The role and regulation of FOXO3a in cancer and chemotherapy resistance

Thesis submitted by

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DECLARATION OF ORIGINALITY

Figure 56, in Chapter 5 was produced by Dr Christina Karadedou.

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

ABSTRACT

Drug resistance is the major impediment to the success of cancer therapy. The PI3K/AKT pathway mediates a plethora of cellular functions, including cell survival, proliferation and differentiation. However, increased activation of this pathway has been correlated with drug resistance mechanisms. This pathway regulates the activity of FOXO transcription factors in a negative manner through AKT-dependent phosphorylations. The modulation of FOXO activity leads to a variety of cellular outputs, including cell cycle arrest and apoptosis that define this transcription factor as a tumour suppressor. Importantly, FOXO has also been shown to mediate the effect of many anti-cancer drugs, suggesting that it has an additional role in drug sensitivity and resistance. With this work, by studying the PI3K/AKT/FOXO axis in breast cancer, I have characterised its impact in drug sensitivity and resistance. I found that this axis is deregulated in breast cancer resistant cells. By extending my *in vitro* findings to clinical samples, I further elucidated the potential role of AKT and FOXO3a as indicators and predictors of treatment response in breast cancer.

In addition, I have also characterised three novel downstream targets of FOXO3a - FOXP1, FOXM1 and VEGF – with important roles towards breast cancer progression and in the development of drug-resistance. By characterising these FOXO3a effectors, I unravelled a potential general mechanism by which FOXO3a represses gene target expression.

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LIST OF ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
ABCB1	ATP-binding cassette, sub-family B, member 1
AI	Aromatase inhibitor
APOBEC3G	Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G
ATM	Ataxia-telangiectasia mutated
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
BCRP	Breast cancer resistant protein
BRCA1	Breast cancer associated gene 1
BRCA2	Breast cancer associated gene 2
BSA	bovine serum albumin
CDC25B	Cell division cycle protein 25b
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
cDNA	complementary DNA
ChIP	Chromatin Immunoprecipitation
CK1	Casein kinase 1
CML	Chronic myelogenous leukemia
Co-IP	Co-Immunoprecipitation
CREB	cAMP response element binding protein
CRM1	Chromosomal region maintenance 1
CtBP1	C-terminal-binding protein 1
CXCR4	C-X-C chemokine receptor type 4
DBCL	Diffuse large B-cell lymphoma
DBD	DNA binding domain
DBE	DNA binding element
DCIS	Ductal carcinomas in situ
DFS	Disease-free survival
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
DYRK1	Dual specificity Yak1-related kinase
E2	Estradiol
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid

EGCG	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERE	Estrogen responsive element
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FHRE	Forkhead responsive element
FOX	Forkhead box
GADD45	Growth arrest and DNA damage 45
GFP	Green fluorescent protein
h	hour
HAT	Histone acetyltransferase
HBV	*Hepatitis B virus*
HDAC	Histone deacetylase
HER	Human Epidermal growth factor Receptor
HIF-1α	Hypoxia-inducible factor-1 α
HIV-1	*Human immunodeficiency virus type 1*
HRE	Hypoxia responsive element
HSC	Haematopoietic stem cells
IARC	International agency for research on cancer
IDC	Invasive ductal breast carcinoma
IGF-IR	Type 1 insulin-like growth factor receptor
IHC	Immunohistochemistry
IRS-1	Insulin receptor substrate-1
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase
KO	Knockout
L19	*Ribosomal* protein *L19*
LAC	Lung adenocarcinoma
LB	Lysogeny broth
LOH	Loss of heterorzygosity
LZ	Leucine-zipper-like motif
MALT	Mucosa-associated lymphoid tissue

MAPK	Mitogen-activated protein kinases
MDM2	Murine double minute 2
MDR	Multidrug resistance protein
MEF	Mouse embryonic fibroblasts
MEK	MAPK/ERK kinase
min	minute
miR	Micro-RNA
MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase
MRP	Multidrug resistant associated protein
MST1	Macrophage stimulating 1
mTOR	Mammalian target of rapamycin
mut	Mutant
NCOR	Nuclear receptor corepressor
NES	Nuclear export sequence
Nfat3	Nuclear factor of activated T cell
NF-κB	*Nuclear factor kappa B*
NLS	Nuclear localisation signal
NSC	Non-specific control
NSCLC	Non-small cell lung cancer
OS	Overal survival
PAGE	Polyacrylamide gel electrophoresis
PARP1	Poly ADP-ribose polymerase1
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-tween
PCNSL	Primary central nervous system lymphoma
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide–dependent kinase 1
P-gp	P-glycoprotein
PI3K	Phosphatidylinositol-3 kinase
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLK1	Polo-like kinase 1
PMSF	Phenylmethylsulfonyl fluorid

PR	Progesterone Receptor
pRB	Retinoblastoma protein
PTEN	Phosphatase and tensin homolog
QRF-1	Glutamine rich factor 1
RAS	Rat Sarcoma
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase
RT-qPCR	real time quantitative polymerase chain reaction
SDF-1	Stromal cell-derived factor-1
SDS	Sodium dodecyl sulphate
sec	second
SERM	Selective estrogen receptor modulator
SGK	Serum and glucocorticoid kinase
SIN1	Stress-activated map kinase-interacting protein 1
siRNA	Small interfering RNA
SKP2	S-phase kinase-associated protein 2
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SRB	Sulphorhodamine B
STAT	Signal transducer and activator of transcription
TAD	Transactivation domain
TBP	TATA-binding protein
TBS	Tris buffered saline-tween
TBST	Tris buffered saline-tween
TCA	Trichloroacetic acid
TGFβ	Transforming growth factor β
TKI	Tyrosine kinase inhibitor
TNBC	Triple negative breast cancer
TNF	Tumour necrosis factor
TOP2A	Topoisomerase II A
TRADD	TNF-receptor-associated death domain
TRAIL	TNF-related apoptosis inducing ligand
USP7/HAUSP	Herpes virus-associated ubiquitin-specific protease
UV	Ultraviolet
VEFGR	Vascular endothelial growth factor receptor
VEGF	Vascular endothelial growth factor

WT	Wild-type

CHAPTER 1

INTRODUCTION

1.1 CANCER

Regardless of sex, type or country, every year there is an increase in the number of diagnosed cancer cases. The more recent estimates from the International Agency for Research on Cancer (IARC) in 2008 indicate that around 12.7 million cancer cases and 7.6 million cancer deaths occurred worldwide (Jemal et al 2011). Lung cancer remains the commonest, accounting for 1.2 million new cases per year, followed closely by breast and colorectal cancers. In 2008, just in the UK 309,500 people were diagnosed with cancer. Breast and prostate cancers are the more prevalent among the population accounting for 23% and 24%, respectively, of all cases of cancers in the UK (UK 2011). The future trend predicts a rise in deaths from cancer worldwide to over 11 million in 2030 (GLOBOCAN 2008 Section of Cancer Information accessed on 1/6/2011). Cancer cells break the most basic rules of behaviour by which multi-cellular organisms are built and maintained. Functional abnormalities of proteins involved in DNA repair, cell signalling, cell cycle control, programmed cell death, and tissue architecture have been identified to lead to cancers (Alberts 2002).

1.1.1 CANCER GENETICS

Most cancers derive from an individual abnormal cell that has undergone serial somatic mutations. Typically, a series of multiple mutations are required to transform a normal cell into a malignant cell. Chemicals, radiation or viruses that insert their DNA into the human genome can cause these mutations (Karakosta et al 2005). Cancers develop in slow stages from mildly aberrant cells and progress involving more rounds of mutation and natural selection. Therefore, most cancer cells are genetically unstable. The uncontrolled proliferation of cancer cells depends on defective control of cell division, death or cell differentiation. Many cancer cells escape a built-in limit to cell proliferation, and gradually gain the ability of a constant proliferative state and evasion of apoptosis (Deshpande et al 2005). In 2000, *Hanahan and Weinberg* reviewed the axiomatic requirements of cancer cells as six behavioural properties which result ultimately in capabilities that allow for cancer cells to proliferate, survive and spread (Hanahan and Weinberg 2000).

Generally cancers arise due to genetic alterations that occur in two types of genes – oncogenes and tumour suppressor genes, such as DNA repair genes. Recent advances in cancer genetics uncover the serial aberrations of proto-oncogenes and tumour suppressor genes (e.g. *RAS, P53* mutations and/or allelic losses, hyper-expression of *c-MYC* and *RB* genes) in most cancer development at various stages. By definition, oncogenes are dominant, activated versions of normal genes (proto-oncogenes). In a normal cancer scenario, mutations in oncogenes drive gene expression activation leading to an

accelerating cell growth, loss of differentiation, avoidance of apoptosis and invasion. The vast majority of oncogenes are involved in pathways that normally serve to regulate cell division and survival. *RAS* and *c-MYC* are two of these oncogenes that have been extensively studied over the past years.

RAS genes encode a family of membrane-associated small G proteins that was the first identified oncogene and approximately 30% of human tumours overexpress RAS proteins which can be activated by point mutations or amplifications. RAS activation is known to potentiate a variety of extracellular stimuli through activation of receptor tyrosine kinases (RTKs) (Downward 2003). Once in the active GTP-bound form, RAS can activate a number of intracellular signalling cascades, most notably the RAF/MEK/ERK signalling pathway. Active RAS binds to and activates the three closely related RAF proteins (c-RAF, B-RAF and A-RAF) (Tidyman and Rauen 2009), leading to its translocation to the plasma membrane where it phosphorylates and activates the mitogen-activated protein kinase kinases (MAPKKs), MEK1 and MEK2. These in turn phosphorylate and activate the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2). In addition to activating RAF/MEK/ERK signalling cascade, RAS also binds directly to and activate the class I PI3K proteins (Shaw and Cantley 2006).

The oncogenic functions of the transcription factor c-MYC play a key role in well-known human cancers, such as Burkitt's lymphoma as a result of chromosomal translocation and is often associated with bad prognosis (Pelengaris et al 2002). Additionally, *c-MYC* activation appears to be crucial at some stage during tumour development. c-MYC deregulation is a consequence of different signalling pathways, including the mitogen activated protein kinases (MAPKs) (Desbiens et al 2003), the phosphatidylinositol-3 kinase (PI3K) (Grumont et al 2002) and the signal transducer and activator of transcription (STAT) (Kiuchi et al 1999) pathways. c-MYC is a "master regulator" of large numbers of genes that virtually play a role in almost all the biological processes of a cell. However, its key biological function is to promote cell cycle progression (Hoffman and Liebermann 2008). This *c-MYC* proto-oncogene is known as an early-response gene and it allows cells to exit G_0 and proliferate. Thus, c-MYC is crucial for normal cell proliferation and without it, cells would have a difficult task replicating themselves.

Tumour suppressors are regarded as the guardians of the cells against the internal and external insults that they are constantly exposed to. They monitor critical checkpoints that drive the mitotic cycle, DNA repair, transcription, apoptosis and differentiation. Generally, tumour suppressors are transcription factors or signalling molecules that inhibit cell proliferation and prevent damaged or abnormal cells from becoming malignant as a result of cellular stress (e.g. hypoxia, ultraviolet radiation, DNA damage, oxidative stress,

telomere shortening or activated oncogenes) (Papazoglu and Mills 2007). Mutations or deletions result in their functional inactivation and creates an imbalance that potentiates tumorigenesis. Two of the most well characterised tumour suppressors are the retinoblastoma protein (pRB) and p53.

The retinoblastoma tumour suppressor gene (*RB*) was one of the first tumour suppressor genes to be discovered and belongs to a family that consists of three genes – RB, p107 and p130. Of these, only RB has been found to be inactivated in a variety of tumours (Sherr and McCormick 2002). Its main function is to prevent excessive cell proliferation by repressing the transcription of genes responsible for the progression through the G1 phase into S phase of the cell cycle thus, the integrity of RB pathway is essential for normal cell growth. Throughout cell cycle progression, RB is negatively regulated by fluctuations of phosphorylation events that culminate with the release of proteins associated with RB (Weinberg 1995). One of the proteins that form a determinant complex with RB is its main downstream target E2F transcription factor. E2F induces transcription of genes necessary for G1 to S phase progression. By interacting with E2F, RB prevents their transcriptional activity and restricts cell cycle progression. RB can also repress genes required for cell cycle progression by recruiting HDACs.

The protein p53, also referred to as the 'guardian of the genome' (Lane 1992), is the most widely studied tumour suppressor gene due to the high frequencies of mutations found in human cancers - ~50% (Vousden and Lu 2002). The loss of *p53* functions in cancer is most commonly achieved through a point mutation in *TP53,* the gene that encodes p53, or alternatively by defects in the signalling pathways that are upstream or downstream of p53, provides a selective advantage for clonal expansion, therefore loss or impaired of p53 function is an integral step in cancer development. Another such example is in the case of Li-Fraumeni syndrome where patients have a high susceptibility to line development of diverse cancer types. In line with this, although viable almost all the *P53* null mice develop cancers before six months of age (Lee et al 2001).

When triggered by cellular stress, p53 is stabilised and activated through phosphorylation and other post-translational modifications to induce the expression of a set of target genes involved in DNA repair, differentiation, senescence, cell cycle arrest or apoptosis (Soussi et al 2006). Phosphorylation of p53 results in its activation through protein stabilisation by disruption of its MDM2 interaction, regulation of *trans*activation activity and nuclear re-localisation (Pollard and Earnshaw 2002, Shieh et al 1999). The E3 ubiquitin ligase murine double minute 2 (MDM2) is a negative regulator of p53 that promotes p53 ubiquitination leading to its nuclear export and proteasomal degradation. Moreover, MDM2 null mice are embryonic lethal due to the elevated levels of p53-induced apoptosis, suggesting that MDM2 oncogenic role is to suppress normal p53 function (Jones et al 1995). Importantly,

p53 has been shown to engage in a negative feedback mechanism with MDM2, indicating that p53 expression is a fine-tuned mechanism (Batchelor et al 2009).

1.1.2 CANCER EPIGENETICS

The genome encodes two forms of information, genetic and epigenetic. In cancer, not all gene or protein expression deregulation arises from mutation in coding DNA; instead, activation of oncogenes and silencing of tumour suppressors can be a result of epigenetic factors. The epigenetic information is not contained in the DNA sequence itself but can still be transmitted from one cell to all its descendants. Epigenetic factors, including DNA methylation and post-translational modifications of histone can direct gene expression and cell fate (Bachman et al 2003).

One of the major forms of epigenetic modification associated with transcriptional silencing in cancer cells is aberrant DNA methylation of CpG islands located in the gene promoter regions of genes. Hypermethylation may silence some tumour suppressor genes whereas hypomethylation may activate specific oncogenes. In normal cells, CpG islands are not usually methylated and can be found in up to 40% of the promoters regions of human genes (Esteller 2008). However, once methylation occurs in or around a gene promoter region, the transcriptional activation machinery can no longer bind and as a result gene expression is silenced. Contrarily, hypomethylation can inappropriately activate gene expression. One such example of a gene affected by hypomethylation includes the *H-RAS* oncogene (Feinberg and Vogelstein 1983).

Another epigenetic factor that controls gene expression is histone post-translational modifications, including acetylation, methylation and phosphorylation. These modifications of histone tails make them key points for chromatin condensation/decondensation. Acetylation can occur on a range of proteins with diverse cellular functions, most notably histones (Yang and Seto 2008). This dynamic process is regulated by histone deacetylases (HDACs) and histone acetyltransferases (HATs) that remove or add acetyl groups from lysine residues on the histone tails, respectively. Acetylation results in a relaxation of the DNA-nucleosome structure, allowing the access of transcription factors and other associated proteins that can initiate gene transcription. On the other hand, deacetylation of histones by HDACs is associated with chromatin condensation and results in transcriptional repression (Marks et al 2001, Struhl 1998). DNA methylation is used as a marker to help define regions of transcriptionally active chromatin from transcriptionally inactive chromatin and determine the epigenetic state of cells. These marks involve methylation of lysine 9 in the tail of histone H3, and methylation of lysine 4 on histone H3, which mark transcriptionally inactive and active chromatin, respectively (Ruthenburg et al 2007). Despite playing a crucial role in altering the epigenetic scenario of a cell, DNA

methylation appears to have a dominant effect over histone acetylation as inhibition of HDAC in cancer cells does not result in reactivation of hypermethylated (ie silenced) genes (Bachman et al 2003).

1.2 BREAST CANCER

Despite being the most prevalent disease affecting women in the UK, breast cancer is a heterogeneous disease that accounts for multiple genetic alterations. However, advances in research led to the identification of hereditary risk factors. Breast cancer type 1 (BRCA1) and breast cancer type 2 (BRCA2) susceptibility proteins have been found to harbour mutations that increase the predisposition to the development of breast as well as ovarian cancer thus, acting like tumour suppressor genes (Miki et al 1994, Wooster et al 1995). Yet, this hereditary form of breast cancer only accounts for a minority of the total number of cases, with the majority of cases being sporadic. The prognosis of patients with breast cancer can be categorised into low, intermediate and high risk, depending on the assessment of clinical and biological factors, including age at diagnosis, tumour size, lymph node status, tumour histological grading, expression of estrogen (ER) and progesterone (PR) receptors and HER-2 status (Goldhirsch et al 1998).

Depending on the diagnosis, a patient will receive treatment, which includes surgery on the primary tumour, hormone therapy, radio- and chemo-therapy and targeted agent-therapy.

1.2.1 ESTROGEN RECEPTOR (ER)

The estrogen receptors (ERs) are members of a superfamily of nuclear hormone receptors, which comprises different transcription factors and transcriptional regulators, including the progesterone (PR) and the androgen receptors (AR) (Lonard et al 2007, Olefsky 2001). ERs are involved in the regulation of development, reproduction and metabolism (Nilsson and Gustafsson 2002). There are two main receptors – ER alpha (ERα) and ER beta (ERβ) – that transmit the physiological signalling in response to 17β estradiol (E2). E2 facilitates transcriptional changes in pathways involved in the promotion of cell proliferation,

inhibition of apoptosis, stimulation of metastasis, and angiogenesis. Since their discovery several ERα and ERβ isoform species have been identified (Herynk and Fuqua 2004). Estrogen has been shown to be essential for normal breast tissue development as a study reported that in female aromatase-deficient patients, which are unable to convert steroids into estrogens, breast development was impaired whereas estrogen administration to the same patients reinstated normal breast development (MacGillivray et al 1998). The role of

estrogen signalling in breast cancer came from a study as surprisingly early as in the 1880s where in post-menopausal women whom have had surgery to remove one or both of their ovaries, tumour regression was noted (Boyd 1899). Currently it is commonly accepted that continuous exposure to both exogenous and endogenous estrogens contributes to breast as well as prostate, ovarian, and endometrial cancer (Bosland et al 2002, Deroo and Korach 2006, Hankinson et al 2004, Henderson and Feigelson 2000). Estrogen is also thought to contribute to the accumulation of genotoxic stress by increasing potentially damaging factors such as oxidative stress, which can contribute to tumour formation.

ERα has six domains that comprise regions of conserved function and sequence. Two of those domains that are crucial for ER transcription activation function are AF-1 and AF-2. AF-1 is localised in the N-terminal region of the receptor and acts in a ligand-independent manner whereas the C-terminal AF-2 is responsible for the ligand-dependent *trans*activation functions of ERα. AF-1 and AF-2 act independently and synergistically in a promoter- and cell-specific manner (Beato et al 1995, Gronemeyer 1991). ERs exert a wide range of biological effects following activation in response to ligand binding or in a ligand-independent manner. In response to ligand-binding, ERα functions as a transcription factor, translocating from the extracellular membrane to the nucleus, binding to the estrogen response elements (EREs), in the promoter regions of ER target genes, and recruiting co-activator or co-repressor machinery that will determine whether gene transcription is activated or repressed (Ali and Coombes 2002). Yet, in the absence of ligand other growth factor receptors, such as epidermal growth factor receptor (EGFR) are able to induce signalling cascades that in turn phosphorylate and activate ERα (Britton et al 2006). Some of ER regulated genes include *Cyclin D1*, involved in cell cycle progression and the anti-apoptotic *Bcl-2*, determinant for apoptosis (Madureira et al 2006).

1.2.2 BREAST CANCER TREATMENT

ERα relevance in breast cancer is well established and around 70% of breast cancer cases exhibit increased ERα expression (ERα positive tumours) and a dependence on ERα-driven proliferation (Hayashi et al 2003). Thus, it is natural that ERα is one of the most targetable proteins in the treatment of breast cancer. Endocrine therapies have become the primary adjuvant treatment for breast cancer. They consist of anti-estrogens such as tamoxifen or fulvestrant, which bind and neutralise ER, and/or aromatase inhibitors (AIs) such as letrozole or exemestane, that will block the endogenous estrogen synthesis (Mauri et al 2006). Tamoxifen is a selective estrogen receptor modulator (SERM) that is used as the main form of adjuvant treatment in ERα positive breast cancer patients

that has been shown to improve relapse-free and overall survival. Several studies showed that the agonistic and antagonistic activity of tamoxifen results from the activation of AF-1 and AF-2, respectively. Tamoxifen binds to ER and the complex dimerises and translocates to the nucleus, where it inhibits co-activator binding and promotes co-repressor binding to the ER ligand-binding domain blocking the transcription of AF2, while AF1 remains active (Xu et al 2002). One third of women with ERα positive tumours treated with tamoxifen for 5 years will recur as incurable within 15 years (Goss et al 2008, Johnston 1997, Yamashita 2008). In around 15% of patients, tamoxifen-resistance is associated with the loss of ERα. However, the majority of tamoxifen-resistant patients remain positive for ERα expression and frequently respond to aromatase inhibitors or pure anti-estrogens, including ICI 182780 (also called fulvestrant and Faslodex). AIs are used to deprive tumours from estrogen; AIs inhibit the aromatase enzyme, which catalyses the conversion of androgens to estrogens (Winer et al 2005). Letrozole, the most potent non-steroidal AI, was shown to reduce cell proliferation and cause tumour regression in estrogen-dependent cancers, showing that their clinical benefits were similar or even superior to tamoxifen (Baum et al 2002, Miller and Jackson 2003). AIs are currently used as first-line therapy for metastatic disease and as second-line agents in cases of tamoxifen resistance.

For breast cancer patients whose tumours fail to respond to endocrine treatment or for those that lack estrogen receptors, cytotoxic chemotherapy agents, including taxanes (Paclitaxel and Docetaxel) and anthracyclines (Epirubicin and Doxorubicin), represent an option for treatment (Ali and Coombes 2002, Wenzel et al 2002).

The first anthracyclines, including doxorubicin, were extracted from the pigment producing *Streptomyces peucetius* in the 1960's are one of the most effective anticancer drugs ever developed. However, the clinical use of doxorubicin, as with other anticancer drugs proved challenging due to its high toxicity effects in healthy tissues, including cardiomyopathy and congestive heart failure. Epirubicin, a semi-synthetic derivative of doxorubicin, was discovered due to the need for novel anthracyclines that showed superior activity and a better cardiac tolerability (Weiss 1992). The pharmacokinetic and metabolic changes introduced in epirubicin approved the clinically use of double the dose of epirubicin compared to doxorubicin without an impact in its activity but with a reduction in cardiotoxicity (Robert 1993). Two key adjuvant breast cancer trials comparing cyclophosphamide–epirubicin–fluorouracil (CEF) with cyclophosphamide–methotrexate–fluorouracil (CMF), and comparing epirubicin (E-CMF) with CMF, both reported a gain in overall survival of 7% after 5 years for patients treated with anthracyclines (Levine et al 2005, Poole et al 2006). One of the main mechanisms associated with the anti-cancer

activity of anthracyclines includes initiation of DNA damage by interference with topoisomerase II.

Topoisomerases are ubiquitous enzymes responsible for maintaining DNA topology by over- or under-winding of DNA with a key role in DNA replication and transcription. These dual-action enzymes cause transient single-stranded (Topoisomerase I) or double-stranded (Topoisomerase II) DNA breaks and after new DNA is generated they covalently reseal it (Froelich-Ammon and Osheroff 1995). Topoisomerase II is particularly important since they are the main target of anthracyclines, specifically doxorubicin. These drugs "explore" the dualistic nature of topoisomerases enzymes by enhancing their rate of DNA cleavage and by impairing their ability to reseal DNA breaks. Anthracyclines are known to intercalate into DNA, induce the formation of reactive oxygen species (ROS) and inhibit topoisomerase II among other actions. Additionally, anthracyclines induce DNA damage, activate ataxia teleangiectasia mutated kinase (ATM), induce p53 nuclear translocation and lead to cell cycle arrest or apoptosis (Minotti et al 2004, Zhou and Elledge 2000). Nevertheless, anthracyclines can also induce apoptosis in a DNA damage or p53 independent manner by inducing the release of cytochrome c from mitochondria and inhibit downstream activity of signalling pathways, including the PI3K/AKT (Clementi et al 2003, Laurent and Jaffrezou 2001).

Paclitaxel (Taxol) was the first natural product shown to stabilise microtubules and is the prototype of the taxane family. Taxanes exert their cytotoxic effects in the G2/M phase of the cell cycle by blocking cell cycle progression through centrosomal impairment, induction of abnormal spindles and suppression of spindle microtubule dynamics. A meta-analysis of 13 clinical trials involving women with early-stage breast cancer showed that the disease-free survival (DFS) and overall survival (OS) were significantly improved with the incorporation of taxanes to the anthracycline-based regimens (De Laurentiis et al 2008). Despite an initial response to taxane and anthracycline-based chemotherapies, tumours eventually become insensitive to treatment and disease recurs.

These chemotherapy regimens are also used for the treatment of patients with metastatic breast cancer; however, treatment options for these group of patients is limited especially for those in which response rates are low or whom resistance against anthracyclines and taxanes has developed (Roozrokh and Stahlfeld 2002). Therefore, it is essential to develop suitable newer predictive factors to select optimal therapy for each subset of patients (Walgren et al 2005).

1.2.3 PREDICTIVE MARKERS

Predictive markers are crucial indicators of the efficacy of a treatment by means of predicting the sensitivity or resistance in response to a particular therapy. The improvements made on breast cancer classification lead to the identification of some predictive markers such as ER, PR and HER-2 that proved useful for predicting prognosis and for establishing guideline-based recommendations. In 2000, Perou *et al* classified breast tumours according to their gene expression patterns using cDNA microarrays (Perou et al 2000). This study grouped breast cancers into five distinct types, including two ER- and PR-positive groups, called luminal A and B, a HER-2 overexpressing group, a basal-subtype group and a normal basal-like group. The majority of basal-like tumours are characterised by the absence of staining for ER, PR, and HER-2 receptors and therefore they are referred as triple negative breast cancers (TNBC) (Sorlie et al 2001). However, a small number of basal-like tumours have been found to express one or more of these receptors thus basal-like tumours are not synonymous with TNBC. Several studies showed that TNBC correlate with a poorer clinical outcome (Abd El-Rehim et al 2005, Sorlie et al 2003). Of all breast cancer cases, 80% are ER- or PR-positive and the remaining cases are HER-2 overexpressing tumours. The presence of ER and/or PR are good predictors of endocrine treatment response in breast cancer patients. ER and/or PR expression is an independent prognostic factor in breast cancer (Burcombe et al 2005). However, in regards to chemotherapy treatment, ER negative tumours respond better than ER positive tumours.

The transmembrane receptor tyrosine kinase HER-2 (neu, C-erbB2), is one of the most extensively studied biomarkers in breast cancer. Its overexpression and amplification is found in approximately 20–25% of early breast cancers (Bartlett et al 2001, Slamon et al 1989) and is commonly associated with increased tumour aggressiveness, increased risk of recurrence and reduced survival. As well as HER-2, overexpression of other HER family members has been shown to increase proliferation *in vivo* (Tovey et al 2004). Therefore, the predictive power of HER-2 elucidated for the requirement of more aggressive treatment. Women with tumours exhibiting high HER2 expression were shown to benefit from increasing doses of adjuvant anthracyclines in terms of relapse-free survival when compared with women with low HER2 expression tumours (Muss et al 1994). The discovery that *HER-2* and *TOP2A* (topoisomerase II) are located in close proximity on chromosome 17q together with the fact that anthracyclines directly target *TOP2A* raised the possibility of assessing TOP2A expression as a predictive marker of anthracycline response (Di Leo et al 2002, Pritchard et al 2008). *TOP2A* amplification is mostly restricted to HER-2 positive breast cancers and is rarely encountered in tumours that lack *HER-2* gene amplification. In fact, some studies reported that *TOP2A* deletion or amplification

linked with *HER-2* amplification leads to a worse prognosis but greater response to anthracycline containing regimens (O'Malley et al 2009).

With the discovery and characterisation of novel molecular targets, targeted treatments that allow differentiating between malignant and benign cells gradually emerged. Consistently, there are current drug targeted therapies for HER-2-positive breast cancers in clinical use, including trastuzumab (Herceptin) and lapatinib (Browne et al 2009), and bevacizumab for HER-2-negative breast cancer.

Currently, there are no standard recommendations in the clinical management of TNBC. Since TNBC is null for all three receptors, ER, PR and HER-2, neither hormonal nor targeted therapies are available options thus, for patients with this subtype of breast cancer the primary treatment relies on conventional systemic chemotherapy. In an attempt to identify new predictive markers related to cell proliferation, cell cycle, angiogenesis and signal transduction, especially in TNBC cases, some candidate genes were shown to play an important role in its pathogenesis, including the epithelial growth factor receptor (*EGFR*), c-KIT, *P53*, poly ADP-ribose polymerase1 (*PARP1*) and vascular endothelial growth factor receptor (*VEGFR*) (Podo et al 2010).

1.3 RESISTANCE TO CANCER THERAPY

A great progress has been made in the development of newer and better tolerated anti-cancer drugs for the treatment of breast cancer during the past years. However, most of the drugs currently used for the treatment of breast cancer are associated with acquired resistance, which is for the time being the major hurdle in cancer research. Resistance to chemotherapy can develop prior to drug treatment (intrinsic resistance) or may develop over time after drug exposure (acquired resistance) (Giaccone and Pinedo 1996).

1.3.1 GENERAL RESISTANCE MECHANISMS

Multidrug resistance is defined by simultaneous cross-resistance to anti-cancer drugs that differ in their chemical structures, modes of action and molecular targets. There are two main mechanisms thought to be involved in multidrug development, reduced drug accumulation and enhanced capacity of the tumour to counteract the cytotoxic effects of the drugs (Figure 1) (Polgar and Bates 2005, Scotto et al 1986).

There are two mechanisms by which cancer cells can reduce drug accumulation based on the strategies of drug uptake. The first mechanism involves decreased expression or activity of transporter molecules that regulate the uptake of hydrophilic chemotherapeutic

drugs such as folate antagonists and cisplatin. The second one encompasses an upregulation of transporters associated with energy dependent efflux of a wide variety of hydrophobic chemotherapeutic agents that enter cells freely by diffusion through the plasma membrane, including taxanes (e.g. paclitaxel and docetaxel), vinca alkaloids and anthracyclines (e.g. doxorubicin, daunorubicin, and epirubicin) (Gottesman et al 2002). Examples of such transporter molecules include P-glycoprotein (P-gp), also called MDR1, the multidrug-resistance-associated protein MRP1 and the breast cancer resistance protein (BCRP). These proteins play a crucial physiologically role in cellular protection from endogenous substrates. These transporters are often found to be overexpressed in cancer (Longley and Johnston 2005). Malignant cells have a further enhanced capacity to counteract the cytotoxic and/or cytostatic effects of therapeutic agents. It involves amplification of proliferation and survival signals (alteration in cell cycle checkpoint, deregulation of apoptotic mechanisms, activation of oncogenes, including *HER-2*, *c-MYC*, *RAS*, *MDM2*, *BCR-ABL*, or mutant *P53*), increased DNA damage repair (e.g. O6-methylguanine-DNA methyltransferase, topoisomerase II, p21WAF1/CIP1), nucleoside and nucleobase salvage pathways, and altered drug metabolism as well as mutation or overexpression of the drug targets (e.g. cytochrome P-450 reductase and BCR-ABL) (Figure 1) (Raguz and Yague 2008, Stavrovskaya et al 1998, Tsuruo et al 2003).

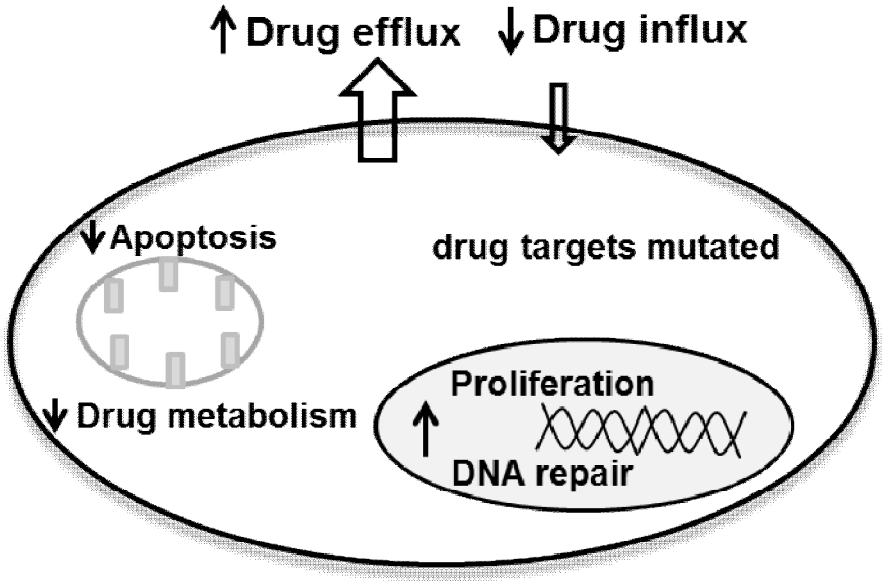


Figure 1. Mechanisms involved in drug resistance.

Reduced drug accumulation can occur by inhibition of membrane transporters used by hydrophilic drugs to access the cell or by active export of hydrophobic drugs from the cell. Other mechanisms include activation of cell repair or detoxification systems and inhibition of apoptosis. [Adapted from (Gottesman et al 2002)].

1.3.2 BREAST CANCER THERAPIES AND RESISTANCE

The multifunctional hormonal action of estrogen as well as the presence of a ligand-independent activation mechanism together with the interaction and cross-talk between estrogen and a variety of growth factors or cytokine driven signal transduction pathways have led to intense investigation into the mechanism of action of estrogen and endocrine resistance. Many mechanisms have been proposed to help understand the loss of therapeutic responsiveness to endocrine therapies. The main mechanism of acquired resistance to tamoxifen is the loss of ERα expression or ERα mutations yet, only 15-20% of breast cancer patients lose ERα expression (Gutierrez et al 2005) and as few cases as less than 1% exhibit mutations in this receptor (Herynk and Fuqua 2004).

Several studies have shown that endocrine resistance is associated with a crosstalk between ER and receptor tyrosine kinase signalling, including HER-2 (De Laurentiis et al 2005). Furthermore, increased expression of EGFR and PI3K activity has also been associated with endocrine resistance (Jordan et al 2004, McClelland et al 2001). Moreover, induction of EGF-mediated growth response and MAPK activation were also shown to compensate for the estrogen-deprivation in MCF-7 cells (Martin et al 2003, Miller et al 1994). Conversely, in the presence of specific tyrosine kinase inhibitors

responsiveness to endocrine treatment was restored and reversed tamoxifen-resistant tumour growth (Gee et al 2003). Deregulation of estrogen target genes is yet another mechanism by which breast cancer cells can overcome endocrine sensitivity. Overexpression of the estrogen-targeted cell cycle regulators c-MYC and Cyclin D1 occurs frequently and has been associated with altered sensitivity to endocrine therapies. Indeed, overexpression of these genes has been shown to suppress anti-estrogen-induced growth arrest (Prall et al 1998) modulating sensitivity to anti-estrogens *in vitro.*

Estrogen co-repressors and co-activators are also crucial for governing the agonistic or antagonistic mode of action of SERM and subsequently have been associated with resistance to anti-estrogens in breast cancer. Decreased levels of nuclear receptor corepressor (NCOR) have been associated with a shorter DFS. Accordingly, the absence of silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) has been correlated with reduced DFS and OS (Girault et al 2003, Green and Carroll 2007).

Another possibility why some ER-positive patients fail to respond to tamoxifen therapy relates to failure of converting tamoxifen to its active metabolite, endoxifen, a reaction catalyzed by cytochrome P4502D6 (CYP2D6). Certain polymorphisms in the *CYP2D6* gene originate different phenotypes that can metabolise with poor to ultra-rapid efficacy (Jin et al 2005). Patients with a poor metaboliser genotype for CYP2D6 have impaired conversion of tamoxifen and thus are less likely to benefit from tamoxifen therapy. Although there is still controversy as to what is the significance of the different CYP2D6 genotypes some studies suggested that patients with a genotype responsible for a low CYP2D6 activity had a higher disease recurrence rate compared to those with a high metabolism genotype (Dezentje et al 2009).

Taxane-resistance has been linked to defects in its various targets, including the class III β-tubulin expression, Aurora-A, Bcl-2, and Tau. Overexpression of Bcl-2 elicits resistance to docetaxel (Noguchi 2006) whereas increased tau expression can be associated with a decrease in response to neoadjuvant paclitaxel. Accordingly, silencing Tau can restore paclitaxel sensitivity (Rouzier et al 2005). Similarly to tamoxifen metabolism, taxanes action is also altered by faster metabolising enzymes activity or defective cytochrome P450 (CYP3A4 and CYP2C8) that culminate in a reduction of the active drug concentration in the plasma.

Anthracycline resistance develops by a multitude of processes, including overexpression of P-glycoprotein and the breast cancer resistance protein (BCRP) (Faneyte et al 2002, Fojo and Menefee 2007), topoisomerase II mutations, overexpression of DNA repair mechanism and deregulation in the apoptotic signalling (e.g. specific mutations in *p53* are associated with doxorubicin resistance and culminate in early relapse in breast cancer)

(Minotti et al 2004). Following the progress made in understanding the molecular basis of cancer a new era has been initiated with the emergence of biologically targeted therapies. Trastuzumab, the recombinant monoclonal antibody that attaches to and blocks HER-2-mediated signalling (Yarden and Sliwkowski 2001) and lapatinib, the small-molecule inhibitor that binds to both HER-2 and EGFR (HER-1), thereby blocking any downstream signalling from these receptors (Moy and Goss 2006) are two such examples of breakthroughs in targeted therapy. Although they have proven useful in the treatment of the more aggressive form of breast cancer, each of these compounds has a response rate of approximately 30% as single agents for the HER-2 positive breast cancer cases. The remaining 70% of patients will not benefit from treatment with either agent. Moreover, for patients who respond to treatment the relapse comes within one year suggesting that these HER-2 positive tumours have resistant mechanisms to counteract the effect of these agents (Nahta et al 2006). Mutation in the *PIK3CA* gene (Berns et al 2007), loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Nagata et al 2004) and overexpression of insulin-like growth factor receptor (IGF-1R) have been suggested to contribute to herceptin rather than lapatinib resistance mechanisms. Indeed, a HER-2/IGF-1R heterodimer has been found in trastuzumab-resistant breast cancer cell lines but not in sensitive cells and disruption of this complex can re-sensitise cells to trastuzumab treatment (Nahta et al 2005). Another potential mechanism of trastuzumab-mediated resistance is the presence of truncated isoforms of the HER2 receptor (e.g. p95 HER-2 and HER2Δ16) lacking the extracellular trastuzumab-binding domain. The p95 HER-2 mutant isoform is found in around 60% of all HER-2 positive tumours (Scaltriti et al 2007). The HER-2 splice variant, HER2Δ16 has enhanced transforming activity when compared to wild-type HER-2 (Esteva et al 2002). The HER family is known for its ability to form dimers amongst different members. For example, the heterodimers EGFR/HER-2 and HER-2/HER-3 were found in aggressive breast cancers and EGFR/HER-2 complex has been shown to associate with worst prognosis (Suo et al 2002). Pertuzumab is a new generation of monoclonal antibodies that binds to a distinct epitope of HER2 and prevents dimerization between different HER members thus, this drug might proof useful for the existing trastuzumab-resistant cases (Franklin et al 2004).

Lapatinib-acquired resistance has been shown to be mediated by enhanced estrogen receptor signalling as a consequence of FOXO3a activation (Xia et al 2006). This example illustrates one of the biggest obstacles in cancer therapy, the immense network complexity and the cross-talks between different transduction pathways. Nevertheless, it appears conflicting that lapatinib resistance arises from its potent proliferative inhibitory effect. Unlike trastuzumab, lapatinib-resistance mechanisms do not appear to involve IGF-1R and

in fact lapatinib has been shown to inhibit IGF-1R signalling in trastuzumab-resistant cells (Nahta and Esteva 2006).

Resistance mechanisms have been described across the anti-cancer therapeutic field for almost every available drug used for the treatment of breast cancer. Therefore, in order to achieve a better clinical management of breast cancer there is a need for the discovery of new markers that can predict both response and resistance to specific treatments and a deeper knowledge of the molecular mechanisms that allow for resistance to develop. Importantly, the comprehension of the mechanistic side of resistance has to be addressed in a wider integrative cellular scale and move towards the implementation of broader therapies that will account for resistance.

1.4 PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) SIGNALLING PATHWAY

Phosphoinositide 3-kinase (PI3K) activity was first characterised over 25 years ago, co-purifying with oncoproteins pp60 v-src and polyoma middle T antigen (Whitman et al 1985). This family of enzymes are dual specificity protein and lipid kinases that are able to phosphorylate proteins on serine/ threonine residues. They catalyse the addition of phosphate to the 3-OH position of the inositol ring within lipids, termed phosphatidylinositols (PtdIns) - which are present on the intracellular space of the cell membrane – converting phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3). Through successive phosphorylations, this family of enzymes provides critical signals for a wide range of biological processes, including regulation of cell size, proliferation, survival, metabolism, glucose transport, ageing, malignant transformation, cytoskeletal reorganisation, angiogenesis, invasion and metastasis, differentiation, membrane trafficking and chemotaxis (Fruman and Cantley 2002, Okkenhaug and Vanhaesebroeck 2003). The diverse range of functional effects reflect the existence of a PI3K enzyme family, which consists primarily of three main classes each containing a range of subunits and isoforms, distinct from each other due to their substrate specificity and lipid products (Engelman et al 2006). The class I PI3Ks are the most studied PI3Ks. These heterodimeric enzymes are composed of a p110 subunit that contains the catalytic domain and a p85 subunit which facilitates the translocation of the catalytic domain and contains the regulatory domain. Class I PI3K enzymes are sub-divided into class IA and class IB, downstream of tyrosine kinases receptors and G-protein coupled receptors, respectively. Class IA enzymes have three types of p110 catalytic subunit (p110α, p110β, p110γ) and five types of regulatory/adaptor subunits (p85α, p55α, p50α, p85β, p85γ). The 3 catalytic isoforms are encoded by 3 separate genes (*PIK3CA* on

chromosome 3q26.3, *PIK3CB* on 3q22.3, *PIK3CD* on 1q36.2 for p110α-γ, respectively). The regulatory subunits p85α, p55α and p50α are derived from a single gene (*PIK3R1* on 5q13.1) by alternative splicing, whereas p85β and p55γ are encoded by distinct genes (*PIK3R2* on 19q13.2-13.4 , *PIK3R3* on 1p34.1). Of these, p85α is the most abundantly expressed and important, as all p110 subunits need p85α to function *in vitro* (Okkenhaug and Vanhaesebroeck 2001). The p85 subunit contains two Src homology 2 (SH2) domains which allow binding to the p110 catalytic subunit. Class IB contains a p110γ catalytic subunit that binds to a different regulatory subunit, termed p101 and is activated by G-protein coupled receptors (Djordjevic and Driscoll 2002). The role of PI3K activity in physiology and pathology has been investigated by studies using knock-out mice models. Of these, *p110*α and *p110*β KO mice died *in utero* between days 9.5 and 10.5, while double heterozygous *p110*$\alpha^{+/-}$ *p110*$\beta^{+/-}$ mice grew normally, suggesting a role in foetal development (Bi et al 1999). Class IA PI3K regulate the proliferation, differentiation and development of various inflammatory cells. They are activated by cytokine and antigen recognition by T cell-, B cell-, Fc receptors. As a result, they regulate inflammatory responses such as chemotaxis, polarisation, migration, phagocytosis, NO synthesis and exocytosis (Koyasu 2003, Stephens et al 2002). Class II PI3K family are monomeric and do not contain a regulatory subunit. They act downstream of various receptors, including EGFR and TNFα receptor. Mammalian class III PI3K is homologous to *Saccharomyces cerevisiae* Vps34p. Similarly to class I PI3K, class III PI3K are heterodimers consisting of a catalytic and regulatory subunit.

The class I PI3K proteins can be triggered by hormones, cytokine or growth factors and result in proliferation, survival and motility of most cell types. They are essential for development and function of inflammatory cells physiologically, and are important in lymphoproliferative and autoimmunity diseases. Constitutive activation drives cell proliferation and protects cells from apoptosis, and this is frequently seen in cancers (Burgering and Medema 2003). The biological effects of PI3Ks are mediated by their lipid products. Therefore, their effects can be antagonised with metabolism of PtdIns regulated by phosphoinositide phosphatases, such as PTEN which counteracts PI3K signalling by dephosphorylating PIP_3. Importantly, PTEN is frequently deleted or mutated in cancers, resulting in constitutive PI3K hyperactivation (Maehama and Dixon 1998). Somatic tumours such as glioblastoma, prostate cancer, endometrial cancer, and ovarian cancer have shown to harbour *PTEN* mutations. Moreover, knockout mice lacking *PTEN* develop cancer, establishing PTEN as a tumour suppressor (Liu et al 1999, Vivanco and Sawyers 2002, Wisniewski et al 1999).

In summary, class I PI3Ks is the most extensively studied due to their defined role in the development of cancer, whereas class II and III proteins have not yet been shown to be linked with oncogenesis (Maiese et al 2008).

1.4.1 AKT AS THE MAIN EFFECTOR

When a growth stimuli is received by the cell through an extracellular receptor, it causes the activation and recruitment of the PI3K heterodimer, to the plasma membrane where it interacts with and phosphorylates its substrate PIP_2, generating PIP_3. PIP_3 accumulation on the plasma membrane acts as a "docking site" for proteins that contain a pleckstrin homology (PH) domain, such as the cAMP-dependent cGMP-dependent and protein kinase C (AGC) family members, including the phosphoinositide–dependent kinase 1 (PDK1), atypical protein kinases C (PKC), phospholipase C-γ (PLC-γ) and the serine/threonine kinase (AKT or PKB) (Cantley 2002, Toker and Newton 2000, Wymann and Pirola 1998). Activation of AKT is strongly associated with the proliferative and pro-survival activities of PI3K. PIP_3 accumulation on the plasma membrane leads to the recruitment of AKT to the plasma membrane. This results in a close proximity with PDK1, which then activates AKT by phosphorylation on threonine residue 308 (T308), within its catalytic site (activation loop), leading to its activation and release into the intracellular space to phosphorylate its downstream targets (Engelman 2009, Fu and Tindall 2008). Additionally, AKT is also phosphorylated on serine residue 473 (S473) within the C-terminal hydrophobic motif (HM), by the mammalian target of rapamycin (mTOR) complex 2 (Jacinto et al 2006). Upon phosphorylation on both sites – T308 and S473 – AKT was shown to have increased catalytic activity *in vitro* (Alessi et al 1996, Scheid et al 2002) highlighting the importance of both phosphorylation sites on its activity. Studies on the implication of differential phosphorylation on these sites have proposed that while the phosphorylation of T308 is essential for AKT activation, phosphorylation of S473 might determine substrate specificity significance. In mouse embryonic fibroblasts that lack mTORC2 component SIN1, AKT is phosphorylated on T308 rather than on S473. AKT activity is still detected although its levels are considerably lower but the effect of impaired phosphorylation on S473 affects a number of AKT downstream targets, most notably the forkhead transcription factors (Brunet et al 1999, Guertin et al 2006, Jacinto et al 2006). Once activated, AKT relocates to the nucleus where it recognises and phosphorylates serine and threonine residues within a consensus motif, which for many targets causes functional inhibition (Figure 2). Among these targets are proteins such as the FOXO forkhead transcription factors (Kops and Burgering 1999), BH3-containing protein BAD

(Datta et al 1997), the E3 ubiquitin ligase MDM2 (Mayo and Donner 2001) and many other of its targets.

Three AKT isoforms or family members, termed AKT1 (PKBα), AKT2 (PKBβ) and AKT3 (PKBγ) have been described in mammals (Toker and Yoeli-Lerner 2006, Vivanco and Sawyers 2002). These isoforms originate from different genes but share more than 80% homology on the protein level. In humans, AKT1 and AKT2 are expressed in all tissue and cell types, whereas AKT3 expression is restricted to the brain and testes. Knockout mice highlighted the different phenotypes that arise from the loss of each AKT isoform. *Akt1*$^{-/-}$ mice displays impaired growth as well as reduced bodyweight, *Akt2*$^{-/-}$ mice are hyperinsulemic and hyperglycaemic and *Akt3*$^{-/-}$ mice have defects in brain development. Yet, all knockout mice are viable suggesting the existence of compensatory mechanisms between the AKT isoforms (Franke 2008).

1.4.2 THE ROLE OF PI3K/AKT PATHWAY IN CANCER

PI3K/AKT signalling pathway is important in cancer development and response to therapy. Overexpression of RTKs, loss of function mutations within *PTEN* and activating mutations within *PIK3CA* (p110α) all lead to increased AKT activity which promotes tumour survival and cancer progression. *PIK3CA* mutations and amplification have been reported in a variety of cancers, including those of colorectal, breast, lung, ovarian and cervical (Ma et al 2000, Samuels and Velculescu 2004, Shayesteh et al 1999). Furthermore, approximately 30% of mutations in this gene have been found in ductal carcinomas *in situ* (DCIS) and invasive ductal breast carcinomas (IDC). *PIK3CA* mutations enhance p110α activity, highlighting its oncogenic potential and its ability to mediate PI3K-dependent proliferation and cancer progression. In addition, *PIK3CA* activation has been correlated with poor prognosis and radiation resistance in glioblastoma multiforme (Chakravarti et al 2004). Somatic mutations within *AKT1* have been found in 8% of breast cancer patients. This mutation allows the binding of AKT to the extracellular membrane bypassing with PIP_3 accumulation, promoting the phosphorylation of AKT on S473 (Carpten et al 2007). Recently, AKT2 and HER2 dual overexpression in mammary epithelial has been shown to markedly increased pulmonary metastasis (Dillon et al 2009). Additionally, amplification of *AKT2* is found in ovarian cancer and *AKT3* in steroid insensitive breast cancers (Hennessy et al 2005). Somatic deletions or mutations in the negative AKT regulator, *PTEN*, are found in 12-60% of a variety of cancers and can result in permanent PI3K/AKT activation (Lian and Di Cristofano 2005).

As a result of the constitutive activation of PI3K/AKT pathway, AKT can inhibit apoptotic signals by phosphorylating BAD, procaspase-9 and FOXO forkhead transcription factors.

On the other hand, AKT can induce anti-apoptotic activity by phosphorylating cAMP response element binding protein (CREB) and IkB kinase (Fresno Vara et al 2004). In addition, it can regulate localisation of substrates, such as with inhibition of GSK3, allowing β-catenin to translocate to the nucleus and induce expression of genes like *cyclin D1*, leading to pRB hyperphosphorylation. Similarly, it phosphorylates anti-proliferative regulators, such as $p21^{Cip1}$ and $p27^{Kip1}$ in the cytoplasm, disabling them from their cell cycle arrest functions. All of these events indicate a tumourigenic role of AKT and thus, drug inhibitors for the components of the PI3K/AKT pathway could be beneficial for cancer treatment. Several studies have shown that increased activity of the PI3K/AKT axis is associated with failure of gefitinib and trastuzumab, EGFR and HER-2-targeted therapies, respectively (Jones et al 2004, Lu et al 2001). Indeed, PI3K inhibitors have been shown to reverse the resistance to chemotherapeutic drugs in various cancers, including in cases of acute leukaemia (Neri et al 2002a).

1.5 FORKHEAD TRANSCRIPTION FACTORS

Forkhead box proteins are a large family of transcriptional regulators that are characterised by a winged-helix domain, through which they bind DNA. The conserved forkhead domain with nuclear localisation signals (NLS) is also referred as winged helix domain because of the 3-D structure of two loops (wings) at the C-terminal, three α-helices at the N-terminal and three β-sheets, forming a structure which is similar in appearance to the wings of a butterfly (Burgering 2008, Weigel et al 1989). The first member of this family was identified in *Drosophila*. The 110 amino-acid DNA binding domain (DBD) is evolutionary conserved and over 100 forkhead genes have been identified from yeast to human, but not in plants. At least 46 members have been identified in the human forkhead box (FOX) gene super-family (Katoh 2004). Since their discovery, a unified nomenclature for the forkhead family members was revised in 2000 and proteins became known as FOXs, named after their 'forkhead box'. Forkhead proteins in chordates were divided into 15 classes or subfamilies and has since been expanded to 19, each subfamily termed A-S (*FOXA-FOXS*), based on their sequence similarity and structure, not their function (Kaestner et al 2000). Three clusters of FOX genes are found on human chromosomes: *FOXD2-FOXE3* locus (1p33), *FOXC1-FOXF2-FOXQ1* locus (6p25.3) and *FOXC2-FOXF1-FOXL1* locus (16q24.1) (Katoh 2004). All forkhead transcription factors bind to DNA with the core consensus sequence RYMAAYA (R=A/G, Y=C/T, M=A/C) (Kaufmann et al 1995, Overdier et al 1994, Pierrou et al 1994) or TRTTTAY (YRKTTRT) in reverse complement (Costa et al 2001). This transcription factor family bind DNA as monomers except for FOXP, which requires dimerization (Li et al 2004b). This super-family is further grouped into two classes, based

on the presence or absence of a C-terminal region in the forkhead domain. The majority of FOX proteins belong to class I with a C-terminal basic region, whereas FOXH, FOXM, FOXN, FOXO and FOXP are in class II (Katoh 2004).

Functionally, forkhead proteins are involved in a variety of biological processes, including cell cycle regulation (FOXM1, FOXO, FOXA1, FOXG1, FOXK1), cellular survival (FOXO), metabolism (FOXA, FOXC, FOXO), immunoregulation (FOXP3, FOXJI, FOXN1, FOXO, FOXD2, FOXP1) and embryonic development (all except FOXB, FOXG, FOXK, FOXO, FOXQ, FOXS). Therefore, deregulation of various FOX gene expression through gene amplification, retroviral integration, chromosomal translocation, or transcriptional regulation can lead to congenital disorders or carcinogenesis (Lehmann et al 2003, Wijchers et al 2006).

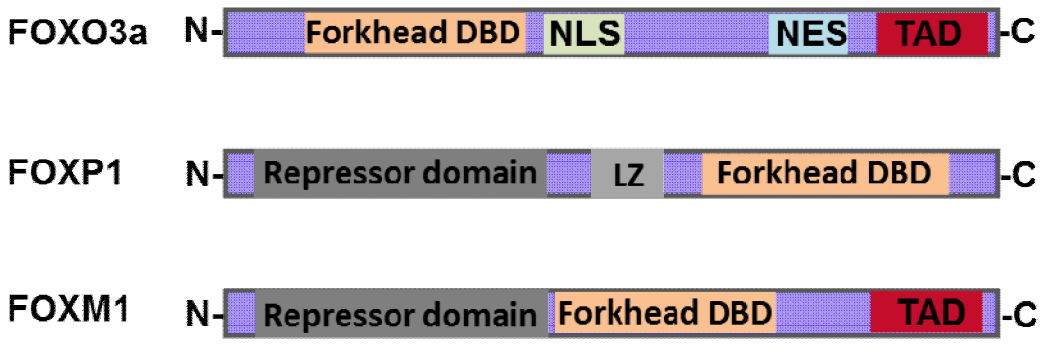


Figure 2. Schematic representation of FOXO3a, FOXP1 and FOXM1.

Depicted is a representation of the structure of FOXO3a, FOXP1 and FOXM1. As shown in the figure, the location of the forkhead DNA-binding domain (DBD) can vary amongst FOX proteins. Also represented are the nuclear localisation sequence (NLS) and nuclear export sequence (NES) that mediate nuclear import and export of FOXO3a, respectively. FOXP1 contains a repressor domain and a leucine-zipper-like motif (LZ), crucial for dimerisation; FOXM1 also contains an N-terminal repressor domain as well as a *trans*activation domain (TAD).

1.5.1 FORKHEAD SUBFAMILY (M) - FOXM1

FOXM1, also known as HNF-3, HFH-11 or Trident, is another member of the Forkhead box superfamily of transcription factors. 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) whereas FOXM1A is transcriptionally inactive due to

disruption of the *trans*activation domain, while retaining a functional DNA binding domain (Figure 2), which might have dominant-negative effects (Ye et al 2007).

FOXM1 is ubiquitously expressed in all embryonic tissues, especially in proliferating cells of epithelial and mesenchymal origin, while in adults its expression is only observed in actively proliferating tissues, including the thymus and testes (Korver et al 1997, Yao et al 1997). *Foxm1*$^{-/-}$ mice die *in-utero*, due to failure to enter mitosis. FOXM1 expression is restricted to cells progressing through the cell cycle being almost undetectable in quiescent cells. Its expression is crucial for the G1/S and G2/M cell cycle phase progression, increasing through S phase into G2 and mitosis. Therefore, FOXM1 is a critical regulator of cell cycle progression through the transcriptional activation of the pro-proliferation genes, such as *cyclin B*, *polo-like kinase-1* (*PLK1*) and *cell division cycle protein 25b* (*CDC25b*), and repression of cyclin-dependent kinase inhibitors (CDKIs), *p21*^{Cip1} and *p27*^{Kip1}, inhibiting their activity and promoting their cellular degradation (Costa 2005, Kim et al 2006, Major et al 2004, Wang et al 2002). FOXM1 transcriptional activity is controlled by its phosphorylation status: FOXM1 phosphorylation increases as cells progress from G1/S to G2/M phases and dephosphorylation of FOXM1 coincides with exit from mitosis (Fu et al 2008). FOXM1 phosphorylation is controlled mainly by the Raf/MEK/ERK signalling cascade (Ma et al 2005) and the CDK2-Cyclin E complex (Major et al 2004). Additionally, pRB phosphorylation by Cyclin D1-CDK4 may be a necessary step to contribute to the relieve of 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 2006b).

The role of FOXM1 in tumorigenesis has also been documented. FOXM1 overexpression has been identified in many different cancer types, including those of the brain, breast, lung, liver and prostate and has been shown to promote tumourigenesis (Myatt and Lam 2007), by driving the cell cycle and proliferation through the regulation of its downstream targets. FOXM1 can also activate genes involved in angiogenesis and metastasis, such as VEGF and matrix metalloproteinases (MMP), MMP2 and MMP-9 (Wang et al 2007). Furthermore, *FOXM1* amplification is also seen in breast cancer suggesting that *FOXM1* is an oncogene (Spirin et al 1996).

1.5.2 FORKHEAD SUBFAMILY (P) - FOXP

In 1993, Li and Tucker identified and cloned the first FoxP1 cDNA and named it QRF-1 (glutamine rich factor 1/ B-cell derived DNA binding protein). At the same time mouse homologues Foxp1, Foxp2 and Foxp4 were identified from a screen for new forkhead family members in mouse lung (Banham et al 2001, Lu et al 2002b, Shu 2001). Human

FOXP3 was later isolated from a patient with IPEX (immuno-dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Chatila et al 2000) and shown to cause the disease scurfy in mice (Brunkow et al 2001, Schubert et al 2001, Wildin et al 2001). FOXP3 shares only 22% identity with FOXP1, while FOXP2 and FOXP4 share more than 50% identity with FOXP1 (Ensemble data). This subfamily binds DNA as homo- or heterodimers with other FOXP subfamily members, unlike other FOX proteins that bind as monomers. FOXP subfamily members contain a highly conserved C-terminal forkhead DNA binding domain. Uniquely, this subfamily contains an N-terminal zinc finger and a leucine zipper essential for DNA binding and transcriptional activity, respectively (Figure 2) (Li et al 2007b, Li et al 2004b, Shu 2001). FoxP forkhead domains are the most divergent amongst all family members; however, there is an 84 amino acid region homology within the DNA-binding domain (Brunkow et al 2001, Chatila et al 2000, Li and Tucker 1993, Shu 2001). All of the FOXP proteins have been proposed to act as transcriptional repressors. For example, Foxp1 and Foxp2 were shown to act as transcriptional repressors in the lung by repressing the mouse *CC10* promoter and the human *SP-C* promoter (Shu 2001). Moreover, characterisation of the N-terminal region of Foxp1, Foxp2 and Foxp4 found the presence of a domain favouring the interactions with corepressor molecules, such as CtBP-1 (Li et al 2004b). In addition, ectopic Foxp3 expression in primary murine T cells has been showed to repress IL-2 expression (Hori et al 2003) and in Jurkat cells FOXP3 expression, resulted in a reduction in IL-2 production following cross-linking of cell-surface CD3 (Schubert et al 2001). The importance of this subfamily of transcription factors in development and disease is better elucidated in knock-out mice models. *Foxp2* knockout causes severe motor impairment and premature death in mice (Shu et al 2005), while *Foxp4* homozygous null mice die at E12.5 (Li et al 2004c). The well-studied *Foxp3*$^{-/-}$ mice phenotype succumbs to a lethal autoimmune syndrome which results from a CD4+CD25+ regulatory T cell deficiency (Fontenot et al 2003). Finally, *Foxp1*-null mice exhibit severe defects in the cardiac valve morphogenesis, which lead to embryonic death at E14.5 (Wang et al 2004), resulting in abnormal B-cell development and differentiation (Hu et al 2006) and impacts, in cooperation with Foxp2, in dramatic morphological defects in the lung (Shu et al 2007). Furthermore, the coordinated actions of Foxp1 and Hox proteins are determinant for motor neuron diversity (Dasen et al 2008). Together these studies reveal the significant contribution of Foxp1 for the heart, B-cell, lung and neuron development.

Importantly, spontaneous mutations in mouse and human diseases affect all the FoxP subfamily members. FOXP1 expression is deregulated in a variety of tumours. It is enhanced in mucosa-associated lymphoid tissue (MALT) lymphoma through several recurrent chromosomal translocations (Streubel et al 2005) or amplifications (Lenz et al 2008). However, its expression is downregulated or cytoplasmic localised in other solid

tumours such as breast tumours, lung carcinomas, head and neck squamous cell carcinomas and early endometrial adenocarcinomas (Banham et al 2001, Banham et al 2005, Giatromanolaki et al 2006). Furthermore, *FOXP1* maps to a region in 3q14.1 that is associated with loss of heterozygosity (LOH) in various tumour types (Banham et al 2001).

FOXP2 expression is diffused in organs such as the brain, lung, heart and gut. Its importance in development has been shown in the lungs by inhibition of genes related to the differentiation of pulmonary epithelial cells (Shu 2001). Furthermore, various studies have demonstrated that FOXP2 is an active and vital factor at several stages of brain development, from embryo to adult in rats (Takahashi et al 2003) and mice and in human embryos (Ferland et al 2003, Lai et al 2003). A single arginine-to-histidine missense mutation (R553H) in the FOXP2 forkhead domain has been linked to abnormal development of neural structures that are important for speech and language (Enard et al 2002, Lai et al 2001).

The seemingly less pleiotropic gene of this group, FOXP3, has been extensively studied in the past and present due to its impact in immune responses in autoimmunity by controlling Treg development and functionality (Fontenot et al 2003, Hori et al 2003). However, FOXP3 expression is also found in epithelial cells from organs such as breast, thymus, prostate and lung (Chang et al 2005, Chen et al 2008). Frameshift mutations in *Foxp3* result in the production of a functionally inactive, truncated protein product that lacks the forkhead DNA binding domain. These mutations were identified to cause the X-linked phenotype of the scurfy mouse, suggesting that FOXP3 is essential for normal immune homeostasis (Brunkow et al 2001). Similarly, mutations in *FOXP3* human ortholog gene result in the fatal, recessive disorder which is characterised by immune dysfunction, polyendocrinopathy, enteropathy and X-linked inheritance (IPEX) (Bennett et al 2001). Another study identified a 3-base-pair deletion in the leucine-zipper dimerisation domain, which might allow FOXP3 to homo- or heterodimerise and therefore its mutation could result in aberrant function (Chatila et al 2000). In addition, a germline mutation of *Foxp3* resulted in a high rate of spontaneous breast cancer and increased susceptibility to carcinogens in the mouse. Moreover, in human breast cancer samples widespread deletion and somatic mutations of *FOXP3* have also been observed (Zuo et al 2007b). Furthermore, in prostate cancer chromosomal deletions, somatic mutations and epigenetic silencing of FOXP3 gene has also been observed (Wang et al 2009). Recent studies have characterised the role of FOXP3 role as a tumour suppressor gene in human cancers through identification of some of its targets. FOXP3 has been shown to repress gene expression through the forkhead DNA-binding element in the *ErbB2* (Zuo et al 2007b) and *Skp2* promoters (Zuo et al 2007a). Furthermore, in a *HER2/ERBB2* negative breast carcinoma cell line (MCF-7), FOXP3 has also been shown to suppress growth and induce

cell death (Zuo et al 2007a). However, FOXP3 does not always act by repressing expression of target genes. FOXP3 has been shown to mediate *p21^{Cip1}* gene induction by disrupting HDAC2 and HDAC4 binding in a specific site in *p21^{Cip1}* locus (Liu et al 2009).

Foxp4 was initially implicated in carcinogenesis where it was shown to be reduced in both kidney and larynx carcinomas (Teufel et al 2003). FOXP4 is expressed in different embryonic and adult tissues including, lung, intestine, heart, liver, kidney, testis and brain (Lu et al 2002b, Takahashi et al 2008, Teufel et al 2003). Various studies have suggested that Foxp4 may have an evolutionarily conserved role in early brain development and patterning amongst different species (Schon et al 2006, Takahashi et al 2008). FOXP4 is beginning to be the subject of studies in human cancers. Sjoblom *et al* identified a single *FOXP4* somatic mutation in breast carcinomas in a highly conserved motif in vertebrates (Sjoblom et al 2006). In addition, a study aiming to identify genetic factors that contribute to prostate cancer risk in the Japanese population, mapped *FOXP4* to a prostate cancer susceptibility region (Takata et al 2010).

1.5.3 FORKHEAD SUBFAMILY (O) - FOXO

Mammalian FOXO proteins are orthologues of the transcription factor abnormal Dauer Formation 16 (DAF-16) identified in the nematode *Caenorhabditis elegans* (*C. elegans*), which was found to be an integral component of metabolic insulin signalling and longevity (van der Horst and Burgering 2007). In mammals FOXO transcription factors consists of four members: FOXO1, FOXO3, FOXO4 and FOXO6. Their structure contains four domains: the highly conserved DNA binding domain (DBD) that defines all forkhead proteins, a nuclear localisation signal (NLS) downstream of the DBD, a nuclear export sequence (NES) and a C-terminal transactivation domain (Figure 2). These FOXO isoforms share sequence homology within the DBD. The human *FOXO1*, *FOXO3* and *FOXO4* genes were first identified as sites of chromosome breakpoints in cancers. Chromosomal translocations disrupting the human FOXO gene have been associated with leukaemia and alveolar rhadomyosarcoma (Anderson et al 1998, Borkhardt et al 1997, Galili et al 1993, Hillion et al 1997). Notably, only FOXOs have been identified in fusion oncoproteins, probably due to their 90-130Kb large introns that exist within the genes and can act as common targets of breakpoints (Anderson et al 1998). Such evidence points to a role of FOXO factors in tumour development and the transforming activity of the forkhead transactivation domain (So and Cleary 2002, So and Cleary 2003).

FOXOs are ubiquitously expressed but with varying expression levels. Of all, FOXO3 (or FOXO3a) has the broadest expression pattern in embryo and adult tissues (Furuyama et al 2000, Greer and Brunet 2005) and FOXO4 is more tissue specific (Biggs et al 2001).

Highest expression levels for FOXO1, FOXO3, and FOXO4 are also found in adipose tissue, brain and heart, respectively. On the other hand, FOXO6 mRNA is found mostly in the developing brain (Jacobs et al 2003). The three *FOXO* genes found in brain, *FOXO1*, -*3a* and -*6*, have different expression patterns suggesting that FOXO isoforms share overlapping as well as distinct roles. Deletion of specific FOXO genes in mice has revealed both redundant and non-redundant effects for FOXO proteins.

Foxo1 null embryos are embryonic lethal due to defective angiogenesis (Furuyama et al 2004), whereas *Foxo3a* and *Foxo4* null mice are viable and normal. *Foxo3a*$^{-/-}$ females have premature ovarian failure, a common cause of infertility and aging in women and suggest that Foxo3 normally helps to maintain a resting follicle pool (Castrillon et al 2003). *Foxo3a* deletion also leads to spontaneous lymphoproliferation and organ inflammation. *Foxo3a*$^{-/-}$ mice also produce hyperactivated helper T cells with increased rates of proliferation and cytokine production (Lin et al 2004). Unlike *Foxo1*- and *Foxo3a*-, *Foxo4*-deficient mice do not exhibit any abnormalities (Hosaka et al 2004). *Foxo6* null mice are not yet generated. Although the gene knock-out studies revealed functional differences between the three FOXOs, the possibility of functional redundancy still remains. The generation of the inducible triple knock-out (*Foxo1/3/4*$^{-/-}$*)* mouse model highlighted this. Following conditional deletion of the three *Foxo* genes, mice developed lymphoblastic thymic lymphomas and haemangiomas yet, the disruption of only two *Foxo* genes originated mild haemangiomas and no lymphomas. These results indicate that FOXOs are functional redundant tumour suppressors (Paik et al 2007).

1.5.4 FOXO TARGETS AND FUNCTIONS

As transcription factors, FOXO proteins elicit their effect on the cellular phenotype by activating the transcription of their target genes. FOXO proteins bind to a promoter with the consensus sequence (termed DBE for DNA binding element), 5'-TT(G/A)TTTAC-3' (Furuyama et al 2000). A large number of gene promoters contain the DBE (Xuan and Zhang, 2005) which can explain their diversity in function. Once bound to DNA, the FOXO transactivation domain initiates gene transcription either as transcriptional activators or repressors, probably depending on the range of associated co-factors that they recruit. Since FOXO proteins regulate a plethora of targets with distinct physiological activities only some of them will be mentioned.

One of the functions that initially was attributed to FOXO activation was the regulation of cell cycle. The cell cycle is regulated by the coordinated action of multiple cyclin/cyclin dependent kinases (CDK), which phosphorylate and regulate a variety of substrates that are indispensable for cell cycle progression. $p27^{Kip1}$ and $p21^{Cip1}$ are members of Cip/Kip

family of CDK inhibitors that bind to both cyclin and CDK subunits inhibiting the activities of cyclin D-, E-, and A-CDK complexes (Besson et al 2008).

Firstly, FOXO blocks G1 progression by repressing cyclin D1 and D2 either directly (Ramaswamy et al 2002) or indirectly through Bcl6/STAT5 (Fernandez de Mattos et al 2004, Schmidt et al 2002). FOXO also promotes cell cycle arrest at the G1/S boundary phase by upregulating the cyclin dependent kinase inhibitors $p27^{Kip1}$ (Medema et al 2000) and $p21^{Cip1}$ (Seoane et al 2004). The overexpression of FOXO3a and FOXO4 were shown to induce the expression of $p27^{Kip1}$, disrupting the cyclin D/CDK4 and Cyclin E/CDK2 complexes causing a cell cycle arrest at the G1 cellular checkpoint (Medema et al 2000). Moreover, the pro-oncogene c-myelocytomaosis (c-MYC) was shown to repress FOXO3a activity leading to loss of $p27^{Kip1}$ expression, an event which is observed in B-cell lymphomagenesis (Dansen and Burgering 2008). The regulation of $p27^{Kip1}$ by FOXO3a was also shown to be important in the induction of apoptosis in both B and T cells through loss of Interleukin 3 (IL-3) and Interleukin 2 (IL-2) mediated survival, respectively (Dijkers et al 2000b, Stahl et al 2002). Additionally, FOXO promotes mitotic entry but not exit by upregulating PLK and cyclin B (Alvarez et al 2001). FOXO can also cause an arrest in cells at the G2/M checkpoint by induction of growth-arrest and DNA-damage inducible protein α of 45 kDa (GADD45α), which interferes with the G2/M transition by inducing the dissociation of cyclin B/CDK1 and by inhibiting CDK1 activity (Tran et al 2002, Wang et al 1999). Thus, the two checkpoints important for stress response can be both mediated by FOXO factors. Moreover, FOXO prevents the re-entry into the cell cycle by transcriptionally upregulating cyclin G2 (CCNG2) (Martinez-Gac et al 2004) and activation of pRB related pocket protein p130 (Kops et al 2002b). p130 associates in a complex with E2F transcription factor 4/5 (E2F4/5) and leads to the exit of cell cycle to a reversible quiescence state.

Programmed cell death, also known as apoptosis, is induced either by intrinsic or extrinsic pathways. In the extrinsic pathway activation of death receptor triggers the formation of a death inducing signalling complex and consequently leads to the activation of caspases (Guicciardi and Gores 2009). In contrast, intracellular stress can induce apoptosis by activating the pro-apoptotic protein Bcl-2, which mediate the release of cytochrome c from the mitochondrial membrane leading to caspase-9 activation and subsequently downstream effector caspases, which will execute the apoptotic programme (Brunelle and Letai 2009). FOXO has been reported to target both death receptor and mitochondrial pathways. The activating ligand, tumour necrosis factor (TNF) cytokine family member, FasL (Fas ligand) was one of the first identified targets of the AKT/FOXO signalling pathway (Brunet et al 1999). Ectopic expression of a constitutively active FOXO3a mutant was found to increase FasL promoter activity in luciferase assays. Furthermore, cells

deficient in components of the FasL signalling cascade failed to undergo apoptosis following expression of FOXO3a, suggesting that the Fas-mediated axis is required for induction of apoptosis by FOXO3a in Jurkat cells (Brunet et al 1999). Overexpression of FOXO1 and FOXO3a also induced apoptosis through upregulation of tumour necrosis factor-related apoptosis inducing ligand (TRAIL). Promoter reported assays further demonstrated that TRAIL is a direct transcriptional target of FOXOs (Modur et al 2002). In addition, FOXO1 was shown to bind and directly regulate the tumour necrosis factor receptor-associated death domain (TRADD) (Rokudai et al 2002). While most reports show that activation of FOXO proteins induces apoptosis, one particular study by Jonsson *et al.* suggested that FOXO may also contribute to increase survival of neutrophils through transcriptional repression of *FasL* promoter (Jonsson et al 2005). Apparently, FOXO activates different genes differently in distinct cell types and environment depending on appropriate cofactors. Nevertheless, the presence of multiple FOXO targets in both cell cycle arrest and apoptosis pathways ensure an efficient result even when target genes are expressed differentially through combinatory effects.

Cytokine deprivation of bone marrow-derived Ba/F3 cells results in the activation of FOXO3a and cytochrome c release, DNA laddering and induction of apoptosis, through the induction of the pro-apoptotic Bcl-2 family member Bim, whereas overexpression of Bcl-2 can rescue cells from FOXO3a-induced apoptosis (Dijkers et al 2000a). The regulation of Bim expression by FOXO3a was also shown to play an important role in survival of activated T cells (Stahl et al 2002). Further studies into the regulation of Bim by FOXO3a have shown that it is possible for the pro-apoptotic function of FOXO proteins to be exploited therapeutically. Treatment of chronic myelogenous leukemia (CML) cell lines, driven by the BCR-ABL chimeric oncogene (Chen et al 2010b) and cell lines overexpressing BCR-ABL, with the BCR-ABL inhibitor STI571 leads to the activation of FOXO3a and increased FOXO3a-dependent Bim expression, resulting in the induction of apoptosis. Moreover, the induction of Bim expression by STI571 treatment was lost when the cellular pool of FOXO3a was abrogated using siRNA. Furthermore, Bim silencing also abrogated the induction of apoptosis in STI571 treated cells (Essafi et al 2005).

Subsequent studies in MCF-7 breast cancer cell lines have shown that FOXO3a also drives the expression of Bim in response to the chemotherapeutic agent paclitaxel, inducing apoptosis (Sunters et al 2003). However, Bim fails to be upregulated by active FOXO mutants in growth arrest cells (Burgering and Medema 2003) and its expression is not induced in the PTEN-deficient cells, such as LNCaP prostate cells, when transduced with a FOXO1-encoding adenovirus (Ramaswamy et al 2002). Nevertheless, a more recent report showed that FOXO3a also upregulates another Bcl-2 family member Puma following IL-2 withdrawal. *Bim*$^{-/-}$ and *Puma*$^{-/-}$ mouse derived T cells have been shown to be

resistant to apoptosis after IL-2 deprivation, demonstrating that regulation of both Bim and Puma by FOXO3a is critical for apoptosis in the absence of cytokines (You et al 2006a).

FOXO proteins have also been found to be key sensors of oxidative stress through the regulation of genes involved with detoxification and DNA repair. Reactive oxygen species (ROS), which include oxygen free radicals, nitric oxide and hydrogen peroxide are produced as by-products from exogenous sources such as, ultraviolet (UV) and ionising radiation (Essers et al 2004), in addition to a result of metabolic processes that take place within the mitochondria. ROS has been shown to be important in some cell signalling pathways and cell cycle progression, but excessive ROS accumulation can be detrimental to the cell and can lead to the induction of cell cycle arrest and apoptosis. When these cellular processes are perturbed, disproportionate ROS accumulation can damage proteins, lipids and DNA leading to cellular transformation and cancer (Benhar et al 2002).

Initial protection against oxidative stress involves FOXO mediated upregulation of antioxidant enzymes manganese superoxide dismutase (MnSOD). Activation of FOXO3a has been shown to increase the expression of MnSOD through direct binding to its promoter in a colon carcinoma cell line (Kops et al 2002a). In addition to MnSOD, increased catalase expression by FOXO3a has been found to decrease oxidative stress resulting in increased survival (Nemoto and Finkel 2002). The analysis of the haematopoietic cells in the bone marrow of *Foxo1/3/4*$^{-/-}$ mice reveals reduced numbers of haematopoietic stem cells (HSCs), while the number of myeloid progenitors in peripheral blood was increased, suggesting that FOXOs are crucial for maintaining HSCs in a quiescent state. In HSCs from *Foxo1/3/4*$^{-/-}$ mice, the levels of ROS and apoptosis were also increased. Treatment of mice with the antioxidant N-acetyl-cysteine was sufficient to rescue the *Foxo1/3/4*$^{-/-}$ HSCs phenotype, demonstrating that FOXO-mediated resistance to oxidative stress is critical for homeostasis of the HSC compartment *in vivo* (Tothova et al 2007). FOXOs can also protect cells from DNA damage by increasing the rates of DNA repair. The G2/M checkpoint is activated following DNA damage, which pauses the cell cycle and allows the cell time to repair the damage before continuing to divide. Gadd45α acts as a sensor to cellular stress and is known to promote the activation of the G2/M cellular checkpoint, allowing time for DNA repair mechanisms to be initiated. Activation of FOXO3a increased Gadd45α at both mRNA and protein levels and restored expression of an UV-damaged luciferase construct, suggesting that FOXO3a upregulates DNA damage repair mechanisms. Moreover, the FOXO3a-induced DNA damage repair response was compromised in *Gadd45α*$^{-/-}$ cells, arguing that Gadd45α expression is indispensable for FOXO3a-mediated DNA repair (Tran et al 2002). A subsequent study reiterated the importance of FOXO3a in regulating Gadd45α expression and showed that FOXO4 could

also promote Gadd45α expression and initiate DNA repair mechanisms (Furukawa-Hibi et al 2002).

Through regulation of multiple transcriptional targets, FOXO proteins modulate various cellular functions, including proliferation, apoptosis and stress resistance. As a result, their loss of function is implicated in predisposition to cancer (Accili and Arden 2004, Hillion et al 1997, Parry et al 1994) and oxidative stress related diseases, such as neurodegenerative diseases (Morris 2005).

1.5.5 REGULATION OF FOXO TRANSCRIPTION FACTORS

To achieve successful regulation of this repertoire of target genes, the activity of FOXO transcription factors is regulated by a range of post-translational processes including phosphorylation, ubiquitination, acetylation and methylation, which allows the activation of specific subsets of genes that influence cell fate (Calnan and Brunet 2008). These can be induced by growth factors, hormones, metals and chemicals, and are important for cell cycle control, development, differentiation and adaptation to environment. Of all modifications, AKT-mediated phosphorylation, which induces cytoplasmic translocation, is the most studied. The PI3K/AKT/FOXO signalling axis is evolutionary conserved and in *C. elegans*. The activation of an orthologue of the insulin receptor, DAF-2, results in activation of the PI3K orthologue, AGE-1, which in turn induces activation of the orthologue of AKT (Dorman et al 1995, Kimura et al 1997). The DAF-2 pathway negatively regulates the induction of a stress-resistant stage known as dauer formation. This larval stage (dauer) in which the worms lower their metabolism and can survive longer is dependent on the FOXO transcription factor DAF-16 as the long-lived phenotype is reverted in *daf-16* deficient organisms (Lin et al 1997). This pathway is homologous to the mammalian PI3K/AKT pathway (Larsen et al 1995). Upon growth factor activation, AKT is recruited to the plasma membrane but also translocates to the nucleus where it phosphorylates its nuclear targets (Burgering and Medema 2003, Meier et al 1997). All FOXO family members, except for FOXO6, contain multiple AKT consensus sequences for phosphorylation by AKT and these sites are conserved throughout evolution, from *C. elegans* to mammals. Three AKT phosphorylation motifs are found in FOXO1, FOXO3a and FOXO4 at residues designated 1, 2 and 3 (Figure 3) (Furukawa-Hibi et al 2002, Kops and Burgering 1999, Lin et al 1997) which are equivalent to T32, S253, S315 of FOXO3a (Takaishi et al 1999). FOXO6 however does not have the third motif and is constitutively localised in the nucleus (Jacobs et al 2003, van der Heide et al 2005).

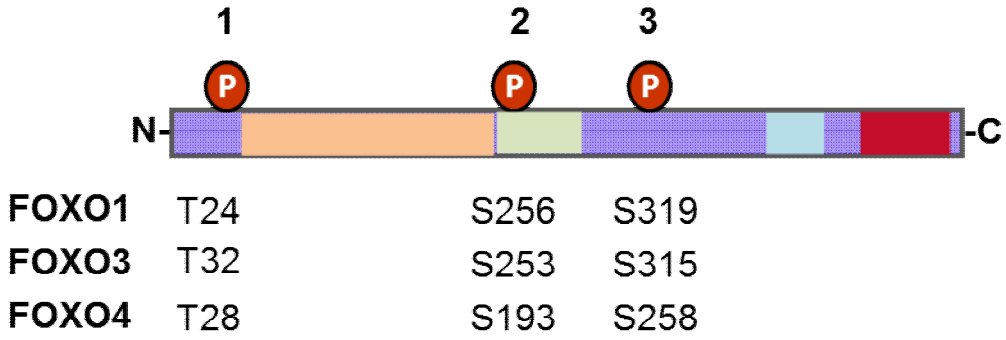


Figure 3. Schematic representation of AKT phosphorylation sites in FOXO.

Representation of the AKT phosphorylation sites in FOXO1, FOXO3a and FOXO4 and their respective amino acid locations. T and S refer to threonine and serine residues, respectively.

The phosphorylation of FOXO3a at T32 and S253 results in an interaction with the chaperone protein 14-3-3, which provides a NES (Lopez-Girona et al 1999) and blocks nuclear import through interfering with the NLS in the forkhead domain (Rena et al 1999), thereby shifting the localisation of FOXOs from the nucleus into the cytoplasm, inhibiting FOXO3a activity (Greer and Brunet 2005). The nuclear export of the FOXO:14-3-3 complex is facilitated by chromosomal region maintenance 1 (CRM1) and Ran GTPase (Burgering and Kops 2002). In the absence of growth factor signalling, AKT and other AGC family members are unable to phosphorylate FOXO3a and the rate of import of unphosphorylated FOXO3a exceeds that of export, resulting in a predominantly nuclear localisation (Figure 4) (Brownawell et al 2001). Similar to FOXO, FOXA2 is the only other forkhead transcription factor that is also regulated by the AKT-mediated translocation control (Wolfrum et al 2003).

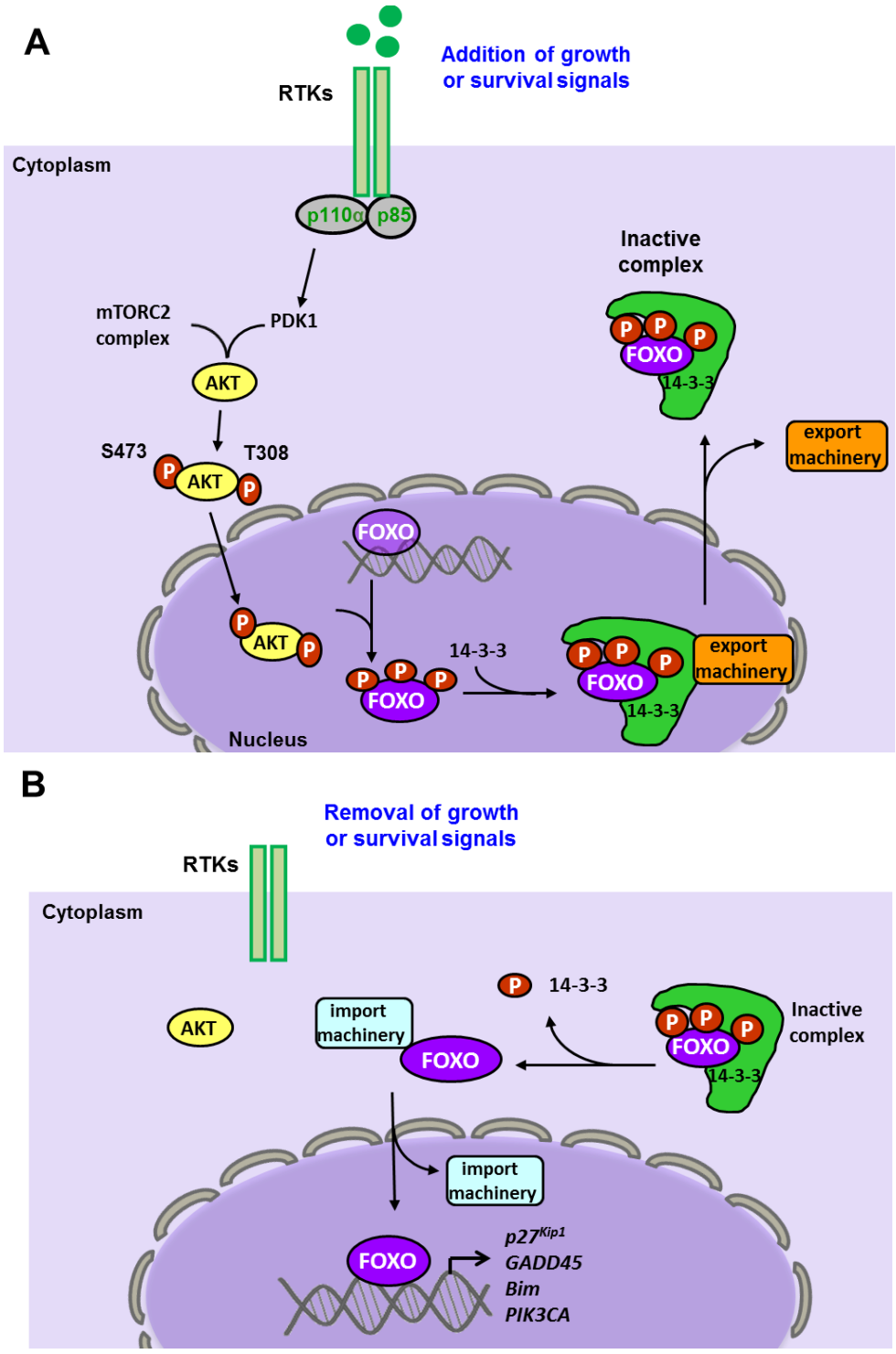


Figure 4. Regulation of FOXO subcellular localisation by AKT.

A) In the presence of stimulatory signals, the activated AKT phosphorylates and inhibits FOXO. This results in nuclear export into the cytoplasm, by association with 14-3-3 protein. **B**) In unstimulated cells, FOXO transcription factors are active in the nucleus and can modulate the expression of genes regulating diverse processes, including proliferation, apoptosis and response to cellular stress.

Other kinases have also been identified to promote the cytoplasmic redistribution of FOXO factors and they include serum and glucocorticoid kinase (SGK) (Brunet et al 2001), casein kinase 1 (CK1) (Rena et al 2002), dual specificity tyrosine-phosphorylated and regulated kinase 1a (DYRK1) (Woods et al 2001) and IKK (Hu et al 2004). Similar to AKT, SGK is also activated by PI3K, translocates to the nucleus (Buse et al 1999) and phosphorylates FOXO3a on the same residues as AKT. CK1 phosphorylates FOXO1 at S322 and S325 only after S319 phosphorylation by AKT/SGK; DYRK1 phosphorylates FOXO1 at S329 independent of AKT. IKKβ has also been shown to phosphorylate FOXO3a on S644 leading to its nuclear exclusion and promoting its degradation by the proteasome. This regulation was independent of AKT, suggesting that multiple mechanisms, such as increased IKK signalling, are increased to inhibit the tumour suppressor function of FOXO family members (Arden 2004, Hu et al 2004). Not all the phosphorylation events that target FOXO represent negative regulation and cytoplasmic retention. In response to oxidative stress, FOXO is phosphorylated at serine or threonine residues not targeted by AKT and yet this results in nuclear accumulation of FOXO. In fact, oxidative stress has been shown to trigger phosphorylation of FOXO4 by the stress kinase JNK. This phosphorylation results in nuclear translocation of FOXO4 and increases its activity; however, this phosphorylation is not conserved in FOXO1, FOXO3a or FOXO6 (De Ruiter et al 2001, Essers et al 2004). One possible explanation is the quenching of 14-3-3 by JNK prevents the association of 14-3-3 with FOXO (Tsuruta et al 2004). Although oxidative stress stimuli can activate FOXO, they do not always confer stress resistance. The upstream activator of MAPK pathway, MST1 phosphorylates FOXO on a conserved site within the DBD, resulting in the disruption with 14-3-3 and promotion of nuclear translocation (Lehtinen et al 2006). Additionally, phosphorylation of FOXOs can induce their poly-ubiquitination and subsequently proteasomal degradation. The phosphorylation of FOXO1 by AKT results in nuclear exclusion but also promotes the interaction with the F-box protein SKP2, which induces both poly-ubiquitination and degradation of FOXO1 (Huang et al 2005).

Another example involves the downstream effector of the RAS/MEK pathway, ERK, which phosphorylates FOXO3a on S294, S344 and S425 to promote its retention in the cytoplasm, where the interaction with MDM2 is increased. Poly-ubiquitination by MDM2 results in the degradation of FOXO3a by the proteasomal pathway (Yang et al 2008). Finally, triggered by oxidative stress, FOXO4 has also been shown to become mono-ubiquitinated resulting in nuclear translocation and increased transcriptional activity. The de-ubiquitinating enzyme herpes virus-associated ubiquitin-specific protease (USP7/HAUSP) was found to negatively regulate this process (van der Horst et al 2006). Indeed, HAUSP had previously been identified as a regulator of p53 ubiquitination,

competing with MDM2 and modulating its post-translational status, reiterating the similarities between FOXO and p53 regulation (Li et al 2002, Li et al 2004a).

Acetylation is another post-translational FOXO regulatory control. Acetylation is required for FOXO functions, especially under oxidative stress and attenuates their transcriptional activity. FOXOs are acetylated by the acetyl transferases p300 and the cyclic-AMP responsive element binding (CREB)-binding protein, while SIRT1 mediates the deacetylation of FOXO proteins (Calnan and Brunet 2008). This post-translational modification is evolutionary conserved as in *C. elegans*, the orthologue Sir2 increases the lifespan of the worm in a *daf-16*-dependent manner, indicating that DAF-16 is positively regulated by acetylation (Tissenbaum and Guarente 2001).

SIRT1 has been showed to bind FOXO1 at LXXLL motif at 459-463 amino acids, enhancing its function for gluconeogenesis, DNA repair, stress resistance and cell cycle arrest, but not apoptosis (Vogt et al 2005). Similarly, expression of SIRT1 increased FOXO3a-induced cell cycle arrest and resistance to oxidative stress but inhibited FOXO3a-induced cell death. Acetylation appears to influence the functional outcome of FOXO proteins by stimulating only a specific subset of FOXO target genes (Brunet et al 2004).

FOXO proteins can interact with other transcription factors contributing to the functional endpoint of FOXO activity. For example, β-catenin of Wnt signalling pathway, a proliferative signalling molecule, binds to FOXO and enhances oxidative stress response and is thus sequestered from its oncogenic effect with T-cell factor (TCF) (Essers et al 2005); FOXO interaction with Smad (similar to mothers against decapentaplegic) is triggered in response to transforming growth factor β (TGFβ) activation. FOXO factors bind to the phosphorylated, nuclear localised Smad protein complexes and result in activation of $p21^{Cip1}$. The $p21^{Cip1}$ *trans*activation is inhibited when FOXG1 binds to the FOXO-Smad complexes in response to PI3K activation (Seoane et al 2004). There are a number of parallels between FOXO proteins and p53. Both proteins induce cell cycle arrest, apoptosis and DNA repair; they also share a considerable number of target genes, including $p21^{Cip1}$, *GADD45α* and *Puma*; finally they are regulated by similar modifications such as phosphorylation and acetylation (Renault et al 2011). All these similarities suggest that both transcription factors may share the same regulatory complex. p53 and FOXO seem to inhibit each other. p53 can induce SGK to inhibit FOXO function (You et al 2004), while FOXO physically interacts with p53 and inhibits its function through suppression of the *SIRT1* promoter (Nemoto et al 2004). Conversely, FOXO3a has been reported to lead to stabilisation of p53 and activate p53-dependent apoptosis (You et al 2006b). In summary, growth factor signalling induces phosphorylation and inactivation of FOXO, while stress stimuli phosphorylate and activate FOXO. Nevertheless, FOXO proteins are

activated in the presence of both growth factors and stress stimuli (Brunet et al 2004, Wang et al 2005), indicating that stress-induced signalling is predominant in the activation of FOXOs. Eventually, the decision on longevity and stress resistance is favoured over apoptosis if SIRT1 is present.

1.5.6 FOXO AND CANCER

Through regulation of distinct intracellular events, FOXO factors have been proposed to act as tumour suppressors. Loss of FOXO function can lead to tumour development due to increased proliferation, decreased DNA repair and thus increased genomic instability. In addition, its interactions with both tumour suppressors and oncogenes are evidence of their involvement in carcinogenesis. The results of interacting with Smad/$p21^{Cip1}$, β-catenin and p53 eventually enhance FOXO functions in cell cycle arrest and stress resistance. Consistently, the PI3K/AKT pathway is frequently hyperactivated in cancer resulting in FOXO inactivation (Engelman 2009). Furthermore, FOXO reactivation either by ectopic expression or by inhibition of PI3K culminated in induction of apoptosis in a variety of cancers (Essafi et al 2005, Sunters et al 2003, Yamamura et al 2006). The analysis of the inducible *Foxo1*$^{-/-}$, *Foxo3*$^{-/-}$ and *Foxo4*$^{-/-}$ mouse model demonstrated FOXO role as tumour suppressors (Paik et al 2007).

The loss of FOXO3a mRNA and protein expression has been observed in lung adenocarcinoma (LAC) and non-small cell lung cancer (NSCLC). In 24.2% of LAC samples, FOXO3a expression was lost due to homozygous deletion and a further 60.6% displayed a significant loss of FOXO3a expression (Mikse et al 2010), further supporting that FOXO3 functions as a tumour suppressor.

The suppression of FOXO3a activity through overactivation of upstream regulatory pathways has been implicated in breast cancer tumour progression; for example, in tumour cell lines harbouring PTEN mutations, FOXO1 and FOXO3a are retained in the cytoplasm resulting in their inhibition. However, reintroduction of functional PTEN or overexpression of FOXO family members results in a G1 cell cycle arrest and induction of apoptosis, highlighting the importance of PI3K hyperactivation in breast cancer and its suppression of FOXO proteins, promoting tumour progression and survival (Medema et al 2000, Nakamura et al 2000).

Since the activity of FOXO proteins is regulated by signalling pathways that are upstream of the transcription factors and that these pathways are frequently hyperactivated in cancer, compounds that impair this hyperactivation are potent activators of FOXO proteins. Probably the best examples are the targeted therapies, including gefitinib, lapatinib and trastuzumab which target EGFR, EGFR and HER-2, and HER-2 respectively, resulting in

the inhibition of the PI3K/AKT axis and subsequently inhibiting tumour growth. In fact, we and others have shown that FOXO3a is indirectly activated in response to lapatinib and gefitinib treatment, and that this activation modulates the induction of apoptosis (Hegde et al 2007, Krol et al 2007, McGovern et al 2009). Because FOXO proteins are commonly the cellular targets of most the anti-cancer drugs, it is logical to assume that the PI3K/AKT/FOXO3a axis is also involved in mediating drug-resistance. Indeed, some studies support the positive correlation of drug efflux transporter activity and PI3K/AKT pathway. Moreover, FOXO3a has also been found to induce MDR1 expression (Hui et al 2008a). Drug resistant cells exhibit increase PI3K/AKT and drug treatment induces AKT activity (Plo et al 1999). This increase in AKT activity is thought to be responsible for cisplatin (Asselin et al 2001) and paclitaxel resistance (Mabuchi et al 2002). ROS induces the insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation and activates $p21^{Ras}$ to interact with PI3K/p110, resulting in AKT activation (Rodriguez-Viciana et al 1996). Since most chemotherapeutic drugs induce activation of the PI3K/AKT pathway and subsequently lead to the inactivation of FOXO proteins, this axis appears to be an inevitable step target for cancer therapy.

1.6 OVERALL AIMS AND THESIS OUTLINE

FOXO transcription factors have been implicated in a variety of cellular functions that are impaired in carcinogenesis. Furthermore, they have also been shown to mediate the effect of many chemotherapeutic drugs. Thus, FOXO activity is expected to have a role in chemotherapy sensitivity. However, when drug-resistance mechanisms develop, survival of cancer cells relates to hyperactivation of AKT and thus removal of FOXO tumour suppressive functions. My hypothesis is therefore that FOXO3a has a role in apoptosis and cell cycle arrest mediated by anti-cancer drugs as well as development of chemoresistance. However, despite the potential importance of FOXO proteins in tumorigenesis and drug resistance, their regulation, modes of action and functions are not clearly defined and understood. In this thesis, I explored the regulation, mechanisms of action and functions of FOXO proteins through studying the regulation of a number of putative FOXO target genes in breast cancer.

Firstly, I explored the role of the PI3K/AKT/FOXO3a axis in drug response and drug-resistance, in sensitive and resistant breast cancer cell lines and validated my *in vitro* results in tumour patient samples (Chapter 3). Among some of the newly identified transcriptional targets of FOXO were FOXP1 and VEGF. I confirmed and validated that these genes are indeed regulated by FOXO3a and may contribute to FOXO3a-mediated resistance in breast cancer cell lines, in Chapter 4 and Chapter 5, respectively.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 CELL LINES

The human breast carcinoma cell lines MCF-7, SKBR3, T47D, MDA-MB-453, MDA-MB-231, MDA-MB-468, BT474, BT549, CAL51, ZR751, ZR7530, HBL-100 and CAMA-1 were cultured in Dulbecco's modified eagle medium (DMEM) (GIBCO-BRL UK). The medium was supplemented with 10% foetal calf serum (FCS) (First Link Ltd, Birmingham, UK), 2mmol/L glutamine and 100U/mL penicillin/streptomycin (Sigma-Aldrich, Poole, UK). Cells were maintained at 37°C in a humidified incubator with an atmosphere of 10% CO_2.

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 cholorotoxin, 20ng/mL epidermal growth factor, 100U/mL penicillin and 100µg/mL streptomycin (all supplements from Sigma, UK).

The mouse embryonic fibroblasts (MEF) were derived from *wildtype* (WT) and *Foxo1/3/4*$^{-/-}$ knockout mice. Cells were cultured in DMEM and supplemented as described above for MCF-7 cells.

The leukaemic naïve K562 and the resistant derivate KD30 cells were obtained from Dr Ernesto Yague (MRC Clinical Sciences Centre, London, UK) these cells were cultured in RPMI (Sigma) with 10% foetal bovine serum (First Link Ltd, Birmingham, UK) supplemented with 2mM L-glutamine, penicillin and streptomycin in 5% CO2. KD30 were generated by one-step exposure of K562 cells to 30nM doxorubicin for over 4 weeks (Yague et al 2003).

The doxorubicin-resistant MCF-7-Dox^R, the MCF-7-Epi^R (also known as MCF-7-Dox^R, cross-resistant to Epirubicin), and the MCF-7-Cis^R cells were derived from the parental MCF-7. MCF-7-Dox^R, MCF-7-Epi^R, and MCF-7-Cis^R were generated previously in the laboratory and were cultured as described above and supplemented with 0.01mg/ml doxorubicin and 1 µM/ml cisplatin, respectively. The lapatinib-resistant BT474 cells (BT474 Lap^R) were derived from the parental BT474 cells and were generated (by myself) by continuous addition of 1µM/ml of lapatinib to the supplemented DMEM described above.

The two estrogen-independent and endocrine-resistant cell lines, LCC2 and LCC9, were derived from MCF-7/LCC1 (Clarke et al 1994) by stepwise *in vitro* selection with prolonged tamoxifen (LCC2) and fulvestrant (ICI 182780) (LCC9) treatment as described (Brunner et al 1993, Brunner et al 1997). Following *in vitro* selection, they were maintained in modified IMEM-phenol red free with 5% charcoal-stripped FBS. R27 is another endocrine-resistant cell line derived from its parental MCF-7 with prolonged tamoxifen treatment *in vitro*. LCC2,

LCC9 and R27 breast cell lines were kindly provided by Dr. Robert Clarke (Georgetown University Medical School, Washington D.C.).

The 4-hydroxytamoxifen (4-OHT) inducible cell lines were grown in phenol-red free DMEM medium (Invitrogen, Paisley, UK) supplemented with 10% double-charcoal stripped serum (Globepharm Ltd).

Adherent cells were grown and split at approximately 80% confluency, twice a week. Media was aspirated and the cell monolayer was washed in warm PBS and then detached by the addition of 1x trypsin-EDTA. After 2 min at 37°C, the trypsin was inactivated by the addition of complete media. The cells were then counted by use of a haemocytometer and seeded in the appropriate media volume into plates. Cells were not cultured for more than 40 passages. Suspension cells were directly diluted into new flasks.

2.1.2 CELL LINE PRESERVATION

Cells were detached from the monolayer with trypsin, as described above, washed in complete media and spun down at 1200rpm for 5 min. The cell pellet was then resuspended in FCS with 10% DMSO. 1ml aliquots were transferred to cryotubes and slowly frozen in a freezing container (VWR International) at -80°C overnight before storage in liquid nitrogen. Suspension cells were collected by centrifugation at 1200rpm for 5 min. The following steps are similar to those described above for adherent cells. When defrosting cells, the cryotubes were defrosted rapidly in a waterbath (~37°C) washed in complete media, then spun at 1200rpm for 3 min to remove any DMSO-containing media and the pellet was resuspended in 10ml of fresh supplemented medium in a 25cm^2 flask.

2.1.3 CHEMICALS

Cisplatin (Onco-tain DBL, Leamington Spa) was maintained as a stock solution (3.3M) at room temperature (RT) and diluted in fresh media prior to treatment. **Paclitaxel** (Imperial College Healthcare, UK) was maintained as a stock solution (6mg/ml) at 4°C. **Lapatinib** (LC laboratories, USA) was dissolved in DMSO and stored at -20°C at a concentration of 1mg/µl. **Epirubicin** (2mg/ml in 0.9% sodium chloride) and **Doxorubicin** (2mg/ml in 0.9% sodium chloride) were obtained from Imperial College Healthcare, UK and stored in solution at 4°C. **Trichostatin A** (TSA; Sigma, Poole, UK).

2.1.4 GENERATION OF STABLE CELL LINES

For the generation of stable cell lines, the optimal concentration of neomycin (G418, GIBCO) was determined with a killing curve for MCF-7 cells. The selected dose was the minimum that killed all cells by day 14, while the maintenance dose was the one that

inhibited cell proliferation. 12µg of the plasmid of interest or empty vector (control) were transfected into cells using Fugene 6 transfection reagent (Roche, UK). MCF-7 cells were then selected with neomycin 24 h after transfection for at least 2 weeks until cell proliferation was established. During this period, medium was changed every 2-3 days.

2.2 PROTEIN ANALYSIS

2.2.1 PREPARATION OF TOTAL PROTEIN LYSATES

Whole cell protein extracts were prepared by initially harvesting cells and washing the cell pellet in PBS before centrifuging at 2000rpm 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 $NaCl_2$, 50mM Tris-HCl (pH 7.6), 5mM EDTA, 1mM DTT, 1mM NaF, 2mM PMSF, 1mM sodium orthovanadate and "Complete" protease inhibitor cocktail, as instructed by the manufacturer (Roche Diagnostics, Burgess Hill, West Sussex, UK)] at 4^oC for 20 min. Insoluble lysate material was removed by centrifugation at 13000rpm for 10 min at 4°C. The supernatant was transferred to a clean eppendorf tube and its protein concentration was determined. Protein concentration was determined using the Bio-Rad D_c protein assay (Bio-Rad Laboratories, CA, USA), as instructed by the manufacturer. Absorbance was read at 750nm for each of the experimental samples as well as the non-protein containing control. Protein concentrations were determined by the equation absorbance x 25 = µg/µl.

2.2.2 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOTTING

To separate the proteins, 20µg of protein lysate was added to an equal volume of 2x sample buffer (200mM Tris-HCl pH 6.8, 6% sodium dodecyl sulfate (SDS), 2mM EDTA, 10% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) and incubated for 10 min. Samples were then spun at 1000rpm for 1 min, loaded into the sacking wells and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) for 2 h at 80V using a Mini-Protean III apparatus (Bio-Rad Laboratories, Hemel Hempstead, UK). The recipe for buffers and gels used in SDS-PAGE is illustrated in Appendix 2. Once the proteins had been separated by SDS-PAGE, they were transferred to 0.45µm Protran nitrocellulose membranes (Schleicher and Schuell, Whatman, Brentford, UK) using a wet tank blotting system (Bio-Rad Laboratories Trans-Blot Cell) in transfer buffer for 90 min at 90V at RT. Membranes were then blocked with 5% (w/v) bovine serum albumin (BSA) in Tris buffered solution with 0.05% Tween-20 (TBST, pH 7.5) for 30 min at RT and then incubated with

the primary antibody of choice (Appendix 1) in 5% BSA-TBST overnight at 4oC. Membranes were then washed 4 times, every 10 min with 50ml of TBST at RT, prior to incubation with either anti-mouse or anti-rabbit secondary antibody coupled to horseradish peroxidase (Dako, Ely, Cambridgeshire, UK) at 1:3000 dilution for 30 min at RT and visualised using the ECL detection system (GE Healthcare, UK). The list of primary antibodies used is included in Appendix 1. Antibodies against Tubulin or Actin were used as a loading control.

2.2.3 SUBCELLULAR FRACTIONATION

The subcellular fractionation assay was done by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Perbio, Belgium). Cells were pelleted and resuspended in 200µl ice-cold CER I solution with protease inhibitors and vortexed vigorously on the highest setting for 15 sec. After incubation on ice for 10min, 11µl ice-cold CER II solution was added to the tube. The tube was then vortexed for 5 sec and incubated on ice for 1 min. This mixture was further vortexed for 5 sec on the highest setting and then centrifuged at maximum speed on a desktop centrifuge for 5 min at 4°C. The supernatant containing the cytoplasmic extract was transferred to a clean pre-chilled tube and the insoluble fraction was further mixed with 100µl ice-cold NER solution with protease inhibitors. The tube was vortexed for 15 sec on the highest setting and incubated on ice for 10 min. The same procedure was repeated for a total of 40 min. Finally, the tube was centrifuged at maximum speed for 10 min and the supernatant was collected for the nuclear fraction.

2.3 RNA ANALYSIS

2.3.1 TOTAL RNA EXTRACTION

Total RNA was isolated from mammalian cells using the RNeasy Kit (Qiagen, Crawley, UK). Frozen cell pellets were disrupted by adding 350µl buffer RLT with 10% β-mercaptoethanol. Samples were homogenised 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 15 sec at 10000rpm. The flow through was discarded and 700µl of buffer RW1 was added to the RNeasy spin column. This was microcentrifuged for 15 sec at 10000rpm and the flow through discarded again. Next, 500µl of buffer RPE was added to the spin column and microcentrifuged at 10000rpm for 15 sec. The flow through was discarded and this step was repeated, followed by a 2 min 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.

2.3.2 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). 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, 1µl RNaseOUT and 1µl Superscript III RT, to a final volume of 20µl 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. By pooling equal amounts of all diluted cDNA samples, a cDNA standard was obtained. Four serial 1:4 dilutions (1/4, 1/16, 1/64 and 1/256) of the standard in water were done for generation of standard curve points, including a blank sample. As a control the established housekeeping genes L19 or TBP were used to normalise gene expression between samples.

The resulting cDNA samples were used as a template for RT-qPCR, which was performed with an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems) using 2x SYBRGreen Mastermix (Applied Biosystems) according to manufacturer's instructions. Gene specific primers were designed using ABI Primer Express Software (Applied Biosystems). Primers with amplicon size 50-150bp spanning intron/exon boundary were selected whenever possible to avoid amplification of contaminating genomic DNA.

The reaction mix contained 2µl of sample and 23µl of SYBR Master Mix and primers to a final volume of 25µl. All measurements were performed in triplicate. The TAQMAN 7900 Real-time PCR machine was used. Efficiency of PCR was obtained from the slope of the standard curve.

2.3.3 PRIMER OPTIMISATION

JAK2, CXCR4, FOXP1, $p27^{Kip1}$ and APOBEC3G primer sets were optimised with 10ng cDNA standard to determine the minimum concentration required for maximum yield and minimum non-specific amplification in each cell line. 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 FOXO3a, FOXM1, VEGF, PIK3CA, IGF1R, L19 and TBP were optimised by previous

members in the lab (E. Lam's laboratory, Imperial College, London). Primers used for RT-qPCR are summarised in **Error! Reference source not found.**.

Human gene	Forward (5'-3')	Reverse (5'-3')
FOXO3a	TCTACGAGTGGATGGTGCGTT	CGACTATGCAGTGACAGGTTGTG
FOXM1	TGCAGCTAGGGATGTGAATCTTC	GGAGCCCAGTCCATCAGAACT
FOXP1	TCAGTGGTAACCCTTCCCTTA	GTACAGGATGCACGGCTTG
JAK2	GGAAGCGACCTGGAAGCATGA	GGCCTCGAATCTGTTGGCTC
CXCR4	ACCAGAAGAAACTGAGAAGCATGA	GAGGAGGTCGGCCACTGA
$p27^{Kip1}$	GAATAAGGAAGCGACCTGCAA	TCTTCTGTTCTGTTGGCTCTTTTGT
APOBEC3G	TGAAAACAAAGGGTCCCTCAAG	GGAATACACCTGGCCTCGAA
IGF1R	GGATATTGGGCTTTACAACCTG	GGCTTATTCCCCACAATGTAGTT
PIK3CA	AAATGAAAGCTCACTCTGGATTCC	TGTGCAATTCCTATGCAATCG
VEGF	GAAGTGGTGAAG TTCATGGATGTC	CGATCG TTCTGTATCAGTCTTTCC
L19	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA
TBP	ACGAACCACGGCACTGAT	AACCCAACTTCTGTACAACTCTAGCA

Mouse gene	Forward (5'-3')	Reverse (5'-3')
FOXO3a	CCGGACAAAGGGCTCACT	GGCACACAGCGCACCAT
FOXP1	GGATTAAGTCTCCCAAGAGGAATG	GCCACTGACACGGGAACCT
$p27^{Kip1}$	GGACCAAATGCCTGACTCGT	CGCTTCCTCATCCCTGGAC

Table 1. List of primers used for RT-qPCR

2.4 DNA ANALYSIS

2.4.1 PLASMID CONSTRUCTS AND EXPRESSION VECTORS

The constitutively active form of FOXM1, pcDNA3-ΔN-FOXM1, was generated by deletion of the FOXM1 N-terminal repressor domain (Wierstra and Alves 2006a) and was cloned by Jimmy Kwok (PhD student, Prof Eric Lam's lab).

The constitutively active FOXO3a mutant vectors, pcDNA3-FOXO3a(A3) and pcDNA3-FOXO3a(A3):ER were cloned by previous lab members.

The $HDAC2^{WT}$ and $HDAC2^{C262A/C274A}$ expression vectors were a kind gift from Dr Antonella Riccio (University College London, UK).

FOXP1 was cloned into pcDNA3 vector (Invitrogen, Paisley UK) (pcDNA3-FOXP1) by previous lab members.

The FOXP1 cDNA fragment generated by PCR from pcDNA3-FOXP1 vector was cloned into pEGFP-C1 (Clontech, CA) vector, generating the final pEGFP-C1-FOXP1 vector (FOXP1:GFP).

The *VEGF* promoter was cloned by Dr Alexa Stavropoulou; the VEGF promoter constructs FHRE mutant 1 (VEGF pro-mut1) and FHRE mutant 2 (VEGF pro-mut2) were generated by introduction of mutations into the forkhead response elements (FHRE) using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA).

2.4.2 DNA QUANTIFICATION

The concentration of DNA was measured by using a ND-1000 spectrophotometer (NanoDrop Technologies) which measures instantly the DNA concentration upon the addition of 1µl of sample.

2.4.3 SUBCLONING

2.4.3.1 RESTRICTION DIGESTS

Available restriction enzyme sites were examined with NEB cutter version 2. Approximately 1µg of plasmid DNA was digested by restriction endonucleases (New England Biolabs, Hertfordshire, UK) for 3 h at room temperature (RT) or overnight with appropriate buffers at suggested temperature according to protocols supplied by New England Biolabs.

2.4.3.2 LIGATION

DNA fragments with compatible over-hanging ends were covalently joined by T4 DNA ligase (New England Biolabs) simultaneously at room temperature for 1 hour or overnight at 16°C. The vector: insert molar ratio was usually 1:3 or 1:4 in a 10µl final volume reaction. 1-2µl of the ligation reaction was used for transformation.

2.4.3.3 AGAROSE GEL ELECTROPHORESIS

DNA fragments mixed in a final 1x Orange G loading dye (6x, Promega, Southampton, UK) were separated by electrophoresis in 1-2% (w/v) agarose gel (incorporated with 0.5µg/ml ethidium bromide) in Tris-acetate–EDTA (TAE) buffer. 1% gel was used to visualise DNA fragment of size 500bp to 10kb; 2% gel for 50bp to 2kb. The gel image was recorded with a UVIpro Gel Documentation system (Uvitec, Cambridge, UK).

2.4.3.4 EXTRACTION AND PURIFICATION OF DNA

Where applicable, DNA was purified with PCR purification Kit, Reaction cleanup Kit or Gel extraction kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instruction. Ethanol precipitation was used when concentration of DNA was desired; 0.1 volumes of 3M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol were added to the DNA solution. The mixture was stored at -80^{o}C for at least 20 min and then centrifuged at 14000rpm for 30 min at 4^{o}C. The pellet was washed with 70% ethanol, re-centrifuged for 5 min, air-dried and resuspended in an appropriate volume of 10mM Tris buffer or sterile water.

2.4.3.5 PCR SUBCLONING

Two restriction enzyme sites capable of digestion in the same buffer and preferably with incompatible ends were selected from the multiple cloning region in the vector plasmid. They were then added into the 5'- end of the PCR primers with one in the forward primer and one in the reverse primer. Three additional bases were added in front of the restriction recognition site for efficient cleavage at the end of DNA fragment. PCR was performed according to Taq PCR Handbook (Qiagen) using 1ng plasmid DNA. The PCR product was then digested and subcloned into the desired plasmid cloning vector in a desired orientation.

2.4.3.6 BACTERIA TRANSFORMATION AND CULTURE

1µl of DNA (<10ng) and 20-50µl competent cells were incubated on ice for 30 min. XL-1 Blue supercompetent cells from Stratagene (Amsterdam Zuidoost, The Netherlands) were used for subcloning ligated DNA. The cells were then heat shocked at 42^{o}C for 45 sec and returned to ice for 2 min. The transformed cells were then spread on agar plates with appropriate selection antibiotics (ampicillin 100µg/ml in water or kanamycin 50µg/ml in water).

A single colony was picked from a plain LB agar plate and inoculated in 5ml of LB broth (Sigma) with suitable selection antibiotics, at 37^{o}C for 6 h in a shaking incubator. 1ml of this culture was then added to 250ml LB in a 500ml flask and left to shake in the 37^{o}C incubator overnight.

2.4.3.7 PLASMID MINIPREP AND MAXIPREP

DNA was purified from the overnight-grown culture using a Miniprep Plasmid Purification Kit (Qiagen). For isolation of larger amounts of DNA a Maxiprep Plasmid Purification Kit (Qiagen) was used. Both Kits were used according to the manufacturer's instructions.

2.4.3.8 SITE-SPECIFIC MUTAGENESIS

Mutagenesis of the two potential forkhead binding sites on the *VEGF* promoter cloned into a pGL3-Basic vector was achieved by using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). According to the protocol provided, oligonucleotides complementary to the *VEGF* promoter template were designed to contain alterations to enable nucleotide changes as appropriate.

	5'-Oligonucleotide-3'
Site 1 (-178)	ATCCCTCTTCTTTTTTCTTGGGCATTTTTTTTTAAAACTGTATTGT
Site 2 (-319)	TTGCTCTACTTCCCCGGGTCACTGTGGATTTTGGGGGCCAGCAGA

The mutant strand synthesis reaction was performed using PCR with the following cycling parameters: (1) 95°C 30 sec; (2) 95°C 30 sec; (3) 55°C 1 min; (4) 68°C 7 min; (5) Go to (2) ×29; (6) 4°C for ever. Generally, each reaction was prepared in a final volume of 25µl, containing 100ng of non-mutated template plasmid, 2.5µl of 10×reaction buffer, 0.7µl of both forward and reverse primers (100ng/µl), 1µl of dNTP mix, 3µl of Quick solution and 1µl of *Pfu Ultra* HF DNA polymerase (2.5U/µl).

2.4.4 POLYMERASE CHAIN REACTION (PCR)

PCR amplification was performed using a Taq DNA polymerase Kit (Qiagen) with a Gene Amp® PCR system 9700 (Applied Biosystems). Reaction solutions contained 1µg DNA template, 0.5µM primers, 200µM dNTP, 0.5U Taq DNA polymerase in a 25µl reaction. The PCR was performed on a Gene Amp® PCR system 9700 (Applied Biosystems) using the following program: denaturation step for 1 min at 94^{o}C, annealing for 1-2 min at 55-65^{o}C depending on the oligonucleotides used, extension at 72^{o}C for 1 min for 33 cycles followed by a final extension for 10 min at 72^{o}C. Optimisation of the primer annealing temperatures was performed by a gradient of increasing temperatures from 50-65^{o}C for 35 cycles, followed by change of magnesium concentration.

2.5 TRANSFECTION OF PLASMID DNA AND EXPRESSION VECTORS

Cells were seeded into six well plates or 10cm^3 dishes to a confluency of approximately 60%. Plasmid DNA was transfected using a ratio of Fugene 6 (µl) to DNA (µg) of 3:1. After 15 min of incubation, the fugene/DNA mixture was added directly to each well or plate and the cells were incubated for 6h at 37°C, after which the cells were washed in warm PBS and fresh media was added. 24 h after transfection, cells were either directly harvested for western blot and RT-qPCR analysis or re-plated for further experiments.

2.6 LUCIFERASE REPORTER ASSAYS

Luciferase assays were performed with the LucLite Luminescence Reporter Gene Assay System (Perkin ElmerTM Life Sciences, Milan, Italy) coupled with coelenterazine (Lux Biotechnology, Edinburgh, UK), according to manufacturer's instructions. Cells were lysed in 1xLucLite Reagent for 15 min at RT. 100µl of lysate were transferred to a 96 well microplate and firefly luciferase activity was read with TopCount Luminometer (Perkin ElmerTM Life Sciences). Afterwards, 25µl of RenLite reagent [Coelenterazine (50µg/ml) in Renilla Buffer (0.5M HEPES, pH7.8, 40mM EDTA)] was added and left in the dark for 20 min at RT before measurement for Renilla luciferase activity. The relative luciferase activity was determined by dividing firefly luciferase activity by renilla luminescence values. Reporter assays were performed in triplicates and repeated at least three times.

2.7 RNA INTERFERENCE

For the work in this thesis, genes were silenced using the SMARTpool TM product Insert – Version 2.0 (DHARMACON, RNA technologies). The SMARTpool siRNAs used were: FOXO3a (L-003007-00), siHDAC1 (L-003493-00), siHDAC2 (L-003495-00), FOXP1 (L-004256-01) and GFP (P-002048-02-20). The transfection reagent used was Oligofectamine (Invitrogen Ltd, UK).

Cells were plated into 6-well plates at 60% confluency 16 h prior to transfection. For each well, 70µl of optiMEM (Life Technologies Gibco/BRL, Paisley, UK) was mixed with 5µl of oligofectamine and incubated at RT for 10 min. 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 25 min incubation, 160µl optiMEM were added to each mix and 500µl were then added to the wells, which had been washed immediately before with warm PBS. After 4 h of incubation at 37°C, 2ml of normal media, with 10% FCS, were added 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. 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. 24-48 h post-transfection, cells were either harvested or re-plated for further protein and mRNA analysis by Western blotting or RT-qPCR.

2.8 FLUORESCENCE ACTIVATED CELL SORTING (FACS) ANALYSIS

Cells were harvested, washed and resuspended in 1ml PBS. Cells were fixed by resuspension in 90% ethanol in PBS and stored at -20°C until analysis. For analysis, samples were centrifuged at 2000rpm to remove the ethanol, washed twice in PBS, and stained with propidium iodide (50µg/ml) and RNase A (5µg/ml) for 1 h at RT in the dark. Samples were analysed on a FACSDiva flow cytometer (BD Biosciences) using the FACSDiva^{TM} (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).

2.9 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. Cells were 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 and then 100µl of SRB solution (0.4% SRB in 0.1% acetic acid) were added. Plates were left at RT for 1 h, then washed with 1% acetic acid to remove any unbound dye. The plates were left to air-dry overnight and then stored. To read the plates, 100µL Tris (10mmol/L) was added to each well to solubilise the bound sulforhodamine B dye and placed on a rotator for 30 min to evenly distribute the dye. The plates were then read at 492nm using the Sunrise plate reader (Tecan Group Ltd, Mannedorf, Switzerland).

2.10 IMMUNOHISTOCHEMICAL (IHC) STAINING

In the tissue microarray used in this thesis, 133 breast cancer cases which were diagnosed between the years 1992 to 2001 with clinical follow-up data, were retrieved from the records of the Department of Pathology, Queen Mary Hospital, Hong Kong. The age of patients at diagnosis ranged from 30 to 90 years old, with a mean of 53 years. Histological

sections of all cases were reviewed by pathologists. The representative paraffin tumour blocks were chosen as donor block for each case and used for construction of tissue microarray blocks. Of all cases, 38 tumour cases had non-tumour tissues available for comparison with their tumour counterparts in protein expression studies. A total of 120 cases could be assessed and scored for FOXO3a, P-AKT, VEGF, FOXM1 and FOXP1 expression. The expression pattern and subcellular localisation were correlated with clinical stage, histological type, histological grade, lymph node metastasis, survival time, HER-2 overexpression status, and ER and PR status. 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 and finally in tap water. The slides were then placed in 1l of pre-warmed citrate buffer (0.01M sodium citrate, pH 6) and then heated for 15 min at full power 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 dish containing warmed PBS and washed twice in PBS. To block endogenous peroxidase activity, which may result in high, non-specific background staining, slides were then immersed in 3% H_2O_2 in methanol for 10 min at room temperature. This resulted in the irreversible inactivation of endogenous peroxidase. Slides were then washed again twice in PBS for 5 min. The dilution of antibody used was determined by first performing a titration of antibody dilutions, to optimise tissue staining whilst eliminating excessive background or non-specific staining. For example, for optimisation of FOXO3a staining with the FOXO3a anti-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. The same procedure was used for optimisation of P-AKT, FOXP1, FOXM1 and VEGF antibodies.

The primary antibodies were added to each sample and the slides were incubated overnight at 4°C in a closed humidified incubation chamber. On the following day, the slides were washed three times in PBS. Then 4-5 drops of DAKO Polymer was applied on each section and incubated at room temperature for 30 min. The slides were washed again in PBST for two changes. Chromogen DAB/substrate reagent solution (DAKO REAL EnVision Detection System, DAKO, Denmark) (one drop of DAB in 1ml substrate reagent) was freshly prepared and 200µl of the reagent were added onto each section for 3 min incubation until colour development could be observed. Colour development was stopped by washing the slides in tap water. The sections were counterstained in Haematoxylin

(DAKO, Denmark) for 5 min and de-stained in acid-alcohol for 5 sec. The slides were placed in Scott tap water until the sections grossly turned bluish, followed by washing them in running tap water. Finally the slides were dehydrated with increasing concentrations of ethanol followed by clearing in xylene. 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.

The staining was assessed at high power (×40) under a light microscope. The intensity and percentage of staining either in the cytoplasm or in the nucleus were scored in a semi-quantitative method. The intensity of staining was scored as follows: 1 = weak, 2 = moderate, 3 = strong, while the percentage of cells positively stained was scored as follows: 1 = < 25%, 2 = < 50%, 3 = 75%, 4 = > 75%. The final score for each case was obtained by multiplying the score of intensity with the score of percentage.

2.11 IMMUNOFLUORESCENT STAINING

Cells were seeded in four-well chamber culture slides (BD Falcon, Erembodegem, Belgium). 24 h following plating, the appropriate treatments were added to the wells and left for the required time. Cells were then washed twice with warm PBS and fixed with 4% formaldehyde in PBS for 10 min at RT. The fixative was removed and the cells were washed four times with PBS for 5 min. The cells were then permeabilised with 0.2% triton in PBS for 10 min, washed again three times with PBS, followed by blocking with 5% goat serum in 10% BSA for 30 min at RT. The primary anti-rabbit FOXO3a (H144, Santa Cruz Biotechnology), anti-rabbit FOXP1 (ab16645, Abcam) or anti-rabbit ERα (sc-543, Santa Cruz) antibodies were added at a dilution of 1:100 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 488 anti-rabbit, Invitrogen) was then added (1:500 dilution in 10% BSA) and the cells were incubated in the dark for 45 min. The cells were washed again three times in PBS for 5 min each. Cells were then incubated in DAPI (Invitrogen) for nuclear staining (1:1000 dilution in 10% BSA) for 20 min in the dark. The chamber was removed from the slides and one drop of Vectashield mounting medium (Vector laboratories Inc., Burlingame, CA) was added on each well, covered with a glass coverslip and sealed with clear nail polish. The slides were analysed using a Zeiss confocal laser scanning microscope with the respective software (Zeiss Ltd).

2.12 CHROMATIN IMMUNOPRECIPITATION (ChIP)

Cells at 90% confluence in 100mm culture dishes with or without drug treatment were cross-linked for 10 min at 37°C with fomaldehyde (Sigma). Cells were then rinsed in ice-cold PBS and scraped of the dish in 1ml of scraping buffer (recipes for the buffer used, see Appendix 3). After washing in PBS, Buffer I and Buffer II sequentially, the cell pellet was resuspended in 300µl Lysis buffer and sonicated (12 min in total with 30 sec on and 30 sec off). After removing the insoluble fraction, the cell lysate was mixed with 300µl buffer D and 100µl was then taken as the INPUT control. Dynabeads Protein A (Invitrogen Dynal A.S., Oslo, Norway) were first washed with TSE I buffer three times before binding with the primary antibody (1µg) overnight at 4ºC, while dynabeads with IgG were used as negative control. The primary antibodies used for immunoprecipitation were the anti-FOXO3a (H-144), anti-FOXM1 (C-20), anti-ERα (sc-543), anti-H4K16 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-H3K9 (Cell Signalling Technologies), anti-HDAC2 and anti-FOXP1 (both from Abcam), and the anti-IgG control (DAKO, USA). A total of 500µl sonicated lysate was then added to the dynabeads and rotated overnight at 4ºC. After washing the dynabeads five times with TSE I and twice with TE buffer, 100µl of Elution buffer were added to the dynabeads and the mixture was rotated at RT for 1 h. The eluted supernatant was then transferred to a collection eppendorf while 100µl fresh elution buffer was added to elute one more time at RT for 1 h. Finally, a total of 200ul of sample were de-crosslinked by incubation at 65ºC overnight. Qiagen PCR Purification Kit (QIAGEN, UK) was used to extract DNA from the de-crosslinked sample. Approximately 30µl of DNA were eluted from each sample and 3µl of each were used in one PCR reaction later. The primer sequences used in the PCR are listed below.

FOXO binding sites	**Forward (5'-3')**	**Reverse (5'-3')**
VEGF promoter	TCCGGGTTTTATCCCTCTTC	TCTGCTGGTTTCCAAAATCC
FOXP1 promoter site1	TGCAGAATCAGATTAGCAGCAATC	CAATTCATCCTCTGATCTTGTTTTTC
FOXP1 promoter site2	ACGGGAGAGAGGGAGAAAGG	GTTTTGCCATCAGTGACAGTTGA
FOXP1 promoter site3	CCCCGCTCTCCTTCAACTG	GGACAGGCTAGAATGTGAGTTTCC

Analysis of the PCR products was performed on a standard 2% (w/v) agarose gel by electrophoresis in Tris-acetate-EDTA buffer. After amplification, PCR products were visualised on an ethidium bromide-stained 2% agarose gel, using a Transiluminator.

2.13 PULL-DOWN USING BIOTIN-LABELLED OLIGONUCLEOTIDES

Biotinylated oligonucleotides (Invitrogen) were coupled to streptavidin beads (Sigma) in a solution containing 100µl PBS, 10µl FCS, 20µl of a 50% slurry of streptavidin-agarose beads (Sigma) and 1µl of annealed biotinylated oligonucleotide. Beads were left to incubate with the oligonucleotides while rotating for 2 h at RT. Following incubation, the oligo-coupled beads were spun down at 12000rpm on a bench top centrifuge for 2 min. The supernatant was discarded and 50 µg of nuclear lysate was diluted in 2x volumes of low salt and added to each of biotinylated oligo-coupled beads along with 1µg of sheared salmon sperm DNA and competitor non-biotinylated double stranded oligonucleotides containing a wild-type, high affinity or mutant consensus site for prospective binding proteins. Samples were incubated with rotation at 4^{o}C for 1 h and subsequently incubated at 30^{o}C for 10 min. The beads were then washed 6 times in PBS containing protease inhibitors. Beads were separated from the washing buffer by centrifugation at 3000rpm for 2 min, loading buffer was added and the samples were then loaded in an SDS-PAGE gel and the prospective binding proteins detected by western blotting. The sequences of oligonucleotides used for pull down assays are summarised below.

Oligonucleotides	**Sequence**
WT FOXO3a F Biotin	Biotin-GTTTTATCCCTCTTCTTTTTTCTTAAACATTTTTTAAA
WT FOXO3a F	GTTTTATCCCTCTTCTTTTTTCTTAAACATTTTTTAAA
WT FOXO3a R	TTTAAAAAAAATGTTTAAGAAAAAAGAAGAGGGAT
MT FOXO3a F Biotin	Biotin-GTTTTATCCCTGTTCTTTTTTCTTGGGCATTTTTTAAA
MT FOXO3a F	GTTTTATCCCTCTTCTTTTTTCTTGGGCATTTTTTAAA
MT FOXO3a R	TTTAAAAAAAATGCCCAAGAAAAAAGAAGAGGGAT

2.14 ENZYME-LINKED IMMUNO SORBANT ASSAY (ELISA)

VEGF and FGF7 concentrations were measured by a quantitative sandwich enzyme immunoassay (Quantikine ELISA R&D). Microplates were pre-coated with a specific VEGF or FGF7 monoclonal antibody and 50µl of assay diluent RD1W were added to the wells. 200µl of either cell supernatants or standards were loaded in triplicates to the wells and were left to incubate at room temperature in order for the soluble VEGF or FGF7 to bind to the immobilised antibody. After washing four times with 400µl of washing buffer, 200µl of VEGF or FGF7 conjugate (an enzyme-linked VEGF or FGF7 polyclonal antibody solution) was added to the wells. The plates were incubated for 2 h at RT. After washing, 200µl of substrate solution were added to the wells and colour was developed. After 20 min incubation, 50µl of top solution were added. The colour change was then stopped and its optical density was measured at 450nm (Sunrise-Tecan) within 30 min.

2.15 CO-IMMUNOPRECIPITATION

Co-immunoprecipitation (Co-IP) was performed to detect protein-protein interactions. The proteins were extracted using a Co-IP lysis buffer (50mM Hepes pH 7.5, 150mM NaCl, 2mM EDTA, 10% glycerol (v/v), 0.5% Triton-X (v/v), 10mM NaF, 10mM β-Glycerophosphate pH7.3, 0.1mM Na_3VO_4, 100U/ml aprotinin, 10µg/ml leupeptin and 1mM PMSF), which has a suitable formulation to perform the precipitation of the target protein and co-precipitation of binding partners/protein complexes. The lysates were pre-cleared with agarose beads (15µl per sample) for 30 min. After centrifugation, the supernatant was transferred to a new eppendorf tube. Protein concentration was then determined and 0.3-0.5mg of protein extract was incubated with the antibody and beads for 2-3 h rotating at 4˚C. The antibodies used in this step were the same as the ones used for ChIP except for the FOXP1 antibody. Immunoprecipitates were then washed, by centrifugation, 3 times with the same lysis buffer and samples were prepared for separation on a SDS-PAGE gel.

CHAPTER 3

PI3K/AKT/FOXO3A AXIS IN CHEMORESISTANCE

3.1 INTRODUCTION

The PI3K/AKT signalling pathway has been well documented to regulate a plethora of fundamental biological processes including those involved in enhanced cell proliferation and growth. Thus, it is not surprising that this signalling axis has also been implicated in tumorigenesis (Hennessy et al 2005, Jiang and Liu 2008). In cancer cells, in addition to various receptor tyrosine kinases (RTKs), such as HER-2, many other upstream molecules feed into the PI3K/AKT activity and culminate in increased cell proliferation, cell survival, angiogenesis, invasion and metastasis (Browne et al 2009, Tokunaga et al 2006). As a consequence of its aberrant expression in cancer cells, many reports have showed that the PI3K/AKT pathway is associated with therapeutic resistance (Campbell et al 2001, Clarke et al 2001a, Clarke et al 2001b). FOXO3a is one of a panoply of downstream targets, negatively regulated by AKT, which functions as an obstacle for cell proliferation and growth (Brunet et al 1999, Kops et al 2002b). FOXO3a has been showed to serve as therapeutic target in various cancers by mediating the cytotoxic and cytostatic effects of several chemotherapeutic drugs. For example, in breast cancer cells paclitaxel has been shown to induce the nuclear translocation and accumulation of FOXO3a to induce apoptosis (Sunters et al 2003). Furthermore, ER-negative breast cancer cells are sensitised to tamoxifen by OSU-03012, a small-molecule inhibitor of PDK1, through activating FOXO3a (Weng et al 2008). Various studies have shown that FOXO3a mediates the cytotoxic effects of cisplatin in oral squamous cancer (Mauri et al 2006), colon cancer (Ligibel and Winer 2005), ovarian cancer (Baum et al 2002) and bladder cancer (Giaccone and Pinedo 1996). Similarly, in CML cells, the cytotoxic effect of doxorubicin is also mediated by the nuclear accumulation of FOXO3a (Hui et al 2008a, Hui et al 2008b). However, sustained FOXO3a activation by doxorubicin also promotes drug-resistance and survival of leukaemic cells by activating the expression of genes important for drug efflux (e.g. ABCB1) and survival (e.g. PIK3CA) (Hui et al 2008a, Hui et al 2008b, Yang and Hung 2009).

3.2 AIMS & OBJECTIVES

FOXO3a is undoubtedly a transcription factor preferentially targeted by cancer therapeutic drugs. Furthermore, in cancer cells FOXO3a expression mediates the drug impact and determines the cellular decisions that eventually lead to survival or death. However, the molecular mechanisms behind FOXO3a-mediated sensitivity and chemoresistance are currently unclear. As a consequence, it is reasonable to study the PI3K/AKT/FOXO pathway and its impact on drug sensitivity or resistance in breast cancer and explore its use as prognostic clinical tools.

3.3 RESULTS

3.3.1 Tamoxifen-resistant breast cancer cell lines are sensitive to doxorubicin treatment

In order to assess the resistance profile of different breast cancer cell lines *in vitro*, I analysed the proliferation rate of a panel of chemotherapy sensitive and resistant breast cancer cell lines treated with tamoxifen or doxorubicin. LCC2, LCC9 and R27 are MCF-7 (LCC1) endocrine-resistant derivate cell lines. Cell proliferation was determined by SRB assays following the incubation of cells with increasing amounts of tamoxifen for six days. The results showed that both the parental MCF-7 and the R27 derivative cell lines are sensitive to tamoxifen while LCC2 and LCC9, as well as the BT474 cells are resistant to tamoxifen treatment up to concentrations of 5μg/ml (Figure 5). I also generated a cytotoxic chemotherapy resistant MCF-7 cell line through continuous exposure up to 10μM doxorubicin. The proliferative analysis of this MCF-7Dox^R cell pool, confirmed that its proliferation is not significantly affected by doxorubicin treatment when compared to the parental MCF-7 cells proliferation which was severely inhibited when incubated with ＞5μM of doxorubicin (Figure 6A). Similarly, the multidrug resistant breast carcinoma cell line MDA-MB-231 also displayed resistance to 1μM of doxorubicin, a dose that effectively inhibited the parental MCF-7 cell proliferation (Figure 6B). Notably, the tamoxifen-resistant BT474 cells as well as the MCF-7 derived cell lines, including LCC2, LCC9 and R27, were all found to be sensitive to doxorubicin treatment at 1μM (Figure 6C).

These results showed that the MCF-7 Dox^R and MDA-MB-231 cell lines represent advanced breast cancers of poor prognosis with limited treatment options. This finding corroborates with the clinical evidence that breast cancers which failed endocrine treatments are often intrinsically resistant to cytotoxic chemotherapy (Greenberg et al 1996).

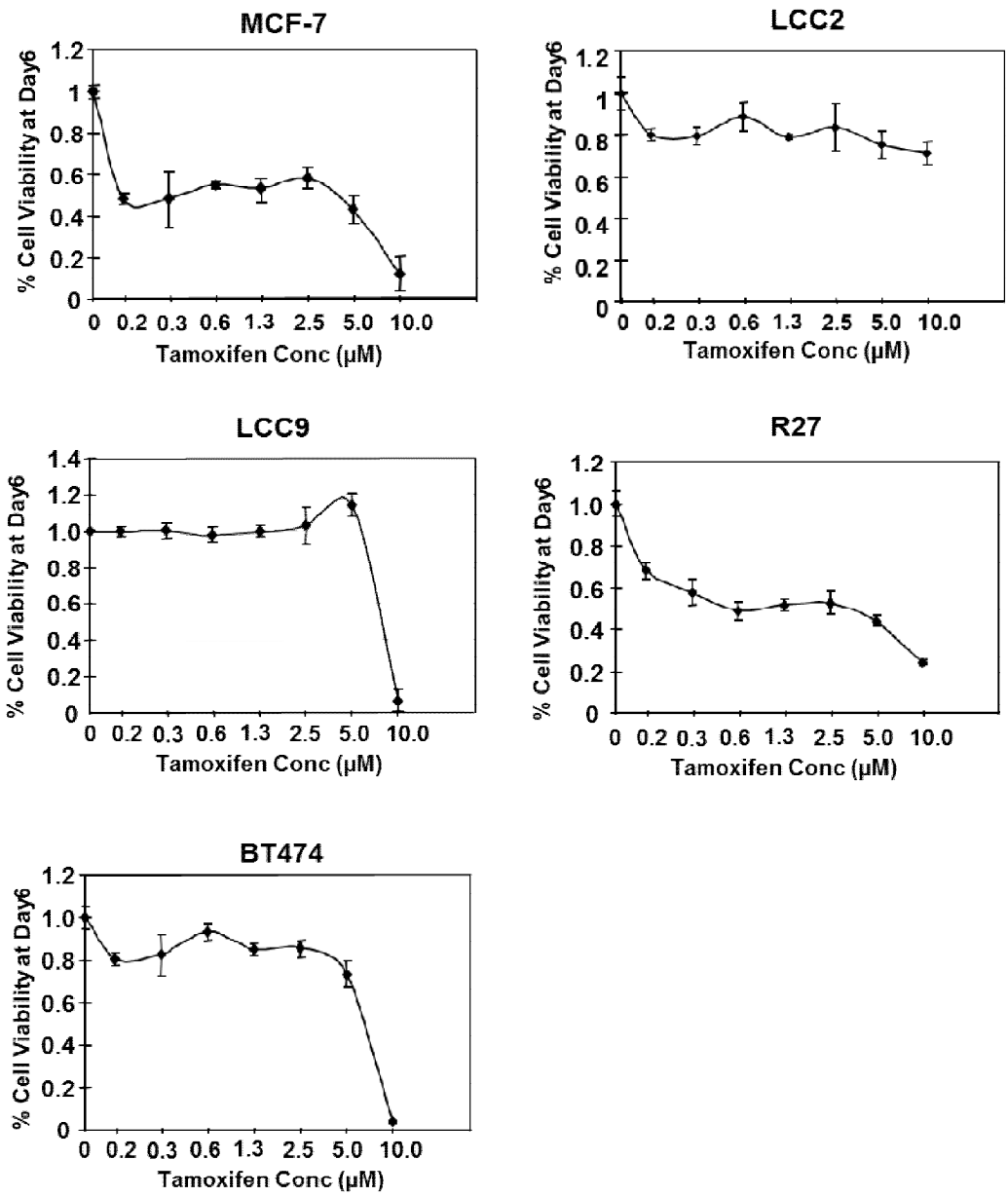


Figure 5. Effects of tamoxifen on cell proliferation of a panel of breast cancer cell lines.

MCF-7(LCC1), LCC2, LCC9, R27 and BT474 cells were treated with 0 to 10μM of tamoxifen for 6 days. Cell proliferation was determined by SRB assay. Points, mean of three independent experiments; bars, SD.

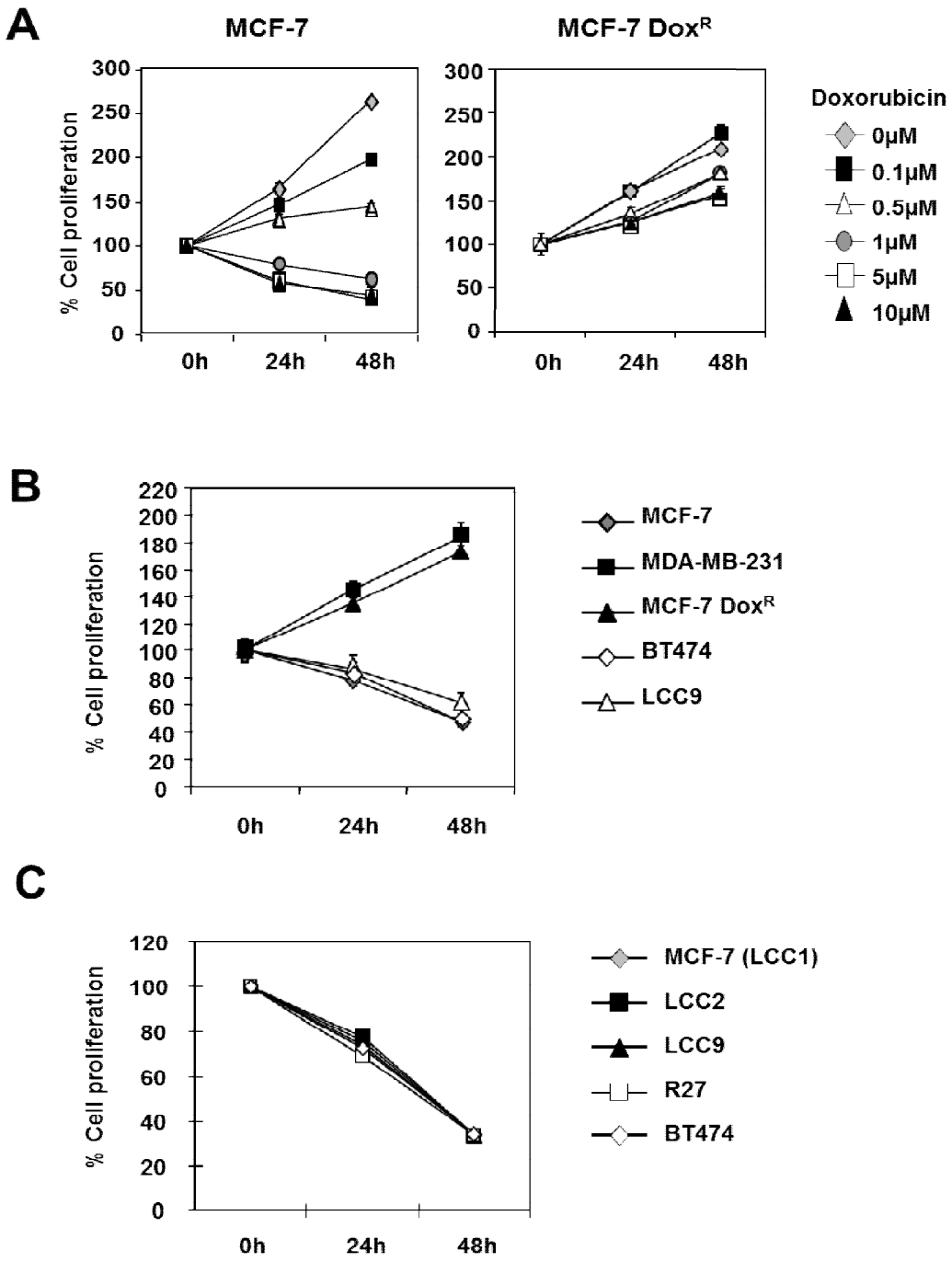


Figure 6. Effects of doxorubicin on cell proliferation of a panel of breast carcinoma cell lines.

A) MCF-7 and the derived MCF-7 Dox^R cells were treated with 0 to 10μmol/L of doxorubicin for 0, 24, and 48 h. **B**) MCF-7, MCF-7 Dox^R, MDA-MB-231, BT474, and LCC9 cells were treated with 1μmol/L of doxorubicin for 0, 24, and 48 h. **C**) MCF-7(LCC1), LCC2, LCC9, R27 and BT474 cells were treated with 1μmol/L of doxorubicin for 0, 24, and 48 h. Cell proliferation was determined by SRB assay. Points, mean of three independent experiments; bars, SD.

3.3.2 Predominant nuclear FOXO3a localisation in doxorubicin but not tamoxifen-resistant cell lines

To explore the potential role of FOXO3a and its upstream regulator AKT in the development of drug-resistance, I examined the basal expression levels of both the total and phosphorylated forms of FOXO3a and AKT in the tamoxifen and doxorubicin drug sensitive as well as resistant breast cancer cell lines. Western blot analysis showed that the basal levels of P-AKT (S473), FOXO3a and P-FOXO3a (T32) were higher in the tamoxifen-resistant cell lines compared to the sensitive parental MCF-7 (LCC1) cells (Figure 7A), suggesting that PI3K/AKT activity is enhanced in endocrine resistant cells.

Interestingly, P-AKT and total AKT were expressed at similar levels in the doxorubicin sensitive MCF-7 and resistant MCF-7 Dox^R and MDA-MB-231 cells. By comparison, the P-FOXO3a expression was low in the MCF-7 Dox^R while in MDA-MB-231 cells P-FOXO3a expression was higher. The reason for this decrease in FOXO3a activity in MDA-MB-231 is unclear. Nevertheless, the expression levels of FOXO3a were high in both the MCF-7 Dox^R and MDA-MB-231 cells (Figure 7B). These results indicate that in doxorubicin resistant cells, especially in MCF-7 Dox^R, there is a substantial amount of active FOXO3a and this non-phosphorylated FOXO3a escapes the negative regulation of AKT further suggesting that the AKT-FOXO3a axis is uncoupled in doxorubicin resistant cells. Because of this result, I next examined the subcellular localisation of FOXO3a in the cytoplasmic and nuclear extracts of these tamoxifen and doxorubicin drug sensitive and resistant breast cancer cell lines (Figure 8A). Tubulin and Lamin B were used as controls for cytoplasmic and nuclear fractions, respectively. Western blotting results showed that FOXO3a was present at considerably higher levels in the cytoplasmic compartment compared with the nuclear fractions in all doxorubicin sensitive breast cancer – MCF-7, BT474, LCC2, LCC9 and R27. In contrast, in the doxorubicin-resistant breast cancer cells, MCF-7 Dox^R and MDA-MB-231, FOXO3a expression was higher in the nuclear compartment of these cells (Figure 8A). These results suggest that FOXO3a expression is predominantly nuclear in the doxorubicin-resistant cells whereas in the doxorubicin-sensitive cells and also in the tamoxifen-resistant BT474, LCC2 and LCC9 cells lines, FOXO3a expression is primarily cytoplasmic.

To confirm the subcellular fractionation results and to directly visualise the subcellular distribution of FOXO3a in the two doxorubicin-sensitive and in the drug-resistant, I performed immunofluorescence staining (Figure 8B). Consistently with my previous results, FOXO3a staining was primarily located in the cytoplasm of the doxorubicin-sensitive MCF-7 and BT474 cell lines whereas this localisation shifted to the nucleus in doxorubicin-resistant MCF-7 Dox^R and MDA-MB-231 cells (Figure 8B). This result further

indicates that nuclear FOXO3a localisation could be associated with the development of cytotoxic drug-resistance.

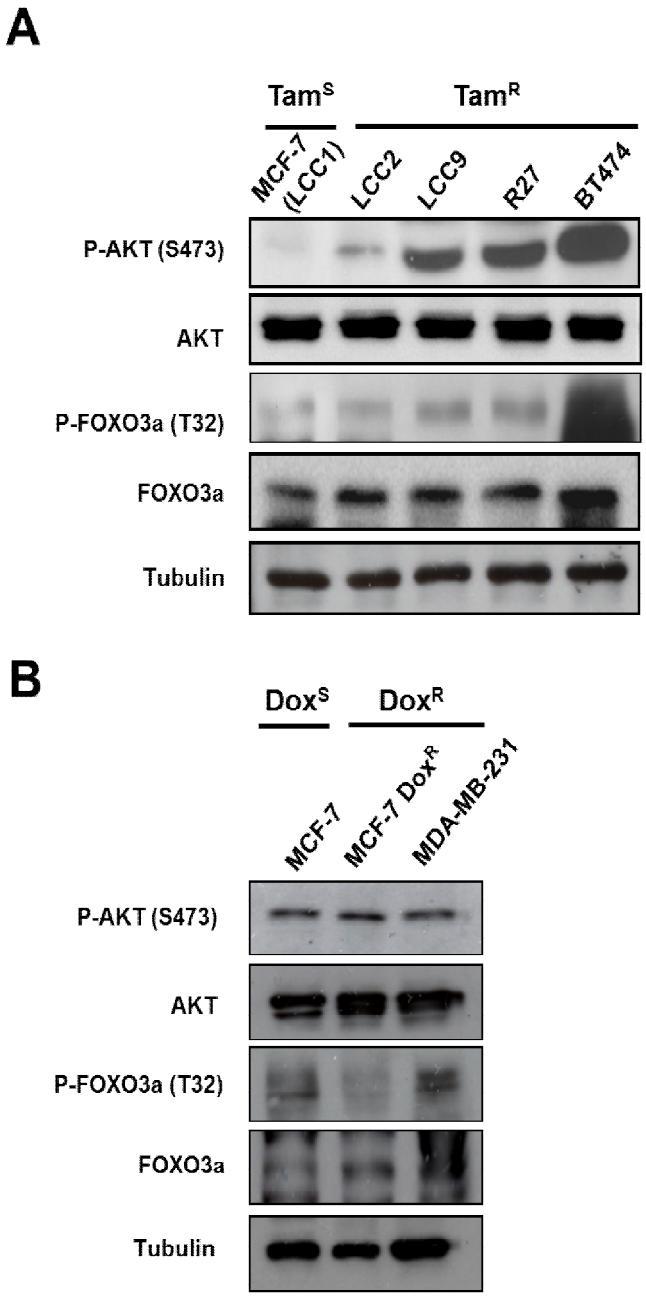


Figure 7. Expression of total and phosphorylated AKT and FOXO3a in a panel of tamoxifen and/or doxorubicin sensitive and resistant breast carcinoma cell lines.

A) The tamoxifen sensitive MCF-7 (LCC1), and resistant LCC2, LCC9, R27 and BT474 cells as well as the **B**) doxorubicin sensitive MCF-7 and resistant MCF-7-Dox^R and MDA-MB-231 cells were cultured in normal growth medium and used for western blot analysis for P-AKT (S473), total AKT, P-FOXO3a (T32), total FOXO3a and Tubulin.

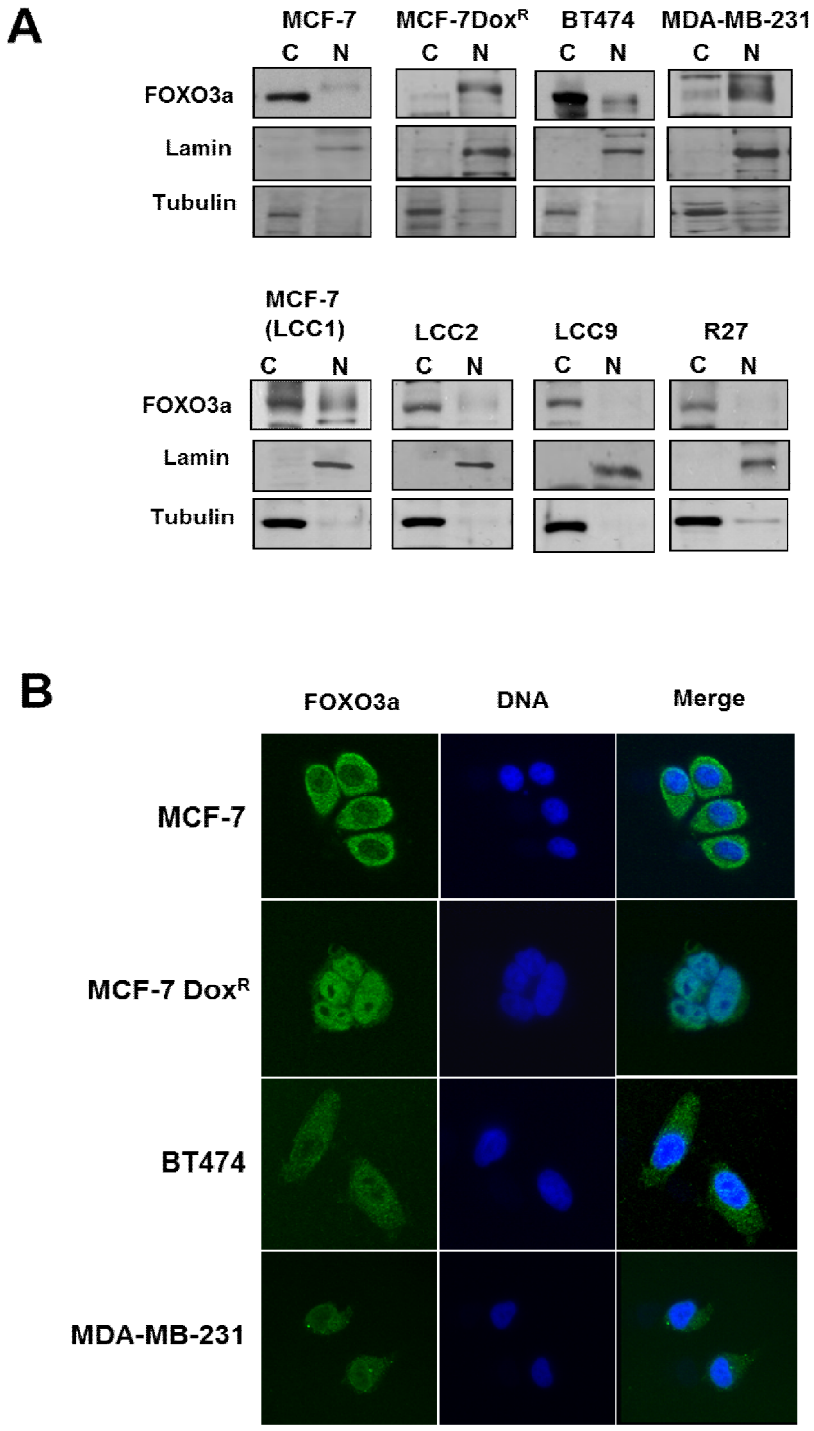


Figure 8. Subcellular location of FOXO3a in a panel of tamoxifen and/or doxorubicin sensitive and resistant breast carcinoma cell lines.

A) Cytoplasmic and nuclear protein lysates were prepared and protein expression levels were analysed by Western blotting using specific antibodies against against FOXO3a, Lamin B and Tubulin. **B**) Cells were stained for FOXO3a with ALEX488 (green). DAPI (blue) were also applied to visualise the nuclei.

3.3.3 Doxorubicin induces FOXO3a nuclear relocation in drug-sensitive cells

To explore the potential role of FOXO3a in drug response and resistance, I assessed FOXO3a expression in the cytoplasmic and nuclear fractions of the sensitive MCF-7 and the resistant MCF-7 Dox^R and MDA-MB-231 cells. Cell extracts were analysed by Western blot following doxorubicin treatment over a 24 h timecourse. Tubulin and Lamin B were probed as controls for effective nuclear and cytoplasmic fractionation. As shown in Figure 9. Doxorubicin treatment causes a nuclear relocation of FOXO3a expression.FOXO3a expression levels increased in the nucleus, and this was accompanied by a reciprocal decrease in levels in the cytoplasm in the sensitive cells, 12 and 24 h following doxorubicin treatment (Figure 9. Doxorubicin treatment causes a nuclear relocation of FOXO3a expression.A). In contrast, FOXO3a expression levels remained unchanged in both the cytoplasmic and nuclear fractions of the resistant MCF-7 Dox^R and MDA-MB-231 cells in response to doxorubicin. Notably, FOXO3a nuclear accumulation was more pronounced in MCF-7 Dox^R than in MDA-MB-231.

Again, to support my subcellular fractionation results, I performed immunofluorescence to determine the subcellular distribution of FOXO3a in both doxorubicin-sensitive and the drug-resistant cells in the absence or presence of doxorubicin for 16 h (Figure 9B). Consistent with the results in Figure 5A, FOXO3a relocated to the nucleus in the doxorubicin-sensitive MCF-7 cells following doxorubicin treatment, while in the doxorubicin-resistant cells FOXO3a subcellular distribution remained largely unchanged even upon doxorubicin treatment (Figure 9B).

Together these results suggest that doxorubicin causes FOXO3a to relocate to the nuclear compartment in the drug-sensitive breast cancer cells and confirmed my previous data in Figure 8A, that FOXO3a expression is predominantly nuclear in the resistant cells.

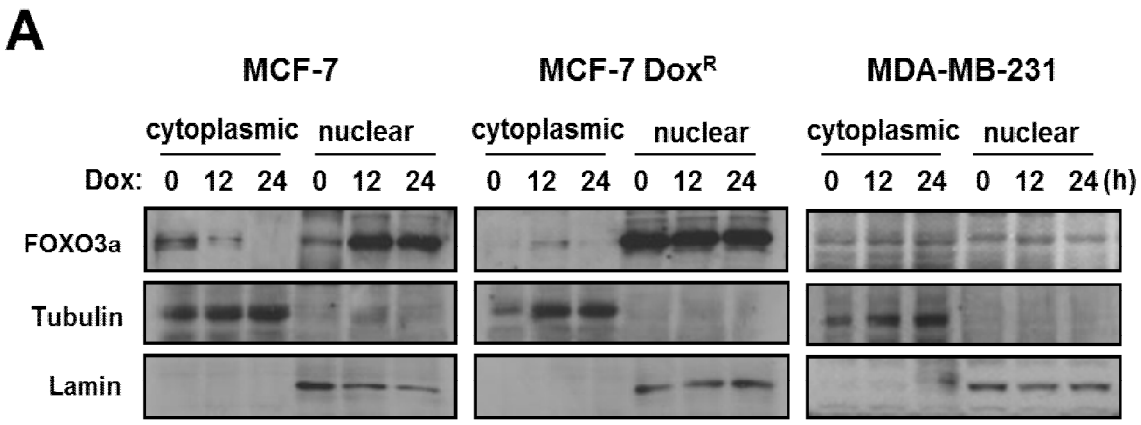


Figure 9. Doxorubicin treatment causes a nuclear relocation of FOXO3a expression.

The doxorubicin sensitive MCF-7 and resistant MCF-7-Dox^R and MDA-MB-231 cells were treated with 1µmol/L doxorubicin. **A**) Cytoplasmic and nuclear protein lysates were prepared at the times indicated after doxorubicin treatment and protein expression levels were analysed by Western blotting using specific antibodies against FOXO3a, Lamin B and Tubulin. **B**) The cells were cultured in four-well chamber culture slides and treated for 16 h with or without 1µmol/L doxorubicin, before being fixed in 4% formaldehyde. FOXO3a expression was visualised with a specific antibody against FOXO3a followed by the addition of ALEX488 (green) labeled anti-rabbit antisera. DAPI (blue) was also applied to visualise the nuclei.

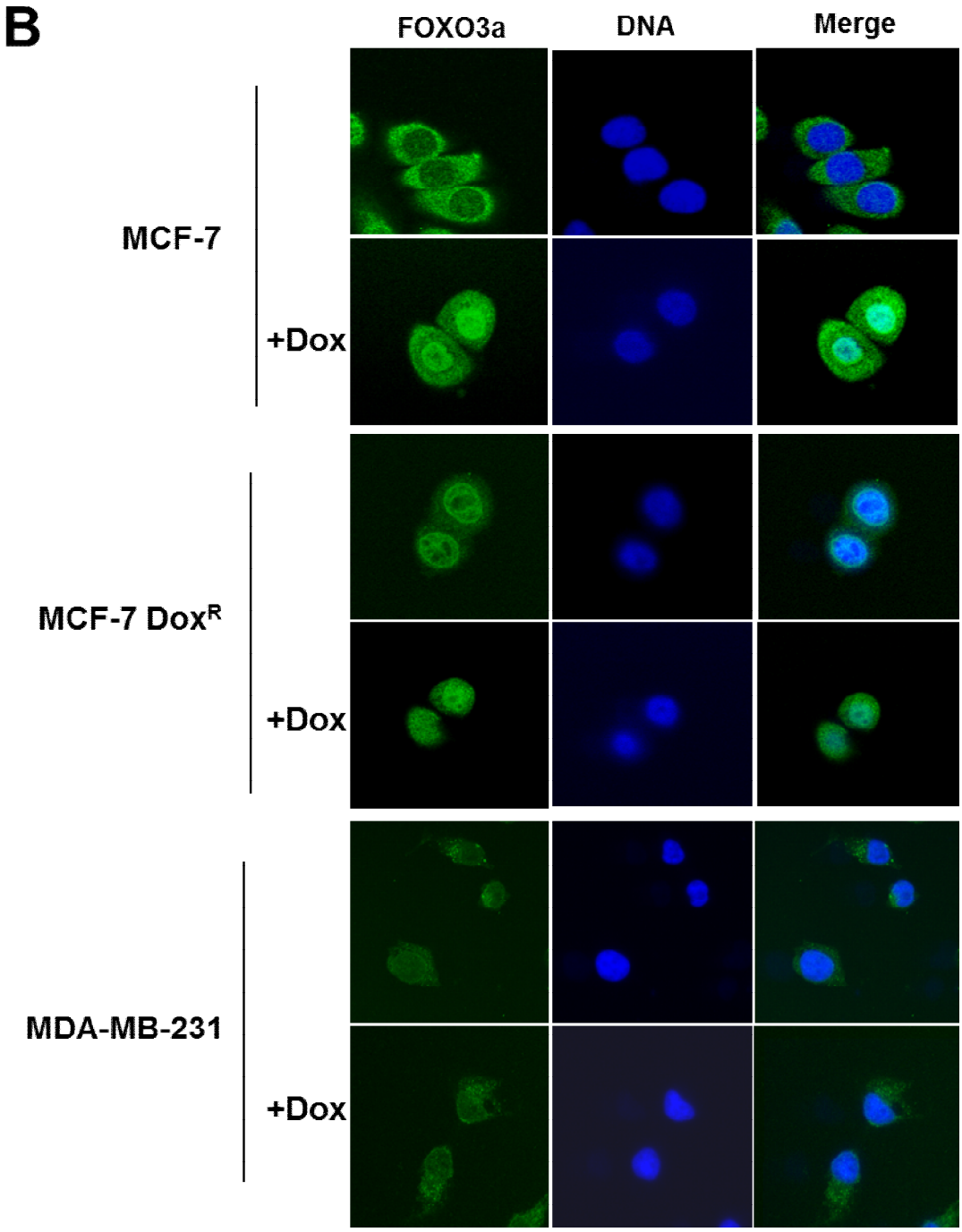


Figure 9. Continued.

3.3.4 Ectopic FOXO3a expression results in proliferative arrest and enhances AKT phosphorylation in cytotoxic drug sensitive cells

To determine the role of FOXO3a in drug response, I investigated the effects of the overexpression of FOXO3a in the drug sensitive MCF-7 cell line. To this end, I transiently transfected MCF-7 cells with empty vector (pcDNA3) or an expression vector encoding a constitutively active FOXO3a(A3), in which all three AKT-phosphorylation sites were mutated to alanine. FOXO3a overexpression was confirmed by Western blot and RT-qPCR results (Figure 10A & 10B). The results also showed that FOXO3a overexpression resulted in enhanced AKT phosphorylation without altering total AKT expression levels. This is consistent with a previous report from our laboratory, where FOXO3a was shown to engage in a negative feedback mechanism to increase PI3K/AKT activity through induction of *PIK3CA* gene in leukaemic cells (Hui et al 2008b). Furthermore, this result also supports the notion that FOXO3a enhances the expression of *PIK3CA* transcriptionally (Figure 10B). I also examined the effect of overexpressing FOXO3a on cell proliferation in MCF-7 cells by SRB assay. FOXO3a overexpressing cells displayed an attenuated cell proliferation, indicating that doxorubicin inhibition of cell proliferation is at least in part FOXO3a-mediated in the cytotoxic chemotherapeutic resistant breast cancer cells (Figure 10C).

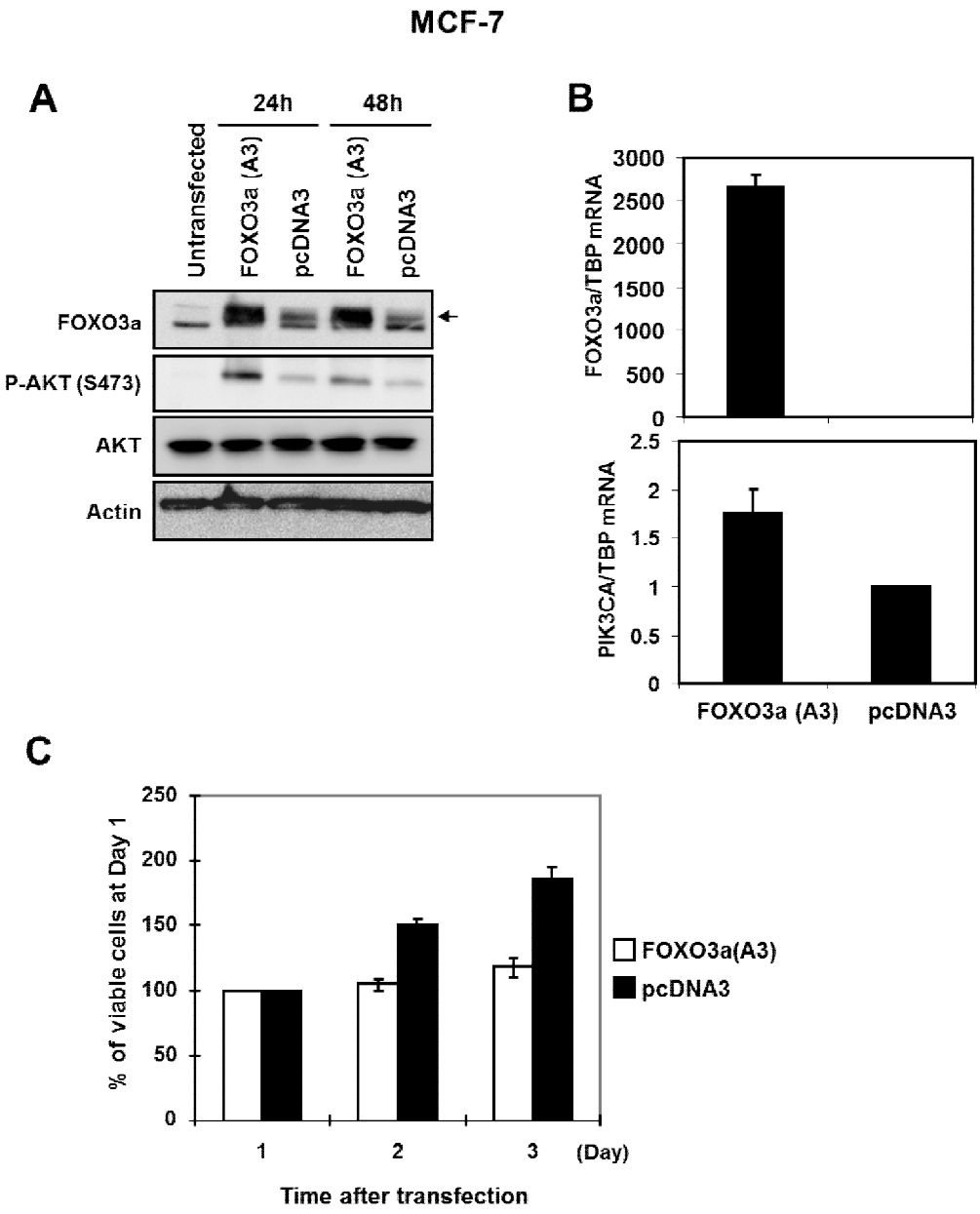


Figure 10. Overexpressed active FOXO3a results in cell proliferation arrest and AKT phosphorylation in drug-sensitive breast cancer cell line MCF-7.

MCF-7 cells were transiently transfected with the constitutively active FOXO3a(A3) or control vector. **A**) Western blot analysis for FOXO3a, P-AKT (S473), AKT and Actin. **B**) The transfected cells were also examined by RT-qPCR analysis for FOXO3a and PIK3CA mRNA expression. TBP mRNA was used as an internal control. **C**) SRB assays were performed on these transfected cells, indicating that the ectopic expression of FOXO3a(A3) decreases the cell proliferation rate.

In order to confirm the observation that FOXO3a regulates AKT activity, scramble control and FOXO3a-specific shRNAs were used to knockdown the expression of the endogenous FOXO3a in the doxorubicin-sensitive BT474 cell line which exhibited enhanced PI3K/AKT activity as previously shown in Figure 7. The effect of silencing FOXO3a with shRNAs in BT474 cells was confirmed at protein level by Western blot analysis (Figure 11A) and mRNA level by RT-qPCR (Figure 11B). Importantly, silencing FOXO3a dramatically reduced the P-AKT levels (Figure 11A) and induced a reduction in the levels of *PIK3CA* transcripts (Figure 11B).

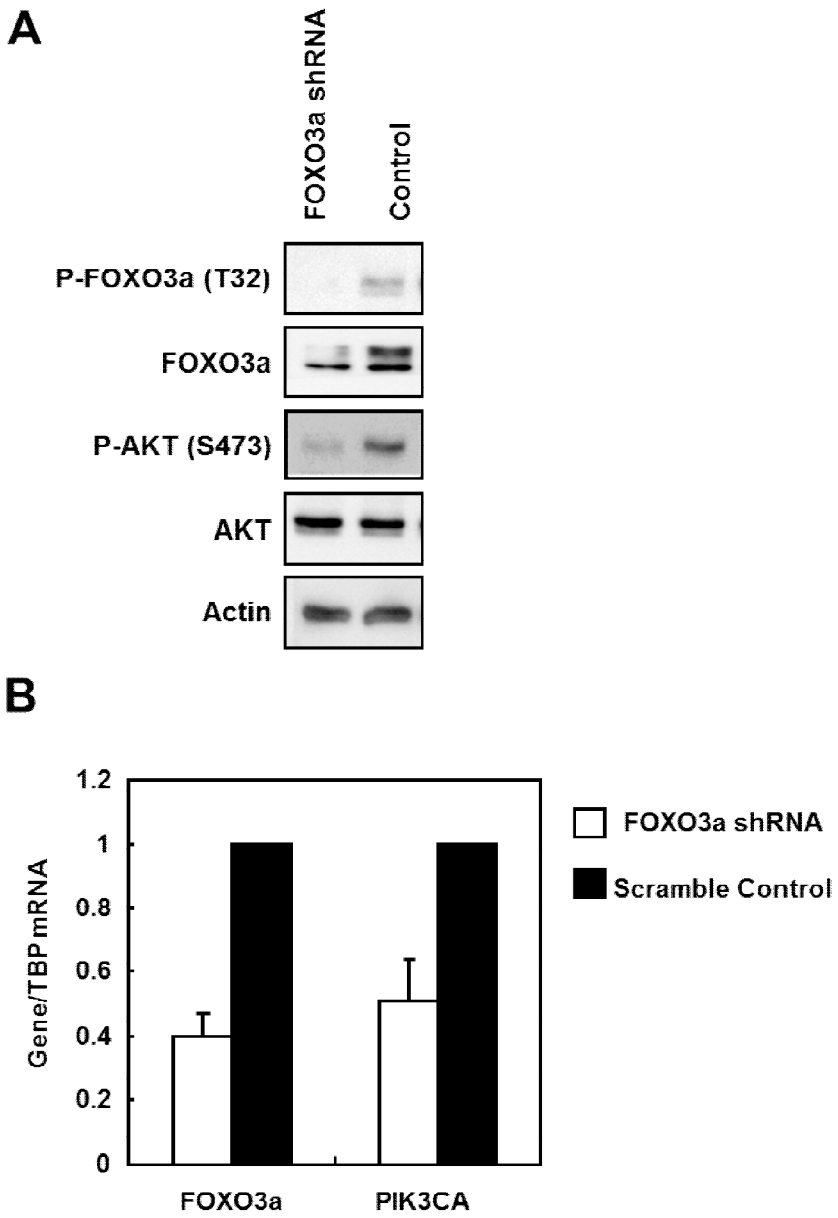


Figure 11. Knockdown of FOXO3a expression decreases AKT phosphorylation and PIK3CA mRNA expression.

BT474 cells were transiently transfected with FOXO3a or control shRNA, and 24 h after transfection cells were analysed by **A**) Western blot using specific antibodies as indicated and by **B**) RT-qPCR.

3.3.5 FOXO3a activation promotes AKT phosphorylation but not proliferative arrest in drug-resistant breast cancer cells

To study the effects of FOXO3a induction and activation in drug-resistant breast cancer cells, I made use of a stably transfected MDA-MB-231 cell line with an expression vector that encodes for the hormone binding domain of the estrogen receptor (ER) fused to FOXO3a(A3), the constitutive active FOXO3a mutant described previously. In these MDA-MB-231-FOXO3a(A3):ER cells, FOXO3a activity can be conditionally induced by treatment with 4-OHT. MDA-MB-231 cells were used as a control. Whole cell lysates as well as nuclear extracts were analysed by Western blotting and RT-qPCR (Figure 12A & 12B). Treatment of MDA-MB-231-FOXO3a(A3):ER cells with 4-OHT induced the relocation of FOXO3a from the cytoplasm to the nucleus fractions. Furthermore, this FOXO3a induction and nuclear relocation resulted in enhanced AKT phosphorylation without significantly altering its total expression levels and enhanced PIK3CA (p110α) levels. This increase in AKT phosphorylation, and thus in PI3K/AKT activity can be attributed to FOXO3a induction, as this response was absent in the control MDA-MB-231 cells (Figure 12A). Consistent with this, RT-qPCR results further showed that treatment with 4-OHT induced the expression of the FOXO3a target genes, PIK3CA and IGFR1 (Figure 12B). The nuclear relocation of FOXO3a(A3):ER in response to 4-OHT induction was further validated by immunofluorescence staining of ER in MDA-MB-231-FOXO3a(A3):ER cells (Figure 13A). The staining results confirmed that following 4-OHT addition FOXO3a(A3):ER relocalised from the cytoplasm into the nucleus. I also studied whether the induction of FOXO3a affects the cell proliferative rate of the drug-resistant MDA-MB-231 cell line. Cells were stimulated with 4-OHT for 0, 24 and 48 h and SRB assay performed for both MDA-MB-231 and MDA-MB-231-FOXO3a(A3):ER cells. As showed in Figure 13B, there were no significant changes in the rate of cell proliferation on these MDA-MB-231 cells, suggesting that the anti-proliferative function normally attributed to FOXO3a is deregulated in the cytotoxic drug-resistant cells.

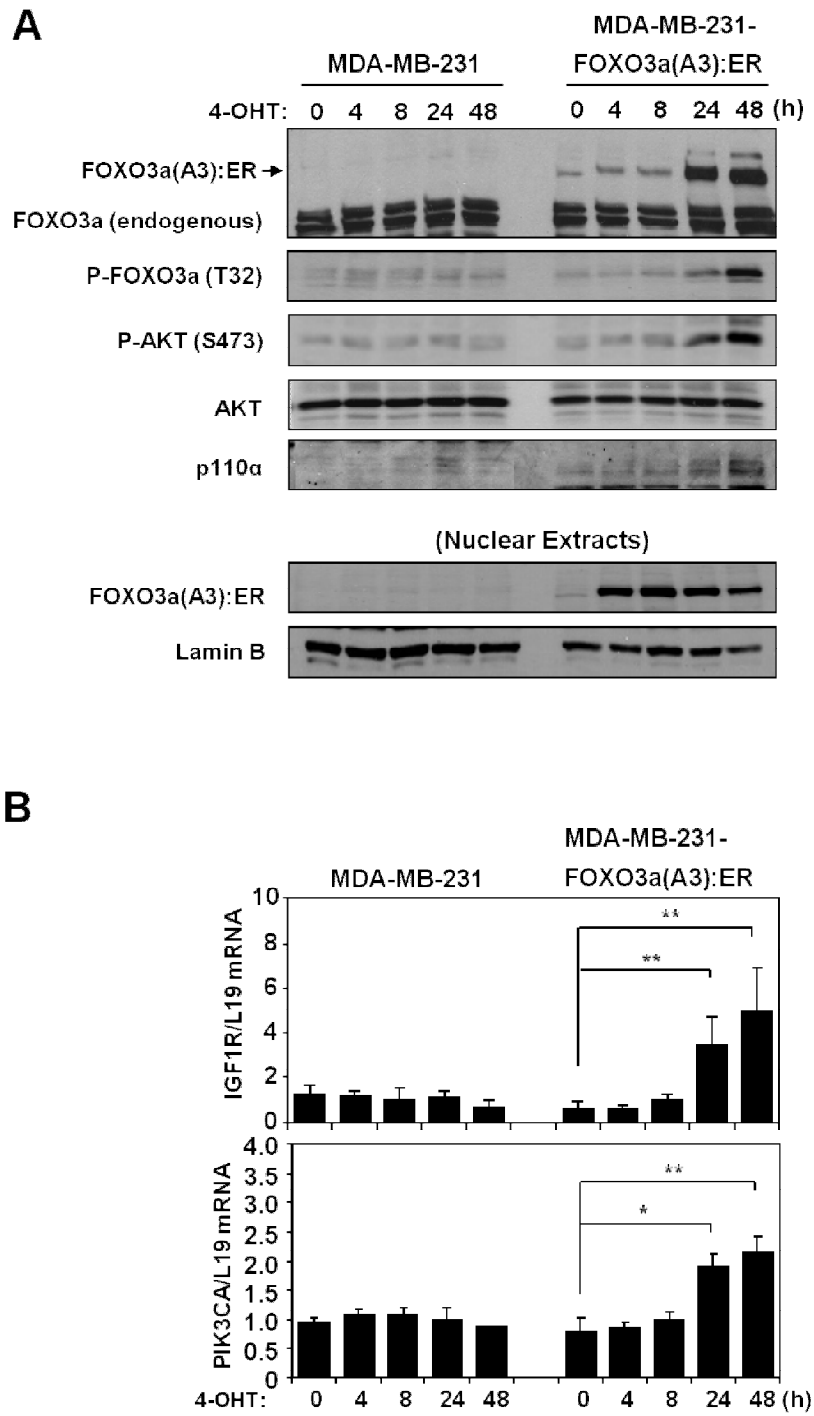


Figure 12. FOXO3a induces AKT phosphorylation, *PIK3CA* and *IGFR1* gene expression in the drug-resistant MDA-MB-231 breast carcinoma cells.

MDA-MB-231-FOXO3a(A3):ER and MDA-MB-231 cells were treated with 1μM of 4-OHT for the indicated times. **A**) Total and nuclear extracts were prepared at the times indicated, and the expression levels determined for the indicated antibodies by Western blotting. **B**) mRNA expression was analysed for *PIK3CA*, and *IGFR1* using RT-qPCR and normalised with L19 mRNA. All data shown represent the averages of data from three experiments, and the error bars show the standard deviations.

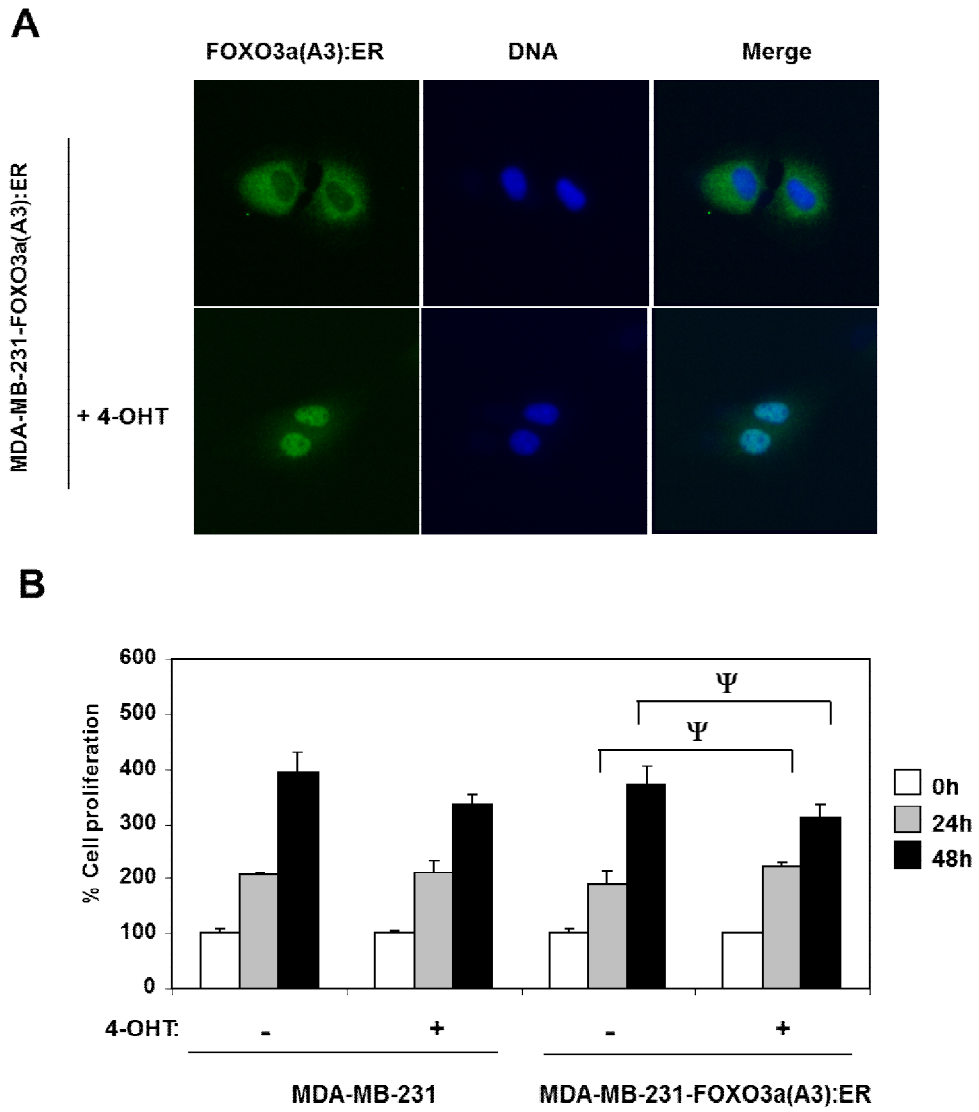


Figure 13. FOXO3a induction does not result in proliferative arrest in the drug resistant MDA-MB-231 breast carcinoma cells.

A) MDA-MB-231-FOXO3a(A3):ER cells were cultured in 4-well chamber slides and treated for 16 h with or without 1μM of 4-OHT as in the previous Figure, before being fixed in 4% formaldehyde. FOXO3a(A3):ER was visualised with a rabbit polyclonal antibody against ERα followed by the addition of ALEX488 (green) labeled anti-rabbit antisera. DAPI (blue) were also applied to visualise the nuclei. **B**) SRB assays were performed on these cytotoxic resistant MDA-MB-231 cells, indicating that the induction of FOXO3a(A3) has little effect on the cell proliferation rate of the drug resistant breast carcinoma cells. Statistical analysis was done using t-test Ψ, P=non-significant.

3.3.6 FOXO3a function is deregulated in the drug-resistant breast cancer cells

To further test and confirm that in drug-resistant cells there is a deregulation of the anti-proliferative function of FOXO3a, I transiently transfected the doxorubicin-resistant MCF-7 Dox^R cells with either the empty vector or the vector encoding for the constitutively active FOXO3a(A3). The cells were then incubated with increasing amounts of doxorubicin ranging from 0 to 10µM and cell proliferation was determined by SRB assay for 0, 24 and 48 h. Overexpression of the active FOXO3a was confirmed at protein level by Western blotting (Figure 14A) and did not result in any significant decreases of the cell proliferation rate (Figure 14B), indicating that this known anti-proliferative FOXO3a function is deregulated in drug-resistant cells.

To further assess the effects of FOXO3a activation on drug-resistance in breast cancer cells, MDA-MB-231-FOXO3a(A3):ER cells were subjected to subcellular fractionation following incubation with 4-OHT over a timecourse and the cytosolic and nuclear protein fractions were assessed by Western blotting (Figure 15A). The result showed that after 4 h of 4-OHT incubation there was an increase in the nuclear levels of FOXO3a accompanied by a decrease on its cytoplasmic levels, indicating that FOXO3a activity increased (Figure 15A). FOXO3a activation also resulted in an increase of AKT activity as revealed by its readout, P-FOXO3a (T32) expression which was also increased. It was noticeable that at 24 and 48 h after FOXO3a(A3):ER induction, there was an accumulation of the endogenous levels of FOXO3a which is consistent with the late induction in AKT activity. MDA-MB-231-FOXO3a(A3):ER cells were then incubated with both the vehicle (ethanol) or 4-OHT and treated with a dose titration of doxorubicin for 48 h. The proliferative rate for both conditions was determined by SRB assay. As shown in Figure 15B, doxorubicin doses ≥ 2µM, elicited a significantly decrease in the proliferation rates of the vehicle-treated cells in comparison with 4-OHT-treated cells, suggesting that the vehicle-treated cells are sensitive to higher doses of doxorubicin. Importantly, the result also showed that activation of FOXO3a(A3):ER by 4-OHT protected MDA-MB-231-FOXO3a(A3):ER cells against the anti-proliferative effects of higher doxorubicin levels (Figure 13). These data (Figure 15A) confirms that FOXO3a function is deregulated in drug-resistant breast cancer cells.

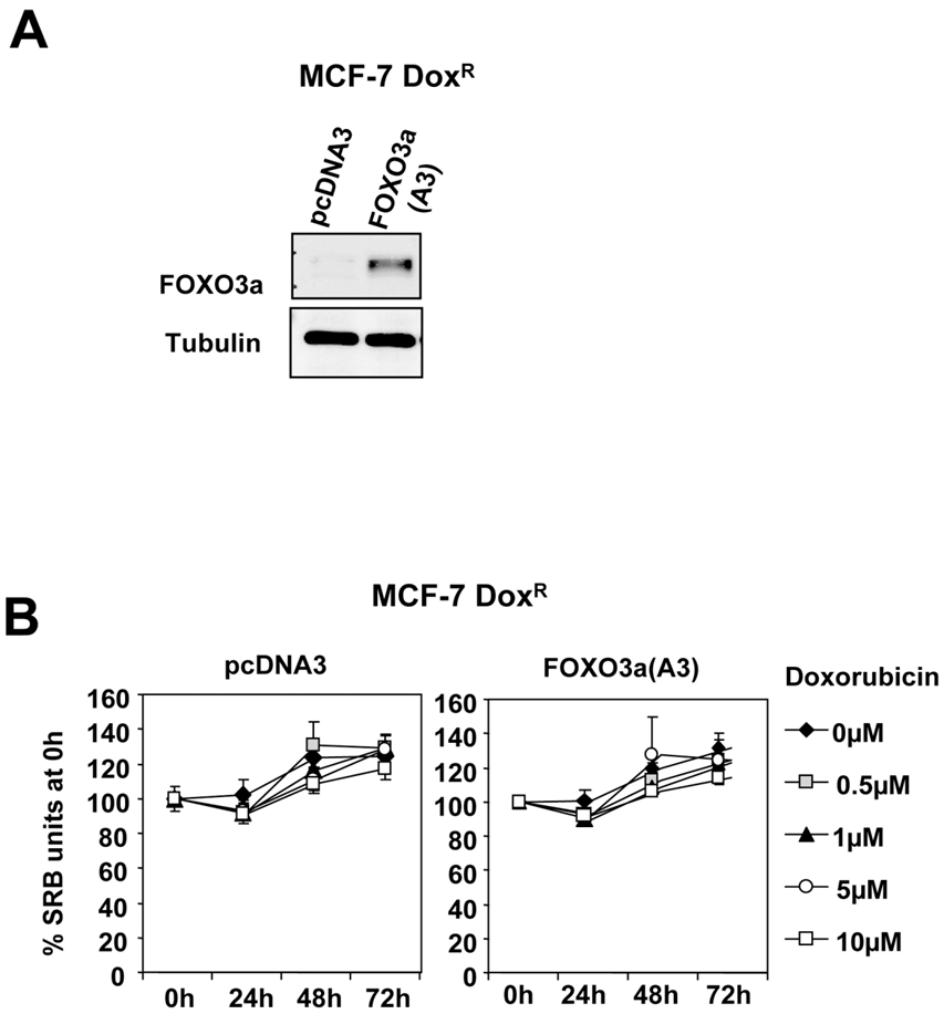


Figure 14. Overexpressed active FOXO3a has little effect on cell proliferation and drug sensitivity of the drug resistant breast cancer cell line MCF-7 Dox^R.

MCF-7 Dox^R cells were transiently transfected with the constitutively active FOXO3a(A3) or control pcDNA3. **A**) The transfected cells were analysed by Western blot for FOXO3a and Tubulin expression. **B**) The cells were then treated with 0 to 10μM of doxorubicin for 0, 24, 48 and 72 h and SRB assays were performed on these cells after 48 h.

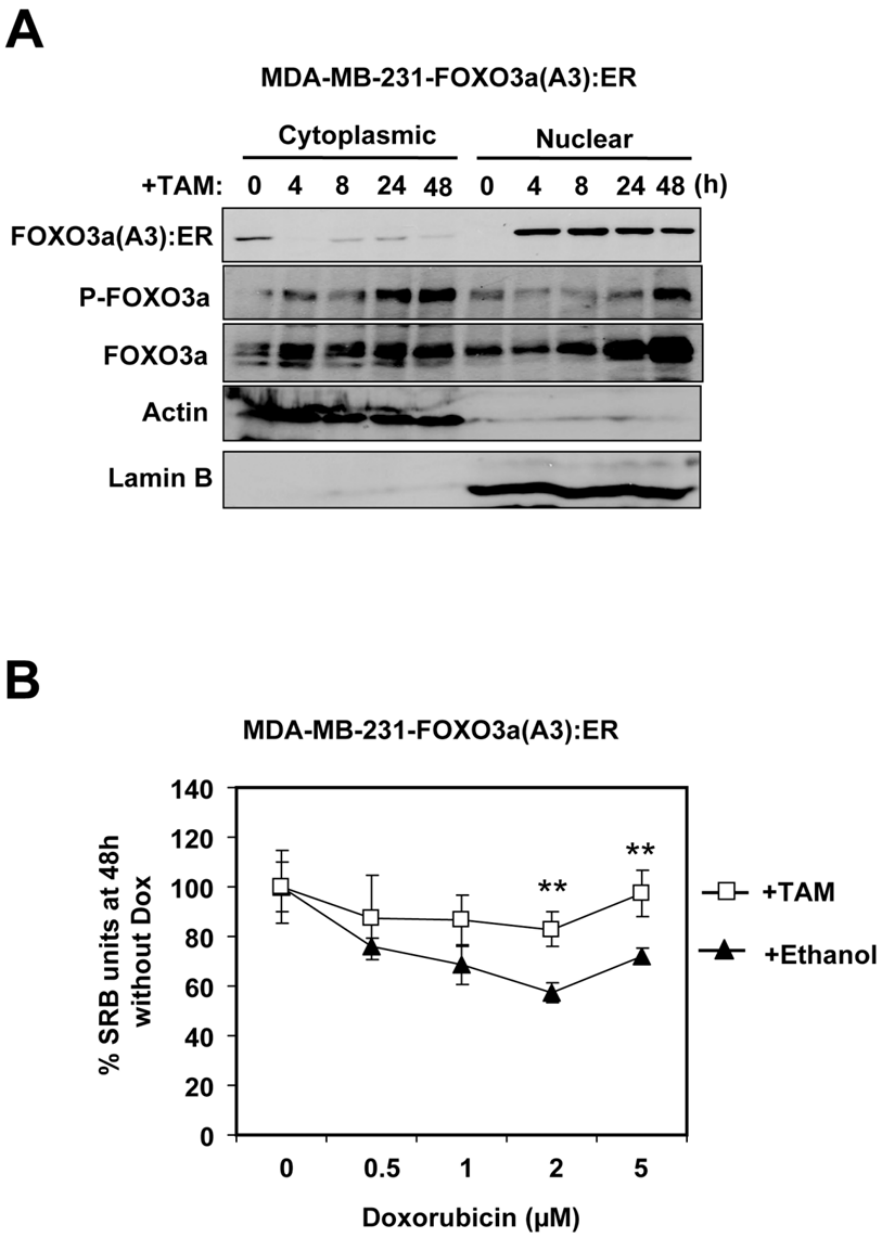


Figure 15. Induction of FOXO3a can confer resistance to the MDA-MB-231 cells.

Cells were treated with 200nM of 4-OHT and collected at 0, 4, 8, 24 and 48 h. **A**) Cytoplasmic and nuclear extracts were prepared and analysed by western blotting for the indicated antibodies. **B**) SRB assays were performed on untreated and 4-OHT-treated cells after 48 h of treatment with various concentration of doxorubicin. Statistical analysis was done using t-test. **, $P<0.01$, significant.

3.3.7 Nuclear FOXO3a expression in breast cancer tissue is significantly associated with lymph node metastasis and poor survival in invasive ductal carcinoma.

Not many studies have addressed the significance of FOXO3a expression in breast cancer patient samples; however, the very few that did produced conflicting results. Hu *et al* (Hu et al 2004) have found that cytoplasmic expression of FOXO3a correlated with phosphorylated AKT and associated with poor survival in breast cancer. Yet, another study found that nuclear rather than cytoplasmic FOXO3a was significantly associated with lymph node metastasis (Jin et al 2004). In order to confirm and validate my *in vitro* findings in breast cancer patient samples and to explore the potential use of the AKT/FOXO3a signalling axis as prognostic markers in breast cancer, immunohistochemical staining was performed to assess the expression patterns of FOXO3a and P-AKT. The results, summarised in Table 2, showed that in tumours the staining of FOXO3a was found localised in both nucleus and cytoplasm. Two representative cases, in Figure 16A, show the expression patterns of FOXO3a staining in both tumour and non-tumour samples. Example of two tumour cases showing corresponding FOXO3a and P-AKT staining patterns are shown in Figure 16B. FOXO3a cytoplasmic staining ($p<0.0001$, Chi-Square test) was also significantly associated with P-AKT staining, confirming that the activated P-AKT negatively regulates FOXO3a and relocates it from the nucleus to cytoplasm in most breast cancer samples.

As the majority of tumour samples are invasive ductal carcinoma, the above parameters were re-analysed for cases of invasive ductal carcinomas only. This new analysis showed that the nuclear staining of FOXO3a marginally correlated with lymph node positivity ($p=0.052$, Chi-Square test). Furthermore, FOXO3a nuclear staining was also significantly associated with shorter survival time ($p=0.014$, log-rank test) (Figure 17). Interestingly, using the comparison scoring system $N \geqq C$ or $C > N$ previously described (Hu et al 2004), the $N \geqq C$ FOXO3a staining of all cases irrespective of histological types was positively associated with the P-AKT staining ($p=0.039$, Chi-square test). This suggests that the AKT/FOXO3a signalling axis uncoupling is associated with lymph node metastasis and poor survival in invasive ductal carcinoma. Together these data indicate that the function of FOXO3a and its control by AKT have been deregulated in breast cancer associated with lymph node metastasis and poor survival, and this indicates cancer progression and treatment failure, respectively.

Tumour cases			
Nuclear staining	**No. of cases**	**Cytoplasmic staining**	**No. of cases**
negative	85	negative	4
N score ≤6	25	C score ≤6	24
N score >6	10	C score >6	92
Total	120	Total	120
Non-tumour cases			
Nuclear staining	**No. of cases**	**Cytoplasmic staining**	**No. of cases**
negative	0	negative	17
N score ≤6	27	C score ≤6	14
N score >6	11	C score >6	7
Total	38	Total	38

Table 2. FOXO3a immunoreactivity in all cases.

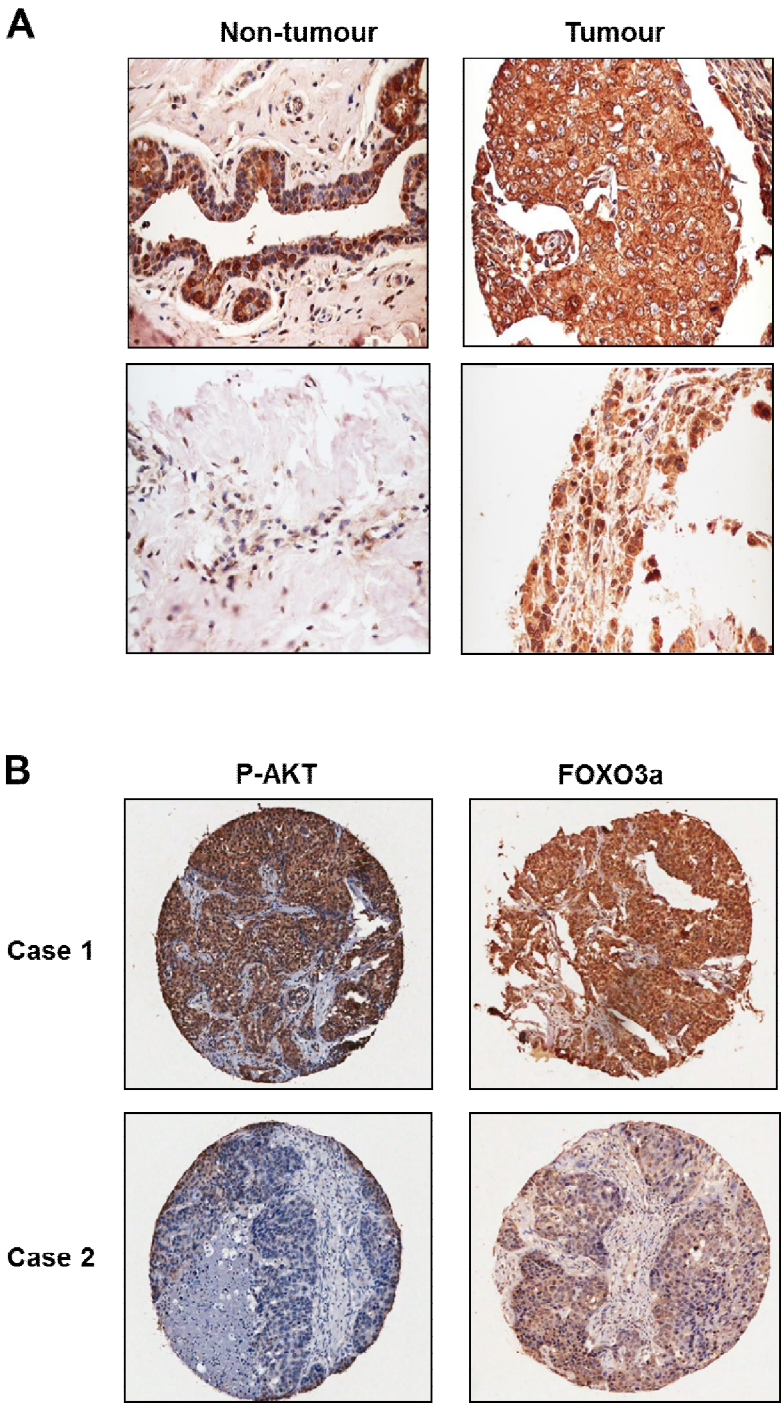


Figure 16. Representative expression patterns of FOXO3a and P-AKT in tissue microarray.

Tumour tissue samples obtained from breast cancer patients that had been formalin-fixed and paraffin-embedded were immunohistochemically stained with FOXO3a and P-AKT (T308) antibodies. **A**) Representative FOXO3a staining patterns in both tumour and non-tumour cases. **B**) Two representative tumour cases showing corresponding FOXO3a and P-AKT staining patterns (magnification x 170).

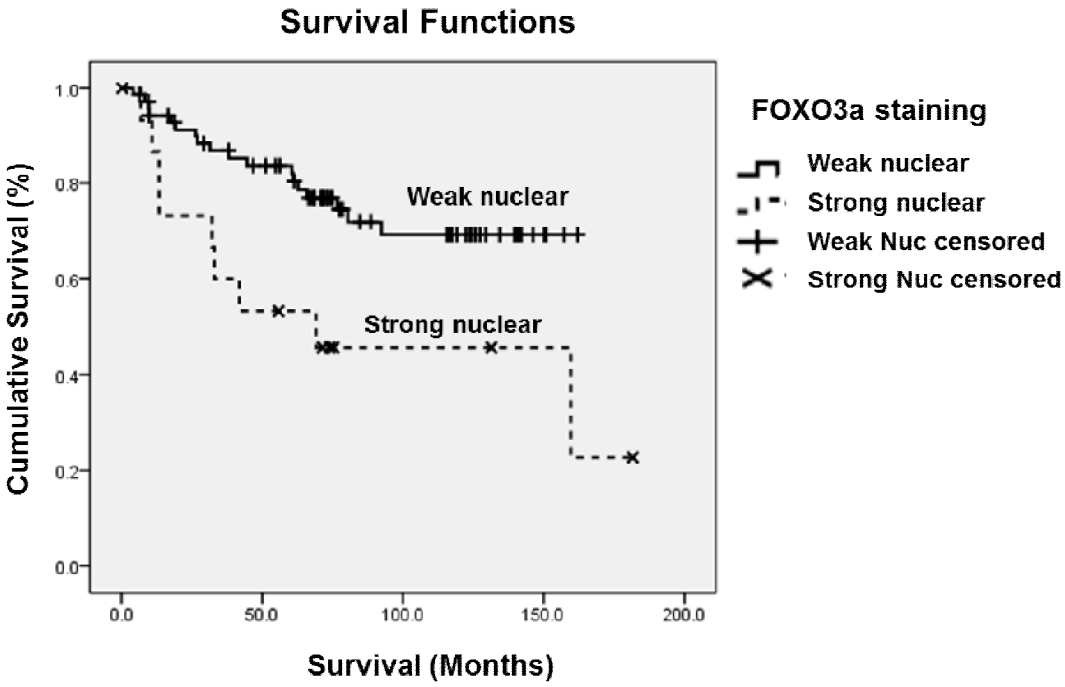


Figure 17. Survival analysis with nuclear FOXO3a staining in invasive ductal carcinoma cases.

Of the 120 cancer samples assessed, there were 94 cases of invasive ductal carcinoma. The correlation between nuclear FOXO3a expression and survival was studied using Kaplan Meier analysis (P=0.014) and was considered significant at $p<0.05$.

3.4 DISCUSSION

Although anti-cancer therapy produces an initial overall reduction in cell proliferation, in the long-term there is residual cell cycle progression and survival that suffice to maintain tumour growth. There are multiple mechanisms whereby breast cancer cells can bypass normal endocrine responsiveness and develop resistance to both endocrine- and chemo-therapy (Geisler and Lonning 2001). Indeed, it has been shown that signalling pathways such as EGFR, HER2, MAPK, HIF-1α and PI3K/AKT are key regulators of cell cycle and cell proliferation events that circumvent ER-mediated gene transcription and growth in resistant breast cancer cells (Martin et al 2003, Nicholson et al 1999). Yet, the knowledge regarding the involvement of these signalling pathways in such processes comes mainly from *in vitro* cell line models, which not always reflect the heterogeneity of breast cancers and thus are limited in their clinical relevance. In this chapter, I explored not only the impact of the PI3K/AKT/FOXO axis in different resistant cell line breast cancer models but I further validated my *in vitro* results in breast cancer patient samples.

The PI3K/AKT signalling pathway has already been shown to play a vital role in tamoxifen and cytotoxic chemotherapeutic drug-resistance (Campbell et al 2001, DeGraffenried et al 2003, Faridi et al 2003, Frogne et al 2005). For instance, enhanced AKT activity has been reported to promote resistance to tamoxifen and cytotoxic drugs by promoting cell proliferation and survival (Clark et al 2002) and to increase resistance to paclitaxel (Page et al 2000, VanderWeele et al 2004), further emphasising the importance of PI3K/AKT/FOXO pathway in determining drug sensitivity. The downstream AKT negative effector, FOXO3a, has been shown to mediate cytotoxic response of chemotherapeutic drugs such as anthracyclines (doxorubicin), taxanes (paclitaxel) and cisplatin (Fernandez de Mattos et al 2008, Hui et al 2008a, Hui et al 2008b, Krol et al 2007, Kwok et al 2008, McGovern et al 2009, Sunters et al 2003, Sunters et al 2006). Not many studies have addressed the predictive value of AKT-mediated FOXO3a subcellular localisation. However, one of the first reports that investigated this was by Hu *et al*, who reported that FOXO3a is localised mainly in the cytoplasm of breast cancer cells in a study of 113 breast cancer cases and that predominant cytoplasmic FOXO3a localisation is inversely associated with patient survival (Hu et al 2004). Contrary to these findings, my immunohistochemistry results showed that nuclear FOXO3a expression in invasive ductal carcinomas is significantly associated with lymph node metastasis ($p=0.052$) and shortened overall survival time ($p=0.014$). These data also indicate that consistent nuclear FOXO3a expression in patient samples is associated with shorter survival time after treatment, as most if not all of these patients were treated and monitored after diagnosis. Nevertheless, my findings are in keeping with another report showing that predominant

nuclear-targeted FOXO3a is significantly correlated with lymph node metastasis in invasive ductal breast carcinoma (Jin et al 2004). Furthermore, using the same scoring system as that of Hu et *al* (Hu et al 2004), I found that cases with higher nuclear to cytoplasmic FOXO3a expression ratio are also positively associated with P-AKT expression (p=0.039, Chi-square test). Consistent with this, predominant nuclear expression of FOXO3a has also been shown to correlate with enhanced PI3K/AKT activity and acquisition of chemoresistance in chronic myeloid leukaemia (CML) cell lines (Hui et al 2008a, Hui et al 2008b).

In this chapter, I investigated FOXO3a subcellular localisation in a panel of endocrine and cytotoxic chemotherapy resistant and sensitive breast cancer cell lines in order to establish the significance of my findings *in vivo* and to test the hypothesis that active FOXO3a is associated with resistance to chemotherapy. Similar to that observed in doxorubicin-resistant CML cell lines (Hui et al 2008a, Hui et al 2008b), my tissue culture models showed enhanced basal phosphorylated AKT in tamoxifen-resistant cell lines compared with the parental sensitive cell line MCF-7. Interestingly, high levels of nuclear FOXO3a were only detected in doxorubicin (e.g. MCF-7 Dox^R and MDA-MB-231) but not tamoxifen (e.g. LCC2, LCC9 and BT474) resistant breast cancer cell lines. After breast cancer patients develop resistance to the first line agent tamoxifen, other endocrine therapeutic (e.g. fulvestrant and aromatase inhibitors, including anastrozole, letrozole and exemestane) alternatives are available. In fact, approximately 30% of ERα positive breast patients do not respond to tamoxifen treatment, which is defined as *de novo* resistance. Furthermore, the majority of tumours that initially respond to tamoxifen treatment will acquire resistance over time even with maintained ERα expression (Riggins et al 2005a, Riggins et al 2005b). However, these endocrine resistant tumours still respond, at least initially, to cytotoxic chemotherapy regimens. Consistent with this notion, all the tamoxifen-resistant cell models tested in this chapter, including LCC2, LCC9, BT474, are sensitive to doxorubicin treatment, whereas the doxorubicin resistant MCF-7 Dox^R and MDA-MB-231, are not sensitive to tamoxifen. Notably, the MDA-MB-231 cells are also resistant to paclitaxel treatment (Sunters et al 2003, Sunters et al 2006). Although these cell line models cannot extrapolate into breast cancer types in the clinic, it is reasonable to think that, these tamoxifen-resistant breast cancer cells may model breast cancers that have failed endocrine therapy but remain sensitive to cytotoxic drug-based treatments, while the resistant cells may represent breast cancers that have progressed and become resistant to anthracyclines and taxanes. In fact, these advanced drug-resistant breast cancers could be thought as metastatic disease to which treatment options are extremely limited. The cytotoxic chemotherapeutic resistant cell lines and the invasive ductal breast carcinoma have numerous significant overlapping clinical and pathological features. In here, I showed

that they at least share a common molecular phenotype of sustained nuclear FOXO3a expression. Taken together my data from the cell culture models support and explain the immunocytochemistry findings that in advanced breast cancers FOXO3a is predominantly nuclear localised despite the presence of phosphorylated AKT. This also provides *in vitro* evidence suggesting sustained nuclear FOXO3a expression predicts poor survival and chemotherapy resistance.

My *in vitro* data confirmed that overexpression of active FOXO3a(A3) in both MCF-7 and MDA-MB-231 could indeed enhance AKT activity, supporting the feedback mechanism via the PI3K/AKT pathway. Yet, overexpression of the active FOXO3a(A3) can efficiently inhibit cell proliferation in the chemosensitive breast cancer MCF-7 but has little effect in the resistant MDA-MB-231 cells. Two of the very best characterised FOXO3a targets - $p27^{Kip1}$ and Bim – have also been shown to be induced by FOXO3a in MCF-7 breast cancer cells which resulted in an induction of proliferation arrest and apoptosis, respectively. In line with this, I have also shown that doxorubicin treatment of the sensitive MCF-7 cells also resulted in re-localisation of FOXO3a to the nucleus. Thus, the enhanced P-AKT expression resulting from FOXO3a induction in MCF-7 was insufficient to counteract the action of activated FOXO3a, resulting in an overall decrease in cell viability. However, in the chemoresistant cell line MDA-MB-231, the cells may have already adopted as yet unknown mechanisms to avoid the anti-proliferative effects of activated FOXO3a, remaining unresponsive to cell cycle arrest and apoptosis under drug treatment. It is important to note that the two chemoresistant cell lines used in my *in vitro* experiments – MCF-7 Dox^R and MDA-MB-231 – may represent two different stages in the chemoresistance process in respect of FOXO3a activation and localisation. MDA-MB-231 cells are already long-term intrinsic resistant cell lines whereas MCF-7 Dox^R resistance reflects the process of acquired resistance by exposure to increasing amounts of the drug. This could help explain that despite both cell lines showing higher FOXO3a nuclear accumulation, upon treatment with doxorubicin the nuclear accumulation of FOXO3a in the nucleus is more pronounced in MCF-7 Dox^R than in MDA-MB-231 cells. The fact that MDA-MB-231 are also ER and HER-2 negative cell lines draws a resemblance to the TNBC that are unresponsive to almost all available therapeutic drugs. Although this was not explored in this chapter, it would be relevant to further study the different mechanisms regulating FOXO3a expression in these two cell lines. It could be that in these cell lines that model intrinsic and acquired resistance, FOXO3a is targeted by different post-translational modifications. The sustained nuclear localisation of FOXO3a may also contribute to the increased PI3K/AKT activity observed in the chemoresistant breast cancer cells. Treatment of MDA-MB-231-FOXO3a(A3):ER cells but not the MDA-MB-231 with 4-OHT was sufficient to induce P-AKT expression, suggesting that the increase in

FOXO3a activity is causative for the induction of PI3K/AKT activity. Moreover, induction of FOXO3a activity also resulted in the induction of expression of PIK3CA and IGFR1, two key components of the PI3K/AKT signalling pathway. The increase in expression levels of PIK3CA and IGFR1 as well as other components of PI3K/AKT signalling pathway by FOXO3a may be sufficient for the induction of AKT phosphorylation and activity if their expression levels are limiting in these breast cancer cells. FOXO3a activity is likely to be deregulated in both the invasive ductal breast carcinoma associated with poor survival, as well as the anthracycline-resistant breast cancer cell lines as in both cell-types nuclear-localised FOXO3a failed to elicit its anti-proliferative effects. Consistently, induction of FOXO3a activity and nuclear localisation using 4-OHT in the drug-resistant MDA-MB-231-FOXO3a(A3):ER cells did not have an appreciable effects on the cell proliferation. The molecular basis for this is unclear but can be due to post-translational modifications and/or differential recruitment of co-factors. Nevertheless, sustained nuclear localisation of FOXO3a may be a crucial molecular marker for chemotherapy resistance and poor prognosis in breast cancer. Together these data suggest that lymph node metastasis and poor survival in invasive ductal breast carcinoma are linked to an uncouping of the AKT/FOXO3a signalling axis, as in these breast cancers active AKT fails to inactivate and re-localise FOXO3a to the cytoplasm and that nuclear-targeted FOXO3a does not induce cell death or cell cycle arrest.

In summary, sustained nuclear FOXO3a expression in breast cancer may culminate in cancer progression and the development of an aggressive phenotype similar to that observed in cytotoxic chemotherapy resistant breast cancer cell models and, can significantly attenuate their response to tamoxifen and cytotoxic chemotherapy. Studying the expression profiles of the components of the AKT/FOXO3a axis, in breast cancer patients might help predict and monitor their response to chemotherapy. A better understanding of the mechanism by which AKT and FOXO3a are regulated, as well as their roles in cancer progression, drug sensitivity and resistance, may turn these proteins into crucial therapeutic targets and prognostic markers for breast cancer and other malignancies.

CHAPTER 4

NEW POTENTIAL FOXO3A TARGETS

4.1 INTRODUCTION

Over the past years, a plethora of studies have demonstrated that FOXO proteins play critical roles in a wide variety of cellular processes. Identification of their target genes will help unravel their functional outcome in these cellular processes. FOXOs regulate multiple genes such as, *Bim* and *FasL*, involved in apoptosis (Brunet et al 1999, Dijkers et al 2000a); $p27^{Kip1}$ and *Cyclin D*, essential for the cell cycle regulation (Dijkers et al 2000b, Schmidt et al 2002); B-cell lymphocytic leukaemia proto-oncogene 6 (*Bcl-6*), indispensable for the integrity of mitochondrial outer membrane (Tang et al 2002); *GADD45a*, important for DNA damage repair (Tran et al 2002) and manganese superoxide dismutase (*MnSOD* or *SOD2*), involved in stress responses (Kops et al 2002a). FOXO3a is a transcription factor that impacts in almost all different cell functions. However, the full extent of FOXO3a functions are yet to be understood. For this reason, it is imperative to further identify and characterise new putative FOXO3a targets that can reveal more information regarding its downstream roles. Some of the potential new targets are, JAK2, CXCR4, APOBEC3G, and FOXP1.

4.1.1 JAK2

JAK2 is a member of a tyrosine kinase family of proteins with an essential role in haematopoietic growth factors signalling pathways promoting cell growth, proliferation and differentiation (De Laurentiis et al 2008). Indeed, *Jak2* knockout mice exhibit a lethal phenotype, which has been attributed to a block in definitive erythropoiesis (Vasileiadis et al 2011). The most common somatic alteration of JAK2 is a gain-of-function mutation (JAK2 V617F) that results in a constitutively active JAK2 protein which mediate oncogenic transformation in a wide array of myeloproliferative disorders (Fromantin et al 2011, Milman et al 2011, Petoukhova et al 2011). Furthermore, *JAK2* is also involved in chromosomal translocations with the *TEL* gene, generating a constitutively active fusion gene *TEL-Jak2* in lymphomas. However, in other malignancies including, colon, breast, and lung cancers, *JAK2* amplification or mutations have not been found yet (Ho et al 2011). Following the binding of cytokines, JAKs associate with the intracellular tails of cytokine receptors and activate members of the signal transducer and activator of transcription (STAT) family of transcription factors; STATs translocate into the nucleus, acting as transcriptional activators (Bao et al 2011). As a result of its activation, JAK2 recruits STAT proteins, including STAT5, which translocate into the nucleus where they regulate expression of target genes that include *Bcl-X*, *Mcl-1* and *Cyclin D1/2*, associated with survival and proliferation in CML cells (Brennan et al 2007, Gajalakshmi and Peto 2007, Lewington et al 2007, Peto et al 2007). Moreover, constitutively active Jak2/Stat5

fusion protein was shown to be sufficient to induce neoplastic transformation of mammary epithelial cells *in vivo* (Giezen et al 2011).

4.1.2 CXCR4

Chemokines are expressed in various cell types and tissues and have a central role in the development and maintenance of the immune and hematopoietic systems (Go and Pollard 2008). Stromal cell-derived factor-1 (SDF-1), also known as CXCL12, is a homeostatic chemokine highly expressed by bone marrow stromal cells (Pollard 2008). SDF-1 binds to a G-protein coupled seven-span transmembrane receptor (GPCR) - CXCR4 (Petersen et al 2008). Chemokines were initially identified as being constitutively expressed within lymphoid organs. These chemokines and their receptors were found to play an important role in the homing of leukocytes to sites of inflammation (Kirshbom et al 1999). Similarly, in non-haematopoietic neoplasias, chemokines were also found to be constitutively expressed. Functionally active CXCR4 receptors mediate cell migration and survival *in vitro* of cancer cells, including those of colorectal cancer (Unger 1999), prostate cancer (Min et al 1999) and melanoma (Lajtai et al 1999). The CXCL12/CXCR4 ligand/receptor pair is critical in bone marrow hematopoiesis and plays important roles in vascularisation and in heart and brain development. Thus, both *CXCR4* and *CXCL12* knockouts are embryonic lethal (Cully et al 2006, Huang et al 1999). Breast cancer metastasis typically involves spread of tumour to regional lymph nodes, lung, liver, and bone marrow. Stromal fibroblasts within these tissues were found to have higher expression of CXCR4 ligand, CXCL12 (Bianco et al 2006, Fresno Vara et al 2004). Contrary to normal breast tissues, breast cancer cells were shown to specifically express functionally CXCR4 receptors that direct chemotaxis and invasiveness. Blockage of CXCR4 with anti-CXCR4 monoclonal antibodies inhibited the metastatic spread to target organs *in vivo* (Fresno Vara et al 2004). Furthermore, high CXCR4 expression was found to be associated with poor overall survival in breast cancer patients (Neri et al 2002b). These findings suggest that CXCL12 and its receptor CXCR4 play a crucial role in breast cancer metastasis. Moreover, CXCR4 has also been shown to be involved in the metastasis of prostate cancer to the bone marrow (Taichman et al., 2002) and of colon cancer to the liver (Zeelenberg et al., 2003). The involvement of the CXCL12/CXCR4 axis in chemotaxis, tumour progression, metastasis and survival is mediated by activation of different signalling pathways including the PI3K (Fukami et al 2004) and the JAK/STAT cascades (Ii et al 2004).

4.1.3 APOBEC3G

Apolipoprotein B mRNA-editing complex (APOBEC) constitutes a superfamily of cytidine deaminases which converts cytosine to uracil and includes 11 enzymes that edit RNA or single-stranded DNA (Lopes Ferreira et al 2006). This family of cytidine deaminases have been extensively studied for their important roles in the adaptive and innate immune systems (Vanchiere et al 2005). The different family members have diverse cellular functions. APOBEC1 plays a role in mRNA editing (Costanzo et al 2001), activation-induced deaminase (AID) is required for antigen-specific antibodies (Greer and Shannon 2005, Ren et al 2006) and APOBEC3 (A-H), especially APOBEC-3G and -3F, regulate innate immunity by protecting cells against important viral pathogens, including human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV) (Greer et al 2006, Wagner et al 2006, Wu et al 2005). The function of APOBEC2 and APOBEC4 remains unknown. APOBEC3G (A3G) has been found to block the replication of a specific HIV-1 strain. This strain lacks its viral infectivity factor (Vif) protein. However, when Vif is present, it counteracts A3G by targeting it for proteasomal degradation (Calderwood and Greer 2005). A3G importance in HIV-1 infection is demonstrated by the fact that its locus is screened for genetic variation to predict disease progression in HIV-positive patients (Buntain et al 2005). Enzymatic cytidine deamination is one of the sources of DNA mutations, which can eventually lead to carcinogenic DNA damage when the repair mechanisms fail. Thus, it is conceivable that APOBEC members in general, by their ability to generate mutations that are potentially tumorigenic, could have a role towards carcinogenesis (Ackerman et al 2005, Egea et al 2005, Zhang et al 2005). So far, there are no publications that show APOBEC3G as having a role in carcinogenesis; however, a study showed that overexpression of the family related AID resulted in T cell lymphomas in mice (Ackerman et al 2005).

4.1.4 FOXP1

FOXP1 was isolated and characterised in 2001 by Banham *et al* (Banham et al 2001). It shares 93% identity with the mouse homologue (Ensemble data). Interestingly, human FOXP1 has also been isolated from different sources by several groups. Shi *et al* identified the same FOXP1 (677 a.a.) as the 85kD MFH1 (Mac-1 regulated forkhead) that is down-regulated in monocytic THP1 upon adhesion to fibrinogen due to Mac-1 clustering (Shi 2004). Yamada *et al* identified the antigen thyroid and eye muscle shared protein G2s (55kD) as a fragment of FOXP1 (Yamada et al 2002). The full-length FOXP1 protein (677 a.a.) contains 2 glutamine rich regions for transactivation, 2 coiled coils, 1 leucine zipper and 1 zinc finger domain for dimerisation; 2 NLS around the forkhead domain for nuclear

localisation; and an acidic region for ubiquitin-mediated proteolysis. Unlike FOXO, there are no AKT phosphorylation sites, but a few potential Cyclin-Cdk, protein kinase C, casein kinase and p70S6-kinase phosphorylation sites. Therefore, FOXP1 might be involved in cell cycle progression and nuclear factor kappa B (NF-κB) signalling. Of interest, two PKC and S6Kinase phosphorylation sites are close to the NLS, implying a possible role in regulating the subcellular localisation of the protein (Banham et al 2001). FoxP1 represses transcription through direct binding to FoxP consensus DNA-binding sequences (T**TATTTRT**RTTKKYKWKTWT, R=G/A, W=A/T, K=T/G, Y= T/C) (Wang 2003). The molecular mechanisms by which the transcription factor FOXP1 functions would be better understood by the identification of its target genes; however, only few have been identified so far. Its targets include the promoters of *SV40*, *IL2* (Wang et al 2003), *c-fms* (encodes M-CSF) (Shi 2004), mouse *CC10* and human surfactant protein C (Shi 2004, Shu 2001). Although direct binding has not been confirmed, in *Foxp1* knockout mice, there is differential expression of *Sox4*, $p21^{Cip1}$ and $p27^{Kip1}$ mRNA in cardiac tissues (Wang et al 2004). It also binds to Erag enhancer in proB cells but not thymocytes, to upregulate gene expression but not promoter activity (Hu et al 2006). Recently, Foxp1 has also been shown to negatively regulate IL-7Rα expression critical for regulation of T cell quiescence and homeostasis (Feng et al 2011). Apart from interacting with other FoxP family members, FoxP1 has been reported to interact and bind DNA with other transcription factors, including the androgen receptor (Takayama et al 2008), and Nfat3 (Bai and Kerppola 2011). Its interaction with co-repressor proteins has also been shown for SMRT and CtBP1 (Jepsen et al 2008, Li et al 2004b). So far, there is a lack of information describing post-translational modifications for FOXP1. However, FOXP1 expression in hepatocellular and lung cancer has been shown to be regulated by miR-1 (Datta et al 2008, Nasser et al 2008). Additionally, miR-34a and miR-9 have also been found to regulate FoxP1 expression in B cell development and motor neuron specification, respectively (Otaegi et al 2011, Rao et al 2010).

4.1.4.1 THE ROLE OF FOXP1 IN CANCER

In humans, FOXP1 has been shown to be expressed in a variety of B cell lymphomas including MALT and diffuse large B cell lymphoma (DLBCL). Its strong nuclear expression has been noted in both these tumour types, which is associated with poor patient prognosis (Banham et al 2001, Banham et al 2005, Barrans et al 2004, Streubel et al 2005). A recent study also correlates the presence of FOXP1 overexpression, in a considerable number of primary cutaneous large B-cell lymphomas (PCLBCL), with unfavourable prognosis (Espinet et al 2011). *FOXP1* gene is frequently the target of

chromosomal translocations in DLBCL and MALT lymphomas (Wlodarska et al 2005). One such example is the translocation of t(3;14)(p14.1;q32), which renders the *FOXP1* gene under the control of the *IGH* gene enhancer, and has been found to be responsible for FOXP1 aberrant expression in some occasions (Streubel et al 2005). This is detected in 10% of MALT lymphoma outside the stomach (Streubel et al 2005) and in extra nodal presentation B cell lymphomas (Haralambieva et al 2006). In these lymphomas, increased FOXP1 expression has been suggested to result in the constitutive activation of the NF-κB pathway (Farinha and Gascoyne 2005). However, not all FOXP1 overexpressing DLBCL and MALT cases display *FOXP1* gene rearrangements or duplication, suggesting that mechanisms other than genetic alterations can upregulate FOXP1 expression (Flossbach et al 2011, Haralambieva et al 2006). One possible explanation could come from the discovery of 9 alternatively spliced transcripts for the human *FOXP1* gene (Brown et al 2008a, Brown et al 2008b). Most of these truncated isoforms lack the N-terminal coiled-coil domain as well as their second poly-glutamine domain that has been shown to correlate with transcriptional repressor activity. However, this is not the case for isoform 1, which encodes the full-length protein (Brown et al 2008a, Wang et al 2003). These isoforms have been found to be significantly increased in follicular lymphoma when compared to the full-length forms. Thus, these isoforms may inhibit the repressor function of the full-length protein on NF-κB signalling by competing for binding on target sequences enhancing this signalling pathway (Green et al 2009). Moreover, some of these isoforms are also overexpressed in primary central nervous system lymphomas (PCNSL), where the FOXP1 full-length form is downregulated (Courts et al 2009).

As yet, no clinical significance was found for these isoforms in lymphomagenesis. However, it seems likely that FOXP1 isoforms may play a determinant role in human malignancy. In a variety of B cell disorders, FOXP1 has been described as a potential oncogene due to its overexpression and subsequently poor prognosis predictor risk. However, FOXP1 has also been proposed to act as a tumour suppressor gene in solid tumours. Indeed, differentially FOXP1 mRNA expression has been confirmed between normal tissue and tumours in breast, prostate, pancreatic, colon and stomach, lung, head and neck squamous cell carcinomas as well as early endometrial adenocarcinomas. FOXP1 alterations in solid tumours are generally the result of a complete loss of expression or nuclear to cytoplasmic relocation (Banham et al 2001, Banham et al 2005, Giatromanolaki et al 2006). *FOXP1* location on chromosome 3p14.1 maps to a locus, which is often disrupted and displays loss of heterozygosity (LOH) in a range of solid tumours. In addition, other tumour suppressor genes including *FHIT*, *MLH1* and *VHL* (Banham et al 2001, Kok et al 1997) also reside in this location. Allelic loss at 3p or 3p12-14 are reported in various pre-malignant solid tumours of breast (Euhus et al 1999, Moinfar

et al 2000), cervical (Fouret et al 1998) and lung (Wistuba et al 2000). Recent studies have revealed some of the elusive roles of FOXP1 in different human cancers. Patients with stage III serous ovarian cancer have downregulated FOXP1 expression (Kim et al 2010). In prostate cancer, FOXP1 is commonly associated with TMPRSS2-ERG fusion-positive tumours (Taylor et al 2010) and identified as a novel partner to ETV1 (Hermans et al 2008). Interestingly, FOXP1 protein expression has been found to be associated with estrogen receptor alpha (ERα) in MCF-7 cells and with improved prognosis in breast tumours. However, its upstream regulation by ERα could not be confirmed. Since FOXP1 contains a nuclear receptor box LXXLL (a.a. 126-130) at its N-terminus, it is conceivable that FOXP1 could be a co-regulator of ERα (Fox et al 2004). Furthermore, a correlation between FOXP1 and ERα is also documented in deep myometrium invasion in endometrial cancers along with cytoplasmic FOXP1 (Giatromanolaki et al 2006). Similar to ERα, FOXP1 is also found to be associated with nuclear but not cytoplasmic ERβ expression, and has eventually been established that nuclear ERβ rather than ERα are more related to FOXP1 expression (Bates et al 2008). In keeping with a role of FOXP1 as a tumour suppressor gene, the expression of FOXP1 has been correlated with survival in familial breast cancers. Loss of FOXP1 is associated with a shorter relapse free and overall survival (Rayoo et al 2009). As a result of the reported relationship between FOXP1 with ERα and ERβ, interest has also emerged on the potential association of FOXP1 with the androgen receptor (AR). In prostate cancer, nuclear rather than cytoplasmic expression of FOXP1 correlates with AR expression (Banham et al 2007). Furthermore, FOXP1 is regulated on an androgen-dependent manner in LNCaP cells at both protein and transcriptional levels. In addition, FOXP1 has been also shown to directly interact with AR in the nucleus and negatively modulate AR transcription (Takayama et al 2008). There are still a number of questions to answer regarding the role of FOXP1 role in breast cancer. Additionally, to fully understand the function of FOXP1 and its mechanisms of action, it is pertinent to identify more of its physiological target genes in the context of cancer development.

4.2 AIMS & OBJECTIVES

FOXP1, JAK2, CXCR4 and APOBEC3G have been proposed to be potential FOXO3a targets. These putative targets were identified in two DNA microarrays using overexpressed constitutive active FOXO3a in human colon carcinoma cell lines (Delpuech et al 2007). Little is known about the regulation and significance of these proteins in breast cancer. The aim of this chapter is therefore to investigate the regulation of these proteins by FOXO3a and their potential involvement in FOXO3a-mediated chemoresistance in breast cancer.

4.3 RESULTS

4.3.1 FOXO3a and its targets expression in a panel of breast cancer cell lines

To address whether FOXO3a expression correlates with FOXP1, APOBEC3G (A3G), CXCR4 and JAK2, I decided to build a cell panel with most of the breast cancer cell lines available in the laboratory. MCF-10A is a non-malignant human mammary epithelial cell line and was used as a non-tumour control. Western blotting analysis (Figure 18A) showed that, in general, FOXO3a was expressed in all cell lines investigated, although there was a large amount of heterogeneity amongst the cell lines. The levels of inactive phosphorylated FOXO3a vary between cell lines, with the highest in BT474 and MDA-MB-468. In general the results showed that, APOBEC3G, CXCR4 and JAK2 proteins were expressed throughout the whole cell panel. HBL-100, ZR751, ZR7530, CAMA1 and MDA-MB-453 exhibited higher levels of A3G expression; CXCR4 was broadly expressed in all cell lines, whereas JAK2 expression was not detected in MCF-7, BT474 and T47D cell lines. This could indicate a negative correlation between JAK2 and ERα, as JAK2 expression was only found in ERα negative cell lines. In line with this, a study showed a negative correlation between P-JAK1 and ER status in breast cancer tissues (Yeh et al 2007). Furthermore, the *in vitro* results confirmed that the expression levels of P-JAK1 are inversely correlated with the ER presence in breast cancer cell lines (Yeh et al 2007). Despite a great heterogeneity between cell lines, a negative correlation between the inactive phosphorylated FOXO3a, and A3G, CXCR4 and JAK2 expression levels was noted. For example, in BT474 and MDA-MB-468 cells, phosphorylated FOXO3a correlated with lower levels for A3G, CXCR4 and JAK2. Conversely, HBL-100 and ZR751 cell lines, which displayed higher FOXO3a activity, assessed by the lower levels of P-FOXO3a (T32), had higher levels of all three proteins - A3G, CXCR4 and JAK2 (Figure 18A). Analysis of the transcript levels of JAK2 and CXCR4 also reflected the great heterogeneity between these cell lines and no obvious correlation can be drawn from this result. However, CXCR4 mRNA levels were lower when compared to JAK2 mRNA levels throughout the cell panel (Figure 18A). FOXP1 expression levels negatively correlated with P-FOXO3a levels for the majority of cell lines. For example, MCF-10A and MDA-MB-468 expressed high levels of P-FOXO3a (T32) and lower levels of FOXP1, whereas HBL-100, ZR751, ZR7530 and CAMA-1 expressed lower levels of P-FOXO3a (T32) and higher levels of FOXP1. However, it is notable that BT474 and MDA-MB-231 expressed high levels of P-FOXO3a (T32) and high levels of FOXP1. The reason for this is not understood. Yet, in BT474 the higher expression of FOXP1 may be related to a strong ERα expression as ERα expression has been reported to have a correlation with FOXP1 in a previous study (Fox et al 2004). I also examined the transcript levels of both FOXO3a

and FOXP1 to assess whether they are regulated at the transcriptional level (Figure 18B). Study of the FOXO3a transcript levels showed that the non-cancerous cell line, MCF-10A exhibited higher levels of FOXO3a when compared to all other breast cancer cell lines in accordance of the tumour suppressor role of FOXO3a. FOXP1 transcript levels correlated well with its protein expression. In fact, there was a good correlation between FOXO3a activity, as revealed by the lack of P-FOXO3a (T32), and FOXP1 transcript levels. Together, these results suggest that, all the target proteins studied are in general expressed in breast cancer cell lines and notably that, FOXP1 expression correlates with FOXO3a activity in breast cancer.

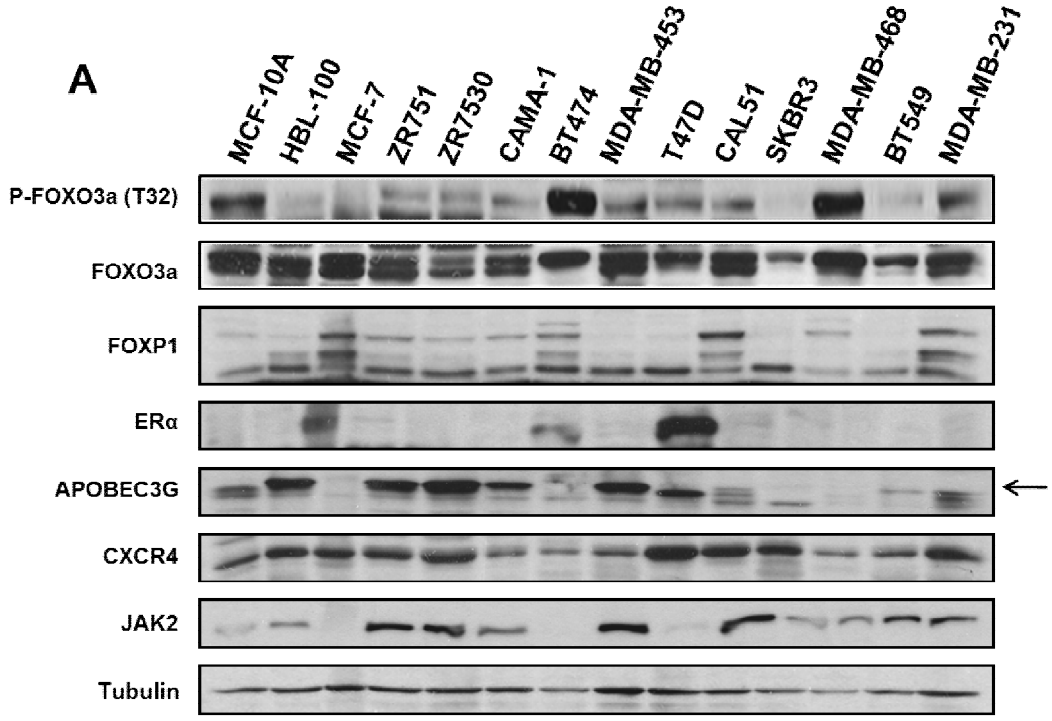


Figure 18. The expression of FOXO3a and its potential targets in breast cell panel.

Whole cell lysates were prepared from the panel of breast cancer cell lines. **A**) The expression of the indicated proteins was analysed by Western blotting. Tubulin was used as a loading control. **B**) The mRNA levels of FOXO3a, FOXP1, JAK2 and CXCR4 were determined by RT-qPCR and normalised to the level of L19.

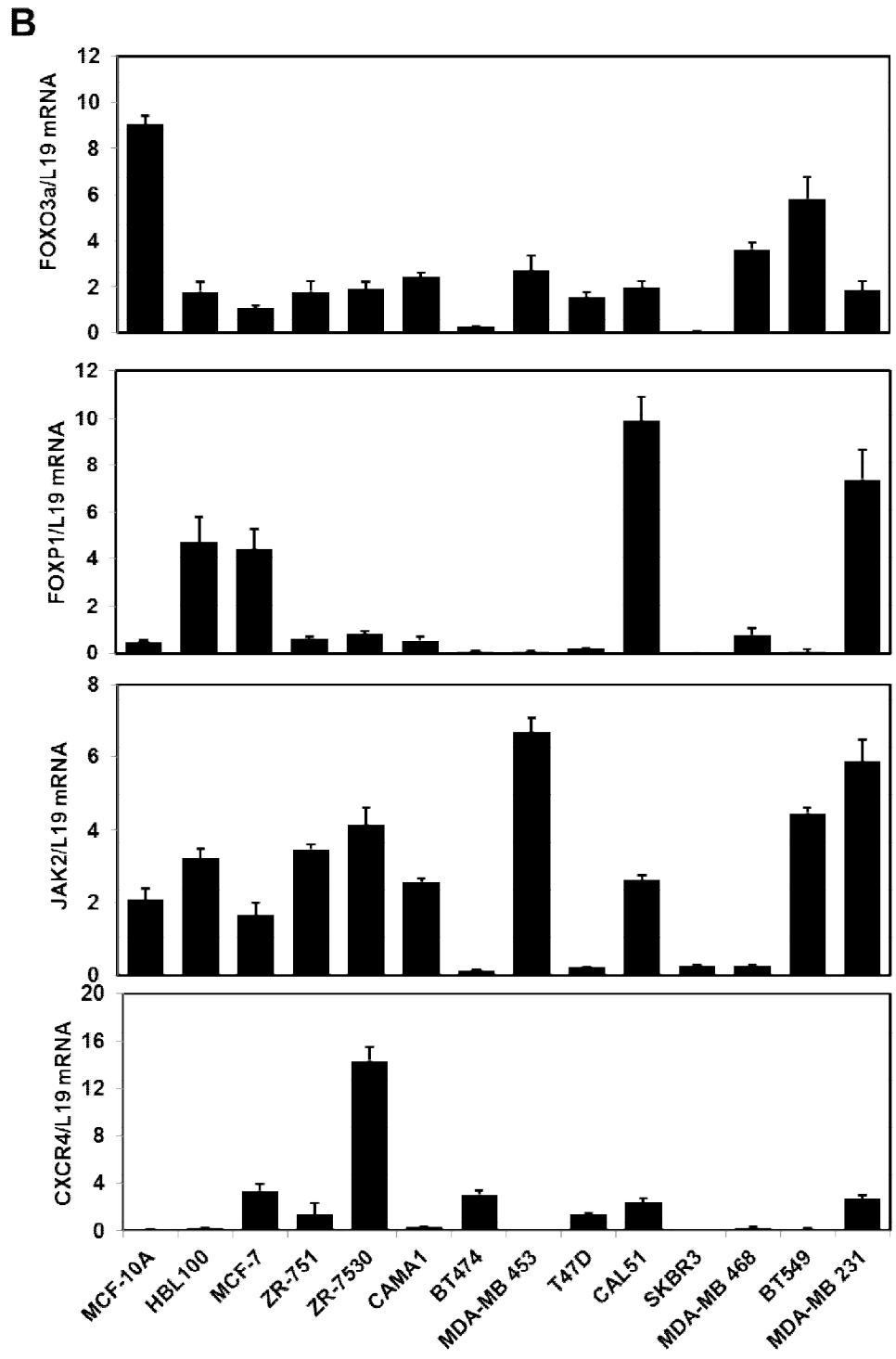


Figure 18. Continued.

4.3.2 Effect of FOXO3a induction and activation on its targets

To further study the effect of FOXO3a induction on FOXP1, A3G, CXCR4, and JAK2 expression levels, I used a stably transfected MDA-MB-231 cell line with an expression vector that encodes for the hormone binding domain of the estrogen receptor (ER) fused to FOXO3a(A3), a constitutive active FOXO3a mutant. In these MDA-MB-231-FOXO3a(A3):ER cells, FOXO3a activity can be induced by treatment with 4-OHT. In these experiments MDA-MB-231 cells were used as a control. Both MDA-MB-231 cells and MDA-MB-231-FOXO3a(A3):ER stably transfected cells were treated with increasing amounts of 4-OHT, ranging from 0-100nM, for 24 h. Protein expression was determined by western blotting for FOXO3a, FOXP1, A3G, CXCR4, JAK2, $p27^{Kip1}$ and Tubulin. FOXO3a(A3):ER induction was revealed by a mobility shift to a slower migratory band (indicated by the arrow). FOXP1 and CXCR4 expression was enhanced with 4-OHT treatment in MDA-MB-231-FOXO3a(A3):ER but not in the control cells. JAK2 levels were generally higher in FOXO3a induced cells, whereas A3G expression was abrogated by FOXO3a induction (Figure 19A). This result suggests a potential negative regulation of A3G by FOXO3a. The increased CXCR4 levels in MDA-MB-231-FOXO3a(A3):ER cells were also found to be significantly higher at the transcriptional level compared to MDA-MB-231 cells (Figure 19B).

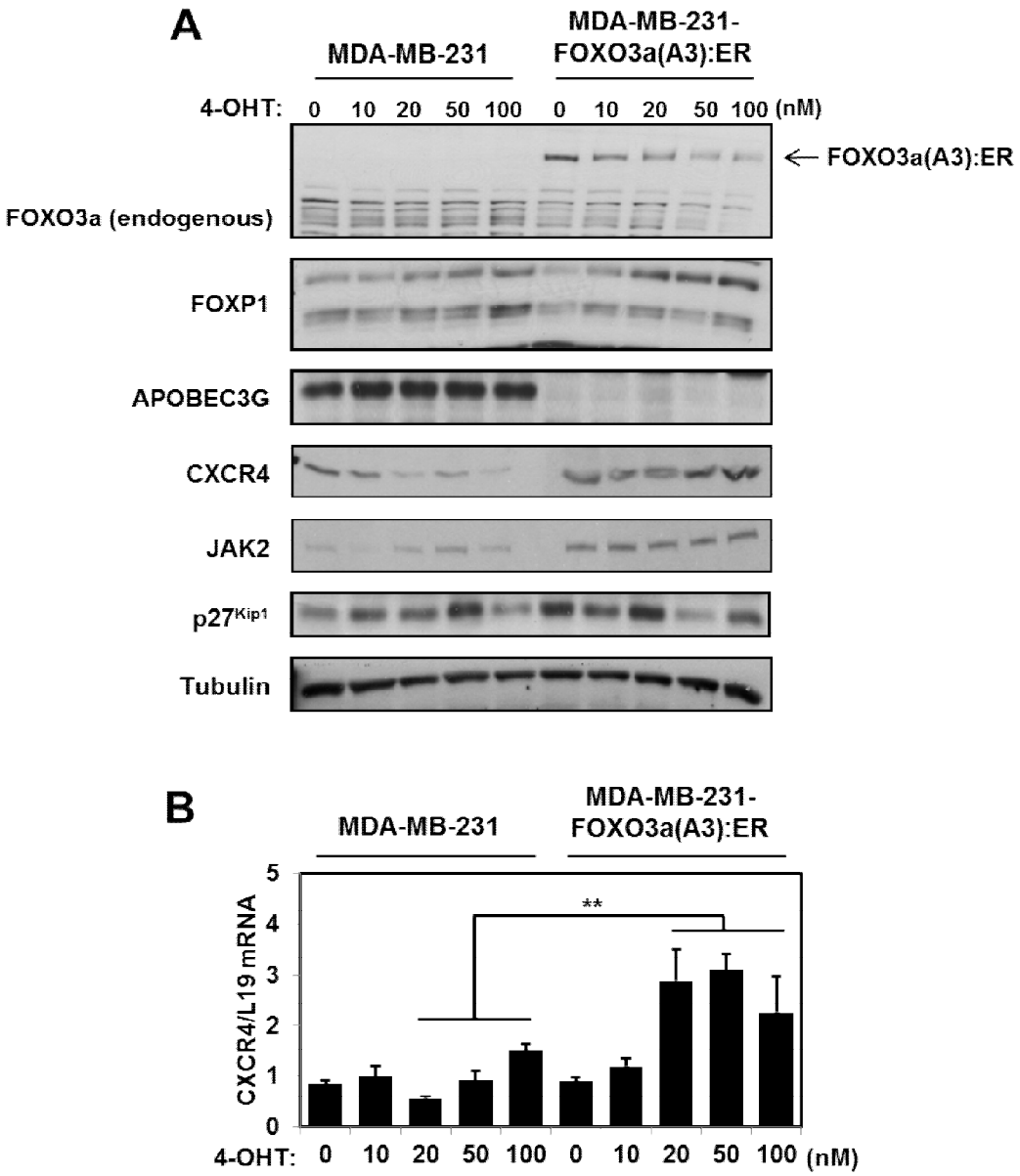


Figure 19. FOXO3a inducible system dose titration in breast cancer cells.

MDA-MB-231-FOXO3a(A3):ER and MDA-MB-231 cells were treated with increasing amounts of 4-OHT, ranging from 10-100nM, for 24 h. **A**) Expression levels of FOXO3a, FOXP1, APOBEC3G, CXCR4, JAK2, $p27^{Kip1}$ and Tubulin were assessed by Western blotting. **B**) RT-pPCR was performed for CXCR4 transcript levels. L19 was used to normalised the mRNA levels. Error bars show the standard deviations. Statistical analysis was done using t-test. **, P<0.01, significant.

Next, I investigated the effect of the activation of FOXO3a over a timecourse of 0, 4, 8, 24 and 48 h following addition of 4-OHT. Induction of FOXO3a was confirmed at protein level after 24 h by the increased levels of FOXO3a(A3):ER and P-FOXO3a (T32), respectively. FOXP1, CXCR4 and JAK2 expression levels were induced in MDA-MB-231-FOXO3a(A3):ER, reaching a maximum level at 48 h, similarly to what was observed for FOXO3a. Moreover, the known FOXO3a downstream target, $p27^{Kip1}$, was also increased after 24 h (Figure 20). Consistent with the previous result in Figure 19A, there was a marked decrease of A3G expression in MDA-MB-231-FOXO3a(A3):ER when compared to the control MDA-MB-231 cells, further confirming the idea that A3G is negatively regulated by FOXO3a. At transcriptional level, neither JAK2 nor CXCR4 transcript levels were increased in MDA-MB-231-FOXO3a(A3):ER. In fact, a decrease in CXCR4 mRNA levels was noted (Figure 20B).

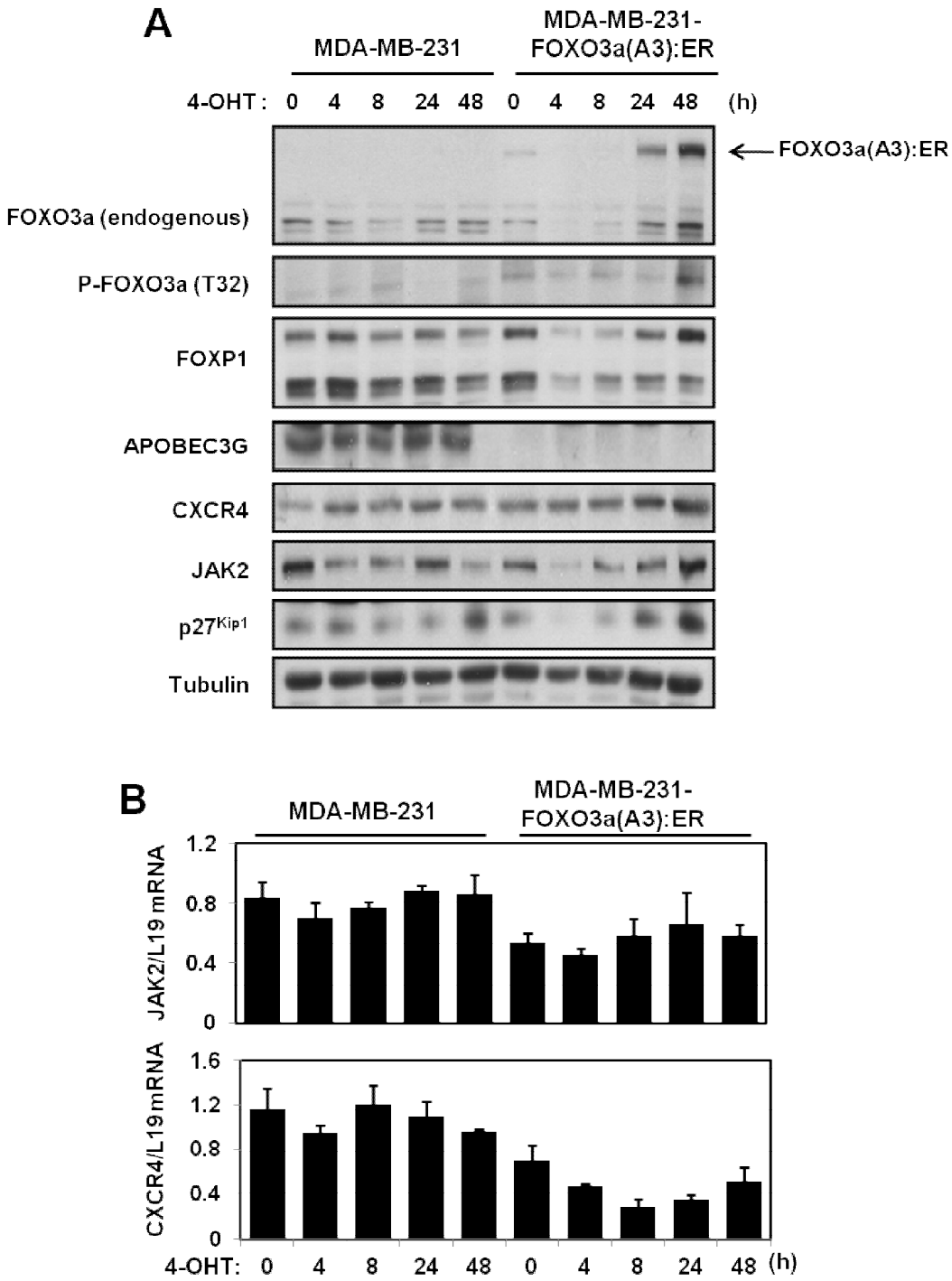


Figure 20. FOXO3a induces some targets in a timecourse manner.

MDA-MB-231 untransfected and FOXO3a(A3):ER transfected were treated at 1µM of 4-OHT for the indicated times. **A**) Western blotting was performed to assess the protein changes of FOXO3a and the targets throughout the timecourse for the indicated antibodies. **B**) RT-qPCR was performed to measure the transcript levels of JAK2 and CXCR4 using L19 to normalise the mRNA.

4.3.3 FOXO3a induces FOXP1 expression

In order to examine the nuclear and cytoplasmic localisation of FOXO3a and its putative targets, I performed subcellular fractionation with the MDA-MB-231 and MDA-MB-231-FOXO3a(A3):ER cells. The western blot results showed that, upon 4-OHT addition, FOXO3a(A3):ER accumulated in the nuclear compartment in MDA-MB-231-FOXO3a(A3):ER cells. Importantly, FOXO3a nuclear accumulation resulted in an upregulation of both FOXP1 and $p27^{Kip1}$ expression levels. This upregulation of FOXP1 and $p27^{Kip1}$ was specifically mediated by FOXO3a as no such effect was observed in the MDA-MB-231 control cells. No substantial difference was noted for CXCR4 and JAK2 levels after FOXO3a induction. The result also indicated that, JAK2 nuclear expression was increased in MDA-MB-231-FOXO3a(A3):ER cells after 4-OHT induction (Figure 21). This increase could reflect nuclear/cytoplasmic cross contamination as a result of ineffective fractionation as confirmed by the presence of tubulin expression in this set of nuclear extracts. Notably, A3G expression showed the same pattern of negative correlation upon FOXO3a induction in MDA-MB 231-FOXO3a(A3):ER cells (Figure 21). This result further suggests that FOXP1 expression is regulated by FOXO3a in MDA-MB-231-FOXO3a(A3):ER cells.

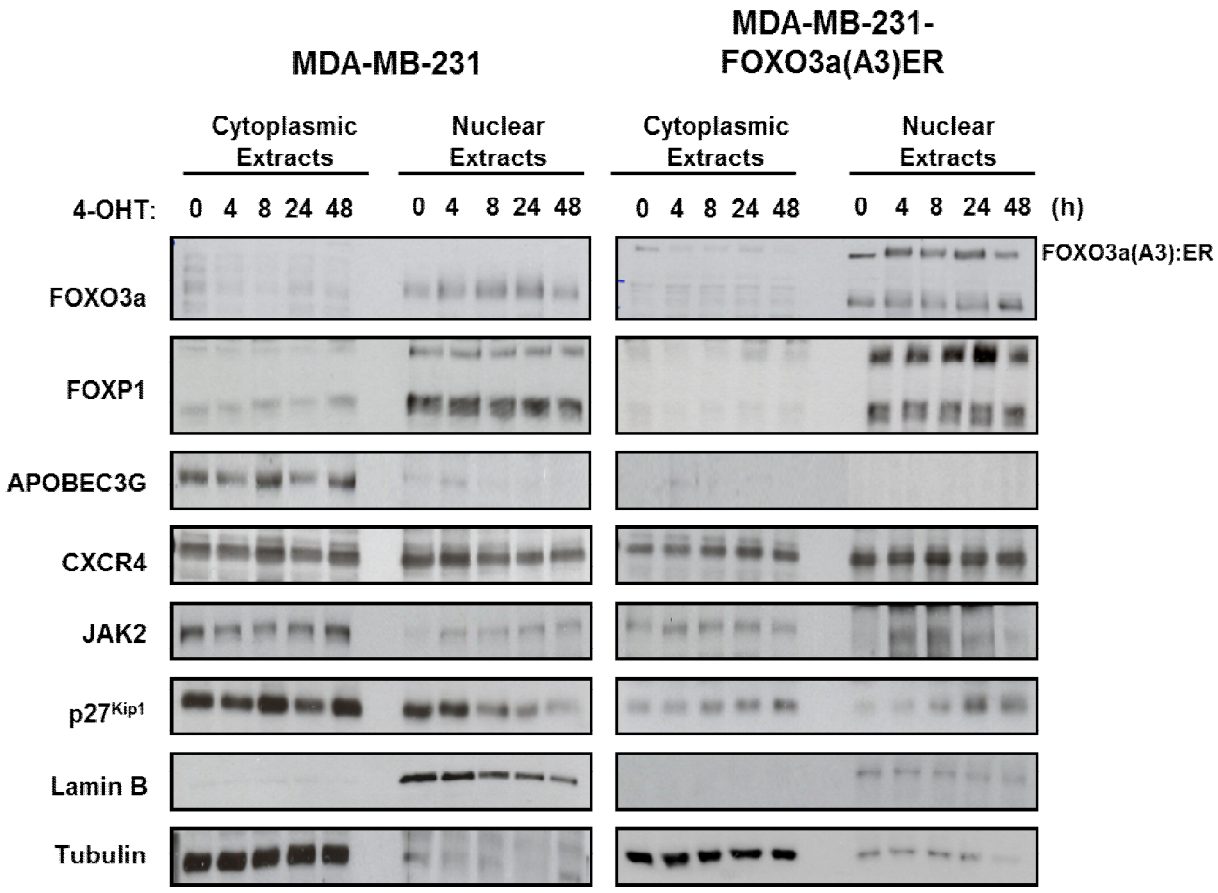


Figure 21. Subcellular localisation of FOXO3a in MDA-MB 231 cells.

Control and FOXO3a(A3):ER transfected MDA-MB-231 cells were treated in the absence or presence of 1μM of 4-OHT for the indicated times. Subcellular fractionation was performed and subsequent protein analysis was performed by western blotting. Tubulin and Lamin B were used as loading controls for cytoplasmic and nuclear fractions, respectively.

4.3.4 Impact of silencing FOXO3a on the targets

To address the effect of silencing FOXO3a on its potential targets, MCF-7 cells were untransfected or transfected with a non-specific (NSC) or a FOXO3a-specific siRNA pool. Since NSC siRNA treatment also resulted in the knockdown of FOXO3a in this cell line, I compared the effect of Mock transfection with FOXO3a siRNA transfected cells. The results showed that FOXP1, JAK2 and $p27^{Kip1}$ levels were markedly decreased following silencing of FOXO3a. However, neither CXCR4 nor A3G were affected by FOXO3a knockdown (Figure 22A). To study the effect of FOXO3a on putative target regulation in a FOXO null background, I used the wild-type (WT) and *Foxo1/3/4* null mouse embryonic fibroblasts (MEFs). The expression levels of Foxo1, Foxo3a, Foxo4, Foxp1, A3g, Cxcr4, Jak2, $p27^{Kip1}$, and Tubulin were assessed by western blotting. As expected, the expression of Foxo1, -3a and -4 was absent in the *Foxo1/3/4* null MEFs. Consistently, CXCR4 levels were abolished in *Foxo1/3/4*$^{-/-}$ cells. However, A3G and JAK2 expression were not differentially expressed between WT and *Foxo1/3/4*$^{-/-}$ MEFs (Figure 22B). The results also showed that in *Foxo1/3/4*$^{-/-}$ cells both $p27^{Kip1}$ and FOXP1 expression levels were markedly reduced. This effect was not observed in WT MEFs and so it was fully attributed to the absence of FOXO isoforms in these cells further confirming that the potential newly identified FOXP1 transcription factor is indeed a target of FOXO transcription factors. This regulation by FOXO was also confirmed at transcriptional level as demonstrated in Figure 22. Similar to *Foxo3a*, both *p27*^{Kip1} and *Foxp1* mRNA levels were significantly reduced in comparison to WT cells, suggesting that the regulation of FOXP1 by FOXO3a is at both protein and transcriptional levels (Figure 22C).

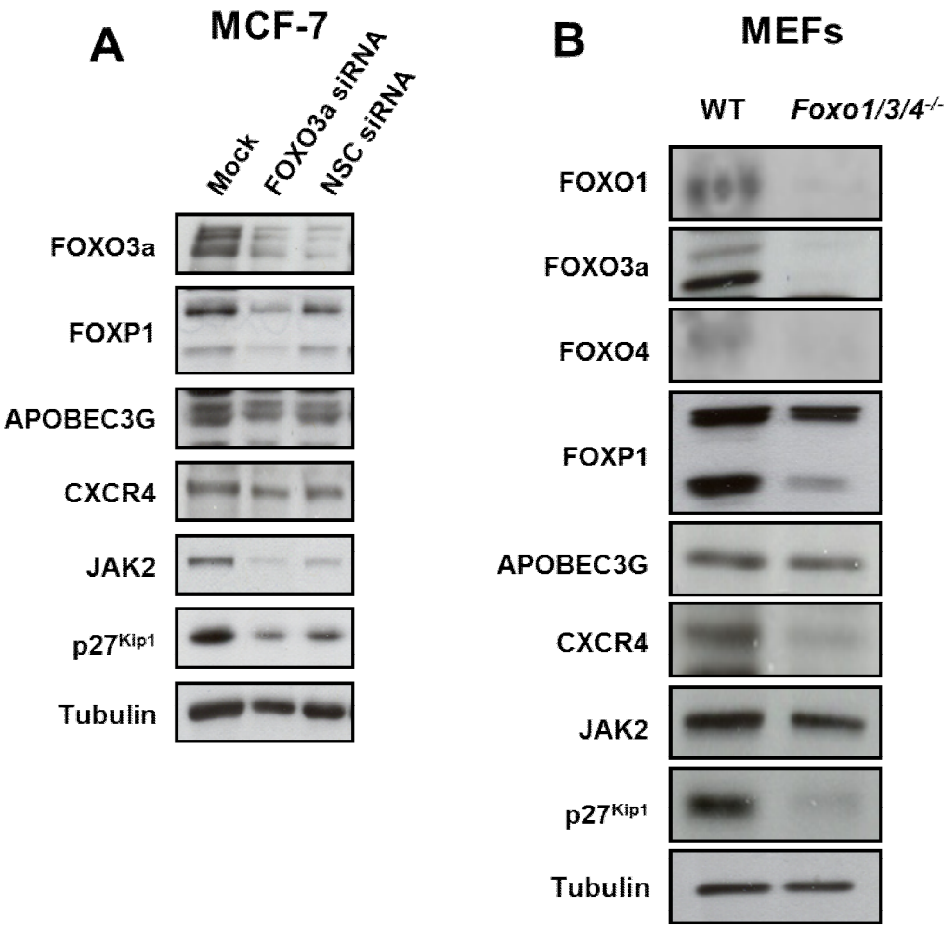


Figure 22. FOXO3a silencing in MCF-7 cells and *Foxo1/3/4*$^{-/-}$ MEFs confirm downregulation of FOXO targets.

A) MCF-7 cells were transfected with a FOXO3a specific siRNA, NSC and Mock. Western blot analysis confirmed knockdown of endogenous of FOXO3a. The expression levels of FOXP1, APOBEC3G (A3G), CXCR4, JAK2, p27^{Kip1} and Tubulin were also assessed. **B**) WT and *Foxo1/3/4* null mouse embryonic fibroblasts cell lysates were collect to assess the expression levels of FOXO1, FOXO3a, FOXO4 and the targets mentioned above by western blotting. Tubulin was used as a control. **C**) Cells were collected and total RNA was isolated. Transcript levels of *Foxo3a*, *Foxp1* and *p27^{Kip1}* were analysed by real-time quantitative PCR normalised with mouse *L19* mRNA levels. Error bars show the standard deviations. Statistical analysis was done using t-test. ***, P≤0.001, significant.

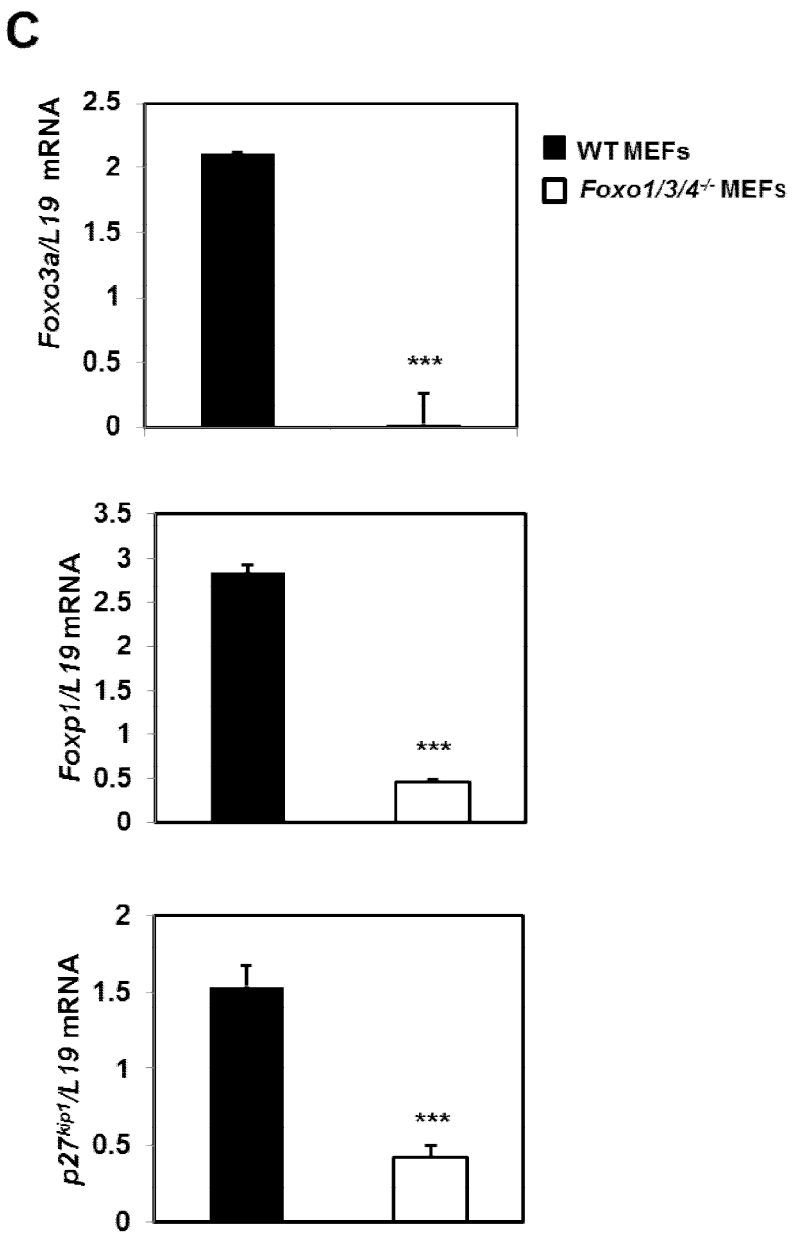


Figure 22. Continued.

To further study the effect of silencing FOXO3a on these targets regulation in other breast cancer cell lines, I silenced FOXO3a in BT474 cells. The efficiency of the FOXO3a siRNA transfection was confirmed at protein and transcript levels (Figure 23A & B). FOXP1 and $p27^{Kip1}$ expression levels were decreased following FOXO3a knockdown, nevertheless A3G and CXCR4 levels were not affected. As previously observed in Figure 18A, JAK2 was not expressed in BT474 cells (Figure 23A). There was no difference observed for the transcript levels of FOXP1 and CXCR4 following FOXO3a silencing (Figure 23B).

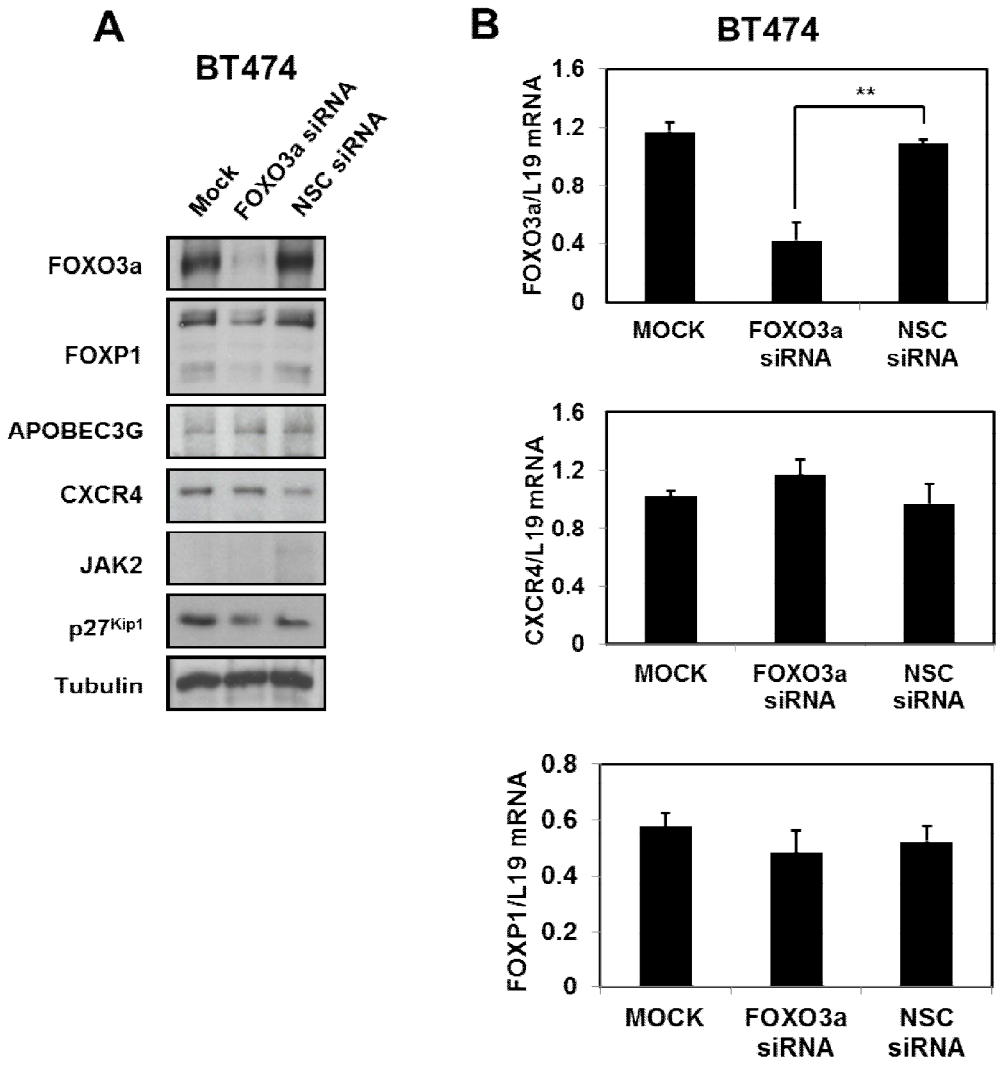


Figure 23. Effect of FOXO3a silencing in BT474 cells.

A) A specific FOXO3a and a non-specific siRNA was transfected into BT474 cells. After 24 h, cell lysates were collected and protein expression of the indicated antibodies determined by western blotting. **B**) FOXO3a, CXCR4 and FOXP1 mRNA levels were determined by RT-qPCR and normalised to L19 levels. Error bars show the standard deviations. Statistical analysis was done using t-test. **, P<0.01, significant.

4.3.5 FOXO3a regulation of FOXP1 is not confined to breast cancer cells

To investigate whether the regulation of the target proteins by FOXO3a is a universal mechanism and not cell type-dependent, I studied the effect of FOXO3a overexpression in a K562 leukaemic cell line. Similarly to MDA-MB-231-FOXO3a(A3):ER cells, these cells are stably transfected with an expression vector that encodes the hormone binding domain of a mutant ER fused to a constitutive active FOXO3a mutant - FOXO3a(A3). K562:ER cells were used as a control. Cell lysates from each cell line were collected and western blotting performed. FOXO3a overexpression was confirmed at protein level and this was accompanied by increases in FOXP1, JAK2 and $p27^{Kip1}$ expression levels, whereas CXCR4 levels remained unchanged. Consistent with my previous observations, following FOXO3a overexpression A3G levels were downregulated in K562 leukaemic cells (Figure 24). This result further suggests that FOXP1 is a FOXO3a target and this regulation is not exclusive to breast cancer cell lines.

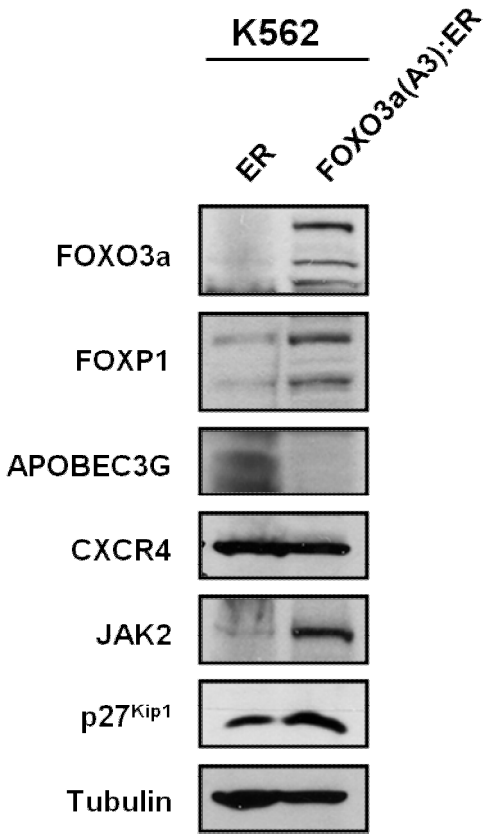


Figure 24. FOXO3a overexpression regulates its targets.

Untreated K562 ER and FOXO3a(A3):ER cells were subjected to western blotting for FOXO3a, FOXP1, APOBEC3G, CXCR4, JAK2, $p27^{Kip1}$ and Tubulin.

4.3.6 FOXO3a has a role in doxorubicin-mediated resistance in leukaemic cells

We have previously reported that FOXO3a plays an essential role in mediating doxorubicin resistance in leukaemic cell lines (Hui et al 2008b). In line with this, I made use of sensitive and resistant K562 and KD30 cell lines, respectively, to examine the protein expression of FOXO3a and its potential targets in a chemoresistant context. As expected, FOXO3a expression was upregulated in the resistant KD30 cells. Accordingly, FOXP1 and JAK2 levels were also found to be upregulated. Surprisingly, $p27^{Kip1}$ levels negatively correlated with FOXO3a whereas A3G showed for the first time an increase in its expression (Figure 25A). A significant increase in JAK2 mRNA levels was also observed. CXCR4 mRNA levels were substantially reduced and no significant changes were detected for A3G transcripts (Figure 25B).

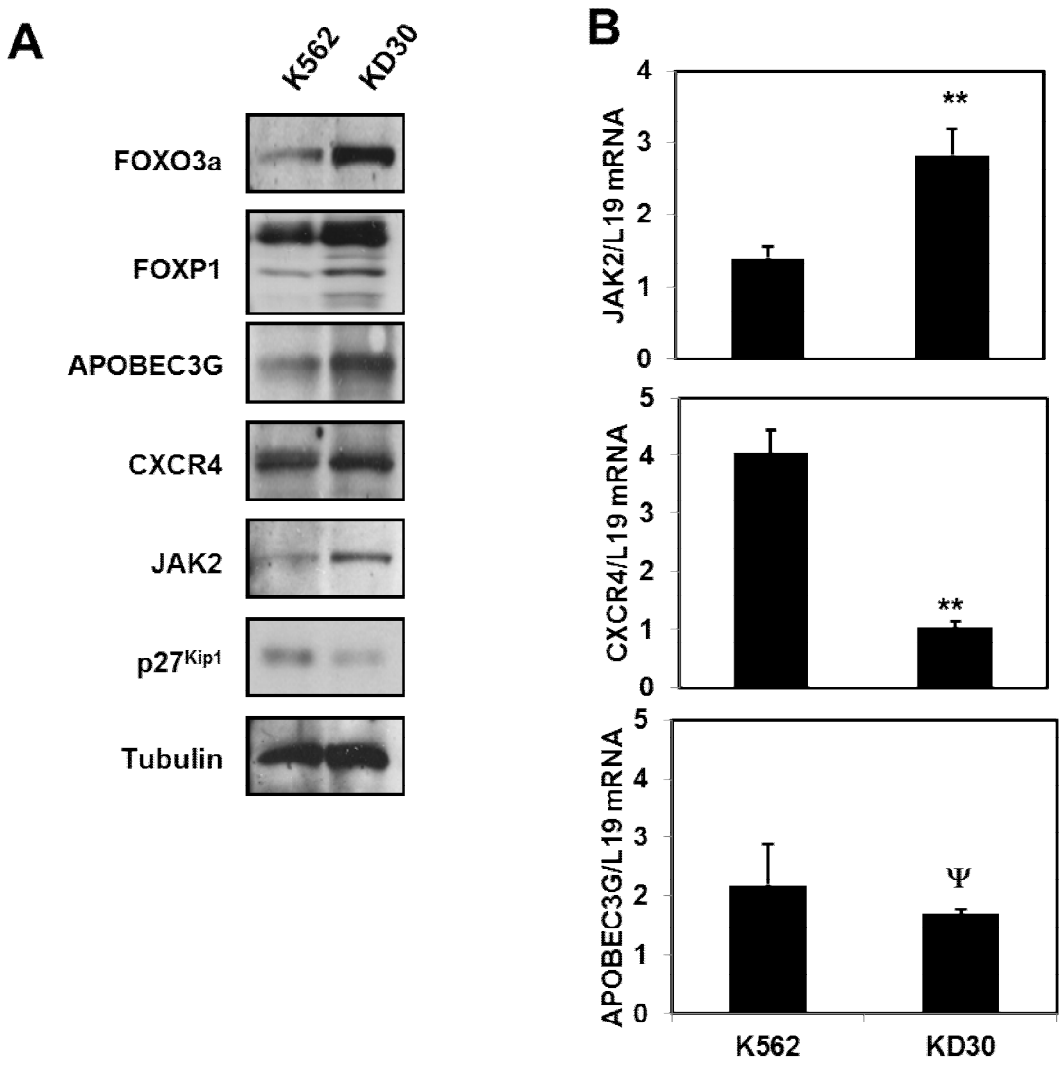


Figure 25. FOXO3a is upregulated in leukemic resistant cells.

Both sensitive and resistant K562 and KD30, respectively, CML cell lines were analysed for FOXO3a and its targets expression. **A**) Western blotting was performed for the indicated antibodies. **B**) The transcripts levels of JAK2, CXCR4 and A3G were analysed by RT-qPCR. mRNA levels were all normalised with L19. Error bars show the standard deviations. Statistical analysis was done using t-test. **, P<0.01; Ψ, non-significant.

4.3.7 FOXO3a is targeted by epirubicin in both WT and epirubicin resistant (Epi^R) MCF-7 breast cancer cells

To establish the impact of FOXO3a activation in response to epirubicin treatment, on the putative target proteins expression, I analysed both sensitive (WT) and epirubicin-resistant (Epi^R) MCF-7 breast cancer cell lines. Cells were treated with epirubicin or remained untreated for 24 h before harvested for western blotting analysis. In Epi^R MCF-7 cells total FOXO3a levels were lower although these cells also displayed higher FOXO3a activity as seen by the lower levels of P-FOXO3a (T32). Although MCF-7 cells had higher levels of total FOXO3a, they also exhibited higher levels of inactive FOXO3a. Nevertheless, in both cell lines, following epirubicin treatment there was a notable increase in total FOXO3a levels. Upon treatment with epirubicin, FOXP1 and p27^{Kip1} expression levels increased in Epi^R but not in WT MCF-7 cells. These proteins were already expressed at high levels in WT MCF-7 cells and probably FOXO3a cannot induce them further. Interestingly, A3G, JAK2 and to a less extend CXCR4, appeared to be upregulated in Epi^R MCF-7 when compared to WT MCF-7 cells (Figure 26). This might suggest a role for these proteins in epirubicin-mediated resistance independent from FOXO3a. This result indicates that epirubicin elicited an increase in FOXO3a expression in both sensitive and resistant breast cancer cell lines. Furthermore, both FOXP1 and p27^{Kip1} displayed the same expression pattern as FOXO3a and therefore, FOXP1 is by far the most consistent FOXO3a target.

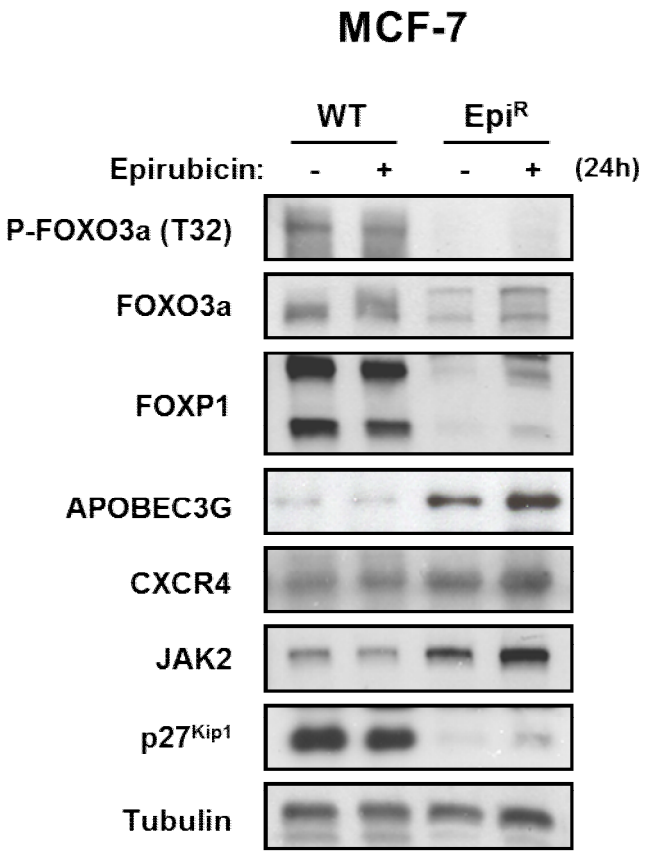


Figure 26. FOXO3a is targeted by epirubicin in sensitive and resistant breast cancer cell lines.

MCF-7 and Epi^R cells were treated with or without 1μM of epirubicin for 24 h. Cells were harvested and western blotting performed to examine the expression levels for the proteins represented by the indicated antibodies. Tubulin served as a control.

4.3.8 FOXO3a is targeted by lapatinib in BT474 and SKBR3 cells

To determine the effects of other anti-cancer drugs, such as lapatinib, on FOXO3a, I examined the expression of FOXO3a and its putative targets in lapatinib responsive breast cancer cell lines – BT474 and SKBR3. Cells were treated with 1μM of lapatinib for the indicated timepoints and western blot analysis performed. Lapatinib treatment caused a decrease in the phosphorylation levels of FOXO3a although total FOXO3a levels remained relatively constant, suggesting FOXO3a activation. As expected, following FOXO3a activation, $p27^{Kip1}$ levels were also increased. Furthermore, FOXP1 also showed an induction in both cell lines, although in SKBR3 cells after 8 h of treatment there was a reduction of its levels. A3G expression was decreased after FOXO3a induction, despite an increase at 4 h. In SKBR3 cells, A3G expression remained higher up to 24 h when compared to BT474 cells, in which A3G expression decreased 8 h after treatment. CXCR4 levels remained unchanged in both cell lines throughout the timecourse whilst JAK2 expression, confined solely to SKBR3 cells, appeared to peak at 4 h, after which the level remained unchanged (Figure 27A). FOXO3a transcript levels were also upregulated as a result of treatment with lapatinib in SKBR3 cells (Figure 27B). Together these results further support that FOXO3 is targeted by various anti-cancer drugs and upregulates some of its target genes, including FOXP1.

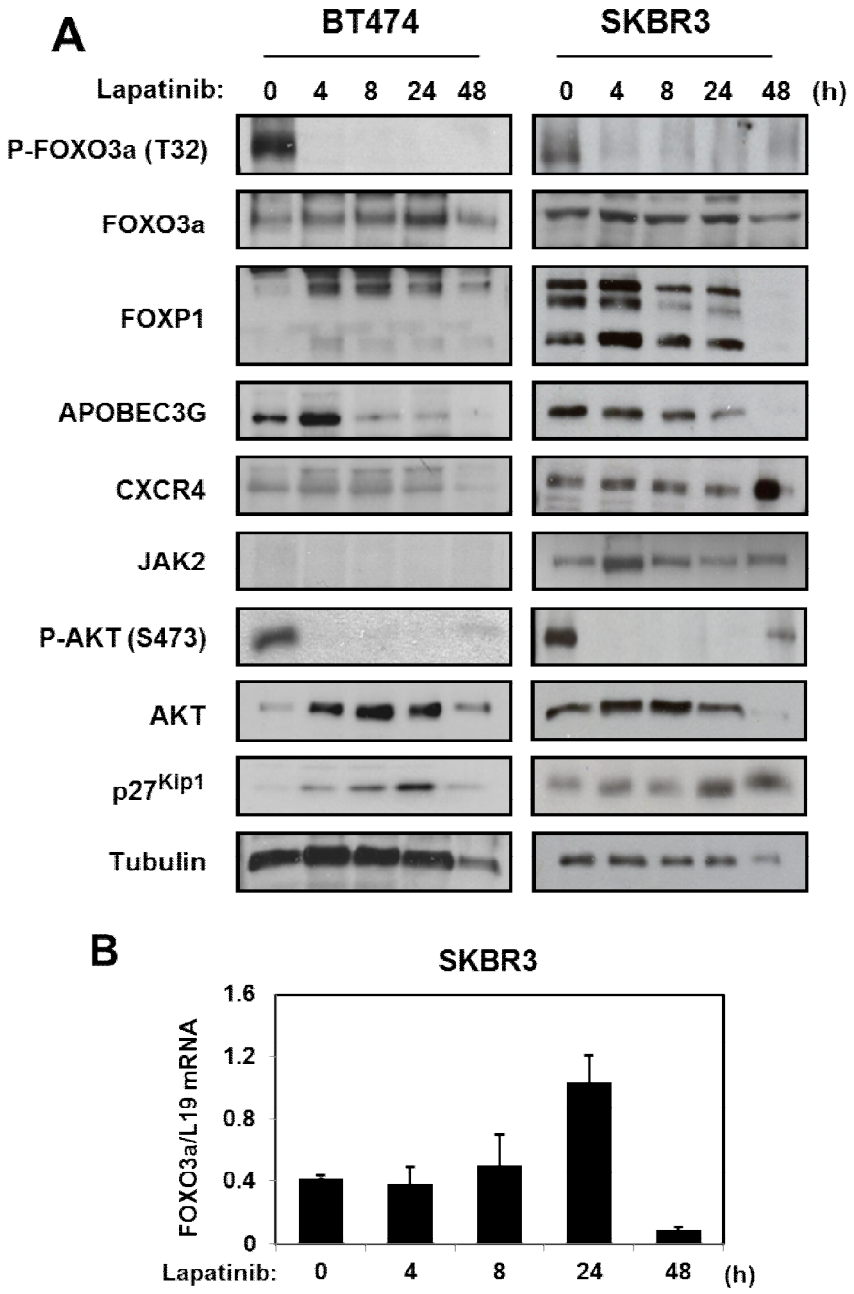


Figure 27. FOXO3a is activated by lapatinib in BT474 and SKBR3 cells.

A) BT474 and SKBR3 cells were incubated with and without 1µM of lapatinib. Cell lysates were collected, 24 h after and prepared for western blotting. The expression levels of all the indicated antibodies were assessed. **B**) Total RNA was extracted from SKBR3 cells and FOXO3a mRNA levels were determined by RT-qPCR. L19 was used to normalise mRNA levels.

4.3.9 BT474 Lap^R cells express higher FOXO3a levels

We and others have shown that FOXO3a is a downstream cellular target of both lapatinib and gefitinib and that FOXO3a activation results in cell cycle arrest and cell death in sensitive breast cancer cells (Hegde et al 2007, Krol et al 2007, Xia et al 2006). Moreover, we have previously confirmed the role of FOXO3a in promoting drug-resistance and survival through MDR1 activation in leukaemic cells (Hui et al 2008a). As a consequence, I hypothesised that FOXO3a also has a role towards lapatinib resistance. To test this idea, I generated a lapatinib resistant (Lap^R) BT474 cell line. BT474 cells were continually exposed to increasing concentrations of lapatinib for 8 weeks. After cell proliferation was established in the presence of 1μM lapatinib, cells were considered resistant. I then determined FOXO3a activity and expression after lapatinib addition in both WT and Lap^R BT474 cells by performing western blot analysis. As predicted, in sensitive WT BT474 cells, AKT is inactivated following addition of lapatinib, resulting in an increase in FOXO3a activity (Figure 28A). Nevertheless, total FOXO3a levels also decreased, probably due to degradation as a result of cell death, as indicated by the decrease in tubulin levels after 24 h of lapatinib treatment. FOXP1 showed once again a similar expression pattern to that of FOXO3a. A3G expression was higher when compared to CXCR4 expression, which is almost undetectable. The expression levels of p27^{Kip1} remained relatively unchanged. In resistant Lap^R BT474 cells following lapatinib addition, an increase in levels of phosphorylation of FOXO3a were observed, consistent with the enhanced AKT activity. Total FOXO3a levels were also increased after lapatinib treatment. Accordingly, FOXP1 expression levels were also found to be increased in response to lapatinib. p27^{Kip1} expression remained relatively unchanged, despite a marginal decrease at 48 h (Figure 28A). This could be a reflection of the decreased FOXO3a activity in these cells and the reason for this is not fully understood. As shown earlier for Epi^R resistant cells in Figure 26, the expression levels of A3G, CXCR4 and JAK2 were substantially higher in Lap^R BT474 resistant cells, suggesting that these targets are involved in common drug-resistance mechanisms that are not only restricted to epirubicin. To further confirm the resistance of the generated Lap^R cells to lapatinib, SRB assays were performed to compare cell proliferation of Lap^R with WT BT474 cells over a timecourse in the presence and absence of lapatinib. For untreated Lap^R cells there was a significant increase in the proliferative rate compared to WT BT474 cells (Figure 28B). This finding is supported by the decreased activity of FOXO3a, which can ultimately favour increased cell proliferation. Yet, when both cell lines were treated with lapatinib the difference between proliferative rates was not as clear. Lap^R BT474 cells showed a decreased proliferation up to 48 h that was recovered at 72 h, whereas WT BT474 cell proliferation was decreased throughout the timecourse (Figure 28B). To examine these results further, I also determined the cell viability of both

cell lines at 72 h following treatment with a dose titration of lapatinib. Lap^R BT474 cells showed that 1µM of lapatinib was the best concentration to differentiate their degrees of resistance. At lower concentrations, although resistant cells had a better advantage they still showed an overall decrease in their survival rate. However, at higher lapatinib concentrations (1.5µM) both cell lines had their survival rates decreased to a minimum (Figure 28C). These data indicate that FOXO3a has a role in lapatinib-mediated resistance in BT474 cells. Although in Lap^R BT474 cells FOXO3a activity is decreased, its total levels are substantially increased. Thus, it is reasonable to argue that the net FOXO3a activity is better assessed by its *bona fide* targets, such as $p27^{Kip1}$ and FOXP1, rather than its expression.

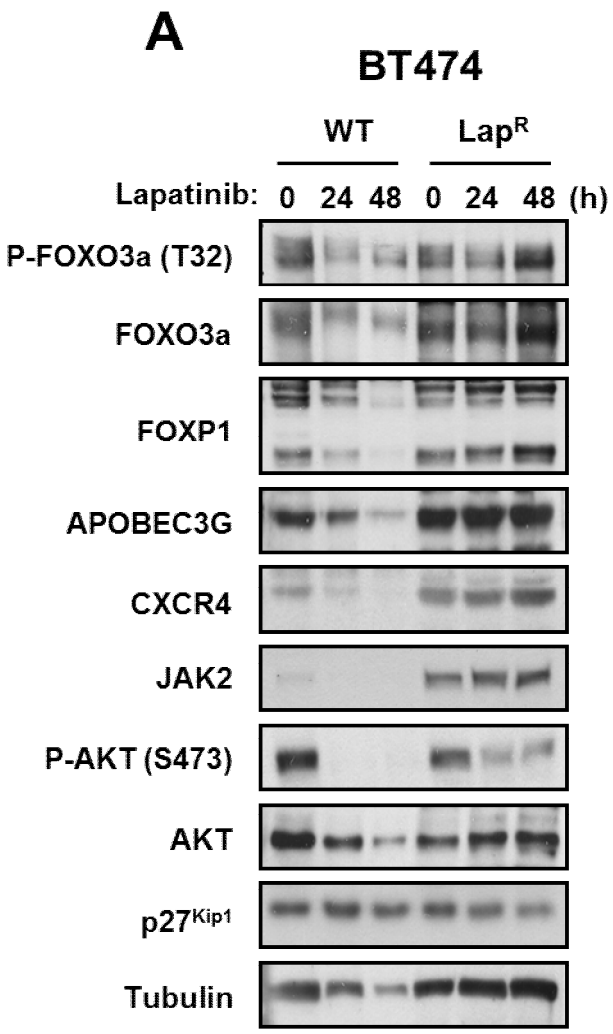


Figure 28. BT474 Lap^R cells have higher inactivation of FOXO3a.

BT474 WT and Lap^R cells were treated with and without 1µM of lapatinib for 24, 48 and 72 h. **A**) Cell lysates were collected at 24 and 48 h for subsequent western blot analysis for the indicated antibodies. **B**) Cell proliferation was analysed by SRB assay in the absence or presence of lapatinib for the given time points. Results are the mean ±SD of eight replicates. **, P<0.01; ***, P < 0.001. **C**) Cells were treated with a dose titration of lapatinib and cell survival at 72 h analysed by SRB assay.

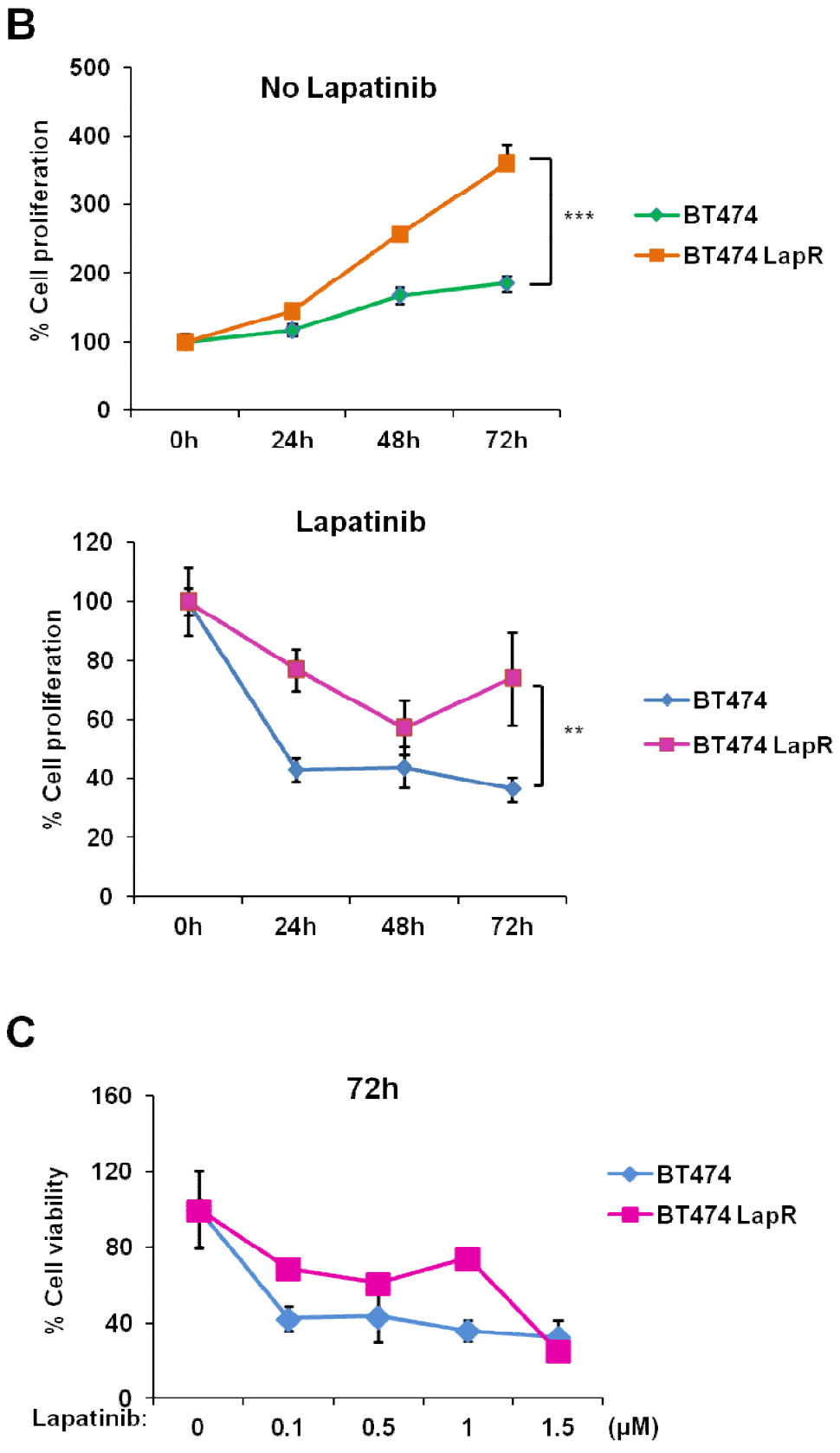
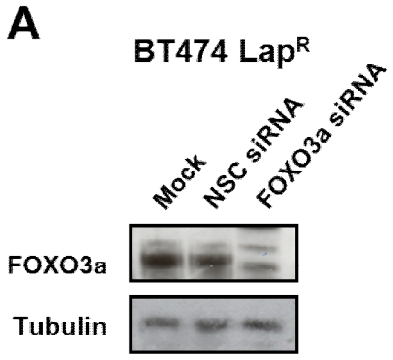


Figure 28. Continued.

4.3.10 Silencing of FOXO3a does not alter the proliferation of BT474 Lap^R cells

To further understand the role of FOXO3a in lapatinib-mediated resistance, the effects of lapatinib treatment of resistant Lap^R BT474 cells following the knockdown of FOXO3a were investigated. Lap^R BT474 cells were untransfected (Mock) or transfected with a NSC or FOXO3a specific siRNA. Western blot analysis confirmed the efficient knockdown of endogenous FOXO3a (Figure 29A) and the subsequent SRB assays were performed to analyse the effects of this knockdown on Lap^R BT474 cells proliferation in the presence or absence of lapatinib (Figure 29B). It is notable, that the transfection itself had an effect in the proliferative rates of the cells. This can be demonstrated by the overall positive proliferative effect of both NSC and FOXO3a siRNA transfected cells in comparison to Mock untransfected cells. As observed in Figure 29B, FOXO3a silencing did not have discernable effect on the proliferation of Lap^R BT474, whereas when FOXO3a siRNA transfected cells were compared with Mock there was a marginal increase in the proliferative rate of these cells. Following lapatinib addition there was a substantial decrease in proliferation of Mock cells when compared to both NSC and FOXO3a siRNA transfected cells, suggesting that the lapatinib-induced decrease in cell proliferation was overcome in cells transfected with NSC or FOXO3a siRNA. This could indicate that FOXO3a is involved in the mediation of lapatinib-resistance in BT474 cells.



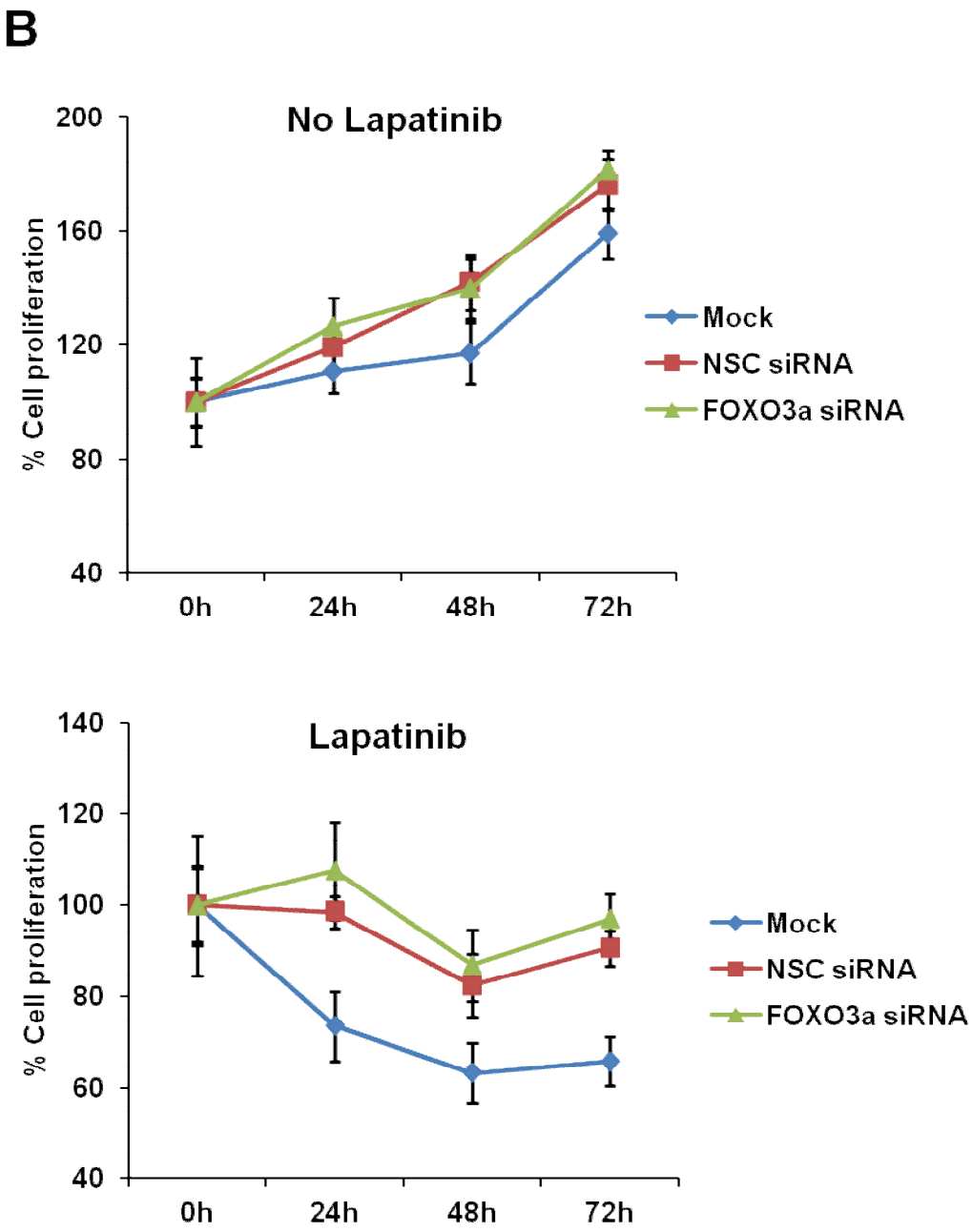


Figure 29. FOXO3a silencing increases proliferation in BT474 Lap^R cells.

BT474 Lap^R cells were transfected with a NSC and FOXO3a specific siRNA and Mock. **A**) Western blot analysis confirmed knockdown of endogenous FOXO3a. Tubulin levels were also analysed. **B**) Following siRNA FOXO3a transfection, cells were re-plated into 96 well plates for 24 h and cell proliferation measured by SRB assay for the given time points with and without lapatinib.

4.3.11 FOXO3a expression in sensitive and resistant breast cancer cells

Taking in consideration all the results obtained thus far, FOXP1 showed to be the most likely FOXO3a downstream target. For this reason all the experiments hereafter were focused on characterising FOXP1 regulation by FOXO3a. The experiment on Figure 3018 enabled me to choose a cell line that expresses relatively high levels of FOXO3a and FOXP1. This could prove useful for studying the potential FOXO3a-mediated regulation of FOXP1 in the context of sensitivity and resistance by using MCF-7 as a breast cancer cell line model which is sensitive to chemotherapeutic drug treatment.

To study the role of FOXO3a in the development of drug-resistance in breast cancer, I determined the expression and phosphorylation levels of AKT and FOXO3a in sensitive wild type (WT), cisplatin-resistant (Cis^R), and epirubicin-resistant (Epi^R) MCF-7 cells. Western blot analysis showed that FOXO3a activity and expression levels were higher in sensitive WT and in Cis^R MCF-7 cells compared with Epi^R cells. Epi^R cells showed a reduction in the levels P-FOXO3 (T32), as revealed by the increased levels of phosphorylated FOXO3a, as well as a reduction in its expression. The increased levels of phosphorylated FOXO3a in Epi^R cells, were associated with the increased levels of P-AKT (S473) compared to MCF-7 and Cis^R cells. FOXP1 expression is higher in WT and Cis^R MCF-7 cells and it was greatly reduced in Epi^R MCF-7 cells (Figure 30).

This result supports the previous finding that the MCF-7 breast cancer cell line has an increased activity and expression of FOXO3a and consequently FOXP1 expression (Figure 18A). Furthermore, these data also suggest that FOXP1 has a role in mediating drug-resistance, at least for epirubicin-resistance, in a FOXO3a dependent manner.

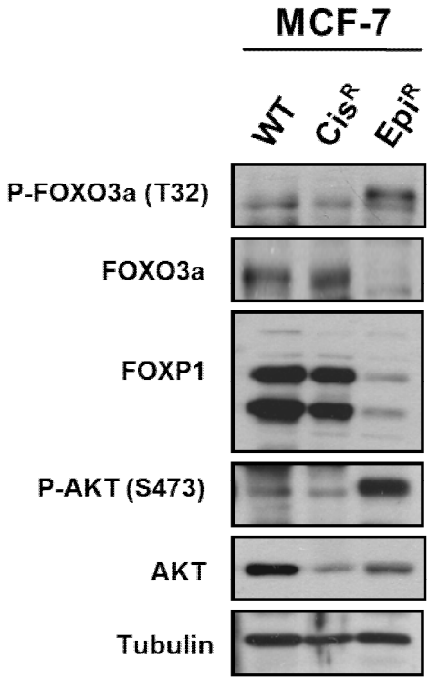


Figure 30. FOXO3a activity and expression levels are downregulated in epirubicin-resistant MCF-7 (Epi^R) cells.

Western blotting was performed to compare the basal expression levels of P-FOXO3a (T32), FOXO3a, FOXP1, P-AKT (S473) and AKT in sensitive MCF-7 (WT), cisplatin-resistant MCF-7 (Cis^R) and epirubicin-resistant MCF-7 (Epi^R) cells. Tubulin was used as a loading control.

4.3.12 No correlations are found between FOXO3a expression and cisplatin resistance in MCF-7 cell lines

To examine the potential role of FOXO3a and FOXP1 in cisplatin resistance, I further determined the effect of 1μM cisplatin on total and phosphorylated FOXO3a and FOXP1 expression over a 48 h period in both MCF-7 and Cis^R cell lines (Figure 31A). Western blot analysis showed little difference in FOXO3a and FOXP1 expression pattern during the timecourse between sensitive and cisplatin-resistant cell lines. Moreover, treatment with cisplatin did not affected the expression levels of FOXO3a, FOXP1 and AKT. Despite this, an increase in P-FOXO3a (T32) was noted at 4 h in MCF-7 cells. I also determined FOXP1 transcriptional levels by performing RT-qPCR. Similar to what was observed in Figure 31A, there were no significant changes on FOXP1 transcript levels throughout the cisplatin timecourse and between the cell lines (Figure 31B). In accordance to the previous result shown in Figure 30, no difference was observed between MCF-7 and Cis^R cell lines.

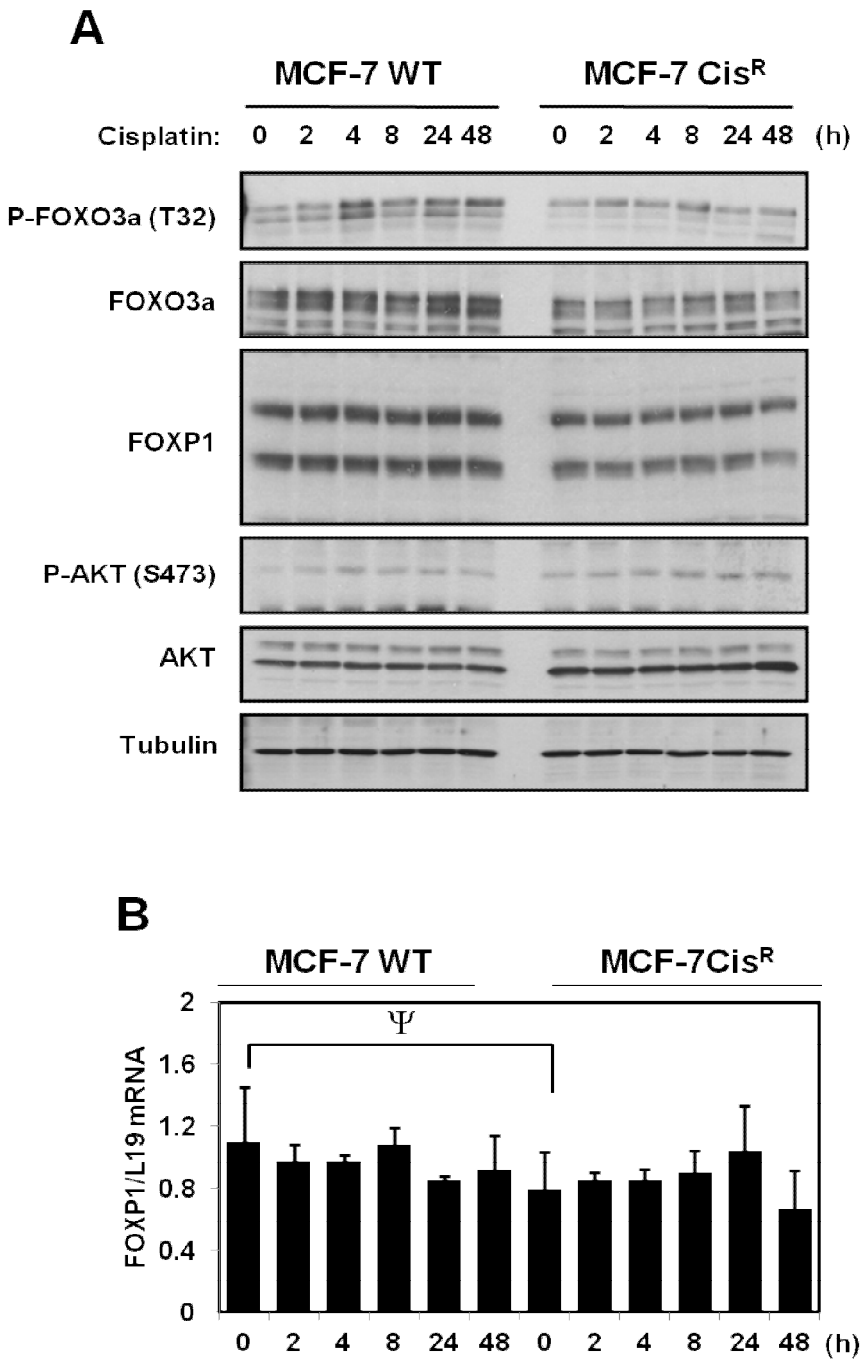


Figure 31. FOXO3a and FOXP1 expression levels remain constant throughout a cisplatin timecourse in sensitive and resistant cells.

MCF-7 and Cis^R cells were treated with 1μM of cisplatin for the times indicated. **A**) The expression levels of P-FOXO3a (T32), FOXO3a, FOXP1, P-AKT (S473), AKT and Tubulin were assessed by Western blotting. **B**) Total RNA was isolated in parallel and the expression of FOXP1 transcripts was measured by RT-qPCR and normalised to the level of L19. All data shown represent the averages of data from three experiments, and the error bars show the standard deviations. Statistical analysis was done using t-test Ψ, P=non-significant.

4.3.13 FOXO3a is differentially expressed in sensitive and epirubicin-resistant cell lines

To further investigate the role of FOXO3a in epirubicin-mediated resistance I subjected both MCF-7 and Epi^{R} cells to treatment with 1µM epirubicin and performed Western blotting to assess the activity and expression of FOXO3a, AKT and FOXP1 in a timecourse over 48 h. As shown in MCF-7 cells (Figure 32A), there was a reduction in the phosphorylated forms of FOXO3a following 24 h of treatment. This suggests that epirubicin treatment was activating FOXO3a by increasing its activity, whilst total FOXO3a levels remained relatively constant throughout the timecourse. Furthermore, p27^{Kip1} - a direct target of FOXO3a - was also downregulated at 8 h which was accompanied by a decrease in the activity of FOXO3a. FOXP1 expression decreased after 24 h of treatment although at transcriptional level there was an increase in FOXP1 mRNA levels after 24 h (Figure 32B). It is notable that FOXP1 expression mirrored the activity of FOXO3a, further suggesting that FOXP1 is regulated by FOXO3a. In Epi^{R} MCF-7 cells, FOXO3a activity and expression levels were lowered when compared to sensitive cells. In line with our previous result in Figure 30, this result showed a pronounced difference in FOXP1 expression in both cell lines, which correlates with FOXO3a expression levels. In Epi^{R} cells, FOXO3a activity was higher and relatively constant after treatment, consistent with an increase in activity of the PI3K pathway, as revealed by the higher levels of P-AKT (S473). Moreover, p27^{Kip1} displayed the same expression pattern as the potential FOXO3a downstream target FOXP1, almost undetectable in this cell line. Examination of FOXP1 transcript levels by RT-qPCR revealed that similarly to protein levels, FOXP1 transcripts were significantly lower in Epi^{R} MCF-7 cells and remained at low levels after epirubicin treatment (Figure 32B). The results above further support the idea that FOXP1 is a transcriptional target of FOXO3a in breast cancer cells and that both FOXO3a and FOXP1 are associated with epirubicin sensitivity.

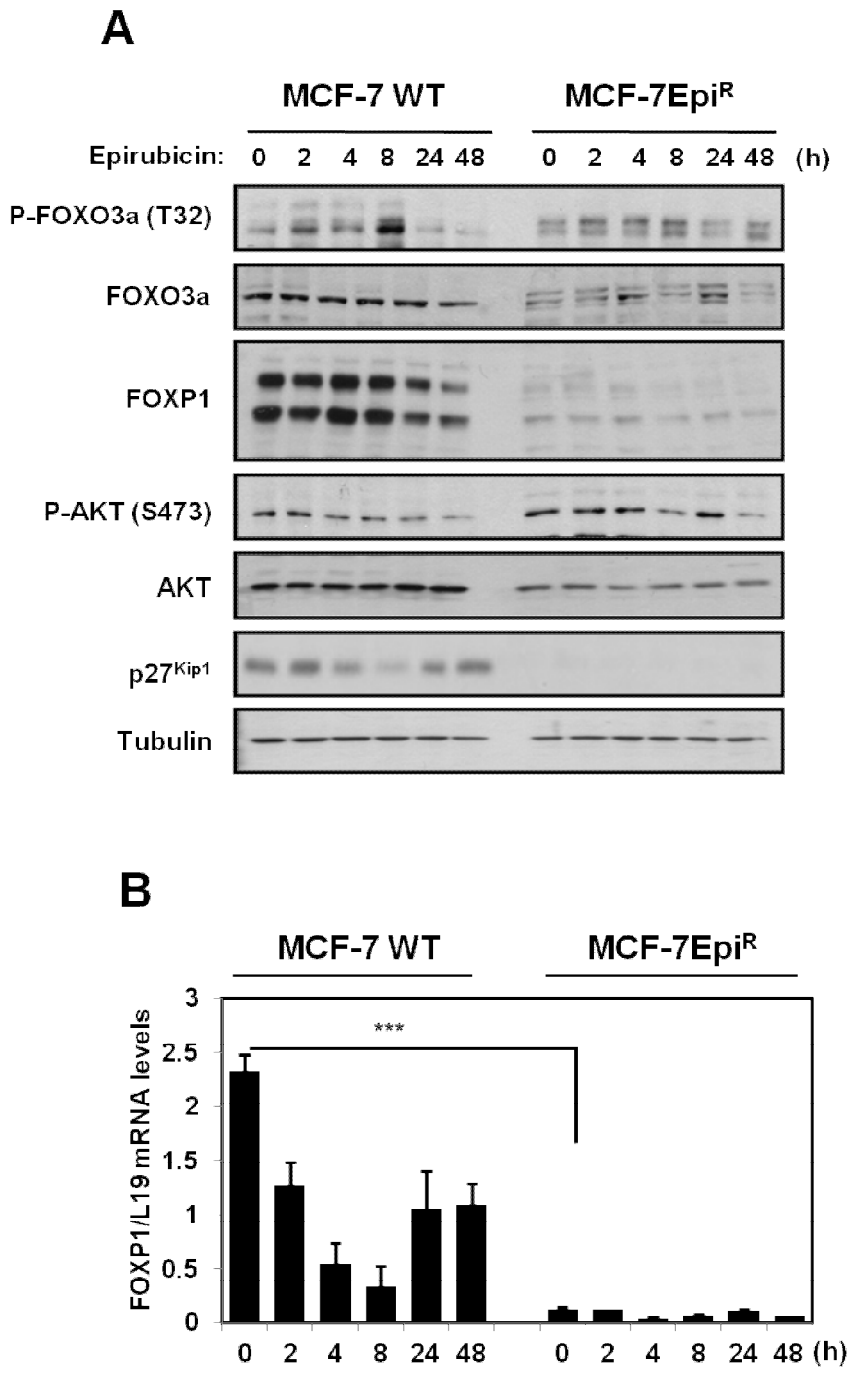


Figure 32. Epi^R cell line shows a sustained reduction in FOXP1 protein and mRNA levels.

MCF-7 and Epi^R were treated with 1µM of epirubicin for the indicated times. **A**) P-FOXO3a (T32), FOXO3a, FOXP1, P-AKT (S473), AKT and p27^{Kip1} expression levels were determined by Western blotting. Tubulin was used as a loading control. **B**) Total RNA was isolated in parallel and the expression of FOXP1 transcripts was measured by RTq-PCR and normalised to L19 levels. All data shown represent the averages of data from three experiments and the error bars show the standard deviations. Statistical analysis was done using t-test ***, P≤0.001, significant.

4.3.14 Nuclear FOXO3a and FOXP1 increases upon epirubicin treatment in MCF-7 and Epi^R cells

Since FOXO3a activity is dependent on its localisation within the cell, I performed subcellular fractionation on both sensitive and resistant cell lysates to study the nuclear and cytoplasmic localisation of FOXO3a and FOXP1 in response to epirubicin treatment. Both WT and Epi^R MCF-7 cells were treated with 1µM of epirubicin or remained untreated for 24 hours. Western blot analysis showed that nuclear FOXO3a expression increases upon treatment with epirubicin in both cell lines. Concordantly, FOXP1 and p27^{Kip1} levels also increased in the nuclear compartment following epirubicin treatment although in Epi^R cells their levels are lower. It is noteworthy that equal amounts of nuclear and cytoplasmic proteins were analysed by western blotting. As a result, high levels of nuclear proteins compared with cytoplasmic proteins will be represented in the western blot (Figure 33). This could explain the considerable amounts of Tubulin found in the nuclear fractions. Consistent with what had been seen before in Figure 30 & 32, FOXP1 levels are lower in Epi^R cells compared to MCF-7 cells. Importantly, this result further supports that FOXO3a is targeted by epirubicin and this leads to an increase in FOXP1 expression in both sensitive and resistant breast cancer cell lines.

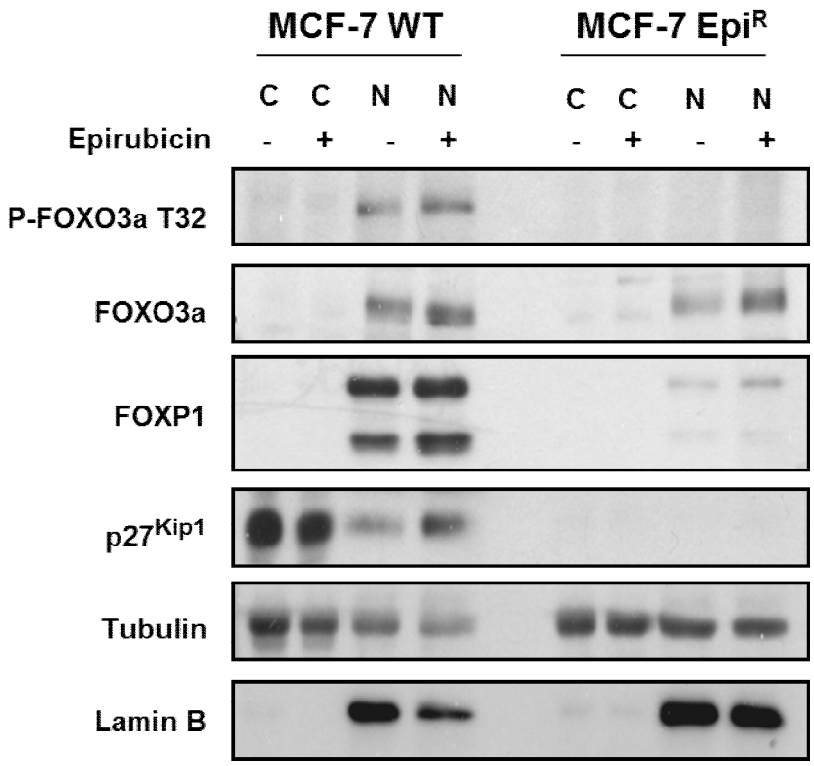


Figure 33. Nuclear FOXO3a and FOXP1 increases upon epirubicin treatment in MCF-7 and Epi^{R} cells.

MCF-7 and Epi^{R} cells were treated with or without 1µM of epirubicin for 24 h. Cell pellets were subject to subcellular fractionation for each cell line. P-FOXO3a, FOXO3a, FOXP1 and p27^{Kip1} expression levels were assessed by western blotting. Tubulin and Lamin B were used as loading controls for cytoplasmic and nuclear fractions, respectively.

4.3.15 FOXO3a activation enhances downstream FOXP1 expression

I next explored whether FOXO3a induction has an effect on FOXP1 expression. I made use of a stably transfected MDA-MB-231 cell line with an expression vector that encodes for the hormone binding domain of the estrogen receptor (ER) fused to FOXO3a(A3), a constitutive active FOXO3a mutant. In these MDA-MB-231-FOXO3a(A3): ER cells, FOXO3a activity can be induced by treatment with of (4-OHT. MDA-MB-231 cells were used as a control. As shown in Figure 34A, 4-OHT treatment of MDA-MB-231-FOXO3a(A3):ER cells, but not of control cells, induced the expression of FOXO3a target $p27^{Kip1}$. The result also confirmed the previously described PI3K/AKT negative feedback mechanism loop, in which FOXO3a enhances AKT phosphorylation through inducing p110α (Hui et al 2008b). Importantly, FOXP1 induction was observed in MDA-MB-231-FOXO3a(A3):ER cells and no such effect was seen in the control cells, suggesting that this induction of FOXP1 expression is specifically mediated by FOXO3a. I also examined whether FOXO3a induction would regulate FOXP1 at the transcriptional level. As illustrated by the results in Figure 34 B, FOXP1 increased following treatment with 4-OHT in the cells transfected with the active form of FOXO3a, whereas in control cells FOXP1 levels remained unchanged. This result further supports the notion that FOXP1 is regulated by FOXO3a at both transcriptional and translational levels in MDA-MB-231 cells.

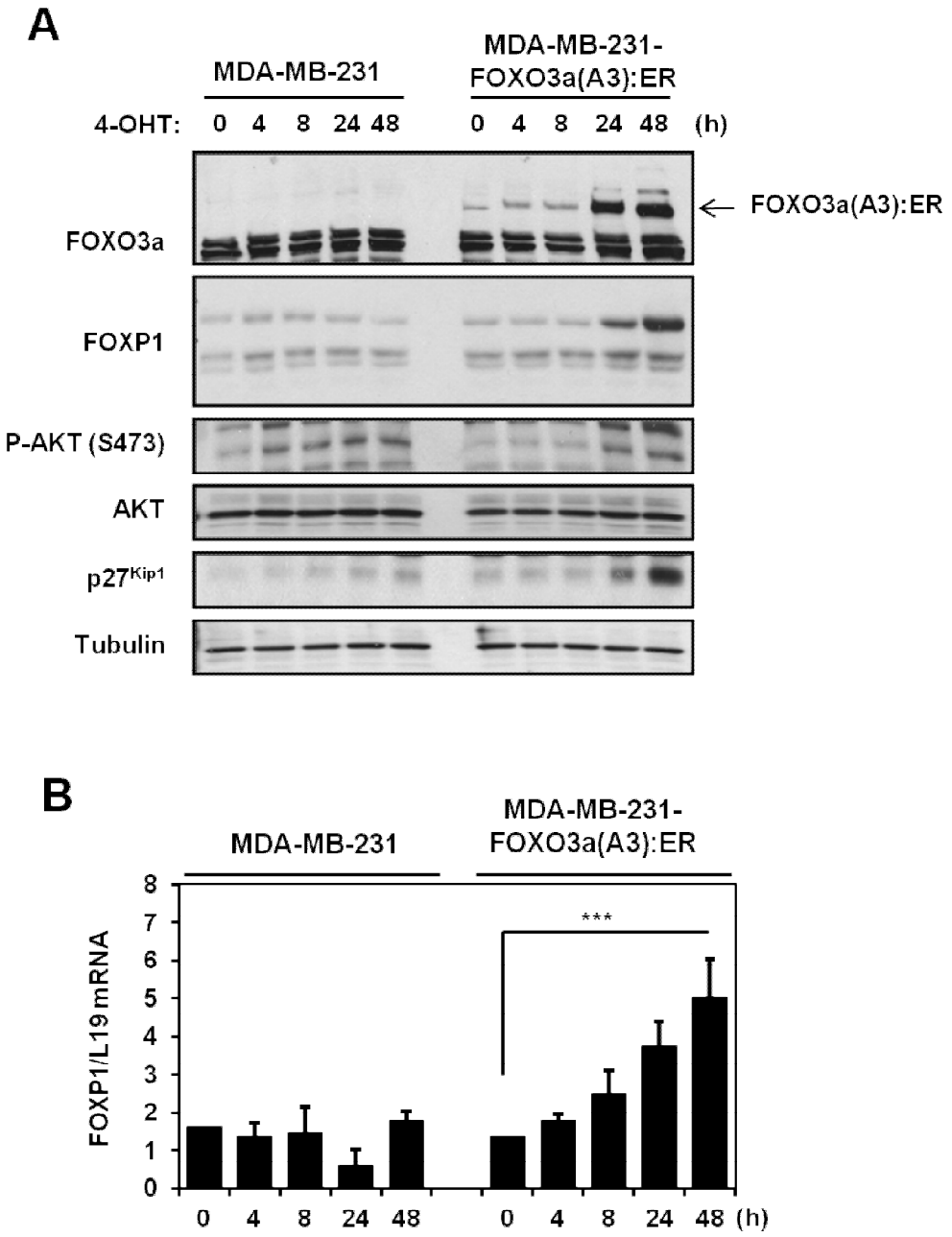


Figure 34. FOXO3a induces FOXP1 at protein and mRNA levels in MDA-MB-231 cells.

MDA-MB-231-FOXO3a(A3):ER and MDA-MB-231 cells were treated with 1µM 4-OHT for the indicated times. **A**) Whole cell extracts were prepared at the times indicated and subjected to immunoblotting. Expression levels of FOXO3a, FOXP1, P-AKT (S473), AKT, $p27^{Kip1}$ and Tubulin were analysed by Western blotting. The known FOXO3a target ($p27^{Kip1}$) was used as a readout of the inducible system. **B**) Total RNA was extracted from these cells and analysed for FOXP1 mRNA expression using RT-qPCR and normalised to the level of L19. All data shown represent the averages of data from three experiments, and the error bars show the standard deviations. Statistical analysis was done using t-test ***, P≤0.001, significant.

4.3.16 FOXP1 is transactivated by FOXO3a in vivo

Next, I investigated whether FOXO3a regulates FOXP1 expression at the gene promoter level. To this end, I studied *FOXP1* endogenous promoter occupancy by FOXO3a using chromatin immunoprecipitation (ChIP). MCF-7 cells were cultured in the absence or presence of epirubicin for 24 h and the chromatin was precipitated with a specific FOXO3a antibody and analysed by PCR. Three sets of primers were used to target three potential forkhead DNA binding sequences. These primers were optimised using genomic MCF-7 DNA. The ChIP result confirmed that FOXO3a binds to the *FOXP1* promoter within the forkhead containing region (-634/-557) following epirubicin treatment. However, using the same precipitated DNA with the other two sets of primers failed to amplify a PCR product, showing that the corresponding two sites are not occupied by FOXO3a in response to epirubicin (Figure 35). This result showed that FOXO3a regulates FOXP1 expression through binding and transactivation of its promoter region in MCF-7 cells.

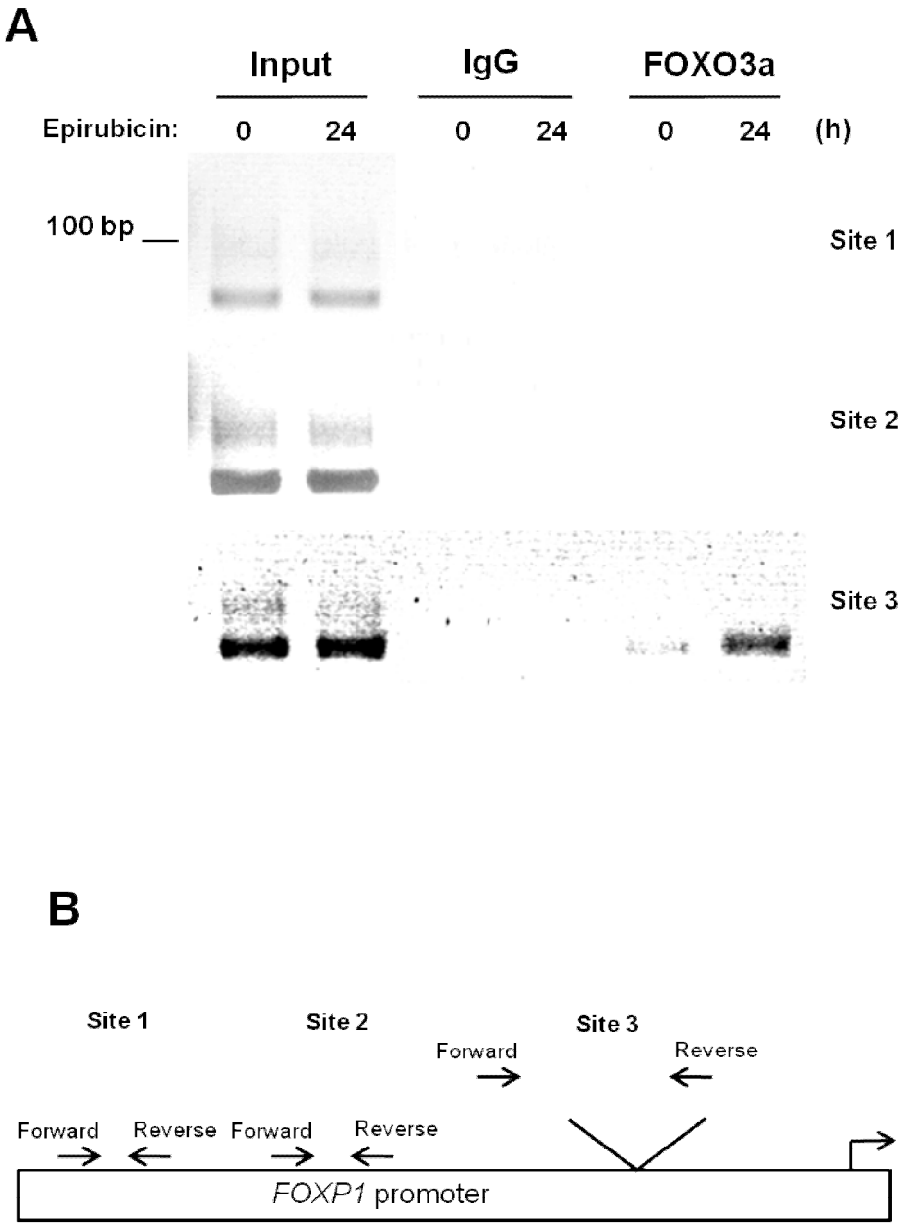


Figure 35. FOXO3a transactivates *FOXP1* promoter *in vivo*.

A) Chromatin immunoprecipitation (ChIP) was performed in MCF-7 cells either in the absence or presence of epirubicin to analyse the human *FOXP1* promoter. A FOXO3a specific antibody (H-144) was used. After crosslink reversal, the co-immunoprecipitated DNA was amplified by PCR using primers amplifying the FOXP1 FHRE containing region (-634/-557) and resolved in 2% agarose gel. Representative data from three independent experiments are shown. **B**) Schematic representation of the three putative FHRE binding sites on *FOXP1* promoter.

4.3.17 Effect of FOXO3a silencing on FOXP1 expression levels

To further confirm that FOXP1 is a target of FOXO3a I decided to investigate the effect of FOXO3a silencing on FOXP1 expression in MCF-7 breast cancer cell line.

To this end, cells were Mock transfected or transfected with NSC or FOXO3a siRNA and harvested at 24 h post-transfection. Western blot results showed that FOXO3a knock-down was partially achieved, and the effects on FOXP1 expression levels were only marginal. There was a reduction in FOXP1 levels when comparing Mock against both NSC and FOXO3a siRNA (Figure 36).

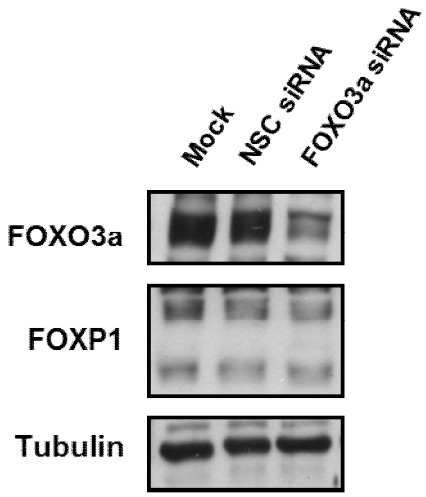


Figure 36. Silencing of FOXO3a does not impact on FOXP1 expression levels.

Cells were transfected with Mock or siRNA specifically targeting FOXO3a or a NSC. 24 h post-transfection, Western blot analysis was performed to confirm efficient FOXO3a knockdown and FOXP1 expression levels.

4.3.18 FOXP1 overexpression induces p27^{Kip1} in Epi^{R} cells

To investigate the function of FOXP1, I transiently transfected Epi^{R} cells with FOXP1 expression vector. Previously, I showed that FOXP1 protein was expressed at lower levels in Epi^{R} cells when compared to Cis^{R} and MCF-7 cells (Figure 30 & 32). Therefore, MCF-7 Epi^{R} cells is a more suitable model to examine the effects of FOXP1 overexpression. I assessed the expression patterns of FOXO3a, AKT and p27^{Kip1} in cells transfected with FOXP1 construct (pcDNA3-FOXP1) and with the control empty vector (pcDNA3) in the absence or presence of 1μM epirubicin treatment for 24 h (Figure 37). The results confirmed FOXP1 overexpression. The exogenous FOXP1 protein was expressed at a higher molecular weight band compared with the endogenous protein. FOXP1 overexpression had no effect on total FOXO3a and AKT levels. However, a marginal increase was observed in P-FOXO3a (T32) and P-AKT (S473) levels suggesting that FOXP1 may function in a similar manner as FOXO3a possibly by engaging in feedback mechanisms with PI3K. Surprisingly, FOXP1 overexpression resulted in an induction of p27^{Kip1}. This induction was mainly due to FOXP1 as FOXO3a activity was decreased, as revealed by the increased levels of P-FOXO3a (T32). Previously, loss of FOXP1 has been showed to increase p21^{Cip1} and decrease p27^{Kip1} levels in the development of the heart. Therefore, this result suggests that FOXP1 may share common downstream targets with FOXO3a.

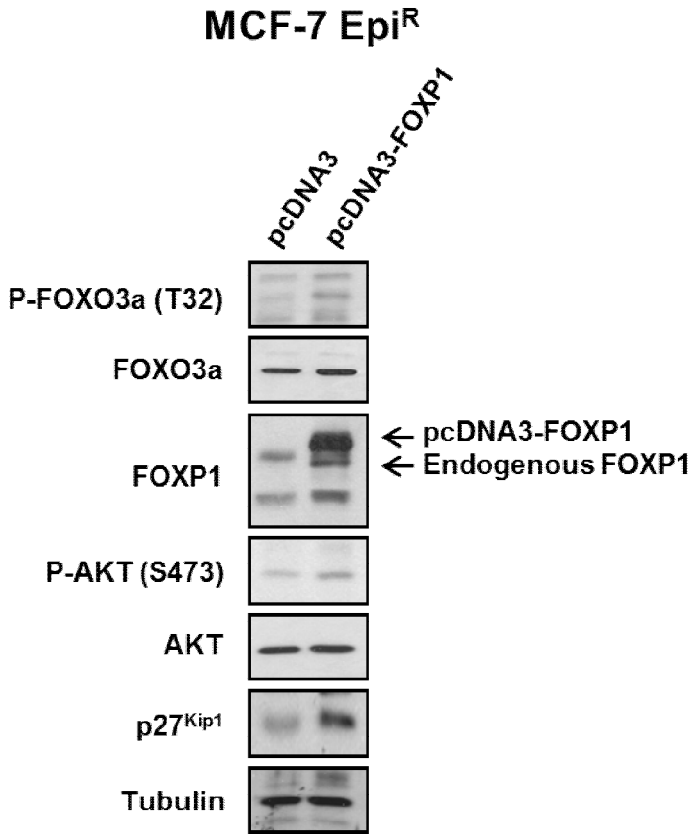


Figure 37. FOXP1 overexpression induces $p27^{Kip1}$ levels in Epi^R cell line.

Epi^R cells were transiently transfected with FOXP1 overexpression vector (pcDNA3-FOXP1) or empty vector pcDNA3 (pcDNA3) for 24 h. Cells were treated with and without epirubicin for 24 h. 24 h after treatment and analysed by Western blotting against P-FOXO3a (T32), FOXO3a, FOXP1, P-AKT (S473), AKT and $p27^{Kip1}$ expression levels were determined. Tubulin was used as a loading control.

4.3.19 FOXP1 overexpression in Foxo1/3/4$^{-/-}$ MEFs leads to an induction of p27^{Kip1}

Since previously I have observed an induction of p27^{Kip1} by FOXP1 in Epi^{R} cells, I overexpressed FOXP1 in *Foxo1/3/4$^{-/-}$* MEFs to show definitely that FOXP1 has a role in inducing p27^{Kip1} independently of FOXO3a. To this end, WT and *Foxo1/3/4$^{-/-}$* MEFs were transfected with 0, 4, 6 and 8µg of FOXP1 expression vector (pcDNA3-FOXP1). Overexpression was confirmed at protein levels (Figure 38A) and at mRNA levels (Figure 38B). In *Foxo1/3/4* deficient MEFs, p27^{Kip1} and also FOXP1 expression levels were lower when compared to the same condition in WT MEFs. However, when FOXP1 was titrated into cells lacking all three FOXO isoforms, an increase in p27^{Kip1} was observed suggesting that FOXP1 by itself can induce p27^{Kip1} expression levels (Figure 38A).

At transcriptional level, FOXP1 overexpression was also confirmed in both cell lines although this overexpression was higher in WT MEFs probably, due to the presence of FOXO3a. In *Foxo1/3/4$^{-/-}$* cells, FOXP1 overexpression was able to restore the induction of p27^{Kip1} mRNA levels, as observed in WT MEFs. However, there was a reduction in p27^{Kip1} mRNA levels (Figure 38B). This can be due to an increase in global protein and mRNA degradation as a result of cell death in the *Foxo1/3/4* deficient cells. It is possible that in WT MEFs, FOXO3a and FOXP1 act together to regulate p27^{Kip1} expression. In summary, this result showed that like FOXO3a, FOXP1 can also induce p27^{Kip1} expression.

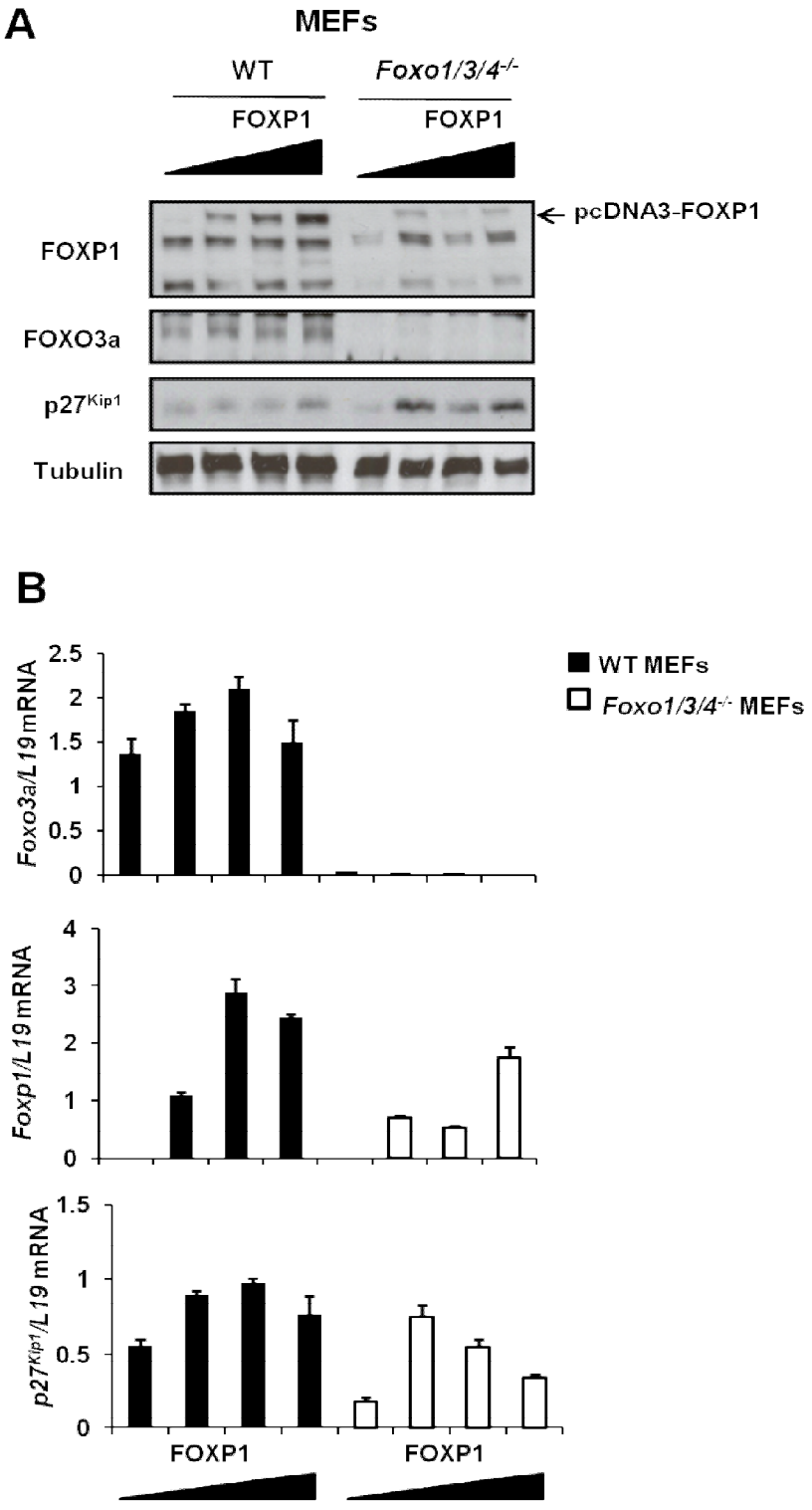


Figure 38. Transfection of FOXP1 induces p27^{Kip1} expression in *FOXO1/3/4* null mouse embryonic fibroblasts.

WT and *Foxo1/3/4* null MEFs were transfected with increasing amounts of empty pcDNA3 vector and pcDNA3-FOXP1. 24 h post-transfection whole cell lysates were collected. **A**) Western blotting was performed to determine the expression levels of FOXP1, FOXO3a, p27^{Kip1} and Tubulin. **B**) RNA was extracted and RT-qPCR performed to evaluate the transcript levels of *foxo3a*, *foxp1* and *p27Kip1*. *L19* was used for normalisation.

4.3.20 FOXP1 silencing enhances cell cycle progression in MCF-7 cells

In order to provide insights into the functional role of FOXP1 in breast cancer cells, MCF-7 cells were either Mock-transfected, or transfected with a non-specific control siRNA pool (NSC) or a FOXO3a-specific siRNA pool. Cells were transfected in dishes, seeded into different format plates after a period of approximately 16 h and harvested for western blotting and RT-qPCR, cell proliferation and FACS analysis. Western blotting and RT-qPCR analysis confirmed knockdown of endogenous FOXP1 (Figure 39A & B). Since the role of FOXP1 in breast has not yet been elucidated, I assessed the impact of its silencing on different cell cycle regulator proteins. Interestingly, $p27^{Kip1}$ levels were higher in the NSC siRNA transfected cells suggesting that the transfection itself induced stress in the cell and caused this upregulation, whereas in FOXP1 siRNA transfected cells, its levels were decreased in comparison with the non-specific siRNA. For this reason, the effect of FOXP1 silencing henceforth was compared with that of the non-specific siRNA transfected cells. FOXP1 silencing increased the phosphorylation levels of P-pRB (S807/811), P-pRB (S795) and also the total pRB levels, indicating an increase in proliferation. Accordingly, there was also an increase in CyclinD1, CDK4, CyclinE1 and CDK2, as well as a decrease in $p27^{Kip1}$ expression (Figure 39A). Together these results indicate that FOXP1 is necessary for cell cycle arrest and that its downregulation may result in the promotion of cell cycle progression.

To establish the effect of FOXP1 knockdown on the sensitivity of the MCF-7 cell line to epirubicin, SRB assays were performed to assess cell proliferation with or without epirubicin treatment over a timecourse of 72 h (Figure 39C). In non-treated MCF-7 cells, the non-specific control siRNA caused a decrease in the rate of proliferation compared to Mock-transfection. This was associated with the increased $p27^{Kip1}$ levels observed in Figure 39A. Knockdown of FOXP1 enhanced cell proliferation significantly when compared to the non-specific control siRNA, and this was again associated with a decrease in $p27^{Kip1}$ levels. Following treatment with epirubicin, the proliferation of both the Mock and siRNA transfected cells was impaired whereas FOXP1 knockdown initially enabled cells to proliferate better although that effect disappeared after 48 h of treatment (Figure 39C). Next, to study the effects of FOXP1 silencing on the cell cycle profile, FACS analysis was performed (Figure 39D). The cell cycle profile showed that FOXP1 silencing caused more cells to enter S and G2/M phases. Moreover, when FOXP1 siRNA transfected cells were treated with epirubicin, in comparison with NSC siRNA transfected cells, there were fewer cells in G1. It was notable that there was an increase in the number of cells in G2/M phase. This can be attributed to the treatment with epirubicin. Unexpectedly, an increase in cell death is also noted following 72 h of epirubicin treatment in FOXP1 siRNA transfected cells (Figure 39D).

In summary, the proliferative effect of knocking-down FOXP1 in MCF-7 cells suggests a role in cell cycle and cell proliferative arrest for FOXP1. Silencing FOXP1 leads to phosphorylation and inactivation of pRB, which in turn results in cell cycle progression. The proliferation assay further supports that FOXP1 is necessary for cell proliferation arrest. Together these results suggest that FOXP1 may be necessary for cell cycle arrest and cell proliferation inhibition in response to epirubicin in drug-sensitive breast cancer cells.

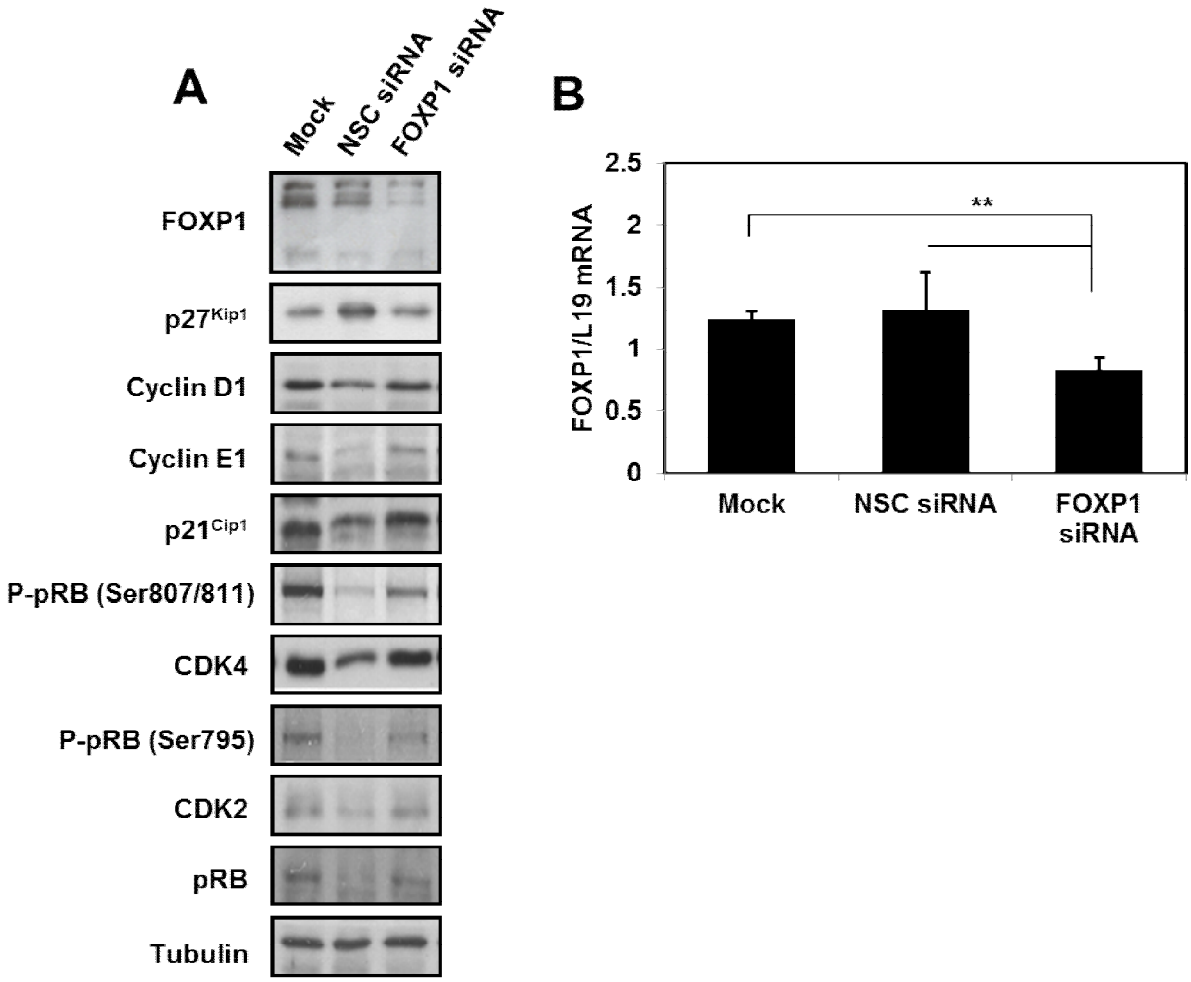


Figure 39. FOXP1 silencing enhances cell cycle progression and proliferation in MCF-7 cells.

MCF-7 cells were transfected either with Mock, NSC siRNA or FOXP1 siRNA. **A**) Whole cell lysates were collected 24 h post-transfection for the indicated timepoints and western blotting was performed for the indicated antibodies. **B**) Total RNA was extracted 24 h post-transfection to confirm FOXP1 knock-down at mRNA level by RT-qPCR. L19 was used for normalisation. Error bars show the standard deviations. Statistical analysis was done using t-test **, P≤0.01, significant. **C**) Cells were counted and seeded into 96 well-plates and treated for 0, 24, 48 and 72 h in the absence and presence of 1µM of epirubicin. Cell proliferation was determined by SBR assay. Each point represents the mean of three independent experiments, each done at least in triplicate; bars, SD. Statistical analysis has been done using Student's t test. ***, P < 0.001; **, P<0.01; Ψ, non-significant. **D**) 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 (<G1, G1, S and G2/M).

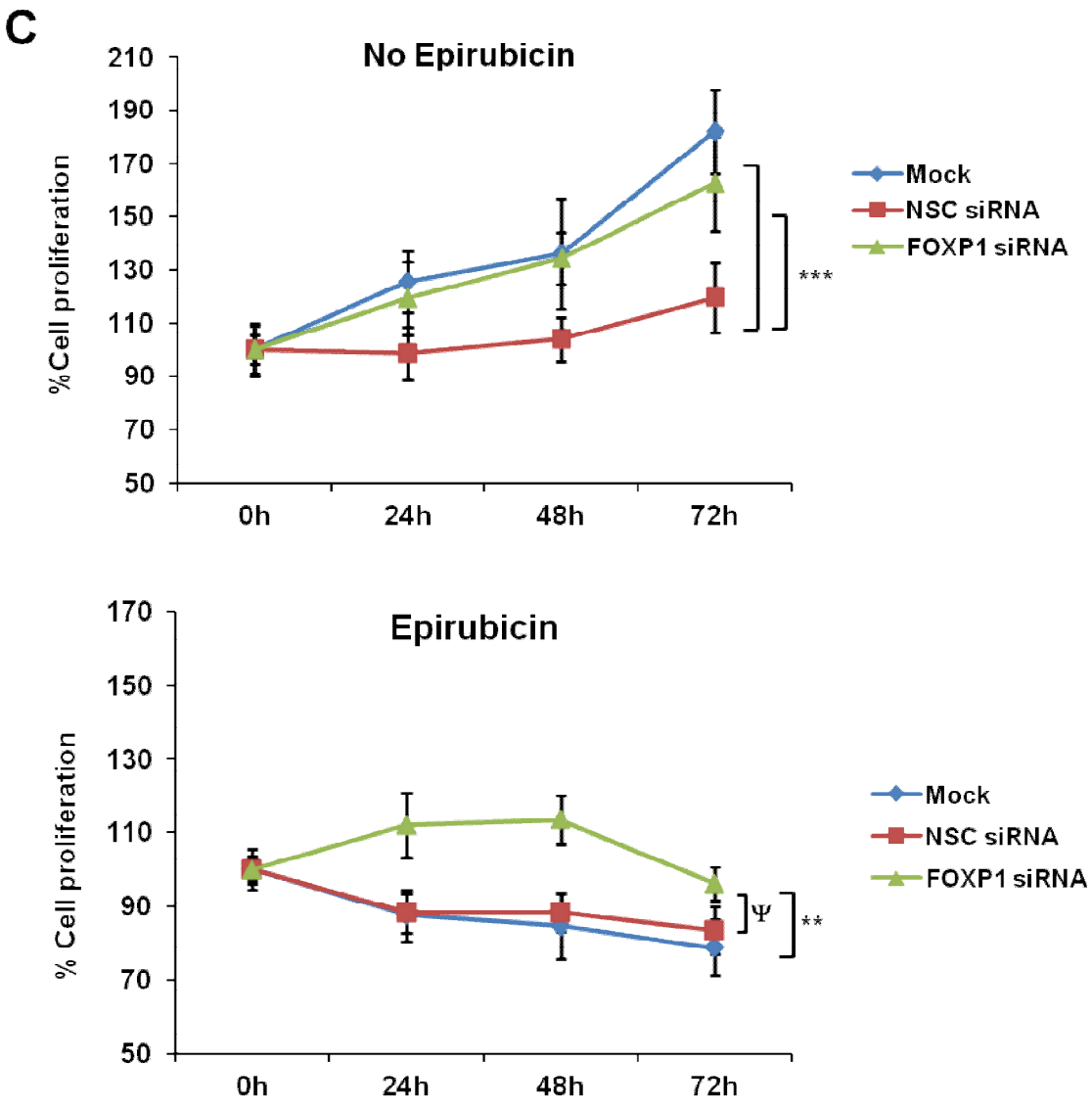


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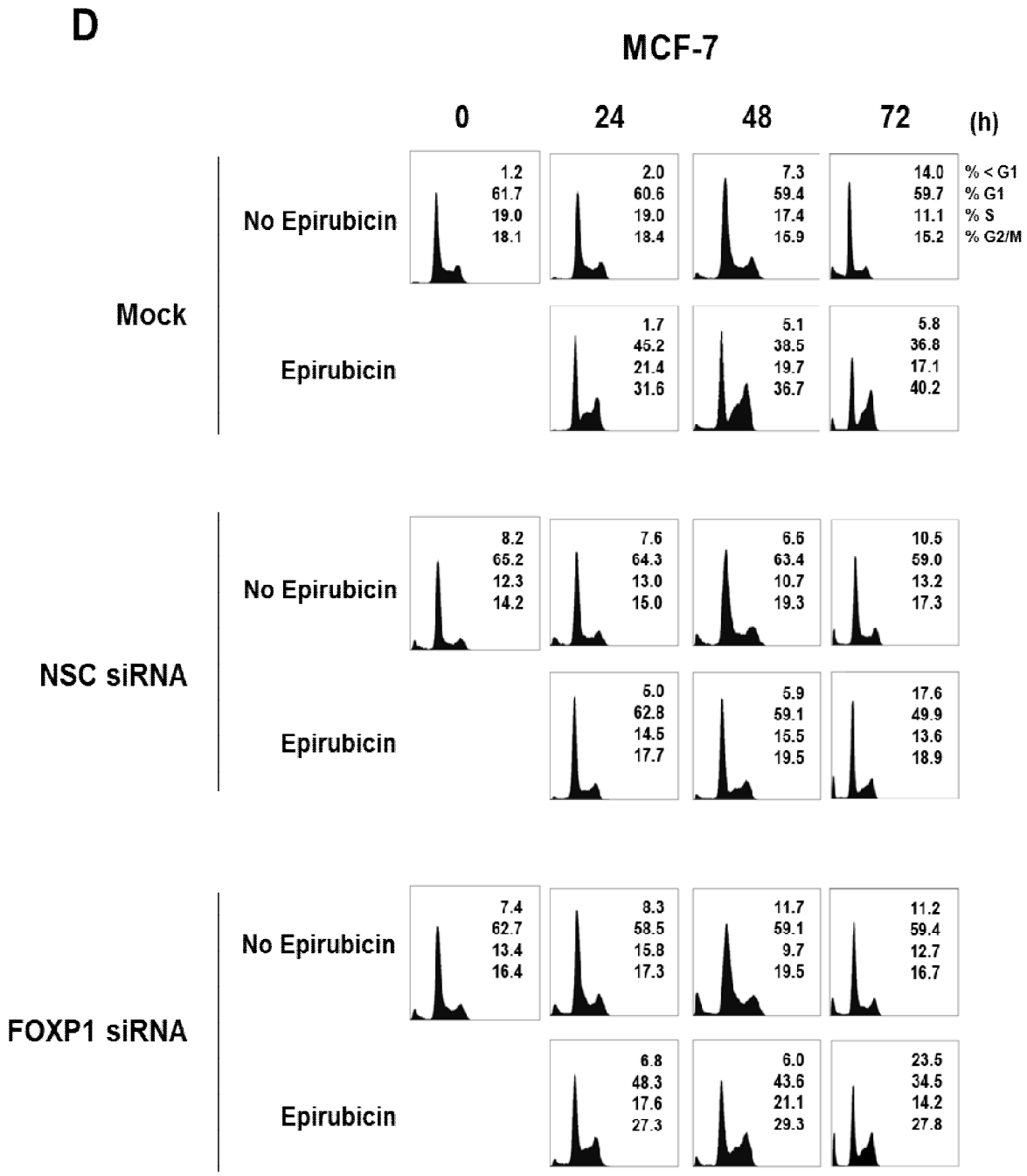


Figure 39. Continued.

4.3.21 Effect of FOXP1 silencing in MDA-MB-231 breast cancer cell line

To further characterise the effect of silencing FOXP1 in breast cancer cells, I performed FOXP1 knockdown by siRNA in a different breast cancer cell line – MDA-MB-231. Western blotting analysis confirmed FOXP1 silencing as indicated by the arrow showing the loss of the slower migrating FOXP1 band. Importantly, loss of FOXP1 caused an abrogation of $p27^{Kip1}$ levels (Figure 40). Unlike what was observed previously in MCF-7 cells, phosphorylation of pRB along with total pRB expression levels were decreased in MDA-MB-231 cells after FOXP1 depletion. One possible explanation is that the loss of FOXP1 could facilitate pRB degradation by regulating MDM2 expression. Accordingly, pRB has been shown to be targeted by MDM2 proteosome-dependent degradation (Sdek et al 2005). Although there was a decrease in CDK4 and CyclinD1 levels, little change was observed in CyclinE1 levels and a marginal increase was seen in CDK2 levels. In line with my previous observations in MCF-7 cells, silencing of FOXP1 in MDA-MB-231 cells, the silence of FOXP1 favoured cell cycle progression.

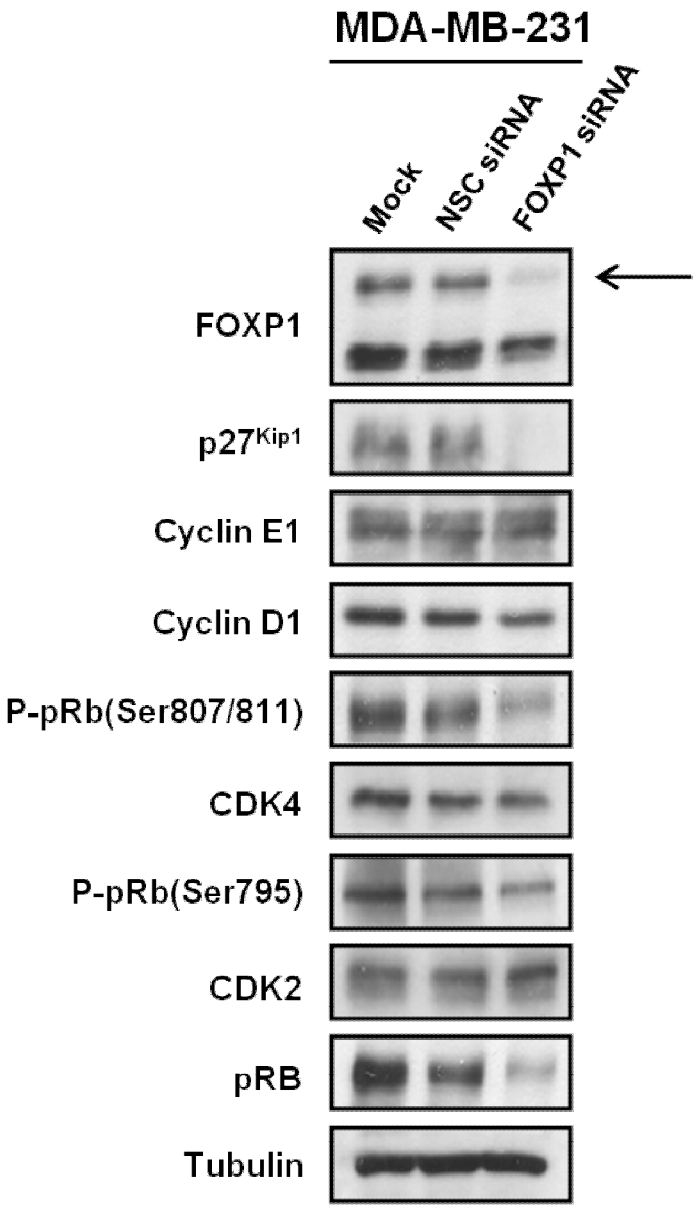


Figure 40. FOXP1 knock-down in MDA-MB-231 cells impairs proteins necessary for cell progression.

MDA-MB-231 cells were transfected with a FOXP1 or NSC specific siRNA and Mock. Western blot analysis confirmed knockdown of endogenous FOXP1. The expression levels of various cell cycle protein regulators were analysed and Tubulin was used as a loading control.

4.3.22 Effects of FOXP1 overexpression in Epi^R cells

To delineate the effect of FOXP1 overexpression in epirubicin-resistant breast cancer cells, Epi^R cells were either transfected with empty vector (pcDNA3) or pcDNA3 encoding FOXP1 cDNA (pcDNA3-FOXP1). Cells were transfected for 24 h and later harvested for FACS analysis (Figure 41A) and Western blotting (Figure 41B). FACS results showed that, in untreated cells over the timecourse, there were no significant differences between untransfected, empty vector-transfected and FOXP1 overexpressing cells. Furthermore, when cells were treated with epirubicin for the different times, no changes were again observed. However, at 0 h, FOXP1 overexpressing Epi^R MCF-7 cells exhibited a higher percentage in S phase and fewer G2/M phase cells in comparison with untransfected and empty vector transfected cells. Western blot analysis showed that there was no difference in FOXP1 levels between the control and FOXP1 transfected cells. An increase in p27^{Kip1} levels was observed in FOXP1 overexpressing cells, although in the transfected control cells a slight decrease was noted (Figure 41B). The reason for this is unclear. Due to Epi^R cells lower transfection efficiency, I performed FOXP1 stable rather than transient transfections.

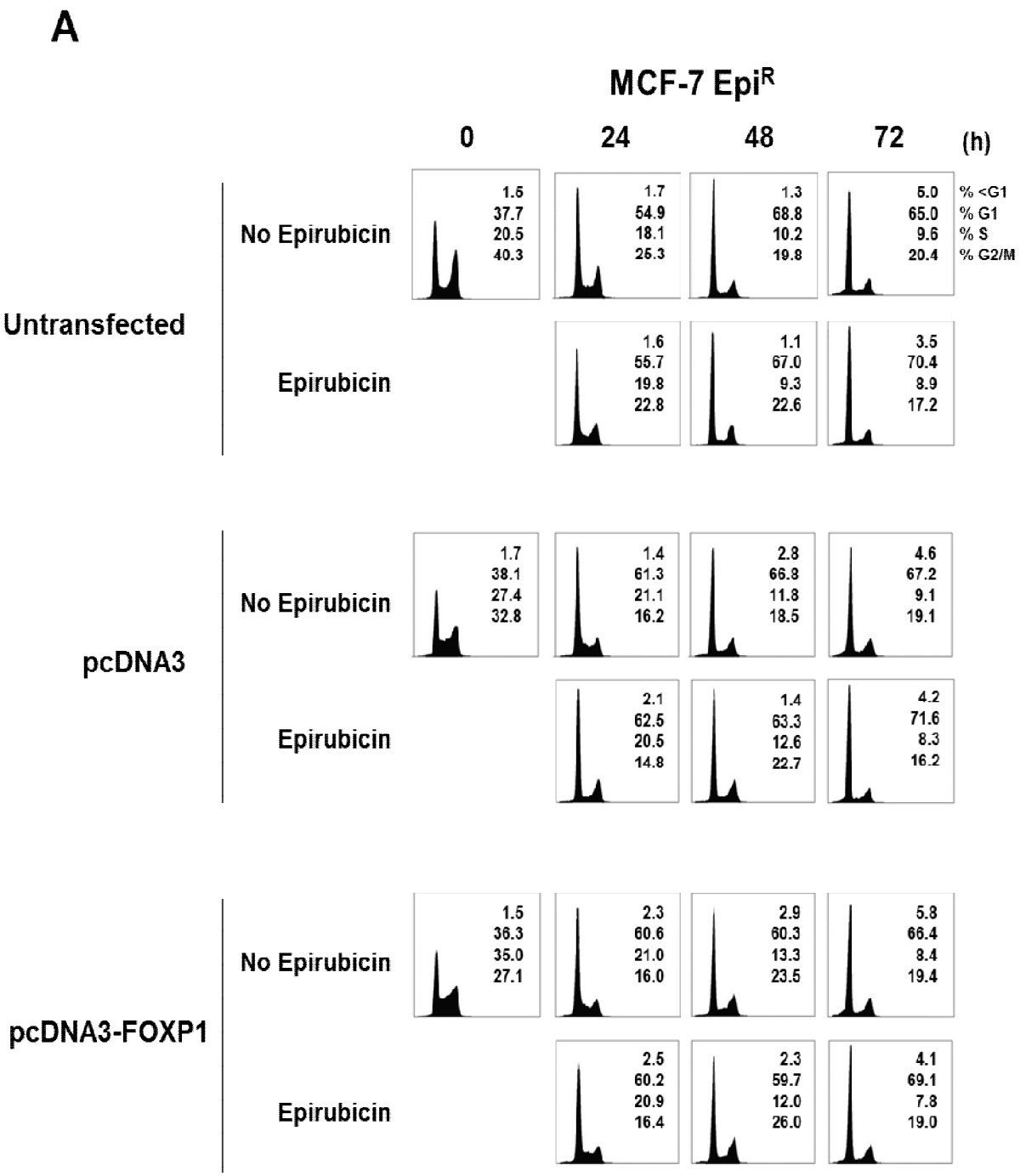


Figure 41. Effects of FOXP1 overexpression in Epi^R cells in the cell cycle profile.

Cells were either untransfected or transfected with a FOXP1 overexpressing vector (pcDNA3-FOXP1) and the control vector (pcDNA3). **A**) 24 h post-transfection, cells were fixed in ethanol and stained with propidium iodide then analysed by FACS. The % of cells in each phase of the cell cycle is indicated. **B**) Whole cell lysates were also collected to assess the overexpression of FOXP1 at protein level by Western blotting. The expression levels of FOXP1, p27^{Kip1} and Tubulin were analysed.

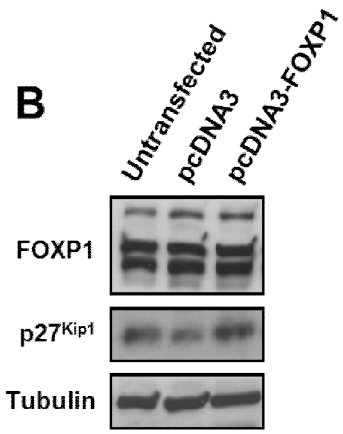


Figure 41. Continued.

4.3.23 Generation and characterisation of Epi^{R}FOXP1 stable cell line

As it proved difficult to transiently overexpress FOXP1 in Epi^R cells, I decided to generate MCF-7 Epi^R cell line stably overexpressing FOXP1. For this purpose, two different pools of Epi^R cells were transfected with pcDNA3-FOXP1 vector and then selected with an optimised concentration of neomycin. After 3 weeks, cells were pooled and subjected to western blotting to assess FOXP1 expression. As observed in Figure 42 the two different pools of transfected cells showed higher FOXP1 expression levels when compared to control cells transfected only with empty pcDNA3 vector. Additionally, an induction in p27^{Kip1} levels was observed, further supporting our hypothesis that FOXP1 upregulates this cell cycle regulator (Figure 42).

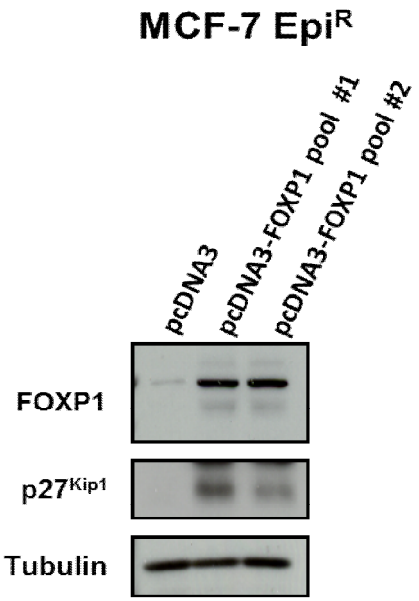


Figure 42. FOXP1 stable cell line generation.

Cells were transfected with the empty control vector (pcDNA3) or with the neomycin-resistant (pcDNA3-FOXP1) vector. 24 h post-transfection cells were selected with neomycin for a period of at least 2 weeks until cell proliferation was established. Cells were tested for the stable overexpression of FOXP1 by western blotting. The expression of the FOXP1, p27^{Kip1} and Tubulin was analysed.

4.3.24 Functional effect of Epi^R FOXP1 stable cell lines

Next, in order to study the effect of FOXP1 on cell proliferation and cell cycle, I used the MCF-7 Epi^R-FOXP1 stable cell line and compared them to the empty vector transfected stable cell lines (Epi^R pcDNA3). To this end, cells were treated with a dose titration of epirubicin ranging from 500nM to 10μM for 0, 24, 48 and 72 h. MCF-7 Epi^R-FOXP1 and the control stable cell lines continued to proliferate even in the presence of concentrations as high as 10μM. Across all doses, no significant cytotoxic effects were observed, further confirming the resistant profile of these cells (Figure 43A, B, C). I also examined the viability of these cells in terms of cell survival at 72 h. MCF-7 cells were included as the drug-sensitive control. Surprisingly, the FOXP1 stably overexpressing cells showed an increased cell proliferative rate in comparison to control cells (Figure 43C). To study the effects of FOXP1 on the cell cycle, both the control and FOXP1 overexpressing cells were analysed by FACS (Figure 43D). Cells were treated for 0, 24 and 48 h with the same range of epirubicin used for the proliferation assays. For the untreated cells, the cell cycle profile showed that sustained FOXP1 overexpression led to a decrease of cells in G1 phase – 54.4% compared to 33.6% in the controls - and an accumulation in G2/M phase – 23.8% compared to 39.3% in the controls. The epirubicin doses used did not result in a substantial difference in the proliferative rate between the two cell lines. Nevertheless, a reduction was noted in the percentage of cells in G1 phase and an increase in the percentage of G2/M cells, which was more prominent at 24 h. At 48 h with both 5 μM and 10μM of epirubicin, the controls had higher numbers of cells in G2/M, whilst MCF-7 Epi^R-FOXP1 maintained their cell cycle profile as the untreated cells (Figure 43D).

Taken together, these results suggest that the stable cell line overexpressing FOXP1 is more resistant to epirubicin than the MCF-7 Epi^R. This is contradictory to my earlier data and it appears that these cells can cope better with the cytotoxic effects of epirubicin. The MCF-7 Epi^R-FOXP1 cells generated may represent a pool of cells that can tolerate high levels of FOXP1 and may have downstream signalling mechanisms deregulated. To circumvent this problem, I decided to clone FOXP1 cDNA into a GFP-vector so that FOXP1 can be analysed by FACS after sorting for GFP expression.

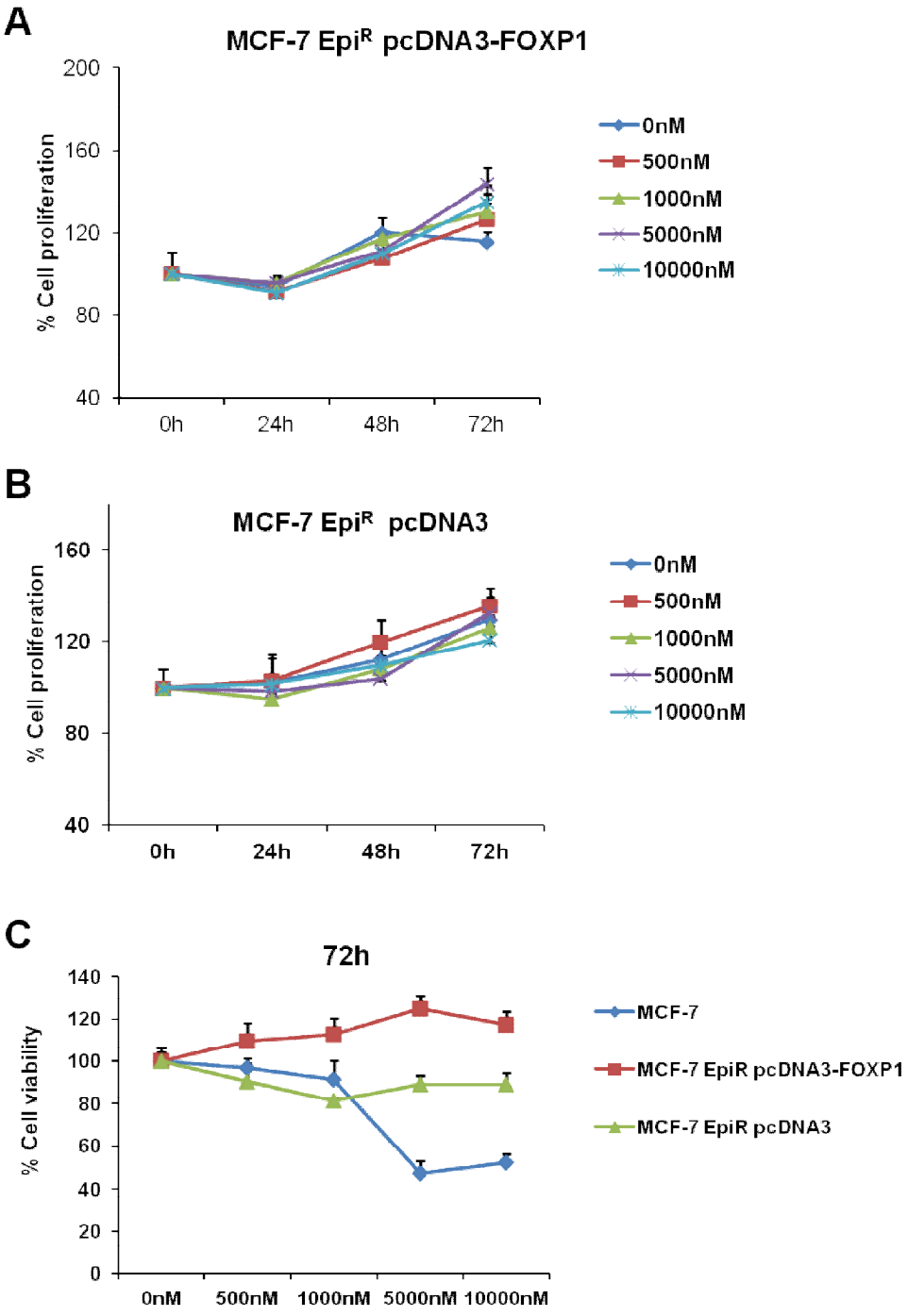


Figure 43. FOXP1 stable overexpression confers a protective effect to Epi^R cells.

Epi^R pcDNA3 and Epi^R FOXP1-pcDNA3 cells were treated with a dose titration of 0, 500, 1000, 5000 and 10000nM of epirubicin for 0, 24, 48 and 72 h. **A**, **B**) Cell proliferation was determined by SRB assay. **C**) Cell survival at 72 h was assessed. The sensitive MCF-7 cell line was included. **D**) Cell cycle analysis of the same samples was performed after propidium iodide staining for 0, 24 and 48 h.

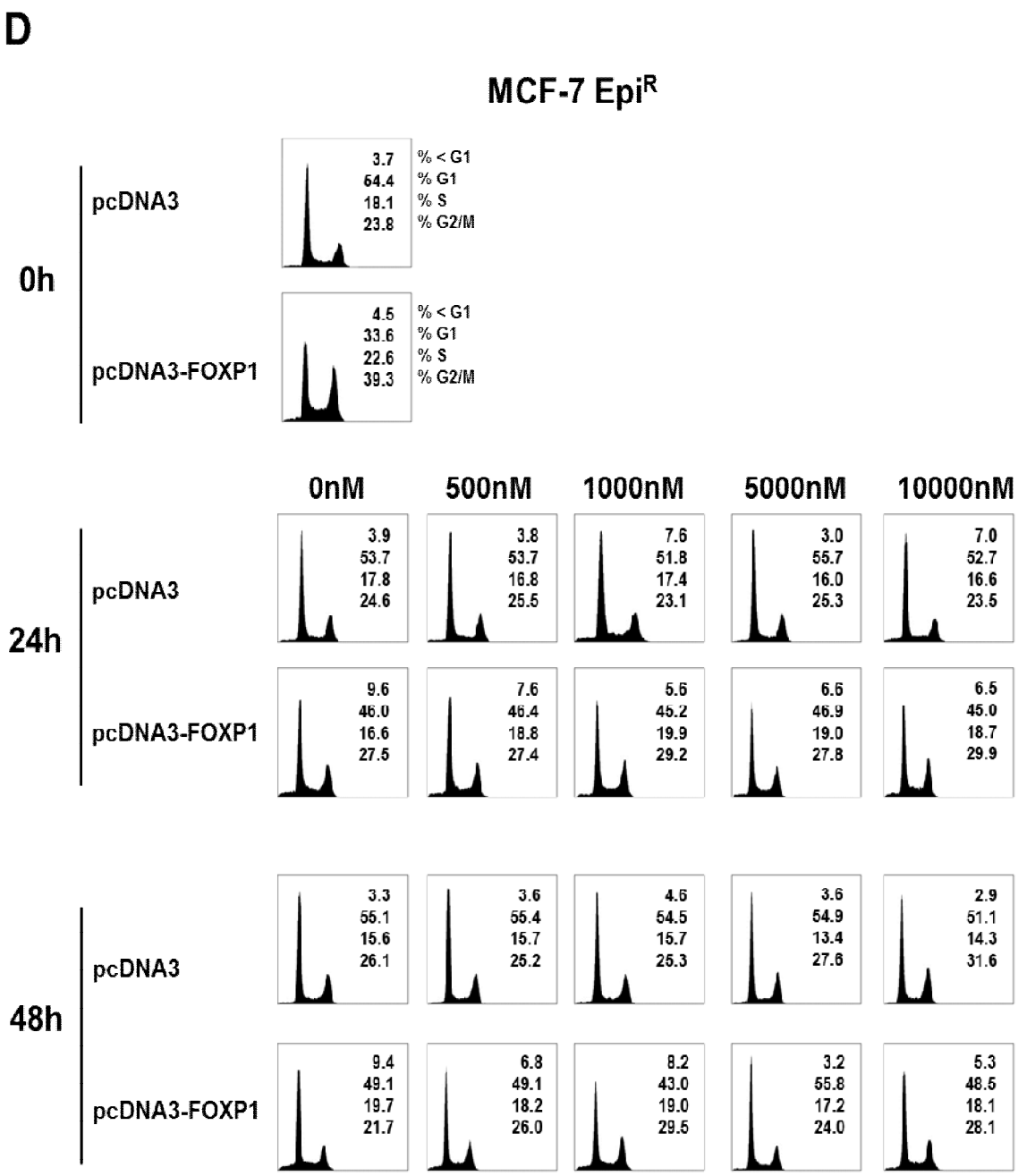


Figure 43. Continued.

4.3.25 Sub-cloning FOXP1 into a GFP vector

To generate the GFP-FOXP1 vector, a cDNA fragment (270-2297) encoding the full length FOXP1 protein was amplified by PCR from the parent vector (pcDNA3-FOXP1) (see Appendix 4, Appendix 5 and Appendix 6 for detailed sequence and vectors used). The primers were designed to clone FOXP1 cDNA in frame with the receiver vector (pEGFP-C1). The PCR product was analysed and purified on an agarose gel (Figure 44A), and sub-cloned into pCR 2.1-TOPO vector. The insert was then excised from the TOPO vector and sub-cloned into the XhoI/HindIII digested pEGFP-C1 vector (Appendix 5). After sub-cloning into pCR 2.1-TOPO (Appendix 6), the plasmid was then digested with XhoI/HindIII to confirm for the presence of FOXP1 in the different TOPO clones (indicated by the arrow) (Figure 44B). Clone number 3 was selected for sub-cloning into pEGFP-C1 vector. To confirm that I had successfully cloned FOXP1 into the pEGFP-C1 vector, both TOPO:FOXP1 and pEGFP-C1:FOXP1 were double digested with XhoI and HindIII and the digests resolved in a 1.5% agarose gel. The sizes of the empty vectors, TOPO and pEGFP-C1, 3.9kb and 4.7kb respectively, were confirmed in the agarose gel.

The FOXP1 fragment released by the double digestion, from both vectors harbouring FOXP1 construct is indicated by an arrow (Figure 44C).

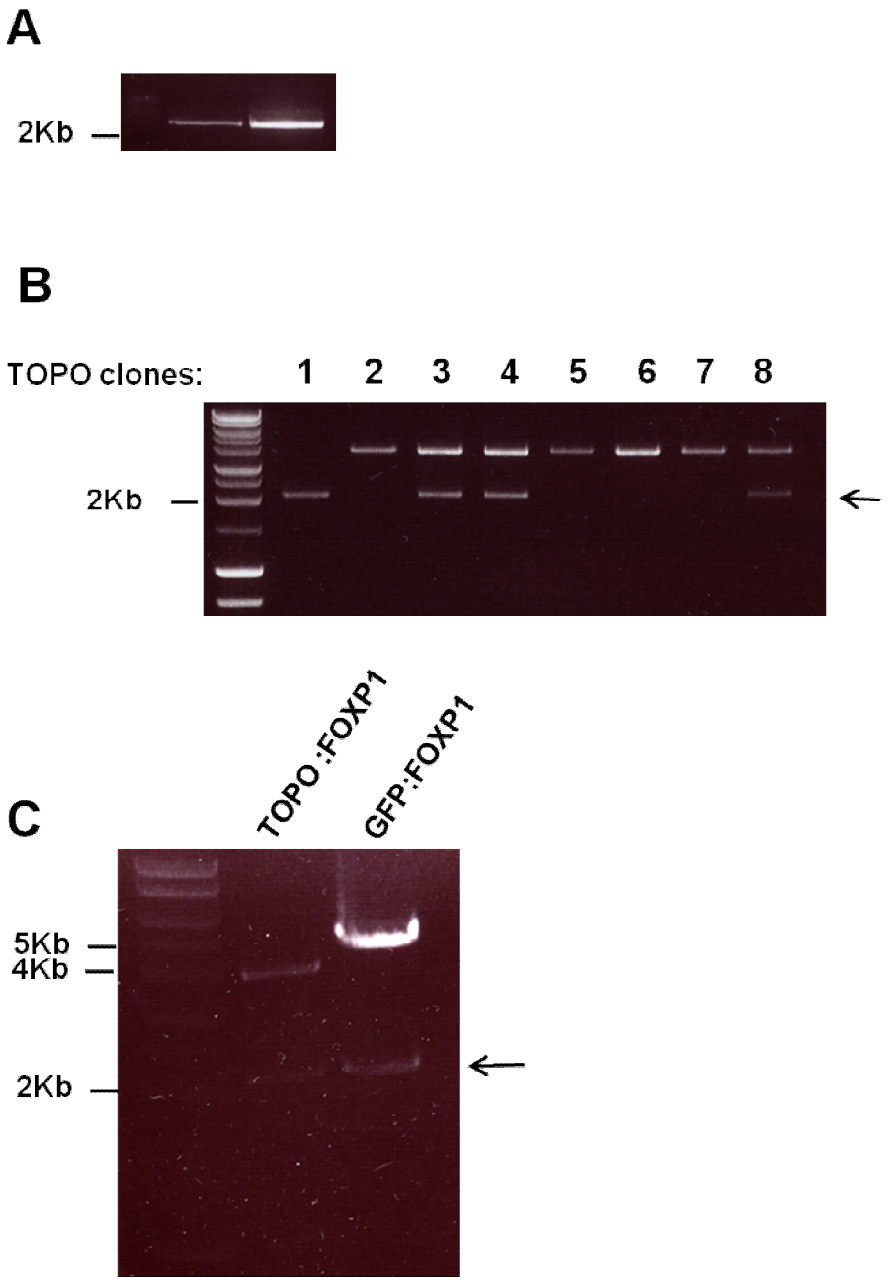


Figure 44. Subcloning FOXP1 into a GFP vector.

A) Agarose gel with the fragments originated from the PCR amplification of the parental pcDNA3-FOXP1. Lanes 1 and 2 result from the amplification of 0.5µg and 1µg of the parental vector, respectively. **B**) Following subcloning of FOXP1, TOPO vector was double digested with XhoI/HindIII to confirm for the presence of insert in different colonies ranging from 1 to 8. Arrow indicates the colonies positive for FOXP1. **C**) An agarose gel was run to confirm for the presence of construct in the final receiver vector. Both vectors, TOPO 2.1 and pEGFP-C1, harboring FOXP1 construct were double digested with XhoI/HindIII.

Next, I transfected both MCF-7 and Epi^R cells with GFP and pEGFP-C1:FOXP1 vectors. Following 24 h of transfection, cells were analysed by western blotting and immunofluorescence. FOXP1 overexpression was confirmed at the protein level by the presence of a higher molecular weight band as indicated by the arrow (GFP:FOXP1) (Figure 45A). The Western blot results were confirmed by confocal microscopy, where the transfected MCF-7 and Epi^R cells were co-stained with DAPI. GFP:FOXP1 was detected in the nucleus in both cell lines in contrast to the control GFP which was confined to the cytoplasmic compartment (Figure 45B). This result further supports the previous finding shown in Figure 33 that FOXP1 protein is preferentially localised in the nucleus. In line with this, a previous immunohistochemical staining study of solid tumours, including breast cancer, showed that FOXP1 expression is predominantly nuclear (Banham et al 2001).

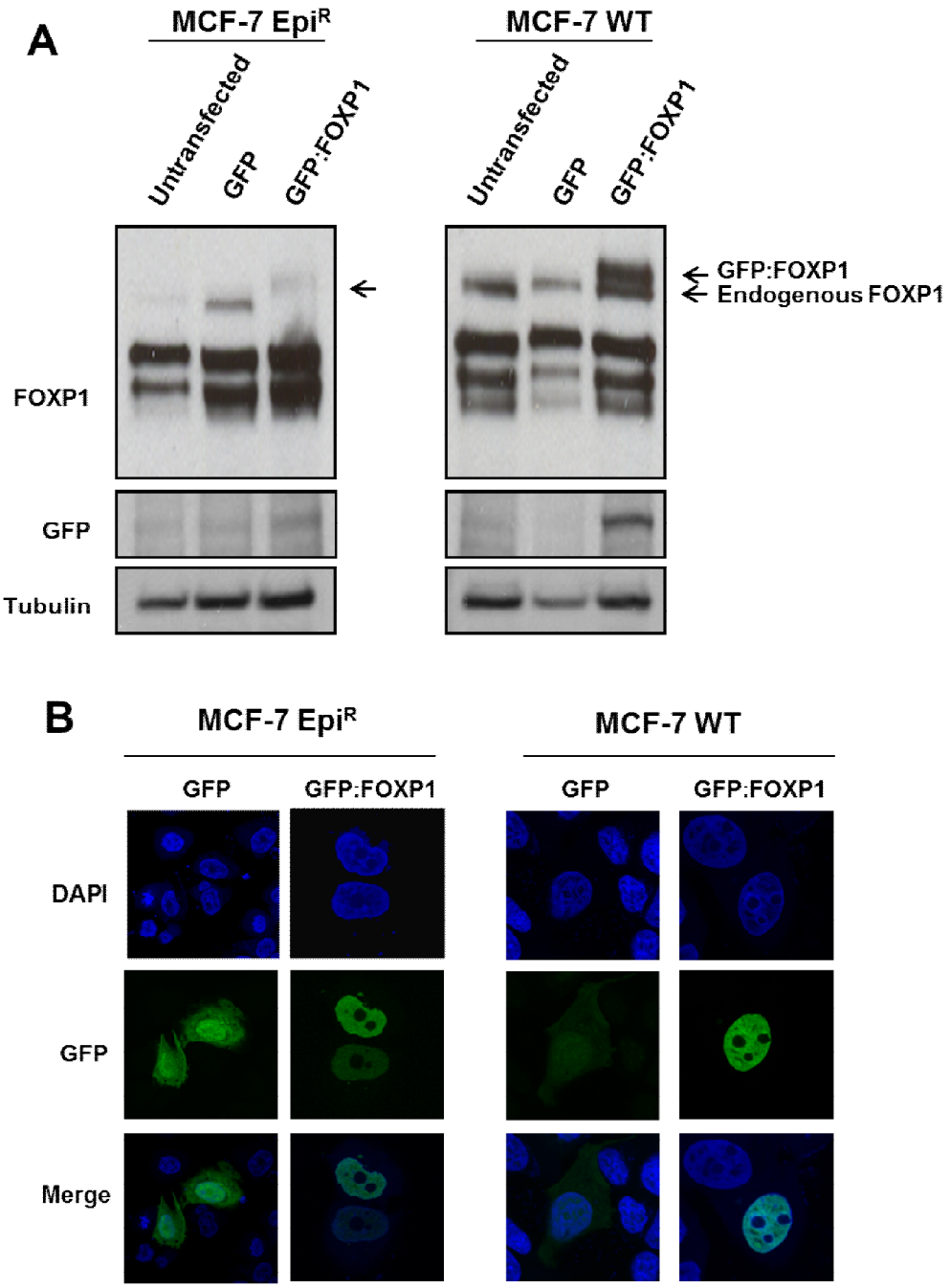


Figure 45. FOXP1 overexpression shows its nuclear subcellular localisation in both Epi^R and MCF-7 cell lines.

MCF-7 and Epi^R cells were transiently transfected with FOXP1 overexpression vector pEGFPC1-FOXP1 (GFP:FOXP1) or empty vector pEGFP-C1 (empty GFP) for 24 h. **A**) Cell lysates were collected 24 h post-transfection for western blot analysis to determine the expression levels of FOXP1. Tubulin was used as a loading control. **B**) MCF-7 and Epi^R cells were seeded in four-well chamber culture slides, fixed with formaldehyde and mounted with vectashield into a slide. Cell nuclei was stained with DAPI (blue) and GFP positive cells were visualised using the 488nm excitation laser.

4.3.26 FOXP1 overexpression results in a G1 arrest in MCF-7 Epi^R cells

In order to study the effect of FOXP1 on cell proliferation and cell cycle, the GFP:FOXP1 transfected cells were sorted for GFP expression by FACS analysis. For this purpose, MCF-7 Epi^R cells were Mock transfected or transfected with the GFP:FOXP1 overexpression vector. After 24 h post-transfection, cells were incubated in the absence or presence of epirubicin. FOXP1 overexpression was confirmed both at protein and transcriptional levels (Figure 46 A & B, respectively). Western analysis showed that for untreated cells, p27^{Kip1} levels were upregulated in FOXP1 overexpressing cells, in agreement to what was seen previously. FOXP1 overexpression resulted in a decrease in p21^{Cip1}, CDK4 and CyclinE1 levels but increased PLK and CyclinB1 levels. Although CDK4 were decreased, a marginal increase was observed for CyclinD1 levels. However, in epirubicin treated cells, there was little difference in the expression levels of p27^{Kip1}, CyclinD1, PLK, and CyclinB1 between untransfected and transfected cells. This suggests that these cell cycle regulators are induced by epirubicin. An increase was noted for CyclinE1, CDK4 and p21^{Cip1} levels (Figure 46A). This may be due to the fact that these genes can further be induced by FOXP1 expression.

In parallel, SRB assays and FACS analysis were performed to examine the impact of epirubicin on FOXP1 overexpressing cells. For this purpose, cells were transfected with GFP and GFP:FOXP1 following incubation in the absence or presence of epirubicin over a timecourse of 0, 24, 48 and 72 h.

In untreated cells, there was little difference in the growth rate of Mock transfected and GFP transfected cells. Nonetheless, a marginal but not significant increase was noted for GFP:FOXP1 expressing cells. Following epirubucin treatment, no substantial difference in the proliferative rate was observed between Mock transfected and GFP or GFP:FOXP1 transfected cells (Figure 46C). This is supported by the previous western blotting results, showing that, in epirubicin treated cells FOXP1 expression is induced.

GFP and GFP:FOXP1 transfected cells were analysed by FACS and the total and the GFP positive cell populations (GFP+) were studied (left- and right-hand side, respectively). The cell cycle profile of untreated cells at 0 h, showed a dramatic increase in the percentage of cells in G1 phase in the GFP:FOXP1 transfected cells (81.1%) compared to the GFP controls (68.6%). In addition, a substantial decrease was noted in the number of cells accumulated in the G2/M phase in GFP:FOXP1 transfected cells (5.2%) when compared to GFP transfected cells (12.2%). Notably, there was an enhanced G1 accumulation, in GFP:FOXP1 expressing cells, especially at 72 h (66.6%) in comparison with the GFP transfected cells (75.7%). However, when cells were treated with epirubicin, the differences in the G1 phase between GFP and GFP:FOXP1 transfected cells, became less obvious (Figure 46D). This further supports the previous western blotting and SRB results

showing that FOXP1 expression/activity is induced by epirubicin. The total cell population cell cycle profiles did not show much difference between the GFP and GFP:FOXP1 transfected cells. This could be due to the high percentage of untransfected cells. Moreover, there were no obvious differences between Mock transfected and GFP or GFP:FOXP1 transfected cells in the presence or absence of epirubicin (Figure 46D).

Figure 46E illustrates a simpler representation of the percentage of the GFP positive cells in G1 phase. This graph is relative to the GFP and GFP:FOXP1 transfected cells. The graph shows a clearer G1 accumulation for GFP:FOXP1 overexpressing cells in comparison to GFP cells. This result was further supported by Figure 46F where there was a significant increase in G1 phase accumulation caused by FOXP1 overexpression.

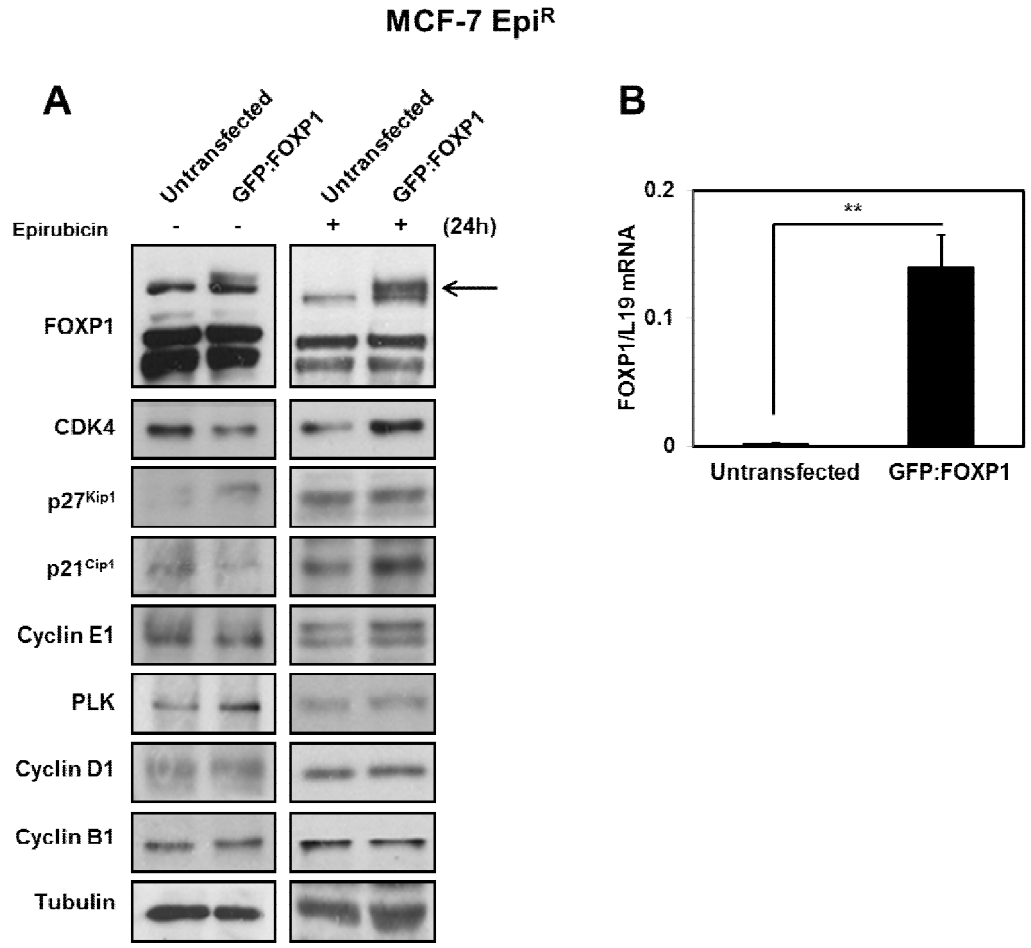


Figure 46. FOXP1 overexpression results in a G1 accumulation without affecting the proliferation of Epi^R cells.

Epi^R cells were either untransfected or transfected with GFP:FOXP1 vector and then for each condition cells were either non-treated or treated with epirubicin. **A**) Protein expression of FOXP1 and various cell cycle proteins was elucidated using western blot analysis. **B**) Total RNA was isolated. FOXP1 mRNA levels were analysed by RT-qPCR and normalised with L19 mRNA levels. Data shown represent the averages from two experiments. **C**) 24 h post-transfection, cells were counted, seeded into 96 well-plates and treated for 0, 24, 48 and 72 h in the absence and presence of epirubicin. Cell proliferation was determined by sulforhodamine B assay. Each point represents the mean of three independent experiments, each done at least in triplicate. **D**) Cell cycle profile was analysed by FACS for 0, 24 and 48 h in the presence and absence of epirubicin. Profiles acquired reflect the general cell population and the GFP positive population (GFP cells). **E**) Graphic illustration of the GFP cells accumulated at the G1 phase for both control (empty GFP) and GFP:FOXP1 cells throughout the timecourse and **F**) percentage of untreated cells at G1 phase, 24 h post-transfection. Error bars show the standard deviation. Statistical analysis has been done using Student's t test. **, P≤0.01.

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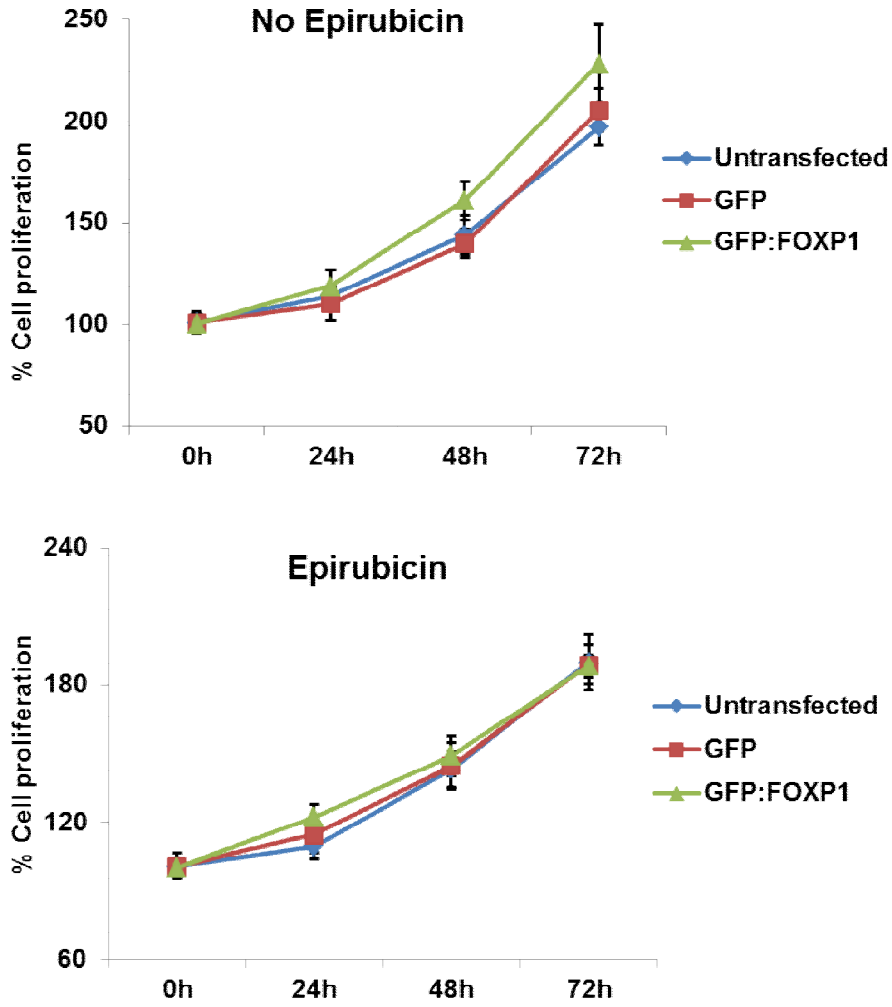


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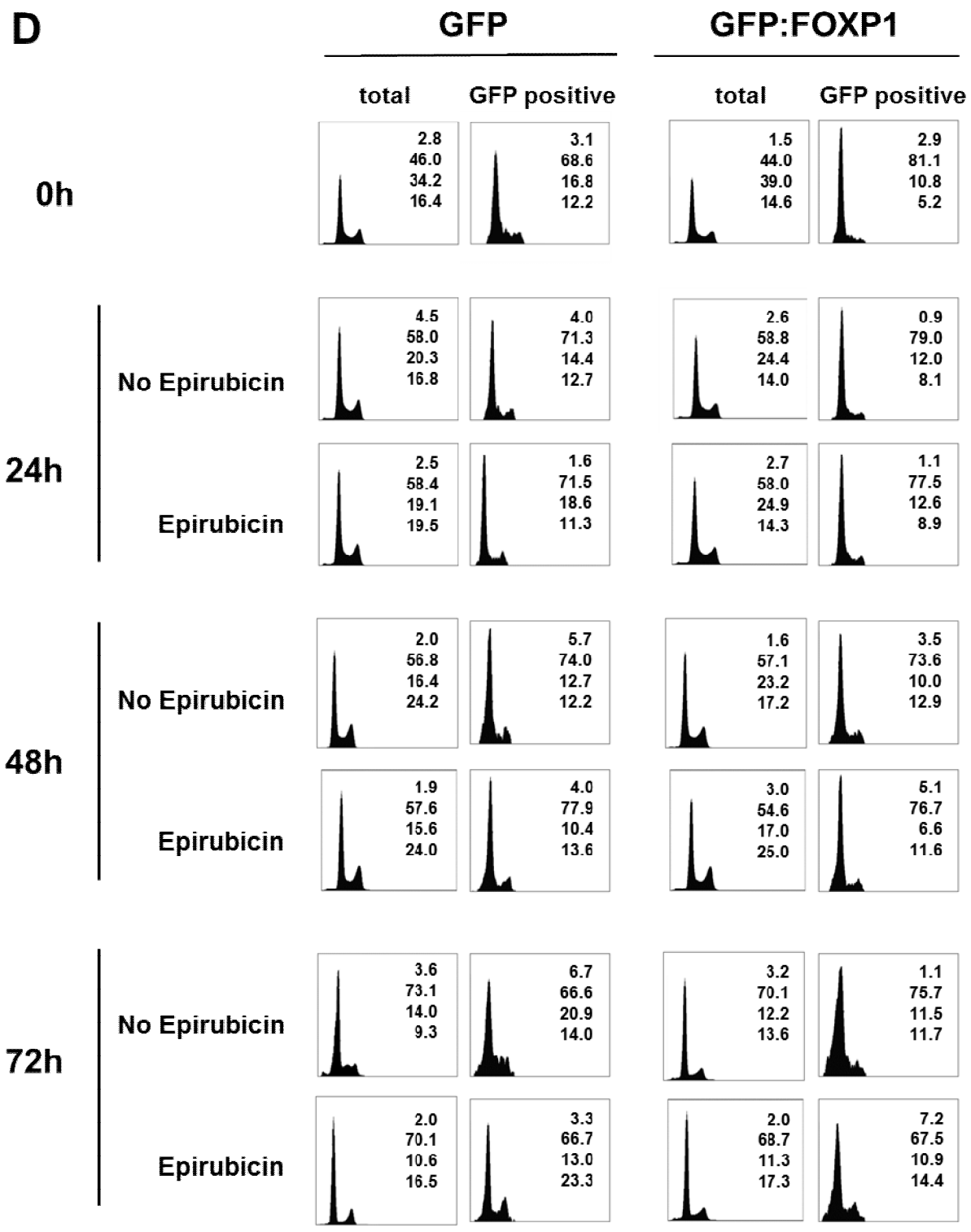


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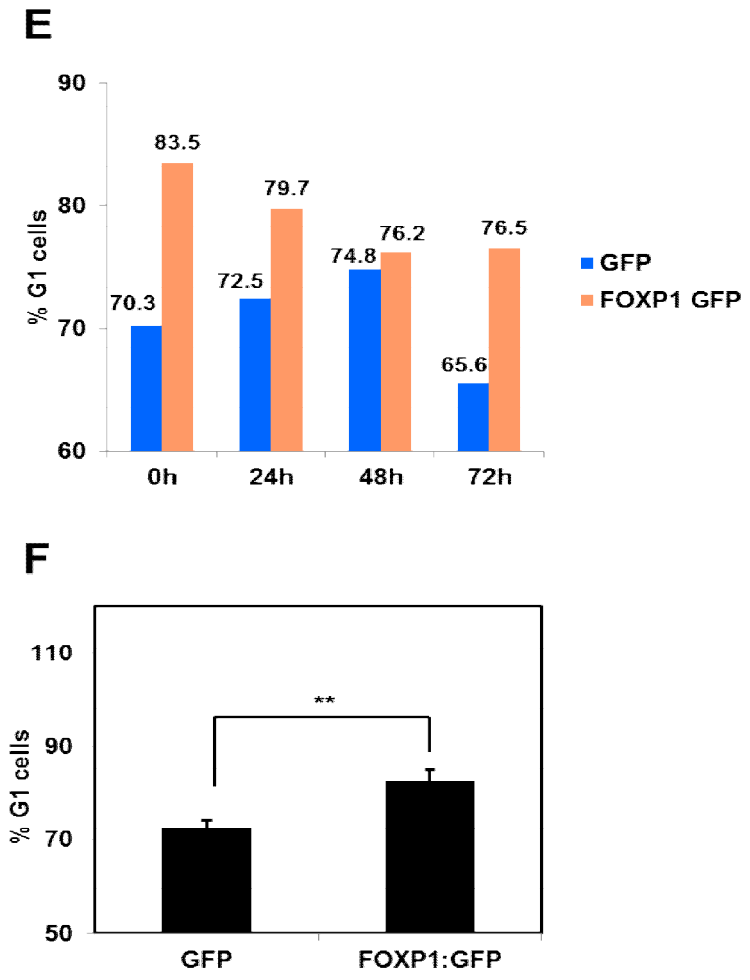


Figure 46. Continued.

4.3.27 FOXO3a and FOXP1 are inversely correlated in clinical samples

To determine the physiological significance of our cell culture findings, I analysed the expression patterns of FOXO3a and FOXP1 by IHC staining in breast cancer patient samples. Samples were obtained from Queen Mary Hospital in Hong Kong and together with our collaborators, IHC staining was performed on formalin-fixed, paraffin-embedded tissue sections using specific FOXO3a and FOXP1 antibodies. The intensity of staining for FOXO3a and FOXP1 was scored as low = 0 and high = 1. Figure 47 represents FOXO3a and FOXP1 patterns of staining. The majority of the cases analysed exhibited positive FOXO3a cytoplasmic staining (n=69) compared to nuclear FOXO3a staining (n=28). Stronger FOXP1 staining was also observed in the cytoplasm compared to the nucleus (62 versus 59). The correlation between FOXP1 and other clinical-pathological parameters, such as ER and PR status, tumour-grade, age and lymph-node involvement, was not statistically significant in invasive ductal cases.

The correlation between cytoplasmic FOXO3a and FOXP1 was not statistically significant (P=0.419, Chi-square test) however, nuclear FOXO3a was negatively associated with nuclear FOXP1 in a significant manner (P=0.012, Chi-square test) (Appendix 12). The correlation between FOXP1 expression and disease specific survival was studied using Kaplan Meier analysis in invasive ductal cases only. The result showed that high FOXP1 staining was significantly associated with a better survival time (p=0.032, log-rank test) whereas low FOXP1 expression was associated with poor survival (Figure 48). In accordance with my *in vitro* results, this further suggests that FOXP1 acts as a tumour suppressor. In summary, an inverse correlation was identified between FOXO3a and FOXP1 as high nuclear FOXO3a correlates with low nuclear FOXP1. In line with this, our previous report showed that the constitutively expression of nuclear FOXO3a predicts a worse prognosis. This suggests that, in order for cancer cells to proliferate and, at the same time, sustain a prolonged nuclear accumulation of FOXO3a, the downstream signalling regulation of FOXO3a targets, including FOXP1, must be disabled. Consistently, FOXP1 association with improved survival further corroborates the idea that FOXO3a-FOXP1 axis is deregulated in cancer cells.

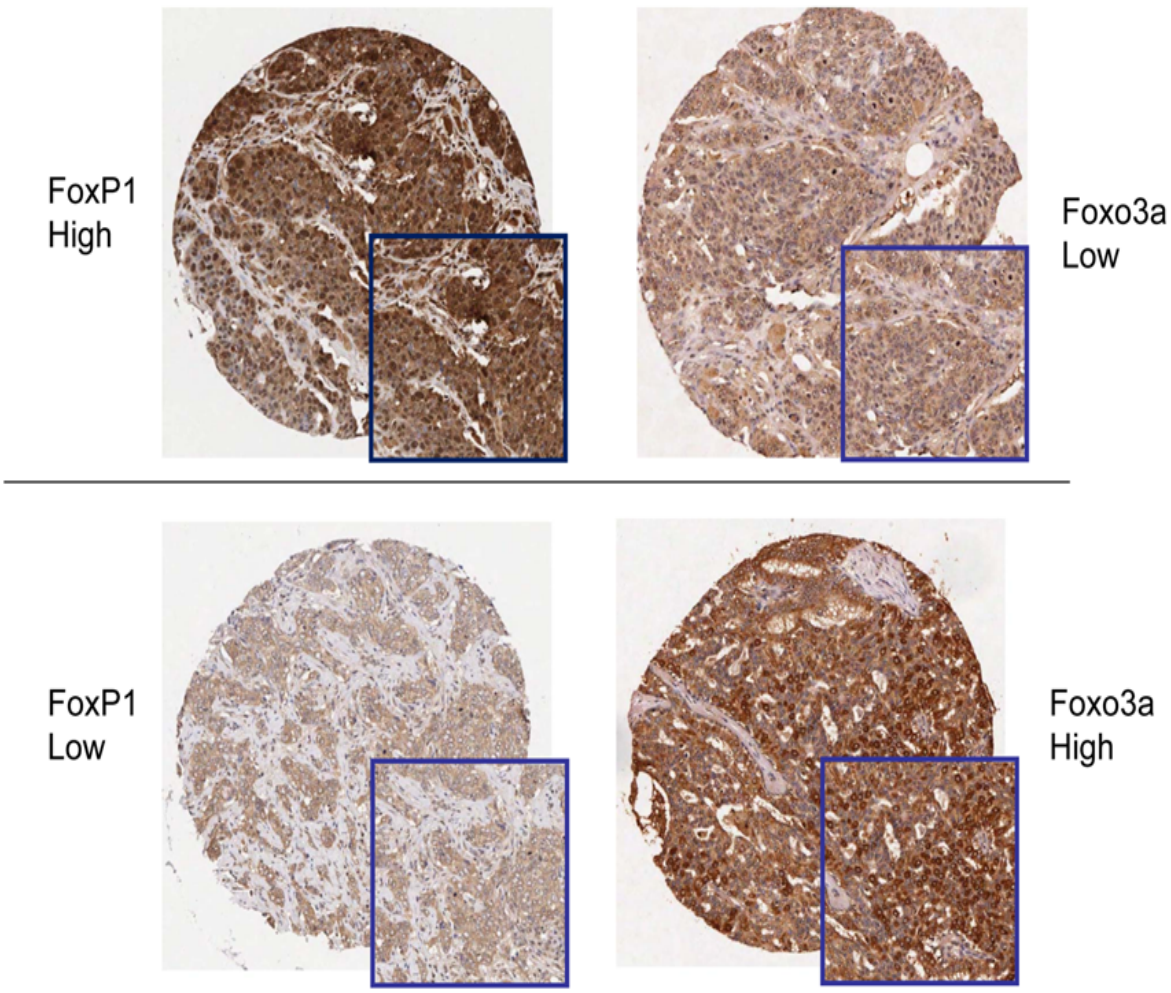


Figure 47. FOXO3a and FOXP1 have a negative correlation in breast cancer patient samples.

IHC staining was performed on tumour tissue samples obtained from breast cancer patients that had been formalin-fixed and paraffin-embedded were immunohistochemically stained with FOXO3a and FOXP1 antibodies using the streptavidin-biotin-peroxidase technique. Representative expression patterns corresponding to FOXO3a and FOXP1 illustrate the findings.

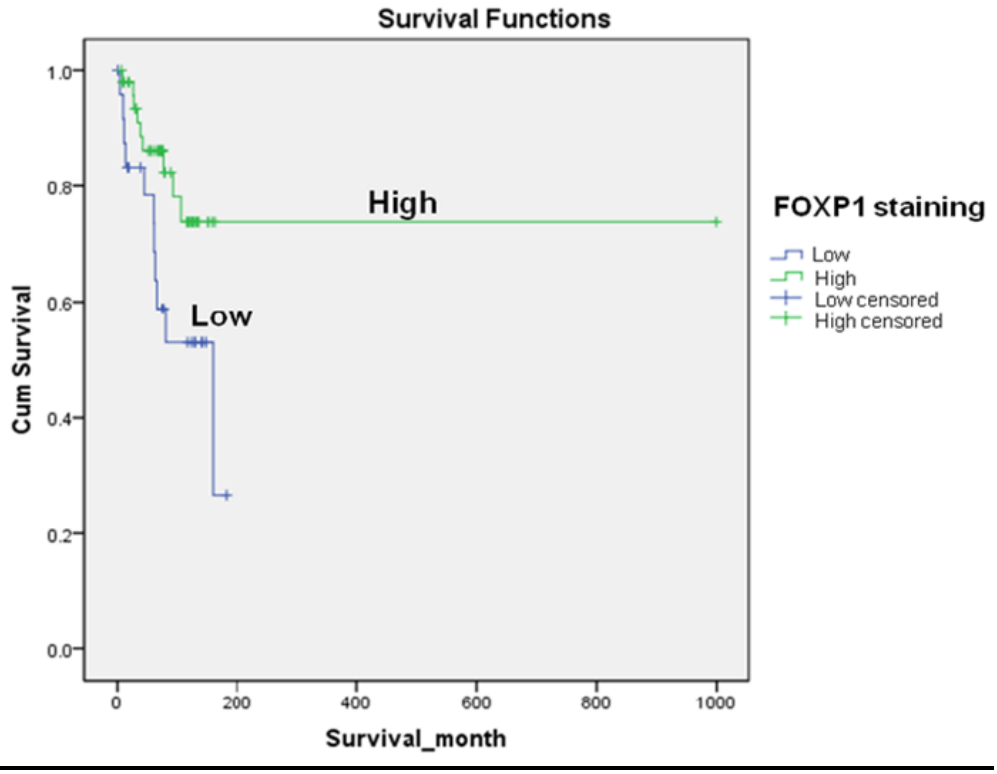


Figure 48. Survival analysis with cytoplasmic FOXP1 staining in invasive ductal carcinoma cases.

Of the 120 cancer samples assessed, there were 94 cases of invasive ductal carcinoma. The correlation between nuclear FOXO3a expression and survival was studied using Kaplan Meier analysis (P=0.032) and was considered significant at $p<0.05$.

4.4 DISCUSSION

The aim of this chapter is to validate new transcriptional targets of FOXO3a. From all the four targets investigated - FOXP1, JAK2, CXCR4 and APOBEC3G – only FOXP1 is consistently shown to be a target of FOXO3a. CXCR4 was found to be consistently upregulated in MDA-MB-231-FOXO3a(A3):ER cells in comparison to MDA-MB-231 cells. In addition, in *Foxo1/3/4*$^{-/-}$ MEFs CXCR4 levels were abrogated. However, FOXO3a depletion by siRNA in MCF-7 and BT474 cells did not have an effect on CXCR4 expression. Furthermore, there was not a difference in expression of CXCR4 between sensitive and Epi^{R} MCF-7 cells nor between K562 and the resistant leukaemic KD30 cells. As for JAK2 and A3G, the only notable difference was found in resistant cells. JAK2 levels were found upregulated in the leukaemic K562 cells overexpressing FOXO3a, however no such effect was observed when FOXO3a was inducibly expressed in MDA-MB-231 cells. In all resistant cell line models used – MCF-7 Epi^{R}, KD30 and BT474 Lap^{R} – JAK2 levels were consistently upregulated. The same result was observed for A3G. This could suggest that these proteins are in general involved in the development of resistant mechanisms probably independent of FOXO3a. A3G levels were abrogated when FOXO3a was constitutively induced in MDA-MB-231 cells. However, no changes in A3G levels were found in other FOXO3a overexpression or knock-down experiments. It is likely that the expression of these targets is differentially regulated but in a cell type-dependent manner. Because of the inconsistent results regarding these targets regulation by FOXO3a, I decided to focus on the study of FOXP1.

Over the last decade, there has been increasing evidence for a role for FOXP1 in human cancers. However, FOXP1 expression has been shown to predict different clinical outcomes in different human cancers. A number of studies have identified FOXP1 as an oncogene, especially in B cell lymphomagenesis, whereas studies of solid tumours, especially those of the breast and prostate, pointed to FOXP1 acting as a tumour suppressor. However, the molecular mechanisms that regulate FOXP1 expression, particularly in breast cancer, are unclear. Therefore, further characterisation of the function of FOXP1 and its impact on drug sensitivity would prove essential for determining whether FOXP1 may be a therapeutic target or may be used to guide therapy.

One objective of this chapter was to validate whether or not FOXP1 is a downstream target of FOXO3a in breast cancer. FOXP1 has previously been shown to be a potential FOXO3a target in a study using colon cancer cells expressing an inducible FOXO3a (Delpuech et al 2007). This work shows that FOXP1 is targeted by epirubicin, FOXO3a regulates FOXP1 expression, and FOXP1 has a role in cell cycle arrest.

In this chapter, I confirm that FOXP1 is a downstream target of FOXO3a in breast cancer cell lines by a number of different experiments. Firstly, FOXP1 protein and mRNA levels were impaired in *Foxo*-null MEFs and there was a slight decrease in FOXP1 protein after silencing FOXO3a in MCF-7 cells. Secondly, FOXO3a activation by 4-OHT was sufficient to increase FOXP1 protein expression in MDA-MB-231-FOXO3a(A3):ER cells. Moreover, in sensitive and resistant breast cancer cell lines, FOXO3a protein expression mirrored that of FOXP1.

My results also show that FOXO3a upregulates FOXP1 at mRNA and protein levels (Figure 34A & 3B). In addition, I have also shown that FOXO3a binds to *FOXP1* promoter *in vivo* (Figure 35). With regards to a possible role of FOXP1 in chemoresistance, FOXP1 expression is found to be downregulated in epirubicin-resistant cells in comparison with sensitive cells. Therefore, FOXP1 function was further analysed in the context of epirubicin-mediated resistance. I also observed that silencing FOXP1 in both MDA-MB-231 and MCF-7 cells resulted in a downregulation of $p27^{Kip1}$ levels. It is possible that this accounts for the enhanced cell proliferation and cell cycle progression observed following the knock-down of FOXP1. Moreover, in resistant cells, FOXP1 overexpression not only enhanced $p27^{Kip1}$ expression levels but also resulted in a substantial G1 arrest of the cell cycle. The finding that $p27^{Kip1}$ is a target of FOXP1 was surprising as FOXP1 has previously been described as a transcription repressor (Banham et al 2001, Shu et al 2007), and there are no publications as yet to suggest that FOXP1 can also be a *transactivator*. Concomitant with my findings, the related member, FOXP3 has been shown to act as a transcriptional activator of $p21^{Cip1}$ (Liu et al 2009). One could argue that FOXP1 regulates $p27^{Kip1}$ expression directly as a transactivator or indirectly through repressing an inhibitor of $p27^{Kip1}$. It is well established that FOXO3a regulates $p27^{Kip1}$ expression (Dijkers et al 2000b) and it could be that the regulation of $p27^{Kip1}$ by FOXP1 is FOXO3a-independent or that FOXO3a and FOXP1 cooperate to regulate $p27^{Kip1}$ expression. Further studies are required to decipher this regulation mechanism. Indeed, FOXP family members have the ability to heterodimerise, adding even more complexity to how these transcription factors regulate gene expression. Several studies have now shown that Foxp1, Foxp2, and Foxp4 expression overlaps in various tissues, including the lung and brain (Banham et al 2001, Lu et al 2002a, Shu 2001). Thus, it is possible that one FOXP member affects the activity of another through binding cooperately at promoter level or by acting as a partner either with a positive or negative regulation purpose via direct protein interaction (Wang 2003). It would be interesting to assess whether FOXP1 and FOXO3a could form heterodimers and study their impact on breast cancer chemoresistance. With this work, I also provided evidence that FOXP1 levels in breast cancer cell lines is preferentially nuclear regardless of FOXP1 basal levels, in agreement with previous publications

(Banham et al 2001, Takayama et al 2008). Unlike FOXO transcription factors, which have their localisation negatively regulated by the PI3K/AKT pathway, FOXP1 lacks AKT phosphorylation sites. This suggests that FOXP1 escapes direct regulation by PI3K/AKT pathway. However, FOXP1 can be indirectly regulated by PI3K/AKT via FOXO3a. The FOXO3a/FOXP1 axis appears to have a role in breast cancer chemoresistance. I have shown that FOXP1 is a good prognostic marker in breast cancer survival. In line with our previous report, where nuclear FOXO3a predicts a worse prognosis (Chen et al 2010a), I have also shown that FOXO3a and FOXP1 expression are inversely correlated in breast cancer patient samples, suggesting that for cancers which are resistant to chemotherapy, the FOXO3a/FOXP1 axis has been uncoupled, resulting in cell cycle progression and proliferation. This suggests that FOXP1 plays a key role in the control of the cell cycle and cell proliferation. The fact that FOXP1 overexpression fails to impair the proliferation of epirubicin-resistant MCF-7 cells, indicates that in these clonal FOXP1 overexpressing cells, the downstream signalling is deregulated, resulting in a further defection in cell proliferation control. These data suggest that FOXP1 could be useful as an additional marker to predict clinical outcomes. Another aspect that was not addressed is the presence and significance of FOXP1 isoforms in breast cancer. The majority of these isoforms are N-terminal truncated species of the full-length protein, which have been implicated as having an oncogenic role in DLBCL (Brown et al 2008a). It would be of informative to identify them in breast cancer cell lines as well as ascertain their clinical implications. It would also be interesting to test whether FOXP1 regulates other known FOXO3a targets. Moreover, one could test whether FOXP1 and FOXO3a cooperate for the binding of potential common targets. ChIP-Sequencing (ChIP-Seq) would prove a useful technique to map and sequence these FOX protein interaction binding sites within the genome of cancer cells. This will inform us if FOXO and FOXP1 share overlapping gene target and their identities.

CHAPTER 5

VEGF IS A TARGET OF FOXO3A

5.1 INTRODUCTION

5.1.1 ANGIOGENESIS

Angiogenesis is by definition the process that generates new blood vessels. Angiogenesis is not only crucial during development but it also occurs in adulthood, after injury during wound healing. This process occurs daily in our body and so it is essential for it to be tightly regulated. It comes as no surprise that, when this regulation fails, the abnormal blood vessel formation sets stage for "feeding" numerous diseases such as cancer (Folkman and Shing 1992). By supplying tumour cells with oxygen and nutrients, angiogenesis becomes indispensable for tumour growth, invasion and metastasis (Folkman 1990). Angiogenesis is triggered by different angiogenic factors that are secreted by a variety of cells, including tumour cells. Interleukins, fibroblast growth factor (FGF), transforming growth factor β (TGF-β), tumour necrosis factor α (TNF-α) and platelet-derived growth factor (PDGF) are some of the different soluble inducers of angiogenesis. However, the angiogenic factor most intensively studied is the vascular endothelial growth factor (VEGF) (Papetti and Herman 2002, Presta et al 2005).

5.1.2 VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) FAMILY

The Vascular Endothelial Growth Factor (VEGF) is a family of growth factors that includes 5 proteins: VEGF-A (herein referred as VEGF), -B, -C, -D, and Placenta Growth Factor (PLGF) (Ferrara 2004, Lohela et al 2009). Once secreted these growth factors mediate their biological roles by binding to three distinct tyrosine kinase primary receptors: Flt-1 or VEGFR-1, Flk-1 or VEGFR-2 and Flt-4 or VEGFR-3. VEGFR-1 is expressed in monocytes, macrophages and vascular endothelial cells (ECs) (Jakeman et al 1992) whilst VEGFR-2 is mainly expressed in the cell surface of vascular ECs and have been showed to be involved in blood vessel growth (Koch et al 2011). VEGFR-3 is restricted to lymphatic EC and has been showed to be involved in haematopoiesis and lymphangiogenesis (Partanen et al 1999, Rockson 2001). These receptors trigger VEGFs mediated cellular responses and have different affinities for the different VEGF ligands. For example, VEGFR-1 mediates the responses of VEGF, VEGFB and PLGF. Although VEGF binds with higher affinity to VEGFR-1, its tyrosine activity is weaker than VEGFR-2 (Shinkai et al 1998). Therefore, VEGFR-1 have been considered a "trap" for VEGF by negatively regulating its binding to VEGFR-2 (Chappell et al 2009). VEGFR-2 is expressed ubiquitously on almost all endothelial cell types (Quinn et al 1993) and binds to VEGF, VEGFC and VEGFD (McColl et al 2003) whereas VEGFR3 is activated by the binding of VEGFC and VEGFD (Makinen et al 2001). Due to the similarity of phenotypes between *Vegfr2*$^{-/-}$ and *Vegfa*$^{-/-}$

mice, VEGFR-2 is considered to be the main receptor of VEGF (Ferrara et al 1996). The neuropilin-1 and -2 receptors are co-receptors that can enhance the binding affinity of the various VEGF ligands to the previously mentioned primary receptors. For example, neuropilin-1 receptor has been shown to complex with VEGFR-2 to enhance VEGFR-2 mediated permeability (Becker et al 2005). Upon ligand-binding, VEGF receptors trigger downstream signalling cascades, including the PI3K, the p38-MAPK, and the Raf pathways, which in turn control the endothelial cell survival, proliferation and migration (Lamalice et al 2006, Lentzsch et al 2004, Pytel et al 2009).

5.1.3 VEGF

The importance of VEGF in angiogenesis has been demonstrated in knockout mice where disruptions of a single allele of the VEGF gene resulted in abnormal angiogenesis and led to embryonic lethality (Carmeliet et al 1996, Ferrara et al 1996). VEGF gene is located on 6p21.3 and is organised in eight exons that by alternative exon splicing generate different mRNA species (Vincenti et al 1996). Until recently only seven $VEGF_{xxx}$ isoforms had been identified, where xxx refers to the number of amino acids within a given isoform. These isoforms have 121, 165, 145, 148, 183, 189 and 206 amino acids, respectively (Charnock-Jones et al 1993, Neufeld et al 1999, Tischer et al 1991). From these VEGF splice variants, many were found to be overexpressed in a variety of cancers, including renal-cell (Tomisawa et al 1999), lung and colon (Cheung et al 1998) carcinomas and act as pro-angiogenic factors. The first of these isoforms to be identified was $VEGF_{165}$ which is the most abundant splice variant and is considered to be the predominant regulator of angiogenesis (Ferrara and Henzel 1989). The finding of different isoforms have drawn the attention to the misconception that $VEGF_{165}$ is a measure of total VEGF and many antibody-based assays have been developed specifically to detect $VEGF_{165}$. The $VEGF_{165}$ isoform is essential for cell migration and proliferation *in vitro* and is upregulated in many cancers such as colorectal (Uthoff et al 2002) and hepatocellular (Li et al 2006) carcinomas. Moreover, $VEGF_{165}$ expression is correlated with tumour progression and poor prognosis in breast cancer (Gasparini 2000). Comparative studies of the different VEGF isoform properties have led to the concept that indeed, $VEGF_{165}$ is the major VEGF isoform (Houck et al 1992). Aside from the above mentioned isoforms, another family of unconventional VEGF isoforms - $VEGF_{xxxb}$ – has been found to exist and have anti-angiogenic properties, counterbalancing the pro-angiogenic activity by other VEGF isoforms (Woolard et al 2004). In 2002, Bates *et al* identified and characterised $VEGF_{165b}$. $VEGF_{165b}$ was found downregulated in renal cell (Bates et al 2002), prostate (Woolard et al 2004), and colon (Varey et al 2008) carcinoma and malignant melanoma (Pritchard-Jones et al 2007). Additionally, $VEGF_{121b}$ isoform has also been identified and reported to inhibit cell migration and tumour growth in colorectal cancer (Rennel et al 2009), further suggesting that this entire family of $VEGF_{xxxb}$ acts as a brake for excessive angiogenic activity. It is now well established that both oxygen and nutrient deprivation are the main processes that drive angiogenesis so it is not surprising that under hypoxia or hypoglycaemia, cells respond by stimulating VEGF mRNA expression (Gogitidze Joy et al 2010, Shweiki et al 1992, Shweiki et al 1995). In fact, hypoxia is such an important event that a low oxygen gradient is necessary for wound-healing angiogenesis and whenever this gradient is destroyed, angiogenesis ceases (Knighton et al 1981). Whenever hypoxia occurs, the hypoxia-inducible factor-1 α (HIF-1α) binds to hypoxia-response elements

(HREs) in their target gene promoters restoring oxygen homeostasis. One of the genes that is transcriptionally activated by HIF-1α is VEGF (Semenza 2003). Several studies have shown that tumour vascularisation is launched by HIF-1α-dependent upregulation of VEGF. In different cell lines hypoxia has been found to both increase VEGF transcription mRNA expression and at the same time decrease degradation mechanisms of VEGF mRNA (Carmeliet et al 1998, Ikeda et al 1995, Liu et al 1995). VEGF upregulation by HIF-1α has been found not only in hypoxic niches in solid tumours (Stein et al 1995) but also in the context of other tissue ischemia *in vivo* (Banai et al 1994, Pe'er et al 1995). Although hypoxia is the most important regulator of VEGF expression other factors, including oncogene expression can also stimulate VEGF expression (Rak et al 1995). Interestingly, inactivation of the tumour suppressor von Hippel-Landau (vHL) also leads to overexpression of VEGF in tumour cells and consequently increased angiogenesis (Siemeister et al 1996). This tumour suppressor gene has also been shown to regulate VEGF expression by transcriptional and post-transcriptional mechanisms (Iliopoulos et al 1996). Cytokines and growth factors such as transforming growth factor α (TGF-α), insulin-like growth factor (IGF-I) and IL-6 were also shown to stimulate VEGF mRNA expression (Ferrara and Davis-Smyth 1997, Neufeld et al 1999).

5.1.4 VEGF THERAPIES

Neovascularisation stimulated by VEGF occurs in several important clinical contexts, including myocardial ischemia, retinal disease, and importantly in tumour growth. In fact, the growth of tumour vessels is one of the hallmarks of cancer (Hanahan and Weinberg 2011) and so, VEGF has been extensively studied in human cancers. VEGF and its receptors have been frequently found to be overexpressed in human tumours such as breast (Schneider and Sledge 2007), non-small cell lung (Heist et al 2008), colorectal (Yamaguchi et al 2007), and prostate cancers (Ferrer et al 1998). Moreover, VEGF expression has been associated with tumour metastasis and increased levels of VEGF have been shown to correlate with poor prognosis in colon (Takahashi et al 1995), non-small cell lung (Imoto et al 1998) and breast (Gasparini et al 1997) cancers. In line with all these data, VEGF has become an attractive target in the clinical development of pharmacologic agents. Different approaches have been developed to block VEGF signalling, including anti-VEGF antibodies and VEGF tyrosine kinase inhibitors. Bevacizumab (trade name Avastin) is a recombinant monoclonal antibody that binds and neutralises VEGF (Yang et al 2003) and has been shown, in combination with chemotherapy to increase survival in patients with metastatic colorectal (Hurwitz et al 2004), HER-2 negative breast (Miller et al 2007) and lung cancer (Sandler et al 2006).

Another VEGF neutralising monoclonal antibody, 2C3, selectively blocks VEGF from VEGFR-2 but no VEGFR-1 and inhibits growth of ECs *in vitro* (Brekken et al 1998). Similarly, other antibodies, including Sunitinib and Sorafenib, target VEGF receptors or downstream signalling pathways. These two small molecule inhibitors target a variety of tyrosine kinases and are used in patients with solid tumours such as renal cell carcinomas (Escudier et al 2007, Motzer et al 2007). Sunitinib-mediated inhibition of the VEGF signalling has been shown to decrease proliferation of different cancer cells *in vitro* (Ikezoe et al 2006a, Ikezoe et al 2006b). Despite all the advances in VEGF directed therapy, VEGF signalling is still not fully understood so elucidation of the mechanisms that fine-tune VEGF production is needed.

5.2 RESULTS

5.2.1 FOXO3a activation correlates with downregulation of FOXM1 and VEGF expression

FOXO3a is known to activate gene expression; however, the data from the cDNA microarray that identified potential novel FOXO3a targets revealed that VEGF is repressed by this transcription factor. Consistent with this, FOXM1 has recently been suggested to control VEGF expression (Zhang et al 2008) and to be regulated by FOXO3a (Francis et al 2009). This led me to hypothesise that FOXO3a could regulate VEGF expression by a mechanism involving FOXM1. To this end, I assessed the expression of cytoplasmic and nuclear fractions of VEGF, FOXM1, and FOXO3a upon lapatinib treatment of responsive and resistant breast cancer cell lines. I have also investigated the expression levels of FGF7, another mitogenic growth factor as a control. Western blot analysis showed that lapatinib treatment of sensitive BT474 and SKBR3 cells caused a decline in the phosphorylation levels of FOXO3a but an increase in nuclear FOXO3a levels, indicating activation of this transcription factor (Figure 49A). In both BT474 and SKBR3 cells, FOXO3a activation upon lapatinib treatment was accompanied by a decrease in VEGF and FOXM1 levels. In contrast, in the lapatinib-resistant MDA-MB-231 cells FOXO3a phosphorylation levels were higher and consequently FOXM1 expression was increased in comparison with sensitive cells. The result also showed that FGF7 was not downregulated by lapatinib, suggesting that the repression of VEGF expression by lapatinib and FOXO3a was specific (Figure 49A). Notably, all factors were downregulated in sensitive BT474 cells after 48 h of treatment, probably reflecting global protein degradation and cell death. The total levels of VEGF, FOXM1, and FOXO3a also recapitulated the fractionation results. FOXO3a was activated by lapatinib treatment in lapatinib-sensitive BT474 and SKBR3 cells and VEGF and FOXM1 levels were decreased. Instead, in the lapatinib-resistant MDA-MB-231 cells there were no appreciable changes in the total levels of P-FOXO3a (T32), nuclear FOXO3a, FOXM1, or VEGF levels upon treatment.

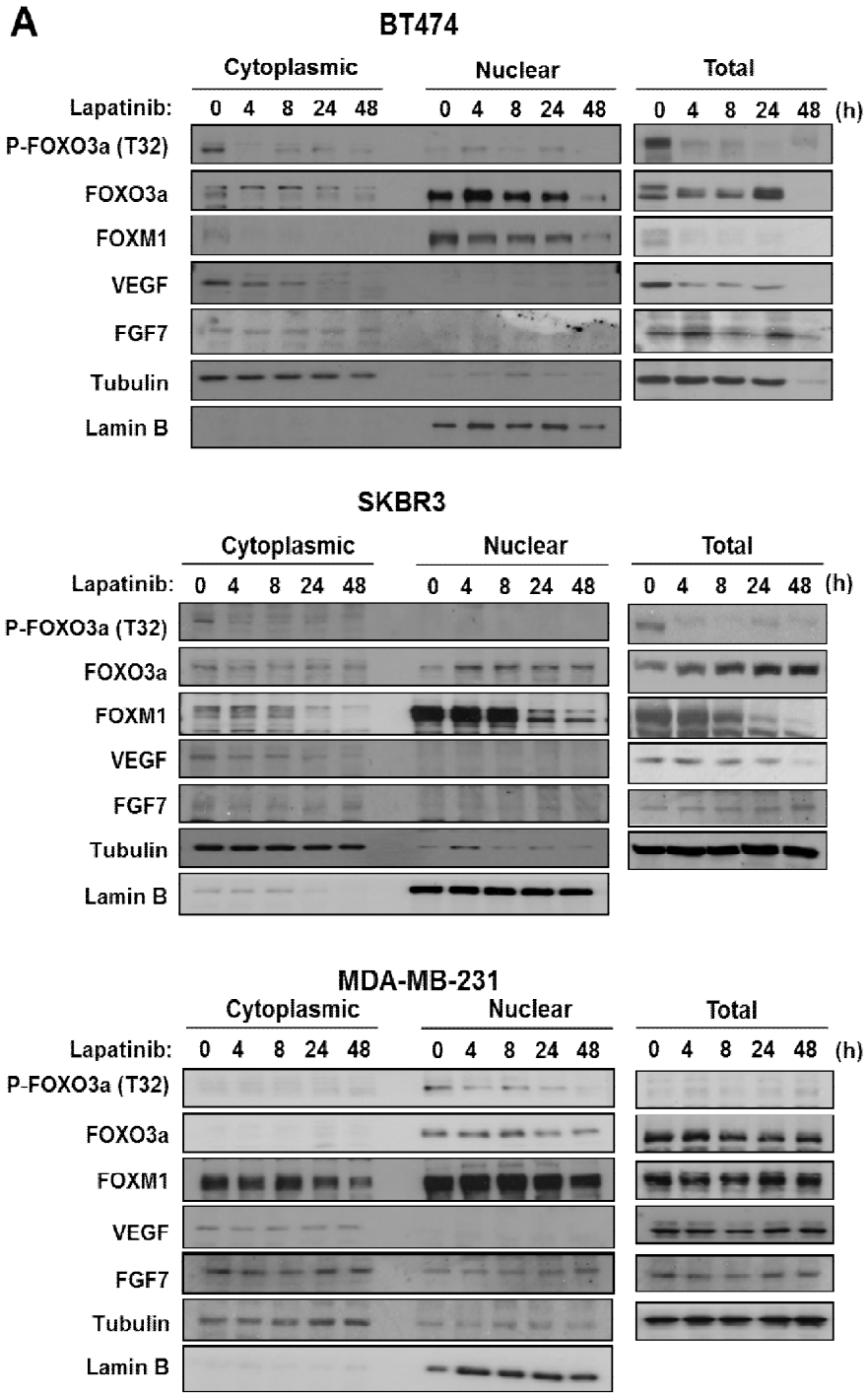


Figure 49. Expression of FOXO3a, FOXM1 and VEGF in response to lapatinib treatment in breast cancer cell lines.

A) Cells were collected and analysed for P-FOXO3a (T32), FOXO3a, FOXM1, VEGF, FGF7, Lamin B and Tubulin expression by western blotting of the nuclear/cytoplamic (left panel) and total (right panel) lysates. **B**) VEGF and FGF-7 concentrations in supernatants were measured by ELISA and the concentrations normalised using standard curves. **C**) In parallel, VEGF, FOXM1 and FOXO3a mRNA levels were also analysed by RT-qPCR and normalised to L19 RNA expression. All experiments were performed in triplicate.

To confirm that lapatinib represses VEGF expression, I determined the secreted levels of VEGF by ELISA in the three previously used cell lines. Whereas secreted VEGF levels remained unchanged upon lapatinib treatment of resistant MDA-MB-231 cells, its levels declined markedly after 24 h of treatment of the lapatinib-sensitive BT474 and SKBR3 cells. The secreted levels of FGF7 were included as a negative control (Figure 49B). The results showed that the concentrations of the control FGF7 did not alter significantly following lapatinib treatment in BT474, SKBR3 and MDA-MB-231 cells, suggesting that the repression of VEGF by FOXO3a and lapatinib is specific. In order to test whether lapatinib treatment regulates VEGF, FOXM1 or FOXO3a expression at the transcriptional level, I performed RT-qPCR analysis (Figure 49C). In accordance to the Western blot results, lapatinib inhibited VEGF and FOXM1 mRNA expression in the sensitive BT474 and SKBR3 cells but not in the lapatinib-resistant MDA-MB-231 cells. Notably, FOXO3a transcript levels were also upregulated in sensitive BT474 and SKBR3 cells (Figure 49C). Together these results demonstrate that lapatinib treatment of sensitive breast cancer cells induces and activates FOXO3a but inhibits FOXM1 and VEGF expression.

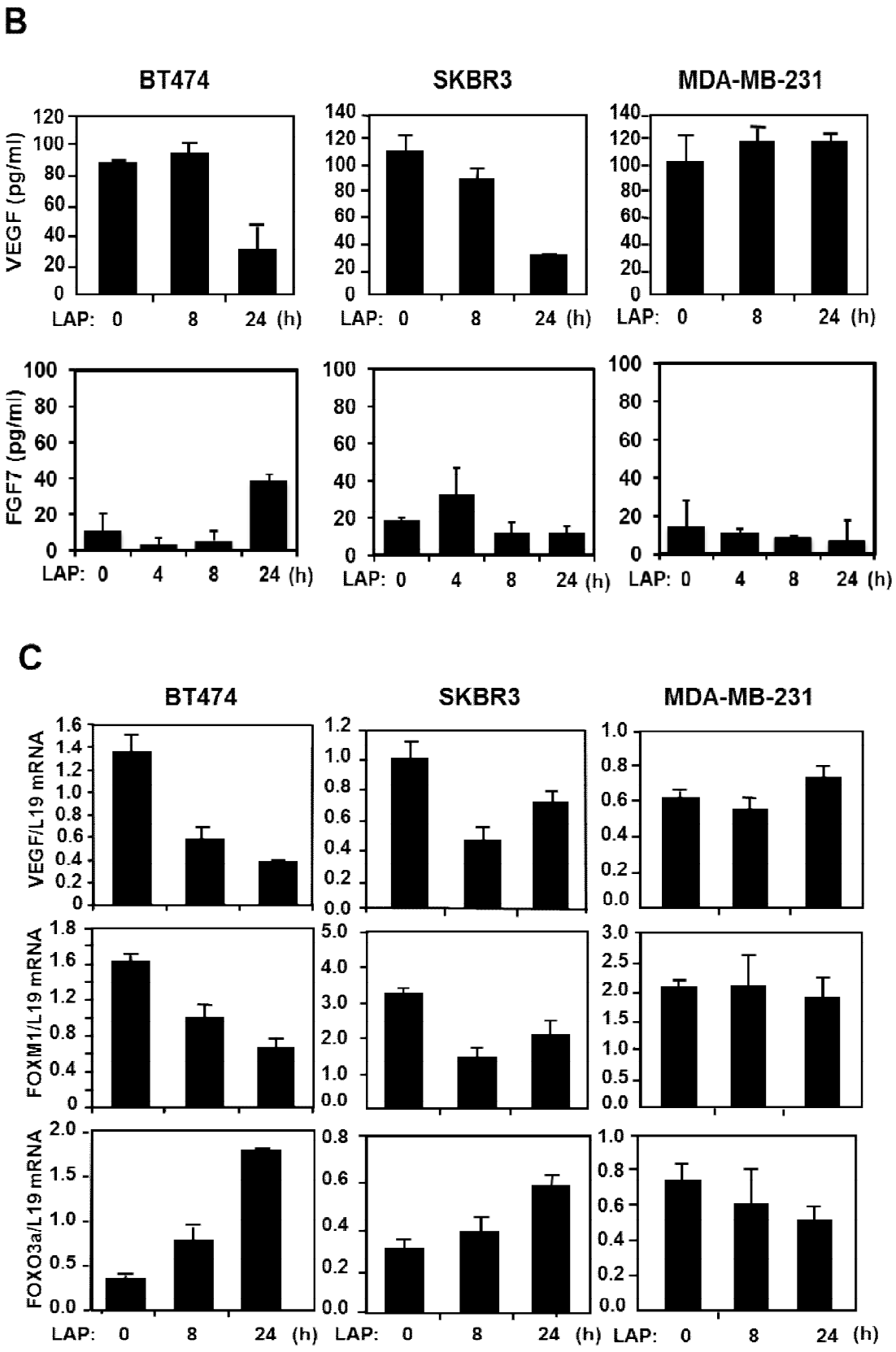


Figure 49. Continued.

5.2.2 FOXO3a represses VEGF and FOXM1 expression

In order to study the mechanism underlying the reciprocal relationship between FOXO3a activation and VEGF and FOXM1 inhibition, I used the 4-OHT dependent FOXO3a(A3):ER inducible system in MDA-MB-231 cells described in the previous chapters. Subcellular fractionation was performed with control MDA-MB-231 and MDA-MB-231-FOXO3a(A3):ER cells treated with 4-OHT over a timecourse. As shown in Figure 50A, 4-OHT not only induced nuclear accumulation of activated FOXO3a but also inhibited expression of both VEGF and FOXM1. This downregulation of VEGF and FOXM1 upon 4-OHT treatment was dependent upon FOXO3a activation, as no such response was observed upon treatment of control MDA-MB-231 cells (Figure 50A). I also determined the impact of FOXO3a activation in these cell lines in the secreted levels of VEGF by ELISA. Expectedly, induction of FOXO3a activity also decreased secreted VEGF levels, apparent after 8 h of 4-OHT stimulation, whereas this response was absent in control MDA-MB-231 cells (Figure 50A). FOXM1 and VEGF transcript levels were also measured by RT-qPCR analysis upon 4-OHT stimulation. Importantly, 4-OHT stimulation resulted in a downregulation of VEGF and FOXM1 mRNA levels in MDA-MB-231-FOXO3a(A3):ER cells, relative to control MDA-MB-231 cells (Figure 50B). These results suggest that FOXM1 and VEGF expression are negatively regulated by FOXO3a at both transcriptional and translational levels.

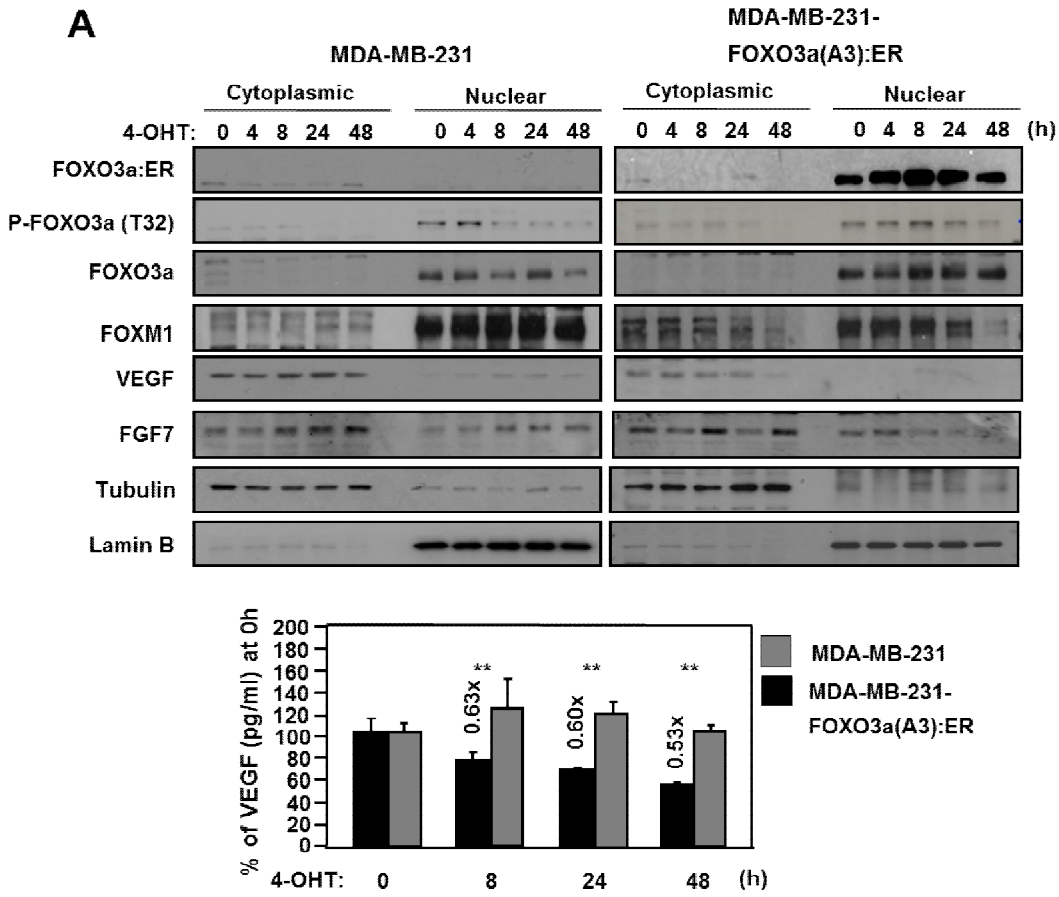


Figure 50. FOXO3a represses the expression of FOXM1 and VEGF in the breast carcinoma cells MDA-MB-231 and MCF-7.

MDA-MB-231-FOXO3a(A3):ER and MDA-MB-231 cells were treated with 200nmol/l of 4-OHT for the indicated times. **A**) Nuclear and cytoplasmic extracts were prepared at the times indicated. The expression levels of FOXO3a, P-FOXO3a (T32), FOXM1, VEGF, FGF7, Tubulin and Lamin B were analysed by western blotting. The supernatants of the cells were collected and VEGF concentrations were measured by ELISA. **B**) Total RNA was extracted and the mRNA expression of FOXM1 and VEGF was determined by RT-qPCR and normalised to the level of L19. The FOXO3a, FOXM1, VEGF and Tubulin proteins levels were assessed in MCF-7 cells by western blotting following the **C**) transient transfection with the constitutively active FOXO3a(A3) or control vector and the **D**) transient transfection with a specific FOXO3a and NSC siRNA, or Mock. **C**, **D**) VEGF and FOXM1 mRNA expression was determined by RT–qPCR. All data shown represent the averages of data from three experiments, and the error bars show the standard deviations. Statistical analyses were done using Student's t test. *P<0.05, significant; **P<0.01, significant; Ψ, non-significant.

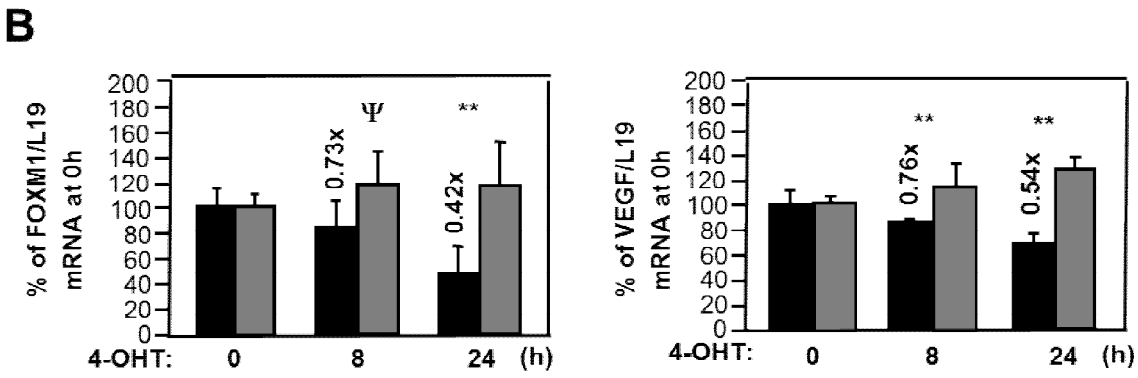


Figure 50. Continued.

To corroborate these observations, I transiently transfected MCF-7 cells with the constitutively active FOXO3a(A3) or control empty expression vectors, and determined both VEGF and FOXM1 expression levels. Western blot and RT-qPCR analyses demonstrated that the FOXO3a(A3) mutant inhibited FOXM1 and VEGF expression, at protein and mRNA levels, respectively (Figure 50C). This was further supported by the reverse experiment of transiently transfecting MCF-7 cells with a FOXO3a targeting siRNA pool or NSC siRNA. Silencing FOXO3a increased VEGF and FOXM1 expression at both protein and mRNA levels (Figure 50D).

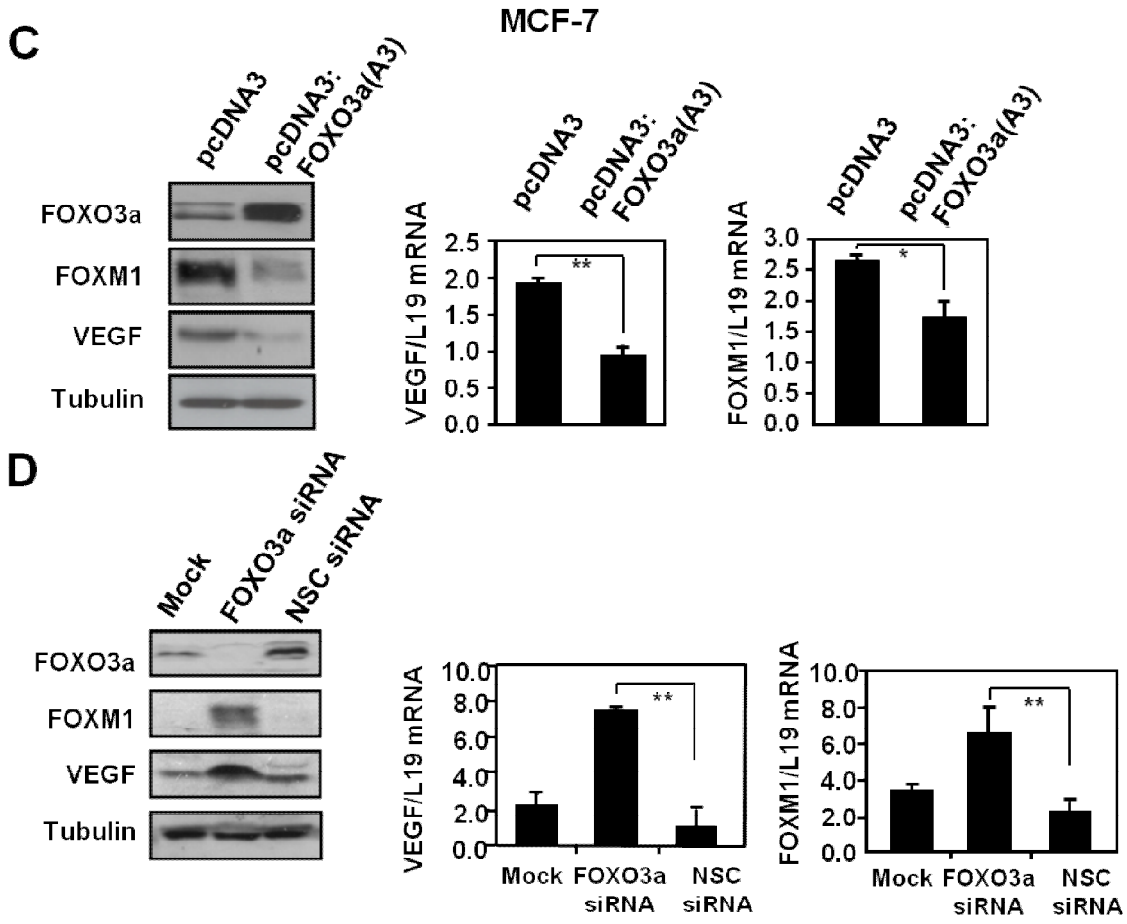


Figure 50. Continued.

To demonstrate further that FOXO3a has a role in the downregulation of FOXM1 and VEGF by lapatinib treatment, I silenced FOXO3a in BT474 cells. Cells were transfected with either a FOXO3a-specific or a NSC siRNA pool and the expression levels of VEGF and FOXM1 were determined following lapatinib treatment (Figure 51). Western blot analysis confirmed the efficient knock-down of the endogenous FOXO3a in BT474 cells. Consistent with previous data (Nelson and Dolder 2006) lapatinib treatment led to a decrease in P-HER2 levels in both NSC and FOXO3a siRNA cells. Silencing of FOXO3a resulted in increased basal levels of FOXM1 and VEGF, and alleviated the downregulation of FOXM1 and VEGF by lapatinib (Figure 51). Notably, the expression levels of FOXM1 and VEGF eventually declined at 48 h after lapatinib treatment, which could be due to the functional compensation by other FOXO isoforms or the fact that FOXM1 and/or VEGF are also repressed by lapatinib through other transcription factors or at the post-transcriptional level. Together these data further confirmed that FOXO3a negatively regulates VEGF and FOXM1 expression, through a mechanism likely to involve transcriptional inhibition.

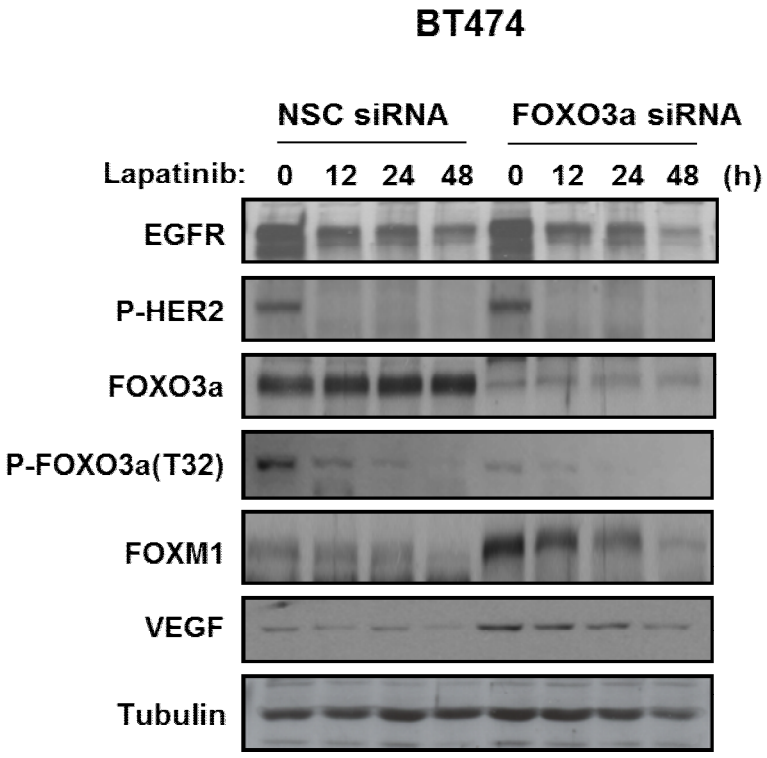


Figure 51. Silencing FOXO3a induces the expression of FOXM1 and VEGF in BT474 cells.

BT474 cells were transfected with a specific FOXO3a and NSC siRNA, or Mock transfected and treated with lapatinib for 0, 16, 24 and 48 h. The expression levels of the indicated antibodies was analysed by western blot.

5.2.3 FOXO3a and FOXM1 modulate VEGF promoter activity

Next, I hypothesised that FOXO3a could suppress *VEGF* transcription, either by modulating promoter activity or, indirectly, by inhibiting FOXM1 expression. To differentiate between these scenarios, the *VEGF* promoter luciferase reporter was analysed (Figure 52A). Sequence analysis identified two consensus forkhead transcription response elements (FHREs) in the proximal *VEGF* promoter region (see Appendix 7). Each of these sites, designated as FHRE1 and FHRE2 were then mutated and used to study *VEGF* promoter activity in the presence of a constitutively active form of FOXO3a or FOXM1, FOXO3a(A3) or FOXM1(ΔN) expression vectors, respectively. MCF-7 cells were co-transfected with the wild-type (VEGF pro-WT), the mutant FHRE1 (VEGF pro-mut1) or mutant FHRE2 (VEGF pro-mut2) *VEGF* promoter together with a titration of FOXO3a(A3) and FOXM1(ΔN) expression vectors. The results showed that the expression of the FOXO3a(A3) mutant represses the activity from the putative *VEGF* WT promoter whereas exogenous expression of FOXM1 *trans*activated the reporter construct in a dose-dependent manner (Figure 52B). Mutation of the distal (-319) but not the proximal (-178) FHRE abrogated the ability of FOXO3a(A3) and FOXM1 to inhibit and activate, respectively, this promoter-reporter construct. Thus, a single response element, designated FHRE2, appears to mediate the effects of both transcription factors on the *VEGF* promoter.

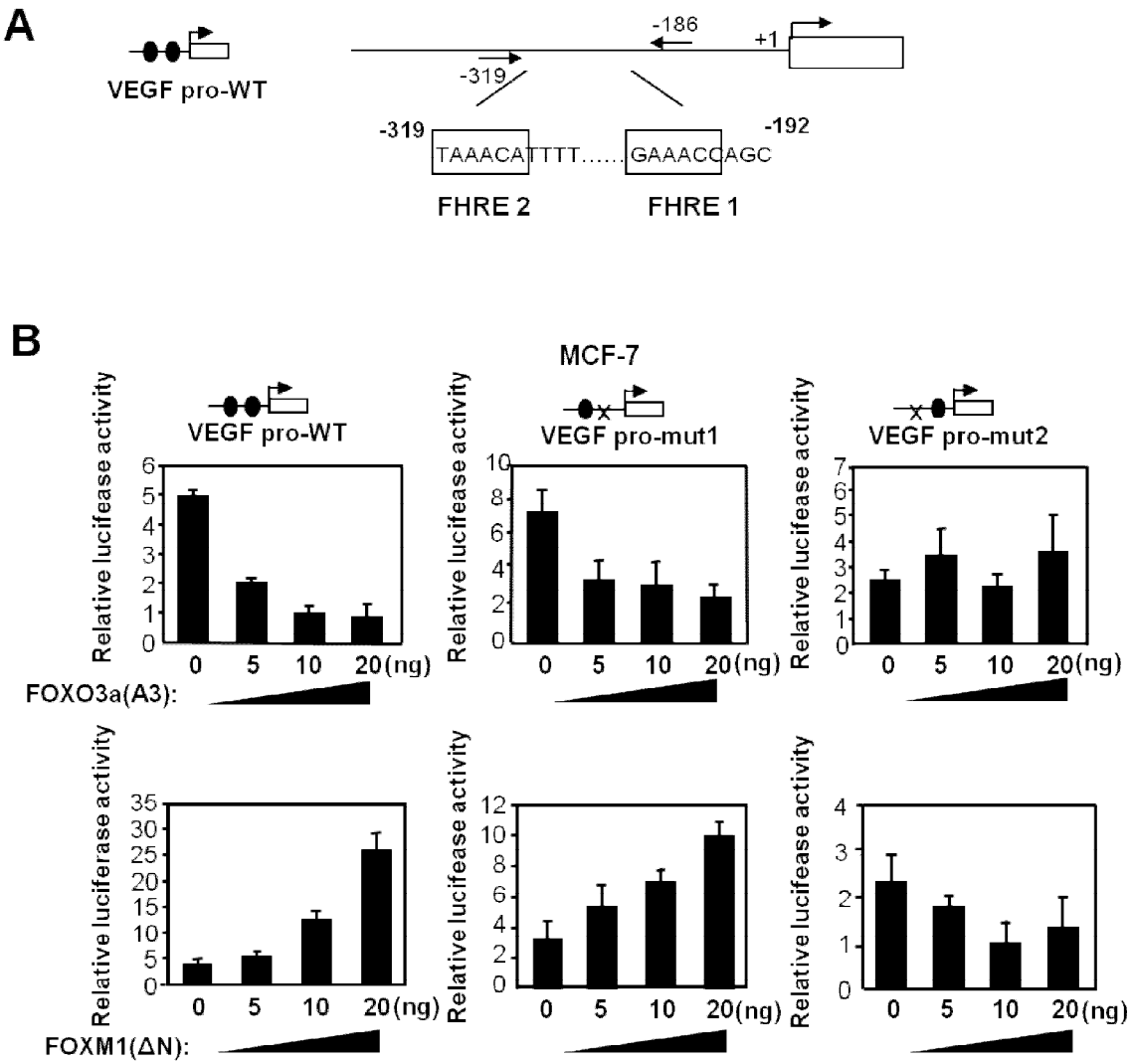


Figure 52. FOXO3a represses and FOXM1 induces *VEGF* promoter through a FHRE consensus site proximal to the transcription start site.

A) Schematic representation of the VEGF-luciferase reporter construct, showing the two consensus FHRE sequences. **B**) MCF-7 cells were transiently transfected with 20ng of the WT, mutant FHRE1 or mutant FHRE2 VEGF promoter/reporter and 0, 5, 10 or 20ng of either the constitutively active FOXO3a(A3) or FOXM1(ΔN) expression vector. Cells were assayed for luciferase activity. All relative luciferase activity values are corrected for co-transfected Renilla activity. All data shown represent the averages of data from three independent experiments, and the error bars show the SD.

5.2.4 FOXO3a and FOXM1 compete for binding to FHRE2

To get more insights into the mechanism by which FOXO3a and FOXM1 regulate *VEGF* promoter transcription, I next performed oligonucleotide pull-down assay with nuclear lysates from MDA-MB-231-FOXO3a(A3):ER and MDA-MB-231 cells untreated or treated with 4-OHT for 8 and 24 h. The nuclear lysates were incubated with biotinylated wild-type (WT) or mutant FHRE2 (mut) oligonucleotides . Western blot analysis of the pulled-down complexes showed that both FOXO3a and FOXM1 bind to the wild-type FHRE2 of *VEGF*, but not the mutated FHRE2 site (Figure 53A). The binding of FOXO3a and FOXM1 to the FHRE2 could be competed off by excess amounts of the wild-type but not mutated FHRE2 oligonucleotides, indicating that both transcription factors bind directly to this response element (Figure 53A). The results also revealed that FOXM1 is constitutively bound to FHRE2 in untreated MDA-MB-231-FOXO3a(A3):ER and the MDA-MB-231 cells. However, FOXM1 was replaced by the FOXO3a(A3):ER in response to 4-OHT stimulation of MDA-MB-231-FOXO3a(A3):ER but not of MDA-MB-231 cells, suggesting that activated FOXO3a downregulates *VEGF* expression by competitive displacing FOXM1 bound to FHRE2.

This FHRE pull-down experiment was repeated in the BT474 cells following lapatinib treatment in the presence of molar excess of mutated FHRE oligonucleotides (Figure 53B). Parallel Western blot analysis of nuclear and cytoplasmic lysates showed that lapatinib induces nuclear accumulation of FOXO3a after 2 to 4 h, concomitant with the downregulation of VEGF expression but without discernible change in FOXM1 levels at these timepoints (Figure 53B). The pull-down results, however, could indicate that the lapatinib-activated FOXO3a displaces FOXM1 from the FHRE2 of the *VEGF* promoter at these timepoints. Thus, although prolonged activation of FOXO3a will downregulate FOXM1 levels, inhibition of *VEGF* expression is an early event and mediated, at least in part, by displacing FOXM1 and binding to FHRE2.

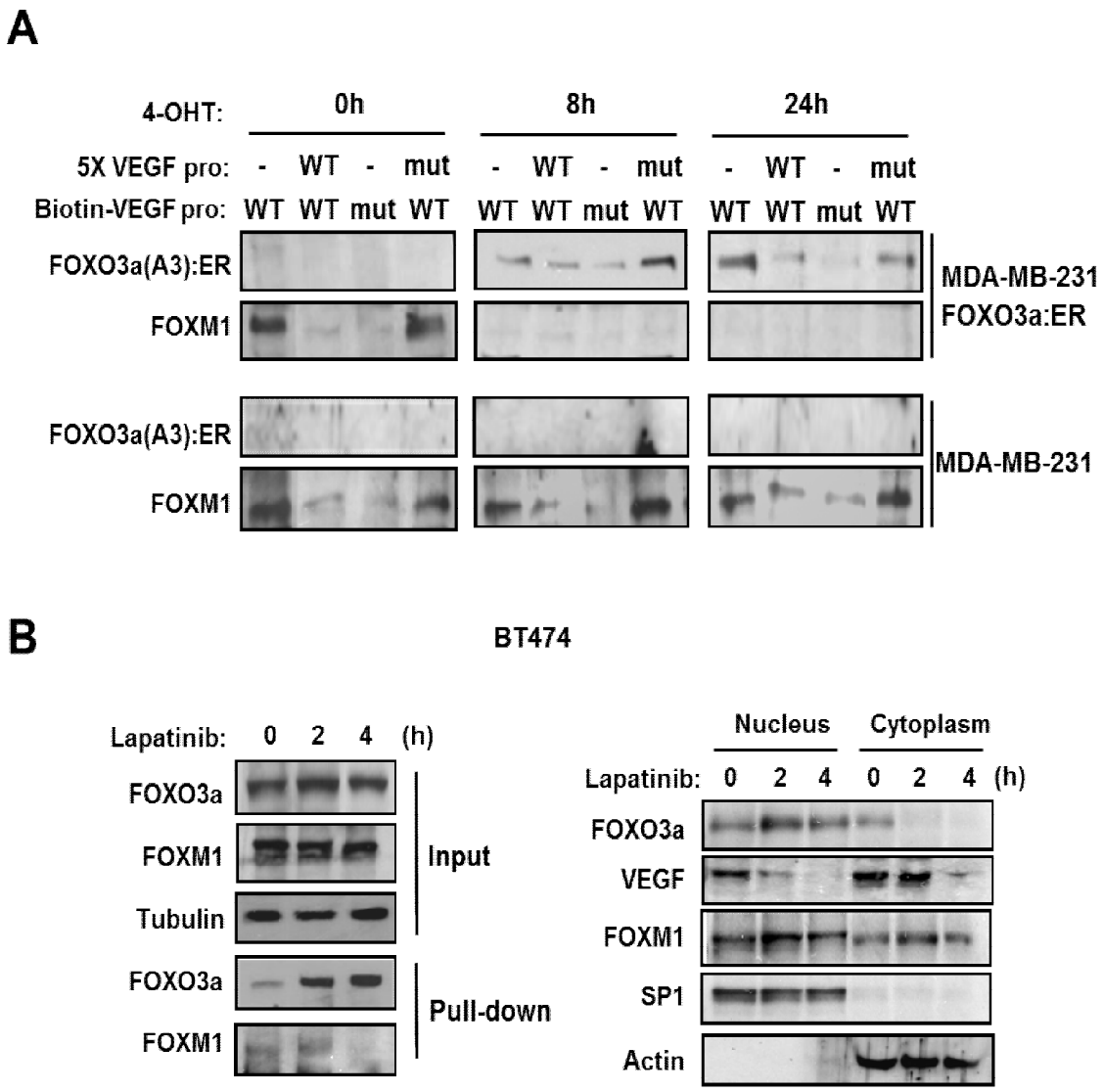


Figure 53. FOXO3a competes off FOXM1 from the *VEGF* promoter.

A) MDA-MB-231-FOXO3a(A3):ER and MDA-MB-231 cells were treated with 200nmol/L of 4-OHT for the indicated times. Pull-down assays were performed and protein expression was analysed by western blot using specific antibodies as indicated. **B**) The nuclear and cytoplasmic extracts prepared from BT474 cells treated with lapatinib for 0, 2 and 4 h were western blotted for the indicated proteins (right panel). The nuclear extracts from the lapatinib-treated cells were also examined by pull-down assays using biotinylated WT or mutant FHRE2 oligonucleotides.

To further study the binding of FOXO3a and FOXM1 to FHRE2 on *VEGF* promoter, I have performed FHRE pull-down and ChIP assays in MCF-7 cells. Cells were transiently transfected with or without FOXO3a(A3) expression vector and nuclear extracts were incubated with biotinylated wild-type (WT) or mutant FHR2 (mut) oligonucleotides with or without 5x molar excess of non-biotinylated wild type (WT) or mutant FHR2 (mut) oligonucleotides. Proteins bound to the biotinylated oligonucleotides were pull-down and analysed by Western blotting (Figure 54A). The results showed that FOXO3a was able to attenuate FOXM1 binding to the FHR2 site on *VEGF* promoter. To definitely show that FOXO3a competes FOXM1 off *VEGF* promoter, ChIP analysis of *VEGF* promoter was performed. MCF-7 cells were transfected with increasing amounts of FOXO3a(A3) expression vector and used for ChIP assays and western blotting (Figure 54B). The results showed that an increase in FOXO3a-binding caused a decrease in the binding of FOXM1 on *VEGF* promoter, further suggesting that the two transcription factors compete for binding. To test whether FOXM1 was able to compete FOXO3a off the *VEGF* promoter, I transfected MCF-7 cells with a fixed amount of FOXO3a(A3) and increasing amounts of FOXM1 expression vectors. The ChIP assay and Western blot results showed that, although FOXO3a can displace FOXM1 from binding *VEGF* promoter, the opposite is not true (Figure 54C).

The finding that FOXO3a can displace FOXM1 from the *VEGF* FHRE2 and not vice versa is further supported by a recent structural study of the FOXM1 DNA-recognition domain, showing that FOXM1 has a lower DNA-binding affinity to the consensus 'TAAACA' recognition sequence compared with other forkhead proteins (Littler et al 2010).

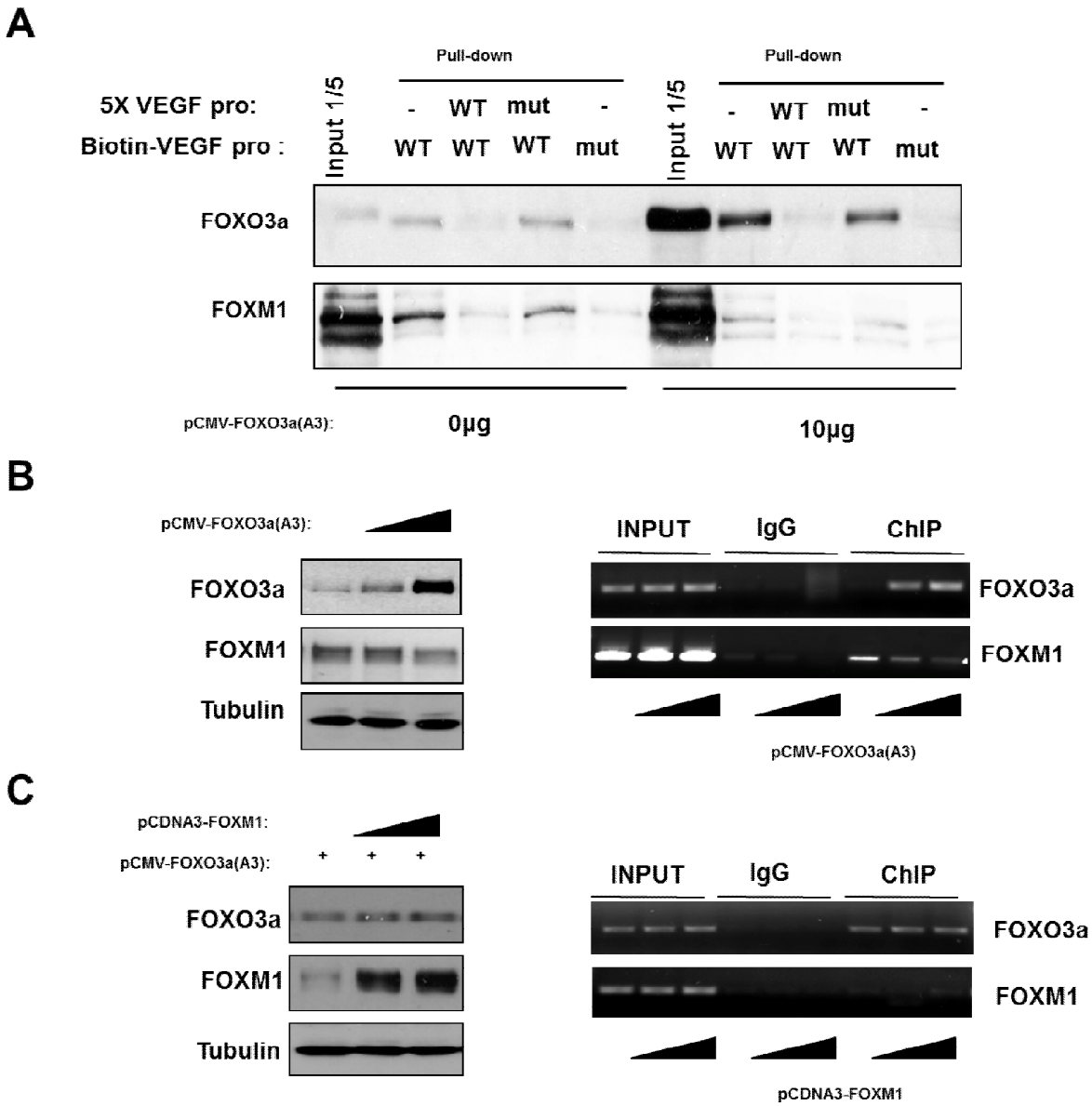


Figure 54. DNA pull-down and ChIP assays showing that FOXO3a can displace FOXM1 in MCF-7 cells.

A) Cells were transfected with 0 and 10µg of FOXO3a(A3) expression vector. Nuclear extracts incubated with the WT or mutant FHRE2 oligonucleotides in the presence or absence of 5x molar excess of non-biotinylated WT or mutant FHRE2. Proteins were pull-down and analysed by western blot using the indicated antibodies. **B**) Cells were transfected with 0, 2, 10µg of the constitutively active FOXO3a(A3) vector and ChIP assays were performed (right). The transfected cells were also western blotted for FOXO3a, FOXM1 and Tubulin expression (left). **C**) Cells were transfected with 0, 2 or 10µg of FOXM1 and 2µg of the FOXO3a(A3) vector, ChIP was performed the indicated antibodies (right). FOXO3a, FOXM1 and Tubulin expression was assessed by Western blot analysis (left).

5.2.5 FOXO3a is recruited to the proximal region of the VEGF promoter in vivo

I then performed chromatin ChIP assays to determine the *in vivo* occupancy of the *VEGF* promoter by FOXO3a and FOXM1 in the BT474 cells in response to lapatinib treatment and in the MDA-MB-231 control and FOXO3a(A3) transfected cells in response to 4-OHT. The anti-FOXO3a antibody, but not the control antibody (IgG), precipitated the proximal region, encompassing FHRE2, of the *VEGF* promoter in BT474 cells (Figure 55). The amount of precipitated DNA increased significantly following 2 h of lapatinib treatment, reflecting an enhanced FOXO3a occupancy of this region of the *VEGF* promoter *in vivo*, consistent with the previous DNA pull-down results. In contrast, the binding of FOXM1 decreased at 2 h following lapatinib treatment. Notably, the binding of both the FOXO3a and FOXM1 to the *VEGF* promoter decreased substantially by 4 h, probably suggesting decreased accessibility to the proximal region of the *VEGF* promoter (Figure 55). This observation suggested that FOXO3a could play a role in recruiting chromatin remodelling enzymes, such as HDACs, to repress the *VEGF* transcription.

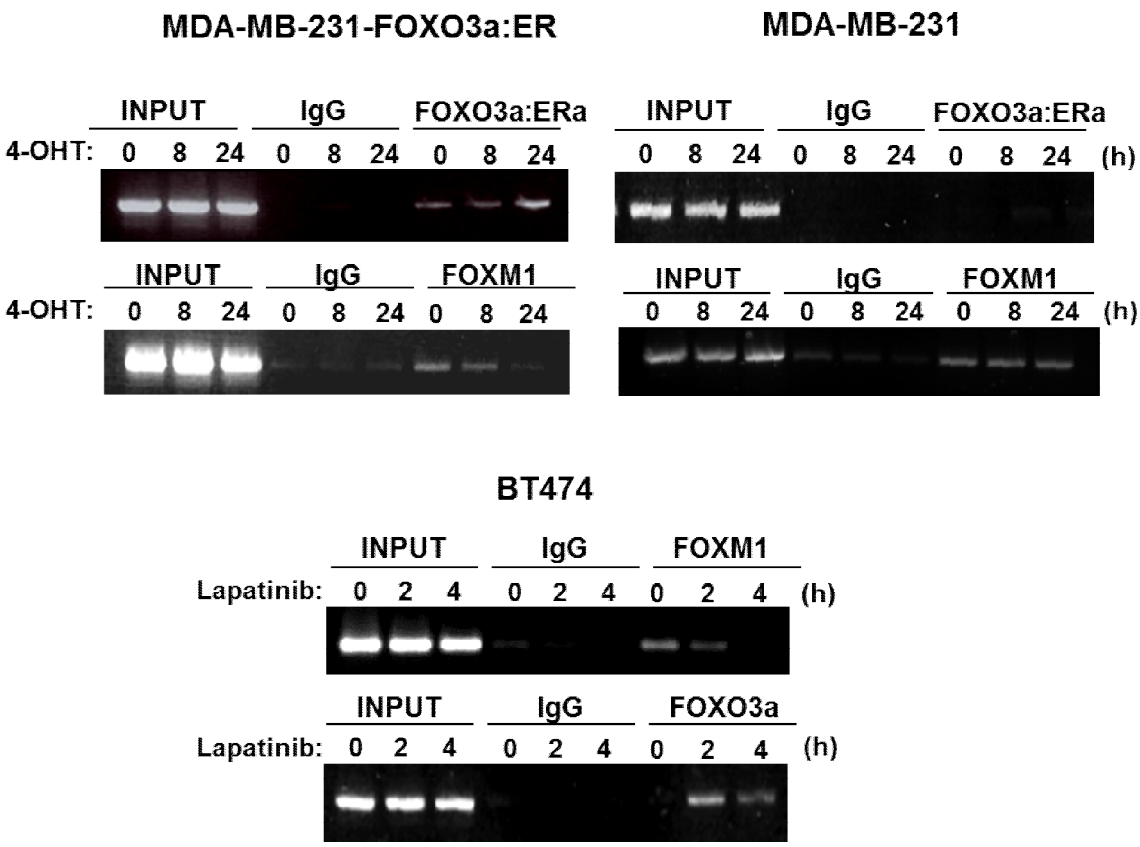


Figure 55. FOXO3a is recruited to *VEGF* promoter.

MDA-MB-231-FOXO3a(A3):ER, MDA-MB-231 and BT474 cells were treated with 200nmol/L of 4-OHT and 1μM of lapatinib for the indicated times. ChIP assays were performed using immunoglobulin G (IgG), anti-FOXO3a, anti-ERα and anti-FOXM1 antibodies as indicated. Representative data from three independent experiments are shown.

5.2.6 FOXO3a recruits HDAC2 to the VEGF promoter

To test the hypothesis that FOXO3a recruits HDACs to repress VEGF transcription, I treated MCF-7 cells with the HDAC inhibitor TSA and monitored VEGF expression. RT-qPCR and Western blot analyses showed that following TSA incubation, VEGF mRNA and protein levels were strongly enhanced (Figure 56A). In order to investigate the impact of TSA and HDAC2 expression on *VEGF* promoter activity, MCF-7 were transfected with the WT, mutant FHRE1, or mutant FHRE2 *VEGF* promoter. The cells were either untreated or treated with TSA, or co-tranfected with increasing amounts of either the wild-type ($HDAC2^{WT}$) or constitutively active HDAC2 ($HDAC2^{C262A/C274A}$) expression vector. The luciferase assay showed that TSA triggered a marked induction in *VEGF* promoter activity, which was abolished upon mutation of the FHRE2, but not FHRE1, site. In contrast, overexpression of the dominantly active $HDAC2^{C262A/C274A}$ mutant, but not the WT HDAC2, repressed *VEGF* promoter activity in a dose-dependent manner. This ability of HDAC2 to repress *VEGF* promoter activity was again dependent on a functional FHRE2 (Figure 56B). The inability of WT HDAC2 to repress *VEGF* promoter activity could be due to the high levels of endogenous HDAC2 in MCF-7 cells. ChIP assay was performed in MCF-7 cells untreated or treated with TSA for 24 h to determine HDAC2 binding on *VEGF* promoter. The assay further demonstrated that TSA induced a decrease in HDAC2 binding to the proximal *VEGF* promoter (Figure 56C). Finally, to study which HDAC species contributes the most to regulate VGEF expression, MCF-7 cells were transfected with NSC and smart pool siRNA against either HDAC1 or HDAC2 and analysed by western blotting. HDAC2 knockdown using siRNA significantly upregulated VEGF expression whereas silencing of HDAC1 had little or no effect on VEGF expression (Figure 56D). All together, these data provides compelling evidence that HDAC2 mediates transcriptional inhibition of the *VEGF* promoter in breast cancer cells.

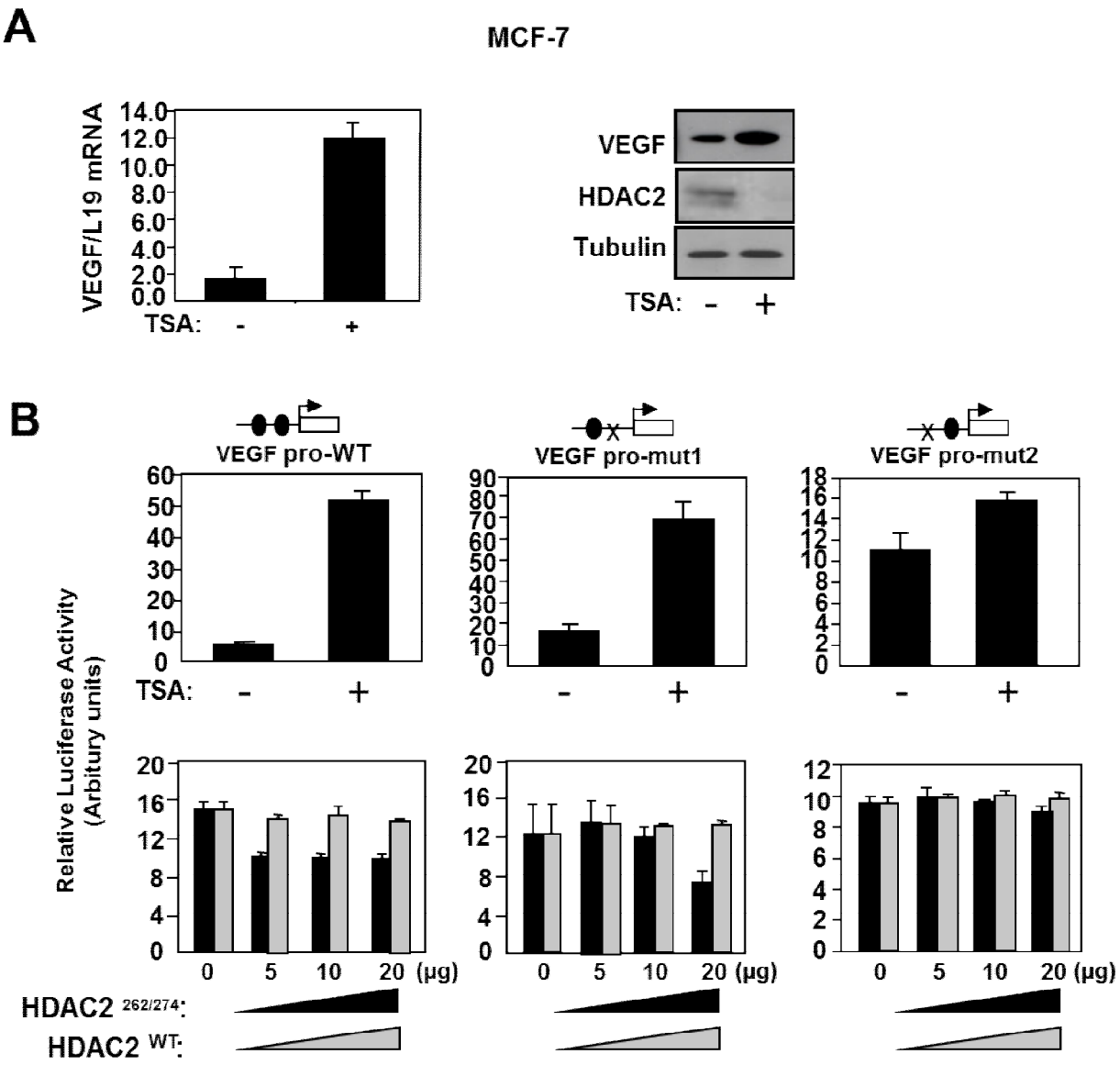


Figure 56. Effects of HDAC2 overexpression and depletion on the expression of VEGF in MCF-7 cells.

A) Cells were treated with vehicle or 100nM TSA for 24 h and harvested for analysis of VEGF expression by RT-qPCR (left) and western blot analysis for VEGF, HDAC2 and Tubulin expression (right). **B**) Cells were transfected with 20ng of the WT, mutant FHRE1 or mutant FHRE2 VEGF promoter/reporter and either untreated or treated with 100nM TSA, or co-transfected with 0, 5, 10 or 20ng of either the WT or constitutively active HDAC2 expression vector. The cells were harvested for luciferase assays after 24 h. **C**) Cells were untreated or treated with TSA for 24 h were analysed for HDAC2 binding on the VEGF promoter by ChIP assays. **D**) Cells were transiently transfected with NSC and specific siRNA against either HDAC1 or HDAC2 and were analysed by western blotting for protein expression as indicated.

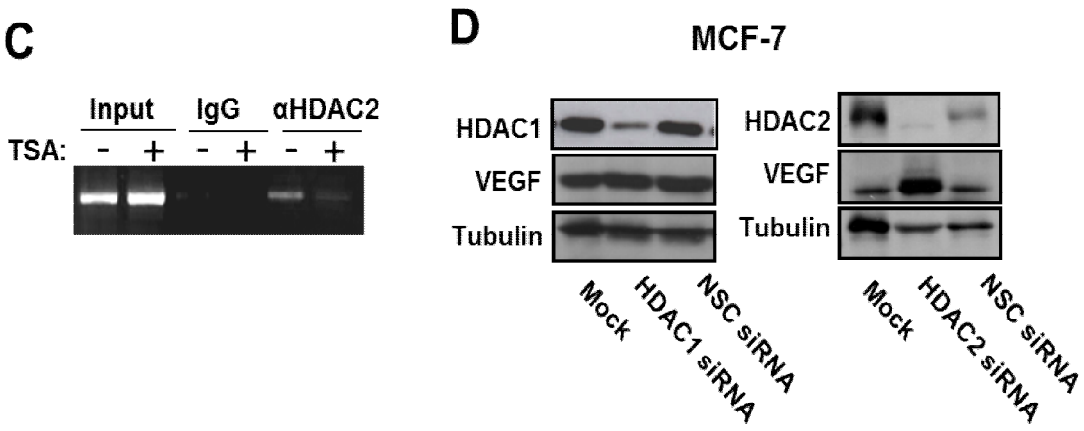


Figure 56. Continued.

To examine if FOXO3a recruits HDAC2 to the *VEGF* promoter, I performed immunoprecipitation and ChIP experiments on BT474 cells treated with lapatinib. HDAC2 and FOXO3a co-immunoprecipitated and this interaction was enhanced upon lapatinib treatment, probably reflecting the previously observed nuclear translocation of FOXO3a in Figure 53B. The ChIP assays showed increased recruitment of HDAC2 to the proximal *VEGF* promoter following 2 h of treatment with lapatinib (Figure 57B). Since acetylated histone H3 and H4 are epigenetic marks associated with activated promoters (Bernstein et al 2005, Davie and Candido 1978), I examined whether the HDAC2 recruitment coincided with a decrease in acetylated histones H3 and H4. As seen in the two lower panel of Figure 57B, HDAC2 was recruited only after a decrease in bound acetylated histones H3 and H4 was noticed, indicating active chromatin remodelling and compaction of the proximal *VEGF* promoter. Further, siRNA-mediated FOXO3a knockdown (Figure 57C, right and left panels) in BT474 cells abolished the recruitment of HDAC2 to the proximal *VEGF* promoter upon lapatinib treatment as well as the concomitant decrease in acetylated histones H3 and H4. These findings demonstrate that FOXO3a activation in breast cancer cells results in displacement of DNA-bound FOXM1 from FHRE2, recruitment of HDAC2, and transcriptional repression of *VEGF*.

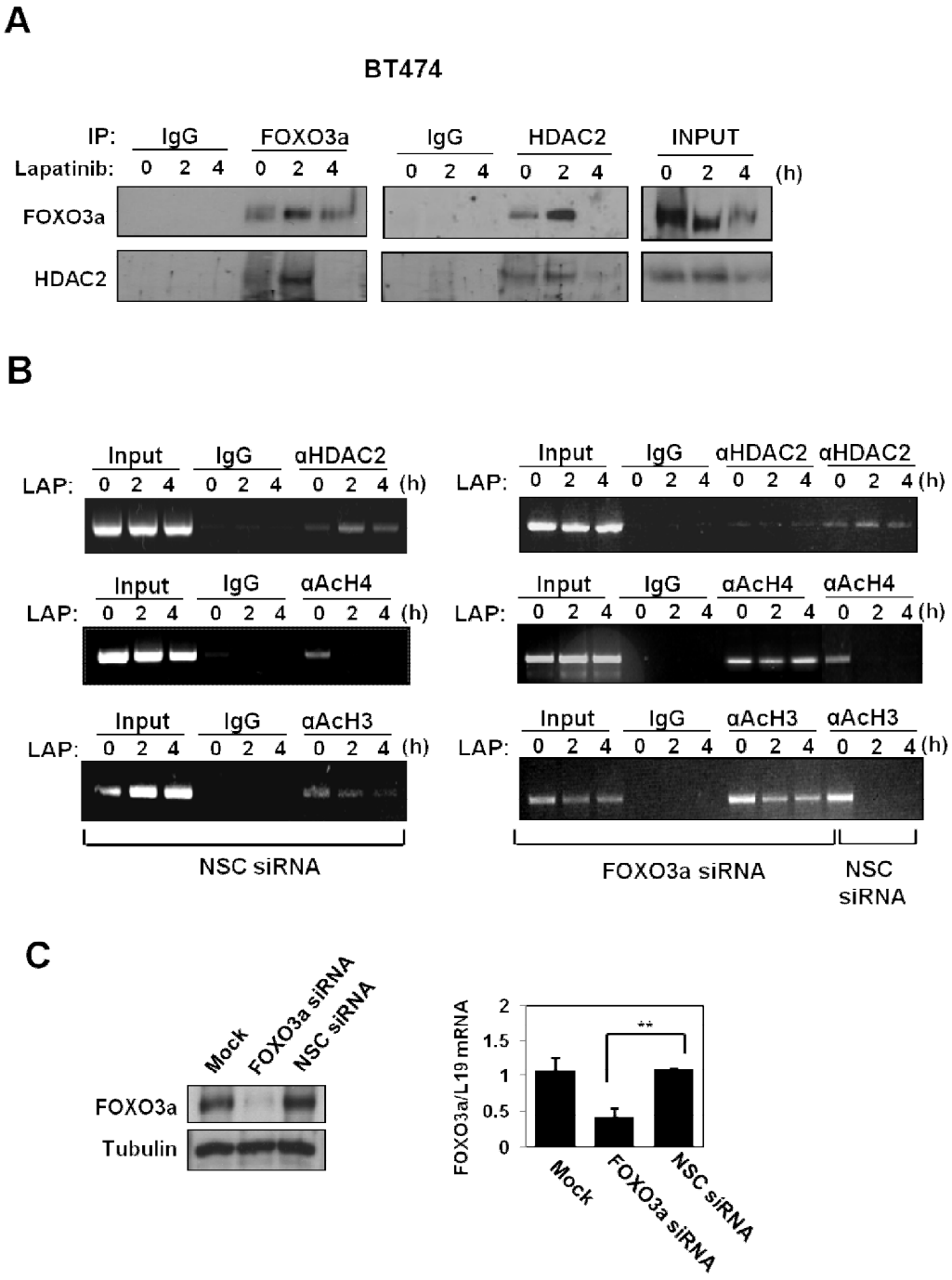


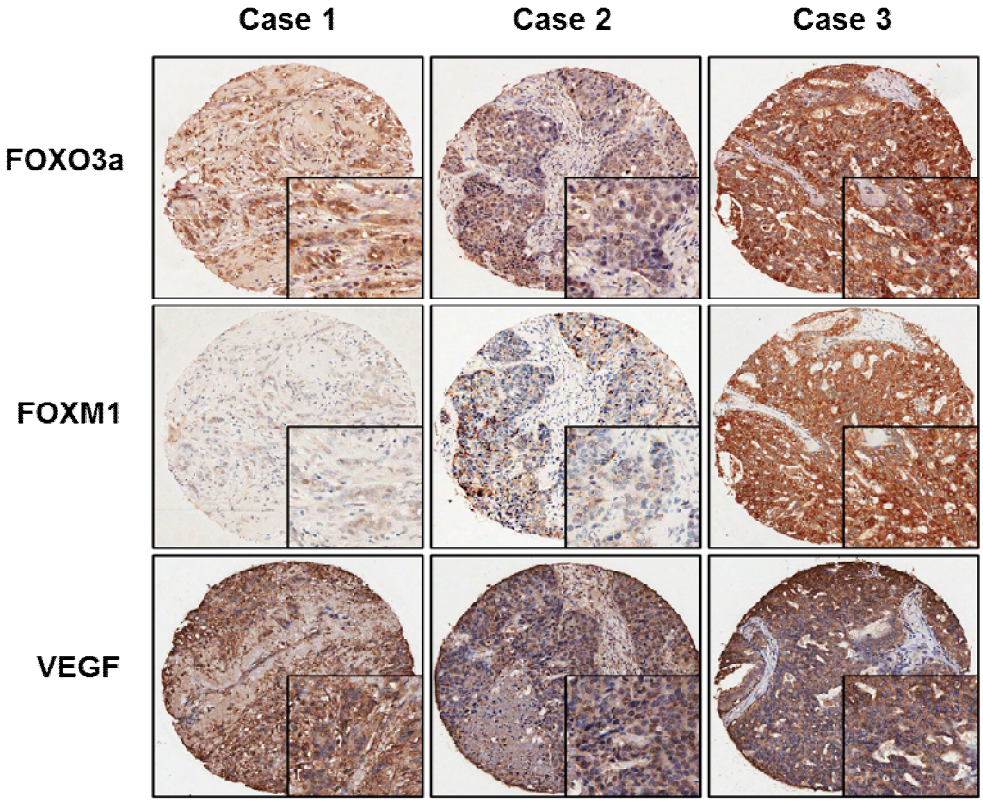
Figure 57. FOXO3a recruits HDAC2 to the *VEGF* promoter in response to lapatinib in BT474 cells.

A) Cells were treated with 1μM lapatinib for 0, 2 and 4 h. Cells were immunoprecipitated and examined for FOXO3a and HDAC2 expression. **B**) Cells were transfected with specific siRNA against FOXO3a or NSC siRNA for 24 h, treated with lapatinib and then analysed for HDAC2, acetylated histone H3 and H4 binding on the *VEGF* promoter by ChIP assays. **C**) Western blot and RT–qPCR analyses were performed to confirm effective and specific FOXO3a knockdown. Statistical analyses were done using Student's t test, and were considered significant at **P<0.01.

5.2.7 Inverse correlation between FOXO3a and VEGF expression in breast cancer

To determine the regulation of VEGF by FOXO3a *in vivo*, I analysed the expression patterns of FOXO3a, FOXM1 and VEGF in a panel of breast cancer samples by IHC, together with our collaborators from Queen Mary Hospital in Hong Kong. Representative patterns of staining are shown in Figure 58. FOXO3a immunoreactivity was predominantly cytoplasmic in most tumour samples and correlated positively with VEGF (P = <0.001, Chi-square test) and FOXM1 (P = 0.011, Chi-square test) staining irrespective of histological type, suggesting that the activated nuclear FOXO3a inhibits FOXM1 and VEGF expression *in vivo* in most breast cancer samples. Notably, there was also an inverse association trend between nuclear FOXO3a and VEGF expression but it was not statistically significant. Moreover, FOXM1 expression also significantly correlated with the expression of VEGF (P=0.015, Chi-square test) (Figure 58; see also Appendix 9, Appendix 10 and Appendix 11), suggesting FOXM1 promotes VEGF expression in breast cancer cells.

A



B

Total Case No. : 116	FOXO (cytoplasmic staining)	FOXO (nuclear staining)
VEGF	**<0.000****	0.118
FOXM1	**0.011****	0.773

Total Case No. : 116	VEGF
FOXM1	**0.015***

Figure 58. Representative expression patterns of FOXO3a, FOXM1 and VEGF in tissue microarray.

Tumour tissue samples were stained by IHC with FOXO3a, FOXM1 and VEGF antibodies. **A**) Three representative tumour cases showing corresponding FOXO3a FOXM1 and VEGF staining patterns (magnification x100). The three cases 1, 2 and 3 represent low, medium and high FOXO3a cytoplasmic staining. **B**) Correlation analysis of FOXO3a, FOXM1 and VEGF staining in 116 breast carcinoma cases. The correlation between predominant nuclear/cytoplasmic FOXO3a expression with FOXM1 and VEGF expression and FOXM1 with VEGF expression was studied using Chi-square tests and was considered significant at *P<0.05 and very significant at **P<0.01.

5.3 DISCUSSION

VEGFs and their receptors are essential for breast cancer carcinogenesis, angiogenesis and metastasis (Lohela et al 2009). VEGF signals through different pathways including the PI3K/AKT, the p38-MAPK, and Raf however, the molecular mechanisms regulating VEGF expression in cancer cells are not fully understood. Yet, two studies have shown that inhibition of PI3K/AKT and MEK/ERK pathways lead to activation of FOXO factors which in turn mediated the anti-angiogenic effects of the chemopreventive agent epigallocatechin gallate (EGCG) present in green tea (Shankar et al 2008) and enhanced the anti-angiogenic effects of resveratrol in HUVEC cells (Srivastava et al 2010). The previously described cDNA microarray study suggested that VEGF is a potential negatively regulated target of FOXO3a (Delpuech et al 2007). The analysis of breast cancer patient samples showed that FOXO3a nuclear localisation is significantly but inversely associated with VEGF expression, suggesting FOXO3a negatively regulates VEGF expression *in vivo* in breast cancer.

Anti-angiogenic therapies that target both VEGF and its main receptor (VEGFR-2) are effective adjuvants to the treatment of solid tumours. Currently, the anti-VEGF antibody - bevacizumab - is being used for treatment of different carcinomas in combination with cytotoxic chemotherapy and has been shown to prolong the progression-free survival (Hurwitz et al 2004, Miller et al 2007, Sandler et al 2006). In addition, some tyrosine kinase inhibitors (TKIs) have also been developed to target VEGFR, including sunitinib and sorafenib. Despite the promising results of these VEGF and VEGFR targeting drugs, evidence of resistance has been described. This resistance mechanism may involve other receptor tyrosine kinases (RTKs) such as EGFR and HER-2. Antibody-therapies against these RTKs have been shown to decrease VEGF expression (Petit et al 1997) whereas tumour cells with enhanced HER-3/HER-4 ligand expression increased the production of VEGF (Yen et al 2000). Similarly, the EGFR ligand – EGF – has been shown to induce the expression of VEGF in glioma cells (Goldman et al 1993). Importantly, the EGFR specific monoclonal antibody cetuximab, decreased VEGF expression in a dose-dependent manner in squamous carcinoma cells (Petit et al 1997). Moreover, similar results were obtained with gefitinib and erlotinib (Ciardiello et al 2001, Pore et al 2006). The link that promotes the cross-talk between EGFR and VEGF is most likely HIF-1, as this transcription factor is a known regulator of VEGF and moreover, was showed to engage in a bidirectional regulation with EGFR (Swinson et al 2004). It has been shown that in tumour ECs, EGFR-specific TKIs switch from EGFR to VEGFR signalling in a feedback mechanism (Amin et al 2008) thus, indicating that EFGR inhibition enhances VEGF signalling (Bianco et al 2008, Naumov et al 2009, Viloria-Petit et al 2001). Whether VEGF

inhibition can promote EGFR signalling is not known. In fact, vandetanib, a new small molecule TKI of both EGFR and VEGFR may prove a useful tool for overcoming this mode of resistance (Heymach et al 2007). In this chapter, the dual inhibitor lapatinib was used to block the downstream signalling cascades of both EGFR and HER-2, such as the PI3K/AKT, which in turn results in FOXO3a activation (Hegde et al 2007). Using the lapatinib sensitive breast cancer cell lines BT474 and SKBR3 as models for FOXO3a activation, I tested and examined the underlying mechanism of VEGF regulation by FOXO3a. Lapatinib treatment results in nuclear translocation and activation of FOXO3a and ultimately reduction in VEGF expression at protein, mRNA and gene promoter levels. Transient transfection and inducible FOXO3a expression experiments show that FOXO3a represses while FOXM1 activates VEGF expression through a proximal FHRE site of the *VEGF* promoter, as mutation of this FHRE abrogated the regulation by FOXO3a and FOXM1. ChIP and oligonucleotide pull-down assays further demonstrate that both FOXO3a and FOXM1 bind directly to the FHRE of the *VEGF* promoter and that activated FOXO3a can displace FOXM1 from the FHRE, suggesting that FOXO3a can repress VEGF expression through competing off the transcriptional activator FOXM1. Consistently, FOXO3a accumulated and replaced FOXM1 at the FHRE as early as 2 h after lapatinib treatment; however, it was also noted that neither FOXO3a nor FOXM1 bound to the FHRE by 4 h. The lack of occupancy of the proximal *VEGF* promoter region by FOXO3a and FOXM1 at 4 h suggests that FOXO3a accumulation might lead to exclusion of transcription factors through chromatin remodelling. Histones are known targets of post-translational modifications, including methylation and acetylation (Kouzarides 2007). The later chromatin modifications have been shown to regulate transcription factor binding (Li et al 2007a). Indeed, some studies have shown that the promoter regions of *VEGFR-1* and *VEGFR-2* are targets of epigenetic alterations and this leads to altered gene expression (Kim et al 2009, Yamada et al 2003). Histone acetylation decondenses the chromatin, making nucleosomal DNA more accessible to transcription factors, whereas inhibition of histone deacetylase activity by HDACs leads to condensation of the chromatin and exclusion of transcription factors (Strahl and Allis 2000). Consistently, I found that upon activation, FOXO3a recruits HDAC2 to the proximal region of the *VEGF* promoter, as revealed by ChIP analysis. Recently, the levels of acetylated histones H3 and H4 located in the nucleosomes have been proposed to be proportional to the levels of transcriptionally active DNA (Pokholok et al 2005). Consistently, the ChIP assays showed that in response to lapatinib treatment in BT474 cells, there was an increase in FOXO3a and HDAC2 binding, concomitant with a decrease in acetylated histones H3 and H4 levels. In order to examine the amount of HDAC2 binding to FOXO3a in response to lapatinib in BT474 cells, I also performed co-immunoprecipitation experiments. The results show that the amount of

HDAC2 interacting with FOXO3a increased substantially at 2 h but declined by 4 h following lapatinib treatment. The increase in FOXO3a binding to HDAC2 in response to lapatinib is likely to be due to the relocation of FOXO3a to the nucleus, while the declined in FOXO3a binding to HDAC2 was probably a result of the disassociation in binding between the two proteins as well as a decline in HDAC2 levels, as revealed by immunoprecipitaion and western blot analyses, respectively.

Overexpression of FOXM1 has been implicated with metastasis and angiogenesis in a number of malignancies, including glioma (Dai et al 2007), colon (Uddin et al 2011) and pancreatic (Wang et al 2007) cancer. Consistent with my findings, a recent study has also demonstrated that FOXM1 transcriptionally regulates *VEGF* expression in glioma cells (Zhang et al 2008). However, the FOXM1 responsive sites identified previously are located over 500 bp 5'-upstream of the FOXO/FOXM1 binding site defined in this study and neither of these sites appears to have a consensus FOXO-binding element. Importantly, deletion of site 2 in the present *VEGF* promoter abolished responsiveness to FOXO3a, FOXM1 and HDAC, suggesting this FHRE is targeted by both FOXO3a and FOXM1. Consistent with a previous report (Francis et al 2009), I further demonstrated that FOXM1 functions downstream of FOXO3a, and its activity and expression are negatively regulated by FOXO3a. Nevertheless, FOXM1 is not the sole mediator of FOXO3a function. FOXO3a can also negatively regulate gene expression through FOXM1 independent mechanisms, such as by means of HDAC recruitment. The ability of FOXO proteins to repress VEGF expression has been documented in *Foxo1* null cells where VEGF is overexpressed and angiogenesis deregulated (Furuyama et al 2004, Park et al 2009). In fact, the loss of FoxO1 causes embryonic lethality as a result of severe defects in vascular development (Hosaka et al 2004). The notion that FOXO proteins are involved in the regulation of VEGF is now further supported by my finding that expression of nuclear FOXO3a expression significantly correlates with VEGF expression in breast cancer patient samples. Moreover, the fact that FOXO3a activity is enhanced in a HIF1-dependent way in response to hypoxia (Bakker et al 2007) suggests that HIF-1 could serve a role in another level of regulation of VEGF by a mechanism FOXO3a-dependent.

In summary, together these results suggest that FOXO3a can potentially repress *VEGF* expression, through at least two mechanisms. First, activated FOXO3a can compete off the transcription activator FOXM1 from binding to the FHRE of the *VEGF* gene promoter. Second, FOXO3a can recruit HDACs to the *VEGF* promoter to induce chromatin condensation and transcription factor exclusion. Furthermore, FOXO3a has also been shown previously to be able to repress *FOXM1* expression at the transcriptional level (Francis et al 2009). Consequently, FOXO3a can repress *VEGF* expression indirectly via regulating FOXM1 expression. The mechanisms by which FOXO3a represses VEGF

expression may represent common means whereby FOXO3a negatively regulates target gene expression. Thus, these data also provide novel understanding on the mechanisms by which FOXO transcription factors repress target gene expression. In addition, the findings from this chapter further suggest that therapeutic strategies targeting FOXO3a or FOXM1 can be used as an alternative or in parallel with anti-VEGF targeted agents as well as conventional chemotherapy in rational and effective treatment of tumours (Fernandez de Mattos et al 2008, Gomes et al 2008, Srivastava et al 2010).

CHAPTER 6

FINAL DISCUSSION

Over the last two decades, advances have been made in the development of new and more effective drugs for different cellular targets in the treatment of cancer. However, cancer cell clones are always one step ahead, responding by generating new mechanisms to tolerate, evade or compensate the lethal effect of drugs.

One of the signalling pathways most commonly deregulated during tumorigenesis and in the development of cancer drug-resistance is the PI3K/AKT/FOXO signalling axis. This signalling cascade has been found to be frequently hyperactivated in cancer, leading to FOXO inactivation. The PIK3/AKT/FOXO signalling pathway plays a critical role in a wide variety of cellular processes. One such example is the well characterised role that this pathway plays towards increased resistance to apoptosis and thus, in mediating drug-resistance responses. Indeed, enhanced AKT activity has been showed to promote resistance to endocrine and cytotoxic drugs, further emphasising the importance of PI3K/AKT/FOXO axis in determining the outcome of drug sensitivity. Furthermore, many studies showed that the PI3K/AKT/FOXO cascade mediates both directly and indirectly the anti-survival and anti-proliferative effects of cancer drugs, such as paclitaxel, doxorubicin, cisplatin, gefitinib and lapatinib (Fernandez de Mattos et al 2008, Hui et al 2008b, Sunters et al 2003, Xia et al 2006). However, the functional role, the mechanism of action and the control of this signalling axis in mediating cancer drug sensitivity and resistance have not been fully elucidated.

Several studies have provided insights into the mechanisms of FOXO regulation by AKT; however, not much is known regarding the relationship between FOXO and AKT, and its contribution to the development of breast cancer drug-resistance as well as the potential clinical relevance of the AKT/FOXO axis as both prognostic and predictive tools that could be used to “sort” patients in order to provide the most beneficial treatment combinations. This was explored in chapter 3, where I investigated not only the activity, subcellular localisation and impact of the PI3K/AKT/FOXO axis in different breast cancer drug sensitive and resistant cell lines but I further validated my *in vitro* results in breast cancer patient samples. I have shown that PI3K/AKT has definitely a role in mediation of cytotoxic chemotherapeutic drug-resistance, similarly to what has been described in numerous literature reports (Knuefermann et al 2003, Neri et al 2003, Tsuruo et al 2003, West et al 2002). However, in contrast to a previous publication (Hu et al 2004), I have shown that in breast cancer patient samples, nuclear FOXO3a localisation predicts poor survival as well as chemotherapy resistance and associates with bad prognosis in breast cancer. Moreover, I found that higher nuclear FOXO3a expression, *in vitro* as well as in advanced breast cancer patient samples, is positively associated with P-AKT expression. This finding is contradictory to the well-established mechanism of regulation of FOXO proteins by AKT. Nonetheless, a negative feedback mechanism loop, in which FOXO3a upregulates

PI3K/AKT activity, has been previously described by our group in leukeamic cell lines. Consistently, I confirmed that this FOXO/AKT negative feedback mechanism is in place in both sensitive and resistant breast cancer cell lines, yet with different functional outcomes to what concerns FOXO mode of action. In sensitive cells FOXO could efficiently inhibited cell proliferation whereas in resistance cells, although nuclear FOXO expression is higher, its tumour suppressive function is deregulated (**Error! Reference source not found.**). The mechanism that allows FOXO3a to evade PI3K/AKT-dependent cytoplasmic sequestration, resulting in higher nuclear FOXO3a expression, is yet unknown but it could involve different post-translational modifications. For example, JNK pathway has been shown to phosphorylate FOXO and mediate its nuclear accumulation and promote the recruitment of SIRT1, leading to the activation of a specific subset of genes (Brunet et al 2004). In accordance, SIRT1 has been shown to mediate FOXO activity by deacetylating FOXO3a resulting in the activation of genes involved in survival. For instance, it is possible that in sensitive breast cancer cells FOXO is enabled to activate a pro-apoptotic and anti-proliferative gene programme, thus acting as a tumour suppressor whilst in resistant breast cancer cells, the PI3K/AKT-insensitive FOXO3a is regulated by different co-factors that act on FOXO to deregulate their known pro-apoptotic and anti-proliferative functions and favour the expression of genes determinant for cell survival and drug-resistance (Figure 59).

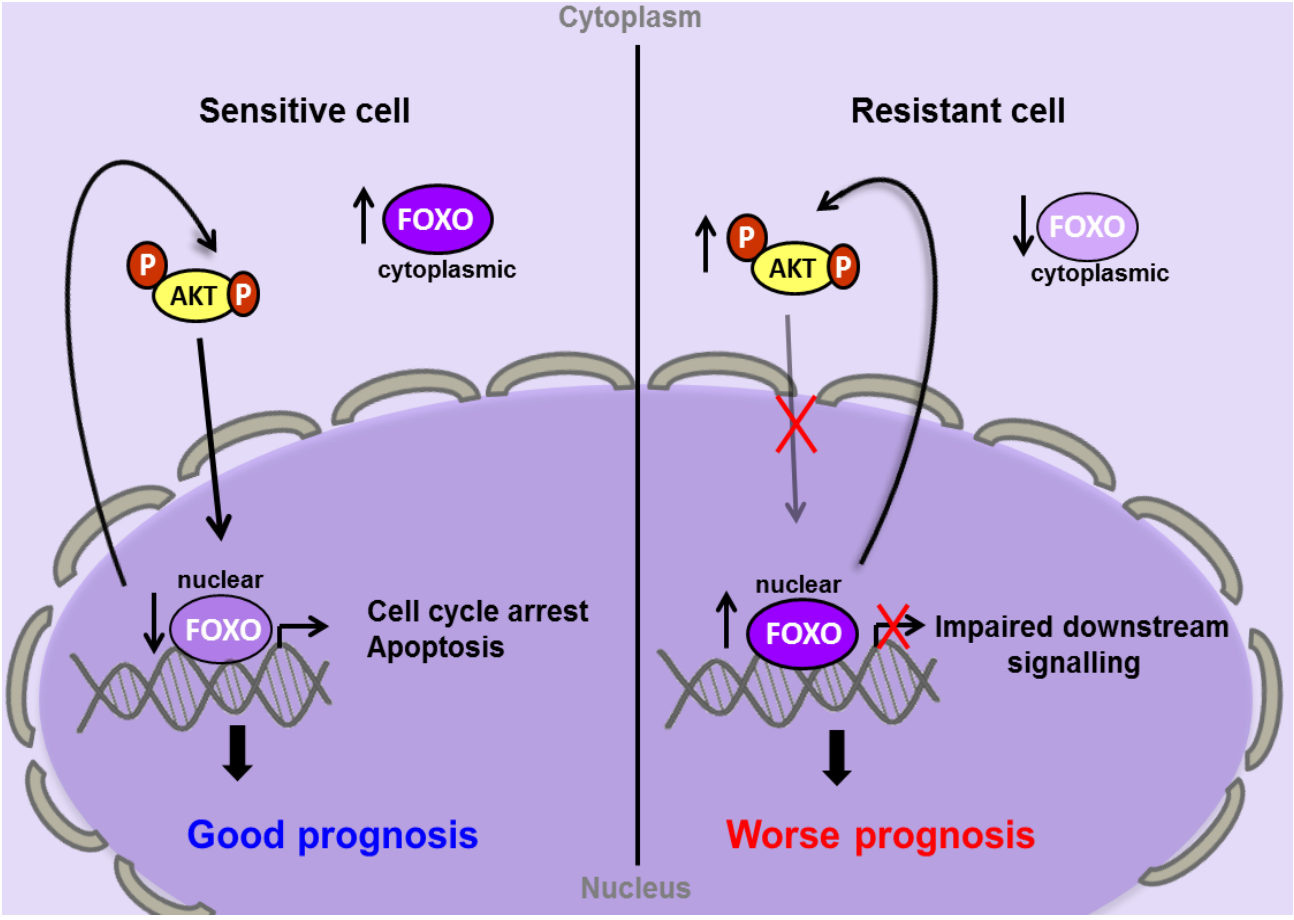


Figure 59. Proposed model of FOXO subcellular localisation, differential effects and clinical impact in sensitive and resistant breast cancer cells.

This work provides evidence that sustained nuclear FOXO3a expression may attenuate the response to endocrine and cytotoxic chemotherapy. Therefore, the study of the phosphorylation status and subcellular localisation of both AKT and FOXO proteins are two parameters that could determine whether these two proteins would be useful prognostic and predictive markers of chemotherapy response in breast cancer. The mechanism responsible for the differential regulation of FOXO3a activity in sensitive and resistant cells is still unknown; however it is likely that FOXO3a activity is regulated by other posttranslational modifications, including their acetylation status. As previously mentioned, deacetylation of FOXO by SIRT1 is known to shift FOXO-induced responses away from apoptosis towards survival (Brunet et al 2004). Moreover, SIRT1 has been shown to have a role in tumorigenesis. Overexpression of SIRT1 is found in a variety of cancers, including leukaemia and lymphomas, prostate, lung and colon carcinomas (Fraga et al 2007, Fraga and Esteller 2007, Lim 2007). Importantly, SIRT1 has been shown to be overexpressed in drug-resistant cancer cell lines and mediate cisplatin resistance (Liang et al 2008, Olmos et al 2011, Shiota et al 2010). In fact, cisplatin treatment has been shown

to induce SIRT1-dependent deacetylation of FOXO3a (Shiota et al 2010). This has led to the development of small synthetic molecules that can specifically target SIRTs influencing their activity, including sirtinol (Napper et al 2005), salermide (Grozinger et al 2001) and EX527 (Lara et al 2009) which have been shown to inhibit cancer cell growth. For example, treatment with sirtinol resulted in increased efficacy of cisplatin, in part by the reactivation of FOXO1 and induction of a growth arrest in prostate cancer cells (Jung-Hynes et al 2009). It would be interesting to use these inhibitors to model FOXO3a acetylation status and restore the pro-apoptotic and anti-proliferative functions of FOXO3a in resistant breast cancer cells, which ultimately would result in overcoming drug-resistance.

To better understand the functional significance of the PIK3/AKT/FOXO signalling pathway and its role in tumorigenesis, it is also important to define the downstream network of FOXO targets. The identification of FOXO targets will help decipher its function, for example in sensitive as well as resistant cancer cells. Additionally, by identifying and characterising novel downstream targets of FOXO, which are differentially regulated in sensitive and resistant cancer cells, could also represent new therapeutic targets for the development of anti-cancer drugs. In chapters 4 and 5, I focused on characterising some of the new potential FOXO targets in breast cancer, previously validated on a microarray study (Delpuech et al 2007), including, FOXP1, FOXM1 and VEGF.

I have shown not only that FOXO3a regulates FOXP1 expression but also that FOXP1 is targeted by the chemotherapeutic drug epirubicin and plays a crucial role towards cell cycle arrest. Furthermore, FOXP1 expression was differentially regulated in sensitive and epirubicin-resistant breast cancer cell lines, resembling the one of FOXO3a. This clearly suggests that the FOXO3a-dependent FOXP1 regulation could have a determinant role towards drug-resistance in breast cancer. Importantly, I have also found that $p27^{Kip1}$ is a target of FOXP1. This finding was unexpected and raises some possibilities regarding the regulation of $p27^{Kip1}$ by FOX proteins, specifically FOXO and FOXP. FOXO3a has been shown in a definite way to be a regulator of $p27^{Kip1}$ expression. Since I validated FOXP1 as a downstream target of FOXO3a in breast cancer, two pertinent questions were raised. Is $p27^{Kip1}$ expression regulated directly by FOXP1, in a FOXO3a-independent manner? Or do FOXO3a and FOXP1 cooperatively regulate the expression of $p27^{Kip1}$? Unfortunately, my time-scale did not allow me to further investigate these conjectures however, the two scenarios are possible. In arguement of a potential FOXP1 direct regulation of $p27^{Kip1}$, lays the fact that FOXP1 is not under the negative regulation of AKT, i.e., no AKT phosphorylation sites were found in FOXP1 sequence to date. Additionally, the tumour patient samples showed that FOXO3a is inversely correlated with FOXP1. Therefore, in cases where PI3K/AKT pathway is activated leading to FOXO3a cytoplasmic

sequestration, FOXP1 can proclaim the role of the principal regulator of $p27^{Kip1}$. In support of this conjecture is also the fact that FOXP1 is mainly nuclear localised. On the other hand, FOXP family are known to form homo- and hetero- dimers (Wang et al 2003). It could be that a complex of FOXO3a:FOXP1 would cooperatively regulate $p27^{Kip1}$ expression. Yet, FOXO proteins have been described to bind DNA as monomers. Nevertheless, I have demonstrated that FOXP1 has an anti-proliferative through induction of $p27^{Kip1}$ expression. Furthermore, in epirubicin-resistant cells characterised by low levels of FOXP1 and $p27^{Kip1}$, overexpression of FOXP1 was able to restore $p27^{Kip1}$ expression while silencing FOXP1 in sensitive MCF-7 cells abrogated $p27^{Kip1}$ expression. Consistent with the role of FOXP1 in the cell cycle, FOXP1 is associated with a good prognosis, increasing the survival time of breast cancer patients. Nevertheless, the mechanism of regulation of FOXP1 expression and activity is still remains poorly understood. However, the family member FOXP3 has been shown to be targeted by post-translational modifications, including acetylation. In fact, SIRT1 has been shown to deacetylate Foxp3, resulting in its proteasomal degradation (van Loosdregt et al 2011).

Similarly to FOXP1, in Chapter 5 I also validated VEGF as a downstream target of FOXO3a in breast cancer. In this chapter, lapatinib was used as a drug that blocks the PI3K signalling pathway and subsequently activates FOXO3a expression. Consistent with this notion, I found that lapatinib treatment induces FOXO3a nuclear translocation and activation. Contrary to what I found for FOXP1, FOXO3a negatively regulates VEGF expression at protein, mRNA and promoter levels. Consistently, IHC staining showed that nuclear FOXO3a expression significantly correlates with VEGF expression in breast cancer patient samples. Additionally, I have elucidated a mechanism of regulation of VEGF that encompasses a competition between FOXO3a and FOXM1 binding to *VEGF* promoter. These two transcription factors are able to recognise and bind the same FHRE sequence site on VEGF promoter. While the constitutively active FOXO3a overexpression results in downregulation of VEGF levels, FOXM1 was found to induce VEGF expression. I found that upon lapatinib treatment, FOXO3a becomes activated and competes off FOXM1 from the common FHRE site. However, four hours upon lapatinib treatment neither FOXO3a nor FOXM1 were found bound to the FHRE site. This led me to hypothesise that the early FOXO3a-mediated occupancy of *VEGF* promoter could serve as a recruitment base for transcriptional repressor complexes. In a global perspective, I have shown that lapatinib promotes the nuclear accumulation and activation of FOXO3a, which in turn recruits HDAC to the *VEGF* promoter, resulting in transcriptional repression through chromatin remodelling. In line with previous observations, I have further demonstrated that FOXM1 activity and expression is negatively regulated by FOXO3a in breast cancer cell lines. My findings lead to three potential FOXO3a-mediated

mechanisms of VEGF regulation: the first encompasses regulation of target expression indirectly via repression of FOXM1 expression; secondly, FOXO3a can recruit HDACs to target promoters to induce chromatin condensation and transcription factor exclusion, including FOXM1; finally, activated FOXO3a can displace FOXM1 from binding the FHRE site on target gene promoters. To my knowledge this is the first study demonstrating how class I HDACs are involved in gene repression through FOXO3a. It is also possible that the proposed mechanism of regulation of VEGF by FOXO3a may well represent the general mechanism by which FOXO transcription factors repress gene expression.

In summary, this work has highlighted the importance of the FOXO/FOXM1 axis in tumorigenesis and drug-resistance. I have also elucidated the mechanism of action of FOXO and FOXM1, through studying the downstream effectors, FOXP1 and VEGF. Furthermore, my work has revealed that these forkhead transcription factors, FOXM1, FOXO3a and FOXP1, are not only frequently deregulated in cancer but also in the cancer resistant clones. Finally, my data suggests that these transcription factors could be useful targets for therapeutic interventions to treat cancer and ultimately to revert resistance. Since these transcription factors are regulated by post-translational modifications, such as acetylation one could potentially explore HDAC inhibitors to model their activity. The indisputable role of the PI3K/AKT/FOXO pathway in mediating many anti-cancer drug effects makes both this pathway and the newly identified effectors network an attractive axis to use in the diagnosis of cancer, prognosis to treatment as well as for monitoring the drug effects. However, the clinical potential of these proteins should not be assessed independently. For example, the phosphorylation status of both AKT and FOXO should be integrated with its expression. Ultimately, the use of these proteins as biomarkers and therapeutic tools in breast cancer will warrant further studies regarding their mechanisms of action and regulation as well as the expression and function of their downstream components, and this will be subject of future projects seeking to obtain further success on cancer therapy and avoiding drug-resistance.

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APPENDICES

Antibody	Product number	Species	Company
EGFR	2232	Rabbit	Cell Signallirg Technologies
P-HER-2 (y1248)	44-900	Mouse	Biosource
AKT	9272	Rabbit	Cell Signallirg Technologies
P-AKT (S473)	9271	Rabbit	Cell Signallirg Technologies
P-AKT (T308)	9275	Rabbit	Cell Signallirg Technologies
FOXO3a (H-144)	sc-11351	Rabbit	Santa Cruz Biotechnology, CA
P-FOXO3a (T32)	9464	Rabbit	Cell Signallirg Technologies
FOXO1 (C29H4)	2880	Rabbit	Cell Signallirg Technologies
FOXO4	834	Rabbit	Gift from Prof. BM Burgering
HDAC1	2062	Rabbit	Cell Signallirg Technologies
HDAC2	2540	Rabbit	Cell Signallirg Technologies
JAK2 (D2E12)	3230	Rabbit	Cell Signallirg Technologies
CXCR4	AB1847	Rabbit	Millipore
SP1 (IC6)	sc-420	Mouse	Santa Cruz Biotechnology, CA
p27 (C-19)	sc-528	Rabbit	Santa Cruz Biotechnology, CA
p21 (F-5)	sc-6246	Mouse	Santa Cruz Biotechnology, CA
H4K16	sc-8662	Goat	Santa Cruz Biotechnology, CA
H3K9	9671	Rabbit	Cell Signallirg Technologies
FGF7 (C-19)	sc-1365	Rabbit	Santa Cruz Biotechnology, CA
VEGF	ab46154	Rabbit	Abcam
FOXM1 (C-20)	sc-502	Rabbit	Santa Cruz Biotechnology, CA
Actin (I-19)	sc-1616	Goat	Santa Cruz Biotechnology, CA
Erα (HC-20)	sc-543	Rabbit	Santa Cruz Biotechnology, CA
p110a (H-201)	sc-528	Rabbit	Santa Cruz Biotechnology, CA
pRB	9313	Rabbit	Cell Signallirg Technologies
P-pRB (Ser807/811)	9308	Rabbit	Cell Signallirg Technologies
P-pRB (Ser795)	9301	Rabbit	Cell Signallirg Technologies
CDK2	ab236	Mouse	Abcam
CDK4 (Ab-2)	DCS32.1	Mouse	Abcam
Cyclin E1	sc-481	Rabbit	Santa Cruz Biotechnology, CA
Cyclin D1 (HD11)	sc-246	Mouse	Santa Cruz Biotechnology, CA
Cyclin B1 (H-433)	sc-752	Rabbit	Santa Cruz Biotechnology, CA
PLK (3F8)	sc-53751	Mouse	Santa Cruz Biotechnology, CA
GFP	sc-9996	Mouse	Santa Cruz Biotechnology, CA
Tubulin (H-235)	sc-9104	Rabbit	Santa Cruz Biotechnology, CA
Lamin B (C-20)	sc-6216	Goat	Santa Cruz Biotechnology, CA
APOBEC3G		Rabbit	Gift from Dr Naveenan Navaratnam, MRC-CSC Unit, London

Appendix 1. List of primary antibodies.

SDS-PAGE minigel

x1 gel

	resolving gel					stacking
	5%	7%	10%	12%	14%	5%
dH2O-ml	5.68	5.02	4.02	3.35	2.68	3.67
1.5M **Tris** pH 8.8	2.5	2.5	2.5	2.5	2.5	-
1.5M **Tris** pH 6.8	-	-	-	-	-	0.42
30% **Acrylamide**/ 0.8% **Bis** mix (37.5:1)	1.67	2.33	3.33	4.00	4.67	0.83
10% **SDS-µl**	100	100	100	100	100	50
25% **APS-µl**	40	40	40	40	40	20
TEMED-µl	10	10	10	10	10	10
total-ml	10	10	10	10	10	5

Resolving gel

0.375M Tris pH 8.8, acrylamide, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.1% (v/v) TEMED

Stacking gel

0.126M Tris pH 6.8, acrylamide, 0.1% (w/v) SDS, 0.1% (w/v) APS, TEMED

SDS-PAGE Electrophoresis Buffer (1x)

25mM Tris base, 250mM glycine, 0.1% (w/v) SDS

10x stock

Transfer Buffer (1x)

24mM Tris base, 193mM glycine, 20% (v/v) methanol

10x stock

Appendix 2. Gel and buffer recipes for SDS-PAGE.

TAE Buffer (1X)

40mM Tris·acetate, 1mM EDTA [50x stock (57.1ml glacial acetic acid in 1L)]

0.5 M EDTA, pH 8

Adjust pH to 8 with NaOH~20g

TE, pH 8

10mM Tris-HCl, pH 8, 1mM EDTA

TBS-Tween Buffer pH 7.5

40mM Tris-HCl, 300mM NaCl, 0.05% (v/v) Tween 20 (20x stock)

Buffer I

0.25% Triton X-100; 10mM EDTA; 0.5mM EGTA; 10mM HEPES pH 6.5

Buffer II

200mM NaCl; 10mM EDTA; 0.5mM EGTA; 10mM HEPES pH 6.5

Lysis Buffer (ChIP)

1% SDS; 10mM EDTA; 50mM Tris-HCl pH 8.1

TSE I

0.1% SDS; 1% Triton X-100; 2mM EDTA; 20mM Tris-HCl pH 8.1; 150mM NaCl

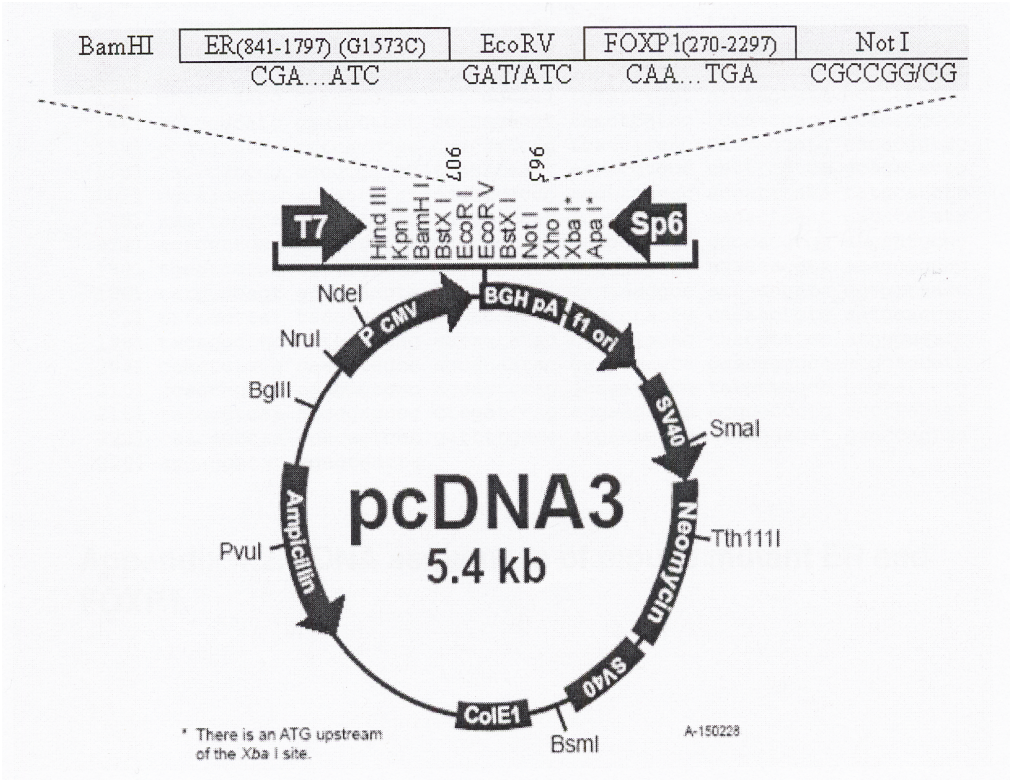
Scraping Buffer

100mM Tris-HCl pH 9.4; 0.1% SDS

Elution Buffer

0.1M $NaHCO_3$; 1% SDS

Appendix 3. Buffer recipes.



XhoI

FOXP1 Forward primer: CTCGAGGGCAAGAATCTGGGACTGAGAC

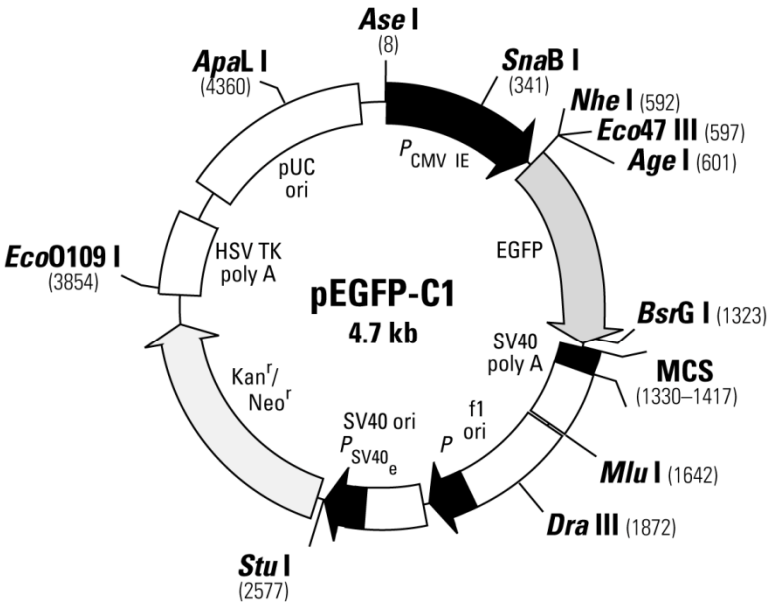
FOXP1 Reverse primer: GGATCCTCACTCCATGTCCTCGTTTAC

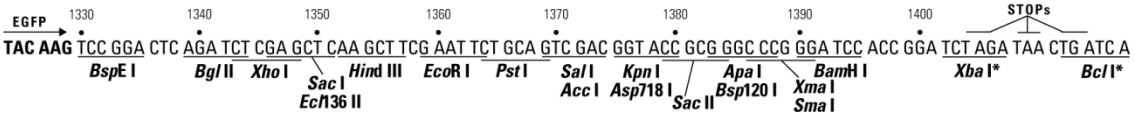
BamHI

Appendix 4. Map of the FOXP1 cDNA in pcDNA3 vector and primer sequence used for sub-cloning.

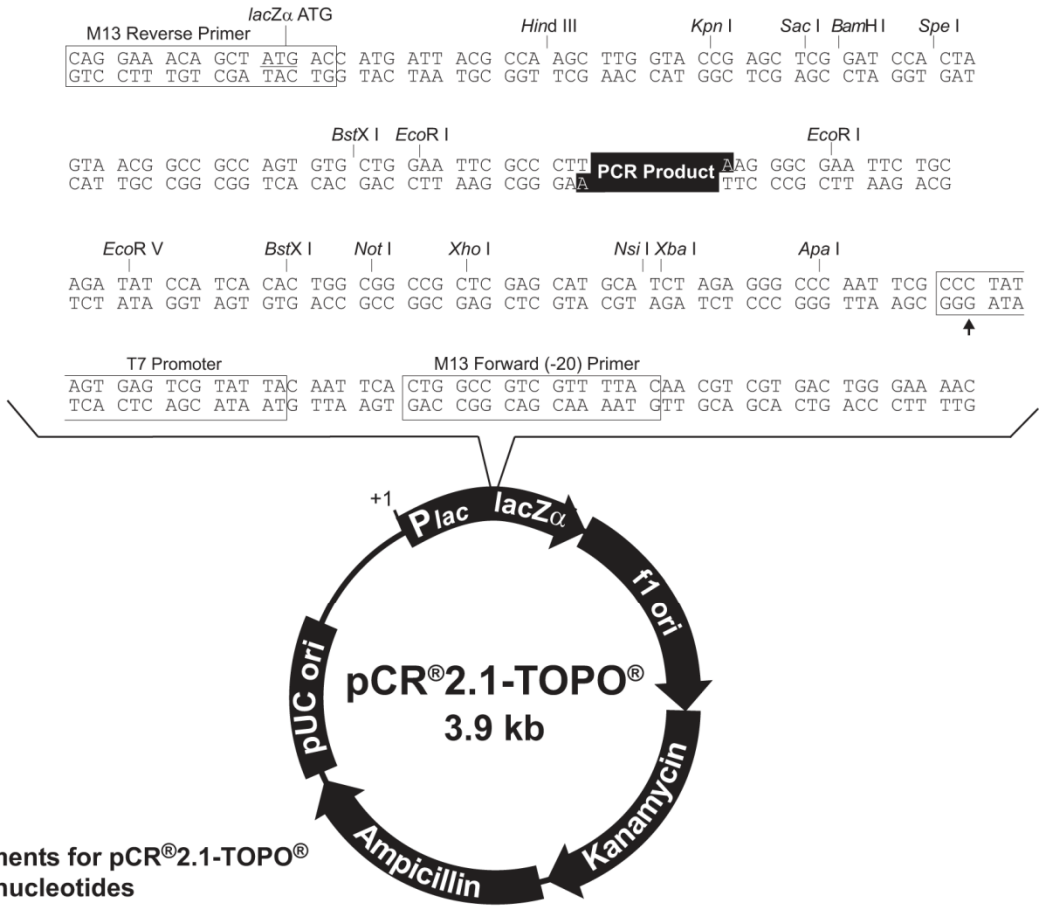
pEGFP-C1 Vector Information PT3028-5

GenBank Accession #: U55763 Catalog #6084-1





Appendix 5. pEGFP-C1 vector map.



Comments for pCR®2.1-TOPO®
3931 nucleotides

*Lac*Zα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Appendix 6. pCR2.1 TOPO vector.

VEGF promoter

aggctatgcc agctgtaggc cagaccctgg caagatctgg gtggataatc
agactgactg gcctcagagc cccaactttg ttccctgggg cagcctggaa atagccaggt
cagaaaccag ccaggaattt ttccaagctg cttcctatat gcaagaatgg gatgggggcc
tttgggagca cttagggaag atgtggagag ttggaggaaa agggggcttg gaggtaaggg
aggggactgg gggaaggata ggggagaagc tgtgagcctg gagaagtagc caagggatcc
tgagggaatg ggggagctga gacgaaaccc ccatttctat tcagaagatg agctatgagt
ctgggcttgg gctgatagaa gccttggccc ctggcctggt gggagctctg ggcagctggc
ctacagacgt tccttagtgc tggcgggtag gtttgaatca tcacgcaggc cctggcctcc
acccgccccc accagccccc tggcctcagt tccctggcaa catctggggt tgggggggca
gcaggaacaa gggcctctgt ctgcccagct gcctcccccc ttgggttttg ccagactcca
cagtgcatac gtgggctcca acaggtcctc ttccctccca gtcactgact aaccccggaa
ccacacagct tcccgttctc agctccacaa acttggtgcc aaattcttct cccctgggaa
gcatccctgg acacttccca aaggacccca gtcactccag cctgttggct gccgctcact
ttgatgtctg caggccagat gagggctcca gatggcacat tgtcagaggg acacactgtg
gcccctgtgc ccagccctgg gctctctgta catgaagcaa ctccagtccc aaatatgtag
ctgtttggga ggtcagaaat agggggtcca ggagcaaact ccccccaccc cctttccaaa
gcccattccc tctttagcca gagccggggt gtgcagacgg cagtcactag ggggcgctcg
gccaccacag ggaagctggg tgaatggagc gagcagcgtc ttcgagagtg aggacgtgtg
tgtctgtgtg ggtgagtgag tgtgtgcgtg tggggttgag ggtgttggag cggggagaag
gccaggggtc actccaggat tccaacagat ctgtgtgtcc ctctccccac ccgtccctgt
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ggccctgtcc gcacgtaacc tcactttcct gctccctcct cgccaatgcc ccgcgggcgc
gtgtctctgg acagagtttc cgggggcgga tgggtaattt tcaggctgtg aaccttggtg
ggggtcgagc ttccccttca ttgcggcggg ctgcgggcca ggcttcactg ggcgtccgca
gagcccgggc ccgagccgcg tgtggagggg ctgaggctcg cctgtccccg ccccccgggg
cgggccgggg gcggggtccc ggcggggcgg agccatgcgc cccccccttt ttttttaaa
agtcggctgg tagcggggag gatcgcggag gcttggggca gccgggtagc tcggaggtcg
tggcgctggg ggctagcacc agcgctctgt cgggaggcgc agcggttagg tggaccggtc
agcggactca ccggccaggg cgctcggtgc tggaatttga tattcattga tccgggtttt
atccctcttc ttttttctta aacattttt tttaaaactg tattgttctt cgttttaatt **FHRE2**
tatttttgct tgccattccc cacttgaatc gggccgacgg cttggggaga ttgctctact
tccccaaatc actgtggatt ttggaaacca gcagatgcct agactgactg gcctcagagc **FHRE1**
cccaactttg ttccctgggg cagcctggaa atagccaggt acacttccca aaggacccca

FHRE1: 5'-TTGCTCTACTTCCCCGGGTCACTGTGGATTTTGGGGGCCAGCAGA-3'

FHRE2: 5'-ATCCCTCTTCTTTTTTCTTGGGCATTTTTTTTTTAAAACTGTATTGT-3'

Appendix 7. VEGF promoter sequence.

Histological types	No. of Cases (% of 116 cases)
Pure ductal cell carcinoma in situ (pure DCIS)	10 (8.62%)
Invasive ductal carcinoma	90 (77.58%)
Invasive lobular carcinoma	2 (1.72%)
Pure tubular or cribriform	1 (0.86%)
Tubular mixed	5 (4.31%)
Pure mucinous carcinoma	2 (1.72%)
Medullary or atypical medullary	1 (0.86%)
Others*	5 (4.31%)
Total	116 (100%)

Variables	No. of cases (% in 116 cases)
PR Status	
Positive	42 (36.21%)
Negative	52 (44.83%)
N.A.	22 (18.97%)
ER Status	
Positive	58 (50%)
Negative	35 (30.17%)
N.A.	23 (19.83%)
Tumour Grade	
I	10 (8.62)
II	27 (23.28%)
III	56 (48.28%)
N.A.	23 (19.83%)
Tumour Stage	
I	29 (25%)
II	64 (55.17%)
III & IV	17 (14.66%)
N.A.	6 (5.17%)

Appendix 8. Histological types of tumour samples and characteristics of the 116 patients with breast cancer used in tissue microarray (TMA) study.

Crosstab				
Count		VEGF		
		0	1	Total
cyto_FOXO3a	0	16	19	35
	1	12	69	81
	Total	28	88	116

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square		1	.000		
Continuity Correction[h]	11.111	1	.001		
Likelihood Ratio	11.999	1	.001		
Fisher's Exact Test				.001	.001
Linear-by-Linear Association	12.633	1	.000		
N of Valid Cases	116				
a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 8.45. b. Computed only for a 2x2 table					

Crosstab				
Count		FOXM1		
		0	1	Total
cyto_FOXO3a	0	25	10	35
	1	37	44	81
	Total	62	54	116

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square		1	.011		
Continuity Correction[b]	5.519	1	.019		
Likelihood Ratio	6.695	1	.010		
Fisher's Exact Test				.015	.009
Linear-by-Linear Association	6.467	1	.011		
N of Valid Cases	116				
a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 16.29. b. Computed only for a 2x2 table					

Appendix 9. Analysis of correlations between cytoplasmic FOXO3a and VEGF or FOXM1 staining (using SPSS programme).

Crosstab				
Count		VEGF		
		0	1	Total
Nuclear FOXO	0	19	72	91
	1	9	16	25
	Total	28	88	116

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square		1	.118		
Continuity Correction[b]	1.693	1	.193		
Likelihood Ratio	2.299	1	.129		
Fisher's Exact Test				.185	.099
Linear-by-Linear Association	2.428	1	.119		
N of Valid Cases	116				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 6.03.
b. Computed only for a 2x2 table

Crosstab				
Count		FOXM1		
		0	1	Total
Nuclear FOXO	0	48	43	91
	1	14	11	25
	Total	62	54	116

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square		1	.773		
Continuity Correction[b]	.004	1	.950		
Likelihood Ratio	.084	1	.773		
Fisher's Exact Test				.824	.476
Linear-by-Linear Association	.083	1	.774		
N of Valid Cases	116				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.64.
b. Computed only for a 2x2 table

Appendix 10. Analysis of correlations between nuclear FOXO3a and VEGF or FOXM1 staining (using SPSS programme).

Crosstab				
Count		VEGF		
		0	1	Total
FOXM1	0	37	25	62
	1	20	34	54
	Total	57	59	116

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square		1	.015		
Continuity Correction[b]	5.048	1	.025		
Likelihood Ratio	5.974	1	.015		
Fisher's Exact Test				.017	.012
Linear-by-Linear Association	5.868	1	.015		
N of Valid Cases	116				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 26.53.
b. Computed only for a 2x2 table

Appendix 11. Analysis of correlations between VEGF and FOXM1 staining (using SPSS programme).

Count		Nuclear Foxo3a		
		0	1	Total
Nuclear FoxP1	0	24	17	41
	1	48	11	59
Total		72	28	100

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.248	1	.012		
Continuity Correctionb	5.168	1	.023		
Likelihood Ratio	6.193	1	.013		
Fisher's Exact Test				.023	.012
Linear-by-Linear Association	6.186	1	.013		
N of Valid Cases	100				

Count		Cytoplasmic Foxo3a		
		0	1	Total
Cytoplasmic FoxP1	0	13	24	37
	1	17	45	62
Total		30	69	99

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.653	1	.419		
Continuity Correctionb	.339	1	.560		
Likelihood Ratio	.647	1	.421		
Fisher's Exact Test				.499	.279
Linear-by-Linear Association	.647	1	.421		
N of Valid Cases	99				

Appendix 12. The correlation between nuclear FOXO3a and FOXP1 and cytoplasmic FOXO3a and FOXP1 was assessed by using SPSS program.