THE UNIVERSITY *of* EDINBURGH

Transcriptional and Epigenetic Regulation of Oestrogen Signalling in Breast Cancer Cells

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Declaration

I declare that all of the work in this thesis was performed personally. No part of this work has been submitted for consideration as part of any other degree or award.

Jing Bi

2012

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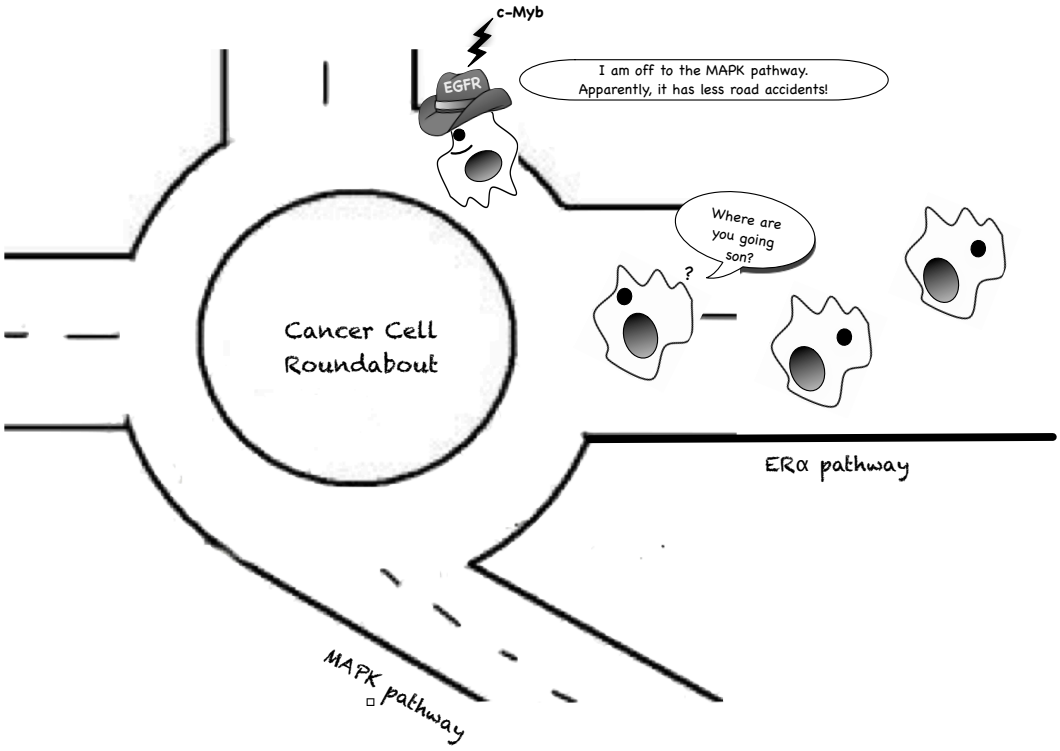
感谢亲爱的妈妈，感谢你们我的外公外婆，没有你们的付出就没有今天的我！

Trailer



A brief history of the Acquired drug resistance

JING BI, 2012

Abstract

Breast cancer is a common disease in women and has major impacts on health and quality of life. About 70% of breast cancers over express ERα, and are classified as ER positive breast cancer. Oestrogen receptor alpha (ERα) belongs to the nuclear receptor superfamily and is responsible for many effects of oestrogen on normal and cancerous breast tissue. Endocrine therapies that block the function of ERα or the synthesis of oestrogen have been a mainstay of ERα positive breast cancer treatment. However, their efficacy is limited by intrinsic and acquired drug resistance overtime, and endocrine resistance remains one of the biggest challenges in breast cancer treatment.

In order to investigate the underlying mechanisms of acquired drug resistance, and to develop new strategies for breast cancer therapy, I generated a novel long-term oestrogen deprived cell line (DH) in serum-free condition. As DH cells are cultured in a defined media with known concentrations of growth factors, it provides an ideal system to identify and dissect changes in signalling pathways in response to hormones and inhibitors *in vitro*. At the same time, DH cells are representative of ER positive breast cancers treated with drugs that reduce the level of oestrogen. It enables the identification of survival pathways that could be activated during oestrogen deprivation.

By using this cell model, I find that oestrogen stimulation enables cells to up-regulate the EGFR level and simultaneously reduces ERα expression at both mRNA and protein levels. Once up-regulated, EGFR expression is maintained despite oestrogen withdrawal indicating a stable transcriptional re-programming at the *EGFR* promoter. By using the whole genome expression microarrays, I identified a list of genes that also show stable changes in gene expression in response to oestrogen, suggesting that the oestrogen promotes transcriptional re-programming at multiple pathways in cells.

In terms of signalling pathways, oestrogen activates the growth promoting MAPK pathway in an EGFR dependent manner and a 5-day oestrogen pulse substantially increases the resistance of cells to tamoxifen, while cells remain sensitive to the EGFR inhibitor, demonstrating a functional switch between ERα and EGFR survival pathways. Furthermore, microarray analysis of ERα and EGFR downstream target genes shows that there is a general activation of MAPK gene signature after 5 days of oestrogen stimulation in DH cells.

In this thesis, I also investigate the molecular mechanism of oestrogen induced EGFR up-regulation in ER positive breast cancer cells. c-Myb is an oestrogen responsive transcription factor whose expression is regulated by ERα in breast cancer cells. I demonstrate that oestrogen treatment leads to ERα dependent c-Myb up-regulation in DH cells. I also find that c-Myb transiently locates upstream of the *EGFR* promoter to enhance its expression. As the up-regulation of EGFR in ER positive breast cancer could lead to survival pathway switching and endocrine therapy resistance, c-Myb could be a good drug target to prevent the likelihood these switches and subsequent relapse on endocrine therapies.

The expression of EGFR remains high after the removal of oestrogen suggesting there may be epigenetic changes, which maintain the transcriptional re-programming stimulated by c-Myb. Bisulphite sequencing however demonstrates *EGFR* promoter DNA methylation pattern is not affected by oestrogen. Meanwhile, ChIP microarrays with four different histone modifications show no significant changes around the promoter area of *EGFR* in response to oestrogen. These observations suggest that alternative epigenetic modifications or epigenetic alternations at other genes may subsequently lead to the stable expression of EGFR in response to oestrogen.

Abbreviations

APS	ammonium persulfate
bp	base pair
CDK	cyclin dependent kinase
CGI	CpG island
ChIP	chromatin immunoprecipitation
CK	cytokeratin
DCIS	ductal carcinoma in situ
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ERE	oestrogen response element
ERα	oestrogen receptor alpha
ERβ	oestrogen receptor beta
FCS	foetal calf serum
GPCR	G protein coupled receptor
GTR	general transcription factor
HAT	histone acetyltransferase
HDAC	histone deacetylase
HMT	histone methyltransferase
IF	immunofluorescence
IGFR	insulin like growth factor receptor
IHC	immunohistochemistry
MAPK	mitogen-activated protein kinase
MOPS	3-morpholinoprropane-1-sulfonic acid
ºC	degree centigrade
OD	optical density
PBS	phosphate buffered saline
PcG	polycomb group proteins
PCR	polymerise chain reaction
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PIC	pre-initiation complex
PKA	protein kinase A
PR	progesterone receptor
PRC1	polycomb repressor complex 1
PRC2	polycomb repressor complex 2

PRMT	protein arginine methyltransferase
PVDF	polyvinylidene fluoride
Ras	rat sarcoma
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SAM	S-adenosyl-L-methionine
SDS	sodium dodecyl sulphate
SERM	selective oestrogen receptor modulator
SRB	sulphorhodamine B
TAF	TATA box-binding protein associated factors
TBP	TATA box-binding protein
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TGF-α	transformming growth factor alpha
TSA	trichostatin A
X-Gal	5-bromo-4-chloro-3-indolyl-D-galactopytanoside

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Chapter 1 Introduction

1.1 Breast cancer

Breast cancer is by far the most common malignancy in women and constitutes nearly a quarter of all cancer diagnosed worldwide according to the world cancer report 2008 (www.iarc.fr/en/publications/pdfs-online/wcr/2008/wcr_2008.pdf). As breast cancer is often a classic hormone-dependent tumour, endocrine therapies are widely used as effective treatments for hormone sensitive early breast cancers or as an adjuvant therapy to prevent relapse for patients who have had their primary tumours surgically removed. Despite well-documented benefits, it is known that not all patients who are hormone receptors positive respond to endocrine therapies due to intrinsic resistances. Additionally, patients who do respond initially often have cancer recurrent while on therapy due to acquired resistances. The occurrence of endocrine resistances is still one of the major challenges in breast cancer therapy.

1.1.1 Histopathological Classification of breast cancer

Breast cancer classification divides breast cancers into several categories aiming to help the clinical diagnosis and select the appropriate treatment with increased efficacy and low toxicity for each cancer patient. Classification of breast cancer is usually based on the histological appearance of tissue in the tumour. Breast cancer begins in normal breast tissue, which is made up of glands for milk production, called lobules, and ducts that connect the lobules to the nipple (Donegan and Spratt, 2002). These lobular and ductal units extend far into the adjacent fatty, connective, and lymphatic tissues to make up the mammary gland. As the ducts branch deeper into the breast parenchyma, they form smaller ductal units called the terminal duct-lobular units that are lined by an inner luminal epithelial cell layer and a outer mesenchymal myoepithelial (basal) cell layer (Wellings et al., 1975). Interestingly, the percentage of cells that stain positively with Ki67 antibody reveals that proliferating cells are predominantly found in the epithelial layer and less frequently in the myoepithelial cell layer (Russo et al., 2000). Cancers originating from the ducts are knows as ductal carcinomas, while those arising from lobules are known as lobular carcinomas. Breast cancers can also be classified as either in situ (non-invasive) or invasive cancer where cancer started in the lobules or ducts of the breast

invades into the surrounding tissue. The majority of in situ breast cancers are ductal carcinoma in situ (DCIS). DCIS is an early stage breast cancer that can be treated successfully with lumpectomy followed by radiation therapy or tamoxifen as adjuvant therapy (Virnig et al., 2010). However, if left untreated, there is a high chance of DCIS to become invasive breast cancer. Among the diagnosed breast cancers, approximately 80% of the cases are invasive ductal carcinomas making it the most common type of breast cancer followed by invasive lobular carcinomas. The seriousness of invasive breast cancer is strongly influenced by the stage of the diseases, which describes the tumour size (T), whether or not the tumour has spread to the lymph nodes (N), and the presence or absence of distance metastases (M) (Edge SR, 2010). Once the TNM is determined for a certain tumour, a stage of 0, I, II, III, or IV is assigned, with stage 0 being in situ disease, stage I to III being cancer within the breast or regional lymph nodes, and stage IV being a metastatic cancer. (www.cancerstaging.org/staging/posters/breast8.5x11.pdf)

1.1.2 Molecular profiling of breast cancer

Breast cancer patients with the same diagnostic profile can have strikingly different clinical outcomes. This difference is possibly due to the limitation of classifying breast cancer based on histopathological characteristics, which can group molecularly distinct breast cancers into the same clinical disease. Perou et al. and Sorlie et al. were the first to use DNA microarray analysis to show that breast cancers can also be subdivided based on gene expression profile (Perou et al., 2000). They used unsupervised hierarchical clustering analysis to group genes on the basis of similarity in gene expression pattern. A subset of 496 genes termed the “intrinsic” gene set was selected to show the variation in expression between different tumours rather than between paired samples (n=22) before and after chemotherapy from the same tumour. Using this approach, breast carcinomas can be subdivided into 4 groups according to different molecular features. The largest difference in overall gene expression profile is observed between ER positive and ER negative tumours. Tumours in the ER positive group are characterised by the relatively high expression of genes associated with breast luminal cells, therefore it is classified as luminal-like.

ER negative tumours can be further divided into basal-like and HER2-overexpressing tumours. The forth group is the normal-like tumours. Follow-up studies that using the intrinsic gene set in conjunction with a gene subset that correlated with patient survival further divided the ER positive / luminal-like breast cancer into at least two subgroups, each with a distinctive expression profile. A difference in disease outcome was observed for tumours classified as luminal A versus luminal B. Luminal A tumours generally have a higher ER protein level and better disease outcome comparing with the luminal B tumours (Sorlie et al., 2001). Subsequently, DNA microarray analysis has been used to identify gene signatures for poor prognosis, which provides a novel strategy to select patients who would benefit from adjuvant therapy (van 't Veer et al., 2002). Later, the composition of the intrinsic gene set and breast cancer molecular classification was refined in a study with larger number of tumour samples (n=268) from three independent data sets. The refined intrinsic gene subset contains 534 genes, which confirms the five molecular subtypes of breast cancer (luminal A, luminal B, basal-like, HER2-overexpressing and normal-like). This study also included tumours from 18 carriers of *BRCA1* and 2 carriers of *BRCA2* mutations. The results indicate that tumours with mutations in the *BRCA1* gene predisposes for the basal-like subtype, which are ER negative and associated with poor prognosis (Sorlie et al., 2003).

1.2 Oestrogen signalling mediated by oestrogen receptors

1.2.1 ERα and ERβ

As discussed in the previous section, the biggest difference in the overall gene expression pattern across various breast cancer subtypes was the level of ER. At the same time, ER is expressed in more than 70% of the primary breast cancers making it one of the most important therapeutic targets for breast cancer (Stierer et al., 1993). ER belongs to the nuclear receptors super family which have the ability to bind directly to the DNA and regulate gene expression upon oestrogen stimulation (Mangelsdorf et al., 1995). ERα and ERβ are the two main isoforms of human ER, encoded by two separate genes termed *ESR1* and *ESR2*, respectively (Enmark et al., 1997). Although ERα and ERβ are the products of independent genes, they share homology at the DNA and ligand binding domains (96% and 58%, respectively) (Mosselman et al., 1996). Both receptor subtypes bind oestrogen with a similar affinity and activate the expression oestrogen responsive reporter system in an oestrogen dependent manner (Kuiper et al., 1997). However, they display distinct tissue distributions and have different functions among cells (Gustafsson, 1999, Younes and Honma, 2011). In terms of human breast, both receptors were shown to be expressed in the normal and cancerous human mammary gland by immunohistochemistry. The staining of ERα was restricted to the cell nucleus of luminal epithelial cells lining ducts and lobules, while ERβ staining was detected in the nucleus of a variety of cells including myoepithelial, surrounding stromal as well as luminal epithelial cells (**Figure 1.1**) (Speirs et al., 2002). For breast cancer, majority of the studies have been focused on the functions of ERα, which is vital for normal mammary development, breast tumorigenesis and progression. In comparison, little is known about the function of ERβ in breast cancer (Murphy and Leygue, 2012). It has been suggested as tumour suppressor in ovarian cancer (Lazennec, 2006). Furthermore, studies done with human breast cancer cell lines showed that ERβ was able to block cell proliferation and tumour formation by causing cell cycle arrest (Paruthiyil et al., 2004). Therefore, activation of ERβ expression has been suggested as an alternative therapeutic approach to treat breast cancer (Williams et al., 2008). Interestingly, there are also published data suggesting

ERβ expression correlates with pro-proliferation activity as well as anti-proliferative activity in normal mammary cells (Cheng et al., 2004, Helguero et al., 2005). At the transcriptional level, ERβ acts like a modulator of ERα mediated gene transcription through unknown mechanisms (Lindberg et al., 2003).

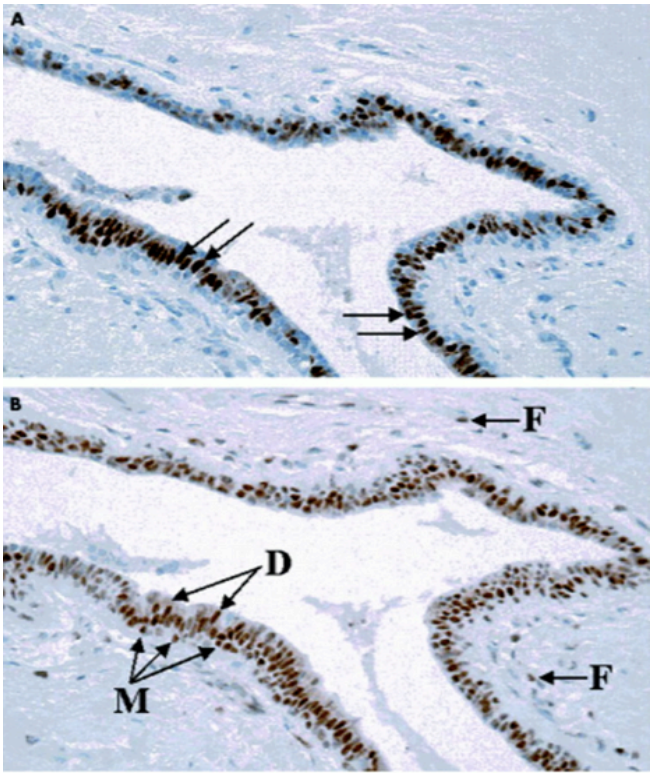


Figure 1.1 Immunochemistry staining of ERα and ERβ in normal mammary gland.

A) ERα is localised to the luminal epithelial cells lining the breast ducts (arrows). B) Serial section of A) stained with ERβ. ERβ staining is observed in myoepithelial cells (M) and stromal fibroblasts (F) as well as luminal epithelial cells (D) (arrows). (Picture taken from Speirs et al., 2002)

1.2.2 ERα structure

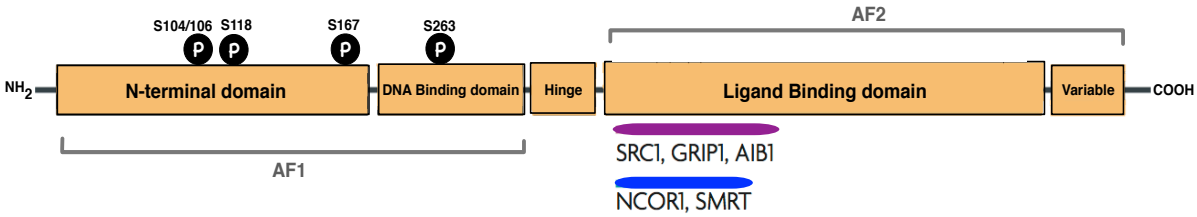


Figure 1.2 Structure of oestrogen receptor.

The ERα is composed of a N-terminal domain, a DNA binding domain, a hinge region, a ligand-binding domain, and a variable domain. The transactivation function (AF-1) region contains the N-terminal domain and the DNA binding domain, which can by phosphorylated at multiple sites through various pathways. The AF-2 region of ligand-binding domain directly interacts with co-activators, SRC1, GRIP1 or AIB1 in the presence of oestrogen (purple), while it binds to the co-repressors NCOR1 and SMRT in the presence of tamoxifen (blue).

Like other nuclear receptors, ERα and ERβ are comprised of several conserved functional domains including a N-terminal domain (NTD), a DNA binding domain (DBD), a hinge region, a ligand-binding domain and a variable domain (**Figure 1.2**) (Beato et al., 1995). The NTD of ERα harbours a ligand independent activation function (AF-1), which was originally identified by its ability to stimulate transcription in an ERα deletion mutant containing only the NTD and DBD (Lees et al., 1989). The ligand independent activity of AF-1 is mediated by phosphorylation (Lannigan, 2003), which will be discussed in details in section 1.2.2. The NTD varies between ERα and ERβ. For example, ERα can directly bind to the TATA box-binding protein (TBP) while ERβ cannot (Warnmark et al., 2001). The difference in TBP binding between the receptors may imply ERα and ERβ have different sets of target genes.

Adjacent to the N-terminal transactivation domain is a conserved DBD of ER, which is composed of two non-equivalent cysteine rich zinc fingers that are responsible for DNA binding (Green et al., 1988). The DBD of both ERα and ERβ bind to the oestrogen response element (ERE) composed of a palindromic DNA sequence (GGTCAnnnTGACC) (Klinge, 2001). The ERE can reside either proximally to the

target gene promoter area or enhancer region that is distant from the transcription initiation site (Tsai and O'Malley, 1994).

The hinge domain serves as flexible region connecting the DBD with LBD. It contains nuclear localisation signal, which gets exposed during the ligand induced conformational change of the receptor (Kumar et al., 2011). The LBD contains another transactivation function (AF-2) region that is ligand dependent. The hinge region of ERα promotes the synergy between AF-1 and AF-2 in response to oestrogen (Zwart et al., 2010).

Despite low sequence homology in LBDs among the nuclear receptor superfamily, the 3 dimensional structures of the LBD monomers are similar (Bain et al., 2007). Upon ligand binding, the receptors undergo conformational change and forms dimers that are recruited to target gene sites. The LBD of ER consists of 12 α helices that dock the ligand-binding pocket for both agonist like oestrogen and antagonist like tamoxifen (Brzozowski et al., 1997). After oestrogen is bound to the receptor, helices 3 and 11 are held together by a network of hydrogen bonds to keep the ligand in the binding pocket, while helix 12 is positioned to interact with co-activators to form an active transcription complex (Celik et al., 2007). However, when tamoxifen is bound to the receptor, helix 12 is placed in a position that blocks the co-activators recognition region (Shiau et al., 1998). The region within LBD that is responsible for co-activators binding is AF-2. It was identified by its ability to stimulate transcription only in the presence of ligand (Webster et al., 1988, Lees et al., 1989). Upon oestrogen binding, helices 3, 5 and 12 form a surface to interact with co-activators like the p160 protein family through the LxxLL motif (Heery et al., 1997, Plevin et al., 2005). The p160 protein family is the most characterised of ER co-activators that consists of 3 member proteins, which are SRC1 (also known as p160-1, N-CoA1), GRIP1 (also known as SRC-2, TIF2, N-CoA1) and AIB1 (also known as SRC-3, P/CIP, ACTR, RAC3, TRAM1) (Nilsson et al., 2001). They are recruited by ER onto oestrogen responsive genes in a cyclic manner to activate transcription (Shang et al., 2000). In contrast, the partial antagonist recruits co-repressors like NCOR1 and SMRT.

The ER co-activators, p160 protein family, contain two intrinsic transcriptional activation domains (AD1 and AD2) (Xu and Li, 2003). The AD1 region is responsible for the interaction with other co-activators like CBP and p300 that facilitate histone acetyltransferase (HAT) activity (Li et al., 2000), while AD2 is responsible for interaction with histone methyltransferase like CARM1 and PRMT1 (Strahl et al., 2001, Koh et al., 2001). Although the C-terminal domains of SRC-1 and AIB-1 also have HAT activity, it is much weaker than those in CBP and P300 (Chen et al., 1997, Spencer et al., 1997). These chromatin-remodelling enzymes are recruited to oestrogen responsive genes in a complex with the p160 protein family co-activators and ER to activate the transcription in a ligand dependent manner. Members of the p160 protein family have also been implicated in breast cancer. It has been shown that tumours with increased expression of SRC-1 together with high expression of the epidermal growth factor receptor, HER2, have a greater probability of recurrence on tamoxifen treatment (Fleming et al., 2004, Redmond et al., 2009). AIB-1 is involved in normal breast development and breast cancer tumourgenesis. It is amplified in around 10% and overexpressed in 64% of primary breast cancers (Anzick et al., 1997). Together, these observations indicate that altered ER co-activators may contribute to development of breast cancer.

Next to the LBD is the variable domain, which contains 42 amino acids. It was found to modulate transcription and also impact the receptor dimerisation in the presence of oestrogen (Koide et al., 2007, Yang et al., 2008).

1.2.3 ERα activation

ERα can function through three different pathways in response to oestrogen, which are the genomic pathway, non-genomic pathway and ligand independent pathway (**Figure 1.3**).

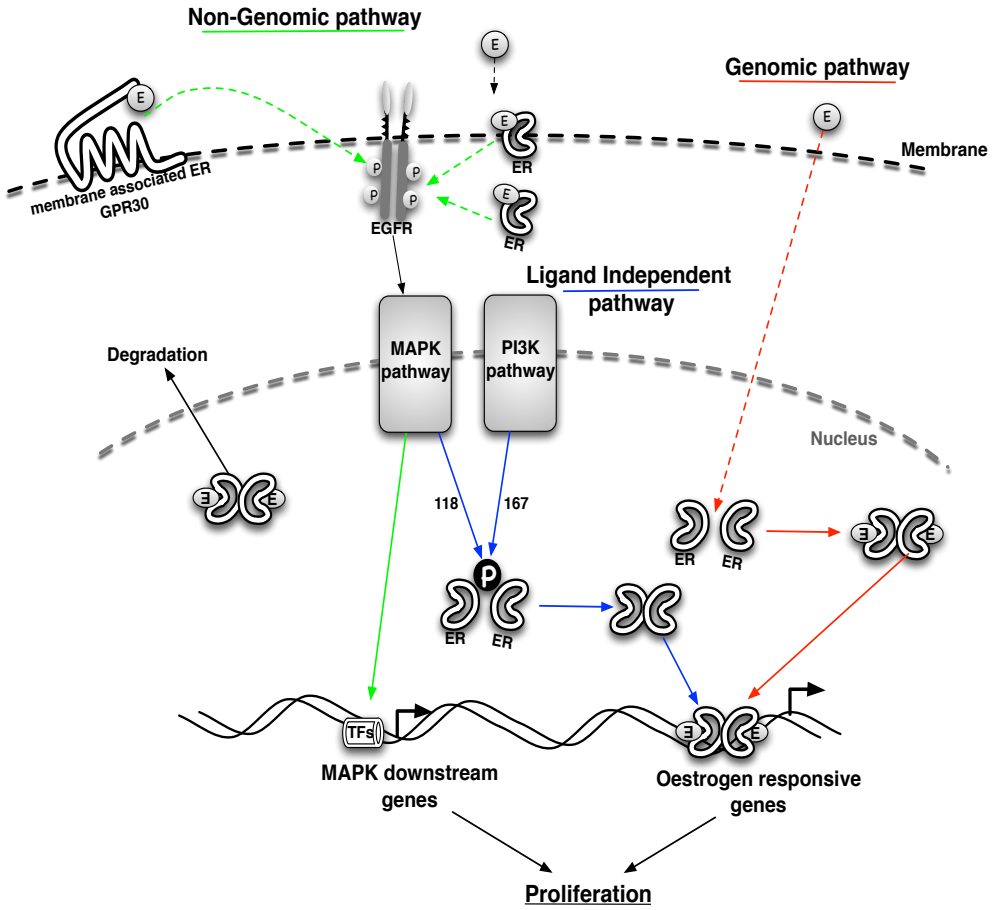


Figure 1.3 Different pathways of ERα activation.

The genomic pathway is illustrated with red arrows. Oestrogen diffuses through the membrane to bind to ERα in the nucleus. Oestrogen causes conformational changes throughout the receptor leads to receptor dimerisation, followed by binding onto the oestrogen responsive genes. The ligand independent pathway is demonstrated with blue arrows. In the absence of ligand, ERα is phosphorylated at Ser 118 and Ser 167 by MAPK pathway and PI3K pathway, respectively. The phosphorylation is able to facilitate conformational changes and causes un-liganded ERα to dimerise and transactivate oestrogen responsive genes. In addition to the genomic and ligand independent pathway, there are a series of non-genomic pathways of ERα facilitated by the newly identified membrane associated ER, GPR30, and cytosolic or membrane associated classic ERα (green arrows). Oestrogen causes rapidly activation of several signalling transduction pathways like the MAPK pathway. Activated receptors are recycled by proteasome degradation.

1.2.3.1 The genomic pathway

ERα proteins are found primarily in the nucleus, where they form complex with heat shock proteins like hsp90 to inhibit their action (Picard, 2006). In the genomic

pathway, the oestrogen diffuses through the membrane and binds to the nuclear ERα, which causes conformational changes lead to displacement of the heat shock protein and dimerisation of the receptors. This is followed by co-activators recruitment and chromatin binding at the promoter or enhancer regions of the oestrogen responsive genes (**Figure 1.3** red arrows) (Carroll and Brown, 2006). The conformation changes also reveal several phosphorylation sites at the AF-1 region of ERα. ERα is predominantly phosphorylated on the serine (Ser) residues, where Ser 118 is the major phosphorylation site in response to oestrogen (Joel et al., 1995). It is phosphorylated by TFIIH and cyclin dependent kinase (CDK) 7 (Chen et al., 2000). Ser 104 and Ser 106 are phosphorylated to a lesser extent by CDK2 (Rogatsky et al., 1999). Although multiple phosphorylation sites have been reported in ERα, the exact role of phosphorylation at individual or multiple sites is still underexplored. However, it has been shown that a combination of phosphorylation sites within ERα rather than any individual site may be important for the transcriptional activity of the receptor (Le Goff et al., 1994). Meanwhile, a wide range of effects have been demonstrated in the cell culture system such as transcription regulation, receptor dimerisation, interactions with ligand or DNA and co-activators recruitment (Murphy et al., 2011).

1.2.3.2 The ligand independent pathway

In the ligand independent pathway, ERα is activated through post-translational modifications such as phosphorylation by cell signalling transduction pathways including MAPK pathway and PI3K pathway (**Figure 1.3** green arrows) (Lannigan, 2003). The receptor can also be phosphorylated at Ser 236 by protein kinase A (PKA), which is important for receptor dimerisation (Chen et al., 1999b). However, its role in the ligand independent pathway of the receptor is unknown. Upon activated by phosphorylation, the un-liganded ERα is able to dimerise and transactivate oestrogen responsive genes (Bunone et al., 1996, Maggi, 2011).

1.2.3.2.1 EFG receptor family and MAPK pathway

The action of ERα can be coupled to epidermal growth factor (EGF) signalling, where the activation of epidermal growth factor receptor (EGFR) leads to ERα phosphorylation at Ser 118 via the MAPK pathway in the absence of oestrogen. (Bunone et al., 1996, Kato et al., 1995). In the presence of ligand, the ERα phosphorylation of Ser 118 is not dependent on the p42/44 MAPK pathway as shown in MCF-7 cells (Joel et al., 1998). Interestingly, oestrogen can also lead to rapid activation of MAPK (within minutes) (Improta-Brears et al., 1999), and MAPK pathway inhibitors are able to prevent oestrogen induced mitogenesis in MCF-7 cells (Lobenhofer et al., 2000). All these suggest a bidirectional crosstalk between the ERα and MAPK pathway mediated signalling, which will be discussed further in **section 1.2.3.3**.

In general, MAPK pathway is activated through growth factors receptors like EGFR. EGFR is a receptor tyrosine kinase (RTK) belonging to the transmembrane EGF family, which contains 3 other members that are HER2, HER3 and HER4. The receptors are made up of an extracellular ligand-binding region, a transmembrane spanning region and a cytosolic tyrosine kinase domain (Wells, 1999). A variety of ligands have been shown to activate EGF receptors. For instance, EGF and TGF-α are specific to EGFR whilst heregulin binds to HER3 and HER4. However, the ligand for HER2 is undefined. Both EGFR and HER4 are autonomous receptors, which undergo dimerisation followed by producing intracellular signals in the presence of the ligand. In the meantime, these receptors can interact with other EGF receptors and regulate their downstream effectors. For example, EGFR monomer will pair with HER2 and HER3 but not HER4 upon stimulation, but HER4 is able to pair with HER2. In contrast to HER2, HER3 can be recognised by extracellular ligands but has an impaired cytosolic kinase domain. Therefore, it serves as a docking partner for EGFR and HER2.

In the absence of growth ligands, the extracellular ligand-binding region of EGFR is locked in an inactive conformation by a β-hairpin loop, called the dimerisation loop, which prevents the formation of a high affinity ligand-binding site composed of domain I and III (**Figure 1.4**) (Bublil and Yarden, 2007). Upon binding of the ligand,

the receptor monomer is stabilised in an active conformation to expose the dimerisation loop and mediate receptor dimerisation. Binding of the ligand also induces conformational changes at the cytosolic kinase domain. The EGFR has a bi-lobular kinase structure consisting of a N-terminal lobe (N-lobe) and a C-terminal lobe (C-lobe). In the inactive conformation, the two leucine (L) residues from the activation loop are packed against the helix α-C from the N-lobe. After dimerisation, the kinase domain from two receptors are brought close to each other, where the C-lobe of one kinase attaches to the N-lobe of the other. The conformational change allows re-orientation of the activation loop and relief of the auto-inhibited kinases activity. The kinase activity causes phosphorylation of multiple tyrosine (Y) residues at the EGFR C-terminus including Y992, Y1045, Y1068, Y1148 and Y1173 (Jorissen et al., 2003).

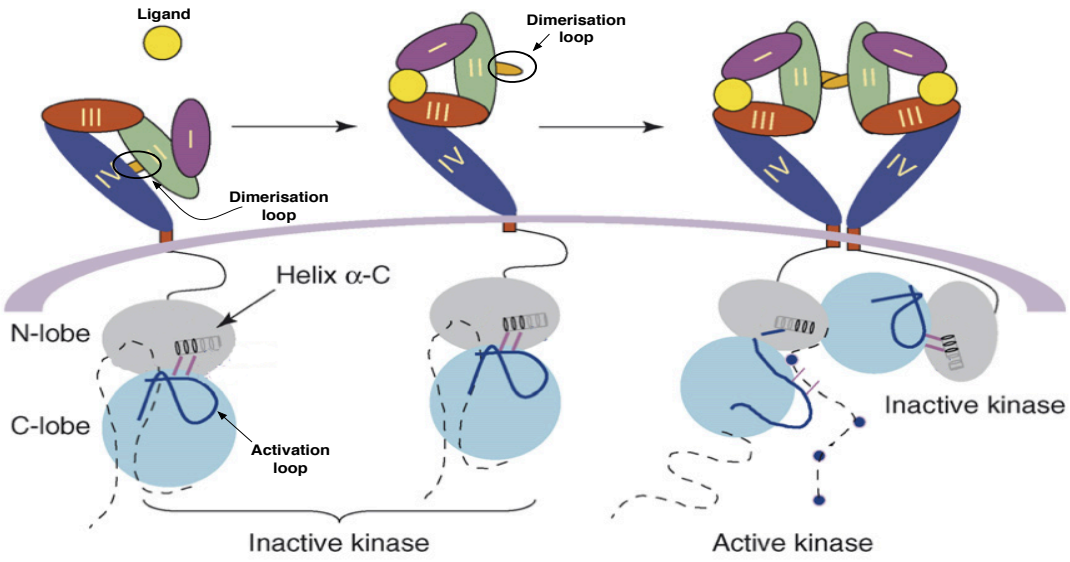


Figure 1.4 The structure of inactive and active EGF receptors.

A schematic representation of the EGF receptor structure is shown. Before binding of ligand, domain II and IV from the extracellular domain of the receptor are locked together by the dimerisation loop. In the intracellular kinase domain, L837 and L834 from the activation loop are packed against the helix α-C to inhibit the kinase activity of the receptor. Upon binding of ligand (yellow), major conformational changes happen throughout the receptor. The dimerisation loop is exposed to mediate receptor dimerisation in the extracellular domain. The close proximity of two kinase domains allows the C-lobe from one kinase domain to interact with the N-lobe from another, which actives the kinase domain by relief of auto-inhibition. The activated

kinase phosphorylates tyrosine residues (purple) on the C-terminal tail of its dimerisation partner. The figure was modified from figure 2 of reference, Bublil and Yarden, 2007.

In the growth promoting MAPK pathway, the phosphorylated residues at the C-terminal of EGFR trigger recruitment of adaptor proteins such as SHC, Grb2 and SOS to activate the small GTPase Ras (Pearson et al., 2001). Raf kinase is then activated by active Ras to initiate the signalling cascade. The three components of the pathway (Raf-1, MEK1/2 and ERK1/2) are held together by the scaffolding protein kinase suppressor of Ras1 (KSR1) at the cell surface (Roy et al., 2002). At the end of the cascade, the phosphorylated ERK1/2 leaves the plasma membrane and enters into the nucleus, where it phosphorylates and activates a number of genes that lead to cell survival and proliferation.

Altered EGFR expression has been reported in a variety of cancers including breast cancer (Jorissen et al., 2003). A number of mechanisms of EGFR up-regulation have been proposed in the literature such as gene amplification, overexpression of receptor/ligand protein (Bhargava et al., 2005). However, no EGFR mutations have been identified in the breast cancer so far. EGFR expression is significantly associated with loss of sensitivity towards endocrine therapy and overall survival in breast cancer (Nicholson et al., 1994, Rimawi et al., 2010). In breast cancer, the increased expression of EGFR is generally seen in ER negative, progesterone receptor (PR) negative and HER2 negative (triple negative) breast cancers (Rakha et al., 2008). These findings are consistent with gene expression profiling studies, which identify basal-like ER negative breast cancer apart from HER2 overexpressing breast cancer (Perou et al., 2000). In addition, EGFR is a positive marker for the basal-like breast carcinomas (Rakha et al., 2007). Another subtype of breast cancers that have high expression of EGFR is the metaplastic breast cancer, which is a rare but severe form of invasive ductal breast cancer (Reis-Filho et al., 2005). However, studies have suggested that this form of cancer is also a subtype of basal-like breast tumours (Reis-Filho et al., 2006). Aberrant EGFR and HER2 receptor signalling has been shown to hyperactive the MAPK pathway (Kurokawa and Arteaga, 2003). Furthermore, activation of MAPK pathway has been shown to associate with

oestrogen unresponsiveness and endocrine therapy resistance using cell model system (McClelland et al., 2001).

1.2.3.2.2 PI3K/Akt pathway

Activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is often implicated in human breast cancer (Perez-Tenorio and Stal, 2002). Similar to the MAPK pathway, the PI3K/Akt signalling pathway is also a downstream target of RTK like EGFR and is well known to regulate cell proliferation and survival (Franke et al., 2003). RTK mediated activation of PI3K leads to the conversion of phosphatidylinositol-4, 5-biphosphate (PIP_2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP_3). PIP_3 then recruits Akt to the plasma membrane, where it becomes phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) at threonine (Thr) 308 and by PDK2 at Ser 473 (Cantley, 2002). In the ligand independent pathway of ERα, the receptor can be activated through the PI3K/Akt signalling pathway by phosphorylation at Ser 167 (Sun et al., 2001). Moreover, Akt overexpression can lead to up-regulation of oestrogen responsive genes, and protect cells from tamoxifen-induced apoptosis (Campbell et al., 2001). Recently, it has been shown that ER positive breast cancer cells that grew in low oestrogen environment have an enhanced sensitivity towards a combination of PI3K/Akt pathway inhibitors such as rapamycin and LY294002 (Ray et al., 2011). Collectively, these data suggest targeting PI3K/Akt pathway can be an alternative strategy in treating endocrine resistant breast cancers.

1.2.3.3 The non-genomic pathway

Oestrogen also has rapid stimulatory effects on a variety of signal transduction proteins (**Figure 1.3** green arrows). This non-genomic pathway of ERα is mediated by a small fraction of ERα that is localised near or at the plasma membrane as well as a newly identified transmembrane G protein-coupled receptor (GPCR) GPR30 (Prossnitz et al., 2008). The classical steroid receptor, ERα, is predominantly localised in the nucleus, where it functions as a transcription factor that regulates gene expression. In the non-genomic pathway, it acts as a component of a signalling transduction cascade. As ERα does not have intrinsic kinase activity, it is likely to

function like an adaptor protein to mediate the rapid oestrogen induced signalling responses, like stimulation of growth factor receptors and activation of MAPK and PI3K/Akt signalling cascades (Revankar et al., 2005, Song et al., 2002, Haynes et al., 2003). ERα localisation to the membrane can be mediated by several mechanisms. Palmitoylation is a post-translational modification of ERα, which involves the attachment of a long palmitic acid to the receptor. This long fatty acid chain increases protein hydrophobicity and thereby allows ERαs to anchor into the plasma membrane, where they are found to associate with caveolin-1 (Marino and Ascenzi, 2008). The membrane localisation of ERα can also be mediated by interaction with a membrane adaptor protein, Shc (Song et al., 2004). Shc also gets activated in response to oestrogen stimulation, leading to Shc-Grb2-Sos complex formation and activation of the MAPK pathway (Song et al., 2002). In the meantime, oestrogen induces complex formation of ERα with non-RTK, Src, and the PI3K subunit p85 to activate two important pathways that are the Src/Ras/MAPK pathway and PI3K/Akt signalling (Simoncini et al., 2000, Moriarty et al., 2006).

GPR30 is a classic GPCR that is found to be involved in the oestrogen induced MAPK pathway activation in breast cancer cells that lack ERα (Filardo et al., 2000). Therefore, it is identified as a membrane associated oestrogen receptor. Interestingly, ERα antagonists like tamoxifen and ICI 182,780 can also induce the GPR30 dependent activation of MAPK pathway. The mechanism of the oestrogen induced activation of MAPK pathway is through the transactivation of EGFR receptor by GPR30 (Daub et al., 1996).

Although the genomic, ligand independent and non-genomic pathways are present as separate activation pathways of ERα, these signalling pathways are not mutually exclusive but rather complementary, with many interactions between them. For example, the bidirectional signalling between the ERα and EGFR, where the activated EGFR signalling cascade causes the ligand independent activation of ERα, and membrane associated ERα is able to transactivate the EGFR through the non-genomic pathway. This enables a broader range of genes to be regulated by ER in response to oestrogen, and making breast cancer a difficult disease to treat.

1.3 Transcriptional and epigenetic regulation of oestrogen signalling in breast cancer cells

1.3.1 Role of transcription in gene regulation

Many biological processes are regulated, spatially and temporally, at the level of transcription. Physiological signals or stimulus outside cells are transduced to the nucleus through a wave of complex molecular interactions, which can either cause activation or attenuation of gene expression by altering the interactions of transcriptional factors with their target genes. The appearance and behaviour of cancer cells is a consequence of failure of normal regulation of genes involved in cell growth and differentiation.

Transcription occurs in the context of chromatin, where a part of the DNA carrying the genetic code is copied into corresponding messenger RNA (mRNA) (Alberts et al., 2002). Chromatin is a highly organized structure, which is generally inhibitory to transcription. Therefore, a number of steps are required to activate transcription from a nucleosomal template. One of the first steps is the recruitment of chromatin remodelling proteins and co-activators by specific transcription factors to target genes. Collectively, they set an appropriated chromatin environment for transcription initiation and also facilitate the recruitment of pre-initiation complex (PIC) (Orphanides and Reinberg, 2002).

In eukaryotic cells, genes are transcribed by three different DNA-dependent RNA polymerase enzymes (Blackwell and Walker, 2006). Only RNA polymerase II (RNAP II) is responsible for transcribing protein-coding genes (mRNA) and several micro RNAs. As RNAP II on its own is not able to initiate transcription in a selective and effective manner, other factors are required to convert it from a transcriptionally inert form to a highly processive elongating form (Kornberg, 2007). These factors are called the general transcription factors (GTRs), which include TFII (transcription factor for RNA polymerase II) A, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. Among GTRs, TFIID is a key player that is involved in the promoter recognition (Nikolov and Burley, 1997). TFIID is multi-subunit complex composed of the TATA binding

protein (TBP) and at least 13 TBP associated factors (TAFs). Upon stimulation, TBP binds to DNA together with TAFs to form the TFIID complex. The complex is stabilized by the binding of TFIIA and TFIIB. TFIIF then recruits Pol II to the complex, which follows by the association of TFIIE and TFIIF. Together, they form the PIC to pre-initiate the transcription. After pre-initiation, it needs to pass a stage called promoter clearance before engaging into productive elongation. During this stage, the PIC is partially disassembled to allow the recycling of GTRs (Zawel et al., 1995). Once the promoter is cleared, the next round of transcription can be reinitiated. Several histone modifications are deposited on nucleosomes during this stage to mark active transcription, such as H3K4 trimethylation and H2B monoubiquitylation (Weake and Workman, 2010).

Following transcription initiation, the carboxy terminal domain (CTD) of RNAP II is phosphorylated on Ser5 by TFIIH (Sims et al., 2004). The resulting RNAP II complex is found to be paused at most promoters by negative elongation factors (Saunders et al., 2006). Other cofactors are required to stimulate further CTD phosphorylation and transcription elongation. For active genes, CTD is further phosphorylated at Ser2 by positive transcription-elongation factor-b (p-TEFb) to produce a stable elongation complex (Ni et al., 2004). Chromatin modification also plays an important role during elongation. For instance, a productive elongation is usually associated with methylation on H3K36 (Krogan et al., 2003). Termination is the final stage of transcription cycle, during which the mRNA is cleaved, polyadenylated and transported out of nucleus (Proudfoot et al., 2002).

1.3.2 Epigenetic maintenance of gene expression

Transcriptional regulation is responsible for the rapid activation of gene expression in response to stimuli. Epigenetic modifications not only help transcription regulators to facilitate the changes in gene expression after stimulation but also stably maintain the expression pattern after initiation of transcriptional re-programming. For example, the maintenance of *Hox* genes expression pattern after embryo development. *Hox* genes are expressed with precise boundaries along the anterior-

posterior axis of the drosophila embryo (Francis, 2009). Unlike many patterning genes expressed transiently, *Hox* genes expression is maintained through to adult stages by chromatin remodelling proteins called polycomb group proteins (PcGs) (Cao et al., 2002).

1.3.2.1 Histone Modification

In eukaryotic cells, the hereditary information is stored in the form of DNA that is packed in various levels of condensation in the nucleus. DNA is first wound around a core histone octamer, two each of H2A, H2B, H3 and H4 (Kornberg, 1977). This core structure sealed with linker histone is then held together by linker DNA to form a protein DNA complex called a nucleosome. The nucleosome core particles are predominantly globular except their un-structured N-terminal "tails." These histone tails are subjected to several different types of covalent post-translational modifications (Kouzarides, 2007). Although the histone tail modifications have little direct effect on individual nucleosomes, they dynamically shift the chromatin fibre to a more open or closed state by affecting the contact between different histones in adjacent nucleosomes or by recruiting other binding partners to the chromatin (Goldberg et al., 2007).

1.3.2.1.1 Histone acetylation

Among all the histone modifications, acetylation has the most potential to create an open chromatin conformation since it neutralises the negative charges of the lysine molecules. Acetylation on histone is often found to be enriched at promoter regions of active genes, and the enrichment significantly correlates with transcription activity (Pokholok et al., 2005). A genome wide study of histone modifications in five human cell lines showed that H3Ac modifications are tightly associated with TSS in actively transcribed genes, while H4Ac modifications have more widespread distributions (Koch et al., 2007). Acetylation at H3K9 (H3K9Ac) and H3K14 (H3K914Ac) has also been shown to be important for TFIID recruitment and transcription initiation of the human interferon-β gene (Agalioti et al., 2002). H3K9 is an interesting position

that embraces two histone modifications. H3K9Ac is a marker for gene activation, while lysine 9 is often methylated in heterochromatin. Histone acetylation is catalysed by histone acetyltransferases (HAT) enzyme families, which can be divided into three major groups, GNAT, MYST, and CBP/p300 (Sterner and Berger, 2000). HATs do not directly interact with DNA but rather with DNA binding activators, and function as transcriptional co-activators to facilitate the activation of GTFs and initiation of RNAP II (Wade et al., 1997).

In contrast, histone de-acetylation induces compaction of the chromatin fibre and leads to repression of expression. Therefore, they generally function as transcriptional repressors (Kuo and Allis, 1998). Histone acetylation is removed by histone deacetylases (HDACs). HDAC activity can be pharmacologically modified using HDAC inhibitors such as Trichostatin A (TSA) (Yoshida et al., 1995). Studies using these agents show that changes in acetylation patterns can activate specific genes that are responsible for cell cycle alterations and induction of apoptosis, which makes HDAC inhibitors attractive candidates for cancer therapy (Taddei et al., 2005).

1.3.2.1.1 Histone methylation

Histone methylation is catalysed by histone methyltransferases (HMT), which happens at various sites on histones. It can be broadly divided into lysine and arginine methylation (Zhang and Reinberg, 2001). Protein arginine methyltransferases (PRMTs) catalyse the transfer of methyl groups from S-adenosyl-L-methionine (SAM) to the guanidino nitrogens of arginine residues. Lysine methylation is catalysed by SET domain containing HMTs. Unlike acetylation, histone methylation can either be activator or repressor for transcription depending on the chromodomain-containing proteins recruited by these methylation marks (Kouzarides, 2002). For example, methylated H3K4 recruits chromodomain helicase DNA-binding protein 1 (CHD1) to the site of modification, which is an activator for transcription (Pray-Grant et al., 2005). While, H3K9me3 recruits binding of HP1 and is associated with gene silencing (Smallwood et al., 2007).

Modifications of mono-, di- and tri-methylated H3K4 are all linked to gene

activation. All three of these modifications peak at TSS of active transcribed genes. While, H3K4 dimethylation (H3K4me2) will also be carried into the coding region (Barski et al., 2007). Furthermore, the distribution of these H3K4 methylation markers progressively gets more localised to the vicinity of TSSs as the modification moves from mono- to di- and tri-methylation in highly expressed genes. Meanwhile, high levels of H3K4me1 combined with low H3K4me3 was suggested as a signature for active enhancers (Heintzman et al., 2007).

H3K27 trimethylation is an interesting repressive histone modification that is mediated by PcGs (Lee et al., 2006). The PcGs in mammalian consist of two main multimeric protein complexes called polycomb repressor complex 1 (PRC1) and polycomb repressor complex 2 (PRC2) (Simon and Kingston, 2009). EZH2 of PRC2 contains the HMT activity, which catalyses the methylation on H3K27. Interestingly, EZH2 can also be recruited by H3K27 trimethylation, indicating a potential role of this histone modification in epigenetic cellular memory (Hansen et al., 2008). Meanwhile, a specific modification pattern called bivalent domains is found to associated with development genes in embryonic stem cells (Bernstein et al., 2006). They consist of both repressive H3K27me3 marks and active H3K4me3 marks. It was proposed that the exist of bivalent domains poised differentiation genes in a silenced state while keeping them ready for activation (Vastenhouw et al., 2010). Therefore, mis-regulation of PcG proteins can also trigger genomic instability and re-programming of gene expression, and ultimately leads to diseases like cancer.

1.3.2.1 DNA methylation

DNA methylation is a covalent modification of DNA that provides a direct mechanism to regulate gene expression (Bird, 2002). In mammalian cells, nearly all DNA methylation occurs on cytosine at position C5 of CpG dinucleotides. DNA methylation is carried out by enzymes called DNA methyltransferases (DNMTs) (Robertson et al., 1999). Three enzymatically active DNMTs have been identified in mammalian cells: DNMT1, DNMT3a, and DNMT3b. DNMT1 is the most abundant in somatic cells, which helps to maintain the methylation patterns. While, DNMT3a

and DNMT3b are *de novo* DNMTs that establish the primary methylation patterns on the genome following embryonic fertilization (Okano et al., 1999). Another key player in the DNA methylation is the methyl-CpG binding proteins, which are involved in recognising of methylation markers (Bird and Wolffe, 1999, Hendrich and Bird, 1998). Methyl-CpG binding proteins such as MBD2 and MeCP1 function as transcriptional repressors by interacting with co-repressor complexes containing HDACs (Ng et al., 1999, Feng and Zhang, 2001).

Regions of the genome that have high concentration of CpGs are defined as CpG islands (CGIs) (Saxonov et al., 2006). CGIs are generally unmethylated, and are on average between 200-1000 bp in length around the gene promoter area, but may also extend into gene coding regions. Approximately 50% of annotated genes' TSSs are associated with a CGI, making this the most common type of promoter and a key feature in promoter recognition (Deaton and Bird, 2011). DNA methylation of these islands controls gene activity either at a local level through effects on a single gene's promoter and enhancer or at global level through mechanisms that influence many genes within an entire chromosome (Jaenisch and Bird, 2003). Hypermethylation of CGIs are usually associated with transcriptional repression such as silencing of developmentally regulated and imprinted genes, while actively transcribed genes tend to have a hypomethylated promoter (Reik, 2007). Therefore, DNA methylation provides a stable epigenetic effect on transcription regulation, which allows the transcriptional machinery to focus on genes that are essential for the maintenance of the normal physiological functions of the cell.

DNA methylation is used by the cell to guard gene expression and chromosomal stability. In diseases like cancer, alterations in global and regional methylation patterns happen during early development of the pathogenesis such as the promoter hypermethylation at tumour suppressor genes (Egger et al., 2004). In breast cancers, genes inactivated by methylation largely fall into several categories including cell cycle regulation, steroid receptor signalling, cancer cell viability, cell adhesion and invasion process (Yang et al., 2001b). For instance, the cyclin dependent kinase inhibitor, $p16^{INK4a}$, is a tumour suppressor gene that regulates the transition from G1

to S phase. Hypermethylation is frequently detected at the 5' promoter and the first exon site in both breast cancer cell lines and primary breast cancers (Herman et al., 1995, Woodcock et al., 1999). The classic example for promoter methylation of a steroid receptor is ERα (Lapidus et al., 1996). ERα level is an important marker for endocrine therapy. Most ER-positive breast cancer lines as well as normal breast tissue are unmethylated at ERα CGIs. In contrast, most ER-negative cancer cell lines have reduced ERα expression caused by promoter methylation, and are resistant to endocrine therapy (Lapidus et al., 1998). Furthermore, the expression level of DNMTs, especially DNMT3b, is elevated in ER-negative cancer cell lines and is significant correlated with ERα negativity in breast cancer patients (Girault et al., 2003). Treating both DNMT and HDAC inhibitors to ER-negative breast cancer cell lines can trigger re-expression of ERα mRNA and protein, suggesting an epigenetic regulation of ERα transcription in ER-negative breast cancer (Yang et al., 2001a). However, it is still not very clear whether DNA methylation is the driving force of gene silencing or a secondary effect recruited by the initial trigger.

1.3.3 Oestrogen dependent ERα mediated transcription

As discussed above, chromatin is generally packed in state that is inhibitory to transcription. Therefore, a variety of transcriptional co-factors are recruited by liganded ERα to de-condense the chromatin at the TSS in the oestrogen mediated transcriptional activation. They work together to set an appropriate chromatin environment for the transcription initiation. These co-factors includes co-activators complexes that harbours HAT and HMT activity, chromatin remodelling complexes such as the ATP-dependent nucleosome remodelling complex, the TRAP/DRIP/ARC complexes that facilitate the recruitment of RNAP II (**Figure 1.5**) (Rosenfeld and Glass, 2001).

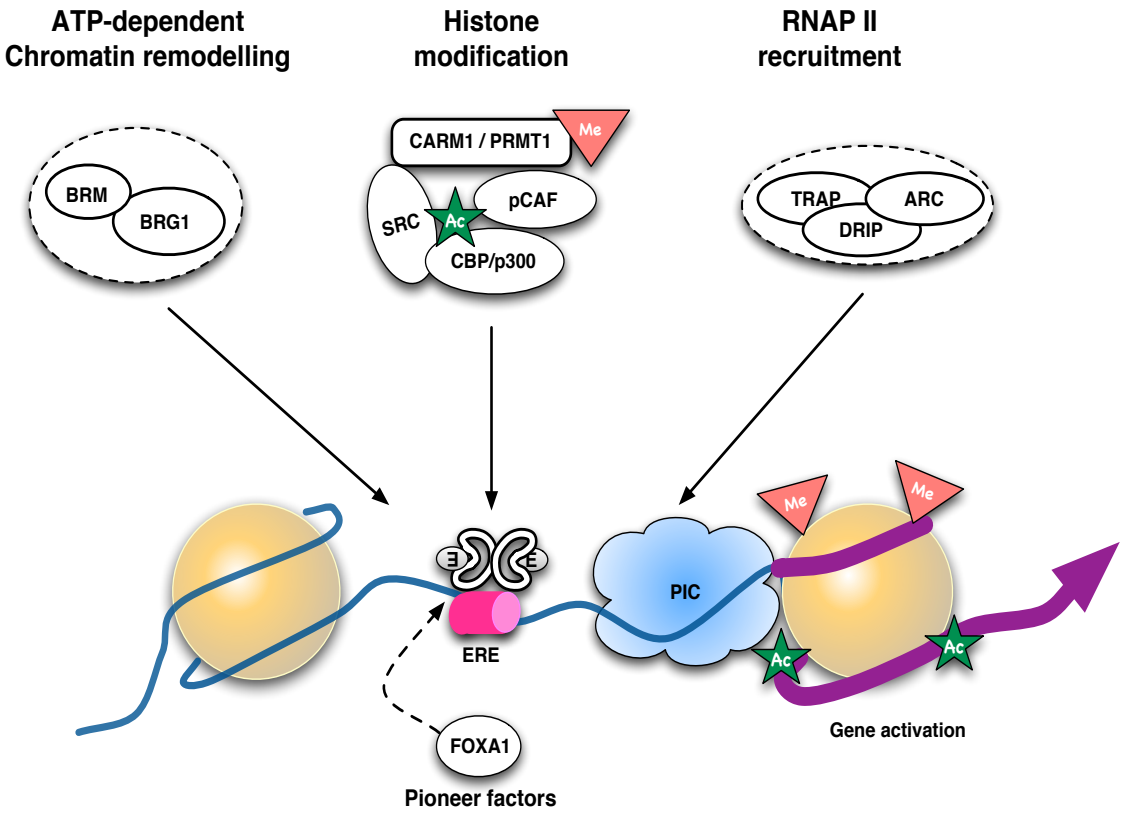


Figure 1.5 Oestrogen induced ERα dependent transcription is mediated by multiple co-factor complexes.

Co-factor complexes include ATP-dependent chromatin remodelling complex, HAT and HMT associated p160 protein family co-activator complex, and the TRAP/DRIP/ARC complex. Pioneer factors like FOXA1 contributes to ERα chromatin binding.

During the transcriptional activation of oestrogen responsive genes, the liganded ERα together with a number of co-factors dynamically bind to the chromatin in cycles of approximately 45 minutes (Metivier et al., 2003). At the begin of the oestrogen cycle, the binding of ERα can be directed by pioneer factors like FOXA1 (Laganiere et al., 2005). Upon binding of oestrogen, liganded ERα undergoes dimerisation and binds to the promoter of oestrogen responsive genes, like pS2. Subsequently, the binding of the receptor attracts multiple combinations of co-factor complexes to the promoter to initiate the transcription. For example, there is a network of 46 transcription factors in 6 different co-factor complexes presents on the

pS2 promoter during the oestrogen induced transcriptional activation (Metivier et al., 2003). Liganded ERα is required in all complexes but other co-factors are not always part of the complex. The HAT and HMT associated p160 protein family co-activator complex is one of the co-factors complexes recruited to the promoter area. Members of the p160 protein family act like adaptor proteins to recruit HAT like CBP/p300/pCAF and HMTs like CRAM1/PRMT1. CBP and p300 contain intrinsic HAT activity for lysine residues on primary histones as well as on histone2A and 2B subunits (Schiltz et al., 1999). Another ERα associated HAT is the p300/CBP-associated factor pCAF, which is acetylated by itself and by p300 (Santos-Rosa et al., 2003). The acetylation of histone results in the modification of chromatin and increases the access of the DNA to other components of transcription apparatus. Overexpression of p160 protein family as well as HATs has been reported in the literature. For example, AIB-1 overexpression is detected in both ER positive and ER negative breast cancer (Bautista et al., 1998, Bouras et al., 2001). Moreover, AIB-1 and HER overexpression are associated with tamoxifen resistance and poor survival rate in patients (Osborne et al., 2003). This can be due to the enhanced cyclin D1 expression promoted by AIB-1 binding (Planas-Silva et al., 2001). In the meantime, both p300 and CBP have found to be preferentially co-overexpressed in breast carcinomas with high tumour grade and in invasive ductal carcinomas (Hudelist et al., 2003). HMTs like co-activator-associated arginine methyltransferase 1 (CRAM1) and protein arginine methyltransferase 1 (PRMT1) have been identified to interact with ER co-activators and enhance its activity (Chen et al., 1999a, Wang et al., 2001). CARM1 preferentially methylated histone 3 and PRMT1 preferentially methylated histone 4, either in a bulk histone preparation or individual purified form. Further experiments show that PRMT1 methylates arginine 3 on histone 4 *in vivo*, and methylation at H4R3 facilitates subsequent acetylation of H4 tails by p300 (Strahl et al., 2001). Together, these histone modifications happen early on the pS2 promoter to assist the oestrogen-induced transcriptional activation (Wagner et al., 2006).

Another co-factor complex recruited by ERα is the ATP-dependent chromatin remodelling complex. Multiple proteins are involved in the complex including BRG1

and BRM (Kadam and Emerson, 2003). BRG1/BRM complex is essential for ERα activity, which interacts with the AF-2 domain of the ERα and facilitates chromatin remodelling in an ATP dependent manner (Ichinose et al., 1997, DiRenzo et al., 2000). BAF57 is another subunit of the ATP-dependent chromatin remodelling complex, which binds to the hinge region of ERα (Garcia-Pedrero et al., 2006).

The TRAP/DRIP/ARC complex is a multimeric mediator complex that supports ERα activity in a ligand dependent manner (Rosenfeld and Glass, 2001). The complex is recruited to the nuclear receptor via TRAP220 or DRIP/ARC subunit (Ito et al., 1999, Rachez et al., 1999). Other factors within the protein complex function to interact GTFs and co-activate target gene transcription (Fondell et al., 1999, Naar et al., 1999).

1.4 Current therapies in Breast cancer and acquired endocrine resistance

Before the discovery of intracellular oestrogen receptors demonstrating that breast cancers are hormone-dependent for growth, surgery was the only effective means of treating the disease. Nowadays, apart from the primary surgical treatment, patients with ER positive breast cancers are often treated with drugs that block the activity of ERα as adjuvant endocrine therapies to prevent recurrence of the cancer. Anti-ERα drugs can be broadly divided into two classes: drugs that block the activity of the receptor and drugs that block ligand synthesis (**Figure 1.6**). As ER negative breast cancer can be sub-divided to basal-like and HER2 overexpression according to the molecular profile, different treatments are used for patients belong to each ER negative subtype.

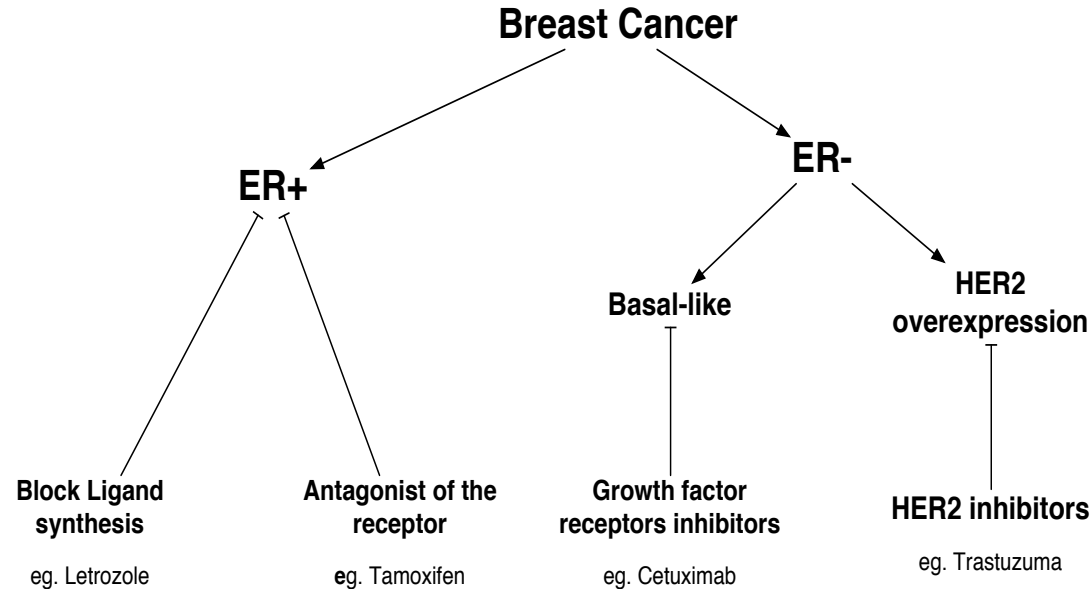


Figure 1.6 Different therapies for ER positive and negative breast cancer.

Two classes of drugs are used to block the activity of ERα mediated oestrogen signalling in ER positive breast cancer patients. ERα antagonist like tamoxifen is used to inhibit the function of the receptor, and aromatase inhibitors like letrozole is used block the synthesis of oestrogen. Different treatment strategies are employed for patients with ER negative diseases with distinctive molecular subtypes. Patient with HER2 overexpression are generally treated with HER2 inhibitors, like trastuzuma. Growth factor receptors inhibitors like EGFR inhibitor (Cetuximab) have been proposed as a potential treatment for the triple negative basal-like breast.

1.4.1 ERα Antagonists

Tamoxifen is one of the most widely used endocrine treatments for breast cancer patients. Clinical studies from patients with five years tamoxifen treatment show that tamoxifen reduces the risk of recurrence by 47% and reduces the risk of death by 26% (Group, 1998). It is a chemically diverse compound that lacks the steroid structure of oestrogen but has a tertiary structure that allows it to bind to ERα (**Figure 1.7**). Tamoxifen competes with oestrogen for binding at the AF-2 region of the receptor. As tamoxifen only inhibits the transcriptional activity of ERα through the AF-2, it may enable transcription through AF-1. After binding of the tamoxifen, ERα is locked in a position that not only represses the binding of co-activators complex but also promotes the binding of co-repressors complex (detailed in **section 1.2.2**). Thus, it serves as an antagonist of the ER in breast tissue. However, in other tissue like bone and endometrium, it can function as an agonist through binding of unknown co-activators (Riggs and Hartmann, 2003). Therefore, tamoxifen is often referred as a selective oestrogen receptor modulator (SERM). The agonist effect on the endometrium puts treated patients in high risk of developing endometrial cancer. In fact, tamoxifen treatment is associated with a 2.53 fold increase in endometrial carcinoma in elderly women (Fisher et al., 1998). The aromatase inhibitors are an alternative to tamoxifen for treating ER positive breast cancer patients and, unlike tamoxifen, are not associated with an increased risk of endometrial cancer and other side effects of tamoxifen like thromboembolic disease and vaginal bleeding (Goss and Strasser, 2001). However, aromatase inhibitors elevate the rate of bone lost in postmenopausal women (Heshmati et al., 2002).

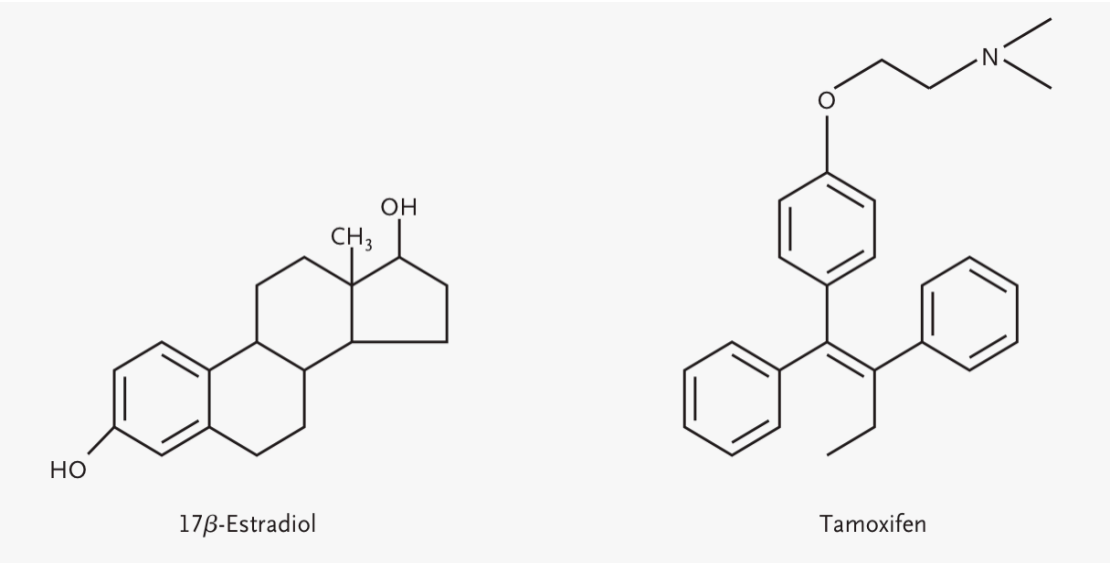


Figure 1.7 Chemical structures of 17 β-oestradiol and tamoxifen.

Picture taken from reference, Riggs and Hartmann, 2003.

Unlike tamoxifen, ICI 182,780 and ICI 164,384 are considered as pure ERα antagonists devoid of any agonist activities (Howell et al., 2000), as they prevent the transcriptional activation of the receptor from both AF1 and AF2 (Ali and Coombes, 2002). Structurally, both drugs are analogues of oestradiol, but ICI 182,780 is far more potent than ICI 164,384 (Wakeling and Bowler, 1992). Like tamoxifen, ICI 182,780 also promotes the binding of co-repressor to the receptor (Webb et al., 2003). Meanwhile, the pure ERα antagonist has been shown to block receptor dimerisation preventing DNA binding of the receptor (Fawell et al., 1990). It can also disrupt the ER shuttling between nucleus and cytosol (Dauvois et al., 1993). Using mouse ER, Dauvois et al showed that ICI 182,780 will cause the receptors to accumulate in the cytosol, while the partial antagonist of ERα, tamoxifen, retains the receptors in the nucleus. Moreover, ICI 164,384 can increase the ER turnover (Dauvois et al., 1992). These pure ERα antagonists are recommended for treatment of metastatic ER positive breast cancer, particular for patients who have relapsed on tamoxifen treatment (Howell et al., 1996). The effectiveness of pure ERα antagonists on tamoxifen resistant patients suggests a lack of cross-resistance between drugs. Furthermore, since these pure ERα antagonists do not have any agonist effect it has not been associated with an increase in endometrial cancer (Addo et al., 2002).

Collectively, these results suggest that pure ERα antagonists can be good second line treatment for patients with advanced breast cancers.

Despite the success of these ERα antagonists in treating ER positive breast cancer, acquired endocrine resistance is always an area of concern. A trial conducted in 1996 demonstrates that women who continued to receive tamoxifen after five years of treatment had worse outcomes than women in whom it was discontinued at five years (Fisher et al., 2001, Fisher et al., 1996). The development of resistance towards tamoxifen can be due to it agonist activity through the AF-1 region of ERα, which may become exaggerated over time. Resistance to pure ERα antagonist, ICI 182,780, has also been reported in cell line model (Brunner et al., 1997). Resistant cell line shows an increased dependence on EGFR mediated MAPK pathway for growth (McClelland et al., 2001). In general, resistance to endocrine therapy could result from genetic and epigenetic changes within the primary tumour that activate other pathways of ERα such as the ligand independent pathway.

1.4.2 Aromatase Inhibitors

The use of aromatase inhibitor is limited in premenopausal women, as it leads to incomplete oestrogen suppression and ovarian stimulation (Pritts, 2010). In post-menopausal women, local oestrogen synthesis relies on aromatase. Drugs that reduce peripheral oestrogen synthesis by inhibiting aromatase are frequently used in post-menopausal women with ER positive breast cancer (Goss et al., 2003). Aromatase inhibitors reduce the level of oestrogen by inhibiting or inactivating aromatase, which is a key enzyme in oestrogen biosynthesis. Aromatase is a member of the cytochrome p450 superfamily that catalyses the conversion from androgen to oestrogen. Aromatase inhibitors are generally classified as Type 1 (steroidal) or Type 2 (non-steroidal) inhibitors. Type 1 aromatase inhibitors are also known as inactivators as they bind irreversibly to the enzyme. In contrast, type 2 inhibitors bind reversibly to the enzyme (Smith and Dowsett, 2003). Letrozole and anastrozole are both type 2, third-generation aromatase inhibitors that were developed in the early 1990s with enhanced specificity and potency (Bhatnagar, 2007). Multiple clinical trials have evaluated the effectiveness of both drugs as a second line

treatment after tamoxifen and as a first line adjuvant treatment for breast cancer patients (Goss et al., 2003, Coates et al., 2007, Forbes et al., 2008, Mouridsen et al., 2009, Litton et al., 2012). Overall, all studies have shown aromatase inhibitors with excellent activity in enhancing disease-free survival and preventing of recurrence, especially when compared to tamoxifen. In cases of using them as neoadjuvant treatment (before surgery to reduce the tumour size), letrozole achieved a significantly higher tumour response rate than tamoxifen (Eiermann et al., 2001). Furthermore, studies done with MCF-7 breast cancer xenograft model reveal that oestrogen deprivation is more efficient in induction of apoptosis and inhibition of proliferation than tamoxifen (Johnston and Dowsett, 2003).

Despite the advantages of aromatase inhibitors, acquired endocrine resistance will also happen with the treatment. Several mechanisms have been proposed for the development of the resistance such as oestrogen hypersensitivity and activation of growth factor receptors. An enhanced sensitivity to oestrogen (oestrogen hypersensitivity) has been used to describe the aromatase inhibitor sensitivity in premenopausal women who relapse after oestrogen withdrawal after ovarian ablation (Santen et al., 1990). Long-term oestrogen deprived (LTED) cells is a model system derived from MCF-7 in a low oestrogen environment (Masamura et al., 1995). In using this cell model, the authors found that breast cancer cells can adapt to low oestrogen environment by up-regulating ERα expression to maximise its activity (Santen et al., 2004). As ERα remains functional after the treatment of aromatase inhibitor, ligand independent and non-genomic pathway may play important role in resistance to aromatase inhibitors. Evidence to support this is provided by activated MAPK pathway together with HER2 and insulin like growth factor receptor (IGFR) signalling in LTED cells (Jeng et al., 2000, Stephen et al., 2001).

1.4.3 Treatments for ER negative breast cancers

1.4.3.1 HER2 overexpression

HER2 overexpression is usually caused by gene amplification (Pauletti et al., 1996). It is associated with lack of response to endocrine treatments and reduced survival in breast cancer patients (Borg et al., 1994, Sjogren et al., 1998). HER2 is a RTK that

can be inhibited by monoclonal antibodies targeted to the extracellular domain and kinase inhibitors target the intracellular domain. HER2 overexpression tumours are mainly treated with monoclonal antibodies like trastuzumab and pertuzumab. Trastuzumab (herceptin) is a humanised anti-HER2 monoclonal antibody that was approved by FDA to treat HER2 overexpressing breast cancer (Hudis, 2007). It exhibits its anti-tumour activity by antibody dependent cell-mediated cytotoxicity, dis-regulation of receptor turnover or inhibition of receptor signalling due to impaired dimerisation (Valabrega et al., 2007). Pertuzumab is another HER2 targeting monoclonal antibody in Phase II clinical trail (Gianni et al., 2010). As it binds to different positions on the receptor, it is used among women refractory to trastuzumab treatment (Baselga et al., 2010).

1.4.3.2 Basal-like

Basal-like breast cancers often have a triple-negative (ER-, PR-, HER2-) phenotype. As a result, they cannot be managed effectively with existing endocrine treatments and HER inhibitors, and are usually associated with a poor outcome (Banerjee et al., 2006). Efforts on looking for basal-like gene signature and biomarkers for immunohistochemistry (IHC) have revealed several potential targets for treating basal-like breast cancer. EGFR is expressed in more than 60% of basal-like breast cancer, and is therefore used together with cytokeratin (CK) 5/6 as biomarkers for identifying this breast cancer type (Nielsen et al., 2004). In the meantime, the activation of EGFR signalling and its downstream targets have been mentioned repetitively as causes of acquired endocrine resistance in ER positive breast cancer, therefore EGFR inhibitors could be effective for both treating basal-like breast cancers and preventing the occurrence of endocrine resistance.

Gefitinib, tarceva and tyrphostin (AG1478) are all quinazoline based small molecule tyrosine kinase inhibitors that reversibly inhibit the kinase activity of EGFR by competing with the binding of ATP (Dowell et al., 2005, Ellis et al., 2006). In contrast, CI-1033 is a pan inhibitor of EGF receptors, which is able to irreversibly inhibit the kinase activity of intracellular domain (Hynes and Lane, 2005). All EGFR

inhibitors have been shown to influence a variety of cellular processes that lead to cell cycle arrest, reduced proliferation and elevated apoptosis, etc. (Ciardiello and Tortora, 2008). EGFR inhibitors alone achieve little effects in cancer therapy, however, when used in combination with HER2 inhibitors produces more pronounced growth inhibitory effect (Friess et al., 2005, Normanno et al., 2002). Cetuximab is a human-mouse chimeric monoclonal antibody of EGFR. A phase II trial is undergoing to assess the effectiveness of cetuximab together with paclitaxel in treating basal-like breast cancer (http://clinicaltrials.gov/ct2/show/study/NCT00353717).

1.5 Aim of the thesis

ER positive breast cancers account for the majority of breast cancer incidence worldwide. Drugs like tamoxifen and aromatase inhibitors are effective treatments to manage the disease initially. However, the subsequently development of acquired endocrine resistance remains one of the biggest challenge in breast cancer therapy. Studies focussing on acquisition of tamoxifen resistance and resistance to oestrogen deprivation have all indicated an important role of growth factor mediated survival pathways such as the activation of EGFR and its downstream signalling pathways in resistant cancer cells. Therefore, the aim of this study is to investigate the molecular mechanism that links the oestrogen mediated ERα signalling and growth factor signalling pathways in endocrine resistant cells.

Most in vitro ER positive breast cancer models employed to date have been developed in the presence of serum growth factors. Furthermore, the so-called oestrogen deprived cell line models to mimic the effects of aromatase inhibitors are generally cells grown in a low oestrogen environment (phenol red-free and charcoal-stripped serum conditions) rather than a true oestrogen-free environment. Therefore, it is important to generate and characterise a new oestrogen deprived cell line model grown in the presence of defined growth factors. Using this new cell model, I want to investigate i) the importance of different signalling pathways for breast cancer cells survival, ii) factors that control the cross-talks between different pathways, and iii) epigenetic mechanisms that maintain the transition between different pathways.

Chapter 2 Material and Methods

2.1 Materials

Materials are listed by technique. All chemicals are purchased from Sigma-Aldrich unless otherwise stated. All laboratory plastics are from TPP (Techno Plastic Products AG) unless otherwise stated. Primers are all ordered from Invitrogen Life Technologies.

2.2 Tissue culture

2.2.1 Cell lines

T47D and ZR75 cells were kindly donated by Dr. Arkadiusz Welman (Edinburgh Cancer Research Centre, Edinburgh). The parental MCF-7 and HEK293T cells were obtained from the European Tissue Culture Collection. DH and DHe cells were derived from MCF-7 cells by gradually replacing the normal growth medium with a defined serum free medium. Details of the derivation are described in Chapter 3, **section 3.1**.

Table2.1 HMM medium for DH/DHe cells

	Concentration		*Concentration*
DMEM/F12	1x	Hydrocortisone	0.5µg/ml
HEPES	10mM	Transferrin	5µg/ml
Penicillin/Streptomycin	10µg/ml	Isoproterenol	0.1mM
EGF	5ng/ml	Ethanolamine	1mM
Insulin	10µg/ml	O-Phosphoethanolamine	1mM
Oestrogen	1nM	B27 supplement	1x

Table 2.2 Composition of B27 Medium Supplement			
Chemicals			*Proteins*
Biotin	Glutathione (reduced)	Retinyl acetate	Albumin, bovine
L-Carnitine	Linoleic acid	Selenium	Catalase
Corticosterone	Linolenic acid	T3 (triodo-l-thyronine)	Insulin
Ethanolamine	Progesterone	DL-α-tocopherol (vitamin E)	Superoxide dismutase
D(+)-glactose	Putrescine	DL-α-tocopherol acetate	Transferrin

2.2.2 Tissue culture and reagents

T47D, ZR75 and HEK293T cells were cultured in DMEM (Dulbecco's modified eagle medium, Gibco, Invitrogen). This was supplemented with 10% heat-inactivated foetal calf serum (FCS, Harlan Sera-Lab) and 1% penicillin and streptomycin (Gibco, Invitrogen). Stable DH cells were cultured in human mammosphere medium+ (HMM+), adapted from (Duss et al., 2007). HMM+ medium composition is detailed in **Table 2.1**. One of the components, B27, is a serum-free medium supplement containing antioxidants, vitamins, growth factors and hormones including progesterone. Detailed composition of this supplement can be found in **Table 2.2** (Brewer et al., 1993). DH and DHe cells poorly adhered to the tissue culture plastics when cultured in 100% serum free medium, therefore plates and flasks were coated with collagen (3~4 mg/ml rat tail collagen, BD Bioscience) before use, see below. All cell lines were maintained in a humidified atmosphere at 37^{o}C and 5% CO_2.

2.2.3 Collagen coating of tissue culture dishes

Collagen purchased from BD Bioscience was diluted 1/1000 in 0.02 M acetic acid. Tissue culture dishes were coated with 1-10 ml of diluted collagen solution depending on size. Dishes were incubated at 37^{o}C for an hour or 4^{o}C overnight followed by two washes with pre-warmed PBS.

2.2.4 Cell harvesting and counting

Cells were washed in phosphate buffered saline (PBS, pH 7.3) to ensure all media was removed. Cells were detached by adding an appropriate volume of 1 x Trypsin-EDTA (Invitrogen) and incubating at 37^{o}C for 3~5 minutes. Trypsin was inactivated by adding growth media and the cell suspension was centrifuged at 200 g for 4 minutes. The cell pellet was then resuspended in growth media. HyQTase (HyClone, Thermo Scientific) rather than trypsin was used for DH and DHe cells as they are less well adhered. However, HyQTase cannot be inactivated by serum so an extra PBS wash was required after pelleting down the cells. Cell pellets were resuspended in an appropriate volume of growth media and counted using a haemocytometer. Cells were then diluted to achieve the desired cell number for each experiment.

2.2.5 Cryopreservation and liquid nitrogen cell recovery

Cells to be stored in liquid nitrogen were harvested as described above and a T75-flask of cells were typically resuspended in 3 ml of freezing mix (10% DMSO (dimethyl sulphoxide) in foetal calf serum). 1ml of cell suspension was transferred to a cryovial (Nuncleon, Fisher Scientific) and frozen immediately at -80^{o}C before being transferred to liquid nitrogen after ~24 hours.

Cells removed from liquid nitrogen were rapidly defrosted in a 37^{o}C waterbath for a minute then resuspended in prewarmed growth media. Cells were pelleted by centrifugation at 200 g for 4 minutes, resuspended in culture media and plated as required.

2.2.6 Transfection and selection of stable cell lines

Cells were transfected either by Lipofectamine 2000 (Invitrogen, Life technologies) or electroporation using Amaxa Necleofector (Lonza). For Lipofectamine, cells were freshly plated in a 6-well plate 24 hours before the transfection. 5 µl of Lipofectamine and 1~2 µg of plasmid DNA or shRNA construct were diluted separately in 100 ml of Opti-MEM for 5 minutes at room temperature (Gibco,

Invitrogen). The two solutions were mixed together for another 10 minutes at room temperature. The mixture was then added dropwise to the cells and incubated for 24 hours. Cells were then either lysed to extract protein and RNA samples or transferred into a 100 mm dish with selective media. Cells were closely monitored during the selection process. For electroporation at least 1 million cells were required for each reaction. Cell pellet together with 1~2 μg of plasmid DNA was resuspended in 100 μl of Nucleofector solution. An appropriate Nucleofector program was selected for each cell line. After transfection, cells were transferred into pre-warmed media. Selective media was added 24 hours after transfection.

2.2.7 RNAi

RNAi experiments were carried out on cells at low confluence as this gives a greater knockdown effect (10~30% confluence). ON-TARGETplus SMARTpool siRNAs purchased from Thermo Scientific were used for all siRNA experiments (**Table 2.3**) and siRNAs were transfected into cells with Oligofectamine (Invitrogen, Life technologies). Oligofectamine and 100 nM siRNA were diluted in Opti-MEM in two separate tubes and incubated at room temperature for 5 minutes, the contents of both tubes were then combined and mixed together by gentle tapping followed by incubation for a further 15 minutes in a 37^{o}C waterbath. Cells to be transfected were washed with PBS and replaced with Opti-MEM. The siRNA transfection mix was added dropwise to cells and incubated for 4 hours after which time normal growth media with desired serum concentration was added back to the cells. In order to achieve a better knockdown, cells were usually transfected with freshly prepared siRNA again 48 hours later prior to protein and RNA extraction or further experiments.

Table 2.3 Target sequence for siRNA	
Gene	*Target sequence*
Human c-Myb	CCGAAACGUUGGUCUGUUA
	CAGUCAAGCUCGUAAAUAC
	CCAAUUAUCUCCCGAAUCG
	UCCAUACCCUGUAGCGUUA
Human ESR1	GCCAGCAGGUGCCCUACUA
	GAUGAAAGGUGGGAUACGA
	GAAUGUGCCUGGCUAGAGA
	GAUCAAACGCUCUAAGAAG

2.3 Cell assays

2.3.1 Growth assays

2.3.1.1 SRB assay

Sulphorhodamine B (SRB) assay was used to test the proliferation and chemosensitivity of cells. 1000 DH cells or 800 MCF-7 cells were seeded in a 96-well tissue culture plate. Cells were left overnight in the incubator to allow attachment to the plate, at which point day 0 plates were fixed. For growth assays, the media was then supplemented with specific drugs (**Table 2.4**) and incubated for a specific number of days depending on the experiment, prior to cells being fixed by adding 50 µl ice-cold 50% TCA to the 200 µl culture media to give a final TCA concentration of 12.5%. The plates were left at 4 ^{o}C for an hour before washing 10 times with water. The plates were then left to dry. Cells were stained with 50 µl SRB dye (0.4% w/v SRB in 1% v/v acetic acid (Fisher Scientific)) for 1 hour at room temperature before being washed 4 times with 1% v/v acetic acid and dried at 37^{o}C overnight. SRB stain was solubilised in 150 µl 10 mM Tris pH 10.5 for 1 hour at room temperature with shaking. The optical density (OD) was measured at 540 nm on a plate reader (Biohit). Cell number at each time-point was calculated as an average of six wells. Statistically significant differential growth was assessed by unpaired two-tailed Student's T-test using a p-value of significance < 0.05.

Table 2.4 Drugs used in Cell Assays		
Drug	*Function*	*Concentration*
Tamoxifen	ERα antagonist	1 μM
ICI 182,780	ERα antagonist	0.1 μM
AG1478	Blocks EGFR phosphorylation	10μM
UO126	MEK1 and MEK2 inhibitor	10μM
LY294002	P13 Kinase inhibitor	10 μM
TSA	HDAC Inhibitor	100nM

2.3.1.2 Colony formation assay

Methylene blue colony formation assay was used to assess the colony forming ability of cultures grown under control and test conditions. 10,000 cells were plated in a 6-well plate and allowed to attach for 24 hours before drug addition. Fresh media containing drugs was replaced every 48 hours. After 10 days colonies were stained in 2% methylene blue dissolved in 50% ethanol for 10 minutes at room temperature and rinsed with water and air dried. Plates were pictured using a Canon 350D with a 20mm fixed lens.

2.3.2 Apoptosis assay

PE Annexin V Apoptosis Detection Kit I (BD Bioscience) was used to assay apoptotic cells according to manufacturer's instructions. Briefly, a million cells were resuspended in 1 ml 1 x binding buffer. 100 μl of the cell suspension was transferred into a fresh tube with 5 μl of PE Annexin V and 7-AAD, respectively. The solution was incubated in the dark for 15 minutes at room temperature. 400 μl of 1 x binding buffer was added to each sample before analyzing with flow cytometry. PE Annexin V positive staining is used to look at cells that are actively undergoing apoptosis. It relies on the property of cells to lose membrane integrity in the early stages of apoptosis, while the nuclear acid dye 7-AAD staining allows the identification of cells that are in late stage apoptosis or already dead. Therefore, cells that are in early

apoptosis are PE Annexin V positive and 7-AAD negative and cells that are in late apoptosis are positive for both.

2.4 Protein Analysis

2.4.1 Protein extraction

In experiments where cell numbers were equal, cells were washed in PBS before being lysed with 1 x SDS loading buffer (50 mM Tris pH 6.8, 1% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol (Fisher Scientific)). Cells were then scraped from tissue culture dishes into eppendorf tubes using cell scrapers. Lysates were sonicated briefly on ice (5 seconds at 5Aμ (Soniprep 150, MSE) and denatured at 100^{o}C for 3 minutes and the debris cleared by centrifugation at 16,000 g for 5 minutes.

In experiments where cell numbers varied e.g. timecourse experiments, cells were lysed with RIPA buffer on ice for 10 minutes (25 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 1% NaDoc, 0.1% SDS, 250 μM PMSF). Protein concentration was measured by Precision Red Advanced Protein Assay kit (Cytoskeleton, Inc). An appropriate amount of 5 x SDS loading buffer and water was added to RIPA buffer lysed samples to achieve desired protein concentrations. Lysates were sonicated on ice (5 seconds at 5Aμ) and denatured at 100^{o}C for 3 minutes followed by centrifugation at 16,000 g for 5 minutes.

2.4.2 Acrylamide gel electrophoresis

Protein lysates were run on 10% Bis-Tris acrylamide gels (1 x Bis-Tris pH 6.7, 10% acrylamide (19:1, Severn Biotech Ltd.), 0.1% APS, TEMED) in MOPS running buffer (50 mM MOPS, 50 mM Tris (Melford), 1 mM EDTA and 0.1% SDS) at 150V for 1~2 hours depending on the size of the target protein. Protein integrity was analysed by Coomassie blue staining the gel (10% acetic acid, 45% methanol (VWR) and 0.3 mM Coomassie brilliant blue) at room temperature for 1 hour before being destained (10% acetic acid and 45% methanol) at room temperature overnight.

2.4.3 Western blotting

For subsequent antibody probing, protein gels were transferred onto PVDF (polyvinylidene fluoride) membrane (Millipore) using either wet transfer for large proteins (100V 2hrs in cold room) or semi-dry transfer for smaller histone proteins (100mA per gel for an hour at room temperature). The PVDF membrane was wet in the methanol first before washing either with wet (25 mM Tris, 192 mM glycine, 10% methanol, pH8.3) or semi-dry (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol, pH9.2) transfer buffer.

After the transfer, the membrane was blocked in 4% Marvel/TBST (137 mM NaCl, 2.7 mM KCl, 25 mM Tris, 0.1% Tween® 20 (Riedel-de Haën), pH7.4) for 1 hour at room temperature. Primary antibody was added at a suitable dilution (**Table 2.5**) in either 4% Marvel/TBST or 5% BSA (bovine serum albumin)/TBST depending on the working conditions of the primary antibody. The membrane was incubated with primary antibody at 4^{o}C overnight. The primary antibody was washed off by 3 x 5 minute washes in TBST and then incubated with anti-rabbit (Sigma, A0545) or anti-mouse (Thermo Scientific, 31444) secondary antibody conjugated to HRP (horseradish peroxidase) at a 1:5,000 dilution in 4% Marvel /TBST for 1 hour at room temperature. Membranes were then washed 3 x 5 minute washes in TBST before being incubated with SuperSignal® West Pico Chemiluminescent Substrate (ECL) (Thermo Scientific) for 3 minutes. Membranes were exposed to X-Ray film (Fuji) and developed.

To re-probe a membrane with a different primary antibody the first antibody signal was removed by firstly rinsing the membrane under water to wash away the ECL, followed by 1 x 15 minute wash in TBST. The membrane was then incubated in 100 mM glycine pH 2.5 at room temperature for 30 minutes. Membranes were then washed twice for 15 minutes each in TBST before being blocked in 4% Marvel /TBST for 1 hour at room temperature and then incubated with the new primary antibody.

Table 2.5 Primary Antibodies for western blot.		
Antibody	*Dilution factor*	*Supplier*
Total ERα	1:10,000	Santa Cruz (F10) # sc-8002
Phospho-ER Ser 167 Phospho-ER Ser 118	1:100 1:100	Cell Signalling Tech. #2514 Cell Signalling Tech. #2515
EGFR	1:1000	Dako #M7298
Phospho-EGFR	1:500	Abcam #ab5644
ERK1/2 (p44/p42)	1:1000	Cell Signalling Tech. #9102
Phospho-ERK1/2 (pp44/42)	1:1000	Cell Signalling Tech. #9101
c-Myb	1:1000	Millipore # 05-175
Total HER2	1:500	Cell Signalling Tech. #2242
Akt	1:1,000	Cell Signalling Tech. #9272
Phosphor-Akt Ser473	1:1,000	Dako #3628
GAPDH	1:1,000	Cell Signalling Tech. #2118
Pan H4	1:10,000	Millipore #05-858

2.4.4 Immunofluorescence (IF)

Cells seeded on glass slides were fixed by 4% paraformaldehyde (PFA) in PBS for 10 minutes at room temperature before being permeabilised by 0.2% Triton X-100 in PBS for 12 minutes. Slides were rinsed 3 times with PBS and blocked for 15 minutes at room temperature with PBS/5% horse serum. Primary antibodies diluted in PBS/5% horse serum were incubated overnight in a humidified chamber followed by secondary antibody incubation (anti-mouse or anti-rabbit FITC (1:150) or Texas Red (1:100)) for 1hour at room temperature. Slides were mounted in Vectashield and Dapi (0.5 μg/ml).

2.5 RNA Analysis

2.5.1 RNA extraction

Two different methods of RNA extraction were used depending on the size of tissue culture flask/plate used. For small (<T75) flasks/plates, RNA was purified using the Absolutely RNA Miniprep Kit (Stratagene #400800) following the manufacturer's protocol. In brief, cells were lysed with 350 μl of lysis buffer containing 2.5 μl of fresh β-Mercaptoethanol. The mixture was then sonicated very briefly (5 seconds at

5Aμ) to ensure the viscosity of the sample was low. The RNA and DNA mixture was isolated by centrifuging through a prefilter spin cup, and then the RNA purified through an RNA binding spin cup. On-column digest with DNase I (Roche, #04716728001) was performed on each sample for 15 minutes at 37^{o}C. RNA samples were washed and then eluted in 30 μl of elution buffer. For larger tissue culture flasks (T75 and T125) total RNA was extracted using Tri-reagent (Invitrogen). After homogenisation using a 25-gauge needle, the RNA was cleaned by standard phenol-chloroform procedure (**section 2.6.2**) and treated with DNase I. RNA was then ethanol precipitated and the pellet was washed with 80% ethanol and then resuspended in RNAse-free water. RNA was quantified using a Nanodrop spectrophotometer. A small aliquot of RNA was also run on a 2% formaldehyde gel (20 mM MOPS, 5 mM NaOAc, 1 mM EDTA, 1.2% agarose, 2% formaldehyde) in RNA running buffer (20 mM MOPS, 5 mM NaOAc, 1 mM EDTA, pH7.0) to check the integrity of the RNA.

2.5.1 Quantitative RT-PCR

QIAGEN one step RT-PCR was used to perform quantitative PCR from RNA samples. 100 ng of RNA was added to 7.5 μl of SYBR Green Master Mix, 0.375 μl primer mix (20 μM each primer), 0.15 μl RT-enzyme, and RNase-free water in a total reaction volume of 15 μl. All samples were run in triplicate. The quantitative PCR was run on a Rotor-Gene RG-3000 (Corbett Research) at 95^{o}C for 15 minutes, then 50 cycles of 94^{o}C for 15 seconds, 55~60^{o}C for 30 seconds (using specific annealing temperatures for different primer sets) and 72^{o}C for 30 seconds; followed by extension at 72^{o}C for 5 minutes. A standard curve was generated for each primer set using serial dilutions of cDNA. Relative expression values were normalized to β-actin. Primers were designed using Primer3 and are listed in **Table 2.6**.

Table 2.6 Primer sequences used in QRT-PCR		
Gene	*Forward primer*	*Reverse primer*
ERα	CCACCAACCAGTGCACCATT	GTCTTTCCGTATCCCACCTTTC
pS2	TTGTGGTTTTCCTGGTGTCA	CCGAGCTCTGGGACTAATCA
EGFR	TGCACTCAGAGAGCTCAGGA	CAGCGCTACCTTGT
HER2	CTGAATGGGTCGCTTTTGTT	CTCGTTGGAAGAGGAACAGC
HER3	CTCCTTTGTGCACAGTTCCA	GCTTTTGGCATTCACCTATG
HER4	CACCCAGACTACCTGCAGGA	GGAAATTGGAGCAGGTGTGT
IGFR	GTTGGGAAGGGGATCATTTT	ATGAAAACCATTGGCTGTG
b-Myb	GCCACTTCCCTAACCGCAC	CCCTTGACAAGGTCTGGATTCA
c-Myb	GCCAATTATCTCCCGAATCGA	ACCAACGTTTCGGACCGTA
β-actin	CTACGTCGCCCTGGACTTCGAGC	GATGGAGCCGCCGATCCACACGG
Cyclophilin A	GGCAAATGCTGGACCCAACACAAA	CTAGGCATGGGAGGGAACAAGGAA

2.5.2 Human whole genome expression array

RNA was extracted as described in **section 2.5.1**. RNA integrity (RIN) was assessed on an Agilent 2100 Bioanalyzer by Angie Fawkes at the Wellcome Trust Clinical Research Facility (WTCRF, Edinburgh). Only RNA with a RIN number >9 was reverse transcribed and biotin-labelled using the Illumina® TotalPrep RNA Amplification kit (Ambion) according to the manufacturer's instructions. Briefly, 500 ng of total RNA was reverse transcribed at 42°C to synthesize the first strand of cDNA followed by second strand synthesis at 16°C. The cDNA was then purified and in vitro transcribed to biotin labelled cRNA. The cRNA was purified and assessed again on an Agilent 2100 Bioanalyzer before array hybridisation. Array hybridisation was performed on the Human HT-12 Gene Expression Bead Chip (Illumina®) by Angie Fawkes at WTCRF, Edinburgh. Raw expression results were analyzed by Andy H. Sims (Breakthrough Research Unit, Edinburgh Cancer Research Center). Normalized data were transferred to an excel spreadsheet for further analysis.

2.6 DNA Cloning

2.6.1 DNA gel electrophoresis

DNA was run on agarose gels in 1 x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 90V for 1 hour. Genomic DNA was run on a 0.7% agarose gel and PCR products on 1 ~ 2.5% agarose gels depending on the product size.
DNA was visualised on gels by staining with ethidium bromide in 1 x TBE with gentle shaking at room temperature. Bands were visualised on an UV transilluminator (GelDoc-IT, BioImaging Systems).

2.6.2 Phenol-chloroform extraction

An equal volume of the buffered phenol chloroform (Invitrogen) was added to the DNA or RNA sample. After mixing the sample by vortexing, it was spun at 16,000 g for 3 minutes. The supernatant was transferred into a clean tube and mixed with an equal volume of chloroform. The mixture was vortexed and spun again at 16,000 g for 3minutes. The supernatant was transferred into a clean tube. 1/10 volume of 3M NaAc pH 5.2, 2 volume 100% ethanol and 1 µl glycogen were added to the supernatant to precipitate the DNA or RNA samples at -80^{o}C for 30 minutes. The sample was then spun down at 16,000 g for 10 minutes. The pellet was washed twice in 70 % ethanol (DNA sample) or 80% ethanol (RNA sample) before resuspending in TE buffer.

2.6.3 DNA gel extraction

After electrophoresis through an agarose gel the band of interest was visualized and excised using a clean scalpel on a UV light-box. DNA was purified by gel extraction using the MinElute Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. In brief, the gel was dissolved in buffer QG at 50 ^{o}C for 10 minutes. After the gel slice has dissolved completely, the mixture was applied to a MinElute column. The extracted DNA was washed and eluted 10 µl of elution buffer.

2.6.4 Plasmid DNA dephosphorylation

1μg of DNA was dephosphorylated with 10U calf intestinal phosphatase (New England Biolabs) at 37°C for 1 hour. DNA was then purified by gel extraction.

2.6.5 DNA ligation

DNA ligation was usually performed in a 10 μl reaction mix with 1 μl T4 DNA ligase (New England Biolabs) and 1 μl of 10x T4 DNA ligase buffer provide with the enzyme. The remaining volume was vector and insert DNA mixture at a 1:3 molar ratio. Sticky-end ligations were incubated at room temperature for 1 hour and blunt-end ligations at 16°C overnight.

2.6.6 Transformation into competent cells

Plasmids were transformed into competent *E. coli* cells (New England Biolabs) by heat shocking at 42°C for 30 seconds and then left on ice for 2 minutes. Cultures were grown in 250 μl SOC medium (New England Biolabs) at 37°C with shaking for 1 hour, before being plated on LB (Luria broth)-agar with ampicillin (100 μg/ml) overnight at 37°C.

2.6.7 Plasmid DNA extraction

Colonies were picked from agar plates using a sterile p200 pipette tip into 3 ml LB with ampicillin (100 μg/ml) and grown with shaking at 37°C overnight. Plasmid DNA was extracted using the PureLink Quick Plasmid Miniprep Kit (Invitrogen) or QIAfilter Plasmid Midi kit (QIAGEN) according to the manufacturer's instructions.

To provide a high, pure yield of DNA, expression vectors were maxi-prepped by cesium chloride (CsCl) gradient ultracentrifugation and ethanol precipitation. Briefly, a colony was picked and grown up in 1ml LB with ampicillin (100 μg/ml) by shaking at 37°C for ~6 hours. This inoculation was then added to 400 ml LB with ampicillin in a 2 L conical flask and grown in a shaker at 37°C overnight. Each 400 ml culture was split into two 200 ml bottles and cells pelleted at 5,000 rpm at 4°C for 10 minutes using a Sorvall® RC6 centrifuge (Thermo). The pellet was resuspended in

40 ml of buffer P1 (50 mM Tris pH8.0, 10 mM EDTA and 100 µg/ml RNase A (Invitrogen)), to which an equal amount of buffer P2 (200 mM NaOH and 1% SDS) was added and incubated at room temperature for 10 minutes. Next 40 ml of buffer P3 (3 M CH_3CO_2K pH5.5) was then added and incubated on ice for 15 minutes. After centrifuging at 10,000 rpm at 4^{o}C for 30 minutes, the supernatant was clarified by decanting through damp muslin. DNA was precipitated by adding two-thirds volume of isopropanol and incubating at room temperature for 1 hour. After centrifugation at 10,000 rpm at 4^{o}C for 10 minutes, the pellet was washed in 70% ethanol before being resuspended in 2.5 ml of TE. To perform CsCl extraction, 2.5 g CsCl was dissolved in the DNA by incubating in a water bath at 37^{o}C. 370 µl ethidium bromide was then added and the sample was centrifuged at maximum speed for 10 mins on a spin out rotor to remove any precipitate. The solution was transferred into an ultracentrifuge tube (Quick-seal® polyallomer bell-top centrifuge tube, Beckman), sealed and centrifuged at 80,000 rpm at room temperature for 24 hours in an Optima™ MAX-XP ultracentrifuge (Beckman Coulter®). The ultracentrifuge tube was pierced near the top to allow the pressure to escape before the dense red band containing the DNA was removed using a 25-gauge needle attached to a 1 ml syringe. Ethidium bromide was removed by progressive washing with water-saturated butanol until the aqueous layer was clear. DNA was then precipitated using phenol-chloroform extraction and then resuspended in TE.

2.6.8 Blue/white screening and sequencing

E.coli cells transformed with the pGEM-T® Easy vector (Promega, **Figure 2.1**) were plated on LB-agar with ampicillin (100µg/ml), IPTG (isopropyl-β-D-thiogalactoside, 0.5 mM) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 80µg/ml) overnight at 37^{o}C. Colonies containing PCR products appear white. At least 10 white colonies were picked from each plate using a sterile p200 pipette tip into 3 ml LB with ampicillin and incubated at 37^{o}C overnight with shaking. Plasmid DNA was purified from the bacteria using the PureLink Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's instructions. The DNA was then sent

for sequencing, using the T7 and/or Sp6 primer, by technical services at the MRC Human Genetics Unit (Edinburgh).

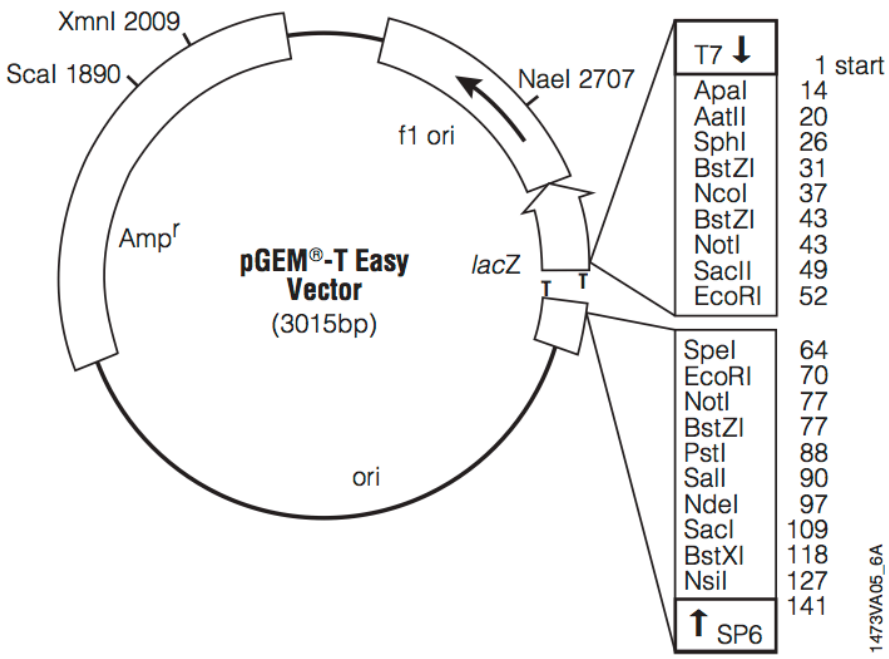


Figure 2.1 pGEM®-T Easy Vector (Promega) showing multiple cloning sites.

2.6.9 Cloning of human myb empty vector

The human myb cDNA was purchased from OriGene Technologies, Inc. The myb cDNA was inserted between the EcoRI and SalI restriction sites within the multiple cloning site of vector pCMV6-XL5. However, the SalI site was destroyed during the cloning. In order to create an empty vector control, the plasmid was digested with NotI to release a 3.2kb cDNA insert from the 4.5kb vector backbone as shown in **Figure 2.2**. The vector backbone was gel purified and re-ligated using T4 DNA ligase. The plasmid DNA was transformed into competent *E. coli* cells and purified using the QIAfilter Plasmid Midi kit (QIAGEN).

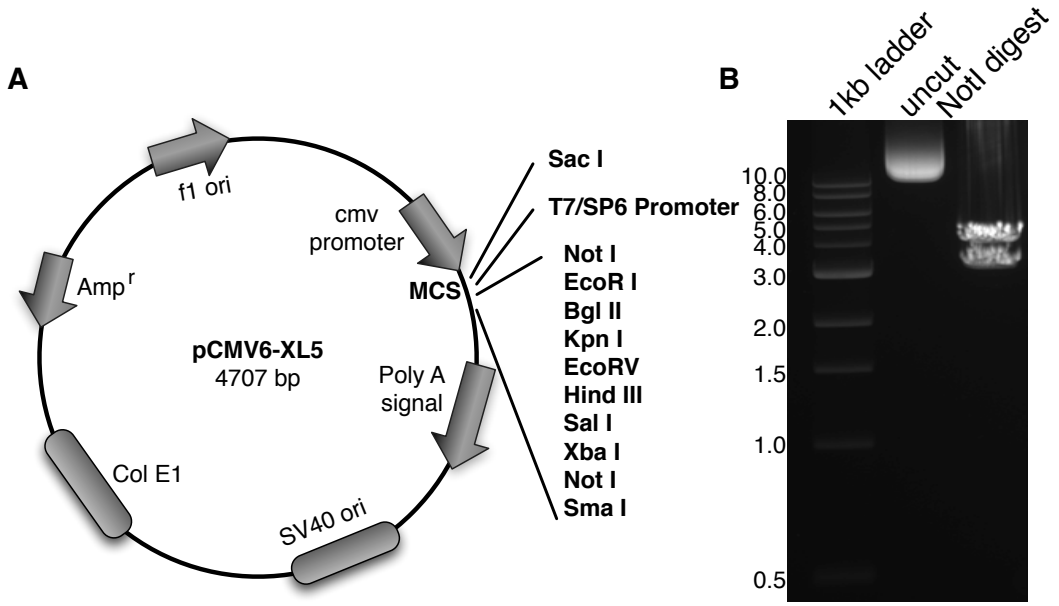


Figure 2.2 Cloning of human c-myb empty vector.

The map of the empty vector pCMV6-XL5 was shown (A). NotI digest of human myb cDNA was run on a 1% agarose gel with 1kb ladder and uncut cDNA (B).

2.7 DNA Analysis

2.7.1 Genomic DNA extraction

Cells were harvested and pelleted as described in **section 2.2.4**. The pellet was resuspended in PBS. An equal amount of 2 x genomic lysis buffer (300 mM NaCl, 20 mM EDTA pH 8.0, 1% SDS) was added to the cell suspension. After homogenisation using a 25-gauge needle, the mixture was incubated with 300µg proteinase K (Roche) at 37^{o}C overnight. DNA was then phenol-chloroform extracted, RNAse-treated with RNase A/T1 (Ambion) at 37^{o}C for 1 hour and then phenol-chloroform extracted again. DNA was then precipitated using ethanol and a one-tenth volume of 3 M sodium acetate, washed with 70% ethanol and then resuspended in 1 x TE (10 mM Tris pH8.0 and 1 mM EDTA). The genomic DNA was quantified using the Nanodrop spectrophotometer. The integrity of the genomic DNA was examined by electrophoresis on a 0.7% TBE gel. The gel was stained in

TBE buffer containing ethidium bromide at room temperature overnight. Bands were visualised on an UV transilluminator (GelDoc-IT, BioImaging Systems).

2.7.2 Bisulphite sequencing at EGFR promoter

Using the EZ DNA Methylation Gold Kit (Zymo Research) according to the manufacturer's instructions, 500 ng of genomic DNA was chemically deaminated by sodium bisulphite. This converts unmethylated cytosines to uracils whilst methylated cytosines remain unaltered. In brief, 20 µl (500 ng) of genomic DNA was mixed with 130 µl CT Conversion Reagent. The reaction mixture was incubated at the following temperature steps: 98 ^{o}C for 10 minutes, 64 ^{o}C for 2.5 hours, then hold at 4 ^{o}C. After the incubation, the DNA was desulphonated, washed and recovered in 10 µl of elution buffer.

Primers for bisulphite sequencing were designed using MethPrimer available at http://www.urogene.org/methprimer/index1.html. Gene promoters were amplified from bisulphite-treated DNA using the primers and conditions listed in **Table 2.7**. As shown in **Figure 2.3**, the EGFR promoter has a large region of CpG islands. Primers were designed at two regions to cover both the transcription start site and the translation start site of the promoter. PCR was carried out in a 20µl volume containing around 200ng DNA, 1 x PCR buffer with $MgCl_2$, 2mM dNTPs, 1µM primers and 1 unit Taq DNA Polymerase (Roche) for 94^{o}C for 5 minutes, then 40 cycles of 94^{o}C for 30 seconds, appropriate annealing temperature for 30 seconds and 72^{o}C for 45 seconds; followed by 72^{o}C for 5 minutes. Due to the size of the second amplicon (800 bp), nested PCR was used to get a clear product on the gel. This required taking 5µl of the PCR product obtained using bisulphite sequencing primer set 2 and re-amplifing it using primer set 3 to get a smaller product (643 bp). PCR products were analysed on a 2% agarose gel and the DNA was gel purified (**section 2.6.2)** and cloned into the pGEM-T® Easy Vector (**section 2.6.7**) and then transformed into competent *E. coli* cells (**section 2.6.5**). Colonies were selected through blue/white screening. Plasmid DNA was extracted from white colonies containing the PCR product and sequenced using the T7 and/or Sp6 primer by

technical services at the MRC Human Genetics Unit (Edinburgh). Sequencing results were analysed using BiQ Analyzer (Bock et al., 2005).

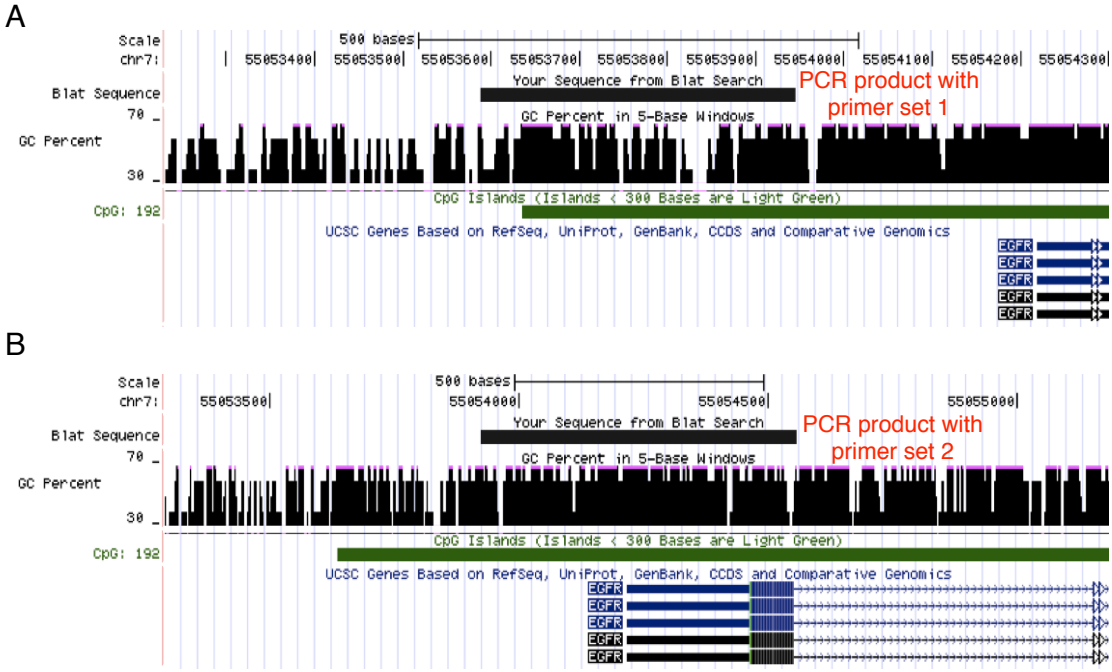


Figure 2.3 Primers designed for EGFR promoter bisulphite sequencing.

Figures were produced by UCSC browser. Relative position of the PCR product with bisulphite sequencing primer set 1 on EGFR promoter was shown in A, and PCR product with bisulphite sequencing primer set 2 was shown in B.

Table 2.7 Primer sequences for Bisulfite Sequencing at EGFR promoter

Set	*Forward primer*	*Reverse primer*	*Size*	*Mt*
1	GGTGTTTGATAAGATTTGAAGGATT	CAACACTACCCCTCTAAACC	356bp	55°C
2	TTGGATATAGGTTGGGTTTGTAAGT	ACTAATCTCAAAAAAACAAAAAAAA	800bp	50°C
3	GGG TTT AGA GGG GTA GTG TT	CCTTACCTTTCTTTTCCTCCAAAAC	643bp	50°C

2.8 Chromatin Analysis

2.8.1 Cross-linked ChIP

Chromatin immunoprecipitation (ChIP) is a technique for studying interactions of specific proteins with defined genomic regions. Here, it was used to determine whether a transcription factor interacts with a candidate target gene. Cells were grown to ~70% confluency in 10 cm dishes. Cells were cross-linked with 1% formaldehyde for 10 minutes at 37^{o}C and unreacted formaldehyde was quenched by gentle agitation at room temperature for 10 minutes with 0.125 M glycine. Cells were then washed twice with ice-cold PBS, collected into PBS containing protease inhibitors (Roche), and centrifuged for 4 minutes at 1000 g at 4°C. The pellets were resuspended in 200µl lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1), and 1× protease inhibitor cocktail), and duplicate plates were combined to give a total volume of 400µl. The chromatin was sheared by sonication on ice (12 x 20 seconds for MCF-7 cells; 16 x 20 seconds for DH cells) at 2Aµ (Soniprep 150, MSE) with 1-minute intervals to avoid overheating. Following centrifugation for 15 minutes at 8000 g and 4°C, supernatants were divided into 4 x 100 µl aliquots and only 100µl of each crossed-linked chromatin sample was used in an IP experiment.

To verify the DNA fragment size after sonication, 5 µl of 4M NaCl was added to one of the 100µl chromatin aliquots and incubated at 65^{o}C for 4 hours. The DNA was purified by phenol-chloroform extraction and ethanol precipitated. Following a 70% ethanol wash the DNA was resuspended in 50 µl of TE. Fragments were visualized by electrophoresis on a 1.5% TBE gel.

To proceed with the immunoprecipitation, 800 µl of dilution buffer (16.7 mM Tris pH 8.1, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton and 0.01% SDS) was added to the 100 µl chromatin sample, after which 30 µl was removed as the input. Protein-A agarose/magnetic beads (Millipore/NEB) were prepared by washing three times with 1ml dilution buffer. The chromatin mixture was firstly pre-cleared by incubation with 50 µl protein A beads, 1µg mouse/ rabbit IgG (Santa Cruz Biotechnology) and 2 µg salmon sperm DNA (Invitrogen) for at least 3 hours at 4^{o}C rotating at 15 rpm.

After centrifugation at 1000 g at 4°C for a minute, the supernatant was removed to a new tube containing freshly washed beads, 5 µl of primary antibody and 2 µg of salmon sperm DNA. This was then incubated overnight at 4°C with rotation of 15 rpm.

To recover the bound DNA, the supernatant containing unbound, non-specific DNA was removed and the beads were washed by rotation at 20 rpm for 5 minutes at 4°C with 1 ml each of TSE I (20 mM Tris pH 8.1, 2 mM EDTA, 150 mM NaCl, 1% Triton and 0.1% SDS), TSE II (20 mM Tris pH 8.1, 2 mM EDTA, 500 mM NaCl, 1% Triton and 0.1% SDS), Buffer III (10 mM Tris pH 8.1, 250 mM LiCl, 1 mM EDTA, 1% NP40 and 1% deoxycholate) and finally with TE twice. Bound chromatin was eluted in 250 µl elution buffer (100 mM $NaHCO_3$ and 1% SDS) by rotating at 15 rpm for 30 minutes at room temperature. This elution step was repeated with another 200 µl of elution buffer by rotating at 15 rpm for 15 minutes at room temperature. Samples and inputs were incubated with 0.2M NaCl for 6 hours at 65°C to reverse cross-linking. The DNA was then treated with proteinase K for 1 hour at 45°C, followed by QIAGEN PCR purification following the manufacturer's instructions. All samples were eluted in 30 µl of TE and 2 µl of the ChIP or input sample was used in a 15 µl QRT-PCR reaction containing: 7.5 µl FastStart Universal SYBR Green Master Mix (Roche), 0.9 µl primer (forward + reverse at 10 µM each) and 4.6 µl nuclease-free water. All samples were run in triplicate. The PCR was run on a Rotor-Gene RG-3000 (Corbett Research) at 95°C for 15 minutes, then 50 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds; followed by 72°C for 5 minutes. Data was normalised to the input DNA and the fold-enrichment of antibody-bound DNA versus IgG control was calculated.

Table 2.8 Primer sequences used in ChIP		
PCR target	*Forward primer*	*Reverse primer*
EGFR promoter set 1	GCACGGCGACCTCCTCAG	GGCACCGACGGGGAAACT
EGFR promoter set 2	GGTCCAGAGGGGCAGTGCT	CCCCGCGGGACCTAGTCT
EGFR promoter set 3	TGGCACAGATTTGGCTCGAC	GGTGCCCTGAGGAGTTAATT
Myb binding at EGFR	CTGTACAGCTGGTGGCAGTT	TGATGGGTTGATTCCCTTGT
pS2 promoter	GACGGAATGGGCTTCATGAGC	CTGAGACAATAATCTCCACTG
ERα binding at Myb	AAAGAGCGTGGGTGGAGAC	GCAGTCGGGTTTCTCTTCC

2.8.2 Native ChIP

Histones wrap around DNA to form nucleosomes, therefore, they are naturally linked. Native ChIP was used to analysis histone proteins and their modified forms at target regions, as it offers major advantages in terms of antibody specificity. Several histone histone modifications (H3K4me2, H3K9Ac, H3K27me3, H3K9me3) were examined in DH cells at different time points (**Figure 2.5**), as these histone modifications can occur with different kinetics during oestrogen treatment. A T75-flask of cells were harvested as described previously (**section 2.2.4**), and the pellet was resuspended in 4 ml of NB-A (85 mM KCl, 10 mM Tris pH7.6, 0.5 mM spermidine, 0.2 mM EDTA, 250 μM PMSF, 5.5% Sucrose). Equal volume of NB-B buffer (NB-A supplemented with 0.1% NP40) was added to the mixture to lyse the cells. The mixture was then incubated on ice for 3 minutes. Cell nuclei were collected by centrifugation, washed once with 10 ml of NB-R2 (85 mM KCl, 10 mM Tris pH7.6, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 250 μM PMSF, 5.5% Sucrose) and resuspended in 0.5 ml of NB-R2. The concentration of the nuclei was measured in sonication buffer (5M Urea, 2M NaCl). The nuclei were diluted to 10 A_{260} units/ml in NB-R2 buffer. In a 1 ml aliquot of the nuclei, 3 μl of RNaseA/T1 and 80 unit/ml micrococcal nuclease were added to the mixture for 10 minutes at room temperature. A small aliquot of the digest was purified using QAIGEN PCR purification kit following the manufacturer's protocol. Purified DNA was run on a 1% TBE gel to test the digest. The rest of the nuclei were spun down at 8000 g for 30 seconds to the first supernatant (SN1). The pellet was resuspended in 0.5 ml of $TEEP_5N$ (10mM

Tris pH7.5, 0.5mM EDTA, 0.5mM EGTA, 250μM PMSF, 0.05%NP40, 5mM NaCl) to release the chromatin overnight on ice.

The next day, the sample was spun at 16,000 g for 5 minutes to take the second supernatant (SN2). Again, the concentration of SN1 and SN2 was measured, and a small aliquot of each fraction was purified by QAIGEN PCR purification kit, and run on a 1% TBE gel to check the quality of the released chromatin. For each ChIP, 2.5 μg of SN1 and 2.5 μg of SN2 was diluted in 1ml of TEEP50N (10 mM Tris pH7.5, 0.5 mM EDTA, 0.5 mM EGTA, 250 μM PMSF, 0.5%NP40, 50 mM NaCl), and incubated with magnetic beads pre-bound with 5 μg of antibodies or IgG control at 4^{o}C overnight. 150 μl of the chromatin suspension was taken as input before addition of antibodies. Chromatin-bound magnetic beads were wash with ice-cold TEEP 140 (10 mM Tris pH7.5, 0.5 mM EDTA, 0.5 mM EGTA, 250μM PMSF, 0.5%NP40, 140 mM NaCl, 0.5% deoxycholate), TEEP 200 (10 mM Tris pH7.5, 0.5 mM EDTA, 0.5 mM EGTA, 250 μM PMSF, 0.5%NP40, 200 mM NaCl, 0.5% deoxycholate) and TE before eluting in 450 μl elution buffer (100 mM $NaHCO_3$ and 1% SDS) for 30 minutes at room temperature with rotation. The released chromatin fragments were treated with proteinase K at 45^{o}C overnight. The DNA was purified using a QAIGEN PCR purification kit according to manufacturers' instructions. The efficiency of the enrichment was tested by QRT-PCR as described previously with crosslinked ChIP (**Figure 2.4**). Samples were then amplified and hybridized onto a custom 4 x 72K Nimblegen arrays (Roche).

Table 2.9 Primary Antibodies for Cross-linked ChIP and Native ChIP.

Antibody	*Supplier*
Anti - Total ERα	Santa Cruz (F10) # sc-8002
Anti - c-Myb	Millipore # 05-175
Anti - Pan acetyl H4	Millipore # 05-858
Anti - H3K4 di-Methylation	Millipore # 07-030
Anti - H3K9 Acetylation	Abcam #Ab10812
Anti - H3K27 tri-Methylation	Millipore # 07-449
Anti - H3K9 tri-Methylation	Millipore # 07-442

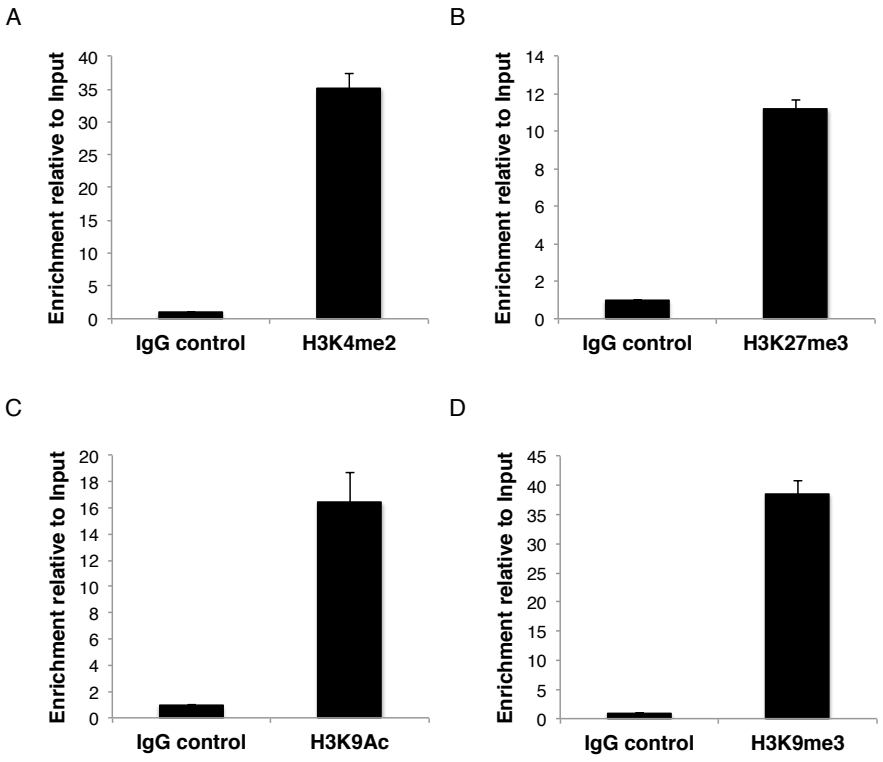


Figure 2.4 Native ChIP with different histone modification antibodies in DH cells.

The enrichment of H3K4me2 (A), H3K27me3 (B), H3K9Ac (C) and H3K9me3 (D) relative to IgG control at EGFR promoter (primers shown in **Table 2.8**) in DH cells were measured to test the efficiency of the native ChIP.

2.8.3 ChIP-on-chip

2.8.3.1 Design of the custom array for ChIP-on-chip

The 4 x 72K Nimblegen arrays were designed according to the results of expression array data obtained from DH cells at different time points as shown in **Figure 2.5**. Genes were grouped into several categories according to their change in expression in response to oestrogen over time. These categories were, Group 1: genes whose expression goes up/down on day 2 (early response genes), Group 2: genes whose expression goes up/down on day5 (later response genes), Group 3: genes whose expression stays up/down after oestrogen removal, Group 4: genes which had the most variable expression, and Group 5: genes whose expression remained

unchanged. Theses groups were then sorted by p value to find out the most significant changes. Data were also analysed using a pfp (probability of false prediction) test with a threshold value of 0.01 to look for genes that have a stepwise increased or decrease during the oestrogen treatment. At the same time, the stepwise increased or decrease was maintained after the removal of the oestrogen. A certain amount of genes were chosen from each group to create a list of 757 genes to put on the array. The genomic coordinates of these 757 genes were taken from the commercially available human 2.1M Deluxe Promoter array (Roche, #06532985001).

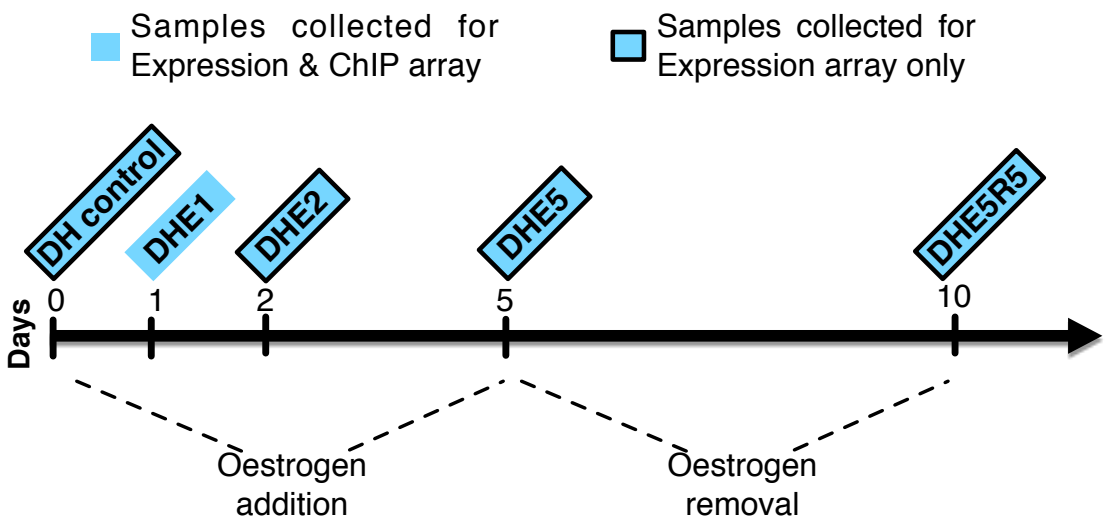


Figure 2.5 Time course experiments of oestrogen treatment in DH cells.

DH cells were treated with oestrogen for 5 days. Two biological samples were collected at day0 (DH control), day1 (DHE1), day2 (DHE2) and day5 (DHE5) for ChIP array to capture the kinetics of histone modifications during oestrogen treatment in DH cells. After 5 days of oestrogen treatment, oestrogen was washed away from the media. Two biological samples were collected again 5 days after the removal of the oestrogen (DHE5R5). Three biological samples were collected for expression array at four different time points, which were DH control, DHE2, DHE5, and DHE5R5.

2.8.3.2 Amplification, labelling and hybridisation of ChIP material onto custom array

The amplification, labelling and hybridisation of ChIP materials onto the custom arrays were done along with the help of Dr. Catherine Naughton. 10 µl of native sample was used as input material for each amplification reaction using the GenomePlex® whole genome amplification (WGA) kit (Sigma-Aldrich) according to manufacturers' instructions. In brief, 2 µl 1x library preparation buffer and 1 µl of library stabilisation buffer was added to the input material for 2 minutes at 95 ^{o}C before cooling on ice immediately. 1 µl of library preparation enzyme was then added to the reaction mix to incubate 16^{o}C for 20 minutes, 24^{o}C for 20 minutes, 37^{o}C for 20 minutes and 75^{o}C for 5 minutes. The mixture was chilled at 4^{o}C at the end of the library preparation. The sample was then amplified twice in a reaction mix of 7.5 µl of 10x amplification master mix, 5 µl of WGA DNA polymerase and 47.5 µl of nuclease-free water at 95^{o}C for 3 minutes, then 14 cycles of 94^{o}C for 15

seconds followed by 65°C for 5 minutes. The amplified materials were purified using the QIAGEN PCR purification kit and stored at -20 °C.

Labelling of amplified samples was performed using the CGH Labeling Kit for Oligo Arrays (ENZO) according to manufacturers' instructions. In brief, 500 ng of DNA together with 20 µl the Primers/Reaction buffer in a total volume of 39 µl was denatured at 98 °C for 10 minutes and placed on ice for 5 minutes to allow the annealing of random primers. 10 µl of the appropriate cyanine dye-labeled nucleotide mix together with 1 µl Klenow Exo-DNA polymerase was then added to the mixture to allow primers extension at 37 °C for 4 hours. The reaction was terminated with adding 5 µl of Stop buffer. The samples were then cleaned with QIAGEN PCR purification kit and stored at -20 °C.

Labelled samples were hybridised into the 4 x 72K Nimblegen arrays using the Nimblegen Hybridisation System (Roche) according to manufacturers' instructions. In brief, labelled DNA samples were dried in a DNA vacuum concentrator on low heat and resuspended in 3.3 µl of nuclease-free water. The resuspended sample was mixed with 8.7 µl of hybridisation solution master mix. The reaction mix was incubated at 95 °C for 5 minutes and then placed at 42 °C for at least 5 minutes. The mixture was then loaded into the array and hybridised at 42 °C for 16 ~ 20 hours. After the hybridisation, arrays were washed with the Nimblegen wash buffers provided with the kit and spin dry immediately. Arrays were then scanned at 2 µm resolution using the Nimblegen MS 200 microarray scanner. Raw data obtained from the ChIP arrays were analysed by Dr Nick Gilbert and Sam corless. Data at different time points with different histone modifications were then converted to bed files and analysed by the UCSC genome browser.

Chapter 3 Generation and Characterizations of an oestrogen and serum deprived ER positive breast cancer cell line to study mechanisms of endocrine resistance

3.1 Introduction

ERα is a transcription factor that can be activated through multiple pathways (detailed in **section 1.2.3**). In the absence of ligand, the nuclear receptor is locked in an inhibitory complex with heat shock proteins (Pratt and Toft, 1997). Upon binding of the oestrogen, liganded ERα undergo conformational changes to cause receptor dimerisation and recruitment of co-activators. The resulting complex binds to their target genes and stimulate the proliferation of cancer cells (Tsai and O'Malley, 1994). Furthermore, ERα can be activated through phosphorylation in a ligand-independent manner via a variety of intracellular signalling events including MAPK and PI3K/Akt pathways (Bunone et al., 1996, Kato et al., 1995). The activated ERα complex binds to its downstream target genes and activate transcription. The ligand dependent and independent pathways are the genomic actions of ERα in response to stimulus. Meanwhile, ERα can work as part of the signalling cascades to activate other transcription factors. This is called the non-genomic function of ERα. As oestrogen receptor α (ERα) is overexpressed in majority of breast cancers, interfering with oestrogen receptor signalling has been the main mainstay for breast cancer therapy.

The activity of the ERα can be blocked through oestrogen deprivation or by antagonists of the receptor (**section 1.4)**. Tamoxifen is an ERα antagonist that was used widely as a first-line treatment for breast cancer patients with good initial responses (**section 1.4.1**). While, the pure ERα antagonist, ICI, is more often used as a second-line therapy due to its cost and disadvantages in route of administration. As ICI can only be administered as injections, tamoxifen is taken orally. Furthermore, ICI has shown to be effective after cells become resistant to tamoxifen. However, cells were insensitive to tamoxifen after they developed resistant to ICI (Osborne et al., 1995). Oestrogen deprivation is achieved clinically using aromatase inhibitors in postmenopausal women. Clinical data with aromatase inhibitors have shown their advantages when comparing with tamoxifen in managing breast cancer progression (**section 1.4.2**). However, as with other endocrine strategies, the subsequently development of endocrine resistance remains one of the biggest challenges in breast

cancer therapy. The diverse functions of ERα and pathways it was involved in plays a main part in the development of endocrine resistance. Indeed, it has been shown with xenograft and cell models that aberrant activation of growth factors signalling and their downstream pathways are associated with resistance after tamoxifen treatment and oestrogen deprivation (Jelovac et al., 2005, Massarweh et al., 2008). Therefore, it is important to study the crosstalk between different survival pathways and underlying molecular mechanisms that trigger them in order to understand the development of resistance towards anti-ERα endocrine therapy.

Human breast cancer cell lines play vital roles in the discovery of mechanisms that lie behind the development of the endocrine resistance. Several ER-positive breast cancer cell lines have been developed to study the acquisition of resistance. MCF-7 cells are commonly used in ER-positive breast cancer research, which represents the early stages of the disease and is a model for oestrogen dependent and anti-ERα sensitive breast cancer. Oestrogen independent and endocrine therapy resistant cells were then derived from MCF-7 cells to provide an adequate model mimicking the clinical effects of primary hormonal therapy in cancer patients (discussed in **section 3.7**). For instance, LCC1 is an oestrogen independent but responsive cell line that was derived from MCF-7 cells using ovariectomized mice. LCC1 cells are sensitive to ERα antagonist, tamoxifen and ICI, but do not require oestrogen to grow. Long-term estradiol-deprived (LTED) is another breast cancer cell line model that derived from MCF-7 cells. They were made by culturing cells in media treated specifically to remove substantial amounts of oestrogen (**Figure 3.1**). After a period of proliferative quiescence lasting 1~3 months, LTED cells are the returning population of proliferative cells. LTLT-Ca is a cell line model for aromatase inhibitors derived from cells that survived from long-term letrozole treatment in a xenograft tumour model (Jelovac et al., 2005). In this model, MCF-7 cells that stably transfected with human aromatase gene (MCF-7Ca) were inoculated into ovariectomized mice. Thus, tumours were served as autocrine sources of oestrogen. Mice were then treated with letrozole over a period up to 56 weeks before cells were collected for analysis.

Most of these oestrogen independent breast cancer cell models developed to data

have derived from cells in serum or stripped serum containing media, which prevents us to have a clear view of crosstalk between different signalling pathways. In order to have a better understanding of the different actions of ERα and growth factor mediated signalling; and their contributions towards endocrine resistances, it will be beneficial to generate a novel breast cancer cell line with defined growth factors and known level of oestrogen. So we can investigate the role of different signalling pathways and their response to change in hormone concentrations in a controlled environment.

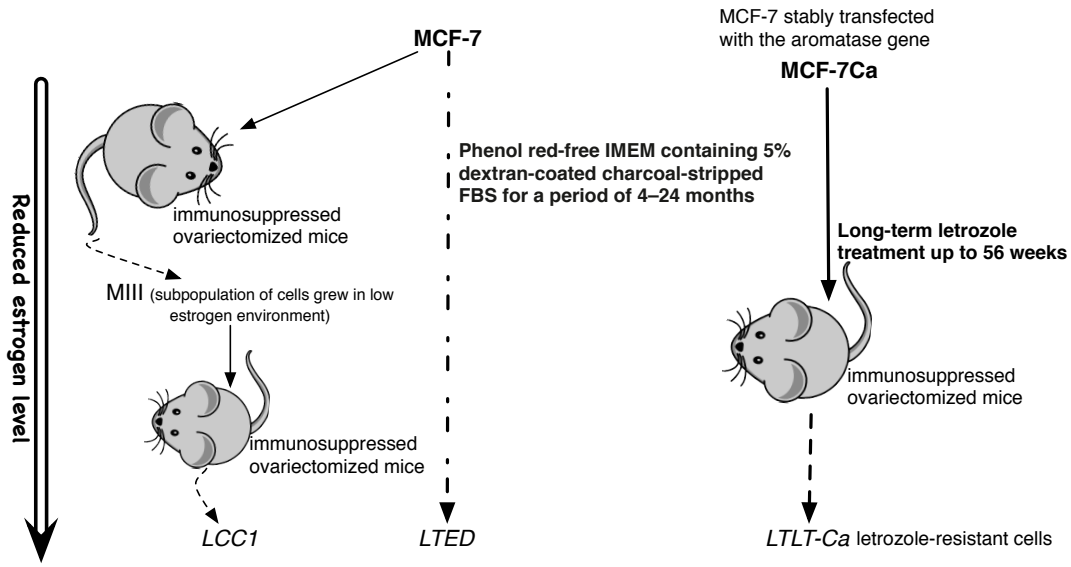


Figure 3.1 Derivation of different oestrogen independent and endocrine therapies resistant breast cancer cell lines from MCF-7 cells.

3.2 Generation of a long-term serum free and oestrogen deprived breast cancer cell line

MCF-7 cells are well characterized ERα positive breast cancer cell lines which was isolated from a pleural effusion in a postmenopausal patient with metastatic breast cancer in 1973 (Soule et al., 1973). They were routinely cultured in phenol red containing complete DMEM with 10% FCS. To generate a serum free and oestrogen deprived ER positive cell line, the complete DMEM of MCF-7 was gradually replaced with a defined serum free medium HMM over a two-month period (**Figure 3.2A**). The HMM medium was first developed as a serum free medium for culturing hippocampal neurons (Brewer et al., 1993), which has also been adopted for culturing human mammary epithelia cells (HMECs) (Duss et al., 2007). This defined serum free media contains a known concentration of oestrogen and EGF (see detailed HMM media recipe in **section 2.1**). When cells were cultured in a 100% HMM medium, they developed adhesion defects and could no longer attach to the uncoated tissue culture plate. This could due to the inactivation of extracellular matrix production in serum free condition, as cells attached well on collagen or fibronectin coated plates. At this point, oestrogen was withdrawn from a proportion of cells to produce the serum free and oestrogen deprived Die Hard (DH) cells. DH cells were continuously maintained in this environment for over a month to allow the expansion of cell population. SRB assay was used to investigate the growth rate of DH cells and shown no growth cessation when comparing with parental MCF-7 cells (**Figure 3.3**). One surprising result is that cells kept under the serum free environment in the presence of oestrogen all died out over the selection period. However, when oestrogen was added back to the adapted DH cells no obvious cell death was observed. Furthermore, the presence of oestrogen in the media was able to promote the proliferation DH cells (**Figure 3.5A**). DHe cells were DH cells that were cultured in 1nM of oestrogen over a long period (over one month). They can be used as a control when assaying the effects of oestrogen deprivation in DH cells. In turns of morphology, parental MCF-7 cells look flatter than the serum free DH cells (**Figure 3.2B**). When oestrogen was initially added to the media, long and thin protrusions were observed around the cell membrane (**Figure 3.4B**). However, the protrusions diminished after pro-longed oestrogen treatment.

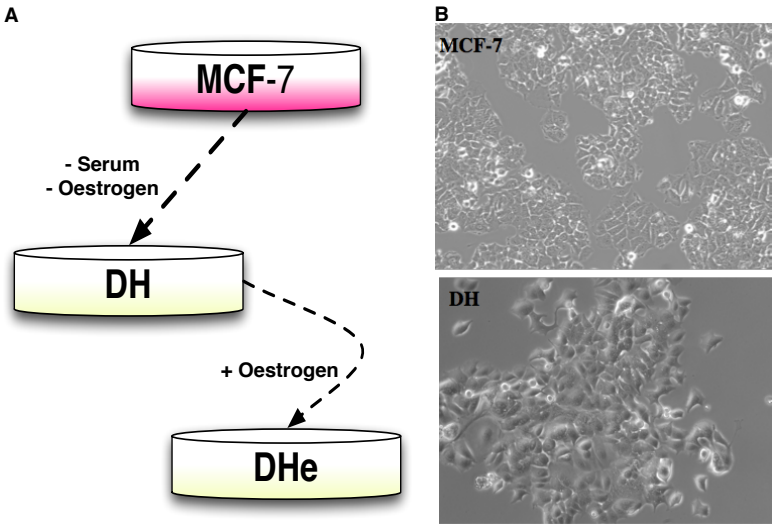


Figure 3.2 Generation of DH cells.

(A) DH were derived from ER positive breast cancer cell line, MCF-7, by progressively removing serum and oestrogen from the culture environment over two months. DHe cell were subsequently made by addition of 1nM of oestrogen back to the culture medium. (B) 10x image for MCF-7 cells and DH cells that were cultured on collagen-coated plates.

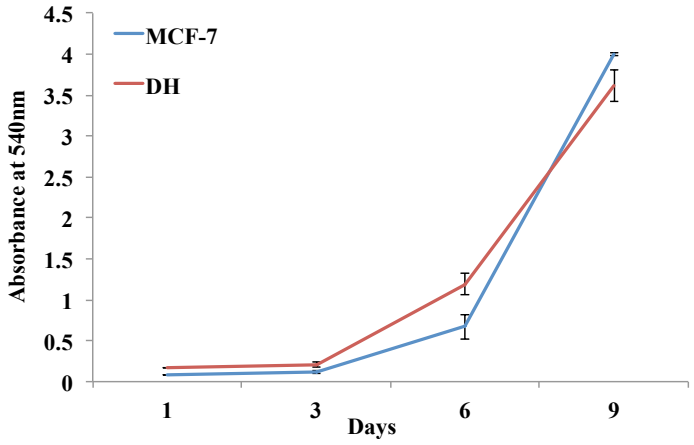


Figure 3.3 Growth characterisation of DH cells and MCF-7 cells.

SRB assay was used to monitor the proliferation of DH and MCF-7 cells. 1000 cells were plated on collagen coated well over nine days. MCF-7 cells were cultured in complete DMEM media, and DH cells were cultured in oestrogen and serum free HMM media during the assay. By this assay no significant difference was found in proliferation between the DH and MCF-7 cells. Data plotted represent means of six repeats. Error bars=SD, n=6.

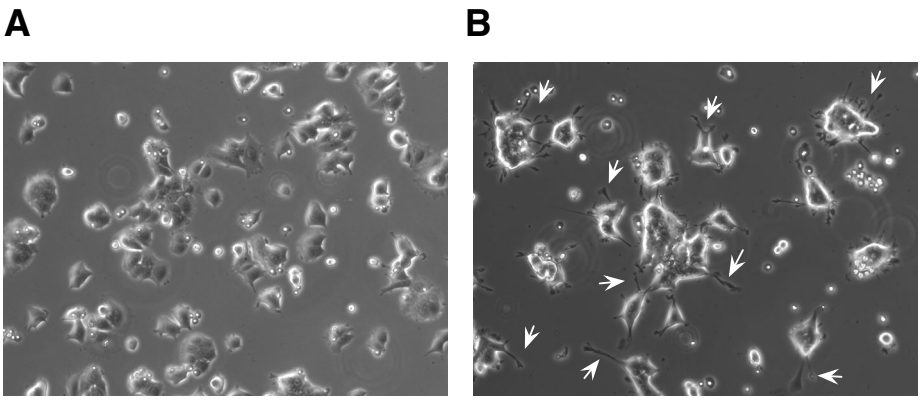


Figure 3.4 Cellular morphology of DH cells in the presence of oestrogen.

(A) 10x image of control DH cells. (B) DH cells stimulated with 1nM of oestrogen for 24h. Arrows indicated cell protrusions after oestrogen stimulation.

3.3 DH cells proliferation is responsive to ERα agonist and antagonist

In order to characterise whether DH cells are oestrogen sensitive or ERα dependent for growth, cells were treated with different ERα agonist and antagonists (**Figure 3.5**). Although DH cells grew well in the absence of oestrogen, they remain responsive to ERα agonist and antagonist in growth assays. Oestrogen stimulation gave a 35% increase in the value of absorbance in DH cells (**Figure 3.5A**). DH cells were then treated with two ERα antagonists, tamoxifen and ICI (ICI 182,780) (**Figure 3.5B**). ICI is a "pure" inhibitor of the receptor, as it can block the ERα transactivation coming from both AF1 and AF2 domains (Wakeling et al., 1991). Furthermore, it also induced degradation of the receptor (Dauvois et al., 1992). ICI demonstrated a similar result in restraining the proliferation of MCF-7 and DH cells suggesting both cell types were ERα dependent for growth. Interestingly, when compared with parental MCF-7 cells, SRB assay shown that DH cells are more sensitive to the partial antagonist tamoxifen. This was further confirmed by using a colony formation assay (**Figure 3.6**). 0.2~0.4μM of tamoxifen was sufficient to significantly inhibit the growth of DH cells. Whilst, 1~2μM was required for MCF-7 cells demonstrating that DH cells were highly sensitive to tamoxifen induced cell death. As tamoxifen competes with oestrogen for binding at the ERα AF-2 region, it inhibits the transcriptional activity of AF-2 (**section 1.4.1**). As DH cells were highly sensitive to tamoxifen, it implied that ERα were being transactivated through AF-2 region in DH cells. In addition, apoptosis assay was used to compare the effectiveness of tamoxifen and ICI in triggering apoptosis in DH cells (**Figure 3.7**). Tamoxifen was more efficient in triggering apoptosis in DH cells. After 48 hours of tamoxifen treatment, almost half of the cell population were in early apoptosis.

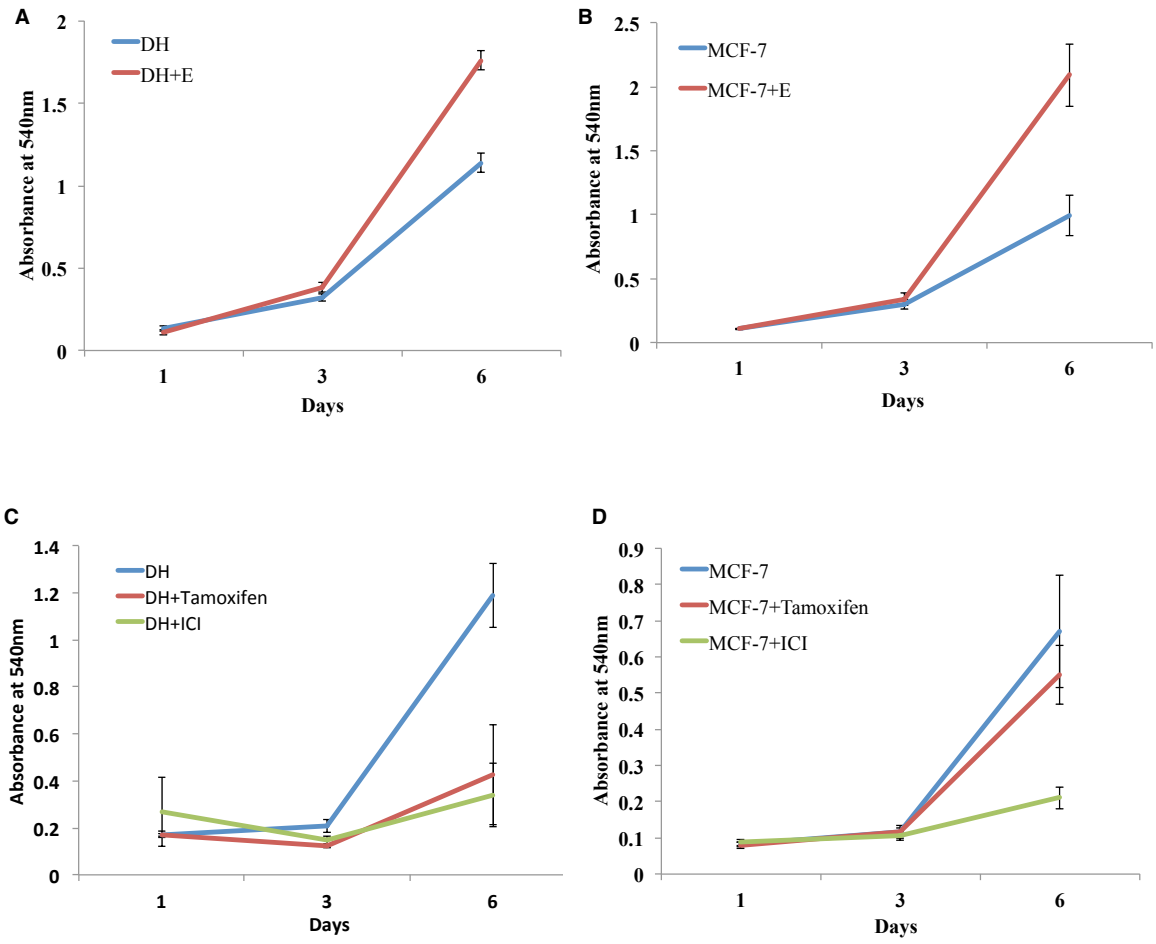


Figure 3.5 Growth characterisation of DH cells with ERα agonist and antagonist.

SRB assay was used to investigate the proliferation of DH in response to ERα agonist and antagonist. MCF-7 cells were cultured in complete DMEM media, and DH cells were cultured in oestrogen and serum free HMM media during the assay. DH cells were treated with 1nM oestrogen (A), while 5nM oestrogen was used for MCF-7 cells (B). 1μM tamoxifen and 0.1μM ICI was used in both cell lines (C & D). The absorbance was measured at Day 1, 3 & 6. Data plotted represent means of six repeats. Error bars=SD, n=6.

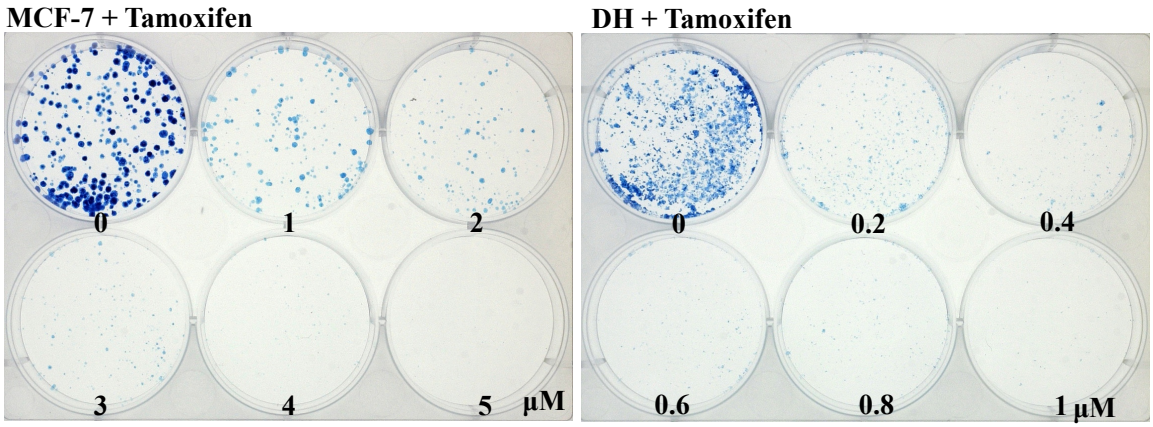


Figure 3.6 Colony formation assays of MCF-7 and DH cells with tamoxifen.

Cells were allowed to attach for 24 hours before addition of different concentration of tamoxifen. MCF-7 cells were cultured in complete DMEM media, and DH cells were cultured in oestrogen and serum free HMM media during the assay. Plates were fixed and stained using methylene blue solution on day 10. 1~5µM of tamoxifen was chosen to put on MCF-7, where 4µM of tamoxifen was required to stop the formation of any colonies. When the same concentration range of tamoxifen was applied to DH cells, no colonies were formed in any concentration. Therefore, lower concentration of tamoxifen was used (0.2~1µM). 0.6µM of tamoxifen is sufficient to stop the formation of colonies for DH cells on day 10.

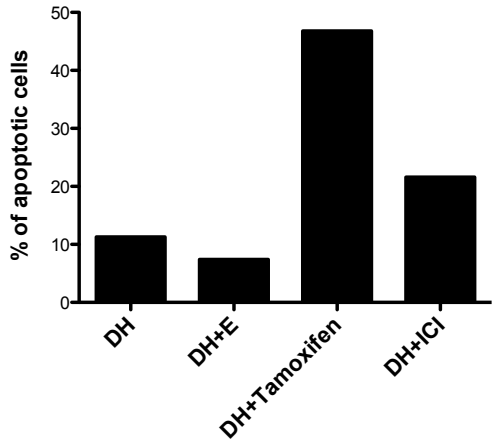


Figure 3.7 Apoptosis assay of DH cells with agonist and antagonist of ER*α*.

DH cells were treated with oestrogen, tamoxifen and ICI for 48 hours before preparing for apoptosis assay as described in section 2.3.3. Cells were sorted according to the PE Annexin V and the 7-AAD staining. Non-apoptotic cells will present in a population with low PE Annexin V and 7-AAD staining. Early apoptotic cells will show positive for PE Annexin V staining and negative for 7-AAD staining. Late apoptotic (dead) cells will be positive for both Annexin V and 7-AAD staining. The figure above shows the percentages of early apoptotic cells after each treatment. Control and oestrogen treated DH cells were mainly non-apoptotic cells. Both tamoxifen and ICI significantly triggered apoptosis in DH cells.

3.4 The effects of oestrogen on transcription in DH cells

To investigate whether DH cells response to oestrogen at the transcription level, the classic oestrogen response gene, pS2, was used to test the transcriptional activity of ERα in DH cells. In MCF-7 cells, oestrogen induces massive expression of pS2, but only weakly by tamoxifen (Berry et al., 1989). It was therefore used as a marker for measuring the level of oestrogen mediated transcription in cells. DHe cells were used as control to assay the oestrogen responses for long-term oestrogen deprived DH cells rather than the parental MCF-7 cells. As MCF-7 cells were cultured in serum-supplemented media, oestrogen was already existed in the growth environment. Therefore, it is difficult to interpret the result using MCF-7 cells. DHe cell were derived from DH cells, which had 1nM oestrogen putting back to the media for over a month. Unlike DH cells, DHe cells were oestrogen dependent for growth (**Figure 3.8**). Removal of oestrogen from DHe cells gave a more than 50% reduction in cell density according to the SRB assay.

To assay the transcription response, DH and DHe cells were treated with oestrogen, tamoxifen and ICI. The expression level of pS2 gene was measured by qRT-PCR. The results indicated that DH cells were highly sensitive to oestrogen at transcriptional level, though they have been passaged in an oestrogen free environment (**Figure 3.9**). A significant induction of pS2 gene expression (>6 fold *vs.* basal DH cells, >3 fold *vs.* DHe cells) was observed in DH cells after 48hs oestrogen treatment. DHe cells shown only a less than two fold reduction of pS2 expression when oestrogen was removed from the media for 48hs. Therefore, long term oestrogen deprived DH cells were highly sensitive to the oestrogen mediated transcription. Tamoxifen is a partial agonist for the receptor. It demonstrated a weak induction of the pS2 gene expression in DH cells. A reduction of pS2 gene expression with ICI treatment was observed in both cells. To sum up, the results suggested that DH cells were ERα agonist and antagonist responsive at the transcription level despite the oestrogen-free growth environment.

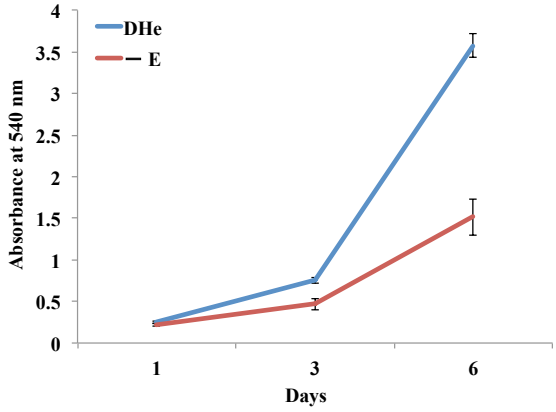


Figure 3.8 Growth characterisation of DHe cells with oestrogen.

SRB assay was used to investigate the proliferation of DHe cells. DHe cells were cultured in HMM media containing 1nM oestrogen. Oestrogen was removed from the growth environment for 6 days, to assay whether DHe cells are oestrogen dependent for growth. 2000 cells were plated in each 96 well. The absorbance was measured at Day 1, 3 & 6. Data plotted represent means of six repeats. Error bars=SD, n=6.

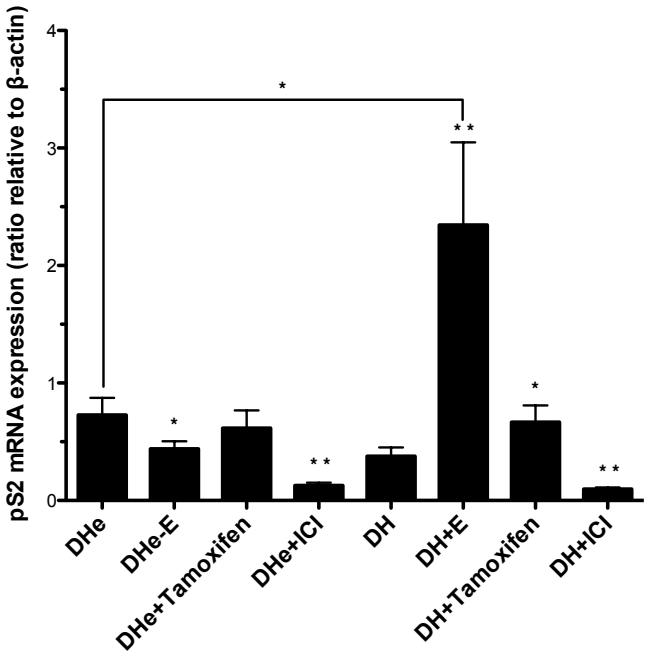


Figure 3.9 Expression of the pS2 gene in DH and DHe cells.

Cells were treated with 1nM oestrogen, 1μM tamoxifen or 0.1μM ICI for 48hs before collecting for RNA extraction. Representative experiment is shown of at least two experiments carried out. Each column presents mean of triplicate qRT-PCR analysis for each sample relative to β-actin expression. Error bars=SD, n=3. Statistical significance noted for different treatments vs matched control (Unpaired student's t-test *P<0.05; **P<0.01).

3.5 Cell signalling pathways in DH cells

It has been shown in the previous two sections that DH cells are ERα but not oestrogen dependent for growth. Furthermore, they are oestrogen responsive at the transcriptional level. I then went on to study the signalling pathways in DH cells. As DH cells were routinely cultured in the absence of oestrogen, I hypothesis the presence of ligand independent ERα pathways in DH cells. Therefore, multiple cell signalling pathways together with the phosphorylation status of ERα were examined in DH cells.

In the classic genomic pathway of ERα, the receptor undergoes conformational changes to reveal phosphorylation sites upon binding of the ligand (**section 1.2.3.1**). In the ligand independent pathway, ERα can also be activated through phosphorylation at multiple sites including Ser 118 and Ser 167 by a variety of intracellular signalling pathways. For example, the action of ERα can be coupled to growth factor receptors signalling, where the activation of extracellular receptors like EGFR leads to ERα phosphorylation at serine 118 and 167 via the MAPK and Akt/PKB pathway respectively (**section 1.2.3.2**). Therefore, the level of ERα phosphorylation can be an important indicator for different ERα mediated signalling pathway in cells.

The phosphorylation level of ERα and the activities of MAPK and PI3K/Akt pathways in MCF-7, DHe, and DH cells were analysed using western blotting (**Figure 3.10**). Among the three different cell lines, MCF-7 cells that cultured in the serum containing media had the highest phosphorylated level of ERα at both Ser 118 and Ser 167 position (**Figure 3.10A**). DHe that grew in the oestrogen containing serum free media had a reduced level of phosphorylated ERα as well as the total level of ERα. Comparing with MCF-7 and DHe cells, the oestrogen and serum deprived DH cells overexpressed ERα. However, DH cells showed the lowest level of phosphorylated ERα at serine 118. Nevertheless, the presence of phosphorylation at Ser 118 in DH cells evidence the involvement of ligand independent phosphorylation of the ERα. Therefore, the activity of MAPK pathway was examined in DH cells.

Phosphorylated ERK (p-ERK) was used to study the activity of MAPK pathway in MCF-7, DHe and DH cells (**Figure 3.10B**). DH cells had an increase in the level of p-ERK versus MCF-7 cells. However, DHe cells that had oestrogen added back to the growth media showed a much bigger increase the p-ERK level as well as EGFR protein level, suggesting that the activity of MAPK pathway was likely be triggered by oestrogen signalling in DH cells. PI3K/Akt signalling pathway was also tested in this three cell lines. Serine 473 phosphorylation of Akt was used as a marker to exam the activity of the pathway. Both DH and DHe cells have shown an increased phosphorylated level of Akt. However, the phosphorylation of ERα at Ser 167 was completely absent in DH cells indicating the activation of PI3K/Akt signalling pathway did not contribute to the ERα ligand independent pathway.

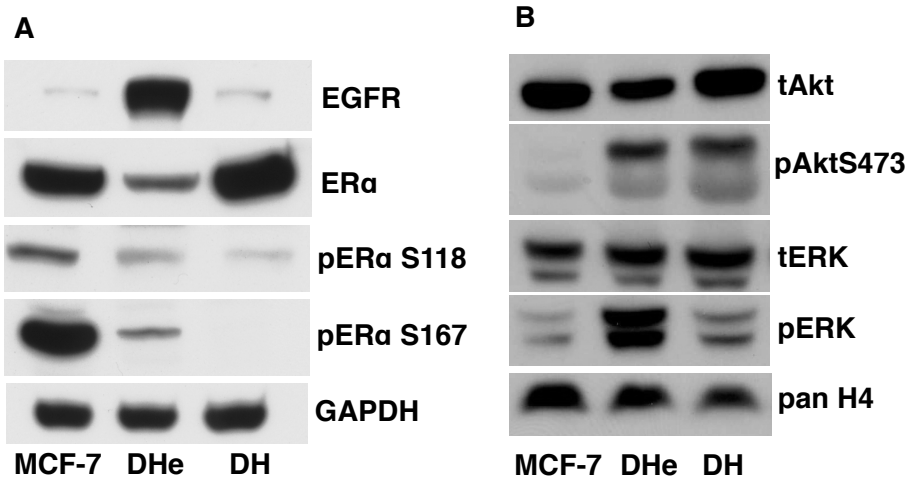


Figure 3.10 Western Blot analysis of ERα, MAPK and Akt pathways in MCF-7, DHe and DH cells.

MCF-7 cells were cultured in complete DMEM media, DH cells were cultured in oestrogen and serum free HMM media, and DHe cells were cultured in HMM media with 1nM of oestrogen before lysing. Whole cell lysates were prepared from MCF-7, DHe, and DH cells. Total and phosphorylated ERα levels were measured (A). The activation of MAPK and Akt pathways were examined using specific phosphorylated antibodies. GAPDH and panH4 were used as loading controls.

3.6 DH cells bypass oestrogen deprivation through high ERα occupancy at oestrogen response gene

Here, the mechanism of how the ER positive DH cells can survival in the absence of oestrogen was investigated. Firstly, I questioned whether DH cells were survived on autocrine production of oestrogen. Colony formation assay with aromatase inhibitor, letrozole, was performed on both MCF-7 cells and DH cells. If DH cells were survived on autocine oestrogen production, letrozole treatment should significantly impair the proliferation of DH cells and result less colonies. 10μM of letrozole significantly inhibited the formation of colonies in MCF-7 cells. While, 30μM of letrozole only slightly affect the number of colonies formed by DH cells (**Figure 3.11**). Therefore, DH cells were unlikely to survive on the autocrine production of oestrogen.

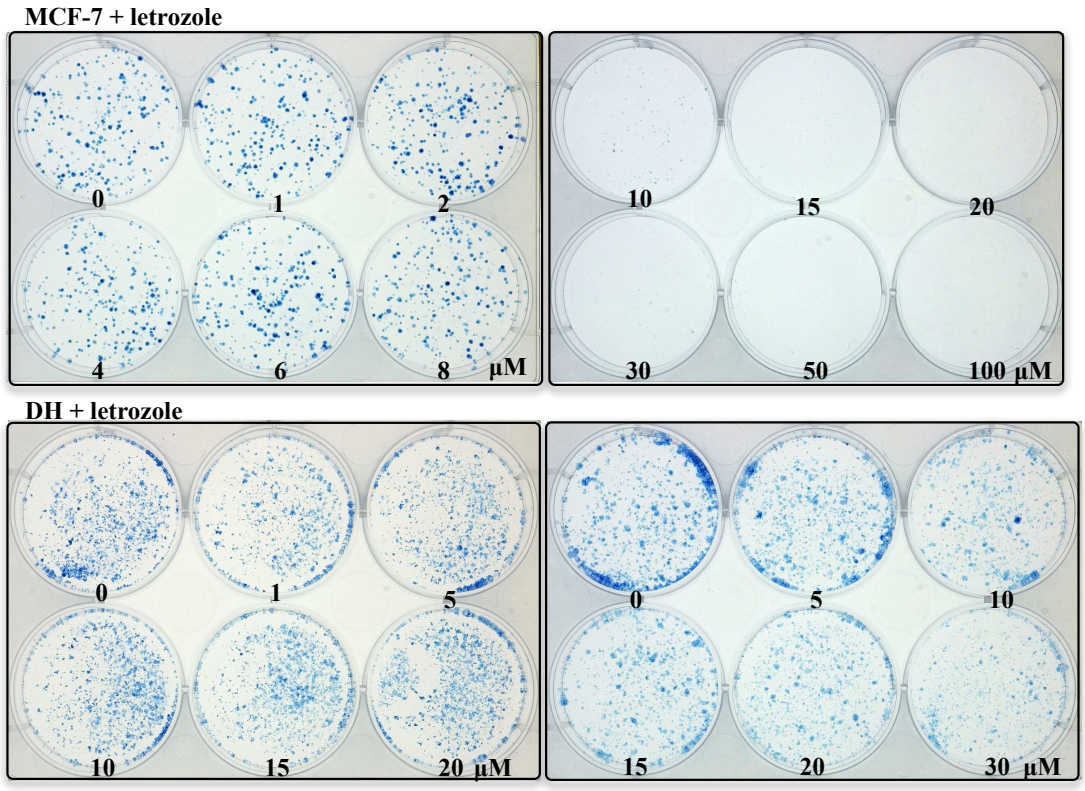


Figure 3.11 Colony formation assays of MCF-7 and DH cells with letrozole.

Cells were allowed to attach for 24 hours before addition of different concentration of letrozole. MCF-7 cells were cultured in complete DMEM media, and DH cells were cultured in oestrogen and serum free HMM media during the assay. Plates were fixed and stained using methylene blue solution on day 10. 1~100µM of letrozole was chosen to put on MCF-7 cells, where 15µM of letrozole was required to stop the formation of any colonies. Therefore, 1~30µM of letrozole was chosen to put on DH cells. Even with the highest concentration of letrozole (30µM), DH cells formed significant amount of colonies on day 10.

When comparing the expression level of ERα among MCF-7, DHe and DH cells, DH cells showed an up-regulation of the total ERα protein level (**Figure 3.10**). As ERα is a nuclear receptor that is found mainly in the nucleus, ERα immunofluorescence was performed in DH cells. Indeed, the result showed that ERα was presented mainly in the nucleus in DH cells (**Figure 3.12**). In response to external stimulations, liganded ERα assembles to the oestrogen response elements (ERE) of target genes, and induces transcriptional activation (**section 1.3.3**). Therefore, un-liganded ERα should have low occupancy at its target genes without stimulation. Due to the increased amount of ERα found in the nucleus, I hypothesised that un-liganded ERαs might

pre-occupy oestrogen response genes in DH cells to compensate the absence of ligand.

Using pS2 as an example of oestrogen responsive gene, the basal ERα occupancy was analysed by ChIP at pS2 promoter and distal regions (**Figure 3.13**). Indeed, DH cells had a high ERα promoter occupancy at pS2 gene in the absence of oestrogen. The basal ERα promoter occupancy of control DH cells was about 9 fold greater than control DHe cells (15.71 vs. 1.76). In the meantime, addition of oestrogen gave a further 4 fold increase in ERα binding to pS2 promoter. Similarly, oestrogen withdrawal from DHe cell for 48 hours also caused a 4-fold decrease in ERα pS2 promoter binding. This suggested that the magnitude of ligand induced receptor binding remained the same, though DH cells had a higher basal ERα binding at oestrogen response genes. Collectively, the results suggested that DH cells bypassed oestrogen deprivation through overexpression of ERα. The increased receptor proteins bound to the oestrogen responsive genes in the absence of ligand to maintain their expression and therefore maintain the ERα mediated pathways in DH cells.

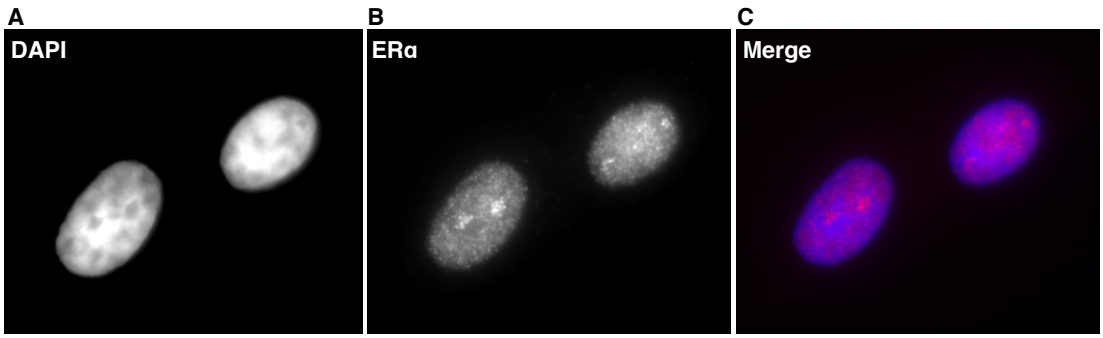


Figure 3.12 Immunofluorescence of DH cells.

DAPI staining was used to mark the nuclear region of the cell (A). Total ERα staining was shown in (B). A merge image of DAPI (blue) and ERα (red) was shown in (C).

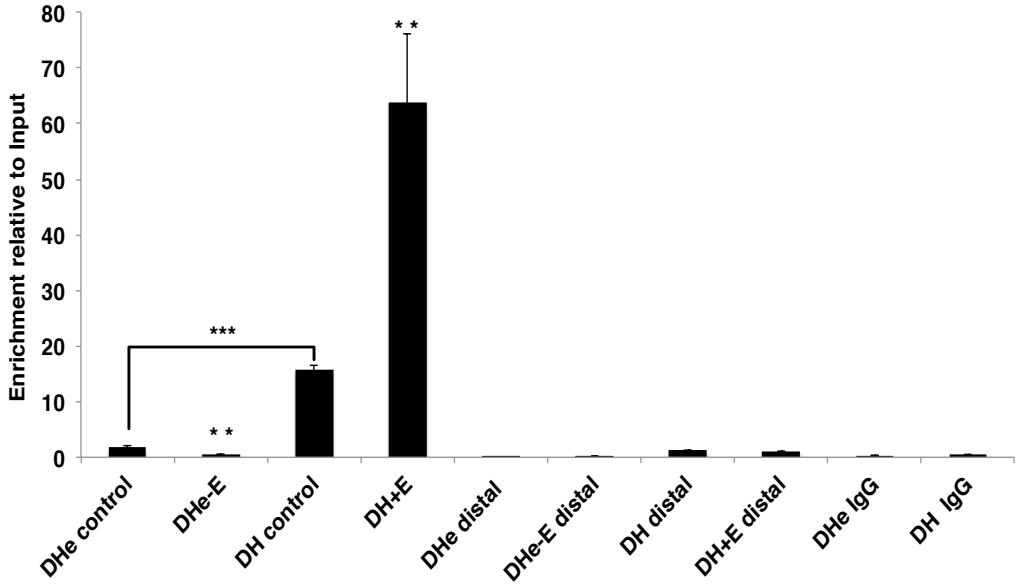


Figure 3.13 ChIP analysis of ERα at pS2 promoter in DHe and DH cells.

Oestrogen induced ERα recruitment to the pS2 promoter (-353 to -30) and distal region (-3000 to -2700) were examined in DH and control DHe cells. 1nM oestrogen was added to DH cell, while oestrogen was completely removed from DHe cells for 48hs. Results were quantified by QPCR using specific primers that cover the promoter and distal sites of pS2 gene. Data were presented as means ± SD of input-corrected values from triplet samples. Statistical significance noted for different treatments vs matched control (Unpaired student's t-test **P<0.01; ***P<0.001).

3.7 Discussion

DH was a novel oestrogen and serum deprived breast cancer cell line derived from MCF-7 cells. The survival pathways and intracellular signalling pathways of DH cells were characterised in this chapter. After culturing in oestrogen-free environment over a long period (>3 months), DH cells no longer required oestrogen for growth but remained sensitive to ERα inhibitors (**Figure 3.5**). This suggested that ERα was vital for the proliferation of DH cells even in the absence of ligand stimulation. Therefore, the activation of ERα ligand independent pathway was tested in DH cells by investigating the basal phosphorylation status of ERα (**Figure 3.10**). Serine 118 and 167 were the two main ERα phosphorylation sites that played important role in the ERα ligand independent pathway, as they could be phosphorylated by MAPK and PI3K respectively in the absence of oestrogen (Bunone et al., 1996, Kato et al., 1995). Phosphorylated ERα could then cause receptor dimerisation and activation of its downstream targets in the absence of ligand. DH cells showed little phosphorylation at Ser 118 and virtually no phosphorylation at Ser 167 of ERα (**Figure 3.10A**). Although the level of ERα phosphorylation at Ser 118 was really low, it was likely to be mediated by the activation of MAPK pathway in the serum-free and oestrogen deprived DH cells (summarised in **Figure 3.14**). On the other hand, despite the high phosphorylation level of Akt in DH cells when comparing with parental MCF-7 cells, activation of PI3K pathway did not trigger ERα phosphorylation at Ser 167 (**Figure 3.10B**)

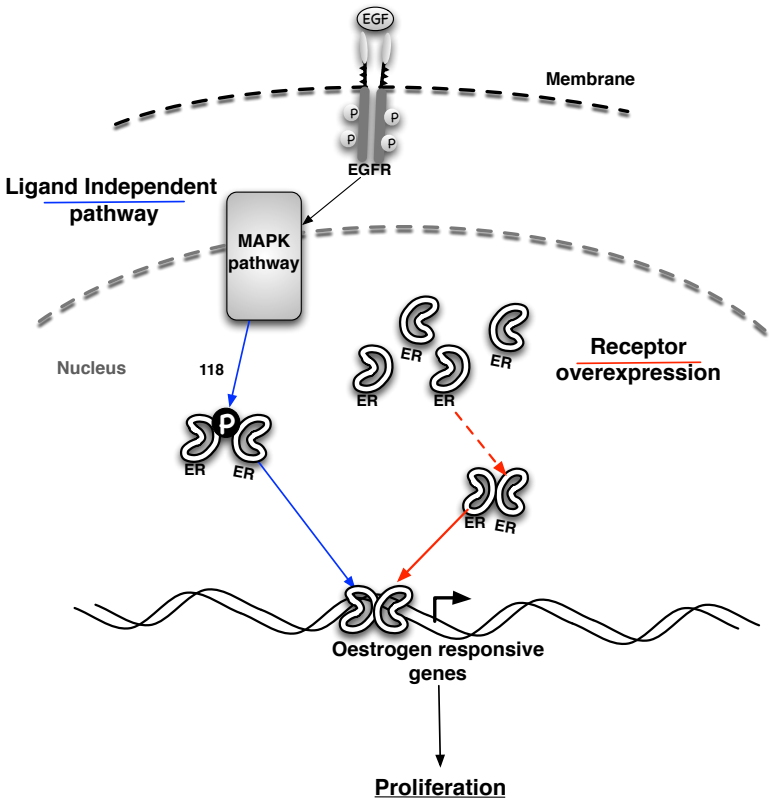


Figure 3.14 ERα mediated survival pathways in DH cells.

ERα ligand independent pathway and ERα overexpression were present in DH cells to help cells bypassed oestrogen deprivation. In the ligand independent pathway, ERα is phosphorylated by MAPK pathway. The phosphorylated ERα is able to drive the transcription of oestrogen responsive genes. At the same time, DH cells overexpress ERαs to accumulate at the promoter of oestrogen responsive genes. However, the mechanism triggers conformational changes and receptor dimersation is unknown.

Apart from the activation of ERα ligand independent pathway, DH cells also overexpressed ERα to compensate the loss of ligand stimulation. Using the oestrogen responsive pS2 gene as a reporter, I found that DH cells had high basal promoter occupancy in the absence of oestrogen, which allowed DH cells to bypass oestrogen deprivation (**Figure 3.13**). In ER positive breast cancer cells, the expression of ERα is tightly regulated through a balance of protein synthesis and degradation. Constitutive oestrogen stimulation will cause a rapid reduction of ERα protein level through the ubiquitin-proteasomal degradation pathway (Saceda et al., 1988, Read et al., 1989). On the other hand, protein kinase like glycogen synthase kinase-3 (GSK-3) has been reported to protect ERα from degradation and mediate ERα

phosphorylation at Ser 118 in the presence of oestrogen (Grisouard et al., 2007). Therefore, culturing ER positive breast cancer cells in an oestrogen-free media like the HMM media were likely to abolish the homeostasis of ERα regulation and promote changes that allow cells to adapt to the new growth environment.

Since the isolation and characterization of the MCF-7 cell line in 1973, ER-positive breast cancer cells lines have become standard models to mimic the effects of endocrine therapies on breast cancer patients. Throughout the time, several oestrogen independent and endocrine therapy resistant cells have been developed from MCF-7 cells to study the effects of therapies against ERα (detailed in **Section 3.1**). Like DH, LCC1 and LTED were other oestrogen independent cell lines derived from MCF-7 cells (**Figure 3.1**). Up-regulation of the total ERα level was observed in LCC1 cells. Furthermore, LCC1 cells respond to oestrogen at transcriptional level and have enhanced binding of ERα to pS2 promoter in the absence of oestrogen (Kuske et al., 2006). Similarly, LTED cells also have increased expression of ERα and increased basal transcriptional activation of ERα target genes (Jeng et al., 1998). Therefore, up-regulation of the total ERα protein level in response to the lost of ligand could be a general mechanism for cells to survive through oestrogen deprivation in ER-positive breast cancer cells.

However, an increase in tamoxifen sensitivity has not been reported in either LCC1 or LTED cells. This might due to both LCC1 and LTED cells were cultured in media that supplemented with stripped serum. Although the majority of the oestrogen and other growth factors have been removed during the stripping process, the remaining continue to stimulate the growth through oestrogen induced pathway and other unknown signalling pathways. Therefore, LCC1 and LTED cells might not that heavily dependent on the ERα mediated survival pathway as DH cells.

Removal of oestrogen from cells was essentially like treating cells with aromatase inhibitors. In the LTLT-Ca cell model, the protein level of ERα increases initially during the treatment. However, it decreases after 56 weeks when cells became resistant to letrozole. The resistant LTLT-Ca cells have elevated MAPK pathway activity. As a consequent, growth of LTLT-Ca cells is inhibited by MAPK kinase

inhibitors but not ERα inhibitors. Furthermore, the EGFR inhibitor, gefitinib, can not only inhibit the growth of LTLT-Ca cells but also restore their sensitivity to tamoxifen indicating direct cross-talks between ERα and growth factor signalling pathways.

Although these oestrogen deprived cell models were derived individually, they all highlighted the involvement of alterations of ERα protein level or elevation of intracellular signalling pathways. The defined culture condition of DH cells will enables us to clearly dissect different ERα pathways and interactions with other intracellular pathways in ER positive breast cancer cells. Thus, the relationship of ERα and growth factors mediated transcription regulation in DH cells was investigated further in the next chapter.

Chapter 4

Oestrogen mediated transcriptional regulation of breast cancer cell signalling

4.1 Introduction

The growth factor receptors signalling crosstalks with ERα signalling at multiple levels through the ligand independent pathway and non-genomic pathway (detailed in **section 1.2.3**). The EGFR mediated MAPK pathway is a common player involves in both pathways of ERα. The difference between the two modes of ERα actions is that ERα signalling is activated by the EGFR mediated MAPK pathway in the ligand independent pathway, while, the EGFR mediated MAPK pathway is activated by the membrane associated ERα in the non-genomic pathway. Apart from their different working mechanisms, a key characteristic of the non-genomic pathway is that it happens rapidly in response to oestrogen in ER positive breast cancer cells.

The non-genomic role of ERα was first suggested because of the observations that ERαs was on or near the plasma membrane in cancer cells (Simoncini and Genazzani, 2003, Falkenstein et al., 2000). In the non-genomic pathway, the classic hormone receptor ERα as well as the membrane associated oestrogen receptor GPR30 induces a rapid (within seconds to minutes) activation of growth factor receptors such as IGF-1R and EGFR signalling in response to oestrogen, which have common downstream pathways including MAPK and Akt (Song et al., Revankar et al., 2005, Filardo, 2002).

For the genomic pathway, ERα mediates the effects of oestrogen through altering gene expression following hormone binding. Alterations in the transcriptional states of genes usually involve an orchestrated recruitment of basal transcription factors and other co-factors to generate a local change in chromatin environment (**section 1.3.1**). Therefore, unlike the quick signalling responses of the non-genomic pathway, the genomic effects usually happen more slowly. Using pS2 gene as an example, ERα undergoes major structural rearrangements on association of ligand to expose binding surfaces that recruit transcription cofactors and a array of other proteins include the p68 RNA helicase, ATP-dependent chromatin remodeling complexes (SWI/SNF), HATs and HMTs, components of the TRAP/mediator complexes and basal transcriptional factors to the pS2 promoter. These different groups of protein form distinct combinations of protein complexes that cycle on and off the pS2 gene

to monitor the enzymatic activities of transcription factors and the epigenetic status of the promoter (Metivier et al., 2003). Therefore, it usually takes hours to see the induction of the pS2 gene in ERα positive breast cancer cell lines depending on the culture condition and concentration of oestrogen used (Naughton et al., 2007, Masiakowski et al., 1982). Although the genomic and the non-genomic pathway have diverged into separate pathways of ERα, they can indirectly affect each other. For instance, the non-genomic pathway may influence gene expression through the activation of signal transduction pathways that eventually act on target transcription factors (Bjornstrom and Sjoberg, 2005). As transcription factors can be regulated through protein kinase-mediated phosphorylation, and these transcription factors as well as kinases may thus be targets for the non-genomic actions of oestrogen.

In the previous chapter, DHe cells that have oestrogen added back to the growth media demonstrates a high EGFR level and an up-regulation of MAPK pathway activity (**Figure 3.10**) as well as a reduced ERα level suggesting that oestrogen is able to alter the balance between ERα and growth factor signalling in DH cells. As DH cells were cultured in a defined serum free media, it provided a controlled environment to study the dynamics of how the two pathways interacted and changes in survival pathways in oestrogen deprived breast cancer cells.

4.2 Oestrogen induces a slow but stable up-regulation of EGFR expression in DH cells

As DHe cells displayed a high protein level of the EGFR (**Figure 3.9**), 1nM oestrogen was added back to DH cells to investigate the dynamics of EGFR up-regulation in response to oestrogen in DH cells. Cells were treated with oestrogen for four weeks to find out when EGFR was up-regulated. The level of EGFR went up robustly in the first week of oestrogen treatment with little subsequent changes afterwards (**Figure 4.1**). Therefore, a 5-day time course was used in later experiments. Oestrogen treatment caused a gradual increase in EGFR protein level, while the level of ERα went down progressively. The decrease in ERα protein level is triggered by oestrogen, as it has been shown in the literature that oestrogen promotes ERα degradation through the ubiquitin proteasome pathway (Alarid et al., 1999). As the level of EGFR only significantly went up on day 3, the up-regulation is unlikely to be caused by the genomic effect of oestrogen.

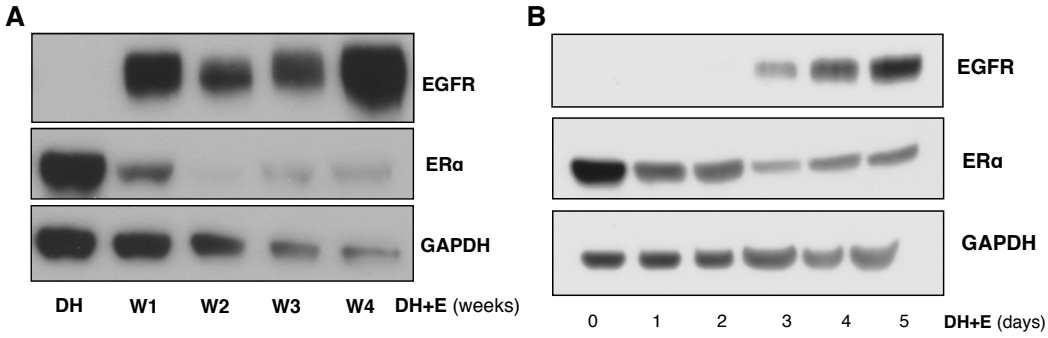


Figure 4.1 Western blot analysis of ERα and EGFR level in DH cells after oestrogen treatment.

Fresh whole cell lysate was prepared each week for four weeks (A) and over a 5-day period (B) from DH cells cultured in 1nM oestrogen. The control DH cells were left untreated. The level of EGFR and ERα was detected at each time points. GAPDH was used as a loading control.

qRT-PCR was used to look at the mRNA level of EGFR at different time points. Likewise, the expression level of EGFR did not go up immediately. It showed a "staircase" increase corresponding to the protein level of EGFR at each time point. Unlike the steady loss of protein level, the mRNA level of ERα went down sharply. As discussed previously (**section 3.7**), DH cells overexpress ERα to bypass

oestrogen deprivation and they are ERα dependent for growth. In the presence of oestrogen, I hypothesised that oestrogen might trigger a shift from an ERα dependent to an EGFR dependent survival pathway in DH cells.

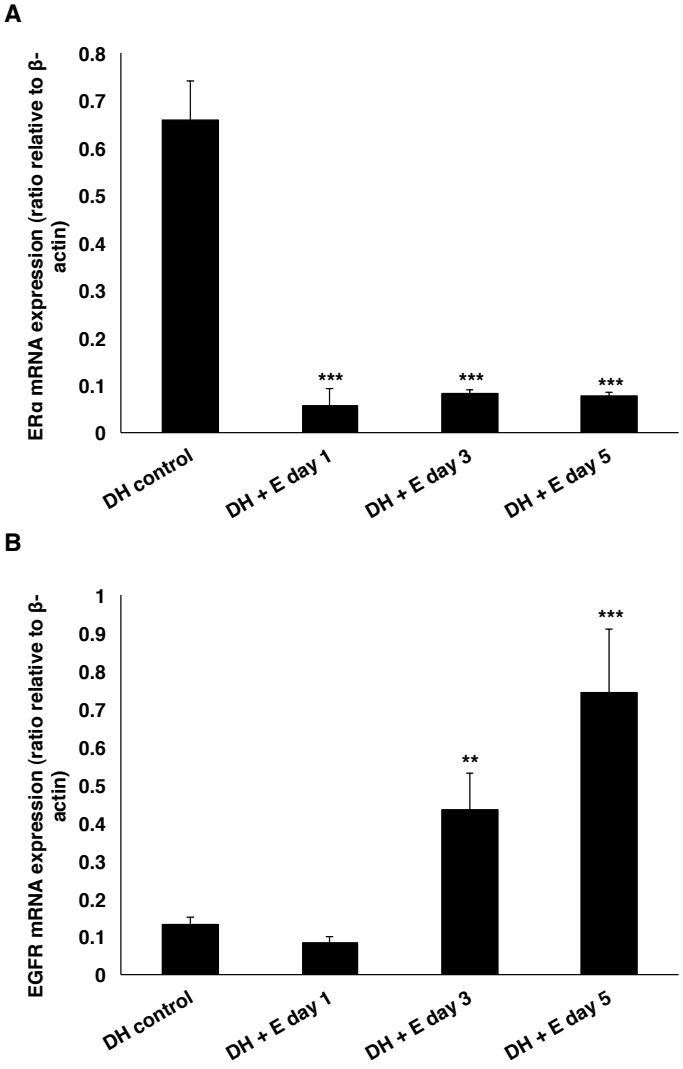


Figure 4.2 Expressions of ERα and EGFR gene in DH cells during 5 days oestrogen treatment.

DH cells were plated with 1nM oestrogen over a 5-day period to look at the expression level of ERα (A) and EGFR (B). RNA was extracted at different points and stored at -80°C for QRT-PCR analysis. DH control represented cells that were cultured in HMM serum free media without oestrogen during the 5-day time course. Representative experiment is shown of at least two experiments carried out. Each column presents mean of triplicate QPCR analysis for each sample relative to β-actin expression. Error bars=SD. Statistical significance noted for different oestrogen time points vs untreated control (Unpaired student's t-test **P<0.01; ***P<0.001).

A surprising result was that the EGFR level was stably maintained when oestrogen was removed from the growth media after a 5-day time course. Both the mRNA and the protein level of EGFR remained high for at least another 5 days after oestrogen was withdrew from the media (**Figure 4.3**). Furthermore, the level of EGFR continuous to went up after the removal of oestrogen. A group of cells were treated with ICI when oestrogen was removed from DH cells on day 5. The expression data showed that ICI did not inhibit the stable expression of EGFR. The results indicated that the transcription factor, ERα, was no longer required after the initial oestrogen stimulation to maintain the expression of EGFR in DH cells. As the change of EGFR expression was stably maintained after the withdrawal of oestrogen, epigenetic modifications could explain the effects at the EGFR locus. For example, local changes of the chromatin environment caused by histone modifications or DNA methylation at the EGFR gene promoter have the ability of stably maintaining the expression of genes (**section 1.3.2**).

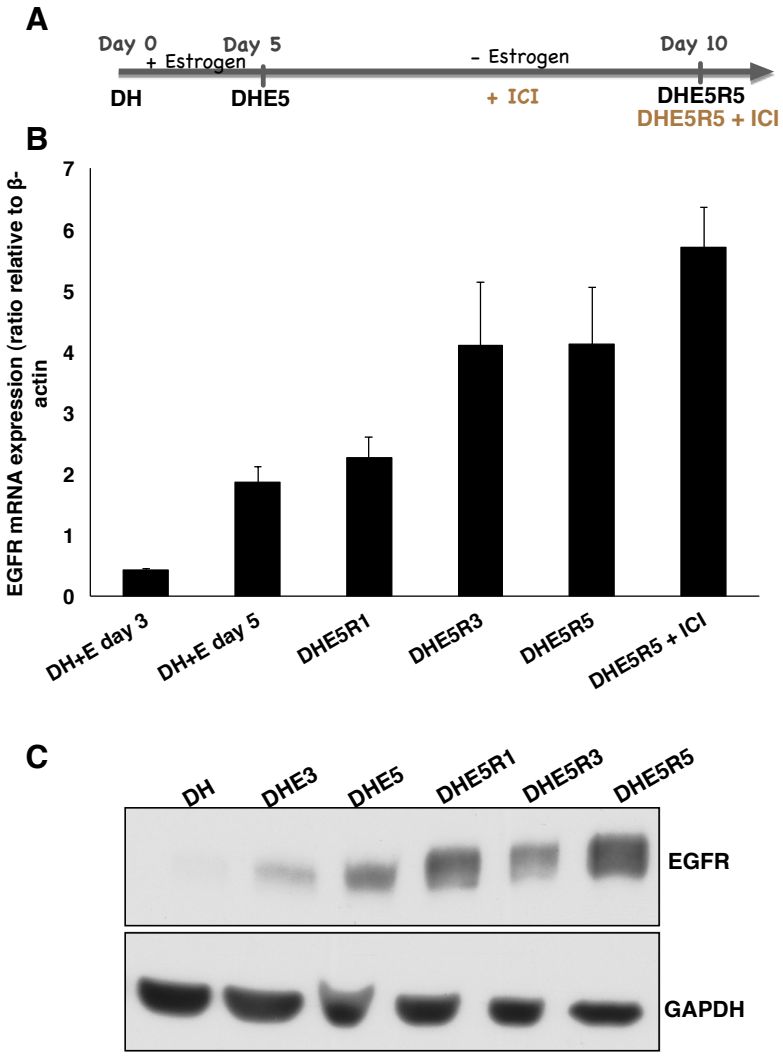
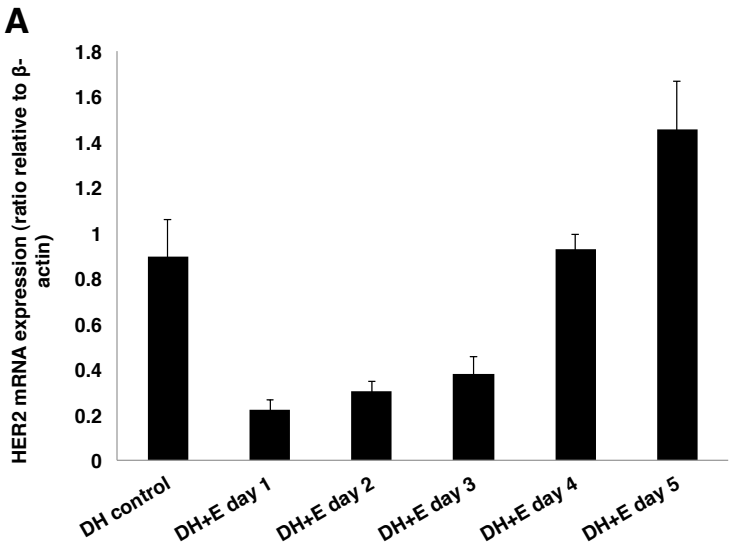


Figure 4.3 The mRNA and protein level of EGFR remained high after removal of oestrogen in DH.

(A) Scheme for DH cells time course experiment. DH cells were pre-treated with 1nM oestrogen for 5 days (DH~DHE5). Oestrogen was then washed away from the growth media for another five days (DHE5R1~DHE5R5). After removal of oestrogen, one group of cells were treated with 0.1μM ICI for 5 days (DHE5RE + ICI). RNA (B) and Protein (C) samples were extracted at different points. The mRNA level of EGFR was analysed by QRT-PCR. Each column presents mean of triplicate QPCR analysis for each sample relative to actin expression. Error bars=SD. The protein level was shown by western blot. Representative experiment is shown of at least two experiments carried out.

4.3 The effects of oestrogen on other tyrosine kinase receptors in DH cells

As EGFR is a member of the erbB family of RTK proteins, which also includes HER2 (gene name ERBB2), HER3 (ERBB3), and HER4 (ERBB4) (Herbst, 2004), the expression levels of other family proteins were analysed during 5 days of oestrogen treatment. Unlike EGFR, oestrogen did not cause HER2, HER3 or HER4 mRNA levels to go up in DH cells. Rather, their expression was down-regulated in the presence of oestrogen (**Figure 4.4**). The mRNA levels of HER2, HER3 and HER4 went down on day 1, however, the expression then gradually recovered after 5 days of oestrogen treatment. HER2 and HER3 expression increased beyond control on day 5, though the increased level did not reach significance. The mRNA level of HER4 was still significantly lower than control after 5 days of oestrogen treatment. Therefore, the transcriptional regulations of HER2, HER3 and HER4 were affected by oestrogen in DH cells. However, these alterations in expression were not stable changes like EGFR.



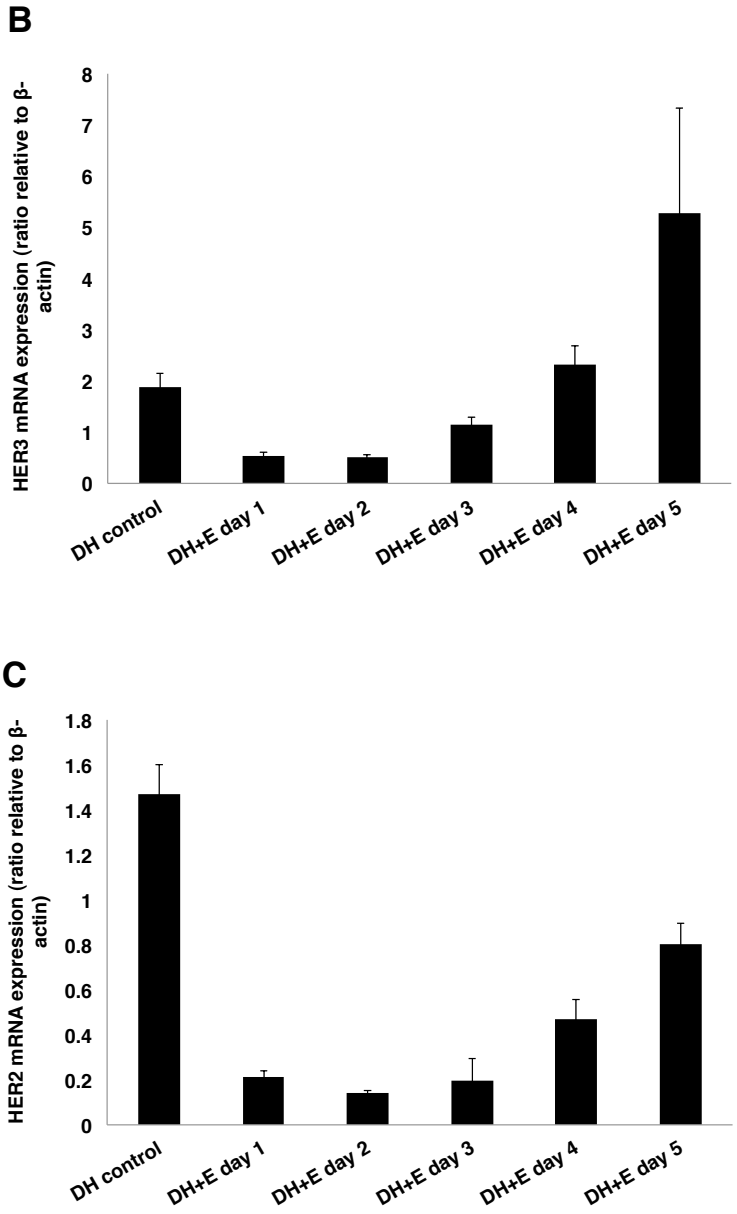


Figure 4.4 HER2, HER3 and HER4 expression levels of DH cells after the addition of oestrogen.

Expression was measured by qRT-PCR. Each column presents mean of triplicate QPCR analysis for each sample relative to actin expression. Error bars=SD, n=3.

4.4 Oestrogen causes EGFR up-regulation in other ER positive breast cancer cells

In order to demonstrate whether the oestrogen induced EGFR up-regulation is a general mechanism in ER positive breast cancer cells, oestrogen was added to two other ER positive breast cancer cell lines for 5 days. Initially the experiment was undertaken in the parental MCF-7 cells that were routinely cultured in complete DEME media. The result indicated that a higher amount of oestrogen was required to up-regulate EGFR expression (**Figure 4.5A**). MCF-7 cells required at least 5nM of oestrogen to up-regulate EGFR. ZR75-1 was the other cell lines used in the experiment. Like MCF-7 cells, ZR75-1 cells were ER positive breast cancer cells that were isolated from a pleural effusion obtained from different female patients with ductal carcinoma (Neve et al., 2006). The level of EGFR rose gradually with the increased concentration of oestrogen in ZR75-1 cells (**Figure 4.5B**). Again, a higher concentration (7~ 10nM) of oestrogen was needed to see a significant increase in EGFR level for ZR75-1 cells.

Higher concentrations of oestrogen were required to activate EGFR expression in ER positive breast cancer cells that grew in serum rich media. This leads us to consider whether DH cells have become hypersensitive to oestrogen mediated EGFR up-regulation. Therefore, DH cells were treated with lower doses of oestrogen to determine the minimal concentration required to up-regulate EGFR expression (**Figure 4.6B**). Among the tested concentrations, 50pM of oestrogen was sufficient to cause EGFR up-regulation, which was 100 times less than the parental MCF-7 cells.

Oestrogen hypersensitive has been characterised previously using long-term estradiol-deprived (LETD) cells. LETD cells are hypersensitive to oestrogen in turns of proliferation. They require a 4-log lower concentration of oestrogen (10^{-14} M) to maximally stimulate the growth when comparing with the parental MCF-7 cells (Santen et al., 2004). According to their data, they suggest that the oestrogen hypersensitive in LETD cells does not occur at the transcription level through the genomic pathway of ERα. Rather, it is mediated by the rapid non-genomic pathway

of ERα and activation of MAPK pathway. Here, my results show that DH cells are hypersensitive to oestrogen induced EGFR up-regulation. As the protein level as well as the expression level of EGFR takes at least 5 days to increase significantly, it is unlikely to mediated by the non-genomic pathway of ERα. Collectively, the data indicate that long-term oestrogen deprivation may sensitise ER positive breast cancer cells to up-regulate EGFR.

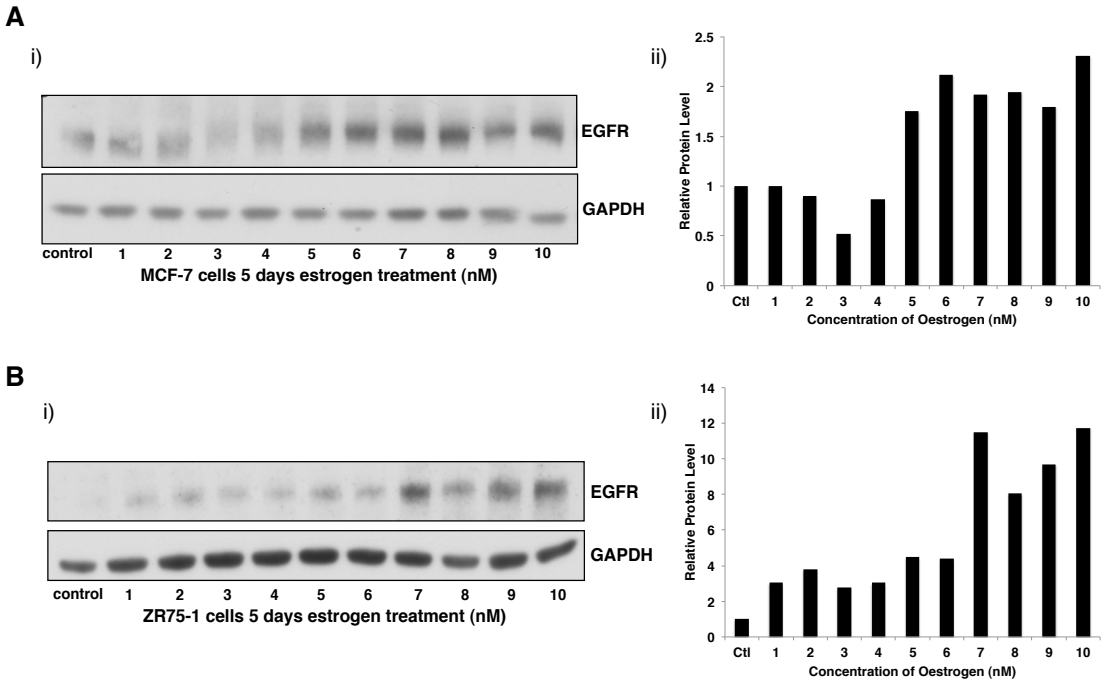


Figure 4.5 EGFR protein level in MCF-7 and ZR75-1 cells treated with different oestrogen concentrations for 5 days.

MCF-7 (A) and ZR75-1 (B) cells routinely cultured in complete DMEM media were treated with 1 to 10 nM of oestrogen for 5 days. Whole cell lysates were obtained at the end of the experiment and analysed using western blots. GAPDH was used as a loading control. Data quantification representing the GAPDH normalised EGFR levels were shown along with the western blots.

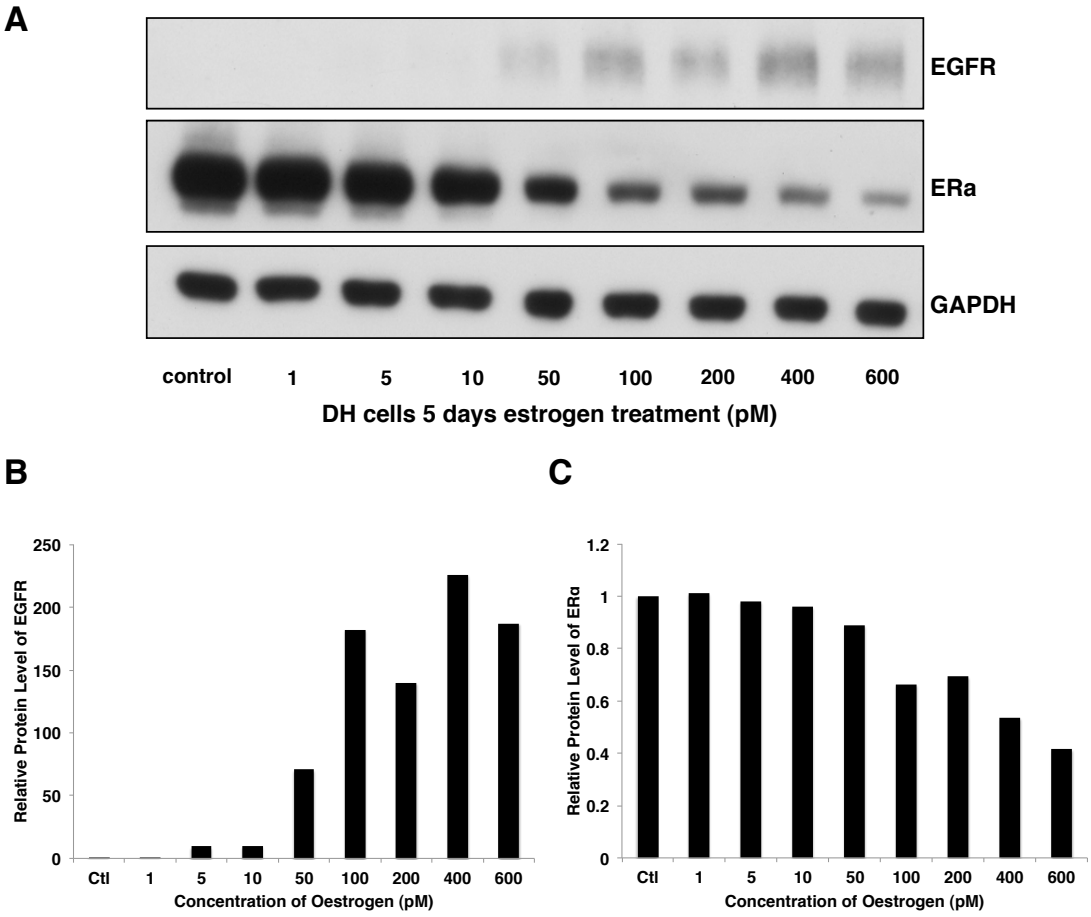


Figure 4.6 DH cells become hypersensitive to oestrogen induced EGFR up-regulation.

DH cells were treated with a concentration range (0 to 600 pM) of oestrogen as indicated in the figure above for 5 days. EGFR and ERα protein level were measured by western blotting (A). 50pM oestrogen was sufficient to cause EGFR up-regulation in DH cells. GAPDH was used as a loading control. Data quantification representing the GAPDH normalised EGFR (B) and ERα (C) levels were shown along with the western blot.

4.5 Genome-wide expression profiling of DH cells

Whole genome expression analysis was used to assay the global changes in gene expression after oestrogen stimulation in DH cells. Four time points were chosen aimed to capture early, late and stable changes of gene expression after oestrogen stimulation. They are control DH cells, DH cells with 2 days (DHE2) and 5 days (DHE5) oestrogen treatment, and cells that have 5 days oestrogen treatment and oestrogen was removed from the media for 5 days (DHE5R5). Total RNA was extracted and labelled from three biological replicates at these different time points (**Section 2.5.2, Figure 2.5**). Labelled RNA samples were analysed using the Illumina® Human HT-12 Gene Expression Bead Chip which has 48,804 probes representing 36,157 of annotated transcripts. Raw data were normalised by quantile normalisation by Andy Sims from the Breakthrough research unit using the lumi package designed especially to process the illumine microarray data (Du et al., 2008) (**Figure 4.7B**). To compare biological replicates on the array, samples were clustered by hierarchical Ward clustering and shown in a heat map. The results indicated that all biological replicates were clustered together in the heat map (**Figure 4.7C**). Normalised data were transferred to a excel sheet for further analysis.

Firstly, the changes in expression of EGF receptor family proteins (gene names EGFR, ERBB2, ERBB3), ERα (ESR1), pS2 (TFF1) as well as β-actin (ACTB) in response to oestrogen were examined using data from the expression array to validate results collected previously from using the qRT-PCR (**Figure 4.8**). 5 days of oestrogen treatment significantly reduced the expression level of ERα (fold change (fc) = 3.91, p = 2.04^{E-7}), which agreed with previous qRT-PCR result (**Figure 4.2**). The expression of EGFR (fc =1.62, p = 3.95^{E-5}) and pS2 (fc = 2.97, p = 2.63^{E-7}) went up in response to oestrogen stimulation. The expression of HER2 and HER3 decreased on day 2 but recovered after 5 days of oestrogen treatment. β-actin expression was used as a control, which has no significant changes during oestrogen treatment (fc = 1.15, p = 0.421).

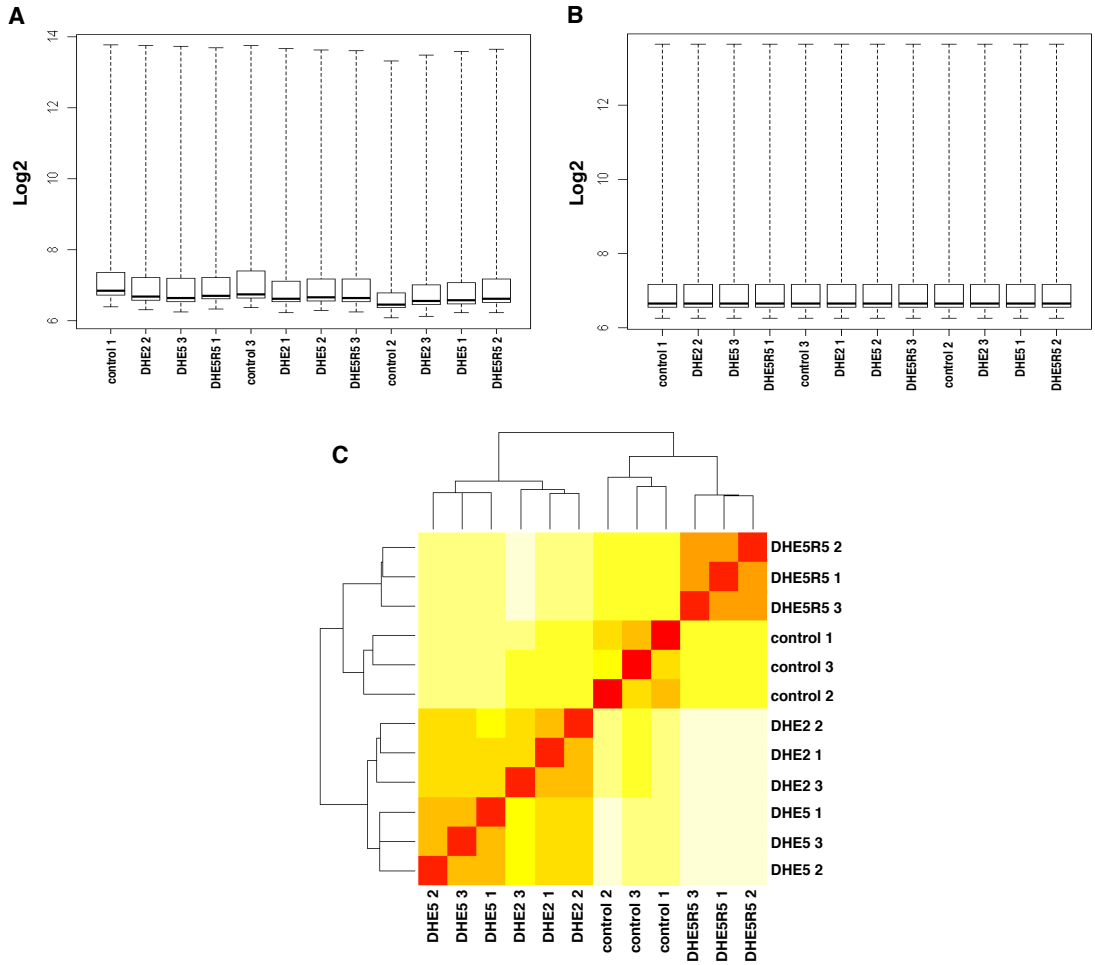


Figure 4.7 Normalisation and clustering of samples on expression array.

Raw data (A) were normalized by quantile normalization as shown in (B). The correlation between different samples on the array was analysed using the ward clustering. The data was presented as a heat map (C). Heat map colouring from white to red indicates greater similarity.

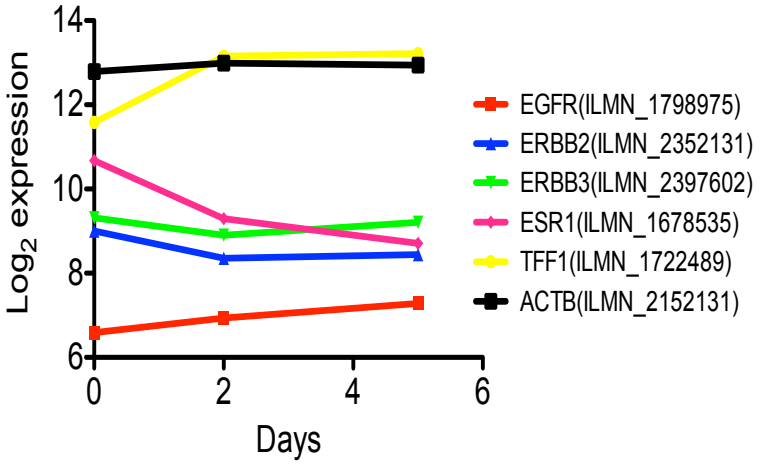


Figure 4.8 Expression array value of genes in response to oestrogen.

Normalised log_2 expression value of EGFR, ERBB2, ERBB3, ESR1, TFF1 and ACTB genes after 2 and 5 days of oestrogen treatment were shown in the figure above. Corresponding microarray probes are indicated in the inset. Each data point represents mean of three biological replicates. Error bars = SEM.

Rank products analysis rather than t-test was used to identify genes that were differentially expressed from control after oestrogen treatment with a pfp (probability of false prediction) value ≤ 0.01, as rank products analysis is a more stringent way to analyse the expression data than t-test for a small data set of biological samples (Hong et al., 2006). After 2 days of oestrogen stimulation (DHE2), 428 genes were found significantly different from the control. Among the 428 genes, 211 of which were significantly up-regulated, whilst, 217 of them were down-regulated. DHE5 had 426 genes significantly differentially expressed from control. The 426 genes included 225 up and 201 down-regulated genes. In addition, 380 genes including 218 up and 162 genes were found to be differentially expressed compared to control in DHE5R5 cells (**Figure 4.9**).

Oestrogen responsive genes at different time points were examined using the Gene Ontology (GO) term analysis to find enriched biological processes after oestrogen stimulation. Among the 211 up-regulated genes in DHE2, significant GO terms (q-value < 0.02) were terms related to regulation of locomotion including 'cellular component movement' (26 genes out of the total 488 genes associate with the

cellular component movement GO term), 'regulation of cell migration' (14/228), as well as genes related to 'protein polymerization' (7/41) and 'response to steroid hormone stimulus' (11/176). For the 225 up-regulated genes in DHE5 cells, the significant GO terms contained a broad range of biological processes included 'regulation of developmental process' (36/791), 'positive regulation of angiogenesis' (7/43), 'positive regulation of cell adhesion' (9/63), 'negative regulation of apoptotic process (20/386), etc. Significant GO terms for genes up-regulated in DHE5R5 included 'response to chemical stimulus' (70/1535), 'cellular response to type I interferon' (14/49), 'signal transduction' (68/2183), cell surface receptor signalling pathway (42/1146), etc. The GO term 'response to stimulus' was significantly enriched in all three time points (DHE2: 76/3561, DHE5: 89/3561, DHE5R5: 102/3561). No significant GO terms were found in the 217 down-regulated genes in DHE2 and the 201 down-regulated genes in DHE5 cells.

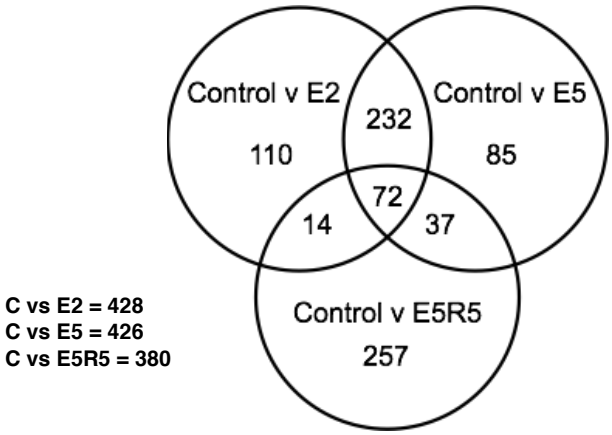


Figure 4.9 Number of genes whose expression is significantly altered (pfp ≤ 0.01 after oestrogen at different time points.

4.5.1 Genes with stable changes after oestrogen stimulation

A 5-day oestrogen pause has been shown to induce a stable EGFR up-regulation in DH cells. Using data from the expression array, we were looking for other genes, like EGFR, that harboured stable changes during the oestrogen treatment. Genes with progressive and significant increases or decreases at each time points were extracted from the data set. A list of 50 genes including EGFR were found (**Figure 4.10**).

Among these genes, 22 of them were continuously up-regulated and 28 genes were continuously down-regulated. Pathway analysis was performed on these genes using g:Profiler (http://biit.cs.ut.ee/gprofiler/index.cgi), which revealed several pathways were affected by oestrogen in DH cells. This included kinase mediated cellular stresses response pathway (STK29), proteins regulated cell adhesion and spreading (FGG, FGB), membrane proteins promoted cell proliferation (EGFR, CAV1), adaptor protein involved in vesicle trafficking (AP2S1), transcription activator that is important for cell viability (STAT1), etc. The stable changes of these genes suggested a re-programming at the transcriptional level in response to oestrogen. The ability of cells to re-program its genome in order to adopt changes in extracellular signals and proliferation stresses is one of the most important and fundamental driving forces of acquired drug resistance in cancer patients. As a result, the survived cancer cells become insensitive to the initial treatment and begin to proliferation with the presence of drugs. For example, patients will no longer benefit from tamoxifen after 5 years of treatment (Fisher et al., 1996, Fisher et al., 2001). Therefore, it is important to study the molecular mechanisms that trigger the initial genomic re-programming in cancer cells.

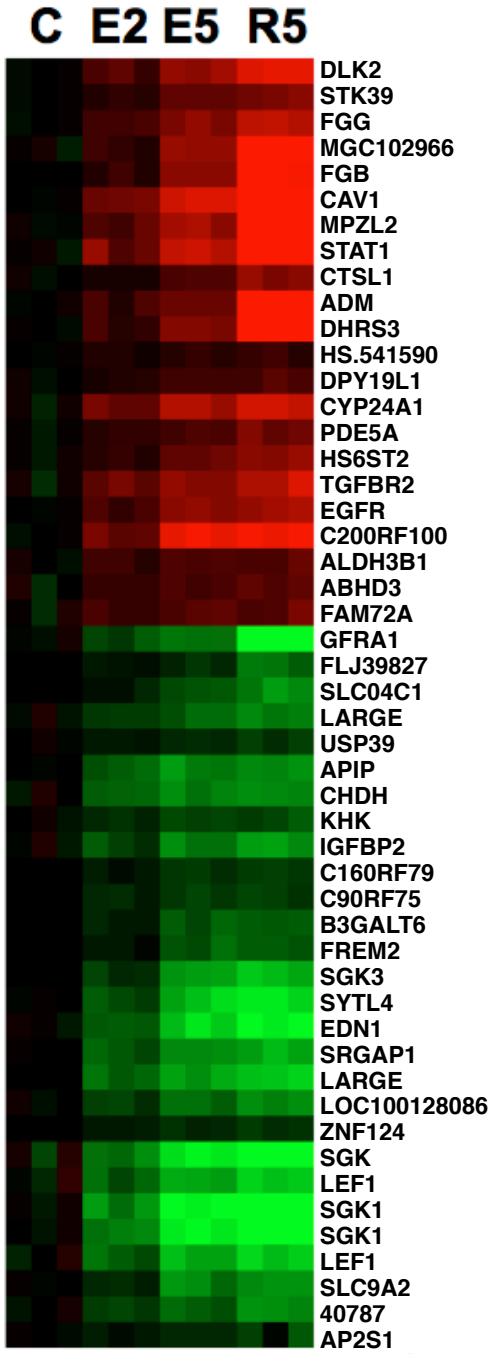


Figure 4.10 Genes with progressive changes after oestrogen treatment.

Three biological replicates were collected at each time points including DH control (C), 2 days after oestrogen treatment (E2), 5 days after oestrogen treatment (E5), and cells that have oestrogen removed from the media for 5 days after 5 days of oestrogen treatment (R5). Expression data from DH cells are graphically represented as average $\log_2$ intensity ratios (treatment / control). Intensities of red squares (genes induced with treatment) and green squares (genes repressed with treatment) correlated with the relative expression level of control. 50 genes with significant and progressive increases from control to E2, E5 and R5 were listed in figure above.

4.5.2 Oestrogen triggers a stable up-regulation of MAPK pathway in DH cells

EGFR is a membrane-associated tyrosine kinase receptor which has the potential to activate a series of downstream signalling cascades (**section 1.2.3.2.1**). It has been demonstrated in section 4.2 that oestrogen can induce a stable EGFR up-regulation in DH cells. Therefore, EGFR activity and its downstream signalling pathways were examined after oestrogen stimulation.

4.5.2.1 Oestrogen induced activation MAPK pathway in DH cells

Previous analysis using DH and DHe cells shown that long term oestrogen treatment did not alter the phosphorylation level of Akt at Serine 473 (**Figure 3.10**). However, an increase level of phosphorylated ERK was observed. Hence, the activity of MAPK pathway in response to oestrogen was investigated in DH. The level of phosphorylated EGFR (pEGFR) and phosphorylated ERK (pERK) were used to measure the activity of EGFR signalling and downstream MAPK pathway after a 5-day oestrogen time course. The result showed that oestrogen gave a significant increase in both the total and pEGFR. As a consequence, the level of pERK that is downstream of EGFR mediated MAPK pathway has also increased (**Figure 4.11**). In contrast, a reduction of the total and phosphorylated ERα at Serine 118 was observed in DHE5. Collectively, the data suggested that oestrogen caused activation of MAPK signalling pathway in companied with the down-regulation of ERα signalling pathway.

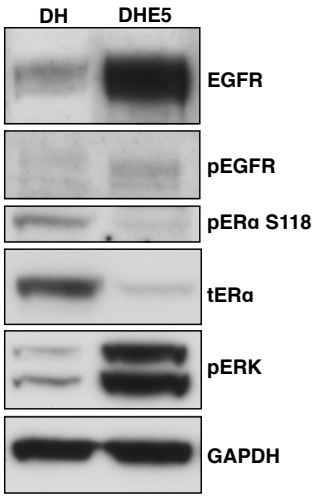


Figure 4.11 Signalling pathways in DH after 5 days oestrogen treatment.

Western blot analysis was used to measure the total and phosphorylated ERα and EGFR together with the phosphorylated ERK protein levels in control DH cells and cells treated with 1nM oestrogen for 5 days (DHE5). GAPDH was used as a loading control.

4.5.2.2 Oestrogen induced a stable up-regulation of MAPK gene signature

It has been shown that DH cells harboured a high MAPK activity after 5 days of oestrogen stimulation. Therefore, downstream target genes of MAPK signalling pathway were studied to investigate whether there was a reprogramming at the transcriptional level after oestrogen treatment. Furthermore, expression data from DHE5R5 cells will be used to analyse whether the oestrogen induced transcription reprogramming is stable.

An activation of "MAPK gene signature" in ER positive breast cancer cells was obtained from a study done by Creighton et al (Creighton et al., 2006). In this study, different MAPK initiators such as Raf-1, MEK, HER2 or EGFR were stably overexpressed in the ER positive MCF-7 cells to activate the signalling pathway. Alterations in the global expression profiles due to the activation of MAPK signalling pathway were characterised using microarray analysis in these four cell lines when comparing with control MCF-7 cells. The MAPK gene signature is made

up of 469 genes that were consistently up-regulated or down-regulated in all four of the MAPK activated cell lines.

Among the 469 genes, 153 were commonly up-regulated and 316 were commonly down-regulated during the activation of MAPK pathway. Within the 153 up-regulated MAPK genes, 64% of them showed an increase in expression after 5 days of oestrogen (DHE5) in DH cells. At the same time, 62% of the 316 down-regulated genes showed a decrease in expression in DHE5. The result indicated a clear overall activation of MAPK pathway and its downstream target genes after 5 days of oestrogen treatment in DH cells.

In addition, 133 from the 469 genes were differentially expressed between control and DHE5R5 which we took for further analysis. A heatmap was used to analysis the trend of change in gene expression during and after a 5-day oestrogen treatment **(Figure 4.12)**. In general, it showed a gradual change in expression profile with genes either went down or up progressively during the 5 days time course and stayed down or up in DHE5R5. Amongst the 133 genes, 86% of the genes that Creighton showed down-regulated (blue) in MAPK active cells were down-regulated in DHE5R5 cells ($p<0.0001$). Likewise, 66% of the genes that Creighton showed up-regulated (yellow) in MAPK active cells were up-regulated in DHE5R5 cells ($p<0.001$). These results indicated that the oestrogen induced activation of MAPK pathways and its downstream target genes were stable at the transcriptional level.

Nevertheless, differences in expression pattern were observed among the 133 genes. There were genes that were only induced transiently during the oestrogen time course such as ARHGEF1, an important Rho GTPase which is fundamental for many GPCR mediated signalling pathways in cells, as well as its downstream effectors such as DIAPH1 and FHOD1 that are important for actin cytoskeleton. There were also genes that were further up-regulated after the removal of oestrogen such as PLSCR1, an enzyme that is responsible for transferring phospholipids across the cell membrane. Furthermore, PLSCR1 has been shown to interact with EGFR in the lipid rafts in response to mitogens (Sun et al., 2002). Interestingly, there were a number of

ERα target genes like *ESR1*and *IGFBP5*, as well as oestrogen responsive genes like *MYB*, *CREB1* and *TFF1* in the constitutively down-regulated MAPK gene signature, suggesting the activation of MAPK pathway might generally be accompanied with the down-regulation of ERα signalling pathway in ER positive breast cancer cells.

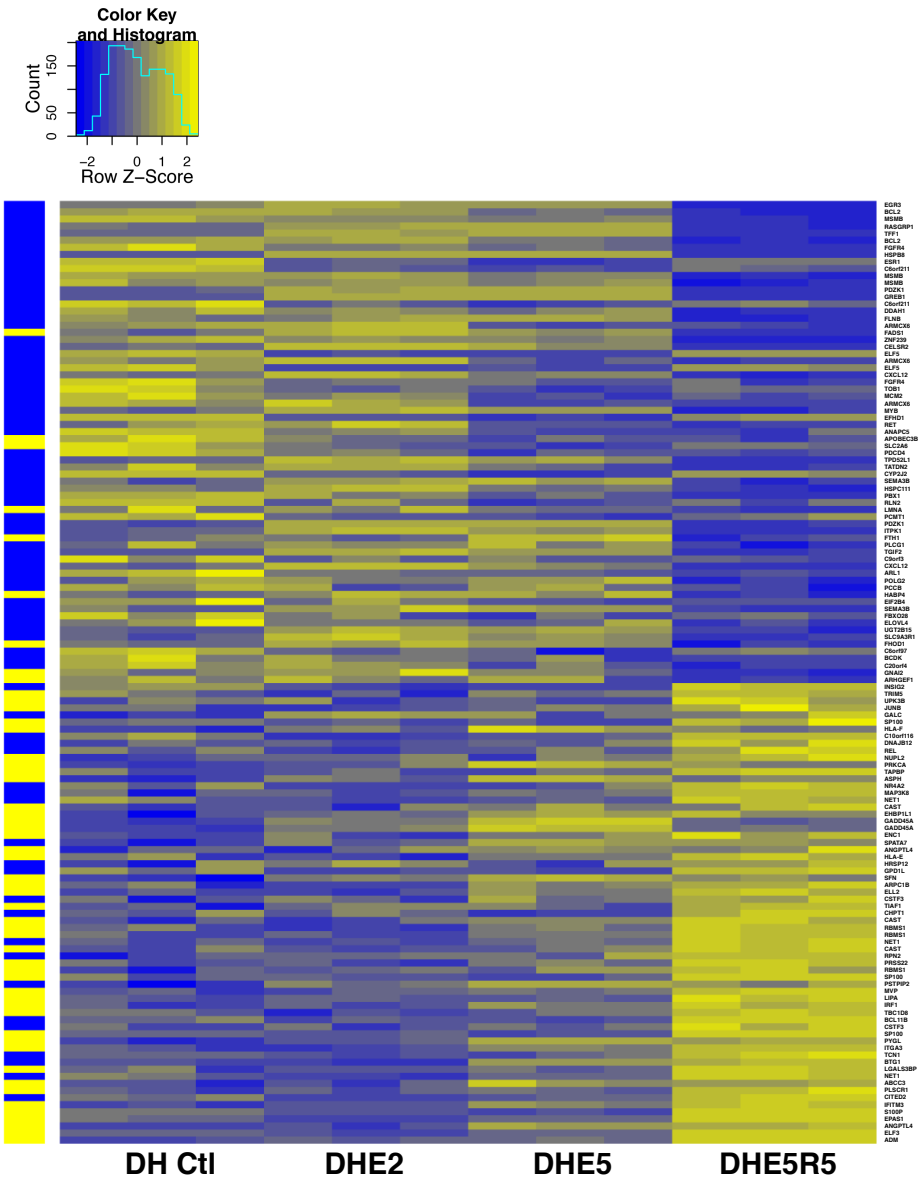


Figure 4.12 Activation of MAPK gene signature after oestrogen stimulation in DH cells.

A MAPK gene signature was taken from Creighton et al, 2006. The gene signature contained genes that were up-regulated and down-regulated during the activation of MAPK pathway. The bar on the left-hand side corresponded to the gene direction change in the Creighton paper. Blue represented genes that were down-regulated and yellow represented up-regulated genes. Normalised expression values of genes in the MAPK gene signature were extracted from the expression data of DH cells. Three biological replicates were collected at each time points including DH control (DH Ctl), 2 days after oestrogen treatment (DHE2), 5 days after oestrogen treatment (DHE5), and cells that have oestrogen removed from the media for 5 days after 5 days of oestrogen treatment (DHE5R5). The heat map represented ordered changes in gene expression of these genes during oestrogen treatment.

4.6 Oestrogen promotes a functional switch between ERα and EGFR survival pathways in DH cells

It has been shown previously that DH cells overexpress ERα and are highly sensitive to ERα inhibitors in turns of proliferation (**Figure 3.5C, Figure 3.10A**). When DH cells were stimulated with oestrogen for 5 days, the protein and expression level of EGFR went up stably in company with a lost of ERα expression (**Figure 4.2**). Therefore, proliferation assay were used to investigate whether 5 days of oestrogen treatment could cause any functional changes in DH cells such as an increase in drug sensitivity towards EGFR inhibitors.

AG1478 is a tyrosine kinase inhibitor specific to EGFR intracellular kinase domain, which works by competing for ATP binding sites on the catalytic domain of EGFR (Han et al., 1996). AG1478 treatment significantly reduced the phosphorylation of EGFR and ERK in the serum free and oestrogen deprived DH cells (**Figure 4.13**). As the level of p-ERK was markedly reduced after the addition of EGFR inhibitor suggesting that MAPK pathway was mainly activated by EGFR in the un-stimulated DH cells. DH cells stimulated with oestrogen showed a significant increase in both the pEGFR and pERK level. However, the oestrogen induced EGFR and ERK phosphorylation was completely lost in the presence of AG1478.

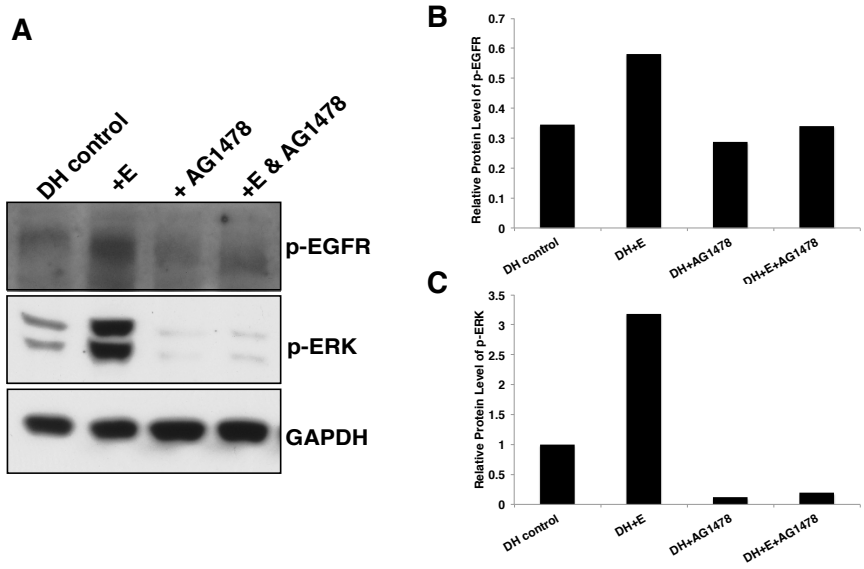


Figure 4.13 Oestrogen induced activation of MAPK pathway can be blocked by EGFR inhibitor.

Cells were treated with AG1478 with or without the presence of oestrogen as indicated in the figure for 48 hours before being lysed for western blot analysis (A). GAPDH was used as a loading control. Data quantification representing the GAPDH normalised p-EGFR (B) and p-ERK (C) levels were shown along with the western blot.

The drug sensitivity towards ERα and EGFR inhibitors in un-stimulated DH cells and DHE5 cells that had high EGFR level was examined using a SRB assay. Tamoxifen was used in the assay rather than ICI, as patients are usually treated with tamoxifen as a second line endocrine therapy after aromatase inhibitors. DH cells were more sensitive to tamoxifen than to AG1478 before the oestrogen pre-treatment (**Figure 4.14A**). Tamoxifen gave an approximately 56% reduction in cell density on day 6 ($p<0.001$). In contrast, AG1478 only inhibited the proliferation of cells by 21%. A combined treatment of tamoxifen and AG1478 almost completely blocked the growth of DH cells on day 6. Hence, the control DH cells relied more on the ERα mediated survival pathway, which was consistent with the high expression level of ERα in DH cells.

After 5 days of oestrogen treatment, tamoxifen only gave an 11% reduction in cell density on day 6 (**Figure 4.14B**). In addition, DHE5 cells became more sensitive to AG1478. Nearly a 50% reduction of absorbance according to the SRB assay was

observed with AG1478 treatment on day 6 after oestrogen pre-treatment ($P<0.01$). The data suggested that oestrogen stimulation was able to promote a shift from ERα to EGFR dependent survival pathway in DH cells.

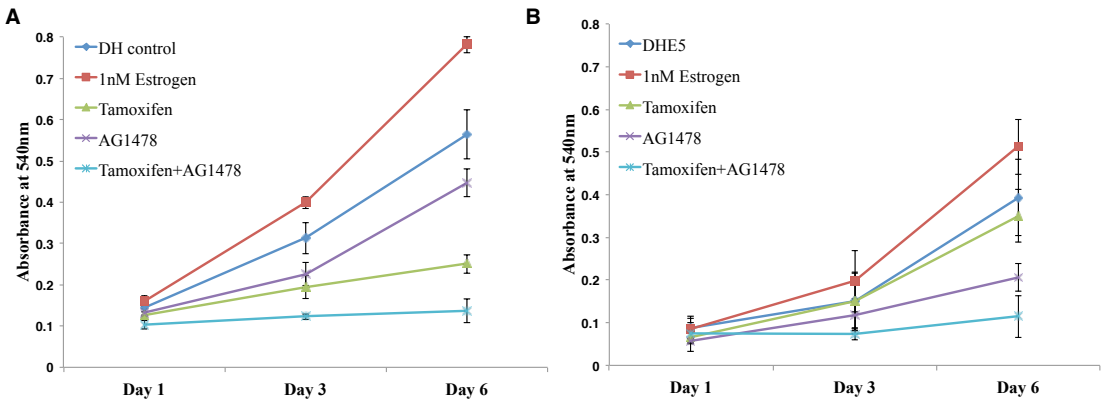


Figure 4.14 SRB assay of DH cells after 5 days of oestrogen pre-treatment.

DHE5 cells were DH cells pre-treated with 1nM oestrogen for 5 days in prior to the SRB assay. DH (A) and DHE5 (B) were then treated with 1 nM oestrogen or 1 μM tamoxifen or/and 10 μM AG1478 over a 6-day period. Cells were fixed at different time points as indicated in the figure. Data plotted represent means of six repeats. Error bars =SD, n=6.

4.7 Discussion

In this chapter, I find oestrogen induced a slow but stable increase in EGFR expression in DH cells (**Figure 4.2**) as well as other ER positive breast cancer cell lines such as MCF-7 and ZR75-1 cells (**Figure 4.5**). Furthermore, DH cells that are normally growth in serum and oestrogen deprived media became hypersensitive to oestrogen induced EGFR up-regulation, and required only 50pM of oestrogen to alter the expression of EGFR (**Figure 4.6**).

The MAPK pathway downstream of EGFR signalling was also activated as a result of oestrogen stimulation. Compared with the gradual up-regulation of EGFR expression, EGFR and ERK were phosphorylated after 48 hours of oestrogen stimulation. In the un-stimulated DH cells, MAPK pathway was mainly mediated by the canonical EGF stimulated EGFR signalling, as the level of ERK phosphorylation was completely blocked by the EGFR kinase inhibitor, AG1478 (**Figure 4.13**). After oestrogen stimulation, the activation of MAPK pathway was likely to be caused by the non-genomic action of ERα.

Oestrogen induced rapid activation of MAPK pathway has been observed in various different cell models (Endoh et al., 1997, Migliaccio et al., 1996). The non-genomic pathway of ERα has been proposed to be responsible for the effects. Oestrogen acts through the membrane associated GPR30, to transactivate EGFR and MAPK pathway (Filardo et al., 2000). Furthermore, oestrogen can cause phosphorylation of the ERα membrane adaptor protein Shc. The phosphorylated Shc binds to Grb-2 and Sos, which results in the rapid activation of the MAPK pathway (Santen et al., 2005). Another paper suggests that oestrogen activates the MAPK pathway through a rapid increase in cytosolic calcium (Improta-Brears et al., 1999). Therefore, the quick activation of EGFR and MAPK pathways that I have observed could be through the non-genomic pathway of ERα in DH cells. As blocking of EGFR phosphorylation by AG1478 was sufficient in inhibiting the oestrogen induced activation of MAPK pathway (**Figure 4.13**), the non-genomic pathway of ERα must function upstream of the EGFR mediated phosphorylation of ERK. However, the up-regulation of EGFR expression was not dependent on the phosphorylation of EGFR, as AG1478 did not

prevent the up-regulation of EGFR expression (data not shown). The up-regulation of EGFR expression was likely to be the genomic function of ERα, which fed back to the EGFR signalling pathway and caused continuous activation of EGFR mediated MAPK signalling (summarised in **Figure 4.15**). Furthermore, global expression analysis revealed that oestrogen was able to trigger a stable activation of MAPK gene signature in DH cells (**Figure 4.12**).

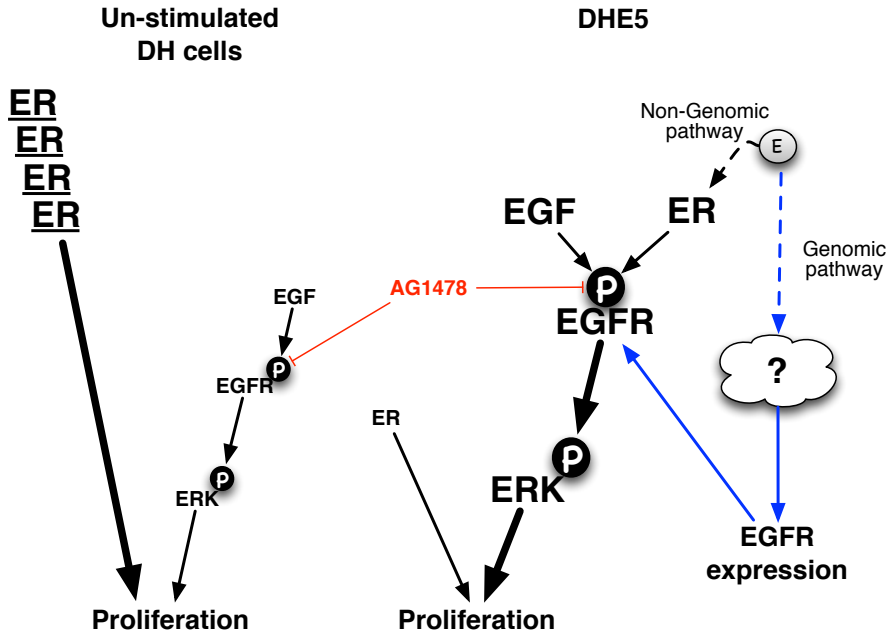


Figure 4.15 Summary of survival signalling pathways in DH and DHE5 cells.

In un-stimulated DH cells, cells overexpressed ERα to compensate the loss of oestrogen stimulation. Therefore, DH cells were highly relied on the ERα pathway for growth. The EGFR mediated MAPK pathway did not play a major role for the overall survival of DH cells. After oestrogen stimulation, EGFR was first phosphorylated by the non-genomic action of ERα. Furthermore, the expression of EGFR was up-regulated by the genomic function of ERα to maintain a continuous activation of EGFR and its downstream MAPK pathway. Therefore, DHE5 cells became more sensitive to the EGFR inhibitor, AG1478.

Despite the stable up-regulation of EGFR and its MAPK signalling pathway induced by oestrogen, the expression of other erb family members were not stably altered suggesting that these receptors were regulated differently in ER positive breast cancer cells (**Figure 4.5**). HER2 is a member of the erb family, which is frequently overexpressed in breast cancer patients. HER2-positive breast cancers account about

20% of patients with early stage breast cancer (Ross et al., 2009). Targeted therapies against HER2 have been developed in recent years, which significantly improved the outcome of patients (Arteaga et al., 2012). However, like many other cancer therapies, the acquired drug resistance remains a big challenge. It has been showed that inhibition of HER2 by trastuzumab can induce constitutive activation of PI3K/Akt signalling pathway (Nahta and Esteva, 2006). This demonstrated an interesting preference of the membrane associated erb family receptors as initiators of downstream signalling pathways in drug resistant breast cancer cells.

In terms of proliferation, DH cells became less sensitive to tamoxifen after 5 days of oestrogen pre-treatment but remained sensitive to the EGFR inhibitor, implying a functional switch from ERα to EGFR-MAPK dependent survival pathway after a 5-day oestrogen pulse. The reduced sensitivity towards endocrine therapies, reduced ERα protein level, and increased growth factor receptor signalling showed an interesting phenotype similar to ER negative breast cancer cells. Furthermore, global expression analyses showed that apart from EGFR, a range of genes have also been stably up-regulated or down-regulated in DHE5R5 (**Figure 4.10**). This suggested that a pulse of oestrogen had the potential to cause transcriptional re-programming in ER positive breast cancer cells grew in low oestrogen environment. In order to prevent the occurrences of transcriptional re-programming during breast cancer therapy, it is important to understand the mechanism that triggers the initial change in gene expression after oestrogen stimulation. Therefore, the molecular mechanism of oestrogen induced EGFR up-regulation in DH cells was discussed in the next chapter.

Chapter 5 The transcriptional and epigenetic regulation of EGFR up-regulation

5.1 Introduction

Having made the observation that oestrogen induces EGFR expression in the long-term oestrogen deprived DH cell, the mechanism of EGFR up-regulation was investigated in this chapter. EGFR is a membrane protein that belongs to the EFG receptors protein family of receptor tyrosine kinases (RTKs) (Jorissen et al., 2003). It can be activated through a range of growth factors including EGF, transforming growth factor-α (TGF-α), and the neuregulins (Yarden and Sliwkowski, 2001). Ligand binding brings EGFR molecules to close proximity forming homodimers or heterodimers with other family members. The resulting dimers undergo auto-phosphorylation at specific tyrosine residues, and activate a series of downstream signalling pathways leading to cell proliferation, blocking apoptosis, activating invasion and promoting metastasis (Hynes and Lane, 2005).

Aberrant EGFR activation has been identified in many types of cancer cells, which can be caused by gene amplification, overexpression of ligands or receptors, and mutations in the kinase domain (Ciardiello and Tortora, 2008). In terms of breast cancer, multiple studies have examined whether EGFR was overexpressed in clinical samples. The results show a substantial variation in the EGFR overexpression rate from 15% to 45% among breast cancer samples (Ferrero et al., 2001, Fox et al., 1994, Klijn et al., 1992, Pawlowski et al., 2000, Tsutsui et al., 2002, Rimawi et al., 2010). Interestingly, an inverse relationship between ERα expression and EGFR expression was reported in several studies and high EGFR expression was associated with poor prognosis (Nicholson et al., 1994, Mizukami et al., 1991). However, the molecular detail of how EGFR expression was regulated in breast cancer cells and the role of ERα was unclear.

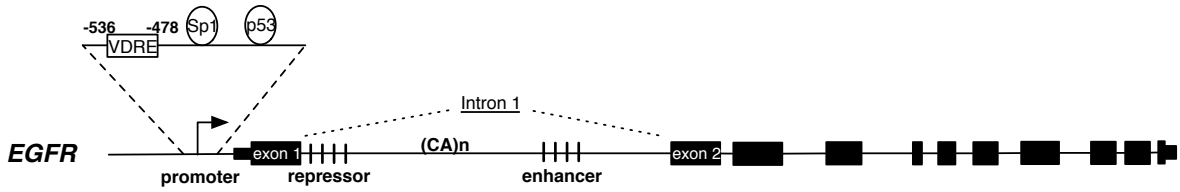


Figure 5.1 A schematic representation of the *EGFR* gene and its regulatory elements.

Exons are shown as boxes connected by introns. DNase I hypersensitive sites in intron 1 are displayed as lines. A repressor element is found at the exon 1/intron1 boundary. Whilst, an enhancer element is located within the first intron. The first intron also contains a $(CA)_n$ dinucleotide polymorphism, which is important for the transcriptional regulation of EGFR.

The *EGFR* gene has a TATA-less promoter with high GC content, and is located on chromosome 7p11.2 (Ishii et al., 1985). The expression of EGFR can be regulated through a number of transcription factors and response elements identified around the promoter and intron 1 area of the gene across different cell types (**Figure 5.1**). For example, Sp1 and p53 have been identified as positive transcriptional regulators that bind directly to the EGFR promoter (Kageyama et al., 1988a, Kageyama et al., 1988b, Ludes-Meyers et al., 1996). In contrast, the vitamin D response element (VDRE) found between -536 and -478 of the promoter negatively regulates the transcription of EGFR in breast cancer cells. Liganded vitamin D receptors (VDRs) bind to the VDRE through an unknown nuclear partner to causes displacement of the positive regulator, Sp1, from the EGFR promoter (McGaffin and Chrysogelos, 2005).

Furthermore, the expression of EGFR can also be influenced by the chromatin structure of its first intron. In ER positive breast cancer cells with low EGFR expression, DNase I hypersensitive sites are preferentially found around the exon 1/intron 1 boundary. Whilst, in ER negative breast cancer cells, DNaes I hypersensitive sites detected within intron 1 are associated with high EGFR expression (Chrysogelos, 1993). The first intron of the *EGFR* gene also contains a $(CA)_n$ dinucleotide polymorphism comprising of 14 to 21 repeats (Chi et al., 1992). An inverse relationship has also been shown between the number of CA dinucleotide

repeats in intron 1 of EGFR and the expression level of EGFR (Gebhardt et al., 1999). Furthermore, in a study with 112 cancerous and noncancerous breast tumours, high EGFR expression was found to be correlated with more advanced breast cancer samples and short CA repeats suggesting a direct link between the EGFR transcriptional regulation and disease outcomes (Buerger et al., 2000). Collectively, a complex transcriptional regulation of EGFR expression has been reported in the literature. However, how oestrogen up-regulates EGFR expression in DH cells is not clear.

In this chapter, I show that the oestrogen induced EGFR up-regulation in DH cells is mediated by an oestrogen responsive transcriptional activator, c-Myb. c-Myb belongs to the *MYB* gene family, which consists of *MYBL1* (A-Myb), *MYBL2* (B-Myb) and *MYB* (c-Myb). They all encode proteins that function as transcriptional activators. A-Myb is mainly expressed in the central nerve system during embryogenesis, male germ cells of adult mice and mammary gland of pregnant mice (Trauth et al., 1994, Mettus et al., 1994). c-Myb is a proto-oncogene which is expressed in most hematopoietic tissues (Hoffman et al., 2002). In contrast, B-Myb is ubiquitously expressed in cells and plays important roles in cell cycle progression (Lam et al., 1995, Catchpole et al., 2002).

The MYB family proteins consist of three major functional domains, a N-terminal DNA binding domain, a transactivation (TA) domain and a C-terminal regulatory domain (**Figure 5.2**). The DNA binding domain of Myb proteins contain three evolutionary conserved tandem repeats termed R1, R2, and R3, where R2 and R3 are essential for Myb DNA binding while R1 stabilise the Myb-DNA complex formation (Oh and Reddy, 1999). Downstream of the DNA binding domain is a TA domain that is required for the activation of target gene transcription. The TA domain of Myb is able to recruit co-activator such as CBP/p300 that has HAT activity (Dai et al., 1996, Ogryzko et al., 1996). The C-terminal regulatory domain of c-Myb contains a leucine rich motif that negatively regulates the transactivation activity of the protein (Kanei-Ishii et al., 1992). The transactivation activity of Myb proteins can also be regulated by post-translational modifications such as phosphorylation.

Phosphorylation at the N-terminus by casein kinase II was shown to be inhibitory to c-Myb DNA binding (Luscher et al., 1990). Furthermore, c-Myb can also be phosphorylated on Ser 528 at the C-terminus through the MAPK pathway. Mutation of serine to alanine enhances the transactivation ability of c-Myb as shown by a c-Myb reporter gene construct (Aziz et al., 1995).

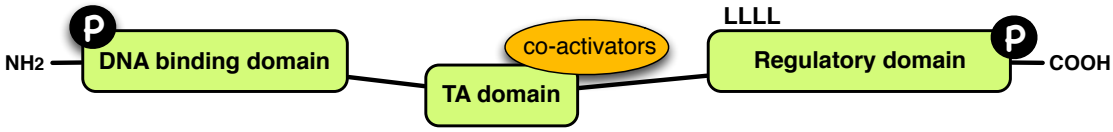


Figure 5.2 Myb protein structure.

The Myb protein is mainly composed of a DNA binding domain, a transactivation domain, and a regulatory domain. The N and C terminus of the protein are subjected to phosphorylation. The TA domain is able to recruit co-activators during transcriptional activation. c-Myb contains a leucine rich motif at the regulatory domain, which negatively regulates the transcriptional activity.

5.2 Role of ERα in oestrogen induced EGFR up-regulation

ERα is a transcription factor that is recruited to target genes in response to oestrogen stimulation (**section 1.3.3**). As ERα is responsible for many of the oestrogenic effects on normal and cancerous breast tissue, ERα siRNA was used to investigate its role in oestrogen induced EGFR up-regulation. As DH cells express high level of ERα, cells were transfected with two rounds of ERα siRNA to achieve sufficient knockdowns (**Figure 5.3A**). After the second round of siRNA treatment, oestrogen was added to cells, and samples were analysed over a 5-day period by western blotting (**Figure 5.3B**). ERα depletion by siRNA reduced the level of oestrogen dependent EGFR up-regulation suggesting that ERα played an important role in the regulation of EGFR expression.

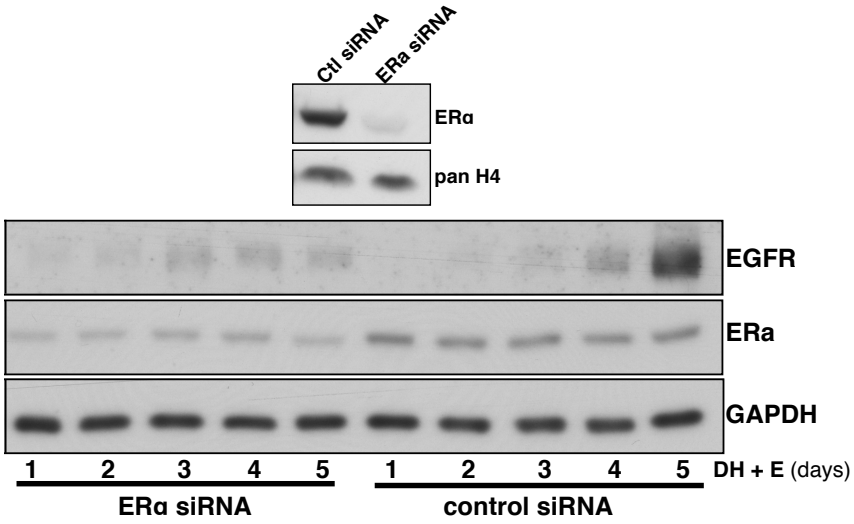


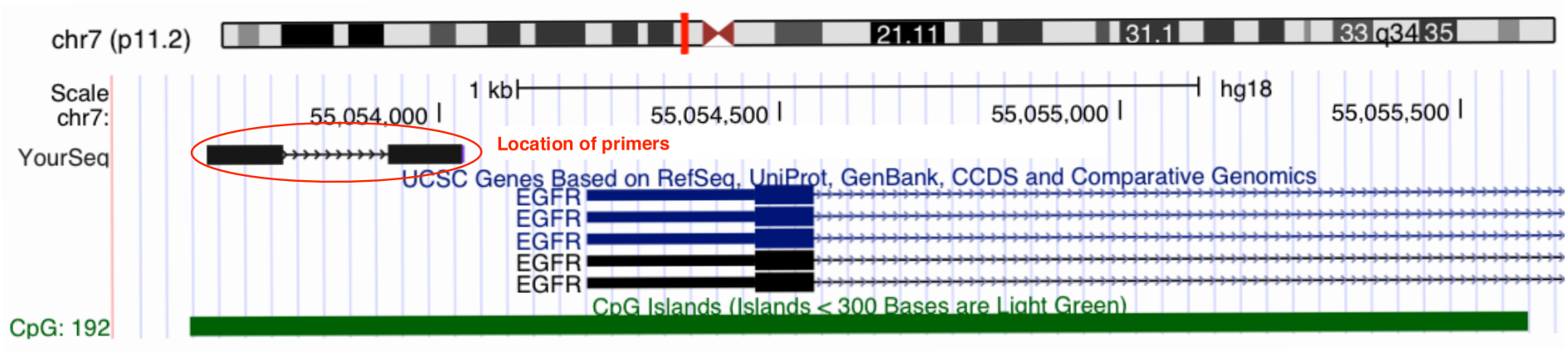
Figure 5.3 ERα knockdown abrogats oestrogen induced EGFR up-regulation.

(A) DH cells were transfected with control and ERα siRNA. Western blot analysis demonstrated effective ERα reduction after the ERα siRNA. (B) Following the siRNA treatment, 1nM of oestrogen was added to cells for 5 days. EGFR and ERα levels were measured at each time points by western blots. As a control, DH cells were treated with non-targeting control siRNA.

To investigate whether ERα directly interacts with the EGFR promoter region, ChIP analysis was performed. The same ERα ChIP protocol used at the pS2 promoter was employed to examine the presence of ERα enrichment after oestrogen stimulation at the EGFR promoter (**Figure 3.11A**). The start of the EGFR promoter was determined by the location of CpG islands (CGIs), as CGIs are generally found around the gene promoter area (**section 1.3.2.1**). Multiple primers were designed around the EGFR promoter region from -751 to -451 (**Figure 5.4A**). ChIP analysis indicated that ERα did not accumulate at the EGFR promoter in response to oestrogen. Instead, there was a decrease in ERα binding at the promoter with the presence of oestrogen. However, the binding of ERα observed at the EGFR promoter could be non-specific binding, as it did not show a significant enrichment relative to IgG control. A representative ChIP result with one set of primer is shown (**Figure 5.4B**).

The binding status of ERα across the *EGFR* gene was further investigated by data mining a genome-wide map of ERα binding sites in MCF-7 cells (Carroll et al., 2006). Classic ERα target gene, for example TFF1, shows vast enrichment of ERα around its promoter and enhancer regions (**Figure 5.5A**). In contrast, careful examination ERα binding upstream, downstream, and across the *EGFR* gene body fails to demonstrate any enrichment (**Figure 5.5B**). Together, the data showed that ERα played an important role in oestrogen induced EGFR up-regulation, though ERα did not directly bind to the EGFR promoter to control its expression.

A


chr7 (p11.2)
21.11
31.1
33
q34
35
Scale
chr7:
1 kb
hg18
55,054,000
55,054,500
55,055,000
55,055,500
YourSeq
Location of primers
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
EGFR
EGFR
EGFR
EGFR
EGFR
CpG Islands (Islands < 300 Bases are Light Green)
CpG: 192

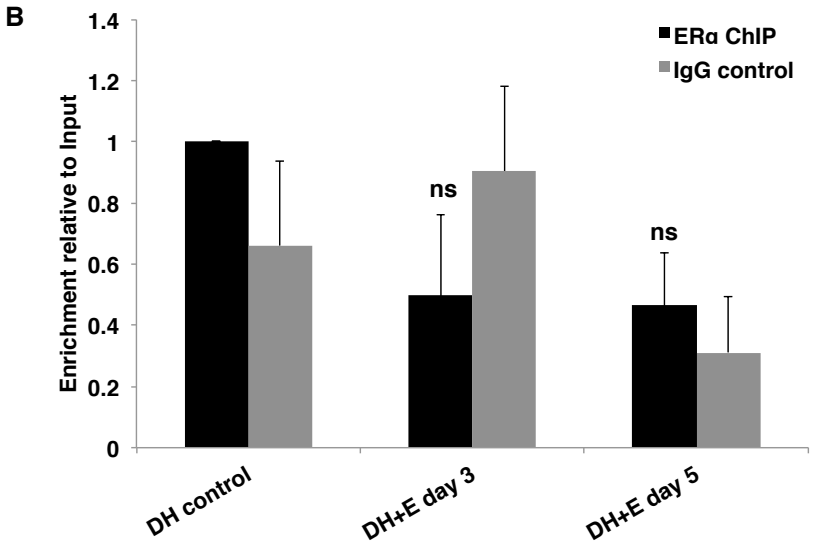
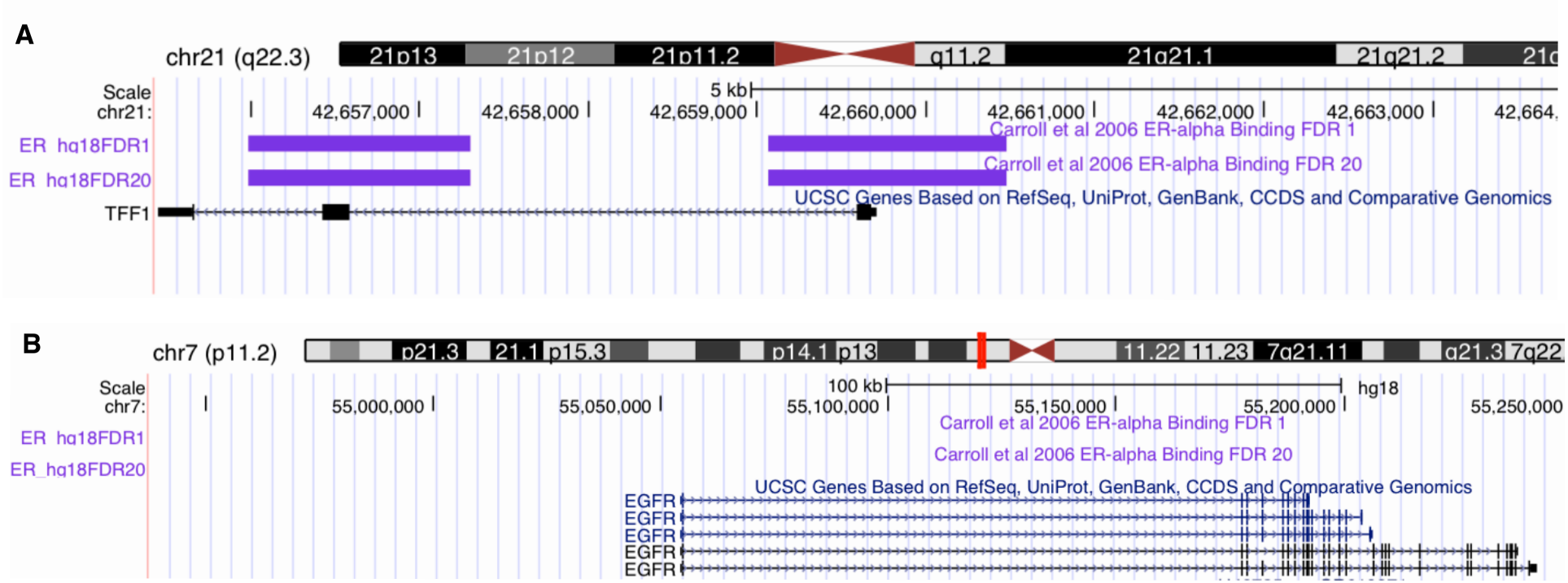


Figure 5.4 ERα ChIP at the EGFR promoter region.

(A) Location of primers designed for ERα ChIP at the EGFR promoter. The figure was produced by the blat function of UCSC genome browser (http://genome.ucsc.edu/). (B) ERα ChIP with the EGFR promoter primer set 3 (**Table 2.8**) on untreated DH cells and cells with 3 or 5 days oestrogen treatment. Each column presents mean of triplicate qPCR analysis for each sample relative to input. Error bars=SD, n=3. Unpaired student's t-test was used to compare ERα ChIP samples between DH+E at different time points vs DH control and showed no significant difference (ns).


A
chr21 (q22.3)
21p13
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ER hg18FDR1
ER hg18FDR20
TFF1
Carroll et al 2006 ER-alpha Binding FDR 1
Carroll et al 2006 ER-alpha Binding FDR 20
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
B
chr7 (p11.2)
p21.3
21.1
p15.3
p14.1
p13
11.22
11.23
7q21.11
q21.3
7q22
Scale
chr7:
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hg18
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55,200,000
55,250,000
ER hg18FDR1
ER hg18FDR20
Carroll et al 2006 ER-alpha Binding FDR 1
Carroll et al 2006 ER-alpha Binding FDR 20
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
EGFR
EGFR
EGFR
EGFR
EGFR

Figure 5.5 ERα binding status across the *TFF1* and *EGFR* gene.

Whole genome ERα ChIP microarray data (ER_hg18FDR1 & ER_hg18FDR20) were obtained from (Carroll et al., 2006). The data were then analysed on UCSC genome browser (http://genome.ucsc.edu/). The oestrogen responsive gene *TTF1* (pS2) was used as a control, where binding of ERα at the promoter and within the gene was observed (purple bars) (A). In contrast, no signals have been detected across the *EGFR* gene body or areas upstream and downstream of the gene (B).

5.3 Oestrogen responsive transcription factors in ER positive breast cancer cells

As ERα played an indirect role in the oestrogen induced EGFR up-regulation, I suspected that there was another transcription factor downstream of ERα, which was able to regulate the expression of EGFR in ER positive breast cancer cells. I therefore looked for a transcription factor in DH cells, which could fit three conditions: oestrogen responsive, downstream of ERα pathway, and directly regulates the expression of EGFR.

A list of oestrogen responsive genes was generated from expression array data performed previously in our lab by Ben Skerry using LCC1 cells. LCC1 is an oestrogen independent ER positive breast cancer cell line derived from MCF-7 cells (detailed in **section 3.7**) LCC1 cells were treated with oestrogen for 4 hours and 24 hours. Genes that were significantly ($p < 0.01$) and consistently up-regulated at both time points was used to produce the oestrogen responsive gene list. GO term analysis was used to find a list of genes involved in transcriptional regulation (http://cbl-gorilla.cs.technion.ac.il/). The oestrogen responsive gene list was combined with the transcription GO term list (GO:0006350) to produce a list of 25 genes (**Figure 5.6**).

A

B

A list of estrogen responsive transcription factors

CARM1, MYB, PHF5A, E2F2, PTTG1, CTNNB1, TYMS, KLF10, ZNF138, THOC4, SMARCC1, CRABP2, DNMT1, RRM2, PRDX3, CSTF1, SNRPD3, SUV39H1, PPM1A, ECD, AGER, SNRPB, PMF1, ARID4A, NFATC2IP

Figure 5.6 Venn diagram of oestrogen responsive transcription factors.

Oestrogen responsive gene list (grey) was combined with the transcription factor GO term (silver) to get a list of 25 genes (A), which was shown in figure B.

Among the 25 genes, *MYB* was identified as a particularly interesting gene, as its expression significantly correlates with the presence of ERα in breast cancer (Guerin et al., 1990). *MYB* is an oestrogen responsive gene whose transcription appears to be predominantly regulated by elongation control. In ER positive MCF-7 cells, liganded ERα binds directly to a site adjacent to the attenuation region to relieve the elongation arrest of *MYB* transcription (Drabsch et al., 2007). Therefore, the expression of c-Myb is downstream of ERα in response to oestrogen making c-Myb a promising transcription factor for linking ERα dependent EGFR expression in DH cells.

5.4 *MYB* is an ERα regulated oestrogen responsive gene in DH cells

To confirm that *MYB* was an oestrogen responsive gene in DH cells, cells were treated with 1nM of oestrogen for 5 days and changes in expression were measured by qRT-PCR (**Figure 5.7A**). The mRNA level of c-Myb went up 24 hours after the oestrogen treatment, which kept increasing for two days before falling off on day 4. Changes in c-Myb expression as well as A-Myb and B-Myb were also analysed by DH cells expression arrays at different time points (**Figure 5.7B**). Consisting with the qRT-PCR result, the data showed that A-Myb and c-Myb were up-regulated after 2 days of oestrogen treatment. After 5 days of oestrogen stimulation, the expression of both transcription factors began to fall back to the basal level. In contrast, the expression of B-Myb did not vary significantly in response to oestrogen.

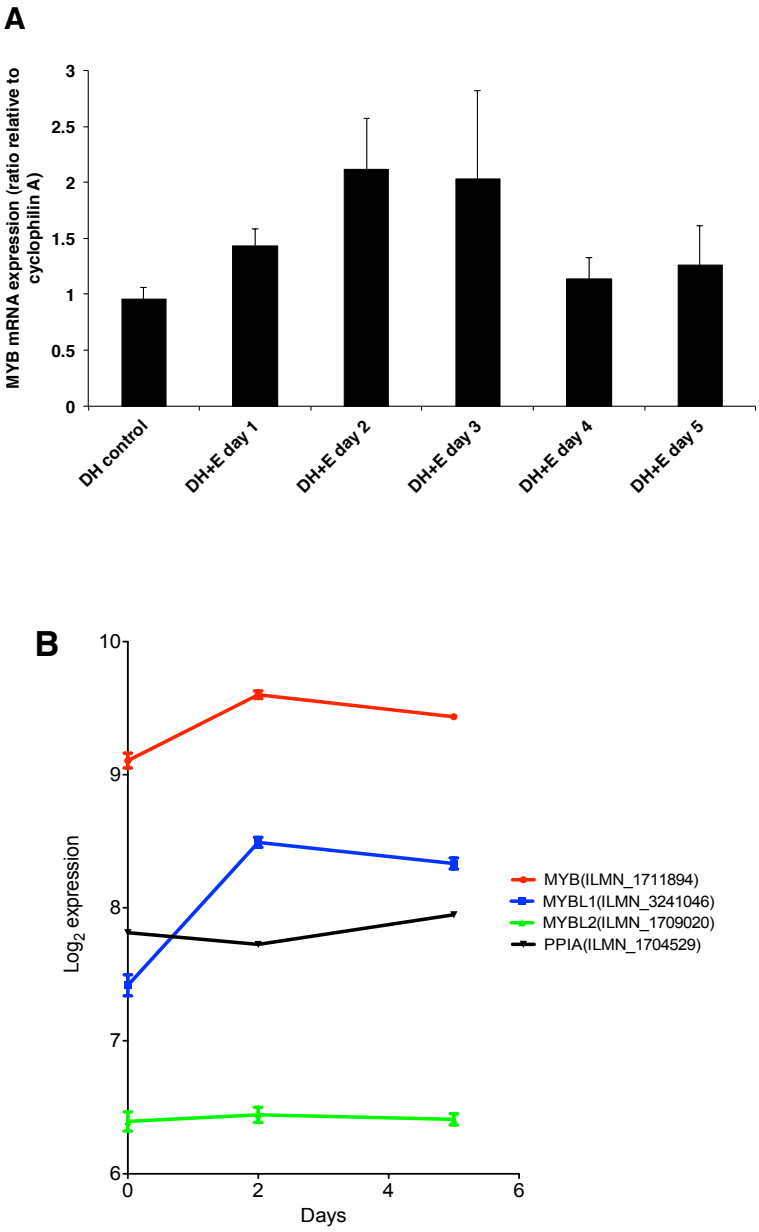


Figure 5.7 Expression of *MYB* in DH.

(A) DH cells were plated with 1nM oestrogen for 5 days and samples were taken to investigate the expression level of c-Myb using qRT-PCR. Each column presents mean of triplicate qPCR analysis for each sample relative to cyclophilin A (gene name PPIA) expression. Error bars=SD. (B) Log_2 expression data of *MYB, MYBL1, MYBL2,* and *PPIA* from the expression array (**section 4.5**). Corresponding microarray probes are indicated in the inset. Error bars=SD.

In order to test whether ERα was required for c-Myb expression in DH cells, cells were treated with different ERα inhibitors (**Figure 5.8C**). The 'pure' ERα inhibitor, ICI, significantly down-regulated the level of ERα level after 48 hours treatment. In

contrast, treating cells with the partial agonist, tamoxifen, was not able to reduce the level of ERα in cells. Therefore, ICI was used to exam the function of ERα in oestrogen responsive c-Myb expression in DH cells. 1 and 10 nM of oestrogen was added to DH cells with or without the presence of ICI, and c-Myb protein level was analysed by western blotting (**Figure 5.8A**). Samples were collected 24 and 48 hours after different treatments. The protein level of c-Myb increased progressively after 24 and 48 hours of oestrogen treatment. However, 10nM of oestrogen did not provide any further increase in c-Myb expression, suggesting 1 nM of oestrogen might be sufficient to saturate ERα in cells. As expected, the c-Myb up-regulation was significantly blocked by the presence of ICI when DH cell were treated with 1 nM of oestrogen for 48 hours (highlighted by dashed line in **Figure 5.8A** and quantified in **Figure 5.8 B**). While, the c-Myb level in DH cells treated with 10 nM of oestrogen was not affected to the same extent by ICI as cells in 1 nM of oestrogen. This may because the excess oestrogen has the ability to competitively display ICI from ERα and reactivate to a transcriptionally active form (Wardell et al., 2011).

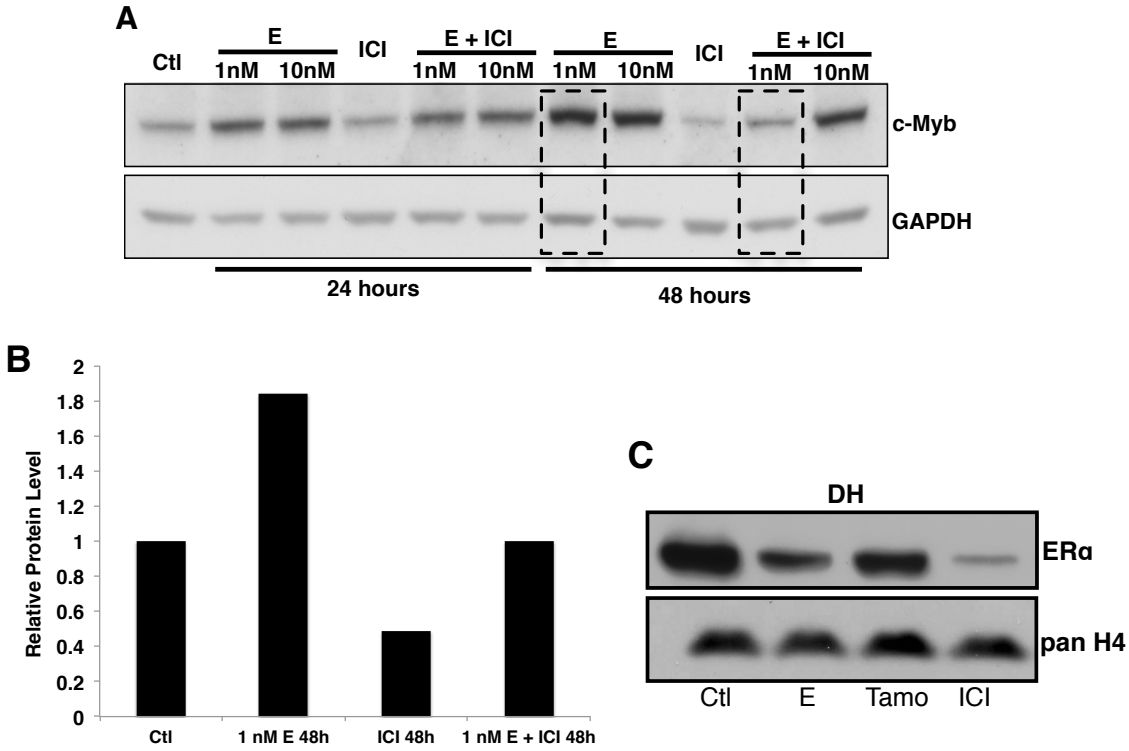


Figure 5.8 ERα was required for c-Myb expression in ER positive breast cancer cells.

(A) DH cells were treated with 1 and 10nM of oestrogen with or without the presence of 100 nM of ICI. Protein samples were collected 24 and 48 hours of the treatment as indicated in the figure. Ctl represents un-treated control DH cells. Western blotting was used to analyse the protein level of c-Myb. GAPDH was used as a loading control. (B) Quantification of the western blot showing the c-Myb protein levels in control DH cells and cells with 48 hours of treatment with 1 nM oestrogen and/or 100 nM of ICI. (C) Western blots showing the protein level of the total ERα after treating DH cells with 1 nM of oestrogen or 1 of μM tamoxifen or 100 nM of ICI for 48 hours.

It has been reported in the MCF-7 cells that the transcription of c-Myb is blocked in a region between 1.4 kb and 2.2 kb away from the start of intron 1. This region contains a SL-poly(dT) motif that blocks the elongation of the RNAP II. After oestrogen stimulation, ERα binds directly to the attenuation site in order to relieve the paused c-Myb transcription (Drabsch et al., 2007). ERα ChIP was performed in DH cells at different time points after oestrogen treatment (**Figure 5.9**). The result confirmed that ERα gradually accumulated at the previously reported attenuation site to stimulate the transcription of c-Myb. Together, this data demonstrated that *MYB* was an oestrogen responsive gene whose expression was dependent on the presence

of ERα in DH cells. Furthermore, ERα bound to the c-Myb transcriptional attenuation site in response to oestrogen to stimulate its expression in DH cells.

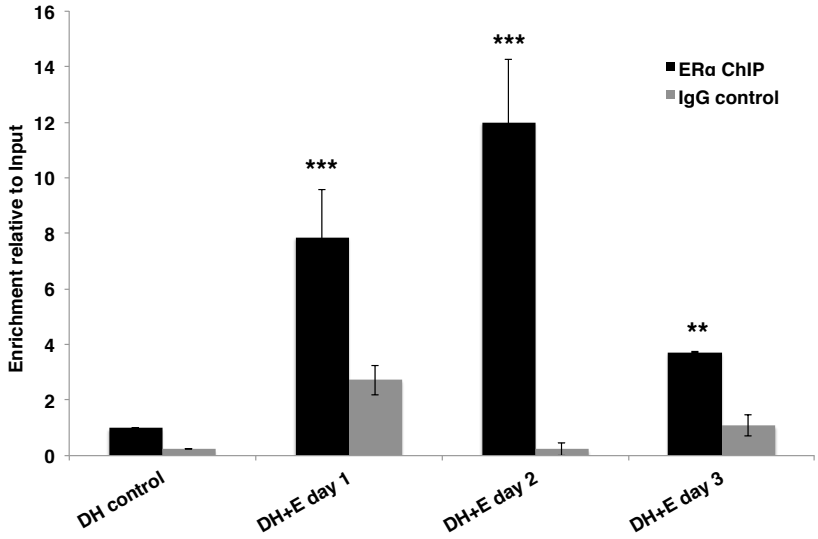


Figure 5.9 ERα ChIP at *MYB* gene intron 1 site.

ERα ChIP was performed on oestrogen treated DH cells adjacent to the *MYB* transcriptional attenuation site. IgG antibody was used as a negative control for the ChIP. Data were presented as mean of triplicate qPCR analysis for each sample relative to input. Error bars=SD, n=3. Unpaired student's t-test was used to compare ERα ChIP samples between DH+E at different time points vs DH control, **P<0.01; ***P<0.001.

5.5 c-Myb siRNA abrogates the oestrogen induced EGFR up-regulation

To test whether oestrogen induced EGFR up-regulation was c-Myb dependent, siRNA was employed to reduce the c-Myb protein level before treating cells with oestrogen. c-Myb siRNA efficiently knocked down the basal c-Myb protein level in DH cells (**Figure 5.10A**). Oestrogen was then added to cells for 5 days after the siRNA treatment. The protein levels of EGFR and c-Myb were measured every day by western blotting (**Figure 5.10B**). The results showed that the level of c-Myb remained low over the oestrogen time course, though an increase in c-Myb protein level on day 2 was observed in both control and c-Myb siRNA treated cell. Furthermore, c-Myb depletion by siRNA abrogated the oestrogen induced EGFR up-regulation in DH cells (**Figure 5.10C**). As expected, control siRNA treated cells showed a gradual increase in EGFR level upon oestrogen treatment. Collectively, the

results indicated the oestrogen induced EGFR up-regulation in DH cells was c-Myb dependent.

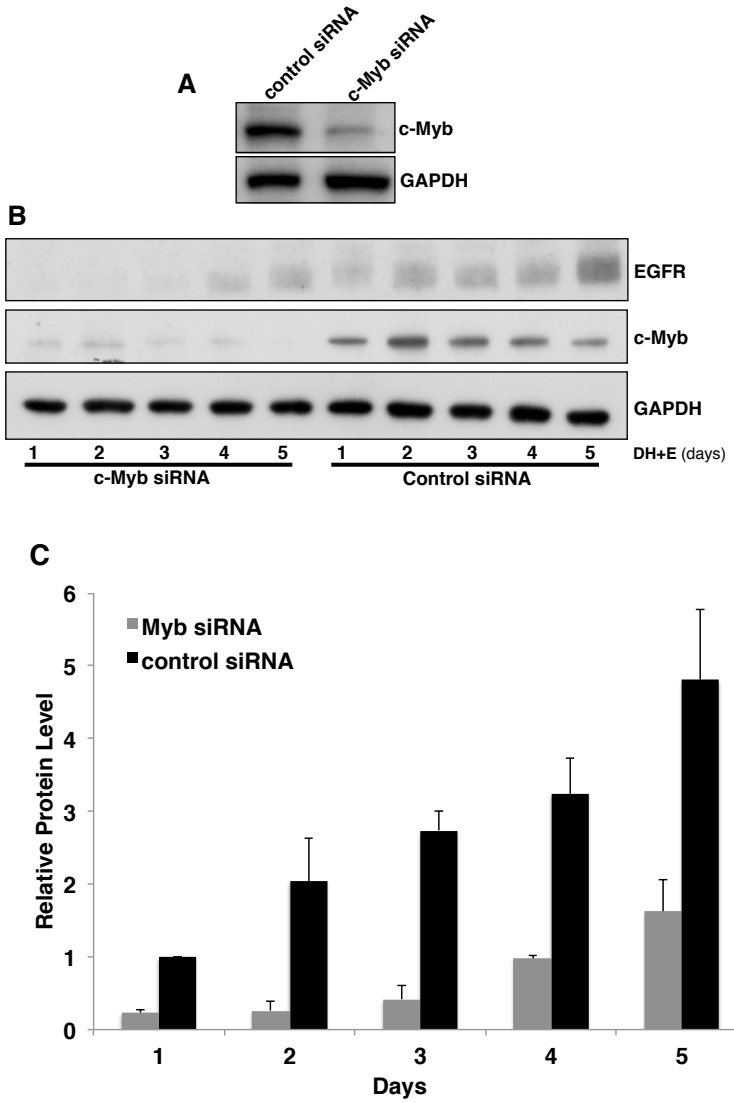


Figure 5.10 Effects of c-Myb siRNA in oestrogen induced EGFR up-regulation in DH cells.

(A) DH cells were transfected with control or c-Myb siRNA. Western blot analysis demonstrated effective reduction of c-Myb protein level after siRNA treatment. (B) Following the siRNA treatment, DH cells were plated with 1nM oestrogen for a 5-day time course experiment. Protein levels of EGFR and c-Myb levels were measured at each time points using western blots. GAPDH was used as a loading control. (C) Quantification of western blots showing relative change in EGFR level in control and c-Myb knockdown cells after oestrogen stimulation. Data are shown as means ± SD, n=3.

5.6 Overexpression of c-Myb causes up-regulation of EGFR in DH cells

To determine whether c-Myb up-regulation was sufficient to promote EGFR expression in DH cells without the presence of oestrogen, DH cells were transiently transfected with human c-Myb cDNA and the empty vector control (**Figure 5.11A**). Overexpression of c-Myb resulted in an increase in the endogenous EGFR protein level 24 hours post transfection (**Figure 5.11B**). The data further supported the hypothesis that c-Myb was a novel regulator of *EGFR* gene expression in DH cells. However, compared with the massive increase in c-Myb protein level after the transient transfection, c-Myb overexpression did not cause a vast increase in EGFR protein level. This implied that other factors might be involved in the transcriptional regulation of EGFR expression in response to oestrogen. However, longer time points will be required for future experiments, as a 24-hour of c-Myb overexpression might be too short to see a significant up-regulation of EGFR.

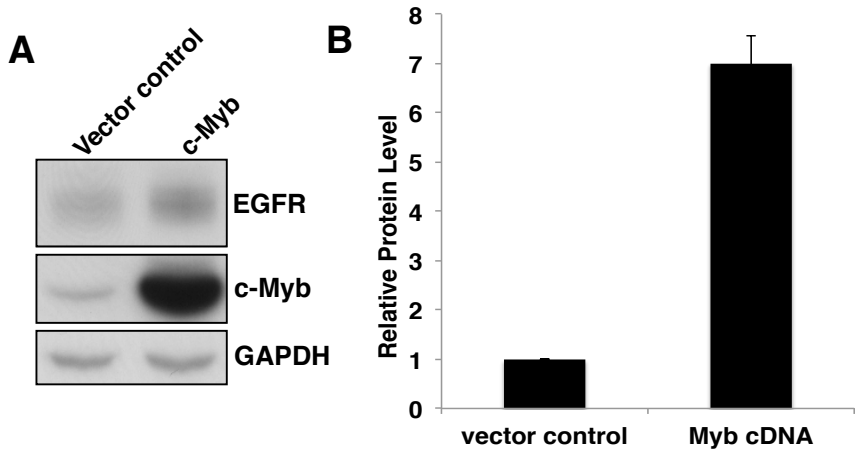


Figure 5.11 Overexpression of c-Myb in DH cells.

(A) DH cells were transiently transfected with vector control and human c-Myb cDNA. Cells lysates were collected 24 hours after the transfection to measure the level of EGFR and c-Myb using western blot. GAPDH was used a loading control. (B) Relative protein levels of EGFR after c-Myb overexpression. Data are shown as means ± SD.

5.7 c-Myb binds upstream of EGFR promoter in response to oestrogen

To investigate whether c-Myb binds directly to the EGFR promoter, c-Myb ChIP was performed on DH cells after the addition of oestrogen. Primers designed close to the EGFR promoter failed to show significant enrichment for c-Myb in response to oestrogen (data not shown). Recently, endogenous c-Myb binding sites were mapped in MCF-7 cells by a genome wide ChIP-on-chip study (Quintana et al., 2011). Data from their study were analysed using the UCSC genome browser to detect c-Myb binding sites around the EGFR promoter. The result indicated that c-Myb bound to a region 80 kb upstream of the EGFR promoter (**Figure 5.12**). Primers were designed within this region to look for c-Myb binding. The ChIP results in DH cells and MCF-7 cells showed that c-Myb transiently localized upstream of the EGFR promoter to regulate its expression on day 2, which was then lost from the binding site after 5 days of oestrogen stimulation (**Figure 5.13**). Interestingly, the binding of c-Myb around the EGFR promoter area showed similar kinetics compared to the binding of ERα at *MYB* intron 1 site (**Figure 5.9**) and the change in expression level of c-Myb in response to oestrogen (**Figure 5.7**). These data therefore implied a dynamic recruitment of transcription factors during the oestrogen induced EGFR up-regulation in DH cells. Firstly, ERα accumulated at the *MYB* transcriptional attenuation site to activate its expression. The expression of c-Myb peaked on day 2 after oestrogen addition. c-Myb was then loaded onto a region upstream of EGFR promoter to activate its transcription. After the initial stimulation by c-Myb, the expression of EGFR was maintained, despite loss of the c-Myb. This implies that other mechanisms are employed in the maintenance of EGFR expression.

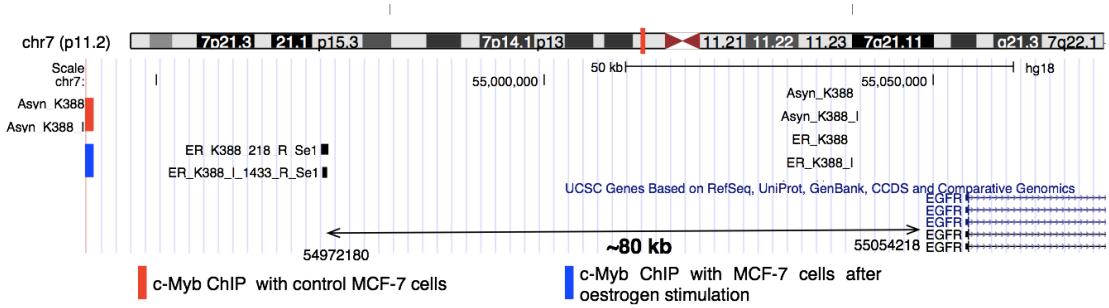


Figure 5.12 Binding of c-Myb around EGFR promoter area in MCF-7 cells.

c-Myb binding site according to the c-Myb ChIP-on-chip data was analysed (Quintana et al., 2011). Duplicate results were obtained from control MCF-7 cells (red bar) and cells stimulated with 10 nM of oestrogen for 24 hours after 48 hours of oestrogen deprivation (blue bar). The results showed that c-Myb bound 80 kb upstream of EGFR promoter after oestrogen stimulation. Black bars in the figure represented the position of predicated c-Myb binding site. c-Myb binding was not detected in control MCF-7 cells. The data were analysed on UCSC genome browser (http://genome.ucsc.edu/).

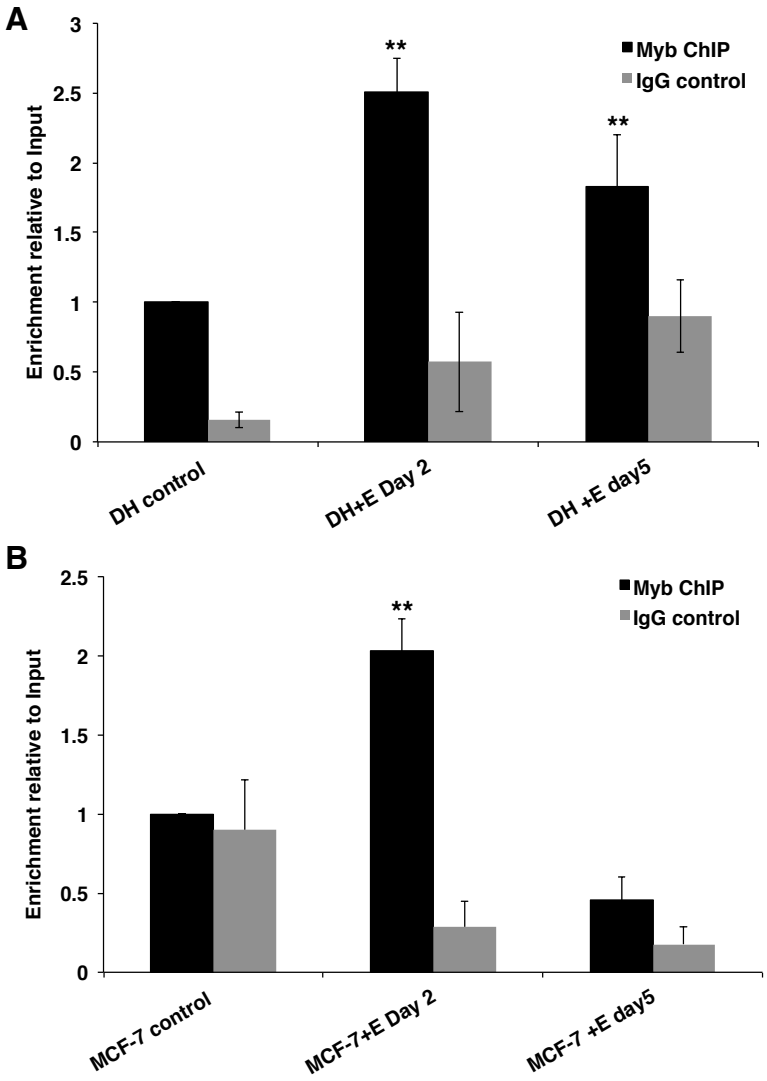


Figure 5.13 c-Myb ChIP at the EGFR upstream binding region in response to oestrogen.

c-Myb ChIP was performed on DH (A) and MCF-7 (B) cells 2 and 5 days after oestrogen stimulation. IgG antibody was used as a negative control for the ChIP. Data were presented as mean of triplicate qPCR analysis for each sample relative to input. Error bars=SD, n=3. Unpaired student's t-test was used to compare c-Myb ChIP samples between DH+E at different time points vs DH control, **P<0.01.

5.8 Epigenetic regulations at EGFR locus

In order to survive, cells need to response quickly to changes in their external growth environment. This is partially regulated at the transcriptional level, which results in rapid alterations in gene expression after stimulation. However, once the stimulus is removed, the induced changes in gene expression should quickly return to its basal

state unless there are mechanisms involved to maintain the changes in gene expression. Epigenetic modifications such as histone modifications or DNA methylation around the promoter or enhancer area of target genes not only have the ability to facilitate transcription initiation but also stably maintain the expression pattern after the initial stimulation (**section 1.3.2**). In the previous section, c-Myb was shown to be a transcriptional activator which was required for the oestrogen induced EGFR up-regulation in DH and MCF-7 cells. c-Myb bound transiently to a region upstream of EGFR promoter to stimulate its expression. Interestingly, once up-regulated, EGFR expression was maintained despite oestrogen withdrawal and displacement of the transcriptional activator indicating a possible involvement of the epigenetic reprogramming at the EGFR locus to maintain the high expression of EGFR in DH cells. In this section, I discuss the possible mechanisms that could lead to a stable EGFR up-regulation upon oestrogen stimulation.

5.8.1 Histone modifications at the EGFR locus after oestrogen stimulation

Histone acetylation was the first histone modification that I investigated in DH cells after oestrogen treatment. ChIP analysis was used to study the change in pan histone H4 acetylation at the EGFR promoter during the oestrogen treatment. Oestrogen gave a transient increase in the level of pan acetylated H4 on day 3. However, the increase in H4 acetylation was not sustained on day 5 (**Figure 5.14**). The transient increase in H4 acetylation may be due to the recruitment of co-activators containing HAT activity by c-Myb after oestrogen stimulation. I then went on to investigate other alterations in histone modifications that might lead to the stable oestrogen induced EGFR up-regulation in DH.

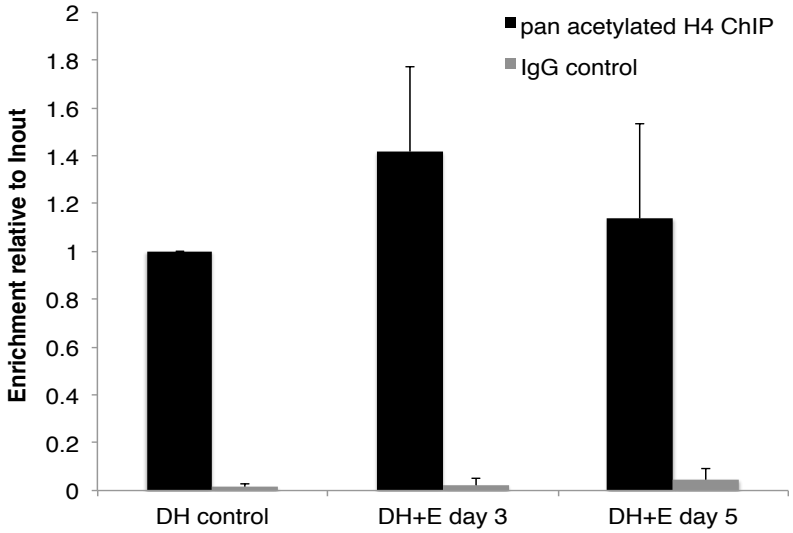


Figure 5.14 Change of H4 acetylation at the EGFR promoter in response to oestrogen.

H4 acetylation at the EGFR promoter was determined by ChIP on untreated DH cells and cells treated with oestrogen for 3 and 5 days. IgG antibody was used as a negative control for the ChIP. Data were presented as mean of triplicate qPCR analysis for each sample relative to input. Error bars=SD, n=3.

Native ChIP was used to analyse different histone modifications at the EGFR locus during the oestrogen time course. Samples collected 1 and 2 days after the oestrogen treatment (DHE1 and DHE2) were used to detect early changes in the histone modifications induced by oestrogen. Whilst, data collected from DHE5 and DHE5R5 samples should uncover late and stable changes in histone modifications. Four histone marks represent active, repressive and bivalent histone modifications were studied at the EGFR promoter region and the c-Myb binding region upstream of EGFR promoter.

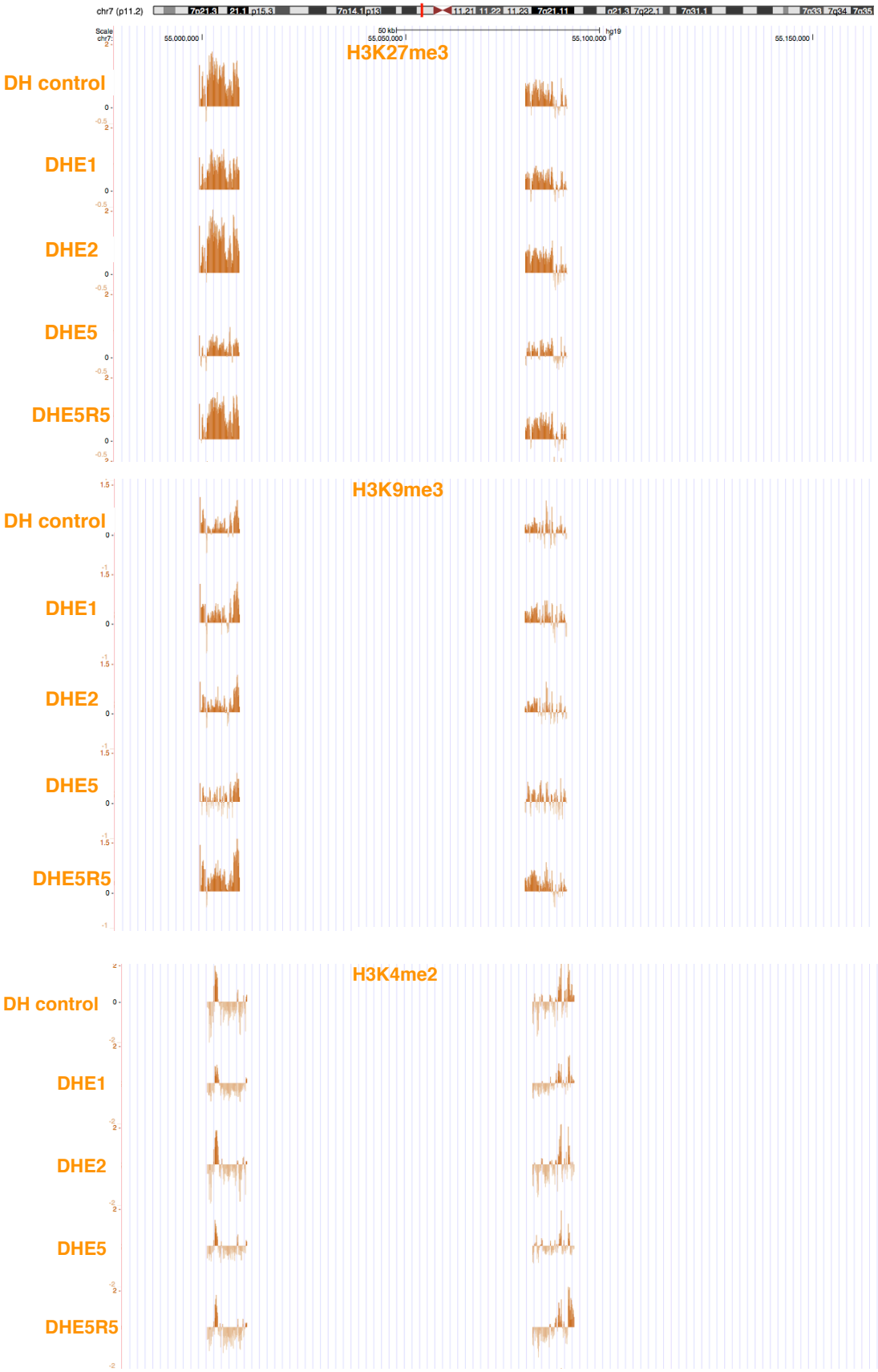
H3K4me2 is an active histone mark, which gives a bimodal distribution at the transcription start site of active promoters. Interestingly, there was a decrease rather than increase of H3K4me2 modification at the EGFR promoter after 5 days of oestrogen treatment (**Figure 5.15**). However, the H3K4me2 peak came back after oestrogen was removed from DH cells for 5 days. In the meantime, H3K4me2 gave a single peak at the c-Myb binding site that did not vary significantly during the

oestrogen treatment. H3K9Ac is another active histone mark that was studied at the EGFR locus. DH cells had low H3K9Ac modification at the c-Myb binding site. Again, a gradual decrease in H3K9Ac modification at the EGFR promoter was observed in DH cells with oestrogen, which did not recover when oestrogen was removed from the culture media. The decrease in active histone marks did not fit well with the increase in EGFR expression observed after oestrogen stimulation. Further investigations are required to determine whether there was a general decrease in active histone modifications at the EGFR promoter in response to oestrogen, or it was only specific to these two histone modifications.

H3K27me3 is a bivalent histone mark, which is associated with gene repression (Lee et al., 2006). Furthermore, a higher H3K27me3 histone modification is detected at silent promoters than at active promoters (Barski et al., 2007). A transient reduction was detected at both the promoter and c-Myb binding site after 5 days of oestrogen treatment.

H3K9me3 at the promoter region is associated with transcriptional repression. Methylation at H3K9 recruits binding of HP1 that is important for the maintenance of the heterochromatin state (Zhang and Reinberg, 2001). No significant changes was detected at the EGFR promoter region for the repressive histone mark, H3K9me3. Whilst, a significant reduction in H3K9me3 modification was observed at the c-Myb binding site after 5 days of oestrogen treatment. Like the decrease in H3K27me3 at the c-Myb binding site on day 5, the decrease in H3K9me3 was not maintained after the removal of oestrogen.

To summarise, DHE5 cells showed a decrease in the examined active histone modifications at the promoter region of EGFR, and a reduction of bivalent and repressive histone mark were observed at the c-Myb binding region upstream of EGFR promoter. However, none of the four histone modifications examined at the EGFR locus were stably maintained after the removal of oestrogen.


chr7 (p11.2)
H3K27me3
DH control
DHE1
DHE2
DHE5
DHE5R5
H3K9me3
DH control
DHE1
DHE2
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DHE5R5
H3K4me2
DH control
DHE1
DHE2
DHE5
DHE5R5

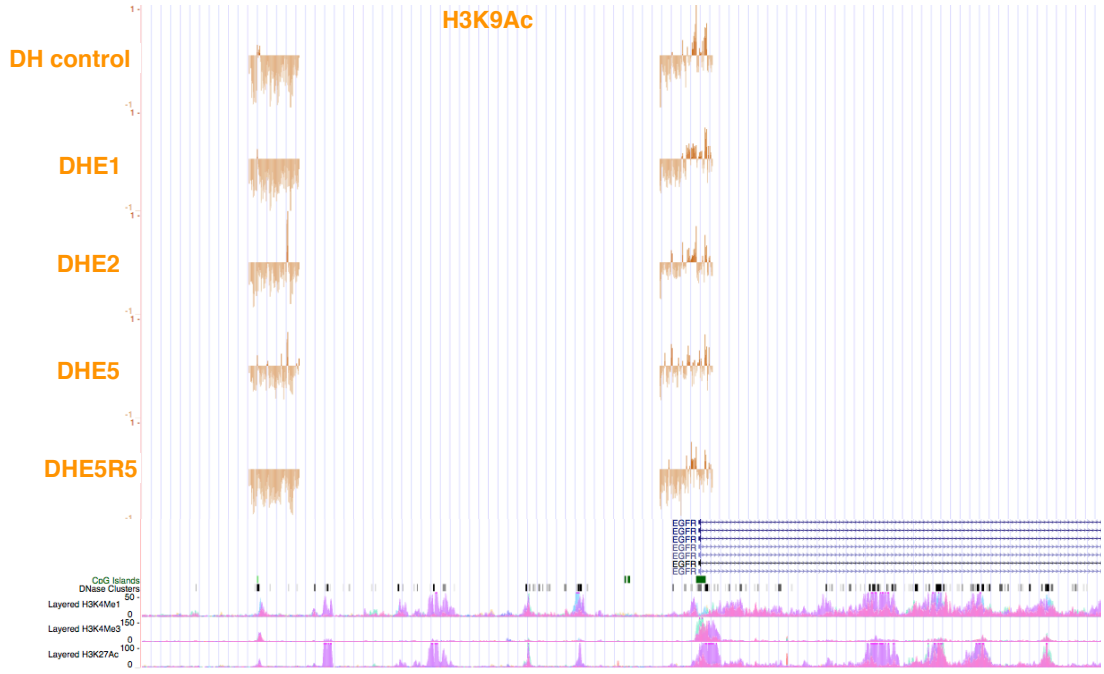

Figure 5.15 Histone modification patterns of the EGFR gene.

Native ChIP analysis with H3K4me2, H3K9Ac, H3K27me3, and H3K9me3 were performed at the EGFR promoter and the c-Myb binding site 80 kb upstream of the EGFR gene on untreated DH cells as well as cells with 1 or 2 or 5 days of oestrogen treatment, and cells have oestrogen removed from the culture media for another 5 days after 5 days of oestrogen treatment. The data were shown as mean of duplicate ChIP results, which were analysed as custom tracks on the UCSC genome browser with indicated histone modification and time points (orange).

5.8.2 DNA methylation of the EGFR promoter after oestrogen addition in DH cells

As a subtle increase in EGFR expression was observed after oestrogen stimulation, I investigated whether a decrease in EGFR promoter methylation could be responsible for the up-regulation of EGFR expression in DH cells. The EGFR promoter DNA methylation status in control and DH cells with 5 days of oestrogen treatment (DHE5) were investigated by bisulphite sequencing. Primers were designed to cover around a 1 kb region from the beginning of the CpG islands to the end of first exon. The EGFR promoter was mostly methylated at the edge of the CpG islands in both control DH cells and DHE5 cells (**Figure 5.16A**). Whilst, the EGFR promoter became mostly unmethylated when moving towards the transcription and translation start site regardless the presence of oestrogen (**Figure 5.16B**). Therefore, overall there was not a change in the promoter methylation status to maintain the high

expression of EGFR after oestrogen stimulation in DH cells. One un-expected result was that two of the cytosine residues in the EGFR promoter became methylated after 5 days of oestrogen treatment according to the bisulphite sequencing. This result is difficult to explain, as an increase in gene expression is usually associated with a decrease in promoter methylation rather than an increase.

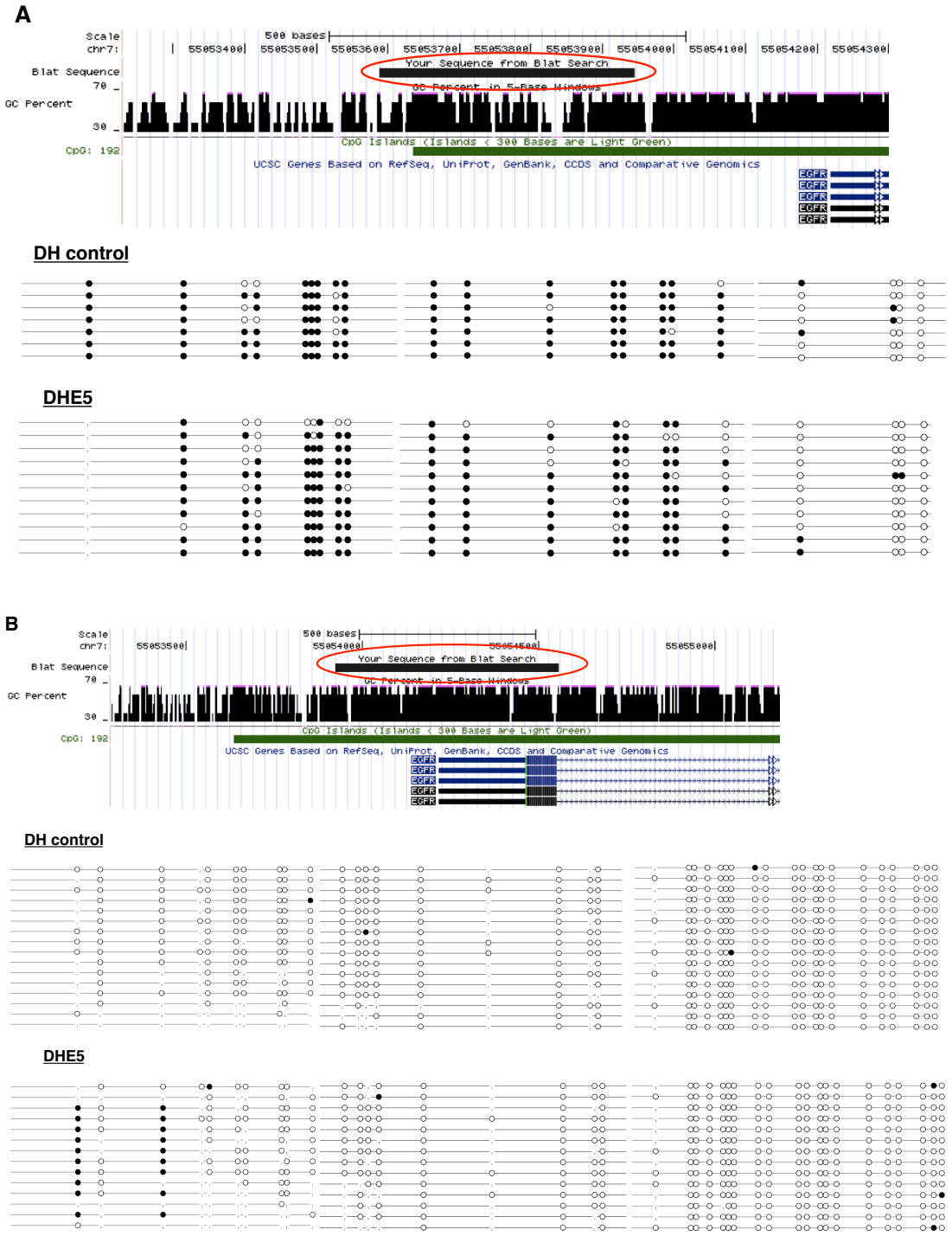

Figure 5.16 DNA methylation at the EGFR promoter in DH cells.

Bisulphite sequencing was performed at two different places around the CpG island of the EGFR promoter on untreated DH cells and cells with 5 days of oestrogen treatment. The area covered by the bisulphite sequencing was circled in red and showed by the UCSC genome browser track. Methylation data at the EGFR promoter was presented as lollipop diagram with each lollipop represented an individual CpG dinucleotide. Filled and open circles represented methylated sites and unmethylated sites respectively.

5.9 Discussion

In this chapter, the molecular mechanism of the oestrogen induced EGFR up-regulation in DH cells was investigated. c-Myb was identified as a novel transcription factor that regulated the expression of EGFR in long-term oestrogen deprived DH cells. In addition, ChIP analysis revealed a direct interaction between c-Myb and the *EGFR* gene, where c-Myb transiently bound to a region upstream of the EGFR promoter in response to oestrogen (**Figure 5.13**). It has been shown in the literature that the expression of c-Myb was regulated by ERα in ER positive breast cancer cells (Ramsay and Gonda, 2008). This was confirmed using DH cells, as ERα was enriched at previously reported c-Myb transcriptional attenuation site after oestrogen stimulation (**Figure 5.9)**; and the pure ERα inhibitor ICI was able to block the oestrogen induced expression of c-Myb in DH cells (**Figure 5.9)**. Collectively, the data outline a novel mechanism of how the expression of EGFR was regulated in ER positive breast cancer cells (**Figure 5.17**).

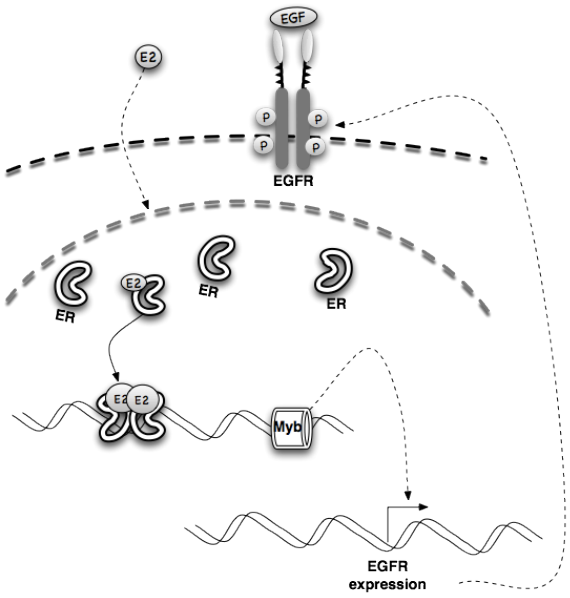

Figure 5.17 Mechanism for oestrogen induced EGFR up-regulation in DH cells.

After oestrogen stimulation, liganded ERα stimulates the transcription of c-Myb. c-Myb then binds transiently to a region upstream of EGFR promoter to promoter its expression.

As EGFR expression was maintained when oestrogen was no longer present in the growth media, epigenetic modifications at the EGFR locus were suspected to be responsible for the stable expression of EGFR after 5 days of oestrogen stimulation. Histone modifications and DNA methylation were studied at multiple oestrogen time points at the EGFR promoter and the c-Myb binding region upstream of the promoter. However, the data I collected so far failed to identify the epigenetic mechanism that could explain the high EGFR expression after oestrogen stimulation. Therefore, further experiments are required to clarify the mechanisms involved in the oestrogen induced stable up-regulation of EGFR in DH cells.

The c-Myb binding site upstream of *EGFR* promoter was further analysed using the UCSC genome browser. Enhancer associated histone modifications were mapped around the c-Myb binding region using database available on the genome browser. The result indicated that the c-Myb binding site is located in close proximity to an active enhancer region upstream of EGFR (**Figure 5.18**), as high level of H3K4me1 and H3K27Ac combines with low H3K4me3 is suggestive of a signature for an active enhancer (Heintzman et al., 2007, Creyghton et al., 2010). Enhancers are DNA sequences that play important roles in transcriptional regulation. They serve as platform for a range of transcription factors to bind. Although they can act independent of their location, distance or orientation with respect to the promoters of genes, transcription factor bound enhancers will generally loop close to the promoter to interact with the PIC during gene activation (Maston et al., 2006). Apart from being a docking site for transcription factors in response to stimuli, enhancers can also carry epigenetic information as distinctive histone modifications that may serve as marks for differentiation and future gene activation (Heintzman et al., 2009, Ong and Corces, 2012).

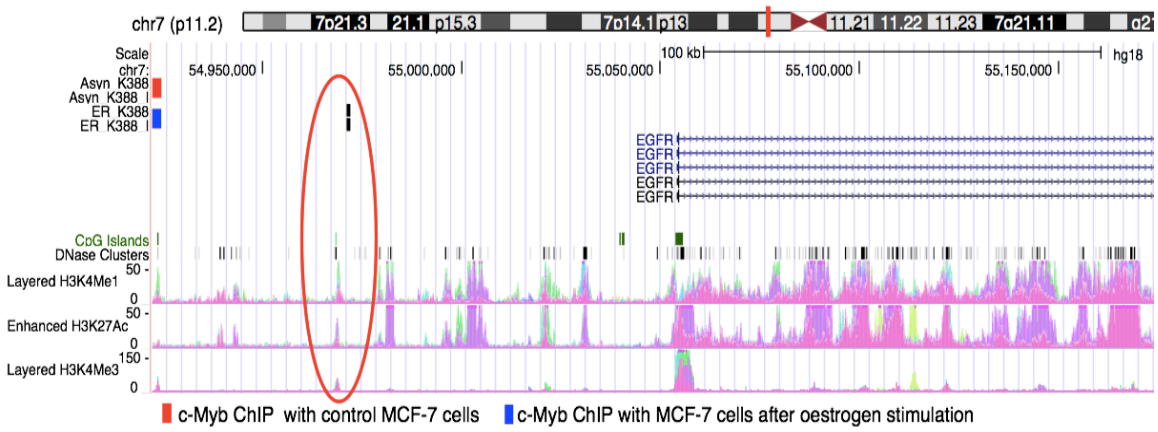

Figure 5.18 Bioinformatic analysis of the c-Myb binding site upstream of EGFR.

Oestrogen induced c-Myb binding site around the *EGFR* gene according to the c-Myb ChIP-on-chip data was described in figure 5.12. The data was further analysed using the UCSC genome browser to map the locations of the enhancer associated histone modifications (H3K4me1 and H3K27Ac and H3K4me3) in 8 different cell lines (http://genome.ucsc.edu/). The result indicated the c-Myb binding sat closely to active enhancer upstream of the EGFR gene (red oval).

A pulse of oestrogen was used to trigger the c-Myb and EGFR expression in DH cells. However, patients resistant to aromatase inhibitors would unlikely start to produce oestrogen. Other mechanisms must be involved in the ER positive breast cancer cells to up-regulate the expression of c-Myb and its downstream target in a low oestrogen environment. Expression data collected from MCF-7 cells deprived of oestrogen between 0 and 180 days reveal that the expression levels of ERα as well as oestrogen responsive genes like *TFF1* and *MYB* are able to recover after a initial decrease caused by the removal of oestrogen (**Figure 5.19**). This evidences the presence of oestrogen independent c-Myb up-regulation in ER positive breast cancer cells (Aguilar et al., 2010). Meanwhile, mechanisms have been proposed in other tissue compartment to regulate the expression of c-Myb. During T cell activation, the expression of c-Myb is induced by activation of IL-2 signalling, which is acting through the PI3K pathway (Lauder et al., 2001). Furthermore, c-Myb plays important role in hematopoietic cell differentiation. High c-Myb expression is essential for the proliferation of hematopoietic progenitor cells whereas the c-Myb level decreases when cells undergo differentiation (Mucenski et al., 1991). In hematopoietic

progenitor cells, the high expression level of c-Myb is maintained by the nuclear NF-*k*B complexes (Suhasini and Pilz, 1999).

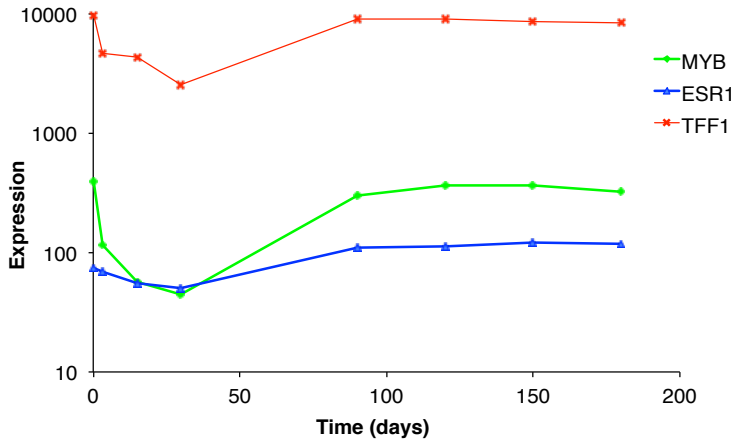

Figure 5.19 Change in gene expression during oestrogen deprivation in MCF-7 cells.

Expression profiles of *MYB*, *ESR1* and *TFF1* were obtained from a study performed in MCF-7 cells during the acquisition of oestrogen independent growth (Aguilar et al., 2010). Oestrogen was taken away from MCF-7 cells for a period of 180 days. The expression data were collected at multiple time points during the process to look at the dynamics of transcriptomic changes. The raw expression values of each gene were plotted on a $\log_{10}$ scale.

I have demonstrated in the previous chapter that EGFR up-regulation in the oestrogen deprived DH cells could lead to survival pathway switching and tamoxifen resistance (**section 4.6**). As the up-regulation of EGFR expression is c-Myb dependent, c-Myb could be a good drug target to prevent the acquired drug resistance through activation of growth factor signalling. Moreover, inhibition of *MYB* expression with shRNA or antisense oligonucleotides (Opalinska et al., 2004) reveals that c-Myb is necessary for the oestrogen dependent proliferation of ER positive but not ER negative breast cancer cell lines by blocking cell cycle progression from the G_1/S and G_2/M phases (Drabsch et al., 2007). This further supports the idea that c-Myb inhibitors could be used as a supplementary treatment for ER positive breast cancers to avoid tumour relapse.

Chapter 6 Conclusions

6.1 Summary

Acquired drug resistance occurring during endocrine therapy remains one of the biggest challenges in breast cancer treatment. Despite extensive studies, the precise molecular mechanisms driving the drug resistance remain unclear. The activation of extracellular growth factors signalling has been suggested in the literature as one of the underline reasons of acquired drug resistance in breast cancer cells. In this study, I have generated a novel oestrogen and serum free ER positive breast cancer cell line called DH. As DH cells were cultured in a defined serum-free medium with known oestrogen concentration, it enabled me to investigate the molecular mechanism that triggered pathways crosstalks between ERα and extracellular growth factors signalling in a controlled environment. However, culturing cells in a serum-free environment made DH cells a less favourable model for mimicking the responses in patients.

By studying DH cells, I found that long-term oestrogen deprivation caused ERα overexpression in cells (**Figure 3.10)**. Furthermore, a high basal level of un-liganded ERα was found around the promoter of oestrogen responsive gene in DH cells (**Figure 3.13**). As a result, DH cells were highly relied on the ERα pathway for growth (**Figure 3.5**). The same observation has also been reported using other oestrogen deprived models suggesting up-regulation of the steady-state ERα protein level could be a general mechanism for cells to survive through oestrogen deprivation in ER-positive breast cancer cells.

Using DH cells, I demonstrated that signalling pathways in breast cancer cells were dynamically regulated in response to alterations in extracellular oestrogen concentration. A 5-day oestrogen pulse could cause a stable up-regulation of EGFR expression together with the down-regulation of ERα in DH cells. As a consequence, DH cells became less dependent on the ERα survival pathway but more dependent on the EGFR mediated signalling pathway. Global expression analysis of EGFR mediated MAPK pathway downstream target genes revealed a stable increase in MAPK pathway gene signature 5 days after the oestrogen stimulation (**Chapter 4**). DH cells could be considered as a model of breast cancer patients receiving

aromatase inhibitors to reduce the oestrogen level in their body. Interestingly, it has been suggested in the literature to use oestrogen as a treatment for advanced anti-ERα resistant breast cancer (Yao et al., 2000). This was based on the studies using long-term oestrogen deprived and tamoxifen resistant cell line models. When cells were cultured under long-term oestrogen deprived conditions, they showed that cells lost their dependency on oestrogen for proliferation but maintained expression of ERα. Furthermore, oestrogen paradoxically inhibited the proliferation of cells by inducing apoptosis (Ariazi et al., 2011). However, I have shown that oestrogen has the potential to cause stable transcriptional re-programming and activation of growth factor receptor signalling using my long-term oestrogen deprived DH cell model. This questioned the feasibility of using oestrogen as a second-line treatment for tamoxifen or AI resistant patient as this might promote activation of growth factor signalling pathways in breast cancer cells. Furthermore, this study also emphasised the potential of using EGFR inhibitors such as cetuximab and gefinib in combination with anti-ERα drugs to prevent the occurrence of endocrine resistance.

c-Myb was identified in this study as a novel transcriptional activator which is responsible for EGFR expression (**Chapter 5**). It bound transiently to a region upstream of EGFR promoter in response to oestrogen, and c-Myb siRNA showed that it was vital for oestrogen induced EGFR up-regulation in DH cells. Bioinformatics analysis demonstrated that the c-Myb binding region upstream of EGFR promoter might sit in close proximity to a known active enhancer region.

c-Myb expression was regulated by oestrogen in ER positive breast cancer cells, as liganded ERα is able to relieve the elongation block at the first intron of the *MYB* gene. At the same time, c-Myb expression could also be activated without oestrogen stimulation, as the expression level of c-Myb recovered after an initial dip during the making of LTED cells (**Figure 5.19**). The increase in c-Myb expression could be due to the up-regulation of ERα after removal of oestrogen from culture media. As ER positive breast cancer cells were likely to up-regulate ERα during the acquisition of oestrogen independent growth in order to compensate the loss of oestrogen stimulation as showed with DH cells (**Chapter 3**). In this process, ERα gradually

accumulated at oestrogen responsive genes like *MYB,* which might be sufficient to drive its expressions in a low concentration of oestrogen. Alternatively, *MYB* expression was activated by other signalling pathways like PI3K or NF-*k*B as discussed in chapter 5. Furthermore, the expression of c-Myb can also be up-regulated by ERα inhibitor, tamoxifen (Hodges et al., 2003). The increased c-Myb expression could cause up-regulation of EGFR expression and activation of growth factors signalling observed in endocrine resistant breast cancer cells. Transcription factors other than c-Myb might also be recruited to the upstream response element or to the EGFR promoter area during the process, as overexpression of c-Myb alone did not significantly up-regulate EGFR expression (**Figure 5.12**). So far, my data suggested a potential mechanism of how EGFR expression was up-regulated during aromatase inhibitors treatment in ER positive breast cancer cells. Therefore, c-Myb could be a novel drug target to prevent the up-regulation of growth factor signalling in cells treated with aromatase inhibitors.

As a transcription factor, c-Myb is not a conventional drug target. Nevertheless, several potential methods have been proposed either to directly target c-Myb expression or to target proteins involved in its transcription machinery. The use of nuclease-resistant antisense oligodeoxynucleotides (ODNs) against c-Myb was a direct approach to alter the expression of c-Myb in a highly specific manner. Its implication has been investigated in cells taken from patients with chronic myelogenous leukaemia (CML), where they found c-Myb ODNs effectively inhibited the proliferation and viability of CML cells over normal cells (Gewirtz, 1993). In ER positive breast cancer cells, the expression of c-Myb was regulated by transcription elongation as discussed in chapter 5. Hence, inhibitors could be developed to prevent the interactions between ERα with proteins in the elongation complex. 6-dichloro-1-beta-D-ribofurano-sylbenzimidazole (DRB) is an inhibitor of pTEFb (Yamaguchi et al., 1998). In order to produce a stable elongation complex, the RNAP II of active transcribed genes were phosphorylated by pTEFb (**section 1.3.1**). Therefore, DRB can be used to block the expression of c-Myb in breast cancer cells. However, the use of DRB might have many potential side effects, as it was not specific to c-Myb.

As the up-regulation of EGFR expression was maintained after the removal of initial stimulation in DH cells, this indicated an epigenetic-based mechanism. One possibility was an epigenetic switch at the EGFR promoter, but my analysis did not reveal any suitable changes that could cause the stable up-regulation of EGFR in response to oestrogen (**section 5.8**). An alternative possibility was that the EGFR expression was maintained by stable changes at other genes. However, identification of this is beyond the scope of this study.

6.2 Further directions

c-Myb was identified as a novel regulator for EGFR in DH cells. EGFR is one of the most important membrane receptors in cells that is vital for a range of cellular processes including signal transduction, proliferation, migration, adhesion and so on. Although c-Myb is vital for EGFR up-regulation, it might not be the only transcription factor required to regulate the expression of EGFR in DH cells. Therefore, it will be valuable to study other transcription factors that regulate the expression of EGFR in ER positive breast cancer cells.

As c-Myb is a master transcription activator in cells, it is responsible for the transcriptional regulations of a range of other genes. Especially for genes that are important for cancer cell survival. Thus, it will be interesting to comparing the c-Myb binding sites across the genome before and after the oestrogen treatment to see how it shapes the expression profile in DH cells that are cultured in oestrogen deprived environment. One interesting observation during the 5-day oestrogen stimulation is the dynamic formation of cell protrusions and activation of genes involved in cell motility pathway. However, wound healing migration assay as well as matrigel invasion assay did not show an increase in migration or invasion ability of DH cells in the presence of oestrogen or after oestrogen pre-treatment before the assay (data not shown). This can due to the nature of the DH cells, as it is derived from a non-invasive early stage breast cancer cells, MCF-7. Nevertheless, it emphasise the complex regulation of the oestrogen signalling in ER positive breast

cancer cells. Furthermore, c-Myb has been shown to regulate the expression of slug, which is a gene involved in the activation of epithelial-mesenchymal transition (EMT) (Tanno et al., 2010). A 2-fold increase in slug expression was observed in DHE5R5 cells according to the expression microarray data (data not known), which can be a downstream effect of c-Myb binding after oestrogen stimulation. However, the expression of slug as well as another important EMT initiator snail can also be switched on through the Ras activated MAPK pathway (Thiery, 2002). The fact that only slug was up-regulated in DH cells after oestrogen stimulation stressed the involvement of c-Myb. Therefore, c-Myb may also trigger EMT in response to oestrogen in ER positive breast cancer cells.

To study the significance of c-Myb function in the clinical setting, it is necessary to examine the level of c-Myb in patients who relapse on aromatase inhibitors to see if there is a correlation between EGFR overexpression and c-Myb level. If so, c-Myb inhibitor can be used as a second line treatment to prevent the endocrine drug resistance due to the activation of growth factor signalling pathways.

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