

FOXO3a AND ID1 AS TARGETS OF CHEMOTHERAPY IN BREAST CANCER

Thesis submitted by

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To

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DECLARATION OF ORIGINALITY

Unless otherwise stated in the text, this thesis is the result of my own work.

ABSTRACT

Cancer is one of the leading causes of death and more than 40000 women are diagnosed every year in the UK with breast cancer. Chemotherapy regimens containing the anthracycline epirubicin are often used in the medical management of breast cancer, but the cellular mechanisms of action and the molecular pathways underlying response to treatment are not fully understood.

The FOXO family of transcription factors are downstream targets of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway and play a role in a variety of processes including cellular differentiation, metabolism, cell-cycle arrest, cell death and protection from stress. This work identifies the transcription factor FOXO3a as a cellular target of epirubicin. Treatment of the MCF7 breast cancer cell line with epirubicin causes G₂/M cell cycle arrest and leads to upregulation of FOXO3a at the transcriptional, translational and gene promoter level. FOXO3a accumulates in the nucleus following treatment with epirubicin and silencing of FOXO3a by RNA interference confers protection against the cytotoxic effects of the drug. FOXM1, another member of the Forkhead family, is repressed at the protein, RNA and gene promoter level by epirubicin. This repression is relieved when FOXO3a is silenced or prevented from binding the FOXM1 promoter in gene promoter assays, suggesting FOXO3a regulates the repression of FOXM1.

This work also identifies Id1 as a target of epirubicin. Id1 is highly expressed in chemoresistant cell lines and is rapidly downregulated following treatment of MCF7 cells with epirubicin in sensitive cell lines. Overexpression of Id1 confers resistance

to epirubicin and knockdown of Id1 increases the sensitivity of MCF7 cells to a number of chemotherapeutic agents. Id1 activity is independent of FOXO3a regulation.

Analysis of clinical samples confirms the changes seen in cell lines in FOXO3a, FOXM1 and Id1 following chemotherapy and treatment with the EGFR inhibitor gefitinib. Thus, in the future, FOXO3a, FOXM1 and Id1 could be used as indicators and predictors of response in the treatment of breast cancer.

PUBLICATIONS

Francis RE, Myatt SS, Krol J, Hartman J, Peck B, **McGovern UB**, Wang J, Guest SK, Filipovic A, Gojis O, Palmieri C, Peston D, Shousha S, Yu Q, Sicinski P, Coombes RC, Lam EW. FoxM1 is a downstream target and marker of HER2 overexpression in breast cancer. *Int J Oncol.* 2009;35(1):57-68.

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Essafi A, Gomes AR, Pomeranz KM, Zwolinska AK, Varshochi R, **McGovern UB**, Lam EW. Studying the subcellular localization and DNA-binding activity of FoxO transcription factors, downstream effectors of PI3K/Akt. *Methods Mol Biol.* 2009;462:201-11.

In Memory of a dearly loved Mother

**Louisa McGovern
1941-2006**

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TABLE OF CONTENTS

DECLARATION OF ORIGINALITY	2
ABSTRACT	3
PUBLICATIONS	5
ACKNOWLEDGEMENTS	7
TABLE OF FIGURES	13
LIST OF TABLES	16
ABBREVIATIONS.....	17
CHEMOTHERAPY REGIMENS	19
 CHAPTER 1 INTRODUCTION	 20
1.1 CANCER – an overview	21
1.1.1 Oncogenes and tumour suppressors	21
1.1.2 Epigenetics in cancer	26
1.1.3 Chromosomal abnormalities and cancer	28
1.1.4 DNA damage and cancer	30
1.2 BREAST CANCER.....	33
1.2.1 Presentation and diagnosis	35
1.3 MEDICAL MANAGEMENT OF BREAST CANCER.....	36
1.3.1 The oestrogen receptor (ER).....	36
1.3.2 Endocrine treatment.....	37
1.3.3 The anthracyclines	39
1.3.4 The anthracyclines and early breast cancer.....	42
1.3.5 Neo-adjuvant chemotherapy.....	45
1.3.6 Predictors of response to chemotherapy	46
1.3.7 Chemotherapy resistance	49
1.4 THE CELL CYCLE.....	51
1.4.1 Cdk inhibitors.....	54
1.4.2 The cell cycle and cancer	55
1.5 APOPTOSIS	57
1.5.1 Bcl-2 proteins	59

1.5.2	Apoptosis and chemoresistance	60
1.6	THE PHOSPHATIDYLINOSITOL-3-KINASE PATHWAY	62
1.6.1	Class 1 PI3K proteins.....	64
1.6.2	Akt/Protein kinase B (PKB)	65
1.6.3	The PI3K pathway and cancer.....	67
1.6.4	Therapeutic approaches targeting the PI3K pathway	70
1.7	FOXO TRANSCRIPTION FACTORS	72
1.7.1	The structure and function of FOXO proteins	74
1.7.2	FOXO activity and signaling pathways	77
1.7.3	Transcriptional targets of FOXO proteins	81
1.7.4	FOXO proteins and cancer	85
1.8	FOXO1	88
1.8.1	FOXO1 as a therapeutic target.....	91
1.9	ID PROTEINS	92
1.9.1	Id1	97
1.9.2	Id1 and cancer	99
THESIS AIMS AND OBJECTIVES		101
CHAPTER 2 MATERIALS AND METHODS		102
2.1	CELL CULTURE	103
2.1.1	Sub-culturing of adherent cells	104
2.1.2	Preservation of cells	104
2.2	CHEMICALS.....	105
2.3	PROTEIN ANALYSIS.....	105
2.3.1	Preparation of total protein extracts	105
2.3.2	Determination of protein concentration	105
2.3.3	(Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) SDS-PAGE	106
2.3.4	Western blotting of SDS-PAGE.....	107
2.4	RNA MANIPULATION.....	109
2.4.1	Total RNA extraction	109

2.4.2	Real-time quantitative PCR (RT-qPCR)	110
2.4.3	Primer optimization	111
2.5	DNA TRANSFECTIONS OF MAMMALIAN CELLS	111
2.5.1	Plasmid constructs	111
2.5.2	Promoter assays	112
2.5.3	Transfection of plasmid DNA	113
2.5.4	Luciferase reporter assay	113
2.6	FLUORESCENCE ACTIVATED CELL SORTING (FACS) ANALYSIS	114
2.7	SULPHORHODAMINE B (SRB assay)	115
2.9	SUBCELLULAR FRACTIONATION	117
2.10	IMMUNOHISTOCHEMICAL (IHC) STAINING	117
2.11	IMMUNOFLUORESCENT STAINING	122
2.12	STATISTICAL ANALYSIS	123
CHAPTER 3 FOXO3a AS A TARGET OF EPIRUBICIN		124
3.1	INTRODUCTION AND OBJECTIVES	125
3.2	RESULTS	127
3.2.1	FOXO3a expression in a breast cancer cell line panel...	127
3.2.2	FOXO3a is highly expressed in growth arrested cells ...	127
3.2.3	The sensitivity of a panel of breast cancer cell lines to epirubicin	130
3.2.4	Epirubicin causes G ₂ /M arrest in MCF-7 cells	132
3.2.5	Epirubicin induces FOXO3a protein and mRNA expression	135
3.2.6	Epirubicin induces FOXO3a nuclear sublocalisation in MCF-7 cells	139
3.2.7	FOXO1 expression in a panel of breast cancer cell lines	142
3.2.8	FOXO1 is repressed by epirubicin at the protein, mRNA level and gene promoter level	144

3.2.9	Dose dependent effects of epirubicin on FOXM1 and targets	144
3.2.10	Epirubicin represses FOXM1 promoter activity	145
3.2.11	Silencing of FOXO3a by siRNA confers protection to the cytotoxic effects of epirubicin	149
3.2.12	Expression of a constitutively active FOXM1 (Δ N-FOXM1) abrogates the cytotoxic effects of epirubicin	151
3.3	DISCUSSION	153
3.4	FUTURE WORK	158

CHAPTER 4 CHARACTERISING THE ROLE OF ID1 IN

BREAST CANCER CELLS		160
4.1	INTRODUCTION AND OBJECTIVES.....	161
4.2	RESULTS	162
4.2.1	Id1 expression in breast cancer cell lines.....	162
4.2.2	Id1 expression is highest in proliferating cells	164
4.2.3	Epirubicin rapidly downregulates Id1 expression.....	166
4.2.4	Epirubicin represses Id1 promoter activity	166
4.2.5	Id1 expression is higher in aggressive and resistant cell lines	170
4.2.6	Silencing of Id1 slows proliferation of breast cancer cells	174
4.2.7	Id1 silencing can reduce the growth of resistant and aggressive cell lines and increase sensitivity to epirubicin.....	177
4.2.8	Id1 knockdown increases epirubicin cytotoxicity in MCF7 cells	180
4.2.9	Cyclin D1 is downregulated following Id1 silencing.....	184
4.2.10	Overexpression of Id1 confers protection to epirubicin.	186
4.2.11	FOXO3a does not regulate Id1 in breast cancer cells ...	188
4.3	DISCUSSION.....	193
4.4	FUTURE WORK	199

CHAPTER 5 THE EXPRESSION OF FOXO3a AND TARGETS

IN CLINICAL SAMPLES	201
5.1 INTRODUCTION	202
5.2 RESULTS	204
5.2.1 The expression of FOXO3a, FOXM1 and Id1 in normal breast tissue and invasive carcinoma.....	204
5.2.2 The expression of FOXO3a, FOXM1 and Id1 in different histological subtypes of breast cancer.....	206
5.2.3 The effects of FEC chemotherapy in clinical samples. ..	210
5.2.4 A dose titration of gefitinib induces FOXO3a activity and represses FOXM1.....	212
5.2.5 Gefitinib induces FOXO3a nuclear accumulation and FOXM1 downregulation in breast cancer patient samples	214
5.3 DISCUSSION	219
5.4 FUTURE WORK	222

CHAPTER 6 FINAL DISCUSSION

6.1 Overview	224
6.2 Clinical relevance	227
6.3 Id1 as a therapeutic target	229
6.4 Future perspectives	231

TABLE OF FIGURES

Figure 1.1 Mechanisms of cancer development	33
Figure 1.2 Age standardised incidence and mortality rates of female breast cancer 1975-2006.....	35
Figure 1.3 Structure of the anthracyclines doxorubicin and epirubicin	39
Figure 1.4 Mammalian cell cycle.....	52
Figure 1.5 Signalling through the PI3K/AKT pathway.....	63
Figure 1.6 Structure and phosphorylation sites of FOXO3a	76
Figure 1.7 FOXO subcellular localisation.	79
Figure 1.8 FOXM1 regulation of the cell cycle.	89
Figure 1.9 Id proteins as dominant negative regulators of bHLH transcription factors.	94
Figure 2.1 Optimisation of the FOXO3a antibody for tissue staining	120
Figure 3.1 The expression of FOXO3a in a breast cancer cell line panel	128
Figure 3.2 FOXO3a expression is highest in growth arrested cells.....	129
Figure 3.3 The sensitivity of breast cancer cells to epirubicin. ...	131
Figure 3.4 Epirubicin causes G2M arrest in MCF-7 cells	133
Figure 3.5 Dose dependent effects of epirubicin.....	134
Figure 3.6 The effects of epirubicin on FOXO3a and targets.....	137
Figure 3.7 Epirubicin activates FOXO3a at the transcriptional and gene promoter level.....	138
Figure 3.8 Epirubicin induces FOXO3a nuclear sublocalisation in MCF-7 cells.....	141

Figure 3.9 FOXM1 expression in a panel of breast cancer cell lines	143
Figure 3.10 Epirubicin represses FOXM1 protein and mRNA.....	146
Figure 3.11 Dose dependent effects of epirubicin on FOXM1 and targets.....	147
Figure 3.12 Epirubicin represses FOXM1 promoter activity	148
Figure 3.13 Silencing of FOXO3a protects cells against the cytotoxic effects of epirubicin	150
Figure 3.14 Expression of a constitutively active FOXM1 (Δ N-FOXM1) abrogates the cytotoxic effects of epirubicin.	152
Figure 4.1 The expression of Id1 in a breast cancer cell line panel.	163
Figure 4.2 Expression of Id1 in growth arrested cells.....	165
Figure 4.3 Epirubicin downregulates Id1 transcriptionally and translationally	168
Figure 4.4. Epirubicin represses Id1 at the gene promoter level	169
Figure 4.5 Id1 expression is highest in aggressive cell lines.....	172
Figure 4.6 Id1 expression following epirubicin treatment in Dox-R MCF-7 cells	173
Figure 4.7 Silencing of Id1 slows proliferation of MCF-7 cells	176
Figure 4.8 Silencing of Id1 slows the proliferation of MDA-MB-231 cells treated with epirubicin	178
Figure 4.9 Silencing of Id1 in Dox-R MCF-7 cells introduces some sensitivity to epirubicin	179
Figure 4.10 Silencing of Id1 increases the cytotoxicity of epirubicin	182
Figure 4.11 Silencing of Id1 increases the sensitivity of MCF-7 cells to cisplatin and docetaxel.....	183
Figure 4.12 Cyclin D1 is downregulated following silencing of Id1.	185

Figure 4.13 Overexpression of Id1 confers resistance to epirubicin.	187
Figure 4.14 FOXO3a silencing does not affect Id1 mRNA levels.	190
Figure 4.15 Epirubicin represses both the wildtype and mutated Id1 promoter.....	191
Figure 4.16 Id1 repression by epirubicin is independent of FOXO3a	192
Figure 5.1 The expression of FOXO3a, FOXM1 and Id1 in normal and malignant breast tissue.....	205
Figure 5.2 The expression of FOXO3a, FOXM1 and Id1 in invasive ductal and lobular carcinomas.	207
Figure 5.3 the expression of FOXO3a, FOXM1 and Id1 in ER positive and ER negative invasive ductal carcinoma	208
Figure 5.4 The expression of FOXO3a, FOXM1 and Id1 in HER2 positive and HER2 negative breast cancers.....	209
Figure 5.5 Pre and post FEC chemotherapy staining	211
Figure 5.6 Dose titration of gefitinib in SKBR3 cells.	213
Figure 5.7 Gefitinib treatment correlates with upregulation of nuclear FOXO3a and downregulation of Ki67 and FOXM1 expression in breast cancer patient samples	216
Figure 5.8 Analysis of the relationship between Ki67, nuclear FOXO3a, and FOXM1 expression in breast cancer patient samples	218
Figure 6.1 Schematic representing the role of FOXO3a and Id1 as targets of chemotherapy.....	231

LIST OF TABLES

Table 2.1	List of breast cancer cell lines used	104
Table 2.2	Proportions of the constituents of SDS-PAGE resolving and stacking gels	106
Table 2.3	Antibodies used for Western blotting	108
Table 2.4	Primers used for RT-qPCR.....	111
Table 2.5	Primers used for FOXM1 promoter mutation.	112
Table 2.6	Antibodies and dilutions used for IHC staining	121
Table 5.1	Immunohistochemical staining results with H-scores on Ki67, nuclear FOXO3a and FOXM1.	217

ABBREVIATIONS

APS	Ammonium persulphate
AR	Androgen receptor
bHLH	Basic helix loop helix
β-ME	β-mercaptoethanol
BSA	Bovine Serum Albumin
CDK	Cyclin dependent kinase
CKI	Cyclin dependent kinase inhibitors
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
dNTP	Di-nucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ERα	Oestrogen receptor α
FNA	Fine needle aspiration
5-FU	5-flurouracil
h	Hour
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IKK	IkappaB kinase
JNK	C-Jun-N-terminal kinase
m	Milli
M	Molar
MAPK	Mitogen activated protein kinase
min	Minutes
MnSOD	Manganese superoxide dismutase
NES	Nuclear export signal
NHL	Non Hodgkins Lymphoma
NLS	Nuclear localization signal
NSC	Non-specific control
OS	Overall survival
PBS	Phosphate buffered saline
pCR	Pathological complete response
PK1	Phosphoinositide-dependent kinase
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PLK1	Polo-like kinase 1
pRB	Retinoblastoma protein
PTEN	Phosphatase and tensin homologue detected on chromosome ten
RFS	Relapse free survival

ROS	Reactive oxygen species
RT	Room temperature
s	Seconds
SCLC	Small cell lung cancer
TCA	Trichloroacetic acid
TNF	Tumour necrosis factor
μ	Micro
v/v	volume:volume ratio
w/v	weight:volume ratio

CHEMOTHERAPY REGIMENS

AC	Doxorubicin and cyclophosphamide
CMF	Cyclophosphamide, methotrexate and 5-flurouracil
FEC	5-flurouracil, epirubicin and cyclophosphamide
FAC	5-flurouracil, doxorubicin and cyclophosphamide

CHAPTER 1

INTRODUCTION

1.1 *CANCER – an overview*

Cancer is the second largest cause of mortality in the Western world after cardiovascular disease and was responsible for 27% of all deaths in the UK in 2005 - 29% for males and 25% for females (Mortality Statistics: Cause England & Wales, 2006.) More than 200 distinct varieties of cancer are recognised but the four most common causes of cancer - lung, bowel, breast and prostate cancer account for the majority of deaths seen.

Cancers are heterogeneous diseases, where multiple genetic alterations within cells leads to transformation of normal cells into cancer cells. At the core of this process are cancer causing or oncogenic mutations in critical genes, which cause sustained proliferation in cells and leads to escape from the normal cues that will inhibit cell growth or promote cell death (Luo and Elledge, 2008). Tumourigenesis and tumour progression in humans is a multistep process, requiring a number of genetic alterations that drive the progressive transformation of normal cells, via a series of premalignant states, into highly invasive derivatives. These steps include self sufficiency to growth signals, insensitivity to growth inhibitory signals, limitless replicative potential, evasion of programmed cell death (apoptosis), sustained angiogenesis, tissue invasion and metastasis - all of which can give rise to the cancer genotype that collectively dictates malignant growth (Hanahan and Weinberg, 2000).

1.1.1 *Oncogenes and tumour suppressors*

Oncogenes are genes whose inappropriate activation can lead to cancerous or oncogenic transformation of cells. Their protein products are often regulators of cell growth and proliferation

and are involved in signalling pathways controlled by feedback mechanisms (Pollard and Earnshaw, 2008). The first oncogene identified was *RAS* and the *RAS* gene is now known to be mutated in more than 30% of human cancers. The small GTPase proteins of the *RAS* family are signalling molecules which are normally activated by growth factor receptors to promote cell proliferation – single nucleotide mutations in their coding sequences can lead to constitutive activation, resulting in deregulated cell growth and cell death and the ability to induce new blood vessel formation (Downward, 2003). Three members of the *RAS* family are found to be activated by mutation in human tumours – *HRAS*, *KRAS* and *NRAS*. These proteins are normally highly expressed, with *KRAS* being found in virtually all cell types and it is in *KRAS* that point mutations are most frequently seen (85% of total *RAS* mutations). *RAS* signalling pathways are commonly activated in cancers, especially those which overexpress growth factor receptor tyrosine kinases, the most common examples being *ERBB2* (human epidermal growth factor receptor 2/*HER2/neu*) and *EGFR* (epidermal growth factor receptor 1). Downstream of *RAS* is the protein serine/threonine kinase *RAF*. GTP-bound *RAS* binds to and activates *RAF*, causing its relocation to the plasma membrane where it can initiate the mitogen-activated protein kinase (*MAPK*) cascade, leading to *ERK* activation. *RAS* can also activate the phosphatidylinositol-3-kinase (*PI3K*) pathway (Downward, 2003). Therefore, aberrant *RAS* signalling can lead to continuous activation of pathways promoting cell cycle progression and survival, a key component of malignant transformation.

Myc has been identified as another potent oncogene and deregulated expression of *c-Myc* has been identified in a broad

range of human cancers and is often associated with a poor prognosis (Pelengaris et al., 2002). The transcription factor c-Myc orchestrates the expression of up to of 15% of all genes and its targets are involved in the cell cycle, protein synthesis, metabolism and survival (Hoffman and Liebermann, 2008). C-Myc is widely expressed during embryogenesis and is found in tissues with high proliferative rates such as the skin and gut, where its expression strongly correlates with cell proliferation (Pelengaris et al., 2002). With multiple extracellular and intracellular signalling cascades converging to regulate Myc, activation of these pathways, often seen in many cancers, can induce Myc overexpression and lead to the transcription of its target genes, promoting genomic instability, proliferation, angiogenesis and blocking differentiation (Ponzielli et al., 2005).

Tumour suppressors are genes whose inactivation can lead to cancerous transformation as their protein products typically inhibit products of oncogenes or negatively regulate cell proliferation. Two of the most well characterised tumour suppressors are the retinoblastoma protein (pRb) and p53. The Rb tumour suppressor has a central role in enforcing cell cycle checkpoints, which are activated in response to intra and extracellular stresses, including DNA damage, growth factor deprivation and nucleotide depletion (Macleod, 2008). There are three mammalian Rb-related proteins – pRb, p107 and p130 – and these proteins regulate a family of transcription factors known as E2F. Rb regulates the ability of E2F to activate genes required for progression through the cell cycle as complexes between Rb family members and E2Fs actively repress gene transcription by associating with the promoter regions of target genes, blocking transcription (Sherr and McCormick, 2002). Rb can also recruit histone deacetylases (HDACs), which cause

compaction of chromatin structure, thus repressing genes required for cell cycle progression. However, phosphorylation of Rb by cyclin dependent kinases (Cdks) prevents the association of Rb with both HDACs and E2Fs, enabling E2F dependent gene transcription, particularly those required for DNA replication. A cell that can phosphorylate Rb can pass through the cell cycle, whereas cells that cannot remain arrested (Alberts et al., 2008).

Rb is one of the most characterised tumour suppressors – loss of Rb can lead to inappropriate cell cycle progression, rendering cells independent of growth factor stimulation and insensitive to antigrowth factors, such as TGF β , that can block the cell cycle. Mutations occur frequently in the Rb pathway through mutation, deletion or epigenetic silencing. Individuals who inherit one defective *Rb* gene in the germ line and then develop a somatic mutation in the remaining normal allele often develop retinoblastomas as children and osteosarcomas as adults, the classic example of a cancer caused by loss of a tumour suppressor gene. Rb inactivation is seen in a variety of cancers, including breast, prostate, colon and lung cancer (Sherr and McCormick, 2002).

p53 is a transcription factor that plays a central role in the cellular response to a variety of stress signals, including DNA damage, hypoxia and the presence of oncogenic activation, including mutant RAS and Myc. p53 prevents cells from entering or progressing through the cell cycle in conditions that could perpetuate or generate DNA damage and it is essential for effective tumour suppression in humans (Toledo and Wahl, 2006). Mutations in *TP53*, the gene that encodes p53, are found in 50% of human cancers and in those tumours that do not

have mutated p53, a large proportion have inactivated p53 function via other mechanisms, including disrupted p53 signalling pathways. A germline mutation in p53 can cause Li Fraumeni syndrome, characterised by an abnormally high and early incidence of cancer. *p53* null mice are viable but nearly all develop cancers before six months of age (Horn and Vousden, 2007).

In contrast to Rb, most cells have minimal levels of p53 protein under normal conditions, as it is rapidly degraded and is negatively regulated by MDM2 ubiquitin ligase which binds it and targets it for destruction by proteosomes. However, under conditions of cellular stress or DNA damage, the protein kinase ATM stabilises and activates p53 by phosphorylation and the nuclear levels of p53 increase dramatically, where it becomes a potent transcriptional activator (Pollard and Earnshaw, 2008). Depending on the cell type, cell environment and oncogenic alteration, this activation of p53 leads to induction of senescence, inhibition of cell cycle progression, differentiation or apoptosis – thus allowing p53 to exert its tumour suppressor function (Pietsch et al., 2008).

Over time, cells lacking the p53 regulated damage response accumulate cancer causing mutations. The most common mutations observed in p53 in human tumours are in its DNA binding domain, preventing p53 from binding to its DNA target sequences. p53 dependent stress responses are abolished and cells can escape apoptosis and continue to proliferate with a corrupted genome. This can lead to loss of other tumour suppressor genes, such as p21^{Cip1} and p27^{Kip1}, and to activation of oncogenes. Loss of p53 can also make cancer cells less sensitive to irradiation and cytotoxics. Drugs and radiation used

commonly in the treatment of cancer can induce p53 in normal cells, contributing to their own destruction and hence treatment related toxicity. However, $p53^{-/-}$ cells are resistant to drug induced apoptosis and mutations in p53 or in the p53 pathway can produce multidrug resistance *in vitro* and *in vivo* (Johnstone et al., 2002).

1.1.2 Epigenetics in cancer

Epigenetics is defined as heritable changes in gene expression that are not due to an alteration in the DNA sequence. The abnormal epigenetic silencing of genes is equally as important as mutations in DNA sequences in the development of many cancers (Alberts et al., 2008). Genetic and epigenetic changes can cause oncogenes to be activated by chromosome gains, amplification of genes, chromosomal translocations and activating point mutations. Similarly, tumour suppressor genes can become inactivated by loss of whole chromosomes, point mutations and deletions.

Epigenetic changes can involve covalent modifications of the amino acid residues found in histones wrapped around DNA but the best known epigenetic marker is DNA methylation. During embryonic development, DNA methylation patterns are important in cellular growth and differentiation and occur in cytosine residues that precede guanines – dinucleotide CpGs, with the “p” referring to the phosphodiester bond between the cytosine and guanine nucleotides. These CpG sites are not just randomly distributed but are found in CpG rich regions called CpG islands, spanning the 5′ regulatory end of many genes. In normal cells, these islands are not usually methylated and can be found in up to 40% of the promoters of human genes (Esteller, 2008).

CpG methylation plays an important role in maintaining normal gene silencing, and methylated cytosines in gene promoters are associated with chromatin from which transcription is blocked – essential for monoallelic expression of genes and X-chromosome inactivation (Kirsten, 2007). Modification of histone proteins also occurs with DNA methylation. Core histones can normally be modified post-translationally in a variety of ways, including acetylation, phosphorylation, methylation and ubiquitination (Pollard and Earnshaw, 2008). Their long amino terminal ends protrude outside the nucleosome and can interact with various nuclear factors which bind modified histones and dictate whether particular regions of chromatin are transcribed or held in an inactive state. This is essential for the normal physiological activity of cells and tissues. However, in cancers, methylated cytosine residues can alter the coding regions of genes by introducing point mutations and causing genomic instability. Focal hypermethylation of gene promoters can inactivate gene transcription, thus silencing key genes involved in the cell cycle, DNA repair, angiogenesis, metabolism of carcinogens and apoptosis, allowing neoplastic transformation (Esteller, 2008). Changes in the DNA methylation pattern of the cell result in aberrant histone modification profiles. Hypoacetylation and hyperacetylation of histones and the appearance of repressive histone markers resulting from hypermethylated CpG islands, can lead to the silencing of genes with tumour suppressor like functions and promote the development of tumours (Esteller, 2008; Esteller and Almouzni, 2005).

CpG hypermethylation at the promoters of tumour suppressor genes is likely to be the most common mechanism of tumour suppressor inactivation in cancer. There are many classical

tumour suppressor genes which are mutated in the germline of patients with hereditary cancers, giving rise to specific cancer syndromes and these genes can also be inactivated by methylation in sporadic tumours. Examples include *RB1* and *BRCA1*, where hypermethylation gives rise to the sporadic variant of retinoblastoma and breast cancer (Kirsten, 2007). Promoter hypermethylation is seen in a wide variety of other cancers, including colon, pancreas, lung and ovarian cancers and may have potential use in the development of novel cancer therapies. DNA methylation and histone modifications are reversible and demethylating agents have been used to re-express DNA methylated genes in cancer cell lines: the drug decitabine, a DNA demethylator, has been approved in the treatment of leukaemia and myelodysplastic syndrome. Similarly, a HDAC inhibitor, SAHA, which can induce cell cycle arrest and promote apoptosis *in vitro*, has been approved for the treatment of cutaneous T-cell lymphoma (Mack, 2006)

1.1.3 Chromosomal abnormalities and cancer

Cytogenetic abnormalities are a characteristic often seen in cancer cells and clonal chromosome aberrations are found in most major tumour types. Chromosomal abnormalities in cancer can be broadly classified into two main types – chromosomal rearrangements and chromosomal imbalances (Frohling and Dohner, 2008).

Chromosomal rearrangements seen in cancer include translocations, inversions and insertions. These rearrangements often have functional consequences, leading to the formation of a chimeric fusion gene with altered or new activity or chromosomal changes that cause altered expression of a normal gene, by bringing genes closer to enhancer or promoter

elements (Nambiar et al., 2008). Initially seen most commonly in haematological cancers, rearrangements can also play a role in the pathogenesis of epithelial cancers.

The most common chromosomal translocations result in the formation of a chimeric gene, via the fusion of parts of two genes. The genes most commonly involved are those coding for tyrosine kinases and transcription factors. The most well described chimeric fusion gene is the Philadelphia chromosome, found in most patients with chronic myeloid leukaemia (CML) where there is a reciprocal translocation between chromosome 9 and 22, in which sequences of the *Bcr* gene are joined to portions of the gene encoding the Abl tyrosine kinase. This results in the chimeric fusion protein Bcr-Abl, resulting in constitutive activation of tyrosine kinase activity. The discovery of the Philadelphia chromosome led to one of the biggest breakthroughs in cancer therapeutics over the last twenty years, namely the development of the selective tyrosine kinase inhibitor imatinib, a drug which has revolutionised the treatment of CML.

Chromosomal imbalances, where there is gain or loss of genetic material, are also seen in cancers. Changes in the number of whole chromosomes lead to genetic instability and gains (polysomy) and losses (aneusomy) are seen in a number of epithelial cancers, leading to activation of oncogenes and inactivation of tumour suppressors (Reinholz et al., 2009). Genomic gains can enhance the activity of genes in the affected chromosomal region. One of the most well known gains is amplification of the gene on band 17q21.1 encoding the ERBB2 receptor kinase – of interest in particular in breast cancer, as a specific target for the monoclonal antibody herceptin

(trastuzumab). Other examples of gene amplifications include those encoding for EGFR and KRAS, which are seen in a variety of cancers (Frohling and Dohner, 2008). Extensive genomic deletions, which can affect multiple genes, are also frequently seen in tumours, including deletions affecting *PTEN*, the PI3K pathway inhibitor, and the previously mentioned *Rb* and *TP53*.

1.1.4 DNA damage and cancer

Human cells experience a large amount of DNA damage events every day and cell division must not occur with damaged genomes or inaccurate replication as this can lead to cell death and mutations. Cells have evolved a number of systems to repair damaged DNA and the activity of these pathways depends on the type of DNA damage inflicted on the cell (Pollard and Earnshaw, 2008). In response to stress or DNA damage, cells possess a number of DNA damage checkpoints, which can arrest the cell cycle allowing cells the chance to repair their genome. Cancer is associated with a disrupted DNA damage response and impaired DNA repair pathways, making cells dependent on a reduced and more error prone set of DNA repair mechanisms (Helleday et al., 2008).

There are a number of mechanisms by which cells can repair DNA damage. When both strands of the double DNA helix are broken – double strand breaks – there is no intact template left to ensure accurate repair. This can be caused by radiation, free radicals or chemicals. Two mechanisms exist to overcome this: non homologous end joining and homologous recombination. In the former, there is connection and resealing of the two ends of a DNA double strand break by DNA ligation, often with the loss of one or more nucleotides at the site of joining (Alberts et al., 2008). Homologous recombination is a much more accurate

type of repair, which can copy the DNA sequence, using the sister chromatid as a template, thus repairing or bypassing replication lesions (Helleday et al., 2008).

Mismatch repair is a process which occurs during DNA replication to correct base pairing errors, such as insertion or deletions, not detected by the proofreading activity of DNA polymerase. When a mismatch is detected, a complex of mismatch repair proteins can identify this mismatch, excise the DNA strand involved and resynthesize the missing strand. Defects in mismatch repair, where defects go unrepaired, can lead to microsatellite instability. Microsatellites are repeated sequences of DNA, varying in length and appearing normally throughout the genome. When DNA repair genes are mutated, these sequences can accumulate errors, become shorter, longer and unstable, hence the term microsatellite instability. Mutations cause replication errors to go unfixed, and the resulting frameshift mutation or epigenetic changes can switch off key tumour suppressor genes, promoting tumourigenesis (de la Chapelle, 2003).

Damage to individual DNA bases can also be repaired by base excision repair, where the altered base is removed and replaced by a DNA glycosylase enzyme, or by nucleotide excision repair, where sections of the damaged DNA strand is removed as an oligonucleotide from the double helix and the opposite strand used as a template for repair (Alberts et al., 2008).

DNA repair pathways are not only important in the development of cancer, but can also be targeted in the treatment of cancer. Several chemotherapeutic agents produce excessive DNA damage which can cause cell cycle arrest and death either directly or following S phase DNA replication, where lesions can

obstruct replication fork progression leading to lethal double strand breaks (Helleday et al., 2008). Drugs such as the platinum agent cisplatin and carboplatin can cause chemical modifications of DNA bases, known as adducts. By cross-linking DNA with proteins or cross-linking two DNA bases within the same strand of DNA, replication forks are blocked. Antimetabolites, such as 5-fluorouracil (5-FU), inhibit nucleotide metabolism pathways, by depleting cells of dNTPs by mimicking nucleotides, their precursors or co-factors which are required for normal nucleotide biosynthesis (Swann et al., 1996).

DNA strand breaks can arise naturally during DNA synthesis and these can also be exploited and targeted by anticancer agents. The topoisomerase enzymes induce transient DNA breaks, relaxing supercoiled DNA which allows resolution of torsional strains imposed on the DNA double helix during replication. Topoisomerase inhibitors, such as the anthracyclines, can prevent resealing of these breaks by trapping the enzymes in complexes with the DNA (Wang, 2002). Thus, tumour development can be associated with disrupted DNA damage repair pathways and response, but these pathways can also be exploited by chemotherapeutic agents to produce excessive cell damage and subsequent death of tumour cells.

In summary, there are multiple mechanisms and events that can lead to the transformation of normal cells into cancerous cells (Figure 1.1).

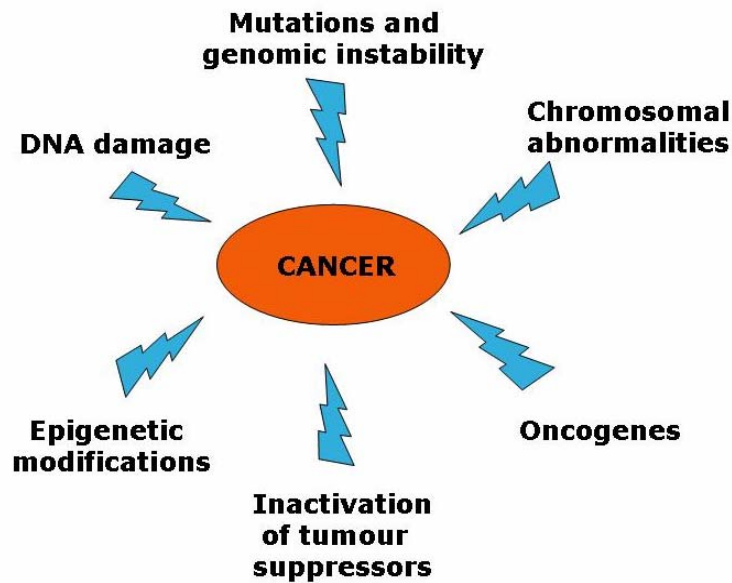


Figure 1.1 Mechanisms of cancer development

1.2 BREAST CANCER

Breast cancer has now become the most prevalent cancer in the UK, despite its low incidence in men. In 2005, over 45000 new cases were diagnosed and in women there is a one in nine lifetime risk of developing the disease (Cancer Research UK). Several risk factors for breast cancer development have been identified, many of which relate to reproductive events. These include early menarche, nulliparity, late age at first birth and late natural menopause. These risks may arise from the cumulative exposure of breast tissue to endogenous oestrogens. The use of exogenous hormones may also increase the risk of breast cancer, with an association seen between the use of hormone replacement therapy and the development of breast cancer. The risk of breast cancer for current or recent users of HRT increases per year of use. A strong family history of breast

cancer is another known risk factor, particularly when first degree relatives have been diagnosed, although in most cases there is no family history of the disease. Perhaps the most well defined genes where mutations have been found to substantially increase the risk of breast cancer are *BRCA1* and *BRCA2*, both discovered in the mid 1990's (Futreal et al., 1994; Wooster et al., 1995). These cancers tend to be associated with high histological grade, inactivation of p53 and *BRCA1* tumours in particular are often ER negative (Tutt and Ashworth, 2008). These two genes however are very different in terms of their structure and function. *BRCA1* is involved in chromatin remodelling, regulation of transcription and also has a key role in the DNA damage response, whereas *BRCA2* maintains genome stability through DNA repair by homologous recombination (Ashworth, 2005; Gudmundsdottir and Ashworth, 2006). When the function of these genes is lost, and defective DNA repair and genomic instability results, mutation associated breast and ovarian cancers can develop. However, most breast cancers do not have a known genetic predisposition and by far the strongest risk factor for breast cancer (after gender) is age: the older the woman, the higher her risk and more than 80% of breast cancer cases are seen in women aged 50 and over.

The incidence of breast cancer has continued to increase over the last twenty years, but survival rates are improving and now more than 80% of newly diagnosed patients can expect to survive beyond five years. The earlier the stage at diagnosis, the better the outcome - hence the importance of detecting cancers at an early stage, when treatment is more likely to be curative (Figure 1.2).

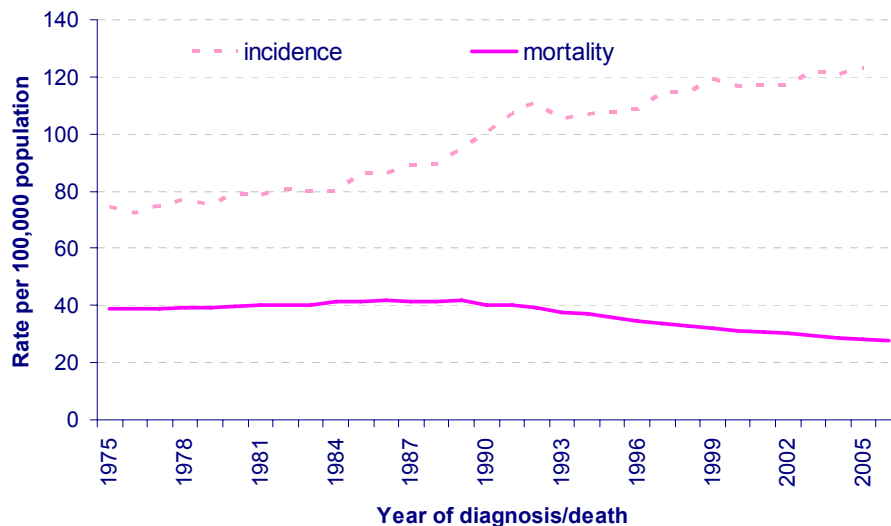


Figure 1.2 Age standardised incidence and mortality rates of female breast cancer 1975-2006. (Cancer Research UK)

1.2.1 Presentation and diagnosis

The breast cancer screening programme was set up in 1988 and uses mammography to screen all women between 50 and 70 who are registered with a GP in the UK. The screening programme diagnoses around 10000 cases of breast cancer each year (Cancer Research UK). However, the vast majority of breast cancers are self-detected.

A diagnosis is usually made following triple assessment – clinical examination, imaging and core biopsy/fine needle aspiration (FNA). This allows a decision to be made about the appropriate primary medical treatment, whether it be surgery in the form of wide local excision or mastectomy, or in the case of large or locally advanced cancers, neo-adjuvant treatment. Breast cancers can be categorised into low, intermediate and high risk depending on a number of predictive and prognostic factors. Patients at low risk of recurrence include those with small, low grade tumours, lymph node negative, HER2 negative and age >

35 years. Risk of relapse increases with higher histological grade, tumour size, nodal involvement, HER2 positivity and younger age (Smith and Chua, 2006). Thus, the medical management a particular patient receives will depend on a number of factors, including the characteristics of the tumour itself and the risk of recurrence.

1.3 MEDICAL MANAGEMENT OF BREAST CANCER

The management of breast cancer involves a multimodality approach with many women receiving a combination of medical options including surgery, endocrine treatment, chemotherapy, radiotherapy and targeted agents. Amongst these, it is undoubtedly the use of endocrine treatment and adjuvant chemotherapy that has contributed most to the improved survival rates seen in breast cancer over the last twenty years.

1.3.1 The oestrogen receptor (ER)

The ER belongs to a superfamily of nuclear hormone receptors, which classically function as transcription factors when bound to their ligands in the nucleus. The two main forms of ER - ER α and ER β - have similar structures but are the products of different genes (Osborne and Schiff, 2005). ER plays a role in the proliferation, differentiation and survival of both normal and malignant breast epithelial cells (Madureira et al., 2006). Classical (genomic) activity of ER involves protein activation by phosphorylation when oestrogen binds to ER α . This also causes a conformational change and dissociation of chaperone proteins such as heat-shock protein 90. Hormone bound ER can then dimerize with another receptor, interact with coregulatory proteins and bind to oestrogen response elements (ERE) found

in the promoter area of target genes leading to the transcription of these oestrogen responsive genes (Osborne and Schiff, 2005). Many ER regulated genes are involved in cell cycle progression, including *cyclin D1* and in survival, such as the anti-apoptotic *Bcl-2* (Madureira et al., 2006).

ER can also have non-genomic activity and can mediate transcription through ligand independent effects. Several kinases in the growth factor signaling pathways can phosphorylate ER, largely on serine residues in the AF1 region of ER α creating crosstalk between ER and other signalling pathways. This may be important in the development of endocrine resistance – increased growth factor signalling may lead to the loss of oestrogen dependence and an increase in ligand independent effects. Endocrine resistance may also develop due to ER mutations or disrupted coregulator expression or recruitment (Pearce and Jordan, 2004).

1.3.2 Endocrine treatment

Oestrogens have been implicated in the development of breast cancer for many years and endocrine therapy has become an integral part in the treatment of cancers expressing the oestrogen receptor (ER). These drugs block ER function, but have different mechanisms to achieve their effects. Tamoxifen, a selective ER modulator (SERM), binds ER and partially antagonizes its activity. The third generation aromatase inhibitors, such as anastrozole and exemestane, block the peripheral synthesis of oestrogen and inhibit ligand-induced activation of ER and steroidal antioestrogens such as fulvestrant bind ER and can induce receptor degradation (Osborne and Schiff, 2005).

Historically, tamoxifen has been the most commonly used endocrine agent in the adjuvant setting and is suitable for both pre and post menopausal women. The Oxford overview of 2005 confirmed that in ER positive breast cancers, five years of adjuvant tamoxifen reduces the annual breast cancer death rate by 31%, largely irrespective of the use of chemotherapy and of age (EBCTCG, 2005). Additional benefits were seen in patients receiving both chemotherapy and 5 years of tamoxifen, as discussed later in this chapter.

The emerging role of aromatase inhibitors has been addressed by a number of influential trials. In postmenopausal women, the Arimidex, Tamoxifen Alone or in Combination (ATAC) trial compared 5 years of anastrozole with five years of tamoxifen – initially this showed a small disease free survival benefit, but the 100 month follow-up analysis failed to show a survival advantage in favour of anastrozole (Forbes et al., 2008). However, a recent update of the Breast International Group (BIG) 1-98 trial has shown a trend towards better overall survival in patients receiving letrozole as compared with tamoxifen (Coates et al., 2007). The Intergroup Exemestane Study (IES) compared exemestane versus 2-3 years of tamoxifen followed by exemestane. The results suggested early improvements in disease free survival in the patients who switched over to an aromatase inhibitor, translating into a modest reduction in risk of death (Coombes et al., 2007). Further studies are ongoing to elucidate the optimum endocrine treatment strategy.

1.3.3 The anthracyclines

The first anthracyclines, including doxorubicin, were extracted from the pigment producing *Streptomyces peucetius* in the 1960's. They have a characteristic four ring structure (rings A-D), linked to an amino sugar via a glycoside bond (Moro et al., 2004). Epirubicin, a semi-synthetic derivative of doxorubicin, is structurally modified, with an axial to equatorial epimerization of the hydroxyl group at C-4' (Minotti et al., 2004) (Figure 1.3).

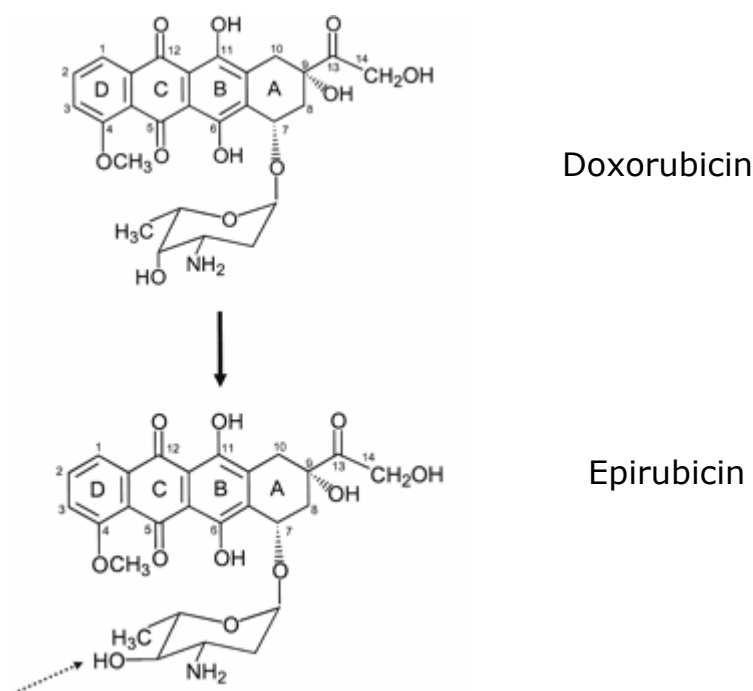


Figure 1.3 Structure of the anthracyclines doxorubicin and epirubicin (Minotti et al, 2004).

Although this positional change does not have much of an effect of the mode of action of epirubicin when compared to doxorubicin, it does introduce metabolic and pharmacodynamic changes such as increased volume of distribution and shorter terminal half-life (Danesi et al., 2002; Robert and Gianni,

1993). These differences may contribute in part to the fact that greater cumulative doses of epirubicin can be tolerated by patients than those of doxorubicin and that epirubicin is less cardiotoxic.

The anthracyclines are some of the most widely prescribed chemotherapy agents and are used in the treatment of a variety of cancers. Their primary cellular target is the topoisomerase enzymes. These are ubiquitous enzymes with a key role in DNA replication and transcription as well as chromosome structure and segregation (Froelich-Ammon and Osheroff, 1995). Topoisomerases can modify DNA topology without any alterations in sequence or deoxynucleotide structure. There are two main classes – topoisomerase I and topoisomerase II, with the former causing transient single strand DNA breaks and the latter leading to double strand breaks, both of which can be resealed. DNA double helix supercoiling is modulated throughout the different phases of the cell cycle and depends on transcriptional activity, hence the important role of topoisomerases in DNA replication (Binaschi et al., 2001).

Topoisomerase II in particular appears to be the main target for the anthracyclines. As a multi-subunit protein, it requires ATP for its catalytic activity and creates breaks in the DNA backbone, by passing an intact DNA helix through a transient double strand break, thereby regulating winding of the double helix and resolving nucleic acid tangles and knots (Froelich-Ammon and Osheroff, 1995). The anthracyclines are topoisomerase II poisons, stabilising a reaction where DNA strands are cut and linked covalently to topoisomerase II by tyrosine residues. Thus, an anthracycline-DNA –topoisomerase complex is formed, preventing DNA resealing – in effect, a transient double strand

break becomes permanent. The resulting unrepaired breaks lead to a wide range of toxic effects on the cell and damage to the genome, including mutations, insertions, deletions and chromosomal aberrations (Minotti et al., 2004).

Following topoisomerase II inhibition, the subsequent mechanisms of anthracycline induced cytotoxicity remain unclear, but it is likely a number of mechanisms are involved. The proteasome plays a crucial role in the normal turnover of cytosolic and nuclear proteins, including those involved in cell growth and metabolism. The proteasome has a role in modulating anthracycline activity, by transporting the drug from the cytoplasm into the nucleus, where it can then bind DNA and exert its cytotoxic effect (Adams, 2003; Kiyomiya et al., 2002). Once in the nucleus the anthracyclines can activate p53-DNA binding, thus leading to cell cycle arrest and p53 mediated apoptosis (Ruiz-Ruiz et al., 2003). Reactive oxygen species (ROS) are generated early on following anthracycline administration and these can also modulate the activity of several kinases and transcription factors associated with cell cycle progression and apoptosis (Laurent and Jaffrezou, 2001). Anthracyclines can directly release cytochrome c from mitochondria inducing apoptosis and they can also activate downstream cell death effector-mediated pathways and downregulate activity of the PI3K/Akt pathway. Thus, anthracycline induced apoptosis brings into play a wide, complex network of cellular events, many of which are not yet fully understood (Laurent and Jaffrezou, 2001; Martin et al., 2002).

1.3.4 *The anthracyclines and early breast cancer*

For nearly thirty years, the anthracyclines have been pivotal in the management of early stage, operable breast cancer. Improvements have been seen in adjuvant chemotherapy as a result of their incorporation into non-cross resistant regimens such as cyclophosphamide, methotrexate and fluorouracil (CMF), their combination with the taxanes, dose-dense administration and new scheduling approaches (Gianni and Valagussa, 2009).

Evidence for the benefit of adjuvant chemotherapy in breast cancer first emerged in the 1970's, where Bonnadonna and colleagues first published data on the benefits of CMF as adjuvant treatment for node positive breast cancer. Their data showed that twelve months of postoperative chemotherapy with CMF decreased the risk of recurrence in patients with positive axillary lymph nodes (Bonnadonna et al., 1976). Long term follow up (median observation period 28.5 years) from this initial pivotal trial has confirmed that both relapse free survival and overall survival remained significantly superior in women receiving adjuvant CMF (29% and 25%) than in those treated with surgery alone (22% and 16%). CMF contributed to reducing the relative risk of disease relapse by 34% and of death from all causes by 22% (Bonadonna et al., 2005). The main therapeutic effect of adjuvant CMF was to reduce the incidence of distant metastases (absolute difference of 11% in long term analysis). Thus developed the theory that micrometastases, which are not clinically detectable, may be present at the time of surgery on the primary tumour and are unaffected by local treatment. Operable breast cancer could therefore be considered a systemic disease at presentation and

therapy directed at the primary tumour fails to affect micrometastases which may disseminate, ultimately leading to metastatic disease. It is these micrometastases that are targeted by adjuvant chemotherapy.

Once a clear benefit for the use of adjuvant chemotherapy had been established, the additional benefits of including an anthracycline were addressed. The use of doxorubicin, which had good activity in advanced breast cancer, was investigated in combination with cyclophosphamide and proved to have equivalent efficacy and greater tolerability than CMF. The National Surgical Adjuvant Breast and Bowel project (NSABP) studies B-15 and B-23 found equivalence between four cycles of doxorubicin and cyclophosphamide (AC) and six cycles of CMF in terms of event free survival, relapse free survival (RFS) and overall survival (OS), regardless of nodal status, age or ER status. However, AC offered the advantages of fewer side effects and a shorter treatment course (Fisher et al., 2001). Subsequent trials looked at the combination of 5-fluorouracil, epirubicin and cyclophosphamide (FEC), replacing doxorubicin with the less cardiotoxic epirubicin. In the MA5 trial, pre- or post-menopausal women received either FEC or CMF and there was a statistically significant improvement in RFS and OS in the FEC arm, which has subsequently been maintained throughout a ten year follow up period (Levine et al., 2005). The meta-analysis reported by the Early Breast Cancer Trialists' Collaborative Group in 1998 confirmed that anthracycline containing regimens were associated with significant reductions in the rates of recurrence and death when compared to CMF (EBCTCG, 1998).

Up until this point, many trials had looked at the incorporation of an anthracycline, whether doxorubicin or epirubicin, into a CMF regimen. The National Epirubicin Adjuvant Trial (NEAT) and the BR9601 trial were designed to determine the value of anthracyclines in the adjuvant setting, paying particular importance to their scheduling. The NEAT trial compared four cycles of epirubicin followed by four cycles of CMF with six cycles of CMF alone. BR9601 compared four cycles of epirubicin followed by four cycles of CMF with eight cycles of CMF alone. The primary end points were RFS and OS. Both trials, involving 2391 women in total, confirmed a superiority of epirubicin plus CMF over CMF alone. 5 year RFS was 76% vs. 69% and overall survival was 82% vs. 75% ($p < 0.001$). The incidence of adverse effects was higher with epirubicin plus CMF than with CMF alone, but this did not affect the dose delivered intensity or quality of life (Poole et al., 2006).

Numerous other trials have looked at the benefits of adjuvant therapy, whether involving CMF, FEC or FAC. The Oxford overview from 2005, performed by the EBCTCG, perhaps gives the most up-to-date analysis of all the trial data available. Collaborative meta-analyses were undertaken of 194 unconfounded randomised trials of adjuvant chemotherapy that began by 1995. The results combined three chemotherapy meta-analyses: anthracycline-based versus no chemotherapy (8000 women); CMF-based versus no chemotherapy (14000 women) and anthracycline-based versus CMF-based chemotherapy (14000 women). The findings of the overview confirmed that six months of adjuvant anthracycline based chemotherapy (FAC or FEC) reduces the annual breast cancer death rate by 38 % for women <50 years of age and by 20%

for those of age 50-69. Such regimens were significantly more effective than CMF chemotherapy (EBCTCG, 2005).

1.3.5 *Neo-adjuvant chemotherapy*

The use of chemotherapy before definitive surgery (neo-adjuvant chemotherapy) began in the late 1970's and has now become a well established treatment option in the management of large and potentially operable locally advanced breast cancers. The rationale behind this treatment is to allow more women to have breast conserving surgery - if large tumours can be downsized with pre-operative chemotherapy, the need for mastectomy may be avoided. Most patients respond to treatment (60-90%). Also, the tumour itself can be used as a measure of response to treatment in vivo and achievement of a complete pathological response (pCR), which is seen in 3-16% of patients, is a good predictor of long term survival (Jones and Smith, 2006). Randomised clinical trial data has shown that women who have large, operable tumours and who receive neo-adjuvant chemotherapy have similar survival to patients treated with adjuvant chemotherapy. One of the largest of these trials, the National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-18, randomised 1523 women to either pre-operative or post-operative doxorubicin and cyclophosphamide (Fisher et al., 1998) and at a follow up point of nine years, no significant difference was seen between the two groups in terms of either disease free survival or overall survival. A 2005 overview has confirmed that neo-adjuvant and adjuvant treatment are equivalent in terms of survival and overall disease progression. (Mauri et al., 2005)

With the development of the taxanes, trials have been designed to look at the addition of paclitaxel and docetaxel in the neo-adjuvant setting. In the NSABP B-27 trial, 2411 women were randomised to either four cycles of pre-operative doxorubicin and cyclophosphamide, followed by four cycles of docetaxel either pre or post-operatively (Bear et al., 2006). Outcome was best in the group receiving both the anthracycline and taxane containing regimen pre-operatively, with a higher clinical complete response (63.6%) and pCR (26.1%) when compared to the other two groups (40.1% and 13.7% respectively).

Detection of response to neo-adjuvant chemotherapy is useful for a number of reasons. Early signs of response can be used as a predictor of pCR, a predictor of long-term outcome and in a decision aid to identify patients who may require a switch in systemic treatment (Untch and von Minckwitz, 2009)

1.3.6 *Predictors of response to chemotherapy*

Decision making regarding the use and benefit of both adjuvant and neo-adjuvant chemotherapy depends on a set of clinical criteria including axillary lymph node status, tumour size, lymphovascular invasion, grade and hormone receptor status. However, with the advent of new biological and targeted treatments and the development of new molecular techniques it still remains difficult to clearly identify those patients who will benefit from chemotherapy whilst on the other hand heavily over treating individuals who may derive little benefit from systemic therapy (McGovern and Stebbing, 2009).

The presence of the oestrogen receptor (ER) predicts to some extent the response of breast cancer to endocrine treatment.

Patients with tumours which are also strongly positive for the progesterone receptor (PgR) have the highest likelihood of response (Ravdin et al., 1992). ER and/or PgR expression is an independent prognostic factor in breast cancer (Burcombe et al., 2004). However, in terms of benefit from chemotherapy, it appears that ER negative tumours tend to respond better to chemotherapy, rather than ER positive tumours. Studies have also shown that the likelihood of achieving a pathological complete response (pCR) with primary chemotherapy, a good surrogate for long-term survival in the neo-adjuvant setting, is also significantly higher in ER negative tumours (Tewari et al., 2008). Thus, patients who have ER negative cancers likely derive the most benefit from adjuvant chemotherapy.

HER2 expression can be used as a predictor of response to chemotherapy. Some studies have shown that patients who have *HER2* positive tumours will benefit particularly from an anthracycline containing chemotherapy regimen although the exact mechanism for this remains unclear. There is close proximity of the *HER2* gene to the *TOP2A* gene located on chromosome 17q and topoisomerase II is the main target of the anthracyclines, amongst other proteins (Pritchard et al., 2008). A number of studies have suggested that deletion or overamplification of *TOP2A*, perhaps closely associated with coamplification of *HER2*, leads to a worse prognosis but greater response to anthracycline containing regimens. Using samples from the MA.5 trial, which compared CEF chemotherapy to CMF, patients who showed *TOP2A* alterations (either amplifications or deletions) had an increased responsiveness to the anthracycline containing regimen, similar to that seen with *HER2* amplification (O'Malley et al., 2009).

The absence of the expression of certain receptors can also be a predictor of chemotherapy response. A number of different pathological subclasses of breast cancer have been identified, based on gene expression profiles, including triple negative breast cancers i.e. breast cancers which do not express ER, PgR or HER2. This subtype comprises approximately 15-20% of breast cancers and the majority of triple negative cancers have basal like expression profiles, expressing cytokeratins 5, 14 and 17, normally found in the cell layer lying closest to the epithelial basement membrane (Irvin Jr and Carey, 2008). Similar markers are also expressed in BRCA1-associated cancers. Tissue microarray studies have shown a high rate of EGFR overexpression in these triple negative cancers (Colleoni et al., 2008) and c-kit expression is also higher. Interestingly, these tumours are sensitive to chemotherapy, and the pCR rate may be higher in these cancers than some of the other subtypes. However, they have a high risk of relapse in contrast to other cases with pCRs. The association of these triple negative tumours with BRCA1 mutation carriers may have implications for choice of therapeutic agent. Tumours with BRCA1 dysfunction have deficient double-stranded DNA repair mechanisms and evidence is now emerging that these cancers may be more sensitive to DNA damaging agents such as the platinum (Taniguchi et al., 2003).

Ki67 is a proliferation marker that has become a useful means of monitoring cell proliferation. Ki67 is expressed and can be detected in cells in the G₁ phase of the cell cycle, but not in quiescent G₀ cells (Jones et al., 2008). MIB-1, a monoclonal antibody, has been used to assess the expression of Ki67 in formalin fixed, paraffin embedded tissue sections and correlates well with Ki67 measurement (Cleator et al., 2002;

Urruticoechea et al., 2005). Studies have shown that a significant fall in Ki67 occurs as a result of neo-adjuvant chemotherapy and that higher pre-treatment proliferation is associated with a better response to treatment (Archer et al., 2003; Ellis et al., 1998). Also, tumours that show a >75% reduction in Ki67 post-chemotherapy were more likely to achieve a pathological response (Burcombe et al., 2004).

Therefore, the type of chemotherapy offered to patients may in the future not just depend on their risk or recurrence but also the particular characteristics of their tumour. ER negative cancers and cancers that overexpress *HER2* and have *TOP2A* alterations may particularly benefit from the use of anthracyclines in the adjuvant setting and patients still displaying elevated proliferation markers, such as Ki67, post chemotherapy may benefit from other non cross-resistant regimens.

1.3.7 Chemotherapy resistance

Resistance to chemotherapy remains one of the biggest limiting factors in the treatment of all cancers. Chemotherapy resistance can be intrinsic, where no response to initial treatment is seen, or acquired, where failure to respond to treatment develops over time, perhaps after multiple lines of therapy (Perez, 2009). There are multiple mechanisms by which cells can develop drug resistance including apoptosis evasion, membrane transporters, defects in DNA repair and intracellular drug inactivation (Raguz and Yague, 2008).

The exact mechanism by which many chemotherapeutic agents are taken up by cells remains unclear. However, more is known

about drug efflux, which is mediated by the ATP-binding cassette (ABC) transporters. These membrane proteins function as drug efflux pumps, reducing intracellular concentrations of drug in an energy dependent manner, thereby leading to drug resistance (Chien and Moasser, 2008). This family includes the P-glycoprotein (P-gp) membrane protein encoded by *MDR1* (or *ABCB1*) and the multidrug-resistance-associated protein MRP1 and the breast cancer resistance protein (BCRP). *In vivo*, these proteins function physiologically by clearing excessive intracellular concentrations of toxins and they are thought to play a vital role in cellular protection from endogenous substrates. For example, P-gp is found on the luminal surface of endothelial cells involved in the formation of the blood brain barrier, thus protecting the central nervous system from cytotoxins. Other “sanctuary” sites include testicular tissue and the developing foetus (Gottesman et al., 2002). ABC transporter genes are often overexpressed in cancer and the anthracyclines are known to be substrates for both P-gp and MRP1. These proteins can actively transport the drug out of the cell, and seem particularly effective at targeting hydrophobic drugs such as the anthracyclines (Longley and Johnston, 2005).

Other mechanisms of resistance can include inactivation of cytotoxics. For example, up to 80% of the anti-metabolite 5-FU is metabolised in the liver, by dihydropyrimidine dehydrogenase (DPD). Studies have shown that high levels of DPD mRNA in colorectal tumours correlate with 5-FU resistance, likely to be due to drug degradation of 5-FU by DPD (Salonga et al., 2000). Other drugs, such as the platinum agents, can form conjugates with the thiol glutathione (GSH) and the resulting complex inactivates the drug, which then becomes a substrate for the ABC transporter proteins and ultimately leads to efflux from the

cell (Longley and Johnston, 2005). Cytochrome P450 enzymes, an inducible oxidative enzyme from the liver, can be overexpressed in solid tumours, and can inactivate drugs such as irinotecan and altered activity of enzymes such as topoisomerase II can prevent anthracycline binding to the topoisomerase-DNA complex, thus allowing broken DNA strands to be repaired and bypassing the toxic effects of the drug (Raguz and Yague, 2008).

Other factors that lie downstream of the initial cytotoxic insult also contribute to drug resistance. Following chemotherapy, there is a critical balance between cell cycle arrest, which allows for DNA repair and survival, and cell death. Mutations in these downstream events can lead to drug resistance. Loss of normal cell cycle checkpoints, mutated DNA repair pathways and an ability to evade apoptotic death are all mechanisms by which tumour cells can develop resistance to chemotherapy (Johnstone et al., 2002) and are discussed later in this chapter.

1.4 THE CELL CYCLE

A cell reproduces by performing an orderly sequence of events, producing two daughter cells that are accurate copies of the parent. This cycle of division and duplication is known as the cell cycle and consists of a series of steps allowing replication of DNA and the segregation of the duplicated chromosomal DNA equally to the two daughter cells. A key aspect of cancer is dysregulated cell cycle control.

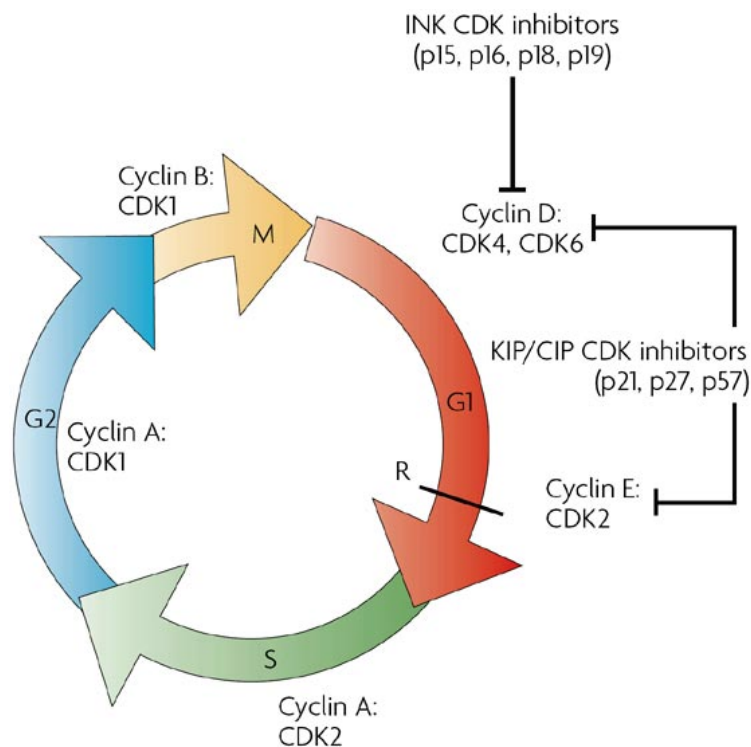


Figure 1.4 Mammalian cell cycle (Dehay and Kennedy, 2007)

There are four main phases of the cell cycle: G₁, S, G₂ and M phase (Figure 1.4). Chromosomal duplication occurs during S phase (DNA synthesis), occupying almost half the cell cycle time in mammalian cells. Chromosomal segregation and cell division occurs in M phase, including nuclear division (mitosis) and cytoplasmic division (cytokinesis). More time is required for growth and protein synthesis and the gap phases of G₁ and G₂ allow for this: G₁ between M phase and S phase and G₂ between S phase and mitosis. In a typical cell in culture, G₁, S, G₂ (also known as interphase) may last for 23 hours of a 24 hour cycle, with 1 hour for M phase (Alberts et al., 2008).

G₁ is the phase during which cells respond to extracellular stimuli that ultimately determine whether cells make the

decision to divide and replicate DNA or, alternatively, to exit the cell cycle and enter a quiescent state known as G_0 . However, once cells have made the commitment to begin DNA replication, a time late in G_1 known as the restriction point, they are irreversibly committed to complete the cycle, even if the extracellular signals that stimulate cell growth and division are removed (Sherr, 2000). The restriction point is the first of three checkpoints in the cell cycle. The second is the G_2/M checkpoint, where the early mitotic events that lead to chromosome alignment on the spindle in metaphase is triggered and the third at the metaphase to anaphase transition, where sister chromatid separation takes place, leading to the completion of mitosis and cytokinesis (Schafer, 1998). These checkpoints can block cell-cycle progression and also trigger cell death by apoptosis.

The key components of the cell cycle control system are members of a protein kinase family known as the cyclin dependent kinases (Cdks) and their activity fluctuates as the cell progresses through the cell cycle, resulting in cyclical changes in the phosphorylation of intracellular proteins which initiate and regulate the major cell cycle events. These serine/threonine kinases are dependent on a discrete protein partner, a cyclin, for activity. The catalytic activity of Cdks is determined by the binding of this regulatory cyclin subunit. Cyclin binding and phosphorylation can stimulate activity, whilst phosphorylation of residues adjacent to the Cdk ATP-binding site and binding of inhibitory proteins can inhibit Cdk activity (Pollard and Earnshaw, 2008).

The cyclins are defined by the stage of the cell cycle at which they bind Cdks and by their function. The G_1/S cyclins (cyclins

D and E) activate Cdk2, Cdk4 and Cdk6 late in G₁ and help trigger progression through the restriction point, leading to commitment to entry to the cell cycle. The S-cyclins (cyclin A) bind Cdk1 and Cdk2 to promote chromosome duplication and some early mitotic events. The M-cyclins (cyclin B) activate Cdk1 to stimulate entry into mitosis at the G₂/M checkpoint (Alberts et al., 2008).

The cyclin D kinases, in combination with their catalytic partners Cdk4 and Cdk6, are the critical integrators of mitogenic signals for the cell cycle and cyclin D1 expression is seen early following cell cycle entry. Its assembly with Cdk4 is key to allowing cells to progress through the restriction point following mitogenic stimulation. Cyclin D dependent kinases catalyse the phosphorylation dependent inactivation of Rb, releasing E2F complexes which in turn transcriptionally activate genes whose products are critical for cell cycle progression, such as cyclin E (Diehl, 2002).

A number of mechanisms can inactivate Cdks. Phosphorylation of threonine and tyrosine in the roof of the kinase active site can inhibit the activity of cyclin-Cdk complexes. A protein kinase known as Wee1 can phosphorylate these sites – dephosphorylation by the Cdc25 phosphatases can reverse this. Another strategy for inactivating Cdks involves the binding of cyclin-dependent kinase inhibitors (CKIs) and inhibitors of Cdk4 (INK4).

1.4.1 Cdk inhibitors

Binding of CKIs regulates cyclin-Cdk complexes, by stimulating a rearrangement in the structure of the Cdk active site, thus

rendering it inactive. There are two main families of inhibitors – INK4 proteins, named after their ability to inhibit Cdk4 (INK4A, INK4B, INK4C AND INK4D) and the Cip/Kip family, composed of p21^{Cip1}, p27^{Kip1} and p57^{kip2} (Malumbres and Barbacid, 2009). INK4 proteins target the cyclin D dependent kinases and sequester Cdk4/6 proteins, which in turn liberates Cip/Kip proteins, thereby inhibiting cyclin E-Cdk2 to ensure cell cycle arrest in G₁ (Sherr, 2000).

p27^{Kip1} is an important Cdk inhibitor that regulates cell proliferation, motility and apoptosis. A variety of signal transduction networks can regulate p27^{Kip1} and its activity is controlled by its concentration, subcellular localisation and phosphorylation status (Chu et al., 2008). In quiescent cells, p27^{Kip1} is nuclear, its levels are high and it is mobilised to inhibit cyclin E-Cdk2, arresting cell cycle progression. However, when cells enter the cell cycle after mitogenic stimulation and accumulate cyclin D dependent kinases, the Cip/Kip proteins are sequestered in complexes with these cyclin D –dependent Cdk, eventually targeting them for destruction in proteosomes (Sherr, 2000).

1.4.2 The cell cycle and cancer

A variety of genetic and epigenetic events can cause overactivity of the cell cycle in human cancers – cells are no longer subject to normal proliferative and inhibitory effects, with signal transduction pathways overactive and their normal inhibitory mechanisms switched off. Transformed cells often exhibit reduced growth factor requirements and a number of cell cycle regulators are known to be mutated, deleted or overexpressed in human cancers (Schafer, 1998). p53,

responsible for promoting G₁ arrest after DNA damage and with a key role in the DNA damage checkpoint, is mutated in up to 50% of malignancies. After DNA damage, p53 is stabilised and initiates the transcription of p21^{Cip1}, leading to G₁ arrest. However, when p53 is mutated or in *p53* null cells, the DNA damage checkpoint is abrogated, allowing cells to continue through the cell cycle, ensuring that after mitosis the damage is fixed in the genome (Schafer, 1998). Deletions and missense mutations, resulting in a truncated, non-functioning pRb are also seen in human cancers. Phosphorylation of pRb, leading to its inactivation, releases E2F and the cell cycle is initiated. Abrogation of pRb function in cancers can again lead to loss of restriction point control and cell cycle inhibitory mechanisms (Schafer, 1998).

Cyclin D1 is frequently overexpressed in tumours, particularly breast cancer. This can result from chromosomal translocations and gene amplification. Its role in malignancy is multifactorial – it can drive the cell cycle through interaction with its catalytic partners Cdk4 and 6, prevent the inhibitory functions of the Cip/Kip family by sequestration, promote phosphorylation of pRb and may even have a role as a transcriptional co-factor for steroid receptors such as the androgen receptor (AR) and the oestrogen receptor (ER), deregulating hormone dependent gene expression (Diehl, 2002).

p27^{Kip1} is rarely mutated or deleted in tumours, but protein levels are often reduced or mislocalised in many human cancers – this is associated with a poor prognosis. Oncogenic activation of the receptor tyrosine kinase pathways, such as PI3K and Ras-MAPK can also enhance inactivation of p27^{Kip1} and accelerates its proteolysis in human cancers. (Chu et al., 2008).

1.5 APOPTOSIS

An organism has two main mechanisms for the elimination of cells – necrosis and apoptosis. Cell death is an essential part of embryonic development, tissue homeostasis, immunity and regulation of cell viability by growth factors and hormones. It is disruption of these pathways that allow cancer cells to survive. Necrosis results from irreversible injury to the cell and a collapse of normal cell physiology – the plasma membrane is damaged and ruptures, spilling cytoplasmic contents into the surrounding tissues. This produces a local, inflammatory reaction, activating phagocytic cells which ingest the debris (Pollard and Earnshaw, 2008).

Apoptosis is a form of programmed cell death that allows organisms to remove damaged, senescent or unwanted cells and is the best-defined mechanism for counteracting tumour growth (Russo et al., 2006). It is characterised by morphological changes, such as shrinkage of the cell, cytoskeletal collapse, condensation of nuclear chromatin and the formation of small membrane enclosed fragments, called apoptotic bodies, that can be engulfed without initiating inflammation (Jaattela, 2004). Cells can recognise damage and can kill themselves by undergoing apoptosis. In the case of DNA damage, where cancer promoting mutations may occur if not repaired, cells can detect this damage and undergo apoptosis. These cells display characteristic biochemical changes which allow the identification of apoptotic cells, for example cleavage of chromosomal DNA by endonucleases produce DNA fragments of distinctive sizes, generating multiple new DNA ends which can be identified by the addition of labelled deoxynucleotide (dUTP) to the 3'-OH ends of DNA fragments – the TUNEL

technique. These cells also express negatively charged phospholipids on their outer leaflet, which can be visualised with a labelled form of the *Annexin V* protein, which specifically binds to this phospholipid.(Alberts et al., 2008; Pietsch et al., 2008).

Apoptosis is carefully regulated and the caspases, specialized proteases with a cysteine in their active site, have a key role in inactivating cellular survival pathways and activating factors promoting cell death. Caspases are selective enzymes, responsible for cleaving a limited subset of cellular proteins. They can cleave protein kinases, producing constitutively active enzymes that can promote cell death. They can also cleave and inactivate proteins that normally function in the detection and repair of DNA damage (Pollard and Earnshaw, 2008). Once activated, caspases cleave and activate other procaspases, resulting in an amplifying proteolytic cascade, which eventually leads to activation of a nuclease, ultimately destroying the chromosomal DNA of the cell.

Two principle pathways lead to cell death by apoptosis – the extrinsic and intrinsic pathways. Extracellular signal proteins which bind to cell surface death receptors activate the extrinsic pathway. These death receptors are transmembrane proteins, consisting of an extracellular ligand-binding domain and an intracellular 'death domain', essential for the receptors to activate apoptosis (Alberts et al., 2008). The receptors belong to the tumour necrosis factor (TNF) receptor family, including a receptor for TNF itself and the Fas death receptor. Activation of these receptors by means of specific ligands, such as TNF α and TRAIL, bring about the recruitment of other family members, resulting in the chain activation of caspases 8, 3 and 7 (Dempsey et al., 2003). Death receptor activation can also

trigger intracellular signalling pathways which do not result in apoptosis. For example, TNF receptors can also activate the NF κ B pathway, responsible for cell survival and the activation of genes involved in the inflammatory response (Shen and Tergaonkar, 2009).

In the intrinsic pathway, there is release of mitochondrial proteins that can activate a proteolytic caspase cascade in the cytoplasm, leading to apoptosis. These mitochondrial events are regulated by the Bcl-2 family of proteins. A crucial protein released from the mitochondria is cytochrome C, which associates with the scaffolding protein Apaf-1, forming an apoptosome, which in turn can bind caspase 9 and recruit and activate caspase 3, a key effector of both the intrinsic and extrinsic pathways (Li et al., 1997). Further downstream executioner procaspases are then activated to induce apoptosis.

1.5.1 Bcl-2 proteins

The human *Bcl-2* homologues make up the major apoptosis regulatory gene family and like the caspases family have been conserved in evolution from worms to humans. The apoptosis suppressing *Bcl-2* gene was initially discovered in low grade B-cell non Hodgkin's lymphoma (NHL), at the breakpoints of t(14;18) chromosomal translocation, moving the *Bcl-2* gene into the immunoglobulin heavy-chain gene cluster, a site of active gene transcription. (Tamm et al., 2001). Overexpression of Bcl-2 and other antiapoptotic proteins inhibits cell death induced by growth factor deprivation, oxidative stress and hypoxia (Yip and Reed, 2008). The Bcl-2 family consists of more than 30 anti- and proapoptotic molecules which can bind to each other, forming homo- and heterodimers. They are characterised by

the presence of one to four conserved Bcl-2 homology (BH) domains, termed BH1 to BH4. The antiapoptotic members, including Bcl-2 and Bcl-XL are primarily localised to the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear membrane, where they prevent the release of cytochrome C and other proapoptotic proteins into the cytoplasm and nucleus. The pro-apoptotic members can be divided into two sub groups – the multidomain groups, such as BAX, and the BH3 only proteins, including BIM and BAD. These act as sensors of cellular damage and can initiate cell death (Pommier et al., 2004). In response to cellular insults, BH3-only family members are activated, through increased transcription, proteins stabilisation and post-translational modifications. This induces mitochondrial permeabilisation, leading to release of cytochrome C and other proapoptotic proteins from the mitochondrion (Letai, 2008). BH3-only proteins are the crucial link between apoptotic stimuli and the intrinsic pathway of apoptosis. When cells are deprived of extracellular survival signals, the transcription of the gene encoding the BH3-only protein Bim can then activate the intrinsic pathway. Similarly, when DNA damage occurs, the tumour suppressor p53 accumulates, promoting the intrinsic pathway and thus eliminating a potential cancerous cell (Alberts et al., 2008).

1.5.2 Apoptosis and chemoresistance

Apoptosis is key to cell signalling pathways, the cell cycle, cell structure but also to the development of human diseases, in particular – cancer. A block in apoptosis is essential for cancer maintenance. The regulation of genes encoding Bcl-2 family proteins is altered in a variety of cancers. As well as the

chromosomal translocations observed in NHL, *Bcl-2* gene amplification is seen in other lymphomas and small cell lung cancer (SCLC). Other mechanisms of elevated gene expression include loss of endogenous microRNAs which normally repress *Bcl-2* gene expression and gene hypomethylation (Cimmino et al., 2005; Hanada et al., 1993). Overexpression of Bcl-2 and other antiapoptotic proteins has been implicated in resistance to chemotherapy. Most cytotoxic agents and radiation kill cancer cells by inducing apoptosis and there is emerging evidence that Bcl-2 is a multidrug resistant protein, preventing induction of apoptosis, regardless of the mechanism of action of the drug (Yip and Reed, 2008). This is distinct from other forms of drug resistance, namely mechanisms involving drug metabolism, drug efflux and drug inactivation. Whether it be through DNA breaks (platinum), microtubule depolymerisation (taxanes), topoisomerase inhibition (anthracyclines) or receptor inhibition (anti-androgens and oestrogens), most drugs appear to depend on Bcl-2 dependent mechanisms to exert their cytotoxic effect. Cancer cells may escape apoptosis through a number of Bcl-2 mediated mechanisms. This includes eliminating the activation of pro-death BH3-only proteins such as Bim or increased expression of Bcl-2 itself, leading to bound and sequestered anti-apoptotic proteins. Defective expression of pro-apoptotic Bcl-2 family members can also occur when p53 function is lost, as *Bcl-2* genes are regulated by p53.

Bcl-2 has been therapeutically targeted, with the most advanced approach utilizing an antisense oligonucleotide to down regulate Bcl-2 expression (Genasense or G3139). In preclinical studies, antisense inhibition of Bcl-2 could induce death in cancer cell lines, although clinical studies have failed to reach their designated endpoints. In a recent study however in chronic

lymphocytic leukaemia (CLL), an improvement in response rate was seen in relapsed patients when treated with G3139 in combination with fludarabine and cyclophosphamide (O'Brien et al., 2007). These drugs may have use in the future, synergising with more traditional cytotoxic agents.

1.6 THE PHOSPHATIDYLINOSITOL-3-KINASE PATHWAY

The phosphatidylinositol 3-kinase (PI3K) family is a large family of lipid and serine/threonine kinases, a highly conserved group of intracellular lipid kinases which phosphorylate the 3'-hydroxyl group of phosphatidylinositol and phosphoinositides (Engelman et al., 2006). This pathway is activated downstream of a number of extracellular signals and PI3Ks generate second messengers that have a key role in cell growth, proliferation and cell survival –activation or inactivation of components in this pathway can disrupt normal cellular signalling pathways leading to malignant transformation and this pathway is frequently dysregulated in human cancers and targeted by genomic aberrations such as mutations, amplifications and rearrangements (Dillon et al., 2007; Hennessy et al., 2005).

The PI3K pathway is important in internalising the effects of extracellular growth factors and of membrane tyrosine kinases. Ligand binding activates receptor tyrosine kinases by bringing together a pair of kinase domains on the cytoplasmic surface of the membrane. This dimerisation allows kinase domains to activate each other by phosphorylation on tyrosine residues, creating phosphotyrosine binding sites for downstream effectors and adaptor proteins (Pollard and Earnshaw, 2008) (Figure 1.5).

Once activated, PI3K catalyses phosphorylation at the 3'-position of the inositol ring of phosphatidylinositol (PI), generating several phosphoinositides second messengers such as PI 3,4 bisphosphate (PIP₂) and then PI 3,4,5 -trisphosphate (PIP₃), which can then recruit and serve as docking sites for signaling proteins with PIP₃-binding pleckstrin-homology (PH) domains, including one of the main effectors - AKT (protein kinase B) (Alberts et al., 2008). PIP₃ is also a substrate for the phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) which dephosphorylates PIP₃ to generate PIP₂, thus negatively regulating PI3K signalling, switching off the pathway and acting as a tumour suppressor. Many human cancers also show inactivation of PTEN, which results in constitutive activation of the PI3K pathway (Bader et al., 2005).

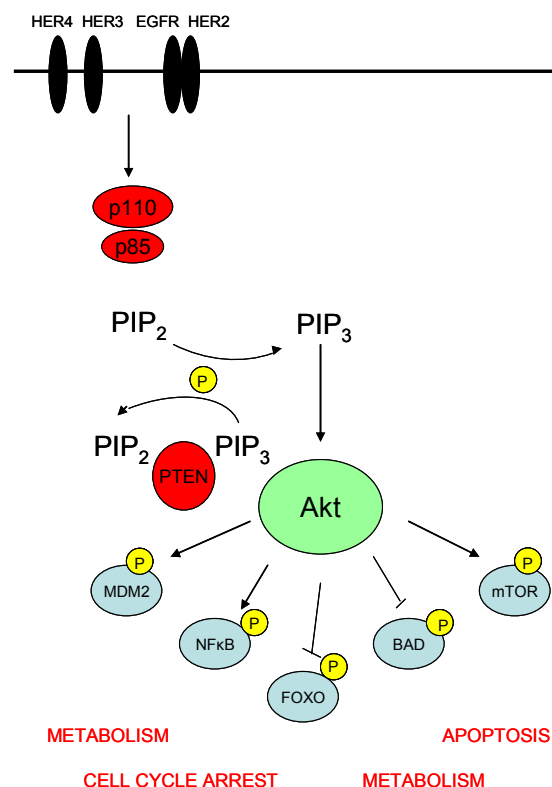


Figure 1.5 Signalling through the PI3K/AKT pathway
(Adapted from Hennessy et al., 2005)

1.6.1 Class 1 PI3K proteins

There are multiple isoforms of the PI3K superfamily and these are divided into three main classes, based on their lipid substrate specificity and sequence homology. Class I PI3Ks are comprised of a p110 catalytic subunit and a p85 regulatory adaptor subunit, Class II PI3Ks are monomeric, consisting of only a catalytic subunit and lack adaptor subunits and preferentially phosphorylate PtdIns and PtdIns(4)P and class III phosphorylate only PtdIns and are implicated in autophagy (Cantrell, 2001).

The activity of class I PI3Ks is tightly controlled, with extracellular signals recruiting them to the plasma membrane. They are activated by extracellular stimuli via receptor tyrosine kinases or G-protein-coupled-receptors - only Class I PI3Ks have been implicated in cancer, with no known link between class II and III PI3Ks and malignancy (Zhao and Vogt, 2008). The production of the second messenger PIP₃ is a unique feature of Class I PI3Ks, which has a key role in cell growth and replication and it is this that is likely to contribute to their oncogenic potential. Class I PI3Ks have both protein and lipid kinase activity but it is the latter which appears critical for their role in cancer development (Zhao and Vogt, 2008). Class I PI3Ks are heterodimeric proteins. There are four isoforms of the catalytic subunit: p110 α , p110 β , p110 δ and p110 γ . These isoforms share similar domain compositions, including an adaptor binding domain, a Ras binding domain, and a C2 (protein-kinase-C- homology-2) domain with affinity for lipid membranes (Walker et al., 1999).

Class I PI3Ks can be further subdivided into Class IA and Class IB, according to their regulatory subunits and connection to upstream signalling. Class IA, which encompasses p110 α , p110 β and p110 δ are activated by receptor tyrosine kinases and associate with the p85 regulatory subunits of which there are five species; p85 α , p85 β , p55 α , p55 γ and p50 α . p85 has two Src homology 2 (SH2) domains, responsible for linking the p85-p110 PI3K enzyme complex to upstream signals from receptor tyrosine kinases (Kok et al., 2009). Class IB, which consists of p110 γ , bind the non p85 regulatory subunit p101 as well as p84 and p87^{PIKAP} and can be activated by small G-proteins (Denley et al., 2007). When upstream signals are absent, the regulatory subunits stabilize p110 and suppresses its catalytic activity (Luo et al., 2005; Yu et al., 1998).

Specific roles have been identified for the multiple isoforms of PI3Ks. Mouse knockout models have shown that p110 α and p110 β knockouts are embryonically lethal, whereas p110 δ and p110 γ knockouts are viable, but have defects in immune response components (Bi, 2002; Bi et al., 1999; Sasaki et al., 2000).

1.6.2 Akt/Protein kinase B (PKB)

One of the most important downstream effectors of PI3K is the serine/threonine kinase Akt (also known as protein kinase B or PKB). Akt is the primary downstream mediator of PI3K, regulating a variety of cellular processes through the phosphorylation of a diverse set of substrates. Akt has a role in cell cycle regulation by phosphorylating the cell cycle inhibitors p21^{Cip1} and p27^{Kip1}, leading to their cytoplasmic retention and can also cause increased translation and stabilisation of cyclin

D1 (Diehl et al., 1998; Viglietto et al., 2002; Zhou et al., 2001). Akt plays a role in cell survival, by inhibiting the proapoptotic Bcl2 family member Bad and by inhibition of the Forkhead family of transcription factors, which activate proapoptotic related genes (Datta et al., 1997). Akt also leads to NFκB mediated expression of prosurvival genes and can activate mTOR (Kane et al., 1999; Li et al., 2004).

All Akt isoforms share similar structures, containing an N-terminal regulatory domain including a pleckstrin homology (PH) domain, a hinge region where the PH domain is connected to a serine/threonine kinase domain and a C-terminal region responsible for its kinase activity (Franke, 2008). Three isoforms of Akt have been identified in mammalian cells – Akt1, Akt2 and Akt3, all of which are encoded for by different genes but share a high degree of amino acid homology (West et al., 2002). Akt1 is the most ubiquitously expressed, Akt2 is found at high levels in insulin-sensitive tissues such as the liver and adipose tissue and Akt3 is expressed at highest levels in the brain and testes (Hill et al., 1999; Nakatani et al., 1999; Paul J. Coffer, 1991).

Akt is activated by a dual regulatory mechanism. PIP₃ is generated on the inner leaflet of the plasma membrane following PI3K activation and this recruits Akt by direct interaction with its PH domain. Once at the plasma membrane, another PH-domain containing serine/threonine kinase, called 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates Akt on Thr308 (Vanhaesebroeck and Alessi, 2000). Maximal activation of Akt however requires further phosphorylation at Ser473, although the exact mechanism of this second phosphorylation event is not fully understood, but is

likely to occur via the kinase PDK2 (Vivanco and Sawyers, 2002). Once phosphorylated at Ser473 and Thr308, Akt is highly active and can translocate to the cytoplasm and nucleus to phosphorylate a diverse set of substrates (Thompson and Thompson, 2004).

Mechanisms of inactivation of Akt remain poorly understood, with no specific Akt phosphatase having been identified. However, treatment of phosphorylated Akt with a serine-threonine phosphatase PP2A, has been shown to dephosphorylate Akt and abolish its activity (Andjelkovic et al., 1996).

1.6.3 The PI3K pathway and cancer

The PI3K pathway has been implicated in cancer and it is one of the most frequently targeted pathways in human tumours, with up to 30% of all cancers estimated to have mutations in PI3K pathway components (Luo et al., 2003). Chromosomal amplification of PI3K or Akt, constitutive activation of Akt and *PTEN* deletions are seen in a wide variety of cancers and play a role in the tumorigenic process (Thompson and Thompson, 2004). Other transforming events that do not directly modify PI3K, Akt or PTEN can still cause constitutive activation of the PI3K pathway, including EGFR and HER2 overamplification, as these receptors signal through the PI3K pathway.

The gene encoding for the p110 α catalytic subunit of PI3K, *PIK3CA*, is mutated in a wide variety of human cancers, often arising from single nucleotide substitutions. These mutations are seen in around 30% of common epithelial tumours, including breast, colon and prostate cancer (Zhao and Vogt,

2008). Point mutations in p110 α often cluster around two hotspots – E545 in the helical phosphatidylinositol kinase homology domain and H1047 near the end of the catalytic domain (Engelman et al., 2006). In cell lines, the expression of these p110 α mutants leads to Akt activation in the absence of growth factor stimulation and a gain in function in p110 α with significant upregulation of the lipid kinase activity of the mutant protein (Kang et al., 2005; Samuels et al., 2004). Overamplification of *p110 α* (or PIK3CA) is seen in bowel, ovarian, head and neck and other cancers, where the subsequent overexpression of a structurally normal protein is likely to contribute to malignant transformation, analogous to HER2 overexpression in breast cancer (Vivanco and Sawyers, 2002). Studies have shown also that amplification of the *PIK3CA* locus during lung cancer progression occurs in high grade, but not low grade pre-invasive lesions, further evidence that gain in PI3K function represents commitment to tumour progression (Massion et al., 2004). Interestingly, mutations in p110 α and other mechanisms of activating PI3K signalling seem to be mutually exclusive. Cancers that frequently harbour p110 α mutations, such as breast and bowel cancer, rarely show *PIK3CA* amplification and in tumours where *PIK3CA* amplification is common, such as gastric cancer, mutations are much less frequently observed (Campbell et al., 2004). Thus, only one mutation may be sufficient for tumour development and double mutations may in fact have a negative effect (Yuan and Cantley, 2008).

p85 mutations and translocations are rare but have been identified in transformed cell lines and in human tumour samples. Mutated p85 leads to constitutive activation of PI3K signalling and expression of mutant p85 in mouse models leads

to the development of solid tumours (Bader et al., 2005; R.-Borlado et al., 2000).

Akt itself is another target of genomic and epigenetic changes in cancer and amplification in multiple Akt isoforms have been seen in ovarian, breast and gastric cancers (Engelman et al., 2006). Some of these changes may be isoform specific. Mutations in the PH domain of Akt1 result in prolonged constitutive activation of Akt and prolonged association with the plasma membrane sufficient to transform cells in culture (Carpten et al., 2007). The expression of Akt2 and Akt3 is pathologically increased in human cancers and elevated Akt2 activity can increase cell motility and metastatic potential, a phenotype not exhibited in cells expressing activated Akt1 (Arboleda et al., 2003; Hutchinson et al., 2004).

Finally, the tumour suppressor PTEN has been shown to be mutated or deleted in many human tumours. The normal function of PTEN is critical for cells to maintain homeostasis and the absence of functional PTEN in cancer cells results in elevated levels of PIP₂ and PIP₃, causing constitutive activation of the PI3K signalling pathway through downstream components such as Akt and mTOR (Cantrell, 2001; Sansal and Sellers, 2004). There is growing evidence that inactivation of *PTEN* may be as frequent as *p53* mutations in many adult epithelial cancers. Germline mutations in the *PTEN* gene result in Cowdens disease, a familial disease characterised by a high risk of hamartomas and cancers (Sansal and Sellers, 2004). Knockout mice rendered heterozygous for *PTEN* develop cancers, particularly endometrial cancers and up to 50% of human endometrial cancers have been shown to harbour *PTEN* mutations. Interestingly, *PTEN* mutations are also seen in early preinvasive

endometrial conditions such as endometrial hyperplasia, suggesting loss of PTEN may be an early event in cancer development (Maxwell et al., 1998; Mutter et al., 2000; Podsypanina et al., 1999; Stambolic et al., 2000). Somatic mutations, gene deletion or gene inactivation of *PTEN* occur in glioblastomas, prostate and breast cancer and malignant melanoma (Engelman et al., 2006). Loss of PTEN protein is seen in about one fifth of prostate cancers and this correlates with Gleason score and advanced stage, suggesting accumulation of mutations and progressive loss of PTEN is associated with advanced disease (McMenamin et al., 1999).

1.6.4 Therapeutic approaches targeting the PI3K pathway

Many cancers depend on a persistently activated PI3K signalling pathway to promote proliferation, survival and migration thus making this pathway an attractive target for therapeutic intervention by disrupting PI3K signalling. For example, the Rak tyrosine kinase interacts with PTEN, leading to its phosphorylation on Tyr336. Ectopic expression of Rak can suppress breast cancer cell proliferation and invasion, whilst knockdown of Rak promotes PTEN protein degradation, thus suggesting Rak may be an interesting target to modulate activity of the PI3K pathway (Yim et al., 2009). Similarly, overexpression of Inositol Polyphosphate 4-Phosphatase Type II (INPP4B), which can target PIP₂ hydrolysis, reduces tumour growth and promotes cellular senescence, whilst knockdown of INPP4B results in enhanced Akt activation in breast cancer cells, again identifying a potential therapeutic target to disrupt PI3K signalling (Gewinner et al., 2009). Modulation of the PI3K pathway is seen after chemotherapy administration *in vitro*.

Inhibition of Akt is seen in chemotherapy induced cytotoxicity – treatment of MCF7 cells by paclitaxel is accompanied by Akt inactivation (Sunters et al., 2006). However the effects of chemotherapy are often non-specific and the development of more specific agents is likely to lead to greater efficacy and less toxicity. Wortmannin and LY294002 are both PI3K inhibitors that have been used in cell lines with good effect, by inhibiting the catalytic activity of the p110 subunit of PI3K, but these have not been developed for clinical applications, in part due to unfavourable pharmacokinetic properties and a narrow therapeutic index (Vivanco and Sawyers, 2002).

Receptor tyrosine kinase inhibitors have had clinical success, at least in part by shutting off upstream signalling to the PI3K pathway. Drugs such as trastuzumab (herceptin) which targets the Erb2/HER2 receptor and imatinib (Gleevec) which inhibits the Bcr-Abl, c-KIT and PDGF-R tyrosine kinases are licensed for use in breast cancer and CML respectively, but resistance does develop. This could be due to gene amplifications or activating mutations of downstream components, such as Akt, p110 and p85 or the inactivation of PTEN as a negative regulator, suggesting targeting one single component of the PI3K pathway is insufficient to achieve long term clinical success (Luo et al., 2003). Mutated kinases may be more specific targets – in NSCLC, tumours that have mutated forms of the tyrosine kinase EGFR are more responsive to EGFR inhibitors such as erlotinib and gefitinib when compared to tumours expressing wild-type EGFR (Lynch et al., 2004).

Isoform specific inhibitors of p110 or Akt would appear to be an attractive therapeutic option and may be less toxic and have a higher therapeutic index than “pan-PI3K” inhibitors – p110 α

inhibitors in particular may be useful in treating cancers that have *PIK3CA* mutations. However, specific isoform inhibition may have unwanted effects on other cellular pathways. For example, p110 α has a key role to play in insulin signalling and any anti-cancer effect may be outweighed by interference with glucose metabolism and a diabetic effect (Engelman et al., 2006).

mTOR is another attractive target in cancer treatment. The serine/threonine kinase mTOR is involved in cell growth control, functioning as a molecular sensor regulating protein synthesis and both the Ras and PI3K pathways converge to activate it (Shaw and Cantley, 2006). mTOR is a direct target of Akt. Rapamycin, the prototype inhibitor of mTOR, induces G₁ cell cycle arrest, prevents CDK activation and accelerates cyclin D1 turnover (West et al., 2002). Results have been promising in clinical trials, with rapamycin and its analogues showing anti-tumour activity, including tumour regression and prolonged stable disease in patients with non-small cell lung cancer (Gridelli et al., 2008).

It is likely that activation of the PI3K pathway, due in part to its many downstream effectors, has a role to play in resistance to chemotherapy. Therefore, the development of new compounds to target particular proteins in this pathway which can be used alone or in combination with existing therapies, may enhance the efficacy of current cytotoxic treatment.

1.7 FOXO TRANSCRIPTION FACTORS

Transcription factors are modular proteins and have a distinct function in the expression of protein-encoding genes by binding

to their regulatory elements. Once bound, additional factors are recruited to the initiation complex, resulting in either the initiation or repression of transcription. The DNA-binding domains of transcription factors tend to be highly conserved, thus they can be grouped and classified on the basis of their shared structural domains that bind DNA in a sequence specific manner (Arden and Biggs, 2002; Carlsson and Mahlapuu, 2002).

FOXO transcription factors belong to the large Forkhead protein family of proteins, transcriptional regulators found in all eukaryotes and characterized by a conserved DNA-binding domain, known as the 'forkhead box', named after the founding gene member in *Drosophila* (Kaestner et al., 2000). There are at least 39 distinct members of the Forkhead family in humans and these have been further divided into 19 subgroups (FOX for 'Forkhead Box' A to S) (Greer and Brunet, 2005). The 'forkhead box' is a conserved 100 amino acid DNA binding domain, forming a butterfly shaped structure, comprising three tightly packed α helical domains at the N-terminal region, three β -sheets and two loop regions located at the C-terminal end, giving it a three dimensional "wing-like structure" – hence the Forkhead box is sometimes referred to as the winged-helix motif. It is the third helix (H3) that interacts with DNA bases within the major groove of double stranded DNA, allowing Forkhead proteins to bind to DNA (Burgering and Medema, 2003).

Within the larger Forkhead family are a subfamily of transcription factor named FOXO (Forkhead box, class O), consisting of the members FOXO1, FOXO3a, FOXO4 and FOXO6. These are homologous to the transcription factor DAF-16, found

in the nematode worm *C. elegans* and known to have a critical role in metabolism and longevity (Birkenkamp and Coffey, 2003). These transcription factors bind as monomers to a consensus DNA-binding sequence, determined as TTGTTTAC (Furuyama et al., 2000).

1.7.1 The structure and function of FOXO proteins

FOXO proteins are expressed in a wide variety of tissues. FOXO1 mRNA is abundant in adipose tissue, FOXO3a is highly expressed in the brain and FOXO4 in the heart. The most recently identified, FOXO6, is mainly expressed in the developing brain, suggesting it may have a role in the central nervous system (Fu and Tindall, 2008; Jacobs et al., 2003). Initially FOXO proteins were identified in fusion genes occurring as a result of chromosomal translocations in human cancers and leukaemias – in particular alveolar rhabdomyosarcomas (Maiese et al., 2008). These chromosomal translocations resulted in a chimeric protein in which the FOXO transactivation domains were fused to the DNA-binding domains of other transcriptional regulators resulting in constitutive activation and nuclear retention – the first insight into the potential role of FOXO factors in cancer (Fu and Tindall, 2008).

FOXO transcription factors play a critical role in a number of physiological processes. From the early stages of development, FOXO proteins are crucial components in the development of the immune, reproductive and haematopoietic systems. *Foxo3a*^{-/-} null mice are infertile, with ovarian follicle depletion. Lymphoproliferation, organ inflammation and enhanced T-helper cell activity is seen in these mice (Maiese and Chong, 2003; Maiese et al., 2008). FOXO proteins also play a role in cell

metabolism and are linked to the prevention of diabetic complications by preserving cellular energy and mitochondrial integrity (Maiese and Chong, 2003). The release of reactive oxygen species (ROS) during oxidative stress can contribute to a wide variety of pathological states such as ischaemia, Alzheimer's disease and cerebrovascular disease (Chong et al., 2005) and as discussed later, FOXO has a key role to play in repairing the damage caused by ROS.

FOXO proteins consist of four domains – the highly conserved Forkhead DNA-binding domain (DBD), a nuclear localization signal (NLS) located downstream of the DBD, a nuclear export sequence (NES) and a C-terminal transactivation domain. FOXO1, FOXO3a and FOXO6 are all of similar length – approximately 650 amino acid residues, whereas FOXO4 is shorter, with approximately 500 amino acid residues. Highly conserved regions between FOXO proteins include not only the Forkhead DBD but also the N-terminal region containing an Akt/PKB phosphorylation site, the NLS and part of the C-terminal transactivation domain (Obsil and Obsilova, 2008). It is FOXO3a that is the main focus of work in this thesis (Figure 1.6).

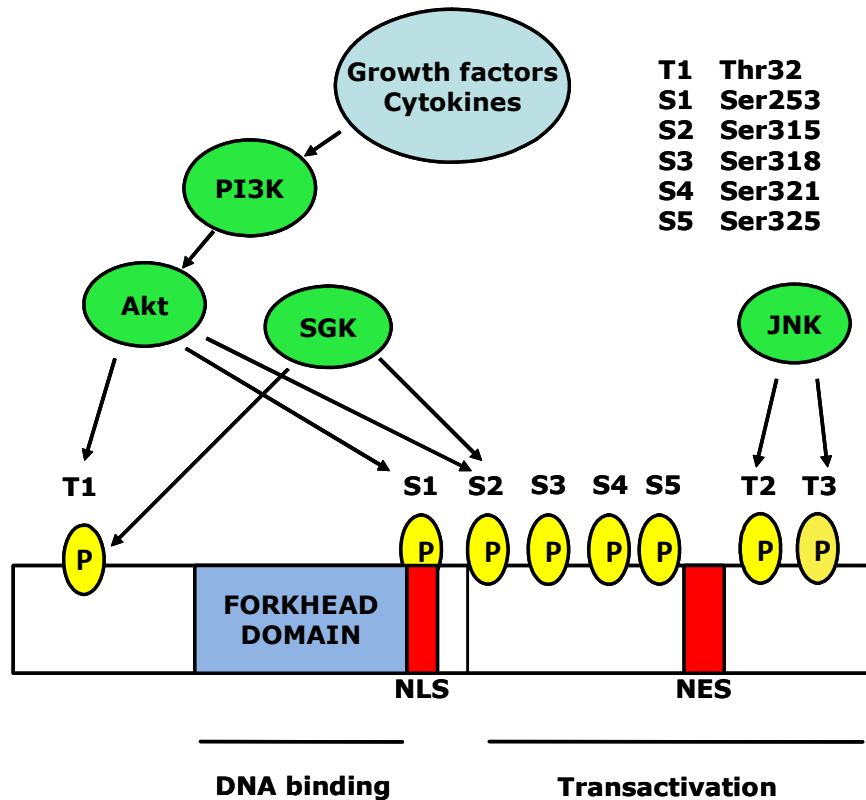


Figure 1.6 Structure and phosphorylation sites of FOXO3a. Phosphorylation sites of FOXO3a, as indicated by the specific amino acid positions. The position of the nuclear localization site (NLS), nuclear export sequence (NES) and transactivation domains are indicated. (Adapted from Arden, 2006).

FOXO proteins function predominantly as transcription factors in the nucleus, binding their DNA-targeting sequences. They can exert their cellular function via different mechanisms and can bind directly to the promoter of target genes, recruiting other co-activators or transcription factors to initiate transcription. FOXO proteins can repress transcription by competing with other transcription factors at promoter binding sites or recruit other co-activators which can initiate or repress transcription via promoters lacking FOXO binding sites (Fu and Tindall, 2008).

1.7.2 FOXO activity and signaling pathways

The FOXO family is one of the best characterized substrates of the protein kinase Akt in response to extracellular stimulation by growth factors or insulin. When growth factors or insulin bind to their tyrosine kinase receptors, PI3K is recruited and activated, which in turn activates Akt and also the related SGK (serum and glucocorticoid inducible kinase) family of proteins (Greer and Brunet, 2005). Evidence that the PI3K pathway was important in FOXO activity came initially from studies in the nematode worm *C.elegans*. As mentioned previously, the transcription factor DAF-16 was identified as a Forkhead transcription factor in this organism and was found to have a critical role in metabolism and longevity – analysis of the amino acid sequence of DAF-16 identified three sites corresponding to the consensus Akt/PKB phosphorylation sites, suggesting that PI3K activity suppresses the function of DAF-16 and that this and other Forkhead transcription factors may be direct targets of Akt (Birkenkamp and Coffey, 2003). It has subsequently been shown that Akt can directly phosphorylate FOXO transcription factors on multiple threonine and serine residues, triggering the rapid relocalisation of FOXO proteins from the nucleus into the cytoplasm. Akt can phosphorylate FOXO1, FOXO3a and FOXO4. On FOXO3a there are three key sites – Thr 32, Ser 253 and Ser 315 – sites which are conserved between species (Alessi et al., 1997). SGK can also phosphorylates FOXO factors, but preferentially at Ser 315, whereas Akt preferentially phosphorylates Ser 253 – Thr 32 is phosphorylated by both kinases (Greer and Brunet, 2005). A number of growth factors can trigger phosphorylation via Akt and SGK, including insulin and EGF, suggesting FOXO transcription factors can integrate a wide variety of external stimuli and selectively respond to these

stimuli depending on which site is phosphorylated (Furukawa-Hibi et al., 2005).

The main consequence of these phosphorylation events is a change in the subcellular localization of FOXO transcription factors. Shuttling of proteins between the cytoplasm and the nucleus is a highly regulated process, requiring accessory proteins that recognize and bind to specific sequences on their targets, including the nuclear localisation signal (NLS) and the nuclear export signal (NES). A 14-3-3 protein, which harbours a NES, has been shown to bind FOXO proteins in a phosphorylation dependent manner (Burgering and Kops, 2002). 14-3-3 proteins belong to a highly conserved family of molecules involved in regulating intracellular signal transduction events within cells and form homo- and heterodimeric U-shaped structures that are able to bind to phosphorylated signalling molecules through a large central channel located in the centre of the U. 14-3-3 protein localizes to the nucleus in the absence of ligand binding and associates with nuclear phosphorylated FOXO3a, with the NES functioning as a binding interface between the 14-3-3- protein and FOXO3a (Brunet et al., 2002). Under conditions of low Akt activity, FOXO is located in the nucleus, anchored to DNA but AKt activation, following growth factor stimulation for example, leads to phosphorylation of FOXO - probably at multiple sites Thr32, Ser253 and Ser315 - inducing the binding of 14-3-3 to FOXO and thus releasing it from the DNA. Once shuttled back into the cytosol, 14-3-3 can prevent nuclear re-entry by masking the NLS and preventing binding of accessory transport proteins (Burgering and Kops, 2002) (Figure 1.7).

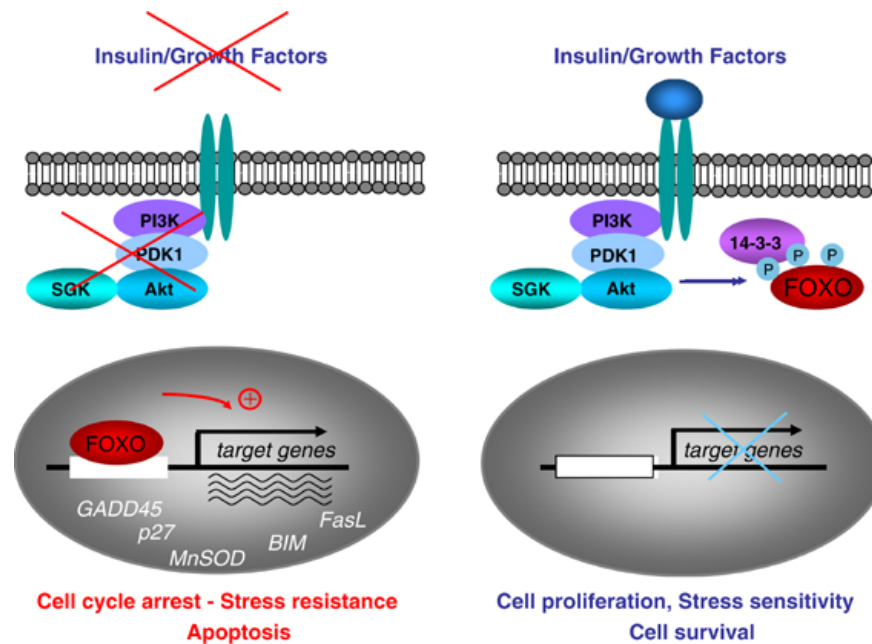


Figure 1.7 FOXO subcellular localisation. In the absence of growth factors, FOXO transcription factors are localized in the nucleus, upregulating key target genes. In the presence of growth factors, the PI3K/SGK pathway is activated, FOXO factors are phosphorylated and sequestered in the cytoplasm (Greer and Brunet, 2005).

Akt mediated phosphorylation of FOXO may regulate its transcriptional activity via other mechanisms. Phosphorylation may prevent FOXO interacting with other co-factors essential for transcription – for example, phosphorylation of FOXO1 prevents binding of the histone acetylase p300, essential to activate transcription (Mahmud et al., 2002).

Phosphorylation is key to FOXO localization, but there are other post-translational modifications that occur which play an important role in FOXO function. Binding of co-activator or co-repressor complexes can alter FOXO acetylation levels. Acetylation of FOXO factors can have an inhibitory effect on their transactivation activity and may reduce their DNA-binding activity. Many FOXO acetylation sites are located in the DNA binding domain and acetylation of FOXO may interfere with

FOXO-DNA binding and prevent transcription. SIRT1, a deacetylase which requires NAD^+ as a co-factor, can deacetylate FOXO, promoting FOXO dependent transcription of genes involved in stress resistance whilst repressing transcription of genes involved in apoptosis (Greer and Brunet, 2005; Motta et al., 2004).

The degradation of FOXO involves an irreversible post translational modification – ubiquitination mediated by the ubiquitin-proteasome pathway. Akt is involved in degradation of FOXO3a and FOXO1 – in particular phosphorylation of Ser256 is the primary site for FOXO1 ubiquitination. Subcellular localization of FOXO is also important in terms of degradation – when all three Akt phosphorylation sites are mutated to alanines and the NLS is mutated, FOXO1 is forced to localize to the cytoplasm and ubiquitination is reduced. Similarly, when phosphorylated FOXO1 is maintained in the cytoplasm due to NES mutation, ubiquitination is also decreased. Thus, for successful degradation, FOXO1 needs to be located in the cytoplasm. The F-box protein Skp2 has been identified as the key E3 ubiquitin ligase responsible for this polyubiquitination of FOXO1, although neither FOXO3a or FOXO4 interact with Skp2, suggesting different ubiquitin ligases may be involved (Arden, 2006; Huang et al., 2005). I KappaB kinase β (IKK β) can also contribute to proteasomes dependent degradation of FOXO. The IKK β signaling pathway has antiapoptotic activity and IKK β can phosphorylate FOXO3a at Ser644, found at the C-terminal end of the molecule. This inhibits FOXO3a activity, promoting proteolysis of FOXO3a by the ubiquitin dependent proteasome pathway (Hu et al., 2004).

1.7.3 Transcriptional targets of FOXO proteins

FOXO proteins have a wide range of cellular roles and can induce the transcription of target genes involved in differentiation, glucose metabolism, protection from oxidative stress, cell death, DNA repair and cell cycle arrest (Greer and Brunet, 2005).

FOXO plays a role in the cellular resistance to oxidative stress. ROS are required for normal cell proliferation but also have the ability to induce apoptosis. In quiescent cells, which lack the protective mechanism of Akt mediated signalling, FOXO3a can protect the cell from oxidative stress by inducing the expression of manganese superoxide dismutase (MnSOD), a free radical scavenging antioxidant enzyme leading to a reduction in ROS, and protection from apoptosis (Kops et al., 2002a). FOXO3a is phosphorylated on a number of serine or threonine residues, excluding those targeted by Akt, in response to oxidative stress by as yet unidentified kinases. It has been postulated that some of these phosphorylation events may interfere with 14-3-3 binding of FOXO, thus allowing nuclear accumulation of FOXO. Alternatively, the stress activated protein kinase (MAPK) c-Jun NH₂-terminal kinase (JNK) may phosphorylate 14-3-3 and allow translocation of FOXO to the nucleus (Furukawa-Hibi et al., 2005). Growth factor-activated Akt and stress-activated JNK appear to be in a tight balance regulating the subcellular localization of FOXO. Akt can inhibit FOXO dependent transcription by phosphorylation of FOXO and cytoplasmic sequestration. However, in response to stress stimuli, JNK can phosphorylate FOXO, leading to nuclear sequestration of FOXO and cause FOXO mediated transcription of target genes involved in the stress response, promoting longevity (Greer and Brunet,

2005). JNK phosphorylated nuclear FOXO can also recruit SIRT1 and SIRT1 deacetylation of FOXO3a can lead to the transcription of stress resistant genes, such as *GADD45* and *p27^{Kip1}*, whilst preventing the transcription of the pro-apoptotic genes *Bim* and *Fas* ligand. The transcriptional co-activator p300 can also interact with FOXO and acetylates it on several lysine residues, leading to inhibition of FOXO transactivation activity. However, SIRT1 can bind to and deacetylate FOXO, reversing the inhibitory effects of p300 (Furukawa-Hibi et al., 2005). Therefore, deacetylation protects against apoptosis and promotes survival under stress through FOXO-mediated transcription (Reagan-Shaw and Ahmad, 2007).

FOXO family members are involved in cell cycle control and have roles at both the G₁/S and G₂/M checkpoints. Ectopic expression of FOXO1, FOXO3a or FOXO4 can block cell cycle progression through G₁, predominantly by inducing the transcription of the cell cycle inhibitor *p27^{Kip1}* (Weidinger et al., 2008). FOXO can directly activate the transcription of the *p27^{Kip1}* gene, the promoter of which contains multiple consensus FOXO binding motifs. FOXO can also increase the expression of a reporter gene controlled by the *p27^{Kip1}* gene promoter and can stabilize *p27^{Kip1}* protein (Nakamura et al., 2000). Upregulation of *p27^{Kip1}* and subsequent binding to the cyclin E/cdk2 complex can prevent entry of cells into S phase (Weidinger et al., 2008).

FOXO can also inhibit cell cycle progression in a *p27^{Kip1}* independent mechanism. Progression from G₁ to S phase of the cell cycle is regulated by the cyclin/cdk complexes cyclin D/Cdk4/6 and cyclin E/Cdk2. Activation of FOXO reduces protein expression of cyclin D1 and D2, resulting in impaired ability of Cdk4 to inactivate the S-phase suppressor pRb. Conversely,

ectopic expression of cyclin D1 is able to overcome FOXO induced cell cycle arrest (Schmidt et al., 2002).

FOXO proteins play a role at other cell cycle checkpoints. FOXO3a functions at the G₂M checkpoint, triggering the repair of damaged DNA. Microarray analysis has shown that FOXO3a modulates the expression of genes involved in regulating the cellular responses to stress at G₂/M, including the cyclin G2 and growth arrest and DNA damage inducible protein 45 (GADD45). In response to stress stimuli, FOXO3a induces a delay in G₂/M, contributing to the repair of damaged DNA in a GADD45 dependent manner (Tran et al., 2002). FOXO can also contribute to exit from the cell cycle and quiescence of cells (G₀). FOXO3a can directly control transcription of the retinoblastoma-like p130 protein, binding directly to consensus binding motifs in the p130 promoter and upregulating its protein expression. In quiescent cells, p130 is hypophosphorylated and bound to the transcription factor E2F-4, which results in repression of the expression of genes required for re-entry into the cell cycle and maintenance of cells in G₀ (Kops et al., 2002b). Therefore, the ability of FOXO3a to induce cell cycle arrest at multiple checkpoints and to control cell cycle re-entry makes it a likely candidate as a tumour suppressor.

In addition to a role in cell cycle arrest, FOXO has a role in programmed cell death. Under low stress conditions, FOXO may be able to regulate the resistance of cells to stress by promoting DNA repair, but with higher levels of cellular insult, cell death may be inevitable and FOXO is integral to commitment to the induction of apoptosis. Target genes of FOXO that mediate apoptosis include *Bim*, a member of the pro-apoptotic Bcl-2 family member and the death cytokines, including Fas ligand

(FasL) and TRAIL (Greer and Brunet, 2005). Bim functions at the mitochondria, disrupting mitochondrial integrity and promoting the release of cytochrome c, triggering the cascade of proteolytic events which are hallmarks of apoptosis (Tran et al., 2003). Apoptosis induced by PI3K inactivation is reduced in lymphocytes from Bim deficient mice (Greer and Brunet, 2005) and FOXO3a activation causes induction of Bim expression in Ba/F3 cells, a mouse pro-B cell line – with an active mutant of FOXO3a inducing Bim expression at both mRNA and protein level (Dijkers et al., 2000). FOXO3a can also induce the expression of Bcl-6, a potent transcriptional repressor, which in turn negatively regulates the expression of the antiapoptotic protein BCL-X_L, suggesting that FOXO may induce apoptosis by Bcl-2 mediated repression of BCL-X_L (Tang et al., 2002). Thus, FOXO may trigger apoptosis by modulating the ratio of prosurvival and prodeath Bcl-2 family members.

As well as regulating transcription by direct FOXO/DNA interactions, FOXO proteins can have a DNA-binding independent effect on transcription through interaction with nuclear receptors – leading to changes in the activity of both proteins (Van Der Heide et al., 2004). FOXO can interact in a ligand dependent manner with the AR, ER, the thyroid hormone receptor and the glucocorticoid steroid receptors amongst others. These receptors are normally localised in the cytoplasm, but when ligand binding occurs, they are released from their chaperones and dimerise, entering the nucleus and binding to the specific response elements of their target genes, which initiates transcription (Rochette-Egly, 2003). Non-steroid receptors are primarily located in the nucleus and are associated with HDAC-containing complexes in the absence of ligand binding, resulting in chromatin compaction and silencing of the

promoter region of target genes. When ligand binding occurs, this complex dissociates and the nuclear receptor can bind to their responsive elements and initiate transcription. All FOXO factors contain an LxxLL domain located at the C-terminus end of the Forkhead DNA binding domain and it is thought that FOXO may interact with nuclear hormone receptors through this motif – regions flanking the LxxLL motif differ between FOXO family members and may play a role in receptor selectivity (Van Der Heide et al., 2004).

The physiological roles of FOXO proteins in stress, cell cycle progression and apoptosis are perhaps the most well characterised, but FOXO proteins have other important cellular roles. They are linked to the prevention of diabetic complications through their role in preserving mitochondrial integrity and cellular energy reserves (Maiese and Chong, 2003). They are protective during ageing and promote longevity – in cultured fibroblasts, silencing of FOXO3a leads to senescence, slows proliferation and the generation of ROS (Kyoung Kim et al., 2005). In summary, FOXO transcription factors are important signalling integrators and by regulating their stability, cellular localization and transcriptional activity, they play a wide role in growth, cell cycle regulation, metabolism, differentiation and survival of normal cells.

1.7.4 FOXO proteins and cancer

The ability of FOXO proteins to control cell survival and cell death suggests that FOXOs may function as tumour suppressors. Their role in cell cycle arrest, DNA repair and apoptosis suggests that loss of FOXO function may lead to a

decreased ability to arrest the cell cycle and promote genomic instability, resulting in tumour development.

A number of studies have pointed to a role for FOXO transcription factors in cancer regulation. As mentioned previously, FOXO1, FOXO3a and FOXO4 are present at chromosomal breakpoints in human cancers – rhabdomyosarcoma for FOXO1 and acute myeloid leukaemia for FOXO3a and FOXO4, resulting in fusion proteins no longer controlled by Akt and constitutively present in the nucleus (Greer and Brunet, 2005). The signalling pathways modulating FOXO activity have all been implicated in cancer – Ras, PI3K and Akt all have oncogenic activity whilst PTEN acts to suppress tumour formation (Tran et al., 2003). In prostate cancer, early studies observed that PTEN is mutated in up to 80% of tumours and this was associated with loss of FOXO1 and FOXO3a activity. Suppression of FOXO3a activity promotes prostate cancer cell growth and the progression of prostate cancer from androgen sensitive to androgen resistant involves decreased FOXO3a expression and reduced p27^{Kip1} promoter activity (Lynch et al., 2005).

Ras is a powerful negative regulator of FOXO and can promote oncogenesis by two independent pathways. Ras can activate the PI3K pathway, leading to phosphorylation of FOXO and nuclear export. Ras can also activate the ERK-MAPK pathway, which targets FOXO and leads to ERK phosphorylated FOXO being targeted for polyubiquitination by the MDM2 E3 ligase and subsequent proteosomal degradation (Yang et al., 2008).

More recent studies have provided further evidence for a role of FOXO proteins as tumour suppressors – conditional deletion of

FOXO1, FOXO3a and FOXO4 simultaneously induces spontaneous tumour formation in mice and thymocytes from FOXO triple-knockout mice show increased proliferation and decreased apoptosis (Dansen and Burgering, 2008). FOXO3a has been shown to interact in the nucleus with the tumour suppressor p53 in response to stress or nutrient deprivation. Combined with the findings that FOXO and p53 share some target genes, such as *p21^{Cip1}* and *GADD45*, this would suggest that these two proteins may coordinate tumour suppression. In fact, activation of FOXO3a in p53 mutated cancer cells is sufficient to induce tumour suppression (Hu et al., 2004; Yang et al., 2008). p53 and FOXO are both phosphorylated and acetylated in response to UV radiation and oxidative stress and both can bind to SIRT1 deacetylase (Luo et al., 2001). FOXO proteins are also known to interact in glioblastoma cells with the tumour suppressor SMAD, which promotes transforming growth factor- β (TGF β) dependent activation of *p21^{Cip1}* and subsequent G₁ cell cycle arrest (Seoane et al., 2004).

FOXO factors also affect oncogenic activity. When active, FOXO can inhibit induction of a number of genes targeted by the oncogene Myc as well as Myc-induced proliferation and transformation and loss of FOXO function allows myc to activate target genes that induce transformation (Bouchard et al., 2004). The oncogene *β -catenin* has also been shown to bind to FOXO factors and this can enhance the ability of FOXO to inhibit cell cycle progression. In colorectal cancer *β -catenin*, in combination with T-cell factor, has been implicated in cancer progression suggesting that FOXO may sequester *β -catenin* away from T-cell factor and inhibit cell cycle progression and subsequent tumour progression (Essers et al., 2005).

With respect to breast cancer in particular, the presence of cytoplasmic FOXO3a in breast cancer tissue sections has been shown to correlate with poor overall survival in breast cancer patients. Cytoplasmic FOXO3a correlates also with expression of IKK β and Phosphorylated Akt, again a poor prognostic factor. In breast cancer cell lines, constitutive expression of IKK β promotes cell proliferation and tumourigenesis which can be overridden by FOXO3a, suggesting negative regulation of FOXO promotes tumour development (Hu et al., 2004).

In summary, inactivation of FOXO appears to be an important step in malignant transformation. The signaling pathways regulating FOXO activity are known to be implicated in cancer development and loss of the tumour suppressor role of FOXO activity may play a crucial role in many common cancers, making the restoration of FOXO activity an attractive therapeutic strategy.

1.8 FOXM1

FOXM1 is another member of the Forkhead box superfamily of transcription factors. The human *FOXM1* gene is a 10 exon structure spanning 25kb on chromosome 12. There are three splice variants of the human FOXM1 protein: A, B and C. Both FOXM1B and FOXM1C are the transcriptionally active variants (Laoukili et al., 2007). FOXM1 is found in all embryonic tissues, especially in proliferating cells of epithelial and mesenchymal origin, but in adult tissue is only found in high levels in the thymus and testes and at moderate levels in the gut and lung – tissues which all have a high proliferative index. Loss of FOXM1 is embryonically lethal in mice, due to failure to enter mitosis, and overexpression of the FOXM1B isoform potentiates the

development and growth of tumours in mouse models of prostate cancer (Kalin et al., 2006). FOXM1 expression is also seen in cancer derived cell lines and it is a proliferation specific protein. Protein and mRNA levels are low in quiescent cells but FOXM1 expression is induced when cells re-enter the cell cycle following serum starvation (Korver et al., 1997). FOXM1 is critical for G₁/S and G₂/M cell cycle phase progression and also for mitotic spindle integrity (Myatt and Lam, 2007). It can regulate the G₁/S transition by diminishing levels of nuclear p21^{Cip1} and p27^{Kip1}, the inhibitory cell cycle regulators, and can activate Cdc25A transcription. An increase in FOXM1 expression is seen at the onset of S phase and continues throughout G₂ and mitosis, where it can activate transcription of Cdc25B, which in turn activates the Cdk-cyclin B complex (Costa, 2005) (Figure1.8).

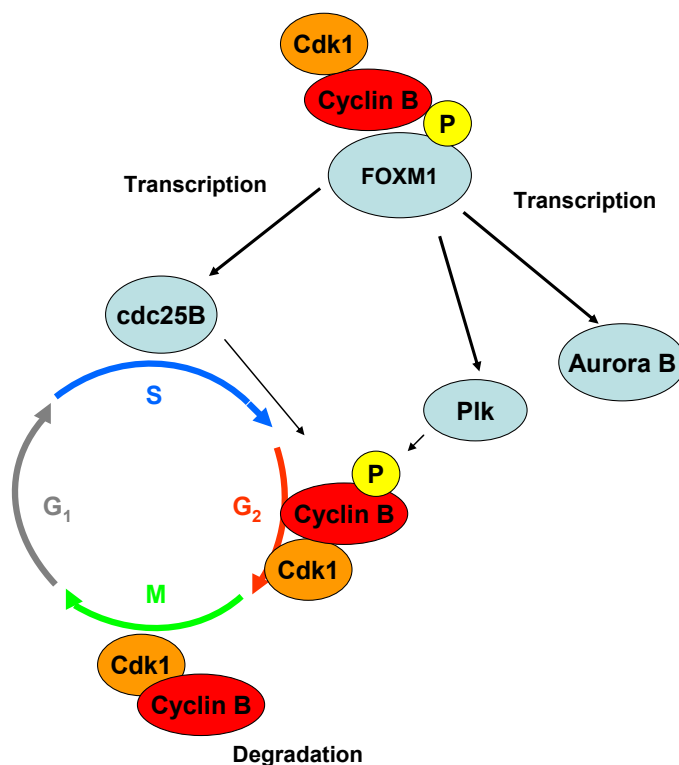


Figure 1.8 FOXM1 regulation of the cell cycle. (adapted from (Costa, 2005)).

FOXM1 is activated by phosphorylation and this requires the presence of appropriate mitogenic signals – MAPK mediated phosphorylation of FOXM1 can regulate subcellular localisation and transcriptional activity and Raf/MEK/MAPK signalling and cyclin E-Cdk2 promotes nuclear translocation of FOXM1 through phosphorylation before entry into the G₂/M phase of the cell cycle, suggesting that multiple kinases may be required to phosphorylate FOXM1 at different stages of the cell cycle (Laoukili, 2007; Ma et al., 2005). pRb phosphorylation by cyclin D1-Cdk4 may also be necessary to relieve FOXM1 repression by disrupting their direct interaction and increased expression of cyclin D1, often observed in breast cancer, may further drive the phosphorylation of FOXM1 (Wierstra and Alves, 2006).

An increase in expression and activity of FOXM1 is associated with the development and progression of human cancers, including breast, prostate, pancreatic and lung cancer (Kwok et al., 2008). Overamplification of the *FOXM1* gene locus on chromosomal band 12q13 is also seen in breast cancer suggesting *FOXM1* may be an oncogene (Spirin et al., 1996). Increased FOXM1 activity may promote tumourigenesis by driving the cell cycle and proliferation by regulating down stream targets such as cyclin B1, CDC25b and Polo-like kinase-1 (Plk1), promoting undifferentiated cells towards malignant transformation (Costa, 2005). FOXM1 can also activate genes involved in angiogenesis and metastasis.

In *Foxm1* knock-out (KO) mouse models, mice die *in-utero*, shortly before birth. Pathologically, these mice show abnormal liver development, with reduced hepatocyte proliferation, reduced liver perfusion and loss of intrahepatic bile ducts.

Pulmonary defects are also seen in *foxm1*^{-/-} mice, with hypertrophic smooth muscle cells and defective pulmonary capillaries, suggesting FOXM1 is required during organogenesis (Laoukili et al., 2007). This is accompanied by highly polypoid (>4*n*) cells in the heart and liver. FOXM1 also has a role in coordinating genes that are important in spindle assembly and chromosome segregation. Pleiotropic cell cycle defects are seen with loss of FOXM1, including a delay in G₂, mis-segregation of chromosomes and frequent failure of cytokinesis (Laoukili et al., 2005). Thus, FOXM1 is crucial for the proper execution of mitosis.

In pancreatic cell lines, downregulation of FOXM1 inhibits cell growth and inhibits the expression of vascular endothelial growth factor (VEGF) and the matrix metalloproteinase-2 (MMP-2) and MMP-9, resulting in the inhibition of migration, invasion and angiogenesis (Wang et al., 2007). Knock-out of FOXM1 in prostate cell lines slows cell proliferation and progression into S phase. This is accompanied by an increase in nuclear p27^{kip1} and reduced expression of cyclin A2, required for S phase promotion and reduced cyclin B1, required for M-phase entry (Kalin et al., 2006). FOXM1 expression has also been shown to be up-regulated in gastric cancer – constitutive expression of FOXM1 is required for the proliferation of gastric cancer cells and knock down of FOXM1 leads to cellular senescence and impaired clonogenesis (Zeng et al., 2009).

1.8.1 FOXM1 as a therapeutic target

FOXM1 has been identified as a potential target for anticancer agents. FOXM1 has been shown to be a physiological regulator of oestrogen receptor α (ER α) in breast cancer cells. FOXM1 and

FOXO3a co-operate to regulate *ERα* gene transcription and ectopic expression of FOXM1 can upregulate the expression of ERα at the protein and transcript level (Madureira et al., 2006). This may have a role in the response of cancers to anti-oestrogens such as tamoxifen and also to the development of endocrine resistance in tumours.

The thiopeptide antibiotic thiostrepton has been shown to have anticancer properties and can cause cell cycle arrest and cell death in the MCF-7 breast cancer cell line, accompanied with a reduction in the invasiveness and transforming ability of these cells. Thiostrepton can induce a rapid and sustained loss of FOXM1 protein and mRNA expression, leading to cell cycle arrest at G₁ and S phase and overexpression of a constitutively active FOXM1 mutant, ΔN-FOXM1, can abrogate the antiproliferative effect of the drug (Kwok et al., 2008). No such effect is seen in the untransformed MCF-10A breast epithelial cells.

1.9 ID PROTEINS

Inhibitor of DNA binding (Id) family members are key regulatory proteins involved in a wide range of developmental and cellular processes. They belong to the helix-loop-helix (HLH) family of transcription factors, which comprises over 200 family members and have been identified in organisms from yeast to man (Norton, 2000).

HLH proteins coordinate cell type specific gene expression and play a role in cell lineage determination, cell proliferation, cell death and differentiation in most tissues. They are grouped into seven classes (I-VII) based on their tissue distribution, their

ability to dimerise and their DNA binding specificities (Coppé et al., 2003). One of the most characteristic features of HLH proteins, which is essential to their normal function, is their ability to form homo- and heterodimers with each other through their highly conserved HLH domain. Most of these proteins belong to a further subclass – the basic HLH (bHLH) family, which share a common sequence motif of a stretch of basic amino acids. bHLH proteins act as transcriptional enhancers or inhibitors of various genes. The basic region within the protein is required for binding to DNA, commonly to a region that includes a specific sequence motif (CANNTG) known as the E-box in the promoter region of target genes (Sikder et al., 2003).

The Id proteins belong to Class V HLH proteins, and are characterized by the fact that they do not possess a basic DNA binding domain. Thus, whilst Id proteins are capable of dimerizing with ubiquitous and tissue specific bHLH factors, the heterodimers formed are unable to bind DNA, ensuring Id proteins function as dominant negative regulators of bHLH transcription factors (Desprez et al., 2003) (Figure 1.9).

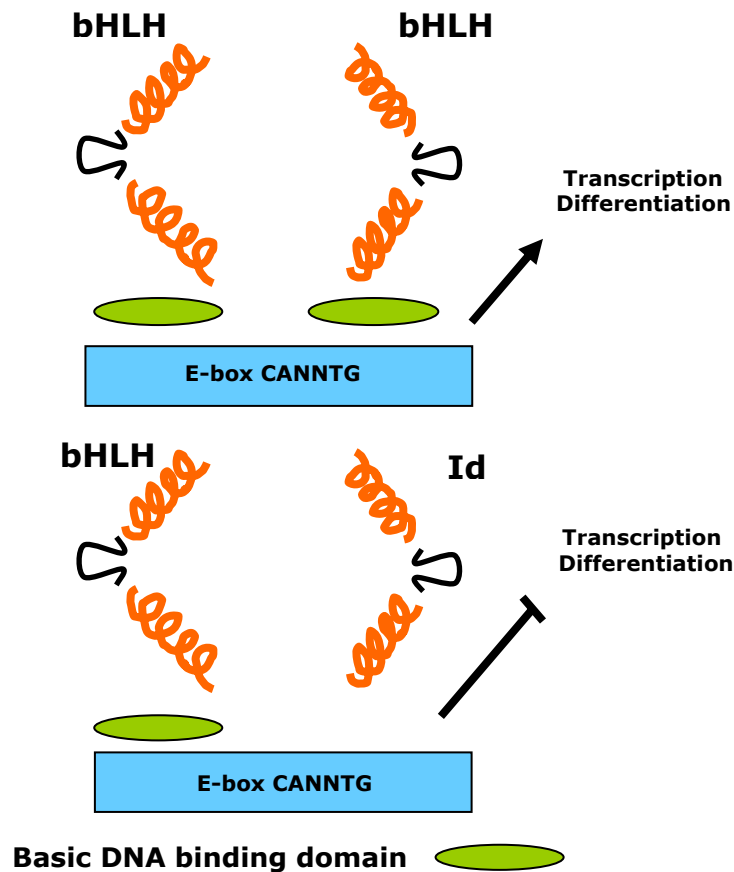


Figure 1.9 Id proteins as dominant negative regulators of bHLH transcription factors. (adapted from Sikder et al, 2003).

As well as their interaction with the bHLH E-protein family, Id proteins can also bind to other important cell cycle regulatory proteins, such as pRb and the Ets family of transcription factors, suggesting they may have a key role in cell cycle regulation and transformation (Zebedee and Hara, 2001).

In mammals, four members of the Id protein family have been identified to date, Id1 through to Id4. They derived their name from their ability to inhibit both DNA binding and act as inhibitors of differentiation. The genes encoding for the four Id proteins are located on different chromosomes and although the proteins share the highly conserved HLH region and have similar sizes of between 13-20KDa, they display extensive sequence

differences and show distinct expression patterns (Wong et al., 2004). Id proteins have been shown to have a role in development. During mouse development, there is widespread expression of Id1, Id2 and Id3 from early gestation through to birth, with considerable overlap between the expression of Id1, Id2 and Id3, whilst Id4 appears limited to the nervous system. In knock-out animal models, deletion of the *Id1* gene alone does not lead to any phenotypic abnormalities, whereas mice that are null for Id2 and Id3 possess defects in immunity (Yakota, 2001). However, mice null for both Id1 and Id3 possess embryonic lethality, with aberrant neuronal differentiation and angiogenesis (Lyden et al., 1999). Increasing evidence has pointed to a role for Id proteins in differentiation and development. Differentiation of a number of cell lineages is accompanied by downregulation of Id expression whilst overexpression of Id in keratinocytes, myeloid precursor cells and mammary epithelial cells inhibits their ability to differentiate (Lasorella et al., 2001).

Id proteins are thought to have a critical role in the cell cycle, promoting G₁-S cell cycle transition. Id proteins are mitogen responsive – quiescent cells express low or undetectable levels of Id proteins. However, when fibroblasts are stimulated by mitogens, Id expression is rapidly induced, within 1-2h, as part of a cascade of early response genes, and levels are sustained through G₁ until the onset of S phase (Norton, 2000). Only some of the cell cycle targets of Id proteins have been characterized. Id2 and Id4 (but not Id1 or Id3) are known to directly interact with pRb. Id2 can bind to pRb and abolish its growth suppressing activity – binding is dependent on the HLH domain of Id2 and the pocket domain of pRB as growth arrest by pRb is not antagonized *in-vitro* using Id2 lacking the HLH

domain (Lasorella et al., 1996). In addition to binding to pRb, Id proteins may promote cell cycle progression by downregulation of other key cell cycle inhibitors. The bHLH protein E2A can block G₁ to S phase transition through the transcriptional activation of *p21^{Cip1}*. Id proteins can heterodimerise with E2A, repressing the transcription of *p21^{Cip1}*, ensuring cell cycle progression (Prabhu et al., 1997).

Id proteins may also play a role in promoting normal cell survival by overcoming replicative senescence - *id1^{-/-}* primary mouse embryonic fibroblast undergo premature senescence and conversely overexpression of Id1 in normal primary cells overcomes replicative senescence in several cell types (Alani et al., 1999). Transfection of human primary keratinocytes with ectopically expressed Id1 results in prolonged survival of normal cells and cotransfection of Id1 with the antiapoptotic factor Bcl-2 leads to immortalization of primary rodent fibroblasts (Norton and Atherton, 1998).

Id proteins are regulated at multiple levels with transcriptional regulation, proteins stability and post translational modifications all contributing to their function. However, very little is known about the upstream signaling pathways that couple Id protein expression to extracellular signals. Like many cell cycle regulators, Id proteins are immediate early genes, and generally have a very short half-life, although they are stabilized by the formation of dimers with bHLH factors (Bounpheng et al., 1999). Id proteins lack a nuclear localization sequence, but as small proteins with molecular weights less than 40kDa can freely pass through the nuclear pore, it has been suggested that their subcellular distribution may be determined in part by passive diffusion (Makita et al., 2006). Alternatively, the E

proteins they bind may act as nuclear chaperones (Ruzinova and Benezra, 2003).

Id proteins can undergo phosphorylation and Id2, Id3 and Id4 (but not Id1) are substrates for Cdk2-dependent phosphorylation during late G₁ and early S phase. Phosphorylation alters the bHLH dimerisation specificity of Id2 and Id3 and appears essential for cell cycle progression – Id2 and Id3 mutants lacking Cdk2 phosphorylation sites elicit arrest in S phase and cell death (Hara et al., 1997).

The rapid turnover and degradation of Id proteins is thought to be due to proteolysis through the ubiquitin-proteasome pathway. Id1, Id2 and Id3 are ubiquitinated and degraded by the 26S proteasome, a large protein complex which cellular proteins are targeted to for degradation by the covalent addition of multiple molecules of ubiquitin (Bounpheng et al., 1999). The rapid degradation of Id proteins is necessary to ensure stringent control of their signalling activity and their role in the cell cycle.

1.9.1 Id1

Id1 was the first Id protein to be identified and is the most well characterized of all the Id proteins and is the focus of the work in Chapter 4 of this thesis. Early evidence supporting Id1 as an inhibitor of differentiation came from studies in haematopoietic cell lineages, where high Id1 expression was detected in precursor cells but decreased during progression through differentiation (Kreider et al., 1992). Id1 blocks erythrocyte differentiation in cultured K562 cells and upregulation of Id1 in CD34⁺ primary cells correlates with inhibition of erythrocyte

differentiation and induction of granulocyte differentiation (Cammenga et al., 2003) and Id1 also has a role in cell fate determination choice, with overexpression of Id1 in embryonic and postnatal cerebral cortex resulting in inhibition of neuronal development in favour of glial cell fate (Cai et al., 2000).

Although *Id1*^{-/-} mice do not produce an obvious phenotype, double knockouts of Id1 and Id3 are lethal, suggesting Id1 expression alone is not essential for embryonic development (Lyden et al., 1999). However, forced expression of Id1 in adult mouse small intestine results in the formation of adenomas, suggesting that although the function of Id1 may overlap with other Id proteins in development, the role it plays in promoting cell survival and malignant transformation could be unique (Wice and Gordon, 1998).

Id1 expression is seen in normal mouse mammary gland development and during early pregnancy, when epithelial ductal cells proliferate and invade the stroma, high expression is seen. During mid pregnancy and the lactating period, Id1 is downregulated and undetectable, corresponding to a highly differentiated state (Parrinello et al., 2001). The SCp2 cell line, a mouse mammary epithelial cell line, which was developed from a midpregnant mouse mammary gland, gave the first insight into a potential role for Id1 in tumour development – transfection of SCp2 cells with a constitutively expressed *Id1* gene lead to failure of the cells to differentiate and lead to cell proliferation and invasion, suggesting Id1 can induce an invasive phenotype (Fong et al., 2003).

1.9.2 *Id1 and cancer*

Id1 overexpression results in three key events that can contribute to the phenotype of a malignant cell – inhibition of differentiation, enhanced proliferation and invasiveness (Perk et al., 2005). There is now much evidence to suggest an oncogenic role for Id1 in human cancers. In a number of epithelial cancers, correlation has been seen between levels of Id1 and malignant potential. In ovarian tumours, strong expression of Id1 correlates with poorly differentiated and more aggressive tumours, resulting in a worse clinical outcome for patients (Schindl et al., 2003). A similar pattern is seen in early stage cervical cancer, where strong or moderate expression of Id1 in tumours is found in patients with shorter disease free survival and overall survival (Schindl et al., 2001). In colorectal adenocarcinomas, Id1 expression is elevated in tumours when compared to normal bowel mucosa, and correlates with mitotic index and p53 immunoreactivity – a marker of mutational inactivation of p53 function (Wilson et al., 2001).

Further evidence for the oncogenic role of Id1 comes from the observation that upregulation of Id1 protein is mediated by well established oncogenes such as Myc and Ras – induction of Id1 by Myc is required for breast cancer cells to enter S phase in culture and overexpression of Myc in breast epithelial cell results in increased expression of Id1 mRNA (Swarbrick et al., 2005). Mitogenic signals driven by oestrogens and insulin growth factor can induce expression of Id1 and some of the extracellular signals that elevate Id1 levels may be mediated by the Ras/Erk/MAPK cascade and the PI3K pathway (Lasorella et al., 2001).

With respect to breast cancer in particular, Id1 is highly expressed in aggressive breast cancer cell lines with loss of serum dependent regulation. When a constitutively expressed *Id1* cDNA is introduced into a non aggressive cell line (T47D), this conferred a more aggressive phenotype as measured by growth and invasiveness. In breast cancer biopsies a similar pattern was seen, with higher expression of Id1 in invasive carcinomas when compared to ductal carcinoma *in situ* (Lin et al., 2000). In lymph node negative breast cancer, patients with strong or moderate Id1 expression have a significant shorter disease free and overall survival compared to those patients with low/absent Id1 expression (Schoppmann et al., 2003). Id1 may also have an impact on the development of metastases, with an increase prevalence of Id1 positivity among lung metastases when compared to primary breast tumours – consistent with its role in invasiveness and sustained proliferation (Gupta et al., 2007).

In summary, Id1 is overexpressed in a variety of cancers and may act as a marker of progression, metastases and outcome. Id1 therefore could be an attractive therapeutic target as it is expressed at low levels in normal tissues but reactivated in many cancers and plays a key role in proliferation, differentiation and invasiveness – events that promote malignant transformation.

THESIS AIMS AND OBJECTIVES

The aim of this thesis was to explore the hypothesis that FOXO3a and its putative downstream targets FOXM1 and Id1 have a role in breast cancer development and progression and are therefore targets for anticancer therapeutics.

Experiments were conducted to demonstrate if:

- 1) FOXO3a was a target of the commonly used chemotherapeutic agent epirubicin
- 2) FOXO3a was involved in the regulation of FOXM1 in breast cancer cells
- 3) Id1 was a target of epirubicin and was regulated by FOXO3a

Finally, results obtained in cell line work were then explored to demonstrate if changes observed were reproducible in clinical samples.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE

The human breast carcinoma cell lines MCF-7 and MDA-MB-231 were cultured in Dulbecco's modified eagle medium (DMEM) (Life Technologies Gibco/BRL, Paisley, UK). The medium was supplemented with 10% foetal calf serum (FCS) (First Link Ltd, Birmingham, UK), 2mmol/L glutamine and 100 U/mL penicillin/streptomycin (Sigma-Aldrich, Poole, UK). Cells were maintained at 37°C in a humidified incubator with an atmosphere of 10% CO₂. Other breast cell lines used for cell panel work were cultured by a similar technique and are listed below (table 2.1). Cell lines were originally all obtained from the American Type Culture Collection (ATCC).

Doxorubicin resistant MCF-7 cells, generated previously in the laboratory, were cultured in a similar way and maintained with the addition of 0.01mg/ml doxorubicin to the culture media (Pfizer, UK).

MCF-10A cells were cultured in DMEM/Ham's F-12 (1:1 mix; Sigma) supplemented with 5% (v/v) horse serum, 10ng/mL insulin, 5mg/mL hydrocortisol, 100ng/mL cholera toxin, 20ng/mL epidermal growth factor, 100U/mL penicillin and 100µg/mL streptomycin (all supplements from Sigma, UK).

ΔN-MCF-7 cells were generated in the laboratory by Mr Jimmy Kwok (Kwok et al., 2008) by transfecting the selection plasmid pBABApuro (Addgene) containing a puromycin selection marker and a ΔN-FOXN1 plasmid. The cell line was maintained in 0.75µg/ml of puromycin.

CELL LINE	SOURCE
MCF-10A	ATCC - fibrocystic mammary gland
MCF-7	ATCC - pleural effusion
ZR751	ATCC - ascites
BT474	ATCC - invasive ductal carcinoma
T47D	ATCC - pleural effusion
MDA-MB-231	ATCC - pleural effusion
MDA-MB-453	ATCC - pericardial effusion
MDA-MB-468	ATCC - pleural effusion

Table 2.1 List of breast cancer cell lines used

2.1.1 Sub-culturing of adherent cells

Cells were grown and split at 80% confluency. Media was aspirated and the cell monolayer was washed in warm PBS and then detached by the addition of 1x trypsin-EDTA. After 5 min at 37°C, the trypsin was inactivated by the addition of complete media. The cells were then resuspended in a flask at a defined ration (1:5) or counted by use of a haemocytometer and seeded in the appropriate plates.

2.1.2 Preservation of cells

Cells were trypsinized, washed in complete media and spun down at 500x g for 5 min. The cell pellet was then resuspended in FCS with 10% DMSO (dimethyl sulphoxide). 1ml aliquots were transferred to cryotubes and frozen at -80°C overnight before transfer to storage in liquid nitrogen.

When defrosting cells, the cryotube solution was added to complete media, then spun at 1200rpm for 3 min to remove any DMSO-containing media and the pellet was resuspended in 12ml of media in a 25cm² flask.

2.2 CHEMICALS

Epirubicin (2mg/ml in 0.9% sodium chloride) and doxorubicin (2mg/ml in 0.9% sodium chloride) were obtained from Pfizer, UK and stored in solution at 4°C.

2.3 PROTEIN ANALYSIS

2.3.1 Preparation of total protein extracts

Whole cell protein extracts were prepared by initially harvesting cells, washing the cell pellet in PBS before centrifuging at 250 x g for 5 min. The supernatant was discarded and pellets were frozen at -80°C until lysis was performed. Frozen pellets were lysed in 2 volumes of NP40 lysis buffer (1% (v/v) Nonidet P-40, 150mM NaCl₂, 50mM Tris-HCl (pH 7.8), 5mM EDTA, 1mM DTT, 1mM NaF, 1mM PMSF, 1mM Na₃VO₄ and protease inhibitors (complete protease inhibitor mixture, as instructed by the manufacturer (Roche Applied Sciences, UK) on ice for 15 min. Insoluble material was removed by microcentrifugation at 13000rpm at 4°C for 10 min. The supernatant was transferred to a clean eppendorf tube and its protein concentration was assayed.

2.3.2 Determination of protein concentration

Protein concentration was determined using the Bio-Rad D_c protein assay (Bio-Rad Laboratories, CA, USA). 20µl of reagent S was added to 1ml of reagent A. A standard curve was established by assaying 5 dilutions of a protein standard within the range of 0.2mg/ml to 1.5mg/ml protein. 100µl of reagent A was added to 2µl of each dilution and mixed with 800µl of

reagent B. After 15 min, absorbance was read at 750nm and a standard curve was established. The absorbance of the experimental samples were read at 750nm and their protein concentration determined by multiplying the absorbance of the sample by the standard curve's regression co-efficient.

2.3.3 (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) SDS-PAGE

Gels for resolution of proteins were made using a 37.5% (w/v) acrylamide/bis stock solution. Ammonium persulphate (APS) and TEMED were used as polymerisation catalysts. The percentage of gel used depends on the size of the protein being isolated - 7 and 14% gels predominantly. Gels consisted of a lower resolving gel and a 5 % upper stacking gel, according to the following table:

Resolving gel	7%	14%	Stacking gel	5%
dH ₂ O	5.02ml	2.68ml	dH ₂ O	3.67ml
1M Tris-HCl (pH 8.8)	2.5ml	2.5ml	1M Tris-HCl (pH 6.8)	0.42ml
Acrylamide: Bisacrylamide (37.5:1)	2.33ml	4.67ml	Acrylamide: Bisacrylamide (37.5:1)	0.83ml
10% SDS	100µl	100µl	10% SDS	50µl
25% APS	40µl	40µl	25% APS	20µl
TEMED	10µl	10µl	TEMED	10µl
TOTAL	10mls	10 ml	TOTAL	5 ml

Table 2.2 Proportions of the constituents of SDS-PAGE resolving and stacking gels

To separate the proteins, 25µg of lysate was added to 1 volume of 2x SDS loading buffer (4% (w/v) SDS, 62.5mM Tris-HCL (pH 6.8), 1% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 10% (v/v) β-mercaptoethanol) and boiled at 100°C for 3 min. Samples were then loaded into the stacking gel wells and fractionated by electrophoresis on the SDS-PAGE gels and run at a constant voltage of 100V in running buffer (25mM Tris-base, 250mM glycine, 0.1% (w/v) SDS). Proteins were separated alongside an Amersham rainbow marker (GE Healthcare Ltd, UK) to monitor progress of protein electrophoresis and the molecular weight of the separated proteins.

2.3.4 Western blotting of SDS-PAGE

Once separated by SDS-PAGE, proteins were then transferred to a nitrocellulose membrane (Whatman, Germany) by electrophoretic transfer in a wet tank blotting system (Bio-Rad Laboratories Trans-Blot cell) in transfer buffer (25mM Tris, 190mM glycine and 20% (v/v) methanol) for 90 min at 90V. The nitrocellulose membrane was then blocked at RT for 1 h in 5% (w/v) skimmed milk powder (Marvel, Premier Brands, UK) dissolved in Tris Buffered Saline/Tween (TBS-T) (20mM Tris (pH 7.6), 136mM NaCl, 0.05% (v/v) Tween) with shaking. The membrane was then incubated overnight with the primary antibody of choice (Table 2.3) in 25ml 5% BSA TBS-T at dilutions recommended by the supplier. The next day, the membrane was washed five times in TBS-T for 10 min each at RT, prior to incubation with the secondary anti-mouse, anti-rabbit or anti-goat horseradish peroxidase (HRP) conjugated secondary antibody (Dako (UK) Ltd, Ely, UK). The membrane was then exposed to an auto-radiographic film for varying

lengths of time and bands visualized using the ECL detection system (GE Healthcare, UK).

Antibody	Supplier
Actin (L-19) Goat polyclonal	Santa Cruz Biotechnology, CA
Akt (5G3) Mouse monoclonal	Cell Signalling Technologies
Bim (Y36) Rabbit polyclonal	Abcam Ltd, UK
CDC25b (C-20) Rabbit polyclonal	Santa Cruz Biotechnology, CA
Cyclin B1(H-433) Rabbit polyclonal	Santa Cruz Biotechnology, CA
Cyclin D1 (HD11) Mouse monoclonal	Santa Cruz Biotechnology, CA
FOXO3a (H-144) Rabbit polyclonal	Santa Cruz Biotechnology, CA
FOXO1(C-20) Rabbit polyclonal	Santa Cruz Biotechnology, CA
Id1 (C-20) Rabbit polyclonal	Santa Cruz Biotechnology, CA
Phospho-Akt(Thr ³⁰⁸) 92755 Rabbit polyclonal	Cell Signalling Technologies
Phospho-Akt(Ser ⁴⁷³) 9271L Rabbit polyclonal	Cell Signalling Technologies
Phospho-FOXO3a(Thr ³²) 9464C Rabbit polyclonal	Cell Signalling Technologies
p21 (F-5) Mouse monoclonal	Santa Cruz Biotechnology, CA
p27 (C-19) Rabbit polyclonal	Santa Cruz Biotechnology, CA
Plk (3F8) Mouse monoclonal	Santa Cruz Biotechnology, CA
Sp1 (IC6) Mouse monoclonal	Santa Cruz Biotechnology, CA
Tubulin (H-235) Rabbit polyclonal	Santa Cruz Biotechnology, CA

Table 2.3 Antibodies used for Western blotting

2.4 RNA MANIPULATION

2.4.1 Total RNA extraction

Total RNA was isolated from cells using buffers from the RNeasy kit (Qiagen, Crawley, UK). The kit allows samples to be lysed and homogenized by a guanidine-thiocyanate-containing buffer which is highly denaturing. This inactivates RNases to ensure purification of intact RNA. Ethanol provides appropriate binding conditions and the samples are then applied to an RNeasy mini spin column, where high quality total RNA binds to the membrane and contaminants are washed away.

Frozen cell pellets were disrupted by adding 350µl buffer RLT with 10% β-mercaptoethanol. Samples were homogenized by pipetting, then 350µl 70% ethanol was added and 700µl of total sample was added to a spin column placed in a 2ml collection tube and microcentrifuged for 15s at 10000rpm. Flow through was discarded and 700µl of buffer RW1 was added to the RNeasy spin column. This was microcentrifuged for 15s at 10000rpm and the flow through again discarded. Next, 500µl of buffer RPE was added to the spin column and microcentrifuged at 10000rpm for 15s. The flow through was discarded and this step was repeated, followed by 2 min of centrifugation at 10000rpm. The RNeasy column was then transferred to a new collection tube. 30µl of RNase-free water was added directly to the spin column membrane and the column was microcentrifuged for 1 min at 10000rpm to elute RNA. The purity and concentration of RNA was determined by measuring the spectrophotometric absorption at 260nm and 280nm.

2.4.2 *Real-time quantitative PCR (RT-qPCR)*

2µg of total RNA was reversed transcribed to first strand cDNA using the Superscript III first strand synthesis system (Invitrogen, Paisley, UK). 1µl of random primers and 1µl 10mM dNTP mix was added to 2µl of total RNA and made up to a total volume of 13µl with sterile RNase free water. The sample was heated at 65°C for 5 min to denature then placed on ice for at least 1 min. The samples were then mixed with 4µl 5x first strand buffer, 1µl 0.1M DTT, 1 µl RNaseOUT and 1 µl Superscript III RT, to a final volume of 20 µl per sample. The mixture was incubated at 25°C for 5 min then heated at 50°C for 50 min. The reaction was terminated by heating at 70°C for 15 min.

The resulting cDNA samples were used as a template for RT-qPCR, which was performed using the TAQMAN 7700 machine and 2x SYBRGreen Mastermix (Applied Biosystems) according to manufacturers instructions. Gene specific primer pairs were designed using the ABI Primer Express Software (Applied Biosystems) or obtained from the literature. Analysis was performed using the relative standard curve method, where 1-2µg cDNA from each sample was mixed, diluted 1:2 and then 1:4 for four serial dilutions. As an internal control, the L19 ribosomal housekeeping gene was used to normalize input RNA. The reaction mix contained 2µl of sample and 23 µl of SYBR Master Mix and primers to a final volume of 25µl. All measurements were performed in triplicate. The TAQMAN 7700 Real-time PCR machine was used. Efficiency of PCR was obtained from the slope of the standard curve.

2.4.3 Primer optimization

Each primer set was optimized with 10ng cDNA standard to determine the minimum concentration required for maximum yield and minimum non-specific amplification. Dilutions of 50-900nM of forward and reverse primers in different combinations were tested. The concentration that gave the lowest Ct value (10-35) with standard DNA and highest Ct value (40) with water was selected for use in further experiments. The concentrations of primers used to amplify FOXM1 and L19 were optimized by Dr Demetra Constantinidou and Dr Rosaline Hui (E.lam's laboratory, Imperial College, London.)

Primers used for RT-qPCR were as follows:

Primer	Sequence 5'-3'
Human FOXO3a-sense	TCTACGAGTGGATGGTGCGTT
Human FOXO3a-anti-sense	CGACTATGCAGTGACAGGTTGTG
Human FOXM1-sense	TGCAGCTAGGGATGTGAATCTTC
Human FOXM1-anti-sense	GGAGCCCAGTCCATCAGAACT
Human Id1-sense	CTGGACGAGCAGCAGGTAAA
Human Id1-anti-sense	AGCTCCTTGAGGCGTGAGTAA
Human L19-sense	GCGGAAGGGTACAGCCAAT
Human L19-anti-sense	GCAGCCGGCGCAAA

Table 2.4 Primers used for RT-qPCR

2.5 DNA TRANSFECTIONS OF MAMMALIAN CELLS

2.5.1 Plasmid constructs

The full length (-1480/-25) human *FOXO3a* promoter was cloned previously by Dr Karen Pomeranz and used in this study, having been introduced into a pGL3 basic vector (Promega, UK).

The human *FOXM1* promoter construct was a gift from Professor Rene H. Medema (University Medical Center Utrecht, the Netherlands). The *FOXM1* promoter/ApaI fragment with mutations of forkhead binding sites were generated by Dr Maja Petkovic by performing two rounds of PCR using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) following the manufacturers instructions. The PCR primers used were as follows:

First round	primer1F:cgccggcgccaatttcaccacgcggaacaaactgaaagc primer1R: cgtttcagtttggtccgcgtggtgaaattggcgccggcg
Second round	primer2F: ccaatttcaccacgcggccacaactgaaagctccggtg primer2R: caccggagcttcagttgtggccgcgtggtgaaattgg.

Table 2.5 Primers used for FOXM1 promoter mutation.

The wild-type and mutant *Id1* promoters were a gift from Professor Paul Coffey (Department of Molecular Medicine, University of Utrecht, The Netherlands). The promoter spanned the region upstream of the transcription start site (+1 to -1692). Using site directed mutagenesis, Dr Kim Birkenkamp generated the FHRE (at position -131) mutated *Id1* promoter which was cloned into pGL3-Basic vector (Promega, Southampton, UK) resulting in pGL3*Id1*^{FHREMUT}. The pLZRS-Id1 vector was also a gift from Professor Paul Coffey.

2.5.2 Promoter assays

24h prior to transfection, MCF-7 cells were cultured in a 96 well plate at a density of 3000 cells/well in 100µl of complete media and left overnight. Cells were then transfected using Fugene 6 transfection reagent (Roche, UK) according to the manufacturers instructions. The transfections were performed

in triplicate. 20ng of pGL3 firefly-luciferase reporter plasmid, containing the appropriate cloned promoter sequence, and 5ng of pRLTK renilla plasmid (Promega, Southampton UK) was added to 0.075µl of Fugene diluted in 5µl of serum free DMEM and incubated at RT before addition to the cells.

Cells were then left overnight, and drug added at the appropriate time points. On completion of the time course, wells were then washed three times in PBS and stored at -80°C until ready for analysis.

2.5.3 *Transfection of plasmid DNA*

24h prior to transfection, MCF-7 cells were plated in a six well plate at a density of 200,000 cells per well. Transfections were performed using a ratio of Fugene 6 (µl) to DNA (µg) of 3:1. After 15min incubation, the fugene/DNA mixture was added directly to each well and the cells were incubated for 5h at 37°C after which time the cells were washed in warmed PBS and fresh media added.

2.5.4 *Luciferase reporter assay*

Luciferase assay was performed with the LucLite Luminescence Reporter Gene Assay System (Perkin Elmer™ Life Sciences, Milan, Italy) coupled with coelenterazine (Lux Biotechnology, Edinburgh, UK) according to manufacturers instructions.

Cells were lysed in 1xLucLite Reagent for 15min at RT. 100µl of lysate was transferred to a 96 well microplate and firefly luciferase activity was read with TopCount Luminometer (Perkin

Elmer™ Life Sciences) – two wells are read simultaneously for 1s and counts per second are determined.

To measure renilla activity, 25µl of RenLite reagent - coelenterazine (50µg/ml) in renilla buffer (0.5M HEPES, pH7.8, 40mM EDTA) – was added to each well and incubated in the dark for 20min at RT, before measurement of renilla activity with the TopCount Luminometer.

The relative luciferase activity was determined by dividing firefly luciferase activity by renilla luminescence values.

2.6 FLUORESCENCE ACTIVATED CELL SORTING (FACS) ANALYSIS

Cells were treated as indicated and then collected by centrifugation and washed in PBS. Cells were fixed by resuspension in 90% ethanol with 10% PBS and stored at -20°C until analysis.

For analysis, samples were microcentrifuged at 10000rpm to remove the ethanol and then resuspended in PBS and stained with propidium iodide (50µg/ml) and RNase A (5µg/ml) for 30min at 4°C. The cell suspension was then aspirated through a fine bore needle to generate a single cell suspension.

Samples were analysed on a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK) using the FACSDiva™ (BD Biosciences) acquisition software. Graphical results represent the % of cells (Y-axis) in each phase of the cell cycle determined by their DNA content (X-axis).

2.7 *SULPHORHODAMINE B (SRB assay)*

The SRB assay is used for examination of cell proliferation/viability as the colorimetric measurement of the bound dye allows a measurement of cellular protein content. SRB is a water-soluble dye that can bind to the basic amino acids of cellular proteins. Cells were initially seeded in a 96 well plate at a density of 3000 cells/well and incubated overnight. On the day of harvesting, cells were fixed with 100µl cold 40% (w/v) trichloroacetic acid (TCA) and incubated for 1 h at 4° C. Plates were washed five times with slow running tap water then 100µl of SRB solution (0.4% SRB in 0.1 % acetic acid) was added. Plates were left at room temperature for 1 h, then washed with 1% acetic acid to remove unbound dye and allowed to dry overnight.

To read the plates, 100 µl of 10mM TRIS base solution was added to each well, solubilising the protein bound dye. The plates were then left on a shaker for 30 min. Optical density (OD) at 492nm was measured on an Anthos 2001 microplate reader.

2.8 *RNA INTERFERENCE (RNAi)*

RNA interference is used to suppress gene expression in mammalian cells. RNAi is triggered by an externally introduced double strand RNA (dsRNA). The dsRNA is cleaved by the RNase-III enzyme, Dicer, into small interfering RNAs (siRNA) that are approximately 21-23 nucleotide fragments. The siRNA then loads into an RNA-Induced Silencing Complex (RISC) which

facilitates the separation of the two siRNA strands and cleavage of the target mRNA.

For the work in this thesis, genes were silenced using the SMARTpool™ product Insert – Version 2.0 (DHARMACON, RNA technologies). The SMARTpool siRNAs used were siFOXO3a (L-003007-00) and siId1 (L-005051-00). The transfection reagent used was Oligofectamine (Invitrogen Ltd, UK).

MCF-7 cells were plated at a density of 200,000 cells per well in a six-well plate 24h prior to transfection. For each well, 70µl of optiMEM (Life Technologies Gibco/BRL, Paisley, UK) was mixed with 5µl of oligofectamine and incubated at room temperature for 10min. This solution was then mixed with 250µl optiMEM and 7.5µl of the specific siRNA oligonucleotide for each gene, with a resulting final concentration of 50nM. After a further 25min incubation, 160µl optiMEM was added to each mix and 500µl was then added to the wells, which had been washed immediately beforehand with warmed PBS. After 4h of incubation at 37°C, 2ml of normal media, with 10% FCS, was added back to each well.

Two types of controls were used for the siRNA experiments. As a negative control, cells were transfected with a non-specific/non-targeting siRNA (NSC D-001210-01-05, Dharmacon). This served to distinguish sequence specific silencing from non-specific effects. These siRNAs have an impaired ability to be processed by the RISC and share little or no homology with known mRNA targets in the cell. Another control used was the untreated or mock control, where cells were cultured without any siRNA treatment. This allows

detection of any cellular effects caused by the transfection event or delivery process and determines target gene level.

48-72h post-transfection, cells were harvested and protein and mRNA levels were analysed by Western blotting or RTqPCR as described previously.

2.9 SUBCELLULAR FRACTIONATION

The NE-PER nuclear-cytoplasmic kit (Perbio, Belgium) was used to separate and prepare cytoplasmic and nuclear extracts from MCF-7 cells, according to the manufacturers instructions. Two kit reagents, named CERI and CERII were added to the cell pellet, causing disruption of the cell membrane and release of cytoplasmic contents. The intact nuclei were then recovered from the cytoplasmic extract by centrifugation and lysed with the NER kit reagent to yield the nuclear extract.

Extracts were then used for Western blot analysis. Efficient fractionation was confirmed by analyzing the purity of the cytoplasmic fraction by blotting for tubulin and for the nuclear fraction with Sp1.

2.10 IMMUNOHISTOCHEMICAL (IHC) STAINING

Patients receiving neo-adjuvant chemotherapy at Charing Cross Hospital, London were identified from a clinical data-base. A formal application to the Trust's Tissue Management Committee (authorised by the Wales MREC to release tissue to researchers) was made and ethical approval granted to access the surgical blocks from the identified patients.

Tissue blocks from pre-chemotherapy core biopsies and post - chemotherapy surgical specimens were matched for individual patients and collected from the tissue bank at Charing Cross Hospital. 5µm slides were cut from the blocks by Ms Mahrokh Nohadani, Research Laboratory Manager in the histopathology department at Hammersmith Hospital and immunohistochemical staining was performed on these formalin-fixed, paraffin embedded sections.

Additional slides from a 2005 neo-adjuvant study of gefitinib were also stored in the laboratory and immunohistochemical staining was performed on these formalin-fixed, paraffin embedded sections. Ethical approval was obtained at the time of the study (Polychronis et al., 2005).

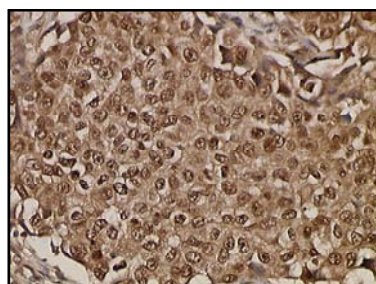
Slides were loaded into a rack and placed in an oven at 70°C for 1 h to melt the paraffin around the tissue sample. To remove the embedding material, the samples were deparaffinised and rehydrated. This was done by sequentially placing the slides and rack in xylene twice for 10 min, 100% ethanol for 10 min, 70% ethanol for 10 min then finally tap water. The slides were then placed in 1l of pre-warmed citrate buffer (0.01M sodium citrate, pH 6) and then heated at full power for 15 min in a microwave for antigen retrieval. This allowed recovery of the antigenicity of the tissue which had been masked by formalin fixation. After 20 min of cooling at RT, the slides were transferred rapidly to a cuvette containing warmed PBS and washed a further two times in PBS. To block endogenous peroxidase activity, which may result in high, non-specific background staining, slides were then immersed in 3% H₂O₂ in methanol for 10 min. This resulted in the irreversible

inactivation of endogenous peroxidase. Slides were then washed again twice in PBS for 5 min.

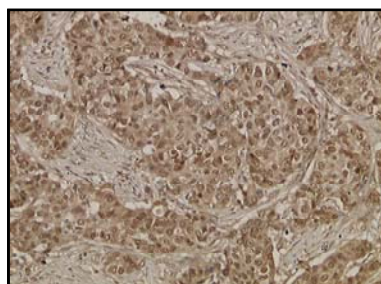
Next, the slides were placed on a PBS moistened incubation tray and the tissue samples were outlined with a hydrophobic pen. Five drops of V block (Thermo Fisher Scientific, CA, USA) was added to each sample. This further reduced nonspecific background staining. After 5 min the primary antibody was added.

The dilution of antibody used was determined by first performing a titration of antibody dilutions, to optimize tissue staining whilst eliminating excessive background or non specific staining. For example, for optimization of FOXO3a staining with the upstate FOXO3a rabbit polyclonal antibody, the following dilutions were used: 1:100, 1:200, 1:400, 1:800 and 1:1600. Omission of primary antibody was used as a negative control. Sensitivity and staining quality was then imaged on light microscopy and the most appropriate dilution selected for subsequent staining. In the case of FOXO3a, 1: 400 was used as the optimal dilution for staining of the breast cancer samples (Figure 2.1). Optimization of FOXM1 and Id1 staining was performed by the same method.

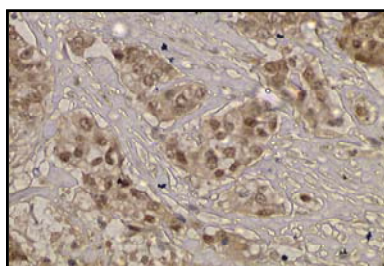
The primary antibody was diluted in Lab vision UltraAntibody diluent (Lab Vision Corporation, CA, USA) at the optimized dilutions (table 2.6) and 200µl was added to each sample and the slides were incubated overnight with the primary antibody at 4°C.



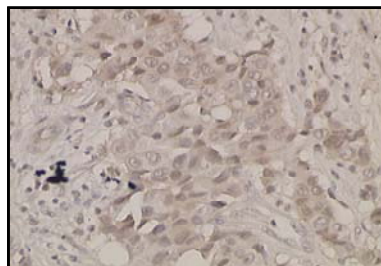
1: 100



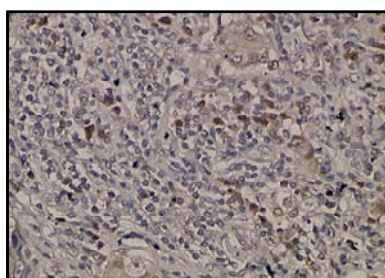
1 : 200



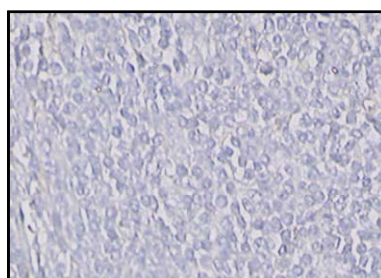
1 : 400



1 : 800



1 in 1600



**Negative
control**

Figure 2.1 Optimisation of the FOXO3a antibody for tissue staining. For optimization of FOXO3a staining with the upstate FOXO3a rabbit polyclonal antibody, the following dilutions were used: 1:100, 1:200, 1:400, 1:800 and 1:1600. Omission of primary antibody was used as a negative control. The dilution giving optimum staining with minimal background was used for subsequent experiments.

Antibody	Dilution
FOXM1(C-20) Rabbit polyclonal Santa Cruz Biotechnology,CA	1:400
FOXO3a (07-1719) Rabbit polyclonal Upstate	1:400
Id1 (C-20) Rabbit polyclonal Santa Cruz Biotechnology,CA	1:100

Table 2.6 Antibodies and dilutions used for IHC staining

The following day, the slides were washed three times in PBS for 5 min and incubated with biotinylated goat antipolyvalent secondary antibody (Lab Vision Corporation, CA, USA) for 15 minutes. The slides were then washed twice in PBS for 5 min, dried off by gentle shaking and then streptavidin peroxidase (Lab Vision Corporation, CA, USA) was added for 15 min. Slides were then wash twice in PBS.

Chromagen DAB/ substrate reagent solution was prepared from a DAKO kit (DAKO REAL EnVision Detection System, DAKO, Denmark) adding 20ul DAB chromagen to 1ml DAB buffer. (In the presence of a peroxidase enzyme, DAB will produce a brown precipitate that is insoluble in alcohol, thus allowing the slides to be dehydrated through alcohols to xylene in the final steps). The chromagen/DAB substrate was applied to the slides and the tissue was monitored for staining development and intensity. Staining was terminated by washing the slides in tap water for 5 min. 4-5 drops of Mayer's haematoxylin counterstain (DAKO, Denmark) was added to each sample, and removed after 30 s by washing in tap water.

The final steps involve dehydrating the slides by sequential washing in tap water for 10 min, 70% ethanol for 10 min, 100% ethanol for 10 min and xylene for 10 min. Slides were mounted by adding one drop of mounting media (Histolab Products, Gothenburg, Sweden) to each sample, then covering the sample with a coverslip. Slides were allowed to dry before examining under the microscope.

For the tissue slides analysed from the gefitinib neo-adjuvant study, intensity of FOXM1, Ki67 and FOXO3a staining was evaluated using a 0 to 3 score system. A score of zero indicated no staining relative to background, 1+ = weak staining, 2+ = moderate staining, 3+ = strong staining. For comparison of staining, the results were quantified by calculation of H-scores that consider both staining intensity and the percentage of cells stained at a specific range of intensities. A complete H-score was calculated by multiplying the percentage cells stained at a given staining intensity (0–100) and the staining intensity itself (0–3).

2.11 IMMUNOFLUORESCENT STAINING

MCF-7 cells were plated in six well plates containing 2cm coverslips. 24 h after plating, media was removed, the cells were washed in warmed PBS and media containing a final concentration of 1 μ M epirubicin was added back to the wells. Cells were incubated with epirubicin containing media for the required time then the coverslips were removed with forceps and fixed with 4% formaldehyde in PBS for 10 min at room temperature (RT). The fixative was removed and the cells were washed four times with PBS for 5 min. The cells were then permeabilized with 0.2% triton in PBS for 5 min, washed again

with PBS for 10 min, followed by blocking with 5% goat serum in 10% BSA for 30 min. The primary antibody (FOXO3a, H144, Santa Cruz Biotechnology) was then added at a dilution of 1:100 dissolved in 10% BSA blocking solution and the cells were incubated for 1 h, before further washing with PBS three times for 5 min. The secondary antibody (Alexa-Fluor 555 anti – rabbit, Invitrogen) was then added (1:500 dilution in 10% BSA) and the cells were incubated in the dark for 1h. The cells were washed again three times in PBS for 5 min each. Cells were then incubated in DAPI for nuclear staining (1:1000 dilution in 10% BSA) for 1h in the dark. The coverslips were placed on microscope slides, sealed with clear nail varnish and the slides analyzed on a Zeiss confocal microscope with LSM meta 510 software.

2.12 STATISTICAL ANALYSIS

T-test was used to determine statistical significance of the differences between the mean of two groups using the excel programme. A *p* value of less than 0.05 was considered significant (*).

In chapter 5, to test for differences between Ki67, nuclear FOXO3a and FOXM1 expression pre- and post-gefitinib treatment and FOXM1 expression in treated and control groups before and after treatment, Dunnett's two-tailed t-test was performed and the mean difference considered significant at the $p < 0.05$ level. The relationship between fold changes in H-score in FOXM1 and Ki67 or nuclear FOXO3a was statistically analysed using Pearson's correlation test based upon their H-scores and was considered significant at $p < 0.05$. All statistical analysis was performed with SPSS v.15 (SPSS inc, Chicago, IL, USA).

CHAPTER 3

FOXO3a AS A TARGET OF EPIRUBICIN

3.1 INTRODUCTION AND OBJECTIVES

Improvements have been seen in the last twenty years in the outcome of patients who develop breast cancer. The use of adjuvant chemotherapy has undoubtedly contributed to this and central to many of the chemotherapy regimens that patients receive is the anthracycline epirubicin. Given usually in combination with 5-fluorouracil and cyclophosphamide, epirubicin remains one of the most widely prescribed drugs for breast cancer in the UK. The anthracyclines inhibit the topoisomerase enzymes, interfering with the ability of the enzyme to repair transient double strand breaks in the DNA helix. However, following topoisomerase inhibition, the subsequent mechanisms of anthracycline induced cytotoxicity are not fully understood.

The FOXO family of transcription factors function downstream of the PI3K pathway and have a key role in a range of cellular processes. A number of studies have also pointed to a role for FOXO in cancer regulation – their ability to control cell survival and cell death suggests they may function as tumour suppressors. FOXO factors also appear to be cellular targets for anti-cancer drugs. In leukaemic cells, treatment with imatinib and cell death is accompanied by activation of FOXO3a (Birkenkamp et al., 2007) and in breast cancer cells, FOXO3a has been shown to be a crucial target for the EGFR inhibitor gefitinib (Krol et al., 2007). With respect to chemotherapy, paclitaxel mediated cell death is accompanied by increased FOXO3a activity and increased expression of its pro-apoptotic target *Bim* (Sunters et al., 2003; Sunters et al., 2006). The effects of epirubicin on FOXO3a function and regulation have not previously been characterized and the work in this chapter aimed to determine whether the cellular effects of epirubicin are

via FOXO3a and to clarify the role of FOXO3a and its targets in epirubicin mediated cytotoxicity.

3.2 RESULTS

3.2.1 *FOXO3a expression in a breast cancer cell line panel*

To establish the expression of FOXO3a amongst breast cancer cells, Western blotting of 10 different breast cancer cell lines was performed (Figure 3.1). FOXO3a was expressed in all the cell lines investigated, although there was a large amount of heterogeneity amongst the cell lines. Interestingly, the non-transformed MCF-10a also expressed significant amounts of FOXO3a, consistent with a role for FOXO3a not just in malignant cells but also in untransformed cells growing in culture.

3.2.2 *FOXO3a is highly expressed in growth arrested cells*

The breast cancer cell line panel confirmed the expression of FOXO3a in all cell lines investigated. To further characterize the expression pattern of FOXO3a, MCF-7 cells were serum starved for 24h, then serum containing media was reintroduced and the cells analysed at various timepoints. The highest expression of FOXO3a protein and mRNA was found in growth arrested cells, consistent with a role for FOXO3a in cell cycle progression and arrest (Figure 3.2a and 3.2b). As cells re-enter the cell cycle, FOXO3a levels fall. A similar effect was observed at the gene promoter level, with FOXO3a promoter activity being highest in serum starved cells and gradually reducing as the cells are stimulated with full serum (Figure 3.2c).

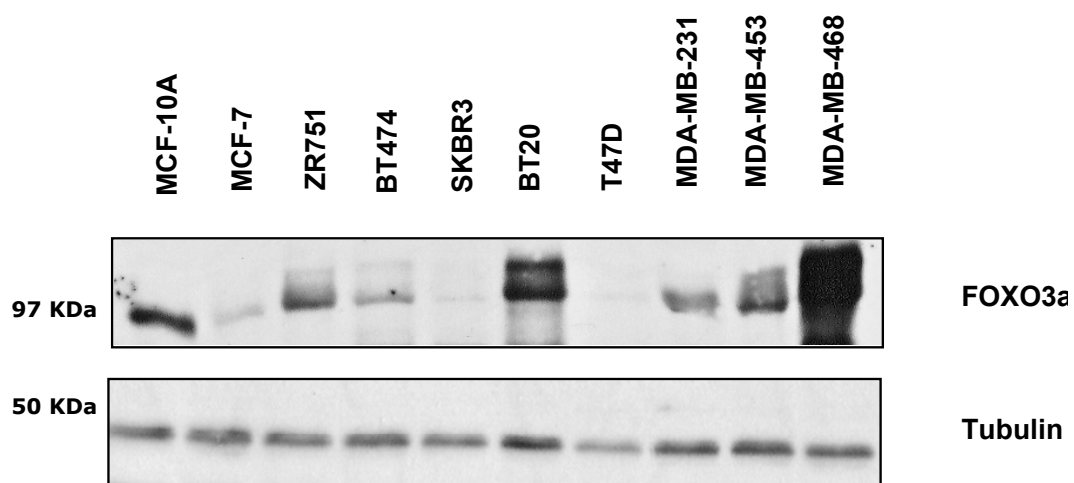


Figure 3.1 The expression of FOXO3a in a breast cancer cell line panel. Whole cell lysates were prepared from the panel of breast cancer cell lines as indicated. The expression of total FOXO3a was analysed by Western blotting. Tubulin was used as a loading control.

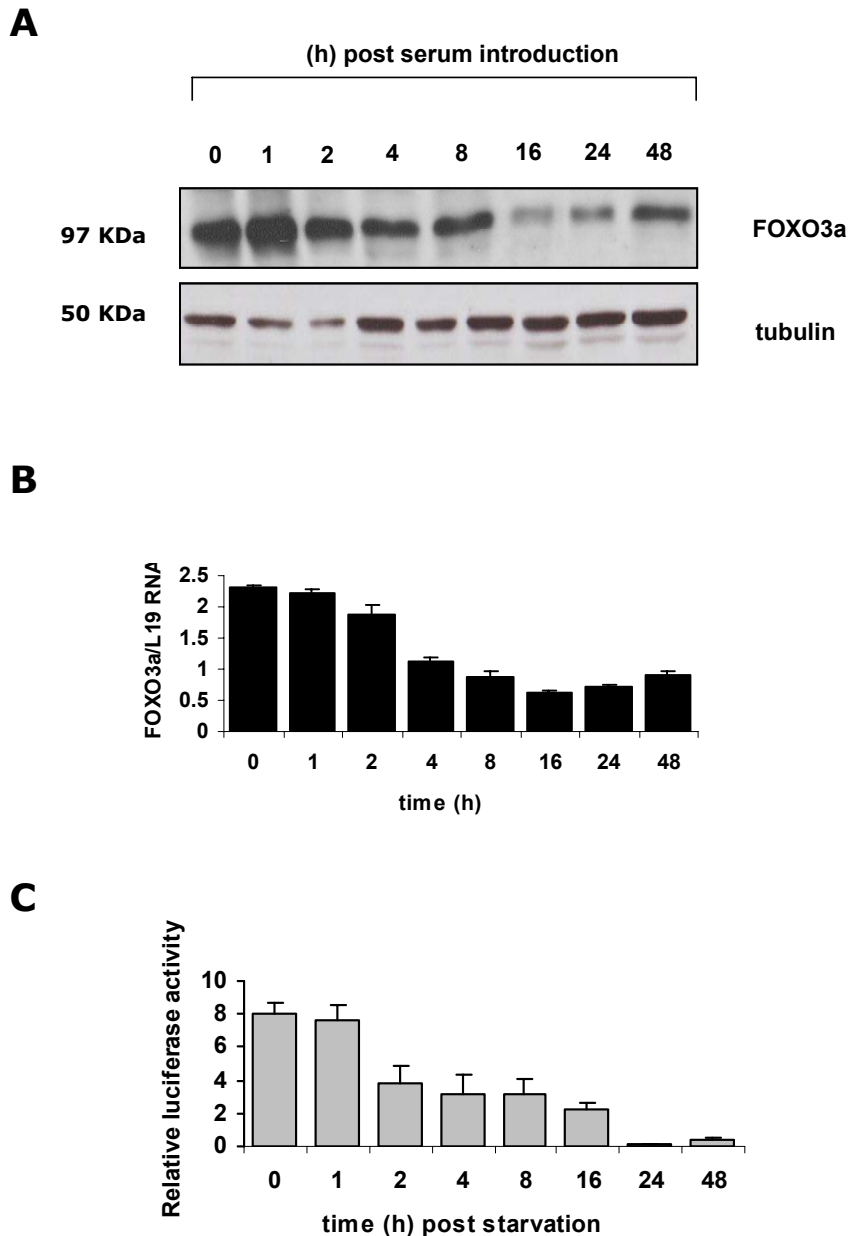


Figure 3.2 FOXO3a expression is highest in growth arrested cells. A) MCF-7 cells were grown for 24 h in the absence of serum before readdition of complete media with 10% FCS for the indicated timepoints. The expression of FOXO3a was analysed by Western blotting. **B)** Total RNA was isolated in parallel and the expression of FOXO3a mRNA was measured by RTq-PCR and normalised to the level of L19. **C)** Cells were transfected with the WT FOXO3a promoter, then incubated in serum free medium for 24h. Following starvation, complete media was added at the indicated timepoints. Promoter activity was measured and normalised to renilla. The results represent the average $\pm$ SD of three independent experiments.

3.2.3 *The sensitivity of a panel of breast cancer cell lines to epirubicin*

To establish the sensitivity of the MCF-7 breast cancer cell line to epirubicin, SRB assays were initially performed to assess cell survival following treatment with a dose titration of epirubicin (Figure 3.3a). Cells were treated with a dose titration of epirubicin ranging from 100nM to 10 μ M for 48h. Cells continued to proliferate at doses of 100nM and 500nM and a dose of 1 μ M and above was required to show an effect on cell survival and promote cell death. 1 μ M epirubicin was therefore chosen as a treatment dose for subsequent experiments. A panel of cell lines was then treated with 1 μ M epirubicin, confirming sensitivity of the breast cancer cell lines MCF-7, ZR75-1, SKBR3 and BT474 to the cytotoxic effects of epirubicin. MDA-MB-231 cells were resistant to the effects of 1 μ M epirubicin and continued to proliferate after 48 h of treatment (Figure 3.3b).

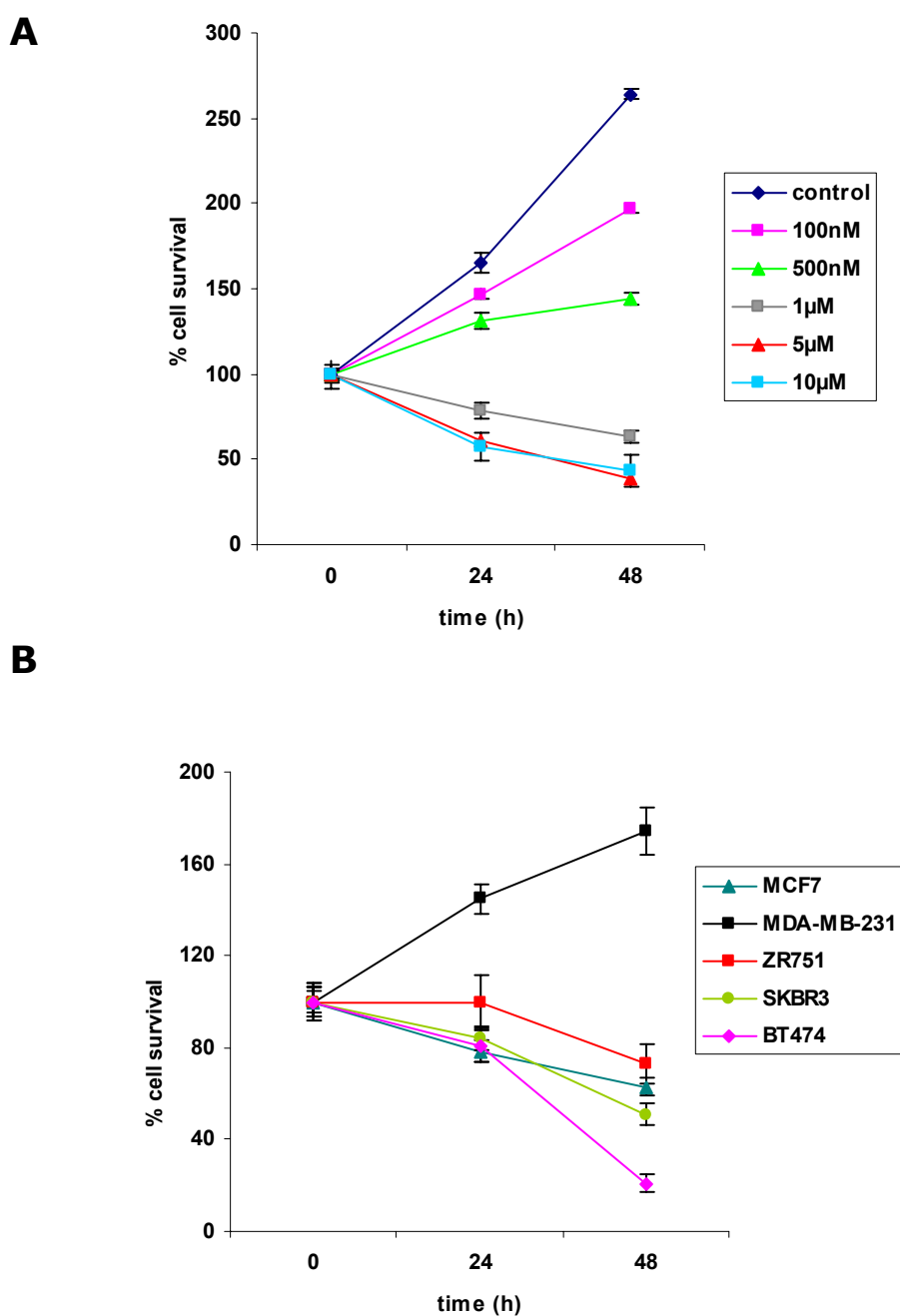


Figure 3.3 The sensitivity of breast cancer cells to epirubicin. A) MCF-7 cells were treated with a dose titration of epirubicin and cell survival measured by SRB assay. **B)** The breast cancer cell lines MCF-7, MDA-MB-231, ZR751, SKBR3 and BT474 were plated in 96 well plates and 24 h later treated with 1µM epirubicin for 48 h and cell survival measured by SRB assay. Results are the mean $\pm$ SD of three independent experiments that included 6 replicates each.

3.2.4 *Epirubicin causes G₂/M arrest in MCF-7 cells*

The anthracyclines are topoisomerase poisons but the exact mechanisms by which they induce cell death are not fully understood. To characterize the effects of epirubicin on the cell cycle, MCF-7 cells were treated with 1 μ M epirubicin and their cell cycle profile was analysed by FACS at certain timepoints after treatment. This confirmed that epirubicin induces G₂/M cell cycle arrest (Figure 3.4). Changes in the cell cycle profile appeared after 16h of treatment and by 24h more than 50% of the cell population had arrested – by 48h a significant amount of cell death was observed. The effects of epirubicin on the cell cycle appeared to be dose dependent. At low doses, little effect was seen on the cell cycle profile, with higher doses causing G₂/M arrest and significant cell death, even after 24h of treatment (Figure 3.5a).

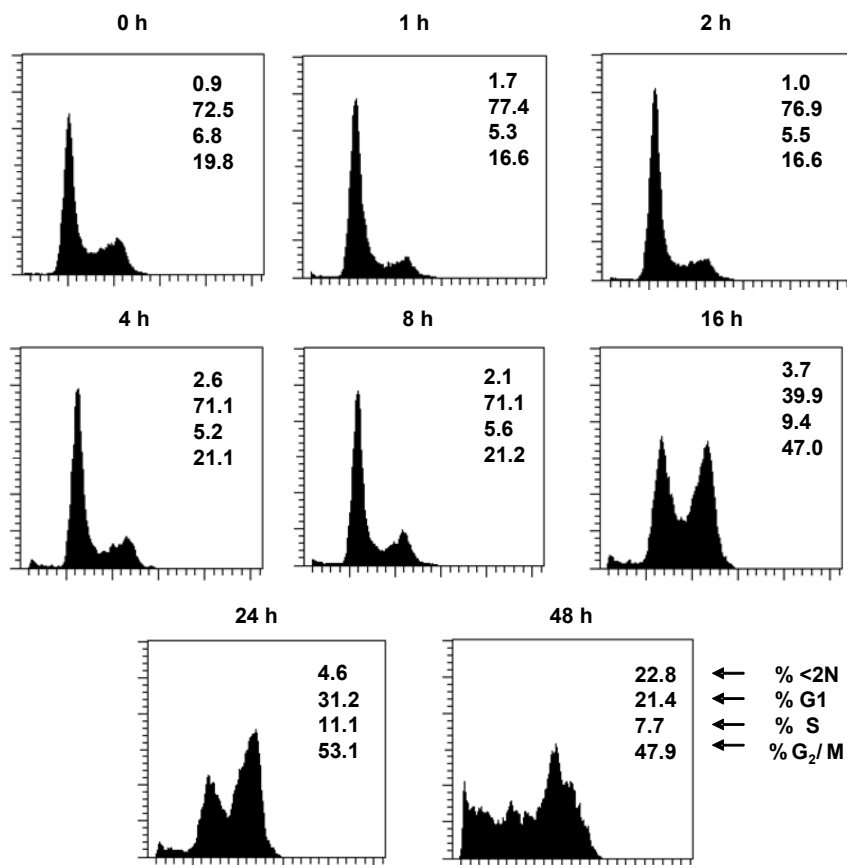


Figure 3.4 Epirubicin causes G2M arrest in MCF-7 cells. MCF-7 cells were treated with 1μM epirubicin and harvested after 24h. Cells were fixed with 90% ethanol and stained with propidium iodide. DNA content was then analysed by flow cytometry. The percentage of cells in each phase of the cell cycle is indicated (sub-2N, G1, S and G2/M).

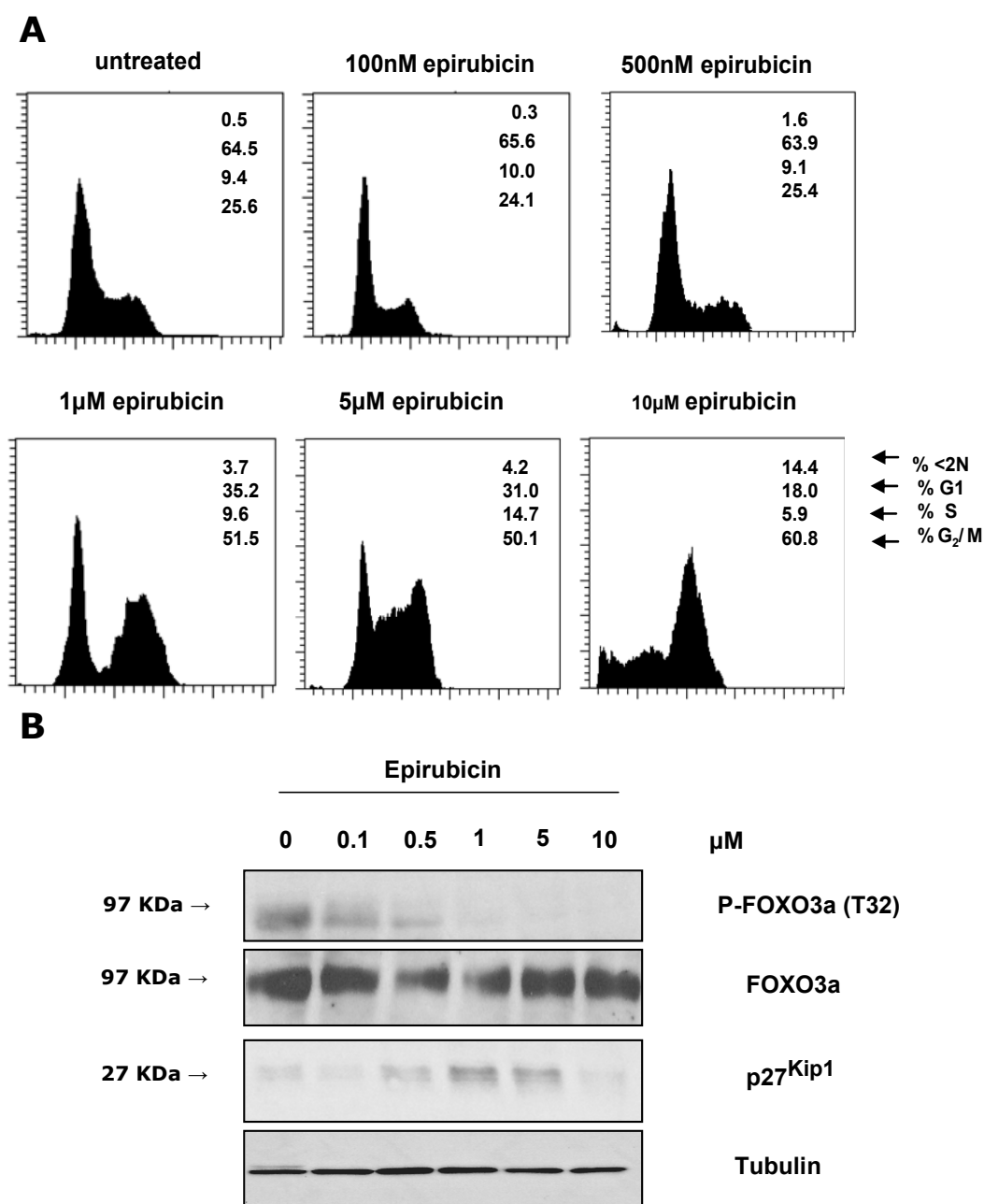


Figure 3.5 Dose dependent effects of epirubicin. A) MCF-7 cells were treated with a dose titration of epirubicin and harvested at 24h. Cells were fixed with 90% ethanol and stained with propidium iodide. DNA content was then analysed by flow cytometry. The percentage of cells in each phase of the cell cycle is indicated (sub 2N, G₁, S and G₂/M). **B)** Cells were treated in parallel with a dose titration of epirubicin for 24h and whole cell lysates prepared and analysed by Western blotting with the indicated antibodies.

3.2.5 *Epirubicin induces FOXO3a protein and mRNA expression*

The effects of epirubicin on targets both upstream and downstream of FOXO3a were next investigated. The PI3K pathway is known to regulate FOXO3a and when MCF-7 cells were treated with epirubicin, phospho-Akt initially increased up to 4h after treatment, but then decreased significantly by 48h (Figure 3.6). Total Akt remained essentially unchanged. Total FOXO3a protein did not change significantly following epirubicin treatment, but there was a marked reduction in phospho-FOXO3a protein levels, almost undetectable by 48h. This would suggest that epirubicin may be activating FOXO3a. Phospho-FOXO3a is the inactive, cytosolic form of the transcription factor and levels were decreasing whilst total levels of FOXO3a remain unchanged, implying that FOXO3a activity is rising following epirubicin treatment. This is supported by changes in the downstream targets of FOXO3a, p27^{Kip1} and Bim, which increase following epirubicin treatment, suggesting that epirubicin activates FOXO3a, which in turn promotes the transcription of the cell cycle inhibitor p27 and the pro-apoptotic factor Bim, resulting in cell cycle arrest and apoptosis. Another downstream target of FOXO3a, GADD45a, also showed an increase in protein levels following treatment with epirubicin. GADD45 plays a key role at the G₂/M checkpoint and the cell cycle arrest and DNA damage induced by epirubicin is likely to lead to its upregulation. The effects of epirubicin are dose dependent, with increasing amounts leading to downregulation of phosphorylated FOXO3a, whilst upregulating p27^{Kip1} (Figure 3.5b).

FOXO3a was also upregulated at the transcriptional level by epirubicin. RTq-PCR analysis confirmed that FOXO3a mRNA

increased following drug treatment (Figure 3.7a), correlating with the changes seen at protein level. This upregulation in protein and mRNA was accompanied by increased FOXO3a promoter activity. When the human *FOXO3a* promoter was transfected into MCF-7 cells and the cells were then treated with 1 μ M epirubicin results confirmed that the *FOXO3a* promoter is activated by epirubicin (Figure 3.7b). The longer timepoint of 48h was not analysed in the promoter assays as a large amount of cell death had occurred by this point, causing cells to detach and be unavoidably lost in the washing steps of the luciferase assays. However, the results confirm that by 24h, epirubicin upregulates FOXO3a at the protein, mRNA and gene promoter levels.

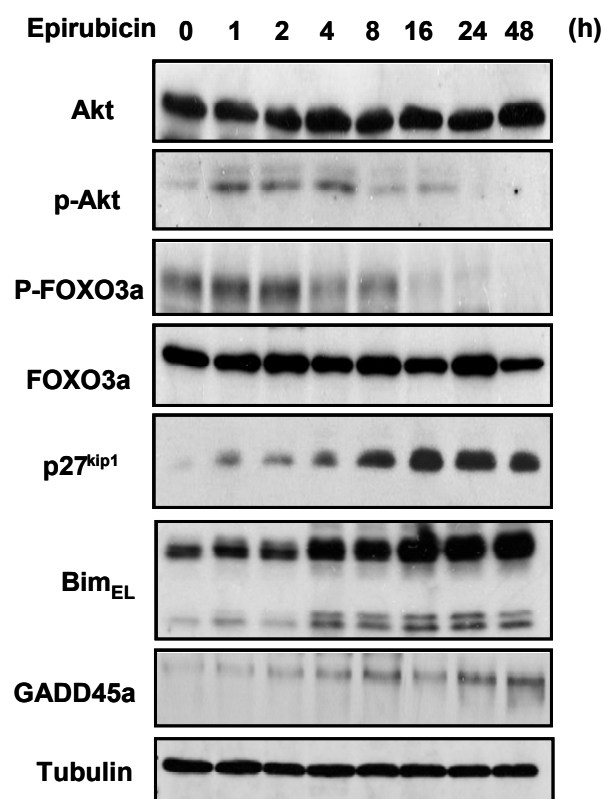


Figure 3.6 The effects of epirubicin on FOXO3a and targets. MCF-7 cells were treated with 1 μ M epirubicin and protein lysates were obtained at the times indicated. Protein expression was analysed by Western blotting using the indicated antibodies.

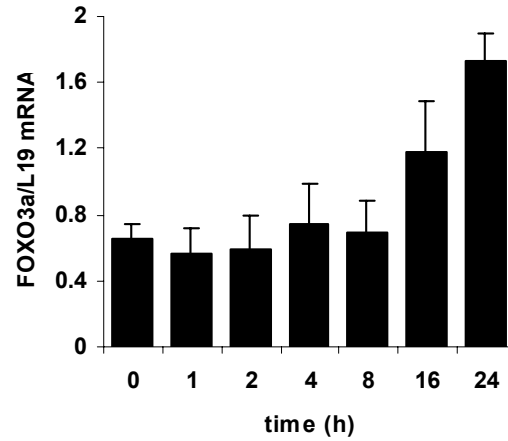
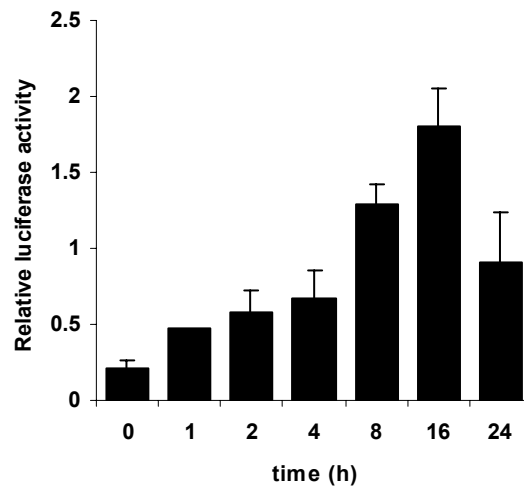
A**B**

Figure 3.7 Epirubicin activates FOXO3a at the transcriptional and gene promoter level. A) MCF-7 cells were treated with 1 μ M epirubicin for the indicated time points. Cells were collected and total RNA was isolated. FOXO3a mRNA levels were analysed by real-time quantitative PCR and the results normalised with L19 mRNA levels. **B)** MCF-7 cells were transfected with 10ng of the *FOXO3a* promoter and treated with 1 μ M epirubicin for the indicated time points before cells were collected for luciferase reporter assays. The relative luciferase activity values were corrected for cotransfected *renilla* activity. Results are the average $\pm$ SD of three independent experiments.

3.2.6 *Epirubicin induces FOXO3a nuclear sublocalisation in MCF-7 cells*

The transcriptional activity of FOXO proteins is regulated by their subcellular localisation. When phosphorylated, FOXO proteins are sequestered in the cytoplasm, where they are inactive. When activated, they are shuttled into the nucleus, where they are then able to activate their downstream effectors involved in cell cycle arrest and apoptosis. To investigate the effects of epirubicin on subcellular localisation of FOXO3a, MCF-7 cells were treated with 1µM epirubicin, harvested at 0, 8 and 24h and then nuclear and cytoplasmic components were separated using a fractionation kit. Western blot analysis confirmed that epirubicin treatment causes a nuclear accumulation of FOXO3a protein over 24h and this is accompanied by a reduction in cytoplasmic phosphorylated FOXO3a, consistent with shuttling of FOXO3a from the cytoplasm to the nucleus and a shift from the inactive to transcriptionally active form of FOXO3a (Figure 3.8a). Phospho-FOXO3a is found in the cytosolic fraction only, whereas FOXM1 is found in both the cytosolic and nuclear fractions and FOXM1 protein in both subcellular compartments decreases following epirubicin treatment. Efficient fractionation was confirmed with the controls – tubulin for the cytoplasmic compartment and Sp1, a transcriptional regulator, for the nuclear compartment.

The Western blot findings were confirmed by confocal microscopy, where MCF-7 cells were treated with epirubicin and then stained with a FOXO3a specific antibody. In the untreated MCF-7 cells, endogenous FOXO3a was visualised predominantly in the cytoplasm, but following 24h treatment with epirubicin, FOXO3a had relocated to the nucleus (Figure 3.8b). Together,

these results suggest that epirubicin is inducing dephosphorylation of FOXO3a and causing its nuclear translocation and accumulation and subsequent transcriptional activity.

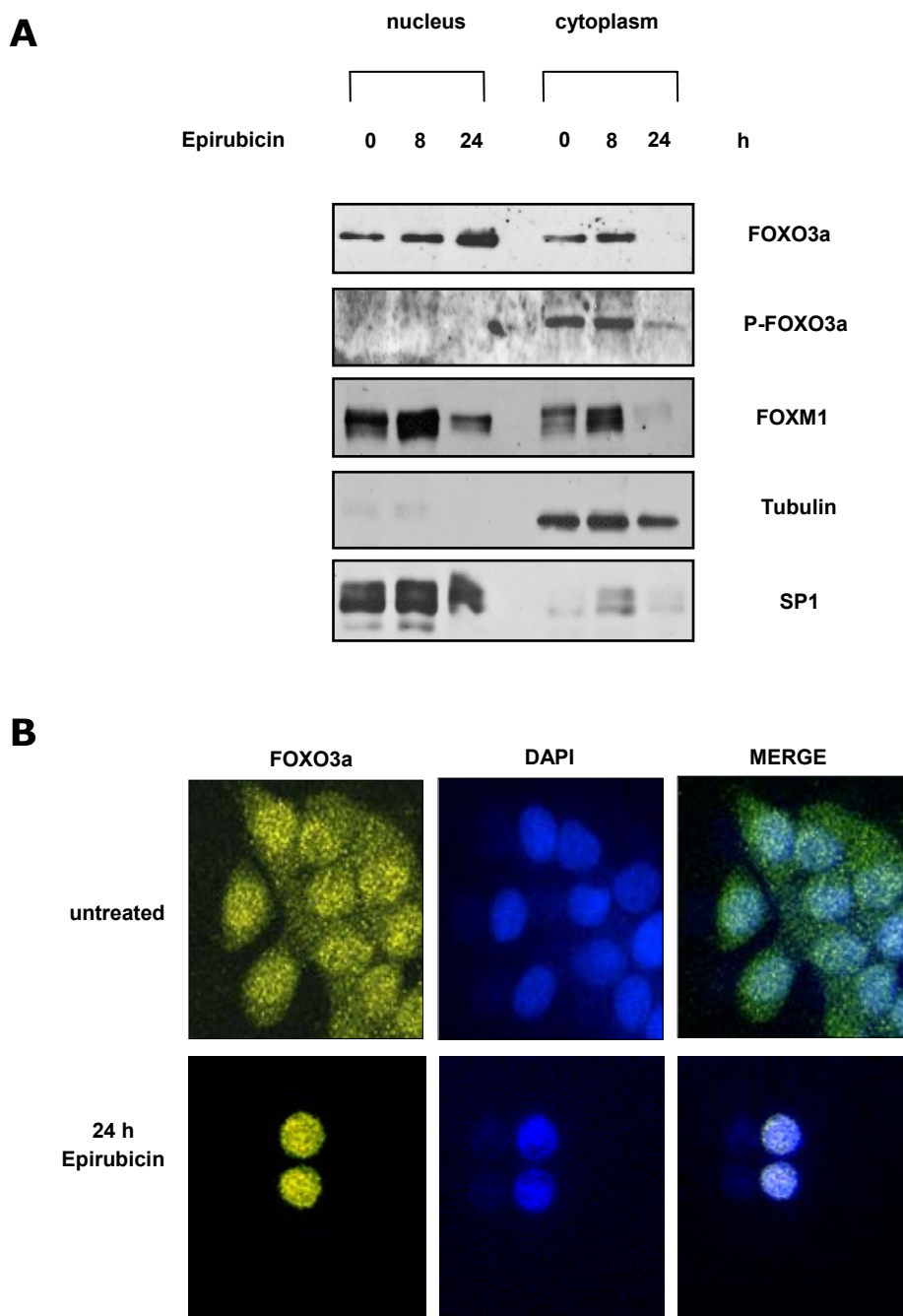


Figure 3.8 Epirubicin induces FOXO3a nuclear sublocalisation in MCF-7 cells. **A)** MCF-cells were treated with 1 μ M epirubicin at the indicated time points. Nuclear and cytoplasmic fractions were separated and protein expression was analysed by Western blotting. **B)** Cells were cultured on coverslips, treated with 1 μ M epirubicin and fixed in 4% formaldehyde. FOXO3a was visualised with a rabbit polyclonal antibody followed by the addition of Alexa Fluor 488 labelled anti rabbit sera. DAPI was also applied to visualise the nuclei.

3.2.7 *FOXM1 expression in a panel of breast cancer cell lines*

As FOXO3a was identified as a target of epirubicin, another member of the Forkhead family, FOXM1, was also studied and the effects of epirubicin on its expression investigated. Firstly, the expression of FOXM1 in a panel of cell lines was established. FOXM1 was found to be expressed in all cancer cell lines, although again there was a degree of heterogeneity between different cell lines (Figure 3.9). Interestingly, the untransformed cell line MCF-10A did not express FOXM1, consistent with higher FOXM1 expression being found in cancer cells as opposed to normal epithelial cells. FOXO3a expression did not correlate with FOXM1 expression. In fact, cells with high FOXM1 expression tended to have lower levels of FOXO3a expression and vice versa.

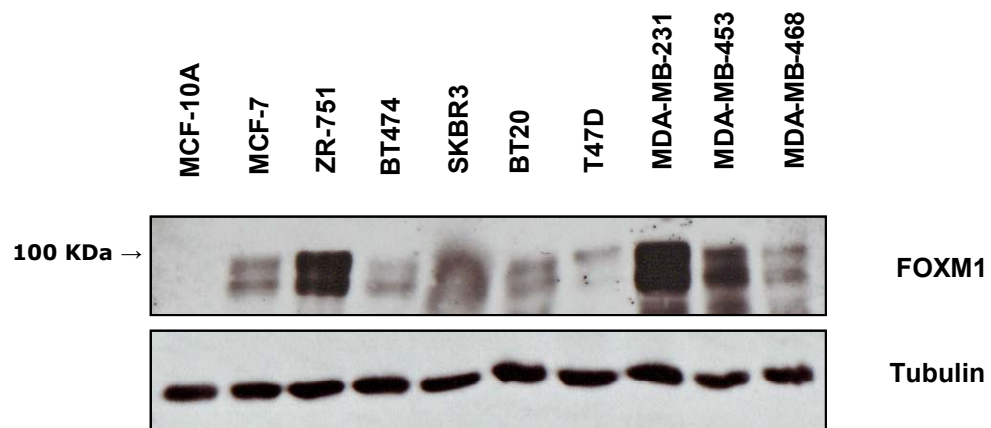


Figure 3.9 FOXM1 expression in a panel of breast cancer cell lines. Whole cell lysates were prepared from the panel of breast cancer cell lines as indicated. The expression of total FOXM1 was analysed by Western blotting. Tubulin was used as a loading control.

3.2.8 *FOXM1 is repressed by epirubicin at the protein, mRNA level and gene promoter level*

Work in the laboratory has previously shown that FOXM1 can be repressed by FOXO3a following treatment with gefitinib in sensitive breast cancer cell lines (McGovern et al., 2009). The effect of other chemotherapeutic agents on FOXM1 has not been characterised. Therefore, it was next of interest to look at FOXM1 expression following treatment with 1 μ M epirubicin.

Western blot analysis confirmed that treatment of MCF-7 cells with epirubicin led to a decrease in FOXM1 protein levels as well as a decrease in its downstream targets, cyclin B1 and PLK1 (Figure 3.10a). RTQ-PCR confirmed these findings at the transcriptional level with a marked reduction in FOXM1 mRNA transcript by 24 and 48h (Figure 3.10b), suggesting FOXM1 is also a downstream signalling target of epirubicin in MCF-7 cells.

3.2.9 *Dose dependent effects of epirubicin on FOXM1 and targets*

Increasing doses of epirubicin downregulated proteins levels of phosphorylated FOXO3a and increased levels of the FOXO3a downstream target p27. Similarly, the dose dependent effects of epirubicin on FOXM1 and its downstream targets were investigated. FOXM1 protein was downregulated by epirubicin in a dose dependent manner, as were the downstream targets PLK1 and cyclin B1 (Figure 3.11).

3.2.10 Epirubicin represses *FOXM1* promoter activity

As the previous data suggested that both FOXO3a and FOXM1 are targets of epirubicin treatment, the mechanisms involved in the repression of FOXM1 by epirubicin were investigated further. A full-length *FOXM1* promoter reporter construct was transiently transfected into MCF-7 cells and the cells were then treated with epirubicin. A significant decrease in promoter activity was observed (Figure 3.12a). However, when a *FOXM1* promoter construct was used where two FOXO3a binding sites were mutated, the repression of promoter activity by epirubicin was much less (Figure 3.12b). This would suggest that the repression of FOXM1 by epirubicin is mediated, at least in part, by FOXO3a. Epirubicin can activate FOXO3a and can repress FOXM1, and this repression appears to be dependent on FOXO3a – if FOXO3a is prevented from binding to the FOXM1 promoter, the repression of FOXM1 by epirubicin is abrogated.

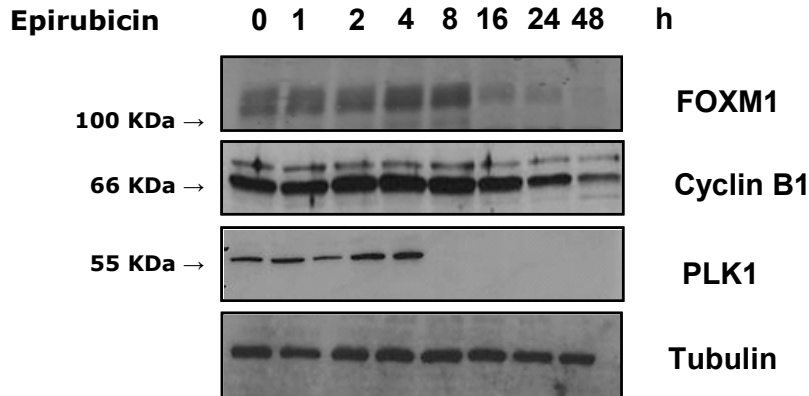
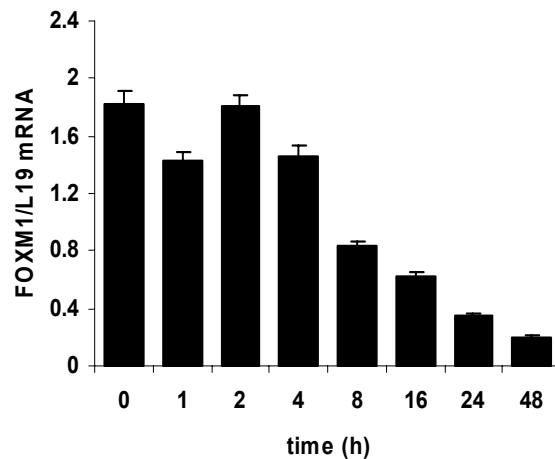
A**B**

Figure 3.10 Epirubicin represses FOXM1 protein and mRNA. **A)** MCF-7 cells were treated with 1 μ M epirubicin and protein lysates were obtained at the times indicated. Protein expression was analysed by Western blotting using the indicated antibodies. **B)** MCF-7 cells were treated with 1 μ M epirubicin for the indicated time points. Cells were collected and total RNA was isolated. FOXM1 mRNA levels were analysed by real-time quantitative PCR and the results normalised with L19 mRNA levels. Results are the average $\pm$ SD of three independent experiments.

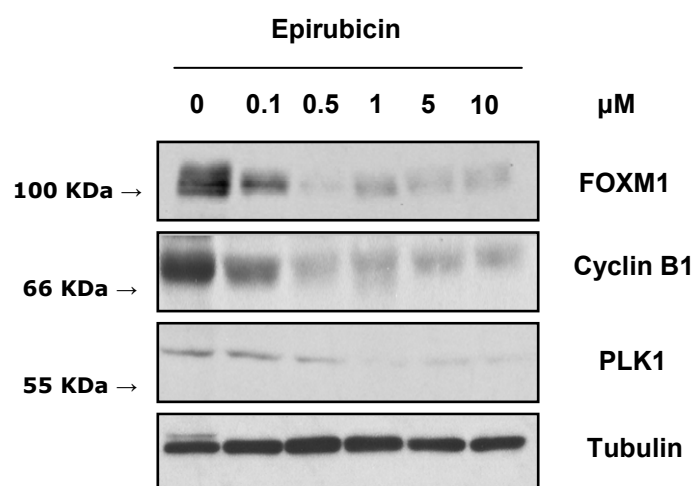


Figure 3.11 Dose dependent effects of epirubicin on FOXM1 and targets. MCF-7 cells were treated with a dose titration of epirubicin and harvested after 24h of exposure to the drug. Whole cell lysates were prepared and analysed by Western blotting with the indicated antibodies.

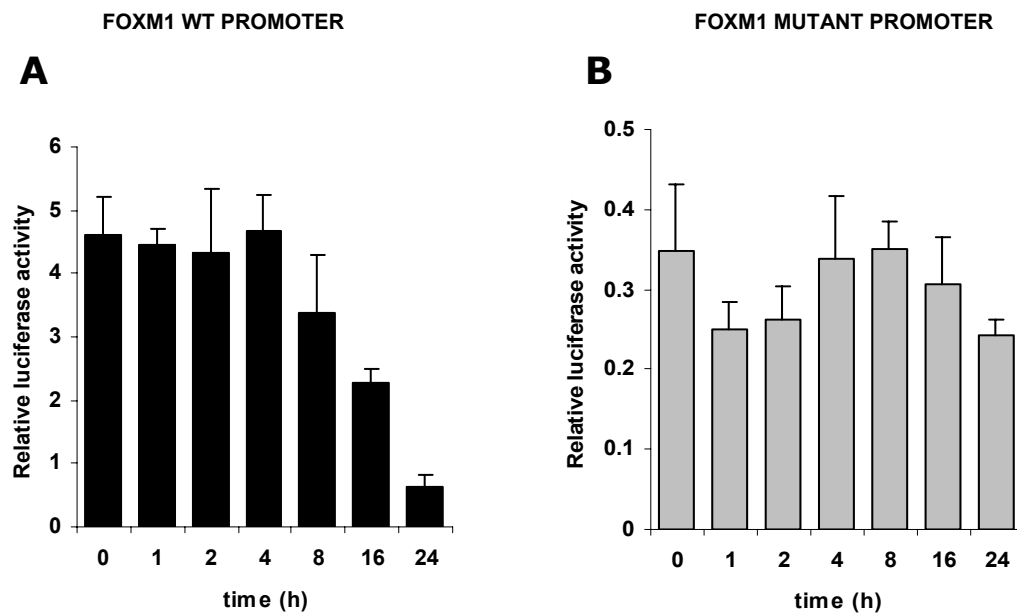


Figure 3.12 Epirubicin represses FOXM1 promoter activity. **A)** MCF-7 cells were transfected with 10ng of the *FOXM1* promoter or **B)** a *FOXM1* promoter with mutated FOXO3a binding sites and treated with 1 μ M epirubicin for the indicated time points before cells were collected for luciferase reporter assays. The relative luciferase activity values were corrected for cotransfected *renilla* activity. Results are the average $\pm$ SD of three independent experiments.

3.2.11 *Silencing of FOXO3a by siRNA confers protection to the cytotoxic effects of epirubicin*

To further examine the role of FOXO3a in epirubicin mediated cytotoxicity, the effects of treating cells with epirubicin following knockdown of FOXO3a was investigated. MCF-7 cells were transfected with a FOXO3a specific siRNA or a non specific control siRNA. Western blot analysis confirmed efficient knockdown of endogenous FOXO3a (Figure 3.13a) and subsequent SRB assay analysing the effects of this knockdown on MCF-7 survival following drug treatment confirmed that the cytotoxic effects of epirubicin were less in the FOXO3a siRNA cells than in the control cells (Figure 3.13b). This suggests that loss of FOXO3a confers some protection against epirubicin and that FOXO3a is required in mediating cell death induced by epirubicin in MCF-7 cells. To characterise the relationship between FOXO3a and FOXM1, changes in FOXM1 following epirubicin treatment when FOXO3a was silenced by siRNA was looked at. In the control transfected cells, epirubicin lead to down regulation of FOXM1 and its targets, but in the FOXO3a knockout cells, FOXM1 levels were essentially unchanged and the downregulation of the FOXM1 targets cyclin B1 and PLK1 was also much less (Figure 3.13a). This would suggest that FOXO3a is required for the repression of FOXM1 following epirubicin treatment and when FOXO3a is silenced, derepression of FOXM1 leads to MCF-7 cells being less sensitive to the effects of epirubicin, conferring some protection against drug treatment.

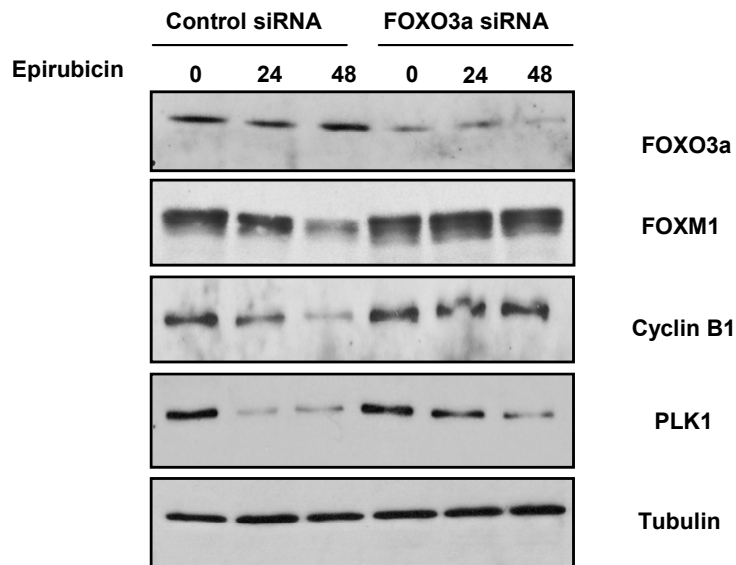
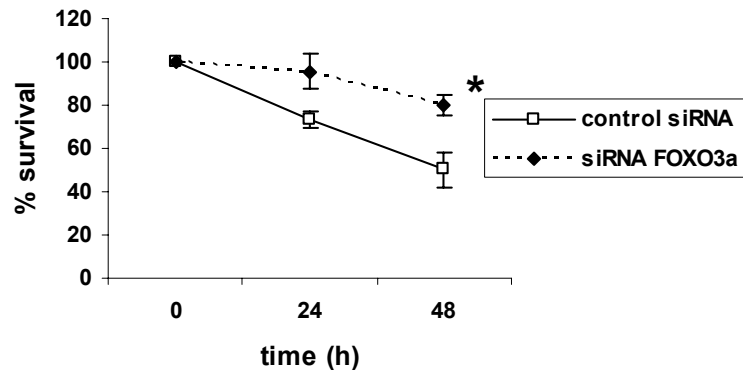
A**B**

Figure 3.13 Silencing of FOXO3a protects cells against the cytotoxic effects of epirubicin. A) Cells were transfected with siRNA specifically targeting FOXO3a or a NSC. 48h post transfection, Western blot analysis was performed to confirm efficient FOXO3a knockdown and FOXM1 expression levels. **B)** 24h post siRNA transfection, cells were replated in 96 well plates, treated with 1 μ M epirubicin for the given time points and cell proliferation analysed by SRB assay. Results are the mean $\pm$ SD of six replicates. * $p < 0.05$.

3.2.12 *Expression of a constitutively active FOXM1 (Δ N-FOXM1) abrogates the cytotoxic effects of epirubicin*

To confirm that FOXM1 is a downstream target of epirubicin, MCF-7 cells were transfected with an expression vector encoding for a constitutively active NH₂-terminal truncated form of FOXM1, Δ N-FOXM1. The Δ N-FOXM1 cells were treated with 1 μ M epirubicin and their cell cycle profile following treatment was analysed by FACS. Epirubicin causes G₂M arrest in MCF-7 cells but in the Δ N cells, this effect was abrogated and minimal cell cycle arrest or cell death was seen (Figure 3.14a). Western blot analysis of cell lysates from epirubicin treated wild-type MCF-7 cells and the Δ N-FOXM1 transfected cells confirmed repression of endogenous FOXM1 levels by epirubicin in the wild type cells, corresponding with cell cycle arrest and cell death, but in the Δ N-FOXM1 transfected cells, the constitutively active Δ N-FOXM1 was not repressed by epirubicin and the downstream target of FOXM1, cyclin B1, was repressed to a much lesser extent than in the wild-type cells (Figure 3.14b). This would suggest that overexpression of a constitutively active form of FOXM1 can overcome the antiproliferative and cytotoxic effects of epirubicin.

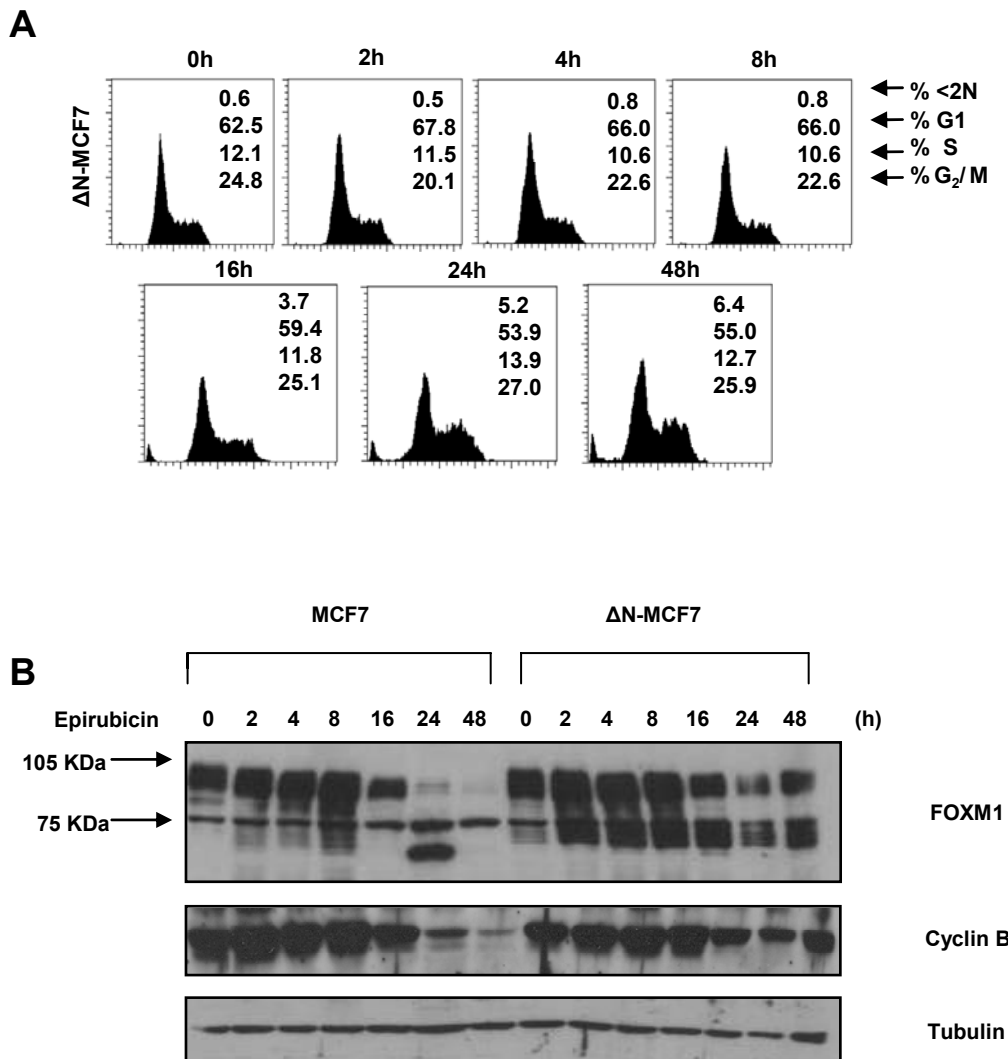


Figure 3.14 Expression of a constitutively active FOXM1 (Δ N-FOXM1) abrogates the cytotoxic effects of epirubicin. **A)** MCF-7 cells were transfected with a vector containing a constitutively active Δ N-FOXM1 and treated with epirubicin. Cells were fixed at the indicated timepoints and cell cycle phase distribution was analysed by flow cytometry after propidium iodide staining. Percentage of cells in each phase of the cell cycle are indicated (< 2N, G₁, S and G₂M.) **B)** Wild type MCF-7 cells and the transfected Δ N-FOXM1 cells were treated with 1 μ M epirubicin at the indicated timepoints and the expression of FOXM1 and its targets was analysed by Western blotting.

3.3 DISCUSSION

To continue to improve outcomes in breast cancer, a greater understanding of the mechanisms through which chemotherapy exerts its cytotoxic effects is required, allowing better selection of patients for individual treatments and identifying patients who may be resistant to particular drugs or treatment regimens. Epirubicin is used widely in the clinical setting for the treatment of breast cancer, but not all patients will show a response. Thus, identifying the cellular pathways involved in mediating the effects of epirubicin could be of clinical benefit in the future.

The work in this chapter demonstrates that FOXO3a plays a key role in the response of MCF-7 cells to the chemotherapeutic agent epirubicin. Epirubicin is highly toxic to most of the breast cell lines tested (Figure 3.3), with only MDA-MB-231 cells being resistant to the drug, an ER and HER2 negative cell line. In the clinical setting, debate is ongoing about which particular subtypes of breast cancer are more sensitive to the anthracyclines. ER negative cancers appear to derive the most benefit from chemotherapy and there is some evidence that HER2 positive cancers are more sensitive to anthracyclines, due to the close proximity of the *HER2* gene to the *TOP2A* gene on chromosome 17 – coamplification of both may contribute to anthracycline sensitivity (Pritchard et al., 2008). Both HER2 positive cell lines tested (BT474 and SKBR3) were epirubicin sensitive.

Doses of at least 1 μ M were required *in vitro* to achieve a significant amount of cell death in MCF-7 cells and this was the dose carried forward for subsequent experiments. 1 μ M is sufficient to induce G₂/M cell cycle arrest, which starts to occur

at 16h post-treatment (Figure 3.4). By 48h, a significant amount of cell death has occurred, suggesting that the damage induced by epirubicin is unrecoverable and cell cycle arrest is followed by cell death. G₂/M arrest is not unique to epirubicin - although chemotherapeutic agents may have different mechanisms of action, a common theme is that they all confer DNA damage, directly or indirectly, and induce cell cycle checkpoints. Paclitaxel, a mitotic spindle poison, also induces G₂/M arrest in MCF-7 cells (Sunters et al., 2003). Both FOXO3a and FOXM1 have roles at the G₂/M checkpoint. In response to stress, FOXO3a can induce a delay in G₂M by regulating cyclin G2 and GADD45a, in an attempt to repair damaged DNA. FOXM1 is critical for G₁/S and G₂M cell cycle progression and also for mitotic spindle integrity (Myatt and Lam, 2007). The activation of FOXO3a and repression of FOXM1 seen after epirubicin treatment is therefore likely to play a significant role in the G₂/M cell cycle arrest observed.

Consistent with its role in the cell cycle and anti-proliferative effects, FOXO3a expression can be induced by serum starvation and FOXO3a protein, mRNA and promoter activity are all highest in serum starved MCF-7 cells (Figure 3.2). FOXO proteins can contribute to exit from the cell cycle, into quiescence (G₀). FOXO3a is known to control transcription of the retinoblastoma-like p130 protein, which is hypophosphorylated and bound to the transcription factor E2F-4, maintaining cell in G₀ (Kops et al., 2002b). When conditions are favourable (i.e. when serum is reintroduced) FOXO3a levels fall as cell reenter the cell cycle and begin to proliferate. This demonstrates the different functions of FOXO3a - serum starvation and epirubicin can both upregulate FOXO3a and lead to cell cycle arrest. However, the decision to reenter the cell

cycle or undergo apoptosis will depend on the damage inflicted on the cell. Cells can tolerate serum starvation in the short term but clearly cannot recover from the DNA damage induced by epirubicin and hence undergo programmed cell death.

Treatment with epirubicin leads to a reduction in levels of phosphorylated Akt, suggesting that the activity of the PI3K pathway is repressed by epirubicin. Akt directly phosphorylates FOXO3a and repression of Akt activity may be an early event in the activation of FOXO3a – low Akt activity following epirubicin treatment renders FOXO3a dephosphorylated and located in the nucleus. Indeed, levels of phosphorylated FOXO3a also fall following epirubicin treatment. Further evidence for FOXO3a activation is the upregulation of p27^{Kip1} and Bim, two known FOXO3a targets, following epirubicin treatment. By 24 and 48h, as cells are arresting and undergoing apoptosis, protein levels of both p27^{Kip1} and Bim increase (Figure 3.6). Bim has been shown previously to be transcriptionally regulated by FOXO3a following treatment of MCF-7 cells with paclitaxel (Sunters et al., 2003). Bim is also unregulated following gefitinib treatment in sensitive cells (Krol et al., 2007). Bim, as a BH3 protein, acts as a sensor of cellular damage and when activated by FOXO3a, can trigger mitochondrial permeabilisation and the release of other pro-apoptotic proteins from the mitochondrion, initiating cell death (Letai, 2008). To some extent, the effects of epirubicin are dose dependent and increasing doses of the drug are associated with greater induction of p27^{Kip1} and decreasing amounts of phosphorylated FOXO3a (Figure 3.5). However, at very high doses of epirubicin (10µM) there is no induction of p27^{Kip1}, which may be just a reflection of a large amount of cell death and poor protein integrity.

The nuclear localization of FOXO3a is a prerequisite for transcriptional activation and when MCF-7 cells are treated with epirubicin, a clear rise is seen in nuclear FOXO3a with an accompanying fall in phosphorylated cytoplasmic FOXO3a (Figure 3.8). This was confirmed with immunofluorescent studies, with little or no cytoplasmic FOXO3a identifiable after 24h treatment with epirubicin. This confirms the hypothesis that epirubicin is activating FOXO3a – treatment with the drug leads to shuttling of FOXO3a from the cytoplasm, where it is transcriptionally inactive and phosphorylated by Akt, into the nucleus where it can activate target genes. FOXM1 is active when nuclear and phosphorylated – epirubicin reduced both the nuclear and cytoplasmic expression of FOXM1.

Silencing of FOXO3a renders MCF-7 cells more resistant to the effects of epirubicin. Cells that would normally undergo apoptosis may instead survive in the absence of FOXO3a, resulting in increased drug resistance. Loss of FOXO3a function leads to a decreased ability to induce cell cycle arrest. The DNA damage induced by epirubicin would normally be expected to trigger the activation of a number of cell cycle regulators, including FOXO3a, which would arrest the cells and prevent them passing through key cell cycle checkpoints with damaged DNA. If the damage cannot be repaired, cells will undergo apoptosis. Silencing of FOXO3a is likely to reduce the ability of cells to arrest in response to epirubicin, resulting in the replication of damaged DNA and increased cell survival. This would further support the role of FOXO3a as a tumour suppressor – the decreased ability to repair damaged DNA due to the absence of FOXO factors results in the survival of

damaged cells, promoting genomic instability, one of the hallmarks of cancer. In other tissues, reduced FOXO3a expression has been associated with increased resistance to therapeutic agents. In prostate cancer, FOXO3a function is compromised in androgen independent prostate cancer cells versus androgen dependent LnCaP cells (Lynch et al., 2005). Thus, as cells progress from androgen sensitive to androgen resistant, FOXO3a expression and function is lost. Therefore, the expression of FOXO3a may be of use in predicting the likelihood of response to treatment and cancers expressing low levels of FOXO3a may be less likely to respond to particular treatments.

This work also identifies FOXM1 as a downstream signalling target of epirubicin. Treatment of MCF-7 cells with epirubicin leads to a reduction in FOXM1 protein levels, which coincides with a decrease in FOXM1 activity, as seen by the concomitant decrease in expression of the FOXM1 targets cyclin B1 and PLK1 (Figure 3.10). This repression of FOXM1 by epirubicin occurs at the transcriptional and gene promoter level, as a reduction in FOXM1 mRNA accompanies the fall in protein levels and a decrease in *FOXM1* promoter activity is seen following treatment (Figure 3.12). The downregulation of FOXM1 expression by epirubicin may be another critical target for the cytotoxic effects of the drug – FOXM1 is a crucial regulator of cell proliferation and regulates the expression of a number of target genes involved in cell cycle progression and survival. An increase in FOXM1 expression is seen at the onset of S phase and continues throughout G₂ and mitosis, activating the transcription of Cdc25B, survivin, PLK1 and the Cdk-cyclin B complex (Costa, 2005). That FOXM1 is a target of the antiproliferative effects of

epirubicin is further confirmed by the observation that overexpression of a constitutively active FOXM1, Δ N-FOXM1, abrogates the proliferative arrest and apoptosis induced by epirubicin, suggesting FOXM1 has a functional role in mediating the effects of the drug (Figure 3.14).

The repression of FOXM1 expression appears to be dependent in part on FOXO3a. FOXO3a is activated by epirubicin, whilst FOXM1 is repressed. Transient transfection of a *FOXM1* promoter reporter construct showed a decrease in promoter activity following epirubicin treatment. However, with the *FOXM1* mutant promoter, where two FOXO binding sites were mutated to make it refractive to FOXO transactivation, no significant decrease in promoter activity was seen. This would suggest that FOXO3a is required for the repression of FOXM1 promoter activity. Similarly, when FOXO3a is silenced by siRNA and cells are then treated with epirubicin, FOXM1 protein levels are not downregulated, again supporting the hypothesis that FOXO3a is involved in the repression of FOXM1 (Figure 3.13). It is also of interest to note the inverse correlation of FOXO3a and FOXM1 expression in the cell line panel – cells with high expression of FOXM1, such as ZR-75-1, MDA-MB-231 and MDA-MB-453, express lower levels of FOXO3a than some of the other cell lines.

3.4 FUTURE WORK

There is scope to further explore the relationship between FOXO3a and FOXM1. Chromatin immunoprecipitation (ChIP) could be used to see whether FOXO3a is associated with the *FOXM1* promoter *in vivo*. FOXO3a may act as a transcriptional repressor of FOXM1 by binding directly to the *FOXM1* promoter.

Further analysis of the FOXO3a promoter would be useful also to try and identify key regions involved in the promoter activation caused by epirubicin, which may identify other important co-activators or repressors involved in the response to epirubicin. Deletion mapping of responsive elements on the FOXO3a promoter would also be useful to identify key regulatory areas involved.

GST pulldown experiments would be useful to identify other binding partners of FOXO3a and the role of other stress signalling molecules such as JNK and NFκB and their interaction with FOXO3a in response to drug treatment will give more insight into cross talk between different signalling pathways in response to drug treatment.

CHAPTER 4
CHARACTERISING THE ROLE OF ID1 IN
BREAST CANCER CELLS

4.1 INTRODUCTION AND OBJECTIVES

Inhibitor of DNA binding (Id) proteins are regulatory proteins that belong to the helix-loop-helix (HLH) family of transcription factors which are involved in a wide range of developmental and cellular processes, including cell lineage determination, cell proliferation and differentiation. Id proteins lack a basic DNA binding domain, but are capable of dimerizing with other basic HLH (bHLH) factors. However, the resulting heterodimers formed are unable to bind DNA and initiate transcription – thus Id proteins function as dominant negative regulators of bHLH transcription factors (Desprez et al., 2003).

Id1 is the most well characterized Id protein. In normal development, Id1 is highly expressed during mouse mammary gland development and in pregnancy, during epithelial ductal cell proliferation, but levels become undetectable post-partum and in the lactating period, corresponding to a highly differentiated state (Parrinello et al., 2001). In haematopoietic cell lineages, high expression of Id1 is seen in precursor cells but decreases as cells progress through differentiation and Id1 is capable of blocking erythrocyte differentiation in cultured K562 cells (Cammenga et al., 2003; Kreider et al., 1992).

There is increasing evidence to suggest an oncogenic role of Id1 in human cancer. Id1 is overexpressed in a variety of cancers and may act as a marker of progression, metastases and outcome. In this chapter, the expression and role of Id1 in breast cancer cells was characterised and the effects of treatment with epirubicin on Id1 was demonstrated. The hypothesis that FOXO3a may regulate Id1 in breast cancer was also investigated.

4.2 RESULTS

4.2.1 *Id1 expression in breast cancer cell lines*

To establish the expression of Id1 in a variety of cell lines, a comparative protein expression analysis was performed by Western blotting to look at the expression of Id1 protein in a panel of 10 breast cancer cell lines, including the non-transformed cell line MCF-10A (Figure 4.1).

Id1 was expressed in all cell lines in the panel, but there was a degree of heterogeneity in Id1 expression amongst the different cell lines. Id1 was expressed in both ER positive and ER negative cell lines, and also in both HER2 positive (BT474, SKBR3) and HER2 negative cell lines. MDA-MB-231 cells had the highest relative expression of Id1 in the panel, with ZR-751 cells expressing low amounts of Id1. Id1 expression was also low in the non transformed breast epithelial cell line MCF-10A, consistent with the observation that Id1 expression is generally higher in malignant cells than in normal epithelial breast cells.

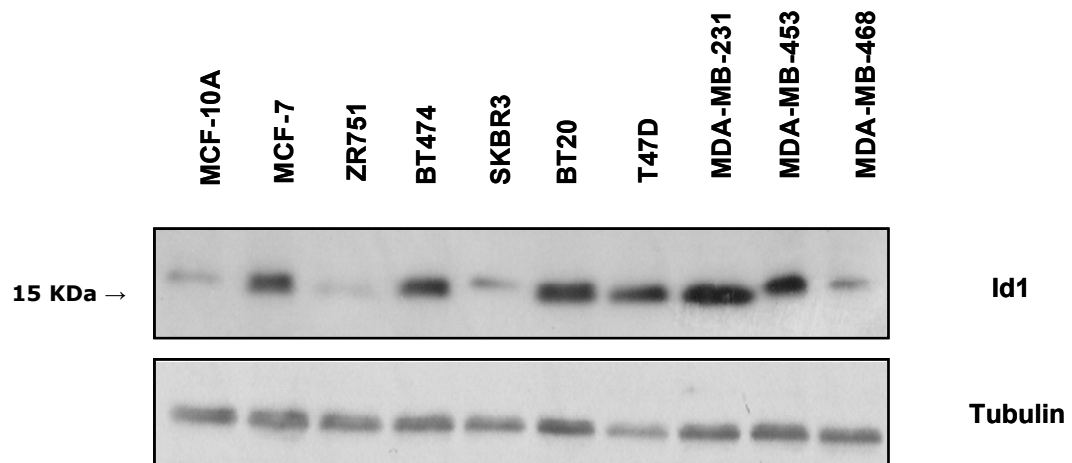


Figure 4.1 The expression of Id1 in a breast cancer cell line panel. Whole cell lysates were prepared from the panel of breast cancer cell lines as indicated. The expression of Id1 protein was analysed by Western blotting. Tubulin was used as a loading control.

4.2.2 *Id1 expression is highest in proliferating cells*

Id proteins have a critical role in the cell cycle, and are known to be mitogen responsive in fibroblasts – quiescent cells express low or undetectable levels. To establish the expression of Id1 in MCF-7 cells and to correlate expression with proliferation, MCF-7 cells were serum starved for 24h. Full medium (with 10% FCS) was then reintroduced to the cells, and cells were harvested at different time points following readdition of serum. Synchronisation and re-entry to the cell cycle was confirmed by FACS analysis (Figure 4.2a). Following 24h of serum starvation, nearly 90% of cells were arrested in G₁. After 16h, cells were seen to be progressing through S phase and into G₂M i.e returning to a normal cell cycle profile. Both Id1 protein and mRNA levels were analysed in parallel with the FACS samples and results confirmed that in growth arrested cells, Id1 protein and mRNA levels are low, but increase significantly by 16h, as cells re-enter the cell cycle (Figure 4.2b and 4.2c). This confirms Id1 as a proliferation dependent protein and identifies Id1 as serum responsive in MCF-7 breast cancer cells.

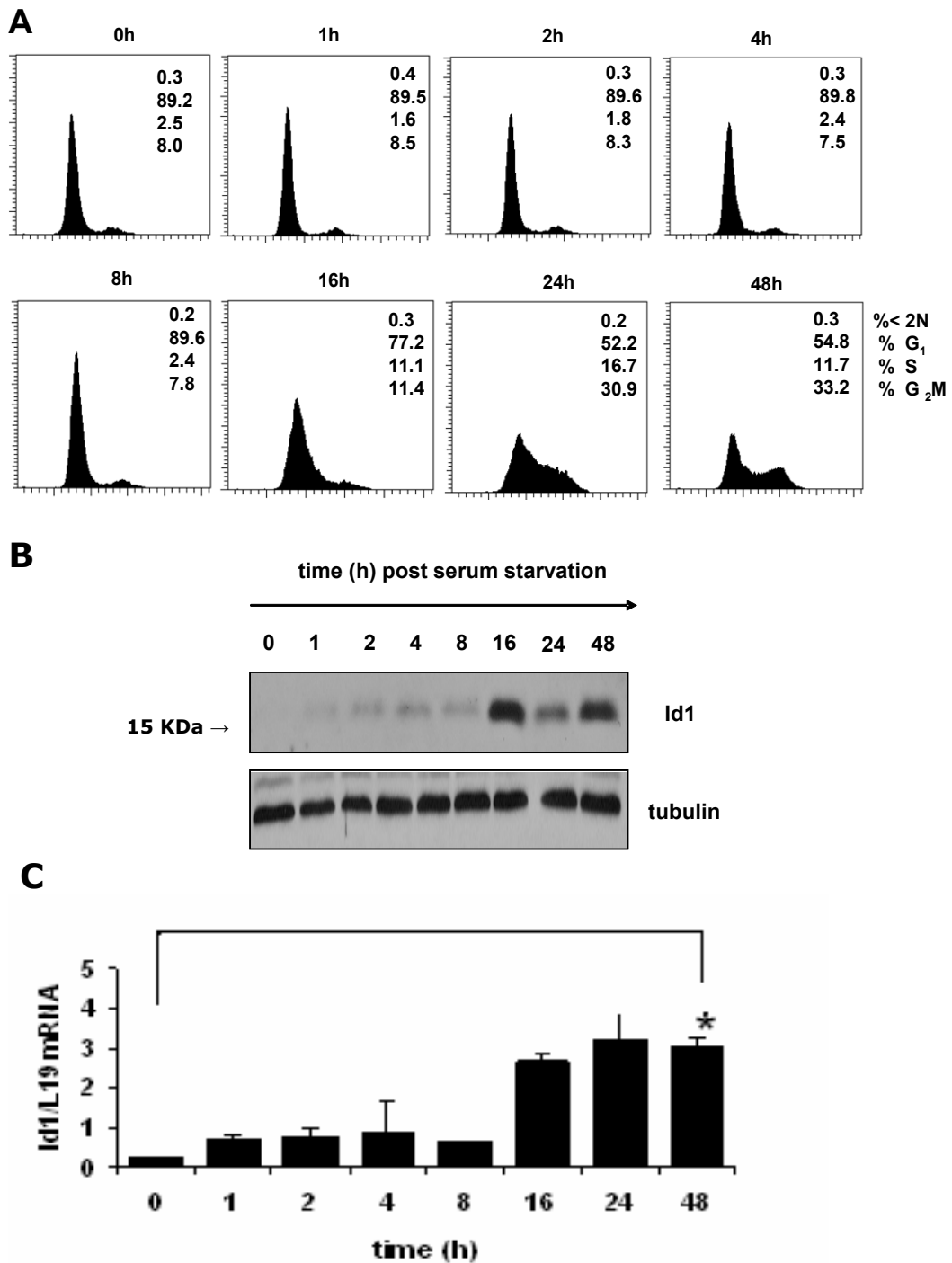


Figure 4.2 Expression of Id1 in growth arrested cells. A) MCF-7 cells were grown for 24 h in the absence of serum before restimulation with complete media containing 10% FCS for the indicated timepoints. Cells were harvested and analysed by FACS **B)** Whole cell lysates were prepared and the expression of Id1 was analysed by Western blotting. **C)** Total RNA was isolated in parallel and the expression of Id1 mRNA was measured by RTq-PCR and normalised to the level of L19. The results represent the average $\pm$ SD of three independent experiments. * $p < 0.05$.

4.2.3 *Epirubicin rapidly downregulates Id1 expression*

In the previous chapter, results showed that both FOXO3a and FOXM1 are targets of the anthracycline epirubicin. Epirubicin upregulates FOXO3a protein and mRNA and activates the *FOXO3a* promoter whilst repressing FOXM1 at the protein, mRNA and gene promoter level. The most significant changes are seen after at least 16h of drug treatment. The effects of epirubicin on Id1 have not previously been characterized.

To investigate whether Id1 is a cellular target of epirubicin, MCF-7 cells were treated with 1 μ M epirubicin and then harvested at the indicated timepoints post treatment to assess changes in Id1 protein and mRNA levels. Downregulation of Id1 at both protein and mRNA level was observed (Figure 4.3a and 4.3b). The changes in protein and mRNA levels were relatively rapid, occurring within a few hours of drug treatment. Id1 would therefore seem to be an early cellular target of epirubicin and repression of Id1 at both protein and mRNA levels appears to precede the changes in FOXO3a and FOXM1 seen in Chapter 3, suggesting Id1 repression is an early upstream event of the cascade of cellular responses to epirubicin.

4.2.4 *Epirubicin represses Id1 promoter activity*

As epirubicin downregulates Id1 protein and mRNA, the effect of epirubicin on the Id1 promoter was next investigated. Id1 has a short half-life and is known to be degraded by proteolysis through the ubiquitin-proteasome pathway (Bounpheng et al., 1999). However, the downregulation of Id1 following epirubicin

treatment cannot simply be due to its degradation. Gene promoter analysis confirmed that epirubicin represses the Id1 promoter and therefore the downregulation of Id1 mRNA observed is due to direct repression of the transcriptional activity of the Id1 promoter (Figure 4.4). The repression of promoter activity lags behind the changes seen in both Id1 protein and mRNA, with maximal promoter repression being seen by 16h of drug treatment. This could be due to comparative longer half life of luciferase in the reporter system or additional mechanisms not yet determined.

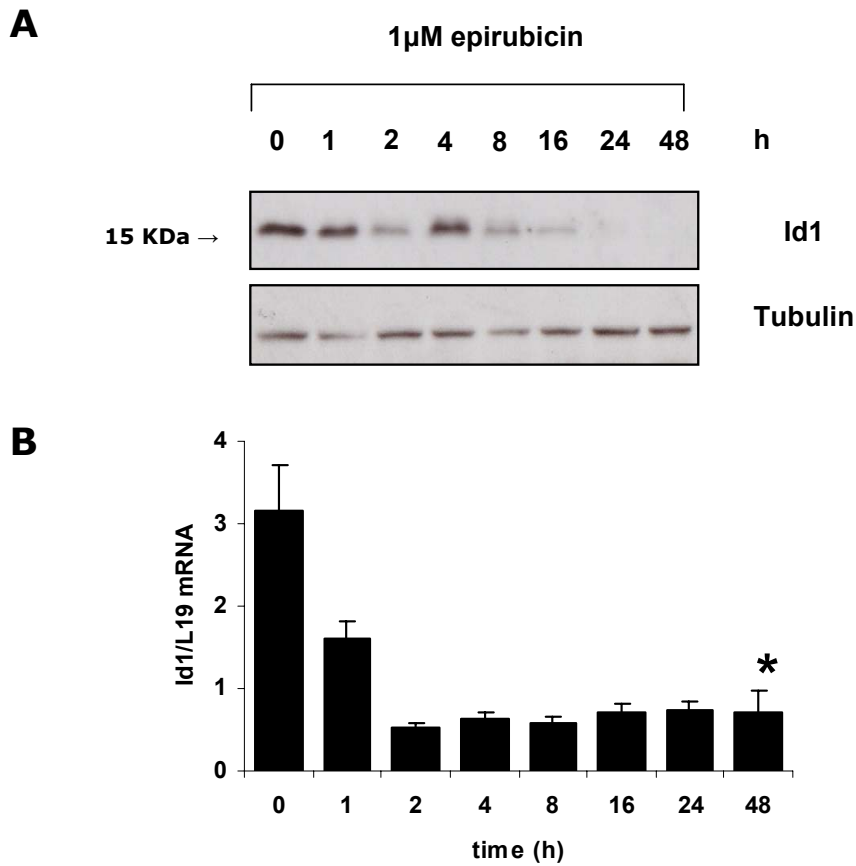


Figure 4.3 Epirubicin downregulates Id1 transcriptionally and translationally. A) Whole cell lysates were prepared from MCF-7 cells treated with 1 μ M epirubicin at the indicated timepoints and the expression of Id1 was analysed by Western blotting. Tubulin was used as a loading control. **B)** Total RNA was isolated in parallel and the expression of Id1 mRNA was measured by RTq-PCR and normalised to the level of L19. The results represent the average $\pm$ SD of three independent experiments. * $p < 0.05$.

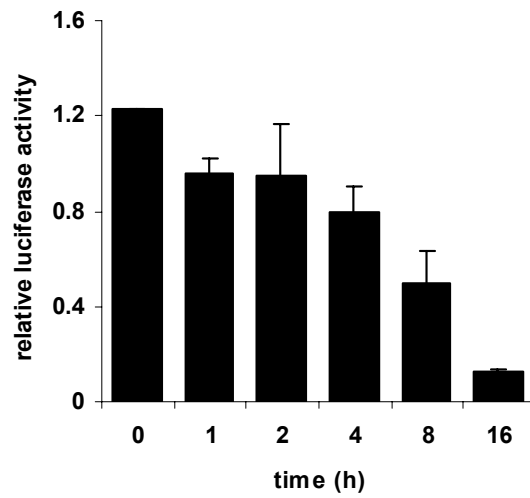


Figure 4.4. Epirubicin represses *Id1* at the gene promoter level. MCF-7 cells were transfected with 10ng of the *Id1* promoter and treated with 1 μ M epirubicin for the indicated time points before cells were collected for luciferase reporter assays. The relative luciferase activity values were corrected for cotransfected *renilla* activity. Results are the average $\pm$ SD of three independent experiments.

4.2.5 *Id1 expression is higher in aggressive and resistant cell lines*

The expression of Id1 varies in breast cancer cell lines, as does the sensitivity of cell lines to the cytotoxic effects of epirubicin. In the previous chapter, SRB assays confirmed the sensitivity of MCF-7 cells to epirubicin, whilst the ER negative cell line MDA-MB-231 was epirubicin resistant and cells continued to proliferate in the presence of the drug. It was therefore of interest to look at the expression of Id1 in sensitive and resistant cells lines and to assess whether there is any correlation between Id1 expression and chemosensitivity. MCF-10a cells, non-tumorigenic immortalized breast epithelial cells, were used for comparison.

Western blot analysis confirmed higher expression of Id1 in the rapidly proliferating and epirubicin resistant MDA-MB-231, when compared to both MCF-7 cells and the untransformed MCF-10a cells (Figure 4.5a). Id1 mRNA expression correlated well with protein levels, again with MDA-MB-231 cells having the highest relative expression of Id1 mRNA (Figure 4.5b).

Another good model for a drug resistant cell line is the doxorubicin resistant MCF-7 cell line (Dox-R), generated in the laboratory by long term culture of MCF-7 cells in the presence of 0.01mg/ml of the anthracycline doxorubicin. These cells grow normally and proliferate in doxorubicin and are therefore a good model for a more aggressive, drug resistant cell line. The expression of Id1 is higher in the Dox-R cells lines than in the wild-type MCF-7 cells, implicating Id1 as a possible contributor to a more drug resistant phenotype (Figure 4.6a.)

To investigate the effects of epirubicin on these Dox-R MCF-7 cells, and to characterize whether Id1 may contribute any part to their resistant profile, a dose titration of epirubicin treatment was performed in the cells, with treatment ranging from 0.1 μ M to 10 μ M epirubicin. Across all doses, the effects of epirubicin were significantly less than compared to WT MCF-7 cells (Figure 3.3a). Even at high doses of 5 and 10 μ M, Dox-R MCF-7 cells were still proliferating, suggesting this is undoubtedly a highly anthracycline resistant cell line (Figure 4.6b). Western blot analysis confirmed that, unlike the epirubicin sensitive wildtype MCF-7 cells, over 48h of drug treatment, no changes in Id1 protein or mRNA levels were observed, confirming that early downregulation of Id1 is specific only to sensitive cells (Figure 4.6c and 4.6d).

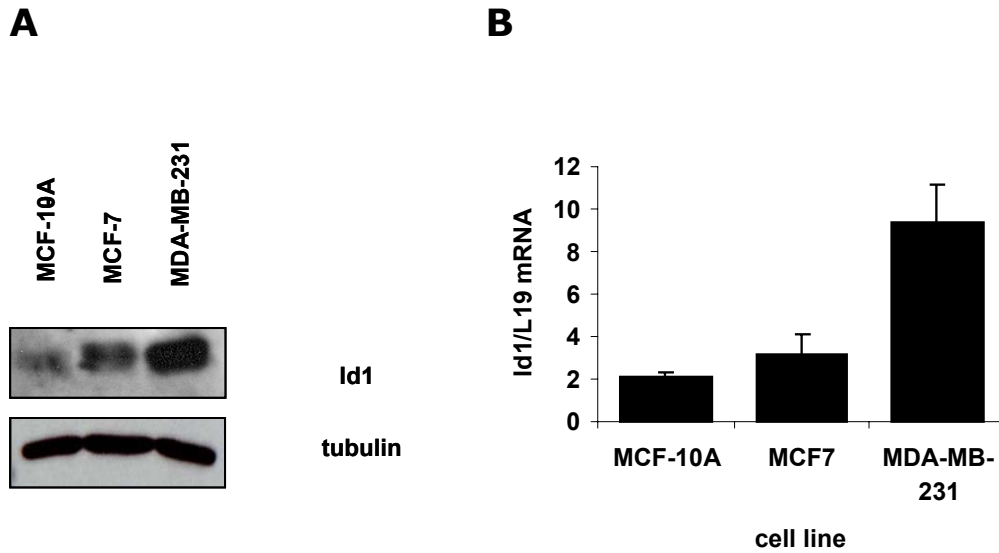


Figure 4.5 Id1 expression is highest in aggressive cell lines. **A)** Whole cell lysates were prepared from MCF-10a, MCF-7 and MDA-MB-231 cells and the expression of Id1 was analysed by Western blotting. **B)** Total RNA was isolated in parallel and the expression of Id1 mRNA was measured by RTq-PCR and normalised to the level of L19. The results represent the average $\pm$ SD of three independent experiments.

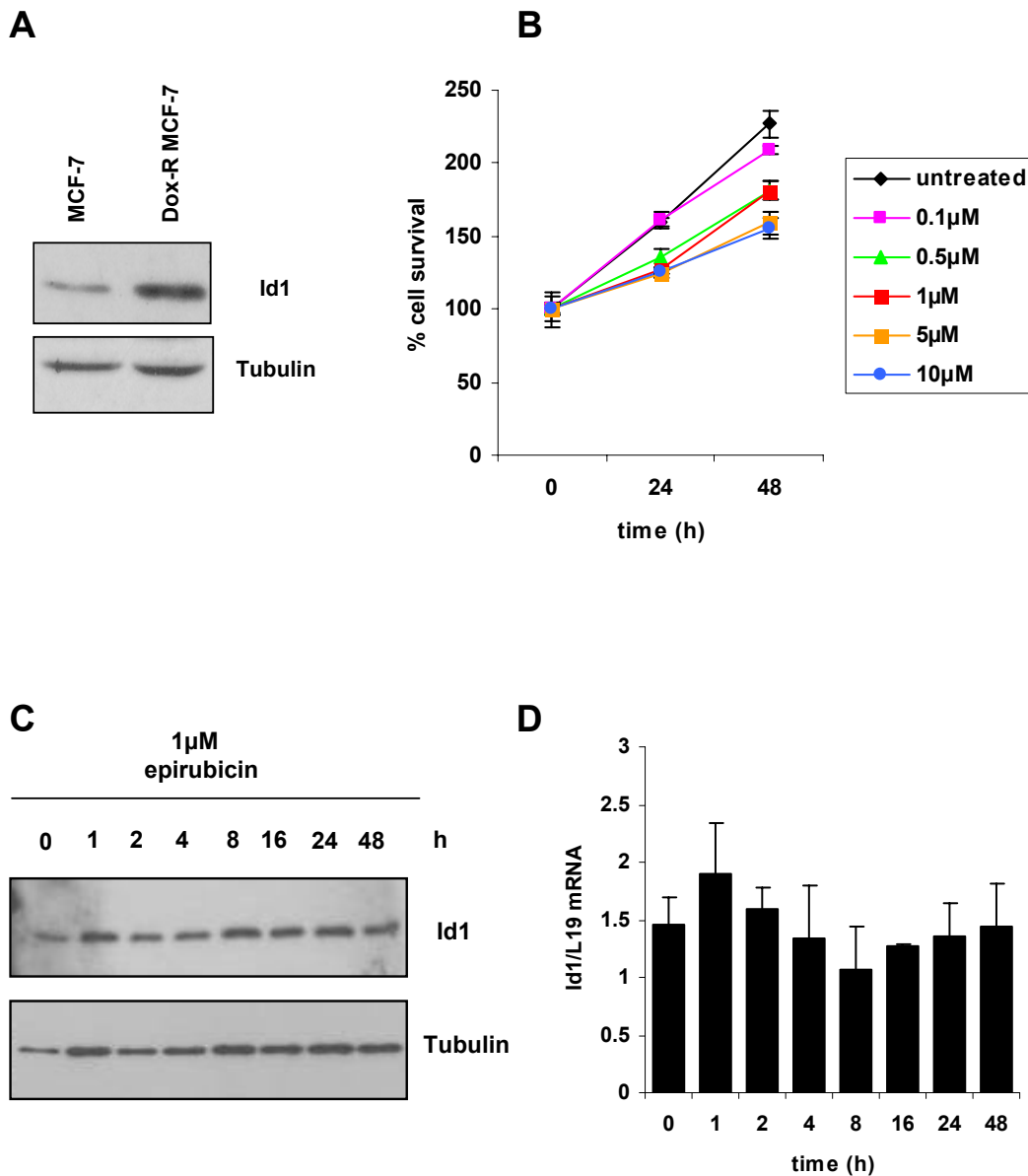


Figure 4.6 Id1 expression following epirubicin treatment in Dox-R MCF-7 cells. **A)** Total cell lysates from MCF-7 cells and Dox-R MCF-7 cells were analysed by Western blotting for Id1 expression. **B)** Dox-R MCF-7 cells were treated with a dose titration of epirubicin and cell survival analysed by SRB assay. **C)** Dox-R MCF-7 cells were treated with 1µM epirubicin for the indicated times and protein lysates were analysed for Id1 expression by Western blotting. **D)** RNA was extracted in parallel and Id1 mRNA was measured by RTq-PCR and normalised to the level of L19. The results represent the average ±SD of three independent experiments.

4.2.6 Silencing of Id1 slows proliferation of breast cancer cells

MCF-7 cells proliferate *in vitro* with a doubling time of approximately 48h (Figure 4.7d). Id1 is a proliferation dependent protein and expression is highest in proliferating cells and lowest in growth arrested cells (Figure 4.2). To investigate more specifically at the effects of Id1 on the cell cycle in MCF-7 cells, cells were transfected with an Id1 specific siRNA, mock transfected and non specific control (NSC) siRNA. Western blot analysis confirmed efficient knockdown of endogenous Id1 protein and RTq-PCR showed similar efficient knockdown of Id1 mRNA (Figure 4.7a and 4.7b).

To study the effects of Id1 silencing on the cell cycle, an Id1 specific siRNA pool was transfected into MCF-7 cells the cell cycle profile was then analysed by FACS analysis 48h post-transfection. The cell cycle profile confirmed that silencing of Id1 leads to a greater accumulation of cells in the G₁ phase of the cell cycle – 75% compared to 63% for the mock and NSC (Figure 4.7c). This is consistent with a role for Id1 in cell cycle progression - in fibroblasts Id proteins have a critical role in promoting G₁-S cell cycle transition. These results suggest that in breast cancer cells, a similar cell cycle role is likely, with more cells accumulating in G₁ and thus less progression through to S phase for DNA synthesis and subsequent mitosis during G₂/M.

This change in cell cycle profile is confirmed by SRB assay (Figure 4.7d). The mock and control siRNA transfected cells continue to proliferate, but the cells transfected with an Id1 specific siRNA show much slower proliferation, confirming Id1 is

required for normal MCF-7 proliferation and cell cycle progression.

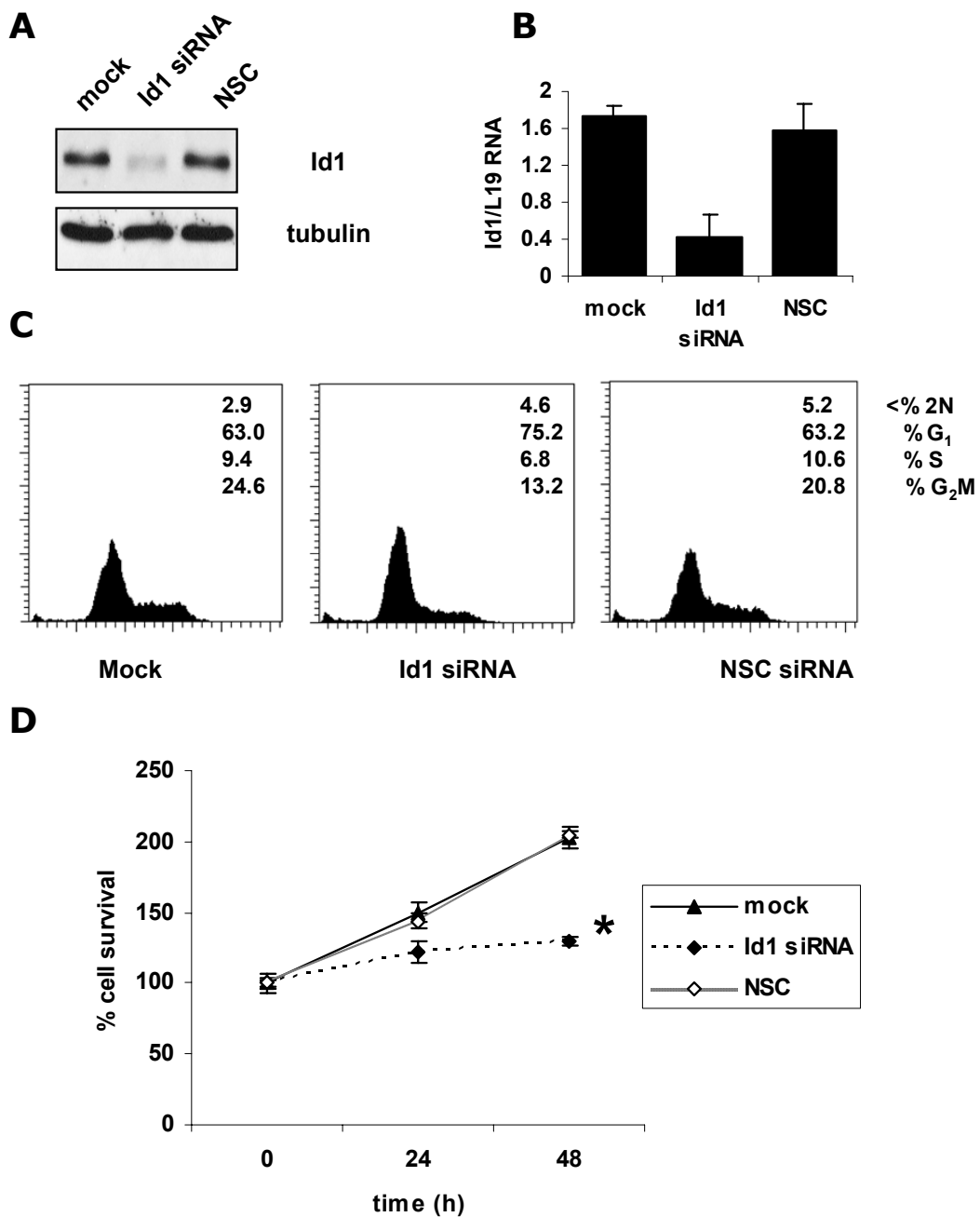


Figure 4.7 Silencing of Id1 slows proliferation of MCF-7 cells. **A)** MCF-7 cells were transfected with an Id1 specific siRNA, mock and NSC. Western blot analysis confirmed knockdown of endogenous Id1. **B)** RNA from cells transfected in parallel was used to monitor siRNA Id1 efficiency at mRNA level. **C)** Cells were transfected with an Id1 specific siRNA, mock and NSC for 48h, fixed in ethanol and stained with propidium iodide then analysed by FACS. The % of cells in each phase of the cell cycle is indicated. **D)** Following siRNA Id1 transfection, cells were replated in 96 well plates for 48h and cell survival measured by SRB assay. Results are the average $\pm$ SD of three independent experiments with 6 replicates. * $p < 0.05$.

4.2.7 *Id1 silencing can reduce the growth of resistant and aggressive cell lines and increase sensitivity to epirubicin*

MDA-MB-231 cells therefore express high levels of Id1 and are resistant to epirubicin. To see if Id1 contributed to this resistant phenotype, knockdown of Id1 was performed using siRNA (Figure 4.8a) and then MDA-MB-231 cells were treated with epirubicin for 48h. SRB assay confirmed that knockdown of Id1 can significantly slow the proliferation of MDA-MB-231 cells and increases the cytotoxic effects of epirubicin (Figure 4.8b). However, in these particular cells, silencing of Id1 is unable to reverse a resistant phenotype.

The other cell line shown to express high levels of Id1 and to be resistant to epirubicin is the Dox-R MCF-7 cell line. An Id1 specific siRNA was transfected into these cells and they were then treated with 1 μ M epirubicin for 48h. Their cell cycle profile was then analysed by FACS. Western blot analysis confirmed good knockdown of endogenous Id1 protein (figure 4.9a). When the cell cycle profile of the siId1 transfected cells was compared to the mock or control transfected cells, the number of sub G₁ cells had doubled (Figure 4.9b). Although the order of change was relatively small, from approximately 5% sub G₁ to 10%, this did suggest that Id1 may contribute to drug resistance in these cells.

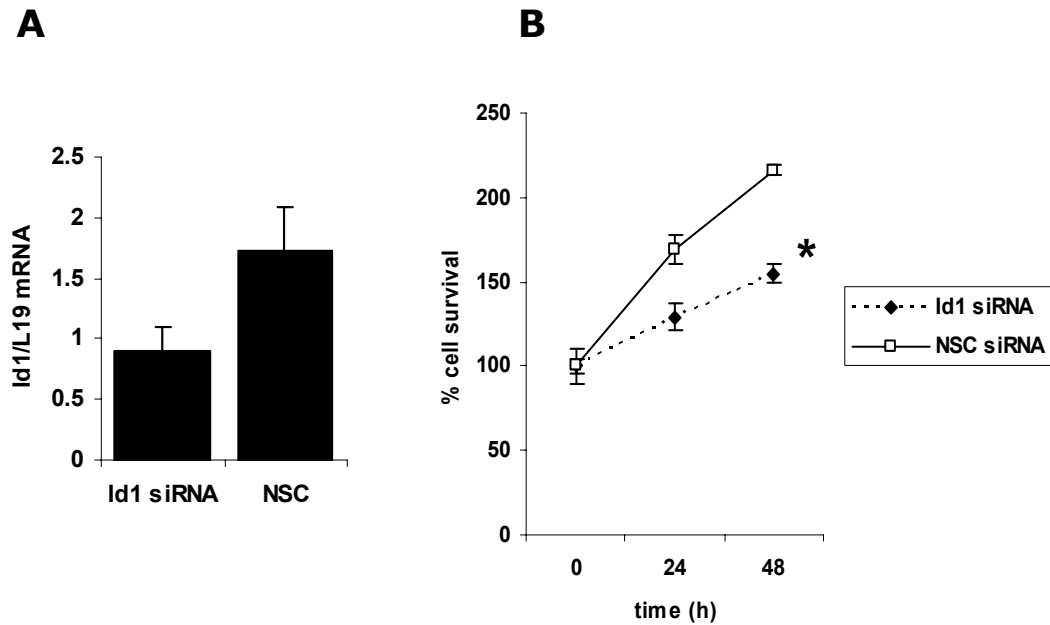


Figure 4.8 Silencing of Id1 slows the proliferation of MDA-MB-231 cells treated with epirubicin. A) MDA-MB-231 cells were transfected with an Id1 specific siRNA pool, and NSC. RNA from cells transfected in parallel was used to monitor siRNA Id1 efficiency at mRNA level. **B)** Following siRNA Id1 transfection, cells were replated in 96 well plates for 48h, treated with 1 μ M epirubicin and cell survival measured by SRB assay. Results are the average $\pm$ SD of three independent experiments with 6 replicates. * $p < 0.05$.

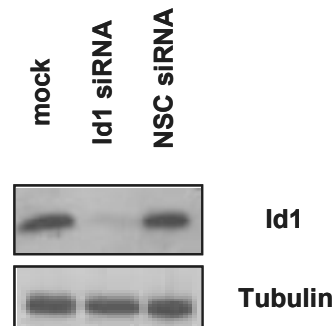
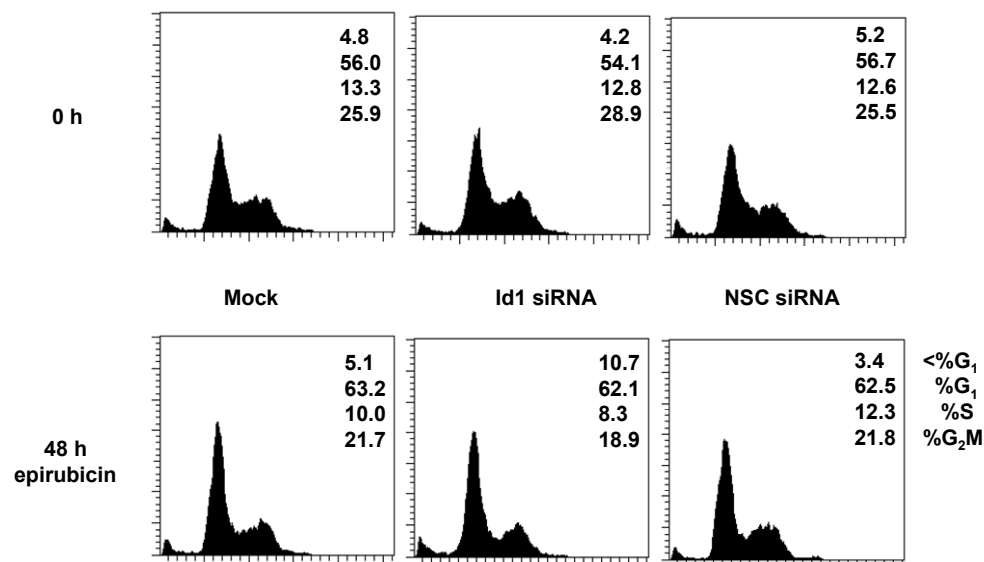
A**B**

Figure 4.9 Silencing of Id1 in Dox-R MCF-7 cells introduces some sensitivity to epirubicin. **A)** Dox-R MCF-7 cells were transfected with an Id1 specific siRNA pool, mock and NSC. Western blot analysis confirmed knockdown of endogenous Id1. **B)** Cells were transfected with an Id1 specific siRNA, mock and NSC and treated with 1 μ M epirubicin for 48h, fixed in ethanol and stained with propidium iodide then analysed by FACS. The % of cells in each phase of the cell cycle is indicated.

4.2.8 *Id1 knockdown increases epirubicin cytotoxicity in MCF7 cells*

Earlier results have shown that Id1 is an early target of epirubicin in breast cancer cells. Treatment with epirubicin leads to downregulation of Id1 protein and mRNA and Id1 promoter activity is repressed following addition of the drug. Silencing of Id1 by siRNA slows the proliferation of MCF7 cells and leads to a greater accumulation of cells in G₁. It was therefore of interest to look at whether Id1 silencing has an effect on the sensitivity of MCF7 cells to epirubicin and whether targeting Id1 can increase the cytotoxic effect of epirubicin.

MCF7 cells were transfected with an Id1 specific siRNA or control siRNA then grown for 48h either with or without the addition of epirubicin. In the control, untreated cells, proliferation was as expected, with a growth doubling time of approximately 48h. In the untreated cells, transfected with an Id1 siRNA, proliferation was slower, as previously shown. However, when cells were treated with epirubicin, silencing of Id1 increased the cytotoxic effects of epirubicin and Id1 knockout cells were more sensitive to epirubicin than their control transfected counterparts (Figure 4.10b).

Thus, not only does silencing of Id1 simply slow proliferation of MCF-7 cells, but it also increases their sensitivity to chemotherapy and targeting Id1 prior to treatment with epirubicin increases the cytotoxic effect of the drug. This effect was not unique to epirubicin. Two other cytotoxic drugs – cisplatin and docetaxel – were also investigated in similar experiments. The same results were observed, in that Id1

knockout cells were more sensitive to both cisplatin and docetaxel than the control transfected cells. Interestingly, the doses used of both drugs on their own (10 μ M cisplatin and 10nM docetaxel) were not particularly toxic to MCF-7 cells and although cell proliferation slowed, a decrease in cell survival was not observed until after 24h of drug treatment (Figure 4.11a and 4.11b). However, in the siRNA Id1 transfected cells, after 24h, a decrease in cell survival was observed, suggesting knockout of Id1 can contribute to making MCF-7 cells more sensitive to sub-lethal doses of chemotherapeutic drugs.

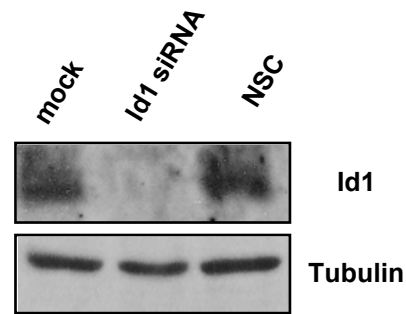
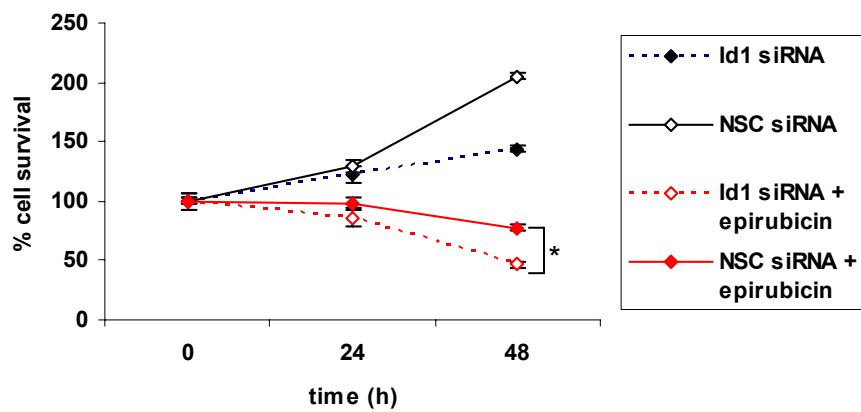
A**B**

Figure 4.10 Silencing of Id1 increases the cytotoxicity of epirubicin. **A)** MCF-7 cells were transfected with an Id1 specific siRNA or NSC. Western blot analysis confirmed efficient knockdown of endogenous Id1. **B)** Cells were untreated or epirubicin was added for 48h. Cell survival was measured by SRB assay. Results are the average $\pm$ SD of three independent experiments with 6 replicates. * $p < 0.05$

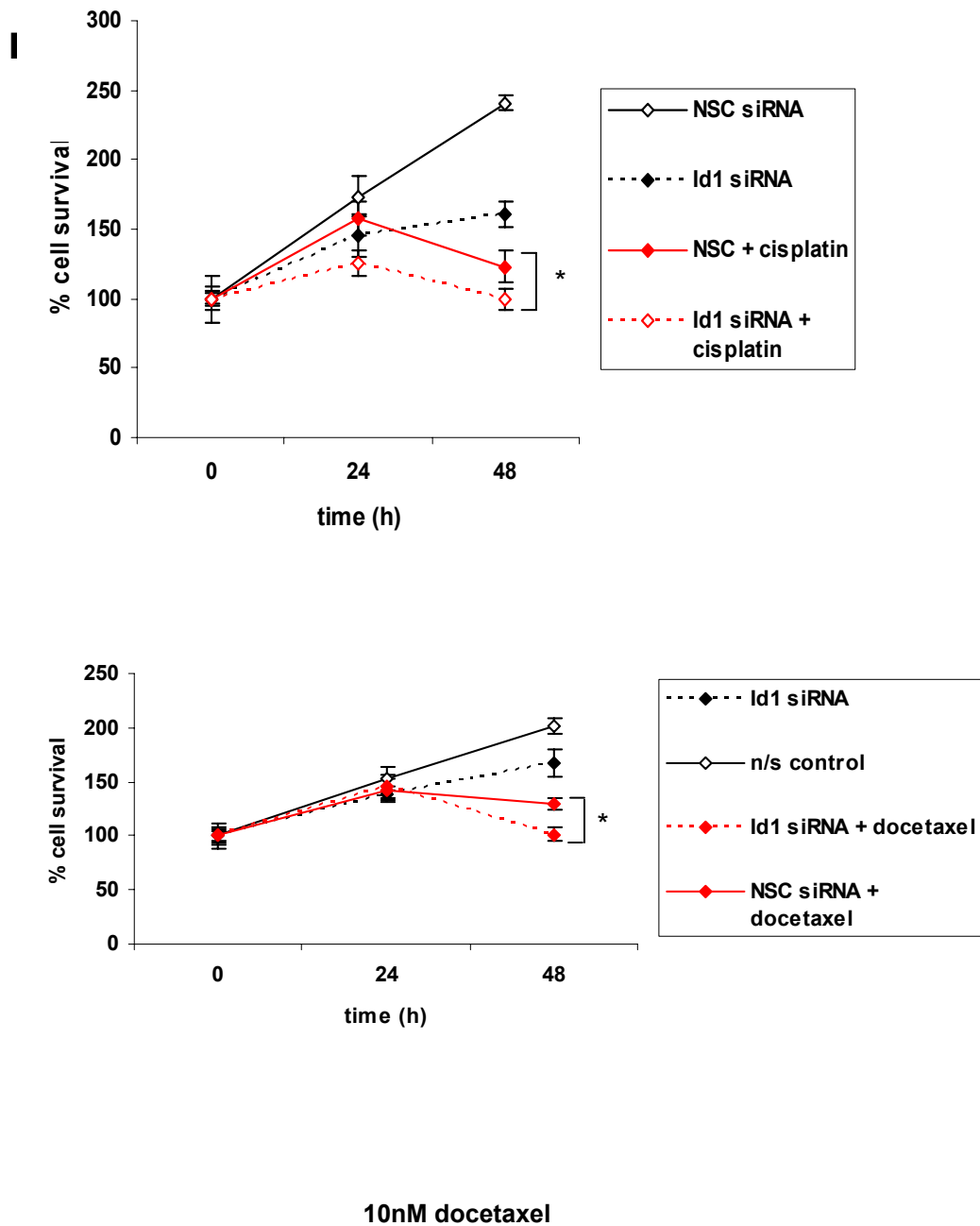
A

Figure 4.11 Silencing of Id1 increases the sensitivity of MCF-7 cells to cisplatin and docetaxel. MCF-7 cells were transfected with an Id1 specific siRNA or NSC. Cells were untreated or **A)** 1 μ M cisplatin or **B)** 10nM docetaxel was added for 48h. Cell survival was measured by SRB assay. Results are the average $\pm$ SD of three independent experiments with 6 replicates. * $p < 0.05$

4.2.9 *Cyclin D1 is downregulated following Id1 silencing*

These results have shown that Id1 is expressed in a variety of breast cancer cell lines and expression is highest in proliferating cells. Id1 is rapidly downregulated by epirubicin at the protein, mRNA and gene promoter level. Silencing of Id1 slows proliferation of cells and leads to a greater accumulation of cells in the G₁ phase of the cell cycle. This effect on the cell cycle appears to increase the sensitivity of MCF-7 cells to chemotherapy, with a greater cytotoxic effect observed following knockdown of Id1.

Another key regulator of the G₁ phase of the cell cycle is cyclin D1. Cyclin D1 expression is seen early following cell cycle entry and its assembly with Cdk4 is key to allowing cells to progress through the restriction point following mitogenic stimulation (Alberts et al., 2008). To see whether any changes were observed in cyclin D1 as a result of Id1 silencing, MCF-7 cells were transfected with an Id1 specific siRNA and mock and non-specific control. Interestingly, Id1 protein knockdown was accompanied by reduced expression of cyclin D1 at both the protein and mRNA level (Figure 4.12a and 4.12b). No changes were seen in the protein levels of p21 or p27, two other key G₁ regulators. This suggests that Id1 may have an effect on the regulation of cyclin D1 expression and the loss of Id1 may in turn reduce the expression of cyclin D1 which would contribute to the inhibition of proliferation observed in these cells.

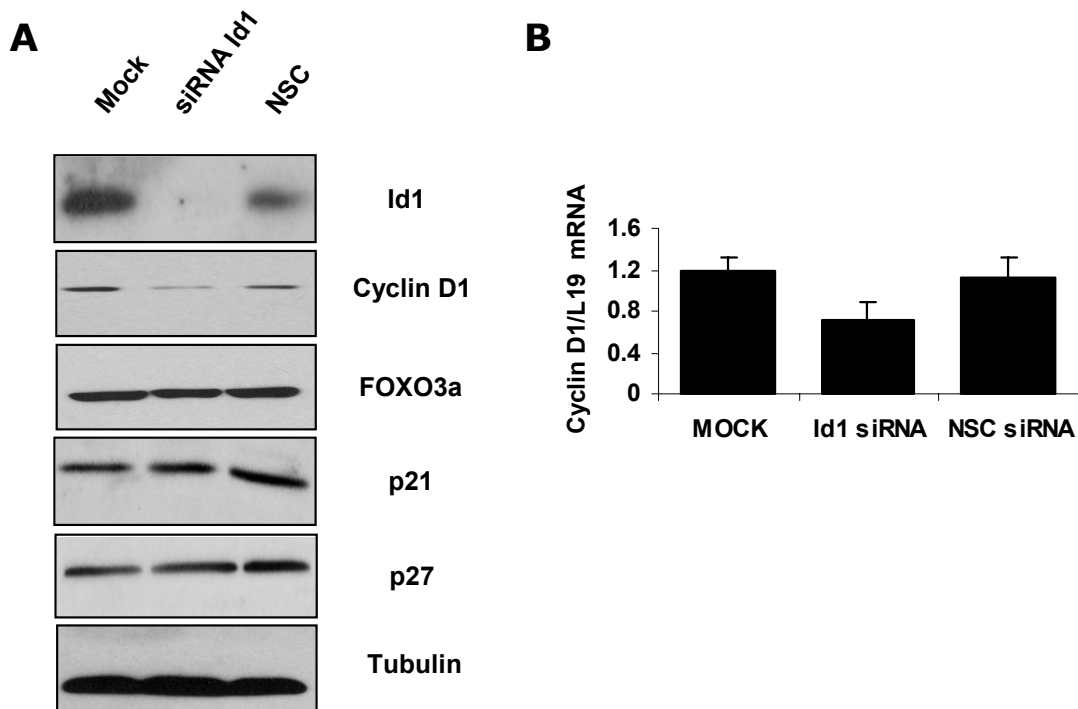


Figure 4.12 Cyclin D1 is downregulated following silencing of Id1. **A)** MCF-7 cells were transfected with an Id1 specific siRNA, mock and NSC siRNA for 48h. Whole cell lysates were prepared and protein expression was analysed by Western blotting using the indicated antibodies. **B)** Total RNA was isolated in parallel and the expression of cyclin D1 mRNA was measured by RTq-PCR and normalised to the level of L19. The results represent the average $\pm$ SD of three independent experiments.

4.2.10 Overexpression of Id1 confers protection to epirubicin

In many epithelial cancers, correlation has been seen between levels of Id1 and malignant potential. Higher levels of Id1 expression have been seen in invasive cancers, compared to ductal carcinoma *in situ* and introduction of a constitutively expressed *Id1* cDNA into the non aggressive T47D cell line confers a more aggressive phenotype (Lin et al., 2000).

An Id1 expression vector was transiently transfected into MCF-7 cells and the effects of epirubicin on cell survival was measured by SRB assay. Western blot analysis confirmed overexpression of Id1 (Figure 4.13a). Interestingly, cyclin D1 levels were unchanged in the Id1 overexpressed cells, even though the earlier reciprocal experiment, where Id1 was silenced, had shown reduced expression of cyclin D1 following Id1 knockdown. This could be because cyclin d1 is already at maximal levels as these are normal cycling cells. No changes were observed in p21^{Cip1}, p27^{Kip1} or phosphorylated Akt. However, the SRB assay showed that overexpression of Id1 confers some protection against the effects of epirubicin and overexpression of Id1 provided MCF-7 cells with some resistance to epirubicin (Figure 4.13b).

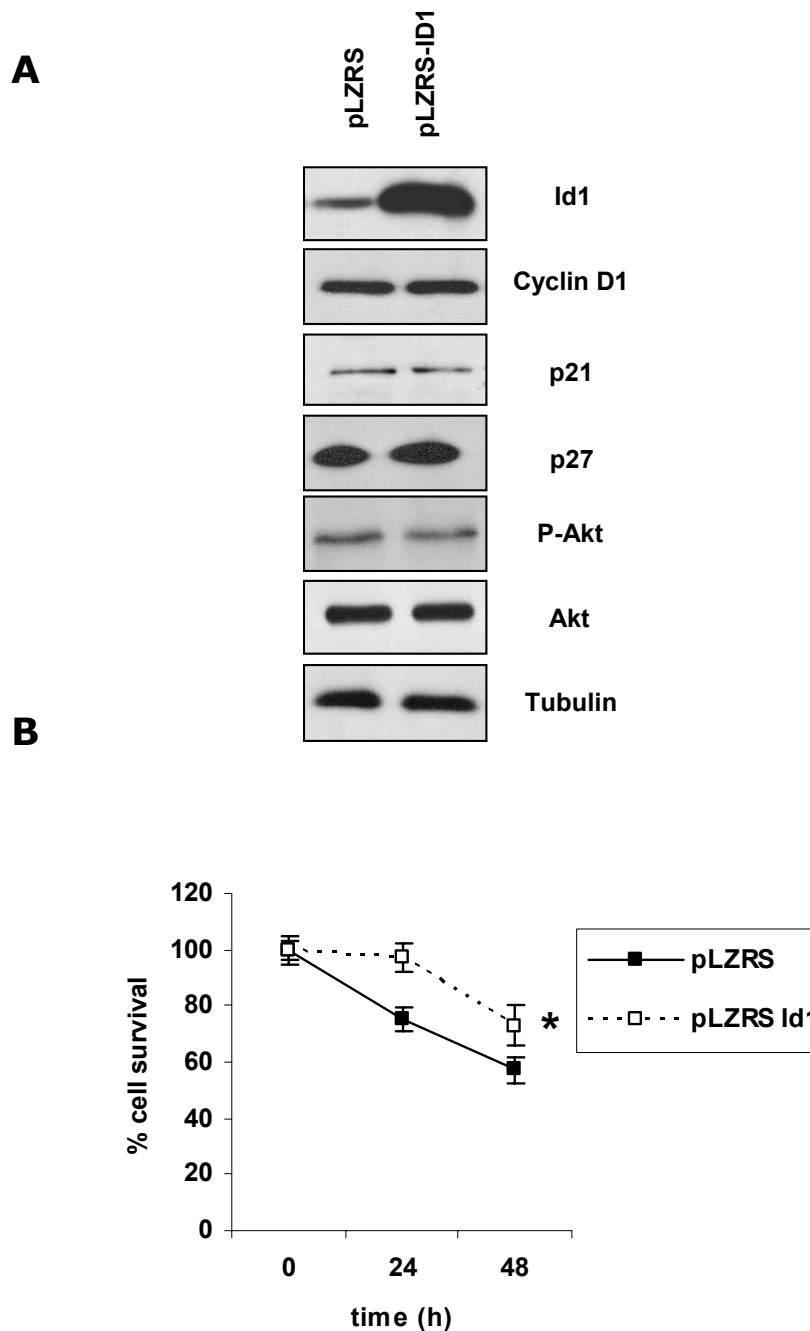


Figure 4.13 Overexpression of Id1 confers resistance to epirubicin. A) MCF-7 cells were transfected with a vector expressing Id1 (pLZRS-Id1) or an empty vector and analysed for the expression of Id1 by western blotting. **B)** MCF-7 cells transfected in parallel were plated in 96 well plates, treated for 48h with epirubicin and cell survival analysed by SRB assay. Results are the average $\pm$ SD of three independent experiments with 6 replicates. * $p < 0.05$.

4.2.11 FOXO3a does not regulate Id1 in breast cancer cells

Id1 has previously been shown to be highly expressed in the Bcr-Abl transformed K562 cell line. Inhibition of Bcr-Abl activity by imatinib resulted in activation of FOXO3a and downregulation of Id1 expression. FOXO3a was shown to be a transcriptional repressor of Id1, by directly binding to the Id1 promoter (Birkenkamp et al., 2007). Myeloid leukaemic cells are characterized by both uncontrolled proliferation but also failure to differentiate and as an inhibitor of differentiation, it is clear to see that Id1 may play a role in the leukaemic process.

Little is known about the regulation of Id1 in solid, epithelial tumours. This work has shown that FOXO3a is activated by epirubicin and Id1 is repressed by epirubicin – it was therefore of interest to investigate whether FOXO3a activation is responsible for Id1 repression in response to chemotherapy, as has previously been shown to be the case in leukaemic cell lines.

MCF-7 cells were transfected with a FOXO3a specific siRNA or control siRNA and relative mRNA levels were measured by RTq-PCR. If FOXO3a is functioning as a repressor of Id1 and if Id1 is a downstream target of FOXO3a, silencing of FOXO3a may be expected to increase Id1 expression as this repression is relieved. However, FOXO3a knockdown did not have any effect on Id1 levels (Figure 4.14a). The reciprocal experiment, where Id1 was silenced, also had no effect on FOXO3a expression (Figure 4.14b).

To further investigate whether Id1 may be a downstream target of FOXO3a, gene promoter studies were performed. In

leukaemic cells, treatment with imatinib has been shown to repress Id1 promoter activity. However, when a key FOXO3a binding site in the Id1 promoter (-134 to -128bp upstream of the ATG start codon) is mutated from a TTT core sequence to CGT, no repression was observed. This lead to the conclusion that binding of FOXO3a to this critical site is required for downregulation of Id1 promoter activity in K562 cells (Birkenkamp et al., 2007).

To test if a similar effect was observed in MCF-7 cells, the cells were transfected with either the wild type or the mutated promoter and then treated with epirubicin. As expected, epirubicin repressed the activity of the wild type promoter (Figure 4.15a). However, a similar pattern of repression was also seen in the mutated promoter (Figure 4.15b). This would suggest that epirubicin is repressing Id1 promoter activity independent of FOXO3a and that mutating a putative FOXO3a binding site has little effect on Id1 promoter repression. Thus, the regulation of Id1 promoter activity differs between haematopoeitic and epithelial cancer cells.

To confirm that Id1 repression by epirubicin is not dependent on FOXO3a, a further knockdown experiment was performed. MCF-7 cells were transfected with a FOXO3a specific siRNA or non-specific control and then treated over 48h with epirubicin. Western blot analysis confirmed good knockdown of endogenous FOXO3a protein. However, in both the knockdown and control cells, Id1 protein levels were downregulated by epirubicin and were undetectable from 8h post treatment (Figure 4.16). This again suggests that Id1 is a target of epirubicin but is not downstream of FOXO3a.

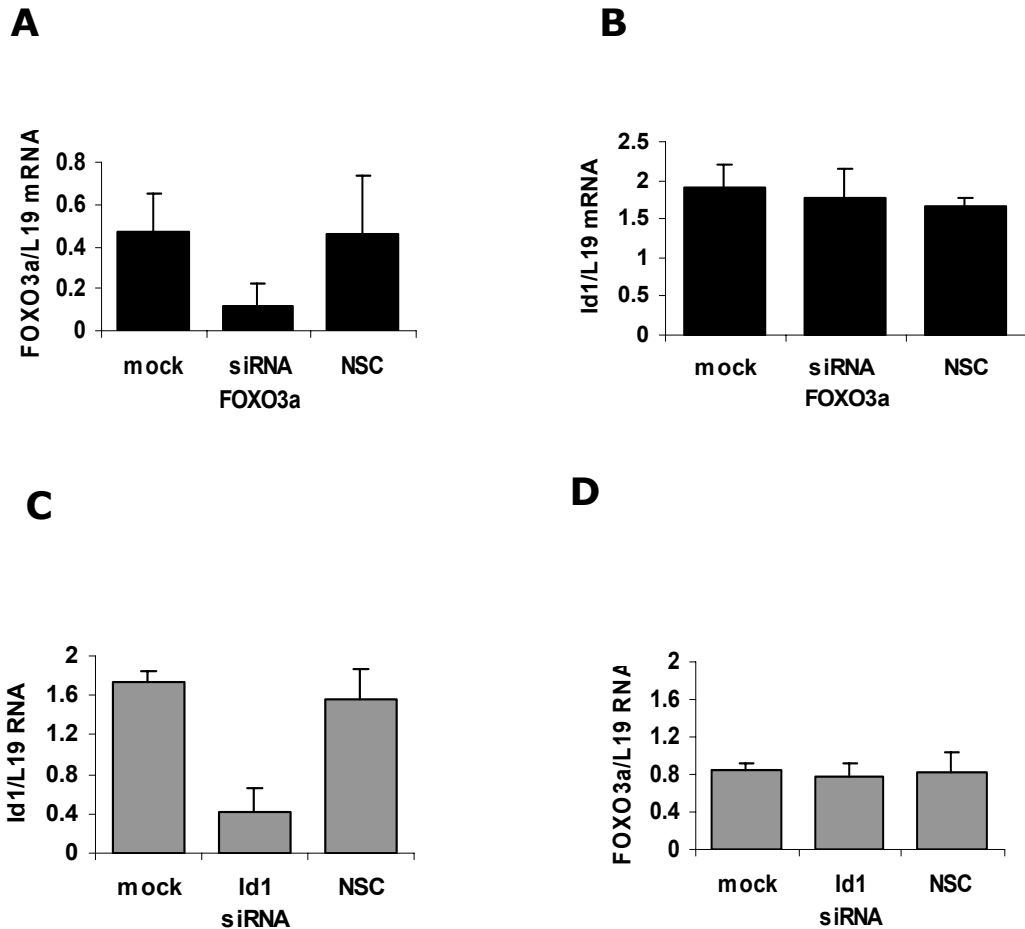


Figure 4.14 FOXO3a silencing does not affect Id1 mRNA levels. MCF-7 cells were transfected with **A)** a FOXO3a specific siRNA, mock and NSC siRNA and **B)** an Id1 specific siRNA, mock and NSC siRNA for 48h. Total RNA was isolated and the expression of FOXO3a and Id1 mRNA was measured by RTq-PCR and normalised to the level of L19. The results represent the average $\pm$ SD of three independent experiments.

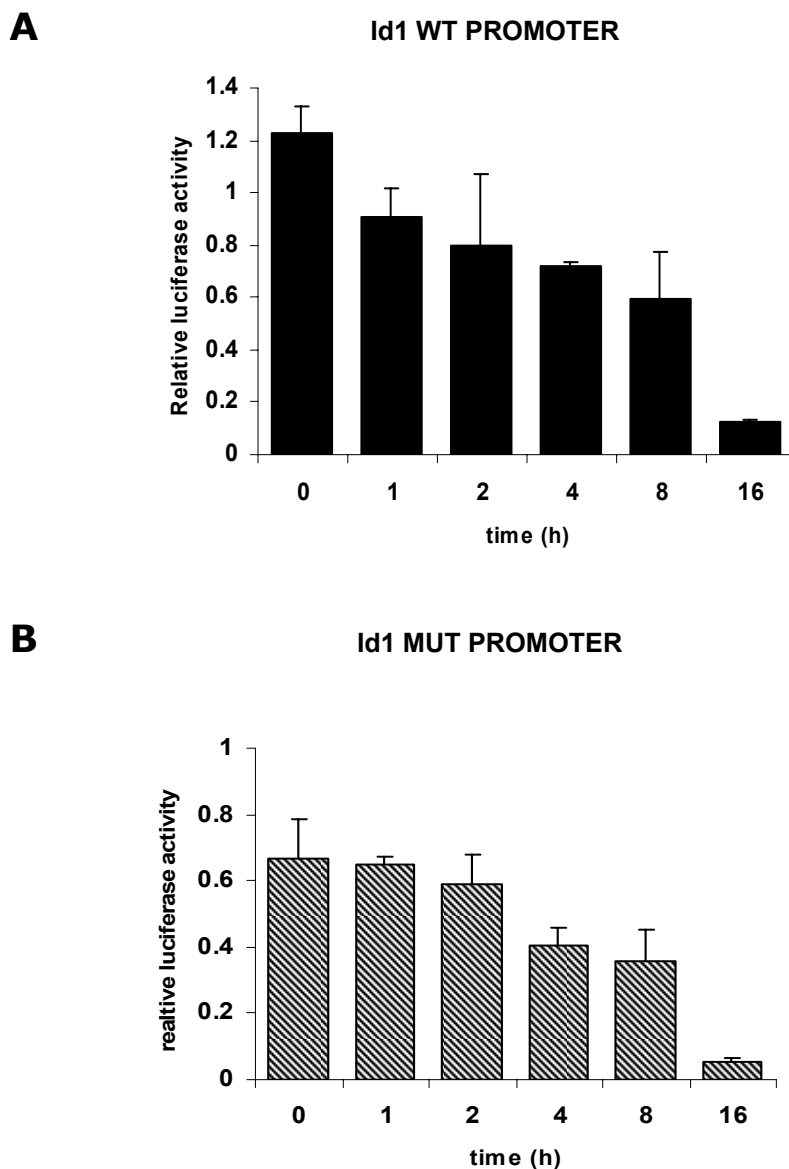


Figure 4.15 Epirubicin represses both the wildtype and mutated *Id1* promoter. MCF-7 cells were transfected with **A)** 10ng of the *Id1* promoter or **B)** an *Id1* promoter with mutated FOXO3a binding sites and treated with 1 μ M epirubicin for the indicated time points before cells were collected for luciferase reporter assays. The relative luciferase activity values were corrected for cotransfected *renilla* activity. Results are the average $\pm$ SD of three independent experiments.

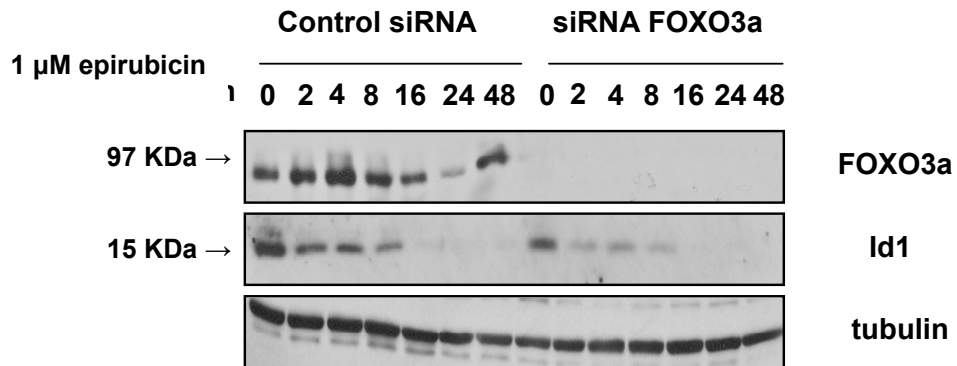


Figure 4.16 Id1 repression by epirubicin is independent of FOXO3a. MCF-7 cells were transfected with a FOXO3a specific siRNA or NSC then treated for 48h with 1 μ M epirubicin. Whole cell lysates were prepared and FOXO3a and Id1 protein expression was analysed by Western blotting. Tubulin was used as a loading control.

4.3 DISCUSSION

Over the last decade, there has been increasing evidence for an oncogenic role for Id1 in human cancers. Correlation has been seen between expression of Id1, malignant potential and clinical outcome. However, the molecular mechanisms responsible for the function of Id1, particularly in breast cancer, are not known. Therefore further characterization of the function of Id1 and the effects of chemotherapy on its expression would provide interesting data in determining whether Id1 may be a useful therapeutic target in cancer.

Id1 protein is expressed in all breast cancer cell lines investigated in this work, and expression correlates with a more aggressive and drug resistant phenotype - both MDA-MB-231 cells and a doxorubicin resistant MCF-7 derivative have relatively higher expression levels of Id1 (Figure 4.5). This confirms results seen in other epithelial cancers - in oesophageal squamous cell carcinoma for example, Id1 expression is higher in cancerous cell lines than the immortalized, non-transformed NE1 cell line (Yuen et al., 2007). It is also evident that Id1 expression is highest in proliferating cells and that serum starvation leads to downregulation of Id1 in MCF-7 cells, confirming Id1 is induced by mitogens (Figure 4.2). MCF-7 cells are ER positive and are therefore sensitive to the effects of oestradiol. Withdrawal of serum and the downregulation of Id1 may be a direct result of steroid hormone deprivation. Studies have shown previously that in the non-aggressive T47D breast cancer cell line, treatment with oestradiol downregulates Id1. However, MDA-MB-231 cells constitutively express high levels of Id1 regardless of the

presence of serum (Lin et al., 2000) and thus one mechanism for linking high expression of Id1 and an aggressive phenotype is the loss of serum dependent regulation of *Id1* gene expression. This could also provide one hypothesis for the high expression of Id1 found in ovarian, endometrial and prostate tumours - cancers which initially arise in hormone dependent tissues.

Id1 protein is downregulated by epirubicin and RTq-PCR analysis showed Id1 transcript levels also decreasing with time, as an early event following epirubicin treatment – correlating well with changes in Id1 protein levels (Figure 4.3). Thus, Id1 appears to be an early target of epirubicin treatment and suggests that epirubicin is directly suppressing gene transcription as *Id1* promoter activity is also suppressed. This is an interesting observation indicating that epirubicin inhibits Id1 expression at the promoter level and not through mechanisms modulating protein and mRNA stability. For example, in prostate cancer, treatment of DU145 and PC-3 cells with TNF α downregulates Id1 protein levels also, but this is not accompanied by changes in Id1 mRNA or promoter activity and TNF α was shown to downregulate Id1 through the activation of the ubiquitin/proteasome degradation pathway (Ling et al., 2006). Therefore, although Id1 may be a target of a number of drugs and cytokines, the mechanisms by which it is repressed can differ and Id1 appears to be a transcriptional target of epirubicin in MCF-7 cells.

To further characterize the role of Id1, a number of experiments were performed to look at the effects of silencing Id1 by siRNA. In particular, the effects of Id1 knockdown on the cell cycle profile and proliferative ability of MCF-7 cells was investigated.

FACS analysis of cells transfected with an Id1 specific siRNA showed that loss of Id1 causes a greater accumulation of cells in G₁ (Figure 4.7). G₁ is the key cell cycle phase required for growth and protein synthesis and is the point at which cells respond to extracellular stimuli and commit to DNA replication. The cell cycle profile was confirmed by SRB assay which showed a slowing in the proliferation rate of MCF-7 cells following Id1 knockdown, suggesting that Id1 must have an important role in cell cycle progression through G₁ and without it, fewer cells are able to commit to progression through G₁ and on into S phase and G₂/M. There is relatively little information known about how Id1 may regulate the cell cycle so to investigate further the mechanisms underlying Id1 mediated cell proliferation, other cell cycle proteins were analysed following Id1 silencing. p21^{Cip1} and p27^{Kip1} are both key cell cycle regulators and both can lead to G₁ cell cycle arrest. In fibroblasts, the regulation of p21^{Cip1} by the HLH transcription factor E2A has been shown to be antagonized by Id1. (Prabhu et al., 1997). However, although silencing of Id1 leads to cells accumulating in G₁, proteins levels of p21^{Cip1} and p27^{Kip1} are unchanged. However, downregulation of cyclin D1 is observed following knockdown of Id1, at both the protein and mRNA level (Figure 4.12). Cyclin D1 expression is usually an early event in cell cycle entry and this allows progression of cells through the restriction point and the phosphorylation and inactivation of Rb. Id1 may require cyclin D1 for its proliferative effects – knockdown of Id1 also results in reduced cyclin D1 protein and mRNA, implicating regulation of cyclin D1 in Id1 mediated inhibition of proliferation. The promoter of the *cyclin D1* gene is known to contain a number of consensus motifs for several transcription factors that can interact with Id proteins (Yates et al., 1999). However, conversely, cyclin D1 levels did not increase following

overexpression of Id1 suggesting that Id1, rather than increasing expression of cyclin D1, may simply potentiate its effect.

Having established the antiproliferative effect of silencing Id1, it was also of interest to see whether this had any implications on the sensitivity of MCF-7 cells to chemotherapy, in particular to epirubicin. Results in chapter 3 confirmed that MCF-7 cells were sensitive to 1 μ M epirubicin, causing G₂/M cell cycle arrest and apoptosis. Earlier work in this chapter identified Id1 as a direct target of epirubicin and the role that Id1 plays in chemosensitivity was characterized further. SRB assays confirmed that silencing of Id1 not only slows proliferation of MCF-7 cells but also increases the sensitivity of MCF-7 cells to chemotherapy (Figure 4.10). Control transfected cells begin to die following treatment with epirubicin but this effect is enhanced in cells where Id1 has been silenced. This effect was not unique to epirubicin but was also seen in cells treated with cisplatin and docetaxel, drugs which have a different mechanism of action to the anthracyclines. Inactivation of Id1 seems to increase a cells susceptibility to chemotherapy. Studies have shown similar findings in prostate cancer cells, where inactivation of Id1 leads to decreased cell viability following treatment with paclitaxel (Zhang et al., 2006). How loss of Id1 increases sensitivity to chemotherapy is unclear. Chemotherapeutic agents are likely to target a number of cell signaling pathways, including the PI3K pathway and MAPK. Similarly, Id protein function is likely to be regulated thorough multiple molecular pathways as they have a diverse range of cellular roles. Id proteins function as dominant negative regulators, binding to other proteins and transcription factors,

rendering them transcriptionally inactive. Perhaps loss of Id1 allows these binding partners to become activated and respond to the cellular stress induced by chemotherapy, triggering activation of the apoptotic pathway and increasing cell death. Interestingly, the reciprocal experiment of overexpressing Id1 confers some protection against the cytotoxic effects of epirubicin. When cells are transfected with an Id1 containing retroviral vector, less cell death was observed following epirubicin treatment (Figure 4.13). Resistant and aggressive cell lines express high levels of Id1 and the transient overexpression of Id1 may similarly introduce some drug resistance.

The protective role of Id1 in drug or cytokine induced apoptosis has previously been reported in LNCaP prostate cells – stable expression of Id1 renders resistance to TNF- α induced apoptosis and this is through activation of the NF κ B signaling pathway (Ling et al., 2004). Similarly, in oesophageal cancer cells, overexpression of Id1 protects cells from TNF- α induced apoptosis and is accompanied by an increased expression of phosphorylated Akt, suggesting Id1 may be activating the PI3K (Carpten et al., 2007). However, there was no evidence of this in the transfected MCF-7 cells and overexpression of Id1 did not upregulate levels of phosphorylated Akt.

One of the principle reasons for studying Id1 in this thesis was because it has been identified as a downstream target of FOXO3a in Bcr-Abl transformed K562 cells. The expression of Id1 is abrogated by FOXO3a activation and FOXO3a was identified as a transcriptional repressor of Id1 and binds to the Id1 promoter in leukaemic cell lines. This does not appear to be the case in breast cancer cell lines, and FOXO3a does not

regulate Id1 in MCF-7 cells. This was confirmed by a number of different experiments. Silencing of FOXO3a did not affect Id1 protein or mRNA levels (Figure 4.14) and epirubicin repressed the activity of both the wild-type Id1 promoter and a promoter where a key FOXO3a binding site is mutated. If FOXO3a was truly a transcriptional repressor of Id1, mutating its binding site should relieve the repression on Id1 promoter activity, but this was not observed (Figure 4.15). Also, the downregulation of Id1 protein following epirubicin treatment is independent of FOXO3a activity, as Id1 levels in control and FOXO3a knockout cells follow a similar trend i.e epirubicin targets Id1 with or without endogenous FOXO3a activity (Figure 4.16).

It would therefore appear that Id1 as a target of FOXO3a may be dependent on cell type and malignancy. Solid, epithelial tumours are very different in characteristics and behaviour to the haematological malignancies. One of the characteristics of diseases such as CML is the eventual progression into blast phase, where haematopoietic differentiation arrests and immature blasts accumulate in the bone marrow. Thus, as Inhibitors of differentiation, Id1 proteins are likely to have a key role in the pathogenesis of the disease. Solid tumours behave differently, and can often be well differentiated and Id1 may have more of a role in proliferation. A differing role for Id1, depending on tumour type, may account for different mechanisms of regulation. Much of the published literature regarding Id1 is based on cells of non-epithelial origin. For example, in cardiac myocytes, ectopic expression of Id1 induces apoptosis and Id3 induces apoptosis in fibroblasts – directly contrasting with the function of Id1 seen in epithelial cells (Ling et al., 2003). FOXO activation also can be cell type specific, with programmed cell death induced in haematopoietic cells but

a block in proliferation seen in other cells. MnSOD is not induced by FOXO activation in haematopoietic cells, but can elicit a cell survival signal in other cells (Birkenkamp and Coffey, 2003). Thus, FOXO3a regulation of Id1 may be cell type specific, occurring in leukaemic cells but not in breast cancer cells.

4.4 FUTURE WORK

To further characterize the role and regulation of Id1 it would be useful to try and identify exactly how Id1 interacts with the apoptotic pathway in response to chemotherapy. The binding partners of Id1 in breast cancer cells remain largely unknown and pulldown experiments may be useful to identify other cellular proteins that Id1 binds to.

Generation of stable Id1 knockout or Id1 expressing cell lines may provide useful models for analyzing the role of Id1 in cell cycle progression and the response to chemotherapy. Much of the work in this chapter involved transient transfections and stable cell lines may allow greater insight into the phenotypic changes associated with loss or overexpression of Id1.

The relationship between cyclin D1 and Id1 should be explored further and luciferase assays may confirm whether silencing of Id1 can repress cyclin D1 promoter activity. Similarly, mapping of responsive sites on the Id1 promoter may give more insight into the epirubicin mediated promoter repression observed.

Also, silencing of FOXO1, FOXO3a and FOXO4 together would be of interest – there may be compensatory mechanisms involved

so that silencing of FOXO3a alone is insufficient to repress Id1, but when all FOXO proteins are silenced, there may yet be a role for FOXO negatively regulating Id1.

CHAPTER 5
THE EXPRESSION OF FOXO3a AND
TARGETS IN CLINICAL SAMPLES

5.1 INTRODUCTION

Although MCF-7 cells are widely used as a good laboratory model of ER positive breast cancer, changes seen within cells in culture may not necessarily be a true surrogate for the physiological behaviour of breast cancer in patients. Therefore, translating *in vitro* findings is an integral component of validating and confirming results obtained in cell line work.

In the clinical management of breast cancer, patients who have large tumours or locally advanced disease at presentation are often treated up front with neo-adjuvant chemotherapy, in an attempt to downstage their disease and avoid the need for mastectomy. All patients have a diagnostic pre-treatment core biopsy and then have their definitive surgery after completion of chemotherapy. Thus, a direct comparison can be made between pre-treatment and post-treatment tissue. To this end, it was possible to obtain clinical samples from breast cancer patients treated in Charing Cross Hospital, London. The first cohort of patient samples was from patients who had received neo-adjuvant FEC chemotherapy and had a good clinical response to treatment. Single agent chemotherapy is not indicated in this setting, so unfortunately patients did not receive epirubicin alone, but had also received 5-FU and cyclophosphamide. Nevertheless, in this chapter, the expression of FOXO3a, FOXM1 and Id1 pre and post chemotherapy was investigated. Tissue was also available from a second cohort of patients who had received neo-adjuvant gefitinib, an epidermal growth factor receptor (EGFR) inhibitor. Work in the laboratory had already established FOXO3a as a target of gefitinib in breast cancer cells. FOXO3a dephosphorylation and nuclear localization was shown to have a direct role in mediating gefitinib induced

proliferative arrest (Krol et al., 2007). In this chapter, the effect of gefitinib on FOXO3a was further investigated and, in particular, the role and regulation of FOXM1 in the response to gefitinib treatment was explored.

(N.B Figure 5.7 was the result of a collaboration of work with Dr Janna Krol and Dr Andreas Polychronis).

5.2 RESULTS

5.2.1 *The expression of FOXO3a, FOXM1 and Id1 in normal breast tissue and invasive carcinoma.*

In chapters 3 and 4, the role of FOXO3a and Id1, and the FOXO3a target FOXM1, was investigated in response to epirubicin in MCF-7 cells. To translate these *in vitro* findings, normal and malignant breast tissue was immunohistochemically stained to compare the expression of each between normal tissue and invasive ductal carcinoma (Figure 5.1). FOXO3a was expressed in both types of breast tissue, but FOXM1 and Id1 expression was higher in invasive carcinoma when compared to normal breast tissue. This was consistent with the findings from the breast cancer cell lines, where FOXM1 and Id1 expression was much higher in the cancer cell lines when compared to the untransformed MCF-10A cell line, and confirmed expression of FOXM1 and Id1 as markers of malignancy.

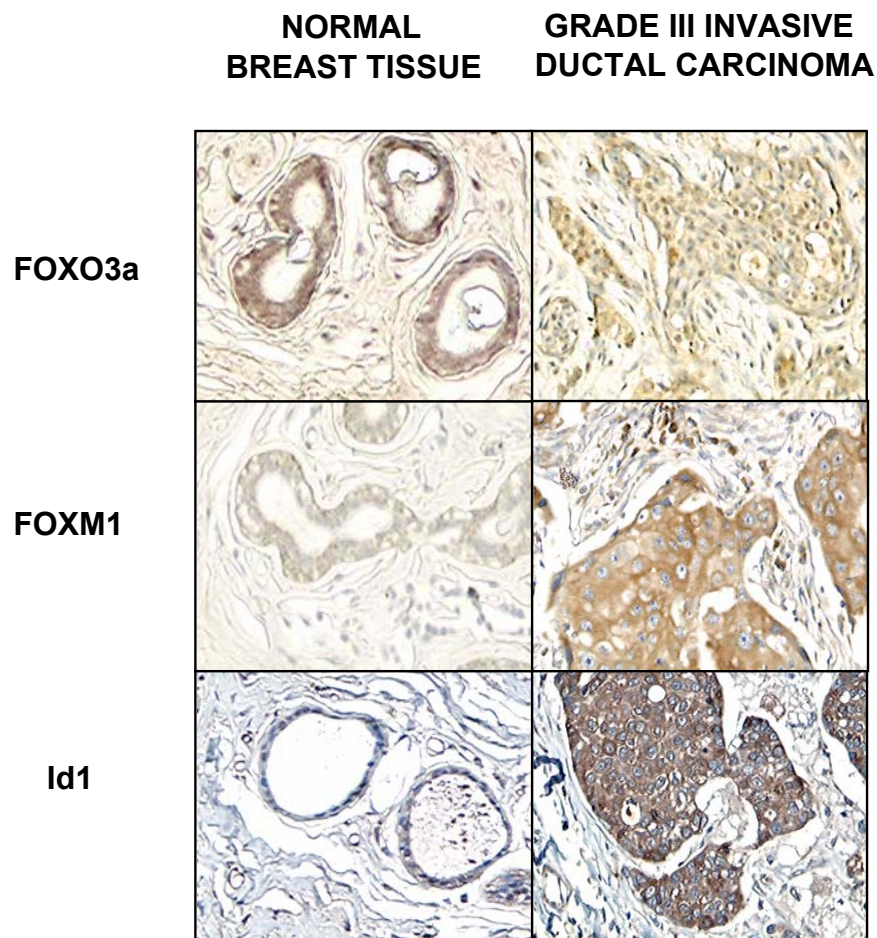


Figure 5.1 The expression of FOXO3a, FOXM1 and Id1 in normal and malignant breast tissue. Normal breast tissue and tumour samples were obtained which had been formalin fixed and paraffin embedded. Tissue was immunohistochemically stained with FOXO3a, FOXM1 and Id1 antibodies using the streptavidin-biotin-peroxidase technique. Examples shown are representative of staining patterns observed (Magnification x200).

5.2.2 *The expression of FOXO3a, FOXM1 and Id1 in different histological subtypes of breast cancer.*

Invasive ductal carcinoma is the most common histological subtype of breast cancer, followed by invasive lobular carcinoma (Cancer Research UK, 2006). Cancers can be further divided into subclasses depending on the expression of ER and HER2. Cancers belonging to different subgroups behave differently clinically. The expression of ER in breast cancer is an independent prognostic factor (Burcombe et al., 2004). Studies have shown that the likelihood of achieving a pathological complete response (pCR) with neo-adjuvant chemotherapy is significantly higher in ER negative tumours (Tewari et al., 2008). There is emerging evidence that HER2 expression can also be used as a predictor of response to chemotherapy and that HER2 positive tumours will benefit particularly from an anthracycline containing regimen (Pritchard et al., 2008). The expression of HER2 is an adverse prognostic factor, with HER2 positive breast cancers behaving more aggressively, with a higher rate of recurrence and shorter disease-free interval than HER2 negative tumours (Gonzalez-Angulo et al., 2006).

Therefore, it was of interest to look at the expression of FOXO3a, FOXM1 and Id1 in clinical samples and compare the expression of each between the different histological sub-types. Staining was positive in all subtypes and there was no discernable difference in expression between ductal and lobular cancers (Figure 5.2), ER positive and ER negative cancers (Figure 5.3) and HER2 positive and HER2 negative cancers (Figure 5.4). Thus, FOXM1 and Id1 are only weakly expressed in normal breast tissue, but are highly expressed in invasive cancers, irrespective of histological subtype. FOXO3a is expressed in both normal and malignant breast tissue.

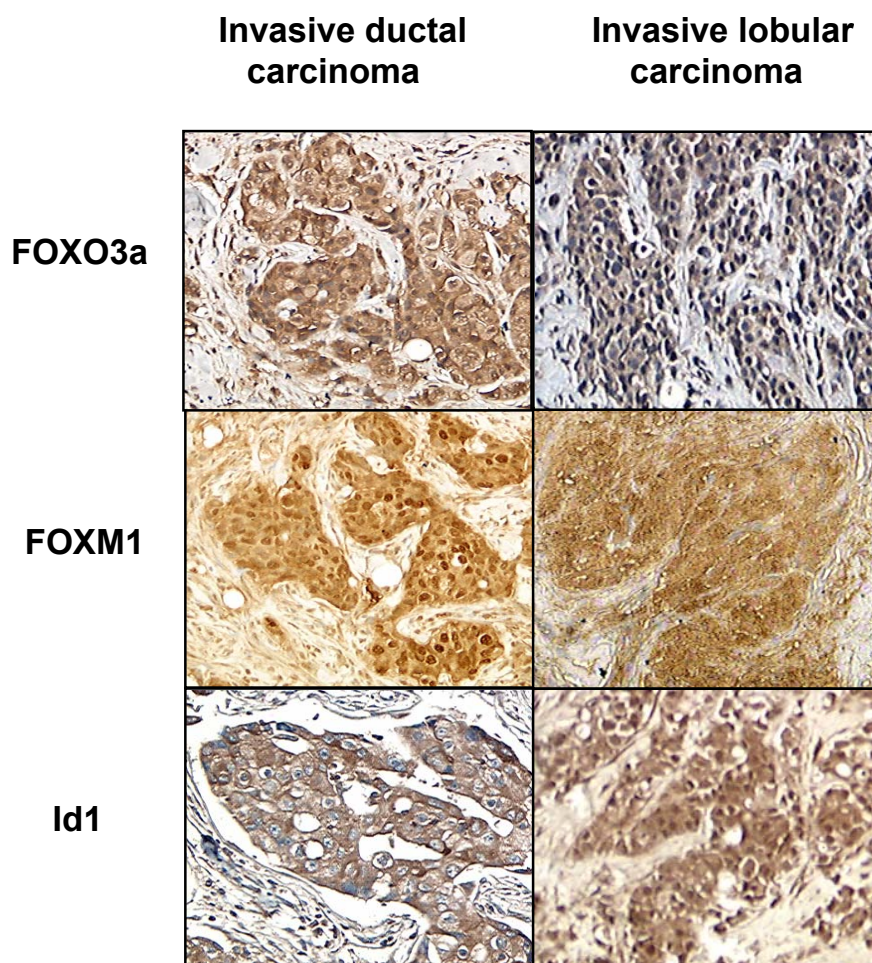


Figure 5.2 The expression of FOXO3a, FOXM1 and Id1 in invasive ductal and lobular carcinomas. Samples from patients with ductal and lobular carcinomas were obtained which had been formalin fixed and paraffin embedded. Tissue was immunohistochemically stained with FOXO3a, FOXM1 and Id1 antibodies using the streptavidin-biotin-peroxidase technique. Examples shown are representative of staining patterns observed (Magnification x200).

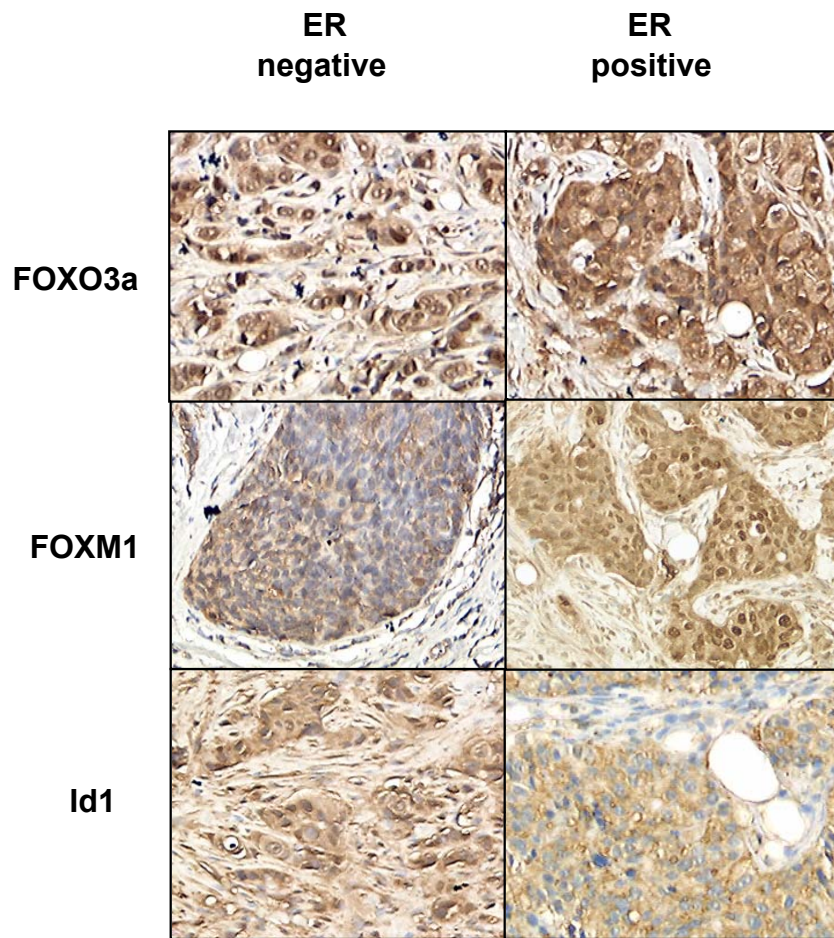


Figure 5.3 the expression of FOXO3a, FOXM1 and Id1 in ER positive and ER negative invasive ductal carcinoma. Samples from patients with ER positive and ER negative ductal carcinomas were obtained which had been formalin fixed and paraffin embedded. Tissue was immunohistochemically stained with FOXO3a, FOXM1 and Id1 antibodies using the streptavidin-biotin-peroxidase technique. Examples shown are representative of staining patterns observed (Magnification x200).

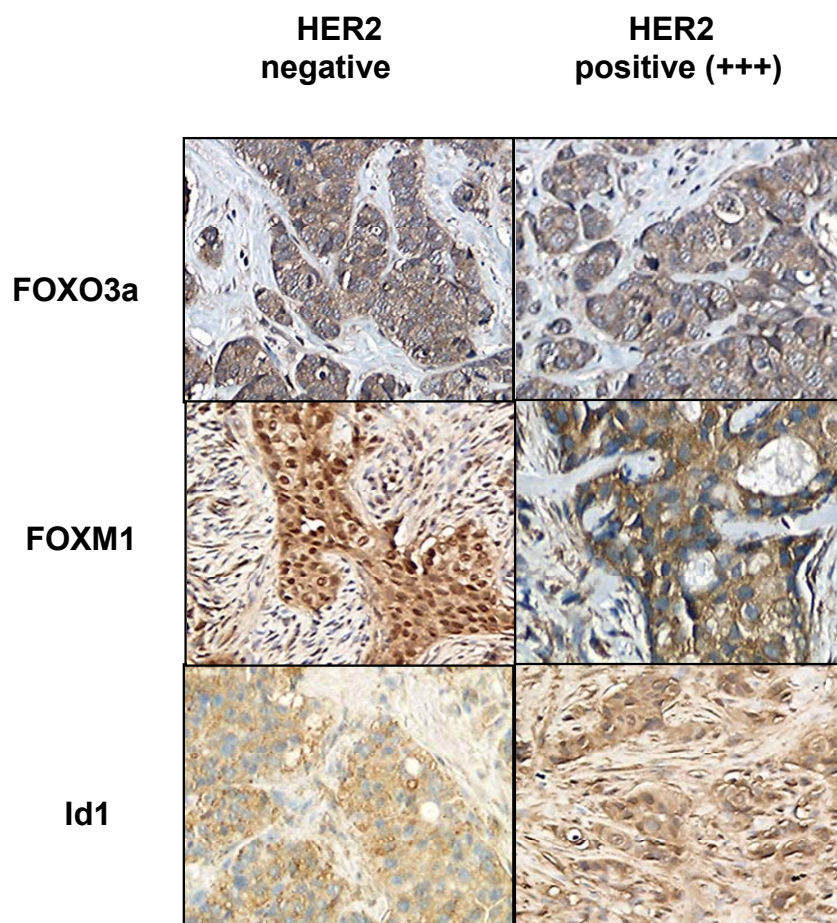


Figure 5.4 The expression of FOXO3a, FOXM1 and Id1 in HER2 positive and HER2 negative breast cancers. Samples from patients with HER2 positive and HER2 negative ductal carcinomas were obtained which had been formalin fixed and paraffin embedded. Tissue was immunohistochemically stained with FOXO3a, FOXM1 and Id1 antibodies using the streptavidin-biotin-peroxidase technique. Examples shown are representative of staining patterns observed (Magnification x200).

5.2.3 The effects of FEC chemotherapy in clinical samples.

To visualize the changes in expression of FOXO3a, FOXM1 and Id1 induced by chemotherapy, samples were analysed before and after neo-adjuvant chemotherapy with FEC (Figure 5.5). Patients received 6 cycles of treatment over a period of 18 weeks. Core biopsies pre-treatment and the corresponding post chemotherapy residual tumour were stained and expression levels compared. FOXO3a expression was found in both pre and post treatment specimens. Interestingly, both Id1 and FOXM1 expression was visibly reduced following chemotherapy, with very little Id1 expression in particular observed following treatment. These findings were consistent with the results obtained in chapters 3 and 4, confirming that chemotherapy downregulates the expression of FOXM1 and Id1 *in vitro* and *in vivo*. FOXO3a was expressed in both the pre and post FEC treatment samples. Post FEC treatment, there was an observed increase in nuclear staining of FOXO3a, and although this was not formally quantified due to small patient numbers, this would be consistent with activation of FOXO3a and its nuclear localization following drug treatment.

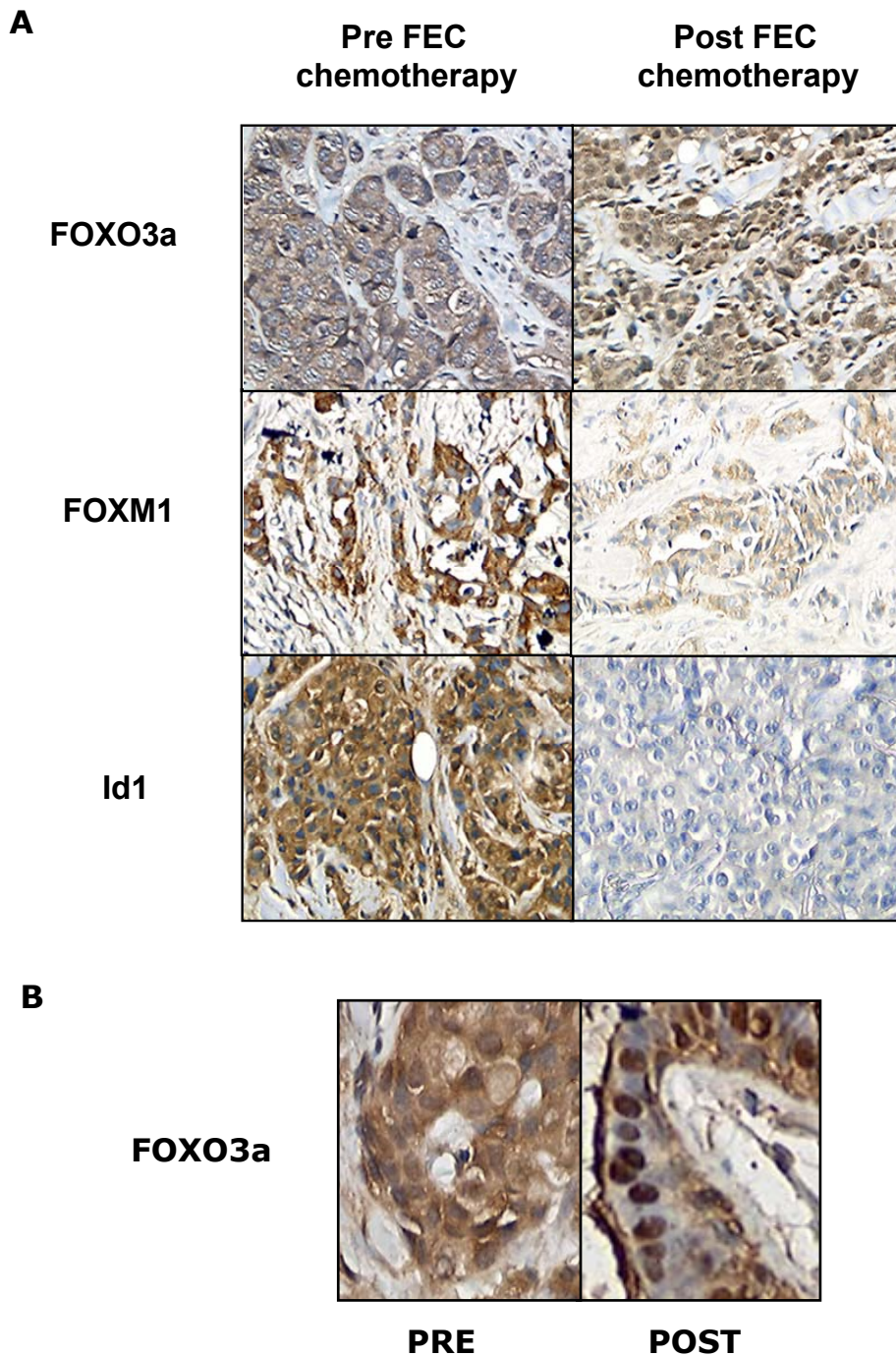


Figure 5.5 A) Pre and post FEC chemotherapy staining. Pre treatment core biopsies and post FEC chemotherapy tissue samples were obtained which had been formalin fixed and paraffin embedded. Patients had received 6 cycles of FEC chemotherapy. Tissue was immunohistochemically stained with FOXO3a, FOXM1 and Id1 antibodies using the streptavidin-biotin-peroxidase technique, pre and post treatment. Examples shown are representative of staining patterns observed (Magnification x200). **B)** Higher magnification FOXO3a to highlight increased nuclear staining post FEC.

5.2.4 *A dose titration of gefitinib induces FOXO3a activity and represses FOXM1.*

The work in chapter 3 of this thesis has shown FOXO3a to be a target of epirubicin. Previous work in this laboratory had also shown that FOXO3a was a critical signaling effector of the EGFR inhibitor, gefitinib (Krol et al., 2007). Thus, signaling via FOXO3a is a key mechanism by which both epirubicin and gefitinib bring about their cellular effects. Analysis of clinical samples in this chapter has so far only been from patients who received neo-adjuvant FEC chemotherapy. However, samples were also available from patients who had received neo-adjuvant gefitinib treatment. Again, this allows a direct comparison between expression of FOXO3a and FOXM1 pre and post treatment. Thus, the following work was performed to contribute to an ongoing project in the laboratory.

To firstly confirm that gefitinib functions via FOXO3a and FOXM1, a dose titration of gefitinib was performed in SKBR3 cells. The effects of gefitinib on FOXO3a, FOXM1 and their downstream targets were comparable to those observed with epirubicin (Figure 5.6). Cells were treated with 0, 0.1, 0.5, 1, 5 and 10 μ M of gefitinib and the results showed a dose dependent induction of FOXO3a activity as shown by a reduction in FOXO3a phosphorylation but not protein level and confirmed by the induction of the FOXO3a targets Bim and p27^{kip1}. This was associated with a dose-dependent decrease in FOXM1 expression and its downstream targets PLK and cyclin B.

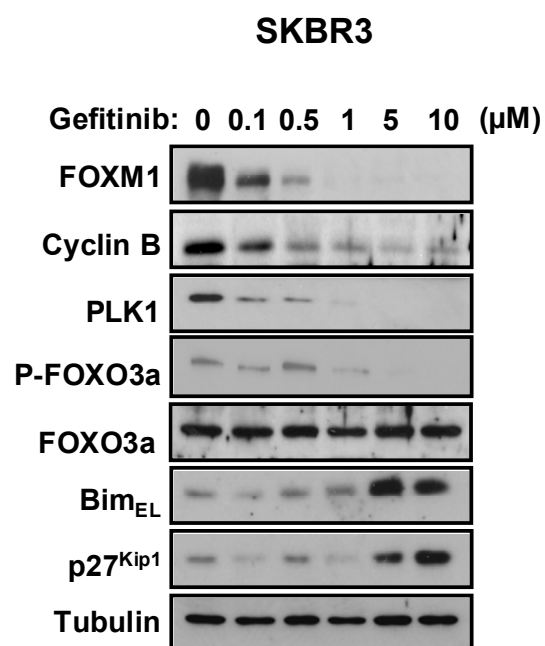


Figure 5.6 Dose titration of gefitinib in SKBR3 cells. SKBR3 cells were treated with 0, 0.1, 0.5, 1, 5 and 10 μM gefitinib and protein lysates prepared after 24 h of drug treatment. Protein expression levels were analysed by western blotting using specific antibodies.

5.2.5 Gefitinib induces FOXO3a nuclear accumulation and FOXM1 downregulation in breast cancer patient samples

Having demonstrated that the use of neo-adjuvant FEC chemotherapy can down-regulate FOXM1 expression in breast cancer samples, immunohistochemical staining was also used to analyse the expression of FOXO3a and FOXM1 in breast cancer patient samples obtained before and after 4 weeks of gefitinib treatment, from a neoadjuvant study in patients with early breast cancer (Polychronis et al., 2005). Samples were also stained for Ki67, a marker for cell proliferation. Staining was performed on 11 cases in patients whose tumour size reduced more than 1 cm after gefitinib treatment as measured by ultrasonography. Tumour tissue sections from 11 patients that had no treatment between biopsy and surgical tumour excision were used as controls. After estimating staining intensity using H-scores for nuclear FOXO3a, FOXM1 and Ki67 expression (Table 5.1, as described in materials and methods) FOXM1 expression was significantly downregulated in response to gefitinib, concomitant with a reduction in Ki67 staining and an increase in the level of nuclear FOXO3a (Figure 5.7). T-test analysis also indicated that a significant difference in FOXM1 staining between the treated group before and after gefitinib ($p \leq 0.05$) and between treated and control groups after treatment. The relationship between fold changes in FOXM1 and Ki67 or nuclear FOXO3a was statistically analysed using Pearson's correlation test based upon their H-scores, and was considered significant at $p \leq 0.05$ (t-test). The results indicated a significant and strong positive correlation between FOXM1 and Ki67 expression ($n=22$; Pearson coefficient=0.697; $p \leq 0.05$) and a significant inverse correlation between FOXM1 and nuclear FOXO3a expression ($n=20$; Pearson coefficient=-0.669;

$p \leq 0.05$) in response to treatment (i.e. gefitinib or control) (Figure 5.8). These results confirm that FOXM1 and cell proliferation are repressed by gefitinib, and this is accompanied by activation of FOXO3a, a mechanism of action similar to that previously demonstrated in response to epirubicin.

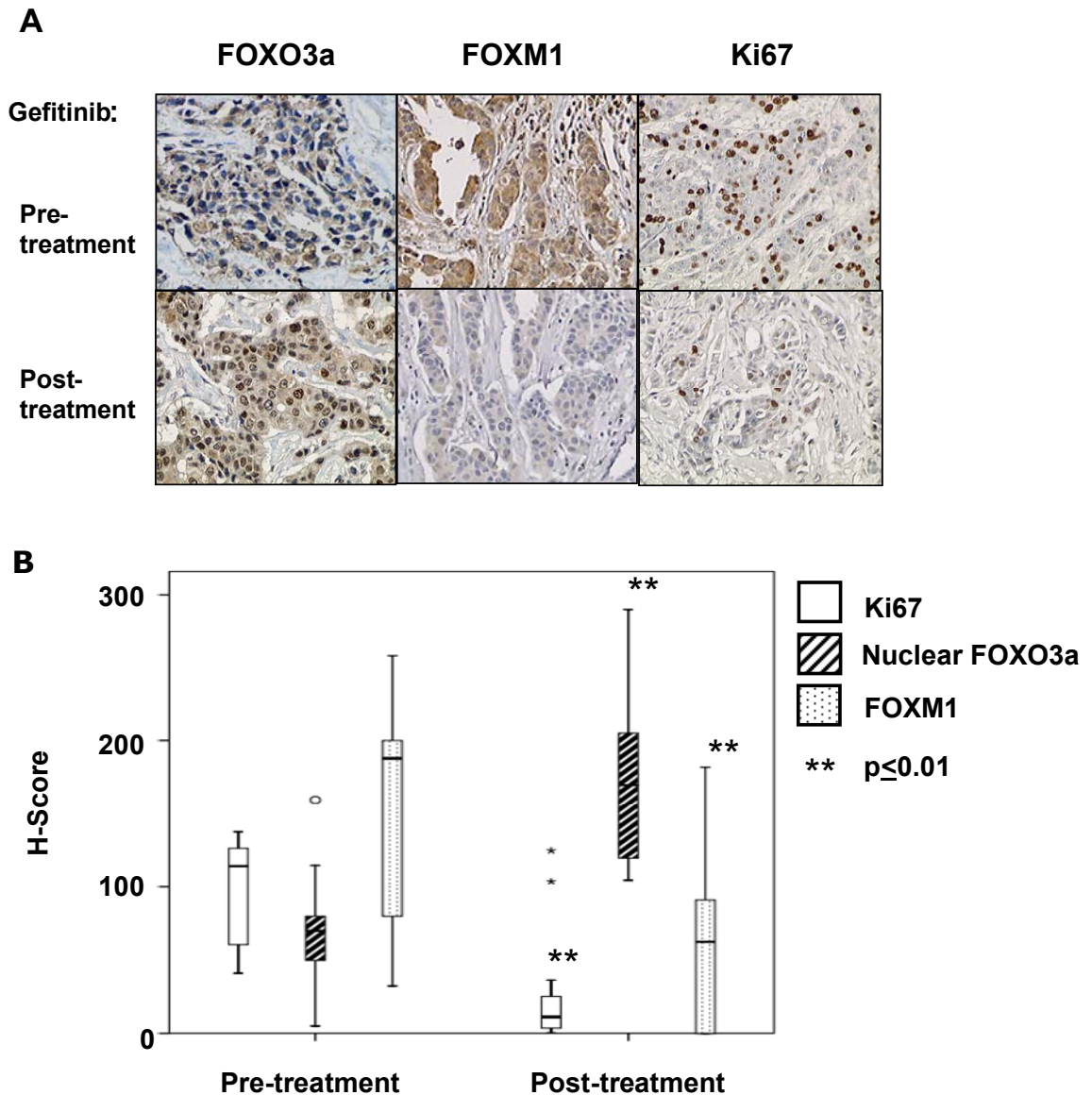


Figure 5.7 Gefitinib treatment correlates with upregulation of nuclear FOXO3a and downregulation of Ki67 and FOXM1 expression in breast cancer patient samples. **A.** Tumour tissue samples obtained from breast cancer patients, formalin fixed and paraffin-embedded were immunohistochemically stained with Ki67, FOXO3a and FOXM1 antibodies using the streptavidin-biotin-peroxidase technique. Control patients received no treatment between primary biopsy and subsequent tumour resection. Examples of staining of samples from a gefitinib treated patient are shown (X200 magnification). **B.** The relationships between Ki67, nuclear FOXO3a or FOXM1 expression before and after gefitinib treatment were analysed using Box-and-Whisker plots illustrating the median values, first and third quartile, and smallest and largest values of Ki67, nuclear FOXO3a and FOXM1 before and after gefitinib treatment.

<i>Gefitinib group patient samples</i>						
	Ki67 H-score		NucFOXO3a H-score		FOXM1 H-score	
	pre	post	pre	post	pre	post
1	57	4	10	115	87	0
2	133	13	5	105	55	50
3	121	3	80	130	26	0
4	71	3	80	170	200	0
5	41	0	40	110	15	0
6	138	104	60	235	188	91
7	115	10	115	185	204	80
8	117	11	70	125	32	25
9	50	36	160	200	258	182
10	136	125	70	210	80	75
11	64	14	80	290	190	160

<i>Control group patient samples</i>						
	Ki67 H-score		NucFOXO3a H-score		FOXM1 H-score	
	pre	post	pre	post	pre	post
1	57	48	40	15	35	75
2	87	108	135	130	53	135
3	84	105	95	130	130	158
4	63	72	70	0	21	53
5	114	129	30	50	289	341
6	39	27	40	10	32	64
7	153	192	130	145	259	300
8	138	153	100	155	228	216
9	45	54	60	45	77	75
10	57	69	85	20	61	103
11	207	216	80	70	179	289

Table 5.1 Immunohistochemical staining results with H-scores on Ki67, nuclear FOXO3a and FOXM1. Intensity of Ki67, FOXM1 and FOXO3a was evaluated using 0 to 3 scoring system. Results were quantified by calculating H-scores that consider both the staining intensity and the percentage cells stained at a specific range of intensities.

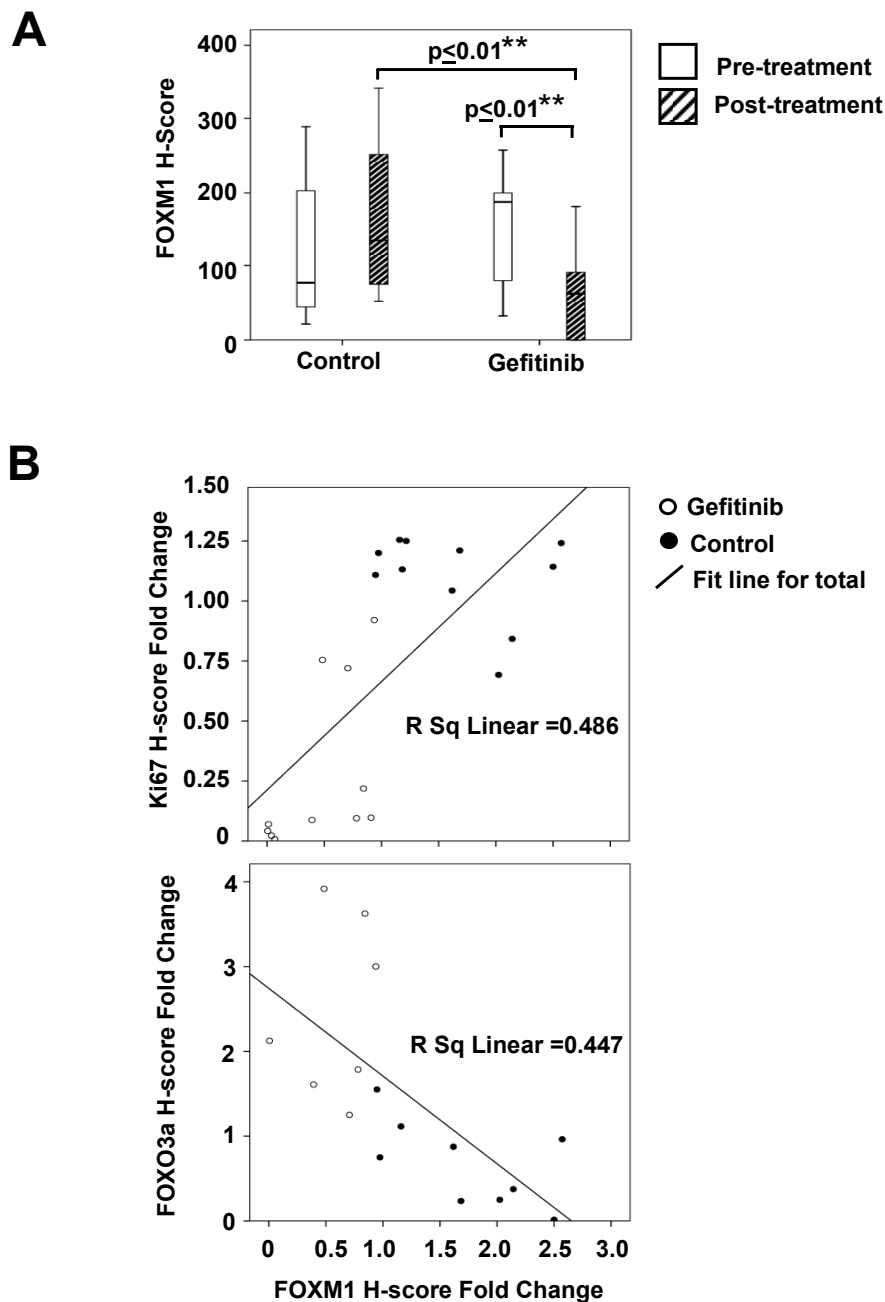


Figure 5.8 Analysis of the relationship between Ki67, nuclear FOXO3a, and FOXM1 expression in breast cancer patient samples. A. To test for differences between FOXM1 expression before and after treatment in gefitinib treated and control groups, Dunnett's t-test was performed, and the mean difference considered significant at the $p \leq 0.05$ level. **B.** The relationship between the fold of change in FOXM1 status and Ki67 or nuclear FOXO3a expression was statistically analysed using Pearson's correlation test based upon the H-scores, and was considered significant at $p \leq 0.05$.

5.3 DISCUSSION

The use of neo-adjuvant chemotherapy in breast cancer has a number of advantages, allowing down-staging of tumours and reducing the need for mastectomy. This also enables the monitoring of treatment efficacy and allows the possibility of identifying markers of response. In this chapter, the expression of FOXO3a, FOXM1 and Id1 was characterised in invasive cancers and the changes induced by neo-adjuvant treatment with FEC chemotherapy and gefitinib treatment was investigated.

Immunohistochemical staining confirmed that FOXO3a, FOXM1 and Id1 are all expressed in invasive ductal carcinomas of the breast. FOXO3a is also expressed in normal breast tissue, however, FOXM1 and Id1 expression is much higher in invasive tumour than in normal breast tissue (Figure 5.1). This is consistent with the known role of both FOXM1 and Id1 in proliferation and the development and progression of cancer. FOXM1 is highly expressed in proliferating cells and although found in all embryonic tissues, it is not normally expressed at high levels in adult tissue. However, overamplification of the *FOXM1* gene locus on chromosome 12 does occur in breast cancer, which could promote overexpression of FOXM1 leading to tumourigenesis by driving the cell cycle and proliferation, and promoting undifferentiated cells towards malignant transformation (Costa, 2005; Spirin et al., 1996). Similarly, Id1 expression has been shown to be high in invasive, high grade tumours, correlating with more aggressive behaviour and worse outcome in patients (Schindl et al., 2001; Schindl et al., 2003). In colorectal cancers for example, Id1 expression is elevated in tumours when compared to normal bowel mucosa (Wilson et al.,

2001). These results suggest a similar expression pattern for both FOXM1 and Id1, with expression being elevated in tumours when compared to normal breast tissue. Neither histological sub-type, ER or HER2 appears to influence expression of FOXO3a, FOXM1 or Id1 – levels are high in invasive cancers, whether ductal or lobular and regardless of ER or HER2 status, suggesting that FOXO3a, FOXM1 and Id1 expression are not specific to any particular subtype of breast cancer but are expressed as a marker of malignancy (Figures 5.2, 5.3 and 5.4).

Some care does need to be taken when interpreting these results. Despite optimising staining techniques, the Id1 antibody in particular did show a high degree of non-specific staining and could be considered a “dirty” antibody in terms of giving high background staining. The use of positive and negative controls, for example tissue containing high levels of Id1 such as cerebral cortex, could be used to corroborate immunohistochemical findings.

In response to FEC chemotherapy, FOXM1 and Id1 expression is downregulated (Figure 5.5). The changes in FOXO3a expression following chemotherapy are not so easy to characterise as changes in subcellular localisation as opposed to expression levels *per se* are more important in determining FOXO3a activity and function. However, neo-adjuvant chemotherapy inflicts significant changes on the histological appearances of tumour cells, disrupting the normal architecture of the epithelial cells including distortion of glandular structure, enlarged cells due to an increased cytoplasm, pleomorphic and bizarre nuclei and decreased mitotic activity (Sahoo and Lester, 2003). Thus, a direct comparison between pre and post chemotherapy FOXO3a

expression is difficult to interpret. However, in the gefitinib treated patients, the cellular architectural changes following treatment are much less dramatic - patients received only 4 weeks of drug as opposed to 18 weeks of chemotherapy. This allowed a formal staining score to be applied and it was possible to estimate staining intensity using H-scores, enabling quantification of the changes in expression of FOXO3a and FOXM1. FOXM1 was significantly downregulated following gefitinib treatment, concomitant with an increase in the level of nuclear FOXO3a. The downregulation of FOXM1 expression was associated with a decrease in cell proliferation as revealed by a reduction in the level of Ki-67, a marker for cell proliferation (Figure 5.7).

EGFR inhibitors are a relatively new class of drug and gefitinib is a selective EGFR tyrosine kinase inhibitor, which acts through competitive inhibition of ATP binding to the catalytic domain of EGFR tyrosine kinase (Campos, 2008). Recent phase II trials have shown that gefitinib has antitumour activity in breast cancer when used as a monotherapy or in combination with other drugs, such as docetaxel and anastrozole (Polychronis et al., 2005). Identifying the downstream targets of gefitinib, such as FOXM1 and FOXO3a, may allow the selection of patients more likely to benefit from anti-EGFR inhibitors. If tumours expressing high levels of FOXO3a or FOXM1 respond well to EGFR inhibition, drugs such as gefitinib could be used concurrently with standard chemotherapy regimens to increase efficacy or used on their own to avoid chemotherapy related toxicity.

5.4 FUTURE WORK

There is much scope for a more in depth analysis of patient samples, to try and identify prognostic and predictive factors in patients treated with neo-adjuvant chemotherapy. For the work in this chapter, it was particularly difficult to obtain matched patient samples (i.e pre and post FEC chemotherapy) which limited the amount of quantitative analysis that could be undertaken. With a larger number of complete patient samples, there would be scope for a much more detailed analysis. Formal H-score assessment and pathological evaluation would give a more quantitative assessment of response to chemotherapy. Furthermore, retrospective and prospective analysis of clinical data may provide some predictive data, for example, correlation between changes in FOXO3a, FOXM1 and Id1 expression and time to disease progression. Significant changes in FOXM1 and Id1 after treatment may equate to longer disease free survival and overall survival, and provide a useful prognostic tool for the future.

CHAPTER 6

FINAL DISCUSSION

6.1 Overview

Early stage breast cancer is increasingly accepted as a systemic disease and as such, the use of adjuvant treatment, including endocrine treatment and chemotherapy, has increased significantly over the last twenty years. Epirubicin remains one of the most widely used chemotherapeutic agents in the management of breast cancer. Epirubicin, administered usually in combination with 5-Fluorouracil and methotrexate (FEC), has a well established and proven role in improving outcome and survival in breast cancer. Though known to inhibit topoisomerase II and introducing irreversible DNA damage, the exact cellular mechanisms via which epirubicin exerts its cytotoxic effects are not yet fully understood.

One of the main aims of this thesis was to identify cellular targets of epirubicin and to characterize how these targets are regulated and play a role in cell death. The work explored the hypothesis that FOXO3a was a potential target of epirubicin and that FOXM1 and Id1 may also be targets of epirubicin and could be regulated by FOXO3a.

In chapter 3, epirubicin was shown to induce a dose dependent G₂/M arrest in MCF-7 cells and this was accompanied by a decrease in levels of phosphorylated FOXO3a protein. Together with the upregulation of the FOXO3a target p27^{Kip1}, this suggested that FOXO3a was indeed a target of epirubicin. This was confirmed by time course experiments, where 48h of treatment with epirubicin in MCF-7 cells suppressed the activity of the PI3K pathway, downregulated phospho-FOXO3a and upregulated the FOXO3a targets p27^{Kip1}, Bim and GADD45a. FOXO3a mRNA levels also increased following epirubicin

treatment and promoter assays confirmed epirubicin was activating FOXO3a promoter activity and identified FOXO3a as a cellular target of epirubicin.

Experiments investigating the subcellular localization of FOXO3a gave further insight into the mechanism via which epirubicin targets FOXO3a. After 24h of drug treatment, nuclear levels of FOXO3a rise, whereas cytoplasmic FOXO3a and phospho-FOXO3a levels fall. Fluorescent microscopy confirmed this, with cytoplasmic and nuclear FOXO3a visualised before treatment, but only nuclear FOXO3a visualized after 24h of treatment. This suggests that epirubicin induces shuttling of FOXO3a from the cytoplasm to the nucleus, where it can be transcriptionally active.

Having established FOXO3a as a target of epirubicin, FOXM1 was next investigated as an epirubicin target and also the hypothesis that FOXO3a may regulate FOXM1 was tested. Epirubicin downregulated FOXM1 at the protein, mRNA and gene promoter level, inversely correlating with the changes seen in FOXO3a. The FOXM1 promoter repression induced by epirubicin appeared to be dependent on FOXO, as mutation of the FOXO3a binding sites within the FOXM1 promoter relieved the repression induced by epirubicin. Further evidence for the regulation of FOXM1 by FOXO3a was shown when FOXO3a was silenced – epirubicin treatment downregulated FOXO3a in control cells, but in the FOXO3a knockdown cells, no such repression of FOXM1 was seen. This suggests that FOXO3a is required for the repression of FOXM1 following epirubicin treatment. Failure of FOXO3a to repress FOXM1 also provides cells with a survival advantage and knockdown of FOXO3a renders cells more resistant to epirubicin.

Overexpression of FOXM1 also contributes to a drug resistant phenotype – when MCF-7 cells were transfected with a constitutively active truncated form of FOXM1, Δ N-FOXM1, the cytotoxic effects of epirubicin were abrogated, implicating FOXM1 as an important mediator of drug resistance. Interestingly, the two cell lines with the highest relative expression of FOXM1 – ZR751 and MDA-MB-231 cells – were the least sensitive to epirubicin, providing further evidence for a role of FOXM1 in chemotherapy resistance.

In chapter 4, epirubicin was shown to rapidly down-regulate Id1 protein, mRNA and promoter activity. Id1 expression was found to be high in chemotherapy resistant cell lines and silencing of Id1 by siRNA slowed proliferation of cells and increased the sensitivity of MCF-7 cells to epirubicin, docetaxel and cisplatin. Unlike haematopoietic cells, FOXO3a does not appear to regulate Id1 in breast cancer and when FOXO3a is silenced, Id1 is still downregulated as an early event in response to epirubicin. This would suggest that the role of Id1 pertains more to proliferation of cells and cell cycle progression as opposed to a direct role in the apoptotic pathway. Loss of Id1 leads to downregulation of cyclin D1, a key mediator of cell cycle entry, and may “prime” cells into being more susceptible to the effects of chemotherapy. It is likely that other, as yet unidentified, proteins may also interact with or bind to Id1 and there may be cross-talk with other signaling pathways, so that once Id1 is silenced, the effects of chemotherapy are potentiated.

The tissue staining performed in Chapter 5 corroborated the findings in the cancer cell lines. FOXO3a, FOXM1 and Id1 were all highly expressed in breast cancer samples. No obvious difference was seen in expression when comparing ER positive

and ER negative tumours, HER2 positive and HER2 negative tumours and between invasive lobular and ductal carcinomas. However, with regards to Id1 and FOXM1 expression, normal breast tissue had much lower expression of both when compared to invasive ductal carcinomas. There was also a marked reduction in Id1 and FOXM1 expression following neo-adjuvant treatment with FEC chemotherapy, confirming the downregulation observed in MCF-7 cells following drug treatment. It is not possible to study the effects of epirubicin alone in clinical samples as single agent epirubicin is not a standard treatment in the neo-adjuvant setting. However, assessing the effects of gefitinib as a single agent treatment in the neo-adjuvant setting was possible. As had been shown with epirubicin, gefitinib also targeted FOXO3a and a titrating dose of gefitinib in the sensitive SKBR3 cell line downregulated phospho-FOXO3a, whilst upregulating the FOXO3a targets Bim and p27^{kip1} as well as downregulating FOXM1 and its downstream targets cyclin B and PLK1, suggesting epirubicin and gefitinib share a similar FOXO3a dependent mechanism of inducing cell death. FOXM1 expression is downregulated following gefitinib treatment in patient samples and this is accompanied by nuclear accumulation of FOXO3a. Similarly, in cell lines, silencing of FOXO3a upregulates FOXM1 and its targets and rescued cells from gefitinib induced cell proliferative arrest.

6.2 Clinical relevance

Identifying the cellular mechanisms via which chemotherapeutics achieve their toxic effects may allow better selection of both patients and drug combinations in the treatment of cancer. One of the most significant problems with

chemotherapy encountered in the clinical setting is chemoresistance. Some patients fail to respond up-front to chemotherapy – inherent or *de novo* drug resistance, whilst many others eventually develop resistance after multiple lines of treatment. To be able to identify patients who are more likely to respond to specific drugs and to be able to predict outcome would be of huge benefit in the clinical setting. Just as the expression of ER and HER2 are used to guide treatment choices in breast cancer, the expression of FOXO3a, FOXM1 and Id1 may be useful in the future as predictors of response to particular drugs. If the anthracyclines function via FOXO3a, perhaps patients with tumours which express little or no FOXO3a may benefit from alternative non-anthracycline containing regimens such as CMF or the taxanes. Similarly, if high expression of FOXM1 confers resistance to treatment, targeting FOXM1 specifically may be useful in improving the chances of response. As mentioned previously thiostrepton, a thiopeptide antibiotic, has been shown to selectively target FOXM1 in breast cancer cells, and this could be given in combination with standard chemotherapy to improve outcome (Kwok et al., 2008). This approach has been adopted successfully with other tumour types, where targeted treatments are given in combination with well established chemotherapy regimens and good outcomes have been observed. The anti-EGFR monoclonal antibody cetuximab and bevacizumab, an anti-VEGF monoclonal antibody, have both been used to improve outcome in colorectal cancer when given in combination with oxaliplatin, irinotecan and 5-FU containing chemotherapy regimens (Cassidy et al., 2008; Saltz et al., 2007). The use of poly ADP ribose polymerase (PARP) inhibitors, currently in clinical trials, exploits a similar therapeutic strategy, where inhibition of the base excision repair pathway by PARP

inhibitors leads to increased drug sensitivity in patients with BRCA mutations (Tuma, 2009). In the future this may be a further mechanism for sensitizing patients to the effects of chemotherapy.

Also of clinical relevance is the potential role of FOXO3a, FOXM1 and Id1 as markers of response to chemotherapy. FOXM1 and Id1 expression was downregulated by FEC chemotherapy, in samples from patients who had a good clinical response to neo-adjuvant treatment. Similarly, cellular FOXO3a localization differed pre and post gefitinib treatment, with post treatment nuclear accumulation of FOXO3a occurring. It is currently difficult to predict how a patient will respond to chemotherapy. Studies looking at expression of Bcl-2, ER, p53, HER2 and Ki67 pre and post neo-adjuvant chemotherapy have failed to identify any good predictors of response – all markers, except Ki67, were similar before and after chemotherapy (Faneyte et al., 2003). Thus, FOXO3a, FOXM1 and Id1 expression could provide new biomarkers in assessing outcome and predicting response to chemotherapy.

6.3 *Id1 as a therapeutic target*

The work in Chapter 4 identified Id1 also as a target of epirubicin, something never previously characterized in the literature. Treatment of MCF-7 cells lead to a rapid down-regulation of Id1 protein and mRNA and there was a direct effect at the gene promoter level, with epirubicin repressing Id1 promoter activity. The expression of Id1 was highest in more aggressive cell lines, including MDA-MB-231 cells and the anthracycline resistant Dox-R MCF-7 cells. Interestingly,

silencing of Id1 had a negative effect on proliferation but also, perhaps more importantly, could increase the sensitivity of MCF-7 cells to a variety of cytotoxic agents.

From a clinical viewpoint, the increased sensitivity of cells to chemotherapy following silencing of Id1 could be very interesting. Over the last two decades, a number of new drugs have been developed which have specific molecular targets, such as PI3K and mTOR (Markman et al., 2009). By particularly targeting key events in signaling pathways, these drugs may prove to have significant anti-cancer properties, whilst avoiding much of the toxicity associated with standard chemotherapy. Targeted agents may also improve the efficacy of chemotherapy, for example, phase I clinical trials are now ongoing to study the effects of PI3K inhibitors, which have shown activity in breast, lung and cancer models and enhanced anti-tumour activity when combined with chemotherapy with paclitaxel and carboplatin (Papadopoulos et al., 2008). If Id1 expression can be inhibited by siRNA to reduce proliferation and increase the sensitivity of cells to cytotoxics, this presents a novel therapeutic strategy for cancer treatment. Drugs specifically targeting Id1 may have multiple effects on tumour progression as Id1 plays a key role in growth, invasion, metastases and angiogenesis (Carpten et al., 2007). One study has shown this to be a potential viable therapeutic strategy, where targeting Id1 expression with antisense Id1 cDNA reduced the metastatic spread of 4T1 breast cancer cells in mice (Fong et al., 2003). Also, as shown in this work, Id1 expression is highest in aggressive cell lines and high grade breast cancer tumours, when compared to non-transformed cells or normal breast tissue. This could therefore be an advantage for systemic therapy, as non-tumour tissue, expressing only low levels of

Id1, may show little effect to inhibition of Id1, thus sparing the normal tissue from any drug related toxicity.

6.4 *Future perspectives*

The work presented in this thesis has demonstrated the important function of FOXO transcription factors and Id1 in response to chemotherapy in breast cancer (Figure 7.1). FOXO3a has a role in mediating the effects of epirubicin in breast cancer cells and transcriptional activation of FOXO3a following drug treatment is a key event in the cell cycle arrest and cell death induced by epirubicin. The regulation of FOXM1, another cellular mediator of epirubicin, by FOXO3a is a key step in defining drug sensitivity and loss of FOXO3a or overexpression of FOXM1 contributes to a more drug resistant phenotype in breast cancer cells.

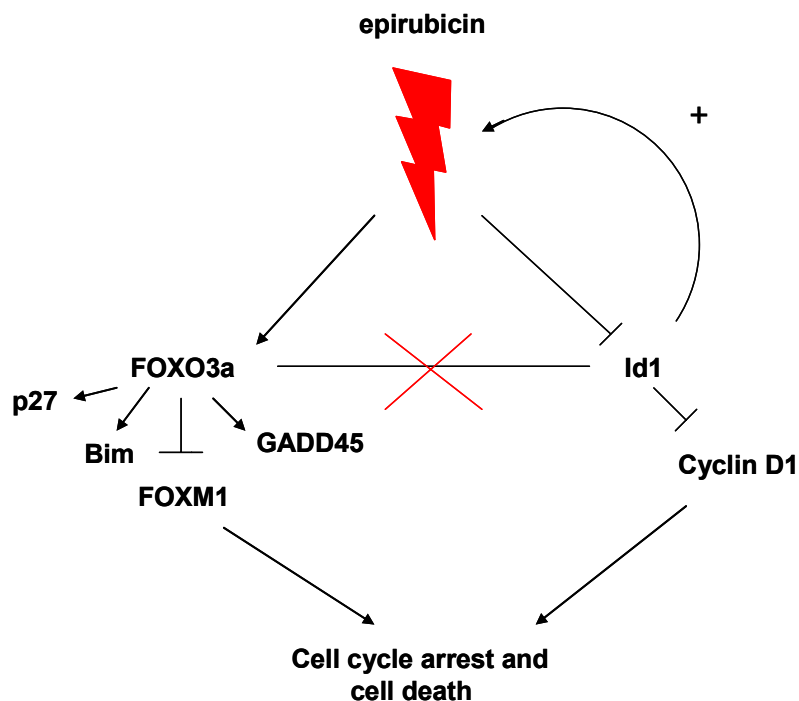


Figure 6.1 Schematic representing the role of FOXO3a and Id1 as targets of chemotherapy.

This is likely to be a mechanism shared with other therapeutic drugs as treatment of gefitinib sensitive breast cancer cells also activates FOXO3a, whilst repressing FOXM1, leading to proliferative arrest and cell death. Although Id1 does not appear to be regulated by FOXO3a in breast cancer, this work has shown it also to be an important target of epirubicin and Id1 plays a role in sensitivity to chemotherapy, as silencing of Id1 increases the cytotoxic effects of epirubicin (figure 7.1).

Expression of FOXO3a, FOXM1 and Id1 in clinical samples suggests that in the future, there could be a role for their use as biomarkers of response to chemotherapy. Both FOXM1 and Id1 are expressed at low levels in normal breast tissue and at much higher levels in high grade breast cancers. Treatment of patients with FEC chemotherapy or gefitinib down-regulates FOXM1 expression and FEC chemotherapy also down regulates Id1 expression. Therefore, there may be a role for developing specific targeted treatments against FOXM1 and Id1 to increase drug efficacy, avoid resistance and improve sensitivity to chemotherapy. Furthermore, the expression of FOXO3a, FOXM1 and Id1 in clinical samples could be used to refine patient selection, assess responders from non responders and increase sensitivity to chemotherapy to ultimately improve the outcome of patients with breast cancer. Future research into the FOXO3a-FOXM1 axis may ultimately give more insight into the mechanisms underlying sensitivity and resistance to chemotherapy in breast cancer.

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