# ERα, HOXC11 and Breast Cancer: The Good, the Bad and the Ugly

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CITATION

Cocchiglia, Sinéad (2012): ERα, HOXC11 and Breast Cancer: The Good, the Bad and the Ugly. Royal College of Surgeons in Ireland. Thesis. https://doi.org/10.25419/rcsi.10819268.v1

DOI

10.25419/rcsi.10819268.v1

# ERα, HOXC11 and Breast Cancer:

# The Good, the Bad and the Ugly


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Thesis submitted to the Royal College of Surgeons in Ireland for the Degree of Master of Science

November 2012

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree, Master of Science (MSc) is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed Sinead Cocchiglia

RCSI Student Number: 07212836

Date:

# Acknowledgments

There are many people that I would like to thank who have helped and encouraged me throughout the course of this research.

First and foremost I wish to thank my supervisor, Dr. Leonie Young to which I am forever grateful. Thank you for your patience and guidance and the opportunity for allowing me to carry out this research in the first place. Without your faith in me and encouragement this thesis would never have come into existence.

I would also like to thank Professor Arnold Hill for his support and especially to his patients who kindly provide us with invaluable samples to further our studies. A special thank you also, to Breast Cancer Ireland who has supported this research from the very beginning.

To the posties- firstly a heart warming thanks to Dr. Jean McBryan. I am in awe of your patience and ability to make sense of out of absolutely everything scientific and unscientific alike! Words cannot thank you enough for the input and help you have given me throughout the final months. Dr. Marie McIlroy, your scientific thinking is something I could only ever wish to aspire to. You are a fountain of HOX knowledge and have been there from the very beginning to answer all of my questions over and over again. I appreciate the help you have given me throughout this entire research and have enjoyed all of our nerdy HOX-y, epigenetic-y conversations...no matter how confusing they may get!

Sudipto, thank you for the time and effort you gave me and for being at the end of every phone call and text message to answer all of my methylation questions.

To the Wolfpack (honorary member Claire included!). If it's one thing that got me over the final hurdle, it's the fantastic coffee break times we all shared together. There have been tears and stress and a lot of laughter (and slaggings), actually mainly bullying and slagging, but still, I couldn't have shared these times with better colleagues just kidding, friends! Long lab weekends were never lonely when you saw Claire Bear's (Dr. Claire Walsh) friendly face at the bench. HOX blots were more fun

when competing for the Olympic gold HOX medal against Amy (Dr. Éamon Hughes). Stress levels were always washed away when hanging out with Dolly, ok, ok Big D (Damir Vareslija). Break times have never been tastier since jLo (Jarlath Bolger) got hit with the baking bug. Keep it up and you may win a Golden sieve this year!
Special thank you to Fiona T. Bane, you are the core and hub of lab daily life. Without you, the lab would surely fall apart. To lab friends past and present who have each helped me along the way, a heart warming thank you to Dr. Christopher Byrne, Dr. Sarah Theissen and Dr. Aisling Redmond.

To my family, grazie per l'ncoraggiamento per finire, sopratutto grazie a Brunella che non lasciarmi sofrire di fame quando scrivevo! Finalmente, dedico questa tesi a la mia cugina Annamaria, che a demonstrato forza e coraggio durante la sua malattia con cancro al seno. Lei è fonte di ispirazione per tutte le donne. Xx

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# Abbreviations

| | |
|---|---|
| **4-OHT** | 4-hydroxytamoxifen |
| **AD** | Activation domain |
| **AF** | Activation function |
| **AI** | Aromatase Inhibitor |
| **AR** | Androgen receptor |
| **ATCC** | American Tissue Type Collection |
| **bp** | Base pair |
| **BRCA** | Breast cancer susceptibility gene |
| **BSA** | Bovine serum albumin |
| **CBP** | CREB-binding protein |
| **cDNA** | Complementary DNA |
| **CDS-FCS** | Charcoal dextran-stripped fetal calf serum |
| **ChIP** | Chromatin Immunoprecipitation |
| **Co-IP** | Co-Immunoprecipitation |
| **DAPI** | 4',6-diamidino-2-phenylindole dihydrochloride |
| **DBD** | DNA binding domain |
| **DCIS** | Ductal carcinoma *in situ* |
| **DFS** | Disease free survival |
| **dH2O** | Distilled water |
| **DMEM** | Dulbecco's modified Eagle's medium |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **DNMT** | DNA methyltransferase |
| **dNTP** | Deoxyribonucleotide triphosphate |
| **ECL** | Enhanced chemiluminescent reagent |
| **ECM** | Extracellular matrix |
| **EGF** | Epidermal growth factor |
| **EGFR** | Epidermal Growth Factor Receptor |
| **EMT** | Epithelial mesenchymal transition |
| **ER** | Estrogen Receptor |
| **Erb** | Erythroblastic Leukemia Viral Oncogene |
| **ERK** | Extracellular signal-regulated kinase |

| | |
|---|---|
| **ERE** | Estrogen response element |
| **EZH2** | Enhancer of Zest 2 homologue |
| **FBS** | Foetal bovine serum |
| **FCS** | Foetal calf serum |
| **FDR** | False discovery rate |
| **FGF** | Fibroblast growth factor |
| **G-418** | Geneticin sulphate |
| **GFR** | Growth factor receptor |
| **GR** | Glucocorticoid receptor |
| **HAT** | Histone acetyltransferase activity |
| **HDAC** | Histone Deacetylase |
| **HER2** | Human Epidermal Growth Factor Receptor 2 |
| **HMT** | Histone methyltransferase |
| **HRP** | Horseradish peroxidase |
| **Ig** | Immunoglobulin |
| **IGF** | Insulin Growth Factor |
| **IHC** | Immunohistochemistry |
| **IMS** | Industrial methylated spirits |
| **kD** | Kilodalton |
| **LB** | Lysis buffer |
| **LBD** | Ligand binding domain |
| **LetR** | Letrozole resistant Aro cells |
| **LiCl** | Lithium Chloride |
| **lncRNA** | long non coding RNA |
| **MAPK** | Mitogen activated protein kinase |
| **MEM** | Minimum essentials medium |
| **ml** | millilitre |
| **MgCl2** | Magnesium chloride |
| **mRNA** | messenger RNA |
| **N** | Experimental number |
| **NR** | Nuclear receptor |
| **NRID** | Nuclear receptor interacting domain |
| **p** | p-value |
| **PBS** | Phosphatase buffered saline |

| | |
|---|---|
| **PCR** | Polymerase Chain Reaction |
| **PRC2** | Polycomb repressor complex 2 |
| **PI3K** | Phosphatidylinositol-3 kinase |
| **PR** | Progesterone Receptor |
| **qPCR** | Quantitative PCR |
| **RNA** | Ribonucleic acid |
| **RTK** | Receptor tyrosine kinase |
| **RT-PCR** | Reverse transcriptase polymerase chain reaction |
| **SDS** | Sodium dodecyl sulphate |
| **SDS-PAGE** | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| **siRNA** | small interfering RNA |
| **SERD** | Selective ER downregulators |
| **SERM** | Selective ER Modulators |
| **SRC** | Steroid receptor coactivator |
| **TAE** | Tris acetate-EDTA |
| **TBS** | Tris buffered saline |
| **TF** | Transcription factor |
| **TK** | Tyrosine kinase |
| **TKI** | Tyrosine kinase inhibitor |
| **TMA** | Tissue microarray |
| **TSS** | Transcriptional start site |
| **Tyr** | Tyrosine |
| **v/v** | Volume by volume |
| **VEGF** | Vascular endothelial growth factor |
| **w/v** | Weight by volume |
| **WHO** | World Health Organisation |
| **µl** | Microlitre |
| **µM** | Micrometer |
| **%** | Percent |

# Abstract

**Introduction:**

In Ireland breast cancer one of the most common causes of cancer related death for women. Upon diagnosis approximately two thirds of patients will present with a hormone receptor positive tumour (ER+). The ER pathway plays a pivotal role in breast cancer development and progression. Consequently endocrine therapies to block ER signalling are one of the most prevailing and effective treatment methods to date. However, a growing body of evidence suggests that within a heterogeneous breast tumour a sub-set of pluri-potent cells are capable of evading therapeutic treatment. Subsequently tumours metastasise and patients develop *de novo* or acquired resistance to endocrine therapies. Continuous activation of growth factor signalling pathways can provide tumours with altered proliferative and survival stimuli. Deregulated epigenetic machinery has also been implicated in the development of resistance. Translational studies from our group and others, has identified the steroid receptor co-activator SRC-1 to be master transcriptional regulator of breast cancer disease progression. Further studies identified the developmental protein and transcription factor HOXC11 as an interacting partner of SRC-1. Interactions between HOXC11 and SRC-1 in breast cancer cell lines and tissue have previously been reported. Their relationship has been shown to be important in tumour progression and metastasis in tamoxifen treated patients. Aberrant expression of HOXC11 has been associated with endocrine resistance. HOXC11 is known to drive the expression of the secreted protein S100β. Both HOXC11 and S100β have been described as tissue and serum biomarkers which can predict response to endocrine therapy. To date however, a functional role for HOXC11 in breast cancer has not been established.

**Hypothesis:**

The hypothesis of this thesis is to investigate a functional role for HOXC11 in an endocrine resistant breast cancer phenotype. It will investigate how this developmental transcription factor evades endocrine therapies in order to promote malignant transformation and it will explore the relationship between HOXC11 and ERα and the significance of this relationship at a clinical level.

**Results:**

Aberrant expression of HOXC11 we found confers a malignant phenotype similar to that of the known LY2 tamoxifen resistant cells. We found MCF7 endocrine sensitive breast cancer cells with overexpressed HOXC11 displayed a loss of differentiation and cellular polarity. HOXC11 alone induces proliferation, cell motility and anchorage independence. We observed a novel relationship between HOXC11 and the estrogen receptor alpha (ERα) and found that ERα could tame the aggressive malignant transformations induced by HOXC11. We observed that HOXC11 overexpression downregulates transcriptional regulation of ERα and propose a mechanism of epigenetic regulation in which HOXC11 may be silencing ERα activity. S100β a secreted protein and known HOXC11 target gene was also found to be a target of ERα and we observed HOXC11 and ERα compete for the regulation of S100β depending on the cellular microenvironment.

**Conclusion:**

HOXC11 can mediate malignant transformation and ERα in part can compete with HOXC11 to tame this aggressive phenotype. Tumour transformation is due in part to the altered therapeutic target. At a clinical level this is of significance as we have opened up new avenues in which resistant tumours could be targeted with alternative drug therapies.

# Chapter 1

# Introduction to Breast Cancer and Homeobox genes

## 1.1 Breast Cancer epidemiology

Breast cancer incidence fluctuates widely throughout the world and recent figures confirm Europe and the U.S to have the highest incidence rate (Jemal, Center et al. 2010). Although genetics may play a role, the high prevalence of breast cancer in the western world is thought to be a reflection of the increased risk factors associated with the disease in these countries (Parkin and Fernandez 2006). At the same time however, breast cancer mortality rates in these western countries has been stabilising over the past 25 years due to early detection methods and improved treatment care (Jemal, Center et al. 2010).

In Ireland we rank fourth amongst the 27 European countries for incidence and mortality rates of breast cancer, (Figure 1.1) (National Cancer Registrar Ireland, 2011 (NCRI)). According to the latest report from the NCRI in 2011, breast cancer is the foremost diagnosed cancer in Ireland accounting for 23% of all female invasive cancers (excluding non-melanoma skin cancer). After lung cancer, it has the second highest mortality rate (NCRI 2011). Current treatments are increasing life expectancy after diagnosis but advances in personalised medicine would enhance survival rates far better. A greater need to understand the inner workings of the disease is warranted to improve targeted therapies.

## 1.2 Risk factors involved in Breast Cancer

The female mammary gland is an extraordinary plastic organ, in that unlike other organs it does not fully develop during embryogenesis, instead reaches maturity post-pubescently. Mammary gland maturity is divided into many phases of development and differentiation and these processes are all under tight molecular supervision. Deregulation of these processes can ultimately lead to a malignant tumour in the breast. Breast cancer is a heterogeneous disease that originates from the epithelial cells lining the milk ducts (Polyak 2011). Cancer progression is a dynamic process, arising from one single cancer cell, which undergoes clonal expansion and constant genetic and epigenetic alterations, until finally a clinical diagnosis of a tumour is made (Almendro and Fuster 2011). The nature of cellular diversity within a tumour itself is a reflection of the many risk factors at play.

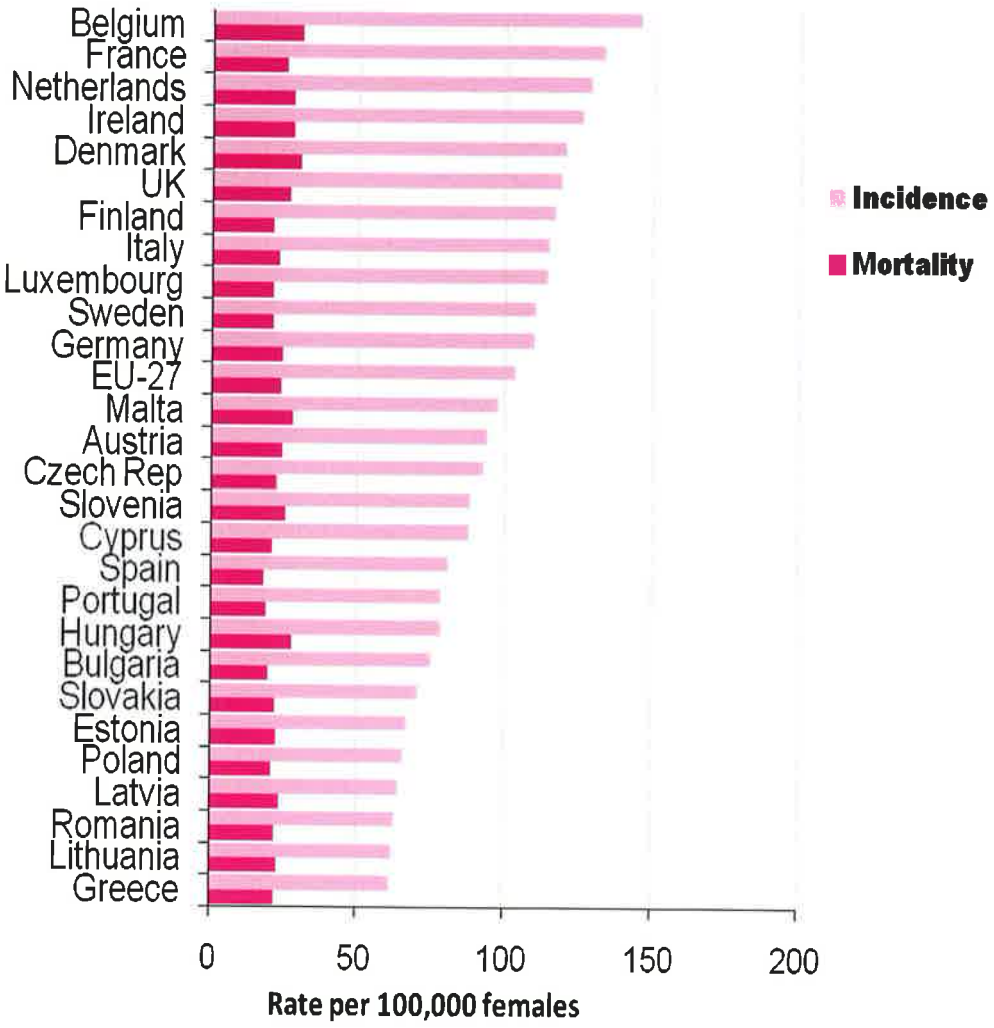


***Figure 1.1 European comparisons of incidence and mortality rates in female breast cancer*** *(National Cancer Registrar Ireland, 2011).*

A number of the risk factors such as, genetics, age, lifestyle, obesity and family history, are thought to play a role in the development of breast cancer (Table 1.1). Some of the higher associated risks for developing the disease include increased levels of reproductive hormones. This is very often associated with early menarche, late child baring age, fewer pregnancies and use of hormonal therapies (Parkin and Fernandez 2006). High levels of endogenous estrogen or testosterone in postmenopausal women doubles the chance of acquiring breast cancer compared to women with lower circulating levels (Hulka and Moorman 2001). Oral contraceptive and hormone replacement therapy users are also at higher risk compared to non-

users. Women who breast feed are thought to acquire a protective barrier against breast cancer due to lower levels of ovarian hormone production (IARC, 2008, Lacey et al., 2009). Obesity in postmenopausal women increases the risk of breast cancer as estrogen is produced mainly in adipose fat tissue in these women (American Cancer Society, 2011-2012). Specific germline mutations like the BRCA1 and BRCA2 mutations account for 15-20% of the observed risk of breast cancer. Other mutated genes such as p53 or PTEN (phosphatase and tensin) both tumour suppressor genes, account for an even smaller fraction of the risk (Venkitaraman 2002). Men can also be affected by breast cancer although incidence rates are rare and only account for approximately 1% of all cases (NCRI).

***Table 1.1 Factors that increase the risk of breast cancer in women.*** *(Breast cancer Facts and Figures- 2011-2012 (American Cancer Society, 2012).*

| Relative Risk | Factor |
|---|---|
| >4.0 | • Age (65+ vs. <65 years, although risk increases across all ages until age 80)<br>• Biopsy-confirmed atypical hyperplasia<br>• Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2)<br>• Mammographically dense breasts<br>• Personal history of breast cancer |
| 2.1-4.0 | • High endogenous estrogen or testosterone levels<br>• High bone density (postmenopausal)<br>• High-dose radiation to chest<br>• Two first-degree relatives with breast cancer |
| 1.1-2.0 | • Alcohol consumption<br>• Ashkenazi Jewish heritage<br>• Early menarche (<12 years)<br>• Height (tall)<br>• High socioeconomic status<br>• Late age at first full-term pregnancy (>30 years)<br>• Late menopause (>55 years)<br>• Never breastfed a child<br>• No full-term pregnancies<br>• Obesity (postmenopausal)/adult weight gain<br>• One first-degree relative with breast cancer<br>• Personal history of endometrium, ovary, or colon cancer<br>• Recent and long-term use of menopausal hormone therapy containing estrogen and progestin<br>• Recent oral contraceptive use |

## 1.3 Breast Cancer subtypes

Breast cancer is a complex heterogeneous disease which harbours distinct clinical, morphological and molecular characteristics. Clinical parameters such as tumour size, lymph node metastasis, histological grade and receptor status such as ER (estrogen receptor) PR (progesterone receptor) and HER2 are no longer adequate for identification of a particular tumour type (Guedj, Marisa et al. 2011). Over the past decade technological advances and high throughput technologies have given us the ability to study large numbers of breast cancer samples and our understanding of the biology of the disease has therefore improved immensely. Classification of tumours is now joined by gene expression profiling. This classification system refines the old

histopathological system and adds a molecular perspective. Expression profiling allows for the analysis of thousands of genes in one sample and creates a molecular tumour profile. Some of the more well known platforms include: MammaPrint®, Oncotype DX®, the wound response model, the rate of two genes model (HOXB13:IL17BR) and the intrinsic subtype model (Eroles, Bosch et al. 2012). None of these platforms share common gene profiles however a comparative study of the top five recognised platforms display similarities. They all predict disease free survival (DFS) except for the rate of two genes model (Eroles, Bosch et al. 2012). The biggest drawbacks to these platforms are cost and the need for fresh-frozen tumour samples for some platforms. Therefore, not all of these gene classification systems are clinically relevant, as standardised practises are not yet in place.

### 1.3.1 Clinical breast cancer subtypes

A pathological staging system for grading breast tumours is used in the clinic to help practitioners make decisions for the appropriate therapy to be administered. The most common system followed is the American Joint Committee on Cancer (AJCC) which set up the "TNM staging system" for breast cancer. It classifies breast tumours into T, N or M (tumour, lymph node and metastasis status, respectively) with numbers allocated after the letter to indicate severity of the tumour (Table 1.2). An example for the primary tumour, T stage can be seen in Figure 1.2.

Therapeutic decisions are also made according to receptor status (ER/PR/HER2), menopausal status and general health of the patient. Histoclinical breast cancer classification however is still somewhat inadequate for grading such heterogeneous tumours. Today the most biologically valid molecular tumour classification system in place is the intrinsic subtype model. It classifies tumours into five molecular subtypes.

***Table 1.2 TNM staging system in breast cancer.*** *(AJCC- Breast Cancer Staging)*

| Tumour Status | | Status of the Lymph Nodes | |
|---|---|---|---|
| T0 | No palpable tumour | N0 | No palpable axillary nodes |
| T1 | Tumour <2cm with no fixation to underlying muscle | N1a | Palpable nodes not thought to contain tumour |
| T2 | Tumour >2cm but <5cm with no fixation | N1b | Palpable nodes thought to contain tumour |
| T3 | Tumour maximum diameter >5cm | N2 | Nodes >2cm or fixed to one another and deep structures |
| T4 | Tumour of any size with fixation to the chest wall or ulceration of skin | N3 | Supraclavicular or infraclavicular nodes |
| Metastases | | | |
| M0 | No clinically apparent metastases | | |
| M1 | Distant metastases are present | | |

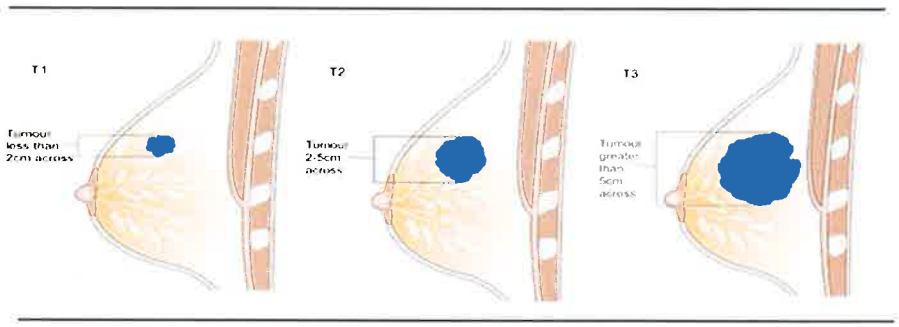


***Figure 1.2 TNM classification (T) stage in a primary breast tumour.*** *(Adapted from Cancer Research UK http://cancerhelp.cancerresearchuk.org)*

### 1.3.2 Molecular breast cancer subtypes

For clinicians to provide personalised treatment, the need to identify specific molecular markers is warranted. Even though tumours initially respond to anti-cancerous drugs, very often tumour cells reach a point of drug resistance. Advances in technology have allowed us to delve deeper into a tumour at a molecular level and the five major breast cancer molecular subtypes revealed are:

- **Luminal A:** The most common subtype, characterised by ER+, PR+, Bcl-2 and cytokeratin 8/18 expression and are HER2-negative. These tumours have a low proliferation rate as measured by ki67 and high GATA3 expression. Patients usually have a good prognosis and recurrent patients have a distinct pattern of bone metastasis. Treatment of choice for this subtype are, hormonal therapies including, aromatase inhibitors (AI) and selective estrogen receptor modulators (SERMS).
- **Luminal B:** This subtype makes up for about 10-20% of patients. Characterised by a more aggressive phenotype compared to luminal A tumours with a higher proliferative index and histological grade. Tumours are ER positive and PR positive or negetive. The main difference between the luminal A and B subtype is the higher level of expression in proliferative genes (MK167 and cyclin B1). Tumours often express EGFR (epidermal growth factor) and HER2; however some tumours (6%) are ER-/HER2-. Luminal B tumours have a worse prognosis; recurrent patterns are similar to the Luminal A type however they show a distinct pattern of liver metastasis. Treatment of choice for this subtype is neoadjuvant chemotherapy.
- **HER2 positive** Representing 15-20% of breast cancers these tumours have amplification and high expression of the ErbB2 gene (and genes associated with the ErbB2 pathway). Characteristically these tumours have a high histological grade and are highly proliferative with 40% of them having p53 mutations. Patients have a poor prognosis (although anti-HER2 treatment has improved this). Treatment of choice is also neoadjuvant chemotherapy.
- **Basal -like** Representative of 10-20% of tumours. These are hormone receptor negative, ER-, PR- and HER2- (termed triple negative). They express normal myoepithelial genes such as cytokeratins CK5, CK17, P-cadherin, CD44 and EGFR and express genes characteristic of luminal epithelial cells, CK8/18 and Kit. Often these tumours have a high rate of p53 mutations.

Clinically tumours present at an early age in women of African origin, where the tumours are generally large at diagnosis, with high histological grade and manifest with lymph node metastasis. Patients have a worse prognosis despite responding well initially to chemotherapy. Mutated BRCA1 tumours are also classed as Basal-like. Promising treatments against these aggressive tumours include the PARP-1 inhibitors.

- **Claudin-low** Newly characterised in 2007 (Hulit, Suyama et al. 2007) making up about 12-14% of breast tumours. They express low levels of genes associated with tight junctions and intercellular adhesion (such as claudin 3/4/7 and E-cadherin). Characteristically this subtype is similar hierarchically to the basal subtype. They express low levels of HER2 and clinically they are a high histological grade infiltrating ductal carcinoma displaying poor prognosis to neoadjuvant chemotherapy.

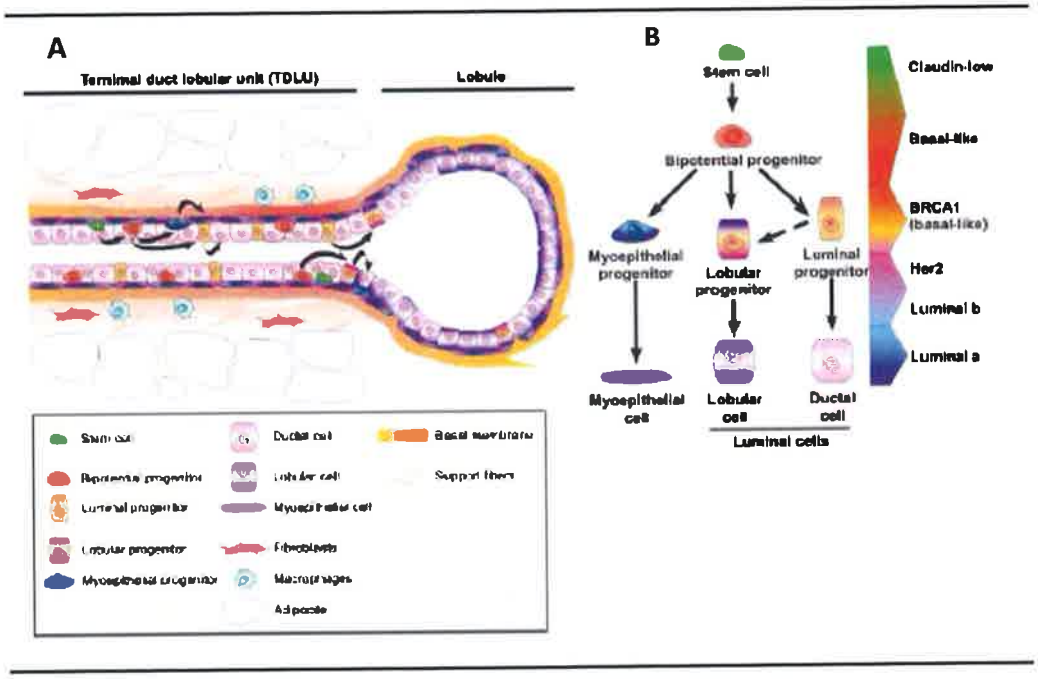


***Figure 1.3 Hierarchy of the normal mammary epithelium and proposed origin for different breast cancer cell subtypes.*** A) The mammary gland composed of branching networks of ducts and lobules. The epithelium contains a luminal layer of cells (ductal and lobular) surrounded by a basal layer of myoepithelial cells. Black arrows indicate areas that can differentiate into mature ductal, lobular

and myoepithelial cells. The surrounding basal membrane is rich in stroma, support fibres and other cell types. B) Breast cancer stem cells will give rise to potential progenitors, which differentiate further into lineage restricted progenitors generating new ductal, lobular and myoepithelial cells. Cells from the mammary gland at different stages of differentiation will be the cells of origin for the different breast cancer subtypes, adapted from (Almendro and Fuster 2011).

## 1.4 Endocrine Regulated Signalling

Mammary gland development occurs predominantly after birth and every cycle of proliferation and differentiation thereafter is under control of steroid and peptide hormones mainly estrogen and progesterone (produced mostly by the ovaries) (Shyamala 1997). Estrogen exists in three major forms, estrone (E1), estradiol (E2) and estriol (E3). Each form dominating at various periods of a women's lifecycle. E1 is the predominant circulating estrogen in menopausal women, E2 is the major form during the reproductive years and E3 is dominant during pregnancy (Shyamala 1997). The hormone estradiol (from now on will be referred to as estrogen or E2) and the ER pathway plays a vital role in the progression of breast cancer. This is due to the majority of breast tumours starting off as estrogen dependent (Saha Roy and Vadlamudi 2011). The biological effect of estrogen is mediated via its binding to the ER and as a result activates a multitude of signalling pathways. The ER is a nuclear receptor protein, belonging to the steroid receptor family (Class I). There are two forms of ER, ERα and ERβ. These receptors act as nuclear ligand activated transcription factors and both lead to the activation or repression of specific target genes involved in cell proliferation and differentiation (Renoir 2012). Estrogen binds to both receptors and whilst ERα has been studied extensively, less has been studied of ERβ. ERβ shares homology with ERα yet it differs in tissue distribution and physiological function, (Figure 1.4) (Palmieri, Cheng et al. 2002). It is thought that ERβ can act as a tumour suppressor (Lazennec, Bresson et al. 2001). ERα is the major subtype of ER and is composed of an N-terminal AF1 domain, a DNA binding domain (DBD) and a C-terminal ligand binding domain, (Figure 1.4). This nuclear receptor and its downstream signalling processes plays an extremely important role in the development of breast cancer (Saha Roy and Vadlamudi 2011).

### 1.4.1 ER signalling

In the breast ER signalling is composed of two main pathways, the genomic (or nuclear) pathway and the non-genomic pathway (Levin and Pietras 2008). The genomic pathway occurs when estrogen binds to its receptor ER and activates target genes (also known as the classical pathway). Upon E2 binding, the ER releases chaperone proteins (Hsp90) and the E2-ER complex translocates to the nucleus. The

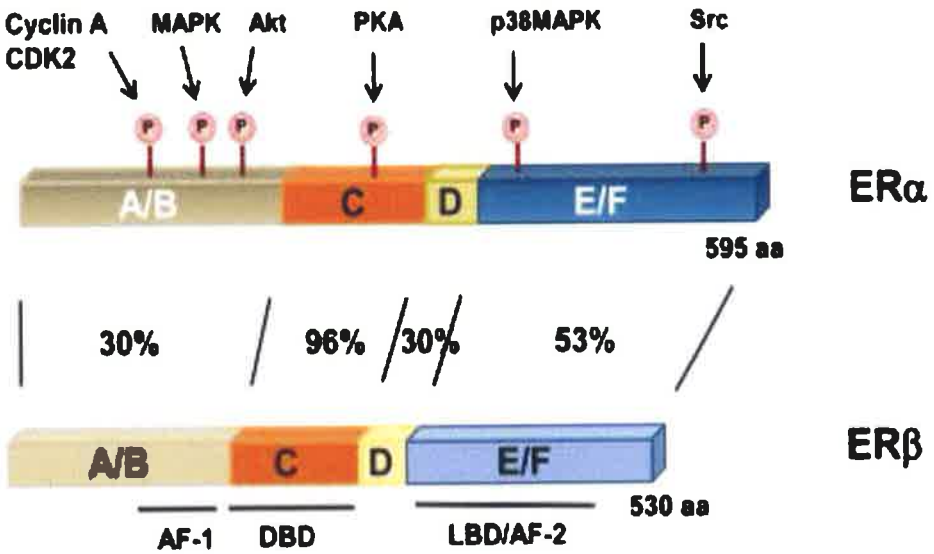


***Figure 1.4 Structural compositions of ERα and ERβ.*** Both receptors have four functional domains, DNA-binding domain (DBD), a ligand-binding domain (LBD) and two transcriptional activation functions (AF-1 and AF-2). The percentage homology between ERα and ERβ domains is indicated, as well as the location of several phosphorylation sites in ERα whereby this receptor is activated by important kinases that modulate a wide variety of cellular events, (Roman-Blas, Castaneda et al. 2009).

complex undergoes conformational change followed by dimerisation. Dimers of the E2-ER complex bind to imperfect estrogen response elements (EREs) (AGGTA) in the promoter region of target genes, where associations or dissociations of co-regulators takes place. Co-regulators include CBP/p300 and the steroid coactivator protein, SRC-1 both of which enhance transcriptional activity. Co-regulators act as either activators (CoAc) or repressors (coR) of transcription depending on cell or promoter context (Al Saleh, Sharaf et al. 2011). The p160 co-activator family (to which SRC-1

belongs) serve as a platform for the recruitment of HATs (histone acetyltransferases) and methyltransferases which induce chromatin remodelling and facilitate ER transcription, (Figure 1.5). Other P160 members include SCR-2 (NCOA2) and SRC-3 (AIB-1, NCOA3). All of which have been shown to co-activate the nuclear receptors ER, GR (glucocorticoid receptor) and PR (progesterone receptor) (Onate, Tsai et al. 1995).

Ligand activated ER signalling can also induce transcription via indirect ER-DNA binding through protein-protein interactions with transcription factors (TFs). ER signalling such as this is known as the 'non-classical pathway'. It occurs when ER complexes bind directly to DNA target sequences. For example, ER can bind to AP-1 (activator protein-1), SP-1 (specificity protein-1) or cyclic AMP-response elements on target DNA resulting in transcription of target genes such as cyclin D1 and IGFR-1 (Figure 1.5) (Zilli, Grassadonia et al. 2009; Osborne and Schiff 2011).

As mentioned above ER signalling can also be regulated by non-genomic actions and is usually mediated via receptor tyrosine kinases (RTKs) (Figure 1.5). This mechanism also known as membrane initiated steroid signalling (MISS) utilizes membrane/cytoplasmic ER. Upon E2 binding to the ER, the complex dimerises and directly interacts with adaptor proteins such as SRC and G-proteins resulting in activation of growth factor receptors (GFRs) such as EGFR, HER2, IGF-R1 (insulin growth factor) and cytoplasmic kinases such as MAPK (p42/p44 mitogen-activated protein kinase), PI3K (phosphatidylinositol 3 kinase/protein kinase B (AKT) and p38 MAPK pathways which all result in activation of nuclear ER transcription (Zilli, Grassadonia et al. 2009).

In the absence of estrogen, the AF1 domain of cytoplasmic ER is activated through phosphorylation of serine residues resulting in activation of MAPK, PI3K, AKT and p38 MAPK. These kinases are triggered by GFRs which in turn regulate ER and transcription of ER target genes. The activation of ER target genes in this manner is thought to be due to lack of efficacy of some endocrine treatments, this will be discussed in further detail below (Zilli, Grassadonia et al. 2009; Osborne and Schiff 2011), (Figure 1.4 and Figure 1.5).

## 1.5 Estrogen receptor targeted therapies

Breast cancer is primarily an estrogen driven disease and the suggestion of targeting estrogen signalling was first introduced in 1936 by Lacassagne (Lacassagne 1936). Today anti-estrogen therapy along with surgery is still regarded as the best treatment of choice for ER+ tumours. In the clinic ER status is used as a predictive factor for response to adjuvant hormone therapy.

### 1.5.1 Estrogen receptor modulators

SERMs (selective estrogen receptor modulators) are a class of drugs designed to antagonise ER function. They competitively inhibit binding of estrogen to the ER and affect both genomic and non-genomic ER transcription. Tamoxifen for example, binds to nuclear ER whilst competing with estrogen. It induces dimerisation but obstructs the AF-2 domain which results in inhibition of interactions with co-activators associated with AF-2 transcription (Figure 1.4). ERα target genes that regulate cell cycle and apoptosis are disrupted and the tumour regression occurs. Tamoxifen bares agonistic properties too and this action primarily involves the AF-1 domain. How tamoxifen activates the AF-1 domain remains to be fully established but it is thought to involve phosphorylation of ER (similar to estrogenic activity). It is well established that tamoxifen exhibits its effect in a tissue specific and cellular context depending on the availability of activators or repressors (Osborne 1998; Smith and O'Malley 2004). It acts as an anti-estrogen in cells where estrogen is the dominant force in gene transcription and behaves as an agonist in cells driven mostly though non-classical MISS signalling networks (Zilli, Grassadonia et al. 2009). Tamoxifen therapy has improved breast cancer management significantly and even though it was first intended as treatment for metastatic disease (regression is seen in at least 30% of patients), efficacy has been identified in patients after surgical removal of the primary tumour (Ali and Coombes 2002).

SERDs (selective estrogen downregulators) on the other hand such as Fulvestrant work by inhibiting estrogen signalling though inactivation of both AF domains in the ER. Fulvestrant binds to the LBD and obstructs ER dimerisation resulting in ER degradation, (Figure 1.4) (Zilli, Grassadonia et al. 2009).

### 1.5.2 Aromatase inhibitors (AIs)

The use of AIs is more relevant in post-menopausal women, where the principal source of estrogen is no longer the ovaries. In this sub-set of women estrogen is produced in extragonadal sites and acts locally in tissue. In the breast, mesenchymal cells in adipose tissue synthesise estrogen and in fact, elevated estrogen levels are usually seen locally when compared to circulating estrogen levels produced by the ovaries (Simpson 2003). So drugs targeting local estrogen inhibition have been developed. In breast adipose tissue, androstenedione and testosterone is converted to estrone and estradiol respectively via the enzyme cytochrome p450 aromatase (CYP19). Today third generation aromatase inhibitors exhibit their effect by blocking CYP19. Both steroid and non-steroidal AIs exist, including exemestane and letrozole respectively (Figure 1.4). Letrozole in particular has shown great efficacy in a large clinical trial (ATAC trial) and has even superseded tamoxifen as adjuvant therapy for breast cancer treatment (Mouridsen, Gershanovich et al. 2001).

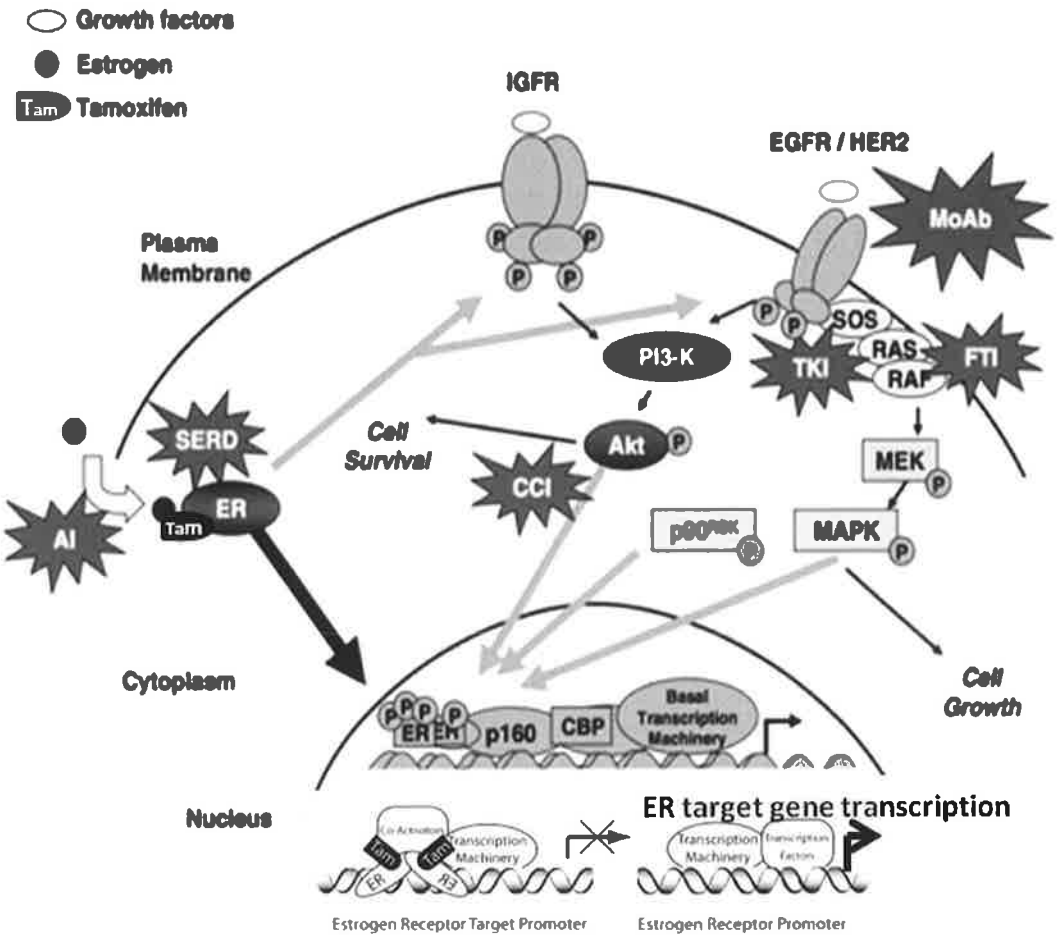


***Figure 1.5 Estrogen signalling and crosstalk in breast cancer.*** *Diagram displays both the genomic and non-genomic pathways of estrogen signalling with targeted intervention for both anti-estrogen tamoxifen and aromatase inhibitors (AI) to inhibit estrogen signalling. E2-liganded ER activates E2-regulated genes (black arrow), but following long-term tamoxifen therapy resistance can develop with bidirectional crosstalk (grey arrows) between ER and growth factor receptors, with association of membrane bound ER with growth factor receptors, and/or IGFR or EGFR/HER2 activation of ER phosphorylation. Stars show various targeted therapies, AI, SERDs, MoAb,TK, FTI, and CCI, adapted from Johnston, Martin et al. 2005; Thomas and Munster 2009.*

## 1.6 Resistance to Endocrine Therapies

Despite the remarkable advances in therapeutic management of breast cancer a considerable number of women will relapse and develop metastasis. Almost 30% of the women treated with tamoxifen over 5 years will have recurrence of the disease within 15 years due to *de novo* or intrinsic endocrine resistance (Ali and Coombes 2002; Riggins, Schrecengost et al. 2007). Breast cancer recurrence remains a major clinical challenge and considerable effort has been made to elucidate the mechanisms that underlie drug resistance with the main focus on defining and understanding ER biology.

### 1.6.1 Mechanisms behind endocrine resistance

In the clinic there are many predictors to help determine occurrence of endocrine resistance. Some of these include: a decrease or loss of ER, upregulation of HER2, loss of PR, reduced response to endocrine treatment leading to lower clinical benefit to therapy (resulting in a highly proliferative high grade tumour) (Osborne and Schiff 2011). At a molecular level many perturbed ER signalling networks have been established in relation to endocrine resistance.

#### 1.6.1.1 Loss of ER function/expression

One mechanism behind de novo resistance is lack of ER expression. This very often is due to histone deacetylation or aberrant methylation of the ER gene at CpG specific sites (Ottaviano, Issa et al. 1994; Parl 2003). Mutations in ER tumours have been identified and often these tumours show enhanced binding of ER co-regulators due in part to hypersensitivity of the ER receptor (Herynk and Fuqua 2004). Luminal ER+ tumours have recently been found to have greater heterogeneity in terms of the various mutations present in these breast tumours (The Cancer Genome Atlas Network 2012). However, the role of these mutations in luminal and other breast cancer subtypes is yet to be fully defined. The emergence of other ER variants has been suggested as seen by the truncated ER36 which displays reduced response to endocrine therapy (Shi, Dong et al. 2009).

#### 1.6.1.2 Estrogen hypersensitivity

Evidence provided from breast cancer cell lines suggests that some breast tumours ultimately acquire a hypersensitivity to estrogens. LTED or long term estrogen deprived cells (usually derived from MCF7 cells) display significantly higher levels of growth factor signalling. Estrogen stimulation in these cells often result in rapid ER signalling and upregulation of various kinase cascades, such as RAS/RAF/MEK/MAPK (Song, Santen et al. 2002; Santen, Song et al. 2004). Phosphorylation at serine$^{118}$ is also stimulated along with upregulation of the IGF-1R and ERBB2 pathways. (Santen, Fan et al. 2009).

#### 1.6.1.3 Altered expression of estrogen co-regulators

Considering ER signalling is tightly regulated via co-activators and co-repressors, it is widely accepted that these transcriptional modulators are associated with breast cancer resistance. AP1 and NFKB have been linked to endocrine resistance and similarly overexpression of AIB1 (SRC-3) and SRC-1, p160 proteins are known to mediate ER signalling in vitro. In the patient population overexpression of these proteins show a reduced response to tamoxifen (Al-azawi, Ilroy et al. 2008; Redmond, Bane et al. 2009).

#### 1.6.1.4 Increased growth factor signalling

Very often elevated levels of various growth factors are seen in tumours that do not respond to endocrine treatment. These pathways are extremely important in resistant breast cancer as they provide direct alternatives for tumours to proliferate and survive when typical ER signalling pathways are inhibited (Massarweh, Osborne et al. 2008).

Under normal circumstances estrogen is a positive regulator of the cell cycle by downregulating genes involved in cell proliferation and survival (HER2/EGFR). Anti-estrogens have the opposite effect, as estrogen is inhibited proliferative pathways are unleashed, (Figure 1.6) (Shou, Massarweh et al. 2004). With induction of signalling through growth factors, nuclear ER activity is constantly switched on via activation MAPK and AKT which phosphorylate ER at serine $^{118}$ and $^{167}$ (Arpino, Green et al. 2004; Schiff, Massarweh et al. 2005; Riggins, Schrecengost et al. 2007). The consequent increased expression of ER sensitive target genes, such as EGFR creates a

cellular "short-circuit" that maintains growth and survival during endocrine treatment, a so called "signalling loop" (Figure 1.6) (Zilli, Grassadonia et al. 2009). This consistent expression of pro-proliferative and survival genes thereby limits the efficacy of hormonal therapy.

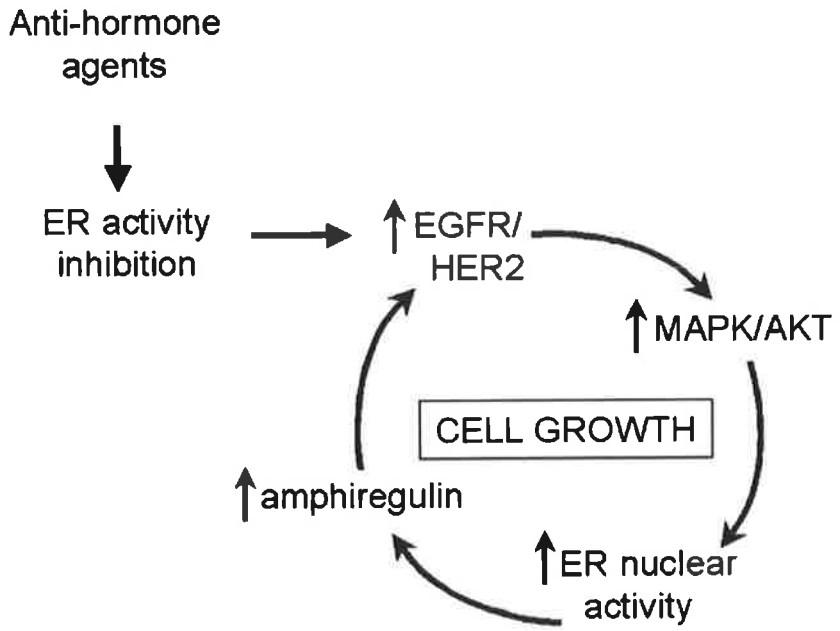


***Figure 1.6 Autocrine signalling loop limiting the efficacy of anti-hormonal therapy.*** *Estrogen-activated ER down-regulates the transcription of EGFR and HER2 genes, anti-estrogen agents promote an increase of EGFR/HER2 expression. These receptor tyrosine kinases activate the downstream MAPK/AKT cascade which, in turn, phosphorylates and activates nuclear ER, counteracting the inhibitory effect of the anti-hormonal therapy. As a consequence, the persistent ER activation leads to the transcription of ER-sensitive genes and an autocrine proliferative loop is established adapted from, Zilli, Grassadonia et al. 2009.*

ER signalling is a complex system of intertwining cellular signal transductions. Membrane ER can activate HER2 and induce kinase cascades resulting in activation of ER co-regulatory proteins (Shou, Massarweh et al. 2004). Tamoxifen treatment can also induce this action via its agonistic effect on membrane ER. Crosstalk between ER and GFRs (EGFR, HER2 and IGF-R1) is further enhanced in tamoxifen resistant breast

tumours through up-regulation of ER non-genomic activity (Shou, Massarweh et al. 2004; Osborne, Shou et al. 2005). Proteins downstream of these GF signals are then upregulated through activation of various kinase cascades, such as ERK1/2 and AKT (Britton, Hutcheson et al. 2006). This particular mechanism is thought to be related to the distribution of nuclear ER to the cytoplasm which in turn enhances EGFR activity (Fan, Wang et al. 2007).

## 1.7 Homeobox genes

Homeobox genes belong to a superfamily of regulatory genes encoding transcription factors that are primarily involved during embryogenesis and development (Cillo, Faiella et al. 1999). However, today there is a large body of supporting evidence to suggest that aberrant HOX gene expression is associated in breast cancer (Cillo 1994; Cillo, Faiella et al. 1999; Cantile, Pettinato et al. 2003; Grier, Thompson et al. 2005). Observed first in Drosophila back in the 1900s, HOX genes have come a long way from the humble fruit fly, (Figure 1.7). Since then, HOX genes have been intensely studied and today are considered one of the master regulators of spatial limb and organ development during embryogenesis and also maintain tissue and organ specificity in adult tissue (Krumlauf 1994; Shah and Sukumar 2010; He, Hua et al. 2011). HOX genes are involved in multiple cellular processes throughout the body including, morphogenesis, cell growth, differentiation and they are responsible for lineage specific expression during haematopoiesis (Kongsuwan, Webb et al. 1988; Grier, Thompson et al. 2005).

In more recent times it has been suggested that HOX proteins can sequester other proteins to activate or repress target gene expression (Shen, Krishnan et al. 2001; Norris, Chang et al. 2009). Direct HOX target genes however have remained somewhat elusive. This is mainly due to the fact that these genes activate such a multitude of downstream processes that it is hard to pin point direct HOX targets.

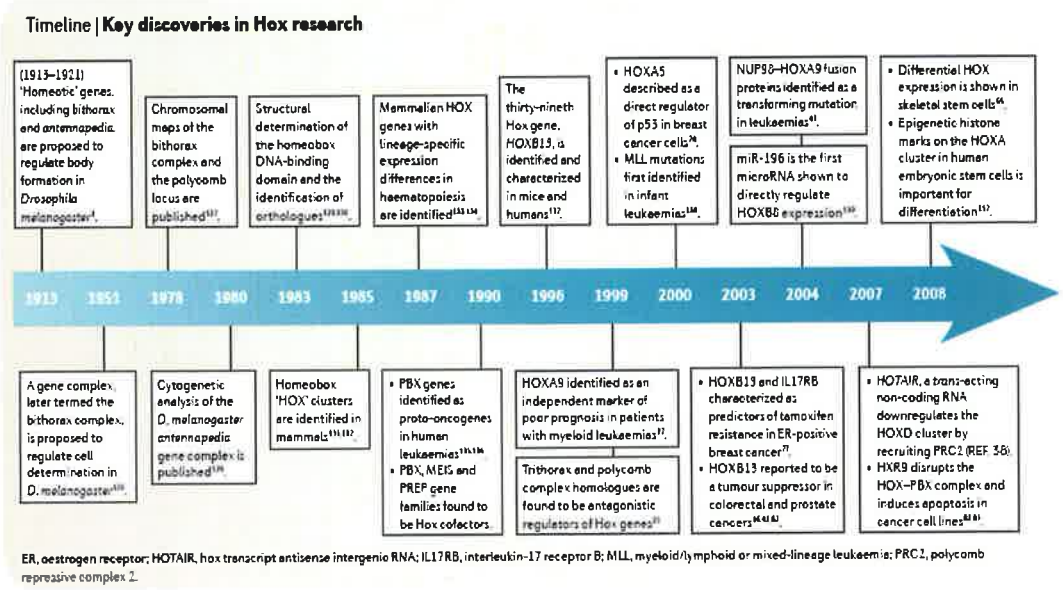


***Figure 1.7 Timeline of key homeobox research discoveries from 1913 to 2008.*** *HOX genes and their roles in oncogenesis, (Shah and Sukumar 2010).*

### 1.7.1 Homeobox gene structure

Homeobox genes are highly evolutionary conserved. They contain a defined 183 nucleotide sequence within exon 2 known as the "homeobox" (Shah and Sukumar 2010). The homeobox encodes a 61 amino acid domain, called the homeodomain (HD) which is responsible for recognising and binding target DNA motifs. A helix-turn-helix motif in the HD contains a consensus core binding sequence, *(5'-TAAAT-3')* which allows binding of target gene enhancers. The specificity of target gene binding permits homeoproteins to activate or repress target genes (Figure 1.8) (Abate-Shen 2002).

There are 39 mammalian HOX genes and they are categorised into four clusters, A, B, C and D located on chromosomes 2, 7, 12 and 17. Each cluster contains 9 to 13 genes and the alignment of each gene along the chromosome parallels their expression pattern in the body axis. This phenomena is known as "colinerality" and it explains one mechanism by which the homeobox genes define segment structure and organ

development along the anterior -posterior vertebrate axis, (Figure 1.9) (Shah and Sukumar 2010).

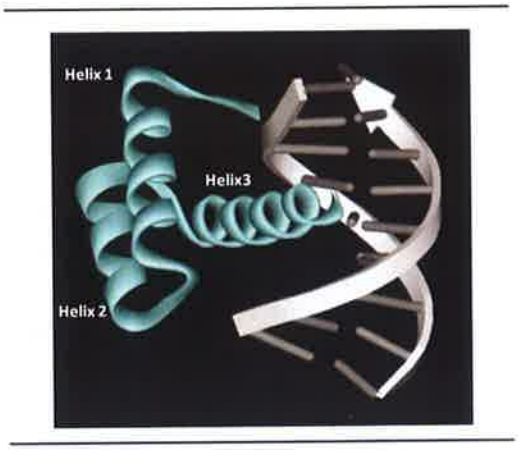


***Figure 1.8 Structure of the homeodomain.*** *The 3 dimensional structure of the homeodomain is conserved in nature. It corresponds to 3 α helices essential for target DNA binding (Abate-Shen 2002).*

### 1.7.2 Homeobox genes, master regulators of embryonic development

During embryogenesis HOX genes expressed at the 3' end of the cluster, primarily groups 1-4, are expressed earliest during development and correspond to the hindbrain area (anterior axis). The cluster groups 5-8, usually control the thoracic portion of the body and the genes located in the 5' region, groups 9-13, control the lumbosacral region (posterior axis) and are expressed later during embryogenesis, (Figure 1.9). The entire HOX gene family is also expressed in the embryonic central nervous system. The linear and physical arrangement of the HOX genes is essential for the direction of temporal and spatial gene expression (Cillo, Faiella et al. 1999; Daftary and Taylor 2006).

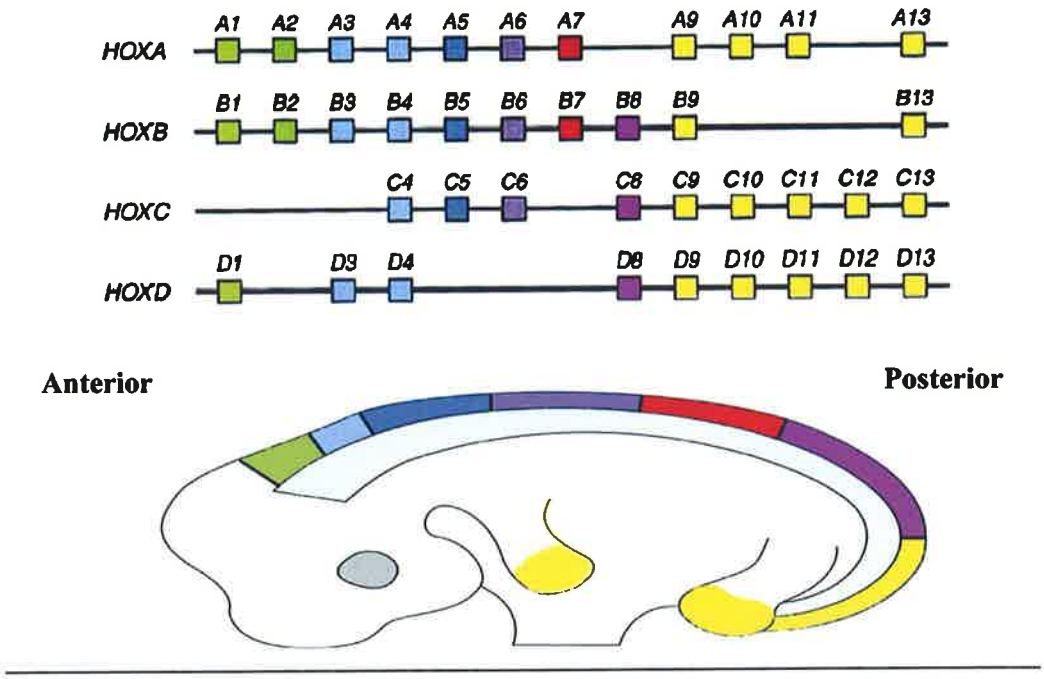


***Figure 1.9 Schematic representations of the HOMEOBOX gene clusters and their embryonic expression pattern.*** *Top panel: HOX clusters A, B C and D. Every gene along each cluster is aligned in a collinear fashion. Bottom panel: mammalian embryonic expression pattern of the HOX genes, 3' end cluster genes are expressed in the more anterior regions and the 5' genes are expressed more in the posterior region (Daftary and Taylor 2006).*

### 1.7.3 Homeobox genes in the adult

Although HOX genes have been extensively studied in embryogenesis, a role for homeobox proteins in adult tissue is now widely accepted. Global HOX expression patterns, from each HOX gene cluster (A,B,C and D), can be seen in organs such as, the kidney, lung and colon (Cillo 1994). Considering HOX genes play such an important role in cell fate during embryogenesis it is thought that they can also regulate adult functional differentiation through similar mechanisms. Some studies suggest that HOX genes retain their developmental plasticity and utilize this characteristic in adult tissue organs that undergo rapid adult developmental change (Lewis 2000; Samuel and Naora 2005; Daftary and Taylor 2006).

Today many studies discuss the role of HOX genes in the mammary gland (Friedmann, Daniel et al. 1994; Duboule 1999). Maturity of the mammary gland is mostly postpubertal and development is generally characterised as having both a linear and cyclical growth phase, (Figure 1.10). The linear phase starts from embryonic development lasting until puberty and the cyclical phase leads to breast maturity, pregnancy and regression. During each specific phase, important cell differentiation and apoptotic decisions are made. The homeobox family of genes are considered to regulate many of these developmental processes (Lewis 2000; Hens and Wysolmerski 2005; Garin, Lemieux et al. 2006). Identifying clear functional roles for the HOX proteins in mammary gland development is complicated as HOX knockout mouse models tend not to survive due to embryonic lethality. However, the regulation of expression of the HOX genes can give clues to the involvement at specific stages of development. A number of murine hox genes with specific expression or function during stages of mammary gland development are shown in Figure 1.10.

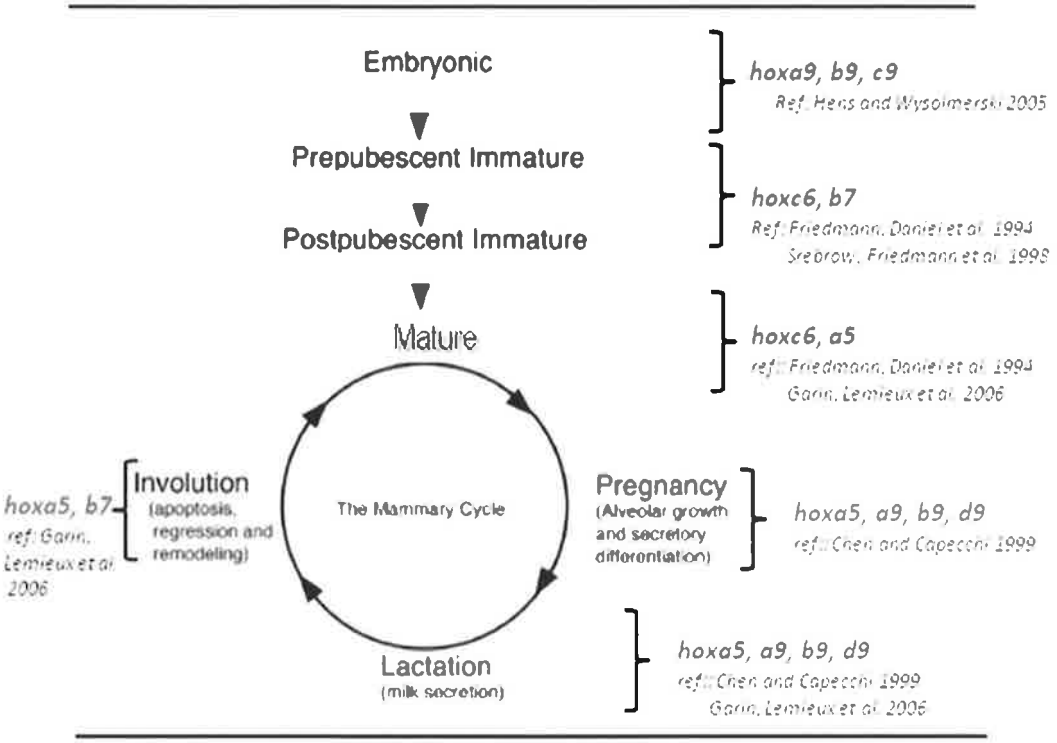


***Figure 1.10 Phases of mammary gland development.*** *Proliferative development is represented in the linear phase and maturity is represented in the cyclical phase. Corresponding murine mammary hox genes are also displayed with each mammary gland development phase (adapted from Lewis 2000).*

### 1.7.4 Endocrine regulation of homeobox genes

Mammary gland development is tightly regulated by hormonal control. Friedman and colleagues observed that the HOX genes could be directly regulated by estrogen (Friedmann, Daniel et al. 1994). Retinoic acid regulates expression of the 3' cluster HOX genes (anterior segments) during early embryogenesis. Estrogen in contrast influences the posterior axial patterning. Progesterone also regulates HOX genes and in particular regulates the HOXA gene in the adult uterus and it is thought to modulate endometrial functional differentiation (Ma, Benson et al. 1998; Taylor, Arici et al. 1998; Daftary and Taylor 2006). Endocrine regulated signalling whether activated during embryogenesis or in the adult can effect overall HOX gene expression. Further investigations led by Friedmann et al, demonstrated that Hoxa1 and Hoxb7 activity could be altered via basement membrane extracellular signals,

indicating homeobox proteins were not only responsive to hormones, but could also interact with extracellular components in the microenvironment (Srebrow, Friedmann et al. 1998).

### 1.7.5 Aberrant expression of homeobox genes

Increasing evidence suggests that the molecular pathways involved in embryogenesis are similar to those involved in neoplasia. They share similar intracellular processes such as; cell proliferation, cell motility, invasion of surrounding tissue and apoptosis (Lewis 2000). Pathways involved in both embryogenesis and tumourigenicity are not unheard of. For instance it is well known that both Wnt and Hedgehog (Hh) signalling take part in both normal and tumourigenic cell growth (Taipale and Beachy 2001). Likewise in a similar fashion the homeobox genes are thought to play contrasting roles from normal cellular growth during healthy development through to adult function to abnormal growth during neoplasia (Cillo, Faiella et al. 1999).

Given that the mammary gland under normal conditions undergoes many cellular transformations involving endocrine stimulated cyclical phases of proliferation, differentiation and apoptosis, it is not surprising that so many dysfunctional HOX proteins have been linked with mammary tissue. Friedmann and colleagues identified several genes, HOXC6, HOXC8, HOXD9, HOXD10, in 1994 to be expressed in normal mouse mammary gland and neoplastic tissue (Friedmann, Daniel et al. 1994). Since then, numerous HOX genes have been associated with breast cancer and the expression patterns of a wide number of HOX genes have been analysed in breast cancer cell lines and tumour tissues.

Quantitative real time PCR revealed that whilst some HOX genes were uniformly expressed across normal and cancerous cell lines/ tissue, such as HOXA13, HOXB5 and HOXC4, other HOX genes, manifested in altered expression between normal and cancerous cell/tissue, such as HOXB9, HOXC13 and HOXD3 (Cantile, Pettinato et al. 2003; Svingen and Tonissen 2003). Thus, suggestive those HOX genes are involved not only in ordinary breast organogenesis, but also in deregulation of cancer.

A study from Australia in 2003 found that some HOX genes (HOXB13, HOXB9 and HOXA4) were completely absent in normal breast tissue but were upregulated in malignant cells. On the other hand they also observed other HOX genes expressed in normal breast (HOXD4) where completely absent in malignant cells and re-expressed in highly invasive cells (Svingen and Tonissen 2003).

Abate-Shen tried to classify some of the mechanisms behind HOX deregulation and proposed 3 main mechanisms.

1. Temporospatial deregulation; where HOX gene expression patterns arise in tumours that are not seen in normal healthy tissue.
2. Gene dominance; this occurs when HOX genes are expressed at higher levels than normally found in a specific tissue type.
3. Epigenetic deregulation; silencing or downregulation of HOX genes when they should typically be expressed in a particular tissue (Abate-Shen 2002).

An attempt to classify groups of HOX genes was made by Cantile et al, who focused solely on *in vivo* expression of the whole HOX gene network in normal and malignant breast tumours. They took a broad approach and looked at the differentiated HOX expression patterns along the temporal-spatial 5' to 3' axis. They surmised that the thoracic HOX genes bare similar expression profiles in normal and malignant breast tumours and on the other hand the cervical (3' anterior axis) and lumbo-sacral, 5' posterior axis, (Figure 1.9) expression pattern was altered in the primary tumour tissue with respect to the normal breast tissue. Supporting a role for the thoracic HOX genes in normal breast organogenesis and the anterior (cluster groups 1-4) and posterior genes (cluster groups 9-13) involvement in breast cancer development (Cantile, Pettinato et al. 2003).

In other cancers upregulated expression of HOX genes can promote an oncogenic phenotype through upregulated growth factor signals leading to increased proliferation and tumourigenicity. One example of this aberrant upregulated HOX expression can be seen in the primary tumours of some lung carcinomas, where overexpression of the 5' cluster of the HOXA and HOXD locus is apparent (Shah and Sukumar 2010). Similarly in ovarian cancer, overexpression of HOXB13 is thought to mediate cancer progression. HOXB13 overexpression *in vitro* activates the ER and can induce tamoxifen resistance (Miao, Wang et al. 2007).

In other breast cancers aberrant HOX expression is linked with loss of tumour suppressors. In the case of HOXA10, loss of this HOX gene results in downregulation of the well known tumour suppressor, p53 causing a metastatic and invasive phenotype (Chu, Selam et al. 2004). Epigenetic modifications to the HOXA5 promoter can lead to downregulation of p53, inhibition of apoptosis and promotion of tumourigenicity (Raman, Martensen et al. 2000). Marks and Sukumar, observed a loss HOXA5 expression, correlated with loss of p53 expression in human breast tumours. A 5-10 fold loss of p53 mRNA expression levels were found in breast tumours in comparison to normal tissue and HOXA5 mRNA levels were concomitantly found reduced in breast cancer cells. Further studies revealed HOX putative binding regions along the promoter of the p53 gene and subsequent experiments (gel electrophoretic mobility shift assays (EMSA) and luciferase reporter assays) confirmed that HOXA5 is a positive regulator of p53. Breast cancer cell lines containing wildtype p53 and transfected with full length HOXA5, displayed no colony forming ability. Conversely transfection with a mutated HOXA5 plasmid, lead to loss of HOXA5 function and colony formation. HOXA5 signalling under normal conditions stimulates p53 expression to activate growth suppressor signals and induce apoptosis (Raman, Martensen et al. 2000).

The promiscuous nature of deregulated HOX expression in cancer shows just how vast and convoluted the HOX signalling networks can be. When the delicate equilibrium between embryogenesis and organogenesis, driven by normal HOX expression is disturbed, perturbed HOX expression can cause tissue to change into a more undifferentiated early-embryonic like state or can induce cells to escape normal regulatory pathways, guiding the way to carcinogenesis and metastasis, (Figure 1.11) (Shah and Sukumar 2010). Homeobox aberrant gene function has previously been described as, "an extension of their normal function". Under normal circumstances HOX genes are global regulators of growth and differentiation but disruption of this delicate process unleashes a whole host of possible perturbed signalling networks that drive tumour growth (Abate-Shen 2002).

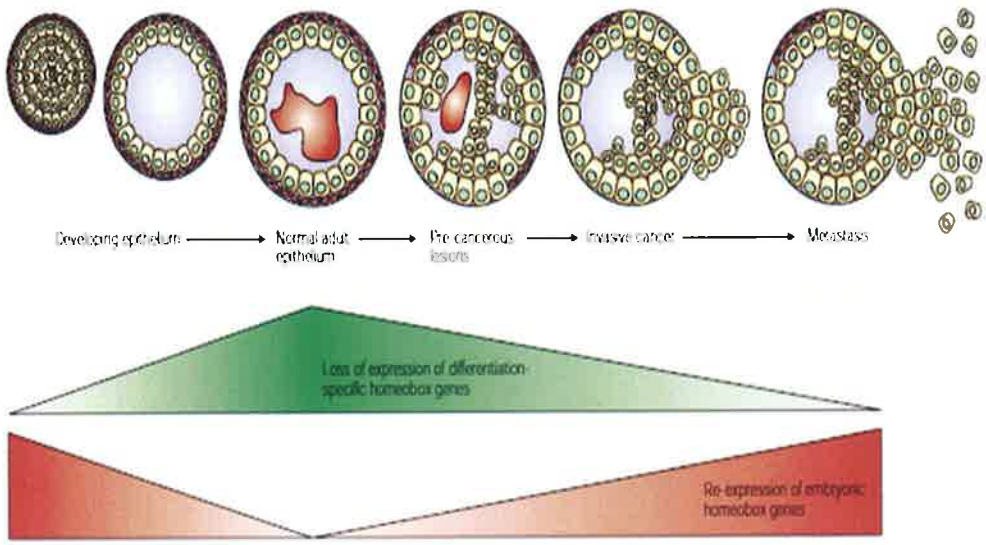


***Figure 1.11 The relationship between Homeobox gene expression and epithelial development and cancer.*** *Hox genes normally expressed in developing tissues and downregulated in differentiated tissue cell types are often re-expressed in cancer. Conversely Hox genes expressed in differentiated tissue are often downregulated in cancer progression (Abate-Shen 2002).*

### 1.7.6 Aberrant expression of homeobox genes in resistant breast cancer

Deregulation of a number of HOX genes are now emerging to be involved in drug resistance, see Table 1.3 for a list of the more known HOX genes to be involved in breast cancer. Probably one of the most widely studied HOX genes is HOXB13. Initially Svingen et al, observed HOXB13 expression in the non-invasive MCF7 cells when compared to normal breast tissue. Interestingly they detected no expression in the invasive MDA-MB-231 cell line (Svingen and Tonissen 2003). Today HOXB13 has been studied extensively and it is now used as a gene predictive biomarker for endocrine resistance. More recent studies have observed differentially expressed HOXB13 levels in patients who relapsed on tamoxifen, when compared to disease free patients post treatment (Sieuwerts et al. 2007). Ma et al, showed HOXB13 expression to enhance motility and invasion in the non-tumourigenic MCF-10A cells. They established that the two gene-expression signature for high HOXB13 to IL17BR

(interleukin 17B receptor) accurately predicted tumour recurrence in adjuvant tamoxifen monotherapy and proposed that this ratio could be used to identify patients in need of alternative therapy in early stage breast cancer (Ma, Wang et al. 2004; Jansen, Sieuwerts et al. 2007).

***Table 1.3 List of the more common HOX genes within each HOX cluster known to be involved in resistant breast cancer.***

| HOX Cluster | HOX gene | Perturbed Function in Breast Cancer | Reference |
|---|---|---|---|
| HOX A | HOXA1<br>HOXA5<br><br>HOXA10 | Oncogene, required for anchorage independence<br>Loss of HOXA5 correlates with loss of p53 and gain of tumour invasiveness<br>Loss of HOXA10 correlates with loss of p53 and tumour invasiveness | Zhang et al. 2006<br>Raman, Martensen et al. 2000<br>Chu, Selam et al. 2004 |
| HOX B | HOXB7<br>HOXB9<br><br>HOXB13 | Inducer of EMT<br>Inducer of angiogenic factors, such as VEGF, BFGF & IL8, induces metastasis<br>Overexpression is a marker of endocrine resistance, induces cell motility and invasion | Wu, Chen et al. 2006<br>Hayashida, Takahashi et al. 2009<br>Sieuwerts et al. 2007, Ma et al 2004 |
| HOX C | HOXC5<br>HOXC8<br>HOXC10<br>HOXC11 | Reduced mRNA levels correlate with mutated p53<br>Induces migration & metastasis<br>Overexpressed, regulated by MLL3 & MLL4,<br>Interacting partner of SRC-1, overexpression mediates endocrine resistant breast cancer | Makiyama, Kokonoe et al 2005<br>Yong Li et al 2010<br>Khairul I Ansari et al 2012<br>McIlroy, McCartan et al. 2010 |
| HOX D | HOXD4 | Transcriptionally repressed by miRNA-10a via DNA methylation in MCF7 cells | Yuliang Tan et al 2009 |

HOXB7 has also been implicated in resistance to tamoxifen treatment. Wu and colleagues observed that, HOXB7 mRNA expression levels were 3 fold higher in bone metastasis, when compared to the primary tumour and 18 fold higher when compared to normal breast tissue. When HOXB7 was overexpressed in cell lines and in the mammary fat pad of mice, they could induce EMT. It is thought that HOXB7 promotes tumour invasion through activation of Ras/Rho pathway, via upregulation of bFGF (basic fibroblast growth factor) (Wu, Chen et al. 2006). It is thought that HOXB7 functions through the EGFR (epidermal growth factor receptor) pathway resulting in stimulation of growth factor signals thus leading to disease progression (Jin, Kong et al. 2011).

Aberrant HOX expression and endocrine resistance is not exclusive to breast cancer. Drug resistance in ovarian and prostate cancer has been intently studied (Kelly,

Michael et al. 2011) (Miller, Miller et al. 2003) . In prostate cancer in particular, Miller et al., found aberrant expression of the HOXC cluster to induce metastasis, migration and invasion. In particular HOXC8 mRNA levels were highly expressed in prostate cell lines and lymph node metastases compared to benign cells. Overexpression of HOXC8 however in the LNCaP prostate cells, was found to suppress androgen signalling. Miller, noting, that HOXC8, was suppressing a growth promoting signalling pathway, hypothesized that if increased aberrant expression of HOXC8 gene was to occur at an early stage of prostate cancer development, then a tumour must adapt to the diminished androgen signalling, prompting the tumour to be partially androgen resistant thus suggesting a role for HOXC8 gene expression in androgen resistant cancer (Miller, Miller et al. 2003).

It is clear that HOX genes play a critical role in breast cancer initiation and disease progression, however as with all cancer, in order to treat the disease we need to fully understand the complex signalling networks and target genes involved in order to design better drugs to fight the disease (Figure 1.12).

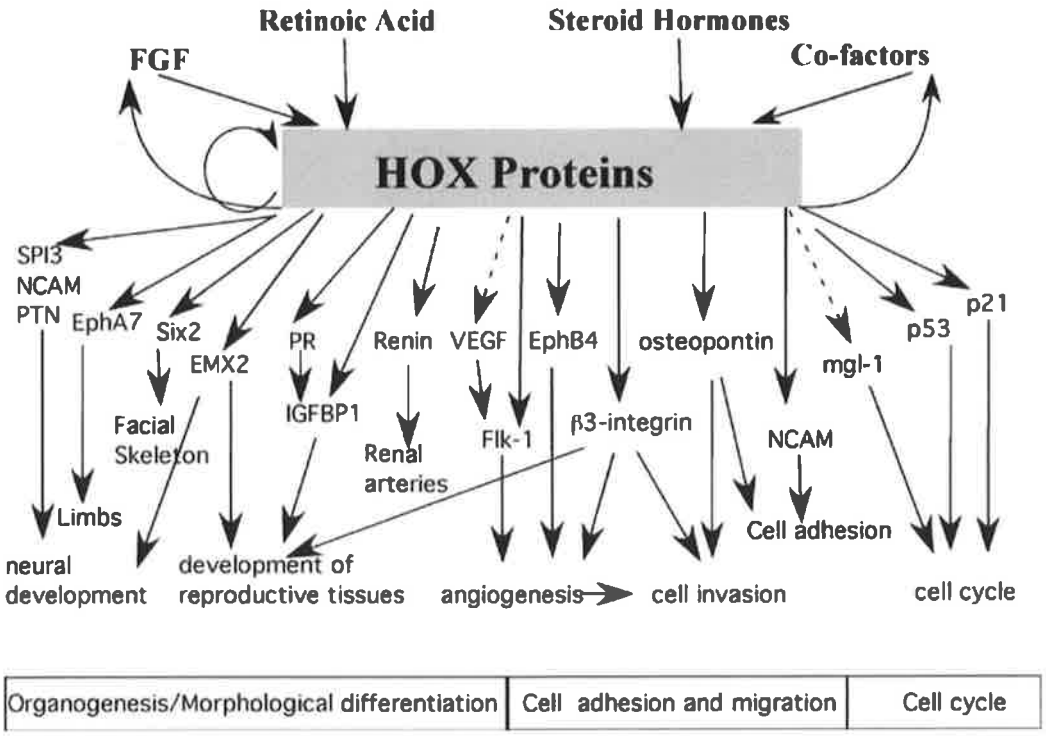


***Figure 1.12 Homeobox downstream targets.*** *HOX target genes are involved in numerous cellular processes. Many downstream targets are implicated in several molecular pathways making it difficult to elucidate direct target genes (Svingen and Tonissen 2006).*

Of particular interest to us is the transcription factor HOXC11. Differential expression of HOXC11 is associated with several cancers such as; leukaemia, renal carcinoma, melanoma and in the breast (Cillo, Faiella et al. 1999; Makiyama, Hamada et al. 2005; Samuel and Naora 2005; deBlacam, Byrne et al. 2010; McIlroy, McCartan et al. 2010). Previous studies from our group have identified that HOXC11 is strongly expressed in endocrine resistant breast cancer. HOXC11 first came to our attention when we identified it as an interactive binding partner for SRC-1 in endocrine resistant breast cancer. SRC-1, a steroid co-activator has now been well established to be a mediator of endocrine resistance (Walsh, Qin et al. 2012). To date a functional role for HOXC11 in breast cancer has not yet been fully elucidated. We want to investigate HOXC11 and its role in mediating tamoxifen resistance breast cancer.

## 1.8 Hypothesis

HOXC11 has previously been implicateded in endocrine resistant breast cancer. However a functional role in cancer remains to be elucidated. We propose that enhanced expression of this transcription factor evades cancer targeted therapies and confers an endocrine resistant phenotype through regulating pro-proliferative and anti-differentiating molecular activities. We suggest a novel relationship between HOXC11 and ERα and propose a mechanism for their role in breast cancer.

### 1.8.1 Thesis outline

The primary objective of this study is to establish a functional role for HOXC11 in breast cancer and to elucidate a functional relationship between HOXC11 and ERα.

The secondary objective of this study will focus on elucidating HOXC11 target genes. Specifically to validate ERα as a HOXC11 target gene and explore the mechanism in which HOXC11 regulates ERα expression.

The final portion of this thesis will focus on the regulation of S100β by both HOXC11 and ERα in a promoter context and at the protein expression level. Finally we investigate the significance of HOXC11 and ERα together in a clinical setting.

These objectives will be supported through the use of functional, *in vitro* applications that will utilize various molecular techniques such as, gene silencing, overexpression studies, western blotting, chromatin immunoprecipitation, DNA methylation array and TMA (tissue microarray) studies.

# Chapter 2

# Materials and Methods

## 2.1 Cell culture

### 2.1.1 Cell lines

Four human breast cancer cell lines were used throughout this study, MCF7, HOXC11 MCF7 stables, LY2 and LetR cells (Figure 2.1). The MCF7 cell line was obtained from the American Type Culture Collection (ATTC, Manassas, VA, USA). They are an epithelial adherent cell line derived from a pleural effusion; they express ER and PR and are endocrine sensitive. MCF7s were cultured in complete growth media, Eagles Minimum Essential Medium (MEM) (Sigma Aldrich) supplemented with foetal bovine serum (FBS) (Sigma Aldrich) and 200mM L-glutamine (Sigma Aldrich).

The HOXC11 over-expressing stable cell line was created by transiently transfecting MCF7s with an expression vector containing the full length HOXC11 gene. 72 hours post transfection, G418, (Geneticin) (Sigma) a selective antibiotic, was added to the media at concentration of 800mg/ml. The pcDNADest47 expression vector confers a resistant gene to this antibiotic, therefore only MCF cells successfully transfected with the expression vector were able to proliferate. Within two weeks of selection, cells which did not confer the resistant gene died off and just the HOXC11 stable cells continued to proliferate. The cell line was then routinely grown in MEM culture media supplemented with a lower concentration of G418 (200 mg/ml). A control cell line containing the expression vector alone with no HOXC11 insert was also created simultaneously and cultured in an identical manner.

The LY2's were a kind gift from Dr. Robert Clarke, Department of Oncology, Georgetown University, DC, USA. They are a stable hormone resistant cell line model derived from MCF7s and were produced by treating with an increasing dose of the selective estrogen modulator LY117018. They are ER positive and PR negative, they respond to estrogen in cell proliferation assays however treatment with 4-hydroxytamoxifen (4-OHT) (an anti-estrogen) does not inhibit cell proliferation (Bronzert, Greene et al. 1985). LY2's were cultured in MEM supplemented with 10% charcoal dextran stripped FBS (CDS-MEM) (Sigma Aldrich), 2mM L-glutamine and $10^{-8}$M 4-hydroxytamoxifen (4-OHT) (Sigma Aldrich). Stripping FBS with charcoal reduces

the amount of circulating hormones in the serum (essential in cell culture steroid studies).

The LetR cell line is derived from MCF7 cells, they were created in house by Dr. Jane O'Hara. They stably overexpress aromatase and were made resistant to the aromatase inhibitor letrozole (Novartis). These cells are ER+, PR+ and Her2+, they represent an endocrine resistant model which respond less to steroid treatment and are more growth factor dependent (McBryan, Theissen et al. 2012).


***Figure 2.1 Breast cancer cell lines used in this study.*** *From left to right, endocrine sensitive MCF7, HOXC11 overexpressing MCF7, endocrine resistant LY2 and letrozole resistant LetR cells.*

All cell lines were routinely tested every 6 months for mycoplasma with the MycoAlert Mycoplasma detection kit (Lonza). Mycoplasma are small prokaryotes (0.2-0.8µm in diameter), which make them hard to detect. Mycoplasma does not kill mammalian cells directly but instead interferes with cellular function such as cell growth, metabolism and apoptosis. Routine testing is important for consistency in experimental results and positive detection in cell lines were replaced with an earlier passage that was mycoplasma free or by treating the cells with a course of antibiotics; BM- cyclin 1 and BM-cyclin 2 (Roche Applied Science). The antibiotics were given once a week per antibiotic every 3 days for 3 consecutive weeks and the cells were then re-tested for mycoplasma.

### 2.1.2 Routine cell culture

All cell lines were maintained in T75 culture vessels (Cruinn) with 10 ml of culture media in a humid incubator at 37°C 5% (V/V) $CO_2$. Cells were passaged routinely every 3-4 days in a sterile laminar airflow cabinet. Sub-culturing was carried out by

rinsing the cells twice in sterile phosphate buffered saline (PBS) (Oxoid Limited), adding 2 ml of 1X trypsin/EDTA solution (Sigma Aldrich) and incubating the cells at 37°C for 5 min to allow the adherent cells detach from the culture vessel. To neutralise the trypsin, 5 ml of culture media was added and the suspension was transferred into a 15 ml falcon tube (Greiner-Bio-One). Cells were centrifuged at 270 x g for 4 min, supernatant was discarded and the cell pellet re-suspended in fresh appropriate culture media to the cell density required.

### 2.1.3 Long term storage of cells

To maintain long term use of the cells, they were stored in cryovials (Greiner) under liquid nitrogen (~ -196°C) at early passage numbers in complete media. DMSO was added to the freeze down media as a cryoprotective agent. It acts by lowering the freezing point of the media which reduces the number of ice crystal formation and helps to maintain cell integrity. Cell pellets from one T75 culture vessel were collected and re-suspended in 1 ml of freeze down media. Freeze down media consisted of, culture media supplemented with 10% DMSO (dimethyl sulphoxide) (Sigma) and an extra 10% FBS. The cryovial was then placed into Mr. Frosty (a cyrovial container submerged in isopropanol) and placed overnight at -80°C. Use of Mr. Frosty reduces the temperature in the cells in a slow and controlled manner allowing the movement of water out of the cells before being placed in liquid nitrogen.

To recover cells from liquid nitrogen, a cryovial was thawed as quickly as possible and the cells were resuspended in fresh culture media to avoid cell death. Cells were centrifuged at 270 x g for 4 min, the supernatant discarded and the pellet resuspended in 10 ml fresh culture media and pipetted into a new T75 culture vessel which was then placed into the incubator at 37°C overnight. The media was replenished once again the next day to clear away any dead cells that did not survive recovery from cryopreservation.

### 2.1.4 Cell culture treatment conditions

Cells were steroid depleted for 72 hrs before commencing any steroid treatments. Tissue culture vessels were rinsed twice in PBS and the cells were re-suspended in

CDS-phenol free media with no steroids. Steroid treatment time points varied depending on the type of experimental procedure (Table 2.1). Vehicle control was used at 0.01% ethanol in media.

***Table 2.1 Cell culture treatment concentrations and time points***

| | | Treatment time | |
|---|---|---|---|
| **Steroid** | **[Concentration]** | **ChIP** | **RNA** |
| Estrogen | 10-8 M | 45' -2hr | 4hr |
| Tamoxifen | 10-7M | 45' -2hr | 4hr |

### 2.1.5 Cell counting

Cell counts were carried out using a haemocytometer. 10 µl of cell suspension was pipetted onto the haemocytometer and the cells on the 4 outer corner larger squares (containing 16 smaller squares) and the central square were counted, (Figure 2.2). 1 large square is equivalent to: 1 $mm^2$ X 0.1 mm = $10^4$ $cm^3$ or $10^{-4}$ ml. Therefore the total number of cells counted were divided by 5 and multiplied by $10^4$ to give the number of cells per ml.

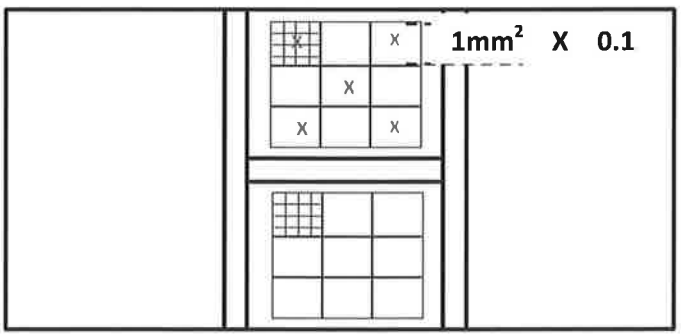


***Figure 2.2 Schematic representation of a haemocytometer.*** X marks each of the squares where cells were counted and the dimension of a counting square is shown.

## 2.2 Transient Transfections

### 2.2.1 Protein overexpression

Protein over-expression studies were performed using Lipofectamine 2000® (Invitrogen) transfection reagent. This reagent contains cationic lipids that allow DNA be delivered into the cell. Negatively charged DNA binds spontaneously to positively charged lipids, forming a DNA/liposome complex. This complex facilitates movement of the DNA through the cell membrane, where gene expression of the protein of interest occurs in the nucleus, (Figure 2.3).

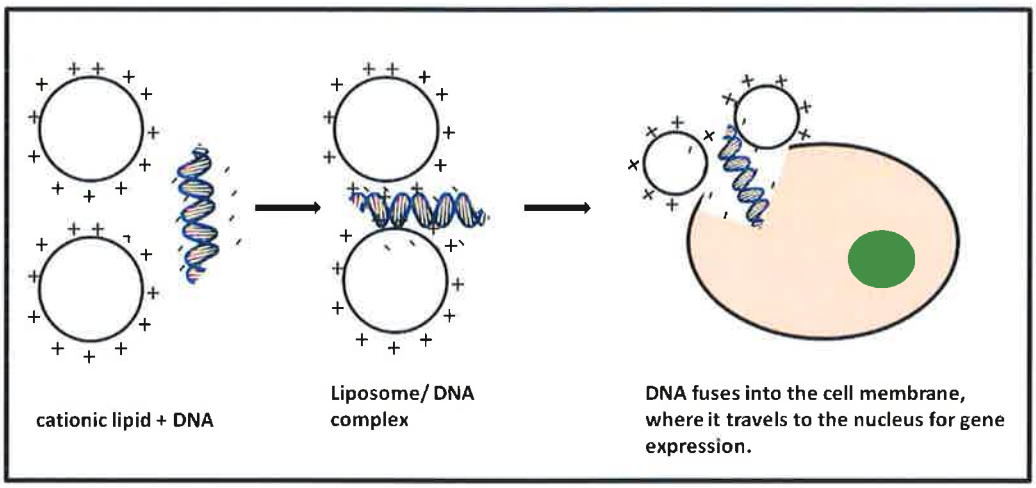


***Figure 2.3 Schematic diagram of DNA transfection with a cationic lipid.***

2 X $10^5$ cells were seeded onto a 6 well dish in appropriate culture media containing no antibiotics. Cells were left to adhere overnight and were approximately 90% confluent the next day. 2 µg of plasmid, either ERα-hego/pSG (a kind gift from Professor Pierre Chambon, University of Strasbourg) or HOXC11-dest47 or both plasmids in combination (1 µg of plasmid each) were mixed with 250 µl of opti-mem (Gibco) in a microcentrifuge tube (Figure 2.4). In a separate tube, 10 µl of lipofectamine and 250 µl opti-mem were mixed together. The individual tubes were incubated for 5 min combined and mixed together the total volume was 500 µl. The transfection mix was incubated for a further 20 min after which, the media on the cells was aspirated, washed twice in PBS and then 500 µl of transfection mix was carefully pipetted onto the cells. The well was then topped up to 2 ml with opti-mem and the cells incubated for 5 hours at 37°C, 5% $CO_2$. Afterwards the opti-mem was

replenished with full culture media and cells were left to incubate for a further 24hrs. Transfections were carried out in either duplicate or triplicate for each plasmid.

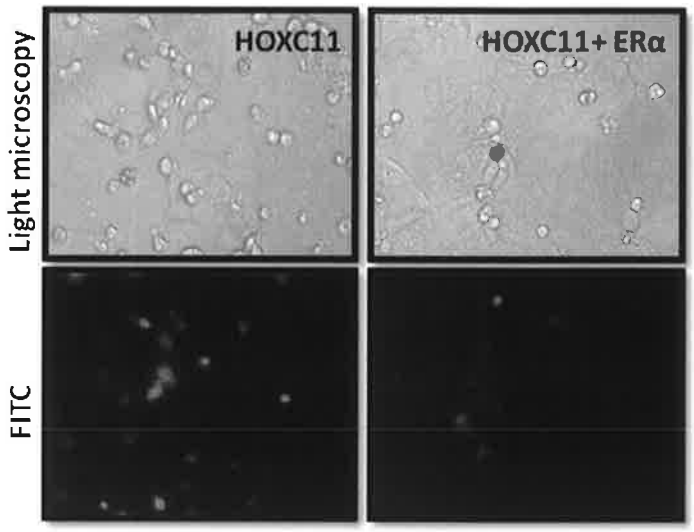


***Figure 2.4 Transient transfection of LY2 cells with HOXC11-dest47 alone and in combination with ERα-hego/pSG over expression.*** *Images demonstrate transient transfection with 1 μg of plasmid HOXC11-dest47 plasmid (left panel) and co-transfection with HOXC11 and ERα-hego (2 μg of plasmid DNA in total). Transfection efficiency can be seen by GFP expression with reduced expression in the combination transfection.*

### 2.2.2 Gene Silencing

Gene silencing was carried out using an RNA interference technique (RNAi). It involves introducing a small double stranded RNA (dsRNA) molecule into a cell. The dsRNA separates into a single stranded RNA (ssRNA) molecule and the antisense strand binds to RNA induced silencing complex (RISC). The RISC/ssRNA complex bind to its target mRNA and nucleases from the RISC complex degrade the target mRNA, thus silencing gene expression.

Silencing or "gene knockdown" was carried out with pre-validated human siRNA against HOXC11 (Qiagen) (Hs_HOXC11_2) and a scrambled siRNA (Ambion) (AM4635) was used as an off target control. 1X $10^5$ cells were plated into a 6 well dish with culture media containing no antibiotics. Cells were approximately 40-50% confluent

the next day. Lipofectamine 2000® reagent was used for transfection into the LY2 cell line as describe in section 2.2.1. The appropriate amount of HOXC11 siRNA and control are listed in Table 2.2. 5 hours after transfection the media was replenished with full culture media and incubated for 72 hours at 37°C, 5% $CO_2$.

***Table 2.2 siRNA details used in gene silencing experiments.***

| siRNA | Supplier | Stock Concentration | Final Concentration |
|---|---|---|---|
| HOXC11 | Qiagen | 20µM | 30nM |
| scrambled | Ambion | 50µM | 30nM |

## 2.3 Nucleic Acid biochemistry

### 2.3.1 RNA purification

Total RNA was purified from harvested cell pellets using the RNeasy Kit (Qiagen). Experiments and centrifugation steps were carried out at room temperature. 10 µl of β-mercaptoethanol (β-ME) (Sigma) was added per 1 ml of RLT buffer (kit). 350 µl of this solution was added to each cell pellet. Cells were homogenised by vortexing for 10 sec and passing the lysate 5 times through a 24-gauge needle, fitted to a 1 ml syringe. One volume of 70% ethanol was added to each sample and pipette mixed. The sample was transferred to the RNeasy spin column and centrifuged at ≥8000 g for 15 sec. Flow through was discarded and the column was washed once with 700 µl of RW1 buffer and twice with 500 µl RPE buffer, followed by a 2 minute centrifugation step, ≥8000 x g. Flow through was discarded and the column was centrifuged once more to ensure no residue was left behind. The column was placed in a clean centrifuge tube and the RNA was eluted in 40 µl of nuclease free $H_2O$. RNA concentration and quality was quantified using a NanoDrop spectrophotometer and the RNA was stored at -80°C until ready for use.

### 2.3.2 Reverse transcription

mRNA was transcribed into complementary DNA (cDNA) using the Superscript III First Strand Synthesis System for RT-PCR (Life Technologies). 1 µg of RNA was primed with 1 µl Oligo $(dT)_{20}$ and 1 µl 10 mM DNTP's to a final volume of 10 µl with nuclease free water. The sample was heated to 65°C for 5 min and placed on ice immediately after to cool. 10 µl of cDNA synthesis mix (Table 2.3) was added to each sample. The RNA/synthesis mix was then heated to 25°C for 10 min, followed by 50°C for 50 min. A control sample, without superscript enzyme (–RT) was also included. cDNA was stored at -20°C until ready for use.

***Table 2.3 Components of the cDNA synthesis mix, per 1 reaction.***

| cDNA synthesis mix | volume (µl) |
|---|---|
| 10 X RT buffer | 2 |
| 25mM $MgCl_2$ | 4 |
| 0.1M DTT | 2 |
| RNase OUT (40U/ µl) | 1 |
| SuperscriptIII (200U/µl) | 0.5 |
| $H_2O$ | 0.5 |

### 2.3.3 DNA extraction

Genomic DNA was purified using the DNeasy Blood & Tissue Kit (Qiagen). Cell pellets were harvested and resuspended in 200 µl PBS, to which 20 µl of proteinase K was added followed by 200 µl Buffer AL and the suspension was heated at 56°C for 10 min. 200 µl of ethanol was then added to the sample and the total suspension was piptted into the spin column provided and centrifuged at >6000 x g. The flow through was discarded and the column was washed once with 500 µl AW1 and once with 500 µl AW2 and centrifuged at >20000 x g for 3 min. The column was placed into a clean microcentrifuge tube and the DNA was eluted in 200 µl Buffer AE. The DNA was quantified on a nanodrop and the DNA stored at -20°C until ready for use.

### 2.3.4 Polymerase chain reaction

The polymerase chain reaction (PCR) is a technique by which a single copy of a DNA molecule can be exponentially amplified into millions of copies of a specific DNA target sequence. The reaction is catalysed by the enzyme DNA polymerase and utilises a primer of a known nucleotide sequence which binds to the target DNA. PCR is a thermal cycling reaction, consisting of repeated heating and cooling cycles which melt DNA and enzymatically replicate the DNA. The thermal cycling takes place in 3 stages; denaturation, annealing and extension which are repeated for number of specified cycles.

An initial **denaturation step** at 95°C allows DNA polymerase to be heat activated.***Denaturation*** breaks double stranded DNA into single stranded DNA. This is performed at high temperatures of 94°C.

***Annealing*** is performed at lower temperatures of about 50-64°C, depending on the primer sequence design. The primers bind to the target DNA and DNA polymerase begins to synthesise new DNA.

***Extension***, DNA polymerase elongates the DNA template by binding DNTPs in the 5' to 3' direction. This is commonly carried out at 72°C for different lengths of time depending on the size of the product. A final elongation step is carried out to ensure the entire DNA strand has been synthesised. This occurs for approximately 5-15 min.

Relative levels of ChIP (chromatin immunoprecipited) DNA products were assessed by QuantiTect SYBR green (Qiagen) technology on the ABI PRISM 7500 platform. A primer dissociation step was included to ensure one specific product was amplified. Primer sequences, PCR reaction components and cycling conditions are displayed in tables 2.4, 2.5 and 2.6 respectively. The comparative delta $C_T$ ($\Delta\Delta C_T$) method was used for calculating relative gene expression.

***Table 2.4 Primer sequences for ChIP PCR***

| Gene | Region of amplification | Forward primer 5' to 3' | Reverse primer 5' to 3' |
|---|---|---|---|
| S100β | promoter | TGGCAGAGGAGAGAAGCTC | TTCCTGAGCGTCCTCTTGG |
| ERα | Exon 1 | GTGTACCTGGACAGCAGCAAG | CTCGGAGACACGCTGTTGAGT |
| ERα | Exon 2 | GACAAGGGAAGTATGGCTATGGA | CCTCACAGGACCAGACTCCATAAT |

***Table 2.5 Master Mix for SYBR green PCR***

| **Reagent** | **Volume (µL) per reaction** |
|---|---|
| DNA | 2 |
| Primer mix F/R 10µM | 1 |
| SYBR Green | 10 |
| Nuclease free $H_2O$ | 7 |
| Total | 20 |

***Table 2.6 SYBR Green thermocycling conditions***

| Thermocycling step | Tm °C | Time | |
|---|---|---|---|
| *initial denaturation* | *95* | *15 min* | |
| *Denaturation* | *94* | *15 sec* | ***50 cycles*** |
| *Annealing* | *62* | *34 sec* | |
| *Extention* | *72* | *34 sec* | |
| *Final extention* | *72* | *10 min* | |
| **Dissociation step** | | | |
| *Primer melting* | *95* | *1 min* | |
| *Primer annealing* | *60* | *1 min* | |
| *Primer melting* | *95* | *1 min* | |

Relative levels of HOXC11, ERα, SRC-1, and ELAV-4 mRNA were assessed using TaqMan probe technology (Applied Biosystems), on the ABI PRISM 7500 platform. The comparative $C_T$ ($\Delta\Delta C_T$) method was also applied to analyse relative gene expression levels. Probe details, PCR master mix volumes and cycling conditions are outlined in tables 2.7, 2.8 and 2.9 respectively.

***Table 2.7 TaqMan probe sets***

| Gene | TaqMan probe |
|---|---|
| HOXC11 | Hs00204415_m1 |
| ERα | Hs00174860_m1 |
| βactin | HuACTB 4333762 |

***Table 2.8 Master Mix for TaqMan PCR***

| Reagent | Volume (µL) per reaction |
|---|---|
| cDNA | 0.5 |
| Probe | 1 |
| TaqMan master mix | 10 |
| Nuclease free $H_2O$ | 8.5 |
| Total | 20 |

***Table 2.9 TaqMan PCR thermocycling conditions***

| Thermocycling step | Tm °C | Time | |
|---|---|---|---|
| *Hold* | *50* | *2 min* | |
| *Denaturation* | *95* | *10 min* | |
| *Denaturation* | *95* | *15 sec* | ***50 cycles*** |
| *Annealing* | *60* | *1 min* | |

## 2.4 Protein Biochemistry

### 2.4.1 Lysate preparation

Cells were harvested from tissue culture flasks as per section 2.1.2. Cell pellets were re-suspended in 60-100 µl lysis buffer (RIPA buffer) (Appendix 1) supplemented with 1% protease inhibitor cocktail (Sigma) to prevent protein degradation. Cell lysates were transferred to a microcentrifuge tube (Sarstedt) and kept on ice. The cell suspension was vortexed for 15 sec every 10 min and this was repeated 3 times, followed by centrifugation at >10,000 x g for 20 min at 4°C. The supernatant was transferred to a clean microcentrifuge tube and the pellet was discarded. Protein lysate was stored at -20°C until further use.

### 2.4.2 Protein quantification

Protein quantification was determined using the BCA kit from Pierce (Thermo Scientific). This is a colorimetric assay that quantifies total protein concentration via a chemical reaction. A protein sample reduces $Cu^{+2}$ (copper) to $Cu^{+1}$ in an alkaline solution of bicinchoninic acid (supplied as Reagent A, in the kit). The protein sample is converted into a purple coloured by- product, of which the optical absorbance can be read on a spectrophotometer. The more concentrated the protein is, the greater the absorbance.

A set of albumin standards were made ranging from 0-1400µg/ml as per Table 2.10. 25 µl of each standard was aliquoted in duplicate into a 96 well dish (Greiner Bio-One). Dilutions (1:20) were made of each protein sample to be assayed and 25 µl of diluted lysate was pipetted in duplicate onto a 96 well dish. In a clean sterilin (Greiner Bio-One) 1 part reagent B was mixed with 49 parts reagent A (kit). 200 µl of this solution was added to each standard and sample. The samples were incubated at 37°C for 30 min, before reading the absorbance in a plate reader (KC4, BioTek) at 560nm.

A standard curve was prepared, with the y-axis represented as absorbance and x-axis as concentration. The average absorbance of each standard was plotted (Figure 2.5)

and the protein concentrations of each sample were quantified using linear regression.

***Table 2.10 BCA albumin standards***

| Albumin stock (µl) | dH2O(µl) | [µg/ml] |
|---|---|---|
| 0 | 100 | 0 |
| 10 | 90 | 200 |
| 20 | 80 | 400 |
| 30 | 70 | 600 |
| 40 | 60 | 800 |
| 50 | 50 | 1000 |
| 60 | 40 | 1200 |
| 70 | 30 | 1400 |

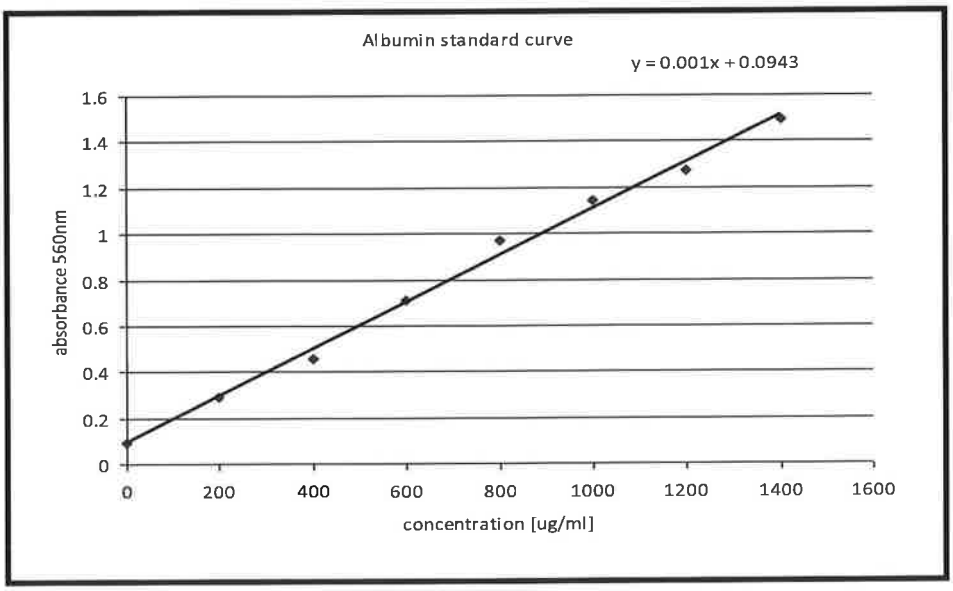


***Figure 2.5 Albumin standard curve with the equation of the line displayed.***

### 2.4.3 Western blotting

Western blotting is a universal technique which uses Sodium dodecyl sulphate (SDS) and polyacrylamide gel electrophoresis (PAGE) to separate proteins according to their molecular weight and utilizes specific antibodies to detect the proteins of interest.

SDS-PAGE gels are formed from the polymerisation of two compounds; acrylamide and bis-acrylamide. Ammonium persulfate (APS) (Sigma) and TEMED (tetramethylethylenediamine) (Sigma) are added to the gel to catalyse the polymerisation reaction. Protein samples are loaded onto the gel and can be resolved via an electric current.

SDS - polyacrylamide gels were set in ATTO gel cast plates (Atto Corporation, Tokyo Japan). A 12% resolving gel, suitable for separating proteins of 10-70 kDa was poured between the glass plates, overlayed with isopropanol (Sigma) and allowed to polymerise. Once polymerised, the isopropanol was removed, a 5% stacking gel was poured on top, a 1.5 mm comb was inserted into the top of the gel and it was left to set for a further 30 min. Components of in the resolving and stacking gels are outlined in Table 2.11.

***Table 2.11 Components for making SDS-PAGE gels***

| **Components** | **12%<br>Resolving gel<br>Total volume 10ml** | **5%<br>Stacking gel<br>Total volume 4ml** |
|---|---|---|
| H2O | 3.3 | 2.7 |
| 30% bis-acrylamide | 4.0 | 0.67 |
| 1.5M Tris pH8.8 | 2.5 | 0.5 |
| 10% SDS | 0.1 | 0.04 |
| 10% APS | 0.1 | 0.04 |
| TEMED | 0.004 | 0.004 |

Gels were placed in an ATTO electrophoresis tank, filled with 500ml of 1 X running buffer (Appendix 2). 50 µg of protein sample was mixed with an equal volume of 2 X Laemelli buffer (Sigma), briefly vortexed and heated at 95°C for 5 min. The Laemelli buffer contains SDS which denatures the protein causing them to be negatively charged. The prepared samples were then loaded onto the gel and a broad range molecular weight marker (Fermentas) was included alongside the samples to estimate protein molecular weight. The gels were run at a constant voltage of 130V for approximately 2 1/2 hours until the proteins were clearly resolved.

Proteins were then transferred onto a nitrocellulose membrane (BioRad). The gel was removed from the glass plates; a sandwich layer of 5 sheets of whatman® filter paper, nitrocellulose, gel and 5 sheets of whatman® filter paper was assembled and submerged into 1X transfer buffer (Appendix 2). The assembled gel sandwich was then placed into a semi-dry electronic transfer rig (ATTO) and a constant current of 250 mA was applied for 60 min.

Protein transfer was confirmed using Ponceau S protein stain solution (Sigma). The Ponceau S was washed off with water and the membrane was blocked for 1 hour at room temperature in 5% non-fat dried milk in 1X tris-buffered saline (TBS) buffer (Appendix 2) containing 0.1% Tween (TBST) (Sigma). After blocking, the membrane was incubated in primary antibody diluted in 4 ml TBS-T and rocked overnight at 4°C. Antibody conditions and concentrations for each protein of interest are outlined in Table 2.12 (suppliers are outline in Appendix 2). Following primary antibody incubation, the membrane was washed for 10 min, 3 times in TBS-T buffer. Horseradish peroxidise (HRP)-conjugated secondary antibody was diluted in 4 ml of 5% non-fat dried milk in TBS-T, the membrane was incubated in secondary antibody, for 1 hour at room temperature followed by three 10 minute washes in TBS-T. The membrane was developed and visualised using a chemiluminescent substrate, either ECL or SuperSignalWest FEMTO (Pierce). Both utilize a luminol and peroxidise solution, at a ratio of 1:1 to detect HRP activity. The substrate was incubated on top of the membrane for 1 min and the membrane was then placed into an x-ray cassette and exposed to an x-ray film (Fuji) in a dark room after which the bands were visualised.

***Table 2.12 Western blot antibody conditions and detection reagents***

| *1° Antibody* | *Dilution* | *MWkDa* | *Secondary Antibody* | | *Detection reagent* |
|---|---|---|---|---|---|
| ERα | 1:200 | 66 | anti-mouse | 1:2000 | ECL |
| HOXC11 | 1:1000 | 33.7 | anti-mouse | 1:4000 | FEMTO |
| S100β | 1:250 | 21 | anti-mouse | 1:4000 | ECL |
| βActin | 1:7500 | 42 | anti-mouse | 1:7500 | ECL |

## 2.5 Chromatin Immunoprecipitation Studies

Chromatin immunoprecipitation (ChIP) is a powerful technique used to analyse DNA - protein interactions, specifically, DNA sequences that are bound to regulatory proteins. Typically, DNA-protein complexes are chemically fixed by crosslinking, followed by sonication, immunoprecipitation, purification and the DNA sequence of interest is then amplified by PCR.

LY2 cells (1 X $10^6$) were seeded in 2 X 10cm culture dishes (per treatment), in 10ml CDS-MEM media and steroid depleted for 72 hours. Cells were treated, (as per treatment conditions in Table 2.1), crosslinked with 1% formaldehyde (Sigma), for 10 min, followed by quenching with 1 M glycine (Sigma) to a final concentration of 100mM. The culture dishes were washed once with PBS, 2 ml of PBS supplemented with protease inhibitor (PI) (Roche) was added to the cells and they were removed with a cell scraper (Sarstedt). Cells were transferred to a 15 ml falcon tube and centrifuged at >3000 x g for 5 min at 4°C. The supernatant was discarded and the cell pellets were stored at -80°C until ready for sonication shearing.

The immunoprecipitation step was carried out using the Dynabead® Magnetic bead system (Invitrogen). Dynabeads (anti-rabbit or anti mouse) were vigorously vortexed and 50 µl per sample, was transferred to a microcentrifuge tube. The beads were washed three times in PBS supplemented with 5µg/ml BSA using a magnetic stand and then resuspended in 350 µl of PBS/BSA. Either ERα, HOXC11 or EHZ2 antibody (6 µg, 12 µg and 2 µg respectively) (Table 2.12) was added to the beads and rotated overnight at 4°C.

The cell pellets were defrosted on ice and resuspended in 1 ml lysis buffer 1 (Appendix 3) and PI, rotated for 10 min at 4°C, centrifuged >3000 x g for 5 min. The supernatant was removed and the pellet was resuspended in lysis buffer 2, rotated for 5 min and centrifugation was repeated as above. The pellet was then resuspended in lysis buffer 3, followed by sonication (Branson, USA) of each sample at output power 4 and 90% duty cycle, for 10 second pulses, 1 minute intervals on ice and repeated 8 times (Figure 2.6). After which, 60 µl of 10% triton X-100 (Sigma) was

added to the sonicated lysate and the samples were centrifuged at 20,000 x g for 10 min at 4°C. The DNA was quantified on a nanodrop spectrophotometer.

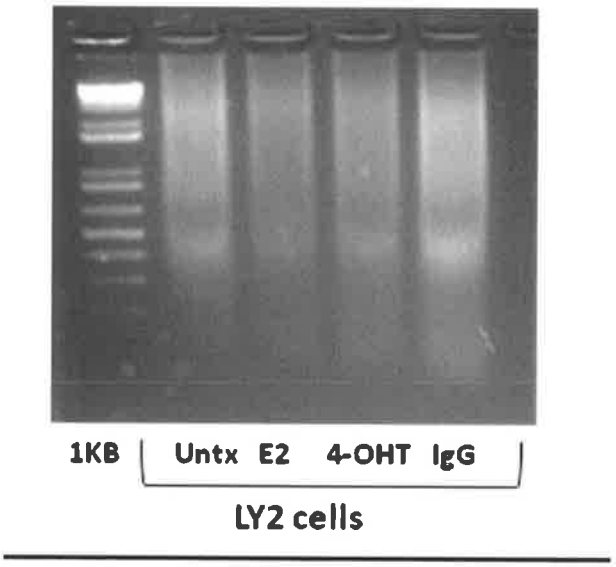


***Figure 2.6 LY2 sheared DNA after sonication.*** *10 µl of sheared DNA from each treatment was run out on a 1.5% agarose gel. The majority of sheared DNA is between 300-1000bp.*

300 µg of DNA, per sample was diluted up to 1 ml with lysis buffer 3. 40 µl of each sample was removed and stored at -80°C as input DNA. The previously prepared antibody complexed Dynabeads were retrieved and washed three times in PBS/BSA, using the magnetic stand, to remove unbound antibody. The bead/antibody complex was resuspended in 200 µl of PBS/BSA and added to the 300 µg of sheared DNA. The beads/antibody/ DNA mix was rotated overnight at 4°C. To reverse crosslink the DNA-protein complex from the beads, the beads were captured using the magnetic rack, the supernatant removed and the beads then resuspended in 1 ml ChIP RIPA buffer (Appendix 3) and mixed gently. The beads were captured again on the magnetic rack and washed a total of 8 times this way. After the final wash beads were resuspended in 1 ml TE buffer (Appendix 3), washed gently by inverting the microcentrifuge tube, and captured on the magnetic rack. The supernatant was removed; the microcentrifuge tube containing the beads was centrifuged at 1000 x g for 3 min. Excess buffer was removed with a pipette. 200 µl of Elution buffer

(Appendix 3) was added to the beads and to the input DNA. The samples were place in a water bath at 65°C for 16 hours to reverse crosslink.

To harvest the DNA, 150 µl of proteinase K mix (Appendix 3) was added to each ChIP sample and input control, mixed well and incubated for 2 hours at 37°C. Samples were extracted once with 300 µl chloroform: isoamyl alcohol (24:1), at 4°C. The pellet was dried at 37°C for 10-15 min (ensuring no residual ethanol remained) and the DNA pellet for both ChIP samples and inputs was resuspended in 40 µl of nuclease free water and stored at -20°C until further use.

Enrichment of the DNA to ERα exon 1, exon 2 and S100β promoter was analysed by semi-quantitative PCR, using SYBR green technology as per section 2.3.4. ChIP PCR primers, master mix and thermocycling conditions are outlined in Tables 2.4, 2.5, and 2.6.

## 2.6 Methylation Studies

DNA methylation is an important aspect of epigenetic regulation in eukaryotic development and cell differentiation. In cancerous cells, abnormal methylation patterns can lead to altered expression of both protein coding and non-coding genes, leading to an enhanced disease state.

To further elucidate a mechanism of HOXC11 signalling in endocrine resistant breast cancer, HOXC11 was silenced in the LY2 cell line, (as per section 2.2.2), and differential methylation patterns were assessed in genes that HOXC11 was previously found to bind directly to the DNA.

### 2.6.1 Methylated immunoprecipitated (MeDIP)

Methylated immunoprecipitated (MeDIP) DNA, reaction was carried out in the Cancer Genetics Department, in The Royal College of Surgeons, with the help of Dr. Sudipto Das under the supervision of Professor Ray Stallings.

Genomic DNA was purified from the LY2s as previously described in section 2.2.3. 5 µg DNA in a volume of 100 µl of nuclease free water was sonicated at amplitude 50% for 10 sec for 8 rounds. The DNA was sheared from 300-1000 bp and this was confirmed by resolving the DNA on a 2 % agarose gel. The DNA was then immunoprecipitated with anti-5' methylcytidine (5mC) antibody (Eurogentec) overnight, followed by selective immunoprecipitation using the Invitrogen Dynabeads System (as per section 2.5).

MeDIP and Input DNA were labelled with Cy5 and Cy3 fluorescent dyes (Roche Nimblegen) respectively. The labelled DNA was mixed together and then hybridised to a Roche Nimblegene CpG Island Plus Promoter Microarray (Human Meth. 385K Prom Plus CpG: 05543622001) overnight. These arrays consist of all known human gene promoters, that is 385, 000 probes and 22, 728 CpG Islands, sourced from UCSC genome browser, *HG18* build. The promoter regions are covered by 1KB of tiled sequence, while the CpG Islands extend at both 5' and 3' ends, with a total coverage of 700bp.

The arrays were then scanned and analysed using the GenePix pro software and results were visualised using Signal Map application. A general workflow of the methylation study can be seen in Figure 2.7. The experiment was carried out with a biological duplicate.

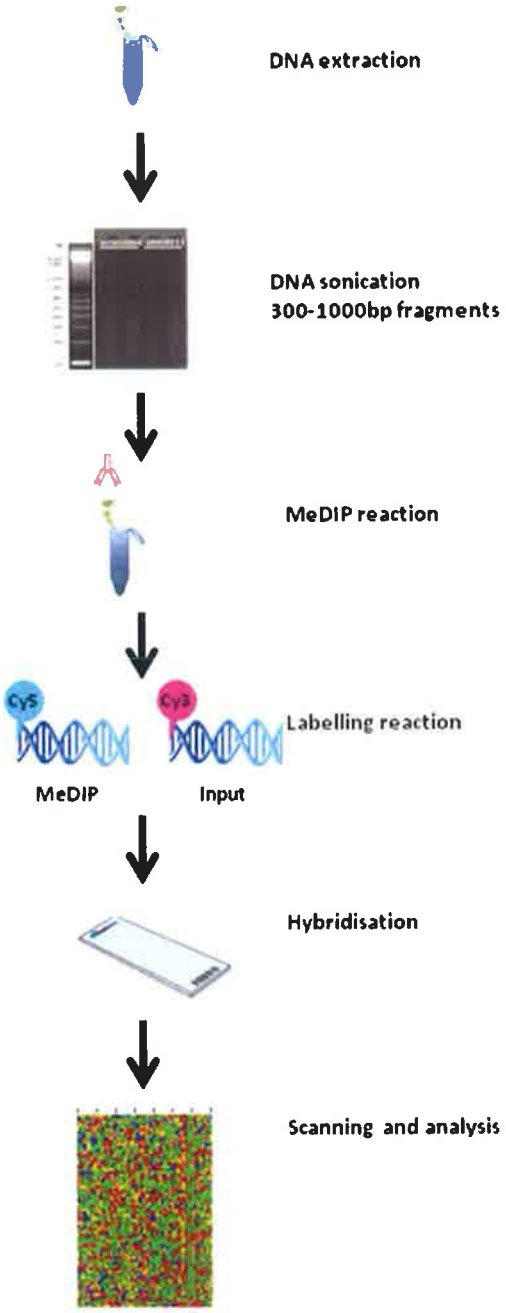


***Figure 2.7 Workflow of the MeDIP (methylation immunoprecipitation) experiment.*** *Genomic DNA is purified from cells and sheared to 300-1000bp, shearing is confirmed by resolving the DNA on a 2% agarose gel. The DNA is immunoprecipitated with a 5' methylcytidine (5mC) antibody (MeDIP). The MeDIP DNA and Input DNA are then fluorescently labelled followed by hybridisation to the arrays. The arrays are then scanned and analysed using the SignalMap software.*

## 2.7 Functional Studies

### 2.7.1 Cell motility assays

Cell motility (or migration), is an important process of normal cell growth activity. It is especially important for biological processes such as embryonic development, inflammation and angiogenesis. Migration depends on a network of specialized cell signals, adhesion molecules and cytoskeleton activity, all which can be regulated by intracellular and extracellular matrix proteins, growth factors and cell signal cascades. Cell migration, however, is also a key element of a cancerous cell's ability to invade and metastasise.

In this study, the Cellomics Cell Motility kit (Thermo Scientific) was used to quantify cell motility by measuring track areas formed by migrating cells on a specialized matrix.

Cells (MCF7, LY2's and/or HOXC11 stables) were transfected with either ERα over expression vector or empty vector as previously described in section 2.2. 24 hours post transfection a 96 well plate was coated in collagen (Sigma) and incubated for 1 hour at room temperature. The wells were washed twice with 200 µl of 1 X wash buffer, 75 µl of blue fluorescent microspheres (Kit) was pipetted into each well (vortexing after each well, to maintain a homogeneous mixture). The plate was covered in tin foil and incubated at 37°C for 1 hour in the incubator. The cells were trypsinised and a suspension of 5000 cells/ml was prepared. The plate was subsequently washed three times with 1X wash buffer (Kit) and 100 µl of cell suspension was seeded onto each well in triplicate and the plate was incubated for 24 hours, at 37°C, covered in tin foil. Cells were then fixed with 200 µl of formaldehyde staining solution for 1 hour. The formaldehyde was aspirated and cells were permeabilised with 100 µl of 1X permeabilisation buffer (kit), for 15 min at RT. The wells were washed and the cells were stained with 100 µl Rhodamine Phallodin staining solution (kit), for 30 min at RT. The plate was washed three times in wash buffer, after the third wash the wells were filled with 100 µl PBS and the plate was sealed with a sticky coversheet. Images were taken on an inverted microscope (Olympus) and track areas were measured using Cell $^{F}$ imaging software.

### 2.7.2 Proliferation assays

Proliferation assays were performed on MCF7, HOXC11 stables and LY2 cells using the MTS CellTiter 96 AQ $_{ueous}$ ® Non radioactive Proliferation Assay (Promega). The MTS assay is a colourmetric assay that determines the number of viable cells. MTS is a tetrazolium salt compound that is bioreduced by dehydrogenase enzymes found in metabolically active cells. Upon enzyme reduction, MTS is converted to aqueous formazan and the amount of formazan produced can be measured by absorbance at 490nm and is directly proportional to the number of living cells.

$5X10^3$ cells were seeded in triplicate into a 24 well dish and overlayed with 500 µl of appropriate media. The cells were incubated for 8 days and media replenished every 3 days, after which 100 µl MTS solution was added to each well. The plate was incubated for 3 hours and the absorbance read at 490nm in a spectrophotometer.

### 2.7.3 3D polarisation assays

3D polarisation assays were carried out to examine the formation and architecture of tumour cells in a luminal space. Well differentiated cells organise themselves in a highly polarised fashion with a hollowed out lumen. In contrast, lesser differentiated cells are unable to form these well organised polar structures and often contain incomplete hollowed lumens, surrounded by very disorganised cells.

Basement membrane matrigel (BDBiosciences) was thawed out overnight on ice and in the fridge. 50µl of matrigel was used to coat each well of an 8 well chamber slide (BDBiosciences). The chamber slide was incubated at 37°C to solidify for at least 30 min. $7 X 10^4$ cells were mixed with 400 µl of culture media and 10% matrigel. Cells were seeded into the chamber slide and incubated at 37°C 5% $CO_2$ for 14 days, media was replenished every 3 days containing 2% matrigel. Cells were fixed in 4% paraformaldehyde (PFA) (Sigma), permeabilised in 0.5% triton X (Sigma) in PBS for 10 min and blocked in 10% goat serum (Vector Lab) containing 1% bovine serum albumin (BSA) (Sigma) for 1 hour, then washed very gently 3 times in PBS and stained with Phalloidin Alexa Fluor 594 (Invitrogen) in the dark for 20 min. Cells were stained with DAPI (nuclear stain) for 5 min and a cover slip was mounted with DAKO (Invitrogen). Slides were visualised using a confocal microscope (Carl Zeiss S Live) coupled to LSM software.

### 2.7.4 Anchorage independent studies

A malignant cell that is able to proliferate independently of internal or external signals is a hallmark of tumourgenesis. To assess this, *in vitro* anchorage independent studies were carried out in a soft agar matrix.

A 0.6% (low melting point) agarose (Promega)/ culture media mix was prepared by heating in a water bath at 65°C for 3 hours. Once melted, 2 ml of this agararose/media mix was pipetted onto a 6 well dish and allowed to solidify in the tissue culture hood for 30 min. Excess agarose/media mix was kept at 37°C until ready for further use.

4 X $10^5$ cells for each cell line was prepared and re-suspended in 4 ml of appropriate culture media. A further 4 ml of the agarose/media mix was added to the cells and mixed thoroughly but quickly and 3 ml of this suspension was pipetted onto the prepared solidified base layer, forming a 0.3% layer. Each cell line was prepared in duplicate (~20000 cells / well). The top layer was allowed to rest for 20 min at room temperature and a final layer of 500 µl of culture media was added. The plates were kept in the tissue culture incubator at 37°C and the media was replenished once a week. At 14 days the colonies were stained with 400 µl of p-Iodoinitrotetrazolium chloride (Sigma) (1mg/ml) for 24 hours at 37°C and then stored in the fridge until ready for counting.

Viable colonies were stained brown and a graticule was used to visualise the size of the colonies. Each cell line was counted in duplicate under a light phase microscope, counting the number of colonies formed through the layers of the agarose, at 8 different points in each well.

## 2.9 Bioinformatics

Bioinformatic analysis was carried out with our collaborators Dr. P. O'Gaora and Miss Yuan Hao in the Department of Bioinformatics, The Conway Institute, UCD, Dublin 4.

## 2.10 Statistical analysis

Statistical analysis was performed using STATA 10 data software (Stata Corp. LP, Texas, USA). Survival times between patient groups were analysed by Kaplan Meier and statistical associations were examined using Fishers exact tests.

## 2.11 Construction of tissue micro-array and immunohistochemistry

TMA (tissue microarray) construction was previously carried out in the lab. Archival paraffin-embedded breast cancer tissue from the Department of Pathology, St Vincent's University Hospital, Dublin, was attained. A total 560 patients were used Patients received either, no endocrine treatment (n = 200; estrogen receptor–positive 68%) or tamoxifen (n = 360; estrogen receptor–positive 74%). Follow-up data, (median 7.72 years) was collected on the patients to determine DFS (disease free survival) and overall survival. Briefly, three 0.6-mm punch biopsies were taken from each specimen, and transplanted into a recipient block. The block was cut into 5-mm sections, mounted on Superfrost Plus slides (BDH), and baked in an oven for 1 hr at 60°C. Immunohistochemisty on the TMA sections was also previously carried out in the lab using the VectaStain Elite kit (Vector Labs). Primary antibodies for staining were as follows: chicken anti-HOXC11 (3 μg/mL) or S100β (3 μg/mL) and the TMA was counterstained with hematoxylin.

# Chapter 3

# A Functional Role for HOXC11 and ERα in Breast Cancer

## 3.1 Introduction

Homeobox genes have a distinct and fundamental role during embryogenesis and normal adult development. It is widely accepted, that signalling pathways utilized by HOX genes are subsequently perturbed in cancer. The spectrum, of deregulated HOX networks is vast and aberrantly expressed HOX genes have been found in multiple tissue organs, such as the breast, prostate, ovaries and skin. Disrupted HOX signalling pathways lead to uncontrolled proliferation, enhanced cell survival and incomplete cell differentiation (Samuel and Naora 2005).

Of particular interest to us, is the transcription factor, HOXC11. Higher expression levels of HOXC11 have been observed in invasive ductal carcinomas compared to benign tissue (Makiyama, Hamada et al. 2005). Work from our group has established that HOXC11 predicted poor disease free survival in breast cancer. Translational studies, further characterised HOXC11, as a novel SRC-1 interacting protein, (McIlroy, McCartan et al. 2010). MALDI-TOF mass spectrometry identified HOXC11 and SRC-1, to be highly expressed in LY2 endocrine resistant cells compared to MCF7 endocrine sensitive cells. Subsequently, immunofluorescent studies uncovered that HOXC11 was localised primarily in the cytoplasm, in the MCF7 cells and had a more nuclear expression, in the LY2 cells (Figure 3.1, top panel). HOXC11 is a transcription factor and nuclear expression in the resistant cell line would indicate that it is more constitutively active in the LY2 cells in comparison to the MCF7 cells where HOXC11 is found in the cytoplasm. Treatment with estrogen depleted nuclear HOXC11 in the LY2 cells whereas tamoxifen enhanced nuclear expression (Figure 3.1). We also established, that HOXC11 within a breast cancer population of 560 patients, predicted poor disease free survival ($p=0.0001$), (McIlroy, McCartan et al. 2010). These results suggest a significant role for HOXC11 in breast cancer, particularly in terms of tamoxifen resistance. Results however have so far been at an observational level and a comprehensive functional role for HOXC11 in breast cancer is yet to be explored. HOX genes are known to interact with ligand activated nuclear receptors through binding of DNA response elements resulting in trans-activation or repression of target genes (Daftary and Taylor 2006). Taking into consideration that HOXC11 is regulated by estrogen and the important interactions

between HOX proteins and hormone receptors an investigation into the interaction between HOXC11 and the estrogen receptor is also warranted.

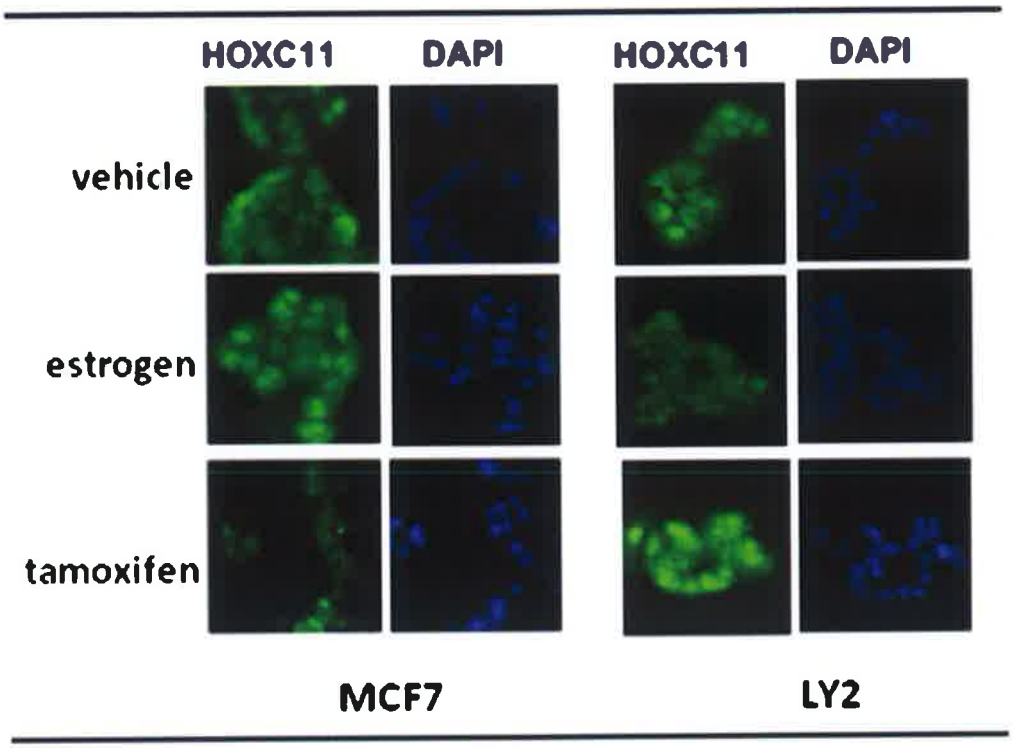


***Figure 3.1 Immunofluorescent localisation of HOXC11 (TRITC) (X100) in MCF7 and LY2 cells.*** *Cells were treated with estrogen and tamoxifen for 45 min, then immunostained with anti-HOXC11 antibody using the Vectastain Elite kit (Vector Labs) and counterstained with haematoxylin. Nuclei were counterstained with DAPI. Adapted from, McIlroy, McCartan et al. 2010 .*

HOX proteins interacting with hormone receptors is a common mechanism observed in a number of cancer types. In prostate cancer, HOXB13 for instance, has been found to be a key factor in regulation of gene transcription in response to androgen stimulation. HOXB13 can interact with the AR in several different ways, resulting in either repressive or active gene functions. Both HOXB13 and the AR can manipulate their respective DNA binding domains (DBD) to mediate interaction with each other directly, activating target genes that contain a HOXB13 binding site. Indirectly, HOXB13 can recruit AR co-regulators to androgen response elements (ARE) to activate target genes. HOXB13 can act as a repressor, by interacting with the AR

DBD, (this weakens AR association with the chromatin) and in turn causes repression of AR target genes. The biological function of silencing HOXB13 in prostate cells resulted in, reduced proliferative capacity; this was in response to androgen stimulation. Likewise, HOXB13 was also found critical for androgen stimulated cellular migration. HOXB13 ultimately can act as a repressor or as a co-activator, by differentially modulating AR target genes (Norris, Chang et al. 2009). As ER activity plays such a critical role in breast cancer and in particular in endocrine resistance, it is important to investigate the functional interaction between HOXC11 and ER. It has been established that estrogen signalling results in depleted nuclear HOXC11 in cell models of endocrine resistance. Furthermore, ChIP sequencing revealed HOXC11 enrichment on DNA of the ERα gene (unpublished data). Establishing the functional interplay between these two proteins will be a key aspect to understanding the functional role of HOXC11 in endocrine resistance.

## 3.2 Aims

(A) To establish a functional role for HOXC11 in breast cancer

and

(B) To elucidate a functional relationship between HOXC11 and the ERα.

These objectives were supported through the use of functional *in vitro* applications specifically looking at cell differentiation, proliferation, anchorage independence and migratory studies.

## 3.3 Results

### 3.3.1 HOXC11 overexpressing cells confer a phenotype similar to that of less differentiated breast cancer cell.

Epithelial cancer is not only associated with aberrant cell growth but also with changes to the organisation of the cells within the 3D structure of the effected tissue. The ability of an epithelial cell to undergo an oncogenic transformation is in part due to epithelial mesenchymal transition (EMT). EMT confers a loss of cell adhesion, an increase in cell motility and failure of a cell to differentiate, leading to irregular cell shape and disorganised polarisation (Liu, Chen et al. 2010). On the other hand in vitro, a well differentiated glandular epithelial cell will form spherical acini with a hollow lumen surrounded by a single layer of polarised cells (Debnath, Mills et al. 2002).

To assess whether HOXC11 could induce a more aggressive cell phenotype alone, 3D polarisation assays were carried out. The HOXC11 stable overexpressing cells were compared to the parental MCF7 endocrine sensitive cell model. Cells were seeded into a chamber slide containing a layer of extracellular matrix (matrigel) and incubated for 14 days, before fixing and staining. Nuclei were stained with DAPI (blue) and F-actin was stained with Phalliodin (red).

The MCF7 cells formed spherical acini, displayed a degree of polarisation and started to form a lumen (Figure 3.2). The HOXC11 overexpressing cells on the other hand show no degree of polarisation (Figure 3.2). Cells are visible within the entire acini structure and there is a complete lack of organisational architecture. HOXC11 in the stable cell line is clearly driving the cells to become more disorganised and less differentiated than their parental counterpart.

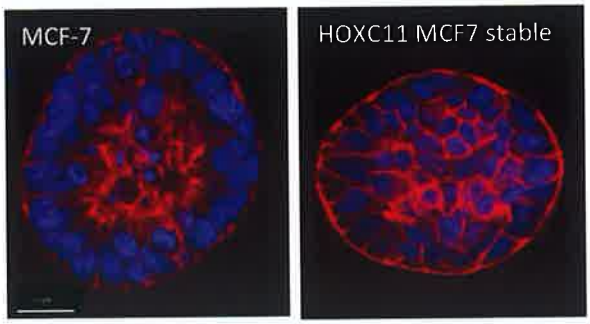


***Figure 3.2 HOXC11 overexpressing cells display a more disorganised structure and loss of any polarisation in comparison to its parental cell line, the MCF7s.*** *7 X10$^4$ cells from each cell line were seeded onto a chamber slide with matrigel. After 14 days incubation, cells were fixed and stained. Nuclei are stained with DAPI (blue) and F-actin stained with Phalloidin (red). Scale bar 200 µM, Results are representative of 3 individual experiments (N=3).*

### 3.3.2 HOXC11 overexpressing cells show a greater capacity to proliferate.

Cell growth, development and tissue formation, under normal growth conditions are tightly controlled within the cell cycle process. This environment creates a delicate balance between cell proliferation and programmed cell death. Uncontrolled proliferation however, evades and disrupts this delicate balance and is a mark of tumorigenicity.

Increased HOXC11 basal protein levels have been seen previously in the LY2 cells and silencing HOXC11 and its interacting partner SRC-1, has been shown to reduce cell proliferation in the LY2 resistant cells (McIlroy, McCartan et al. 2010). To examine, whether HOXC11 alone could contribute to cellular transformation, we explored the effect of HOXC11 on cell growth.

The MTS, colourmetric assay was employed to assess the proliferative capabilities of the MCF7, HOXC11 MCF stables and the LY2 cell lines. Over an 8 day period, HOXC11 alone was found to increase proliferation nearly 2 fold higher in comparison to the parental cell line. The LY2's had a proliferative capacity nearly 3 fold higher than the parental MCF7's (Figure 3.3). These results, suggest that HOXC11 has the potential to enhance tumorigenicity through cell growth and that this is one of the mechanisms involved in endocrine resistance. The enhanced proliferative capacity of LY2 cells suggest that whilst HOXC11 alone can effect proliferation, it is likely not the only mechanism involved in conferring an endocrine sensitive cell into a more resistant phenotype.

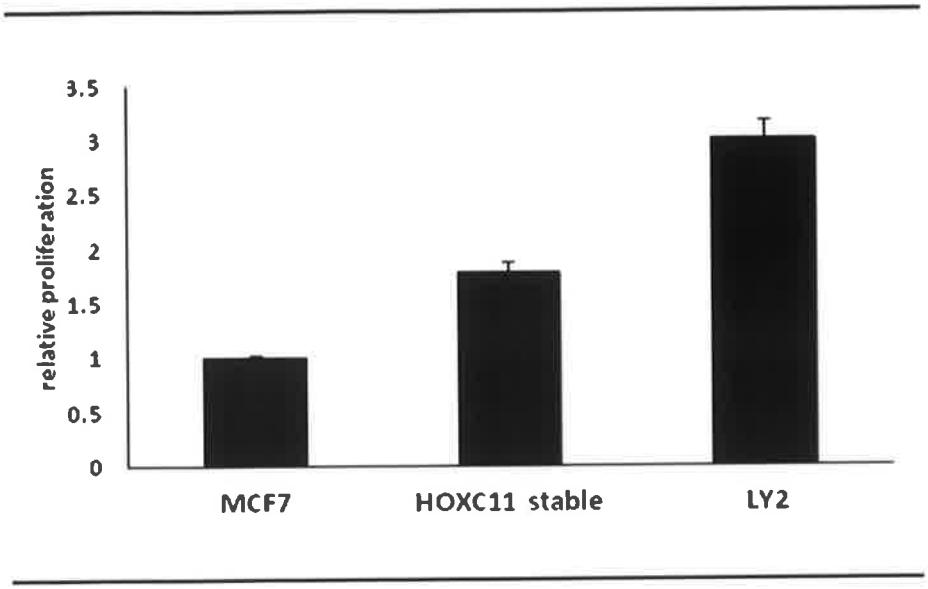


***Figure 3.3 HOXC11 MCF7 stable cells proliferate more than their parental MCF7 cells, whilst the LY2 cell line proliferate 3 fold more than their endocrine sensitive counterpart.*** *5 X10$^3$ cells were seeded in triplicate into a 24 well dish. Cells were incubated for 8 days, after which MTS solution was added and the absorbance read at 490nm. Error bars show SEM. Results are representative of 2 individual experiments (N=2).*

### 3.3.4 HOXC11 stable cell line is advancing towards an anchorage independent phenotype.

Most non-malignant cells are anchorage-dependent, that is, cells need to anchor themselves to the extracellular matrix (ECM) or solid surface for normal growth and cell division. In the development of cancer, a cell loses the need to grow on an adherent surface and is able to proliferate regardless (Thullberg and Stromblad 2008). During oncogenesis the loss of anchorage control allows for a cell in primary tumours to detach metastasis and invade other tissues. This complex process is due in part to a cancer cells ability to adapt to its microenvironment and utilize activation of extracellular proteases. In general, loss of cell-cell adhesion molecules (CAMS) and loss of integrin factors (important for cell attachment to the ECM) all play a role in malignant cellular transformation from an anchorage-dependent phenotype to an anchorage-independent phenotype (Hanahan and Weinberg 2000).

The ability of a cell to grow on soft agar is a routine method to test anchorage-potential. Cells are seeded onto a dish containing an agarose/media basement layer and left for a period of time to proliferate. Malignant metastatic cells are able to form colonies within the agarose "jelly like" matrix, compared to, well differentiated cells that are incapable of forming colonies without the availability of an adherent surface. The MCF7 cells formed very few colonies, on average 13.1 colonies per well (graph, Figure 3.4). They were very small and inconsistent in spherical shape with most colonies less than 50 μM wide (top panel, Figure 3.4). The HOXC11 overexpressing cells on the other hand formed larger colonies than the MCF7s (top panel, Figure 3.4) reaching up to 100 μM wide with an average of 26.5 colonies per well. The HOXC11 overexpressing cells formed less very small colonies of less than 50 μM in size (graph, Figure 3.4). In contrast the LY2s formed lots of colonies, on average 64.1 per well within the 3 different layers of the agar. These colonies were highly structured and very large in comparison to the MCF7 derived cells, ranging on average from 200 μM and upwards (Figure 3.4).

Our results show that the HOXC11 overexpressing cells, though not as anchorage independent as the endocrine resistant LY2 cells have the potential to proliferate without the need of a solid matrix unlike the parental MCF7 endocrine sensitive cells.

The HOXC11 stables are displaying signs of malignant transformation of a cell line with a more aggressive phenotype.

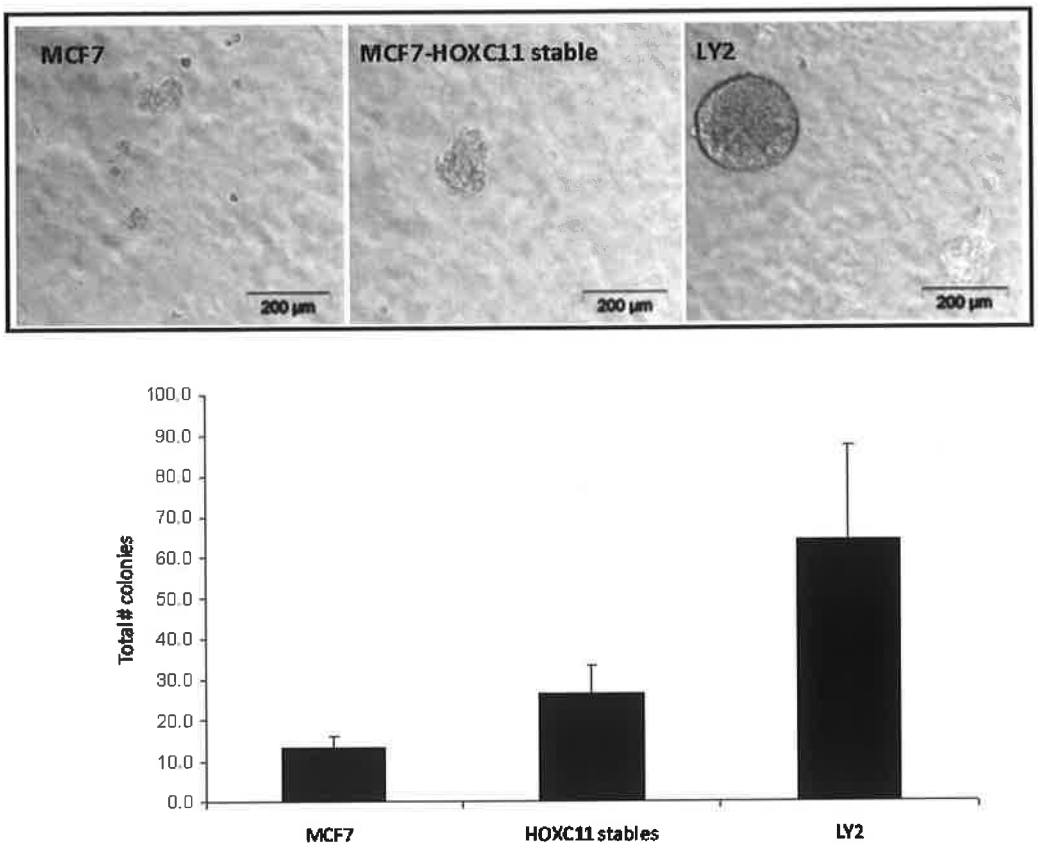


***Figure 3.4 HOXC11 stable overexpressing cells are beginning to display an enhanced anchorage independent phenotype.*** *In a 6 well dish a 0.3% agarose/cell suspension mix was pipetted on top of a 0.6% agarose/media basement layer. The plate was incubated for 14 days, followed by fixing and staining with p-Iodoinitrotetrazolium chloride. Upper panel displays an image of the individual colonies under a light microscope and the lower panel displays a graph with the total number of colonies counted. Each cell line was plated in duplicate. Graph displays SEM. Results are representative of 3 individual experiments (N=3).*

### 3.3.5 HOXC11 induces cell motility.

Tumourigenicity is characterised by a cell's ability to spread out and migrate from the primary tumour site, metastases and invade secondary tissue. The migratory capacity of a cell is therefore an important factor in deducing whether a cancer cell has metastatic potential or not.

Results thus far, indicate that HOXC11 expression, induces a loss of polarisation, enhances proliferative capacity and drives anchorage-independence. The drift towards a more aggressive characteristic drove us to explore whether HOXC11 could induce cell migration.

MCF7 cells and HOXC11 overexpressing cells were seeded on a surface coated with collagen and blue fluorescent beads. As the cells migrate, they phagocytose and push away the beads, leaving behind a track area. The track area is directly proportional to the degree of cell movement. The HOXC11 over expressing cells had nearly double the capacity to migrate over the MCF7's alone, as seen from both the microscope images and bar graph of relative migration (Figure 3.5). This result demonstrates that overexpression HOXC11 confers a more metastatic and invasive characteristic.

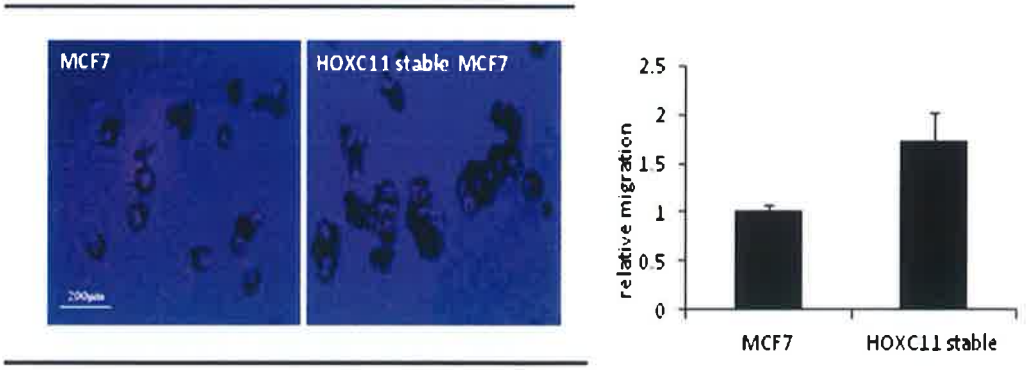


***Figure 3.5 HOXC11 overexpression increases cell motility.*** Cells were seeded onto a lawn of blue fluorescent beads, incubated for 24 hours, then fixed and stained with Rhodamine. Figure displays an overlayed image of DAPI (blue) and TRITC (red). Scale bar 200 μM. Graph shows relative migration per cell. Results are representative of 3 individual experiments (N=3).

### 3.3.6 Knockdown of HOXC11 reduces migratory capacity of resistant cells.

Previous studies in our lab have shown that the LY2 and LetR endocrine resistant cell models have higher protein levels of HOXC11 (McIlroy, McCartan et al. 2010). In addition, the LetR cells were seen to have 5 fold more, migratory capacity than their parental cell line (McBryan, Theissen et al. 2012). With this in mind, we wanted to investigate, to what effect HOXC11 knockdown would have on the migratory capacity of a cell.

LY2 and LetR cells were transfected with an siRNA targeted at HOXC11. 24 hours post transfection the cells were trypsinised, counted and seeded onto a lawn of collagen and blue fluorescent beads, as described previously above. The cells were incubated for a further 48 hours before fixing and staining.

Both the LY2 and LetR cells are highly motile, as seen by the large tracks formed (Figure 3.6). These results are consistent with our previous studies. Silencing HOXC11 hindered migration in both cell lines, by at least 50% in the LY2s and 80% in the LetR cells. Notably, silencing HOXC11 expression has a greater effect in hindering the migratory capacity of the most aggressive metastatic cell line the LetRs.

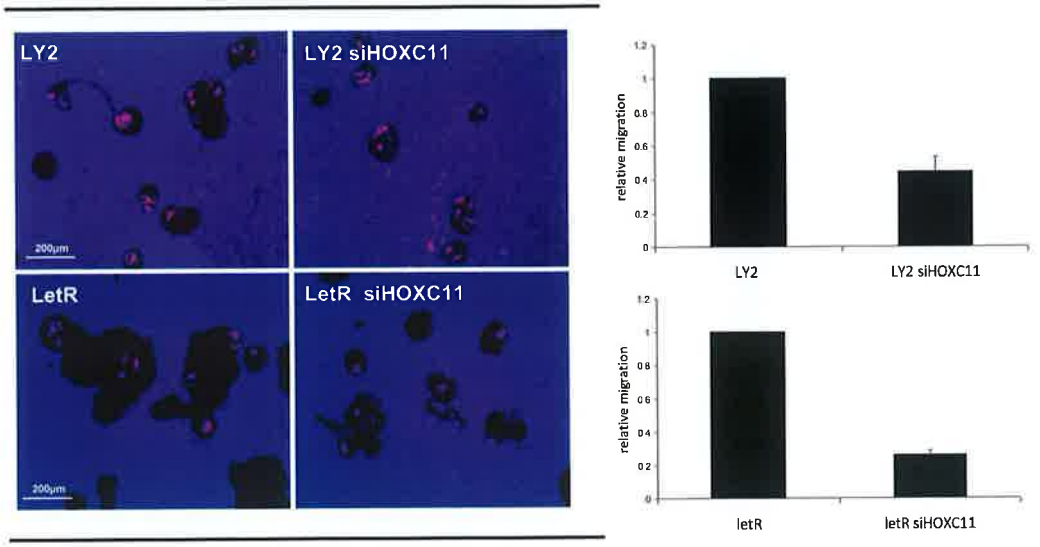


***Figure 3.6 Knockdown of HOXC11 decreases cell motility in the LY2 and LetR endocrine resistant cell lines.*** *Cells were transiently transfected with siRNA against HOXC11. 24 hours post transfection, cells were trypsinised and seeded onto a lawn of blue fluorescent beads, incubated for a further 48 hours and then fixed and stained with Rhodamine. Figure displays an overlayed image of DAPI (blue) and TRITC (red). Scale bar 200 μM. Graph shows relative migration per cell. Results are representative of 3 individual experiments (N=3).*

### 3.3.7 Forced expression of ERα in MCF7 and LY2 cells can improve differentiation.

Both ERα and ERβ are present in normal breast tissue. The absence of either receptor is not lethal but it can induce physiological consequences (Zilli, Grassadonia et al. 2009). ERα expression alone is responsible for ductal mammary growth, as seen by ERα KO mice which display primitive ductal growth in comparison to WT mice that undergo normal ductal morphogenesis with terminal end bud branching (Korach, Couse et al. 1996). ERα is not only important in mammary tissue formation but its expression in a breast tumour is also one of the main predictors to endocrine therapy response (Zilli, Grassadonia et al. 2009). Considering these very important roles for ERα in breast tissue, we wanted to explore to what effect overexpression of a functional ERα could have on differentiation in the endocrine sensitive model versus the endocrine resistant model.

To achieve this, we carried out 3D polarisation assays on the MCF7 and LY2 cells. Firstly ERα was transiently transfected into each cell line, 24 hours post transfection, the cells were trypsinised, counted and seeded onto the chamber slide containing matrigel. The cells were incubated, fixed and stained as previously described in section 3.3.1.

ERα overexpression did not have a significant impact on polarisation of the MCF7 cells. By contrast ER overexpression did appear to have an effect on the LY2 cells which normally have no distinct polarised characteristics and form highly disorganised spheres (A, Figure 3.7). The ERα overexpressing LY2 cells are a little more organised and are beginning to polarise towards the outer layer of the sphere compared to the control empty vector LY2 cells. Although, there is still no visible sign of lumen formation, this result is indicative that ERα may have the potential to rescue a cell, with a metastatic endocrine resistant phenotype, to becoming a more, well differentiated cell type. Confirmation of the ERα overexpression was carried out by western blot analysis, (B, Figure 3.7).

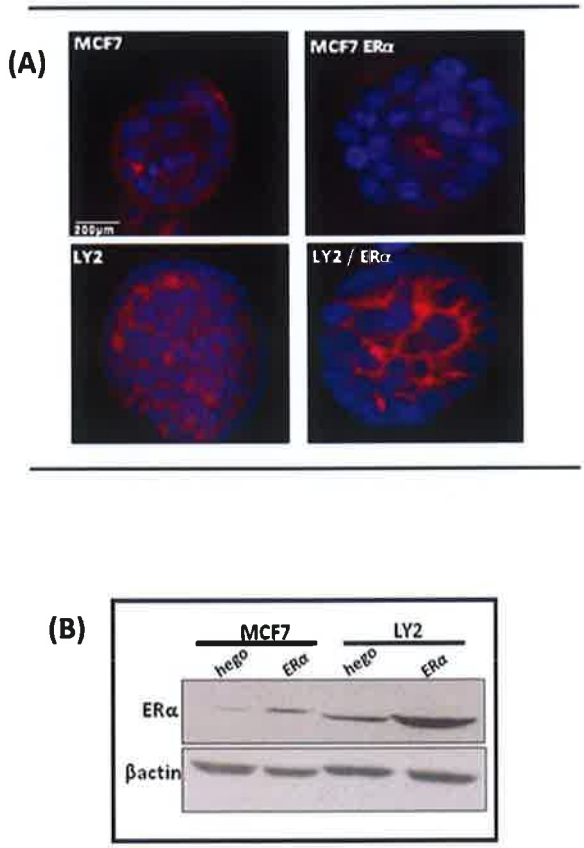


***Figure 3.7 In 3D polarisation assays MCF7's form better organised spheres in comparison to the LY2 cell line. Forced overexpression of ERα displays enhanced polarisation and a more organised structure than their empty vector counterparts.*** ***(A)*** *MCF7 and LY2's were transiently transfected with an ERα overexpression vector. 24 hours post transfection; cells were trypsinised, counted and seeded onto a chamber slide containing matrigel. Cells were incubated for 14 days before fixing and staining. Nuclei are stained with DAPI (blue) and F-actin stained with Phalloidin (red). Scale bar 200μM.* ***(B)*** *ERα overexpression in the MCF7 and LY2 cell line was confirmed by western blot analysis. 30μg of protein was loaded onto a 12% acrylamide gel. ERα (66kDa) was successfully overexpressed in both cell lines. Protein loading control was assessed by βactin levels (42kDa). Results are representative of 3 individual experiments (N=3).*

### 3.3.8 Forced expression of ERα in HOXC11 stable cells can greatly enhance differentiation.

Earlier results show HOXC11 expressing cells do not form complete acini structures with hollowed out lumens and they have no form of organised polarisation (Figure 3.1) and having seen that ERα may have the potential to rescue an LY2 cell from an un-differentiated cell to a more well differentiated cell (Figure 3.7), we thought to investigate whether the effects of HOXC11 overexpression could also be rescued by ERα.

The HOXC11 stable cell line was transiently transfected with ERα or empty vector (hego-PSG) for 24 hours. The cells were then seeded onto matrigel and left to incubate as previously described. Once again, the HOXC11 expressing cells did not form structurally ordered acini; however the cells co-transfected with ERα show a significant degree of improvement in organised architecture, (Figure 3.8). The extent to which ERα transformed a badly disorganised, more metastatic cell, into a well-differentiated cell, can be seen by the formation of a lumen beginning to emerge (Figure 3.8). This finding suggests that HOXC11 and ERα may be working in an opposing manner, where one protein (HOXC11) confers to malignant transformation and the other protein (ERα) can reverse these effects of malignancy into a more differentiated cell type.

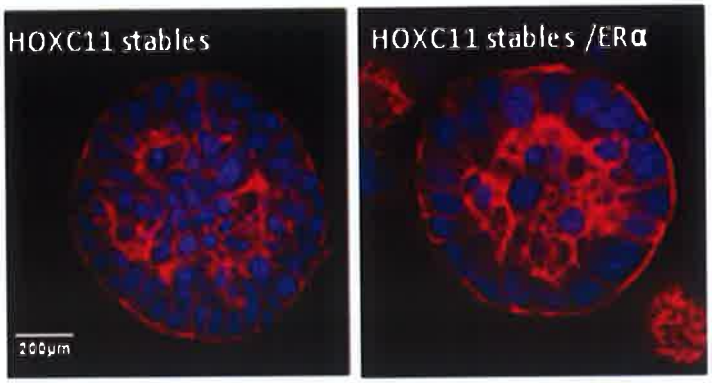


***Figure 3.8 Overexpression of ERα can help rescue a poorly differentiated unorganised HOXC11 MCF7 cell into a more well-differentiated polarised cell phenotype.*** *The HOXC11 stable cell line was transiently transfected with an ERα overexpression vector or an empty vector, Hego. 24 hours post transfection; cells were trypsinised, counted and seeded onto a chamber slide containing matrigel. Cells were incubated for 14 days before fixing and staining. Scale bar 200 µM. Results are representative of 3 individual experiments (N=3).*

### 3.3.9 Forced expression of ERα in HOXC11 stable cells can significantly reduce cell motility compared to MCF7 cells.

Taking into account that the HOXC11 expressing cells have a higher migratory capacity than their parental MCF7 cell line (Figure 3.4), we thought to explore whether ERα overexpression could have any impact on migratory capacity of this cell line.

As described previously, ERα was transiently overexpressed, along with its empty vector counterpart, in the HOXC11 stable MCF7. Once again, ERα did not have a significant impact on the MCF7s alone, whereas ERα forced expression greatly reduced migration in the HOXC11 stables, by close to 50% as seen by the graph displaying relative migration (Figure 3.9).

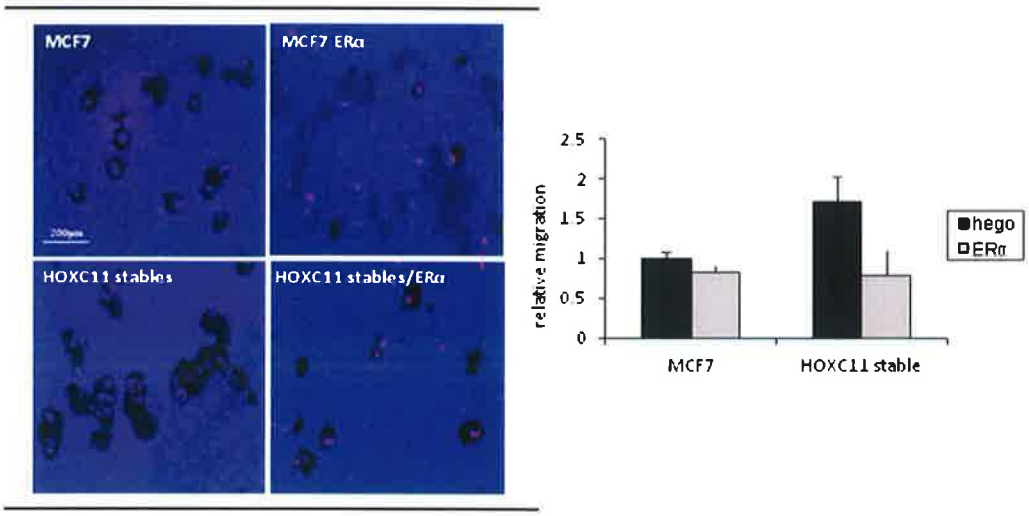


***Figure 3.9 HOXC11 overexpressing cells are more motile than MCF7 cells alone. Transiently transfecting ERα reduces cell motility in both cell lines with greater effect in the HOXC11 overexpressing stables.*** *Cells were transiently transfected with either empty hego or ERα overexpression vector. 24 hours post transfection; cells were trypsinised and seeded onto a lawn of blue fluorescent beads, incubated for 24 hours, followed by fixing and staining with Rhodamine. Scale bar 200 μM. Graph shows relative migration per cell, SEM. Results are representative of 3 individual experiments (N=3).*

## 3.4 Discussion

Our group has characterised a particular homeobox transcription factor HOXC11 that co-operates with SRC-1 and drive an endocrine resistant phenotype and in breast cancer patients HOXC11 was found to be a strong predictor of poor disease free survival (McIlroy, McCartan et al. 2010). On the whole associations between HOXC11 and endocrine resistance have been merely observational and a functional role for HOXC11 in breast cancer has not been described. Here we discuss, for the first time a definitive role for HOXC11 within the cell microenvironment and reveal its relationship with ERα.

### 3.4.1 HOXC11 in breast cancer

We have identified HOXC11 as a driving factor of malignant transformation. In order for a cancer cell to become metastatic it must undergo a transition from a normal epithelial cell into a mesenchymal like cell type, in a process known as EMT (epithelial mesenchymal transition) (Micalizzi, Farabaugh et al. 2010). EMT consists of the breakdown of tissue architecture through disintegration of cell-cell adhesion junctions, loss of cellular apico-basal polarity and anchorage independence. All of these molecular processes are required before a cell can finally undergo metastasis and invasion into new cell tissue (Fidler 2003).

Our findings show that breast cancer cells expressing higher levels of HOXC11 display a phenotype closer to that of cells undergoing EMT. We found that HOXC11 promotes loss of polarisation and encourages a more disorganised structure in a 3D matrix. HOXC11 alone can induce increased proliferative and migratory activity. As seen by our colony forming assays HOXC11 can drive cells to grow in a more anchorage-independent fashion. Examples of HOXC11 and its role in breast cancer are few and far between. However other homeobox transcription factors have been implicated in breast cancer (Makiyama, Hamada et al. 2005). One particular HOX protein that can induce a metastatic phenotype is the transcription factor GOOSECOID. Elevated GOOSECOID expression levels in breast tumours were found to promote a metastatic and invasive phenotype. Found partially due to the

concordant loss of epithelial markers, E-cadherin, α-catenin and γ-catenin (Hartwell, Muir et al. 2006).

Anchorage independence is one of the hallmarks of malignant transformation. In order for a cell to metastasise it typically develops alterations in cell shape as well as attachment to other cells and to the extracellular matrix (ECM) (Hanahan and Weinberg 2011). Our study is not too dissimilar to that of previous study by Toi and colleagues. They found that tamoxifen resistant MCF7 cells conferred a more aggressive phenotype in soft colony agar. These endocrine resistant clones were the only cells able to form distinguishable colonies in comparison to normal MCF7 cells (Toi, Harris et al. 1993). We also observed that our tamoxifen resistant LY2 cells could grow on soft agar and found that HOXC11 alone could induce a similar phenotype in the MCF7 cells (although to a lesser extent).

There are numerous studies involving other HOX proteins which have been implemented in breast cancer metastases. The aggressive phenotype induced by HOXC11 overexpression bares resemblance to the aggressive behaviour of many other HOX genes. HOXB9 for instance induces several angiogenic factors such as VEGF (vascular endothelial growth factor), bFGF and IL8 (interleukin 8) which leads to increased cell motility and lung metastasis (Hayashida, Takahashi et al. 2009). HOXB7 overexpression can promote tumourigenesis through increased invasion and migration (Wu, Chen et al. 2006). On the other hand, reduced levels of certain other HOX proteins, such as HOXA5 and HOXA10 can also induce tumour progression via the downregulation of p53 resulting in reduced apoptotic signalling pathways (Raman, Martensen et al. 2000; Chu, Selam et al. 2004). In accordance to the 5' deregulated HOX genes in cancer we found that the posterior HOX protein, HOXC11 can induce tumourigenicity through increased proliferation and migration.

Our studies show that overexpression of HOXC11 in particular confers to a more malignant characteristic than the original parental MCF7 breast cancer cell line. Proliferation, differentiation and cell death are fundamental components of tumourigenesis (Hanahan and Weinberg 2000) and HOXC11s' ability to deregulate these complex cellular processes is reflective of the fact that it can drive progressive oncogenic transformation to a phenotype similar to that of the resistant LY2 cell line.

### 3.4.2 HOXC11 and ERα

Findings published from our group have uncovered an important role for HOXC11 in endocrine resistant breast cancer (McIlroy, McCartan et al. 2010). Our group found that tamoxifen stimulation enhanced nuclear HOXC11 in the LY2 cells. In contrast estrogen abrogated this effect. In this study we focused on elucidating a functional role for HOXC11 and the estrogen receptor and see to what effect their relationship may have in breast cancer tumourigenesis. In cells overexpressing HOXC11 and ERα, ERα overexpression can result in a reduction of HOXC11 expression (as would be expected due to increased estrogen signalling). However, overexpression of ERα did not abrogate HOXC11 expression levels completely (Figure 5.3, second panel from the top). Therefore indicating functional interactions between HOXC11 and ERα are not purely at a transcriptional level.

We found that forced expression of ERα, improved cellular differentiation in breast cancer cell lines, as seen by 3D polarisation and motility assays. Enhanced effects of ERα were seen primarily in the HOXC11 overexpressing cells in comparison to the parental cell line. It would appear that the more aggressive phenotype induced by HOXC11 can be somewhat reversed by the ERα forced expression.

A substantial amount of evidence suggests that endocrine resistance is, in part due to reduced ER expression or function (Creighton, Hilger et al. 2006). From our studies we can infer that the consequence of reduced ER function can enhance the aggressive phenotype induced by HOXC11. Thus the loss of ER function in endocrine resistance results not only in tumour cells becoming resistant to endocrine disruptors but also forces the promotion of an aggressive phenotype through HOXC11 signalling.

In all, the findings presented in this chapter, indicate that HOXC11 plays an important role in breast cancer. We focused on the functional aspects of HOXC11 overexpression as they relate to human breast cancer cells. We describe a role for HOXC11 which supports several oncogenic functions relevant to malignant transformation and we demonstrate a previously unrecognised relationship between HOXC11 and ERα.

# Chapter 4

# HOXC11 Regulates Target Genes, including ERα

## 4.1 Introduction

HOX genes are ubiquitous in nature and considering they regulate an abundance of developmental and normal cellular pathways it is not surprising that they have numerous target genes (Daftary and Taylor 2006). Whilst these genes have been extensively studied in embryogenesis remarkably in adult tissue HOX targets remain somewhat elusive (Svingen and Tonissen 2006). HOX genes regulate numerous downstream cellular processes and aberrant expression is a foundation for mass deregulation for many of these molecular pathways. For this reason alone it is difficult to identify individual HOX targets (see Figure 1.12). Uncovering HOX target and teasing out the regulatory elements that drive HOX expression will help us understand their role in the development of breast cancer.

### 4.1.1 Uncovering HOXC11 target genes

Understanding how proteins interact with the DNA is important if we are to appreciate the complexity of transcriptional regulation. One prevailing method around today to help study this field of molecular biology is a technique known as chromatin immunoprecipitation sequencing or ChIP-seq for short. This technology allows us to identify new target genes from scanning global protein-DNA binding patterns.

As HOXC11 is a DNA binding protein a ChIP-seq experiment was carried out in our lab in order to help us identify new HOXC11 target genes. This experimental work was carried out previously in our lab by Dr. Damian McCartan. Published studies from our group revealed that the transcription factor HOXC11 was upregulated in the LY2 endocrine resistant breast cancer cell line (McIlroy, McCartan et al. 2010). Therefore a HOXC11 ChIP -seq experiment was undertaken in this cell line. The cells were treated with either vehicle or tamoxifen ($10^{-7}$ M), followed by fixating and shearing of the chromatin into fragments of about 200-300 bp. Chromatin enrichment was carried out by immunoprecipitating the cells with an antibody against HOXC11. Library preparations of the target DNA were primed and deep sequencing was carried out on the Illumina/Solexa Genome platform, by Genpathway (Figure 4.1).

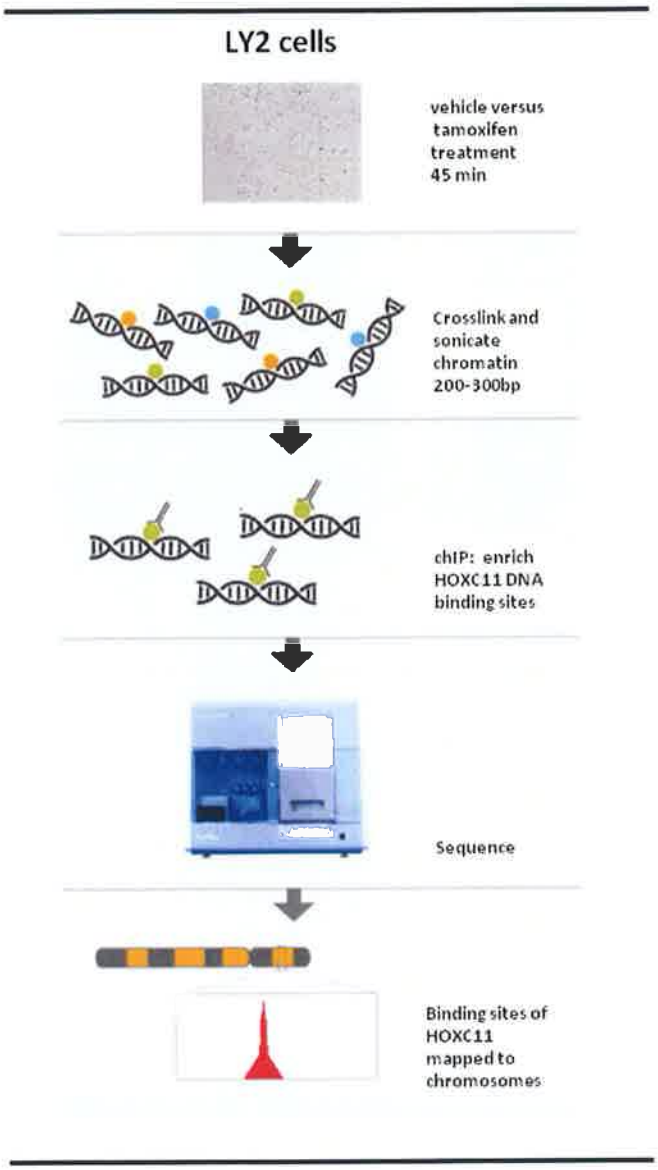


***Figure 4.1 ChIP sequencing workflow overview.*** *DNA-protein interactions are crosslinked and the cells are sheared into fragments of about 200-300 bp. The chromatin was immunoprecipitated with a HOXC11 antibody. The final DNA prep was sequenced and mapped to the genome. (Image adapted from the Ilumina® website)*

HOXC11 global binding patterns were then mapped to each chromosome in the human genome.

More than 1000, putative DNA-protein binding events were differentially visualised between the vehicle and tamoxifen treated LY2 cells. 49% of HOXC11 binding events were found in the protein coding region, 19% were in pseudogenes (genes that have lost protein coding function) and 32% were found in non-coding regions. Potential HOXC11 targets were associated with binding regions that were overlapping genes directly or regions associated nearest to a gene downstream of the binding event. The results of the ChIP seq analysis suggest that HOXC11 can induce transcription via direct binding to gene promoters or distally by modulating enhancer regions, which induce chromatin re-modelling in order to activate target genes (He, Hua et al. 2011).

Interestingly a number of genes were highlighted which contained multiple HOXC11 binding peaks within the gene body. The peaks were not randomly dispersed within the gene but rather, the binding peaks were specifically localised to exon regions of these genes. The peak calling for these genes was particularly strong and these peaks were significantly higher in the tamoxifen treated sample compared to the vehicle treated sample. In total, five genes were identified which displayed this particular pattern of HOXC11 binding, ERα, SRC-1, ELAV-4, TFF1 and HOXC11 itself. Our group had already identified SRC-1 as an interacting binding partner for HOXC11. ELAV-4 also known as HuD, is an RNA binding protein, whose main function is in neuron plasticity regulation during brain development (Lazarova, Spengler et al. 1999). TFF1, trefoil factor 1 (or pS2) is a known ER target gene involved in transcriptional regulation and cell proliferation and it is often upregulated in ER+ breast cancer epithelial cells (Carroll and Brown 2006). HOXC11 was also on this list and it therefore appears that it might regulate itself. ERα was also identified as a HOXC11 target and HOXC11 peaks were enriched in all 8 of the protein coding exons. The multiple anchoring pattern combined with the strength and specificity of these binding peaks is indicative of a strong association between HOXC11 and these 5 target genes. For the purpose of this study we focused on the ability of HOXC11 to bind to the DNA and regulate expression of ERα.

### 4.1.2 Epigenetics: methylation of ERα in breast cancer

Having established that HOXC11 binds to a number of target genes in endocrine resistant cells, our next goal was to identify if HOXC11 upregulates or downregulates expression of those target genes and to investigate the mechanisms by which HOXC11 induces this regulation. A lot of research today would suggest that epigenetic regulation may play a large role in the progression of breast cancer resistance (Pathiraja, Stearns et al. 2009). We were particularly interested to see if HOXC11 plays a role in epigenetic regulation of ERa in endocrine resistant breast cancer cells.

Epigenetic regulation literally describes the phenotypical changes that occur in our genome by anything other than changes in the DNA sequence (Pathiraja, Stearns et al. 2009). Such processes that can induce epigenetic changes include DNA methylation and histone modification. Until recently it was thought that only protein coding genes were capable of inducing epigenetic changes. Now it is known that both microRNAs and lncRNAs (long non-coding RNAs) can also induce gene silencing through DNA hypermethylation (Gibb, Brown et al. 2011). All of these processes are normal functions of gene regulation and deregulation of this epigenetic machinery is considered a marker of oncogenesis (Szyf 2008).

DNA methylation consists of the addition of a methyl group to the carbon 5 nitrogenous base, cytosine in a DNA strand. Methyl groups are added only when a cytosine is adjacent to guanine in a positioning system known as CpG (Szyf 2008). Areas densely populated with CpGs are known as CpG islands and they are often located in the proximal promoter regions close to the TSS of a gene, (Figure 4.2) (Jones and Baylin 2002). DNA methylation in CpG rich regions usually induces gene silencing and un-methylated CpGs are often associated with gene activation. Methylation itself is a tightly regulated process catalysed by a group of enzymes called DNA methyltransferases or DNMTs (Agrawal, Murphy et al. 2007).

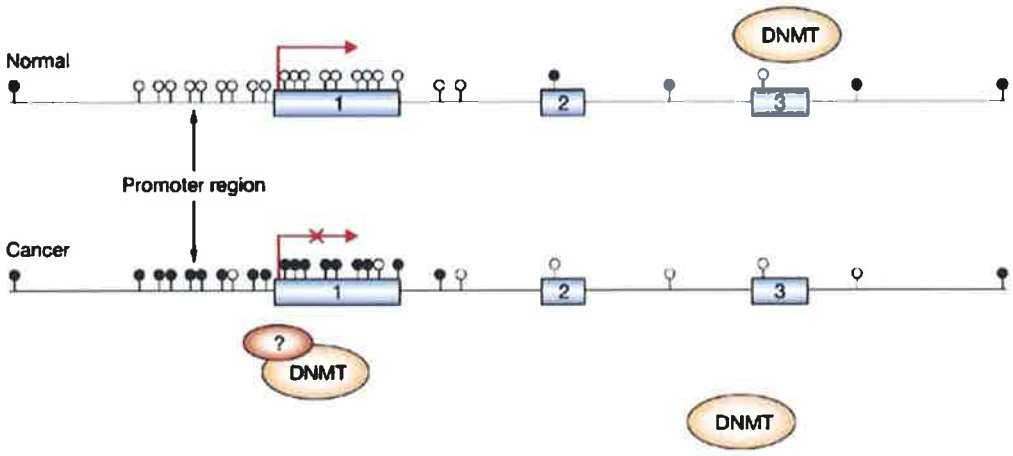


***Figure 4.2 DNA methylation in normal and cancerous cells.*** Schematic representation of methylation patterns in normal and cancerous cells. CpG islands (white lollipops) are usually unmethylated in normal cells and hypermethylated in cancerous cells (black lollipops). 1, 2 and 3 represent the exonic region of the gene. Densely populated CpG sites are primarily found in the promoter and proximal exonic regions. Methylation results in the disassociation of DNMTs (Baylin 2005).

***Table 4.1 Genes known to be hypermethylated in breast cancer*** *(adapted from Agrawal, et al. 2007).*

| | |
|---|---|
| BRCA1 | Breast cancer |
| CDH1 | Cadherin 1 |
| CDKN2A | Cyclin-dependent kinase inhibitor 2A |
| ER | Estrogen receptor |
| FOXA2 | Forkhead box A2 |
| HOXD11 | Home box D11 |
| LATS1/LATS2 | Large tumor suppressor 1 and 2 |
| MGMT | Methylguanine methyltransferase |
| p57KIP2 | Cyclin-dependent kinase inhibitor 1C |
| PCDH10 | Protocadherin 10 |
| PR | Progesterone receptor |
| TIMP-3 | Tissue inhibitor of metalloproteinases-3 |
| TMS1 | Target of methylation-induced silencing 1 |

Numerous studies have evaluated the methylation status of the ERα gene (Ottaviano, Issa et al. 1994; Parl 2003; Agrawal, Murphy et al. 2007). In ER negative breast cancer cells, prominent methylation has been detected along the CpG island 5' promoter region of the ERα. It is hypothesised that this abnormal methylation could account for transcriptional silencing of ERα and subsequent hormone resistance in some breast tumours (Ottaviano, Issa et al. 1994). More recently the methyltransferase SET7 has been directly linked with ERα protein methylation. SET7 can methylate lysine 302 on the ERα, resulting in the stabilisation of ER protein and encouraging the ERα to become hypersensitive to estrogen activity (Zhou, Shaw et al. 2009). The reported magnitude of which ERα is methylated varies widely amongst different studies. This is often thought to be due to the differences in technical methods and the degree of variation between the sensitivity of these methods (Pathiraja, Stearns et al. 2009). However it is clear that further understanding in the epigenetic regulation of the ERα may ultimately lead to improved treatment care options.

Probably one of the more important aspects of epigenetic regulation is that its effects can be reversed. Therefore novel drugs targeting the enzymes which catalyse methylation have been developed to reverse their silencing effects. Of the more popular class of drugs are the HDAC inhibitors and DNMT enzyme inhibitors (HDACIs and DNMTIs). HDACIs target histone methylation and DNMTIs on the other hand target DNA methylation. HDACIs work by altering chromatin structure, resulting in loosening or opening of the histones allowing for regulatory transcriptional machinery to enter and bind to the DNA and can re-activate the expression of inappropriate silenced genes (Huang, Nayak et al. 2011). DNMTIs on the other hand, target direct methylation of the DNA. These drugs directly inhibit methyltransferases from silencing genes. Promising research into the efficacy of these inhibitors demonstrates that both HDACIs and DNMTIs in combination have a greater effect (Cameron, Bachman et al. 1999). Specifically in breast cancer, ER negative cells treated with both inhibitors in combination, show increased response to tamoxifen treatment (Fan, Yin et al. 2008). In the clinic this poses significant hope for patients that are ER negative and for those who develop acquired or de novo resistance to tamoxifen treatment.

To date it has been well established that DNA methylation in the 5' promoter region silences gene expression. However, the role of gene methylation within gene bodies

remains unclear and is yet to be fully explored (Maunakea, Nagarajan et al. 2010). Current thinking and new found evidence would suggest that methylation patterns seen within gene bodies are found in highly expressed human genes (Ball, Li et al. 2009)and intragenic methylation is thought to have a functional and profound effect on gene transcription (Ball, Li et al. 2009; Maunakea, Nagarajan et al. 2010; Friedlander, Roy et al. 2012). Our ChIP-seq data revealed that HOXC11 enrichment lies solely along the intragenic region of the ER. This enrichment was specifically only seen after stimulation with tamoxifen.

We believe ERα is a true HOXC11 target gene and hypothesise that aberrant expression of HOXC11 in an endocrine resistant model might lead to loss of ER function via a mechanism of hypermethylation in the intragenic region of ER. Identifying ERα as a HOXC11 target gene and teasing out the interplaying mechanisms between HOXC11 and ER will help us elucidate the role in which HOXC11 plays in promoting endocrine resistance.

## 4.2 Aims

The focus of this chapter was to elucidate the molecular mechanism of HOXC11 regulation of target genes. The following specific objectives were defined:

(A) Identify HOXC11 target genes.

(B) Validate ERα as a HOXC11 target gene.

(C) Identify the mechanism by which HOXC11 can regulate ERα

## 4.3 Results

### 4.3.1 ChIP seq. analysis reveals binding peaks from the transcription factor HOXC11 exclusively along the exons of 5 genes.

HOXC11 ChIP sequencing was previously carried out by Dr. Damian McCartan in conjunction with GenPathway (external company who carried out deep sequencing).

The experiment was performed on the LY2 endocrine resistant cell line model and the cells were treated with either vehicle or tamoxifen ($10^{-7}$M) for 45 min. There were 1147 reads in the vehicle sample alone (FDR ≤10%, p≤$10^{-5}$) compared with 1541 reads in the tamoxifen treated sample (FDR ≤5%, p≤$10^{-5}$). The peaks were mapped to UCSC Genome Browser, HG19 and bioinformatic analysis was carried out with our collaborators Dr. P. O'Gaora (Department of Bioinformatics, The Conway Institute, UCD, Dublin 4), by Miss Yuan Hao.

HOXC11 peaks were found to bind in the multiple exon regions of 5 different genes (Table 4.2). Interestingly among these genes was the steroid receptor ERα. Notably, HOXC11 is also on the list, suggesting that HOXC11 can regulate itself. What was also intriguing was that HOXC11, not only sat on the exons of these genes, but it did so, in a very specific manner. The peaks exhibit a "double hump" pattern within all the exons. When we examined exon 1 of the ER gene we found that this peak pattern was far more apparent (Figure 4.3).

Considering the preliminary functional molecular studies where we observed that HOXC11 has an opposing relationship with ERα, we decided to investigate a possible mechanism in which HOXC11 could be regulating ERα.

***Table 4.2 Genes containing HOXC11 binding peaks on multiple exons***

| Gene | Transcript ID | TSS | Total Peaks | Exon No. |
|---|---|---|---|---|
| ELAV4 | ENsT000000371824 | Chr1: 50574605 | 5 | 2,3, 4,5,7 |
| SRC-1 | ENTST000000405141 | Chr2: 24714927 | 14 | 7, 8, 9, 10, 11, 12, 14, 15, 17, 18, 20, 22, 24, 25 |
| ESR1 | ENTST000000440973 | Chr 6: 152011631 | 8 | 1, 2, 3, 4, 5, 6, 7, 8 |
| HOXC11 | ENTST000000546378 | Chr2: 54366910 | 2 | 1, 2 |
| TFF1 | ENTST000000291527 | Chr2: 43782391 | 2 | 1,3 |

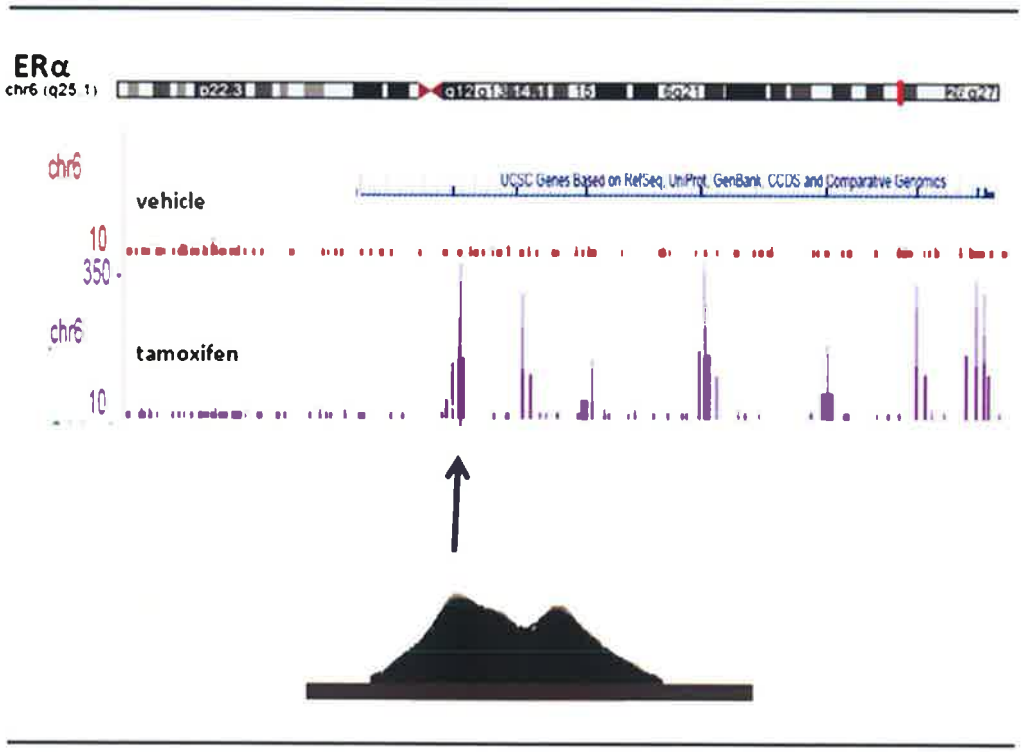


***Figure 4.3 ChIP sequencing analysis displaying HOXC11 peaks in the tamoxifen treated sample, along the exonic regions of the ESR1 gene (ERα).*** *A closer look reveals that HOXC11 binds in a very specific manner; peaks exhibit a "double hump" pattern along each exon. LY2 cells were treated with tamoxifen (10-7M) or vehicle for 45 min. Next generation sequencing was performed on the Illumina/Solexa Genome analyser platform, by Genpathway. The reads were mapped to the UCSC HG19 sequence.*

### 4.3.2 HOXC11 binds directly to exons 1 and 2 of the ERα gene

Preliminary results from the HOXC11 ChIP sequencing showed HOXC11 binding to the exonic region of the ER gene. To validate this finding at a molecular level we carried out a ChIP experiment. Bearing in mind, that HOXC11 peaks were found on the exons of the ERα gene, ChIP PCR primers were designed in the exonic region of ER exon 1 and 2.

LY2 cells were steroid depleted for 72 hours, followed by treatment with tamoxifen ($10^{-7}$M) for 2 hours. The DNA was crosslinked to the protein and the cells were sheared, followed by an immunoprecipitation with a HOXC11 antibody and semi-quantitative PCR was carried out using Sybr- green technology, on the ABi PRISM platform. HOXC11 was recruited to both exon 1 and exon 2 of ERα over the untreated LY2 cells alone. Tamoxifen treatment doubled recruitment of HOXC11 to exon 1 and there was a 9 fold enrichment over the untreated sample in exon 2 (Figure 4.4 and Figure 4.5). Basally, there is less recruitment to exon 2 in the untreated LY2 samples when compared to the IgG control. Upon stimulation however, tamoxifen induces a greater enrichment to exon 2 when compared with recruitment to exon 1 (Figure 4.4 and Figure 4.5).

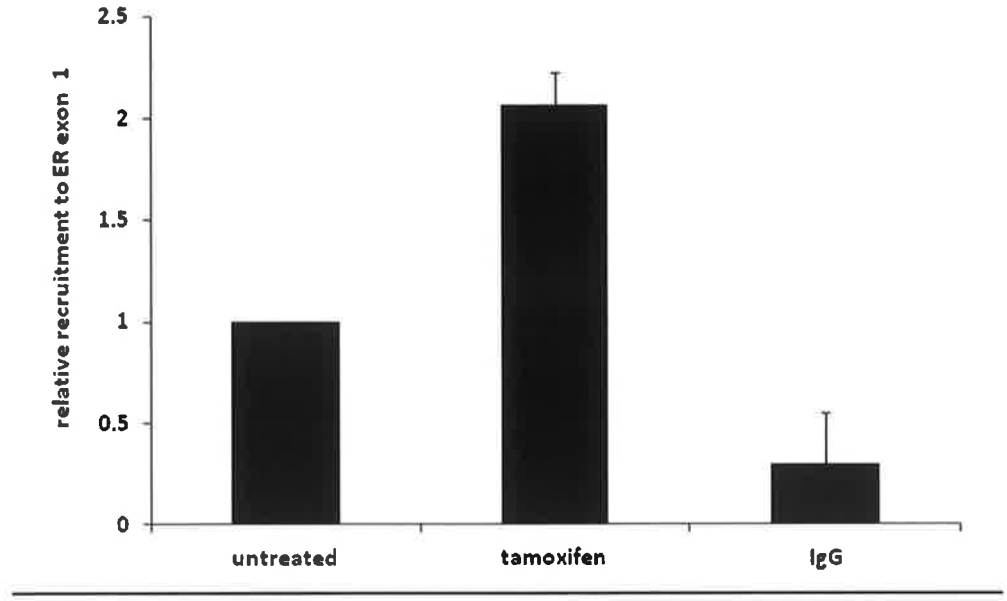


***Figure 4.4 HOXC11 is recruited to exon 1 of the ERα gene upon tamoxifen stimulation.*** *ChIP analysis in LY2 cells. Cells were either treated with tamoxifen (10-7M) or untreated for 2 hours. Enrichment over untreated control was calculated using the $\Delta c_T$ comparative method. Graph shows semi-quantitative relative enrichment. Results are representative of 3 individual experiments (N=3).*

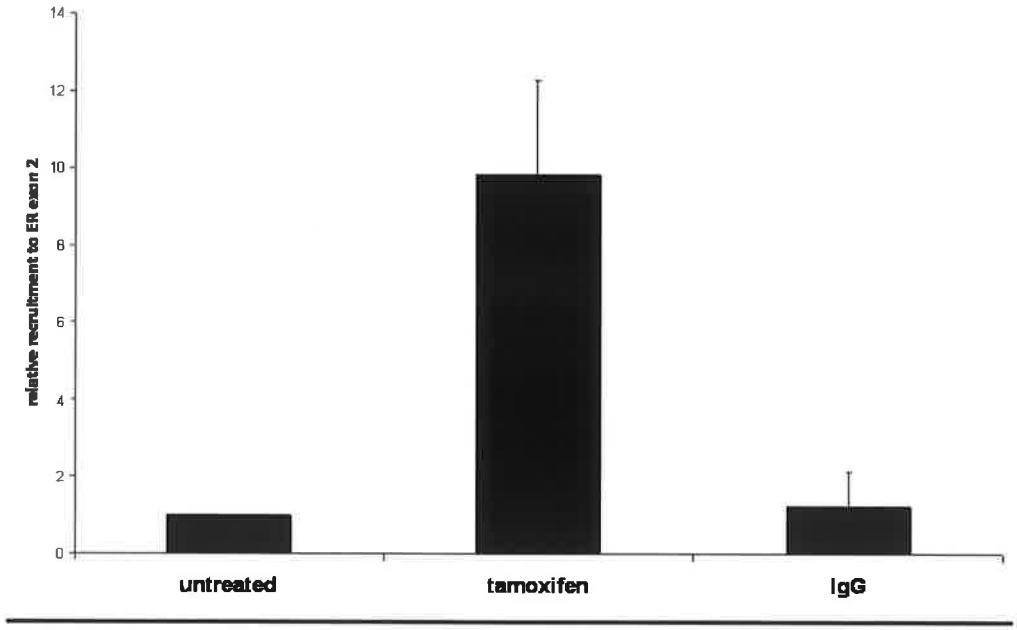


***Figure 4.5 HOXC11 is recruited to exon 2 of the ERα gene upon tamoxifen stimulation.*** *ChIP analysis in LY2 cells. Cells were either treated with tamoxifen (10-7M) or untreated for 2 hours. Enrichment over untreated control was calculated using the $\Delta c_T$ comparative method. Graph shows semi-quantitative relative enrichment. Results are representative of 3 individual experiments (N=3).*

### 4.3.3 HOXC11 directly regulates ERα protein expression levels

As this pattern of HOXC11 binding to exons has not previously been reported, it was important to assess the functional relevance of HOXC11 binding to the ERα gene in this way. To assess whether HOXC11 expression could impact on ERα protein levels directly, western blotting was performed. HOXC11 silencing and overexpression studies were carried out on the resistant LY2 cells and the stable HOXC11 MCF7 overexpressing cells respectively. Silencing of HOXC11 in the LY2s resulted in a substantial increase of ERα protein levels. βactin was used as a protein loading control and HOXC11 silencing, was confirmed by at the mRNA level using semi-quantitative taqMan PCR (Figure 4.6).

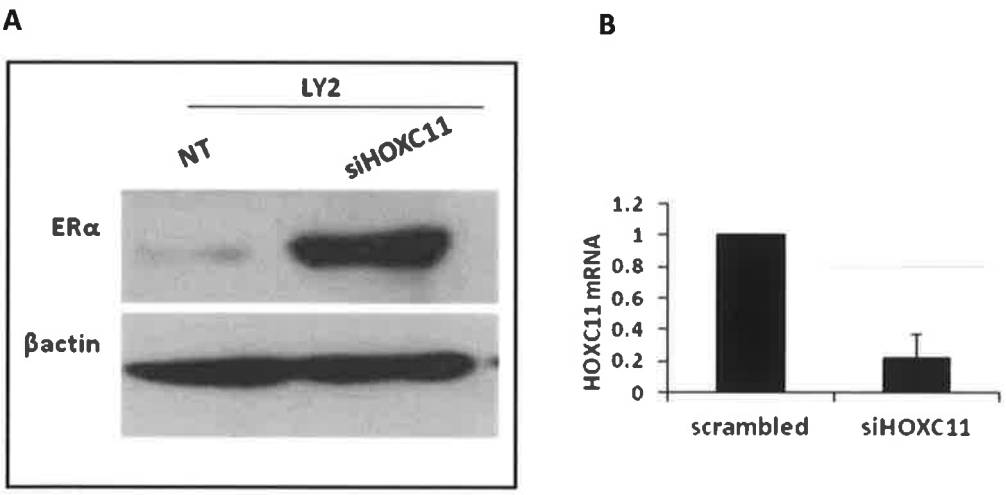


***Figure 4.6 Silencing HOXC11 results in increased protein levels of ERα.*** *(A) HOXC11 was knocked down in the LY2 cells, resulting in ERα protein levels (66 kDa) to be up-regulated by western blot analysis. βactin was used as a loading control (42 kDa). (B) Graph displays confirmation of HOXC11 silencing, by semi-quantitative taqMan PCR. Real time was analysed by the $\Delta c_T$ comparative method. Results are representative of 3 individual experiments. Results are representative of 3 individual experiments (N=3).*

Conversely overexpression of HOXC11 in the MCF7 cells also regulates ERα protein levels. Western blot analysis resulted in nearly complete down-regulation of ERα

protein expression. HOXC11 overexpression was confirmed by semi-quantitative taqMan PCR (Figure 4.7). The results so far indicate that not only does HOXC11 bind directly to the estrogen receptor but that can also directly effects transcriptional regulation of the target gene itself.

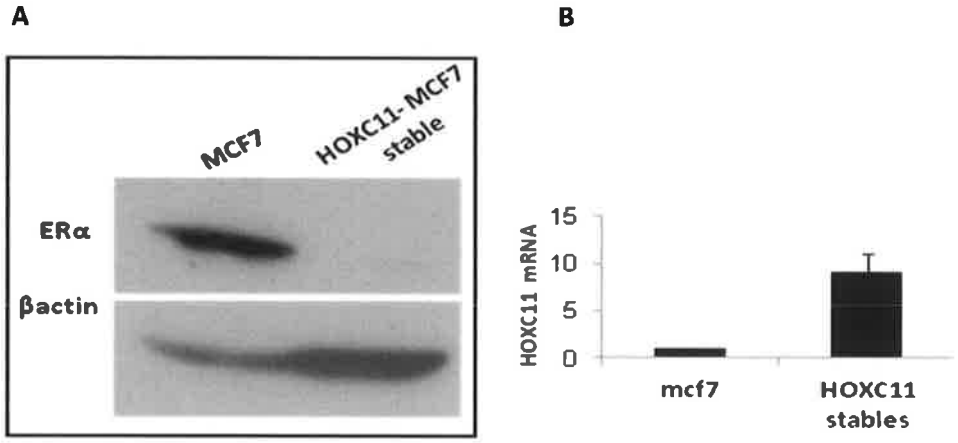


***Figure 4.7 Overexpression of HOXC11 results in decreased protein levels of ERα.*** *(A) MCF7 cells stably overexpressing HOXC11 were steroid depleted for 72 hours. Western blot analysis shows ERα protein levels (66 kDa) to be down-regulated with HOXC11 overexpression. βactin was used as a loading control (42 kDa). (B) Graph displays confirmation of HOXC11 overexpression by semi-quantitative taqMan PCR. Real time was analysed by the $\Delta c_T$ comparative method. Results are representative of 3 individual experiments (N=3).*

### 4.3.4 A potential mechanism for HOXC11 regulating ER

A genome wide-methylation microarray was carried out and this allowed us to investigate a possible mechanism for HOXC11 regulation of ERα. This work was carried out in conjunction with Dr. Sudipto Das, Cancer Genetics Department, in The Royal College of Surgeons in Ireland under the supervision of Professor Stallings.

A MeDIP (methylated DNA immunoprecipitation) reaction was carried out on LY2 cells that were transiently transfected with either an siRNA against HOXC11 or scrambled control siRNA. The MeDIP labelled DNA was hybridized onto the Nimblegene CpG island (plus promoter) microarray. The arrays were scanned and GFF (genomic feature format) files were produced from the array images. The ratio of immunoprecipitated DNA to total input DNA was plotted against genomic position to identify areas of increased signal (methylated DNA enrichment). The files were then analysed with GenePix pro software and the results were visualised using the SignalMap application program from Nimblegen®.

For this study in particular we were interested in the ESR1 gene (ERα) and a Signal Map image was created by analysing the array data generated from the scrambled and HOXC11 silenced LY2 samples. From the image, CpG islands are visible just downstream of the TSS start site (Figure 4.8). Differential peaks, as seen by the green bars are distinguishable between the scrambled and the knocked down HOXC11 sample within this CpG enriched region. The scrambled sample, displays methylation peaks in the tiled region downstream from the transcription start site (TSS) and the siHOXC11 sample illustrates a diminution of peaks in this same region. Stringent analysis within the MeDIP array software allows for a peak height score above two to be of significant methylation. Our peaks were just below the peak score cut off. Nonetheless, there is clear disparity between the scrambled and knockdown sample. Notably, the peaks visible are only within the intragenic region of ER (downstream from the TSS of ER variant 1). This is the same region where HOXC11 was observed to bind to the DNA.

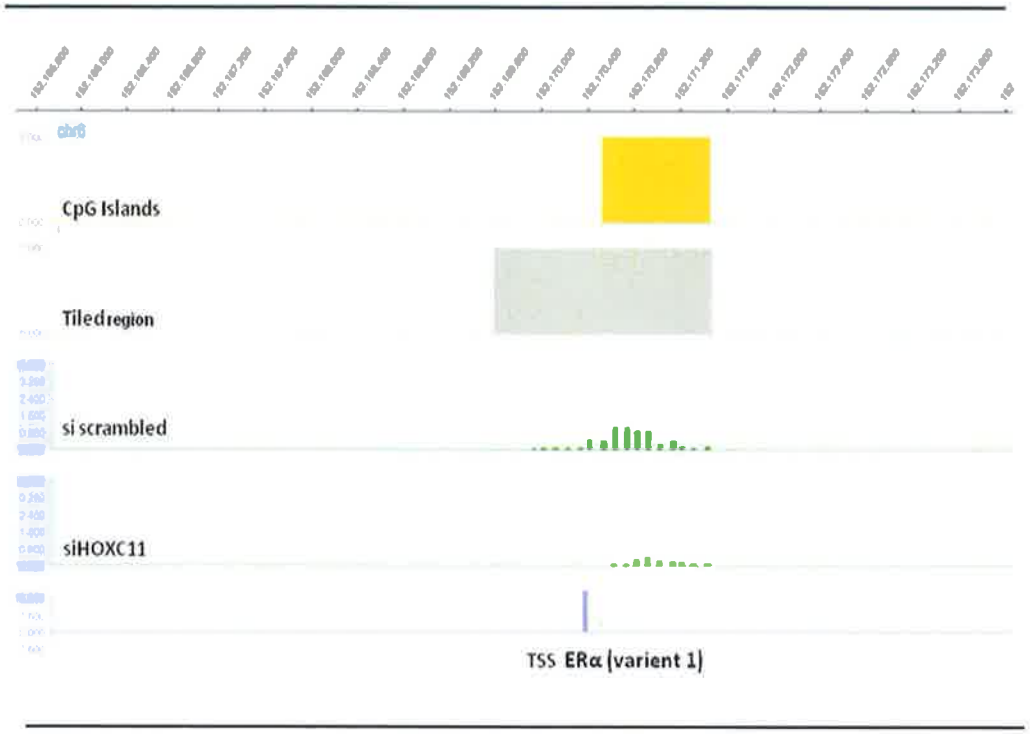


***Figure 4.8 SignalMap image displaying typical MeDIP-ChIP data generated from SignalMap software.*** *The raw data represents the fluorescence ratio (Cy5/Cy3). Methylation peaks in the control siScrambled sample are visible downstream from the TSS in the exon 1 region. Silencing HOXC11 reduces these peaks. HOXC11 was knocked down in LY2 cells for 72 hours. Genomic DNA was extracted, followed by a MeDIP reaction and hybridisation onto* the *Nimblegene CpG island plus promoter microarray. The various tracks include; the genomic co-ordination of the ERα transcript (chromosome 6), location of CpG islands, tiled array region and the transcription start site (TSS).*

### 4.3.1 The polycomb protein EZH2 is recruited to the ERα exonic region

To further investigate a possible mechanism behind ER regulation we carried out an EZH2 ChIP. EZH2 (Enhancer of zeste homolog 2) is a member of the polycomb family and has been implicated in various malignancies including breast cancer. EZH2 is a transcription factor known primarily to be involved in keeping genes in a transcriptionally repressive state. EZH2 can directly interact with DNMTs and facilitate CpG island methylation (Jin, Li et al. 2011).

The HOXC11 stable overexpressing cells and control MCF7 cells were subjected to the EZH2 pull down followed by a PCR for ERα exon 1. Initial results would suggest that the HOXC11 cells had a significant amount of EZH2 enrichment in the exonic region of ER. A 38 fold difference was detected between the HOXC11 stables and the control MCF7s (Figure 4.9).

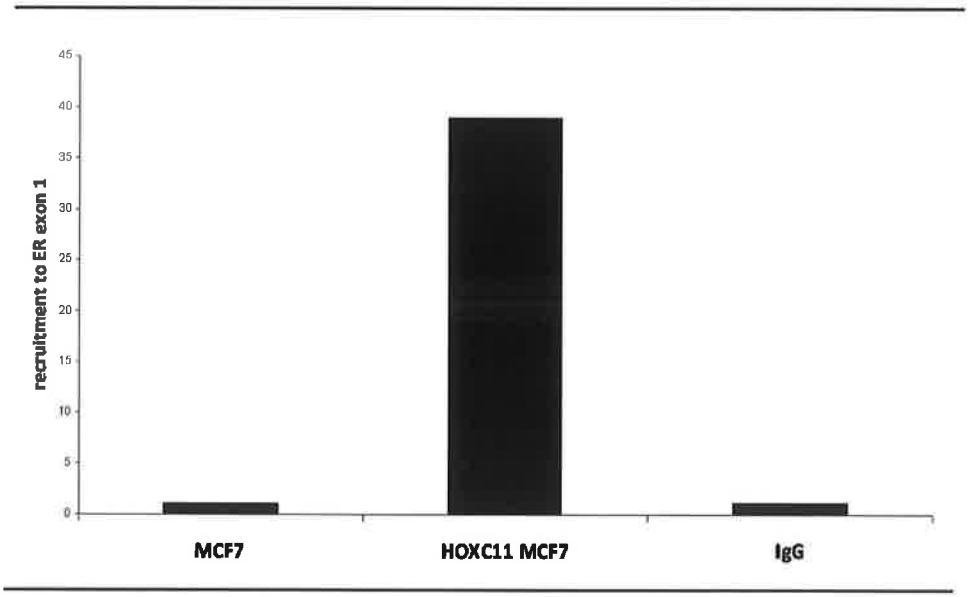


***Figure 4.9 EZH2 is enriched at exon 1 of the ERα gene in the HOXC11 stable MCF7.*** *ChIP analysis in MCF7 and HOXC11 MCF7 stable cells. EZH2 is enriched to the ER exon 38 fold higher than the MCF7 cells alone. Enrichment over rabbit IgG control was calculated using the $\Delta c_T$ comparative method. Graph shows semi-quantitative relative enrichment, this result is preliminary, N=1.*

## 4 .4 Discussion

ChIP-seq is a powerful tool for examining protein-DNA interactions. For this study we were fortunate enough to have a HOXC11 ChIP seq experiment which had been previously carried out by a member of our group. From this experiment we were able to identify new HOXC11 target genes that were specifically related to endocrine resistant breast cancer. One bioinformatic observation was that HOXC11 binds to a small number of genes in a very specific intragenic manner. The unusual binding pattern whereby HOXC11 binds specifically to multiple exons of the one gene has not previously been reported. Close-up analysis of the binding peaks revealed a "double-hump" shape to the binding peaks. Although each peak is localised to an exon, the peaks do not end sharply at the exon-intron boundary. In other words, the shape of the peak is in keeping with that of a ChIPseq peak and is distinct from peaks observed during RNAseq experiments, excluding the possibility that experimental contamination might have been responsible for the observation of exon-specific peaks. The significance of the exon binding pattern remains unclear however the 5 genes which display this pattern are genes of known interest to endocrine resistance. Thus, it would appear that HOXC11 binding specifically to exons of genes is an important mechanism in its role in endocrine resistance.

To validate the ChIP seq experiment and confirm that ERα was a true target gene for HOXC11 we undertook a chromatin immunoprecipitation (ChIP) experiment. ChIP was carried out on the same endocrine resistant cells as the ChIP-sequencing experiment was carried out in (LY2 cells). We choose two exons from the ERα gene (exons 1 and 2) in which to test for recruitment of HOXC11 to ER. In the ChIP-seq experiment the cells were stimulated for 45 min with tamoxifen. However, when we tried to replicate this treatment time point we did not find any recruitment of HOXC11 to the exons (data not shown). When we increased tamoxifen treatment to two hours we did see that HOXC11 was recruited to ER and to a greater extent under tamoxifen treatment. The discrepancy between the two treatment time points is more than likely due to the technical variation between the two experimental methods.

The endocrine resistant LY2 cells have previously been shown to have more HOXC11 protein basal levels than the MCF7 endocrine sensitive cells alone (McIlroy, McCartan et al. 2010). Therefore to investigate whether HOXC11 recruitment to the ER exons could affect ERα activity directly, we silenced HOXC11 in the LY2 cells and looked at ERα protein expression levels. We found HOXC11 expression levels can directly regulate ER protein expression. Conversely we found that the HOXC11 stable cell line had depleted amounts of ERα protein (this effect was only seen after 72 hours of steroid depletion). From our general understanding of DNA methylation in breast cancer we postulated a possible mechanism in which HOXC11 could downregulate ERα function. The MeDIP CpG microarray experiment although not conclusive, did show promising results indicative that ER hypermethylation may be induced by aberrantly expressed HOXC11. Further investigation to confirm this hypothesis will have to be carried out.

There are numerous methods available today to investigate DNA methylation patterns. The MeDIP microarray consists of tiled regions of DNA and although it was useful to examine exon 1 of ERα, it does not span all exons of interest for HOXC11 binding. We therefore propose to confirm our methylation experiment with bisulfite modification followed by PCR and sequencing or by methylation specific PCR alone (MSP). Bisulfite conversion is one of the more common techniques used to identify DNA methylation patterns. DNA treated with bisulfite results in unmethylated cytosines converting to uracil and methylated cytosines remaining unchanged (Figure 4.10). Converted DNA can then be analysed either by sequencing the regions of interest or by designing primers specific to methylated or unmethylated sequences. Either method should be able to detect noticeable differences in the percentage of methylated DNA when we compare our LY2 cells with our HOXC11 knocked down cells. To further investigate the role of HOXC11 in methylation, we also propose to examine methylation status in both stable HOXC11 overexpressing cells and stable HOXC11 silenced cells which have now been generated in our lab. The advantage of using stable cell lines is the identification of more long term methylation events than can be detected by transient transfections.

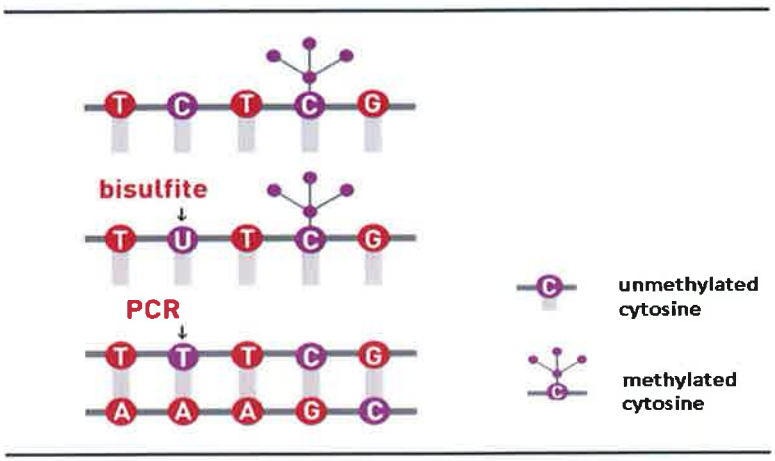


***Figure 4.10 Schematic representation of bisulfite conversion of DNA.*** *DNA is treated with bisulfite. Methylated cytosines remain unchanged and unmethylated cytosines are converted to uracil. Image is adapted from the Diagenode website http://www.diagenode.com/en/applications/bisulfite-conversion.php.*

EZH2 (enhancer of zest homologue 2) is a transcription factor known to facilitate CpG island methylation. ChIP studies revealed that the HOXC11 overexpressing cells had more than 35 fold EZH2 enrichment to the ER exon when compared to MCF7 cells alone. The results we found here are still in their preliminary stage and must be further repeated in order to be validated. However, this result does provide further indication that HOXC11 can function to methylate ERα DNA methylation and proposes a novel relationship between HOXC11 and EZH2 in breast cancer. From the current literature it is widely accepted that the polycomb group protein EZH2 can be frequently overexpressed in breast cancer (Kleer, Cao et al. 2003; Gong, Huo et al. 2011). Aberrant expression of EZH2 has been linked to cycle progression and tumourigenesis and decreased expression is thought to mediate favourable outcome to tamoxifen (Kleer, Cao et al. 2003; Collett, Eide et al. 2006; Reijm, Jansen et al. 2011). We believe EZH2 in partner with HOXC11 may be orchestrating the transcriptional silencing of ERα through a possible mechanism of DNA hypermethylation. Each of these transcription factors independently have been linked to breast cancer progression and further investigations between these two proteins may lead to new insights into the process of deregulated ER gene expression in breast cancer.

HOXC11 is not alone in utilising EZH2 in DNA methylation. One of the more recently discovered markers of epigenetic silencing is the HOX ncRNA called *HOTAIR (*HOX transcript antisense RNA). *HOTAIR* resides on the HOXC locus but it enforces its silencing properties in trans across the entire HOXD locus (Rinn, Kertesz et al. 2007). In breast cancer induced *HOTAIR* expression re-programs PRC2 binding of an epithelial cell to a binding pattern resembling an embryonic fibroblast. The PRC2 utilizes both subunits EZH2 and SUZ12 to induce epigenetic alterations that result in HOTAIR driven tumour invasiveness (Gupta, Shah et al. 2010). In general, breast cancer is associated with many epigenetic alterations and hypermethylation patterns can be seen across multiple genes in relation to breast tumours (Agrawal, Murphy et al. 2007) (Table 4.1).

At a transcriptional level it is now clear that not only does estrogen signalling through ER, silence HOXC11 expression, but in addition HOXC11 can silence ERα expression. As seen in Chapter 3, HOXC11 can drive an aggressive endocrine resistant phenotype as seen by 3D polarisation assay, migration and anchorage independent assays. This aggressive phenotype can be tamed by the addition of ERα. However, the ability of HOXC11 to downregulate ERα expression at a transcriptional level presumably has functional consequences. It would appear that HOXC11 has developed a mechanism not only to promote an endocrine resistant phenotype but also to switch off control mechanisms which would normally exist within a cell to regulate and limit HOXC11 expression. At a clinical level, the results from our methylation studies would suggest that the use of DNMT enzyme inhibitors may be a useful approach for tackling HOXC11 driven endocrine resistance.

# Chapter 5

# The competitive nature of HOXC11 and ERα's regulation of S100β

## 5.1 Introduction

S100 proteins belong to a family of small calcium binding proteins of which there are at least 25 members. They are found in a diverse range of tissue and have the ability to interact with various partners in a calcium dependent manner (Sedaghat and Notopoulos 2008). Expression levels vary widely depending on a cell specific context. In general S100 proteins are thought to be involved in many differential intracellular and extracellular processes including; phosphorylation, cell growth and motility, cell cycle, transcription and differentiation (Heizmann, Fritz et al. 2002). In disease, altered expression of several S100 proteins has been implicated in numerous diseases such as neurological disorders, neoplastic, inflammatory and cardiac disorders (Sedaghat and Notopoulos 2008). In particular the protein we are interested in, S100β, is thought to be involved in neurological disorders and neoplastic cancer (Santamaria-Kisiel, Rintala-Dempsey et al. 2006; McIlroy, McCartan et al. 2010; deBlacam, Byrne et al. 2011). S100β has been reported to act as both a stimulator of cell proliferation and migration and an inhibitor of apoptosis and differentiation during brain development, cartilage and skeletal muscle development and repair as well as melanomagenesis (Donato, Sorci et al. 2009). S100β is a secreted protein and in high stage malignant melanoma elevated levels observed in patient serum is considered a prognostic method for detecting disease recurrence or metastasis (Mohammed, Abraha et al. 2001; Sedaghat and Notopoulos 2008).

Our group were the first to report an association between S100β and resistance to endocrine therapies in breast cancer (McIlroy, McCartan et al. 2010). Through molecular and clinical studies, S100β was identified as a direct target gene of SRC-1 and HOXC11 interactions. ChIP analysis confirmed that HOXC11 could be recruited to the S100β promoter and reporter assays showed that tamoxifen stimulation increased HOXC11 recruitment and consequently increased S100β expression. *Ex vivo* studies, showed HOXC11 and S100β were both strong predictors of poor disease free survival ($P \leq 0.0001$). In a cohort of 560 breast cancer patients, those who presented with HOXC11 and S100β positive tumours associated with local and distant metastasis. Given that S100β is a secreted protein, it can be detected in protein serum and is therefore more easily monitored than HOXC11 expression.

Serum levels of S100β in our breast cancer patient cohort were also found to be a marker of disease recurrence. Thus S100β offers potential as a biomarker to identify some of those patients with elevated HOXC11 signalling who may be at risk of endocrine resistance. This could have important implications for improved patient therapeutic care in the future (McIlroy, McCartan et al. 2010).

Our investigations of the S100β promoter region previously led us to a HOXC11 putative binding site within the S100β promoter region. Interestingly further analysis also revealed a putative estrogen response element (ERE) to be located just upstream from the HOXC11 binding site (Figure 5.1). Considering the complex nature of other HOX genes and their interactions with nuclear receptors, such as HOXB13 or HOXC8 and the androgen receptor (Axlund, Lambert et al. 2010) (Norris, Chang et al. 2009), we believe further investigations into the role of ERα in HOXC11 mediated expression of S100β are warranted. We know from our published studies, tamoxifen stimulation can induce HOXC11 expression to promote S100β production. Investigations observed in this study, illustrate that HOXC11 can functionally induce a malignant phenotype in breast cancer cells and we revealed a novel relationship between HOXC11 and ERα. We propose deregulated endocrine signalling involving HOXC11, ERα and S100β may play a role in the development of an endocrine resistant tumour. We postulate that tamoxifen signalling (via ERα) can induce a HOXC11 malignant phenotype resulting in elevated S100β production in an endocrine resistant setting. However considering there is also an ERE adjacent to the HOXC11 binding site on S100β promoter we also wanted to investigate whether estrogen signalling (via ERα) could also have an effect in the endocrine resistant setting.

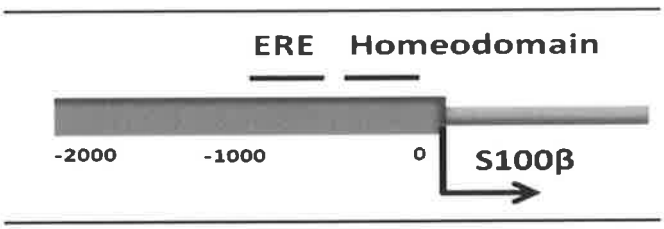


***Figure 5.1 Cartoon representing putative HOXC11 and ERα binding DNA interactions in the promoter region of S100β.*** *Putative binding sites were located using Genomatrix MAtInspector (http:www.genomatix.de. (Image is adapted from McIlroy, et al. 2010.)*

## 5.2 Aims

The aims of this chapter were to:

(A) To investigate the regulation of S100β by both HOXC11 and ERα

(B) Investigate the significance of HOXC11/ERα in a clinical setting.

## 5.3 Results

### 5.3.1 Both HOXC11 and ER are recruited to the S100β promoter

Bioinformatic analysis uncovered a putative HOXC11 and ERE binding site within the promoter region of the S100β calcium binding protein. S100β has previously been described from our group as a HOXC11 target gene in endocrine resistant breast cancer. It has been previously shown HOXC11 stimulates S100β production under tamoxifen treatment (McIlroy, McCartan et al. 2010). However we also wanted to investigate to what effect estrogen may have in this endocrine resistant phenotype. Therefore we performed ChIP studies in the LY2 resistant cells. The cells were treated with either estrogen ($10^{-8}$M) or tamoxifen ($10^{-7}$M) followed by a HOXC11 immunoprecipitation. Recruitment to the S100β promoter was then investigated using semi-quantitative PCR and sybr green technology.

We confirmed HOXC11 recruitment to the S100β promoter upon tamoxifen stimulation and interestingly we observed estrogen treatment significantly reduced HOXC11 recruitment to the S100β promoter compared to tamoxifen and the untreated sample (Figure 5.1, A).

A similar ChIP experiment was carried out with the same treatments, only this time, ERα was immunoprecipitated. In this case we saw that estrogen enhanced recruitment of ERα to the S100β promoter (at least 2.5 fold over the untreated sample) and tamoxifen treatment visibly decreased recruitment to the promoter to below basal untreated levels (Figure 5.1, B).

Taken together these results clearly show that HOXC11 and ERα are both recruited to the S100β promoter. Estrogen drives a pattern of strong ERα and weak HOXC11 recruitment. By direct contrast, tamoxifen drives strong HOXC11 and weak ERα recruitment.

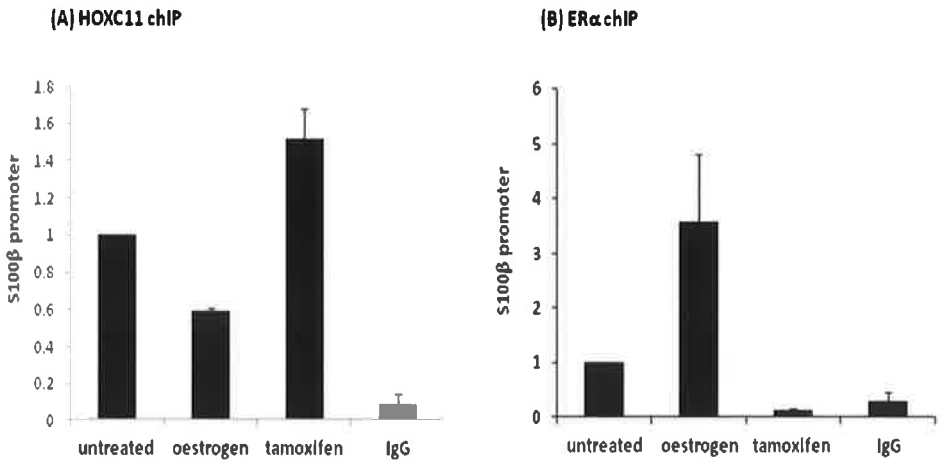


***Figure 5.2 HOXC11 recruitment to the S100β promoter is enriched upon tamoxifen stimulation and ERα recruitment is enriched to the S100β promoter upon estrogen stimulation.*** *ChIP analysis in LY2 cells. Cells were treated with estrogen (10-8M), tamoxifen (10-7M) or untreated for 45 min. Enrichment over untreated control was calculated using the $\Delta c_T$ comparative method. Graph shows semi-quantitative relative enrichment, SEM. Results are representative of 3 individual experiments (N=3).*

### 5.3.2 HOXC11 and ERα have opposing effects on S100β protein expression

To establish whether HOXC11 and ERα had any implication on S100β protein expression levels, western blot analysis was performed. In LY2 cells, overexpression of HOXC11 and ERα both alone and in combination were transiently transfected. Their empty vector counterparts were also transfected as controls. HOXC11 overexpression resulted in increased expression of S100β, whereas ERα overexpression could down-regulate S100β (Figure 5.2, top panel). Co-overexpression of HOXC11 and ERα resulted in decreased expression of the target gene S100β. It would appear ERα competitively participates with HOXC11 for control over S100β production (Figure 5.2, top panel).

On the other hand we see that HOXC11 overexpression can down-regulate ERα protein levels (3$^{rd}$ panel from the top, Figure 5.2), (similar results were also found in the HOXC11 overexpressing stable cell line (Figure 4.8)). ERα overexpression modestly induced HOXC11 protein (2$^{nd}$ panel from the top, Figure 5.2), however, when HOXC11 and ERα were co-expressed, HOXC11 protein expression was reduced closer to basal levels (2$^{nd}$ panel from the top, Figure 5.2.).

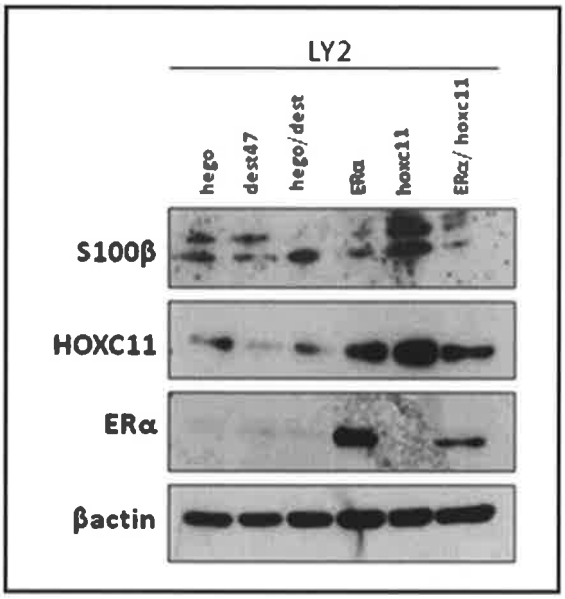


***Figure 5.3 Overexpression of HOXC11 down-regulates ERα protein levels directly, ERα down-regulates HOXC11's target protein S100β, whilst forced co-expression of HOXC11 and ERα results in a competitive protein production of each other.*** *Western blot analysis from transient transfections of HOXC11 and ERα alone and in combination in LY2 cells. βactin was used as a loading control (42 kDa).*

### 5.3.3 Clinical associations of HOXC11 and ERα in breast cancer

To determine the significance of HOXC11 and ERα in the breast cancer patient population, we analysed 560 patients for the expression of HOXC11 and ERα.

Kaplan Meier survival graphs indicate a significant association between HOXC11 expression and disease free survival. Testing positive for HOXC11 is a strong predictor of poor disease free survival, (p≤0.000) (Figure 5.3, A).
Within the HOXC11 positive patient population, patients presenting with HOXC11 and ERα positive tumours, had a better disease free survival rate when compared to patients who were HOXC11 positive alone, (p≤0.000) (Figure 5.3, B).

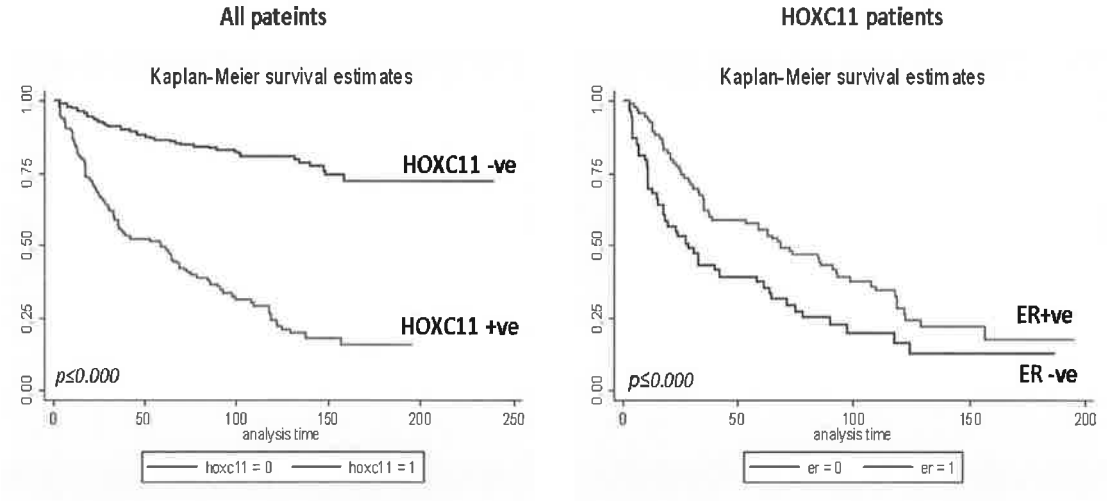


***Figure 5.4 Patients that are HOXC11 positive alone have a poor disease free survival in comparison to patients who express ERα and HOXC11 together.*** *Kaplan-Meier survival estimates in (A) all patients with or without HOXC11 expression, (p≤0.000), (McIlroy, McCartan et al. 2010). (B) HOXC11 positive patients alone or patients expressing HOXC11 and ERα together, (p≤0.000). Analysis time = months.*

## 5.4 Discussion

Elevated S100β serum levels have long since been implicated in a variety of disorders including both melanoma and neurological diseases. In melanoma in particular, high S100β levels are well established to be a marker of metastasis and disease recurrence. Research carried out by our group has now implicated S100β in breast cancer and we have shown that elevated levels can also be a predictor of disease recurrence. In addition our group recognised S100β as a direct target gene for the transcription factor HOXC11 (McIlroy, McCartan et al. 2010).

In this study through ChIP analysis we confirmed S100β as a target gene for HOXC11. Enrichment of HOXC11 to the S100β promoter was seen under tamoxifen stimulation and conversely we found that estrogen stimulation could abrogate this affect. To investigate a possible relationship between S100β and the ER we examined recruitment of ERα to the S100β promoter. Results disclosed that S100β is also a direct target gene for ERα. However we observed unlike our HOXC11 results, estrogen stimulation increased enrichment to the promoter and tamoxifen practically diminished binding. Thus occupancy of the S100β promoter is largely dependent on the microenvironment and external stimuli to determine whether HOXC11 or ERα is the major transcription factor recruited at this location.

To establish the functional significance of ERα or HOXC11 occupancy of the S100β promoter, it was important to establish if these two transcription factors were working in concert or to oppose each other in the regulation of S100β expression. Protein overexpression studies demonstrated that HOXC11 forced expression resulted in up-regulation of S100β expression, confirming previously published results from our group (McIlroy, McCartan et al. 2010) and in line with other studies in neuronal cells that showed HOXC11 overexpression could also induce S100β production (Zhang, Hamada et al. 2007). By contrast, we show here that ERα overexpression can decrease S100β protein expression. Thus, HOXC11 and ERα have opposing effects on expression of S100β. Moreover, combined overexpression of ERα and HOXC11 suggests that ERα is sufficient to quench HOXC11-driven expression of S100β. Although ERα overexpression is capable of opposing HOXC11 by reducing

HOXC11 expression, it is clear from this chapter that ERα can also oppose HOXC11 by competing with it for binding at the S100β promoter.

The competitive nature of this transcription factor and nuclear receptor for the production of S100β is a reflection of the heterogeneous nature of breast cancer. Depending on the tumour microenvironment S100β can recruit either HOXC11 or ERα. The significance of this finding is mirrored in what we see at a clinical level. Previous work from our group has shown that patients presenting with HOXC11 positive tumours alone have significantly poor disease free survival (P≤0.0001) (McIlroy, McCartan et al. 2010). Findings presented here, show that patients with HOXC11 and ERα positive tumours have significantly better outcome than HOXC11 positive alone (p=0.000). Biomarkers such as S100β are important so that we can predict patient response to endocrine therapy and can also help us predict when HOXC11 is more likely to drive malignant transformation.

The results in this chapter successfully illustrate that ERα is also a regulator of S100β. We have shown in a promoter context and at a protein expression level that HOXC11 and ERα can compete for S100β production (as seen by Figure 5.2 and 5.3). At a clinical level we demonstrate how patients presenting with both HOXC11 and ERα have longer disease free survival than patients with HOXC11 positive tumours alone (P≤0.0001) *(N=560)*.

# Chapter 6

# General Discussion

Breast cancer is a highly heterogeneous disease. This is evident because breast tumours display numerous different oncogenic characteristics, with varying degree of angiogenic, invasive and metastatic potential. These differences are detected not only between patient tumours but also intracellularly within tumours themselves (Almendro and Fuster 2011). The complex nature of the disease is reflective in the variability of clinical outcome following therapeutic intervention (Perez 2011). Today approximately 85% of breast cancer patients will reach a 5 year disease free survival rate (NCRI). This high success rate is mainly due to improved individual treatment strategies. Historically histo-pathological tumour classification methods (such as receptor status, tumour size and lymph node status) were the only clinical systems set in place to help clinicians decide the best treatment available. Today these methods have been complemented with modern molecular classification systems (Eroles, Bosch et al. 2012). It is these technological advances that have immensely improved therapeutic outcome for the majority of patients. Nevertheless a persistent problem faced by clinicians today is the acquired resistance to endocrine therapies (NCRI).

One of the most important aspects of drug resistance in ER+ breast cancer is reflected in the ability of a tumour to circumvent the inhibitory aspects of estrogen deprivation (Sabnis, Jelovac et al. 2005). Estrogen under normal circumstances is required for regular cell growth and differentiation. In a cancerous environment enhanced estrogen signalling via the ER sustains the growth of ER+ tumours. For the past 100 years endocrine therapy blocking estrogen driven ER signalling was one of the most effective treatments against tumour growth. Although for the most part effective, the main disadvantage of endocrine therapy is the development of endocrine resistance. Mechanistic studies in ER biology have increased our understanding and highlighted the role of estrogen induced extracellular and ligand free ER activated signalling in resistant tumours. ER activation often results in the gain of growth factor signalling in a process known as cell signalling crosstalk. Gain of growth factor signalling is associated with the activation of target genes involved in metastasis and invasion leading to an aggressive and resistant phenotype.

Previous studies from our group have identified the transcription factor HOXC11 to be involved in endocrine resistance. HOXC11 was found to be an independent predictor of poor disease free survival in breast cancer patients and in this study we

have identified a functional role for HOXC11 in breast cancer. Specifically HOXC11 was shown to induce a poorly differentiated phenotype, increasing cell proliferation, inducing anchorage independent growth and rendering cells more motile (see schematic representation Figure 6.1). Conversely we observed that forced expression of ERα could reverse or modify these effects induced by HOXC11 overexpression.

HOX genes have been studied intently since 1921 when they were first observed in drosophila fruit flies (Bridges 1921). Today patterns of deregulated HOX expression have now been well established in cancer (Cillo, Faiella et al. 1999; Grier, Thompson et al. 2005). The sheer volume of HOX genes and their complex involvement in numerous regulatory pathways has meant that a huge number of HOX genes and their function in cancer are yet to be uncovered. Although as yet there is little research directly involving HOXC11 in breast cancer, a compelling amount of evidence implicating HOX genes directly with malignant transformation does exist. Whilst some perturbed HOX proteins are overexpressed in breast cancer others are downregulated and can promote tumourigenesis via upregulation of GFs or downregulation of tumour suppressors (Raman, Martensen et al. 2000; Jin, Kong et al. 2011).

Evidence suggests that HOX genes are also thought to regulate steroid receptor activity. Probably one of the most studied to date is HOXB13 and its interaction with the androgen receptor in prostate cancer (Jansen, Sieuwerts et al. 2007; Norris, Chang et al. 2009). HOXC8 is also known to interact indirectly with the androgen receptor. It blocks recruitment of SRC-3 and CREB binding protein to the AR, resulting in downregulation of androgen-regulated genes via the inhibition of histone acetylation. Overexpression of HOXC8 was also found to induce invasion in non-tumourigenic prostate cells ((Miller, Miller et al. 2003).

The second portion of this study was to elucidate HOXC11 target genes. Specifically we validated ERα as a HOXC11 target. We observed that HOXC11 can directly bind to the coding region of the ER gene and impart direct regulation of ER transcription. Conversely silencing HOXC11 induced upregulation of ER transcription. Given that in ER biology genetic mutations such as polymorphisms and deletions exert little effect on endocrine resistance, one can surmise that epigenetic inactivation of the ER might

be the prevailing mechanism at play. DNA methylation in cancer is a well recognised mechanism of gene regulation (Jones and Baylin 2002). It is widely accepted that promoter methylation controls transcriptional regulation by inducing gene silencing (Jones and Baylin 2002). However, DNA methylation in the gene body (intragenic DNA methylation) is still a relatively novel area of investigation. Some studies show that DNA methylation within the gene body can activate gene transcription (contrary to promoter methylation) (Maunakea, Nagarajan et al. 2010). Current research in prostate cancer from Friedlander supports this contemporary theory. They surmised in metastatic castrate-resistant prostate cancer patients some patients with a deletion of the CYP17A1 gene (a critical gene for AR synthesis and a target of many AR inhibitors) could circumvent drug treatment through upregulation of CYP17A1 and its target genes via methylation in the gene body (Friedlander, Roy et al. 2012). Other studies have found that gene body methylation can be interpreted dissimilarly in different regions of the gene body. Brenet et al, carried out a genome-wide analyses of DNA methylation and suggest that first exon methylation (downstream from the TSS) is highly related to gene silencing, in comparison to DNA methylation within introns and 3' downstream exons, which are thought not to be associated with transcriptional silencing (Brenet, Moh et al. 2011). In line with these studies we have found we found perturbed HOXC11 expression resulted in ER downregulated activity and propose that exon body methylation may be a mechanism of which HOXC11 can regulate ER. In turn ER intragenic DNA methylation may induce an increase of ER independent gene activation through crosstalk with growth factor receptors and thus could promote a more aggressive phenotype (Figure 6.1).

In breast cancer the general consensus is that the degree of ER DNA methylation varies widely, this may be due to the varying extent of methylation between different patients but also due to diverse technical sensitivities (Pathiraja, Stearns et al. 2009). In this study we observed a decrease in the amount of DNA methylation on the ESR1 (ERα) gene body in the absence of HOXC11, suggestive that ESR1 gene expression is regulated in a dynamic fashion by HOXC11 levels. There are numerous methods which can be employed to detect methylation status and future studies will need to include methods with greater sensitivity. Bisulfite conversion followed by MSP (methylation specific PCR) may be a superior technique to adequately detect subtle differences between cancer cells and cancerous cells which have overexpressed or silenced HOXC11.

Preliminary results from our group indicate that the HOXC11 overexpressing MCF7 cells recruit significantly more EZH2 (a polycomb group protein), in the exonic region of ERα than the MCF7 cells alone (Figure 4.9). The polycomb proteins or PcGs are transcriptional repressor proteins that regulate lineage decisions during embryogenesis and differentiation and today deregulated PcGs are considered key regulators of malignant transformation (Bracken and Helin 2009). EZH2 is a direct histone methyltransferase and is a member of the PRC2 (polycomb repressor complex 2). One particular study found EZH2 to be highly deregulated in breast cancer. The increased levels of EZH2 strongly associated with poor clinical outcome and EZH2 levels were also an independent predictor of breast cancer recurrence. EZH2 overexpression is thought to induce a neoplastic phenotype characterized by anchorage-independent growth and cell invasion in normal breast epithelial cell lines (Kleer, Cao et al. 2003). From our initial observations we can see that a possible correlation between high EZH2 levels and the HOXC11 induced aggressive phenotype may exist (Figure 6.1). Further investigations would be necessary however in order to validate a direct link between HOXC11 and EZH2 in breast cancer. Inhibiting EZH2 in resistant breast cancer would have beneficial prospects. However even though EZH2 inhibitors have been developed (such as DZNeP) and show anti-tumour properties in breast cancer cell lines (Hayden, Johnson et al. 2011), at a pharmacological level they disappoint as they have a short half life and in particular behave like global methyltransferase inhibitors and are not specific to H3 lysine 27 (Miranda, Cortez et al. 2009). Specific EZH2 inhibitors are urgently needed in order to improve anti-tumour activity and in the mean time alternative inhibitors such as the HDAC inhibitors have been employed.

The final portion of this study focused on the regulation of S100β by both HOXC11 and ERα. S100β was previously identified from our group as a predictive serum biomarker for endocrine resistance in breast cancer patients (McIlroy, McCartan et al. 2010). In a heterogeneous cancer such as the breast, biomarkers are clinically useful. Breast tumours have a high degree of plasticity and the clinical advantage of predictive patient endocrine response would have a huge impact on strategic treatment decisions. The ability to switch or change targeted therapies in order to adapt to a tumour status at a given time point would no doubt increase longevity of patient disease free survival. In this study we confirmed the regulation of S100β by

HOXC11 and found that ERα can directly compete with HOXC11 for the production of S100β (Figure 6.1). In a tamoxifen resistant setting HOXC11 overexpression is dominant and its aggressive phenotype is induced. Nevertheless this mode of action can be overridden with forced re-expression of functional ERα.

In conclusion, it is well known that ER+ patients respond better to endocrine treatment. Resistance to tamoxifen in particular is often joined by the concomitant loss or dysfunction of ER. We propose that increased S100β serum levels in patient blood could indicate an aberrantly high degree of HOXC11 expression. We observed overexpression of HOXC11 leads to a loss of ER production. We hypothesized that HOXC11 may be silencing ER function in an epigenetic manner through a mode of methylation. Verification of this mechanism will need to be confirmed, however if it holds true it may lead to new treatment strategies in the fight against endocrine resistance. Novel DNMTIs (DNA methyltransferase inhibitors) are a relatively new potential class of anticancer agents. CpG island methylation occurs infrequently in normal cells, the modulation of this post-translational modification may provide a selective tumour-specific therapeutic target. DNMTIs work by mediating hypomethylation of DNA. However toxicity is observed at higher concentrations. For this reason this class of drug works especially well when used at lower doses and in combination with other agents or with other treatments. (Gravina, Festuccia et al. 2012). DNMTIs work by modulating many different cellular processes such as differentiation, apoptosis, cytostasis and tumour angiogenesis (Jones and Baylin 2002; Herman and Baylin 2003). Although these drugs are still relatively new if clinical trials prove efficacy in the future, they may help the effectiveness of long term tamoxifen use. The goal in combining different treatments in the management of cancer is to prolong disease free survival for the patient especially in particular for patients that would ordinarily develop resistance.

Investigations in this study have illustrated for the first time a direct function for HOXC11 in endocrine resistant breast cancer. HOXC11 can promote invasiveness through downregulation of ERα which under normal healthy circumstances is associated with fully differentiated functioning breast epithelia. A greater understanding of HOXC11 and its interactions with the estrogen receptor in breast cancer is warranted to further elucidate mechanisms which drive the progression of endocrine resistance. The HOX regulatory network in general is vast and elucidating

the function of each perturbed HOX cluster and every gene within is nothing short of a daunting task. The consequences however will be of huge benefit. Breast cancer is a complex disease and resistance to endocrine therapies is an ongoing challenge for both clinicians and researchers alike. Only by teasing out the intricate molecular details will new therapeutic strategies be uncovered. The findings presented here represent a somewhat minute portion of a very complex network of HOX deregulation in breast cancer. What is certain is the scope for research into HOX genes and breast cancer will continue far beyond into the future.

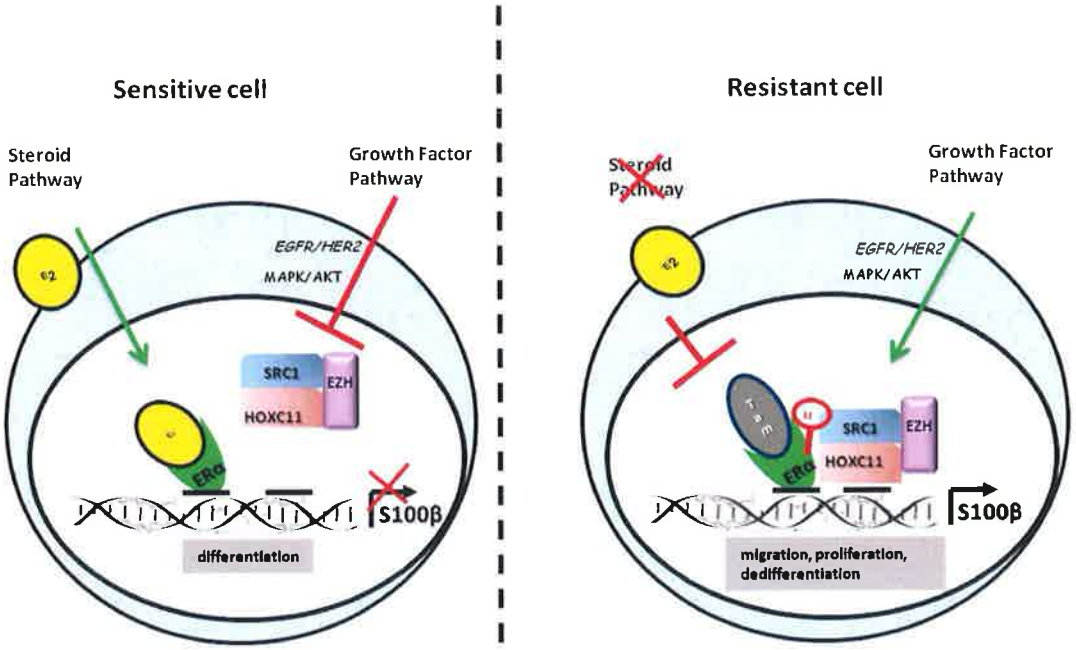


***Figure 6.1 Schematic representations of ERα and HOXC11 signalling in an endocrine sensitive cell versus and endocrine resistant cell.*** *In the endocrine sensitive cell, ERα stimulated by estrogen is recruited to the S100β promoter Inhibiting S100β production. Tamoxifen in the endocrine resistant cell stimulates a more growth factor pathway signalling mechanism. HOXC11 and ERα are recruited to the S100β promoter. HOXC11 in combination with EZH and accompanied by SRC-1, silences ERα activity via ERα DNA methylation (donated by the red lollipop). S100β production is activated inducing cell proliferation, migration and leads to a more aggressive dedifferentiated breast cancer phenotype.*

# Appendix

## Appendix 1

### Cell culture reagents

| Reagent | Cat # | Supplier |
|---|---|---|
| Minimum Essential Media (MEM) | M4526 | Sigma |
| Foetal Bovine serum (FCS) | F7524 | Sigma |
| L-Glutamine | G7513 | Sigma |
| Trypsin -EDTA 10X | T4174 | Sigma |
| Charcoal Dextran | C6241 | Sigma |
| OPTIMEM | 11058 | Invitrogen |
| PBS tablets | BR0014G | Oxoid |

## Appendix 2

### Western blotting buffers

**RIPA buffer:**

150mM sodium chloride

1.0% NP-40

0.5% sodium deoxycholate

0.1% SDS (sodium dodecyl sulphate)

50 mM Tris, pH 8.0

**20% SDS**

20g SDS dissolved in 100ml $dH_20$

**10% Ammonium persulphate**

100mg/ml dissolved in $dH_20$, stored at -20°C

**Running Buffer**

1.92M Glycine

250mM Trizma base

1% SDS

dH20 to 1L

**Tris Buffered Saline (TBS) 20X**

121.1g Tris

175.5g NaCl

dH20 to 1L, pH8.3

Use at 1X concentration

**TBS-T wash buffer**

1X TBS

0.05% Triton X-100

pH 7.6

**Blocking buffer**

1X TBS

0.05% Triton X-100

5% skimmed milk

**1M Tris-HCL, pH 6.8**

157.6g Tris-HCL

dH20 to 1L

**1.5M Tris-HCL, pH 8.8**

236.4g Tris -HCL

dH20 to 1L

**Semi dry Transfer Buffer**

390mM Glycine

480mM Trizma base

0.37% SDS

20% methanol by volume

dH20 to 1L

***Western blotting antibody concentrations and suppliers***

| Antibody | Clonality | Concentration | Supplier | Cat # |
|---|---|---|---|---|
| **ERα** | mouse monoclonal | 75 µg/ml | Novocastra | 6F-11 |
| **HOXC11** | Mouse monoclonal | 100 µg/ml | Santa Cruz | sc-81293 |
| **S100β** | mouse monoclonal | 2 mg/ml | Abcam | ab14849 |
| **βActin** | mouse monoclonal | N/A | Sigma | A1978 |
| **Mouse IgG** | HRP conjugated | N/A | GE-healthcare | NXA931 1ML |

## Appendix 3

### ChIP buffers

| **Lysis buffer 1** | *per 100 ml* |
|---|---|
| 50 mM Hepes–KOH, pH 7.5 | *5ml (of 1M)* |
| 140 mM NaCl | *2.8 ml (5M)* |
| 1 mM EDTA | *0.2 ml (0.5M)* |
| 10% Glycerol | *20 ml (50%)* |
| 0.5% NP-40/Igepal CA-630 | *5 ml (10%)* |
| 0.25% Triton X-100 | *2.5 ml (10%)* |
| ddH2O | *64.5 ml* |

| **Lysis Buffer 2** | |
|---|---|
| 10 mM Tris–HCL, pH8.0 | *1 ml (1M)* |
| 200 mM NaCl | *4 ml (5M)* |
| 1 mM EDTA | *0.2 ml (0.5M)* |
| 0.5 mM EGTA | *0.1 ml (0.5M)* |
| ddH2O | *94.7 ml* |

| **Lysis Buffer 3** | |
|---|---|
| 10 mM Tris–HCl, pH 8 | *1 ml (1M)* |
| 100 mM NaCl | *2 ml (5M)* |
| 1 mM EDTA | *0.2 ml (0.5M)* |
| 0.5 mM EGTA | *0.1 ml (0.5M)* |
| 0.1% Na–Deoxycholate | *1 ml (10%)* |
| 0.5% N-lauroylsarcosine | *2.5 ml (20%)* |
| ddH2O | *93.2 ml* |

**RIPA buffer**

| RIPA Buffer | Stock | [Final] | Volume for 50ml |
|---|---|---|---|
| 50mM Hepes, pH 8.0 | 1M | 50mM | 2.5ml |
| 500mM EDTA, pH 8.0 | 0.5M | 1mM | 100µl |
| 10% NP-40 | 10% | 1.00% | 5ml |
| 10% DOC | 10% | 0.70% | 3.5ml |
| dH2O | | | 34.8ml |
| 8M LiCl | 8M | 0.5M | 3.125ml |
| 1 PI Tablet in 2ml dH2O | | 1X | 1ml |
| Total | | | 50ml |

**TE buffer**

18.6mg EDTA

78.8 mg Tris HCL

50 ml $dH_2O$, pH 8.0

**Elution buffer**

| Elution Buffer | Stock | [Final] | Volume for 50ml |
|---|---|---|---|
| 1M Tris, pH 8.0 | 1M | 10mM | 500µl |
| 500mM EDTA | 0.5M | 1mM | 100µl |
| 10% SDS | 10% | 1.00% | 5ml |
| dH2O | | | 44.35ml |
| Total | | | 50ml |

**Proteinase K Mix**

| Proteinase K Mix | 1 Sample | 10 Samples |
|---|---|---|
| 1X TE | 140µl | 1.4ml |
| 10 mg/ml Glycogen | 3µl | 30µl |
| Proteinase K 20mg/ml | 7µl | 70µl |
| Total | 150µl | 1.5µl |

# Publications

Metastatic progression with resistance to aromatase inhibitors is driven by the steroid receptor coactivator SRC-1. *McBryan J, Theissen SM, Byrne C, Hughes E, **Cocchiglia S**, Sande S, O'Hara J, Tibbitts P, Hill AD, Young LS. Cancer Res. 2012 Jan 15;72(2):548-59. Epub 2011 Nov 22.*

RuvBl2 cooperates with Ets2 to transcriptionally regulate hTERT in colon cancer. *Flavin P, Redmond A, McBryan J, **Cocchiglia S**, Tibbitts P, Fahy-Browne P, Kay E, Treumann A, Perrem K, McIlroy M, Hill AD, Young LS. FEBS Lett. 2011 Aug 4;585(15):2537-44. Epub 2011 Jul 13.*

PGC-1alpha is coupled to HIF-1alpha-dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells. *O'Hagan KA, **Cocchiglia S**, Zhdanov AV, Tambuwala MM, Cummin EP, Monfared M, Agbor TA, Garvey JF, Papkovsky DB, Taylor CT, Allan BB. Proc Natl Acad Sci U S A. 2009 Feb 17;106(7):2188-93. Epub 2009 Jan 28.*

Ets-2 and p160 proteins collaborate to regulate c-Myc in endocrine resistant breast cancer. *Al-azawi D, Ilroy MM, Kelly G, Redmond AM, Bane FT, **Cocchiglia S**, Hill AD, Young LS. Oncogene. 2008 May 8;27(21):3021-31. Epub 2007 Dec 3.*

As acknowledged contributor

Valerie B. O'Leary et al, and Members of the Birth Defects Research Group. Reduced folate carrier polymorphisms and neural tube defect risk. Molecular Genetics and Metabolism,Volume87,Issue4,April2006,Pages364-369

Valerie B. O'Leary et al. Screening for new MTHFR polymorphisms and NTD risk. American Journal of Medical Genetics Part A 2005 Oct 1;138(2):99-106.

Valerie B. O'Leary et al. Analysis of methionine synthase reductase polymorphisms for neural tube defects risk association. Molecular Genetics and Metabolism, Volume 85,Issue3,July2005,Pages220-227.

Valerie B. O'Leary et al. Analysis of the human folate receptor β gene for an association with neural tube defects. Molecular Genetics and Metabolism, Volume 79,Issue2,June2003,Pages129-133.

# Bibliography


Abate-Shen, C. (2002). "Deregulated homeobox gene expression in cancer: cause or consequence?" Nat Rev Cancer **2**(10): 777-85.

Agrawal, A., R. F. Murphy, et al. (2007). "DNA methylation in breast and colorectal cancers." Mod Pathol **20**(7): 711-21.

Al-azawi, D., M. M. Ilroy, et al. (2008). "Ets-2 and p160 proteins collaborate to regulate c-Myc in endocrine resistant breast cancer." Oncogene **27**(21): 3021-31.

Al Saleh, S., L. H. Sharaf, et al. (2011). "Signalling pathways involved in endocrine resistance in breast cancer and associations with epithelial to mesenchymal transition (Review)." Int J Oncol **38**(5): 1197-217.

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

Almendro, V. and G. Fuster (2011). "Heterogeneity of breast cancer: etiology and clinical relevance." Clin Transl Oncol **13**(11): 767-73.

Arpino, G., S. J. Green, et al. (2004). "HER-2 amplification, HER-1 expression, and tamoxifen response in estrogen receptor-positive metastatic breast cancer: a southwest oncology group study." Clin Cancer Res **10**(17): 5670-6.

Axlund, S. D., J. R. Lambert, et al. (2010). "HOXC8 inhibits androgen receptor signaling in human prostate cancer cells by inhibiting SRC-3 recruitment to direct androgen target genes." Mol Cancer Res **8**(12): 1643-55.

Ball, M. P., J. B. Li, et al. (2009). "Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells." Nat Biotechnol **27**(4): 361-8.

Baylin, S. B. (2005). "DNA methylation and gene silencing in cancer." Nat Clin Pract Oncol **2 Suppl 1**: S4-11.

Bracken, A. P. and K. Helin (2009). "Polycomb group proteins: navigators of lineage pathways led astray in cancer." Nat Rev Cancer **9**(11): 773-84.

Brenet, F., M. Moh, et al. (2011). "DNA methylation of the first exon is tightly linked to transcriptional silencing." PLoS One **6**(1): e14524.

Bridges, C. B. (1921). "Current Maps of the Location of the Mutant Genes of Drosophila Melanogaster." Proc Natl Acad Sci U S A **7**(4): 127-32.

Britton, D. J., I. R. Hutcheson, et al. (2006). "Bidirectional cross talk between ERalpha and EGFR signalling pathways regulates tamoxifen-resistant growth." Breast Cancer Res Treat **96**(2): 131-46.

Bronzert, D. A., G. L. Greene, et al. (1985). "Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY 117018." Endocrinology **117**(4): 1409-17.

Cameron, E. E., K. E. Bachman, et al. (1999). "Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer." Nat Genet **21**(1): 103-7.

Cantile, M., G. Pettinato, et al. (2003). "In vivo expression of the whole HOX gene network in human breast cancer." Eur J Cancer **39**(2): 257-64.

Carroll, J. S. and M. Brown (2006). "Estrogen receptor target gene: an evolving concept." Mol Endocrinol **20**(8): 1707-14.

Chu, M. C., F. B. Selam, et al. (2004). "HOXA10 regulates p53 expression and matrigel invasion in human breast cancer cells." Cancer Biol Ther **3**(6): 568-72.

Cillo, C. (1994). "HOX genes in human cancers." Invasion Metastasis **14**(1-6): 38-49.

Cillo, C., A. Faiella, et al. (1999). "Homeobox genes and cancer." Exp Cell Res **248**(1): 1-9.

Collett, K., G. E. Eide, et al. (2006). "Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer." Clin Cancer Res **12**(4): 1168-74.

Creighton, C. J., A. M. Hilger, et al. (2006). "Activation of mitogen-activated protein kinase in estrogen receptor alpha-positive breast cancer cells in vitro induces an in vivo molecular phenotype of estrogen receptor alpha-negative human breast tumors." Cancer Res **66**(7): 3903-11.

Daftary, G. S. and H. S. Taylor (2006). "Endocrine regulation of HOX genes." Endocr Rev **27**(4): 331-55.

deBlacam, C., C. Byrne, et al. (2010). "HOXC11-SRC-1 regulation of S100beta in cutaneous melanoma: new targets for the kinase inhibitor dasatinib." Br J Cancer **105**(1): 118-23.

deBlacam, C., C. Byrne, et al. (2011). "HOXC11-SRC-1 regulation of S100beta in cutaneous melanoma: new targets for the kinase inhibitor dasatinib." Br J Cancer **105**(1): 118-23.

Debnath, J., K. R. Mills, et al. (2002). "The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini." Cell **111**(1): 29-40.

Donato, R., G. Sorci, et al. (2009). "S100B's double life: intracellular regulator and extracellular signal." Biochim Biophys Acta **1793**(6): 1008-22.

Duboule, D. (1999). "No milk today (my Hox have gone away)." Proc Natl Acad Sci U S A **96**(2): 322-3.

Eroles, P., A. Bosch, et al. (2012). "Molecular biology in breast cancer: intrinsic subtypes and signaling pathways." Cancer Treat Rev **38**(6): 698-707.

Fan, J., W. J. Yin, et al. (2008). "ER alpha negative breast cancer cells restore response to endocrine therapy by combination treatment with both HDAC inhibitor and DNMT inhibitor." J Cancer Res Clin Oncol **134**(8): 883-90.

Fan, P., J. Wang, et al. (2007). "Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells." Cancer Res **67**(3): 1352-60.

Fidler, I. J. (2003). "The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited." Nat Rev Cancer **3**(6): 453-8.

Friedlander, T. W., R. Roy, et al. (2012). "Common structural and epigenetic changes in the genome of castration-resistant prostate cancer." Cancer Res **72**(3): 616-25.

Friedmann, Y., C. A. Daniel, et al. (1994). "Hox genes in normal and neoplastic mouse mammary gland." Cancer Res **54**(22): 5981-5.

Garin, E., M. Lemieux, et al. (2006). "Stromal Hoxa5 function controls the growth and differentiation of mammary alveolar epithelium." Dev Dyn **235**(7): 1858-71.

Gibb, E. A., C. J. Brown, et al. (2011). "The functional role of long non-coding RNA in human carcinomas." Mol Cancer **10**: 38.

Gong, Y., L. Huo, et al. (2011). "Polycomb group protein EZH2 is frequently expressed in inflammatory breast cancer and is predictive of worse clinical outcome." Cancer **117**(24): 5476-84.

Gravina, G. L., C. Festuccia, et al. (2012). "Biological rationale for the use of DNA methyltransferase inhibitors as new strategy for modulation of tumor response to chemotherapy and radiation." Mol Cancer **9**: 305.

Grier, D. G., A. Thompson, et al. (2005). "The pathophysiology of HOX genes and their role in cancer." J Pathol **205**(2): 154-71.

Guedj, M., L. Marisa, et al. (2011). "A refined molecular taxonomy of breast cancer." Oncogene **31**(9): 1196-206.

Gupta, R. A., N. Shah, et al. (2010). "Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis." Nature **464**(7291): 1071-6.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-74.

Hartwell, K. A., B. Muir, et al. (2006). "The Spemann organizer gene, Goosecoid, promotes tumor metastasis." Proc Natl Acad Sci U S A **103**(50): 18969-74.

Hayashida, T., F. Takahashi, et al. (2009). "HOXB9, a gene overexpressed in breast cancer, promotes tumorigenicity and lung metastasis." Proc Natl Acad Sci U S A **107**(3): 1100-5.

Hayden, A., P. W. Johnson, et al. (2011). "S-adenosylhomocysteine hydrolase inhibition by 3-deazaneplanocin A analogues induces anti-cancer effects in breast cancer cell lines and synergy with both histone deacetylase and HER2 inhibition." Breast Cancer Res Treat **127**(1): 109-19.

He, H., X. Hua, et al. (2011). "Epigenetic regulations in hematopoietic Hox code." Oncogene **30**(4): 379-88.

Heizmann, C. W., G. Fritz, et al. (2002). "S100 proteins: structure, functions and pathology." Front Biosci **7**: d1356-68.

Hens, J. R. and J. J. Wysolmerski (2005). "Key stages of mammary gland development: molecular mechanisms involved in the formation of the embryonic mammary gland." Breast Cancer Res **7**(5): 220-4.

Herman, J. G. and S. B. Baylin (2003). "Gene silencing in cancer in association with promoter hypermethylation." N Engl J Med **349**(21): 2042-54.

Herynk, M. H. and S. A. Fuqua (2004). "Estrogen receptor mutations in human disease." Endocr Rev **25**(6): 869-98.

Huang, Y., S. Nayak, et al. (2011). "Epigenetics in breast cancer: what's new?" Breast Cancer Res **13**(6): 225.

Hulit, J., K. Suyama, et al. (2007). "N-cadherin signaling potentiates mammary tumor metastasis via enhanced extracellular signal-regulated kinase activation." Cancer Res **67**(7): 3106-16.

Hulka, B. S. and P. G. Moorman (2001). "Breast cancer: hormones and other risk factors." Maturitas **38**(1): 103-13; discussion 113-6.

Jansen, M. P., A. M. Sieuwerts, et al. (2007). "HOXB13-to-IL17BR expression ratio is related with tumor aggressiveness and response to tamoxifen of recurrent breast cancer: a retrospective study." J Clin Oncol **25**(6): 662-8.

Jemal, A., M. M. Center, et al. (2010). "Global patterns of cancer incidence and mortality rates and trends." Cancer Epidemiol Biomarkers Prev **19**(8): 1893-907.

Jin, B., Y. Li, et al. (2011). "DNA methylation: superior or subordinate in the epigenetic hierarchy?" Genes Cancer **2**(6): 607-17.

Jin, K., X. Kong, et al. (2011). "The HOXB7 protein renders breast cancer cells resistant to tamoxifen through activation of the EGFR pathway." Proc Natl Acad Sci U S A **109**(8): 2736-41.

Jones, P. A. and S. B. Baylin (2002). "The fundamental role of epigenetic events in cancer." Nat Rev Genet **3**(6): 415-28.

Kelly, Z. L., A. Michael, et al. (2011). "HOX genes in ovarian cancer." J Ovarian Res **4**: 16.

Kleer, C. G., Q. Cao, et al. (2003). "EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells." Proc Natl Acad Sci U S A **100**(20): 11606-11.

Kongsuwan, K., E. Webb, et al. (1988). "Expression of multiple homeobox genes within diverse mammalian haemopoietic lineages." EMBO J **7**(7): 2131-8.

Korach, K. S., J. F. Couse, et al. (1996). "Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes." Recent Prog Horm Res **51**: 159-86; discussion 186-8.

Krumlauf, R. (1994). "Hox genes in vertebrate development." Cell **78**(2): 191-201.

Lacassagne, A. (1936). "Hormonal Pathogenesis of Adenocarcinoma of the Breast " The American Journal of Cancer **XXVII**(2): 217-228.

Lazarova, D. L., B. A. Spengler, et al. (1999). "HuD, a neuronal-specific RNA-binding protein, is a putative regulator of N-myc pre-mRNA processing/stability in malignant human neuroblasts." Oncogene **18**(17): 2703-10.

Lazennec, G., D. Bresson, et al. (2001). "ER beta inhibits proliferation and invasion of breast cancer cells." Endocrinology **142**(9): 4120-30.

Levin, E. R. and R. J. Pietras (2008). "Estrogen receptors outside the nucleus in breast cancer." Breast Cancer Res Treat **108**(3): 351-61.

Lewis, M. T. (2000). "Homeobox genes in mammary gland development and neoplasia." Breast Cancer Res **2**(3): 158-69.

Liu, Y., N. Chen, et al. (2010). "The protein kinase Pak4 disrupts mammary acinar architecture and promotes mammary tumorigenesis." Oncogene **29**(44): 5883-94.

Ma, L., G. V. Benson, et al. (1998). "Abdominal B (AbdB) Hoxa genes: regulation in adult uterus by estrogen and progesterone and repression in mullerian duct by the synthetic estrogen diethylstilbestrol (DES)." Dev Biol **197**(2): 141-54.

Ma, X. J., Z. Wang, et al. (2004). "A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen." Cancer Cell **5**(6): 607-16.

Makiyama, K., J. Hamada, et al. (2005). "Aberrant expression of HOX genes in human invasive breast carcinoma." Oncol Rep **13**(4): 673-9.

Massarweh, S., C. K. Osborne, et al. (2008). "Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function." Cancer Res **68**(3): 826-33.

Maunakea, A. K., R. P. Nagarajan, et al. (2010). "Conserved role of intragenic DNA methylation in regulating alternative promoters." Nature **466**(7303): 253-7.

McBryan, J., S. M. Theissen, et al. (2012). "Metastatic progression with resistance to aromatase inhibitors is driven by the steroid receptor coactivator SRC-1." Cancer Res **72**(2): 548-59.

McIlroy, M., D. McCartan, et al. (2010). "Interaction of developmental transcription factor HOXC11 with steroid receptor coactivator SRC-1 mediates resistance to endocrine therapy in breast cancer [corrected]." Cancer Res **70**(4): 1585-94.

Miao, J., Z. Wang, et al. (2007). "HOXB13 promotes ovarian cancer progression." Proc Natl Acad Sci U S A **104**(43): 17093-8.

Micalizzi, D. S., S. M. Farabaugh, et al. (2010). "Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression." J Mammary Gland Biol Neoplasia **15**(2): 117-34.

Miller, G. J., H. L. Miller, et al. (2003). "Aberrant HOXC expression accompanies the malignant phenotype in human prostate." Cancer Res **63**(18): 5879-88.

Miranda, T. B., C. C. Cortez, et al. (2009). "DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation." Mol Cancer Ther **8**(6): 1579-88.

Mohammed, M. Q., H. D. Abraha, et al. (2001). "Serum S100beta protein as a marker of disease activity in patients with malignant melanoma." Med Oncol **18**(2): 109-20.

Mouridsen, H., M. Gershanovich, et al. (2001). "Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: results of a phase III study of the International Letrozole Breast Cancer Group." J Clin Oncol **19**(10): 2596-606.

Norris, J. D., C. Y. Chang, et al. (2009). "The homeodomain protein HOXB13 regulates the cellular response to androgens." Mol Cell **36**(3): 405-16.

Onate, S. A., S. Y. Tsai, et al. (1995). "Sequence and characterization of a coactivator for the steroid hormone receptor superfamily." Science **270**(5240): 1354-7.

Osborne, C. K. (1998). "Tamoxifen in the treatment of breast cancer." N Engl J Med **339**(22): 1609-18.

Osborne, C. K. and R. Schiff (2011). "Mechanisms of endocrine resistance in breast cancer." Annu Rev Med **62**: 233-47.

Ottaviano, Y. L., J. P. Issa, et al. (1994). "Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells." Cancer Res **54**(10): 2552-5.

Palmieri, C., G. J. Cheng, et al. (2002). "Estrogen receptor beta in breast cancer." Endocr Relat Cancer **9**(1): 1-13.

Parkin, D. M. and L. M. Fernandez (2006). "Use of statistics to assess the global burden of breast cancer." Breast J **12 Suppl 1**: S70-80.

Parl, F. F. (2003). "Multiple mechanisms of estrogen receptor gene repression contribute to ER-negative breast cancer." Pharmacogenomics J **3**(5): 251-3.

Pathiraja, T. N., V. Stearns, et al. (2009). "Epigenetic regulation in estrogen receptor positive breast cancer--role in treatment response." J Mammary Gland Biol Neoplasia **15**(1): 35-47.

Perez, E. A. (2011). "Breast cancer management: opportunities and barriers to an individualized approach." Oncologist **16 Suppl 1**: 20-2.

Polyak, K. (2011). "Heterogeneity in breast cancer." J Clin Invest **121**(10): 3786-8.

Raman, V., S. A. Martensen, et al. (2000). "Compromised HOXA5 function can limit p53 expression in human breast tumours." Nature **405**(6789): 974-8.

Redmond, A. M., F. T. Bane, et al. (2009). "Coassociation of estrogen receptor and p160 proteins predicts resistance to endocrine treatment; SRC-1 is an independent predictor of breast cancer recurrence." Clin Cancer Res **15**(6): 2098-106.

Reijm, E. A., M. P. Jansen, et al. (2011). "Decreased expression of EZH2 is associated with upregulation of ER and favorable outcome to tamoxifen in advanced breast cancer." Breast Cancer Res Treat **125**(2): 387-94.

Renoir, J. M. (2012). "Estradiol receptors in breast cancer cells: Associated co-factors as targets for new therapeutic approaches." Steroids **77**(12): 1249-61.

Riggins, R. B., R. S. Schrecengost, et al. (2007). "Pathways to tamoxifen resistance." Cancer Lett **256**(1): 1-24.

Rinn, J. L., M. Kertesz, et al. (2007). "Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs." Cell **129**(7): 1311-23.

Roman-Blas, J. A., S. Castaneda, et al. (2009). "Osteoarthritis associated with estrogen deficiency." Arthritis Res Ther **11**(5): 241.

Sabnis, G. J., D. Jelovac, et al. (2005). "The role of growth factor receptor pathways in human breast cancer cells adapted to long-term estrogen deprivation." Cancer Res **65**(9): 3903-10.

Saha Roy, S. and R. K. Vadlamudi (2011). "Role of estrogen receptor signaling in breast cancer metastasis." Int J Breast Cancer **2012**: 654698.

Samuel, S. and H. Naora (2005). "Homeobox gene expression in cancer: insights from developmental regulation and deregulation." Eur J Cancer **41**(16): 2428-37.

Santamaria-Kisiel, L., A. C. Rintala-Dempsey, et al. (2006). "Calcium-dependent and -independent interactions of the S100 protein family." Biochem J **396**(2): 201-14.

Santen, R. J., P. Fan, et al. (2009). "Estrogen signals via an extra-nuclear pathway involving IGF-1R and EGFR in tamoxifen-sensitive and -resistant breast cancer cells." Steroids **74**(7): 586-94.

Santen, R. J., R. X. Song, et al. (2004). "Adaptive hypersensitivity to estrogen: mechanism for sequential responses to hormonal therapy in breast cancer." Clin Cancer Res **10**(1 Pt 2): 337S-45S.

Schiff, R., S. A. Massarweh, et al. (2005). "Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators." Cancer Chemother Pharmacol **56 Suppl 1**: 10-20.

Sedaghat, F. and A. Notopoulos (2008). "S100 protein family and its application in clinical practice." Hippokratia **12**(4): 198-204.

Shah, N. and S. Sukumar (2010). "The Hox genes and their roles in oncogenesis." Nat Rev Cancer **10**(5): 361-71.

Shen, W. F., K. Krishnan, et al. (2001). "The HOX homeodomain proteins block CBP histone acetyltransferase activity." Mol Cell Biol **21**(21): 7509-22.

Shi, L., B. Dong, et al. (2009). "Expression of ER-{alpha}36, a novel variant of estrogen receptor {alpha}, and resistance to tamoxifen treatment in breast cancer." J Clin Oncol **27**(21): 3423-9.

Shou, J., S. Massarweh, et al. (2004). "Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer." J Natl Cancer Inst **96**(12): 926-35.

Shyamala, G. (1997). "Roles of estrogen and progesterone in normal mammary gland development insights from progesterone receptor null mutant mice and in situ localization of receptor." Trends Endocrinol Metab **8**(1): 34-9.

Simpson, E. R. (2003). "Sources of estrogen and their importance." J Steroid Biochem Mol Biol **86**(3-5): 225-30.

Smith, C. L. and B. W. O'Malley (2004). "Coregulator function: a key to understanding tissue specificity of selective receptor modulators." Endocr Rev **25**(1): 45-71.

Song, R. X., R. J. Santen, et al. (2002). "Adaptive mechanisms induced by long-term estrogen deprivation in breast cancer cells." Mol Cell Endocrinol **193**(1-2): 29-42.

Srebrow, A., Y. Friedmann, et al. (1998). "Expression of Hoxa-1 and Hoxb-7 is regulated by extracellular matrix-dependent signals in mammary epithelial cells." J Cell Biochem **69**(4): 377-91.

Svingen, T. and K. F. Tonissen (2003). "Altered HOX gene expression in human skin and breast cancer cells." Cancer Biol Ther **2**(5): 518-23.

Svingen, T. and K. F. Tonissen (2006). "Hox transcription factors and their elusive mammalian gene targets." Heredity (Edinb) **97**(2): 88-96.

Szyf, M. (2008). "The role of DNA hypermethylation and demethylation in cancer and cancer therapy." Curr Oncol **15**(2): 72-5.

Taipale, J. and P. A. Beachy (2001). "The Hedgehog and Wnt signalling pathways in cancer." Nature **411**(6835): 349-54.

Taylor, H. S., A. Arici, et al. (1998). "HOXA10 is expressed in response to sex steroids at the time of implantation in the human endometrium." J Clin Invest **101**(7): 1379-84.

The Cancer Genome Atlas 2012. " Comprehensive molecular portraits of human breast tumours Nature **490** | 6 1

Thullberg, M. and S. Stromblad (2008). "Anchorage-independent cytokinesis as part of oncogenic transformation?" Cell Cycle **7**(8): 984-8.

Toi, M., A. L. Harris, et al. (1993). "cDNA transfection followed by the isolation of a MCF-7 breast cell line resistant to tamoxifen in vitro and in vivo." Br J Cancer **68**(6): 1088-96.

Venkitaraman, A. R. (2002). "Cancer susceptibility and the functions of BRCA1 and BRCA2." Cell **108**(2): 171-82.

Walsh, C. A., L. Qin, et al. (2012). "The function of steroid receptor coactivator-1 in normal tissues and cancer." Int J Biol Sci **8**(4): 470-85.

Wu, X., H. Chen, et al. (2006). "HOXB7, a homeodomain protein, is overexpressed in breast cancer and confers epithelial-mesenchymal transition." Cancer Res **66**(19): 9527-34.

Zhang, X., J. Hamada, et al. (2007). "HOXC6 and HOXC11 increase transcription of S100beta gene in BrdU-induced in vitro differentiation of GOTO neuroblastoma cells into Schwannian cells." J Cell Mol Med **11**(2): 299-306.

Zhou, Q., P. G. Shaw, et al. (2009). "Epigenetics meets estrogen receptor: regulation of estrogen receptor by direct lysine methylation." Endocr Relat Cancer **16**(2): 319-23.

Zilli, M., A. Grassadonia, et al. (2009). "Molecular mechanisms of endocrine resistance and their implication in the therapy of breast cancer." Biochim Biophys Acta **1795**(1): 62-81.