

THE UNIVERSITY OF HULL

**Regulation of hypoxia responsive gene expression by
Specificity protein family transcription factors in breast
cancer**

being a Thesis submitted for the Degree of
Doctor of Philosophy in the University of Hull

By

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“Almost all aspects of life are engineered at the molecular level, and without understanding molecules we can only have a very sketchy understanding of life itself.”

Francis Crick (1988)

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Figure Index

Figure 1.1: The hallmarks of cancer	10
Figure 1.2: Diagram of HIF-1 α , HIF-2 α and HIF-1 β domain structures	20
Figure 1.3: Oxygen dependent HIF regulation	23
Figure 1.4: Diagram of Structural motifs in Sp factors.....	30
Figure 1.5: Interaction of Sp1 protein in basal transcriptional apparatus.....	32
Figure 2.1: Summary of Surface plasmon assay development.....	65
Figure 3.1: mRNA expression of Sp1 in breast cancer cells under severe hypoxia	76
Figure 3.2: mRNA expression of Sp3 in breast cancer cells under severe hypoxia	77
Figure 3.3: mRNA expression of Sp4 in breast cancer cells under severe hypoxia	78
Figure 3.4: Effect of severe and moderate hypoxia on Sp1 protein levels in MCF-7 cells.....	80
Figure 3.5: Effect of severe and moderate hypoxia on Sp1 protein levels in MDA-MB-231 cells	81
Figure 3.6: Effect of severe and moderate hypoxia on Sp1 protein levels in T47D cells.....	82
Figure 3.7: Effect of severe and moderate hypoxia on Sp3 protein levels in MCF-7 cells.....	84
Figure 3.8: Effect of severe and moderate hypoxia on Sp3 protein levels in MDA-MB-231 cells	85
Figure 3.9: Effect of severe and moderate hypoxia on Sp3 protein levels in T47D cells.....	86
Figure 3.10: Effect of severe hypoxia on binding activity of Sp proteins in MCF-7 cells	88
Figure 3.11: Effect of severe hypoxia on binding activity of Sp proteins in MDA-MB-231 cells	89
Figure 3.12: Effect of severe hypoxia on binding activity of Sp proteins in T47D cells	90
Figure 3.13: Effect of severe hypoxia on binding activity of Sp proteins in MCF-7 cells	92
Figure 3.14: Effect of severe hypoxia on binding activity of Sp proteins in MDA-MB-231 cells	93
Figure 3.15: Effect of severe hypoxia on binding activity of Sp proteins in T47D cells	94

Figure 3.16: Effect of severe hypoxia on binding activity of Sp proteins in MCF-7 cells	95
Figure 3.17: Effect of severe hypoxia on binding activity of Sp proteins in MDA-MB-231 cells	96
Figure 3.18: Effect of severe hypoxia on binding activity of Sp proteins in T47D cells	97
Figure 3.19: Effect of Mithramycin A and severe hypoxia on Sp1 gene expression in breast cancer cells.....	100
Figure 3.20: Effect of Mithramycin A and severe hypoxia on Sp3 gene expression in breast cancer cells.....	101
Figure 3.21: Effect of Mithramycin A and severe hypoxia on Sp4 gene expression in breast cancer cells.....	102
Figure 4.1: Heatmap and profile plot represent Genes with 1.5-fold change expression and Sp1 binding sites after 0.5% O ₂ hypoxic exposure in MDA-MB-231 cells.....	117
Figure 4.2: mRNA expression of CA9 in breast cancer cells under severe hypoxia	124
Figure 4.5: mRNA expression of GLUT1 in breast cancer cells under severe hypoxia.....	125
Figure 4.6: mRNA expression of PFKFB4 in breast cancer cells under severe hypoxia	127
Figure 4.7: mRNA expression of ARTN in breast cancer cells under severe hypoxia	128
Figure 4.8: mRNA expression of VEGFA in breast cancer cells under severe hypoxia.....	129
Figure 4.9: mRNA expression of CCNG2 in breast cancer cells under severe hypoxia	131
Figure 4.10: mRNA expression of IRS2 in breast cancer cells under severe hypoxia	132
Figure 4.11: mRNA expression of TPBG in breast cancer cells under severe hypoxia.....	133
Figure 4.12: mRNA expression of FUT11 in breast cancer cells under severe hypoxia	135
Figure 4.13: mRNA expression of PPP1R3B in breast cancer cells under severe hypoxia	136
Figure 4.14: mRNA expression of CCL28 in breast cancer cells under severe hypoxia	139
Figure 4.15: mRNA expression of NR2F2 in breast cancer cells under severe hypoxia.....	140
Figure 4.16: mRNA expression of JUN in breast cancer cells under severe hypoxia	141
Figure 4.17: mRNA expression of PDLIM2 in breast cancer cells under severe hypoxia.....	142

Figure 4.18: mRNA expression of SLC11A2 in breast cancer cells under severe hypoxia	145
Figure 4.19: mRNA expression of AHCTF1 in breast cancer cells under severe hypoxia.....	146
Figure 4.20: mRNA expression of PNPT1 in breast cancer cells under severe hypoxia.....	147
Figure 4.21: mRNA expression of AKIRIN1 in breast cancer cells under severe hypoxia	148
Figure 4.22: mRNA expression of THBD in breast cancer cells under severe hypoxia	150
Figure 4.23: mRNA expression of TOX2 in breast cancer cells under severe hypoxia.....	151
Figure 4.24: mRNA expression of TNFRSF21 in breast cancer cells under severe hypoxia ...	153
Figure 4.25: mRNA expression of CIRBP in breast cancer cells under severe hypoxia.....	154
Figure 5.1: Sp protein inhibitors	176
Figure 5.2: Immobilization of biotinylated ligands to Sensor chip (SA chip) surface.....	179
Figure 5.3: Solvent correction.....	180
Figure 5.4: Variation of DMSO concentration in samples.....	181
Figure 5.5: Interaction of Mithramycin A with Sp consensus sequence in the presence of divalent cation Mg ²⁺	184
Figure 5.6: Interaction of Mithramycin A with Sp consensus sequence in absence of divalent cation Mg ²⁺	185
Figure 5.7: Interaction of Chromomycin A with Sp consensus sequence in the presence of divalent cation Mg ²⁺	187
Figure 5.8: Interaction of Chromomycin A with Sp consensus sequence in absence of divalent cation Mg ²⁺	188
Figure 5.9: Interaction of Terameprocol with Sp consensus sequence.....	189
Figure 5.10: Interaction of Mithramycin A with Bak gene promoter ABC region in the presence of divalent cation Mg ²⁺	191
Figure 5.11: Interaction of Mithramycin A with Bak gene promoter ABC region in absence of divalent cation Mg ²⁺	192

Figure 5.12: Interaction of Chromomycin A with Bak gene promoter ABC region in the presence of divalent cation Mg ²⁺	194
Figure 5.13: Interaction of Chromomycin A with Bak gene promoter ABC region in absence of divalent cation Mg ²⁺	195
Figure 5.14: Interaction of Terameprocol with Bak gene promoter ABC region.....	196
Figure 5.15: Interaction of Mithramycin A with Bak gene promoter B region in the presence of divalent cation Mg ²⁺	200
Figure 5.16: Interaction of Mithramycin A with Bak gene promoter B region in absence of divalent cation Mg ²⁺	201
Figure 5.17: Interaction of Chromomycin A with Bak gene promoter B region in the presence of divalent cation Mg ²⁺	202
Figure 5.18: Interaction of Chromomycin A with Bak gene promoter B region in absence of divalent cation Mg ²⁺	203
Figure 5.19: Interaction of Terameprocol with Bak gene promoter B region	204
Figure 5.20: Mode of interaction between DNA and grove binders.	214
Figure 6.1: Effect of Sp1 knockdown on transcript levels of Sp1 in breast cancer cell under hypoxia.....	222
Figure 6.2: Effect of Sp1 knockdown on transcript levels of Sp3 in breast cancer cells under hypoxia.....	223
Figure 6.3: Effect of Sp1 knockdown on transcript levels of Sp4 in breast cancer cells under hypoxia.....	225
Figure 6.4: Effects of Sp1 gene knockdown and Mithramycin A on CA9 gene expression in breast cancer cells under severe hypoxia.....	226
Figure 6.5: Effects of Sp1 gene knockdown and Mithramycin A on GLUT1 gene expression in breast cancer cells under severe hypoxia.....	228

Figure 6.6: Effects of Sp1 gene knockdown and Mithramycin A on PFKFB4 gene expression in breast cancer cells under severe hypoxia	229
Figure 6.7: Effects of Sp1 gene knockdown and Mithramycin A on ARTN gene expression in breast cancer cells under severe hypoxia	231
Figure 6.8: Effects of Sp1 gene knockdown and Mithramycin A on VEGFA gene expression in breast cancer cells under severe hypoxia	232
Figure 6.9: Effects of Sp1 gene knockdown and Mithramycin A on CCNG2 gene expression in breast cancer cells under severe hypoxia	234
Figure 6.10: Effects of Sp1 gene knockdown and Mithramycin A on IRS2 gene expression in breast cancer cells under severe hypoxia	235
Figure 6.11: Effects of Sp1 gene knockdown and Mithramycin A on TPBG gene expression in breast cancer cells under severe hypoxia	237
Figure 6.12: Effects of Sp1 gene knockdown and Mithramycin A on FUT11 gene expression in breast cancer cells under severe hypoxia	238
Figure 6.13: Effects of Sp1 gene knockdown and Mithramycin A on PPP1R3B gene expression in breast cancer cells under severe hypoxia	241
Figure 6.14: Effects of Sp1 gene knockdown and Mithramycin A on CCL28 gene expression in breast cancer cells under severe hypoxia	242
Figure 6.15: Effects of Sp1 gene knockdown and Mithramycin A on NR2F2 gene expression in breast cancer cells under severe hypoxia	243
Figure 6.16: Effects of Sp1 gene knockdown and Mithramycin A on Jun oncogene expression in breast cancer cells under severe hypoxia	245
Figure 6.17: Effects of Sp1 gene knockdown and Mithramycin A on PDLIM2 gene expression in breast cancer cells under severe hypoxia	246
Figure 6.18: Effects of Sp1 gene knockdown and Mithramycin A on SLC11A2 gene expression in breast cancer cells under severe hypoxia	248

Figure 6.19: Effects of Sp1 gene knockdown and Mithramycin A on AHCTF1 gene expression in breast cancer cells under severe hypoxia	249
Figure 6.20: Effects of Sp1 gene knockdown and Mithramycin A on PNPT1 gene expression in breast cancer cells under severe hypoxia	251
Figure 6.21: Effects of Sp1 gene knockdown and Mithramycin A on AKIRIN1 gene expression in breast cancer cells under severe hypoxia	252
Figure 6.22: Effects of Sp1 gene knockdown and Mithramycin A on THBD gene expression in breast cancer cells under severe hypoxia	254
Figure 6.23: Effects of Sp1 gene knockdown and Mithramycin A on TOX2 gene expression in breast cancer cells under severe hypoxia	255
Figure 6.24: Effects of Sp1 gene knockdown and Mithramycin A on TNFRSF21 gene expression in breast cancer cells under severe hypoxia	257
Figure 6.25: Effects of Sp1 gene knockdown and Mithramycin A on CIRBP gene expression in breast cancer cells under severe hypoxia	258
Figure 6.26: Possible modes of inhibition of Sp mediated transcription by Mithramycin A (MTR).....	268
Figure 7.1: Potential molecular mechanism of Sp driven hypoxia responsive gene regulation.	275

Table Index

Table 1.1: High and moderate-risk genes predisposing to breast cancers	7
Table 1.2: Known interactions of the Sp1 transcription factor.....	35
Table 2.1: Summary of cell properties used in this study.....	47
Table 2.2: Cycling protocol for TaqMan real-time PCR.....	52
Table 2.3: Summary of TaqMan qPCR assays of gene targets.....	52
Table 2.4: Composition of 10% acrylamide gels for SDS gel electrophoresis.....	55
Table 2.5: Composition of SDS gel electrophoresis buffers (10x).....	56
Table 2.6: Primary antibodies used in this study.....	56
Table 2.7: DNA oligomers used in this study	60
Table 2.8: Composition of representative EMSA gel reaction mixtures.....	61
Table 2.9: BROAD gene sets used for GSEA analysis.....	69
Table 4.1: NCBI microarray data sets retrieved for analysis.....	114
Table 4.2: Gene enrichment analysis for Sp1 promoter sites.....	116
Table 4.3: Gene enrichment analysis for HIF-1 sites.....	120
Table 4.4: Differentially expressed genes in MCF-7 cells.....	121
Table 4.5: Potential Sp-mediated hypoxia responsive genes selected for qPCR analysis	122
Table 4.6: Fold changes in mRNA expression of hypoxia responsive genes in MCF-7 cells relative to the zero-hour control	155
Table 4.7: Fold changes in mRNA expression of hypoxia responsive genes in MDA-MB-231 cells relative to the zero-hour control	157
Table 4.8: Fold changes in mRNA expression of hypoxia responsive genes in T47D cells relative to the zero-hour control	159
Table 5.1: Binding kinetics of Mithramycin A with different gene promoters at 25 °C.....	205
Table 5.2: Binding kinetics of Chromomycin A with different gene promoters at 25 °C.....	206

Table 6.1: Expression changes (mean difference) of hypoxia responsive genes in response to <i>Sp1</i> gene knockdown and Mithramycin A treatment in MCF-7 cells	259
Table 6.2: Expression changes (mean difference) of hypoxia responsive genes in response to <i>Sp1</i> gene knockdown and Mithramycin A treatment in MDA-MB-231 cells.....	260
Table 6.3: Expression changes (mean difference) of hypoxia responsive genes in response to <i>Sp1</i> gene knockdown and Mithramycin A treatment in T47D cells.....	261

List of Abbreviations

ARNT	Aryl hydrocarbon receptor nuclear translocator
ATM	Ataxia-telangiectasia mutated
BRCA1	Breast cancer 1, early onset
EBNA	Epstein-Barr nuclear antigen
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptors
GH	Growth hormone
GSEA	Gene Set Enrichment Analysis
HDAC	Histone deacetylases
HER-2	Human epidermal growth factor receptor 2
HIF	Hypoxia inducible factor
HRE	Hypoxia response element
KLF	Krüppel-like factors
MAPK	Mitogen activated protein kinases
MMPS	Matrix metalloproteins
MTR	Mithramycin A
NDGA	Nordihydroguaiaretic acid
PDGF	Platelet-derived growth factor
PHD	Prolyl hydroxylases
PI3K	Phosphoinositide 3-kinase
PR	Progesterone receptor
RNA	Ribonucleic acid
Sp	Specificity protein
TAF	TATA-binding protein associated factor
TBP	TATA-binding protein
TCA	Tricarboxylic acid cycle
TGF	Transforming growth factor

TMP	Terameprocol
TNBC	Triple negative breast cancer
TNF- α	Tumour necrosis factor alpha
VHL	Von Hippel-Lindau

List of materials

Consumables	Supplier
Tissue culture plastics	Fisher Scientific UK, NUNC
Glass pipettes	Sarstedt, UK
Cell scrapers	Fisher Scientific UK
Chemicals	
Ethanol	Fisher Scientific UK
Methanol	Fisher Scientific UK
Tris	Sigma
Sodium hydroxide pellets	Fisher Scientific UK
EDTA	Sigma
Equipment	
Olympus CK x41 microscope	Scientific Laboratories, UK
Stratalinker® UV Crosslinker	Northumbria Biological Ltd, South Nelson Industrial East Cramlington
Gel electrophoresis Kit	Bio-Rad

Table of Contents

ACKNOWLEDGEMENTS.....	iii
Thesis associated communications.....	iv
Figure Index.....	v
Table Index.....	xi
List of Abbreviations	xiii
List of materials.....	xv
<i>ABSTRACT</i>	1
Chapter 1. Introduction	3
1. Introduction	4
1.1. Breast cancer.....	4
1.2. Tumour development	9
1.3. Tumour Hypoxia.....	15
1.4. The Sp transcription factor family.....	26
1.5. The role of Sp transcription factors in cancer	37
1.6. Aims and Objectives.....	42
Chapter 2. Materials and Methods.....	45
2. Materials and Methods.....	46
2.1. Cell culture	46
2.2. Hypoxia treatment	48
2.3. Cell transfection with silencing siRNA.....	49
2.4. Mithramycin A treatment	50
2.5. Transcript analysis.....	50
2.6. Protein analysis	53
2.7. Analysis of binding activity of Sp proteins using electromobility shift assay (EMSA).....	57
2.8. Binding and kinetic analysis of Sp inhibitors.....	62
2.9. Microarray data set analysis	67
2.10. Statistical Analysis.....	70

Chapter 3. Effect of hypoxia on Specificity protein transcription factors.....	71
3. The effect of hypoxia on Specificity protein transcription factors and their activity	72
3.1. Introduction	72
3.2. Results	75
3.3. Discussion.....	103
Chapter 4. Transcriptional regulation of hypoxia responsive genes	110
4. Transcriptional regulation of hypoxia responsive genes	111
4.1. Introduction	111
4.2. Results	113
4.3. Discussion.....	161
Chapter 5. Binding analysis and kinetic evaluation of Specificity protein family inhibitors..	172
5. Binding analysis and kinetic evaluation of Specificity protein family inhibitors.....	173
5.1. Introduction	173
5.2. Results	178
5.3. Discussion.....	207
Chapter 6. Effective inhibition of Sp mediated transcription of hypoxia responsive genes..	218
6. Effective inhibition of Sp mediated transcription of hypoxia responsive genes	219
6.1. Introduction	219
6.2. Results	221
6.3. Discussion.....	262
Chapter 7. General discussion	269
7. General discussion	270
7.1. Summary of the work presented in this thesis	270
7.2. Potential effects of hypoxia on Sp family protein.....	271
7.3. Hypoxia responsive gene regulation.....	272
7.4. Significance of Sp transcription factors as a therapeutic target.....	276
7.5. Future directions.....	279

ABSTRACT

Cancer accounts for the highest amounts of disease related premature deaths worldwide with 8.1 million of deaths being associated with malignancy. In the cancer microenvironment, particularly in solid tumours, hypoxia plays a significant role in progression and metastasis by altering signal transduction and gene regulation which leads to aggressive phenotypes, poor prognosis and lower survival rates. Hypoxia-Inducible Factor (HIF) driven gene regulation is well established and believed to promote survival of tumour cells under the hypoxic microenvironment. Accumulating evidence also suggests that Specificity protein (Sp) family transcription factors might also play a role in the hypoxic microenvironment by regulating transcription of key hypoxia responsive genes such as *VEGFA* in either a HIF dependent or independent manner. In normal cells, Sp transcription factors are ubiquitously expressed and known to regulate numerous genes involved in vital cellular pathways such as cell cycle, apoptosis and angiogenesis. In tumour environments, deregulated Sp protein levels have been demonstrated and shown to correlate with poor prognosis and treatment response. However, the exact role of Sp transcription factors in hypoxic microenvironment is not fully understood.

This study aimed to identify the effect of severe (chronic) hypoxia on Sp transcription factors and hypoxia-responsive gene regulation using breast cancer as a cell model. Initial studies measured the expression levels and binding activity of Sp transcription factors. Subsequently, an integrative genomic analysis was performed to identify Sp driven hypoxia-responsive genes in breast cancer cells. The study was further extended to analyse the binding kinetics of Sp protein inhibitors using surface plasmon resonance spectroscopy. Finally, transcriptional changes of hypoxia responsive genes were examined after addition of Sp inhibitors or knockdown of *Sp1* level under the hypoxic environment.

Expression analysis of Sp family members (Sp1-4) showed that the transcript levels of Sp genes were unaffected due to chronic hypoxic exposure whilst Sp protein levels were induced in all three cell lines. However, expression patterns were dependent on tissue type and severity of hypoxia. Twenty genes were identified as potential Sp driven hypoxia responsive genes which consist of GC-rich putative Sp binding sites in their promoters. Gene expression analysis validated the hypoxic induction of these

genes and their dependency on Sp protein-mediated transcription. Affinity studies of Sp protein inhibitors prove binding of antibiotic derivatives, Mithramycin A and Chromomycin A to GC-rich regions with different binding affinities and kinetics. Different Equilibrium constants (K_d) of Mithramycin A and Chromomycin A were identified which varied according to the promoter sites (10^{-3} to 10^{-6} M range). Furthermore, novel data also confirm the Mithramycin-DNA interaction is independent of cation Mg^{2+} which has been considered obligatory for DNA interaction. Interestingly, nordihydroguaiaretic acid (NDGA) derivative, Terameprocol exhibits no detectable interaction with linear DNA consisting of Sp binding sites.

These results emphasise the importance of Sp proteins as regulators of hypoxia-mediated gene transcription. Sp-regulated transcription is vital in altering hypoxia-related cellular pathways and has a potential as biomarkers for solid tumours. Moreover, these results suggest the potential use of Sp antagonists to inhibit expression of key hypoxic genes in the cancer microenvironment. Results also provide solid background knowledge on pharmacokinetics of Sp inhibitors which will be useful in synthesis of new derivatives which can be used in novel therapeutic strategies for cancer and perhaps other diseases. Understanding the molecular mechanisms of Sp mediated hypoxic gene regulation can be further extended to elucidate other cellular stress and cellular adaptive mechanism.

Chapter 1.
Introduction

1. Introduction

1.1. Breast cancer

Cancer is responsible for more premature deaths worldwide than any other disease. In the UK alone, nearly 0.4 million new cancer cases and 0.2 million deaths were reported and recorded respectively in 2014 (Cancer Research UK). Breast cancer is the fourth most common form of cancer in the world whilst it remains as the most common cancer in women. In the United Kingdom, breast cancer contributes to nearly 30% of all types of cancers in women with 55,000 new cases identified in the year 2014 and also 11,433 recorded mortalities attributed to breast cancer (Cancer Research UK) (Department of Health, 2014). Whilst breast cancer is common in females, 390 cases of breast cancer in males were also recorded in the year 2014 in the UK (Cancer Research UK).

1.1.1. Risk factors

Despite the actual causes of breast cancer still being largely unknown, several risk factors have been identified which are associated with increased risk of developing a breast cancer in an individual. These include sex, age, genetics, family history, life style choices, endocrine and reproductive factors (McPherson et al., 2000). Breast tumours have been diagnosed in both men and women despite its prepotency in females. The lifetime risk of developing a breast cancer is 1 in 8 for women and 1 in 870 for men (Cancer Research UK). Age is also an important factor that contributes to breast cancer development where 80% of cases are recorded in women who are over the age of 50 years. Lifestyle choices and environmental factors also believed to have increased the risk of developing breast cancer. It is estimated that 27% of female breast cancer in the UK are linked with various lifestyle choices some of which are potentially avoidable such as obesity (9%), alcohol consumption (6%), physical inactivity (3%) and oestrogen exposure (Cancer Research UK) (Parkin & Boyd, 2011). In addition, oral contraceptives, ionisation radiations, and hormone replacement therapies are also identified as potential risk factors (Banks et al., 2003; Parkin & Darby, 2011). Family history is also an important factor to be considered in the breast cancer development where inherited genetic conditions play a vital role (The National Institute for Health and Care Excellence, August 2015).

1.1.2. Aetiology

The majority of breast cancers are identified as sporadic ($\approx 95\%$) whilst 5-10% of incidents are linked with pre-disposing genetic conditions (Hereditary breast cancers) such as gene mutations and DNA damage repair abnormalities (Couch et al., 2017). Since the discovery of the first high risk breast cancer gene Breast cancer susceptibility gene1 (*BRCA1*) in mid-1990 (Hall et al., 1990; Miki et al., 1994), several other genes which confers an increased risk of developing breast cancer have been identified and clinically utilized to assess the risk of developing a familial breast cancer (Walsh et al., 2006; Gage et al., 2012; Apostolou & Fostira, 2013; Couch et al., 2014). According to their relative risk of causing breast cancer, these genes have been categorised as high and moderate penetrance genes.

BRCA1 and *BRCA2* genes have been identified as two major genes that cause hereditary breast cancer (Miki et al., 1994; Wooster et al., 1995). Pathogenic mutations in *BRCA1* and *BRCA2* genes confer a lifetime risk of breast cancer of 60-85% in women (Evans et al., 2008). Mutations in *BRCA1* and *BRCA2* genes are often found throughout the coding sequence (with the majority of frameshift mutations) which leads to truncated proteins. In addition, mutations due to rearrangements (up to 27%) and missense mutations (2%) are also common which associate with higher risk of breast cancer (Hogervorst et al., 2003). Both *BRCA* gene products are involved in DNA repair mechanisms including homologous recombination, nucleotide excision repair and double strand break repair (Venkitaraman, 2001; Davis & Lin, 2011; Roy et al., 2012). Moreover, *BRCA1* and *BRCA2* genes have widely been used for genetic testing of breast cancer. However, the large number of unclassified gene sequence variants (not disease related) have limited the use of these genes in clinical aspects. Tumour suppressor gene *TP53* has also been identified as a high penetrance gene for breast cancers where somatic mutations of this gene are common in most solid tumours. Even though inherited germline mutations are rare, nearly 30% of carriers with inherited germline mutations (Li-Fraumeni syndrome) have developed an early onset of breast cancer before the age of 30. These mutations also increase the risk of developing an early onset breast cancer (before the age of 45) by up to 60-fold compared to non-carriers (Olivier et al., 2003; Gonzalez et al., 2009). Potential high-risk genes such as *PTEN* and *STK11* have also been identified. Similar to high

penetrance genes, germline mutations in *PTEN* (Cowden syndrome) and *STK11* (Peutz-Jeghers syndrome) genes also increase the lifetime risk of developing a breast cancer up to 50% (Li et al., 1997) (van Lier et al., 2010).

Moreover, moderate penetrance genes such as *CHEK2*, *PALB2*, *ATM* and *BARD1*, are also associated with an increased lifetime risk of breast cancer development (Thompson et al., 2005; Rahman et al., 2007; Casadei et al., 2011). Particular germline mutations of *CHEK2* gene such as *CHEK2* c.1100delC and *CHEK2* p.I157T increases the risk of developing both female and male breast cancer (Easton et al., 2004; Weischer et al., 2008; Cybulski et al., 2009). In addition to genes discussed in the previous section, more genetic variants that predispose to breast cancer have been identified where mutation of these genes may increase the risk of developing the disease (Couch et al., 2015). Details of these genes have been summarised in Table in 1.1.

1.1.3. Subtypes

Breast cancers can be classified into different subtypes based upon expression status of three hormone receptors; oestrogen receptor (ER+), progesterone receptors (PR+) and human epidermal growth factor (HER-2) (Prat et al., 2015). The majority of breast cancers exhibit ER+ or PR+ subtypes (nearly 70% of all breast cancer) where absence of any of these receptors are known as triple-negative breast cancer (TNBC) (Ovcaricek et al., 2011). This classification is widely used for therapeutic aspects. In addition, different treatment strategies and survival rates were also evaluated for these subtypes.

Each subtype exhibits specific tumour metabolism and sensitivity towards chemo and radiation therapies (Carey et al., 2007). In fact, the presence of these receptors facilitates different cellular pathways which lead to specific proliferation and metastasis potential of these subtypes. Therefore, identification of different receptor expression is vital in prediction of chemotherapy responses and treatment plans. For an example, oestrogen and progesterone are required for growth proliferation of ER+ and PR+ tumours (Heldring et al., 2007). Hence the use of hormone analogues (oestrogen and progesterone analogues) have been extensively efficient for inhibition of proliferation of ER+ and PR+ subtypes. On the other hand, endocrine therapies showed minimal effect on TNBC (Puhalla et al., 2012).

Table 1.1: High and moderate-risk genes predisposing to breast cancers

Gene	Chromosomal location	Neoplasm	Proportion of breast cancer (%)	Lifetime risk in women
Genes with high-penetrance mutations				
<i>BRCA1</i>	(17q12–21)	Female breast, ovarian cancer	1.5	40–80%
<i>BRCA2</i>	(13q12-13)	Male and female breast, ovarian, prostate, and pancreatic cancer	1.5	20–85%
<i>TP53</i>	(17p13.1)	Breast cancer, sarcomas, leukaemia, brain tumours, adrenocortical carcinoma, lung cancers	0.02	56–90%
<i>PTEN</i>	(10q23.3)	Breast, thyroid, endometrial cancer Other: benign hamartomas, macrocephaly	0.004	25–50%
<i>STK11</i>	(19p13.3)	Breast, ovarian, cervical, uterine, testicular, small bowel, and colon carcinoma	0.001	32–54%
Moderate-penetrance mutations				
<i>ATM</i>	(11q22.3)	Breast and ovarian cancers	0.5	15–20%
<i>CHEK2</i>	(22q12.1)	Breast, colorectal, ovarian, bladder cancers	0.5	25–37%
<i>PALB2</i>	(16p12.1)	Breast, pancreatic, ovarian cancer, male breast cancers	0.1	20–40%

Source: (Shiovitz & Korde, 2015)

This classification has also been used for evaluating survival rates of breast cancer patients (Onitilo et al., 2009; Haque et al., 2012). Patients with metastatic expression of ER/PR receptor status displayed better survival rates, whereas TNBC patients showed lowest survival rates compared to other subtypes (Lower et al., 2005; Onitilo et al., 2009). In addition, HER-2 negative phenotypes with single hormone receptors also demonstrated lower survival rates and aggressive behaviour similar to TNBC (Bae et al., 2015).

In addition, breast cancers can also be categorised depending on their size, invasive ability and proliferative stages. Four stages (stages I-IV) have been defined to grade breast tumours depending on size, number of lymph nodes and metastasis nature. Stages I-III are considered as primary cancers where stage IV is referred to as a secondary breast cancer. Survival rates are considerably decreasing with higher grades/stages and the sub type of the cancer. Stage IV TNBC shows the lowest survival rate compared to others types and stages (Polyak & Metzger Filho, 2012).

1.1.4. Diagnosis, treatment and survival

Similar to other cancer types, early diagnosis of breast cancer is vital for better survival rates of patients and treatment strategies. Symptoms of breast cancer include lumps, changes in the breast (size, shape, skin or feel), pain in the breast and fluid leaking from nipples. These symptoms are also found under other medical conditions, hence often linked with potential breast cancers (Cancer research UK, 2016). Breast cancers are typically diagnosed through a mammogram, MRI scan, breast ultrasound and various biopsy techniques such as needle and punch biopsies. Biopsy samples or surgical tissue samples are often analysed for receptor status in order to grade the stage of the tumour and assess the effective therapeutic strategy (NHS, 2016). Hormone receptors (PR, ER) and HER-2 protein levels are organised in order to determine effective treatment plans (Cancer Research UK, 2016). In England, the NHS offers a mammogram breast screening program for high risk age group (over 50s) every three years to improve early diagnosis of breast cancer. This screening program has been shown to reduce the number of mortalities associated with breast cancer in the UK by approximately 1300 deaths per year (NHS, 2016, Cancer Research UK, 2016). Treatment for breast cancer typically involves one or combination of surgical procedures like chemo, radiation, endocrine or biological therapies. The treatment

plan entirely depends on the type of the cancer, stage and size and metastasis status. In addition, patient's health conditions and age are also considerable factors for treatment choices. However, most treatments start with surgical interventions such as mastectomy or lumpectomy followed by chemo and radiation therapies. Survival rates of breast cancer patients have doubled over the past 40 years in the UK where around 65% of women diagnosed with breast cancer survive 20 years or more (Cancer research UK, 2011).

1.2. Tumour development

Cancer research is largely focused on elucidating the underlying mechanisms of tumour development, improving diagnosis and therapies. Mechanisms underlying tumorigenesis and metastasis are still not fully understood, due to the heterogeneity of different cancer types, it has been a complex and challenging aspect to generalise the exact mechanisms of tumour formation and progression. However, a review published by Hanahan & Weinberg has identified major events that contribute to cancer formation and progression which are known as cancer hallmarks (Hanahan & Weinberg, 2000; 2011). Key alterations that cause tumour progression include: Sustained proliferative signalling, evading growth suppressors, resisting to cell death, enabling replicative immortality, inducing angiogenesis, activation of invasion and metastasis, deregulation of cellular energetics, and evading immune destruction (See Figure 1.1). Even though the sequence of these events may vary in case of different types of tumour as suggested by the authors, these key stages have been used to identify underlying basal mechanisms of cancer.

Sustained proliferative signalling: Normal cells maintain homeostasis of cell number and tissue architecture by extensively regulated cell cycle and proliferative signalling. In cancer cells, growth promoting signalling cascade is deregulated enabling to sustain chronic proliferation (Hanahan & Weinberg, 2011; Feitelson et al., 2015). Phenotypic changes of cells are common in carcinogenesis which are acquired through alterations of various cellular pathways such as cell cycle regulation, autophagy and tumour microenvironment (Feitelson et al., 2015). Autophagy is widely involved in maintaining cell homeostasis by removing damaged cell components and contribute to tumour suppression.

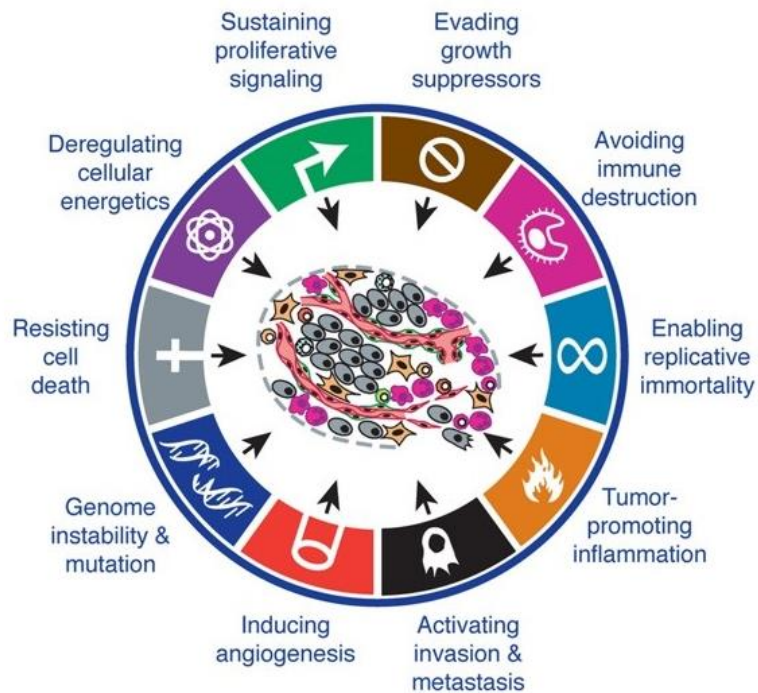


Figure 1.1: The hallmarks of cancer

Eight hallmarks and two enabling characteristics of cancer (Tumour-promoting inflammation and genome instability and mutations). Tumour growth and progression may acquire one or all of these essential pathways. Figure taken from (Hanahan & Weinberg, 2011).

However, impaired level of apoptosis and autophagy contribute sustained proliferation of tumours (Yang et al., 2011). Similarly, mitogenic signalling cascades are also altered in cancer cells which leads to the transformation of cells from a quiescent state to active state. Growth promoting signals mainly consist of growth factors which typically interact with tyrosine kinase receptors (Lemmon & Schlessinger, 2010). Cancer cells are capable of producing their own growth factors as well as various signalling molecules (receptor molecules) which can induce production of growth factors from other cells in tumour associated stroma (Gleave et al., 1993; Cheng et al., 2008; Witsch et al., 2010). In addition, cancer cells become hyper-responsive to mitogenic signalling due to the increased levels of growth factor receptor on their cell surface (Gullick, 1991). Similarly, certain somatic mutations may directly activate downstream signalling pathways (e.g. PI3K/AKT and Notch) and constitute growth signalling in cancer cells (Witsch et al., 2010). For instance, the B-Ras protein is mutated in 40% of human melanomas and is known to constitutively activate Mitogen-activated protein (MAP) kinase pathway (Davies & Samuels, 2010).

Evasion of growth suppression: Persistent cell proliferation is usually repressed by various tumour suppressor genes which are capable of inducing cell cycle arrest or apoptosis in normal cells (Larsson, 2011; Chan et al., 2014). Well studied tumour suppressors *TP53* and *RB* (retinoblastoma associated) play the central role in cellular proliferation and apoptotic pathways alongside large network of other downstream regulators (Sherr & McCormick, 2002; Burkhardt & Sage, 2008). Mutations of *TP53* and *RB* are frequently observed, which lead to the evasion of apoptosis induction as well as reducing contact inhibition (Hickman et al., 2002; Fry et al., 2017). In late stage tumours, TGF- β ; a well-known antiproliferative protein is capable of redirecting TGF- β signalling to activate tumour growth which is believed to be due to, associated mutation, elevated levels of TGF- inhibitors as well as activation of epithelial-to-mesenchymal transition (EMT) (Bhowmick et al., 2001; Ikushima & Miyazono, 2010; Principe et al., 2014; Syed, 2016).

Cell death resistance: Tumour cells employ various mechanisms to circumvent apoptosis by regulating pro and anti-apoptotic factors. Loss of TP53 function is common in most tumours which eliminate apoptosis inducing signalling mechanism (Soengas et al., 1999; Hickman et al., 2002; Lowe et al., 2004; Larsson, 2011). Similarly,

overexpression of anti-apoptotic factors such as Bcl-2 and Bcl-x_L or downregulation of pro-apoptotic factors (*Bax*, *Puma*) also increases resistance which ultimately leads to survival of cancer cells (Hemann et al., 2004; Paul-Samojedny et al., 2005; Adams & Cory, 2007; Singh et al., 2015).

Evasion of immune destruction: The role of the immune system in preventing early tumour formation and controlling progression of established tumours has been recently identified. Studies carried out using mouse models have shown that both innate and adaptive immune systems act as a significant barrier to tumour formation and progression. Mice immunodeficient in T cells and NK cells are susceptible to more frequent and rapid tumour formation compared to the immunocompetent controls (Kim et al., 2007; Teng et al., 2008). Cancer cells derived from immunodeficient mice also have a reduced ability to metastasise and initiate secondary tumour formation. In addition, studies carried out by Strauss and Thomas have shown that immunosuppressed organ transplant recipients are more susceptible to developing donor-derived cancers which implicate the role of immune system in preventing cancer development (Strauss & Thomas, 2010).

Replicative immortality: Normal cell lineages in the body are capable of undergoing a limited number of successive cell divisions and growth cycles. This process is believed to be regulated by telomeres (length of telomeric DNA) which dictate the number of cell cycles before the cell undergoes apoptosis and cellular senescence (Harley et al., 1990; Sherr & DePinho, 2000; Blasco, 2005). Telomeres are largely eroded in an irreversible manner due to absence of functional telomerase activity in normal cells. However, significant levels of telomerase are expressed in cancer cells enabling extension of telomeric DNA which leads to immortalise cells. Modifications of telomerase activity are also associated with poor prognosis and aggressive phenotypes (Akinçilar et al., 2016; Kubic et al., 2016).

Induction of angiogenesis: Adult vasculature is mostly quiescent except in physiological conditions such as wound healing and female reproductive cycle. During tumour progression, angiogenic switch is activated by several angiogenic factors enabling continuous vessel formation and sprouting (Hanahan & Folkman, 1996). Nevertheless, tumour neovasculature is aberrant due to precarious capillary sprouting, distorted and enlarged vessels, leakiness, erratic blood flow and excessive

branching (Dvorak et al., 1988; Brown & Giaccia, 1998; Nagy et al., 2010). This unorganised and ineffective nature of tumour vasculature leads to formation of hypoxic regions in tumour microenvironment which induces angiogenic switch as well as other hypoxia-related cellular pathways in tumours (Brown & Giaccia, 1998).

Invasion and metastasis activation: Cancer cells develop various mechanisms to activate invasion and metastasis by altering cell morphology, adhesion mechanisms and extracellular matrix (ECM) (Christofori, 2003; Wirtz et al., 2011). Cancer cells are known to acquire morphological transition through epithelial to mesenchymal transition (EMT) whilst losing their epithelial characteristics (e.g. intercellular junctions, apico-basal polarity) (Nistico et al., 2012). These changes are mainly governed by altering the expression of adhesion molecules. Genes which regulate cell adhesion to ECM are typically downregulated whilst cell migration factors are upregulated. Expression of cell adhesion molecule E-cadherin is reduced where, N-cadherin, an adhesion molecule associated with cell migration are upregulated in most tumours (Cavallaro & Christofori, 2004; Yang & Weinberg, 2008). This phenotypic changes also lead to degradation of ECM by secreting metalloproteins and reorganising the collagen network (Nistico et al., 2012). Mesenchymal phenotypes are highly flexible, motile and invasive in nature which are capable of invading the ECM and also the neighbouring tissues (Nistico et al., 2012).

Deregulation of cellular energetics: Cancer cells are capable of reprogramming their energy metabolism in order to provide the energy required for uncontrolled cell proliferation. Glycolytic metabolism is predominant in cancer cells despite the oxygenic state of the cells, and produces pyruvate and lactate as by-products (Warburg, 1956; Denko et al., 2000). Hence, glucose metabolism through glycolysis is 18-fold less efficient compared to the oxidative phosphorylation, cancer cells upregulate glucose transporters such as GLUT1 and other glycolysis enzymes in order to uptake and utilise more glucose (Mathupala et al., 2001; Jones & Thompson, 2009). Moreover, this metabolic switch facilitates diversion of glycolytic intermediates to different biosynthetic pathways which leads to rapid proliferation and assembling of new cells. Relying on glycolytic metabolism ensures ability to proliferate regardless of oxygen availability especially in tumour hypoxia which is common tumour microenvironment for most solid tumours (Taylor & Colgan, 2007; Tuder et al., 2007;

Grammas et al., 2011). Tumour hypoxia and Ras oncoproteins collectively increase expression of HIF transcription factors which ultimately upregulate glycolysis (Semenza et al., 1994; Dang & Semenza, 1999).

Tumour microenvironment: Progression of cancer is associated with multi-component approach where alteration of tissue microenvironment is predominant. Tumour microenvironment has been identified as one of the major contributors to tumorigenesis and metastasis (Hanahan & Weinberg, 2011). Understanding the mechanisms of different signalling cascades and interactions of malignant cells (with different cell types and surrounding environment) are essential when attempting to identify the contribution of tumour microenvironment.

The tumour microenvironment is highly complex due to presence of numerous different types of cell. Cell types including endothelial cells, cancer-associated fibroblasts, immune inflammatory cells and smooth muscle cells can be commonly found in the tumour microenvironment. These cells collectively regulate the localised tissues stroma and their signalling cascades. Using host tissue microenvironment and vasculature, cancer cells continuously activate different signalling cascades which allow them to progress into proliferation and metastasis (Mbeunkui & Johann, 2009; Mathias et al., 2013). For instance, endothelial cells (typically associated with angiogenesis) exhibit various signal transducing receptors which are involved in different signalling pathways such as VEGF, Angiopoetin, Notch and FGF signalling cascades (Dejana et al., 2009). Similarly, immune cells such as macrophage, lymphocytes (T and B) and neutrophils are also found in tumour stroma which contributes to tumorigenesis (Johansson et al., 2008). All of these cells collectively regulate tissue stroma under normal circumstances. However, under pathological or neoplastic conditions, signalling mechanism and cellular interactions are altered (Lorusso & Rugg, 2008).

Moreover, it has been shown that cancer cells are also capable of remodelling extracellular matrix (ECM) which is the first barrier for metastasis (Liotta & Kohn, 2001). In non-cancerous cells, interactions and signalling of cells are limited to specific tissues due to presence of ECM where altered ECM facilitates continuous progression and invasive abilities to cancer cells. In addition, various signalling molecules that are secreted by host cells in the tumour microenvironment including several growth

factors, cytokines, inflammatory factors, immune system suppressors and proteolytic enzymes such as metalloproteinases (MMPS) also contributes to the degradation of ECM leading to tumour metastasis (Mathias et al., 2013).

Physical and chemical components of the tumour microenvironment include different gas tensions such as O₂ concentrations, pH levels, acidosis, and extracellular matrix (Lorusso & Rugg, 2008). Both physical and chemical components are also maintained at homeostatic levels despite neoplastic conditions such as tumorigenesis, cause disruption of these regulation mechanisms due to altered signalling mechanisms and pathways. These modifications lead to the physical and chemical changes in the tumour stroma such as lowering pH and formation of tumour hypoxia (Lorusso & Rugg, 2008).

1.3. Tumour Hypoxia

Breast cancer is considered as one of the most hypoxic and metastatic cancer types compared to other types such as lung and bone cancer (Bennewith & Dedhar, 2011; McKeown, 2014). Hypoxic tumour microenvironment leads to a poor prognosis of tumours which ultimately results in poor clinical outcome (Chia et al., 2000; Yoshimura et al., 2004). In general, oxygen tension in tissues varies among different organs and infrequently exceeds 10% O₂ (Ivanovic, 2009; Hancock et al., 2015). Oxygen availability depends on tissue type and usually decreases with the proximity where the majority of O₂ being utilised within 150 µm distance of the blood vessels (Thomlinson & Gray, 1955; Rademakers et al., 2008). This leads to the formation of low-oxygen areas known as hypoxic regions in tissues (Vaupel & Mayer, 2007; McKeown, 2014). Oxygen tensions in hypoxic regions usually lie below 2.0% and may decrease up to 0.02% O₂ (anoxic levels) (Thomlinson & Gray, 1955; Bennewith & Dedhar, 2011; Strese et al., 2013). Different terms have been used to define hypoxic conditions such as severe and mild hypoxia. However, based on the duration of hypoxia and origin, it can be classified as acute, chronic and anaemic hypoxia. Low oxygen conditions that last for short period of time are referred to as acute hypoxia whereas chronic hypoxia results due to the prolonged periods. Similarly, low blood oxygen conditions in blood veins are described as anaemic hypoxia which is common due to therapeutic interventions (Vaupel & Harrison, 2004).

In the tumour microenvironment, formation of hypoxic regions is a common phenomenon due to structural abnormalities of tumour vasculature and high demand for oxygen. Rapidly growing tumours are usually associated with non-uniform abnormal vasculature (Brown & Giaccia, 1998; Brown & William, 2004) which consist of irregular, leaky vasculature leading to poor perfusion of nutrients and oxygen supply resulting in low oxygen level regions known as tumour hypoxia (Vaupel & Mayer, 2007; McKeown, 2014). This is a prevalent condition in most of the solid-state tumours such as lung, colon and breast tumours. However, the level of hypoxia and extent of hypoxic tumour regions vastly depend on tissue type and characteristics of the tumour where some tumours may contain up to 50% hypoxia of tumour mass. Nevertheless, the oxygen concentration of malignant tissue/cells is also significantly lower compared to the normal tissues (Brown & William, 2004). For example, tumour oxygenation measured using polarographic O₂ needle electrodes have shown that in breast cancer; one of the most hypoxic tumours, tumour pO₂ (oxygen partial pressure) of malignant cells are 10.0 mmHg whereas normal cells (non-cancerous breast tissue) exhibit around 55-65 mmHg (Vaupel et al., 1991; Hohenberger et al., 1998). This was also observed in other tumour types (lung cancer: tumour pO₂ 7.5 mmHg, normal lung tissue 38.5 mmHg, cervical cancer; tumour pO₂ 5.0 mmHg, normal cervical tissue 51.0 mmHg. (Lyng et al., 1997; Vaupel et al., 2002; Brown & William, 2004).

Tumour hypoxia is a vital concept in therapeutic aspects of solid tumours especially in hypoxic tumours such as breast cancer. The hypoxic microenvironment often induces a plethora of changes within the tumours and their behaviour, which are correlated with aggressive phenotypes, increased metastases and lower survival rates (Hockel et al., 1999; Hockel & Vaupel, 2001a; 2001b; Rademakers et al., 2008; Yamamoto et al., 2008; Semenza, 2012b). Furthermore, hypoxic tumours become less sensitivity to chemo and radiotherapy strategies due to induction of hypoxia-related alterations in the tumour microenvironment (Harrison et al., 2002; Shannon et al., 2003; Cosse & Michiels, 2008; Harada, 2011; Strese et al., 2013). One of the most striking observations is the reduction of apoptosis potential in tumours due to the hypoxic environment (Finger & Giaccia, 2010). Contradictory to this observation, several HIF (Hypoxia-inducible factor) dependent and independent signal cascades which are known to induce apoptosis are also activated during hypoxia (Greijer & van der Wall,

2004). However, this induction is counteracted by the expression of anti-apoptotic factors during hypoxia conferring greater tumour resistance. Hence cells are capable of surviving under adverse conditions and tumour hypoxia is associated with higher rates of metastasis (Hockel et al., 1999; Cosse & Michiels, 2008). Studies have also suggested that the irregular nature of tumour vasculature results in poor diffusion of chemotherapeutic agents which in turns fail to reach its target locations (Brown & Giaccia, 1998; Brown & William, 2004). Furthermore, overexpression of some membrane transporters also facilitates outward transportation of drugs which makes the use of chemotherapeutic strategies more challenging (Fletcher et al., 2010). Glycolysis is the main source of energy production in hypoxic conditions and glycolytic products mainly contribute to lowering pH and acidification. Acidification due to hypoxia changes the chemical dynamics of the tumour microenvironment. For example, pH changes may reduce the efficiency of chemo agents by altering their molecular conformations and binding activities (Yamagata et al., 1998; Wykoff et al., 2000; Santi et al., 2013). Genomic instability and DNA repair deficiencies are common in tumours which lead to development of resistance towards chemotherapeutics (Bristow & Hill, 2008; Pires et al., 2010; Bouwman & Jonkers, 2012). Moreover, tumour cells exhibit lower levels of proliferation due to hypoxia and cells are usually in a dormant non-proliferative state (Cosse & Michiels, 2008). Therefore, hypoxic conditions can affect the efficiency of the majority of chemotherapeutics which are designed to target rapidly proliferative cells (Cosse & Michiels, 2008). Similarly, radiotherapy applications are also inefficient in hypoxic tumours compared to normoxic conditions (Harrison et al., 2002). It has been shown that presence of lower number of oxygen molecules vastly decreases the radiosensitivity of tumour cells. Since ionisation by radio ions require oxygen molecules it was shown that higher doses of radiations are required to induce tumour DNA damages (Moeller et al., 2005; Rockwell et al., 2009; McKeown, 2014). A study carried out by Vaupel and colleagues have shown that nearly 40% of breast malignancies exhibited hypoxic regions with lower oxygen concentrations required for half-maximum radio sensitivity ($pO_2 < 2.5$ mmHg) (Vaupel et al., 1991).

As mentioned previously, the success of chemotherapy and radiotherapy outcomes largely depends on oxygen tensions in tumour tissues. These challenges have

highlighted the importance of the tumour hypoxia related cellular pathways and signalling cascades in tumour progression and metastasis. Hypoxia induces a range of biological processes and signalling cascades such as proliferation, angiogenesis, metabolism, apoptosis, immortalisation and metastasis. Tumours are capable of using these induction mechanisms along with other oncogenic mechanisms to survive and proliferate in the hypoxic microenvironment. It is well established that Hypoxia-inducible factors (HIF-1 and HIF-2) which are often overexpressed in tumours, play a central role in hypoxia-induced cellular mechanism (Semenza, 2000; Semenza, 2004). Therefore, it is vital to understand the mechanisms of these key transcriptional modulators in cellular/tissue hypoxia.

1.3.1. Hypoxia inducible factors (HIFs)

The key modulator of hypoxia induction, the hypoxia-inducible factor (HIF) is an oxygen-sensitive transcription factor that regulates most of the hypoxia-responsive genes in tumour microenvironment (Liu et al., 2012). HIF is a heterodimer consisting of two subunits, HIF- α and aryl hydrocarbon receptor nuclear translocator (ARNT) (also known as HIF- β). HIF- α subunit is approximately 120 kDa in size and the ARNT subunit is approximately 90 kDa in size where both *HIF- α* and *HIF- β* are constitutively expressed at mRNA levels. However, only HIF- β protein is constitutively expressed in the cytoplasm under both normoxic and hypoxic conditions where it is only activated during the hypoxic condition (Wang et al., 1995; Wang & Semenza, 1995). On the other hand, HIF-1 α protein is stable only in hypoxic condition and stabilisation is predominantly regulated by posttranslational modifications such as hydroxylation and ubiquitination.

HIF- α protein has three family members known as HIF-1 α , HIF-2 α , and HIF-3 α . HIF-1 α was first identified as a transcription regulator of the human erythropoietin gene (Semenza & Wang, 1992). Subsequently, its transcriptional activity in glycolysis was also highlighted (Semenza et al., 1994). Both HIF-1 α and HIF-2 α are known to behave in a similar manner in gene regulation whereas HIF-3 α is believed to repress the HIF driven transcriptional pathways. The functions of HIF-1 α and HIF-2 α were initially investigated in embryonic development using HIF null mice models (HIF-1 $\alpha^{-/-}$, HIF-2 $\alpha^{-/-}$). Knockdown of HIF resulted in lethal abnormalities and defects in embryonic development such as aberrant vasculogenesis (Iyer et al., 1998; Peng et al., 2000). Both

HIF-1 α and HIF-2 α exhibited different phenotypes suggesting their distinct roles in embryonic development and hypoxic induction (Loboda et al., 2010; Florczyk et al., 2011). However, both HIF-1 α and HIF-2 α share structural similarity between them whilst sharing 48% homology of amino acid sequence (Tian et al., 1997). Basic helix loop helix (bHLH) domain (constitute of HIF- α DNA binding domain) and two PER-ARNT-Sim (PAS) domains can be found in N-terminal region of both HIF- α and HIF- β (see Figure 1.2) (Wang & Semenza, 1993). PAS domain facilitates protein-protein interactions which is used for dimerisation of HIF- α and HIF- β (recognised by HIF-1 β) (Jiang et al., 1996). In addition, Transactivation domains (TAD) can be found on both C and N terminals which are linked by an inhibitory domain (ID). Contrastingly HIF-1 β does not contain any transactivation domains (Jiang et al., 1997; Pugh et al., 1997). Both HIF-1 α and HIF-2 α constitute the oxygen-dependent degradation domains (ODDD) which are necessary for stability of HIF proteins in normoxic and hypoxic conditions (undergo post-translationally modifications in hypoxia) (Huang et al., 1998). Moreover, these domains are highly conserved between HIF-1 and HIF-2 particularly bHLH domain (nearly 85%). In addition, some key amino acids which are required for post translation modifications of HIF- α proteins are shared between them, explaining their similarities in their regulatory mechanisms (Loboda et al., 2010). Nevertheless, TAD domains of HIF-1 and HIF-2 have some heterogeneity leading to differential roles of each protein (Keith et al., 2012). It is believed that these differences in TAD domains confer interactions with specific gene targets of HIFs (Hu et al., 2007). As a result, some genes are specifically regulated by either HIF-1 or HIF-2 proteins despite the majority of hypoxic gene are regulated by both HIF-1 and HIF-2 where. For an example, some glycolytic genes are only regulated by HIF-1 α protein (Hu et al., 2003).

Distinct roles of HIF- α have been demonstrated in different cell types and oxygen tensions (Ratcliffe, 2007). A study carried by Bracken and colleagues highlighted the cell specific differential regulation patterns of HIF-1 and HIF-2 in six different cell types (Bracken et al., 2006). HIF-1 α mediated regulation is prominent in early hypoxic responses under severe or chronic hypoxia where protein is believed to be induced as soon after the hypoxic exposure.

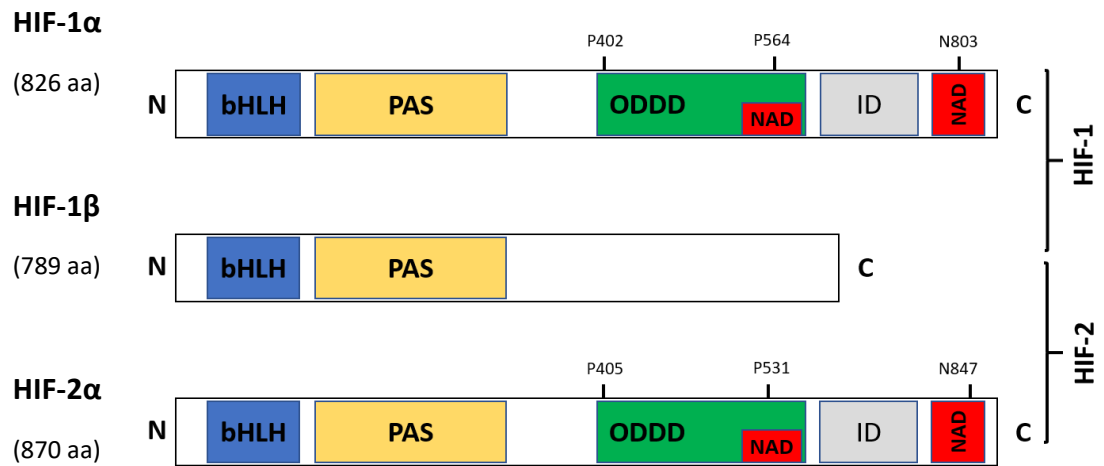


Figure 1.2: Diagram of HIF-1 α , HIF-2 α and HIF-1 β domain structures

HIF subunits contain basic helix loop helix (bHLH) domains, PER-ARNT-SIM (PAS) domains (DNA binding domains). Only HIF-1 α and HIF-2 α contain oxygen-dependent degradation domains (ODDD) and transactivation domains (TAD) which are linked by Inhibitory domain (ID).

Optimal activity of HIF-1 α is observed at 0.5% O₂ where it gradually decreases in higher oxygen tensions (Bertout et al., 2008). On the other hand, HIF-2 α is induced in moderate hypoxia (oxygen tensions below 10%) suggesting its potential role in physiological normoxia (Holmquist-Mengelbier et al., 2006). In addition, some studies have also shown that HIF-2 α upregulation tend to last longer (prolonged hypoxia) compared to the HIF-1 (Wiesener et al., 2002; Koh & Powis, 2012).

ARNT (HIF- β) was first identified as a complex with arylhydrocarbon receptor and HIF- α interaction and transcription mechanisms were established later (Wood et al., 1996). Both HIF-1 α and HIF-2 α protein, interact with ARNT at PAS domains to form heterodimer protein complex in hypoxia. These protein complexes then translocate to the nucleus where in turn regulate the transcription of hypoxia responsive genes. Two other family members, known as ARNT2 and ARNT3 were also discovered later which are structurally homologous to ARNT despite their functional activities (Jain et al., 1998). However, the functional role of ARNT2 and ARNT3 is poorly understood in hypoxia. ARNT2 is known to be involved in the neural development whereas ARNT3 (BMAL) is involved in circadian rhythms (Takahata et al., 1998).

However, ARNT2 interaction with HIF-1 α and HIF-2 α has also been reported previously. In addition, using MCF-7 cells, Qin et al., have suggested a possible role of ARNT2 in HIF mediated gene signalling and metabolism (Qin et al., 2011).

1.3.2. Hypoxic induction

HIF molecules are capable of sensing hypoxia and act as central modulators of all hypoxia signalling cascades. Under the normoxic environment, proline residues of HIF- α subunit (Pro402 and Pro564 of oxygen depended degradation domain (ODDD) are subject to hydroxylation by prolyl hydroxylases (PHDs) (Jaakkola et al., 2001; Masson et al., 2001). Three prolyl hydroxylases have been identified till date. These enzymes require O₂, Fe²⁺ and 2-oxoglutarate for their enzymatic activity (Bruick & McKnight, 2001). Hydroxylation of HIF- α subunits facilitates direct interaction with pVHL protein (von Hippel-Lindau protein) which is capable of ubiquitinating HIF- α subunit via E3 ligase activity (Maxwell et al., 1999; Ivan et al., 2001; Yu et al., 2001). Furthermore, a study carried out by Jeong et al., in 2002 has shown that acetylation of HIF-1 α at Lys532 by acetyl transferase ARD1 also increases the VHL binding (Jeong et al., 2002). The ubiquitinated product is identified by the proteasome and subsequently degraded

by proteolytic activity (Maxwell et al., 1999). Enzymatic activity of PHDs is significantly decreased in absence or low tension of O₂ (below 5% O₂) which decreases hydroxylation of HIF- α (Bruick & McKnight, 2001). This leads to the stabilisation of HIF- α by preventing proteasomal degradation (Schofield & Ratcliffe, 2004). Unhydroxylated HIF- α subunit forms a heterodimer with constitutively expressed HIF- β subunits. Studies have shown that HIF- α possessed nuclear localisation signals in N-terminus which allows translocation of HIF subunits to the nucleus (Chilov et al., 1999). Co-factors, p300 and CBP (Histone acetyltransferases) directly interact with HIF- α at TAD domains in the nucleus (Arany et al., 1994; Arany et al., 1996). This interaction is also tightly regulated by post-translational modification at asparagine residue 803/851 of HIF-1 α and HIF-2 α respectively. Hydroxylation also prevents interaction of these cofactors in normoxic conditions (Lisy & Peet, 2008) (see Figure 1.3). In addition, two cellular kinase pathways are known to regulate HIF by posttranslational modifications. Mitogen-activated protein kinases (MAPKs) phosphorylate HIF-1 α in hypoxic conditions which increases the stability of HIF subunits (Hahm et al., 2014). Similarly, phosphoinositide-3-kinase (PI3K) phosphorylates HIF-1 α increasing their transcriptional activity and stability (Richard et al., 1999). However, the effect of phosphorylation of HIF- α subunits on transcriptional activity is not entirely understood. This HIF-ARNT complex directly associates with hypoxia response elements (HRE) in gene promoter sites of HIF targets.

1.3.3. HIF mediated gene transcription

HIF transcription factors recognise conserved DNA consensus region, known as hypoxia responsive element (HRE) which are located upstream of gene promoter of target genes. The transcriptional mechanism is carried out by direct interaction with the DNA promoter region facilitated by p300 and CREB binding proteins (CBP) (Arany et al., 1996). The consensus sequence of HRE site has been identified as A/GCGTG (Semenza et al., 1996). Variants of HRE regions were also reported with larger motifs (Wenger et al., 2005). A vast array of HIF mediated gene targets have been identified which are widely spread among various cellular pathways such as angiogenesis, glucose metabolism, growth proliferation and apoptosis.

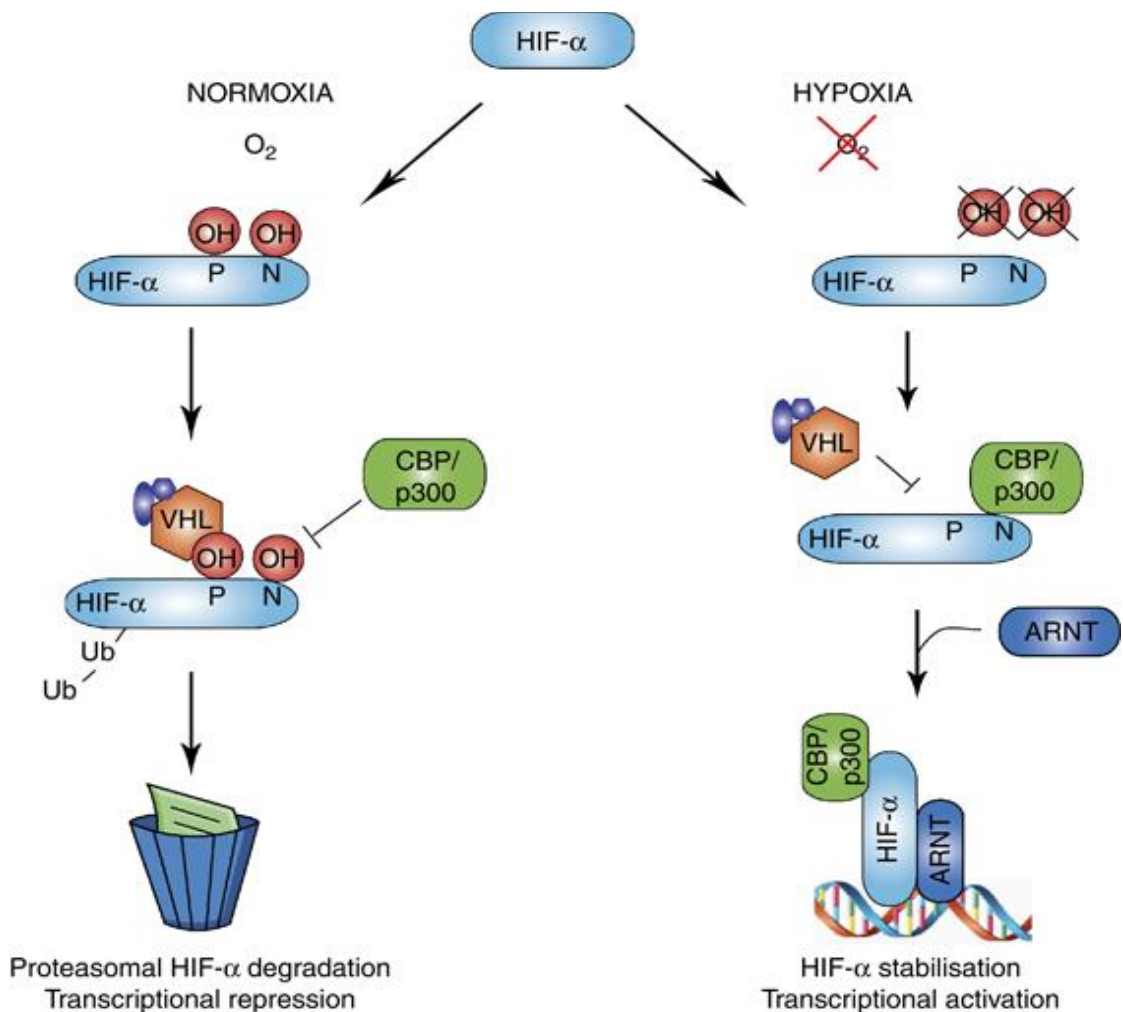


Figure 1.3: Oxygen dependent HIF regulation

In normoxia, HIF-1 α is hydroxylated by proline hydroxylases (PHD1, 2 and 3) in the presence of O_2 , Fe^{2+} , 2-oxoglutarate (2-OG) and ascorbate. Hydroxylated HIF-1 α is recognised by VHL protein which ubiquitinates HIF-1 α allowing recognition by the proteasome and subsequent degradation. Acetylation of HIF-1 α also promotes pVHL binding. In response to hypoxia, proline hydroxylation is inhibited. VHL is no longer able to bind and target HIF-1 α for proteasomal degradation, which leads to HIF-1 α accumulation and translocation to the nucleus. In the nucleus, HIF-1 α dimerises with HIF-1 β , binds to hypoxia-response elements (HREs) within the promoters of target genes and recruits transcriptional co-activators such as p300/CBP for full transcriptional activity. Abbreviation: CBP, CRB binding protein; Ub, ubiquitin. Figure taken from (Martin et al., 2011)

Angiogenesis is one of the key cellular pathways induced in hypoxic microenvironments. HIF proteins play a central part in angiogenesis by regulating the transcription of key regulator of vasculature; *VEGF*. HIF driven VEGF transcription is well established and exploited in several cancer cell lines (Ferrara, 2009). In addition, the *EPO* gene which encodes for erythropoietin is also mediated by HIF regulation (Wang & Semenza, 1993). Erythropoietin is an essential component for the formation of erythrocytes.

Glycolytic metabolism is predominant in hypoxic environment and is the primary mechanism for ATP production in hypoxic cancer cells (Dang & Semenza, 1999). Cancer cells are capable of switching aerobic metabolism to anaerobic metabolism which is also known as the Warburg effect (Warburg, 1956). Transcription of glycolytic enzymes as well as glucose transporters is mainly regulated by HIF-mediated gene transcription. Expression of glucose uptake transporters such as *GLUT1* and *GLUT3* (*SLC2A1* gene) are also regulated by HIF-1 mediated transcription (Chen et al., 2001a).

Furthermore several other hypoxia responsive genes including Aldolase A (ALDOA), Carbonic anhydrase 9,12 (CA9, CA12), Lactate dehydrogenase (LDH-A), Hexokinase-2 (HK2) are also regulated by HIF-1 transcription factors (Semenza et al., 1994; Dang & Semenza, 1999; Mathupala et al., 2001; Wykoff et al., 2001) (Koukourakis et al., 2005). Lactic acid formation as a by-product of anaerobic glycolysis also leads to the decrease in pH which is a common phenomenon observed due to tumour hypoxia (Yamagata et al., 1998; Chia et al., 2000).

Hypoxia promotes growth and proliferation by inducing expression of several growth factors which are also involved in tumour proliferation and metastasis. Studies have shown that transforming growth factor β (TGF- β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) are all induced in hypoxia (Ryuto et al., 1996; Kieran et al., 2012). Studies have also shown that activity of several signalling cascade kinases such as phosphatidylinositol-2-OH kinases (PI3K) and mitogen-activated protein kinases (MAPKs) are also increased due to hypoxic response in a HIF dependent manner (Richard et al., 1999) (Chen et al., 2001b).

Induction of apoptosis is another major process by which cells response to hypoxic environment. Hypoxia-induced apoptosis and its downstream protein production is mainly regulated by HIF-mediated genes. HIF complex is known to activate

transcription of pro-apoptotic genes while inhibiting anti-apoptotic genes. HIF mediated transcriptional activation of pro apoptotic genes such as *BAX* and *BAD* genes ultimately lead to activation of apoptosis by releasing caspases (caspase 3 and 9) and cytochrome c respectively. In addition, HIF-1 α participates in p53 mediated apoptosis by direct interaction with the dephosphorylated form of HIF-1 α (Suzuki et al., 2001). On the other hand, the hypoxic response may downregulate the anti-apoptotic gene such as *BCL-2* in cells (Suzuki et al., 2001).

1.3.4. HIF-independent gene regulation in hypoxia

HIF driven transcriptional regulation is well established and plays a central role in the hypoxic response. However recent gene array studies have revealed that presence of large cohort of hypoxia induced genes which are alternatively regulated in HIF-independent manner. Even though underlying mechanisms are still yet to be validated, studies have shown that transcription mediated regulation is responsible for these inductions. Furthermore, some of the HIF-mediated hypoxia responsive genes were also alternatively regulated by these transcription factors emphasising their importance in hypoxia. For example, several studies have shown that the **Nuclear factor kappa-B (NF κ B)** mediated regulation of hypoxia-induced genes includes, cyclooxygenase-2 (*COX-2*), Tumour necrosis factor α (*TNF- α*) and interleukin-6 (*IL-6*) (Schmedtje et al., 1997; Leeper-Woodford & Detmer, 1999; Matsui et al., 1999; Chandel et al., 2000a). Interestingly it has also been suggested that there is a potential role of NF κ B in regulation of HIF expression as well (van Uden et al., 2008). Similarly, hypoxia is known to induce **Activating protein 1 (Ap-1)** transcription factor which in turn regulates genes involved in tumour angiogenesis and invasion such as tyrosine hydroxylase and endothelial NOS (*eNOS*) in collaboration with of one or more other transcription factors like HIF-1 α (Millhorn et al., 1997; Hoffmann et al., 2001).

In addition, **Specificity protein (Sp)** transcription factors are also involved in transcription of hypoxia responsive genes in HIF dependent/independent manner suggesting its potential role in hypoxic mechanisms.

1.4. The Sp transcription factor family

Sp protein transcription factors are DNA-binding nuclear proteins which are known to interact with GC-rich gene promoter sequences (Lania et al., 1997). The presence of at least 12,000 Sp binding sites within the human genome emphasises the importance of Sp family proteins in transcriptional regulation. In addition to their basal transcription role, Sp family members are known to play essential roles in various cellular mechanisms in response to different physiological conditions (Oleaga et al., 2012). Sp transcription factors are involved in several key cellular pathways such as cell cycle, proliferation, apoptosis and DNA damage repair mechanisms (Black et al., 2001; Grinstein et al., 2002; Deniaud et al., 2006; Wang et al., 2008; Deniaud et al., 2009b). In addition, Sp mediated gene regulation is highlighted in invasion and metastasis of most solid tumours including breast cancer (Wang et al., 2008; Kajita et al., 2013). In general, Sp proteins are aberrantly expressed in most tumours nevertheless, the expression patterns vary greatly among different tumour types (Safe & Abdelrahim, 2005; Hsu et al., 2012; Sankpal et al., 2012; Safe et al., 2014). Expression levels of Sp proteins are strongly correlated with tumour progression and survival rates of several cancers. It was also noted that Sp protein levels are related with cancer type, stage, invasive and metastatic potential. Overexpression of Sp proteins is considered to be a negative prognostic factor and usually associated with poor survival rates and prognosis of several cancers such as lung, breast, pancreatic and gastric cancers (Wright et al., 1987; Wang et al., 2003; Yao et al., 2004; Yuan et al., 2007; Hsu et al., 2012; Sankpal et al., 2012; Jiang et al., 2015). It has been shown that knocking down or inhibiting the Sp proteins decreases the tumour formation, proliferation and metastasis of several cancer cell lines (Jiang et al., 2004; Yuan et al., 2007; Previdi et al., 2010). In contrast to other cancer types, the role of Sp protein in breast cancer is still controversial. Several studies have suggested that Sp1 is overexpressed in breast cancer patients where silencing of *Sp1* gene is also observed in more advanced breast cancer (Li et al., 2016).

1.4.1. Functional and structural characteristics of Sp proteins

Specificity protein (Sp) family is a subgroup of Sp/KLF (Krüppel-like factors) family characterised by Cys2His2 zinc fingers which form the DNA binding domains of these factors. Additionally, both Sp and KLF transcription factors recognise GC (GGGGCGGGG) and GT boxes (GGTGTGGGGG) sequences for DNA binding (Briggs et al., 1986). However, Sp family members exhibit different specificity and binding affinities compared to KLF family due to conserved amino acids in the zinc finger.

Sp1 was the first member of the Sp transcription factor family to be characterised and identified to interact with the SV40 early promoter (Dyran & Tjian, 1983). The Sp family now consists of 9 genes (Sp1-9), each located adjacent to a *HOX* gene cluster (Philipsen & Suske, 1999). Sp family members can be separated into two different subgroups depending on their domain structures. Sp1-4 shares more similarity in their domain structures whereas Sp5-9 are more similar in structure to Krüppel-like factors. The Zinc finger, DNA binding domains of Sp1-4 are highly homologues and preferentially binds to GC boxes (Nagaoka et al., 2001). However, binding affinities are greatly varying among each protein. For an instance, each of the three zinc fingers of Sp1 protein have different affinities to GC sequences suggesting that, each zinc finger may have different preferences and binding affinities towards promoter sites (Narayan et al., 1997). Furthermore, it has been implicated that these zinc fingers are also essential for nuclear localisation in addition to DNA binding function (Ito et al., 2009; Ito et al., 2010).

The Sp1-4 subset has two glutamine-rich transactivation domains in N-terminal (Domain A and B) except for Sp2 which has only one transactivation domain. Transactivation domains play a major role in transcription activity (see Figure 1.4). These domains have significantly less homology (despite same number of amino acids) which may facilitate different functions and specificities of each protein. These transactivation domains are also associated with adjacent serine/threonine-rich regions which believed to be targeted for post-translational modifications and therefore suggests post translational modifications may regulate their activity (Courey & Tjian, 1988). Domain C, which is referred to as a highly charged region is also shared with Sp1-Sp4 subfamily. This region lies next to transactivation domains towards

C-terminal suggesting it may support DNA binding and transactivation (Courey & Tjian, 1988).

In contrast to other members, domain D of Sp1 protein has the ability of binding to multiple sites due to the presence of multi-dimerisation domain (Mastrangelo et al., 1991). Moreover, Sp3 protein contains an inhibitory domain located in N-terminus which enhances its activity as a transcriptional inhibitor (Dennig et al., 1996). Sp1 and Sp3 proteins are also similar in nature where both proteins exhibit equal affinity for GC box binding. However, genes with multiple Sp1 binding sites may require multimerisation of Sp1 proteins in transactivation. A study carried out with human topoisomerase II promoter have demonstrated that multiple binding of Sp1 at distal and proximal sites activate transcription. However, competition between Sp1 and Sp3 may lead to repression of the transcription activity (Williams et al., 2007).

In addition to the main domain structures, other conserved regions/domains have also been identified which are dedicated for specific cellular signalling and interactions. In the year 2000, a study carried out by Harrison et al., have shown two conserved stretches namely Buttonhead box (Btd) (11 amino acid region) and Sp box (Harrison et al., 2000). Even though the exact functions of these domains are not fully understood it is believed that they are possibly involved in transactivation (Murata et al., 1994; Harrison et al., 2000).

Sp5-8 proteins share similarities in C-terminal regions most noticeably in DNA binding domains. However, N-terminal regions are very different to those of Sp1-4. Proline-rich regions dominate the N terminal portion of Sp5-8 despite the lack of details that are available about this subgroup in terms of biochemical properties. Schematic representation of different domains of Sp family proteins can be found in Figure 1.4.

1.4.1.1. *Sp1*

Sp1 protein is considered as a prototype of the subfamily. The human *Sp1* gene is located on chromosome 12q13 which encodes a 785 amino acid protein (approximately 81 kDa) with few splice variants (Gaynor et al., 1993). Sp1 protein has been extensively studied compared to other members of the family. Marin et al. have demonstrated that Sp1 is vital in early development using knockout experiments (Marin et al., 1997) as Sp null mice did not survive or showed slow growth development and broad range of abnormalities (Kruger et al., 2007). In addition,

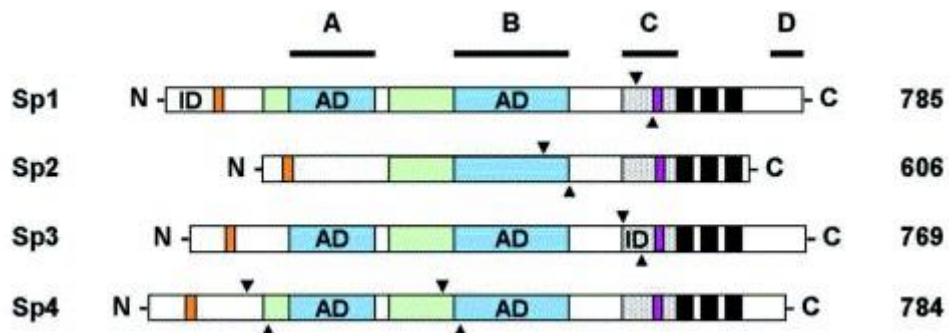
involvement of Sp1 protein has been extensively studied in transcriptional regulation in basal transcription mechanism and expression of housekeeping genes. The role of Sp1 protein in cancer progressions will be discussed later in this chapter.

1.4.1.2. *Sp2*

Sp2 gene is located on chromosome 17q21.3-q22 which encodes 613aa protein (Schohy et al., 1998). Sp2 protein is approximately 65 KDa in size and exhibit different splice variants. Sp2 protein is differently expressed in various tissues and is different from other sub family members due to absence of transactivation domain (Kingsley & Winoto, 1992). Earlier, it was believed that Sp2 transcription factors are unable to interact with GC boxes, but hinder transcription activity. However, recent studies have shown that human ROR γ T and Cd14 promoters are regulated by Sp2 protein suggesting its transcriptional regulation (Ratajewski et al., 2016; Zschemisch et al., 2016).

1.4.1.3. *Sp3*

Sp3 exhibits the most structural and functional homology to Sp1 protein. Sp3 protein is approximately 82 KDa in size where three of its isoforms have been identified (781 aa- 82KDa, 738aa- 78KDa, 713aa- 75KDa). The human *Sp3* gene is located in chromosome 2q31 (KalffSuske et al., 1996). Sp3 also plays a vital role during early development and knockout experiments identified lethal embryonic abnormalities (Bouwman et al., 2000; Gollner et al., 2001b; Kruger et al., 2007). It has also been reported that transactivation activity of Sp3 is weaker compared to that of Sp1. Sp3 can act as a gene repressor or activator depending on gene promoter structure (Hagen et al., 1994; Li & Kellems, 2003; Zhao et al., 2012). Sp3 repression activity can be found where Sp1 multimerisation is prominent. In addition, it has also been reported that acetylation of Sp3 protein plays a vital role in transcription activation (Braun et al., 2001). Several mammalian genes have been reported to be regulated by Sp3 proteins such as metastasis marker of sarcoma *AFAP1L1* gene (Kajita et al., 2013).



■ Sp box
 ■ S/T-rich region
 ■ Q-rich region
 ■ Highly charged region
■ Btd box
 ■ Zinc finger
 ■ P-rich region
 ▼▲ potential PEST sequence

Figure 1.4: Diagram of Structural motifs in Sp factors.

Structural domains of Sp1-Sp8. Sp and buttonhead (Btd) boxes, serine/threonine-rich, glutamine-rich and highly charged regions, and zinc fingers are indicated, as well as activation (AD) and inhibitory (ID) domains. A, B, C, and D domains of Sp1 are marked with black bars. Each pair of arrow heads points at potential PEST domains. On the right: lengths in amino acids according to accession numbers P08047 (Sp1), M97190 (Sp2), CAC34575 and Q02447 (Sp3), CAA48563 (Sp4). Figure taken from (Bouwman & Philipsen, 2002).

1.4.1.4. Sp4

The *Sp4* gene is located in chromosome 7p15.3-p21 and encodes a 784 aa protein (approximately 82 KDa) (Kalffsuske et al., 1995). Similar to Sp1 and Sp3 proteins, expression of Sp4 is essential for growth and development of the embryo (Supp et al., 1996). Expression of Sp4 is limited to tissue-specific pattern where higher levels can be found in brain and central nervous system. Due to similar domain structures Sp4 shares similar transactivation potential compared to the other sub family members. However, transactivation is reported to be more additive (work alongside with other TFs) in nature. Several gene promoters are reported to be activated by Sp4 mediated transcription such as *GluA2* gene in brain tissues. (Hagen et al., 1995; Lerner et al., 2005; Priya et al., 2014; Sun et al., 2015).

1.4.2. Sp mediated transcriptional regulation

The involvement of Sp1 transcription factors in basal transcription machinery has been previously identified (Lee et al., 2005). The basal transcription apparatus of eukaryotic cells contains a promoter element with one or more enhancer or repressor regions which facilitate formation of multi-sub unit transcription complex (Ranish & Hahn, 1996). Sp proteins were originally thought to be only capable of regulating promoters with TATA elements which facilitate binding of TATA-binding protein (TBP) (Butler & Kadonaga, 2002). However, it was later found that Sp proteins are also capable of regulating genes that lack a TATA box in the promoter. Sp proteins directly interact with TBP/ TATA binding protein-associated factors (TAFs) (TAF(II)130 and TAF(II)55) as well as upstream activating sequences (UAS) via activation domains A and B which in turn regulate TBP binding by formation of multi-subunit transcription complex (Emili et al., 1994; Dunah et al., 2002). This complex recruits accessory factors such as TFIIA (Transcription factor for RNA polymerase II A), with others co-factor complexes in the basal transcription machinery (Juven-Gershon et al., 2008; Juven-Gershon & Kadonaga, 2010) (see Figure 1.5).

Growing evidence suggests that Sp proteins are also involved in transcriptional regulation of a vast number of genes in addition to its primary role in basal transcriptional machinery (Lee et al., 2005). However, it is yet to be elucidated the

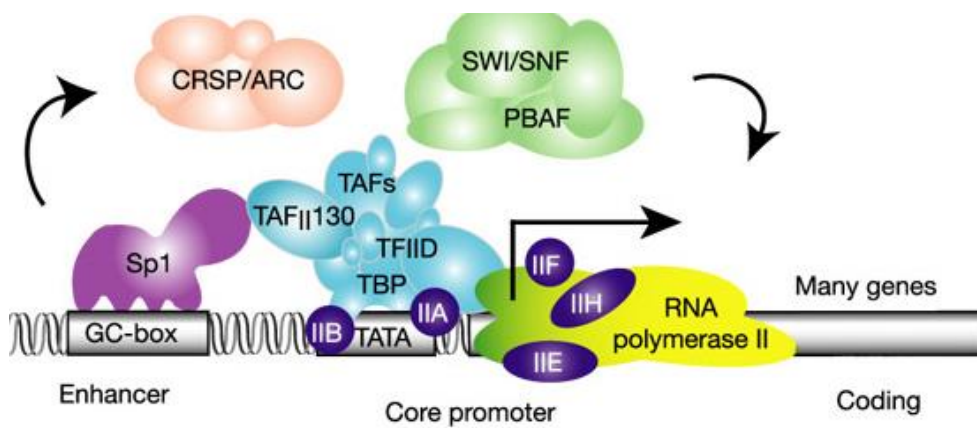


Figure 1.5: Interaction of Sp1 protein in basal transcriptional apparatus

Sp1 interacts with TPB and other TATA-binding protein associated factors (TAF) via activation domains A and B along with RNA polymerase II core complex and associated general transcription factors. CRSP cofactor complex promotes activation of transcription. Figure taken from (Levine & Tjian, 2003).

exact mechanism of how Sp proteins are capable of regulating genes under different conditions. Nevertheless, it has been shown that altered levels of Sp protein, binding and specificity changes governed by post-translational modifications, and direct molecular interaction with other co-factors are the main driving mechanisms of the transcriptional mediation.

Sp proteins are also known to interact with other proteins, transcription factors, chromatin remodelling molecules, sequence-specific DNA binding proteins by direct or indirect interactions. Table 1.2 summarises some of the key genes that interact with Sp1 protein. These key interactions are required for regulation of specific genes in addition to basal transcription mechanism.

1.4.2.1. Relative abundance of Sp proteins

The expression of Sp proteins is regulated at mRNA level up to certain extent by alternative splicing and transcriptional regulation (Saffer et al., 1991). Sp1 and Sp3 proteins are ubiquitously expressed in mammalian cells where upregulation is common in most tumours. It has been shown that relative levels of Sp proteins vary throughout the cell cycle. Higher Sp protein levels were observed in the G1 phase in HBL 100 cells (Grinstein et al., 2002). Similarly, it has been observed that transcription of certain genes is achieved by altering protein levels. Studies carried out by Wong *et al.*, have shown that upregulation of monoamine oxidase (MAO) is achieved by decreasing Sp3 protein levels in CACO2 cell differentiation (Wong et al., 2003).

However, effect of hypoxia on abundance of the Sp family proteins is not yet clear. Several studies have attempted to address this issue where conflicting results were observed due to different oxygen conditions and tissue types. Discher *et al.* have shown depletion of Sp3 due to hypoxia conditions in pyruvate kinase M and β -enolase expression analysis (Discher et al., 1998). However, Sp1 levels remained unchanged suggesting that repression action of Sp3 protein. Involvement of Sp protein-mediated regulation in hypoxia responsive gene *COX-2* has been identified. In the experiment, nuclear localisation of Sp1 increased where no change was detected in Sp3 protein levels (Xu et al., 2000).

1.4.2.2. Regulation of Sp1 affinity and binding

Binding affinities were also believed to play a part in transcriptional regulation of Sp proteins. It has been identified that expression of Specific genes and altered activity under different physiological conditions are achieved by different affinities of Sp proteins towards their cognate sites. Mutations in consensus sites greatly alter affinity of Sp protein. Substitution of Central C base in consensus region with G base may decrease binding of Sp1 protein by at least 30 fold in naked DNA (Letovsky & Dynan, 1989). Furthermore, regulation of certain other genes also resulted decrease of Sp binding activity. Decrease of Sp1 and Sp3 binding activity was reported towards *COL2A1* gene promoter site where expression of the gene is repressed by interleukin-1 β (Poree et al., 2008). Alterations of binding activity of Sp proteins possibly play a significant role in regulating gene expression in hypoxic microenvironment. However, only a few studies have focused on identification of effect of hypoxia on Sp protein binding up to date. Following hypoxic exposure, decrease of binding of Sp1 protein to UDP-glucose dehydrogenase (UGDH) promoter was observed (Bontemps et al., 2003). In contrast, Discher *et al.* have shown decrease of binding activity of Sp3 protein in regulation β -enolase where Sp1 levels were unchanged (Discher et al., 1998).

1.4.2.3. Post-translational modifications of Sp proteins

Sp1 is highly modified by post-translational modifications suggesting that transcriptional regulation, binding and stability may depend on these modifications. Furthermore, translocation of Sp proteins also depends on post-translational modifications such as phosphorylation and O-linked glycosylation (Black et al., 1999). It has been shown that phosphorylation, O-linked glycosylation, acetylation, SUMOylation and ubiquitylation are common types of modifications (Waby et al., 2008). Two major types of modification of Sp family; phosphorylation and O-linked glycosylation are discussed below.

Phosphorylation: Sp proteins can undergo phosphorylation under various conditions at several sites of the protein (possibly serine/threonine-rich regions) by different kinases (Samson & Wong, 2002; Tan & Khachigian, 2009). Phosphorylation may result in changes in DNA binding leading to expression changes in certain genes (Arinze & Kawai, 2003). DNA damage may cause phosphorylation of Sp1 at serine 101 by Ataxia

Table 1.2: Known interactions of the Sp1 transcription factor

Protein	References
Transcription factors	
HIF-1 α	(Koshiji et al., 2005)
HIF-2 α	(Koshiji et al., 2005)
p53	(Lagger et al., 2003)
E2F1	(Lin et al., 1996)
c-MYC	(Gartel et al., 2001)
c-Jun	(Kardassis et al., 1999)
EGR1	(Zhang & Liu, 2003)
SMAD2	(Moustakas & Kardassis, 1998)
Transcription co-factors	
TBP	(Emili et al., 1994)
TAF4	(Dunah et al., 2002)
TAF7	(Chiang & Roeder, 1995)
dTAFII110	(Yang et al., 2001)
TAF-1 α/β	(Suzuki et al., 2003)
DNA repair factors	
Nbs1	(Beishline et al., 2012)
Brca2	(Tapias et al., 2008)
Rad51	(Tapias et al., 2008)
ATM	(Gueven et al., 2003)
Cell cycle	
p21	(Koutsodontis et al., 2005)
CDK4	(Tapias et al., 2008)
Cyclin A	(Wang et al., 2006)
Chromatin remodelling factors	
p300	(Suzuki et al., 2000; Hung et al., 2006)
SWI/SNF /Brg-1	(Xu et al., 2010)
HMGA1, HMGA2	(Aiello et al., 2010; Li et al., 2011)
HDAC1/HDAC2	(Doetzlhofer et al., 1999; Won et al., 2002)

Table 1.2: (Continued)

Protein	References
Tumour suppressors	
Brca1	(Abramovitch et al., 2003)
SKP2	(Tapias et al., 2008)
MDM2	(Johnson-Pais et al., 2001)
VHL	(Cohen et al., 1999; Rafty & Khachigian, 2002)
β -TCRP	(Han & Kudlow, 1997)
Nuclear receptors	
SMRT	(Lee et al., 2005)
AR	(Curtin et al., 2001)
ER α	(deGraffenried et al., 2004; Tu et al., 2013)

Telangiectasia-Mutated (ATM) kinase (Olofsson et al., 2007). Mitogen-activated protein kinases (MAPK) pathway has also been reported to phosphorylate Sp1/Sp3 (Tang et al., 2002). Particularly by p42/p44 MAPK at threonine 453 and 739 of Sp1 protein (Milanini-Mongiat et al., 2002).

Furthermore, protein kinase CK2 is capable of phosphorylating the C-terminus of Sp protein resulting reduction in DNA binding and transactivation (Zhang & Kim, 1997) (Zhu & Liao, 2000). Furthermore Yang *et al.*, have reported that phosphorylation by CDK-1 also decreases Sp1 DNA binding activity in mitosis (Yang et al., 2014).

Glycosylation: O-GlcNAcylation of Sp1 proteins at serine and threonine can be found very often where it may act as a stimulator or repressor of gene expression. A study carried out by Yang et al., has shown that O-GlcNAcylation prevents Sp1 and TATA-binding protein-associated factor (TAF110) association in basal transcription machinery. Similarly, it has suggested that decreased glycosylation may lead to proteolytic degradation of Sp1 proteins resulting in alteration of gene transcription (Han & Kudlow, 1997; Su et al., 1999; Walgren et al., 2003).

1.5. The role of Sp transcription factors in cancer

Many studies have implicated the vital role of Sp transcription factors in cancer development and progression. Sp transcription factors, particularly Sp1, is ubiquitously expressed in most cells where elevated levels of Sp1 protein is predominant in different tumours such as breast and lung tumours (Fulciniti et al., 2011; Deacon et al., 2012). Sp transcription factor driven gene expression can be identified in all of the hallmarks of cancer described by Hanahan and Weinberg in 2011. Sp proteins primarily regulate genes involved in the key cellular pathways by direct DNA binding to their promoter sites. Furthermore, it has other functional capabilities such as localisation of essential factors to the transcriptional complex and transcription of other cofactors.

Transcription of several essential growth signalling molecules in cancer and normal cells are regulated by the Sp protein. Well-studied examples include epidermal growth factor and its receptor (EGF and EGFR), insulin-like growth factor and its receptor (IGF/IGFR), fibroblast growth factor (FGF) (Kageyama et al., 1988; Kitadai et al., 1992). Studies carried out by (Werner et al., 1990) have shown that the *IGF1R* promoter, which contains eight Sp binding sites, is regulated by Sp1 protein (Kaytor et al., 2001).

Cancer cells exhibit great resistance to growth suppressions, cellular stresses and DNA damage. Usually, these cellular events cause normal cell populations to suppress their growth or lead to apoptosis. CDK inhibitor p21 plays a key role in cell cycle arrest which is regulated by Sp transcription factors particularly Sp1 and Sp3 (Pagliuca et al., 2000). Sp1 and Sp3 have converse effects on transcriptional regulation of *p21* gene where decrease of Sp3 enhances Sp1 dependent activation. In cancer cell, p21 activation is suppressed due to post translational modifications of Sp proteins which mediate transcription induction and binding activity. Additionally transforming growth factor β (TGF- β) which is known to promote tumour progression is also regulated by Sp transcription factors (Principe et al., 2014). Transcriptional regulation of Ataxia telangiectasia mutated (ATM) gene, the key player in DNA damage response is also regulated by direct association of Sp1 protein to its kinase domain (Gueven et al., 2003).

The role of Sp proteins has been emphasised in several pro and anti-apoptotic factors such as BCL-2 antagonist killer 1 (BAK) and cell surface TRAIL receptors DR4/DR5. The change in expression of these factors are mediated by Sp proteins which promote cell resistance against immune system destruction.

Cancer cells are capable of overcoming cellular senescence by stabilisation of telomeres which usually decrease in length over the replication process (Harley et al., 1990). Sp1 protein remains the key transcriptional regulator of telomerase subunits. For an example *hTERT* gene catalytic subunit includes five Sp binding sites suggesting Sp mediated gene transcription (Takakura et al., 1999). Sp1 and Sp3 are also responsible for the recruitment of histone deacetylases in hTERT expression (Won et al., 2002). The transcription factor p53 and the cyclin dependent kinase (CDK) inhibitor p16 are also essential for cellular senescence. It has been shown that Sp family proteins, particularly Sp1 regulate these key components. Promoter region of p16 gene contains multiple Sp binding sites which implies activity of Sp protein (Wang et al., 2007; Wu et al., 2007). Furthermore, Sp1 is also known to regulate *MDM2* gene which is a key regulator of p53 in cancer cells (Knappskog et al., 2011).

The role of Sp proteins in inflammatory signalling is also well studied. Yu *et al.*, have shown that T cell specific T-box transcription factor (*TBET*) is regulated by Sp1 transcription factor (Yu et al., 2007). TBET is responsible for regulating key

inflammatory components such as interferon γ in T, B and dendritic cells. It has also been reported that non-steroidal anti-inflammatory drugs (NSAIDs) decrease Sp protein levels and Sp mediated transcriptional activity in different tumours implicating necessity of Sp proteins in inflammation (Abdelrahim & Safe, 2005; Pathi et al., 2012). Sp proteins are correlated with cell invasion and metastasis in different tumour types such as breast and pancreatic cancers. Regulation of pro and anti-invasive factors are regulated by Sp proteins such as matrix metalloproteinases, E-cadherin and integrin $\alpha 5$. Studies have shown that Sp1 is a key regulator of MMPs and MMP-like proteins (Qin et al., 1999). It was shown that these regulations are mediated by direct interaction, localisation cofactors or post-translational modifications of Sp protein (Wang et al., 2006). On the other hand, Sp proteins also regulate anti-migratory factors which are dysregulated in cancer cells. Decreased levels of E-cadherin are associated with higher metastasis. A study carried out by Hsu *et al.*, have shown that Sp1 increases expression of *CDHI* gene which in turn support metastasis by downregulating E-cadherin in human lung cancer (Hsu et al., 2012).

Interestingly Sp transcription factors are the key mediator of a number of pro and anti-angiogenic genes. Well studied examples are vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and thrombospondin 1 (TSP-1). Sp1 protein regulates VEGF transcription in several different mechanisms. The VEGF promoter site contains Sp binding sites which imply direct interaction with Sp proteins in transcription (Abdelrahim et al., 2004). It has also shown that association of other co-factors such as ER- α is dependent on Sp1/3 interaction which leads to estrogen dependent VEGF upregulation (Eisermann et al., 2013). In addition, post translational modification like O-GlcNAcylation of Sp1 has been reported in transcriptional modulation of VEGF (Santra et al., 2008).

Transcriptional regulation of anti-apoptotic genes is also regulated by Sp transcription factors. Expression changes in factors such as B-cell CLL/lymphoma 2 (Bcl-2, BCL-2 antagonist/killer1 (BAK), tumour necrosis factor receptor 10 (TRAIL) and its receptors are well studied in different tumours. These apoptosis and survival factors are mainly regulated by Sp transcription factors. It has been shown that down regulation of TRAIL receptors by Sp proteins make cancer cells more resistance to extrinsic apoptotic signals (Maksimovic-Ivanic et al., 2012) (French & Tschopp, 2002). Moreover, the

glycolytic fuelling process is usually associated with other oncogenes and tumour suppressors which are regulated by Sp transcription factors. Examples include *TP53*, *RAS* and *MYC* genes (Jones & Thompson, 2009).

1.5.1. Relevance of Sp transcription factors in hypoxia responsive gene regulation

Involvement of Sp mediated gene transcriptions are well studied in cancer environment (as previously discussed in section 1.4). Highly diverse, Sp driven gene regulation has also been identified in cellular adaptive mechanism such as hypoxic induction of tumours. Some key hypoxia responsive genes were induced by Sp1 transcription factor in HIF depended/independent manner suggesting its potential role in hypoxic induction. In addition, Sp mediated transcription has been identified as an alternative mechanism in the absence of HIF regulation. Some key hypoxia responsive genes were identified with Sp consensus sites in their promoter sites suggesting possible role of Sp driven transcription. Therefore, it is obvious that Sp proteins play a significant role in tumour hypoxia. Till date, several Sp mediated genes which are induced in a hypoxic microenvironment have been identified. These genes are associated with diverse cellular pathways such as angiogenesis, glucose metabolism, cell cycle regulation, invasiveness and survival.

Interestingly, recent studies have also shown that expression of ***HIF-1A*** gene which encodes HIF-1 α is also regulated by Sp mediated transcription. HIF-1A gene promoter consists of Sp consensus sites and direct DNA interaction of Sp1 is believed to drive this transcriptional mechanism (Vlaminck et al., 2007). In addition, HIF-1 α -Sp1 interaction is also identified via N terminal of PAS domain of HIF-1 α (Koshiji et al., 2005). Some studies have suggested inverse correlation with two transcription factors where HIF-1 α inhibit Sp1 promoter interaction which leads to downregulation of these genes (Culver et al., 2011). However, these mechanisms are yet to be elucidated.

Expression of ***VEGFA*** is widely studied in different tumour types as a key hypoxia induced gene. Sp transcription factors (Sp1) regulate *VEGFA* expression in a HIF/ HRE dependent and independent manner (Abdelrahim et al., 2004; Pore et al., 2004; Lee et al., 2014) (Deacon et al., 2012). The presence of Sp binding sites on the *VEGF* promoter facilitates direct interaction of Sp proteins. Similarly, it has also been shown that expression of the erythropoietin (***EPO***) gene is regulated by Sp1 in hypoxic conditions (Sanchez-Elsner et al., 2004).

Sp protein regulates transcription of enzymes which are involved in hypoxic response and glucose metabolism. A study carried out by Higashimura *et al.*, have shown that glyceraldehyde-3-phosphate dehydrogenase gene (**GAPDH**) is upregulated through Sp regulation in MCF-7 breast cancer cells (Higashimura et al., 2011). Knockdown of Sp1 significantly reduces the hypoxic induction of **GAPDH** in breast cancer in a specific manner. In addition, a key hypoxia marker, carbonic anhydrase 9 (**CA9**) is also regulated by Sp mediated transcription (Kaluz et al., 2003). Both of these genes were identified with Sp consensus sites in their promoters adjacent to HRE sites. It is believed that Sp1 collaboratively regulate these genes with HIF-1 α /HRE dependent manner.

Similarly, HIF and Sp1 proteins collectively up-regulate **CD147**, a transmembrane glycoprotein in hypoxia (Ke et al., 2012). This synergistic regulation was also identified in transcription of sulfonyleurea receptor 1 (SUR1) (Gene **ABCC8**) (Woo et al., 2012). It implies the ability of Sp proteins to act as an alternative regulator or synergetic regulation with HIF transcription factors.

Matrix metalloproteins (MMPs) which are known to regulate invasiveness of tumours by destruction of extracellular matrix, are induced in Sp mediated gene expression in hypoxia. Genes such as **MMP14** and **MMP9** are identified as Sp driven hypoxic genes (Petrella et al., 2005; Chakraborty et al., 2006; Li et al., 2015). The gene promoter of **MMP14** has HRE sites suggesting HIF-2 α depended Sp regulation is identified as transcriptional regulation mechanism (Petrella et al., 2005). In 2013, Xi *et al.*, showed HIF-2 α depended, Sp mediated copper transporting ATPase (**ATP7A**) induction in intestinal epithelial cells in hypoxia (Xie & Collins, 2013).

A study carried out by Discher *et al.*, have demonstrated that β -enolase (**ENO3**) and pyruvate kinase M (**PKM**) regulation by Sp proteins due to hypoxia in muscle cells in a HRE independent manner (Discher et al., 1998). Similarly, chromatin immunoprecipitation studies conducted in glioma cells have shown the HRE independent, Sp regulation of **ADAM17** in hypoxia (Szalad et al., 2009). HRE independent regulation is believed to be driven by alteration of Sp protein expression levels or activity. However, exact mechanisms are not fully understood.

These examples indicate an important role of Sp protein mediated gene transcription in hypoxia responsive cellular pathways. In contrast to HRE mediated transcription, Sp

dependent mechanisms are not fully understood. More gene profile studies are required to identify all the key hypoxia responsive genes, specifically genes which are mediated by Sp transcription factors. Although HIF-ARNT mediated transcription is well established, it is evident that HIF targets are also directly or indirectly (HRE independent) mediated by Sp proteins. Identification of hypoxia responsive Sp-regulated gene targets are vital for elucidating the mechanisms of hypoxic induction and downstream cellular pathways.

Moreover, Sp protein mediated genes can be potential therapeutic targets whereas inhibition of Sp mediated hypoxia responsive genes can be an effective way of overcoming hypoxic related challenges. Inhibition Sp regulated gene targets may be an effective therapeutic aspect in hypoxia. Since most of the studies have been focused on HIF mediated genes therapeutic aspects these gene will give new level of synergetic approach on targeting downstream (Castro-Gamero et al., 2013). Sp protein inhibitors, Mithramycin A and Chromomycin A₃ have been widely examined in different tumours and clinically proven tested as a therapeutic for various cancers. In addition, DNA binding small molecules such as Terameprocol is also in phase I/II clinical trials for treatment of solid tumours (Parker et al., 1960; Kuru, 1961; Khanna et al., 2007).

1.6. Aims and Objectives

Breast cancer is one of the most common malignancies found in women worldwide. Identification of the molecular mechanisms underlying the tumour formation, invasion and metastasis are vital for the development of therapeutic aspects and better the survival rates. Understanding the exact mechanisms will facilitate advance prognosis and early diagnosis of patients. Furthermore, enhanced understanding will support advancement of drug discovery which can be effective in devising prevention strategies.

Tumour hypoxia, a common phenomenon in solid tumours, has been identified as the key player in tumour development, progression and metastasis which also leads to poor prognosis, aggressive phenotypes and lower survival rates. Due to the elevated levels of chemo and radio resistance of hypoxic tumours, therapeutic strategies are significantly challengeable compared to normoxic environment. Since breast cancer is one of the most hypoxic tumours, investigation of hypoxia in breast cancer is vital for

elucidating hypoxic related cellular mechanisms and pathways. Identification of novel gene profiles of hypoxia induction can be useful as therapeutic targets. In addition, key hypoxia responsive genes can be used as potential bio markers for early detection of hypoxic solid tumours.

Several studies have also implicated that Sp proteins are aberrantly expressed in different tumours which leads to poor prognosis. Under the hypoxic microenvironments, Sp expression levels may undergo further changes in response to oxygen tension. It was also identified that Sp1 protein transcription and translation might correlate with tissue type which highlights the necessity of investigating the effect of hypoxia on Sp family transcription family proteins in breast cancer. In addition to expression changes, Sp proteins might undergo activity changes in order to response to hypoxic induction in tumour cells. Binding activity changes and expression changes may facilitate transcriptional regulation of Sp protein itself as well as its downstream gene targets.

Moreover, HIF mediated gene transcription has been identified as a key modulator of hypoxic induction. However, there is growing evidence that presence of regulatory mechanism alternative to HIF mediated gene transcription in hypoxic microenvironment which is regulated by other transcriptional regulators such as Sp transcription factors. It is believed that Sp1 mediated transcription is important in both HIF dependent and independent hypoxia response which can be a promising therapeutic target for hypoxic tumours. In addition to Sp driven *HIF1A* gene transcription, several other key genes in hypoxic induction are also regulated by Sp mediated regulation. Overall, both development of hypoxic microenvironment and dysregulated expression of *Sp1* are associated with poor prognosis of solid tumours which leads to higher resistance to chemotherapy and radiotherapy. Therefore, it is important to further investigate the role of Sp1 under the hypoxic microenvironment in breast cancer.

The work in this thesis aims to identify whether Sp1 has a driving role in the hypoxia response observed in cancer development, particularly in breast cancer. The significance of chronic hypoxia on expression of Sp transcription family and their DNA binding activity will be investigated using breast cancer cell models. Potential downstream targets of key hypoxia genes which are regulated by specificity protein

transcription factors in breast cancer cells will be identified and further examined to understand the transcript changes of those genes in chronic hypoxia. In addition, dependency of these genes on Sp mediated gene regulation will be assessed by inhibition with Sp1 gene knockdowns as well as Sp protein inhibitor Mithramycin A. Finally, the study will be further expanded to determine the kinetics and binding activity of three well known Sp protein inhibitors in real time binding analysis towards biological promoter sites. Kinetic data produced in this thesis may provide insight into the synthesis of novel drug moieties for prevention of Sp protein family and HIF mediated cellular pathways of solid tumours in normoxic and hypoxic environments.

1.6.1. Key objectives

Key objectives of this study are as follows:

- Investigating the key changes in Sp family transcription factors due to hypoxic microenvironment (**Chapter 3**)
- Identifying hypoxia responsive genes which are regulated by Sp1 in breast cancer cell lines (**Chapter 4**)
- Characterisation of the binding kinetics of Sp protein inhibitors (**Chapter 5**)
- Investigating the effect of Sp protein inhibition on hypoxia responsive gene transcription (**Chapter 6**)

Chapter 2.

Materials and Methods

2. Materials and Methods

2.1. Cell culture

2.1.1. Cell lines

Three human breast cancer cell lines were used throughout this study. MCF-7 (Pleural effusion of metastatic adenocarcinoma), MDA-MB-231 (Pleural effusion of metastatic adenocarcinoma) and T47D (Pleural effusion of ductal carcinoma). Properties of cell lines have been summarised in Table 2.1. Cell lines were originally purchased from ATCC or ECACC and regularly tested for mycoplasma contamination.

2.1.2. Sub culture

MCF-7 and T47D cells were grown in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen) medium supplemented with 2 mM L-glutamine (Gibco Life Technologies, UK) and 10% (v/v) heat-inactivated foetal calf serum (FCS) (Gibco Life Technologies). MDA-MB-231 cells were grown in 4.5 g/L Glucose Dulbecco's Modified Eagle's medium (DMEM, from PAA) supplemented with 4 mM L-glutamine (Gibco Life Technologies, UK) and 10% (v/v) heat-inactivated FCS (Gibco Life Technologies).

Cells were passaged twice a week at 70-80% confluency in order to maintain exponential growth phase. The media was discarded and the cell monolayers were washed gently with 1x Phosphate-Buffered Saline, w/o Ca^{2+} or Mg^{2+} (PBS) (Lonza). Cells were then incubated in 1x Trypsin/EDTA (0.05% (w/v) trypsin, 0.5 mM EDTA) (PAA, UK) for 4-5 minutes at 37 °C to dissociate the cells from the plastic. Detached cells were re-suspended in excess of fresh growth media and an appropriate fraction of volumes were transferred to fresh flasks. Cells were maintained in a humidified, 5% CO_2 incubator at 37 °C (Nuair IR Direct Heat CO_2 Incubator, Red Laboratory Technology USA).

Table 2.1: Summary of cell properties used in this study

Cell	MCF-7	MDA-MB-231	T47D
Cancer type	Adenocarcinoma	Adenocarcinoma	Ductal carcinoma
Classification	Luminal A	Basal	Luminal A
Development	Metastatic	Metastatic	Metastatic
Origin	Pleural effusion	Pleural effusion	Pleural effusion
Culture type	Adherent	Adherent	Adherent
Culture media	Roswell Park Memorial Institute RPMI 1640	Dulbecco's Modified Eagle Medium (DMEM)	Roswell Park Memorial Institute RPMI 1640
Age	69	51	54
Ethnicity	Caucasian	Caucasian	Unknown
PR	+	-	+
ER	+	-	+
HER2	-	-	-

PR; Progesterone receptor, ER; oestrogen receptor, HER2; Human epidermal growth factor receptor 2

2.1.3. Viable cell counting

For experiments, known number of cells were plated to improve reproducibility. The number of viable cells present was determined using the vital dye Trypan blue. A small amount of cell suspension (50 μ l) was diluted with 1:1 of 0.4% Trypan blue solution (Gibco, UK) and cells were counted using Neubauer haemocytometer (Marienfeld Superior, Germany). Four quadrants (16 corner squares) of the haemocytometer were counted and final cell concentration (cells per millilitre) were calculated by multiplying average cell count per quadrat by 10^4 and dilution factor. A different number of cells were seeded according to the cell line and experimental specification.

2.1.4. Freezing down and thawing cells

To maintain healthy cells in their exponential growth phase cells were not passaged beyond 15 passages. Cells purchased from ECACC/ATCC were expanded and frozen down after few passages to generate early passage stocks.

Cells were detached as described in section 2.1.2. After resuspending cells in fresh media, cells were centrifuged at 300 x *g* followed by resuspension in 1x PBS. The cell suspension was centrifuged at 300 x *g* and the supernatant was discarded. The cell pellet was re-suspended in a small amount of freezing media (10% Dimethyl sulfoxide (DMSO, Sigma, UK) in heat inactivated FCS (Gibco Life Technologies, UK). Cell suspension in freezing media was aliquoted into cryovials (Nunc, Thermo Scientific). Cryovials were then placed in a freezing container (Mr Frosty, Nalgene, Thermo Scientific) and gradually frozen at -80 °C for 48 hours prior to transfer to liquid nitrogen for long term storage.

When required, fresh vials were thawed quickly in a 37 °C water bath and placed in pre-warmed media for culturing. An appropriate number of cells were initially grown in T25 flasks (recommended cell number or 1/3 of a T25 flask of cells) and then passage as described earlier. Cells were sub cultured at least two passages until they acquire exponential and stable growth before being used for experiments.

2.2. Hypoxia treatment

Hypoxia treatments were carried out using H35 Hypoxystation hypoxia chamber (Don Whitley Scientific, UK). Temperature and CO₂ concentrations were maintained at 37 °C and 5% respectively, whilst humidity was maintained at 75%. Different oxygen concentrations (0.5-10.0% (v/v) O₂) were defined accordingly to the experimental specification. Culture media was changed prior to hypoxic exposure in order to maintain effective O₂ concentration. All the hypoxic samples were lysed inside the chamber to prevent reoxygenation. Normoxic samples were maintained in parallel to hypoxic samples in a conventional incubator with same experimental conditions except atmospheric oxygen concentration (~20% (v/v) O₂).

2.3. Cell transfection with silencing siRNA

Transfection experiments were performed using siPORT *NeoFX* lipid-based transfection reagent (Thermo Scientific). siRNA assays were purchased including recommended controls (Fisher). Silencer® Select Sp1 siRNA (Human) (assay ID s13318), Silencer® Select GAPDH Positive Control siRNA (Human, Mouse, Rat) and Silencer® Select Negative Control #2 siRNA were used. All the assays were reconstituted in nuclease free water (Supplied with transfection reagent-Thermo Scientific) in order to prepare 2 μM stock solutions of siRNA. Optimisation of cell number, siRNA concentration and the amount of *NeoFX* transfection reagent was carried out using ranges suggested by the manufacturer. Knockdown efficiency was assessed at 24-72 hours post transfection with *GAPDH/SP1* qPCR assays to determine the optimal duration. The optimised protocol as described below, was used throughout the experiments.

Briefly, healthy growing adherent cells of MCF-7, MDA-MB-231 and T47D were rinsed with PBS prior to being trypsinized with Trypsin/EDTA. Trypsin/EDTA was neutralised with an excess amount of media and the cell pellet was obtained by centrifuging at 300 x *g*. Cells were counted (see section 2.1.3) and re-suspended in complete growth media (without antibiotics) to 5 x 10⁶ cells/ml. Transfection agent and Opti-MEM serum free media (Gibco Life Technologies, UK) were brought to room temperature prior to use. For each well to be transfected, 5 μL siPORT *NeoFX* was diluted with 295 μL of Opti-MEM serum free media and incubated for 10 minutes at room temperature. Similarly, 45 μL of each 2 μM siRNA stock solutions were diluted to 30 nM final concentration in 255 μL of Opti-MEM media prior to use. Diluted siPORT *NeoFX* and siRNA solutions were mixed together and further incubated for 10 minutes at room temperature. For each well to be transfected, 600 μL of siRNA/ siPORT *NeoFX* complex were dispensed into empty wells of 6 well plate (Greiner Bio-One, UK). Opti-MEM media was also used to prepare of non-transfected controls without siRNA and no transfection agent.

For transfection, 1 x 10⁵ cells/ well (2.0 ml cell suspension) were plated in 6 wells plate with siPORT transfection mixture. The transfection mixture was incubated at 37 °C in normal growth conditions for 24 hours followed by replacement of fresh growth media. At this point, the visual appearance of cells was checked for any evidence of

cell necrosis/apoptosis due to cytotoxic effects. Cells were incubated for a further 24 hours in normal growth conditions followed by 12 hours incubation in a hypoxic/normoxic environment. After 60 hours of total incubation, total RNA was extracted using Bio-Rad Aurum total RNA extraction kit for qPCR analysis (see section 2.5).

2.4. Mithramycin A treatment

Mithramycin A (Abcam, UK) was purchased as a dry powder and reconstituted in 100% DMSO to prepare 10 mM concentrated stock solutions. Cells were seeded into six-well plates (Greiner Bio-One, UK) and left to adhere overnight. The next morning, media was replaced with fresh complete media supplemented with 1 μ M Mithramycin A. Controls contained only fresh media and DMSO. Cells were then placed in hypoxia (0.5% v/v O₂) or normoxia (21% v/v O₂) for 12 hours prior to total RNA extraction using Bio-Rad Aurum total RNA extraction kit.

2.5. Transcript analysis

2.5.1. Total RNA extraction

The Aurum Total RNA mini extraction kit (Bio-Rad) was used for all the RNA extraction procedures according to manufacturer's instructions. Following a PBS rinse of adherent cells, lysis buffer was added directly to cell dishes and cells were scraped into the lysis solution. Lysates were then collected into 2 ml capped tube and an equal volume of 70% (v/v) ethanol was added. Lysates were mixed thoroughly until bilayers disappear completely. Then cell lysates were transferred to 1.5 ml uncapped centrifuged tube containing RNA binding column followed by centrifugation at 20000 x *g* for 2 minutes at room temperature. The RNA column was washed with low stringency buffer followed by centrifugation at 20000 x *g* for 30 sec. After all the washing steps, DNA bound to the column was digested with 80 μ l DNase solution for 15 minutes at room temperature. After incubation, the column was washed with high stringency solution, followed by a low stringency solution wash, before finally the RNA was eluted into fresh centrifuge tube using elution buffer. Total RNA was quantified using Nanodrop light spectrometer (Thermo Scientific) and stored at -80 °C.

2.5.2. cDNA synthesis

cDNA synthesis was carried out using the iScript cDNA synthesis kit (Bio-Rad). One microgram of total RNA was placed in PCR tubes with 1 μ l of iScript reverse transcriptase (RT) and 4 μ l of 5x master mix and reverse transcribed using a thermal cycler (Bio-Rad C1000, Bio-Rad, UK). Nuclease-free water was used for adjusting volumes, so that final reaction mixture was 20 μ l. A no-RT sample was also performed by addition of nuclease free water as a replacement for the RT enzyme. The reaction mixture was incubated initially for 5 minutes at 25 °C followed by 30 minutes at 42 °C. Finally incubated for 5 minutes at 85 °C with a final hold at 4 °C.

2.5.3. Quantitative PCR analysis using TaqMan assays

Real-time qPCR analyses were performed using a standard protocol (see Table 2.2) on a StepOnePlus (Applied Biosystems, Life Technologies) real-time PCR system. FAM labelled pre-designed TaqMan assays for genes of interest (GOI) and VIC labelled Human beta 2 globulin (*B2M*) assay (Life Technologies) were purchased (see Table 2.3). *B2M* was used in duplex reactions as an endogenous reference for all samples. Before carrying out fold change analyses, 6-point standard curves were prepared using a 1:3 serial dilution of cDNA to check PCR efficiency.

One microliter of cDNA was incubated in a 10 μ l reaction with 1x TaqMan Fast Advance master mix (Applied Biosystems, Life Technologies), 1x *B2M* VIC labelled TaqMan assay and 1x GOI FAM labelled TaqMan assay. Nuclease-free water was used to adjust the final reaction volume. Each cDNA sample was tested in triplicate. No RT and no template control (NTC) samples were assayed in parallel. C_T (C_Q) values were generated using the StepOne software (Applied Biosystems, Life Technologies) and expression changes were calculated according to relative expression C_T method (Schmittgen & Livak, 2008). Expression changes of gene of interest (GOI) were determined by ddCT fold change (FC) analysis and presented as mean difference of fold changes against the untreated control sample.

Table 2.2: Cycling protocol for TaqMan real-time PCR

Stage 1			Stage 2		
50 °C	2 min	1x	95°C	0.15 min	Repeat 40x
95°C	10 min	1x	60 °C	1.0 min	

Table 2.3: Summary of TaqMan qPCR assays of gene targets

Entrez Gene ID	Gene Symbol	Gene name	Assay reference ID	Amplicon length
6667	<i>Sp1</i>	Sp1 transcription factor	Hs00916521_m1	72
6670	<i>Sp3/SPR2</i>	Sp3 transcription factor	Hs01595811_m1	138
6671	<i>Sp4/HF1B</i>	Sp4 transcription factor	Hs00162095_m1	110
5210	<i>PFKFB4</i>	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	Hs00190096_m1	70
901	<i>CCNG2</i>	Cyclin G2	Hs00171119_m1	85
9048	<i>ARTN</i>	Artemin	Hs00754699_s1	78
8660	<i>IRS2</i>	Insulin receptor substrate 2	Hs00275843_s1	70
7162	<i>TPBG</i>	Trophoblast glycoprotein	Hs00272649_s1	57
170384	<i>FUT11</i>	Fucosyltransferase 11 (alpha (1,3) fucosyltransferase)	Hs00543033_m1	69
79660	<i>PPP1R3B</i>	Protein phosphatase 1, regulatory (inhibitor) subunit 3B	Hs00226322_m1	143
56477	<i>CCL28</i>	Chemokine (C-C motif) ligand 28	Hs00219797_m1	84
7026	<i>NR2F2</i>	Nuclear receptor subfamily 2, group F, member 2	Hs00819630_m1	145
64236	<i>PDLIM2</i>	PDZ and LIM domain 2 (mystique)	Hs00222154_m1	104
3725	<i>JUN</i>	Jun oncogene	Hs01103582_s1	91

7422	<i>VEGFA</i>	Vascular endothelial growth factor A	Hs00900055_m1	59
4891	<i>SLC11A2</i>	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	Hs00167206_m1	59
25909	<i>AHCTF1</i>	AT hook containing transcription factor 1; AT hook containing transcription factor 1 pseudogene	Hs01628527_s1	191
87178	<i>PNPT1</i>	Polyribonucleotide nucleotidyltransferase 1	Hs01105971_m1	135
79647	<i>AKIRIN1</i>	Akirin 1	Hs00607590_m1	129
7056	<i>THBD</i>	Thrombomodulin	Hs00264920_s1	91
84969	<i>TOX2</i>	TOX high mobility group box family member 2	Hs00262775_m1	96
27242	<i>TNFRSF21</i>	Tumour necrosis factor receptor superfamily, member 21	Hs01560899-m1	65
1153	<i>CIRBP</i>	Cold inducible RNA binding protein	Hs00154457_m1	103
768	<i>CA9</i>	Carbonic anhydrase IX	Hs00154208_m1	78
6513	<i>SLC2A1/Glut1</i>	Solute carrier family 2(facilitated glucose transporter) member 1	Hs00892681_m1	76

2.6. Protein analysis

2.6.1. Cell lysis and protein extraction

Cells were seeded into plastic tissue culture dishes (Nunc) depending on experimental strategy. Incubation conditions were also defined according to the experiment (O₂ concentration and duration of hypoxia exposure). After each time point, media was removed and cell monolayers were washed with 1x PBS followed by scraping of cells into 1ml of PBS. The cell suspension was centrifuged at 20, 000 x *g* and supernatant was carefully discarded. The cell pellet was re-suspended in cell lysis buffer (UTB

buffer: 9M Urea, 75mM Tris-HCl pH 7.5, 0.15M β -mercaptoethanol). Volumes of lysis buffer used were adjusted to the size of the pellet and desired concentration.

Cell lysates were sonicated using a Bioruptor (chilled with ice and water) (Diagenode, Belgium) for 15 minutes (5 x 3 minutes) at high setting with 30 sec on/off intervals. Finally, cells lysates were centrifuged at 20,000 x *g* for 15 minutes at 4 °C and the supernatant was removed and stored at -80 °C.

2.6.2. Protein quantification

Prepared cell lysates were quantified using a Nanodrop light spectrometer (Thermo Scientific). UTB buffer was used to dilute all the samples to the same concentration (3-5 $\mu\text{g}/\mu\text{l}$) so that equal loading of proteins can be obtained with a fixed volume.

2.6.3. SDS-PAGE

Protein analyses were carried out by gel electrophoresis. Protein samples were separated by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The stacking gel (4% Acrylamide) consisted of 1.5 M Tris-HCl pH6.8, 30% acrylamide/0.8 bisacrylamide, 10% SDS, 10% TEMED and 10% APS for appropriate volumes and 10% separating gel consisted of 1.5 M Tris-HCl pH 8.8, 30% acrylamide/0.8 bisacrylamide, 10% SDS, 10% TEMED and 10% APS (see table 2.4)(Adapted from (Green & Sambrook, 2012). Reducing sample buffer (3.3% SDS; 6M Urea, 17 mM Tris-HCl pH 7.5; 0.01% (w/v) bromophenol blue; 0.07 M β mercaptoethanol) was added to each sample and heated at 95°C for 5 minutes on a heat block. Depending on protein expression levels 30-75 μg of protein lysates were loaded per well and electrophoresis was carried out for 60-90 mins at 100-120 V in 1x running buffer (See Table 2.5). A molecular weight ladder was run parallel with samples to identify molecular weights. (Bio-Rad precision plus. Bio-Rad, USA).

Table 2.4: Composition of 10% acrylamide gels for SDS gel electrophoresis

Reagent	Stacking gel*	Separation gel*
1.5M Tris pH 8.8	-	3.75 ml
0.5 M Tris pH 6.8	2.5 ml	-
Acrylamide	1.3 ml	4.95 ml
10% SDS	100 μ l	150 μ l
10% APS	100 μ l	75 μ l
TEMED	20 μ l	18 μ l
MilliQ H2O	6.1 ml	6.1 ml

*Sufficient for two 1mm thick mini gels

2.6.4. Western Blotting

Gel electrophoresis was carried out until migration dye front reached around 1 cm from the bottom. Then the gel was carefully removed from running buffer and placed on a stack of blotting papers followed by polyvinylidene difluoride (PVDF) membrane (GE Healthcare). PVDF membrane was activated in methanol for 10 seconds prior to use. The transfer cassette was placed in a gel tank containing 1x transfer buffer with 20% (v/v) of methanol (see Table 2.5) and run at 100V for 60-90min with ice block. After the transfer, PVDF membranes were blocked with blocking buffer (5% (w/v) fat-free milk powder in TBST (10% 10x TBS (Tris-buffered saline) (88g NaCl; 24g Tris base; pH7.4 in final volume of 1 L molecular grade H₂O and 5% Tween) on shaking at room temperature for 1 hr or at 4°C overnight. Membranes were then incubated at 4°C overnight in primary antibody diluted in 1% (w/v) Milk-TBST. Primary antibodies were diluted as recommended (See Table 2.6). After incubation with primary antibody, membranes were washed with 10 ml of TBST for 3 x 10 minutes washes on rolling. Secondary antibody conjugated to Horseradish peroxidase (HRP) (Anti-Mouse HRP and Anti-Rabbit HRP (IgG)) (Abcam) was also diluted 1:2000 dilution in 1% (w/v) Milk-TBST as recommended dilutions and membranes were incubated for one hour at room temperature. After incubation with secondary antibody, membranes were washed again with TBST for 3 x 10 min washes whilst rolling, prior to incubation in ECL

developing (Millipore, UK) solution for 5 minutes. A Bio-Rad Chemidoc XRS+ system was used for imaging using Image Lab software (Bio-Rad). Densitometry analyses were also carried out with Image Lab software (Bio-Rad).

Table 2.5: Composition of SDS gel electrophoresis buffers (10x)

Reagent	Running Buffer		Blotting Buffer	
	mass	Concentration	mass	Concentration
Tris base	30.2 g	250 mM	30.2 g	250 mM
Glycine	144 g	1.92 M	144 g	1.92 M
SDS	10 g	1% (w/v)	-	-
MilliQ H ₂ O	Volume to 1 L	-	Volume to 1 L	-

2.6.5. Coomassie blue gel staining

Even loading for western blotting was verified by staining duplicate gels for total protein using Coomassie blue. Duplicate gels were incubated in Instant blue Coomassie blue stain (Expedeon, UK) for 1 hour at room temperature with shaking followed by a wash with molecular biology grade water. Gel images were captured using the Bio-Rad Chemidoc XRS+ system.

Table 2.6: Primary antibodies used in this study

Target	Manufacturer	Reference number	Dilution	Origin	Clone	Band size (kDa)
β- Actin	Santa Cruz	Sc-69879	1:10000	M	Mono	42
GAPDH	Ambion	AM4300	1:2000	M	Mono	36
HIF-1α	BD Biosciences	610958	1:500	M	Mono	120
Sp1	Millipore UK	07-645	1:1000	R	Poly	95
Sp2	Santa Cruz	Sc-166575	1:2000	M	Mono	80
Sp3	Abcam	Ab72594	1:1000	R	Poly	82
Sp4	GeneTex	GTX117167	1:500	R	Poly	82
Sp4	Abcam	Ab83373	1:500	R	Poly	82

R: Rabbit, M: Mouse, Mono: monoclonal, Poly: polyclonal

2.7. Analysis of binding activity of Sp proteins using electromobility shift assay (EMSA)

2.7.1. Nuclear extractions

For preparation of nuclear extracts, cell monolayers were washed with warmed 1x PBS. Cells were then scraped into 1x PBS and centrifuged at 200 x *g* for 5 minutes. The cell pellet was then re-suspended in 1x PBS and centrifuged at the same speed. All extraction buffers were prepared from stock solutions, with DTT and PIC added just prior to use. The protocol used was based on the published protocol by (Dignam et al., 1983). After washing, the cell pellet was suspended in approximately 3 ml of cold nuclear extraction buffer A (NEBA: 10 mM HEPES pH7.9; 1.5 mM MgCl₂; 10 mM KCl; 1 mM DTT; 1 mM PMSF and 1x Sigma protease inhibitor cocktail). Cells were pelleted by centrifuging at 100,000 x *g* for few seconds and washing with NEBA was repeated at least two times.

Cell lysis buffer was prepared by adding 0.1% (v/v) NP-40 to NEBA buffer. The cell pellet was re-suspended in lysis buffer (1 ml per 1.5 ml tube) and incubated on ice for 5 minutes with occasional agitation. After the incubation, cell lysate was centrifuged at 300 x *g* for 15 minutes at 4 °C and cell supernatant (cytosolic extract) was discarded. Nuclear lysis was performed with nuclear lysis buffer (NEBC: 20 mM HEPES pH 7.9; 1.5 mM MgCl₂; 420 mM NaCl; 1 mM DTT; glycerol 25% v/v; 1 mM PMSF and 1x Sigma protease inhibitor cocktail). The pelleted nuclei were re-suspended in 300 µl cold (4 °C) NEBC. Genomic DNA was sheared by passing the extract through a yellow tip while lysis on ice for minimum 15 minutes. Sheared DNA and insoluble materials were removed by centrifugation at 20,000 x *g* for 15 minutes at 4 °C. The supernatant (nuclear extract) was carefully collected to a fresh tube without any insoluble materials. Prepared nuclear extracts were quantified using Nanodrop light spectrometer (Thermo Scientific) before being aliquoted and stored at -80 °C.

2.7.2. Preparation of labelled DNA oligos for EMSA

Target DNA sequences were synthesised with a 3'-biotin tag on both positive and negative strands (Sigma-Aldrich). Oligos were reconstituted in molecular grade water to a concentration of 100 µM and then diluted to 0.1 µM concentration in 10 mM Tris, 1 mM EDTA, 50 mM NaCl (pH 8.0) buffer (TEN buffer). Single-stranded DNA oligomers

were annealed in TEN Buffer by mixing in 1:1 ratio of forward and reverse strands together followed by 5 minutes incubation at 95 °C on a heat block. After the incubation, oligomers were left overnight on the same heat block without heating so that temperature will gradually decrease allowing single stranded oligomers to be annealed. The effectiveness of annealing was checked with gel electrophoresis and samples were aliquoted and stored in -20 °C freezer. Details of oligomers can be found in Table 2.7.

2.7.3. DNA binding assay procedure

The LightShift Chemiluminescent EMSA kit (Thermo Scientific) was used for binding analysis of Sp proteins. The assay protocol was carried out according to the manufacturer's guidelines with minor modifications. Briefly, 0.5x Tris, boric acid, EDTA (TBE) buffer was prepared using 5x TBE stock buffer (450 mM Tris, 450 mM Boric acid, 10 mM EDTA pH 8.3). Polyacrylamide native gel (4.0% acrylamide) was prepared using 0.5x TBE or purchased as precast gels (Bio-Rad). Gels were pre-run at 100 V using 0.5x TBE buffer for 20 minutes. Binding reactions were prepared as 20 µL reaction per well. Binding reactions were incubated for 20 mins at room temperature. The composition of reaction mixtures can be found in Table 2. 8. Five microliters of 5x Loading Buffer were added to each of 20 µL binding reaction by pipetting up and down gently to mix. Vigorous mixing or vortexing was avoided to prevent disruption of complex. Control reactions were carried out as per manufacturers conditions using Epstein-Barr virus nuclear antigen (EBNA) DNA.

The entire reaction mixture was loaded into 1 well and separated by electrophoresis at 100V for approximately 45 minutes until the bromophenol dye had travelled 3/4 of way through the gel. Nylon membranes (Thermo Scientific) were soaked in 0.5x TBE buffer prior to preparation of transfer cassette. The gel was sandwiched with blotting papers and nylon membrane and the electrophoretic transfer was carried out in 0.5x TBE 100V for 30-60 mins with an ice block. Clean forceps were used all of the time and membranes were handled only from edges to minimise contamination.

Upon completion of the transfer, membranes were carefully removed and placed on a paper towel with the bromophenol side on top and the crosslinking step was immediately carried out. Care was taken to make sure that membranes were not

allowed to dry out before crosslinking. Crosslinking was carried out at 120 mJ/cm² using a commercial UV-light crosslinking instrument equipped with 254 nm illumination (45-60 seconds exposure using the auto crosslink function) (Stratalinker® UV crosslinker, Northumbria Biological Ltd, South Nelson Industrial East Cramlington). Blocking and washing of membranes were carried out as recommended by the manufacturer.

Biotin labelled DNA-protein complexes were detected using streptavidin horseradish peroxidase (HRP). After all the washing steps membranes were incubated in 12 ml of detection solution (6.0 ml Luminol/Enhancer solution + 6.0 ml Stable peroxide solution) as recommended. A Chemidoc XRS+ system and Image Lab software (Bio-Rad) were used for imaging of the chemiluminescent signal. Densitometry analyses were also carried out with Image Lab software (Bio-Rad).

Table 2.7: DNA oligomers used in this study

Name	Size (bp)	Sequence	Molecular weight (KDa)
Sp consensus	30	5' AAT TCG ATC GGG GCG GGG CGA GCA TGC TCA 3'	18574.94
Mutant Sp consensus	30	5' AAT TCG ATC GGT TCG GGG CGA GCA TGC TCA 3'	18572.98
Bak ABC	60	5' GCC TGG GAG GTG GGG TGG GGA AAG TGG GCG GGA CAT GCT CCT GGG CCT GGC CCA CCC AGA 3'	37120.76
Bak B	20	5' AAA GTG GGC GGG ACA TGC TC 3'	6286.06
Mutant Bak B	20	5' AAA GTG AGC GTG ACA TGCTC 3'	6245.05
<i>TP53</i> Non-promoter	60	5' TCC TCC AAA ATG ATT TCC ACC AAT TCT GCC CTC ACA GCT CTG GCT TGC AGA ATT TTC CAC 3'	37106.06
Control I	30	5' AAG ACT GAT ACT GAA TCG CTA CTC TAA GC 3'	17950.67
Control II	60	5' AGA CGT GAT ATT GTA ATG CTT ATC TTC AGA TTG CAG ATA GCT GCA TCG TTT TTA TAA TCT 3'	37098.22
Control III	20	5' AGA CGT GAT ACT GAA TCG CT 3'	6220.04

Table 2.8: Composition of representative EMSA gel reaction mixtures

Component	Final amount	Samples				
		Probe only	Probe +NE	Unlabelled comp	Probe +NE+ Sp1	Unlabelled+ Sp1
10x binding buffer	1x	2.0 µl	2.0 µl	2.0 µl	2.0 µl	2.0 µl
1 M NaCl		1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
1 µg/µl Poly (dl.dC)	50 ng/µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
1% NP-40		1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
100 mM MgCl2		1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
NF water to volume	--					
Unlabelled Target DNA	6 pmol	---	---	6 µl	---	6 µl
Nuclear extract	30 µg					
Biotin Labelled target DNA	10 fmol	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl
Antibody	1.5 µg	---	---	---	1.5 µl	1.5 µl
Total volume	---	20 µl	20 µl	20 µl	20 µl	20 µl

2.8. Binding and kinetic analysis of Sp inhibitors

2.8.1. Selection of Sp promoter sites

Surface Plasmon Resonance (SPR) was used to examine the binding kinetics of known Sp inhibitors. DNA oligomers of Sp consensus sequence and gene promoter sequences with known Sp protein binding sites were used for this experiment. Promoter sequences were carefully selected to avoid complex formation and multiple interactions. To test for specificity of binding to the consensus/promoter sequences, oligos were designed containing base substitutions in GC rich regions (mutant sites). Control DNA oligomers of the same length and molecular weight but without any Sp binding sites were also designed.

To characterise the binding kinetics of Sp inhibitors towards known promoter sequences containing Sp binding sites, previously established *Bak* gene promoter sequence was used. A sixty base pair long *Bak* promoter sequence (termed Bak ABC) was used. As a biologically relevant negative control, an upstream region from TP53 gene promoter sequence was used which does not contain any Sp binding sites or GC-rich regions. The *Bak* ABC region contains 3 known Sp binding sites. According to the literature, the central site (*Bak* B) has the highest affinity for Sp protein binding when compared to others two sites (Waby et al., 2010). Therefore, specificity of Sp inhibitor binding was analysed using a 20 base pair DNA oligomer homologous to the of the Bak B promoter sequence. A Bak B mutant, containing substitutions within its GC rich region was used to check the specificity of binding. Negative control DNA sequences were designed of similar length but lacking GC regions (Control I/II/III). Full details of DNA oligomers used can be found in Table 2.10.

2.8.2. Preparation of target DNA sequences

Target DNA sequences were synthesised as single strand oligos with a 3' biotin tag on the positive strand (Sigma-Aldrich) to allow capture on a Streptavidin on the sensor chip (GE Healthcare). Annealing of the complementary strands of the oligos were carried out as described in section 2.7.2. In this experimental setup, only the positive strand was labelled with Streptavidin to facilitate single interaction on the sensor chip.

2.8.3. General Assay development

Binding and Kinetic analyses were carried out on the Biacore T200 (GE Healthcare). Biacore T200 control software was used to develop the assay method. A standard small molecule kinetic template was used to build the assay method. Modifications were made accordingly. A summary of assay development can be found in Figure 2.1.

2.8.4. Sample and surface preparation

All buffers for SPR experiments were prepared using SPR grade 10 x PBS (GE Healthcare) and HPLC grade water. 1.05x PBS was used as working buffer solution where 1x PBS buffer supplemented with 0.05% (v/v) P20 surfactant and 5% (v/v) DMSO was used as running buffer. Mithramycin A (Abcam, UK), Chromomycin A3 and Terameprocol (Sigma) were purchased as dry powders and 10 mM stock solutions were prepared in DMSO. The stock solution was diluted to appropriate concentration with 1x PBS or 1x PBS+ 5% (v/v) DMSO. The concentration of the final DMSO was carefully maintained at 5% (v/v).

A Biacore T200 Surface Plasmon Resonance instrument was primed with 1.05 x PBS for a minimum of three consecutive injections for 7 minutes prior to any surface preparation or kinetic assay with maintenance chip.

As the biotin-streptavidin capture method was used for ligand immobilisation, SA sensor chips were used throughout all the experiments. SA sensor chips (GE Healthcare) were allowed to reach room temperature prior to use in order to prevent formation of a humid layer inside the chamber. Activation of sensor surface was carried out with three consecutive injections of 1 M NaCl/50 mM NaOH for 60 seconds at 30 $\mu\text{l min}^{-1}$.

After activation of sensor chip surface, double-stranded biotin labelled DNA oligomers were immobilised onto the surface. Manual injection method was used for immobilisation. R_{Max} value of 50 response units (RU) was selected (Karlsson et al., 1991) in order to prevent steric hindrance or any mass transfer limitations (Oshannessy & Winzor, 1996; Edwards & Leatherbarrow, 1997). The amount of ligand to be immobilised was calculated for each oligonucleotide as described by (Glaser, 1993) using Equation 1 (see below). Consecutive injections at 30 $\mu\text{l min}^{-1}$ flow rate were carried out until the desired response units were captured onto the surface. It

was essential to maintain a constant number of response units on every flow cells in order to compare the binding kinetics towards each sequence. Flow cell one was always left without immobilisation, so that binding responses were subtracted from the response of flow cell one (core responses were accounted for binding analysis and kinetic constant determination). Flow cell arrangement was 2-1 core for single oligomer experiments where 2-1, 3-1, 4-1 core multi-flow cell arrangements were used for more than one oligomer.

Equation 1: Calculating Immobilization amounts

$$R_L = \frac{\text{Molecular weight of Ligand}}{\text{Molecular weight of Analyte}} \times R_{Max} \text{ (RU)}$$

RU; Response units, R_{Max} ; Maximal response in RU, R_L ; Amount of ligand to be immobilised (RU)

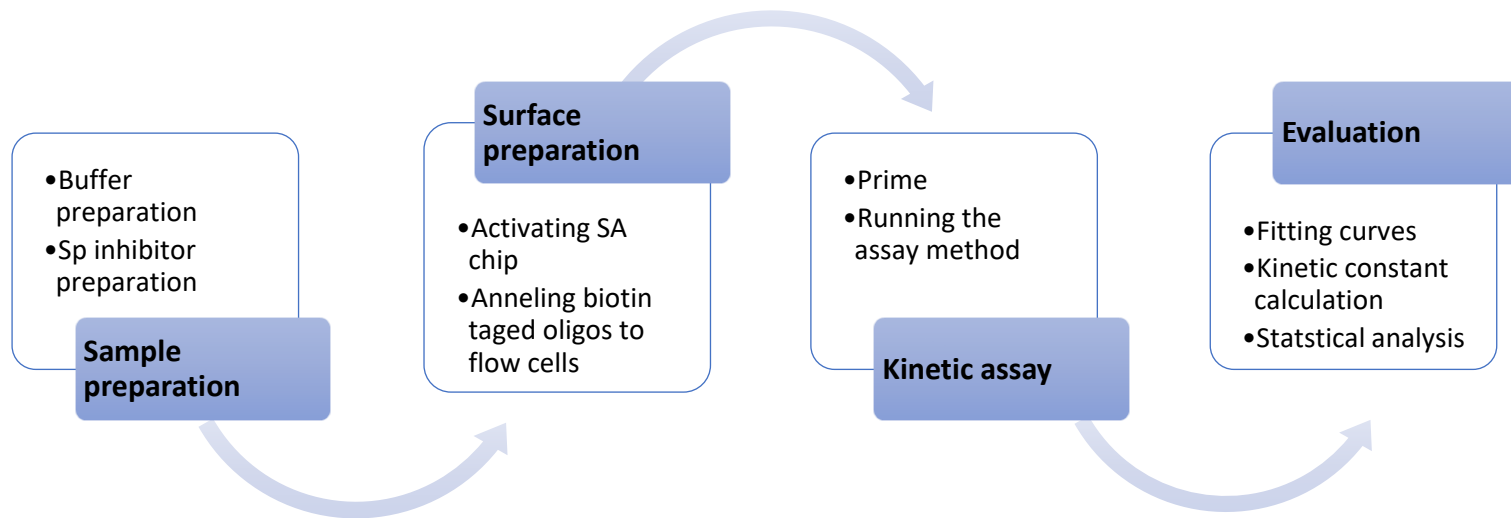


Figure 2.1: Summary of Surface plasmon assay development

2.8.5. Solvent correction

A solvent correction was used to minimise any errors associated with preparations of samples as well as to minimise bulk effect of buffer components. Since DMSO may have bulk effect on samples, it was beneficial to use a solvent correction. Solvent correction solutions were prepared to vary from 4.5-5.8% (v/v) DMSO concentrations and eight solvent samples were included as recommended (pre- assigned in software). The solvent correction step was included in assay step and consecutively injected at the beginning and end of the sample injections. Solvent correction curves were used to analyse the kinetics using Biacore evaluation software (GE Healthcare).

2.8.6. Binding Assay for Sp inhibitors

For all the binding experiments 1x PBS + 5% (v/v) DMSO + 0.05% (v/v) surfactant p20 was used as the running buffer. Optimisation and validation of method were carried out with 1x PBS + 5% (v/v) DMSO in the absence of any surfactant to establish binding conditions. The optimised method was used for binding experiments with p20 surfactant (GE Healthcare) to minimise any nonspecific interaction on sensor chip surfaces. Binding experiments were carried out at 25 °C and flow rate was 30 μmin^{-1} .

To study binding kinetics, Sp inhibitors were injected at the concentration indicated in the sensorgrams for 60 seconds followed by 120 seconds' dissociation. Any drug remaining on the surface was removed by an extra wash with 50% (v/v) DMSO for 60 seconds following the dissociation phase. Each drug was tested in at least 3 independent experiments with a minimum of 4 different concentrations of 3-500 μM . Possible bulk effects from DMSO were subtracted using solvent correction within the assay steps.

2.8.7. Data evaluation and kinetic analysis

Any background signal from a sensor surface (reference flow cell) was subtracted from each data set. Binding kinetics were determined using different fitting models for sensorgrams. Fitting models were selected according to the experimental strategy. Dual subtracted data were fit to 1:1 binding model (Langmuir interaction model) for global fitting. Final dissociation constants (K_d) values were calculated for each inhibitor

towards different DNA oligonucleotides. Bivalent analyte fitting model was used for some occasions where more than one analyte or divalent analyte is predicted.

2.8.8. Single cycle kinetic assay

The single kinetic approach was used on several occasions where regeneration was either very slow or not achievable. Single cycle kinetic model for small molecules temple (Biacore) was used with modifications. A concentration series with five different concentrations were injected consecutively so that amounts of drug and ligand complexes are built on the surface followed by single dissociation. Drugs were injected at 15-250 μM concentration with serial injections followed by 600 seconds' dissociation.

2.9. Microarray data set analysis

2.9.1. Collection of NCBI microarray data

Publicly available microarray data sets were collected from NCBI GEO database. Searches for data sets were carried out using the search terms "cancer and hypoxia" to archive wide range of data sets. Data sets were then filtered as detailed below.

To identify the expression of Specificity protein transcription factor mediated genes under hypoxic microenvironment, only mRNA microarray data sets were retained while microRNA data sets and tissue arrays were excluded. The data sets were further filtered by cell type and hypoxic exposure and only the data sets that were based on breast cancer cells with exposure to acute hypoxia (0.1-2.0% O_2 concentration) were selected.

Selection criteria were extended further in order to limit the data analysis to reliable data sets that had biological replicates ($n \geq 3$). Data sets that had used more than one microarray platforms in a single experiment were also omitted from further analysis due to technical limitations of the software. The data sets, which fulfilled all selection criteria, were used for gene expression analysis. These data sets were downloaded and imported to GeneSpring GX 12.1 using the data-importing wizard for further analysis.

2.9.2. Experimental grouping and quality control of data sets

Following the import into the GeneSpring GX Software, the new experiment wizard was used to define experimental conditions such as experiment name, analysis type

(Expression) and workflow (Analysis biological significance). Occasionally, only partial data sets were used for analysis where more than one cell type had been used in an experiment.

Baseline transformation was used to minimise systematic non-biological variations such as unequal starting materials of RNA and differences in hybridisation. This involves setting a median value for all samples, so that all gene intensities were rescaled to a similar relative abundance level centred on zero. Box-whisker plots were used to identify median values, quintiles and sample distribution, as well as any outliers prior to any further analysis.

The experimental grouping step was used to define samples with the same conditions as well as biological replicates. Experimental parameters were added using this window. Furthermore, experimental conditions were also specified. In this study probe sets were grouped according to the hypoxia treatment (O_2 tension) and exposure time with biological replicates. In addition to experimental grouping, data sets were arranged in ascending order of hypoxia exposure for convenience.

Probe set filtration was conducted to assess the quality control of entities. Filtration of probe sets was carried out using expression values; particularly percentile values were selected for probe set filtrations. Raw data of probe set data were filtered with cut-off values of 20-100 of expression of non-averaged samples

Filtered data sets were subjected to quality control and statistical analysis. Distribution of samples and expression values were checked for any outliers and relevant statistical methods were used depending on no of samples and parameters. One-way ANOVA analysis was used to compare more than two sets of samples. P value calculation was done with asymptotic computation with cut-off p-value of 0.05. Benjamini-Hochberg's model was used for post hoc analyses (multiple testing corrections).

2.9.3. Gene expression analysis using GeneSpring GX 12.1

Fold change analyses were carried out for filtered probe sets. Fold change cut-off values were selected dependent on the cell type and overall expression values. A cut-off value of 1.5 FC was selected as the standard for all the data sets unless stated otherwise. All hypoxia exposure conditions were compared with the normoxic condition. Different oxygen concentrations were paired and one out three pairs, which fulfils the cut off fold change, were selected as gene expression changes.

2.9.4. Gene Set Enrichment Analysis (GSEA) for Specificity protein 1 and Hypoxia-inducible factor (HIF)

Following identification of genes which showed a 1.5 FC in hypoxia, gene list was subjected to GSEA analysis using GSEA wizard in GeneSpring GX. To identify involvement of transcription factors in observed expression changes, standard algorithm parameters for GSEA analysis (minimum number of gene sets = 15; maximum number of permutations = 100) were used. BROAD gene sets (See Table 3.2) were searched with q value cut off 0.3. Gene sets GGGCGGR_V\$SP1_Q6 (M1474) (Molecular Signatures Database v5.2) and ELVIDGE_HIF1A_TARGETS_DN (M2513) (Elvidge et al., 2006) were used for analyses. List of genes with altered expression and HIF or Sp sites were generated.

Table 2.9: BROAD gene sets used for GSEA analysis

Name	Description
C1	Cytogenetic sites
C2	Functional sites
C3	Regulatory –Motif sets
C4	Neighbourhood sets
C5	Gene Ontology sets
C6	Oncogenic signature
C7	Immunologic signature

2.9.5. Pathway analysis and gene ontology analysis

Genes with differential expression in hypoxia which were identified by GSEA contain HIF1A and/or Sp1 binding sites were subjected to pathway and gene ontology analyses. Selected gene lists were processed with GeneSpring software in order to identify most represented pathways and biological process. Organism and pathway organism parameters were restricted to *Homo sapiens* and curated Wiki pathways were used for analysis.

2.10. Statistical Analysis

Statistical analysis was carried out where appropriate. Each experiment was performed at least in three independent occasions unless otherwise stated. Suitable statistic tests were selected according to the number of samples and type of samples (e.g. parametric and non-parametric data sets). The standard deviation (SD) and standard error of mean (SEM) were plotted alongside with sample data. The significance analysis was carried out where the difference was observed among data sets. Student t-test was used to compare two groups where One-way ANOVA/Two-way ANOVA was performed to compare more than two groups followed by appropriate post hoc test. Prism 5 (GraphPad, USA) software was used for all the statistical analysis unless stated otherwise.

Chapter 3.
Effect of hypoxia on Specificity protein transcription
factors

3. The effect of hypoxia on Specificity protein transcription factors and their activity

3.1. Introduction

The role of Sp family transcription factors in tumours has been extensively investigated in terms of different gene regulations which are widely spread in most of the cellular pathways (Archer, 2011). Growing evidence suggests that Sp proteins function in the hypoxic microenvironment as a central mediator. Importantly the key regulator of hypoxic induction, HIF-1 α has Sp1 binding sites in its promoter indicating *HIF-1 α* transcription is mediated by Sp1 (Iyer et al., 1998; Minet et al., 1999; Vlamincx et al., 2007; Zawacka-Pankau et al., 2011). This was further confirmed by Kim and *et al.*, who showed that *HIF-1 α* transcription depends on Sp1 protein in PC3 human prostate cancer cells (Zelko & Folz, 2004; Kim & Park, 2010). In addition, several other studies have also demonstrated that expression of *HIF-1 α* as well as *HIF-2 α* depends on Sp1 and Sp3 proteins (Minet et al., 1999; Wada et al., 2006; Lafleur et al., 2014). Moreover, several gene specific studies have further investigated the function of Sp proteins under hypoxic microenvironment. For instance, genes involved in angiogenesis and glucose metabolism, two important cellular pathways induced in hypoxia (*VEGF*, *CA9* and *EPO*), have Sp binding site on their promoters suggesting the possible role of Sp proteins in hypoxia (Pal et al., 2001; Kaluz et al., 2003; Lee et al., 2004b; Sanchez-Elsner et al., 2004; Deacon et al., 2012). These key findings emphasise the importance of Sp transcription factors in hypoxic induction as vital transcription mediators.

Mechanisms underlying transcriptional mediation in the hypoxic microenvironment and hypoxia-related modifications of Sp proteins are not clearly understood. Accumulating evidence suggests that Sp mediated transcription is regulated mainly via four mechanisms; transcriptomic changes, translational changes (protein level), activity changes (binding activity) and post-translational modifications. In the hypoxic microenvironment, Sp proteins may undergo one or more these alterations in response to lower oxygen tensions.

Altered expression levels of Sp proteins, particularly Sp1 and Sp3 may contribute to hypoxia-driven gene regulation in tumours. Some studies have shown that Sp1/Sp3 levels are significantly upregulated under severe hypoxia (chronic hypoxia) where HIF-

mediated induction is predominant. Kong *et al.*, have reported upregulation of Sp1 protein levels in lung cancer cells due to hypoxic exposure (Kong et al., 2010). Similarly, Sp1 protein levels were upregulated due to hypoxia in neuronal cells where the generation of reactive oxygen species is believed to play a vital part under ischemic conditions (Yeh et al., 2011). Contrastingly, some studies have shown no alteration in Sp1 protein levels in response to hypoxia. For an example, a study carried out by Xu *et al.*, investigated the regulation of *COX -2* gene, another hypoxia responsive gene, and showed that Sp levels remained unchanged even though nuclear localisation of Sp1 increased (Xu et al., 2000). Bontemps et al., have also shown that Sp1 is capable of regulating UDP-glucose dehydrogenase (*UGDH*) while protein levels were unchanged under hypoxic conditions (Bontemps et al., 2003). It has been suggested that these contrary observations are associated with tumour/cell type specific nature of Sp proteins. However, actual mechanism and related modifications are yet to be elucidated.

Binding activity changes of Sp proteins due to hypoxic condition have also been investigated by several studies in different cell types. A study carried out by Bontemps *et al.*, has shown that binding to the promoter of glycolytic enzyme *UGDH* is decreased due to hypoxic condition (Bontemps et al., 2003). An increased binding activity of Sp1 protein was also reported towards *Smad3* where EPO protein is induced under hypoxic condition through TGF β pathway (Sanchez-Elsner et al., 2004). Under the hypoxic microenvironment, increased binding activity of Sp1 was also reported towards *CCL2* promoter in melanoma cells (Kunz et al., 2002).

Therefore, it is becoming evident that Sp proteins play a necessary role in induction of the hypoxic response via multiple mechanisms. Sp family members may undergo transcriptional, translational, and activity alterations in response to the hypoxic microenvironment. However, further investigations are required to elucidate the extent of the role of Sp mediated gene regulation in the hypoxic response.

3.1.1. Aims and objectives of the chapter

The main objective of this study is to investigate the effect of the hypoxic microenvironment on Sp transcription family members in breast cancer cell lines *in-vitro*. It is hypothesised hypoxic exposure may cause alterations at transcript, translational level and binding activity in addition to post translational modifications. These modifications can be correlated with severity of hypoxia as well as the tumour type.

Key questions answered from this chapter;

- Do expression levels (mRNA and protein) of Sp proteins change in response to severe hypoxia?
- Are Sp protein levels affected by hypoxia in breast cancer cells?
- Does hypoxia alter the binding activity of Sp1 protein?
- What is the effect of inhibitor Mithramycin A on transcription of Sp proteins?

3.2. Results

3.2.1. Effect of hypoxia exposure on transcript levels of *Sp1*, *Sp3* and *Sp4* in breast cancer cell lines

The objective of this section was to investigate the effect of severe hypoxia (0.5% (v/v) O₂) on transcript levels of Sp protein family members in a breast cancer cell model. MCF-7, MDA-MB-231 and T47D cells were exposed to hypoxia at 0.5% (v/v) O₂ tension for 24 hours followed by RNA extractions. Transcript levels of *Sp1*, *Sp3* and *Sp4* genes were analysed using specific TaqMan assays and ddCT fold change (FC) analysis (see section 2.5).

Hypoxic exposure caused alterations in transcription of Sp genes in breast cancer cell lines. As can be seen from Figure 3.1, cells cultured in 0.5% (v/v) O₂ hypoxia showed induction of *Sp1* gene mRNA levels. The hypoxic response was prominent at 24 hours of exposure where it was increased by 1.04 FC (+/- 0.63 SD) in MCF-7 cells and 0.86 FC (+/- 0.52 SD) in T47D cells. Changes were significant compared to the control. Contrary, no significant changes were observed for MDA-MB-231 cell line for any time points.

Transcript levels of *Sp3* gene were unaffected due to the hypoxic exposure as expression levels were consistent at all the time points with few exceptions. As can be seen in Figure 3.2, cell type specific changes were observed at some time points such as 0.82 FC (+/- 0.28 SD) increase at 24 hours normoxic sample of MCF-7 and 0.49 FC (+/- 0.17 SD) of decrease in MDA-MB-231 cells compared to the control.

Transcript levels of *Sp4* gene were altered due to the hypoxic exposure at 0.5% (v/v) O₂. Both MCF-7 cells and T47D cells grown in hypoxia exhibited increase of transcript levels compared to the control and normoxic counterparts. A significant increase of 0.68 FC (+/- 0.23 SD) was observed in MCF-7 cells compared to the control. Changes were only visible in T47D cells. On the other hand, MDA-MB-231 cells, expression levels were visibly decreased at time points of 3 and 24 hours of hypoxic exposure compared to the control. As evident from Figure 3.3, 24-time point showed significant reduction of 0.39 FC (+/- 0.07 SD) against its normoxic counterpart.

Sp1 and *Sp4* genes were induced in severe hypoxia whilst *Sp3* gene lacks any significant changes. Overall, expression changes of Sp family genes followed a cell type specific manner without common changes after exposure to severe hypoxia.

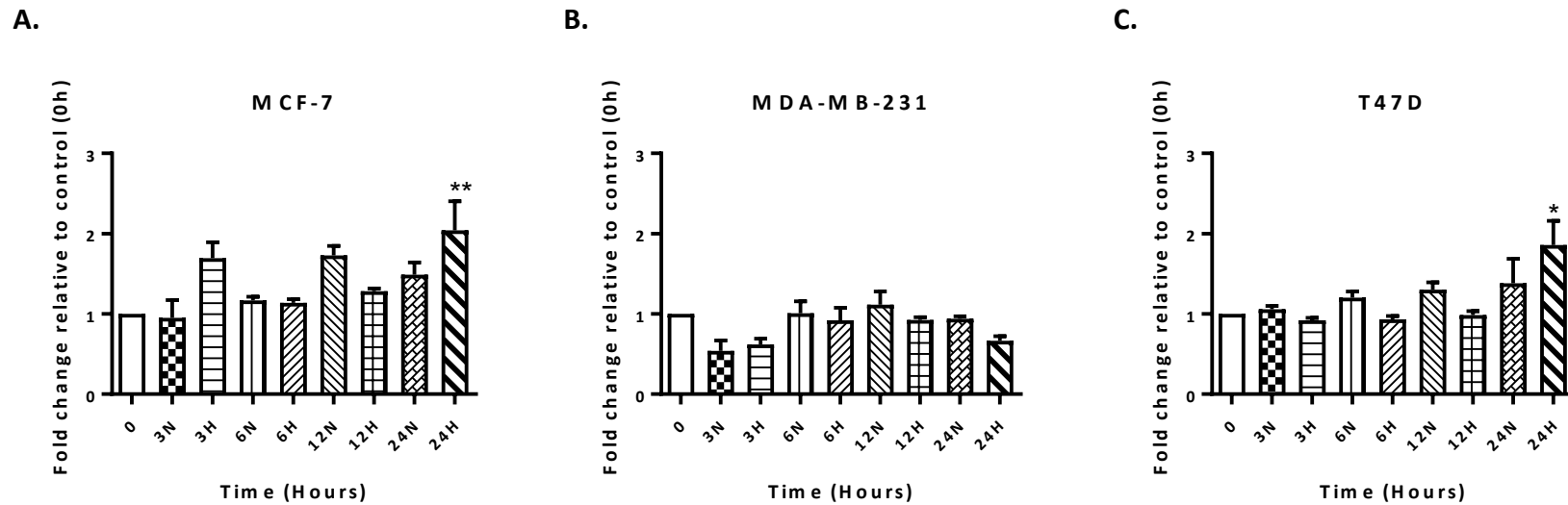


Figure 3.1: mRNA expression of *Sp1* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represent +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Results were compared against the control (Black coloured *) as well as the normoxic counterparts. *, P< 0.05; **, P< 0.01. (Abbreviations: N: Normoxia, H: Hypoxia).

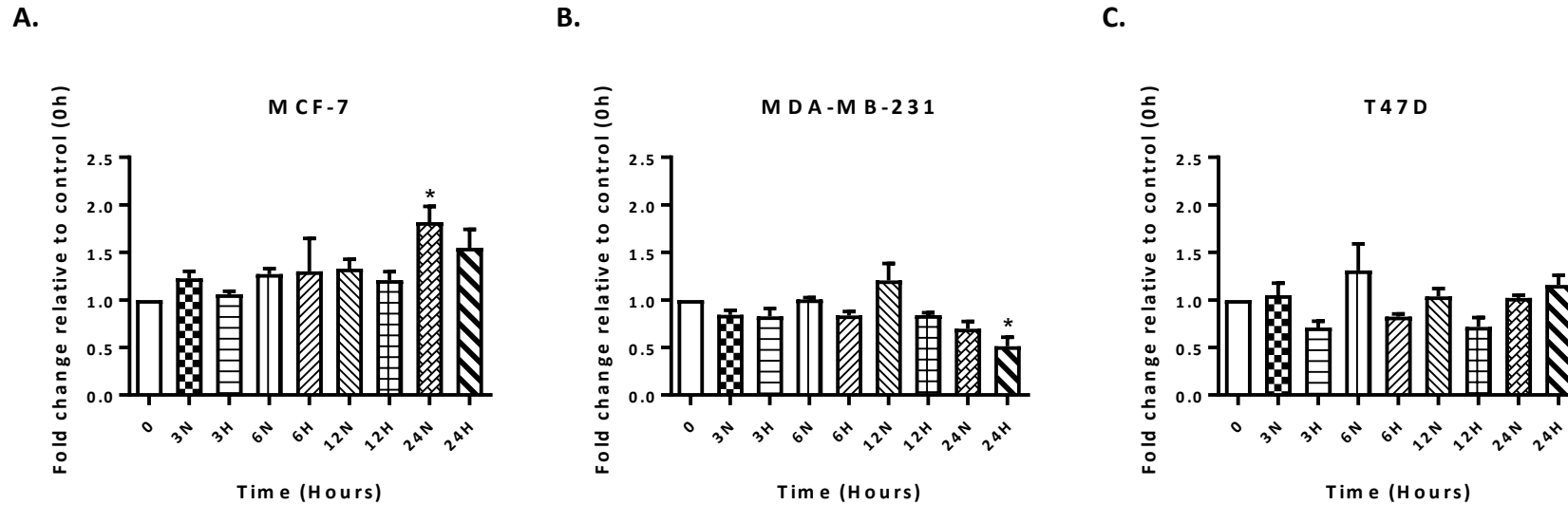


Figure 3.2: mRNA expression of *Sp3* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represent +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Results were compared against the control (Black coloured *) as well as the normoxic counterparts. *, P< 0.05 (Abbreviations: N: Normoxia, H: Hypoxia).

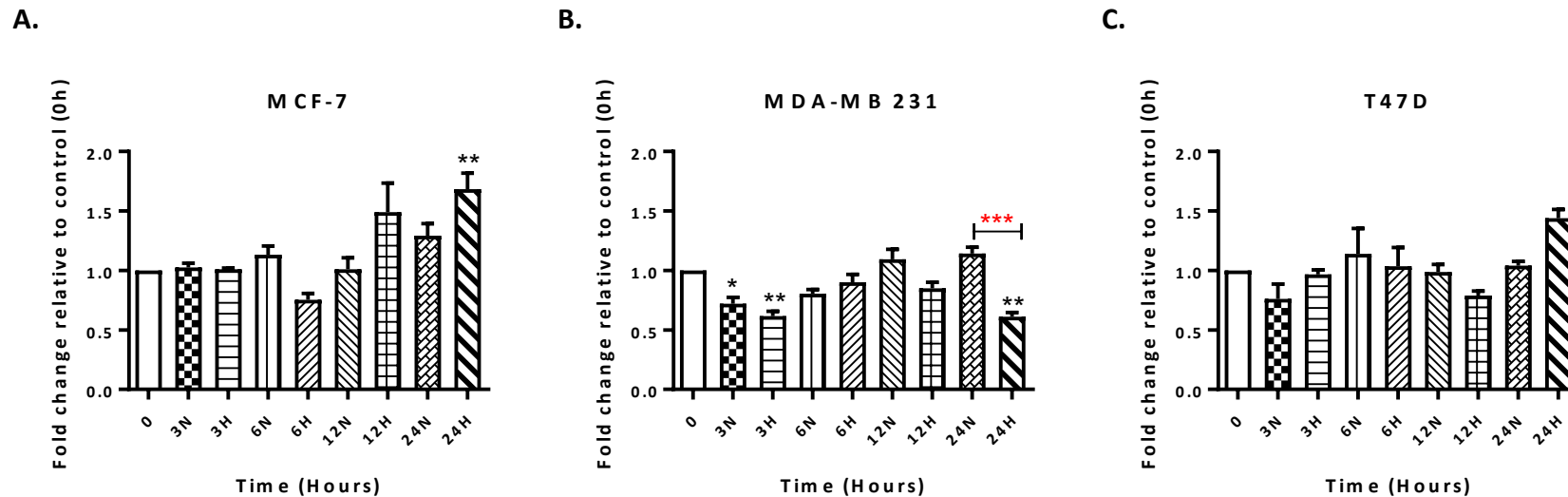


Figure 3.3: mRNA expression of *Sp4* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represent +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P< 0.05; **, P< 0.01; ***, P< 0.001. (Abbreviations: N: Normoxia, H: Hypoxia).

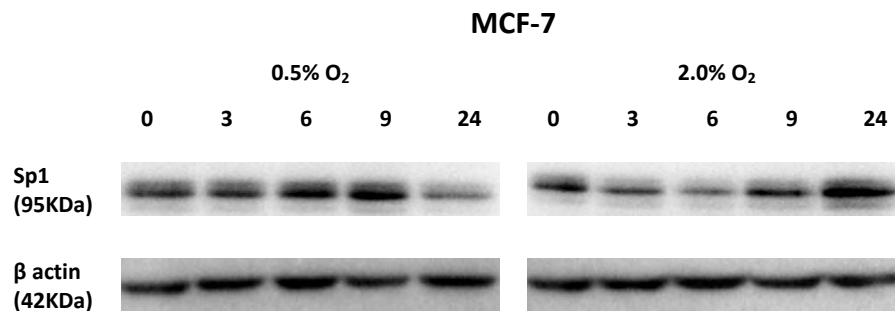
3.2.2. Effect of hypoxic exposure on protein levels of Sp1, Sp3 and Sp4 in breast cancer cell lines

In this section, it was aimed to identify the effect of severe hypoxia on protein levels of Sp family transcription factors. Expression levels of Sp proteins were analysed using the western blotting technique with specific antibodies for Sp1, Sp3 and Sp4. Cells were exposed to two different hypoxic conditions (0.5 and 2.0% (v/v) O₂) over a period of time points. Densitometry was performed for quantification and identification of absolute changes in blots.

Sp1 protein level was upregulated due to severe and moderate hypoxia in breast cancer cell lines. As Figure 3.4 represents, expression of the Sp1 protein in MCF-7 cells was significantly upregulated by 0.56 FC (+/- 0.31 SD) at 9 hours of exposure. However, at 2.0% O₂ exposure, this increase in Sp1 was seen later at 24 hours (1.02 FC (+/- 0.33 SD)). In addition, changes in expression levels were also observed at 6 hours for 0.5% O₂ and at 9 hours for 2.0% O₂ which were statistically insignificant. MDA-MB-231 cells responded to hypoxia in a slightly different way compared to MCF-7 cells. After 3 hours of exposure, Sp protein levels were significantly increased by 0.74 FC (+/- 0.26 SD) in severe hypoxia (0.5% O₂) whereas only a visible increase was detected in mild hypoxia (see Figure 3.5). However, no significant changes were detected at any other time. In T47D cells, hypoxic responses were different between two conditions. After 0.5% O₂ exposure, protein levels were decreasing after 9 hours in a significant manner. In contrast, significant increases were identified at 3 (0.31 FC (+/- 0.06 SD) and 6 hours (0.77 FC (+/- 0.23 SD) of hypoxic exposure of 2.0% O₂ (see Figure 3.6).

Sp3 protein was also induced due to the hypoxic exposure in breast cancer cell lines. Under severe hypoxia (0.5% O₂), increase in protein levels was visible in MCF-7 cells, despite no significant changes were identified (bands were visible at 3 and 9 hours' time points). Similarly, Sp3 levels were increased by (0.44 FC (+/- 0.10 SD) at 6 hours' time point for 2.0% O₂ exposure (see Figure 3.7). In MDA-MB-231 cells, Sp3 protein levels were visibly increased after 3 hours of hypoxic exposure. Significant changes were observed at 3-hour time points, which was (0.44 FC (+/- 0.11 SD). Proteins levels remained constant at 2.0% O₂ where no changes were detected (see Figure 3.8).

A.



B.

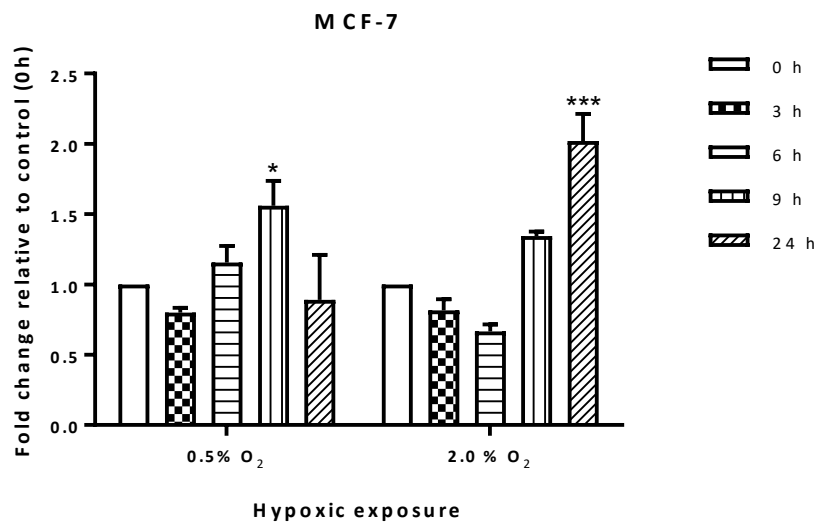
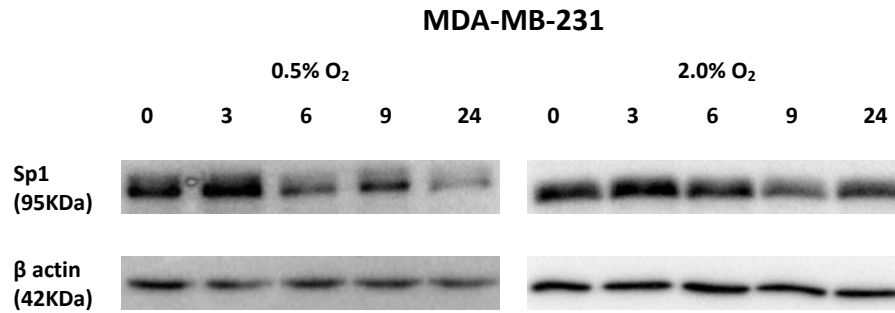


Figure 3.4: Effect of severe and moderate hypoxia on Sp1 protein levels in MCF-7 cells

Panel A: MCF-7 cells were exposed to hypoxia followed by protein lysis at different time points. 30 µg of whole cell lysates were separated by SDS-PAGE and further analysed by Western blotting with antibodies against Sp1 protein. β-Actin was used as a loading control. n=3 experiments.

Panel B: protein levels were analysed by densitometry analysis using Image Lab software. All results were presented as relative fold change compared to zero-hour control. Error bars represent +/- SEM. Statistical analysis were carried out with Two-way ANOVA with Bonferroni post hoc correction. *, P < 0.05; ***, P < 0.001.

A.



B.

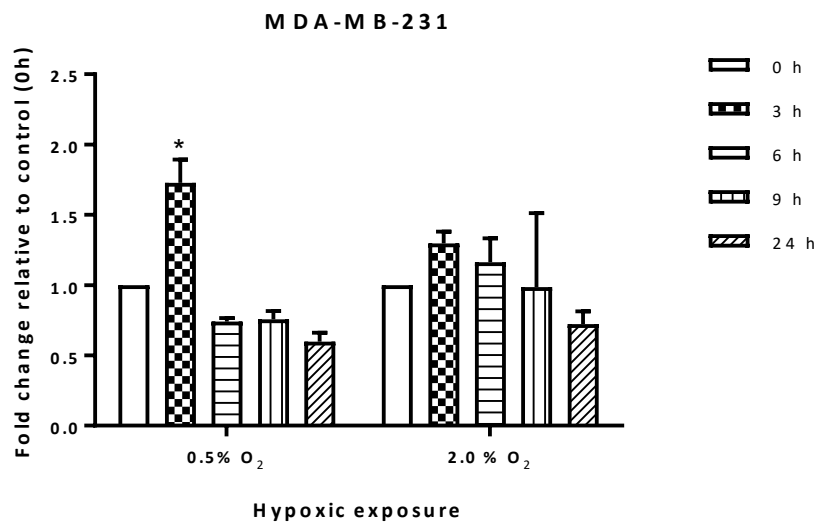
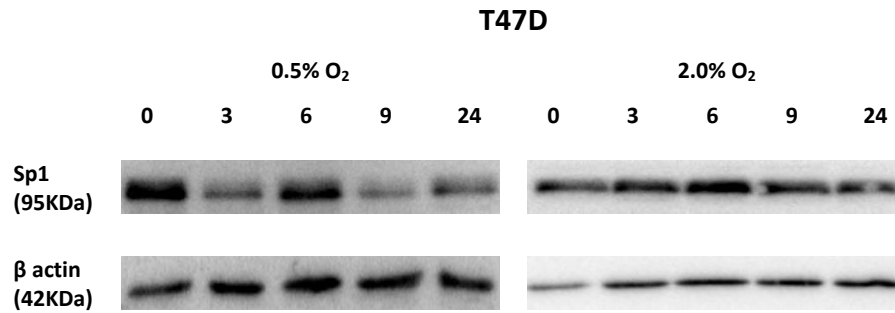


Figure 3.5: Effect of severe and moderate hypoxia on Sp1 protein levels in MDA-MB-231 cells

Panel A: MDA-MB-231 cells were exposed to hypoxia followed by protein lysis at different time points. 30 μ g of whole cell lysates were separated by SDS-PAGE and further analysed by Western blotting with antibodies against Sp1 protein. β -Actin was used as a loading control. n=3 experiments.

Panel B: protein levels were analysed by densitometry analysis using Image Lab software. All results were presented as relative fold change compared to zero-hour control. Error bars represent +/- SEM. Statistical analysis were carried out with Two-way ANOVA with Bonferroni post hoc correction. *, P< 0.05.

A.



B.

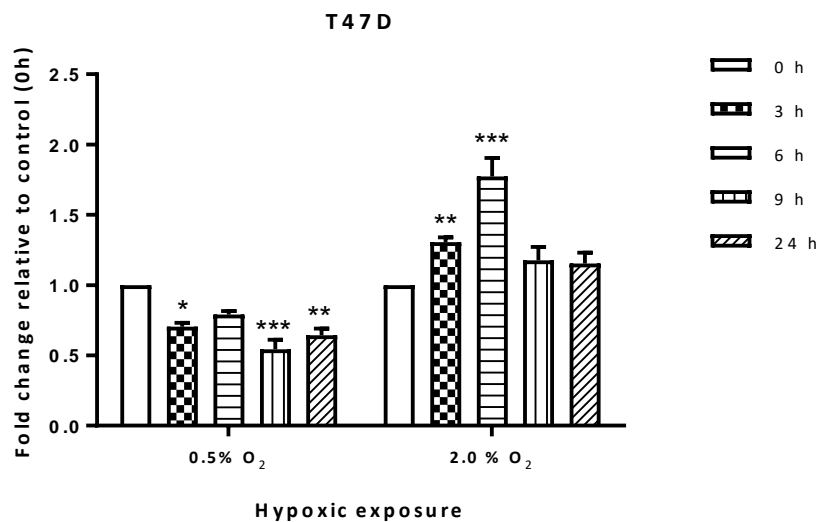


Figure 3.6: Effect of severe and moderate hypoxia on Sp1 protein levels in T47D cells

Panel A: T47D cells were exposed to hypoxia followed by protein lysis at different time points. 30 μ g of whole cell lysates were separated by SDS-PAGE and further analysed by Western blotting with antibodies against Sp1 protein. β -Actin was used as a loading control. n=3 experiments

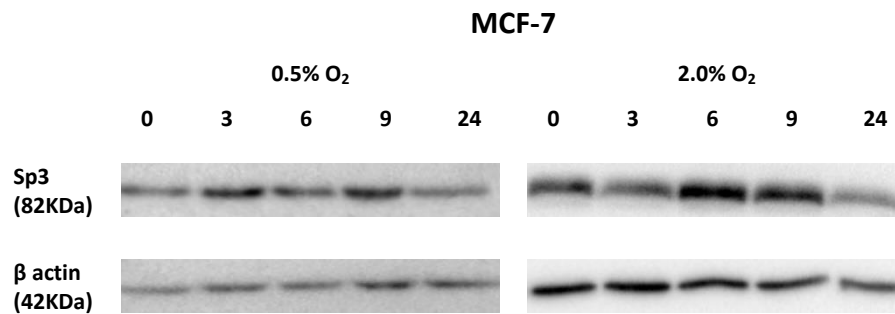
Panel B: protein levels were analysed by densitometry analysis using Image Lab software. All results were presented as relative fold change compared to zero-hour control. Error bars represent +/- SEM. Statistical analysis were carried out with Two-way ANOVA with Bonferroni post hoc correction. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Sp3 protein levels in T47D cells were significantly decreased after 3 hours of severe hypoxia. Highest decrease of Sp3 protein levels was recorded at 24 hours of exposure which was (0.48 FC (+/- 0.20 SD)). In contrast, at 2.0% O₂ exposure, upregulation at 6 (0.37 FC (+/- 0.05 SD)) and 9 (0.39 FC (+/- 0.15 SD)) hours' time points were identified which were statistically significant (see Figure 3.9).

No detectable bands were observed for Sp4 protein at any given conditions. Several antibodies were tested which resulted absence of specific band for Sp4 with precise molecular weight (data not shown).

Overall results indicate the hypoxic induction of Sp proteins in breast cancer cell lines. Severe hypoxic exposure induced upregulation of Sp1 and Sp3 proteins in both MCF-7 and MDA-MB-231 cells whereas in mild hypoxia only MCF-7 showed induction of both proteins. However, T47D cells behaved contrary to other two cell lines. Sp protein levels were downregulated in severe hypoxia whilst they were upregulated in mild conditions. Expression changes were also dependent on oxygen concentrations (severity of hypoxia) and tissue types.

A.



B.

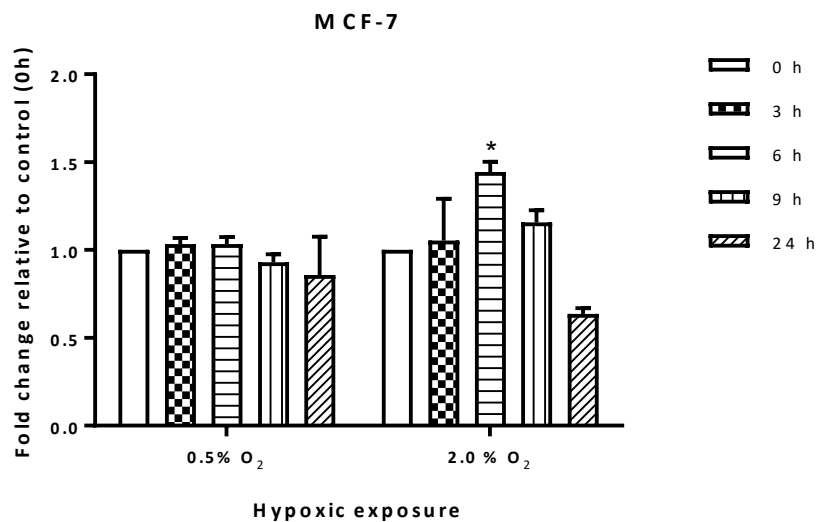
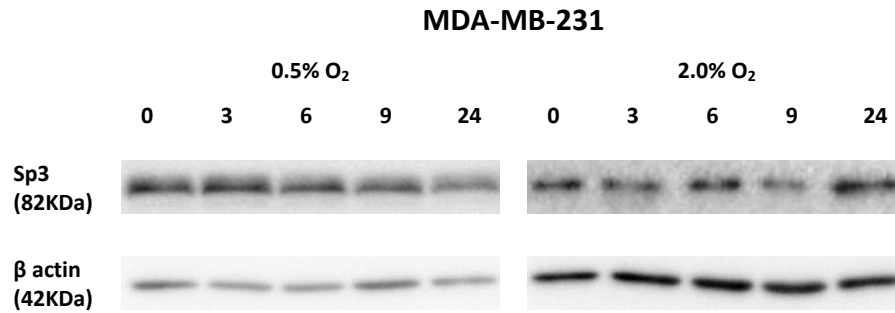


Figure 3.7: Effect of severe and moderate hypoxia on Sp3 protein levels in MCF-7 cells

Panel A: MCF-7 cells were exposed to hypoxia followed by protein lysis at different time points. 30 μ g of whole cell lysates were separated by SDS-PAGE and further analysed by Western blotting with antibodies against Sp1 protein. β -Actin was used as a loading control. n=3 experiments.

Panel B: protein levels were analysed by densitometry analysis using Image Lab software. All results were presented as relative fold change compared to zero-hour control. Error bars represent +/- SEM. Statistical analysis were carried out with Two-way ANOVA with Bonferroni post hoc correction. * P< 0.05.

A.



B.

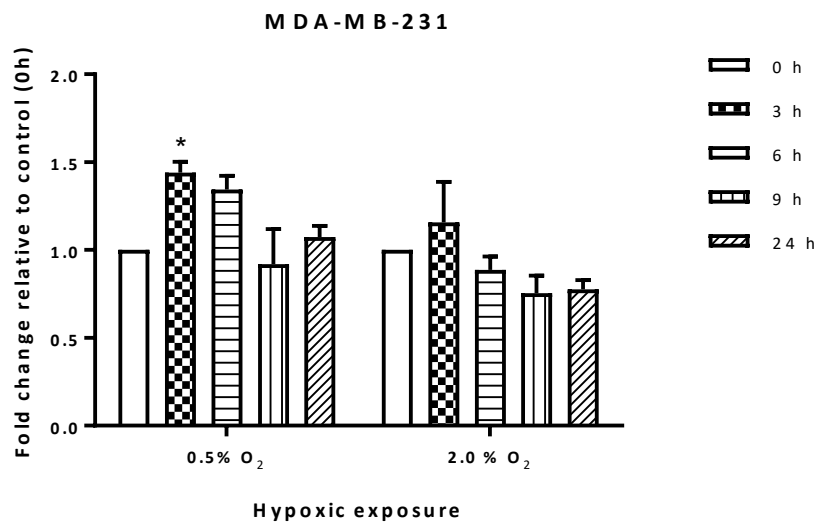
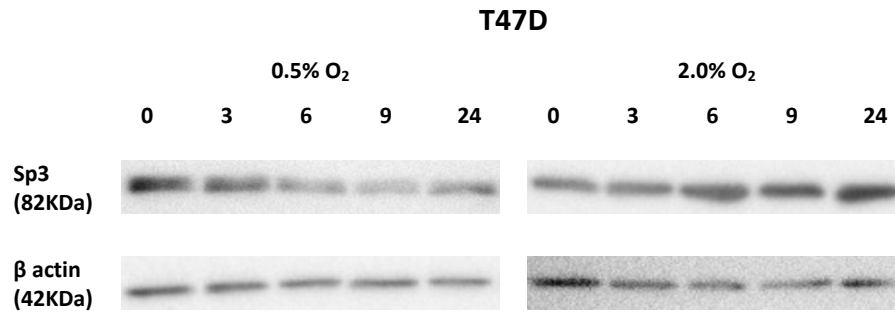


Figure 3.8: Effect of severe and moderate hypoxia on Sp3 protein levels in MDA-MB-231 cells

Panel A: MDA-MB-231 cells were exposed to hypoxia followed by protein lysis at different time points. 30 μ g of whole cell lysates were separated by SDS-PAGE and further analysed by Western blotting with antibodies against Sp1 protein. B-Actin was used as a loading control. n=3 experiments.

Panel B: protein levels were analysed by densitometry analysis using Image Lab software. All results were presented as relative fold change compared to zero-hour control. Error bars represent +/- SEM. Statistical analysis were carried out with Two-way ANOVA with Bonferroni post hoc correction. * P< 0.05.

A.



B.

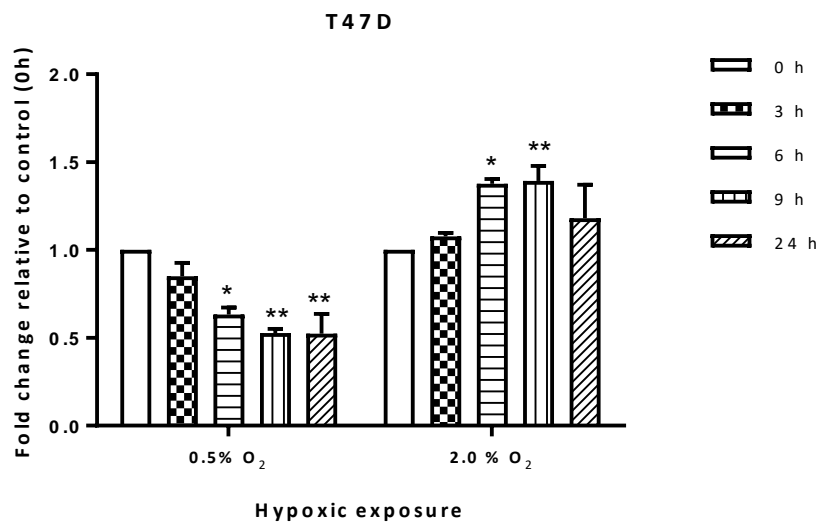


Figure 3.9: Effect of severe and moderate hypoxia on Sp3 protein levels in T47D cells

Panel A: T47D cells were exposed to hypoxia followed by protein lysis at different time points. 30 μ g of whole cell lysates were separated by SDS-PAGE and further analysed by Western blotting with antibodies against Sp1 protein. β -Actin was used as a loading control. n=3 experiments.

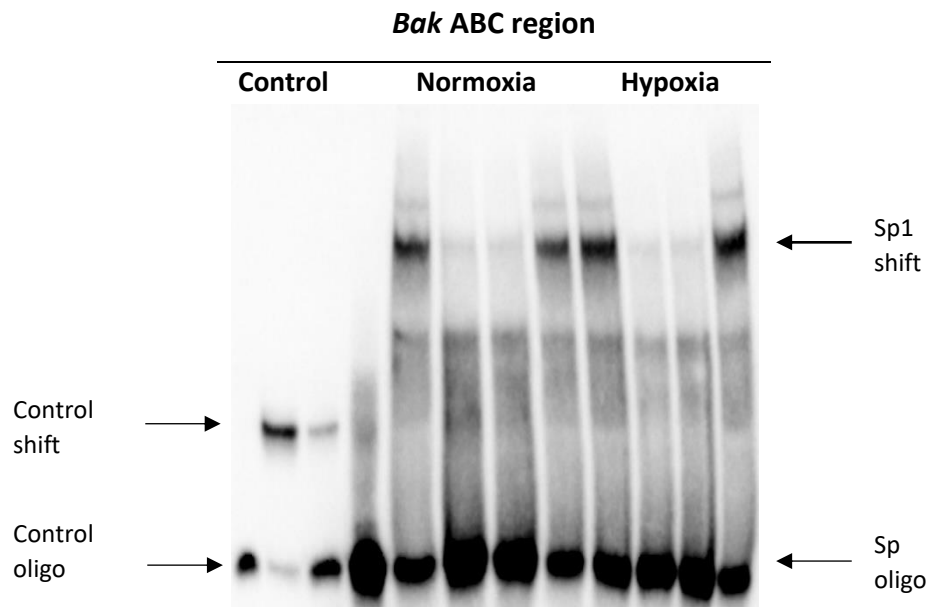
Panel B: protein levels were analysed by densitometry analysis using Image Lab software. All results were presented as relative fold change compared to zero-hour control. Error bars represent +/- SEM. Statistical analysis were carried out with Two-way ANOVA with Bonferroni post hoc correction. *, P < 0.05; **, P < 0.01.

3.2.3. Binding activity changes of Sp protein/complex due to hypoxic exposure

During hypoxic induction, Sp protein transcription factors may undergo functional and structural modifications such as binding activity changes which is vital in transcriptional regulation of Sp mediated genes. In order to assess the binding activity changes of Sp proteins due to hypoxic microenvironment, the Electro Mobility shift assay (EMSA) technique was used. Cells were exposed to 0.5% (v/v) O₂ hypoxic condition followed by nuclear protein extraction. Normoxic counterparts were also treated in the same way in a conventional chamber. Extracted nuclear proteins were then subjected to EMSA assay with commercially purchased EBNA DNA controls in order to validate the assay procedure. The binding analysis was carried out with known Sp regions of *Bak* gene promoter (ABC and B region) and Sp consensus region (as described in Chapter 2). The binding pattern of Sp proteins/complex was assessed by analysing the appearance of shifted band on the EMSA blots. Densitometry was also carried out for quantification purposes.

As seen in figures 3.10-18 EBNA controls showed expected binding patterns where binding of labelled DNA and nuclear extracts were detected in band 2 (lane b) whereas in the presence of excess unlabelled DNA detected band was disappeared (lane c). Similarly, no binding patterns were detected either for control DNA (DNA without any GC-rich regions) (Results not shown). This validated the assay procedure and experimental conditions.

Binding of Sp protein/complex to *Bak* gene promoter ABC region was detected in both normoxic and hypoxic conditions as shifted bands on EMSA gel (Figures 3.10-12). Nuclear extracts of all three cell lines, MCF-7; Figure 3.10, MDA-MB-231; Figure 3.11 and T47D; Figure 3.12) showed similar type of binding patterns towards *Bak* gene promoter ABC regions. Clear bands were detected in samples containing nuclear extract and the labelled DNA (lane 2 and 6) where no bands were detected in the presence of unlabelled DNA (lane 3 and 7). It was clearly visible that Sp complex interacts with DNA in both normoxic and hypoxia conditions. However, no significant alteration under hypoxic conditions were observed compared to the normoxic counterparts despite some visible changes in band intensities. In addition, Sp1 protein specific antibody resulted no super shifted band (lane 5 and 9).



Lane	a	b	c	1	2	3	4	5	6	7	8	9
Biotin labelled EBNA DNA	+	+	+	-	-	-	-	-	-	-	-	-
EBNA nuclear extract	-	+	+	-	-	-	-	-	-	-	-	-
Unlabelled EBNA DNA	-	-	+	-	-	-	-	-	-	-	-	-
Biotin labelled DNA	-	-	-	+	+	+	+	+	+	+	+	+
Nuclear extract	-	-	-	-	+	+	+	+	+	+	+	+
Competitor (Unlabelled)	-	-	-	-	-	+	+	-	-	+	+	-
Sp1 antibody	-	-	-	-	-	-	+	+	-	-	+	+

Figure 3.10: Effect of severe hypoxia on binding activity of Sp proteins in MCF-7 cells
MCF-7 cells were exposed to severe hypoxia (0.5% (v/v) O₂) for 12 hours. Normoxic samples were also prepared in parallel to hypoxic samples. Nuclear proteins were extracted following the procedure described in method section (section 2.7). Biotinylated DNA oligomers of *Bak* gene promoter ABC region containing Sp binding sites were used to assess the binding of Sp protein/complex. EMSA protocol was carried out with Light shift EMSA kit and EBNA control DNA was used as a positive control for EMSA. 30 µg of nuclear proteins were separated by electrophoresis in a non-denaturing gel in TBE buffer. Blots are representative of n=4 experiments.

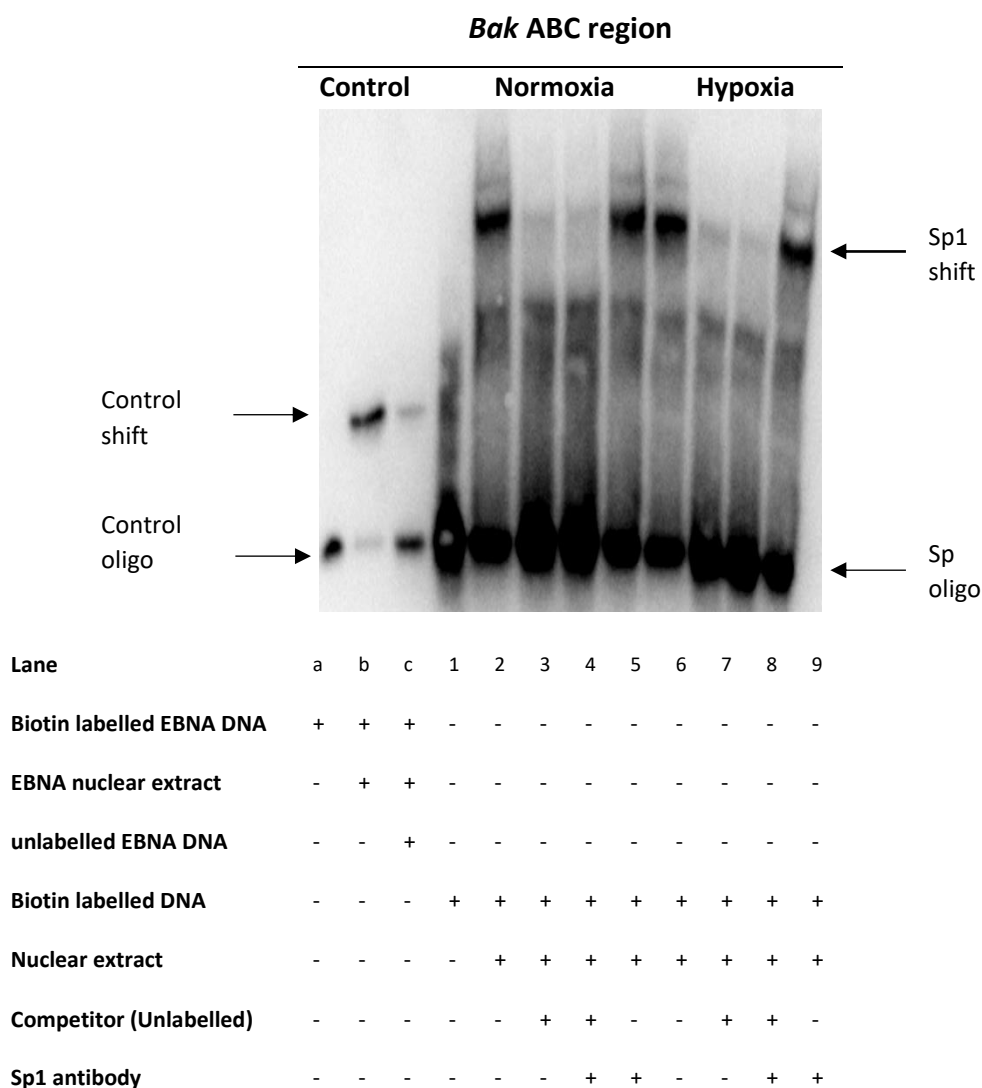


Figure 3.11: Effect of severe hypoxia on binding activity of Sp proteins in MDA-MB-231 cells

MDA-MB-231 cells were exposed to severe hypoxia (0.5% (v/v) O₂) for 12 hours. Normoxic samples were also prepared in parallel to hypoxic samples. Nuclear proteins were extracted following the procedure described in method section (section 2.7). Biotinylated DNA oligomers of *Bak* gene promoter ABC region containing Sp binding sites were used to assess the binding of Sp protein/complex. EMSA protocol was carried out with Light shift EMSA kit and EBNA control DNA was used as a positive control for EMSA. 30 µg of nuclear proteins were separated by electrophoresis in a non-denaturing gel in TBE buffer. Blots are representative of n=4 experiments.

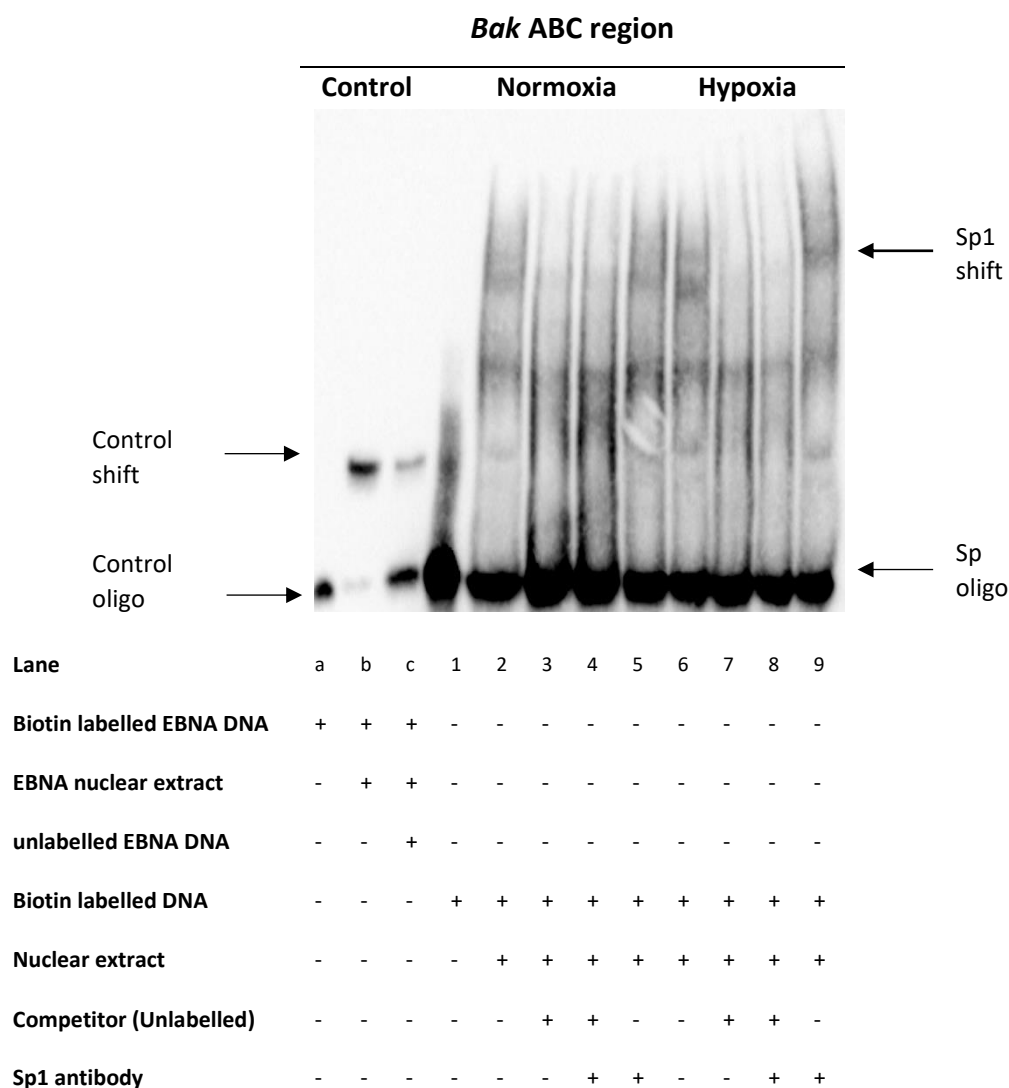


Figure 3.12: Effect of severe hypoxia on binding activity of Sp proteins in T47D cells
T47D cells were exposed to severe hypoxia (0.5% (v/v) O₂) for 12 hours. Normoxic samples were also prepared in parallel to hypoxic samples. Nuclear proteins were extracted following the procedure described in method section (section 2.7). Biotinylated DNA oligomers of *Bak* gene promoter ABC region containing Sp binding sites were used to assess the binding of Sp protein/complex. EMSA protocol was carried out with Light shift EMSA kit and EBNA control DNA was used as a positive control for EMSA. 30 µg of nuclear proteins were separated by electrophoresis in a non-denaturing gel in TBE buffer. Blots are representative of n=4 experiments.

Even though it was possible that hypoxia may cause higher binding of the particular protein complex, densitometry analysis also showed that changes are insignificant in nature (data not shown).

Binding of the Sp protein complex towards *Bak* gene B region was also identified. A similar type of binding patterns was detected in all three cell lines. As can be seen in Figures 3.13-15, samples containing nuclear extracts and labelled DNA showed clear evidence of binding of the protein complex where bands disappeared in the presence of unlabelled DNA. Similar to the previous occasion, Sp1 antibody did not affect the binding of the protein complex. Similar to *Bak* ABC region hypoxic samples showed a slight increase of binding compared normoxic counterparts. However, changes were insignificant in densitometry analysis.

In contrast, binding analysis towards Sp consensus region resulted in lack of clear bands (smears were detected). Even though it was visible that binding of a similar protein complex is apparent, all three cell lines showed smears type of bands. Due to lack of clear bands, further analysis and quantification was not feasible (see Figures 3.16-18)

Gel shift assays indicate the DNA-Sp protein complex interaction in both normoxic and hypoxic conditions in addition to possible increase of binding activity of Sp proteins/Sp protein complex towards biologically relevance promoter sites. Due to the hypoxic induction, visibly intense bands were observed (compared to the normoxic condition) which can be interpreted as higher association of the protein complexes with DNA. However, due to the limitations of the EMSA technique and insignificant nature of densitometry analysis, more work is required to draw a conclusion.

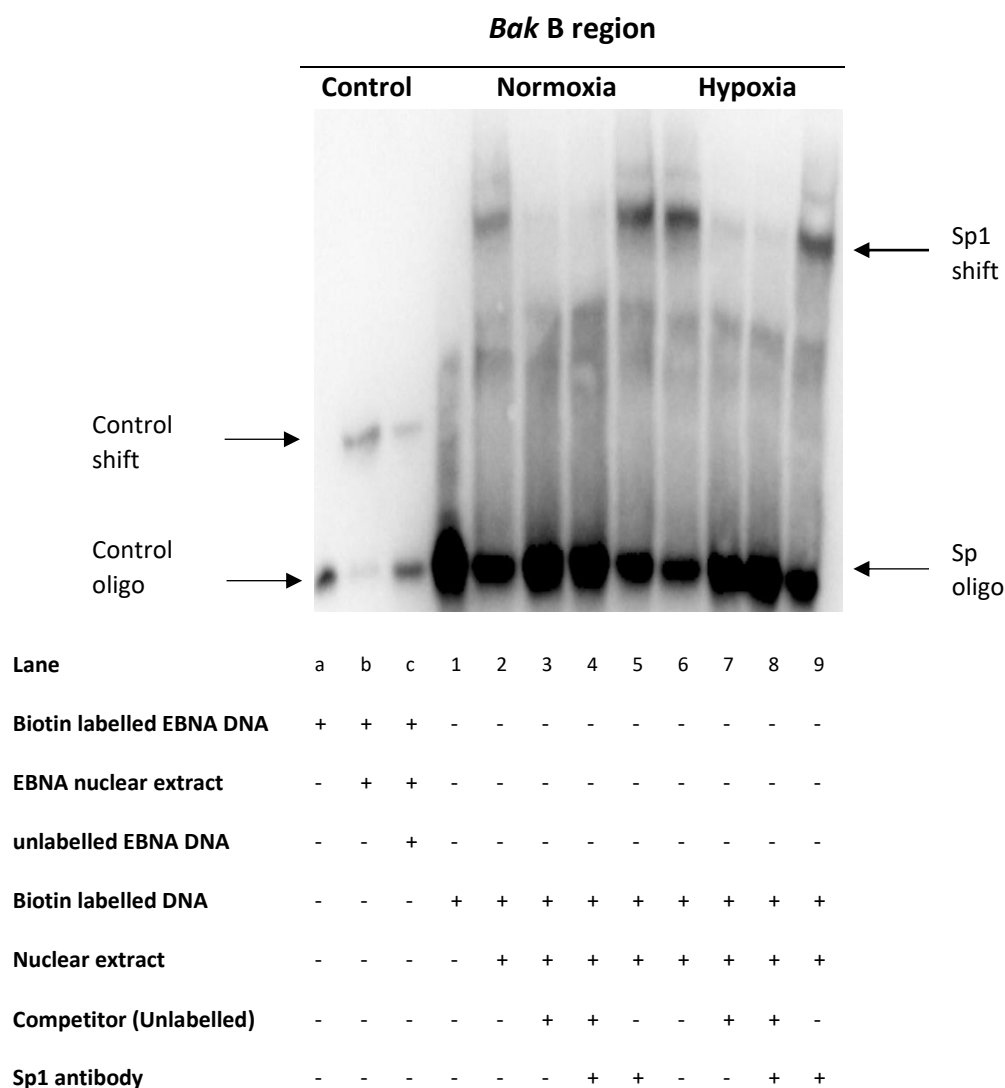


Figure 3.13: Effect of severe hypoxia on binding activity of Sp proteins in MCF-7 cells
MCF-7 cells were exposed to severe hypoxia (0.5% (v/v) O₂) for 12 hours. Normoxic samples were also prepared in parallel to hypoxic samples. Nuclear proteins were extracted following the procedure described in method section (section 2.7). Biotinylated DNA oligomers of *Bak* gene promoter B region containing Sp binding sites were used to assess the binding of Sp protein/complex. EMSA protocol was carried out with Light shift EMSA kit and EBNA control DNA was used as a positive control for EMSA. 30 µg of nuclear proteins were separated by electrophoresis in a non-denaturing gel in TBE buffer. Blots are representative of n=4 experiments.

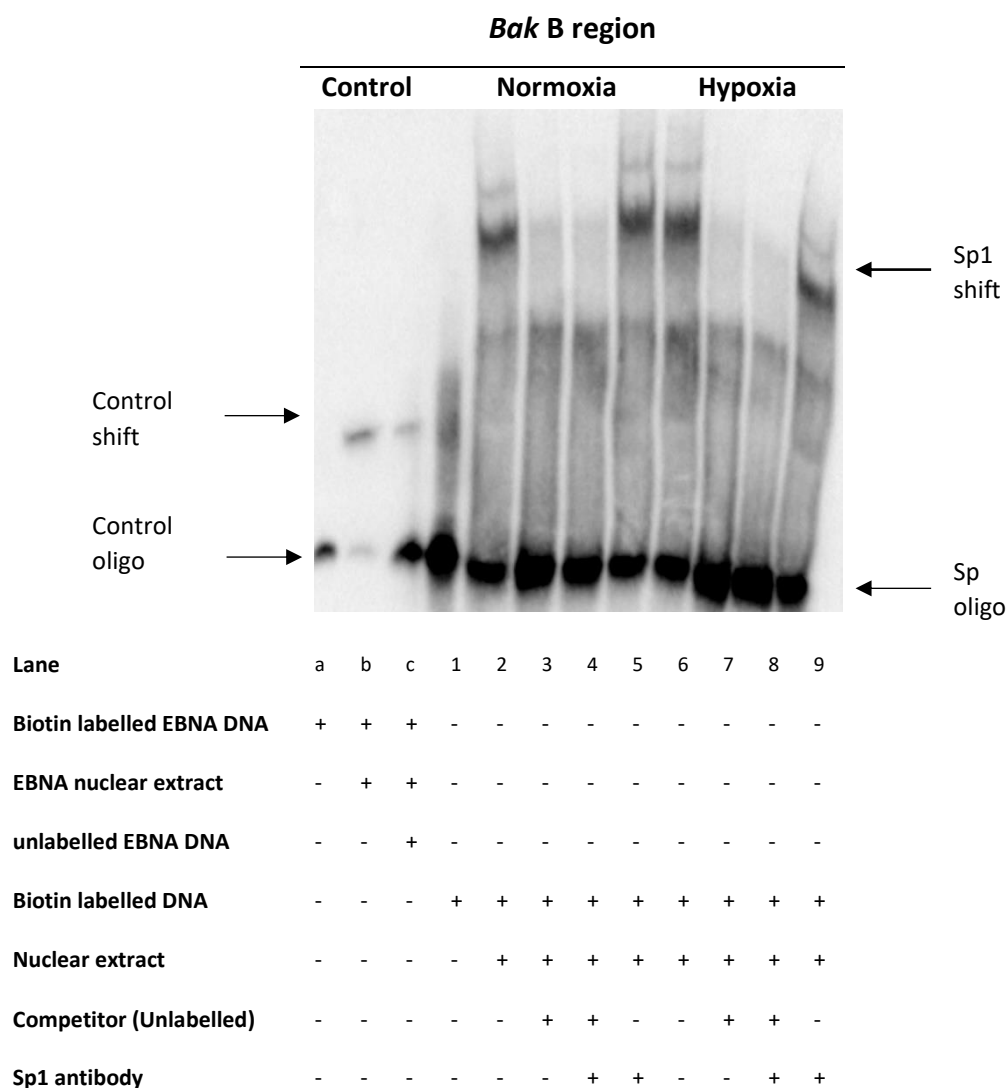


Figure 3.14: Effect of severe hypoxia on binding activity of Sp proteins in MDA-MB-231 cells

MDA-MB-231 cells were exposed to severe hypoxia (0.5% (v/v) O₂) for 12 hours. Normoxic samples were also prepared in parallel to hypoxic samples. Nuclear proteins were extracted following the procedure described in method section (section 2.7). Biotinylated DNA oligomers of *Bak* gene promoter B region containing Sp binding sites were used to assess the binding of Sp protein/complex. EMSA protocol was carried out with Light shift EMSA kit and EBNA control DNA was used as a positive control for EMSA. 30 µg of nuclear proteins were separated by electrophoresis in a non-denaturing gel in TBE buffer. Blots are representative of n=4 experiments.

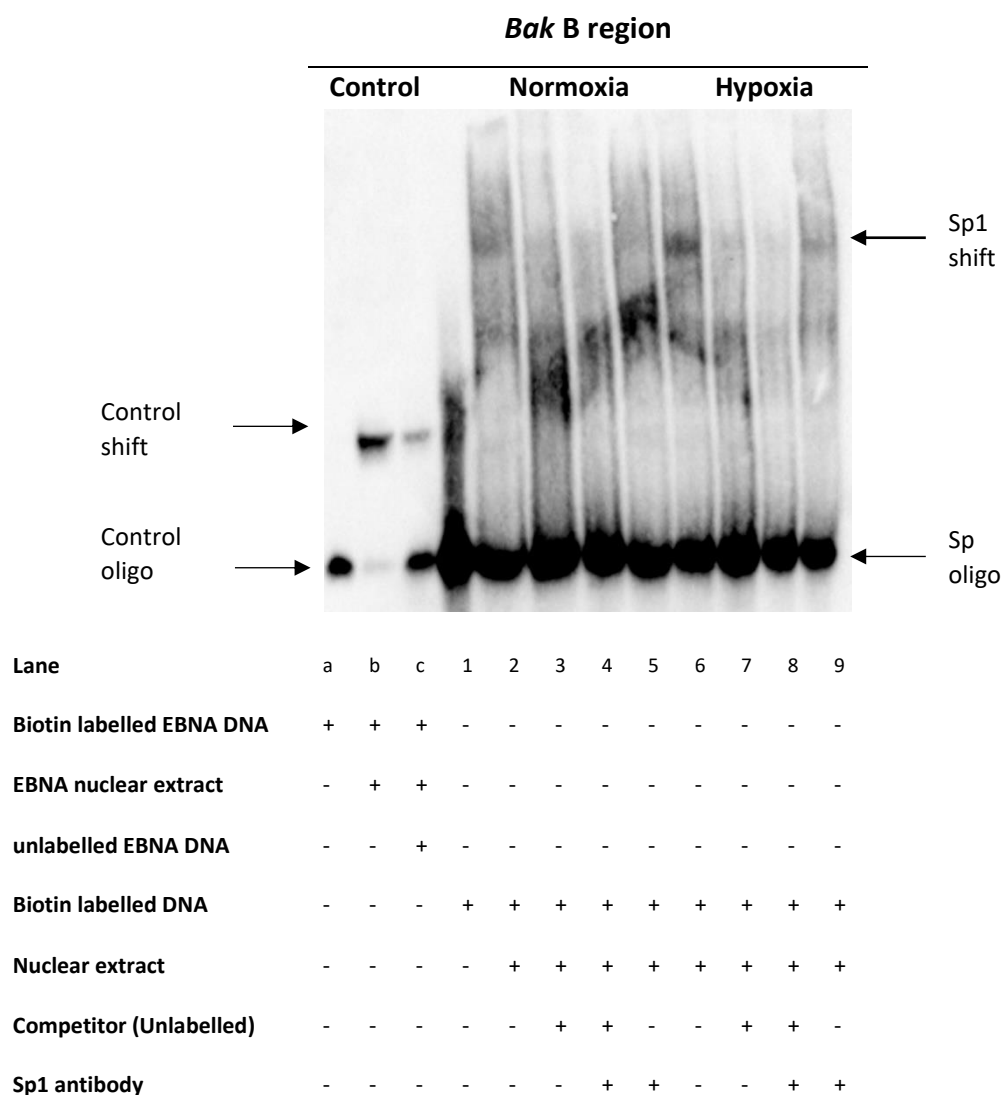


Figure 3.15: Effect of severe hypoxia on binding activity of Sp proteins in T47D cells

T47D cells were exposed to severe hypoxia (0.5% (v/v) O₂) for 12 hours. Normoxic samples were also prepared in parallel to hypoxic samples. Nuclear proteins were extracted following the procedure described in method section (section 2.7). Biotinylated DNA oligomers of *Bak* gene promoter B region containing Sp binding sites were used to assess the binding of Sp protein/complex. EMSA protocol was carried out with Light shift EMSA kit and EBNA control DNA was used as a positive control for EMSA. 30 µg of nuclear proteins were separated by electrophoresis in a non-denaturing gel in TBE buffer. Blots are representative of n=4 experiments.

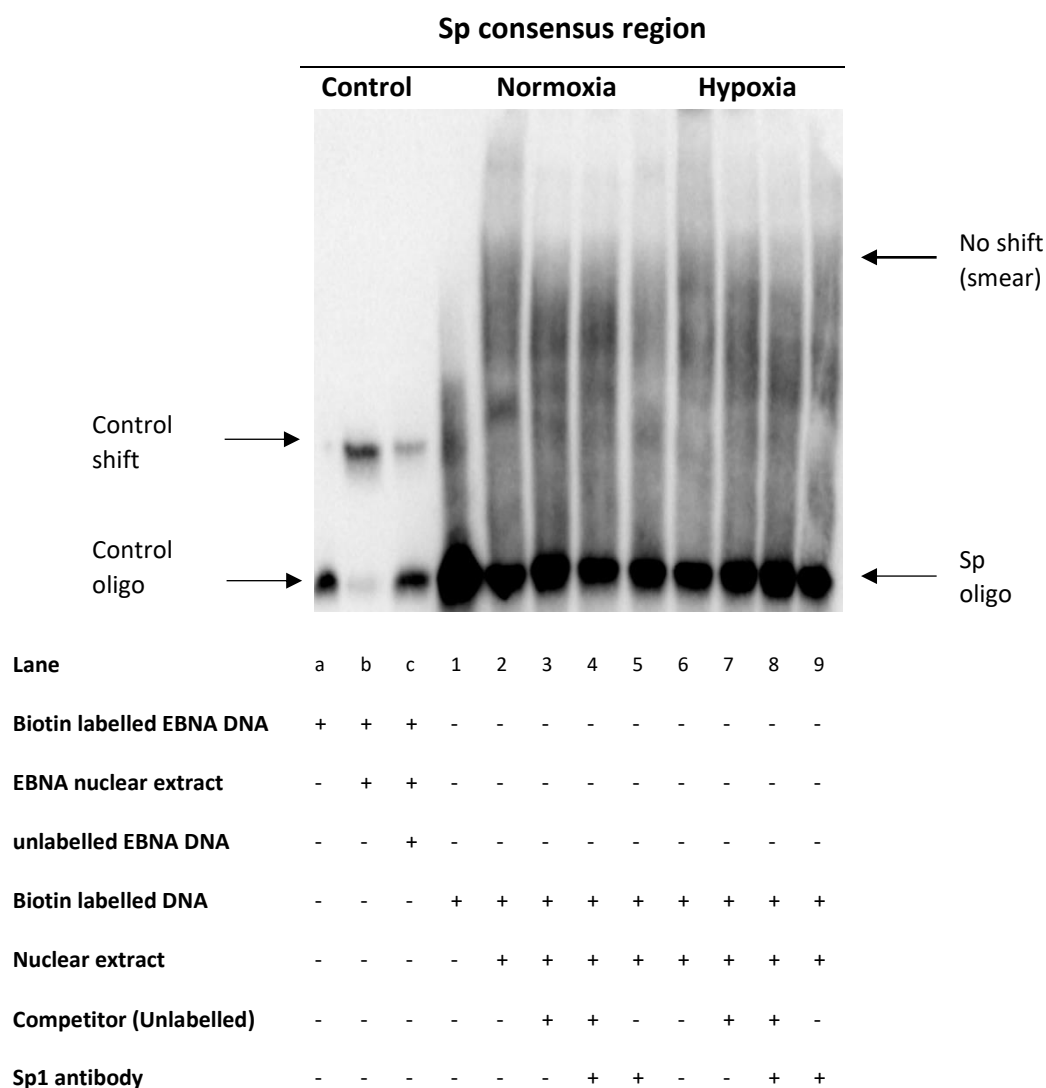


Figure 3.16: Effect of severe hypoxia on binding activity of Sp proteins in MCF-7 cells
MCF-7 cells were exposed to severe hypoxia (0.5% (v/v) O₂) for 12 hours. Normoxic samples were also prepared in parallel to hypoxic samples. Nuclear proteins were extracted following the procedure described in method section (section 2.7). Biotinylated DNA oligomers representing Sp consensus region were used to assess the binding of Sp protein/complex. EMSA protocol was carried out with Light shift EMSA kit and EBNA control DNA was used as a positive control for EMSA. 30 µg of nuclear proteins were separated by electrophoresis in a non-denaturing gel in TBE buffer. Blots are representative of n=4 experiments.

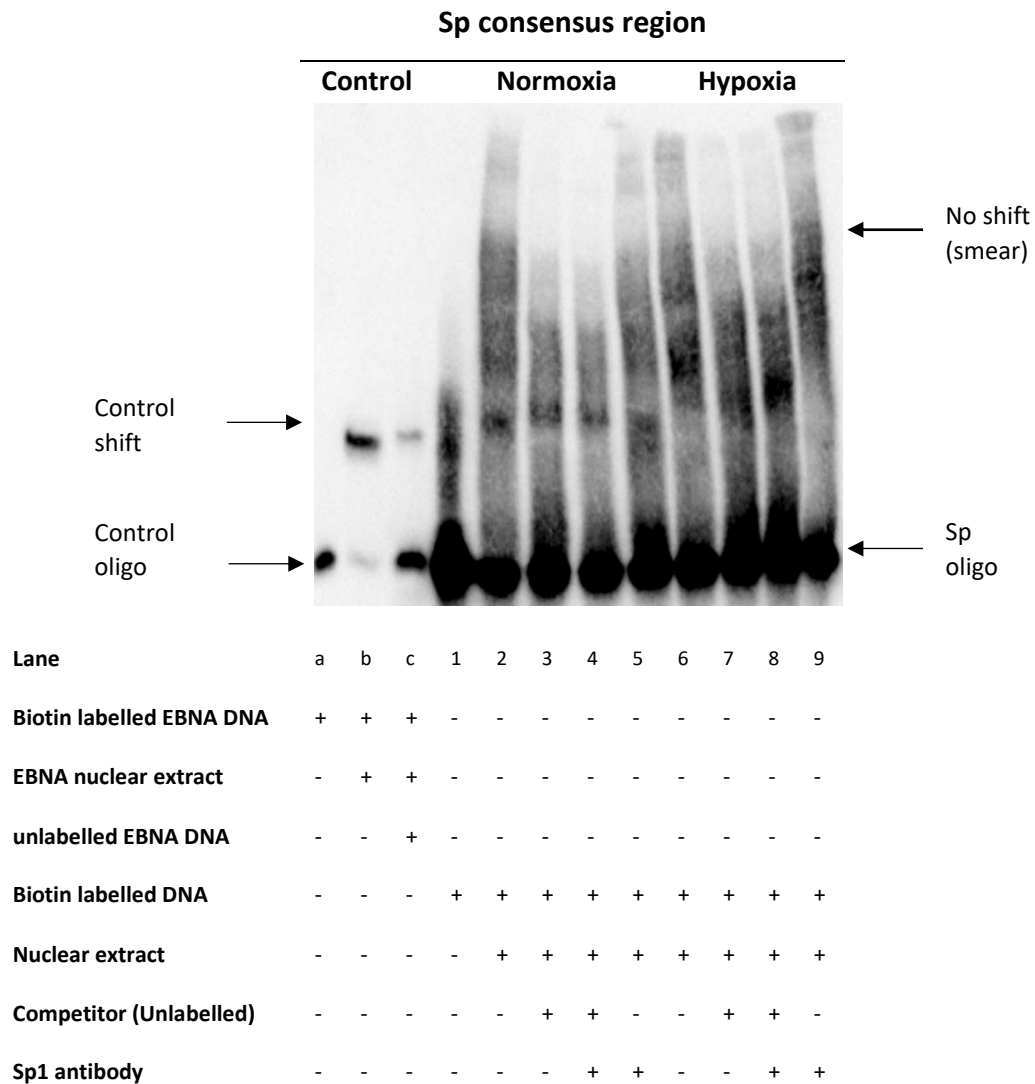


Figure 3.17: Effect of severe hypoxia on binding activity of Sp proteins in MDA-MB-231 cells

MDA-MB-231 cells were exposed to severe hypoxia (0.5% (v/v) O₂) for 12 hours. Normoxic samples were also prepared in parallel to hypoxic samples. Nuclear proteins were extracted following the procedure described in method section (section 2.7). Biotinylated DNA oligomers representing Sp consensus region were used to assess the binding of Sp protein/complex. EMSA protocol was carried out with Light shift EMSA kit and EBNA control DNA was used as a positive control for EMSA. 30 µg of nuclear proteins were separated by electrophoresis in a non-denaturing gel in TBE buffer. Blots are representative of n=4 experiments.

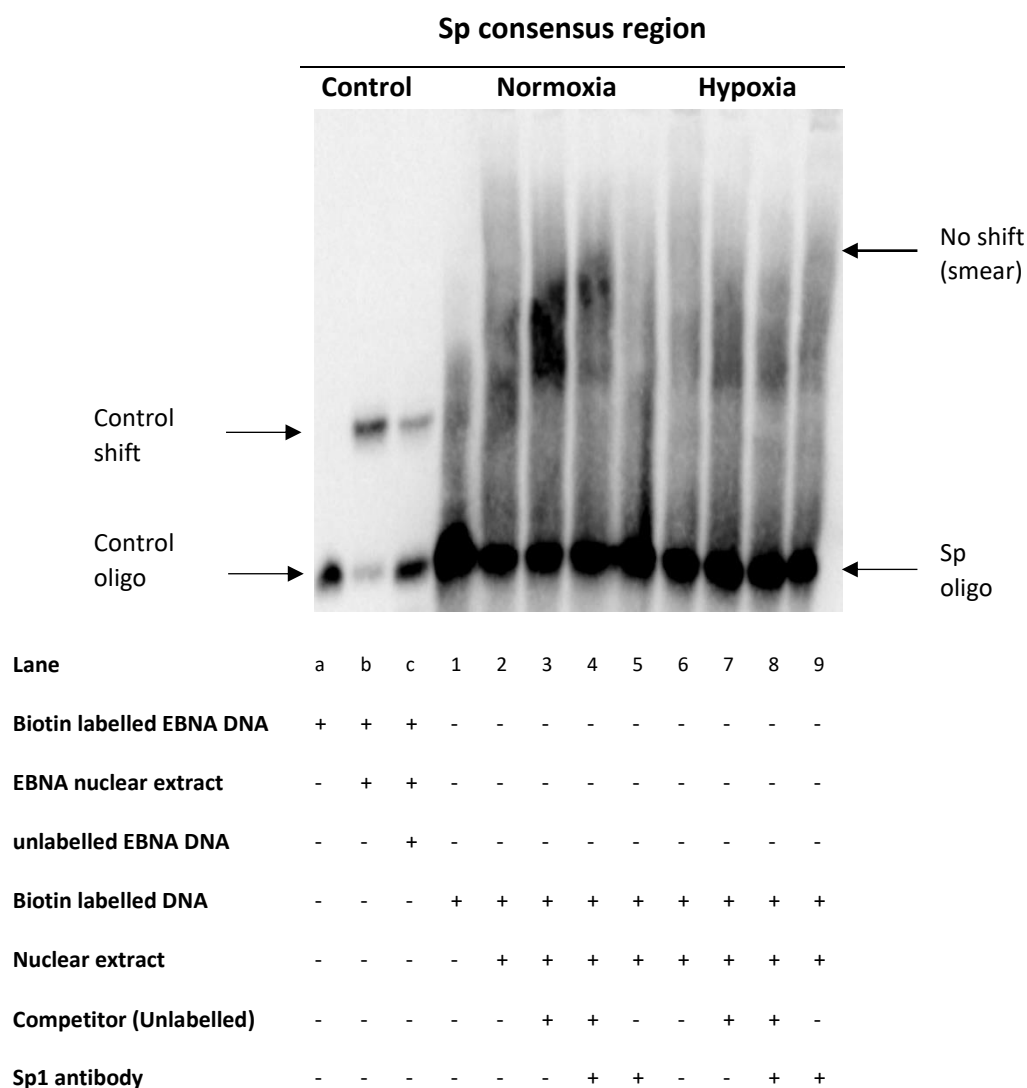


Figure 3.18: Effect of severe hypoxia on binding activity of Sp proteins in T47D cells

T47D cells were exposed to severe hypoxia (0.5% (v/v) O₂) for 12 hours. Normoxic samples were also prepared in parallel to hypoxic samples. Nuclear proteins were extracted following the procedure described in method section (section 2.7). Biotinylated DNA oligomers representing Sp consensus region were used to assess the binding of Sp protein/complex. EMSA protocol was carried out with Light shift EMSA kit and EBNA control DNA was used as a positive control for EMSA. 30 µg of nuclear proteins were separated by electrophoresis in a non-denaturing gel in TBE buffer. Blots are representative of n=4 experiments.

3.2.4. Impact of Mithramycin A on transcription of Sp protein family

Mithramycin A is a GC-promoter specific, DNA-binding inhibitor which is known to inhibit Sp mediated gene transcription (Miller et al., 1987). In this study, the effect of Mithramycin A on Sp family transcription was investigated in the hypoxic microenvironment. MCF-7, MDA -MB-231 and T47D cells were treated with 1 μ M Mithramycin A for 24 hours including 12 hours of hypoxic exposure. Transcripts were analysed using TaqMan qPCR assays with *B2M* as a house keeping gene. Normoxic counterparts were analysed in parallel to hypoxic samples. Data represented as a fold change against the untreated normoxic sample.

Mithramycin A treatment caused a significant decrease in transcript levels of *Sp1* gene in all three cell lines. Expression inhibition was prominent under the hypoxic microenvironment where changes were significant compared to the normoxic control as well as the untreated hypoxic samples. As evident from Figure 3.19, decrease of 1.15 FC (+/- 0.05 SD) in MCF-7, 1.14 FC (+/- 0.13 SD) in MDA-MB-231 and 0.83 FC (+/- 0.09 SD) in T47D cells were observed against the untreated hypoxic counterparts. These changes were also significant against the control. However, Mithramycin A treatment had no significant effect on normoxic samples of MCF-7 and MDA-MB-231 cells compared to the control except for T47D cells where it was significantly decreased by 0.69 FC (+/- 0.11 SD).

Sp3 gene was downregulated upon Mithramycin A treatment in the hypoxic condition in all three cell lines (see Figure 3.20). Changes were significant against untreated hypoxic sample as well the control. MCF-7, MDA-MD-231 and T47D cells showed 0.65 FC (+/- 0.08 SD), 0.47 FC (+/- 0.04 SD) and 0.41 FC (+/- 0.03 SD) decrease in expression levels of *Sp3* against the untreated hypoxic counterparts in particular order. The effects of Mithramycin A treatment on normoxic sample were varied among cell lines where MCF-7 cells showed significant upregulation (0.43 FC (+/- 0.40 SD) while it decreased in T47D cells (0.73 FC (+/- 0.06 SD). However, MDA-MB-231 cells showed no fold changes.

Mithramycin A treatment caused similar pattern of changes in *Sp4* transcripts as other family members. Treated samples in hypoxia showed significant reduction in expression levels compared to the control as well as the untreated hypoxic sample. As evident from Figure 3.21, in MCF-7 cells 1.30 FC (+/- 0.06 SD), in MDA-MB-231 cells

0.44 FC (+/- 0.18 SD) and in T47D cells 0.89 FC (+/- 0.04 SD) decreases were identified against the untreated hypoxic counterparts. In addition, MDA-MB-231 and T47D cells showed significant reduction in normoxic samples compared to the normoxic control.

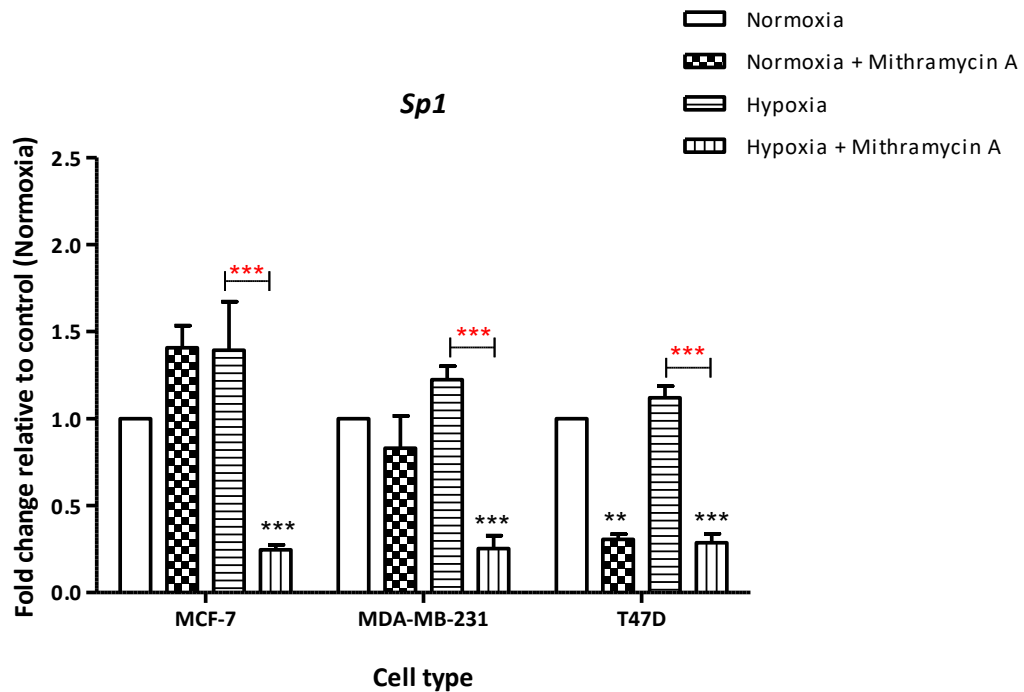


Figure 3.19: Effect of Mithramycin A and severe hypoxia on *Sp1* gene expression in breast cancer cells

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were treated with 1 μ M Mithramycin for 24 hours followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as Fold change relative normoxic sample. n=3 experiments: Error bars represent +/- SEM: Statistical analysis by Two-way ANOVA with Bonferroni post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). **, P< 0.01; ***, P< 0.001.

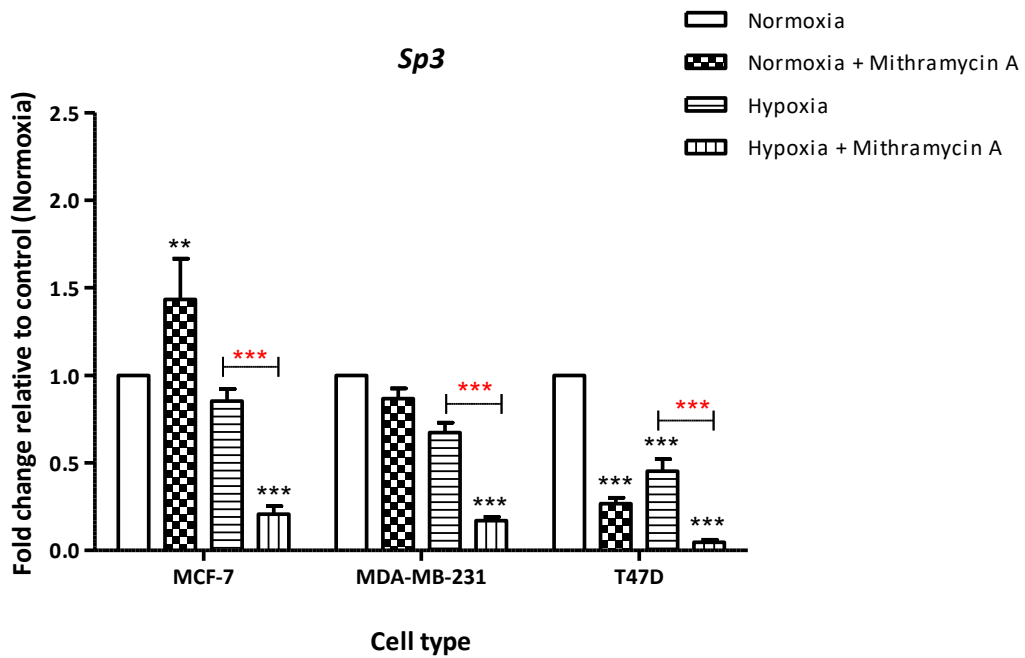


Figure 3.20: Effect of Mithramycin A and severe hypoxia on *Sp3* gene expression in breast cancer cells

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were treated with 1 μ M Mithramycin for 24 hours followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as Fold change relative normoxic sample. n=3 experiments: Error bars represent +/- SEM: Statistical analysis by Two-way ANOVA with Bonferroni post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). **, P< 0.01; ***, P< 0.001.

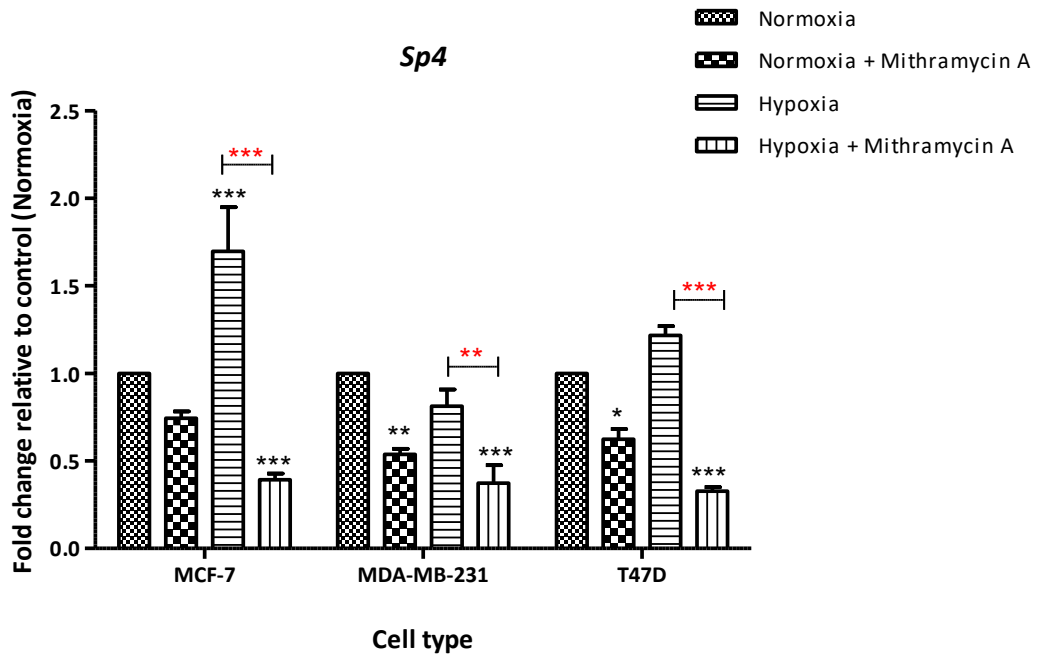


Figure 3.21: Effect of Mithramycin A and severe hypoxia on *Sp4* gene expression in breast cancer cells

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were treated with 1 μ M Mithramycin for 24 hours followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as Fold change relative normoxic sample. n=3 experiments: Error bars represent +/- SEM: Statistical analysis by Two-way ANOVA with Bonferroni post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P< 0.05; **, P< 0.01; ***, P< 0.001.

3.3. Discussion

This chapter aimed to investigate the effect of hypoxic exposure on Sp family transcription factors in breast cancer cell lines. Alterations in expression levels (transcript and protein level) and binding activity changes were selected as potential areas that these proteins may response to hypoxia. It was essential to elucidate these key aspects in order to identify the effect of hypoxia and related cellular pathways.

To evaluate transcriptomic changes, TaqMan qPCR assays were used with specific assays for *Sp1*, *Sp3* and *Sp4* genes. Cells were exposed to severe hypoxia for variable lengths of time (0-24 hours) followed by qPCR analysis. In addition, western blotting method was used to assess the effect of hypoxia on protein levels of Sp family members. Activity changes of Sp proteins (in terms of binding activity) towards promoter sequences were also examined using Gel shift assay technique (EMSA) with three known DNA sequences. Finally, the inhibitory action of Mithramycin A on transcription of Sp genes was also tested in hypoxic environment.

Key findings of this chapter are summarised as below.

1. mRNA expression levels of *Sp1*, *Sp3* and *Sp4* were changed due to severe hypoxic condition in a cell type specific manner.
2. Alterations in protein levels were observed in severe and moderated hypoxic conditions where upregulation of *Sp1* and *Sp3* proteins were prominent. However, hypoxic induction varied depending on cell type and severity of hypoxia.
3. No significant changes in binding level of Sp protein/complex were observed after hypoxic exposure.
4. Mithramycin A significantly inhibited transcription of *Sp1*, *Sp3* and *Sp4* genes in all three cell lines under hypoxia.

3.3.1. The effect of severe hypoxia on expression levels of Sp proteins

In this section, alteration in expression of Sp transcription factors was investigated using breast cancer cell lines. Both MCF-7 and T47D cells showed upregulation of *Sp1* and *Sp4* gene transcripts while *Sp3* transcript levels remained unchanged. Similarly, in MDA-MB-231 cells, *Sp1* transcripts showed no changes where expression of *Sp3* and *Sp4* genes were slightly decreased.

Sp transcription factors are ubiquitously expressed in most cell types where gene upregulation can be found in pathological conditions and tumours. Till date, it is not clear how exactly and to what extent hypoxia affects the expression of Sp proteins. It has been identified that Sp proteins expression levels were altered under both in mild and severe hypoxic conditions. Two potential mechanisms have been proposed which drives expression changes of Sp proteins in the hypoxic microenvironment. Generation of reactive oxygen species in low oxygen environments may upregulate Sp expression levels. Reactive oxygen species may activate translation of Sp proteins at 5'-untranslated region of Sp1 mRNA (Yeh et al., 2011). Another possible mechanism is HIF-1 α mediated expression changes of Sp proteins. A study carried out by Woo *et al.*, has shown the presence of HRE elements in rat Sp promoter sites suggesting the HIF-mediated transcription changes of Sp proteins (Woo et al., 2012). A similar mechanism was established by Jeong et al., suggesting HIF-1 α mediated Sp1 transcription in neurone cells (Jeong & Park, 2012). However, lack of data is available in cancer cell context and detailed mechanisms of Sp protein induction is not fully understood.

3.3.1.1. The effect of hypoxia on Sp transcription factor protein levels

It is hypothesised that protein levels of Sp transcription factors might be upregulated due to hypoxic exposure which in turn facilitates the regulation of hypoxic induction at the transcriptional level. Expression level changes were analysed using the western blotting technique with specific antibodies. Protein level changes in Sp family transcription factors were identified at two different hypoxic conditions 0.5% (v/v) (severe hypoxia) and 2.0% (v/v) (moderate hypoxia). The present study confirms that under severe hypoxic condition, levels of Sp1 and Sp3 proteins were upregulated at different time points suggesting cell type specific expression changes. Moreover, this study further confirms the correlation between Sp protein expression and hypoxic exposure in MCF-7, MDA-MB-231 and T47D cells. *In-vitro* studies confirm that different oxygen tensions affect expression changes in specific manners where overall Sp proteins showed upregulation in hypoxia. This emphasised the importance of the Sp proteins as a valuable transcription factor in severe hypoxia.

Alteration in protein levels of Sp transcription factors has been reported in several occasions due to different cellular stresses including tumour hypoxia and nutrient starvation (Bouwman & Philipsen, 2002). Previous studies which include various

cancer cell lines such as lung, colon and ovarian cancers have shown the aberrant regulation of Sp protein expression in the hypoxic microenvironment. Conflicting ideas have been presented where some studies have suggested that only the severe hypoxia has an effect on protein levels where some studies have shown that both severe and moderate hypoxia have an effect on protein levels of Sp family transcription factors. For instance, a study carried out by Kong *et al.*, have reported the upregulation of Sp1 protein levels in lung cancer cells in severe hypoxia where overexpression of Sp protein lead to expression of *CD147* gene (Kong et al., 2010). Similarly, Kaluz *et al.*, have reported that both Sp1 and Sp3 proteins are upregulated under severe hypoxia where moderate hypoxia had no significant effect (Kaluz et al., 2003). Moreover, a study carried out by Deacon *et al.*, have shown that levels of elevated levels of Sp1 levels in lung cancer cells (NSCLC cells) due to severe hypoxic exposure (1.0% (v/v) O₂) (Deacon et al., 2012). Contrastingly, a study carried out by Discher *et al.*, noted that Sp1 levels were unchanged whilst Sp3 protein levels decreased in muscle cells (Discher et al., 1998).

Several studies have attempted to define the link between transcription of Sp family genes and hypoxia. HIF mediated transcription has been identified as the central hypoxic induction mechanism where key hypoxic genes are regulated by HIF mediated mechanisms (Lee et al., 2004a; Miki et al., 2004). The presence of HRE elements in Sp promoter sites suggests dependency of Sp gene transcriptions on HIF (HIF-1 α and HIF-2 α) mediated transcription (Florczyk et al., 2011; Woo et al., 2012). However, none of these studies were able to elucidate the actual relationship of hypoxia and Sp genes. In this study, we confirm that both moderate and severe hypoxic conditions alter the expression levels of Sp1/Sp3 proteins in breast cancer cells. Expression patterns also depended on the tissue type and the severity of the hypoxic exposure.

3.3.1.2. Antibody optimization

Different antibodies specific to Sp proteins were optimised for experimental conditions. Preliminary, hypoxic/normoxic conditions were used to optimise different antibodies. Antibodies specific to Sp1 and Sp3 were reliable in both standard RIPA buffer based cell lysates as well as Urea based cell lysates.

As HIF protein is unstable at normoxic conditions, Urea (CH₄N₂O) based buffer was required to detect stable HIF proteins. Unfortunately, no Sp4 antibodies used in this

study were reliable to detect a specific protein band at any conditions stated above. Utmost best was done to try all the commercially available antibodies. However, no detectable protein was detected at any conditions such as different antibody concentrations/different protein concentrations etc. Positive controls provided/specified by the provider were also used in conjunction with actual experimental samples where band were detected only in the control lysates. Presence of low level of Sp4 protein in these cell line may have obscured the detection on visible bands. Therefore, protein level analysis was limited to Sp1 and Sp3 proteins.

Preliminary studies carried out with Sp proteins indicated that Sp proteins could also be induced by confluency of the cells and nutrient starvation (Bouwman & Philipsen, 2002). This is a common phenomenon for hypoxia-induced proteins. In order to minimise the confluency effect, it was made sure that cell monolayers would not reach the confluency by the end of the experimental procedure. In addition, media was replenished prior to the hypoxia exposure.

3.3.2. Hypoxia-driven activity changes on Sp DNA binding

Alteration in activity changes of Sp transcription factors are yet fully understood in hypoxic microenvironment. In this study, binding activity changes of Sp proteins were analysed after severe hypoxic exposure in breast cancer cell model. In these experiments, nuclear proteins were extracted after 12 hours of hypoxia exposure (0.5% O₂) and binding activity of Sp protein/Protein complexes were analysed using EMSA assay. Present experimental strategy successfully identified the interaction of Sp protein complex towards its binding sites in both normoxic and hypoxic conditions. Both Bak gene promoter sequences have shown that binding of a protein complex whilst some occasions higher binding was detected in hypoxic samples compared to the normoxia (only visible in band densities).

During hypoxic induction, it is believed that Sp proteins undergo a variety of post translational modifications which allow them to alter their binding activity. Common types of post-translational modifications of Sp proteins have been summarised in review published by Waby and colleagues (Waby et al., 2008). Phosphorylation has been identified as the most common modification, and enhances the interaction of Sp proteins with double-stranded DNA. Studies carried out by Pal *et al.*, have shown suppression of Sp1 activity by p53 mediated pathways in hypoxic microenvironment

(Pal et al., 2001). However contrasting results were presented by Choi *et al.*, who showed that increased activity of Sp1 protein due to hypoxia in liver (Hep3B), lung (A549) and head and neck (SQ2OB) cancer cells where *VEGF* expression is upregulated via Akt-PI3K pathway (Choi et al., 2011). Acetylation and SUMOylation of Sp1 have also reported in hypoxia which activate *TNXB* (breast cancer) and *SIRT1* genes (lung and ovarian cancers) respectively (Kato et al., 2008; Sun et al., 2013a; Sun et al., 2013b). The EMSA technique identifies binding activity of particular proteins to the promoter region of interest. This method can be used to investigate binding activity of proteins towards different promoter sequences in cost effective way with minimum optimisation (Hellman & Fried, 2007). Three promoter regions were selected for this study (as described in Chapter 2) including Sp consensus region. Alterations in binding activity were compared against normoxic condition. Binding activity of Epstein Bar Nuclear Antigen (EBNA) DNA was used as a positive control for the assay. DNA oligomer without any GC-rich regions or Sp binding sites were also analysed to validate Sp protein binding. *Bak* gene promoter is known to regulate by Sp mediated transcription. Previously published data have shown that *Bak* gene promoter contains three Sp1 binding sites where each binding site has different affinity for binding. The whole sequence was selected and named as ABC region (60 bp), while highest affinity region has been named as B region (20 bp) (Waby et al., 2010; Yu et al., 2010). These two regions were used to identify the binding of Sp proteins in normoxic and hypoxic conditions.

These results are in line with previous literature where increased binding activity has been recorded towards *CCL2* promoter sites in melanoma cells (Kunz et al., 2002). Hence, it is surprisingly, no clear bands were detected for Sp consensus region. All three cell lines showed similar behaviour in analysis where smears were detected. It is possible that some other proteins also interact with this DNA which makes it more inconsistent with particular complex. It is believed that interaction of antibody and protein complex results formation of a large complex in terms of molecular weight. This can be seen as “super shift” in the corresponding blot. However, incorporation of Sp1 protein specific antibody had a contrasting effect on binding of the complex. Hence, in these experiments, no “super shift” was observed at any occasion which may be due to following reasons. Interaction DNA may prevent binding of antibodies due

to steric hindrance or antibody interaction destructs the complex formation. The other possible explanation would be that molecular weight of the (antibody + protein + DNA) complex is much higher in size which is not feasible to be detected. It is widely accepted that multiple complexes of EMSA super shift assays are notoriously difficult to be interpreted. Nevertheless, this technique was essential to identify any preliminary changes before further characterisation of the identified protein complex using immunoprecipitation techniques.

3.3.2.1. Limitation of the Gel shift assays (EMSA)

Even though the gel shift assays are commonly used in binding analysis, there are several limitations associated with the EMSA technique. Gel shift assays are typically used to detect protein-DNA interactions. Mainly it is more of a qualitative technique where quantification is not widely practised. Densitometry analysis was carried out to quantify the EMSA gels in order to determine the binding activity changes of Sp proteins. Despite some visible alterations of the band densities in normoxic and hypoxic conditions, changes were negligible to claim any significant changes. Moreover, all the densitometry results of EMSA analysis were deemed insignificant in nature. It is also a well-known fact the lack of reliability of densitometry technique for quantitative analysis of proteins (Gassmann et al., 2009; Taylor & Posch, 2014). Data derived from densitometry analysis varied among replicates and due to less sensitivity of the technique no significant changes were identified at this juncture.

Alternatively, chromatin immunoprecipitation could be a better technique for the quantitative analysis of DNA-protein interactions. A similar approach has been used by Stoner *et al.*, in ZR-75 breast cancer cell lines (Stoner et al., 2004). However, this may require higher level of optimisation of the standard protocol prior to quantification.

In this study, it was assumed that Sp proteins are interacting with specific DNA sequences. However, it is possible that hypoxic exposure may have caused other cofactors and interacting proteins to associate with Sp proteins which in turn bind to the DNA as whole complex to the DNA. *Drosophila* SL2 cells which lack Sp family transcription factors could have been an alternative option for *in-vitro* studies (or Sp^{-/-} mice models) to minimise the formation of Sp protein complexes especially to prevent the competition between Sp1 and Sp3 proteins (Courey & Tjian, 1988). When

using SL2 cells, cells can be transfected with desired vector constructs and nuclear extracts can be analysed with specific antibodies against Sp proteins. A study carried out by Chandran *et al.*, have used similar techniques to identify the interaction of Sp protein interaction towards GC-Box (Chandran et al., 2008). Furthermore, Sp1/Sp3 associate with DNA in a chromatin context where steric hindrance and other intermolecular forces play some vital roles. Nevertheless, most of the studies use linear DNA with desired promoter sites. Naked DNA and nuclear extracts which are commonly used may not reflect the in-situ situation. Moreover, binding of Sp proteins to naked DNA affinity is shown to be higher than binding to chromatin structures.

3.3.3. Inhibitory effect of Mithramycin A on transcription of Sp proteins

Mithramycin A is known to inhibit Sp mediated transcription by interacting with GC rich promoter regions. This study aimed to investigate the role of Mithramycin A on transcription of Sp family proteins particularly in hypoxic microenvironment. The present study agrees with previous studies where Mithramycin A leads to decreased expression of three Sp family members (Xie & Collins, 2013). In addition, combination of hypoxia and Mithramycin A resulted in further reduction in transcription. This is the first time that this combination has been used to investigate the effect of Sp family transcriptions. This lead to further characterise kinetics of Mithramycin A and the effect of Mithramycin A on other hypoxia responsive genes later on this study.

3.3.4. Conclusion

Data presented in this study identifies main modifications that Sp transcription factors undergo in hypoxic microenvironment. Hypoxia induces transcriptomic changes of Sp genes particular *Sp1* and *Sp4* genes in breast cancer cells. In addition, both Sp1 and Sp3 protein levels were significantly upregulated in hypoxia suggesting hypoxic induction of these proteins and their potential role in gene regulation. Induction of Sp proteins may possibly tissue-specific and depend on severity of hypoxia. In addition, Sp protein inhibitor, Mithramycin A suppresses the expression of all three Sp genes in hypoxia. Therefore, Mithramycin can be useful in inhibition of Sp driven gene regulation in hypoxia.

Chapter 4.

Transcriptional regulation of hypoxia responsive genes

4. Transcriptional regulation of hypoxia responsive genes

4.1. Introduction

Tumour hypoxia is associated with higher tumour survival and metastasis leading to aggressive phenotypes and poor prognosis (Tsai & Wu, 2012; Chang & Epler, 2014; Semenza, 2016). Formation of hypoxic microenvironments induce transcriptomic changes of numerous genes involved in key hypoxia mediated cellular pathways (Benita et al., 2009). Genes involved in early metastatic changes such as epithelial-mesenchymal transition (EMT), invasion, migration, angiogenesis, immune suppress and tumour resistance are also induced in tumour hypoxia. In addition, a study carried out by Wong *et al.*, have shown that secreted factors such as LOX and LOX like promoters which play a key role in premetastatic niches in secondary organs in later metastasis are also produced in hypoxia (Wong et al., 2011). Activation of metastatic cascades (mainly by hypoxia signalling) is a major challenge in therapeutic strategies and often linked with higher mortality in patients (Yamamoto et al., 2008).

Transcription factors play a vital role in hypoxic induction by regulating transcriptomic changes of key hypoxia responsive genes. To date, several transcription factors have been identified and are well characterised. Hypoxia inducible factors (HIFs) (Heterodimeric transcription factor comprised of HIF-1 α and HIF-2 α) (Keith et al., 2012; Liu et al., 2012)) play the central role in the hypoxic signalling by regulating the transcription of the major hypoxia inducible genes (Dengler et al., 2014). In addition to HIF, there are other transcription factors which are also involved in hypoxia induced transcriptional changes. Studies suggest potential transcriptional regulation roles of cyclic AMP response element binding protein (CREB) (Beitner-Johnson & Millhorn, 1998), Activating protein-1 (AP-1) (Fantozzi et al., 2003) , Nuclear factor kappa-B (NF-kB) (van Uden et al., 2008), p53 (Chandel et al., 2000b) and Early growth response - 1(Egr-1) (Yan et al., 1999) in hypoxic induction *in-vitro* and *in-vivo*.

Importantly, recent investigations have shown that Sp transcription factors which are also known to regulate transcription of the *HIF1A* gene, are involved in regulating key hypoxia responsive genes in HIF dependent and independent manner (Archer, 2011; Zawacka-Pankau et al., 2011). In fact, some hypoxia responsive genes are known to bear binding sites which are similar to Sp transcription factor promoter consensus

sequence suggesting a potential role of Sp proteins. Hypoxia responsive genes; *CA9* (Kaluz et al., 2003), *GAPDH* (Higashimura et al., 2011), *CD147* (Ke et al., 2012) and *MDR1* (Comerford et al., 2002) are regulated by the Sp1 transcription factors in HIF dependent manner in hypoxia microenvironment. In addition, studies conducted by Pore *et al.*, have shown that HIF independent regulation of *VEGF* is controlled by the Sp1 transcription factor (Pore et al., 2004). Nevertheless, hypoxic signalling pathways and not fully elucidated, especially HIF independent mechanisms. Lack of studies has been conducted to identify the Sp mediated hypoxia responsive genes in the tumour hypoxia. Identification of Sp mediated hypoxia responsive genes is vital to elucidate cellular pathways of hypoxic induction and the exact role of Sp transcription family in the hypoxic tumour microenvironment. Furthermore, novel hypoxia responsive genes can be potentially used as biomarkers for hypoxic tumours including breast cancers.

4.1.1. Aims and objectives of the chapter

Sp transcription factors may regulate genes involved in the hypoxic induction and downstream cellular pathways alongside HIF mediated gene regulation. This work hypothesis that the Sp1 transcription factor in particular, may play a key role in a number of HIF independent transcription changes in response to hypoxia. This study, therefore aims to use publicly available microarray data to:

- Identify hypoxia induced changes in gene expression in different breast cancer lines (Gene expression changes will be verified by qPCR analyses)
- Determine if these hypoxia responsive genes have Sp and /or HIF promoter binding sites
- Conduct pathway analysis to identify key pathways which are altered in hypoxia and have Sp binding sites for gene regulation

4.2. Results

4.2.1. Analysis of microarray data sets

In order to identify hypoxia responsive genes, publicly available NCBI microarray data sets which fulfilled the search criteria (see section 2.9) were downloaded and analysed using GeneSpring 12.1X software (see Table 4.1). Due to some technical limitations, gene expression analysis was further narrowed to limited number of data sets. Two data sets were further analysed to identify changes in gene expression. Expression analysis and gene enrichment analysis were carried out accordingly followed by pathway analysis.

4.2.1.1. *Microarray data set GSE18494*

NCBI microarray data set, GSE18494 was downloaded and analysed using GeneSpring 12x software. Affymetrix genechip, HG-U133_Plus_2 microarray platform had been used for the initial experiment. In the original experiment, MDA-MB-231 cells have been subjected to 0.5% O₂ hypoxic exposure for different durations (0, 4, 8 and 12 hours) before gene expression analysis. All 54675 entities were subjected to probe set filtration and experimental grouping followed by one-way ANOVA test. The number of entities was narrowed down to 2050 after statistical analysis, which was then subjected to fold change analysis. The number of genes with the minimum cut-off value of 1.5 were selected for further analyses (460 out of 2050 entities). To determine the presence of genes with Specificity protein (Sp1) or HIF protein binding sites in their promoters, Gene Set enrichment analyses were carried out with gene set GGGCGGR_V\$SP1_Q6 and ELVIDGE_HIF1A_TARGETS_DN separately. According to the GeneSpring GSEA algorithm, 58 genes were found to contain Sp sites and 51 genes contained HIF binding sites (out of 460 genes). As seen in Figure 4.1, expression changes in genes which have putative Sp1 sites were presented as a heat map representing fold changes at different time points. Genes identified with Sp and HIF binding sites are also listed in Table 4.2 and 4.3 respectively. Pathway analysis identified 90 pathways where one or more genes with Sp1 sites and 51 pathways for genes with HIF sites (*Appendix I*).

Table 4.1: NCBI microarray data sets retrieved for analysis

Database Accession No	Microarray platforms	Experimental design and cell line	Reference
GSE49955	Affymetrix Human Genome U133 Plus 2.0 Array GPL570	Two breast cancer cell lines (T47D and MDA-MB-231) exposed to hypoxia (0.1% O ₂ for 24h) and untreated conditions (3 replicates each)	(Chen et al., 2014b)
GSE30019	Illumina HumanWG-6 v3.0 expression bead chip GPL6884	MCF-7 were collected respectively at 0, 1, 4, 8, 12 and 24 hours after reoxygenation (3 replicates each)	(Lai et al., 2011)
GSE17188	Agilent-014850 Whole Human Genome Microarray 4x44K G4112F GPL6480	Two MDA-MB-231 sublines, SCP2 and LM2 (4175), were applied to low oxygen (1%) for 0, 6 and 24 hours. (Two replicates each)	(Lu et al., 2010)
GSE18494	Affymetrix Gene Chip Human Genome U133 Plus 2.0 Array GPL9419	MDA-MB231 breast cancer cells were collected under normoxic conditions (~19% O ₂ , 0 hours) and after 4, 8 and 12 hours of hypoxia treatment (0.5% O ₂) (3 replicates each)	(Xia & Kung, 2009)
GSE49999	Affymetrix Multispecies miRNA-3 Array GPL16384	MCF7 cells were exposed to hypoxia (0.1% O ₂) and Normoxia for 48 h (3 replicates each)	(Bandara et al., 2014)
GSE47533	Illumina HumanWG-6 v3.0 expression bead chip GPL6884	MCF-7 exposed to hypoxia (1% Oxygen) for 16h, 32h, 48h and normoxia (3 replicates each)	(Camps et al., 2014)

GSE47009	Affymetrix Human Gene 2.0 ST Array [transcript (gene) version] GPL16686	MCF-7 cells exposed to 90 hours in either normoxic or hypoxic conditions (2 replicates for normoxia and 1 sample of hypoxia)	No citation
GSE3188	[HG-U133A] Affymetrix Human Genome U133A Array GPL96 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array GPL570 Sentrix Human-6 Expression Bead Chip GPL2507	MCF7 breast cancer cell lines were grown under either normoxia (21% oxygen) or hypoxia (1% oxygen) conditions for 16 hours (3 replicates each)	(Elvidge et al., 2006)

Table 4.2: Gene enrichment analysis for Sp1 promoter sites

Entrez Gene	Gene Symbol	Entrez Gene	Gene Symbol
1832	<i>DSP</i>	6676	<i>SPAG4</i>
1153	<i>CIRBP</i>	9314	<i>KLF4</i>
694	<i>BTG1</i>	55689	<i>YEATS2</i>
1843	<i>DUSP1</i>	79365	<i>BHLHE41</i>
8553	<i>BHLHE40</i>	11000	<i>SLC27A3</i>
6513	<i>SLC2A1</i>	79660	<i>PPP1R3B</i>
2026	<i>ENO2</i>	8862	<i>APLN</i>
3725	<i>JUN</i>	79896	<i>THNSL1</i>
4739	<i>NEDD9</i>	51062	<i>ATL1</i>
5209	<i>PFKFB3</i>	81555	<i>YIPF5</i>
901	<i>CCNG2</i>	85301	<i>COL27A1</i>
4891	<i>SLC11A2</i>	87178	<i>PNPT1</i>
7162	<i>TPBG</i>	22936	<i>ELL2</i>
7056	<i>THBD</i>	25909	<i>AHCTF1</i>
8987	<i>STBD1</i>	139596	<i>UPRT</i>
8819	<i>SAP30</i>	150864	<i>FAM117B</i>
4300	<i>MLLT3</i>	9180	<i>OSMR</i>
7490	<i>WT1</i>	84725	<i>PLEKHA8</i>
5210	<i>PFKFB4</i>	84969	<i>TOX2</i>
1907	<i>EDN2</i>	170384	<i>FUT11</i>
5033	<i>P4HA1</i>	56477	<i>CCL28</i>
9048	<i>ARTN</i>		
10957	<i>PNRC1</i>		
7026	<i>NR2F2</i>		
8660	<i>IRS2</i>		
5727	<i>PTCH1</i>		
7422	<i>VEGFA</i>		
55818	<i>KDM3A</i>		
23210	<i>JMJD6</i>		
144699	<i>FBXL14</i>		
115123	<i>Mar-03</i>		
9520	<i>NPEPPS</i>		
1294	<i>COL7A1</i>		
79647	<i>AKIRIN1</i>		
51315	<i>KRCC1</i>		
27242	<i>TNFRSF21</i>		
64236	<i>PDLIM2</i>		

Heat map key

Colour variation in relation to fold changes.

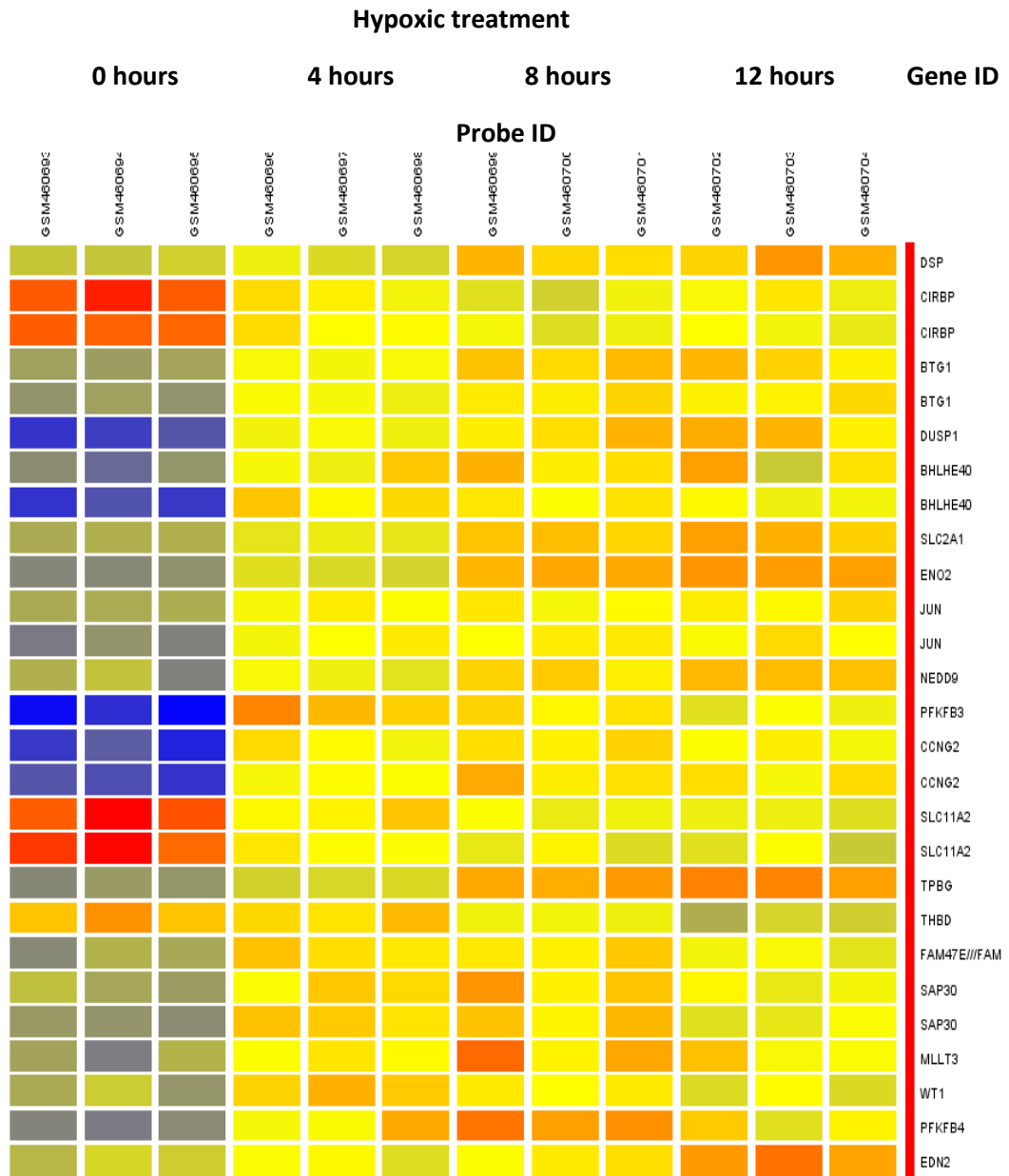
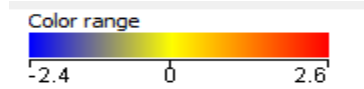


Figure 4.1: Heatmap and profile plot represent Genes with 1.5-fold change expression and Sp1 binding sites after 0.5% O₂ hypoxic exposure in MDA-MB-231 cells.

Microarray data (GSE18494) set was retrieved from NCBI GEO data base and analysed using GeneSpring GX 12.1 bioinformatics software for gene expression patterns. MDA-MB-231 cells were incubated in hypoxic condition (0.5% v/v O₂) for 4, 8 and 12 hours. Probe sets (GSM***) were filtered according to expression levels (cut off values 20-100% expression). One-way ANOVA analysis was carried out with p cut off = 0.05 and multiple testing correction with Benjamini-Hochberg. Fold change cut off value was set as 1.5 where samples were compared with normoxic (0 hour) condition.

Heat map key

Colour variation in relation to fold changes.

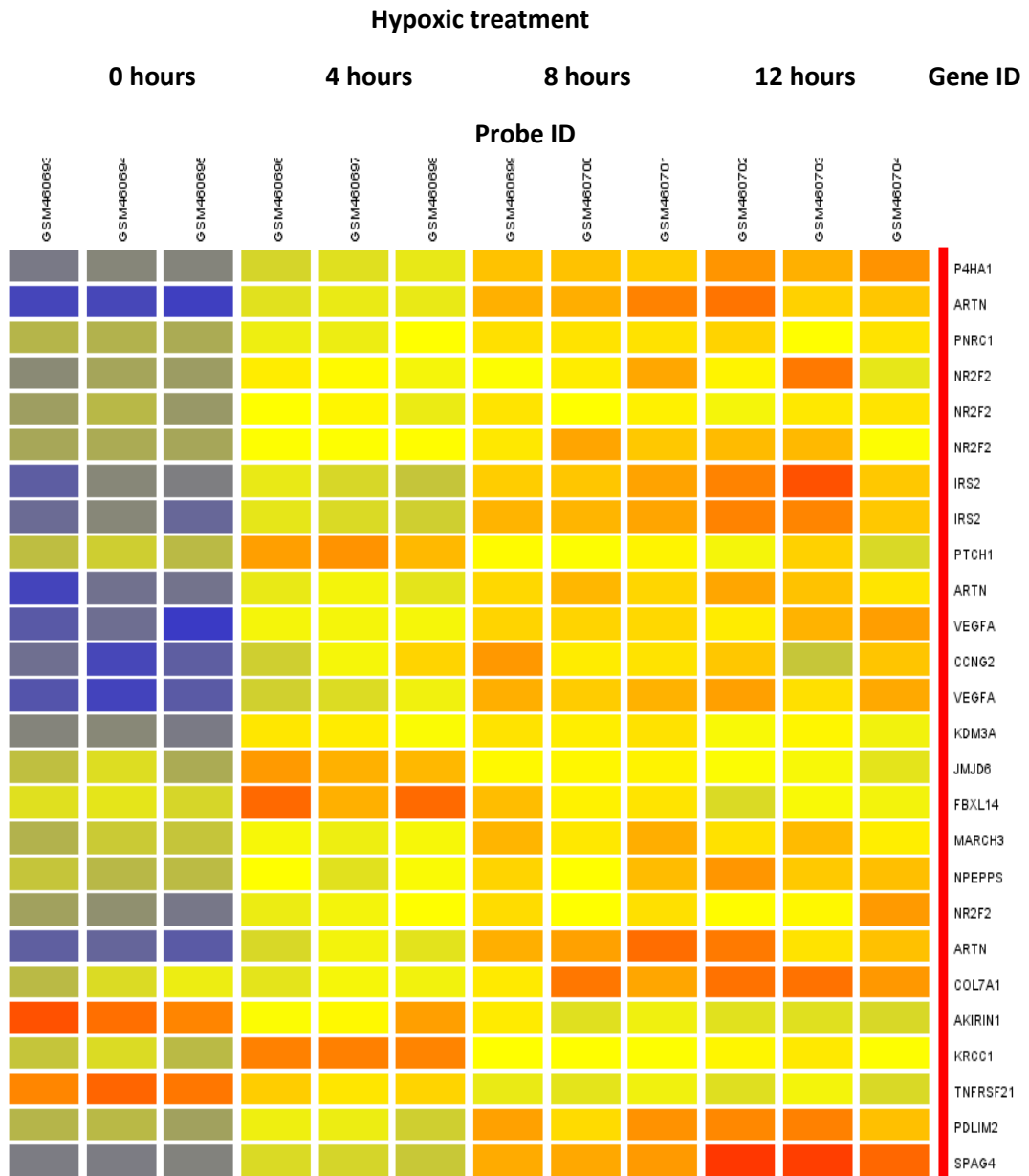


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Heat map key

Colour variation in relation to fold changes.

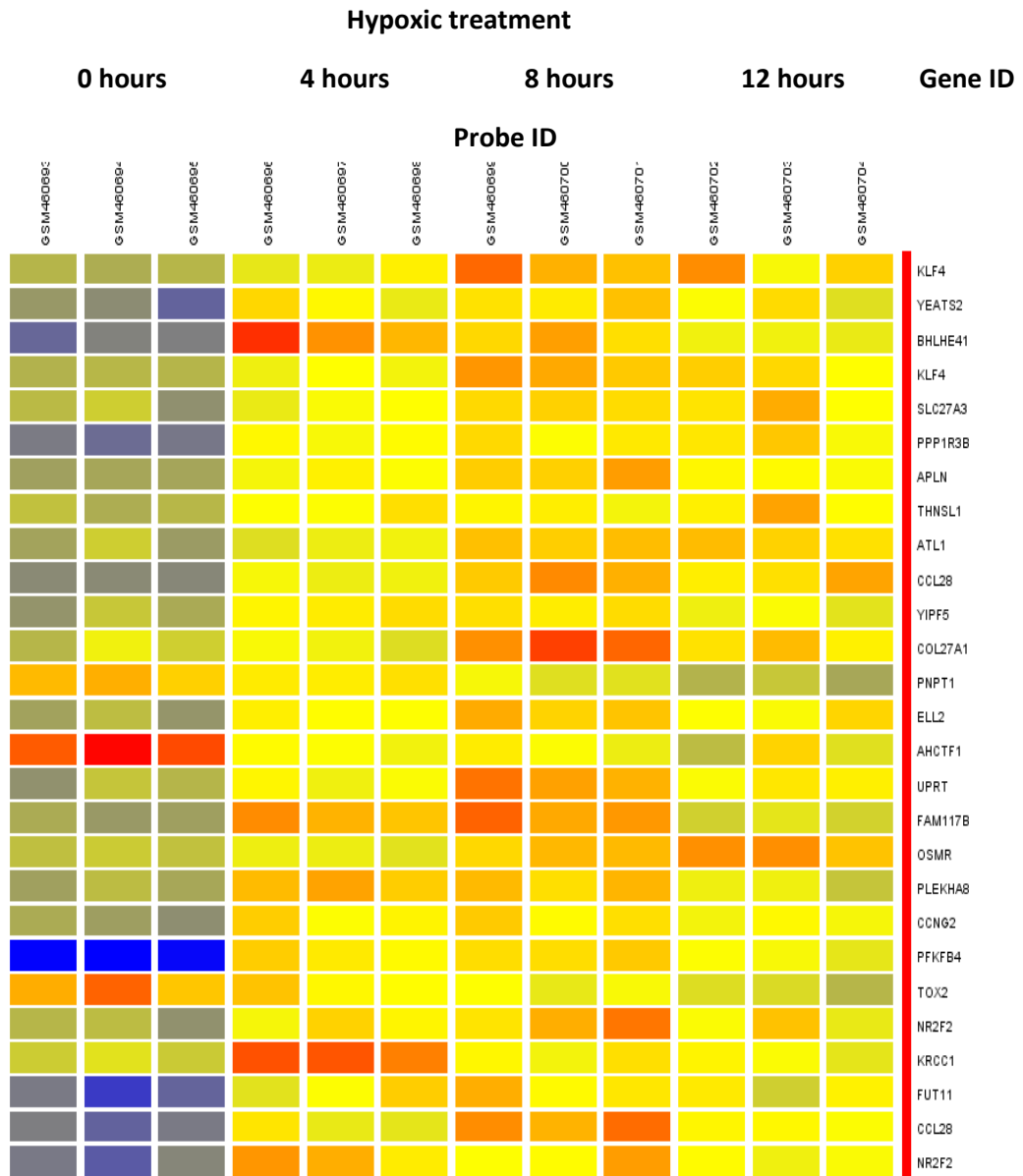
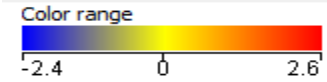


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Microarray data (GSE18494) set was retrieved from NCBI GEO data base and analysed using GeneSpring GX 12.1 bioinformatics software for gene expression patterns. MDA-MB-231 cells were incubated in hypoxic condition (0.5% v/v O₂) for 4, 8 and 12 hours. Probe sets (GSM***) were filtered according to expression levels (cut off values 20-100% expression). One-way ANOVA analysis was carried out with p cut off = 0.05 and multiple testing correction with Benjamini-Hochberg. Fold change cut off value was set as 1.5 where samples were compared with normoxic (0 hour) condition.

Table 4.3: Gene enrichment analysis for HIF-1 sites

Entrez Gene	Gene Symbol	Entrez Gene	Gene Symbol	Entrez Gene	Gene Symbol
10397	<i>NDRG1</i>	6781	<i>STC1</i>	81855	<i>SFXN3</i>
5230	<i>PGK1</i>	283	<i>ANG</i>	55893	<i>ZNF395</i>
6513	<i>SLC2A1</i>	6038	<i>RNASE4</i>	55139	<i>ANKZF1</i>
26118	<i>WSB1</i>	768	<i>CA9</i>	30001	<i>ERO1L</i>
2026	<i>ENO2</i>	2745	<i>GLRX</i>	112399	<i>EGLN3</i>
664	<i>BNIP3</i>	1466	<i>CSRP2</i>	55076	<i>TMEM45A</i>
1956	<i>EGFR</i>	5033	<i>P4HA1</i>	6676	<i>SPAG4</i>
230	<i>ALDOC</i>	123	<i>PLIN2</i>	51129	<i>ANGPTL4</i>
4601	<i>MXI1</i>	51141	<i>INSIG2</i>	55689	<i>YEATS2</i>
5209	<i>PFKFB3</i>	7436	<i>VLDLR</i>	665	<i>BNIP3L</i>
8974	<i>P4HA2</i>	3486	<i>IGFBP3</i>	54583	<i>EGLN1</i>
133	<i>ADM</i>	23036	<i>ZNF292</i>	8996	<i>NOL3</i>
3099	<i>HK2</i>	23030	<i>KDM4B</i>	55893	<i>ZNF395</i>
10144	<i>FAM13A</i>	23363	<i>OBSL1</i>	26355	<i>FAM162A</i>
2632	<i>GBE1</i>	113146	<i>AHNAK2</i>	205	<i>LOC100507</i>
8987	<i>STBD1</i>	8497	<i>PPFIA4</i>	5163	<i>PKD1</i>
4015	<i>LOX</i>	7852	<i>CXCR4</i>	157574	<i>FBXO16</i>

4.2.1.2. Microarray data set GSE47533

In the original experiment, Generic single colour (gpl6884) microarray platform had been used for analysis. MCF-7 cells were subjected to hypoxic exposure for 16, 32, 48 hours' time durations and results were normalised to normoxic condition (0 hours). Here, 48803 entities were subjected to probe set filtration and experimental grouping followed by one-way ANOVA test. Probe set filtration resulted in 38883 genes which were narrowed down to 12585 after statistical analysis. Gene lists were then subjected to fold change analysis. Only 4 genes were identified with minimum cut off value of 1.5 FC (see Table 4.4). Hence lack of expression values of gene set expression, the analysis was also carried out with 1.25 FC (resulting 130 genes). Gene functions were identified using DAVID Bioinformatic sources 6.7. However, gene enrichment analysis was not feasible due to lack of annotations.

Table 4.4: Differentially expressed genes in MCF-7 cells

ID_REF	Entrez Gene	Gene Symbol
ILMN_1756417	353322	<i>ANKRD37</i>
ILMN_1725139	768	<i>CA9</i>
ILMN_1756573	56901	<i>NDUFA4L2</i>
ILMN_1667791	8497	<i>PPFIA4</i>

4.2.1.3. Microarray data set GSE47009

Affymetrix Human Gene 2.0 ST Array [transcript (gene) version] had used for analysis. MCF-7 cells have been exposed to 90 hours of hypoxia or normoxia. Nevertheless, this data set lack the minimum three replicates which meant statistical analysis was not feasible. Thus, no further analysis was carried out due to extensive hypoxia exposure and lack of replicates.

4.2.2. Validation of hypoxia responsive genes identified from microarray data sets

In order to validate expression changes of hypoxia responsive genes with potential Sp1 binding sites, identified genes from GSEA (Gene Set Enrichment Analysis) for both Sp1 promoter sites and HIF-1 sites were grouped. Genes with Sp1 sites (lacking HIF sites) were filtered and arranged according to the fold change. Twenty genes (12 up-regulated and 8 down-regulated) with highest fold changes were selected for qPCR analysis.

Table 4.5: Potential Sp-mediated hypoxia responsive genes selected for qPCR analysis

A) Genes up-regulated in hypoxia

Gene Symbol	Gene name	Fold change
<i>PFKFB4</i>	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	3.04
<i>ARTN</i>	Artemin	2.80
<i>VEGFA</i>	Vascular endothelial growth factor A	2.59
<i>CCNG2</i>	Cyclin G2	2.44
<i>IRS2</i>	Insulin receptor substrate 2	2.40
<i>TPBG</i>	Trophoblast glycoprotein	2.09
<i>FUT11</i>	Fucosyltransferase 11 (alpha (1,3) fucosyltransferase)	2.01
<i>PPP1R3B</i>	Protein phosphatase 1, regulatory (inhibitor) subunit 3B	1.98
<i>CCL28</i>	Chemokine (C-C motif) ligand 28	1.91
<i>NR2F2</i>	Nuclear receptor subfamily 2, group F, member 2	1.85
<i>JUN</i>	Jun oncogene	1.79
<i>PDLIM2</i>	PDZ and LIM domain 2 (mystique)	1.78

B) Genes down-regulated in hypoxia

Gene Symbol	Gene name	Fold change
SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	-1.73
AHCTF1	AT hook containing transcription factor 1; AT hook containing transcription factor 1 pseudogene	-1.70
PNPT1	Polyribonucleotide nucleotidyltransferase 1	-1.60
AKIRIN1	Akirin 1	-1.58
THBD	Thrombomodulin	-1.52
TOX2	TOX high mobility group box family member 2	-1.52
TNFRSF21	Tumour necrosis factor receptor superfamily, member 21	-1.50
CIRBP	Cold inducible RNA binding protein	-1.50

4.2.3. Evaluation of transcript levels of identified hypoxia responsive genes

The microarray data analysis in the previous section indicated that Sp transcription factors may regulate some of the hypoxia responsive genes in the absence of HIF mediated regulation. In this section, expression levels of those hypoxia responsive genes were analysed over variable time points of severe hypoxia exposure by qPCR assays in breast cancer model.

Expression of Carbonic anhydrase 9 (*CA9*) and Glucose Transporter Type 1 (*GLUT1/SLC2A1*) genes are well studied in hypoxia and have been used as biomarkers for hypoxia induction. Due to severe hypoxic exposure, all three breast cancer cell lines showed significant upregulation of *CA9* gene (see Figure 4.2). MCF-7, MDA-MB-231 and T47D cell lines showed highest fold changes of 43.84 (+/- 8.60 SD), 52.87 FC (+/- 13.53 SD) and 35.59 (+/- 7.71 SD) at 24 hours of hypoxic exposure against the control. These changes were also significant compared to the normoxic counterparts. Expression levels of *GLUT1* were also significantly increased due to severe hypoxia (see Figure 4.3). In MCF-7 and MDA-MB-231 cell lines, mRNA levels were increased after 6, 12 and 24 hours' hypoxic exposure. The highest fold changes were detected after 6 hours in MCF-7 cells (2.58 FC (+/-1.05 SD) and 24 hours in MDA-MB-231 cells (1.84 FC (+/- 0.38 SD) against the control. Similarly, hypoxic induction of T47D cells was only significant after 12 and 24 hours' hypoxic exposure where the highest increase was 5.64 FC (+/-0.79 SD) recorded at 12-hour exposure. These changes were also significant against the normoxic counterparts. These results demonstrate the effective hypoxic exposure of samples.

4.2.3.1. Transcript analysis of genes up-regulated (with Sp1 binding sites) in hypoxia

Expression patterns of twelve upregulating genes were analysed. Fold changes were compared to zero-hour exposure as well as normoxic samples. Fold changes of these transcripts are described below (summarised in Table 4.6-8).

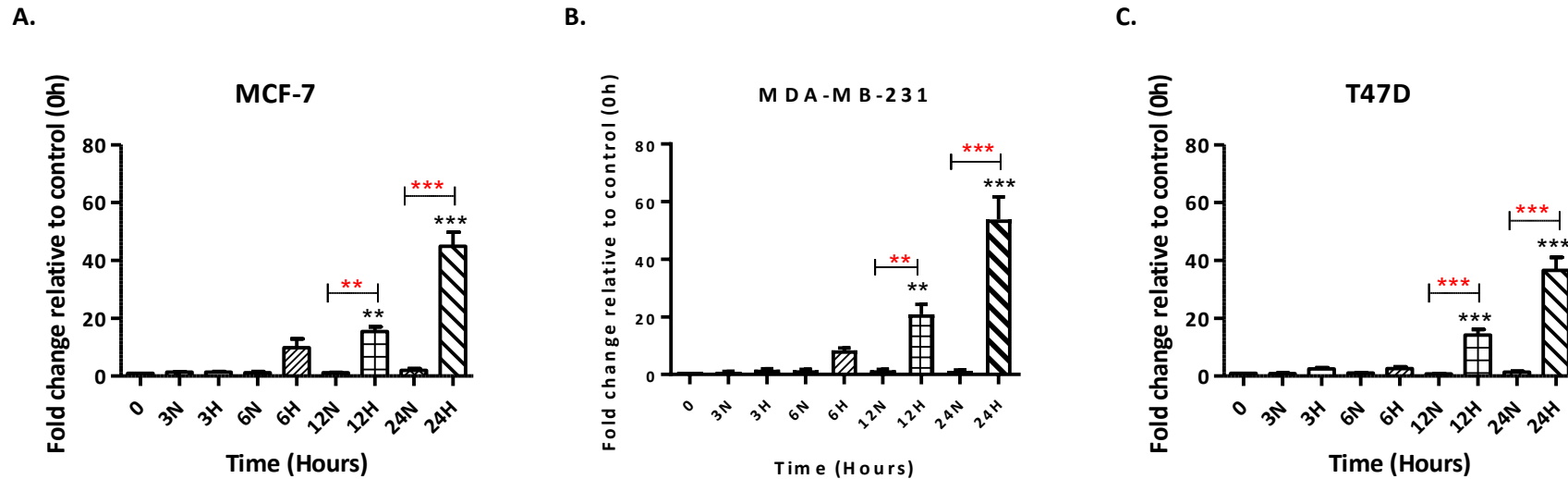


Figure 4.2: mRNA expression of CA9 in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

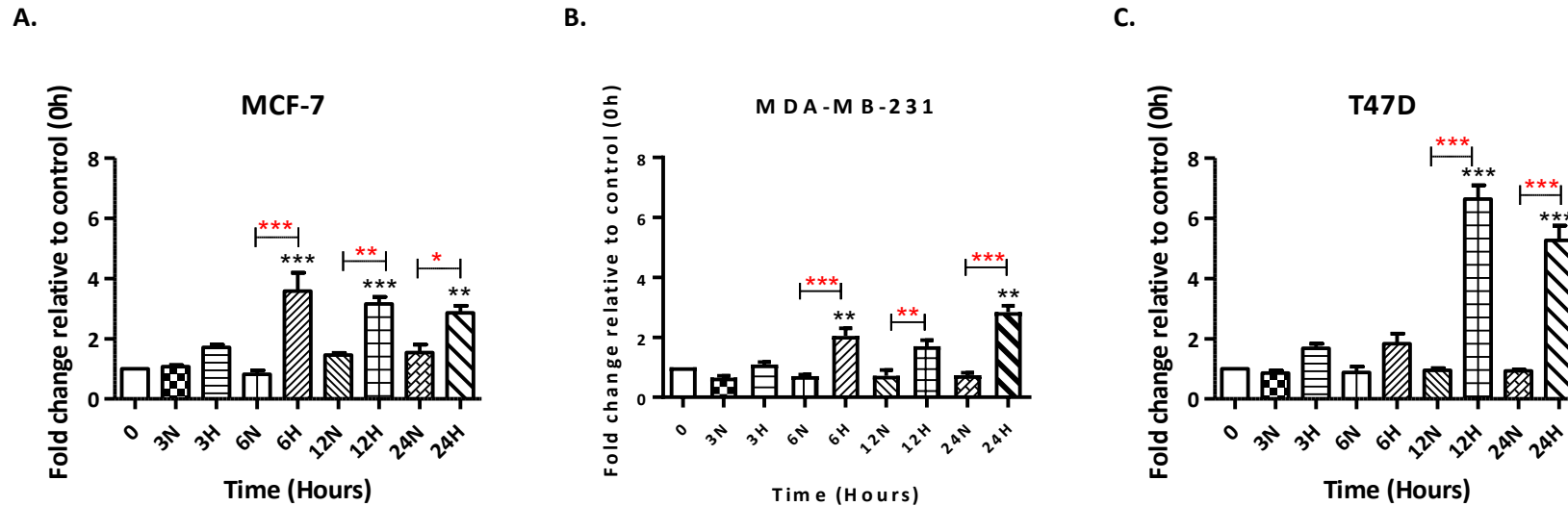


Figure 4.3: mRNA expression of *GLUT1* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

1. 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4)

Transcript levels of *PFKFB4* gene were upregulated due to hypoxic exposure in breast cancer cells. As shown in Figure 4.4 hypoxia samples of all three cell lines showed significant changes compared to normoxic counterparts as well as the zero-hour control. In MCF-7 cells, mRNA levels were increased by 6.03 FC (+/- 0.58 SD), 9.81 FC (+/- 1.51 SD) and 7.74 FC (+/- 1.23 SD) at 6, 12 and 24-hour time points against the control respectively. Similarly, 3.03 FC (+/- 0.52 SD) and 4.15 FC (+/- 0.64 SD) were detected for MDA-MB-231 cell lines at 6 and 24 hours. In T47D cells, hypoxic response was prominent after 12 hours of hypoxic exposure. Expression levels were increased by 6.73 FC (+/- 0.54 SD), 5.46 FC (+/- 0.97 SD) at 12 and 24 hours. These changes were also significant against the normoxic counterparts.

2. Artemin (ARTN)

Expression of *ARTN* gene was unchanged due to hypoxic exposure in breast cancer cells. MCF-7, MDA-MB-231 cells and T47D cells culture in hypoxia showed no significant changes of *ARTN* expression at any condition despite slight variations visible at some time points (see Figure 4.5).

3. Vascular endothelial growth factor A (VEGFA)

Transcript levels of *VEGFA* were significantly changed due to exposure of severe hypoxia in all three cell lines (see Figure 4.6). *VEGFA* was upregulated after three hours to hypoxic exposure in MCF-7 cells and continued to increase with the duration of the experiment. Highest fold change of 1.95 FC (+/- 0.27 SD) was observed at 12 hours against the control. Expression changes in MDA-MB-231 cells were prominent after 24 hours where 5.83 FC (+/- 0.96 SD) was observed. In T47D cells, mRNA levels were increased by 2.94 FC (+/- 0.65 SD) and 3.67 FC (+/- 0.80 SD) at 12 and 24 hours respectively. Changes detected in all the cell lines were also significant against normoxic counterparts.

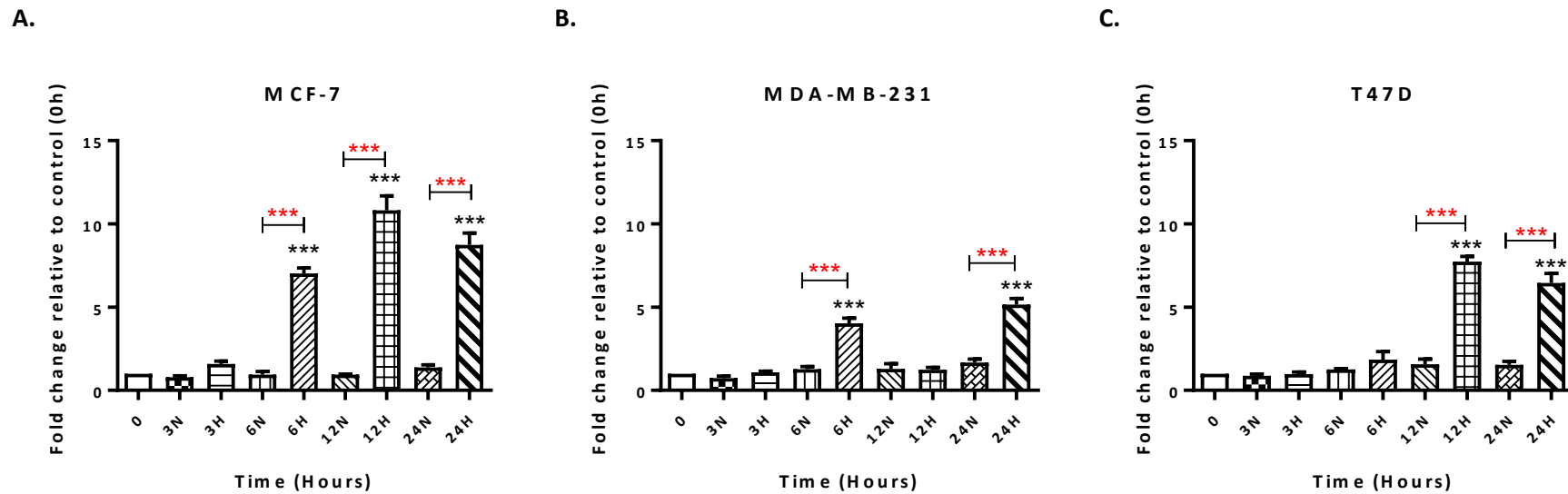


Figure 4.4: mRNA expression of *PFKFB4* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

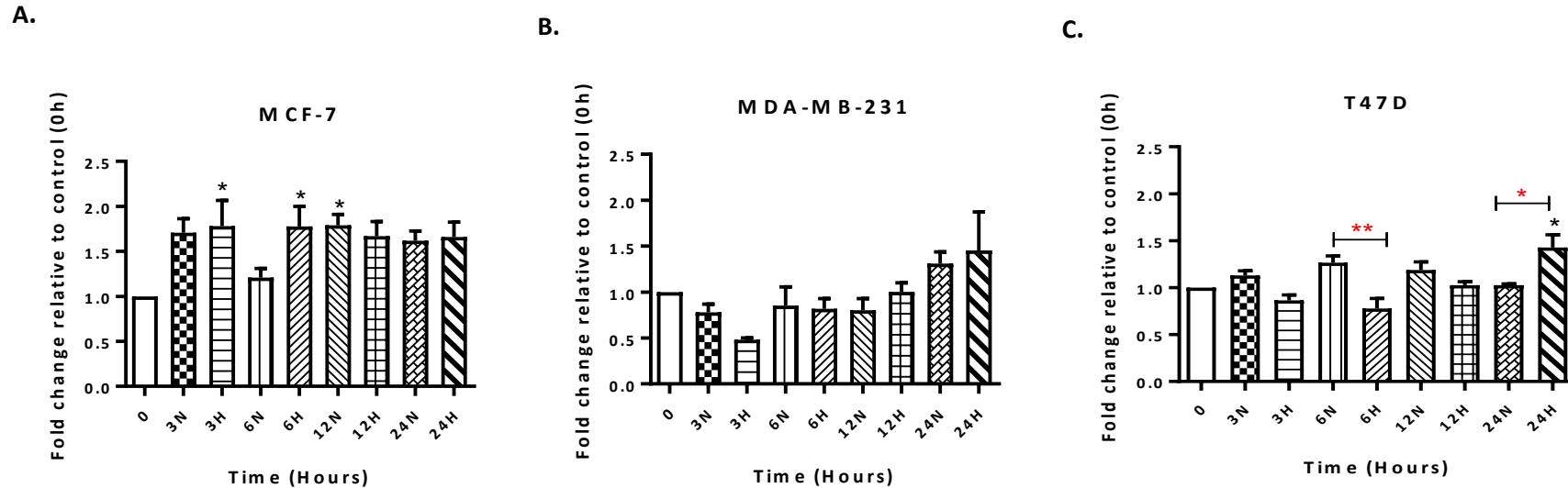


Figure 4.5: mRNA expression of *ARTN* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; **, P < 0.01 (Abbreviations: N: Normoxia, H: Hypoxia).

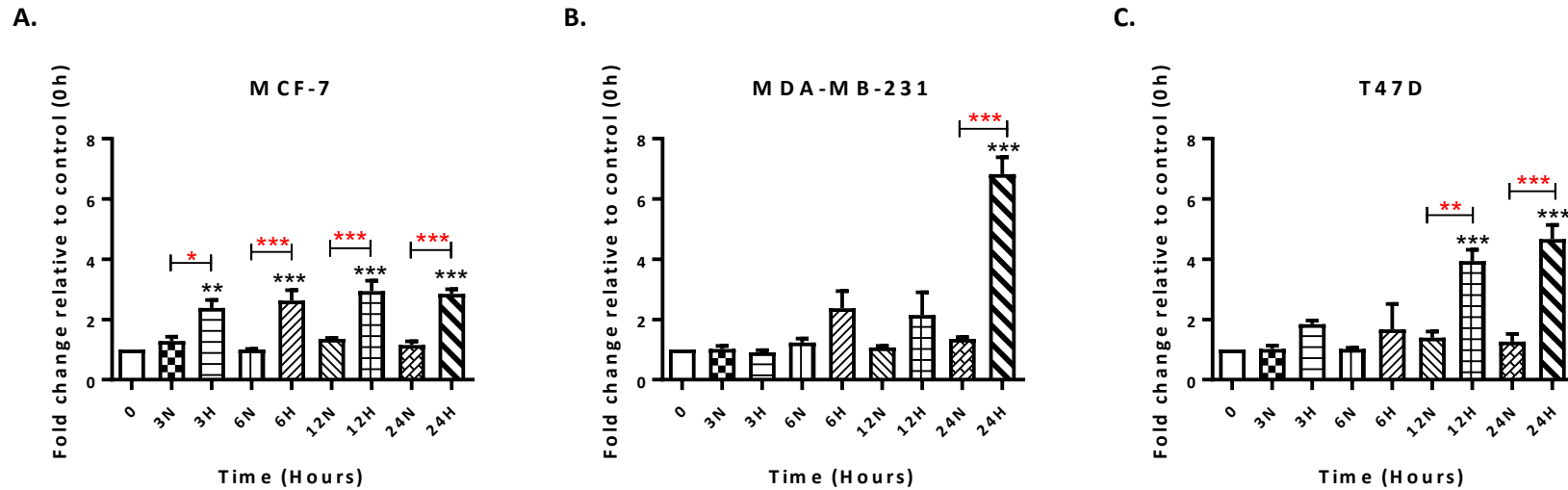


Figure 4.6: mRNA expression of VEGFA in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P< 0.05; **, P< 0.01; ***, P< 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

4. Cyclin G2 (CCNG2)

The *CCNG2* gene was up-regulated due to hypoxic exposure in all three breast cancer cell lines (see Figure 4.7). MCF-7 cells showed 1.77 FC (+/- 0.32 SD) and 3.60 FC (+/- 0.69 SD) increases compared to the control at 12 and 24 hours' exposure. Similarly, MDA-MB-231 cells showed the highest change of 6.56 FC (+/- 0.86 SD) change after 24-hour exposure. mRNA levels of T47D cells were significantly increased by (4.07 FC (+/- 1.16 SD)) and (5.03 FC (+/- 1.72 SD)) at 12 and 24 hours' exposure compared to the control. Expression changes were also significant against the normoxic counterparts.

5. Insulin receptor substrate 2 (IRS2)

Expression levels of *IRS2* gene remained unchanged in all three cell lines after exposure to severe hypoxia with some exceptions. As seen in Figure 4.8, a slight increase in expression levels were observed at 12 hours in MCF-7 cells and at 24 hours in MDA-MB-231 and T47D cells. However, changes were statistically insignificant. A significant reduction of *IRS2* expression (0.55 FC (+/- 0.13 SD)) was also detected at 12 hours in T47D cells compared to the control. However, this change was insignificant against the normoxic counterpart.

6. Trophoblast glycoprotein (TPBG)

TPBG gene showed increased expression levels after hypoxia exposure for all three cell lines (see Figure 4.9). For MCF-7 cells, expression levels were increased by 1.75 FC (+/- 0.52 SD) at 24 hours against the control. Similarly, MDA-MB-231 cells and T47D cells showed 1.57 FC (+/- 0.32 SD) and 0.82 FC (+/- 0.35 SD) increases due to 24-hours hypoxic exposure respectively. Changes were significant against the control as well as against the normoxic counterparts.

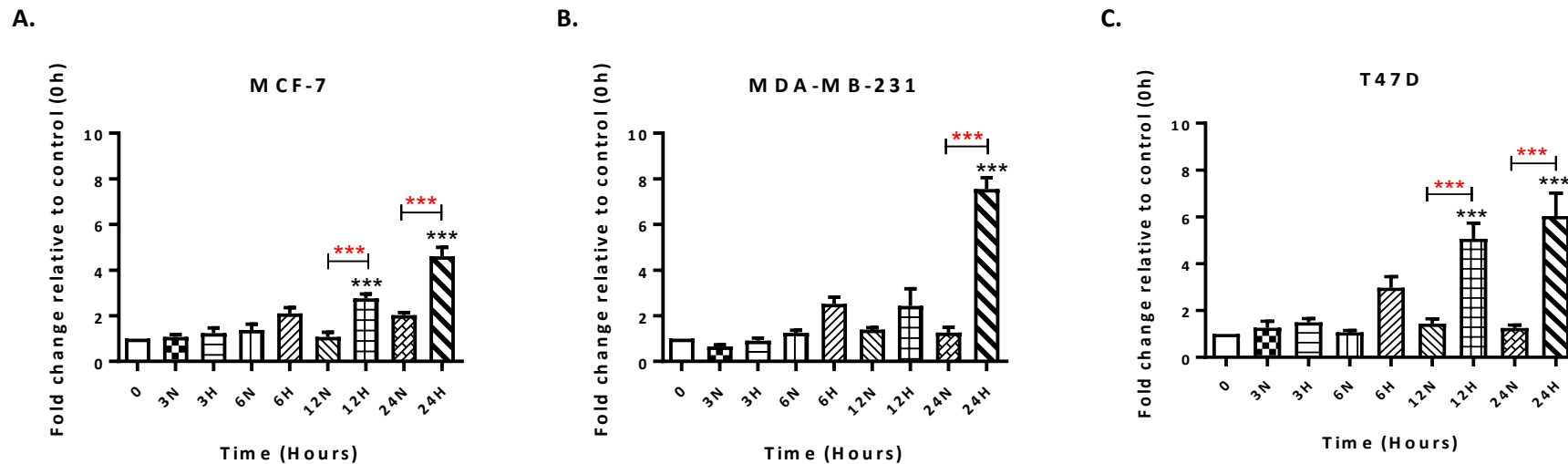


Figure 4.7: mRNA expression of *CCNG2* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% O₂ (v/v) for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P< 0.05; ***, P< 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

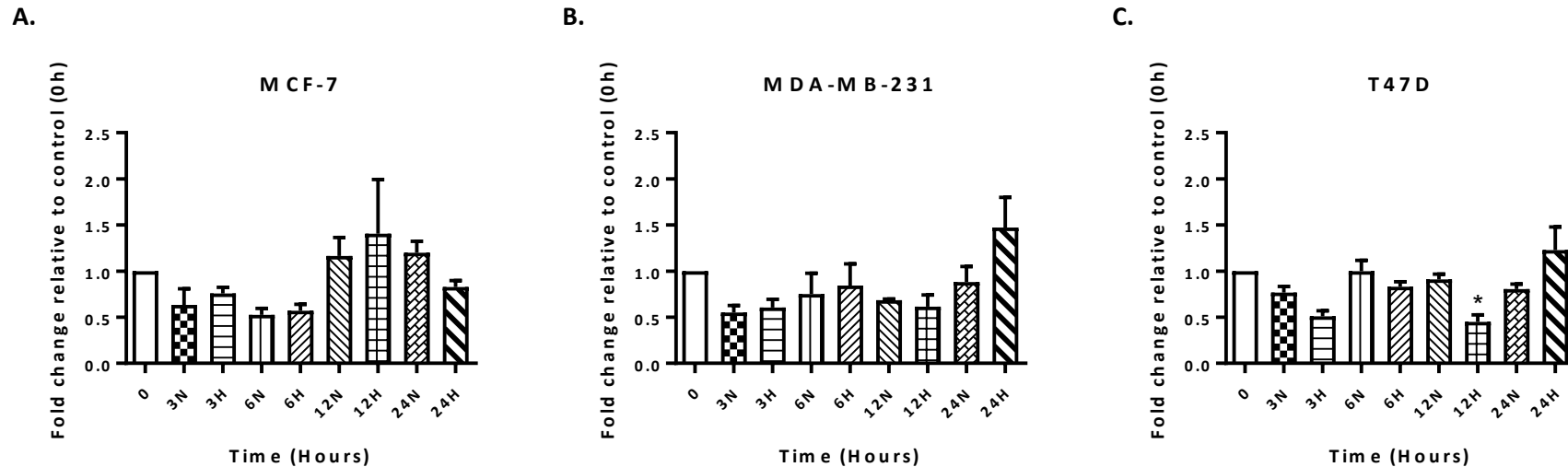


Figure 4.8: mRNA expression of *IRS2* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts. *, P< 0.05. (Abbreviations: N: Normoxia, H: Hypoxia).

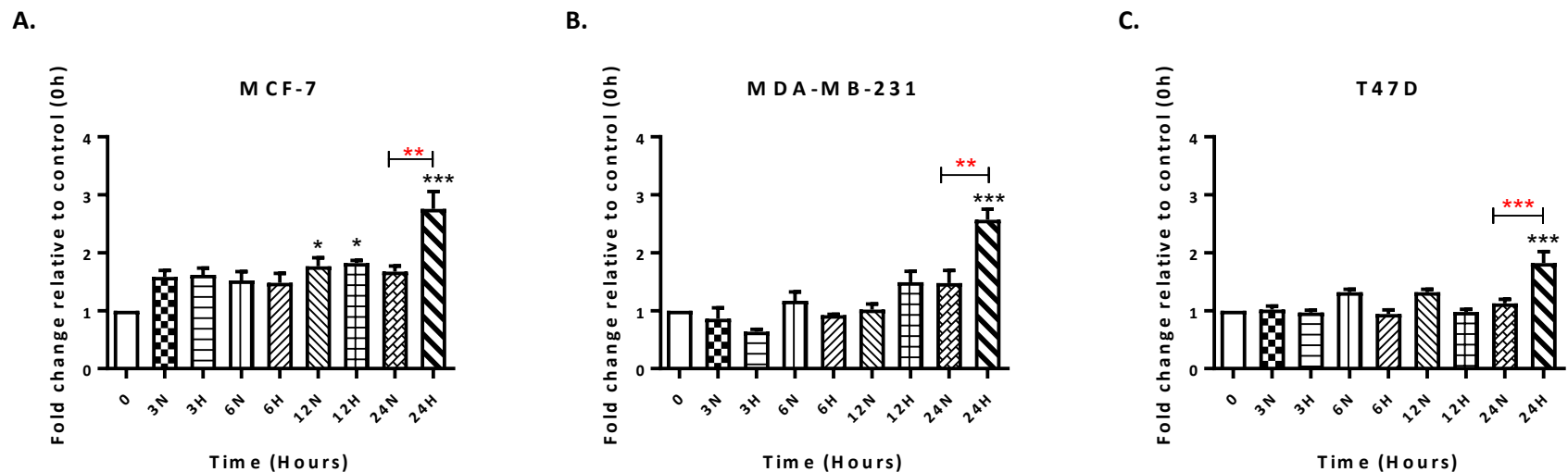


Figure 4.9: mRNA expression of *TPBG* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

7. *Fucosyltransferase 11 (alpha (1,3) fucosyltransferase) (FUT11)*

All three cell lines subjected to hypoxic exposure exhibited significant increase of *FUT11* gene expression compared to the control and normoxic samples. In MCF-7 cells significant changes were visible after 3 hours of hypoxia exposure with the highest expression levels detected at 12 hours (5.74 FC (+/- 0.63 SD) compared to the control. MDA-MB-231 cells showed a 3.07 FC (+/- 0.88 SD) and a 2.63 FC (+/- 0.46 SD) increases at 6 and 24-hour time points. mRNA levels of T47D cell line were also increased by 5.82 FC (+/- 0.32 SD) at 12-hour and 5.37 FC (+/- 0.45 SD) at 24 hours' hypoxic exposure (see Figure 4.10). All these expression changes were also significant against the normoxic counterparts.

8. *Protein phosphatase 1, regulatory (inhibitor) subunit 3B (PPP1R3B)*

PPP1R3B expression changes varied across the cell lines with different patterns were observed at different time points. As can be seen in Figure 4.11, transcript levels of MCF-7 cells visibly increased in both normoxic and hypoxic conditions where significant increase in expression levels were detected at 6 and 12 hours of hypoxic exposure against the control. At 24-hour time point, expression of *PPP1R3B* in normoxic sample was increased by 1.75 FC (+/- 0.57 SD) resulting in significant difference between hypoxic and normoxic sample (-1.27 FC (+/- 0.25 SD)).

In MDA-MB-231 cells, the only significant change in expression level was a 1.53 FC (+/- 0.17 SD) at 24 hours' hypoxic exposure compared to the control. However, changes were insignificant compared to normoxic condition.

In contrast to other cell lines, mRNA levels in T47D cell line decreased by 0.64 FC (+/- 0.09 SD) at 3 hours and 0.53 FC (+/- 0.15 SD) at 24-hour hypoxic exposure against the control. A significant increase of expression in normoxic samples were also observed compared to the control as well as the hypoxic sample.

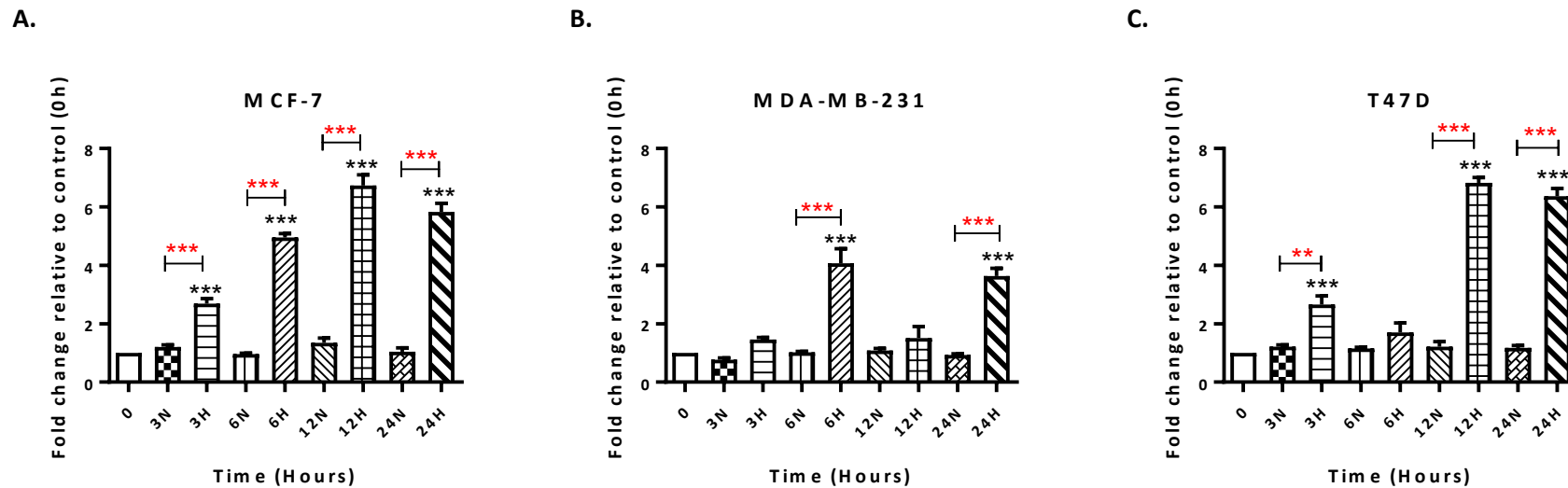


Figure 4.10: mRNA expression of *FUT11* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia)

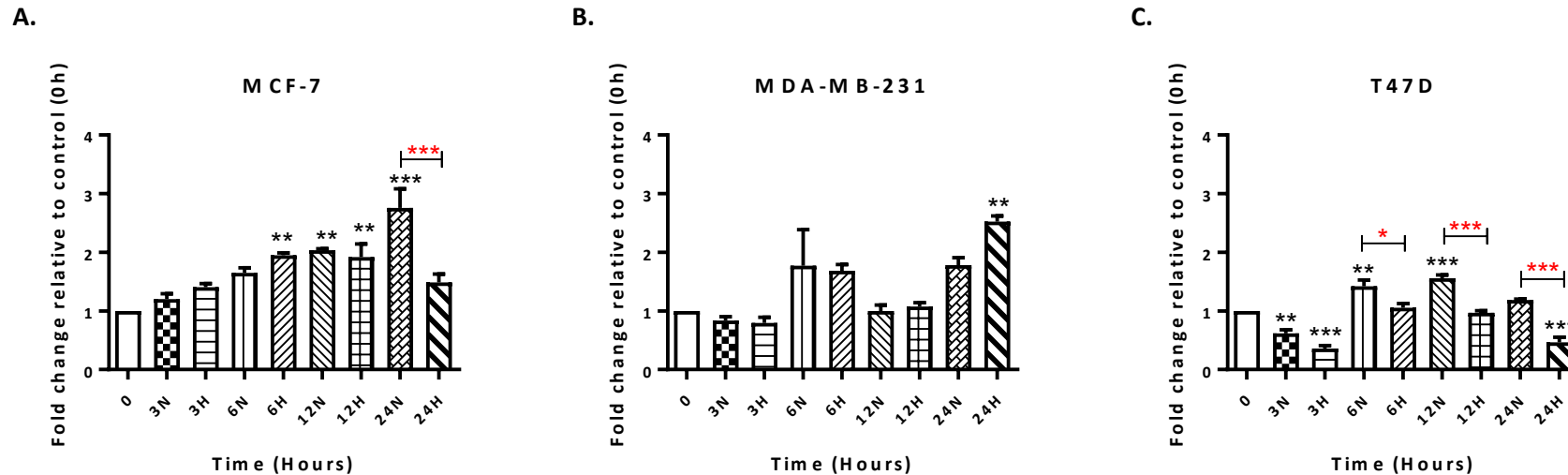


Figure 4.11: mRNA expression of *PPP1R3B* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P< 0.05; **, P< 0.01; ***, P< 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

9. Chemokine (C-C motif) ligand 28 (CCL28)

MCF-7 and MDA-MB-231 both displayed upregulation of *CCL28* gene expression in severe hypoxia. In MCF-7 cells, hypoxic induction was prominent after 6 hours of exposure with the highest change of 2.57 FC (+/- 0.65 SD) was detected compared to the control at 24 hours. In MDA-MB-231 cells expression levels were increased by 1.01 FC (+/- 0.27 SD) and 1.84 FC (+/- 0.11 SD) at 6 and 24 hours of hypoxic exposure. Expression changes were also significant against the normoxic counterparts. In contrast, T47D cells did not show any significant upregulation due to hypoxia. Changes in normoxic and hypoxic samples were insignificant compared to control. Interestingly hypoxic samples showed a significant decrease in expression at 6,12,24 hours against their normoxic counterparts (see Figure 4.12).

10. Nuclear receptor subfamily 2, group F, member 2 (NR2F2)

The *NR2F2* gene expression was up-regulated in MCF-7 and MDA-MB-231 cells due to hypoxia exposure. Transcript levels were significantly increased by 1.78 FC (+/- 0.56 SD) at 24 hours in MCF-7 cells compared to the control. MDA MB cells showed 0.97 FC (+/- 0.30 SD) increase at 6 hours' hypoxic exposure. Expression changes were also significant against the normoxic counterparts. In contrast, no significant upregulation was observed at any point for T47D cells. Six-hour hypoxic exposure caused significant reduction of mRNA levels compared to normoxic counterpart which was insignificant against to the control (See Figure 4.13).

11. Jun oncogene (JUN)

Jun oncogene was upregulated due to severe hypoxia in breast cancer cells. The hypoxic response in MCF-7 cells was prominent at 3 hours of exposure where it was increased by 0.59 FC (+/- 0.41 SD) against the control in MCF -7 cells. However, after 3 hours expression levels have decreased back to basal levels where no significant changes were observed at later time points. Similarly, 1.03 FC (+/- 0.31 SD) change were displayed in MDA-MB-231 cells at 12 hours' hypoxic sample compared to the control. In T47D cells, 1.01 FC (+/- 0.44 SD) upregulation was detected only after 24 hours in the hypoxic sample. All these changes were also significant against the normoxic counterparts. Similar levels of mRNA expression were observed all other time points and this was common for all three cell lines (see Figure 4.14).

12. PDZ and LIM domain 2 (*mystique*) (*PDLIM2*)

As evident from Figure 4.15, the *PDLIM2* gene also exhibited different expression patterns in the cell lines following exposure. Some variation in expression was seen in the MCF-7 cell lines. However, no significant changes were detected. Expression levels in MDA-MB-231 normoxic samples decreased at 3,6 12 hours compared to the zero-time point. However, only the 6-hour hypoxia time point showed slight increase against its counterpart which was insignificant when compared to the control. At 12, 24-hour time points, mRNA levels of the hypoxic sample decreased compared to the control. However, this change was insignificant when compared to the normoxic counterpart. T47D cells showed a significant increase in transcript levels at 12 and 24-hour exposure compared to the control and the normoxic counterparts. mRNA levels were increased by 0.47 FC (+/- 0.21 SD) and 0.63 FC (+/- 0.21 SD) compared to the control respectively. These changes were also significant against the normoxic counterparts.

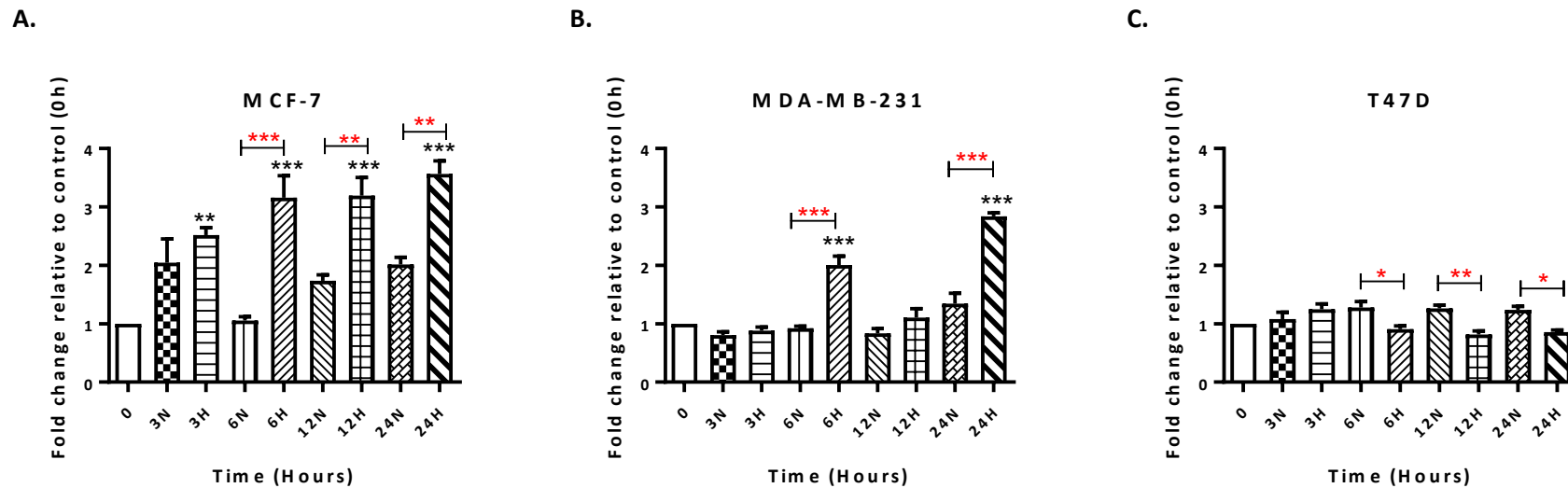


Figure 4.12: mRNA expression of *CCL28* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

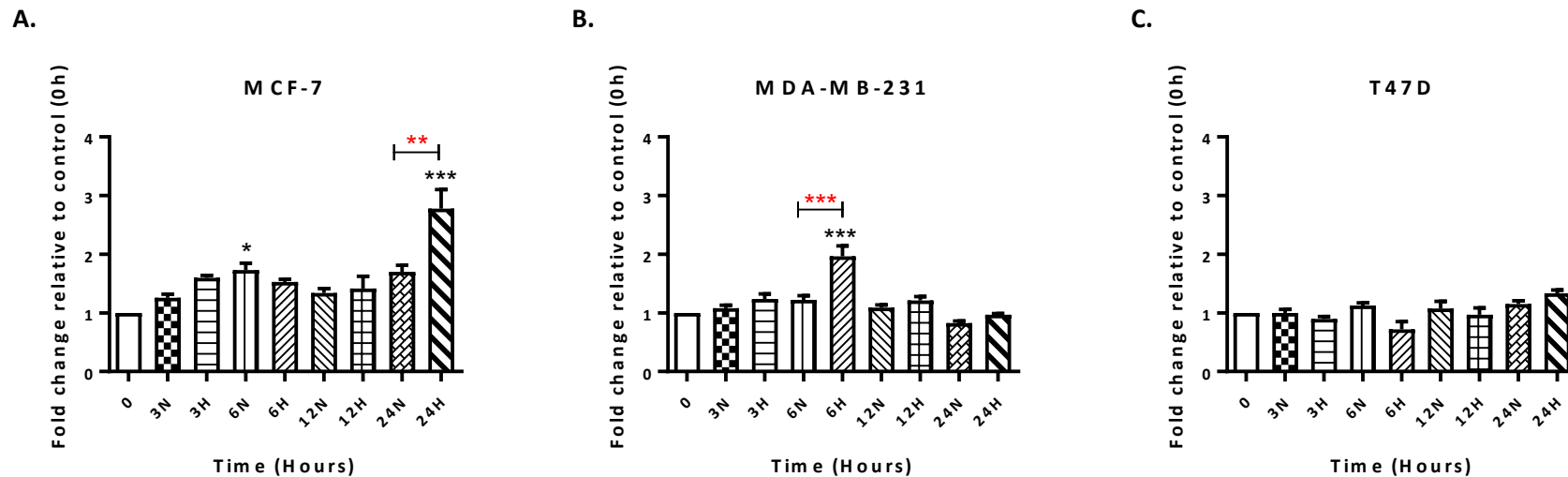


Figure 4.13: mRNA expression of *NR2F2* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P< 0.05; **, P< 0.01; ***, P< 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

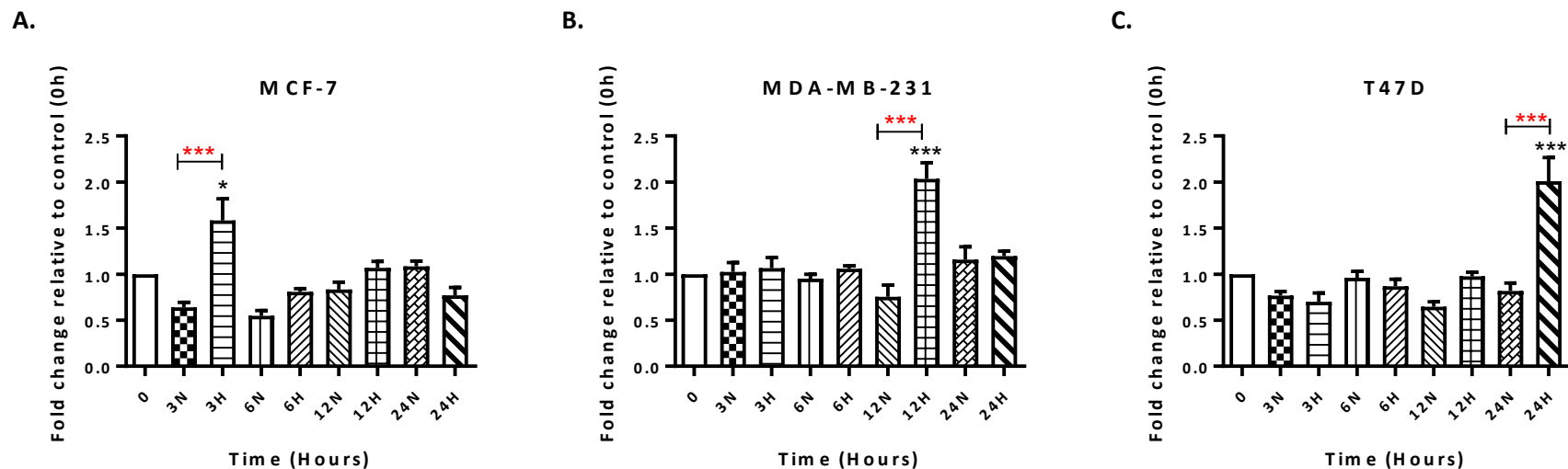


Figure 4.14: mRNA expression of *JUN* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

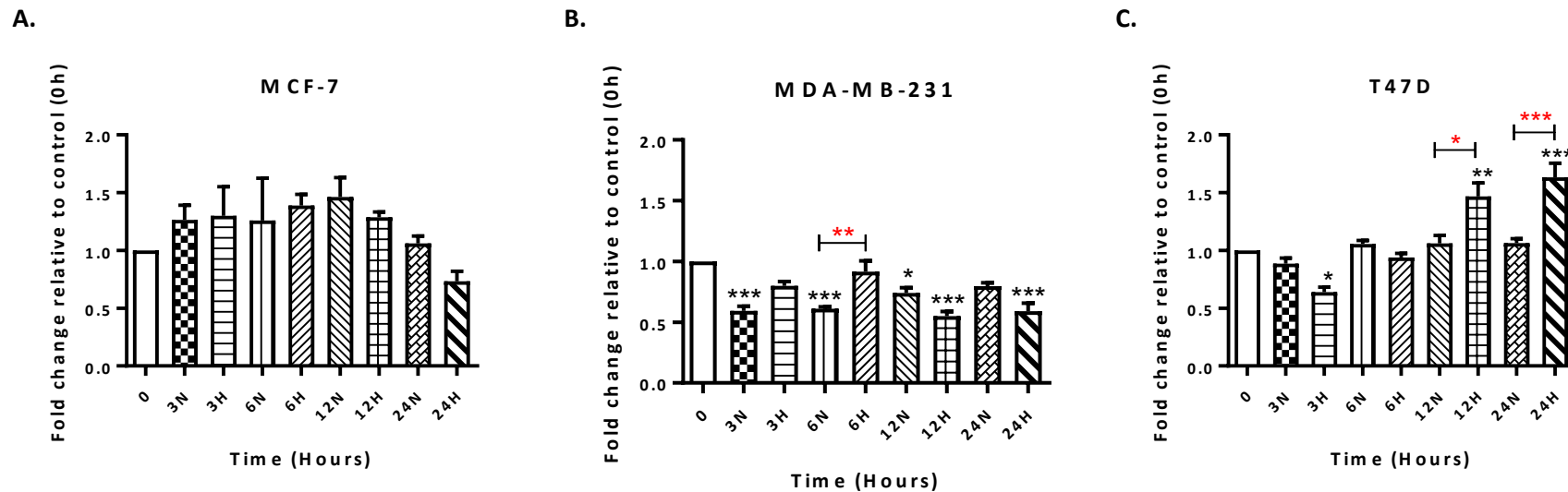


Figure 4.15: mRNA expression of *PDLIM2* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

4.2.3.2. Transcript analysis down-regulated genes with putative Sp1 binding sites

Expression patterns of eight down-regulating genes were also analysed. Fold changes were compared to zero-hour exposure as well as normoxic samples and presented as positive values against zero-hour control. A summary of fold changes of these transcripts can be found in Table 4.6-8.

1. Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (SLC11A2)

Expression of the *SLC11A2* gene was significantly decreased in MCF-7 and MDA-MB-231 cells due to severe hypoxic exposure. Changes in MCF-7 cells were significant at 12 and 24 hours against its normoxic counterparts. mRNA levels of normoxic samples increased at both time points resulting in nearly 0.50 FC (+/- 0.10 SD) difference. However, these changes were insignificant when compared to the control. Hypoxia treated MDA-MB-231 cells showed significant decreases against the control for all the time points. Whereas these changes were insignificant compared to normoxic counterparts except for 24 hours. Cells exposed to 24 hours of hypoxia displayed 0.61 FC (+/- 0.13 SD) decrease compared to the normoxic counterpart. As evident in Figure 4.16 transcript levels of T47D cells exposed to hypoxia were visibly decreasing compared to normoxic counterparts despite no significant changes were observed at any time point.

2. AT hook containing transcription factor 1; AT hook containing transcription factor 1 pseudogene (AHCTF1)

Cells exposed to hypoxia showed decreased expression of *AHCTF1* compared to the control. At 12 and 24 hours, MCF-7 cell cultured in normoxic conditions showed a significant increase in *AHCTF1* expression levels compared to the zero-hour control and cells cultured in hypoxic conditions for the same duration (0.86 FC (+/- 0.20 SD) (see Figure 4.17 panel A). MDA-MB-231 cells showed a similar difference in expression in 24 hours hypoxic samples where 1.25 FC (+/- 0.08 SD) decrease observed compared to the normoxic condition. Six hours and 12 hours hypoxic samples showed a similar decrease compared to the normoxic pair but this was not statistically significant. However, no significant changes were detected in the T47D cells (see Figure 4.17).

3. Polyribonucleotide nucleotidyltransferase 1 (PNPT1)

Expression of *PNPT1* gene was affected by exposure to severe hypoxia in breast cancer cell lines. Figure 4.18 represents fold changes of *PNPT1* of all three cell lines. MCF-7 and MDA-MB-231 cells showed a significant reduction in *PNPT1* expression in the hypoxic samples of 24 hours against normoxic counterpart. MDA-MB-231 cells also showed decrease of expression levels in hypoxic samples at 3 and 12. MCF-7 cells showed a significant increase in expression at 12 hours normoxia against the control. However, aside from 12 hours normoxia samples, all other MCF-7 and MDA-MB-231 normoxia samples were similar in expression levels. T47D cells did not show any detectable significant changes in *PNPT1* expression.

4. Akirin 1 (AKIRIN1)

Under severe hypoxia, MCF-7 cells showed no significant changes in *AKIRIN1* expression. However, MCF-7 cells cultured in normoxic conditions showed a significant increased expression of *AKIRIN1* at 12 and 24-hour time points compared to the zero-hour control. The increase in *AKIRIN1* expression in MCF-7 cells cultured in 24 hours normoxia was also significantly higher (0.56 FC (+/- 0.13 SD)) to the expression observed at 24 hours hypoxia (see Figure 4.19). Both MDA-MB-231 and T47D cells were lack of any expression changes of *AKIRIN1* gene in hypoxia.

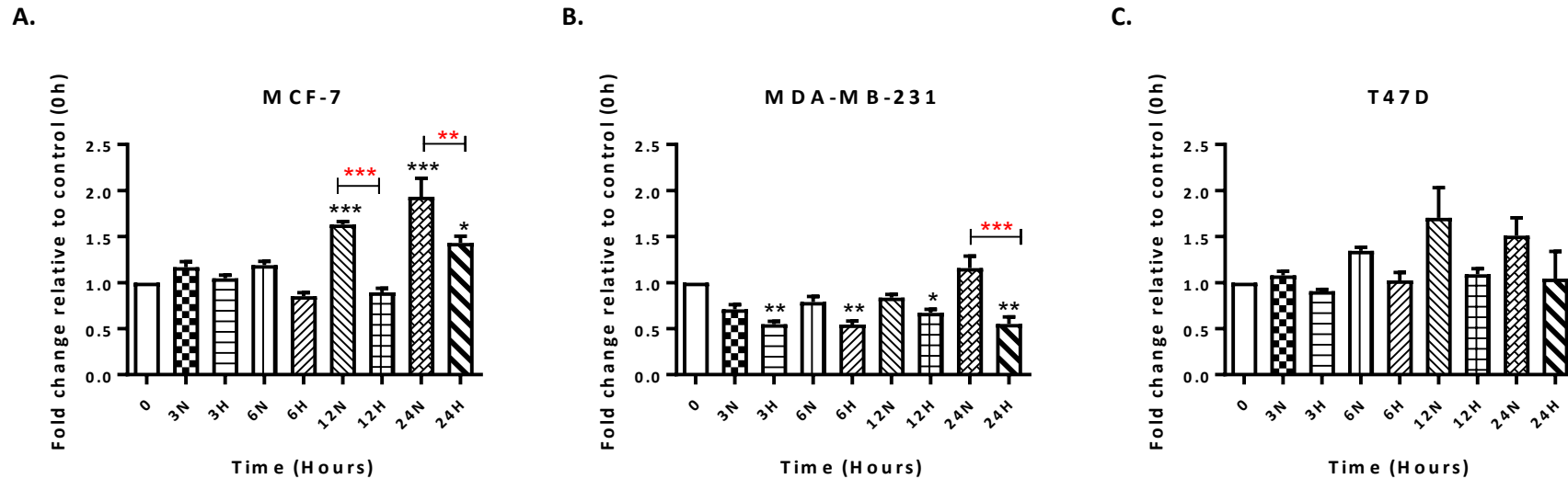


Figure 4.16: mRNA expression of *SLC11A2* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

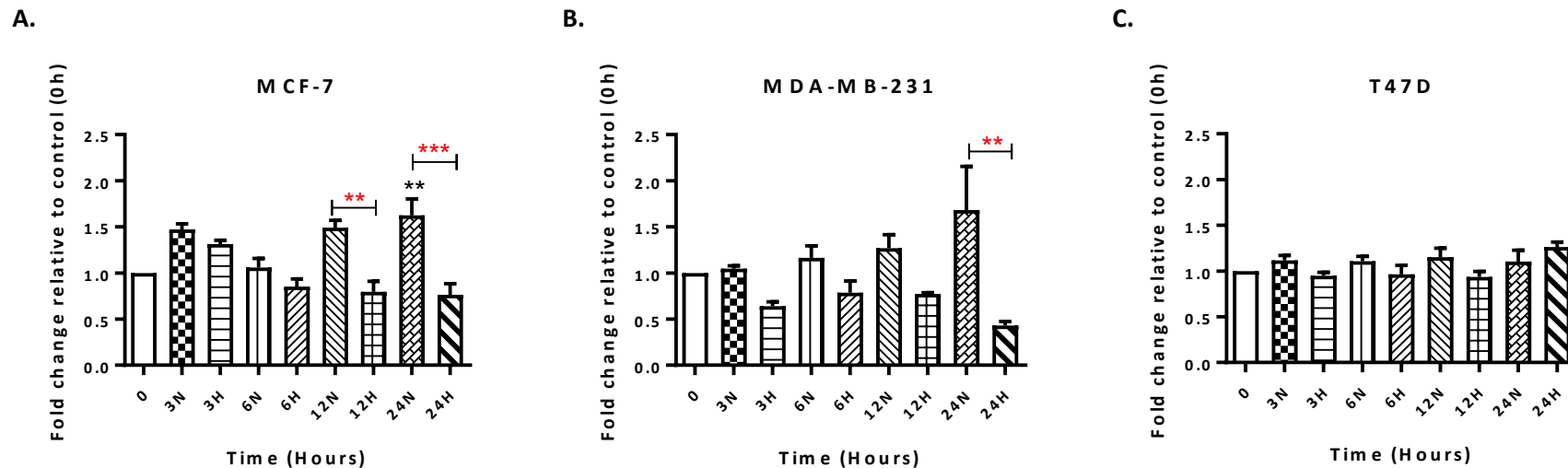


Figure 4.17: mRNA expression of *AHCTF1* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P< 0.05; **, P< 0.01; ***, P< 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

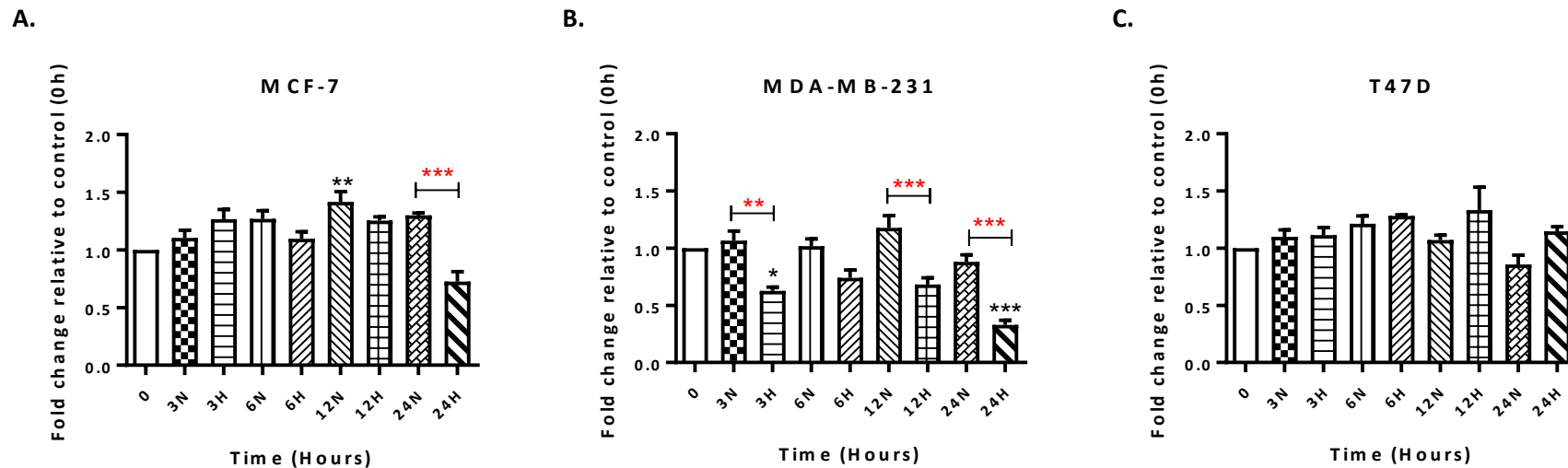


Figure 4.18: mRNA expression of *PNPT1* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

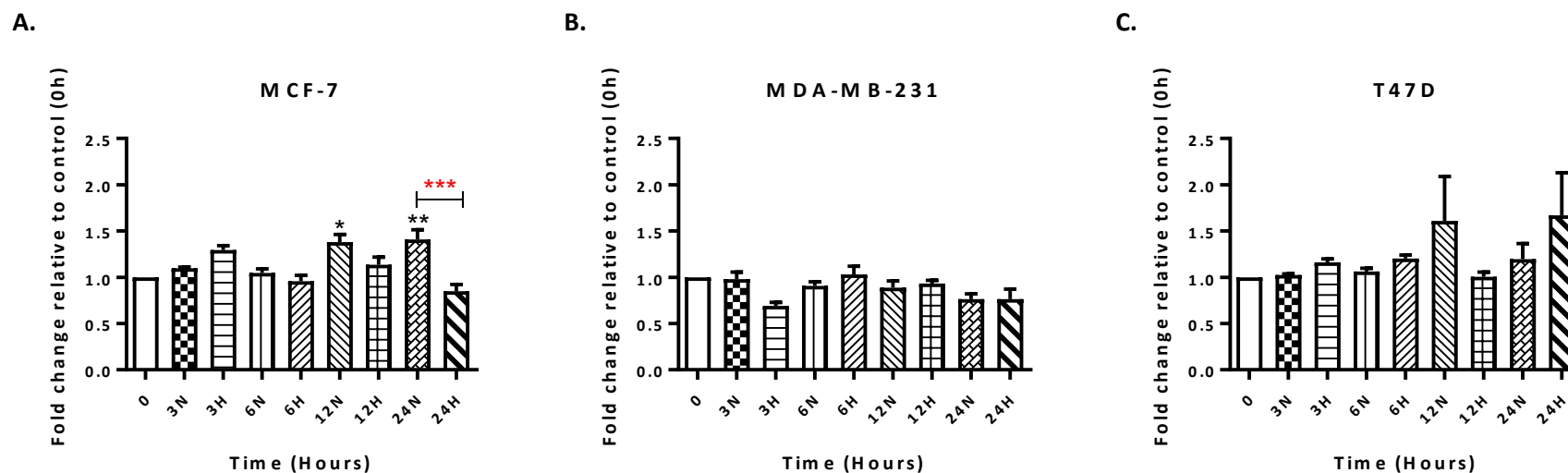


Figure 4.19: mRNA expression of *AKIRIN1* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P< 0.05; **, P< 0.01; ***, P< 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

5. *Thrombomodulin (THBD)*

MCF-7 cells showed a significant decrease of *THBD* expression at 3 and 6 hours of hypoxic exposure compared to the normoxic counterparts by 0.60 FC (+/- 0.08 SD) and 0.73 FC (+/- 0.13 SD) respectively. However, these changes were not significant compared to the zero-hour control. A 0.63 FC (+/- 0.28 SD) increase in *THBD* expression was detected in hypoxic sample of 24-hour time point compared to the control. Transcript levels of *THBD* in MDA-MB-231 cells decreased due to hypoxic exposure throughout the experimental duration and significant reductions were observed after 6 hours. At 6-hour, 0.68 FC (+/- 0.06 SD) decrease was identified against the control which was significant compared to the normoxic counterpart. Expression levels of both normoxic and hypoxic samples decreased significantly in T47D cells compared to the control. No significant changes were detected between each counterpart at any time point (see Figure 4.20).

6. *TOX high mobility group box family member 2 (TOX2)*

Expression of *TOX2* gene decreased significantly in breast cancer cell lines due to severe hypoxia. In MCF-7 cells, all the hypoxic samples showed significant decrease in expression levels compared to both counterparts and the control. Highest decrease of 0.58 FC (+/- 0.10 SD) was observed at 6-hour time point against the control (see Figure 4.21). MDA-MB-231 cells also followed the exact same pattern as MCF-7 cells where 3 and 6 hours' samples showed significant decrease compared to the control (0.5 FC (+/- 0.07 SD)). In addition, mRNA levels decreased by nearly 0.5 FC (+/- 0.07 SD) at 6, 12 and 24-hours compared to the normoxic counterpart. Transcript levels of T47D cells also decreased significantly at all time points where prominent hypoxic response was observed at 12 hours of hypoxic exposure. Expression of hypoxic sample decreased by 2.9 FC (+/- 0.16 SD) against the normoxic counterpart.

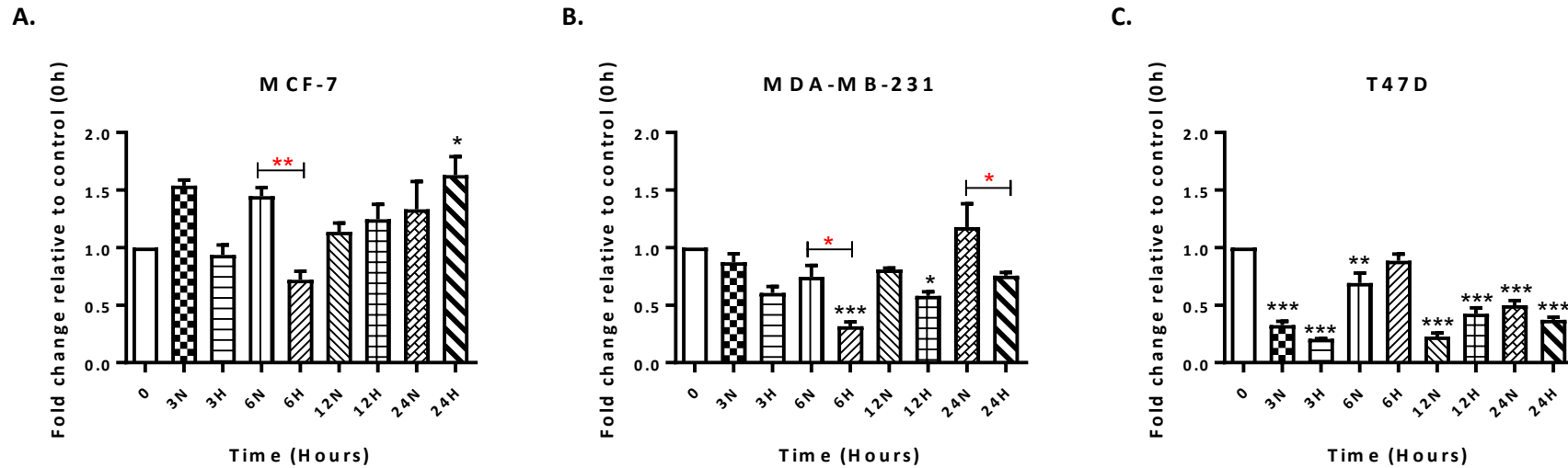


Figure 4.20: mRNA expression of *THBD* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

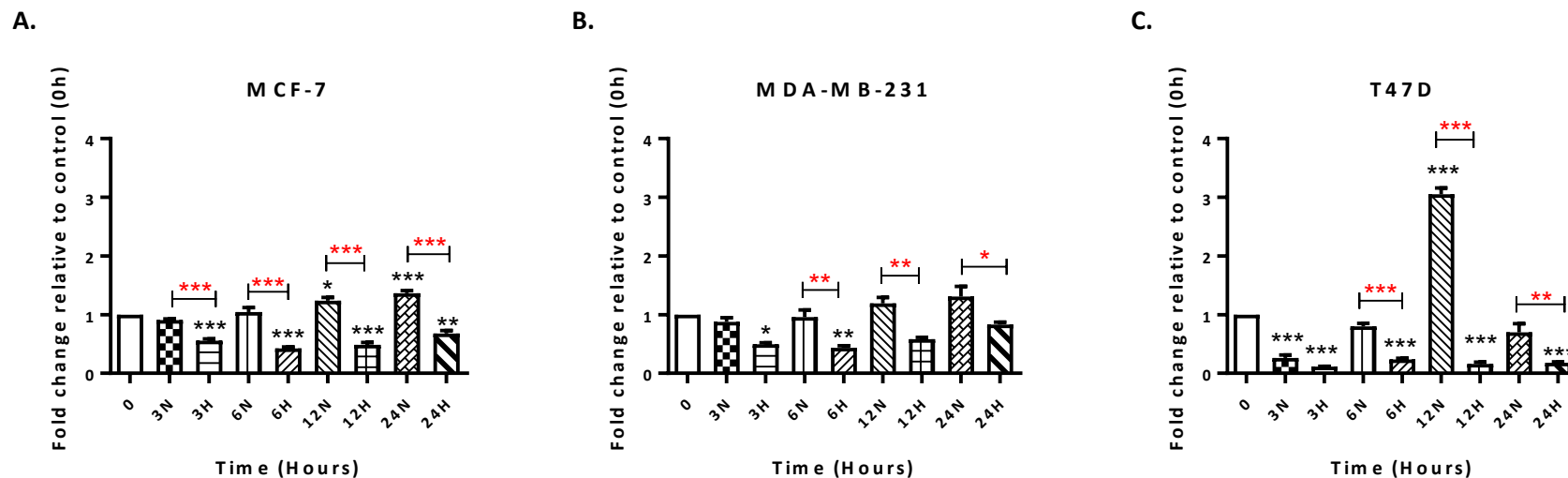


Figure 4.21: mRNA expression of *TOX2* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

7. Tumour necrosis factor receptor superfamily, member 21 (TNFRSF21)

As seen in Figure 4.22, panel A, MCF-7 cells showed a significant increase in normoxia at 12 and 24-hour time points compared to the control and hypoxic counterparts. At both time points expression levels were 0.50 FC (\pm 0.07 SD) higher in normoxia treated samples compared to hypoxic condition. In MDA-MB-231 cells, *TNFRSF21* levels were significantly decreased at 6 and 24-hour time points (see in Figure 4.22) compared to the control. At 24-hour time point hypoxic sample showed 0.55 FC (\pm 0.13 SD) decrease against the control.

In contrast, T47D cells displayed significant increase of expression due to hypoxic exposure after 6 hours with further increases detected at 12 and 24 hours. After 12 hours of hypoxic exposure mRNA levels increased by 0.48 FC (\pm 0.07 SD) and at 24-hour time point it was 1.18 FC (\pm 0.16 SD) against the control.

8. Cold inducible RNA binding protein (CIRBP)

CIRBP gene showed low levels of expression in all three cell lines. MCF-7 cells exhibited increase of CIRBP mRNA levels in both normoxic and hypoxic conditions compared to the control except 6 hours normoxic and 12 hours hypoxic samples. Even though they were statistically significant, changes were less than 0.5 FC. In MDA-MB-231 cells, significant decrease of expression was recorded after 12 (0.41 FC (\pm 0.07 SD) and 24 hours (0.47 FC (\pm 0.09 SD) against the control as well as against the normoxic counterpart. No significant changes in CIRBP expression were observed for T47D cells (see Figure 4.23).

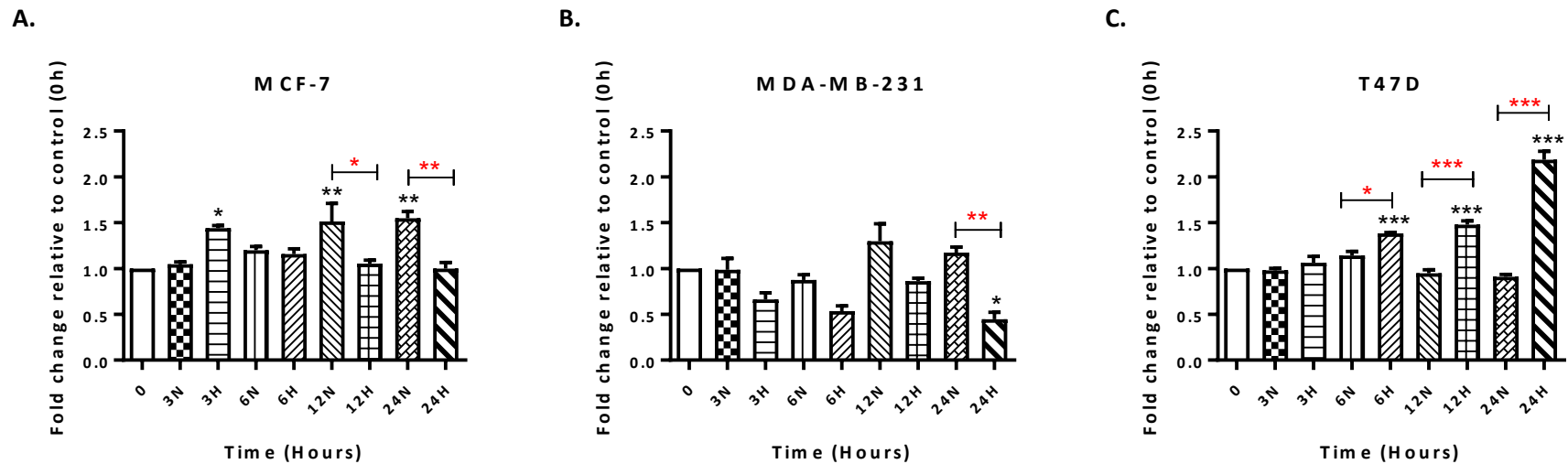


Figure 4.22: mRNA expression of *TNFRSF21* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P< 0.05; **, P< 0.01; ***, P< 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

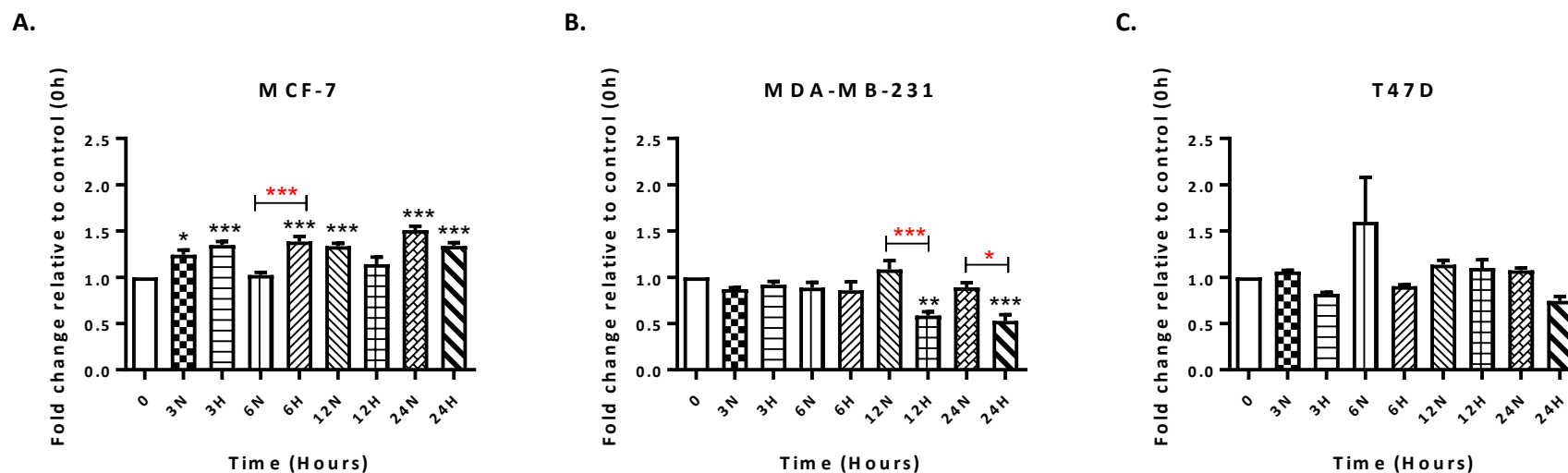


Figure 4.23: mRNA expression of *CIRBP* in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) cells were exposed to 0.5% (v/v) O₂ for a range of time points. Normoxia counterparts were incubated at 21% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and reverse transcribed. Transcript levels were analysed by TaqMan RT qPCR (as described in section 2.5). All results were normalised to the housekeeping gene *B2M*. Results are expressed as fold change relative to 0-hour control (normoxic sample). n=3 experiments: Error bars represents +/- SEM: Statistical analysis by One-way ANOVA with Tukey's post-hoc correction. Significance were compared against the control (Black coloured *) as well as the normoxic counterparts (Red coloured *). *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Abbreviations: N: Normoxia, H: Hypoxia).

Table 4.6: Fold changes in mRNA expression of hypoxia responsive genes in MCF-7 cells relative to the zero-hour control

Gene Symbol	Fold change relative to normoxic control (0h)															
	3 N	p-value	3 H	p-value	6 N	p-value	6H	p-value	12 N	p-value	12 H	p-value	24 N	p-value	24 H	p-value
<i>PFKFB4</i>	0.79	>0.9999	1.58	0.9424	0.95	>0.9999	7.03	<0.0001	0.94	>0.9999	10.81	<0.0001	1.38	0.9955	8.74	<0.0001
<i>ARTN</i>	1.71	0.0603	1.78	0.0308	1.21	0.9770	1.78	0.0317	1.79	0.0280	1.67	0.0835	1.62	0.1284	1.66	0.0912
<i>VEGFA</i>	1.29	0.9692	2.38	0.0023	1.00	>0.9999	2.63	0.0004	1.36	0.9081	2.94	<0.0001	1.16	0.9995	2.85	<0.0001
<i>CCNG2</i>	1.08	>0.9999	1.24	0.9964	1.38	0.9432	2.10	0.0592	1.07	>0.9999	2.77	0.0010	2.02	0.0918	4.60	<0.0001
<i>IRS2</i>	0.63	0.9512	0.76	0.9960	0.53	0.8331	0.57	0.8910	1.16	0.9998	1.41	0.9167	1.20	0.9990	0.83	0.9996
<i>TPBG</i>	1.58	0.1202	1.62	0.0883	1.52	0.2035	1.48	0.2813	1.77	0.0202	1.82	0.0117	1.67	0.0512	2.76	<0.0001
<i>FUT11</i>	1.20	0.9905	2.69	<0.0001	0.96	>0.9999	4.96	<0.0001	1.35	0.8146	6.74	<0.0001	1.04	>0.9999	5.83	<0.0001
<i>PPP1R3B</i>	1.20	0.9869	1.41	0.6207	1.65	0.1261	1.95	0.0094	2.03	0.0043	1.92	0.0123	2.76	<0.0001	1.49	0.4067
<i>CCL28</i>	2.05	0.1340	2.52	0.0108	1.06	>0.9999	3.16	0.0003	1.74	0.4955	3.20	0.0003	2.02	0.1550	3.57	<0.0001
<i>NR2F2</i>	1.26	0.9311	1.60	0.1527	1.73	0.0493	1.53	0.2699	1.34	0.7602	1.42	0.5430	1.70	0.0646	2.78	<0.0001
<i>JUN</i>	0.64	0.2758	1.58	0.0164	0.55	0.0924	0.81	0.9007	0.83	0.9489	1.07	0.9998	1.08	0.9994	0.77	0.7743
<i>PDLIM2</i>	1.26	0.9583	1.30	0.9176	1.26	0.9611	1.39	0.7447	1.46	0.5597	1.29	0.9344	1.06	>0.9999	0.73	0.9553

SLC11A2	1.17	0.8651	1.05	>0.9999	1.19	0.7687	0.85	0.9265	1.63	0.0013	0.89	0.9879	1.93	<0.0001	1.43	0.0367
AHCTF1	1.47	0.0952	1.31	0.4932	1.06	>0.9999	0.85	0.9812	1.49	0.0778	0.80	0.8912	1.62	0.0142	0.76	0.7910
PNPT1	1.10	0.9607	1.27	0.1577	1.27	0.1484	1.10	0.9731	1.42	0.0075	1.25	0.2001	1.30	0.0838	0.73	0.1396
AKIRIN1	1.10	0.9794	1.29	0.1227	1.05	0.9999	0.96	>0.9999	1.38	0.0232	1.14	0.8723	1.41	0.0127	0.85	0.8099
THBD	0.94	0.1014	1.54	>0.9999	0.72	0.2510	1.45	0.7698	1.14	0.9951	1.25	0.8633	1.33	0.5900	1.63	0.0386
TOX2	0.91	0.8618	0.56	<0.0001	1.04	0.9985	0.42	<0.0001	1.24	0.0399	0.48	<0.0001	1.37	0.0008	0.68	0.0033
TNFRSF21	1.05	>0.9999	1.44	0.0255	1.20	0.6940	1.16	0.8848	1.51	0.0072	1.05	0.9999	1.55	0.0039	1.00	>0.9999
CIRBP	1.24	0.0255	1.35	0.0009	1.02	>0.9999	1.39	0.0003	1.34	0.0014	1.14	0.3926	1.51	<0.0001	1.34	0.0013

(Abbreviations: N: Normoxia, H: Hypoxia)

Table 4.7: Fold changes in mRNA expression of hypoxia responsive genes in MDA-MB-231 cells relative to the zero-hour control

Gene Symbol	Fold change relative to normoxic control (0h)															
	3 N	p-value	3 H	p-value	6 N	p-value	6H	p-value	12 N	p-value	12 H	p-value	24 N	p-value	24 H	p-value
<i>PFKFB4</i>	0.73	0.9825	1.08	>0.9999	1.27	0.9837	4.03	<0.0001	1.30	0.9695	1.23	0.9930	1.66	0.3402	5.15	<0.0001
<i>ARTN</i>	0.78	0.9734	0.48	0.3049	0.85	0.9980	0.82	0.9912	0.80	0.9864	1.00	>0.9999	1.31	0.8474	1.45	0.4654
<i>VEGFA</i>	1.03	>0.9999	0.91	>0.9999	1.24	0.9999	2.38	0.1876	1.07	>0.9999	2.15	0.3698	1.36	0.9975	6.83	<0.0001
<i>CCNG2</i>	0.65	0.9966	0.91	>0.9999	1.25	0.9997	2.52	0.0759	1.40	0.9918	2.43	0.1079	1.26	0.9995	7.56	<0.0001
<i>IRS2</i>	0.55	0.5343	0.61	0.6867	0.75	0.9586	0.84	0.9978	0.68	0.8652	0.61	0.7041	0.88	0.9997	1.47	0.4732
<i>TPBG</i>	0.86	0.9974	0.64	0.5967	1.17	0.9876	0.92	>0.9999	1.02	>0.9999	1.49	0.2436	1.48	0.2787	2.57	<0.0001
<i>FUT11</i>	0.77	0.9974	1.46	0.8604	1.02	>0.9999	4.07	<0.0001	1.08	>0.9999	1.51	0.7970	0.93	>0.9999	3.63	<0.0001
<i>PPP1R3B</i>	0.84	0.9998	0.79	0.9991	1.77	0.3696	1.68	0.5183	1.00	>0.9999	1.08	>0.9999	1.78	0.3596	2.52	0.0064
<i>CCL28</i>	0.81	0.9158	0.88	0.9957	0.92	0.9997	2.00	0.0001	0.84	0.9655	1.11	0.9976	1.35	0.3753	2.84	<0.0001
<i>NR2F2</i>	1.08	0.9971	1.24	0.4524	1.22	0.5226	1.97	<0.0001	1.09	0.9937	1.21	0.5771	0.83	0.7887	0.97	>0.9999
<i>JUN</i>	1.02	>0.9999	1.07	>0.9999	0.95	>0.9999	1.06	>0.9999	1.63	0.7688	1.07	<0.0001	1.16	0.9746	1.20	0.9138
<i>PDLIM2</i>	0.59	0.0006	0.80	0.1686	0.61	0.0010	0.92	0.9460	0.74	0.0360	0.55	0.0002	0.80	0.1557	0.59	0.0006

SLC11A2	0.71	0.1017	0.55	0.0035	0.79	0.3991	0.54	0.0032	0.84	0.6915	0.67	0.0491	1.16	0.7328	0.55	0.0037
AHCTF1	1.05	>0.9999	0.64	0.8750	1.16	0.9989	0.79	0.9934	1.27	0.9718	0.77	0.9902	1.50	0.2247	0.43	0.4239
PNPT1	1.07	0.9979	0.63	0.0229	1.02	>0.9999	0.74	0.2004	1.18	0.6358	0.68	0.0710	0.88	0.9247	0.33	<0.0001
AKIRIN1	0.98	>0.9999	0.69	0.0951	0.91	0.9842	1.03	>0.9999	0.89	0.9517	0.93	0.9983	0.76	0.3240	0.76	0.3240
THBD	0.87	0.9596	0.61	0.0535	0.75	0.4124	0.32	0.0004	0.81	0.7384	0.58	0.0342	1.18	0.8015	0.76	0.4610
TOX2	0.88	0.9836	0.49	0.0222	0.96	>0.9999	0.43	0.0089	1.19	0.8425	0.58	0.0795	1.31	0.3273	0.83	0.9050
TNFRSF21	0.99	>0.9999	0.66	0.2729	0.88	0.9866	0.54	0.0526	1.30	0.4163	0.86	0.9751	1.17	0.9107	0.45	0.0143
CIRBP	0.87	0.8648	0.92	0.9889	0.89	0.9200	0.86	0.7943	1.08	0.9857	0.59	0.0055	0.89	0.9311	0.53	0.0016

(Abbreviations: N: Normoxia, H: Hypoxia)

Table 4.8: Fold changes in mRNA expression of hypoxia responsive genes in T47D cells relative to the zero-hour control

Gene Symbol	Fold change relative to normoxic control (0h)															
	3 N	p-value	3 H	p-value	6 N	p-value	6H	p-value	12 N	p-value	12 H	p-value	24 N	p-value	24 H	p-value
<i>PFKFB4</i>	0.88	>0.9999	0.97	>0.9999	1.25	0.9984	1.85	0.4034	1.57	0.8211	7.73	<0.0001	1.53	0.8762	6.46	<0.0001
<i>ARTN</i>	1.13	0.9497	0.86	0.9345	1.27	0.3354	0.64	0.5470	1.19	0.7413	1.23	>0.9999	1.03	>0.9999	1.40	0.0275
<i>VEGFA</i>	1.02	>0.9999	1.85	0.6104	1.03	>0.9999	1.68	0.8238	1.40	0.9897	3.94	0.0001	1.27	0.9993	4.67	<0.0001
<i>CCNG2</i>	1.27	0.9999	1.49	0.9923	1.07	>0.9999	2.97	0.0603	1.44	0.9962	5.07	<0.0001	1.25	>0.9999	6.03	<0.0001
<i>IRS2</i>	0.77	0.8048	0.51	0.0792	1.00	>0.9999	0.83	0.9569	0.91	0.9993	0.45	0.0374	0.81	0.9159	1.23	0.8266
<i>TPBG</i>	1.02	>0.9999	0.97	>0.9999	1.32	0.2543	0.94	>0.9999	1.32	0.2543	0.98	>0.9999	1.12	0.9796	1.82	0.0001
<i>FUT11</i>	1.21	0.9916	2.66	0.0001	1.15	0.9992	1.70	0.1711	1.21	0.9916	6.83	<0.0001	1.17	0.9982	6.37	<0.0001
<i>PPP1R3B</i>	0.62	0.0117	0.36	<0.0001	1.42	0.0052	1.05	0.9993	1.56	0.0003	0.97	>0.9999	1.19	0.5032	0.47	0.0005
<i>CCL28</i>	1.08	0.9890	1.25	0.1758	1.28	0.0981	0.91	0.9720	1.26	0.1364	0.82	0.5129	1.24	0.2242	0.86	0.7757
<i>NR2F2</i>	1.00	>0.9999	0.90	0.9901	1.13	0.9664	0.72	0.3338	1.07	0.9990	0.97	>0.9999	1.15	0.9082	1.34	0.1564
<i>JUN</i>	0.77	0.7081	0.70	0.4186	0.96	>0.9999	0.87	0.9829	0.65	0.2374	0.98	>0.9999	0.82	0.8904	2.01	<0.0001
<i>PDLIM2</i>	0.89	0.9621	0.64	0.0497	1.06	0.9996	0.94	0.9994	1.06	0.9994	1.47	0.0066	1.06	0.9991	1.63	0.0003

SLC11A2	1.08	>0.9999	0.91	>0.9999	1.35	0.8668	1.02	>0.9999	1.70	0.1557	1.09	>0.9999	1.51	0.4956	1.04	>0.9999
AHCTF1	1.12	0.9664	0.95	0.9998	1.11	0.9800	0.96	>0.9999	1.15	0.8782	0.94	0.9994	1.10	0.9835	1.26	0.3106
PNPT1	1.10	0.9941	1.11	0.9869	1.21	0.7045	1.28	0.3758	1.07	0.9995	1.33	0.2047	0.86	0.9491	1.15	0.9425
AKIRIN1	1.02	>0.9999	1.16	0.9996	1.06	>0.9999	1.20	0.9980	1.61	0.4987	1.01	>0.9999	1.20	0.9984	1.67	0.3857
THBD	0.33	<0.0001	0.21	<0.0001	0.69	0.0054	0.89	0.7134	0.23	<0.0001	0.43	<0.0001	0.50	<0.0001	0.37	<0.0001
TOX2	0.26	<0.0001	0.11	<0.0001	0.80	0.5179	0.24	<0.0001	3.05	<0.0001	0.16	<0.0001	0.70	0.1044	0.18	<0.0001
TNFRSF21	0.98	>0.9999	1.06	0.9805	1.14	0.4162	1.38	0.0004	0.95	0.9933	1.48	<0.0001	0.91	0.8747	2.19	<0.0001
CIRBP	1.06	>0.9999	0.82	0.9963	1.60	0.2727	0.90	>0.9999	1.14	0.9994	1.10	>0.9999	1.08	>0.9999	0.74	0.9663

(Abbreviations: N: Normoxia, H: Hypoxia)

4.3. Discussion

This chapter focused on identification of hypoxia responsive genes which are regulated by Sp transcription factors. Publicly available microarray data sets were analysed using GeneSpring GX software to identify a cohort of possible hypoxia responsive genes with Sp binding sites. Expression changes identified by data analyses were further validated using qPCR analyses.

Summary of the findings is listed below in relation to objectives of the study.

- Identification of hypoxia induced changes in gene expression in different breast cancer lines

A large cohort of genes was identified which exhibited different expression patterns due to hypoxia in breast cancer cell lines.

- Determine if these hypoxia responsive genes have Sp and /or HIF promoter binding sites

Hypoxia responsive genes were identified with Sp and HIF binding sites.

Twenty genes with Sp binding sites and absence of HIF binding sites were selected and expression patterns of these potential Sp regulated genes were validated using qPCR analyses.

- Identify any common cellular pathways which are altered in hypoxia and may be regulated by Sp transcription factors

Ninety-one cellular pathways were linked with potential Sp regulated genes. The role of Sp transcription factors and Sp mediated genes are widely distributed in most of the vital cellular pathways including apoptosis, angiogenesis and cell cycle regulation (*Appendix I*).

4.3.1. NCBI Data set analysis

Retrieving data sets from NCBI data base was an economical and reliable method of collecting data sets. Since microarray technique is associated with large costs, it was decided to approach thus cost-effective way to collect publicly available data sets which are relevant to experimental criteria. These data sets were accessible with bioinformatics software such as GeneSpring GX or IPA. This method allowed to broaden the study into other cells lines as well as different hypoxic conditions without any limitations.

However, data collection was limited to a small number of data sets due to availability and experimental conditions. Search criteria used for collection of data sets include few parameters. Briefly, studies that had been focused on breast cancer cells were selected while MCF-7, MDA-MB-231 and T47D cells were selected with preference. Situations where studies had used more than one cancer cell lines, only breast cancer cell lines were selected. Other key criteria for data set selection was hypoxia exposure and time durations. Data sets fulfilled both search criteria were preliminary analysed using the GeneSpring 12x software. The study was also limited to mRNA data sets and omitted all other types of data sets such as microRNA data. In addition, some data sets were lack of appropriate controls and replicates. Data sets with minimum number of three biological replicates were selected for further investigation so that appropriate statistical analysis can be performed.

4.3.2. Limitations of the GeneSpring 12X software.

GeneSpring 12x software is widely used for bioinformatics analysis. This software allows analysing different types of data including analysis of microarray data. However, there were few limitations which restrict some analysis of this study. The software was not capable of analysing data sets which had used more than one microarray platform. Data sets such as *GSE3188* had used more than one platforms in the original experiment. This problem was overcome by separating different platforms. However, in some occasions separation of NCBI data sets to each different categories of data sets with different platforms were not feasible. Another limitation liaised with lack of annotations. Some annotations were missing or not compatible with the software which prevented identification of Gene ID and Gene set enrichment analysis. Nevertheless, those genes were identified with external web references (David Bioinformatics database) wherever possible even though GSEA was not feasible.

4.3.3. Hypoxia exposure

Since it was the main objective to identify hypoxic induction of responsive genes experimental parameters and time points were carefully selected. Some studies have suggested that Sp1 proteins are involved in gene regulation in severe hypoxic conditions where Sp protein and mRNA levels were unchanged in mild hypoxia.

Therefore, severe hypoxia was selected over mild hypoxic conditions. Selection of different time points was also critical due to large number of genes. Time points were chosen in accordance with microarray data sets to allow validation of microarray data. Hypoxic exposure was limited to a maximum of 24 hours in order to maintain effective O₂ tension throughout the time. Even though most of the previous studies have not included normoxic counterparts, in this study, normoxic samples were maintained parallel to hypoxic samples. Comparable analysis with normoxic samples has enhanced the reliability of data. It was prominent that some genes were altering their expression due to hypoxia as well as the cell proliferation and confluency.

Absolute quantification method is usually used for direct quantification of target gene without standards. Since the objective of this study was to measure expression changes in hypoxia, relative quantification was selected over absolute quantification where normoxic control was used as a reference. *B2M* gene was selected as housekeeping gene which was used for normalization of fold changes. Common housekeeping genes such as *GAPDH* was not suitable for hypoxic conditions where *GAPDH* expression is also changed due to hypoxia. Some studies have utilised 18s RNA as a housekeeping gene. Since duplex assays were used for analysis, *B2M* was more suitable as a housekeeping gene. mRNA expression levels were analysed at different time points of hypoxic exposure and results were presented as relative fold changes.

4.3.4. Alteration of gene expression due to hypoxic microenvironment

In this study, the key objective was to identify Sp protein dependent hypoxia responsive genes in breast cancer cell lines and their expression patterns in severe hypoxia. Using integrative genomic analysis approach with publicly available microarray data sets, it was possible to pool large cohort of genes which are potentially hypoxia responsive and Sp dependent. However, only 20 genes were further investigated and validated using qPCR analysis after exposure to severe hypoxia for variable time intervals. As predicted by microarray data set analysis, all the genes subjected to analysis showed alterations in their expression due to severe hypoxic exposure suggesting the hypoxia responsive nature. Despite some exceptions and contrary expression patterns (which will be discussed later in this section), the majority of the genes showed predicted expression patterns. Genes subjected to qPCR analysis were initially identified with the presence of putative Sp1 binding sites in their

promoter sites. Therefore, it is possible to infer that gene identified in this section are hypoxia responsive and potentially regulated by Sp transcription factors particularly in the hypoxic microenvironment. Similar approaches have been previously employed for identifying hypoxia responsive genes in different cancer cell lines. Most of the studies have been focused on identification of genes which exhibit altered expression in various oxygen tensions and reoxygenation conditions. A study carried out by Benita *et al.*, showed core hypoxia responsive genes which are HIF dependent (Benita *et al.*, 2009). Bando *et al.*, has employed a similar approach to identify hypoxia responsive genes in MCF-7 and MDA-MB-231 cells where 26 genes were identified as commonly upregulated in 1.0% O₂ hypoxic condition (Bando *et al.*, 2003). In the present study, analysis of hypoxia responsive genes was further extended to identify potential Sp transcription regulated genes in breast cancer under severe hypoxia. TaqMan qPCR analysis was carried out with newly prepared samples from three breast cancer cell lines in order to validate expression patterns of selected genes. These genes were predicted to be regulated by Sp proteins and have different expression patterns due to hypoxia. Alteration in mRNA expression of 20 genes at variable time points of hypoxic exposure was validated. Overall, some genes exhibited expression changes exactly as predicted by GeneSpring data analysis. However, some of the gene expression patterns have deviated from expectations. Some genes exhibited only minute changes due to the lower level of expression.

Both **CA9** and **GLUT1** genes showed upregulation after exposure to hypoxia, validating O₂ tensions in sample preparation (Figure 4.2-3). Hypoxic induction was identified in all three cell lines and prominent after 12 hours of hypoxic exposure. These two genes have been widely used as hypoxia biomarkers in ample occasions which are shown to induced by HIF (Wykoff *et al.*, 2000; Olive *et al.*, 2001; Chung *et al.*, 2009). Involvement of Sp1 mediated transcription on these gene expression has also been reported (Chen *et al.*, 2001a; Kaluz *et al.*, 2003; Kaluz *et al.*, 2009). It is possible that both HIF and Sp transcription factors collaboratively regulate the expression as both HIF and Sp1 binding sites can be found in promoter sites.

PFKFB family enzymes are known to play a significant role in hypoxic induction particularly in the glycolytic switch (Hennipman *et al.*, 1988; Bartrons & Caro, 2007). The protein of this gene is known to regulate the concentration of glycolytic by-

product fructose-2,6-bisphosphate where an increased amount of fructose-2,6-bisphosphate and ATP can be found as the primary energy source for cancer cells in the hypoxic microenvironment (Chesney et al., 2014). Hypoxic induction of **PFKFB4** has been reported in several studies including breast and colon cancers (Minchenko et al., 2005). Expression of **PFKFB4** is increased in most of the cancers which is essential for the survival of cancer cells under hypoxia (Yun et al.; Goidts et al., 2012). In line with previous studies, the **PFKFB4** gene was upregulated due to hypoxic exposure in breast cancer cell lines (Figure 4.4). All three cell lines showed similar expression changes at 24 hours of hypoxic exposure. Microarray results suggest that transcriptome of **PFKFB4** gene is regulated by Sp1 mediated transcription in hypoxic microenvironment due to the presence of putative Sp consensus binding sites in its promoter.

Artemin gene encodes for a ligand which binds to various TGF- β receptors. This protein is known to regulate tumour growth and metastasis in several cancer types including breast cancer (Kang et al., 2009; Zhang et al., 2015; Zhang et al., 2016). In contrast to previous studies, expression levels of **ARTN** gene remained unchanged after the hypoxic exposure (Figure 4.5). Despite the presence of Sp promoter binding sites, some studies have also suggested that expression of **ARTN** gene might be regulated by HIF mediated transcription (HIF-1 α) (Zhang et al., 2016). However, no expression changes were identified in this study. Further analysis is required to elucidate hypoxic induction of **ARTN** gene in breast cancer cell environment.

VEGFA is one of the well-studied genes of hypoxic response which plays a major role in angiogenesis. It was proven that transcription **VEGFA** is regulated by HIF dependent/independent manner where Sp1 plays a central role in HIF independent regulation (Abdelrahim et al., 2004; Deacon et al., 2012). In line with previous studies, **VEGFA** expression was also increased due to hypoxic exposure in all breast cancer cell lines used in this study. Hypoxic induction was varied among cell types such as MCF-7 showed the earliest response just after 3 hours of exposure (Figure 4.6). Hypoxic induction was prominent after 24 hours of exposure. In addition, microarray results confirm the presence of Sp promoter sites and the Sp1 mediated regulation of **VEGFA**. Expression of **CCNG2** is correlated with Sp1 protein levels and may lead to poor prognosis of cancers (Deniaud et al., 2009a; Hasegawa et al., 2015). Cyclins play an essential role in cell cycle by regulating cyclin-dependent protein kinases (CDKs). It is

also suggested that hypoxic exposure induces overexpression of *CCNG2* in malignant tumours such as glioblastomas (Fujimura et al., 2013). In contrast, *CCNG2* was downregulated in some tumour types such as colorectal and oesophageal cancers (Chen et al., 2014a; Sun et al., 2014). However, the effect of hypoxia has not been investigated. Results presented in this section clearly indicate that the *CCNG2* is a hypoxia responsive gene and expression levels were significantly increased (at 24 hours hypoxic exposure in particular) in all three breast cancer lines after severe hypoxic exposure (Figure 4.7).

IRS2 is a member of IRS protein family which function as intermediate signalling molecules for cytokines and growth factors such as IGF-1 and insulin receptors, growth hormone (GH) and VEGF receptors. Elevated levels of *IRS2* are known to promote aggressive tumour behaviour especially in hypoxic microenvironment (Nagle et al., 2004; Gibson et al., 2007). Some studies have identified elevated levels of *IRS2* in HIF dependent manner in hypoxia (Mardilovich & Shaw, 2009). Contrary to the predictions, expression levels of *IRS2* in breast cancer cell lines showed no significant changes (Figure 4.8). However, the presence of putative Sp sites suggests a possible role of Sp1 transcription factors.

TPBG encodes for a transmembrane glycoprotein that involved in cell adhesion. In tumours, altered expression of *TPBG* is reported which may lead to poor prognosis (He et al., 2015). Results suggest that regulation of *TPBG* gene might be Sp dependent and induced due to severe hypoxia in breast cancer cell lines. Hypoxic induction was significant at 24 hours of hypoxic exposure in all three cell lines (Figure 4.9)

Only a few studies has focused on the investigation of ***FUT11*** gene products. It is believed that the protein product may be involved in protein glycosylation and fucosylation. Microarray results suggested that expression of *FUT11* is Sp dependent in hypoxia. Exposure to severe hypoxia highly induced *FUT11* expression in all three cell lines (Figure 4.10). All three cell lines showed a similar pattern of expression changes whereas changes at 24-hour time point was prominent. Upregulation of *FUT11* was identified as early as 3 hours in MCF-7 and T47D cells.

PPP1R3B gene product, Protein phosphatase 1 is believed to play a key role in cancer metastasis and *PPP1R3B* gene mutations and other gene alterations are commonly associated with malignant phenotypes. (Takakura et al., 2001; Hayashida et al., 2005).

Results presented in this chapter suggest that the regulation of *PPP1R3B* may be governed by Sp mediated transcription where hypoxic microenvironment causes alternative expression of this gene. MCF-7 and T47D cells showed decrease of *PPP1R3B* expression at 24 hours. On the contrary, MDA-MB-231 cells showed opposite expression changes. Due to cell line specific nature, further individual gene analysis is required to confirm its expression patterns (Figure 4.11).

CCL28 gene expression is known to be induced by hypoxic microenvironment which in turns plays a vital role in angiogenesis and metastasis of tumours (Dimberg et al., 2006; Facciabene et al., 2011; Huang et al., 2016). In line with previous studies, results identify the hypoxic response of *CCL28* gene in breast cancer cell lines. In addition, the *CCL28* expression may be regulated by Sp mediated transcription due to the presence of putative Sp promoter binding sites. Significant induction of *CCL28* was common for both MCF-7 and MDA-MB-231 cell lines (Figure 4.12). However, T47D cells exhibited contrasting results where expression levels decreased compared to the normoxic counterparts.

Altered expression levels of nuclear receptor **NR2F2** have been implicated in most tumours including breast, ovarian, colon and lung cancers (Navab et al., 2004; Le Dily et al., 2008; Shin et al., 2009). Multiple studies have suggested the potential role of *NR2F2* in vasculogenesis and angiogenesis (Navab et al., 2004; Schafer et al., 2008; Qin et al., 2010a). *NR2F2* gene has been reported to be up-regulated in cancers such as prostate and pancreatic tumours leading to poor prognosis and higher metastasis (Qin et al., 2010b; Qin et al., 2013; Zhang et al., 2014). Upregulation of *NR2F2* in breast cancer showed a negative effect on epithelial-mesenchymal transition (EMT) and invasiveness (Zhang et al., 2014). Results presented in this study further confirm upregulation of *NR2F2* levels in breast cancers particularly induction of the gene due to severe hypoxic exposure (Figure 4.13). Upregulation was common for both MCF-7 and MDA-MB-231 cells lines whereas T47D cells behaved differently (no significant increase).

JUN gene encodes for the protein c-Jun which heterodimerise with pro-oncogene c-Fos protein to form Activator -1 (Ap-1) transcription factor. Regulation of *JUN* transcription is believed to be mediated by autoregulation of Ap-1 protein. It was implicated that AP-1 plays a regulatory role in hypoxia microenvironment in tumour

models as a HIF independent transcription factor (Michiels et al., 2001; Fantozzi et al., 2003). Both HIF dependent and independent regulation of *JUN* gene is identified in chronic hypoxia (severe hypoxia) (Laderoute et al., 2002). Upregulation of *JUN* gene is recorded in several tumours under chronic hypoxia (Ausserer et al., 1994). In addition, c-Jun is believed to interact with HIF-1 α in conjunction with other proteins to facilitate transcriptional mediation of some genes in a cooperate mechanisms (Alfranca et al., 2002). Results presented in the current study suggests the potential Sp mediated transcription of *JUN* gene due to the presence of putative Sp binding sites in its promoter. Hypoxic induction of JUN oncogene in MCF-7 cells was identified after 3 hours of hypoxic exposure. In MDA-MB-231 and T47D cells it was after 12 and 24 hours respectively (Figure 4.14). Both HIF and Sp transcription factors may regulate transcription of *JUN* gene in a collaborative manner.

Upregulation of ***PDLIM2*** is reported in highly metastatic cancers and known to play a vital role in NF- κ B transcription factor regulation (Qu et al., 2010). Tumour suppression activity of *PDLIM2* has also been identified in breast cancer cell models where NF κ B activity is inhibited by *PDLIM2*. Yet, no evidence is available on its role in hypoxic induction nor Sp mediated transcriptional regulation. Results indicate the possible role of Sp driven hypoxic induction of *PDLIM2* gene in severe hypoxia (Figure 4.15).

SLC11A2 gene encodes the protein for NRAMP2 (DCT1), an iron transporter which involved in iron absorption (Fleming et al., 1997; Jiang et al., 2010). The effect of hypoxia on *SLC11A2* has not been identified in hypoxia nor the Sp mediated regulation except a single study carried out by Xue *et al.* In this study it has been suggested that *SLC11A2* is regulated in HIF-2 α mediated transcription (Xue et al., 2016). Regulation of other iron transporters in the hypoxic environment has also been reported previously. Results presented in this study confirm that *SLC11A2* is a potential hypoxia responsive gene which is potentially mediated by Sp transcription factors. Figure 4.16, Panel A and B clearly indicates the significant downregulation of *SLC11A2* gene in both MCF-7 and MDA-MB-231 cell lines.

AHCTF1 encodes for a transcription factor ELYS which is believed to play a role in nuclear pore assembly (Rasala et al., 2006). Nevertheless, expression of *AHCTF1* and hypoxia has not yet been investigated. However, it is possible that it is a hypoxia responsive gene which is transcribed in Sp mediated manner in breast cancer cell lines.

Both MCF-7 and MDA-MB-231 cells showed alteration (lower expression levels) of *AHCTF1* in hypoxia (Figure 4.17). Also, it is noticed that the expression of *AHCTF1* is increased in normoxia.

The role of ***PNPT1*** have not reported in hypoxia related cellular pathways up to date. The gene encodes for a polynucleotide phosphorylase (hPNPase^{old-35}) which is known to play a central role in cell cycle, apoptosis and mRNA degradation (Sarkar et al., 2007; Das et al., 2011; Sokhi et al., 2013). In MCF-7 and MDA-MB-231 cells, expression levels of *PNPT1* were significantly decreased at 24 hours of hypoxic exposure suggesting its hypoxic nature (Figure 4.18). In addition, it is highly likely that Sp driven transcriptional activation of *PNPT1* due to the presence of putative Sp sites.

The role of ***AKIRIN1*** in the hypoxic environment is barely understood. Up to date, only a single study has suggested NF- κ B mediated gene expression in mice and *Drosophila* cells. Microarray results suggested that *AKIRIN1* expression can be hypoxia depended. However, only the MCF-7 cells showed significant downregulation of the gene expression of *AKIRIN1* after 12 and 24 hours of hypoxic exposure (Figure 4.19). Both MDA-MB-231 and T47 cells showed slight decreases at some points nevertheless lower level of expression changes of the gene could possibly mask any changes.

The ***THBD*** gene, Thrombomodulin is known as a specific endothelial cell receptor and its involvement in hypoxic induction is yet to be discovered. A study carried out by Zheng et al., has shown regulation of cadherin expression by thrombomodulin in lung cancer cells suggesting the possible role in epithelial-mesenchymal transition (EMT) which is identified as a hypoxia induced transition mechanism (Zheng et al., 2016). PCR analysis of the *THBD* expression validated the hypoxia driven expression alterations and results clearly indicate the downregulation of the *THBD* gene in severe hypoxia (Figure 4.20). Both MCF-7 and MDA-MB-231 cell lines showed significant decreases of the gene expression in severe after 6 hours of exposure. However, T47D showed slight different pattern of expression compared to other two cell lines.

The potential role of ***TOX2*** gene in cancer microenvironment has been recorded in lung and breast cancer cell models where expression levels were significantly decreased compared to the normal cells (Tessema et al., 2012; Yu & Li, 2015). However, the actual role of *TOX2* in hypoxic microenvironment remains unspecified. Results presented in this section confirm hypoxic response of *TOX2* gene in breast

cancer cell lines. In line with previous results, all three cell lines used in this study showed a significant downregulation of the *TOX2* gene, particularly after 6 hours of hypoxic exposure (Figure 4.21). Expression levels continued to decrease throughout the experiment duration.

Involvement of ***TNFRSF21*** in hypoxia has been identified in microarray analysis of adipocytes (Mazzatti et al., 2012). Nevertheless, the role of *TNFRSF21* is not fully understood in cancer models and lack of details were available about its expression changes in hypoxic microenvironment. As predicted from microarray analysis, a decrease of the gene expression was detected in both MCF-7 and MDA-MB-231 cells which was prominent at 24 hours hypoxic exposure (Figure 4.22). However, T47D cells behaved completely opposite to the predictions and other two cell lines where exposure to severe hypoxia lead to significant up regulation of the *TNFRSF21* gene. Hypoxic responsive nature of *TNFRSF21* is evident, although discrepancies of expression patterns among the cell lines is likely due to *TNFRSF21* is mediated in tissue specific manner.

CIRBP is believed to be up-regulated under several cellular stress conditions such as cold shock, radiation and hypoxia (Lee et al., 2015). Autoregulation of *CIRBP* transcription has been reported due to activation of alternative promoters (Al-Fageeh & Smales, 2009). However, the involvement of the *CIRBP* gene in the hypoxic induction mechanisms are barely investigated. Presence of the putative Sp sites in *CIRBP* promoter suggests the potential role of Sp mediated transcription regulation. Despite some alterations identified, data is not sufficient to claim the hypoxic nature of the *CIRBP* at this juncture.

In general, all three breast cancer cell lines exhibited similar expression patterns with some exceptions (*ARTN*, *IRS2*, *PPP1R3B*, *PDLIM2*). All cell lines followed the common trend of hypoxic induction (upregulation/downregulation) despite their different hormone receptor status (Properties of these cell lines have been summarised in Chapter 2, Table 2.1). Therefore, Sp driven hypoxic induction of above identified genes may be independent of hormone receptors status/related pathways or cell types suggesting the importance of these induction mechanisms on other solid tumour types.

4.3.5. The possible mechanisms of Sp protein driven transcriptional regulation of hypoxia responsive genes

There are few possible mechanisms that explain how Sp proteins mediate the transcription of hypoxia responsive genes. Similar to HIF mediated transcription, it is possible that Sp transcription factors may bind to the distal promoter sites of the target gene promoters, which in turn regulate the transcription of the genes. It is also suggested that possible synergistic collaboration of HIF mediated transcription and Sp transcription factors in gene regulation. The presence of Sp binding sites adjacent to HRE sites may facilitate the enhanced expression of hypoxia responsive genes. Several studies have demonstrated the presence of Sp1 sites adjacent to HRE regions in hypoxia responsive genes such as *GAPDH* which is essential for full promoter activity (Higashimura et al., 2011). Moreover, it is also hypothesised that HRE and Sp sites can be potentially shared with Sp proteins and other transcription factors at some gene promoter sites. Nevertheless, this speculation has not been widely accepted, it is believed that Homology with the HRE and Sp sites may facilitate occupation of Sp proteins which in turn regulates transcription (Zhang et al., 2007; Rezvani et al., 2010).

4.3.6. Conclusion

Results presented in this section identifies hypoxia responsive genes in breast cancer cell lines. Some of the genes are newly identified as hypoxia responsive genes. Moreover, these results suggest the vital role of Sp transcription factors in hypoxic response. Important hypoxia responsive genes such as *PFKFB4*, *CCNG2* and *FUT11* genes are potentially regulated by Sp transcription factors. Pathway analysis also suggests that role of Sp transcription factors are widely dispersed in most of the cellular pathways empathising its importance as a key player in transcription. These genes can be possibly used as potential biomarkers for hypoxic tumours such as breast cancers.

Chapter 5.
Binding analysis and kinetic evaluation of Specificity
protein family inhibitors

5. Binding analysis and kinetic evaluation of Specificity protein family inhibitors

5.1. Introduction

The potential of Specificity protein (Sp) inhibitors, particularly DNA-binding small molecules, as anti-cancer therapeutic agents has been identified and tested in different cancer types such as lung, colon and breast cancers (Yuan et al., 2007; Chadalapaka et al., 2010). Effective blockade of Sp mediated transcription using small DNA binding molecules might be an attractive target for cancer therapeutics (Safe et al., 2014; Vizcaino et al., 2015). Since, Sp transcription factors directly interact with DNA and DNA polymerase complex, use of small DNA binding moieties to compete for Sp binding sites and /or mimic Sp binding might be a vital approach to inhibit Sp mediated transcription while keeping gene toxicity levels at a minimum (Kadonaga et al., 1988; Gill et al., 1994).

Vast variety of DNA binding molecules such as antibiotic derivatives have been used to inhibit transcription of many Sp mediated genes. Moreover, the pharmacological inhibition of Sp family mediated gene regulation by some DNA binding molecules such as Celecoxib, Mithramycin A, Curcumin, and Tolfenamic acid have shown promising results in pre-clinical studies. Also, these natural and synthetic inhibitors have demonstrated the downregulation of Sp protein levels (Sp1-4) and their gene targets which ultimately inhibits cellular proliferation, tumorigenesis as well as cancer progression (Abdelrahim & Safe, 2005; Chintharlapalli et al., 2007; Jutooru et al., 2010; Pathi et al., 2014).

Mithramycin A and Chromomycin A are well studied antibiotic derivatives, and proven to inhibit transcription of Sp regulated genes. The structure of Mithramycin and Chromomycin A were determined in the 1960s and ever since these antibiotic derivatives and modified moieties have been tested for their inhibitory action in cellular process on ample occasions (Bakhaeva et al., 1968; Montanari & Rosazza, 1988; Blume et al., 1991; Devi et al., 2009) .

The early stage investigation of Mithramycin A and Chromomycin A have mainly focused on identification of their DNA interaction. Structural determinations have

been carried out with NMR, DNA foot print and spectroscopy techniques in several studies in order to determine their binding activity (Bakhaeva et al., 1968; Chakrabarti et al., 2000; Barcelo et al., 2010). These conventional studies have proven that both Mithramycin A and Chromomycin A can interact with GC-rich regions of DNA. In addition, a DNase footprint study carried out by Carptener *et al.*, showed that Mithramycin A preferably binds to GC rich DNA compared to CG regions (Carptener et al., 1994).

Studies were further extended to identify the nature of DNA-Mithramycin/Chromomycin interaction. Barcelo *et al.*, suggested that both Chromomycin and Mithramycin bind reversibly to the minor groove of G/C-rich regions in DNA whilst study carried out by Chakrabarti *et al.*, showed that groove size of the DNA oligomer may also affect the binding (Chakrabarti et al., 2000; Barcelo et al., 2010). Another study by Banerjee *et al.*, adds that Mithramycin has the ability to interact with DNA as well as core histone proteins (Banerjee et al., 2014).

Moreover, these studies have focused on assessing the effect of different metal chelates on binding activity. Most of the studies have suggested that Mithramycin A requires Mg^{2+} as an obligatory cation for DNA interaction. However, in contrast to previous studies, Banerjee *et al.*, suggested that Mithramycin may not need presence of any essential metal chelating ions for DNA interaction (Banerjee et al., 2014). The requirement of metal chelating for Chromomycin A is barely reported. A study carried out by Lahiri *et al.*, suggested that the presence of Cu^{2+} may suppress the binding ability of Chromomycin a towards the DNA (Lahiri et al., 2012).

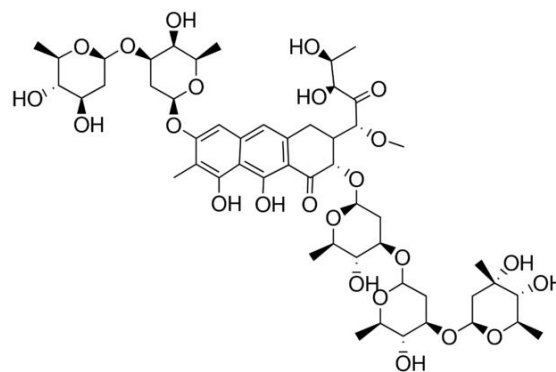
Similarly, Nordihydroguaiaretic acid (NGDA) derivative, Terameprocol (Tetra-O-methyl nordihydroguaiaretic acid, TMP) has been identified as a Sp protein inhibitor (Lopez et al., 2007a; Smolewski, 2008). NDGAs and their derivatives have shown promising applications in treatment of multiple diseases, including cardiovascular diseases, neurological disorders and cancers (Arteaga et al., 2005). The role of NDGAs has been identified in several cancer types such as breast, lung, prostate and skin cancers (Huang et al., 2004). The exposure to NDGAs resulted in cell growth inhibition of lung and breast cancer cell lines (Soriano et al., 1999). In addition, a study carried out by Kubow *et al.*, has shown that the breast cancer cells exposed to NDGAs exhibit significantly lower transformation and proliferation rates (Kubow et al., 2000). The

most promising derivative was methyl substituted form which was named as Terameprocol or M4N (McDonald et al., 2001; Ho & Go, 2013). Similar to NDGAs, Terameprocol exhibits growth inhibitory action on most of the tumours and growth inhibitory properties have also been clinically tested (Park et al., 2005; Meyers et al., 2009; Ho & Go, 2013) (see the Figure 5.1).

Different approaches have been used in designing and synthesising specific moieties, that can be used to enhance/inhibit, desired DNA related cellular functions. For an example, recent studies have attempted to synthesise new derivatives of Mithramycin and tested for their activity (Malek et al., 2012; Fernandez-Guizan et al., 2014). Similarly, new derivatives of NDGAs with slight modifications such as substitution of methyl groups to cyclic ring has been tested for their anti-tumour activity (Li et al., 2013). However, all these primary investigations have mainly focused on identification of their anti-tumour activity on different cancer types. In addition, DNA-Mithramycin/Chromomycin interaction has only been tested using conventional techniques. Interaction of these drugs were tested towards GC-rich DNA sequences in presence of different cations. Even though previous studies confirm the interaction with DNA, binding towards biologically relevant promoter sites have not been investigated. In addition, requirement of other co-factors such as obligatory cation has been suggested hence yet to be proven. Similarly, actual DNA-Terameprocol interaction has not been studied. Furthermore, binding kinetics of NDGA derived Sp inhibitors, genotoxicity and DNA damaging properties are still unknown.

C₅₂H₇₆O₂₄

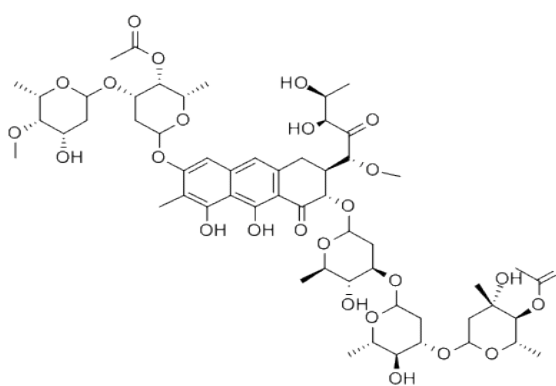
Molecular Weight :1085.15



Mithramycin A

C₅₇H₈₂O₂₆

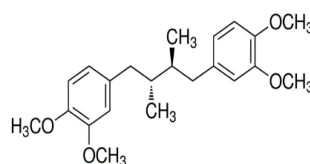
Molecular Weight :1183.25



Chromomycin A3

C₂₂H₃₀O₄

Molecular Weight :358.47



Terameprocol

Figure 5.1: Sp protein inhibitors

Chemical Structures of **A.** Mithramycin A, **B.** Chromomycin A3, **C.** Terameprocol (Structures taken from Sigma).

5.1.1. Aims and objectives of the chapter

Present work attempts to determine the interaction of Mithramycin A, Chromomycin A and Terameprocol towards linear DNA of Sp consensus sequence and known Sp promoter sites using real-time Surface Plasmon Resonance technique. In addition, binding kinetic of these drugs will be determined in terms of on and off rates.

This chapter aims to answer the following questions:

- What is the nature of DNA interaction of Mithramycin, Chromomycin and Terameprocol in terms of DNA binding and specificity?
- What are the effects of Mg^{2+} cation on Mithramycin A and Chromomycin A binding?
- What are the kinetics of interaction including on/off rates?

5.2. Results

In this section, it was aimed to evaluate different Sp inhibitors and their binding efficiencies using real-time Surface Plasmon Resonance technology. DNA-binding small molecules Mithramycin A, Chromomycin A and Terameprocol which are known Sp inhibitors were used for analysis. In general binding analysis of each drug was carried out with known Sp binding sites followed by a kinetic evaluation.

Streptavidin-biotin capture method was used for ligand immobilisation where double-stranded specific DNA oligomers were immobilised on to Sensor chip SA (Series S) using the manual injection method. Figure 5.2 represents an example of surface preparation procedure. Since samples and buffers contained 5% (v/v) DMSO, solvent correction range was selected as 4.5-5.8% (v/v) DMSO and included in each experiment (see Figure 5.3-4).

5.2.1. Kinetic analysis

All the data presented here adhered to following experiment strategy unless stated otherwise. Binding analysis was performed using *Biacore T200* (GE Healthcare) equipped with Series S SA sensor chip. Biotinylated, double stranded DNA oligomers were captured using streptavidin-biotin capture chemistry. In order to generate kinetic data, Mithramycin A, Chromomycin A and Terameprocol were injected over the flow cells. Injections were performed for 60 seconds followed by 120 seconds of buffer flow at 30 $\mu\text{l}/\text{min}$ flow rate at 25 °C. Binding analysis was performed in binding buffer (1x PBS; 5% (v/v) DMSO; 5% (v/v) p20 with or without 5 mM MgCl_2). Since the streptavidin-biotin capture method was used, regeneration was not applicable for these experiments. Data were collected at the rate of 60 Hz. Data evaluation was performed using *Biacore T200* evaluation software. A solvent correction was added to each experiment and *corr* response was used for the fitting curves. A Langmuir 1:1 interaction model was used for sensorgrams fitting with global data analysis. In addition, visual inspection was carried out after each assay in order to identify any random or systemic deviations. Residual plots were also inspected and residual levels were maintained within the instrument noise (within the width of the band in residual plots) level all the time. Best fitting curves were prepared with minimum Chi^2 values.

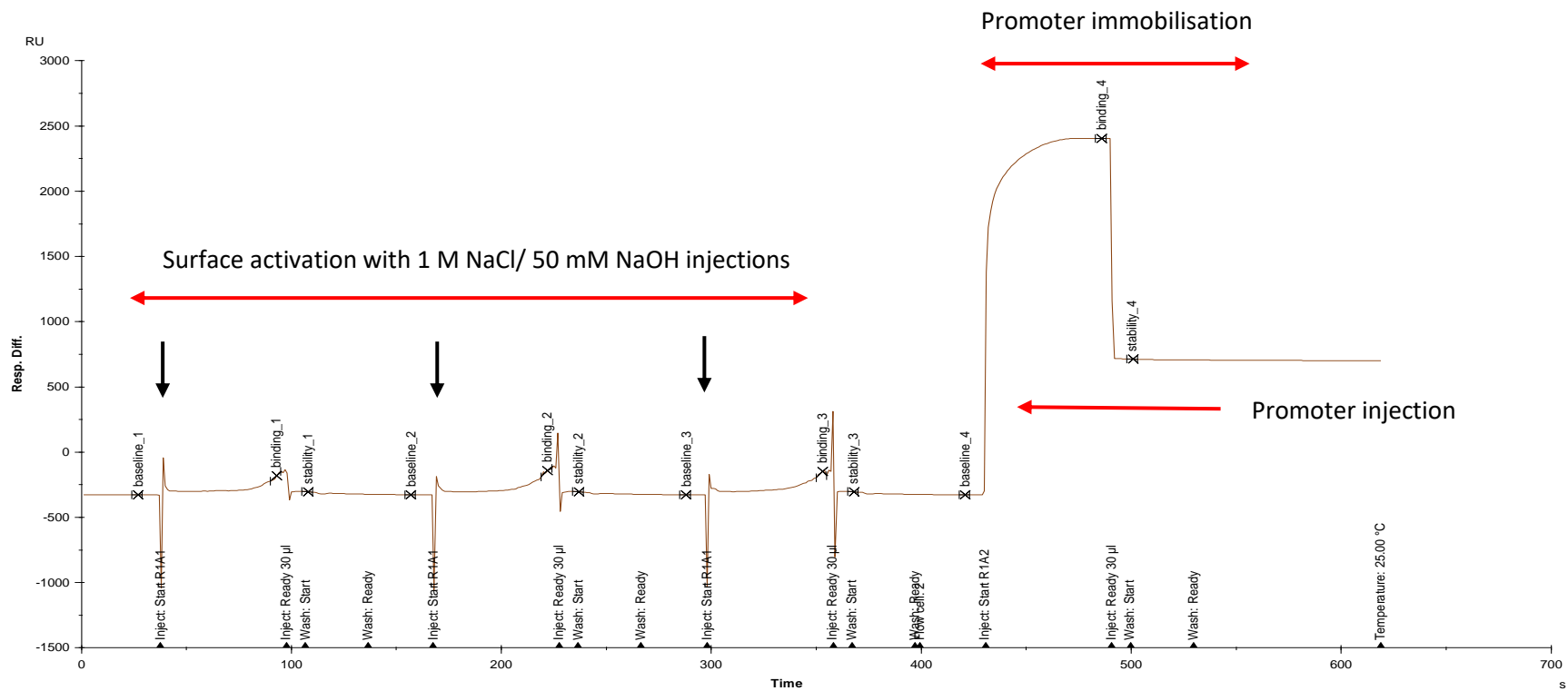


Figure 5.2: Immobilization of biotinylated ligands to Sensor chip (SA chip) surface.

Sensor chip surface was activated using injection of 1 M NaCl/ 50 mM NaOH solution for three consecutive times at rate of 30 $\mu\text{l}/\text{sec}$ for 60 seconds. Double stranded promoter sequences were prepared as of 1 pmol/ μl solutions and were manually injected until they achieved the desired immobilization level (~ 1000 RU).

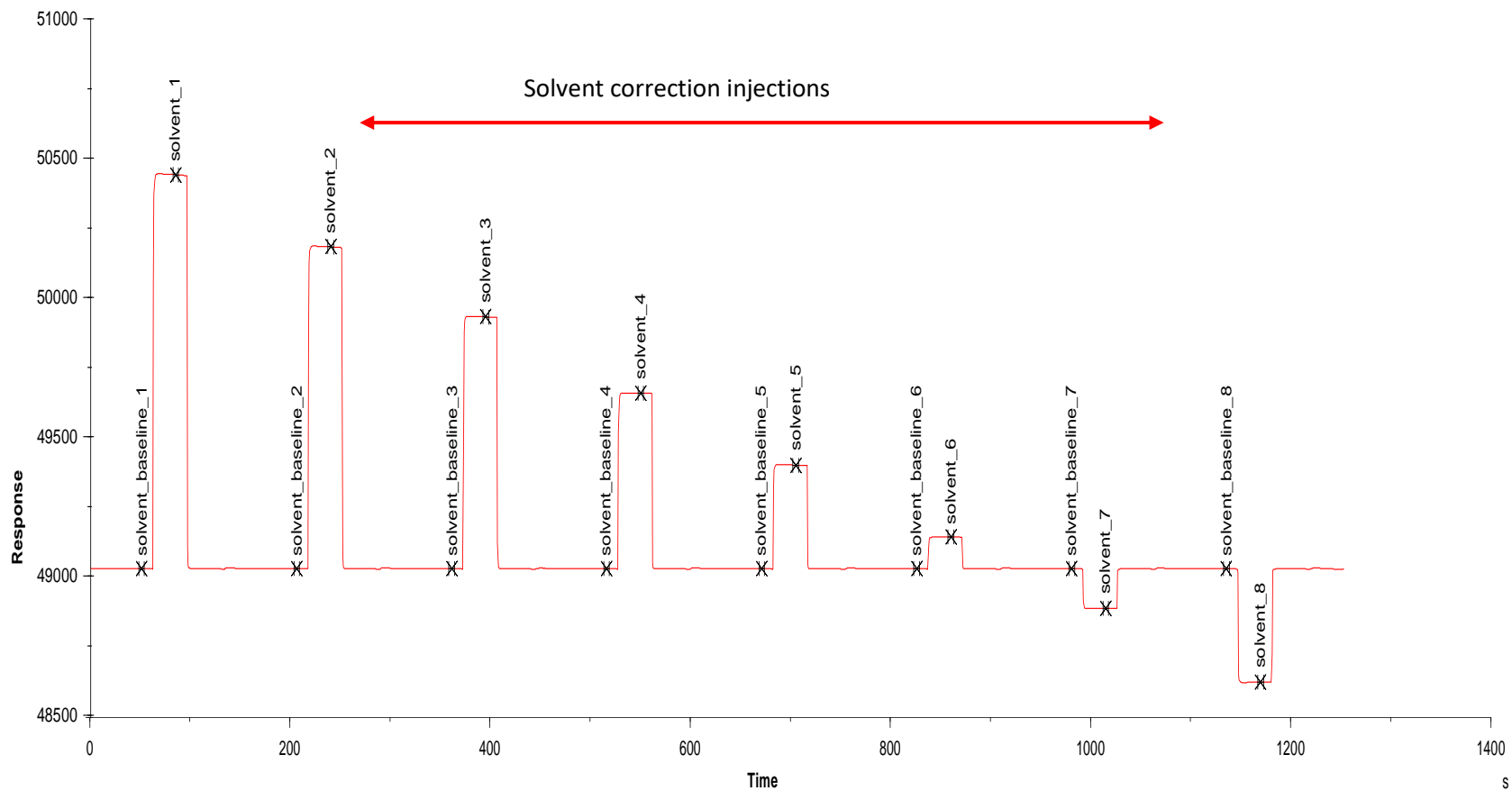


Figure 5.3: Solvent correction

The solvent correction was prepared using DMSO solution. Concentration gradient of DMSO was prepared using 4.5% -5.8% (v/v) DMSO solutions and eight solvent correction samples were prepared as standard. A solvent correction step was run at the beginning and the end of each assay as well as after 20 cycles. The graph represents an example of experimental assay.

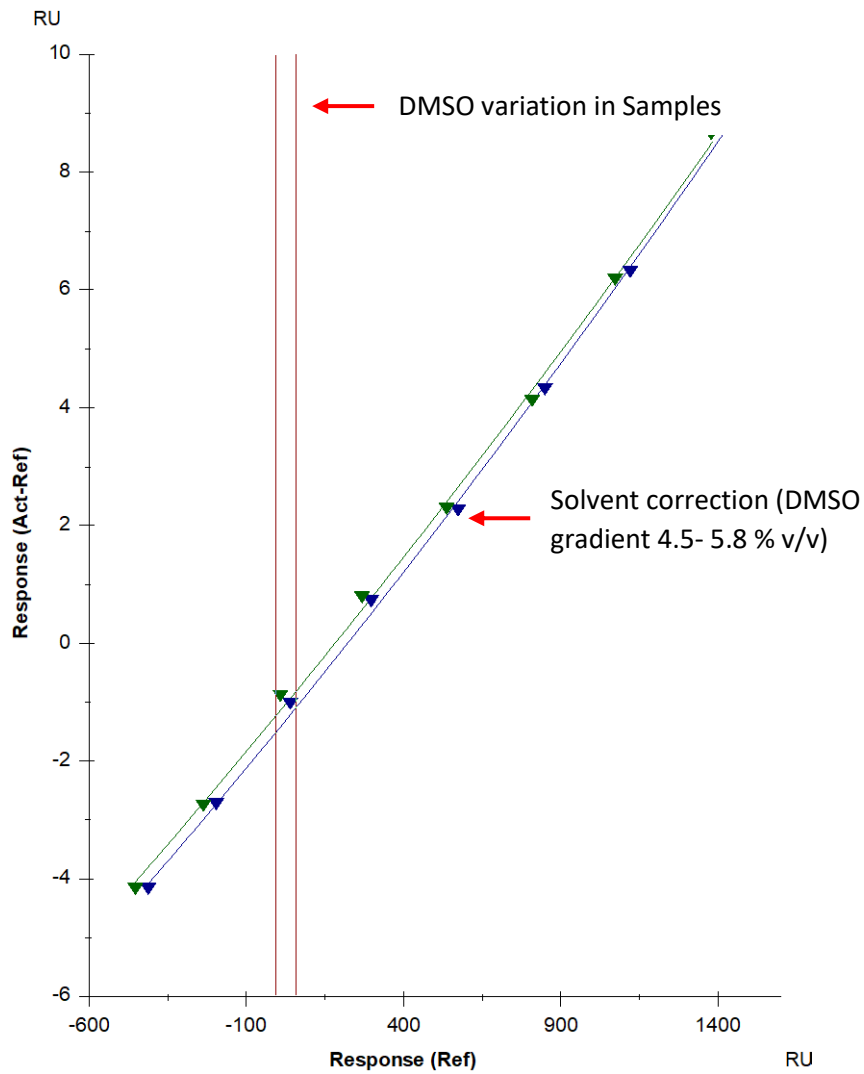


Figure 5.4: Variation of DMSO concentration in samples

Solvent correction was prepared using DMSO solution. The graph indicates the solvent correction and sample variation. Red lines represent variation of DMSO concentration in samples. The graph shows an example of an experimental assay.

5.2.2. Interaction of Mithramycin A towards Sp consensus sequence

Sp transcription factors directly bind to the GC-rich consensus sequence (GC box element) of DNA via zinc fingers (Cys₂/His₂). It is hypothesised that DNA binding Sp protein inhibitors also occupy the same binding sites and inhibit Sp mediated gene transcription. In this study, 30 base pairs (bp) long double-stranded DNA oligomer consisting Sp consensus sequence was used for binding analysis (5' **AAT TCG ATC GGG GCG GGG CGA GCA TGC TCA** 3'). Binding analysis was carried out using a concentration series of Mithramycin A (3-125 μ M) and running buffer contained 1x PBS + 5% (v/v) DMSO + 5% (v/v) p20 surfactant and 5 mM MgCl₂ as previously described (section 5.3.1).

As evident from Figure 5.5, Mithramycin A specifically binds to DNA oligomer of Sp consensus region in the presence of cation Mg²⁺, where no bindings were detected for the control sequence. Kinetics of Mithramycin A interaction with Sp consensus region was determined to have an association constant (K_a) of 5.92 (+/- 0.92 SD) x 10³ (M⁻¹s⁻¹) and dissociation constant (K_b) of 1.85 (+/- 0.03 SD) x 10⁻² (s⁻¹). Equilibrium constant was determined to be K_d = 3.18 (+/- 0.56 SD) x 10⁻⁶ (M) at 25 °C. Rmax 2.93 (+/- 0.85 SD) x 10² (RU) was observed at 31.25 μ M Mithramycin A concentration (see Figure 5.5 panel A). Similarly, Mithramycin A was interacting with mutant Sp consensus region (5' **AAT TCG ATC GGT TCG GGG CGA GCA TGC TCA** 3') with K_a of 5.53 (+/- 0.52 SD) x 10³ (M⁻¹s⁻¹) and K_b of 3.43 (+/- 0.12 SD) x 10⁻² (s⁻¹). In addition, K_d was identified as 6.24 (+/- 0.36 SD) x 10⁻⁶ (M) at 25 °C. However, Rmax value decreased to 1.63 (+/- 0.37 SD) x 10² RU at same concentration (see Figure 5.5 panel B).

Binding analysis of Mithramycin A was further extended to investigate the dependency of the cation requirement for DNA interaction. Identical experimental conditions were used except running buffers composition which lacks Mg²⁺ ions. Interestingly Mithramycin A was specifically interacting with Sp consensus DNA in the absence of Mg²⁺ ions. As evident from Figure 5.6, DNA-Mithramycin A interaction was specific to Sp consensus regions where no detectable binding was reported for the control sequence. Kinetics were reported as K_a = 9.63 (+/- 0.39 SD) x 10² (M⁻¹s⁻¹) and K_b = 1.60 (+/- 0.21 SD) x 10⁻² (s⁻¹). Equilibrium constant was reported as K_d = 1.67 (+/- 0.26 SD) x 10⁻⁵ (M) at 25 °C. Rmax of 2.32 (+/- 0.11 SD) x 10² (RU) was observed at concentration of 125 μ M concentration of Mithramycin A (see Figure 5.6 panel A). Due to the changes

of base pairs in binding sequence with similar GC content (mutant Sp consensus sequence) resulted decrease in Rmax value for same concentration. Kinetics were reported as $K_a = 1.54 (+/- 0.22 \text{ SD}) \times 10^2 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ and $K_b = 2.12 (+/- 0.15 \text{ SD}) \times 10^{-2} \text{ (s}^{-1}\text{)}$. Equilibrium constant was reported as $K_d = 1.39 (+/- 0.92 \text{ SD}) \times 10^{-4} \text{ (M)}$ at 25 °C. Rmax of $8.92 (+/- 0.12 \text{ SD}) \times 10^{-1} \text{ (RU)}$ was observed at concentration of 125 μM Mithramycin A (see Figure 5.6 panel B).

5.2.3. Interaction of Chromomycin A towards Sp consensus sequence

Binding analyses were performed using running buffer with 5 mM MgCl_2 and a concentration series of Chromomycin A (up to 125 μM). As seen in Figure 5.7, Chromomycin A preferably interacts with GC rich regions where lower bindings were detected for the control sequences. Kinetics of Chromomycin A interaction with DNA of Sp consensus region was marked by association constant (K_a) $5.51 (+/- 0.56 \text{ SD}) \times 10^2 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ and dissociation constant of (K_b) $5.55 (+/- 0.44 \text{ SD}) \times 10^{-3} \text{ (s}^{-1}\text{)}$. Equilibrium constant was reported as $K_d = 1.02 (+/- 0.18 \text{ SD}) \times 10^{-5} \text{ (M)}$ at 25 °C. Rmax of $1.95 (+/- 0.08 \text{ SD}) \times 10^2 \text{ (RU)}$ was observed at concentration of 62.50 μM concentration of Chromomycin (see Figure 5.7 panel A). Similarly, Chromomycin was interacting with mutant Sp consensus region with $K_a = 5.58 (+/- 0.63 \text{ SD}) \times 10^2 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ and dissociation constant of $K_b = 8.16 (+/- 0.20 \text{ SD}) \times 10^{-3} \text{ (s}^{-1}\text{)}$. Equilibrium constant was reported as $K_d = 1.48 (+/- 0.21 \text{ SD}) \times 10^{-5} \text{ (M)}$ at 25 °C. However, Rmax value decreased to $1.13 (+/- 0.19 \text{ SD}) \times 10^2 \text{ RU}$ at same concentration (see Figure 5.7 panel B).

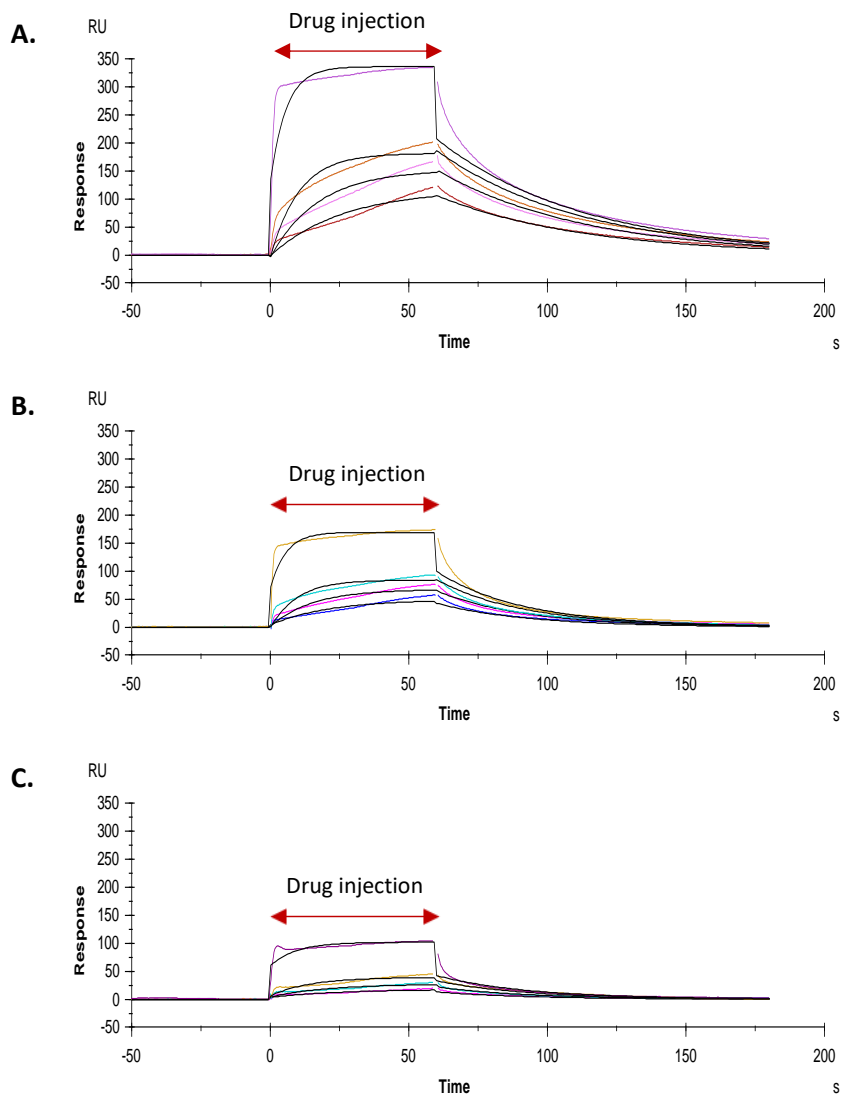


Figure 5.5: Interaction of Mithramycin A with Sp consensus sequence in the presence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Mithramycin A with 30bp DNA oligomers: Sp consensus (panel A); Sp consensus mutant (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Mithramycin A was injected over the surface (3-31.25 μM). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 $\mu l/min$ flow rate at 25 $^{\circ}C$. Sensorgrams are representative of n=4 experiments.

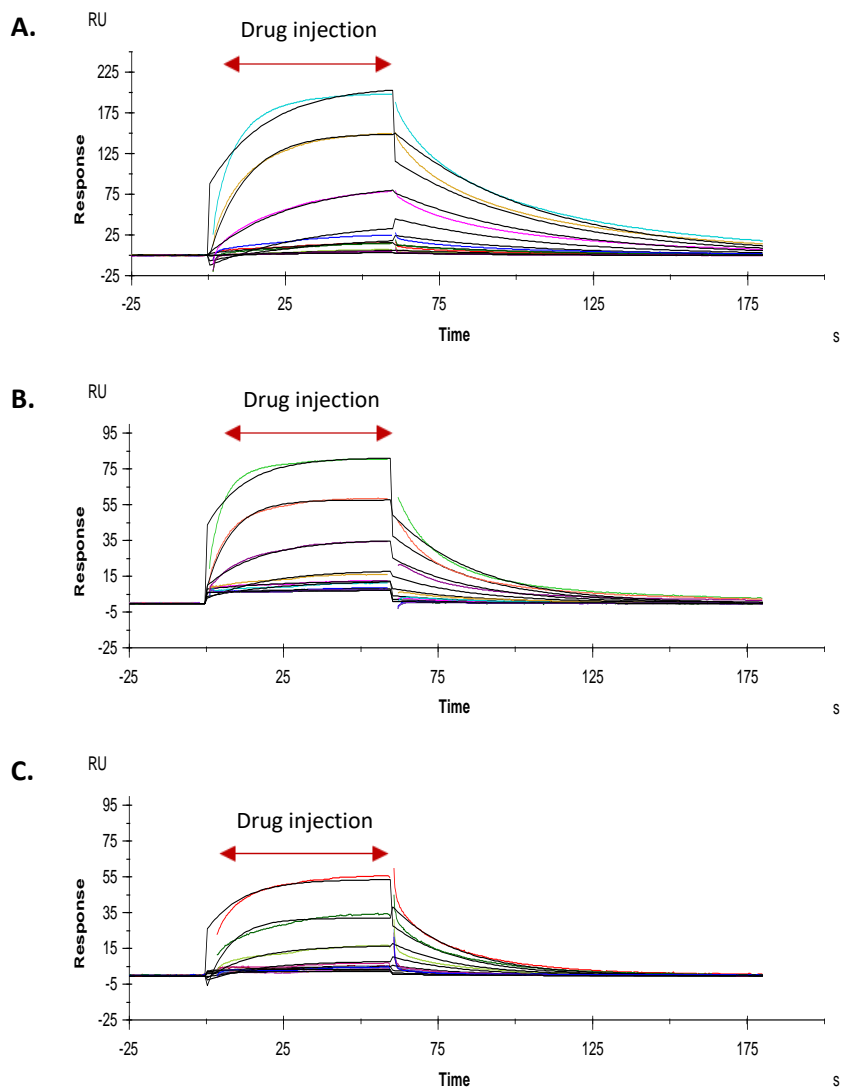


Figure 5.6: Interaction of Mithramycin A with Sp consensus sequence in absence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Mithramycin A with 30bp DNA oligomers: Sp consensus (panel A); Sp consensus mutant (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Mithramycin A was injected over the surface (3-125 μ M). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 μ l/min flow rate at 25 $^{\circ}$ C. Sensorgrams are representative of n=4 experiments.

As evident from Figure 5.8, DNA-Chromomycin interaction was also identified in the absence of Mg^{2+} ions. Kinetics were reported as association constant of $K_a = 6.4 (+/- 0.73 \text{ SD}) \times 10^1 \text{ (M}^{-1}\text{s}^{-1})$ and dissociation constant of $K_b = 8.33 (+/- 1.20 \text{ SD}) \times 10^{-3} \text{ (s}^{-1})$. Equilibrium constant was reported as $K_d = 1.30 (+/- 0.06 \text{ SD}) \times 10^{-4} \text{ (M)}$ at 25 °C. R_{max} $8.24 (+/- 0.8 \text{ SD}) \times 10^1 \text{ (RU)}$ was observed at concentration of 125 μM concentration of Chromomycin (see Figure 5.8 panel A). Due to the Changes of base pairs in binding sequence with similar GC content (mutant Sp consensus sequence) resulted in decrease in R_{max} value for same concentration. Kinetics were reported as association constant of $K_a = 5.30 (+/- 0.69 \text{ SD}) \times 10^1 \text{ (M}^{-1}\text{s}^{-1})$ and dissociation constant of $K_b = 1.56 (+/- 0.10 \text{ SD}) \times 10^{-2} \text{ (s}^{-1})$. Equilibrium constant was reported as $K_d = 3.07 (+/- 0.37 \text{ SD}) \times 10^{-4} \text{ (M)}$ at 25 °C. R_{max} $6.64 (+/- 1.64 \text{ SD}) \times 10^1 \text{ (RU)}$ was observed at concentration of 125 μM concentration of Chromomycin (see Figure 5.8 panel B).

5.2.4. Interaction of Terameprocol towards Sp consensus sequence

No detectable binding was observed between Terameprocol and double stranded DNA oligomer of Sp consensus region. Concentration serious of 3 μM to 500 μM of Terameprocol was used for binding analysis. No detectable binding was observed at any given concentrations. Less than 10 RU response was detected for all the oligomers including the control sequence (see Figure 5.9).

Results clearly indicated the specific interaction of Mithramycin A and Chromomycin A with GC box of Sp binding sites without interference from Mg^{2+} cation. It is prominent that mutations in the binding sites alter the affinities.

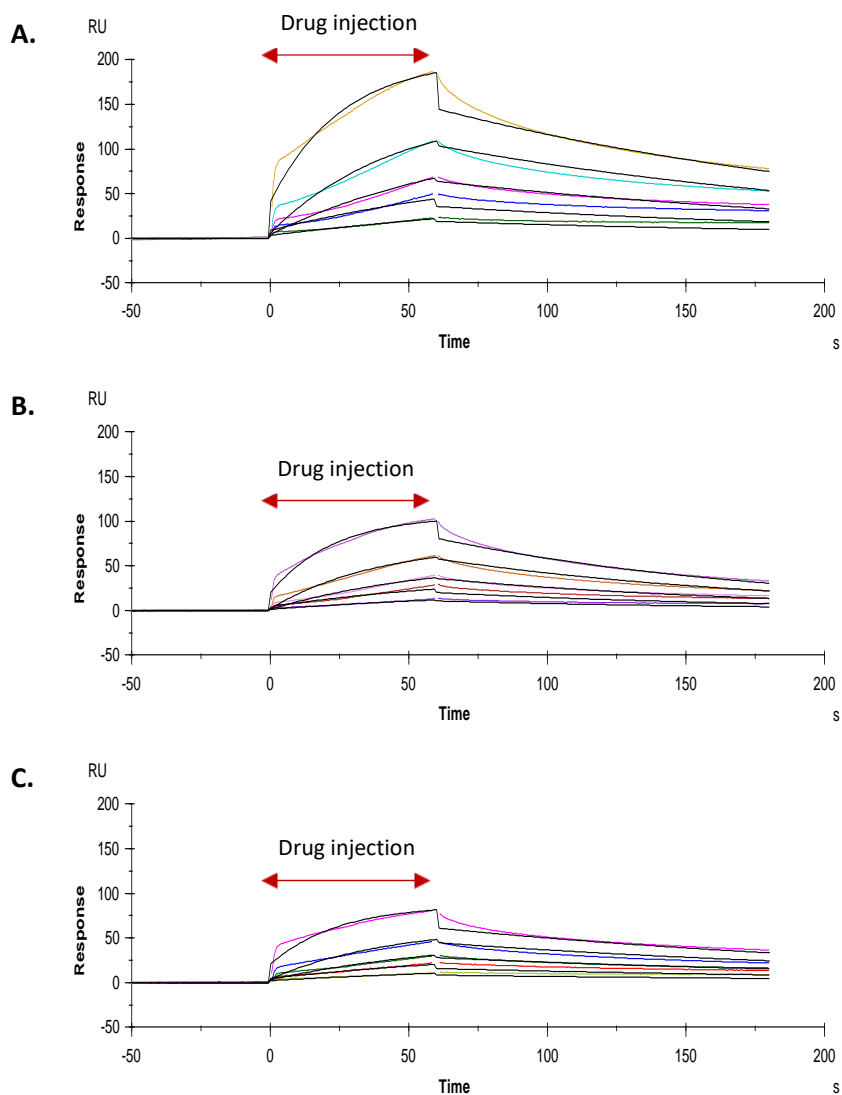


Figure 5.7: Interaction of Chromomycin A with Sp consensus sequence in the presence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Chromomycin A with 30bp DNA oligomers: Sp consensus (panel A); Sp consensus mutant (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Chromomycin A was injected over the surface (3-62.50 μM). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 $\mu l/min$ flow rate at 25 $^{\circ}C$. Sensorgrams are representative of n=4 experiments.

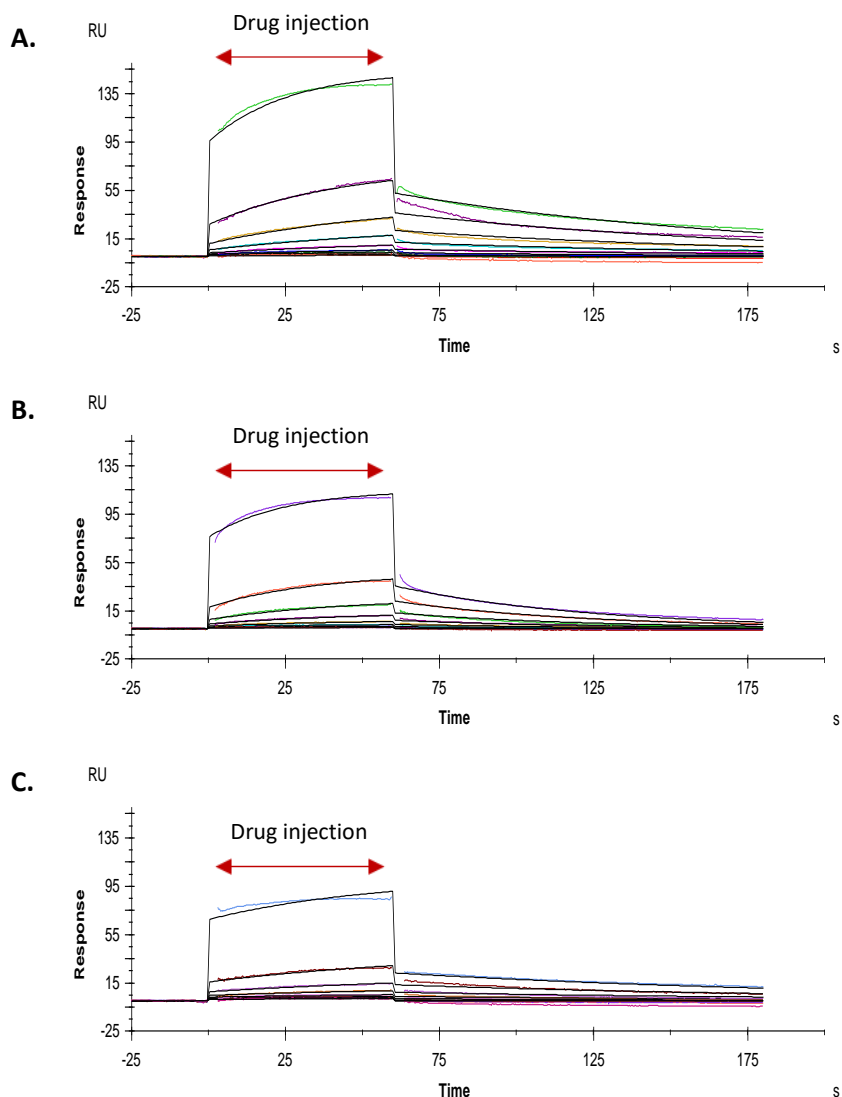


Figure 5.8: Interaction of Chromomycin A with Sp consensus sequence in absence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Chromomycin A with 30bp DNA oligomers: Sp consensus (panel A); Sp consensus mutant (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Chromomycin A was injected over the surface (3-125 μ M). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 μ l/min flow rate at 25 $^{\circ}$ C. Sensorgrams are representative of n=4 experiments.

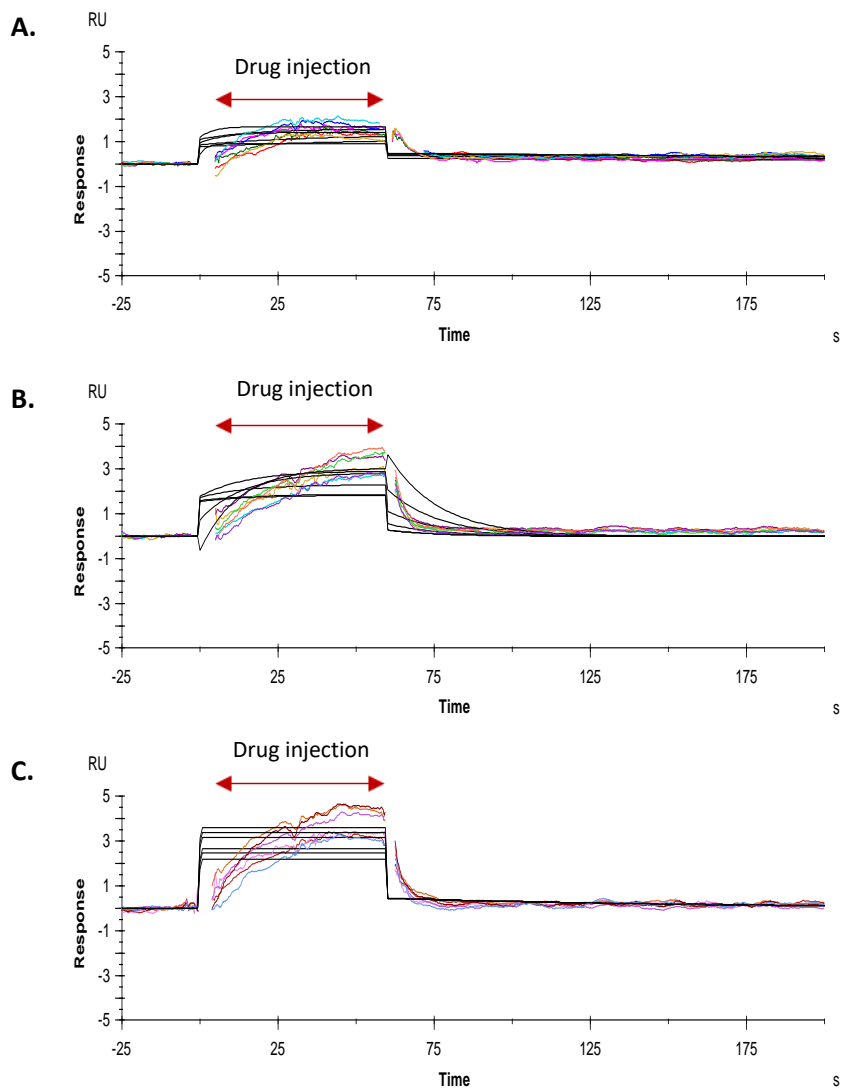


Figure 5.9: Interaction of Terameprocol with Sp consensus sequence

Sensorgrams represent binding curves for interaction of Terameprocol with 30bp DNA oligomers: Sp consensus (panel A); Sp consensus mutant (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Terameprocol was injected over the surface (3-500 μM). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 $\mu\text{l}/\text{min}$ flow rate at 25 $^{\circ}\text{C}$. Sensorgrams are representative of $n=4$ experiments.

5.2.5. Interaction of Mithramycin A towards *Bak* gene promoter

The *Bak* gene promoter consists of three known Sp binding sites and has widely been studied as a Sp mediated gene promoter. Specific DNA-Sp protein interaction has also been well established by our group previously (Waby et al., 2010). It is believed that Mithramycin A specifically binds to *Bak* gene promoter region, preferably to Sp binding sites. Mithramycin A interaction with 60 bp DNA oligomer of *Bak* gene promoter (*Bak* ABC region) was analysed in the presence of cation Mg^{2+} (-194 **GCC TGG GAG GTG GGG TGG GGA AAG TGG GCG GGA CAT GCT CCT GGG CCT GGC CCA CCC AGA** – 134). Running buffer contained 1x PBS + 5% (v/v) DMSO + 5% (v/v) p20 surfactant and 5 mM $MgCl_2$. As seen in Figure 5.10, Mithramycin A specifically interacts with GC rich linear DNA where no binding was detected for the control sequence. Kinetics of Mithramycin A interaction with *Bak* gene promoter ABC region was marked by association constant of $K_a = 6.44 (+/- 0.53 SD) \times 10^3 (M^{-1}s^{-1})$ and dissociation constant of $K_b = 3.26 (+/- 0.48 SD) \times 10^{-2} (s^{-1})$. Equilibrium constant was reported as $K_d = 5.13 (+/- 1.15 SD) \times 10^{-6} (M)$ at 25 °C. R_{max} of $1.99 (+/- 0.36 SD) \times 10^2 (RU)$ was observed at concentration of 31.25 μM concentration of Mithramycin A (see Figure 5.10 panel A). No specific binding was identified for the DNA oligomer of the non-promoter sequence which lacks any GC rich regions or Sp binding sites (see Figure 5.10 panel B).

Interaction of Mithramycin A with *Bak* gene promoter was also tested in the absence of Mg^{2+} cation. Mithramycin A specifically interacted with DNA of *Bak* gene promoter ABC region in the absence of Mg^{2+} ions. As evident from Figure 5.11, DNA-Mithramycin A interaction was specific to *Bak* ABC regions where no detectable binding was reported for the control sequences. Kinetics were reported as association constant of $K_a = 9.70 (+/- 0.36 SD) \times 10^2 (M^{-1}s^{-1})$ and dissociation constant of $K_b = 4.63 (+/- 0.27 SD) \times 10^{-2} (s^{-1})$. Equilibrium constant was reported as $K_d = 4.63 (+/- 0.27 SD) \times 10^{-5} (M)$ at 25 °C. R_{max} of $1.75 (+/- 0.43 SD) \times 10^2 (RU)$ was observed at concentration of 250 μM concentration of Mithramycin A (see Figure 5.11 panel A). No detectable bindings were recorded for DNA of non-promoter sequence which lacks any GC rich regions (see Figure 5.11 panel B).

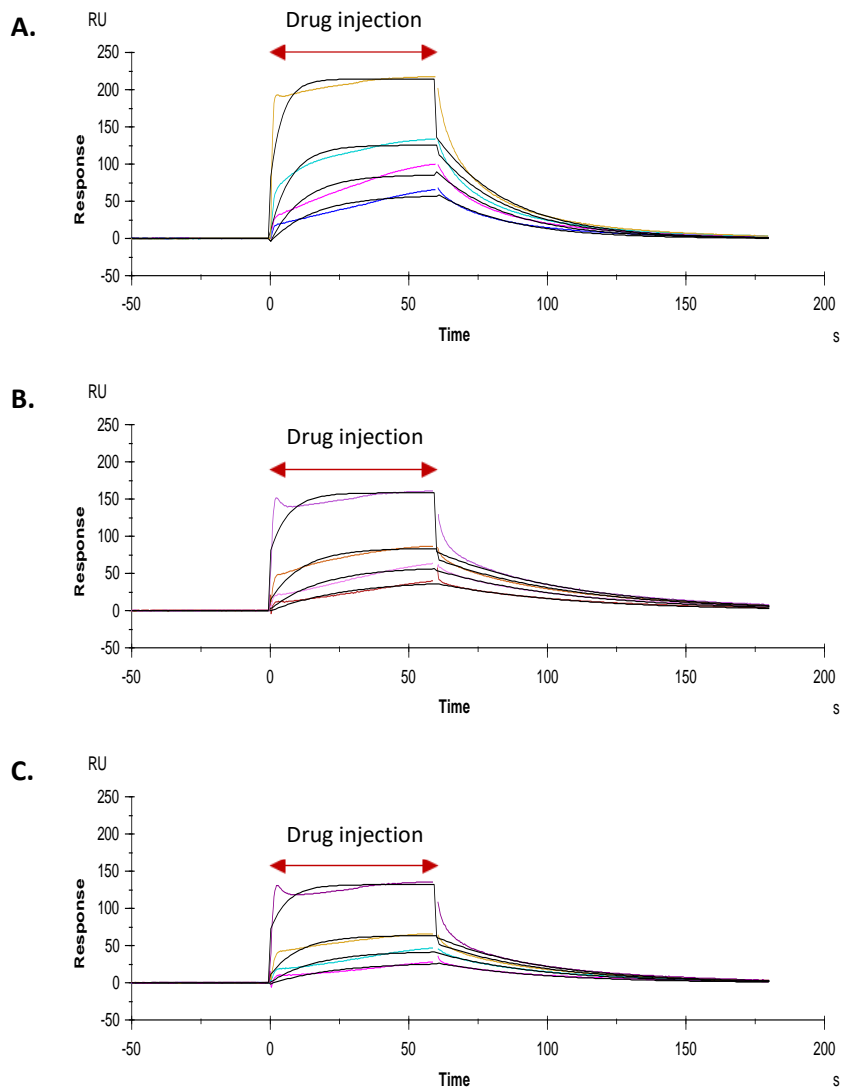


Figure 5.10: Interaction of Mithramycin A with *Bak* gene promoter ABC region in the presence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Mithramycin A with 60bp DNA oligomers: *Bak* ABC (panel A); non-promoter region (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Mithramycin A was injected over the surface (3-31.25 μM). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 $\mu l/min$ flow rate at 25 $^{\circ}C$. Sensorgrams are representative of $n=4$ experiments.

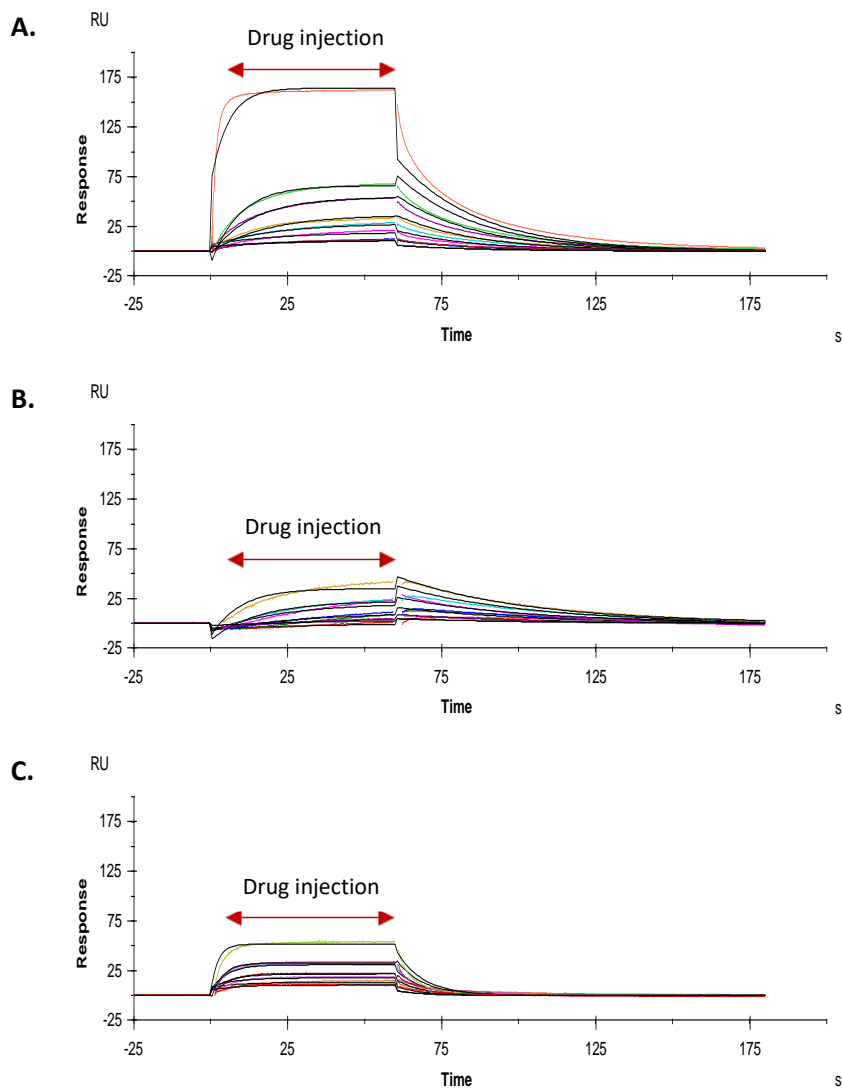


Figure 5.11: Interaction of Mithramycin A with *Bak* gene promoter ABC region in absence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Mithramycin A with 60bp DNA oligomers: *Bak* ABC (panel A); non-promoter region (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Mithramycin A was injected over the surface (3-250 μ M). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 μ l/min flow rate at 25 °C. Sensorgrams are representative of n=4 experiments.

5.2.6. Interaction of Chromomycin A towards *Bak* gene promoter

Chromomycin A preferably binds towards double stranded DNA oligomer of *Bak* gene promoter ABC region in the presence of Mg^{2+} cation. Binding analyses were performed using the running buffer with 5 mM $MgCl_2$. As seen in Figure 5.12, Chromomycin A preferably interact with GC rich regions where no specific bindings were detected for the control sequences. Kinetics of Chromomycin A interaction with DNA of *Bak* gene promoter was marked by association constant of $K_a = 8.72 (+/- 0.51 \text{ SD}) \times 10^2 \text{ (M}^{-1}\text{s}^{-1})$ and dissociation constant of $K_b = 2.62 (+/- 0.14 \text{ SD}) \times 10^{-3} \text{ (s}^{-1})$. Equilibrium constant was reported as $K_d = 3.01 (+/- 0.30 \text{ SD}) \times 10^{-6} \text{ (M)}$ at 25 °C. Rmax of $1.22 (+/- 0.22 \text{ SD}) \times 10^2 \text{ (RU)}$ was observed at concentration of 62.50 μM concentration of Chromomycin (see Figure 5.12 panel A). Interaction with non-promoter sequence was non-specific in nature (see 5.12 panel B).

Specific interaction of Chromomycin A and DNA oligomer of *Bak* gene promoter ABC was also detected in the absence of divalent cation Mg^{2+} . Kinetics were reported as $K_a = 3.58 (+/- 0.29 \text{ SD}) \times 10^2 \text{ (M}^{-1}\text{s}^{-1})$ and dissociation constant of $K_b = 3.63 (+/- 0.14 \text{ SD}) \times 10^{-3} \text{ (s}^{-1})$. Equilibrium constant was reported as $K_d = 1.01 (+/- 0.02 \text{ SD}) \times 10^{-5} \text{ (M)}$ at 25 °C. Rmax of $1.08 (+/- 0.16 \text{ SD}) \times 10^2 \text{ (RU)}$ was observed at concentration of 125 μM concentration of Chromomycin A (see Figure 5.13 panel A). No detectable binding was observed for DNA of non-promoter sequence (see Figure 5.13 panel B).

5.2.7. Interaction of Terameprocol towards *Bak* gene promoter

No detectable binding was observed between Terameprocol and double stranded DNA oligomer of *Bak* gene promoter. Concentration series of 3 μM to 500 μM of Terameprocol was used for binding analysis. No detectable binding was observed at any given concentrations. Less than 5 RU response was detected for all the oligomers including the control sequence (see Figure 5.14).

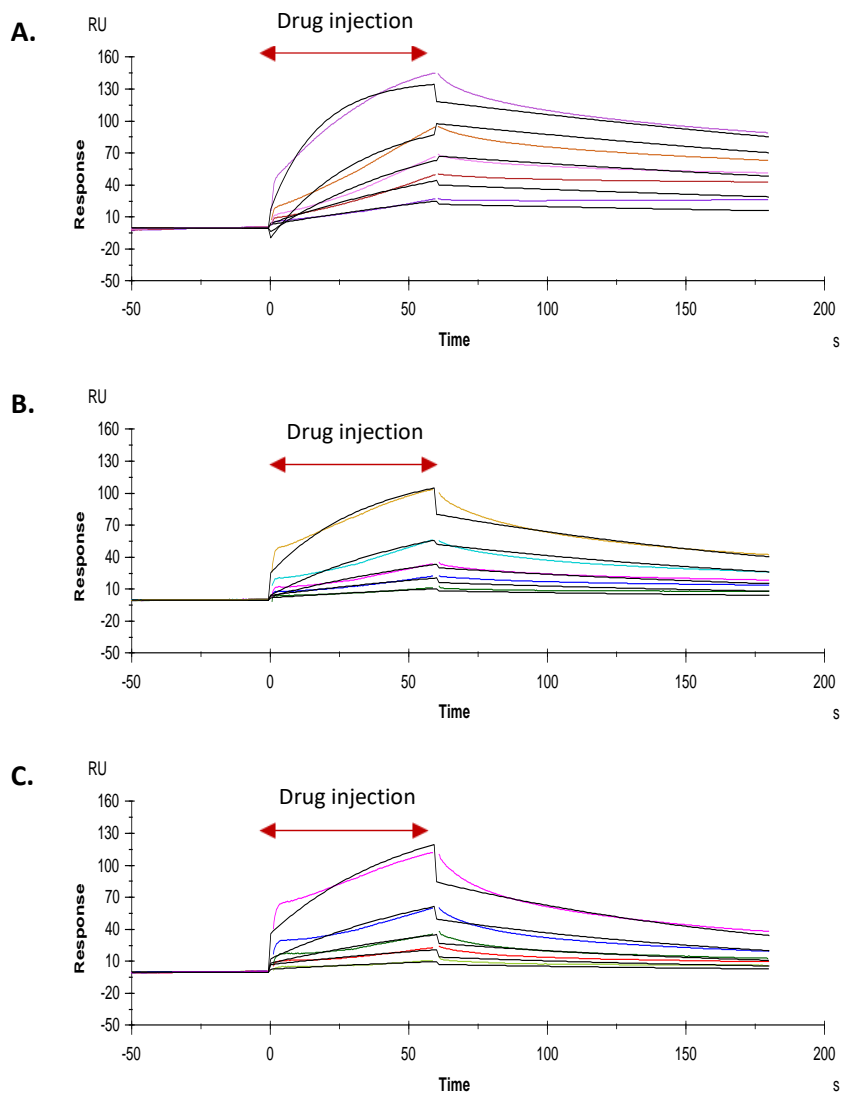


Figure 5.12: Interaction of Chromomycin A with *Bak* gene promoter ABC region in the presence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Chromomycin A with 60bp DNA oligomers: *Bak* ABC (panel A); non-promoter region (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Chromomycin A was injected over the surface (3-62.50 μ M). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 μ l/min flow rate at 25 °C. Sensorgrams are representative of n=4 experiments.

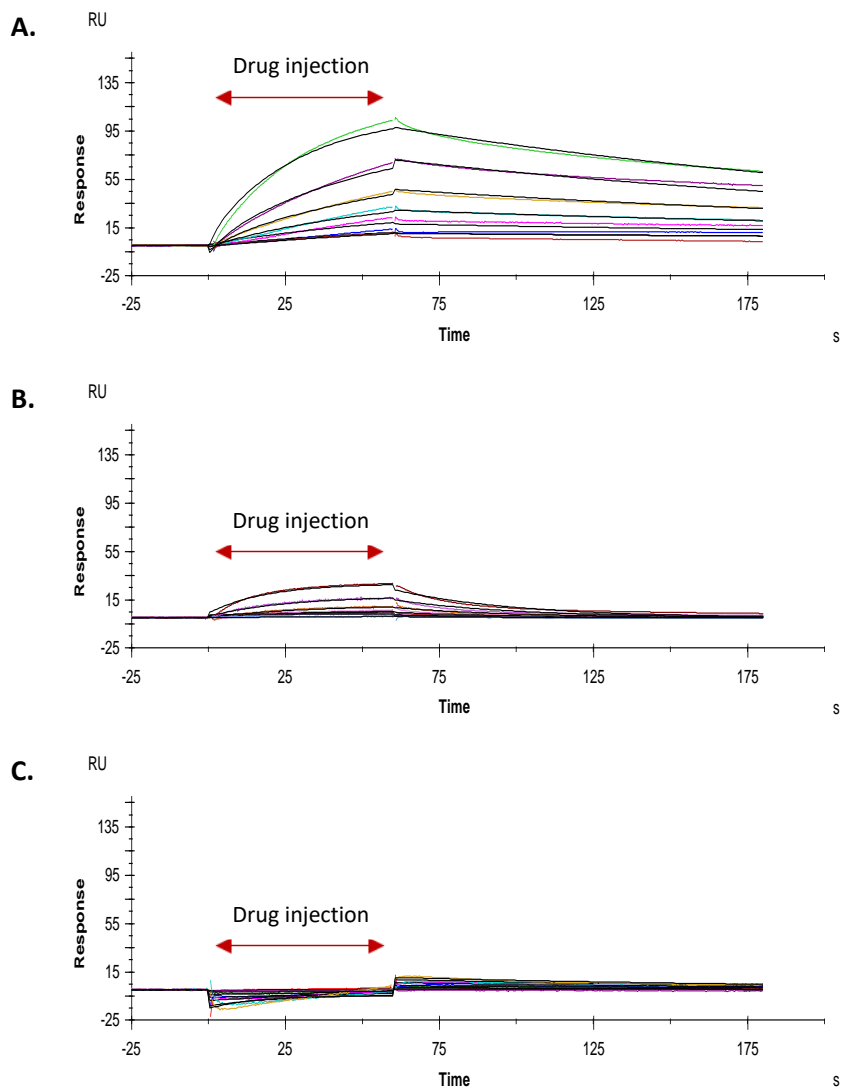


Figure 5.13: Interaction of Chromomycin A with *Bak* gene promoter ABC region in absence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Chromomycin A with 60bp DNA oligomers: *Bak* ABC (panel A); non-promoter region (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Chromomycin A was injected over the surface (3-125 μ M). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 μ l/min flow rate at 25 $^{\circ}$ C. Sensorgrams are representative of n=4 experiments.

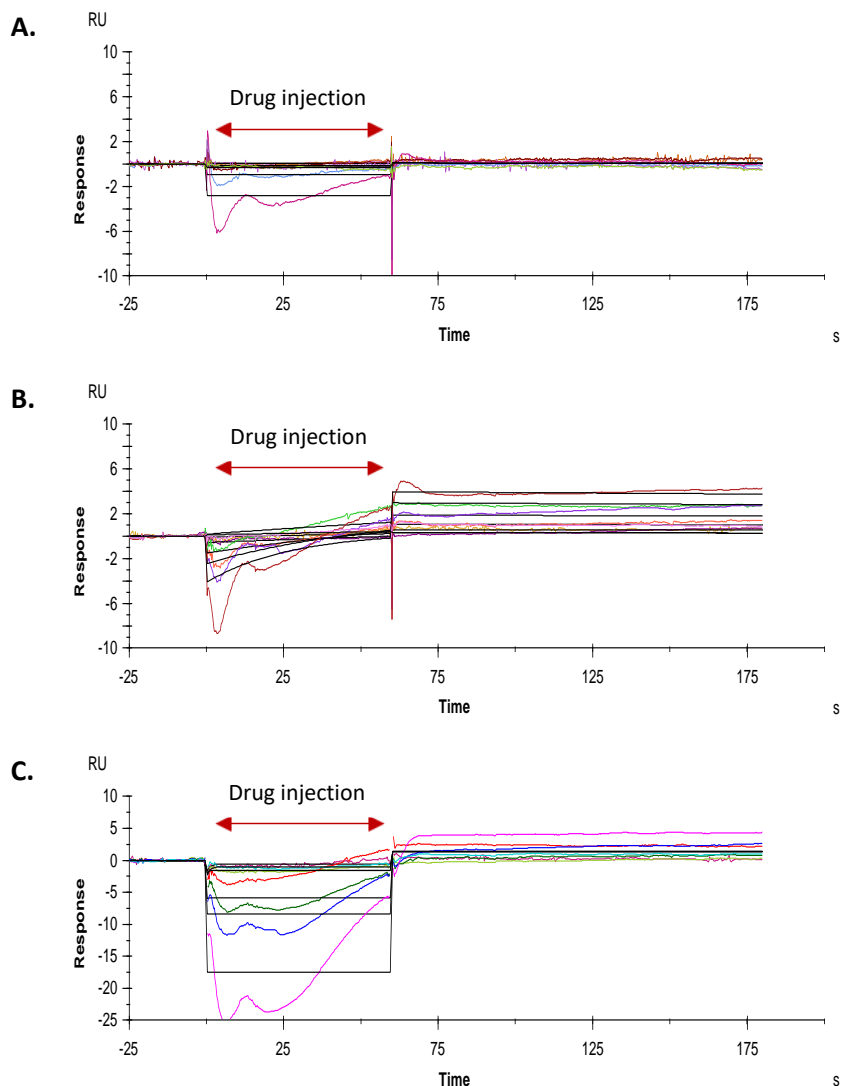


Figure 5.14: Interaction of Terameprocol with *Bak* gene promoter ABC region.

Sensorgrams represent binding curves for interaction of Terameprocol with 60bp DNA oligomer: *Bak* ABC (panel A); non-promoter region (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Terameprocol was injected over the surface (3-500 μ M). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 μ l/min flow rate at 25 $^{\circ}$ C. Sensorgrams are representative of n=4 experiments.

5.2.8. Interaction of Mithramycin A towards *Bak* gene promoter B region

Bak gene promoter B region was identified as the highest affinity site for Sp1 protein binding compared to the other adjacent sites. Mithramycin A interaction with *Bak* gene promoter B region was further investigated with 20 bp long DNA oligomer (5' **AAA GTG GGC GGG ACA TGC TC** 3'). Running buffer contained 1x PBS + 5% (v/v) DMSO + 5% (v/v) p20 surfactant and 5 mM MgCl₂.

As seen in Figure 5.15, Mithramycin A specifically interacts with linear DNA which consists of Sp1 binding site where, no binding was detected for the control sequence. Kinetics of Mithramycin A interaction with *Bak* gene promoter B region was marked by association constant of $K_a = 9.49 (+/- 0.48 \text{ SD}) \times 10^3 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ and dissociation constant of $K_b = 3.40 (+/- 0.39 \text{ SD}) \times 10^{-2} \text{ (s}^{-1}\text{)}$. Equilibrium constant was reported as $K_d = 3.60 (+/- 0.53 \text{ SD}) \times 10^{-6} \text{ (M)}$ at 25 °C. Rmax 1.30 (+/- 0.29 SD) x 10² (RU) was observed at concentration of 31.25 μM concentration of Mithramycin A (see Figure 5.15 panel A).

Similarly, Mithramycin A interacted with mutant *Bak* B region (5' **AAA GTG AGC GTG ACA TGC TC** 3') with $K_a = 6.84 (+/- 0.93 \text{ SD}) \times 10^3 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ and dissociation constant of $K_b = 2.30 (+/- 0.02 \text{ SD}) \times 10^{-2} \text{ (s}^{-1}\text{)}$. Equilibrium constant was reported as $K_d = 3.42 (+/- 0.46 \text{ SD}) \times 10^{-6} \text{ (M)}$ at 25 °C. However, Rmax value decreased to 8.34 (+/- 0.61 SD) x 10² RU at same concentration (see Figure 5.15 panel B). Mithramycin A was specifically interacting with DNA of *Bak* gene promoter B region in the absence of Mg²⁺ ions. Binding analyses were carried out using identical conditions except for running buffer which lacks metal chelator Mg²⁺. As can be seen in Figure 5.16, DNA-Mithramycin A interaction was specific to *Bak* B regions where no detectable binding was reported for the control sequences. Kinetics were reported as association constant of $K_a = 6.58 (+/- 1.79 \text{ SD}) \times 10^2 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ and dissociation constant of $K_b = 2.75 (+/- 0.23 \text{ SD}) \times 10^{-2} \text{ (s}^{-1}\text{)}$. Equilibrium constant was reported as $K_d = 4.35 (+/- 0.99 \text{ SD}) \times 10^{-5} \text{ (M)}$ at 25 °C. Rmax of 2.10 (+/- 0.17 SD) x 10² (RU) was observed at concentration of 125 μM concentration of Mithramycin A (see Figure 5.16 panel A).

Changes in Sp binding sites of *Bak* gene promoter B region decrease the DNA-Mithramycin A interaction. Mithramycin A interacted with mutant *Bak* B region with $K_a = 4.12 (+/- 1.33 \text{ SD}) \times 10^2 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ and dissociation constant of $K_b = 2.13 (+/- 0.63 \text{ SD}) \times 10^{-2} \text{ (s}^{-1}\text{)}$.

Equilibrium constant was reported as $K_d = 5.67 (+/- 2.62 \text{ SD}) \times 10^{-5} \text{ (M)}$ at 25 ° C. However, R_{max} value decreased to $1.07 (+/- 0.35 \text{ SD}) \times 10^2 \text{ RU}$ at the same concentration (see Figure 5.16 panel B).

5.2.9. Interaction of Chromomycin towards *Bak* gene promoter B region

Chromomycin preferably binds towards double stranded DNA oligomer of *Bak* gene promoter B region in the presence of Mg^{2+} cation. Binding analyses were performed using the running buffer with 5 mM MgCl_2 . As seen in Figure 5.17, Chromomycin preferably interacts with GC rich regions where no specific bindings were detected for the control sequences. Kinetics of Chromomycin interaction with DNA of *Bak* gene promoter B region was marked by association constant of $K_a = 8.77 (+/- 0.87 \text{ SD}) \times 10^2 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ and dissociation constant of $K_b = 4.38 (+/- 0.22 \text{ SD}) \times 10^{-3} \text{ (s}^{-1}\text{)}$. Equilibrium constant was reported as $K_d = 5.03 (+/- 0.64 \text{ SD}) \times 10^{-6} \text{ (M)}$ at 25 °C. R_{max} of $9.72 (+/- 0.44 \text{ SD}) \times 10^1 \text{ (RU)}$ was observed at concentration of 62.50 μM concentration of Chromomycin (see Figure 5.17 panel A).

Nucleotide changes in the Sp binding sites of *Bak* gene promoter B region leads to decreased interaction of Chromomycin A with linear DNA. As seen in Figure 5.16 panel B, R_{max} decreased to $6.88 (+/- 1.04 \text{ SD}) \times 10^1 \text{ (RU)}$ at concentration of 62.50 μM concentration of Chromomycin A. Kinetics were marked by association constant of $K_a = 1.908 (+/- 0.87 \text{ SD}) \times 10^3 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ and dissociation constant of $K_b = 6.70 (+/- 0.26 \text{ SD}) \times 10^{-3} \text{ (s}^{-1}\text{)}$. Equilibrium constant was reported as $K_d = 3.53 (+/- 0.33\text{SD}) \times 10^{-6} \text{ (M)}$ at 25 °C (see Figure 5.17 panel B).

Chromomycin A specifically interacts with DNA oligomer of *Bak* gene promoter B region in the absence of divalent cation Mg^{2+} . Kinetics were reported as $K_a = 3.15 (+/- 0.37 \text{ SD}) \times 10^2 \text{ (M}^{-1}\text{s}^{-1}\text{)}$ and dissociation constant of $K_b = 7.44 (+/- 0.41 \text{ SD}) \times 10^{-3} \text{ (s}^{-1}\text{)}$. Equilibrium constant was reported as $K_d = 2.38 (+/- 0.23 \text{ SD}) \times 10^{-5} \text{ (M)}$ at 25 °C. R_{max} of $1.47 (+/- 0.17 \text{ SD}) \times 10^2 \text{ (RU)}$ was observed at concentration of 125 μM concentration of Chromomycin (see Figure 5.18 panel A).

DNA oligomer representing mutant *Bak* gene promoter B region showed decreased binding due to nucleotide changes in the Sp binding sites. As seen in Figure 5.18 panel B, R_{max} decreased to $4.17 (+/- 0.65 \text{ SD}) \times 10^1 \text{ (RU)}$ at concentration of 125 μM concentration of Chromomycin. Kinetics were marked by association constant of $K_a =$

3.0 (+/- 0.60 SD) ($M^{-1}s^{-1}$) and dissociation constant of $K_b = 5.00 (+/- 0.79 SD) \times 10^{-3} (s^{-1})$. Equilibrium constant was reported as $K_d = 1.92 (+/- 0.33 SD) \times 10^{-3} (M)$ at 25 °C.

5.2.10. Interaction of Terameprocol towards *Bak* gene promoter B region

No detectable binding was observed between Terameprocol and double stranded DNA oligomer of *Bak* gene promoter B region. Concentration series of 3 μM to 500 μM of Terameprocol was used for binding analyses. Less than 15 RU response was detected for all the oligomers including the control sequence (see Figure 5.19).

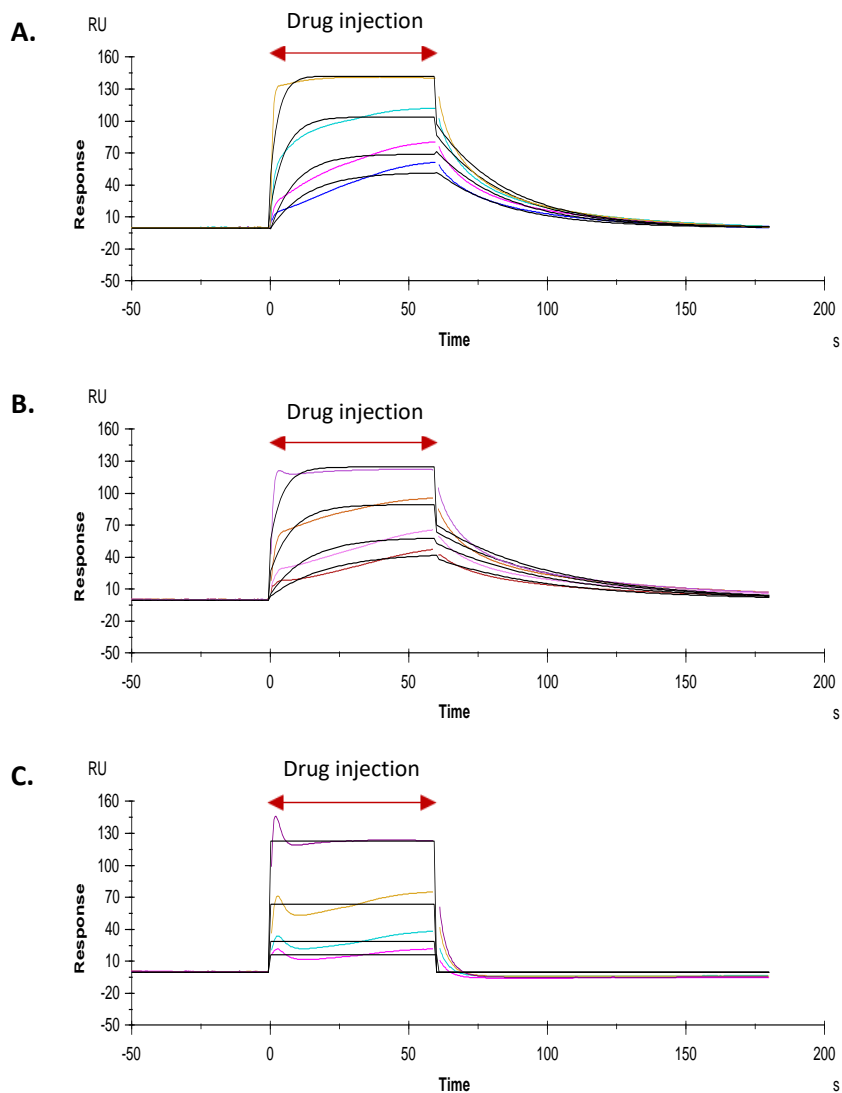


Figure 5.15: Interaction of Mithramycin A with *Bak* gene promoter B region in the presence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Mithramycin A with 20bp DNA oligomers: Bak B (panel A); Bak B mutant (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Mithramycin A was injected over the surface (3-31.25 μ M). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 μ l/min flow rate at 25 $^{\circ}$ C. Sensorgrams are representative of n=4 experiments.

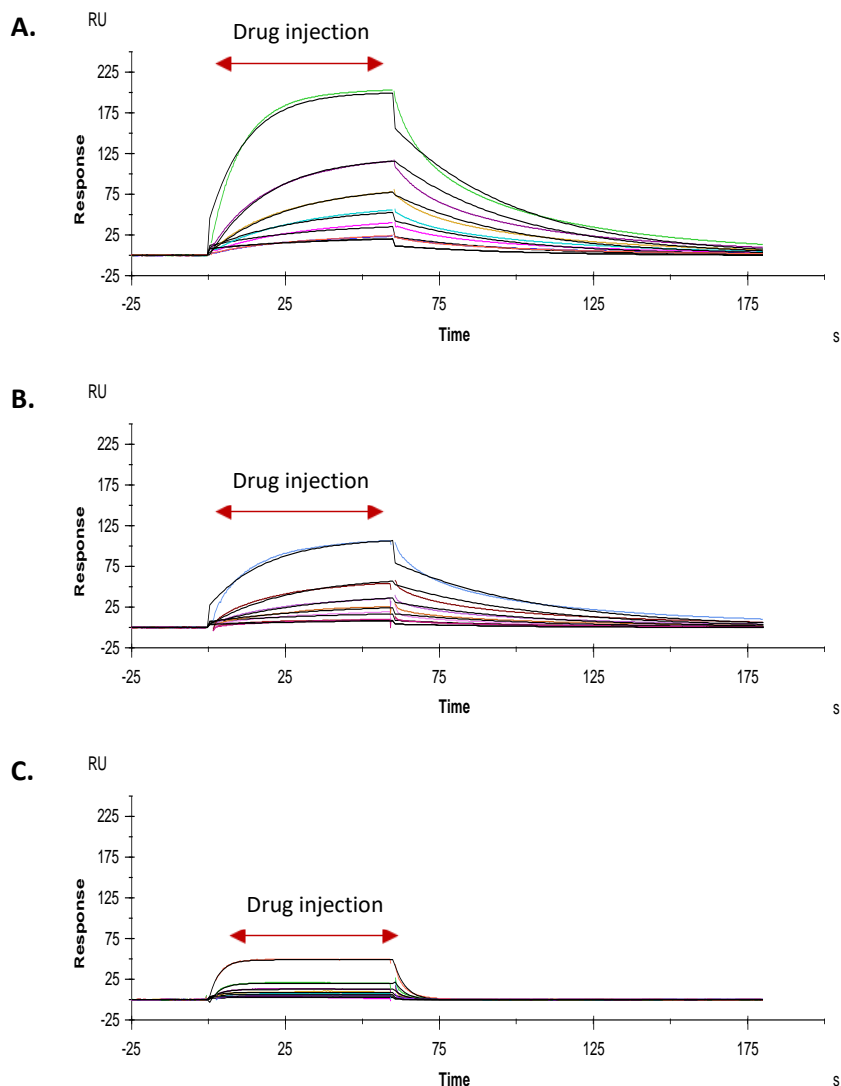


Figure 5.16: Interaction of Mithramycin A with *Bak* gene promoter B region in absence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Mithramycin A with 20bp DNA oligomers: Bak B (panel A); Bak B mutant (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Mithramycin A was injected over the surface (3-125 μ M). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 μ l/min flow rate at 25 $^{\circ}$ C. Sensorgrams are representative of n=4 experiments.

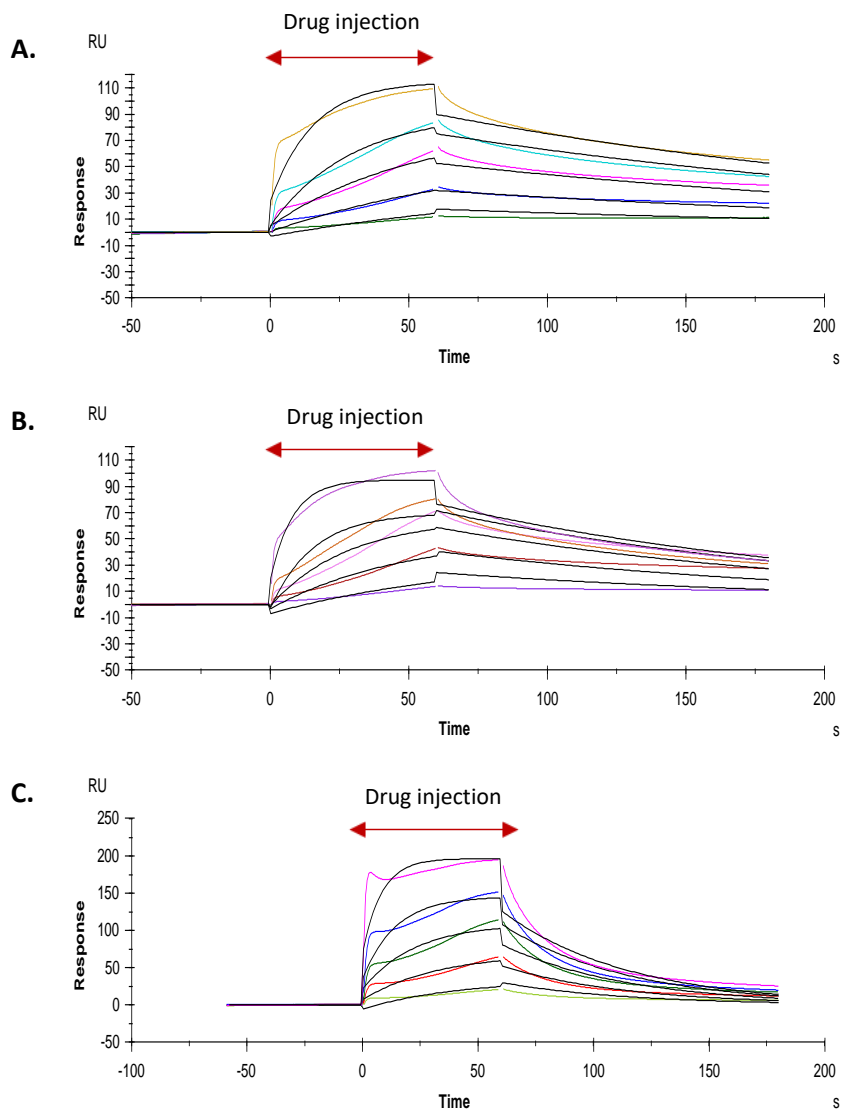


Figure 5.17: Interaction of Chromomycin A with *Bak* gene promoter B region in the presence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Chromomycin A with 20bp DNA oligomers: Bak B (panel A); B mutant (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Chromomycin A was injected over the surface (3-62.50 μM). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 $\mu l/min$ flow rate at 25 $^{\circ}C$. Sensorgrams are representative of n=4 experiments.

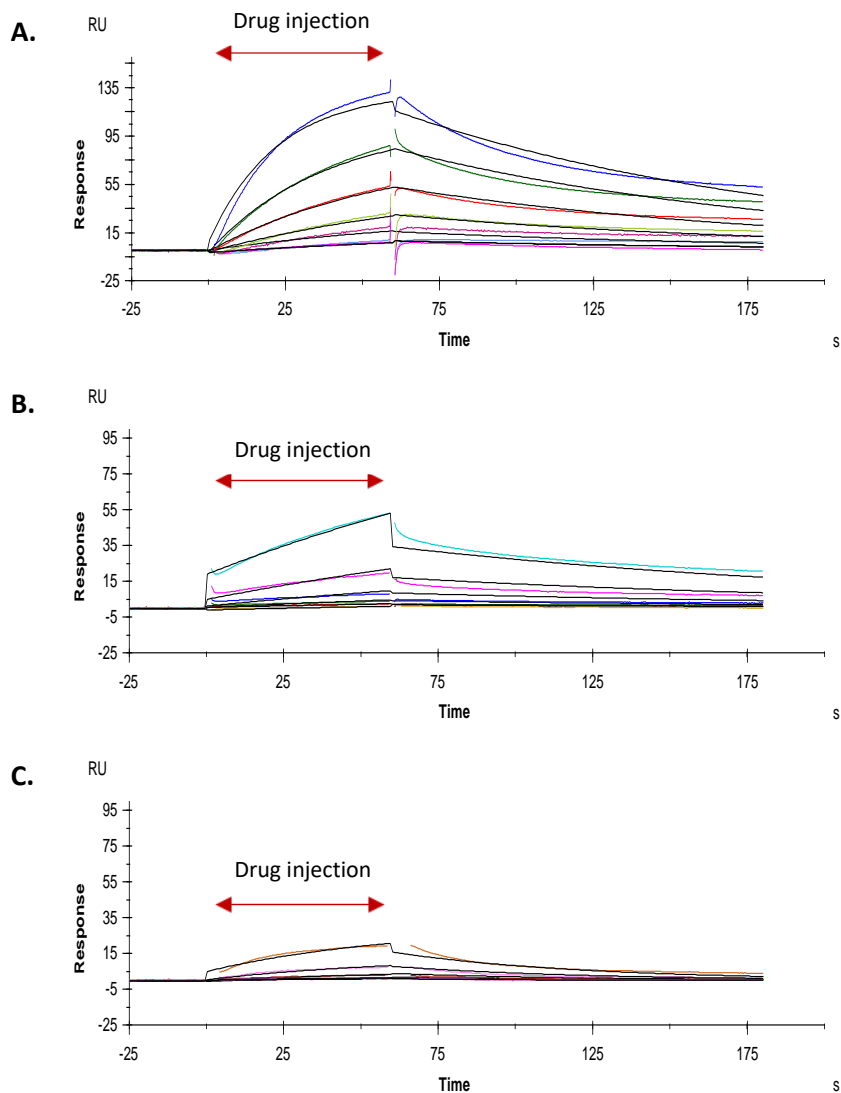


Figure 5.18: Interaction of Chromomycin A with *Bak* gene promoter B region in absence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Chromomycin A with 20bp DNA oligomers: Bak B (panel A); B mutant (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Chromomycin A was injected over the surface (3-125 μ M). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 μ l/min flow rate at 25 $^{\circ}$ C. Sensorgrams are representative of n=4 experiments.

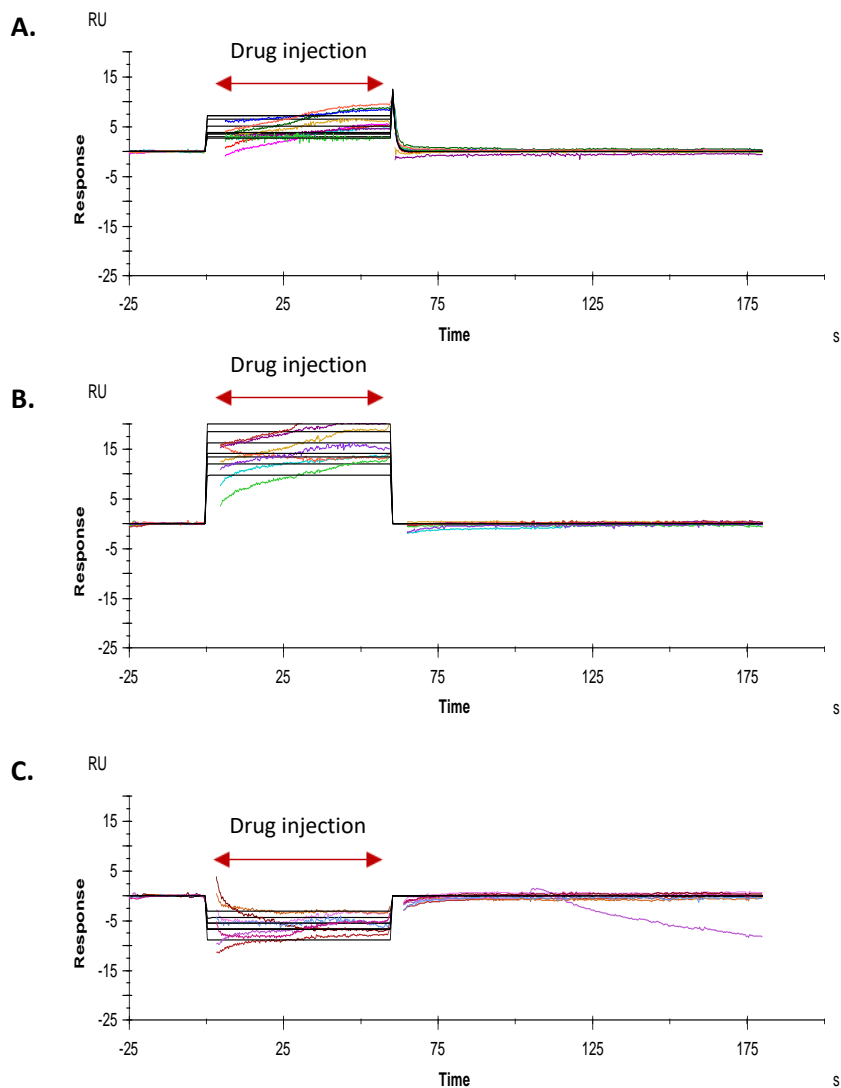


Figure 5.19: Interaction of Terameprocol with *Bak* gene promoter B region

Sensorgrams represent binding curves for interaction of Terameprocol with 20bp DNA oligomers: Bak B (panel A); Bak B mutant (panel B) and control sequence without GC regions (panel C). Biotinylated DNA oligomers were captured on a SA sensor chip, and increasing concentrations of Terameprocol was injected over the surface (3-500 μ M). The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 60 s followed by 120 s of buffer flow at 30 μ l/min flow rate at 25 $^{\circ}$ C. Sensorgrams are representative of n=4 experiments.

Table 5.1: Binding kinetics of Mithramycin A with different gene promoters at 25 °C

Promoter sequence		Binding Kinetics					
		K_a ($M^{-1}s^{-1}$)	K_b (s^{-1})	K_d (M)	Conc. (μM)	Rmax (RU)	Chi ²
Presence Mg ²⁺	Sp1 consensus region	5.92 (+/- 0.92 SD) x 10 ³	1.85 (+/- 0.03 SD) x 10 ⁻²	3.18 (+/- 0.56 SD) x 10 ⁻⁶	31.25	2.93 (+/- 0.85 SD) x 10 ²	16.25
	Sp1 consensus mutant region	5.53 (+/- 0.52 SD) x 10 ³	3.43 (+/- 0.12 SD) x 10 ⁻²	6.24 (+/- 0.36 SD) x 10 ⁻⁶	31.25	1.63 (+/- 0.37 SD) x 10 ²	2.82
	<i>Bak</i> B region	9.49 (+/- 0.48 SD) x 10 ³	3.40 (+/- 0.39 SD) x 10 ⁻²	3.60 (+/- 0.53 SD) x 10 ⁻⁶	31.25	1.30 (+/- 0.29 SD) x 10 ²	15.80
	<i>Bak</i> B mutant	6.84 (+/- 0.93 SD) x 10 ³	2.30 (+/- 0.02 SD) x 10 ⁻²	3.42 (+/- 0.46 SD) x 10 ⁻⁶	31.25	8.34 (+/- 0.61 SD) x 10 ²	15.10
	<i>Bak</i> ABC region	6.44 (+/- 0.53 SD) x 10 ³	3.26 (+/- 0.48 SD) x 10 ⁻²	5.13 (+/- 1.15 SD) x 10 ⁻⁶	31.25	1.99 (+/- 0.36 SD) x 10 ²	12.6
Absence of Mg ²⁺	Sp1 consensus region	9.63 (+/- 0.39 SD) x 10 ²	1.60 (+/- 0.21 SD) x 10 ⁻²	1.67 (+/- 0.26 SD) x 10 ⁻⁵	125	2.32 (+/- 0.11 SD) x 10 ²	9.82
	Sp1 consensus mutant region	1.54 (+/- 0.22 SD) x 10 ²	2.12 (+/- 0.15 SD) x 10 ⁻²	1.39 (+/- 0.92 SD) x 10 ⁻⁴	125	8.92 (+/- 0.12 SD) x 10 ⁻¹	6.4
	<i>Bak</i> B region	6.58 (+/- 1.79 SD) x 10 ²	2.75 (+/- 0.23 SD) x 10 ⁻²	4.35 (+/- 0.99 SD) x 10 ⁻⁵	125	2.10 (+/- 0.17 SD) x 10 ²	17.5
	<i>Bak</i> B mutant	4.12 (+/- 1.33 SD) x 10 ²	2.13 (+/- 0.63 SD) x 10 ⁻²	5.67 (+/- 2.62 SD) x 10 ⁻⁵	125	1.07 (+/- 0.35 SD) x 10 ²	4.93
	<i>Bak</i> ABC region	9.70 (+/- 0.36 SD) x 10 ²	4.63 (+/- 0.27 SD) x 10 ⁻²	4.63 (+/- 0.27 SD) x 10 ⁻⁵	250	1.75 (+/- 0.43 SD) x 10 ²	7.5

Table 5.2: Binding kinetics of Chromomycin A with different gene promoters at 25 °C

Promoter sequence		Binding Kinetics					
		K_a ($M^{-1}s^{-1}$)	K_b (s^{-1})	K_d (M)	Conc. (μ M)	Rmax (RU)	Chi ²
Presence Mg ²⁺	Sp1 consensus region	5.51 (+/- 0.56 SD) x 10 ²	5.55 (+/- 0.44 SD) x 10 ⁻³	1.02 (+/- 0.18 SD) x 10 ⁻⁵	62.50	1.95 (+/- 0.08 SD) x 10 ²	3.22
	Sp1 consensus mutant region	5.58 (+/- 0.63 SD) x 10 ²	8.16 (+/- 0.20 SD) x 10 ⁻³	1.48 (+/- 0.21 SD) x 10 ⁻⁵	62.50	1.13 (+/- 0.19 SD) x 10 ²	7.04
	<i>Bak</i> B region	8.77 (+/- 0.87 SD) x 10 ²	4.38 (+/- 0.22 SD) x 10 ⁻³	5.03 (+/- 0.64 SD) x 10 ⁻⁶	62.50	9.72 (+/- 0.44 SD) x 10 ¹	12.00
	<i>Bak</i> B mutant	1.908 (+/- 0.87 SD) x 10 ³	6.70 (+/- 0.26 SD) x 10 ⁻³	3.53 (+/- 0.33 SD) x 10 ⁻⁶	62.50	6.88 (+/- 1.04 SD) x 10 ¹	2.29
	<i>Bak</i> ABC region	8.72 (+/- 0.51 SD) x 10 ²	2.62 (+/- 0.14 SD) x 10 ⁻³	3.01 (+/- 0.30 SD) x 10 ⁻⁶	62.50	1.22 (+/- 0.22 SD) x 10 ²	3.89
Absence of Mg ²⁺	Sp1 consensus region	6.4 (+/- 0.73 SD) x 10 ¹	8.33 (+/- 1.20 SD) x 10 ⁻³	1.30 (+/- 0.06 SD) x 10 ⁻⁴	125	8.24 (+/- 0.8 SD) x 10 ¹	0.51
	Sp1 consensus mutant region	5.30 (+/- 0.69 SD) x 10 ¹	1.56 (+/- 0.10 SD) x 10 ⁻²	3.07 (+/- 0.37 SD) x 10 ⁻⁴	125	6.64 (+/- 1.64 SD) x 10 ¹	2.66
	<i>Bak</i> B region	3.15 (+/- 0.37 SD) x 10 ²	7.44 (+/- 0.41 SD) x 10 ⁻³	2.38 (+/- 0.23 SD) x 10 ⁻⁵	125	1.47 (+/- 0.17 SD) x 10 ²	9.40
	<i>Bak</i> B mutant	3.0 (+/- 0.60 SD)	5.00 (+/- 0.79 SD) x 10 ⁻³	1.92 (+/- 0.33 SD) x 10 ⁻³	125	4.17 (+/- 0.65 SD) x 10 ¹	2.73
	<i>Bak</i> ABC region	3.58 (+/- 0.29 SD) x 10 ²	3.63 (+/- 0.14 SD) x 10 ⁻³	1.01 (+/- 0.02 SD) x 10 ⁻⁵	125	1.08 (+/- 0.16 SD) x 10 ²	9.32

5.3. Discussion

In this chapter, the binding kinetics of three known Sp inhibitors: Mithramycin A, Chromomycin A and Terameprocol were investigated using Surface Plasmon Resonance. Kinetics were determined towards DNA oligomers representing Sp consensus sequence as well as biological gene promoters which contain Sp transcription factor binding sites.

Findings of this chapter are summarised below, answering the initial research questions.

- What is the nature of DNA interaction of Mithramycin, Chromomycin and Terameprocol in terms of DNA binding and specificity?
 - a. Are they DNA binding molecules?

Surface plasmon (Biacore T200) analysis confirm the direct interaction of Mithramycin A and Chromomycin A and GC rich DNA oligomers. Under experimental conditions described in this chapter, no detectable binding was observed for Terameprocol towards any DNA oligomer.

- b. Specificity towards GC rich DNA regions?

Both Mithramycin A and Chromomycin A interacted with GC rich DNA oligomers where no binding patterns were detected towards the control sequences (DNA oligomers which lacks GC rich regions)

- c. Specificity towards Sp promoter binding sites?

Random changes in the Sp binding sites regions have decreased the binding activity of both Mithramycin A and Chromomycin A suggesting potential higher affinity towards Sp promoter sites

- d. How they interact with different DNA regions

Data evaluation with *Biacore T200* evaluation software suggests that interaction of both Mithramycin A and Chromomycin A towards linear DNA is 1:1 ratio (best fitting mode for the sensograms).

- What are the effects of Mg²⁺ cation on Mithramycin A and Chromomycin A binding?

In the presence of Mg²⁺ divalent cation, both Mithramycin a and Chromomycin A interact with linear DNA. In addition, these antibiotic derivatives are cable of binding to linear DNA in the absence of any chelating cations.

- What are the kinetics of interaction including on/off rates?

Kinetics of Mithramycin A and Chromomycin A interaction with DNA were determined for all the promoter sequences.

5.3.1. Limitations associated with Surface plasmon resonance (SPR) technique

Label-free SPR analysis is capable of detecting molecules as small as 100 Da. In addition, real time binding analysis provides robust kinetic data on ligand interactions, concentration and affinity in a label-free detection method. Developed assay method in this experiment can be used to investigate binding of any DNA binding molecule with any piece of promoter or interested DNA oligomer with modifications. Since the sensitivity of this technology is remarkably higher than any other binding assay technique available up to date, it will allow analysing small concentrations even up to femto moles. This assay system provides details of

- Binding nature of compound
- Mode of interaction
- Affinity and kinetics of each compound towards interested DNA oligomer.

However, there are some caveats and limitations associated with the SPR technology. In general, SPR technique is associated with mass transport limitation. Analyte concentration near the ligand and the analyte concentration of the bulk solution may vary due to the presence of limited analytes near the surface area. Similarly, nonspecific binding on the surface leads to the mass transport effect. In this study, this problem was minimised by selecting an appropriate flow rate and association times for binding assays (Schuck & Zhao, 2010).

Another fundamental limitation is maintaining ligand configuration and its accurate orientation. Since ligand orientations vital for ligand-analyte interaction, immobilisation procedures should be carefully designed. In this experimental setup, double stranded linear DNA was immobilised using a biotin-streptavidin capture method. Use of a biotin tag allows capturing DNA in specific direction. It was made sure that DNA is labelled only on one end (5' end) so that steric hindrance for analyte interaction is minimised (commercially purchased). In addition, DNA oligomers were carefully handled to prevent excessive freeze-thawing which may lead to generation of free biotin molecules. In addition, the assay procedure was optimised using detergent free buffer solutions in order to minimise possible ligand misconfigurations.

Once the assay method was established, detergent (p20 surfactant) was incorporated to reduce any possible non-specific interactions.

Moreover, there are technical limitations associated with the developed assay procedure. Since Biotin-streptavidin capture method is irreversible, regeneration of the sensor surface is restricted. Therefore, sensor chips can be used to immobilise limited number of DNA oligomers. Nevertheless, due to irreversible nature of the ligand capture, the sensor chip is reusable for multiple interaction analysis.

Well designed and optimised assay protocol is essential for precise data generation and data interpretation. In this experimental setup, optimisation of assay procedure was carried out in order to address following parameters.

1) Binding stability

Binding stability of the analyte was optimised so that stable binding of the analytes is achieved. Start-up cycles were increased up to 10 cycles in order to stabilise the binding of ligands.

2) Binding to reference

Binding to the reference and nonspecific interactions were checked at each assay time. Using Biacore evaluation software, separate graphs (with RU values) were generated and analysed visually for any non-specific interactions.

3) Solvent correction

Solvent corrections were incorporated in each and every assay in order to identify the variants in the sample preparation especially with bulk agents such as DMSO.

5.3.2. Promoter selection

In order to analyse the interaction of DNA-Antibiotic complex, oligomers of double stranded DNA were used. Mithramycin A and Chromomycin A are known to recognise GC-rich regions of the promoter sequences and inhibit Sp mediated transcription. DNA representing; Sp consensus sequence and *Bak* gene promoter sequence were subjected to analysis with modification. Consensus sequences of Sp transcription factors are identified as 5'-(G/T)GGGCGG(G/A) (G/A)(C/T)-3' which is also known as GC box (Dyran & Tjian, 1983; Letovsky & Dyran, 1989). However, in this study, Sp consensus sequence was extended up to 30bp so that biotin-streptavidin capture method has minimal steric hindrance for DNA-drug association. In parallel, *Bak* gene promoter was widely used in this study for kinetic analysis. *Bak* gene promoter was an

ideal selection kinetic studies since it has three distinctive Sp promoter binding sites. This gene promoter has been widely investigated by our group previously and have identified that, Sp proteins (Sp1 particularly) exhibited different affinities towards each binding sites. It has also been identified that B region (Binding site located in the middle of the sequence) has the highest affinity (Waby et al., 2010).

This study was limited to naked DNA where synthetic oligomers of double stranded were used with different lengths and promoter sites. Since the primary objective was to identify the direct association of DNA-drug interaction, naked DNA was an ideal substitute. This will provide a good understanding of chemistry of the interaction as well as the stoichiometry of the binding. Moreover, lack of knowledge of Terameprocol was available (yet to date) about the actual interaction of DNA-TMP association. Therefore, naked DNA was the ideal candidate to elucidate possible interaction of Terameprocol.

However, there is a potential that this developed assay method can be extended to investigate the interaction of the drugs with tertiary structures of DNA and histone proteins. A study carried out by Rutigliano *et al.*, have used triple helix forming oligomer of *Ha-ras* promoter of Sp1 binding site to investigate the Chromomycin A interaction (Rutigliano et al., 1998). Some studies have also examined the interaction of Mithramycin A and Chromomycin A using chromatin and Chromatin-histone complexes (Banerjee et al., 2014).

5.3.3. Interaction Mithramycin A

Structure of Mithramycin A (Aureolic acid) was vastly studied using different biophysical techniques, particularly by (Bakhaeva et al., 1968; Banville et al., 1990; Carptener et al., 1994; Wohler et al., 1999; Chakrabarti et al., 2000; Banerjee et al., 2014). In this present study, interaction of Mithramycin A has been analysed using comparative analyses (Real time label free analysis) with biologically relevant promoters. Mithramycin A specifically interacted with DNA oligomers of Sp consensus sequences and *Bak* gene promoter regions. In addition, more specific bindings were observed towards B region of the *Bak* gene promoter. Interaction of the Mithramycin A was in micromolar range (10^{-6} M) where similar binding range was observed for all the DNA oligomers used in this study. Kinetic results presented in this study were in line with previously published data such as using UV melting studies, Barcelo and

colleagues have reported that Mithramycin-DNA interaction occurs at the range of 8.0×10^{-6} M towards salmon testes DNA at 25° C (Barcelo et al., 2007). Similar results were presented where Mithramycin A is interacting with naked DNA with K_d of 3.3×10^{-7} M at 20° C (Dasgupta et al., 2012). It was also apparent that changes in the GC sequences affect their binding where lower binding responses were recorded for mutant sequences (random base pair changes in the binding sites) of the gene promoters.

In line with previous studies, binding interactions were investigated in the presence of the chelating metal ion Mg^{2+} . Different concentrations of Mg^{2+} ions were tested (up to 10 mM) in optimisation process where 5 mM concentration was used for the assay procedures. Binding levels (Response values (RU) and binding constants were not affected with different Mg concentration. Similarly, Ca^{2+} ions were also tested (Data not shown) in preliminary studies where no significant changes were observed. However, it has been demonstrated that Mithramycin A is capable of binding to other metal ions such as Ca^{2+} , Mn^{2+} , Cd^{2+} , Tb^{3+} and Gd^{3+} (Demicheli & Garniersuillerot, 1993). It was predicted that Mithramycin A might interact with DNA without any requirement of metal chelators. Till date, no studies have been carried out to investigate the requirement of the cations in Mithramycin A binding. In this study, DNA-Mithramycin A interaction was also investigated in the absence of Mg^{2+} and Ca^{2+} . Interestingly Mithramycin A was specifically interacting with all the DNA oligomers (Sp consensus sequence, Bak gene promoters). As evident from the results, 1:1 interaction between Mithramycin A and DNA was identified with different binding constants. In the absence of Mg^{2+} ions, the association rates were significantly (nearly 10 times) decreased. Hence sensorgrams for all the DNA oligomers followed the same shapes regardless the cation status.

In parallel with previous literature, this study further confirms that antibiotic derivative Mithramycin A bind to GC rich regions specifically. In addition to that these results suggest that these drugs have increased binding patterns towards Sp protein binding sites.

Furthermore, studies have shown that Mithramycin A is possibly binding with DNA as a dimer (Demicheli et al., 1991; Sastry & Patel, 1993; Weidenbach et al., 2016). In this study, it was tried to identify both single and dimer interaction of Mithramycin A. Both

one to one and bivalent ligand interaction modes were used to evaluate the results. Nevertheless, no significant differences were observed at any concentration of mithramycin A. Contrary to previous studies, it was clear that one to one ligand interaction is the predominant form of Mithramycin-DNA interaction (depending on best fitting curves). Using *Biacore T200* evaluation software, K_a / K_b values for possible bivalent ligand interaction was also determined. Even these evaluation results presented no significant differences. Therefore, it is possible that Mithramycin A is capable of single molecule interaction with linear DNA. Another possibility is, the dimer of the molecule has only a single mode of binding to the linear DNA with identical K_a / K_b values.

5.3.4. Interaction of Chromomycin A

Chromomycin A behaved as predicted and specific interaction of Chromomycin A and DNA oligomers of gene promoters were identified.

It was also apparent that in the absence of Mg^{2+} cation, Chromomycin interaction is more specific to GC rich regions/Sp binding sites. As can be seen in Figure 5.12 and Figure 5.17, changes in binding sites (mutant sequences) has hindered the DNA interaction of Chromomycin A. However, in the presence of 5 mM Mg^{2+} , lower level of bindings was detected for DNA of mutant regions compared to the promoter sequences (Figure 5.12 and 17). Possible complex formation with Mg^{2+} may reduce specificity of Chromomycin A towards its binding sites.

The single kinetic approach was also used for binding analysed of the Chromomycin A due to slow off rates. In single kinetic approach amounts ligands were build up to 5 cycles before being regenerated with running buffer. This method is recommended when regeneration of the analyte compound is required. However similar kinetic results were observed under both single and multiple kinetics methods. Representation of Single kinetics assay of Chromomycin is included in *Appendix II*.

5.3.5. Mode of interaction of Mithramycin A and Chromomycin with DNA

DNA interacting small molecules can be identified as DNA intercalators and DNA groove binders. DNA intercalators usually interact with DNA via insertion between adjacent base pairs. Planer heteroaromatic ring structures of intercalators are typically oriented parallel to base pairs. Stabilisation of association is supported by π - π stacking

interactions and conformation changes of the helix structure (possible unwinding at the intercalating site) (E.g. Actinomycin and doxorubicin) (Welch et al., 1994; Ferguson & Denny, 2007). On the other hand, DNA groove binders particularly minor groove binders consist of multiple aromatic rings such as pyrol, furan or benzene (see Figure 5.19). Van der Waals interactions, hydrogen bonds and hydrophobic interactions are the major stabilisation forces of the drug moiety and the DNA interaction. It is shown that drug moieties of DNA groove binders recognise the hydrogen bond donors and acceptors in order to recognise the sequence specificity (Nelson et al., 2007).

According to the previously published literature, magnesium complexes of both Mithramycin A and Chromomycin A bind to minor groove of the DNA in a GC-selective manner. A study carried out by Dasgupta *et al.*, with binding isotherms has shown that Mithramycin A interact with naked DNA at K_d of 3.3×10^{-7} M and 5.4×10^{-7} M at pH 8.0 at 20 °C (Dasgupta et al., 2012). This interaction was independent of the DNA source. In line with these results, K_d values presented in this study has shown that Mithramycin A and Chromomycin can interact with double stranded DNA in 10^{-6} M affinity (see Table 5.1 and 5.2). Both antibiotics also interact with chromatin leading to aggregation of chromatin and DNA release (Mir et al., 2003). Previous studies have also shown the interaction of Mithramycin A with core histone proteins which also leads to the alteration of post-translational modifications of histones (inhibit histone H3 acetylation) (Banerjee et al., 2014). Therefore, it is obvious that Mithramycin A process the dual binding properties which enable them to interact with DNA as well as the histone protein structures.

5.3.6. Interaction of Terameprocol

Nordihydroguateric acid derivatives were discovered as naturally occurring lignan and had been used as therapeutic agents in indigenous medicines for years. Recently modified methyl derivative, Terameprocol (dimethyl substitute) is noted as specific inhibitor of Sp protein mediated transcription (Smolewski, 2008). Initially, TMP was an anti-viral drug, used for HIV treatments (Pollara et al., 2010). After identifying its potential in cancer, therapeutics several attempts have been done to further investigate its mode of action upon several tumours (Lopez et al., 2007a; Smolewski, 2008).



Figure 5.20: Mode of interaction between DNA and groove binders.

Both Mithramycin A and Chromomycin A3 are known as minor groove binders. Multiple aromatic ring structures of minor groove binders associate with DNA as a characteristic curved shaped (isohelical with groove) structures. Induced structural changes in DNA and drug molecules may facilitate stabilisation of the interaction which is also supported by different types of bonds such as hydrogen, hydrophobic and Van der Waals interactions (Spolar & Record, 1994; Chaires, 1997). Figure adapted from Dasgupta et al., (2012).

Several biological studies have been speculated that action of TMP as a Sp protein inhibitor where specific gene inhibition was identified such as *Survivin* and *VEGF* gene transcription (Lopez et al., 2007b; Moretti et al., 2009; Sun et al., 2011). Furthermore Oyegunwa *et al.*, have shown that inhibitory action of Terameprocol on NFκB dependent TNF-α expression which suggests the direct TMP-DNA interaction (Oyegunwa et al., 2010). However, lack of knowledge behind the chemistry of Terameprocol has limited the application it as a therapeutic target. In this study, it was aimed to identify the nature of the interaction between Terameprocol and DNA. As a DNA binding molecule, it was expected to see specific binding of TMP towards double stranded DNA, interestingly towards Sp consensus region as well as other gene promoters. Surprisingly it was unable to detect any binding activity of TMP towards DNA oligomers used in this study. Under the conditions used for the experimental setup, only less than 5 RU units were detected. Even at higher concentrations (up to 1 mM) and longer DNA oligomers with more Sp binding sites (up to 6), no detectable binding was observed (Data not shown). It is convinced no detectable association with any DNA oligomers that have used for experiments. In accordance with the results and observation, this study proposes following possible explanations for lack of TMP binding.

- i. TMP is lacking a factor that essential for binding such as chelating ion

In this study, various concentrations of Mg²⁺ and Ca²⁺ ions have been tested as an obligatory cation requirement. Mg²⁺ and Ca²⁺ are the common chelating cations which are proven to assist DNA interactions of most of the moieties. Therefore, it is highly unlikely that lack of chelating ions prevents the formation of DNA-TMP interaction at this juncture. However, it is possible to check for other cations such as Zn²⁺ and Mn²⁺ which have been used with inhibitory molecules. Due to the lack of knowledge available on Terameprocol this study was limited to check for only two common cations.

- ii. Requirement of a different pH and buffer conditions

Throughout the experiments, pH7.4 which is known to facilitating DNA binding activity has been maintained. Similar pH value can be found in the cellular environment. In addition to PBS buffer, other common SPR buffers with

different pH, cations were also tested whilst no detection of bindings were identified.

iii. Linear DNA conformation may prevent binding of TMP

Steric hindrance of linear naked DNA is believed to be minimum which may facilitate the easier approach of the drug moieties as well as direct interactions. However, it is possible that TMP may require tertiary DNA helix structures for stable DNA-TMP interaction.

iv. Structure of the TMP lacks essential moieties which enable direct DNA interaction such as aromatic ring structures

Methyl substitution of the quinin ring structure may not be sufficient to access the double stranded helix DNA. Stabilisation of the interactions may need structural conformations (bond formation ability depends on the type of the interaction). Lack of information is available about mode of interactions of the TMP. According to the methyl substitution nature, it is possible that TMP is designed to be a groove binder which can be stabilised by H-bonding. Nevertheless, apart from methyl groups, it lacks any other functional groups which can interact with DNA. Further analysis with biophysical methods is required to elucidate structural analysis.

However, present results confirm that TMP may not directly interact with linear DNA at given conditions. However, no literature data is available till date to elucidate its DNA binding properties. Further analyses are required in order to examine the binding of TMP. Use of tertiary DNA structures and mimicking of cellular environment may be helpful to detect its possible DNA binding. Hence natural lignan TMP has shown anti-tumour activity and potential application as HIV and cancer drug it is worth to further discover the chemistry of the TMP with available techniques. Moreover, it is also possible that anti-tumour activity of TMP is governed by an alternative mechanism such as TMP-protein interaction.

5.3.7. Conclusion

This study provides knowledge of kinetics of two known Sp inhibitors; Mithramycin A and Chromomycin A in relation to biological relevant promoters. This study also proves that both aureolic acid derivatives can interact with DNA in the absence of divalent

metal ion Mg^{2+} . Binding kinetics of these inhibitors provide a better understanding of affinity and specificity of current molecules in pharmacokinetic aspects. In addition, results have shown that NDGA acid derivative TMP is unable to form direct interaction under the experimental conditions used in this study.

Assay method developed in this study provides real time accurate and quantitative evaluation of common Sp protein inhibitors. When combined with toxicology and pharmacokinetic data these kinetic values might be useful in determining the efficiency of these small molecules and their activity in biological context. Moreover, this method of detection can be used in parallel with synthetic process to check their binding ability and kinetics of natural and synthetic moieties for disease treatment.

Chapter 6.
**Effective inhibition of Sp mediated transcription of
hypoxia responsive genes**

6. Effective inhibition of Sp mediated transcription of hypoxia responsive genes

6.1. Introduction

Sp mediated transcription is widely investigated in different tumour models by specific inhibition of Sp genes. Knockout mouse models have demonstrated the vital role of individual Sp proteins in embryonic development and postnatal development, emphasising the necessity of Sp transcription factors and their regulated pathways (Marin et al., 1997; Bouwman et al., 2000; Gollner et al., 2001a). Gene knockdown of Sp proteins in cancer cells have exhibited induced apoptosis, inhibition of cell cycle progression (G_0/G_1 to S) and decrease proliferation (Hedrick et al., 2016). In addition, a study carried out Hsu *et al.*, has shown *Sp1* knockdown in lung cancer cells leads to increased migration and invasion potential (Hsu et al., 2012). Similarly, in colon and prostate cancer cells *Sp1* knockdown resulted G_0/G_1 to S phase inhibition and induced apoptosis (Ishibashi et al., 2000; Borgatti et al., 2003). Moreover, *Sp3* and *Sp4* knockdown studies on pancreatic and colon cells also showed same effects which emphasise the necessity of Sp proteins in key cellular pathways in cancers (Chintharlapalli et al., 2009; Luo et al., 2009).

On the other hand, different types of inhibitors have been used to inhibit Sp mediated transcription and cellular pathways. These inhibitors have been tested as anti-tumour drugs in different cancer types. Sp protein inhibitors such as Sp decoys, antibiotic derivatives, have shown promising results in integrative/*in-vivo* gene expression studies (Chintharlapalli et al., 2007; Pathi et al., 2012). Mithramycin A, a DNA binding small molecule is widely identified as a specific Sp protein inhibitor which is known to interact with GC-rich gene promoter sequences. Inhibitory effect of antibiotic derivatives Mithramycin A has been reported several times in different cancers (Duverger et al., 2004; Yuan et al., 2007; Otjacques et al., 2013). In addition, there are ample numbers of different genes that are effectively inhibited by Mithramycin A and its synthetic derivatives (Previdi et al., 2010; Malek et al., 2012; Choi et al., 2013; Fernandez-Guizan et al., 2014). Common examples include; dihydro folate reductase (DHFR) gene (Blume et al., 1991), copper transporting ATPase (*Atp7a*) (Xie & Collins, 2013), cancer associated molecule CD147 (Kong et al., 2010). These studies have

suggested that Mithramycin A inhibits gene transcription by Sp-dependent manner and potential use as an anti-tumour drug. Some clinical studies also provide importance of Sp targets in chemotherapy strategies (Kennedy, 1970; Khanna et al., 2007; Grossman et al., 2009).

The role of Sp proteins in hypoxia is barely understood. Nevertheless, most of the therapeutic aspects of hypoxic response have been focused on HIF mediated regulation (Lee et al., 2016; Wigerup et al., 2016). However, few studies have focused on inhibition of downstream products of hypoxia induced pathways. Studies have shown that some hypoxia inducible genes also contain promoter sites which are similar to Sp protein binding sites. Therefore, inhibition of Sp protein binding and its activity may be a vital target for regulating downstream gene targets of hypoxia induction. Sp protein might be a vital candidate as therapeutic target, particularly in hypoxia microenvironment.

6.1.1. Aims and objectives of the chapter

The main objective of this chapter is to study the effect of Sp protein blockade on transcription of hypoxia responsive genes in breast cancer cell models. In order to inhibit Sp mediated transcription, *Sp1* gene has been knockdown using specific siRNA in breast cancer cell lines. In addition, DNA binding small molecule Mithramycin A was selected to inhibit Sp protein binding to its cognate sites. Effect of both knockdowns and Mithramycin A treatments were assessed in both hypoxic and normoxic conditions. This will allow identifying effective inhibition of Sp mediated transcription of hypoxia mediated genes.

Key questions answered from this chapter;

- The dependency of hypoxia responsive genes on Sp mediated transcription?
- The effectiveness of Mithramycin A as a Sp antagonist?

6.2. Results

6.2.1. Effect of Sp protein blockade

In the previous chapter (Chapter 4), hypoxia responsive genes with Sp binding sites were identified and their expression changes were further examined under hypoxic environments. As predicted by microarray data analysis, expression levels were changed due to hypoxia exposure at different time points. Moreover, it was clearly demonstrated that Mithramycin A, Sp protein inhibitor binds to DNA, preferably to Sp binding sites, in turn prevent binding of Sp proteins to its cognate sites.

This chapter identifies the effect of Sp blockade by Mithramycin A and *Sp1* gene knockdown on expression of previously identified (Chapter 3) hypoxia responsive genes. Cells were treated with 1 μ M Mithramycin or specific siRNA for *Sp1* gene followed by 12 hours' of hypoxia exposure. Expression levels were analysed using TaqMan qPCR assays. Expression of *CA9* and *GLUT1* were used as hypoxia markers. In addition, expression levels of Sp family proteins (*Sp1*, *Sp3* and *Sp4*) were also examined.

Sp1 expression levels were significantly decreased due to siRNA knockdowns. MCF-7 cells showed 0.89 FC (+/- 0.05 SD) and 1.23 FC (+/- 0.46 SD) decreases in *Sp1* mRNA levels in normoxic and hypoxic conditions respectively. In MDA-MB-231 cells, knockdown resulted 0.91 FC (+/- 0.04 SD) and 1.66 FC (+/- 0.72 SD) decreases in normoxic and hypoxic conditions. Similarly, *Sp1* mRNA levels in T47D cells decreased by 0.80 FC (+/- 0.06 SD) and 1.52 FC (+/- 0.82 SD) respectively. However, changes in only hypoxic sample was statistically significant in MDA-MB-231 and T47D cell lines (See Figure 6.1).

Sp1 knockdowns had a different effect on *Sp3* and *Sp4* transcripts. As seen in Figure 6.2, *Sp3* expression levels were unchanged for all the cell lines except the hypoxic sample of T47D cells where 0.42 FC (+/- 0.19 SD) increase was observed due to knockdown.

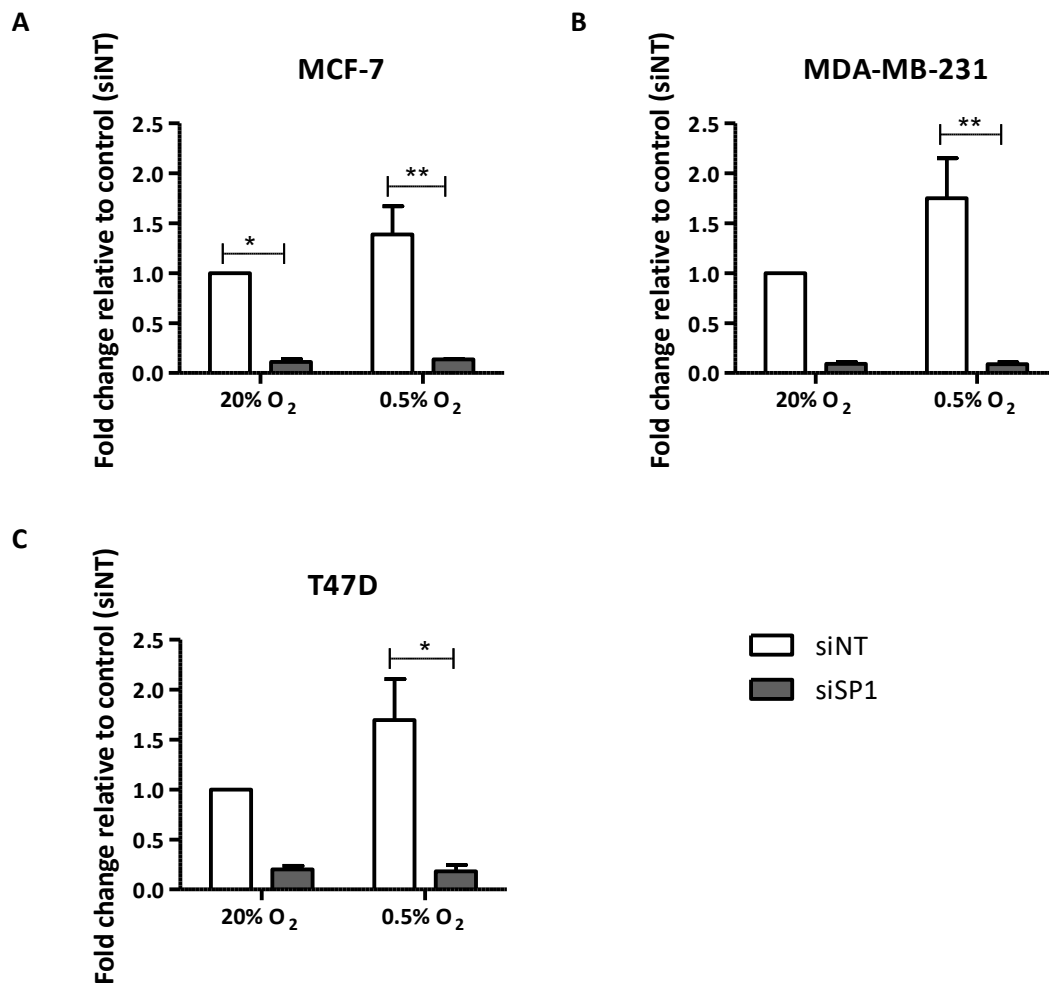


Figure 6.1: Effect of *Sp1* knockdown on transcript levels of *Sp1* in breast cancer cell under hypoxia

MCF-7 (panel A), MDA-MB-231 (panel B) and T47D (panel C) cells were treated with siRNA to knockdown *Sp1* (siSP1) or control non-targeting siRNA (siNT). Cells were incubated in normoxia (20% (v/v) O₂) or hypoxia (0.5% (v/v) O₂) for 12 hours followed by total mRNA extraction. Transcript level changes were analysed by qPCR analysis by TaqMan assays. *B2M* was used as housekeeping gene. Results are expressed as fold change relative to control (siNT). n=3 experiments: Error bars represents +/- SEM and statistical significance was determined by Two-way ANOVA with Bonferroni post-hoc correction. *, P< 0.05; **, P< 0.01.

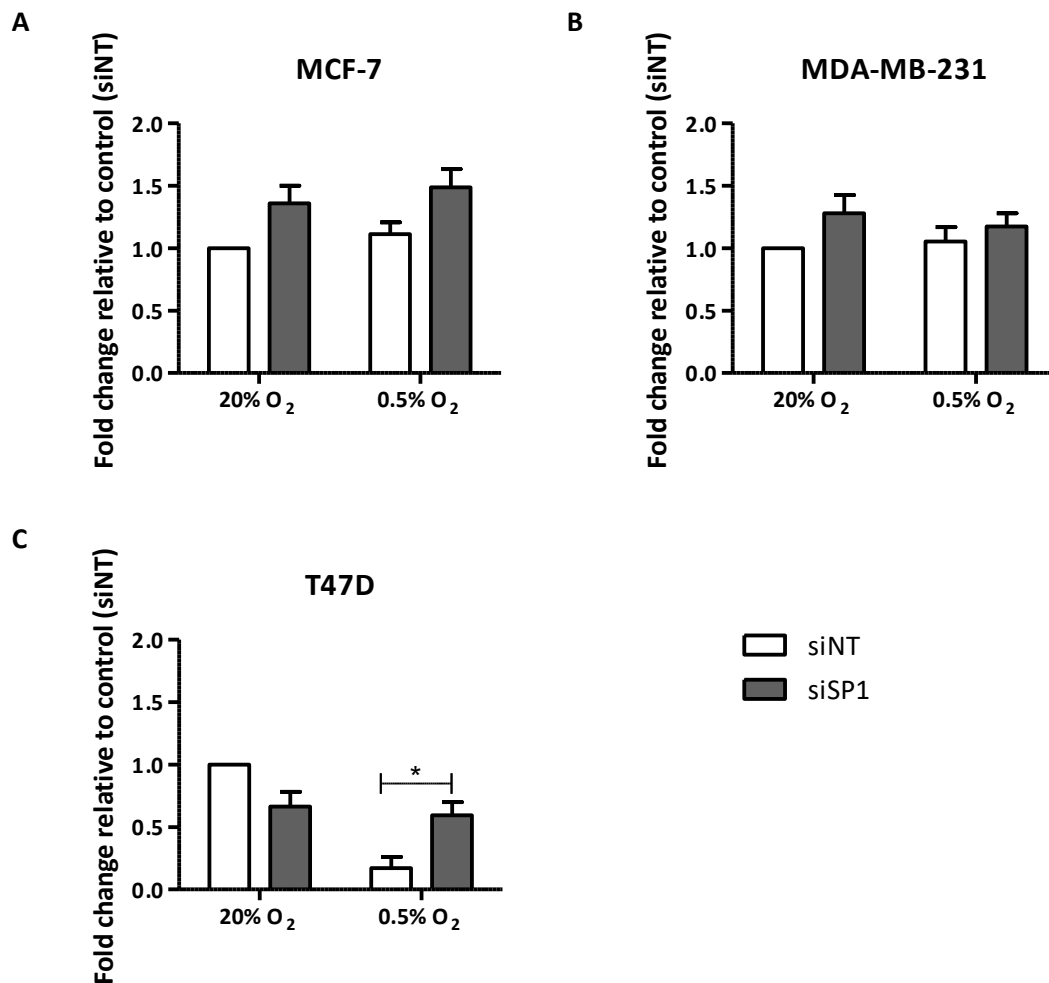


Figure 6.2: Effect of *Sp1* knockdown on transcript levels of *Sp3* in breast cancer cells under hypoxia

MCF-7 (panel A), MDA-MB-231 (panel B) and T47D (panel C) cells were treated with siRNA to knockdown *Sp1* (siSp1) or control non-targeting siRNA (siNT). Cells were incubated in normoxia (20% (v/v) O₂) or hypoxia (0.5% (v/v) O₂) for 12 hours followed by total mRNA extraction. Transcript level changes were analysed by qPCR analysis by TaqMan assays. *B2M* was used as housekeeping gene. Results are expressed as fold change relative to control (siNT). n=3 experiments: Error bars represents +/- SEM and statistical significance was determined by Two-way ANOVA with Bonferroni post-hoc correction. *, P < 0.05.

On the other hand, *Sp4* transcript levels were increased due to siRNA knockdowns in both hypoxic and normoxic conditions. In MCF-7 cells, 1.05 FC (+/- 0.33 SD) and 1.99 FC (+/- 0.33 SD) increases were detected for both normoxic and hypoxic conditions respectively. MDA-MB-231 cells showed similar pattern and 0.96 FC (+/- 0.11 SD) increase was detected in normoxic condition and 1.28 FC (+/- 0.60 SD) in hypoxic condition. *Sp4* mRNA levels of T47D cells were also increased in both conditions, although changes were statistically insignificant (see Figure 6.3).

The expression level of *CA9* was also checked after *Sp1* knockdown. Hypoxic induction was prominent in all three cell lines where MCF-7, MDA-MB-231 and T47D cells showed increased expressions. Interestingly, *CA9* transcript levels of MCF-7 and T47D cells decreased in *Sp1* knockdown samples. Expression levels of *CA9* decreased by 11.32 FC (+/- 2.90 SD) and 9.70 FC (+/- 1.75 SD) against the untreated hypoxic sample in both cell lines respectively (see Figure 6.4 Panel A).

In parallel, the effect of Mithramycin A on *CA9* expression was also analysed. All three cell lines treated with Mithramycin A under normoxic condition were lack of any significant changes compared to the control. In contrast to normoxic condition, Mithramycin A had an inhibitory effect on *CA9* expression under severe hypoxia where all cell lines treated with Mithramycin A showed a significant reduction (inhibition of hypoxic induction) of expression levels of *CA9* gene. MCF-7 showed 16.46 FC (+/- 1.87 SD) decrease compared to the untreated hypoxic control. Similarly, MDA-MB-231 and T47D cells showed 12.28 FC (+/- 2.71 SD) and 7.03 FC (+/- 2.03 SD) reduction respectively (see Figure 6.4 Panel B).

Hypoxia marker *GLUT1* also showed a similar pattern of expression after Mithramycin treatment and *Sp1* gene knockdown. Under normoxic condition transcript levels of *GLUT1* were unchanged without any significant changes due to knockdown of *Sp1* gene. Hypoxic exposure has increased mRNA levels of *GLUT1* in all three cell lines. Expression levels decreased by 3.98 FC (+/- 1.58 SD) in MCF-7 cells when compared against the untreated hypoxic control (see Figure 6.5 Panel A).

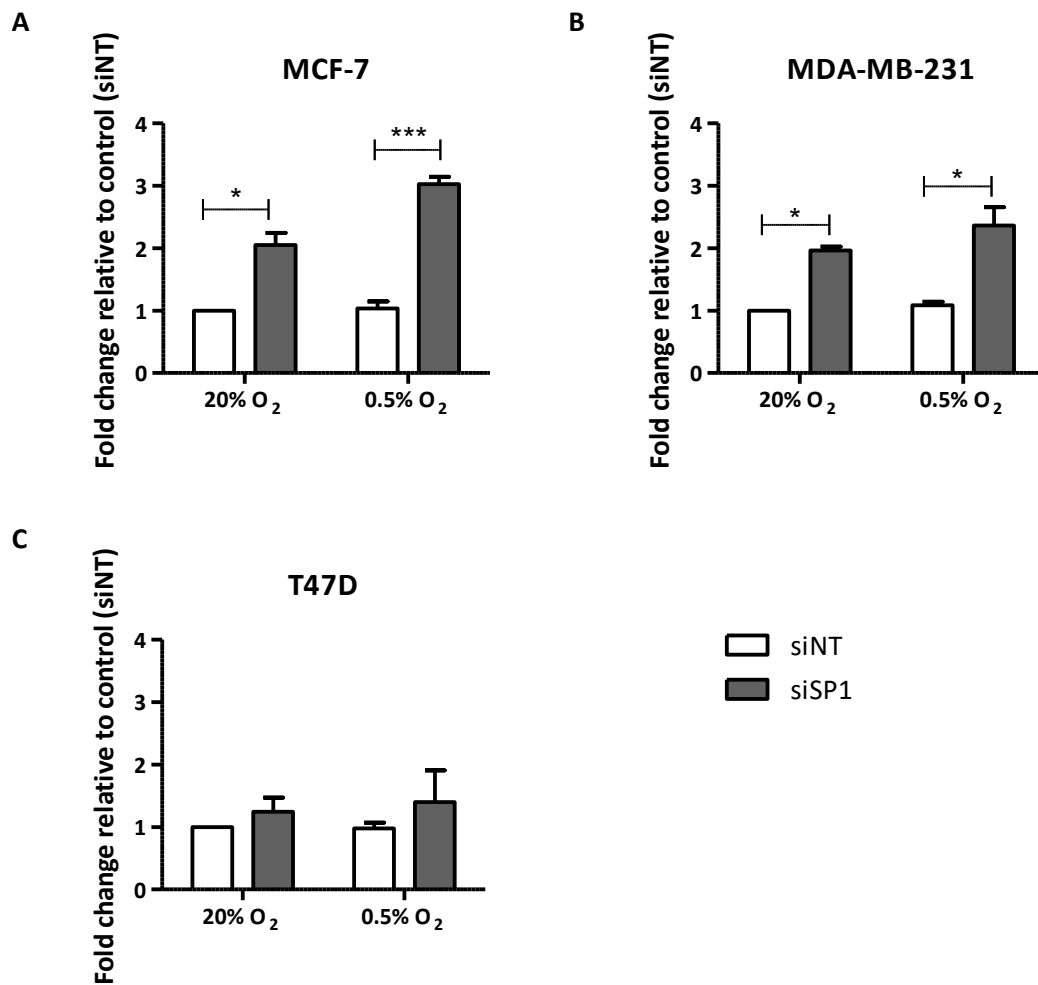


Figure 6.3: Effect of *Sp1* knockdown on transcript levels of *Sp4* in breast cancer cells under hypoxia

MCF-7 (panel A), MDA-MB-231 (panel B) and T47D (panel C) cells were treated with siRNA to knockdown *Sp1* (siSp1) or control non-targeting siRNA (siNT). Cells were incubated in normoxia (20% (v/v) O₂) or hypoxia (0.5% (v/v) O₂) for 12 hours followed by total mRNA extraction. Transcript level changes were analysed by qPCR analysis by TaqMan assays. *B2M* was used as housekeeping gene. Results are expressed as fold change relative to control (siNT). n=3 experiments: Error bars represents +/- SEM and statistical significance was determined by Two-way ANOVA with Bonferroni post-hoc correction. *, P < 0.05; ***, P < 0.001.

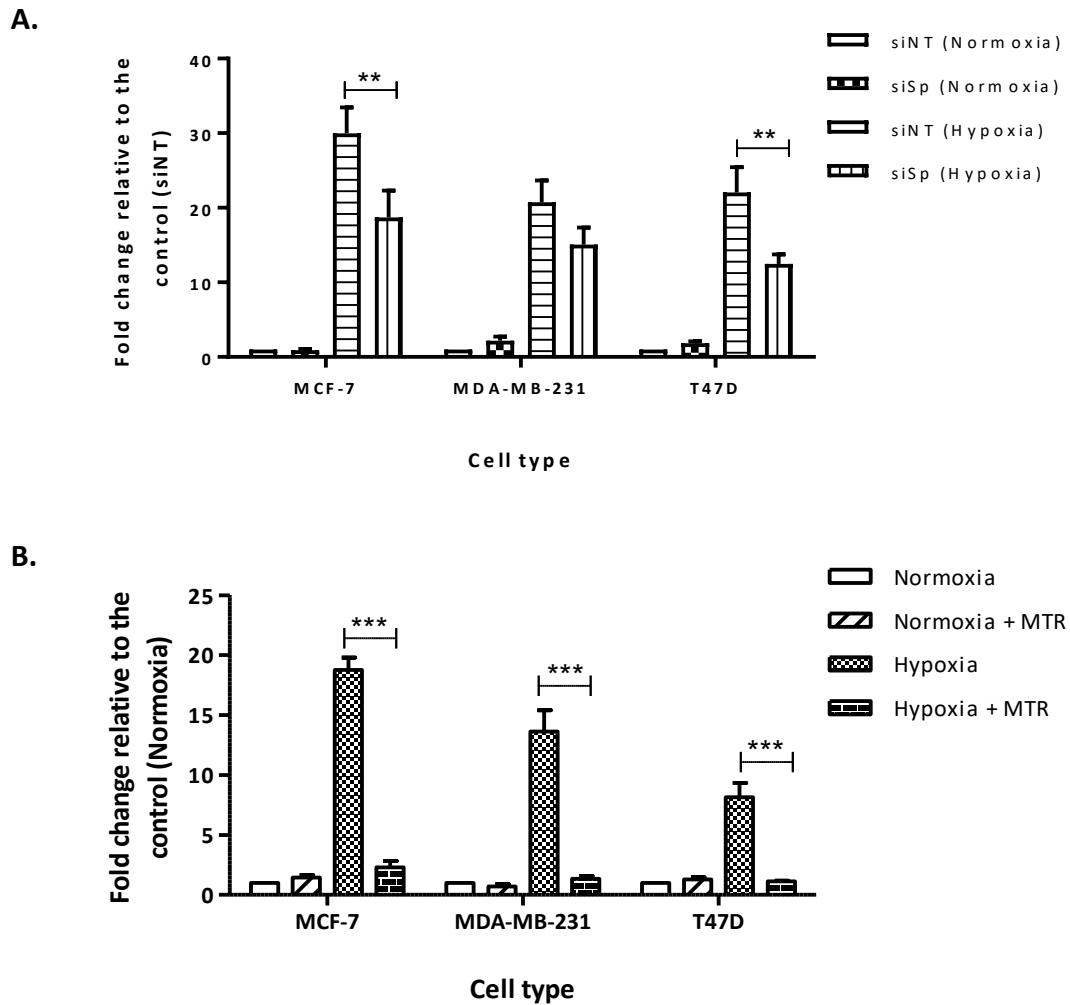


Figure 6.4: Effects of *Sp1* gene knockdown and Mithramycin A on *CA9* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. **, P< 0.01; ***, P< 0.001

Sp1 gene knockdown did not change *GLUT1* expression in MDA-MB-231 and T47D cells despite some insignificant variations (see Figure 6.5 Panel A). In MCF-7 cells Mithramycin A treatment increased *GLUT1* expression levels by 0.83 FC (+/- 0.35 SD) under normoxic condition. Hence no significant changes were detected for other cell lines. *GLUT1* gene was upregulated in all three cell lines due to hypoxic exposure. As seen in Figure 6.5, Panel B, *GLUT1* expression levels were dramatically reduced by mithramycin A treatment in the hypoxic environment. Upregulation observed in hypoxic condition was absent in Mithramycin A treated hypoxic samples where expression levels were further decreased compared to the control. Expression levels decreased by 2.54 FC (+/- 0.78 SD), 4.65 FC (+/- 0.24SD) and 1.63 FC (+/- 0.53 SD) in MCF-7, MDA-MB-231 and T47D cells respectively compared to the untreated hypoxia control.

6.2.2. The effect of Mithramycin A and siRNA knockdown on hypoxia responsive genes

Twenty genes which were investigated in the previous chapter were further analysed after treatment of Mithramycin A and siRNA for *Sp1* gene.

1. 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4)

Cells cultured in normoxic condition showed no significant expression changes in *PFKFB4* gene due to Knockdown of *Sp1* gene or Mithramycin A treatment. As evident from Figure 6.6 Panel A, *PFKFB4* gene expression was up-regulated in all three breast cancer cell lines under severe hypoxia. However, when *Sp1* gene was knockdown by siRNA, hypoxia induction of *PFKFB4* gene decreased by 6.20 FC (+/- 1.65 SD) in MCF-7 cells compare to sample treated with siNT control. Decreased expression levels were also visible in MDA-MB-231 and T47D cells despite insignificant in statistical analysis (see panel A). Similarly, hypoxic induction was completely inhibited by Mithramycin A treatment in breast cancer cells. Under severe hypoxia, expression levels decreased by 14.33 FC (+/- 3.63 SD), 4.94 FC (+/- 1.68 SD) and 4.27 FC (+/- 0.28 SD) in MCF-7, MDA-MB-231 and T47D cell lines respectively when compared to the untreated hypoxic sample (see panel B).

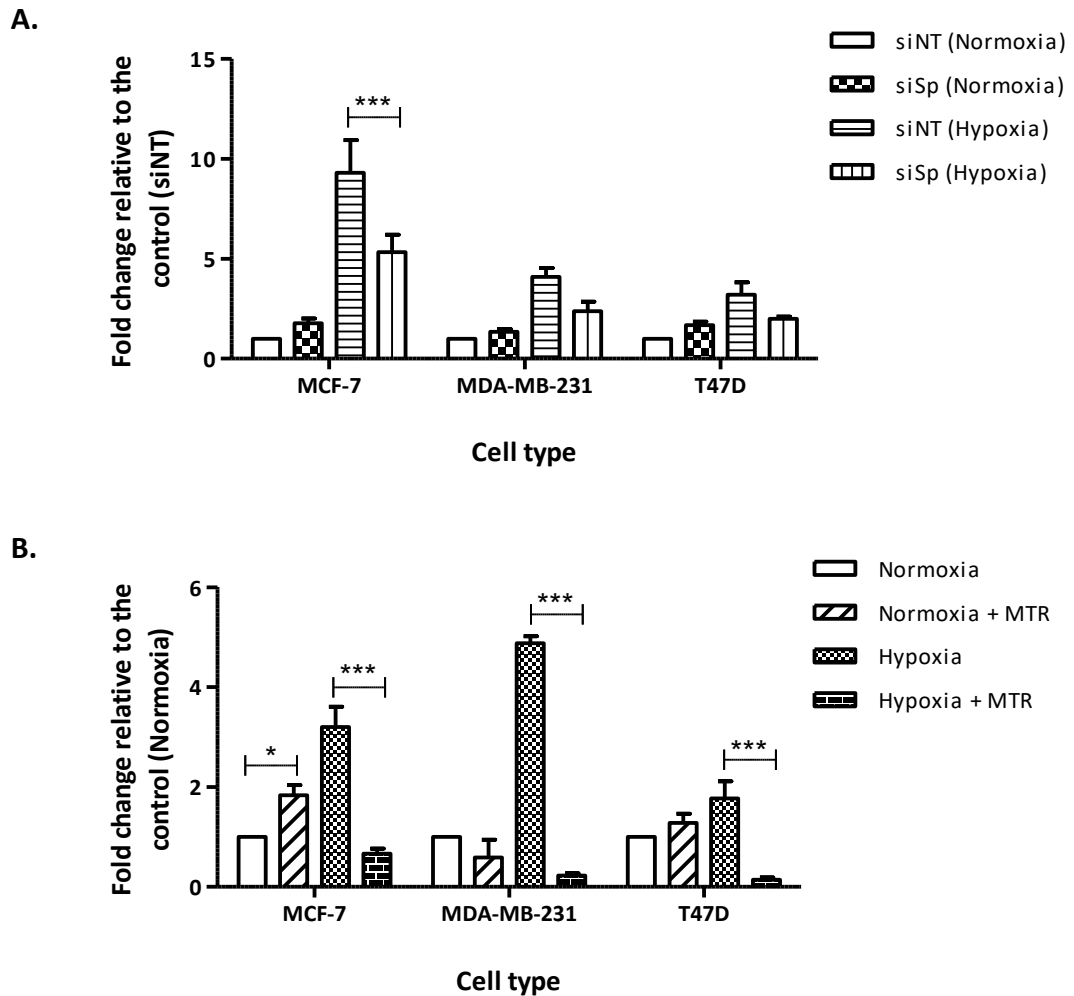


Figure 6.5: Effects of *Sp1* gene knockdown and Mithramycin A on *GLUT1* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; ***, P< 0.001.

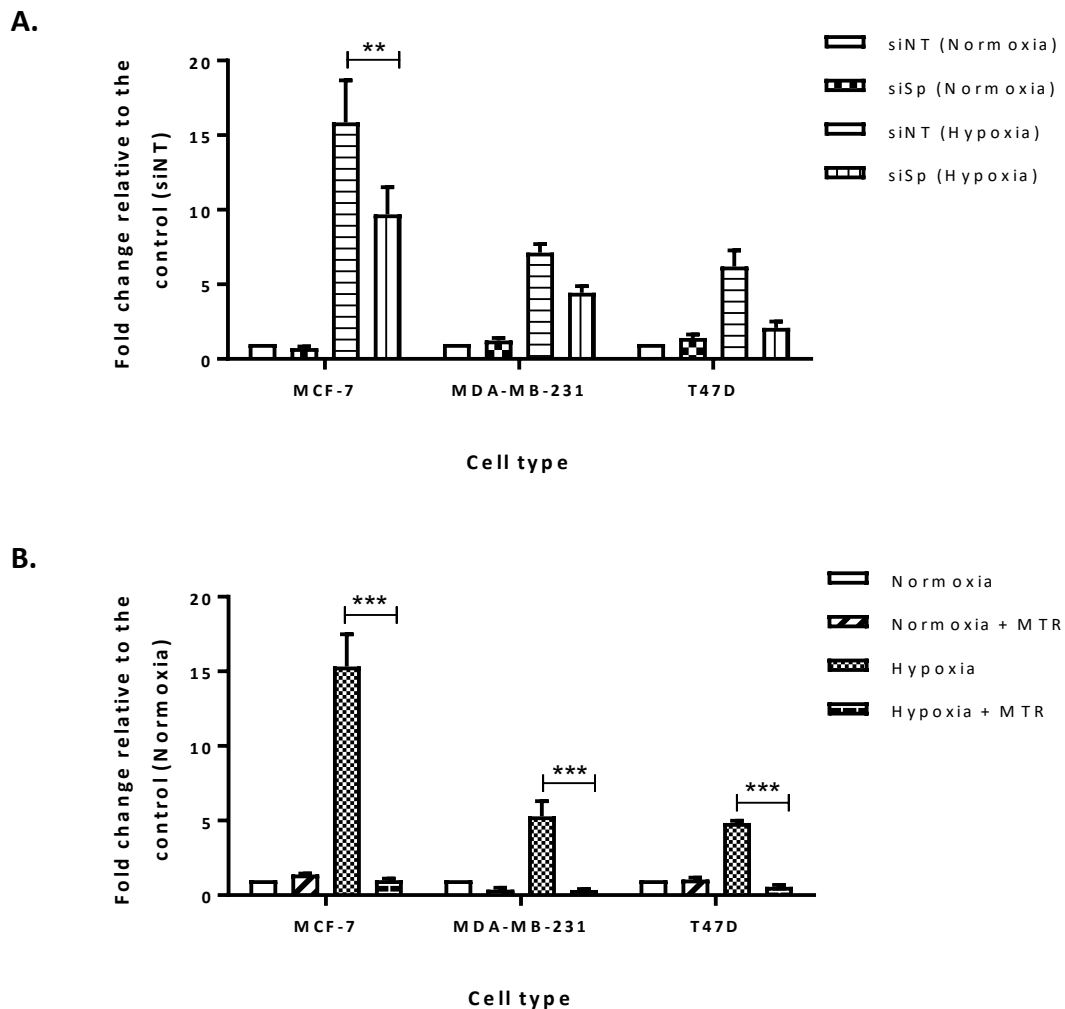


Figure 6.6: Effects of *Sp1* gene knockdown and Mithramycin A on *PFKFB4* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. **, P< 0.01; ***, P< 0.001.

2. Artemin (ARTN)

Exposure to severe hypoxia showed no clear expression changes of *ARTN* gene in breast cancer cells (see Figure 6.7). Similarly, expression of *ARTN* gene was unchanged due to knockdown of *Sp1* gene where no significant changes were observed at any condition for all three cell lines despite some apparent decreases (see panel A). On the other hand, Mithramycin A treatment significantly increased mRNA levels of *ARTN* gene under hypoxic and normoxic conditions. In MCF-7 cells 2.98 FC (+/- 0.13 SD) and 3.81 FC (+/- 0.28 SD) increases were detected at normoxic and hypoxic samples respectively. Similarly, MDA-MB-231 cells showed 1.74 FC (+/- 0.39 SD) increase in normoxia and 3.59 FC (+/- 0.95 SD) in hypoxia. Expression levels of *ARTN* were increased by 4.70 FC (+/- 1.55 SD) and 6.77 FC (+/- 0.63 SD) in both normoxic and hypoxic conditions respectively (See panel B).

3. Vascular endothelial growth factor A (VEGFA)

As evident on Figure 6.8, hypoxia induction of *VEGFA* was prominent in severe hypoxia. Under normoxic condition cells treated with siRNA (siSp1 and siNT) or Mithramycin A exhibited similar levels of mRNA expression without any significant changes. Hypoxic samples of MCF-7, MDA-MB-231 and T47D cells treated with siNT showed upregulation of mRNA levels where hypoxic induction was inhibited by *Sp1* gene knockdown. Expression levels were significantly decreased in MCF-7 by 2.00 FC (+/- 0.96 SD), in MDA-MB-231 by 1.20 FC (+/- 0.86 SD) and in T47D cells by 1.55 FC (+/- 0.96 SD) against the control (see panel A). Similarly, Mithramycin A treatment effectively inhibit hypoxic induction of *VEGFA* gene expression in all three cell lines. Expression levels significantly decreased by 1.79 FC (+/- 0.75 SD), 4.20 FC (+/- 1.16 SD) and 2.51 FC (+/- 0.41 SD) for MCF-7, MDA-MB231 and T47D cells respectively compared to the untreated hypoxic sample (see panel B).

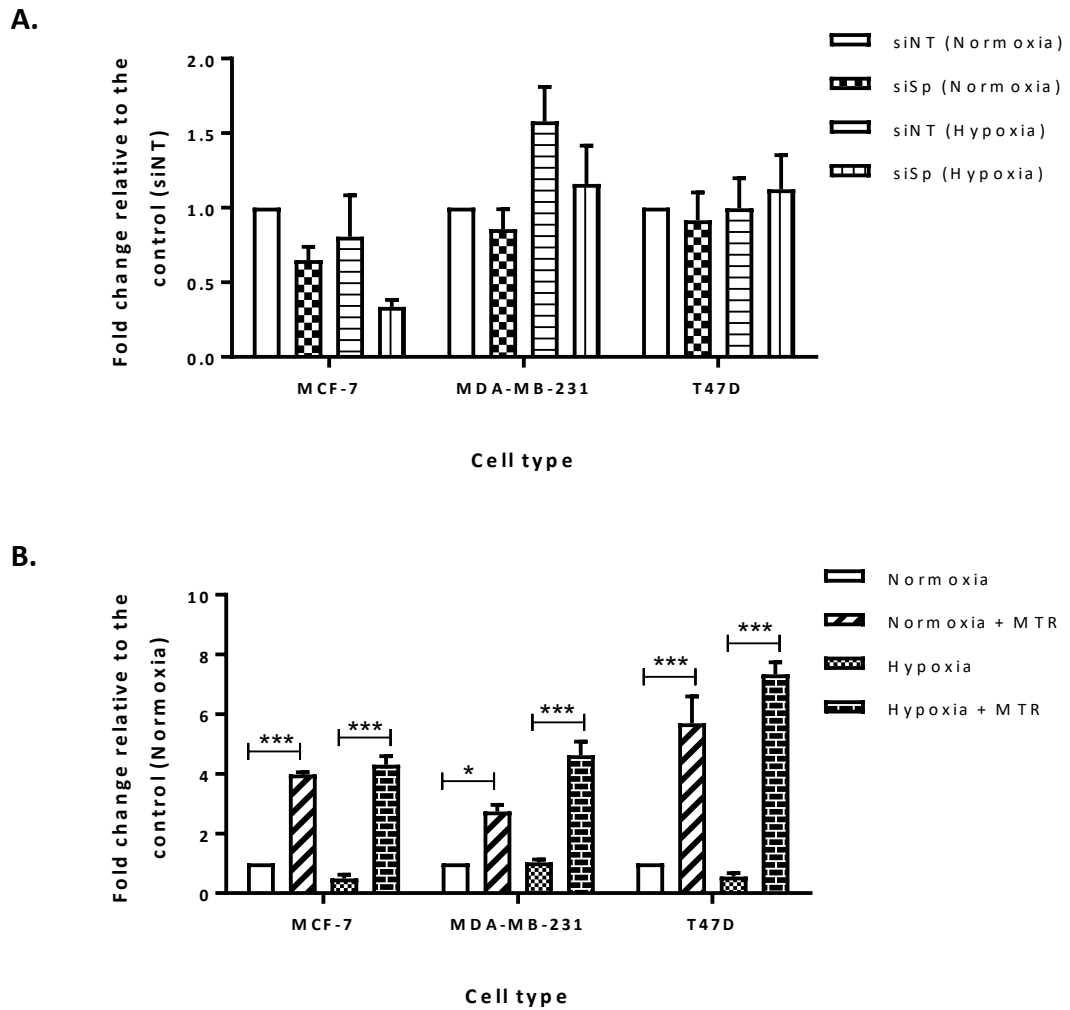


Figure 6.7: Effects of *Sp1* gene knockdown and Mithramycin A on *ARTN* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P < 0.05; ***, P < 0.001.

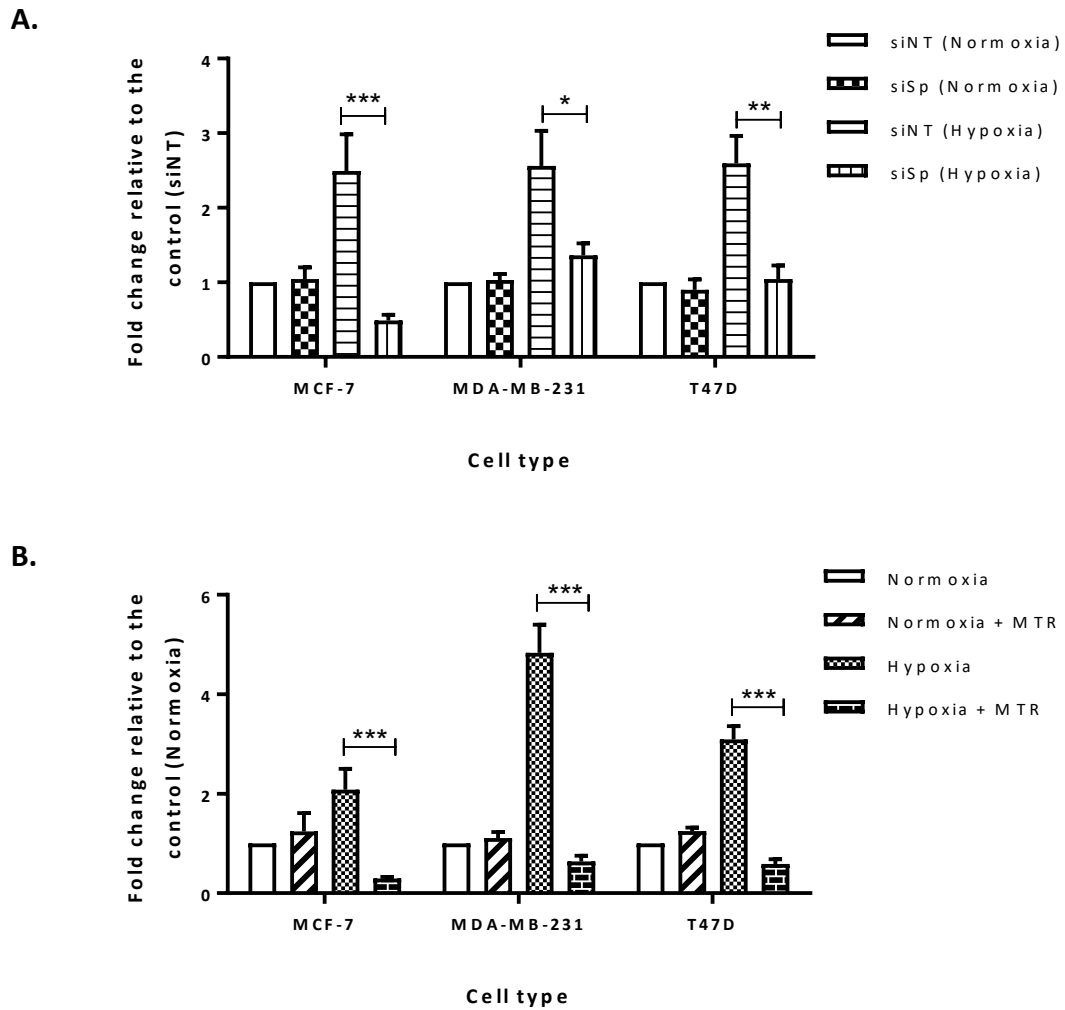


Figure 6.8: Effects of *Sp1* gene knockdown and Mithramycin A on *VEGFA* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

4. cyclin G2 (CCNG2)

Cells cultured in normoxic conditions showed no expression changes of the gene *CCNG2* due to the knockdown of *Sp1* gene (see Figure 6.9 panel A). Alteration of mRNA levels was clearly visible due to Mithramycin A treatment in all cell lines. However, changes were statistically insignificant in normoxia (panel B). Hypoxic induction of *CCNG2* gene was clearly visible in all three cell lines where both *Sp1* gene knockdown and Mithramycin A treatments significantly inhibit the upregulation. *Sp1* gene knockdown caused mRNA levels to decrease by 2.43 FC (+/- 1.50 SD), 1.47 FC (+/- 0.76 SD) and 2.34 FC (+/- 1.51 SD) in MCF-7, MDA-MB-231 and T47D cells respectively compared to the untreated control (siNT) (see panel A). When cells were treated with Mithramycin A hypoxic induction of *CCNG2* was repressed and expression levels decreased significantly compared to the untreated counterpart. As seen in Figure 6.9, expression levels decreased by 2.94 FC (+/- 1.08 SD) in MCF-7, 1.75 FC (+/- 0.93 SD) and 2.29 FC (+/- 0.16 SD) in MDA-MB-231 and T47D cells respectively (see panel B).

5. Insulin receptor substrate 2 (IRS2)

As evident from Figure 6.10, cell cultured in both hypoxic and normoxic conditions showed a similar level of *IRS2* gene expression in all three cell lines. Similarly, *Sp1* gene knockdown showed no effect on expression of *IRS2* gene in breast cancer cells. No significant alterations in mRNA levels of *IRS2* gene were detected in any cell line under normoxic or hypoxic conditions except some insignificant variations among the cell lines (see panel A). However, Mithramycin A treatment decrease *IRS2* expression levels in both hypoxic and normoxic conditions. Expression levels of MDA-MB-231 and T47D cells decreased by 0.35 FC (+/- 0.09 SD) and 0.45 FC (+/- 0.15 SD) respectively under normoxic conditions where it was only visible in MCF-7 cells. In the hypoxic environment, expression levels of *IRS2* gene were significantly decreased in MCF-7 and MDA-MB-231 cell lines compared to the untreated control. Expression changes were reported as 0.77 FC (+/- 0.20SD) and 0.81 FC (+/- 0.03 SD) respectively. Expression patterns in T47D followed the same pattern where reduction of *IRS2* levels was clearly visible (see panel B).

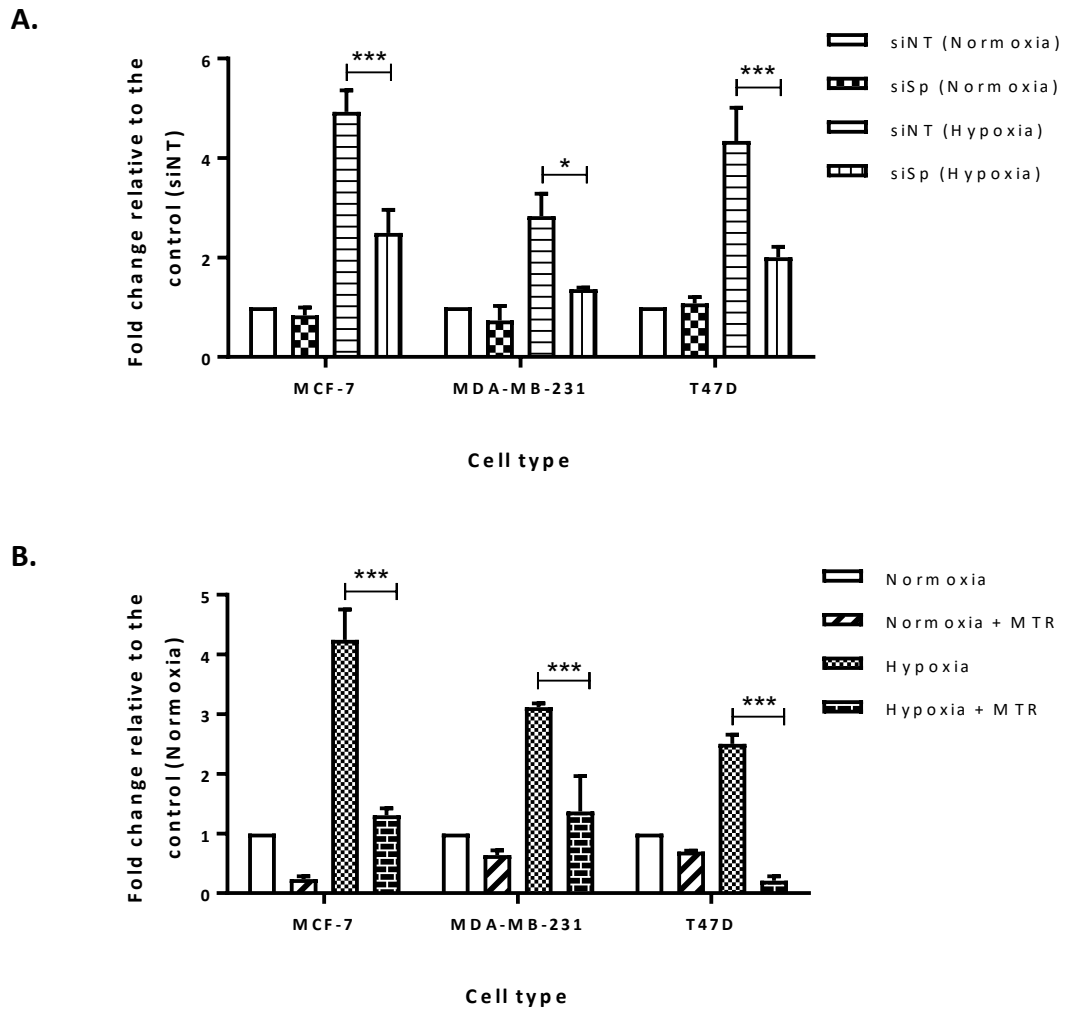


Figure 6.9: Effects of *Sp1* gene knockdown and Mithramycin A on *CCNG2* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; ***, P< 0.001.

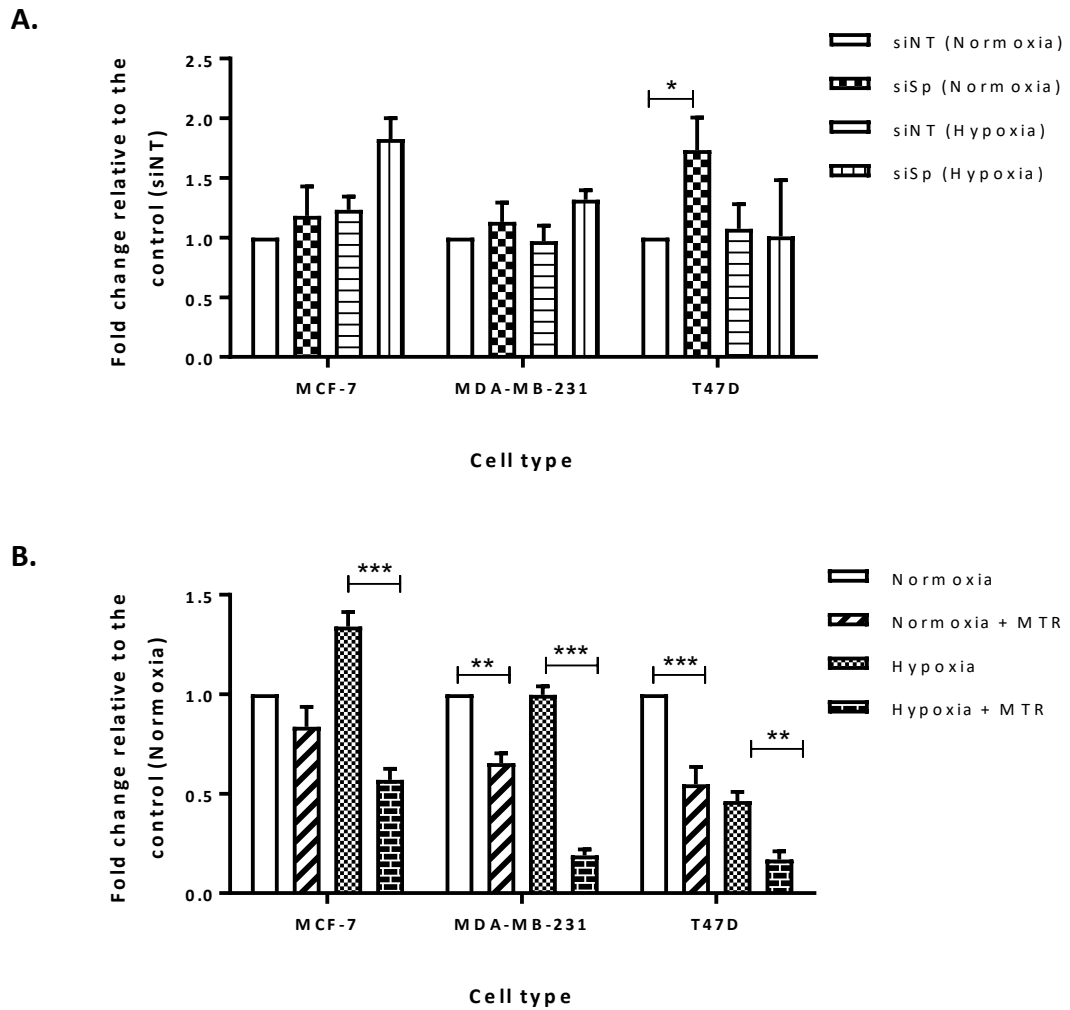


Figure 6.10: Effects of *Sp1* gene knockdown and Mithramycin A on *IRS2* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

6. Trophoblast glycoprotein (TPBG)

Hypoxic induction of *TPBG* gene was clearly identified in all three breast cancer cell lines (see Figure 6.11). Under normoxic condition, all three cell lines treated with *Sp1* gene specific siRNA showed similar level of expression without any significant changes. However, knockdown of *Sp1* gene decreased hypoxic induction of *TPBG* in breast cancer cells. These changes were clearly visible in MCF-7 and significant in MDA-MB-231 cells and T47D cells. mRNA levels decreased by 1.07 FC (+/- 0.50 SD) in both MDA-MB-231 and T47D cells compared to the siNT hypoxic samples (see panel A).

Mithramycin A showed inhibitory effect *TPBG* gene expression in both hypoxic and normoxic conditions. Under normoxic conditions expression levels decreased by 0.63 FC (+/- 0.13 SD) in MCF-7 and 0.51 FC (+/- 0.09 SD) in T47D cells (see panel B). A decrease was also visible in MDA-MB-231 cells even though it was insignificant in nature. In hypoxia, mRNA levels decreased compared to the untreated hypoxic samples and fold changes were reported as 2.35 FC (+/- 0.60 SD) in MCF-7 and 0.81 FC (+/- 0.32 SD) in MDA-MB-231 and 1.25 FC (+/- 0.34 SD) in T47D cells.

7. Fucosyltransferase 11 (alpha (1,3) fucosyltransferase) (FUT11)

FUT11 gene expression was highly induced in severe hypoxia in all three breast cancer cell lines. Cells grown in normoxic conditions showed no expression changes of *FUT11* gene due to *Sp1* gene knockdown or Mithramycin A treatment (see Figure 6.12). However, hypoxic induction of *FUT11* gene was significantly decreased by *Sp1* gene knockdown in hypoxia. mRNA levels decreased by 2.09 FC (+/- 0.89 SD) in MCF-7, 2.32 FC (+/- 1.51 SD) in MDA-MB-231 and 2.45 FC (+/- 1.61 SD) in T47D cells compared to hypoxic samples treated with siNT (see panel A). Similarly, treatment of Mithramycin A effectively inhibits hypoxic induction of *FUT11* in breast cancer cells. In MCF-7 cells mRNA levels decreased by 3.15 FC (+/- 1.58 SD) compared to untreated hypoxic sample. Similarly, 1.74 FC (+/- 0.76 SD) and 2.84 FC (+/- 0.68 SD) were reported for both MDA-MB-231 and T47D cells respectively (see panel B).

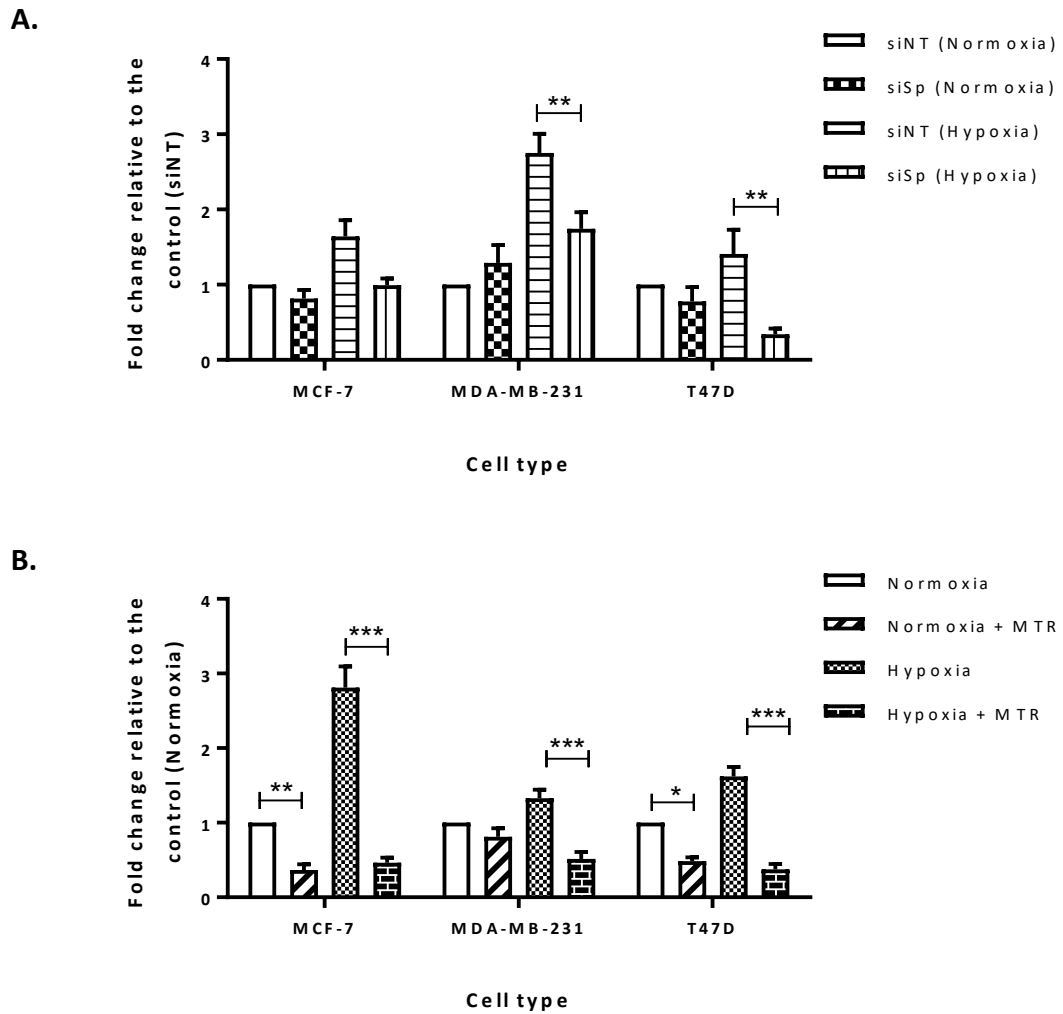


Figure 6.11: Effects of *Sp1* gene knockdown and Mithramycin A on *TPBG* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

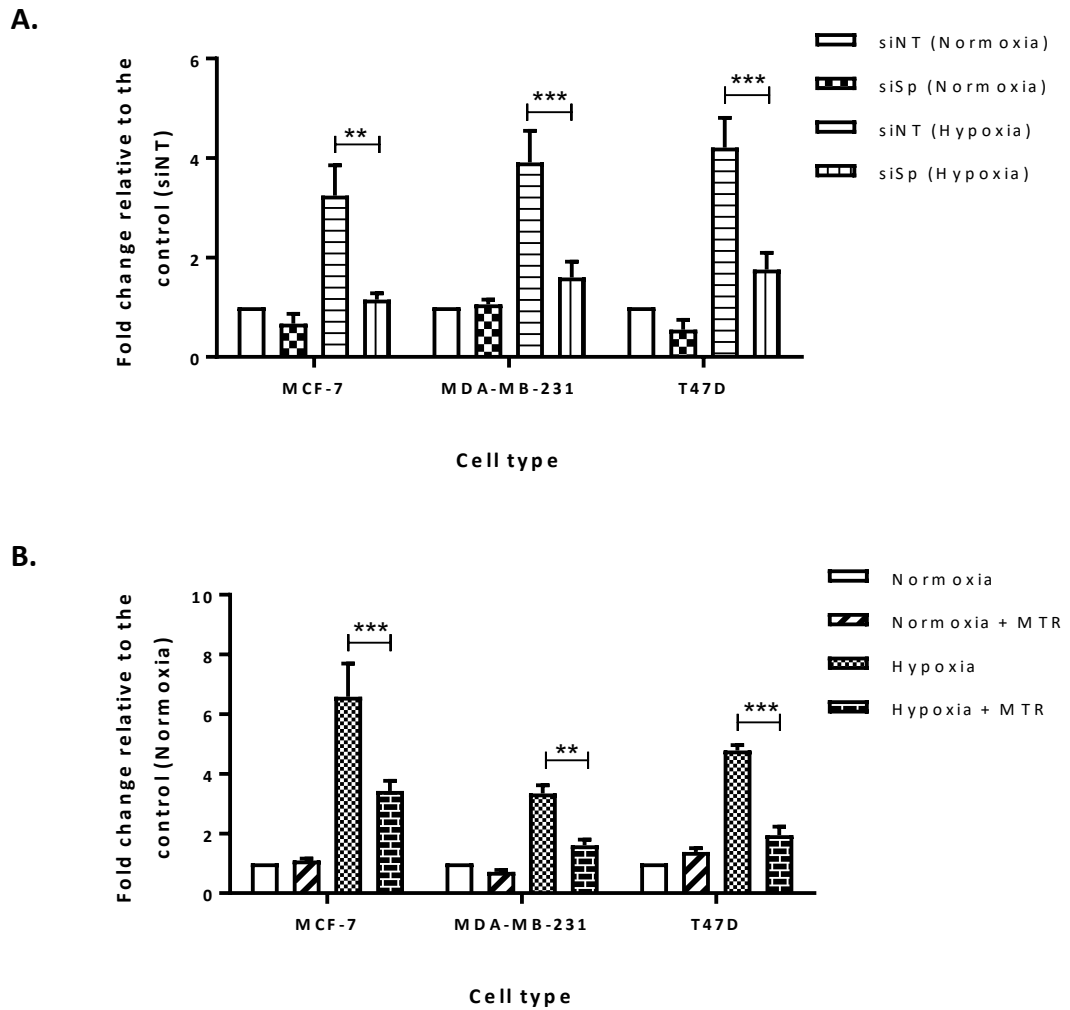


Figure 6.12: Effects of *Sp1* gene knockdown and Mithramycin A on *FUT11* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. **, P< 0.01; ***, P< 0.001.

8. Protein phosphatase 1, regulatory (inhibitor) subunit 3B (PPP1R3B)

Hypoxic induction of *PPP1R3B* gene decreased by *Sp1* gene knockdown in breast cell lines. A similar level of mRNA expression was reported under normoxic conditions in all three cell lines without any significant changes. Due to *Sp1* gene knockdown, mRNA expression levels were significantly decreased in MDA-MB-231 cells by 1.23 FC (+/- 0.56 SD) and 1.34 FC (+/- 0.50 SD) in T47D cells compared to the cells treated with siNT in hypoxia. A decrease of expression levels in MCF-7 cells were also detected (see panel A). Mithramycin A significantly decreases *PPP1R3B* expression levels in hypoxia. Under normoxic conditions, only MCF-7 cells showed a significant decrease of 0.63 FC (+/- 0.19 SD) due to Mithramycin A treatment. Hypoxic exposure increased the expression levels of *PPP1R3B* gene in all three cell lines whilst Mithramycin A treatment has significantly decreased mRNA expression levels in hypoxia compared to untreated samples. As evident in Figure 6.13 panel B, expression levels decreased by 0.85 FC (+/- 0.24 SD) in MCF-7, 0.48 FC (+/- 0.28 SD) in MDA-MB-231 cells and 0.85 FC (+/- 0.18 SD) in T47D cells.

9. Chemokine (C-C motif) ligand 28 (CCL28)

Expression of *CCL28* gene was induced in severe hypoxia in all three breast cancer cell lines (see Figure 6.14). Under the normoxic condition, *CCL28* expression was unchanged due to *Sp1* gene knockdown or Mithramycin A treatment. However, when cells treated with *Sp1* gene specific siRNA in hypoxia, mRNA levels decreased in all cell lines compared to the untreated control. Fold changes were detected as 0.90 FC (+/- 0.21 SD), 0.60 FC (+/- 0.17 SD) and 1.04 FC (+/- 0.39 SD) in MCF-7, MDA-MB-231 and T47D cells respectively (see panel A).

Mithramycin A inhibits hypoxic induction of *CCL28* in hypoxia. Expression levels of normoxic samples of all three cell lines remained without any significant changes. However, an increase of mRNA levels was visible due to Mithramycin A treatment in MDA-MB-231 cells. *CCL28* mRNA levels were significantly decreased due to Mithramycin A treatment in hypoxia in all cells lines. In particular order, 1.01 FC (+/- 0.41 SD), 2.07 FC (+/- 0.73 SD) and 1.16 FC (+/- 0.61 SD) were identified in MCF-7, MDA-MB-231 and T47D cell lines compared to untreated hypoxic samples (see panel B).

10. nuclear receptor subfamily 2, group F, member 2 (NR2F2)

Hypoxic induction of *NR2F2* gene was significantly repressed by *Sp1* gene knockdown in breast cancer cells. Under the normoxic condition, no significant changes were recorded due to *Sp1* gene knockdown (see Figure 6.15). However, hypoxic exposure increased mRNA expression in MCF-7 and MDA-MB-231 cells where no changes were observed in T47D cells. Knockdown of *Sp1* gene resulted significant decrease of mRNA levels in hypoxia where 1.55 FC (+/- 0.68 SD) in MCF-7, 1.12 FC (+/- 0.54 SD) in MDA-MB-231 and 0.98 FC (+/- 0.32 SD) in T47D cells were detected compared to the cells treated with non-targeting siRNA (siNT) (see panel A).

Mithramycin A inhibits mRNA expression of *NR2F2* gene in breast cancer cells. Under normoxic environment, mRNA levels of both MCF-7 and T47D cells decreased by 0.66 FC (+/- 0.18 SD) and 0.69 FC (+/- 0.23 SD) respectively. In contrast expression levels in MDA-MB-231 cells remained unchanged. Nevertheless, a significant reduction in mRNA levels was identified when cells were treated with Mithramycin A under hypoxia. Compared to the untreated hypoxic condition, mRNA levels decreased by 1.62 FC (+/- 0.25 SD) in MCF-7, 0.56 FC (+/- 0.83 SD) in MDA-MB-231 and 1.02 FC (+/- 0.16 SD) in T47D cells (see panel B).

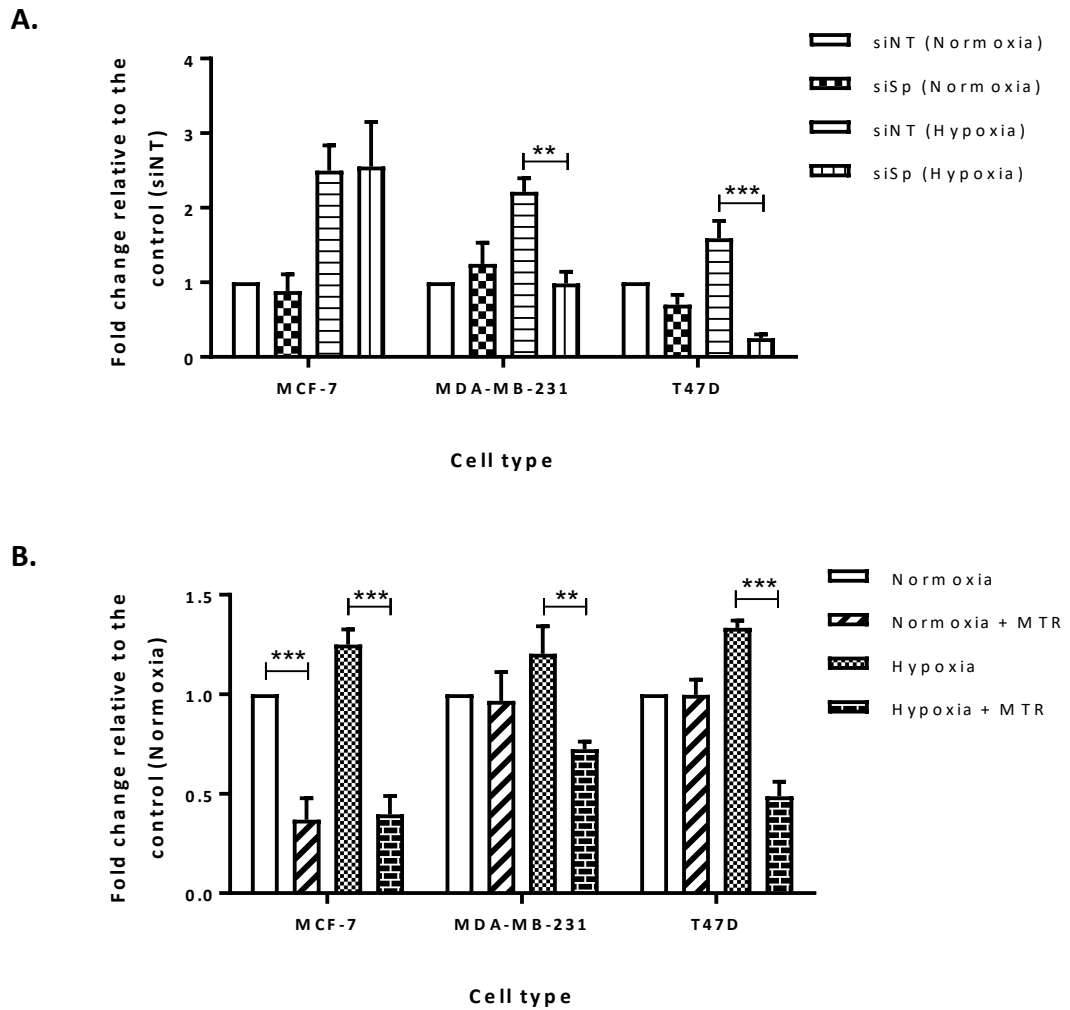


Figure 6.13: Effects of *Sp1* gene knockdown and Mithramycin A on *PPP1R3B* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

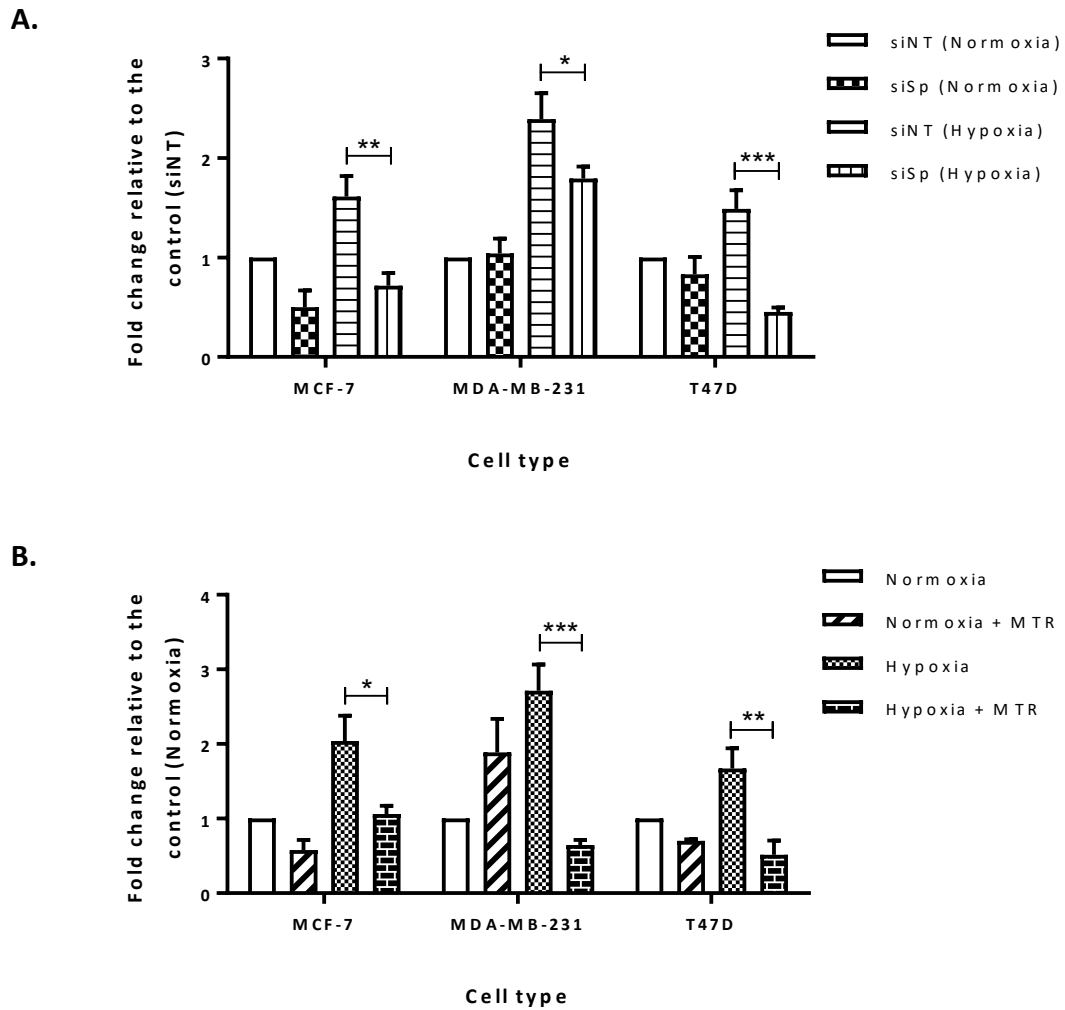


Figure 6.14: Effects of *Sp1* gene knockdown and Mithramycin A on *CCL28* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

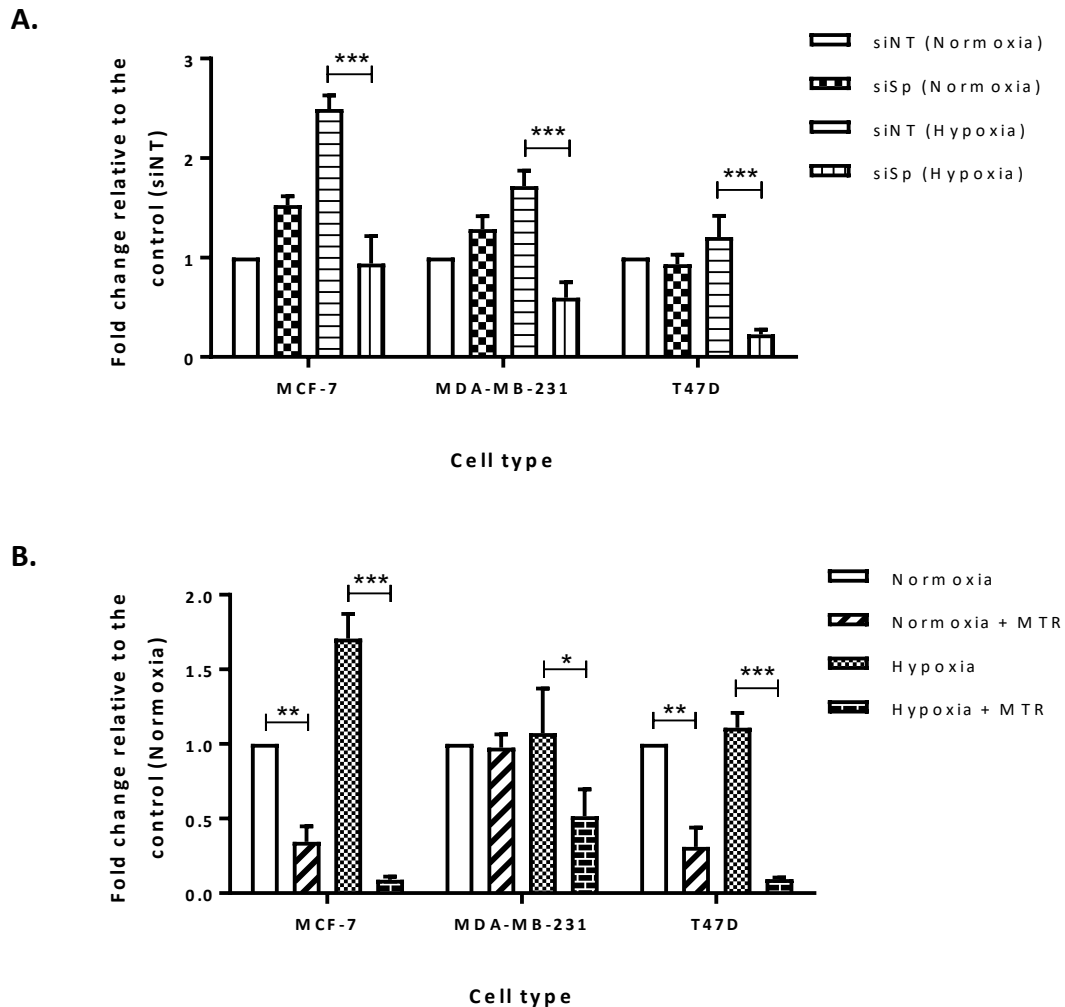


Figure 6.15: Effects of *Sp1* gene knockdown and Mithramycin A on *NR2F2* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

11. *Jun* oncogene (*JUN*)

Expression levels of *JUN* gene oncogene decreased due to *Sp1* gene knockdown in both hypoxic and normoxic condition (see Figure 6.16). Under normoxic conditions, decrease of mRNA levels of *Jun* oncogene was clearly visible in all three cell lines. Expression levels were significantly decreased in hypoxia by 1.13 FC (+/- 0.64 SD), 0.85 FC (+/- 0.31 SD) and 0.79 FC (+/- 0.27 SD), in MCF-7, MDA-MB-231 and T47D cells respectively (see panel A).

As evident from Figure 6.16, panel B, Mithramycin A significantly decreases *JUN* oncogene expression under hypoxic condition in all three cell lines. Under the normoxic condition, only MDA-MB-231 cells showed significant reduction of *JUN* expression level due to Mithramycin A treatment (0.52 FC (+/- 0.14SD)). However, compared to the untreated hypoxic condition, mRNA levels decreased by 1.23 FC (+/- 0.08 SD) in MCF-7, 1.13 FC (+/- 0.15 SD) in MDA-MB-231 and 0.92 FC (+/- 0.28 SD) in T47D cells due to Mithramycin A treatment.

12. *PDZ* and *LIM* domain 2 (*mystique*) (*PDLIM2*)

PDLIM2 gene was upregulated in the hypoxic environment where *Sp1* gene knockdown significantly decreases expression levels compared to the control sample (siNT). Fold changes were detected as 0.91 FC (+/- 0.20 SD) in MCF-7, 1.09 FC (+/- 0.40 SD) in MDA-MB-231 cells and 0.81 FC (+/- 0.15 SD) in T47D cells (see Figure 6.17, panel A). Similarly, Mithramycin A treatment decreased *PDLIM2* expression in all three cell lines. Under the normoxic conditions, mRNA levels decreased due to Mithramycin A and it was only significant in T47D cells (0.57 FC (+/- 0.14SD)). In hypoxic condition, all three cell lines exhibited a significant reduction of *PDLIM2* expression due to Mithramycin A treatment compare to the untreated samples. As can be seen in panel B, 1.22 FC (+/- 0.57SD) in MCF-7, 0.83 FC (+/- 0.11SD) in MDA-MB-231 and 1.26 FC (+/- 0.22SD) in T47D cells were recorded.

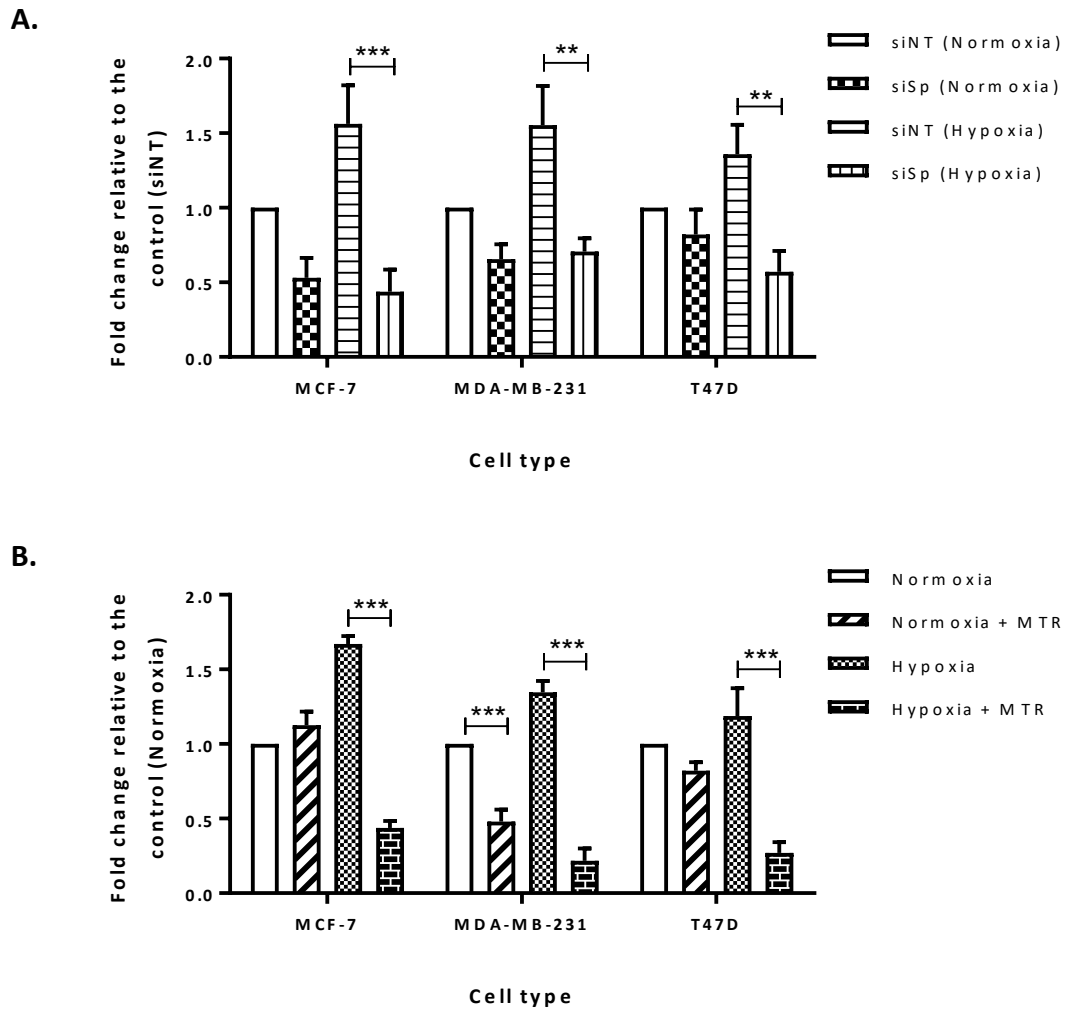


Figure 6.16: Effects of *Sp1* gene knockdown and Mithramycin A on *Jun oncogene* expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. **, P< 0.01; ***, P< 0.001.

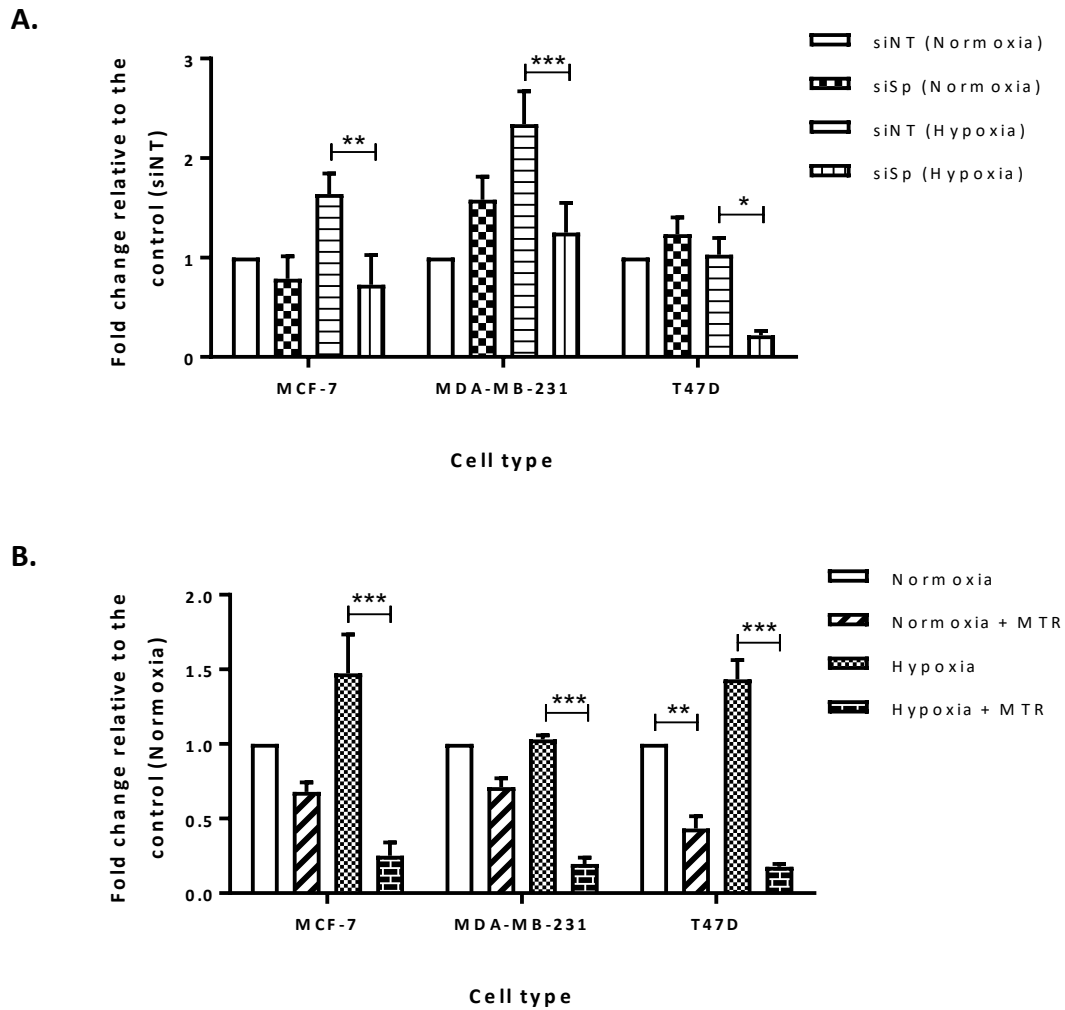


Figure 6.17: Effects of *Sp1* gene knockdown and Mithramycin A on *PDLIM2* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

13. solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (SLC11A2)

Sp1 gene specific knockdown by siRNA had no significant inhibitory action on *SLC11A2* gene expression in normoxic condition. Cells treated with siNT and siSp showed similar level of *SLC11A2* expression in normoxia. However, inhibition of *Sp1* gene by siRNA knockdown increased expression of *SLC11A2* gene in breast cancer cells under hypoxia. MCF-7, MDA-MB-231 and T47D cell lines followed a similar pattern of expressions, where under hypoxic conditions, significant increase of 0.92 FC (+/- 0.52SD) in MCF-7, 0.90 FC (+/- 0.35SD) in MDA-MB-231 and 0.80 FC (+/- 0.37 SD) in T47D cells were detected compared to the untreated hypoxic counterpart (siNT (see Figure 6.18, panel A).

SLC11A2 gene expression decreased in both MCF-7 and T47D cells due to Mithramycin A treatment under normoxic conditions. These changes were only significant in MCF-7 cells (0.49 FC (+/- 0.23SD)). Contrastingly, MDA-MB-231 cells showed a significant increase of 0.48 FC (+/- 0.13SD) under same experimental conditions. Nevertheless, all three cell lines behaved in a similar pattern after hypoxic exposure where significant level of reductions were observed due to Mithramycin A treatment. MCF-7, MDA-MB-231 and T47D cells showed 0.87 FC (+/- 0.20SD), 0.48 FC (+/- 0.09SD), 0.64 FC (+/- 0.40 SD) changes respectively (see panel B)

14. AT hook containing transcription factor 1; AT hook containing transcription factor 1 pseudogene (AHCTF1)

Expression of *AHCTF1* gene was unaffected by knockdown of *Sp1* gene under normoxic conditions. However, after hypoxic exposure slight increase in mRNA levels were observed in all three cell lines compared to the control (siNT). Despite changes were statistically insignificant (see Figure 6.19, panel A).

However, expression levels of *AHCTF1* gene were significantly reduced due to Mithramycin A treatment under hypoxic and normoxic condition in all three cell lines. MCF-7 cells showed 0.45 FC (+/- 0.09 SD) reduction in normoxic condition where it was 0.96 FC (+/- 0.65SD) after hypoxic exposure. MDA-MB-231 cells showed 0.74 FC (+/- 0.13 SD) change in normoxia and 0.66 FC (+/- 0.17 SD) change in hypoxic condition compare to the untreated samples.

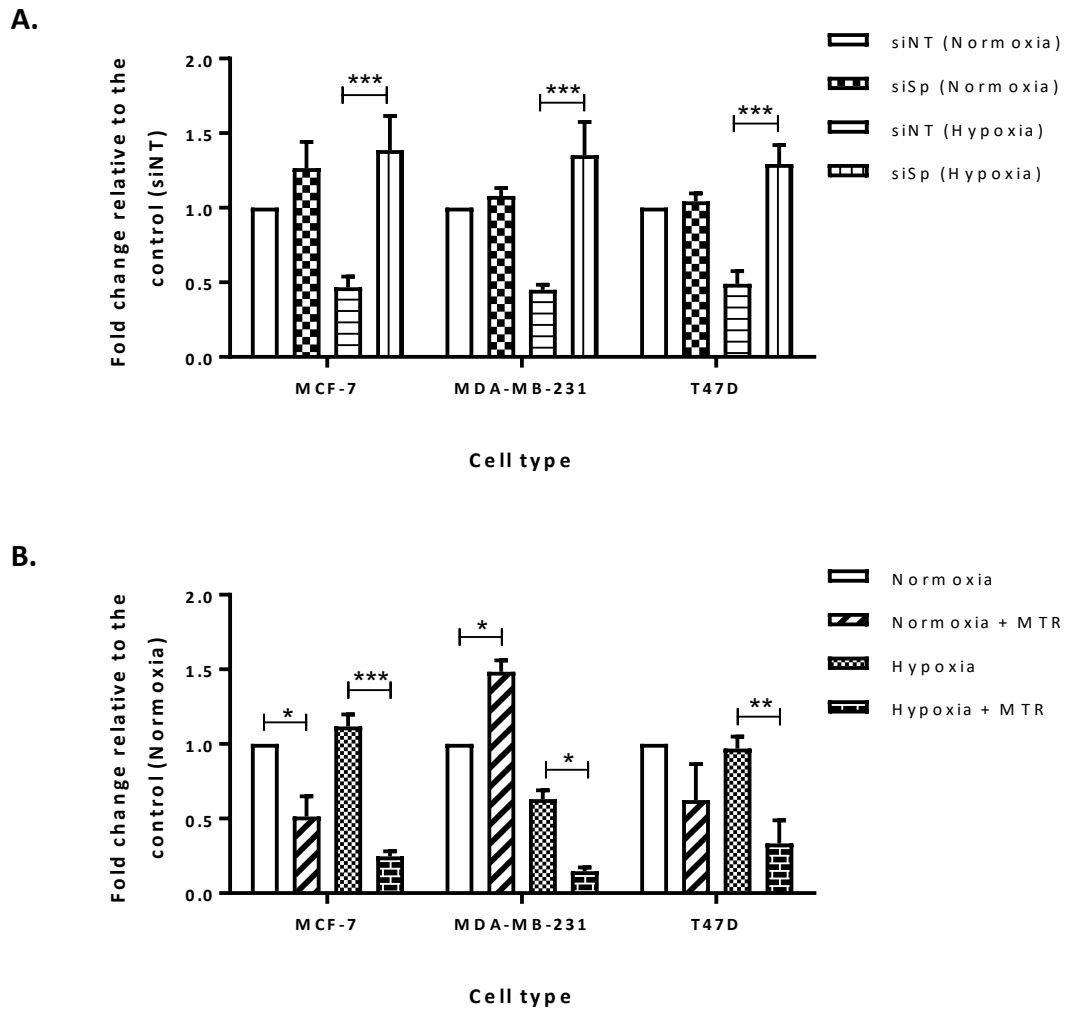


Figure 6.18: Effects of *Sp1* gene knockdown and Mithramycin A on *SLC11A2* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

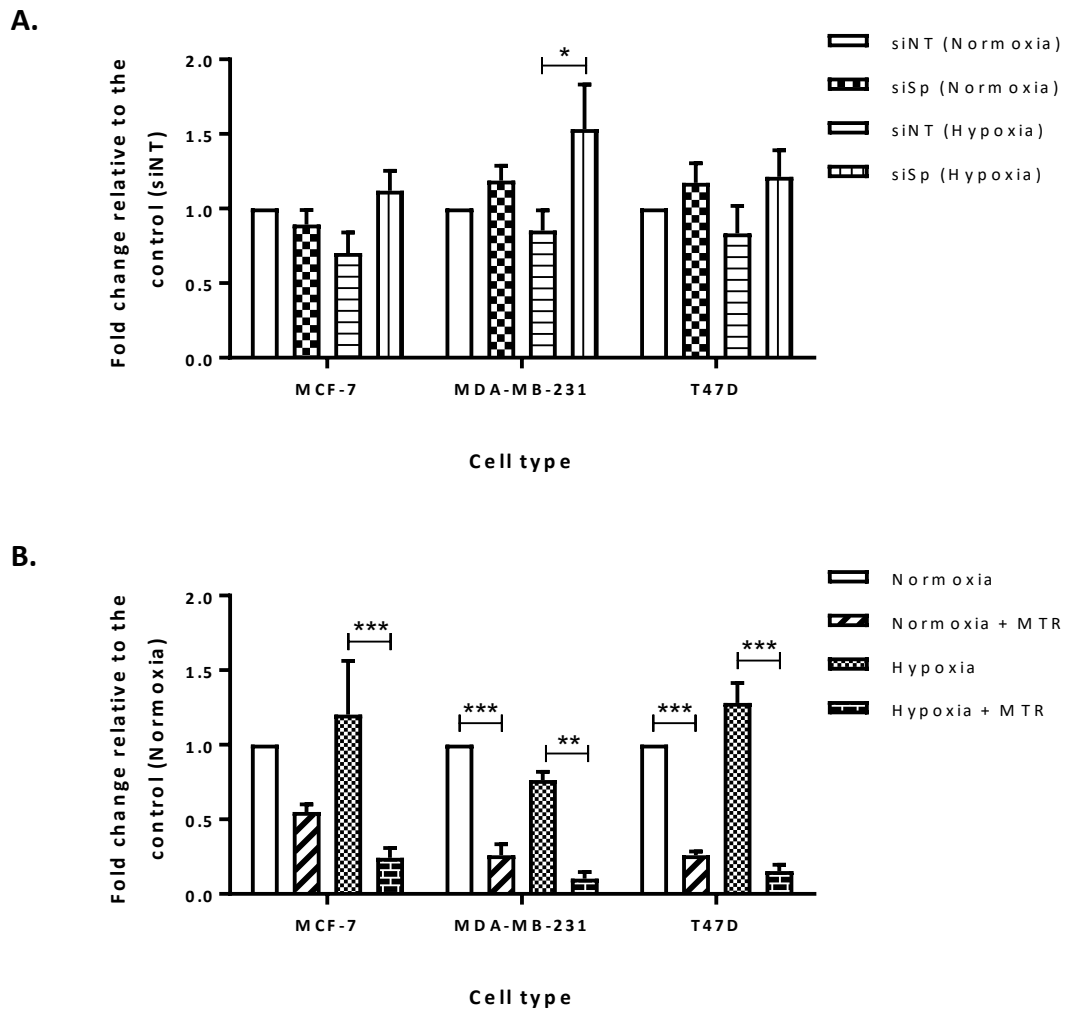


Figure 6.19: Effects of *Sp1* gene knockdown and Mithramycin A on *AHCTF1* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Similarly, 0.74 FC (+/- 0.04 SD) and 1.13 FC (+/-0.16 SD) changes were observed in T47D cells under normoxia and hypoxia respectively (see panel B).

15. Polyribonucleotide nucleotidyltransferase 1 (PNPT1)

Expression of *PNPT1* gene remained without any significant changes due to *Sp1* gene knockdown in normoxic conditions. Under hypoxic environment, all three cell lines showed slight upregulation of the gene compared to the hypoxic control (siNT) (see Figure 6.20, panel A).

Mithramycin A represses the expression of *PNPT1* gene under normoxic environment. The change was clearly visible in both MCF-7 and T47D cells and it was significant in MDA-MB-231 cells (0.48 FC (+/- 0.10 SD)). Mithramycin A treatment also further decrease the expression of *PNPT1* gene expression in all three cell lines. Expression levels decreased by 0.67 FC (+/- 0.18 SD) in MCF-7, 0.72 FC (+/- 0.37 SD) in MDA-MB-231 and 0.57 FC (+/- 0.04 SD) in T47D cells (see panel B).

16. Akirin 1 (AKIRIN1)

AKIRIN1 gene expression was unaffected upon *Sp1* gene knockdown in both normoxia and hypoxia (see Figure 6.21, panel A). Similarly, expression levels of *AKIRIN1* were unchanged in both MCF-7 and MDA-MB-231 cells after treatment of Mithramycin A in normoxic condition. However significant reduction was detected in T47D cell line 0.59 FC (+/- 0.09 SD). Expression levels significantly decreased compared to the untreated samples for all three cell lines after hypoxic exposure. Expression changes were recorded as 0.70 FC (+/- 0.10 SD), 0.47 FC (+/- 0.14 SD) and 0.98 FC (+/- 0.29 SD) for MCF-7, MDA-MB-231 and T47D cells respectively (see Figure 6.21, panel B).

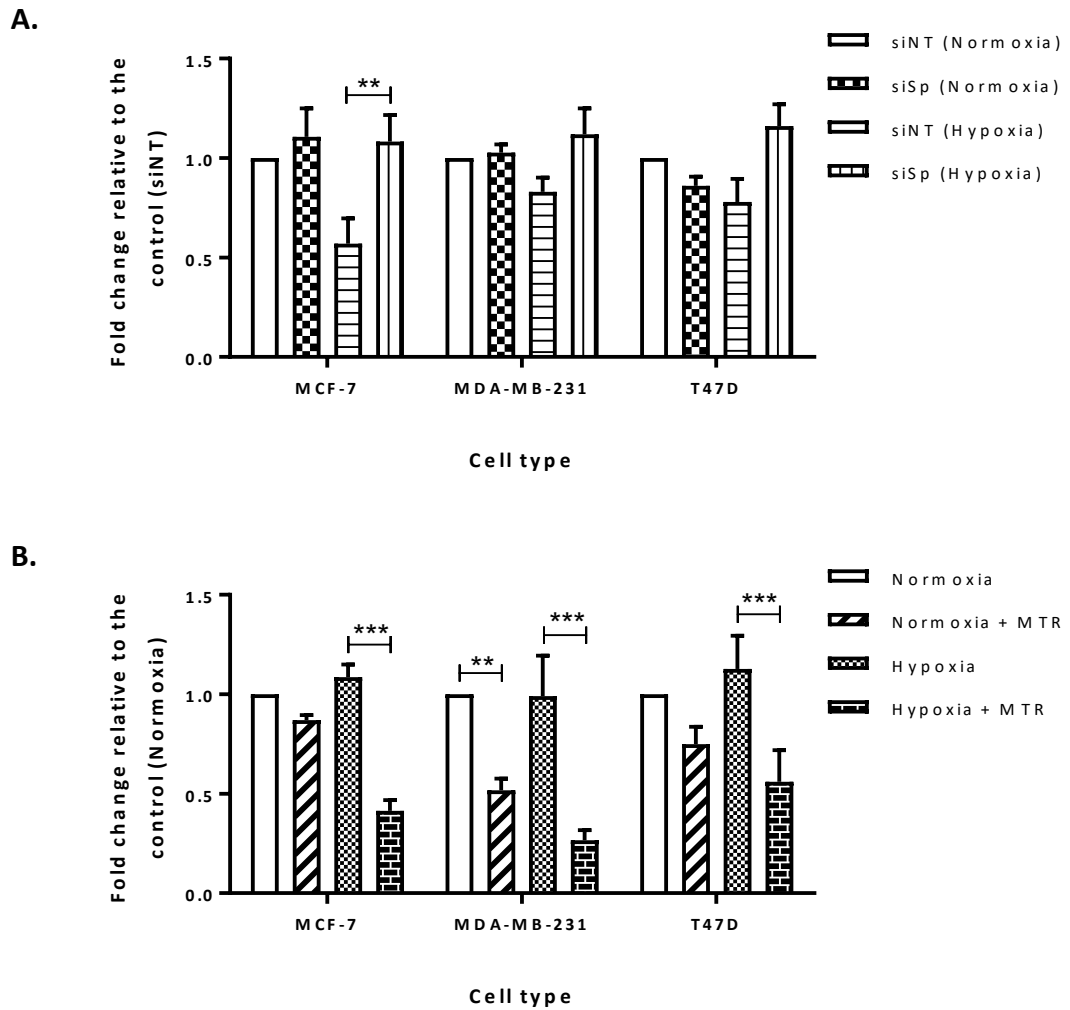


Figure 6.20: Effects of *Sp1* gene knockdown and Mithramycin A on *PNPT1* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. **, P < 0.01; ***, P < 0.001.

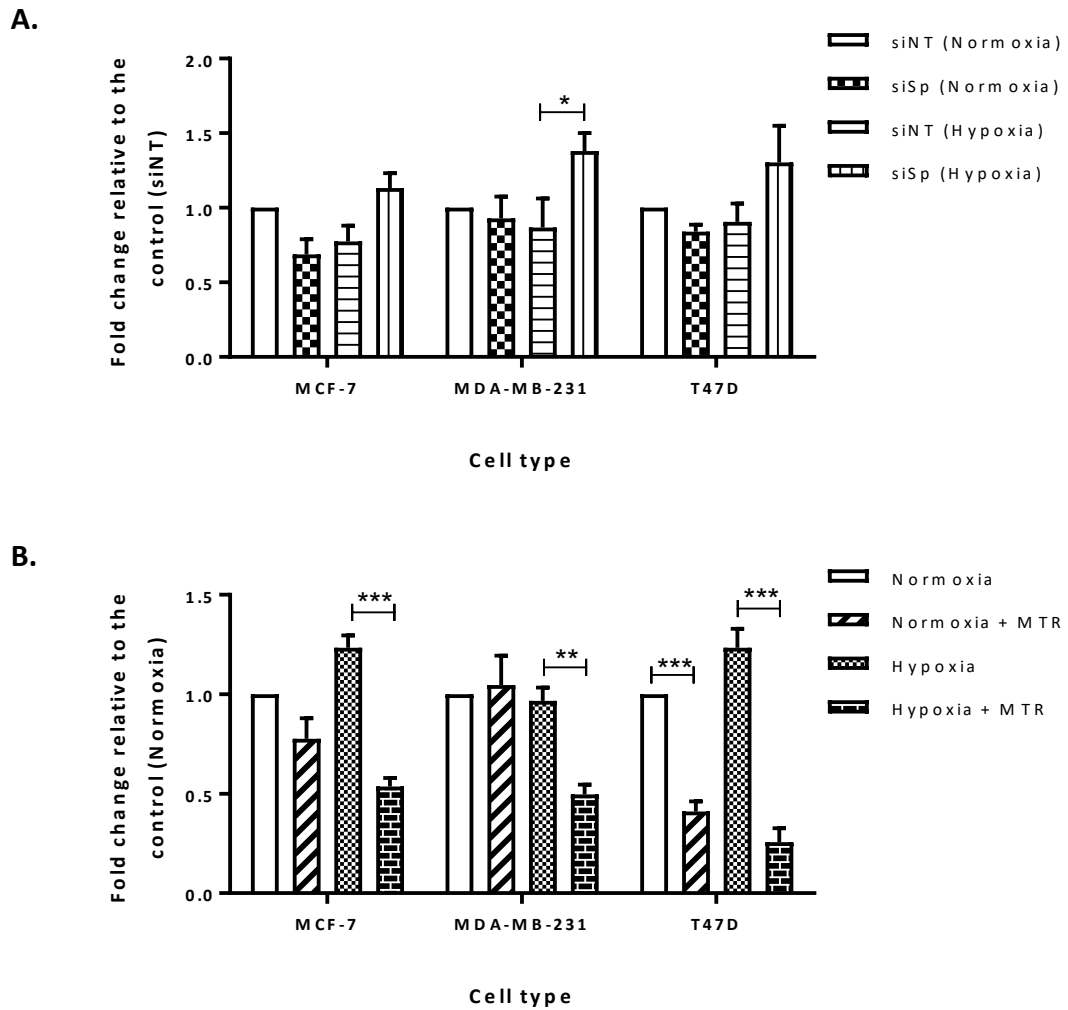


Figure 6.21: Effects of *Sp1* gene knockdown and Mithramycin A on *AKIRIN1* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

17. *Thrombomodulin (THBD)*

As seen in Figure 6.22, expression of *THBD* gene remained without any significant changes due to *Sp1* gene knockdown in normoxic conditions. Under hypoxic environment, all three cell lines showed decrease of *THBD* expression compared to the hypoxic control (siNT). mRNA levels of MCF-7 and MDA-MB-231 cells significantly increased by 0.69 FC (+/- 0.41 SD) and 0.58 FC (+/- 0.19 SD) respectively (see panel A). Mithramycin A treatment increased *THBD* gene expression in MCF-7 cells in normoxia whilst expression levels of both MDA-MB-231 and T47D cells decreased. MCF-7 cells showed 1.00 FC (+/- 0.71 SD) increase where 0.61 FC (+/- 0.16 SD) reduction was identified in MDA-MB-231 cells. Similarly, in hypoxic environment, no significant changes were observed except significant reduction in MCF-7 cells (1.08 FC (+/- 0.75 SD) (see panel B).

18. *TOX high mobility group box family member 2 (TOX2)*

As seen in Figure 6.23, *TOX2* gene expression decreased in all three cell lines as a hypoxic response. Knockdown of *Sp1* gene leads to significant upregulation of *TOX2* gene in both MCF-7 and MDA-MB-231 cells by 0.82 FC (+/- 0.53 SD) and 0.65 FC (+/- 0.34 SD) respectively. An increase in expression levels was also visible in T47D cells (see panel A). In normoxia, expression levels of *TOX2* gene remained unchanged after the treatment of siRNA.

In contrast, Mithramycin A treatment and hypoxic exposure exhibited cell type specific changes in *TOX2* gene expression. Under normoxia, MCF-7 cells showed significant upregulation of *TOX2* gene by 0.99 FC (+/- 0.27 SD) where 0.66 FC (+/- 0.21 SD) decrease was detected in MDA-MB-231 cells. After exposure to hypoxic stress and Mithramycin A, *TOX2* expression decreased in both MCF-7 and MDA-MB-231 cells by 0.86 FC (+/- 0.20 SD) and 0.42 FC (+/- 0.21 SD) respectively. No significant changes were identified in T47D cells under any conditions (see panel B).

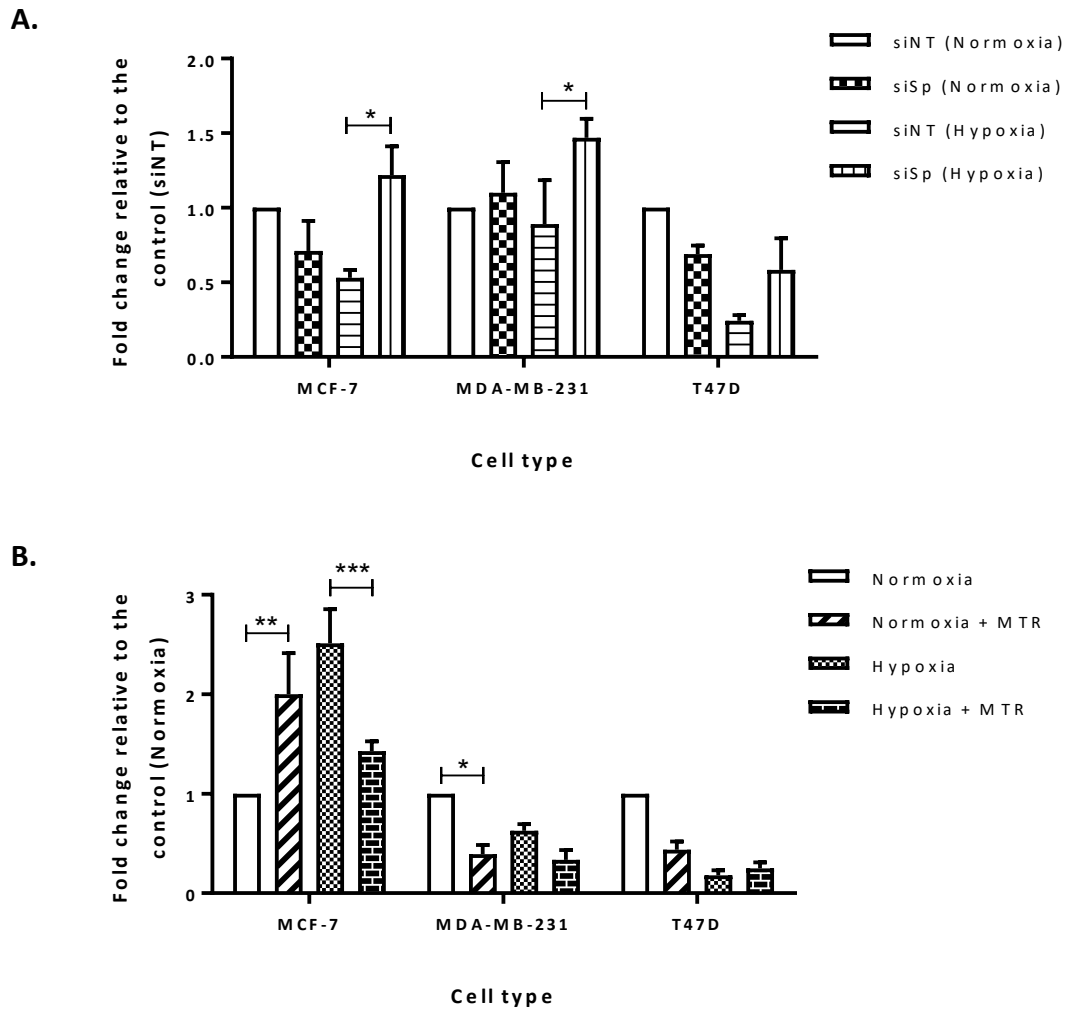


Figure 6.22: Effects of *Sp1* gene knockdown and Mithramycin A on *THBD* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

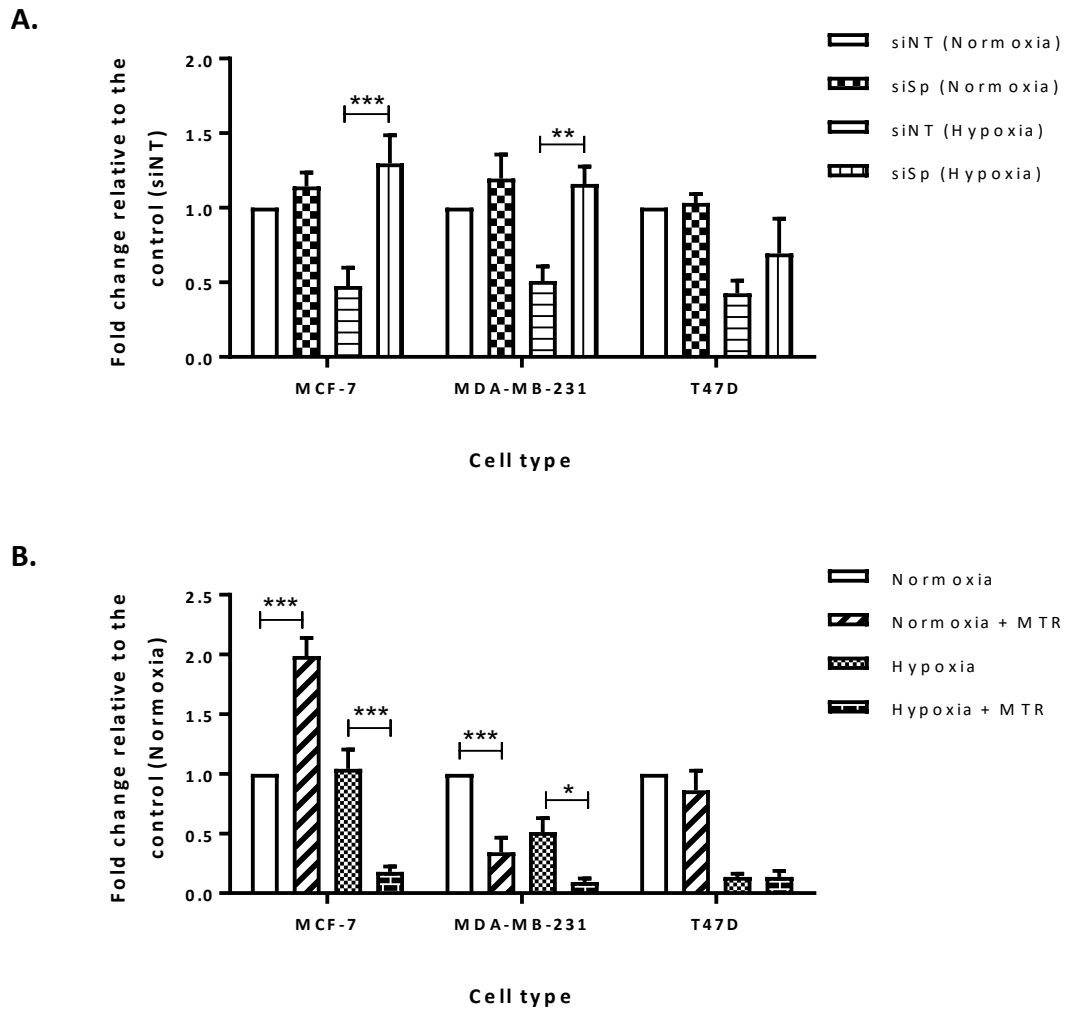


Figure 6.23: Effects of *Sp1* gene knockdown and Mithramycin A on *TOX2* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

19. Tumour necrosis factor receptor superfamily, member 21 (TNFRSF21)

When cell exposed to severe hypoxia, expression levels of *TNFRSF21* decreased as a response to hypoxic stress in all breast cancer cell lines used in this experimental process (see Figure 6.24). However, inhibition of *Sp1* gene repressed hypoxic response of *TNFRSF21* gene in breast cancer cell lines whilst restoring expression levels to basal levels. Under normoxia, no detectable changes were identified for any cell line (see panel A). Transcript levels of *TNFRSF21* gene increased by 0.75 FC (+/- 0.28 SD) in MCF-7, 0.60 FC (+/- 0.38 SD) in MDA-MB-231 and 0.52 FC (+/- 0.22 SD) in T47D cells compared to the untreated hypoxic sample (see panel A). In contrast, expression of *TNFRSF21* gene decreased due to Mithramycin A treatment under both normoxic and hypoxic conditions. Under normoxia, significant reduction was observed in MDA-MB-231 cells (0.35 FC (+/- 0.06 SD) and T47D cells 0.53 FC (+/- 0.06 SD). Similarly, under hypoxic condition, when cells were treated Mithramycin A, transcript levels of *TNFRSF21* gene decreased by 0.69 FC (+/- 0.12 SD) in MCF-7, 0.37 FC (+/- 0.04 SD) in MDA-MB-231 and 0.54 FC (+/- 0.12 SD) in T47D cells compared to the untreated sample (see panel B).

20. Cold inducible RNA binding protein (CIRBP)

Breast cancer cell lines cultured in hypoxia showed downregulation of *CIRBP* gene expression (see Figure 6.25). When cell treated with *Sp1* gene specific siRNA, *CIRBP* mRNA levels increased in hypoxia whereas expression levels in normoxic samples remained unchanged. In hypoxia, mRNA levels of *CIRBP* gene increased by 0.67 FC (+/- 0.27 SD) in T47D cells compared to the untreated counterpart (siNT) (see panel A). In hypoxia, *CIRBP* gene expression level significantly decreased, when MCF-7 and T47D cells treated with Mithramycin A (see Panel B). However, only the MCF-7 cells showed significant change which was 0.83 FC (+/- 0.43 SD). In contrast MDA-MB-231 cells showed up-regulation *CIRBP* expression in normoxia against the control (0.87 FC (+/- 0.17 SD) (see panel B).

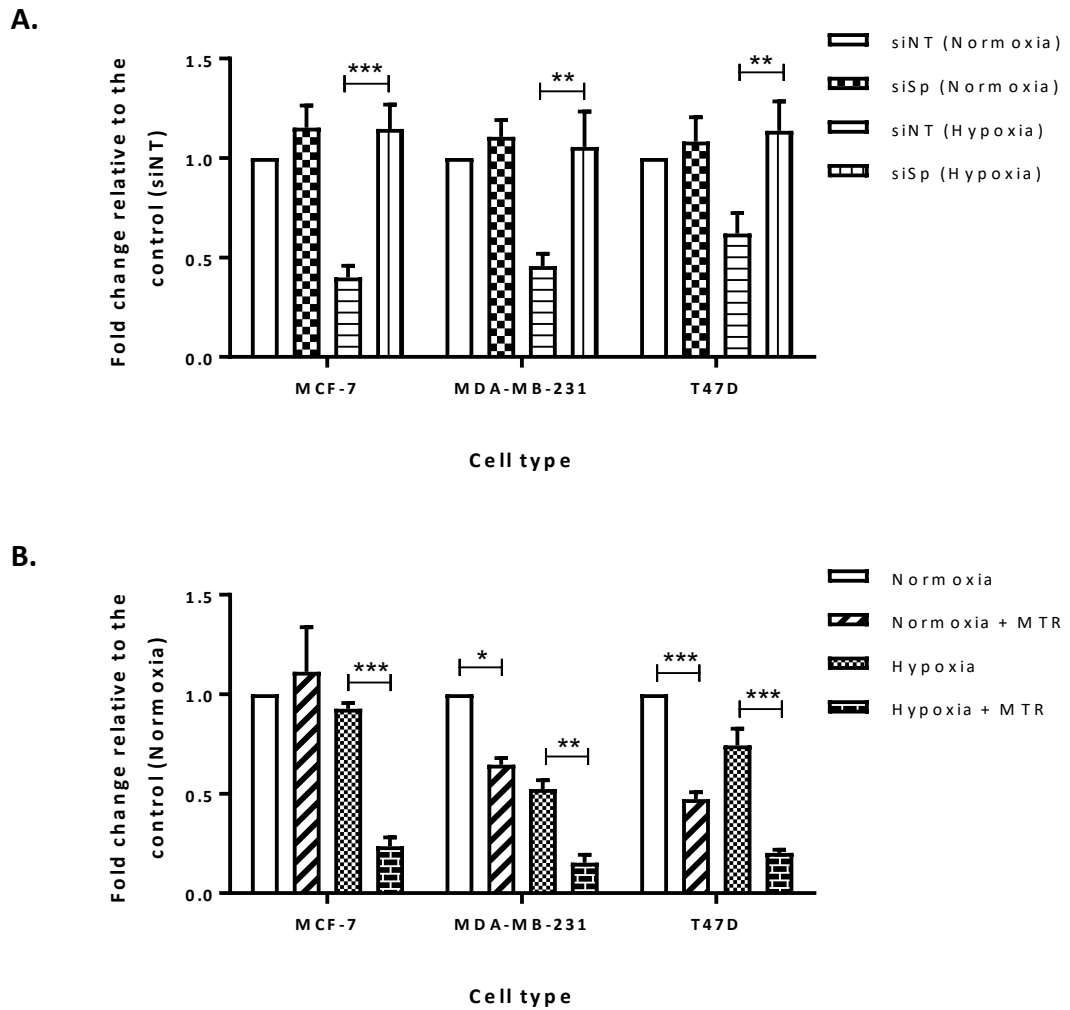


Figure 6.24: Effects of *Sp1* gene knockdown and Mithramycin A on *TNFRSF21* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O₂ for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O₂ parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, P< 0.05; **, P< 0.01; ***, P< 0.001.

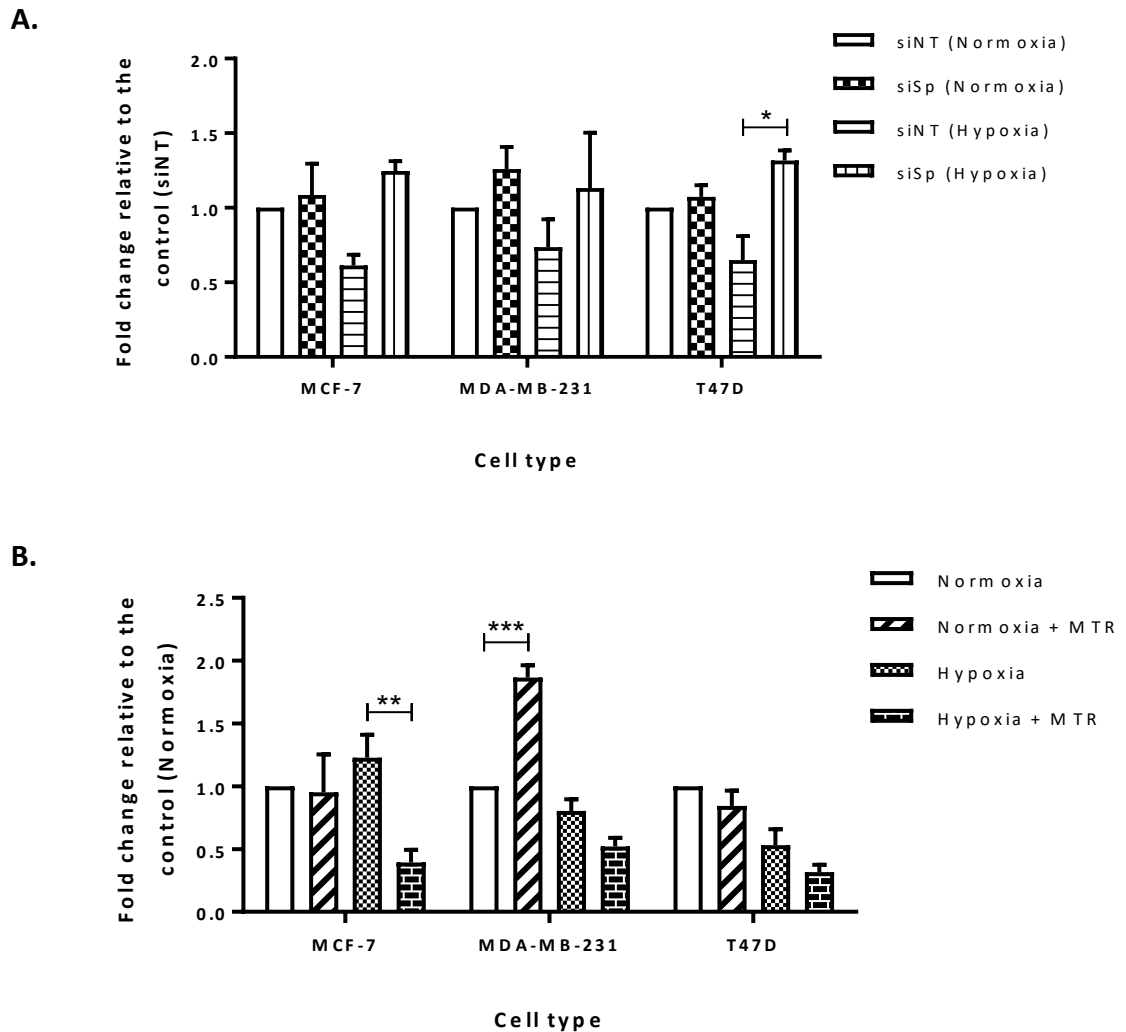


Figure 6.25: Effects of *Sp1* gene knockdown and Mithramycin A on *CIRBP* gene expression in breast cancer cells under severe hypoxia

Breast cancer cells (MCF-7, MDA-MB-231 and T47D) were treated with siRNA to knockdown *Sp1* gene (siSp1) or control non-targeting siRNA (siNT) (panel A) or 1 μ M Mithramycin A (MTR) (panel B) followed by hypoxic exposure at 0.5% (v/v) O_2 for 12 hours. Normoxia counterparts were incubated at 20% (v/v) O_2 parallel to hypoxic samples. mRNA was extracted and transcript levels were analysed by TaqMan qPCR (as described in section 2.5). All the results were normalised to housekeeping gene *B2M*. Results are expressed as fold change relative to normoxic sample. n=3 experiments: Error bars represents +/- SEM: Statistical analysis by Two-way ANOVA with Tukey's post-hoc correction. Significance of the siRNA/Mithramycin A treated samples were compared against the untreated samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 6.1: Expression changes (mean difference) of hypoxia responsive genes in response to *Sp1* gene knockdown and Mithramycin A treatment in MCF-7 cells

Gene ID	<i>Sp1</i> gene knockdown (siSp1)				Mithramycin A treatment			
	N	p-value	H	p-value	N	p-value	H	p-value
<i>PFKFB4</i>	-0.27	>0.9999	-6.17	0.0088	0.38	0.9776	-14.33	<0.0001
<i>ARTN</i>	-0.35	0.5498	-0.47	0.3048	+2.98	<0.0001	+3.81	<0.0001
<i>VEGFA</i>	+0.04	0.9994	-2.00	0.0002	+0.24	0.9177	-1.79	0.0008
<i>CCNG2</i>	-0.16	0.9877	-2.43	0.0007	-0.76	0.1346	-2.94	<0.0001
<i>IRS2</i>	+0.18	0.8993	+0.59	0.1514	-0.16	0.2178	-0.77	<0.0001
<i>TPBG</i>	-0.18	0.9075	-0.65	0.1191	-0.63	0.0073	-2.35	<0.0001
<i>FUT11</i>	-0.33	0.9183	-2.09	0.0038	+0.10	0.9975	-3.15	<0.0001
<i>PPP1R3B</i>	-0.12	0.9793	+0.06	0.9975	-0.63	0.0001	-0.85	<0.0001
<i>CCL28</i>	-0.50	0.1225	-0.90	0.0026	-0.42	0.6185	-0.98	0.0474
<i>NR2F2</i>	+0.53	0.0902	-1.55	<0.0001	-0.66	0.0140	-1.62	<0.0001
<i>JUN</i>	-0.47	0.1842	-1.13	0.0004	+0.13	0.5523	-1.23	<0.0001
<i>PDLIM2</i>	-0.21	0.8186	-0.91	0.0077	-0.32	0.1232	-1.22	<0.0001
<i>SLC11A2</i>	+0.27	0.4086	+0.92	0.0002	-0.49	0.0172	-0.87	<0.0001
<i>AHCTF1</i>	-0.11	0.9634	+0.42	0.2791	-0.45	0.0562	-0.96	<0.0001
<i>PNPT1</i>	+0.11	0.8878	+0.51	0.0134	-0.13	0.6504	-0.67	<0.0001
<i>AKIRIN1</i>	-0.31	0.3741	+0.36	0.2615	-0.22	0.2013	-0.70	<0.0001
<i>THBD</i>	-0.29	0.5359	+0.69	0.0211	+1.00	0.0019	-1.08	0.0009
<i>TOX2</i>	+0.14	0.8609	+0.82	0.0015	+0.97	<0.0001	-0.86	<0.0001
<i>TNFRSF21</i>	+0.15	0.7474	+0.75	0.0006	+0.11	0.7233	-0.69	<0.0001
<i>CIRBP</i>	+0.08	0.9810	+0.63	0.0568	-0.05	0.9951	-0.83	0.0025

(Abbreviations: N: Normoxia, H: Hypoxia)

Table 6.2: Expression changes (mean difference) of hypoxia responsive genes in response to *Sp1* gene knockdown and Mithramycin A treatment in MDA-MB-231 cells

Gene ID	<i>Sp1</i> gene knockdown (siSp1)				Mithramycin A treatment			
	N	p-value	H	p-value	N	p-value	H	p-value
<i>PFKFB4</i>	+0.23	>0.9999	-2.68	0.7274	-0.63	0.9127	-4.94	0.0004
<i>ARTN</i>	-0.14	0.9455	-0.42	0.3982	+1.74	0.0165	+3.59	<0.0001
<i>VEGFA</i>	+0.03	0.9998	-1.2	0.0203	+0.11	0.9909	-4.20	<0.0001
<i>CCNG2</i>	-0.26	0.9522	-1.47	0.0415	-0.36	0.7023	-1.75	0.0003
<i>IRS2</i>	+0.13	0.9575	+0.35	0.5710	-0.35	0.0023	-0.81	<0.0001
<i>TPBG</i>	+0.29	0.7186	-1.01	0.0086	-0.19	0.6911	-0.81	0.0007
<i>FUT11</i>	+0.06	0.9995	-2.32	0.0015	-0.28	0.9431	-1.74	0.0145
<i>PPP1R3B</i>	+0.25	0.8428	-1.23	0.0035	-0.03	0.9906	-0.48	0.0024
<i>CCL28</i>	+0.04	0.9975	-0.60	0.0516	+0.89	0.0792	-2.07	<0.0001
<i>NR2F2</i>	+0.29	0.5316	-1.12	0.0002	-0.02	0.9993	-0.56	0.0414
<i>JUN</i>	-0.34	0.4307	-0.85	0.0063	-0.52	0.0002	-1.13	<0.0001
<i>PDLIM2</i>	+0.58	0.1185	-1.09	0.0016	-0.29	0.1809	-0.83	<0.0001
<i>SLC11A2</i>	+0.08	0.9632	+0.90	0.0002	+0.48	0.0181	-0.48	0.0181
<i>AHCTF1</i>	+0.19	0.8379	+0.68	0.0327	-0.74	0.0013	-0.66	0.0037
<i>PNPT1</i>	+0.03	0.9978	+0.29	0.2401	-0.48	0.0019	-0.72	<0.0001
<i>AKIRIN1</i>	-0.07	0.9821	+0.51	0.0624	+0.05	0.9722	-0.47	0.0020
<i>THBD</i>	+0.1	0.9646	+0.58	0.0604	-0.61	0.0699	-0.29	0.5932
<i>TOX2</i>	+0.1	0.7086	+0.65	0.0111	-0.66	0.0009	-0.42	0.0339
<i>TNFRSF21</i>	+0.11	0.8955	+0.6	0.0048	-0.35	0.0201	-0.37	0.0145
<i>CIRBP</i>	+0.26	0.6741	+0.40	0.3379	+0.87	0.0017	-0.28	0.4896

(Abbreviations: N: Normoxia, H: Hypoxia)

Table 6.3: Expression changes (mean difference) of hypoxia responsive genes in response to *Sp1* gene knockdown and Mithramycin A treatment in T47D cells

Gene ID	<i>Sp1</i> gene knockdown (siSp1)				Mithramycin A treatment			
	N	p-value	H	p-value	N	p-value	H	p-value
<i>PFKFB4</i>	+0.40	>0.9999	-4.10	0.1357	+0.06	>0.9999	-4.27	0.0016
<i>ARTN</i>	-0.08	0.9883	+0.13	0.9613	+4.70	<0.0001	+6.77	<0.0001
<i>VEGFA</i>	-0.1	0.9927	-1.55	0.0026	+0.25	0.9115	-2.51	<0.0001
<i>CCNG2</i>	+0.08	0.9987	-2.34	0.0010	-0.30	0.7972	-2.29	<0.0001
<i>IRS2</i>	+0.73	0.0567	-0.06	0.9950	-0.45	0.0001	-0.29	0.0095
<i>TPBG</i>	-0.22	0.8468	-1.07	0.0052	-0.51	0.0326	-1.25	<0.0001
<i>FUT11</i>	-0.45	0.8168	-2.45	0.0009	+0.38	0.8732	-2.84	0.0001
<i>PPP1R3B</i>	-0.3	0.7507	-1.34	0.0015	-0.01	>0.9999	-0.85	<0.0001
<i>CCL28</i>	-0.17	0.8532	-1.04	0.0006	-0.30	0.8218	-1.16	0.0157
<i>NR2F2</i>	-0.07	0.9884	-0.98	0.0010	-0.69	0.0096	-1.02	0.0002
<i>JUN</i>	-0.18	0.8479	-0.79	0.0108	-0.18	0.2626	-1.0	<0.0001
<i>PDLIM2</i>	+0.23	0.7761	-0.81	0.0184	-0.57	0.0030	-1.26	<0.0001
<i>SLC11A2</i>	+0.04	0.9937	+0.80	0.0008	-0.38	0.0792	-0.64	0.0019
<i>AHCTF1</i>	+0.17	0.8651	+0.38	0.3539	-0.74	0.0013	-1.13	<0.0001
<i>PNPT1</i>	-0.14	0.7812	+0.38	0.0829	-0.25	0.1457	-0.57	0.0004
<i>AKIRIN1</i>	-0.16	0.8319	+0.40	0.1917	-0.59	0.0002	-0.98	<0.0001
<i>THBD</i>	-0.31	0.4810	+0.34	0.4032	-0.56	0.1001	+0.07	0.9897
<i>TOX2</i>	+0.03	0.9978	+0.27	0.4818	-0.14	0.7611	-0.0	>0.9999
<i>TNFRSF21</i>	+0.08	0.9461	+0.52	0.0156	-0.53	0.0006	-0.54	0.0005
<i>CIRBP</i>	+0.07	0.9883	+0.67	0.0425	-0.16	0.8541	-0.21	0.7014

(Abbreviations: N: Normoxia, H: Hypoxia)

6.3. Discussion

This chapter aimed to examine the effect of Sp gene/protein inhibition on hypoxia responsive genes in selected breast cancer cell lines. For the investigation, MCF-7, MDA-MB-231 and T47D cells were reverse transfected with specific siRNA for *Sp1* gene. Once transfected with siRNA, cells were exposed to severe hypoxia (0.5% (v/v) O₂) and expression patterns of selected genes were analysed using qPCR analysis. Fold changes were calculated compared to the normoxia and non-targeting siRNA (siNT) transfected counterparts. Furthermore, inhibitory effects of Sp protein binding were investigated using specific Sp transcription factor inhibitor Mithramycin A. Cell lines were treated with 1 µM Mithramycin A and exposed to severe hypoxia prior to fold change analysis by qPCR.

Following is a summary of key findings in relation to key objectives of the chapter:

- Effect of inhibition of *Sp1* gene on expression of hypoxia responsive genes?

Twenty genes, previously identified to be hypoxia responsive were subjected to this investigation. Specific inhibition of *Sp1* gene by siRNA transfection repressed the hypoxic induction of those genes under hypoxia. However, there were a few genes, the exemption of which did not change to due to *Sp1* gene inhibition.

- Effect of Sp transcription factor inhibitor Mithramycin A on expression of hypoxia responsive genes?

Under hypoxic environment, Mithramycin A treatment exhibited inhibition of hypoxia responsive gene regardless of their expression patterns. Under the hypoxic environment, hypoxic induction of the genes was completely abolished by Mithramycin A treatment.

6.3.1. siRNA reverse transfection for inhibition of specific gene targets

The effect of *Sp1* gene knockdowns on the expression levels of hypoxia responsive genes was investigated to identify the Sp1 targets and their dependency on Sp mediated transcription. Knockdown efficiencies were determined by analysing mRNA levels of *Sp1* gene after transfection which were significantly reduced in all the cell lines (Figure 6.1). Consequently, it was hypothesised that inhibition of *Sp1* gene may in turn affect the expression levels of other Sp genes and target proteins.

Since Sp family members have exhibited synergetic gene regulation, specially Sp1 and Sp3, the effect of Sp silencing on other Sp family members (*Sp3* and *Sp4*) was also investigated (Figure 6.2 and 6.3) (Zhang et al., 2003; Li et al., 2004; Zhao et al., 2012). mRNA levels of *Sp3* gene remained without any significant changes in all three breast cancer cell lines tested in this study. Since Sp3 protein is capable of repressing the Sp1 activity, inhibition of Sp1 may not affect the transcription of *Sp3* genes (Hagen et al., 1994). On the other hand, several studies have shown that Sp3 protein is capable of regulating Sp1 mediated genes although Sp3 is a weaker transactivator compared to the Sp1 protein (Pagliuca et al., 2000; Li et al., 2004). Therefore, levels of Sp3 may remain unchanged when *Sp1* gene is inhibited. Interestingly, *Sp4* gene showed up-regulation when *Sp1* gene was inhibited under normoxic and hypoxic environments. Only a few studies have focused on identification of the correlation between Sp1 and Sp4 proteins. However, these results suggest that decrease of Sp1 proteins may affect the expression of *Sp4* genes. Since *Sp1* gene regulation is believed to be regulated by Sp1 proteins itself alongside with other proteins by loop mechanisms, transactivation of Sp family members might also be governed by Sp1 proteins (Nicolas et al., 2003). Expression patterns of hypoxia markers *CA9* and *GLUT1* were also investigated alongside with other genes (see Figure 6.4 and 6.6). Interestingly, both *CA9* and *GLUT1* expression were further increased due to the *Sp1* knockdown in hypoxia environment suggesting the possible involvement of Sp proteins in regulation of these proteins. Studies have shown that expression of *CA9* is mainly governed by HIF mediated transcription where Sp1 and Sp3 proteins are also required in mild and severe hypoxia (Kaluz et al., 2003; Kaluz et al., 2009). Similarly, inhibition of *Sp1* further increased the expression of *GLUT1* expression in hypoxia suggesting the involvement of Sp proteins in transcriptional regulation of *GLUT1* (Vinals et al., 1997). Contrary to Sp1 protein action, studies have shown that Sp3 protein is capable of repressing the expression (Fandos et al., 1999). Since *GLUT1* and *CA9* promoter sites contain Sp consensus binding sites, the hypoxic response of these two genes may also be regulated by Sp proteins alternative to HIF protein (Kaluz et al., 2003; Hwang & Ismail-Beigi, 2006). Genes subjected to this study were previously identified with Sp1 binding sites in their promoters (see chapter 3). It was hypothesised that Sp1 transcription factors play a key role in their transcription. Inhibition of Sp mediated transcription may lead to the

suppression of hypoxic induction of these genes. Results presented in this chapter further confirm the hypoxic induction of selected genes which was presented in chapter 3. *Sp1* gene knockdown showed significant impact on the expression changes of genes particularly in hypoxia. Expression of all the genes which showed upregulation under hypoxia showed decreased mRNA levels due to the *Sp1* gene knockdown with some exceptions. It was clearly visible that inhibition of *Sp1* gene had minimal effect on basal transcription level of genes where no significant changes were observed under the normoxic environment such as *PFKFB4* gene. Similarly, genes which were identified as down-regulating genes under the hypoxic environment exhibited an increase in expression in all three cell lines such as *SLC11A2* gene. A significant increase in expression levels was detected compared to the non-transfected samples of all three cell lines with some exceptions. In addition, gene knockdown had no significant effect under normoxic condition where both treated and untreated samples showed a similar level of expression for all the genes.

These results suggest the dependency of identified hypoxia responsive genes on Sp mediated transcription. However, it was evident that under the normoxic condition, the effect of *Sp1* gene knockdown has minimal effect suggesting that basal transcription of these genes may not entirely depend on Sp mediated transcription. Since Sp proteins are known to undergo vast variety of post-translational modifications, it is highly possible that hypoxic exposure may lead to activity changes of Sp1 proteins which in turn enable them to regulate hypoxia inducible genes (Waby et al., 2008).

6.3.2. Mithramycin A as a hypoxia responsive gene inhibitor

A selective Sp1 protein inhibitor, Mithramycin A has been widely tested in several cancer cell lines including breast cancers. Functional role of Mithramycin A has been investigated in numerous genes with GC-rich promoter sequences. In this study, Mithramycin A was selected to inhibit Sp mediated gene transcription of hypoxia responsive genes in breast cancer cell lines. Experimental conditions (concentration, duration of the treatment) were carefully chosen to minimise toxicity and cells were visibly checked for any cell death during the experiment. In addition, β actin levels were also monitored for any changes in cellular integrity.

Results presented in this chapter clearly demonstrate the inhibitory effect of Mithramycin A on expression of hypoxia responsive genes. Hypoxia markers *CA9* and *GLUT1* were induced after exposure to 0.5% (v/v) O₂ hypoxia in all their cell lines. Interestingly this induction was inhibited by Mithramycin A treatment where expression levels returned to basal levels (Figures 6.5 and 6.7). As previously mentioned these results suggest the dependent of *CA9* and *GLUT1* expression on Sp mediated transcription. In line with gene knockdown studies, hypoxic inductions of both genes completely disappeared due to Mithramycin A treatment.

As evident from results presented in this section, treatment of Mithramycin A inhibited expression of all the genes identified as hypoxia responsive genes (except *ARTN* and *IRS2* genes).

Treatment of Mithramycin A inhibited transcription of all the genes regardless the nature of their hypoxic induction (upregulation/downregulation). Inhibition was clearly significant under hypoxic environment. Specific interaction of Mithramycin A to Sp binding sites may prevent binding of Sp proteins to its cognate sites which in turn reduce transcription. Hypoxia induced gene upregulation was clearly suppressed by treatment of Mithramycin A where under hypoxic environment inhibitory effect was significantly higher compared to the normoxic environment. This is may be due to inhibitory effect of hypoxia itself on RNA synthesis and protein. Interestingly genes which are downregulated due to hypoxic exposure showed further reduction in expression due to Mithramycin A treatment. It is apparent that Mithramycin A inhibits RNA synthesis in hypoxic induction regardless the expression patterns.

6.3.2.1. Possible modes of action

According to the results presented in this chapter, it is obvious that Mithramycin A has inhibitory effect on expression changes of identified genes. Interestingly, response to treatment of Mithramycin A resulted in inhibition of both upregulated and downregulated genes in severe hypoxia. Therefore, it can be speculated that inhibitory action of Mithramycin A may be governed by multiple mechanisms (Figure 6.26 summarises the possible mechanisms of Mithramycin A). It is possible that all these mechanisms collectively contribute for selective inhibition of Sp mediated transcription.

A. Inhibition of Sp protein-DNA interaction by occupying Sp promoter sites (direct DNA binding)

Mithramycin A has been identified as a DNA binding small molecule which specifically interacts with GC-rich regions of the gene promoter site. Since Sp consensus sites are GC-rich DNA binding sites, it was believed that Mithramycin A could specifically interact with Sp promoter sites which in turn suppress the Sp mediated gene transcription. Mithramycin A -DNA interaction has been identified in previous studies by several biophysical methods such as NMR analysis, DNA footprint analysis and spectroscopic analysis (Demicheli et al., 1991; Banerjee et al., 2014). As a DNA groove binder, Mithramycin A is able to interact with DNA as well as core histone proteins (Demicheli et al., 1991; Mir & Dasgupta, 2001; Barcelo et al., 2007; Banerjee et al., 2014; Weidenbach et al., 2016).

It has shown that Mithramycin A can inhibit RNA synthesis which may affect the transcription (Miller et al., 1987). Mithramycin is a DNA interacting RNA inhibitor (other type interacts with DNA-dependent RNA polymerase E.g. α -amanitin and Rifamycin) which binds to minor groove of double stranded DNA thus preventing it from being a template for RNA synthesis.

However, it is also suggested that Mithramycin A can inhibit transcription of Sp factors also due to the presence of GC-rich regions in their promoters. However, this mechanism is believed to be regulated by loop mechanism where higher concentration of Mithramycin A (<500 nM) has no effect on transcription of these factors.

B. Promoting proteasomal degradation of Sp1 proteins

It has also been shown that treatment of Mithramycin A might induce proteasomal degradation of Sp proteins, particularly Sp1 proteins by ubiquitination. Another study carried out by Chou *et al.*, also suggested that Mithramycin A leads to degradation of Sp1 protein in pancreatic cancers. However, the exact mechanism is yet to be elucidated (Chou, 2011; Choi et al., 2014).

6.3.3. Conclusion

In this study expression of 20 hypoxia responsive genes were investigated after *Sp1* gene knockdown followed by hypoxic exposure. Hypoxic induction of these genes was suppressed due to *Sp1* gene knockdown in all three breast cancer cell lines. Interestingly, the effect of gene knockdown was more effective and significant under the hypoxia where no significant changes were observed in normoxia. This concludes that Sp transcription factors, particularly *Sp1* may have a minimum effect on basal transcription of these genes under normoxic environment. However, hypoxic induction of these genes might be driven by Sp mediated transcription. Mithramycin A inhibits transcription of hypoxia mediated genes in breast cancer cell model. The treatment of drug effectively suppresses the hypoxic induction of identified genes. It is possible that inhibition of Sp mediated transcription by Mithramycin A might be governed by multiple mechanisms which include direct DNA-Mithramycin A interaction, enhanced proteasomal degradation of Sp proteins and repressed RNA synthesis. This evidence emphasises the potential role of Sp mediated transcription as a therapeutic target for hypoxic tumours such as breast cancers. Moreover, Sp protein inhibitors particularly Mithramycin A can be a potential drug for combinational therapies.

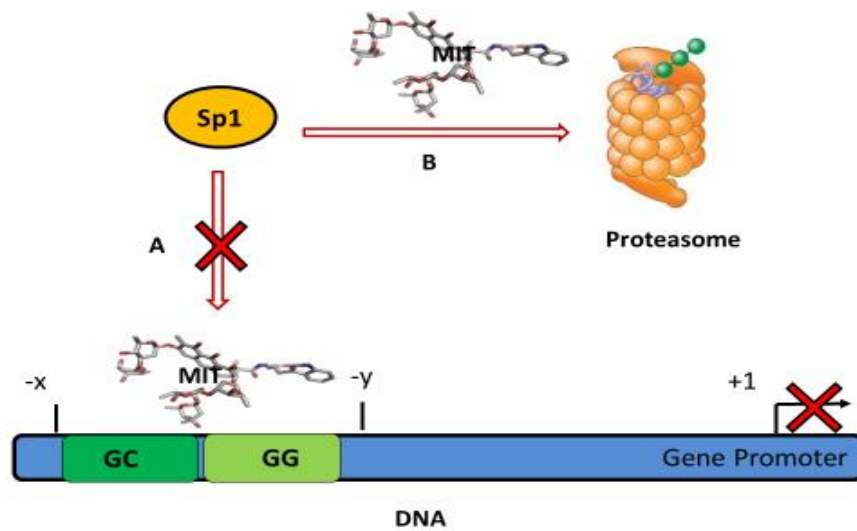


Figure 6.26: Possible modes of inhibition of Sp mediated transcription by Mithramycin A (MTR)

Inhibition of Sp mediated transcription might govern by two possible pathways. **A:** Direct DNA interaction of Mithramycin A and GC rich regions of gene promoters' sites (Sp binding sites) may prevent Sp binding to its cognates sequences. **B:** Mithramycin A promotes proteasomal degradation of Sp proteins due to post translational modifications.

Chapter 7.
General discussion

7. General discussion

7.1. Summary of the work presented in this thesis

Development of therapeutic strategies for cancer has been focused on various aspects of the malignant process including cell cycle regulation, cell death, immune evasion and metastasis. Tumour microenvironment has been identified as one of the major contributors to tumorigenesis and metastasis where mechanisms of different signalling cascades, interactions of malignant cells and physical/chemical alterations play an essential role in tumour development (Hanahan & Weinberg, 2011). Formation of low oxygen environments is a common condition in solid tumours such as Breast tumours which are known to be one of the highly hypoxic tumours. Tumour hypoxia leads to several challenges in disease management such as poor diagnosis, prognosis and survival rates. It has been shown that chemo and radiation therapies are inefficient in the hypoxic environment due to poor perfusion of drugs. In fact, more invasive and metastasis tumours are prominent in hypoxic conditions.

HIF-ARNT, the well-established regulator of the hypoxic environment, mediated transcription has been studied in numerous cancer types whilst HIF mediated gene targets were used for several therapeutic targets. However, emerging evidence suggests that some key hypoxia responsive genes (e.g. *VEGFA*) are regulated in HIF dependent and independent manner suggesting the involvement of other transcription regulators (Pore et al., 2004; Deacon et al., 2012; Lee et al., 2014). Importantly, studies have suggested the potential role of Specificity protein (Sp) transcription factors in transcriptional regulation of HIF genes (*HIF1A*) as well as other hypoxia responsive genes (Koshikawa et al., 2009). The role of Sp transcription factors was extensively studied in related to numerous gene transcriptions which are involved in most of the cellular pathways such as apoptosis, cell cycle, tumorigenesis and DNA damage-repair mechanisms. High levels of Sp transcription factors were recorded in most of the cancer types including breast cancers, which is correlated with poor prognosis and diagnosis (Wright et al., 1987; Hsu et al., 2012; Sankpal et al., 2012). However, the role of Sp proteins under hypoxic microenvironment is poorly studied. Several studies conducted on different cancer types have confirmed that Sp proteins might play an obvious role in hypoxia, potentially similar to HIF capacity. However,

results were more often contrary to each other where function of Sp proteins depended on tumour type and severity of hypoxia. Therefore, it is essential to further investigate the effects of hypoxia and related cellular responses in breast cancer in order to elucidate mechanisms governed under hypoxic stress.

The objective of this study was to understand the role of Sp transcription factors and Sp mediated gene transcription under severe hypoxic microenvironment in breast cancer cell model. Each of the previous chapters focused on identifying the importance of Sp proteins in hypoxic microenvironment. In **Chapter 3**, the effect of hypoxia on Sp transcription family members was identified. Hypoxic induction of Sp proteins was prominent at protein level and Sp1 protein levels were significantly increased in breast cancer cells. In **Chapter 4**, expression of potential Sp mediated, hypoxia responsive genes were identified and validated in breast cancer cell lines. Furthermore, data presented in **Chapter 5** provide kinetic and binding analysis of Sp protein inhibitors in label free real-time SPR analysis. Results also showed that Sp protein inhibitor, Mithramycin A directly interact with GC rich DNA in absence of any cation requirements. Finally, in **Chapter 6** the effects of inhibition of Sp mediated gene transcription on hypoxia responsive gene were investigated. Hypoxia induction of hypoxia responsive genes were effectively suppressed by Sp protein inhibitor; Mithramycin A and gene knockdown by siRNA.

7.2. Potential effects of hypoxia on Sp family protein

Growing evidence suggests that Sp transcription factors are involved in transcriptional regulation of hypoxia responsive genes in different tumour types. However, due to the complex and diverse nature of Sp proteins, their contribution to hypoxic induction is still not fully understood. Moreover, potential mechanisms that Sp proteins undergo in malignant phenotypes are not clearly investigated. Hypoxia driven expression alterations in Sp family transcription factors have been previously reported in tumour types such as lung and colon cancers. In this study effect of severe (chronic) hypoxia on Sp transcription factors (Sp1, Sp3 and Sp4) in breast cancer cells were investigated (Chapter 3). Results clearly indicated the hypoxic induction of Sp proteins under severe hypoxia, where of Sp1 protein levels were significantly increased in all three breast cancer cell lines. However, Sp3 protein expression and transcript levels of all Sp genes

remained unchanged. In addition, increased binding activity of Sp protein complex was also detected under severe hypoxia.

Alterations in Sp protein expression levels have been identified as a common mechanism which Sp protein regulates its transcriptional activity. Increased levels of Sp proteins were detected under several pathological conditions including tumours. Hypoxia driven expression changes have been reported in several tissue types hence detailed mechanisms of Sp protein induction are unclear (Szalad et al., 2009; Jeong & Park, 2012). It has been shown that generation of reactive oxygen species under severe hypoxic stress may activate translation of Sp1 protein in neuronal cells. This mechanism was governed via an internal ribosome entry site in Sp1 mRNA (5' untranslated region) (Yeh et al., 2011). In addition, HIF-1 α and HIF-2 α driven expression changes were also been reported. The presence of HRE sites has been identified in Sp promoter sites in rat cells suggesting the possibility of HRE depended transcription Sp genes (Woo et al., 2012). However, these induction mechanisms are not fully investigated in cancer cell models. Results presented in this section confirms the induction patterns of Sp proteins in cancer cells which may arise from similar molecular mechanisms. Hence, further investigations are required in order to elucidate exact mechanism of hypoxic induction of Sp proteins.

7.3. Hypoxia responsive gene regulation

Therapeutic strategies of breast cancer are largely relying on stage of the tumour, different sub types and receptor status. Since different sub types response to treatments in a specific manner, molecular subtypes are important in prediction of cancer mortality and treatment strategies (Prat et al., 2015). For example, tumours with HER-2 positive status showed poorest survival rates compared to other sub types (Haque et al., 2012) However, it has shown that these classification and treatment strategies are not sufficient in prediction of chemo and radio therapy responses (Prat et al., 2015). Furthermore, even though *BRCA1* and *BRCA2* still remain as two major genes that contribute for breast cancer formation alongside with other contributing genes, these biomarkers were identified only in small percentage of cancers (Couch et al., 2017). Therefore, more comprehensive gene profiles are still required in order to identify biomarkers and prediction of therapeutic responses and survival rates (Weigelt et al., 2005).

In this study, Sp-driven hypoxia responsive genes were identified using publicly available microarray data sets (NCBI GEO data base) and analysed using bioinformatics software GeneSpring 12X (Chapter 4). As hypothesised, a large number of genes were identified with potential Sp binding sites and HIF binding sites. Since previously recorded gene profile studies have mainly exploited HIF mediated gene expressions, this study aimed on identification HIF independent genes. All three cell lines showed hypoxia responsive nature of these genes whereas expression levels were varying with cell lines. Therefore, regardless of different properties of these cell lines, overall expression patterns of these genes were common for all three-cell lines. This may suggest that regulation of these genes is independent of different receptor status which highlights their importance in all types of breast cancers. In addition, hypoxic induction of identified genes was effectively inhibited by *Sp1* gene knockdown and Sp protein inhibitor Mithramycin A treatment in all three cell lines (Chapter 6). In addition, pathway analysis of these identified genes showed (as listed in *Appendix 1*) Sp mediated genes are widely spread in different cellular pathways supporting the fact that wider array of functions and its impotence. Results also highlighted that importance of investigation on Sp transcription factors and their pathways in order to elucidate overall function of Sp proteins in tumour progression under hypoxic conditions. These results further validate use of Sp inhibitors to inhibit hypoxia related cellular pathways.

A review published by Koizume and colleagues have summarised possible mechanisms of Sp driven hypoxic gene induction (Koizume & Miyagi, 2016). It was speculated that numerous molecular mechanisms that undergo in hypoxic micro environment might lead to transcriptional activity of Sp proteins. It was widely accepted that Sp1 and HIF-1 α collaboratively regulate gene transcription by direct interaction with HRE and Sp binding sites of the distal promoter sites of the genes. Some instances HRE sites were believed to be shared with Sp proteins (GC box like HRE sequences). Post translation modifications of Sp proteins are widely examined and well established. Common modifications such as phosphorylation and acetylation are known to increase Sp protein binding activities while some modifications like SUMOlation may suppress its activity. Several studies have shown that severe hypoxia may also lead to different post translational modifications which facilitate transcriptional activation of some

genes. In addition, some studies have also suggested that interaction between Sp transcription factors and HIF proteins may be a possible alternation that undergoes under hypoxic microenvironment. HIF-1 α directly interact with Sp1 via its N terminal PAS domain which may enhance the transcriptional regulation. HIF-2 α interaction is believed to be hindered due to phosphorylation of which in turn negatively regulate expression of some genes such as *MSH2* and *MSH6*. However, some of the hypoxia genes were regulated in absence HIF-ARNT regulation suggesting the vital role of Sp transcription factors. Interestingly it is also possible that Sp1 protein targets *HIF-1 α* gene expression in cooperation with other co factors such as p53. Possible molecular mechanism of Sp driven hypoxic gene regulation are summarised on Figure 7.1.

Identification of these genes can be vital in future therapeutic aspects. Since most solid tumours experience hypoxic stress at some stage of proliferation, hypoxia induced pathways are critical in treatment strategies. There is a potential of using these genes as biomarkers of solid tumours. In addition, these genes can also be crucial in other cellular stresses and adaptive mechanisms. Moreover, targeting Sp mediated hypoxia induction can overcome HIF independent alternative mechanisms of key hypoxia responsive genes.

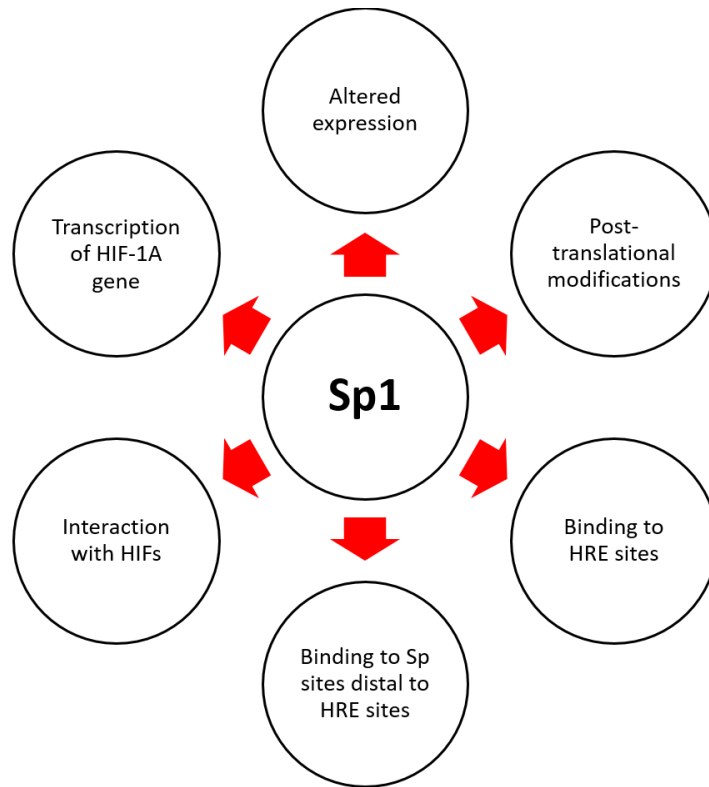


Figure 7.1: Potential molecular mechanism of Sp driven hypoxia responsive gene regulation.

Sp-driven transcriptional regulation of hypoxia responsive genes is believed to be governed by various molecular mechanisms.

- I. The presence of Sp binding sites in gene promoters may facilitate direct regulation of hypoxia responsive genes in HIF independent manner. Altered expression levels and binding activity changes of Sp proteins may facilitate hypoxic induction.
- II. Sp proteins may undergo post translational modification which determines the binding activity of Sp protein.
- III. Sp1 and HIF complex may compete for HRE sites in gene promotes sites.
- IV. HIF-ARNT dependent gene regulation is enhanced by binding of Sp proteins to distal Sp sites.
- V. Protein-protein interaction of Sp1 and HIF-1 at promoter sites may regulate transcription.
- VI. Sp proteins target *HIF1A* gene expression.

7.4. Significance of Sp transcription factors as a therapeutic target

Hypoxic tumours exhibit greater resistance to chemo and radiation therapies in addition to their aggressive phenotypes, higher metastasis and invasive nature. These unique features have been exploited to develop selective therapeutic approaches for hypoxic tumours. Different approaches have been developed over the years which includes hypoxia activated pro-drugs, hypoxia selective gene therapy as well as inhibition of HIF mediated transcription mechanisms. Hypoxia activated pro-drugs such as Tirapazamine, has been clinically tested and demonstrated its anti-tumour activity (Brown, 1993; 2000). However, since the effective oxygen tensions in normal cells fluctuate (2.0 - 9.0%) depending on tissue type hypoxia activated pro-drugs usually associated severe cytotoxic effects on normal cells (Bertout et al., 2008). Similarly, gene therapy approaches (transfection of antisense or shRNA) were also difficult to apply in clinical setup (Yeo et al., 2004).

Transcription factors have been targeted for therapeutic aspects in various disease types including cancer (Darnell, 2002). Inhibition of the key transcriptional regulator in hypoxia adaptive mechanisms, HIF has been proposed and persuaded. Different approaches such as inhibition of HIF-DNA interaction, HIF-ARNT dimerisation and protein- protein interaction has been tested (Melillo, 2007). In addition, small DNA binding molecules have been tested to target HIF mediated transcription alongside with other molecules such as metabolic enzymes whereas some of them are in clinical trials (Wilson & Hay, 2011; Semenza, 2012a; 2013). Small DNA binding molecules such as HSP90 inhibitors (e.g. Geldanamycin and Radicicol analogues), Echinomycin have been tested to inhibit DNA binding of HIF-1 (Vlaminck et al., 2007). Targeting HIF mediated gene targets such as *VEGF*, *CXCR4* has also shown some promising results (Schioppa et al., 2003; Ferrara, 2005). However potential harmful and side effects on normal cells have limited the application of HIF mediated therapeutic targets. This is also believed to be associated with chemo sensitivity and requirement of higher doses due to ineffectiveness of tumour vasculature. Therefore, targeting HIF as therapeutic molecule may not be an effective strategy in hypoxic induction.

Data presented in this study have empathised that vital role of Sp proteins regulating hypoxia responsive gene in HIF dependent/independent regulation. In fact, HIF transcription is also regulated by Sp1 transcription factors. Therefore, targeting of Sp

mediated transcription can be useful for inhibition of not only hypoxic induction, but other tumorigenic pathways and different cellular stress mechanisms. Nevertheless, the main challenge associated with using Sp protein as a therapeutic target is inhibition of Sp proteins can also lead to severe cytotoxic effects in the cellular environment. This is due to the fact that involvement of Sp transcription factors in diverse functions and most of the vital cellular pathways such as cell cycle, apoptosis and growth proliferation. Inhibition of Sp mediated transcription will effectively block the majority of cellular pathways (or their products) in normal cells as well as the tumour cells. Therefore, direct inhibition of Sp protein mediated transcription has not been widely tested in clinical therapies. However, specific Sp protein inhibitors which can only target tumour DNA can be an ideal candidate for inhibitions of hypoxic induction and other cellular networks.

Genetic changes occur in normal cells prior to transformation to tumour cells may cause structural level changes in DNA which facilitate unregulated behaviour of tumours (Galloway, 1989). Numerous studies have revealed the differences in primary structure and secondary structure of tumour DNA where. Studies have shown that the GC content and degree of methylation can be higher in tumour cells compared to normal cells (Rao et al., 1989; Jasem & Tothova, 1993). Similarly, studies have shown that tumour DNA has different melting curves compared to normal cell DNA due to altered structural regions of tumour DNA resulted by specific methylation such as hypermethylation of cytosine (Babaian & Garibian, 1990). Furthermore, changes in physical structure of DNA has also been also reported by Andronikashvili *et al.*, suggesting that secondary structures may contain higher number of defects in tumour DNA structure (Andronikashvili, 1987). Tumour cells exhibit greater degree of gene mutations as compared to normal cells. In addition, they have higher dependence on DNA-related processes, over normal cells due to deficits in checkpoint control and DNA repair. Rapid replication and proliferation also increase DNA dependency of tumour cells.

Adverse side effects and toxicity on normal cells can be minimised by taking the advantage of structural differences of tumour DNA such as higher degree of methylation and conformation of DNA (Gurova, 2009). In addition, the more specific interactions will result minimal structural damage and increase effectiveness, which is

vital in prevention of cancer formation and progression. Initially, TMP emerged as a tumour DNA specific small DNA binding molecule. Even though lack of published literature is available, it was speculated that TMP may utilise open conformation of tumour DNA, so that specific interaction is feasible only at tumour DNAs. Contrary, in this study it has shown that TMP may require further modification in order to establish direct DNA interaction. However, modification of current small DNA binding small molecules of Sp inhibitors/synthesising new moieties which target only tumour DNA and Sp binding sites can be useful in anti-cancer therapies. Gene mutations in Sp mediated genes and their binding sites should be explored in order to identify the changes in tumours. The method developed in this study can be ideal approach for designing and synthesising of new DNA binding small molecules, especially screening large libraries of DNA binding molecules (Chapter 5). When combined with other techniques (computer programming for narrowing down the potential candidates is a cost-effective way of screening a library of new moieties) it can be very useful which provides label free direct kinetics of the moieties in enhanced sensitivity.

In addition to conventional DNA binding inhibitors, synthetic DNA binding protein has been previously used to modulate the natural transcription mechanism and eukaryotic transcription factors (Khalil et al., 2012). DNA binding domains derived from prokaryotic origins such as TetR (mainly bacterial origin) has been used in eukaryotic gene modulation (Gossen & Bujard, 1992; Urlinger et al., 2000). These domains were used to recruit gene activators and suppressors to promoter sites. In addition, synthetic transcription factors based on Zinc fingers and transcriptional Activator-like Effector (TALEs) have also shown promising results in eukaryotic cells (Morbitzer et al., 2010; Maeder et al., 2013). However, both approaches were linked several limitations such as specificity, metabolic burden on cells and precise programming. However, emerging knowledge on Clustered Regularly Interspaced Short Palindromic Repeats/Cas system (CRISPR/Cas9) has shown the potential of programmed modulation of (direct suppression and repression) transcription factors in eukaryotic cells. Sp transcription mediated gene targets would be a highly potent candidate for gene editing given the involvement of major cellular pathways. Modulating the Sp1 transcription factor in tumour microenvironment would obviously inhibit not only one cellular pathway, but major cellular pathways (Farzadfard et al., 2013).

7.5. Future directions

Results presented in this thesis have provided solid background knowledge of Specificity proteins and their role in regulating hypoxia responsive genes in hypoxic microenvironment. Moreover, kinetic characterisation of Sp inhibitors has revealed future prospect of Sp targets as a therapeutic target. These results also direct to further investigations in to elucidate role of Sp mediated transcription in hypoxia.

- Results presented this study clearly suggest that vital role of Sp protein in the hypoxic environment. It is also possible that Sp proteins may undergo specific post translational modifications under hypoxia. Hypoxia driven post translational modifications such as phosphorylation can be investigated in different oxygen potentials and cancer cell models.
- Gel-shift assay (EMSA) results indicated possible binding activity changes of Sp proteins in hypoxia. Further investigation of Sp protein binding can be analysed using Chromatin immunoprecipitation (ChIP) which allows the identification of other cofactors (protein-protein interactions) involved in transcription of hypoxia responsive genes as well as other Sp proteins mediated genes.
- In this study, the only knockdown effect of *Sp1* gene was analysed at mRNA level. However, identification of knockdown effect of other Sp family members especially Sp3 protein which is known to play inhibitory action on Sp1 mediated transcription is essential for elucidating Sp protein signalling cascades in tumour hypoxia. Furthermore, Mithramycin A has been used in this study for inhibition analysis. Investigation of other potential inhibitors, natural compounds and their derivatives such as Curcumin in the hypoxic environment would be beneficial.
- Further investigations are necessary to identify Sp driven hypoxia responsive genes in tumour models. Generation of microarray data on different cell lines can be useful in identification of novel genes in other cancer types. RNA-Seq technique will be also useful. Identification of Sp binding sites using promoter assays such as Luciferase assay will facilitate investigation of importance and dependability of Sp binding sites on expression of these novel genes.
- In this study binding kinetics of Mithramycin, Chromomycin and Terameprocol were analysed using linear oligomers of DNA. However, the method developed

in this study could be easily used to analyse other potential Sp inhibitors which are yet to be identified in terms of their binding kinetics and mode of actions. It is also possible to analyse binding patterns towards chromatin structure which mimics actual steric hindrance of drug-DNA interaction in the cellular environment. This can be used in both SPR as well as binding analysis.

- Results presented in this study has clearly indicated the Sp driven hypoxia gene regulation in breast cancer model. However, tumour specific Sp inhibitors such as Terameprocol requires further characterisation and modification. Synthesising novel derivatives which inhibit both Sp and HIF mediated transcription can be also vital therapeutics for solid tumours.
- In this study, all the experiments were carried out using breast cancer cell lines. The investigation can be further extended to breast cells as well as *in-vivo* models. Finally, investigating the role of Sp proteins in other types of tumours such as lung, colon cancers are important to elucidate overall function in solid tumours.

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Appendix I

Table 1: Cellular pathways of genes with putative Sp1 binding sites

Pathway	p-value	Genes
Hs_IL-1_signaling_pathway_WP195_74010	0.08	<i>JUN</i>
Hs_Adipogenesis_WP236_72082	0.18	<i>IRS2</i>
Hs_RANKL-RANK_Signaling_Pathway_WP2018_74133	0.08	<i>JUN</i>
Hs_TGF_Beta_Signaling_Pathway_WP366_69026	0.08	<i>JUN</i>
Hs_TWEAK_Signaling_Pathway_WP2036_72733	0.06	<i>JUN</i>
Hs_Aryl_Hydrocarbon_Receptor_WP2586_76318	0.06	<i>VEGF</i>
Hs_Insulin_Signaling_WP481_72080	0.01	<i>JUN</i> , <i>IRS2</i> , <i>SLC2A1</i>
Hs_IL-4_Signaling_Pathway_WP395_74009	0.08	<i>IRS2</i>
Hs_Apoptosis_WP254_72110	0.01	<i>TNFRSF2</i> <i>1</i> , <i>JUN</i>
Hs_EPO_Receptor_Signaling_WP581_67199	0.04	<i>IRS2</i>
Hs_Oncostatin_M_Signaling_Pathway_WP2374_73668	0.09	<i>OSMR</i>
Hs_Physiological_and_Pathological_Hypertrophy_of_the_Heart_WP1528_68953	0.04	<i>C-JUN</i>
Hs_Wnt_Signaling_Pathway_and_Pluripotency_WP399_74897	0.13	<i>JUN</i>
Hs_BDNF_signaling_pathway_WP2380_71549	0.02	<i>JUN</i>
Hs_TSH_signaling_pathway_WP2032_75384	0.10	<i>JUN</i>
Hs_AMPK_Signaling_WP1403_75220	0.03	<i>PFK2</i>
Hs_Apoptosis_Modulation_and_Signaling_WP1772_63162	0.12	<i>AP-1</i>
Hs_TGF_Beta_Signaling_Pathway_WP560_68944	0.08	<i>JUN</i>
Hs_Hypertrophy_Model_WP516_71358	0.03	<i>VEGF</i>
Hs_IL-3_Signaling_Pathway_WP286_72139	0.07	<i>JUN</i>

Hs_Signaling_of_Hepatocyte_Growth_Factor_Receptor_WP313_71094	0.05	<i>JUN</i>
Hs_IL-5_Signaling_Pathway_WP127_70017	0.06	<i>JUN</i>
Hs_FAS_pathway_and_Stress_induction_of_HSP_regulation_WP314_71366	0.06	<i>JUN</i>
Hs_Notch_Signaling_Pathway_WP61_70097	0.09	<i>SAP30</i>
Hs_ErbB_Signaling_Pathway_WP673_69914	0.08	<i>JUN</i>
Hs_Myometrial_Relaxation_and_Contraction_Pathways_WP289_72107	0.21	<i>JUN</i>
Hs_Gastric_cancer_network_2_WP2363_76329	0.05	<i>AHCTF1</i>
Hs_Angiogenesis_WP1539_75222	0.02	<i>VEGFA</i>
Hs_MAPK_Signaling_Pathway_WP382_72103	0.03	<i>DUSP1, JUN</i>
Hs_Complement_and_Coagulation_Cascades_WP558_67786	0.07	<i>THBD</i>
Hs_Hedgehog_Signaling_Pathway_WP47_70092	0.02	<i>PTCH1</i>
Hs_Estrogen_signaling_pathway_WP712_77341	0.03	<i>C-JUN</i>
Hs_B_Cell_Receptor_Signaling_Pathway_WP23_72101	0.13	<i>JUN</i>
Hs_Glycolysis_and_Gluconeogenesis_WP534_74524	0.00	<i>SLC2A1, ENO2</i>
Hs_Heart_Development_WP1591_73381	0.00	<i>VEGFA, BHLHE40</i>
Hs_Toll-like_receptor_signaling_pathway_WP75_72133	0.15	<i>JUN</i>
Hs_Arrhythmogenic_Right_Ventricular_Cardiomyopathy_WP2118_71265	0.11	<i>DSP</i>
Hs_Interferon_type_I_signaling_pathways_WP585_73488	0.08	<i>IRS2</i>
Hs_DNA_Damage_Response_(only_ATM_dependent)_WP710_70109	0.07	<i>JUN</i>
Hs_AGE-RAGE_pathway_WP2324_71701	0.10	<i>JUN</i>
Hs_Focal_Adhesion_WP306_71714	0.25	<i>JUN</i>
Hs_Endochondral_Ossification_WP474_72122	0.00	<i>PTCH 1, VEGFA</i>
Hs_TCR_Signaling_Pathway_WP69_72111	0.13	<i>JUN</i>

Hs_Senescence_and_Autophagy_WP615_71375	0.14	<i>JUN</i>
Hs_PDGF_Pathway_WP2526_76331	0.02	<i>AP1 (C-JUN)</i>
Hs_MAPK_Cascade_WP422_72129	0.04	<i>JUN</i>
Hs_Nuclear_Receptors_WP170_71083	0.06	<i>NR2F2</i>
Hs_G1_to_S_cell_cycle_control_WP45_71377	0.10	<i>CCNG2</i>
Hs_Wnt_Signaling_Pathway_WP428_71365	0.09	<i>JUN</i>
Hs_TNF_alpha_Signaling_Pathway_WP231_72093	0.12	<i>JUN</i>
Hs_Alpha_6_Beta_4_signaling_pathway_WP244_72056	0.05	<i>IRS2</i>
Hs_Diurnally_Regulated_Genes_with_Circadian_Orthologs_WP410_69903	0.00	<i>BTG1, GENX 3414</i>
Hs_IL-2_Signaling_Pathway_WP49_75383	0.06	<i>JUN</i>
Hs_Androgen_receptor_signaling_pathway_WP138_72130	0.13	<i>JUN</i>
Hs_RB_in_Cancer_WP2446_76353	0.13	<i>SAP30</i>
Hs_Regulation_of_toll-like_receptor_signaling_pathway_WP1449_72114	0.15	<i>JUN</i>
Hs_EGF-EGFR_Signaling_Pathway_WP437_72106	0.22	<i>JUN</i>
Hs_Integration_of_energy_metabolism_WP1831_77011	0.12	<i>SLC2A1</i>
Hs_Apoptotic_execution_phase_WP1784_76813	0.07	<i>DSP</i>
Hs_Metabolism_of_carbohydrates_WP1848_76833	0.00	<i>SLC2A1, PFKFB3</i>
Hs_Mitotic_Metaphase_and_Anaphase_WP2757_77009	0.21	<i>AHCTF1</i>
Hs_MAPK_targets- _Nuclear_events_mediated_by_MAP_kinases_WP1845_76899	0.02	<i>JUN</i>
Hs_Cell_surface_interactions_at_the_vascular_wall_WP1794_77039	0.13	<i>THBD</i>
Hs_Formation_of_Fibrin_Clot_(Clotting_Cascade)_WP1818_76965	0.05	<i>THBD</i>
Hs_NGF_signalling_via_TRKA_from_the_plasma_membrane_WP1873_76973	0.08	<i>IRS2</i>
Hs_Pre-NOTCH_Expression_and_Processing_WP2786_77076	0.05	<i>JUN</i>

Hs_Gastrin-CREB_signalling_pathway_via_PKC_and_MAPK_WP2664_76844	0.19	<i>EDN2</i>
Hs_Regulation_of_Lipid_Metabolism_by_Peroxisome_proliferator_activated_receptor_alpha_(PPARalpha)_WP2797_77088	0.15	<i>TNFRSF21</i>
Hs_NCAM_signaling_for_neurite_out-growth_WP1866_76927	0.07	<i>ARTN</i>
Hs_Circadian_Clock_WP1797_76871	0.00	<i>BHLHE40/41</i>
Hs_PIP3_activates_AKT_signaling_WP2653_76821	0.10	<i>IRS2</i>
Hs_Signaling_by_Insulin_receptor_WP1913_77046	0.11	<i>IRS2</i>
Hs_Fc_epsilon_receptor_(FCER1)_signaling_WP2759_77017	0.12	<i>JUN</i>
Hs_Signaling_by_VEGF_WP1919_76864	0.02	<i>VEGFA</i>
Hs_Transport_of_glucose_and_other_sugars,_bile_salts_and_organic_acids,_metal_ions_and_amine_compounds_WP1935_76949	0.08	<i>SLC11A2</i>
Hs_Signaling_by_Type_1_Insulin-like_Growth_Factor_1_Receptor_(IGF1R)_WP2677_76867	0.08	<i>IRS2</i>
Hs_GPCR_downstream_signaling_WP1824_76910	0.12	<i>APLN, EDN2</i>
Hs_Hexose_transport_WP1828_77015	0.05	<i>SLC2A1</i>
Hs_GPCR_ligand_binding_WP1825_76977	0.10	<i>EDN2, APLN</i>
Hs_Mitotic_Prometaphase_WP2652_76819	0.14	<i>AHCTF1</i>
Hs_Regulation_of_Hypoxia-inducible_Factor_(HIF)_by_Oxygen_WP2727_76947	0.03	<i>VEGFA</i>
Hs_Iron_uptake_and_transport_WP2670_76854	0.15	<i>SCL11A2</i>
Hs_Transcriptional_Regulation_of_White_Adipocyte_Differentiation_WP2751_76992	0.11	<i>NR2F2</i>
Hs_Hair_Follicle_Development-_Cytodifferentiation_(Part_3_of_3)_WP2840_77312	0.12	<i>JUN</i>
Hs_Prolactin_Signaling_Pathway_WP2037_75382	0.01	<i>JUN</i>
Hs_Prostate_Cancer_WP2263_73838	0.15	<i>JUN</i>
Hs_Regulation_of_toll-like_receptor_signaling_pathway_WP1449_77378	0.15	<i>JUN</i>
Hs_TNF_alpha_Signaling_Pathway_WP2808_75336	0.12	<i>JUN</i>
Hs_Hair_Follicle_Development-_Organogenesis_(Part_2_of_3)_WP2839_77315	0.05	<i>PTCH1</i>

Appendix II

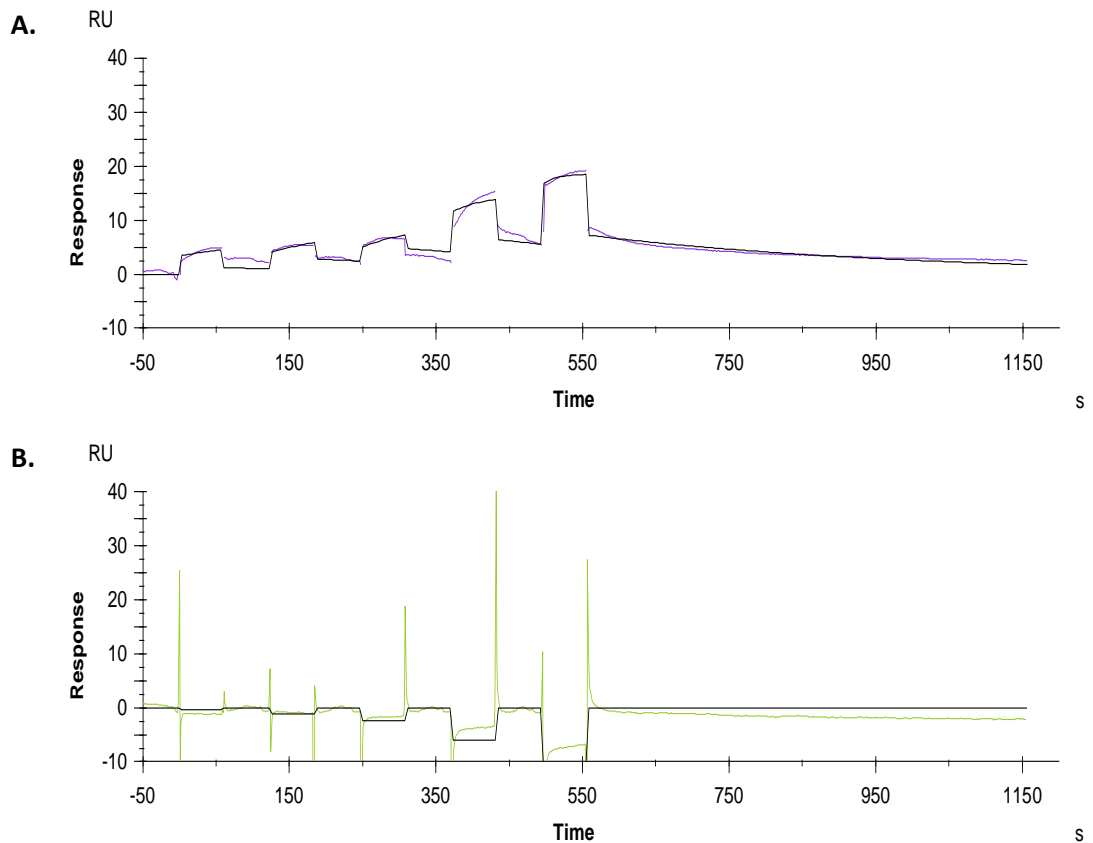


Figure 1: Single kinetic assay for interaction of Chromomycin A with *Bak* gene promoter B region in absence of divalent cation Mg^{2+}

Sensorgrams represent binding curves for interaction of Chromomycin A with 20bp DNA oligomers: Bak B (panel A) and control sequence without GC regions (panel B). Five consecutive concentrations (ascending 3-30 μM) of Chromomycin A was injected over the surface. The coloured lines represent drug injections where black lines represent the global fit of the entire data set to a single site interaction model including a term for mass transport component. Injections were performed for 600 s followed by 600 s of buffer flow at 30 $\mu l/min$ flow rate at 25 $^{\circ}C$.