Crosstalk of TTC5 Cofactor and the Estrogen Receptor in Breast Cancer Cells

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List of Abbreviations

A549	Adenocarcinomic human alveolar basal epithelial cells
AD	androstenedione
AF	Activation fucntion
AIB1	amplified in breast cancer 1
Ais	Aromatase inhibitors
AP-1	activator protein-1
AR	Androgen receptor
ATM	Ataxia telangiectasia mutated
BRCA1	Breast cancer type1 susceptibility protein
BRCA2	Breast cancer type2 susceptibility protein
CARM1	coactivator-associated arginine methyltransferase 1
cDNA	Complementary DNA
DBD	DNA bindind domian
DMSO	Dimethyl sulphoxide
E1	Estrone
E2	17b-estradiol
E2	Estriol
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen Receptor
ERa	Estrogen Receptor alpha
ERb	Estrogen Receptor beta
ERE	Estrogen response element
FOXA1	forkhead transcription factor1
FSH	Follicle-stimulating hormone

GATA3	GATA Binding Protein 3
GR	Glucocorticcoide receptor
HER2	Human epithelial growth factor receptor 2
HMT	histone methyltransferases
HRE	Hormone response elements
HSP70	Heat Shock Protein 70
HSP90	Heat Shock Protein 90
IDC	Invasive ductal carcinoma
JMY	Junction Mediating And Regulatory Protein
LBD	Ligand binding domain
LH	Luteinizing hormone
LHRH	Luteinising hormone releasing hormone
MDM2	Mouse double minute 2 homolog
MIF	Macrophage migration inhibitory factor
MRI	magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NCBI	National centre for biotechnology informatio
NR	Nuclear receptor
PBS	Phosphate buffered saline
PI3K	phosphoinositide-3-kinase
PMD	Percent mammographic density
PP5	Protein Phosphatase 5
PR	Progesterone Receptor
pRb	Retinoblastoma protein
PRMT1	protein arginine methyltransferase 1

PTEN	phosphatase and tensin homologue
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
Ser	Serine
SERMs	Selective estrogen receptor modulators
SP-1	specificity protein-1
TEMED	N,N,N',N'–retraethylethylenediamine
Testosterone	testosterone
TTC5	Tetratricopeptide reprat domain 5
TPR	Tetratricopeptide repeat
μM	Micromolar
WTTTC5	Wild type tetratricopeptide repeat domain 5

Declaration of Originality

This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy. I declared that the work reported in this thesis in my own, or if not, it is stated and fully acknowledged.

This thesis is dedicated to my parents
for their constant selfless support, I wouldn't have come this far if it wasn't for you.

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Abstract

The estrogen receptor (ER) is a transcription factor that regulates a wide array of genes and whose activity is a determinant for the prognosis of diseases such as breast cancer. ER, through L-X-X-L-L (L=leucine, X=any amino acid) motifs, interacts with cofactors such as TTC5 (tetratricopeptide repeat domain 5) which is a 440 amino acid protein, predicted to have 6 TPR (tetratricopeptide repeat) motifs and 4 LXXLL motifs, which are known to mediate protein-protein interactions. TTC5 has been shown previously to interact with and regulate the glucocorticoid receptor, which belongs to the same family of nuclear hormone receptors as ER. Thus, this provided the basis for the investigation of the role of TTC5 in ER signalling.

This investigation revealed that TTC5 interacts with estrogen receptor alpha (ERα) in MCF-7 and T47D breast cancer cells, and that ER transcriptional activity on an estrogen response element was regulated by different TPR and LXXLL motifs of TTC5 in a cell-specific manner. Partial knockdown of TTC5 via siRNA led to a decrease of proliferation in cells treated with 17β-estradiol (E2), raloxifene and tamoxifen, indicating a potential role for TTC5 in breast cancer progression and survival. Effects of TTC5 silencing on cyclin D1 makes this molecule a candidate for inhibiting cyclin D1 gene expression

In summary, this thesis uncovers TTC5 as a novel regulatory factor of ER function, and identifies effects of TTC5 on cell proliferation. These key findings enhance our understanding of ER signalling which, given its importance for diseases such as breast cancer, may have important clinical implications for instance through targeting of TTC5.

Chapter 1 Introduction

1.1 Cancer

Acquisition of certain epigenetic or genetic mutation events can result in cancer, which leads to deregulation in normal function of cellular process involved in DNA repair, proliferation, motility and cell survival. As a result, healthy cells accumulate mutations leading to uncontrolled proliferation. In early stages of life, cells usually divide more frequently allowing growth whereas adult cells commence dividing only to replace worn-out or dying cells to repair injuries. Cancer cells grow continually, as they have lost control at the cell cycle checkpoints which will continue the growth of abnormal cells. Cancer cell progression due to loss of cell cycle control, usually happens when a tumour suppressor gene loss its function, or when an oncogene becomes activated (Lane, 1992).

Typical oncogenes include human epidermal growth factor receptor 2 (Her2), Myc whereas, retinoblastoma protein (pRb) and p53 are typical tumour-suppressor genes. Invading other tissue is another feature of cancer cells, which normal cells do not have; this process is known as metastasis (Chaffer and Weinberg, 2011). According to cancer research UK (CRUK), in 2014, there were approximately 356,860 of new patients diagnosed with cancer, which led to 163,000 cancer related deaths (CRUKa, 2014) Among all types of cancer; lung, bowel breast and prostate cancers are the most predominant ones in the UK (CRUKb, 2014). Cancer is one of the major cause of death in the UK and throughout the world. Depending on the cell and tissue of origin there are more than 200 different types of cancer (CRUKb, 2014).

Cancer is a complex disease and tumorigenesis involves a number of steps and transformation processes in which a normal cell transforms to a cancerous cell have been classified as hallmarks of cancer (Hanahan and Weinberg, 2011). These hallmarks are shown in Figure 1.1. The question is how many regulatory disruptions can a cell endure before the cell becomes cancerous. Hanahan and Weinberg proposed a set of characteristic properties of cancer in 2000, which are considered as hallmarks of cancer.

One of these hallmarks is that cancer cells become resistant to signals that would normally halt cell growth and proliferation. Another hallmark is the independency of cancer cells from external proliferative signals as a result of mutation in their growth factor signalling cascade. Moreover, evading stimuli that causes apoptosis is another ability of cancer cells. An increase in ultimate replication potential is another hallmark of cancer. Another very important characteristics of cancer is cell migration and invasion also known as metastasis, where cancer cells can invade into blood and lymph vessels (Hanahan and Weinberg, 2011). Cancer cells not only survive under stress conditions but also have the ability to grow under conditions such as oxygen starvation, which contributes to tumorigenic potential. A common feature of the tumour microenvironment is deprivation of oxygen, which leads to induction of new blood vessels known as tumour angiogenesis. What facilities the invasion and metastasis of growing tumours is tumour angiogenesis.

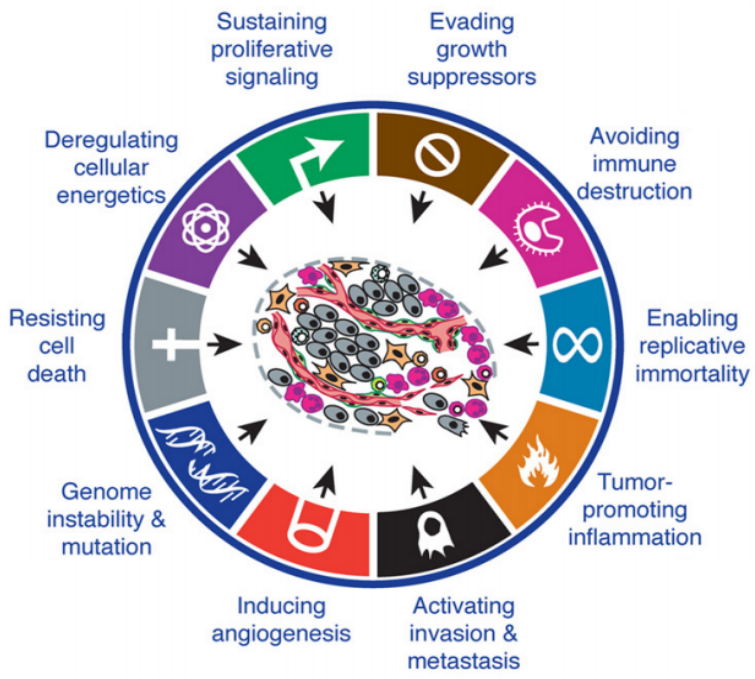


Figure 1.1. The hallmarks of cancer.

Schematic diagram of hallmarks of cancer (Adapted from Hanahan and Weinberg, 2011). These hallmarks feature key underlying reason of rapid evolution of cancer cells as well as tumour features.

1.2 Cell cycle regulation

The proliferative activity of cells in healthy tissues is closely regulated to avoid neoplastic growth and maintain homeostasis (Rhind and Russell, 2012). There are checkpoint controls known as cyclin proteins and the associated cyclin-dependent kinases whose job is to monitor the four phases of cell cycle. For cancer cells to prosper they need to overcome anti-proliferative signalling. The Retinoblastoma (RB) pathway funnels the majority of these signals, however cyclin Ds and their partners CDK4/6 may inactive the RB proteins. Inactivation of RB pathway may be the result of amplification in Cylin D1, CDK4 and CDK6 or direct loss of RB which results in perturbation of the RB pathway and have been associated with breast cancer (Herschkowitz et al., 2008; Knudsen and Knudsen, 2008; Rhind and Russell, 2012).

1.3 The normal human breast anatomy

Normal breast is situated over the pectoral muscles of the chest, which is comprises of, glandular, fatty and fibrous tissues. It is during three phases: embryonic, adolescent and adult phase that developmental of mammary gland occurs and each phase is regulated differentially. While embryonic branching is hormone dependent, estrogen and ERα are required for adolescent branching, and progesterone for adult side-branching (Bocchinfuso et al., 2000). Production of the milk for new born is through a highly-specialized organ, the breast (also known as mammary gland) (Sternlicht, 2006). The three main components of the breast are lobules, ducts and the stroma (Figure 1.2). Production of milk is through the lobules and the tube-like structures which called ducts are responsible for carrying milk from the lobule to the nipple within the breast tissue.

The initiation of human breast development which is a progressive process, starts during embryonic life, further growth depends on the high levels of estrogen that are produced by ovary and progesterone during puberty. Processes such as proliferation and invasion that happens during normal mammary development also occurs in malignant diseases, and development or progression of cancer have been linked to pathways that influence ductal branching (Sternlicht, 2006).

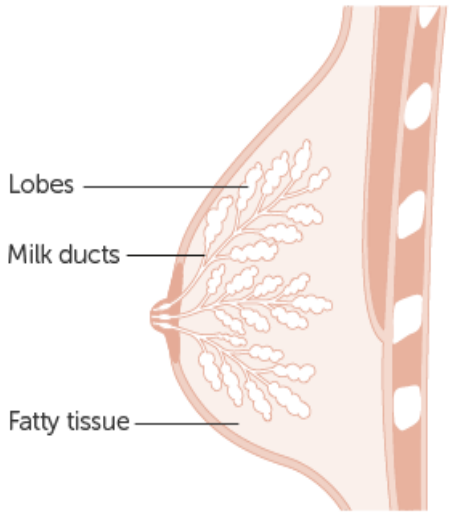


Figure 1.2.Schematic representation of mature human mammary glands.
[figure adapted from(Society, 2013)].

1.4 Breast cancer

Breast cancer is the most common cancer in women in the United Kingdom, and second most common cause of cancer death. There are 150 cases of breast cancer being diagnosed every day in UK according to CRUK, which accounts for 15% of all new cancer cases in the UK (CRUKb, 2014). It has been estimated by CRUK that 1 in 8 women in the UK will develop breast cancer during their life time. Since 1970, the incidence of breast cancer has been rising however the survival rate has been increasing over the last twenty years. Reduction in risk of relapse and long-term survival has been achieved through, advances in approaches to treat breast cancer including, radiation, chemotherapy and hormone therapy (Jones and Buzdar, 2004). Compared to 1970 survival rate in UK that was 5 out of 10 in breast cancer patient in the UK, survival rate has been improved to 8 out 10 (CRUKd, 2012).

1.5 Breast cancer risk factors

Risk of developing cancer in a person depends on various different factors. Although estrogen exposure remains the main potential risk factor of developing breast cancer, obesity, alcohol consumption, and genetic susceptibility are counted as risk factors. It was shown in 19th century that in women with advanced breast cancer, bilateral oophorectomy (removal of ovaries) resulted in reduction in tumour size (at least in a proportion of women) (Ali and R Charles Coombes, 2002). So, the connection between estrogen and breast cancer has been well stablished. In 1930s estrogen was identified as the ovarian hormone that promoted mammary tumorigenesis in animal models (Södersten, 2015).

1.5.1 Hormonal factors

Extensive exposure to estrogen is directly associated with risk of breast cancer. Late menopause, nulliparity and early menarche are factors that are related to increase in estrogen exposure throughout a woman's lifetime (Clemons and Goss, 2001). High levels of endogenous sex hormones like androgens and estrogen in the circulation have been associated with developing breast cancer (Key et al., 2002). Not only endogenous estrogen, but the use of exogenous hormones like contraceptive pills and hormone replacement therapy that contain estrogen, have been linked with significant increase in developing breast cancer. It is believed that during pregnancy the breast epithelial cells are converted into a more stable state hence, a reduction in breast cancer risk have been linked to younger age at first pregnancy (Amir et al., 2010). A lower risk of developing breast cancer has been associated with breast feeding, possibly due to the loss of estrogen in breast milk and ovulatory delay (Millikan et al., 2008; Amir et al., 2010).

1.5.2 Family history and age

It has been demonstrated in many studies that women with a family history of breast cancer have higher risk of developing breast cancer, approximately twice the risk (Nelson et al., 2012). According to the age at which the relative developed cancer, type and

number of relatives affected the extent of this risk varies. Also, mutations in one of the breast cancer susceptibility genes results in breast cancer (Mangia et al., 2011).

Exposure to carcinogenic agents or epigenetic changes can cause DNA damage, when certain mutations in DNA cause loss of function of tumour suppressor genes and uncontrolled oncogenes activation can cause normal breast to become cancerous. In case of breast cancer, a mutation in BRCA1 (Breast cancer type1 susceptibility protein) and BRCA2 (Breast cancer type2 susceptibility protein) genes, can be inherited from a parent. BRCA1 and BRCA2, are involved in DNA damage repair and transcriptional regulation and both are tumour suppressor genes. Mutated version of BRCA1 and/or 2 genes, are unable to perform their normal function of homologous recombination and DNA repair properly, hence promoting breast cancer occurrence (Scully and Livingston, 2000; Welcsh and King, 2001).

BRCA1/2 proteins both play a pivotal role in repairing DNA damage by utilising identical sister chromatids through a process called homologous recombination (HR). When BRCA1/2 proteins lose their normal function due to mutations, cells become susceptible to follow alternative repair pathways such as single-strand annealing or non-homologous end joining (Turner, Tutt and Ashworth, 2004). BRCA1 is known to regulate estrogen metabolism through many different enzymes, one example is CYP1A1 hence loss of BRCA1 may particularly result in estrogen-driven tumours (Savage et al., 2014).

1.5.3 Mammographic density

One of the recognised risk factor of breast cancer is percent mammographic density (PMD), which reflects the proportions of fat, epithelial and stromal tissue that shows a dense appearance at mammography (Boyd et al., 2011). A study by Boyd et al in 2011 showed that indeed the mammographic density is associated with risk of breast cancer. In mentioned study, they compared two groups of patients, one group with more than 50% PMD and the other with less than 10% and it turned out that an increase in risk of developing breast cancer can be seen in women with more than 50% PDM.

1.6 Stages of breast cancer

According to diverse criteria breast cancer can be classified by stages or subtypes, such as pathological state, tumour and molecular subtype classification (Reis-Filho and Pusztai, 2011). When a patient is diagnosed with breast cancer, doctors will assess the size of the tumour, and whether it has metastasised (staging (Figure 1.3)). To identify the stage of a cancer TNM (tumour, modes, metastasis) system is usually used, which often categorises breast cancer into four stages.

Stage 1: means that the cancer hasn't spread outside of the breast, and is relatively small. Stage 2: is when the size of the tumour is often larger than stage 1, and sometimes the cancer has been spread into lymph nodes under the arm. Stage 3 is when cancer cells can be found in quite few lymph nodes under the arm. Stage 4 is when the cancer has metastasised into other parts of the body, such as lungs, liver, bone or brain (Breast cancer grades and stages | Breast Cancer)

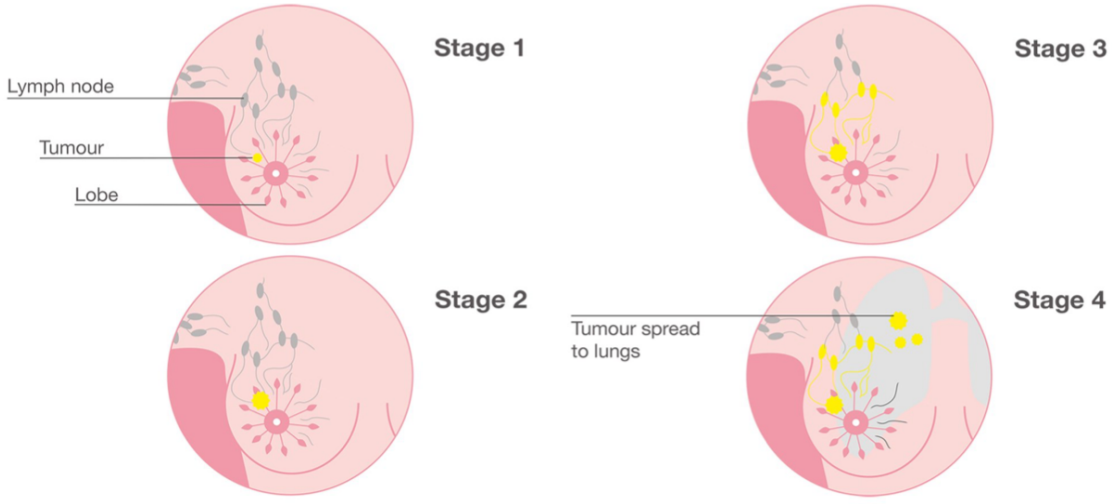


Figure 1.3.Stages of breast cancer.
[figure adapted from (Breast cancer grades and stages | Breast Cancer Now)]

1.7 Molecular sub-types of breast cancer

A patient is diagnosed with breast cancer, usually through presentation at symptomatic breast clinics with a lump or screening programme (Simpson et al., 2005). However, some patients present with other symptoms rather than lump such as nipple discharge, skin dimpling and inflammation. Based on tumour size, lymph node and metastasis breast cancer can be classified into different stages. Accurate staging is important to achieve optimal treatment surgery and determining patient prognosis. Breast cancer arises either from ductal or lobular tissues, primarily classification of breast cancer is carried out through histopathology.

Breast cancer is subdivided into non-invasive and invasive according to histopathological classification (Reiner et al., 1988) (Figure 1.4). Non-invasive breast cancer patients are those that, the cancerous cells remain within the breast tissue, in other word the cancer hasn't metastasised. In case of invasive breast cancer, cancer cells have the ability to spread outside the membrane of the milk duct or lobule into the surrounding tissues, and can then migrate even beyond to other organs as well as the lymph nodes in the armpit. 70% to 80% of all breast carcinomas are invasive ductal carcinoma (IDC), which is the most common type of invasive breast cancer, whereas invasive lobular carcinoma is less common and accounts for about 10% (Reiner et al., 1988). In addition to these two major types, there are other types of breast cancer which are rare such as inflammatory breast cancer, tubular carcinomas and medullary breast cancer (Mallon et al., 2000).

In clinical practice biomarkers are important for early detection, prognosis and therapy of breast cancer patients. Three important biomarkers of breast cancer include: Estrogen Receptor (ER), Progesterone Receptor (PR) and HER-2 receptors, which are important in treatment decision. Breast cancer is classified into luminal, HER-2 positive, and triple negative subtype (van de Ven et al., 2010). Approximately 70-75% of breast cancer cases are ER positive which means they express the estrogen receptor, which makes estrogen inhibition an important target for breast cancer therapy (Fisher et al., 2001).

1.7.1 Luminal A

Approximately 50-60% of all breast cancer cases are luminal A subtype. Identification of luminal A tumour subtype can be achieved through immunohistochemistry by positivity for ER or PR and negativity for Her2 antibody (Blows et al., 2010). This subtype expresses luminal clusters of genes including forkhead box A1 (FOXA1) and GATA-binding protein 3 (GATA3). Generally luminal subtype express estrogen receptor, which responds to endocrine therapy such as tamoxifen and aromatase inhibitors.

1.7.2 Luminal B

About 10-20% percent of all breast cancer cases are luminal B tumour (Blows et al., 2010), this subtype just like luminal A subgroup are ER and/or PR positive, however overexpression of Her2 can be observed in this subtype (Cheang et al., 2008). Compare to luminal A this sub-group has more aggressive phenotype, treatment for this subtype is the combination of tamoxifen and neoadjuvant chemotherapy (Eroles et al., 2012).

1.7.3 Her2 positive tumours

About 10-25% of all breast cancers accounts for this subtype (Eroles et al., 2012). As the name suggests in HER2 subtype, high expression of human epithelial growth factor receptor 2 (HER2) is detected and are ER and PR negative, these patients respond to anti-HER2 therapy (Weinberg, 1995), these patients often have a poor prognosis, with about 52-55% survival rate after 10 years (Cheang et al., 2008).

1.7.4 Basal-like tumours

The absence of all three breast cancer biomarkers ER, PR and HER2 is the most important characteristic of this subtype. Hence, they usually are referred to as triple negative breast cancer (TNBC). TNBC, which has much more aggressive phenotype and it is the most difficult one to treat, and treatment is usually with chemotherapy (Milioli et al., 2017). This type approximately accounts for 15% of breast cancers (Foulkes, Smith and Reis-

Filho, 2010), the immunohistochemical staining for this group of tumours are negative for ER, PR and Her2 overexpression.

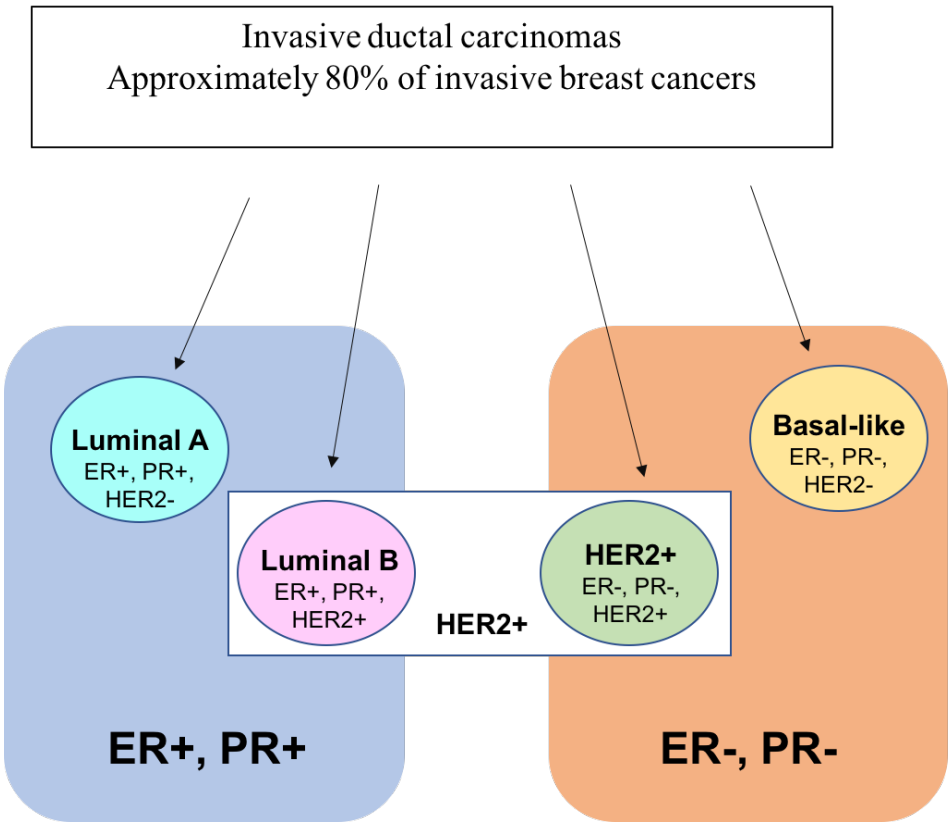


Figure 1.4.Breast cancer classification and its subtypes.
[modified version (Sandhu et al., 2010)].

1.8 Oncogenes and tumour suppressor genes of breast cancer

In approximately 25% of patients with primary breast tumours somatic activating mutation in the PIK3CA oncogene have been observed. A transducer of growth factor signalling known as phosphoinositide-3-kinase (PI3K) is encoded by PIK3CA oncogene. Cancer cell will grow independent of external stimulus when PIK3CA continuously activates PI3K (Lee and Muller, 2010). Also, an increase in invasive and metastatic potential of breast cancer have been monitored through PI3K signalling via Akt kinases (Dillon, White and Muller, 2007). Therefore, acquisition of several necessary hallmarks for progression and initiation of breast cancer is likely through PIK3A oncogene. Antagonising PIK3 signalling through tumour suppressor phosphatase and tensin homologue (PTEN) leads to induction of apoptosis and repression of angiogenesis and invasion (Dillon, White and Muller, 2007). Loss of function germ line mutations in PTEN gene have been associated with higher risk of developing breast cancer.

TP53 is another tumour suppressor gene, encoding p53. Stimulating cell cycle checkpoints and response to pro-apoptotic signals and controlling the expression of numerous other genes are governed by p53. Hence loss of function in p53 function leads to harmful mutations and uncontrolled cell cycle entry of cells (Lee and Muller, 2010).

As mentioned before an increase in risk of developing breast cancer is associated with inheriting mutations in the tumour suppressor genes *BRCA1* and *BRCA2*. Mutations in BRCA1 or BRCA2 tumour suppressor genes have been associated with higher risk of developing breast cancer. BRCA1 and BRCA2, are involved in DNA damage repair and transcriptional regulation and both are tumour suppressor genes. Mutated version of BRCA1 and/or 2 genes, are unable to perform their normal function of homologous recombination and DNA repair properly, hence promoting breast cancer occurrence (Scully and Livingston, 2000; Welcsh and King, 2001).

Approximately 15-30% of breast cancer cases have been seen to contain overexpressed HER2 expression, *Erb2* breast cancer associated oncogene encodes HER2. Increase

recurrence, lymph node metastases and poor prognosis has been linked to HER2 overexpression (Burstein et al., 2007).

1.9 Breast cancer diagnosis and therapies

Several diagnostic procedures could be taken into account for a woman at normal risk of breast cancer. Apart from the clinical breast exam by health care professionals, there are number of imaging techniques of paramount importance in early detection of breast cancer such as X-ray mammography, ultrasound and magnetic resonance imaging (MRI) (Wruk, 2008). The possibility of cancer occurring increases if an abnormality is seen in any of the above-mentioned imaging techniques, however the only definitive way to determine whether cancer is really present is to do biopsy. An abnormal growth or changes in breast tissue before it can even be felt, can be detected with mammography, hence it is gold standard for early detection and screening breast cancer (Nelson et al., 2009). Screening and diagnostic are two different type of mammography, as the name suggests the former (screening) is done to improve the chance of detection. The latter (diagnostic) is performed in patients who have symptoms and the main purpose of this type of mammography is to determine the size and location of the tumour as well as looking for any migration to surrounding tissues.

The most appropriate treatment can be determined based on stage and grade of the tumour. Removal of the tumour mass by surgery is usually offered to patients having localised breast cancer. To manage the risk of relapse surgery is often combined with radiation, adjuvant chemotherapy and endocrine therapy. In case of metastatic breast cancer systemic therapies such as chemotherapy and/or endocrine therapy are also used (Makhoul and Kiwan, 2011). To penetrate cancer tissues, radiotherapy is used. Depending on the type of cancer, stage, grade, and its location, radiotherapy treatment varies. Sometimes in order to increase the effectiveness of the surgery, radiotherapy is administered pre-surgery to reduce the tumour size.

Chemotherapeutic drugs mode of action is to target rapidly dividing cancer cells rather than normal mammary epithelial cells, by affecting pathways of cell growth and DNA

synthesis. Alkylating agent cyclophosphamide is often used as a chemotherapeutic agent in combination with doxorubicin which is a DNA intercalating antineoplastic drug. Also, sometimes paclitaxel is administered along with these drugs to block mitosis; to prevent pyrimidine incorporation during DNA synthesis antimetabolite fluorouracil is advised to be taken by breast cancer patients (Berry et al., 2006).

Targeting estrogen signalling pathway to block its proliferative effect on breast cancer is carried out by endocrine therapies. In pre-menopausal women estrogen is secreted from ovaries in response to luteinising hormone releasing hormone (LHRH), hence blocking the secretion of estrogen activity in pre-menopausal women can be achieved by LHRH antagonists. Surgical oophorectomy or ovarian ablation is a way of inhibiting oestrogen synthesis, in premenopausal women. Ovarian ablation is an effective adjuvant treatment, with high survival rate compare to patients who receive chemotherapy alone (Lancet (London, England), 1996). The permanent loss of ovarian function is the major disadvantage of surgical ovarian ablation. Hence, in order to temporarily supress ovarian estrogen production in ovaries, agonists of LHRH have been developed. One example of these agonists, is goserelin which inhibits the estrogen synthesis in ovaries by reducing the pituitary release of gonadotropins through downregulating LHRH receptors in the pituitary (Emens and Davidson, 2003). In premenopausal women with ER positive breast cancer goserelin is an effective alternative to chemotherapy.

Compared to other breast cancer treatments there are relatively less side effects associated with surgery. In case of radiotherapy during the treatment patients won't feel any pain, however cumulative doses result in tenderness and short-term pain in the localised area. Breast cancer patients may experience sickness and weight loss as well. Depending on the hormone administered the biological side effects of hormone therapy can vary. Few commonly reported side effect includes, diarrhoea, joint pain, nausea. All proliferating cells in the body are targeted by chemotherapy agents, this means cancerous cells as well as healthy cells, hence adverse side effects can be seen in breast cancer patients undergone chemotherapy (nail loss, alopecia, changes in taste and diarrhoea) (Boehmke and Dickerson, 2005). Depending on the dose and format of administration the range of side effects experienced is different. Two to three weeks following the start of chemotherapy

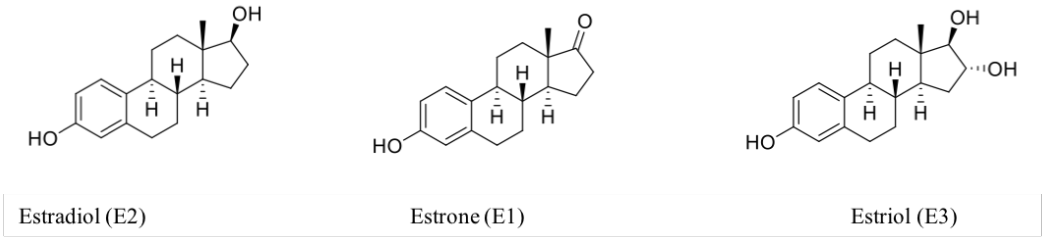
is when the side effects commence. To help manage nausea and vomiting antiemetic drugs can be administered (Hesketh, 2009).

In post-menopausal women aromatase inhibitors are used to block the synthesis of adrenal androgen to estrogen. Last but not least selective estrogen receptor modulators (SERMs) such as tamoxifen are used in both pre-and post-menopausal breast cancer patient to block the action of ER.

1.10 Estrogens

It was in 1920's that estrogen was first described by Edward Doisy (Tata, 2005). Estrogen hormones play an essential role in fertility, and are involved in ovulation and pregnancy maintenance in females. In addition to female reproduction, the expression of wide range of genes in reproductive tract and other areas is known to be influenced by estrogen, like maintaining physiological functions of skeletal tissue, cardiovascular and central nervous system. Estrogens are implicated in the maintenance of bone density, spermatogenesis and cardiovascular health in males (de Ronde and de Jong, 2011).

The three related hormones that make up the estrogen families in humans are: estrone (E1), estradiol (E2) and estriol (E3) (Figure 1.5), which the most biologically active one is 17-β-estradiol (E2). E1, is known to be secreted by ovaries during menstrual cycles. E2, is produced by ovaries, is the most abundant form of estrogen hormone in the blood and is thought to contribute to development of female cancers. Highest levels of E1 and E2 are detectable during the follicle maturation and pre-ovulation stages. E3, is only produced in significant quantities during pregnancy, being produced by placenta (Gruber et al., 2002).



Estradiol (E2) Estrone (E1) Estriol (E3)

Figure 1.5..Structures of normal estrogens.
Estradiol the most potent intracellular estrogen in human body, estrone and estriol are the weaker agonists on estrogen receptor. [structures drawn in ChemDraw]

In the elderly, delayed healing process has been linked to estrogen deficiency (Hardman et al., 2008). Macrophage migration inhibitory factor (MIF) regulates estrogen anti-inflammatory effects in the skin, however in a study by (Campbell et al., 2010) it has been shown that estrogen promote wound healing independent form its anti-inflammatory effects. In both female and male, the systematic levels of estrogen decreases with age. Estrogen beneficial effects on healing became more evident, when taking hormone replacement in post-menopausal women resulted in a quicker healing process (Ashcroft et al., 1997).

1.11 Estrogen biosynthesis

Fundamentally estrogens are synthesised from cholesterol (Figure 1.6). The formation of androgens such as androstenedione and testosterone is through various enzymes that metabolises cholesterol. Next in the endoplasmic reticulum of estrogen producing cells, the P540 enzyme aromatase convert these androgens to estrogen (SIMPSON et al., 1994). During the menstrual cycle in premenopausal women, E1 and E2 are primarily secreted by the ovaries. Hypothalamic-pituitary-gonadal tightly regulate the secretion of ovarian estrogen. In anterior pituitary glands release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) is stimulated by LHRH which is produced in hypothalamus.

In the theca cells in the ovary production of the steroid hormone androstenedione (AD) and testosterone (T) is stimulated by LH, whilst in granulosa cells FHS stimulates the P540 enzyme, which converts AD to E1 and T into E2.

Estrogens that are produced in ovaries circulates in the body and act on their target tissues after being released into the blood stream. Estrogen can inhibit hypothalamic LHRH and pituitary LH through a negative loop (Auchus and Auchus, 2012). Following the decline in ovarian function in postmenopausal women the serum level of estrogen in lowered in the body. In post-menopausal women estrogen biosynthesis involves aromatization of circulating adrenal in tissues such as brain, adipose tissue, the cardiovascular system and bone (Gruber et al., 2002).

Estrogen biosynthesis occurring in the ovaries which is the systematic source of estrogen production that changes to local synthesis in the peripheral tissue after menopause. Although the local estrogen synthesis occurs at younger age, it is only when the systematic loss its function that the local estrogen synthesis becomes important. However, the amount of estrogen produced by local tissues is not enough compensate the shortage of estrogen in systemic levels. Aromatase is believed to play an important role in providing supply of estrogen in adipose tissue of skin of the elderly of both genders (Purohit and Reed, 2002).

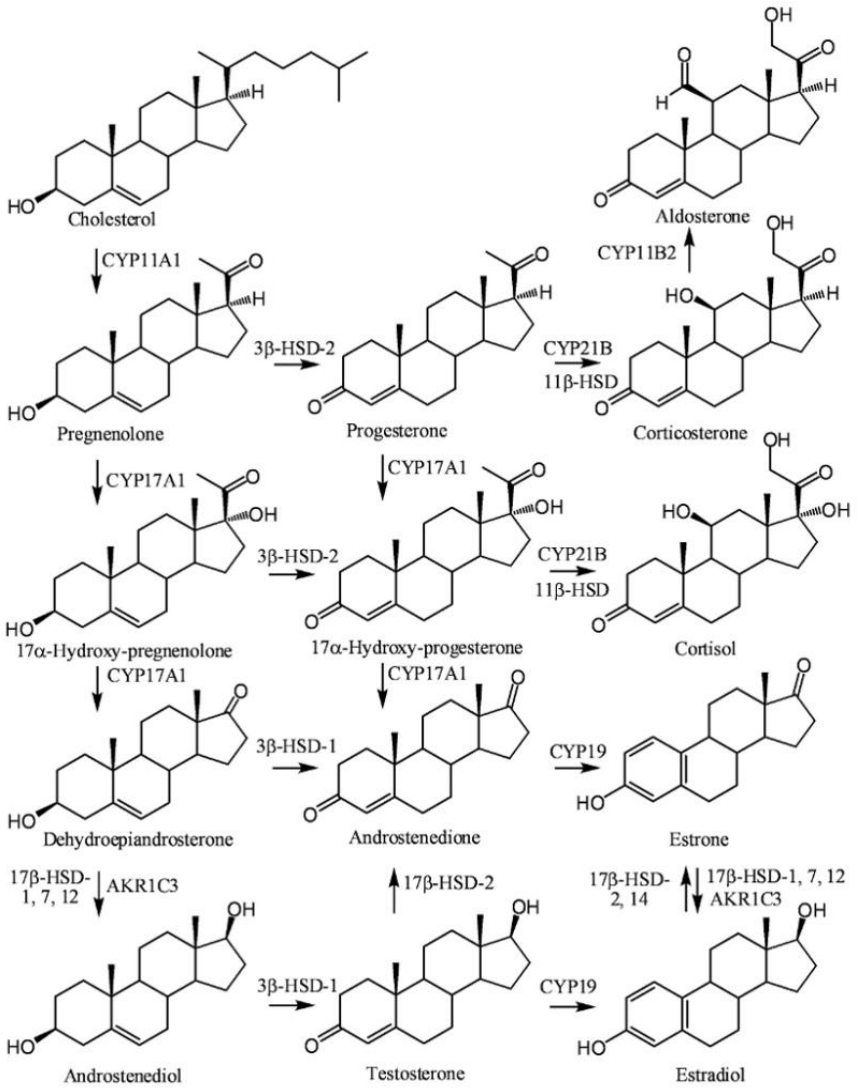


Figure 1.6.The biosynthesis of estrogen.

Hormone synthesis facilitated by members of Cytochrome P450 (CYP), Aldo-keto reductase (ARK) and Hydroxysteroid dehydrogenase (HSD) enzyme family [figure adapted form Blair, 2010].

1.12 Breast cancer and anti-estrogen agents

The development of endocrine therapies to inhibit receptor activation, either through use of estrogen antagonists or inhibition of estrogen biosynthesis started when researchers realised that estrogen regulates breast cancer (Lewis and Jordan, 2005). Significant improvement has been seen in life expectancy of many breast cancer patients since the discovery of tamoxifen. Tamoxifen downregulates estrogenic signalling within the cells

by inhibiting the binding of estrogen to ERα. Tamoxifen is used routinely in ER positive breast cancer patients in clinics (Jordan, 2003; Osborne and Schiff, 2011). It was in 1950s that tamoxifen was discovered, and classified in a group of drugs called selective estrogen receptor modulators (SERMs) (Riggs and Hartmann, 2003; Cuzick et al., 2013). SERMs (Figure 1.7) are able to preserve beneficial action of estrogen in tissues like bone, cardiovascular system and brain, while antagonizing the undesirable effects of it. (Jordan, 2004; Osborne and Schiff, 2005). For past three decades' tamoxifen has been used as the primary adjuvant endocrine agent. Reduction of mortality in ERα positive breast cancer patient by a third has been shown in patients that received 5 years of adjuvant tamoxifen treatment. Use of tamoxifen has been linked to some beneficial effects such as, lowered cholesterol level, reduced heart attack and atherosclerosis (Fisher et al., 2005). Common side effect of tamoxifen treatment is the occurrence of hot flushes which is due to antagonising estrogen function in the brain (Benson, 2002). Primary function of tamoxifen is to antagonise estrogen function, however in many other tissues that are targeted by estrogen such as cardiovascular system, bone and endometrium it has agonist properties (Lewis and Jordan, 2005).

Although an enhanced risk in endometrial proliferative disorders, like hyperplasia in breast can patients undergoing endometrial assessment prior to tamoxifen administration have been monitored, tamoxifen ability to increase the chances of a patient of developing endometrial lesions is one of the most detrimental side effect of it in postmenopausal females. It is the accumulative longer duration of usage that has been linked to endometria cancer rather than the daily dosage of the drug (Hu, Hilakivi-Clarke and Clarke, 2015).

Raloxifene is another SERM that has been used an estrogen antagonist in breast tissues, and it is been used in postmenopausal women to prevent osteoporosis. The binding mode of raloxifene and estradiol was compared by (Brzozowski et al., 1997), and revealed that, since both estradiol and raloxifene can make key hydrogen binding interaction through their hydroxyl group, and that although they have different binding mode but they both bind within the same region of LBD domain of ERα (Brzozowski et al., 1997). An antagonist conformation occurs by ERα, upon raloxifene binding which_results in

blocking the estrogen-induced DNA transcription in both breast and the endometrium (Grese et al., 1997).

Compared to tamoxifen treatment that observed an increase in uterine cancer these findings were a clear improvement. A comparison treatment on women at high risk of developing breast cancer for five years showed that although raloxifene is not as effective as tamoxifen it can reduce the incidence of breast cancer (Voegel et al., 1996). Furthermore, women who received raloxifene suffered from less side effects (uterine hyperplasia, lower incidence of endometrial cancer) compared to those who received tamoxifen. Compared to tamoxifen raloxifene seems to exert less agonist side effects on the same tissue (Voegel et al., 1996).

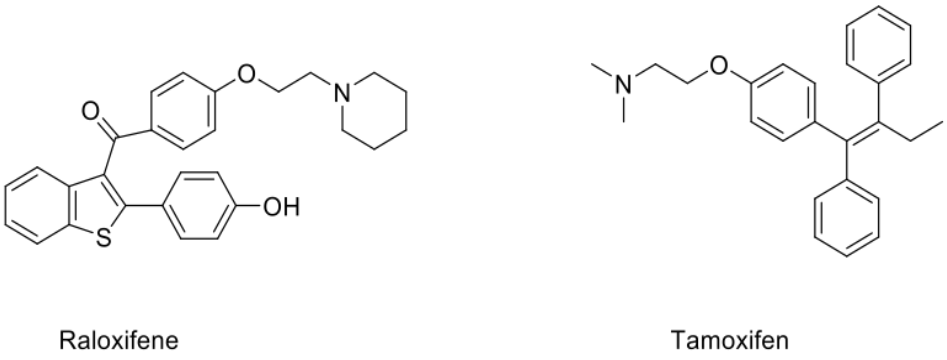


Figure 1.7.Chemical structure of SERMs.
Structure of two common SERMs raloxifene and tamoxifen [structures drawn in ChemDraw].

A newer class of drugs are aromatase inhibitors (AIs), often used in post-menopausal female breast cancer patients, there are two types of these classes of drugs: type I (steroidal or irreversible) and type II (non-steroidal reversible) (Brueggemeier, 1994) (Figure 1.8). Aromatase is an enzyme expressed in several tissues including, liver, muscle, mammary adenocarcinoma, and normal breast tissue. Aromatase is product of the CYP19 gene which is part of cytochrome P-450 super family. AIs mode of action involves inhibiting the enzyme aromatase, which is involved in catalysing androstenedione conversion to estrogen in peripheral tissues; these tissues are the predominant source of

estrogen in postmenopausal women who have undergone ovarian ablation (Johnston and Dowsett, 2003).

According to potency of the AIs to inhibit the aromatase enzyme they are classified as first, second and third generation. Anastrozole, letrozole and exemestane (
Figure 1.8) are from third generation of AIs known to reduce circulating estrogen up to 98% (Geisler et al., 2001). By binding to the cytochrome P450 site in the aromatase complex, letrozole and anastrozole inhibit aromatase function. Whereas exemestane, which is analogous of androstenedione, in competition with natural substrates binds strongly to the aromatase enzyme (Johnston and Dowsett, 2003).

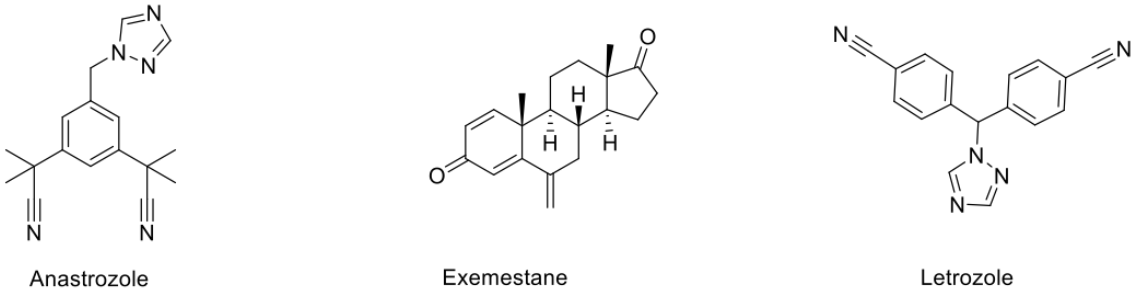


Figure 1.8.Chemical structure of Aromatase Inhibitors.
Anastrozole, Exemestane and Letrozole three Aromatase Inhibitors that are often used in clinical practice [structures drawn in ChemDraw].

Hormonal therapies especially tamoxifen, have been widely used in breast cancer treatments, however what limits the efficacy of the treatment is tamoxifen resistance. As well as becoming resistant to the treatment, developing side effects of the medications is another problem (Ring and Dowsett, 2004). Therefore, the need for better therapeutic efficacy and overcoming endocrine resistance continues.

1.13 Introduction to the nuclear receptor (NR) superfamily

The nuclear receptor (NR) family includes a diverse but related range of transcription factors, which includes nuclear hormone receptors and orphan nuclear receptors (Olefsky, 2001). One of the major classes of transcriptional activators are nuclear hormone receptors; they regulate gene expression through interacting with specific DNA sequences of target genes. The nuclear hormone receptors form a super-family of intracellular transcription factors, activated by ligand binding (Aranda and Pascual, 2001). It has been shown that abnormalities in NR signalling cascade can cause diseases such as diabetes, cancer, obesity and infertility (Mangelsdorf et al., 1995; Gronemeyer, Gustafsson and Laudet, 2004).

Based on receptor function, cellular localization and evolutionary relatedness nuclear hormone superfamily can be dived into two types. Type I nuclear hormone receptors include the steroid hormone receptors: androgen (AR), glucocorticoids (GR), estrogen (ER) and progesterone (PR) receptors. Translocation of some of these receptors from cytoplasm to nucleus is facilitated through association with co-chaperons (PP5, FKBP52) (Pratt et al., 2004), where they associate with DNA response elements of target genes (Mangelsdorf et al., 1995). Type II nuclear hormone receptors include: Vitamin D, retinoic acid, and retinoid X receptor. Type II nuclear hormone receptors are located in the nucleus unlike type I, and in the absence of hormone they act as transcriptional repressors (Germain et al., 2006).

All members of nuclear hormone receptors share common structural features: the transcriptional activation function called AF-1 which is generally referred as A/B domain, the DNA binding domain (DBD), the hinge region and the ligand binding domain (LBD) (Dahlman-Wright et al., 1995). The DBD consists of two unique motifs to NRs known as zinc-fingers, they target specific DNA sequences called hormone response elements (HRE) (Kumar and Thompson, 1999). Depending on the nature and context of the ligand that binds to the receptor, LBD promotes chromatin remodelling and chromatin modification by recruiting transcriptional coregulatory complexes hence gene activation

or repression happens. The C-terminal of LBD consists 12 helices and contain the AF-2(Sonoda, Pei and Evans, 2008) (Figure 1.9).

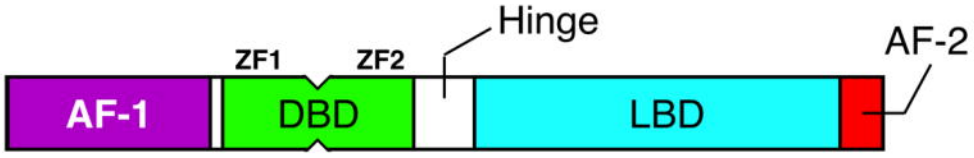


Figure 1.9.Schematic presentation of the functional domains of the steroid hormone receptor group.
The nuclear hormone receptors have a common functional structure containing 2 activation functions: AF-1 and AF-2 located in the N terminal and ligand binding domains respectively. The DNA binding domain (DBD) is located towards the centre and is next to a hinge region. The ligand binding domain is found towards the C terminus (adapted from (Sonoda, Pei and Evans, 2008).

More detailed mechanism of ER in particular ERα, LBD and DBD are explained below, which is the main interest of this study.

1.14 Structure and mechanism of action of estrogen receptor

Binding of two estrogen receptors, ERα and ERβ which are encoded on chromosomes 6q25.1 and 14q23-2.1 (Figure 1.10) respectively, mediates the biological action of estrogen. It was in 1996 that oncologist became aware of estrogen receptor beta (ERβ), its widely expressed in prostate the expression of ERβhas been seen in both normal and malignant breasts (Palmieri et al., 2002). ERα and ERβ have high similarity in the DNA binding and hormone binding domain though they are transcribed from different genes also they have different effect in the cells, while ERβactivity is more relevant in non-reproductive development, ERα is essential for reproductive development (Maggi, 2011). It has been proposed that ERβ has lower transcriptional activity, which might be due to a repression domain in ER-beta amino-terminus, which lowers the efficiency of this receptor (Maggi, 2011). In ERα knockout mice a very little mammary ductal growth was observed whereas normal development of mammary gland with regular ductal branching were observed in ERβ knockout mice, this highlights the importance of ERα in mammary

ductal growth (Zilli et al., 2009). Similar knockout studies in other tissues like bone and uterus also showed ERα plays an important role in their development than ERβ (Couse and Korach, 1999).

It was in 1986, that human ERα gene was cloned (Pinzone et al., 2004). The predominant mediator of estrogen action is ERα, and as ERα expression has been seen in breast cancer patients makes the importance of ERα significant in breast cancer. In breast cancer estrogen receptor alpha (ERα) has been of special interest among all the others steroid hormone receptors, because elevated level of ERα has been seen in premalignant and malignant breast cancer compared to normal tissue (Sommer and Fuqua, 2001).

The C region of both ERα/ERβ encodes the DBD, which comprised of two zinc finger motifs. In promoters of estrogen-regulated genes, DBD mediates the binding of estrogen to specific DNA sequence known as estrogen response element (ERE). The specificity of receptors for its response element, is determined by the so-called P-box which comprises of the amino acids located in the C-terminal end of the first zinc finger (Green et al., 1988). The receptor dimerization between DBDs is mediated by the second zinc finger, containing amino acid residues so-called D-box (Schwabe et al., 1993) (Figure 1.10). ERα and ERβ either as homodimers or heterodimers bind to palindromic DNA response elements matching to the 5'-AGGTCANNNTGACCT-3' consensus ERE sequence (Nilsson and Gustafsson, 2002). Region D or the hinge region of ERα seems to be involved in coregulatory protein binding, and also a site for post-translational modifications (Sentis et al., 2005).

One of the poorly conserved regions between ERα and ERβ is the A/B region, this region is required for transcriptional activation by ERα, as a reduction in ERα activation of reporter genes in a cell specific manner have been monitored in cells lacking A/B region (Kumar and Thompson, 1999). Activation function 1 (AF-1) actives gene expression in an estrogen-independent manner in the absence of LBD (Berry et al., 1990). However, ERα and ERβ require functional synergy with a transcription activation function in the LBD to complete transcriptional activation (Ascenzi, Bocedi and Marino, 2006).

The LBD is the carboxy-terminal E/F domain, and through its activation function 2 (AF-2) mediates ligand binding and transactivation of target gene expression (Evans, 1988). The second most conserved domain between ERα and ERβ is the LBD domain. A short α-helix motif with the consensus sequence LXXLL (where L is leucine and X is any amino acid), mediates the interaction of many coactivators with LBD of NR superfamily (Feng et al., 1998).

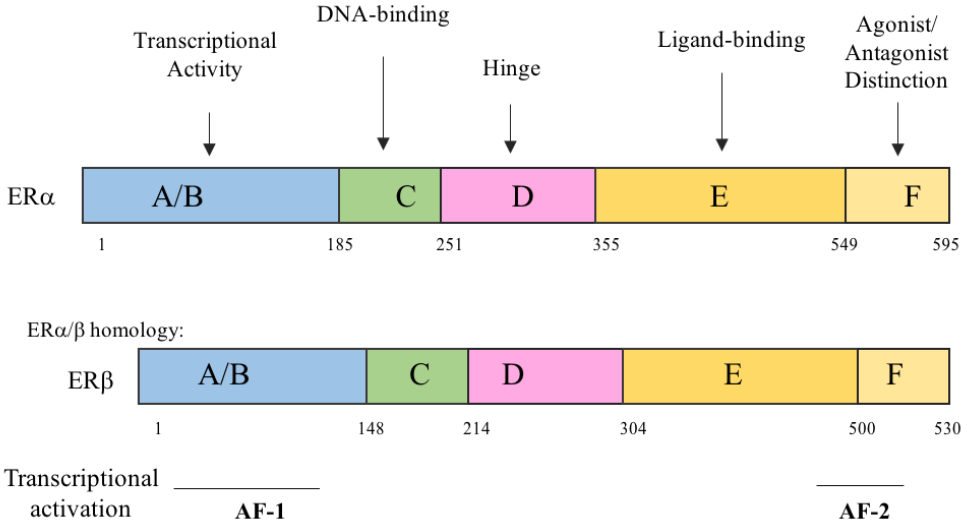


Figure 1.10.Schematic representation of ERα and ERβ common structural and functional domains. Domains of the ERα and ERβ receptor are indicated from A to F. Different regions are indicated as, transcriptional activity, DNA binding, hinge region, ligand binding and interaction with co-activators and co-repressors.

1.15 Control of gene expression

Gene expression is the main regulatory mechanism that ensures controlled and appropriate cellular response to external signals. DNA is transcribed into RNA which is translated into protein, this process is called gene expression. The production rate at which functional proteins are produced, is regulated during the gene expression process at levels of transcription and also post-translational levels.

Three enzymes RNA polymerase I, II and III are responsible for gene transcription. All three enzymes rely on transcription factors to recognize the promoter sequences, in order to start transcription of their specific target genes. Transcribing large ribosomal RNA genes is through RNA polymerase I, whereas RNA polymerase II transcribes mRNA and small nuclear RNA (snRNA) (Nikolov and Burley, 1997). Ribosomes are molecules which are involved in synthesis of proteins, RNA Pol I transcribes ribosomal RNAs into ribosomes. The most abundant class of RNAs in the eukaryotic cells are rRNAs (Stults et al., 2008).

RNA polymerase II targets gene promoters that contain one core and one regulatory region. Generally, the core promoter domain includes a TATA box, an initiator and promoter elements. There are so called "TATA less" promoters as not all promoters contain TATA box. Generally, RNA polymerase III transcribes genes which encode structural or catalytic RNAs such as the components of protein synthesis, and tRNA processing complexes (tRNAs are responsible for transferring amino acids to growing polypeptides) (Nikolov and Burley, 1997).

Several proteins are needed to facilitate and start the transcription, by RNA polymerase. These proteins bind to promoter region of every genes are called general transcription factors (GTFs). Coordinated action of GTFs initiates transcription, which includes TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH and the RNA polymerase core enzyme. For pre-initiation complex (PIC) formation to form, binding of GTFs to the core promoter occurs in an ordered manner and as result facilitate the recruitment of RNA polymerase to the promoter and transcription start site (TSS) (Figure 1.11) (Nikolov and Burley, 1997).

TATA promoter element which is situated 25 bp upstream of the TSS is the core promoter element that controls the positioning of Pol II and TFIID complex, which is the first transcription factor recruited to the promoter (Nikolov and Burley, 1997).
Recognition and binding of TFIID to the TATA box, is through TFIIDs TATA box binding protein (TBP). TFIIF and non-phosphorylated Pol II then bind, followed by TFIIE and TFIIH recruitment (Nikolov and Burley, 1997).

Once the PIC is assembled, the next step is initiated once NTPs become available, which leads to strand separation at the TSS to give an open complex. The large subunit of Pol II is then phosphorylated, resulting in transcription initiation, subsequently followed by Pol II promoter release (Nikolov and Burley, 1997).

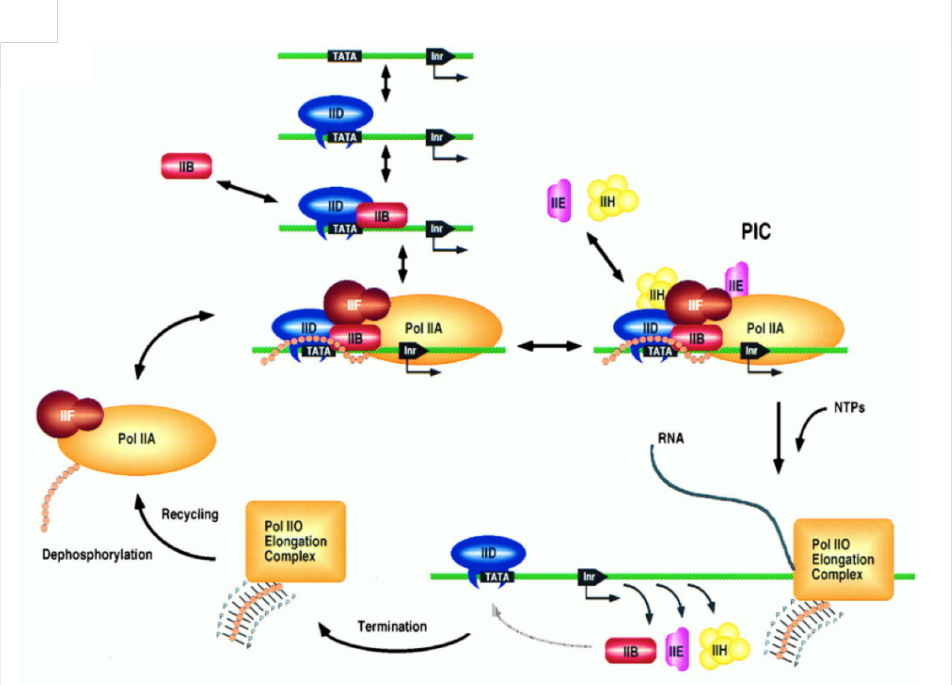


Figure 1.11.Process of transcription.

Diagram showing the process of transcription initiation that involves the recruitment of general transcription factors (TFIID, B, F, E and H) to the DNA to form the PIC (pre-initiation complex) [Adapted from (Nikolov and Burley, 1997)n].

Only low levels of transcriptional activity (basal levels of expression) are produced through the binding of GTFs. There are other sequence-specific TFs that increase or decrease the transcription by binding to regions of DNA celled enhancers and silencers (Trapnell et al., 2010).

Usually a general mediator complex, and a number of proteins that do not bind to DNA themselves so called cofactors, aid the interaction between specific TFs and the factors assembled at the promoter. The common parts of every transcriptional machinery are the mediator complex and the GTFs, however cofactors and TFs can vary for each gene.

1.16 Transcriptional regulation by nuclear hormone receptors

Gene transcription through nuclear hormone receptors occurs either by directly binding to DNA or by protein-protein interactions, with direct DNA binding being a more common mode of nuclear hormone receptors gene targeting. Specific hormone binding to its cognate nuclear hormone receptor activates the protein and allows subsequent transcriptional regulation. In case of type I nuclear hormone receptors, ligand binding induces conformational change that exposes the nuclear localization signals, which then leads to nuclear hormone receptor mediated recognition of the specific DNA sequence in target genes. Whereas in type II, the interaction between hormone and nuclear receptors are in an unliganded manner, which means transcription starts with help of coactivators (Roeder, 2005; Wolf et al., 2008).

1.16.1 Estrogen signalling

1.16.2 Estrogen signalling genomic pathway

ER classical paradigm involves three steps: ligand binding, receptor dimerization and DNA binding at ERE containing the gene promoter, which then leads to initiating transcription by recruiting transcriptional coregulatory protein and RNA polymerase II. Upon entering the cells estrogen binds to the ligand binding domain of the target receptor, and receptor dissociates from chaperon protein by undergoing a conformational change (Pratt et al., 2004). A genomic interaction occurs between the estrogen receptor (ER) and specific sequence of DNA known as estrogen response elements (ERE) (Marino, Galluzzo and Ascenzi, 2006) and this is known as ER classical pathway (Figure 1.12).

ERE classic sequence was found to be composed of two palindromic half-sites 5'AGGTGAnnnTGACCT3'where n can be any nucleotide (Klein-Hitpass et al., 1986).

However, there are many estrogen responsive genes that do not contain EREs. This is when E2 controls gene expression through a non-classical pathway (Figure 1.12). ERα can alter gene expression not only in a classic pathway but also in a non-classical manner at alternative DNA sequences such as, activator protein-1 (AP-1), specificity protein-1 (SP-1) and cyclic AMP response elements (CREs) which bind to their specific transcription factors, c-jun/c-fos, c-jun/ATF2 and SP-1 respectively (Kushner et al., 2000; Saville et al., 2000). Several genes that are involved in growth factor signal transduction pathways are regulated by ER non-classical pathway. Cyclin D1, myc and Bcl-2 are among the genes whose transcription is induced by estrogen in non-classical pathway (Dong et al., 1999).

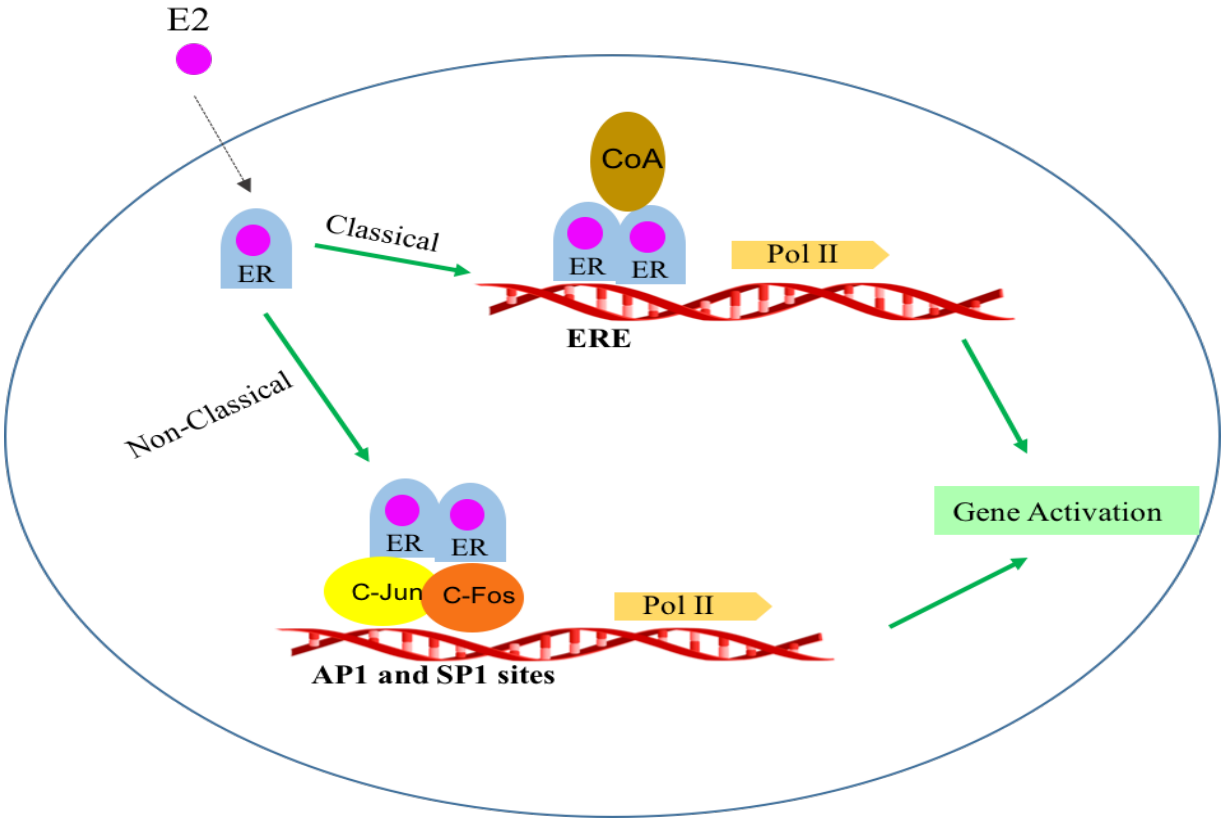


Figure 1.12.Signalling pathway of estrogen receptor.
In classical pathway when estrogen binds to ER in the nucleus the activated receptor then binds to estrogen responsive element of target gene where recruits co-activators to active gene expression. Alternatively, in non-classical pathway binding of activated receptor to AP-1 and SP-1 sites, which binds to c-jun and c-fos and enhances gene transcription.

1.16.3 Ligand-independent activation of estrogen receptor

However, it is presumed that ERα activation requires ligand binding, several growth factors have been shown to stimulate ERα transactivation in absence of ligand, these include: epidermal growth factor (EGF), transforming growth factor α (TGFα), heregulin (HRG) and insulin like growth factor 1 (IGF-1). Ligand-independent activity also occurs as a result of phosphorylation in particular serine of ERα located in AF-1 domain such as serine 118 and 167. Phosphorylation of ERα at these residues occurs as a result of activation of phosphatidyl-inositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) which are growth factor downstream signalling cascade. Indirect modulation of

ERα activity through these growth factor signalling pathways can also happen through enhancing coactivators activity or inhibiting correpressors activities (Ali and R Charles Coombes, 2002; Green and Carroll, 2007).

1.16.4 Estrogen signalling non-genomic pathway

Certain estrogen induced biological effects are simply too rapid, hence it cannot involve altered gene transcription or protein synthesis. Direct estrogenic response on the cell membrane is the result of rapid estrogenic action. GRP30, which is a G protein coupled receptors (GPCRs), have estrogen binding site located on the cell membrane of the cells, and non-genomic action of estrogen is believed to be mediated through these proteins. Downstream intercellular signal transduction pathways such as AKT and MAPK, gest activated as a result of estrogen binding to GPCRs (Simoncini, Rabkin and Liao, 2003; Vrtačnik et al., 2014)

1.17 Post-translational modifications of ER

ER post-translational modifications (PTMs) influences its transcriptional activity, subsellular localization and stability (McKenna and O'Malley, 2002). Phosphorylation, methylation, acetylation and sumolytaion are among the known PTMs (Le Romancer et al., 2011).

ERα phosphorylation leads to an increase between ERα an its coactivators (Zwart et al., 2011), one example is phosphorylation at Y537 of ERα by MAPK kinases, which increases cofactor binding efficiency as a result of helix loop conformation. It has been suggested that Endocrine resistance could be linked to post-translational modification of ERα (Jordan, 2004; Gururaj et al., 2006). Within its AF1 domain (as well as other domain) ERα can be phosphorylated at number of different sites including S167 (Yamashita et al., 2008)S118 (Murphy et al., 2004; Yamashita et al., 2008) S305 (Bostner et al., 2013) and threonine-311. Clinical correlation with tamoxifen resistance and S305 phosphorylation has been shown. It is believed that this is due to increased sensitivity for

E2 and decreased inhibition of tamoxifen (Tharakan et al., 2008). Breast cancer patients with ERα-S305 positive phosphorylation tend to be resistant to adjuvant tamoxifen treatment, however a better recurrence-free survival with tamoxifen treatment have been linked to ERα-S305 phosphorylation negative breast cancer (Holm et al., 2009; Kok et al., 2011).

Acetylation is another major post-translational modification. Promoting transcriptional activation by recruiting several transcription factors and chromatin remodelling complexes appears to be a vital role of histone acetylation. Histone Acetyl-Transferases (HATs) mediates histone acetylation, CBP, p300 and PCAF are among some of the notable HATs (O'Malley et al., 1997a). Importantly, ERα-induced transcriptional activity which is associated with histone acetylation is through activity of CBP and p300. The formation of a complex between p160 with CBP/p300 and PCAF helps recruiting the whole activator complex to the EREs which in turn activates estrogen-responsive transcription (Kim, Hsiao and Kraus, 2001; Demarest et al., 2002). In breast cancer patients, high expression of p300 is correlated with poor prognosis (Xiao et al., 2011). These findings highlight the importance of HATs activity under estrogen-stimulation which results in activation of gene expression by interacting with ERα.

1.18 Interaction of ERα with co-repressors and co-activators

It has been shown *in vitro* that ERα interacts with wide variety of nuclear proteins. Coactivators are proteins that can modulate the functional activity of steroid hormone family of ligand-dependent transcription factors upon binding to them. The protein family of co-activators and co-repressors are unable to bind to DNA by themselves; instead they are recruited to specific gene promoters by transcription factors that are coupled to specific genes. Mediating activation or repression of estrogen mediated transcription depends on coactivator and corepressor proteins. SERMs like tamoxifen can have agonist or antagonist effects depending on balance of coactivator and corepressor proteins in a cell. At least 19 different ERα coactivator proteins have been discovered over the past 5 years (Klinge, 1999). Co-activators are proteins that act with transcription factor to increase the rate of transcription, and are defined according to the nature of

interaction with the nuclear factors and their ability to augment gene transcription. Co-activators are important for the regulation of transcription since they exert positive effects on activators, they can be classified into classes, depending on whether they modify chromatin or interact with RNA polymerase II and other general transcription factors.

Among the large family of ERα co-regulators, the p160 family is known to be the key group. The interaction between ERα and p160 family of proteins, is mediated by interaction between the AF-2 region of ligand binding domain of ERα and the p160 family of coactivators such as SRC-1, SRC-2, SRC-3 and AIB1. There are other coactivators of ERα transcription which includes the CREB binding proteins (CBP), p300 and vitamin-D-receptor-interacting proteins (Nilsson and Gustafsson, 2002).

1.18.1 The p160 coactivator family

The p160 family of coactivator proteins are highly homologous and well-characterised coactivators. This family of coactivator constitute the most well characterised family of nuclear receptor coactivators and consists of three members: SRC1 (NCOA1), SRC2 (GRIP1, NCOA2, TIF2), SRC3 (RAC3, p/CIP, ACTR, AIB1) (Oñate et al., 1995; McKenna and O'Malley, 2002). The three LXXLL motifs that are located within their relatively conserved central region of p160 coactivators are required for interaction between this family of coactivator and LBD domain in nuclear receptors (Xu, Glass and Rosenfeld, 1999). For example, GRIP1's (SRC-2) second LXXLL motif is important in ER binding, whereas, the third LXXLL motif is more important for AR and GR binding (Ding et al., 1998). The SRC-1 family in the presence of agonist has been shown to associate with both ERα and progesterone receptor whereas this association was inhibited by antagonists. Several different proteins bind to the SRC family, and as a result of this binding recruitment of basal transcriptional machinery or molecules that are involved in chromatin remodelling such as, coactivator-Associated Arginine Methyltransferase-1 (CARM1) and p300/CBP associated factor (PCAF) occurs. Enhancement of the transcription is the general effect of these coactivators (Glass and Rosenfeld, 2000).

SRC-1 and ACTR which are from family of p160 family of protein, have been reported to have intrinsic HAT activity (Lynch et al., 2013) however other coactivators like p300 or P/CAF have weaker HAT activity compare to SRC-1 and ACTR. There are two transcriptional activation domains (AD1 and AD2) that are located in C-terminus of p160 family (O'Malley et al., 1997b) . The recruitment of CBP/p300 carried out by AD-1 domain that contains additional LXXLL motifs (Voegel et al., 1996) whereas the AD-2 domain, recruits co-activator associated CARM1 and protein arginine methyltranferase 1 (PRMT1) interact with the SRC family members and increase transcriptional activity of AR, ER and TR through modifying chromatin (Chen et al., 1999; Koh et al., 2001).

1.18.2 CBP/p300 co-activators

Ubiquitously expressed proteins CBP/p300 have been shown to be involved in many signalling pathways, and known as general coactivators. Their role is to possess histone acetyl transferase activity. Several studies have reported the co-activatory role of this family of proteins for different transcription factors. Also, they can directly interact with basal transcription machinery components (Dilworth and Chambon, 2001). Interaction of CBP/p300 and nuclear receptors have been reported in many studies, particularly CBP/p300 interaction with ERα results in an increase in transcriptional activity of ERα (Kraus and Kadonaga, 1998). Some other proteins with HAT activity, such as pCAF may be recruited by CBP and p300. HAT activity of CBP and p300 which enhances the gene transcription by chromatin remodelling may be through recruitment of other proteins such as p/CAF and GCN5L2 (Hamamori et al., 1999).

1.18.3 SWI/SNF chromatin remodelling complex

The SWI/SNF (switch/sucrose non-fermenting) complexes can regulate cellular process including transcription and DNA repair by mediating ATP dependent chromatin remodelling (Tang, Nogales and Ciferri, 2010). This complex was originally identified in yeast, however human homologues of this group have been shown to interact in a ligand dependent manner with ERα, such as SNF2α (BRM1) or SNF2β (BRG) (Belandia et al.,

2002). Recruitment or activity of chromatin remodelling complexes is sometimes required for function of some co-activators, for instance for SRC-1 and CBP to mediate estrogen receptor activation BRG1 is required. Whereas in case of SWI/SNF complexes in order to target them to nuclear receptor, additional bridging molecules are required. For example, a subunit of SWI/SNF which is BAF5 is required to interact with Flightless-I (Fli-I) coactivator, which in return recruits SWI/SNF to estrogen receptor target genes (Jeong, Lee and Stallcup, 2009).

1.18.4 Histone methyltransferases

The second most common histone-modifying enzymes that have been reported to regulate nuclear hormone receptor mediated transcription are histone methyltransferases (HMT). Two of the arginine methyltransferases associated with ER are; coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1). These two proteins are known to methylate histones and nuclear receptors and other coactivators (Chen et al., 1999; Ma et al., 1999). Through methylation of histone H3 at Arg17 and Arg26 residues, CARM1 was found to enhance nuclear receptor transcriptional activation. For instance, it has been shown that glutamate receptor-interacting protein 1 (GRIP1), causes a synergistic increase in ER transcriptional activity by binding to p300 through its AD1 (activation domain 1) and to CARM1 through (AD2) activation domain 2. In MCF-7 breast cancer cells CARM1 has been shown to be crucial for estrogen-induced cell cycle progression, which makes CARM1 potentially important in cancer (Frietze et al., 2008).

Another HMT that has been shown to interact with p160 protein family through their AD2 domain is protein arginine methyltransferase 1 (PRMT1) (Koh et al., 2001). Another PRMT (PRMT2) has been shown to bind to ER via AF-1, the DBD and the LBD in a ligand dependent manner (Qi et al., 2002).

1.18.5 The TRAP/DRIP/SMCC complex

In a ligand dependent manner thyroid-hormone-receptor-associated protein (TRAP) and vitamin-D-receptor-interacting protein (DRIP) binds to nuclear receptor, including ERα, this interaction is through direct interaction with a subunit of TRAP/DRIP complex known as TRAP220/DRIP205 (Burakov et al., 2000). It has been reported that TRAP/DRIP subunits are important for recruitment of Poll II to the gene promoters, as these subunits have been found in the mediator complex associated with RNA polymerase II (Burakov et al., 2000).

1.19 Co-repressors

Repressing gene expression in an unliganded manner by many receptors is through recruiting transcriptional corepressors (Xu, Glass and Rosenfeld, 1999; Ali and R. Charles Coombes, 2002). It has been shown in many studies that ERα can repress a large number of estrogen regulated genes (Frasor, Jeanne M Danes, et al., 2003), for instance upon tamoxifen binding, ERα can represses the expression of some responsive genes. As mentioned above coactivators may relax the chromatin by acetylating histones, whereas transcription repression might follow the recruitment of Histone deacetylases (HDACs), which condense the chromatin by deacetylating lysine residues on histone proteins. Transcriptional corepressors like nuclear corepressor (NCoR) and silencing mediator (SMRT) (Perissi et al., 2010)facilitate gene repression by recruiting HDACs. In order to repress ERα gene expression in absence of ligand, corepressors like NCoR and SMRT, interact with AF-2 and LBD domain of ERα (Lazar, 2003), as when ligand binding happens corepressors becomes dissociated and co-activators are recruited to the site (Ali and R. Charles Coombes, 2002).

Co-activators and co-repressors are essential not only for breast cancer progression but also for the response and resistance to endocrine therapy (Figure 1.13. For example, amplified in breast cancer 1 (AIB1), is overexpressed in 50% of the breast tumours, also in breast tumours overexpression of HER-2 and AIB1 has been associated with tamoxifen resistance (Osborne et al., 2003). In the case of co-repressors, a decrease in rate of survival

and also shorter disease-free interval have been associated with low levels of NCoR and SMRT (Girault et al., 2003). As mentioned before tamoxifen acts as an antagonist in breast cancer, and agonist in other tissues like uterus and bone. Tissue specific action of tamoxifen and other SERMs is due to different expression patterns of ERα coregulatory proteins (Jordan, 2004). Change in expression levels of coactivators and corepressors appears to contribute to the mechanism of resistance. encouraging. Agonist activity of tamoxifen has shown to enhanced by overexpressing co-activator or silencing co-repressor (Shang and Brown, 2002).

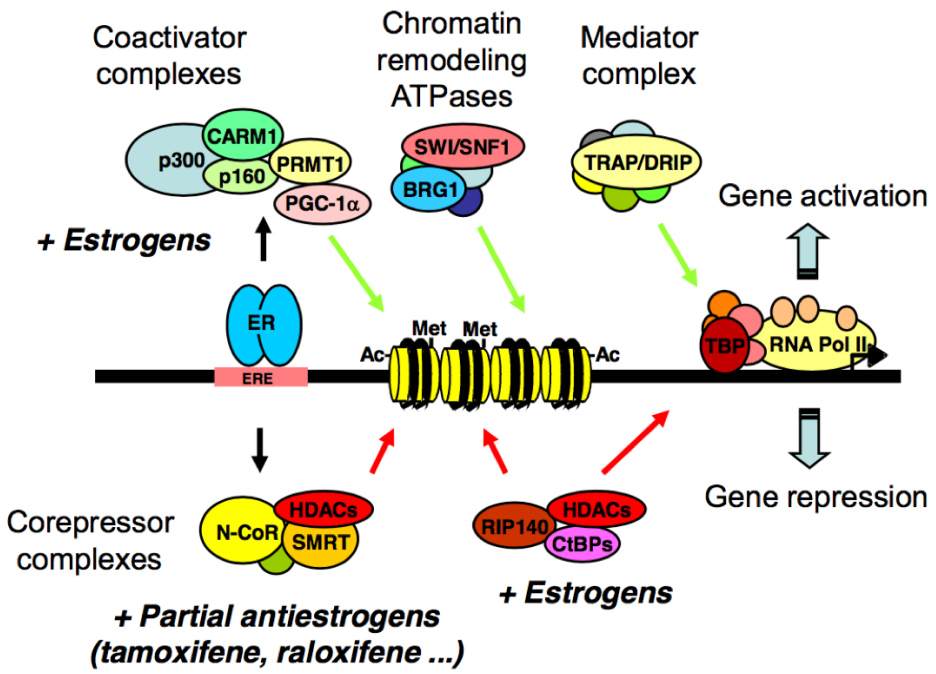


Figure 1.13.The coregulatory complexes of ERα.

Depending on the type of ligand bound to ERα, the recruitment of corepressors or coactivators varies. [figure adapted from (Teyssier et al., 2010)]

1.19.1 Recruitment of ERα to the DNA with aid of pioneer factors

While some transcription factors interaction with DNA requires a euchromatin structure, some have the capacity to associate with condensed chromatin, hence modulating chromatin accessibility. Where these factors bind it facilitates binding of other transcription factors hence these factors have been termed "pioneer factor" (Figure 1.14) (Jozwik and Carroll, 2012; Magnani and Lupien, 2014).

The most studied pioneer factor for ERα signalling is the forkhead transcription factor (FOXA1) (Eeckhoute et al., 2006). Displacing linker histones, which leads to de-compaction of chromatin by FOXA1, is through its unique structure. The winged helix of FOXA1 has a similar structure to histone H1, which allows its interaction with histone H3 and H4 (Cirillo et al., 1998). In other words, when FOXA1 binds to compact chromatin, displaces histone H1 and disrupts H3/H4 hence creating an open chromatin (Cirillo et al., 1998). The FoxA1 binding site has been seen at large number of estrogen responsive genes in close proximity to the mapped ERE of these genes (Carroll et al., 2005). Recruitment of ERα to DNA of these EREs seems to be dependent on FoxA1. Inhibition of ERα and estrogen-dependent gene expression was observed when FoxA1 gene was knockdown, this suggested that FoxA1 is important in the recruitment of ERα to EREs in estrogen responsive genes (Laganière et al., 2005). GATA3, TLE and PBX1 are among other pioneer factors that have been associated with hormone dependent cancer (Jozwik and Carroll, 2012).

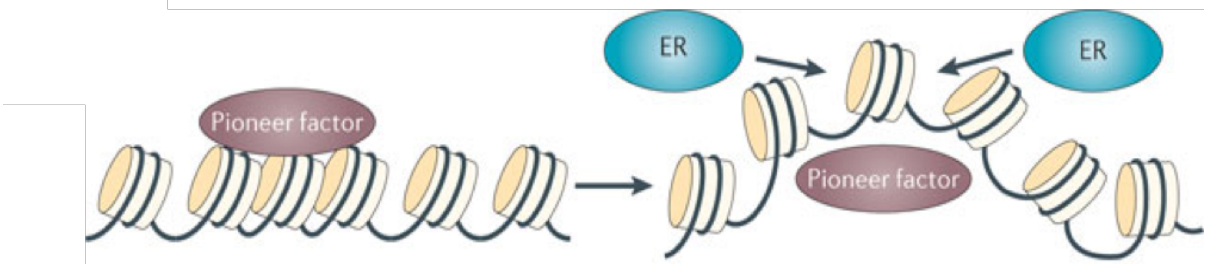


Figure 1.14.The pioneer factors functions.
Once bounded to condensed chromatin, pioneer factors facilitate ERα chromatin interactions by increasing accessibility to estrogen response elements (EREs). [adapted from (Jozwik and Carroll, 2012)].

1.20 Tetratricopeptide repeat domain 5 (TTC5) a novel cofactor of nuclear receptors

The focus of this study is the 440 amino acid (in mouse) protein TTC5 (tetratricopeptide repeat domain 5) previously known as STRAP (Stress responsive activator of p300). The gene encoding TTC5 is located on chromosome 14, at the locus 14q11.2. TTC5 is widely expressed in mammalian tissues, with the strongest expression detected in brain, lung and kidney (Demonacos, Krstic-Demonacos and La Thangue, 2001), the sequence conservation between human, rat and mouse TTC5 is greater than 90%.

Analysis of TTC5 protein predicted the presence of six tandem tetratricopeptide repeat motifs (TPR motifs) throughout the protein structure (Demonacos et al., 2001). Interestingly, TTC5 contains two putative LXXLL motif as well as the six TPR motifs, which makes TTC5 a potential cofactor for nuclear receptor binding (Figure 1.15 and Figure 1.16). It has been shown that TTC5 has an oligonucleotide (OB) domain at its C terminal, it is known that OB containing proteins are critical for various DNA related function such as DNA replication, repair, transcription and translation (Adams et al., 2008).

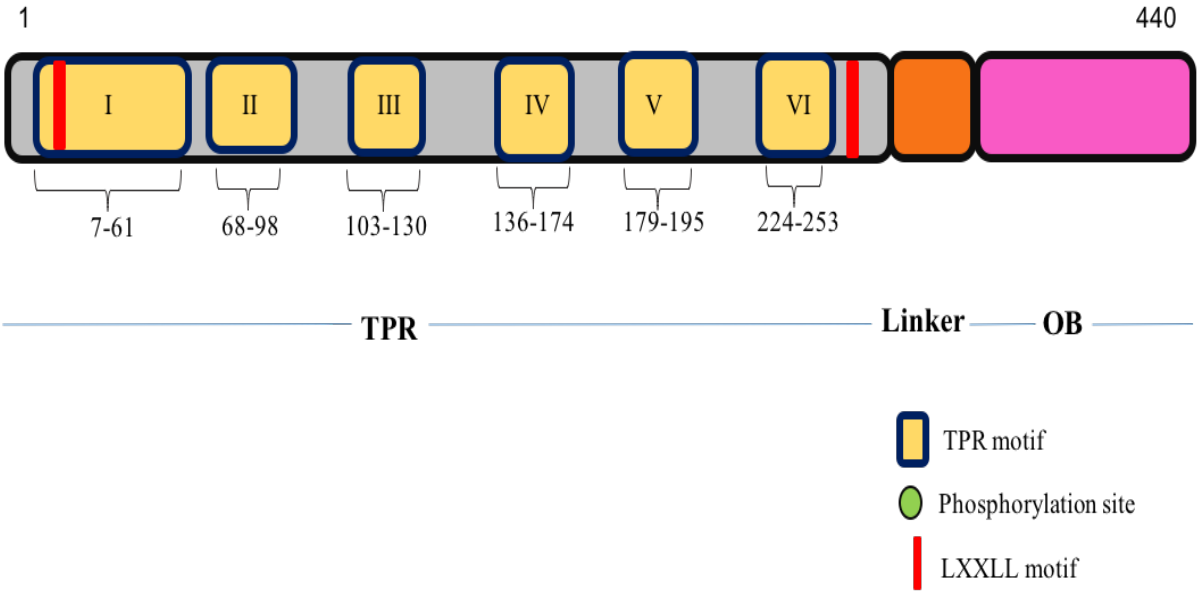


Figure 1.15.TTC5 structure.

Illustration of six TPR motifs distribution within TCC5, highlighted in yellow and labelled I to VI, a linker shown in orange in addition to OB domain shown in pink. Red shows putative LXXLL motifs.

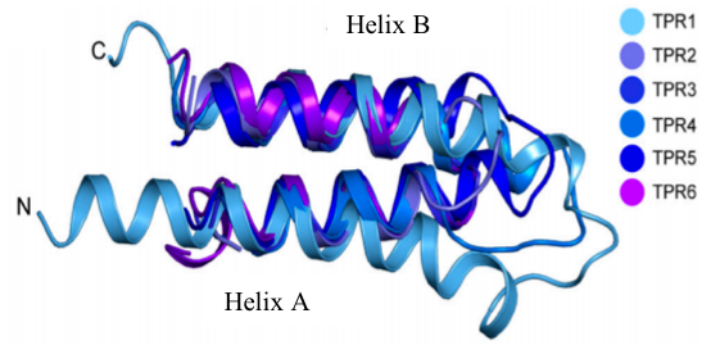


Figure 1.16.Positioning of the six TPR motifs in TTC5.

Approximately each TPR motif consists of 34 amino acid residues assembled as antiparallel alpha helices (A and B) which are connected by a short loop. [modified from (Adams et al., 2012)]

1.21 Protein-protein interaction motifs

Some proteins are important in mediating protein-protein interaction, and they contain a specific motif, among which are the WD40 (Stirnimann et al., 2010) PDZ (Jeleń et al., 2003) SH3 (Li, 2005) and TPR motifs ((Blatch and Lässle, 1999)WD40 domain are implicated in various biological processes such as cell division, chemotaxis, and other signal transduction pathways and are highly abundant in eukaryotic proteins; this domain consists of 44-60 residues (Jeleń et al., 2003). PDZ domain binds to the C termini of interacting proteins such as transmembrane receptors, channel proteins and other PDZ domain containing proteins, and it contains 80-100 amino acid residues (Jeleń et al., 2003). Recognition and binding to phosphorylated tyrosine containing sequence is part of the function of SH2 domain, SH3 which is approximately 60 amino acid long, binds to peptides containing consensus sequence PxxP (x being any other amino acid) (Li, 2005).

TPR motifs are another example of a protein interaction motif, these motifs often occur in tandem arrays, and are made up of 34 amino acids, which are not generally conserved but show similarities in size and spacing (Blatch and Lässle, 1999). TPR motifs are found in various proteins in different sub-cellular locations such as nucleus, mitochondria and cytoplasm, and are evolutionary conserved form prokaryotes to eukaryotes (Blatch and Lässle, 1999). Characterisation of TPR motifs has been revealed through X-ray crystallography of TPR proteins such as pp5 (Lamb, Tugendreich and Hieter, 1995; Das, Cohen and Barford, 1998). Further observation of the secondary and tertiary structures of this motif has shown that TPR motifs consist of two alpha helices forming an anti-parallel hairpin structure, this hairpin structure consist of two anti-parallel alpha helical domains A and B. Each TPR motif is parallel to each other and there is an angle of about 4 degrees between helices A and B of TPR motifs (Lamb, Tugendreich and Hieter, 1995; Das, Cohen and Barford, 1998).

Proteins that contain this motif are often found as part of multi-protein complexes, as this motif is important for protein-protein interactions. TPR motifs are involved in many protein functions, including protein folding, protein transport and regulatory phosphate

turnover. Sequence alignments of different TPR proteins identified amino acid residues W (tryptophan), L (leucine), G (glycine), Y (tyrosine), A (alanine), F (phenylalanine), A (alanine) and P (proline) at position 4, 7, 8,11,20,24,27 and 32 to be highly conserved (Blatch and Lässle, 1999). TPR motifs have been shown to be involved in DNA damage repair. For example, PP5 is an ubiquitously expressed serine threonine phosphatase that can bind to Hsp90. PP5 has a C terminal and an N terminal TPR motif (Das, Cohen and Barford, 1998).

It has been shown that TPR proteins are involved in transcription, the interaction between Tfc4 of TFIIC and Brf1 of TFIIB is an important initiating step in RNA polymerase III transcription. For the process of RNA polymerase III transcription, this has been shown to be the rate-limiting step (Liao, Willis and Moir, 2003) Tfc4 has 11 TPR motifs which are important in mediating Tfc4 interaction with Brf1 and Bdp1 needed for the initial assembly of TFIIB onto DNA. Mutations within specific TPR regions have been shown to disrupt Polymerase III reporter gene transcription and impair interaction between Brf1 and Tfc4 (Liao, Willis and Moir, 2003).

The protein tetratricopeptide repeat domain 4 (TTC4) is also a TPR protein and was originally identified within the gene region implicated in breast cancer (Crevel et al., 2008). It has been shown that TTC4 interacts with Hsp70, Hsp90 and with the replication initiation protein Cdc6 through TTC4 TPR motif (Crevel et al., 2008). An increase in the level of TTC4 was seen in melanoma and TTC4 has been shown to be implicated in cancer progression as increased level of TTC4 protein are detected in various tumour cell lines (Crevel et al., 2008).

1.22 TTC5, p300 and JMY

TTC5 has shown to form a complex with p300 and JMY in response to DNA damage.
It was back in 1986 that p300 was discovered as a phospho-protein with molecular size of 300kDa localized to the nucleus. It was also found that p300 interacts with E1A (Adenovirus early region 1A) protein, through an independent N-terminal binding site

(Dallas, Yaciuk and Moran, 1997). E1A was shown to repress transcription however p300 free from an interaction with E1A was able to overcome this repression (Rikitake and Moran, 1992). An increase in identity of E1A binding site region of p300 was revealed when the sequencing analysis revealed a 63% homology between the human orthologue of p300/ CBP (CREB binding protein) (Dallas, Yaciuk and Moran, 1997). When both CBP and CREB proteins were cloned it was discovered that they were 85% homologous to one another, and also that CBP identified as a CREB binding protein (Arany et al., 1994). Both proteins can acetylate histones and other proteins such as p53 due to their intrinsic HAT activity (Ogryzko et al., 1996).

Through further experiments it was predicted that p300 and CBP have similar functions and that they are implicated in several diseases, highlighting their importance in homeostasis and growth (Kalkhoven, 2004). p300 seems to act as a tumour suppressor as alterations in the protein have been seen in solid tumours (Ozdağ et al., 2002). p300 protein and CBP are transcriptional co-activators, acting as in adaptor molecules between the transcriptional machinery and DNA binding factors (Wang et al., 1993; Zhu et al., 2001).P300 is implicated in wide array of cellular activities, such as DNA repair, proliferation, apoptosis and cell cycle regulation (Wang et al., 1993; Zhu et al., 2001)P300 also interacts with both positive and negative regulators of p53, meaning it is implicated in p53 response (Yuan and Giordano, 2002)Mdm2 which is a negative regulator of p300 binds to p300 through residues 102-22, mutation in these regions means that Mdm2 cannot bind to p53, hence degradation of p53 through Mdm2 cannot happen (Yuan and Giordano, 2002)

Initially JMY was discovered through a yeast two-hybrid assay, a 110 kDa protein which interacts with p300 (two binding domains present between amino acid residues 1-119 and 469-558) (Shikama et al., 1999a) and named according to its function as “junction mediating regulatory protein”. JMY and p300 were found as a multi component coactivator that interact with one another (Shikama et al., 1999b). Further analysis of JMY sequence, reviled a number of interesting features; among which is a central area conserved adenovirus E1A CR2 homology region, a residue of proline rich string at its C termini and three consensus sites for CdK in the N-terminal region (Shikama et al.,

1999b). Initial experiments were carried out to identify JMY chromosomal location, to deduce whether it could be implicated in cancer or not. The region that JMY is located at (chromosome 5 in band 5q 13.2) has been implicated in various malignancies such as leukaemia.

1.23 TTC5 functions

Under stress conditions levels of TTC5 increase, as well as the interaction between p300 and JMY (Demonacos, Krstic-Demonacos and La Thangue, 2001). Facilitating the interaction between JMY and p300 through TTC5 was demonstrated through overexpressing TTC5 in U2OS cells; in these cells p300 was coimmunoprecipitad with JMY and in presence of TTC5, this interaction between p300 and JMY increases. Since, in absence or presence of TTC5, p300 protein levels stayed the same in the immunocomplex, the coimmunoprecipitated JMY levels most likely increased from the influence of TTC5 on the recruitment of JMY to p300 complex. The interaction between TTC5 and JMY is through TTC5 N-terminal domain (residue 1 to 205) and the C-terminal region interacts with p300 (residue 206 to 438) (Demonacos, Krstic-Demonacos and La Thangue, 2001) TTC5 has been reported to stabilize the interaction between JMY/p300 complex (Figure 1.17).

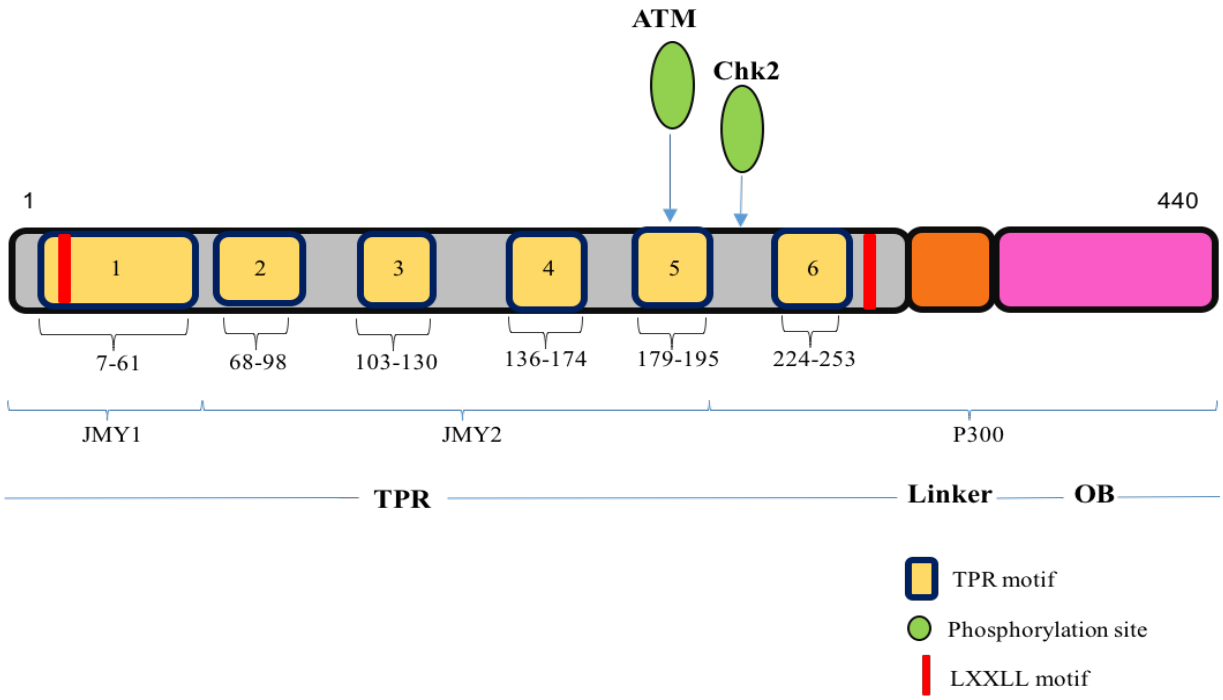


Figure 1.17.TTCR interaction with JMY and p300 through various TPR motifs.
Residues 1-205 of TTC5 are implicated in JYM interaction and residues 206-438 are implicated with p300 interaction. ATM phosphorylation site and Chk2 phosphorylation site are pointed at with arrows.

1.23.1 TTC5 and DNA damage response

Either from within the cells or from the environment (UV, various chemicals) cells are exposed to DNA damaging agents. These agents can cause mismatched bases, single or double strand break hence damaging the DNA. When DNA damage occurs, cells identify and repair the damage as well as preventing the propagation of the mutations (Branzei and Foiani, 2008). Failure of the cells to elicit the DNA damage response, results in genetic disease, cancer and severe immunodeficiency (Bartek, Bartkova and Lukas, 2007). The phosphatidylinositol 3-kinase like family (PIKK) members which includes: ATM, ATR, and DNA-PK identifies the DNA damage. ATM and DNA-PK gets activated by double strand break whereas ATR gets activated as a result of single strand DNA breaks. Downstream targets including the tumour suppressor p53, checkpoint kinases Chk1 and Chk2 are then phosphorylated once these signalling cascades are active (ATM, ATR). This signalling cascade aims to repair DNA damage before mutations are passed

to the next generation of cells (Cimprich and Cortez, 2008). Depending on the type of damage and the phase of the cell cycle different repair mechanism are recruited to the site. These repair mechanisms include: non-homologous end joining (NHEJ), homologous recombination (HR), and mismatch repair (Branzei and Foiani, 2008). Cell death by apoptosis can happen as failure to repair the DNA damage.

In response to DNA damage p53 triggers cell cycle arrest and apoptosis, and its known to be frequently mutated in human cancers (Ko and Prives, 1996). The DNA damage signalling cascade that is initiated by ATM, ATR and Chk family, stimulates the transcriptional activity and stabilization of p53 (Shiloh, 2006). The p300/CBP coactivator plays a role in p53 regulation by interacting with it activation domain (Lill et al., 1997; Lee and Goldberg, 1998).

It has been reported that, TTC5 activates p300 coactivator complex and affect the p53 response. When DNA damage occurs due to ionising radiation or chemical agents such as etoposide, ATM phosphorylates TTC5 at position Ser203, which is within TPR3 of TTC5, resulting in TTC5 nuclear localization (Figure 1.18). Chk2 which is downstream of ATM then phosphorylates TTC5 at position Ser221, (Demonacos et al., 2004; Adams et al., 2008). This region is between TPR3 and TPR4, and phosphorylation leads to TTC5 stabilization, which then results in assembly of the TTC5, p300 and JMY complex, causing p53 acetylation, and activation of the DNA damage response (Demonacos et al., 2004). It has been shown that JMY interacts directly with TTC5 and p300, hence aiding the p53 activation by this complex (Shikama et al., 1999a; Demonacos, Krstic-Demonacos and La Thangue, 2001; Demonacos et al., 2004). TTC5 has also been reported to stabilises p53 by reducing Mdm2 mediated degradation (Demonacos, Krstic-Demonacos and La Thangue, 2001).

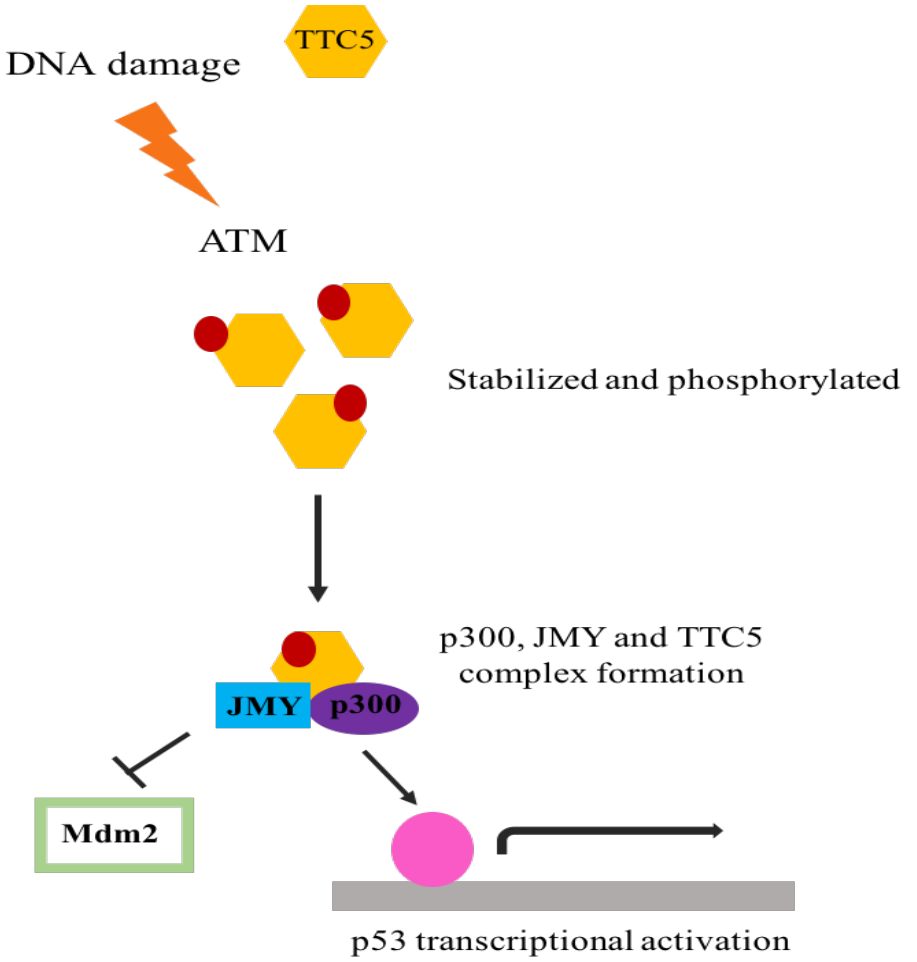


Figure 1.18.TTC5 and DNA damage response pathway.

When DNA damage occurs, ATM becomes activated and phosphorylates TTC5 at S203 and causes its stabilization. Then TTC5 translocate in to nucleus and forms a complex with p300 and JMY. The complex reduces Mdm2 mediated degradation of p53 and increases p53 transcriptional activation, resulting in cell cycle arrest or apoptosis.

TTC5 remains in the cytoplasm in ataxia-telangiectasia (AT) cell lines (1BR and GM02530) tested, both of which have non-functional ATM. Mutated version of TTC5 also remains in the cytoplasm which cannot be phosphorylated by ATM. In these conditions translocating TTC5 into the nucleus while ATM is defective, results in restoring TTC5 stabilization and the DNA damage response (Demonacos et al., 2004; Adams et al., 2008).

1.23.2 TTC5 role in the heat shock response

There are many stress responses pathways that enable cells to live under a variety of environmental stresses (Xu and La Thangue, 2008). Activation of a set of chaperons called heat shock proteins are one outcome of cellular response to heat shock stress. Under normal conditions HSF1 exist as a monomer in the cytoplasm, the heat shock pathway involves HSF1.

It has been shown that TTC5 interacts with HSF1 when cells are heat shocked, and the TTC5/HSF1/p300 complex forms which then binds to heat shock elements (HSEs) of target shock protein genes like Hsp70 (Xu and La Thangue, 2008). p300 then acetylates histones of Hsp70 and results in Hsp70 transcriptional activation (Xu and La Thangue, 2008). Inhibition of apoptosis through inhibition of caspase activation and cytochrome C release is the result of Hsp70 activation (Figure 1.19).

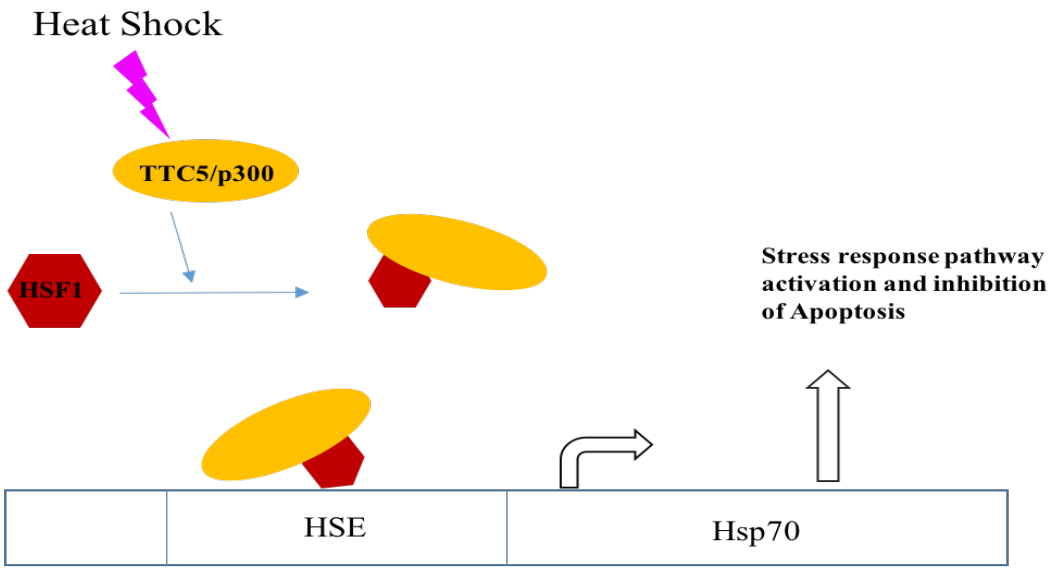


Figure 1.19.TTC5 and the stress response pathway.

TTC5 interacts with HSF1 under heat shock which results is its phosphorylation and formation of HSF1/TTC5/p300 complex. This complex then binds to heat shock elements (HSE) of target genes like Hsp70 (heat shock protein 70), and causes its transcription to activate.

TTC5 has also been shown to prevent apoptosis in human and murine acute myeloid leukaemia (AML) cells. Induction of apoptosis in both human and murine AML cells was observed when TTC5 was knocked down and a, similar effect was observed with knockdown of p300 (Lynch et al., 2013). Also, knockdown of both p300 and TTC5 resulted in increased expression of pro-apoptotic genes and a reduction in expression of BCL2 which is an anti-apoptotic gene. The study show that through the interaction of TTC5 and p300, Myc becomes acetylated and regulates transcription of anti-apoptotic genes (Lynch et al., 2013). Myc is an acetylation target for p300 and knockdown of TTC5 and p300 leads to accumulation of Myc. Excess levels of Myc have the ability to sensitize cells to apoptosis. It is believed that the interaction between p300 and TTC5 prevents the excessive accumulation of Myc in AML cells (Lynch et al., 2013).

1.23.3 TTC5 role in GR regulation

Furthermore, it has been shown that TTC5 is also implicated in glucocorticoid receptor (GR) regulation, under cellular stress (Davies et al., 2011). Through interaction with glucocorticoids under stress GR becomes activated. Diverse sets of genes that are implicated in immune response, inflammation and metabolism are regulated by active GR, in a both positive and negative manner (Davies et al., 2011). GRs activity is regulated through various mechanisms due to its multifunctional property; for instance, interactions with cofactors, protein stability and post-translational modifications. GR is a target for degradation through the process of ubiquitination hence protein stability is a critical regulator of GR. Interaction of GR with cofactors such as p300 and heat shock proteins affects chromatin architecture and interact with the basal transcriptional machinery (Figure 1.20) (Davies et al., 2011). As result of GR interaction with hormones a significant conformational change occurs within GR, results in exposing a surface that have the ability to bind to LXXLL motif of target co-factors.

In A549 cells (lung cancer cells) it has been shown that TTC5 interacts with GR, and that TPR2, TPR3, TPR6 and LTSAL (LXXLL motif) are the most critical motifs in this

interaction. This was determined, because mutations of these motifs especially TPR6 and LTSAL seemed to reduce the GR binding to TTC5. TTC5-GR interaction inhibits GR degradation hence TTC5 is an important regulator of GR stability and its stabilization by increasing GR half-life (Davies et al., 2011).

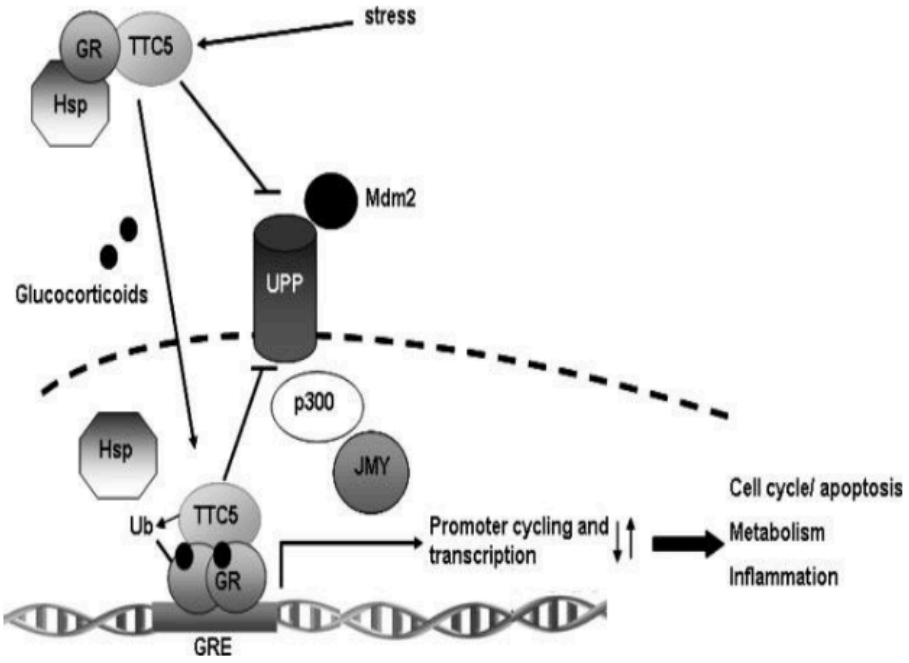


Figure 1.20.Regulation of GR by TTC5.

A tri-meric GR/Hsp/TTC5 complex is formed under stress, resulting in GR stability, through Mdm2 inhibition. By preventing the action of the ubiquitin proteasome pathway (UPP), possibly through ubiquitin ligase Mdm2, TTC5 stabilizes GR. After hormone binding the receptor translocate into nucleus where it binds to glucocorticoid response element (GRE) of its target gene. TTC5, Hsp, JMY and p300 are all implicated in the regulation of GR associated related gene transcription. [Table adapted from (Davies et al., 2011)]

1.23.4 TTC5 role in AP-1 pathway

TTC5 also regulates another transcription factor function. The pathway of transcription

factor activator protein-1 (AP-1) has been shown to play an important role in transformation, proliferation and death. It has been shown that AP-1 is composed of proteins similar to c-Jun and c-Fos and activating transcription factor (ATF) families (Xiong et al., 2013). AP-1 ability to regulate the expression of cell cycle regulators such as p53, cyclin D1 and p21, makes it an important protein to control cell proliferation and cell death. AP-1 can be both antioncogenic and oncogeneic which either induces apoptosis or sends signals which lead to cell survival. TTC5 has been shown to inhibit AP-1 and c-Jun transcriptional activity in HEK293T, HeLa and HCT116 cells through stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) MAPK pathway (Xiong et al., 2013). This discovery happened through observing Ser63 protein level status which is a phosphorylated c-Jun when TTC5 were overexpressed. Indeed, TTC5 overexpression lead cells to have lower level of phosphorylated c-Jun and c-Jun compared to control group. Furthermore, the protein expression of three main subgroups of MAPK family ERK1/2, JNK/SPAK and p38 MAPK were measured when TTC5 was transfected, and the results revealed that TTC5 overexpression only had effects on JNK/SPAK phosphorylation but not ERK1/2 and p38 pathway.

1.23.5 TTC5 regulates ATP synthase

In response to DNA damage mitochondria are the central death regulator, and critical for p53-depedent death (Mihara et al., 2003). The outer mitochondrial membrane (OMM) undergoes permeabilization when mitochondria receive a death signal, as a result potent death factors such as cytochrome *c* are released form intermembraneous space into the cytosol (Mihara et al., 2003).

More recent a new role for cofactor TTC5 in mitochondria has been discovered; it has been shown that TTC5 through ATP synthase, regulates oxidative phosphorylation and p53 mitochondrial activity (Maniam et al., 2015). For intracellular pathways, mitochondrial ATP production is the main source of energy. It has been shown that, reduction in ATP-dependent ion pumps can be due to defective ATP production or excessive consumption of ATP, which can lead to apoptosis. To trigger caspase activation and favouring apoptosome formation during apoptosis a sufficient supply of ATP is

necessary, and the mode of programmed cell death is usually determined by the severity of reduction in cellular ATP (Maniam et al., 2015a).

Wide variety of metabolic pathways like glycolysis and oxidative phosphorylation are modified by p53 activity, which enables cells to respond to metabolic stress. Induction of apoptosis through p53 can occur both in transcription-dependent or transcription-independent fashion. The transcription-independent activation of apoptosis by p53 is through is localisation to mitochondria; where the interaction of p53 with Bcl2 family of genes occurs (Bax and Bak) and regulates their activity. This interaction results in caspase activation in cytosol which is a consequence of realising soluble proteins like cytochrome *c* as a result of p53 influencing the intrinsic pathway of apoptosis (Maniam et al., 2015b).

As it was discovered that p53 and TTC5 interact with each other, a derivative form of TTC5 that was exclusively directed to mitochondria was made to facilitate the analysis of TTC's mitochondrial role in ATP synthesis. Through mass spectrometry the ATP synthase β-subunit was identified as the predominant interacting protein with TTC5. β-subunit is localised at the inner mitochondrial membrane which is an essential component of ATP synthase. Also, the cellular ATP levels seemed to be affected by TTC5, as enhanced cellular ATP levels were observed when TTC5 was knocked. The mitochondrially localised TTC5 seemed to significantly reduce levels of cellular ATP hence sensitising cells to apoptosis which is dependent on p53 activity(Maniam et al., 2015).

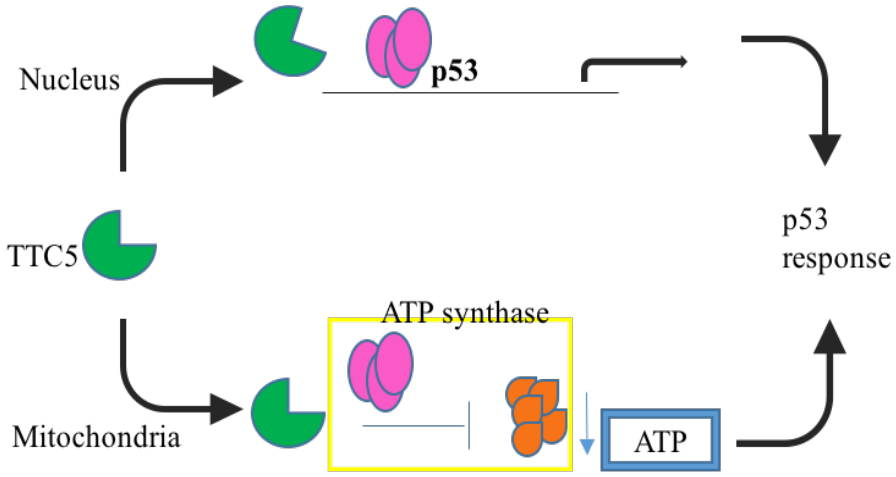


Figure 1.21.TTC5 role in nucleus versus mitochondria.

Model illustrating role of TTC5 in p53 response, where TTC5 (green) stimulate apoptosis in mitochondria, by downregulating ATP synthase (orange) while inhibiting ATP production [modified version form Maniam et al., 2015].

1.24 Study aims

ER is known to regulate the transcription of many genes; hence one major question is how the activity of this transcription factor is controlled. Coactivators and corepressors that are available in different cell types and physiological conditions have been shown to modulate ER activity. These findings within this field are likely to be of importance in the gene expression area, especially since ER is a transcription factor. Findings could also be important for drug design, since and understanding of ER modulation could lead to the discovery of novel treatments, with a faster approach on breast cancer treatments.

It has become evident that TTC5 has a rare structural characteristic, that it harbours six predicted consecutive TPR domains and two LXLL throughout its sequence. Presence of these TPR and LXLL domains within the sequence of TTC5 protein possibly aids this protein to interact with several different binding partners, hence being involved in multiple signalling pathway. These domains may also be critical to amplify particular signal within the cells.

Previous studies in our laboratory established that TTC5 both positively and negatively regulates GR activity in a gene specific manner (Davies et al., 2011). The interaction between hormone and GR causes a significant conformational change within GR, which expose a surface that binds to the LXXLL motif of target co-factors. Due to the fact that ER and GR are from same family of nuclear hormone receptor and have similar structure, hypothesis is that TTC5 co-regulates ER transcriptional activities. Therefore, the aims of my project are

1) To investigate the role played by different motifs of TTC5 on ER transcriptional activity,
2) Determine whether TTC5 interacts with ERα in breast cancer cells,
3) Monitor the effect of estrogen agonist and antagonists on TTC5 protein levels in breast cancer cells,
4) Monitor the effect of TTC5 knockdown on cell viability of breast cancer cells
5) Follow ERα target gene

Together these studies, aim to define the mechanism by which TTC5 regulates ERα activity in breast cancer cells and identify which surface of TTC5 is more important in this interaction, for development of potential new therapeutic agents for breast cancer treatment.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Reagents and materials

General chemicals and reagents were obtained from Sigma-Aldrich (UK), VWR Ltd (UK), and Fisher Scientific (UK). Molecular biology reagents such as protein ladders were obtained from Invitrogen (UK), SYBER Green real-time PCR reagents and kits for DNA and RNA preparation were obtained from Bioline (UK). Transfection reagent PolyFect was obtained from Qiagen (UK), and small interfering RNAs (siRNA) oligos and Dharmafect1 were obtained from Dharmacon (UK). DH5α competent cells (subcloning efficiency) were from Invitrogen. mmobilon-P PVDF membrane was from Millipore. Marvel milk powder was used for antibody incubation and mouse monoclonal and rabbit polyclonal horseradish peroxidase (HRP) linked secondary antibodies were from Amersham. Western blot signals were detected using Pierce supersignal west pico chemiluminescent HRP substrate and Fujifilm medical X ray film. Protein-A sepharose beads and Flag beads were both from Sigma. Luciferase reporter lysis buffer and luciferase reagent were from Promega (UK). Glycine, glycerol, sodium chloride, lithium chloride, potassium chloride and Tris base were purchased from VWR. Antibodies used were as follows: rabbit polyclonal TTC5 antibody was purchased from Abcam (UK), and β-Actin loading control was from Abcam.

2.1.2 Cell culture equipment and reagents

Cells were maintained in a Thermo Scitific (UK) CO_2 incubator at 37^o in a humidified air atmosphere supplied with 5% CO_2. Cell culture manipulations were carried out in Class II biological safety cabinet (Esco, UK), Dulbecco's Modified Eagle's Medium (DMEM), L-Glutamine (200mM), Penicillin-Streptomycin solution and trypsin solution were supplied by Sigma-Aldrich (UK). Fetal bovine serum (FBS), Dextran coated charcoal treated FBS (DCC FBS) was purchased from Life Technologies (UK).

2.2 Cell lines and culturing medium

Dulbecco's Modified Eagle's medium (DMEM) (Sigma, UK) supplemented with 10% foetal bovine serum (FBS) (Sigma, UK) and 1% penicillin and streptomycin (P/S) (Sigma, UK) and 1% Non Essential Amino Acids (NEAA) (Sigma, UK) was used to maintain the human breast cancer cell lines MCF-7 (ER-alpha positive, p53 wild-type) and T47D (ER-alpha positive, p53 mutated) and MDA-MB-468 (ER-negative, p53 mutated) Table 2.1. All three cell lines were purchased from ATCC. The variation in ER-alpha status of these cell lines provides a suitable system to study the crosstalk between ER-alpha and transcription factors.

Table 2.1.Human cell lines used in this study.

Cell line	Source	Tumor Type	Passage No.
MCF-7	ATCC	Derived from metastatic site of a 69 years old Homo sapiens female.	16
T47D	ATCC	Derived from metastatic site of a 54 years old Homo sapiens female.	6

2.3 Cell maintenance

Cells were maintained in 75 cm^2 vented tissue culture flasks (SLS, UK) and subcultured when they reached 70-80% confluency. In order to provide safe and sterile environment cell culture work was carried out in Class II Biological Safety Cabinets (Esco, UK). In order to split the cells, the media was aspirated from the flask and then washed with

Phosphate buffered saline (PBS: 170mM NaCl, 3.3 mM KCl, 1.8 mM Na2HPO4, 10.6 mM KH2PO4; pH 7.4), then the PBS was aspirated out and replaced with 1 ml of trypsin (Sigma, UK). Cells were incubated at 37 °C for 2 to 3 minutes in order to detach from the flask surface. Fresh DMEM medium with FBS and P/S was added to dilute trypsin and cells were passaged at a ratio of 1:5 and flasks were left at 37^{o}C in humidified atmosphere containing 5% CO_2. Cells were seeded into 6 well plates for Western blot, RNA extraction and luciferase assay, 96 well plates for siRNA and 100mm plates for co-immunoprecipitation (co-IP).

2.4 Cell counting

A ruled haemocytometer (Labtech, UK) was used to perform cell count under a light microscope (Olympus, UK) (Figure 2.1). The cells were detached from the flasks and suspended well in order to avoid cell clumps. 100µl of trypan blue (Sigma, UK) (trypan blue was used to assess viability of cells), solution was mixed with 100µl of cell suspension. 10µl of cell suspension was loaded into the haemocytometer and the cell count was performed according to manufacturer's guide line.

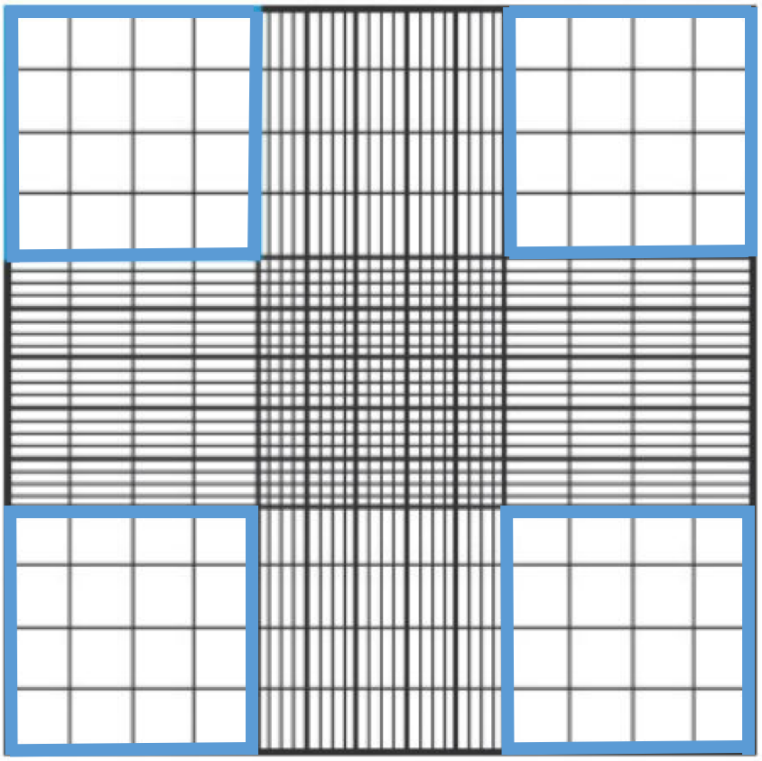


Figure 2.1.Heamocytometer gridlines.
The cells in blue areas were counted and the total number of cells was calculated.

In order to use the appropriate number of cells, cell counting was carried out prior to performing experiments. The $C_1V_1 = C_2V_2$ equation was used to calculate how to obtain an appropriate number of cells/ml for specific experiments.

2.5 Cryogenic storage of cells

To freeze the cells for future use, cells were detached using trypsin-EDTA. The detached cells were then washed with appropriate amount of medium and pelleted by centrifugation at 1,200 rpm for 4 minutes and resuspended in 1ml of FBS (Labtech, UK) and 10% DMSO (Sigma, UK). Adding DMSO prevents the formation of ice crystals, DMSO + FBS was added drop by drop to the cell pallet and the cell suspension was then aliquoted into cryovials (SLS, UK), and stored at -80^{o}C.

2.6 Thawing cells

Vials of frozen cells were removed from -80 ºC freezer to restore the cell culture, 1ml of warm DMEM media was added to the vial rapidly, to reduce the formation of damaging ice crystals during rehydration. Cells were then resuspended in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and transferred into 25 cm^2 culture flask and incubated for 3-4 hours. After cells were attached to the surface of flask the media was changed to fresh media. After reaching 70 – 80% confluency, cells were transferred to 75cm^2 flasks.

2.7 Cell treatments

17 beta-estradiol (Sigma, UK) and two selective estrogen modulators, tamoxifen (Sigma, UK) and raloxifene (Sigma, UK) been used. Prior to treating cells with above mentioned hormones, medium was substituted with DMEM supplemented with, 1% penicillin streptomycin (P/S) 10% Dextran coated charcoal treated FBS (DCC FBS) (Labtech, UK) since normal FBS contains hormones and other reagents which could interfere with E2 activity. Breast cancer cells (MCF-7 and T47D) were incubated for a number of hours depending on the requirements of each experiment with 100nM final concentration of each hormone.

2.8 DNA transformation and isolation

2.8.1 Preparation of LB agar plates and LB broth

LB agar plates were prepared by dissolving 12.5 grams of Luria-Bertani (LB) (Invitrogen, UK) powder in 500 ml of distilled water followed by addition of 7 grams of agar (Sigma, UK). The solution was then autoclaved at 15psi and 121^oC. The solution was then left to cool down under aseptic condition and ampicillin (100 µg/ml) was added. Approximately 10ml of the cooled mixture was poured in sterile 90cm petri dishes and stored at 4^oC.

LB broth was prepared by adding 12.5gr of LB into 50ml of distilled water which was then autoclaved. After the solution was cooled down ampicillin (100 μg/ml) (Sigma, UK) was added to the solution under aseptic conditions.

2.8.2 Transformation

The process of bacterial cells taking up naked DNA molecules is called transformation. Since the bacterial genome is made up of circular DNAs and plasmids it has the ability to replicate foreign DNA alongside their own DNA when the foreign plasmid DNA has an origin of replication recognized by the host cell DNA polymerases.

Per transformation 20 μl of DH5α strain of *Escherichia coli* (*E. coli*) (Invitrogen, UK) were thawed on ice, 200 ng of DNA was added to the cells and carefully mixed by flicking the tube. Then cells were left on ice for 30 minutes and then heat shocked at 42^{o}C for 45 seconds then placed on ice for 2 minutes. 500 μl of LB broth was added to the cells and they were incubated in a shaker for 1 hour at 37^{o}C. 200 μl of the resultant mixture was streaked on an LB agar plate containing ampicillin. Then the plate was incubated for 16 hours at 37^{o}C.

2.8.3 Plasmid purification: maxi preps

Plasmid DNA that was obtained from DH5Aα (Invitrogen, UK) cells colonies through transformation was inoculated in LB-Amp overnight at 37° C with shaking. The samples were centrifuged at 4000g for 10 minutes and the supernatant was discarded. Maxi preps were carried out using the protocol and buffers provided with the Invitrogen Maxi prep Kit. First the filtration cartridge was inserted in the PureLink HiPure Maxi Column. Then 15ml of Equilibrium Buffer (EQ1) were directly added to the filtration catridge, the solution was left to drain by the gravity flow. Next 10 ml of resuspension buffer (R3) with RNAase A were added to the pellet until the cell suspension became homogenous. Next 10ml of lysis buffer (L7) was added to the pellet and left at room temperature for 5 minutes until the mixture became homogenous. Then 10 ml of precipitation buffer (N3) was added, in order to mix the solution, the tubes were inverted. Finally, the precipitated

lysate was transferred into the column, the solution was left to flow through the column by gravity . The inner cartridge was then discarded, and the column was washed with 50 ml Wash Buffer (W8). A 50 ml centrifuge tube was placed underneath the HiPure Filter Column. 15 ml of elution buffer (E4) were added to the column, again the solution was left to follow through column by gravity. The DNA was then precipitated with isopropanol and collected by centrifugation at 15, 000 rpm at 4 °C for 30 minutes. The pellet was then washed with 70% ethanol, and then the pellet was resuspended in 300 µl of TE buffer. The concentration of DNA was then measured using 1 µl of the maxi prep on a Nanodrop at 260 nm.

2.8.4 Plasmid purification: mini prep

A single colony from the agar plate was inoculated in 3ml of LB containing ampicillin and left in a 37 °C shaker for 15 hours. The DNA was then purified using the mini prep kit from Bioline, following the manufacturers guidelines.

The inoculation was centrifuged for 30 second at 11,000g, and the supernatant was discarded. Pellet was re-suspended in 250 µl of P1 buffer by vortexing, then a further 250 µl of lysis buffer P2 were added to the pellet. The solutions were then mixed by gently inverting the tubes 6 to 8 times. Then the tubes were left at room temperature for 5 minutes until the lysate appears clear. 300 µl of neutralization buffer P3 was added and then the lysate was centrifuged at 11,000 g for 5 minutes. The ISOLATE II Plasmid Mini spin column was place onto a 2ml collection tube. Then 750 µl of the clarified sample was pipetted onto the column. The flow-through was discarded and the solution was centrifuged at 11,000 g for 1 minute. 500µl of PW1 buffer that was preheated at 50°C was then added to the pellet and the lysate were centrifuged at 11,000g for 1 minute. Then 600µl of PW2 buffer was added followed by centrifugation for 1 minute at 11,000g was carried out. The flow-through was discarded at this point, and the collection tube was centrifuged for 2 minutes at 11,000g. 50µl of elution buffer P was directly added onto centre of silica membrane, and was left at room temperature for 1 minute. A final centrifugation step for 1 minute at 11,000g was carried out and then the concentration of DNA was measured using 1 µl of the mini prep on a Nanodrop at 260 nm.

2.8.5 Measurement of DNA concentration

Determining nucleic acid concentration can be achieved by UV light as the nucleic acids absorb the UV light. UV light also is used to measure the purity of the DNA. DNA yield determination is based on its ability to absorb light at maximally 260nm. Presence of excess salt, contaminating proteins or organic solvents may affect DNA purity, the ratio of the absorbance at 260nm to 280nm provides an estimation of the purity. For the DNA sample to be considered pure the ratio should be close to 1.8±0.05. The DNA concentration and its purity was determined using NanoDrop ND-1000 UV visible spectrophotometer. Instrument was calibrated using 1 µl of distilled water, after the calibration step 1 µl of sample was loaded to measure the concertation of desired sample.

2.9 Site directed mutagenesis

Quick lightning site directed Mutagenesis Kit from Technologies was used to introduce one or more mutations into desired DNA sequence. A summary of the procedure is shown in Table 2.2.

Table 2.2.The components and volumes required for site directed mutagenesis

	Control	F26A	A88G	A243G	LI16A
10X Reaction Buffer (µl)	5	5	5	5	5
dsDNA template(µl)	5	0.5	0.5	0.5	0.5
Forward primer (µl)	1.25	0.97	1.22	1.22	1.11
Reverse primer (µl)	1.25	0.97	1.22	1.22	1.11
dNTP mix(µl)	1	1	1	1	1
Quick solution reagent(µl)	1.5	1.5	1.5	1.5	1.5
Distilled water(µl)	34	39.06	38.56	38.56	38.78
Quick change enzyme (µl)	1	1	1	1	
Total (µl)	50	50	50	50	50

After sample preparation, each reaction samples were left on thermos cycler with following details:

Segment	Cycles	Temperature	Time
1	1	95°C	2 minutes
2	18	95°C	20 seconds
		60°C	10 seconds
		68°C	30 seconds
3	1	68°C	5 minutes

2 µl of the Dpn I restriction enzyme that was provided was directly added to each amplification reaction. The reaction was then mixed by pipetting and were then immediately incubated at 37 ^{o}C for 5 minutes. 45µl of XL10-Gold competent cells was thawed on ice and 2µl of the β-ME that was provided with the kit was added to the competent cells; the contents of the tube were gently swirled and left on ice for 2 minutes.

2µl of the Dpn I-treated DNA was then added to aliquots of the ultracompotent cells, the transformations reactions were mixed by swirling and left on ice for 30 minutes. The NYZ^{+} was preheated in 42^{o}C water bath for 30 seconds and 0.5 ml of it were added to each tube, then incubated at 37^{o}C for 1 hour with shaking at 225-250 rpm. The appropriate volume of each transformation reaction was plated. Mutations were then confirmed by sequencing.

2.10 Luciferase assay

PolyFect reagent was used to transfect cells with appropriate plasmids for luciferase assay; polyFect has positive net charge and is spherical in shape. Because of its properties polyFect facilitates cellular uptake of plasmid DNA. MCF-7 or T47-D cells were split in 6-well plates and were incubated for 24 hours. Into the recommended amount of DNA 8 µl of polyFect reagent (Qiagen, UK) was added in 15 ml centrifuge tubes containing required volumes of plain DMEM (following the manufacturer's guidelines (Figure 2.2). The mixture was vortexed and then incubated at room temperature for 5-10 minutes. While the DNA mixtures were left for incubation, cells were washed twice with sterile PBS, and 3 ml of complete media (10% FBS, 1% P/S) was added to the cell well. During the incubation time of the mixture, the media was aspirated from cells, and then the cells were rinsed twice with sterile PBS. At the end of the incubation time 600 µl of DMEM (10% FBS, 1% P/S) medium was added to the transfection mixture, which was then added to the specific well. Plates were kept in 37ºC (5% CO2) incubator and when treatment was required, the media was changed to DMEM/DCC treated FBS with antibiotics after 24 hours.

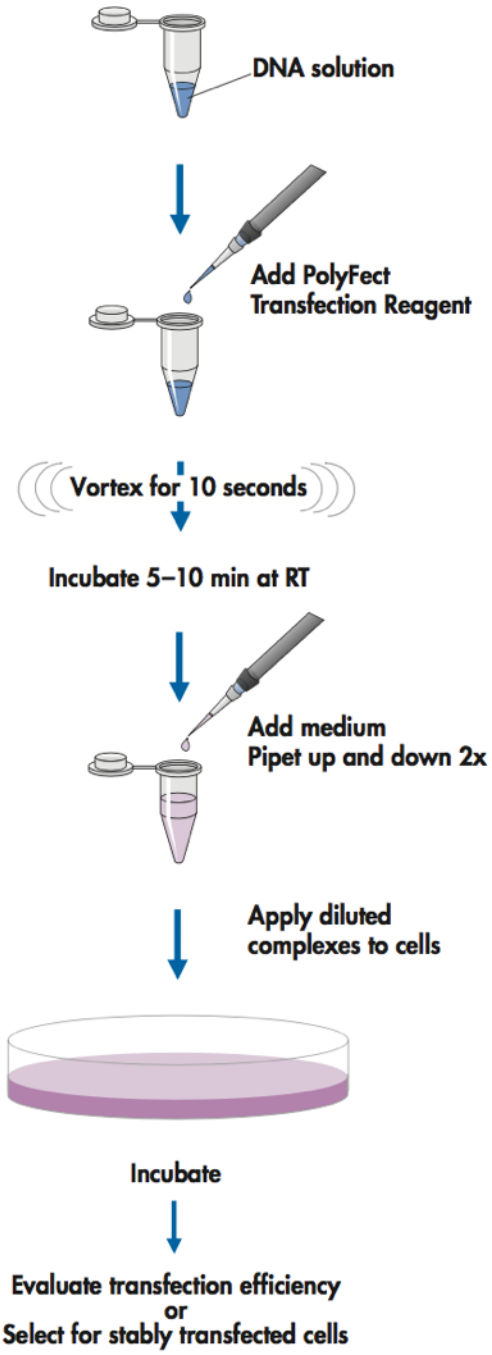


Figure 2.2.Summary of transfection using PolyFect solution.

[Figure adapted from QIAGEN Polyfect transfection protocol]

2.10.1 DharmaFECT

Small interfering RNA (siRNAs) that are produced by cleavage of longer RNAs, are non-coding, double stranded RNA molecules (Hamilton and Baulcombe, 1999; Elbashir et al., 2001). One of the two strands of siRNA separate and integrates into the RNA induced silencing complex (RISC) leading to reduced gene expression through siRNA. It is via the siRNA single strand that RISC binds to a complementary sequence of mRNA, and as a result the active component of RISC Argonaute then cleaves the mRNA preventing translation (Liu et al., 2004).

To transfect MCF-7 and T47D cells with siRNA, DharmaFECT 1 reagent was used and the manufacturers guidelines were followed. A 2μM concentration of each siRNA was made using RNase free water. 100 μl of each siRNA was mixed with 100μl of DMEM (serum and antibiotics free). In a separate tube 6μl of DharmaFECT reagent was mixed with 194μl of DMEM (serum and antibiotics free). Both tubes were left at room temperature for 15 minutes, after incubation time the siRNA/media mixture was added to DharmaFECT/media mixture. In order to form complex tubes were left at room temperature for 20 minutes. 2ml of DMEM with 10% FBS (no antibiotics) was added to the transfection samples. Media was removed from culture plates and the entire transfection reagent was gently added to the well. Plates were then incubated in 37^{o}C.

2.11 Beta-galactosidase assay

In order to measure the transfection efficiency β-galactosidase assay was performed. β-galactosidase is an enzyme and its known to be encoded by the *lac*Z gene of the *lac* operon in *E.Coli.* Cleaving lactose to glucose and galactose is done by β-galactosidase. o-nitrophenyl-β-D-galactoside (ONPG) is a synthetic compound that is known to cleave to yield galactose and o-nitrophenol that has a yellow colour which its absorbance can be measured at 420nm. The concentration of β-galactosidase is proportional to the conversion of ONPG to o-nitrophenol.

Cells were grown in 6 well plates and transfected with desired plasmids using Polyfect transfection reagent. After 24 hour cells were either treated with E2 for 24 hours or left untreated. Then cells were harvested using Reporter lysis buffer (Promega, UK) then centrifuged at 13,000 rpm for 20 minutes at 4°C. 30µl of the supernatant was added to βgal buffer (Table 2.3) samples were then left in 37°C incubator for 1 hour. The absorbance was measured at 420nm after adding 500µl of distilled water to the samples.

Table 2.3.The components and volumes required to make βgal buffer.

Component	Volume/weight
2-Mercaptoethanol (Sigma, UK)	70 µl (from 14.3 M stock)
Magnesium chloride ($MgCl_2$) (Sigma, UK)	20 µl (from 1 M stock)
o-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma, UK)	13.3 mg
Sodium phosphate (pH 7.3) (Sigma, UK)	2 mL (from 1 M stock)
Deionised water	Up to 10 mL

2.12 Immunoblotting

2.12.1 Preparation of whole cell extract

For western blotting when cells reached 60-70% confluency they were seeded in 100mm culture plates. After 24 hours of leaving the plates in 37°C incubator wherever necessary cells were transfected with Polyfect or DharmaFECT transfection reagent, or media was changed to DCC media and cells were treated with appropriate hormones for periods mentioned in each experiment. The media then was removed and cells were washed in 1X PBS (170mM NaCl, 3.3 mM KCl, 1.8 mM Na2HPO4, 10.6 mM KH2PO4; pH 7.4) then 300µl of High Salt Lysis buffer or TNN buffer was added to cells on ice, then cells were scraped and samples collected in 1.5ml centrifuge tubes.

Centrifuge tubes were then left rotating at 4^{o}C for 20 minutes followed by centrifugation at 4^{o}C for 20 minutes at 12,000 rpm. Supernatant were carefully transferred into fresh centrifuged tubes and pellets were discarded.

2.12.2 Determination of protein concentration

The Bradford protein assay was used for determining protein concentration from cell extracts. The Bradford's assay is a colorimetric assay which is based on Bradford dye-binding method to measure total protein concentration. The principle behind this assay is based on colour change of Coomassie brilliant blue G-250 dye after binding to different concentration of proteins. The concertation of protein samples then can be measured by measuring the absorbance at 595 nm.

The Bio-Rad working solution was prepared by adding 800 µl of distilled water to 200 µl of the Bio-Rad reagent (1 part of water to 4 part of the Bio-Rad reagent) in a 1.6 ml, 1 cm path visible cuvette. In order to calibrate 2 µl of distilled water was used and the spectrophotometer was set at 595 nm and was used as blank. To normalise the protein samples concentration 2 µl of samples was added to diluted dye and mixed and the absorbance of the solution was measured. Equal amounts of protein were mixed with 3 X SDS sample buffer (1M Tris pH 6.95 (Sigma, UK), 10% glycerol (Sigma, UK), 5% 2-Mercaptoethanol (Sigma, UK), 0.6g SDS (Sigma, UK)) and either loaded on SDS PAGE on the same day or left at -20^{o}C.

2.12.3 SDS polyacrylamide gel electrophoresis

The first step in studying proteins by western blot is to separate them on SDS gel by means of the electrophoresis technique. SDS is an anionic detergent that binds to polypeptide chains of proteins in a manner that is proportional to molecular mass of the polypeptide. Smaller molecules move faster than the larger molecules on a poly acrylamide gel as it contains pores. In order to make sure that the charge to mass ratio is the same among the samples, protein samples were denatured incubating at 95^{o}C for 5

min in SDS sample buffer (187mM Tris, 30% Glycerol, 6% SDS,15% 2-mercaptoethanol, 0.01% bromophenol blue).

To detect proteins SDS-PAGE was performed using 10% gels, components of 10% gel and volumes are shown in Table 2.4.

Table 2.4.The components and volumes required to create the resolving gel for one 10% acrylamide.

Component	Volume
Deionised water	5.24 mL
Acrylamide (Fisher, UK)	4.67 mL
1.5 M Tris (pH8.95) (Fisher, UK)	3.5 mL
10% SDS (Fisher, UK)	0.14 mL
10% Ammonium persulphate (Sigma, UK)	0.075 mL
0.2 M EDTA(Fisher, UK)	0.14 mL
TEMED(Sigma, UK)	0.0085 mL

Table 2.5.The components and volumes required to create 6% stacking gel for one stacking gel.

Component	Volume
Deionised water	3.37 mL
Acrylamide (Fisher, UK)	0.835 mL
1.5 M Tris (pH6.95) (Fisher, UK)	0.625 mL
10% SDS (Fisher, UK)	0.050 mL
10% Ammonium persulphate (Sigma, UK)	0.075 mL
0.2 M EDTA (Fisher, UK)	0.050 mL
TEMED (Sigma, UK)	0.0085 mL

According to manufacturer's guide line the gel casting apparatus was set-up. The components in Table 2.4 were prepared and the mixture was poured between the plates. To prevent gel from drying and removing the air bubbles after the resolving gel was poured into the gel casting apparatus, 500 µl of distilled water was poured on top. Once the gel was set the water was removed stacking gel mixture was poured on top of resolving gel and 1.5 mm wide well combs were inserted into the stacking gel solution (Table 2.5). Since the stacking gel has lower percentage of acrylamide and lower pH comparing to resolving gel it allows the proteins to be stacked before they leave the stacking gel and entering resolving gel. When the stacking was set the gels were fitted into electrophoresis mini buffer tank and the tank was filled with 1x SDS-PAGE running (25 mM Tris, 190 mM glycine, 3 mM SDS). Protein samples were boiled at 95^{o}C for 5 minutes and loaded into the wells of gel. 5 µl of prestained protein ladder (Fisher, UK) was loaded in every gel and the samples were run at 90V until they enter the resolving gel and then at 110V.

2.12.4 Western transfer

When proteins resolved on the gels they were transferred to a polyvinylidene fluoride PVDF membrane (Millipore, UK) using western transfer method. Western transfer apparatus (Bio-Rad, UK) was set up according to manufacturer's guideline and the tank was filled with western transfer buffer (28 mM Tris, 0.15 M glycine, 20% methanol). The transfer was carried out for 90 minutes in 0.4amps with ice pack changed every 45 minutes. When the transfer was completed the PVDF membranes were blocked in 5% dried milk in 1x phosphate buffered saline (PBS) (170mM NaCl, 3.3 mM KCl, 1.8 mM Na2HPO4, 10.6 mM KH2PO4; pH 7.4) for 1 hour. The membrane then was incubated with desired antibody in 2.5% milk/0.1% PBS Tween (PBS + 0.1% Tween-20) overnight on a rocking platform at 4^{o}C.

The day after incubation membranes were washed three times each time 10 minutes in PBS 0.1% tween, and then membrane was incubated in relevant secondary anti-rabbit or anti-mouse IgG horseradish peroxidise conjugated secondary antibody in 2.5% milk PBS/tween.

Three further washes with PBS Tween (each wash for 10 minutes) were carried out. In order to visualise the proteins SuperSignal™ West Femto (Thermo Fisher, UK) or SuperSignal™ West Pico (Thermo Fisher, UK) Chemiluminescent reagent was added on to the membrane and proteins were visualised on medical X-ray films (Fujifilm, UK) using developer.

2.12.5 Western blot quantification

Image J 1.48 was used to quantify western blot results with readings of each protein band normalized to their corresponding actin band. Readings were then expressed relative to the control lane and results were then presented as bar charts. As shown below in Figure 2.3 a square was drawn around the protein bands, and by selecting a specific tool protein bands were represented as separate peaks. The software in a different window then calculated the density represented by peaks.

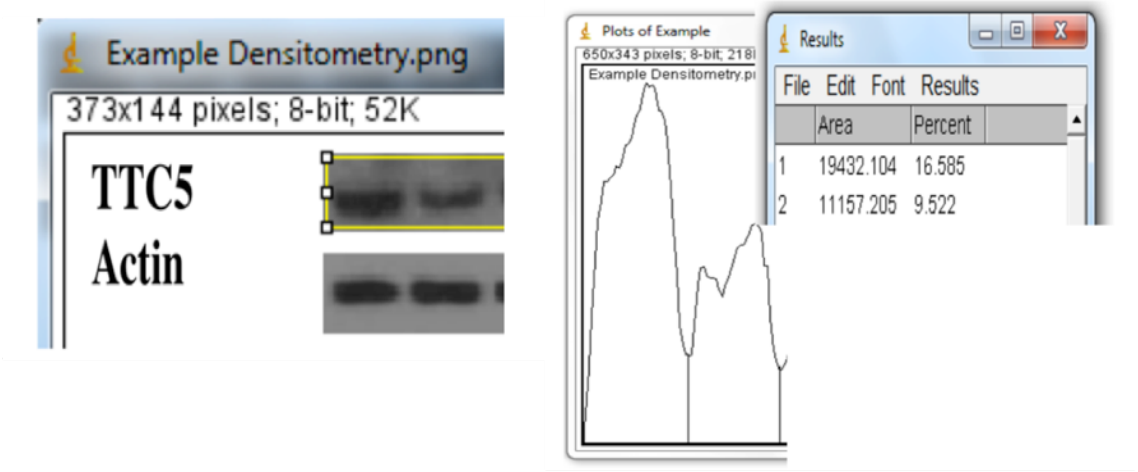


Figure 2.3.Summary of ImageJ quantification.

2.12.6 Striping the membranes

In order to strip the antibody off the membranes after detecting the first protein whenever necessary, the membranes were incubated in striping buffer (100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) for 30 minutes at 50^{o}C and washed three times in 0.1% PBS tween for 10 minutes. Membranes were then blocked with the desired primary antibody.

2.13 Co-Immunoprecipitation (Co-IP)

Co-IP is a popular technique used widely to identify protein-protein interactions and validation. The principle of Co-IP is fairly straight forward, an antibody will be added to cell lysate against target protein, then they will form a complex and this complex is then precipitated through binding to the beads and the proteins that are not precipitated on the beads are washed away. When suitable antibody is added to the samples it will bind to protein of interest and it will form a complex with beads.

Protein-A sepharose (Sigma, UK) beads were washed 3 times in TNN (50 mM Tris pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% Igepal, 1 μg/ml protease inhibitor cocktail, 1 mM DTT, 1 mM PMSF, 2 mM sodium orthovanadate, 5 mM sodium pyrophosphate and 20 mM β glycerophosphate) buffer and once with PBS samples were lysed in TNN buffer and saved for the input, and to the rest of the extract the antibody of interest was added as well as 15 μl protein-A sepharose rotated at 4˚C overnight. Samples were then washed with TNN buffer 3 times and once with PBS and were pelleted at 4˚C by centrifugation. Then before loading the samples onto the gels they were boiled for 3 minutes at 95°C in 30 μl 3 x SDS sample buffer, and the beads were pelleted out with centrifugation 13,000 rpm for 2 minutes. In order to analyse the immunoprecipitated proteins western blotting was used.

2.14 Quantitative Real Time PCR (qRT-PCR)

2.14.1 RNA isolation

The total RNA was extracted using ISOLATE II RNA Mini Kit (Bioline, UK). Approximately 5 x 10^6 cultured cells were lysed using 350μl Lysis Buffer RLY and 3.5 2-mercaptoethanol (Sigma, UK). Protocol was carried out according to manufacturer's guideline. A Bioline silica column was then used to further purify the eluted RNA, DNase digestion as part of the RNA extraction kit was carried out according to manufacturer's instructions. Silica membrane was the washed once with 200μl of wash buffer 1 (RW1) and another wash was carried out with 600μl of wash buffer 2 (RW2). RNA was finally eluted in RNase free eater and stored in -80^{o}C until further use.

2.14.2 cDNA synthesis

From the isolated RNA, the cDNA was generated in a process called reverse transcription. Synthesis of cDNA was carried out using SensiFAST cDNA Synthesis Kit (Bioline, UK). Synthesis was performed in a total volume of 20μl the master mix preparation as shown below Table 2.6:

Table 2.6Components of cDNA master mix.

Total RNA (up to 1 µg)	n µl
5x TransAmp Buffer	4 µl
Reverse Transcriptase	1 µl
DNase/RNase free-water	Up to 20 µl

Mastermix was then mixed gently, and left on thermocycler with the following PCR cycle:

- 25^{o}C for 10 min (primer annealing)
- 42^{o}C for 15 minutes (reverse transcription)
- 85^{o}C for 5 min (inactivation)
- 4^{o}C hold

2.14.3 qPRT-PCR

cDNA levels of each corresponding RNA content of specific genes were determined by quantitative real-time PCR. The SensiFAST^{TM} SYBER dye (Bioline, UK) interacts only with double strand PCR products, so the fluorescence signal is proportional to the number of amplified copies. The components used for qRT-PCR reactions are shown in table below Table 2.7.

Table 2.7.Components of SYBR green master mix.

Reagent	Volume
2x SensiFAST SYBR No ROX Mix	10µl
10µM forward primer	0.8µl
10µM reverse primer	0.8µl
Template	up to 8.4µl
H_2O	As required
	20µl Final volume

16 µl of specific master mix with desired forward and reverse primer (Table 2.7 and Table 2.8) was loaded in the corresponding wells of 96 well plate, followed by addition of 4µl of cDNA in duplicate. Lids were attached and the plate centrifuged at 1,000 rpm for 1 minute. Opticon 3.1 software on a chromo 4 machine was used to perform the PCR reaction with the following cycle:

10 minutes at 45^{o}C
2 minutes at 95^{o}C
5 seconds at 95^{o}C
10 seconds at 60^{o}C
5 seconds at 72^{o}C

Melting curve 72 ºC-95 ºC, reading every 1 ºC, with a 30 second hold. The Opticon 3.1 software was used to carry out analysis, quantifying samples using the standard curve method. RPL19 housekeeping gene was used to normalise the values of each sample.

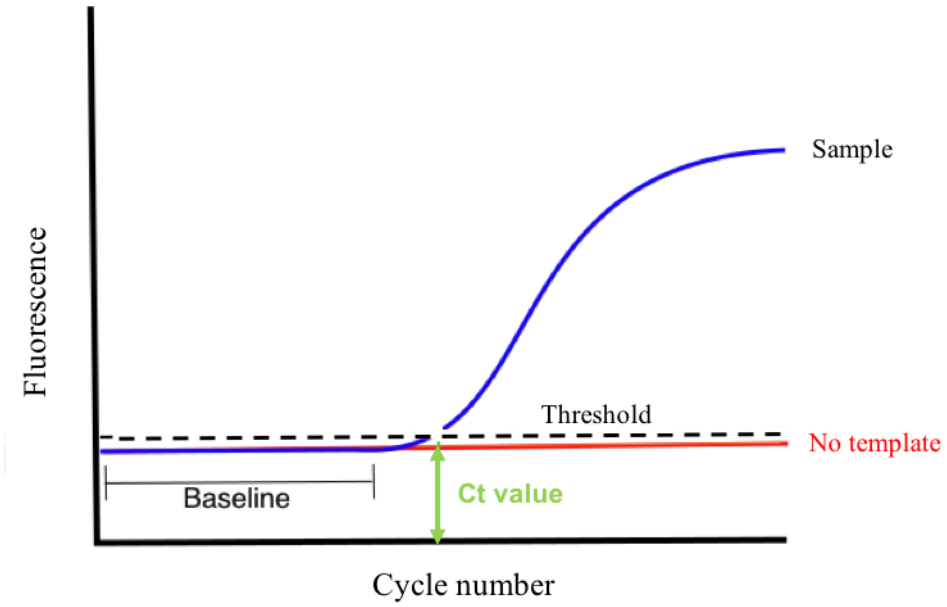


Figure 2.4.Principle involved in qPCR assay

Table 2.8.List of qRT-PCR primers.

Gene	Primer	Sequence
RPL19	Forward	ATG TAT CAC AGC CTG TAC CTG
	Reverse	TTC TTG GTC TCT TCC TCC TTG
TTC5	Forward	CAG CGG TGC CGT CAT CCT G
	Reverse	GCA CTC CCC AGC TCT GCA C
CyclinD1	Forward	CCG TCC ATG CGG AAG ATC GTC
	Reverse	GAA GAC CTC CTC CTC GCA CTT C

Where there is a significant increase in fluorescence that determines the threshold cycle (Ct value). The baseline area is where there is no significant fluorescence early in the cycle. The intensity of SYBR green florescence at end of every cycle determines the copy number of amplified DNA.

2.15 MTT assay

MTT is a colorimetric assay which determines the number of viable cells. In metabolically active cells the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by the action of dehydrogenase enzymes, to generate reducing equivalent such as NADH and NADPH. The intracellular purple formazan that is produced as a result can be solubilized and quantified by spectrophotometric means (Mosmann, 1983)

Cells were seeded at concertation of 2500 cells/well in 100µl of appropriate media on 96-well plate (SLS, UK) and incubated at 37^{o}C containing 5% CO_2 incubator. Following day transfection of siRNA was done as described previously, 24 hours after the transfection cells were washed twice in 1xPBS and dosed with the relevant concentration of each drug and incubated for 24 or 48 hours, this was carried out in triplicate to increase accuracy. Following the end of each incubation time 50µl of MTT (Sigma, UK) working solution (3 mg/ml in 1xPBS) was then added to each well and the plates were then incubated in the 37^{o}C for a further 3 hours. After the 3 hour incubation period, the solution in each well was carefully aspirated, avoiding aspirating the purple crystals formed at the bottom of the walls. Crystals were then dissolved in 200 µl of Dimethyl sulfoxide (DMSO) (Sigma, UK) and the absorbance was then read using an Ascent Plate reader. The difference between the two measurements one at 540 nm and one at 690 nm is what the computer software shows as the final data.

2.16 Identification of transcription factor binding sites

The QIAGEN Champion Chip Transcription Factor Search Portal was used in order to search for putative ERE on TTC5. A list of potential regulators of TTC5 were then listed.

2.17 Kaplan-Meier Analysis

Kaplan-Meier is a non-parametric estimator that incorporates censoring for estimating survival probabilities (Klein 1997). The Kaplan-Meier test was used to analyse the survival data for the entire breast cancer patient group based on TTC5 expression. Using publicly available database (KM plotter; www.kmplot.com).

TTC5 was entered as the gene symbol, the auto selects best cut off box on the main page was ticked as well as exclude outlier arrays in the quality control tab. And high quality images the TIF was ticked in more options tab. In order to restrict the analysis to ERα positive patients the ER status was marked as positive.

2.18 High-performance Integrated Virtual Environment (HIV) analysis

The public domain of HIV through the George Washington University was accessed to identify the single-nucleotide variations of TTC5 among all cancer type, as well as mutations that are known to be associated with breast cancer.

2.19 Statistical analysis

Statistics Statistical analysis was performed in Microsoft Excel. In all figures * = $p < 0.05$, two tailed T-Tests were performed where specifically mentioned.

Chapter 3 Results

3.1 TTC5 levels in MCF-7 and T47D breast cancer cell lines

As mentioned before role the of cofactors in regulating estrogen receptor activity is vital and that activity of a protein is often correlated with its level in the cell. As the area of interest in this thesis is to investigate the role of TTC5 as a cofactor, this section focus is on monitoring TTC5 protein expression in breast cancer cell lines of interest (MCF-7 and T47D) and how hormone treatment affects TTC5 protein levels, hence western blot analysis (completed by densitometry) was employed on MCF-7 and T47D cells under E2, tamoxifen and raloxifene treatment.

The relative levels of TTC5 expression in MCF-7 and T47D cells were determined by western blotting, and followed by qPCR analysis of TTC5 gene expression. For western blotting actin was used as a protein loading control, and the antibodies used were specific to either actin or TTC5 as the corresponding bands aligned with the marker at their known molecular weight, namely 42 kDa and 50kDa respectively.

As for the qRT-PCR, total mRNA was extracted from breast cancer cells and subjected to reverse transcription and the cDNA was used as template for qRT-PCR for the amplification of TTC5. Rpl119 primers were used for amplification of Rpl19 cDNA levels and used as internal control. The ratio of TTC5 mRNA levels with the Rpl19 mRNA levels in the non-treated cells was arbitrarily set to 1.

To ensure that the changes in TTC5 expression were not a result of slightly altered protein loading within different lanes, each TTC band was normalised against its corresponding actin band. Following quantification by densitometry, the untreated values were set as 100% and the TTC5 treated with hormones (E2, tamoxifen or raloxifene) values expressed as a percentage of the untreated value. Since triplicate experiments were not carried out on the same gel or membrane hence the normalisation step was imposed.

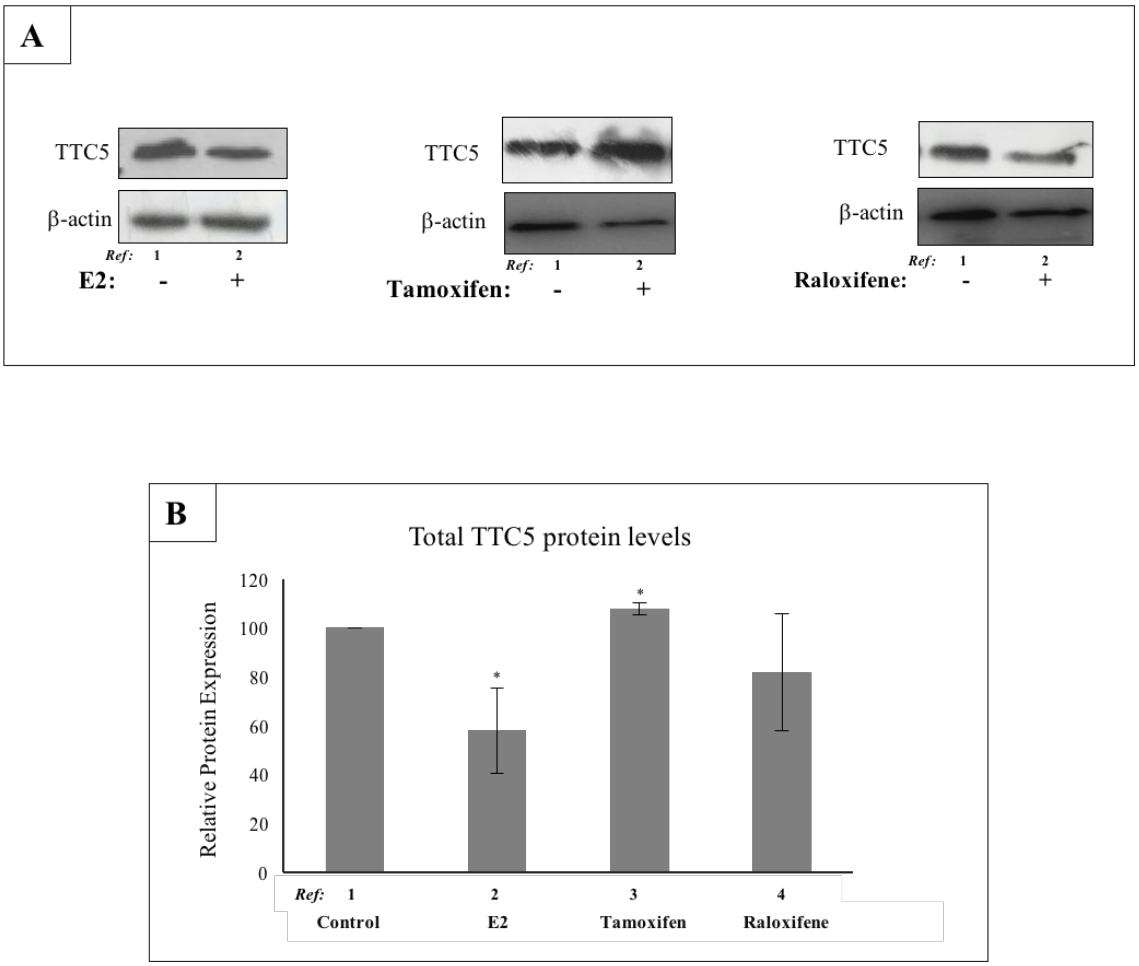


Figure 3.1.TTC5 expression in MCF-7 cells assessed by western blot.

A: MCF-7 cells were either left untreated or treated with E2 (100nM), tamoxifen (100nM) or raloxifene (100nM) for 24 hours. Whole cell lysate was then probed against TTC5 and β-actin in MCF-7 cells. β-Actin was used as control protein loading. **B:** densitometry analysis of western blot data by ImageJ. The data of each treated sample were normalised to each corresponding β-Actin band. The band intensity of TTC5 protein was normalized to the band intensity of Actin. The values of untreated were considered 100 and the values form E2, tamoxifen and raloxifene treated cells calculated accordingly. Error bars present standard errors of the mean form triplicate experiments. (P-value < 0.05 is indicated by *).

Analysis of immunoblotting data by densitometry confirmed that TTC5 is expressed in MCF-7 cells (Figure 3.1A). E2 treatment seems to cause a significant decrease in TTC5 protein levels after 24 hours when compared to control (lane 2 to lane 1, Figure 3.1B).

Interestingly the two SERM's tamoxifen and raloxifene seems to have different effect on TTC5 protein levels in MCF-7 cells. Comparing lane 3 to lane 1 in Figure 3.1B shows that treating MCF-7 cells with 100nM of tamoxifen for 24 hours slightly increased the TTC5 protein levels in these cells. Raloxifene seemed to decrease TTC5 protein levels in MCF-7 after 24 hours however this observation was not significant (comparing lane 4 to lane 1 in Figure 3.1B).

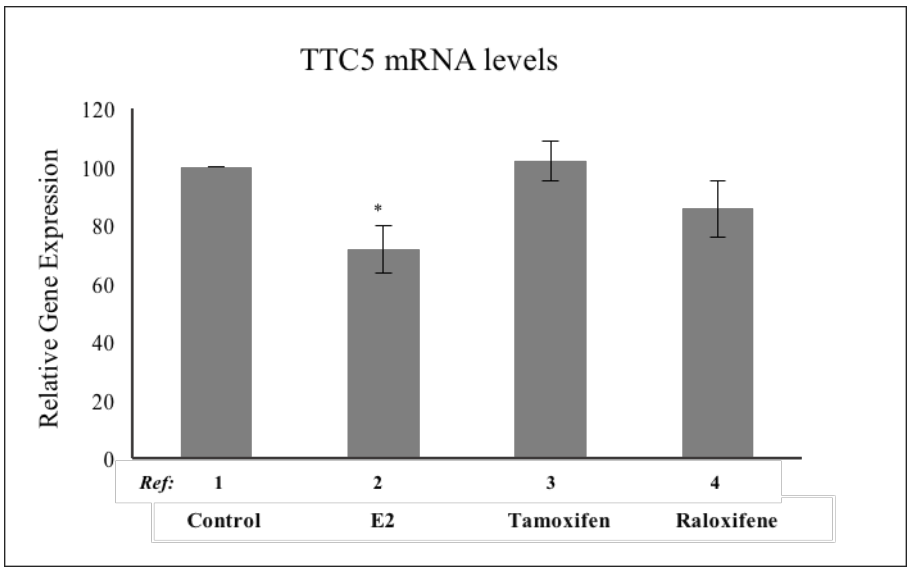


Figure 3.2.qRT-PCR analysis of the effect of hormone on the mRNA levels of TTC5 in MCF-7 cells. MCF-7 cells were either treated with 100nM of E2, tamoxifen or raloxifene for 24 hours or left untreated (control). RNA was extracted from cells, reverse transcribed, before gene expression analysis was performed using qRT-PCR. Graph shows relative expression of TTC5 mRNA from qRT-PCR using TTC5 specific primers with data normalised to a housekeeping gene Rpl19.The ratio of TTC5 mRNA levels with the Rpl19 mRNA levels in the non-treated cells was arbitrarily set to 100 (control). values form E2, tamoxifen and raloxifene treated cells calculated accordingly. Error bars present standard errors of the mean from two independent experiments performed in duplicates (P-value < 0.05 is indicated by *).

Furthermore, effect of hormone on TTC5 mRNA levels were analysed using qRT-PCR. The aim of qRT-PCR analysis was to measure TTC5 mRNA levels and monitor the effect of hormone on TTC5 gene expression, and also to see whether the effect of indicated hormones on TTC5 protein levels correlates with mRNA levels of TTC5 after treatments or not.

Based on ΔΔCt values, the statistical analysis of qPCR data was done where, ΔΔCt = ΔCt of sample - ΔCt of housekeeping gene, ΔCt value was calculated as following Ct of target gene (gene of interest, in this case TTC5 – Ct of reference gene (internal control, Rpl19 was used in this study). Rather than showing the raw data as ΔΔCt values the relative expression is given as $2^{-\Delta\Delta Ct}$.

If mRNA levels were compared with the results from western blotting; the gene expression of TTC5 decreased significantly after treatment with 100nM of E2 in MCF-7 cells (comparing lane 2 to lane 1Figure 3.2) which shows correlation to western blot data (Figure 3.1B comparing lane 2 to 1). Next the qRT-PCR result obtained from treating MCF-7 cells with tamoxifen illustrates that tamoxifen treatment slightly increased TTC5 expression (comparing lane 3 to lane 1 Figure 3.2). This effect didn't seem to be significant, however it does correlate with western blot data obtained from treating MCF-7 with tamoxifen (Figure 3.1B comparing lane 3 to 1). Next monitoring the effect of raloxifene on levels of TTC5 gene expression indicated that raloxifene treatment resulted in slight decrease (comparing lane 4 to lane1 Figure 3.2) on TTC5 expression which although it isn't significant shows the same trend as western blot data.

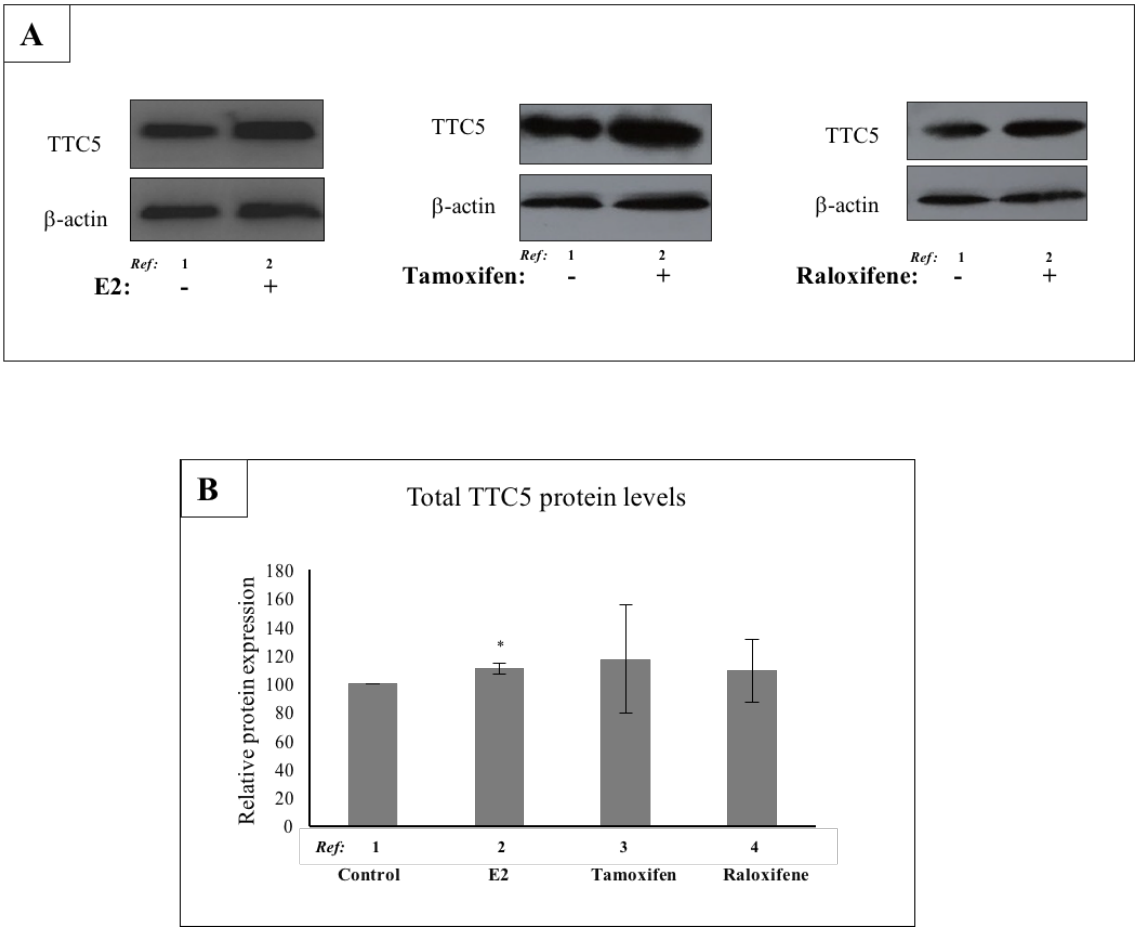


Figure 3.3.TTC5 expression in T47D cells asses by western blot.

A: T47D cells were either left untreated or treated with 100nM of E2, tamoxifen or raloxifene for 24 hours. Whole cell lysate was then probed against TTC5 and β-actin in T47D cells. β-Actin acts to control protein loading. **B:** densitometry analysis of western blot data by ImageJ. The data of each treated sample were normalised to each corresponding β-Actin band. The band intensity of TTC5 protein was normalized to the band intensity of Actin. The values of untreated were considered 100 and the values form E2, tamoxifen and raloxifene treated cells calculated accordingly. Error bars present standard errors of the mean from at least triplicate experiments. (P-value < 0.05 is indicated by *)

Next, we were interested to investigate the effect of E2 (100nM), tamoxifen(100nM) and raloxifene(100nM) on T47D breast cancer cells. For this reason, T47D cells were treated with desired hormone (E2, tamoxifen or raloxifene) for 24 hours. Whole cell lysate was

extracted from tissue culture plates with high salt lysis buffer and western blotting was performed.

As shown in Figure 3.3, TTC5 protein was successfully detected in T47D cells and hormone treatment had modest effect the levels of this protein in T47D cells. Comparing lane 2 to lane 1 of Figure 3.3A, indicates that E2 treatment slightly but significantly increased the TTC5 protein level, however E2 appeared to have the opposite effect in MCF-7 cells on TTC5 protein levels (Figure 3.1A comparing lane 2 to lane 1).

The two SERM's (tamoxifen and raloxifene) seemed to have the same effect on TTC5 protein levels in T47D cells. Comparing bar 3 of Figure 3.3 to lane 1 shows that tamoxifen slightly increases the TTC5 protein levels however this didn't seem to be statistically significant, same pattern have been monitored in MCF-7 cells (Figure 3.1A comparing lane 3 to lane 1). Raloxifene had the same effect as tamoxifen on TTC5 protein levels in T47D cells, comparing lane 4 to lane 1 of Figure 3.3B however raloxifene seemed to have different effect on MC-7 cells (Figure 3.3B comparing lane 4 to lane 1).

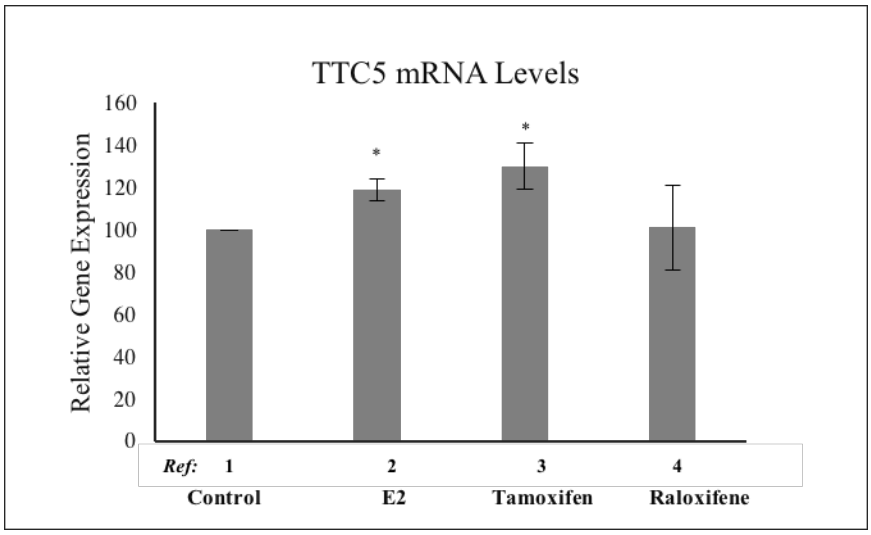


Figure 3.4. qRT-PCR analysis of the effect of hormone on the mRNA levels of TTC5 in T47D cells.
T47D cells were either treated with 100nM of E2, tamoxifen or raloxifene for 24 hours or left untreated (control). RNA was extracted from cells, reverse transcribed, before gene expression analysis was performed using qPCR. Graph shows relative expression of TTC5 mRNA from qRT-PCR using TTC5 specific primers with data normalised to a housekeeping gene Rpl19. The ratio of TTC5 mRNA levels with the Rpl19 mRNA levels in the non-treated cells was arbitrarily set to 100 (control) and the values form E2, tamoxifen and raloxifene treated cells calculated accordingly. Error bars present standard errors of the mean from two independent experiments performed in duplicates (P-value < 0.05 is indicated by *).

The analysis of the qRT-PCR was carried out as explained before. T47D cells were either not treated or treated with 100nM of E2, tamoxifen or raloxifene for 24 hours. RNA was extracted from the cell lysates following the manufacturers instruction described in the materials and method section. Results displayed in Figure 3.4 indicate that E2 causes slight significant increase of TTC5 mRNA levels (comparing lane 2 to lane 1 of Figure 3.4) in T47D cells, which correlates to a great extent with western blot data.

According to data presented in Figure 3.4, in the presence of tamoxifen treatment TTC5 mRNA levels increased in T47D cells (comparing lane 3 to 1 of Figure 3.4). Although western data was not statically significant when T47D cells were treated with tamoxifen the qPCR data seems to be statistically significant. Raloxifene didn't seem to have a

noticeable or significant effect on T47D cells TTC5 mRNA levels, but a slight increase can be seen (comparing lane 4 to lane 1 of Figure 3.4).

The comparison analysis of the effect of E2, tamoxifen and raloxifene on TTC5 protein and mRNA levels on two different cell lines (MCF-7 and T47D) would be as follows:

- E2: resulted in reduction of both protein and mRNA levels of TTC5 in MCF-7 whilst it had the opposite effect on T47D cells.
- Tamoxifen: resulted in an increase in both protein and mRNA levels of TTC5 in both cell lines (MCF-7 and T47D).
- Raloxifene: caused in reduction of both protein and mRNA levels of TTC5 in MCF-7 cells, whilst in T47D cells resulted in slight increase both in mRNA and protein levels.

The most important finding to take away from this section is that TTC5 presence both at the level of protein and gene expression in MCF-7 and T47D, and that hormone treatment can affect these levels.

3.2 ERE identification on TTC5 promoter

At this point we thought of a bioinformatics approach to investigate the possibility of TTC5 being under ER control, hence the sequence analysis of TTC5 by QIAGEN Champion Chip Transcription Factor Search Portal reviled two ERα binding site on TTC5 promoter

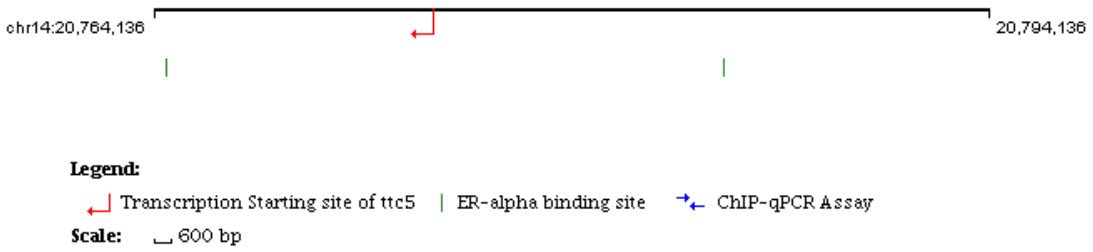


Figure 3.5.Identification of putative EREs on the TTC5 promoter.
[Table adapted from the QIAGEN Champion Chip Transcription Factor Search Portal].

Figure 3.5 shows the two putative ERE binding site on TTC5 promoter hence indicates the possibility of TTC5 being a target for ER.

3.3 TTC5 interacts with ERα in MCF-7 and T47D cells

As mentioned previously TTC5 interacts with GR (Davies et al., 2011), therefore to investigate whether TTC5 could be a common cofactor for multiple members of the nuclear hormone receptor family, co-IP experiment was carried out. If TTC5 acts as a cofactor for the estrogen receptor, it is likely that it would be found in a complex with ERα. In order to assess whether TTC5 interacts with ERα *in vivo*, co-IP assays were performed on MCF-7 and T47D cells.

Appropriate cell lysate was incubated with antibodies against ERα and subsequent immune-complexes were isolated from cell lysates using protein-A agarose beads together with the protein lysate control were subjected to western blotting for TTC5. Also, in order to determine the hormone dependency of the interaction cells were left untreated or treated with 100nM estradiol, tamoxifen or raloxifene for 24 hours.

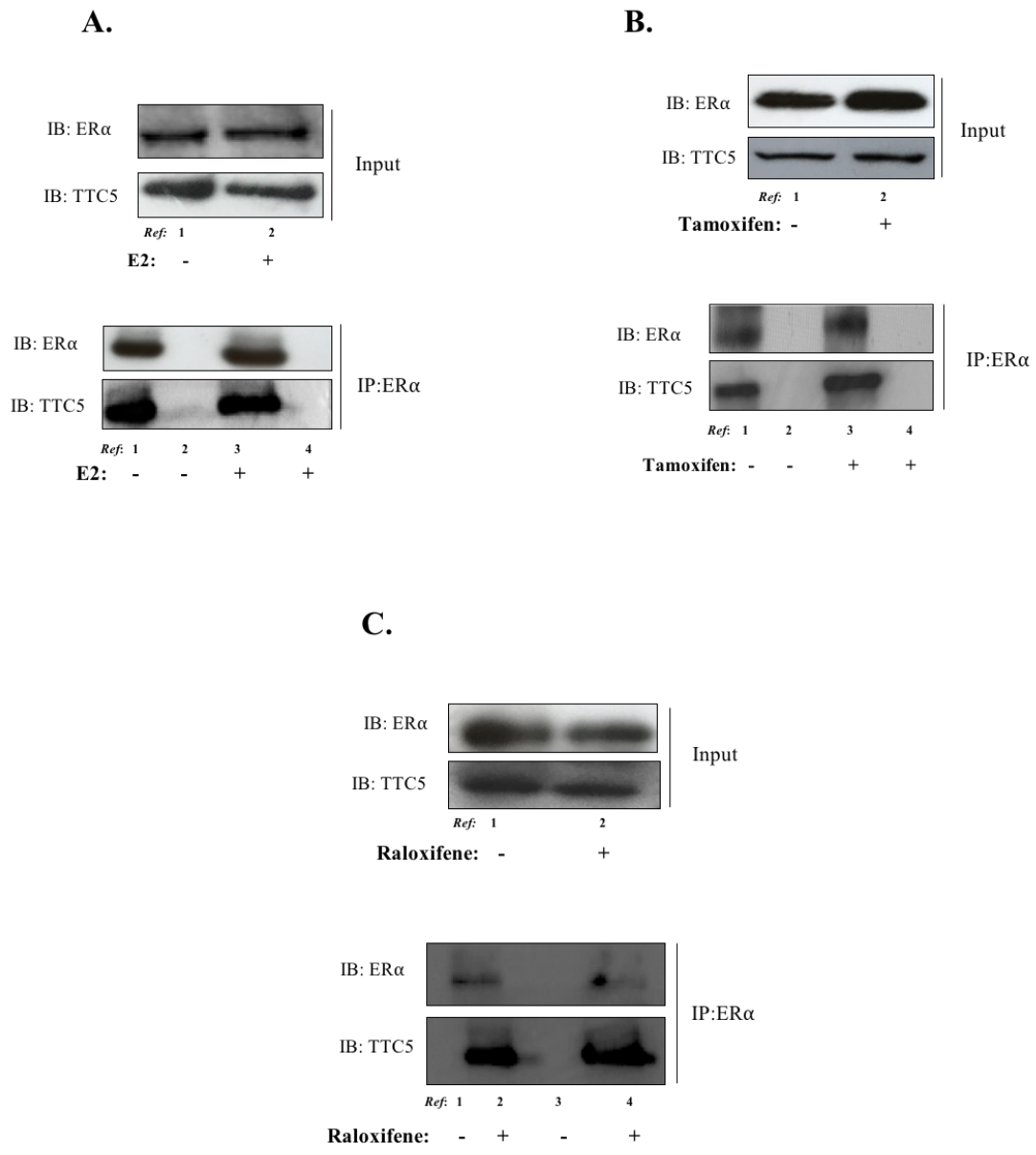


Figure 3.6.ER interacts with TTC5 endogenously in MCF-7 cells.

MCF-7 cells were treated for 24 hours with 100nM of E2 (A), tamoxifen (B) and raloxifene (C), or received no treatment. ERα was immunoprecipitated from cell lysate of MCF-7 cells using ERα monoclonal antibody, TTC5 interaction with ERα was detected by western blot. Mouse IgG was used as negative control, lane 2 and 4 of the IP panel in A and B, and lane 1,3 of IP panel in C. The top 2 panels show input levels of protein and lower 2 panels shows IP results (A, B and C).

Results from Figure 3.6 show that ERα interacts with TTC5 endogenously, in MCF-7 cells both in absence and presence of E2, tamoxifen and raloxifene. Input levels of proteins showed expression of ERα and TTC5 (Figure 3.6 A, B and C input panel) which was the positive control, whilst no proteins were detected in negative control co-IP using control IgG (Figure 3.6A and B IP panels lanes 2 and 4 and Figure 3.6C IP panels lanes 1 and 3). The concept behind analysis is to first monitor weather hormone treatment has any effect on ERα/TTC5, and also compare the proportion of TTC5 that is in complex with ERα to levels of ERα alone.

As shown in Figure 3.6A, TTC5/ERα interaction in MCF-7 didn't seem to change considerably under E2 (100nM) treatment after 24 hours (comparing lane 3 to lane 1 Figure 3.6A lower IP panel). However, it appears that in absence of hormone higher proportion of TTC5 is in ERα/TTC5 complex (comparing lane 1 of IP panel IB: TTC5 to lane 1 IP panel IB: ERα), where this amount increases when treated with hormone (comparing lane 3 of IP panel IB: TTC5 to lane 3 I P panel IB: ERα)

Figure 3.6B, shows that when ERα was immunoprecipated using ERα antibody, TTC5 could be detected as an interacting protein. TTC5 binding to ERα seemed to slightly increase in the presence of tamoxifen treatment (Figure 3.6 B, IP: TTC5 panel comparing lane 3 to lane 1). It seems a higher proportion of TTC5 is in the complex with ER after hormone treatment (comparing lane 3 of IP panel I B: TTC5 to lane 3 of IP panel IB:ERα).

Figure 3.6C, shows the effect of raloxifene on ERα/TTC5 interaction. Raloxifene seems to increase the interaction between ERα and TTC5 in MCF-7 cells (comparing lane 4 of lower IP panel to lane 2).

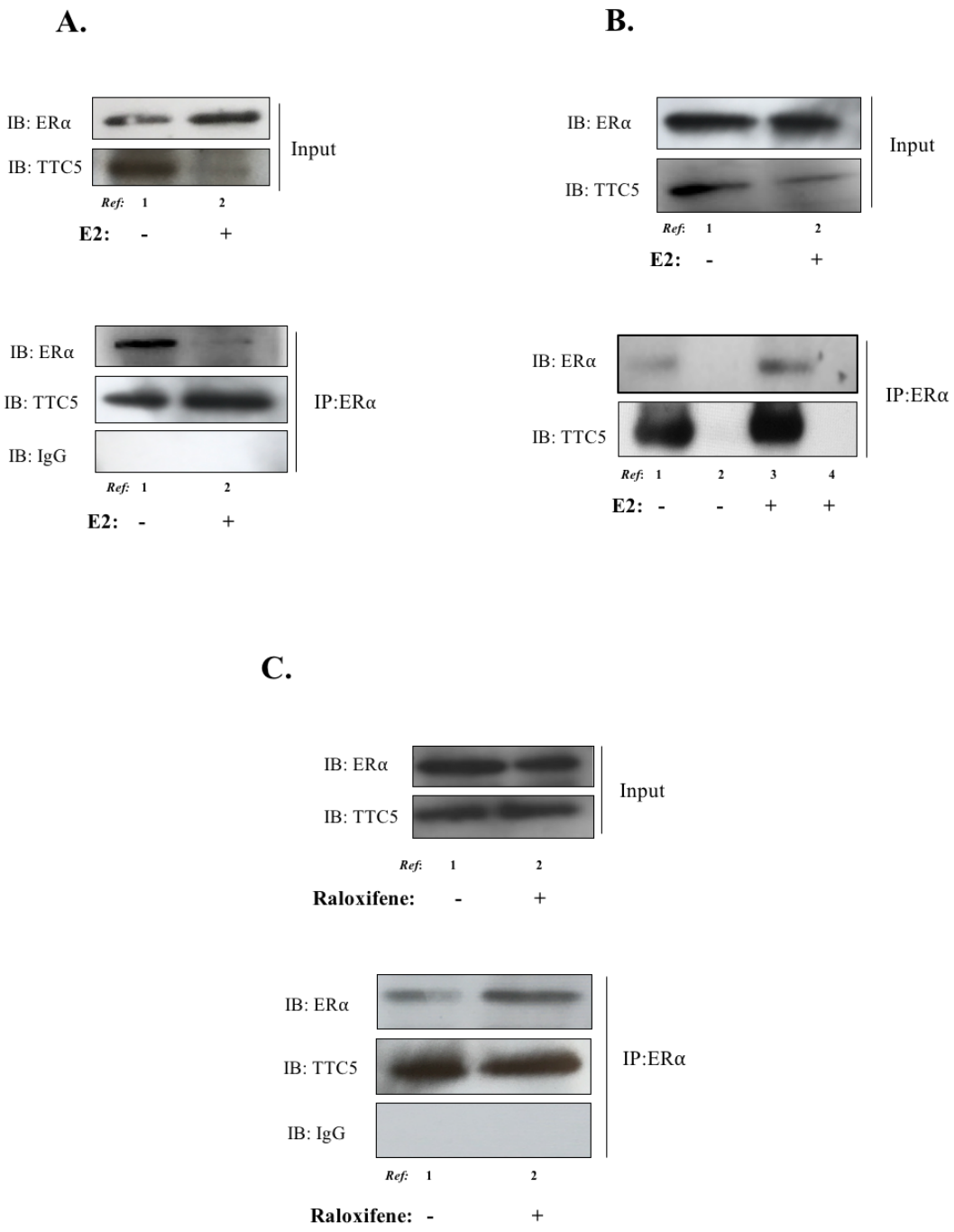


Figure 3.7.ER interacts with TTC5 endogenously in T47D cells.

T47D cells were treated for 24 hours with 100nM of E2 (A), tamoxifen (B) and raloxifene (C), or received no treatment. ERα was immunoprecipitated from cell lysate of MCF-7 cells using ERα monoclonal antibody, TTC5 interaction with ERα was detected by western blot. Mouse IgG was used as negative control, lane 1 and 2 of the lower IP panel in A, lane 2 and 4 and in B, and lane 1 and 2 of the lower IP panel in C. The top 2 panels show input levels of protein and lower panels shows IP results (A, B and C).

In order to further investigate the ERα/TTC5 interaction, co-IP experiment was carried out on T47D cells. The above results present in Figure 3.7 show that ERα interacts with TTC5 in T47D cells in absence and presence of hormones.

In order to assess whether this interaction is affected by E2 treatment, T47D cells were treated with 100nM E2 for 24 hours, and ERα was immunoprecipated and levels of bound TTC5 under E2 treatment are shown in Figure 3.7A. While no bands were detected in negative control sample where IgG (lane 1 and 2 lower IP panel) was used to immunoprecipitate proteins, a noticeable increase can be seen comparing lane 2 to lane 1 of IP panel IB: TTC5 this means in T47D cells the interaction between TTC5 and ERα is hormone dependent. Also noticeably higher proportion of TTC5 is in complex with ERα in presence of hormone (lane 2 of IP panel IB:TTC5 to lane 2 of IP panel IB:ERα).

Next, we monitored if tamoxifen had any effect on TTC5/ERα interaction in T47D cells. Figure 3.7B, shows that tamoxifen increases the interaction between ERα and TTC5 in T47D cells (comparing lane 3 to lane 1 IP panel, IB: TTC5). A very faint line was detected in IP panel immune blot with ERα in absence of hormone compare to hormone treated samples (comparing lane 1 to lane 3 of upper IP panel), it seems this interaction is highly dependent on hormone.

Furthermore, raloxifene effect on TTC5/ERα interaction was observed, as shown in Figure 3.7C, interaction of TTC5 with ERα showed no dependency on raloxifene, as slight increase can be seen (comparing lane 2 IP panel IB: TTC5 to lane 1). However, it appears a higher proportion of TTC5 is in a complex with ERα compared to the levels of ERα detected in IP panel (comparing lane 2 of IP panel IB: TTC5, to lane of IP panel IB: ERα).

Overall, these sets of experiments (Figure 3.6 and Figure 3.7) indicates that TTC5 interacts with ERα, and as previously it was shown that TTC5 interacts with GR, hence TTC5 could be a common cofactor for multiple members of the nuclear hormone receptor superfamily. Following the confirmation of ERα interactions with TTC5, the next

objective of this study is to determine which surface of TTC5 regulated ER's transcriptional activity.

3.4 The role of TPR and LXXLL motifs in regulation ER transcriptional activities

3.4.1 TTC5 plasmid titration in MCF-7 and T47D cells

The working concentration of the TTC5 plasmid for the luciferase assay was first selected as a primary step using a series of plasmid concentrations. Following the effect of WTTTC5 on ERE transcriptional activity can potentially improve understanding of the role of this cofactor in estrogen effects on breast cancer cells. MCF-7 and T47D cells were chosen for purpose of this experiment as they are both estrogen receptor positive, and allow us to follow endogenous ER activity on ERE and assess effect in two different cell lines.

MCF-7 cells were transfected with 200, 400 and 600ng of WTTTC5, 200ng of ERE-luciferase reporter plasmid and 200ng of CMV-β galactosidase, and a suitable concentration of pcDNA3. After 24 hours cells were then incubated with 100nM of E2 for 24 hours and cells were harvested and subjected to analysis of the luciferase and β-galactosidase activity. The control lane of Figure 3.8 contains ERE, β-gal and pcDNA3 plasmid, the transcriptional activity of ER is expected to increase in presence of ERE when treated with E2. Indeed, ER transcriptional activity increased on ERE promoter when cells were treated with 100nM of E2 for 24 hours (comparing lane 2 to lane 1 of Figure 3.8).

Comparing lane 3 to 1 shows transfection of 200 ng of WTTTC5 results in slight increase in ER transcriptional activity which was not statically significant. Transcriptional activity of ER increased significantly in absence and presence of hormone when 400ng of WTTTC5 was transfected into the cells (comparing lane 5 to lane 1 and lane 6 to lane 2). And finally, introduction of 600ng of WTTTC5 into MCF-7 cells, activated the transcriptional activity of ER in absence and presence of hormone, however this result

was statistically insignificant. Based on the observation presented in Figure 3.8, 400ng of WTTTC5 plasmid was selected as the working concentration to be used in reporter gene experiments on MCF-7 cells.

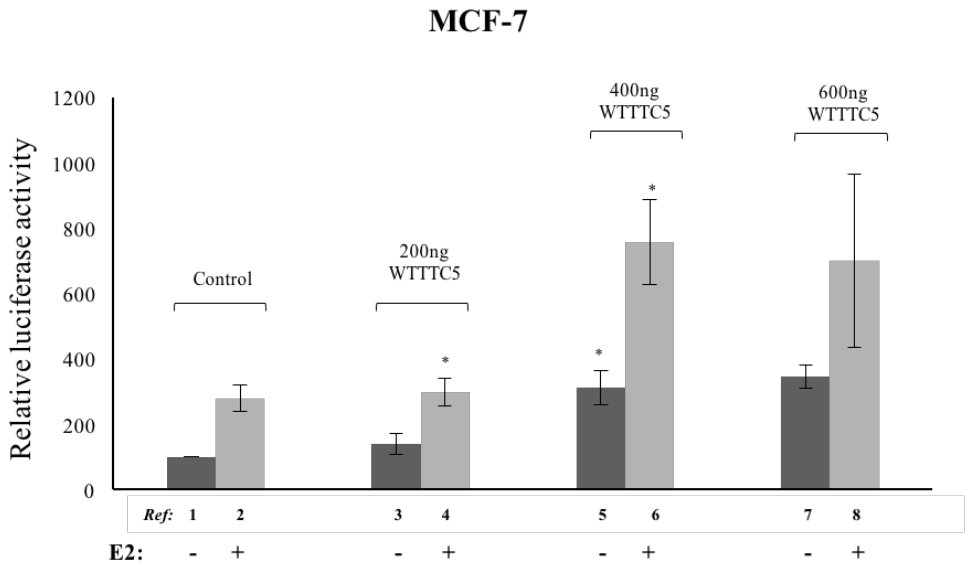


Figure 3.8.Titration of TTC5 plasmid concentration in MCF-7 cells.

Relative luciferase value normalised to beta galactosidase values are displayed. 200ng, 400ng, and 600ng of TTC5 expression plasmid was transfected into MCF-7 cells. MCF-7 cells were transfected with ERE-luciferase reporter plasmid (all lanes), pcDNA3 (all lanes) CMV-βgalactosidase (all lanes) and TTC5 (lane 3,4,5,6,7 and 8). Cells were either treated with 100nM of E2 or received no treatment for 24 hours. Relative luciferase values normalised to β-Galactosidase values are displayed. The value obtained from untreated samples (pcDNA3 and CMV-βgalactosidase) were arbitrarily set to 100 (lane1) and rest of the values were calculated accordingly. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *)

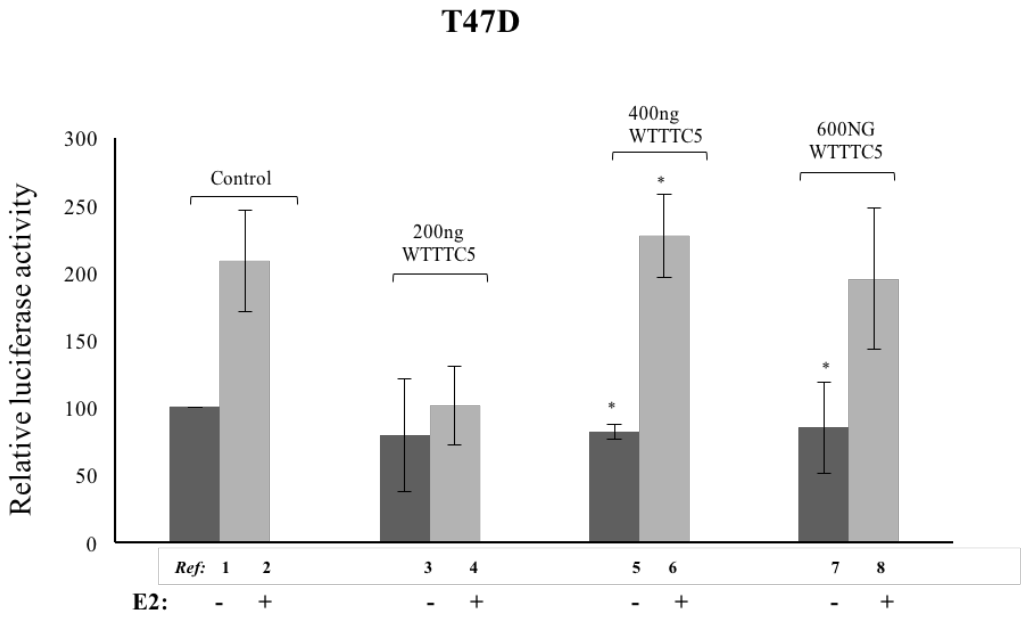


Figure 3.9.Titration of TTC5 plasmid concentration in T47D cells.

Relative luciferase value normalised to beta galactosidase values are displayed. 200ng, 400ng, and 600ng of TTC5 expression plasmid was transfected into T47D cells. T47D cells were transfected with ERE-luciferase reporter plasmid (all lanes), pcDNA3 (lane 1,2) CMV-βgalactosidase (all lanes) and TTC5 (lane 3,4,5,6,7 and 8). Cells were either treated with E2 or received no treatment for 24 hours. Relative luciferase values normalised to β-Galactosidase values are displayed. The value obtained from untreated samples (pcDNA3 and CMV-βgalactosidase) were arbitrarily set to 100 (lane1) and rest of the values were calculated accordingly. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *)

Same experiment with exact same conditions as described above was carried out on T47D cells, as shown in Figure 3.9, ER transcriptional activity increased when T47D cells were treated with 100nM of E2 for 24 hours as expected (Figure 3.9 comparing bar 2 to 1).

Introducing 200ng of WTTTC5 plasmid into T47D cells initiated a repressory effect on ER transcriptional activity (Figure 3.9 comparing lane 3 to lane 1) in absence of hormone and in presence of hormone a slight increase on ER transcription can be observed (Figure 3.9 comparing lane 4 to 1). Furthermore, 400ng of WTTTC5 induced a noticeable significant decrease in ER transcriptional activity in absence of hormone (Figure 3.9 comparing lane 5 to lane 1), however hormone treatment induced significant increase in ER transcriptional activity (Figure 3.9 comparing lane 6 to 1). Finally, 600ng of WTTTC5

was transfected into the cells which in absence of hormone (E2) caused a slight but significant decrease in ER transcriptional activity (comparing lane 7 to 1), however hormone treatment resulted in increasing ER transcriptional activity (Figure 3.9 comparing lane 8 to 7). It appears that, 400ng of WTTTC5 plasmid can be used as working concentration for both MCF-7 and T47D cells in reporter gene experiments.

3.4.2 Site directed mutagenesis of TPR and LXXLL motifs of TTC5

TTC5 was shown to comprise of 6 TPR motifs and two LXXLL motifs. Proteins that contain TPR motifs can interact with various proteins from transcription factors to multi-meric scaffolding proteins (Strauss and Keller, 2008). TTC5 has been shown to regulate GR, which is a member of the nuclear hormone receptors and a steroid receptor like ER. It was shown in A549 cells that TTC5 interacts with GR and the most critical motif in this interaction is known to be TPR 2,3 and 5 and LTSAL (which is a LXXLL motif) motif (Davies et al., 2011).

Though in a short period of time genome sequencing could solve human health issues, complex diseases like breast cancer remain difficult to tackle. However, with advanced high-throughput sequencing (HTS), that provides genomics data by sequencing a large number of tumour samples across cancer types useful information can be achieved. These HTS are the result of several international collaborations such as The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC). Whether a specific gene has been mutated or how a mutation affects protein function can be revealed through such analysis, hence we investigated the abundance or depletion of TTC5 across all cancer type and specially breast cancer (Appendix Figure 5.1). Table 3.1 represent the mutations in TTC5 that have been associated with breast cancer by bioinformatics approaches (Refer to methods section 2.18) . An interesting observation from this data was the missense mutation at serine 221, which correlated with the previous study by Demonacos et al., 2004 where they discovered when DNA damage occurs TTC5 phosphorylates ATM at the Serine 221 (S221) and as a result this activates the p53

response. It becomes apparent that mutations in this residue augments the p53 response hence the mutations are passed on to the next generation of cells.

Table 3.1.TTC5 mutations associated with breast cancer. *Phosphorylation site for chk2 kinase

Type of mutation	Nucleotide	Amino acid
Missense mutation	C662G	S221C*
Missense mutation	C1304T	S435L
Silent mutation	G276	L92L

It was after this observations that we assumed TTC5 is involved in ER regulation, hence in order to investigate the role of TTC5 multiple surfaces in ER mediated transcriptional activation, we performed an ERE-luciferase assay to detect estrogen signalling. In order to determine the role of TTC5, TPR and LXXLL motifs in ER transcriptional activity, site directed mutagenesis was used to mutate crucial residues in these motifs, as shown in Figure 3.10A and Figure 3.10B.

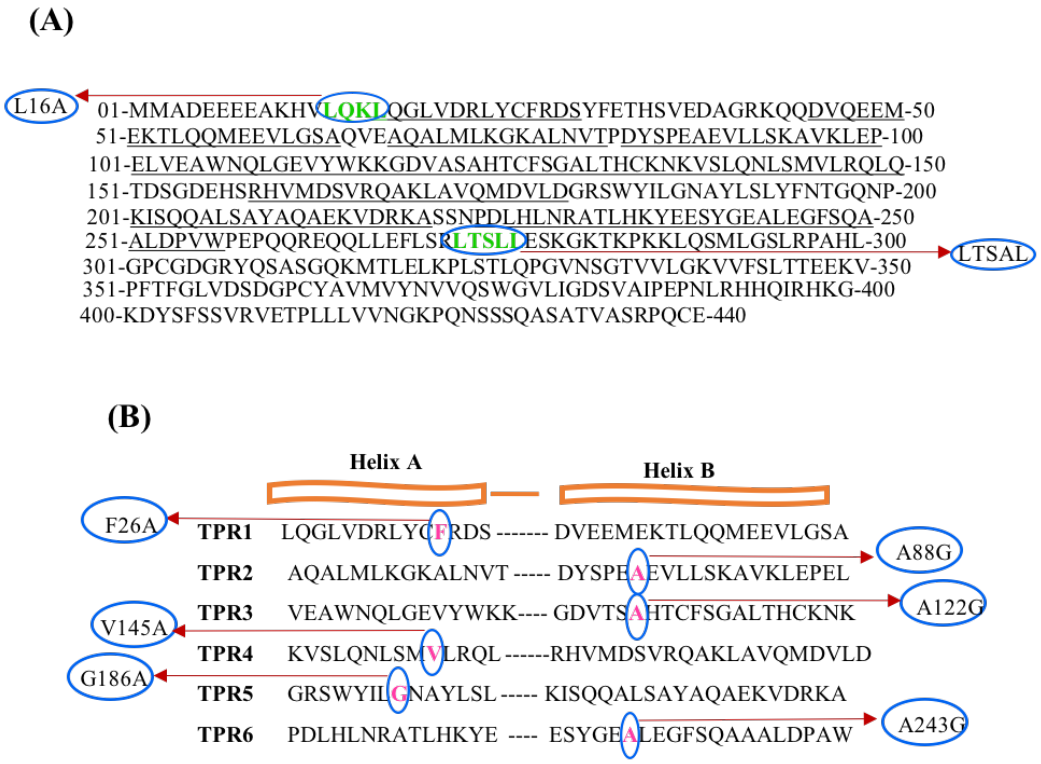


Figure 3.10.Different surfaces of TTC5.

A: TTC5 is a 440 amino acid protein. Amino acid underlined are predicted TPR motifs, and the green fonts are predicted LXXLL motifs. B: TPR motifs show homology in their amino acid sequence, length, spacing and hydrophobicity. TPR motifs consists of two alpha helices forming an antiparallel hairpin structure. TTC5 contains of 6 TPR motifs, a 34 amino acid residue domain. Pink font indicates the position of the chosen amino acid for mutation. TPR1 was labelled as F26A as the phenylalanine residue at position 26 of TTC5 sequence was changed to Alanine (this was done for the rest of the TPR and LXXLL mutants). [B modified from (Adams et al., 2012)]

3.4.3 Different motifs of TTC5 affect the transcriptional activity of ER

In order for transcription to start, ER binds to estrogen response element (ERE), this complex then interacts with coactivator proteins and components of RNA polymerase II transcription initiation. However, details of this process and how its deregulation leads to cancer or drug resistance are still not very clear. Following the effect of TTC5 on ER transcriptional activity can potentially improve understanding of the role of this cofactor in estrogen effects on breast cancer cells. MCF-7 and T47D cells were chosen for purpose

of this experiment as they are both estrogen receptor positive, and allow us to follow endogenous ER activity on ERE-luciferase reporter and assess effect in two different cell lines with identical ER status. The ERE-luciferase reporter was a gift form Professor Arun Rishi (Apendix Appendix Figure 5.2).

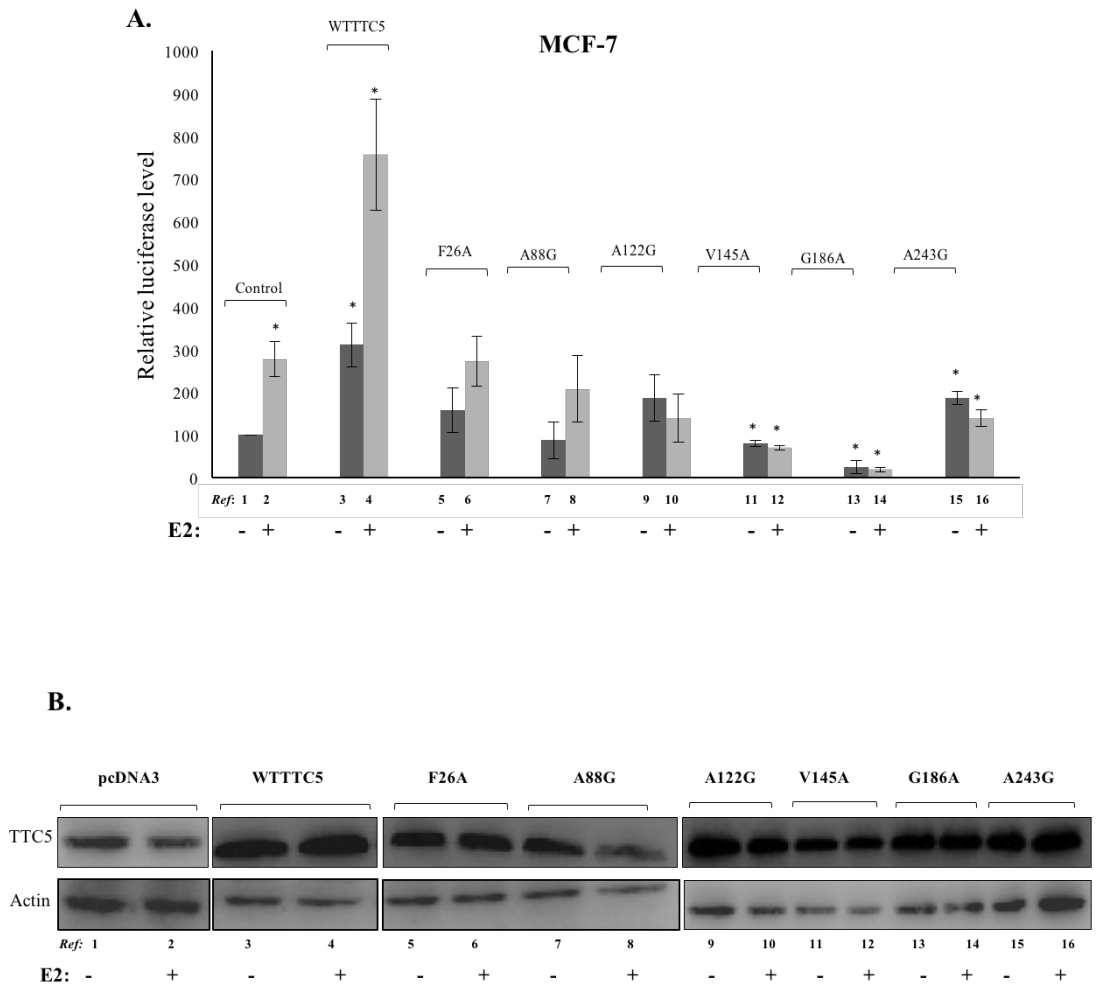


Figure 3.11. Effect of TPR mutants on ER transcriptional activity on ERE promoter in MCF-7 cells.
A. Illustration of relative luciferase of ER on ERE promoter in MCF-7 cells. MCF-7 cells were transfected with ERE-luciferase (all lanes), CMV-β galactosidase (all lanes), pcDNA3 (all lanes) and either WTTTC5 or TPR mutants TPR1 (F26A), TPR2 (A88G), TPR3 (A122G), TPR4 (V145A), TPR5 (G186A), TPR6 (A243G). Relative luciferase values normalised to β-Galactosidase values are displayed. Cells were either treated with E2 (100nM) or received no treatment for 24 hours. Reporter gene activity for the vector control of untreated samples were taken as 100 and all other activities are shown relative to this. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *). B. Western blot for the same experiment was done using MCF-7 cells transfected with pcDNA3, WTTTC5 and the six TPR mutants.

Luciferase assays were performed to measure the activity of endogenous ER by transfecting ERE reporter into MCF-7 cells using PolyFect transfection reagent in the absence and presence of E2. MCF-7 cells were transfected with 200 ng of CMV β-galactosidase, 200ng of ERE-luciferase and 400ng of WTTTC5 or 400ng of TRR1/F26A, TPR2/A88G, TPR3/A122G, TPR4/V145A, TPR5/G186A, and TPR6/A243G and pcDNA3 as required to reach a final concentration of 1.5 μg of total DNA for PolyFect transfection. The cells were then either not treated or treated with 100nM of E2 for 24 hours. While β-gal assay was performed as an internal control for transfection efficiency, western blot shown in Figure 3.11, was also used as a positive control for transfection efficiency. β-actin and TTC5 levels were analysed by means of western blot using primary antibodies against β-actin, and TTC5.

The control lane which measures the ER transcriptional activity on ERE promoter was used as a positive control for this assay as well as the β-gal assay (internal control). As expected E2 treatment increased the transcriptional activity of ER on ERE promoter (comparing lane 2 to lane 1Figure 3.11A). Wild type (WTTC5) increases ER activity on ERE promoter in both absence and presence of hormone (comparing lane 3 to 1 and lane 4 to lane 2) and was shown previously in the titration experiment Figure 3.8. The notion of statistical analysis that was carried out in these sets of experiments, was to compare the effect of each mutant on ER transcriptional activity (in absence and presence of hormone) with control bar and also to the WTTTC5 bar.

In Figure 3.11, it is evident that although F26A (TPR1) induced a slight increase in ER transcriptional activity both in absence and presence of hormone comparing to control (comparing lane 5 and 6 to lane 1). However, in comparison with WTTTC5 this motif activated ER transcription to lesser degree (comparing lane 5 and 6 to lane 3 and 4). As shown in Figure 3.11, A88G (TPR2) didn't appear to have any noticeable effect on ER activity in absence of hormone (comparing lane 7 to lane 1), although hormone treatment induced an coactivatory effect on ER transcription but not above the degree of activation that WTTTC5 caused (comparing lane 8 to lane4).

Results of Figure 3.11 show A122G (TPR3) mutant were overexpressed in T47D cells and indicates, that although both in absence and presence of hormone this mutant induces a slight increase on ER transcription levels compare to control (comparing lane 9 and10 to lane 1), this mutant less activatory effect in comparison with WTTTC5 (comparing lane 9 to lane 3 and 10 to lane 4). In Figure 3.11, it is observed that the V145 (TPR4) is triggering a significantly strong repression on ER transcription both in absence and presence of hormone (lanes 11 and 12 compared to control and WTTTC5 lanes), this reside appeared to be one of the strongest corepressor, which makes this TPR a possible coactivator for ER activity. According to Figure 3.11G186A (TPR5) mutant also is repressing ER activity both in absence and presence of hormone (comparing lane 13 and 14 to both control and WTTTC5 bar) but to a greater degree than V145A mutant. Although A243G (TPR5) mutant may seem to activate ER transcription compare to untreated control group (comparing lane 15 and 16 to lane1) but in comparison with WTTTC5 lanes (comparing lane 15 to lane 3 and lane 16 to lane 4) this mutant activated ER in lesser extent. Hormone treatment doesn't seem to play a noticeable role on this mutant.

Taken together, these luciferase assay results performed on MCF-7 cells (Figure 3.11A), indicates that WTTTC5 and the 6 TPR motifs can activate ER transcriptional activity on ERE promoter, nevertheless the extent of this effect may vary depending on the surface of the TPR motif utilised for ER-TTC5 interaction.

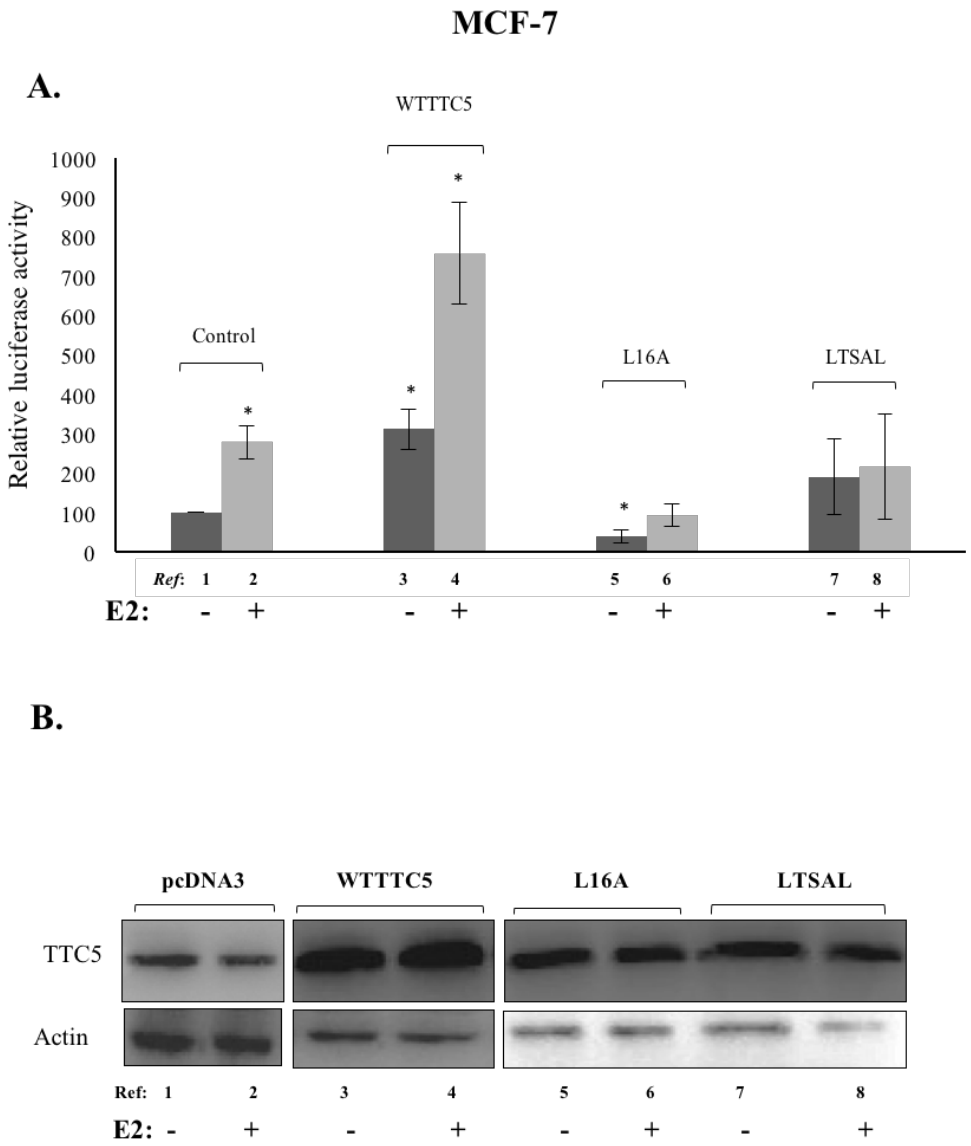


Figure 3.12. Effect of LXXLL mutants on ER transcriptional activity on ERE promoter in MCF-7 cells. A. Illustration of relative luciferase of ER on ERE promoter in MCF-7 cells. MCF-7 cells were transfected with ERE-luciferase (all lanes), CMV-β galactosidase (all lanes), pcDNA3 (all lanes) and either WTTTC5 or LXXLL mutants (LTSAL and L16A). Relative luciferase values normalised to β-Galactosidase values are displayed. Cells were either treated with E2 (100nM) or received no treatment for 24 hours. Reporter gene activity for the vector control of untreated samples were taken as 100 and all other activities are shown relative to this. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *). B. Western blot for the same experiment was done using MCF-7 cells transfected with pcDNA3, WTTTC5 and the two LXXLL mutants.

Next MCF-7 cells were transfected with same ERE reporter, and this time the effect of LXXLL mutants on ER transcriptional activity was observed. MCF-7 cells were transfected with 200ng of CMV β-Galactosidase, 200ng of ERE-luciferase and 400ng of WTTTC5 or 400ng of TTC5/LTSAL, TTCC5/L16A and pcDNA3 as required to reaching a final concentration of 1.5 μg of total DNA for PolyFect transfection. The cells were then either not treated or treated with 100nM of E2 for 24 hours.

The results are summarised in Figure 3.12, where (A) presents the relative luciferase of ER and (B) is the western blot used as positive cell transfection control. Figure 3.12A, revealed that LI16A mutant repressed transcription of ER dramatically both in absence and presence of hormone (comparing lane lanes 5 and 6 to lane 1 and also to WTTC5 lanes). Although LTSAL mutant appears as an activator of ER transcription on ERE promoter (comparing lane 7 to lane 1), this observation wasn't statically significant. Also, the level of activation by this mutant was in lesser degree than WTTTC5 both the absence and presence of hormone (comparing lanes 7 and 8 to lanes 3 and 4).

These results revel that ER transcription is affected by LXXLL motifs of TTC5 on ERE promoter, and that the reduction in ER activity with mutated LXXLL motifs on ERE promoter indicates that perhaps these two LXXLL motifs are required for activating ER transcriptional activity.

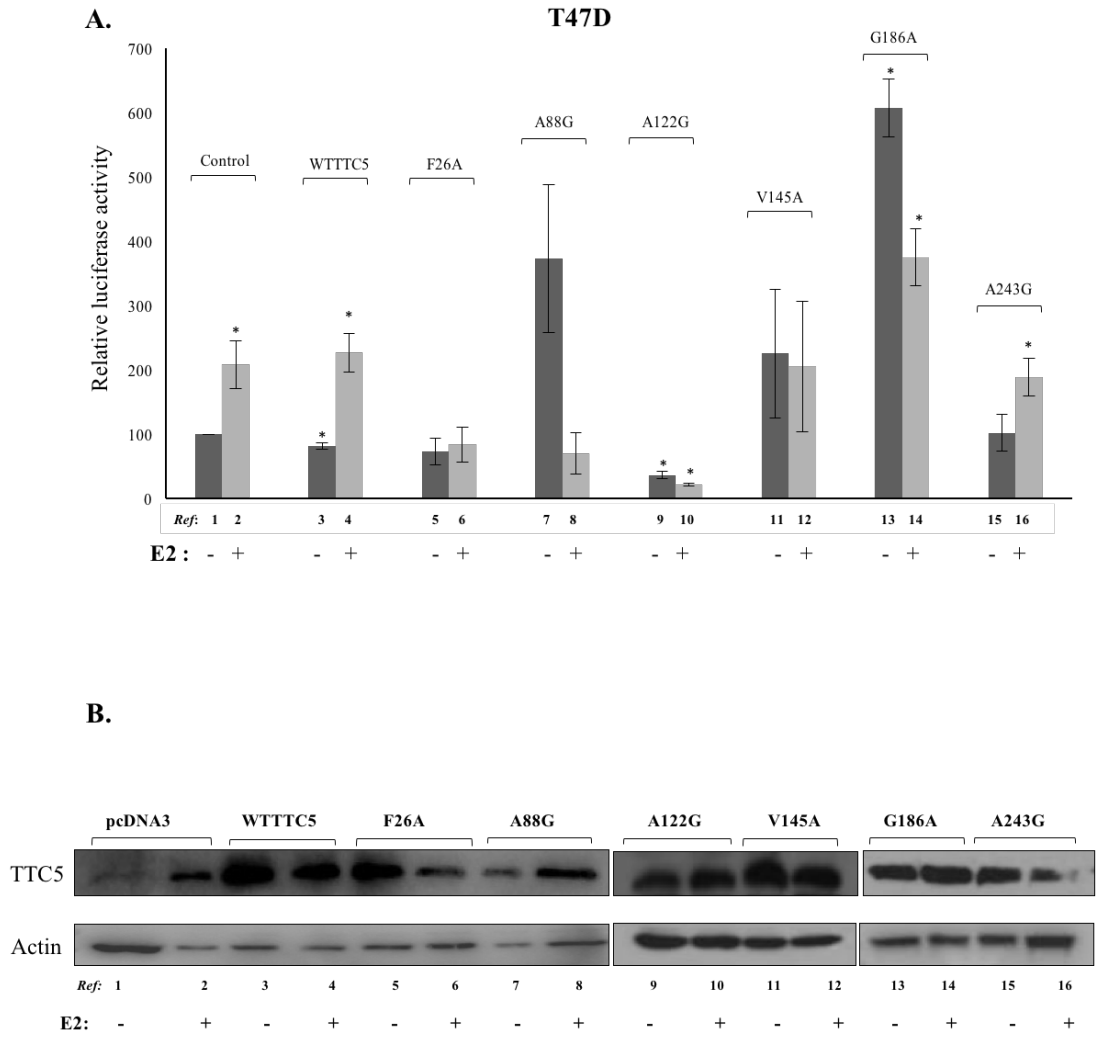


Figure 3.13. Effect of TPR mutants on ER transcriptional activity on ERE promoter in T47D cells.
A. Illustration of relative luciferase of ER on ERE promoter in T47D cells. T47D cells were transfected with ERE-luciferase (all lanes), CMV-β galactosidase (all lanes), pcDNA3 (all lanes) and either WTTTC5 or TPR mutants (F26A, A88G, A122G, V145, G186A, A243G). Relative luciferase values normalised to β-Galactosidase values are displayed. Cells were either treated with E2 (100nM) or received no treatment for 24 hours. Reporter gene activity for the vector control of untreated samples were taken as 100 and all other activities are shown relative to this. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *). B. Western blot for the same experiment was done using MCF-7 cells transfected with pcDNA3, WTTTC5 and the six TPR mutants.

Previous experiment on MCF-7 cells showed that TPR motifs are important for both coactivator or corepressor function of ER activity on ERE promoter. In order to further investigate if these effects are specific to one type of cells, the same luciferase assay was carried out on T47D cells. T47D cells were transfected with 200 ng of CMV β-Galactosidase, 200ng of ERE-luciferase and 400ng of WTTTC5 or 300ng of TTC5/F26A, TTCC5/A88G, TTC5/A122G, TTC5, V145, TTC5/G186A, and TTC5/A243G and pcDNA3 as required to reaching a final concentration of 1.5 μg of total DNA for PolyFect transfection. The cells were then either treated or not treated with 100nM of E2 for 24 hours.

The results are summarized in Figure 3.13 where the relative luciferase levels showed that WTTTC5 significantly induces repression on ER activity on ERE promoter in absence of hormone (comparing lane 3 to lane 1) however hormone treatment caused a slightly increased ER activity but this didn't appear to be greater than in absence of TTC5 (comparing lane 4 to lane 2). According to Figure 3.13A, F26A (TPR1) mutant seems to weakly repress ER activity in T47D cells both in absence and presence of hormone (comparing lanes 5 and 6 to lane 1). As for the TTC5 mutant A88G (TPR2), this mutant activated ER activity on ERE promoter (lane 7) compared to control (lane1), the coactivatory effect of this mutant seemed to be higher than the WTTTC5 (lane 7 to lane 3) however these results were insignificant. On the other hand, hormone treatment caused dramatic decrease of ER transcription (comparing lane 8 to lane 1 and 4). This suggest the hormone dependency of this mutant on T47D cells, which means in absence of hormone this mutant acts as activator while in presence acts as repressor.

Overexpression of A122G (TPR3) motif strongly inhibited ER activity on ERE promoter in T47D cells (comparing lane 9 to lane1and 3) and hormone treatment further decreased this effect (comparing lane 10 to lane 1 and 4). Mutations in amino acid residues of this motif resulted in strong corepressory effect on ER transcriptional activity on ERE promoter, which means this residue is responsible for activating ER transcriptional activity. According to Figure 3.13A, the V145 (TPR4) mutant overexpression in T47D cells triggered an increase on transcription of ER both in absence and presence of hormone (lanes 11 and 12), this mutant didn't seem to be under the influence of hormone.

Results presented in Figure 3.13A, indicates that, ER transcriptional activity at ERE promoter significantly increased when G186A (TPR5) mutant was transfected into T47D cells (comparing lane 13 to lane 1), however E2 treatment decreased this effect but the overall effect was still coactivatory (lane 14 to lane 1 and lane 4). This mutant seems to be the only significant coactivator of ER transcription in T47D cells. A243G (TPR6) motif in absence of hormone didn't have any noticeable effect on ER transcriptional activity (compare lane 15 to lane 1 and lane 3) however hormone treatment caused slight significant increase (comparing lane 16 to lane 1). The effect of this mutant on ER transcriptional activity appeared to be hormone dependent.

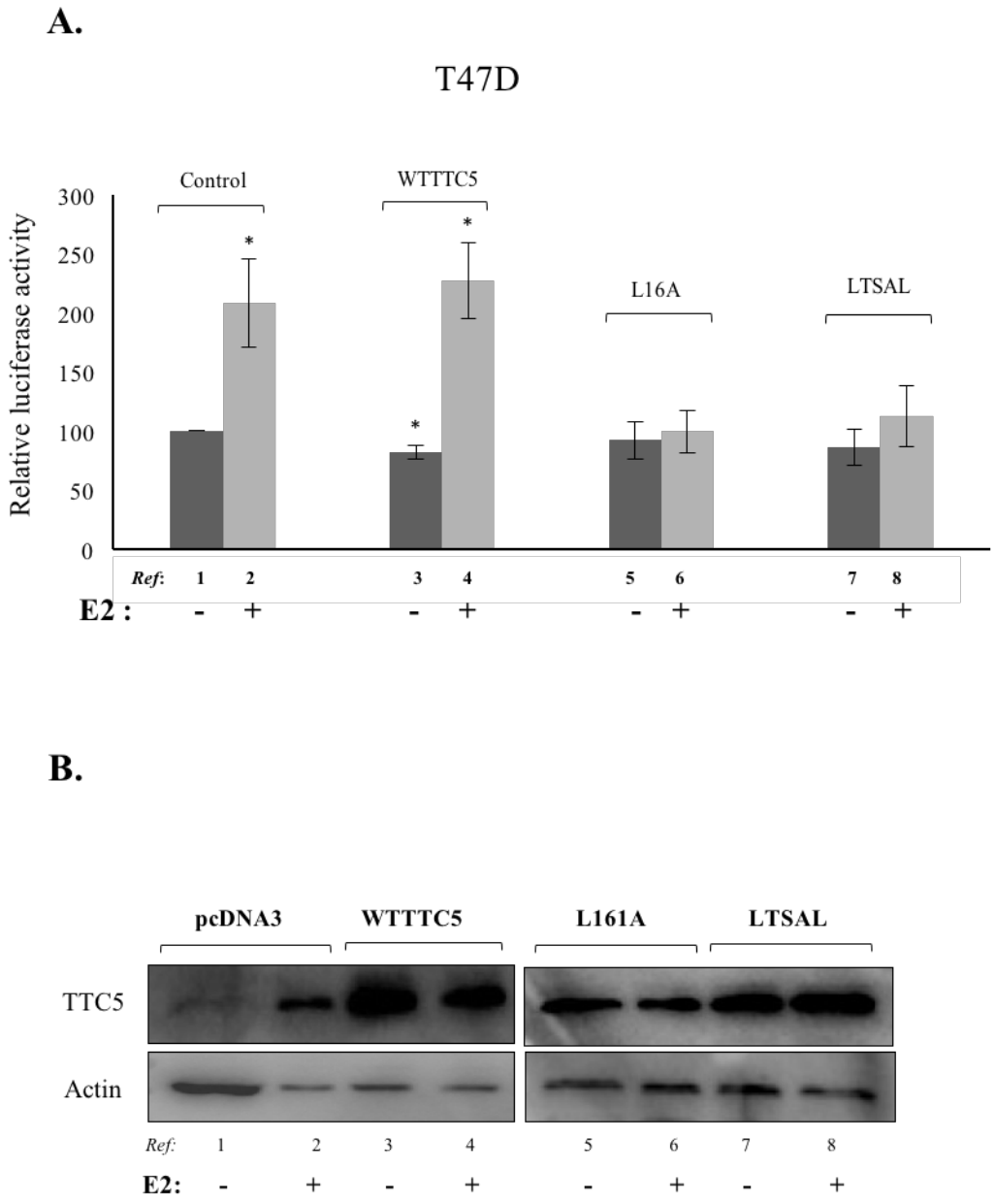


Figure 3.14. Effect of LXXLL mutants on ER transcriptional activity on ERE promoter in T47D cells. A. Illustration of relative luciferase of ER on ERE promoter in T47D cells. T47D cells were transfected with ERE-luciferase (all lanes), CMV-β galactosidase (all lanes), pcDNA3 (all lanes) and either WTTTC5 or LXXLL mutants (LTSAL and L16A). Relative luciferase values normalised to β-Galactosidase values are displayed. Cells were either treated with E2 (100nM) or received no treatment for 24 hours. Reporter gene activity for the vector control of untreated samples were taken as 100 and all other activities are shown relative to this. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *). B. Western blot for the same experiment was done using MCF-7 cells transfected with pcDNA3, WTTTC5 and the six TPR mutants.

T47D cells were transfected with 200 ng of CMV β-Galactosidase, 200ng of ERE-luciferase and 400ng of WTTTC5 or 400ng of TTC5/LTSAL, TTCC5/L16A and pcDNA3 as required to reaching a final concentration of 1.5 µg of total DNA for PolyFect transfection. The cells were then either not treated or treated with 100nM of E2 for 24 hours. As shown in

Figure 3.14A, both LI16A and LTSAL had similar effect on ER transcriptional activity on ERE promoter in T47D cells. They both inhibited ER activity in T47D cells compare to control (comparing LI16A and LTSAL bar to control and WTTTC5 bar). Taken together, luciferase date suggests that TTC5 derivatives carrying TPR and LXXLL mutated motifs can activate, repress or have little effect on ER transcriptional activity inT47D cells depending on the TTC5 surface utilised (Figure 3.13 and

Figure 3.14) The comparison analysis of contribution of each individual TPR and LXXLL motif towards ER transcriptional activity in two different cell lines (MCF-7 and T47D) would be as follows:

- F26A (TPR1) was a corepressor of ER in MC-7 while it was a coactivator in T47D cells.
- A88G (TPR2) was a corepressor of ER transcriptional activity in MCF-7 cells, while it was a coactivator in T47D cells.
- A122G (TPR3) was a coactivator of ER in MCF-7 cells while it was a major corepressor inT47D cells.
- V145A (TPR4) was a major coactivator of ER in MCF-7 while it was a corepressor in T47D cells.
- G186A (TPR5) was the major regulator of ER activity in MCF-7 while it was the most prominent co repressor in T47D.
- A243G (TPR6) motif was showing similar upregulation effects on ER in both cell lines.
- LI16A (LXXLL) motif triggers strong coactivation in MCF-7 cells while a corepressor of ER activity in T47D cells.
- LTSAL (LXXLL) motif was corepressing ER activity in MCF-7 and coactivating in T47D

3.5 Optimisation of siRNA mediated inhibition of TTC5 in MCF-7 and T47D cells

Two specific receptors, estrogen receptor ERα and ERβ mediate the effect of estradiol. As mentioned before in introduction, it is through the classical pathway that ER binds to ERE and activates the expression of target genes. Studies have shown estradiol stimulates the proliferation of estrogen positive breast cancer cells (Glaeser et al., 2006).

The aim of this series of experiments was to determine role of TTC5 and ER crosstalk using TTC5 knockdown/MTT assay and determining potential effects on MCF-7 and T47D cells proliferation when treated with various concentration of E2, tamoxifen and raloxifene.

For successful knockdown specific factors are required such as suitable amount of transfection reagent and number of cells. First step of optimisation was to determine the optimal concentration of DharmaFECT 1 (DF1) transfection reagent that would provide the highest transfection efficiency and lowest cytotoxicity. Using the MTT assay cellular viability was measured with 3 different concentrations of DF1 (0.125µl-0.25µl and 0.5µl). For optimisation purpose a non-targeting (NT) siRNA was used as control. qRT-PCR was performed to confirm the efficiency of knockdown prior to every experiment. Transfection was repeated on MCF-7 and T47D cells at 2500 cells/well before a specific concentration of DF1 was chosen for further studies.

In order to determine experimental conditions where the siRNA mediated TTC5 knockdown was maximal and the viability of the cells was least affected, concentration of the siRNA pool plus the DF1 transfection reagent was varied.

The non-targeting siRNA was recommended by GE Dharmacon company as a control for optimisation. Using a 96-well plate 2500 cells were seeded per well and 2µM stock of each siRNA were made, and transfected into cells with DF1. After 24 hour samples were collected to measure the percentage of TTC5 knockdown before each MTT experiment.

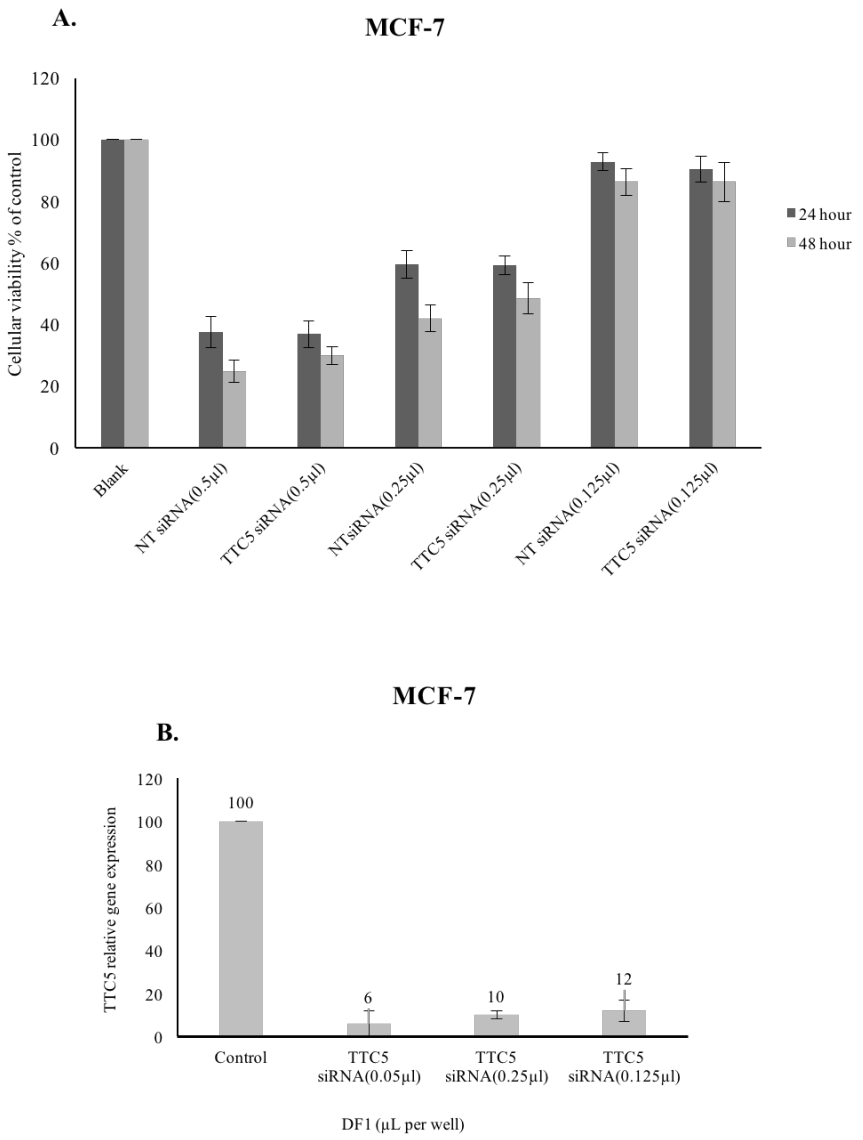


Figure 3.15.Optimisation of MCF-7 cells with various concentrations of DF1 transfection reagent.

A. MTT assay of MCF-7 cells treated with TTC5 siRNA and non-targeting (NT) siRNA with various concentration of DF1, showing cellular viability after 24 and 48 hours (A). Normalised to control (cells with no transfection reagent). Untreated samples were taken as 100 and all other activities are shown relative to this. B. qRT-PCR analysis showing percentage of TTC5 knockdown against non-targeting siRNA after 24 hours in MCF-7 cells. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *).

As shown in Figure 3.15B, the highest recorded knockdown of TTC5 (90%) (Figure 3.15B (DF1 0.5µl/well), also induced a high level of cytotoxicity in MCF-7 cells. Analysis of Figure 3.15A and B, determined that 0.125µl of DF1 was sufficient to provide an efficient level of knockdown with less cytotoxicity compared to other two concentrations of DF1.

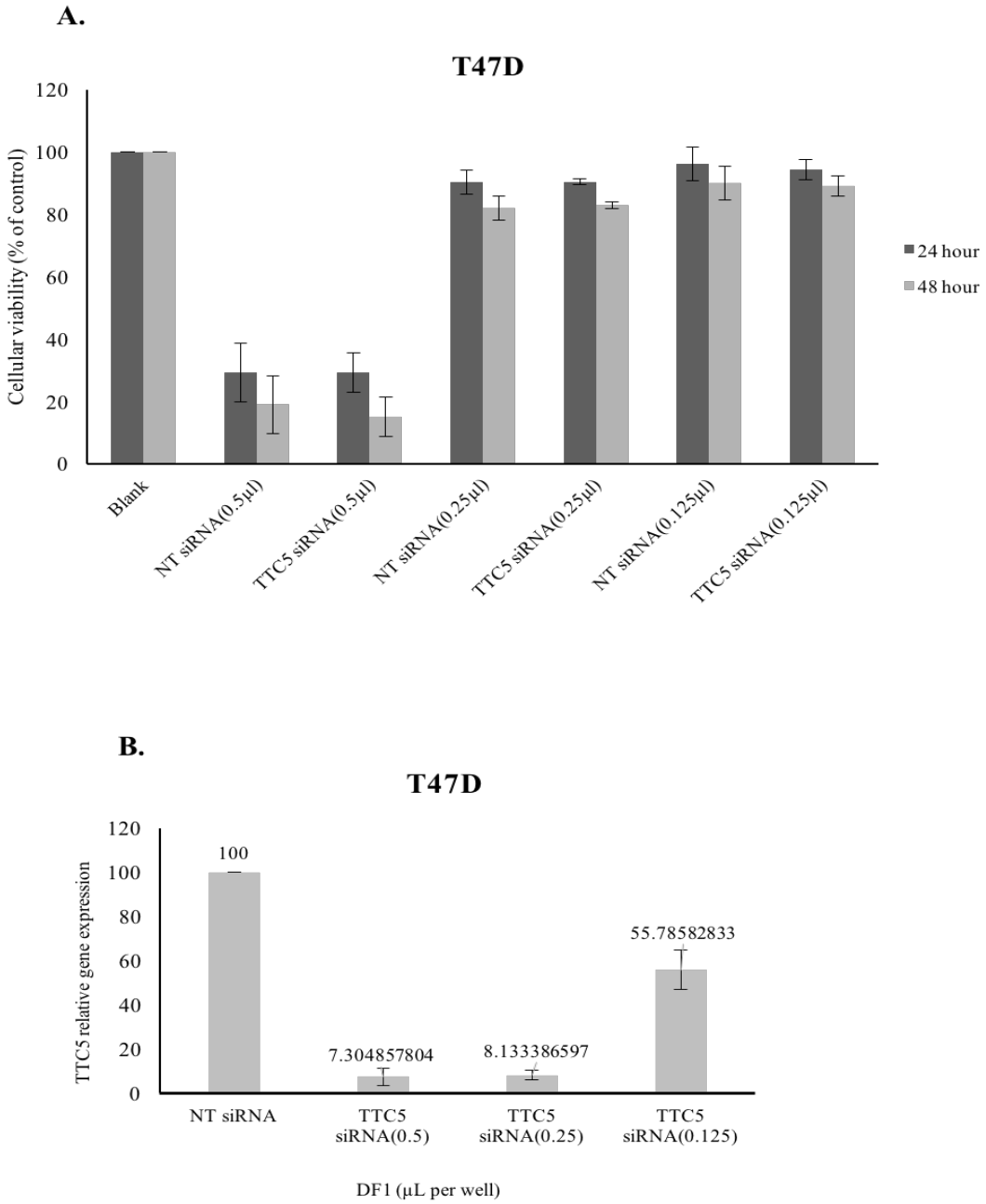


Figure 3.16.Optimisation of T47D cells with various concentration of DF1 transfection reagent**.**

MTT assay of T47D cells treated with TTC5 siRNA and non-targeting (NT) siRNA with various concentration of DF1, showing cellular viability after 24 hours (A). Normalised to control (cells with no transfection reagent). Untreated samples were taken as 100 and all other activities are shown relative to this. qPCR analysis showing percentage of TTC5 knockdown against non-targeting siRNA after 24 hours in T47D cells (B). Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *).

Same sets of experiments were carried out on T47D cells in order to choose the right amount of DF1 that result in highest knockdown percentage with high cell viability. In T47D cells as shown in
Figure 3.16, 0.25µl of DF1 reagent is sufficient to provide about 85% of TTC5 knockdown with minimal cytotoxicity.

3.5.1 Effect of TTC5 knockdown on ERα positive breast cancer cell proliferation

Small interfering RNA (siRNAs) that are produced by cleavage of longer RNAs, are non-coding, double stranded RNA molecules (Elbashir et al., 2001; Hamilton and Baulcombe, 1999). One of the two strands of siRNA separate and integrates into the RNA induced silencing complex (RISC) leading to reduced gene expression through siRNA. It is via the siRNA single strand that RISC binds to a complementary sequence of mRNA, and as a result the active component of RISC Argonaute then cleaves the mRNA preventing translation (Liu et al., 2004). siRNA molecules are designed in a way to be complementary for a gene interest which in this study is TTC5, therefore introducing siRNA into cultured cells prevents translation of TTC5.

Once the optimisation of DF1 and siRNA pools were completed, to evaluate the role of TTC5 and ER in cell apoptosis, TTC5 was then knockdown in ER alpha positive breast cancer cells (T47D and MCF-7) using DF1. To measure the percentage of cell death due to knockdown of TTC5, cells were exposed to different concentrations of drugs (estradiol, tamoxifen and raloxifene) for 24 and 48 hours and the cell viability was determined by MTT assay.

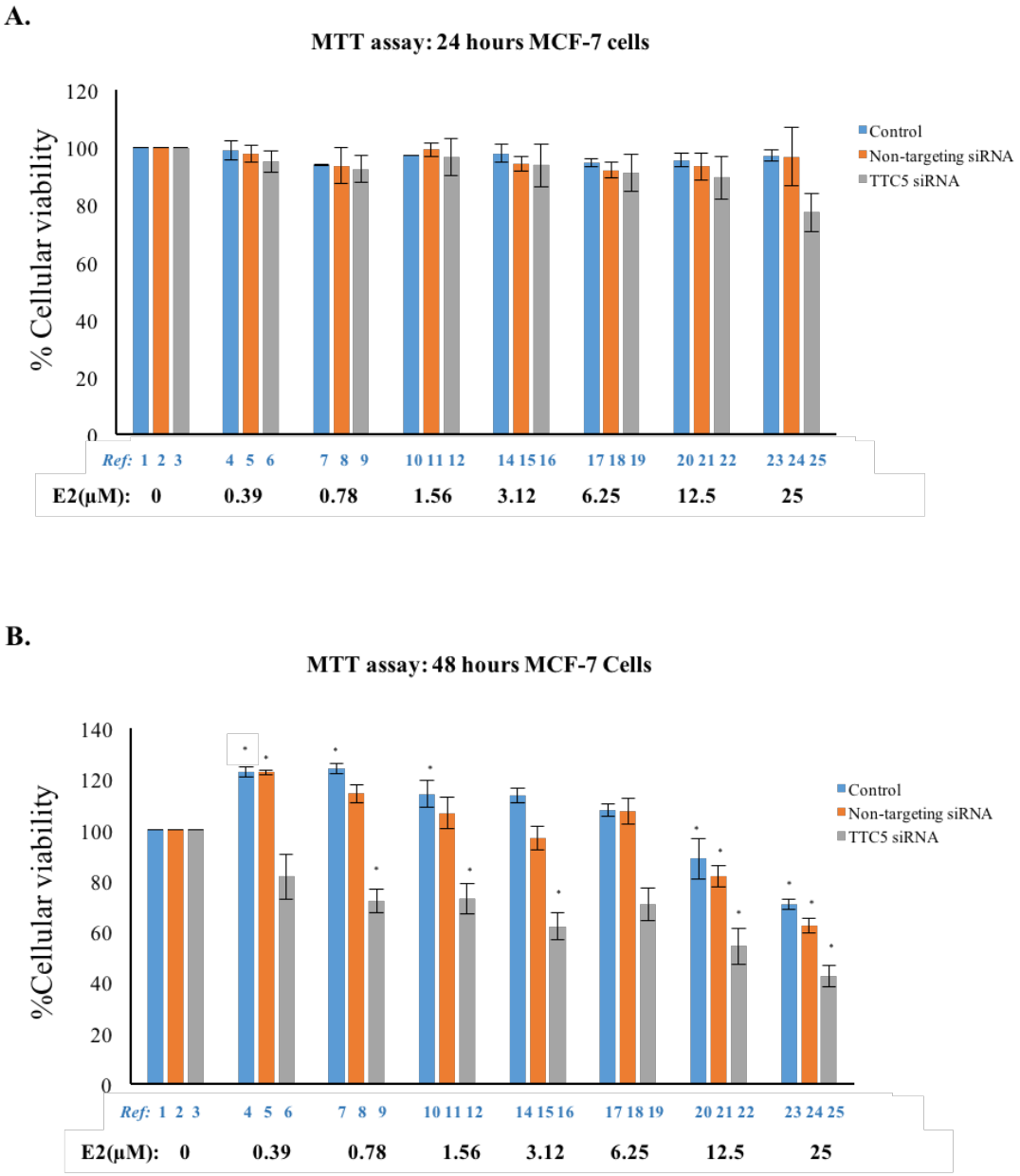
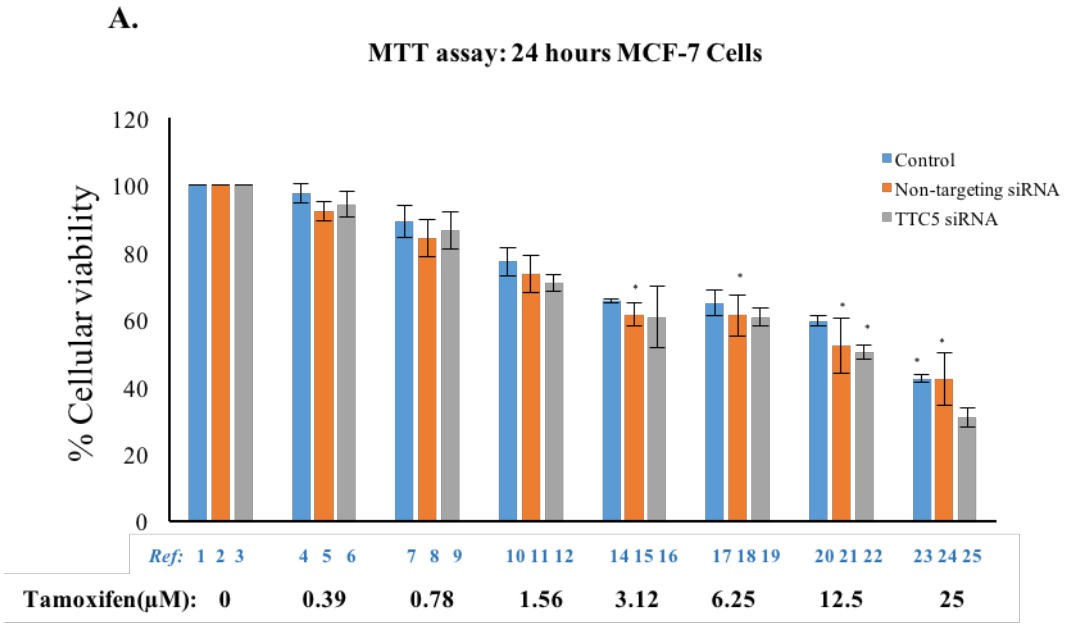


Figure 3.17.Effects of TTC5 silencing on MCF-7 cells viability under various concentration of E2. MCF-7 cells were either left transfected with no siRNA (blue bars), or non-targeting siRNA (orange bars) and TTC5 siRNA (grey bars), using DF1. Cells either received no treatment (0) or were treated with increasing concentrations of E2 (0.39µM-25µM), and cell viability was analysed via MTT assays. A. MCF-7 cells were treated with E2 for 24 hours. B. MCF-7 cells were treated with E2 for 48 hours. The value obtained from untreated samples of each condition were taken as 100 (lane1,2,3) and all other activities are shown relative to this. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *).

The results presented in Figure 3.17 indicates that, the negative control (non-targeting) seemed to not have any effect as the value of orange bars are similar to the control (blue bars). Though at 24 hours, no major difference between control (blue bars) and TTC5 knockdown cells (grey bars) can be seen from 0.39μM to 12.5μM, E2 at its highest concentration (25μM) caused in slight significant decrease in cell viability of siRNA TTC5 transfected cells compared to control can be seen (compare lane 25 to lane 23).

Next MCF-7 cells were left with increasing concentration of E2 for 48 hours and MTT assay was performed (Figure 3.17B). E2 appeared to continue to proliferate MCF-7 cells growth significantly at almost every concentration apart from 3.12μM (lane 15) and 6.25μM (lane 18). The non-targeting seemed to follow the same pattern as control cells, apart from the 3.12μM concentration were the difference between non-targeting and control was noticeable. An interesting observation is the apparent decrease in cellular viability of knockdown cells compared to control Figure 3.17B. The difference between viability of knockdown and control cells seemed to be statically significant from 0.78μM to 25μM (except 6.25μM) after 48 hours. These results highlight that TTC5 silencing inhibits MCF-7 cell growth when estradiol is present at longer time course.



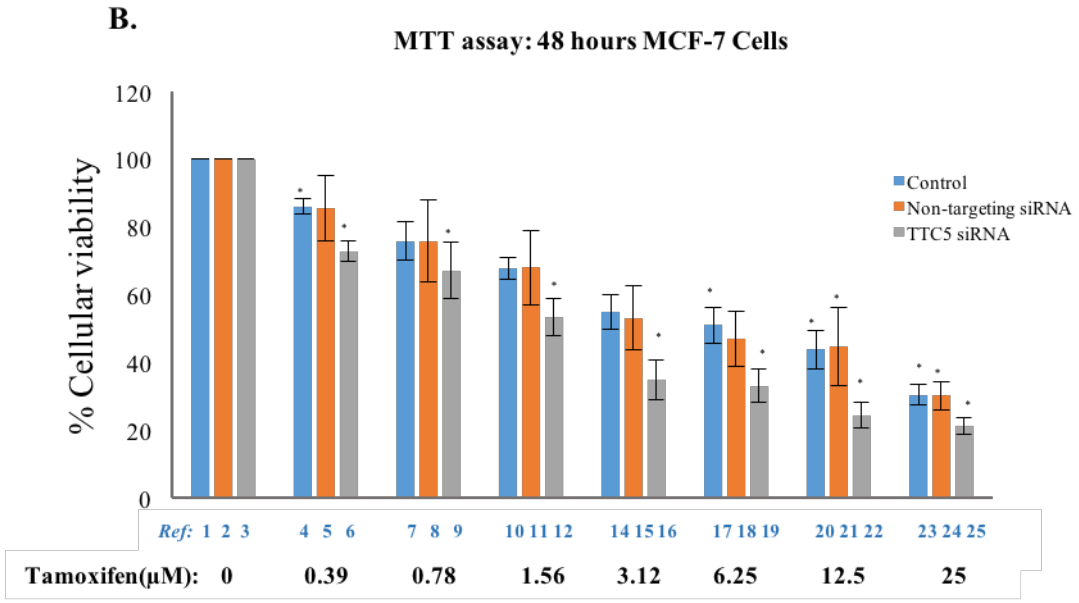


Figure 3.18. Effects of TTC5 silencing on MCF-7 cells under various concentrations of tamoxifen. MCF-7 cells were either left transfected with no siRNA (blue bars), or non-targeting siRNA (orange bars) and TTC5 siRNA (grey bars), using DF1. Cells either received no treatment (0) or were treated with increasing concentrations of tamoxifen (0.39μM-25μM), and cell viability was analysed via MTT assays. A. MCF-7 cells were treated with tamoxifen for 24 hours. B. MCF-7 cells were treated with tamoxifen for 48 hours. The value obtained from untreated samples of each condition were taken as 100 (lane1,2,3) and all other activities are shown relative to this. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *).

To further investigate the role played by TTC5 knockdown on MCF-7 cells, cellular viability; cells were treated with tamoxifen over a wide range of concentration (0.39 to 25μM). Results from 3 to 5 independent MTT experiment were polled and bar char graphs were obtained Figure 3.18. At 25 μM a significant antiproliferative effect was observed (Figure 3.18A, comparing lane 23 to lane 1). TTC5 knockdown appeared to follow the same pattern as control cells, however it appears that tamoxifen at its highest concentration 25μM has slight significant additional inhibitory effect on knockdown cells growth (Figure 3.18A, comparing lane 25 to 23)

Results present in Figure 3.19B indicates, further inhibition of cell growth of MCF-7 by tamoxifen after 48 hours of incubation time. Although no significant effect was observed at 0.78μM, 1.56μM and 3.12μM doses of the control group (lanes 7, 10 and 14), at higher doses of the hormone 6.25μM, 12.5μM and 25μM tamoxifen significantly inhibited cell proliferation up to 70% (lanes 17, 20 and 23). Knockdown of TTC5 in MCF-7 cells resulted in significant further increase in cell death (Figure 3.18B, lanes 19, 22 and 25).

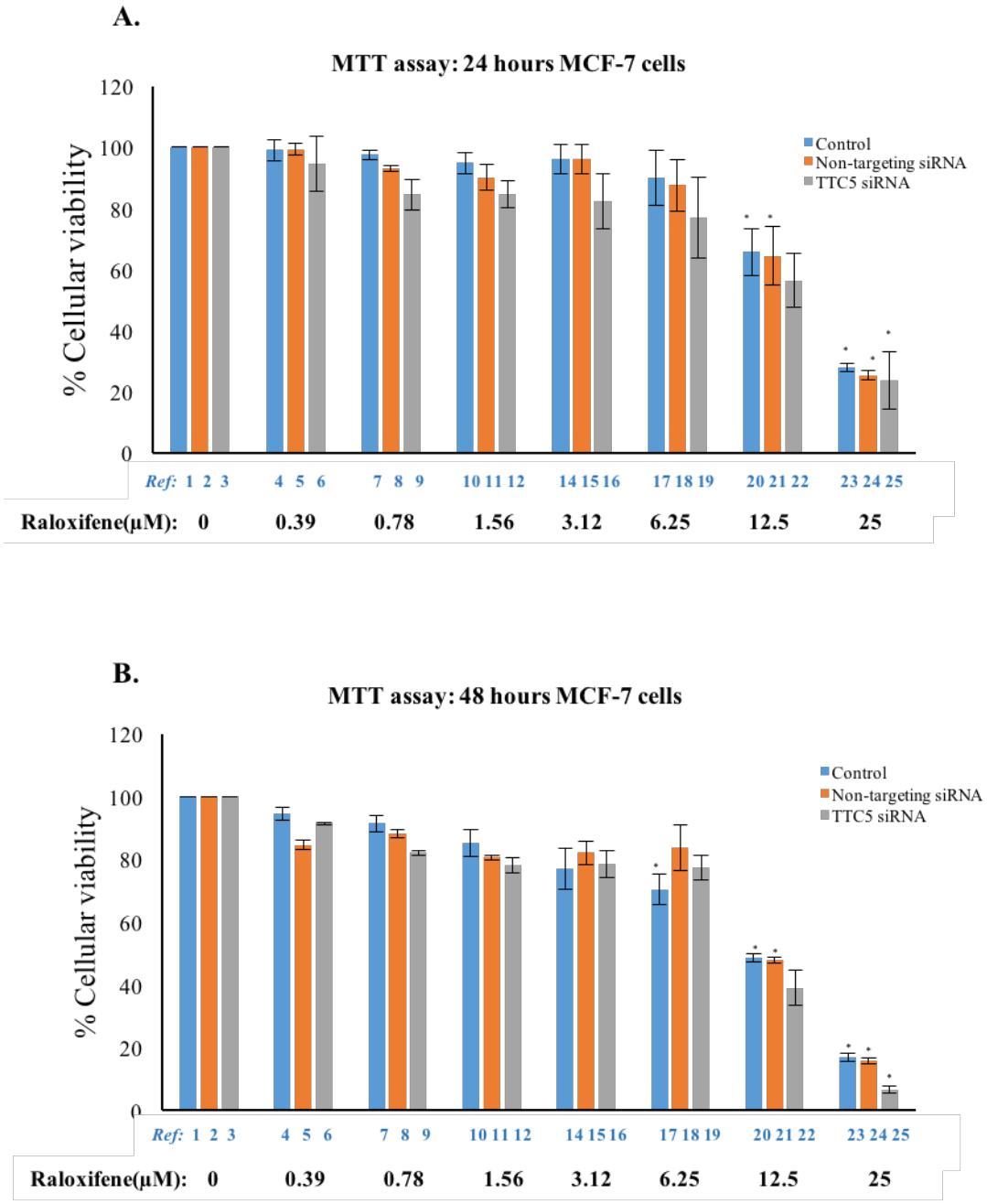


Figure 3.19.Effects of TTC5 silencing on MCF-7 cells under various concentrations of raloxifene. MCF-7 cells were either left transfected with no siRNA (blue bar), or non-targeting siRNA (orange bar) and TTC5 siRNA (grey bar), using DF1 for 24 and 48 hours. Cells either received no treatment (0) or were treated with increasing concentrations of raloxifene (0.39µM-25µM), and cell viability was analysed via MTT assays. A. MCF-7 cells were treated with raloxifene for 24 hours. B. MCF-7 cells were treated with raloxifene for 48 hours. The value obtained from untreated samples of each condition were taken as 100 (lane1,2,3) and all other activities are shown relative to this. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *).

Data presented in Figure 3.19A and B, are results of at least three independent MTT assays performed on MCF-7 cells treated with various doses of raloxifene for 24 and 48 hours. The cytotoxic effect of raloxifene on MCF-7 cells can be clearly seen at 25μM after 24 hours, which is shown to be statically significant (Figure 3.19A comparing lane 23 to lane 1). Moreover, the difference between control cells and knockdown cells didn't appear to be dramatic when treated with various concentration of raloxifene, as shown in Figure 3.19A (comparing grey bars to blue bars).

Figure 3.19B, shows raloxifene effect on MCF-7 cells, and TTC5 knockdown cells after 48 hours incubation. The treatment of the MCF-7 (control group) cells with raloxifene didn't seem to be statically significant at lower doses, although at higher does 6.25μM, 12.5μM and 25μM raloxifene induced cell death on MCF-7 cells (lane 17, 20 and 23 compared to lane 1). Raloxifene appeared to have negligible effect on knockdown cells in comparison to control group (comparing grey bars to blue bars). However, at 0.78μM and 25μM a slight significant difference observed between TTC5 silenced cells and control (comparing lane 9 to 7 and lane 25 to 23 respectively).

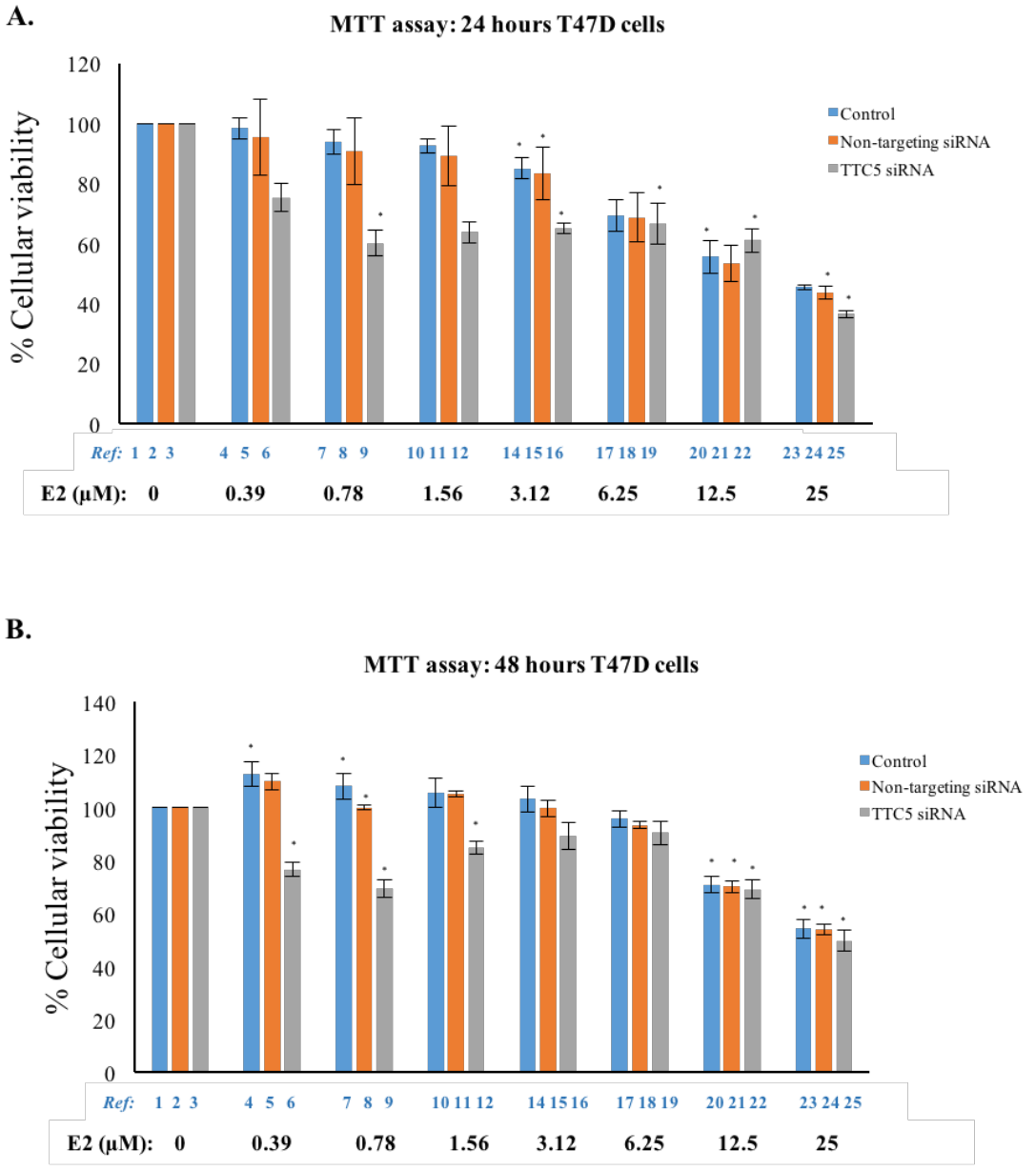


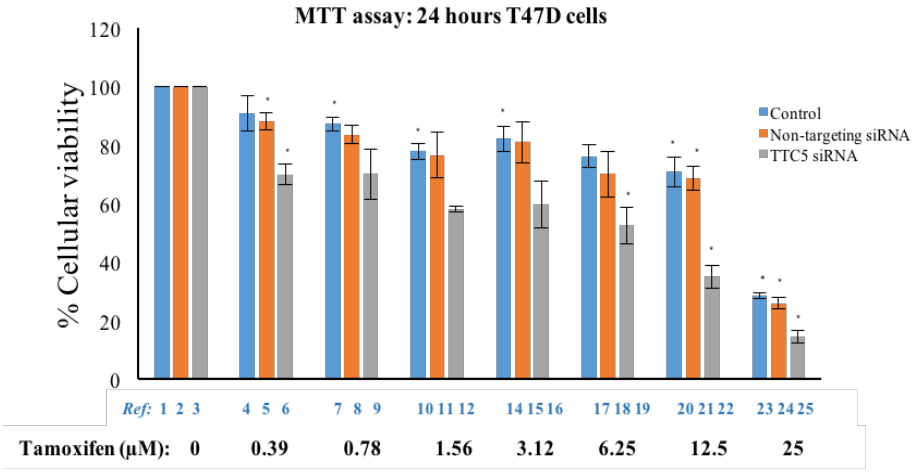
Figure 3.20.Effects of TTC5 silencing on T47D cells under various concentrations of E2.
T47D cells were either left transfected with no siRNA (blue bar), or non-targeting siRNA (orange bar) and TTC5 siRNA (grey bar), using DF1 for 24 and 48 hours. Cells either received no treatment (0) or were treated with increasing concentrations of E2 (0.39µM-25µM), and cell viability was analysed via MTT assays. A. T47D cells were treated with E2 for 24 hours. B. T47D cells were treated with E2 for 48 hours. The value obtained from untreated samples of each condition were taken as 100 (lane1,2,3) and all other activities are shown relative to this. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *).

Following on from the findings, on the effect of TTC5 knockdown on MCF-7 cells viability in presence of E2, tamoxifen and raloxifene, it was important to evaluate these findings in another ERα positive cell line. Therefore, MTT assay was performed, with exact same condition on T47D cells, transfected with non-targeting, and TTC5 knockdown siRNA.

Figure 3.20 represents the result from at least 3 independent MTT experiments that were combined, and a bar chart was obtained. As shown in Figure 3.20A, although E2 didn't seem to exert an effect on T47D cells at lower doses, it didn't cause a noticeable decrease in cellular viability either (0.39μM to 3.12μM lanes 4,7,10 and 14). TTC5 knockdown resulted in inhibition of cell growth when treated with E2 compared to control group at 0.39μM,0.78μM and 1.56μM (comparing lane 6 to 4, 9 to 7 and 12 to 10) these data were not significant. All three different conditions seemed to be statically significant when treated with 3.12μM, and it is clear that TTC5 knockdown results in further increase in cell death (comparing lane 16 to 14).

Interestingly E2 treatment resulted in increased proliferation of T47D cells after 48 hours, from 0.39μM to 6.25 μM (Figure 3.20B comparing lanes 17, 14, 10, 7, 4 to lane 1). The most important observation to note by comparing Figure 3.20B to A, is that E2 at lower doses inhibits cellular growth in TTC5 knockdown cells (Figure 3.20b 0.39μM and 0.78μM, Figure 3.20A 0.39μM, 0.78μM, comparing lane 6 to 4 and 9 to 7 in both figures). However, at higher doses the difference between the control group and TTC5 silenced cells were small (Figure 3.20B, 6.25μM, 12.5μM and 25μM comparing grey bars to blue bars).

A.



B.

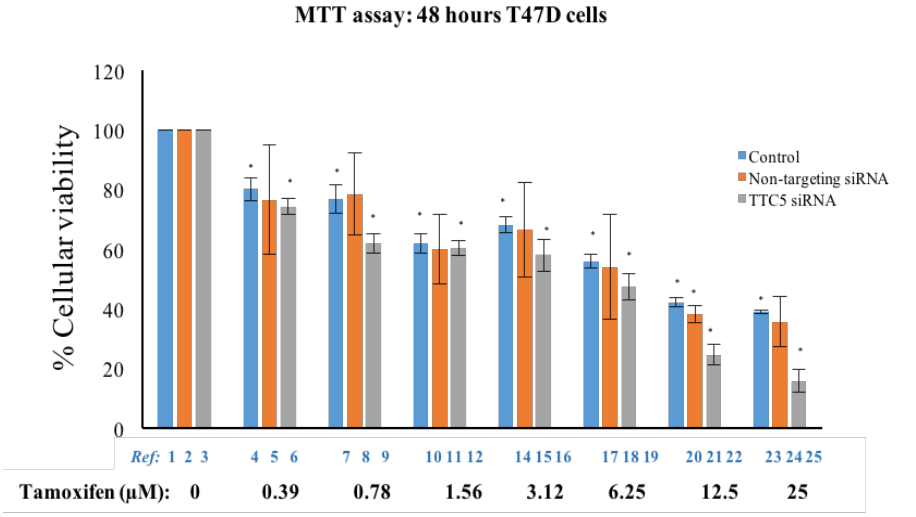


Figure 3.21.Effects of TTC5 silencing on T47D cells under various concentrations of tamoxifen.
T47D cells were either left transfected with no siRNA (blue bar), or non-targeting siRNA (orange bar) and TTC5 siRNA (grey bar), using DF1 for 24 and 48 hours. Cells either received no treatment (0) or were treated with increasing concentrations of tamoxifen (0.39µM-25µM), and cell viability was analysed via MTT assays. A. T47D cells were treated with E2 for 24 hours. B. T47D cells were treated with tamoxifen for 48 hours. The value obtained from untreated samples of each condition were taken as 100 (lane1,2,3) and all other activities are shown relative to this. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *).

T47D cells were transfected with non-targeting siRNA and TTC5 siRNA using DF1, and treated with tamoxifen over a wide range of concentrations (0.39 to 25μM). MTT assay result presented as bar chats Figure 3.21A and B.

Significant antiproliferative effect of tamoxifen can be seen on T47D cells after 24 hours Figure 3.21A, and that TTC5 knockdown further increases this antiprliferative effect (comparing grey bars to blue bars). This effect was shown to be statically significant at 12.5μM and 25μM were the difference between control bar and TTC5 knockdown cells was noticeable (Figure 3.21A comparing lane 22 to 20). Compare to 48 hours treatment (Figure 3.21B), increase in cell death due to TTC5 knockdown, was more dramatic during 24 hours of treatment and especially significant at higher doses of tamoxifen treatment 12.5-25μM Figure 3.21A.

After 48 hours tamoxifen exhibited a complete inhibitory effect on cell proliferation ($P<0.05$) of T47D cells. As shown in Figure 3.21B after 48 hours compared with control, TTC5 knockdown cells displayed a small but significant increase in cell death at lower concertation of tamoxifen treatments (0.39-6.25μM). Furthermore, when cells treated with higher concentrations of tamoxifen (12.5-25μM) knockdown of TTC5 caused significant increase in cell death compared to control ($p<0.05$) (Figure 3.21B)

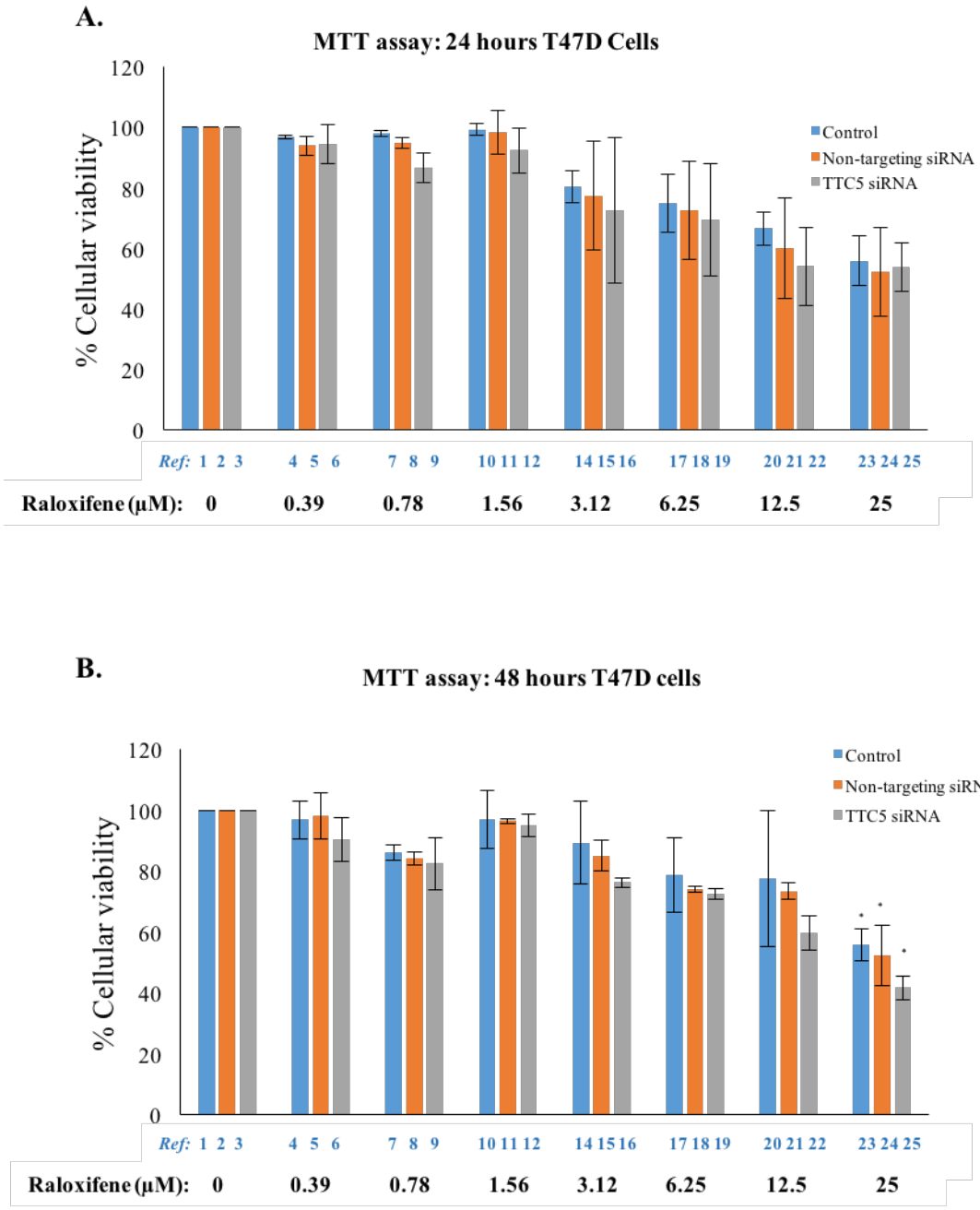


Figure 3.22. Effects of TTC5 silencing on T47D cells under various concentrations of raloxifene.
T47D cells were either left transfected with no siRNA (blue bar), or non-targeting siRNA (orange bar) and TTC5 siRNA (grey bar), using DF1 for 24 and 48 hours. Cells either received no treatment (0) or were treated with increasing concentrations of raloxifene (0.39µM-25µM), and cell viability was analysed via MTT assays. **A.** T47D cells were treated with raloxifene for 24 hours. **B.** T47D cells were treated with raloxifene for 48 hours. The value obtained from untreated samples of each condition were taken as 100 (lane1,2,3) and all other activities are shown relative to this. Error bars present standard errors of the mean from three independent experiments (± SEM) (P-value < 0.05 is indicated by *).

Lastly, the raloxifene effect on T47D knockdown and normal cells were analysed as shown in Figure 3.22A and 3.22B. Raloxifene didn't seem to have any major significant or noticeable effect on T47D cells viability when treated with various concentration of raloxifene after 24. After 48 hours compared with control, raloxifene at its highest concentration (25μM) resulted in a slight but significant decrease in cell viability of T47D cells (comparing lane 25 to 23).

Here is an overall comparison summary of effect of each hormone on MCF-7 and T47D cells:

- E2: TTC5 silencing during the first 24 hours didn't seem to affect the proliferation of MCF-7 cells up until the highest concentration (25μM) however, reduction in cell viability of MCF-7 became more apparent after longer exposure to E2 treatment (Figure 3.17A). TTC5 knockdown in T47D cells exhibited a reduction in cellular viability especially at lower doses of E2 treatment after 24 hours, while it didn't affect the cellular viability at higher doses, this effect persisted after the 48 hours' time point (Figure 3.20A).

- Tamoxifen: while a dose dependent decrease in cell viability was observed in control group in both cell lines after tamoxifen treatment. TTC5 silencing appeared to increase effect of tamoxifen on MCF-7 at higher doses after 48 hours while in T47D cells a noticeable reduction in viability of knockdown cells was observed at time point of 24 hour (Figure 3.18 and Figure 3.21).

- Raloxifene: TTC5 knockdown didn't seem to have any major significant effect on cellular viability of MCF-7 and T47D cells, at 24-hour time point. Slight significant decrease of cellular viability was observed inT47D cells at the highest dose (Figure 3.19 and Figure 3.22).

3.6 TTC5 effect on mRNA levels of cyclin D1

Several methods such as chromatin immunoprecipitation (CHIP) assay and microarray analysis identified several groups of genes that are either positively or negatively regulated by ER. However, the role played by TTC5 on ER regulatory mechanism on those genes is yet to be identified. One of the direct transcriptional target genes of ER is cyclin D1. TTC5 was shown to influence mRNA levels of a number of GR target genes (GILZ, p57 and MCJ) in a study by Davies et al., in 2011. Hence, in this section we aim to study the role of knockdown and overexpression of TTC5 on cyclin D1 mRNA levels and also the effect of, TPR and LXXLL motifs on cyclin D1 gene expression, by qRT-PCR technique. Cyclin D1 is involved in cell cycle progression, and that in 10-15% of invasive breast carcinomas cyclin D1 amplification has been observed.

In the first series of experiments, MCF-7 and T47D cells were transfected with pcDNA3 only (control bars) or 400ng of WTTTC5, or TTC5 containing mutations in TPR or LXXLL motifs and then cells either treated with 100nM E2 or received no treatment for 24 hours. RNA was extracted from the cell lysates and converted to cDNA. control bar represents the endogenous level of cyclin D1 in absence and presence of hormone, rest of the value obtained were normalised to endogenous levels of cyclin D1 in MCF-7 cells in absence of hormone. The idea behind the analysis is to compare the mutant effect to both control group and WTTTC5 group to make an overall conclusion.

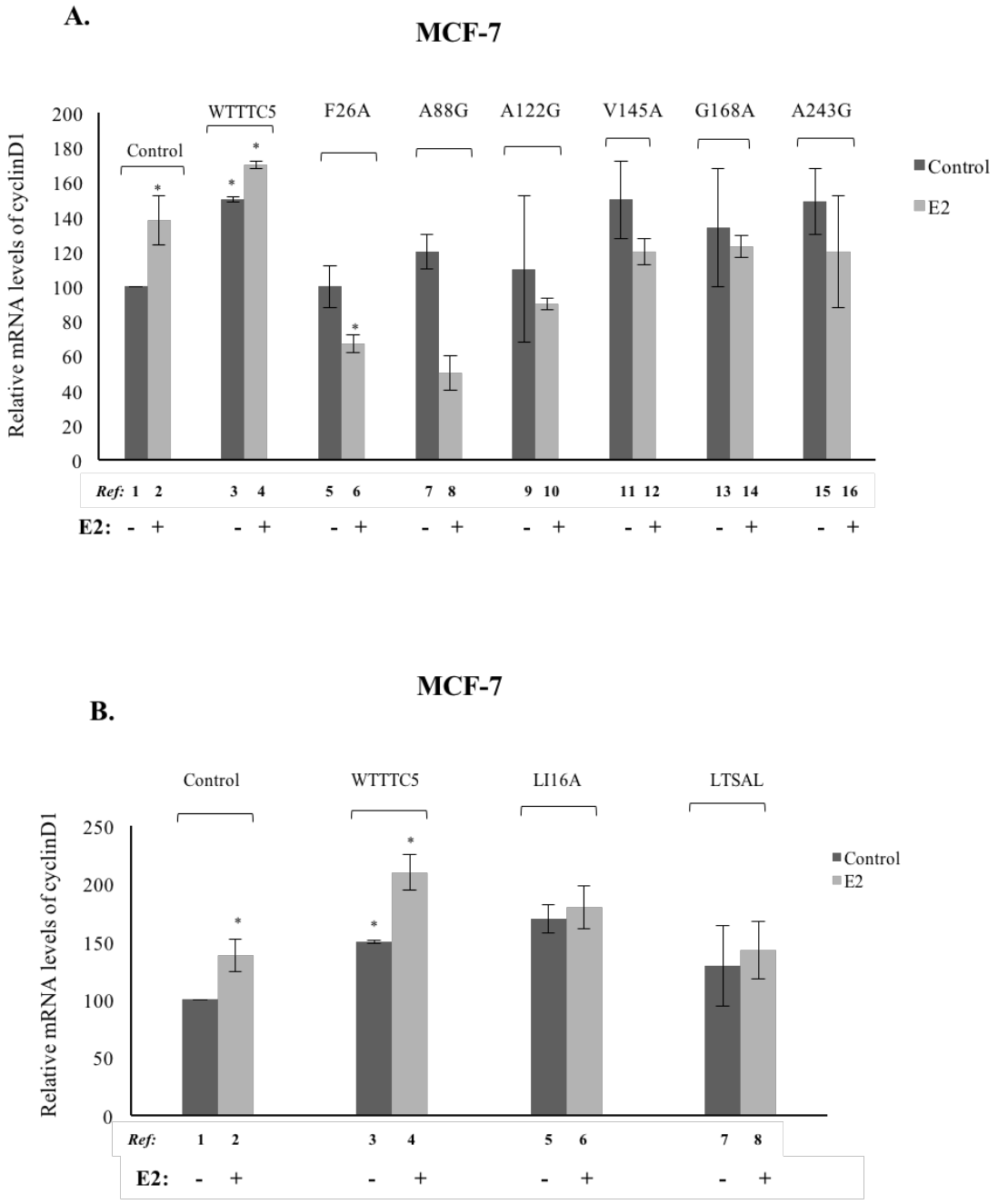


Figure 3.23.Effect of WTTTC5, TPR and LXXLL motifs on cyclin D1 expression in MCF-7 cells.

A. Graph summarizing the effect of TTC5 TPR motifs on mRNA levels of cyclinD1 in MCF-7 cells that were transfected with 400ng of WTTTC5, F26A, A88G, A122G, V145A, G186A and A243G. MCF-7 cells were either left untreated or treated with 100nM of E2. B. diagram summarizing the effect of TTC5 LXXLL motifs on mRNA levels of cyclinD1 in MCF-7 cells that were transfected with WTTTC5, LI16A and LTSAL. Cells were either left untreated or treated with 100nM of E2. The value obtained from untreated samples (pcDNA3) were taken as 100 (lane1) and all other activities are shown relative to this. The average reading from 2 independent experiment in duplicate (± SEM) are presented. (Statistical analysis was performed using t-test *P < 0.05 significant).

Results presented in Figure 3.23A, that E2 increases the mRNA levels of cyclin D1 in MCF-7 cells indicates the positive control worked perfectly (comparing lane 2 to lane1). Next, WTTTC5 overexpression in MCF-7 results in increase in mRNA levels of cyclin D1 both in absence and presence of hormone (comparing lane 3 and 4 to lane 1).

In the absence of E2 the F26A mutant didn't have a noticeable effect on cyclin D1 mRNA levels compare to control (comparing lane 5 to lane 1). In presence of hormone this mutant significantly decreased mRNA levels of cyclin D1 compare to control (comparing lane 6 to lane 1). This means that the normal function of the DNA at F26A leads to increase in gene expression of cyclin D1. As shown in Figure 3.23A, in absence of hormone the TTC5 derivatives carrying TPR motifs; A88G (lane 9) and A122G (lane 11) resulted in increase in cyclin D1 gene expression in comparison with control (lane 1). While in presence of hormone A88G mutant resulted in noticeable decrease in cyclin D1 gene expression (comparing lane 8 to lane 2), A122G also resulted in decrease in mRNA levels of cyclin D1 but in lesser degree than A885 to control (comparing lane 10 to lane 2). There was noticeable increase in cyclin D1 mRNA levels with V145, G186A, and A243G mutants in absence of hormone compared to control (comparing lanes 11, 13 and 15 to 1). It appears hormone treatment had opposite effect and repressed cyclin D1 gene expression compare to control (comparing lane 12, 14 and 16 to lane 2).

Next, effects of mutations in potential LXXLL motifs in TTC5 were tested. As shown in Figure 3.23B the LI16A mutant caused an increase in cyclin D1 mRNA expression both in absence and presence of hormone compare to control (comparing lane 5 to 1 and lane 6 to lane 2). The LTSAL mutant also resulted in an increase in mRNA levels of cyclin D1 compare to control, however to a lesser degree than LI16A (comparing lane 7 to lane 1 and lane 8 to lane 2).

In general, the effect that was caused on cyclin D1 mRNA levels by TTC5 mutants wasn't above the value obtained for WTTTC5. Taking away what was significant these sets of data his indicates that overexpressing WTTTC5 in MCF-7 cells significantly increases in expression of cyclin D1.

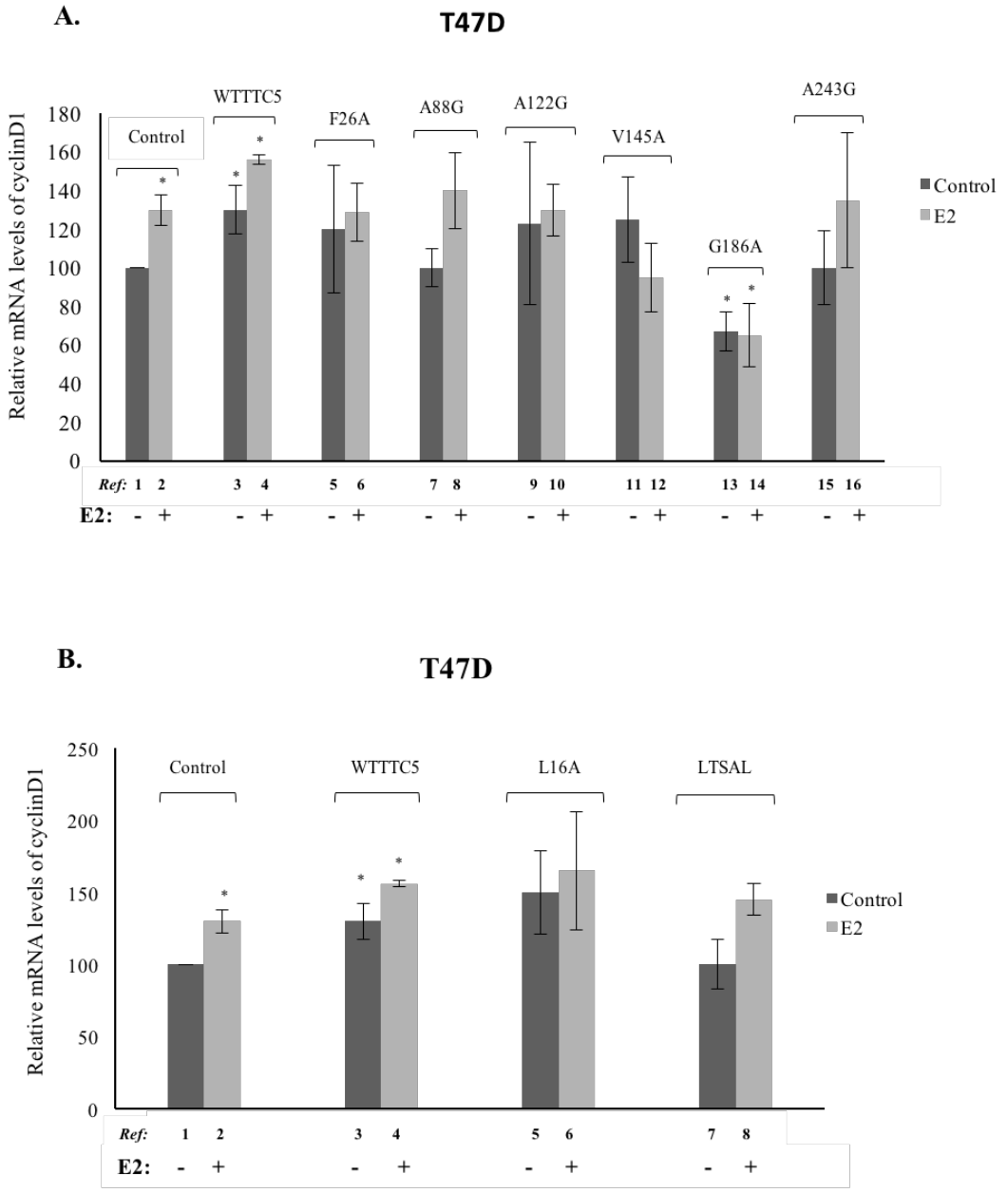


Figure 3.24.Effect TPR and LXXLL motifs on cyclin D1 expression in T47D cells.

A. Graph summarizing the effect of TTC5 TPR motifs on mRNA levels of cyclinD1 in T47D cells that were transfected with pcDNA3, WTTTC5, F26A, A88G, A122G, V145A, G186A and A243G. T47D Cells were either left untreated or treated with 100nM of E2 . B. diagram summarizing the effect of TTC5 LXXLL motifs on mRNA levels of cyclinD1 in T47D cells that were transfected with pcDNA3, WTTTC5, LI16A and LTSAL. Cells were either left untreated (blue bar) or treated with 100nM of E2 (grey bar). The value obtained from untreated samples (pcDNA3) were taken as 100 (lane1) and all other activities are shown relative to this. The average reading from 2 independent experiment (± SEM) are presented (Statistical analysis was performed using t-test *P < 0.05 significant)

In order to shed some more light on the role of WTTTC5 and its surfaces on cyclin D1 expression, qRT-PCR were performed on T47D cells. As shown in Figure 3.24A and Figure 3.24B, WTTTC5 over expression lead to slight significant increase in cyclin D1 expression compared to control (lane 3 to lane1). Hormone treatment caused further activation of cyclin D1 gene expression in T47D cells containing overexpressed WTTC5 (comparing lane 4 to lane 2 in Figure 3.24).

Figure 3.24, shows that F26A mutant results in a slight increase in cyclin D1 gene expression both in absence and presence of hormone compared to control bar (comparing lane 5 and 6 to lane1). Both in absence and presence of hormone A88G didn't seem to have any noticeable effect on cyclin D1 mRNA levels (comparing lane 7 to lane 1 and lane 8 to lane 2). According to Figure 3.24A, A122G and V145A motifs both increased mRNA levels of cyclin D1 in absence of hormone (comparing lane 9 and 11 to lane 1), however in presence of hormone these two mutants decreased cyclin D1 mRNA expression compare to control (comparing lanes 10 and 12 to lane 2). G186A significantly represses cyclin D1 genes expression in T47D cells both in absence and presence of hormone (comparing lane 13 to lane 1 and lane 14 to lane2). A2435 mutant appeared to increase cyclin D1 mRNA level both in absence and presence of hormone in comparison with control (comparing lane 15 to lane 1 and lane 16 to lane 2).

As shown in Figure 3.24B, the two LXXLL motifs seemed to exert different effect on cyclinD1 expression in T47D cells. LI16A mutant seemed to increase cyclin D1 expression both in absences and presence of hormone (comparing lane 5 to lane 3and lane 6 to lane 4). The second LXXLL motif LTSAL didn't have any significant Effet on cyclin D1 gene expression (comparing lane 7 to lane 1), hormone treatment caused and slight increase in cyclin D1 mRNA levels compare to control (comparing lane 8 to lane2).

In comparison with WTTTC5, the 6 TPR mutants and the two LXXLL motif generally repressed cyclin D1 activity in T47D cells.

3.7 Effect of TTC5 knockdown on mRNA levels of cyclin D1

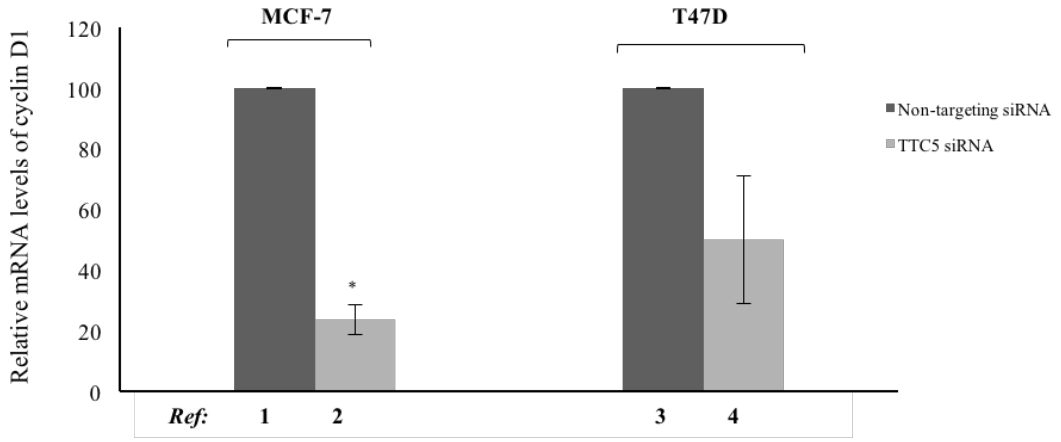


Figure 3.25.TTC5 knockdown effect on cyclin D1 mRNA levels.
MCF-7 and T47D cells were either transfected with non-targeting siRNA (dark grey) and TTC5 siRNA (light grey), using DF1 for 24 hours. RNA was isolated form cells and qPCR with primers specific to cyclin D1 and Rpl19 was performed. The average reading of form 2 independent experiment in duplicate (± SEM) are presented. Values obtained from non-targeting samples were taken as 100 and all other activities are shown relative to this. Statistical analysis was performed using t-test (*P < 0.05 significant).

MCF-7 and T47D were transfected with non-targeting siRNA and TTC5 siRNA using DF1 transfection reagent. After confirming the successful knockdown of TTC5, qRT-PCR was performed with primers specific to cyclin D1 and Rpl19.

As shown in Figure 3.25 when TTC5 is partially knockdown (~89% in this prior to performing qRT-PCR) in MCF-7, cyclin D1 expression noticeably decreased in a significant manner (comparing lane 2 to lane 1). This data correlates with overexpression data presented in Figure 3.23 that shows overexpressing WTTTC5 results in an increase in cyclin D1 expression. Next, we carried on the same experiment on T47D cells, TTC5 knockdown in T47D cells resulted in almost 50 percent reduction in cyclin D1 mRNA levels (comparing lane 4 to lane 3) however this didn't seem to be statistically significant.

3.8 TTC5 gene expression effect on breast cancer patients overall survival

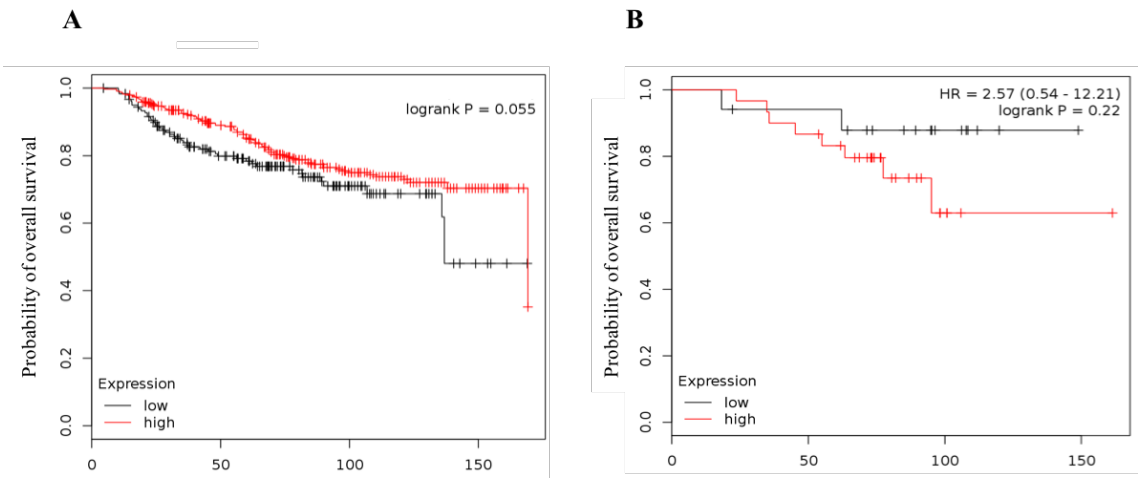


Figure 3.26.Kaplan-Meier survival plots.

A. In patients with breast cancer who had low or high expression of TTC5. B. Overall survival in ERα breast cancer patients who had low or high levels of TTC5.

To evaluate whether there is any correlation between our data and the data obtained from breast cancer patients in clinics, Kaplan Meier survival curve was plotted which shows the probability of survival against time. Figure 3.26A represents data obtained from 523 patients, and Kaplan Meier indicated patients with higher levels of TTC5 gene expression had better survival chance to begin with however at longer period of time the line drops which indicates over longer period of time patients with lower levels of TTC5 gene expression have higher chances of survival. Because the focus of this study was ERα breast cancer cells, Kaplan Meier analysis were carried out to evaluate the effect of TTC5 gene expression on ERα breast cancer patients. Figure 3.26B indicates that although there was a dramatic drop in number of patients in this category, it seems in ERα breast cancer patients lower levels of TTC5 gene expression leads to higher chances of survival.

Chapter 4 Discussion

The initiation and progression of breast cancer is tightly linked to estrogen activity. ERα regulates genes that are known to promote breast cancer cell proliferation and survival, hence ERα is known as the main mediator of estrogen function (Foster et al., 2001; Frasor, Jeanne M. Danes, et al., 2003) Significant improvement in disease free and overall survival in women with ERα-positive breast cancer have been achieved through endocrine therapies aiming at inhibiting ERα action. However, a considerable number of patients do not respond to these therapies, or they develop resistance after initial response to these agents (Ali and R. Charles Coombes, 2002; Ring and Dowsett, 2004). Therefore, there is an urgent need to identify novel cofactors, that can modulate ERα activity; by either repressing or activating its regulation and as a result control estrogens activity.

Previous published data have indicated TTC5 as an important regulator of GR activity (Davies et al., 2011), and is implicated in the DNA damage and p53 function, heat shock, inhibition of AP-1 pathway, and ATP pathway (Adams et al., 2008; Xiong et al., 2013; Maniam et al., 2015a). However, the function of TTC5, TPR and LXXLL domains and motifs in ER activity are poorly defined. Here we investigate the task performed by these TTC5 surfaces on ER activity. Formerly in the introduction chapter, TTC5 was shown to comprise of six TPR and two LXXLL motifs (Davies et al., 2011). TTC5 seems to have dual regulatory function where it both represses and activates GR mediated gene regulation. It is hypothesized that this function may be similar in controlling ER dependent pathways, as GR and ER are from the same family of nuclear hormone receptors.

The primary aim of this study was to shed some light on the nature of TTC5 and ER interaction and the impact of such interactions on ER transcriptional activity and gene regulation. In order to develop or modify treatments for life threating diseases such as breast cancer, the result of such study is vital, as it will contribute to better understanding

of ER mode of actions and the different intracellular pathways that this receptor is involved in.

The TTC5 protein levels were initially investigated in breast cancer cell lines MCF-7, and T47D (Figure 3.1 and Figure 3.3) treated with ER agonist (E2) and antagonists (tamoxifen and raloxifene). As shown in Appendix Figure 5.3 TTC5 protein levels were detected in MDA-MB-468 cells in both absence and presence of hormone; these cells are ER negative this means TTC5 not only in ER positive cells but also ER negative cells is expressed at protein levels.

Then to study the TTC5 gene expression at the transcriptional level the TTC5 mRNA levels were followed in these breast cancer cell lines under the same experimental conditions using qRT-PCR. The obtained data from these experiments suggested that E2 effects TTC5 protein and mRNA levels in a cell specific manner, as a down regulation occurred in levels of TTC5 in MCF-7 cells after hormone treatment while an upregulation was observed in T47D cells. Given that these cell lines do not harbour mutations in ERα these findings suggest that other genetic alterations may be the result of TTC5 cell specific response to E2 hormone. ER agonist tamoxifen resulted in an increase in TTC5 protein and mRNA levels in both cell lines (T47D and MCF-7) while raloxifene didn't have a significant effect on T47D cells and elicited a slight repression on TTC5 mRNA and protein levels in MCF-7 cells. Since we identified two putative ERE on TTC5 gene (Figure 3.5) one hypothesis is that through this binding ER either represses or activates TTC5 activity by recruiting other transcription factors to the binding site. A similar effect has been reported by other cofactors of ER, for example the family of p160 coactivators play gene specific roles in regulating mRNA and protein expression hence their contribution to breast tumorigenesis is unique (Karmakar, Foster and Smith, 2009). As shown in the Appendix Figure 5.3) apart from ERα, there were multiple putative binding sites of ER cofactors known to interact with ER on TTC5 promoter from the family of FOX (Forkhead box) proteins on TTC5 promoter; such as FOXO1, FOXO3 and FOX04. There is a large body of evidence highlighting the interaction between FOXO3 and ER, which as a result inhibits the E2 dependent, ER regulated transcriptional activities (Zou et al., 2008). The dual activity of TTC5 can be explained by different recruitment of these

factors; as upon hormone treatment different coregulatory or co repressory molecules could be recruited to ER TTC5 binding site.

Moreover, taking into account statistically significant results; with reference to the dual effect of E2 on TTC5 expression it is possible that that p53 plays a part in this process since MCF-7 express wild type p53 while T47D cells express mutated p53, however other factors may play a role as well.

After these observations, we became interested to investigate whether TTC5 interacts with ERα in MCF-7 and T47D cells and if so, how does hormone treatment affect this interaction. As shown in Figure 3.6 and 3.7 the two proteins interact endogenously in both cell lines, however the effect of hormones on this interaction wasn't clear. In absence of hormone TTC5 strongly interacted with ERα, as higher levels of TTC5 were detected in the complex. It is well stablished that ER remains in an inactive state in absence of hormone, while after binding to E2, ER dissociates from heat shock protein Hsp90, is phosphorylated and then translocates into nucleus. It has been shown by previous lab members that TTC5 is both nuclear and cytoplasmic, but upon glucocorticoid hormone treatment TTC5 translocated into the nucleus (Sadeq, 2013) from cytoplasm. TTC5 may or may not be associated with ER and the heat shock chaperone complex while in cytoplasm, but the fact that TTC5 is a TPR protein makes it a candidate for binding to heat shock proteins hence to ER. However, since hsp90 only encloses one C-terminal TPR binding site makes the binding to the heat shock proteins competitive (Lee et al., 2011). Previous data has shown that TTC5 forms a complex with GR, our data suggests that TTC5 interacts with ER in *vitro,* hence TTC5 could be involved in the regulation of the superfamily of nuclear hormone receptors and a wide range of other transcription factors (Demonacos et al., 2001, Davies et al., 2011)

Since TTC5 endogenously interacts with ERα in *vitro*, we thought of exploring whether overexpressed TTC5 has any effect on ER's transcriptional activity. TTC5 is composed of 6 TPR motifs, throughout its structure and also contains two potential nuclear receptor interaction motifs, LXXLL. Hence it is very likely that TTC5 binds to ER through one to

more of protein-protein interaction motifs and influences ER's activity through this binding.

The relative luciferase levels confirmed that WTTTC5 is capable of inducing endogenous ER transcriptional activities in MCF-7 and T47D cells in absence and presence of hormone, on ERE promoter using an optimal concentration of 400ng of DNA (Figure 3.8 and Figure 3.9)

Overexpression of WTTTC5 in MCF-7 cells increased ERE promoter luciferase activity, and hormone treatment caused further increase, implying that WTTTC5 binds to ER to facilitate ER mediated transcription. Since E2 treatment led to a clear increase in ER/TTC5 interaction it is possible that, hormone treatment increases levels of TTC5 thus leading to increase in ER mediated transcription. Furthermore, it is also possible that different TTC5 surfaces are employed for hormone independent versus hormone dependent interaction (Figure 3.11A. Nevertheless, WTTTC5 over expression in T47D cells on the same promoter caused a slight but significant repression in ER transcriptional activity in the absence of hormone, whereas hormone treatment increased ER transcriptional activity on ERE promoter in these cells, although not above the values obtained in the absence of TTC5 (Figure 3.13). Since the reporter gene used for the luciferase assay was the same in both cell lines, these results provided further support to the notion to the idea that TTC5 effects ER in a cell specific manner. Moreover, this dual action of TTC5 as coactivator/corepressor has been reported by previous studies on GR, where TTC5 regulated GR transcriptional activity both in positive and negative manner (Davies et al., 2011).

In previous studies, it has been shown that TTC5 stabilizes p53 by reducing Mdm2 mediated degradation, on the other hand transcription of ER has been shown to increase in presence of wild type p53 (Shirley et al., 2009). So, our hypothesis is that in cells expressing wildtype p53 (MCF-7) over expression of WTTTC5 leads to activating p53 hence activating ER transcription, while in T47D cells this effect is reversed as they express a mutated version of p53.

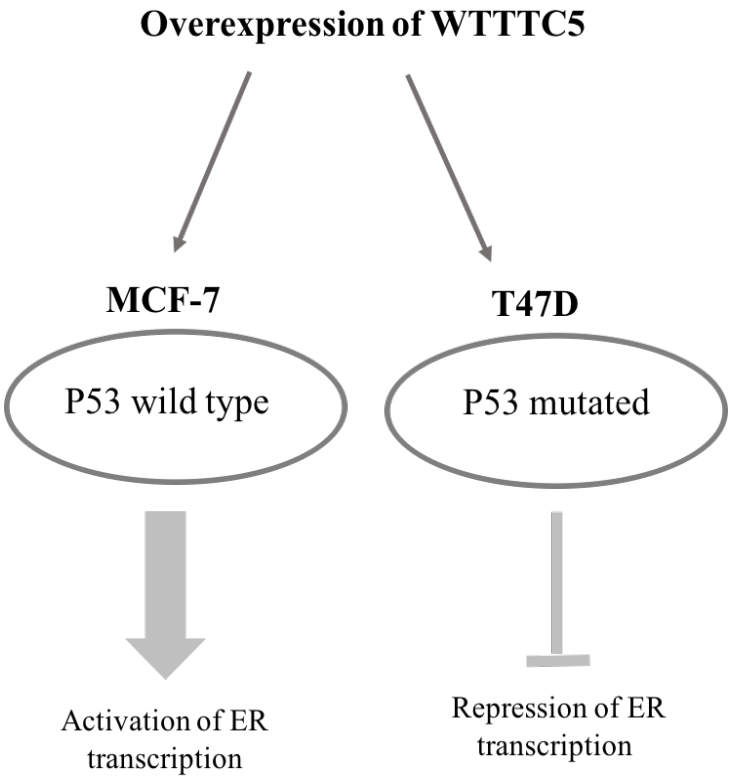


Figure 4.1. Model illustrating the effect of overexpressed WTTTC5 on ER transcriptional activity.

With the purpose of identifying the role of each of the six TPR and putative LXXLL motifs on TTC5 in regulating ER function, each TPR and LXXLL motifs was subjected to one amino acid substitution and that resulted in mutated TPR and LXXLL motifs (apart from the LTSAL motif that was made by previous lab members). The TTC5 mutants were introduced into MCF-7 and T47D cells to assay effect of each mutant on ER transcriptional activity on ERE promoter using luciferase assay. In general, the TTC5 TPR mutants (F26A, A88G, A122G, V145, G186A and A243G) were promoting an induction in ER transcriptional activities on ERE promoter, (Figure 3.11 andFigure 3.13). One interesting observation by taking into account what was significant by carrying the statistical analysis, is the fact that G186A mutant which was the strongest coactivator in MCF-7 cells appeared to be the strongest corepressor in T47D cells (Figure 3.11, Figure 3.13). Davies *et al*., in 2011 reported G186A as one of the major motif that is involved in TTC5 interaction with GR by means of co-IP. Somewhat surprisingly, Serine 221 residue at the Chk2 site (which is involved in TTC5 response during DNA damage) is believed to be between TPR5 (G186A) and TPR6 (A243G) and phosphorylation of these residues

results in dissociation of the hydrogen bonding of TPR5 and 6, which possibly results in protein interaction by widening out the TPR channel (Adams et al., 2012). It is possible that this residue may be the main surface involved in ER TTC5 complex which brings different coregulatory proteins to the complex and effect either represses or activates ER transcriptional activity in ERα positive cells.

These observations would lead to the realization of that TPR motifs were playing the role of possible repressor or coactivator boxes and were either downregulating or upregulating the ER transcriptional activities on ERE promoter depending on cell type. In fact, there is a segregation between the TPRs, that breaks them up into two groups, TPRs1 (F26A), 4 (V145A) and 5(G186A) that were located on the N terminus (Adams et al., 2012) and TPRs 2 (A88G), 3 (A122G) and 6 (A243G) that were on C terminus (Adams et al., 2012). Whether the difference in ER activation levels was correlated to the location of the TPR to the N or C terminus needs more investigation, however previous studies have indicated TTC5 stress dependent regulation of the GR depends on both the TTC5 N and C terminal (Davies et al., 2011).

Another possibility is that individual TPR motifs do induce an induction in ER transcriptional activity on the ERE promoter, that might act a group of motifs to effect transcription. For instance, according to Figure 3.11A, all 6 TPR's seemed to act as strong coactivators hence the overall effect of WTTTC5 on MCF-7 in absence and presence of hormone was to coactivate ER transcription. While in T47D cells 4 TPR's (A88G, V145A, G186A and A243G) out of 6 were strongly acting as corepressors, hence the overall activity of WTTTC5 was to repress ER's transcriptional activity.

Next, each LXXLL motifs were subjected to one amino acid mutation to identify the role played by them on ER transcriptional activity. It has been shown by previous researchers that many proteins with LXXLL motifs exert vital effects on nuclear hormone receptors, and that mutation in these residues may lead to deregulation of this pathway. For example, a mutated LXXLL motif of mediator subunit 1 protein (MED1) in knockout mice resulted in down regulation of ERα target genes (Jiang et al., 2010). The TTC5 mutant LTSAL was promoting repression in both T47D and MCF-7 cell line, however LI16A seemed to

be a strong activator of ER activity in MCF-7 cells while a repressor in T47D cells (Figure 3.12A). The various degrees of repression or coactivation between the different TTC5 LXXLL motifs in ER transcriptional activities could be explained by the fact that certain coregulatory proteins can both activate and repress the same transcription factor. Studies have shown that the histone methyltransferase G9a has a dual and selective role as a coregulatory factor for ERα target genes (PurCell et al., 2011). Another example, is the receptor interacting protein (RIP140) that on different nuclear receptors and different response elements acts as both coactivator and corepressor (Subramaniam et al., 1999).

The luciferase reporter assay provided further endorsement to the view that overexpressed TTC5 affects transcriptional activity of ER in MCF-7 and T47D cells. Next, we evaluated the effect of TTC5 knockdown on cell proliferation of MCF-7 and T47D cells with various concentration of ER agonist (E2) and antagonist (tamoxifen and raloxifene). For this purpose, TTC5 gene was silenced by the introduction of targeted TTC5 RNAi (siRNA) and non-targeting siRNA as control. Generally, the effect of TTC5 knockdown on both cell lines resulted in an decreased cell viability compared to control, in order to make sure this effect wasn't due to the DF1 (the transfection reagent) we carried out series of various titration of the DF1. The choice of assay to use in a siRNA screen of cell viability is a difficult one as different assays have certain disadvantages. The clonogenic survival assay is the 'gold standard' method for assessing intrinsic radio sensitivity. (Franken et al., 2006). This in *vitro* cell survival assay is based on the ability of a single cell to grow into a colony (which is defined as consisting of at least 50 cells). The assay essentially tests every cell in the population for its ability to undergo "unlimited" division. Unfortunately, this assay is not suitable for use in large scale siRNA screens due to the highly labour intensive nature of the assay.

Next taking into account the fact that TTC5 knockdown resulted in decreasing cell viability of breast cancer cells, we monitored cyclin D1 expression levels when TTC5 knockdown to investigate whether TTC5 silencing has any effect on cyclin D1 mRNA levels. In order to address this hypothesis qrt-PCR analysis were carried on MCF-7 and T47D cells to measure mRNA levels of cyclin D1. Interestingly, TTC5 knockdown resulted in repression of cyclin D1 mRNA levels. In order to shed some light on the role

of overexpressed WTTTC5 and also the TPR and LXXLL motifs on cyclin D1 gene expression, mRNA levels of cyclin D1 were measured using the qRT-PCR technique. These results were in agreement with knockdown data where TTC5 silencing resulted in cyclin D1 repression so the overexpression had opposite effect.

Generally, all six TPR mutants and the two LXXLL motifs seemed to repress cyclin D1 activity. However G186A activates the cyclin D1 mRNA levels in MCF-7 cells while it significantly represses cyclin D1 gene expression T47D cells. This mutant appears to be a critical motif in how TTC5 regulates ER activity as it had different regulatory effect on ER transcriptional activity in cell specific manner.

Cyclin D1 plays an important role in cell cycle progression. For a successful transition from the G1 into S phase highly expressed cyclin D1 expression is needed. Cyclin D1 was shown to be induced as a result of estrogen stimulation in MCF-7 cells both at mRNA and protein expression levels (Prall et al., 1997; Sabbah et al., 1999; Silva, Kabil and Kortenkamp, 2010). There is a regulatory pathway between p53 and cyclin D1; it is through induction of p21 that p53 induces cyclin D1 overexpression, this inductive effect of p53 on p21 is abolished by mutations within the p53 gene. Overexpression of Cyclin D1 protein in tumors with wild type p53 and ER positive status have been reported more frequently (Fouladdel et al., 2008). An interesting study by (Grillo et al., 2006)revealed that overexpression of cyclin D1 results in an increase in cell proliferation of MCF-7 cells, while when cyclin D1 expression was silenced the growth rate of cells fell below the control group. They demonstrated that hyperphosphorylation of retinoblastoma gene is associated with cyclin D1/CDK4 activity, this hyperphosphorylation results in an increase passage of the cells through the G1-S phase of the cell cycle division. Hence, they silenced the CDK4 expression with siRNA which in turn resulted in partial G1-S phase arrest.

Our data on TTC5 effects on cyclin D1 gene expression suggests that TTC5 may well be a suitable cyclin D1 inhibitor in MCF-7 and T47D breast cancer cells. It is possible that, the MTT assay results which showed that TTC5 knockdown results in a decrease of cell proliferation is due to partial inactivation of cyclin D1 in these cells. However, the mechanism which TTC5 knockdown leads to results in this repression of Cylin D1 gene

is yet to be studied. More mechanistic studies need to be performed in order to dissect the exact mechanistic pathway of how TTC5 affects cyclin D1 levels and leads to as cell cycle progression.

The data obtained by Kaplan Meier analysis, indicated that low levels of TTC5 gene results in higher survival rate, though this didn't seem to be statistically significant but it indeed correlates with data obtained by this study.

4.1 Conclusion

This thesis demonstrated that TTC5 is a novel cofactor for ER functions in breast cancer cells. In this study, we revealed that TTC5 endogenously interacts with ERα in breast cancer cells. Furthermore, we show through experimental investigations that TTC5 possibly modulates ER mediated gene regulation at various levels including, transcriptional activity and gene expression through its unique TPR motifs.

The results were indication of significance dominancy of TPR5 (G186A) motif, suited to the N-terminal of TTC5 in affecting ER transcriptional activities in different manner. We have also presented evidence to suggest that silencing TTC5 levels inhibits the growth of ERα positive cells, and that TTC5 is possibly a candidate for repressing cyclin D1 gene expression.

4.2 Future directions

This work characterised the role of TTC5 in ER regulation, but the mechanism of action is still not clear. Future work investigating the possible ways in which TTC5 could stabilize ER protein levels and coregulates its transcriptional activity would be very interesting.

- Co-IP assay to identify the different co-activating and corepressing proteins that partner with TTC5 while interacting with ER via the TPR and LXXLL motifs using specific antibodies against TTC5 and ERα.

- Clonogenic assay to examine if partial knockdown of TTC5 increases or decreases the cell viability and colony formation of breast cancer cells. Immunofluorescent microscopy to detect alteration in ER subcellular localization in cells overexpressing TPR and LXXLL mutant derivatives.

- The luciferase assay using a cyclin D1 reporter gene to test the effect of each TPR and LXXLL motifs on transcriptional activity of cyclin D1.

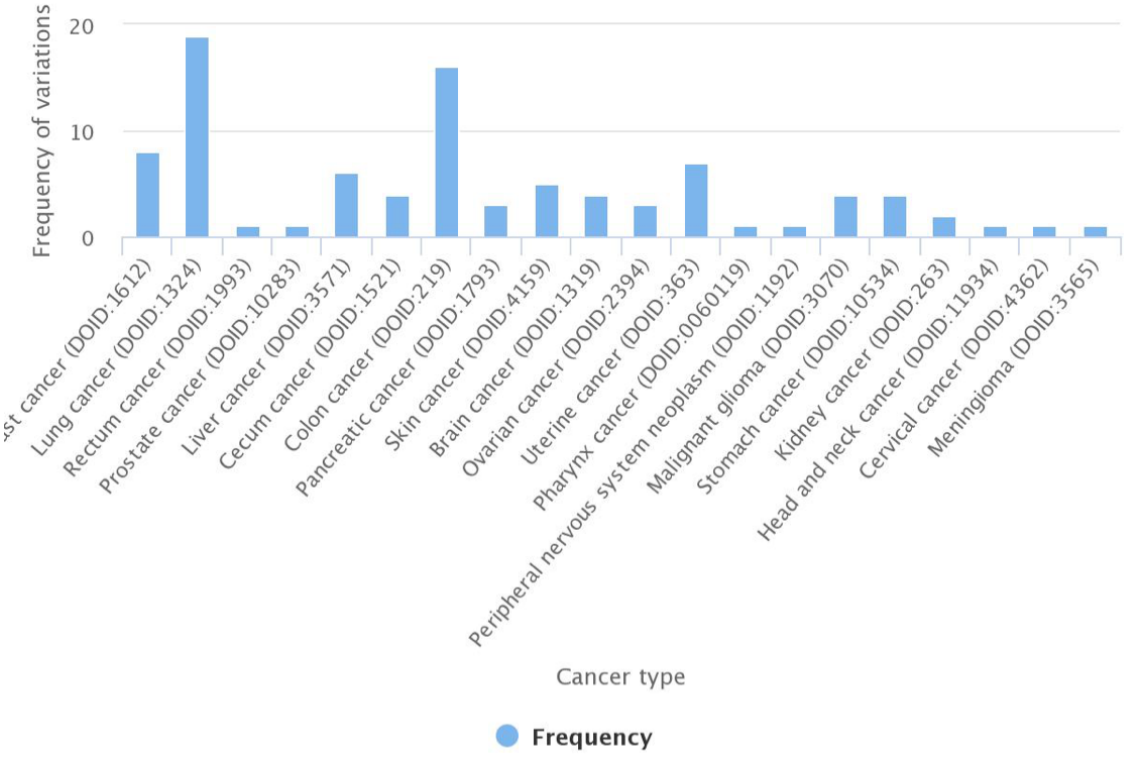
- Flow cytometry technique to investigate the effect of each TPR and LXXLL motifs on cell cycle progression.

- The chromatin immunoprecipitation assay to test ER and TTC5 occupancy on certain gene promoters in cells overexpressing TPR and LXXLL motif mutant.

Chapter 5 Appendices

5.1 Primers that were used for mutating the TPR and LXXLL motifs

TPR/LXXLL motif	Primers
F26A/F	GTAACTGTCTCGAGCACAGTAGAGCCGATCCCACCAGTCC
F26A/R	GGACTGGTGGATCGGCTCTACTGTGCTCGAGACAGTTAC
A88G/F	GAGAGAAGCACCTCGCCCTCAGGGCTATAAT
A88G/R	ATTATAGCCCTGAGGGCGAGGTGCTTCTCTC
A243G/F	GAGAAACCCTCAAGGCCCTCCCCATAACTCT
A243G/R	AGAGGTTATGGGGAGGGCCTTGAGGGTTCTC
LI16A/F	CCAGTCCCTGCGCTTTCTGCACGACGTGCTTCGC
LI16A/R	GCGAAGCACGTCTTGCAGAAAGCGCAGGGACTGG
G186A/F	GCGAAGCACGTCTTGCAGAAAGCGCAGGGACTGG
G186A/R	TCCCAGCATGCTCTGCGCCTTCTTGGGCTTTGTC

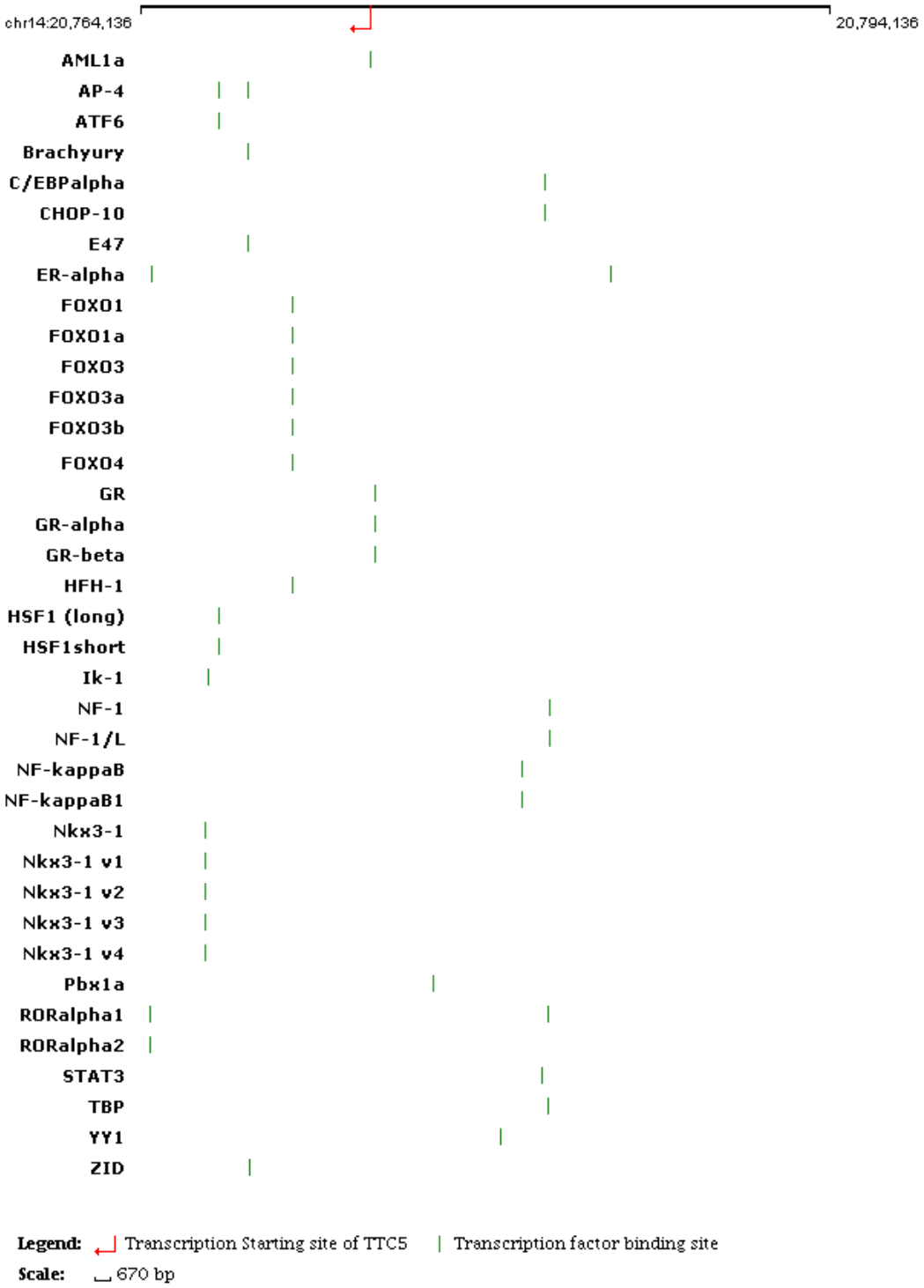


Appendix Figure 5.1.The frequency of single nucleotide variations for TTC5 among all cancer types.

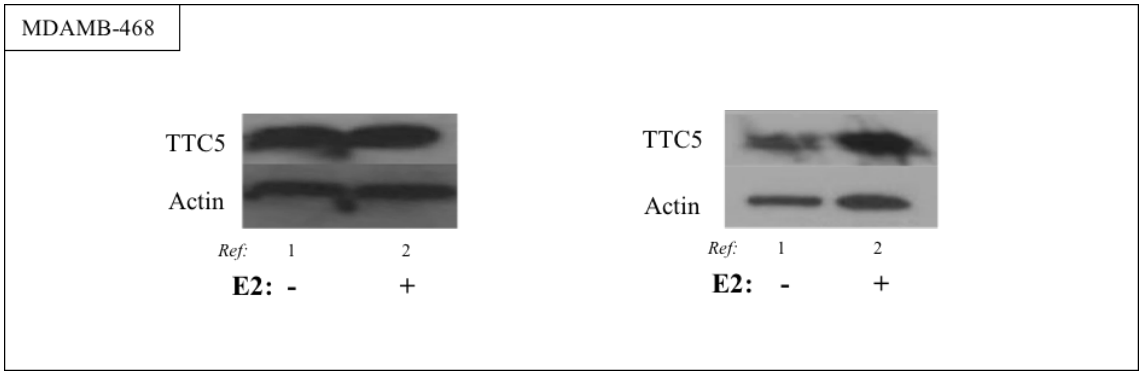
5.2 ERE reporter gene sequence

5’ 3’
CTAGAAAGTCAGGTCACAGTGACCTGAT 5’
3’ TTTCAGTCCAGTGTCACTGGACTACTAGTTA

Appendix Figure 5.2.Sequence of the ERE reporter gene



Appendix Figure 5.3.identification of putative EREs on the TTC5 promoter.



Appendix Figure 5.4. MCF-7 cells were either left untreated or treated with E2 (100nM) for 24 hours. Whole cell lysate was then probed against TTC5 and β-actin in MCF-7 cells. β-Actin was used as control protein loading.

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