THE ROLE OF TREFOIL FACTOR FAMILY-1 (TFF1) IN ESTROGEN-PROMOTED APOPTOSIS RESISTANCE IN BREAST CANCER CELLS

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ABSTRACT

TFF1 is a secreted protein and is expressed in various types of carcinomas. The *TFF1* gene contains an estrogen responsive element in its promoter, due to which its expression could be regulated by estrogen. In addition, estrogen has demonstrated its ability to promote resistance to doxorubicin in estrogen receptor positive MCF-7 breast cancer cell line. This study aims to explain the estrogen induced resistance to chemotherapy by exploring the relationship between the estrogen-induced TFF1 and its role in inferring resistance to apoptosis. Permanent knockdown of *TFF1* gene in MCF-7 cell was generated, to test the sensitivity to doxorubicin in comparison to the parental cell in the presence or absence of 17β-estradiol, which is a potent estrogenic agent. Flow cytometry analysis showed that, 17β-estradiol could rescue MCF-7 cells from doxorubicin induced apoptosis (P = 0.016) but failed to demonstrate this effect in the *TFF1* knockdown MCF-7 cells. Trypan blue cell counting showed that, neutralizing secreted TFF1 resulted in aggravated apoptosis, much higher than just the doxorubicin treated condition (P = 0.029). Moreover reconstituting TFF1 in the knockdown cells refurbished its protective role (P = 0.05). Apoptosis protein array revealed changes in expressions of key apoptosis regulatory proteins under varying treatment conditions, particularly the anti-oxidative enzymes catalase and heme oxygenase 1 (HO-1), on the whole trying to restore homeostasis. These phenomena determine the contribution of TFF1 in estrogen-promoted resistance to apoptosis induced by doxorubicin in MCF-7 breast cancer cells. TFF1 may be a target for manipulation of chemotherapeutic resistance in breast cancer.

KEY WORDS: TFF1 / BREAST CANCER / APOPTOSIS

116 pages

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LIST OF ABBREVIATIONS

Abbreviation	Term
%	Percent
°C	Degree Celsius
α	Alpha
β	Beta
µl	Microliter
µM	Micromolar
µg	Microgram
µg/ml	Microgram per milliliter
ADH	Atypical ductal hyperplasia
ALH	atypical lobular hyperplasia
Avg	Average
cm^2	Centimeter square
DFF	DNA fragmentation factor
DMEM	Dulbecco’s modified eagle medium
Doxo	Doxorubicin
E2	17β-estradiol
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERD	Estrogen receptor down regulator
ERE	Estrogen response element
FADD	Fas-Associated protein with Death Domain

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
Ful	Fulvestrant
g	Gram
HO	Hemeoxygenase
IAP	Inhibitor of apoptosis
IRF-1	Interferon regulatory factor 1
kDa	Kilodalton
mA	Milliampere
mg	Milligram
ml	Milliliter
mM	Millimolar
NPM	IRF-1 inhibitor
ng	Nanogram
nM	Nanomolar
PI	Propidium iodide
PON2	Paraoxonase-2
PBS	Phosphate buffer saline
PCD	Programmed cell death
PI	Propidium iodide
PVDF	Polyvinylidene fluoride
PR	Progesterone receptors

LIST OF ABBREVIATIONS (cont.)

Abbreviation	**Term**
rTFF1	Recombinant TFF1
SERM	Selective estrogen receptor modulator
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide hel electrophoresis
ShRNA	Short hairpin RNA
tBID	Truncated BID
TBST	Tris-buffered saline-Tween 20
TFF1-kd	*TFF1* knocked down
TNF	Tumor necrosis factor
TPA	12-O-Tetradecanoylphorbol 13-acetate
TRADD	TNF receptor type 1-associated DEATH domain
TRAIL	TNF-relate necrosis factor-related apoptosis-inducing ligand
TRAF	TNF receptor associated factor
XIAP	X-linked inhibitor of apoptosis protein
w/v	Weight per volume
v/v	Volume per volume

CHAPTER I
INTRODUCTION

1.1 Background and the research rationale

Breast cancer today, is a common malignancy and one of the leading causes of cancer deaths in women worldwide. In Thailand breast cancer is one of the most common cancer among Thai women with more than 20/100,000 new cases reported every year (1). The incidence has seen a dramatic increase in the last several decades with factors such as genetic, diet, environment and changing living styles contributing to the upsurge (1-2). Although, over the years the early detection and improved treatment regimens led to better survival, the bio-medical side effects were found paramount to their psychological and social lives (3).

Breast carcinomas are characterized by, the loss of normal cellular characteristics resulting in abnormal proliferation of breast epithelial cells. The hormone responsiveness of the breast cancer triggered many researches, in attempt to identify estrogen regulated genes that are of clinical importance and can be of potential therapeutic value. It was suspected that these genes may encode proteins that may help in the propagation of the effects of estrogen on the malignant breast cancer cells. Cathepsin D (4), urokinase type-plasminogen activator, epidermal growth factor receptor (EGFR) (5) and pS2 (6) are some of the breast cancer markers of active research. Elevated level of gene encoding pS2, which is also known as trefoil factor family-1 (TFF1) was found to be frequently detected in almost 50% of the breast cancers with expression mostly seen in estrogen receptor (ER) positive carcinomas (7-8). Teixeira *et al*., reported that estrogen promoted the resistance of the ER-positive breast cancer cells to chemotherapeutic drugs *in vivo* by a mechanism that involved the upregulation of BCL-2 prosurvival gene (9). Normal breasts are known to secrete no or very little amount of TFF1. Therefore, the increase in the serum level have been associated with ER expression and are predictive of response

to hormone manipulation as a first line therapy, with better clinical outcome and increased disease free survival (10).

Most of the researches in TFF1 has been in regard to its tumourigenic properties, hence the role of TFF1 in cancer has long been established (11). Besides its curative effect on the mucosal injury promoting epithelial cell migration, *TFF1* is considered as tumour suppressor gene in gastric cancer (12-13). The anti-proliferative effect of TFF1 is believed to be the factor responsible for the homeostasis of normal gastric epithelium, since the loss of *TFF1* in the gastric cancer is associated with increased proliferation (14). In cancers, outside the gastrointestinal tract, TFFs are usually observed in a state of over-expression (11). Many other cancers express *TFF1* mRNA in the tissues including prostrate, lung, pancreas, endometrium, ovary, bladder, biliary tract, colorectum, esophagus and skin with varying levels of peptides in the serum (15-17). The presence of an estrogen response element (ERE) and other complex enhancer regions, responsive to epidermal growth factor (EGF), tumour promoter (TPA) and the proto-oncogene, *sc-ha-ras* and *c-jun* on the promoter of *TFF1* gene has led to its increased expression in many other malignancies. TFF1 is known to promote angiogenesis (18), metastasis, migration and invasion in the breast cancer cell lines (6). TFF1 has been shown to protect gastrointestinal cells from apoptosis, induced by chemicals, anchorage free growth and pro-apoptotic protein 'Bad'. They also reported decrease in enzymatic activities of several caspase including caspase-3, -8, and -9 after TFF1 treatments (19). Recently, TFF1 has shown to protect the conjunctival epithelial cells from apoptosis, by interfering in the signaling cascade at both pre- and post- mitochondrial levels (20). Amiry *et al.*, demonstrated that TFF1 over-expression could enhance oncogenicity in the mammary carcinoma MCF7 and T47D, and reduce base line of tumour cell apoptosis in xenograft tissue (6). The use of chemotherapeutic agents is an important treatment module for cancers, with the key aim, to manipulate the apoptotic process using cytotoxic drugs. Consequently, the main aim of this study is to investigate the anti-apoptotic property of, estrogen induced TFF1 in ER-positive breast cancer cell model, against doxorubicin induced apoptosis. It is also aimed to identify key apoptotic proteins that could be involved, in the doxorubicin induced apoptosis and

resistance in ER-positive in breast cancer cell line. This will further clarify the role of TFF1 in breast cancer resistance to chemotherapy.

CHAPTER II
RESEARCH QUESTION, HYPOTHESIS AND OBJECTIVES

2.1 Research question:

Does TFF1 play a role in the estrogen mediated protection, in doxorubicin treated MCF-7 breast cancer cell?

2.2 Hypothesis:

TFF1, expressed abundantly in MCF-7 breast cancer cells under estrogen stimulation may protect these cells from apoptosis induced by chemotherapeutic drug, doxorubicin.

2.3 Objectives

2.2.1 To investigate if TFF1 could be a factor, which contributes to resistance in breast cancer chemotherapy by examining the differential ability to resist doxorubicin induced apoptosis in the *TFF1* knockdown and the *TFF1* intact MCF-7 cells.

2.2.2 To determine the major proteins that involve in TFF1-mediated doxorubicin-induced apoptosis resistance mechanism.

CHAPTER III
LITERATURE REVIEW

3.1 Breast cancer

Breast cancer is a heterogeneous disease characterized by the loss of normal cellular characteristics resulting in abnormal proliferation (21). The incidence occurs majorly in females with less than 0.7% of the total breast cancer seen in male population (22). Breast cancer is one of the leading malignancies affecting the female population worldwide, with rising prevalence in the low and middle income countries as a result of better health awareness and development in diagnostic programmes (23). There is a marked geographical difference in the breast cancer incidences, with the rates seen highest in the developed and lower in the developing countries. Therefore the cancers in the developing regions are otherwise diagnosed at a very late stage making it difficult for any clinical intervention (23). By 2020, an estimated 1.7 million women are predicted to be diagnosed with breast cancer in the developing world through enhanced awareness and screening campaigns (23).

3.1.1 Aetiology and risk factors

The variation in cancers like, the clinical presentation, incidence and mortality rate are likely due to complex interactions of non-modifiable risk factors such as, genetic susceptibility, race/ethnicity, aging and modifiable risk factors such as smoking and dietary habits (24-25). Differences in individual behaviors (26), cultural beliefs and practices, socio-economic conditions, use of oral contraceptives (27) and health care systems are some important risk factors among populations (28). The aetiology of breast cancer is multifactorial, with not just the physiology but a significantly greater part being played by the constitutional, environmental and hormonal imbalances (29). Breast cancer is a multistep process where the normal cells undergo hyperplastic transformation, progresses to premalignant stage and to *in situ* carcinoma. These multiple interdependent stages of the breast cancer present

numerous avenues for development of prevention and treatment strategies. Accumulation of various genetic alterations over time, including the amplification of oncogenes and mutation or loss of tumor suppressor genes was revealed during the molecular genetic analysis of breast cancer samples (30). However, there are certain known risk factors that pre-dispose an individual to develop cancer. Prolonged exposure to ovarian hormones was a major risk factor and increased risks were also found to be associated with early menarche, late menopause and also with first full term pregnancy (2, 25). In postmenopausal women, a positive correlation was seen between the weight and the risk of cancer development (26). Genetic factors are known to contribute about 5-10% of the total breast cancer (31), including family history of Klinefelter's syndrome (22). Mutations in BRCA1 and specially BRCA2 (31-33) have been widely established. Wild-type BRCA1 was shown to suppress estrogen dependent transcriptional pathways related to the proliferation of epithelial cells in the breast, therefore mutations in BRCA1 can lead to breast tumorigenesis (24, 34).

3.1.2 Classification of breast cancer

Breast cancers are basically ductal breast carcinoma, the cancer that starts in the cells lining the milk ducts (a common form of breast cancer) and the other form that arises in the glands or the lobes that produce and supply milk to the ducts and is called glandular/lobular carcinoma (clinically also called as adenoid mammary carcinoma). These premalignant lesions are referred to as, atypical ductal hyperplasia (ADH), atypical lobular hyperplasia (ALH), ductal carcinoma *in situ* and lobular carcinoma *in situ* (35). When the cancer has spread out of the site of origin, and started invading the nearby tissues and organs, it is called an invasive cancer (figure 3.1). In certain cases, inflammatory breast cancer, which is a very rare form of breast cancer can start in other areas of the breast (36). Early detection of the breast cancer is essential not only to avoid delay but also to help refine the selection of best treatment corresponding to the cancer subtype, breast cancer cells expresses many surface receptors and markers that help differentiate the type of breast cancers and eventually aid in the choice of therapy (21).

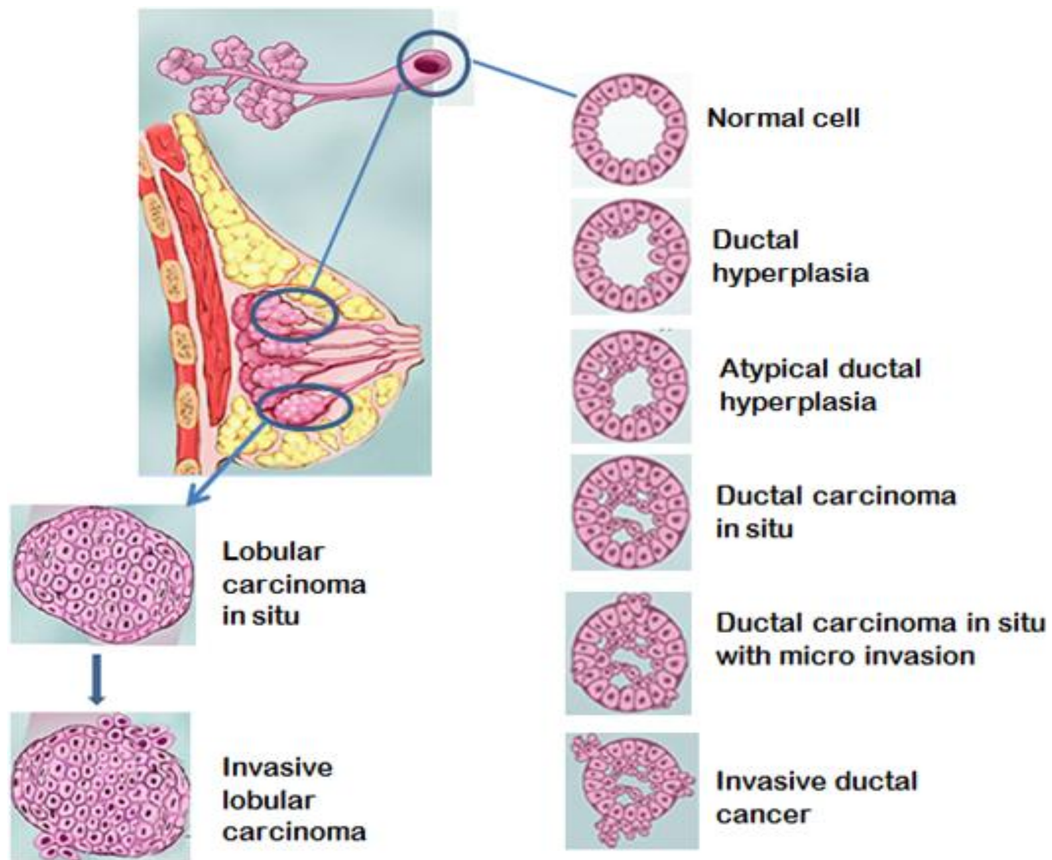


Figure 3.1 Steps in ductal and lobular epithelial breast cancer progression (37)

There are 5 categories of breast cancer based on the expression of different surface receptors and molecular signatures which help in the classification and the choice of treatment (21). These signatures are estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 1 and 2 (HER 1 and 2) and cytokeratins 5/6 (CK 5/6) (21). Breast cancer categories and their signatures are shown in Table 3.1.

Table 3.1 Molecular classification of human breast cancer (21)

Category	Signature	Rx. of choice
Luminal A	ER(+) and /PR(+); HER2(-)	Endocrine Rx; Tamoxifen
Luminal B	ER(+) and /PR(+); ER2(+)	Trastuzumab and Tamoxifen
Basal- like	ER(-); PR(-); HER2(-); CK5/6(+);HER1(+)	Neoadjuvant chemotherapy
HER-2(+)/ER(-)	HER2(+); ER(-); PR(-)	Trastuzumab and adjuvant chemotherapy
Normal breast-like	All markers (-)	

3.1.3 Biomarkers of breast cancer

Screening for breast cancer biomarkers is very important for clinical validation of the cancer before instituting complex therapeutic decisions. Heterogeneity of breast cancers gives rise to multiple biomarkers. Despite intensive research efforts and the use of latest generation technologies, no major biomarkers that could strongly suggest the risk of an individual to develop breast cancer or treatment resistance and relapse were found (38). However, mutations in *BRCA1/2* were successfully carried out to detect heritable breast cancer and preventive measures like mastectomy or salphingo-oophorectomy or both that could be carried out accordingly (38). Cytochrome P450 2D6 *(CYP2D6)* and *HER2* mutations were screened to predict resistances to tamoxifen and trastuzumab respectively (38). *HER2* has been an important prognostic and a predictive marker. *HER2* expressing breast cancer patients presented bad prognosis but it could be altered by treating with trastuzumab. Researchers explored serum protein profiling and suggested macrophage migration inhibitory factor (MIF), matrix metalloprotinases-9 (MMP-9), and myeloperoxidase (MPO), to detect breast lesions in premenopausal women. However, this remains inconclusive as serum protein levels varied with different pathological conditions in the body (39). Serial analysis of gene expression for marker genes *p1B,* trefoil factor family-1 (*TFF1/pS2),* cytokeratin-19 (*CK19*) and epithelial glycoprotein-2 (*EGP2*) was carried out in the blood and bone marrow cells

where at least one of these marker genes were found elevated in 29% of the total cases under study (40). However, some markers expression status such as ER, PR, HER1/2 helps in deciding the best treatment with certain predictable outcome in each subtype (21). In addition, CA 15-3, CA 27.29, carcinoembryonic antigen, urokinase plasminogen activator, plasminogen activator inhibitor 1 were also suggested at one point of time, however these markers demonstrated insufficient evidence to support routine use in clinical practice as yet (41). Building a prognostic model and identifying specific prognostic signatures would still involve years of research owing to the expression of diverse clinical variables and numerous gene expression in the multi-step tumorigenesis and progression of the breast cancer.

3.1.4 Estrogen and breast cancer

As much as estrogen is necessary for the normal growth and differentiation of female secondary sexual characteristics, it has also been used beneficially for hormone replacement therapy, known to prevent hot flashes by diminishing vasomotor instability, preserves the bone mass and has a positive impact on the cardiovascular system. After puberty it controls the menstrual cycles and is essential for reproduction. There are also emerging views that estrogen could delay the onset of Alzheimer's disease (42). However the use of estrogen for its desirable effect was often found responsible for the development of malignant breast and uterine tumors on prolonged exposure. Estrogens from the plants (phytoestrogens) or environment (xenoestrogens) which has structural similarity with the physiological estrogen were also known to increase breast cancer risk (24, 43). Estrogen was found to promote mammary tumor formation in the rodents and exerted proliferative effects either directly or indirectly on the cultured human breast cancer cell lines (44). Estrogen was known to be an endogenous carcinogen for the breast and uterine tumors. It contributes to erroneous DNA replication by promoting excessive proliferation as well as the replication of clones carrying the genetic errors (42). Its proliferative effects have also been attributed to its control over the transcription of many other genes including growth factors, proto-oncogenes and other regulatory molecules (42). Estrogen diffuses passively across the plasma and the nuclear membranes. In the estrogen sensitive cells expressing ER and the Estrogen and the

receptor complex formed, binds to the EREs in the 5'-flanking region of the estrogen responsive genes (figure 2). ERE consists of 5-6 base pairs inverted repeats separated by any 3 base pairs (GGTCAnnnTGACC) or differing in one or more bases) and lead to transcription of genes which regulate the growth and differentiation of the cells (Figure 3.2) (42, 45). Earlier, oophorectomy has been a fairly successful treatment for breast cancer increasing the survival chances (46).

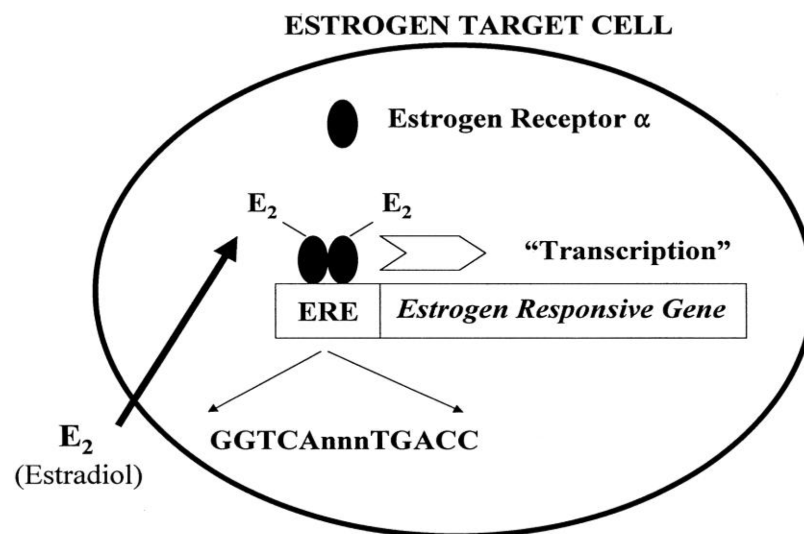


Figure 3.2 A simplified representation of the estrogen action in the estrogen on its target cell (42)

3.2. Estrogen receptors

Estrogen have been known to play a vital role in the development and regulation of both male and the female reproductive systems (47) and requires receptors for the for successful estrogen action. Although estrogen travels through the blood, only those cells in estrogen target tissues that have estrogen receptors, like the breast and uterus, can recognize and respond to estrogen. Cancer cells that have estrogen receptors are called estrogen receptor-positive cancers (ER+) which is possible to be manipulated by hormonal therapy for better survival advantage compared to the ER- negative (ER-) subtype (48). The estrogenic effects are mediated via ER-α and ER-β which belong to nuclear receptor super family, mainly

acting as ligand activated transcription factors (49). ERα, also known as NR3A1 (nuclear receptor subfamily 3, group A, member 1), is encoded by the gene *ESR1* (Estrogen Receptor 1) that is located at chromosome 6 and is mapped at 6q25.1. ERβ or NR3A2 (nuclear receptor subfamily 3, group A, member 2), is encoded by the *ESR2* (Estrogen Receptor 2) gene that is located on chromosome 14 and is mapped at 14q23.2. Structurally ERs have six functional domains namely A/B, C, D, E and F extending from the N-terminal to C-terminal and showing a varying extent of sequence conservation (figure 3.3). A/B domain is highly variable in length and contains the transactivation function-1 (AF-1), which has the potential to transactivate gene transcription in the absence of bound ligand (e.g., the estrogen hormone). However, this activation is weaker and more selective than the activation provided by the E domain. The C domain, also known as the DNA-binding domain (DBD), binds to estrogen response elements in the target DNA sequence via two type II zinc-binding motifs. The D domain is a hinge region that connects the C and E domains. It allows the receptor protein to bend or change conformation for dimerization for nuclear localization. The E domain, known as the ligand binding domain (LBD) also has binding sites for co-activator and co-repressor proteins. The transactivation function-2 (AF-2) contained in the LBD determines the ligand-binding specificity of the ERs. Binding of stereochemically distinct ligands leads to the change in the tertiary structure of the receptors leading to the repositioning of the AF-2 regions, affecting the formation of the multiprotein complex. The E-domain in the presence of bound ligand is able to activate gene transcription. The C-terminal F domain is variable in length and its function not clear (42). Although the ERα and ERβ are different gene products, it binds to the consensus ERE. After estrogen binds to the receptors, the receptors dimerizes to form any of ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers, and binds to the EREs in the promoter of the target genes to regulates its transcription by either recruiting the co-activators or co-repressors (figure 3.4). Transcription is a complex process that requires the interaction of transcription factors, co-repressors, integrator proteins, histone acetyltransferase (HATS) and histone deacetylases (HDACS). In normal breast tissues ER level is lower but increases with age (50). It has been postulated that the loss of tumor suppressor gene could result in the failure to down-regulate ER and this

can be a possible mechanism of breast carcinogenesis (24). About 15% of the breast cancers are known to belong to the triple negative phenotype where the cancer cells do not express any receptors (ER-/PR-/HER2-) and in which no targeted therapy can be given resulting in very poor prognosis (44). Hayashi *et al.,* (51) investigated the role of ERβ and its various isoforms in the ERα-positive breast cancer has been assessed by transfecting MCF-7 cells with ERβ and ERβcx (ERβ with 26 amino acids truncated at the C-terminal region). This the constitutive expression of ERβ and ERβcx in the ERα-positive breast cancer showed reduced cell growth, cell cycle and colony formation in anchorage free culture conditions. Moreover this reduced the estrogen sensitivity in the estrogen responsive genes. It is evident from these findings that the ERα functions could somehow be modulated by ERβ and its isoforms. Hall *et al.,* also demonstrated that ERβ isoform of the ER modulates the transcriptional activity of ERα and regulates the response to estrogens and antiestrogens (52). It is the recruitment of more than 20 co-regulator proteins which are either co-activators or co-repressors at the ERs that modulate the gene transcription. Varying combination of these regulator proteins at the ERs modulate its function in many ways (53).

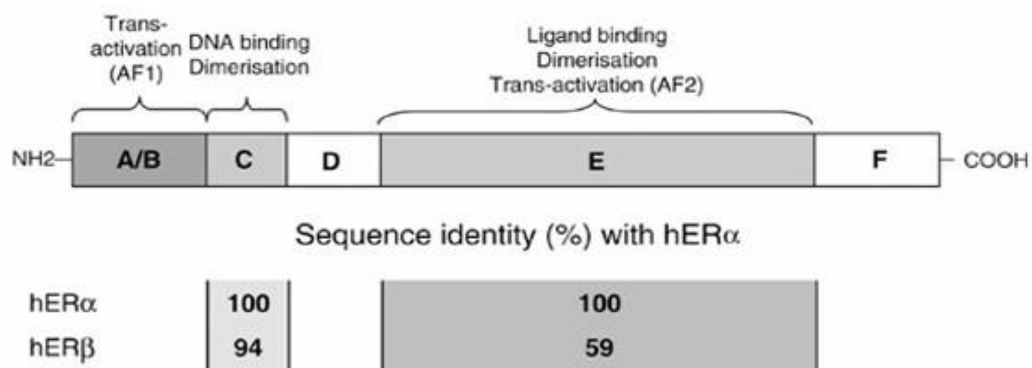


Figure 3.3 Functional domain organization of nuclear receptors with the percentages of identity with human ERα and ERβ in the DBD and LBD (54)

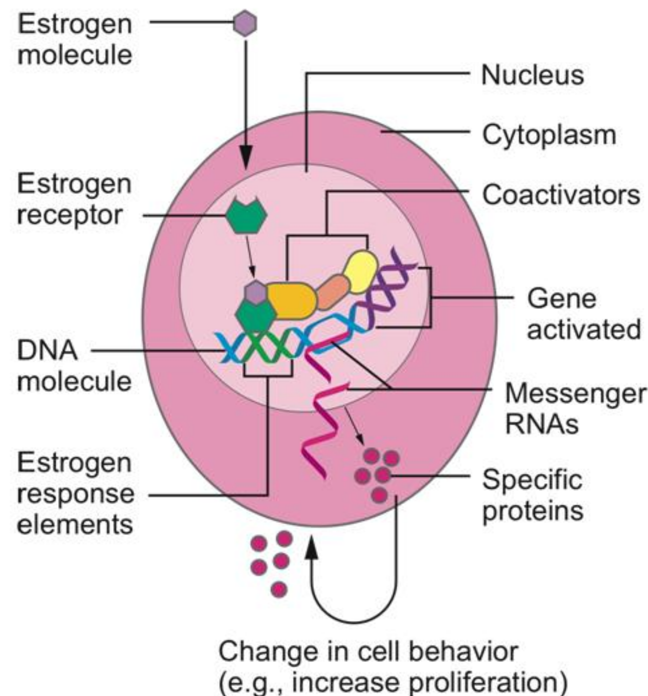


Figure 3.4 Gene activation by ligand bound estrogen receptor (55)

3.2.1 Selective estrogen receptor modulators and down regulators

Targeting estrogen receptor is one of the oldest forms of molecular targeted therapy that has fairly contributed to the cure rate, disease prevention and improved quality of lives in the last 3 decades (56). Selective estrogen receptor modulators (SERMs) and estrogen receptor down regulators (ERDs) are classes of compounds that act on the estrogen receptor. Based on their mode of action these substances can be pure receptor antagonists and agonists, thereby granting the possibility to selectively inhibit or stimulate estrogen-like action in various tissues with their action varying in different tissues. Estrogens are uniformly agonists, and antiestrogens are uniformly antagonists, but the SERMs can either have an agonist or antagonist or mixed agonist-antagonists effects depending on the target tissues. Tamoxifen is a commonly prescribed SERM which was approved for breast cancer treatment and acts as a competitive inhibitor of estrogen receptors owing to its identical conformation that facilitates binding to the LBD of the ER as shown in figure 3.5 (57). Tamoxifen has demonstrated efficacy for the treatment and prevention of ER–positive breast cancer. Adjuvant tamoxifen therapy, has also been

shown to significantly reduce the risk of, recurrence and death from breast cancer in all age groups studied (24). ERDs block the effects of estrogen in breast tissue and works in a similar way to SERMs, such as tamoxifen. ERDs after binding to the estrogen receptors lead to receptor deterioration, bringing down the number of total receptors or change the receptor conformation resulting in the complete blockade of transcription (figure 3.6).

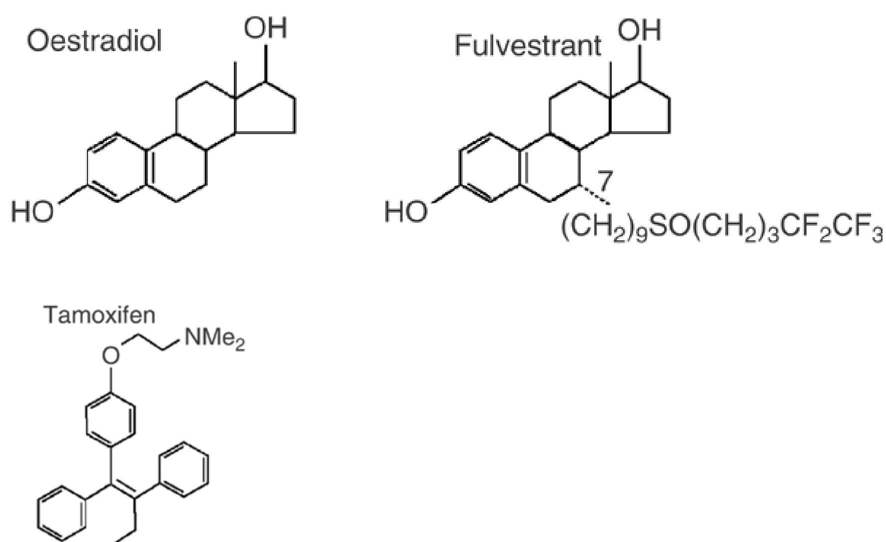


Figure 3.5 Structures of estrogen, fulvestrant and Tamoxifen (57)

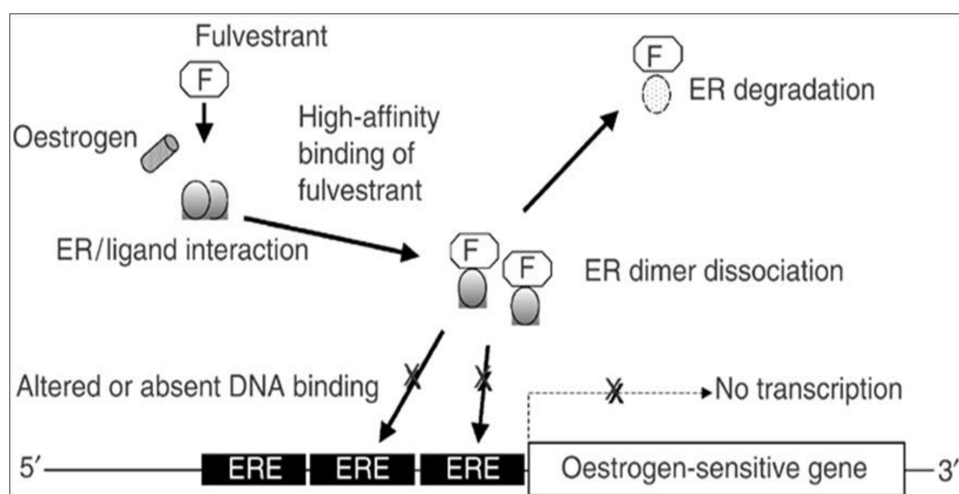


Figure 3.6 Mechanism of action of fulvestrant at the level of transcriptional regulation ERE=estrogen response element; ER= estrogen receptor; F = fulvestrant (57)

Faslodex® (chemical name: fulvestrant) is one ERD available to treat hormone-receptor positive breast cancer. After fulvestrant binds to the ER monomers, it inhibits receptor dimerization, rendering AF1 and AF2 inactive, reducing the nuclear translocation of receptors, and accelerating the degradation of the ER, resulting in pure anti-estrogenic effects (58). Fulvestrant is a new kind of endocrine treatment, well tolerated without much drug related adverse reaction and is found to be a pure estrogen receptor antagonist in every tissue (57). It prevents receptor dimerization and nuclear localization resulting in complete blockade of the ER signalling. Tamoxifen, on the other hand, resulted in drug related adverse actions like, gastrointestinal disorders, hot flashes, vaginitis, thromboembolic events in prolonged use (59) and increased endometrial cancer risk by at least 2-3 folds. The hot flashes observed in some of the tamoxifen treated patients were linked to the possible CNS penetration which was found to be lacking in the fulvestrant treatment (60). However the SERMs and ERDs work via different mechanisms with no cross-resistance, which would enable them to be, combined with other adjuvant hormonal therapies for a prolonged period of time until the institution of chemotherapy (59, 61). Table 3.2 highlights differential effects of SERMs and ERDs on various tissues.

Table 3.2 Target tissue specific agonist/antagonist actions of SERMs and ERDs (59)

	Breast	**Uterus**	**Cardio/Lipid**	**Bone**
TAMOXIFEN SERM	Antagonist (partial agonist)	Agonist	Agonist	Agonist (postmenopausal women only)
FULVESTRANT ERD	Antagonist	Antagonist	Antagonist	Antagonist

3.3 Treatment resistance in breast cancer therapy

The development of drug resistance is the major obstacle leading to reduced efficacy of chemotherapy. Deranged apoptotic pathways in cancers greatly contribute to the failure in chemotherapy since apoptosis is an essential process for

tumor regression (62), which would occur only if the rate of cell proliferation is lower (cytostatic effects) than the rate of cell death (cytostatic effects). Estrogen treatment has been widely reported to favor MCF-7 cell proliferation (44). In addition, estrogen has been associated with the induction of chemotherapeutic resistance in the ER positive breast cancer cells via the upregulation of the Bcl-2 (prosurvival) mRNA expression (9). Doxorubicin is a widely used anthacycline for the treatment of various haematological malignancies and solid tumors including breast cancers. Doxorubicin intercalates, causing DNA strand breakages and inhibits topoisomerase II activity (63). In the endothelial cells and in cardiomyocytes doxorubicin has been shown to induce ROS (H_2O_2) mediated apoptosis where as in the breast cancer cell line MCF-7 it caused early activation of p53 followed by caspase activation and DNA fragmentation (64). However it is not surprising in some cases that the MCF-7 cells undergoing apoptosis fail to exhibit features of apoptosis like the DNA fragmentation as it is not a common phenomenon in all the strains of MCF-7 cell line (65) due to the deletion mutation in exon 3 resulting in the loss of a 47 base pairs in the *CASP3* gene (66). Several studies reported that doxorubicin-induced intracellular oxidative stress was inhibited by over expression and by depleting endogenous antioxidants like glutathione, in the tumor cells. However the apoptosis in tumor cells via ROS induction is still unclear. p53 is a tumor suppressor gene and the most commonly inactivated gene in cancers (67). Normally p53 is activated following DNA damage, hypoxia and other genotoxic stresses to promote cell cycle check points, repair DNA and resume normal cell cycle (68). It holds the cell cycle and allows the DNA repair enzymes to correct the DNA leading to apoptosis at all the damage is unrepairable ensuring genetic homogeneity. Most DNA damaging treatment leads to the accumulation p53. The p53 itself is regulated by the MDM2 oncoprotein via functional inactivation. In normal and tumor cells, doxorubicin induces apoptosis via distinct mechanisms (64). In the MCF-7 cells, one of the reasons for the resistance to doxorubicin was demonstrated due to over expression of multidrug resistance (MDR) transporters like P-glycoprotein or the MDR protein, and also due to ATP-dependent reduction in the intra cellular accumulation of the drug (69). It is evident that the success in breast cancer therapy does not solely depend on targeting the ER signaling system but there is clear

involvement of other estrogen regulated molecules which might as well be targeted to help overcome resistance to endocrine therapy. However the search doesn't end here. Be it endocrine, or chemotherapy, estrogen has shown its role in treatment failure by either up-regulating survival molecules or by engaging in crosstalk with the growth factor signalling pathways. This poses estrogen and/its target genes as an important target to enhance beneficial response in the breast cancer chemotherapy.

3.4 Estrogen and Trefoil Factor Family 1

TFF1 transcription has been reported as a primary and early response to estrogen in the human breast cancer cell line MCF-7, with the transcription apparent within 15 minutes of estrogen treatment (70). Numerous studies reported that estrogen controls the expression of many target genes that possess the ERE in their promoters. Nevertheless, it could also induce ERE independent transcription of some genes via protein-protein interactions. Besides this genomic mechanism, non-genomic mechanisms of ER signalling, where the estrogen can exert its effects by modulating the surface ERs with GPR30 is currently a research focus (71-72). Of several genes that are expressed in breast cancer, *TFF1* is found to be induced abundantly in the breast cancer patients. TFF1 has little or no expression in the normal breasts (73). High correlation was found between the TFF1 and ER expression (74). Therefore TFF1 was considered as a complimentary tool for prediction of ER expression and functionality. It was reported to act as a tumor suppressor in the gastric epithelium but a contradictory role was suspected in the breast cancer progression, with several research groups investigating the biological roles of TFF1 *in vitro* and *in vivo* (6).

3.5 Trefoil Factor Family

Trefoil factor family (TFF) comprises a compact group of secreted peptides encoded by genes, closely linked TFF cluster on chromosome 21q22.3 (75). *TFF1* along with *TFF2* (SP, spasmolytic polypeptide) and *TFF3* (ITF, intestinal trefoil factor) are the members of trefoil factor family (76). Genes encoding all these

three proteins are contained within a single 55 kb genomic DNA fragment with *TFF1* and *TFF2* located 12.5 kb apart (figure 3.7) (77). The 5' untranslated region of all the TFFs are similar and share several sequence motifs which show a common regulation in their expression and these are consensus motif for transcription factors like NF-κB, GATA-6, USF and C-EBP beta that are known to regulate TFFs expression (78).

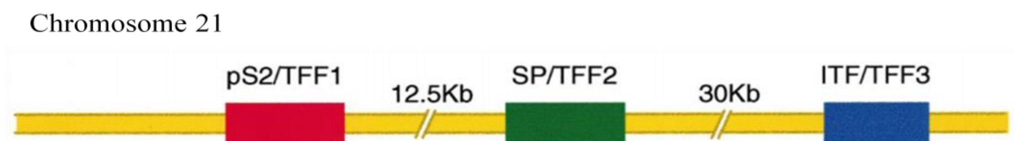


Figure 3.7 Trefoil family cluster on human chromosome 21 (77)

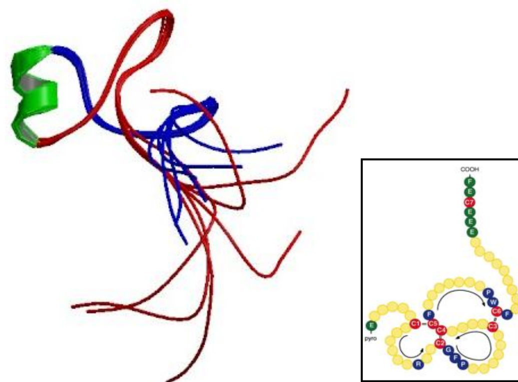


Figure 3.8 Trefoil domain of a monomer showing 3-loop structure with disulphide bonds at cysteine residues 1-5, 2-4 and 3-6, giving a clover appearance (77, 79)

Trefoil peptides are known to be secreted, predominantly by the epithelial cells lining the mucus membrane, in a site specific manner particularly in the normal gastrointestinal lumen and its known to be associated with the mucosal barrier integrity (16). TFF1 is found mainly expressed in the stomach while TFF2 is found in the stomach, duodenum and Brunner's gland. TFF3 is mostly expressed in the small and the large intestines (16). These peptides share a common domain of about 60 amino acids held in a three loop structure resembling a clover leaf, hence the name trefoil peptides and the domain is called 'P' domain (76, 79). These intra domain disulphide linkages are formed between the six conserved cysteine residues, 1-5, 2-4

and 3-6 (figure 3.8), and renders the peptide highly resistant to the proteolytic digestion, an important characteristic required for the peptide to retain its structure and functions in the harsh gastrointestinal environment (76, 80). TFF1 and TFF3 consist of a seventh cysteine residue in the carboxy-terminus which results in the formation of homo or heterodimers of these proteins. These trefoil peptides are co expressed with specific mucins and form a continuous gel on the mucosal surface (81). The expression of these thermo stable protease resistant peptides are elevated during gut inflammation and has been associated with mucosal healing via induction of cell migration and anoikis during the process (82). TFFs has been studied to interfere with the inflammatory processes and the innate immune responses (83). TFFs are known to contribute to organ functions outside the GI tract such as respiratory and ocular tissues, brain and neurosensory cells of the cochlea (83). However no receptors for TFFs have been identified so far though it is known to contribute to the cellular processes (84). Besides their regular sites of expression, trefoil peptides are also found ectopically expressed in many human diseases (85). TFF1 expression is not uniform in all the malignancies but is dependent on the extent of methylation of its promoter in different tissues. It was previously shown that the hypomethylation of CCGG site close to TFF1-ERE in the breasts cancer tissues correlated with its increased expression (86). Whereas promoter methylation resulted in the decreased TFF1 level in the gastric cancer (87). In the knockout mice experiments, generated by targeted-gene disruption of each of the trefoil factors, showed *TFF1* as a tumor suppressor gene (12) where as deleting *TFF2* and *TFF3* did not result in immediate malignancy.

3.5.1 TFF1

TFF1 (previously called pS2, BCEI, D21S21, HP1.A, HPS2, pNR-2) was the first trefoil peptide to be completely sequenced. It was discovered for the first time, during the differential screening of cDNA library, during the search for estrogen regulated mRNAs from MCF-7 breast cancer cell line (88). TFF1 is a small secreted peptide of about 6 kDa and contains 60 amino acid residues, with a single trefoil domain. Extensive studies have been done regarding the *TFF1* as a tumor suppressor gene and its involvement in the development and/progression of the human gastric

cancer (13). Now there are emerging literatures, highlighting the tumorigenic roles in other cancers particularly the breast cancer. In the kidney and colonic epithelial cells that were already engaged in cancer progression, TFF1 was found to be associated with invasion of collagen gels, however, it was unable to induce cellular invasion in premalignant colonic and kidney epithelial cells (89). TFF1 deficiency led to transcriptional upregulation, of GRP78, a member of heat shock protein 70 (HSP70) molecular chaperones, which is found to block protein transport from endoplasmic reticulum by permanently binding to misfolded, or unassembled proteins (90). Bossenmeyer *et al*., 1998, demonstrated the antiproliferative effect of TFF1 in gastric epithelium and preventing anoikis but favouring gastric cell differentiation (19). TFF1 is a mucin stabilizing factor (13), thus have a role in gastric homeostasis. Recently, Buache *et al.,* 2011 (91), demonstrated contradictory findings concerning the roles of TFF1 in tumorigenesis to those demonstrated by Amiry *et al*., 2009 (6). In their study of TFF1 gain-or–loss of function, the former did not observe any oncogenic properties of TFF1 instead found to be having beneficial antitumor properties (91). Therefore these conflicting roles of TFF1 in cancer biology require additional investigations and confirmation.

3.5.2 Gene structure and regulation

The human *TFF1* gene is about 4.5 kilo bases (kb) long, comprising of three exons of, 125, 153 and 212 base pairs with two intervening introns of, 3.1 and 0.77 kb (92). The exons encodes the signal peptide, the TFF domain and a carboxy-terminus acidic domain and the *TFF1* promoter contains an ERE at -420 to -332 base pairs (figure 3.9) and a complex enhancer region responsive to epidermal growth factor (EGF), tumor promoter TPA and the proto-oncogenes *c-Ha-ras* and *c-jun* (78), therefore *TFF1* can be regulated by multiple regulatory factors (84). TFF1 inducing factors are tissue dependent, as in the normal stomach TFF1 is induced by gastrin (93) but in the breast tumors estrogen is the main inducer of TFF1. Therefore, in the ER positive MCF-7 breast cancer cell line, the treatment of estrogen directly controls the expression of TFF1 at the transcriptional level. Functional AP1 motif has been described at the 5’ region upstream from TFF1 promoter (78).

TFF1 is synthesized with a signal peptide of about 21-27 amino acid residues which is cleaved by proteolysis during passage through the endoplasmic reticulum (94). The secreted, matured form of TFF1 displays an N terminal pyro-glutamate residue, a TFF domain of about 39 amino acids and a carboxy-terminal acidic domain (88) which often makes it difficult to be sequenced. The expression of TFF1 is known to be transcriptionally controlled by estrogen in ER rich breast tumors (95). Hypomethylation within the promoter/enhancer sequence of *TFF1,* found in these tumors and the amount of *TFF1* mRNA corresponded with the extent of demethylation, therefore this phenomenon can be involved in the control of TFF1 expression in different tissues under different pathological conditions (86).

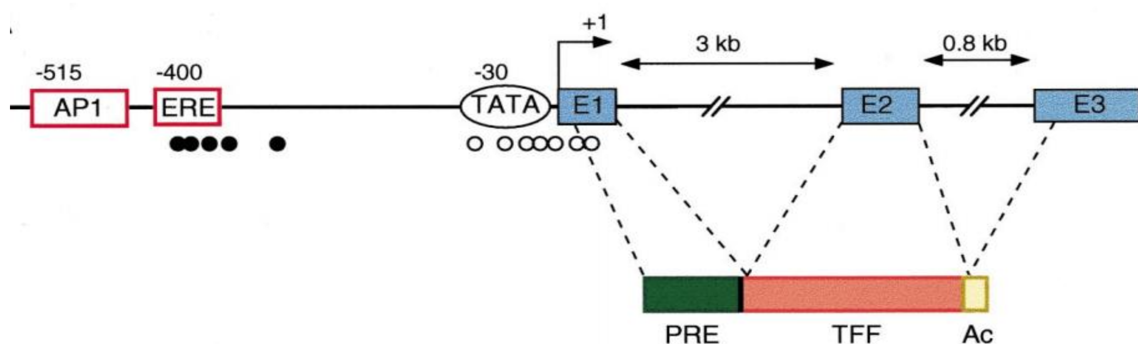


Figure 3.9 Gene organization and regulatory elements in *TFF1* gene (77);
ERE, AP1 (jun/fos) responsive element, TATA box, +1 transcription start site,
○ CpG islands
● CpG islands whose methylation is correlated with TFF1 expression

3.5.3 TFF1 expression and functions in normal tissues

In the TFF1 expressing cells, the intracellular TFF1 peptide is found in the cytoplasm or the peri-nuclear space and the extracellular TFF1 is found in the culture medium or tissue interstitial fluid as a secreted peptide with corresponding autocrine and paracrine effects (19). Besides its predominant expression in the stomach, small normal expression is also seen in the rectum, oesophagus, sublingual gland, pancreas, gall bladder, lung and vagina (16). In the gut epithelium TFF1 is co-expressed with the secreted mucin, MUC5AC (96) suggesting that it could be

involved in the mucosal homeostasis . Focal expression of TFF1 has also been observed in duct luminal cells of normal breasts as well, though no expressions was found in the lactating breast lobules (97). TFF1 is over-expressed during intestinal damage and inflammatory bowel disease and have shown to have beneficial effect like wound healing (98).

3.5.4 TFF1 expression in the pathological tissues

Ectopic expression of TFF1 is seen during the inflammation of the entire gastrointestinal tract (85), though the factors controlling the ectopic expression is still unknown (15). It plays a major role in tissue regeneration during wound healing in ulcerative diseases such as Crohn's disease, duodenal ulcer and haemorrhagic rectocolitis (19). During the inflammation of the gastrointestinal tract, all the TFFs level is elevated suggesting their roles in wound healing and epithelium repair (99-100). Impaired healing leads to chronic inflammation related injury which is a major event in tumorigenesis (11).

3.5.5 TFF1 expression in the malignant tissues

TFF1 peptides in cancers, outside the gastrointestinal tract, are usually observed in a state of over-expression (11). TFF1 generally exhibits two expression patterns. In the tumor of the tissues, where its normally expressed, shows a decrease/loss of expression during malignancy (eg. in stomach), where as in the malignancy of the tissues where TFF1 expression is absent/low, tissues shows abundant expression of the peptide (eg. in breast, gall bladder, prostrate and colon) (73). The ectopic expression of TFF1 in the human carcinoma has been observed in many human diseases (77). Their involvement in the process of metastasis has also been established by many studies (101). TFF1 expression, other than in mammary carcinomas are the carcinomas of the large bowel, pancreas, stomach, endometrium, biliary tract (15, 17), lung, prostrate, endometrium, bladder (15). In the gall bladder carcinoma, immune reactivity was focally observed in the inflamed and the metastatic bladder tissues, but not in the normal non-neoplastic cells (102). The diverse expression of TFF1 in malignant tissues and organs which are normally TFF1-negative suggests an important role during the malignant process.

3.6 TFF1 in breast cancer

Normal breasts are known to secrete no or very little amount of TFF1 (16, 73, 97) therefore, the increase in the serum level was believed to serve as a prognostic tool for breast cancer. In MCF-7 breast cancer cell line, *TFF1* gene was the first *TFF* to be known as an estrogen regulated gene (92). TFF1 was identified as a normal constituent of the breast milk (103) but in the neoplastic transformation of breast epithelium, there are important and reproducible changes in the expression of these peptides (11). The close association between the ER and TFF1 expression in breast cancer has been confirmed by microarray based studies of the primary breast tumors, along with the prediction value for the response to the hormonal therapy (104). The high intrinsic expression of TFF1 in ER-positive breast cancer cell line, like MCF-7 however, has always been controversial with findings correlated to patient survival advantage as well as to the tumor enhancing property (91, 105).

Despite its beneficial effects in its normal physiological level, the over-expressions in certain cancers have been associated with a derogatory role. Tumorigenic roles of TFF1 have been established in many cancers including those of breast and the biliary system (102), both *in vitro* and *in vivo* (6). However, some researchers claim no oncogenic role of TFF1 in mammary cell carcinoma (91, 106). Although no cell surface receptors for TFF1 or its signaling pathways have been elucidated till date, the association of the downstream signaling pathways with several other signal transduction pathways reveals important biological processes like cell survival, differentiation, anchorage independent growth, invasion, motility and angiogenesis (6). It was shown to inhibit BAD induced apoptosis via caspases 3, 6, 8 and 9 (19, 91). Early detection of the breast cancer and prognosis is essential so that patients can be appropriately selected for adjuvant therapies such as chemotherapy and/or hormonal therapy without delay. This can spare some women from the side effects of aggressive chemotherapy. The value of estimation of TFF1 in the breast cancer has always been controversial (105). Microarray based studies found *TFF1* gene expression as one of the informative markers for the detection of micro metastatic breast tumors (101) since it is one of the most abundant estrogen induced mRNA found in breast cancer. TFF1 peptide was highly correlated to the ER and PR (74). There are reports of inverse association of TFF1 with the tumor volume, lymph

node involvement and the histological grade. Yet some researchers reported TFF1 as an independent prognostic marker in the breast cancer and showed no correlation with the tumor size, grade and nodal status (74). In addition to the hormone receptor status in the breast cancer, the anti-proliferative property of TFF1 (14) was also associated with decreased tumor volume and good prognosis (17). Studies in TFF1 knockout mice resulted in the development of gastric tumor but there was no evident effect on the mammary gland development and function. This may be due to the fact that TFFs are expressed in very low levels in the inactive breast (97).

3.7 TFF1 and apoptosis in cancer cells

TFF1 has been extensively studied as a tumor suppressor gene in the gastric mucosa therefore it may have a significant role in gastric cancer development and/progression (12). Most gastric cancers showed, a loss of TFF1 expression either as a result of promoter methylation, deletion and loss of heterozygosity but not as much incidences due to mutation (87). TFF1 has been shown to protect the IEC18, HCT116 and AGS gastrointestinal cells from apoptosis induced by chemicals, anchorage free growth and pro-apoptotic protein ‘Bad’ induced apoptosis (19, 85) and the cells could be consequently rescued from apoptosis on reconstition of recombinant TFF1 (19). TFF1 led to up-regulated expression of cell cycle and cell survival associated proteins such as cyclin D1, cyclin E1, c-MYC, CDK2, ERBB2, Bcl2, MDM2 and transcription factors like c-Jun and c-Fos (6). Anti-apoptotic functions of TFF1 were known to occur as a result of partial or complete inhibition of caspases -3, -6 and -9 activities (19). Recently TFF1 was found to be synthesized abundantly in the pterygium, a small benign growth in the clear, thin tissue that lays over the white part of the eye. TFF1 has shown to protect the conjunctival cells from ultraviolet irradiation and benzalkonium chloride induced apoptosis (20). TFF1 delayed the Caspase-8 activation at the death-inducing signaling complex (DISC) which consists of an adaptor protein and initiator caspases, essential for induction of apoptosis (107). TFF1 was also shown to activate NF-κB which in turn induced X-linked inhibitor of apoptosis protein (XIAP) also known as inhibitor of apoptosis protein 3 (IAP3) (20). XIAP inhibited the caspases 7, -3 and -9. Thus, TFF1 can

inhibit apoptosis both at the pre-mitochondrial and the post-microchondrial levels (20). Growth inhibition and increased apoptosis after the withdrawal of estrogen coincided with TFF1 mRNA level. Oncogenic properties of TFF1 in MCF-7 cells have been elucidated in TFF1 over-expression as well as silenced models both *in vitro* and *in vivo* (6) where apoptosis was decreased and increased respectively. Irrespective of the apoptosis inducing agent, the downstream apoptotic signalling is more or less known to involve similar molecules and mechanism (20). Estrogen have been associated with anti-apoptotic property in the breasts cells (108). TFF1, whose expression is directly controlled by estrogen in the breast cancer cells, may be an explanation, for the anti-apoptotic attribute of estrogen and its involvement in hormone stimulated breast cancer progression.

3.8 Apoptosis

Apoptosis is a neatly executed, efficient cell death process popularly termed as “programmed cell death” (PCD). The process is well regulated for the multi cellular organisms essential during growth and developmental process, cell replacement and in immune system homeostasis, including viral infections and tumor regression (109-110). Inadequate apoptosis is a characteristic feature of cancer. Deregulated apoptosis is linked to the failure in treatment (109). On the other hand, elevated apoptosis are thought to be responsible for neuron degeneration in Alzheimer's disease and in Parkinson’s disease) (111). Necrosis, the other type of cell death on the contrary, is characterized by uncontrolled cell death, accompanied by cell lysis, inflammatory responses and pose serious health problems (112). p53 is a tumor suppressor gene which is frequently found mutated in the human cancer and acts as a sensor of cellular stress and can initiate apoptosis by transcriptionally activating proapoptotic Bcl-2 family (e.g., Bax, Bak, PUMA, and NOXA) and repressing anti-apoptotic proteins (Bcl-2, Bcl-XL) (109, 113-114).

Manipulating the apoptotic pathway, such as reinstating the defective programme as in cancer, autoimmunity and some viral infections and suppression of apoptosis as in AIDS, stroke and neurodegenerative diseases will have enormous impact on the diseases (115). Apoptosis plays critical roles in preventing autoimmune

diseases and in the removal of the dying cells from the system by (a) degradation of harmful DNAs and RNAs, (b) avoiding self-immunization with DNA and RNA, thereby preventing autoimmune diseases, (c) processing of cell corpses and reuse of some cellular components like cholesterol and (d) exposure of nucleosomes on the surface of the dying cells promote phagocytosis (116). In the human body thousands of cells are generated every second and a similar number die by mitosis every second by apoptosis to maintain the tissue homeostasis and proper functioning.

3.8.1 Stages of Apoptosis

The apoptotic cells undergo significant morphological and biochemical changes that are usually (a) cell shrinkage due to volume deregulation due to ion movements (117) (b) breakdown of chromatin (118) and nuclear disintegration (119), (c) proper packaging of the cell and its contents making it easier for the phagocytic cells to engulf by avoiding leakages as seen in necrosis (110) and finally (d) the membrane from blebs or blistering (120).

3.8.1.1 Cell Shrinkage

Cells committed to apoptosis undergo a rapid shrinkage, which is a fundamental characteristic of PCD resulting in considerable loss of its total volume. The loss of intracellular sodium [Na^+] and potassium [K^+] ions causes osmotic imbalance which occurs just after chromatin condensation and DNA fragmentation, and prior to apoptotic body formation are known to contributing to the shrunken morphology (117).

3.8.1.2 Membrane blebbing

Multiple cytoplasmic protrusions extend, and are immediately withdrawn without losing the cellular integrity. This is caused by the depolymerisation and decoupling of the cytoskeleton components of the plasma membrane. The bleb sizes vary depending on bleb growth rates, its actin and other contents (119).

3.8.1.3 Nuclear chromatin (DNA and protein) degradation

DNA fragmentation is a crucial biological event in ensuring the complete destruction of the potentially harmful DNA (121). DNA fragmentation factor (DFF) is a heterodimeric endonucleases, composed of fragmentation factors

(DFFA) 45 and (DFFB) 40. It was found that upon initiation of apoptosis caspase 3 cleaves the heterodimer. The dissociation of DFF45/DFFA, which acts as a molecular chaperone, from DFF40/DFFB endonucleases, is activated and cleaves the chromosomal DNA at the internucleosomal sites into oligosomal sized fragments *in vitro* and is suspected to play similar role in the *in vivo* apoptosis (118, 121). Chromosomal DNA of apoptotic cells undergo strand breaks due to cleavage between nucleosome loops. Nuclear condensation follows the breakdown of chromatin and nuclear structural proteins which is believed to be due to the extrusion of water (110) and is a biochemical hallmark of apoptosis (120).

3.8.1.4 Mitochondria break down with the release of cytochrome c

During early apoptosis the mitochondrial membrane becomes more permeable, which is attributed partly by the opening of the multiprotein complex pore known as permeability transition (PT) pore (122). This result in the leakage of the apoptosis mediators like cytochrome c, which together with Apaf-1 and ATP forms a complex with pro-caspase 9, leading to activation of caspase 9 and the caspase cascade.

3.8.1.5 Formation of apoptotic bodies

Cells continue to shrink, packaging themselves into a form that allows easy clearance by macrophages towards the end stages of apoptosis and are known to occur in the intercellular space (110). This is done by fragmentation into a cluster of sub cellular bodies (apoptotic bodies) each membrane-bounded and containing a variety of compacted cytoplasmic organelles. The plasma membrane changes triggers the macrophage response. One such change is the translocation of phosphatidylserine from the inner leaflet of the cell to the outer surface and is responsible for triggering non-inflammatory phagocytic recognition and clearance of the apoptotic cells (123).

3.9 Apoptotic pathways

Apoptosis involves sophisticated interplay between the factors responsible for suppression and induction of apoptosis and multiple signalling molecules that are genetically encoded for roles in initiation, mediation, execution and regulation of apoptosis. A family of proteins known as caspase is typically activated in the early stages of apoptosis (124). These proteins breakdown or cleave key cellular components required for normal cellular functions, including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus. Apoptotic stimuli can either be from outside or from within the cells but somehow the diverse death signal leads to a common cell death pathway (124). Studies showed that the key molecules of the apoptotic machinery have well been conserved through the evolution. There are 2 separable pathways underlying apoptosis (figure 3.10), the extrinsic and the intrinsic pathways (124).

3.9.1 Extrinsic pathway

It is a very rapid process initiated by the ligation of the transmembrane death receptors (Fas/CD95, TNFR1, DR3, DR4, DR5) leading to receptor clustering and recruitment of intracellular adaptors TRADD and FADD at the death domains leading to the activation of membrane proximal activator caspases -8 and -10, which then lead to the activation of effector caspases-3 and -7 (125). TNF ligation to TNF receptors leads to receptor trimerization and formation of DISC complex after recruitment of pro-caspase 8. The recruitment of TNF-α receptor associated factor-2 (TRAF2) instead of pro-caspase 8 leads to the signalling can lead to the activation of NF-κB and JNK pathway (114). This result in the induction of numerous genes, which includes the inhibitor of apoptosis proteins (IAPs), thus determines the balancing of cell death and cell survival signals (126). This pathway can be triggered within seconds of ligand binding and is regulated by cFLIP, which is a negative regulator of death receptor induced apoptosis and inhibits caspases upstream and also inhibits IAPs, which can regulate both activator and the effector caspases (127-128).

3.9.2 Intrinsic pathway

This pathway involves the generation of apoptotic stimuli following cellular stress after DNA damage, hypoxia, growth factor deprivation or stress following exposure to chemicals and free radicals (109, 114). Mitochondria contains numerous pro-apoptotic proteins including apoptosis inducing factor (AIF), Smac/DIABLO and cytochrome c (129). Cytosolic pro-apoptotic Bcl-2 proteins acts as sensors any cellular stress (124). During stress the pro-apoptotic proteins locate to the mitochondrial membrane and interact with the anti-apoptotic proteins resulting in the formation of permeability transition (PT) pore (122). Through the pore, cytochrome c and other factors are poured out into the cytosol, where it functions with Apaf-1, activates caspase-9 and consequently initiating the apoptotic caspase cascades (124). Smac/DIABLO and HtRA2 binds and antagonizes the IAPs (114). From the proximal death receptor signalling the activated caspase-8 can cleave the cytoplasmic 22 kDa BID pro-apoptotic Bcl-2 protein to a truncated (tBID) 15 kDa which interacts with the anti-apoptotic proteins on the mitochondrial membrane thus amplifying the death receptor signalling. The crosstalk between the extrinsic and the intrinsic pathways at the level of caspase 8 results in the amplification of the apoptotic signals (114).

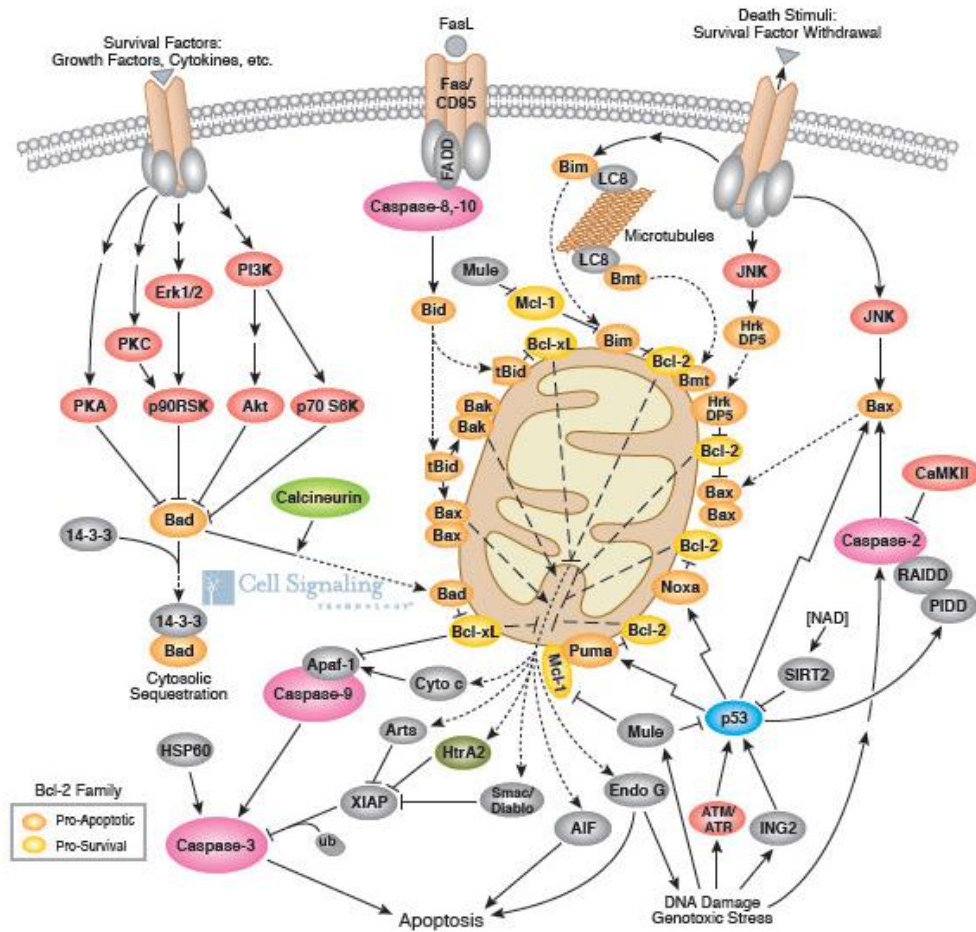


Figure 3.10 A complex signalling network showing extrinsic and intrinsic apoptosis pathways (130)

3.9.3 Apoptosis in cancer and its potential role in therapy

Hyperplasia was speculated to be a result of decreased apoptosis rather than increased mitosis (110). In cancer cells, normal apoptotic process is disabled via mutations, helping the cancer to grow independently, of the growth signals, ignoring the growth inhibitory signals, and constantly conferring resistance to anti-cancer agents (114). Apoptosis is a spontaneous phenomenon that occurs in malignant neoplasms or can be induced for therapeutic purpose (110). Mutations in genes responsible for suppressing tumor development were also seen responsible for treatment failure (113). Treatment of cancer either via chemotherapy or radiotherapy are basically aimed at killing the cells by inducing apoptosis (114, 131-132). Usually in tumors, energetically strained cells are more prone to necrosis than apoptosis

resulting in large number of necrotic tissue in tumors, causing a lot of pain and inflammation. Therefore switching back on apoptosis in tumor cells is at the forefront of cancer therapy. For a cancer cell to propagate it has to overcome several barriers like the anti-tumor immune mechanism, hypoxia, nutritional deprivation, extreme stress, biochemical and/morphological stress (114-115) through altered expressions of genes that encode for the key apoptotic molecules, providing them with intrinsic survival advantage and resistance to therapy (114). With the accumulating knowledge about cancer now it's clear that some oncogenic mutations interrupt the normal apoptotic pathways and leads to tumor initiation, progression and metastasis (109). Apoptosis being an active process, demands a deeper understanding of the signalling pathways that controls the process of cell death and its control in each tumor type. The similarity between the physiological apoptotic programme and the drug induced apoptosis presents an undeniable link between tumor development and development of intrinsic resistance to anticancer therapy, thus aiding tumor progression (114). Since many pathways and molecules control the apoptotic cascade, this presents multiple targets for the development of rationally designed anticancer strategies to check cancer progression (113).

CHAPTER IV
MATERIALS AND METHODS

4.1 Materials

4.1.1 Cell line and growth conditions

Estrogen receptor positive (ER+), malignant human breast adenocarcinoma, MCF-7 cells (figure 4.1) was kindly provided by Prof. Pornchai O-Chareonrat, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University. The MCF-7 cells was grown as monolayer in Dulbecco's Modified Eagle's medium (DMEM) with phenol red, supplemented with 10% heat inactivated fetal bovine serum (FBS), antibiotics and antifungal including 0.1 U/ml penicillin G sodium, 0.1 mg/ml streptomycin and 5mg/ml of amphotericin B to prevent microbial contaminations. Cells were cultured in adhesive sterile culture flasks at 37ºC in a 5% CO_2 in humidified incubator. Upon reaching appropriate confluency, cells were trypsinized using 0.25% trypsin/EDTA. Trypan blue staining was used to evaluate the viability of cells. More than 95% viability was used in the experiments. A part of cells mixed with 5% DMSO and 25% FBS in DMEM was kept in liquid nitrogen as alternative source of MCF-7 cells.

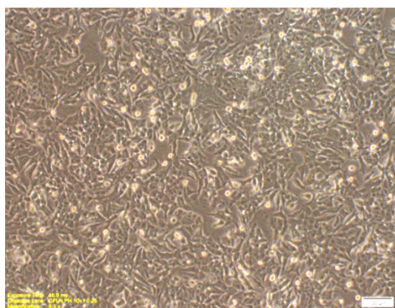


Figure 4.1 ER positive, MCF-7 breast cancer cell

4.1.2 Chemical reagents

All commercial available chemical reagents used in this experiment were of Molecular and culture grades. Some important chemicals and instruments used in this project are listed (Table 4.1- 4.2).

Table 4.1 List of important chemical materials

Chemicals / Reagents	Companies
Cell Culture	
DMEM	Invitrogen, Carlsbad, CA, USA
Fetal bovine serum (FBS)	Invitrogen, Carlsbad, CA, USA
Penicillin, Streptomycin	Invitrogen, Carlsbad, CA, USA
0.25% Trypsin EDTA	Invitrogen, Carlsbad, CA, USA
Phenol red free DMEM	Invitrogen, Carlsbad, CA, USA
Charcoal stripped FBS	Invitrogen, Carlsbad, CA, USA
shRNA transformation	
Plasmid DNA	Sigma Aldrich, St. Louis, MO, USA
5'- CCGGCCTGGTGCTTCTATCCTAATACTCGAGTATTAGGATAGAAGCACCAGGTTTTTG-3'	
5'- CCGGGTGCAAATAAGGGCTGCTGTTCTCGAGAACAGCAGCCCTTATTTGCACTTTTTG-3'	
5'- CCGGCCAGAAGAGGAGTGTGAATTTCTCGAGAAATTCACACTCCTCTTCTGGTTTTTG-3'	
5'- CCGGCCAGAAGAGGAGTGTGAATTTCTCGAGAAATTCACACTCCTCTTCTGGTTTTTG-3'	
5'- CCGGTGAAAGACAGAATTGTGGTTTCTCGAGAAACCACAATTCTGTCTTTCATTTTTG-3'	
5'- CCGGGCCCTCCCAGTGTGCAAATAACTCGAGTTATTTGCACACTGGGAGGGCTTTTTG-3'	
Lipofectamine®2000	Invitrogen, Carlsbad, CA, USA
Opti-MEM I®	Invitrogen, Carlsbad, CA, USA
Puromycin	Sigma Aldrich, St. Louis, MO, USA
Apoptosis study	
Doxorubicin	Pfizer Inc, USA
Annexin V(PI) Kit	BD Biosciences, San Jose, CA, USA

BD FACsFlow™	BD Biosciences, San Jose, CA, USA
Western blot	
Polyvinylidene (PVDF) membrane	Amersham, Buckinghamshire, UK
Rabbit-anti human TFF1	Sigma Aldrich, St. Louis, MO, USA
Goat-anti Rabbit IgG-HRP Ab	Abcam, Canbridge, MA, USA
Mouse-anti human β-actin Ab	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Rabbit-anti mouse IgG HRP	Zymed, Sanfrancisco, CA, USA
Mouse-anti human phospho-AKT Ab	Cell Signalling Technology, Inc, Danvers, MA, USA
Enhanced Chemiluminescense (ECL)	Pierce, Rockford, IL, USA.
Others	
17β-estradiol (E2)	Sigma Aldrich, St. Louis, MO, USA.
rTFF1 (Full length)	Abcam, Canbridge, MA, USA.
Fulvestrant	Sigma Aldrich, St. Louis, MO, USA.

Table 4.2 List of important instruments

Instruments	Companies
Western Blot Analysis	
TE70 Semi dry transfer unit	Amersham, Buckinghamshire, UK
Immunofluorescence	
Olympus U-RFL-T	Shinju-Ku, Tokyo, Japan
Flow Cytometry	
Becton Dickenson FACSort® and CellQuest Software	Becton Dickenson, Franklin Lakes, NJ, USA
Bradford Total Protein Assay	
Coomasie Plus® (Bradford) Assay Kit	Thermo Scientific, Rockford, IL, USA
Statistical Analysis	
Sigma Plot. Version 12.0	Systat software Inc., San Jose, California,,USA

4.1.3 Plasmids and Lipofectamine®2000

Plasmids are double-stranded generally circular DNA sequences, capable of replicating in the host cell. Plasmid vectors consist of, an origin of replication that allows semi-independent replication of the plasmid as well as the transgene insert in the host. Plasmids generally have a "multiple cloning site" which includes nucleotide overhangs for insertion of an insert, and multiple restriction enzyme consensus sites to either side of the insert. Plasmids normally consists of an antibiotic resistance gene which acts as a selective marker and aids in the selection of the positively transfected clones The DNA plasmids were diluted in Opti-MEM I® Reduced Serum medium and Lipofectamine®**2000**. The pLKO.1 vector and the specific sequence of the five different clones of shTFF1 inserts (TRCN_00000033614-18) are summarized in table 4 and figure 12 respectively. These inserts have been derived from the coding region of the TFF1 gene.

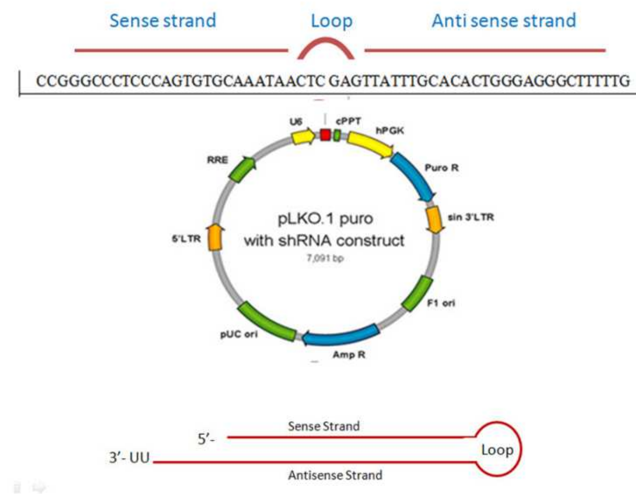


Figure 4.2 Map of pLKO.1 puro, containing shTFF1 plasmid insert

4.1.4 Antibody array

Human Apoptosis Array (Proteome Profiler ™) consisting of 35 apoptosis related proteins was purchased from R&D Systems.

4.1.5 17β-estradiol (E2)

Amorphous water soluble E2, Cyclodextrin encapsulated, (mol. wt 272.38) was supplied in the solid form (45.1 mg E2/gram solid balanced with 2-hydroxypropyl-β-cyclodextrin). A working stock of 10 mM solution was prepared by adding 4 ml PBS to 1 g of E2, gently swirled to dissolve. It was aliquoted and stored at -20°C for preserving efficiency, until further use. For E2 stimulation experiments, 1 nM E2 was further diluted from the working stock as required in the phenol-red free DMEM with 5% charcoal stripped FBS.

4.1.6 Fulvestrant reconstitution (ICI 182,780)

A 25 mg of Fulvestrant (empirical formula: $C_{32}H_{47}F_5O_3S$, mol.wt.: 606.77) was supplied in the powder form. The whole content was dissolved in 1.373 ml of DMSO to obtain a stock concentration of 30 mM from which working stock of 10 mM was prepared, aliquoted and stored at -20°C for preserving efficiency. For the experiments the working stock was further diluted to 10 µM fulvestrant in the appropriate medium.

4.1.7 Doxorubicin

Doxorubicin is a commonly used antineoplastic drug in cancer chemotherapy therapy. It belongs to anthracycline antibiotic isolated from cultures of *Streptomyces peucetius* var. caesius which is a species of soil fungus (figure 4.3). In the breast cancers, doxorubicin has been successfully used as a chemotherapeutic agent. The cytotoxic effect of doxorubicin on malignant cells is presumed to be related to nucleotide base intercalation and cell membrane lipid binding activities of doxorubicin. Intercalation inhibits nucleotide replication and action of DNA and RNA polymerases. The interaction of doxorubicin with topoisomerase II to form DNA-cleavable complexes is an important mechanism of doxorubicin cytocidal activity (63)

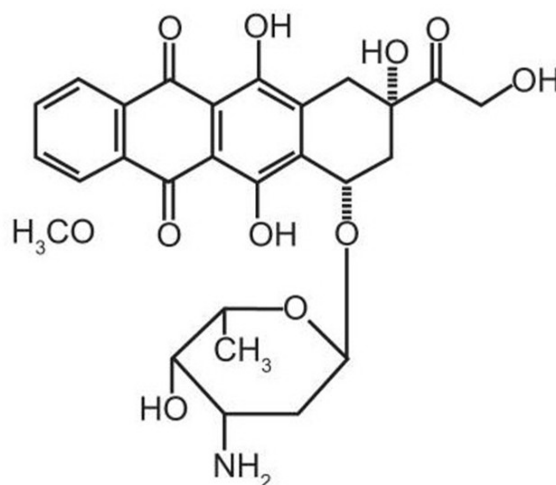


Figure 4.3 Chemical structure of doxorubicin (133)

Injectable doxorubicin hydrochloride (empirical formula: $C_{27}H_{29}NO_{11}\cdot HCl$ mol.wt.: 579.98) was supplied as 10 mg in 5 ml, i.e. 3.4 mM stock was diluted in the phenol red free DMEM with 5% charcoal stripped FBS. It was aliquoted and stored at 4°C for preserving efficiency. Approximately 5×10^5 mock and *TFF1*-knocked down (*TFF1*-kd) MCF-7 cells were seeded in a 24 well plate. After overnight incubation, the wells were treated with different concentrations of doxorubicin ranging from 20, 10, 1, 0.1 µM in complete DMEM. Cell death was determined over the next 24 and 48 hrs by trypan blue dye exclusion method. Dose showing approximately 50% cell death was selected for inducing apoptosis in further experiments (i.e. 1 µM was used for further experiments).

4.2 Methods

The methodology comprises 3 main parts. The first (figure 4.4), to generate stable MCF-7 cells with reduced TFF1 expression. The second, to induce apoptosis using doxorubicin, chemotherapeutic drug and investigate the role of TFF1 in drug induced apoptosis. The TFF1 modulation by E2 was elucidated in the presence of anti-human TFF1 antibody or in the presence of ER antagonist. The reversal of the cell death following reconstitution of full length recombinant TFF1 to the *TFF1*-kd cells was investigated. The third part involved the investigation of changes in expressions of key human apoptosis related proteins under different doxorubicin and E2 treated conditions.

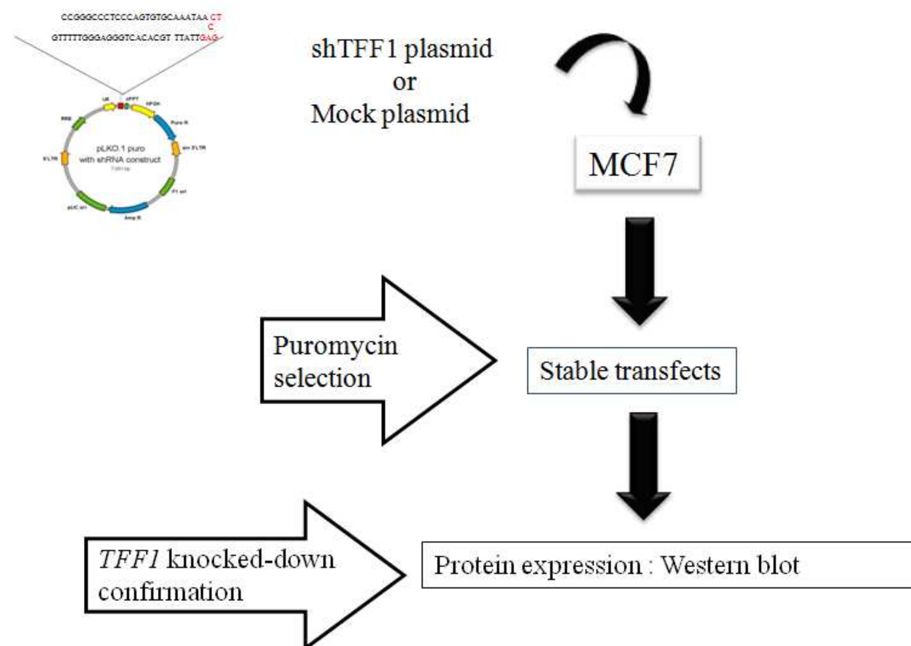


Figure 4.4 Generation of TFF1 knocked down MCF-7 cells

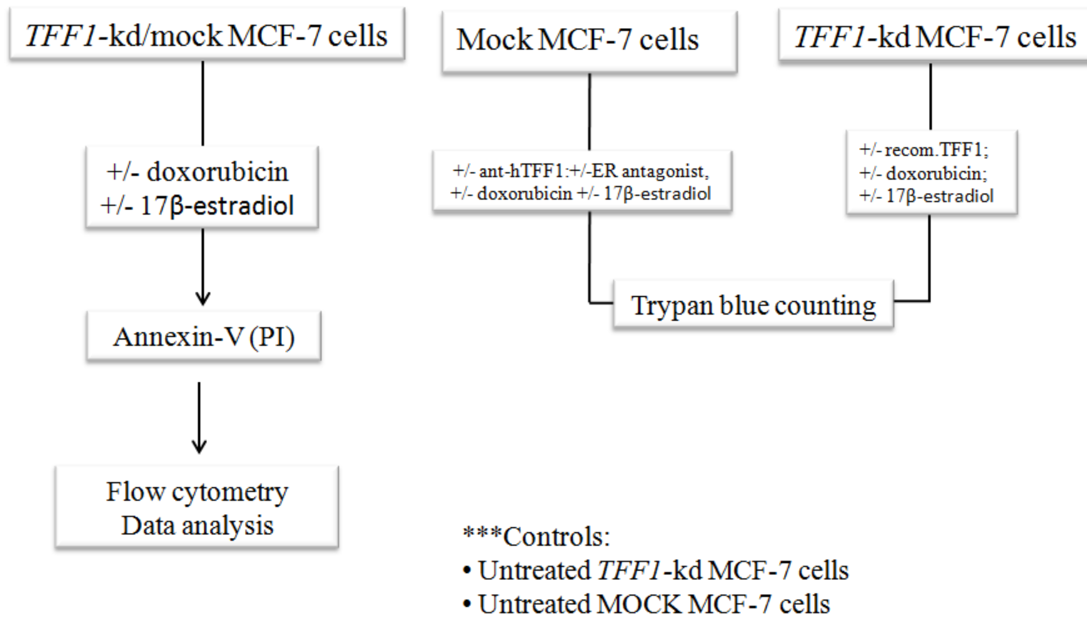


Figure 4.5 Elucidation of anti-apoptotic role of estrogen induced TFF1

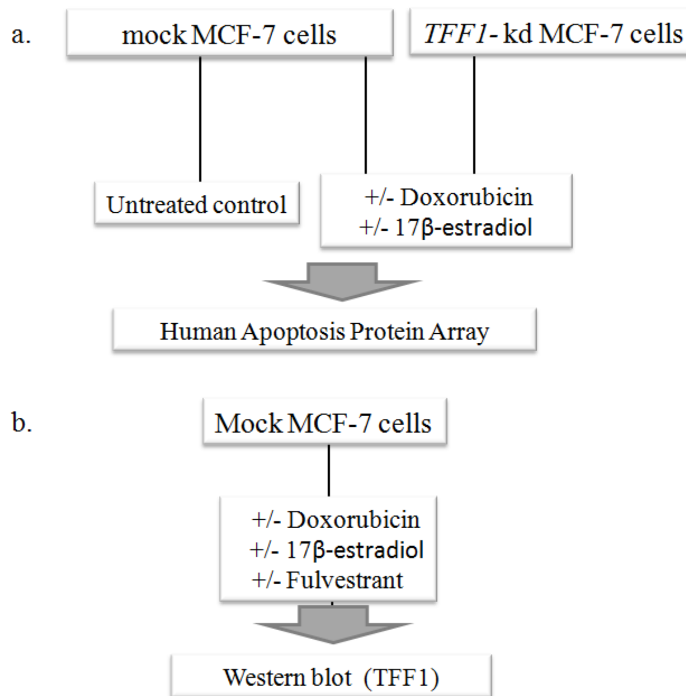


Figure 4.6 Human apoptotsis related protein expression

4.2.1 Growth curve analysis and cell passaging

Approximately $1x10^4$ MCF-7 cells were seeded in complete DMEM onto the 24 well-plate and cultured at 37°C in a 5% CO_2 in a humidified incubator. After 24 hours, fresh DMEM was supplemented. Each day cells were trypsinized in duplicate wells using 0.25% trypsin-EDTA and the viable and the dead cells were counted over the next 12 days using trypan blue staining and the counting chamber.

For cell passaging, the spent medium was removed and the adherent cells were rinsed with 1x PBS. Into well 0.5 ml of 0.25% (v/v) trypsin-EDTA was then added and incubated in CO_2 incubator for 3-5 min. The proteolytic reaction of trypsin was stopped by adding 4.5 ml of the complete medium of each cell type. Cell was suspension was properly mixed and counted using a hemocytometer. 10 µl of cell suspension and 10 µl of trypan blue (1:1 v/v) are mixed and counted under light microscope. Optimal cells were placed into a new 25 cm^2 culture flask containing 5 ml of fresh complete media and incubated in 5% CO_2 incubator at 37°C. Culture medium was changed every 2-3 days after examining the change in phenol red pH indicator.

4.2.1.1 shRNA transfection and gene silencing

Manufacturer's instructions were followed for appropriate dilutions of DNA plasmid pLKO.1 puro, containing shTFF1 plasmid insert and a puromycin selectable, *pac* gene as shown in figure 13. Approximately 5 x 10^4 MCF-7 cells were maintained in 24 well plate in DMEM supplemented with 10% FBS, 0.1 U/ml penicillin G sodium and 0.1 mg/ml streptomycin. As it reached appropriate confluency DMEM containing antibiotics was replaced by the antibiotic-free DMEM for 24 hours before transfection. For transfection, manufacturer's protocol was followed for preparing the reaction mixtures per well. Mixture A was prepared by mixing 0.8 µg of plasmid DNA containing shTFF1 Opti-mem I® to a volume of 50 µl. Mixture B was prepared by mixing Lipofectamine® 2000 at a concentration of 2 µl/50µl of Opti-mem I® per well. Then the Lipofectamine®2000 containing mixture B was added to the plasmid containing mixture A and was further incubated at room temperature for 20 min. Finally total of 100µl of mixture was dispensed into each well. Same protocol was followed for mock (empty vector) transfection as well. After 6 hours fresh DMEM containing 10% FBS was replenished after the spent media was

changed to remove the complexes. Stable transfected cells were selected using appropriate dose of puromycin (10mg/ml). The knockdown of TFF1 was confirmed by western blot analysis.

4.2.1.2 Optimization of puromycin dose for selection of transfects

Puromycin was used against the puromycin resistant gene present in the plasmid vector carrying the shTFF1, to select the positively transfected clones. Approximately 1 $x10^6$ MCF-7 cells were seeded on the 6 well-plate and were treated with different concentrations of puromycin ranging from 10, 5, 2.5, 1.25, 0.625 and 0.3125 µg/ml of puromycin in complete medium. Cell death was observed over the next 7 days and the lowest concentration showing substantial cell death will be selected for further treatment of the cells.

4.2.2 Apoptosis assay (Annexin-V/PI staining)

Apoptosis assay of mock and shTFF1 transfected cells treated with doxorubicin or E2 alone or in combination for 18 hrs was performed using flow cytometer after Annexin-V/propidium iodide (PI) staining. In the apoptotic cells, the membrane phosphotidyl serine (PS) is translocated from inner to the outer leaflet of plasma membrane. PS is a negatively charged phospholipid located in the cytosolic phase of the lipid bilayer in the normal, viable cells (123). Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has high affinity for PS. Annexin V is conjugated to fluorochrome FITC thereby helping in the identification of early apoptosis. Propidium iodide (PI) will intercalate into the DNA helix of fixed and permeabilized cells. The bottom right quadrant (Annexin V^+) of each dot plot represents cells in the early stages of apoptosis and the top right quadrant (Annexin V^+, PI^+) represents cells in late stage apoptosis or cell death. The lower left quadrant represents the viable cells and the upper left represents the necrotic cells (figure 4.7). In the absence or presence of estrogen, both the mock and the *TFF1*-kd cells showed about 95% viability.

Up to 5 X 10^5 mock and *TFF1*-kd MCF-7 cells per reaction were seeded on a 6 well plate in DMEM supplemented with 10% FBS. After allowing adherence for 24 hrs, the media was replaced by phenol red-free DMEM/5% charcoal stripped

FBS with 1 nM E2 and 1 μM doxorubicin, alone or in combination. At appropriate time point, all the wells were microscopically examined for the presence of apoptotic cells. 18 hrs post treatment, both detached and adherent cells were then harvested following standard trypsinization protocol. The cells were centrifuged at 1,500 rpm for 5 min to remove the media followed by an ice cold 1x PBS wash at 1,500 rpm for 5 min. The cells were gently resuspended in 1 ml of Annexin-V binding buffer at an approximate concentration of $1x10^6$ cells. All the processes were performed on ice. 100 μl of this suspension (approximately $1x10^5$cells) was transferred into a 5 ml tube. 5 μl of Annexin-V and 4 μl of PI were added to the cells and were incubated at 20-25ºC in the dark for 15 min. and were suspended in 300 μL of freshly prepared 4% paraformaldehyde. After fixing for 15 min. the cells were spinned down at 1,500 rpm for 5 min and remove the supernatant and the cells were resuspended in 300 μl of Annexin-V binding buffer and mix well. The mean values of fluorescence intensity of 10,000 cells were determined by FACS analysis.

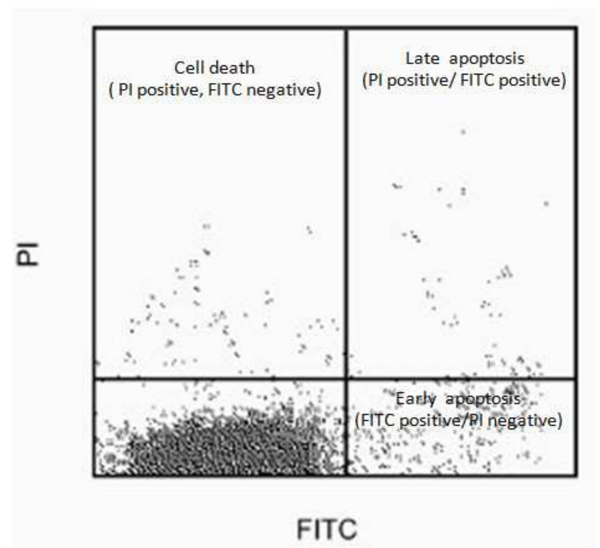


Figure 4.7 Dot plots showing the distribution of cells in different quadrants representing specific stage of apoptosis

4.2.3 Assessment of apoptosis after neutralizing TFF1 with pAb

Approximately 1×10^4 mock MCF-7 cells were seeded in a 96 well plate. After overnight incubation, the complete DMEM was replaced by phenol red-free

DMEM/5% charcoal stripped FBS. After 48 hrs, the respective wells were treated with 1 µM doxorubicin, 1 nM of E2, alone or in combination. Rabbit anti-human TFF1 (100 µg/mL) was used alone or in combination with doxorubicin. Untreated cells were used as background controls. Eighteen hrs after treatment, the cells were trypsinized then stained by trypan blue for counting.

4.2.4 Reconstitution of TFF1 to the *TFF1*-kd cells

Approximately 1×10^4 *TFF1*-kd MCF-7 cells were seeded in a 96 well-plate. After overnight incubation, the complete DMEM was replaced by phenol red free DMEM/5% charcoal stripped FBS. After 48 hrs, the respective wells were treated with 1µM doxorubicin, alone or in combination with 100 µg/ml and 10 µg/ml of full length recombinant human TFF1 in 200 µl of media. Untreated cells were used as background controls. Eighteen hrs after treatment, the cells were trypsinized for trypan blue counting.

4.2.5 Total protein extraction and western blot analysis

Approximately 1×10^6 cells with 95% cell viability were trypsinized as per the standard protocol. The cell pellet was rinsed with cold 1X PBS twice by centrifuging at 400 g for 5 min. After draining out the PBS the pellet was lysed using 50 µl of 2x lysis buffer that is composed of 50 mM tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.05% (w/v) bromophenol blue. Lysates were boiled for 10 min and diluted with 50 µl of H_2O, making the final conc. of the buffer to 1x (i.e. a ratio of 5×10^5 cells/100µl sample buffer). It is then centrifuged at 8,000 g for 1 min to get rid of all the undissolved proteins and debris. Fifteen µl of lysates were loaded into each lane of the 15-20% SDS-gradient polyacrylamide gel with 5% stacking gel separated for 1 hr. 30 min. The proteins were transferred to a pre-wet PVDF membrane at 120V, 0.8 mA/cm^2 using a semi-dry technique for 1 hr. 30 min. The membrane was blocked with 5% skimmed milk in 1X TBST. For TFF1, the membrane was probed with polyclonal anti-human TFF1 (1:2000) at room temperature for 1 hr. After 3 washes of 5 min. each, with TBS-T on a shaker the membrane was probed with horseradish peroxide-labeled goat anti-rabbit secondary antibody (1:2000) at room temperature for 1 hr followed by 3 washes of 5

min. each with TBS-T. Specific protein bands were detected by chemiluminescence**.** β-actin was used as a loading control.

4.2.6 Antagonizing ERs with fulvestrant

Approximately 1×10^4 Mock MCF-7 cells were seeded in a 96 well-plate. After overnight incubation, the complete DMEM was replaced by phenol red free DMEM/5% charcoal stripped FBS. After 48 hrs, the respective wells were treated with 10µM fulvestrant alone or in combination with, 1µM doxorubicin and 1nM E2 by diluting appropriately in the fresh phenol-red free media. Untreated cells were used as background controls. Eighteen hrs after treatment, the cells were trypsinized for trypan blue counting.

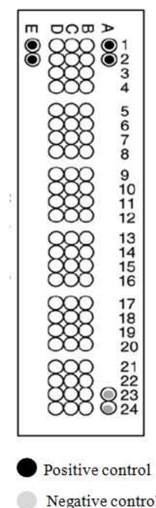
4.2.7 Protein array

Approximately 1×10^6 mock and *TFF1*-kd cells were seeded in 75 cm^2 cell culture flask and after 24 hours, the complete DMEM was replaced by phenol red free-DMEM/5% charcoal stripped FBS. After 48 hrs the media was replaced with fresh phenol red free-DMEM/5% charcoal stripped FBS containing, 1 µM doxorubicin, alone or in combination with 1 nM of E2. Eighteen hrs post treatment; all the cells were microscopically examined for the presence of apoptotic cells. The cells were rinsed with 1X PBS and trypsinized using standard protocol. The cells were then washed with 1X PBS. The apoptotic protein array used in this study was Human Apoptosis Array (Proteome Profiler ™ , R&D Systems). This kit contained 4 membranes with duplicated spots of 35 proteins. Table 4.3 lists the protein according to the position of the spots on the membrane. According to the manufacturer's instructions, all the remaining PBS was removed and approximately, 1×10^7cells/mL was solubilized in Lysis Buffer 15 (provided). The mixture was pipetted up and down and was rocked gently at 2-8ºC for 30 min. followed by centrifugation at 14,000 x g for 5 min. A total protein assay was performed on the supernatant using Bradford assay. Appropriate dilutions of protein in the lysates were prepared as per the maximum allowable volume per array recommended by the manufacturer i.e. about 250 µL/array containing about 200-500 µg of total protein. Each array consisted of 35 human apoptosis related proteins blotted on nitrocellulose membranes in

duplicates with appropriate positive and negative controls. The array required a blocking in the Array Buffer1 (provided) for 1 hr on a shaker at room temperature. Recommended quantity of lysates were diluted with the Array Buffer 1 and was pipetted it onto the arrays and incubated overnight at 2-8º C on a rocking platform shaker. The Arrays were then washed in 1X wash buffer (provided) 3 times for 10 min. each. 1.5 mL of diluted, biotinylated detection antibody cocktail was pipette into each well (provided) and incubated for 1 hr on a rocking platform shaker followed by the same wash process. The array was then incubated with diluted streptavidin-HRP for 30 min. followed by washing process. Figure 4.8 illustrates the schematic interaction between the capture and the detector antibodies with its corresponding proteins in the cell lysate. The membranes were then exposed to chemiluminescent reagents and then exposed to x-ray films for 30 sec. to 10 min. The positive signals (dark spots) from the developed x-ray films were analyzed using Image J, software (NIH).

Table 4.3 Identification of spots to the corresponding proteins

Coordinate	Target/Control	Coordinate	Target/Control
A1, A2	Positive Control	C13, C14	HO-2/HMOX2
A23, A24	Positive Control	C15, C16	HSP27
B1, B2	Bad	C17, C18	HSP60
B3, B4	Bax	C19, C20	HSP70
B5, B6	Bcl-2	C21, C22	HTRA2/Omi
B7, B8	Bcl-x	C23, C24	Livin
B9, B10	Pro-Caspase-3	D1, D2	PON2
B11, B12	Cleaved Caspase-3	D3, D4	p21/CIP1/CDNK1A
B13, B14	Catalase	D5, D6	p27/Kip1
B15, B16	cIAP-1	D7, D8	Phospho-p53 (S15)
B17, B18	cIAP-2	D9, D10	Phospho-p53 (S46)
B19, B20	Claspin	D11, D12	Phospho-p53 (S392)
B21, B22	Clusterin	D13, D14	Phospho-Rad17 (S635)
B23, B24	Cytochrome c	D15, D16	SMAC/Diablo
C1, C2	TRAIL R1/DR4	D17, D18	Survivin
C3, C4	TRAIL R2/DR5	D19, D20	TNF RI/TNFRSF1A
C5, C6	FADD	D21, D22	XIAP
C7, C8	Fas/TNFRSF6	D23, D24	PBS (Negative Control)
C9, C10	HIF-1α	E1, E2	Positive Control
C11, C12	HO-1/HMOX1/HSP32		



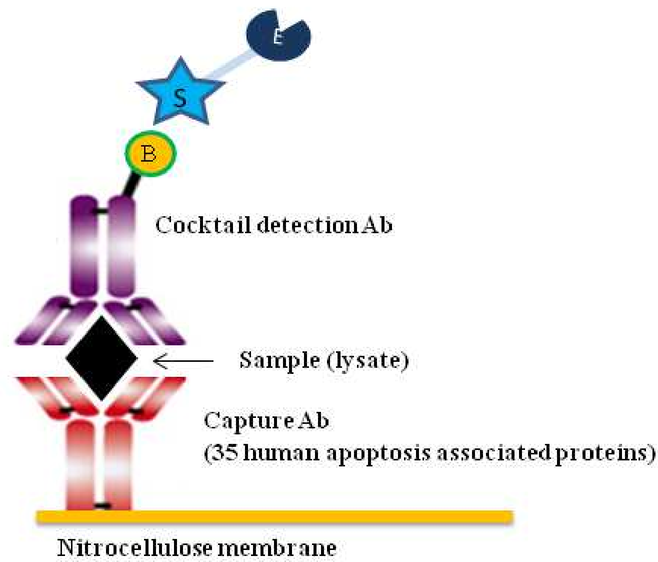


Figure 4.8 Schematic illustration interaction between the capture antibodies and the apoptosis associated proteins in the cell lysates

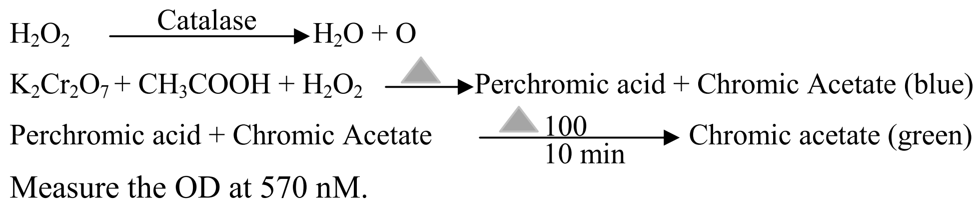
4.2.8 Bradford assay for total protein

After trypsinizing and lysis, 0.05 mL of each lysate were pipetted into properly labeled tubes and mixed with 1.5 mL of Coomassie Plus® Reagent. For consistent results, samples were diluted to 1:30 and 1:60. Absorbance was read spectrophotometrically at 595 nm. The blank replicate measurements were subtracted from individual absorbance of all the samples. Standard curve prepared by plotting the average blank-corrected 595 nm measurements for each diluted BSA standard. This standard curve was used to determine the protein concentration of each unknown sample.

4.2.9 Determination of catalase activity

Dichromate in the presence of acetic acid is reduced to chromic acid when heated in the presence of hydrogen peroxide, with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically. The catalase preparation is allowed to split H_2O_2 and the reaction is stopped at a particular time point by adding dichromate/acetic acid mixture and the

remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture (134).



Measure the OD at 570 nM.

Approximately $1x10^6$ mock and *TFF1*-kd cells were seeded in to the 25 cm^2 culture flask in complete DMEM. After 24 hrs the media was replaced with phenol red free DMEM/5% charcoal stripped FBS and continued culture in the same media for the next 48 hrs. The cells were then treated with/without doxorubicin and E2 with corresponding non treated controls. After 18 hrs the cells were harvested and 3×10^5 cells were gently washed for 2 times with 1x PBS and lysed with 100 µl of chilled 0.1% Triton X-100 solution and vortexed. Approximately 10 µl of the lysates were taken for Bradford assay and 90 µl of each condition was pipette into new tubes. Five mM H_2O_2 was added and incubated at 37°C for 10 min. following which 30 µl of 5% $K_2Cr_2O_7$ in DW and glacial acetic acid (1:3, by volume) was added and heated at 100°C for 10 min. it was then rapidly cooled on ice for ~5 min and centrifuged at 12,000 rpm, 4°C. Optical density was measured at 570 nm in Shimadsu UV-1700® spectrophotometer. Specific activity of catalase (U/mg protein) was determined by using the equation:

$$Specific\ activity = \frac{(S0 - S)}{t \times v \times m}$$

Where S0 was initial concentration of H_2O_2, S was the concentration of H_2O_2 at t min, v was the sample volume added in the reaction and m was the concentration of protein in sample.

4.4 Statistical analysis

All results obtained from these experiments were properly labeled and saved in Microsoft Excel. The comparison of the anti-apoptotic effect of TFF1 between the mock MCF-7 cell and the *TFF1*-kd MCF-7 cells under different treatment conditions were computed by the independent t-test using the Sigma plot® software. *P* value of 0.05 or less was considered as a significant difference.

CHAPTER V
RESULTS

5.1 MCF-7 Characteristics

5.1.1 Growth curve of MCF-7 cell

The MCF-7 cell line was cultured in complete DMEM supplemented with 10% FBS over a 12-day period in a 24-well culture plate. An initial seeding of 10,000 cells per well was used for this experiment. Media was changed after 24 hrs to remove the dead cells that could have resulted from the cell seeding procedure and the cells were counted daily using trypan blue (figure 4.1).

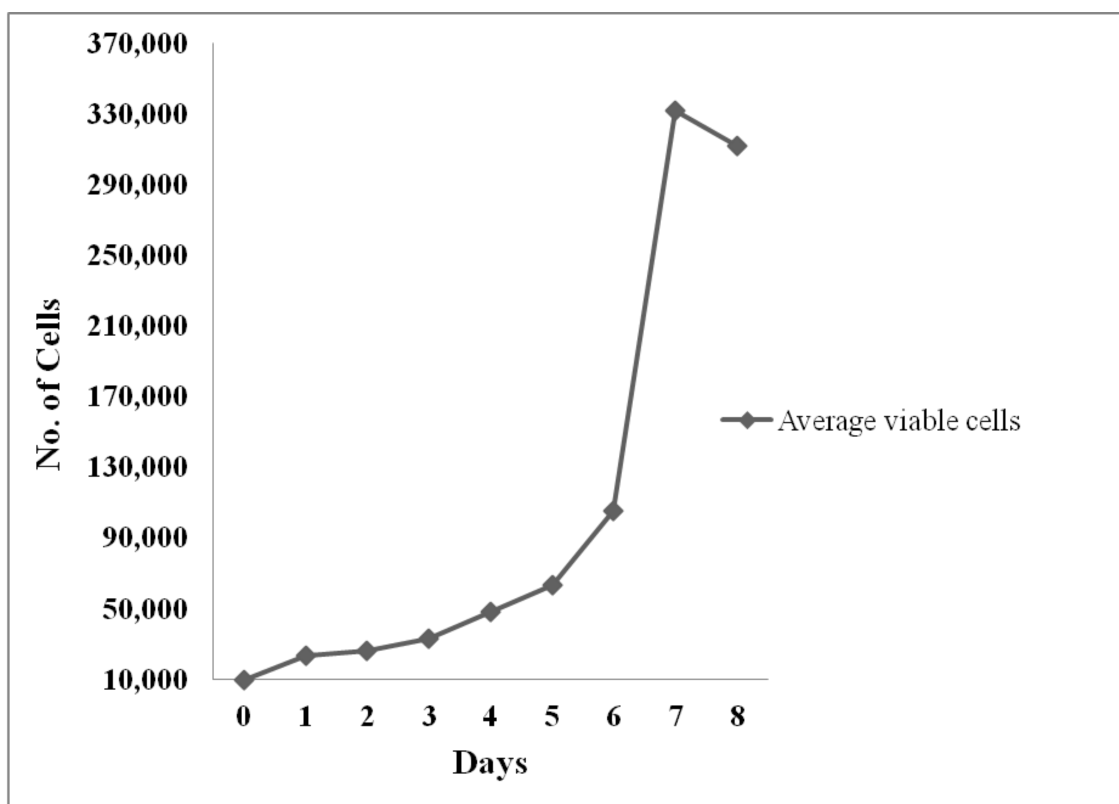


Figure 5.1 The growth curve for MCF-7 cell line

5.1.2 TFF1 expression in parental MCF-7 cells

In this study we took advantage of the fact that *TFF1* is an estrogen regulated gene, therefore it was essential to examine the intrinsic TFF1 expression status of the cells. Our laboratory has already confirmed the expression of both ERα and ERβ in this strain of MCF-7 cells.

The western blot assay confirmed TFF1 (figure 5.2) expression in the cell line cultured in complete DMEM supplemented with 10% FBS. MDA-MB 231, a breast cancer cell line taken as a negative control for TFF1. β-actin was used as the internal loading control.

The western blot assay demonstrated the presence of TFF1 and the estrogen receptors (ERs) thus qualifying this strain of MCF-7 for further manipulation as required for the study.

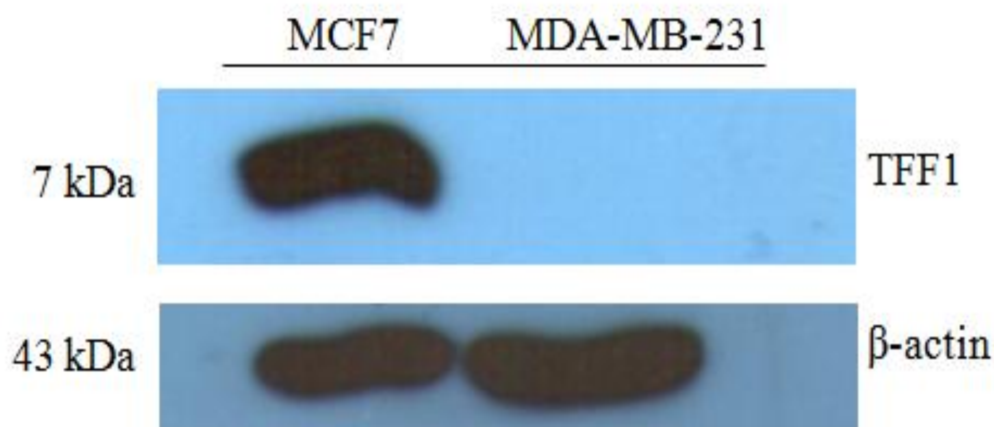


Figure 5.2 TFF1 expressions by the MCF-7 cell compared to MDA-MB-231 breast cancer cells cultured in complete DMEM supplemented with 10% fetal bovine serum

5.2 Generation of MCF7 breast cancer cell, knocked down for endogenous *TFF1* expression

A stable *TFF1* knockdown (*TFF1*-kd) MCF-7 cell was created by knocking down *TFF1* using shRNA strategy. Five potential complementary sequence inserts (shTFF1#14 to shTFF1#18), or *TFF1*-specific shRNA sequences to *TFF1* gene, picked from the TFF1 mRNA (19 nucleotides each) were used to stably modify MCF-7 cells using pLKO.1-puro plasmid DNA vector. Empty vectors (without the *TFF1* specific sequence inserts) were transfected into MCF-7 cells to generate the mock controls for the experiment. The transfected cells were then selected by 1 μg/ml of puromycin in DMEM. The positively transfected cells, resistant to puromycin were collected and propagated until adequate confluency was achieved. No successful gene knock down was achieved with other shTFF1 containing plasmids except for shTFF1#14 (figure 5.3). A knockdown efficiency of about 74% (figure 5.4) has been achieved in comparison to the empty vector transfected mock MCF-7 cells using Image J software analysis of the protein bands. Parental and the mock transfected MCF-7 cells retained its intrinsic expression.

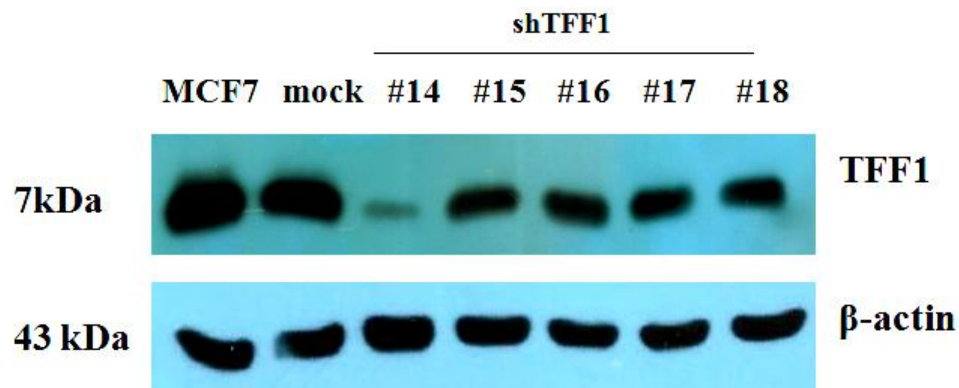


Figure 5.3 SDS-PAGE of lysates from MCF7 cells transfected with plasmid vectors (#14, #15, #16, #17, and #18) carried out on a 15-20% gradient gel and probed by specific antibody to TFF1. The ECL developed x-ray film represents successful knockdown of *TFF1* using clone #14

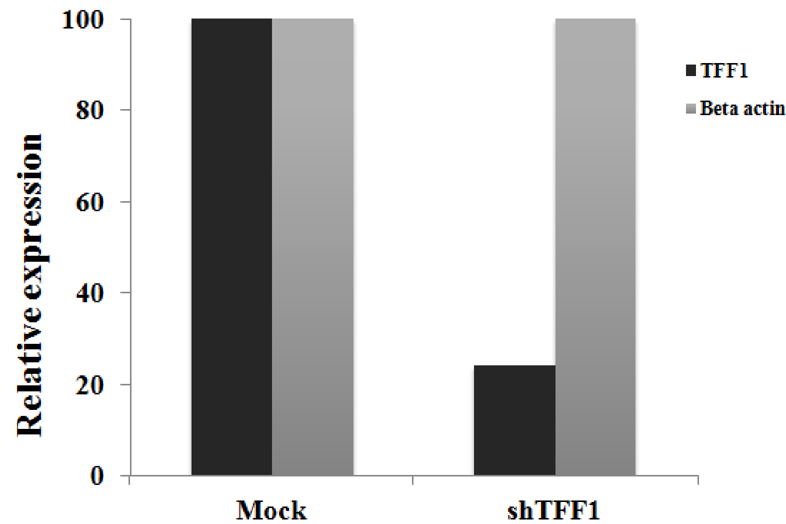


Figure 5.4 Analysis of TFF1 band intensity in the *TFF1*-kd and the mock MCF-7 cells with beta actin as the loading control using Image J software

5.3 Viability of MCF-7 in 1 nM 17β-estradiol

The *TFF1*-kd cells were treated with 1 nM 17β-estradiol (E2) after prolonged culture for 48 hrs in phenol-red free medium, with corresponding controls to examine, the differential effect of estrogen on the viability and/proliferation of mock and *TFF1*-kd cells following 18 hrs treatment. FACS analysis showed an increase in the viable number of mock cells treated with estrogen. However, there was no statistical significance when compared between the untreated controls. The *TFF1*-kd cell was indifferent to E2 treatment as shown in figure 5.5.

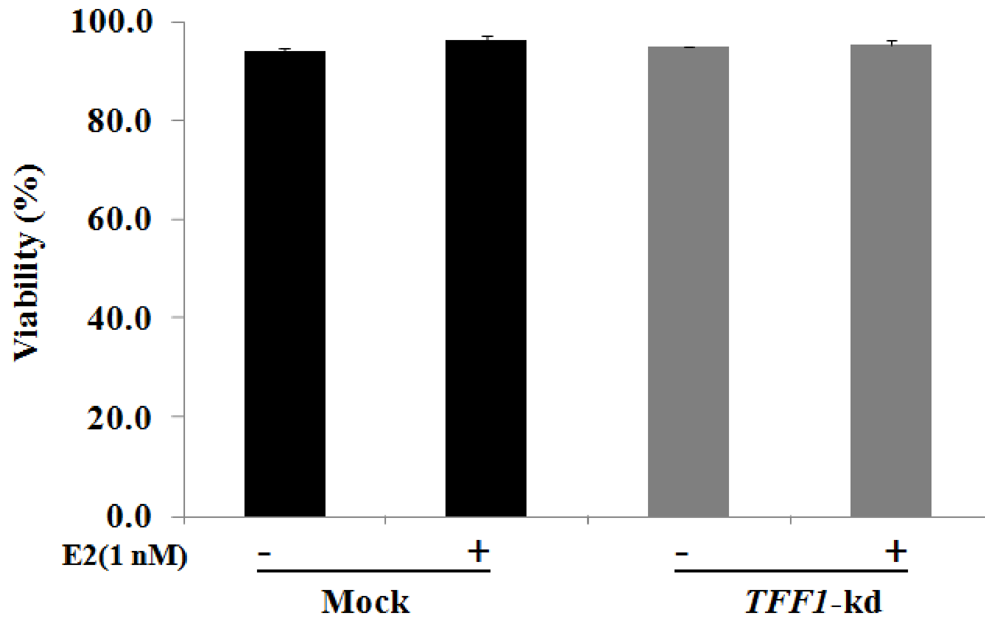


Figure 5.5 Viability of mock and *TFF1*-kd MCF-7 cells in presence of E2 (E2 = 17β-estradiol)

5.4 Apoptosis assay

The mock and the *TFF1*-kd cells incubated with 1 μM doxorubicin and 1 nM E2 in combination or separately for 18 hrs were subjected to FITC-conjugated Annexin V/PI staining and analyzed by flow cytometer as shown by the dot plots in figure 5.6.

Treatment with 1 μM doxorubicin alone showed significant decrease in viability of both the mock (P = 0.004) and the *TFF1*-kd cells (P = 0.003). In the presence of 1 nM E2 alone, the viability of the *TFF1*-kd and the mock cells were indifferent. E2 reversed the doxorubicin induced apoptosis in E2 co-treated conditions (P = 0.016) but was not observed in the *TFF1*-kd cells (P = 0.335). The viability of the *TFF1*-kd cells decreased (P = 0.024) when compared to the mock cells under E2 and doxorubicin co-treatment (figure 5.7).

At the 18th hr of the E2 and doxorubicin co-treatment, mock cells showed increased early apoptosis (P = 0.004) compared to the only E2 treated condition but

the *TFF1*-kd cells showed significantly increased cell population in the early apoptosis (*P* = 0.003) as shown in figure 5.8.

In the mock cells, late apoptosis significantly increased in the doxorubicin treated condition (*P* = 0.035) but the doxorubicin co-treatment with E2 reduced the cell number in the late apoptosis (*P* = 0.005) unlike in the *TFF1*-kd cells where E2 did not reduce the cell number in the late apoptosis phase (figure 5.9).

The necrosis stage had similar trend as in the late phase with significant cell death in the *TFF1*-kd cells even in presence of E2 (*P* = 0.034) as in figure 5.10. The doxorubicin and E2 co-treatment significantly reduced necrosis in the mock cells (*P* = 0.026) but this effect was not seen in the *TFF1*-kd cells.

Figure 5.11 shows the massive apoptosis (early and late) caused by 10 µM doxorubicin, treated for 18 hrs and the estrogen mediated protection of the mock cells from doxorubicin treatment. The estrogen treatment in the *TFF1*-kd cells could not protect from apoptosis with massive cell death when compared to the mock cells under similar treatment (*P* = 0.001).

Taken together, doxorubicin treatment caused significant apoptosis in both the mock and *TFF1*-kd cells but the addition of E2 could protect only the mock cells.

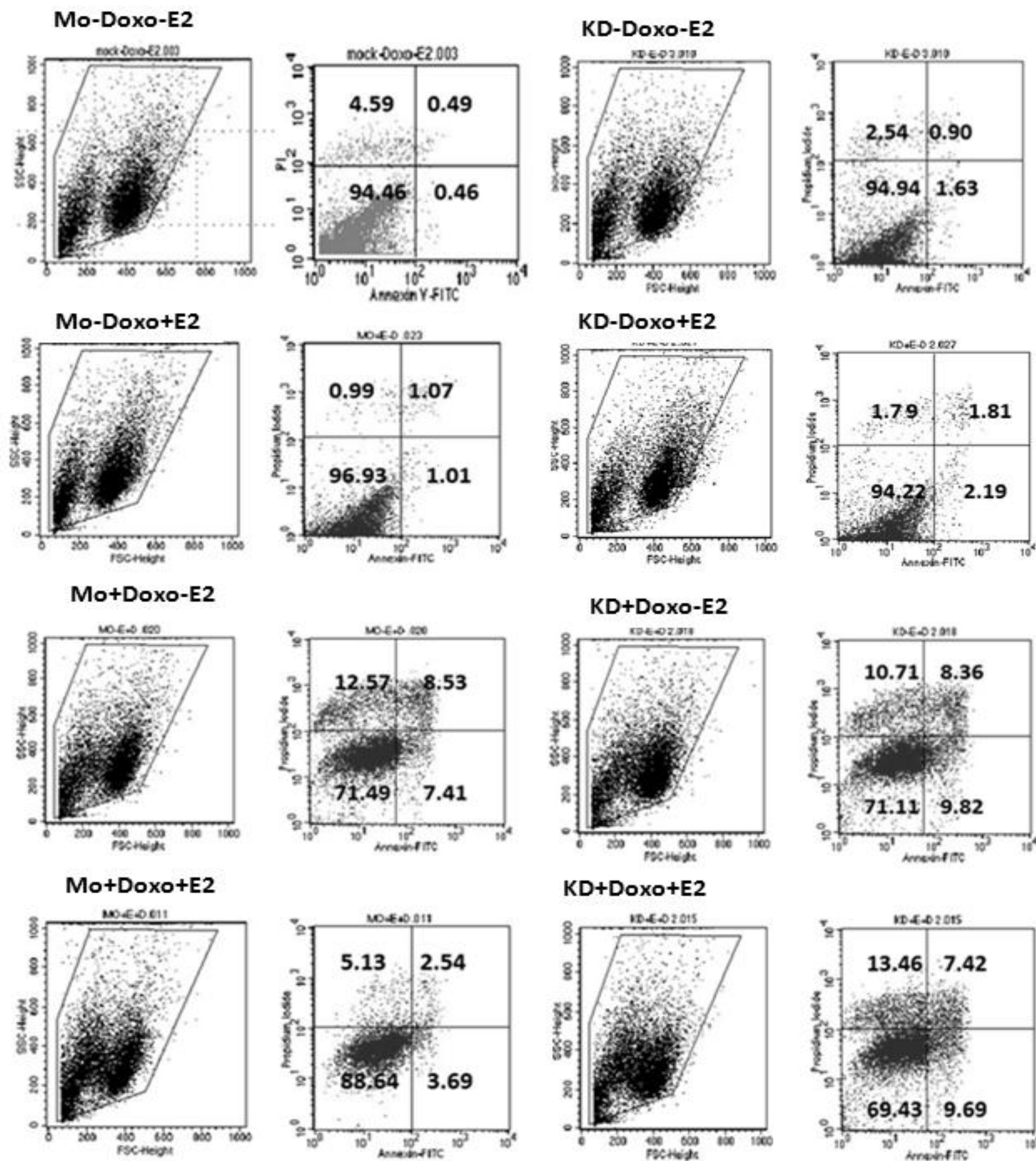
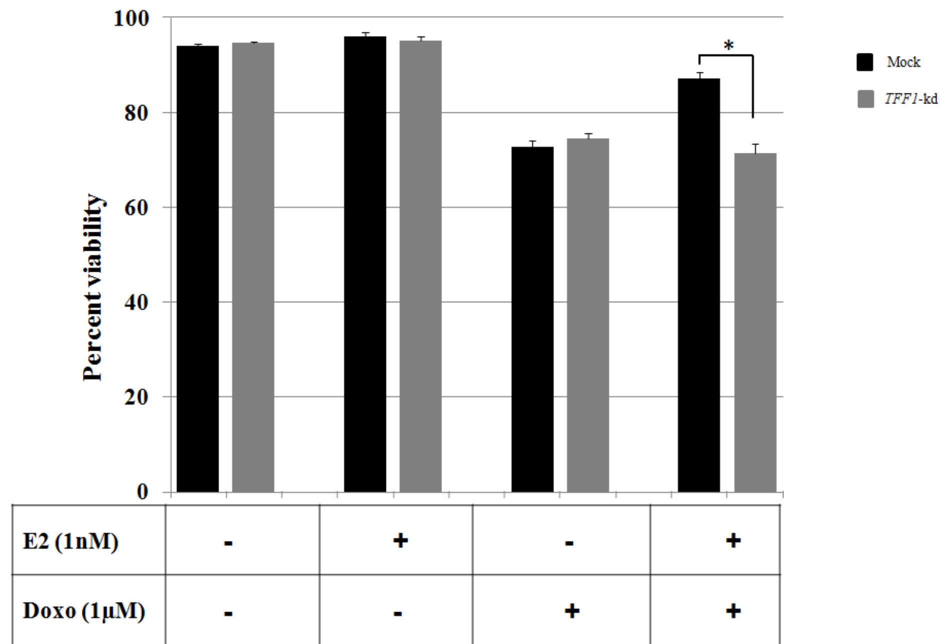


Figure 5.6 Dot plot showing distribution of cells in various stages of apoptosis following treatment with/without 1 μM doxorubicin and 1 nM E2 stimulation for 18 hrs.



E2 (1nM)	-	+	-	+
Doxo (1µM)	-	-	+	+

Figure 5.7 Percentage viability of mock and *TFF1*-kd cells following 18 hrs treatment with/without 1 µM doxorubicin and 1 nM E2 stimulation (*P = ≤ 0.05, NS = Non significant)

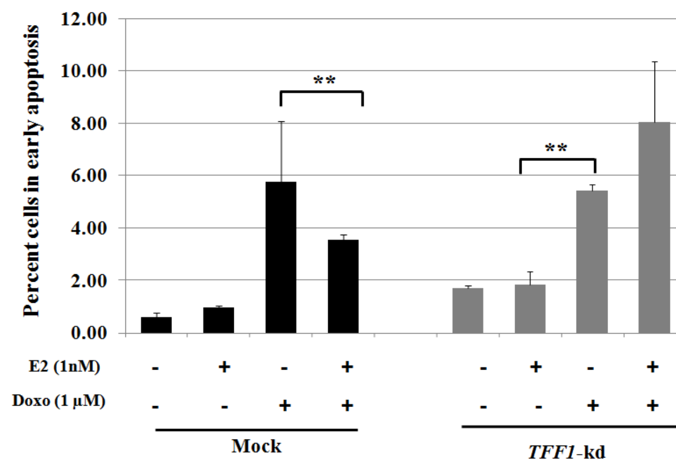


Figure 5.8 Percentage of mock and *TFF1*-kd cells in early apoptosis following 18 hrs treatment with/without 1 µM doxorubicin and 1 nM E2 stimulation (*$P \leq 0.05$, ** $P < 0.001$, NS = Non significant)

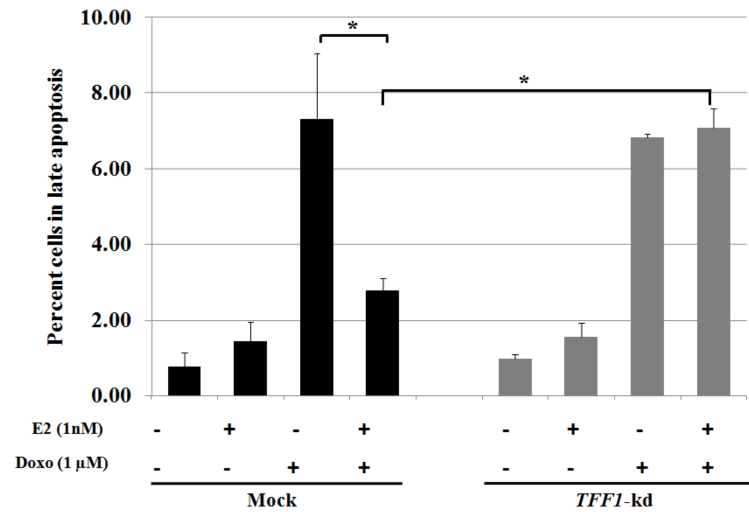


Figure 5.9 Percentage of mock and *TFF1*-kd cells in late apoptosis following 18 hrs treatment with/without 1µM doxorubicin and 1 nM E2 stimulation (**P* ≤ 0.05), NS = Non significant)

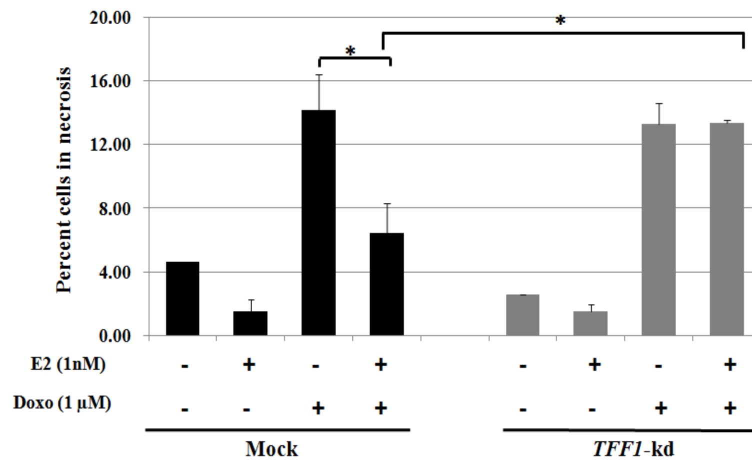


Figure 5.10 Percentage of necrosis in mock and *TFF1*-kd cells following 18 hrs treatment with/without 1 µM doxorubicin and 1 nM E2 stimulation (**P* = ≤ 0.05, NS = Non significant)

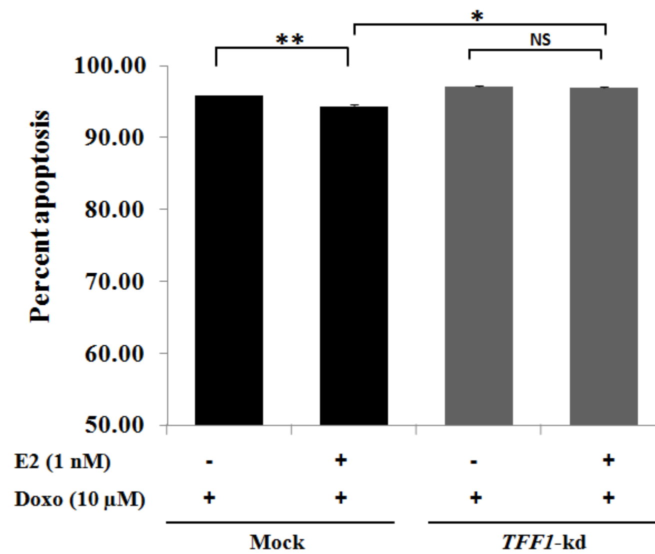


Figure 5.11 The effect of estrogen on the apoptotic effect of 10 µM doxorubicin (**P* < 0.05, ** *P* < 0.01, NS = Non significant)

5.5 Effect of neutralizing secreted TFF1 with anti-human TFF1 antibody

It was necessary to examine if the neutralization of the extracellular/secreted TFF1 had any effect on the doxorubicin induced apoptosis. The TFF1 antibody used here was the same one that used to detect TFF1 in western blot. Cell viability in MCF-7 cells treated with 100 µg/ml of the anti-hTFF1 pAb in presence and/absence of doxorubicin over 18 hrs, was investigated by using trypan blue.

One µM doxorubicin caused significant cell death in the mock cells ($P < 0.001$) but E2 could significantly rescue the cells ($P < 0.001$) from apoptosis. The addition of anti-hTFF1 pAb showed obvious neutralization of TFF1 by increasing the number of cell death significantly ($P = 0.029$) compared to the reversal effect exhibited by the estrogen treatment. The addition of doxorubicin to the condition where the secreted TFF1 has been neutralized aggravated cell death as illustrated in figure 5.12.

For trypan blue, cells were counted in triplicates each time using a cell counting chamber. The floating cells were also collected, mixed with the adherent cells and counted altogether under high power field (40X) to ensure that there would be no loss of the treatment induced dead cells.

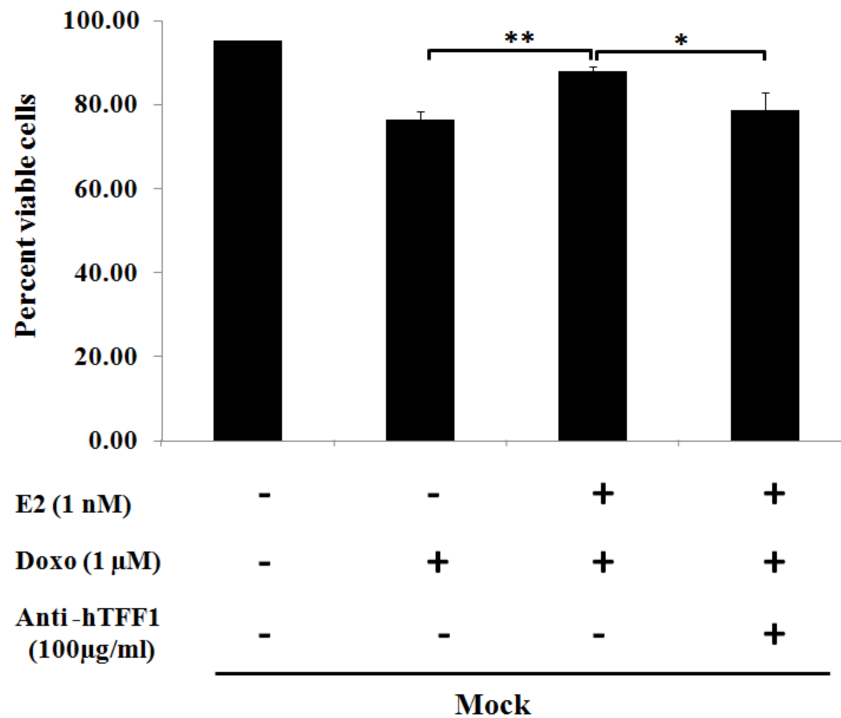


Figure 5.12 The effect of neutralizing secreted TFF1 on the doxorubicin induced apoptosis (*$P \leq 0.05$, ** $P < 0.01$)

5.6 Treatment of the *TFF1*-kd cells with rTFF1 and E2

TFF1 was reconstituted to the *TFF1*-kd cells by treating full length recombinant TFF1 at concentrations 100 µg/ml and 10 µg/ml. The *TFF1*-kd MCF-7 cells were incubated with doxorubicin in the presence and absence of E2. The ability of the rTFF1 to rescue the cells from doxorubicin induced apoptosis was assayed using trypan blue cell counting method and fluorescence microscopy. Anti-apoptotic effect by recombinant TFF1 was evident with maximum at 100 µg/ml ($P = 0.050$) as shown in figure 5.13. Concentration of 10µg/ml could not revert the cell death from

doxorubicin. Since it was not a complete knockdown of *TFF1*, the rescue effect of estrogen was also examined. The result was in total agreement with that of the flow cytometry where the E2 did not affect the viability of the *TFF1*-kd cells under the doxorubicin treated condition ($P = 0.104$).

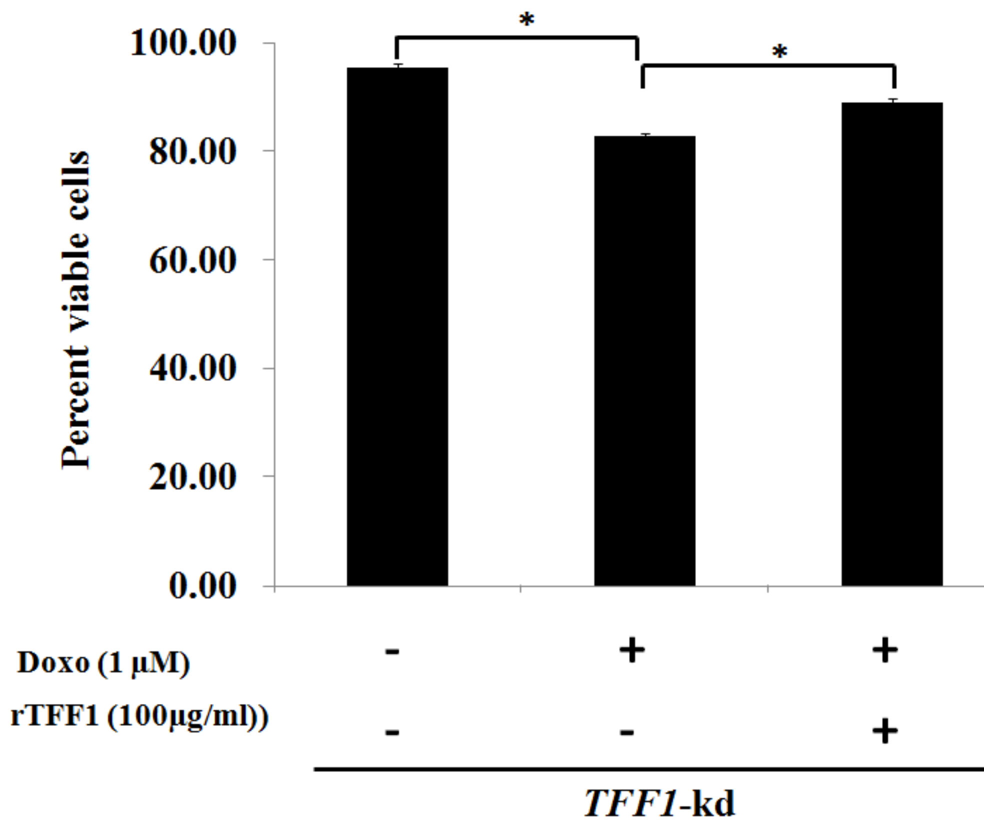


Figure 5.13 The effect of rTFF1 and E2 to rescue the *TFF1*-kd cells from doxorubicin induced apoptosis (*$P \leq 0.05$)

5.7 Effect of estrogen receptor antagonist on the mock cells

To prove that the ER signalling is indispensable, the cells were treated with fulvestrant to inhibit the estrogen receptors and the downstream effect on cell survival was investigated. The toxicity test carried out on the parental MCF-7 cells with concentration ranging from 0.0, 0.1, 1, 10 and 100 µM in a phenol red containing DMEM (figure 5.14). One hundred µM exhibited significant cell death at 18 hrs.

Estrogen has been shown to have proliferative and prosurvival effects on the MCF-7 cells. In this experiment the fulvestrant was expected to block the action of the added estradiol by down regulating the ERs in the MCF-7 cells, to further elucidate the antiapoptotic role of estrogen induced TFF1 following chemotherapeutic drug treatment. Doxorubicin induced apoptosis, was then investigated by comparing the percent viable cells and the apoptotic cells under different treatment conditions including an untreated control, by using trypan blue cell counting method over 18 hrs treatment.

Figure 5.15 shows that the percent viability significantly (P = <0.001) recovered after adding E2 where as the fulvestrant failed to show and appreciable decrease in the viability as expected.

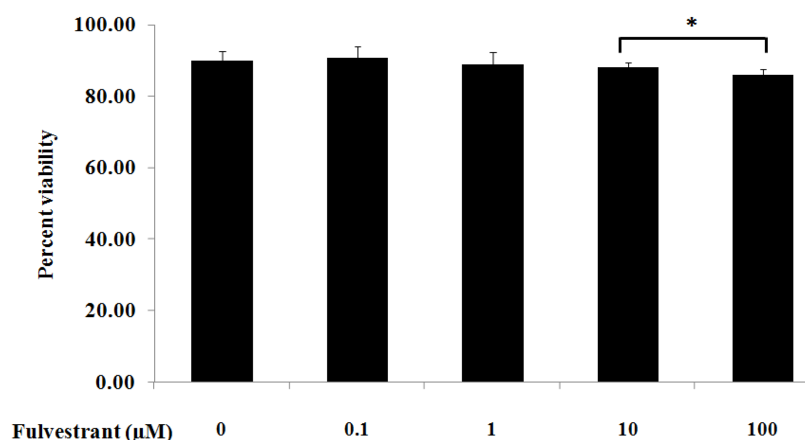


Figure 5.14 Fulvestrant toxicity test (*$P \leq 0.05$)

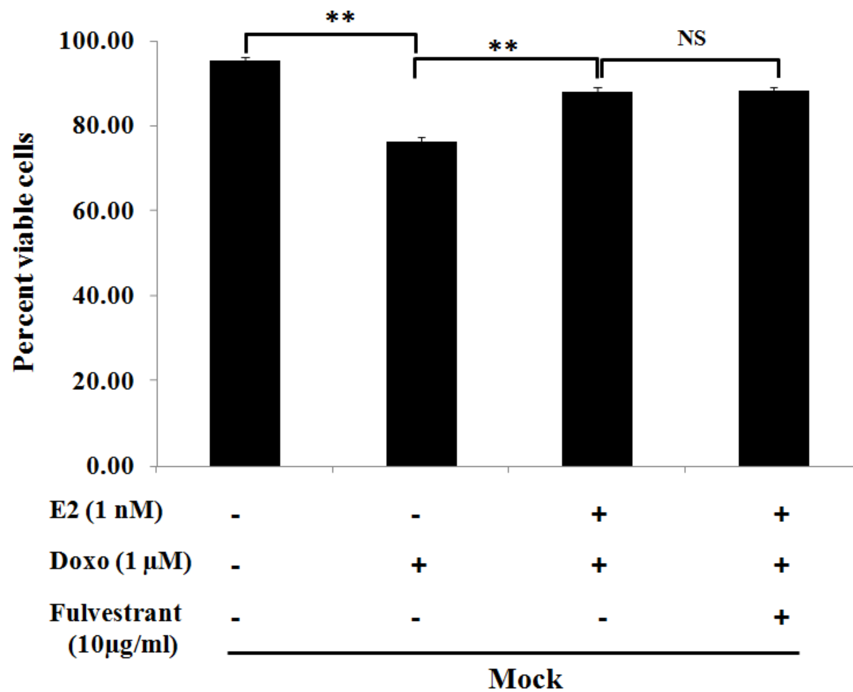


Figure 5.15 The effect of antagonizing ERs on the doxorubicin induced apoptosis (**$P \leq 0.001$, NS = Non significant)

5.8 Proteome Microarrray

5.8.1 Changes in the apoptotic associated protein

To investigate changes in the protein expression under different conditions, four different treatment conditions were set up including a non treated control. Doxorubicin mediated protein expressions were compared to that of the untreated mock. Doxorubicin and E2 co-treated condition was compared to the doxorubicin treated mock to examine the E2 effect. TFF1 effect was compared between the mock and *TFF1*-kd cells co-treated with doxorubicin and E2. Figure 5.16 represents the ECL developed protein array result with mentioned conditions.

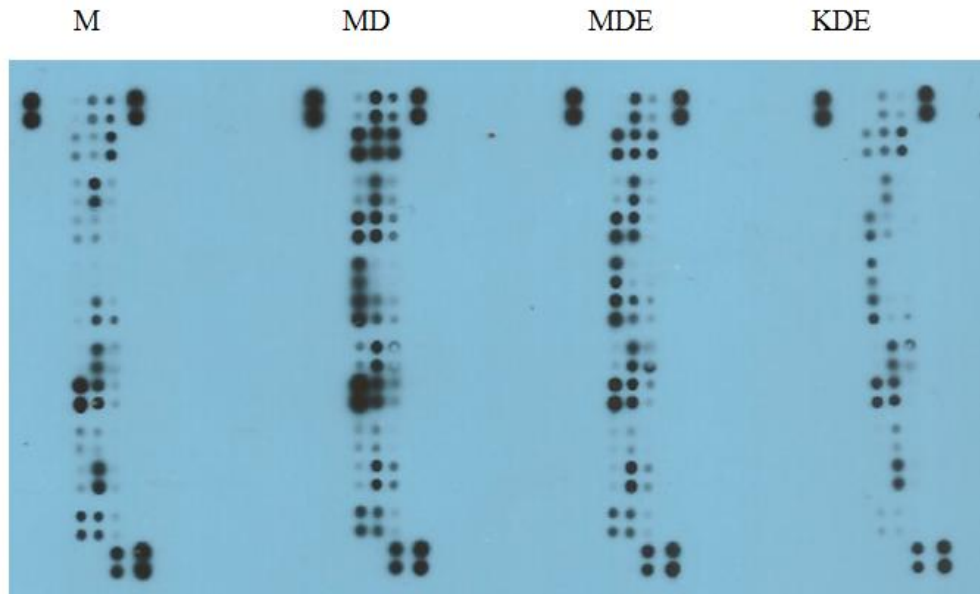
Out of the 35 proteins on the array, doxorubicin significantly upregulated expression of 30 proteins of which E2 reduced 6 and upregulated 9 significantly. The rest showed no evident changes but maintained its upregulated expression. Of the proteins upregulated by doxorubicin, E2 treatment reduced expression of phosphor-Rad17 (P = 0.022), Bad (P = 0.003), Bax (P = 0.006) and upregulated the prosurvival

proteins including Bcl-2 (*P* = 0.032), Bcl-x (*P* = 0.001), cIAP-1 (*P* = 0.039) and cIAP-2 (*P* = 0.019). Table 5.1 shows the folding changes in the protein expression stimulated by doxorubicin, E2 and TFF1.

Some of the E2 upregulated prosurvival proteins could not be expressed in the *TFF1*-kd cells which maintained significantly lower level of expression, such as Bcl-x (*P* < 0.001), Bcl-2 (*P* < 0.001), clusterin (*P* = 0.002), p27/kip1 (*P* = 0.002), p21/CIP1/CDNK1A (*P* = 0.003), XIAP (*P* < 0.001) as well as cIAP-1 prosurvival protein. *TFF1*-kd cells reveal significantly lower level of the survival protein survivin (*P* = 0.045) compared to the mock cells.

Doxorubicin increased clusterin expression (*P* = 0.019), Fas/TNFRSF6 (*P* < 0.001), TRAILR1/DR4 (*P* = 0.008), TRAILR2/DR5 (*P* < 0.001), and FADD (*P* = 0.049), cytochrome c (*P* = 0.049) and smac/Diablo (*P* = 0.013). Figures 5.17 through 5.27 shows the relative expression of proteins under doxorubicin and E2 treatment conditions.

Moreover this experiment also showed significant increase in the expression of anti-oxidative enzyme catalase (*P* = 0.006), and PON2 (*P* = 0.016) following doxorubicin treatment. Heme oxygenase 1 (HO-1/HMOX/HSP32) was another anti-oxidative enzyme whose expression was minimal in the *TFF1*-kd cells (*P* < 0.001), with even catalase showing lower expression compared to the mock.



M; Untreated mock cells

MD; Mock cells + doxorubicin

MDE; Mock cells + doxorubicin + E2

KDE; TFF1-kd cells + doxorubicin + E2

Figure 5.16 The ECL developed apoptotic protein array film for the mock cells and *TFF1-* kd MCF-7 cells treated with doxorubicin and in the presence or absence of E2 for 18 hours.

Table 5.1 Expression of TFF1 and estrogen regulated apoptosis associated proteins

		Fold change			*P* values		
Proteins	**M**	**MD**	**MDE**	**KDE**	**M to MD**	**MD to MDE**	**MDE to KDE**
Bad	0.14	1.59	0.78	0.39	0.048	0.003	0.076
Bax	0.36	1.82	1.23	1.20	0.002	0.006	0.388
Bcl-2	0.01	3.61	2.95	1.19	0.032	0.296	<0.001
Bcl-x	0.00	26.87	5.99	2.26	0.001	0.002	<0.001
Pro-caspase-3	0.00	15.62	18.21	3.56	0.007	0.585	0.065
Cleaved caspase-3	0.06	0.70	1.32	0.80	0.75	0.02	0.401
Catalase	0.03	1.82	9.29	5.53	0.006	0.219	0.487
cIAP-1	0.02	4.48	5.19	1.11	0.039	0.72	0.124
cIAP-2	0.00	2.87	2.51	1.91	0.019	0.628	0.415
Claspin	0.02	6.16	6.81	1.53	<0.001	0.361	0.011
Clusterin	0.01	2.00	3.44	0.72	0.019	0.008	0.002
Cytochrome C	0.53	1.20	1.21	1.00	0.049	0.926	0.198
TRAIL R1/DR4	0.12	5.36	4.68	1.46	0.008	0.29	0.014
TRAIL R2/DR5	0.03	28.01	17.15	9.77	<0.001	0.008	0.014
FADD	0.43	1.37	1.25	0.77	0.049	0.298	<0.001
Fas/TNFRSF6	0.03	24.95	23.43	3.30	<0.001	0.485	0.008
HIF-1a	0.00	17.68	18.13	3.45	0.024	0.91	0.027
HO-1/HMOX/HSP32	0.21	1.34	1.81	0.15	0.236	0.043	<0.001
HO-2/HMOX2	0.39	1.46	1.63	1.28	0.009	0.138	0.049
HSP27	0.47	1.85	1.33	1.19	0.005	0.018	0.388
HSP60	0.02	2.91	4.55	6.03	0.013	0.048	0.524
HSP70	0.60	0.72	1.00	0.97	0.004	0.007	0.223
HTRA2/Omi	0.23	1.15	1.49	0.11	0.123	0.048	0.002
Livin	0.01	1.02	1.40	1.12	0.973	0.603	0.755
PON2	0.01	4.82	1.30	1.14	0.016	0.019	0.576
p21/CIP1/CDNK1A	0.05	18.72	13.68	3.65	0.004	0.049	0.003
p27/Kip1	0.01	4.16	3.17	0.55	0.005	0.036	0.002
Phospho-p53 (S15)	0.02	28.24	33.71	18.36	<0.001	0.294	0.059
Phospho-p53 (S46)	0.00	356.41	318.87	152.93	<0.001	0.214	0.021
Phospho-p53 (S392)	0.01	127.45	112.97	65.98	0.001	0.314	0.044
Phospho-Rad17 (S635)	0.01	6.35	3.94	1.59	0.003	0.022	0.043
SMAC/Diablo	0.79	1.53	1.10	0.69	0.013	0.031	0.043
Survivin	0.02	1.28	1.44	0.39	0.427	0.552	0.045
TNF RI/TNFRSF1A	0.02	0.99	1.36	0.30	0.964	0.026	0.001
XIAP	0.27	1.28	1.23	0.14	0.019	0.353	<0.001

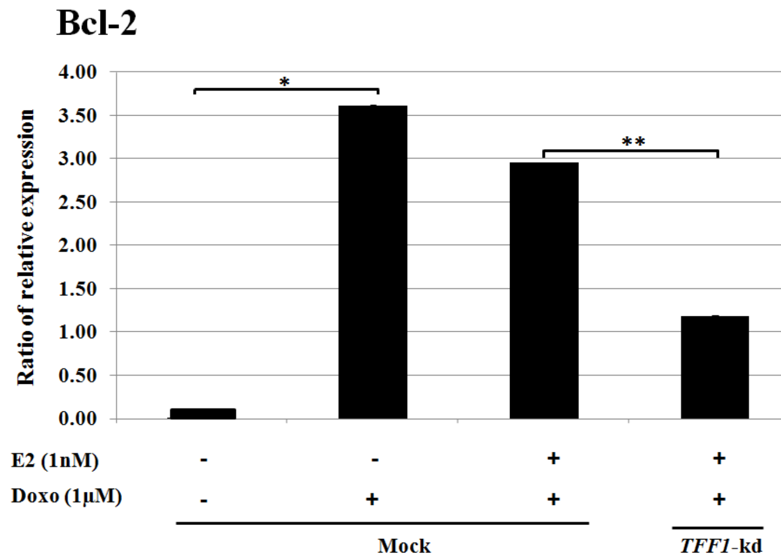


Figure 5.17 Differential expression of Bcl-2 in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (*$P \leq 0.05$, ** $P < 0.01$)

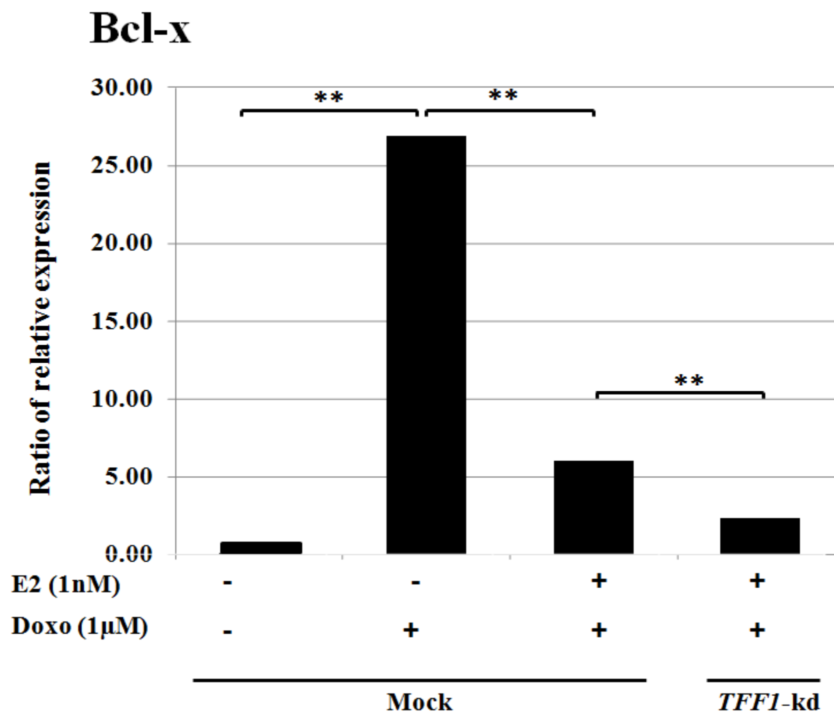


Figure 5.18 Differential expression of Bcl-x in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (** $P < 0.01$)

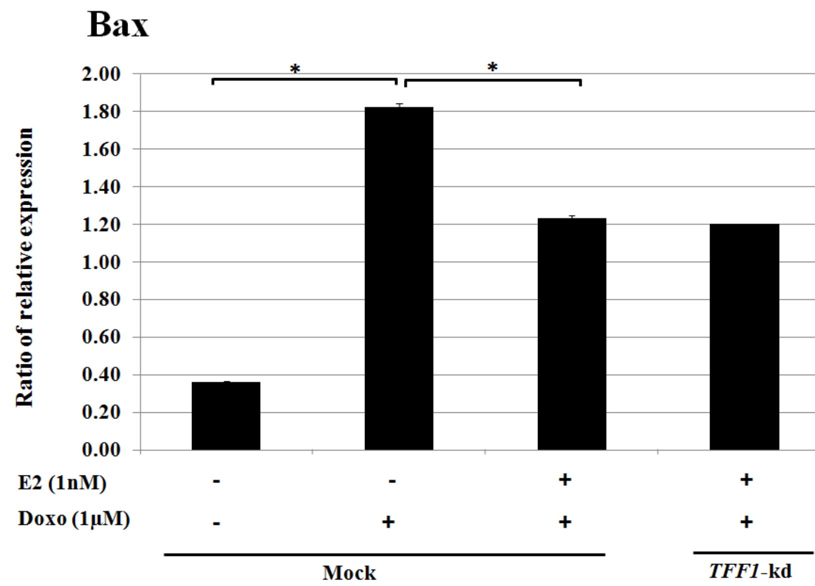


Figure 5.19 Differential expression of Bax in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (*$P \leq 0.05$)

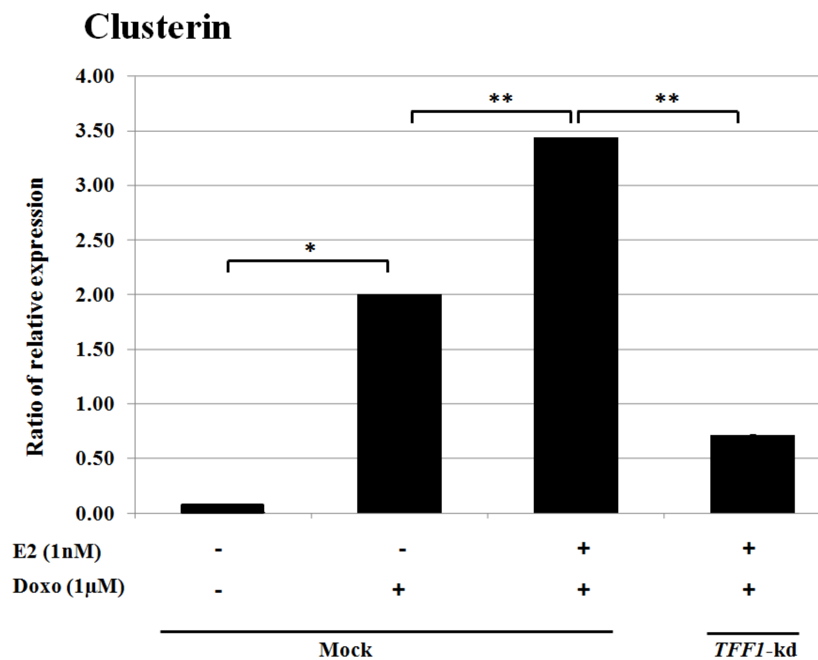


Figure 5.20 Differential expression of clusterin in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (*$P = \leq 0.05$, ** $P < 0.01$)

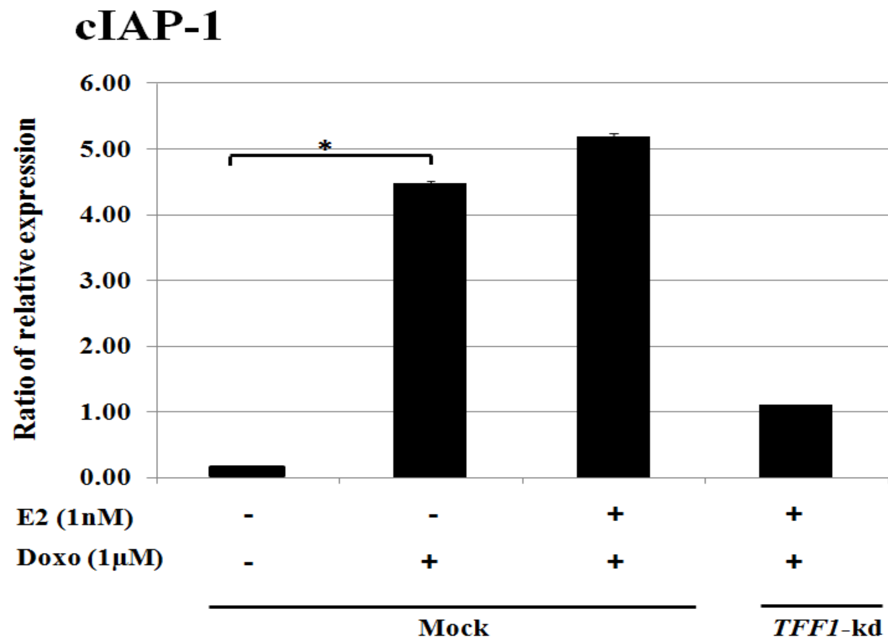


Figure 5.21 Differential expression of cIAP-1 in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (*$P \leq 0.05$)

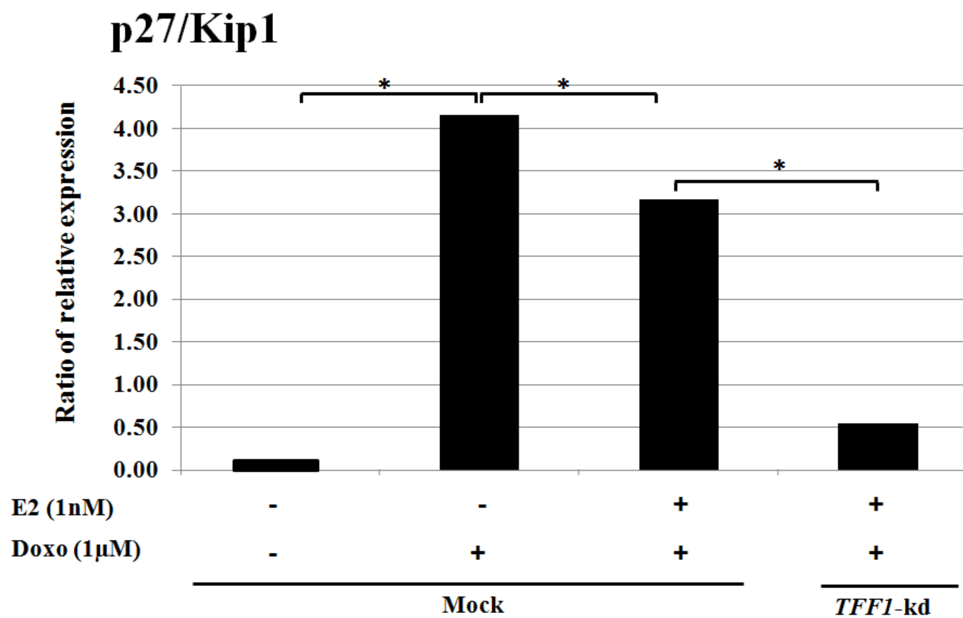


Figure 5.22 Differential expression of p27/Kip1 in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (*$P \leq 0.05$)

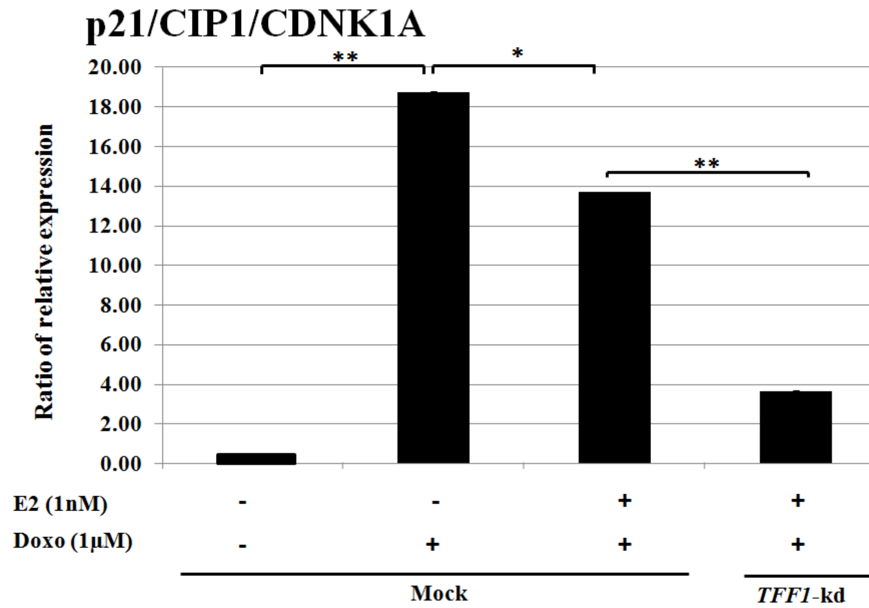


Figure 5.23 Differential expression of p21/CIP1/CDNK1A in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (*$P \leq 0.05$, ** $P < 0.01$)

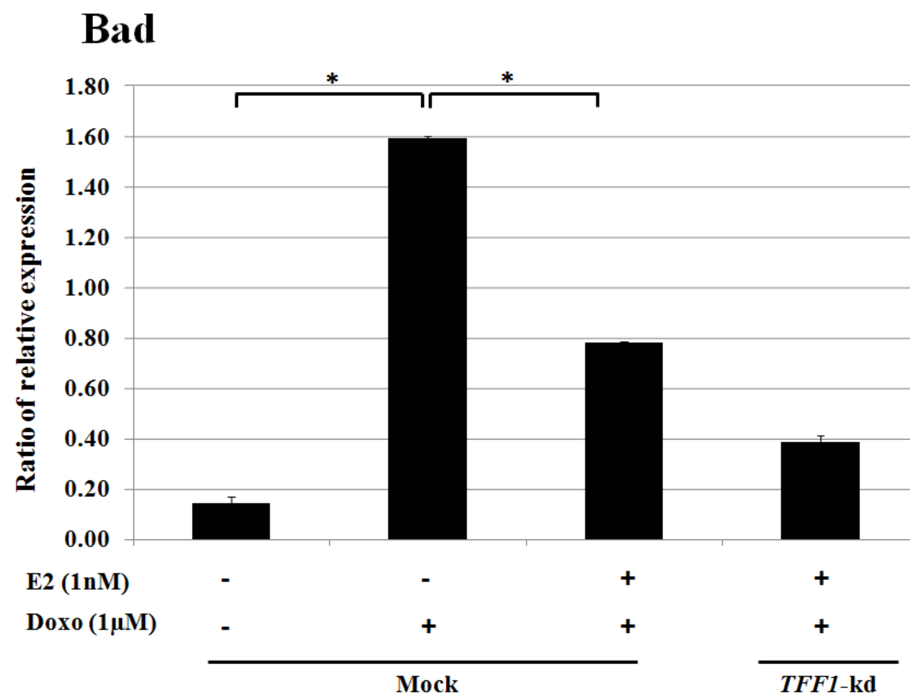


Figure 5.24 Differential expression of BAD in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (*$P = \leq 0.05$)

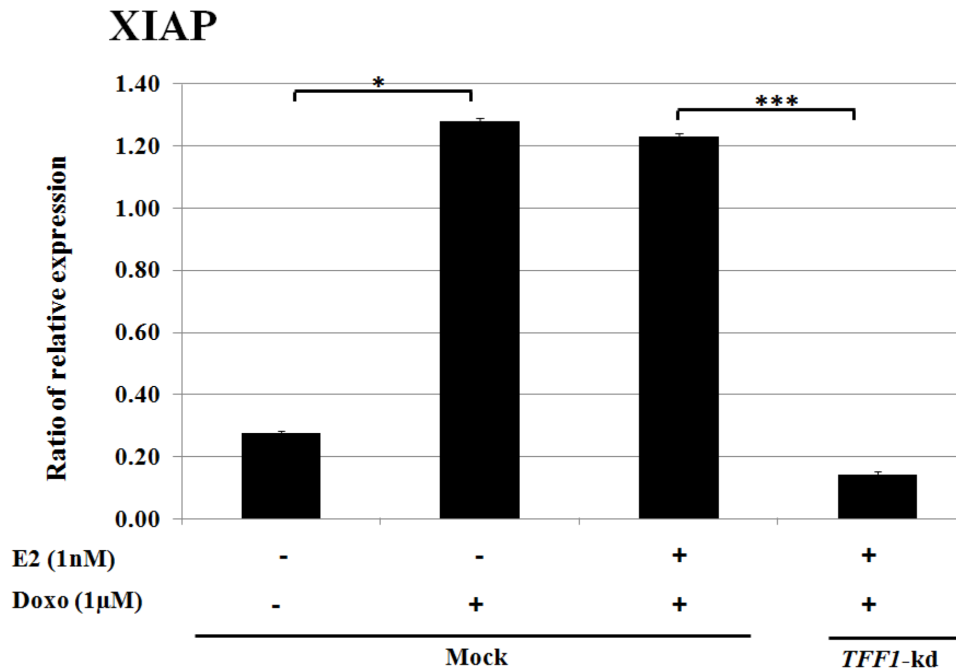


Figure 5.25 Differential expression of XIAP in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (**P* = ≤ 0.05, *** *P* = ≤ 0.001)

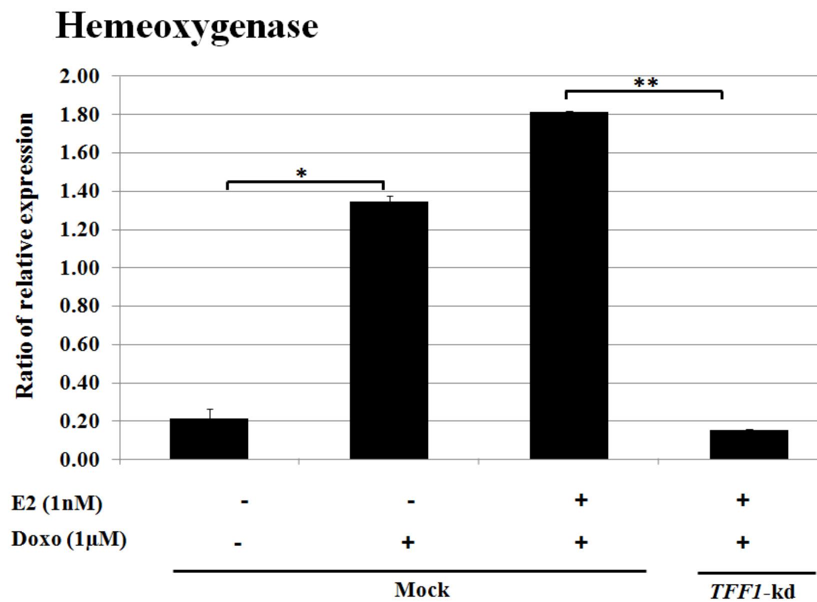


Figure 5.26 Differential expression of hemeoxygenase 1 in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (**P* ≤ 0.05, ** *P* < 0.01)

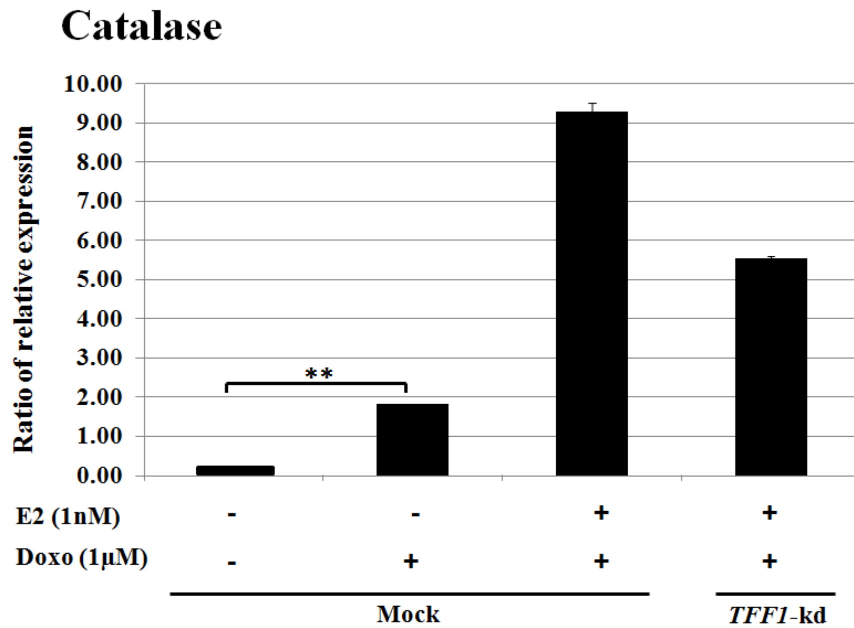


Figure 5.27 Differential expression of catalase in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (**$P = \leq 0.05$)

5.8.2 Catalase Assay

To correlate catalase expression in the protein array (figure 4.27), the cells were treated under similar conditions as that for the array were lysed and their specific catalase activity was determined photometrically (figure 4.28) which generated not just a similar trend but statistically significant catalase activity among different treatment conditions.

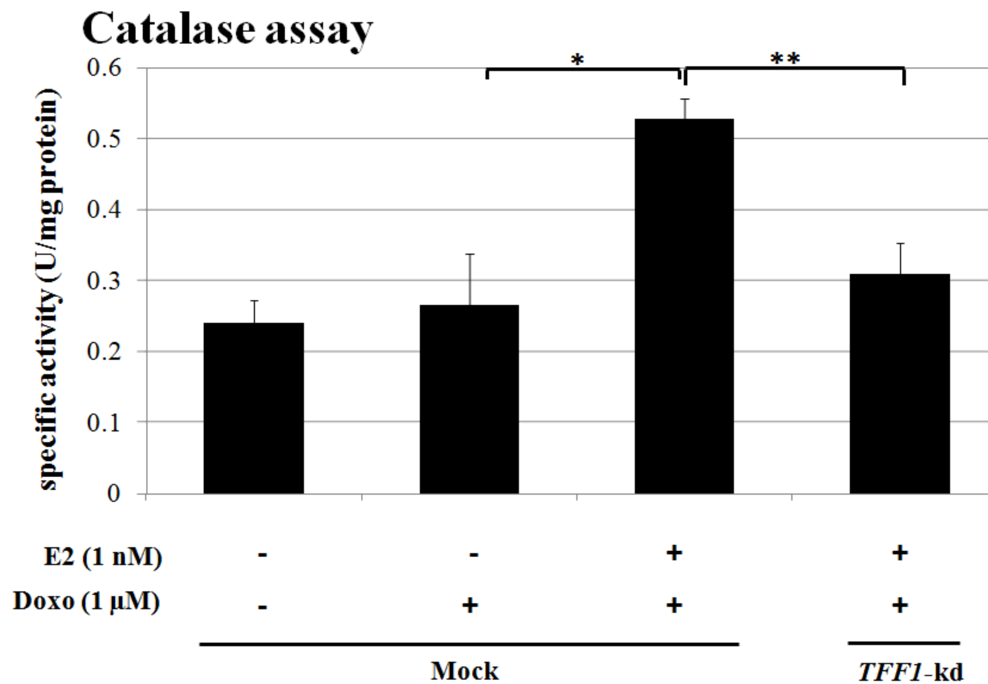


Figure 5.28 Photometrically determined specific catalase activities in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium (**P* ≤ 0.05, ** *P* < 0.01)

5.9 TFF1 expression mock cells under different treatment conditions

As proved earlier (figure 4.2) mock cells cultured in phenol red containing DMEM was able to express TFF1 abundantly. This experiment was aimed at examining the TFF1 induction in the mock cells under different treatment conditions by culturing the cells in the phenol red free media for 48 hrs following initial seeding in the phenol red containing DMEM. Fresh media was supplemented containing 1nM 17β-estradiol, 1µM doxorubicin and 10 µM fulvestrant with respective controls and appropriate combinations. After 18 hrs the cells were harvested and western blot was carried out against specific TFF1 polyclonal antibody as seen in figure 4.29 and 4.30.

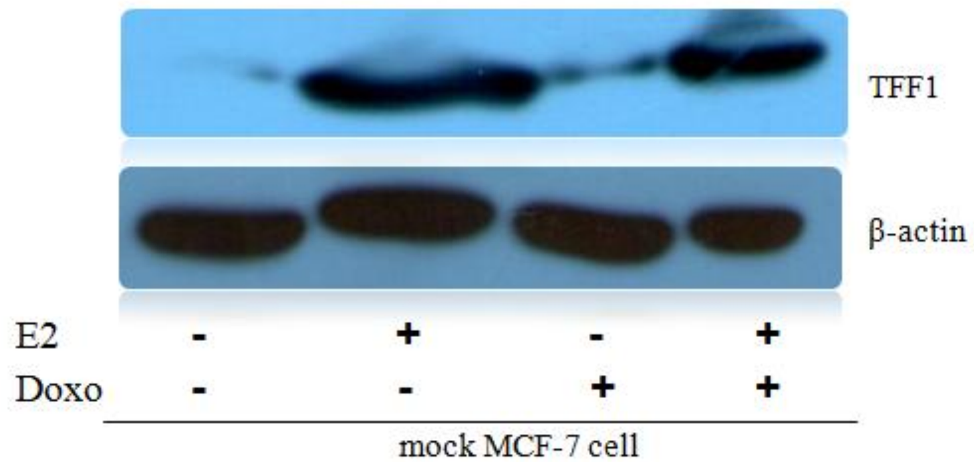


Figure 5.29 TFF1 Expression in mock cells, in response to doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium

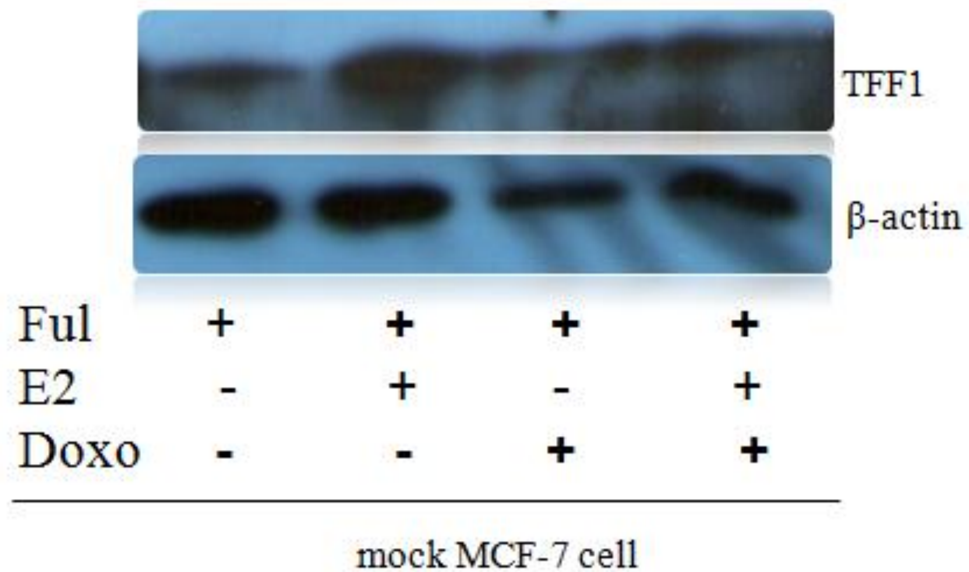


Figure 5.30 TFF1 Expression in mock cells, in response to fulvestrant, doxorubicin and estrogen treatment for 18 hrs in phenol red-free medium

CHAPTER VI
DISCUSSIONS

This study largely involved, 17-β estradiol (E2) stimulation of the MCF-7 cells for TFF1 induction. No obvious change was noticed in the morphology of the MCF-7 cells following transfection with the plasmid bearing the *TFF1* sequence insert or the empty plasmid (mock) from the parental MCF-7 cells. In the absence of any kind of death insult, the mock and the *TFF1*-kd cells exhibited similar health and growth properties, as that of the parental cells. Substantial number of dead cell was noticed each time, on the day after passaging in the transfected cells compared to the mock cells. This in a way showed the susceptibility to cell death in the presence of a slightest disturbance to the *TFF1*-kd cells. Therefore, it's apparent that, under normal culture conditions TFF1 does not play any role in the viability of MCF-7 cells.

6.1 Viability in estrogen

Over the years, studies on estrogenic effects on the cancer cells have been associated with the hormonal control over its growth and proliferation (42) and the breast tumors were found to be regressing rapidly on estrogen ablation (62). In agreement to this, a slightly increased number of viable cells were seen in the mock MCF-7 cells treated with estrogen in this study. However, there was no statistical significance between the estrogen treated and untreated mock cells. As expected the *TFF1*-kd cells showed no difference in the cell number before and after the treatment. It cannot be undermined that the lack of significant proliferation in this experiment maybe due to the fact that estrogen mediated proliferation may require more than 18 hrs of treatment.

6.2 Apoptosis assay

Of various concentrations of doxorubicin used for inducing apoptosis, 10 μM for 18 hrs led to excessive cell death where as 1 μM for 6 hours resulted in increased viable cells. An 18 hrs period was chosen, for treatment of both mock and *TFF1*-kd cells with doxorubicin, as it resulted in acceptable cell death with significant differences among different conditions. Doxorubicin treatment resulted in appreciable apoptosis in both the mock as well as the *TFF1*-kd cells but in the presence of 1 nM E2, the viability was better or similar to that of the mock and the *TFF1*-kd control. In the absence of any insult, minimal or no apoptosis was observed in both the mock and the *TFF1*-kd cells even in the absence of E2. In the mock cells, the apoptosis induced by doxorubicin treatment was reversed in E2 co-treated conditions where as such effect was not evident in the *TFF1*-kd cells. This may be due to reduced targets for the E2 and its receptor complexes in the *TFF1*-kd cells. This undoubtedly explains that the anti-apoptotic role of estrogen is mediated via *TFF1*. It is also clear that *TFF1* does not play any role in the viability of the MCF-7 cells under normal conditions because the *TFF1*-kd cells did no exhibit obvious death in the absence of any death stimuli and was comparable to the untreated controls. There was a significant reduction in the viability of the *TFF1*-kd cells when compared to the mock cells, both subjected to E2 and doxorubicin co-treatment, suggesting that estrogen potentially reversed the apoptosis induced by doxorubicin and which may probably induce resistance to chemotherapy. The accumulation of the cells in the late apoptosis and the necrosis stages are significantly higher in both the mock and the *TFF1*-kd cells at the end of 18 hrs in the conditions that had doxorubicin treatment either with or without E2. The presence of E2 could not resist the doxorubicin toxicity in the *TFF1*-kd cells showing even higher percentages of cells than the mock in all the early, late and the necrosis phases. This also indicates that the process of doxorubicin induced apoptosis is rapid in the *TFF1*-kd cells compared to that of the mock cells. The controls and the only E2 treated conditions demonstrated similar trends with majority of the cells still in the viable stage. Treatment with 10 μM doxorubicin for 18 hrs, forced all the cells to shift to the necrosis phase in all the conditions with only doxorubicin treatment in the mock cells. The *TFF1*-kd cell population was in the necrosis phase despite of the E2

treatment. The apoptosis trend was reproducible in both the cases, except for the treatment time, in the 10 μM doxorubicin condition which was prolonged, to the extent that cell population shifted far past the early and the late apoptotic phases. Altogether, the flow cytometry result explained significant estrogen mediated protection from apoptosis in the mock cells with intact *TFF1*. The presence or absence of estrogen did not have any effect on the survival of the *TFF1*-kd cells under doxorubicin free conditions. Overall, this shows that these pro-survival effects of estrogen could possibly be mediated via TFF1 in the ER positive MCF7 cells. The increase in the percentage of viable mock cells was clearly due to the estrogenic effect mediated via TFF1 as such effect was not shown in the *TFF1*-kd cells under estrogen stimulation. This phenomenon clearly determines the role of TFF1 in estrogen-promoted resistance to apoptosis induced by doxorubicin in MCF-7 breast cancer cell.

6.3 Neutralizing secreted TFF1

TFF1 is among the early response genes that are upregulated within minutes following estrogen treatment (70) and TFF1 being a secreted protein (135) could be neutralized by using polyclonal anti-human TFF1 antibody (pAb). Neutralizing the secreted TFF1 may perhaps result in partial/total elimination of its paracrine and autocrine TFF1 pathways affecting the activation of various pro-survival proteins (6). In agreement with the flow cytometry finding, doxorubicin could cause significant cell death in the mock cells which were reversed by estrogen. Neutralization of the estrogen-induced TFF1, increased the percentage of cell death significantly compared to the reversal effect exhibited by the E2 treatment. This resulted as the *TFF1* downstream signalling was abrogated and the expression of many pro-survival genes was hindered. Thus, aggravating the doxorubicin mediated cell death.

Trypan blue cell count substantiated the findings of the flow cytometry including an anti-human TFF1 pAb treatment condition in the mock cells. However protection from estrogen is undeniable with the control which showed a viability of more than 95 percent. This shows that the abrogation of TFF1 signalling pathways greatly affected the MCF-7 cell viability.

6.4 Reconstitution of TFF1 to the *TFF1* knocked-down MCF-7 cells

The reconstitution of TFF1 to the *TFF1*-kd cells with full length recombinant TFF1 was to investigate the ability of the rTFF1 to restore the functions of the endogenous TFF1 and rescue the cells from doxorubicin induced apoptosis. Trypan blue cell count, demonstrated the reversal of anti-apoptotic property of the *TFF1*-kd MCF-7 cells following treatment with rTFF1, restoring the paracrine and the autocrine pro-survival signaling within the cell at a dose dependent manner with optimal concentration at 100 μg/ml showing significant cell rescue. Ten μg/ml of rTFF1 could not protect the cells from apoptosis. Compounded cell death resulted as a combined effect of reduced survival signalling within the cell and the doxorubicin treatment.

About a third of women with metastatic breast responded positively to estrogen ablation resulting in apoptosis (62), proving the importance of estrogen for the viability of the MCF-7 cells and cancer progression.

6.5 Antagonizing ERs with fulvestrant

Targeting the ER has been an oldest form of molecular intervention in the breast cancer therapy (56) however, the complexity of its signalling network has led to the investigation of molecules and other estrogen regulated gene products. Fulvestrant or ICI 182, 780 is a pure estrogen receptor antagonist (61). The treatment with fulvestrant should possibly engage the ERs and destroy them to an extent that the total ERs in the MCF-7 cells decrease with subsequent reduction in the ER signalling and effects in target cells or tissues. It was expected that the blockade of the ERs would facilitate the doxorubicin mediated apoptosis leading to reduced number of viable cells in doxorubicin treated conditions. Trypan blue cell counting showed that doxorubicin killing could be reversed by E2 significantly. Interestingly, adding fulvestrant appears to have minimal or no effect on the cell viability and did not affect the estrogenic protection of the doxorubicin treated cells. This phenomenon was not understood, could be attributed to the need for carefully optimized treatment conditions for visible effects. It cannot be denied, those estrogen actions on the MCF-7 cells were reportedly very rapid (70). Pre-treatment with fulvestrant before E2 in a time controlled

experiment would shed some light on this finding. It was prolonged that the ER mediated signaling has altered. It was reported that prolonged exposure to fulvestrant led to the loss of ER mediated signalling to apoptosis through interferon regulatory factor 1 (IRF-1) due to subsequent upregulation of its inhibitor NPM and its protein partner NF-kB (136). Changes in response to anti-estrogen stimulated phenotype have also been described in animal models (137). Even the cytotoxicity test failed to exhibit any cytotoxic effects until 10.0 μM. Somehow toxicity was observed at 100 μM (P = 0.002) which is a concentration 10 folds more. This non interference in the cell viability may be explained by an alternate hypothesis where the down regulation of ER led to the up regulation of the growth factor receptors (56), thus maintaining the balance between the pro-apoptotic and the pro-survival signaling events within the cells leading to minimal change in the cell viability. This explains the complexity of the ER signalling and the necessity of the interplay between the ER coregulatory proteins (like the ER subtypes, their ratios, coactivators and corepressors) and the signalling from the growth factors to determine the response to the ER modulators (138). Stabile *et al.*, reported that the EGFR protein expression was down-regulated in response to estrogen and up-regulated in response to fulvestrant *in vitro*, suggesting that the EGFR pathway is activated when estrogen is depleted in the lung cancer cells. Therefore to increase apoptosis both ER and the EGFR had to be blocked in the study (139). Thus the use of anti-estrogen or any ER modulators alone could not determine the therapeutic outcome in ER positive breast cancers calling for more research to stabilize the hypothesis.

6.6 Apoptotic protein array

Human apoptotic protein array revealed a series of proteins that were involved in deciding the fate of the MCF-7 cells under doxorubicin treatment. The proteins expressed by the untreated mock cells were taken as the background to which the relative changes following the addition of doxorubicin and E2 in the mock as well as the *TFF1*-kd cells were compared.

It was apparent that protein expression was specifically triggered based on the fact that the array showed upregulation of certain proteins in response to E2 alone

(e.g. BAD, BAX, HSP60) and some due to TFF1 (Bcl-2, Bcl-x, cIAP1, clusterin, XIAP, TRAIL/DR5, smac/Diablo, p27/Kip1, p21/CIP1/CDNK1A). TFF1 dependent Bcl-2, XIAP, c-IAP, survivin are the most powerful markers of cell survival and chemo resistance (140). Phosphorylation of p53 and phospho-Rad17 (s635) clearly indicated that, doxorubicin interfered with the cell cycle and caused genotoxicity in the cells. Normally p53 is activated following DNA damage and other genotoxic stresses to promote cell-cycle check points, repair DNA and resume normal cell cycle (141). The induction of DNA damage proteins like p21/Cip1, claspin, TNFRSF6 predicted that the cell was responding to DNA damage and p21/Cip1 expression was associated with positive chemo-response in MCF-7 cells (142). However its expressions in *TFF1*-kd cells were relatively lower than in the mock cells subjected to similar treatment supporting increased cell death in the *TFF1*-kd cells.

Doxorubicin increased the expression of pro-apoptotic proteins Bad and Bax while estrogen attempted to counter balance this lethal stress by reducing their expressions. This protection was not demonstrated in the *TFF1*-kd cells. The upregulation of pro-survival proteins including Bcl-2, Bcl-x, cIAP-1, cIAP-2, when treated with doxorubicin was not surprising, as it possibly was an attempt to keep the cell alive and maintain the intracellular homeostasis between the pro and anti-apoptotic signals. Wang *et al*.,(143) reported that, Bcl-2 was expressed as a result of E2-ER ligation and the survival benefit was cross-examined by pre-treating the cells with E2 followed by tamoxifen, which could block the estrogen mediated increase of Bcl-2. When compared to the mock cells under similar treatment, Bcl-2 and Bcl-x expression was negligible. The failure to down regulate Bax expression in the *TFF1*-kd cells indicates that Bax expression could be independent of TFF1 but upregulation could be triggered by the increased Bcl-2 upregulation via another mechanism unknown to us. Wang *et al.,* demonstrated that estrogen controlled the expression of Bcl-2 but not Bax (143). Yu *et.al* reported that the tumors with decreased expression of Bcl-2 were more chemosensitive to various agents (144). An almost no expression of Bcl-2 in the *TFF1*-kd cells in our array agrees with Amiry *et al.,* whose work demonstrated that the forced expression of *TFF1* led to the upregulation of Bcl-2, conferring survival advantage to the MCF-7 cells (6). The significant increase in the smac/Diablo pro-apoptotic protein following doxorubicin treatment could be a

compensatory effect against the increased expressions of IAPs. *TFF1*-kd cell showed a pan suppression of various pro-survival proteins and was unable to express pro-survival proteins like survivin, clusterin and claspin even by E2, indicating its dependence on TFF1. Clusterin suppression reportedly rendered cancers sensitive to chemotherapeutic drugs by interfering with the Bax-pro-apoptotic activities (145). Doxorubicin led increased clusterin expression was apparently a defense signal from the cells. The lack of caspase 3 activity however is in agreement that some strains of MCF7 consisted of mutated *casp3* gene, reportedly existing in certain strains of MCF-7 cells (66). This was supported by the failure to form ladder following tamoxifen treatment. However the lack of DNA fragmentation did not mean that the cell escaped apoptosis (146). The executioner caspases 6 and -7 could substitute caspase 3 finally leading to apoptosis without laddering (147-148).

The array revealed significant upregulation of, the apoptosis initiating receptors Fas/TNFRSF6, TRAILR1/DR4, TRAILR2/DR5, and FADD along with a clear involvement of the mitochondrial pathway. This was also supported by the imbalance in Bax and Bcl-2 protein expression leading to permeabilization of the mitochondrial membrane releasing pro-apoptotic proteins such as cytochrome c and Smac/DIABLO, an antagonist of IAPs (149-150). From the FasL mediated signaling and engagement of the death receptors, it is apparent that the doxorubicin mediated apoptosis in MCF-7 cells involves the extrinsic pathway. But it could also trigger mitochondrial/intrinsic pathway by internal cellular insults like the DNA damage. This shows the involvement of both the extrinsic and the intrinsic apoptotic pathways converging at the mitochondria. Doxorubicin increased the expression of anti-apoptotic HSP60 which was further increased by E2. Equal or sightly higher expression in the *TFF1*-kd cell indicates its independence of TFF1. Anti-apoptotic HSP70 expression was lowered by doxorubicin and E2 could not increase its expression. HSP70 was strongly correlated to TFF1 in colorectal adenocarcinoma (151).

The proliferative effect is not only the basis for estrogen to be a risk factor for the breast carcinogenesis. Oxidative stress has also been postulated as a mechanism, underlying the estrogen carcinogenesis by increasing mitochondrial-derived reactive oxygen species (ROS) by an unknown mechanism (152-153). We found significant increase in the expression of anti-oxidative enzymes catalase and

heme oxygenase 1 (HO-1) and paraoxanases 2 (PON2) following doxorubicin treatment. Catalase is an important enzyme that converts hydrogen peroxide to water and oxygen. In the presence of estrogen, the combined oxidative stress led to further increase in the catalase expression in order to counteract the mounting stress similar to that in the HO-1. PON2 was reported to be an anti-apoptotic, and stabilize tumor cells significantly reduced in the presence of E2 probably because estrogen esters are substrates for the PON2 and E2 was suspected to have modulatory effect on the PONs (154-155). However, it could be the inability of the *TFF1*-kd cells to express adequate level of these anti-oxidants resulting in lower death threshold. Anti-oxidants inhibit the oxidation of other molecules resulting in free radicals which causes cell damage. The cellular oxidative capacity increased as a consequence of using doxorubicin with the effect compounded in the presence of estrogen. In the *TFF1*-kd cells, E2 cannot induce TFF1 which has been shown to regulate the expression of anti-oxidants to balance oxidative stress.

The apoptotic array shows the existence of complicated signalling induced by cellular insults and the compensatory mechanics of the cell attempting to restore the cellular homeostasis and viability. It is finally the magnitude of the insult that would exert the cell to the level that it is unable to counteract the stimuli resulting in apoptosis. The array herein determines the importance of TFF1 in strongly regulating the expression of many pro-survival proteins particularly the two antioxidants, catalase and HO-1 and Bcl-x. As elaborately illustrated by Buron *et. al.,* in the chang conjunctival cells (20), even in the MCF-7 cells the expression of XIAP and cIAP1 could be controlled by TFF1 mediated activation of NF-κB, contributing to the estrogen mediated resistance to doxorubicin.

6.7 TFF1 expression mock cells under different treatment conditions

TFF1 expression is induced by estrogen or estrogenic mimics such as phenol red beyond doubt. The mock cells under prolonged culture in the phenol red free DMEM the cells expressed minimal TFF1, but it did not have much effect on the *TFF1*-kd cells. This selective upregulation of TFF1 by estrogen in the mock cells indicates its role in breast carcinogenesis, which also agrees with the flow cytometry

result (figure 4.1.5b) which represents similarity between the *TFF1*-kd cells and the mock cells under phenol red-free DMEM culture condition. Slight expression of TFF1 was demonstrated under the doxorubicin treated condition in the absence of estrogen. TFF1 promoter consisted of multiple response elements (78) with reports underlining the induction of TFF1 by doxycycline (19), X-rays (73), which are but apoptotic insults to the cells. It cannot be denied, the effort through some unknown mechanism to upregulate TFF1 under doxorubicin condition to maintain the cell viability. Nevertheless, there is no report on doxorubicin inducing TFF1 in the MCF-7 cells and this fact would require further validation.

Additionally, fulvestrant treatment also demonstrated expression of TFF1 in the phenol red free medium. This requires careful validation whether this property was obtained after prolonged culture in the laboratory and is specific to this strain of MCF-7 cells only or the possibilities of such phenomenon in other MCF-7 cells as well.

Fulvestrant is an approved drug used to cure the ER positive breast cancer resistant to tamoxifen (156). However, such breast cancers sometimes become estrogen independent with about 30% of it exhibiting *de novo* or intrinsic resistance with unsuccessful anti hormones treatment (157). Fulvestrant treatment inhibits the ER expression and its transcriptional activities in the MCF-7 cells. Besides its inhibitory effect, fulvestrant consistently induced expression of and activity of ErbB3 in MCF-7 cells (158). ErbB3 has been recently implicated in the resistance of breast cancer to hormone therapy and EGFR and ErbB2 growth factors have been linked to resistance in tamoxifen which maintained the downstream signalling via the mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI3K) cascades. This helps the MCF-7 cells to maintain the survival and proliferative signals while ignoring the pro-apoptotic signals (159-160). Growth factors like EGFR and ErbB3 transmits signals from EGF and EGF-like growth factor ligands to activate a complex array of downstream signaling pathways including the PI3K/Akt and MAPK pathways. Such activation may lead to phosphorylation of multiple downstream targets (161) and ultimately regulate transcription of TFF1 whose promoter also have an EGFR response element. This nevertheless requires further research to trace a connector pathway between the fulvestrant induced growth factor receptors resulting in the transcriptional activity at the specific response element at the *TFF1* promoter. This

study provides a rationale to combine growth factor inhibitors with fulvestrant and TFF1 antagonist for successful breast cancer therapy trials.

6.8 Limitation and suggestions for further studies

6.8.1 Although TFF1 was found to be increased in the breast cancer, data still remain controversial regarding its role in the disease progression requiring further studies (6, 91).

6.8.2 Although fulvestrant is a well-known ER down regulator, there are theories that support tolerance to it. Therefore it would be necessary to explore more to understand the predisposition to ER antagonizing therapies.

6.8.3 The functional assay was limited to the intracellular TFF1 largely excluding the effect of the secreted TFF1 thus, calling for functional and pathways correlation studies.

6.8.4 The apoptotic protein array revealed suppression of many proteins following *TFF1* knock down. This leaves an avenue for future research on pathway tracing, for the series of proteins regulated by or via *TFF1* among which could be possible targets responsible for chemotherapeutic drug resistance.

CHAPTER VII
CONCLUSIONS

The present study has investigated the anti-apoptotic role of estrogen stimulated TFF1 in the doxorubicin induced apoptosis in MCF-7 cells. It has been observed throughout that the *TFF1* knockdown cells do not undergo apoptosis on its own, in the absence of any apoptotic insult. Neither were there any morphological changes between the parental cells. The apoptosis was observed only as a response to stimuli. Therefore, the loss of TFF1 alone does not subject the MCF-7 cells to apoptosis by itself and the protection is clearly a role of estrogen induced TFF1.

The findings can thus be concluded as following:

1. TFF1 is an estrogen induced gene in MCF-7 breast cancer cell line which in turn protects MCF-7 from doxorubicin induced cell death.
2. The inability of the *TFF*-kd cells to upregulate the anti-oxidant enzymes like heme oxygenase 1 and catalase could be a reason for the decreased survival threshold compared to the TFF1 intact mock cells.
3. XIAP might be an important survival protein regulated by TFF1 even in the breast cancer cell.
4. This fact determines the crucial role of TFF1 in estrogen-promoted resistance to apoptosis induced by doxorubicin in MCF-7 breast cancer cell. TFF1 may be a target for enhancing of sensitivity to chemotherapy in breast cancer treatment.

This research findings warrant the hypothesis that estrogen has immense survival effect on MCF-7 cells via TFF1 modulation. TFF1 clearly acts as a link between the hormonal and the chemotherapeutic regimes. Thus, this information highlights the possibility to target TFF1 for enhancing the sensitivity to chemotherapy in breast cancer. A combined approach of antagonizing TFF1 alongside chemotherapy or bypassing TFF1 by ablation of estrogen and growth factor signaling, would contribute to the therapeutic success in the breast cancer patients.

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APPENDICES

APPENDIX A
GENERAL REAGENTS

1) 2X Sample buffer

DW	0.5	ml
Tris-HCl 1.0 M pH 6.8	2.5	ml
Glycerol	2.0	ml
10% SDS	4.0	ml
β-Mercaptoethanol	1.0	ml
0.05% (w/v) Bromophenol blue	0.2	ml

2) 1.5 M Tris-HCl pH 8.8

Tris base	36.33	g
Distilled water	200	ml
pH	8.8	

3) 1.0 M Tris-HCl pH 6.8

Tris base	24.22	g
DW	200	ml
pH	6.8	

4) Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulphate	10.0	g

Dissolve in 100 ml DW

5) Acrylamide/Bis (30%/0.8)

Acrylamide	**60**	**g**
Bisacrylamide	1.6	g

Dissolve in 200 ml DW

6) 10% Ammonium persulfate

Ammonium persulfate	0.5	g
DW	5	ml

Aliquot and store at -20ºC

7) SDS-PAGE running buffer, pH 8.3 (5 X)

Tris base	15	g
Glycine	72	g
SDS	5	g

DW up to 1000 ml (dilute to 1X before use)

8) Transfer buffer (Towbin's solution, 10 X)

Tris base	30.28	g
Glycine	144.13	g
DW up to	1,000	ml

Adjust pH to 8.3

For use, add; 100 ml 10X buffer, 200 ml. methanol, and 5 ml of 20% SDS

9) Tris buffer saline (10 X)

Tris base	7.33	g
NaCl	87.6	g
DW up to	1,000	ml

Adjust pH to 7.4

10) Tween 20 (20 X)

Tween 20	20	ml

Dissolve in 1000 ml of DW

11) Tris Buffer Saline-Tween (TBST)

Tris buffer saline (10 X)	100	ml
Tween 20 (20 X)	50	ml
Distilled water	1000	ml

12) Membrane blocking reagent (5% skimmed milk in 1X TBST)

13) 40% Methanol

Methanol	400	ml

Adjust final volume to 1,000 ml with DW

14) Gel staining reagent

Coomassie blue (G-250) 0.006 % in 10 % Acetic acid

15) Membrane staining reagent

Coomassie blue (R-250)	1.5	g
Acetic acid	30	ml
Methanol	150	ml
Total volume	300	ml

16) 5% $K_2Cr_2O_7$ with glacial acetic acid (1:3, w/v)

$K_2Cr_2O_7$	0.5	g
DW to 10 ml		
Glacial acetic	30	ml

17) 0.1% Triton X-100 solution

Triton X-100	10	µl
10 mM Sodium phosphate buffer pH 7.4	10	ml

18) 1 µM 17β-estradiol working stock (water soluble)

17β-estradiol	0.22	mg

Dissolve in 1,000 µl of sterile DW

19) 30 mM fulvestrant stock dilution

Fulvestrant	25	mg
DMSO	1.373	ml

Aliquot and store it at -20°C

10 mM fulvestrant working stock

30 mM fulvestrant stock	166	µl
Sterile DW up to	500	µl

Aliquot and store it at -20°C

APPENDIX B
BRADFORD ASSAY

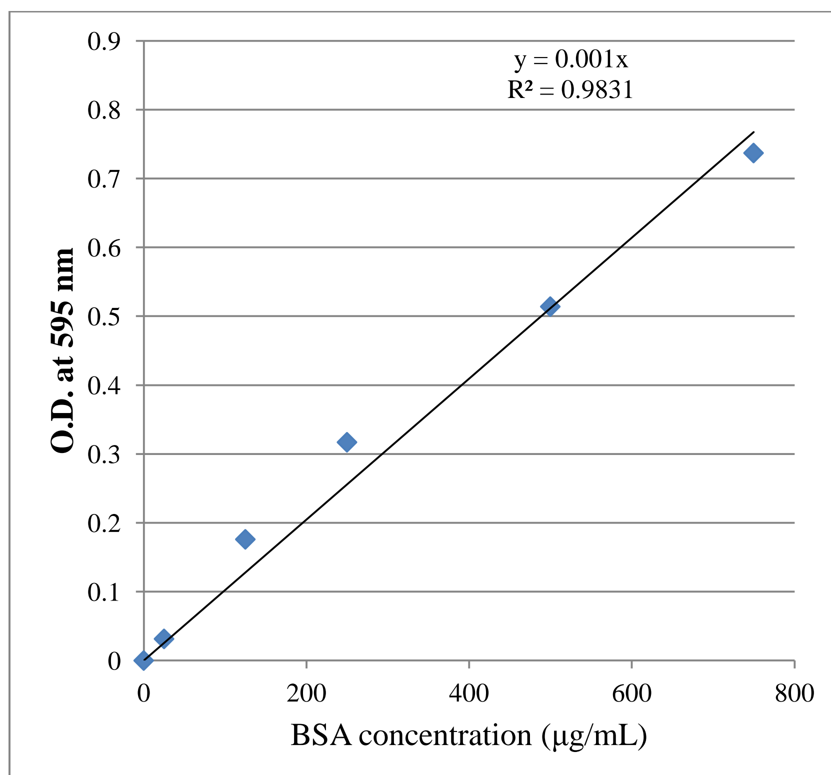

Figure A1 BSA standard curve for Bradford total protein assay

APPENDIX C
CATALASE ASSAY

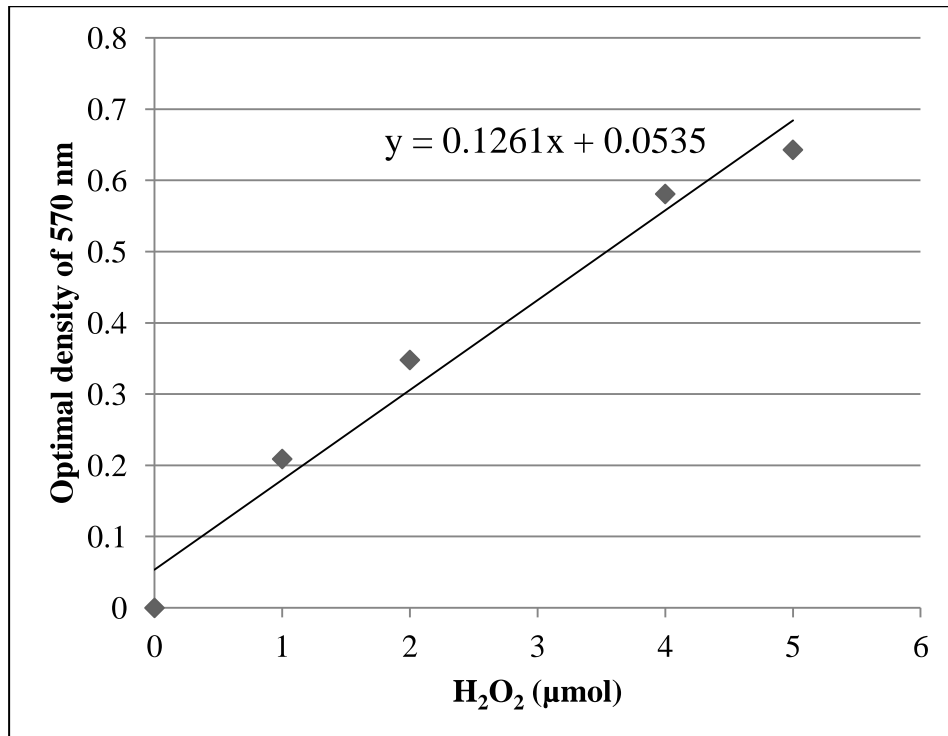

Figure A2 Reduction of H_2O_2, in acetic acid to chromic acetate by H_2O_2

APPENDIX D
HUMAN PROTEIN ARRAY

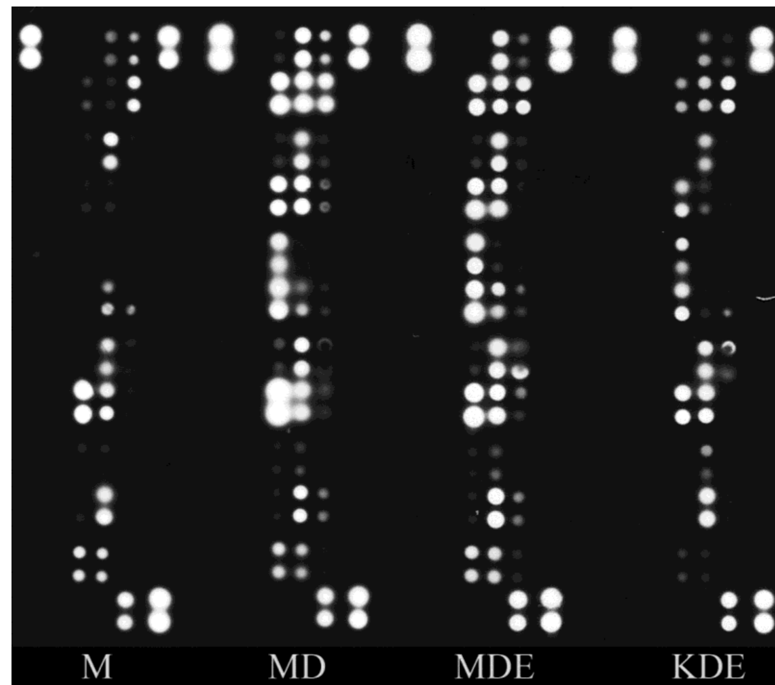

Figure A3 The black and white image of, ECL developed apoptotic protein array inverted using Paint.NET program. This image was further analysed using image J program to compare the protein expression in the mock cells and *TFF1*-kd MCF-7 cells treated with doxorubicin and in the presence or absence of E2

Table 7.1 Image J analysis data of the apoptosis associated protein expression of the mock MCF-7cells

MOCK	proteins in duplicates	Area	Mean	SD	Total	Avg	SD	(-)Background			
E1	Positive control	0.005	209.644	57.461	1.048			0.94555 (+ ctrl. background)			
E2	Positive control	0.005	208.754	58.238	1.044						
A1	Positive control	0.005	210.229	49.933	1.051	1.042	0.047				
A24	Positive control	0.005	218.526	44.017	1.093			Normalized	Avg	n	SD
B1	Bad	0.005	43.092	41.676	0.215	0.233	0.119	0.125	0.144	2	0.027
B2	Bad	0.005	50.193	55.139	0.251		0.154	0.163			
B3	Bax	0.005	87.763	86.413	0.439	0.436	0.342	0.362	0.359	2	0.004
B4	Bax	0.005	86.738	85.914	0.434		0.337	0.356			
B5	Bcl-2	0.005	22.217	3.183	0.111	0.110	0.014	0.015	0.014	2	0.002
B6	Bcl-2	0.005	21.803	1.935	0.109		0.012	0.013			
B7	Bcl-x	0.005	20.173	0.982	0.101	0.100	0.004	0.004	0.004	2	0.001
B8	Bcl-x	0.005	19.998	1.028	0.100		0.003	0.003			
B9	Pro-caspase-3	0.005	19.390	0.912	0.097	0.098	0.000	0.000	0.001	2	0.001
B10	Pro-caspase-3	0.005	19.705	0.855	0.099		0.002	0.002			
B11	Cleaved caspase-3	0.005	21.292	1.933	0.106	0.149	0.010	0.010	0.055	2	0.064
B12	Cleaved caspase-3	0.005	38.362	35.598	0.192		0.095	0.100			
B13	Catalase	0.005	26.358	4.368	0.132	0.130	0.035	0.037	0.035	2	0.003
B14	Catalase	0.005	25.553	3.556	0.128		0.031	0.033			
B15	cIAP-1	0.005	23.941	3.218	0.120	0.116	0.023	0.024	0.021	2	0.005
B16	cIAP-1	0.005	22.633	2.784	0.113		0.016	0.017			
B17	cIAP-2	0.005	20.099	0.860	0.100	0.100	0.004	0.004	0.003	2	0.000
B18	cIAP-2	0.005	19.977	0.840	0.100		0.003	0.003			
B19	Claspin	0.005	22.348	2.757	0.112	0.113	0.015	0.016	0.017	2	0.002
B20	Claspin	0.005	22.861	2.631	0.114		0.017	0.018			
B21	Clusterin	0.005	21.457	2.902	0.107	0.107	0.010	0.011	0.010	2	0.001
B22	Clusterin	0.005	21.246	2.198	0.106		0.009	0.010			
B23	Cytochrome C	0.005	124.464	87.669	0.622	0.600	0.525	0.556	0.532	2	0.033
B24	Cytochrome C	0.005	115.511	83.750	0.578		0.481	0.508			
C1	TRAIL R1/DR4	0.005	42.824	33.703	0.214	0.211	0.117	0.124	0.121	2	0.005
C2	TRAIL R1/DR4	0.005	41.593	28.925	0.208		0.111	0.117			
C3	TRAIL R2/DR5	0.005	25.280	6.160	0.126	0.126	0.029	0.031	0.030	2	0.001
C4	TRAIL R2/DR5	0.005	24.961	6.480	0.125		0.028	0.029			
C5	FADD	0.005	101.273	92.361	0.506	0.506	0.409	0.433	0.433	2	0.000
C6	FADD	0.005	101.303	80.801	0.507		0.410	0.433			
C7	Fas/TNFRSF6	0.005	23.692	3.745	0.118	0.121	0.022	0.023	0.025	2	0.004
C8	Fas/TNFRSF6	0.005	24.714	7.459	0.124		0.027	0.028			
C9	HIF-1a	0.005	19.774	1.199	0.099	0.099	0.002	0.002	0.003	2	0.001
C10	HIF-1a	0.005	19.945	0.919	0.100		0.003	0.003			
C11	HO-1/HMOX/HSP32	0.005	52.003	44.119	0.260	0.296	0.163	0.172	0.210	2	0.053
C12	HO-1/HMOX/HSP32	0.005	66.236	65.070	0.331		0.234	0.248			
C13	HO-2/HMOX2	0.005	93.061	69.991	0.465	0.469	0.368	0.390	0.394	2	0.006
C14	HO-2/HMOX2	0.005	94.681	59.326	0.473		0.376	0.398			
C15	HSP27	0.005	112.277	80.570	0.561	0.539	0.464	0.491	0.468	2	0.033
C16	HSP27	0.005	103.425	91.118	0.517		0.420	0.444			
C17	HSP60	0.005	23.873	5.376	0.119	0.117	0.022	0.024	0.021	2	0.004
C18	HSP60	0.005	22.750	2.593	0.114		0.017	0.018			
C19	HSP70	0.005	131.600	68.199	0.658	0.666	0.561	0.593	0.602	2	0.013
C20	HSP70	0.005	134.986	83.468	0.675		0.578	0.611			
C21	HTRA2/Omi	0.005	63.487	70.460	0.317	0.311	0.221	0.233	0.227	2	0.009
C22	HTRA2/Omi	0.005	61.010	65.213	0.305		0.208	0.220			
C23	Livin	0.005	20.677	1.080	0.103	0.102	0.006	0.007	0.006	2	0.001
C24	Livin	0.005	20.320	0.937	0.102		0.005	0.005			

D1	PON2	0.005	20.644	1.442	0.103	0.105	0.006	0.007	0.008	2	0.002
D2	PON2	0.005	21.307	1.587	0.107		0.010	0.010			
D3	p21/CIP1/CDNK1A	0.005	27.746	11.640	0.139	0.145	0.042	0.044	0.051	2	0.009
D4	p21/CIP1/CDNK1A	0.005	30.265	16.544	0.151		0.054	0.058			
D5	p27/Kip1	0.005	22.632	4.107	0.113	0.111	0.016	0.017	0.015	2	0.003
D6	p27/Kip1	0.005	21.720	2.029	0.109		0.012	0.012			
D7	Phospho-p53 (S15)	0.005	22.785	3.538	0.114	0.118	0.017	0.018	0.022	2	0.006
D8	Phospho-p53 (S15)	0.005	24.431	7.060	0.122		0.025	0.027			
D9	Phospho-p53 (S46)	0.005	19.751	0.933	0.099	0.099	0.002	0.002	0.002	2	0.000
D10	Phospho-p53 (S46)	0.005	19.775	0.962	0.099		0.002	0.002			
D11	Phospho-p53 (S392)	0.005	20.694	1.540	0.103	0.104	0.007	0.007	0.007	2	0.000
D12	Phospho-p53 (S392)	0.005	20.736	1.675	0.104		0.007	0.007			
D13	Phospho-Rad17 (S635)	0.005	20.984	1.155	0.105	0.108	0.008	0.008	0.012	2	0.005
D14	Phospho-Rad17 (S635)	0.005	22.293	1.685	0.111		0.015	0.015			
D15	SMAC/Diablo	0.005	177.598	80.731	0.888	0.845	0.791	0.837	0.791	2	0.065
D16	SMAC/Diablo	0.005	160.257	89.411	0.801		0.704	0.745			
D17	Survivin	0.005	24.313	6.248	0.122	0.116	0.025	0.026	0.020	2	0.008
D18	Survivin	0.005	22.159	1.839	0.111		0.014	0.015			
D19	TNF RI/TNFRSF1A	0.005	22.420	2.298	0.112	0.115	0.015	0.016	0.019	2	0.004
D20	TNF RI/TNFRSF1A	0.005	23.616	4.484	0.118		0.021	0.022			
D21	XIAP	0.005	72.488	78.678	0.362	0.356	0.266	0.281	0.274	2	0.010
D22	XIAP	0.005	69.938	72.597	0.350		0.253	0.267			
D23	Negative control	0.005	19.354	0.834	0.097	0.097					

Table A1 Image J analysis data of the apoptosis associated protein expression of the mock MCF-7 cells after 18 hrs of doxorubicin treatment

MD	Protein in duplicates	Area	Mean	SD	Total	Avg	SD	(-)Background			
E1	Positive control	0.005	233.305	20.492	1.167			0.91502			
E2	Positive control	0.005	233.342	21.061	1.167			(+ ctrl. background)			
A1	Positive control	0.005	187.903	70.878	0.940	1.010	0.128				
A2	Positive control	0.005	196.811	64.180	0.984						
A24	Positive control	0.005	171.458	80.230	0.857			Normalize	Avg	n	SD
B1	Bad	0.005	61.982	65.794	0.310	0.305	0.215	0.235	0.230	2.000	0.008
B2	Bad	0.005	60.005	56.578	0.300		0.205	0.224			
B3	Bax	0.005	136.552	76.103	0.683	0.694	0.588	0.642	0.655	2.000	0.017
B4	Bax	0.005	141.072	76.769	0.705		0.610	0.667			
B5	Bcl-2	0.005	26.963	5.265	0.135	0.141	0.040	0.044	0.050	2.000	0.009
B6	Bcl-2	0.005	29.350	9.226	0.147		0.052	0.057			
B7	Bcl-x	0.005	36.571	23.859	0.183	0.186	0.088	0.096	0.099	2.000	0.005
B8	Bcl-x	0.005	37.766	26.336	0.189		0.094	0.103			
B9	Pro-caspase-3	0.005	21.294	1.056	0.106	0.107	0.012	0.013	0.013	2.000	0.001
B10	Pro-caspase-3	0.005	21.543	0.933	0.108		0.013	0.014			
B11	Cleaved caspase-3	0.005	25.576	4.109	0.128	0.130	0.033	0.036	0.039	2.000	0.004
B12	Cleaved caspase-3	0.005	26.553	8.009	0.133		0.038	0.041			
B13	Catalase	0.005	30.492	12.641	0.152	0.153	0.058	0.063	0.063	2.000	0.001
B14	Catalase	0.005	30.645	7.050	0.153		0.058	0.064			
B15	cIAP-1	0.005	38.481	13.003	0.192	0.179	0.098	0.107	0.092	2.000	0.020
B16	cIAP-1	0.005	33.303	7.933	0.167		0.072	0.078			
B17	cIAP-2	0.005	20.943	1.050	0.105	0.104	0.010	0.011	0.010	2.000	0.001
B18	cIAP-2	0.005	20.637	1.261	0.103		0.008	0.009			
B19	Claspin	0.005	38.153	29.683	0.191	0.191	0.096	0.105	0.105	2.000	0.000
B20	Claspin	0.005	38.201	30.144	0.191		0.096	0.105			
B21	Clusterin	0.005	22.542	2.926	0.113	0.114	0.018	0.019	0.021	2.000	0.002
B22	Clusterin	0.005	23.038	2.778	0.115		0.020	0.022			
B23	Cytochrome C	0.005	136.734	79.981	0.684	0.679	0.589	0.643	0.638	2.000	0.008
B24	Cytochrome C	0.005	134.721	81.912	0.674		0.579	0.632			
C1	TRAIL R1/DR4	0.005	128.534	96.397	0.643	0.686	0.548	0.599	0.646	2.000	0.067
C2	TRAIL R1/DR4	0.005	145.987	90.542	0.730		0.635	0.694			
C3	TRAIL R2/DR5	0.005	172.173	67.888	0.861	0.872	0.766	0.837	0.849	2.000	0.017
C4	TRAIL R2/DR5	0.005	176.683	63.830	0.883		0.789	0.862			
C5	FADD	0.005	120.829	65.434	0.604	0.638	0.509	0.557	0.593	2.000	0.052
C6	FADD	0.005	134.297	76.668	0.671		0.577	0.630			
C7	Fas/TNFRSF6	0.005	136.378	90.666	0.682	0.676	0.587	0.642	0.636	2.000	0.008
C8	Fas/TNFRSF6	0.005	134.193	94.451	0.671		0.576	0.630			
C9	HIF-1a	0.005	25.879	4.807	0.129	0.135	0.035	0.038	0.044	2.000	0.009
C10	HIF-1a	0.005	28.275	6.553	0.141		0.046	0.051			
C11	HO-1/HMOX/HSP32	0.005	66.782	25.329	0.334	0.353	0.239	0.261	0.282	2.000	0.030
C12	HO-1/HMOX/HSP32	0.005	74.428	61.868	0.372		0.277	0.303			
C13	HO-2/HMOX2	0.005	121.041	93.885	0.605	0.620	0.510	0.558	0.574	2.000	0.024
C14	HO-2/HMOX2	0.005	127.134	85.979	0.636		0.541	0.591			
C15	HSP27	0.005	180.040	52.031	0.900	0.887	0.805	0.880	0.866	2.000	0.020
C16	HSP27	0.005	174.738	54.654	0.874		0.779	0.851			
C17	HSP60	0.005	29.427	10.402	0.147	0.150	0.052	0.057	0.060	2.000	0.005
C18	HSP60	0.005	30.665	16.251	0.153		0.058	0.064			
C19	HSP70	0.005	99.815	92.182	0.499	0.493	0.404	0.442	0.436	2.000	0.009

B18	cIAP-2	0.005	20.637	1.261	0.103		0.008	0.009			
B19	Claspin	0.005	38.153	29.683	0.191	0.191	0.096	0.105	0.105	2.000	0.000
B20	Claspin	0.005	38.201	30.144	0.191		0.096	0.105			
B21	Clusterin	0.005	22.542	2.926	0.113	0.114	0.018	0.019	0.021	2.000	0.002
B22	Clusterin	0.005	23.038	2.778	0.115		0.020	0.022			
B23	Cytochrome C	0.005	136.734	79.981	0.684	0.679	0.589	0.643	0.638	2.000	0.008
B24	Cytochrome C	0.005	134.721	81.912	0.674		0.579	0.632			
C1	TRAIL R1/DR4	0.005	128.534	96.397	0.643	0.686	0.548	0.599	0.646	2.000	0.067
C2	TRAIL R1/DR4	0.005	145.987	90.542	0.730		0.635	0.694			
C3	TRAIL R2/DR5	0.005	172.173	67.888	0.861	0.872	0.766	0.837	0.849	2.000	0.017
C4	TRAIL R2/DR5	0.005	176.683	63.830	0.883		0.789	0.862			
C5	FADD	0.005	120.829	65.434	0.604	0.638	0.509	0.557	0.593	2.000	0.052
C6	FADD	0.005	134.297	76.668	0.671		0.577	0.630			
C7	Fas/TNFRSF6	0.005	136.378	90.666	0.682	0.676	0.587	0.642	0.636	2.000	0.008
C8	Fas/TNFRSF6	0.005	134.193	94.451	0.671		0.576	0.630			
C9	HIF-1a	0.005	25.879	4.807	0.129	0.135	0.035	0.038	0.044	2.000	0.009
C10	HIF-1a	0.005	28.275	6.553	0.141		0.046	0.051			
C11	HO-1/HMOX/HSP32	0.005	66.782	25.329	0.334	0.353	0.239	0.261	0.282	2.000	0.030
C12	HO-1/HMOX/HSP32	0.005	74.428	61.868	0.372		0.277	0.303			
C13	HO-2/HMOX2	0.005	121.041	93.885	0.605	0.620	0.510	0.558	0.574	2.000	0.024
C14	HO-2/HMOX2	0.005	127.134	85.979	0.636		0.541	0.591			
C15	HSP27	0.005	180.040	52.031	0.900	0.887	0.805	0.880	0.866	2.000	0.020
C16	HSP27	0.005	174.738	54.654	0.874		0.779	0.851			
C17	HSP60	0.005	29.427	10.402	0.147	0.150	0.052	0.057	0.060	2.000	0.005
C18	HSP60	0.005	30.665	16.251	0.153		0.058	0.064			
C19	HSP70	0.005	99.815	92.182	0.499	0.493	0.404	0.442	0.436	2.000	0.009

Table A2 Image J analysis data of the apoptosis associated protein expression of the mock MCF-7 cells after 18 hrs of doxorubicin and 17-β estradiol co-treatment

MDE	Protein in duplicates	Area	Mean	SD	Total	Avg	SD	(-)Background			
E1	Positive control	0.005	231.45	20.919	1.157			0.98194			
E2	Positive control	0.005	231.55	20.958	1.158			(+ ctrl. background)			
A1	Positive control	0.005	216.748	46.015	1.084	1.074	0.083				
A2	Positive control	0.005	219.296	41.391	1.096						
A23	Positive control	0.005	196.612	61.357	0.983						
A24	Positive control	0.005	193.492	69.393	0.967			Normalize	Average	n	SD
B1	Bad	0.005	41.163	28.061	0.206	0.203	0.113	0.116	0.113	2	0.004
B2	Bad	0.005	40.097	22.338	0.200		0.108	0.110			
B3	Bax	0.005	107.285	85.475	0.536	0.526	0.444	0.452	0.442	2	0.014
B4	Bax	0.005	103.262	93.169	0.516		0.424	0.432			
B5	Bcl-2	0.005	26.383	7.097	0.132	0.133	0.040	0.040	0.041	2	0.001
B6	Bcl-2	0.005	26.65	8.417	0.133		0.041	0.042			
B7	Bcl-x	0.005	22.781	4.771	0.114	0.114	0.022	0.022	0.022	2	0.000
B8	Bcl-x	0.005	22.863	3.622	0.114		0.022	0.022			
B9	Pro-caspase-3	0.005	20.864	1.654	0.104	0.108	0.012	0.012	0.016	2	0.005
B10	Pro-caspase-3	0.005	22.183	3.219	0.111		0.019	0.019			
B11	Cleaved caspase-3	0.005	33.614	23.932	0.168	0.164	0.076	0.077	0.073	2	0.006
B12	Cleaved caspase-3	0.005	32.014	12.135	0.160		0.068	0.069			
B13	Catalase	0.005	53.036	20.324	0.265	0.409	0.173	0.176	0.323	2	0.208
B14	Catalase	0.005	110.697	77.861	0.553		0.461	0.470			
B15	cIAP-1	0.005	45.929	38.511	0.230	0.198	0.137	0.140	0.107	2	0.046
B16	cIAP-1	0.005	33.083	16.249	0.165		0.073	0.074			
B17	cIAP-2	0.005	19.779	1.277	0.099	0.101	0.007	0.007	0.009	2	0.003
B18	cIAP-2	0.005	20.566	1.6	0.103		0.010	0.011			
B19	Claspin	0.005	43.076	36.238	0.215	0.206	0.123	0.125	0.116	2	0.013
B20	Claspin	0.005	39.394	29.167	0.197		0.105	0.107			
B21	Clusterin	0.005	25.535	7.508	0.128	0.127	0.035	0.036	0.036	2	0.000
B22	Clusterin	0.005	25.446	6.616	0.127		0.035	0.036			
B23	Cytochrome C	0.005	154.802	85.707	0.774	0.724	0.682	0.694	0.643	2	0.072
B24	Cytochrome C	0.005	134.812	90.89	0.674		0.582	0.592			
C1	TRAIL R1/DR4	0.005	123.211	88.587	0.616	0.647	0.524	0.533	0.565	2	0.045
C2	TRAIL R1/DR4	0.005	135.594	82.677	0.678		0.586	0.596			
C3	TRAIL R2/DR5	0.005	125.803	84.381	0.629	0.603	0.537	0.547	0.520	2	0.037
C4	TRAIL R2/DR5	0.005	115.446	93.023	0.577		0.485	0.494			
C5	FADD	0.005	125.813	79.621	0.629	0.624	0.537	0.547	0.542	2	0.007
C6	FADD	0.005	123.86	88.414	0.619		0.527	0.537			
C7	Fas/TNFRSF6	0.005	126.839	90.56	0.634	0.678	0.542	0.552	0.597	2	0.064
C8	Fas/TNFRSF6	0.005	144.547	77.611	0.723		0.630	0.642			
C9	HIF-1a	0.005	26.202	5.811	0.131	0.137	0.039	0.039	0.045	2	0.009
C10	HIF-1a	0.005	28.566	8.426	0.143		0.050	0.051			
C11	HO-1/HMOX/HSP32	0.005	92.588	81.555	0.463	0.466	0.371	0.377	0.381	2	0.005
C12	HO-1/HMOX/HSP32	0.005	93.956	62.045	0.470		0.377	0.384			
C13	HO-2/HMOX2	0.005	148.989	70.056	0.745	0.723	0.653	0.665	0.642	2	0.032
C14	HO-2/HMOX2	0.005	140.08	82.149	0.700		0.608	0.619			
C15	HSP27	0.005	134.297	91.349	0.671	0.701	0.579	0.590	0.620	2	0.043
C16	HSP27	0.005	146.115	88.636	0.731		0.638	0.650			
C17	HSP60	0.005	35.665	16.388	0.178	0.185	0.086	0.088	0.095	2	0.010
C18	HSP60	0.005	38.403	25.078	0.192		0.100	0.101			
C19	HSP70	0.005	134.555	95.733	0.673	0.685	0.580	0.591	0.604	2	0.018
C20	HSP70	0.005	139.438	95.176	0.697		0.605	0.616			
C21	HTRA2/Omi	0.005	87.15	76.084	0.436	0.423	0.343	0.350	0.337	2	0.018
C22	HTRA2/Omi	0.005	82.039	70.57	0.410		0.318	0.324			

C23	Livin	0.005	20.594	2.041	0.103	0.100	0.011	0.011	0.008	2	0.004
C24	Livin	0.005	19.57	1.416	0.098		0.006	0.006			
D1	PON2	0.005	20.238	1.758	0.101	0.103	0.009	0.009	0.011	2	0.003
D2	PON2	0.005	20.98	2.272	0.105		0.013	0.013			
D3	p21/CIP1/CDNK1A	0.005	159.119	85.211	0.796	0.776	0.703	0.716	0.696	2	0.029
D4	p21/CIP1/CDNK1A	0.005	151.115	89.668	0.756		0.663	0.675			
D5	p27/Kip1	0.005	27.299	6.731	0.136	0.138	0.044	0.045	0.047	2	0.003
D6	p27/Kip1	0.005	28.018	12.342	0.140		0.048	0.049			
D7	Phospho-p53 (S15)	0.005	149.343	90.725	0.747	0.831	0.654	0.666	0.753	2	0.122
D8	Phospho-p53 (S15)	0.005	183.219	62.624	0.916		0.824	0.839			
D9	Phospho-p53 (S46)	0.005	151.474	75.822	0.757	0.717	0.665	0.677	0.636	2	0.059
D10	Phospho-p53 (S46)	0.005	135.149	90.834	0.676		0.583	0.594			
D11	Phospho-p53 (S392)	0.005	160.705	83.503	0.804	0.872	0.711	0.724	0.794	2	0.099
D12	Phospho-p53 (S392)	0.005	188.064	61.371	0.940		0.848	0.864			
D13	Phospho-Rad17 (S635)	0.005	26.843	5.513	0.134	0.138	0.042	0.043	0.047	2	0.006
D14	Phospho-Rad17 (S635)	0.005	28.55	10.793	0.143		0.050	0.051			
D15	SMAC/Diablo	0.005	178.044	78.991	0.890	0.948	0.798	0.813	0.872	2	0.083
D16	SMAC/Diablo	0.005	201.215	61.657	1.006		0.914	0.931			
D17	Survivin	0.005	25.153	5.127	0.126	0.121	0.033	0.034	0.029	2	0.007
D18	Survivin	0.005	23.317	3.893	0.117		0.024	0.025			
D19	TNF RI/TNFRSF1A	0.005	23.738	5.172	0.119	0.118	0.026	0.027	0.026	2	0.001
D20	TNF RI/TNFRSF1A	0.005	23.481	5.927	0.117		0.025	0.026			
D21	XIAP	0.005	86.056	81.849	0.430	0.423	0.338	0.344	0.337	2	0.010
D22	XIAP	0.005	83.251	74.844	0.416		0.324	0.330			
D23	Negative control	0.005	18.827	1.236	0.094	0.092	0.003				

Table A3 Image J analysis data of the apoptosis associated protein expression of the *TFF*1-kd MCF-7 cells after 18 hrs of doxorubicin and 17-β estradiol co-treatment

KDE	Protein in duplicates	Area	Mean	SD	Total	Avg	SD	(-)Background			
E1	Positive control	0.005	228.233	25.178	1.141			0.95265			
E2	Positive control	0.005	228.242	27.004	1.141			(+ ctrl. background)			
A1	Positive control	0.005	217.162	46.123	1.086	1.046	0.117				
A2	Positive control	0.005	222.907	37.758	1.115						
A23	Positive control	0.005	178.481	77.598	0.892						
A24	Positive control	0.005	180.588	81.439	0.903			Normalize	Average	n	SD
B1	Bad	0.005	26.283	5.839	0.131	0.147	0.038	0.040	0.056	2	0.023
B2	Bad	0.005	32.541	15.843	0.163		0.069	0.072			
B3	Bax	0.005	100.342	92.745	0.502	0.504	0.408	0.428	0.431	2	0.003
B4	Bax	0.005	101.194	93.51	0.506		0.412	0.433			
B5	Bcl-2	0.005	21.917	2.639	0.110	6.277	0.016	0.017	0.016	2	0.000
B6	Bcl-2	0.005	21.84	1.923	0.109		0.016	0.016			
B7	Bcl-x	0.005	20.318	1.324	0.102	0.102	0.008	0.008	0.008	2	0.000
B8	Bcl-x	0.005	20.352	3.044	0.102		0.008	0.008			
B9	Pro-caspase-3	0.005	19.297	0.91	0.096	0.097	0.003	0.003	0.003	2	0.000
B10	Pro-caspase-3	0.005	19.342	0.947	0.097		0.003	0.003			
B11	Cleaved caspase-3	0.005	21.968	2.315	0.110	0.136	0.016	0.017	0.044	2	0.038
B12	Cleaved caspase-3	0.005	32.292	25.287	0.161		0.068	0.071			
B13	Catalase	0.005	64.624	59.081	0.323	0.277	0.229	0.241	0.192	2	0.069
B14	Catalase	0.005	46.074	18.196	0.230		0.137	0.143			
B15	cIAP-1	0.005	23.531	2.751	0.118	0.115	0.024	0.025	0.023	2	0.003
B16	cIAP-1	0.005	22.635	3.092	0.113		0.019	0.020			
B17	cIAP-2	0.005	19.903	1.183	0.100	0.100	0.006	0.006	0.007	2	0.001
B18	cIAP-2	0.005	20.081	1.105	0.100		0.007	0.007			
B19	Claspin	0.005	23.982	4.579	0.120	0.118	0.026	0.028	0.026	2	0.002
B20	Claspin	0.005	23.406	3.623	0.117		0.023	0.024			
B21	Clusterin	0.005	19.929	1.249	0.100	0.101	0.006	0.006	0.007	2	0.002
B22	Clusterin	0.005	20.399	1.629	0.102		0.008	0.009			
B23	Cytochrome C	0.005	125.63	93.903	0.628	0.599	0.534	0.561	0.531	2	0.043
B24	Cytochrome C	0.005	114.03	95.634	0.570		0.476	0.500			
C1	TRAIL R1/DR4	0.005	45.876	33.299	0.229	0.262	0.136	0.142	0.176	2	0.048
C2	TRAIL R1/DR4	0.005	58.727	52.091	0.294		0.200	0.210			
C3	TRAIL R2/DR5	0.005	74.984	71.869	0.375	0.376	0.281	0.295	0.296	2	0.001
C4	TRAIL R2/DR5	0.005	75.38	70.991	0.377		0.283	0.297			
C5	FADD	0.005	81.736	63.015	0.409	0.412	0.315	0.331	0.334	2	0.005
C6	FADD	0.005	83.139	58.121	0.416		0.322	0.338			
C7	Fas/TNFRSF6	0.005	33.468	8.568	0.167	0.174	0.074	0.077	0.084	2	0.010
C8	Fas/TNFRSF6	0.005	36.049	20.949	0.180		0.087	0.091			
C9	HIF-1a	0.005	20.096	1.337	0.100	0.102	0.007	0.007	0.009	2	0.002
C10	HIF-1a	0.005	20.674	2.004	0.103		0.010	0.010			
C11	HO-1/HMOX/HSP32	0.005	23.742	3.07	0.119	0.124	0.025	0.026	0.032	2	0.008
C12	HO-1/HMOX/HSP32	0.005	25.848	6.835	0.129		0.036	0.037			
C13	HO-2/HMOX2	0.005	111.064	82.558	0.555	0.576	0.462	0.485	0.506	2	0.030
C14	HO-2/HMOX2	0.005	119.24	71.943	0.596		0.503	0.527			
C15	HSP27	0.005	134.192	81.633	0.671	0.624	0.577	0.606	0.557	2	0.070
C16	HSP27	0.005	115.453	95.285	0.577		0.484	0.508			
C17	HSP60	0.005	50.109	46.897	0.251	0.213	0.157	0.165	0.125	2	0.056
C18	HSP60	0.005	35.071	18.44	0.175		0.082	0.086			
C19	HSP70	0.005	129.446	82.768	0.647	0.648	0.554	0.581	0.582	2	0.001
C20	HSP70	0.005	129.74	85.768	0.649		0.555	0.583			
C21	HTRA2/Omi	0.005	23.409	5.849	0.117	0.118	0.023	0.025	0.025	2	0.001
C22	HTRA2/Omi	0.005	23.705	5.686	0.119		0.025	0.026			
C23	Livin	0.005	20.692	1.941	0.103	0.100	0.010	0.010	0.007	2	0.005

C24	Livin	0.005	19.306	1.145	0.097		0.003	0.003			
D1	PON2	0.005	20.676	1.349	0.103	0.103	0.010	0.010	0.010	2	0.001
D2	PON2	0.005	20.456	1.213	0.102		0.009	0.009			
D3	p21/CIP1/CDNK1A	0.005	50.648	45.676	0.253	0.271	0.160	0.167	0.186	2	0.026
D4	p21/CIP1/CDNK1A	0.005	57.672	56.076	0.288		0.195	0.204			
D5	p27/Kip1	0.005	20.224	1.399	0.101	0.101	0.007	0.008	0.008	2	0.000
D6	p27/Kip1	0.005	20.324	1.514	0.102		0.008	0.008			
D7	Phospho-p53 (S15)	0.005	94.406	64.028	0.472	0.484	0.378	0.397	0.410	2	0.018
D8	Phospho-p53 (S15)	0.005	99.295	85.128	0.496		0.403	0.423			
D9	Phospho-p53 (S46)	0.005	81.533	82.175	0.408	0.384	0.314	0.330	0.305	2	0.035
D10	Phospho-p53 (S46)	0.005	72.125	55.552	0.361		0.267	0.280			
D11	Phospho-p53 (S392)	0.005	110.14	76.252	0.551	0.535	0.457	0.480	0.464	2	0.023
D12	Phospho-p53 (S392)	0.005	104.02	94.337	0.520		0.426	0.448			
D13	Phospho-Rad17 (S635)	0.005	21.544	2.155	0.108	0.112	0.014	0.015	0.019	2	0.006
D14	Phospho-Rad17 (S635)	0.005	23.137	2.698	0.116		0.022	0.023			
D15	SMAC/Diablo	0.005	129.627	92.945	0.648	0.612	0.554	0.582	0.544	2	0.053
D16	SMAC/Diablo	0.005	115.295	98.082	0.576		0.483	0.507			
D17	Survivin	0.005	20.378	1.514	0.102	0.101	0.008	0.009	0.008	2	0.001
D18	Survivin	0.005	20.099	1.1	0.100		0.007	0.007			
D19	TNF RI/TNFRSF1A	0.005	19.829	1.121	0.099	0.099	0.005	0.006	0.006	2	0.000
D20	TNF RI/TNFRSF1A	0.005	19.856	1.231	0.099		0.006	0.006			
D21	XIAP	0.005	25.17	9.617	0.126	0.131	0.032	0.034	0.039	2	0.008
D22	XIAP	0.005	27.249	13.367	0.136		0.043	0.045			
D23	Negative control	0.005	19.009	1.017	0.095	0.094	0.002				
D24	Negative control	0.005	18.47	1.025	0.092						

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POSTER PRESENTATION	TFF1 mediated, estrogen-promoted resistance to doxorubicin-induced apoptosis in breast cancer cells

Poster Presentation

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MAHIDOL UNIVERSITY
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TFF1 mediated, estrogen-promoted resistance to doxorubicin-induced apoptosis in breast cancer cells

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1. Introduction

TFF1 is a secreted protein expressed in various type of carcinomas. *TFF1* gene contains an estrogen responsive element and therefore its expression could be regulated by estrogen. Estrogen has been demonstrated for its ability to promote resistance to doxorubicin in estrogen receptor positive MCF-7 breast cancer cell line *(1)*. This study demonstrates that this effect was exerted *via* the modulation of TFF1.

2. Method

MCF-7 breast cancer cell
TFF1 stable knocked-down (shTFF1 pLKO.1 puro)
+/- Doxorubicin; +/- 17β-estradiol
Apoptosis assay (Flow cytometry)
+TFF1
Anti-hTFF1
Trypan blue
Apoptotic protein array

3. Result

3a. *TFF1* knocked-down

Stable TFF1 knocked-down MCF-7 cell was generated using pLKO.1 puro. plasmid vector containing the following TFF1 sequence insert :

CCGGCCTGGTGCTTCTATCCTAATACTC GAGTATTAGG ATAGAAG CACCAG GTTTTTG

MCF-7 Mock shTFF1
7 kDa TFF1
43 kDa β-actin

3b. Apoptosis

The TFF1 Knocked down cells were treated with 1μM Doxorubicin and stimulated with 1 nM 17β-estradiol for 18 hours before subjecting to AnnexinV-FITC binding assay by flow cytometry.

3c. TFF1 neutralization and reconstitution

a. Estrogen stimulated mock cells treated with anti-hTFF1 (neutralize secreted TFF1), followed by doxorubicin treatment b. TFF1 was reconstituted to the TFF1 knocked-down cells and treated with doxorubicin. Both the experiments were performed in phenol red-free medium cell counted using trypan blue.

3d. Human apoptotic protein array

Heme oxygenase 1
Catalase

Array showing changes in the expression of Pro survival and proapoptotic proteins under doxorubicin treatment condition.

4. Discussion

1. The mock cells treated with doxorubicin could resist apoptosis with added estrogen (*P*= 0.016) but not the *TFF1*-kd cells.
2. The reconstitution of TFF1 resulted in the regain of the anti apoptotic property in the *TFF1*-kd cells (*P*= 0.05).
3. Neutralization of secreted TFF1 with anti human TFF1 antibody in combination with doxorubicin and estrogen led to increased cell death (*P*= 0.029) compared to doxorubicin and estrogen treatment.
4. Estrogen induced TFF1 modulated the expression of anti-oxidant enzymes (catalase and hemeoxygenase 1) to counteract the oxidative stress caused by doxorubicin which can be a possible mechanism of protecting the MCF-7 cells from apoptosis.

5. Conclusion

TFF1 shows antiapoptotic property and protects the MCF-7 breast cancer cell against apoptosis. This phenomenon determines the crucial role that TFF1 plays in the, estrogen-promoted resistance to apoptosis, induced by doxorubicin in MCF-7 breast cancer cell. TFF1 may therefore be a target for enhancing the sensitivity to chemotherapy in breast cancer treatment.

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Reference

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