Cross-talk between ER and TGFβ Signaling Pathways in Human Breast Cancer

by

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A Thesis submitted to the Faculty of Graduate Studies of

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Cross-talk between ER and TGFβ Signaling Pathways

In Human Breast Cancer

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of

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limiting in Smad3 mediated p3TP-lux expression in the presence of ligand bound ERα, as over-expression of c-Jun reverses the inhibitory effect of estrogen bound ER.

Given these results, a model was proposed in which ligand bound ER sequesters c-Jun away from the promoter region of p3TP-lux, effectively inhibiting Smad3 transcriptional activity. However, EMSA and ChIP experiments were unsuccessful in determining whether alterations in the binding of c-Jun or ERα to the promoter region occur in the presence of estradiol. Alternate models through which ER modulates Smad3 transcriptional activity are discussed.

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LIST OF ABBREVIATIONS

Acronyms/Abbreviations:

4OH-Tam	4-trans-monohydroxytamoxifen
AF-1	Activation function 1
AF-2	Activation function 2
AI	Aromatase inhibitor
AIB1	Amplified in Breast Cancer 1
ANOVA	Analysis of variance
Ap-1	Activator protein-1
AR	Androgen receptor
ATF	Activating transcription factor
BAMBI	BMP and activin membrane bound inhibitor
BMP	Bone morphogenetic protein
BRCA1/2	Breast cancer associated gene 1/2
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol acetyltransferase
CBP	CREB binding protein (CBP/p300)
ChIP	Chromatin immunoprecipitation
CM	Dulbecco's modified essential medium with 5% FCS
CREB	cAMP response element binding protein
CS	Phenol red free dulbecco's modified essential medium with 5% charcoal stripped fetal calf serum
DAX-1	Dosage-sensitive sex reversal, AHC critical region on the X chromosome, gene 1
DBD	DNA binding domain
DMEM	Dulbecco's minimal essential medium
DNA, cDNA	Deoxyribonucleic acid, complementary DNA
E_2, estradiol	17β-Estradiol
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
ER, ERα, ERβ	Estrogen receptor, ER-alpha, ER-beta
ERE	Estrogen responsive element
ERK	Extracellular signal-related kinase
ERKO	Estrogen receptor knockout
FBS	Fetal bovine serum
FRK	Fos regulating kinase
GR	Glucocorticoid receptor
GSK-3	Glycogen synthase kinase 3
GST	Glutathione S-transferase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
hsp	Heat shock protein
ICI	Imperial Chemical Industries, ICI 182,780
IGF-1	Insulin-like growth factor 1

JNK	Jun kinase
K_d	Dissociation constant
LBD	Ligand binding domain
MAF	Musculoaponeurotic fibrosarcoma
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated ERK-activating kinase
N-CoR	Nuclear receptor co-repressor
NF-κB	Nuclear factor kappa B
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone receptor
PRF	Phenol red free
RAR	Retinoic acid receptor
REA	Repressor of estrogen activity
RL-tk-luc	Renilla tk luciferase
RNA, mRNA	Ribonucleic acid, messenger RNA
RT-PCR	Reverse transcription-polymerase chain reaction
RTA	Repressor of tamoxifen activation
SARA	Smad anchor for receptor activation
SBE	Smad binding element
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulator
SHARP	SMRT associated repressor protein
SMRT	Silencing mediator of retinoid and thyroid receptors
SRA	Steroid receptor RNA activator
SRE	Smad responsive element
SRC-1	Steroid receptor coactivator-1
STAR	Study of tamoxifen and raloxifene
TBP	TATA-binding protein
TβR	TGFβ receptor
TGFβ	Transforming growth factor beta
tk	Thymidine kinase
TMA	Tissue microarray
TnT	*In vitro* transcription/translation
TPA	12-O-Tetradecanoylphorbol-13-acetate
TR	Thyroid hormone receptor
TRE	TPA responsive element
VDR	Vitamin D receptor

Amino Acids:

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Glu	Glutamic acid
Gly	Glycine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Pro	Proline
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
Val	Valine

Chemicals:

BES	N,N,bis (2-Hydroxyethyl)-2-aminoethanesulfonic acid
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
DSP	Dithiobis[succinimidylpropionate]
DTT	Dithiotriatol
EDTA	Disodium ehtylenediamine tetraacetate disodium salt
HCl	Hydrocholoric acid
KCl	Potassium chloride
KH_2PO_4	Potassium phosphpate monobasic
NaCl	Sodium chloride
Na_2HPO4	Sodium phosphate
NaOH	Sodium hydroxide
ONPG	o-Nitrophenyl β-D-galacto-pyranoside
RNAsin	Ribonuclease inhibitor
SDS, SDS-PAGE	Sodium dodecylsulfate, SDS-polyacrylamide gel electrophoresis
TPA	12-O-Tetradecanoylphorbol-13-acetate
Tris	Tris (hydroxymethyl) aminomethane
Triton X-100	Octyl phenoxy polyethoxythethanol
Tween 20	Polyoxyethylene-sorbitan monolaurate

Nucleotides:

G	Guanine
A	Adenine
T	Thymine
C	Cytosine

Units:

fmol	Fentomoles
g, mg, µg, ng	Grams, milligrams, micrograms, nanograms
h, hrs	Hour, hours
kb	Kilobases
kDa	Kilodaltons
l, ml, µl	Litre, millilitre, microlitre
^{o}C	Degrees celsius
M, mM, µM, nM	Moles/litre (Molar), millimolar, micromolar, nanomolar
m, cm, mm	metre, centimetre, millimetre
rpm	revolutions per minute
U	Units
V	Volts
v	volume
w	weight

INTRODUCTION

I. Breast Cancer

Breast cancer is the most common cancer diagnosed in Canadian women with the Canadian Cancer Society estimating 22,200 new cases of female breast cancer (approximately 30% of all new cancers) to be reported in 2006 (1). Given this, it is estimated that 1 in 8.9 women are expected to develop the disease in her lifetime while 1 in 26.8 will die from it (1). However, while the incidence rates of breast cancer have stabilized since 1993 after a long period of steady increase, it is comforting to find that mortality rates are declining steadily (Figure 1) (1).

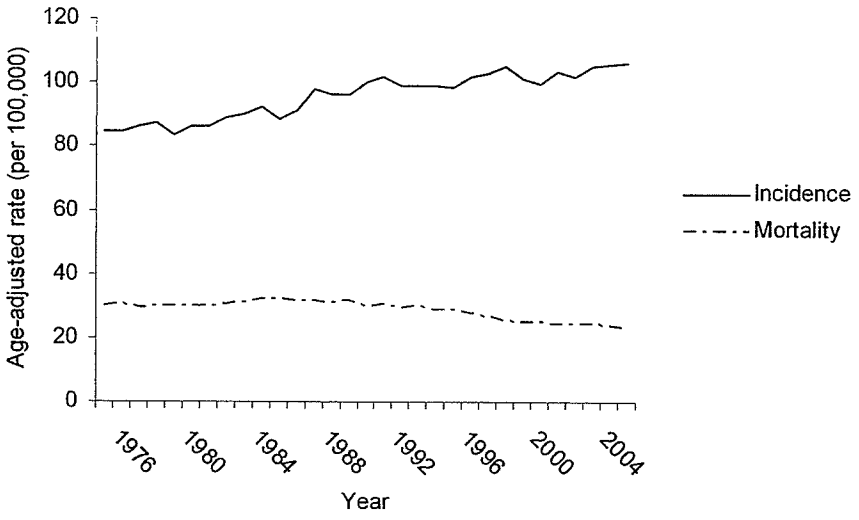


Figure 1: Incidence and mortality rate for breast cancer in Canadian women. Rates are expressed as per 100,000 Canadian women and are standardized to the age distribution of the 1991 Canadian population. Data for 2003-2006 are estimated. Source: Canadian Cancer Statistics, Canadian Cancer Society and the National Cancer Institute of Canada, 2006 (1).

Breast cancer typically arises from the epithelial cells that form the ductal and lobular components of the breast, although cancers associated with extracellular components have also been described but to a lesser extent (2). It is the uncontrolled proliferation of cells within the mammary gland which give rise to a breast tumor or neoplasm – a relentlessly growing mass of abnormal cells. As long as these neoplastic cells remain clustered together and do not penetrate the basal lamina, the tumor is said to be benign. At time of diagnosis, 61% of breast cancer cases are diagnosed while the cancer is still confined to the primary site (3). This stage of the disease represents good prognosis with a 5 year relative survival rate of 94.3% (3). However, if the cells gain the ability to invade the surrounding tissue, the tumor is then termed to be malignant. At first diagnosis, regional metastasis (i.e. regional lymph nodes or directly beyond the primary site) accounts for 31% of cancers while distant metastasis is uncommon, occurring in approximately 6% of newly diagnosed cases (3). Once a tumor becomes invasive, patient prognosis is much poorer, with 5 year relative survival rates of 79.9% and 51.1% for regional and distant metastases, respectively (3). However, about 30% of women initially diagnosed with earlier stages of breast cancer eventually develop recurrent advanced or metastatic disease. The median overall survival of metastatic breast cancer patients is generally 1-2 years (4), although prolonged survival has been observed in a small number of patients (5).

A. Risk Factors in the Development of Breast Cancer

As the number of breast cancer cases steadily increase, people have begun to express concern over the risk factors that may be involved in the development of breast cancer. While several risk factors for the disease are known, whether or not an individual

with one or more of these risk factors will actually develop breast cancer is uncertain. Some women who have one or more breast cancer risk factors may never develop the disease, while many women with breast cancer have no apparent risk factors. Nonetheless, breast cancer is thought to be the result of a combination of many factors, including heredity, environmental agents that influence the acquisition of somatic gene changes and several other systemic and local factors (6).

Hereditary breast cancer accounts for 5-10% of all breast cancers diagnosed (1). Women who have 2 or more relatives with breast or ovarian cancer, especially in a first-degree relative (mother, sister, or daughter) are thought to have an increased risk of developing breast cancer. Hereditary breast cancers tend to develop in younger women 30 to 40 years old (7) as compared to non-hereditary (sporadic) cancers which tend to develop after menopause. Mutations in specific genes that may result in breast cancer have been identified; the most characterized of these being the breast cancer associated gene 1 (BRCA1) and breast cancer associated gene 2 (BRCA2) genes. The first breast cancer susceptibility gene discovered was BRCA1, located on chromosome 17q(8) and later BRCA2, located on chromosome 13q(9), was identified. The BRCA1 and BRCA2 proteins are involved in double strand DNA repair (10), transcriptional regulation mediated by an interaction with RNA helicase and BRCA1 has been implicated as playing a role in cell cycle control (reviewed in (11)). It is interesting to note, however, that both BRCA genes are involved in cellular pathways that are important for all cells and not exclusive for breast cells. The reason why mutations of these genes lead specifically to breast cancer is not understood and is currently under investigation.

While there is no doubt that genetic variations play an important role in the development of breast cancer, the majority of breast tumors occur in women with no familial history. Therefore, the question remains as to what other risk factors may be involved in the development of the disease. A women's age appears to be an important risk factor, in that 78% of breast cancers occur in women over the age of 50 (1). The amount of exposure a woman receives to female sex hormones, especially estrogen, is also an established risk factor. Thus, women who experience early menarche and late menopause have an increased risk as they are exposed to estrogen, one important female sex hormone, for a longer period of time (12). Adipose tissue acts as an estrogen store in postmenopausal women, in that the androgen precursor androstenedione found in peripheral adipocytes, is converted to estrogen within this tissue. While the effects of a high body mass index in relation to breast cancer is complex, it is well documented that adult weight gain is an established risk factor in the development of the disease in postmenopausal women (13, 14). An established correlation between the use of oral contraceptives and the risk of developing breast cancer has not been found, although some studies do suggest that prolonged birth control pill use may increase ones chance of developing the disease (15, 16); this effect appears to be dependent on the dose of estrogen and progestin within the pill (17). Hormone replacement therapy also appears to slightly increase ones risk of developing breast cancer in addition to having detrimental effects on the cardiovascular system in postmenopausal women (18). Nulliparity and late first pregnancy are associated with an increased risk, while an early first pregnancy and multiparity is associated with a reduced risk of breast cancer, probably due to the degree of differentiation of the breast tissue (19, 20).

Life style factors including diet and exercise also may be contributing factors in the development of breast cancer. Physical activity appears to be an important determining factor, as physically active pre-menopausal and post-menopausal women have a lower incidence rate of breast cancer than those who do not exercise (21-23). While studies suggest that alcohol consumption and dietary fat intake slightly increase ones risk for breast cancer development (24, 25), the results are still controversial (26, 27).

B. Treatment of Breast Cancer

Breast cancer is viewed as a systemic disease and the majority of treatments involve a whole body approach. Therefore, optimal treatment of breast cancer often requires several different and combined treatments, including surgery, radiation therapy, chemotherapy, and hormonal therapy.

Surgery procedures to attempt to remove the tumor mass are the first line in managing the disease. As the surgeon removes the tumor mass, they also remove lymph nodes under the arm to determine whether the cancer has spread elsewhere in the body. Indeed, breast cancers often initially spread to the axillary lymph nodes that collect excess lymph fluid from that area of the breast. The presence or absence of cancer cells in these lymph nodes is an essential factor in defining optimal treatment strategies following surgery. Women who have cancer cells present in the axillary lymph nodes are at a higher risk for recurrence and thus, are treated more aggressively following surgery than women who have no cancer cells detected in the axillary lymph nodes. Following surgery, adjuvant therapy, systemic treatment administered post-surgery, is frequently given to patients to remove any remains of the tumor mass that may be present. The type

of adjuvant therapy that is used is dependent upon the type, grade and stage of the cancer as well as the woman's age, general health, menopausal status, hormone receptor levels in the tumor tissue as well as individual choices. Adjuvant therapies, which include chemotherapy, immunotherapy (trastuzumab), radiation and hormone therapy, are used as part of the treatment of breast cancer because they decrease the risk of local cancer recurrence and improve survival. For the purposes of this thesis, I will focus on hormone therapy.

i. *Hormone Therapy in the Treatment of Breast Cancer*

Estrogen is a steroid hormone whose primary function is to stimulate the growth of female reproductive tissues, including the ovaries and breasts (28). Estrogen maintains the uterine tubes, vagina, female external genitalia and breasts and plays an important role in pregnancy. It is involved in bone growth as well as in the eventual cessation of bone growth by closure of the epiphysial plates. In addition, estrogen has been shown to protect against osteoporosis, atherosclerosis and to have a neuroprotective effect in latter life (28). Pathological processes of hormone-dependent diseases, such as breast, endometrial and ovarian cancers are also influenced by estrogens. These biological actions of estrogen are thought to be mainly mediated by estrogen binding to the estrogen receptor (ER).

Hormone therapy aims to either remove hormones from the body or to block their action. As estrogen stimulates breast growth, inhibiting its activity within the breast slows the growth of breast tumors. The decision as to whether or not hormone therapy may be effective in the treatment of breast cancer depends on the ER status of the tumor. At the time of clinical detection, approximately 30% of primary breast cancers contain

ER levels less than 3 femtomoles (fmol)/milligram (mg) protein and are thus considered ER^- (29, 30). These tumors are deemed to be hormone independent in that they will not respond to hormone treatment (30). This represents an aggressive stage of the disease and thus, poor prognosis. However, the remaining 70% of breast cancers are deemed to be ER^+ (contain ER levels equal to or above 3 fmol/mg protein) and hormone dependent, in that they are likely to respond to hormone treatment (30, 31). It has been well established that breast cancers that are ER and/or progesterone receptor (PR), a downstream marker of estrogen action, positive have a more favourable prognosis than ER and/or PR negative tumors (30). Currently, the two most common classes of drugs used for the hormonal treatment of breast cancer are the aromatase inhibitors (AIs) and selective estrogen receptor modulators (SERMs).

a. Aromatase Inhibitors

The aromatase inhibitors are used for the treatment of advanced hormone-dependent breast cancer in post-menopausal women and they function by inhibiting the formation of estrogen in the body. Aromatase is a cytochrome P450 enzyme complex that catalyzes the rate-limiting step in the conversion of androstenedione and testosterone to estrone and estradiol (32). Thus, AIs reduce the level of estrogen in the body that is necessary for the growth of ER expressing breast tumors. While the first AIs (aminoglutethimide) had several side effects which resulted in patients stopping treatment, newer versions, including Anastrazole and Letrozole, are much more specific and have far fewer side effects. While endocrine therapies involving AIs are successfully used to treat breast cancer, for the purposes of this thesis I will focus on the selective estrogen receptor modulators.

b. Selective Estrogen Receptor Modulators

Selective estrogen receptor modulators, or SERMs, function by binding directly to the ER and thereby, effectively block ER function within cancer cells, preventing the growth stimulatory effect of estrogen. Thus, like the AIs, the efficiency of the SERMs in treatment of breast cancer depends on the expression of ER in breast cancer cells. Tamoxifen (Nolvadex) (Figure 2) is presently the most popular choice of hormonal therapy for ER^+ patients. Tamoxifen has been shown to be an effective treatment for both early and advanced breast cancer (33, 34) and may prevent invasive breast cancer (35). Studies from the Early Breast Cancer Trialists' Collaborative Group have shown that the use of tamoxifen as an adjuvant therapy reduces the recurrence of the disease by 30% over a 5 year period and appears to prevent the development of contralateral breast cancers (31). It is an effective treatment for both pre- and post-menopausal women and is considered to be generally safe and well-tolerated (35). In addition, benefits of using tamoxifen in women who do not presently have clinically detectable breast cancer but are at a high risk of developing the disease have been suggested. In a report by the National Surgical Adjuvant Breast and Bowel Project, tamoxifen reduced the risk of developing non-invasive breast cancer by 50% and reduced the risk of developing invasive breast cancer by 49% in women at high risk for developing the disease (35).

While the use of tamoxifen in the treatment of breast cancer has proven to be beneficial, problems do exist. First: tamoxifen targets the ER within a breast cancer cell, and thus, tumors which are ER^- do not respond. Second: not all tumors which are ER^+ initially respond to tamoxifen. Of all breast tumors, 70% are ER^+ (31) and of these, only 40% will initially respond to tamoxifen. This phenomenon is referred to as *de novo*

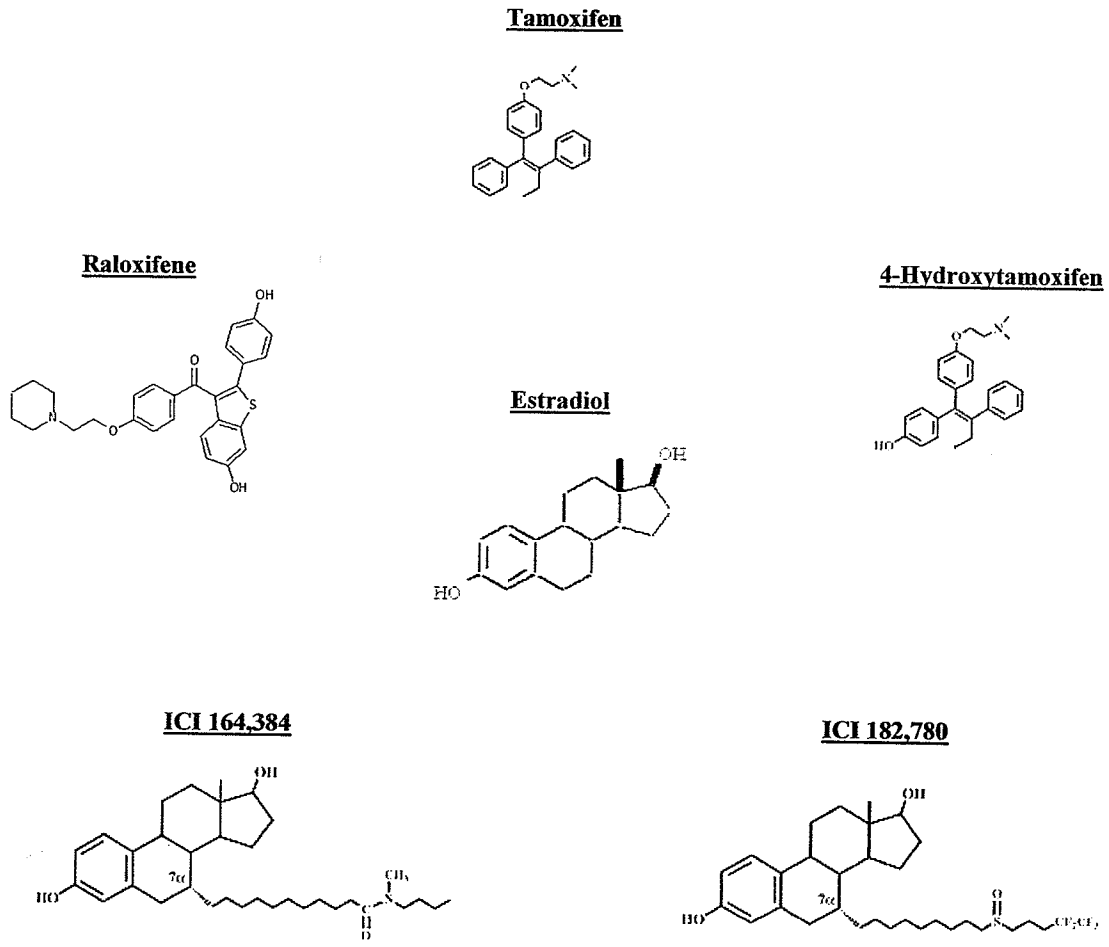


Figure 2: Schematic drawing of estrogen, non-steroidal antiestrogens and steroidal antiestrogens. Non-steroidal antiestrogens, also known as selective estrogen receptor modulators, include tamoxifen, 4-hydroxytamoxifen and raloxifene. Steroidal antiestrogens, named as such as they have a steroidal backbone similar to estradiol but inhibit ER activity, include ICI 164, 384 and ICI 182,780. Adapted from (36, 37).

resistance in that a breast tumor which has never been exposed to tamoxifen is resistant to its effects. Of the ER^+ breast tumors which initially respond to tamoxifen, the majority will eventually acquire hormone resistance and fail to respond to tamoxifen as treatment progresses (acquired resistance). Third: although tamoxifen opposes estrogen activity in the breast, it has estrogenic properties in the uterus (38) and concerns have arisen over the

observed increased incidence of endometrial cancer in patients who receive tamoxifen therapy (31, 35).

As a result of this concern with respect to tamoxifen use, the search for more effective therapies in the prevention and treatment of breast cancer is of utmost importance. However, it is important to note that tamoxifen, like estrogen, has beneficial effects on the bone (39), cardiovascular system (40) and brain (41), especially in post-menopausal women. Thus, SERMs which contain antiestrogenic properties in the breast and uterus (i.e. inhibits breast and endometrial cancers) but estrogen agonistic activities in other tissues are in the process of being developed and evaluated. Raloxifene (LY156758) (Figure 2), a second generation SERM, was originally approved for the treatment (42) and prevention (43) of post-menopausal osteoporosis due to its estrogen agonistic effects in bone. However, it may also be used in the treatment and prevention of breast cancer due to its estrogen antagonistic actions in this tissue (43). Like tamoxifen, raloxifene reduces a women's risk of developing breast cancer (44) and has been shown to be generally safe and well tolerated (44). However, unlike tamoxifen, raloxifene use does not appear to increase the risk for developing endometrial cancer (43, 44), due to its antiestrogenic properties in this tissue. The comparative safety and efficacy of raloxifene and tamoxifen in women at high risk of developing breast cancer was the subject of the Study of Tamoxifen And Raloxifene (STAR). Conducted by the National Surgical Adjuvant Breast and Bowel Project and funded primarily by the National Cancer Institute, STAR is one of the largest breast cancer prevention studies, with a total of 19,737 postmenopausal women enrolled who have an increased risk of breast cancer.

Tamoxifen and raloxifene are considered "partial" non-steroidal antiestrogens as they may act as either an ER agonist or ER antagonist. However, other "pure" antiestrogens have also been developed that do not exhibit any known ER agonist behaviour. These pure steroidal antiestrogens, such as Imperial Chemical Industries (ICI) 164,384 and ICI 182,780 (Figure 2), have proven to be effective in the treatment of breast cancer after a tumor has become resistant to tamoxifen.

II. Nuclear Receptors

The nuclear receptor family modulates a wide range of physiological responses that play important roles in growth, development, metabolism and reproduction. As a result, they are expressed in a broad range of tissues that reflect their biological function. The nuclear receptors are ligand-activated proteins that regulate transcription of selected genes, binding to a particular steroid hormone for activation (45). Despite their diverse range of biological functions, the receptors have a surprisingly similar structure that reflects their ability to act as ligand dependent transcription factors (45). Responding to a diverse range of ligands, the family encompasses not only ER, androgen receptor (AR), glucocorticoid receptor (GR), vitamin D receptor (VDR) and the retinoic acid receptor (RAR), but also a large number of proteins whose function and/or ligands are unknown and are thus termed orphan receptors. Except for GR, they are expressed predominantly within the nucleus of the cell (46). For the purposes of this thesis I will focus on the ER.

A. Estrogen Receptor

The ER family consists of the classical ER, referred to as ERα, and the more recently identified ERβ. ERα and ERβ are products of different genes and exhibit tissue and cell-type specific expression. Both receptors, however, may also be co-expressed in

a number of tissues and can form functional heterodimers. The characterization of mice lacking ERα, ERβ, or both suggests that both receptor subtypes have overlapping but also unique roles in estrogen-dependent action.

i. Tissue and Cellular Expression of ERα and ERβ

Tissue localization studies of the ER have revealed distinct expression patterns for ERα and ERβ, suggesting that the two receptors do not have completely redundant roles in estrogen signaling. In the human, ERα is expressed predominantly in the major target organs and glands necessary for female reproduction, including the endometrium of the uterus, ovary, mammary gland, pituitary and hypothalamus but is also expressed in the liver, kidney, lung, heart and Leydig cells of the testis (47, 48). ERβ mRNA, on the other hand, is expressed primarily in the ovary and isolated granulosa cells, mammary gland, seminiferous tubules of the testis and the epithelium of secretory alveoli in the lung (47). Relatively equal levels of mRNA for the two receptors is found in the thyroid, epididymis, adrenals, bone and various parts of the central nervous system (47, 49), although regional expression differences of the two receptors have been identified in the brain (50). ERβ mRNA expression can also be detected in the mucosal lining of the gastrointestinal tract (i.e. stomach, duodenum, colon and rectum), epithelial lining of the bladder, cortex of the kidney, thymus, spleen, pituitary gland, leukocytes, bone marrow, uterus, epithelium of the prostate and umbilical vein endothelial cells (47). While both ERα and ERβ may be detected in the same tissue, they may be expressed in different cell types. ERβ protein expression is abundant in healthy human mammary glands, but unlike ERα, ERβ expression is not restricted to the epithelium; it is also detected in the myoepithelial and luminal epithelial cells as well as in the nuclei of stromal fibroblasts,

macrophages, lymphocyte infiltrates and in the endothelial cells of blood vessels (51, 52). In the ovary, ERα mRNA and protein is expressed primarily in the thecal and interstitial regions, whereas ERβ is primarily expressed in the granulosa cells (47, 53). Nonetheless, ERα and ERβ proteins have been simultaneously detected in many cell types including the breast (54), and these as well as other cell types that co-express both ER subtypes are targets for potential interplay between the two receptors.

Further evidence of distinct biological functions for the ERs is revealed by contrasting phenotypes observed in ERα (αERKO) and ERβ (βERKO) knockout mice. Female mice carrying a disrupted ERα gene are unresponsive to estrogen induced proliferation of the uterus, cervix, vagina and mammary gland (55), suggesting that ERα mediates the major proliferative effects of estrogen. Male αERKO mice display testicular degeneration and sexual dysfunction (55). These phenotypes combined with severe deficits in sexual behaviour result in complete infertility in both sexes of the αERKO mouse (55). The βERKO mice, on the other hand, exhibit less profound phenotypes than the αERKO. Female βERKO mice have full estrogen responses in the reproductive tract and the mammary gland exhibits normal structure and lactation (55). However, βERKO females have inefficient ovarian function and reduced fertility (56). βERKO females are able to carry pregnancies to term and nurse their offspring, in spite of infrequent pregnancies and small litter sizes (56). Male βERKO mice are fertile and show no obvious phenotypes (56). Mice in which both ERs have been knocked out (αβERKO) exhibit phenotypes that most heavily resemble those of the αERKO (57), indicating the crucial contribution of ERα in mediating reproductive biological events. However, an unique phenotype is observed in the ovary of αβERKO mice, where there is

a loss of granulosa and germ cells and the appearance of sertoli-like cells that are normally found in the male testis (58). This ovarian phenotype suggests that maintenance of the proper differentiation state of the ovary requires both ERα and ERβ.

ii. ERα mRNA

The human ERα gene is located on chromosome 6q25 (59) and spans over 140 kilobases (kb) of DNA (60). It consists of 8 exons and 7 introns which encode a protein of approximately 66 kilodaltons (kDa) in size (Figure 3) (60, 61). The 7 introns of the ERα gene are extremely large, ranging in size from 3.5 to greater than 32 kb (60). The messenger ribonucleic acid (mRNA) that the gene encodes is approximately 6.3 kb in size, with a 5' untranslated region of 232 nucleotides and a long 3'-untranslated region of 4.3 kb (Figure 3) (62). The open reading frame is 1785 nucleotides, corresponding to a polypeptide of 595 amino acids (61, 62).

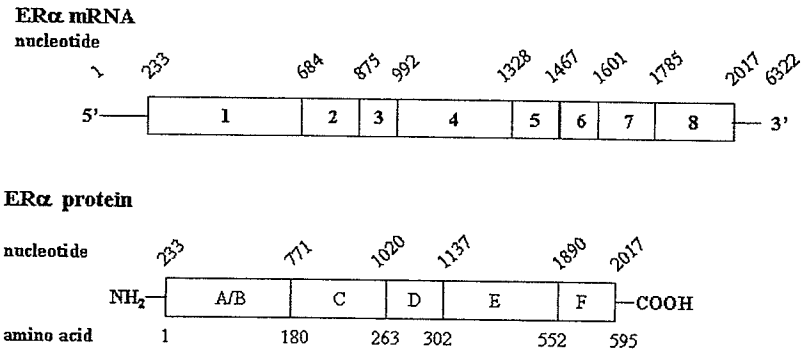


Figure 3: ERα mRNA structure and corresponding protein functional domains. Nucleotide and amino acid numbering is according to (62). The amino-terminal A/B domain contains the AF-1, the centrally located C domain is the site of DNA-binding, D represent the hinge domain and E/F contains the ligand binding domain and the second activator domain, AF-2.

Human ERα mRNA transcripts are generated by a complex process that involves at least seven different promoters, resulting in ERα variants that differ in their 5'-untranslated region (reviewed in (63). These transcripts all encode for the full-length 66 kDa ERα protein. While the question still remains as to the purpose and function of multiple promoters in the ERα gene, it is reasonable to believe that multiple transcripts may allow for tissue-specific regulation of gene promoters. Grandien et al. (64) have shown that two promoters (termed A and C) are used in MCF-7 cells while only one promoter (A) is used in the ZR-75-1 human breast cancer cell line while bone cells utilize the C promoter only. More recently, Fluoriot et al. (65) have described an ERα isoform in MCF-7 breast cancer cells in which exon 1 has been spliced from the transcript and a shorter 46 kDa ERα is generated from an internal start codon. This isoform, termed hERα46, has been shown to heterodimerize with full-length ERα and thereby inhibs full-length ERα activity in a cell type dependent manner (65).

In addition to the use of multiple promoters, multiple ER-like transcripts have been identified which encode proteins that differ in size to the wild-type ER (reviewed in (66). These ERα variants and mutants consist of single or multiple exon deletions; variable sized deletions ranging from a single nucleotide to several hundreds of continuous nucleotides that do not span entire exons; 5' (which have been characterized in rat only) and 3' truncations; insertions ranging from one to two nucleotides to 69 nucleotides or more or complete exon duplications and; point mutations including silent polymorphisms. The function of variant ER proteins, either physiological or pathological, remains unclear.

iii. ERβ mRNA and Protein

ERβ was initially isolated from a rat prostate cDNA library (67) and the full length human complementary DNA (cDNA) was identified in 1998 by Ogawa et. al. (68). The chromosomal localization of the human ERβ gene is 14q22-24 (47) which is different from that of ERα, indicating that ERβ has a distinct identity from ERα. The ERβ gene exon/intron structure is identical to that of ERα, consisting of 8 exons and 7 introns. However, ERβ differs from the ERα gene in its length, 40kb (47) compared to 140 kb (60). The 7 introns of the human ERβ gene range in size from 1.6 kb to greater than 8 kb (47), significantly smaller than those found in ERα. The open reading frame of the ERβ mRNA is 1592 bp in size encoding a 530 amino acid protein with a calculated molecular mass of 59.2 kDa (68).

The first identification of a human ERβ was in 1996 by Mosselman et al. who identified a cDNA encoding a protein of 477 amino acids with high sequence homology to ERα (49). Further cloning of ERβ from human ovarian and prostatic cDNA libraries by Enmark et al. (47) obtained a sequence almost identical to that previously identified except for the presence of extra 5' in-frame sequence which encoded 8 additional N-terminal amino acids ($ERβ_1$-short) (Figure 4). In 1998, Ogawa et al. (68) cloned a cDNA containing an additional 5' sequence which has extended the N-terminus of ERβ resulting in $ERβ_1$-long (Figure 4); it is this form that is currently regarded as the full-length wild-type $ERβ_1$. Variant forms of $ERβ_1$ resulting from alternative splicing have been identified in human breast tumors and other tissues. These variants are exon-deleted $ERβ_1$ mRNAs that encode truncated proteins and use 3' alternative exons (Figure 4).

Screening of a human testis cDNA library resulted in the identification of $ER\beta_2$ (also called ERβcx) and $ER\beta_3$ which are identical to the $ER\beta_1$-long protein except that they differ in their C-terminal extremities (Figure 4) (69, 70), resulting from the use of alternative exon 8 sequences. Human $ER\beta_2$ contains 27 unique amino acid residues which replace the amino acids of exon 8 and encodes a 495 amino acid protein with a calculated molecular mass of 55.5kDa (69, 70). This is in contrast to the rat $ER\beta_2$, in which 54 nucleotides (18 amino acids) have been inserted into the ligand binding domain (71). To date, there is no evidence to suggest that a human homolog of the rodent inserted $ER\beta_2$ variant exists (72). $ER\beta_3$ contains 44 unique amino acid residues which replace exon 8 and therefore, is slightly larger than $ER\beta_2$, containing 513 amino acids and a molecular mass of 57.5kDa (69). Moore et al. (69) also isolated partial cDNA sequences encoding $ER\beta_4$ and $ER\beta_5$ from testis using PCR from a limited region of the gene. The N-terminal sequence of the encoded proteins was not determined until just recently (Figure 4) (73). These variants, like $ER\beta_2$ and $ER\beta_3$, share exons 1 to 7 with $ER\beta_1$ but do not contain exon 8 sequences (69, 73). ERβ variants which have exon 5 and/or exon 6 deletions (ERβ Δ5; ERβ Δ6; ERβ Δ5/6) have been identified in human breast cancers and in various cell lines (72, 74). As the exon 5 or 6 deleted splice variants result in a codon frameshift, they encode C-terminally truncated proteins while deletion of exons 5 and 6 (ERβ Δ5/6) results in an inframe deletion of 91 amino acids. As these variants were obtained by restricted RT-PCR of a region, the N-terminal regions have not been characterized to date.

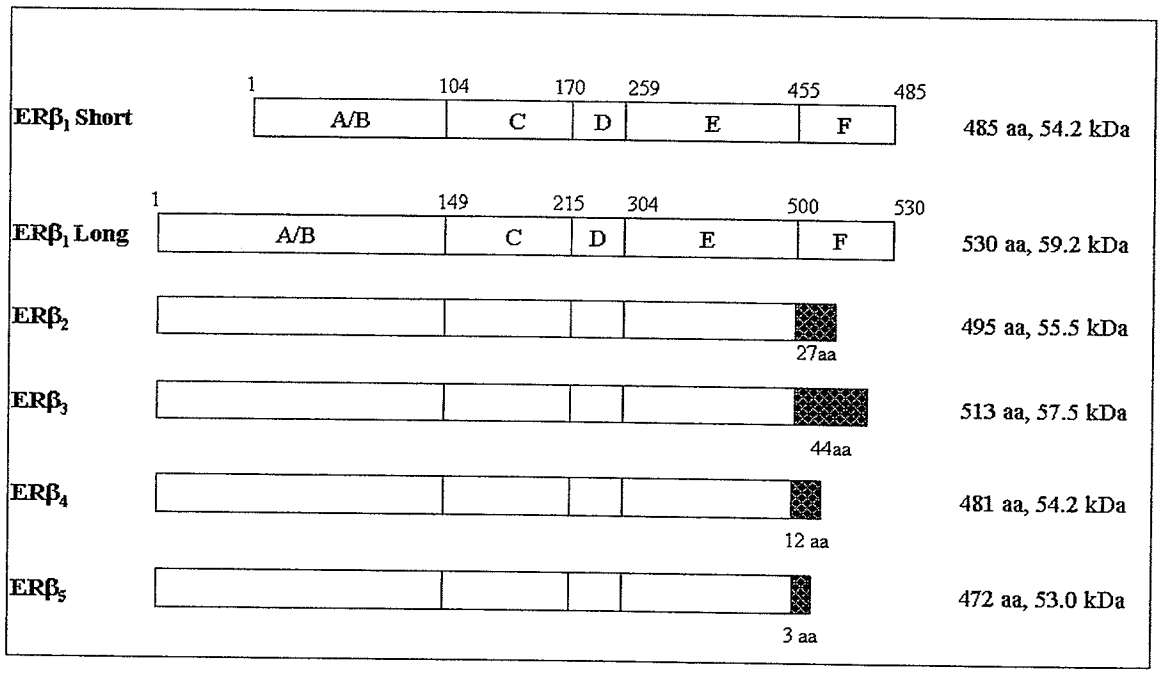


Figure 4: Human ERβ protein isoforms. Amino acid numbering for $ER\beta_1$ functional domains is according to (47). $ER\beta_2$ (Genbank AF051428), $ER\beta_3$ (Genbank AF060555), and $ER\beta_5$ (Genbank AF061055) are truncated at their C-terminal ends that contain different amino acids (cross hatched bars). For each receptor, the length (aa) and the calculated molecular mass (kDa) are indicated.

iv. ERα and ERβ Structural and Functional Domains

As members of the nuclear receptor super-family, ERα and ERβ share the same structural architecture. The ER may be divided into three main independent but interacting functional domains: i) an amino-terminal activation domain, AF-1, or A/B domain; ii) a DNA-binding domain (DBD) or C domain; iii) a hinge region or D domain; and iv) a carboxy-terminal ligand-binding domain (LBD) or domain E, which contains a second activation function, AF-2 (Figure 5).

The amino terminal region of the ER is termed the A/B domain. This region has the lowest degree of similarity amongst members of the nuclear receptor family and within the ER family itself; ERβ has only a 30% homology to ERα in this region (68)

(Figure 5). The A/B domain encodes an activation function (AF-1) that contributes to transcriptional activation in a ligand-independent manner upon phosphorylation in response to growth factors (75, 76). AF-1 may also function in a ligand-dependent manner through its synergism with the AF-2 region of ERα in certain cell types and this is thought to occur through the interaction of the corresponding regions with co-activators (77, 78). The A/B domain contains several phosphorylation sites that are important for efficient transcriptional activity and is the binding site for several co-activators (79-81). Deletion of the amino-terminus of ERβ has been shown to enhance ERβ transcriptional activity in HeLa and HepG2 cells, suggesting that this region may have an intrinsic repressor function on ERβ activity (82, 83). Functional analysis of the AF-1 region of the ER suggest that the transcriptional activity of the AF-1 domain of ERβ is minimal as compared with that of ERα (82, 84). Thus, when transcription from a gene requires both AF-1 and AF-2, the transcriptional activity of ERα will be greater than that of ERβ (84).

The centrally located DBD (C region) encodes a sequence that enables the ER to specifically recognize the estrogen responsive element (ERE) on an estrogen target gene (85). It is through this sequence that allows the ER to bind directly to the promoter region. The DBD of ER consists of two amphipathic helices oriented perpendicular to each other and crossing near their mid-points (86), forming a "scissor-like" structure which is stabilized by the interaction of cysteine residues with a zinc atom at the N-terminus of each helix (87). The crystal structure of the ER bound to DNA demonstrates that residues found in the first zinc finger interact with bases in the DNA, while residues in both zinc fingers make contacts with phosphates in the DNA backbone (87). In addition to DNA binding, this region also contains a dimerization interface that mediates

binding cooperatively between the two zinc fingers (87) and a nuclear localization signal (46). This region is highly homologous between ERα and ERβ and is the most conserved region in the steroid receptor super-family (45). ERα and ERβ share approximately 96% sequence similarity within this region (Figure 5) and thus, both receptors bind similar estrogen responsive elements (88).

C-terminal to the DBD is the D domain or hinge region that is often considered a linker peptide between the DBD and the LBD (Figure 5). While this region has not been well characterized, it has been suggested that its precise structure is not required for a functional ER (85). Nonetheless, this region is involved in the association with the molecular chaperone heat shock protein 90 (hsp90) (89) and is thought to contain a nuclear localization signal (46).

Adjacent to the hinge domain is the LBD or E/F domain. This multifunctional region is involved in binding of agonists (estrogen) and antagonists (90), receptor dimerization (90, 91), nuclear translocation, ER cofactor binding and transactivation of target gene expression. This domain displays a high degree of homology amongst members of the nuclear receptor superfamily; the overall amino acid identity is 53% between ERα and ERβ (68). As the homology of amino acid residues that surround the ligand binding cavity that are involved in contacts with the ligand is high between the two receptors, it is not surprising that ERα and ERβ bind to similar ligands with similar, but not identical, affinity (92, 93). The transactivation function (AF-2) located in the LBD is in most cases dependent on binding of ligand to the receptor for function (85). Unlike AF-1, AF-2 transcriptional activity between ERα and ERβ is very similar (84).

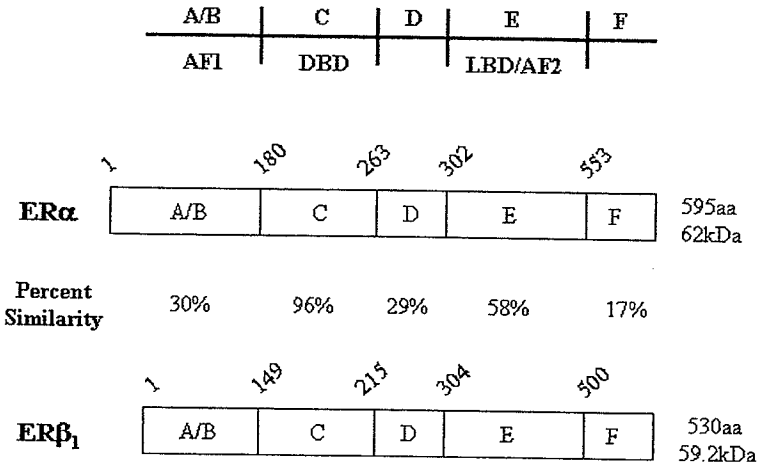


Figure 5: Comparison of human ERα and ERβ$_1$ protein structure. For each receptor, the length (aa) and the calculated mass (kDa) is given. Amino acid numbering is according to (68). The percent (%) similarity between ERα and ERβ$_1$ functional domains is listed below the ERβ$_1$ protein structure. AF1 = activation function 1; DBD = DNA binding domain; LBD/AF2 = ligand binding domain/activation function 2.

The F domains of ERα and ERβ are quite different in both length and sequence (Figure 5). Functionally, the F domain of ERα contains an intrinsic inhibitor of receptor dimerization (94) and may decrease the affinity of the receptor for estrogen (95). Evidence also suggests that the F-region has different structural orientations depending on the type of ligand bound to the receptor that mediates ER transcriptional activity in response to the ligand (96). Conflicting results with respect to the function of the F domain on E_2 bound ER have been reported. Skafar et al. (95) have shown that a point mutation within the F domain that alters its predicated structure greatly increases the transcriptional activity of ERα in HeLa cells. In contrast, Montano et al. (97) have demonstrated that complete deletion of the F domain results in an inhibition of estrogen induced ERα transcriptional activity in HeLa cells. This effect may be cell dependent, as

deletion of the F domain did not affect ERα transcriptional activity in other cell types (97). It is quite clear, however, that the F domain is important in reducing the agonistic and antabonistic effects of tamoxifen bound ERα (95, 97, 98).

v. *Cellular Localization and Protein Associations of Unbound ER*

Immunocytochemical and hormone binding assays suggest that even in the absence of ligand, ER is predominantly located within the nucleus of the cell (99-101), although cytosolic (102) and plasma membrane associated receptors have also been described (discussed in more detail on page 49) (103, 104). Direct visualization of a green fluorescent protein tagged ERα in living cells demonstrates that in the absence of ligand, ERα is diffusely distributed throughout the nucleoplasm being excluded from the nucleoli (101, 105). Upon the addition of ligand, however, a redistribution of ERα occurs such that it becomes localized to discrete regions within the nucleus (101, 105). The distribution pattern of unliganded and liganded ERβ is very similar to that of ERα and studies suggest that when the two receptors are co-expressed within the same cell, they co-localize to discrete nuclear regions (106). Thus, it appears as though the unliganded form of ER is not tightly bound to nuclear matrix components and ligand binding results in a tightly bound form of ER that ultimately initiates target gene transcription.

In the absence of hormone, ERα exists as a monomer within a multiprotein complex consisting of heat-shock proteins (hsp 90 and hsp70), immunophilins and other accessory proteins (107). ERα has been shown to directly interact with the molecular chaperone protein hsp90 (108) through several regions in its ligand binding and DNA-binding domains (89). Hsp90 is the most abundant heat shock protein in eukaryotic cells.

It is localized predominantly to the cytoplasm, although it also may be found in the nucleus bound to steroid receptors (107). Hsp70, another member of the heat-shock family of proteins, is widely distributed throughout the cytoplasm and nucleus of unstressed cells (109). Similar to hsp90, hsp70 has also been detected in unliganded ERα complexes (110), although some researchers have not been able to detect an association between hsp70 and ERα (108). As hsp70 is an essential component in hsp90 heterocomplex assembly (107), it is likely a part of the nonactivated ER complex but does not interact directly with the receptor (107). The association of hsp70 with ERα containing complexes is reduced upon cellular treatment with estrogen or tamoxifen but remains unchanged in the presence of the pure antiestrogen ICI 182,780 (110). While the precise role for accessory proteins bound to unactivated ERα is unclear, it has been suggested that they may be involved in the functional repression of ER in the absence of ligand (111), in preventing ERα degradation (112, 113) and in increasing the efficiency of ERα transcriptional response to hormone (110, 113). The presence of hsp90 and hsp70 results in maximal transcriptional response to ligand bound ERα (112, 113) and hsp70 has been shown to increase the interaction of activated ERα with its cognate DNA binding site (110), although these proteins are not absolutely required for ERα transcriptional activation (114). While the direct interaction between ERβ and heat shock proteins has not yet been described, inhibition of hsp90 activity in cells stably expressing ERβ decreases ERβ transcriptional activity and expression level (113), suggesting that ERβ is likely part of a multiprotein complex involving hsp90.

III. ER Mechanism of Action

Transcriptional activation of the ER usually occurs when ligand (i.e. estradiol) enters the cell through passive diffusion, binding to the ER located within the nucleus of the cell (Figure 6). Upon ligand-binding, the ER undergoes a conformational change, dissociating it from the heat shock protein multicomplex (107, 112, 115). ERα and ERβ then form either homo- or heterodimers (although formation of heterodimers is preferential) that bind to estrogen-responsive elements (ERE) located in the promoter region of target genes (Figure 6) (82, 116-118). The DNA-bound receptors then recruit co-regulators (co-activators or co-repressors) to the promoter region of the target gene that are required for efficient transcriptional regulation. As their name implies, co-activators enhance while co-repressors inhibit ER transcriptional activity. In addition to co-regulators, ER, once bound to DNA, also associates with members of the general transcription apparatus either directly (119) or indirectly via co-activator protein interactions (Figure 6) (119-121). The convergence of these factors onto the receptor-DNA complex ultimately results in chromatin remodelling and, depending on the cell and promoter context, exerts either positive or negative effects on target gene transcription. This direct, genomic interaction between ER and specific sequences of DNA is referred to as the classical mechanism of ER action and is the most studied mechanism by which ER affects gene transcription. ERs can also affect gene transcription independent of ligand binding but still interacting with an ERE. Alternatively, some genes are affected by ER in an estrogenic pathway in which the ER does not necessarily bind to DNA but rather interacts with other DNA-bound transcription factors. More recently, non-genomic actions of ER have also been suggested.

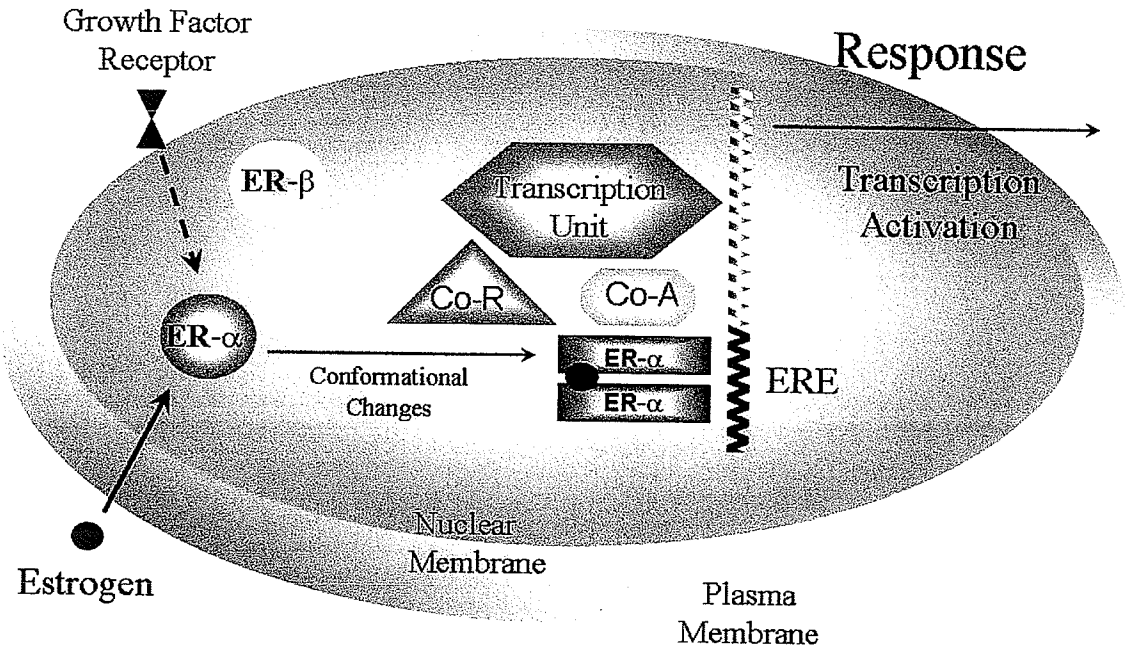


Figure 6: The classical mechanism of ER action. Upon ligand binding, the ER undergoes a conformational change allowing the receptor to form dimers. Alternaltively, the ER may also be activated by growth factor receptor pathways which also allow the ER to dimerize. The dimers then bind to EREs located in the promoter region of target genes. Once bound to DNA, the ER recruits co-activators or co-repressors, depending on the cell type and/or ligand to which it bound, and associates with the general transcriptional machinery. The convergence of these factors onto the DNA-ER complex exerts either positive or negative effects on target gene transcription. See text for more details. ER = estrogen receptor; ERE = estrogen responsive element; Co-R = co-repressors; Co-A = co-activators.

A. Classical Mode of ER Action

i. The Estrogen Response Element

Structural and functional analysis of estrogen regulated genes have revealed a common, palindromic estrogen responsive element (ERE), with the consensus sequence 5'-PuGGTCAnnnTGACCPu - 3', where n = any nucleotide and Pu = purine, that binds to

the ER with a stoichiometry of two molecules of ligand-bound ER per ERE (122). Multiple copies of the consensus sequence (or half palindromes) increase ER binding in a cooperative manner resulting in synergistic transcriptional activation of these estrogen responsive genes (123-125). The ERE consensus sequence has been shown to act in an orientation and distance-independent manner on heterologous promoters *in vitro* (122, 125), although the induction capacity of multiple EREs appear to decrease with increasing distance from the promoter (125). These highly estrogen responsive, perfectly palindromic sequences have been identified in genes encoding the *Xenopus* vitellogenin A1 and A2 genes (126) (Table 1). Most estrogen target genes do not contain a perfect palindrome in their promoter but rather have imperfect, non-palindromic ERE sequences (127). Examples of genes containing perfect and imperfect palindromes are shown in Table 1. Imperfect palindromes are less efficient in mediating transcriptional regulation by the receptor than perfect palindromes (88, 128). Experimental evidence suggests that the more nucleotide changes there are from the consensus within a half-site of the ERE palindrome, the lower the ER binding affinity for the ERE (123, 127). Alterations in nucleotide sequences in both palindromes further reduce ER binding and transcriptional activity (129). An optimal number of nucleotides (i.e. 3) separating the inverted repeat of an ERE is critical for high-affinity ER binding (123) and the DNA sequence immediately flanking the ERE is important in determining ER binding (124, 129). The presence of an AT-rich sequence flanking an ERE increases the binding affinity for ER which may be reflected by a decrease in the dissociation rate of ER from the ERE (124). Synergism between multiple copies of imperfect response elements occurs when they are in close proximity of one another or even when they are separated by hundreds of nucleotides. In

addition, ERE half-sites, which individually have a greatly reduced affinity, can act in synergy with other regulatory elements within the promoter region of a gene (e.g. Ap-1, Sp-1) (130-133). While the exact mechanism of transcriptional synergism between imperfect palindromic elements is still unknown, cooperative recruitment of co-activators, direct interaction between ER dimers, and allosteric modulations between the DNA-ER complexes have been suggested (134).

ERE Consensus Sequence: 5'- Pu G G T C A nnn T G A C C Pu –3'	
Gene	**Sequence**
(A) Perfect Element	
Vitellogenin A1 *(Xenopus)*	5'-GGTCA nnn TGACC -3'
COX7A2L	5'-GGGTCA nnn TGACCC -3'
(B) 1 Mismatch	
Angiotensinogen	5'- AGGgCA nnn TGACCC -3'
Vascular Endothelial Growth Factor (VEGF)	5'- AGGcCA nnn TGACCC -3'
(C) 2 Mismatches	
pS2	5'- AGGTCA nnn TGgCCa –3'
Oxytocin	5'- cGGTgA nnn TGACCC -3'
Cathepsin D	5'- GGGcCg nnn TGACCC -3'
(D) Combination of half-palindromic elements	
Lactoferrin	5'-AGGTCA n AGGTCA

Table 1: Estrogen responsive genes and their respective estrogen responsive elements. The nucleotides in lower case represent changes from the consensus sequence. All sequences correspond to the human genome except for the vitellogenin A1, which is found in *Xenopus*. In the sequence, n = any nucleotide and Pu = purine. COX7A2L = cytochrome c oxidase subunit VIIa polypeptide 2 like. Adapted from (127, 135).

More recently, however, it has also been suggested that the ER binds to sites outside of proximal promoter regions. Using a ChIP-chip approach, in which chromatin immunoprecipitation is used in combination with microarrays, Carroll et al. (136) have

identified ER binding sites in regions far from the transcription start site from putative target genes on chromosomes 21 and 22. ER binding to these sites occurs in an estrogen-dependent manner and is accompanied by RNA PolII and coactivator binding. These authors further showed that these binding sites act as distal enhancers, forming physical interactions with the promoter region of target genes that effectively loop out intervening chromatin (136). This looping out allows for the enhancer to interact with the promoter to initiate gene transcription (136).

ii. ERα and ERβ

The DNA binding and ligand binding domains of ERα and ERβ are highly conserved and thus, it is not surprising that ERα and ERβ bind to similar ligands and EREs (134, 137). The affinity of ERα and ERβ for the consensus ERE is higher than for variant sequences and affinities for many of the imperfect EREs are quite similar (134), although ER transcriptional activity at these promoters may vary (138). ERα homodimers have a higher affinity for consensus and imperfect EREs than ERβ homodimers (117, 134, 137, 139) and as a consequence, ERα is a more potent activator of transcription (128, 134, 138). However, under conditions in which the ligand-dependent activation function predominates, ERα and ERβ may exhibit similar activity (140). While the ERα/ERβ$_1$ heterodimer has a DNA binding affinity similar to that of ERα homodimers (117), the heterodimers have less transcriptional activity than ERα homodimers (117) while ERβ homodimers have the least amount of activity. While the ERβ variants ERβ$_2$, ERβ$_4$ and ERβ$_5$ bind DNA, they do so with less efficiency than ERβ$_1$ (73, 139). The transcriptional activity of the ERβ variants is still unclear. Poola et al. (73) have suggested that ERβ$_4$ and ERβ$_5$ have about a three times higher ligand-

independent activity on an ERE reporter plasmid than $ER\beta_1$ in Cos-7 cells. However, experiments from within our laboratory suggest that the variants do not have any transcriptional activity on their own (139), a direct result of their inability to bind ligand. ERβ and ERβ variants have been shown to function as inhibitors of ERα transcriptional activity on an ERE in cells in which both receptors are expressed with relative activities of $ER\beta_1 > ER\beta_2 > ER\beta_5$ (73, 82, 139, 141). The observation that ERβ knockout (BERKO) animals treated with estrogen do not display a change in the number of dividing cells (as maximal cellular division was already obtained) while estrogen induces a significant increase in proliferating uterine epithelial cells in wild-type littermates (142), further supports this hypothesis.

Once bound to DNA, the ER undergoes specific conformational changes that are dictated by the ligand and the ERE sequence to which it interacts (128). These sequence-induced alterations in ER structure influence not only the specific cofactor that will be recruited to the ER but also the magnitude of this interaction and the level of ER transactivation (128, 138, 143). Interestingly, ERα and ERβ appear to recruit coactivators to varying degrees in response to similar ligands and EREs despite the high degree of sequence homology in the LBD and DBDs (138). As described previously, the transcriptional activity of ERβ is highly dependent on its AF-2 domain as it lacks a functional AF-1 domain (84). Thus, the efficacy of coactivator binding to ERβ potentially determines the magnitude of the transcriptional response through the classical ERE-mediated pathway and as a result, the information within an ERE may have a significant impact on ERβ transcriptional activity. Although ERα possesses a strong constitutive AF-1 function, the magnitude of ERα transcriptional activity on ERE-

containing promoters often correlates with high efficacy of coactivator binding, although the correlation is less apparent than that for ERβ (138).

iii. Influence of Ligand on ER Transcription

a. Estrogen

The natural ligand for the ER is estrogen and 17β-estradiol (estradiol) is considered the major estrogen in the female. Estradiol binds to ERα and $ER\beta_1$ with equilibrium constants (K_d) of approximately 0.06 nM and 0.24nM, respectively (92) and thus, ERα has a slightly higher affinity for estradiol than ERβ. The ERβ variants do not bind ligand due to their C-terminal truncations (73, 139). While ERα and $ER\beta_1$ bind similar ligands, they exhibit different binding affinities; ligands with higher affinity for ERα than for ERβ, and vice versa, have been described (92, 93). One such example is the natural phytoestrogenic compound genistein, that has a 30-fold higher affinity for ERβ than ERα (92) and as a result, has been suggested as being an ERβ selective agonist.

Structural analysis of the LBD of nuclear receptors suggest that the LBD contains common structural motifs that generate a conserved ligand binding pocket, and that agonists and antagonists bind to the same site but induce different conformational changes. The three-dimensional structure of the LBD of ERα and ERβ has been elucidated and the overall structures appear to be quite similar (90, 144). The LBD of the ER is folded into a three-layered structure comprised mainly of anti-parallel α-helices (helices H3-H12) (90). Estrogen binds to the hydrophobic core of ERα through a combination of hydrogen bonds and the complementarity of the binding cavity to the non-polar hormone and is oriented diagonally across the cavity between H11, H3 and H6 (90). Helix 12 fits over the ligand-binding cavity in which estrogen is sitting (Figure 7),

packed against H3, H5/6 and H11, in essence forming a lid over the cavity, exposing the AF-2 domain of ER that interacts with coactivators (90). Antagonists of ER action, including the SERMs raloxifene and tamoxifen, bind to the same site as estrogen within the LBD (90, 144, 145). However, due to their bulky side chains, tamoxifen and raloxifene displace H12 and protrude from the binding cavity between H3 and H11 (Figure 7) (90, 145). The H12 repositioning in the presence of these SERMs partly

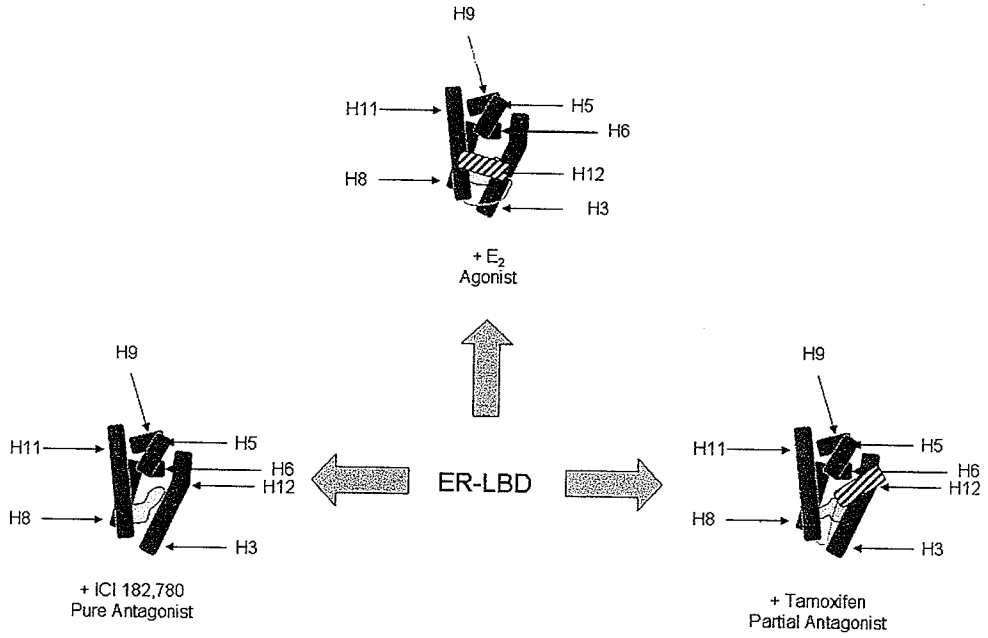


Figure 7: Schematic representation of the conformational states of the ER LBD. The conformations of ER in the presence of an agonist (E_2), pure antagonist (ICI 182,780) and partial antagonist (tamoxifen) are viewed end-on. Selected helices of the LBD that form the ligand binding pocket are shown as black rods. Helix 12 is depicted as the stripped box. See text for more detail. Adapted from (146).

buries the highly conserved lysine 362 that is required for efficient recruitment of certain coactivators to the ER (90, 147). On the other hand, pure antiestrogens, such as ICI 182,780, sterically inhibits the alignment of H12 over the cavity through its long, bulky

side chain, preventing H12 from adopting either its agonist or SERM-induced orientation (Figure 7) (148). These studies thus provide the structural basis for the partial agonistic and antagonistic actions of antiestrogens and demonstrate how different ligands bound to ER potentially affect its transcriptional response.

b. Antiestrogens

In addition to estrogen, ER also binds antiestrogenic compounds that may act as agonists or antagonists depending on the cell and promoter context (149). As a result, antiestrogens are commonly defined according to their abilities to act as either partial agonists (e.g. tamoxifen) or complete antagonists (e.g. ICI 182,780). Both classes of antiestrogens compete with estradiol for ER binding and once bound, sterically hinder correct alignment of the interacting surfaces of the LBD through their bulky side chains (Figure 2 and Figure 7).

Non-steroidal Antiestrogens

Tamoxifen, a non-steroidal antiestrogen (Figure 2), is the most widely used clinical antiestrogen to date. Tamoxifen, or its active metabolite 4-trans-hydroxytamoxifen (4OH-tamoxifen), is a partial ER agonist in that it displays mixed agonist/antagonist properties depending upon the promoter and cell context (149). 4OH-tamoxifen competitively binds estrogen to the ER with a higher affinity for the receptor (92) and thereby, effectively inhibits the growth of ER^+ human breast cancer cells *in vitro* and *in vivo*.

As described previously, tamoxifen binding to ERα and ERβ results in a conformational change that is different from that of the estrogen bound receptor (137). Such changes alter receptor affinity and specificity for binding with ERE sequences.

Thus, while tamoxifen bound ER retains the ability to bind DNA (117, 118, 150), it does so with a significantly lower affinity than that of estrogen bound ER (i.e. K_d is significantly higher for tamoxifen-ER than E_2-ER) (151). Chromatin immunoprecipitation (ChIP) experiments suggest that tamoxifen-ERα complexes bound to an ERE recruit nuclear receptor co-repressors rather than co-activators in MCF-7 human breast cancer cells (121), and this mechanism is thought to be involved in the suppression of transcription. In addition, tamoxifen inhibits co-activator binding to E_2-ER-ERE complexes in a dose-dependent manner in the breast (137).

Tamoxifen inhibits the AF-2 but not the AF-1 activity of ERα and as a result, the partial agonistic effect of tamoxifen-coupled ERα from consensus ERE-driven promoters is thought to be mediated through the amino-terminal AF-1 region (152). Studies have demonstrated that in cell contexts where the AF-2 activity of the ERα may not be required (i.e. endometrial cells), tamoxifen functions as a partial agonist, whereas in cells requiring the AF-2 domain of ERα (i.e. breast cells), tamoxifen can effectively block ER action and function as an antiestrogen. Under conditions in which tamoxifen functions as an agonist of ERα activity, co-expression of ERα and ERβ results in an inhibition of the agonistic effects of tamoxifen on ERα activity, suggesting that ERβ expression inhibits ERα activity (82). As the function of ERβ appears to be completely dependent on its AF-2 region (84), tamoxifen acts as an antiestrogen independent of cell and ERE promoter context.

Steroidal Antiestrogens

Tamoxifen has been the endocrine treatment of choice for treating breast cancer for many years. However, its extended use is associated with the development of

tamoxifen resistant tumors (153) and adverse side effects have been attributed to its partial agonist properties (154). In general, tumors which become resistant to tamoxifen therapy respond to second line endocrine therapy (i.e. ICI 182,780) and retain expression of the ER, suggesting that tumor re-growth may involve a functional ER (155, 156). This led to the search for novel antiestrogens with enhanced antagonistic, and less agonistic, actions that are not cross-resistant with existing endocrine treatment and the development of a series of "pure" steroidal antiestrogens, including ICI 182,780 (fulvestrant; 'Faslodex') and ICI 164,384, resulted. These compounds are derivatives of estrogen but contain an alkylamide extension at the 7α position (Figure 2). Treatment of patients with ER^+ breast tumors with ICI 182,780 has been associated with reductions in ER, PR and Ki67 (a marker of cellular proliferation) expression (157). In clinical trials involving post-menopausal women with hormone receptor positive (either ER or PR), locally advanced or metastatic carcinoma whose disease had progressed after receipt of previous endocrine therapy (primarily tamoxifen), ICI 182,780 was at least as effective as anastrazole (an aromatase inhibitor) for the efficacy endpoints of objective response and time to progression and thus, is currently recommended for use in these patients (158, 159).

ICI 182,780 has been shown to be a much more effective inhibitor of breast cell growth than tamoxifen (160, 161). ICI 182,780 and ICI 164,384 are also pharmacologically distinct from the non-steroidal antiestrogens, in that they act as complete antagonists, inhibiting ER AF-1 and AF-2 functions while still allowing ER dimerization and binding to an ERE (118, 148, 150, 162). As ICI 182,780 inhibits both activation functions of the ER, it is not associated with estrogen agonist activity (160).

Despite a relatively detailed knowledge of the pharmacology of this class of ER antagonists, relatively little is known about the molecular mechanisms that underlie their action. As described previously, ICI 182,780 binding to the ER induces a conformational change that is different from that with tamoxifen or estrogen (Figure 7) (148), preventing the ER from interacting with the co-activators necessary for transcriptional activation (137). Pure steroidal antiestrogens have also been shown to promote nuclear to cytoplasm shuttling of ERα (102). Nonetheless, the major mechanism of ICI 182,780 and ICI 164,384 action is to decrease the half-life of the ER protein such that the receptor is rapidly degraded and cellular levels decrease (163, 164). Thus, the potent antiestrogenic activity of the ICI compounds appears to be the direct result of their ability to inhibit receptor expression that is required for transcriptional activity.

iv. ***Co-regulators***

In order for sufficient basal levels of transcription to occur, the ER, once bound to DNA, must interact with components of the transcription initiation complex (i.e. TATA-binding protein (TBP); transcription factor-II B (TFIIB)) either directly or indirectly in order to recruit RNA polymerase II to the promoter. Direct protein:protein interactions between the general transcription factors TBP and TFIIB with ERα have been described *in vitro* (119, 165, 166). Studies in cell-free systems suggest that the ER forms a stable complex with TBP and TFIIB on DNA and enhances the formation of the preinitiation complex to increase transcription (166). In addition, the TBP-associated factor, $TAF_{II}30$, interacts with the ER LBD in a ligand-independent manner (167) and has been shown to stimulate transcription of an ERE bound ER (168).

In addition to the general transcription factors, the ER also recruits other co-regulators to the promoter region of target genes that are required for highly efficient transcriptional regulation (for a complete review, see references (120, 169)). Nuclear receptor co-regulators that interact with the ER and enhance its transcriptional activity are known as co-activators while those molecules that lower the transcriptional rate of ER are referred to as co-repressors.

a. Co-Activators

Nuclear receptor co-activators consist of a large group of proteins that interact directly with the receptor and enhance transcriptional activity by recruiting members of the general transcription apparatus to target gene promoters. Co-activators of ER are normally recruited in a ligand-dependent fashion, although evidence suggests that they may also be involved in the ligand-independent activation of the ER (see page 43) (170, 171). As described previously, upon estrogen binding to the ER, there is a shift in the position of several helices in the LBD (namely helix 12) such that a hydrophobic groove is exposed to which the co-activator binds (90, 172). Binding of co-activators to the ER involves an amphipathic helix containing the LxxLL sequence (in which L denotes leucine and x denotes any amino acid), termed the nuclear receptor (NR) box, that is conserved on the surface of most co-activators (173). Therefore, the NR box is able to make direct contacts with the LBD that is created upon agonist binding to the ER. Recent evidence also suggests that sequences flanking the NR box may be important in the interaction with ER, thereby determining preferential binding of a specific co-activator to the ER (145, 174). It is important to note, however, that although agonists promote co-activator binding to the LBD of the receptor, interactions between the A/B domain of

ERα and ERβ with co-activators may also occur in a ligand-independent fashion that may not always involve the LxxLL motif (80, 81, 175). Co-activator recruitment to the ER is also dictated by the type of ligand bound to the ER (176, 177). Crystallographic studies of the ER LBD show than when mixed ER agonists/antagonists, such as tamoxifen or raloxifene, occupy the ligand-binding pocket helix 12 is re-oriented to partially occlude the co-activator binding groove, enabling it to block certain AF-2 dependent interaction with co-activators (90, 137, 144, 148). In fact, chromatin immunoprecipitation experiments suggest that tamoxifen bound ERα alters its conformation in such a way that it is able to recruit co-repressors to the promoter of estrogen target genes in MCF-7 cells rather than co-activators (121), thereby effectively repressing gene transcription.

Although the list of ER co-activators is long and still growing, there are only a small number that, to date, have been identified as being potentially clinically relevant to human breast cancer. Among all the co-activators described, the p160/SRC (steroid receptor co-activator) family of proteins, consisting of SRC-1 (also termed p160/NcoA-1/ERAP-160), SRC-2 (GRIP-1/TIF-2) and SRC-3 (AIB1/ACTR/RAC3/p/CIP), has attracted much attention. SRC-1 was first described as a ligand-dependent interacting protein for the progesterone receptor (178) and later was found to interact with and enhance the transcriptional activity of virtually all members of the steroid nuclear receptor super-family (78, 178), as well as other transcription factors including Ap-1 (179). AIB1 (amplified in breast cancer 1) has also been shown to interact directly with endogenous estrogen bound ERα in MCF-7 human breast cancer cells and this interaction is inhibited by tamoxifen and ICI 182,780 (180, 181). Interestingly, AIB1

appears to bind to ERα with a much greater affinity than ERβ and therefore, stimulates ERα transcriptional activity to a greater extent than ERβ (182). Nonetheless, all three members of the p160/SRC family interact with the ER in a ligand-dependent manner, enhancing ER transcriptional activity (183).

The molecular mechanism by which co-activators increase transcription is thought to be the result of their ability to alter chromatin structure and to recruit the general transcription factors through direct or indirect interactions with other co-activators. In accordance with this, SRC-1 has been shown to bind to TBP and TFIIB *in vitro* (184). No direct interaction between AIB1 and the basal transcription machinery has been described. The C-terminal domains of SRC-1 and AIB1 contain intrinsic histone acetyltransferase activity (HAT) (185, 186) and therefore, are able to catalyze the acetylation of histone lysines. The acetylation of N-terminal tail lysines on histones mediates alterations in chromatin structure that further enhance transcriptional activation (187). However, as SRC-1 HAT activity is relatively weak compared to other co-activators (185) and as deletion of the HAT domains do not appear to affect transcription, at least *in vitro* (188), its HAT activity may be dispensable for enhanced transcription. In addition to their interaction with the ER, SRC-1 and AIB1 have also been shown to associate with CREB-binding protein (CBP) and p300 (186, 189), both of which contain intrinsic HAT activity (190, 191), to form an estrogen receptor co-activator complex (189, 192). Although CBP and p300 have been reported to interact with the ER directly (81), the interaction appears to be relatively weak compared to that of ER and SRC-1 (193). However, since the interaction between CBP and SRC-1 is strong, SRC-1 may be responsible for recruiting CBP/p300 to the ER in response to ligand binding (193). Once

in the co-activator complex, SRC-1 acts synergistically with CBP/p300 to enhance ER-mediated transcription (194).

It has now become clear that receptor and co-regulator association with gene promoters occurs in a cyclic and dynamic fashion. For example, ERα and various co-activators appear to repeatedly cycle on and off the pS2 promoter in MCF-7 cells in the presence of continuous E_2 stimulation (121, 195). Shang et al. (195) went on to also demonstrate that ERα rapidly associates with the cathepsin D promoter following hormone stimulation and is accompanied by AIB1 and p300, closely followed by the association of RNA Pol II. The presence of CBP at the promoter appears to occur at a later time, suggesting that it may function at a distinct step in ERα induced transcription (121). Coincident changes in histone acetylation occur to promote a transcriptionally active environment (121). Subsequent rounds of transcription involve a majority of the same proteins, although slight differences exist (121). These observations were later confirmed on the pS2 promoter with few differences in the timing of the recruitment peaks (195).

Although the SRC family of proteins are all considered co-activators of ER-mediated transcription, recent evidence suggests that they may be functionally distinct from one another. Analysis of the two isoforms of SRC-1, SRC-1a and SRC-1e, show that they are functionally distinct from one another in that they differ in their ability to bind to and enhance ER transcriptional activity (196). These two isoforms differ at their C-terminal in that SRC-1a (molecular mass of 156.7 kDa) contains an insert of 56 unique amino acids not found in SRC-1e and is lacking 14 amino acids at its C-terminal that are present in SRC-1e (molecular mass 152.3 kDa) (196). SRC-1a, which contains 4 LxxLL

motifs, binds to the ER in a more stable manner than SRC-1e, which only contains 3 LxxLL motifs (196). However, SRC-1e enhances ligand-dependent ER transcription *in vitro* to a greater extent than SRC-1a due to the suppression of a C-terminal transactivation domain (196). In addition, an isoform of AIB1 has recently been described that enhances the ligand-dependent transcriptional activity of the ER to a greater extent than that of the full-length AIB1 protein (197). Such differences between SRC family proteins and between isoforms of the same family suggest that these proteins may play different roles in ER-mediated transcription.

Another co-activator that has received a great deal of interest is the steroid receptor RNA activator, or SRA. Initial studies suggested that SRA acts as a non-protein coding RNA transcript that when over-expressed, increases steroid receptor ligand-dependent transcriptional activity of the estrogen, progesterone, androgen and glucocorticoid receptors (198). Low resolution RNA structure modelling of SRA led to the identification of several distinct secondary RNA structures that work in concert with one another to convey its co-activator function that are involved in transcriptional up-regulation of the PR (199). More recently, however, it has been shown that some SRA RNA transcripts also encode for a 236 amino-acid SRA protein (200). Subsequent cloning and sequencing of the SRA coding sequence revealed three different SRA transcripts: SRA1, the full coding sequence of the SRA protein; SRA2 that bears two point mutations and SRA3 that contains a point mutation followed by a 3 nucleotide insert (200). Western blot and immunohistochemistry analysis have demonstrated the expression of SRA protein in human breast cancer cell lines and in breast tumor tissue (200, 201). While the exact function of the SRA protein is still unknown, it has been

shown to inhibit E_2-ER activity on an ERE and to increase estradiol-induced PR expression in MCF-7 cells stably transfected with SRA protein (201).

b. Co-Repressors

Co-repressors are factors that repress the transcriptional activity of the ER. Binding of co-repressors to ERα occurs in the presence of antagonists; tamoxifen and raloxifene have been shown to recruit the nuclear receptor co-repressor protein (N-CoR) and SMRT (silencing mediator of retinoid and thyroid receptors) to certain target gene promoters (121, 175). On the other hand, Webb et al. (202) have shown that ERβ interacts with N-CoR and SMRT more strongly in the absence of ligand as well as in the presence of agonists (E_2) than in the presence of SERMs (ICI, raloxifene and tamoxifen), raising the possibility that ERβ may recruit co-repressors to estrogen-regulated promoters. Although the crystal structure of ERα bound to a co-repressor has not been resolved, Huang et al. (203) suggest that helixes 3 and 5 may be important as point mutations within these helixes prevent co-repressor sequence peptides to bind the ER. Like co-activators, co-repressors contain a conserved sequence referred to as the CoRNR box or as an Lxxi/HIxxxI/L helix that recognizes the unliganded and repression competent form of receptors (203) and sequences surrounding the CoRNR box have been suggested to play a role in mediating the specificity of receptor interactions (204). Interestingly, nuclear receptors are able to discriminate between the NR box in co-activators and the CoRNR box in co-repressors while bound to agonist or antagonist ligands, respectively (121). Therefore, SERM binding to the ER may result in a configuration that may recruit either co-activators or co-repressors depending upon the availability of these factors within the cell. Recently, however, the orphan receptor

DAX-1 (dosage-sensitive sex reversal, AHC critical region on the X chromosome, gene 1) has been described as a repressor for estrogen activated ERs that contains an LxxLL motif usually found in nuclear receptor co-activators in its N-terminal domain (205). Co-repressors exist in a complex containing the co-repressor protein Sin3 and the histone deacetylases (HDAC) RPD3 or HDAC1 (206). Histone deacetylases are responsible for deacetylating core histone tails which results in a more condensed chromatin structure that represses gene transcription (207).

As described above, the recruitment of co-repressors to the ER results in active repression of transcription. SMRT/N-CoR binding to either the unliganded receptor or to tamoxifen bound ER recruits histone deacetylases, resulting in transcriptional repression. Recently, a yeast two-hybrid screen using SMRT as bait identified a SMRT/HDAC1 associated repressor protein (SHARP) that binds both SMRT and N-CoR (208). SHARP is a nuclear protein that binds to HDAC1 and HDAC2, and thereby, represses gene transcription. In addition, SHARP associates with the RNA co-activator SRA and represses SRA potentiation of ER transcription on an ERE (208). While the recruitment of histone deacetylases by co-repressors is important in inhibiting ER activity, competition for the LBD also is important (209). REA or repressor of estrogen activity, is a co-repressor that interacts with either the tamoxifen or estrogen bound ER and thereby, potentiates antiestrogen inhibitory activity or inhibits estrogen activity, respectively (209). Although REA expression does not affect ER binding to EREs, it competitively reverses SRC-1 enhancement of ligand-bound ER activity (209). Interestingly, an ER co-regulator that inhibits tamoxifen-mediated partial agonist activity has also been described and accordingly, has been termed repressor of tamoxifen

activation (RTA) (210). When expressed, RTA not only inhibits tamoxifen-mediated agonist activity but also partially inhibits estrogen-mediated transcription (210).

B. Ligand-Independent Activation of ER

In addition to hormone-mediated activation, it is now well accepted that ER function is also modulated in a ligand-independent fashion. While the biological role for the hormone-independent activation of ER is not yet clear, the ability to activate ER in the absence of ligand represents a mechanism by which the cellular environment modulates ER function and may result in a growth advantage to ER positive breast cancer cells.

Peptide growth factors represent a large class of ER activators. Epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) increase ERα and ERβ transcriptional activity in the absence of estrogen (76, 211-213). These findings have also been supported by *in vivo* studies as EGF and IGF-1 mimic the effects of E_2 in the mouse reproductive tract, and pre-treatment of mice with ICI 164,384 greatly diminishes uterine response to EGF (214, 215). In line with the cross-talk between ERα and EGF, ERα knockout mice lack uterine E_2-like responses to EGF even though the EGF signaling pathway is still intact (214, 215).

The molecular mechanism by which the ER is activated in a ligand-independent manner has been characterized and the majority of evidence indicates that modification of the phosphorylation state of the ER by cellular kinases serves as an important mediator. ERα is known to be phosphorylated at multiple sites within the protein while little information exists regarding the phosphorylation of ERβ (Figure 8). It has been well established that serine 118 within the AF-1 domain of ERα is phosphorylated by the

mitogen-activated protein kinase (MAPK) pathway following EGF or IGF treatment, enabling the receptor to bind specific co-activators and increase target gene transcription (75). The same holds true for ERβ, whereby phosphorylation of serines 106 and 124 within the AF-1 domain by MAPK increases its ligand-independent transcriptional activity by stimulating the recruitment of SRC-1 to its N-terminus (213). Extracellular signal-regulated kinase 1/2 (ERK1/2) (downstream signaling proteins of the MAPK pathway) activation also stimulates $pp90^{rsk1}$, resulting in an increase of ERα transcriptional activity via receptor phosphorylation on serine 167 (216). Thus, ERα may be phosphorylated on both serine 118 and serine 167 by two different kinases in an activated MAPK pathway. In addition to phosphorylating ER directly, the MAPK pathway also phosphorylates several ER co-activators, including SRC-1 and AIB1 (217, 218), increasing their transcriptional potential. Other cellular kinases that alter the phosphorylation state of ER and thus, activate ER in the absence of ligand include: i) protein kinase A (PKA), by agents that raise intracellular cyclic adenosine monophosphate (cAMP) (cholera toxin and isobutylmethylxanthine or 8-bromo-cAMP) (76, 212, 219); ii) protein kinase C (PKC), by TPA (12-O-Tetradecanoylphorbol-13-acetate) (211, 220); iii) cytokines (221) and iv) cell cycle regulators, including cyclin D1.

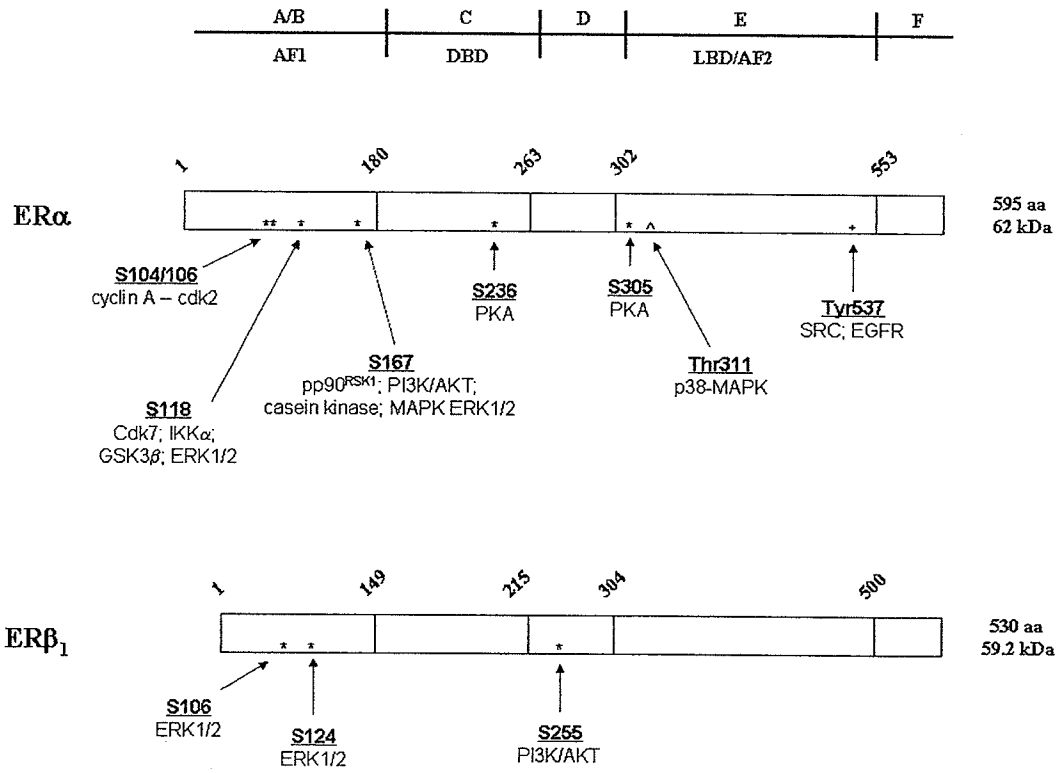


Figure 8: Phosphorylation sites on human ERα and ERβ$_1$. Sites of phosphorylation on the human ERα and ERβ$_1$ is depicted along with the associated kinases. Adapted from (222).

C. Non-Classical Mode of ER Action

A novel mechanism for mediation of an estrogen response was first suggested by the observation that not all genes regulated by ER contain EREs. The mechanism for estrogen action through such "non-classical" pathways is thought to involve protein:protein interactions with other DNA binding transcription factors (e.g. Ap-1 (activator protein 1) (223-225), Sp-1 (131, 226-228) and NF-κB (nuclear factor kappa B) (229, 230)), rather than direct binding of the receptor to regulatory elements in the promoter region (Figure 9). Evidence to support this hypothesis has come from

experiments in which the ER was shown to affect gene expression from promoters containing an Ap-1 site in the absence of an ERE and in ERα mutants either lacking a functional DNA binding domain (231, 232) or bearing a point mutation in the first zinc finger that prevents ERα binding to DNA (225).

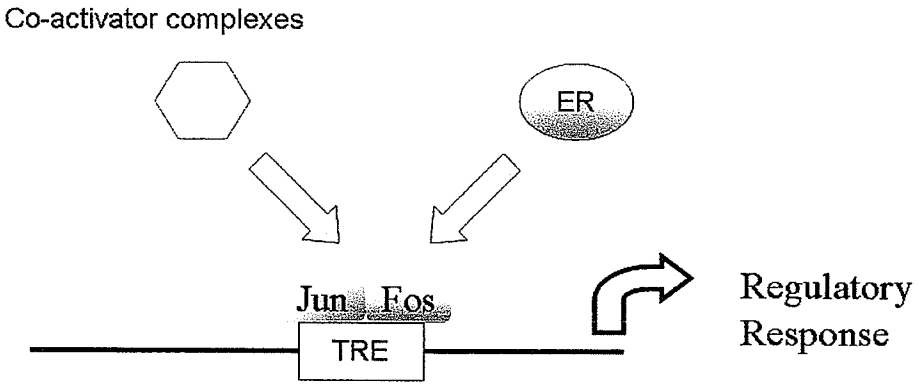


Figure 9: Model of the non-classical mode of ER action. A model of the non-classical mechanism of ER action using Ap-1 as a model. ER binds to Jun/Fos dimers either directly or indirectly through other co-activator complexes (e.g. CBP-p160s) and thereby, modulating target gene transcription. TRE = TPA (12-O-tetradecanoylphorbol-13-acetate) responsive element. Adapted from (223).

i. Activator Protein-1

The transcription factor Ap-1 (activator protein 1) is involved in a wide range of cellular processes, including differentiation, proliferation, survival and death (reviewed in (233). Ap-1 activity is induced by a wide variety of physiological stimuli, including growth factors, cytokines, polypeptide hormones, cell-matrix interactions, bacterial and viral infections as well as by a variety of physical and chemical stresses. The ability of this family of transcription factors to control such a wide range of biological processes is a result of their structural and regulatory complexity. The Ap-1 family of transcription

factors includes the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families. For the purposes of this thesis, I will focus on the Jun and Fos family of proteins.

Fos and Jun proteins were first identified as the viral oncoproteins v-Fos and v-Jun in the Finkel-Biskis-Jinkins osteosarcoma virus (234) and avian sarcoma virus 17 (235), respectively, and their cellular counterparts, c-Fos and c-Jun, were discovered several years later (236, 237). c-Jun, located on human chromosome 1p32 (236), is the most potent transcriptional activator in the Jun family (238). c-Fos, whose gene is located within the region of human chromosome 14q21-q31 (239), enhances the DNA binding activity of Jun proteins through the formation of stable heterodimers (238). The C-terminal region of Jun and Fos contains five leucine residues separated from each other by six amino acids of which form an amphipathic helix (240), in which the leucine residues protrude from one side of the helix forming a hydrophobic surface. Upon protein dimerization, two amphipathic helices fold into a 'coiled-coil' conformation with the leucines aligned along the contact surface (241).

Dimerization of Ap-1 factors is essential for DNA binding in that neither Fos nor Jun can bind DNA as a monomer; they must dimerize prior to binding DNA. Jun has been shown to form homodimers with itself or heterodimers with other proteins containing the leucine zipper structure however, the main dimerization partner of Jun is Fos. X-ray crystallography studies suggest that Jun heterodimers with Fos are more stable than Jun homodimers (242). While Jun-Jun dimers may bind DNA and thereby initiate transcription, Jun-Fos heterodimers have a much higher affinity for the DNA

target sequence than Jun homodimers (243-245). Using electrophoretic mobility shift assays (EMSAs) and *in vitro* translated c-Jun and c-Fos proteins, Halazonetis et al. (243) have shown that Jun heterodimers with Fos bind to an Ap-1 sequence fragment 25 times more efficiently than Jun homodimers. While all Fos proteins increase the binding of c-Jun to Ap-1 sequences, c-Jun-FosB dimers are the most stable and most efficient at binding DNA, followed by c-Jun-Fra-1 and c-Jun-c-Fos (238). Fos cannot form homodimers (242, 243, 245), only heterodimers, due to the presence of several unfavourable contacts in the coiled-coil conformation (242). Due to its failure to homodimerize, Fos cannot bind DNA by itself (243, 246); it must first complex with other leucine zipper proteins, namely Jun, to alter gene transcription. Thus, the dimerization of Jun and Fos is required for DNA binding.

N-terminal to the leucine zipper of Jun and Fos is a basic region, which is involved in DNA binding. The consensus DNA binding sequence for Jun homodimers and Jun-Fos heterodimers is 5'-TGACTCA-3' and is referred to as the 12-O-tetradecanoate-13-acetate (TPA)–responsive element (TRE) (247, 248). Multimers of the TRE increases the Ap-1 transcriptional response dramatically upon Ap-1 activation but also has significant basal activity in the absence of Ap-1 stimulation. This basal activity is thought to be, in part, the result of a low level of active Ap-1 that is present in many cultured cell lines before cellular stimulation.

Phosphorylation plays an important role in modulating Ap-1 transcriptional activity. The N-terminal region of c-Jun is phosphorylated by Jun kinases (JNKs) at serines 73 and 63, enhancing c-Jun transcriptional activation either as a homodimer (249-251) or as a heterodimer with Fos (252). The enhancement of c-Jun activity through

phosphorylation events by JNK has been suggested to be partly due to a higher affinity of phosphorylated c-Jun for the transcriptional co-activator CBP (253). CBP is a potent transcriptional co-activator which was first identified through its strong interaction with phosphorylated CREB (cAMP response element binding protein) (254). CBP has been shown to interact with TFIID, a part of the basal transcriptional machinery, and is important in mediating the transcriptional response of both CREB and Ap-1 (255, 256). In 1996, it was suggested that competition for limiting amounts of CBP by activated nuclear receptors (specifically GR and RAR) and Ap-1 factors is responsible for the inhibition of Ap-1 activity by ligand bound nuclear receptors (257). Indeed, over-expression of CBP through transient transfection of vectors encoding CBP abolished the inhibitory effect of activated RAR and GR on Ap-1 activity on a TRE reporter plasmid (257). More recent evidence suggests, however, that while such a competition may be accurate for RAR (258), it may not be the case for GR (259). De Bosscher et al. (259) suggest glucocorticoid repression of Ap-1 activity occurs irrespective of the amount of co-activator levels present in the cell but rather is the result of JNK inhibition by GR. In these studies by De Bosscher (259), only 100-200 ng of CBP expressing plasmid was transfected into the cells as compared to 2ug in the original study (257). As quantification of CBP levels was not undertaken in either study, it is difficult to determine whether sufficient levels of CBP was obtained to reverse the effect of an activated GR by these authors.

While phosphorylation of c-Jun at its N-terminal extremity is a positive regulator of transcriptional activity, phosphorylation of residues near the C-terminal inhibits transcription. ERK1 and ERK2 (extracellular related kinase 1/2), downstream signaling

proteins in the MAPK cascade, and glycogen synthase kinase-3 (GSK-3) (260, 261) have been shown to phosphorylate c-Jun at Ser243, Ser249 and Thr239 located next to the DNA binding region (262, 263). Phosphorylation at Ser243 inhibits c-Jun binding to DNA (260, 264). Activation of PKC through TPA stimulation results in the rapid dephosphorylation of Thr239 (260) that serves as an important regulatory event in the DNA binding activity of c-Jun to Ap-1 sites (263). The mechanism by which TPA treatment results in c-Jun dephosphorylation is unknown; however it has been suggested that conformational changes in the protein upon phosphorylation at distinct sites exposes the residue to dephosphorylation by phosphatase(s) (263).

The transcriptional activity of c-Fos is also increased by the MAPK family through its phosphorylation at Thr232, the Fos homolog of Ser73 in c-Jun (252). The phosphorylation of c-Fos, unlike that of c-Jun, is not mediated by the JNKs, but rather from a novel MAPK termed FRK (Fos-regulating kinase) (252). Thus, Jun-Fos dimers that have similar DNA binding specificities may differ in their transcriptional activity due to phosphorylation events on both Jun and Fos in domains located outside the DNA binding region.

ii. Interaction between Ap-1 and ER

Evidence for an interaction between ER and Ap-1 was first identified in a search for genes involved in the mitogenic response of cells to estrogens. It was hypothesized that in estrogen growth responsive cells, ER modulates the activity of genes whose products control cell cycle. In line with this hypothesis, it was found that estrogen induces the expression of the proto-oncogene c-*fos* in rat uterus and human breast cancer cells in culture (265, 266). The induction of the c-*fos* gene by estrogen occurs rapidly

after treatment and is not abolished by protein synthesis inhibitors, suggesting that transcriptional activation of c-*fos* is in direct response to the hormone (266). The E_2 responsive sequence in the c-*fos* promoter has been localized to a 240 bp region that contains an imperfect palindromic ERE to which ERα binds in electrophoretic gel mobility shift assays (130). However, induction of c-*fos* was not observed using a construct containing the imperfect palindrome (130, 228) and more recent studies have shown that induction of c-*fos* expression by ER is dependent on the formation of a transcriptionally active Sp1/ER complex that binds to a GC-rich site in the promoter region (228). Up-regulated Fos is then involved in a negative feedback loop in which Fos antagonizes the activation of its own gene promoter (267). In addition to c-*fos*, E_2 activated ER also stimulates the transcription of c-Jun, JunB and JunD in breast and uterine cells (268, 269). Furthermore, the antiestrogen tamoxifen increases c-Fos and Jun-B and to a much lesser extent c-Jun and Jun-D, mRNA expression in intact rat uteri (270, 271) but not in breast tissues (231). The induction of such Ap-1 factors is thought to be partly responsible for the mitogenic effect of estrogen and possibly, tamoxifen, in these tissues.

ER interacts with Ap-1 transcription factors on the promoter region of target genes. As described previously, ER activates transcription either by binding DNA directly on a classical ERE (272) or by binding to other DNA binding proteins at alternate response elements, including Ap-1 (223, 225, 232). Alterations in the expression of collagenase (MMP-1) (225, 231, 273), insulin-like growth factor-1 (IGF-1) (274), progesterone receptor (132), ovalbumin (232, 275), gonadotropin-releasing hormone receptor (276) and cyclin D1 (273, 277) genes by estradiol have all been shown

to involve Ap-1 sites that bind members of the Jun and Fos family of proteins. While the estrogen regulation of Ap-1 activity is generally positive in breast and uterine cells (275), it can also be inhibitory in breast cancer cells (276, 278). In contrast, tamoxifen is generally an agonist of Ap-1 regulated genes in endometrial cell lines but an antagonist in breast cells (231). The description below will focus on the effects of ERα and ERβ on Ap-1 regulated genes in the context of a breast cell background.

The effect of ER on Ap-1 activity in the context of an Ap-1 responsive reporter plasmid containing concatermerized consensus Ap-1 binding fragments is generally stimulatory in ER^+ breast cancer cell lines (278, 279), although a negative effect has also been reported (225). The increase in Ap-1 activity in ER^+ cells is largely attributed to ERα expression, as increased expression of ERβ by transient transfection into MCF-7 cells inhibits the cellular response to estrogen (279). In comparing the effect of E_2 on Ap-1 activity between $ER\alpha^+$ and $ER\alpha^-$ breast cancer cell lines, Philips et al. (278) have suggested that differential expression of the Fos family member, Fra-1, may be responsible for differences in Ap-1 activity upon hormone treatment in $ER\alpha^+$ (MCF7, T47D and ZR75) and $ER\alpha^-$ (MDA-MB-231 and BT20) breast cancer cell lines. These authors have shown that $ER\alpha^+$ cell lines have a positive effect on Ap-1 activity but have a low level of Fra-1 expression while $ER\alpha^-$ cell lines transfected with ERα have a negative effect on Ap-1 activity and have a high expression of Fra-1 (278). Although the mechanism through which Fra-1 inhibits ER activity on Ap-1 promoters remains unknown it may perhaps be due to the specific composition of the Ap-1 dimers present in the cell lines (Jun/Fra-1 rather than Jun/Fos). Reports of the effect of antiestrogen treatment on concatermerized Ap-1 reporter plasmids are few. However, it does appear

as though antiestrogen treatment (tamoxifen and ICI 182,780) increases the Ap-1-mediated response in a dose-dependent manner while having no effect on a consensus ERE plasmid (225, 279).

Responses to ER on the collagenase (MMP-1; matrix metalloproteinase) promoter tends to be quite similar to its effects on the concatermerized Ap-1 plasmid described above. Generally, estrogen treatment of breast cancer cells (MCF-7, MDA 453, ZR75) transiently transfected with a collagenase reporter construct stimulates Ap-1 activity (231, 273, 280) while tamoxifen and ICI 182,780 have no effect (231). However, Jakacka et al. have observed a decrease in collagenase reporter activity in the presence of E_2 and an increase with ICI 182,780. The reasons for the discrepancies between these results is unknown, but may be due to differences in cell culture conditions (presence of E_2 in media during routine upkeep) or to differences in reporter plasmid backbone. Interestingly, MCF-7 cells over-expressing ERβ through transient transfection show a significant increase in collagenase reporter activity with the antiestrogens ICI 164,384, raloxifene and tamoxifen while treatment with E_2 has no effect (280).

The molecular mechanism by which ER affects Ap-1 activity is still under debate. It is unlikely that ER dependent activation at Ap-1 sites is due to changes in the amount of Ap-1 proteins as protein synthesis inhibitors do not diminish the response to estrogen and induction still occurs under conditions in which c-Jun and c-Fos mRNA expression remains unchanged (281). A clue as to the way in which ER functions at Ap-1 sites came from studies mapping the regions of ERα important for its stimulatory effects. Mutations within the AF-1 and AF-2 domains of ERα completely eliminate the Ap-1 response to ERα (231). As the ERα AF-2 domain is involved in co-activator binding in the presence

of estrogens, it is not surprising that GRIP-1, SRC-1 and p300/CBP have all been associated with an increase in ERα activity at Ap-1 sites (223, 225, 273, 282). The co-activators p300/CBP have also been implicated in modulating Ap-1 transcriptional responses to ERα, although they are recruited through distinct protein:protein interactions (282). Furthermore, as Ap-1 factors themselves bind SRC-1 (179) and p300/CBP (253), it thus has been proposed that the inhibitory effects of ER at Ap-1 sites may be the result of direct competition for limiting amounts of transcriptional co-activators within the cell (276). Evidence for this model comes from studies on the gonadotropin releasing hormone receptor gene to which estrogen treatment represses its expression in MCF-7 cells via an Ap-1 motif (276). Transient over-expression of the co-activator CBP reversed the effect of ligand-bound ER in a dose dependent manner, suggesting that CBP was limiting for transcriptional activation (276). Similarly, ER in the presence of SERMs may sequester a transcriptional repressor away from the Ap-1 site, resulting in an increase in Jun/Fos transcriptional activity (283). Another mechanism by which ER has been proposed to affect Ap-1 activity is via direct protein:protein interactions between ER and c-Jun (224, 231). *In vitro* studies suggest that c-Jun and ERα bind to one another in a ligand independent mechanism (224); however the interaction between these two proteins has not been extensively studied *in vivo*.

iii. Ap-1 and the Non-Classical Mode of ER Action

ER affects gene expression from promoters containing an Ap-1 site. A variety of estrogen-responsive genes have been described that lack a palindromic ERE but contain consensus Ap-1 elements (5'-TGAG/CTCA-3'), with or without a degenerate ERE or ERE half-site (5'-GGTCA-3' or 5'-TGACC-3'). The ovalbumin gene, which is induced

by E_2 in chicken oviduct and HeLa cells transiently expressing ERα (232, 275), contains a half-palindromic ERE (5'-TGGGTCA-3') that binds Ap-1 oncoproteins and, to a much lesser extent, ERα (232). Transient transfection experiments in chicken embryo fibroblasts and HeLa cells suggest a synergistic activation between c-Jun, c-Fos and ER on the ovalbumin reporter plasmid and that this effect is independent of ER binding to DNA (232, 275). Estrogen has been shown to increase the expression of collagenase (231, 280), IGF-1 (274) and PR (132) promoters that contain critical Ap-1 elements but lack consensus EREs. The co-operation between Ap-1 and ER has also been shown to be involved in the stimulation of cyclin D1 expression by ERα in human breast cancer cells (277). Further evidence to support the importance of the Ap-1 transcription factor, c-Jun, in mediating the effect of ligand activated ER through protein:protein interactions has recently been described. Using an inducible c-Jun dominant negative mutant in MCF-7 cells, DeNardo et al. (273) show that Ap-1 blockade inhibits estrogen induced expression of Ap-1 dependent genes by preventing the binding of ER to the promoter region. While estrogen-bound ERα typically enhances Ap-1 target genes (277), it is important to note that the Ap-1 response to ligand activated ER varies between different cellular milieus (stimulatory to inhibitory) (223, 225, 231), possibly due to alterations in the relative expression of Ap-1 proteins present (278). ERα and ERβ have been shown to have opposing actions within the same cell type (277, 280) and, when expressed within the same cell, modulate the transcriptional activity of each other on Ap-1 responsive genes (277, 279). Nonetheless, the cellular response to estrogen activated ERα on Ap-1 genes in endometrial and breast cancer cells is generally positive, such that there is an increase in target gene transcription upon E_2 treatment.

Cellular responses on Ap-1 elements in the presence of antiestrogens tend to mimic their effects on cellular proliferation. Alternate responses between breast and uterine cells at Ap-1 sites in the presence of tamoxifen have been observed: tamoxifen bound ERα is stimulatory in uterine cells but has no measurable effects on Ap-1 dependent transcription in breast cells (231, 280). Thus, whereas tamoxifen does not stimulate normal breast tissue growth, it does stimulate uterine growth (284). This effect of tamoxifen bound ERα at Ap-1 sites is in contrast to its response at classical EREs, in which antiestrogen treatment typically inhibits target gene transcription (280). Interestingly, tamoxifen-bound ERβ stimulates Ap-1 activity in both endometrial and breast cell backgrounds (277, 280, 285), suggesting that different ligand actions between ERα and ERβ may be a feature at Ap-1 promoters. Raloxifene, unlike tamoxifen, lacks estrogen-like effects on Ap-1 promoters in uterine cells when bound to ERα but is a strong activator of Ap-1 with ERβ (96, 149, 277). The effects of pure antiestrogens on Ap-1 activity is somewhat controversial, as some researchers suggest that they have no effect on Ap-1 activity while others suggest that ICI 182,780 treatment in the presence of ERα or ERβ potentiates Ap-1 transcriptional activity (277, 280, 285).

Domain requirements for the selective responses of ERα and ERβ at Ap-1 sites are very complex. Deletion studies of ERα show that the AF-1 and AF-2 activation functions of ERα are required for the agonist activity of estrogen at Ap-1 sites (96, 282, 286) while the DBD and regions within the N-terminal domain outside of the AF-1 are required for tamoxifen activation in uterine cells (96, 231, 286). Interestingly, tamoxifen and raloxifene activities appear to be dependent on the amino terminus of ERβ (96), despite the low level AF-1 activity observed on an ERE (84). Deletion of the AF-1

domain of ERα elicits a response to raloxifene that mimics that of ERβ (96, 286), suggesting that ERα AF-1 suppresses the raloxifene activation at the Ap-1 site by ERα. Since ERβ has no constitutive AF-1 function, it has been suggested that ERβ activates Ap-1 by an AF-independent mechanism. However, raloxifene activation at an Ap-1 site by ERβ is diminished when the N-terminal is deleted (96), indicating the need for an activation function located in this region. Thus, different ligands and different ER receptor subtypes utilize different regions to enhance Ap-1 activity.

While the exact mechanism through which ER functions at Ap-1 sites is not completely understood, it has been suggested that the ER binds directly to proteins bound to the Ap-1 site (224). Several investigators have shown that binding of Jun/Fos to the Ap-1 site is necessary for ER action and once bound, ERα appears to act as a co-activator, increasing the intrinsic transcriptional activity of Ap-1 (282). Whether ER binds to Jun and Fos directly (224, 231) or is recruited to Ap-1 sites indirectly through binding to pre-existing Ap-1/co-activator complexes (223) is still unclear. Evidence for a direct interaction between Ap-1 proteins and ERα has been demonstrated both *in vitro* and *in vivo*. GST-pull down assays show that ERα binds to c-Jun and JunB but not to Fos proteins *in vitro* (224, 231) and that the interaction is ligand independent (224). The interaction between ERα and c-Jun *in vivo* has been shown using a mammalian cell two-hybrid assay and by co-immunoprecipitation in MCF-7 and Cos cells transiently transfected with ER and/or c-Jun expression vectors (224, 287). The region of ERα that interacts with c-Jun is unclear. In one study, the ERα DBD and hinge domains were implicated in mediating the interaction between ERα and c-Jun (224) while in other studies, residues within the amino terminus were important as deletion of these regions

abolished the interaction between c-Jun and ERα *in vitro* (231, 287). Cheung et al. (282), using immobilized DNA assays, demonstrate that the DBD is required for the recruitment of ERα to Fos/Jun bound to Ap-1 sites while the AF-1 and AF-2 regions are required for activation. The region of c-Jun that binds to ERα is also uncertain, as both the carboxy (224) and amino terminus (287) have been suggested in protein:protein binding. No physical interaction between ERβ and Ap-1 factors has been described.

iv. The TGFβ$_3$ Promoter and the Non-classical Mode of ER Action

As discussed previously, the transcriptional activation by estrogen of genes that do not contain an ERE requires the interaction of the ER with other signaling proteins that bind DNA, including Sp-1 and Ap-1. However, there are other unidentified factors to which the ER binds that mediate its activity on target genes. In investigating the tissue-selective activity of raloxifene in bone, Yang et al. demonstrated the existence of a DNA response element for raloxifene in the promoter region of the TGFβ$_3$ gene (288). Further studies suggest that the TGFβ$_3$ promoter is activated by estrogen but to an even greater extent by raloxifene, tamoxifen and ICI 164,384, with raloxifene having the greatest activity (289). The raloxifene response element shows no sequence similarities to either the palindromic ERE or the Ap-1 binding site and removal of the DBD of ERα does not prevent activation of the gene, suggesting that protein:protein interactions with an unknown transcription factor occurs (289). Transient transfection of Cos1 cells with a TGFβ$_3$ reporter gene suggests that raloxifene increases ERα and to a lesser extent, ERβ$_1$ transcriptional activity and co-expression of ERβ$_2$ and ERβ$_5$ are able to inhibit raloxifene induced ERα, but not ERβ$_1$, activity (139). While the mechanism through which ER modulates TGFβ$_3$ transcription is unknown, it has been suggested that the Ras-MAPK

signaling pathway may be involved (290) (although these studies have not been confirmed by others).

D. Non-Genomic Mechanism of ER Action

Traditional estrogen signaling pathways involving nuclear transcription usually require hours for maximal gene activation. However, estrogens also have other effects on the cell that cannot be explained by a transcriptional mechanism due to their very rapid onset (seconds to minutes). Multiple studies have shown that these rapid actions take place outside of the nucleus and are initiated at the level of the plasma membrane and thus, have been referred to as 'non-genomic', 'extra-nuclear', or 'membrane-mediated' effects. Some of the effects of estrogen mediated by this pathway include arterial vasodilation (291, 292); induction of cell proliferation and inhibition of apoptosis (293); changes in neuronal excitability (294); and neurotrophic responses (295). These effects may involve: (i) IGF-1 and EGF receptors (296, 297); (ii) MAPK (298-300); (iii) calcium channels (295); or (iv) phosphoinositol-3-kinase/AKT1 (292, 301), to name a few. While membrane receptors distinct from the ER have been suggested to be involved in mediating the non-genomic effects of estrogen (reviewed in (302)), compelling evidence supports the presence of a subpopulation of ERα and ERβ located at the plasma membrane (103, 104), although the definitive identification of a membrane bound ER has not yet been achieved (303).

IV. ER Cross-talk with Other Signaling Pathways

ER cross-talk with other growth factors and their downstream signaling proteins are also important in breast tumorigenesis and breast cancer progression. Similar to ER

binding to co-activators, cross-talk between various signaling pathways amplifies cellular proliferation coming from both the ER and growth factor receptor pathways in breast tumors. While these signaling pathways often exert stimulatory effects on ER signaling, ER also may either increase or decrease the expression of every level of the signaling pathway (304). The signaling pathways to which the ER has been shown to cross-talk with include: PI3K/AKT; Ras-MAPK; stress-induced pathways mediated by JNK and p38 MAPKs; Ap-1; and more recently the TGF-β growth inhibitory pathway.

A. The TGFβ Signaling Pathway

i. TGFβ Receptors

The TGFβ family consists of TGFβ, the bone morphogenetic proteins (BMPs), activins, anti-mullerian hormone, growth and differentiation factor 5 and the Vg1 subfamily. This family of growth factors regulate an array of cellular processes including cell migration, proliferation, adhesion, differentiation and death (305). For the purposes of this thesis, I will focus on the TGFβ signaling pathway.

TGFβ and related factors signal through a family of transmembrane protein serine/threonine kinases referred to as the TGFβ receptor (TβR) family. Based on structural and functional properties, the TβR family consists of two subfamilies: type I (TβR-I) and type II (TβR-II) receptors. TβR-I and TβR-II have the same basic structural elements which include a short, cysteine rich extracellular domain that is involved in ligand binding and an intracellular serine/threonine protein kinase domain (305). In addition, TβR-I contains a conserved glycine and serine region, also known as the GS domain, that precedes the receptor kinase domain (306, 307). Activation of the signaling

cascade begins as TGFβ ligand binds to TβR-II (Figure 10). Ligand binding results in the recruitment of TβR-I to the TβR-II/ligand complex where TβR-I rapidly becomes phosphorylated on serine and threonine residues within the GS domain by the

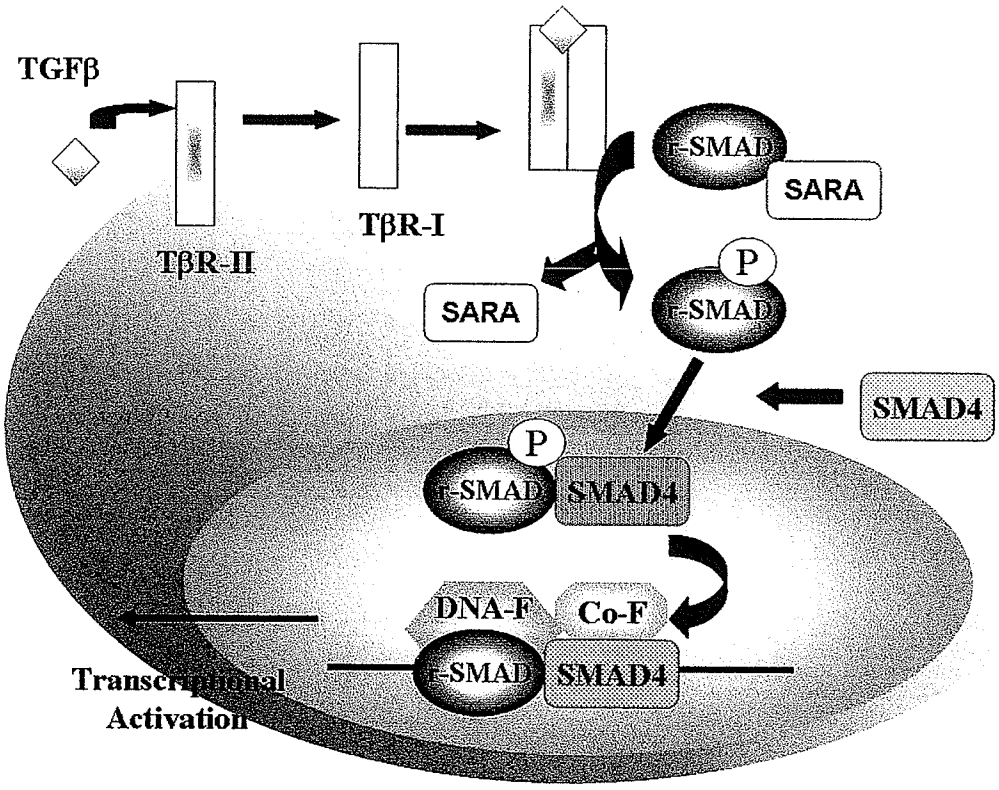


Figure 10: The TGFβ signaling cascade. TGFβ binds to the TβR-II receptor, which then forms a complex with TβR-1 molecules. The TβR-II kinase phosphorylates TβR-I, thereby activating its serine-threonine kinase. In response to receptor activation, receptor Smads (r-Smad), namely Smad2 and Smad3, transiently associate with and are phosphorylated by the TβR-I kinase. After their activation, r-Smads interact with Smad4. The r-Smad/Smad4 complex translocates to the nucleus where it interacts with various co-factors to bind DNA in a sequence specific manner and thereby, modulates target gene transcription. Adapted from (305).

constitutively active kinase of TβR-II (306, 307). These multiple phosphorylations activate TβR-I, which transduces the signal to downstream signaling proteins. Binding of FKBP12 to TβR-I in the basal state protects and stabilizes the TβR-II phosphorylation sites on TβR-I (308) and thereby, regulates TGFβ signaling. Heterodimerization of TβR-I and TβR-II may be disrupted by a BMP and activin membrane-bound inhibitor BAMBI, a naturally occurring truncated type I receptor that lacks a kinase domain (309). BAMBI stably associates with the TβRs and thereby, inhibits TGFβ signaling (309).

ii. Smads

TGFβ binding to TβR-II results in the heterodimerization of TβR-I and TβR-II. Phosphorylation and subsequent activation of the type I receptor stimulates the downstream signaling proteins known as the Smads. The Smad family of proteins, consisting of Smads 1-9, is divided into three functional groups: Smads 1, 2, 3, 5 and 8 are receptor Smads (r-Smads); Smad4 is the common mediator Smad (co-Smad) while Smad 6 and 7 are inhibitory Smads. These proteins are characterized by an N-terminal MAD homology 1 (MH1) domain and a C-terminal MAD homology 2 (MH2) domain which are connected by a proline rich linker region. The MH1 domain is highly conserved among the r-Smads and Smad4 (305). In its activated state, the MH1 domain contains the DNA binding region through which Smads recognize and bind to Smad responsive elements (SREs) (310). The MH2 domain contains receptor phosphorylation sites and is involved in several important protein-protein interactions. This domain also contains the effector function in r-Smads (305).

As previously described, Smads are the downstream signaling proteins in the TGF-β signaling cascade. TβR-I activation results in the recruitment of r-Smads, namely

Smad2 and Smad3, to its kinase domain via Smad interaction with the membrane-associated protein Smad-anchor for receptor activation (SARA) (Figure 10) (311). Confocal microscopy experiments have demonstrated that SARA interacts with Smad2 and Smad3 and co-localizes with TβR-I (311). Therefore, it has been hypothesized that SARA recruits Smad2 and Smad3 into distinct subcellular domains for their interaction with an activated TβR-I. SARA binds to the MH2 domain of Smad2 and Smad3 through a double zinc finger domain (FYVE domain) (311). Activated TβR-I phosphorylates Smad2 and Smad3 on serine residues within the MH2 domain, inducing r-Smad dissociation from SARA (311). Deletion of the FYVE domain in SARA results in r-Smad mislocalization and inhibits the TGF-β dependent transcriptional response. Dissociation of Smad2 and Smad3 from SARA allows Smad binding to Smad4, and the resulting heterodimer translocates to the nucleus (Figure 10). Once in the nucleus, Smad2/Smad4 and Smad3/Smad4 regulate target gene transcription by associating with transcription co-factors (312) and binding to specific DNA sequences, also known as SBEs (Figure 10) (313, 314) through their MH1 domain. Transcriptional factors that cooperate with Smad proteins to regulate transcription include p300/CBP (315, 316), TFE3 (314), FAST-1 (317), Ski and SnoN (318, 319). Yanagisawa et al. (320) have also described a ligand-dependent interaction between the VDR and the MH1 domain of Smad3. Formation of the VDR/Smad3 complex increases the ligand-induced transactivation function of VDR on a reporter plasmid (320) and this effect is abolished by the inhibitor Smad, Smad7 (321). Interactions between the C-terminal activation domain of Smad3 and GR have also been demonstrated *in vitro* and *in vivo* (322).

iii. TGFβ and ER Cross-talk

The interaction of ER and TGFβ pathways has been suggested previously (323, 324). Signal transduction through the TGFβ pathway plays a key role in the negative regulation of breast epithelial cell growth (325-327) and exposure of human breast cancer cells to TGFβ *in vitro* inhibits cell growth (328-330). Treatment of mice with TGFβ has also been shown to protect against mammary tumor formation *in vivo* (331). However, many mammary tumors often become insensitive to the antiproliferative effects of TGFβ (325, 332). Furthermore, it is thought that enhanced secretion of TGFβ protein is associated with the growth inhibitory effects of antiestrogens (323, 324, 333) and arguably implicated in the mechanism by which antiestrogens inhibit the growth of some human breast cancer cells. In contrast, over-expression of TGFβ has been associated with estrogen independence and antiestrogen resistance in human breast cancer (324, 327, 334).

Apart from direct autocrine or paracrine effects of TGFβ on breast cancer cell growth, direct cross-talk between the ER and TGFβ signaling pathways has been potentially identified (335, 336), further adding to the potential complexity of how TGFβ and ER mediate their action. In various model systems, direct physical interactions between ERα and Smad1, Smad2, Smad3 and Smad4 have been suggested to occur in a ligand-dependent manner (335, 336). In addition, direct interactions of Smad3 with other members of the nuclear receptor family have been demonstrated (320-322, 337, 338). The outcomes of these interactions appear to differ depending on the nuclear receptor involved and possibly the cell type background under which the experiments were carried out (320, 335). Nonetheless, the mechanism by which ER modulates Smad

transcriptional activity and the significance of such an interaction in breast cancer tumorigenesis and breast cancer progression is unknown.

TGFβ is involved in the negative regulation of breast epithelial cell growth (325-327) and exposure of human breast cancer cells to TGFβ *in vitro* inhibits their growth (328, 329). However, many mammary tumors often become insensitive to the anti-proliferative effects of TGFβ *in vitro* (325, 332). The mechanism by which breast tumors lose their sensitivity to the growth inhibitory effects of TGFβ is unknown. Alterations in the expression of membrane receptors and downstream Smads are rare as well as mutations within the signaling pathway (339, 340), in spite of a report by Gobbi et al. (341) who reported an increased risk of breast cancer development due to a decrease in the expression of membrane receptors. However, when alterations in the Smad signaling proteins do occur in breast tumors, there is a poorer patient prognosis (342).

iv. TGFβ and Cancer

A strong correlation exists between cancer formation and a loss of sensitivity to the growth inhibitory effects of TGFβ. This insensitivity may be due to several factors, including a loss of functional receptor expression (343-347) or by inactivating mutations of the TGFβ receptors themselves (348, 349). Several researchers have reported a decrease in TβR-II expression in breast tumor cell lines and mammary tumor models (332, 350, 351). Pouliot et al. (330), using a ribonuclease protection assay with a cDNA probe specific for TβR-II, reported that not all human breast cancer cell lines express the TβR-II receptor. Even more intriguing was a report by Gobbi et al. (341), who demonstrated that a loss in TβR-II expression occurs in epithelial cells from patients with atypical breast lesions and further suggested that this loss may be associated with an

increased risk of breast cancer development. Immunohistochemical studies examining TβR-II expression in 178 breast biopsies of normal, benign and malignant breast tissues demonstrated a loss of receptor expression within human breast carcinomas and an inverse relationship between tumor grade and TβR-II expression (352). Thus, a decrease in TβR-II expression is associated with a more aggressive mammary tumor phenotype. The altered expression of the type II receptor in cancer cells may therefore allow these cells to escape the TGFβ growth inhibitory effects (352) that occur in the late stages of breast cancer development and in breast cancer progression. Although TβR expression clearly conveys cellular resistance to TGFβ, mutations within the receptors themselves may also be important. In spite of the fact that mutations involving TβR-II have been reported in breast cancer cell lines (349), no mutations have been found in human breast cancer *in vivo* (339, 340, 353). In contrast, TβR-II mutations are relatively common in colorectal and gastric tumors (348, 354). Chen et al. (353) have reported the presence of a serine to tyrosine mutation (S387Y) in the kinase domain of TβR-I that is responsible for disrupting receptor signaling in human breast carcinomas with axillary lymph node metastases. However, a similar study performed by Anbazhagan et al. (339) revealed no mutations at the S387Y site in 20 breast cancer metastases.

Like the TGFβ receptors, Smad protein mutations have been described in gastric, colorectal and pancreatic cancer cell lines and tumors (355-358) but Smad3 gene mutations are rare in colorectal cancers (359). No mutations have been reported in human breast cancer cell lines (355) although Smad4 is deleted in the MDA MB 468 breast cancer cell line (330) and a splice variant of Smad4 is present in the ZR-75-1

breast cancer cell line (330). Interestingly, no systematic studies have been reported which investigate Smad3 mutations in normal and malignant breast tissues.

V. ER and Breast Cancer

Throughout breast tumorigenesis and breast cancer progression, the expression and function of the ER changes dramatically. In normal breast tissue, the majority of proliferating cells are ERα negative (360), while ERα expression in breast tumors is associated with an increase in cellular proliferation and metastasis (361). ERα expression in normal breast epithelium is relatively low (362) while approximately 70% of all primary breast cancers are ERα positive (363, 364), suggesting that the proportion of cells expressing ERα increases significantly during the initial disease process. While ERα levels are up regulated throughout breast tumorigenesis, they are typically down regulated during breast cancer progression (365, 366). The level of ER expression within a breast tumor provides relevant prognostic information with respect to time to relapse and overall survival as ER^+ patients with metastatic breast cancer survive twice as long as patients with ER^- tumors (367). Tumors that are ER^+ tend to be low grade, well-differentiated tumors that have little or no necrosis or lymphoid cell infiltration (368, 369). Most importantly, however, ER^+ tumors are associated with a significantly higher response rate to endocrine therapies that target the ER (i.e. antiestrogens) than those with low or undetectable levels (370). Furthermore, tumors that express PR, a downstream marker representing a functional ER (371), in addition to ER (ER^+/PR^+) have an increased probability of response to tamoxifen, longer time to treatment failure and longer overall survival than those expressing ER alone (372). Thus, ER and PR are important predictive markers for response to endocrine therapy in breast cancer patients.

Of all primary human breast tumors, approximately 59% co-express ERβ and ERα ($ER\beta^+/ER\alpha^+$) while approximately 17% express ERβ alone ($ER\beta^+/ER\alpha^-$) (373, 374). ERβ appears to be highly expressed in normal mammary glands (51, 52, 54). In contrast to ERα, ERβ expression generally decreases during breast tumorigenesis (51, 52, 375). With respect to the correlation of ERβ with clinicopathological features, conflicting results have been reported. Such contradictory results may in part be due to how ERβ expression was determined, being either RNA or protein, the range and specificity of antibodies used for detecting ERβ, and the scoring systems used to determine ERβ status (376, 377). Nevertheless, some common themes are emerging from the literature involving the expression of ERβ protein, such as correlations with ERα and PR expression (51, 54, 378-380), low tumor grade (378, 381), increased response to endocrine therapy (54), and overall better prognosis than ERβ negative tumors (379, 381). These findings also suggest that ERβ may function as a tumor suppressor within the breast and that loss of ERβ may promote breast tumorigenesis. In support of this hypothesis, ERβ has been shown to inhibit human breast cancer cell proliferation and tumor formation (382-385), possibly by down-regulating components of the cell cycle, including cyclin D1 and c-myc (384, 385). In addition, several lines of evidence suggest that ERβ negatively regulates ERα activity both *in vitro* (82) and *in vivo* (55), such that it decreases cellular sensitivity to estrogens.

ERβ variant mRNA and proteins have also been identified in breast cancer cell lines as well as in both normal and neoplastic human breast tissues (72, 375, 378, 386-388). Unlike the wild-type ER, $ER\beta_1$, evidence suggests that ERβ variant expression,

particularly $ER\beta_{2(cx)}$, may increase in tumor versus normal breast tissue (381, 388, 389). Furthermore, higher expression levels of the ERβ variants as compared to the wild-type ERβ, $ER\beta_1$, occur not only in breast cancer cell lines but also in both tumor and normal breast tissue (388). Few studies have examined the correlations between ERβ variant protein expression and clinicopathological features. Antibodies specific for $ER\beta_2$ have recently been described, but none are currently available for the other ERβ variants. To date, only three studies have examined $ER\beta_2$ protein expression in human breast tissues. Due to the limitation of sample numbers, it is unclear whether $ER\beta_2$ expression is a predictive factor for endocrine therapy. It has been shown, however, that expression of $ER\beta_2$ correlates well with negative PR status in ERα rich cancer foci (390). Saji et al. (390) also undertook a small study of 18 core needle biopsy samples obtained before preoperative tamoxifen treatment. Their results show that $ER\alpha^+$ tumors that are $ER\beta_2$ and PR negative have a better response to tamoxifen therapy than $ER\beta_2^+$ / PR^- tumors (390), suggesting that ERβ variant expression may further define hormonal treatment response in addition to ERα. However, these conclusions need to be evaluated in a larger cohort of patients.

As previously described, the ER does not act alone, but rather is one component in a multi-protein complex that determines the effects of estrogen in a particular tissue or on a particular gene. Co-activators and co-repressors are important in modulating ER activity and their level of expression during breast tumorigenesis may also play an important role in mediating hormonal regulation. In human breast tumors, the ER co-activators SRA, CBP, SRC-1, TIF2 and AIB1 have been shown to be up-regulated as compared to normal breast tissue (366, 391, 392), suggesting that they may have an

important role in breast cancer development and progression. In a study by Anzick et al. (391), AIB1 was found to be over-expressed in four of five ER^+ breast cancer cell lines and amplified in 64% of primary breast tumors as compared to normal mammary epithelium. These studies were later confirmed by several other laboratories (392, 393). Although the level of ER co-activators appears to increase throughout breast tumorigenesis, the expression of ER co-repressors either decrease (366) or do not change (392). Evidence suggests that N-CoR expression may be down-regulated in invasive breast tumors compared to normal breast tissue (366), while REA expression does not change between breast tumor biopsies and adjacent matched normal breast tissue (392, 394). Therefore, the ratio of ER co-activators:co-repressors may be higher in tumor tissue as compared to normal human breast tissue (392) and data from our laboratory demonstrated that the relative expression of SRA and AIB1 to REA increased in ER^+ breast tumors as compared to adjacent normal breast tissue (392). From these results, it was hypothesized that altered relative expression of ER co-activators and co-repressors may dictate the cellular response to estrogen and antiestrogens. Recent data from our laboratory suggests, however, that the relative expression of SRA/AIB1 to REA mRNA is not altered between tamoxifen sensitive and tamoxifen resistant breast tumor biopsies (54).

RESEARCH AIM

Numerous studies have identified physical and functional interactions between the TGFβ signaling pathway and nuclear steroid receptors including VDR (320, 395), AR (338, 396) and GR (322). With respect to ER, it has been suggested that TGFβ, via Smad3, enhances ER transcriptional activity in 293T (human embryonic kidney cells) and MCF-7 (human breast cancer) cells; however others have shown that TGFβ has no effect on ER activity in prostate (DU145) (338), Cos1 (320) and CV-1 (green monkey kidney) (337) cells. Conversely, estrogens have been shown to inhibit TGFβ signaling in 293T and MCF-7 cells (335). The molecular mechanism through which ER modulates TGFβ signaling has been thought to involve direct protein:protein interactions between Smads, including Smad3 (335), and ER; a mechanism similar to that described for the other steroid receptors. As inconsistent data exist in the literature with respect to the potential cross-talk between ER and TGFβ, the goal of this thesis was to characterize the interaction between ER and Smad3 in a mammalian cell environment at the functional and molecular levels.

HYPOTHESIS

The hypothesis of this thesis is that ER modulates the TGFβ signal transduction pathway in human breast tissue through direct protein:protein interactions with Smad3.

OBJECTIVES AND OVERVIEW OF METHODS AND RESULTS

The specific aims were to:

1. Determine if TGFβ, through Smad3, affects ER transcriptional activity.
2. Determine if ER affects Smad3 transcriptional activity.
3. Define the specificity of the interaction between Smad3 and ER, both *in vitro* and *in situ*.
4. Determine the expression of Smad3 as it relates to ER status in human breast tumor tissue.

In order to address the hypothesis, we initially sought to determine if a functional interaction between ER and TGFβ signaling pathways occurs. Specifically, we wanted to determine if Smad3 affects ER transcriptional activity or whether ER affects Smad3 transcriptional activity. Section I of the results addresses this issue. In this section, Cos1 (green monkey kidney; ER-; Smad3-) and MCF-7 (ER+; TGFβ responsive) cells were transiently transfected with various gene expression and reporter plasmids to determine potential functional interactions between TGFβ and ER. Cos1 cells are African green monkey kidney cells that have been transformed with SV40 (Similian virus 40) that promotes continual DNA replication and cell division. These cells grow quite rapidly, are easily transfected with plasmids and, most importantly, have been used in several laboratories to examine the interaction between TGFβ and steroid receptors. These factors make Cos1 cells an ideal cell line to initially address our hypothesis. MCF-7 cells are human breast cancer cells that were derived from a pleural effusion of a patient with metastatic breast cancer. These cells have been used extensively as a cell culture model to study human breast cancer as they are relatively easy to grow and they express a functional ER. The ER reporter plasmids used included ERE-II-luc, consisting of two

repeats of the vitellogenin A_2 ERE cloned upstream of the luciferase expression gene, which represents the classical mechanism of ER action (i.e. ER binds directly to DNA); and the TGFβ_3-luc reporter plasmid, containing an estrogen responsive segment from the TGFβ_3 promoter also cloned upstream to the luciferase expression gene (model of a non-classical mode of ER action). To determine whether ER affects Smad3 transcriptional activity, the TGFβ responsive promoters p3TP-lux and collagen 7(A1) -524/+92 were used. In the majority of the functional studies involving steroid receptors, the p3TP-lux reporter plasmid was used to measure TGFβ transcriptional activity as this reporter is one of the standard plasmids utilized in assessing TGFβ activity. As such, we wanted to use it for this thesis as well. The p3TP-lux reporter plasmid contains three TPA-responsive elements (12-O-tetradecanoylphorbol-13-acetate) of the human collagenase gene that bind Ap-1 transcription factors cloned upstream of a Smad binding element from the plasminogen activator inhibitor 1 gene while the collagen 7(A1) -524/+92 reporter plasmid contains a segment of the human collagen 7(A1) promoter encompassing a Smad binding element.

Having established a functional interaction between ER and TGFβ in section I, we next moved on to determine the molecular mechanism through which ER modulates TGFβ signaling in section II. In the hypothesis, we suggest that ER affects TGFβ signaling by forming direct protein:protein interactions with Smad3. To address this hypothesis, co-immunoprecipitations were performed both *in vitro* and *in situ*. Cos1 cells were transiently transfected with ERα and Smad3 expression vectors in the presence or absence of ligand and the resulting cellular extract was immunoprecipitated with Smad3 or ER specific antibodies. The idea behind these experiments was that Smad3 and ERα

would only co-immunoprecipitate in the presence of estradiol in accordance with the observation that only ligand-bound ER affects Smad3 transcriptional activity. However, the results indicate that Smad3 and ERα do not form direct protein:protein interactions in our model system.

Having disproved the hypothesis that ER modulates TGFβ signaling through direct protein:protein interactions, we sought to explore other possible mechanisms through which activated ER may modulate Smad3 transcriptional activity. These results are also presented in section II. Ap-1 transcription factors, namely c-Jun, have been shown to functionally modulate TGFβ activity through an interaction with Smad3 (397). As it has been suggested that c-Jun also binds to ERα, we hypothesized that, in the presence of estradiol, ER sequesters c-Jun away from TGFβ responsive promoters thereby inhibiting TGFβ transcriptional activity. To address this hypothesis, Cos1 cells were transiently transfected with c-Jun or c-Fos (another Ap-1 transcription factor with which ER does not form direct interactions) expression plasmids with the idea that over-expression of c-Jun, but not c-Fos, would relieve the inhibitory effect of activated ER on TGFβ activity if indeed it was limiting. Having found this to be likely, we further explored the molecular mechanism by which ER, through c-Jun, may affect the transcriptional activity of TGFβ. We examined whether ER and c-Jun interact in the presence of estrogen through co-immunoprecipitations in Cos1 cells transiently transfected with expression vectors and whether ER affects c-Jun binding to the p3TP-lux reporter plasmid via electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP).

In the final section of the results (section III), we have attempted to determine the potential for a functional interaction between ER and Smad3 in human breast cancer. We studied the expression of the relevant factors (i.e. Smad3 and c-Jun) immunohistochemically in human breast tumor tissue microarrays to establish whether these factors could be expressed together in an ER^+ human breast tumor. Having found that Smad3 and c-Jun are co-expressed in ER^+ breast tumors, we next sought to identify an endogenously expressed gene that may be modulated by TGFβ, Ap-1 and ER in a manner similar to that developed in our model system. The reporter plasmids that we utilized in section I of the results were artificially constructed and while their use in determining the presence of a functional interaction between ER and TGFβ signaling plasmids is informative, whether or not this observation occurs on a gene whose protein product is expressed endogenously in human breast cells is unknown. We initially identified two genes whose promoter region contains putative Ap-1 and Smad binding sites and whose expression had been previously shown to be regulated by antiestrogens in a manner similar to that observed in the functional studies described in section I. MCF-7 cells were treated with antiestrogens for various time periods and RT-PCR and/or western blot analysis were performed to determine whether antiestrogen treatment affected the mRNA or protein expression of these two genes.

RELEVANCE TO BREAST CANCER

Tamoxifen has been the mainstay of hormonal therapy in both early and advanced breast cancer patients for approximately 30 years. However, approximately 50% of patients with the disease do not respond to first-line treatment with tamoxifen (31). Furthermore, almost all patients with metastatic disease and approximately 40% of the patients that receive tamoxifen as adjuvant therapy experience tumor relapse and die from their disease. These findings strongly suggest that mechanisms of *de novo* or acquired resistance to tamoxifen occur in breast cancer patients and that this phenomenon might largely affect the efficacy of this treatment. Amongst all the different mechanisms that have been hypothesized to be involved in the resistance of breast cancer cells to hormonal therapy, we were particularly interested in the interaction between ER and growth factors signaling pathways, specifically TGFβ. A more complete understanding of the molecular biology of ER and its interactions with other growth factor stimulatory and/or inhibitory pathways during both breast tumorigenesis and breast cancer progression may aid in the future design of new and/or complementary prevention and treatment strategies.

MATERIALS and METHODS

Materials

Dulbecco's minimal essential medium (DMEM) and phenol red free DMEM (PRF-DMEM) were purchased from GIBCO/BRL (Burlington, Ontario). Penicillin-streptomycin (10,000 units/ml) and L-glutamine (200mM) were purchased from GIBCO/BRL (Burlington, Ontario). Fetal bovine serum (FBS) and activated charcoal were purchased from Sigma Chemical Company (Oakville, Ontario) and dextran T70 from Amersham/Pharmacia (Mississauga, Ontario). Tissue culture flasks and cell culture dishes were from Corning (New York, New York).

The tetracycline-analog doxycycline, 4OH-tamoxifen and 17β-estradiol were purchased from Sigma Chemical Company (Oakville, Ontario). Isoton™ was from Beckman Coulter Incorporated (Mississauga, Ontario). Hygromycin B was obtained from Clontech (Palo Alto, California) and G418 was from Invitrogen Canada (Burlington, Ontario). LY117018 was a gift from Lilly Laboratories (Indianapolis, Indiana) and ICI 182,780 was a gift from Dr. A.E. Wakeling (Zeneca Pharmaceuticals, Macclesfield, England). Effectene transfection reagent was purchased from Qiagen (Mississauga, Ontario). γ-[P^{32}]-ATP and [S^{35}]-methionine were from Perkin Elmer (Boston, Massachusetts). Dithiobis[succinimidylpropionate] (DSP) was from Pierce (Rockford, IL).

The ERα (NCL-ER-6F11, mouse monoclonal)(recognizes full-length ERα) and c-Jun (NCL-c-Jun, mouse monoclonal) antibodies were purchased from NovoCastra Laboratories (Newcastle, United Kingdom). Anti-Xpress (R910-25, mouse monoclonal) and anti-His G (R940-25, mouse monoclonal) antibodies were from Invitrogen

(Burlington, Ontario). Rabbit polyclonal antibodies to Smad3 were from Upstate Biotechnology (06-920) (Lake Placid, New York) and Zymed Laboratories (51-1500) (San Francisco, California). A tubulin antibody (Clone DM1A) (MS-581-P0, mouse monoclonal), used as an internal protein loading control, was from NeoMarkers Inc. (Fremont, California) and an anti-β-galactosidase antibody (Z378A, mouse monoclonal) was from Promega (Madison, Wisconsin). The insulin receptor (insulin R-β, C-19) (sc-711, rabbit polyclonal), ERα (HC-20) (sc-543, rabbit polyclonal)(recognizes C-terminal region of ERα), c-Jun (N) (sc-45, rabbit polyclonal)(recognizes N-terminal region of c-Jun) and phosphorylated c-Jun antibodies (KM-1) (sc-822, mouse monoclonal), which recognizes c-Jun phosphorylation at serine 63, was from Santa Cruz Biotechnology (Santa Cruz, California). AIB1 (611104, mouse monoclonal) was from BD Transduction Laboratories (Oakville, Ontario). Horseradish peroxidase conjugated goat anti-mouse and goat anti-rabbit were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pennsylvania) and Bio-rad (Hercules, California), respectively. Supersignal West Dura western blotting kits were purchased from Pierce (Rockford, Illinois). Prestained molecular weight protein markers were from Bio-rad (Hercules, California). Complete® Protease Inhibitor mixture tablets were from Roche (Laval, Quebec).

CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), ONPG (o-nitrophenyl β-D-galacto-pyranoside), Tween 20 (polyoxyethylene-sorbitan monolaurate), bovine serum albumin (BSA), PIPES (1,4-piperazinediethanesulfonic acid) and Triton X-100 (octyl phenoxy polyehtoxyethanol) were all from Sigma Chemical Company (Oakville, Ontario). I-block was from Tropix (Foster City, California). Nitrocellulose (NitroPlus, 0.45 mircon) paper was purchased from Micron Separations Incorporated (Westborough,

Massachusetts). Protein G sepharose beads were from Amersham International (Baie d'Urfe, Quebec). EDTA (disodium ehtylenediamine tetraacetate disodium salt) was from Fisher Scientific Company (Nepean, Ontario). Rabbit reticulolysate *in vitro* transcription/translation kits (TnT), firefly luciferase reagent and Dual Luciferase® Reporter Assay System were from Promega (Madison, Wisconsin). Micro BCA protein assay kits were from Pierce (Rockford, Illinois), Bradford protein assay reagent was from Bio-rad (Hercules, California) and TRIzol reagent was from GIBCO/BRL (Burlington, ON).

Taq DNA polymerase, M-MLV reverse transcriptase, RNA inhibitor (RNAsin) and random hexamers were from Invitrogen (Burlington, Ontario). dATP, dTTP, dGTP and dCTP were from Promega (Madison, Wisconsin). Custom primers were ordered from Invitrogen (Burlington, Ontario).

ERE-II-Luc containing two vitellogenin A2 (-333/-288) EREs upstream of a luciferase reporter was a gift from Dr. P. Webb (University of California, San Francisco, California) and the TGFβ_3-luc reporter, containing nucleotides -2306/-1848 of the human TGFβ_3 promoter, was a gift from Dr. C. Labrie (Laval University, Quebec). The pCH110 and pcDNA3-β-gal (constitutive β-galactosidase expression vectors) were from Amersham/Pharmacia (Mississauga, Ontario). The renilla luciferase (pRL-tk-luc) internal control plasmid for dual luciferase assays was from Promega (Madison, Wisconsin). pGL3-Col7(A1)-524/+92 and pGL3-Col7(A1)-454/+92 luciferase reporter plasmids were gifts from Dr. A. Mauviel (Institut de Recherche sur la Peau, Hopital Saint-Louis, Paris, France). p3TP-lux reporter was a gift from Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, New York). pCMV5B-Flag-

Smad3 expression plasmid was from Dr. L. Attisano (University of Toronto, Toronto, Ontario). The pcDNA3.1-Flag-Smad3 expression plasmid was constructed by subcloning the KpnI/XbaI Smad3 fragment from pCMV5B-Flag-Smad3 into a KpnI/XbaI digested pcDNA3.1 (Invitrogen). pcDNA4-His/Xpress $ER\beta_1$, $ER\beta_2$, and $ER\beta_5$ expression plasmids were constructed by Dr. B. Peng by directly cloning an ERβ PCR product directly into pcDNA4-His/Xpress TOPO (Invitrogen) (139). pcDNA3.1-ERα (constructed by Mr. Helmut Dotzlaw) and pcDNA4-His/Xpress-ERα (constructed by Dr. Baocheng Peng) were constructed by cloning a full length ERα PCR product into the EcoRI site of pcDNA3.1 (Invitrogen) or pcDNA4-His/Xpress (Invitrogen), respectively. Plasmids encoding the Ap-1 transcription factors c-Jun (pRSV-cJun) and c-Fos (pRSV-cFos) were from Dr. Michael Karin (University of California, San Diego, CA). SRC-1 (pCR3.1-hSRC-1a) and AIB1 (pCMX-F Rac3) expression plasmids were a gift from Dr. Tom Spencer (Baylor College, Houston, Texas) and Dr. Don Chen (University of Massachusetts, Massachusetts), respectively. All constructs were confirmed by sequencing.

Ap-1 oligonucleotide was produced by University of Calgary (Calgary, Alberta). Bio-spin 6 columns were from Bio-rad (Hercules, California) and poly dI/dC was from Amersham International (Baie d'Urfe, Quebec). T4 polynucleotide kinase was from Roche (Laval, Quebec). IGEPAL (octylphenoxy polyethoxyethanol) was from Sigma Chemical Company (Oakville, Ontario).

Methods

i. *Cell Culture*

All cells were routinely grown in 75cm^2 flasks in a humidified incubator at 37°C containing a 5% CO_2 atmosphere. Cos1 cells were obtained from the ATCC (Manassas, VA) (African green monkey kidney cells) and routinely cultured in PRF-DMEM containing 10% v/v twice charcoal dextran stripped FBS (CS), 0.3% w/v glucose, 2mM L-glutamine and 100 units/ml penicillin-streptomycin (10% CS). Cells were passaged at 80-90% confluency using Earle's EDTA solution (5.3mM KCl, 117mM NaCl, 26mM $NaHCO_3$, 1mM NaH_2PO_4, 5.6mM D-glucose and 1mM EDTA). T5 cells, previously called T47D5, were originally thought to be a T47D subline, however, DNA restriction fragment length polymorphism analysis showed that they were a MCF-7 subline (398). MCF-7 (originally from Dr. William McGuire's laboratory in San Antonio, Texas) and T5 cells were cultured in DMEM containing 10nM estradiol (E_2), 5% v/v FBS, 0.3% w/v glucose, 2mM L-glutamine and 100 units/ml (U/ml) penicillin-streptomycin (5% CM). MCF-7 Clone 11 (399) and MCF-7 Clone 47 cells (400) which over-express ERα or ERβ in the presence of doxycycline, were routinely cultured in 5% CM plus 10nM E_2, 500ug/ml G418 and 400ug/ml hygromycin. Cells were passaged at 70-80% confluency using Earle's EDTA solution.

ii. *Transient Transfections*

a) Cos1 Cells

Cos1 cells growing in 150cm^2 flasks were seeded in either 100mm dishes at 1x10^6 cells/dish or in 6 well culture plates at 5x10^5 cells/well in 5% CS the day prior to transfection. The following day, medium was changed to fresh 5% CS containing the

drug of appropriate concentration or vehicle (ethanol) alone. Cells were transiently transfected using the Effectene Transfection Reagent (Qiagen) according to manufacturer's instructions. For each transfection, the DNA/enhancer ratio was kept at 1/8 while the DNA/effectene ratio was kept at 1/15. Forty-eight h post-transfection, media was aspirated off and cells harvested. For reporter gene assays, transfection efficiency was determined by co-transfection of either pCH110 (β-galactosidase expression vector) for β-galactosidase activity (401) or by co-transfection with pRL-tk-luc for renilla luciferase activity. Samples to be analyzed by western blotting were harvested in 100ul of 95°C Joel lysis buffer (0.12M Tris-Cl, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.2M DTT) per well of a 6 well plate, collected in a 1.5ml centrifuge tube, vigorously shaken at 95°C for 20 min and stored at -20°C (402).

b) MCF-7 Cells

Human MCF-7 breast cancer cells were routinely grown in $75cm^2$ flasks in 5% CM + 10nM E_2. To obtain estrogen depleted cells, media was changed to PRF-DMEM supplemented with 5% CS (as phenol red may act as a weak estrogen in tissue culture(403)) once cells obtained 30% confluency. Media was replenished every 2 days and cells were grown for 5 days. Cells were seeded at $5x10^5$ cells/100mm dish one day prior to transfection. Cells were transiently transfected using the calcium phosphate precipitation method (404). Cells were transfected with 3-5ug reporter plasmid, 2-8ug pCMV5B-Flag-Smad3, 1ug pRL-tk-luc and 5ug pcDNA3.1-ERα, unless otherwise stated. Empty pcDNA3.1 was used to ensure equal molar concentrations of plasmid between samples. Each sample was brought up in 1ml BES-buffered saline (25mM BES, pH 6.96, 140mM NaCl, 0.75mM Na_2HPO4) containing 125mM $CaCl_2$, mixed for 10 sec

and incubated at room temperature for 15 min. Fresh PRF-DMEM supplemented with 5% CS was added. The transfection mixture was added drop-wise to plates while being gently swirled and incubated at 37°C and 5% CO_2 overnight. Media was removed and cells were subjected to a 3 min glycerol shock with a 20% v/v glycerol/5% CS solution. Plates were washed 3 times with sterile Isoton™ (Beckman) and new 5% CS media containing vehicle, 10nM E_2, 100nM 4-hydroxytamoxifen (4OH-Tam) or 500nM ICI 182,780 was added, unless otherwise stated. Forty-eight h later, media was removed and cells were washed with 5ml Isoton™ (Beckman). Samples were harvested in 1ml of 1x Passive Lysis Buffer (Promega), as per manufacturer's instructions, collected in 1.5ml tubes and frozen at –80°C until assayed.

Transfection of ERα and ERβ$_1$ over-expressing cells was performed as described above with minor modifications. MCF-7 Clone 11 (ERα inducible clone) and MCF-7 Clone 47 (ERβ inducible clone) cells were routinely cultured in 5% CM plus 10nM E_2, 500ug/ml G418 and 400ug/ml hygromycin B. Five days prior to use, media was changed to 10% CS to obtain estrogen depleted conditions. Cells were set-up at $5x10^5$ cells/100mm dish one day prior to transfection in 5% CS containing 2ug/ml doxycycline. The following day, media was changed (5% CS containing 2ug/ml doxycyline) and cells were transiently transfected using the calcium phosphate precipitation method (404). Twenty-four h post-transfection, cells were subjected to a 5 min glycerol shock (20% glycerol v/v in 5% CS) and new media containing vehicle, 10nM E_2, 100nM 4OH-Tam or 500nM ICI 182,780 was added along with 2ug/ml doxycycline. For dual luciferase assays, samples were harvested in 1ml of 1x Passive Lysis Buffer (Promega). Samples to be used for western blotting were harvested in 300ul of Joel lysis buffer, collected in a

1.5ml centrifuge tube, vigorously shaken at 95°C for 20 min and stored at -20°C until analyzed by SDS-PAGE (402).

iii. *Luciferase and β-Galactosidase Activity*

Cos1 cells transiently transfected with firefly luciferase and β-galacosidase reporter plasmids were scraped into 1ml 100mM K_2HPO_4 pH 7.8, centrifuged briefly at 10,000 x g and supernatant removed. Cell pellets were resuspended in 100ul of 100mM K_2HPO_4, pH 7.8 containing 1mM DTT and cell lysates obtained by 3 cycles of freezing in a dry ice/ethanol bath and thawing at 37°C. Samples were centrifuged at 12,000 x g for 5 min at 4°C and the supernatant was placed in a fresh tube. A 10ul aliquot was used to measure β-galactosidase activity using a standard protocol (401) and a 20ul aliquot was used to determine luciferase activity. Firefly luciferase activity was determined as per manufacturers instructions (Promega) using a plate reading luminometer (Molecular Devices, Sunnyvale, California). To each sample, 100ul of Luciferase Assay Reagent (Promega) was injected into each well and readings were taken every second for 10 sec.

MCF-7 cells transiently transfected with both renilla and firefly reporter plasmids were harvested in 1ml 1x Passive Lysis Buffer (Promega). Samples were placed in 1.5ml tubes and frozen at –80°C until assayed. Samples were thawed at room temperature, mixed briefly and centrifuged at 10,000 x g for 5min. A 20ul aliquot of the supernatant was placed in a well of a 96 well assay plate. Renilla and firefly (dual) luciferase activities were determined as per manufacturers instructions (Dual Luciferase® Reporter Assay System, Promega) using a plate reading luminometer (Molecular Devices, Sunnyvale, California). Dual reporter assays were performed as follows: i) 100ul of Luciferase Assay Reagent II was injected into well, ii) readings for firefly luciferase

activity were taken every second for 10 sec., iii) 100ul of Stop & Glo® Reagent was injected into well and iv) readings for renilla luciferase activity was taken every second for 10 sec.

iv. *In vitro Transcription and Translation*

In vitro transcription/translation (TnT) reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System, Promega). Reactions were performed according to the manufacturer's instructions.

v. *Immunoprecipitation*

Cos1 transiently transfected cells were scrapped into either: i) 1ml ice-cold Triton X-100 immunoprecipitation (IP) buffer containing 1% v/v Triton X-100, 50mM Tris-HCl pH 7.4, 300mM NaCl, 5mM EDTA and Complete Protease Inhibitor (Roche) tablets or ii) 1ml ice-cold NP-40 IP buffer containing 0.5% v/v NP-40, 50mM Tris-HCl pH 8.0, 150mM NaCl, 1mM EDTA and Complete Protease Inhibitor (Roche) tablets. Samples were incubated on ice for 20 min. Cells suspended in NP-40 IP buffer were sonicated for 5 sets of 10 sec each with cooling on ice between sonications. Samples were centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was transferred to a fresh tube containing 50ul of a 1/1 slurry of protein G sepharose beads (Amersham) in Triton X-100 or NP-40 IP buffer and mixed for 1 h at 4°C to pre-clear the supernatant of any non-specific binding proteins. Beads were pelleted by centrifugation at 12,000 x g and 3-5ug of antibody was added to the supernatant. Tubes were incubated overnight at 4°C with rocking. To each sample, 50ul of a 1/1 slurry of protein G sepharose beads (Amersham) in Triton X-100 or NP-40 IP buffer was added and incubated at 4°C for 3 h. Samples

were washed 3 times with 1ml Triton X-100 or NP-40 IP buffer. Final pellets containing beads conjugated to antibody were resuspended in 50ul 5x sample buffer (5% v/v glycerol, 20% v/v 2-β-mercaptoethanol, 0.004% w/v bromophenol blue, 0.8% w/v SDS, 0.125M Tris-HCl pH 6.8). Samples were boiled for 4 min to dissociate antibody from protein G sepharose (Amersham) beads, briefly centrifuged and analyzed on a 10% SDS-PAGE gel (see immunodetection by western blotting).

vi. *DSP Cross-Linking and Immunoprecipitation*

Cos1 cells were seeded at $5x10^5$ cells/100mm dish and transiently transfected as described previously in 5% CS. Forty-eight h post-transfection, proteins were cross-linked for 2 h at 4°C with 1.5mM DSP dissolved in DMSO. To stop the cross-linking reaction, 20mM Tris-HCl was added and incubated for 15 min. Cells were washed twice with ice cold phosphate buffered saline (PBS; 0.1M phosphate, 0.15M NaCl, pH 7.2) and harvested in 0.5 ml ice cold IP buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.5% IGEPAL, 1mM EDTA, 1mM PMSF, 10mM NaF, 1mM Na_3VO_4, 25mM β-glycerophosphate and Complete Protease Inhibitor cocktail), pooling cells from 2 dishes of each treatment. Samples were incubated on ice for 20 min, mixed with a 21 gauge needle, sonicated 8 times at 10 sec each and centrifuged at 10,000 x g for 10 min at 4°C. To preclear the lysate, 50ul of a 1/1 protein G sepharose bead slurry in IP buffer was added and incubated at 4°C for 30 min with agitation. Beads were pelleted by a brief centrifugation at 10,000 x g and the resulting supernatant was placed into a new tube. The supernatant was divided into 2 equal aliquots, one of which was incubated with 3ug of an ERα specific antibody (rabbit polyclonal; Santa Cruz Biotechnology) and one which was incubated with 3ug of the insulin receptor antibody (rabbit polyclonal; Santa

Cruz Biotechnogology). Upon addition of antibody, samples were left overnight at 4°C with agitation. Protein G sepharose beads (50ul of a 1/1 slurry in IP buffer) were added and samples incubated at 4°C for 3 h. Beads were spun down briefly at 10,000 x g and washed 3 times with 1 ml IP buffer. To each sample, 50ul of 5x sample buffer (5% v/v glycerol, 20% v/v 2-β-mercaptoethanol, 0.004% w/v bromophenol blue, 0.8% w/v SDS, 0.125M Tris-HCl pH 6.8) was added and samples were boiled for 5 min, briefly centrifuged and analyzed on a 10% SDS-PAGE gel (see immunodetection by western blotting described below).

vii. *Immunodetection by Western Blotting*

Samples to be used for western blotting were thawed at 37°C and centrifuged briefly at 10,000 x g. Aliquots of the extracts were analyzed using a 10% SDS-PAGE with a 4% stacking gel at 200V for 45 min at room temperature according to the Laemmli method (405). Gels were equilibrated for 5 min at room temperature in ice cold CAPS transfer buffer (10mM CAPS pH 11, 20% v/v methanol) and transferred to nitrocellulose for 1.5 h at 120V and 4°C. Membranes were baked at 65°C for 20 min and blocked for 6 h at room temperature with 0.2% w/v I-block/TBS. Membranes were incubated with: ERα specific antibody (mouse monoclonal) (NovoCastra), Smad3 antibody (rabbit polyclonal) (Upstate Biotechnology), anti-xpress (mouse monoclonal) (Invitrogen) or anti-HisG (mouse monoclonal) (Invitrogen) antibodies at a dilution of 1/1000 in 0.2% w/v I-block/TBS containing 0.5% v/v Tween 20 (TBS-T) overnight at 4°C and subsequently washed 6 times for 5 min each in TBS-T. For AIB-1 immunodetection, samples were run on a 7.5% SDS-PAGE with a 4% stacking gel and membranes were incubated with a 1/250 dilution of mouse anti-AIB-1 (BD Biosciences) overnight at 4°C.

Membranes were incubated with the appropriate peroxidase-conjugated secondary antibody (see Materials) for 1 h at room temperature at a dilution of 1/5000 in 0.2% w/v I-block/TBS-T and washed 6 times for 5 min each in TBS-T. Immunodetection was carried out using the Supersignal West Dura blotting kit (Pierce) according to the manufacturer's instructions and visualized with the Chemidoc System (Bio-rad). The membranes were stripped (0.2M glycine, 0.1% w/v SDS, 1% v/v Tween-20 pH 2.2) for 1 h at room temperature and blocked for 1 h at room temperature with 0.2% w/v I-block/TBS-T. The membranes were probed with a 1/5000 dilution of mouse anti-tubulin (Neomarkers) in 0.2% w/v I-block/TBS-T overnight at 4°C and washed 5 times for 5 min each in TBS-T. Membranes were incubated for 1 h at room temperature with a 1/10000 dilution of a goat anti-mouse horseradish-peroxidase conjugated secondary antibody (Jackson Laboratories) and washed 5 times for 5 min each. Immunodetection was carried out using the Supersignal West Dura blotting kit (Pierce) and visualized with the Chemi-doc system (Bio-rad).

viii. ***Protein Assay***

Protein assays were performed using either the BCA (Micro BCA Protein Assay Reagent kit, Pierce) or Bradford method (Bradford protein assay reagent, Bio-rad) as per manufacturer's instructions.

ix. ***RT-PCR Reactions***

Total RNA was extracted from cells using TRIzol reagent (GIBCO/BRL) as per manufacturer's instructions. Reverse transcription was performed using 1ug of RNA in a final volume of 30ul. RNA was heated to 65°C for 5 min to denature secondary structure

and 1ug of RNA was reverse transcribed in a final volume of 30ul. Each reaction contained 50mM Tris-HCl (pH 8.3), 40mM KCl, 6mM $MgCl_2$, 0.3U/ul RNAsin, 10mM DTT, 5ng/ul random hexamers, 10U/ul M-MLV and 2mM each of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphoshate (dCTP). Samples were left at room temperature for 5 min to allow primers to anneal and incubated at 37°C for 1 h. The reaction was stopped by boiling for 3 min and the resulting cDNA was stored at -20°C until further use.

The primers used for PCR consisted of $TGF\beta_2$-U (5'-CGG AGG TGA TTT CCA TCT ACA ACA G-3'; sense, located in $TGF\beta_2$ 661-685) and $TGF\beta_2$-L (5'-AGG CAC TCT GGC TTT TGG GTT C-3'; antisense, located in $TGF\beta_2$ 1016-1037). Nucleotide positions given correspond to sequences of the human $TGF\beta_2$ mRNA (accession number M19154). Each PCR reaction contained 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.5mM $MgCl_2$, 0.2mM dATP, 0.2mM dTTP, 0.2mM dGTP, 0.2mM dCTP, 4ng/ul of each primer and 0.02U/ul Taq DNA polymerase (Invitrogen). PCR amplifications consisted of 35 cycles with each cycle consisting of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. Primers used for AIB1 PCR consisted of AIB1-U (5'-ATA CTT GCT GGA TGG TGG ACT-3'; sense, located in AIB1 110-130) and AIB1-L (5'-TCC TTG CTC TTT TAT TTG ACG-3'; antisense, located in AIB1 438-458) with nucleotide positions corresponding to the human AIB1 gene (accession number AF012108). Each PCR reaction contained 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.5mM $MgCl_2$, 0.2mM dATP, 0.2mM dTTP, 0.2mM dGTP, 0.2mM dCTP, 4ng/ul of each primer and 0.02U/ul Taq DNA polymerase (Invitrogen) and PCR amplifications consisted of 30 cycles of 30 sec at

95°C, 30 sec at 55°C and 30 sec at 72°C. Amplification of the ubiquitously expressed glyceraldehyde-3-phoshate dehydrogenase (GAPDH) was performed in parallel. The primers used for PCR consisted of GAP-U (5'-ACC CAC TCC TCC ACC TTT G-3'; sense, located in GAPDH 971-989) and GAP-L (5'- CTC TTG TGC TCT TGC TGG G-3'; antisense, located in GAPDH 1148-1130) with nucleotide positions corresponding to the human GAPDH gene (accession number AF261085). Each PCR reaction contained 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.5mM $MgCl_2$, 0.2mM dATP, 0.2mM dTTP, 0.2mM dGTP, 0.2mM dCTP, 4ng/ul of each primer and 0.02U/ul Taq DNA polymerase (Invitrogen). PCR amplifications consisted of 30 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C followed by 7 min at 72°C. PCR products were resolved by electrophoresis on a 1.2% agarose gel containing 5ug ethidium bromide. Gels were visualized with the Geldoc 2000/Chemi-Doc system (Bio-rad). Quantitative densitometry was performed using the Quantity One Software (version 4.2, Bio-rad). Three independent PCR reactions for TGFβ_2, AIB1 and GAPDH were performed for each experiment.

x. ***Electrophoretic Mobility Shift Assay***

Nuclear extracts of Cos1 cells were prepared as described previously by de Moissac et al. (406) with modifications. Cos1 cells were transiently transfected in 100mm dishes with 1ug pCMV5B-Flag-Smad3 and 1ug pcDNA3.1-ERα in the presence or absence of 10nM E_2 in CS5% as described above. Forty-eight h post-transfection, media was removed and cells were gently washed with 2 ml Isoton™ (Beckman). Cells were scrapped with a rubber spatula into 5 ml Isoton™ (Beckman), pooling samples from 3 dishes together. Samples were centrifuged briefly at 3,000 x g and the resulting cell

pellet was resuspended in 200ul buffer A (10mM Hepes, pH 7.9, 60mM KCl, 1.0mM EDTA, 1.0mM dithiotreitol, 0.3% v/v IGEPAL) containing 1 tablet/10ml Complete Protease Inhibitor (Roche). Cells were allowed to swell on ice for 15 min and centrifuged at 1,000 x g for 5 min. Supernatant was removed and stored as the cytosolic fraction at -80°C. The remaining pellet was resuspended in 50ul buffer C (200mM Hepes, pH 7.9, 0.4M NaCl, 1.0mM EDTA, 1.0mM EGTA, 1mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride) and shaken vigorously at 4°C for 15 min. Nuclear extract was the supernatant fraction obtained following centrifugation for 5 min at 10,000 x g. Aliquots of the extract were stored at -80°C. Protein concentration was determined using the Bradford method (Bio-rad) as described above.

DNA binding reactions were performed using the Ap1 site from the TGFβ responsive reporter, p3TP-lux, and consisted of 5'- ATG AGT CAG ACA CCT CTG GCT GTC TGG AAG GG -3' sense and 5'-CCC TTC CAG ACA GCC AGA GGT GTC TGA CTC AT -3' antisense. Oligonucleotides were resuspended in annealing buffer (10mM Tris-HCl pH 7.5, 50mM NaCl and 1mM EDTA) to a concentration of 25pmoles/ul. Equal volumes of sense and antisense oligonucleotides were mixed in a 1.5ml tube and placed in a 95°C heat block for 4 min. Samples were slowly cooled to room temperature yielding a double stranded (ds) oligonucleotide. Oligonucleotides were P^{32} end-radiolabelled with γ-ATP^{32} in a final volume of 50ul. The reaction consisted of 20ng ds-oligonucleotide, 1U/ul T4 polynucleotide kinase, 50mM Tris-HCl, 10mM $MgCl_2$, 100uM EDTA, 5mM DTT, 100uM spermidine pH 8.2 and 2.694MBq γ-ATP^{32}. Samples were incubated at 37°C for 1 h and the reaction was stopped by heating at 65°C for 15 min. Radiolabelled oligonucleotides were purified from the reaction

mixture by centrifuging samples through a Bio-spin 6 column (Bio-rad) at 2250 x g for 5 min.

A gel shift mix consisting of 10ug bovine serum albumin, 2.4ug poly dI/dC, 20mM HEPES, pH 7.9, 1% v/v IGEPAL, 5% v/v glycerol, 1mM EDTA and 5mM dithiothreitol was added to 5 - 20ug Cos1 nuclear extract. Samples were incubated on ice for 15 min and 0.8ng of a P^{32}-radiolabelled duplex oligonucleotide probe was added. This order of probe to sample has previously been shown to reduce non-specific binding between sample and probe (407). To test for the specificity of the protein-DNA complexes formed, competition studies with 25 – 100 times molar excess of unlabelled oligonucleotide were performed. Unlabelled ds-oligonucleotide was added to the P^{32}-radiolabelled duplex oligonucleotide such that the oligonucleotides were added to the samples simultaneously. Reactions were performed on ice for 30 min. To determine which proteins formed complexes with the ds-oligonucleotide probe, antibodies for c-Jun or ERα were added to the gel shift mix prior to being added to the samples. A 5% polyacrylamide gel in 1 x Tris-buffered EDTA (TBE), pH 8.0 was prepared and pre-run for 1 h at 180 V using 1x TBE as running buffer. Nuclear-protein complexes were added and the gel was run for 4 h at 180 V. Gels were dried for 2 h at 80°C on a gel dryer (Bio-rad) and visualized by autoradiography. In addition, gels were exposed to a Phosphorimager screen (Bio-rad) for 24 h, visualized using Storm (Amersham/Pharmacia) and densitometry on specific bands was performed using Quantity One (Bio-rad).

xi. *Chromatin Immunoprecipitation*

Cos1 cells were transiently transfected in 100mm dishes as described previously in this thesis. Thirty-six h post-transfection, media was removed and 8 ml of cold 1% formaldehyde in PBS was added (to cross-link protein and DNA molecules together) and incubated for 10 min at 4°C. To stop further cross-linking, 8 ml of 0.125M glycine solution in PBS was added for 10 min at 4°C. Formaldehyde and glycine were gently removed with suction and cells were harvested into 0.5 ml ice cold PBS, pooling 2 dishes for each treatment. Cells were pelleted by brief centrifugation at 10,000 x g and washed twice with 1ml cold PBS. PBS was gently removed from the tubes by suction and samples were stored at -80°C until further use.

To each sample, 500ul of lysis buffer (5mM 1,4-piperazinediethanesulfonic acid, pH 8.0, 85mM KCl, 0.5% NP-40) containing protease and phosphatase inhibitors (1ug/ml leupeptin, 1ug/ml aprotenin, 1mM PMSF, 10mM NaF) was added. Samples were incubated for 10 min on ice and centrifuged at 13,000 x g for 2 min to pellet nuclei. Nuclear extracts were resuspended in 500ul nuclear lysis buffer (50mM Tris-HCl pH 8.1, 10mM EDTA, 1% SDS) containing protease and phosphate inhibitors (1ug/ml leupeptin, 1ug/ml aprotenin, 1mM PMSF, 10mM NaF) and replicate samples pooled. Extracts were incubated on ice for 10 min, sonicated 3 times for 15 sec each and centrifuged at 13,000 x g for 5 min. Supernatant was transferred to a fresh tube and was diluted with 9 ml ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris, pH 8.1, 167mM NaCl). A 50ul aliquot was taken representing the total amount of DNA sequences available for PCR amplification prior to ChIP (Input). Samples were divided into 2-3 equal volumes (3 - 4.5ml) and 3ug of either an ERα (HC-20) (sc-543; Santa Cruz

Biotechnology), c-Jun (N) (sc-45; Santa Cruz Biotechnology) or insulin receptor (c-19) (sc-711; Santa Cruz Biotechnology) antibody was added. Samples were incubated overnight at 4°C with rotation after which 50ul of a 1/1 slurry of protein G sepharose beads in ChIP dilution buffer were added for 3 h at 4°C. Beads were pelleted by brief centrifugation at 13,000 x g and washed 3 times with 1ml ChIP dilution buffer. To degrade any RNA present in the samples, 100ul of a DNAse free RNAse A solution (100ug/ml) was added and incubated for 30 min at 37°C. Proteins were digested by addition of 100ul proteinase K solution (500ug/ml proteinase K, 0.5% SDS, 10mM Tris, 1mM EDTA, pH 8.0) for 60 min at 55°C. Samples were incubated overnight at 65°C to reverse the formaldehyde cross-links.

DNA was extracted from samples by phenol/chloroform precipitation. Briefly, 3 ml of phenol/chloroform/isoamyl alcohol (25/24/1) (v/v) was added. Samples were mixed vigorously for 10 sec and centrifuged at 13,000 x g for 5 min to enact phase separation. The upper, aqueous layer was carefully removed and placed in a new tube, avoiding proteins contained within the phenol/chloroform interface. To the aqueous phase, 600ul of 3M sodium acetate was added, mixed 10 sec and 12 ml of ice cold 100% ethanol was added and mixed. Samples were incubated on ice for 5 min, centrifuged at 13,000 x g for 5 min and the resulting DNA pellets were washed twice with 1ml 70% (v/v) ethanol to remove any residual salts. Supernatant was discarded and the pellet was allowed to air dry for 15 min. DNA pellets were dissolved in 30ul TE buffer prior to PCR reaction.

Specific DNA fragments isolated through ChIP were analyzed by PCR. For input DNA, 1ul of DNA was amplified while 5ul were amplified from ChIP extracts. Primers

for the PCR reactions were: p3TP-lux-U 5'-TGG AAG GGA TGA GTC AGA CAC C-3' and p3TP-lux-L 5'-CCA GGA ACC AGG GCG TAT CTC-3'. Each PCR reaction contained 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.5mM $MgCl_2$, 0.2mM dATP, 0.2mM dTTP, 0.2mM dGTP, 0.2mM dCTP, 4ng/ul of each primer and 0.02U/ul Taq DNA polymerase (Invitrogen). Samples were incubated at 94°C for 2 min and amplified. PCR amplifications consisted of 35 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. PCR products were resolved by gel electrophoresis on a 1% (w/v) agarose gel containing 5ug ethidium bromide. Gels were visualized with the Geldoc 2000/Chemi-Doc system (Bio-rad). A minimum of three independent PCR reactions were performed for each ChIP.

xii. *Statistical Analysis*

Statistical analysis was performed with Prism Graphpad software (Graphpad Prism v4.00, Graphpad software, San Diego, California). Two-way analysis of variance and Student's t-test were used to determine significant differences. $p<0.05$ was considered significant. Complex analysis of variance was performed by a qualified statistician.

xiii. *Human Breast Tumors*

One-hundred and nine invasive breast carcinomas consisting of 94 ER^+ (defined as >3 fmol/mg of protein) and 80 PR^+ (defined as >10fmol/mg of protein) tumors were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada) (Website: http://www.umanitoba.ca/institutes/manitoba_institute_cell_biology/MBTB/MBTB_Homepage.htm) with the expertise of Dr. Peter Watson, Department of Pathology, University

of Manitoba and tumor tissue microarrays (TMAs) were prepared. Briefly, areas of pathological interest were identified by Dr. Watson and cores typically 3mm long and 0.6mm in diameter were obtained from this region of interest from a paraffin embedded tissue block. Cores were transferred into a recipient standard paraffin block. As a result, the 109 tissue cores all were contained in one paraffin block and referred to as a tissue microarray. Sections are cut from the array block 4-5 μ thick that are used primarily in immunohistochemical analysis (see below).

xiv. *Immunohistochemistry*

Five micron sections were cut from the tissue microarrays and stained using the Discovery XT (Ventana Medical Systems) automated slide staining instrument as per manufacturers' recommendations. Slides were stained for phosphorylated c-Jun (Santa Cruz) and Smad3 (Zymed Laboratories) at a final dilution of 1/300 and 1/30, respectively. Antibodies were detected using the iView DAB Detection Kit (Ventana Medical Systems), with biotinylated immunoglobulin secondary antibodies at concentrations less than 120 ug/ml (mouse) and 180 ug/ml (rabbit) in a phosphate buffered saline containing 0.5% Proclin 300. Slides were counter-stained with Hematoxylin and Bluing Reagent (Ventana Medical Systems) to visualize cellular structures. Levels of expression of phosphorylated c-Jun and nuclear Smad3 were determined by scoring the intensity (0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = intense staining) and the percentage of epithelial tumor cells exhibiting staining within the tissue section. The product of the intensity and the percentage was determined to provide a final semi-quantitative immunostaining score (H score, ranging from 0 to 300). Scoring of TMA immunostaining was performed independently by Tracy

Cherlet and Dr. George Skliris, who has had extensive experience and training in immunohistochemical scoring.

xv. ***Statistical Analysis of Immunohistochemistry***

Statisistical analysis was performed with Prism Graphpad software (Graphpad Prism v4.00, Graphpad software, San Diego, California). Spearman correlations were used to test correlations between c-Jun, Smad3, ER, PR and tumor characteristics. Differences between tumor subgroups were tested using the Mann-Whitney rank-sum test, two-sided. $p<0.05$ was considered statistically significant.

RESULTS

SECTION I. FUNCTIONAL INTERACTION BETWEEN ER AND TGFβ

A. Modulation of ER Activity by Smad3

Rationale

Cross-talk between the TGFβ and ER signaling pathways has been suggested to occur not only by direct autocrine or paracrine effects but also by ER and TGFβ directly modulating one another's transcriptional activity. Matsuda et al. (335) have demonstrated that Smad3 inhibits ER transcriptional activity whereas ER transcriptional activity is enhanced by TGFβ signaling. However, others have shown that TGFβ does not affect ER activity (320). Due to such inconsistencies in the literature, we initially sought to characterize the functional interaction between TGFβ and ER signaling pathways in Cos1 (green monkey kidney) and MCF-7 (human breast cancer) cells.

Results

To test whether Smad3 affects ERα or ERβ transcriptional activity, we utilized an estrogen responsive luciferase reporter plasmid which contains two EREs from the promoter region of the *Xenopus* vitellogenin gene. This reporter plasmid has been previously used to examine ER transcriptional activity. To ensure that the plasmid was ER responsive, we transiently transfected Cos1 cells with ERE, ERα and a β-galactosidase reference gene (pCH110) in the presence or absence of 10nM E_2 and looked for an increase in ERE activity upon addition of ligand. As expected, upon E_2 stimulation there was a 7-fold ($p<0.01$) increase in luciferase activity in the presence of ERα as compared to vehicle-treated cells (Figure 11). When increasing amounts of

Smad3 expression plasmid was co-transfected into Cos1 cells with the ERE, ERα and pCH110 in the presence or absence of 10nM E_2, no significant difference in E_2 induced ERα luciferase activity between samples over-expressing or not over-expressing Smad3 was observed (Figure 11).

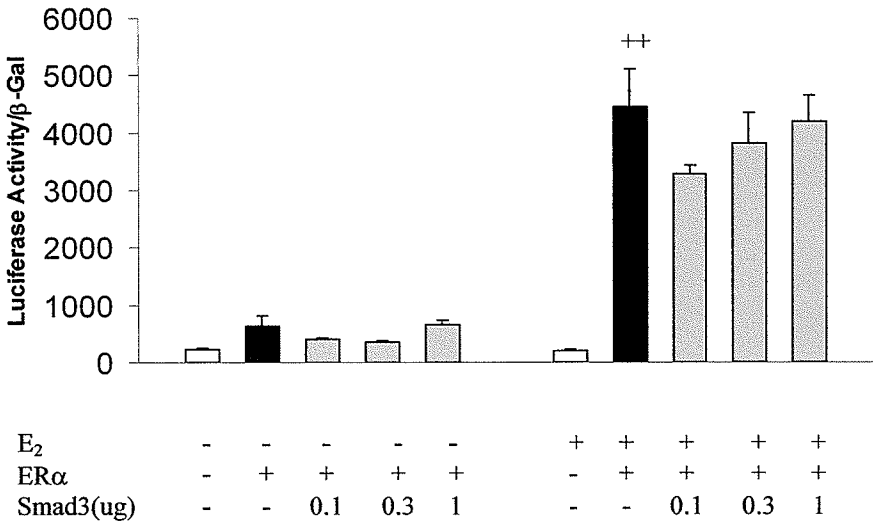


Figure 11. Effect of Smad3 over-expression on ERα transcriptional activity on an estrogen responsive element (ERE) in the presence or absence of 10nM E_2 in Cos1 cells. Cos1 cells were transiently transfected with 100ng pCH110, 200ng ERE-II-luc, 100ng pcDNA4-His/Xpress-ERα and increasing amounts of pCMV5B-Flag-Smad3 (0, 0.1ug, 0.3ug and 1ug) in the presence or absence of 10nM E_2. Equimolar concentrations of DNA were maintained between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and assayed for luciferase and β-galactosidase activity as described in Methods. Results represent the mean ± SEM, n=3 independent experiments. ++ indicates a significant ($p<0.01$) (Student's t-test) difference as compared to untreated cells.

In the presence of $ER\beta_1$, E_2 stimulation also significantly ($p<0.005$) increased ERE activity as compared to vehicle treated samples (Figure 12). Unlike ERα, however,

over-expression of Smad3 in the presence of $ER\beta_1$ resulted in a significant decrease in luciferase activity both in the presence and absence of estradiol (Figure 12), suggesting that this effect was ligand independent. As data from our laboratory suggest that the ERβ variants (i.e. $ER\beta_2$ and $ER\beta_5$) do not have transcriptional activity of their own (139), ERE reporter genes have not been used in conjunction with the variants.

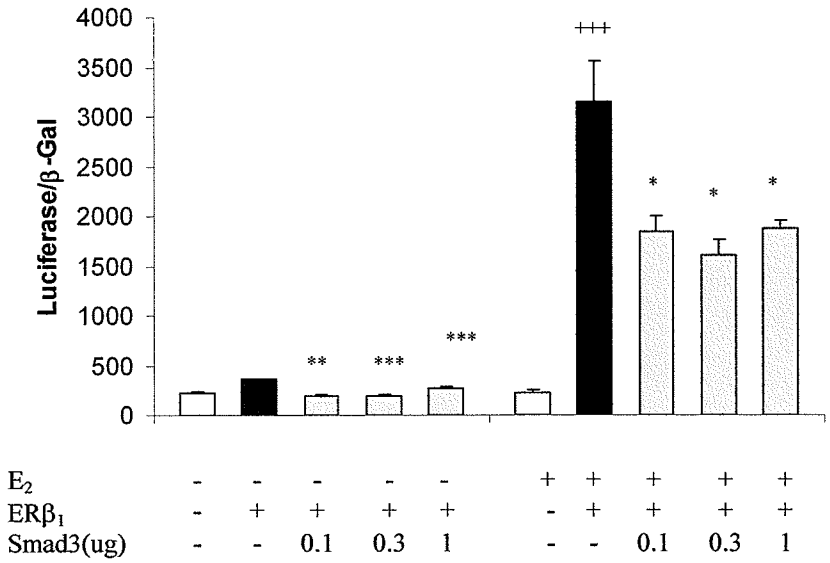


Figure 12. Effect of Smad3 over-expression on $ER\beta_1$ transcriptional activity on an estrogen responsive element (ERE) in the presence or absence of 10nM E_2 in Cos1 cells. Cos1 cells were transiently transfected with 100ng pCH110, 200ng ERE-II-luc, 50ng pcDNA4-His/Xpress-$ER\beta_1$ and increasing amounts of pCMV5B-Flag-Smad3 (0, 0.1ug, 0.3ug and 1ug) in the presence or absence of 10nM E_2. Equimolar concentrations of DNA were maintained between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and assayed for luciferase and β-galactosidase activity as described in Methods. Results represent the mean ± SEM, n=3 independent experiments. +++ indicates a significant difference ($p<0.005$) (Student's t-test) as compared to untreated cells. *, ** and *** indicates significant differences ($p<0.05$, $p<0.01$ and $p<0.005$, respectively) (Student's t-test) as compared to the corresponding sample in the presence of $ER\beta_1$ only.

Modulation of ERα transcriptional activity by Smad3 was also examined on the non-ERE containing TGFβ$_3$-luc reporter gene, where the DNA binding domain of ER is not required for activity (289, 408). The TGFβ$_3$-luc plasmid contains nucleotides -2306/-1848 of the human TGFβ$_3$ promoter, a region that has been implicated in mediating raloxifene bound ERα transcriptional activity (289). This response element, identified as the raloxifene response element, shows no sequence similarities to either the consensus ERE nor the Ap-1 binding site and removal of the DBD of ERα does not inhibit raloxifene induced TGFβ$_3$ expression (289). Thus, the TGFβ$_3$ promoter is an example of a non-classical mode of gene regulation by ER in that no classical ERE sites have been identified. While the mechanism by which ERα modulates TGFβ$_3$ transcription is unknown, the Ras-MAPK pathway has been implicated (290). We have previously shown that both ERα and ERβ increase TGFβ$_3$-luc transcription in the presence of the raloxifene analog Ly117018 and that the ERβ variants, ERβ$_2$ and ERβ$_5$, inhibit the effect of activated ERα (139). Therefore, the effect of Smad3 on ERα transcriptional activity on this promoter was examined. ERα transient transfection into Cos1 cells resulted in a 2.2 x increase ($p<0.05$) in TGFβ$_3$-luc activity in the presence of 100nM Ly117018 (Figure 13). Smad3 over-expression significantly inhibited the up-regulation of TGFβ$_3$-luc transcription by ERα ($p<0.05$) as compared to control (Figure 13). This effect by Smad3 appeared to be ligand-independent as Smad3 similarly inhibited TGFβ$_3$-luc activity in the absence of Ly117018 ($p<0.01$) (Figure 13).

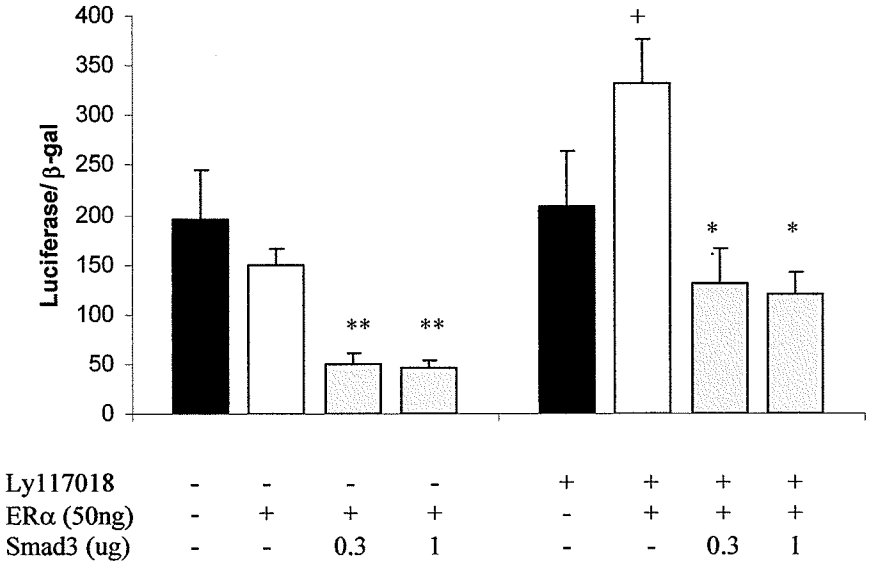


Figure 13. Effect of Smad3 over-expression on ERα transcriptional activity on the TGFβ$_3$-luc promoter in the presence or absence of 100nM Ly117018 in Cos1 cells. Cos1 cells were transiently transfected with 100ng pCH110, 200ng TGFβ$_3$-luc, along with 50ng pcDNA4-His/Xpress-ERα and increasing amounts of pCMV5B-Flag-Smad3 (0, 0.3ug or 1ug) in the presence or absence of 100nM Ly117018 as described in Methods. Equimolar concentrations of DNA were kept between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and assayed for luciferase and β-galactosidase activity as described in Methods. Results represent the mean ± SEM, n=3 independent experiments. + indicates a significant difference ($p<0.05$) (Student's t-test) as compared to corresponding untreated cells. * and ** indicate significant differences ($p<0.05$ and $p<0.01$) as compared to the corresponding sample in the presence of 0ng Smad3.

Upon closer examination of the TGFβ$_3$ luciferase and pCH110 β-galactosidase data, raw luciferase activity values were not affected by Smad3 over-expression while the level of β-galactosidase was significantly increased ($p<0.05$) (Table 2), suggesting that Smad3 affects the expression of our internal control. This increase in β-galactosidase activity corresponded to the degree of inhibition observed by Smad3 over-expression on relative TGFβ$_3$ luciferase activity, suggesting that Smad3 does not affect TGFβ$_3$-luc

transcription in Cos1 cells. To ensure that our internal control would not interfere with relative luciferase activity in future studies, we tested another internal control plasmid, pGL3-β-galactosidase, to see whether Smad3 over-expression affected its activity. Results suggest that, unlike the pCH110 plasmid, Smad3 does not affect pGL3-β-galactosidase activity (Figure 14). Therefore, results from experiments in which Smad3 is over-expressed in the presence of pCH110 will need to be examined closely but utilization of the pGL3-β-galactosidase plasmid as an internal control is preferred.

β-Galactosidase Activity (pCH110)		
Sample (ug Smad3)	**Vehicle**	**100nM Ly117108**
0	0.1428±0.03	0.1454±0.03
0.3	0.3274±0.07 *	0.3792±0.11 *
1	0.4286±0.10 *	0.4020±0.09 *

Luciferase Activity (TGFβ$_3$-luc)		
Sample (ug Smad3)	**Vehicle**	**100nM Ly117018**
0	21.60±3.48	47.37±6.71
0.3	16.03±1.54	44.65±7.26
1	19.41±3.07	45.68±6.35

Table 2: Effect of Smad3 over-expression on pCH110 and TGFβ$_3$-luc activity. Cos1 cells were transiently transfected with 100ng pCH110, 200ng TGFβ$_3$-luc, 50ng pcDNA4-His/Xpress-ERα and increasing amounts of pCMV5B-Flag-Smad3 (0, 0.3ug or 1ug) in the presence or absence of 100nM Ly117018 as described in Methods. Equimolar concentrations of plasmid were kept between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and assayed for luciferase and β-galactosidase activity as described in Methods. Results represent the mean ± SEM, n=3 independent experiments for β-galactosidase (pCH110) and luciferase (TGFβ$_3$-luc) activity. *$p<0.05$, Student's t-test (as compared to 0ng Smad3).

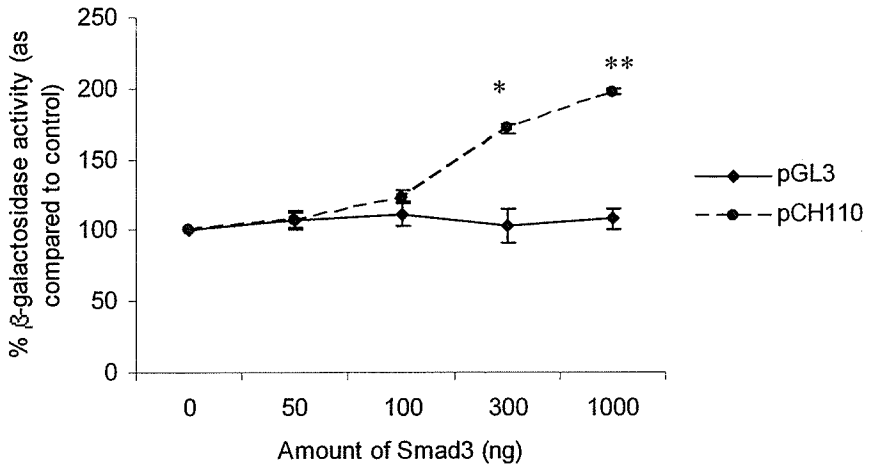


Figure 14: Effect of Smad3 over-expression alone on pCH110 and pGL3-β-Gal activity. Cos1 cells were transiently transfected with 100ng pCH110 or 100ng pGL3-β-gal and increasing amounts of pCMV5B-Flag-Smad3 (0, 50, 100, 300 or 1000 ng) as described in Methods. Equal molar amounts of plasmid were kept between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and assayed for β-galactosidase activity as described in Methods. Values represent percentage β-galactosidase activity with 0ng Smad3 set as 100% and are expressed as the mean ± SEM, n=3 independent experiments. *$p<0.05$, **$p<0.01$ Student's t-test (compared to 0ng Smad3).

As Smad3 over-expression increased the activity of the pCH110 internal control when used with the non-classical TGFβ_3-luc reporter plasmid, we re-examined the effects of Smad3 on the internal control when used in conjunction with the classical ERE-II-luc reporter. Little if any effects of increasing Smad3 on β-galactosidase activity were seen in the presence of the classical ERE luciferase reporter with ER treated with E_2 (data not shown). Thus, although there was a significant effect of increasing Smad3 on β-galactosidase activity in the internal control with TGFβ_3-luc, this did not affect the conclusion that increasing Smad3 had little if any effect on ERα or ERβ_1 activity on the ERE-II-luc reporter gene.

B. Modulation of Smad3 Activity by ER

Rationale

From our experiments in Cos1 cells we have shown that Smad3 has little if any effect on ER transcriptional activity. These results are in accordance with that observed by Yanagisawa et al. (320). While we have determined that TGFβ, via Smad3, does not modulate ER transcriptional activity in our cell system, the question still remains as to whether ER could modulate Smad3 transcriptional activity. To test whether ER affects Smad3 transcriptional activity, we utilized the Smad3 responsive p3TP-lux reporter plasmid, which contains a TGFβ responsive element from the plasminogen activator inhibitor-1 (PAI-1) promoter downstream of three TPA-responsive elements of the human collagenase gene (409).

Results

i. Effect of ERα Expression on Smad3 Transcriptional Activity in Cos1 Cells

Figure 15 is a diagrammatic representation of the p3TP-lux plasmid and Figure 16 illustrates the nucleotide sequence of the human collagenase and PAI-1 promoter insert. The p3TP-lux plasmid has been well characterized as a TGFβ responsive promoter and a dose-dependent increase in its activity occurs in the presence of increasing Smad3 expression (Figure 17) ($p<0.005$) (409-412). There was no significant increase in pCH110 β-galactosidase activity in contrast to that observed in the $TGF\beta_3$-luc reporter assays with increasing amounts of transfected Smad3 plasmid. Therefore, Smad3 significantly increased the TGFβ responsive reporter gene in a dose-dependent manner.

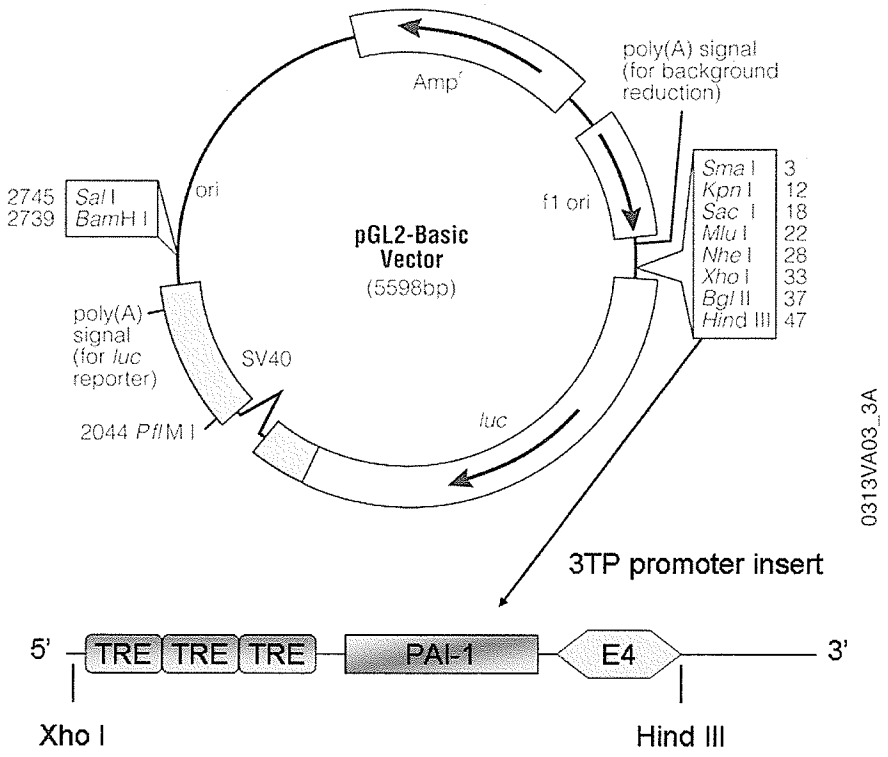


Figure 15: Diagrammatic representation of the basic structure of p3TP-lux. From 5' to 3', the 3TP insert consists of: (1) a multimerized TPA-responsive element corresponding to the -73 to -42 region of the human collagenase gene; (2) a TGFβ responsive element from positions -636 to -740 in the human plasminogen-activator inhibitor-1 promoter; and (3) adenovirus E4 promoter sequences from positions -38 to +38. This 3TP promoter was cloned into Xho-Hind III digested luciferase expression vector (pGL2, basic vector, Promega) generating p3TP-lux. Taken from (409).

Figure 16: Nucleotide sequence of the p3TP-lux insert. The TGFβ responsive reporter plasmid p3TP-lux is composed of three 5' human collagenase promoter sequences upstream of the plasminogen activator inhibitor-1 (PAI-1) promoter region cloned within a pGL2 vector. The sequence in *italics* represents the TPA-responsive elements while the sequence in **bold** represents the Smad3 binding sites in PAI-1 with the sequence **underlined** representing the core consensus sequence. The sequence in red indicates the presence of a half-ERE.

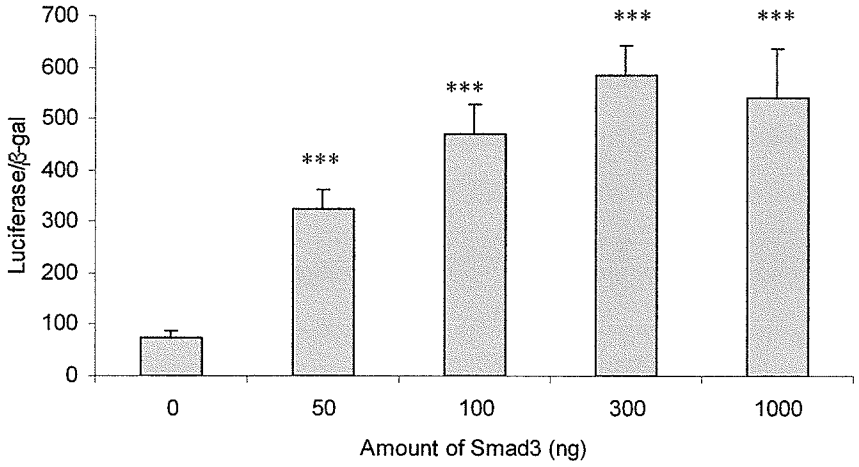


Figure 17. Effect of Smad3 over-expression on p3TP-lux activity in Cos1 cells. Cos1 cells were transiently transfected with 200ng p3TP-lux, 100ng pCH110 and increasing amounts (50-1000ng) of pCMV5B-Flag-Smad3. Empty pcDNA3.1 vector was used to ensure equimolar concentrations of plasmid between samples. Cells were harvested 48 h later and assayed for luciferase and β-galactosidase activity as described in Methods. Values represent the mean ± SEM, n=6 independent experiments. ***$p<0.005$, Student's t-test (compared to 0ng Smad3).

Cos1 transient transfections with p3TP-lux and either a His/Xpress tagged or untagged ERα protein expression vector shows that ERα does not affect p3TP-lux activity on its own in the presence or absence of estradiol (Table 3). Expression plasmids encoding tagged as well as untagged ERα proteins were utilized to ensure that the His/Xpress tag did not alter the activity of ERα on the reporter plasmid. Figure 18 shows the increase in His/Xpress tagged ERα protein expression as more plasmid is transfected into Cos1 cells (note: due to poor transfer to the nitrocellulose membrane, there is a slight shift in the band corresponding to ERα at the far right of the diagram). In the presence of over-expressed Smad3, ERα caused a significant ($p<0.05$) decrease in Smad3 transcriptional activity in a ligand-dependent manner (Figure 19a+b). Similar results

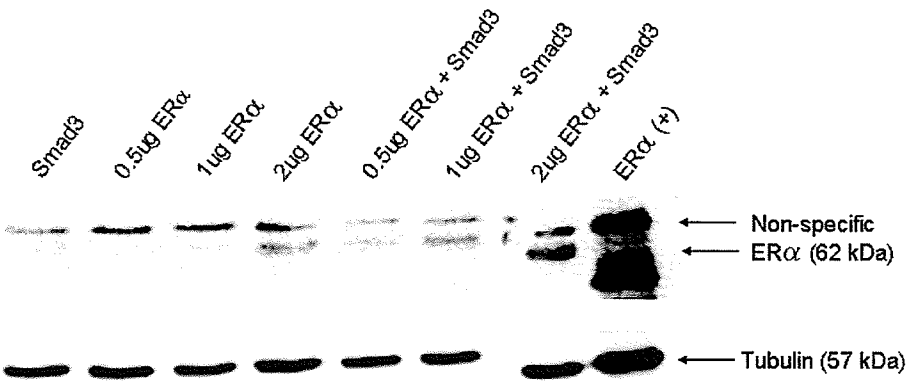


Figure 18. Western blot of Cos1 cells transiently transfected with Smad3 and ERα. Cos1 cells were transiently transfected with 1ug p3TP-lux, 1ug pCH110, 1ug pCMV5B-Flag-Smad3 and increasing amounts of pcDNA4-His/Xpress ERα as described in Methods. DNA was maintained at equimolar concentrations between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and whole cell protein extracts prepared. 22ul of the 300ul whole cell protein extract was run on an SDS-10% acrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-His antibody (Invitrogen) (top panel) and an anti-tubulin antibody (Neomarkers) (bottom panel). The molecular mass of ERα and tubulin is denoted on the left.

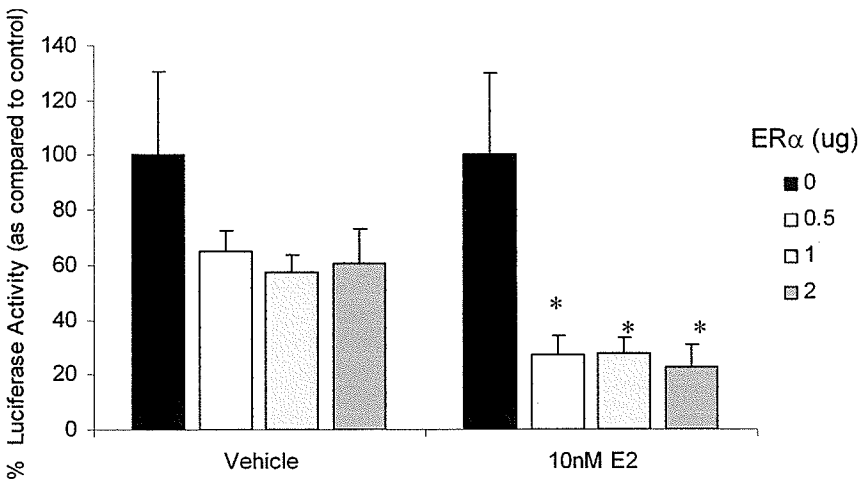
were also observed when a His/Xpress tagged ERα protein was expressed (Figure 19b). These results were not affected by the pCH110 internal control as equimolar concentration of Smad3 expression plasmid was transfected into each test sample. The decrease in Smad3 activity by estradiol bound ERα was inhibited by co-treatment with an excess of either 4OH-tamoxifen ($p<0.01$) or ICI 182,780 ($p<0.001$) (Figure 20). Antiestrogen treatment did not affect p3TP-lux transcription in the absence of estradiol (data not shown). Tamoxifen is known as a selective estrogen receptor modulator (SERM), in that it may either inhibit or stimulate ER activity depending on the promoter sequence and cellular milieu (149). ICI 182,780, on the other hand, is known as a pure

estrogen antagonist, in that it inhibits ER activity independent of cell background and target gene sequences (160).

	pcDNA3.1-ERα		pcDNA4-His/Xpress-ERα	
ug ERα	Vehicle	10nM E_2	Vehicle	10nM E_2
0	403.40 ± 163.29	374.08 ± 193.49	433.88 ± 147.64	446.61 ± 160.39
0.5	388.72 ± 157.07	258.37 ± 101.11	360.61 ± 116.09	225.18 ± 49.93
1	392.48 ± 148.70	253.83 ± 117.44	359.61 ± 84.70	251.41 ± 64.54
2	358.26 ± 112.42	270.76 ± 118.90	391.19 ± 69.98	238.18 ± 34.13

Table 3: Effect of ERα on p3TP-lux activity in Cos1 cells. Cos1 cells were transiently transfected with 1ug p3TP-lux, 1ug pCH110 and increasing amounts (0.5-1ug) of pcDNA3.1-ERα (encodes for an untagged ERα protein) or pcDNA4-Hix/Xpress-ERα (encodes for a His and Xpress tagged ERα protein) in the presence of vehicle or 10nM E_2 as described in Methods. Empty pcDNA3.1 vector was used to ensure equimolar concentrations of plasmid between samples. Cells were harvested 48 h later and assayed for luciferase and β-galactosidase activity and are expressed as described in Methods. Results represent the mean ± SEM, n=3 independent experiments.

A.



B.

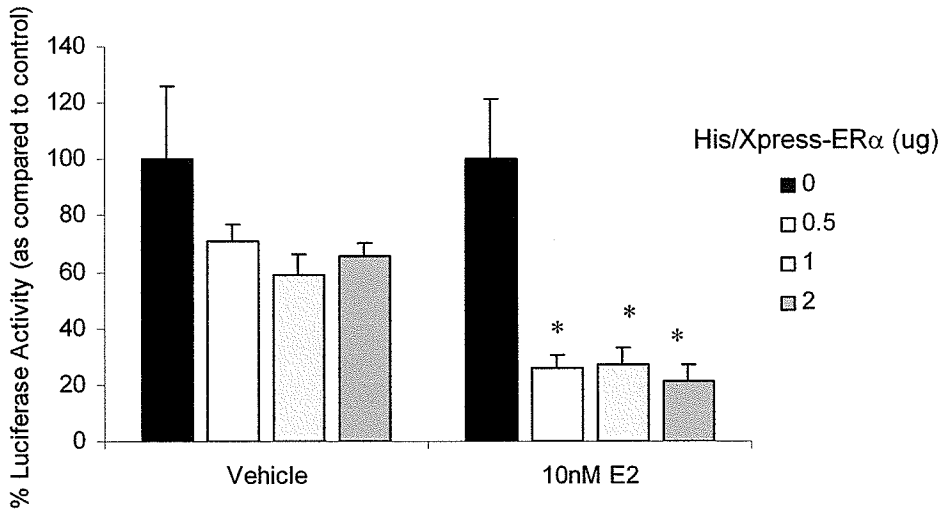


Figure 19. Effect of ERα on Smad3 induced p3TP-lux activity in the presence or absence of 10nM E_2 in Cos1 cells. Cos1 cells were transiently transfected with 1ug p3TP-lux, 1ug pCH110, 1ug Smad3 and increasing amounts (0.5-2ug) of (A) pcDNA3.1-ERα or (B) pcDNA4-His/Xpress-ERα in the presence of vehicle (EtOH) or 10nM E_2. Empty pcDNA3.1 vector was used to ensure equimolar concentrations of plasmid between samples. Cells were harvested 48 h later and assayed for luciferase and β-galactosidase activity as described in Methods. Results represent percentage luciferase activity with 0ug ERα set as 100%. Values represent the mean ± SEM, n=3 independent experiments. *$p<0.05$, Student's t-test (as compared to cells transfected with 0ug ERα).

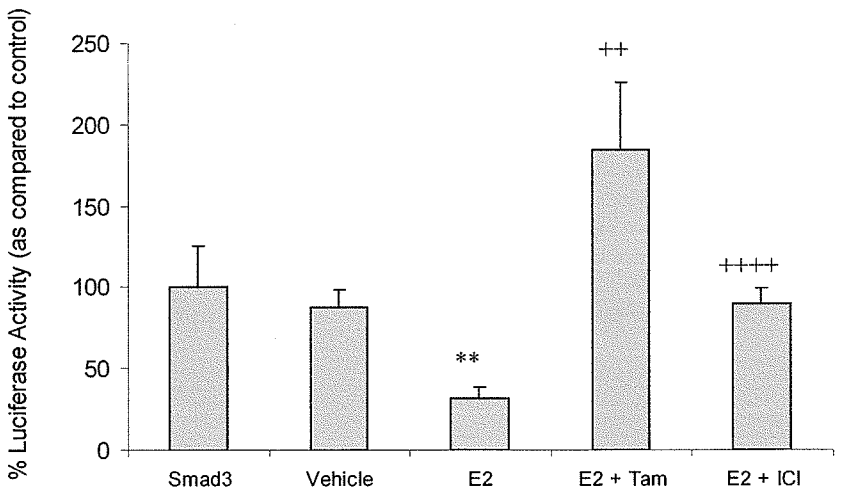
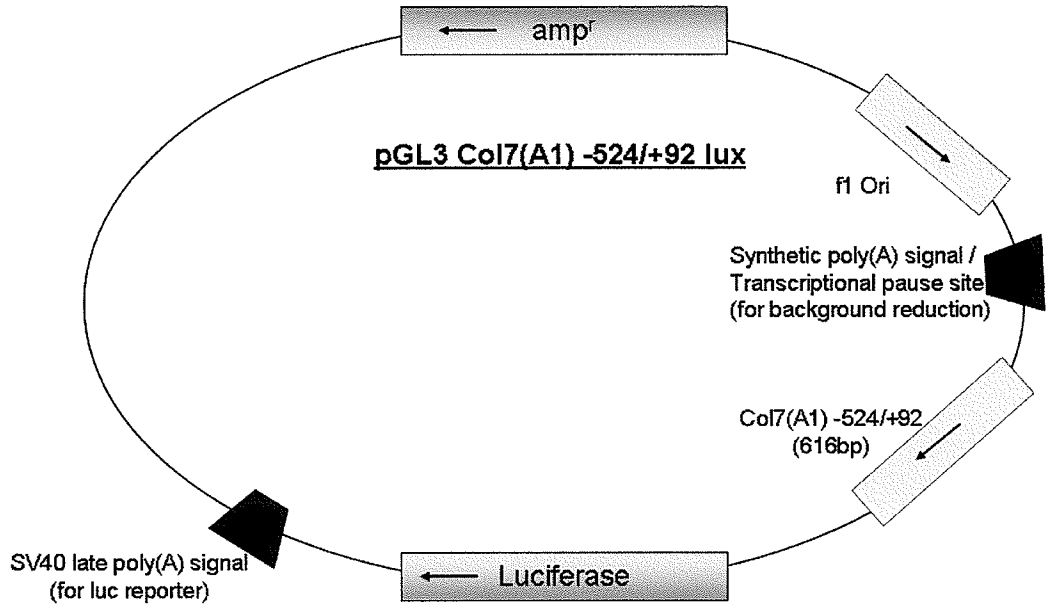


Figure 20. Effect of ERα on Smad3 induced p3TP-Lux activity in Cos1 cells in the presence of vehicle, 10nM E_2, 100nM 40H-tamoxifen plus 10nM E_2 or 500nM ICI 182,780 plus 10nM E_2. Cos1 cells were transiently transfected and assayed for luciferase and β-galactosidase activity as described in Methods. Results are expressed as percentage luciferase activity with Smad3 (control) set as 100%. Cells were transiently transfected with 100ng pCH110, 200ng p3TP-Lux, 1ug pCMV5B-Flag-Smad3 and 1ug pcDNA4-His/Xpress-ERα in the presence of vehicle, 10nM E_2 (E2), 100nM 4OH-tamoxifen plus 10nM E_2 (E2+Tam) and 500nM ICI 182,780 plus 10nM E_2 (E2+ICI). Smad3 (control) samples were transfected in the presence of vehicle and do not contain ERα. DNA was maintained at equimolar concentrations between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and assayed for luciferase and β-galactosidase activity. Results represent the mean ± SEM, n=6 independent experiments. ** indicates significant differences ($p<0.01$, Student's t-test) as compared to vehicle and ++ or ++++ indicates significant differences ($p<0.01$ or $p<0.001$, Student's t-test) as compared to E_2 treated samples.

To determine if this inhibitory effect of estradiol bound ERα on Smad3 transcriptional activity was specific for the p3TP-lux promoter or can potentially be applied to several Smad3 responsive genes, the effect of ERα on another known TGFβ/Smad3 regulated promoter was investigated. Collagen 7(A1) is a type of network forming collagen which helps attach the basal lamina of squamous cell epithelia to the underlying connective tissue and therefore, is especially abundant in the skin. Transcription of collagen 7(A1) has been shown to be increased upon activation of the TGFβ signaling pathway, whereby phosphorylated Smad3 binds to a SBE in the promoter region (413). The SBE of the collagen 7(A1) promoter has been mapped to nucleotides -496/-444 and is composed of two distinct binding sites whose simultaneous presence is required for Smad binding (414). In addition to TGFβ, the Ap-1 signaling pathway also plays an important role in modulating collagen 7(A1) transcription. Two consensus Ap-1 sites have been found in the promoter, one located between the bipartite SBE and one located further downstream of the SBE. A diagrammatic representation of the collagen 7(A1) promoter and the nucleotide sequence of the insert is shown in Figure 21.



5'-CAGGAGGCCCAC**CAGACAGA**TG*GCTGAATCACA*GGAGTGGCCGGCGG
GACCCATGGCCTGAGGGCTT**GTCTGGGCA**CCCCCACTGGATTGGG*GGTGA*
*GTCATC*CCCAACTGCAGCCCCACCCCCCACGGCGCTGCTGCCTTGTGGCTC
TGCAGGAACCTGTCCACTCCTCAGCCTGGTCACTGTGATTGACCTAAAGCA
GCCAAGACCTGTGACCTTAGATGGAGTTAGGGGTACTCCCTCAGCATCTG
CCCATGCAGAACCTTCTGGGAAATTCCCAGAAGCCACGGGGGGTCGGGGG
GTTTATAGTTAAGTGCGTCATATCGTTTGTCTGGGGGAGGGGTGGGGGGG
GCGGCGACCTCTCAGGGATATGGGTGAGGGCGGGTGCCTGGGTTCCCGCC
TGCCGCTCCGCCCCCCGAGATCAGGGACTTTTCTCTGCTCTGCCCGAGAGA
CTGCAGCGGCGGCGGCGGGAGCGGGCGGACGCGCAGGCAAGACCAGGAC
TCGGGCTGGAGGGGCGCTGGGCTCGGACCTGCCAAGGCCACGGGGGAGC
AAGGGACAGAGGCGGGGGTCCTAGCTGACGGCTTTTACTGCCTAGG – 3'

Figure 21: Diagrammatic representation of the pGL3 Col7(A1) plasmid and the nucleotide sequence of the collagen 7(A1) -524/+92 promoter insert. Upper panel: The TGFβ responsive reporter plasmid pGL3-collagen 7(A1)-524/+92-luc consists of nucleotides -524/+92 of the collagen 7(A1) gene cloned into a pGL3 vector. Lower panel: The sequence in *italics* represents a known Ap1 responsive sequence, the sequence in **bold** represents a known Smad3 binding site while the sequence in *italics* or **bold** represent putative Ap1 and Smad3 binding sites, respectively. The sequence in red indicates the presence of half ERE sequences.

To determine whether ERα affects Smad3 signaling on the collagen 7A(1) promoter, we transiently transfected Cos1 cells with a reporter plasmid containing the collagen 7(A1)-524/+92 promoter region upstream of the luciferase gene together with the Smad3 and ERα expression vectors in the presence or absence of ligand. Similar to p3TP-lux, over-expression of Smad3 resulted in a dose-dependent increase ($p<0.05$) in collagen 7(A1) promoter activity (Figure 22). In the presence of Smad3, however, ERα significantly increased collagen 7(A1) transcription in a dose-dependent manner in both the presence ($p<0.05$) or absence ($p<0.01$) of estradiol (Figure 23) while ERα alone, in the absence of Smad3, had no effect (data not shown).

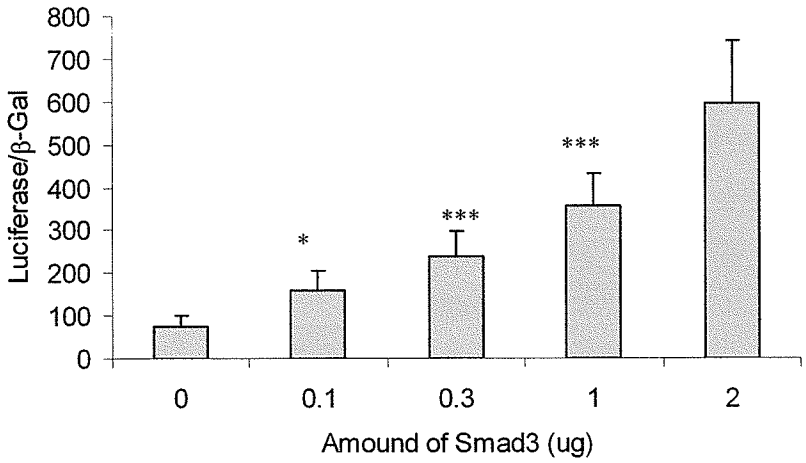


Figure 22. Effect of Smad3 over-expression on collagen 7(A1)-524/+92 activity in Cos1 cells. Cos1 cells were transiently transfected with 100ng pcDNA3-β-gal, 10ng pGL3-collagen7(A1)-524/+92-luc and increasing (0-2ug) amounts of pCMV5B-Flag-Smad3 expression plasmid as described in Methods. Equimolar concentrations of plasmid were kept between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and assayed for luciferase and β-galactosidase activity. Results represent the mean ± SEM, n=4 independent experiments. * and *** indicate significant differences ($p<0.05$ and $p<0.005$, Student's t-test) as compared to control samples.

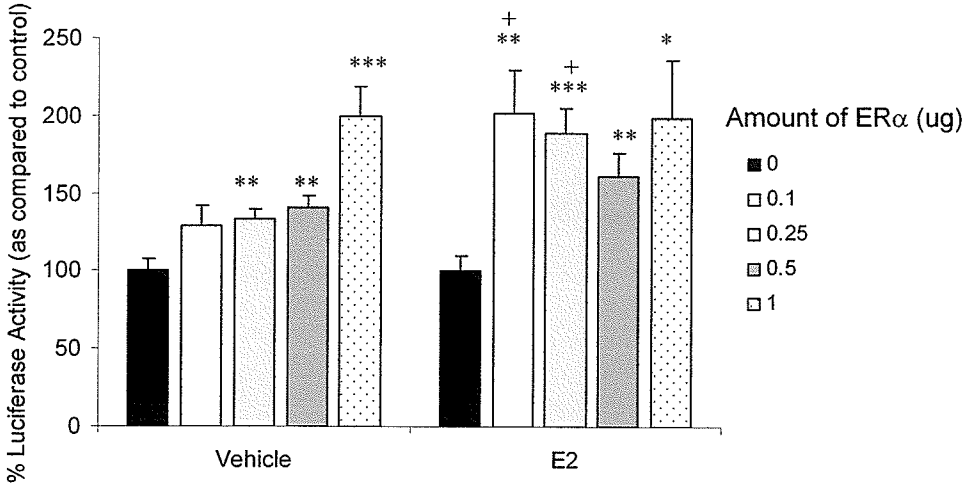


Figure 23. Effect of ERα on Smad3 induced collagen 7(A1)-524/+92 activity in Cos1 cells. Cos1 cells were transiently transfected with 100ng pcDNA3-β-gal, 10ng pGL3-collagen7(A1)-524/+92-luc, 1ug pCMV5B-Flag-Smad3 and increasing (0-1ug) pcDNA4-His/Xpress ERα in the presence or absence of 10nM E_2 as described in Methods. Equimolar concentrations of plasmid were kept between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and assayed for luciferase and β-galactosidase activity. Results represent the mean ± SEM, n=5 independent experiments. *, ** and *** indicates significant differences ($p<0.05$, $p<0.01$ and $p<0.005$, Student's t-test) as compared to control samples. + $p<0.05$ (Student's t-test) as compared to samples treated with the corresponding vehicle.

To further characterize the effects of ERα on Smad3 mediated collagen 7(A1) gene transcription, Cos1 cells were transiently transfected with ERα and Smad3 in the presence of estradiol and the antiestrogens 4OH-tamoxifen and ICI 182,780. Results suggest that ligand bound ERα significantly increases ($p<0.05$) Smad3 induced collagen 7(A1) promoter activity (Figures 23, 24) and this effect is inhibited by 4OH-tamoxifen ($p<0.01$) or ICI 182,780 ($p<0.001$) co- treatment (Figure 24). Antiestrogen treatment in the absence of estradiol had no effect on collagen 7(A1) transcription (Figure 24).

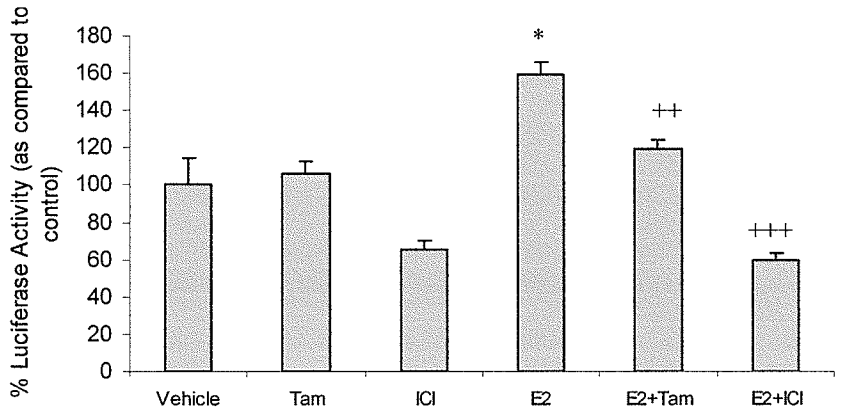


Figure 24. Effect of ERα on Smad3 induced collagen 7(A1)-524/+92 activity in Cos1 cells in the presence of E_2 and/or antiestrogens. Cos1 cells were transiently transfected and assayed for luciferase and β-galactosidase activity as described in Methods. Results are expressed as percent luciferase activity with vehicle control set as 100%. Cells were transiently transfected with 100ng pcDNA3-β-gal, 10ng pGL3-collagen7(A1)-524/+92-luc, 1ug pCMV5B-Flag-Smad3 and 1ug pcDNA4-His/Xpress-ERα in the presence of vehicle, 100nM 4OH-tamoxifen (Tam), 500nM ICI 182,780 (ICI), 10nM E_2 (E2), 100nM 4OH-tamoxifen plus 10nM E_2 (E2+Tam) and 500nM ICI 182,780 plus 10nM E_2 (E2+ICI). 48 h post-transfection, cells were harvested and assayed for luciferase and β-galactosidase activity. Results represent the mean ± SEM, n=3 independent experiments. * indicates a significant difference ($p<0.05$, Student's t-test) as compared to vehicle control. ++ and +++ indicates significant differences ($p<0.01$ and $p<0.005$, Student's t-test) as compared to E_2 treated samples.

ii. Effect of ERβ Expression on Smad3 Transcriptional Activity in Cos1 Cells

$ERβ_1$ has a high degree of sequence homology to ERα, sharing approximately 96% of its amino acids within the DBD and 53% within the LBD (68). Thus, both receptors bind similar ligands and EREs (88). Therefore, we were interested in determining whether differences existed between ERα and ERβ in their ability to modulate Smad3 transcriptional activity. To test this hypothesis, Cos1 cells were transiently transfected with increasing amounts of a His/Xpress tagged $ERβ_1$ expression

plasmid along with Smad3 and the p3TP-lux reporter plasmid in the presence or absence of estradiol. A western blot demonstrating the increase in His/Xpress ERβ$_1$ expression in Cos1 cells is shown in Figure 25. Similar to that observed with ERα, ligand bound ERβ$_1$ significantly ($p<0.05$) inhibited Smad3 transcriptional activity on p3TP-lux (Figure 26a) and this effect was inhibited by co-treatment with excess 4OH-tamoxifen ($p<0.005$) or ICI 182,780 ($p<0.01$) (Figure 26b). Antiestrogen treatment had no effect on p3TP-lux transcription in the absence of estradiol (data not shown).

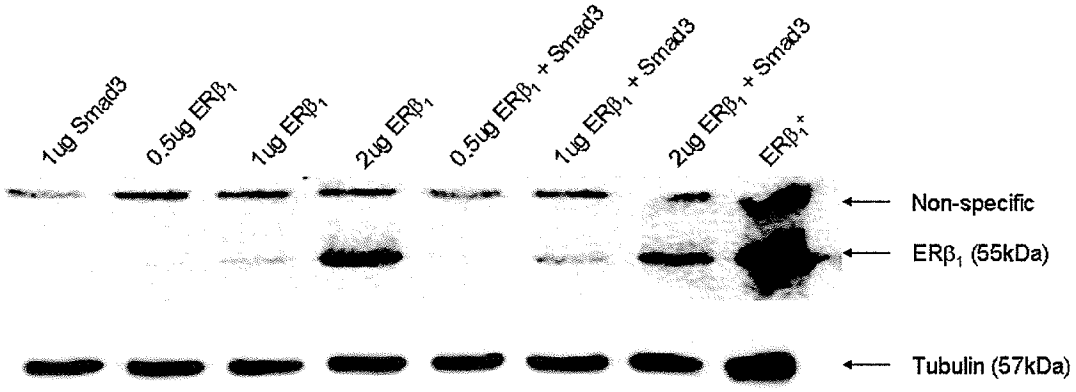
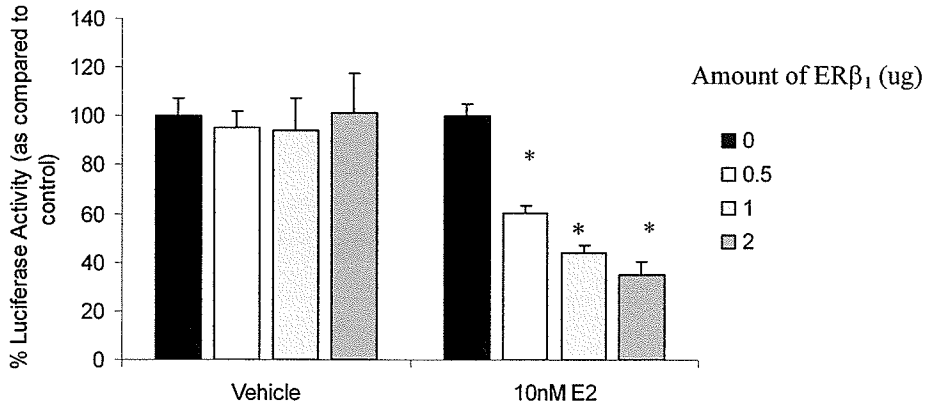


Figure 25. Western blot of Cos1 cells transiently transfected with Smad3 and ERβ$_1$. Cos1 cells were transiently transfected with 1ug p3TP-lux, 1ug pCH110, 1ug pCMV5B-Flag-Smad3 and increasing amounts of pcDNA4-His/Xpress ERβ$_1$ as described in Methods. DNA was maintained at equimolar concentrations between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and whole cell protein extracts prepared. 22ul of the 300ul whole cell protein extract was run on an SDS-10% acrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-His antibody (Invitrogen) (top panel) and an anti-tubulin antibody (Neomarkers) (bottom panel). The molecular mass of ERβ$_1$ and tubulin is denoted on the left.

A.



B.

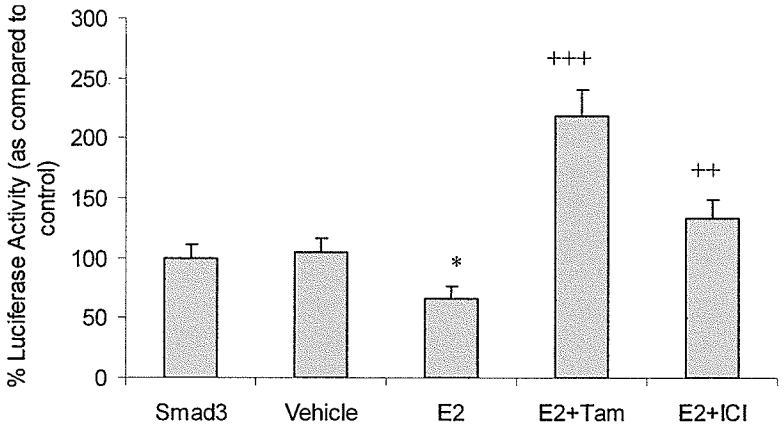


Figure 26. Effect of ERβ1 on Smad3 induced p3TP-Lux activity in Cos1 cells. Cells were transiently transfected as described in Methods. DNA was maintained at equimolar concentrations between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and assayed for luciferase and β-galactosidase activity. A) Cos1 cells transfected with 100ng pCH110, 200ng p3TP-Lux, 1ug pCMV5B-Flag-Smad3 and increasing (0-2ug) amounts of pcDNA4-His/Xpress ERβ1 in the presence or absence of 10nM E_2. Results are expressed as percent luciferase activity with 0ug ERβ1 set as 100% and represent the mean ± SEM, n=3 independent experiments. B) Cos1 cells were transfected with 100ng pCH110, 200ng p3TP-Lux, 1ug pCMV5B-Flag-Smad3 and 1ug pcDNA4-His/Xpress-ERβ1 in the presence of vehicle, 10nM E_2 (E2), 100nM 4OH-tamoxifen plus 10nM E_2 (E2+Tam) or 500nM ICI 182,780 plus 10nM E_2 (E2+ICI). Results are expressed as percent luciferase activity with 1ug Smad3 set as 100% and represent the mean ± SEM, A) n=6 or B) n=5 independent experiments. * indicates a significant difference ($p<0.05$, Student's t-test) as compared to A) Smad3 or B) vehicle control. ++ or +++ indicates significant differences ($p<0.01$ or $p<0.005$, Student's t-test) as compared to E_2 treated samples.

The effect of the ERβ variants, $ER\beta_2$ and $ER\beta_5$, on Smad3 induced p3TP-lux promoter activity was also determined. As mentioned previously, $ER\beta_2$ and $ER\beta_5$ are similar in sequence to $ER\beta_1$ but have a shorter C-terminus and evidence suggests that they do not bind E_2 (139). ERβ variants were over-expressed in Cos1 cells along with Smad3 and the p3TP-lux reporter plasmid in Cos1 cells. Results are shown in Figure 27 and demonstrate no significant effect on Smad3 transcriptional activity. However, Western blot analysis of our transfected cells (Figure 28) suggest that $ER\beta_2$ and $ER\beta_5$ were expressed at much lower levels compared to that of $ER\beta_1$, which may account for the differences. Attempts to attain similar levels of expression by increasing the amount of variant expression plasmids transfected into our cells were unsuccessful (data not shown).

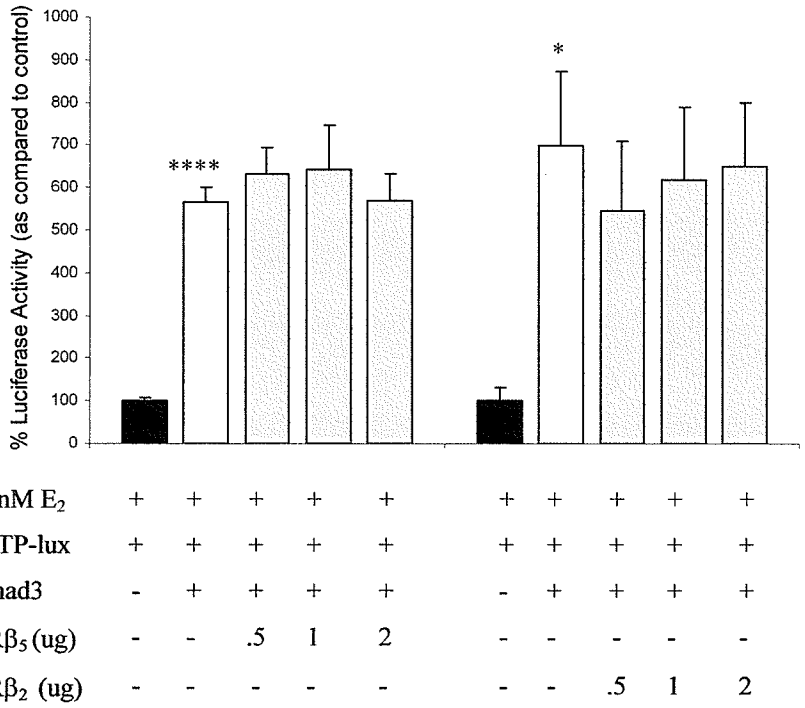


Figure 27. Effect of $ER\beta_2$ and $ER\beta_5$ on Smad3 induced p3TP-Lux activity in Cos1 cells in the presence of 10nM E_2. Cos1 cells were transiently transfected with 1ug p3TP-lux, 1ug pCH110, 1ug pCMV5B-Flag-Smad3 and increasing (0-2ug) amounts of pcDNA4-His/Xpress-$ER\beta_2$ or pcDNA4-His/Xpress-$ER\beta_5$ in the presence of 10nM E_2 as described in Methods. DNA was maintained at equimolar concentrations between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection cells were harvested and assayed for luciferase and β-galactosidase activity as described in Methods. Results are expressed as percent luciferase activity with cells transfected with p3TP-lux only set as 100%. Values represent mean ± SEM, n=3 independent experiments. *$p<0.05$ and ****$p<0.001$ (Student's t-test), as compared to p3TP-lux control.

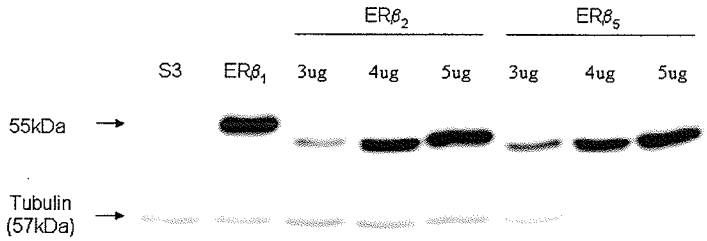
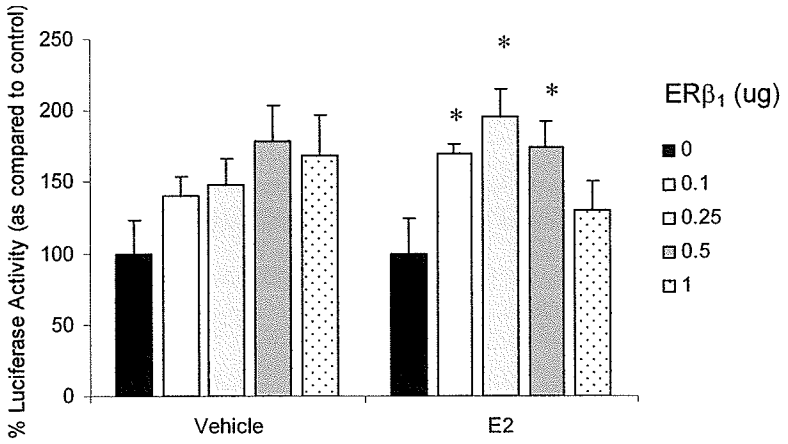


Figure 28. Western blot of Cos1 cells transiently transfected with $ER\beta_2$ and $ER\beta_5$. Cos1 cells were transiently transfected with 1ug p3TP-lux, 1ug pCH110, 1ug pCMV5B-Flag-Smad3 and either 1ug pcDNA4-His/Xpress-$ER\beta_1$ or increasing (0-5ug) amounts of pcDNA4-His/Xpress-$ER\beta_2$ or pcDNA4-His/Xpress-$ER\beta_5$ in the presence of 10nM E_2 as described in Methods. DNA was maintained at equimolar concentrations between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and whole cell protein extracts prepared. 22ul of the 300ul whole cell protein extract was run on an SDS-10% acrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-Xpress antibody (Invitrogen) (top panel) and an anti-tubulin antibody (Neomarkers) (bottom panel). Molecular mass markers are denoted on the left (Bio-rad).

The effect of $ER\beta_1$ on Smad3 transcriptional activity was also tested on the collagen 7(A1)-524/+92 luciferase plasmid. $ER\beta_1$ over-expression significantly ($p<0.05$) increased Smad3 induced collagen 7(A1) promoter activity in a ligand-dependent manner (Figure 29a) (as compared to cells transfected with no $ER\beta_1$ plasmid); no significant effect of $ER\beta_1$ was observed in the absence of ligand although there was a trend towards an increase. In contrast to $ER\alpha$, $ER\beta_1$ did not increase Smad3 induced collagen 7(A1)-524/+92 activity in the presence of E_2 (as compared to cells transfected with $ER\beta_1$ plasmid in the absence of estradiol) although there was a trend towards an increase in activity (Figure 29a+b). However, both 4OH-tamoxifen ($p<0.01$) and ICI 182,780 ($p<0.001$) significantly inhibited the effect of ligand activated $ER\beta_1$ (Figure 29b); antiestrogen treatment alone did not alter promoter activity (Figure 29b).

A.



B.

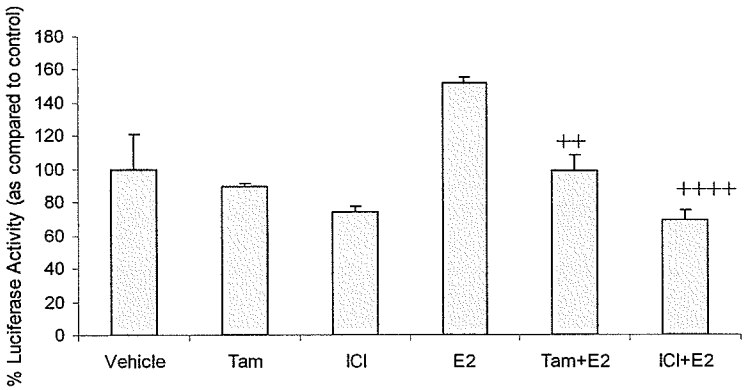
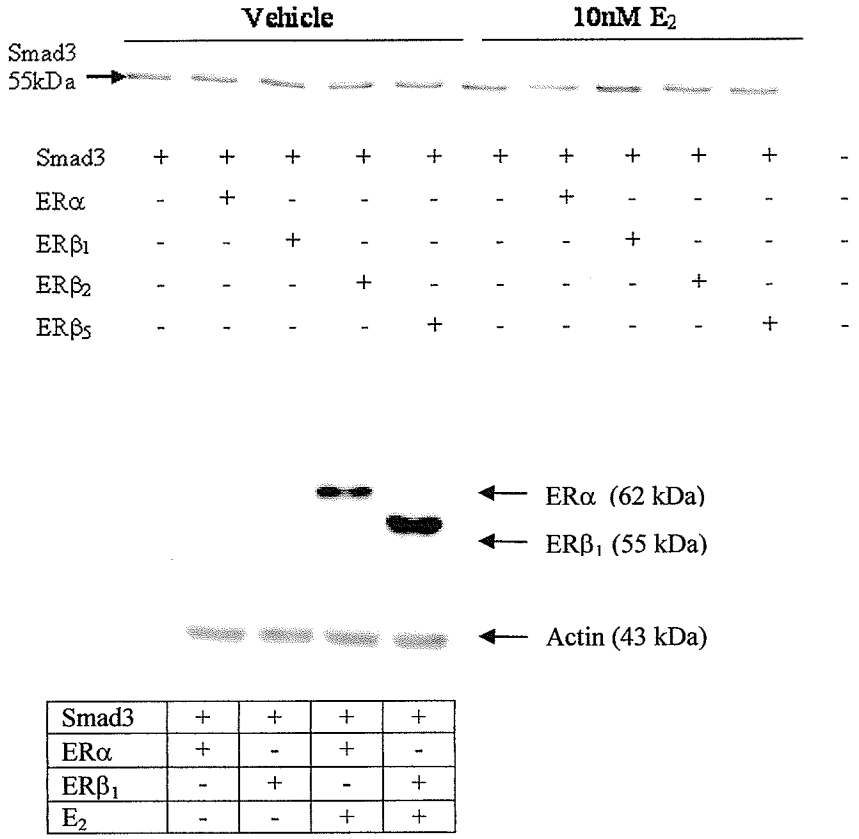


Figure 29. Effect of ERβ$_1$ on Smad3 induced collagen 7(A1)-524/+92 activity in Cos1 cells. Cells were transiently transfected as described in Methods. DNA was maintained at equimolar concentrations between samples by addition of empty pcDNA3.1 vector. 48 h post-transfection, cells were harvested and assayed for luciferase and β-galactosidase activity. A) Cos1 cells transfected with 100ng pcDNA3-β-gal, 10ng pGL3-collagen7(A1)-524/+92-luc, 1ug pCMV5B-Flag-Smad3 and increasing (0-1ug) amounts of pcDNA4-His/Xpress-ERβ$_1$ in the presence or absence of 10nM E_2. B) Cos1 cells transfected with 100ng pcDNA3-β-gal, 10ng pGL3-collagen7(A1)-524/+92-luc, 1ug pCMV5B-Flag-Smad3 and 1ug pcDNA4-His/Xpress-ERβ$_1$ in the presence of vehicle, 100nM 4OH-tamoxifen (Tam), 500nM ICI 182,780 (ICI), 10nM E_2 (E2), 100nM 4OH-tamoxifen plus 10nM E_2 (Tam+E2) or 500nM ICI 182,780 (ICI) plus 10nM E_2 (ICI+E2). Results are expressed as percent luciferase activity with control set as 100%. Results represent the mean ± SEM, n=3 independent experiments. *, ++ and ++++ indicates significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, Student's t-test) as compared to A) corresponding control or B) E_2 treated samples.

One possible mechanism through which ERα/β_1 may inhibit Smad3 signaling is through a decrease in Smad3 protein levels. However, western blot analysis of whole cell extracts from Cos1 cells transiently transfected with ERα/β_1 and Smad3 in the presence or absence of E_2 suggest that activated ER does not affect Smad3 protein expression in Cos1 cells (Figure 30a). Interestingly however, ERα and ERβ_1 expression appeared to be higher in samples treated with 10nM E_2 for 48 h than vehicle control ($p<0.05$) while no significant differences were observed between ERα and ERβ_1 expression (Figure 30b). In this case, both ER expression vectors which were transiently transfected into Cos1 cells encoded proteins which were tagged at the N-terminus with an Xpress epitope, allowing for the simultaneous detection of ERα and ERβ upon immunoblotting with an Xpress antibody. The regulation of ER by estrogen appears to be cell and/or tissue type specific. In the human mammary gland and in cultured MCF-7 breast cancer cells, E_2 has been shown to increase the degradation of ERα protein levels by targeting the ER to the proteasome (408, 415) and thereby decreasing steady state protein levels (416). This observation in cell culture closely resembles studies utilizing human breast biopsy tissue in which ERα expression is greater in tissues taken from postmenopausal women than those from premenopausal women (362, 417-419). In contrast, E_2 increases the expression of ERα in human pituitary, kidney and retinal pigment epithelial cells (420-422). While limited data are available with respect to ERβ turnover, it has been shown that in MCF-7 cells over-expressing ERβ, estradiol decreases the expression of the ERβ protein (423, 424). In contrast, ERβ mRNA and protein expression increases with estradiol treatment in the breast cancer cell line, T47D (424). As Cos1 cells are African green monkey kidney cells, the up-regulation of ER by ligand may be a true observation

in this system. However, one should keep in mind that we are transiently transfecting an ER plasmid into these cells and thus, the normal regulatory mechanism of transcription may be disrupted in part because of the lack of chromatin organization.



Smad3	+	+	+	+
ERα	+	-	+	-
$ER\beta_1$	-	+	-	+
E_2	-	-	+	+

Figure 30. Western blot of Smad3 and ER in Cos1 transient transfections. Cos1 cells were transiently transfected with 1ug p3TP-lux, 1ug pCH110, 1ug pCMV5B-Flag-Smad3 and either 2ug pcDNA4-His/Xpress ERα, 2ug pcDNA4-His/Xpress $ER\beta_1$, 2ug pcDNA4-His/Xpress $ER\beta_2$ or 2ug pcDNA4-His/Xpress $ER\beta_5$ in the absence or presence of 10nM E_2. Equimolar concentrations of plasmid were kept between samples by addition of empty pcDNA3.1. Cells were harvested 48 h later with hot Joel lysis buffer to obtain whole cell protein extracts. 22ul of a 300ul whole cell protein extract was run on an SDS-10% polyacrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-Smad3 antibody (Upstate Biotechnology) or an anti-Xpress antibody (Invitrogen), n=3 independent experiments.

iii. Effect of ER Expression on Smad3 Transcriptional Activity in MCF-7 Cells

To determine the functional interaction between ERα and Smad3 in a breast cell background, we examined whether ERα affects Smad3 transcriptional activity in the MCF-7 human breast cancer cell line. MCF-7 cells are ERα positive human breast cancer cells, which are growth sensitive to E_2 and TGFβ (424-428). MCF-7 cells over-expressing Smad3 had a 5-fold increase in p3TP-lux transcriptional activity as compared to control cells not transfected with Smad3 ($p<0.05$) (Figure 31). Initial experiments with cells grown under estrogen deplete conditions suggest that E_2 treatment had little if any effect on Smad3 activation of p3TP-lux (Figure 31). However, as the expression of ERα in MCF-7 cells is relatively low (20 - 45fmoles/mg cytosolic protein) (unpublished data) as compared to that found in ER positive breast tumors (often >50fmoles/mg cytosolic protein), it is reasonable to assume that an ER response may not be observed under these conditions. Therefore, we transiently over-expressed ERα in MCF-7 cells in order to increase its expression within these cells. This practice has been done previously in order to observe an ER response (281). As MCF-7 cells are difficult to transiently transfect, with generally low transfection efficiencies, western blot analysis of MCF-7 cells transiently transfected with ERα did not demonstrate any increase in ERα protein level (data not shown). However, the expression of ERα may have increased dramatically within a single cell that would not have been reflected under western blot conditions.

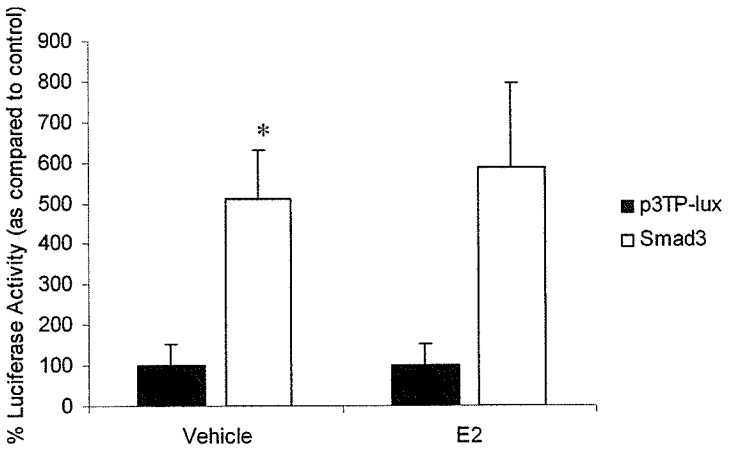


Figure 31: p3TP-lux activity in MCF-7 cells over-expressing Smad3 in the presence or absence of estrogen. MCF-7 cells were transiently transfected with 5ug p3TP-lux, 5ug pCMV5B-Flag Smad3 and 1ug pRL-tk-luc in the presence or absence of 10nM E_2 (E2). Empty pcDNA3.1 plasmid was used to ensure equimolar concentrations of plasmid between samples. 24 h post-transfection, cells were subjected to a 3 minute glycerol shock and new media containing either vehicle or 10nM E_2, was added. Samples were collected 48 h post-transfection and dual luciferase assays were performed as described in Methods. Results represent the mean ±SEM, n=4 independent experiments. * indicates a significant difference ($p<0.05$, Student's t-test) as compared to control cells not transfected with Smad3.

MCF-7 cells over-expressing ERα in the presence of p3TP-lux and Smad3 demonstrated a marked decrease ($p<0.01$) in Smad3 activity that was independent of E_2 (Figures 32 and 33). No significant change in luciferase activity was observed between samples transfected with Smad3 and ERα in the presence or absence of estradiol (Figures 32 and 33). Co-treatment with either 4OH-tamoxifen ($p<0.05$) or ICI 182,780 ($p<0.05$) resulted in an inhibition of the effect of increased ERα expression on p3TP-lux transcription (Figure 33). Thus, while estradiol may not be required to mediate an altered response on Smad3 mediated p3TP-lux transcription in MCF-7 cells, ERα is involved.

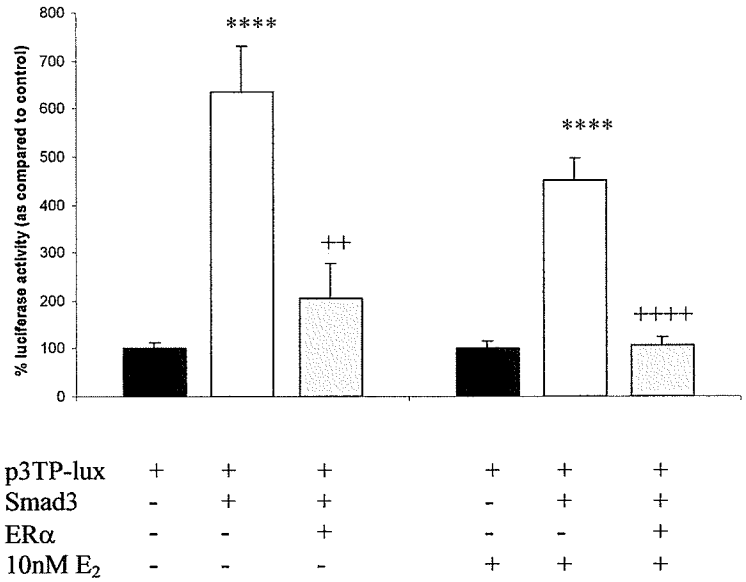


Figure 32: p3TP-lux activity in MCF-7 cells transiently transfected with ERα. MCF-7 cells were transiently transfected with 5ug p3TP-lux, 5ug pCMV5B-Flag Smad3, 1ug pRL-tk-luc and 5ug pcDNA3.1 ERα in the presence or absence of 10nM E_2. Empty pcDNA3.1 plasmid was used to ensure equimolar concentrations of plasmid between samples. 24 h post-transfection, cells were subjected to a three minute glycerol shock and new media containing vehicle or 10nM E_2 was added. Samples were collected 48 h post-transfection and dual luciferase assays were performed as described in Methods. Results represent the mean ±SEM, n=7 independent experiments. ****$p<0.001$ (Student's t-test) as compared to cells transfected with p3TP-lux only. ++ and ++++ indicate significant differences ($p<0.01$ and $p<0.001$, Student's t-test) as compared to cells transfected with Smad3 only in the presence or absence of ligand.

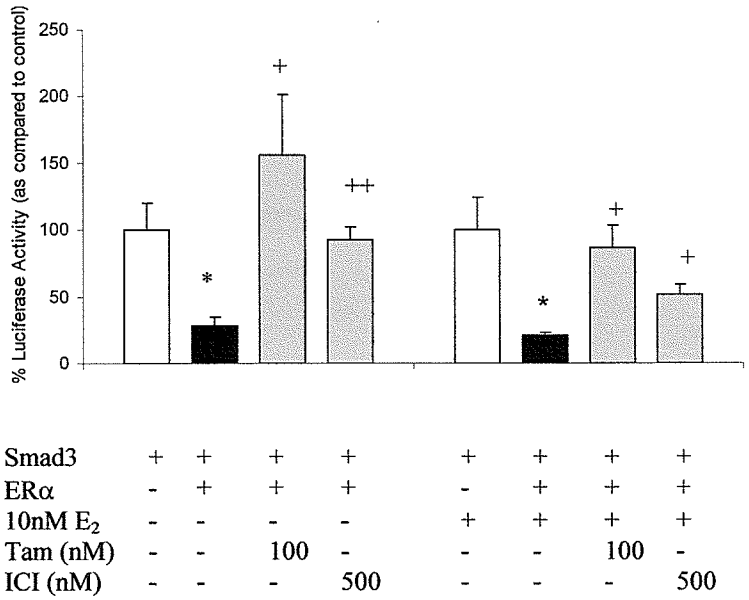
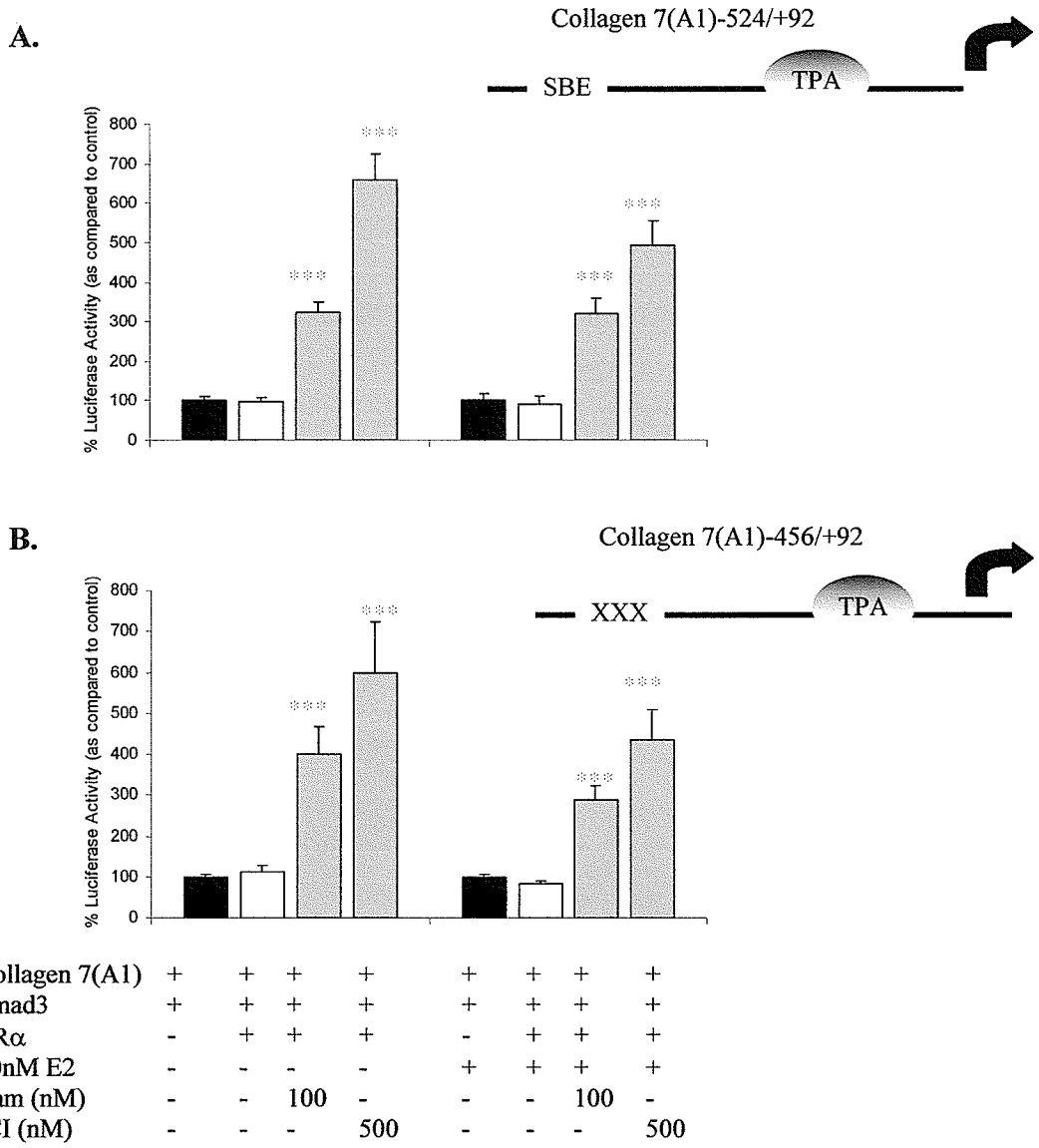


Figure 33: p3TP-lux activity in MCF-7 cells transiently transfected with ERα in the presence of antiestrogens. MCF-7 cells were transiently transfected with 5ug p3TP-lux, 5ug pCMV5B-Flag Smad3, 1ug pRL-tk-luc and 5ug pcDNA3.1 ERα. Empty pcDNA3.1 plasmid was used to ensure equimolar concentrations of plasmid between samples. 24 h post-transfection, cells were subjected to a 3 minute glycerol shock and new media containing vehicle, 10nM E_2, 10nM E_2 + 100nM 4OH-tamoxifen (Tam) or 10nM E_2 + 500nM ICI 182,780 (ICI) was added. Samples were collected 48 h post-transfection and dual luciferase assays were performed as described in Methods. Results represent the mean ±SEM, n=3 independent experiments. *$p<0.05$ (Student's t-test) as compared to samples not transfected with ERα. + indicates significant differences ($p<0.05$, Student's t-test) as compared to samples transfected with ERα in the presence or absence of E_2, respectively.

The effect of over-expressing ERα in MCF-7 cells on the collagen 7(A1) promoter was also tested. Over-expression of Smad3 in MCF-7 cells resulted in a 2-fold increase in collagen 7(A1) -524/+92 transcriptional activity ($p<0.001$) (data not shown). In contrast to the p3TP-lux promoter, over-expression of ERα in MCF-7 cells did not affect promoter activity in the presence or absence of 10nM E_2 (Figure 34a). However,

tamoxifen ($p<0.001$) and ICI 182,780 ($p<0.001$) up-regulated luciferase activity (Figure 34a), suggesting that ERα may affect collagen 7(A1) transcription through antiestrogens. The collagen 7(A1) -456/+92 luciferase plasmid contains a segment of the collagen 7(A1) promoter but lacks the SBE and thus, lacks TGFβ responsiveness (Figure 34b) (413). Therefore, over-expression of Smad3 in MCF-7 transient transfections did not affect promoter activity. However, in the presence of antiestrogens, there was a significant increase in luciferase activity ($p<0.001$) while E_2 had no effect (Figure 34b). This response to antiestrogens mimicked that of the full length promoter sequence in which the SBE was present. Indeed, upon comparing the response to antiestrogens between these two constructs, no significant differences were observed (two-way ANOVA), suggesting that the SBE does not play a significant role in the increase of transcriptional activity by antiestrogens.

The TGFβ signaling pathway is mediated by Smad2 and Smad3, which are phosphorylated by TβRI upon ligand binding, and by Smad4, which heterodimerizes with Smad2/3 (305). It has been suggested that antiestrogen treatment increases the phosphorylation of Smad2 and down-regulates Smad2 protein expression levels in MCF-7 cells (429). Therefore, we wanted to establish whether altered endogenous Smad3 expression is involved in the antiestrogen up-regulation of TGFβ responsive genes. MCF-7 cells were grown under estrogen deplete conditions for 4 days and treated with E_2 in the presence or absence of 100nM 4OH-tamoxifen or 500nM ICI 182,780 for various times. PCR and western blot analysis suggest that Smad3 mRNA (Figure 35) and protein expression levels do not change upon estrogen or antiestrogen treatment (results not shown).



Collagen 7(A1)	+	+	+	+	+	+	+	+
Smad3	+	+	+	+	+	+	+	+
ERα	-	+	+	+	-	+	+	+
10nM E2	-	-	-	-	+	+	+	+
Tam (nM)	-	-	100	-	-	-	100	-
ICI (nM)	-	-	-	500	-	-	-	500

Figure 34: Collagen 7(A1) activity in MCF-7 cells. MCF-7 cells were transiently transfected with 2ug collagen 7(A1)-524/+92 (A), 2ug collagen 7(A1)-456/+92 (B), 3ug pCMV5B-Flag Smad3, 1ug pRL-tk-luc and 5ug pcDNA3.1 ERα. Empty pcDNA3.1 plasmid was used to ensure equimolar concentrations of plasmid between samples. 24 h post-transfection, cells were subjected to a 3 minute glycerol shock and new media containing vehicle, 10nM E_2, 10nM E_2 + 100nM 4OH-tamoxifen (Tam) or 10nM E_2 + 500nM ICI 182,780 (ICI) was added. Samples were collected 48 h post-transfection and dual luciferase assays were performed as described in Methods. Results represent the mean ±SEM, n=6 (A) or n=5 (B) independent experiments. *** indicates significant differences ($p<0.001$, Student's t-test) as compared to control samples.

To ensure that this observation was not limited to one source of MCF-7 cells, we also tested the T5 human breast cancer cell line. Similar to that observed in MCF-7 cells, E_2 and antiestrogen treatment did not affect Smad3 mRNA or protein expression (results not shown). However, these results do not exclude the involvement of Smad2 expression in the antiestrogen up-regulation of TGFβ responsive genes as Smad2 levels were not determined.

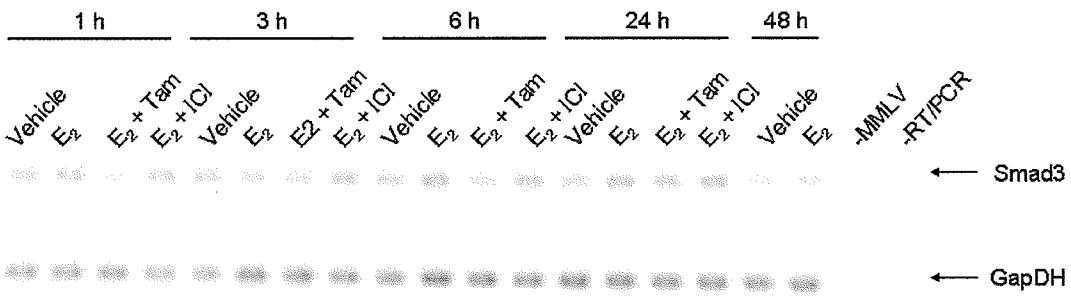
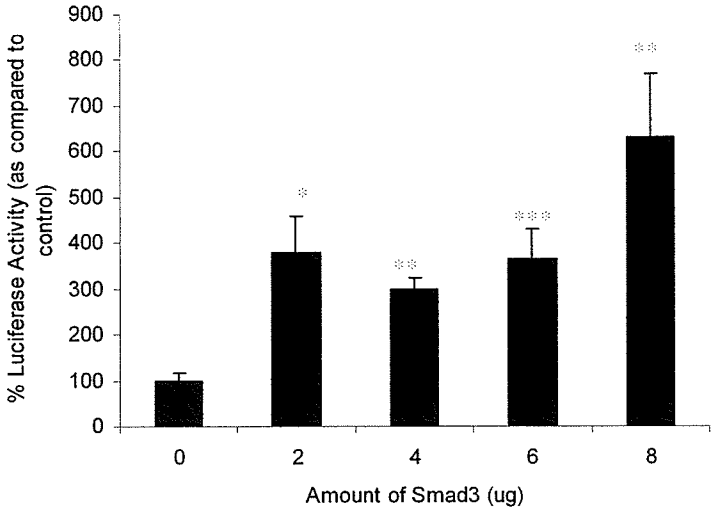


Figure 35: Smad3 mRNA expression in MCF-7 cells. MCF-7 cells were set-up in 6 well plates at $5x10^4$cells/well in CS5%. Four days later, cells were exposed to vehicle, 10nM E_2, 10nM E_2 + 100nM 4OH-tamoxifen or 10nM E_2 + 500nM ICI 182,780 over 1, 3, 6, 24 or 48 hours (h). Cells were harvested and total RNA was extracted, reverse transcribed and PCR amplified using Smad3 and GapDH primer pairs as described in Methods. PCR products were run out on a 1% agarose gel and visualized with the Chemidoc system. Representative gel of n = 3 independent experiments.

To further investigate the effect of increased ER expression in human breast cancer cells on TGFβ/Smad3 transcriptional activity, we have stably expressed tetracycline-inducible (TET-ON) human ERα and $ER\beta_1$ in MCF-7 human breast cancer cells. The generation and characterization of MCF-7 cells stably expressing rTA (reverse tetracycline transactivator, clone 89rTA), the doxycycline-inducible GFP tagged ERα (clone 11) and His/Xpress epitope tagged $ER\beta_1$ (clone 47) have been previously described (399, 424). Maximal induction of ERα and $ER\beta_1$ was achieved by treating the cells with 2ug/mL doxycycline for 48 h, as determined by western blot (data not shown). Ligand binding assays also suggest that the level of ERα and $ER\beta_1$ induction by doxycycline is similar (424). As expected, over-expression of Smad3 in the ERα and $ER\beta_1$ expressing clones resulted in a significant increase ($p<0.05$) in p3TP-lux activity (Figure 36a+b). Transient transfection experiments with p3TP-lux and Smad3 in the $ER\beta_1$ over-expressing cells show that 4OH-tamoxifen ($p<0.005$) and ICI 182,780 ($p<0.01$) significantly increased p3TP-lux activity in samples treated with doxycycline as compared to samples treated with vehicle alone in the absence of estradiol (Figure 37a). No significant effect of antiestrogens in the absence of over-expressed $ER\beta_1$ (i.e. no doxycycline) was observed under estrogen deplete conditions. Estrogen treatment resulted in a significant decrease ($p<0.05$) in p3TP-lux activity in the doxycycline treated samples (compared to vehicle control) and this effect was inhibited by co-treatment with 4OH-tamoxifen ($p<0.005$) or ICI 182,780 ($p<0.01$) (Figure 37a). In the absence of doxycycline, estradiol had no effect on Smad3 transcriptional activity. However, antiestrogen treatment significantly ($p<0.05$) increased p3TP-lux activation in the presence of estradiol but in the absence of doxycycline, albeit to a lesser extent than

A.



B.

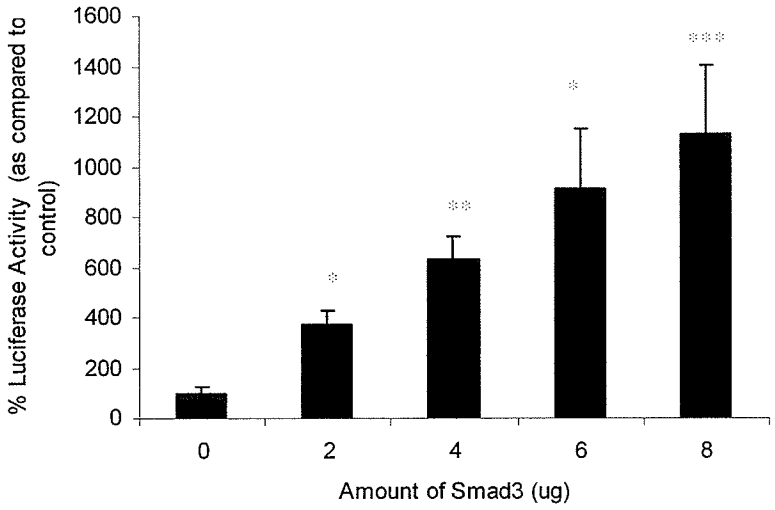
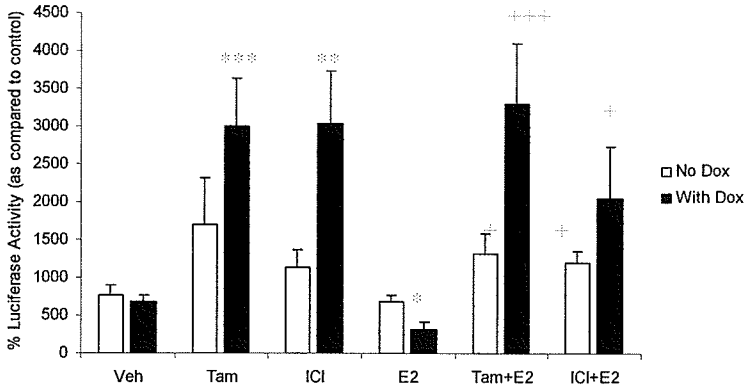


Figure 36. Effect of Smad3 on p3TP-lux activity in ERα and ERβ$_1$ inducible MCF-7 cells. MCF-7 cells over-expressing (A) ERα and (B) ERβ$_1$ were transiently transfected with 5ug p3TP-lux, 1ug pRL-tk-luc and increasing amounts (2-8ug) of pcDNA3.1-Flag-Smad3. Empty pcDNA3.1 plasmid was used to ensure equimolar concentrations of plasmid between samples. 24 h post-transfection, cells were subjected to a 5 minute glycerol shock and new media was added. Samples were collected 48 h post-transfection. Dual luciferase assays of ERα and ERβ$_1$ over-expressing MCF-7 cells were performed as described in Methods. Values represent the percent luciferase activity with 0ug Smad3 set as 100%. Results are expressed as the mean ±SEM, n=4 independent experiments. *$p<0.05$, **$p<0.01$ and ***$p<0.005$ Student's t-test (compared to 0ng Smad3).

that observed in the presence of doxycycline (Figure 37a). In comparing the relative change in luciferase activity between samples treated with doxycycline and those not treated (i.e. plus doxycycline/no doxycycline), there was a significant increase in p3TP-lux activity in samples treated with tamoxifen ($p<0.05$) or ICI 182,780 ($p<0.05$) as compared to vehicle control (Figure 37b), suggesting that ERβ_1 over-expression resulted in an increase in Smad3 mediated transcription in the presence of antiestrogens. A similar effect was also observed in the presence of estradiol (Figure 37b). Western blot analysis of whole cell extracts demonstrate that the ERβ_1 transgene was induced under our conditions and that similar levels of expression were obtained between experimental replicates (Figure 38).

A.



B.

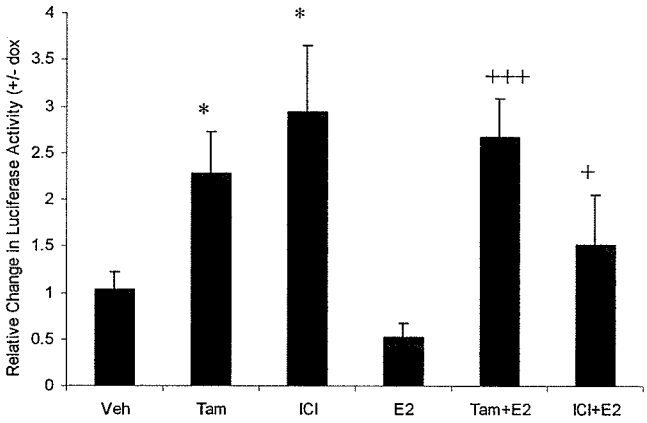


Figure 37: p3TP-lux activity in ERβ$_1$ inducible MCF-7 cells in the presence or absence of doxycycline. ERβ$_1$ over-expressing MCF-7 cells were transiently transfected with 5ug p3TP-lux, 2ug pcDNA3.1-Flag Smad3 and 1ug pRL-tk-luc in the presence or absence of doxycycline. Empty pcDNA3.1 plasmid was used to ensure equimolar concentrations of plasmid between samples. 24 h post-transfection, cells were subjected to 5 min glycerol shock and new media containing vehicle (Veh), 100nM 4OH-tamoxifen (Tam), 500nM ICI 182,780, 10nM E_2 (E2), 10nM E_2 + 100nM 4OH-tamoxifen (Tam+E2) or 10nM E_2 + 500nM ICI 182,780 (ICI+E2) was added. Samples were collected 48 h post-transfection. Dual luciferase assays were performed as described in Methods. A) ERβ$_1$ over-expressing MCF-7 cells treated with or without doxycycline for 48 h. Values represent the percent luciferase activity with cells not transfected with Smad3 set as 100%. B) Relative change in luciferase activity between those samples treated with doxycycline and those not treated with doxycycline (+/- dox). Data represents the mean ±SEM, n=6 independent experiments. *$p<0.05$, **$p<0.01$ and ***$p<0.005$ indicates significant differences (Student's t-test) as compared to vehicle control samples. $^{+}p<0.05$ and $^{+++}p<0.005$ indicates significant differences (Student's t-test) as compared to E2 treated samples.

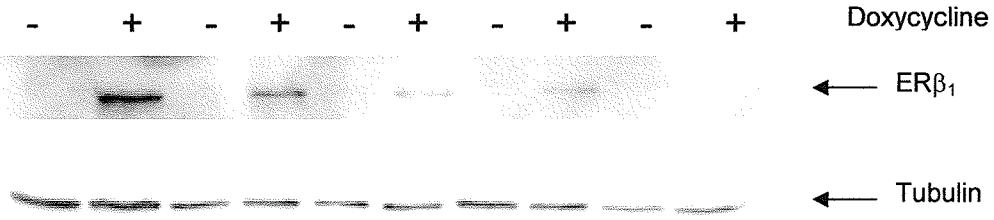
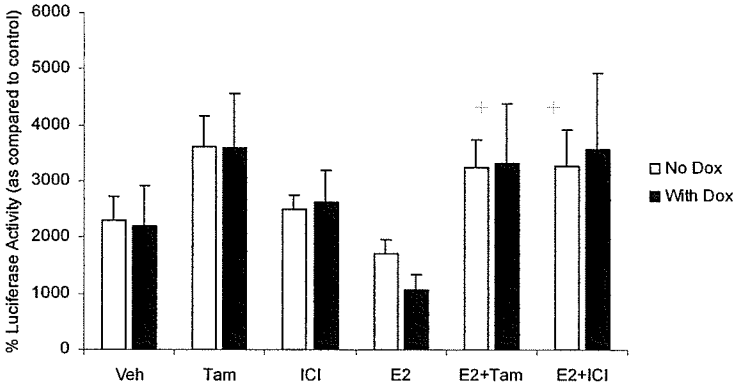


Figure 38: Western blot of ERβ1 in doxycycline inducible MCF-7 cells. ERβ1 over- expressing MCF-7 cells were treated without or with doxycycline for 24 h. Cells were subjected to a 5 min glycerol shock and new media containing vehicle or doxycycline was added. 24 h later, whole cell extracts were prepared as described in Methods. 22ul from a 300ul extract was run on an SDS-10% acrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-xpress antibody (Invitrogen) or an anti-tubulin antibody (Neomarkers). (+) samples treated with doxycycline for 48 h in n=5 independent experiments; (-) samples not treated with doxycycline for n=5 independent experiments.

In the ERα over-expressing clones however, there was only a significant increase in p3TP-lux activity in the absence of doxycycline with antiestrogen treatment in the presence of estradiol (Figure 39a). No significant change in luciferase activity between samples treated with doxycycline was observed either in the presence or absence of ligand (Figure 39a). When comparing the relative change in Smad3 mediated p3TP-lux activation, there was no significant difference between those samples over-expressing ERα and those that did not, suggesting that ERα had little effect on Smad3 mediated activation under these conditions (Figure 39b).

A.



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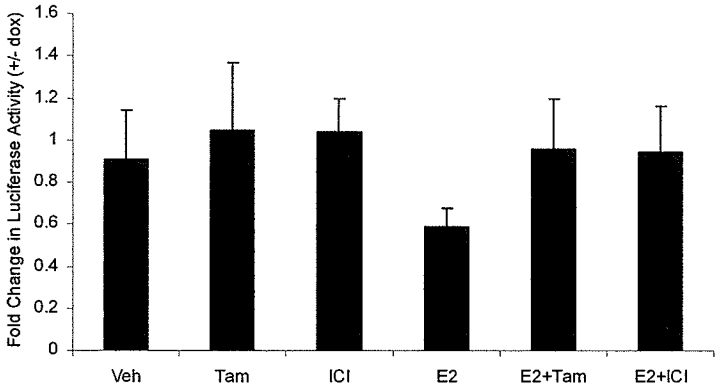


Figure 39: p3TP-lux activity in the ERα inducible MCF-7 cells. MCF-7 cells over-expressing ERα were transiently transfected with 5ug p3TP-lux, 4ug pcDNA3.1-Flag Smad3 and 1ug pRL-tk-luc in the presence or absence of doxycycline. Empty pcDNA3.1 plasmid was used to ensure equimolar concentrations of plasmid between samples. 24 h post-transfection, cells were glycerol shocked and new media containing vehicle (Veh), 10nM E_2 (E2), 100nM 4OH-tamoxifen (Tam), 100nM 4OH-tamoxifen + 10nM E_2 (Tam+E2), 500nM ICI 182,780 (ICI) or 500nM ICI 182,780 + 10nM E_2 (ICI+E2) was added. Samples were collected 48 h post-transfection and dual luciferase assays were performed as described in Methods. A) ERα over-expressing MCF-7 cells treated with or without doxycycline for 48 h. Values represent the percent luciferase activity with cells not transfected with Smad3 set as 100%. B) Relative change in luciferase activity between those samples treated with doxycycline and those not treated with doxycycline (+/- dox). Data represents the mean ±SEM, n=6 independent experiments. $^{+}$ $p<0.05$, indicates significant differences (Student's t-test) compared to samples treated with E2 only in the absence of doxycycline.

Western blot analysis of whole cell extracts show that the level of ERα protein expression varies widely between experiments and the ratio between endogenous ERα and that of the transgene also is altered between replicate samples (Figure 40), even though ligand binding assays demonstrate a significant increase in total ERα expression (382). Thus, the interpretation of these results is difficult.

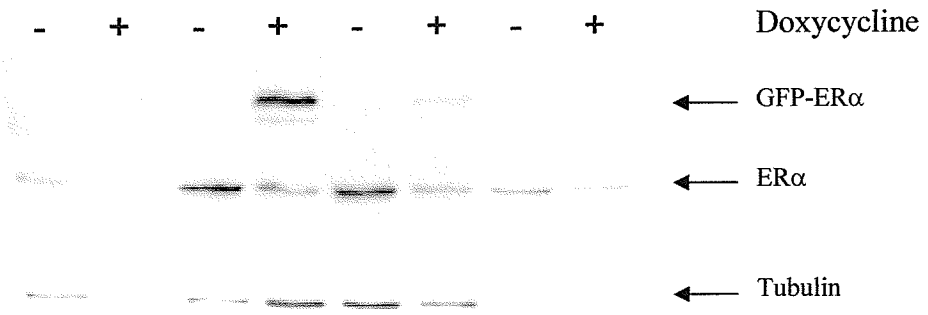


Figure 40: Western blot of ERα in doxycycline inducible MCF-7 cells. ERα over-expressing MCF-7 cells were treated without or with doxycycline for 24 h. Cells were glycerol shocked for 5 min and new media containing vehicle or doxycycline was added. 24 h later, whole cell extracts were prepared as described in Methods. 22ul of a 300ul extract was run on a SDS-10% acrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-ERα antibody (NovoCastra) or an anti-tubulin antibody (Neomarkers), n=4 independent experiments. (+) samples treated with doxycycline for 48 h; (-) samples not treated with doxycycline.

Key Findings from Section I

In Section I of the results for this thesis, we have characterized the functional interaction between Smad3, a downstream signaling protein of the TGFβ signal transduction pathway, and ER in Cos1 and MCF-7 cells. Table 4 summarizes results obtained. In addition, we have also demonstrated:

1. Smad3 has little if any effect on ERα nor $ER\beta_1$ transcriptional activity on an ERE containing reporter plasmid, despite a significant increase in ER transcriptional activity in the presence of estrogen in Cos1 cells.
2. Smad3 does not affect ERα transcriptional activity on the $TGF\beta_3$-luc reporter plasmid (a plasmid that models the non-classical mechanism of ER gene regulation), despite a significant increase in ligand-induced reporter plasmid activity in Cos1 cells.
3. Total Smad3 mRNA and protein expression are not altered upon estrogen or antiestrogen treatment in MCF-7 cells.

Having characterized the functional interaction between ER and Smad3, we next sought to examine the molecular mechanism by which ER modulates Smad3 transcriptional activity in section II of the results.

p3TP-lux Reporter Plasmid		
Expression Plasmid	**Cos1**	**MCF-7**
Smad3	↑ Smad3 overexpression ↑ Smad3 + Ap1	↑ Smad3 overexpression
ERα	ERα alone – no effect	---
Smad3 + ERα	↓ + E_2 ↑ + Antiestrogens (inhibited effect of E_2-ERα)	↓ Ligand-independent (ERα overexpression) ↑ + Antiestrogens (inhibited effect of ERα overexpression)
ERβ$_1$	ERβ$_1$ alone – no effect	---
Smad3 + ERβ$_1$	↓ + E_2 ↑ + Antiestrogens (reversed effect of E_2-ERβ$_1$)	---
ERβ$_2$/ERβ$_5$	No effect	---

Collagen 7(A1)-524/+92 Reporter Plasmid		
Expression Plasmid	**Cos1**	**MCF-7**
Smad3	↑ Smad3 overexpression ↓ Smad3 + Ap1	---
ERα	ERα alone – no effect	---
Smad3 + ERα	↑Ligand-independent ↓ + Antiestrogens (inhibited effect of E_2-ERα)	No effect ↑ + Antiestrogens (independent of SBE)
ERβ$_1$	---	---
Smad3 + ERβ$_1$	↑ + E_2 ↓ + Antiestrogens (inhibited effect of E_2-ERβ$_1$)	---

Table 4: Effects of ERα and ERβ on Smad3 transcriptional activity in Cos1 and MCF-7 cells (summary).

SECTION II. MECHANISM OF CROSS-TALK BETWEEN ER AND TGFβ

A. Direct Interaction between ER and Smad3

Rationale

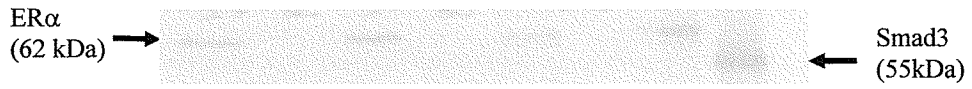
From section I of our results, it is evident that while Smad3 does not affect ER transcriptional activity, ERα and ERβ clearly influence Smad3 transcriptional activity in Cos1 cells. Thus, we next wanted to determine the molecular mechanism(s) by which ER modulates Smad3 transcriptional activity. One possible mechanism is through direct protein:protein interactions between ER and Smad3. Interactions between other steroid receptors, including VDR, AR and GR, and the TGFβ signaling pathway have been demonstrated by other laboratories (320, 322, 396). Thus, we wanted to confirm the observation that a direct interaction between ER and Smad3 occurs in mammalian cells and co-immunoprecipitation (co-IP) experiments were performed both *in vitro* and *in situ*.

Results

To determine whether ER could directly bind to Smad3 *in vitro*, ^{35}S radiolabeled ER and Smad3 were generated by *in vitro* transcription/translation using the TnT® Coupled Reticulocyte Lysate System (Promega) as described in Methods. Proteins were mixed on ice in the presence or absence of 10nM E_2 for 24 h and immunoprecipitated (IP) with antibodies to either ERα, His (recognizes the histidine residues on the tagged ER) or β-galactosidase. The β-galactosidase antibody served as a negative control for the IP as it is an isotype matched antibody to that of ERα (mouse monoclonal IgG). IPs with a Smad3 specific antibody were not performed *in vitro* as this antibody was raised in rabbits and the reticulocyte lysate in which the proteins were produced is also rabbit. IP

products were analyzed by electrophoresis on a 10% SDS-PAGE gel and visualized by autoradiography. No detectable ERα signal was observed when IP was undertaken using the β-galactosidase antibody (Figure 41a, lane 6). However, i*n vitro* translated ERα was detected after IP with either an ERα specific antibody (Figure 41a, lane 5) or with the His antibody (Figure 41a, lanes 1 and 3), which recognizes the His tag on the His/Xpress-ERα. These data provide evidence for the successful IP of *in vitro* translated ERα from solution. However, under these IP conditions, no band corresponding to Smad3 was detected suggesting that Smad3 did not co-IP with ERα either in the presence or absence of estradiol, in spite of Smad3 successfully being *in vitro* translated (Figure 41a, lane 8). Similar results were obtained in attempting to co-IP Smad3 with $ER\beta_1$ and $ER\beta_5$ (Figure 41b). A non-specific band corresponding to Smad3 was found both in the specific and non-specific IP lanes at similar intensities (Figure 41b, lanes 2 and 4), suggesting that Smad3 was not specifically co-IP with $ER\beta_5$ or $ER\beta_1$ under these conditions.

A.



Lane	1	2	3	4	5	6	7	8
ERα	-	-	-	-	+	+	-	-
His-ERα	+	+	+	+	-	-	+	-
Smad3	+	+	+	+	+	+	-	+
10nM E_2	+	+	-	-	+	+	-	-
IP	His	Gal	His	Gal	ERα	Gal	-	-

B.

Lane	1	2	3	4	5	6	7	8
His-ERβ5	+	+	-	-	-	-	-	-
His-ERβ1	-	-	+	+	+	-	-	-
ERα	-	-	-	-	-	+	+	+
Smad3	+	+	+	+	-	+	+	-
10nM E_2	+	+	+	+	-	+	+	-
IP	His	Gal	His	Gal	-	Gal	ER	-

Figure 41: Lack of co-immunoprecipitation of *in vitro* transcribed/translated ER and Smad3 in the presence or absence of 10nM E_2. ER and Smad3 were *in vitro* transcribed/translated as described in Methods. Samples were mixed in the presence or absence of ligand for 24 h and subsequently IP with either His, ERα or β-galactosidase (Gal) antibodies. Samples were run out on a 10% SDS-PAGE gel, dried and visualized by autoradiography. A) *In vitro* transcribed/translated His/Xpress tagged ERα or untagged ERα IP. Lanes 7 and 8 represent the presence of i*n vitro* transcribed/translated His/Xpress tagged ERα or Smad3 before IP, respectively. B) *In vitro* translated His/Xpress tagged $ERβ_5$ and $ERβ_1$ IP. Lanes 5 and 8 represent the presence of *in vitro* transcribed/translated $ERβ_1$ or ERα before IP, respectively. Arrows indicate the molecular mass (kDa) of the bands of interest as determined by a molecular mass standard run in parallel with samples. Results are representative of n=3 independent experiments.

Several attempts were also made to determine if the ER/Smad3 interaction may be observed *in situ* in Cos1 cells. Cells were transiently transfected with ER and Smad3 in the presence or absence of 10nM E_2. Cell lysates were prepared 48 h later and IP for ERα as described in Methods. Smad3 protein is absent upon immunoblot of ERα IP

samples (Figure 42a) while the presence of ERα protein can be detected in ERα transfected, IP cells (Figure 42b). A band corresponding to the molecular mass of Smad3 (55 kDa) was found in untransfected (mock) Cos1 cell samples (Figure 42a), suggesting that this band is not indicative of successful Smad3 co-IP with ER. Similar results were obtained when Smad3 was used to IP and membranes were immunoblotted for ERα (results not shown). Therefore, results from these experiments suggest either that direct binding of ER and Smad3 does not occur *in situ* in Cos1 cells or that the interaction could not be detected using the IP methodology. To ensure that our methodology would successfully co-IP two proteins, we performed the same experiments as outlined above using ERα and $ER\beta_1$. The heterodimerization of these two proteins has been previously demonstrated (82). Similar to results previously published, we were able to successfully co-IP ERα and $ER\beta_1$ in Cos1 cells transiently transfected with ER in the presence of estradiol, suggesting that IP of two proteins under these conditions is possible (Figure 43).

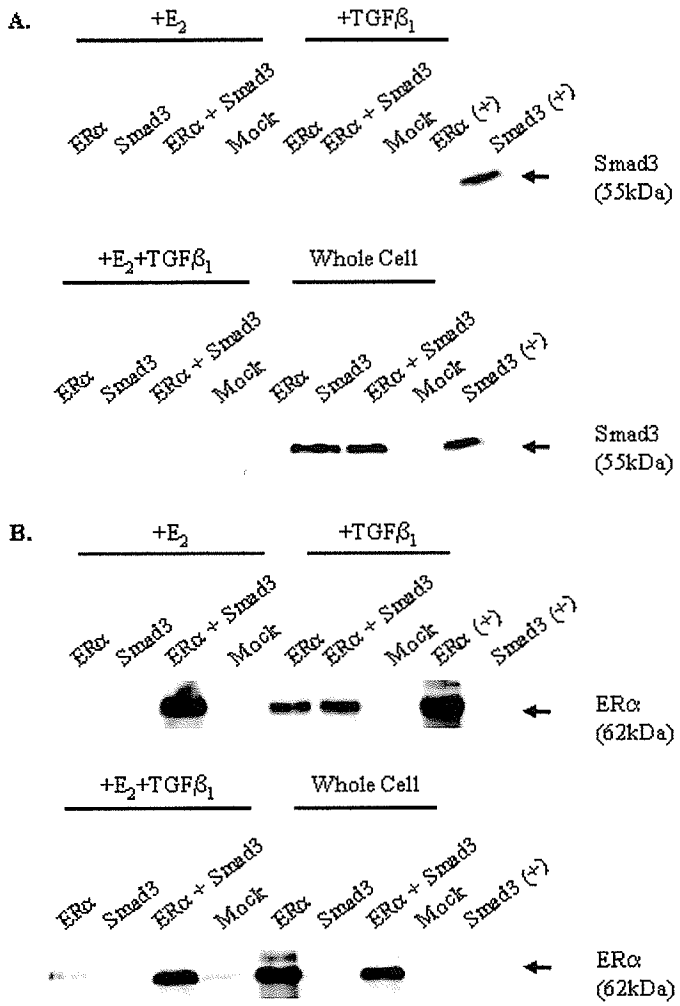


Figure 42: **Lack of co-immunoprecipitation of ERα and Smad3 in Cos1 cells.** Cos1 cells were set-up in 100mm dishes and transiently transfected with 1ug pcDNA3.1-ERα and 1ug pCMV5B-Flag-Smad3 in the presence of 10nM E_2 or 10ng/ml $TGF\beta_1$ as described in Methods. Mock transfected cells were used as negative control. 48 h later, samples were harvested and IP with 3ug mouse monoclonal ERα antibody (NovoCastra). Samples were analyzed by 10% SDS-PAGE and western blotting. (A) Membranes IB for Smad3 (Upstate Biotechnology). Arrows indicate the expected molecular mass of Smad3 (55 kDa). (B) Membranes from A were stripped and subsequently IB for ERα (NovoCastra). Arrows indicate the expected molecular mass of transfected ERα (62kDa). Whole cell is the whole cell lysate proteins present in the sample prior to IP (22ul of a 300ul sample), representing 18% of the IP. Results are representative of n=2 independent experiments.

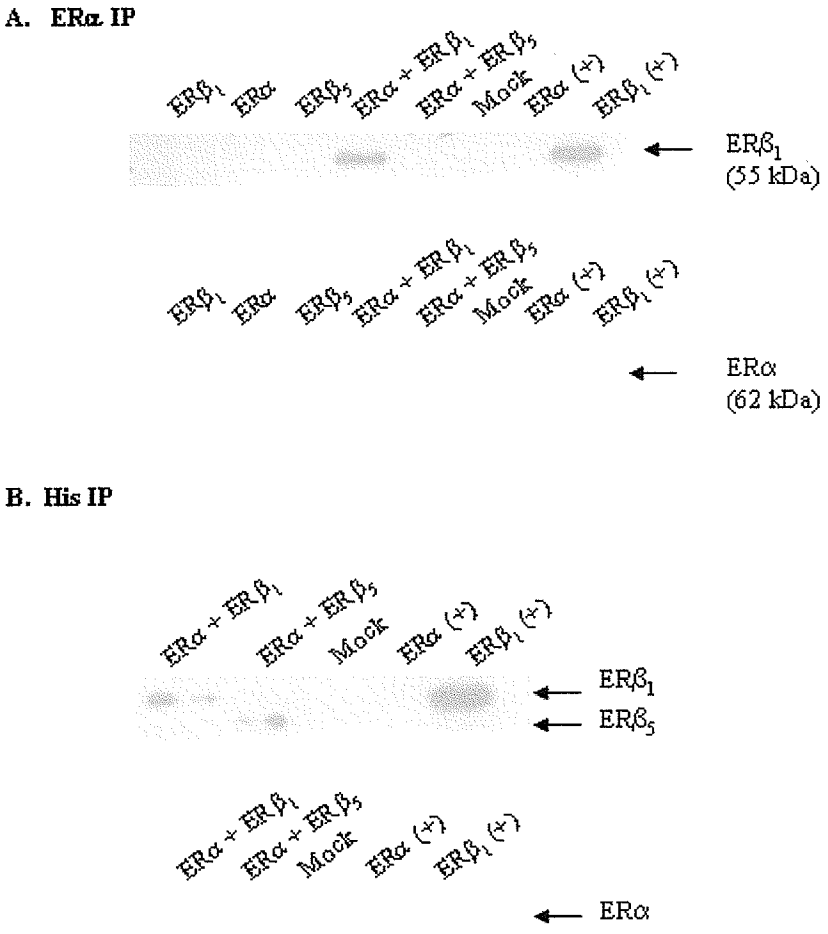


Figure 43: Co-immunoprecipitation of ERα and ERβ$_1$ in Cos1 cells. Cos1 cells were transiently transfected with 1ug pcDNA3.1-ERα, 1ug pcDNA4-His/Xpress ERβ$_1$ or 1ug pcDNA4-His/Xpress ERβ$_5$ as described in Methods. Cells were harvested 48 h later and IP with A) 1ug mouse monoclonal ERα (NovoCastra) or B) 1ug mouse monoclonal His G (Invitrogen). Samples were analyzed by a 10% SDS-PAGE and western blotting. Membranes were immunoblotted for Xpress (Invitrogen), of which recognizes the Xpress tag on ERβ protein, or immunoblotted for ERα (NovoCastra). Results are representative of n=3 independent experiments.

Further experiments within our laboratory have validated the use of cross-linking agents in the determination of protein-protein interactions. DSP (dithiobis[succinimidylpropionate]), is a homobifunctional N-hydroxysuccimide ester that reversibly conjugates proteins through their amino groups (430). Several other laboratories have used this compound in the identification of protein-protein interactions (431, 432) and recent results from our laboratory suggest that the interaction between ERα and $ER\beta_1$ with the ER coactivator, AIB1, can only be detected using this cross-linking agent. Thus, with the technical assistance of Kanyarat Ung, a technician in Dr. Murphy's laboratory, cross-linking experiments using DSP were conducted to determine whether an interaction between ER and Smad3 could be observed in Cos1 cells. Cos1 cells were transiently transfected with ER and Smad3 in the presence or absence of E_2. Forty-eight hours post-transfection, cells were cross-linked with DSP as described in Methods and IPs with ERα and a non-specific antibody of the same class (insulin receptor) were performed. While a band corresponding to the molecular mass of Smad3 was observed upon IP with an ERα specific antibody, a band of similar intensity and mass was also observed with our non-specific antibody (Figure 44). We concluded that Smad3 was being IP from solution in a non-specific fashion (i.e. Smad3 may be "sticky" in that it may bind non-specifically to the protein G sepharose beads or to the IP antibody), clearly demonstrating the need for appropriate negative controls in an IP experiment. These results provide further evidence supporting the observation that ER either does not directly interact with Smad3 under our conditions or that they interact through a site precluding the IP antibody. Interestingly, while these experiments were being conducted, the physical interaction between Smad3 and ER was described by

others (335). Through a series of co-IPs, Matsdua et. al. (335) demonstrated that Smad3 interacts with ERα and $ER\beta_1$ in 293T and MCF-7 cells and that this interaction was dependent on activation of both the estrogen and TGFβ signaling cascades. Further studies by these authors showed that ERα and $ER\beta_1$ interact with Smads 1, 2, 3 and 4 upon stimulation of the ER and TGFβ pathways (335, 336). Therefore, cell type specific factors may influence the interaction of ERs and Smad3.

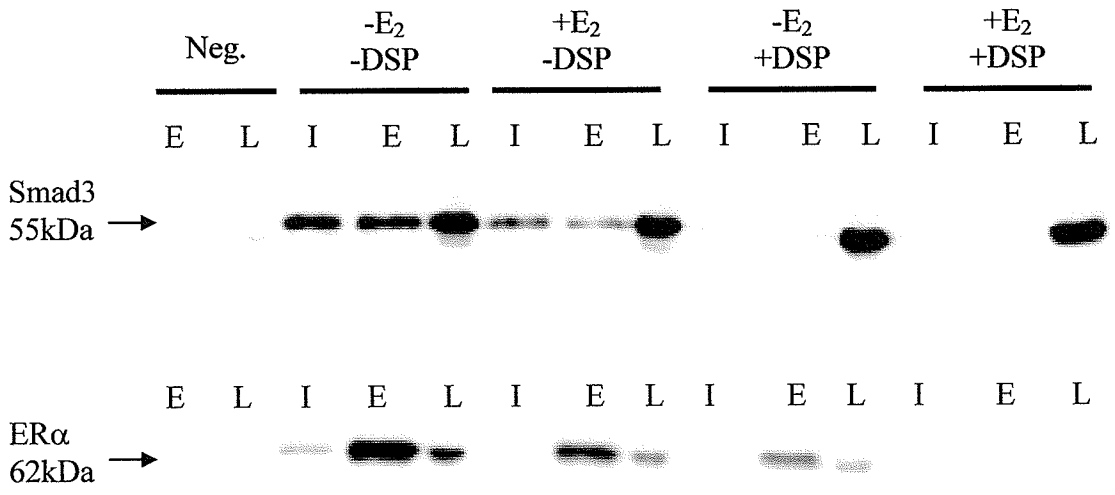


Figure 44: Lack of co-immunoprecipitation of ERα and Smad3 in Cos1 cells using DSP as a cross-linking agent. Cos1 cells were transiently transfected with 1ug pcDNA3.1-ERα and 1ug pCMV5B-Flag-Smad3 in the presence or absence of 10nM E_2 as described in Methods. Mock transfected cells (Neg.) were used as negative control. 48 h post-transfection, proteins were cross-linked with DSP for 2 h, harvested in IP buffer and subsequently IP with either 3ug rabbit polyclonal ERα (Santa Cruz; lanes indicated "E") or 3ug rabbit polyclonal insulin receptor (Santa Cruz; lanes indicated "I"). Samples were analyzed by 10% SDS-PAGE and western blotting. Top panel: membranes IB for Smad3 (Upstate Biotechnology). Arrows indicate the expected molecular mass of Smad3 (55 kDa). Bottom panel: membranes previously IB for Smad3 were stripped as described in Methods and subsequently IB for ERα (NovoCastra). Arrows indicate the expected molecular mass of transfected ERα (62kDa). Whole cell lysates (lanes indicated "L") represent proteins present in the sample prior to IP (30ul of a 300ul sample). Results are representative of n=2 independent experiments.

B. Involvement of Ap-1 Transcription Factors

Rationale

Having disproved the hypothesis that ER modulates TGFβ signaling through direct protein interactions with Smad3, we sought to explore other possible mechanisms through which activated ER may modulate Smad3 transcriptional activity. ERα has been shown to directly bind to and interact with c-Jun but not Fos family members (224, 225, 231, 287) thereby, modulating specific Ap-1 transcriptional responses (225). Stimulation of Ap-1 transcription factors have been shown to increase Smad3 transcriptional activity on the p3TP-lux reporter plasmid but inhibit Smad3 transcriptional activity on the collagen 7(A1)-luc reporter plasmid (397, 412) (Table 4); this modulatory effect is thought to be mediated by Smad3 interacting with the Ap-1 member, c-Jun (433, 434). Therefore, we hypothesized that when Ap-1 transcription factors, specifically c-Jun, are limiting and when ERα is up-regulated and activated there would be an interference with Smad3 transcriptional activity as ERα interacts directly with c-Jun and thereby, altering Ap-1 and TGFβ signaling activity.

Results

To investigate this hypothesis, Cos1 cells were transiently transfected with c-Jun or c-Fos expression vectors. Expression of c-Jun and c-Fos in transiently transfected Cos1 cells is shown in Figure 45. Transient transfection of c-Jun ($p<0.05$) and c-Fos ($p<0.01$) expression plasmids significantly increased Smad3 induced p3TP-lux activity (Figure 46a+b). In the presence of E_2, ERα decreased Smad3 mediated p3TP-lux activity ($p<0.05$) and this effect was reversed by over-expression of c-Jun ($p<0.05$) but not c-Fos (Figure 46a, b). A similar effect was also observed with ERβ (Figure 47a+b), although in

this case the decrease in p3TP-lux transcription in the presence of E_2 was inhibited by both c-Jun ($p<0.001$) and c-Fos ($p<0.001$) transient transfection. The ability of c-Jun to inhibit the effect of activated ERα on Smad3 transcriptional activity was not due to limiting or squelching of other ERα co-activators that can be commonly recruited by ERα or Ap-1 transcription factors as over-expression of SRC-1 or SRC-3 (AIB1) did not affect the ability of ERα to inhibit Smad3 activity (Figure 48). SRC-1 has been shown to form direct protein:protein interactions with ERα (183); interactions between Smad3 and SRC-1 have also been described (although this interaction may not be direct) (320). SRC-3, on the other hand, has been shown to interact directly with ERα (180)but no interactions between Smad3 and TGFβ/Smad3 have been described.

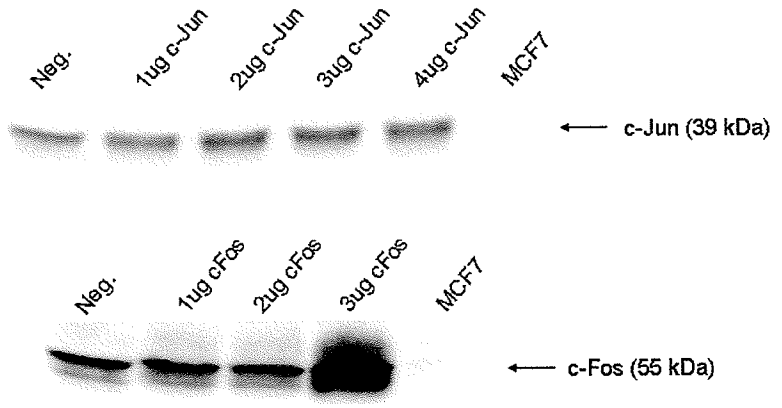
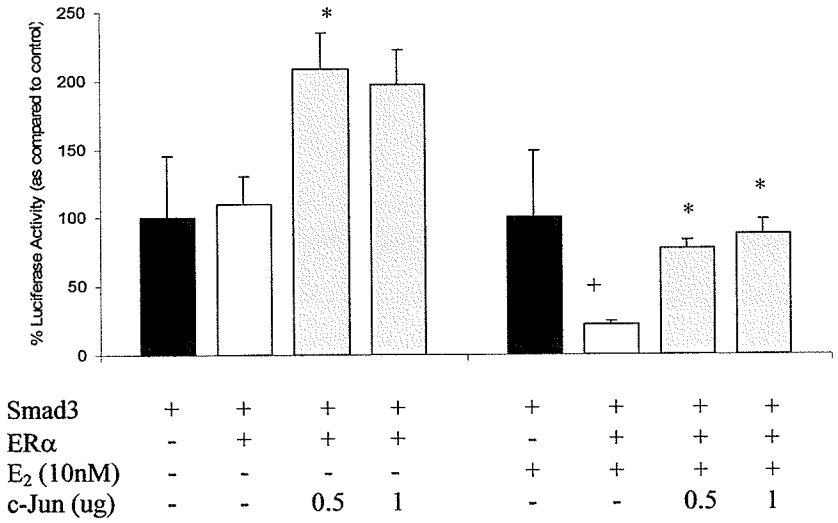


Figure 45: Expression of c-Jun and c-Fos in Cos1 transiently transfected cells. Cos1 cells were transiently transfected with increasing amounts of c-Jun (1- 4ug pRSV c-Jun) or c-Fos (1-3 ug pRSV c-Fos) expression plasmids as described in Methods. Equimolar concentrations of plasmid were kept between samples by addition of empty pcDNA3.1. Neg. represents mock transfected Cos1 cells. Forty-eight h post-transfection, whole cell extracts were prepared as described in Methods. 22ul of a 300ul extract was run on a SDS-10% acrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-c-Jun antibody (Santa Cruz Biotechnology) or an anti-c-Fos antibody (Santa Cruz Biotechnology). Results represent n=1 experiments.

A.



B.

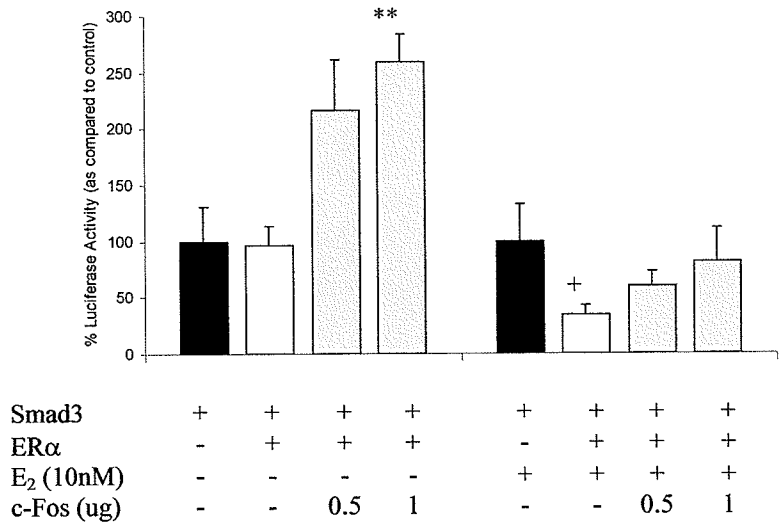
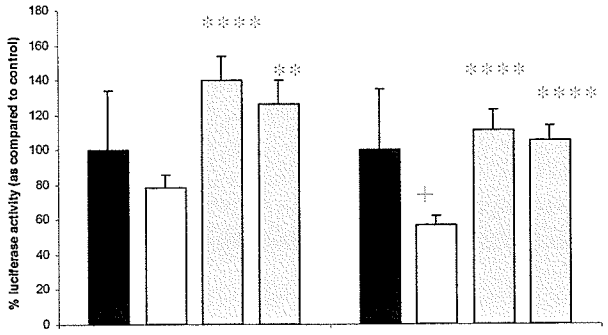


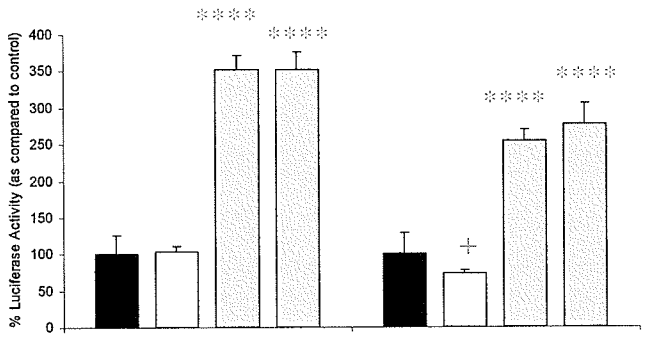
Figure 46: Affect of Ap-1 and ERα on Smad3 transcriptional activity in Cos1 cells. Cos1 cells were transiently transfected with 1ug pcDNA4-His/Xpress ERα, 1ug pCMV5B-Flag-Smad3, 100ng pCH110, 200ng p3TP-lux and 0.5ug or 1ug of either A) pRSV c-Jun or B) pRSV c-Fos in the presence or absence of 10nM E_2. Empty pcDNA3.1 plasmid was used to ensure equimolar concentrations of plasmid between samples. 48 h later, samples were collected and luciferase and β-galactosidase activities were determined as described in Methods. Results represent the mean ±SEM, n=3 independent experiments. * and ** indicate significant differences ($p<0.05$ and $p<0.01$; Student's t-test) as compared to the corresponding sample transfected with ERα and Smad3 in the presence or absence of E_2, respectively. + indicates a significant difference ($p<0.05$) compared to the corresponding sample in the absence of E_2.

A.



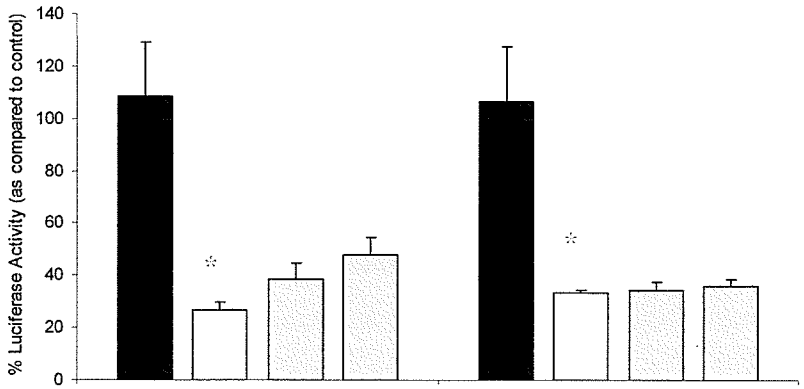
Smad3	+	+	+	+	+	+	+	+
ERβ$_1$	-	+	+	+	-	+	+	+
E$_2$ (10nM)	-	-	-	-	+	+	+	+
c-Jun (ug)	-	-	0.5	1	-	-	0.5	1

B.



Smad3	+	+	+	+	+	+	+	+
ERβ$_1$	-	+	+	+	-	+	+	+
E$_2$ (10nM)	-	-	-	-	+	+	+	+
c-Fos (ug)	-	-	0.5	1	-	-	0.5	1

Figure 47: Affect of Ap-1 and ERβ$_1$ on Smad3 transcriptional activity in Cos1 cells. Cos1 cells were transiently transfected with 1ug pcDNA4-His/Xpress ERβ$_1$, 1ug pCMV5B-Flag-Smad3, 100ng pCH110, 200ng p3TP-lux and increasing amounts (0.5 or 1ug) of either A) pRSV c-Jun or B) pRSV c-Fos in the presence or absence of 10nM E$_2$. Empty pcDNA3.1 plasmid was used to ensure equimolar concentrations of plasmid between samples. 48 h later, samples were collected and luciferase and β-galactosidase activities were determined as described in Methods. Results represent the mean ± SEM n=10 independent experiments. ** and **** indicate significant differences ($p<0.01$ and $p<0.00001$; Student's t-test) as compared to the corresponding sample transfected with ERα and Smad3 in the presence or absence of E$_2$, respectively. + indicates a significant difference ($p<0.05$) compared to the corresponding sample in the absence of E$_2$.



E_2 (10nM)	-	+	+	+	-	+	+	+
Smad3	+	+	+	+	+	+	+	+
ERα	+	+	+	+	+	+	+	+
SRC-1 (ug)	-	-	0.5	1	-	-	-	-
SRC-3 (ug)	-	-	-	-	-	-	0.5	1

Figure 48: Affect of SRC-1 and SRC-3 on Smad3 transcriptional activity in Cos1 cells. Cos1 cells were transiently transfected with 1ug pcDNA4-His/Xpress ERα, 1ug pCMV5B-Flag-Smad3, 100ng pCH110, 200ng p3TP-lux and increasing amounts (0.5 or 1ug) of either SRC-1 or SRC-3 in the presence of 10nM E_2. Empty pcDNA3.1 plasmid was used to ensure equimolar concentrations of plasmid between samples. 48 h later, samples were collected and luciferase and β-galactosidase activities were determined as described in Methods. Results represent the mean ± SEM of n=3 independent experiments. *$p<0.05$ (Student's t-test) as compared to control samples.

The results from our Cos1 transient transfections provide evidence to suggest that c-Jun may be limiting in Smad3 mediated transcription of p3TP-lux in the presence of activated ERα. This premise is on the assumption that upon ligand binding, ERα is able to interact specifically with c-Jun but not c-Fos and thereby, preventing c-Jun binding to Ap-1 sites. Several authors have examined the protein:protein interactions of Ap-1 factors and ER and their results suggest that ERα interacts with c-Jun in a ligand-dependent manner and that this interaction is inhibited by antiestrogen treatment (224). To further support our hypothesis that ER sequesters c-Jun away from the p3TP-lux

promoter, co-IP studies were performed in which Cos1 cells were transiently transfected with ERα and treated either with E_2, the Ap-1 activator TPA, or a combination of E_2 and TPA, as evidence suggests that ERα has a higher binding affinity towards activated c-Jun in MCF-7 cells (287). Our results show that ERα does not form a stable interaction with c-Jun in Cos1 cells, as indicated by the absence of c-Jun protein following IP with an ERα specific antibody (Figure 49). Several attempts were made to optimize the interaction and the conditions of immunoprecipitation, including: (i) various incubation times with E_2 and TPA, (ii) the use of various ERα antibodies which recognize different regions of ER, (iii) the use of various c-Jun antibodies and (iv) in the instance that the ER/c-Jun interaction was weak, formaldehyde or DSP protein-protein cross-linking was used to stabilize potential weak interactions (Figure 49). However, all results obtained were negative in that c-Jun could not be detected despite successful ERα immunoprecipitation (ERα could be detected upon western blotting) (Figure 49). While this was quite disturbing, it does not completely disregard the idea that ERα interacts with c-Jun in a ligand-dependent fashion as the optimal conditions to observe the interaction between ER and c-Jun may not have been achieved, despite our best efforts. Of the authors who observed an interaction between ER and c-Jun, only one described the interaction between endogenously expressed proteins (287), all others utilized either GST pull-down assays (224, 231), over-expressed tagged proteins (224, 287), or *in vitro* transcribed/translated systems (224).

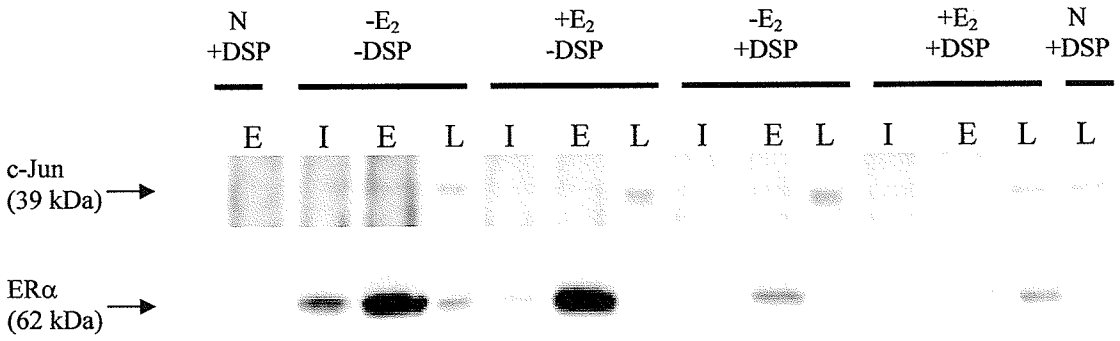


Figure 49: Lack of co-immunoprecipitation of ERα and c-Jun in Cos1 cells using DSP as a cross-linking agent. Cos1 cells were transiently transfected with 1ug pcDNA3.1-ERα in the presence or absence of 10nM E_2 as described in Methods. Mock transfected cells (N) were used as negative control. 48 h post-transfection, proteins were cross-linked with DSP for 2 h, harvested in IP buffer and subsequently IP with 3ug rabbit polyclonal ERα (Santa Cruz; lanes indicated "E") or 3ug rabbit polyclonal insulin receptor (Santa Cruz; lanes indicated "I"). Samples were analyzed by 10% SDS-PAGE and western blotting. Top panel: membranes IB for c-Jun (Santa Cruz). Arrows indicate the expected molecular mass of c-Jun (39kDa). Bottom panel: membranes previously IB for c-Jun were stripped as described in Methods and subsequently IB for ERα (NovoCastra). Arrows indicate the expected molecular mass of transfected ERα (62kDa). Whole cell lysates (L) represent proteins present in the sample prior to IP (30ul of a 300ul sample). Results are representative of n=2 independent experiments.

While the protein-protein interactions between ER and Ap-1 members have been described, whether ER affects Jun DNA binding at Ap-1 sites is unclear. It has been suggested that on the PR promoter, ligand activated ER binds to an ERE half-site immediately adjacent to an Ap-1 site enhancing the binding of Fos and Jun (132). Thus, by forming an active transcription complex with the Fos/Jun heterodimer at an Ap-1 site, ER is capable of modulating Ap-1 gene transcription. The p3TP-lux promoter contains three consecutive Ap-1 sites from the human collagenase promoter that bind Jun and Fos. While p3TP-lux does not contain a consensus ERE binding site, it does contain an ERE

half-site within the PAI-1 promoter region of the plasmid insert approximately 100 bp downstream of the collagenase Ap-1 sites. However, the ability of this ERE half-site to mediate transcription is unknown. Evidence has suggested that the ER interacts with multiprotein complexes containing c-Jun both *in vitro* and *in situ* (224). Thus, it seems reasonable to hypothesize that the ER may sequester c-Jun containing complexes away from the Ap-1 DNA binding site and thereby, inhibit c-Jun transcriptional activity on the p3TP-lux luciferase promoter. Alternatively, binding of ER to complexes containing c-Jun may inhibit the ability of c-Jun to activate target gene transcription either by (i) competitively binding to proteins important in mediating Ap-1 gene transcription by c-Jun or (ii) altering the conformation of c-Jun such that it no longer is able to bind either to DNA or to other factors important for Ap-1 gene transcription. For example, it has previously been shown that c-Jun interacts directly with the general transcription machinery (435, 436) regardless of whether c-Jun is bound to DNA or free in solution as either a homo- or hetero-dimer with Fos (435).

The interaction of Jun with the general transcription machinery has been mapped to the C-terminal region which consists of the basic region and leucine zipper (bZIP region) (435). As the ER also interacts with the C-terminal region of c-Jun (224), the ER may compete with the general transcription machinery for c-Jun binding. On the other hand, ER binding to c-Jun may alter the three dimensional structure of c-Jun such that it no longer is able to interact with the general transcriptional machinery resulting in an inhibition of target gene transcription. To determine whether activation of ER affects Ap-1 binding to its cognate sequence, an oligonucleotide corresponding to the p3TP-lux Ap-1 site was used in EMSAs. Labelled oligonucleotides were incubated with nuclear

extracts from Cos1 cells transiently transfected with ERα in the presence or absence of estradiol as described in Methods. Figure 50 demonstrates the presence of ERα and Smad3 proteins in transiently transfected Cos1 nuclear and whole cell extracts.

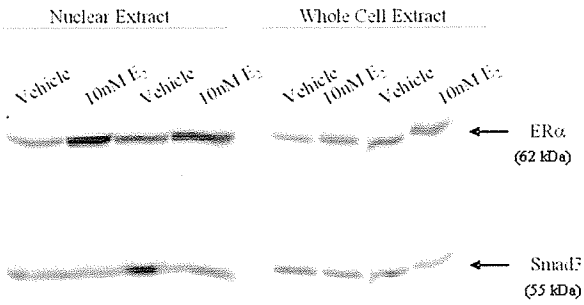


Figure 50: ERα and Smad3 expression in Cos1 nuclear extracts. Cos1 cells were transiently transfected with 1ug pCMV5B-Flag Smad3 and 1ug pcDNA3.1-ERα in the presence or absence of 10nM E_2. 48 h post-transfection, cells were harvested and whole cell or nuclear extracts were prepared as described in Methods. 10ul from a 300ul whole cell extract and 100ug of nuclear extract were run on a 10% SDS-acrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-ERα antibody (NovoCastra) or an anti-Smad3 antibody (Upstate Biotechnology), n=3 independent experiments.

Proteins present in the Cos1 nuclear extract bound to the Ap-1 site in a dose dependent manner (Figure 51). To test for the specificity of the protein-DNA complexes observed, competition studies with 25 – 100 times molar excess of unlabelled oligonucleotide were undertaken (Figure 52). When the excess unlabelled oligonucleotide was added, two DNA-protein complexes diminished in intensity (Figure 52, lanes 7-9), suggesting that these bands correspond to a specific interaction between the Cos1 nuclear extract and probe.

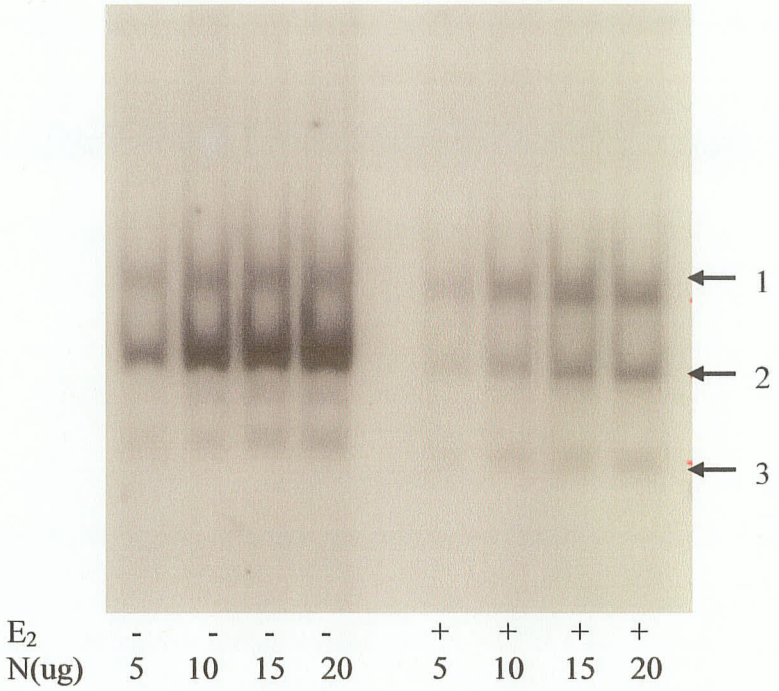
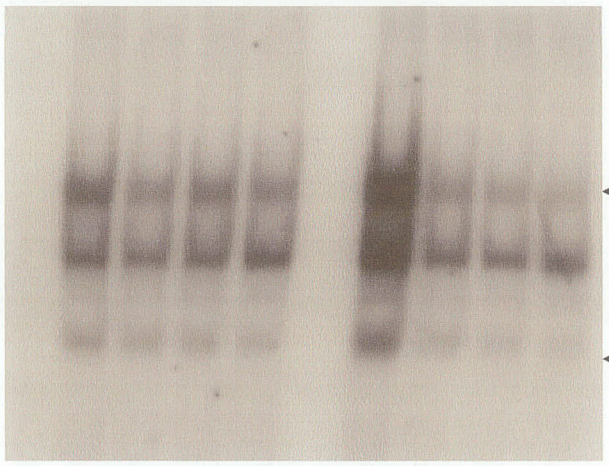


Figure 51: Effect of increasing amounts of nuclear extract on Ap-1 EMSA. Nuclear extracts from Cos1 cells transiently transfected with Smad3 and ERα in the presence or absence of 10nM E_2 were incubated with a [^{32}P] labelled Ap-1 oligonucleotide from p3TP-lux as described in Methods. Increasing amounts of nuclear extract (5-20ug) was used in each instance. E_2 = treatment with 10nM E_2 for 48 h. N(ug) = ug of nuclear extract protein used in assay. Arrows indicate protein-DNA complexes formed.



Lane	1	2	3	4	5	6	7	8	9
Comp (M)	-	-	25	50	100	-	25	50	100

Figure 52: Competition experiments in nuclear EMSA assay. Nuclear extracts from Cos1 cells transiently transfected with Smad3 and ERα in the presence of 10nM E_2 were incubated with a [^{32}P] labelled Ap-1 oligonucleotide from p3TP-lux as described in Methods. 15ug of nuclear extract was used in each instance. Lane 1 = Free probe; Lanes 2-5 = Competitor oligonucleotide added 5 min prior to [^{32}P] labelled oligonucleotide; Lanes 6-9 = Competitor oligonucleotide added at the same time as the [^{32}P] labelled oligonucleotide. Comp (M) = molar excess of competitor oligonucleotide added to reaction. Arrows indicate specific protein-DNA complexes formed.

To determine whether c-Jun binds specifically to the oligonucleotide probe, antibodies raised to various regions of the c-Jun protein by various companies (Upstate Biotechnology, Santa Cruz Biotechnology and NovoCastra Laboratories) were utilized. The presence of c-Jun within the retarded complexes was demonstrated by the ability of specific antibodies to supershift the retarded complexes (Figure 53, lanes 5-9) with a concurrent decrease in band intensity (Figure 53, lanes 5-6). The presence of the supershift band was dependent on the region of c-Jun to which the antibodies were developed as some antibodies did not supershift the complex (Figure 53, lanes 4-6).

These findings demonstrate that c-Jun is part of the DNA-protein complexes formed from Cos1 nuclear extracts.

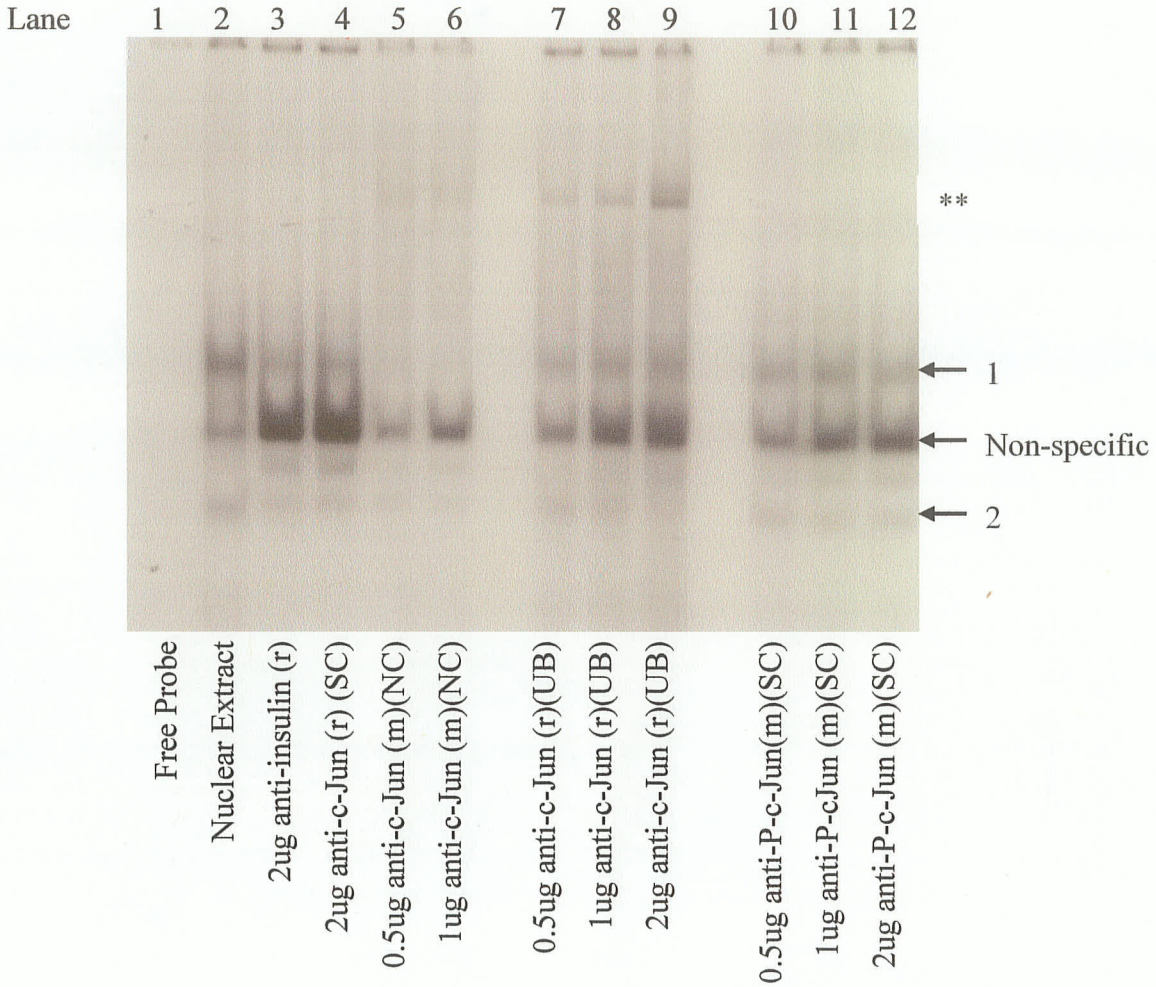
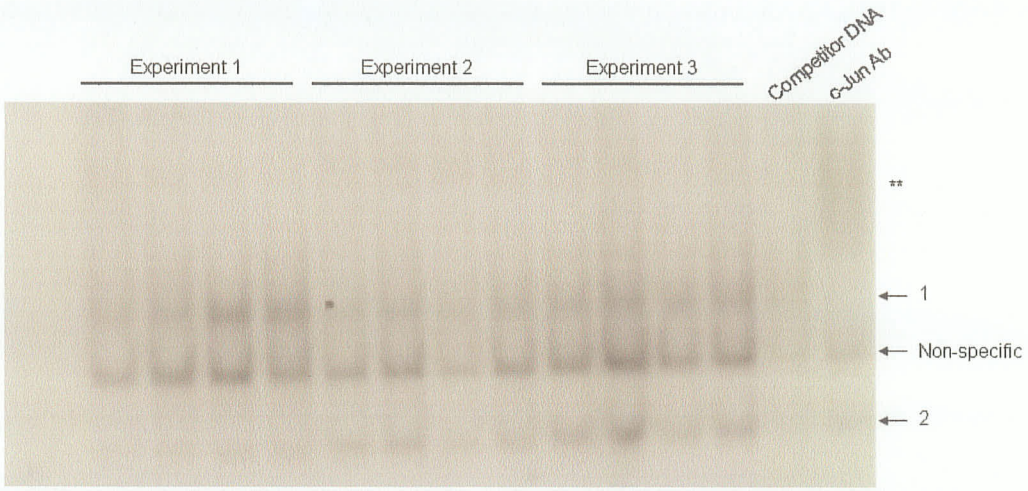


Figure 53: Supershift experiments in nuclear EMSA assay. Nuclear extracts from Cos1 cells transiently transfected with Smad3 and ERα in the presence of 10nM E_2 were incubated with a [^{32}P] labelled Ap-1 oligonucleotide from p3TP-lux as described in Methods. 15ug of nuclear extract was used in each instance and antibodies were added during the pre-incubation step as described in Methods. R = rabbit, m = mouse and p = phosphorylated. Lanes 4 and 10-12 used antibodies from Santa Cruz (SC) Biotechnology, lanes 5-6 were from NovoCastra (NC) and lanes 7-9 were from Upstate Biotechnology (UB). Arrows indicate specific protein-DNA complexes formed. ** indicates presence of supershifted band in the presence of c-Jun antibody.

To determine whether the presence of an activated ER affects c-Jun binding to an Ap-1 site in p3TP-lux, nuclear extracts from Cos1 transient transfections were incubated with the [^{32}P] labelled Ap-1 oligonucleotide as described in Methods. A representative EMSA from three independent assays is shown in Figure 54. Densitometric analysis of DNA-protein specific complexes is shown in Table 5. Comparison of estrogen treated samples to the corresponding control suggests that activated ERα does not affect c-Jun binding to the Ap-1 site found in p3TP-lux (Student's t-test, unpaired). Thus, it does not appear as though ERα sequesters c-Jun away from the promoter as determined by this *in vitro* system. It is important to note, however, that the extraction of proteins from the nucleus may disrupt protein-protein interactions important for DNA binding resulting in a negative result. Furthermore, the oligonucleotide used for these assays consisted of a small fragment of the p3TP-lux plasmid insert and thus, the absence of surrounding promoter sequences may also disrupt protein-DNA binding.



E_2	-	-	-	+	+	-	-	+	+	-	-	+	+	+	+
N(ug)	-	10	15	10	15	10	15	10	15	10	15	10	15	15	15

Figure 54: Effect of activated ERα and Smad3 on Ap-1 EMSA. Nuclear extracts from Cos1 cells transiently transfected with Smad3 and ERα in the presence or absence of 10nM E_2 were incubated with a [^{32}P] labelled Ap1 site from p3TP-lux as described in Methods. 10ug or 15ug of nuclear extract were used in each instance. N(ug) = ug of nuclear extract used in assay. Comp = addition of 100 x molar concentration competitor oligonucleotide. cJun = addition of 1ug cJun antibody (NovoCastra) to assay. Numbers indicate specific protein-DNA complexes formed. ** indicates presence of supershift band in the presence of c-Jun antibody.

10ug NE	**Band 1**	**Non-Specific**	**Band 2**
Control	89.86 ± 20.42	74.81 ± 15.54	94.17 ± 20.39
10nM E2	105.16 ±28.47	72.05 ± 0.35	97.20 ± 16.37
15ug NE			
Control	115.70 ± 31.52	100.32 ± 25.91	106.58 ± 27.04
10nM E2	120.89 ± 18.26	96.55 ± 4.88	111.53 ± 16.30

Table 5: Densitometric analysis of EMSA assays. Nuclear extracts from Cos1 cells transiently transfected with Smad3 and ERα in the presence or absence of 10nM E_2 were incubated with a [^{32}P] labelled Ap1 oligonucleotide from p3TP-lux as described in Methods. 10ug or 15ug of nuclear extract was used in each instance and two independent Cos1 nuclear extracts were prepared. Bands corresponding to specific DNA-protein complexes were quantified by scanning using Quantity One (Bio-rad). Three independent EMSAs were performed. A value of 100 was arbitrarily assigned to one particular sample and all signals are expressed relative to this signal. NE = amount of nuclear extract used in assay.

While ERα does not appear to sequester activated c-Jun away from an Ap-1 oligonucleotide, ER may affect Smad3 transcriptional activity on p3TP-lux by interacting with Ap-1 transcription factors on the oligonucleotide. To determine whether ERα is part of a protein-DNA complex, gel super-shift experiments were performed using antibodies specific for ERα (antibodies recognizing full-length ERα as well as the C-terminal or N-terminal regions were also used). While several protein-DNA complexes were observed upon incubation of the Ap-1 oligonucleotide with Cos1 nuclear extracts from cells transiently transfected with ERα and Smad3, no complexes were super-shifted by the ERα antibodies and no complexes disappeared upon incubation with an antibody (data not shown). Thus, it does not appear as though ERα is part of a protein-DNA complex on an Ap-1 oligonucleotide under these EMSA conditions.

The chromatin immunoprecipitation (ChIP) protocol has recently been developed to study the association of transcription factors with DNA. In this technique, proteins are cross-linked to DNA with formaldehyde and subsequently IP from solution while still bound to their cognate DNA sequences (437). The protein component of the immune complex is then digested away from the DNA sequence and the resulting DNA is amplified with specific primers in a PCR reaction. Thus, protein binding to a specific DNA sequence, in this case the p3TP-lux promoter region, may be determined under varying cellular conditions. Cos1 cells were transiently transfected with p3TP-lux, ERα and Smad3 in the presence or absence of estradiol. ChIP analysis with either an ERα or c-Jun antibody was performed with the technical assistance of Kanyarat Ung on Cos1 cell lysates. The sequence of p3TP-lux that was PCR amplified and the location of the Ap-1 and Smad3 binding sites is depicted in Figure 55. Six independent transient transfections

were carried out with six corresponding ChIP assays. Results were very inconclusive in that some experiments suggested an increase in ERα binding in the presence of ligand; others showed a decrease in ERα binding; while yet others showed no difference. Inconsistent results were also obtained when cell lysates were IP with a c-Jun antibody under these conditions. To determine the effect of Smad3 on c-Jun binding to the promoter sequence in p3TP-lux, Cos1 cells were transiently transfected with p3TP-lux in the presence or absence of Smad3 and ChIP assays were performed with the c-Jun antibody. Amplification of the region by PCR demonstrates that while c-Jun is able to bind the promoter sequence in the absence of over-expressed Smad3, it no longer binds to DNA in the presence of Smad3 (Figure 56), suggesting that Smad3 inhibits c-Jun binding to the Ap-1 sequence in p3TP-lux.

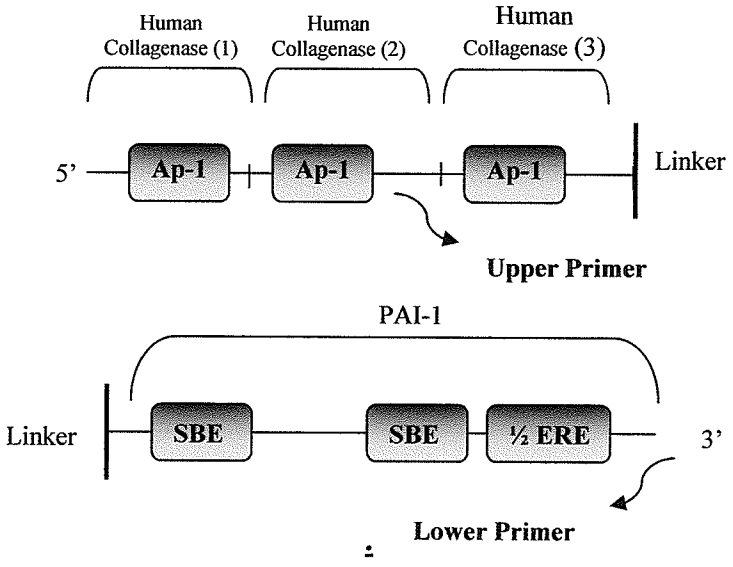


Figure 55: Schematic of p3TP-lux insert showing the location of primers used in ChIP analysis. The TGFβ responsive reporter plasmid p3TP-lux is composed of three human collagenase gene sequences containing Ap-1 binding sites upstream of the plasminogen activator inhibitor-1 (PAI-1) promoter region cloned within a pGL2 vector. Arrows represent the location of the upper and lower primers used in ChIP analysis.

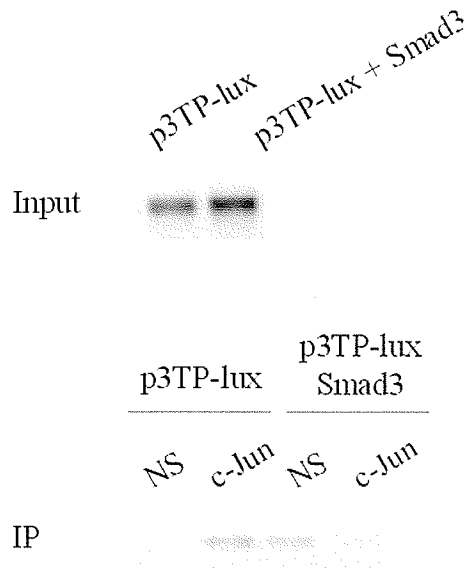


Figure 56: Chromatin immunoprecipitation of c-Jun on p3TP-lux in the presence or absence of Smad3 in Cos1 cells. Cos1 cells were transiently transfected with p3TP-lux along with Smad3. Cells were cross-linked with formaldehyde and cell lysates prepared as described in Methods. Lysates were immunoprecipitated under ChIP conditions with either c-Jun (Santa Cruz Biotechnology) or a non-specific antibody (NS) (insulin receptor, Santa Cruz Biotechnology). Input represents the initial total pool of DNA fragments used in the ChIP assay. IP represents DNA immunoprecipitated with either c-Jun or a non-specific antibody, n = 3 independent experiments.

Key Findings from Section II

In section II of the results, we have explored the mechanism through which ER may modulate Smad3 transcriptional activity. Specifically, we have shown that:

1. ER and Smad3 do not directly interact with one another either *in vitro* or *in situ* indicating that the original hypothesis in which ER modules TGFβ signaling by forming direct protein:protein interactions with Smad3 may not occur in our model system.
2. Over-expression of the Ap-1 transcription factor c-Jun, but not c-Fos, in Cos1 cells relieved the inhibitory effect of activated ERα on Smad3 transcriptional activity. This observation led us to the hypothesis that ligand-bound ERα inhibits Smad3 mediated transcription by binding to and sequestering limiting amounts of c-Jun away from the promoter region of TGFβ responsive genes.
3. Co-immunoprecipitation experiments of ERα and c-Jun demonstrated that these two proteins do not bind with one another despite great efforts to optimize the interaction and IP conditions in this model system.
4. Electrophoretic mobility shift assays and chromatin immunoprecipitation experiments suggest that ERα does not alter c-Jun binding to DNA, suggesting that ligand-activated ERα may not sequester c-Jun away from a Smad3 responsive promoter.
5. Due to the negative results obtained from this section of the thesis, we cannot say for certain what the molecular mechanism is through which ER modulates Smad3 transcription in this model system.

SECTION III. RELEVANCE TO BREAST CANCER

A. Relation of Ap-1, ER and Smad3 in Human Breast Tumors

Rationale

In a study of approximately 50 breast cancer cases, nuclear Smad3 (a surrogate marker for a functional TGFβ signaling pathway) was higher in normal breast tissue than in the adjacent matched invasive breast tumor (438). Conversely, ERα expression increases in breast tumors as compared to matched normal tissues. This suggests that Smad3 activity is decreased in tumors at the same time that ERα activity generally is increased. Evidence suggests that within breast tumors themselves nuclear Smad3 is associated with ER+ status and generally related to good prognostic markers (438). However, ER expression varies widely in human breast cancers and this study only looked at ER status as a binary factor (+ve or –ve), had a small number of tumors and did not evaluate Ap-1 activity in the form of phosphorylated c-Jun expression. Data from this thesis suggest that a complex interplay between ER, Smad3 and Ap-1 factors may be important in breast cancer. As demonstrated in section II, functional modulation of Smad3 transcriptional activity by activated ER involves c-Jun. All three of these factors must be expressed within the same breast tissue in order for this interaction to occur. Therefore, we examined the expression of phosphorylated c-Jun and Smad3 in human breast tumor tissues and determined whether correlations existed between c-Jun, Smad3 and ER protein expression.

Results

To determine whether interactions between ERα, TGFβ and Ap-1 signaling pathways could occur in breast cancer *in vivo*, human breast tumor tissue microarrays (TMAs) were used for immunohistochemical staining of Smad3 and phosphorylated c-Jun. Breast tumor TMAs were produced by and available for our use by the Manitoba Breast Tumor Bank. Summary of the clinicopathological characteristics of the patients used in the tumor TMAs is shown in Table 6. Tumors were identified according to their ER or PR status as defined by ligand-binding assay. Of the 112 tumors on the TMAs, 99 (88%) were ER^+ (ER positive defined as >3 fmol/mg protein) and 13 (12%) were ER^-; 83 (74%) were PR^+ (PR positive defined as >10 fmol/mg protein) and 29 (26%) were PR^-. Of the ER^+ tumors, 74 were also PR^+ (66%) and 9 of the ER^- tumors were PR^+ (8%). In a population of randomly selected breast tumors, one would expect approximately 30% of the tumors to be ER^- and 70% to be ER^+ (29, 30). Therefore, our study sample is slightly skewed towards an ER^+ status. It has been shown that approximately 50% of the ER^+ tumors will also be PR^+, while only a small proportion of ER^- tumors are PR^+ (439); our values are consistent with these. As PR is a downstream marker of a functional ER, it was expected that ER expression positively correlates with PR expression (Spearman r=0.2900; p=0.0019).

Characteristic	Number of Patients	% of Tumors
Total number of patients	112	
ER Status		
ER^+ (>3 fmol/mg protein)	99	88%
ER^- (<3 fmol/mg protein)	13	12%
PR Status		
PR^+ (>10 fmol/mg protein)	83	74%
PR^- (<10 fmol/mg protein)	29	26%
Tumor Size (cm)		
≤2.0	24	22%
>2.0	86	78%
Lymph Node Status		
Positive	61	59%
Negative	42	41%
Histological Grade		
Low Grade (4-6)	19	42%
High Grade (7-9)	26	58%

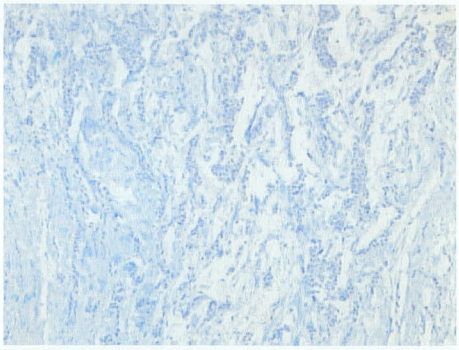
Table 6. Clinicopathological characteristics of patients used in the tumor tissue microarrays.

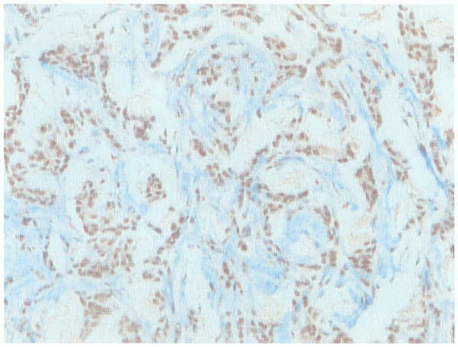
To extend previous data suggesting that Smad3 expression is positively associated with ER, we used a polyclonal antibody that recognizes Smad3 protein (Zymed Laboratories). The immunohistochemical validation of this Smad3 antibody has been previously published within breast tumor tissues (438). The specificity of this antibody for Smad3 is shown in Figure 57. Western blot analysis indicates specific detection for Smad3 in Smad3 transfected Cos1 cells; the corresponding band was not detected in mock transfected cells. Strong nuclear and cytoplasmic staining for Smad3 was observed in Cos1 cells which were transfected with Smad3 (Figure 57, bottom panel) due to its over-expression in these cells. In addition, nuclear and cytoplasmic staining of Smad3 was observed in a formalin-fixed, paraffin-embedded archival breast tissue block while no staining for Smad3 was observed in control sections stained with secondary antibody

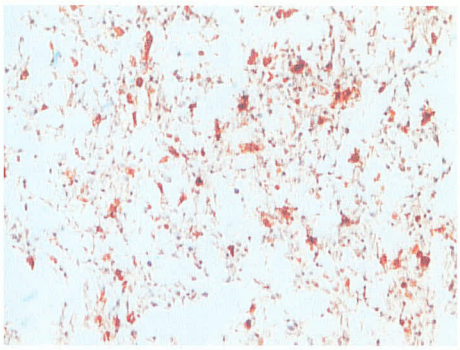
only (Figure 57). Therefore, this antibody was used for additional immunohistochemical analysis of Smad3 in the human breast tumor TMAs.

Immunohistochemical staining of tumor TMAs with Smad3 showed both nuclear and cytoplasmic staining. As the presence of nuclear Smad3 represents a functional and activated TGFβ signaling pathway, only nuclear Smad3 was scored for the purposes of this thesis. For each tumor, the corresponding nuclear Smad3 expression was quantified and expressed as an H-score, as described in Methods. Nuclear Smad3 H-scores ranged from 0-300; median = 90 with 8 tumors displaying no detectable nuclear Smad3 staining. Representative staining of negative, low and high expressing nuclear Smad3 tumors is shown in Figure 58.

When the cohort of cases was considered as a whole (n=112), no correlation was observed between nuclear Smad3 H-score and ER (r=0.1034, p=0.2778, Spearman) or PR levels (r=0.02952, p=0.7573, Spearman). Although there was a trend towards higher levels of nuclear Smad3 in ER^+ (median = 99, n=99) compared to ER^- (median = 70, n=13) tumors, there was no statistically significant difference in Smad3 expression (p=0.5338; Mann-Whitney). Similar levels of nuclear Smad3 were also observed in PR^+ (median = 99, n=83) and PR^- tumors (median = 60, n=29) (p=0.4157, Mann-Whitney). Furthermore, Smad3 levels were not different between ER^+/PR^+ (median = 99, n=74) and ER^-/PR^- (median = 60, n=4) (p=0.6042, Mann-Whitney) tumors and between ER^+/PR^+ (median = 99, n = 74) and ER^+/PR^- (median = 60, n = 25) tumors. Potential relationships between nuclear Smad3 expression and tumor characteristics were tested using nuclear Smad3 H-scores and known tumor characteristics, including tumor size, grade and nodal status. No associations were observed with grade. When tumors were divided into node







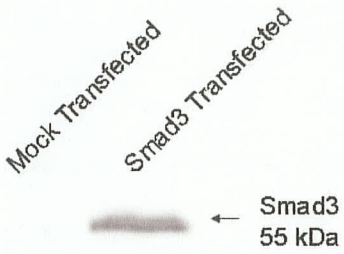


Figure 57: Verification and characterization of Smad3 immunohistochemistry in human breast cancer sections and Cos1 cells. *Top panel*: immunohistochemical analysis of breast tumor tissue stained with secondary antibody only (negative control) (magnification x100); *Middle panel*: human breast cancer biopsy positive for Smad3 (magnification: x200); *Bottom panel*: immunohistochemical analysis (magnification: x100) of Cos1 cells transiently transfected with Smad3. Forty-eight hours post-transfection, cells were embedded in agarose, formalin fixed, paraffin embedded and Smad3 immunohistochemistry was performed on these blocks. Inset: Western blot of Cos1 cells mock transfected or transfected with 1ug pCMV5B-Flag-Smad3. Whole cell extracts were prepared in Joel lysis buffer as described in Methods. 22ul of a 300ul whole cell extract was run on a SDS-10% acrylamide gel. Western blotting and immune detection was performed as described in Methods. Immune detection was using an anti-Smad3 antibody (Zymed Laboratories).

negative (no presence of breast cancer metastasis) and node positive (presence of breast cancer metastasis) groups, nuclear Smad3 expression was significantly lower in the node positive (median = 70, n= 61) cohort than in the node negative cohort (median = 145, n = 42) ($p<0.05$, Mann-Whitney) (Figure 59). No relationship was found between Smad3 expression and small (≤2cm) (median = 135, n = 24) or large (>2cm)(median = 80, n = 86) tumors ($p=0.1004$; Mann-Whitney). While we were unable to confirm the previously reported relationship between Smad3 and ERα, the association we found between Smad3 and node involvement suggests that Smad3 expression may be associated with less aggressive tumors.

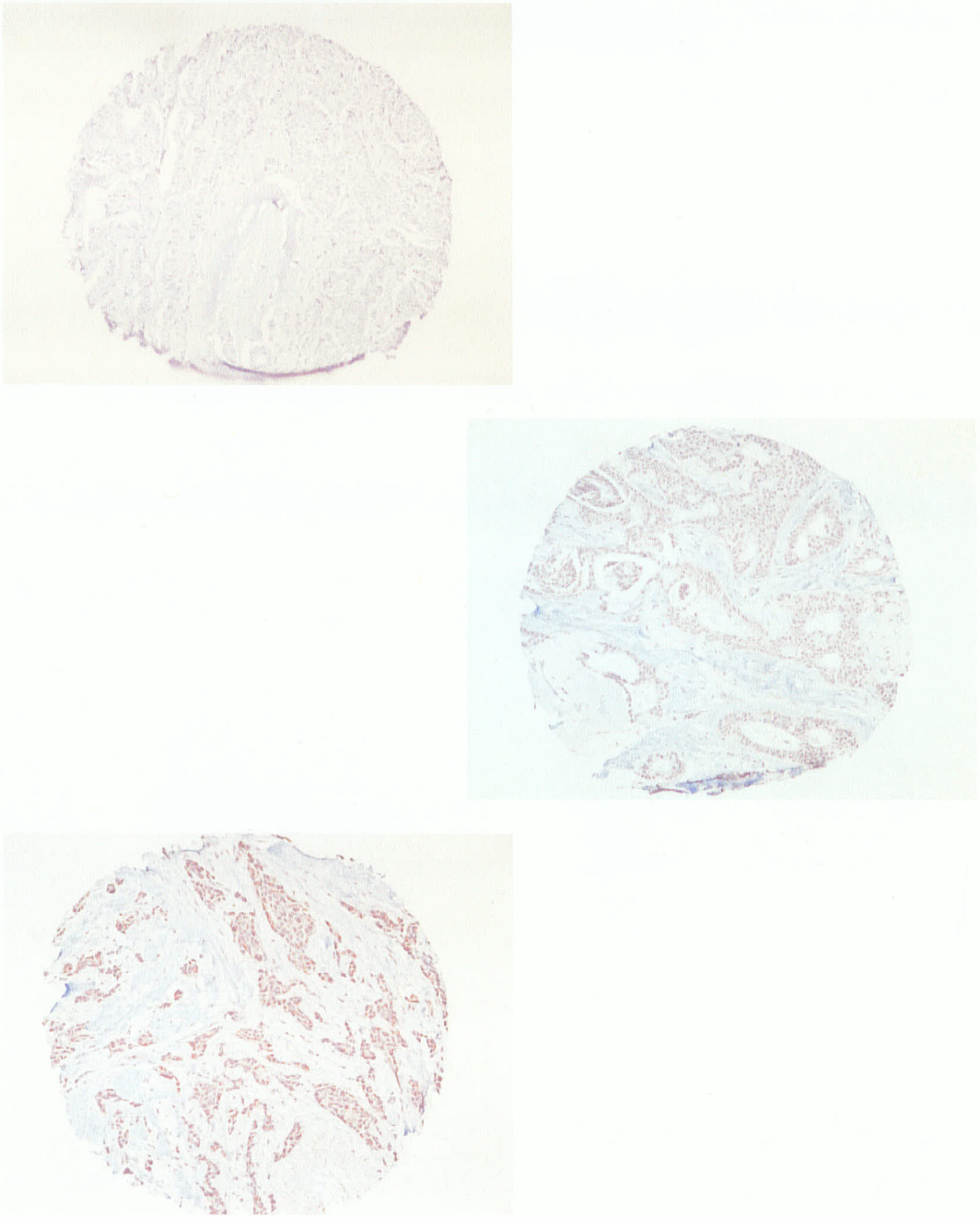


Figure 58: Smad3 immunostaining in human breast tumor tissue microarrays. Immunohistochemistry with rabbit polyclonal antibody to Smad3 (Zymed Laboratories) on primary tumors, showing no staining (top panel), moderate staining (middle panel) and intense staining (bottom panel) for Smad3 (magnification x100).

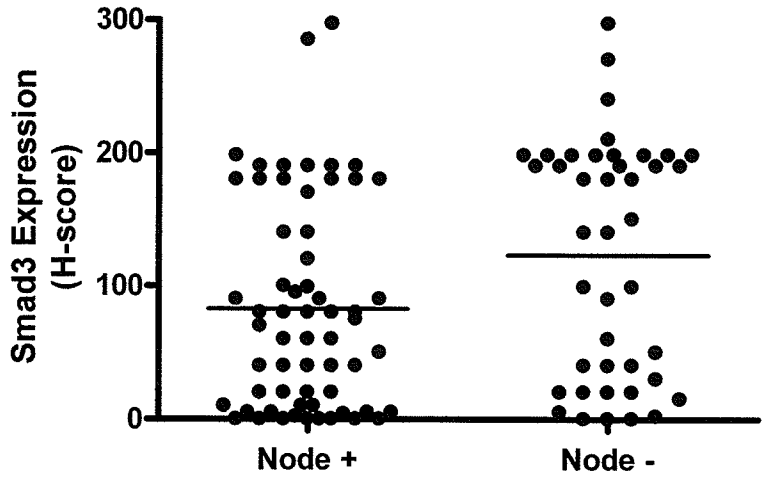
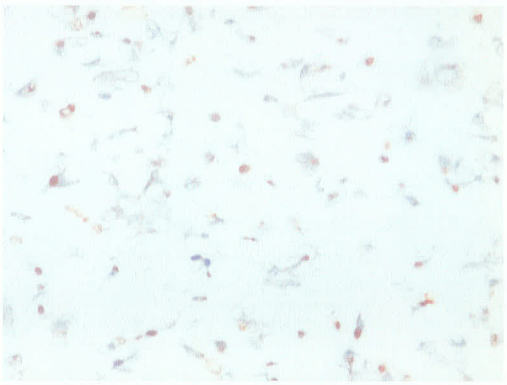


Figure 59: Smad3 H-score in node + and node – tumor tissues. Human breast tumor tissue microarrays were stained with a polyclonal Smad3 antibody (Zymed Laboratories) and expression was quantified by H-score analysis as described in "Methods." The tumors were divided into node + (lymph nodes containing metastatic breast cancer cells) and node – (lymph nodes with no detectable metastatic breast cancer cells). The results are presented as a scatter graph. Lines indicate the median value in each group. Significant differences ($p<0.05$; Mann-Whitney, two-tailed) were observed between the two groups.

Adjacent sections to those stained for Smad3 from the tumor TMAs were stained for phosphorylated c-Jun. To detect the expression of phosphorylated c-Jun, a mouse monoclonal antibody which specifically recognizes c-Jun phosphorylation at serine 63 (Santa Cruz Biotechnology), a marker of Ap-1 activity, was utilized. The c-Jun antibody was validated by determining the expression of phosphorylated c-Jun in Cos1 cells treated with the Ap-1 activator, TPA, for 30 minutes as compared to Cos1 cells not treated with TPA. Upon treatment, cells were pelleted, embedded in agar and formalin fixed prior to IHC. The presence of phosphorylated c-Jun represents an active Ap-1 signaling pathway. Results are shown in Figure 60.

A.



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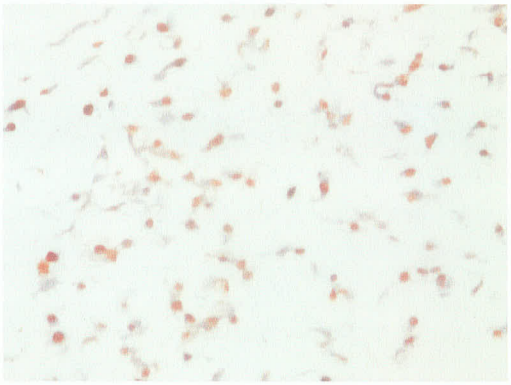

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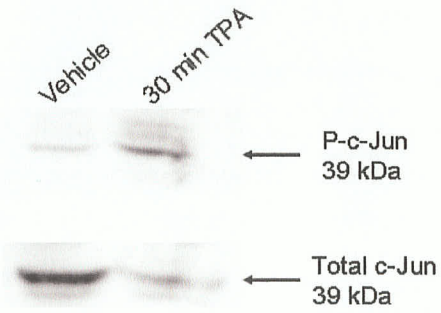


Figure 60: Phosphorylated c-Jun expression in Cos1 cells determined immunohistochemically and by western blot. A) Phosphorylated c-Jun expression in cells not treated with TPA (magnification x500). B) Phosphorylated c-Jun expression in Cos1 cells treated with TPA for 30 minutes, detected using mouse monoclonal antibody (Santa Cruz Biotechnology) (magnification x 500). C) Western blot of Cos1 cells treated with vehicle or TPA for 30 minutes. Whole cell extracts were prepared as described in Methods. 22ul of a 300ul extract was run on a SDS-10% acrylamide gel. Western blotting and immune detection was performed as described in Methods. Immune detection was using an anti-P-c-Jun antibody (Santa Cruz) and an antibody that recognizes total c-Jun expression (Santa Cruz).

Strong nuclear staining for phosphorylated c-Jun was observed in cells treated with TPA for 30 minutes with little nuclear staining observed in untreated cells. Additionally, western blot analysis of whole cell extracts indicate an increase in phosphorylated c-Jun expression in the TPA treated cells as compared to the corresponding vehicle (Figure 60). Therefore, this antibody was used for additional immunohistochemical analysis of activated c-Jun in the human breast tumor TMAs.

Phosphorylated c-Jun was detected in the nucleus of epithelial tumor cells within the biopsy samples and expression was quantified using H-score analysis as described in Methods. Values ranged from 0 - 300; median = 50 with 13 tumors displaying no detectable staining. Representative immunohistochemical staining in the tumor tissues is shown in Figure 61. When the cohort of cases was considered as a whole (n=112), no correlation was observed between phosphorylated c-Jun H-score and ER (r=0.1156, p=0.2247, Spearman) or PR (r=-0.06135, p=0.525, Spearman) levels. Similar levels of phosphorylated c-Jun were found in ER^+ (median = 50, n=99) and ER^- (median = 30, n=13) tumors (p=0.2270, Mann-Whitney), in PR^+ (median = 40, n=83) and PR^- (median = 60, n=29) tumors (p=0.2909, Mann-Whitney), in ER^+/PR^+ (median = 50, n = 74) and ER^-/PR^-(median = 72.5, n = 4) (p=0.7684, Mann-Whitney) tumors as well as in ER^+/PR^+ (median = 50, n = 74) and ER^+/PR^- (median = 60, n = 25) (p=0.4935, Mann-Whitney) tumors. Potential relationships between phosphorylated c-Jun expression and various tumor characteristics were explored and none were found with respect to grade or tumor size. However, phosphorylated c-Jun levels were higher in node negative (median = 60, n = 42) than in node positive (median = 40, n = 61) tumors ($p<0.05$, Mann- Whitney)

(Figure 62). Thus, like nuclear Smad3, phosphorylated c-Jun expression may be associated with a less aggressive tumor.

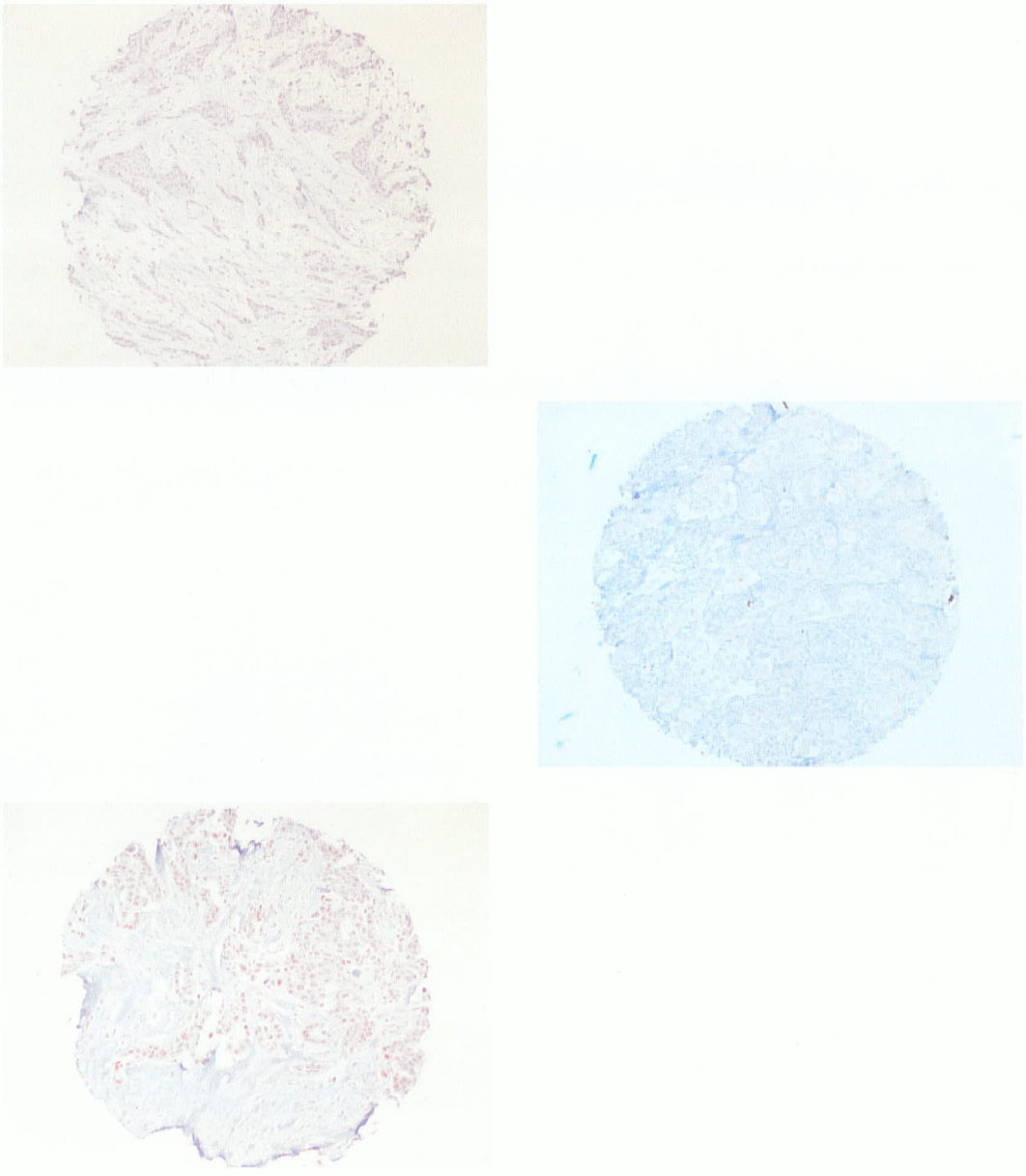


Figure 61: Phosphorylated c-Jun immunostaining in human breast tumor tissue microarrays. Immunohistochemistry with mouse monoclonal antibody (Santa Cruz Biotechnology) on primary tumors, showing no staining (top panel), low staining (middle panel) and high staining tumors (bottom panel) for phosphorylated c-Jun (magnification x100).

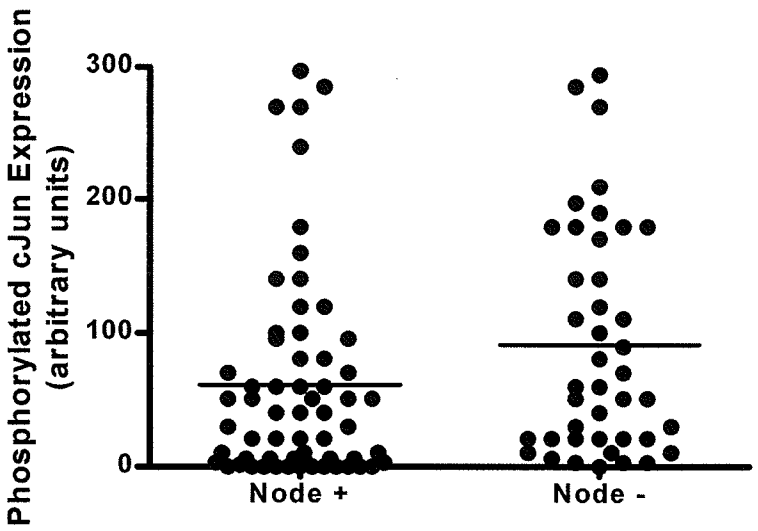


Figure 62: c-Jun H-score in node + and node – tumor tissues. Human Breast Tumor Tissue microarrays were stained with a monoclonal phosphorylated c-Jun antibody (Santa Cruz Biotechnology) and expression was quantitated by H-score analysis as described in "Methods." The tumors were divided into node + (lymph nodes displaying breast cancer metastasis) and node – (lymph nodes not displaying breast cancer metastasis). The results are presented as a scatter graph. Lines indicate the median value in each group. Significant differences ($p<0.05$; Mann-whitney, two-tailed) were observed between the two groups.

The above data suggest that similar patterns of expression between nuclear Smad3 and phosphorylated c-Jun may be observed in breast tumor tissues. To investigate this further, the expression of nuclear Smad3 and phosphorylated c-Jun was compared in ER^+ versus ER^- tumors and in PR^+ versus PR^- tumors. Correlation between nuclear Smad3 and phosphorylated c-Jun expression was tested by calculation of the Spearman co-efficient r using Smad3 and phosphorylated c-Jun H-scores. When the cohort of cases was considered as a whole (n=112), a positive correlation was observed between phosphorylated c-Jun and nuclear Smad3 H-scores (r=0.3060, $p<0.001$, Spearman). The positive correlation between phosphorylated c-Jun and nuclear Smad3 was observed in ER^+ tumors (r=0.3023, $p<0.005$, n=99, Spearman) but not in ER^- tumors (r=0.3736,

p=0.2086, n=13, Spearman). A positive correlation between nuclear Smad3 and phosphorylated c-Jun was also observed in PR^+ (r=0.3743, p=0.0005, n=83, Spearman) but not PR^- tumors (r=0.1999, p=02985, n=29, Spearman).

B. Endogenous Model of ER Affecting TGFβ Signaling

Rationale

Having found that a significant association and possibly cross-talk between the TGFβ and Ap-1 signaling pathways only occurs in the background of an ER^+ tumor, we next sought to identify an endogenously expressed gene in breast cells that is functionally modulated by ER and Smad3 in a manner similar to that observed on our reporter plasmids. All the functional experiments performed in section I relied on the transcriptional properties of synthetic reporter constructs (p3TP-lux and collagen 7) and transient transfections into Cos1 (monkey kidney cells) or MCF-7 (human breast cancer cells) cells. While such data are important in developing a model for an interaction between TGFβ and ER signaling, it does not address the question as to whether such an event occurs on an endogenously expressed gene in breast cancer cells. Thus, we have attempted to identify genes that are up-regulated by antiestrogens in MCF-7 cells that contain Ap-1 and Smad binding sites within their promoter region. In this thesis, we have examined the expression of two such candidate genes, the ER co-activator, AIB1 and $TGF\beta_2$.

AIB1 or SRC-3, enhances the transcriptional activity of the ER in a ligand dependent manner and its expression is up-regulated in breast tumors (391). While little is known about the regulation of AIB1 mRNA and protein expression, it has been shown

that E_2 treatment inhibits AIB1 mRNA and protein expression in MCF-7 cells (440). This inhibition can be reversed by the antiestrogens ICI 182,780 and 4OH-tamoxifen (440). Furthermore, ICI 182,780 and 4OH-tamoxifen can increase the mRNA expression of AIB1 by 2.5 and 2 fold, respectively, and western blot analysis suggests that ICI 182,780 increases AIB1 protein expression (440). Utilizing neutralizing antibodies to TGFβ, Lauritsen et. al.(440) have shown that TGFβ is partly involved in the antiestrogen induced up-regulation of AIB1, possibly through the ability of antiestrogens to stimulate TGFβ. While the exact promoter sequence of AIB1 has not been reported, the nucleotide sequence of chromosome 20q12, 2 kbp upstream of the AIB1 exon 1 (Genbank accession no. AF012108) was determined through the NCBI Map Viewer of the homo-sapiens gene database. This theoretical promoter sequence was then used in the identification of standard transcription factor binding sites using the free online MatInspector software provided by Genomatix. Search for transcription factor binding sites with a similarity cut-off of 0.75 revealed that while no consensus Smad binding sites are found in the sequence 2 kbp upstream of AIB1, an Ap-1 consensus site was found 110 bp upstream of a Fast-1 binding site. Fast-1 is a Smad interacting protein, in that it forms a complex with Smad2 and Smad4 (441). This complex then binds to a specific promoter sequence known as the activin response element and it is the Fast-1 that mediates specific DNA-binding to this response element whereas the Smads act as transcriptional activators and enhancers of DNA binding (310). Therefore, the promoter region of AIB1 may be responsive to TGFβ/activin activation as it contains a Fast-1 binding site. While the AIB1 promoter may also contain Ap-1 transcription factor binding sites, no studies have examined the effects of Ap-1 activation on AIB1 expression. Taken together, it appears

as though AIB1 may represent an endogenous model of p3TP-lux activity in human breast cancer cells, in that it is inhibited by estrogen and up-regulated by antiestrogens in MCF-7 cells, its expression is increased by TGFβ and its promoter contains putative Ap-1 and TGFβ signaling factor elements.

$TGF\beta_2$ mRNA expression has also been shown to be up-regulated by antiestrogens in MCF-7 cells (429). MCF-7 cells treated with 4OH-tamoxifen and ICI 182,780 for 5 days have 18 and 48 fold increases, respectively, in $TGF\beta_2$ mRNA expression as determined by real time PCR analysis. As described in the introduction, $TGF\beta_2$ is a potent negative regulator of breast cancer cell growth. $TGF\beta_2$ is located on chromosome 1q41. As the promoter for $TGF\beta_2$ has not been characterized, putative transcription factor binding sites 2 kbp upstream of the transcription start site were determined using the Genomatix Transcription Factor Search Site. Several Smad3 binding sites with 99% homology were identified downstream of Ap-1 consensus binding sites (>95% homology). Thus, $TGF\beta_2$ was a good candidate in the search for an endogenous gene regulated by TGFβ, Ap-1 and ER.

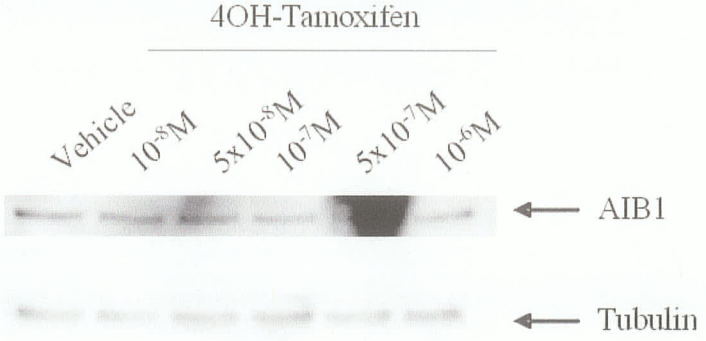
Results

To determine if AIB1 mRNA and protein expression are up-regulated by antiestrogens in MCF-7 cells, cells were grown in CM5% supplemented with 10nM E_2 and subsequently treated with increasing amounts of 4OH-tamoxifen or ICI 182,780 (10^{-8}M - 10^{-6}M), to ensure that saturating levels of antiestrogens were used to compete with the estrogen, for 1-3 days. This co-treatment of estrogen and antiestrogens has previously been shown to be effective in inhibiting the growth of MCF-7 cells after several days. RT-PCR (data not shown) and western blot analysis of whole cell extracts

(Figure 63) suggest that AIB1 mRNA and protein expression is not affected by tamoxifen or ICI 182,780 treatment in MCF-7 cells (note: the apparent increase in AIB1 expression with $5x10^{-7}$M 4OH-tamoxifen (Figure 63a) is an artifact on the membrane that occurred during the western blotting (transfer) procedure). While these results are in contrast to those observed by Lauritsen et. al. (440), it is not surprising as these authors only observed a maximal 2 fold increase in AIB1 expression in MCF-7 cells. Thus, it does not appear as though AIB1 expression is regulated by ER in our model system and cannot be used to further investigate the ER and TGFβ pathway cross-talk.

To determine whether $TGF\beta_2$ expression is increased by antiestrogen treatment, MCF-7 cells were treated with increasing amounts of 4OH-tamoxifen or ICI 182,780 in the presence of 10nM E_2 over a period of 12, 24 and 48 h. RNA was isolated and $TGF\beta_2$ mRNA expression was determined by RT-PCR as described in Methods. Typical PCR reaction products for $TGF\beta_2$ and GAPDH (internal control) in MCF-7 cells treated with 4OH-tamoxifen are shown in Figure 64a. $TGF\beta_2$ mRNA expression increased significantly in the presence of 4OH-tamoxifen in a dose-dependent manner over a period of 24 h ($p<0.005$) and 48 h ($p<0.0005$) (Figure 64b). No significant change in expression was observed at 12 h exposure to 4OH-tamoxifen. ICI 182,780 treatment also significantly ($p<0.01$) increased $TGF\beta_2$ mRNA expression at 48 h in a dose-dependent manner, while no significant effect was observed at 12 and 24 h (Figure 65).

A.



B.

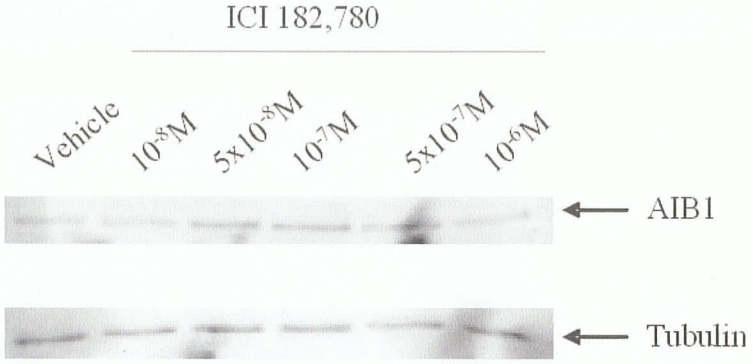
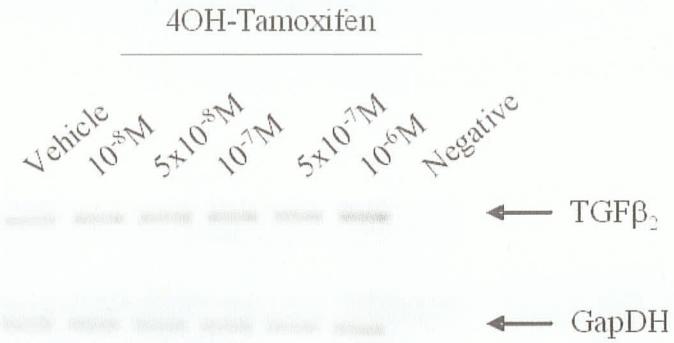


Figure 63: Effect of antiestrogen treatment on AIB1 protein expression in MCF-7 cells. MCF-7 cells were set-up at $5x10^4$ cells/well in 6 well plates in CM5% containing 10nM E_2. Four days later, cells were treated with increasing (10^{-8}-10^{-6}M) concentrations of either A) 4OH-tamoxifen or B) ICI 182,780. Cells were harvested 6, 12, 24 or 48 h later and whole cell extracts were prepared in SDS isolation buffer as described in Methods. BCA protein assays were performed and 23ug of protein was run on a SDS-7.5% acrylamide gel. Western blotting and immune detection were performed as described in Methods. Immune detection was using an anti-AIB1 antibody (BD Transduction Laboratories). Results represent a typical (n=3) western blot for AIB1 upon 24 h treatment with antiestrogens.

A.



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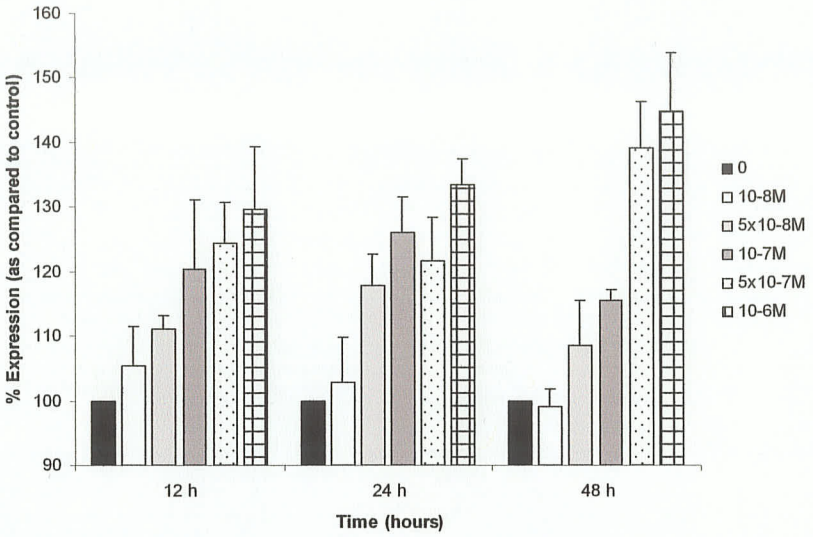
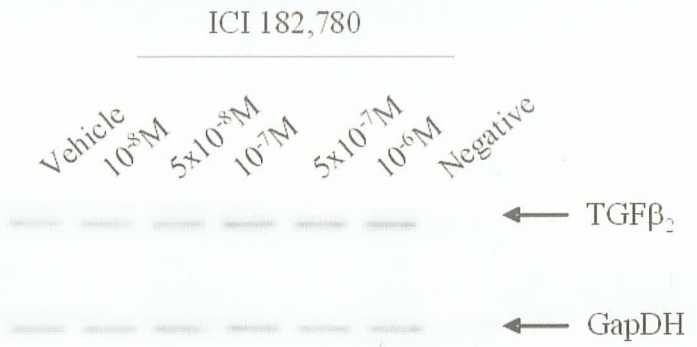


Figure 64: $TGF\beta_2$ mRNA expression in MCF-7 cells treated with 4OH-tamoxifen. MCF-7 cells were set-up in 6 well plates at $5x10^4$cells/well in CM5% containing 10nM E_2. Four days later, cells were exposed to increasing (10^{-8}M – 10^{-6}M) concentrations of 4OH-tamoxifen. Cells were harvested 12, 24 and 48 h later. Total RNA was extracted, reverse transcribed and PCR amplified using $TGF\beta_2$ and GapDH primer pairs as described in Methods. PCR products were run out on a 1.2% agarose gel and visualized with the Chemidoc system. A) Typical agarose gel showing $TGF\beta_2$ and GapDH PCR products from a 24 h treatment with 4OH-tamoxifen. B) Densitometric analysis of $TGF\beta_2$ and GapDH PCR products from MCF-7 cells treated with 4OH-tamoxifen over 12, 24 and 48 h. Results are expressed as the average units of $TGF\beta_2$/GapDH, n=3 independent experiments with each experiment containing n=3 independent PCR reactions. Significant differences in $TGF\beta_2$ mRNA expression were observed over 24 h ($p<0.005$, ANOVA) and 48 h ($p<0.00005$, ANOVA) exposure to 4OH-tamoxifen.

A.



B.

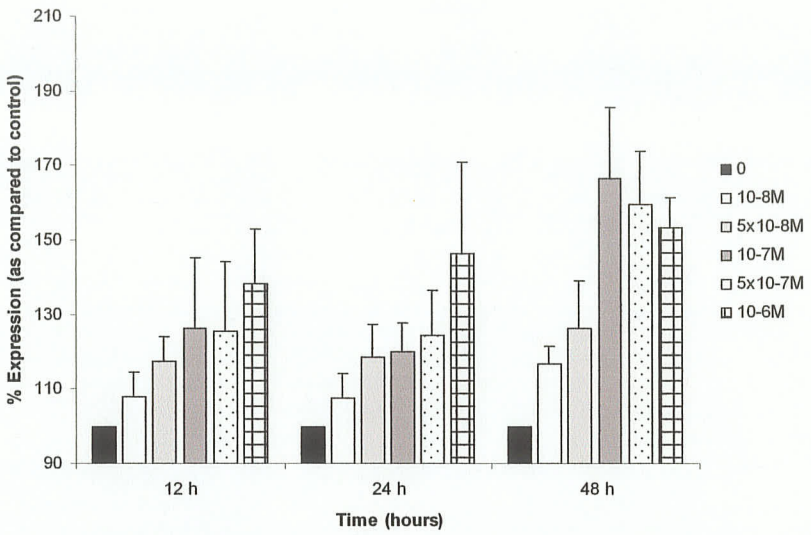


Figure 65: $TGF\beta_2$ mRNA expression in MCF-7 cells treated with ICI 182,780. MCF-7 cells were set-up in 6 well plates at $5x10^4$cells/well in CM5% containing 10nM E_2. Four days later, cells were exposed to increasing (10^{-8}M – 10^{-6}M) concentrations of ICI 182,780. Cells were harvested 12, 24 and 48 h later. Total RNA was extracted, reverse transcribed and PCR amplified using $TGF\beta_2$ and GapDH primer pairs as described in Methods. PCR products were run out on a 1.2% agarose gel and visualized with the Chemidoc system. A) Typical agarose gel showing $TGF\beta_2$ and GapDH PCR products from a 48 h treatment with ICI 182,780 (ICI). B) Densitometric analysis of $TGF\beta_2$ and GapDH PCR products from MCF-7 cells treated with ICI 182,780 over 12, 24 and 48 h. Results are expressed as the average units of $TGF\beta_2$/GapDH, n=3 independent experiments with each experiment containing n=3 independent PCR reactions. Significant differences in $TGF\beta_2$ mRNA expression were observed over 48 h ($p<0.01$, ANOVA) exposure to ICI 182,780.

Key Findings from Section III

Within this section, we have provided evidence that the functional model presented in section I of the results may occur in a human breast cancer cell background. Specifically:

1. Immunohistochemical staining of breast tumor TMAs demonstrated that while neither nuclear Smad3 nor phosphorylated c-Jun expression correlates with ER^+ tumors, there is a positive correlation between nuclear Smad3 and phosphorylated c-Jun protein expression in ER^+ but not ER^- tumors. This suggests that all three proteins can be expressed within the same breast tumor. Thus, a significant association and potential interaction between TGFβ and Ap-1 regulated transcription may only occur in the presence of ER, supporting the hypothesis that all three pathways may cross-talk and have a role in some breast tumors *in vivo*.
2. Endogenously expressed $TGF\beta_2$ mRNA expression is up-regulated by antiestrogens in MCF-7 cells in a manner similar to that observed on the TGFβ responsive reporter plasmid p3TP-lux utilized in section I of the results. As the promoter region of $TGF\beta_2$ contains several putative Smad3 as well as Ap-1 binding sites, this gene may represent an endogenous model on which ER, TGFβ and Ap-1 functionally interact. While $TGF\beta_2$ looks to be a promising candidate in our model system, the regulation of this gene by TGFβ and Ap-1 would be the focus of future studies.

DISCUSSION

The ER is a member of the steroid receptor family and acts as a ligand-inducible transcription factor to modulate target gene transcription. Reports of an interaction between the negative growth regulatory factor TGFβ via Smad3 with the GR (322), AR (337, 338) and VDR (320) suggest that the steroid receptor family can influence TGFβ signaling or, conversely, that the TGFβ pathway may modulate steroid receptor activity. In one study, Smad3, a downstream signaling protein of TGFβ, enhanced the ligand-induced transactivation function of VDR via direct hormone-dependent interactions between Smad3 and VDR (320, 395) while the effect of VDR on Smad3 transcriptional activity has not been assessed to this date. TGFβ/Smad3 has been shown to repress AR transcriptional activity from several AR dependent promoters (337) while in another study, Smad3 increased AR-mediated transcription (338). In contrast, ligand activated AR represses Smad3 transcription in prostate cancer cells (396). Lastly, TGFβ/Smad3 does not affect GR transcriptional activity while activated GR represses Smad3 transcriptional activity (322).

These findings with respect to various members of the steroid receptor family suggest that there may also be cross-talk between the ER and TGFβ signaling pathways. We therefore examined the modulation of ER transcriptional activity in cells over-expressing Smad3. Our results demonstrated that while Smad3 had little if any effect on ER transcriptional activity on promoters representing the classical (represented by the *Xenopus* vitellogenin ERE) or non-classical (as represented by the $TGF\beta_3$ promoter reporter plasmid) mode of ER action, ERα and $ER\beta_1$ effectively inhibited Smad3 transcriptional activity in Cos1 and MCF-7 cells. These observations are in agreement

with several other laboratories (320, 322, 337, 338). However, it had not previously been addressed as to whether the ERβ variants, $ER\beta_2$ or $ER\beta_5$, affect Smad3 transcriptional activity. Results from this thesis suggest that while the wild-type ERβ, $ER\beta_1$, effectively inhibits Smad3 induced p3TP-lux luciferase activity in the presence of estrogen, the ERβ variants do not have any effect, in keeping with the observation that $ER\beta_2$ and $ER\beta_5$ do not bind ligand (139).

Our observation that Smad3 does not affect ER transcriptional activity on an ERE is in contrast to that described by Matsuda et al.(335), who suggest that Smad3 increases ER transcriptional activity on the vitellogenin ERE in MCF-7 and 293T (human embryonic kidney carcinoma) cells. Although the reason behind this discrepancy is unclear, it may be due to different cell backgrounds in which the experiments were performed and/or to differences in reporter genes. Experiments by us and others to assess the effect of TGFβ on ER activity were performed in Cos1 (green monkey kidney cells) (320), human prostate cancer (DU145 and PC-3) (337, 338) and human hepatocellular carcinoma (Hep3B) (322) cells; no studies were performed in MCF-7 or 293T cells as described by Matsuda et al. (335). Contrasting results have previously been reported with respect to the functional interaction between TGFβ and steroid receptors in various cell lines. For example, Yanagisawa et al. (320) demonstrated that in Cos1 cells transiently transfected with AR, activation of TGFβ did not affect AR activity on an androgen responsive promoter while others have reported an inhibitory effect of TGFβ on AR activity in prostate cancer cells (337). Similarly, GR has been shown to inhibit Smad3 transcriptional activity in Hep3B cells (322) while another study demonstrated that GR did not have any effect on TGFβ in 293T cells (335). Therefore, our results

indicate that different functional outcome with respect to the modulation of ERα transcriptional activity by TGFβ occurs in a cell dependent manner.

The TGFβ responsive promoter p3TP-lux used in this thesis consists of a small segment of the human collagenase III promoter cloned in triplicate 5' from that region of the PAI-1 promoter that contains the Smad3 binding site. PAI-1 is a member of the serine protease inhibitor family that contributes to the regulation of endogenous extracellular matrix degradation by tightly binding both tissue-type and urokinase plasminogen activators. Thus, TGFβ, by increasing the expression of PAI-1, effectively inhibits the degradation of the extracellular matrix. Estrogen has been shown to reduce circulating plasma levels of PAI-1 in postmenopausal women (442, 443), an effect thought to partly mediate the cardiovascular protective effects of estrogen. However, Smith et al. (442) have shown that in vascular endothelial cells over-expressing ERα, there is a significant increase in PAI-1 promoter activity in the presence of estrogen while ERβ has little or no effect. Deletion mutations mapped the ERα binding site to a consensus ERE located in the proximal PAI-1 promoter and site-directed mutagenesis of this ERE completely abolished the stimulatory effect of ERα (442). These authors also indicated that intact Ap-1 sites are not essential in the modulation of PAI-1 promoter activity by ER (442). However, the role of the TGFβ consensus sequence was not addressed in these studies. The segment of the PAI-1 promoter used in our studies, while it does include a half ERE site, does not encompass the ERE that was identified by Smith et al. (442) as being essential in modulating ER activity on PAI-1 but rather, encompasses that region surrounding the TGFβ responsive element. The absence of this ERE in the p3TP-lux promoter, in addition to different cell backgrounds, is the most likely

explanation for the discrepancy between our results, in which both ERα and ERβ inhibited promoter activity, and that of Smith et al. (442).

Nonetheless, the half ERE site in p3TP-lux (Figure 16) or the 3 half ERE sites in the collagen 7(A1) -524/+92 (Figure 21) reporter plasmid may play a role in the ability of ER to modulate Smad3 transcriptional activity. Several studies suggest that although ERα cannot bind a single ERE half site, dimeric ERα can bind an ERE half site when stabilized by protein:protein interactions with Ap-1 bound to its DNA binding element (88). In the presence of estrogen, ERα typically acts in synergy with Ap-1 transcription factors in this manner, enhancing Ap-1 transcriptional activity (279), although an inhibitory effect has also been reported (225). It has been suggested that ERα enhances Ap-1 activity through cooperative recruitment of co-activators, namely p160 co-activators, to the promoter region (286) while the inhibitory effect has been hypothesized to be the result of direct competition for limiting amounts of transcriptional co-activators to the promoter (282). By modulating Ap-1 transcriptional activity through the ERE sites in our TGFβ reporter plasmids, ER may indirectly affect Smad3 transcriptional activity as Ap-1 alters Smad3 activity on the reporter plasmids. It may also be possible that ER is able to bind to its half ERE sites through cooperative binding with TGFβ, although this has not been described to date. The putative role of these EREs in our reporter plasmids would have been best illustrated through site directed mutagenesis of the EREs.

The role of estrogen in mediating the ER suppression of p3TP-lux in Cos1 cells is apparent; E_2 inhibits Smad3 activity while co-treatment with the antiestrogens 4OH-tamoxifen or ICI 182,780 suppresses this effect. However, its role in inhibiting TGFβ activity in MCF-7 cells transiently over-expressing ERα is rather unclear as our results

did not show any effect of estrogen treatment on p3TP-lux activity as compared to vehicle treated cells. It has previously been shown by Matsuda et al. (335) that treatment of MCF-7 cells with TGFβ ligand increases p3TP-lux activity 4.5 fold; co-treatment with E_2 significantly inhibited this response (335). While the conditions under which the cells were cultured between the results described in this thesis are quite different from that previously published, transfected cells were exposed to similar E_2 concentrations over similar time periods. It is of concern, however, that Matsuda et al. (335) transfected their cells in the presence of 1% (v/v) fetal calf serum in DMEM that was not free of phenol red. Fetal calf serum and DMEM with phenol red contain growth hormone contaminants, including estrogen, that result in the cells being in a stimulated state. Contaminants of phenol red act as weak estrogens and at concentrations found in common tissue culture media, causes significant stimulation of cell proliferation and protein synthesis in estrogen responsive cells (403). Considerable efforts were made in our experiments to ensure that this was not the case; cells were grown in phenol red free DMEM and in twice charcoal stripped serum (a process that removes a large portion of growth hormones from the serum) to ensure minimal contribution by growth hormones and estrogenic contaminants of phenol red.

Interestingly, the inhibitory effect of ERα over-expression on TGFβ signaling was not observed in a MCF-7 cell line stably expressing GFP-tagged ERα under control of doxycycline. The absence of an effect in these cells is likely due to the lack of total altered ERα expression in these cells. Immunoblot analysis of doxycycline induction of GFP-ERα show that while there is an increase in the expression of GFP-ERα, endogenous levels of ER decreased. This has previously been observed by Zhao et al.

(399). Accordingly, the decline in endogenous ERα protein levels upon doxycycline treatment is accompanied by a decrease in ERα mRNA levels, demonstrating that ER autoregulates its own expression (399). Experiments in our MCF-7 cells suggest that a very high level of ERα expression is required in order to alter Smad3 signaling, as we were unable to observe any effect in these cells without over-expressing ERα through transient transfections. Interestingly, however, whole cell ligand-binding assays show a 2 fold increase in [^{3}H]E_2 binding upon treatment of doxycycline in the GFP-ERα inducible MCF-7 cells (424), suggesting that there is an overall increase in ERα expression. The upregulation of tagged ERα in the presence of doxycycline is sufficient to induce endogenously expressed PR in the MCF-7 cells and a further increase in PR expression may be observed with E_2 treatment (424). Nonetheless, this increase does not appear to be sufficient to modulate TGFβ signaling within these cells.

MCF-7 cells produce and secrete TGFβ isoforms *in vitro* and growth of these cells is inhibited by exogenous TGFβ. It has previously been reported that treatment of MCF-7 cells with the antiestrogen tamoxifen leads to a release of active TGFβ$_1$ from a latent precursor molecule and to increased transcription of TGFβ$_2$ and TβRII (425, 426, 444). Consistent with these observations, we also demonstrated in this thesis that TGFβ$_2$ mRNA expression is increased upon tamoxifen treatment in MCF-7 cells. Thus, TGFβ$_2$ may represent an endogenous model through which ER modulates TGFβ signaling in breast cancer cells. The demonstration by other authors that antiestrogens increase TGFβ$_1$ secretion may explain the increase we observed in p3TP-lux activity upon tamoxifen treatment, as an increase in TGFβ secretion by the cells would in turn activate

Smad signaling pathways, leading to an increase in Smad3/4 phosphorylation and TGFβ transcriptional activity. However, we did not determine the expression of TGFβ ligand upon antiestrogen treatment in this thesis. ICI 182,780 also enhances TGFβ secretion from MCF-7 cells (425) but its inhibitory action on ER activity is primarily due to the ability of ICI to increase ER protein degradation by targeting it to the proteasome (163, 164).

The p3TP-lux luciferase reporter plasmid consists of the Ap-1 containing region of the collagenase promoter concatermerized to a region of the PAI-1 promoter. Mutation of the Ap-1 binding sites in p3TP-lux inhibits induction by Smad3/Smad4 overexpression and reduces, but does not completely eliminate Smad DNA binding (412), suggesting that the Ap-1 sites are important in mediating TGFβ induced p3TP-lux transcription. Furthermore, Smad3/Smad4 co-overexpression activates an Ap-1 reporter containing seven concatermerized Ap-1 sites, firmly establishing a role for Smads in activating transcription from Ap-1 sites (412). In accordance with the Ap-1 sites being active and important in mediating p3TP-lux activity, we have shown that c-Jun or c-Fos over-expression increases Smad3 transcriptional activity in Cos1 cells. Direct interactions between Smad3/Smad4 and Ap-1 factors have been described (412, 433, 434) that mediate the recruitment of Smads to the promoter region.

In addition to TGFβ, Ap-1 factors also interact with and modulate ER transcription through functional and/or physical interactions. ERα cooperates with c-Jun and c-Fos to confer estrogen responsiveness to simple, heterologous promoters (231, 286) and to the ovalbumin (232), *c-fos* (130), collagenase (280, 283), PR (132) and IGF-1 (274) genes. Furthermore, ERα has been shown to interact with promoter bound c-Jun to

mediate transcriptional activation in the absence of its DBD (276) and direct protein:protein interactions between ER and c-Jun have been described both *in vitro* (224, 231) and *in situ* (287). The identification of c-Jun as an important part of the activation complex suggests a molecular mechanism by which ER may modulate Ap-1 activity. The idea that nuclear receptors compete with other transcription factors for binding to a limiting pool of accessory factors necessary for gene expression is not a new one (119, 442). TBP, p300 and CBP co-activators have all been shown to be limiting factors in their ability to activate ER and Smad3 responses (119, 276, 445, 446). Over-expression of the adenovirus E1a protein titrates p300 and CBP from a Smad transcriptional complex inhibiting Smad responses; mutations in E1a that lack co-activator binding had no effect on Smad activity (316, 446). Results from our experiments suggest that Ap-1 factors may also be limiting for TGFβ signaling in the presence of ligand-activated ER as over-expression of c-Jun reverses the inhibitory effect of ERα on p3TP-lux activity. Co-transfection with vectors expressing c-Fos did not significantly diminish the ERα inhibitory effect, a finding which is consistent with ERα not forming protein:protein interactions with c-Fos. This affect does not appear to be common to all co-activators, however, as over-expression of the ER/TGFβ co-activator SRC-1 did not rescue the inhibitory effect of ER on Smad3 transcriptional activity. Nonetheless, it is possible that other transcriptional cofactor(s) may participate in this antagonism, as it has been reported that CBP and p300 are also co-activators for ERα and Smad3 (81, 315).

As c-Jun appears to be a limiting factor in the ability of activated ER to inhibit Smad3 signaling, we proposed the model depicted in Figure 66. In the absence of

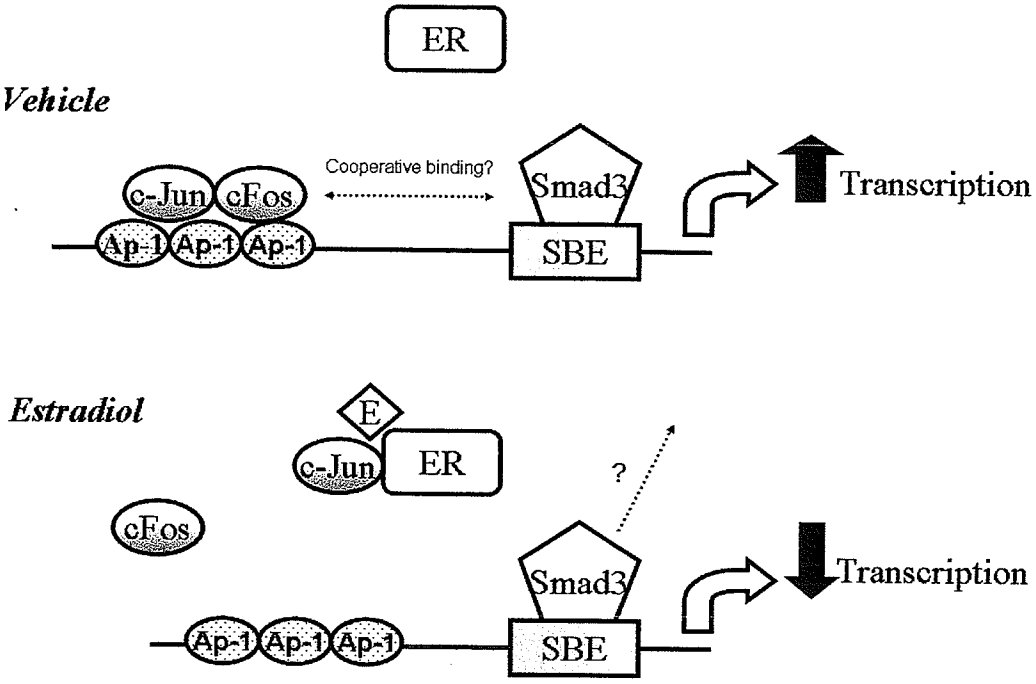


Figure 66: Model of the mechanism through which ER inhibits TGFβ signaling on p3TP-lux. E = estrogen, SBE = Smad binding element.

estrogen, c-Jun homodimers or c-Jun/c-Fos heterodimers bind to the Ap-1 sites located in the human collagenase gene of p3TP-lux and Smad3 binds to the SBE of the PAI-1 region. Whether Smad3 binds to the SBE prior to c-Jun/c-Fos or whether Ap-1 binding enables Smad3 to bind to the SBE is unknown as the transcriptional action of Smad complexes has yet to be understood in terms of sequential recruitment of factors. It is not unreasonable to assume that Smad3 cooperates with Ap-1 factors for effective DNA binding as the affinity of Smad proteins for the SBE is often too low to support binding of the Smad complex to a single SBE (447). Once activated by estrogen, the ER then titrates c-Jun away from the promoter region by specifically binding to Jun and not Fos

proteins, effectively inhibiting Ap-1 factors from binding to the Ap-1 site (as Fos cannot bind DNA itself) (243). The loss of Ap-1 binding may also result in Smad complexes dissociating from the SBE as the cooperative interactions between the proteins are lost. As it has been suggested that ER does not bind to c-Jun in the presence of antiestrogens (224), the ER would be unable to sequester c-Jun away from the promoter region while bound to such ligands. Competition of antiestrogens to estrogen for the ER would effectively reverse the inhibitory effect of E_2-bound ER on TGFβ signaling.

To provide further evidence that a competition between Smad3 and ligand-activated ER is responsible for modulating Smad3 transcriptional activity, we examined the interaction between ER and c-Jun. While the interaction between ERα and c-Jun has been previously described, we were unable to observe a physical interaction between ERα and c-Jun in our model system. In one study, ERα proteins fused to glutathione S-transferase (GST-ER) were shown to interact with *in vitro* translated c-Jun through its amino-terminal domain (231). In contrast, another study using c-Jun fused to GST and *in vitro* translated ERα suggested that the carboxy-terminal region of ERα was required for its interaction with c-Jun while the bZIP region of c-Jun is involved in the interaction (224). No interaction between Fos proteins and ERα has been observed by either of these authors (224, 231). The interaction between ERα and c-Jun in intact cells has been described by Teyssier et al. (224), in which a physical contact between the two proteins was determined by an increase in expression of a GAL4-responsive luciferase reporter plasmid or by co-IP of ERα and c-Jun proteins in transiently transfected Cos1 cells. These authors also suggest that the interaction between ERα and c-Jun is enhanced in the presence of estradiol but is inhibited by tamoxifen or ICI 182,780 treatment (224). Most

importantly, however, the interaction between endogenous ER and endogenous c-Jun has recently been described in MCF-7 cells (287). Immunoprecipitation of MCF-7 cell lysates with an anti-ERα antibody was able to successfully pull-down c-Jun along with ERα; this interaction was enhanced upon c-Jun phosphorylation (287). Thus, while we were unable to observe a physical contact between ERα and c-Jun, evidence strongly suggests that they do; whether this occurs in a ligand-dependent manner, however, is unclear.

To further test our hypothesis that ERα sequesters c-Jun away from the p3TP-lux promoter, we determined if the amount of c-Jun bound to the Ap-1 sites in the promoter decreased in the presence of estrogen. EMSA and ChIP analysis results both suggest that the amount of c-Jun bound to the Ap-1 site does not change in the presence of E_2 bound ER. These results are in agreement to those by Cheng et al. (276) who show that c-Jun and c-Fos binding to the Ap-1 site on the gonadotropin releasing hormone receptor (GnRHR) is not affected by estrogen treatment. Moreover, these authors also determined that ERα was not part of the nucleoprotein complex on the Ap-1 oligonucleotide, as addition of an anti-ERα antibody had no effect on complex formation (276), a finding similar to our own. In contrast, other studies have suggested that the ER is able to interact with c-Jun bound to DNA (224), and that such an interaction results in transcription initiation increasing Ap-1 activity (282). This makes one wonder whether the stimulatory and inhibitory effects of ER are mediated by two distinct mechanisms, as has been hypothesized for the opposing effects of ERα and ERβ at Ap-1 sites (96, 277). Nonetheless, mutations in ER that inhibit its binding to c-Jun significantly diminishes the

inhibitory response of ER on Ap-1 sites, suggesting that the effect of ER is, at least in part, dependent on its ability to bind c-Jun (287).

So, how does ERα suppress Smad3 transcriptional activity? It has been proposed that nuclear receptors may inhibit TGFβ transcriptional activity by decreasing Smad expression levels. Our data exclude the possibility that estrogen represses total Smad3 expression levels, a finding that is in agreement with other laboratories (448). At the time when these experiments were performed, we were unable to determine whether estrogen treatment affects the phosphorylation level of Smad3 as antibodies specific for phospho-Smad3 were unavailable. Recently, however, it has been shown that in the presence of estrogen, phosphorylation of Smad3 was reduced by approximately 60% compared with TGFβ stimulated MCF-7 cells while total Smad3 protein levels were unaffected (448). The decrease in Smad3 phosphorylation by estrogen was shown to be accompanied by a reduction of a functional Smad3/Smad4 complex (448).

It has been proposed by Matsuda et al. (335) that ER inhibits TGFβ transcriptional activity by forming direct physical interactions with Smad3. Co-IP experiments were performed in which 293T cells were transiently transfected with ERα, Flag-tagged Smad3 and TβRI (T204D) (a constitutively active TβRI receptor) expression vectors in the presence or absence of estrogen. Results indicated that ERα-Smad3 interactions occur only in the presence of estrogen; no interaction was detected in the absence of ligand. However, in this thesis, we were unable to co-IP ERα and Smad3 in Cos1 cells transiently transfected with Smad3 and ERα expression vectors. In the study by Matsuda et al. (335), IPs were performed with both an anti-Flag and anti-ERα antibody. However, no negative control antibody (i.e. an antibody of the same class as

the IP antibody) was used in the 293T co-IP experiments. From our experience, a negative control antibody is an essential part in interpreting the results of an IP experiment due to non-specific binding of proteins to antibodies and/or to the protein G sepharose beads (as was observed in several of our co-IP experimental results). In our several attempts to co-IP ERα and Smad3, a band corresponding to the molecular mass of ERα (i.e. 62 kDa) was often observed when cellular extracts were IP for Smad3. However, a band of similar molecular mass and intensity was present when non-specific antibodies of the same class as the Smad3 antibody were used, suggesting that a specific interaction between ERα and Smad3 was not present under those conditions. Further experiments in MCF-7 cells by Matsuda et al. (335) included a control IgG antibody, however, whether this antibody was of the same class as the immunoprecipitating antibody is unknown.

The MH2 domain of Smad3 has been implicated in being involved in the interaction with ER (335). Wu et al. (449) have described the interaction between Smad4 and ERα to entail the MH1 domain of Smad4 and the AF1 domain of ERα while Yamamoto et al. (336) have shown that the DNA binding domain of ERα is essential for binding to Smad1. However, the region of the ER that binds with Smad3 has not yet been identified. The region of the ER that interacts with Smad3 is important as antibodies that recognize the same region as that involved in protein interactions may not be able to successfully IP those two proteins. The ERα antibody used by Matsuda et al. (335) to describe the interaction between ERα and Smad3 is unknown; the authors only indicate that the antibody was obtained from Santa Cruz Biotechnology, a company that has several ERα antibodies available. The IP experiments described in this thesis utilized

2 different ERα antibodies; one that recognized the C-terminal of ERα (rabbit polyclonal; Santa Cruz Biotechnology) and one that recognized the full-length form of the receptor (mouse monoclonal; NovoCastra Laboratories). Both of these antibodies have been used previously to successfully co-IP proteins *in situ*. The conditions under which IPs were performed were very similar between Matsuda et al. and us. However, one significant factor that may impact our the ability to observe an interaction between Smad3 and ERα may involve post-translational modifications. As described previously, Smad3 is phosphorylated by TβRI upon ligand-binding; this phosphorylation event is important in mediating the interaction between Smad3 and the co-Smad, Smad4. In the experiments by Matsuda et al. (335), the constitutively active TβRI receptor, T204D, was transiently transfected into the cells along with Smad3 and ERα or the cells were stimulated with TGFβ ligand. Thus, presumably Smad3 would be in a phosphorylated state (although this was not determined in the study). In our experiments, cells were not typically stimulated with TGFβ and thus, the level of phosphorylated Smad3 may have been low in our cells despite the level of p3TP-lux reporter gene activity. If indeed it is only the phosphorylated form of Smad3 that interacts with ERα, then western blotting of an IP extract may not detect the low level of phosphorylated Smad3 that IP with ERα. At the time of these experiments, however, an antibody towards the phosphorylated form of Smad3 was not available and we were unable to determine the level of phosphorylated Smad3 in our cells.

Another possible mechanism through which ER may affect TGFβ/Ap-1 signaling is by influencing the binding of transcription factors to their cognate recognition sequence (132). In this study, we were unable to observe Smad3 binding to the SBE in

our ChIP analysis, most likely due to the inefficiency of our antibodies to IP Smad3. Thus, whether ERα is able to alter Smad3 binding, either directly or indirectly, to the promoter of target genes is unknown. The affinity of Smad proteins for the SBE is too low to support binding of a Smad complex to a SBE (447). In order for efficient binding to occur, the Smad complex must interact with other DNA-binding transcription factors (450). This allows Smads to bind to promoter regions that contain one SBE in the vicinity of the binding sequence for the other cofactor. Therefore, if ER lowers the expression level of other transcription factors that are important in Smad binding to the p3TP-lux promoter, this would effectively inhibit TGFβ transcription. Alternatively, the ER may activate or even repress another gene whose product modulates TGFβ function, either negatively or positively. One such gene candidate is the protooncogene *c-myc* which has recently been identified as a co-repressor of Smad3 signaling (451). Over-expression of *c-myc* effectively inhibits Ap1/Smad transcriptional synergism through its direct interaction with the Smad nucleoprotein complex on DNA without affecting DNA binding (451). As *c-myc* expression is induced by estrogen in ER^+ cells (452), it may disrupt the functional synergism between Ap-1 and Smad3, thus leading to the inhibition of Smad3 transcriptional activity. In addition, the ER may even titrate a Smad activator other than Ap-1 from the p3TP-lux promoter, resulting in decreased TGFβ activity.

It is important to note that the estrogen repression of Smad3 transcriptional activity was observed after 48 h of treatment. Thus, the repression may be indirect and involve activation of certain early response genes, the products of which can interfere with TGFβ induction. For example, activation of the MAPK pathway has been shown to phosphorylate the Smad2/3 co-repressor TGIF, leading to TGIF stabilization and

formation of a Smad-TGIF complex (453). TGIF has been shown to bind to Smad3 and recruit general transcription co-repressors, including HDAC (454), to the promoter region, effectively inhibiting Smad3 activation. Moreover, the linker region of Smad3 contains multiple serine and threonine consensus sites for ERK MAP kinases and extensive phosphorylation of these sites decrease Smad3 signaling activity (455). In contrast, ER phosphorylation by MAPK increases its transcriptional activity in both a ligand-dependent and ligand-independent manner (75). Thus, the MAPK signaling pathway may serve as a mediator for ER inhibition of TGFβ signaling.

Given the results obtained from this thesis, we strongly believe that ER modulates Smad3 transcriptional activity indirectly through yet another signal transduction pathway. Recent evidence strongly implicates the p38 MAPK pathway in tamoxifen and ICI 182,780 induced cell growth arrest in MCF-7 cells (429). Using the p3TP-lux reporter plasmid in MCF-7 cells, Buck et al. (429) demonstrated that antiestrogen activation of p3TP-lux is mediated by p38 MAPK. It was further shown that p38 MAPK is required for antiestrogen induction of $TGF\beta_2$ and TβRII expression and subsequently, activation of Smad3 dependent TGFβ transcriptional activity (429). These observations certainly help to explain our results in MCF-7 cells but still do not help to explain our results in Cos1 cells where antiestrogens inhibited p3TP-lux activity. It would be interesting to determine if MAPKs mediate the ER modulation of Smad3 transcriptional activity in Cos1 cells. To date, four different MAPKs have been described: the ERKs, c-Jun N-terminal kinases (JNKs), p38 MAPKs and ERK5. By using specific activators and/or inhibitors for each MAPK, it would be possible to determine the putative role, if any, of these MAPKs in the ER modulation of Smad3 transcriptional activity.

The p3TP-lux reporter is an artificial construct designed empirically for maximum TGFβ responsiveness. Although it has been used to aid in the understanding of the TGFβ signaling pathway, the question still remains as to whether ER modulates TGFβ/Smad transcriptional activity on endogenous or native promoter sequences. In our study, we examined several endogenous promoters that are known to be activated by TGFβ; in particular, we focused on the type VII collagen promoter. Type VII collagen expression is up-regulated by TGFβ via rapid and transient binding of a Smad3 containing complex to the promoter region (413). The collagen VII promoter consists of a bipartite binding site for Smad3/Smad4 surrounding a putative Ap-1 site. Fibroblasts over-expressing either Smad3 or Jun proteins show an increase in collagen VII expression mediated at the level of gene transcription (397). However, when Smad3 and Jun are co-expressed, Jun inhibits the Smad3 induced increase in promoter activity. This is thought to be mediated via Smad3 and Jun forming direct off DNA protein interactions with one another (397). Therefore, while the collagen VII promoter contains elements similar to that found on p3TP-lux, the configuration of the TGFβ responsive elements and Ap-1 sites is reversed and TGFβ and Ap-1 regulate the promoter in a different manner. Results from this thesis demonstrate that activation of ER up-regulates transcription from this promoter primarily through the Ap-1 site, relieving the inhibitory effect of c-Jun on collagen VII transcription. This provides further evidence to suggest that the ER, by modulating Ap-1 sites in TGFβ responsive genes, is able to alter Smad3 transcriptional activity in target genes. Examples of other endogenous genes that are repressed by E_2 via Ap-1 sites include the human hepatic lipase (456), murine lipoprotein lipase (457), ovine FSHβ

(458), human choline acetyltransferase (459) and gonadotropin releasing hormone receptor (276) genes.

In order for the interaction between Ap-1, TGFβ and ER to occur, all three of these signaling pathways and the corresponding downstream signaling proteins must be co-expressed within breast tissue. Immunohistochemical analysis of nuclear Smad3 and phosphorylated c-Jun of breast tumor tissue microarrays demonstrate that a significant correlation between nuclear Smad3 and phosphorylated c-Jun occurs in ER^+ tumors (as determined by ligand binding assays), providing *in vivo* evidence that these proteins are co-expressed within the same tissue.

Few studies have examined the expression of Smad3 in breast tumor tissue. In one small study, nuclear Smad3 correlated with ER^+ and PR^+ status (438). In a larger study of 456 breast tumors, neither phosphorylated Smad2 nor Smad4 expression was associated with hormone receptor status (342). However, nuclear Smad3 expression has been associated with small, low grade tumors (438) and loss of phosphorylated Smad2 expression is associated with a decrease in overall patient survival (342). In addition, the majority (90%) of breast tumors were positive for phosphorylated Smad2 (342), suggesting that not only is the TGFβ signaling pathway active in breast carcinomas but also that tumor cells proliferate in the presence of activated TGFβ. However, expression levels of nuclear Smad3 decrease with increasing grade (438) and accordingly, a correlation between the loss of the TβR-II receptor and high tumor grade in breast cancer has been shown (341). Our immunohistochemical results also demonstrate that nuclear Smad3 expression is associated with negative node status, and while not significant, there was a trend toward Smad3 expression being associated with smaller tumors. Taken

together, these results suggest that TGFβ may represent a good prognostic biomarker. As a positive ER status is also associated with good prognosis, it is reasonable to hypothesize that Smad3 expression is associated with ER^+ tumors.

While our results confirm an *in vivo* relationship between Smad3 and ER, we cannot address the relationship of ER transcriptional activity and its effects on Smad3 activity *in vivo*. However, using dual immunofluorescence, Ewan et al. (460) were able to not only co-localize ERα with nuclear R-Smad and active $TGF\beta_1$ in the normal mouse mammary gland, but also demonstrated that depletion of $TGF\beta_1$ results in a significant increase in proliferation of ERα positive cells, suggesting that either TGFβ restrains ERα transcriptional activity or that ERα diminishes the cellular response to TGFβ. Estrogens influence cellular proliferation by positively modulating the transcriptional activation of cyclins, the regulatory phosphorylation of cyclin-dependent kinases and by inducing *c-myc* (461). TGFβ, on the other hand, causes cell-cycle arrest late in G1 by inhibiting cyclin-dependent kinase activity and by repressing *c-myc* expression (462). While Ap-1 factors have been implicated in the regulation of cyclin D1 expression by estrogen (277), there is no evidence suggesting that they modulate *c-myc* expression. ER induces c-*myc* via an ERE (463) while TGFβ reduces *c-myc* expression through co-operative binding with cofactors to a SBE (464). It would be interesting to examine whether the *c-myc* or cyclin D1 promoter response to ER or TGFβ is altered by simultaneous TGFβ or ER activation, respectively.

Only a small number of studies have examined the expression of c-Jun in breast tissues. Gee et al.(465), using a phosphorylated c-Jun specific antibody, did not observe any relationship between activated c-Jun expression and either ER or PR status, results

consistent with those described by others (466-468). Similar results were also obtained for c-Fos (468). In addition, no relationship between c-Fos or phosphorylated c-Jun expression with tumor grade, proliferation, response to endocrine therapy or overall survival has been observed (465, 466, 468); although a trend towards shorter mean survival times for patients expressing high levels of c-Jun has been described (465). Nonetheless, total c-Jun protein levels appear to increase during breast tumorigenesis (466) and a large portion of this c-Jun is in an active state (465).

None of the studies described above addressed the relationship between Ap-1 and TGFβ signaling. Interestingly, however, it has been suggested that high levels of activated c-Jun are associated with distant metastasis to the bone (465), a common site of metastatic foci of breast cancer. Several lines of evidence also suggest that TGFβ acts as a pro-metastatic agent in the late stages of cancer progression; treatment of breast cancer cells in culture with TGFβ increases cellular invasive potential (469) and may even direct metastatic cells to the bone (470, 471). While the role of Smad3 in the migratory response to TGFβ in breast cancer cells is unclear, Smad4 appears to be required for bone metastasis by breast cancer cells in mice (472), clearly indicating an involvement of Smad activation in TGFβ induced migration. Thus, TGFβ action changes during breast cancer progression; during the early stages of breast tumorigenesis, TGFβ effectively inhibits breast tumor cell growth while in the late stages of breast carcinogenesis, TGFβ function becomes pro-metastatic. Moreover, ERα expression has been shown to increase dramatically during breast tumorigenesis but tends to decrease during breast cancer metastasis. Thus, if the effect of ER on TGFβ is relieved during metastasis, this would allow for TGFβ to take over in co-operation with Ap-1 factors. Taken together, these

studies suggest that while an interaction between Ap-1, TGFβ and ER may not play a key role in breast tumorigenesis, the interplay between these proteins may be important during breast tumor progression and metastasis.

Future therapeutic approaches in the treatment and prevention of breast cancer will be based on the precise molecular profile of an individual patient. The more we understand about the signaling mechanisms of the ER and its interactions with other signaling pathways, the better the selection of the most appropriate hormonal intervention. Perhaps by targeting several pathways at once, there will be less chance of recurrence and metastasis to distant organs, resulting in better overall survival rates for this disease.

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