGCACTGGATCTCGTT
INHBA (Check)	F: CCTCCCAAAGGATGTACCCAA
	R: CTCTATCTCCACATACCCGTTCT
PGF (Ashia)	F: acctgccctctatttattagcca
	R: tctitctctcacgttggtgaagg
PDGFB (Check)	F: TGGGCCTCGGGGACCCCAT
	R: CGTGCAGCAGGCGTTGGAGA
PDGFC	F: GGCTTCTCCTGCTGACATCT
	R: TCCGTTCTGTTCTTGTGTC
INHA	F: CGCTCAACTCCCCTGATGTC
	R: GGGTACACGATCCACCGTTC
ARTN	F: ATGAACACTACAGTGGCTGAGG
	R: AGCTCCCATGAGTGAGTACAGG
GDF15	F: GACCCTCAGAGTTGCACTCC
	R: GCCTGGTTAGCAGGTCCTC
BMP2	F: GACTGCGGTCTCCTAAAGGTCG
	R: CTGGGGAAGCAGCAACACTA
TGFB2	F: CTGTCTACCTGCAGCACACT
	R: TGGGACTGTCTGGAGCACAA

2- Western Blot Buffer Recipes

10X TBS (1L)

24g Tris Base

88g NaCl

Dissolve in 900ml dH₂O, adjust pH7.6, make up to 1L final volume.

1X TBS-T (1L)

100ml 10X TBS 900ml dH₂O 1ml Tween-20

1X 5%-BSA-TBS-T [w/v] (100ml)

5g BSA

100ml 1X TBS-T

1X 3%-BSA-TBS-T [w/v] (100ml)

3g Milk Powder 100ml 1X TBS-T

10X Running Buffer (1L)

30.3g Tris Base

144.4g Glycine

10g SDS

Prepare to 1L with dH₂O.

1X Transfer Buffer (1L)

200ml 5X Bio-Rad Transfer Buffer 200ml 100% EtOH

600ml dH₂O

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