

**FORKHEAD TRANSCRIPTION FACTORS
IN THE REGULATION OF VEGF IN
BREAST CANCER**

Thesis submitted by

Christina Theano Karadedou

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Imperial College of London

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Imperial College University, London

Department of Oncology

Division of SORA

Hammersmith Hospital

Du Cane Road

London W12 0NN

ABSTRACT

High levels of the major angiogenic factor VEGF, have been reported in a number of cancer cell lines and in clinical specimens derived from breast. The forkhead transcription factors important for the regulation of many different physiological processes have been implicated in VEGF regulation in breast cancer. In this study, we have shown the interplay between FOXO3a and FOXM1 in breast cancer, with FOXO3a acting as a direct transcriptional repressor of VEGF. The mode of action of FOXO3a on the promoter of VEGF is dictated by events such as the competition with the VEGF transcriptional activator FOXM1, and the subsequent recruitment of a FOXO3a/HDAC2 complex on the exact binding site. This action results in the repression of VEGF transcription and the decrease of VEGF expression and cell migration. Mutating the putative forkhead responsive element affects promoter activity, and silencing FOXO3a results in up-regulation of VEGF expression. Apart overexpression of FOXO3a also results in the repression of FOXM1 expression, by its direct binding to the *FOXM1* promoter. This event is also involved, indirectly, in the regulation of VEGF repression. Apart from FOXO3a and FOXM1, two other forkhead transcription factors that are implicated in breast cancer, FOXA1 and FOXC2, are also involved in the regulation of VEGF. FOXA1, a good prognosis factor in breast cancer, seems to inhibit the expression of FOXC2, a poor prognosis factor. FOXA1 is directly recruited on its binding site of the FOXC2 promoter, affecting its transcription and conferring a significantly low expression. Silencing FOXA1 results in high FOXC2 protein levels. The mode of action of these two factors between them

affects the expression of VEGF. These findings provide information on the cross-talk between different forkhead transcription factors and a crucial factor of tumour migration, invasion, angiogenesis and metastasis.

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DECLARATION

This thesis is the result of my own work, unless stated.

PUBLICATIONS

Jie Chen^{1,2}, Ana R. Gomes², Lara J. Monteiro², San Yu Wong¹, Lai Han Wu¹, Ting-Ting Ng², Christina T. Karadedou², Julie Millour², Ying-Chi Ip¹, Yuen Nei Cheung¹, Andrew Sunters^{2,3}, Kelvin Y. K. Chan¹, Eric W.-F. Lam^{2*}, Ui-Soon Khoo^{1*}

Constitutively Nuclear FOXO3a Localization Predicts Poor Survival and Promotes Akt Phosphorylation in Breast Cancer

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The forkhead transcription factor FOXO3a increases phosphoinositide-3 kinase/Akt activity in drug-resistant leukemic cells through induction of PIK3CA expression.

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ABBREVIATIONS

ABBREVIATIONS USED IN MATERIALS AND METHODS

APS	Ammonium persulphate
BSA	Bovine Serum Albumin
ChIP	chromatin immunoprecipitation
Co-IP	co-immunoprecipitation
ddH₂O	double distilled water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DSS	Double Chacoal Stripped Foetal Calf Serum
ECL	Enhanced Chemi-Luminescence
EDTA	ethylenediamine tetra-acetic acid
FCS	Foetal calf serum
IP	Immunoprecipitation
HRP	Horseradish peroxidase
LB broth	Luria-Bertrani broth
NaF	sodium fluoride
Na₃VO₄	sodium orthovanadate
NS siRNA	Non-specific siRNA
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
P/S/G	Penicillin / Streptomycin / L-glutamine
RPM	Revolutions per minute
RT	Room temperature
RT-QPCR	Real Time Quantitative Polymerase Chain Reaction
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	polyoxyethylene-sorbitan monolaurate

ABBREVIATIONS OF UNITS OF MEASUREMENT

h	hour
μ	micro
M	Molar
min	minutes
n	nano
s	seconds
v/v	volume:volume ratio
w/v	weight:volume ratio

ABBREVIATIONS OF MOLECULES AND AGENTS

CDK	cyclin dependent kinase
CENPA	centromere protein A
CDKI	cyclin-dependent kinase inhibitor
E2	17 β-Estradiol
EGFR	epidermal growth factor receptor
ERα	oestrogen receptor α
ERβ	oestrogen receptor β
EREs	oestrogen Receptor Elements
ERK	extracellular signal-regulated kinase (MAPK)
FKHR	Forkhead Transcription Factor
FOX	Forkhead box
FOXM1	Forkhead Box M1
FOXA1	Forkhead Box A1
FOXO3a	Forkhead Box O 3a
FOXO1	Forkhead Box O 1
FOXO4	Forkhead Box O 4
FOXC	Forkhead Box C
FOXC1	Forkhead Box C1
FOXC2	Forkhead Box C2
HAT	histone acetyltransferase

HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HDACis	histone deacetylase inhibitors
HDLP	histone deacetylase- like protein
HER-2	Human epidermal growth factor receptor 2
Id1	Inhibitor of differentiation 1
JNK	c-Jun NH2-terminal kinase
NES	nuclear export signal
NLS	nuclear localisation signal
PDGF	platelet-derived growth factor
PI3-K	phosphatidylinositol 3-Kinase
PKB	protein Kinase B
PtdIns	phosphatidyl-Inositols
Rb	retinoblastoma protein
ROS	reactive oxygen species
SAHA	suberoylanilide hydroxamic acid
SD	standard deviation
SERMs	selective Estrogen Receptor Modulators
STAT3	signal transducer and activator of transcription 3
SUMO	small ubiquitin-like modifier
TSA	trichostatin A
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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1. INTRODUCTION

1.1 Cancer: An overview

There are historical clues exhibiting that cancer has been affecting people since their existence on the planet. The oldest observations of the disease have been found in Egyptian ancient texts and mummies. Specifically, researchers had found osteosarcoma evidence in the fossilised bones of mummies. (www.lilly.gr) Interestingly, the first description of cancer was found in an Egyptian text circa 1600 BC, where eight cases of tumours were observed in the breast of women. The method of combating the disease was cauterisation with a special “fire” instrument. The disease, however, was described more analytically by the Greek “father of medicine” Hippocrates. Hippocrates stated in his texts, the existence of tumours which grow internally or externally on patients and resembled, due to the metastatic finger-like nature of cancer, the shape of a crab.

Every year, around 3 million people are diagnosed with cancer in Europe and 1.7 million deaths are associated to its diagnosis. The most common type of cancer, with an incidence of 13.2% of all cancers, is lung cancer followed by colorectal (13%) and breast (12.8%) cancer (Fig 1.1). These types of cancer are as well associated to a high incidence of mortality (Boyle* and J. Ferlay, 2005).

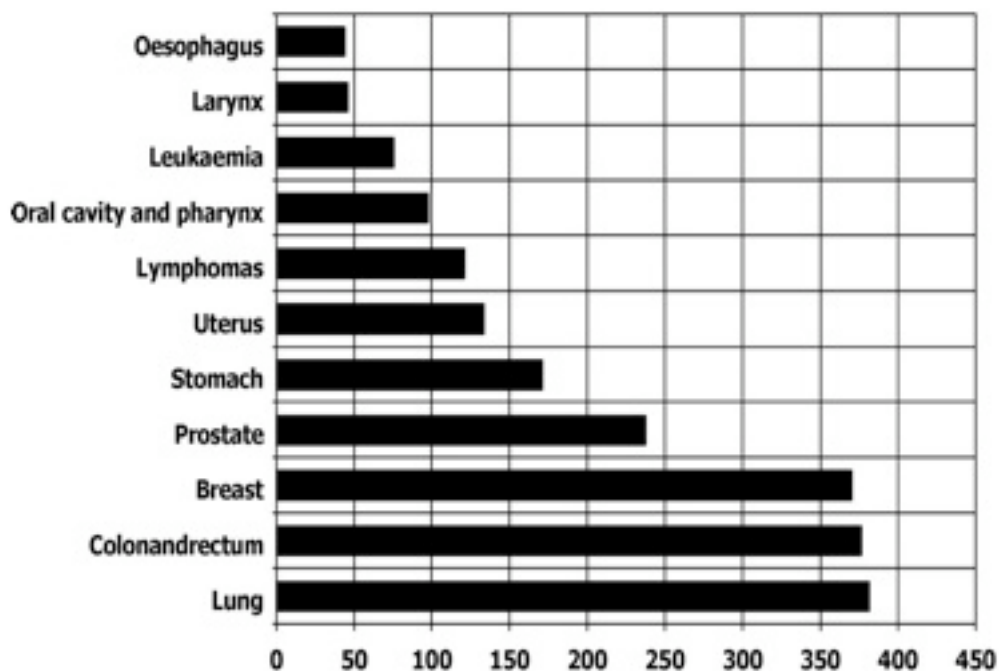


Figure 1.1. Cancer incidence (2004) in Europe (both sexes)

Every year more people are diagnosed with the disease within Europe. One major factor associated to the development of cancer is age and as westernised societies have an overgrowing ageing population, the increase of cancer patients is expected and inevitable. Most of the patients diagnosed with cancer are over 65 years old and only 1% of all cases occur in children.

Cancer accounts as a genetic disease and until now it has not been clearly defined. However it can be accepted as a disease, which is characterised by the pathological expansion of a tissue resulting in uncontrollable spreading and subsequently, death. Ongoing research in the field of cancer has revealed part of the genetics behind the development of the disease. Physical changes such as mutations in gene categories such as tumour-suppressor,

oncogenes and stability genes are nowadays considered to be mostly responsible for tumourigenesis.

Oncogenes are genes that when mutated or over-expressed contribute to converting a normal cell into a cancer cell. When mutated, these genes are constitutively active or active under conditions in which the wild-type gene is not. The activation of an oncogene can result from different reasons, varying from chromosomal translocations, gene amplifications and subtle intragenic mutations affecting crucial residues that regulate the activity of a gene product (Vogelstein and Kinzler, 2004).

Tumour suppressor genes are genes whose protein products inhibit cell division and facilitate survival. Usually, mutations in these genes reduce the activity of the gene product and when mutated, they behave as recessive, which means that as long as the cell contains one wild type allele, tumour suppression continues. This type of genes seems to have a contrary function to oncogenes.

The activation of oncogenes and the inactivation of tumour suppressor genes have similar physiological consequences: they drive the progression and increase in tumour cell numbers via the stimulation of cell proliferation, the inhibition of cell death and cell cycle arrest. This can be achieved through the activation of genes which drive the cell cycle, inhibition of the function of pro-apoptotic genes or by facilitating the provision of nutrients through enhanced angiogenesis (Vogelstein and Kinzler, 2004).

Apart from the two classes of genes mentioned above, there is another class of genes that drive tumourigenesis in a different mode. These genes are called stability genes and are required for the repair of subtle mistakes made during DNA synthesis or those that are induced through the exposure to mutagens. These genes include mismatch repair (MMR), nucleotide - excision repair (NER) and base - excision repair (BER) genes (Charames and Bapat, 2003; Slupphaug et al. 2003). Other stability genes control processes involving large proportions of chromosomes, such as those responsible for mitotic recombination and chromosomal segregation (e.g. BRCA1 and ATM). Stability genes minimise genetic changes and for this reason, their inactivation, leads to a higher rate of accumulation of mutations (Friedberg, 2003). Although the inactivation of the stability genes results in an increased vulnerability to genetic alterations in all genes, only mutations in oncogenes and tumour suppressor genes will confer a growth advantage to the cells. As with tumour suppressor genes, mutations in stability genes behave as recessive and both alleles need to be altered for the inactivation of a specific physiological effect (Vogelstein and Kinzler, 2004).

Nowadays, a big variety of oncogenes, tumour suppressor and stability genes have been identified to contribute in carcinogenesis when mutated. Changes in the expression pattern of these genes leads to subsequent changes in signalling pathways which regulate cell proliferation and cell death. Such signalling pathways are comprised of a complex network of molecules including oncogenic proteins, tumour suppressors, sex hormone receptors,

growth factors and other transcription factors which interact with each other in one or multiple pathways which cross-talk between them.

1.2 Breast Cancer

As mentioned above, breast cancer is one of the most common types of cancer. Within the European Union, every 2.5 minutes a woman is diagnosed with breast cancer. Every 7.5 minutes a woman dies from the disease. Like all cancers, it happens due to a genetic “disregulation” of a cell. Although only 5-10% of the patients developing breast cancer show a hereditary predisposition, the remaining patients develop the cancer due to a cell disregulation.

The risk of a woman developing breast cancer, as with most types of cancer, is increasing with age. The risk of a woman developing breast cancer until the age of 39 is 0.5%, between the ages 40-59 years old is 4% and over 60 years old it is increasing to 7%. Different risk factors of breast cancer have been accounted. Apart from familial predisposition, women whose menstrual cycle started early or late, women who have a late menopause, women who receive estrogens at least 5 years post-menopausaly, women who never gave birth, and finally obese and women who are smoking.

Men can also be affected by breast cancer, however the incidence is very low.

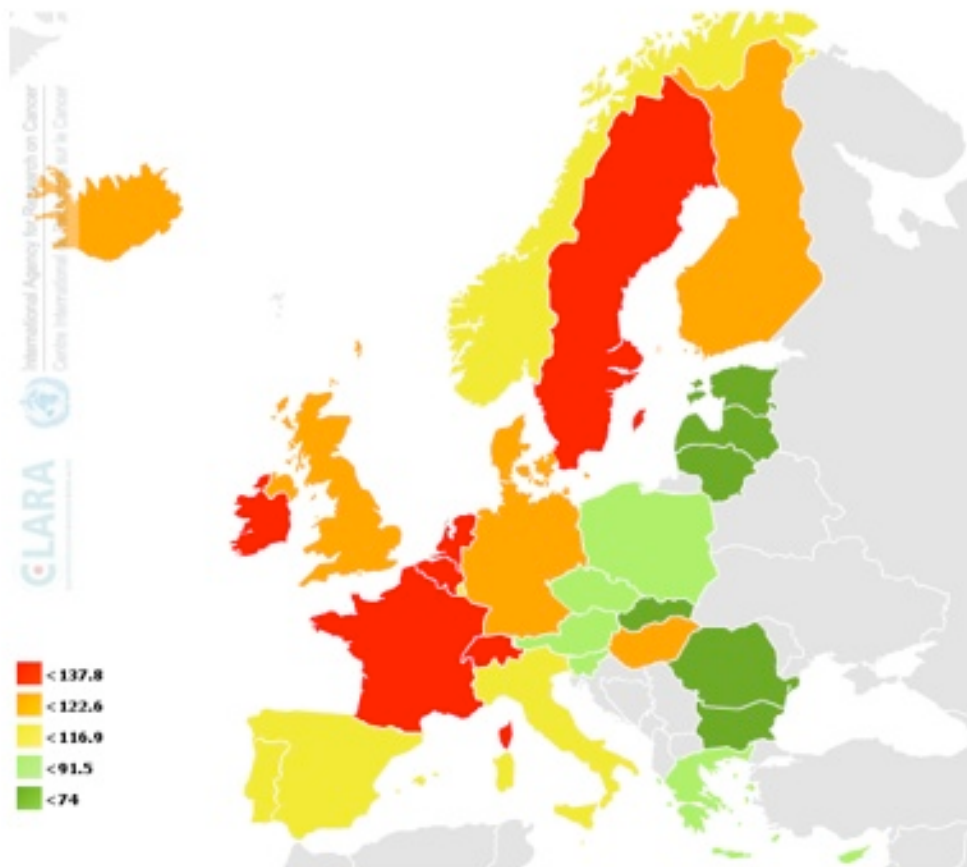


Figure 1.2 Estimated breast cancer incidence in Europe (2006)
(European cancer observatory website)

Although, the percentage of breast cancer patient mortality is still high, the fact of its decline is promising.

The most important regulators of breast cancer growth are the estrogens, the most abundant of which is 17β estradiol. Oestrogens are steroid hormones synthesised mainly by the ovary and the placenta. They act through the nuclear estrogen receptors $ER\alpha$ and $ER\beta$ which control gene expression by

recruiting transcriptional co-factors (Paruthiyil et al., 2004; Sommer and Fuqua, 2001). Although these receptors are very similar, they exert different functions. ER α has a proliferative role and changes in its expression and activity can cause deregulation of various cellular processes, eventually leading to cancer (Sommer and Fuqua, 2001). In contrast to ER α , ER β has anti-proliferative properties. Both receptors play distinct roles in breast development (Paruthiyil et al., 2004). ER α knockout mice have primitive mammary development (Couse and Korach, 1999), whereas ER β knockout mice develop normal mammary glands (Krege et al., 1998). Therefore, only ER α is required for growth and differentiation of the mouse mammary gland. Studies indicate that ER β may function as a tumour suppressor and its loss promotes breast carcinogenesis (Jarvinen et al., 2000; Roger et al., 2001; Skliris et al., 2001); however, the exact role of ER β remains unknown. Other than ER α and ER β , other regulators are involved as well in the growth of breast tumour cells. Several members of the epidermal growth factor (EGFR) family such as EGFR and HER2, have been shown to be over-expressed in breast carcinoma and are mediators of pathogenesis. Also, another growth factor that has been indicated to play a major role in breast cancer is the crucial regulator of physiological and pathological angiogenesis, VEGF-A. VEGF-A has been reported as a strong independent prognostic factor in both node-negative and node-positive breast carcinoma (Linderholm et al., 1998).

1.2.1 Breast Cancer treatment

Systemic treatment for breast cancer can be given after (adjuvant) or before (neoadjuvant) locoregional treatment. Adjuvant therapy has been shown to improve survival and reduce the risk of recurrent breast cancer and is now the standard of care in the treatment of early stage breast cancer. With neoadjuvant therapy, about 50-70% of the patients will have enough tumour regression that mastectomy will be avoided. The choice of adjuvant treatment depends on the risk of relapse, estrogen receptor status, patient performance status and acceptability of treatment. The current treatment for breast cancer involves multimodality therapy including surgery, chemotherapy, radiotherapy, endocrine treatment and molecular therapy (Smith and Chua, 2006).

In the last decades, the endocrine therapy most commonly used for the treatment of breast cancer is tamoxifen (Howell and Dowsett, 1997).

Tamoxifen is a member of the selective estrogen receptor modulators (SERMs), which depending on the type of cancer, may act either as agonists or antagonists (Ali and Coombes, 2002). Tamoxifen is an anti-estrogen that blocks the binding of estrogen to its receptor. ER α is therefore a well-established predictive marker of hormone sensitivity in breast cancer as only ER α -positive tumours can be treated with tamoxifen (Hayes, 2004). In contrast to normal breast cells, most breast cancer cells over-express ER α and are hormone-dependent and thus when oestrogen is removed, tumours shrink. Tamoxifen has antagonistic functions but also agonistic actions on the endometrium and bone. It also causes an increase risk of thromboembolism.

Another disadvantage of this treatment is that after a period of time, patients become resistant to tamoxifen. Tamoxifen is effective in both pre- and postmenopausal women in all age groups and has been shown to be more effective when given after chemotherapy rather than concurrently (Smith and Chua, 2006). Breast cancer patients who become resistant to tamoxifen are sometimes responsive to ICI182, 780 (faslodex), which is a pure anti-oestrogen. In contrast to tamoxifen, it has no agonistic activities as it degrades the ER protein and can therefore target ER dependent tumours that are resistant to endocrine therapy (Horwitz and McGuire, 1980). Research is now focused in understanding the mechanisms that underlie endocrine resistance and the discovery of novel agents for the treatment of breast cancer.

Various clinical trials have shown that aromatase inhibitors are also suitable for the treatment of breast cancer (Buzdar et al., 2007). These agents act by inhibiting oestrogen synthesis and include the non-steroidal anastrozole and letrozole and the steroidal compound exemestane. They are effective only in post-menopausal women and have been shown to improve disease free and metastatic free survival when compared to tamoxifen alone (ATAC trial) (Forbes et al., 2008). Nowadays, patients are switched to an aromatase inhibitor after two or three years of tamoxifen, as it has been shown that sequential treatment with tamoxifen followed by an aromatase inhibitor improves long term survival and reduces the risk of recurrent breast cancer (Dutta and Pant, 2007).

When chemotherapy is used as an adjuvant treatment, younger women tend to have the greatest benefit, particularly when they are lymph node positive and ER negative. The side effects usually include hair loss, fatigue, lethargy, oral mucositis, haematological toxicity and ovarian suppression with potential loss of fertility. The most common chemotherapeutic agents used currently are the anthracyclines doxorubicin and epirubicin. These drugs have been shown to be more effective than the traditionally used cyclophosphamide, methotrexate and fluorouracil (CMF) chemotherapy combination (Smith and Chua, 2006). Furthermore, evidence indicates that the addition of the newer class of chemotherapeutic agents, the taxanes, (paclitaxel and docetaxel) may improve survival in high-risk patients. Clinical trials are ongoing to establish the most effective chemotherapy combination for use in early stage breast cancer. Many of these drugs have already proven efficacy in women with metastatic breast cancer (Friedrichs et al., 2002).

Presence of growth factor receptors has been correlated with poor disease-free survival and resistance to endocrine therapy and chemotherapy and therefore inhibitors of growth factor activity have attracted increasing interest in the treatment of breast cancer (Spigel and Burstein, 2002). Anti-receptor antibodies and tyrosine kinase inhibitors are some of the strategies used to develop drugs to target the epidermal growth factor receptor (EGFR) and the related ErbB2/HER-2/neu. One of these inhibitors is the recently discovered Herceptin or trastuzumab (Slamon et al., 2001). Herceptin is a novel humanized monoclonal antibody that is directed against the external domain of the HER2 receptor. HER2 is over-expressed in 20% of breast cancers

(Stern, 2000). Results have shown that this inhibitor reduces the risk of recurrence by about 50% when given with or after chemotherapy for a year in women across all ages with early stage breast cancer (Engel and Kaklamani, 2007). Clinical trials have also shown that herceptin improves survival when given in combination with paclitaxel or docetaxel in patients with metastatic disease (Azambuja et al., 2008). However, there is an increased risk of cardiotoxicity when given with the anthracyclines; hence Herceptin is given after chemotherapy and not concurrently (Sengupta et al., 2008). Furthermore, because Herceptin only targets one protein, it is limited in the range of patients it can reach.

Recent studies reveal that the novel tyrosine kinase inhibitor Lapatinib (Fig 1.3) may also play a role in the treatment of breast cancer. It targets both the epidermal growth factor receptor (EGFR) and HER2 receptor and has recently been approved by the US Food and Drug Administration (FDA) (Bilancia et al., 2007). Lapatinib (GW572016F) prevents tumour growth by inhibiting intracellular tyrosine kinase activity and in contrast to other EGFR tyrosine kinase inhibitors, such as Erlotinib and Gefitinib, it binds the inactive form of the receptor and therefore dissociates at a slower rate from the receptor resulting in a greater duration of effect on the target site (Wood et al., 2004).

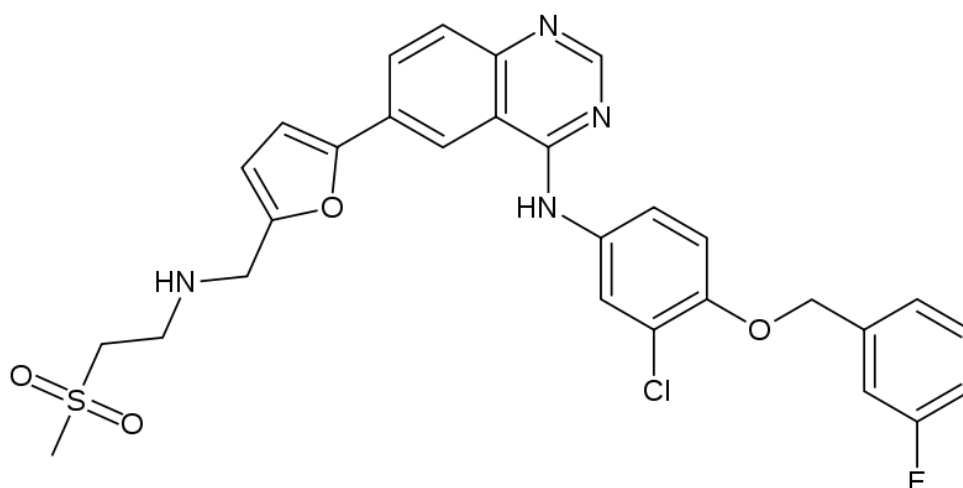


Figure 1.3. Molecular structure of Lapatinib (GW572016F)

(www.lookchem.com)

1.3 Tumour Angiogenesis and VEGF

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing ones. It is a normal process in growth and development, as well as in wound healing. However, this is also a fundamental step in the transition of tumours from a dormant state to a malignant state. Tumours induce blood vessel growth by secreting various growth factors allowing tumour expansion and subsequently the spread of tumour, or metastasis.

Angiogenesis plays a central role in both local tumour growth and distant metastasis in various types of cancer, including breast cancer. Growth factors, such as VEGF, bFGF and PDGF can induce capillary growth into the tumour

allowing its progression. VEGF expression has been reported in a number of cancer cell lines and in several clinical specimens derived from breast.

VEGF is a member of the platelet derived growth factor family of cystine-knot growth factors and its family in mammals consists of five glycoproteins referred to as VEGFA, VEGFB, VEGFC, VEGFD and placenta growth PGF (Ellis and Hicklin, 2008). The most studied and important VEGF variant is the 8 exon-containing VEGFA. VEGFA is expressed in various forms due to alternative splicing (Fig 1.4). The mature products are 121-, 165-, 183-, 189- and 206-amino acid proteins with VEGFA₁₆₅ being the one acting most strongly on endothelial cells and, together with VEGFA₁₂₁, being overexpressed in solid tumours leading to the formation of new capillaries (Ellis and Hicklin, 2008; Price et al, 2001). It is VEGFA, the one that is referred in most publications and in this thesis, commonly as VEGF.

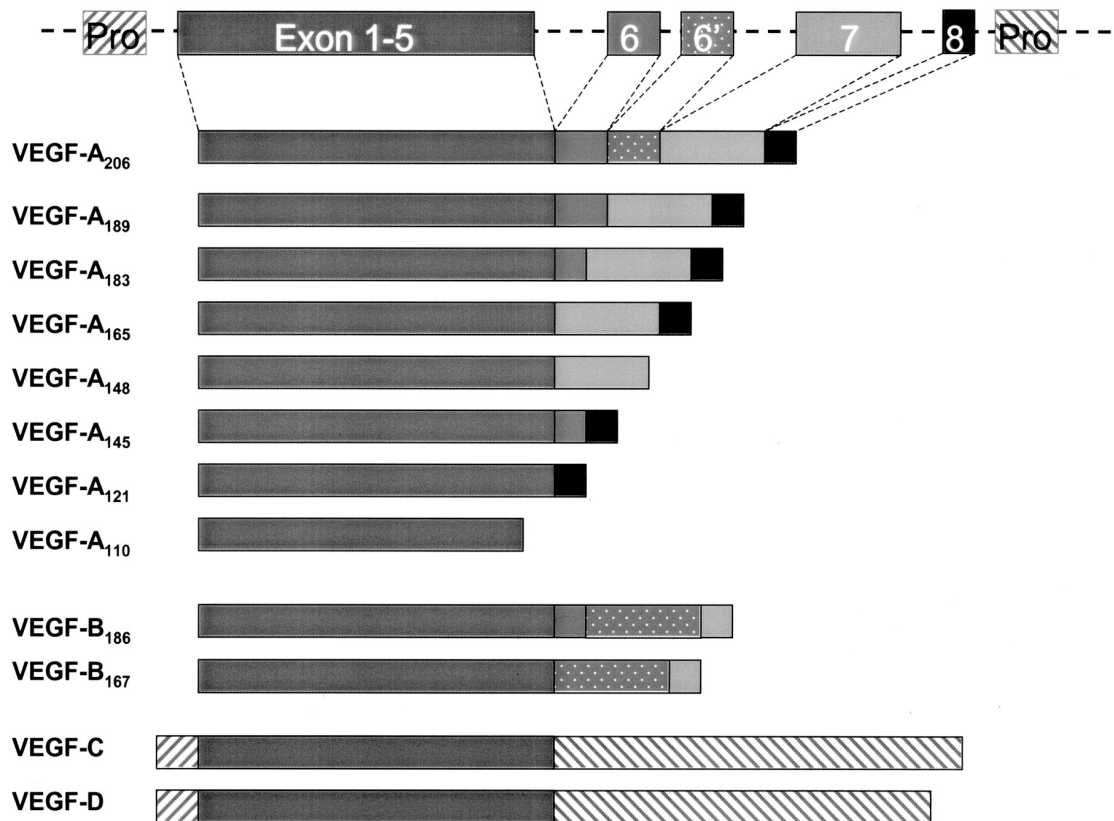


Figure 1.4. Alternative splicing variants of VEGF (Hoeben *et al.* 2004)

VEGF acts in a paracrine and an autocrine mode. When acting in a paracrine way, it is secreted by tumour cells exerting its effect in neighbouring or distant endothelial cells, whereas, when acting in an autocrine way, its action takes place in the cell which is expressed in (Lee *et al.*, 2007). VEGF acts by binding in three different receptors, VEGFR1 (also named FLT1), VEGFR2 (also known as KDR1 or FLK1) and neuropilin 1 (NRP1) (Ferrara *et al.* 2003). From these receptors, VEGFR2 is the one that VEGF mediates initially and predominantly its effects in endothelial cells, since its kinase activity is 10-fold higher than VEGFR1. However, it has been reported that VEGFR1 sustains

angiogenesis and mediates the survival of endothelial cells via VEGF stimulation of the PI3K/Akt signalling pathway (Cai et al, 2003).

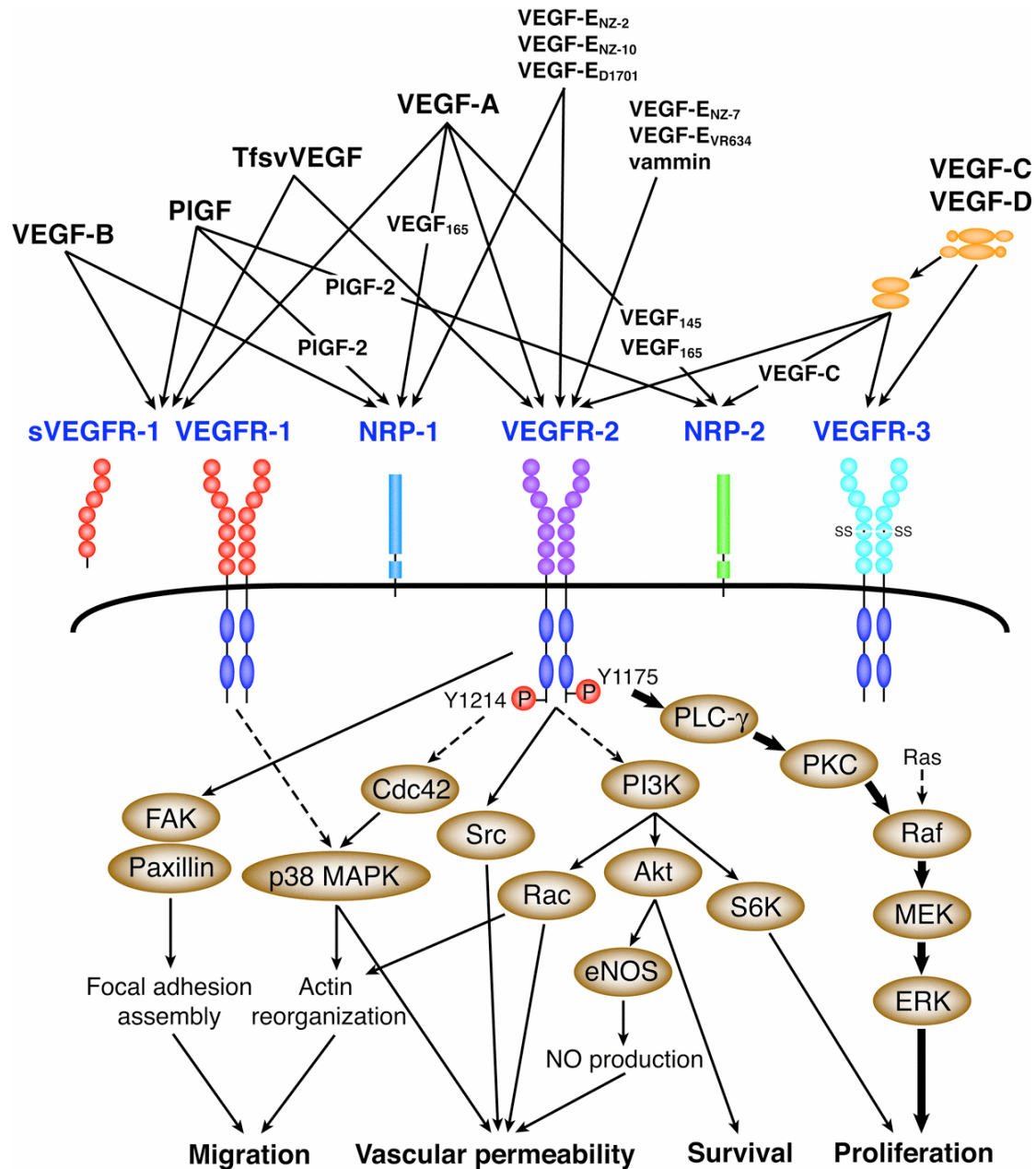


Figure 1.5 VEGF family members are associated to different receptors dictating various cellular functions via downstream signalling cascades.

Although it has been studied mainly in cells of the endothelium, VEGF affects many other cell types. Recently, it has been shown that is expressed in many cell types, including a variety of breast cancers (Schneider et al, 2005).

Overexpression of VEGF only, and not of the other known pro-angiogenic factors, has been reported to be associated with poor prognosis and poor relapse-free survival in breast cancer. VEGF has also been shown to be present in breast tumours at levels that are, on average, 7-fold higher than in normal adjacent tissue (Yoshiji et al. 1996). Moreover, although the clinical significance of it has not been clearly elucidated, VEGF is the only pro-angiogenic factor expressed throughout the breast tumour life cycle. In addition to the overexpression of VEGF in breast tumours with poor prognosis, VEGFR1 has been reported as a marker of aggressiveness in breast carcinoma, when acting in an autocrine manner (Lee et al, 2007).

Recent research has correlated several breast cancer markers with VEGF expression. Tumour markers such as estrogen/estrogen receptor (ER), HER-2 and BRCA-1 have been associated with VEGF expression in breast cancer.

Estrogen modulates VEGF expression at the transcriptional level in breast cancer cells, suggesting a relationship between ER and VEGF. Estrogen-dependent breast cancers show increased VEGF upon estrogen stimulation and furthermore VEGF levels have been associated with response to anti-estrogen therapy in adjuvant treatment (Buteau-Lozano et al., 2008; Linderholm et al., 2000). However, additional studies also show that ER-negative breast tumours have high VEGF expression and poor prognosis as well (Fückar et al., 2006). Additionally, another important breast cancer marker, HER-2, which is amplified in 20%-25% of breast cancers, has also been associated with VEGF upregulation. Patients presenting both HER-2 and

VEGF overexpression, have been reported to have the poorest outcome (Konecny et al., 2004).

VEGF overexpression is an early step in breast cancer progression, and frequently occurs before tumour invasion, for example in ductal carcinomas *in situ* (Guidi et al., 1997). In this context, it is very interesting to note that the role of VEGF in breast cancer seems not to be limited to angiogenesis, as breast cancer cells express both VEGF and its receptors (VEGFR). Thus breast tumour cells may be able to promote their own growth and avoid apoptosis through VEGF. The above hypothesis, suggests an autocrine signalling loop of VEGF in breast cancer cells. Recently, it was proposed that in addition to antiapoptotic effects, autocrine VEGF signalling may contribute to cell migration and progression. An understanding of the VEGF-dependent signalling pathways in breast cancer is important for the complete knowledge and the subsequent development of treatment therapies of breast cancer.

Antiangiogenic therapy is already being developed and explored with various clinical trials exhibiting positive outcome in metastatic breast cancer. The most mature, clinically, antiangiogenic agent is the recombinant, humanised monoclonal antibody against VEGF, named bevacizumab (GW786034B). Bevacizumab binds to all the isoforms of VEGFA and is normally administered in combination with other chemotherapy agents such as taxenes. Specifically the combination of bevacizumab with paclitaxel had given positive results in trials, as a first-line treatment for women with HER2-

negative metastatic disease (Schneider BP and Sledge GW, 2007). Other combinational studies with bevacizumab in breast carcinoma are still ongoing.

Other agents which inhibit VEGF, are studied as possible therapeutic agents of breast cancer. The most prominent are the small molecule receptor tyrosine kinase inhibitors (RTKIs) with specificity to VEGFRs. There are currently many RTKIs being developed and several have already been tested with most studied, sunitinib (SU11248) and sorafenib (BAY43-9006). Phase II trials on previously treated breast cancer patients with either sunitinib or sorafenib, showed promising results in metastatic breast cancer, however single-activity has limited results (Miller KD et al., 2005;). The combination of sorafenib or sunitinib with bevacizumab is regarded to show more efficient results in the ongoing research of breast cancer therapy.

A very interesting approach of antiangiogenic therapy in breast cancer is the combination of VEGF-targeting agents with biological agents such as erbB-targeting drugs. Both HER2 and EGFR appear to regulate VEGF.

Specifically, inhibiting HER2 or VEGF results in decreased VEGF expression (Konecny GE et al., 2004). These observations led to the attempts of combinational trials blocking simultaneously the VEGF and erbB pathways. Positive results were observed in HER2-positive metastatic breast cancer patients who were administered both bevacizumab and trastuzumab (Pegram MD et al., 2004).

Apart from bevacizumab in combination to trastuzumab, there have been studies with the sole use of tyrosine kinase inhibitors which inhibit the VEGF and ErbB pathways. These agents are pazopanib, that inhibits the VEGF tyrosine kinase receptors 1, 2 and 3 (VEGFR-1, -2, and -3), and lapatinib, that inhibits the HER1 and HER2 receptors, as mentioned earlier. Pazopanib and lapatinib, in combination, have shown efficacy in breast cancer patients and a hyperadditive proapoptotic effect in many different carcinoma cell lines, such as a non-small-cell lung carcinoma, a gastric cancer and a colon cancer cell line (Sloan and Scheinfeld, 2008). The antiproliferative and proapoptotic synergist effect of these two agents, was shown to be more potent than either of them alone. Pazopanib and lapatinib were shown to induce major changes in the transcriptome, specifically in proapoptotic mRNA species, and influence the activity of multiple kinases, apart from the ones which are already known to influence solely (Olaussen et al., 2009).

Still, additional anti-VEGF combinational studies are needed and are being developed for metastatic breast cancer. For these studies and the future treatment of the disease, it would be very crucial to identify biomarkers which would guide antiangiogenic therapy in order to establish the optimum dose and to determine which patients might benefit most from a given drug.

1.4. The PI3-K signalling pathway in cancer

The ERs and the phosphatidylinositol 3-kinase (PI3-K)/ protein kinase-B (PKB or Akt) signalling pathways are strongly implicated in the development of breast cancer. Deregulation of the PI3-K pathway has been demonstrated in many human breast cancers and has been linked to poor prognosis and endocrine therapy resistance. Recent evidence has shown that components of the PI3-K signalling cascade and ER α can be cross-regulated (Fry 2001).

PI3-K was first identified as a lipid kinase activity associated with the products of viral oncogenes and with activated protein-tyrosine kinases (Fry 1994) and was then implicated in several human cancers. Alterations in the PI3-K signalling cascades can lead to changes in a number of cell functions that contribute to the transformed phenotype, including cell growth and proliferation, differentiation, cell survival, adhesion and cell motility (Leevers et al. 1999).

There are three classes of PI3-Ks, class I, II and III. Class I PI3-K's are the most studied of them and have been implicated in tumourigenesis. PI3-K is a heterodimer kinase consisting of a p110 catalytic subunit and a p85 regulatory subunit (Sun et al. 2001) which phosphorylates phosphatidylinositols (PtdIns) on the 3' hydroxyl group and converts PtdIns 4,5P₂ to PtdIns 3,4,5P₃ (Leevers et al. 1999). PtdIns 3,4,5P₃ binds to and activates the

Pleckstrin Homology (PH) domain of PDKs which in turn phosphorylates and activates the PH domain of the Protein Kinase B (PKB), also known as Akt, at the Thr-308 and Ser-473 with high affinity, stimulating PKB to protect cells from undergoing apoptosis (Fry 2001).

The PKB (Akt) component of the PI3-K signalling pathway has been found to be a critical regulator of cell survival by phosphorylating a number of downstream proteins including the pro-apoptotic protein Bad, caspase-9 which is also involved in apoptosis, the forkhead transcription factors (FKHR), Raf and p21-activated protein kinase (Sun et al. 2001). Additionally, it has been shown to increase the activity of telomerase, something that constitutes replication unlimited with its major subsequent results in cell biology. Another important effect of the activation of the PI3-K signalling is the regulation of angiogenesis via endothelial nitric-oxide synthase (eNOS) activation, and its role in invasion and metastasis by the stimulation of matrix metalloproteinases (MMPs).

1.5 The Forkhead Transcription Factor family in cancer

1.5.1. Forkhead transcription factors

The Forkhead transcription factors are named after the first gene identified in *Drosophila Melanogaster* (Weigel et al., 1989) where the mutant embryo would show a two spiked-head structure instead of anterior and posterior gut. The 110-amino- acid DNA binding domain is highly conserved and over 100 forkhead genes have been identified from yeast to human. At least 46 members have been identified in the human forkhead box (FOX) gene super-family (Katoh and Katoh, 2004). A unified nomenclature of the genes was revised in 2000 and 18 subfamilies (FOXA to FOXS) are grouped based on structural similarities and conservation levels in the forkhead DNA-binding domain (<http://www.biology.pomona.edu/fox.html>) (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 and Katoh, 2004)

The most conserved forkhead domain with nuclear localization signals (NLS) is also known as 'winged helix' domain because of the 3-D structure of two loops (wings) at the carboxy-terminus, 3 α -helices at the amino-terminus and 3 β -sheets. The third α -helix, also referred to as the recognition helix, is the main DNA binding site, while the second wing influences binding stability and specificity (Wijchers et al., 2006). Therefore all forkhead transcription factors bind to DNA with the core consensus sequence RYMAAYA (R=A/G, Y=C/T,

M=A/C) (Carlsson and Mahlapuu, 2002; Overdier et al., 1994; Pierrou et al., 1994) , or TRTTTAY (YRKTTTRT) in reverse complement (Costa et al., 2001). Based on the presence or absence of a C-terminal basic region in the forkhead domain, the family is further grouped into two classes. While most of the FOX genes are in class 1 with a C-terminal basic region, FOXH, FOXM, FOXN, FOXO and FOXP are in class II (Katoh and Katoh, 2004).

However, several other domains/ motifs have been identified in some subfamilies: transactivation domains, phosphorylation motifs, acetylation motifs, forkhead associated domain (FHA), nuclear receptor interaction motifs (Wijchers et al., 2006). For example, phosphorylation motifs have been shown to allow post-translational modification in FOXO and FOXA2 by Akt, I κ B β or JNK (c-jun N-terminal kinase), resulting in translocation or ubiquitination. Acetylation motifs in the FOXO forkhead domain allow acetylation of lysines by CBP/p300 to reduce transcriptional activity. Nuclear receptor interaction motifs LXXLL are located at the C-terminus of FOXO, FOXM1, FOXK1 and FOXP and mediate the interaction between forkhead transcription factors and nuclear receptors, including the androgen receptor, glucocorticoid receptor, and retinoic acid receptor (Van Der Heide et al., 2004). However, forkheads lacking this motif, such as FOXA and FOXH1, can interact with the androgen receptors too (Chen et al., 2005).

Functionally, forkhead proteins are involved in very diverse biological processes, including cell cycle regulation, survival, metabolism, immunoregulation and embryonic development. Therefore, deregulation of the

activity of various forkhead transcription factors can lead to congenital disorders or carcinogenesis (Lehmann et al., 2003; Wijchers et al., 2006).

1.5.2. FOXO transcription factors

The human FOXO family consists of FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX) and FOXO6, which are the mammalian homologues of daf-16 in *Caenorhabditis elegans*.

The mRNA of FOXO1, FOXO3a, and FOXO4 is ubiquitously expressed in all tissues but in different degrees (Greer and Brunet, 2005). Of the three, FOXO3a has the broadest expression pattern in embryo and adult tissues (Furuyama et al., 2000), and FOXO4 is more tissue specific (Biggs et al., 2001).

The physiological diverse functions of FOXO genes are best demonstrated in knock-out mice (Engstrom et al., 2003). Foxo1 null embryos die on embryonic day 10.5 because of defective angiogenesis (Furuyama et al., 2004). But Foxo3a and Foxo4 null mice are viable and grossly normal. Foxo3a ^{-/-} females are infertile because of premature ovarian failure (Hosaka et al., 2004). In addition, they have defects in glucose uptake, over-proliferation of helper T-cells with multi-organ infiltrative lymphoproliferative disease (Lin et al., 2004), and increased apoptosis of neutrophils (Jonsson et al., 2005). These confirm the involvement of FOXO3a and FOXO4 genes in

development after organogenesis.

FOXO transcription factors bind to a promoter with the consensus sequence (termed DBE for DNA binding element), TT(G/A)TTTAC (Furuyama et al., 2000). An associated element, CTTATCA, is also identified in the promoter region of target genes, implying a presence of cooperation partner. A large number of gene promoters contain the DBE (Xuan and Zhang, 2005) which can explain its diversity in function. Once bound to the DNA, FOXO factors can act as transcriptional activators or repressors. FOXO transcription factors inhibit cell proliferation by regulating cell cycle or apoptosis.

The ability of FOXO factors to induce cell cycle arrest, DNA repair and apoptosis makes them good tumour suppressor candidates. Theoretically, loss of FOXO function can lead to tumour development because of increased proliferation, decreased DNA repair and thus increased genomic instability. In addition, its interactions with both tumour suppressors and oncogenes increase its involvement in carcinogenesis.

There have been observations that the presence of cytoplasmic FOXO3a in breast cancer correlates with poor survival (Hu et al., 2004). Furthermore, cytoplasmic FOXO3a also correlates well with the expression of p-Akt or I κ K β , which are correlated with tumour development and drug resistance. At the same time, over-expression of active FOXO factors reduces tumourigenicity with regards to tumour onset, size and progression in both nude mice transplanted with HER2 over-expressing cells (Yang et al., 2005) or deficient

for PTEN (Yang et al., 2005)). The FOXO-induced tumour suppression is obviously an attractive strategy to treat cancers with hyperactive PI3K/ Akt pathway.

The forkhead box (FOX) transcription factors are an extensive super family of transcription factors, consisting of more than 50 mammalian proteins that share homology in a 100 amino acid monomeric winged helix DNA binding domain (Kaestner et al. 2000). Recent work has identified as targets of PKB (also called Akt) the three members of the forkhead box, group O subfamily forkhead transcription factors FOXO1, FOXO3a and FOXO4. In the presence of growth factors, PI3-K phosphorylates PKB, which in turn phosphorylates and inactivates FOXO proteins.

1.5.3. FOXM1 transcription factor

FoxM1 (HFH-11B, Trident, Win or MPP2) is part of the mammalian Forkhead box transcription factor family is one of the most important cell cycle regulators at both the G₁/S and G₂/M transitions. FOXM1 is localized mainly in the cytoplasm in late G1 to S phases and is translocated in the nucleus during the G2/M phase. At this stage, FOXM1 is phosphorylated by ERK1/2 (Ma et al. 2005).

The consensus binding site of FOXM1 is TAAACA, like a typical forkhead factor binding site on DNA.

In development FOXM1's disruption has been shown to result in postnatal death and histological analysis of the FOXM1 null embryos (day 10) reveal defects in the myocardium. More specifically, the orientation of the myocytes is irregular and their nuclei are polyploid with a very high DNA content. This suggests that FOXM1 is required for normal DNA replication (S phase) and chromosomal segregation (M phase) within the cell cycle (Korver et al. 1998; Laoukili et al. 2005)

Cell cycle is controlled by activation of several cyclin-dependent kinases (CDKs). CDKs are activated when their catalytic subunit is dephosphorylated by the Cdc25-A, -B and -C phosphatases. CDK activity is negatively regulated by CDK inhibitors p21^{Cip1}, p27^{Kip1} and p16^{INK4A}. FOXM1 regulates the G1/S and G2/M transitions, mentioned above, by decreasing the nuclear levels of CDK inhibitors whilst activating Cdc25A and Cdc25B transcription (Wang et al. 2002). FOXM1, additionally, plays a role in mitosis and cytokinesis by transcriptionally regulating cyclin B1, CENP-F, Aurora B and Polo-like kinase 1 (PLK1) (Fig. 1.5) (Costa. 2005)

In cancer, FOXM1's regulation of cell cycle progression suggests that in the absence of FOXM1 there is less likelihood of tumour cells to proliferate and generate large tumours. Moreover, FOXM1, has been shown to transcriptionally regulate the expression of VEGF and promote growth and metastasis in glioma cells (Zhang et al. 2008)

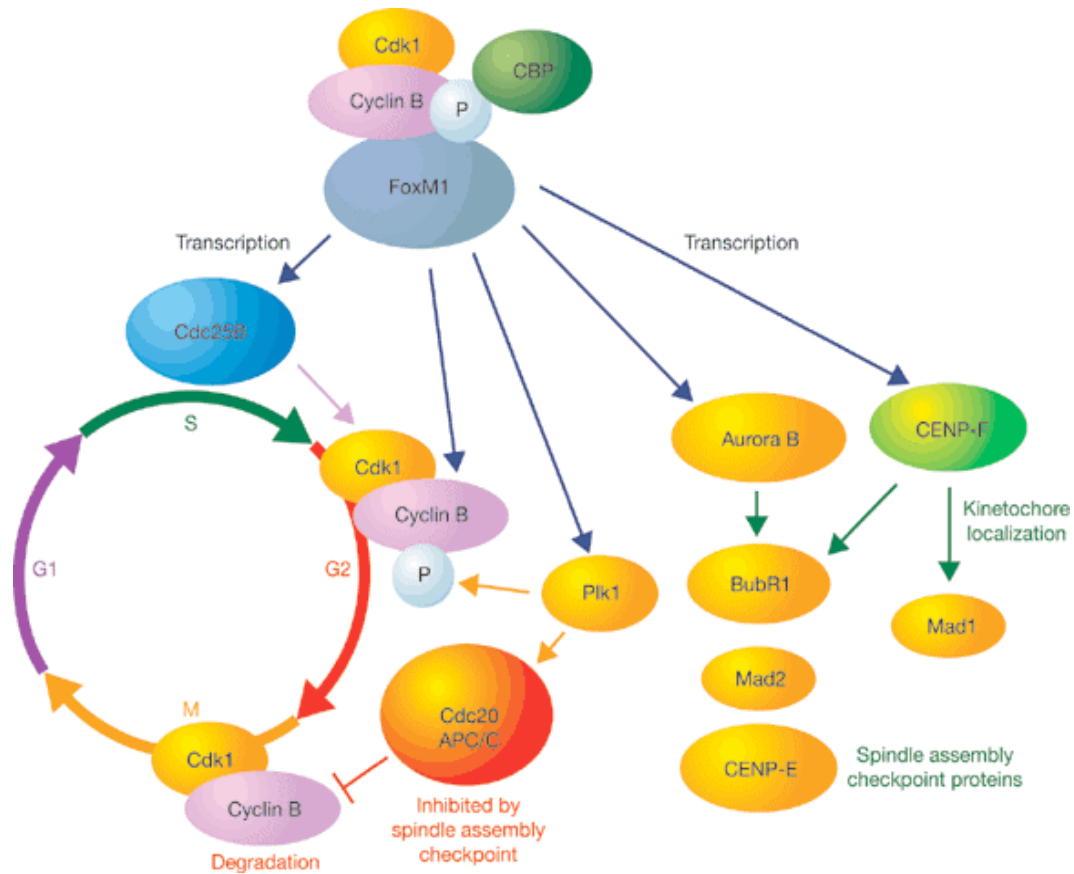


Figure 1.6 FOXM1 activity requires CDKs, its components and downstream targets, for G1/S and G2/M transition. (adapted from Costa, 2005)

1.5.4. FOXA1 transcription factor

FOXA1 (Hepatocyte Nuclear Factor 3 α) is a member of the forkhead transcription factors and plays a role in the differentiation and the development of liver, kidney, lung, prostate and breast (Friedman and Kaestner, 2006).

FOXA1 is observed in breast tumours, being co-expressed with ER α in luminal breast tumours. FOXA1 acts as a good prognosis factor in breast cancer patients with ER α positive tumours as it is associated to sensitivity in endocrine therapy (Badve et al. 2007). FoxA1 has been shown to assist the recruitment of ER α to several *cis*-regulatory elements in the genome followed by transcriptional activation in breast cancer cells (Carroll et al. 2005). FOXA1 is associated to chromatin remodeling in breast cancer cells. The forkhead domain of FOXA1 resembles the globular domain of the linker histone H1 and as a result it replaces the linker histone to de-compact chromatin, in order to promote gene expression. FOXA1 helps the recruitment of ER α on a “loosened” chromatin environment and induces chromatin modifications in p27. Interestingly, it has been shown that FOXA1 is required for optimum expression of around 50% of ER α regulated genes and estrogen-induced proliferation (Thorat MA et al., 2008). Co-expression of GATA3, FOXA1 and ER α in breast cancer has been shown to dictate the phenotype of hormone-dependent breast cancer. FOXA1 is over-expressed in ER-positive breast

cancer cell lines and has been reported as a good prognosis factor in breast cancer.

1.5.5. FOXC transcription factors

The FOXC members of the forkhead transcription factor family, are FOXC1 (Mf1) and FOXC2 (Mfh1, FKHL14). These two forkhead transcription factors, which share the typical forkhead domain in their structure, have been found to play important roles in vascular endothelial and lymphatic development.

FOXC1 and FOXC2 are expressed in the vasculature during development and their importance in development is depicted by the fact that FOXC1/FOXC2 null embryos die during embryonic development with defects in the vascular remodeling of primitive blood vessels and abnormal vascular connections between arteries and veins and arteriovenous malformations in general together with defects in the early sprouting and formation of lymphatic vessels (Kume, 2008; Hosaka et al., 2004; Seo et al., 2006).

FOXC1 plays a role in normal ocular development with null mouse embryos displaying a phenotype of open eyelids, failure of anterior chamber formation, iris hypoplasia, attachment of the lens to the cornea, absence of the corneal endothelium and disorganised corneal stroma. Apart from the ocular development, FOXC1 plays a role in the skeletal and cardiovascular development (Seo et al., 2006).

FOXC2 has been found to play a major role in arteriovenous development, as null mice show a widely defective morphology of blood vessels, FOXC2 as well is implicated in lymphatic development as mice and humans heterozygous for FOXC2/*Foxc2* have lymphedema (Fang et al. 2000). FOXC2 is an important regulator of the cardiovascular development as it has been observed that disruption of its expression in mice causes aortic arch patterning defects (Hayashi et al. 2008). Apart from development, FOXC2 has been shown to act as an activator of epithelial cell differentiation and metastasis in breast cancer (Mani et al. 2007) and as a regulator of angiogenesis via induction of integrin $\beta 3$ (Itgb3) and the subsequent Itgb3-mediated endothelial cell adhesion and migration (Hayashi et al. 2008). Moreover, FOXC2 has been found to activate the epithelial to mesenchymal transition (EMT) of cells which is associated to metastasis of tumours (Hader et al. 2009) triggered by a number of factors, such as TGF- $\beta 1$ and several EMT-inducing transcription factors, such as Snail, Twist, and Goosecoid.

As mentioned above FOXC2 plays a role in metastasis in breast cancer. In this field of research it has been shown that FOXC2 is required for murine mammary cancer cells to metastasize to the lung. Additionally, FOXC2 is correlated with the highly metastatic and aggressive basal-like human breast cancer type (Mani et al. 2007). Concluding, FOXC2 is taken as a marker of aggressiveness and metastasis in tumour development and is associated to poor clinical outcomes.

1.6 Implication of the Forkhead Transcription factors in angiogenesis

Some FOXOs may potentially regulate angiogenesis in human tumours and have been implicated in the regulation of VEGF. VEGF has been shown to activate a number of signalling pathways such as PKC, MAPK/ERK and the PI3-K/Akt pathway. VEGF has been implicated in the regulation of transcription factors such as NF- κ B, Egr-1, NFAT-1, Ets-1 and Stat-3/5. More recently, it was reported that VEGF induces the phosphorylation and inactivation of the forkhead transcription factors via a PI3-K/PKB-dependent mechanism.

Additionally in development, several members of the forkhead transcription factor family take part in the genetic cascade that leads to endothelial differentiation. In the endothelium at least two subclasses of FOXs genes (FOXOs and FOXCs, as mentioned before) have been found to play a relevant role in vasculogenesis and angiogenesis (Dejana et al. 2007).

Genetic inactivation of FOXO1 induces an embryonically lethal vascular phenotype with severe under-development of branchial arteries and dorsal aorta. It has been suggested that part of the vascular defects in the FOXO1 null embryos may be due to a defective arterialisation response to VEGF. Furthermore, the triple conditional inactivation of FOXO1, FOXO3a and

FOXO4 in adult mice leads to large haemangiomata. In addition, FOXO3a null mice, which are viable, present significantly increased neovascularisation. Another significant observation, is that the Akt-1 (PKB α) knock-outs present strongly increased angiogenesis. Moreover, in vivo, FOXO3a deficiency upregulates the angiogenesis related gene eNOS which is essential for postnatal neovascularisation (Potente M et al. 2005). The above interesting observations, may suggest an endothelial cell tumour suppressor role of the FOXO subclass of the forkhead transcription factors.

As mentioned before, FOXM1, has also been implicated in angiogenesis. FOXM1 is an important cell cycle regulator and has been proposed to mediate cellular transformation into cancer cells. In regard to angiogenesis, it has been shown that the downregulation of FOXM1 leads to the inhibition of angiogenesis, migration and invasion in glioma cells (Wang et al. 2007).

1.7 HDACs in breast cancer

Genetic as well as epigenetic events play a key role in carcinogenesis and cancer progression. Epigenetic is a term used to describe changes in gene expression that are not due to changes in the DNA sequence and are inheritable from one generation to the next (Bird, 2002). Unlike genetic changes, epigenetic alterations are reversible. Some of the epigenetic events

include: acetylation, methylation, ubiquitination, phosphorylation, sumoylation and glycosylation.

Acetylation is a chemical modification involving the transfer of an acetyl group from the acetyl co-enzyme to the ϵ -amino group of lysines in target proteins (Lin et al. 2006). Acetylation can occur in histones as well as other proteins and can have many effects. Histone acetylation affects histone structure by destabilizing nucleosomes leading to their dissociation or by inhibiting their stacking into the solenoid structure. Therefore acetylation leads to a less compact structure that allows access of transcription factors and other molecules such as RNA polymerase to the DNA and thus stimulate transcription of genes (Cress and Seto, 2000). Acetylation of a protein affects protein stability as acetylation and ubiquitination occur on lysine residues and so there are cross-talks between these two modifications (Caron et al., 2005). The stability of many known proteins, such as p53 and p73, has been shown to increase after lysine acetylation. The same lysines in these proteins are subject to ubiquitination and acetylation, therefore when acetylation occurs ubiquitination is prevented and stability of these proteins is increased (Caron et al., 2005). In other cases acetylation is needed to stimulate degradation, such as in the case of the Hypoxia-induced factor 1 (Hif-1), where its degradation occurs after binding to pVHL which then mediates its ubiquitination. Acetylation of one of the subunits of Hif-1, Hif-1 α , enhances its binding to pVHL and therefore its degradation (Lee et al. 2004). More complex and indirect processes connecting ubiquitination and acetylation other than just through competition have also been reported (Caron et al., 2005). In

addition, acetylation plays a role in protein-protein interactions and localization. For example, acetylation of the transcription factor signal transducer and activator of transcription 3 (STAT3) induces its dimerization and translocation into the nucleus (Minucci and Pelicci, 2006).

Genomic DNA is wrapped around 8 histones to form nucleosomes, which is the fundamental unit of chromatin. Chromatin contains five types of histones: H1, H2A, H2B, H3, and H4. The nucleosome consists of an octamer of four core histones i.e. two H2A/H2B dimers and an H3/H4 tetramer, surrounded by 146bp of DNA (Yoo and Jones, 2006). H1 histone lies on the outer portion of the nucleosome and occurs in chromatin in about half the amount of the other types of histones. There is also the H5 histone that has similar functions to H1 (Marks et al., 2000). The amino-terminal and the carboxyl-terminal tails of histones protrude out of the nucleosomes and are subject to post-translational modifications that regulate gene expression. Studies on the impact of these modifications in cellular processes are fast growing areas of research. Such a modification is the acetylation of histones by histone acetyltransferases (HATs) and deacetylation by histone deacetylases (HDACs). Acetylation is correlated with an open chromatin structure, allowing transcription factors to access the promoters of target genes, therefore activating transcription (Nelson et al., 2007). Acetylation is involved in the regulation of DNA replication, repair and recombination (Masumoto et al., 2005; Yang and Seto, 2007). Disordered HAT or HDAC activity has been linked to cancer. The fact that acetylation plays a very important role in the

regulation of gene expression has led to the extensive study of HDACs in relation to cancer (Vigushin and Coombes, 2002).

HATs acetylate histones leading to an open chromatin structure where transcription factors can easily reach the DNA and as a result transcriptional activity is induced. There are four families of HATs. The prototype family related to the yeast GCN5 includes human GCN5 and P/CAF (p300/CREB binding protein associating factor). The second family includes CREB binding protein (CBP) and p300 which mainly function as co-activators for many transcription factors. The third family includes the TAF250 protein which is part of the TFIID transcription factor complex that binds the TATA box and finally the fourth family, including SRC-1 and ACTR, function as co-activators for ligand-activated nuclear receptors. Other splice variants or related proteins exist that have HAT activity (Kouzarides, 1999).

HDACs deacetylate histones leading to a very compact chromatin structure where transcription factors and other regulatory complexes cannot access the DNA, thereby gene transcription is repressed. HDACs usually interact as constituents of large protein complexes that downregulate genes through association with co-repressors (NcoR, SMRT), transcription factors (ER, p53, Mad/Max), cell cycle specific regulators (Rb, E2F), but they can also bind their target directly (Cress and Seto, 2000). For example, a decade ago the non-histone tumour suppressor p53 protein was shown to be acetylated by the recruitment of coactivators/HATs, a process very crucial for p53 function and activation of transcription of target genes ; Barlev et al., 2001). Another

example is the thyroid hormone receptor that activates transcription, upon binding of a ligand, by associating with the p300/PCAF histone acetyltransferases (Cress and Seto 2000). The proteins NCoR (Horlein et al., 1995), SMRT (Chen and Evans, 1995), Sin3 and histone deacetylases (Alland et al., 1997) are part of this complex which is usually referred to as the Sin3 complex. Many proteins interact with HDACs directly or through other yet unidentified proteins (Brehm et al., 1998; Luo et al., 1998).

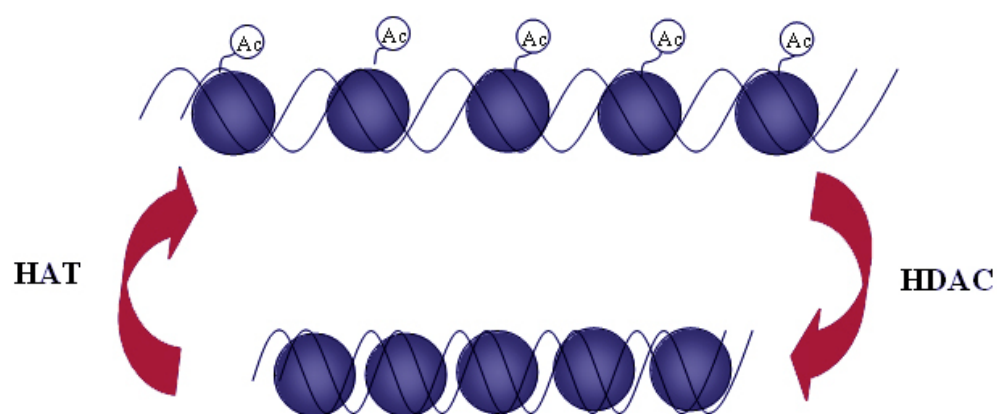


Figure 1.7 HDAC action. Chromatin structure and remodeling by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation of the N-terminal tails of histones leads to a loosening of the histone-DNA structure, allowing access of the transcriptional machinery (above) whereas deacetylation leads to tightening of the histone-DNA complex (below), repressing transcription.

Dependent on sequence similarity and cofactor dependency, HDACs are grouped into two families: the classical HDAC family and the Sirtuin family of NAD⁺-dependent HDACs. Members of the classical HDAC family are divided in three classes. Class I HDACs include HDAC1, 2, 3, 8 and resemble the

RPD3 transcriptional regulator in yeast, class II include 4, 5, 6, 7, 9, 10 HDACs and resemble the yeast HDA1 deacetylase and class IV includes HDAC11. Classes I, II, IV have a similar sequence and are dependent on Zinc for their deacetylase activity. Class I is expressed in most cell types, while class II expression is more restricted. For this reason class II HDACs are thought to be involved in developmental processes and differentiation (de Ruijter et al., 2003). The newly discovered HDAC 11 has conserved regions in the catalytic core region shared by both class I and II HDACs (Gao et al., 2002). Most HDACs contain a nuclear localisation signal (NLS) that targets them to the nucleus where most their substrates locate. Other HDACs can be cytosolic depending on other regulatory domains (de Ruijter et al., 2003). For example HDAC3 has both a NLS and a nuclear export signal (NES) suggesting that it can be also located in the cytoplasm (Yang et al., 2002). In most studies, HDAC3 is localized in the nucleus but this can be cell type specific. Class II HDACs shuttle between nucleus and cytoplasm depending on the signals they receive, while HDAC1 and HDAC2 are always in the nucleus as they lack a NES (Bertos et al., 2001).

Functional HDACs are never found as single polypeptides but, with the exception of HDAC8, they form parts of multiprotein complexes along with other chromatin modifying enzymes or specific co-regulators. Both class I and class II HDACs play roles as transcriptional co-repressors by forming complexes with other DNA binding proteins and target the promoters of genes (Kumar et al., 2005). Although class I and II HDACs have a very similar catalytic site, they have differences in the entrance region of the active site

and the outer charge transfer relay system (Hildmann et al. 2006) Many class II HDACs are subject to posttranslational modifications such as sumoylation. HDAC 7 has been shown to act as a mitochondrial protein playing a role in apoptosis, as it translocates from the mitochondrial membrane to the cytoplasm in response to apoptotic signals (Bakin and Young. 2004). Class II HDACs are able to bind directly and inactivate some transcription factors leading to their transcriptional repression.

The class III HDAC family called the sirtuin family includes seven members SIRT1-7. There are no sequence similarities amongst members and they require NAD^+ as the cofactor important for their enzymatic activity (Nelson et al. 2007).

It was recently shown that the mutational status of HDACs is very important in future pharmacogenetic treatment of cancer patients. Frameshift mutations in HDAC2 causing its loss of expression were seen in sporadic carcinomas with microsatellite instability and in cancer patients with hereditary non polyposis colorectal cancer syndrome. This loss of HDAC2 expression leads to tumours being less sensitive to the anti-cancer effects of HDAC inhibitors (Ropero et al., 2006).

HDAC inhibitors (HDACi) have been proposed as new therapeutic agents in cancer, as they are important modulators of gene transcriptional activity. HDAC inhibitors have been shown to inhibit proliferation, cause cell cycle

arrest, promote differentiation, induce mitotic cell death, senescence and reactive oxygen species-induced cell death (Johnstone et al., 2002)

HDACis are classified into four categories: hydroxamic acids, short-chain fatty acids, benzamide derivatives, and cyclic peptides. The very potent hydroxamic acids include compounds such as Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA) and LAQ-824. They inhibit Class I and II HDACs (Lin et al., 2006). Crystallographic studies have shown that HDACs have a tubular catalytic site containing a zinc atom at its base and the hydroxamic moiety of these inhibitors bind this zinc and fit into this structure (Marks et al., 2000). Short-chain fatty acids are well tolerated by patients and clinical trials are carried out to evaluate them. Their disadvantage is that they have a short plasma half-life due to the rapid rate of metabolism and their non-specific mode of action, as well as the high doses needed for therapeutic effects. Agents like this are Valproate, Butyrate and Phenylbutyrate.. Finally, cyclic peptides, like depsipeptide and apicidin A, are the most structurally complex class of HDACis.

1.8 Objectives

Angiogenesis, although a physiological process in human development, is associated to metastasis and tumour invasiveness in breast cancer. VEGF which is playing a fundamental role in angiogenesis, has been implicated and has been shown to be part of the signaling cascades dictating breast cancer. In these pathways, the family of the forkhead transcription factors are crucial and major players of the transcriptional regulation of several genes associated to breast cancer. The correlation of these factors in the subsequent transcriptional and translational regulation of VEGF could give an insight into the underlying mechanisms governing breast tumourigenesis and potentially help in the therapy of breast cancer.

2. MATERIALS AND METHODS

2.1. Data bank analyses

The NCBI, USCS and ensembl databases and blast software, were used for DNA and protein sequence analyses.

2.2 Mammalian cell culture

Cell Media

The human breast cancer cell lines, MCF7, MDA-MB-231, SKBR3 and BT474 were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% foetal calf serum (FCS) (Globepharm Ltd.), 2mM L-glutamine and 100U/ml of penicillin/streptomycin (Sigma). The MDA-MB-231FOXO3a(A3):ERa cells were maintained in DMEM without phenol red (GIBCO-BRL) supplemented with 10% Double Charcoal Stripped Foetal Calf Serum (DSS) (Globepharm Ltd.). The MCF7 FOXA1 cells were selected and maintained in the presence of Geneticin (Gibco) . MCF7 FOXC2 cells were selected and maintained in the presence of 500 µg/ml Zeocyn (Invitrogen, UK).

MDA-MB-FOXO3a:ERa cells were cultured in DMEM without phenol red supplemented with 5% DSS prior to ELISA and 1% DSS prior to the wound

healing assay. Cells treated with E2 (Sigma) were cultured in DMEM phenol red free whereas cells treated with 4-OHT (Sigma) and ICI (Sigma) were cultured in the DMEM with phenol red.

2.3. Buffers for whole cell extracts from mammalian cell lines.

HSB	400 mM KCl, 20 mM Tris. HCl pH 7.5, 2 mM dithiothreitol (DTT), 1mM EDTA, 20% (v/v) glycerol, 1 tablet of protease inhibitor cocktail (Roche)
PBS pH 7.5	138 mM NaCl, 20.4 mM Na ₂ HPO ₄ , 2.7 mM KCl, 1.47 mM KH ₂ PO ₄

Table 2.1. Buffers used for the whole cell extracts of mammalian cells.

2.4. Protein concentration assay.

The Bio-Rad *DC* protein assay was used to assay the protein concentrations of the samples. 20 µl of Reagent S were added to 1 ml of Reagent A to prepare the working reagent. 2 µl of the lysate were added to 100 µl of the above working reagent, in eppendorf tubes. Finally, 800 µl of Reagent B were added in the same tubes. The eppendorf content for each lysate, was added in disposable polystyrene cuvettes with 1 cm path length. The colour change was measured using a spectrophotometer at a wavelength of 750 nm (Lowry

method), the samples were zeroed against the sample with a control that did not contain any lysate.

2.5 Sodium Dodecyl Sulphate- PolyAcrylamide Gel Electrophoresis (SDS-PAGE).

The protein gels were 7 % (w/v) acrylamide gels. First the running gel was poured to marked line (70%-80% the way up of the small glass plate) and 2ml. of H₂O (or Isopropanol) layered dropwise to give a levelled surface. The gel was left to polymerise for at least 30 minutes. Then, the water was removed, the stacking gel was poured and the combs were inserted. After 5 minutes the gel was topped up. Before assembling the gel tank, the wells were washed using a syringe containing running buffer. 25 µg of protein samples plus an equal volume of 2X Laemmli; were loaded on the gel and were run for about one hour and a half at 100 Volts until the front of the loading buffer reached the end of the gel. In the first well of the gel, 2.5 µl of rainbow ladder (Amersham) were loaded, in order to be able to estimate the molecular weight of the bands. The electrophoresis was performed in 1X running buffer.

7 % Running gel	
ddH ₂ O	3.7 ml
Acrylamide	2.3 ml
1 M. Tris HCl pH 8.8	3.7 ml
10 % SDS	100 µl
25 % Ammonium Persulphate (APS)	33 µl
TEMED (Sigma)	13 µl

5 % Stacking gel	
ddH ₂ O	3.5 ml
Acrylamide	825 µl
1 M. Tris HCl pH 6.8	625 µl
10 % SDS	50 µl
25 % APS	20 µl
TEMED (Sigma)	15 µl

Buffer	Contents
2X Laemmli buffer	50 mM Tris pH 6.8, 15 % glycerol, 2 % SDS, 1mg/ml bromophenol blue dye, 1mM DTT
Running buffer	25 mM Tris Base, 0.25 M glycine, 3.5 mM SDS

Table 2.2. Buffers used for SDS-PAGE.

2.6. Western blotting.

Transfer of the proteins.

The proteins were then transferred on to PROTRAN nitrocellulose transfer membrane for western blotting (Schleicher & Schuell). The transfer took place in 1X transfer buffer for at least 2 hours at 0.6 A.

Then the transfer was checked for equal loading and transfer efficiency by Ponceau-S (Sigma) staining and washed with water and then with Tris Buffered Saline-Tween (TBS-T). The membranes were then blocked in 5 % (w/v) dry milk in TBS-T for about 30 minutes.

Buffer	Contents
1X Transfer buffer	24 mM Tris Base, 0.25 M NaCl
TBS-T	20mM Tris, 150 mM NaCl, 1 % Tween (Sigma)

Table 2.3. Recipe for the preparation of protein transfer buffers.

Probing the membranes.

All membranes were incubated with primary antibodies used at various dilutions in 5 % BSA plus 0.005 % NaAzide, overnight at 4° C.

Primary Antibody	Dilution	Company
FOXM1 (c-20) rabbit polyclonal IgG	1:1000	Santa Cruz Biotechnology
FOXO3a /FKHRL1 (H144) rabbit polyclonal IgG	1:1000	Santa Cruz Biotechnology
ERα (F-10) mouse monoclonal IgG	1:1000	Santa Cruz Biotechnology
FOXA1 (ab23738) rabbit polyclonal IgG	1:1000	Abcam

FOXC1 (ab5079) goat polyclonal IgG	1:1000	Abcam
FOXC2 (F1054) rabbit polyclonal IgG	1:1000	Sigma
β-tubulin (H-235) rabbit polyclonal	1:1000	Santa Cruz Biotechnology
HDAC2 (2540) rabbit polyclonal	1:1000	Cell Signalling
VEGF (Ab46154) rabbit polyclonal	1:1000	Abcam

Table 2.4. List of primary antibodies used in western blotting.

The membranes were then washed with TBS-T for about 60 minutes with changes every 10 minutes. Afterwards, the appropriate secondary antibodies (anti-mouse, anti-rabbit and anti-goat) were added on the membranes at 1:1000 dilution in 5 % (w/v) milk in TBS-T for about 30 minutes. The membranes were washed again with TBS-T for about 60 minutes with changes every 10 minutes.

Developing the membranes.

As soon as the membranes were washed, they were placed onto cling film. Then 2 ml of Enhanced Chemi-luminescence reagent (ECL) (Amersham) were poured over each membrane and left for 1 minute. The ECL reagent was removed and the membranes covered with cling film to avoid the membrane drying out. Finally, the membranes were exposed to ECL hyperfilm (Amersham) to visualise the proteins.

2.7 Tissue culture:

Maintenance of mammalian cell lines for the production of cell extracts for Real Time Quantitative Polymerase Chain Reaction (RTQ-PCR).

The cells were maintained and treated with 4-OHT in the same way stated above and were trypsinised with 1 X trypsin in EDTA (Sigma) and centrifuged at 1200rpm for 5 minutes. The pellet formed was resuspended in 1 ml PBS. The lysate was added in eppendorf tubes and was centrifuged at 5000 rpm for 5 minutes at 4 C. The pellet formed in the tubes was frozen at -80° C.

2.8 RNA extraction and determination.

Total RNA was prepared by harvesting cells and extracted by guanidine isothiocyanate lysis followed by silica gel membrane purification (RNeasy kit, Qiagen Ltd., West Sussex, U.K.). Cells were disrupted by adding buffer RLT (Qiagen) containing 10% β -mercaptoethanol. Samples were homogenized, and 1 volume 70% ethanol was added. 700 μ l of the sample was added to an RNeasy mini column placed in a 2ml collection tube and centrifuged at 10,000rpm for 15s in a microcentrifuge. Flow-through was discarded and 700 μ l of buffer RW1 (Qiagen) was added followed by centrifugation for 15sec at 10,000rpm. Flow-through was again discarded. RNeasy column was transferred to a new collection tube and 500 μ l RPE (Qiagen) buffer added. Centrifugation step was repeated again. Another 500 μ l RPE was added followed by centrifugation at 10,000rpm for 2min. To elute, the RNeasy column was transferred to a new collection tube, 50 μ l RNase free water was added and centrifuged at 10,000rpm for 1 min. The concentration and purity of RNA were determined by measuring the spectrophotometric absorption at 260 nm to 280 nm.

2.9 cDNA synthesis and Real-time Quantitative PCR (RTqPCR)

Total RNA from samples was extracted using the RNeasy kit as explained above. RNA was then converted to cDNA using the Superscript III First-strand synthesis system for RT-PCR (Invitrogen LT). Real time PCR was performed with ABI PRISM 7700 Sequence Detection System using 2x SYBR Green Mastermix (Applied Biosystems) according to manufacturer's instructions. One minute annealing at 60°C was used for 40 cycles. Gene-specific primer pairs were designed using the ABI Primer Express software. Primers were designed so that they produce a fragment of 50-150bp.

Analysis was performed using the relative standard curve method. For the standard curve 1-2µg cDNA from each sample was mixed and diluted 1:2 and then 1:4 for four serial dilutions. As a control, primers for the L19 ribosomal housekeeping protein were used to normalize input RNA. The samples were diluted 1 in 4 and 2µl from each was used per reaction. Reaction mix also contained 22µl of SYBR Master Mix and the appropriate amount of primers which have been previously optimized, to a final volume of 25 µl. Efficiency of PCR was obtained from slope of standard curve ($\text{Eff} = 10^{(-1/\text{slope})} - 1$). 90-110% was expected with optimal primers with slope between -3.1 and -3.6.

Primer optimization

Each primer set was optimized with 10ng cDNA standard to determine the minimum concentration required for maximum yield and minimum non-specific amplification. Dilutions of 50-900nM forward and reverse primers were tested with different combination (Table 2.5). The concentration that gave the lowest Ct value with standard DNA and highest with water was used in further experiments.

	Forward Primer		
Reverse primer	50nM	300nM	900nM
50nM	50/50	300/50	900/50
300nM	50/300	300/300	900/300
900nM	50/900	300/900	900/900

Table 2.5. Different dilutions of primers used in the primer optimisation prior to RTqPCR.

Primer	Sequence
VEGF (Operon)	Forward 5'- GAAGTGGTGAAG TTCATGGATGTC - 3' Reverse 5'- CGATCG TTCTGTATCAGTCTTTCC - 3'
L19 (Invitrogen)	Forward 5'- GCGGAAGGGTACAGCCAAT -3' Reverse 5'- GCAGCCGGCGCAAA -3'
FOXO3a (Invitrogen)	Forward 5' TCTACCAGTGGATGGATGGTGC GTT Reverse 5' CGACTATGCCAGTGACAGGTTGTG
FOXM1 (Invitrogen)	Forward 5'TGCAGCTAGGGATGTGAATCTTC 3' Reverse 5' GGAGCCCAGTCCATCAGAACT 3'
FOXA1 (Operon)	Forward 5' TCTCTTGCTACCAGCATGGCT 3' Reverse 5' CCCGTCCTAAACACTTCCTAGCT 3'
HDAC2 (Invitrogen)	Forward 5' TTGGAGGAGGTGGCTACACAA 3' Reverse 5' ACAATCAAGGGCAACTGCAGT 3'

Table 2.6. List of primers used in RTqPCR

2.10 Transformation of bacteria.

500 ml of LB agar were prepared and autoclaved. When the medium was cooled to 50 ° C, 100 µg/ml of ampicillin antibiotic were added. In sterile conditions maintained by a flame, about 20 ml of the medium were poured into 90 mm plates. The plates were stored at 4 ° C.

1 µl of plasmid construct DNA was added to 50 µl of bacteria in one eppendorf tube. After incubating the tube in ice for 30 minutes, the bacteria were heat shocked in 42° C for 1 minute and then transferred in ice. The samples were then stored for 60 minutes at 37° C. The content of the tubes was spread on the agar plates which were stored overnight at 37° C.

2.11 Plasmid Maxipreparation.

For the plasmid maxiprep, the HiSpeed Plasmid Maxi Kit (QIAGEN) was used. 250ml of LB containing 100µg/ml ampicillin or kanamycin was inoculated with 1 ml of an 8 hour culture of bacteria. Cultures were incubated at 37°C overnight with vigorous shaking.

The bacterial cells were then harvested by centrifugation at 6000x g for 15min at 4°C. Pellet was resuspended in 10ml Buffer P1(50mM Tris-HCl pH 8.0, 10mM EDTA, 100µg/ml RNase A) followed by addition of 10ml of Buffer P2 (200mM NaOH, 1% SDS) and 10ml of Buffer P3 (3mM potassium acetate pH

5.5). The solution was mixed and lysates were poured into the barrel of the QIAfilter cartridge. Incubation at room temperature for 10min was followed. Solution was added to a previously equilibrated HiSpeed Maxi Tip (by addition of 10ml Buffer QBT), washed with 60ml Buffer QC (1M NaCl, 50mM MOPS pH 7.0, 15% ethanol) and DNA was eluted with 15ml Buffer QF (1.25M NaCl, 50mM Tris-HCl pH 8.5, 15% Ethanol). DNA was precipitated by adding 10.5ml isopropanol and incubated at room temperature for 5min. The eluate/isopropanol was filtered through a syringe that is attached to a QIAprecipitator Maxi Module. After 70% ethanol was added, the QIAprecipitator was removed from the syringe as well as the plunger. The QIAprecipitator was then attached again and the plunger was inserted and by pressing air through the QIAprecipitator, the membrane was dried. The QIAprecipitator was then attached to a 5ml syringe and 1ml of buffer TE (10mM Tris-HCl, 1mM EDTA, pH 8) was used to elute DNA into a collection tube.

2.12 DNA quantification

The concentration of DNA was measured by two different methods.

The first was by using a BioRad spectrophotometer (Bio-rad Smart Spec™3000 Spectrophotometer) which measures the optical density (OD) of the samples at 260nm. The formula used to calculate DNA concentration was:
1 OD unit at 260nm = 50µg/ml DNA.

The second method to quantify DNA samples was using a ND-1000 spectrophotometer (NanoDrop Technologies) which measures instantly the DNA concentration upon the addition of 1 µl of sample without the use of cuvettes.

2.13. Luciferase assay

Cells were plated out in 96-well plates and transfected after 24 hours with 20ng of the different luciferase reporter recombinant vectors and 5ng of renilla control constructs per well, using the Eugene 6 transfection reagent. All constructs were made using the PGL3 firefly-luciferase reporter plasmid. Cells on the plates were lysed at the indicated times by firstly removing media, washing with PBS and adding 50µl of lucite lysis buffer together with 50 µl PBS in each well. The SteadyLitePlus, High Sensitivity Luminescence Reporter gene assay system (Perkin Elmer) was used according to the manufacturer's instructions. This system can easily assay the activity of two individual

reported enzymes (the firefly and Renilla luciferases) within a single system. The firefly luciferase reporter is measured first by adding Luciferase assay reagent I and after quantifying it, this reaction is quenched and the Renilla luciferase reaction is initiated with the addition of celenterazine (LuxBiotechnology). Measurement of the luciferase activity is done using a plate reading luminometer. Values from the firefly luciferase activity were normalized by the Renilla values and results were represented as graphs created in Microsoft Excel.

2.14 RNA interference

RNA interference (RNAi) is a post-transcriptional method leading to the silencing of genes. In this study, genes were knocked down using the SMARTpool™ Product Insert-Version 2.0 (DHARMACON, RNA technologies). Oligofectamine (Invitrogen LT) was used as the transfection reagent. 300,000 cells per well of a 6-well plate, were plated 24 hours prior transfection was performed. For each well 70µl of OPTIMEM is mixed with 5µl oligofectamine and incubated at room temperature for 10min. This solution is then mixed with 258µl Optimem and 7.5µl (50nM final concentration) siRNA oligos specific for each gene. After 25 min incubation, 160µl OPTIMEM is added to each one of them, reaching a final volume of 500µl, which is then added to the wells that have been previously washed with PBS. The final concentration of siRNA oligos in each well is 50nM. After 4hours of incubation 2ml of DMEM 10% FCS is added to each well.

As a control reaction for the experiment, cells are subjected to transfection with the 1x Universal Buffer (Mock) (without any siRNA added), in order to detect cellular effects caused by the transfection event or delivery process. Another control used is the transfection with a non-functional, non-targeting siRNA in order to detect off-target effects caused by the introduction of any siRNA. These siRNAs have an impaired ability to be processed by RISC and share little or no homology with known mRNA targets in the cell. It is a more biologically relevant control than the Mock transfection control, as non-targeting siRNAs are expected to have similar properties such as charge density and molecular weight. Their effects in the cell, if any, may be through non-RNAi pathways and for this reason may be used to check for general side effects associated with siRNA delivery.

The SMARTpool siRNAs used were: siFoxM1 (M-009762-00), siHDAC2 (M-003495-02), siFOXO3a (L-00307-00), siFOXA1 (L-010319-00)

2.15. Chromatin Immunoprecipitation (ChIP)

ChIP is an important tool for identifying proteins, including histone proteins and non-histone proteins, associated with specific regions of the genome by using specific antibodies that recognize a specific protein or a specific modification of a protein. The initial step of ChIP is the cross-linking of protein-DNA in live cells with formaldehyde. After cross-linking, the cells are lysed and crude extracts are sonicated to shear the DNA. Proteins together with cross-linked DNA are subsequently immunoprecipitated. Protein-DNA cross-links in the immunoprecipitated material are then reversed and the DNA fragments are purified and PCR amplified.

The cells used for the ChIP experiments were cultured and treated with the usual method stated above, in 10ml plates. When the cells were about 90% confluent, they were crosslinked for 10 minutes at 37 C with 36.5% formaldehyde adjusted to a 1% final concentration (Sigma F8775). After being washed with cold PBS, the cells were sonicated in four 10-second pulses at maximum power in 300 µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1], 1 mM phenylmethyl sulfonyl fluoride, 1x protease inhibitor cocktail [Roche]). The supernatant was cleared by centrifugation at 13,000 x g at 4 C and further diluted with Buffer D, of which 1/6 was retained at -80 C as input control. DNA shearing produced DNA fragments of about 200 to 400 bp. To preclear the chromatin solution, 2 µg of sonicated single-strand herring sperm DNA (Sigma), rabbit IgG (1:1000) and 45 µl of a 50% (vol/vol) slurry of protein G-Sepharose (Sigma) beads were added to the rest of the sample.

After incubation with rotation for 2 hours at 4 C, the beads were removed by centrifugation at 5000 rpm for 1 minute at room temperature in a bench top centrifuge, and the supernatant was collected. The retrieved supernatant was incubated with the antibody of interest overnight at 4 C (Table 2.7). The sample was then incubated with rotation in the presence of 2 µg of sonicated single-strand herring sperm DNA (Sigma) and 45 µl of a 50% (vol/vol) slurry of protein G-Sepharose (Sigma) beads. The beads were then washed sequentially with PBS, Tris-SDS-EDTA buffer (TSE) I, TSE II, TSE III, PBS and EDTA. The immunoprecipitated DNA (or the input control) was extracted with 300 µl of extraction solution (1% SDS and 1.1 M NaHCO₃), incubated at 65°C overnight to reverse the cross-links, and purified with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. PCRs were then performed on the purified DNA using the primers stated below (Table 2.8.). Analysis of the PCR products was performed on a standard 2% (wt/vol) agarose gel by electrophoresis in Tris-acetate-EDTA buffer, using a 100bp molecular marker.

ChIP Antibody	Company
FOXM1 (c-20) rabbit polyclonal IgG	Santa Cruz Biotechnology
FOXO3a /FKHRL1 (H144) rabbit polyclonal IgG	Santa Cruz Biotechnology
ERα (F-10) mouse monoclonal IgG	Santa Cruz Biotechnology
FOXA1 (Ab23738) rabbit polyclonal IgG	Abcam
HDAC2 (Ab7029-50) rabbit polyclonal	Abcam
H3K9 (9671) rabbit polyclonal	Cell Signalling
H4K16 (sc-8662) goat polyclonal	Santa Cruz Biotechnology

Table 2.7. List of antibodies used in ChIP

Primer	Sequence
FOXO3a-VEGFpro	Forward 5'-TCCGGGTTTTATCCCTCTTC-3' Reverse 5'-TCTGCTGGTTTCCAAAATCC-3'
FOXA1-FOXC2pro	Forward 5'-GTGTCTCACTCCTTCAGGGAAAGT-3' Reverse 5'-AGCGACTTGGATCTCCTTCAAG-3'

Table 2.8. List of primers used for the PCR in ChIP.

2.16 PCR

PCR amplification was performed using a Taq DNA polymerase Kit (Qiagen). Reaction solutions contained 3µl DNA template, 0.5µl of forward primer (20µM), 0.5µl reverse primer (20µM), 1x PCR buffer, 1x Q solution, 0.2nM dNTP , 0.5 Taq polymerase and dH₂O to a final volume of 50 µl. The PCR was carried out using a Gene Amp[®] PCR system 9700 (Applied Biosystems) using the following program:

A denaturation step for 1 min at 94 °C, annealing for 1-2 min at 55-65°C according to the oligonucleotides utilised, extension at 72°C for 1 min for 33 cycles followed by a final extension for 10 min at 72 °C.

2.17 Biotinylated oligo pull down

Biotinylated oligonucleotides were coupled to streptavidin beads in a solution containing 100 μ l PBS, 10 μ l FCS, 20 μ l of a 50% slurry of streptavidin-agarose beads (Sigma) and 1 μ l of annealed biotinylated oligonucleotide. Beads were left to incubate with the oligos while rotating on a rotary wheel for 2 hours at room temperature. Following incubation, the oligo-coupled beads were spun down at 12000rpm on a bench top centrifuge for 2 minutes. The supernatant was discarded and 50mg of nuclear lysate was diluted in 2x volumes of low salt and added to each of biotinylated oligo-coupled beads along with 1mg 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 left mixing in a rotary wheel at 4° C for 1 hour and subsequently incubated at 30° C for 10 minutes. The beads were then washed 6 times in PBS containing protease inhibitors.

Beads were separated from the washing buffer by centrifugation for 2 minutes at 3000 rpm and boiled in 20 μ l of 2x SDS-PAGE loading buffer for 10 minutes at 100° C. 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 as following

WTFOXO3AF	Biotin-
Biotin	GTTTTATCCCTCTTCTTTTTTCTTAAACATTTTTTAAA
MTFOXO3AF	Biotin-
Biotin	GTTTTATCCCTGTTCTTTTTTCTTGGGCATTTTTTAAA
MTFOXO3A F	GTTTTATCCCTCTTCTTTTTTCTTGGGCATTTTTTAAA
WTFOXO3A F	GTTTTATCCCTCTTCTTTTTTCTTAAACATTTTTTAAA
WTFOXO3A R	TTTAAAAAAAATGTTTAAGAAAAAAGAAGAGGGAT
MTFOXO3A R	TTTAAAAAAAATGCCCAAGAAAAAAGAAGAGGGAT
WTFOXA1 F Biotin	Biotin- TTCGTCCACAAATAAACAAAC
WTFOXA1 F	TTCGTCCACAAATAAACAAAC
WTFOXA1 R	TTGTGGACTGATTATTTATTC
MTFOXA1 F Biotin	Biotin- TCAGTCCACGGGTGGGCGGGC
MTFOXA1 F	TCAGTCCACGGGTGGGCGGGC
MTFOXA1 R	GCCCGCCCACCGTGGACTGA

Table 2.9. List of biotin- and non- labeled primers used for the biotinylated oligo pulldown assay.

2.18 Nuclear-cytoplasmic fractionation.

Cells were harvested with trypsin-EDTA and then centrifuged at 1200 rpm for 5 minutes. After washing the cell pellets with PBS they were transferred into 1.5 ml eppendorf tubes and pelleted by centrifugation at 5000rpm for 2-3 minutes in a bench top centrifuge at 4° C.

CER I (NE-PER Thermo) was added to the cell pellet. Vortexing was followed for 15 seconds to fully suspend the cell pellet. The tubes were left on ice for 10 minutes and afterwards CER II (NE-PER Thermo) was added to the tubes. The tubes were then vortexed for 5 seconds on the highest setting, left to incubate on ice for 1 minute and then vortexed again for 5 seconds on the highest setting. The cytoplasmic extract was collected after centrifugation of the tubes for 5 minutes at maximum speed in a bench top centrifuge at 4° C. The remaining pellet was then suspended in NER (NE-PER Thermo) and vortexed on the highest setting for 15 seconds. The samples were kept on ice and were vortexed four times, for 15 seconds, every 10 minutes. At the end the tubes were centrifuged at maximum speed in a bench top centrifuge for 10 minutes at 4° C in order to collect the cytoplasmic extract.

2.19 Production of recombinant vector

The cDNA of FOXA1 was cloned into a pCDNA3.1 (Promega) vector. The cDNA of FOXA1 was selected by PCR from MCF-7 cells, as described above and the product ran in a 1.5 % (w/v) agarose gel made up with 1x Tris-acetate buffer (40mM Tris-acetate, 1mM EDTA, pH 8.0) plus 1 μ g of ethidium bromide. Samples were electrophorised by applying a constant current (100V) through the gel. cDNA fragments were visualised using a UV transilluminator. The cDNA fragment from the gel was cut out using a razor blade and used for subsequent purification.

Extraction of DNA from agarose gel

The cDNA fragment that was cut for the agarose gel was purified using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. The gel fragment was dissolved by adding 3 volumes (per weight of gel slice) of QG buffer (QIAquick spin kit, Qiagen) and incubating at 50°C for 10 minutes. The sample was then transferred to a QIAquick spin column and centrifuged for 1 minute after which the DNA in the column was washed with 0.75ml of PE buffer (QIAquick spin kit, Qiagen). Residual wash buffer was removed by centrifugation for 1 minute at full speed. DNA was eluted in a microcentrifuge tube by adding 30 μ l of ddH₂O and centrifugation for 1 minute.

Ligation

Ligation of the cDNA fragment with the pCDNA3 vector was performed using T4 DNA ligase. The ligation reaction included 1.0µl 10x T4 DNA ligase buffer (1x = 50mM Tris-HCl, 10mM MgCl₂, 10mM DTT, 1mM ATP, 25µg/ml BSA, pH 7.5), 1.0µl T4 DNA ligase, 1:3 vector/DNA ratio and dH₂O to a final volume of 10µl. For a control reaction another sample was prepared containing the vector without the T4 ligase. Samples were incubated in water at 4°C overnight.

2.20 Plasmids

For the generation of human VEGF promoter constructs, a 1741 bp VEGF promoter construct was generated using PCR primers 5'-ATCTCGAGGAGGCTATGCCAGCTGTAGG-3' and 5'-GCAAGCTTTCTGCTGGTTTCCAAAATCC-3' from genomic DNA and cloned into the pGL3 basic vector (Promega, Southampton, United Kingdom).

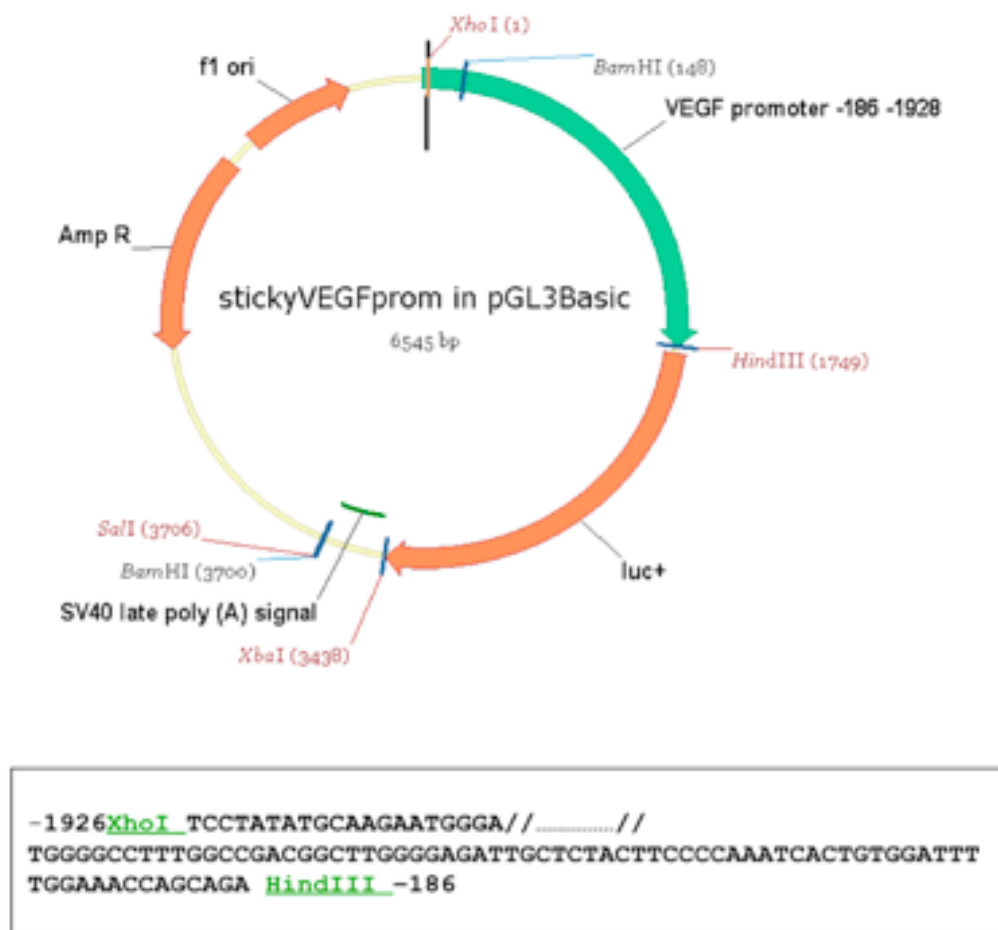


Figure 2.1 The 1741 bp VEGF promoter construct was cloned into a pGL3Basic vector between the XhoI and HindIII restriction enzyme sites.

The HDAC2^{WT} and HDAC2^{C262A/C274A} expression plasmids were kind gifts from Dr. Antonella Riccio (University College London, UK) and the FOXO3a(A3) or FOXM1(Δ N) expression vectors have previously been described (Kwok et al., 2008).

2.21 Site-specific mutagenesis

Mutagenesis of the possible two forkhead binding sites of the *VEGF* promoter cloned into a pGL3-Basic-Luc vector was carried out by using the Quickchange Site-directed mutagenesis kit (Stratagene). Sample reactions were prepared in a total volume of 50 µl containing: 5 µl of 10x reaction buffer (Stratagene), 50ng of double stranded plasmid DNA, 125 ng of each primer, 1µl of 25 mM dNTPs (Stratagene), 1µl of *Pfu* turbo DNA polymerase and ddH₂O.

2.22 Measurement of human VEGF by ELISA

VEGF concentrations were measured by a quantitative sandwich enzyme immunoassay (Quantikine ELISA R&D). Microplates were pre-coated with a specific VEGF monoclonal antibody and upon use 50 µl of assay diluent RD1W were added to the wells. 200 µl of either cell culture supernate samples or standards were loaded in triplicates to the wells and were left to incubate in room temperature in order for VEGF to bind to the immobilised antibody. After washing four times with 400 µl of washing buffer, 200 µl of VEGF conjugate (an enzyme-linked VEGF polyclonal antibody solution) were added to the wells. The plates were left to incubate for 2 hours in room temperature. Following washing, 200 µl substrate solution were added to the wells and colour was developed. After 20 minutes of incubation, 50 µl of stop solution were added. The colour change was then stopped and its optical

density was measured at 450nm (Sunrise-Tecan) within 30 minutes (Sunrise-Tecan).

2.23 Co-Immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed to detect protein-protein interactions. Adherent cells were plated and treated according to the experimental procedure. The proteins were extracted using a Co-IP lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol (v/v), 0.5% Triton-X (v/v), 10 mM NaF, 10 mM β -Glycerophosphate pH 7.3, 0.1 mM Na₃VO₄, 100 U/ml aprotinin, 10 μ g/ml leupeptin and 1 mM PMSF) which has a composition adequate to perform precipitation of the target protein and co-precipitation of binding partners / protein complexes. The lysates were pre-cleared with agarose beads (15 μ l per sample) for 30 minutes. After centrifugation at 0.4xg. for 20-40 seconds at 4 °C, the supernatant was transferred to another eppendorf tube. Protein concentration was then determined and 0.3-0.5 mg of protein extract were incubated with the antibody and beads for 2-3 hours, rotating at 4 °C. The antibodies used at this step were the same as the ones used for ChIP. Immunoprecipitates were then washed 3 times with the same lysis buffer (by centrifugation at 0.4 xg for 20-40 seconds at 4 °C) and boiled for 5 minutes with Laemmli buffer before separation on a SDS-PAGE gel.

2.24 Statistical analysis

To test for the relationship between VEGF, FOXM1 and nuclear/cytoplasmic FOXO3a, statistical analysis was performed using Pearson's correlation test and was considered significant at $p < 0.05$. All statistical analysis was performed with SPSS v.16 (SPSS inc, Chicago, IL, USA).

2.25 Wound healing scratch assay

This assay was done to observe the migration of breast cancer cells .

The cells were plated out on 6 cm dishes and left to grow (10% CO₂ at 37° C) in a confluency of about 90%. Cell were growing in serum starved conditions overnight. The scratch was made using a sterile 2 µl pipette tip and photographed after 24 h.

RESULTS

3. THE ROLE OF FOXO3a AND FOXM1 IN THE REGULATION OF VEGF AND CELL MIGRATION IN BREAST CANCER.

3.1 BACKGROUND AND OBJECTIVES OF CHAPTER 3.

The Forkhead box class O (FOXO) transcription factors are crucial downstream effectors of the PI3K-Akt signalling pathway. Deregulation of FOXO proteins is associated with tumorigenesis and cancer progression. Interestingly, emerging evidence has demonstrated that FOXO proteins, in particular the FOXO3a, has a central role in mediating the cytostatic and cytotoxic effects of chemotherapy (Gomes, 2008; Fernandez de Mattos, 2008; Myatt, 2007; Fernandez de Mattos, 2004; McGovern, 2009).

VEGF plays a crucial role in tissue development and maintenance through regulating the processes of vasculogenesis, angiogenesis and lymphangiogenesis (Lohela et al., 2009). Upon ligand-binding, the VEGF receptors activate downstream signalling cascades, including the phosphatidylinositol 3-kinase(PI3K)-Akt(PKB), which controls the endothelial cell survival, proliferation and migration. VEGF and its receptors are frequently overexpressed in human tumours, especially in breast, non-small cell lung, colorectal, and prostate cancers (Jain et al., 2009). In consequence, VEGF and associated signalling pathways, such as the PI3K-Akt pathway which FOXOs are involved, have been the targets for many novel anti-cancer targeted therapeutics (Margolin, 2002).

A recent cDNA microarray study revealed that FOXO3a can potentially repress VEGF expression in a colon carcinoma cell line (Delpuech et al., 2007). In the present study, we went on to investigate the molecular mechanism by which FOXO3a represses VEGF expression.

3.2 RESULTS OF CHAPTER 3

3.2.1 The activation of FOXO3a in response to Lapatinib in HER2/neu + cells is associated to VEGF repression.

In order to investigate the effect of Lapatinib in the regulation of VEGF expression, the HER2/neu + breast cancer cell lines BT474 and SKBR3 were used. As it has been previously reported in our laboratory, the tyrosine kinase inhibitor Lapatinib is affecting the expression of FOXO3a causing its upregulation and activation in HER2/neu + cells. It would be interesting to know if this effect could be coupled to a possible VEGF response.

For this purpose, the two Lapatinib sensitive (HER2/neu+) cell lines mentioned above and one non-sensitive (HER2/neu-), MDA-MB-231, breast cancer cell line, were used. All were treated with 1 μ M Lapatinib and harvested at different time points of 0, 4, 8, 24 and 48 hours. It was shown by western blotting that the effect of Lapatinib in the expression of FOXO3a, FOXM1 and VEGF is crucial when compared to the non-sensitive cell line.

At 4 hours of Lapatinib treatment, the sensitive BT474 cells showed a decrease in the expression of VEGF which is coupled to a decrease in the phosphorylated FOXO3a and FOXM1 expression levels. The levels of total FOXO3a though were up-regulated, suggesting the time-dependent increase of FOXO3a in the nucleus where it translocates upon its dephosphorylation. This activation of FOXO3a was suggested to be responsible for the further expressional status of FOXM1 and VEGF (Fig. 3.1).

Notably, all factors were down-regulated in BT474 cells after 48 h, probably reflecting global protein degradation and cell death. In contrast, there were no appreciable changes in P-FOXO3a, total FOXO3a, FOXM1, or VEGF levels upon treatment of Lapatinib-resistant MDA-MB-231 breast cancer cells.

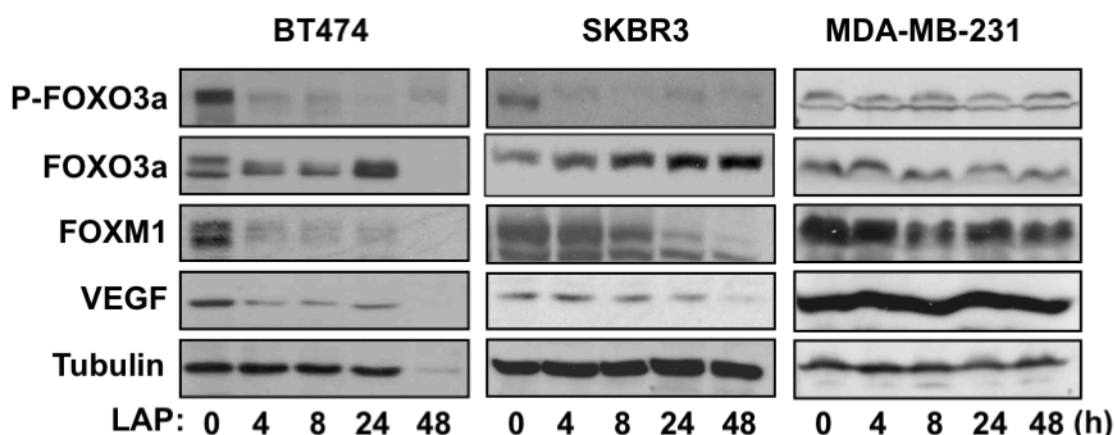


Figure 3.1. FOXO3a activation correlates with down-regulation of FOXM1 and VEGF expression. The (HER2/neu+) BT474 and SKBR3 and the (HER2/neu-) MDA-MB-231 cell lines were plated out on 6 cm dishes, treated with 1 μ M Lapatinib and harvested at 0,4,8,24 and 48 h time points. Cell lysates were separated by SDS-PAGE and immunoblot analysis was done using the indicated antibodies Immunoblotting against Tubulin was used to ensure efficient loading.

To confirm that Lapatinib represses VEGF expression, secreted levels of VEGF were determined by ELISA in the three cell lines (Fig. 3.2). Whereas secreted VEGF levels remained unchanged upon Lapatinib treatment of MDA-MB-231 cells, the levels declined markedly after 24 h treatment of the sensitive BT474 and SKBR3 cells (50% decrease in 24 hours of treatment). The same type of results, occurred in the transcriptional levels of VEGF, which were quantified by RTqPCR and as expected, were down-regulated earlier, by 8 hours of treatment. A significant decrease in FOXM1 transcriptional levels was observed, which could be playing a crucial role to

the down-regulated phenotype of VEGF. Notably, FOXO3a transcript levels were also up-regulated in SKBR3 cells (Fig 3.3).

Together these results demonstrate that Lapatinib treatment of sensitive breast cancer cells induces and activates FOXO3a but inhibits FOXM1 and VEGF expression.

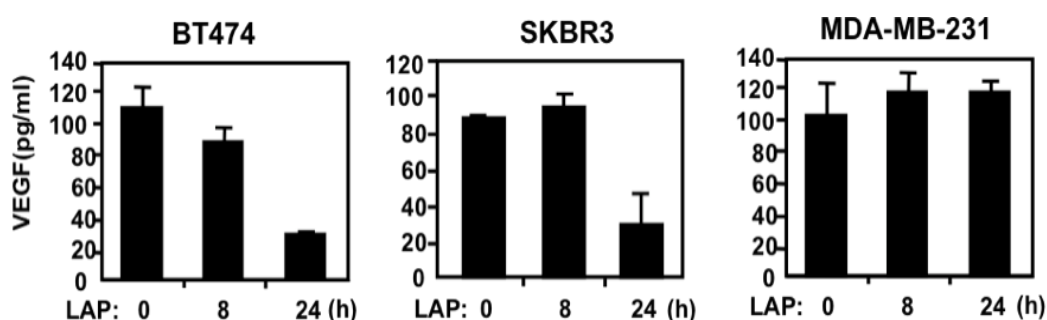


Figure 3.2. Lapatinib treatment of sensitive breast cancer cells inhibits VEGF expression. The (HER2/neu+) BT474 and SKBR3 and the (HER2/neu-) MDA-MB-231 cell lines were plated out on 6 cm dishes, treated with 1 μ M Lapatinib where their supernatant was collected at 0,8, and 24 h time points. VEGF concentration was measured with a human VEGF-ELISA kit.

The transcriptional levels of VEGF did not show any significant change, and as with the secreted VEGF levels, only a small increase of VEGF mRNA was observed. Together with this data, FOXO3a and FOXM1 mRNA levels were unaltered to the addition of Lapatinib in the growing environment of the cells (Fig. 3.3).

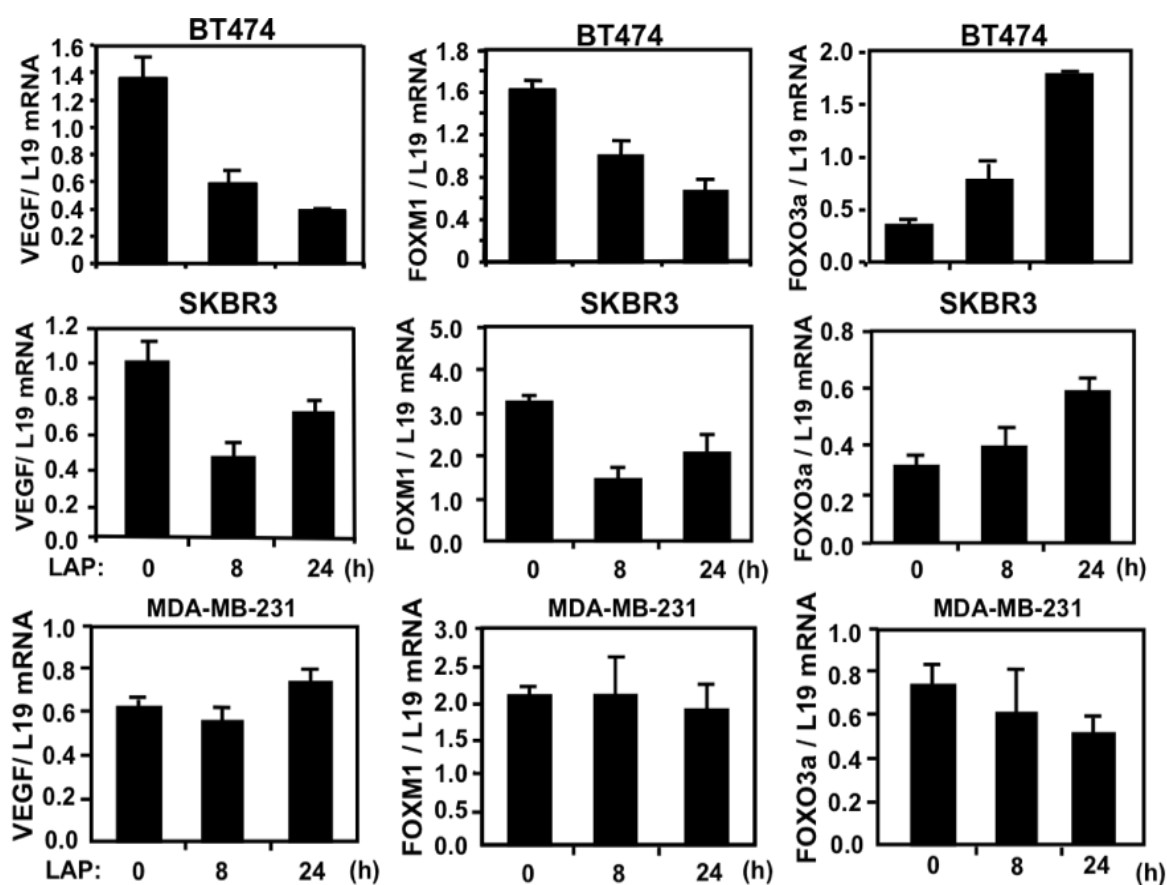


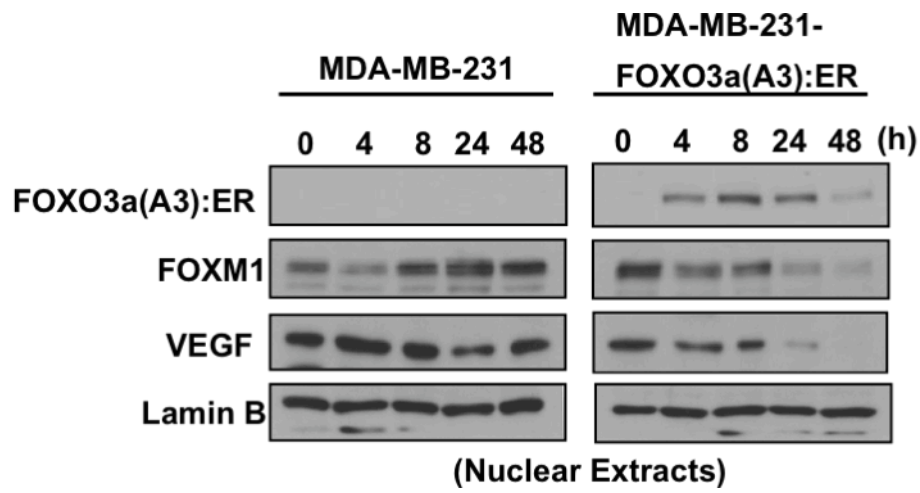
Figure 3.3. Lapatinib inhibits VEGF and FOXM1 mRNA expression in the sensitive SKBR3 and BT474 but not the resistant MDA-MB-231 cells. Both the sensitive and resistant cell lines were plated out on 6 cm dishes, treated with μ M Lapatinib at 0, 8 and 24 h time points. They were harvested for RNA extraction followed by cDNA synthesis. The mRNA levels of VEGF, FOXM1 and FOXO3a were quantified by RTqPCR and normalized by L19.

3.2.2 FOXO3a represses VEGF and FOXM1 expression

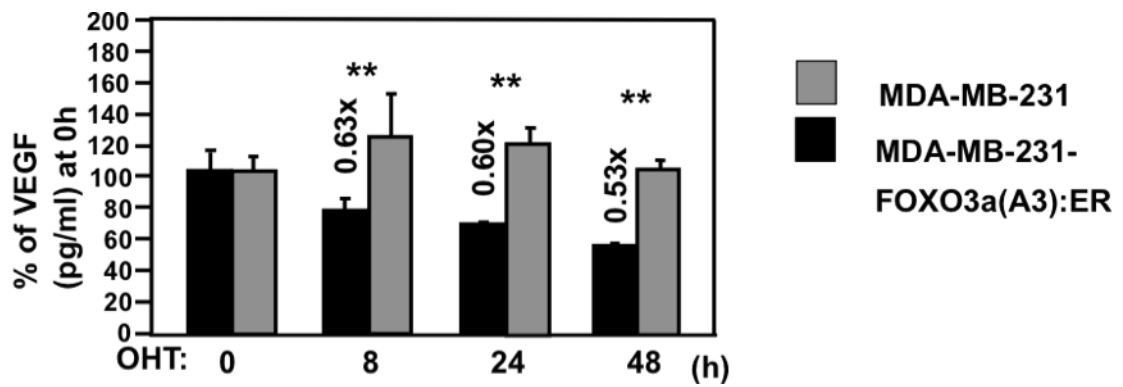
To study the mechanism underlying the reciprocal relationship between FOXO3a activation and VEGF and FOXM1 inhibition, we used an estrogen receptor α (ER)-negative MDA-MB-231 cell line expressing a fusion protein containing a constitutively active FOXO3a(A3) and ligand-binding domain of ER. In MDA-MB-231-FOXO3a(A3):ER cells, FOXO3a can be conditionally activated by 4-hydroxytamoxifen (4-OHT).

Upon 8 hours of 4-OHT treatment, FOXO3a presence in the cell nucleus was increased. This increase was followed by the down-regulation of VEGF expression, both secreted and nuclear, at 8 hours onwards (Fig 3.4A). It was also followed by the decrease of FOXM1 at transcriptional (Fig. 3.4B) and translational level (Fig. 3.4A). The mRNA levels of VEGF showed a small decrease, a fact which was more notably significant when compared to the non-induced control MDA-MB-231 cells in which the mRNA levels of VEGF were increased with time (Fig 3.4B).

A)



B)



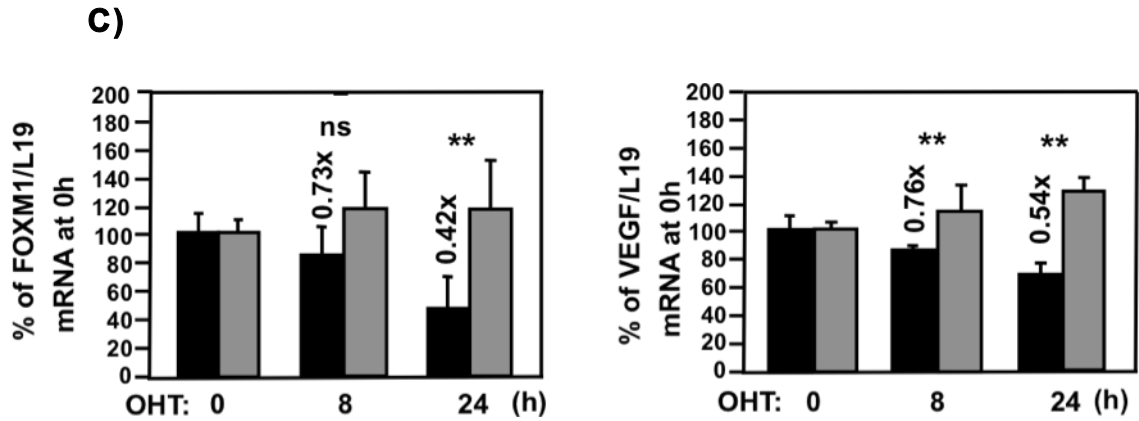


Figure 3.4. Active FOXO3a down-regulates VEGF expression and VEGF and FOXM1 transcription. A) MDA-MB-231-FOXO3a (A3) inducible and control MDA-MB-231 cells were plated out on 6 cm dishes and after 4-OHT treatment for the activation of FOXO3a, were harvested at 0, 4, 8, 24, 48 h time points. Nuclear fractions were prepared with the NE-PER Thermo kit as described in the material and methods section. Cell lysates were separated by SDS-PAGE and immunoblot analysis was done using the FOXO3a, FOXM1 and VEGF antibodies. Immunoblots against Lamin B (nuclear) served to ensure efficient cellular fractionation. **B)** The same cell lines were plated out on 6 cm dishes, treated with 4-OHT and after 0, 8, 24, 48 h their supernatant was collected for secreted VEGF quantification by ELISA. The results were read at 450 nm. **C)** The cell lines mentioned above were plated out on 6 cm dishes, treated with 4-OHT and harvested at 0, 8, 24 h for RNA extraction followed by cDNA synthesis and mRNA quantification by RTqPCR. The results were normalised by L19. Statistical analysis was performed using Pearson's correlation test.

3.2.3 FOXO3a-dependent regulation of VEGF expression.

To assess furthermore the effect of FOXO3a in the regulation of VEGF expression, the constitutively active FOXO3a (A3)-pCDNA3.1 expression vector was transfected and was overexpressed in MCF-7 cells. The effect of this overexpression was a very significant decrease of VEGF expression, together with an, as well, very significant decrease in the expression of FOXM1 (Fig. 3.5A).

Consistent with these results, the mRNA levels of VEGF were increased upon the silencing of FOXO3a in the same cell line (Fig 3.5B). Together with the mRNA levels, the expression of FOXM1 and VEGF was observed to be affected by the silencing of FOXO3a. In the absence of FOXO3a in MCF7 cells, FOXM1 expression seems to be upregulated and VEGF expression (Fig.3.6) as well.

Clearly, these experiments give an insight to the FOXO3a- and FOXM1-dependent regulation of VEGF and the dependence of these two forkhead transcription factors in the regulation of each other.

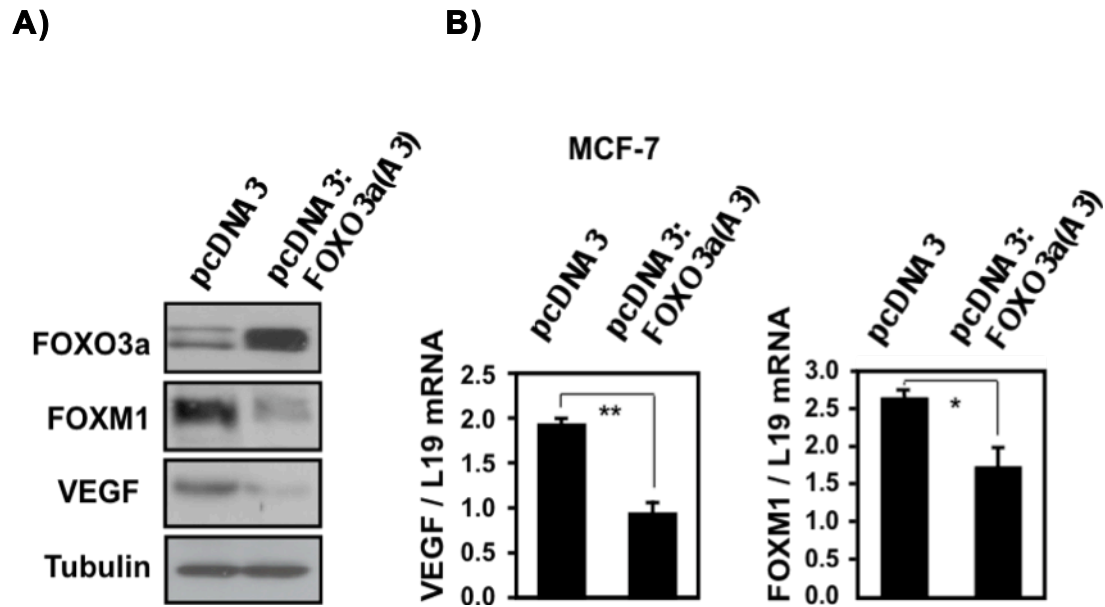


Figure 3.5. Active FOXO3a overexpression causes the down-regulation of FOXM1 and VEGF protein and mRNA levels in MCF-7 cells. A) MCF-7 cells grew on 6 cm dishes and harvested at 48 h after transfection with the FOXO3a(A3)-pcDNA3.1 expression vector. The cells were transfected using oligofectamine. Protein quantification was done after cell lysis followed by western blotting. Tubulin was used as a loading control. **B)** The cells were harvested at 24 h after transfection with the FOXO3a(A3)-pcDNA3.1 expression vector. RNA extraction was followed by cDNA synthesis and mRNA quantification by RTqPCR. The results were normalized by L19. Statistical analysis was performed using Pearson's correlation test.

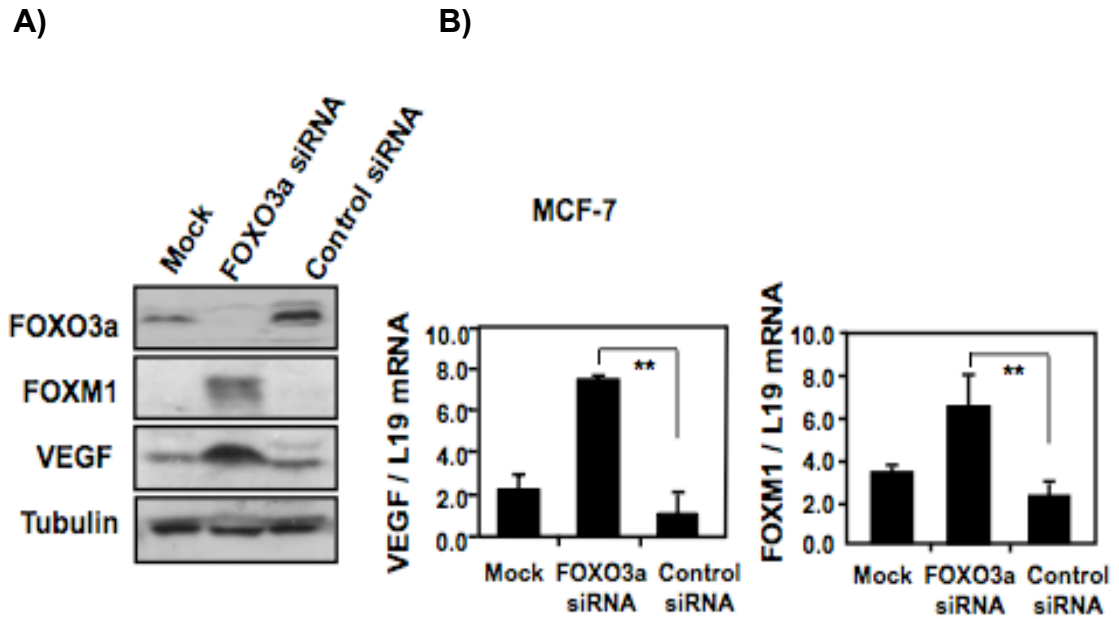


Figure 3.6. FOXO3a silencing is coupled to a FOXM1 and VEGF up-regulation in MCF-7 cells. **A)** MCF-7 cells were plated in a 6-well plate, transfected with FOXO3a siRNA using oligofectamine, harvested after 48 h and lysed. Proteins were separated by SDS-PAGE and immunoblot analysis was done using the indicated antibodies. Immunoblotting against Tubulin was used to ensure efficient loading. Non specific (control) siRNA was used to ensure targeted silencing efficiency. **B)** MCF-7 cells were plated and transfected as above and harvested at 24 h. RNA extraction, cDNA synthesis and subsequently RTqPCR was done for the measuring of mRNA of VEGF and FOXM1 respectively. L19 was used to normalise the results. Statistical analysis was performed using Pearson's correlation test.

3.2.4 FOXO3a and FOXM1 modulate VEGF promoter activity.

We postulated that FOXO3a could suppress VEGF transcription, either by modulating promoter activity or, indirectly, by inhibiting FOXM1 expression. To differentiate between these scenarios, a 1741 bp region of the putative VEGF promoter, representing positions -1,926 to -186 relative to the predominant 5'-transcription start site, was cloned upstream of a luciferase reporter . Co-transfection studies showed that expression of the FOXO3a(A3) mutant represses the activity from the putative VEGF promoter whereas exogenous expression of FOXM1 transactivated the reporter construct in a dose-dependent manner (Fig. 3.7). Sequence analysis identified 2 consensus forkhead transcription response elements (FHREs) in the proximal promoter region. 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.

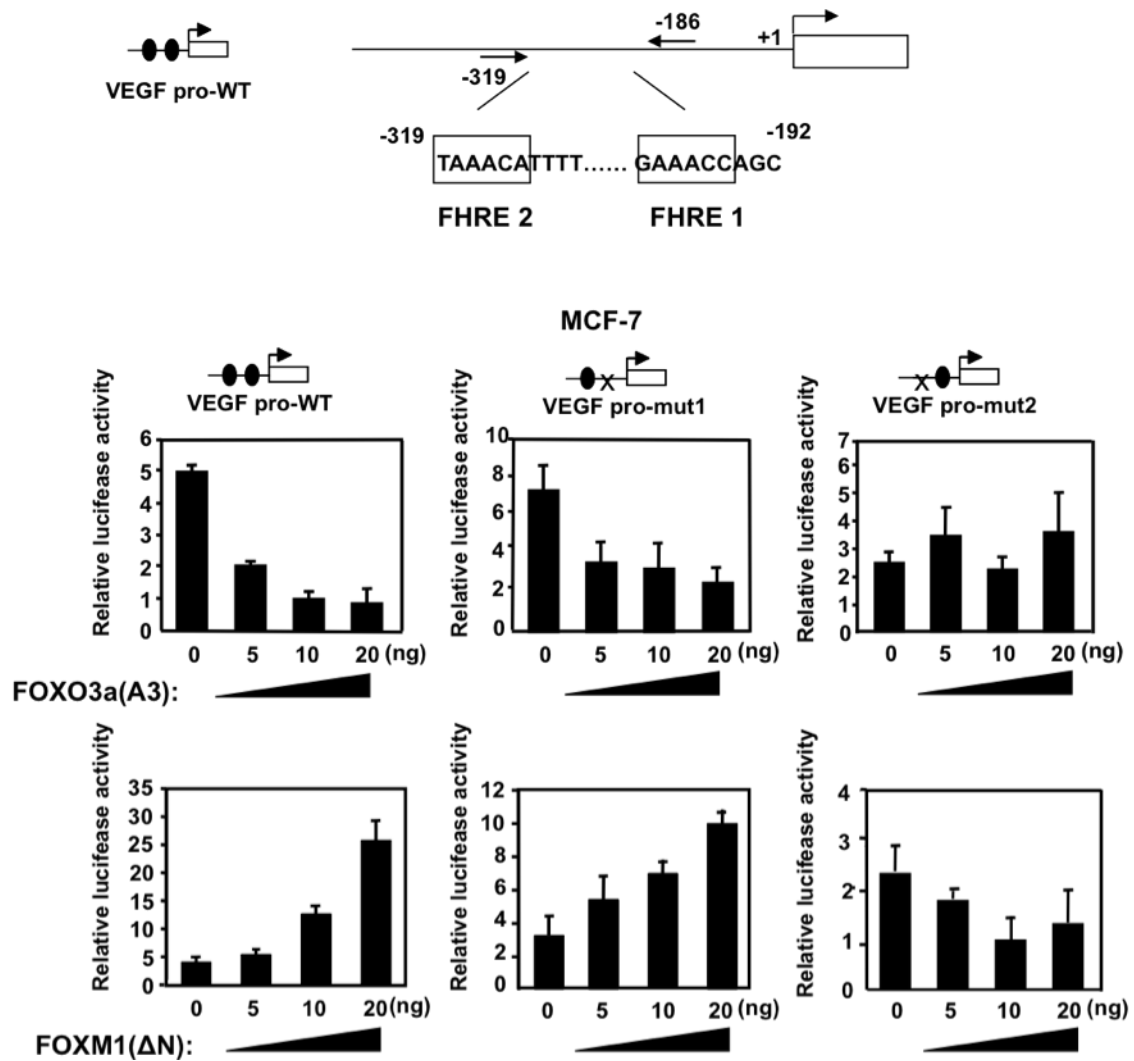


Figure 3.7. The promoter activity of wt VEGF is suppressed by active FOXO3a and increased by active FOXM1 via FHRE2 . MCF-7 cells were plated out in 96 well plates and transiently transfected with 10 ng of the VEGFwt-pGL3b, VEGFmut1-pGL3b and VEGFmut2-pGL3b promoter/reporter constructs respectively. The expression vectors of the active FOXO3A(A3)-pCDNA3.1 and active FOXM1 (ΔN) respectively were transfected in MCF-7 cells, in titrations of 0, 5, 10 and 20 ng harvested after 16 h and assayed for luciferase activity. The VEGF-pGL3b mutated promoter/reporter constructs were formed by site-directed mutagenesis, described in the material and methods section.

3.2.5 FOXO3a antagonises FOXM1 by direct binding at the FHRE binding area of the VEGF promoter.

In order to test further the direct binding of FOXO3a on the FHREs mentioned above, chromatin immunoprecipitation (ChIP) followed. Indeed, it was revealed that FOXO3a is recruited onto the promoter of VEGF.

To determine the mode of action of the two forkhead transcription factors, FOXO3a and FOXM1, on their binding on the *VEGF* promoter, we used two types of assessment. First, BT474 cells were treated with Lapatinib for 2 and 4 hours and immunoprecipitated with the FOXM1 and FOXO3a. We observed that FOXM1 is recruited in non-treated cells on the promoter whereas FOXO3a is not. However, post-treated cells exhibit a different binding status, at 2 hours of Lapatinib treatment, with FOXO3a antagonizing FOXM1 and taking its place on the FHRE binding area of the *VEGF* promoter (Fig. 3.8). 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 (Fig. 3.8). Secondly, to test the consistency of this regulation, the MDA-MB-231 FOXO3a:ERa cell line was used for ChIP.

Again, in those cells, FOXM1 is antagonised by FOXO3a in the FHRE binding area of the *VEGF* promoter (Fig. 3.8). Only this time the effect is not as fast as by treating cells with Lapatinib.

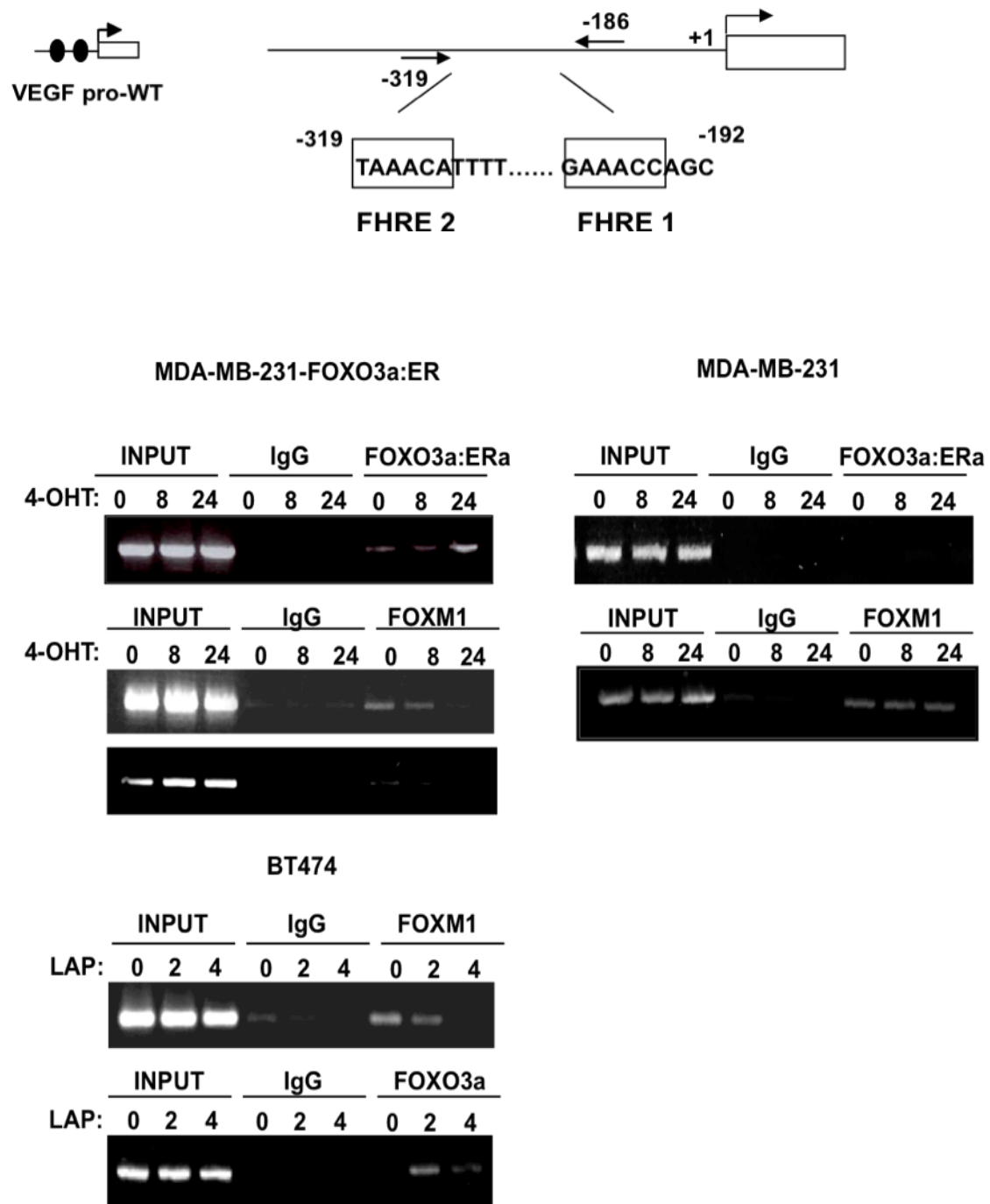


Figure 3.8. FOXO3a is recruited to the proximal region of the VEGF promoter *in vivo*. MDA-MB-231-FOXO3a:ER inducible cells were treated with 4-OHT and were cross-linked using formaldehyde *in vivo*. Chromatin fragments were subjected to immunoprecipitation with antibodies against IgG (non-specific) , FOXO3a or FOXM1. Afterwards, the cross-linking was reversed and the immunoprecipitated DNA was amplified by PCR with primers (-319 to -186) for the indicated region of VEGF's promoter. It was then resolved in a 2% agarose gel and photographed under UV. The same procedure was applied at BT474 cells treated with 1 μ M Lapatinib.

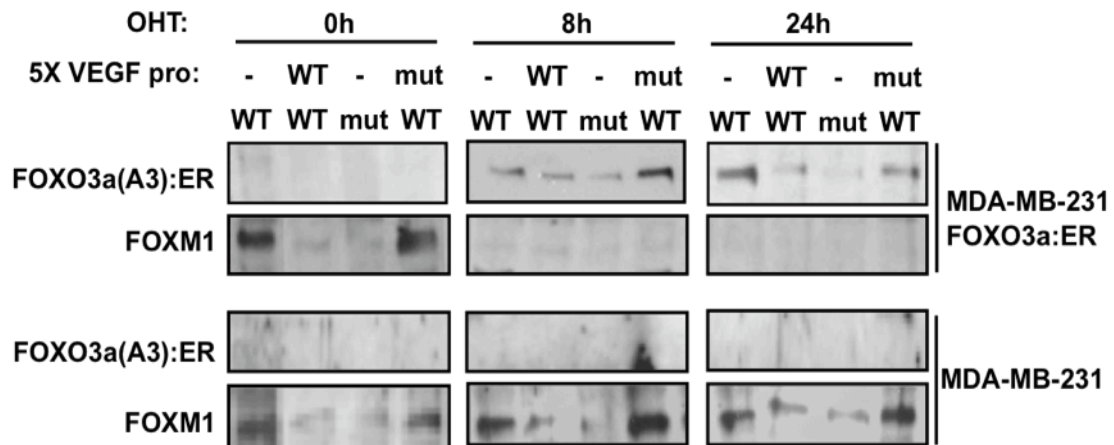
To examine furthermore the mechanism by which FOXO3a and FOXM1 regulates the VEGF promoter, we performed oligonucleotide pull-down assay with nuclear lysates from unstimulated MDA-MB-231-FOXO3a(A3):ER and MDA-MB-231 cells or cells treated with 4-OHT for 8 and 24 h. Western blot analysis of the pulled-down complexes showed that both FOXO3a and FOXM1 bind to the wild-type FHRE2 of VEGF, but not the mutated FHRE2 site (Fig. 3.9). 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 (Fig. 3.9A). 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 down-regulates VEGF expression by competitive displacing FOXM1 bound to FHRE2.

The FHRE pull-down experiment was then repeated in the BT474 cells following lapatinib treatment in the presence of molar excess of mutated FHRE oligonucleotides (Fig. 3.9B). Parallel Western blot analysis of nuclear and cytoplasmic lysates showed that lapatinib induces nuclear accumulation of FOXO3a after 2 to 4 hours, concomitant with the down-regulation of VEGF expression but without discernible change in FOXM1 levels at these time-points (Fig. 3.9C). The pull-down results, however, indicated that the lapatinib-

activated FOXO3a displaces FOXM1 from the FHRE2 of the VEGF promoter at these time-points. Thus, although prolonged activation of FOXO3a will down-regulate FOXM1 levels, inhibition of VEGF expression is an early event and mediated, at least in part, by displacing FOXM1 and binding to FHRE2.

The above observations pointed to the possibility that FOXO3a play a role in recruiting chromatin remodelling enzymes, such as histone deacetylases (HDACs), to repress the VEGF transcription.

A)



B)

C)

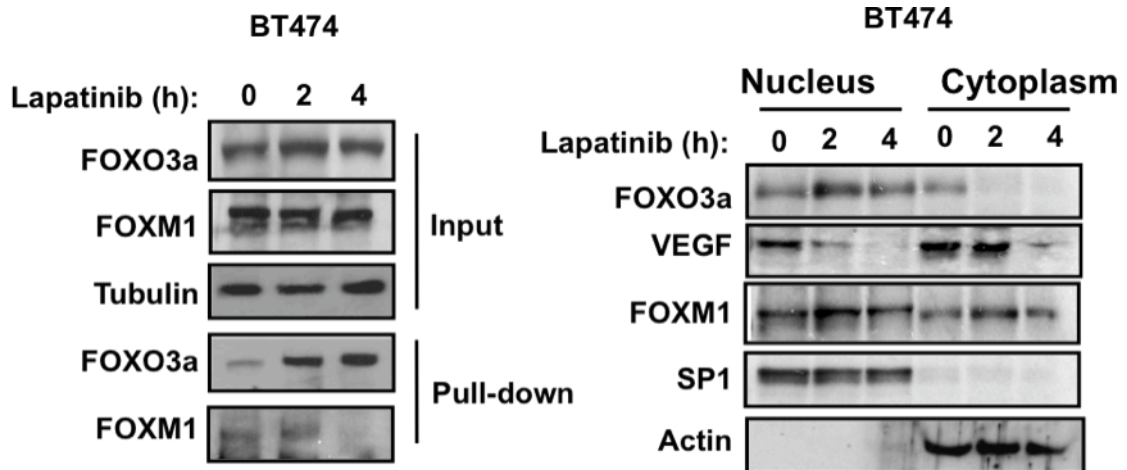


Figure 3.9 FOXO3a and FOXM1 compete for binding to FHRE2. **A)** The MDA-MB-231-FOXO3aER cell line was induced with 4-OHT for 8 and 24h. The extracts were used for pull-down experiments using streptavidin/sepharose beads couples to biotin-conjugated oligos containing the proximal region of the VEGF promoter studied. The pulled down protein complexes were resolved by SDS-PAGE and immunoblotted against the indicated antibodies. MDA-MB-231 cells served as a control. **B)** The same method was applied in BT474 Lapatinib-sensitive cells treated with 1μM Lapatinib for 2 and 4 h. **C)** Cellular fractionation was done in BT474 cells using the NE-PER Thermo kit in order to see the alterations of the proteins interested on, as the whole extracts (B) did not show any major differences. SP1 and Actin were used as loading controls of nuclear and cytoplasmic fractions respectively.

3.2.6 The increased expression of FOXO3a represses cell migration, *in vitro*.

As FOXO3a seems to be a crucial factor for the transcriptional and translational status of VEGF, we were keen on studying the effect of up-regulated FOXO3a expression on breast cancer cell migration. In order to examine this, wound healing assays were performed. Using the MDA-MB-231 FOXO3a:ERa inducible system mentioned above, and the control cell line MDA-MB-231, we made a scratch on the cell monolayer and pictured it, at 0 and 24 hours. For both the cell lines the supernatant applied on the cells was taken from the same cells pre-treated with 4-OHT before, so that, VEGF which is secreted by the cells, would be the sole factor affecting cell migration. The percentage of the wound healing was measured by the ratio of average decrease between the edges of the “wounded” cell monolayer.

Although the control MDA-MB-231 cells managed to heal the wound well exhibiting healing of 75%, the MDA-MB-231 FOXO3a:ERa cells showed a very slow cell migration, healing just 20% of the wound (Fig. 3.10). This decrease in cell migration highlights the importance of FOXO3a in the repression of VEGF and thus its character upon cell migration.

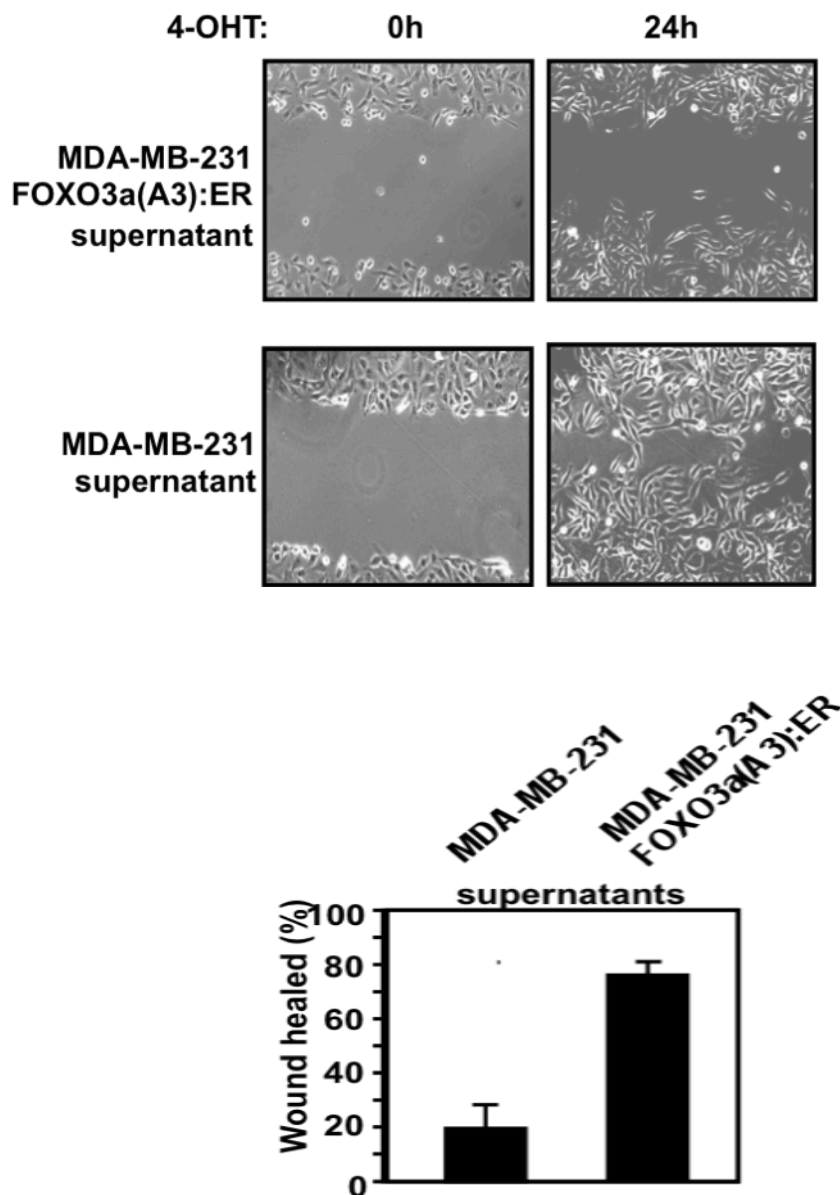


Figure 3.10 Breast cancer cells migrate at slower rates in scratch wound healing assays when cultured in supernatants derived from FOXO3a-induced MDA-MB-231 cells. MDA-MB-231-FOXO3aER cells were plated out on 6cm dishes. When 90% confluent a scratch was done on the monolayer. Supernatant from 4-OHT treated cells was applied on them to grow with, in order to assess the effect of secreted VEGF on the cells. The wound healing was photographed after 24 h. The MDA-MB-231 was used as a control against the inducible cell line.

3.3 DISCUSSION

Signals mediated through VEGFs and their receptors have been shown to be essential for breast cancer carcinogenesis, cell migration (metastasis) and angiogenesis. Yet, the molecular mechanisms regulating VEGF expression in cancer cells are only partially understood.

Using the lapatinib sensitive breast cancer cell lines BT474 and SKBR3 as models for FOXO3a activation, the hypothesis that FOXO3a regulates VEGF expression was examined and the underlying mechanisms involved explored in the present study. Lapatinib treatment resulted in inactivation of the phosphatidylinositol-3-kinase (PI-3K) pathway, nuclear translocation and activation of FOXO3a (Francis, 2009) and ultimately reduction in VEGF expression at protein, mRNA and gene promoter levels. Transient transfection and inducible FOXO3a expression experiments showed 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 demonstrated 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 suggested that FOXO3a accumulation might lead to exclusion of transcription factors through chromatin remodelling. Overexpression of FOXM1 has been implicated with metastasis and angiogenesis in a number of malignancies, including glioma, gastric and pancreatic cancer. Consistent with our findings, a recent study has also demonstrated that FOXM1 transcriptionally regulates VEGF expression in glioma cells (Zhang, 2008). However, in the present study we further demonstrated that FOXM1 functions downstream of FOXO3a, and its activity and expression are negatively regulated by FOXO3a. The ability of FOXO proteins to repress VEGF expression has been documented in Foxo1 null cells where VEGF is overexpressed and angiogenesis deregulated (Park, 2009; Furuyama, 2004).

In summary, together the present results suggest that FOXO3a can potentially repress VEGF expression. Activated FOXO3a can compete off the transcription activator FOXM1 from binding to the FHRE of the VEGF gene promoter. Furthermore, FOXO3a has also been shown previously to be able to repress FOXM1 expression at transcriptional levels (Francis, 2009). Consequently, FOXO3a can repress VEGF expression indirectly via regulating FOXM1 expression. The mechanism by which FOXO3a represses VEGF expression may represent common means whereby FOXO3a negatively regulates target gene expression. Thus, the present study also provides novel understanding on the mechanisms by which FOXO transcription factors repress target gene expression. Furthermore, the findings from this study also 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.

4. THE ROLE OF FOXO3A IN THE RECRUITMENT OF HDAC2 FOR THE REGULATION OF VEGF IN BREAST CANCER.

4.1 BACKGROUND AND OBJECTIVES OF CHAPTER 4

When HDACs deacetylate histones gene transcription is repressed due to the very compact chromatin structure where transcription factors and other complexes cannot access the DNA to promote gene transcription. HDACs interact with other molecules in order to regulate transcription and chromatin remodeling. In this chapter, the idea of HDACs being recruited by FOXO3a for the regulation of *VEGF* transcription is examined.

HDAC inhibitors bind and inhibit HDACs, leaving an open chromatin structure where transcription factors and other elements can bind to the DNA and regulate gene transcription. The HDAC inhibitor TSA could be a powerful tool in the understanding of the transcriptional repression mechanism and the subsequent elucidation of the mode of action of FOXO3a and FOXM1 in the regulation of *VEGF* transcription via HDAC complexing.

4.2 RESULTS OF CHAPTER 4

4.2.1 TSA affects the expression pattern of VEGF.

To test the hypothesis that FOXO3a recruits HDACs to repress *VEGF* transcription, MCF-7 cells were treated with the HDAC inhibitor TSA and the expression of VEGF was monitored. RTqPCR analysis demonstrated that TSA strongly enhances mRNA levels of VEGF (Fig.4A). TSA, as expected, affected in the same manner, the expression pattern of VEGF. VEGF protein levels were increased upon inhibition of HDACs, and more specifically HDAC2 which was used in the experiments below.

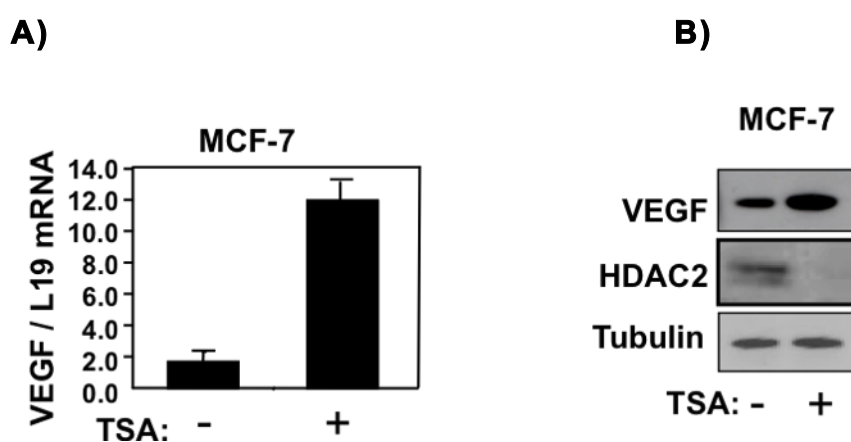


Figure 4.1 TSA treatment increases the transcriptional and translational levels of VEGF. A) MCF-7 cells were plated out on 6 cm dishes, treated with 1 μ M TSA for 8 h and harvested for RNA extraction followed by cDNA synthesis. The mRNA levels of VEGF were quantified by RTqPCR and normalized by L19. **B)** MCF-7 cells were plated out on 6 cm dishes, treated with 1 μ M TSA for 12 h and harvested for protein extraction. Cell lysates were separated by SDS-PAGE and immunoblot analysis was done using the indicated antibodies Immunoblotting against Tubulin served to ensure efficient loading.

4.2.2 TSA alters the promoter activity of VEGF in a FKHR-dependent manner.

TSA also triggered a marked induction in VEGF promoter activity, which was abolished upon mutation of the FHRE2, but not the FHRE1, site (Fig.4.2.A). This result showed that HDACs should be acting in collaboration with forkheads which act on the FHRE2 binding site of VEGF's proximal promoter region. Conversely, overexpression of the dominantly active HDAC2^{C262A/C274A} mutant, but not the wild-type HDAC2, repressed VEGF promoter activity in a dose-dependent manner (Fig.4.2.B)

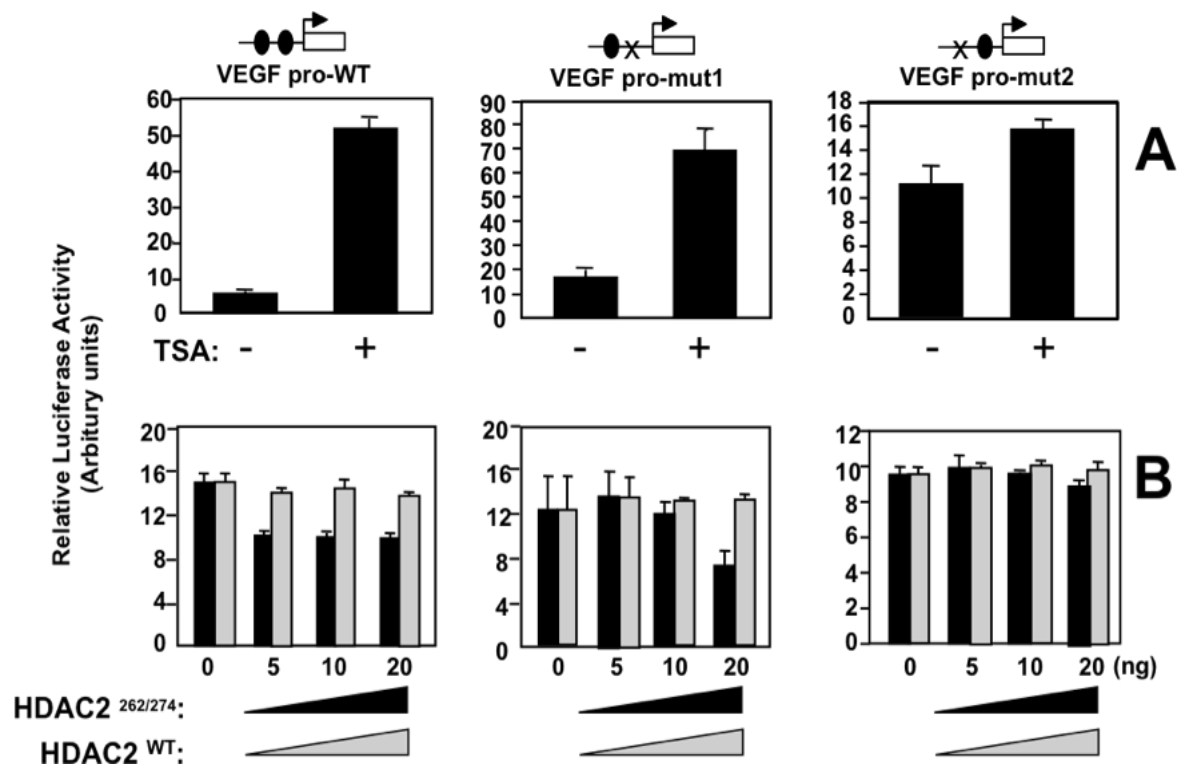


Figure 4.2. The promoter activity of VEGF depends on a correlation between HDACs and FKHR. MCF-7 cells were plated out in 96 well plates and transiently transfected with 10 ng of the VEGFwt-pGL3b, VEGFmut1-pGL3b and VEGFmut2-pGL3b promoter/reporter constructs respectively. **A)** They were treated with 1 μ M TSA for 8h, harvested after 16 h and assayed for luciferase activity, revealing a correlation between HDAC and FKHR for the VEGF promoter activity **B)** MCF-7 cells were treated with the same promoter/reporter constructs, in the same order, and either the dominantly active HDAC2^{C262A/C274A} mutant or the wt HDAC2 expression vectors were titrated into the cells in 5,10, 20 ng doses. The cells were harvested after 16h and assayed for luciferase activity. The promoter of VEGF again confirmed an HDAC2-affected activity via the FKHR binding sites.

This ability of HDAC2 to repress VEGF promoter activity was again dependent on a functional FHRE2. The inability of wild-type HDAC2 to repress VEGF promoter activity could be due to the high levels of endogenous HDAC2 in MCF-7 cells.

4.2.3 HDAC2 binds on the proximal VEGF promoter

In order to examine in more detail the relation between HDAC2 and the FKHR binding sites on the proximal VEGF promoter area, ChIP assays were performed. The cells once more were treated with the HDAC inhibitor TSA and demonstrated that TSA induced a decrease in HDAC2 binding to the proximal VEGF promoter (Fig.4.3).

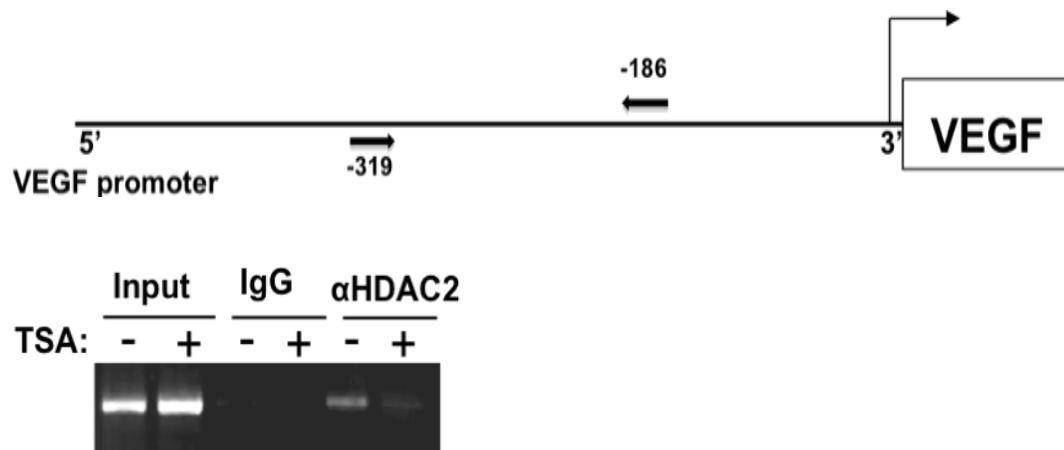


Figure 4.3 ChIP revealed HDAC2 recruitment on FKHR binding site of the VEGF promoter. MCF-7 cells were treated with 1 μ M TSA for 4 h and were undergone formaldehyde cross-linking *in vivo*. Chromatin fragments were subjected to immunoprecipitation with antibodies against IgG (non-specific) or HDAC2. Afterwards, the cross-linking was reversed and the immunoprecipitated DNA was amplified by PCR with primers (-319 to -186) for the indicated region of VEGF's promoter. It was then resolved in a 2% agarose gel and photographed under UV.

4.2.4 VEGF expression increases upon silencing of HDAC2

Moreover HDAC2 was knocked down in MCF-7 cells using siRNA in order to test further the effects of HDAC2 on the expression of VEGF (Fig. 4.4). The protein levels of VEGF were up-regulated significantly, indicating for one more time that HDAC2 plays a crucial role in the regulation of VEGF expression.

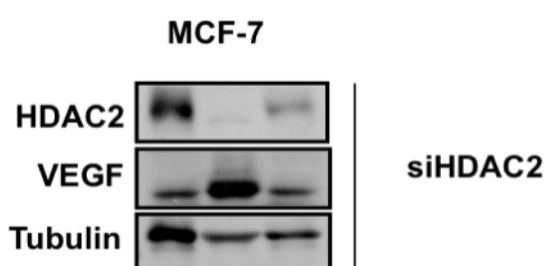


Figure 4.4 HDAC2 silencing induces expression of VEGF. MCF-7 cells were plated out in a 6-well plate, transfected with siRNA against HDAC2 and harvested after 72 h. Lysates were analysed by SDS-PAGE and immunoblotted against HDAC2, VEGF and Tubulin antibodies. Tubulin was used as a loading control.

4.2.5 FOXO3a interacts with HDAC2 on the VEGF promoter causing the deacetylation of histones 3 and 4

To examine if FOXO3a complexes with HDAC2 upon FOXO3a activation, the sensitive BT474 cells treated with Lapatinib were used. HDAC2 and FOXO3a co-immunoprecipitated and this interaction was enhanced upon Lapatinib treatment, probably reflecting nuclear translocation of FOXO3a (Fig. 4.5).

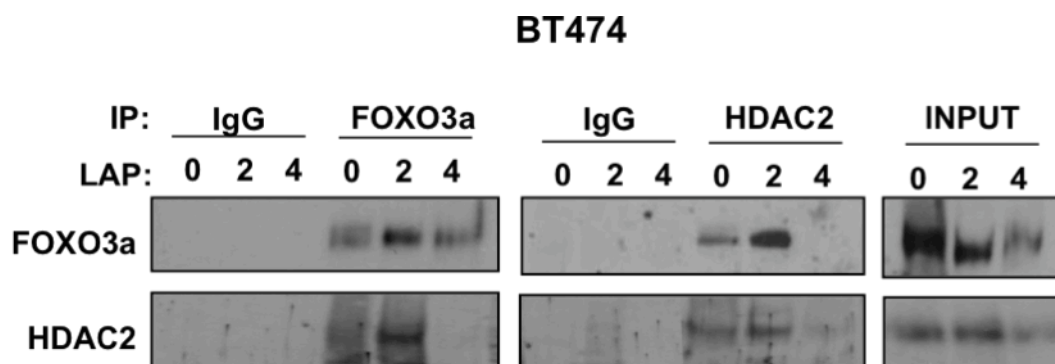


Figure 4.5. HDAC2 and FOXO3a interact and this interaction is enhanced by FOXO3a activation. BT474 cells were plated out on 6 cm dishes and treated with 1 μ M Lapatinib. After harvesting at 0, 2 and 4 h of drug treatment Whole cell extracts were prepared in lysis buffer and immunoprecipitations (IP) were performed using the FOXO3a and HDAC2 antibodies. The IP samples along with INPUT samples from the whole cell lysates were analysed by SDS-PAGE and immunoblotted with the HDAC2 and FOXO3a antibodies.

To assess if the HDAC2-FOXO3a interaction complex is recruited, *in vivo*, on the proximal VEGF promoter on the area characterised from the previous experiments, ChIP was performed. ChIP assays showed increased recruitment of HDAC2 to the proximal VEGF promoter after 2 h of Lapatinib treatment. HDAC2 recruitment coincided with a decrease in bound acetylated histones H3 and H4, indicating active chromatin remodelling and compaction of the proximal VEGF promoter. Further, siRNA-mediated FOXO3a knockdown 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, binding to FHRE2, recruitment of HDAC2, and transcriptional repression of VEGF.

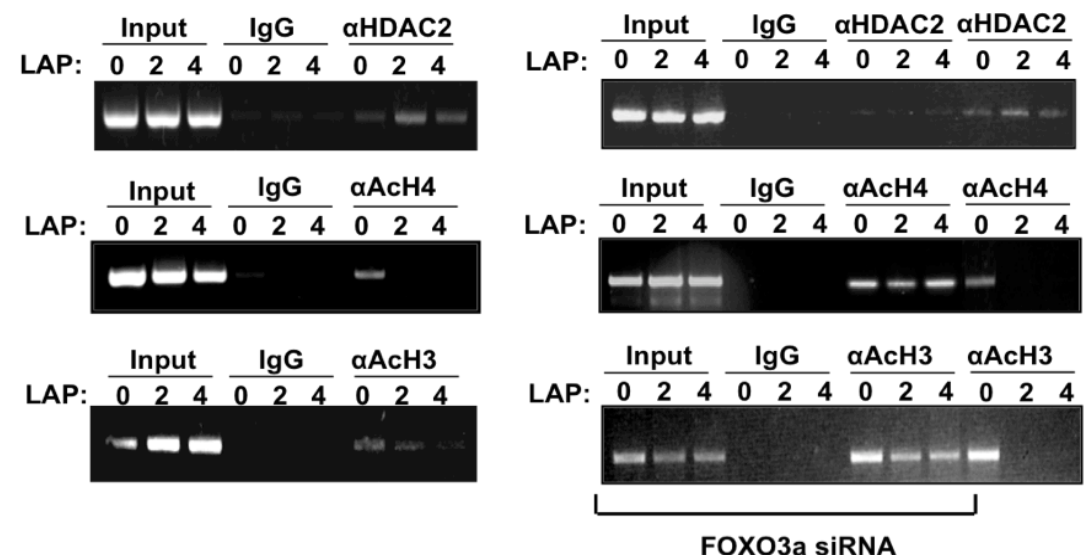


Figure 4.6 HDAC2 is recruited on the proximal VEGF promoter causing deacetylation of H3 and H4 *in vivo*. BT474 cells were treated with 1 μ M Lapatinib and were formaldehyde cross-linked *in vivo*. Chromatin fragments were immunoprecipitated with antibodies against IgG (non-specific) , HDAC2, acH3 or acH4. Afterwards, the cross-linking was reversed and the immunoprecipitated DNA was amplified by PCR with primers (-319 to -186) for the indicated region of the VEGF promoter. Afterwards, it was resolved in a 2% agarose gel and photographed under UV.

4.3 DISCUSSION

As discussed in chapter 3, FOXO3a can repress VEGF, by antagonising FOXM1. It was shown that FOXO3a activation results in the recruitment and the subsequent removal of FOXM1 on a specific forkhead binding site of the proximal promoter of *VEGF*. However, still the potential mechanism of this repression was not elucidated. HDACs which are critical transcriptional regulators were studied and it was shown that HDAC2 is recruited on the VEGF promoter. Upon FOXO3a activation and its recruitment in the nucleus and the VEGF promoter, HDAC2 was also found to be recruited on the same area of the promoter. Additionally HDAC2 complexes with FOXO3a when active. Thus, the recruitment of the HDAC2/FOXO3a on the functional forkhead binding site of the proximal is a crucial regulator of transcriptional regulation. Moreover, this binding on the promoter, causes the de-acetylation of histones 3 and 4 suggesting chromatin remodeling.

Concluding, the model proposed for VEGF transcriptional regulation in breast cancer cells when FOXO3a is activated, is that FOXO3a is recruited on the VEGF promoter in a complex with HDAC2 which causes the deacetylation and the condensation of chromatin. In this way other transcription factors, such as FOXM1 or other inducing factors of VEGF expression are unable to reach the regulatory binding site of VEGF and gene suppression is achieved.

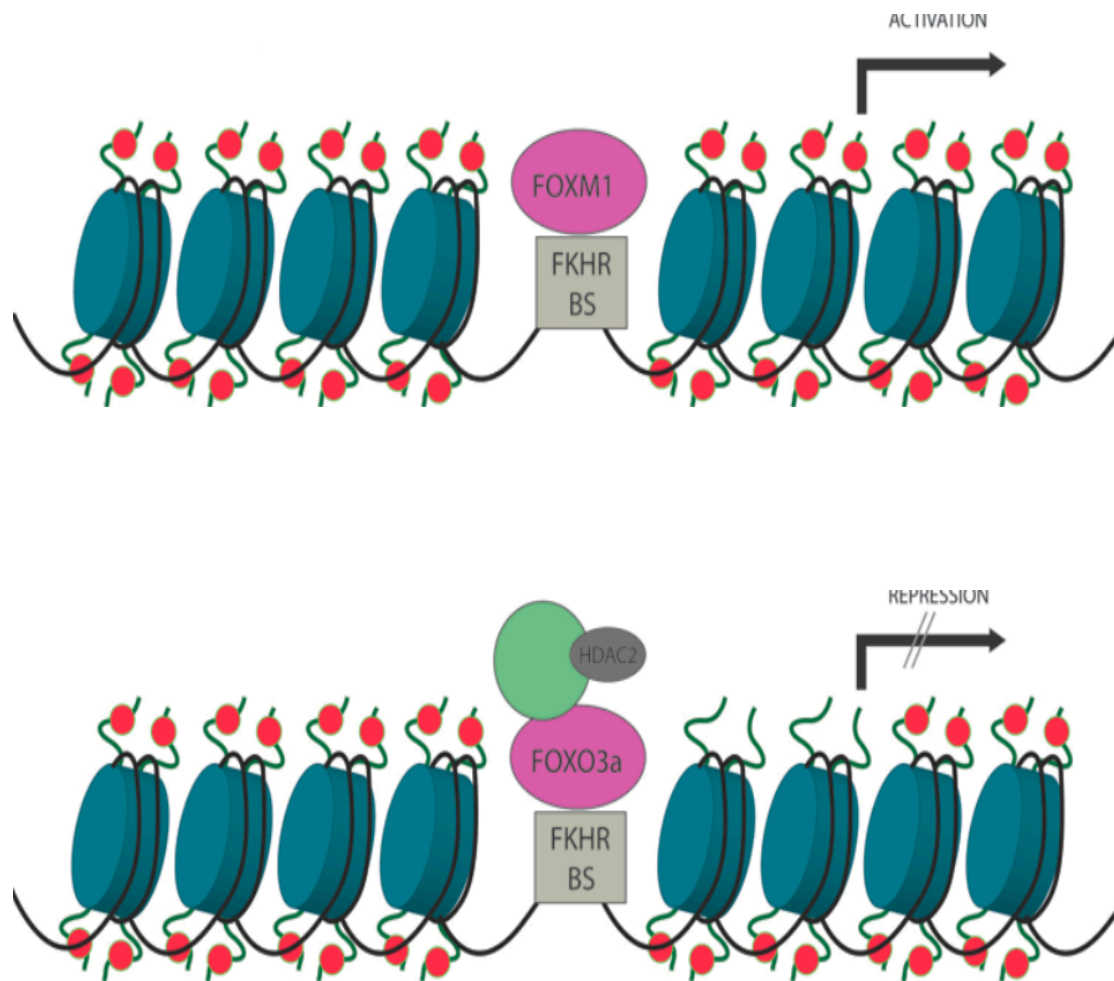


Figure 4.7 FOXO3a complexed with HDAC2 is recruited on the proximal VEGF promoter causing deacetylation of H3 and H4 and subsequent chromatin condensation leading to VEGF repression of transcription.

5. THE REGULATION OF VEGF BY FOXA1 AND FOXC2 IN BREAST CANCER.

5.1 BACKGROUND AND OBJECTIVES OF CHAPTER 5

As we have seen from the previous chapters, two members of the family of the forkhead transcription factors emerged critical for the expression of VEGF and thus, invasion and metastasis of breast carcinoma. However, apart from these forkheads, two other members of that family have been observed to be implicated in breast cancer. FOXA1 has been found to be overexpressed in ER α -positive breast tumours and has been described as a very significant transcription factor, driving a plethora of genes to be transcribed. As noted in the introduction section, FOXA1 is considered a good prognosis factor in breast cancer as it is not present in aggressive and highly metastatic tumours. On the other hand, the forkhead transcription factor that has been found to be overexpressed in ER α -negative breast tumours which are invasive and metastatic is FOXC2. FOXC2 in development is a major factor for proper formation of the vasculature of the embryo and is a regulator of angiogenesis.

Taken together this information, it would be interesting to understand how these two factors regulate VEGF and subsequently angiogenesis and

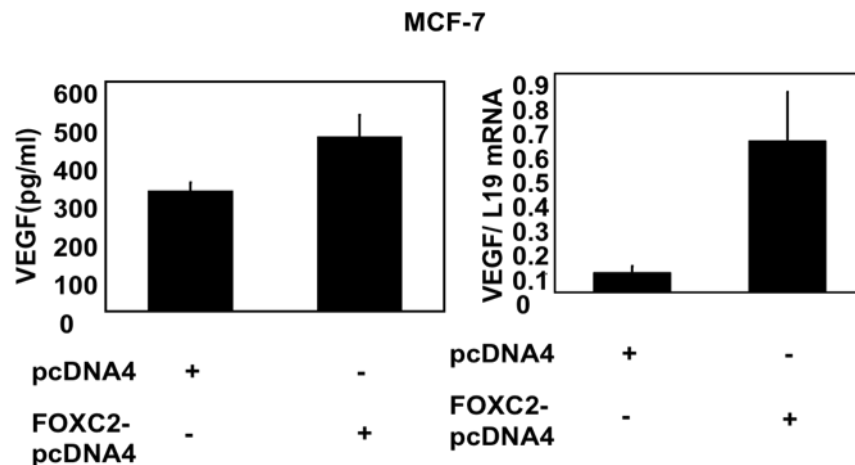
invasiveness in breast cancer. A potential cross-talk relationship between those two transcription factors is studied in this chapter.

5.2 RESULTS OF CHAPTER 5

5.2.1 The transcriptional and translational status of VEGF is affected by FOXA1 and FOXC2.

In order to examine the idea that both FOXA1 and FOXC2 regulate VEGF expression, these two factors were transiently transfected and thus overexpressed in MCF-7 breast cancer cells. The increase of FOXC2 was coupled to the increase of VEGF secreted protein and mRNA levels (Fig.5.1A), whereas FOXA1 had an opposite effect on the cells. FOXA1 overexpression was associated to a decrease of VEGF secreted protein and mRNA levels (Fig. 5.1B).

A)



B)

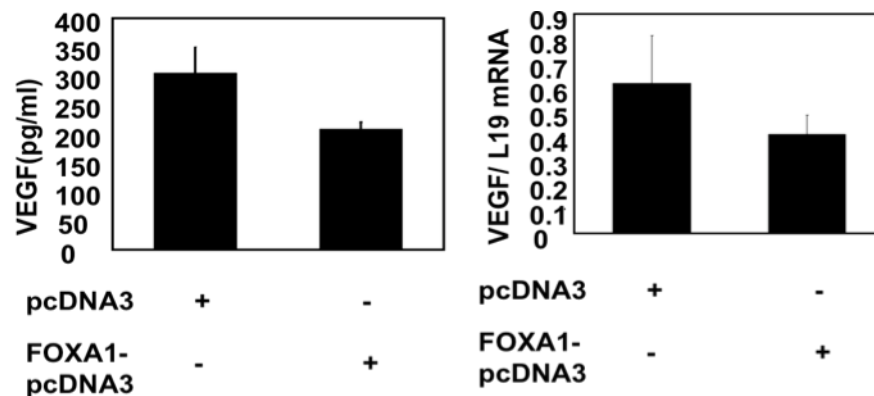


Figure 5.1 FOXC2 overexpression upregulates VEGF mRNA and protein levels in contrast to FOXA1 which downregulates them, in MCF7 cells.

A) MCF-7 cells were plated out on 6 cm dishes, transfected with the FOXC2-pcDNA4 expression vector and harvested after 48 h. ELISA was performed in order to quantify VEGF in the supernatant of the cells. For VEGF mRNA determination, RNA extraction, cDNA synthesis followed by RTqPCR was done. L19 was used to normalise the results. **B)** The exact same procedure was followed for MCF-7 cells transfected with the FOXA1-pcDNA3.1 expression vector. This vector 's construction is described in the materials and methods section 2.19.

It has been shown that FOXA1 and ER α synergize to regulate the transcription of target genes in breast cancer, so, to understand if ER α acts as a regulator of VEGF via or together with FOXA1, the status of ER α was inhibited or activated.

To test this idea, MCF-7-FOXA1 cells which stably overexpress FOXA1 were used. Treatment of those cells with either the ER α activator Estradiol (E2) or the ER α antagonist ICI182,780 did not alter the effect of FOXA1 overexpression in MCF-7 cells. FOXA1, independently of ER α , was shown to decrease the mRNA expression levels of VEGF (Fig. 5.2).

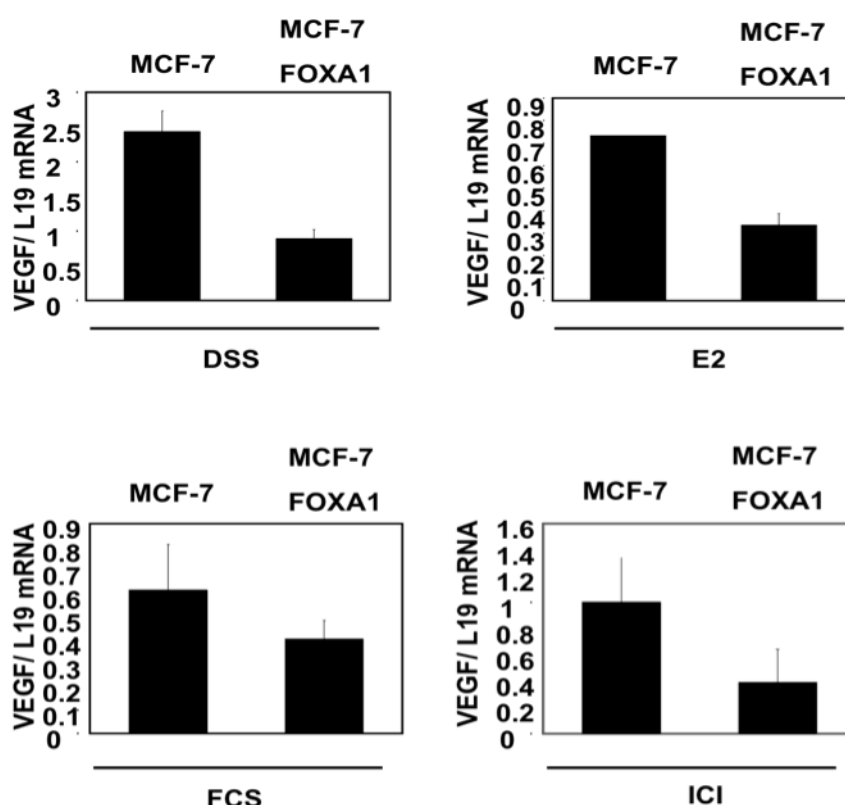


Figure 5.2 FOXA1 represses VEGF mRNA expression independently of ER α . MCF-7-FOXA1 overexpressing cells plated out on 6 cm dishes and treated with either Ethanol (control), E2 (10^{-9} nM) or ICI (10^{-7} nM) for 8 h. The cells treated with E2 were growing in an estrogen-free medium, to control the sole effect of added E2. The MCF-7 cells were used as a control to the FOXA1 overexpressing cells. Cells were harvested, RNA was extracted and after cDNA synthesis, RTqPCR was performed. L19 was used as a normalising control.

5.2.2 FOXC2 protein expression is repressed by FOXA1 in MCF-7 breast cancer cells.

As both FOXA1 and FOXC2 regulate VEGF with the exactly opposite results, we then assessed the possibility of those two factors to be associated in a regulatory relationship between them. It is already known that FOXA1 is a

good prognosis marker in breast cancer, being highly expressed in ER α positive tumours. In contrast FOXC2 is considered a poor prognosis factor and is associated with metastasis and invasiveness of breast tumour.

Indeed, in MCF-7-FOXA1 overexpressing cells, FOXC2 protein expression was significantly repressed. Actually, FOXA1 overexpression, resulted in the total absence of FOXC2 protein levels, depending on the sensitivity of the western blotting technique (Fig. 5.3A). Although there is a known regulatory response of ER α with FOXA1, this response was not a significant in the effect of FOXA1 in the repression of FOXC2 (Fig.5.3A). From the data we can consider ER α not a player of the FOXA1/FOXC2 interplay.

Moreover, to further test the relationship between FOXA1 and FOXC2, FOXA1 was silenced using RNAi. Silencing of FOXA1 in MCF-7 breast cancer cells promoted the induction of FOXC2 protein expression (Fig. 5.3B), reinforcing our hypothesis. Interestingly, VEGF expression followed a FOXC2 dependant expressional status, with the absence of FOXA1 favouring its increased protein levels.

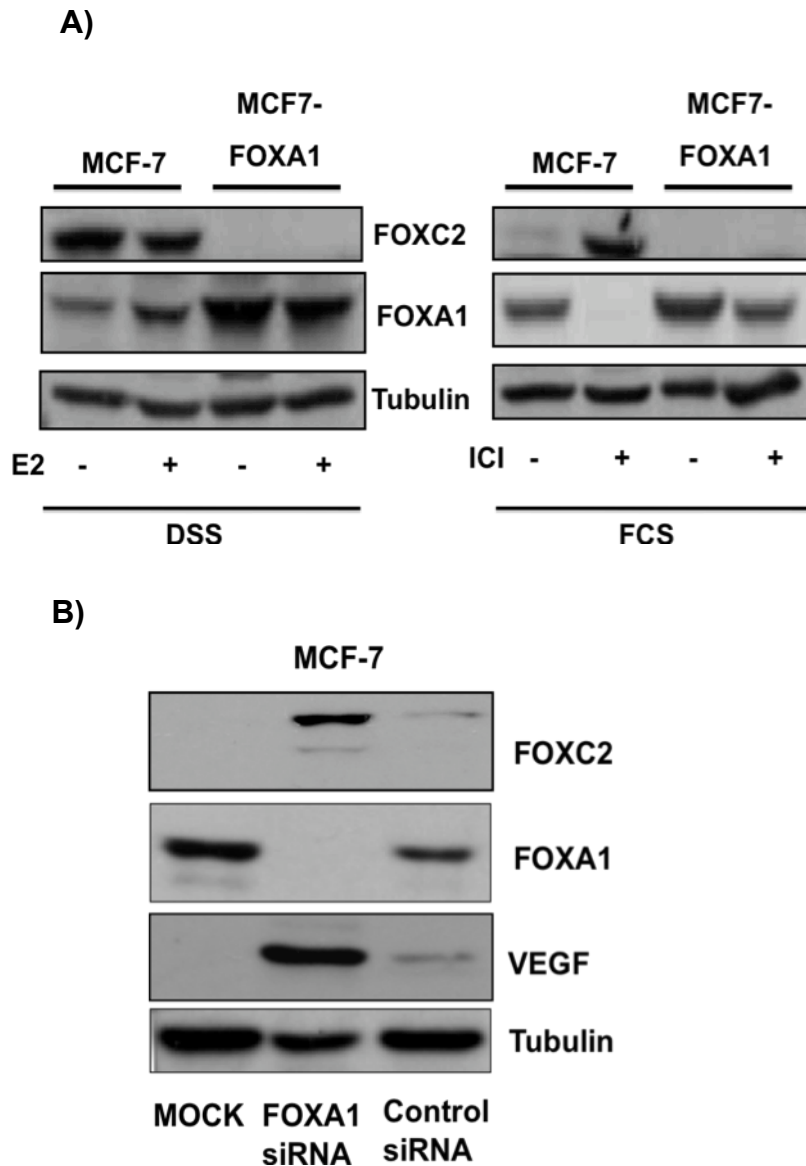


Figure 5.3 FOXA1 overexpression represses FOXC2 protein expression, independently of ER α , whereas its silencing enhances it. **A) MCF-7-FOXA1 and MCF-7 cells were plated out and treated with Ethanol, E2 (10^{-9} nM) or ICI (10^{-7} nM). Cells treated with E2 were growing in a estrogen-stripped medium. After harvesting, cells were lysed and proteins were separated by SDS-PAGE. Then were immunoblotted with FOXA1 and FOXC2 antibodies. Tubulin was used to ensure efficient loading. **B)** MCF-7 cells were plated in a 6-well plate, transfected with FOXA1 siRNA using oligofectamine, harvested after 48 h and lysed. Proteins were separated by SDS-PAGE and immunoblot analysis was done using the FOXA1, FOXC2, VEGF and ER α antibodies. Immunoblotting against Tubulin was used to ensure efficient loading. Non specific (control) siRNA was used to ensure targeted silencing efficiency.**

5.2.3 FOXA1 associates with the promoter of FOXC2, *in vitro* and *in vivo*.

To furthermore study the possible interaction of FOXA1 with FOXC2 we searched for possible FOXA1 consensus binding sites on its promoter. Indeed at the position -683 bp of the FOXC2 promoter the FOXA1 consensus TTTGTTTT was found.

To test this interaction, first, we performed oligonucleotide pull-down assay experiments with nuclear lysates from MCF-7 cells treated with either E2 or ICI. Western blot analysis of the pulled-down complexes showed that FOXA1, which was localized in the nucleus, interacts with FOXA1 binding site of the FOXC2 promoter (Fig. 5.4). A competition assay was then performed using non-biotinylated oligos in excess amounts to compete with the biotinylated oligos in the binding of the complex. The binding could be competed off suggesting that interaction depends on the FOXA1 site. In all cases of treatment FOXA1 was shown to bind on FOXC2, however as expected, the levels of FOXA1 in the cells after ICI treatment, were lower than in the cells treated with estrogen.

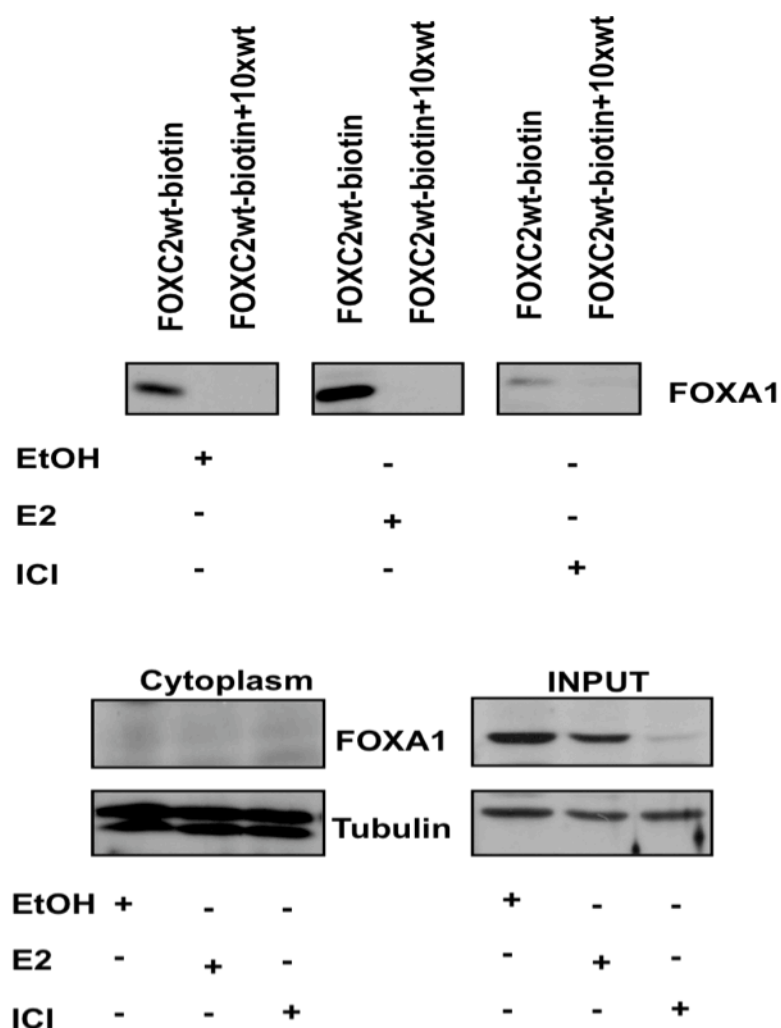


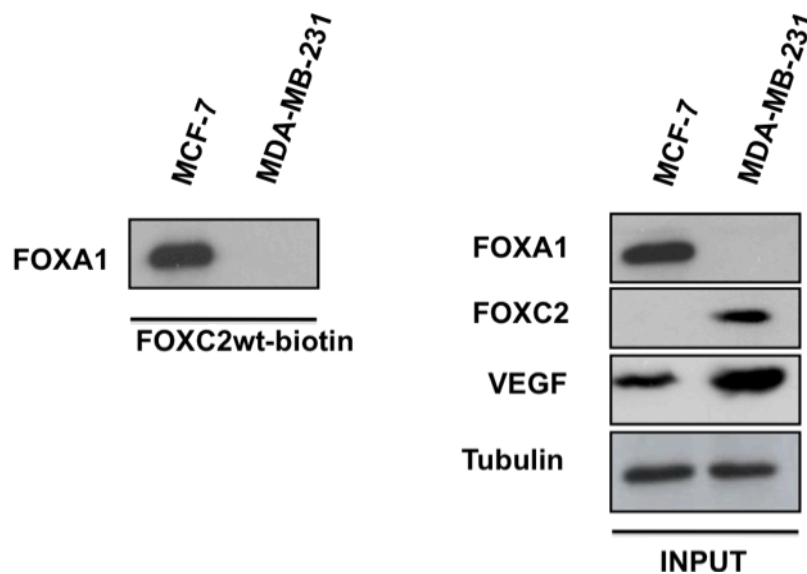
Figure 5.4 FOXA1 binds on the promoter of FOXC2. MCF-7 cells were treated with Ethanol, E2 (10^{-9} nM) or ICI (10^{-7} nM) for 8h, and proteins were pulled down using biotin-labelled oligos coupled to streptavidin/sepharose beads. The pulled-down complexes were then resolved by SDS-PAGE and immunoblotted using a FOXA1 antibody. The amount of FOXA1 in the cytoplasm was determined by western blotting after cytoplasmic/ nuclear fractionation. Tubulin was used as a loading control for both the cytoplasmic and whole cell extracts. Cells treated with E2 were growing in an estrogen-stripped medium. Biotin labeled oligos with excess amounts of site-specific oligonucleotides were used to validate the efficiency of the method.

Additionally, the interaction of FOXA1 with FOXC2 was tested in the ER-negative cell line MDA-MB-231. MDA-MB-231 cells express higher levels of both FOXC2 and VEGF, whereas FOXA1 is downregulated. FOXA1 did not show any interaction with FOXC2 by oligo-pulldown in the MDA-MB-231 cells line (Fig. 5.5A), however this was due to the fact that FOXA1 is repressed in these cells.

In order to determine if the FOXA1-FOXC2-VEGF relationship in MDA-MB-231 cells, FOXA1 was overexpressed by transfection with the FOXA1-pCDNA3 expression vector. Overexpression of FOXA1 resulted in the downregulation of both FOXC2 and VEGF protein expression (Fig 5.5B).

The idea that FOXA1, independently of ER α , is recruited on the FOXC2 promoter, driving its repression was reinforced. A clear connection between the expressional status and an interplay between these two forkhead transcription factors resulting in VEGF regulation, could be suggested.

A)



B)

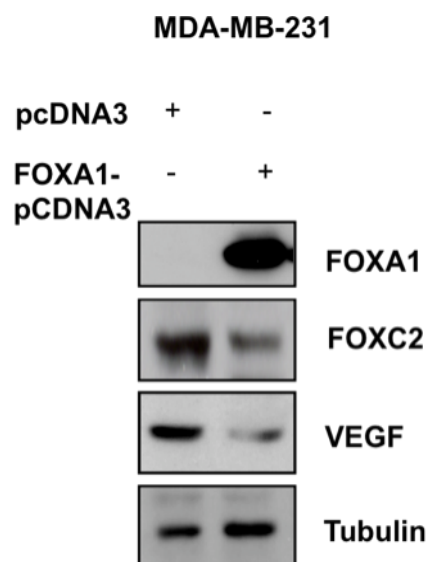


Figure 5.5 The regulation of VEGF by FOXA1 and FOXC2 in MDA-MB-231 cells. **A)** MCF-7 and MDA-MB-231 cells were used for pull down experiments using biotin-labelled oligos coupled to streptavidin/sepharose beads. The pulled-down complexes were resolved by SDS-PAGE and immunoblotted using a FOXA1 antibody. 1/10 of the extracts were used as an input and visualized by western blotting. Tubulin was used as a loading control. Biotin labeled oligos competing with oligos with excess amounts of the FOXA1 site were used to validate the efficiency of the method. **B)** MDA-MB-231 cells were transfected using oligofectamine, with the FOXA1-pCDNA3.1 expression vector. After 48 h the cells were harvested and lysed. After SDS-PAGE separation of the proteins, immunoblotting against FOXA1, FOXC2 and VEGF followed. Tubulin was used as a loading control.

ChIP was then performed to determine the FOXA1 occupancy on the FOXC2 promoter, *in vivo*. FOXA1 was at all different ER α status conditions bound on the promoter of FOXC2. Although as we had seen from previous experiments shown above, FOXA1 acts independently of ER α in the regulation of FOXC2 and subsequently VEGF, still its amounts are affected by ER α . So, FOXA1 binds on the FOXC2 promoter and regulates its transcription, even in small amounts of protein interaction, for example when cells are treated with ICI.

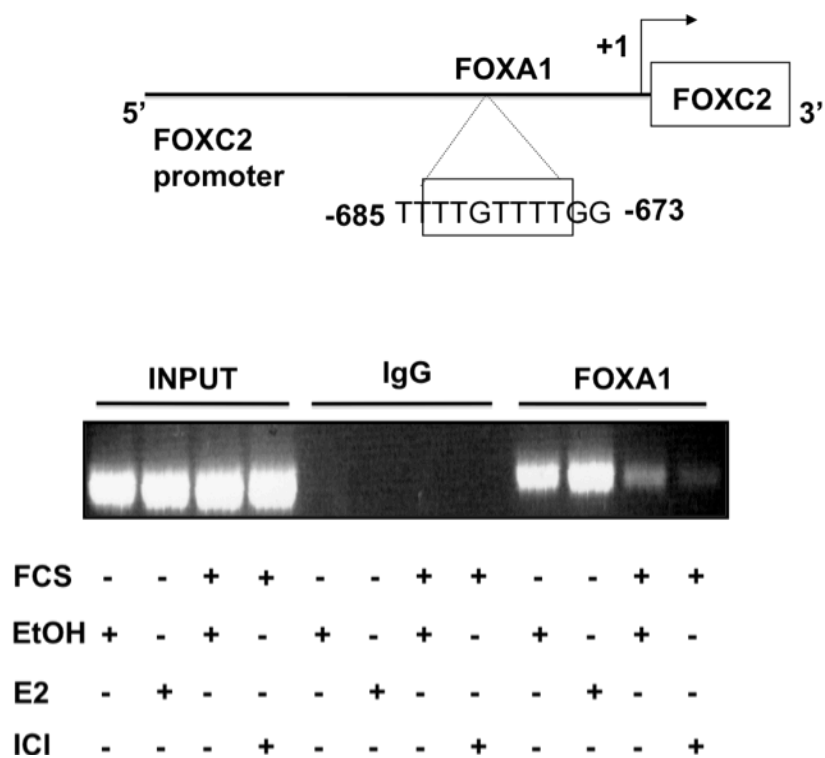


Figure 5.6 FOXA1 binds on the *FOXC2* promoter, *in vivo*. MCF-7 cells were plated out and treated with Ethanol, E2 (10^{-9} nM) or ICI (10^{-7} nM). Cells treated with E2 were growing in a estrogen- stripped medium. Protein-DNA complexes were cross-linked with formaldehyde. Chromatin fragments were undergone immunoprecipitation with a FOXA1 antibody and after de-crosslinking, the co-immunoprecipitated DNA was amplified by PCR and resolved in a 2% agarose gel visualized under UV.

5.3 DISCUSSION

The first steps of trying to understand how the FOXC2 may be regulated in breast cancer have already been made in this project. FOXA1, which is known to be working in close association with ER α in breast cancer and is generally an important transcription factor as it drives the transcription of an extremely high number of genes, was shown to highly associate to the promoter of FOXC2 driving its repression. This hypothesis was shown at both the transcriptional and translation level. This regulation is dependent on the association of FOXA1 to its consensus binding site of the FOXC2 promoter. Although, FOXA1 is associated to ER α status, its importance in the interaction and repression of FOXC2 was not that significant. FOXA1 overexpression was not affected by ER α degradation by ICI and even on the promoter area of FOXC2, FOXA1 was shown to interact, in lower levels however.

The event which was clearly observed is that the presence of FOXA1 in the nucleus of breast cancer cells dictates and is associated with a lower expression of FOXC2, and in reverse, the absence of FOXA1 by siRNA leads to the upregulation of FOXC2. This expressional pattern affects the expression of VEGF in the same exact way, proposing a mechanism of VEGF regulation which potentially could be assessed to target angiogenesis and invasiveness in breast cancer.

REFERENCES

Alland L, Muhle R, Hou H Jr, Potes J, Chin L, Schreiber-Agus N, DePinho RA. Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature*. 1997 May 1;387(6628):49-55.

Ali S, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. *Nat Rev Cancer*. 2002 Feb;2(2):101-12.

Azambuja E, Durbecq V, Rosa DD, Colozza M, Larsimont D, Piccart-Gebhart M, Cardoso F. HER-2 overexpression/amplification and its interaction with taxane-based therapy in breast cancer. *Ann Oncol*. 2008 Feb;19(2):223-32

Badve S, Turbin D, Thorat MA, Morimiya A, Nielsen TO, Perou CM, Dunn S, Huntsman DG, Nakshatri H. FOXA1 expression in breast cancer--correlation with luminal subtype A and survival. *Clin Cancer Res*. 2007 Aug 1;13(15 Pt 1):4415-21.

Barlev NA, Liu L, Chehab NH, Mansfield K, Harris KG, Halazonetis TD, Berger SL. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. *Mol Cell*. 2001 Dec;8(6):1243-54.

Bertos NR, Wang AH, Yang XJ. Class II histone deacetylases: structure, function, and regulation. *Biochem Cell Biol*. 2001;79(3):243-52.

Biggs WH 3rd, Cavenee WK, Arden KC. Identification and characterization of members of the FKHR (FOX O) subclass of winged-helix transcription factors in the mouse. *Mamm Genome*. 2001 Jun;12(6):416-25.

Bilancia D, Rosati G, Dinota A, Germano D, Romano R, Manzione L. Lapatinib in breast cancer. *Ann Oncol*. 2007 Jun;18 Suppl 6:vi26-30.

Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev.* 2002 Jan 1;16(1):6-21.

Boyle P, Ferlay J. Cancer incidence and mortality in Europe, 2004. *Ann Oncol.* 2005 Mar;16(3):481-8

Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T. Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature.* 1998 Feb 5;391(6667):597-601.

Buteau-Lozano H, Ancelin M, Lardeux B, Milanini J, Perrot-Applanat M. Transcriptional regulation of vascular endothelial growth factor by estradiol and tamoxifen in breast cancer cells: a complex interplay between estrogen receptors alpha and beta. *Cancer Res.* 2002 Sep 1;62(17):4977-84.

Buzdar AU, Coombes RC, Goss PE, Winer EP. Summary of aromatase inhibitor clinical trials in postmenopausal women with early breast cancer. *Cancer.* 2008 Feb 1;112(3 Suppl):700-9.

Cai J, Ahmad S, Jiang WG, Huang J, Kontos CD, Boulton M, Ahmed A. Activation of vascular endothelial growth factor receptor-1 sustains angiogenesis and Bcl-2 expression via the phosphatidylinositol 3-kinase pathway in endothelial cells. *Diabetes.* 2003 Dec;52(12):2959-68.

Carlsson P, Mahlapuu M. Forkhead transcription factors: key players in development and metabolism. *Dev Biol.* 2002 Oct 1;250(1):1-23.

Caron C, Boyault C, Khochbin S. Regulatory cross-talk between lysine acetylation and ubiquitination: role in the control of protein stability. *Bioessays.* 2005 Apr;27(4):408-15.

Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoutte J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M.

Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell*. 2005 Jul 15;122(1):33-43.

Charames GS, Bapat B. Genomic instability and cancer. *Curr Mol Med*. 2003 Nov;3(7):589-96

Chen G, Nomura M, Morinaga H, Matsubara E, Okabe T, Goto K, Yanase T, Zheng H, Lu J, Nawata H Modulation of androgen receptor transactivation by FoxH1. A newly identified androgen receptor corepressor. *J Biol Chem*. 2005 Oct 28;280(43):36355-63.

Chen JD, Evans RM. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*. 1995 Oct 5;377(6548):454-7.

Costa RH, Kalinichenko VV, Lim L. Transcription factors in mouse lung development and function. *Am J Physiol Lung Cell Mol Physiol*. 2001 May;280(5):L823-38.

Costa RH. FoxM1 dances with mitosis. *Nat Cell Biol*. 2005 Feb;7(2):108-10.

Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev*. 1999 Jun;20(3):358-417.

Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. *J Cell Physiol*. 2000 Jul;184(1):1-16.

de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J*. 2003 Mar 15;370(Pt 3):737-49.

Dejana E, Taddei A, Randi AM. Foxs and Ets in the transcriptional regulation of endothelial cell differentiation and angiogenesis. *Biochim Biophys Acta*. 2007 Jun;1775(2):298-312

Delpuech O, Griffiths B, East P, Essafi A, Lam EW, Burgering B, Downward J, Schulze A. Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression. *Mol Cell Biol*. 2007 Jul;27(13):4917-30.

Dutta U, Pant K. Aromatase inhibitors: past, present and future in breast cancer therapy. *Med Oncol*. 2008;25(2):113-24

Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer*. 2008 Aug;8(8):579-91.

Engel RH, Kaklamani VG. HER2-positive breast cancer: current and future treatment strategies. *Drugs*. 2007;67(9):1329-41.

Engström M, Karlsson R, Jönsson JI. Inactivation of the forkhead transcription factor FoxO3 is essential for PKB-mediated survival of hematopoietic progenitor cells by kit ligand. *Exp Hematol*. 2003 Apr;31(4):316-23.

Fang J, Dagenais SL, Erickson RP, Arlt MF, Glynn MW, Gorski JL, Seaver LH, Glover TW. Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary lymphedema-distichiasis syndrome. *Am J Hum Genet*. 2000 Dec;67(6):1382-8.

Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003 Jun;9(6):669-76.

Forbes JF, Cuzick J, Buzdar A, Howell A, Tobias JS, Baum M. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial. *Lancet Oncol*. 2008 Jan;9(1):45-53

Friedberg, E. C. DNA damage and repair. *Nature*. 2003 Jan 23;421(6921):436-40.

Friedman JR, Kaestner KH. The Foxa family of transcription factors in development and metabolism. *Cell Mol Life Sci*. 2006 Oct;63(19-20):2317-28.

Friedrichs K, Hölzel F, Jänicke F. Combination of taxanes and anthracyclines in first-line chemotherapy of metastatic breast cancer: an interim report. *Eur J Cancer*. 2002 Sep;38(13):1730-8.

Fry MJ. Structure, regulation and function of phosphoinositide 3-kinases. *Biochim Biophys Acta*. 1994 Jul 18;1226(3):237-68.

Fry MJ. Phosphoinositide 3-kinase signalling in breast cancer: how big a role might it play? *Breast Cancer Res*. 2001;3(5):304-12.

Fuckar D, Dekanić A, Stifter S, Mustać E, Krstulja M, Dobrila F, Jonjić N. VEGF expression is associated with negative estrogen receptor status in patients with breast cancer. *Int J Surg Pathol*. 2006 Jan;14(1):49-55.

Furuyama T, Nakazawa T, Nakano I, Mori N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J*. 2000 Jul 15;349(Pt 2):629-34.

Furuyama T, Kitayama K, Shimoda Y, Ogawa M, Sone K, Yoshida-Araki K, Hisatsune H, Nishikawa S, Nakayama K, Nakayama K, Ikeda K, Motoyama N, Mori N. Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. *J Biol Chem*. 2004 Aug 13;279(33):34741-9.

Gao L, Cueto MA, Asselbergs F, Atadja P. Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. *J Biol Chem*. 2002 Jul 12;277(28):25748-55

Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*. 1997 Aug 22;90(4):595-606.

Guidi AJ, Schnitt SJ, Fischer L, Tognazzi K, Harris JR, Dvorak HF, Brown LF. Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in patients with ductal carcinoma in situ of the breast. *Cancer*. 1997 Nov 15;80(10):1945-53.

Hader C, Marlier A, Cantley L. Mesenchymal-epithelial transition in epithelial response to injury: the role of Foxc2. *Oncogene*. 2010 Feb 18;29(7):1031-40.

Hayashi H, Sano H, Seo S, Kume T. The Foxc2 transcription factor regulates angiogenesis via induction of integrin beta3 expression. *J Biol Chem*. 2008 Aug 29;283(35):23791-800.

Hayes DF. Tamoxifen: Dr. Jekyll and Mr. Hyde? *J Natl Cancer Inst*. 2004 Jun 16;96(12):895-7.

Hildmann C, Wegener D, Riester D, Hempel R, Schober A, Merana J, Giurato L, Guccione S, Nielsen TK, Ficner R, Schwienhorst A. Substrate and inhibitor specificity of class 1 and class 2 histone deacetylases. *J Biotechnol*. 2006 Jun 25;124(1):258-70

Hoeben A, Ann Hoeben, Bart Landuyt, Martin S. Highley, Hans Wildiers, Allan T. Van Oosterom and Ernst A. De Bruijn. *harmacological Reviews* 2004 Dec;56(4):549-580

Horwitz KB, McGuire WL. Studies on mechanisms of estrogen and antiestrogen action in human breast cancer. *Recent Results Cancer Res*. 1980;71:45-58.

Hosaka T, Biggs WH 3rd, Tieu D, Boyer AD, Varki NM, Cavenee WK, Arden KC. Disruption of forkhead transcription factor (FOXO) family members in

mice reveals their functional diversification. *Proc Natl Acad Sci U S A*. 2004 Mar 2;101(9):2975-80.

Howell A, Dowsett M. Recent advances in endocrine therapy of breast cancer. *BMJ*. 1997 Oct 4;315(7112):863-6.

Hu MC, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H, Kobayashi R, Hung MC. I κ B kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell*. 2004 Apr 16;117(2):225-37.

Jain S, Singhal S, Lee P, Xu R. Molecular genetics of hepatocellular neoplasia. *Am J Transl Res*. 2010 Jan 23;2(1):105-18.

Järvinen TA, Peltö-Huikko M, Holli K, Isola J. Estrogen receptor beta is coexpressed with ERalpha and PR and associated with nodal status, grade, and proliferation rate in breast cancer. *Am J Pathol*. 2000 Jan;156(1):29-35.

Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell*. 2002 Jan 25;108(2):153-64.

Jonsson H, Allen P, Peng SL. Inflammatory arthritis requires Foxo3a to prevent Fas ligand-induced neutrophil apoptosis. *Nat Med*. 2005 Jun;11(6):666-71.

Kaestner KH, Knochel W, Martinez DE. Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev*. 2000 Jan 15;14(2):142-6

Katoh M, Katoh M. Human FOX gene family (Review). *Int J Oncol*. 2004 Nov;25(5):1495-500.

Konecny GE, Meng YG, Untch M, Wang HJ, Bauerfeind I, Epstein M, Stieber P, Vernes JM, Gutierrez J, Hong K, Beryt M, Hepp H, Slamon DJ, Pegram MD. Association between HER-2/neu and vascular endothelial growth factor

expression predicts clinical outcome in primary breast cancer patients. Clin Cancer Res. 2004 Mar 1;10(5):1706-16.

Korver W, Schilham MW, Moerer P, van den Hoff MJ, Dam K, Lamers WH, Medema RH, Clevers H. Uncoupling of S phase and mitosis in cardiomyocytes and hepatocytes lacking the winged-helix transcription factor Trident. Curr Biol. 1998 Dec 3;8(24):1327-30.

Kouzarides T. Histone acetylases and deacetylases in cell proliferation. Curr Opin Genet Dev. 1999 Feb;9(1):40-8.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A. 1998 Dec 22;95(26):15677-82.

Kume T. Foxc2 transcription factor: a newly described regulator of angiogenesis. Trends Cardiovasc Med. 2008 Aug;18(6):224-8.

Kwok JM, Myatt SS, Marson CM, Coombes RC, Constantinidou D, Lam EW. Thiostrepton selectively targets breast cancer cells through inhibition of forkhead box M1 expression. Mol Cancer Ther. 2008 Jul;7(7):2022-32.

Laoukili J, Kooistra MR, Brás A, Kauw J, Kerkhoven RM, Morrison A, Clevers H, Medema RH. FoxM1 is required for execution of the mitotic programme and chromosome stability. Nat Cell Biol. 2005 Feb;7(2):126-36.

Lee S, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, Ferrara N, Nagy A, Roos KP, Iruela-Arispe ML. Autocrine VEGF signaling is required for vascular homeostasis. Cell. 2007 Aug 24;130(4):691-703.

Leevers SJ, Vanhaesebroeck B, Waterfield MD. Signalling through phosphoinositide 3-kinases: the lipids take centre stage. Curr Opin Cell Biol. 1999 Apr;11(2):219-25.

Lehmann OJ, Sowden JC, Carlsson P, Jordan T, Bhattacharya SS. Fox's in development and disease. *Trends Genet.* 2003 Jun;19(6):339-44.

Lin L, Hron JD, Peng SL. Regulation of NF-kappaB, The activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity.* 2004 Aug;21(2):203-13.

Linderholm B, Tavelin B, Grankvist K, Henriksson R. Vascular endothelial growth factor is of high prognostic value in node-negative breast carcinoma. *J Clin Oncol.* 1998 Sep;16(9):3121-8.

Linderholm B, Grankvist K, Wilking N, Johansson M, Tavelin B, Henriksson R. Correlation of vascular endothelial growth factor content with recurrences, survival, and first relapse site in primary node-positive breast carcinoma after adjuvant treatment. *J Clin Oncol.* 2000 Apr;18(7):1423-31.

Lohela M, Bry M, Tammela T, Alitalo K. VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. *Curr Opin Cell Biol.* 2009 Apr;21(2):154-65.

Luo RX, Postigo AA, Dean DC. Rb interacts with histone deacetylase to repress transcription. *Cell.* 1998 Feb 20;92(4):463-73.

Ma RY, Tong TH, Cheung AM, Tsang AC, Leung WY, Yao KM. Raf/MEK/MAPK signaling stimulates the nuclear translocation and transactivating activity of FOXM1c. *J Cell Sci.* 2005 Feb 15;118(Pt 4):795-806.

Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, Kutok JL, Hartwell K, Richardson AL, Weinberg RA. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc Natl Acad Sci U S A.* 2007 Jun 12;104(24):10069-74.

Margolin K. Inhibition of vascular endothelial growth factor in the treatment of solid tumors. *Curr Oncol Rep*. 2002 Jan;4(1):20-8.

Marks PA, Richon VM, Rifkind RA. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *J Natl Cancer Inst*. 2000 Aug 2;92(15):1210-6.

Masumoto H, Hawke D, Kobayashi R, Verreault A. A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. *Nature*. 2005 Jul 14;436(7048):294-8.

Miller KD, Chap LI, Holmes FA, Cobleigh MA, Marcom PK, Fehrenbacher L, Dickler M, Overmoyer BA, Reimann JD, Sing AP, Langmuir V, Rugo HS. Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. *J Clin Oncol*. 2005 Feb 1;23(4):792-9.

Nelson RF, Glenn KA, Zhang Y, Wen H, Knutson T, Gouvion CM, Robinson BK, Zhou Z, Yang B, Smith RJ, Paulson HL. Selective cochlear degeneration in mice lacking the F-box protein, Fbx2, a glycoprotein-specific ubiquitin ligase subunit. *J Neurosci*. 2007 May 9;27(19):5163-71.

Olaussen KA, Commo F, Tailler M, Lacroix L, Vitale I, Raza SQ, Richon C, Dessen P, Lazar V, Soria JC, Kroemer G. Synergistic proapoptotic effects of the two tyrosine kinase inhibitors pazopanib and lapatinib on multiple carcinoma cell lines. *Oncogene*. 2009 Dec 3;28(48):4249-60

Overdier DG, Porcella A, Costa RH. The DNA-binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino-acid residues adjacent to the recognition helix. *Mol Cell Biol*. 1994 Apr;14(4):2755-66.

Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL, Leitman DC. Estrogen receptor beta inhibits human breast cancer cell proliferation and

tumor formation by causing a G2 cell cycle arrest. *Cancer Res.* 2004 Jan 1;64(1):423-8.

Pegram MD, Pienkowski T, Northfelt DW, Eiermann W, Patel R, Fumoleau P, Quan E, Crown J, Toppmeyer D, Smylie M, Riva A, Blitz S, Press MF, Reese D, Lindsay MA, Slamon DJ. Results of two open-label, multicenter phase II studies of docetaxel, platinum salts, and trastuzumab in HER2-positive advanced breast cancer. *J Natl Cancer Inst.* 2004 May 19;96(10):759-69.

Pierrou S, Hellqvist M, Samuelsson L, Enerbäck S, Carlsson P. Cloning and characterization of seven human forkhead proteins: binding site specificity and DNA bending. *EMBO J.* 1994 Oct 17;13(20):5002-12

Potente M, Urbich C, Sasaki K, Hofmann WK, Heeschen C, Aicher A, Kollipara R, DePinho RA, Zeiher AM, Dimmeler S. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J Clin Invest.* 2005 Sep;115(9):2382-92.

Price DJ, Miralem T, Jiang S, Steinberg R, Avraham H. Role of vascular endothelial growth factor in the stimulation of cellular invasion and signaling of breast cancer cells. *Cell Growth Differ.* 2001 Mar;12(3):129-35.

Roger P, Sahla ME, Mäkelä S, Gustafsson JA, Baldet P, Rochefort H. Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. *Cancer Res.* 2001 Mar 15;61(6):2537-41.

Ropero S, Fraga MF, Ballestar E, Hamelin R, Yamamoto H, Boix-Chornet M, Caballero R, Alaminos M, Setien F, Paz MF, Herranz M, Palacios J, Arango D, Orntoft TF, Aaltonen LA, Schwartz S Jr, Esteller M. A truncating mutation of HDAC2 in human cancers confers resistance to histone deacetylase inhibition. *Nat Genet.* 2006 May;38(5):566-9

Schneider BP, Miller KD. Angiogenesis of breast cancer. *J Clin Oncol.* 2005 Mar 10;23(8):1782-90.

Schneider BP, Sledge GW Jr. Drug insight: VEGF as a therapeutic target for breast cancer. *Nat Clin Pract Oncol*. 2007 Mar;4(3):181-9.

Sengupta PP, Northfelt DW, Gentile F, Zamorano JL, Khandheria BK. Trastuzumab-induced cardiotoxicity: heart failure at the crossroads. *Mayo Clin Proc*. 2008 Feb;83(2):197-203.

Seo S, Fujita H, Nakano A, Kang M, Duarte A, Kume T. The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. *Dev Biol*. 2006 Jun 15;294(2):458-70.

Skiris GP, Carder PJ, Lansdown MR, Speirs V. Immunohistochemical detection of ERbeta in breast cancer: towards more detailed receptor profiling? *Br J Cancer*. 2001 Apr 20;84(8):1095-8.

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001 Mar 15;344(11):783-92.

Sloan B, Scheinfeld NS. Pazopanib, a VEGF receptor tyrosine kinase inhibitor for cancer therapy. *Curr Opin Investig Drugs*. 2008 Dec;9(12):1324-35.

Slupphaug G, Kavli B, Krokan HE. The interacting pathways for prevention and repair of oxidative DNA damage. *Mutat Res*. 2003 Oct 29;531(1-2):231-51.

Smith I, Chua S. Medical treatment of early breast cancer. *BMJ*. 2006 Jan 28;332(7535):223-4

Sommer S, Fuqua SA. Estrogen receptor and breast cancer. *Semin Cancer Biol.* 2001 Oct;11(5):339-52.

Spigel DR, Burstein HJ. HER2 overexpressing metastatic breast cancer. *Curr Treat Options Oncol.* 2002 Apr;3(2):163-74.

Stern DF. Tyrosine kinase signalling in breast cancer: ErbB family receptor tyrosine kinases. *Breast Cancer Res.* 2000;2(3):176-83.

Thorat MA, Marchio C, Morimiya A, Savage K, Nakshatri H, Reis-Filho JS, Badve S. Forkhead box A1 expression in breast cancer is associated with luminal subtype and good prognosis. *J Clin Pathol.* 2008 Mar;61(3):327-32.

van der Heide LP, Smidt MP. Regulation of FoxO activity by CBP/p300-mediated acetylation. *Trends Biochem Sci.* 2005 Feb;30(2):81-6.

Vigushin DM, Coombes RC. Histone deacetylase inhibitors in cancer treatment. *Anticancer Drugs.* 2002 Jan;13(1):1-13.

Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med.* 2004 Aug;10(8):789-99.

Wang X, Kiyokawa H, Dennewitz MB, Costa RH. The Forkhead Box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. *Proc Natl Acad Sci U S A.* 2002 Dec 24;99(26):16881-6.

Weigel D, Jürgens G, Küttner F, Seifert E, Jäckle H. The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the *Drosophila* embryo. *Cell.* 1989 May 19;57(4):645-58.

Wijchers PJ, Burbach JP, Smidt MP. In control of biology: of mice, men and Foxes. *Biochem J.* 2006 Jul 15;397(2):233-46.

Wood ER, Truesdale AT, McDonald OB, Yuan D, Hassell A, Dickerson SH, Ellis B, Pennisi C, Horne E, Lackey K, Alligood KJ, Rusnak DW, Gilmer TM, Shewchuk L. A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res.* 2004 Sep 15;64(18):6652-9.

Xuan Z, Zhang MQ. From worm to human: bioinformatics approaches to identify FOXO target genes. *Mech Ageing Dev.* 2005 Jan;126(1):209-15.

Yang H, Zhao R, Yang HY, Lee MH. Constitutively active FOXO4 inhibits Akt activity, regulates p27 Kip1 stability, and suppresses HER2-mediated tumorigenicity. *Oncogene.* 2005 Mar 10;24(11):1924-35.

Yang WM, Tsai SC, Wen YD, Fejer G, Seto E. Functional domains of histone deacetylase-3 *J Biol Chem.* 2002 Mar 15;277(11):9447-54.

Yoo CB, Jones PA. Epigenetic therapy of cancer: past, present and future. *Nat Rev Drug Discov.* 2006 Jan;5(1):37-50.

Yoshiji H, Gomez DE, Shibuya M, Thorgeirsson UP. Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. *Cancer Res.* 1996 May 1;56(9):2013-6.

Zhang Y, Zhang N, Dai B, Liu M, Sawaya R, Xie K, Huang S. FoxM1B transcriptionally regulates vascular endothelial growth factor expression and promotes the angiogenesis and growth of glioma cells. *Cancer Res.* 2008 Nov 1;68(21):8733-42.

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