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GRADE / DEGREE

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FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Estrogen Receptor Functions as an Inhibitor of NF-kB

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# The Estrogen Receptor Functions as an Inhibitor of NF-κB

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This thesis is submitted as a partial fulfillment of the requirements for the degree of

**MASTER OF SCIENCE**

in Cellular and Molecular Medicine

University of Ottawa
Ottawa, Canada

September 6, 2006

*Your file Votre référence*
*ISBN: 978-0-494-49207-9*
*Our file Notre référence*
*ISBN: 978-0-494-49207-9*

# *ABSTRACT*

The development of antiestrogen resistance has been correlated with changes in ERα expression/mutation and a rise in NF-κB activity. We believe that NF-κB is activated in both ER-positive and ER-negative breast cancer cells and that it is attenuated by liganded ERα in ER-positive cells by regulating the formation of p50 and p65 complexes. It is possible that mutations in the ER alter its ability to bind and regulate NF-κB activity, which allows constitutive activation of NF-κB and confers estrogen-independence to these cells.

Here, we demonstrate that increased expression of ER in both ER-negative as well as ER-positive cells results in decreased cell viability and decreased NF-κB activity. We also confirm a direct interaction between ERα and NF-κB subunits *in vitro*. We also demonstrate that NF-κB subunits, p65 and p50, are capable of interacting with ERα on an ERE *in vivo* and that this interaction is enhanced by treatment with E2. Moreover we are the first to show that inhibition of NF-κB results in increased ER activity, indicating a possible mutual repression between ERα and NF-κB. Lastly, we demonstrate that treatment with tamoxifen does not have the same inhibitory effects on NF-κB as does estrogen.

Together, these observations suggest that an interaction between ERα and NF-κB have a vital role in the development of breast cancer, estrogen-independent growth and resistance to antiestrogens.

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# *LIST OF ABREVIATIONS*

| | |
|---|---|
| AF | Activation function |
| Amp | Ampicillin |
| BAFF | B-cell-activating factor |
| Bcl-2 | B-cell lymphoma/leukemia-2 |
| Bp | base pair |
| °C | degrees Celsius |
| CAT | chloramphenicol acetyl transferase |
| CBP | cAMP-responsive element-binding protein |
| cDNA | complementary DNA |
| ChIP | chromatin immunoprecipitation |
| CMV | cytomegalovirus |
| $CO_2$ | carbon dioxide |
| CoA | co-activator |
| CoR | co-repressor |
| Cpm | counts per minute |
| DBD | DNA binding domain |
| DCC | dextran-coated charcoal |
| DMEM | Dulbecco's modification of Eagle's Medium |
| DNA | deoxyribonucleic acid |
| DTT | dithiothretol |
| E2 | 17-β-estradiol, estrogen |

| | |
|---|---|
| ECL | enhanced chemiluminescence |
| EDTA | ethylenediaminetetracetic acid |
| EMSA | Electrophoretic mobility shift assay |
| ERAP-160 | estrogen receptor associated protein 160 |
| ERE | estrogen response element |
| ER | estrogen receptor |
| FBS | fetal bovine serum |
| GR | glucocorticoid receptor |
| HAT | histone acetyl transferase |
| HDAC | histone deacetylase |
| HEPES | 4-(2-hydroxylethyl)-1-piperazine ethanesulfonic acid |
| hER | human estrogen receptor |
| Hsp | heat shock protein |
| $I\kappa B^{SR}$ | I kappaB super repressor |
| IAP | inhibitor of apoptosis |
| IgG | immunoglobulin G |
| IκB | inhibitor of κB |
| $I\kappa B\alpha^{SR}$ | IκBα super repressor |
| IKK | IκB kinase |
| IL | Interleukin |
| IPTG | isopropylthioglycoside |
| Kb | kilobase |
| KCl | potassium chloride |

| | |
|---|---|
| kDa | Kilodaltons |
| L | Litre |
| LB | Luria-Bertani |
| LBD | ligand binding domain |
| LiCl | lithium chloride |
| LTβR | lymphotoxin β receptor |
| M | molar |
| mAB | monoclonal antibody |
| mg | milligram |
| min | minute |
| ml | millilitre |
| mM | millimolar |
| MOI | multiplicity of infection |
| MOPS | 3-[N-morpholino] propanesulfonic acid |
| NaCL | sodium chloride |
| NaOH | sodium hydroxide |
| NCoR | nuclear co-repressor |
| NEMO | NF-κB essential modulator |
| Neo | Neomycin |
| NES | nuclear export signal |
| NF-κB | nuclear factor kappaB |
| NLS | nuclear localization sequence |
| OD | optical density |

| | |
|---|---|
| PAGE | polyacrylamide gel electrophoresis |
| PBP | peroxisome proliferator-activated receptor (PPAR)-binding protein |
| PBS | phosphate buffered saline |
| PCD | programmed cell death |
| pCAF | p300/CBP-associated factor |
| PMSF | phenylmethylsulfonyl fluoride |
| PFU | plaque forming units |
| PR | progresterone receptor |
| PRF | phenol red free |
| Puro | puromycin |
| PVDF | polyvinylidenedifluoride |
| RDH | Rel homology domain |
| RIPA | radioimmunoprecipitation assay buffer |
| RIP-140 | receptor interacting protein 140 |
| RNA | ribonucleic acid |
| Rpm | revolutions per minute |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SDS | sodium dodecyl sulfate |
| SERM | selective estrogen receptor modulator |
| SMRT | silencing mediator of retinoid and thyroid hormone receptors |
| SRC | steroid coactivator |
| TAD | transactivation domain |
| TBP | TATA-binding protein |

| | |
|---|---|
| TBS | Tris buffered saline |
| TBS-T | Tris buffered saline-Tween |
| TE | Tris-EDTA |
| µg | microgram |
| µl | microlitre |
| UV | ultraviolet |
| Wt | wild type |

# *ACKNOWLEDGEMENTS*

This thesis is by far the most important scientific achievement in my life, and it would have been unattainable without people who supported me and believed in me. I would like to express my gratitude to all those who gave me the possibility to complete this work.

Most of all I would like to thank my supervisor, **Dr. Christine Pratt**, whose support, guidance, and encouragements helped me completed this work. Her confidence and scientific enthusiasm inspired me. Her greatest qualities were her availability and extensive knowledge.

I am also grateful to acknowledge the other members of my lab, which I will never forget the company and help that they gave me. In particular, I am thankful to **Minying Niu** and **Rosanna Lau**.

I also want to thank my family, who taught me the value of hard work by their own example. They rendered me enormous support during the whole tenure of my research and who always have faith in me.

Finally I would like to give my special thanks to my best friend **Josee Perron**, who provided never ending help and support, offering suggestions for improvement, and providing encouragement. I am lucky to have such a good friend.

# *CHAPTER 1*

# INTRODUCTION

## 1.1 BREAST CANCER

### *1.1.1 Characteristics*

Breast cancer is the most common form of malignant disease in women worldwide, affecting about one in nine women in North America alone (Harrell 1999), with approximately one third dying from the disease (Vahabi 2003). Primary breast carcinomas can present as non-invasive lesions, either intraductural or intralobular carcinomas, or as invasive cancers. Tumors that are invasive can spread rapidly by infiltrating through tissue spaces and can invade both lymphatic and blood vessels. They can produce a high incidence of distant metastases, mostly lung, soft tissues, liver, bones and adrenals glands (Lippman et al. 1988).

Cancer is believed to be the result of multiple genetic events which lead to the loss of growth control and/or inhibition of cell death. The most common risk factors associated with breast cancer are familial association (Plu-Bureau and Thalabard 1998), including mutations in the breast cancer genes BRCA1/2, and the levels of exogenous and endogenous hormones (i.e. estrogen and progesterone) (Soderqvist 1998). The risk of breast cancer is increased by early menarche and late menopause, and by the prolonged administration of high dose estrogens after menopause. All of these factors increase the exposure of breast

epithelium to estrogen and/or progesterone. The first evidence for a connection between estrogen and breast cancer was presented in 1896, when Beaston, a British physician, discovered that by removing the ovaries of postmenopausal women, he could cause a regression of advanced breast tumors. More recent data also points to other factors such as weight, diet and environment as having important roles in the development of breast cancer (Mannisto et al. 1999).

### *1.1.2 Endocrine treatments of Breast Cancer*

Many of the current treatments for breast cancer involve blocking the mitogenic effects of estrogens. Antiestrogens primarily function through their ability to compete with available estrogens for binding to the estrogen receptor (ER). Antiestrogens can be divided into two classes according to their chemical structure and differential functioning in tissues. Steroidal antiestrogens such as ICI 182, 780, act as so called pure antiestrogens with no agonist activity at all. Non-steroidal antiestrogens, such as tamoxifen (Dhingra 1999) and raloxfene (Fuchs-Young et al. 1995), are partial antiestrogens i.e. they exert an estrogen like-function in some tissues and antiestrogen function in others. Antiestrogens with selective estrogenic activities in certain tissues and antiestrogenic in others might be better therapeutic agents because complete blockage of estrogenic activity in all tissues is not desirable as it can lead to loss in bone density and might also have negative effects on cognitive functions. Therefore selective ER modulators (SERMs) would optimally function as estrogens in bone and the cardiovascular system, and as antiestrogens in the breast and uterus.

Currently, the most common SERM used is tamoxifen, which is a classic partial agonist and exhibits both species and tissue specificity. A recent study concluded that adjuvant therapy with tamoxifen decreased the 10-year recurrence risk of patients with ER-positive tumors by 50%, and reduced their risk of death from metastatic disease by 26% (Early Breast Cancer Trialists' Collaborative Group 1998) In addition, the degree of benefit from tamoxifen therapy (defined as the reduction in recurrence and mortality) was directly related to the amount of ER present in the tumor (Early Breast Cancer Trialists' Collaborative Group 1998). The recent decline in breast cancer mortality in western countries is thought to be, in large part, due to the use of tamoxifen therapy that blocks the proliferative effects of estrogen through ERα and perhaps ERβ (Hermon and Beral 1996).

In humans, tamoxifen exhibits antagonist effects in the breast and agonist effects in the bone and endometrium (Morales et al. 2005). The differences in agonistic versus antagonistic activities in various tissues could be related to the ER co-activators and corepressors present in these tissues (Graham et al. 2000). For example, increased levels of co-activators such as SRC3, SRC1 and ErbB2 enhance the agonistic properties of tamoxifen *in vitro* (Smith et al. 1997). The ability to generate these tissue specific effects has prompted the search for other SERMs.

Aromatase inhibitors are another form of antiestrogen treatment and have demonstrated superiority to tamoxifen in controlling the growth of ER-positive breast cancers (Jordan 2004). Most importantly, aromatase inhibitors do not

produce some of the estrogen-like side effects observed in patients treated with tamoxifen, such as the stimulated growth of cells associated with endometrial cancer (Jordan 2004). However, unlike tamoxifen, aromatase inhibitors do not reduce the incidence of osteoporotic fractures.

A new SERM has since been developed that not only maintains bone density, but also reduces the risk of breast cancer by 70% and does not lead to increased incidence of endometrial cancer (Jordan 2004). Raloxifene is now available in the U.S. for the prevention and treatment of osteoporosis in postmenopausal women. Raloxifene has been shown to significantly reduce the number of breast cancer cases, from 1.00% to 0.25% (Cummings et al. 1999).

Interestingly, studies indicate that when some tumors becomes E2-independent E2 acts as an apoptotic agent, rather than a growth stimulus, through an ER-mediated mechanism. E2 treatment is currently being used in trials to treat phase II and phase III tamoxifen-resistant breast tumors (Jordan 2004). Furthermore, adenovirus-mediated expression of the estrogen receptor in the E2-independent T47D cell line has been shown to inhibit cell growth and induce apoptosis (Lee et al. 2001).

Several human breast cancer cell lines are available and are commonly used model systems for the investigation of molecular pathogenesis and disease progression of breast cancers. The MCF-7 cell line represents the ER-positive/hormone-dependent group of cells while MDA-MB-231 and SKBr-3 cell lines represent ER-negative/hormone-independent growth. In addition, Clarke and colleagues have developed the MCF-7(LCC1) cell line, which are ER-

positive but hormone-independent by passaging MCF-7 cells in ovariectomized nude mice (Clarke et al. 1989, Brunner et al. 1993). These cells retain ERα expression but are capable of growing in the absence of E2 both *in vitro* and *in vivo* (Clarke et al. 1989, Brunner et al. 1993). Therefore, the LCC1 subline of MCF-7 cells provides an experimental model of progression from a hormone dependent to a hormone-independent state.

## 1.2 Estrogen Receptor

### *1.2.1 Estrogen Receptor Function*

The Estrogen Receptor (ER) is a member of the nuclear/steroid hormone receptor superfamily, a group of ligand-dependent transcription factors involved in growth, differentiation and development. Estrogen, through its cognate ER, has been found to affect normal development and differentiation of mammary tissues and has been implicated in the progression of many breast cancers.

The ER has been shown to regulate the transcription of various genes either directly by binding specific DNA sequences, known as estrogen response elements (ERE), or indirectly by associating with other transcription factors, including AP-1, SP-1 and possibly NF-κB, and influencing their transcriptional activity.

Until recently, it was believed that a single ER (ERα) existed. However, in 1996, another isoform was cloned from rat prostate cDNA library and named ERβ (Kuiper et al. 1996). ERα and ERβ are separate genes, and do not

represent splice variants. The precise roles of the two isoforms of ER in cellular metabolism are unclear, but tissue specific expression and differential regulation have been observed in several situations. Figure 1 shows the different structural domains (termed A through F) of ERα (Green et al. 1986) and ERβ (Fuqua and Cui 2004). There is a predicted 96% homology in the DNA-binding domain (C), and a 53% homology between the E/F domains, but the A, B and hinge (D) domains are not well conserved between the two ERs (Mosselman et al. 1996, Fuqua and Cui 2004). ERβ is capable of binding EREs and activating the transcription of E2 responsive genes, although it binds with slightly lower affinity to E2 than does ERα (Mosselman et al. 1996, Fuqua and Cui 2004).

It is generally observed that ERβ is a less potent transcription factor than ERα on the majority of ERE promoters (Picard and Yamamoto 1987). It has also been shown that the two ERs can form functional heterodimers on DNA (Ogawa et al. 1998), that the heterodimers may be preferentially formed compared to homodimers (Pettersson et al. 1997), and that the ERα:ERβ heterodimers may have a higher affinity to EREs than do homodimers (Cowley et al. 1997).

### *1.2.2 Estrogen Receptor Structure*

Like all members of the steroid receptor family, the ER consists of six functional domains designated A-F (Kumar et al. 1987): The amino-terminal A/B domain contains the hormone independent activation function (AF-1), the middle C domain is very highly conserved and contains the DNA binding domain (DBD), consisting of two zinc finger motifs that are responsible for ER binding of EREs

and also dimerization of the ER on EREs (Klein-Hitpass et al. 1988). Following the C domain is the D domain, known as the hinge region, which has shown to be involved in co-regulatory protein binding (Jackson et al. 1997) and possible interaction with NF-κB subunit p65 (Stein and Yang 1995). The carboxy-terminal domains E and F contain the ligand binding domain which comprises the ligand-dependent transcription activation functions AF-2 and AF-2a, a hsp90 binding region, a nuclear localization signal, as well as another dimerization domain (Figure 1) (Chambraud et al. 1990, Picard et al. 1990, Montano et al. 1995, Norris et al. 1997).

### *1.2.3 Estrogen Receptor Activation*

Transcriptional activation of ER-regulated genes is mediated by the activation functions AF-1 and AF-2, which represent the two modes of ER activation: ligand-independent and ligand-dependent. The AF-1 domain is hormone-independent, whereas the AF-2 domain is hormone-dependent (Kumar et al. 1987). While both AF-1 and AF-2 are required for maximal ER transcriptional activity, with certain promoters, AF-1 and AF-2 can function independently (Fuqua and Cui 2004). Both the AF-1 and AF-2 domains have also been shown to interact with distinct components of the basal transcription

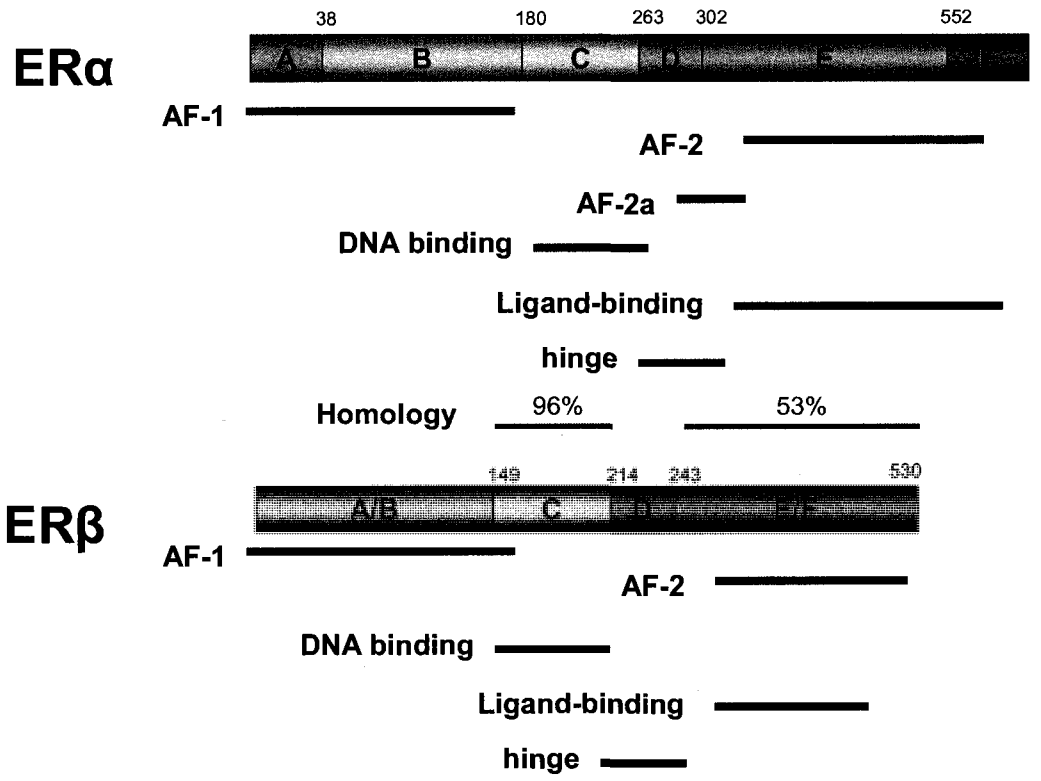



**Figure 1. Estrogen receptor structure.**
The protein domains of ERα and ERβ labelled A-F, amino acid numbers corresponding to the start of each domain are above. Relative positions of some of the known functional domains are represented by solid black bars below, and the regions of high homology are indicated by solid blue bars.

machinery and to mediate the cell-specific agonist and antagonist effects of antiestrogens (McInerney and Katzenellenbogen 1996).

Ligand-independent activation of ER activity occurs via phosphorylation of various serine and tyrosine residues in the AF-1 and AF-2 domains, which can be induced by signaling pathways downstream of growth factor receptors such as EGF, IGF, and insulin receptor as well as ErbB2 (Her2/neu) (Chen et al. 2002). Increased phosphorylation causes ligand-independent activation of ER, enhances estrogenic and antiestrogen-dependent activity, regulates dimerization, and alters the association of the receptor with co-regulatory proteins depending on the phosphorylation site (Chen et al. 2002). The AF-1 domain has been found to be regulated by growth factors acting through the MAP kinase pathway (MAPK) (Kato et al. 1995). MAPK, activated by signaling from EGFR or ErbB2 (Font de Mora and Brown 2000), was shown to phosphorylate a specific serine residue (Ser 118) in the AF-1 region of the human ER$\alpha$ that functionally activates AF-1, thereby establishing a mechanism of crosstalk between membrane type growth factors and nuclear receptors.

Interestingly, patients whose tumors expressed high levels of both steroid coactivator 3 (SRC-3) and ErbB2 had worse outcomes with tamoxifen therapy than all other patients combined (Osborne and Fuqua 1994). High affinity ligands bind the ErbB receptors which may then activate downstream signals such as the MAPK pathway and the AKT pathway (Yarden and Sliwkowski 2001). Activation of these pathways lead to cell division and migration (both associated with tumorigenesis), as well as cell-adhesion, differentiation and apoptosis

(Yarden and Sliwkowski 2001). In many cancers, the ErbB pathway is hyperactivated by a range of mechanisms, including overproduction of ligands, overexpression of receptors or constitutive activation of receptors (Yarden and Sliwkowski 2001). ErbB2 is overexpressed in 15-30% of breast cancers (Ross and Fletcher 1998). Moreover, there is an inverse relationship between ErbB2 and ER expression, in that most tumors overexpressing ErbB2 do not express ERα (Borg et al. 1994). Previous studies have suggested that resistance to antiestrogens in ER-positive tumours is dependent on the overexpression of ErbB2 (Shou et al. 2004). The mechanism of resistance is not yet clear; however, it has been proposed that ligand-independent activation of the ER by MAPK, which themselves are phosphorylated and thereby activated by ErbB2, may contribute to the resistance (Osborne and Fuqua 1994).

There are two models of ligand-dependent activation ERα: the classical activation which is induced by binding to agonist and results in direct DNA binding and subsequent transcriptional stimulation, and a non-classical activation induced by an agonist, which in turn causes the interaction of ER with other proteins that can then bind DNA and activate transcription. Structural analysis indicates that ligands regulate AF-2 activity by directly affecting the structure of the ligand binding domain (LBD) (Brzozowski et al. 1997, Shiau et al. 1998). E2 diffuses across the plasma membrane and binds to the ER, which in the absence of estrogen is normally part of a protein complex consisting of a dimer of hsp90 and hsp70 (Chambraud et al. 1990). Ligand binding induces conformational changes in the ER, which causes dissociation of chaperones such as hsp70 and

hsp90 (Chambraud et al. 1990), and association of co-regulatory proteins. Among these co-regulatory proteins are the p160 type coactivators, including such proteins as steroid coactivator 1 (SRC-1) and steroid coactivator 3 (SRC-3), (Weis et al. 1996, Anzick et al. 1997), as well as estrogen receptor associated protein 160 (ERAP-160) and receptor interacting protein 140 (RIP-140) (Halachmi et al. 1994, Cavailles et al. 1995). In addition, other co-activators such as p300 and the p300/CBP-associated factor (pCAF) have been shown to bind ER and activate transcription (Kamei et al. 1996). Furthermore, interactions with CREB-binding protein (CBP) provide the physical link to basal transcription machinery (Hanstein et al. 1996, Kamei et al. 1996). Interestingly, both the p160 family and CBP/p300 have histone acetyltransferase (HAT) activity (Chen et al. 1997), which modulate the rate of transcription initiation through alterations in the state of chromatin organization at the promoter of target genes. These acetyltransferases add acetyl groups to nucleosomal histones, thereby loosening their interaction with DNA and exposing important residues to the basal transcription machinery (Schiltz et al. 1999). Conversely, deacetylation of chromatin leads to chromatin condensation, which inhibits gene transcription. Interestingly, data suggests that the antagonist function of mixed antiestrogens might, in part, be due to inhibition of interaction with co-activators or by allowing interaction with co-repressor proteins (Smith et al. 1997). The receptor/co-activator complex can then bind its cognate E2 responsive elements.

The classical ERE consists of an inverted repeat of two half sites of the motif GTCA separated by any three nucleotides. The association of the ER to

ERE is determined by the conformation of the receptor and also the availability of co-factor proteins. A number of nonpalindromic sequences without significant homology to classical EREs have also been identified that can potentially function as estrogen response elements (Dana et al. 1994, Norris et al. 1997). Upon ERE binding, the ER:co-factor complex interacts with the basal transcription machinery and also with histones, resulting in either the stimulation or inhibition of gene transcription (Hanstein et al. 1996, McKenna et al. 1999).

In the absence of hormone, the ER interacts with nuclear receptor corepressors silencing mediator for retinoid and thyroid receptors (SMRT) and nuclear receptor corepressor (NcoR) (Smith et al. 1997, Lavinsky et al. 1998, McKenna et al. 1999) to repress transcription. Upon binding of hormone, the corepressors dissociate from the DNA-bound receptor, which can then recruit a nuclear coactivator (NcoA) complex.

The ERα can also alter transcription by binding other transcription factors, such as AP-1, Sp1 or NF-κB (Porter et al. 1997, Cerillo et al. 1998, McKay and Cidlowski 1998, Webb et al. 1998). These complexes can then bind to DNA and activate or inhibit transcription. In addition, ERα/Sp-1 interactions have been characterized in the promoters of several E2-responsive genes, including c-myc, cathepsin D and Bcl-2 among others (Dubik and Shiu 1992, Krishnan et al. 1994, Rishi et al. 1995, Porter et al. 1997, Sun et al. 1998).

Traditionally, it was believed that the ER bound to its response element in target genes and remained bound as long as the stimulus (E2) was present. However, more recent evidence has demonstrated that ligand-bound ER-

transcription complexes assemble and disassemble on target promoters in a cyclic fashion (Shang et al. 2000). Using chromatin immunoprecipitation analysis, Shang *et al.* (2000) found that the endogenous p160 co-activators, CBP, p300 and peroxisome proliferator-activated receptor (PPAR)-binding protein (PBP) are recruited in specific order to the ER transcription complex after stimulation with E2 in MCF-7 breast cancer cells (Shang et al. 2000). In the first cycle of transcription, E2-bound ER first recruits p300 and PBP, followed by CBP and then pCAF (Shang et al. 2000).

### *1.2.4 Estrogen Receptor and Breast Cancer*

Approximately 60-65% of primary breast cancers are initially estrogen receptor (ER) positive (Putti et al. 2005). These tumors tend to be more differentiated and are also associated with relatively better prognosis with better response to antiestrogens such as tamoxifen. In contrast, ER-negative tumors represent a more malignant group of tumors that tend to be associated with poor prognosis. It has been proposed that over time, ER positive breast cancers lose ER expression in their progression towards a more malignant phenotype. ER-negative breast cancers lack ERα mRNA although the ERα gene is generally not mutated (Barrett-Lee et al. 1987, Yaich et al. 1992).

In a normal, nonpregnant, nonlactating human breast, only 7% of the total luminal epithelial cell population express ER and this expression fluctuates with the menstrual cycle (Petersen et al. 1987, Ricketts et al. 1991). Scattered ER-positive cells or small clusters of ER-positive cells are often found in lobules

throughout the mammary parenchyma (Petersen et al. 1987). In contrast, ER expression is significantly increased in premalignant as well as malignant breast lesions (Netto et al. 1990). In some cases, even noncancerous breast tissues from patients with breast tumors show elevated levels of ER expression (Netto et al. 1990).

Since premalignant lesions in the breast are often characterized by intense proliferation of epithelial cells, ER expression may confer a selective growth advantage in the presence of estrogen, and it has been hypothesized that these ER-expressing epithelial cells play a role in the progression of premalignant breast lesions to breast cancer (Fuqua 2001)

It has been shown that the antitumor effects of tamoxifen are directly linked to ER levels in breast tumors (Rutqvist et al. 1989). Nevertheless, many tumors that initially respond to tamoxifen therapy eventually become resistant, and the development of such resistance in advanced breast cancer is a common cause for treatment failure, but the mechanism associated with the development of antiestrogen resistance is poorly understood. Several mechanisms might account for the development of resistance. Loss of ER expression or expression of a mutated ER may lead to the activation of other growth and survival pathways. In addition, alterations of ER cofactor(s) might promote the agonistic effects of tamoxifen (Osborne and Fuqua 1994, MacGregor and Jordan 1998, Clarke et al. 2001). Although loss of ER expression is sometimes observed in tamoxifen-resistant tumors, many tumors remain ER-positive (Johnston et al. 1995). Studies reveal that the ER status of metastases usually correlates with the ER

status of the primary tumor. It has been shown that only 20% of ER-positive tumors will have ER-negative metastasis (Hull et al. 1983, Robertson 1996). Thus, although it is possible that ER-positive tumors lose expression of ER during the progression of the disease to metastatic tumors, it is an infrequent event. The stability of ER expression *in vivo* is also reflected *in vitro*. Only a few cell lines have an ER-negative phenotype. It has been reported that the ER-positive T47D cell line can spontaneously convert to an ER-negative subline (Reddel et al. 1988, Graham 2nd et al. 1990). Studies have also attempted to select for ER-negative sublines from ER-positive T47D and MCF-7 breast cancer cells by growing them in the presence of tamoxifen (Graham 2nd et al. 1990, Montano and Katzenellenbogen 1997) and longterm selection in the absence of estrogen (Katzenellenbogen et al. 1987, Murphy et al. 1990, Pink et al. 1996). However, in the majority of cases, ER expression was maintained despite the conversion to a hormone-independent state (Fuqua 2001).

ER mutations have been observed in some cell lines selected *in vitro* for resistance to antiestrogens, as well as in tumor samples (McGuire et al. 1992, Wolf and Jordan 1994). More recently, a 61kDa variant of ER, corresponding to an exon 3 deletion was found to be expressed in two different sublines of MCF-7 cells: estrogen-independent tamoxifen-sensitive LCC1 cells and estrogen-independent tamoxifen resistant LCC2 cells. It is believed that the exon 3 deletion is a result of aberrant mRNA splicing and studies show that there is increased ERΔ3 expression in response to tamoxifen treatment. Exon 3 encodes the second zinc finger of the DNA binding domain. Therefore, the ERΔ3

protein is not able to bind EREs and does not activate transcription by directly binding DNA. ERΔ3, however, is capable of forming dimers with itself and wild type (wt) ER, and studies have suggested that ERΔ3 functions as a dominant negative variant (Wang and Miksicek 1991). Furthermore, studies have shown that this protein was not sustainable in MCF-7 cells but was maintained for at least 17 passages in LCC1 cells (Han et al. 2004). Numerous studies have also investigated whether genetic changes such as epigenetic silencing of ER gene expression by methylation or mutation of the actual ER coding sequence may account for the loss of ER expression. Studies have shown that ER-negative breast cancer cells are devoid of ER mRNA (Weigel and deConinck 1993) and that the expression of ER can be restored by inhibiting DNA methylation (Ferguson et al. 1995). However, other groups have shown that there is no correlation between ER methylation and ER gene expression in breast tumors (Hori et al. 1999). It is therefore possible that the resistance is not entirely due to a loss of ER, but could also be the result of changes in ER structure and/or function (Fuqua and Cui 2004).

## 1.3 Nuclear Factor Kappa B

Breast cancer cell lines expressing ERα contain lower levels of NF-κB activity in comparison with ER-negative cell lines, which display high constitutive NF-κB activity (Nakshatri et al. 1997, Pratt et al. 2003). Recent studies have also

shown a correlation between NF-κB activity and the acquisition of an estrogen-independent phenotype in breast cancer cells (Pratt et al. 2003).

Nuclear factor Kappa B (NF-κB) is a transcription factor involved in the regulation of immune response (Baeuerle and Baltimore 1996), inflammation (Barnes and Karin 1997), apoptosis (Bours et al. 2000), cell-cycle progression (Karin et al. 2002), and has been implicated in oncogenesis (Chen et al. 2001). Its involvement in the latter three processes is indicative of the role that NF-κB may play in the tumorigenic process as well as the promise of a targeted therapeutic intervention (Baeuerle and Baltimore 1996).

NF-κB was first described as a complex that supershifted DNA fragments containing the DNA sequence motif 5'-GGGACTTTCC-3' in electrophoretic mobility shift assays (EMSAs) (Edbrooke et al. 1989). This NF-κB binding site, known at the time as the B motif, was identified as a B-cell-specific element in the intronic κ light chain enhancer (Lenardo et al. 1987). Shortly thereafter, it was discovered that this element was also functional in other cell types. NF-κB is now recognized as a transcription factor that is found in many cell types and is now considered an important factor in the tumorigenic process mainly because it exerts strong anti-apoptotic functions in cancer cells.

### *1.3.1 Structure of the NF-κB family*

NF-κB was originally identified as a heterodimer of p50 and p65 (Urban et al. 1991, Grimm and Baeuerle 1993), although a variety of other homo- and

heterodimers have since been described. Presently, five members of the NF-κB/Rel family have been identified in mammals including p65 (Rel A), p50/p105 (NF-κB1), p52/p100 (NF-κB2), RelB and cRel (Figure 2). All of the Rel/NF-κB family members share a highly conserved domain called the Rel Homology Domain (RHD) of about 300 amino acids, responsible for DNA binding, dimerization, association with IκB (Inhibitor of κB) and nuclear localization (Gilmore 1999). The NF-κB family of proteins is organized into two classes. The p50/p105 and p52/p100 subunits of the NF-κB family are synthesized as precursors containing multiple copies of ankyrin repeats at their C-termini. Processing of these proteins leads to the production of DNA binding-competent p50 and p52 subunits. The p100 and p105 proteins serve regulatory functions in the cell (described below) and should not be considered solely precursor forms. RelA/p65, c-Rel and RelB are directly synthesized as mature proteins and possess a C-terminal transactivation domain. NF-κB dimers can bind specific κB consensus sequences on DNA (5'- GGGRNYYYCC-3', where R is an unspecified purine, N is any nucleotide and Y is an unspecified pyrimidine) (Ghosh et al. 1990, Kieran et al. 1990). The most common dimer is the p65/p50 heterodimer, which is involved in regulating many κB-dependent genes.

Transactivation functions have been located in the C-terminal regions of p65 (Schmitz and Baeuerle 1991), c-Rel (Bull et al. 1990) and both the C-terminal and N-terminal region of RelB (Dobrzanski et al. 1993). RelA contains two transactivation domains (TADs) within its C-terminus: activation domain TA1, consisting of the 30 C-terminal amino acids, and TA2, located within the 90

amino acids next to TA1 (Moore et al. 1993). Both TADs contain a common sequence motif (Schmitz et al. 1995). Neither p50 nor p52 contain TADs, and are therefore only active as DNA binding partners with p65, c-Rel or RelB. Additionally, both c-Rel and RelA interact with the TATA-binding protein (TBP), and the C-terminal end of RelA interacts with the basal transcription factor TFIIB (Schmitz et al. 1995). Studies *in vivo* and *in vitro* indicate that different NF-κB dimers have different transcriptional activation properties (Schmid et al. 1991, Lin et al. 1995). Evidence also indicates that interactions between NF-κB proteins and other transcription factors influence its ability to regulate gene expression in a selective manner.

### *1.3.2 Regulation of NF-κB activity*

The NF-κB proteins are regulated at several levels, including transcriptional, translational and post-translational processing. In resting cells, NF-κB is maintained in the cytoplasm in an inactive form through its interaction with one of several inhibitory molecules, including IκB -α, IκB-β, IκB-ε, p105, p100, IκB-ζ and Bcl-3 (Baeuerle and Baltimore 1996, Karin and Ben-Neriah 2000, Yamazaki et al. 2001).

The IκB family members are characterized by the presence of five to seven ankyrin repeats that assemble into elongated cylinders that bind the dimerization domain of NF-κB dimers (Figure 2) (Hatada et al. 1992). IκB proteins were originally thought to sequester NF-κB in the cytoplasm by masking its nuclear localization sequence (NLS). However, IκB-α can only mask one NLS

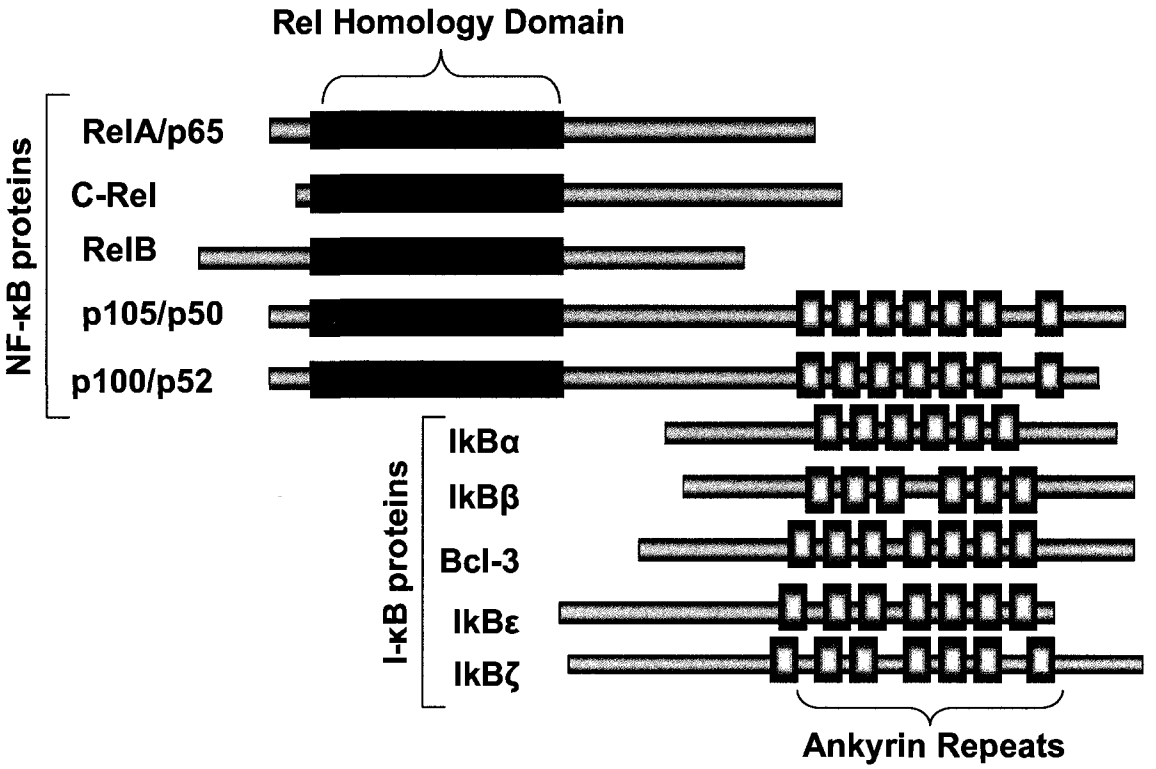



**Figure 2. The NF-κB/Rel and IκB families of proteins.**
The NF- κB family is characterized by the presence of the Rel homology domain (RDH) (red box). The Iκ-B family have multiple copies of ankyrin repeats. P105/p50 and p100/p52 are proteins that contain both the RDH and ankyrin repeats.

in the dimer; so NF-κB -IκBα complexes undergo constitutive nuclear translocation (Malek et al. 2001). Importantly, a nuclear export signal (NES) in IκB-α prevents high steady-state levels of these complexes in the nucleus (Huang et al. 2004). In contrast, IκBβ complexes fail to undergo such shunting as IκBβ is capable of masking both NLSs (Malek et al. 2001). In order to prevent overactivation of NF-κB, newly synthesized IκB molecules migrate to the nucleus via it's NLS to remove DNA bound NF-κB dimers and turn off NF-κB dependent transcription (Sachdev et al. 1998). Subsequently, NF-κB/IκB complexes use a nuclear export sequence (NES) to return to the cytoplasm (Gilmore 1999).

As previously mentioned, both p50 and p52 are produced as p105 and p100 kDa precursors, respectively, and lack transactivational domains. Regulation of p50 and p52 DNA interactions occurs at the level of proteolytic processing from the precursor proteins. Phosphorylation of the precursor proteins signals proteolytic degradation of the C-terminal ends via a ubiquitin-dependent proteosome pathway, therefore allowing the release of active p50 and p52, which can then translocate to the nucleus. The unprocessed forms of p50 and p52 have an IκB-like function in their ability to prevent nuclear translocation of both heterodimers and homodimers NF-κB complexes.

Bcl-3 is a unique member of the IκB family because it exerts a positive effect on gene transcription by displacing inactive p50/p50 or p52/p52 homodimers from DNA, enabling transactivating heterodimers to bind the free κB sites. Bcl-3 can also act by disrupting the association between transcriptionally inactive p50 and p52 homodimers, allowing association of a transactivating

partner. Furthermore, Bcl-3 directly activates transcriptional function by forming a DNA bound complex with p50 and p52 which imparts transactivating ability on these normally inactive dimers (Bours et al. 1993, Fujita et al. 1993).

In response to certain stimuli including cytokines (tumor necrosis factor-α, Interleukin-1), lipopolysacharide, UV radiation and oxidative stress, IκB is phosphorylated and degraded. (Baldwin Jr 1996), This event allows NF-κB complexes to remain in or migrate into the nucleus where they can act as transcription factors, activating cell survival genes such as inhibitors of apoptosis (IAPs) and cyclin D1 (Chu et al. 1997, Gilmore 1999, Hinz et al. 1999, F. Chen et al. 2001) (Figure 3). Thus, certain genes regulated by NF-κB can be transcriptionally activated within minutes following exposure to the relevant inducer. Phosphorylation of IκB is mediated by IκB kinases (IKKs) (Karin 1999). The IKK complex consists of 2 highly homologous subunits, IKKα and IKKβ, and a non-enzymatic regulatory component called IKKγ (Karin 1999). While both IKKα and IKKβ are bona fide kinases that are capable of phosphorylating two serine residues of IκB *in vitro*, IKKβ is the primary kinase that phosphorylates IκB *in vivo* (Li et al. 1999, Park et al. 2005). IKKγ, which is also known as NF-κB Essential Modulator (NEMO), is a crucial regulatory subunit that has no catalytic activity (Rudolph et al. 2000, Karin et al. 2002). Therefore, IKKα and IKKβ along with their regulator subunit NEMO phosphorylate IκB, which is then recognized by a specific ubiquitin ligase that catalyses ubiquitination of IκB. Ubiquinated IκB is then recognized and degraded by the 26S subunit of the proteosome (Zandi

and Karin 1999). NF-κB is now free to translocate to, or remain in, the nucleus and activate the transcription of its target genes.

Two NF-κB activation pathways exist (Figure 3). The first, the classical pathway, is normally triggered in response to microbial and viral infections or exposure to pro-inflammatory cytokines that activate the tripartite IKK complex leading to phosphorylation-induced IκB degradation. This pathway, which mostly targets p50:RelA and p50:cRel dimers, depends mainly on IKKβ activity (Li et al. 1999). Besides the canonical pathway of NF-κB activation, other means of activation have also been described. For example, where IKKβ is mainly responsible for IκB phosphorylation to begin the classical pathway, IKKα phosphorylates the IκB-like C-terminal domain of p100 and p105, resulting in the translocation of p52 and p50 dimers to the nucleus in an IKKβ-independent manner (Figure 3) (Pomerantz and Baltimore 2002). This second pathway is triggered by certain members of the TNF family (B-cell-activating factor (BAFF)), CD40 ligand (CD40L) and lymphotoxin β receptor (LTβR), through selective activation of IKKα homodimers (Karin and Ben-Neriah 2000, Ghosh and Karin 2002, Karin et al. 2002). Both pathways regulate cell survival and death (Senftleben et al. 2001).

### *1.3.3 NF-κB and Cancer*

Based on earlier studies, NF-κB was originally believed to have a mainly pro-apoptotic role because of its rapid induction in cells in response to apoptotic stimuli and its involvement in the expression of some apoptotic genes, including

TNF-α, c-myc, and Fas-L (Hsu et al. 1999, Chen et al. 2001). However, more recent work has revealed a predominantly anti-apoptotic effect of NF-κB in response to a variety of apoptotic stimuli. Candidate anti-apoptotic genes regulated by NF-κB include those encoding the cell cycle regulatory protein cyclin D1, the mitochondrial membrane-stabilizing proteins Blf-1 and Bcl-xl, the caspase inhibitors cIAP1 and XIAP, and the TNF receptor associated factors TRAF1 and TRAF2 (Chen et al. 2001).

Programmed cell death (PCD), or apoptosis, is an essential mechanism for any multicellular organism to eliminate cells that are in excess or potentially dangerous (Strasser et al. 2000). Once the decision to undergo cell death has been made, a proteolytic cascade is triggered in the cell, which ultimately leads to the degradation of chromosomal DNA (Fraser and Evan 1996).

However, in some cases, apoptosis can be evaded by the activation of caspase-independent signaling cascades that function to block the apoptotic responses. A good example of this is TNF-R-mediated activation of NF-κB that induces expression of anti-apoptotic proteins (Chen et al. 2001).

Knock-out studies have shown that RelA-deficient mice are embryonic lethal because of an increased sensitivity of hepatocytes to the apoptotic action of TNFα (Beg et al. 1995). Similar phenotypes were observed after inactivation of genes coding for the NF-κB inhibitors IKKβ and NEMO (Li et al. 1999). Higgins *et al.*. (Higgins et al. 1993) have shown proliferation and tumorigenicity of

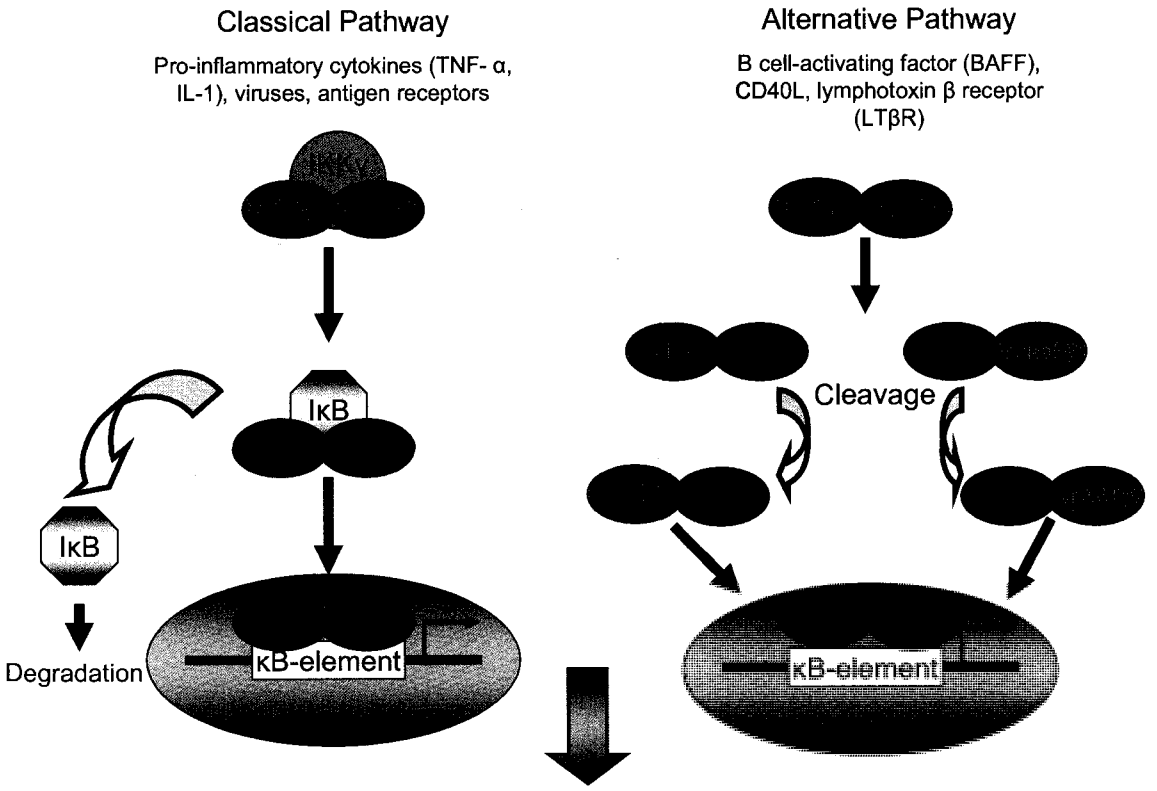



**Figure 3. NF-κB activation pathways.**
The classical pathway is activated by a variety of inflammatory signals, resulting in activation of the IKK complex leading to the phosphorylation and degradation of IκB. This process is dependent mainly on IKKβ and results in p50:p65 and p50;cRel activation. The alternative pathway is strictly dependent on IKKα homodimers. Phosphorylation of the precurser p105 and p100 proteins signals proteolytic degradation of the C-terminal ends allowing release of active p50 and p52, which can then translocate to the nucleus.

several tumor cells lines, including MCF-7 and T47D, to be inhibited by antisense oligonucleotides to RelA. In addition, activation of NF-κB through the disruption of IκBα regulation was shown to result in malignant tumor transformation (Beauparlant et al. 1994). Wang *et al.* (1996) showed that cells with inactivated NF-κB apoptose in response to pro-apoptotic signals other than TNF-α (Wang et al. 1996). Furthermore, the expression of a dominant negative form of the NF-κB inhibitor, IκB, reduced tumorigenic potential and resistance to chemotherapeutic drugs in mouse mammary tumours (Gilmore 1999).

As previously mentioned, the NF-κB signaling pathway regulates the expression of diverse target proteins involved in cell cycle progression and cell survival. The control of the progression of dividing cells through the G1, S, G2 and M phase of the cell cycle depends on a group of regulatory proteins, termed cyclins, which exert their effects by interacting with specific cyclin-dependent kinases (CDKs) (Hinz et al. 1999). The enzymes responsible for allowing entry and passage through the G1 phase are primarily composed of the cyclin D family (D1, D2 and D3), which bind to and activate CDK4 and CDK6. A canonical NF-κB binding site is present within the cyclin D1 and cyclin D2 promoters (Karin et al. 2002), suggesting that NF-κB promotes cell cycle entry through upregulation of cyclin D1. In addition, cyclin D1, which is elevated in 50% of breast cancers, has been suggested to be an essential NF-κB target gene in normal mammary development and breast tumorigenesis (Joyce et al. 2001).

The ability of NF-κB to suppress apoptosis and regulate the cell cycle clearly indicates that NF-κB may participate in many aspects of tumorigenesis.

In fact, elevated NF-κB levels have been found in many cancers including non-small cell lung carcinoma, thyroid cancer, T- and B- lymphocyte leukemia, melanoma, colon cancer, bladder cancer, several virally induced tumors and breast cancer (Chen et al. 2001).

The key role of NF-κB in tumorigenesis makes it a favourable target for therapeutic intervention of cancer. Indeed, experimental evidence has shown that inhibition of NF-κB by anti-sense oligonucleotides to RelA, and degradation-resistant IκB-α can enhance the efficacy of chemotherapies and radiation (Mayo and Baldwin 2000, Baldwin 2001).

### *1.3.4 NF-κB , Breast Cancer and Hormone-Independence*

NF-κB activation regulates normal mammary development and the constitutive activation of NF-κB has been linked with to the development of hormone-independent (ER-negative) breast cancers, in part due to the direct transcriptional control of many genes that direct cell proliferation and invasion (Cao and Karin 2003). NF-κB activity is reported to be minimal in ER-positive cancer cell lines, yet constitutively elevated in ER-negative cancer and cell lines (Nakshatri et al. 1997, Pratt et al. 2003). While breast cancer cell lines show predominantly increased p65 subunit expression and p65/p50 DNA binding activity, breast tumor samples show selective upregulation of p50, p52, Bcl-3 and c-Rel expression and constitutively increased DNA binding by complexes composed primarily of these subunits (Cogswell et al. 2000). In addition, studies

have shown an association with increased NF-κB p50 activation and reduced tamoxifen sensitivity (Zhou et al. 2005).

Clinical findings indicate that high-risk ER-positive breast cancers may be prognostically identified by increased NF-κB p50 DNA-binding activity and suggest that inhibition of NF-κB activation may improve the endocrine responsiveness of high-risk ER-positive breast cancers (Zhou et al. 2005).

## 1.4 ER AND NF-κB CROSSTALK AND THE DEVELOPMENT OF ESTROGEN INDEPENDENCE

### *1.4.1 Transcription Factor Crosstalk*

There is a growing body of evidence supporting estrogen's ability to affect gene expression through a non-classical pathway in which the estrogen receptor modulates the activity of other transcription factors (Discussed briefly in ER Activation section). Interactions between steroid receptors and members of the activator protein 1 (AP-1) family of transcription factors have been studied extensively. AP-1 family members, like c-Jun and c-fos, and the glucocorticoid receptor (GR) have been shown to repress each other's functions (Irgens et al. 1977, Jonat et al. 1990, Schule et al. 1990, Yang-Yen et al. 1990). A direct interaction between GR and AP-1 was shown *in vitro*, resulting in impaired DNA binding (Schule et al. 1990, Yang-Yen et al. 1990). It has clearly been shown that the degree of repression of AP-1 by the GR and the progesterone receptor (PR) is cell type- and promoter-specific (Tzukerman et al. 1991, Zhang et al.

1991, Konig et al. 1992), suggesting that intermediary proteins are likely to be involved, the expression of which can vary between cell types (Kalkhoven et al. 1996). This type of interaction has also been demonstrated for NF-κB repression by ER (Stein and Yang 1995), GR (Caldenhoven et al. 1995), and PR (Kalkhoven et al. 1996). Activation of NF-κB by tumor necrosis factor-α (TNF-α) results in repression of PR, while hormone-induced PR is able to repress TNF-α induced NF-κB activity (Kalkhoven et al. 1996). Furthermore, the mutual repression also requires a direct physical interaction that was found to involve part of the AB domain and the E domain for hormone-independent and –dependent repression, respectively, with both types of repression requiring the C domain (Kalkhoven et al. 1996). Studies have shown that the mechanism by which glucocorticoids inhibit IL-6 gene transcription is by direct physical association and functional antagonism between NF-κB and the GR (Ray and Prefontaine 1994). Importantly there is also evidence that molecular crosstalk occurs between NF-κB and ER, resulting in ER-mediated inhibition of NF-κB as discussed below (Stein and Yang 1995, McKay and Cidlowski 1998).

### *1.4.2 ER/ NF-κB Crosstalk*

Serum levels of estradiol (E2) have been shown to be inversely proportional to serum levels of IL-6 in mice (Masiukiewicz et al. 2000), and humans (McKane et al. 1994), and IL-6 expression has been shown to be repressed by E2-activated ER (Stein and Yang 1995, Ray et al. 1997). Surprisingly, there is no ER binding site in the IL-6 promoter, so it is believed that

the repression is a result of an interaction between the ERα and subunits of NF-κB (Stein and Yang 1995). Galein *et al.* (1997) found that the addition of *in vitro* translated ER interfered with the formation of NF-κB complexes on the IL-6 NF-κB enhancer element (Galien and Garcia 1997). ERα has also been shown to bind p65 *in vitro*, but this interaction has yet to be reported *in vivo* (Ray et al. 1997). Cvoro *et al.* (2006) was able to coimmunoprecipitate ERα with p65 and p50 in U2OS cells expressing ERα (Cvoro et al. 2006). In addition, evidence indicates that ER inhibits the ability of p50 homodimers to bind DNA (Ray et al. 1997).

RelA (p65) has been shown to be the subunit of NF-κB primarily responsible for gene activation and that interacts directly with ER (McKay and Cidlowski 1998, Valentine et al. 2000). A more recent study has shown that both ERα and ERβ are capable of inhibiting NF-κB-mediated expression in a hormone-dependent manner and that repression is also dependent on dimerization of p65 to p50 (Liu et al. 2005).

Ghisletti *et al.* (2005), using EMSA, showed that p65 DNA binding is prevented by a 10-minute exposure to E2. In addition, it was found that lipopolysaccharide (LPS) -induced recruitment of p65 to MIP-2 and IκBα promoters was significantly reduced by treatment with E2. Furthermore, it was shown that the decrease in promoter occupancy by p65 was not a result of reduced nuclear translocation or increased degradation in response to treatment with E2 in MCF-7 cells (Ghisletti et al. 2005). Studies have shown that E2 can inhibit apoptosis in cardiac myocytes, and that this effect is associated with a

reduction in NF-κB activity (Pelzer et al. 2001). This study also reports that, despite reduced NF-κB DNA binding, there is no change in expression or reduction in nuclear content of p65, p50 or IκB in response to treatment with E2 (Pelzer et al. 2001).

Many studies have aimed at identifying the domains of the ER that are involved in the repression of NF-κB. Galien *et al.* (1997) showed that a HE14 ERα mutant containing only the E and F domains (hormone binding domain and AF-2 domain) was unable to inhibit NF-κB activity (Galien and Garcia 1997). In addition, a mutant lacking the DNA-binding domain was unable to repress IL-6 promoter activation (Ray et al. 1997, Liu et al. 2005). These same domains of the GR have been shown to be required for repression of NF-κB by GR (Wissink et al. 1997). Interestingly, mutations in the dimerization domain of ER have also been shown to abolish repression of GR, suggesting that homodimerization may be necessary despite the absence of interaction with DNA (Valentine et al. 2000).

An ER mutant devoid of the DNA binding region is unable to repress IL-2 promoter activation (Ray et al. 1997), however the DNA binding domain is not required for the protein-protein interaction of ER with NF-κB (Stein and Yang 1995). It was also shown that internal deletion of the hinge region D did not impair the ability of ER to inhibit IL-6 promoter activation, however it did diminish the ability of ER to bind p65 *in vitro* (Stein and Yang 1995, Ray et al. 1997). These results indicate potential roles for the DNA-binding domain, and the hormone-binding domain as well as the D domain (hinge region) for ER/NF-κB

complex formation and the inhibition of NF-κB activity (Figure 4) (Stein and Yang 1995, Galien and Garcia 1997, Ray et al. 1997)

### *1.4.3 ER and NF-κB crosstalk and the development of E2-independence.*

ER/NF-κB interactions have also been implicated in the progression of breast cancer. Nakshatri and co-workers (1997) show that the progression of mammary carcinoma cell lines from ER-positive to ER-negative correlates with constitutive activation of NF-κB and IκB (Nakshatri et al. 1997). They hypothesized that if ER represses both constitutive and inducible NF-κB activity, overexpression of NF-κB-inducible genes in ER-negative cells may contribute to malignancy and resistance to Tamoxifen (Nakshatri et al. 1997).

Studies have shown that MCF-7 cells are capable of forming tumors in ovariectomized nude mice in the presence of an estrogen (E2)-release pellet, and that removal of the pellet leads to rapid tumor regression (Shafie and Grantham 1981). Moreover, studies from our lab have demonstrated that withdrawal of E2 from either MCF-7 cells *in vitro* or from MCF-7 tumors in ovariectomized nude mice results in activation of p50:p50 DNA binding (Pratt et al. 2003). This increase in DNA binding was also found to coincide with an increase in Bcl-3 protein expression, which can provide a transcriptional activation function for p50 (Bours et al. 1993, Pratt et al. 2003). It is therefore possible that under E2-free selective pressure, ER-positive breast cancer cells may eventually lose both their requirement and responsiveness for E2, possibly

due, in part, to the rise in NF-κB activity which provides sufficient growth and survival signals to a subset of cells.

Together these observations implicate an interaction between ERα and NF-κB in having a vital role in the development of breast cancer and estrogen independence.

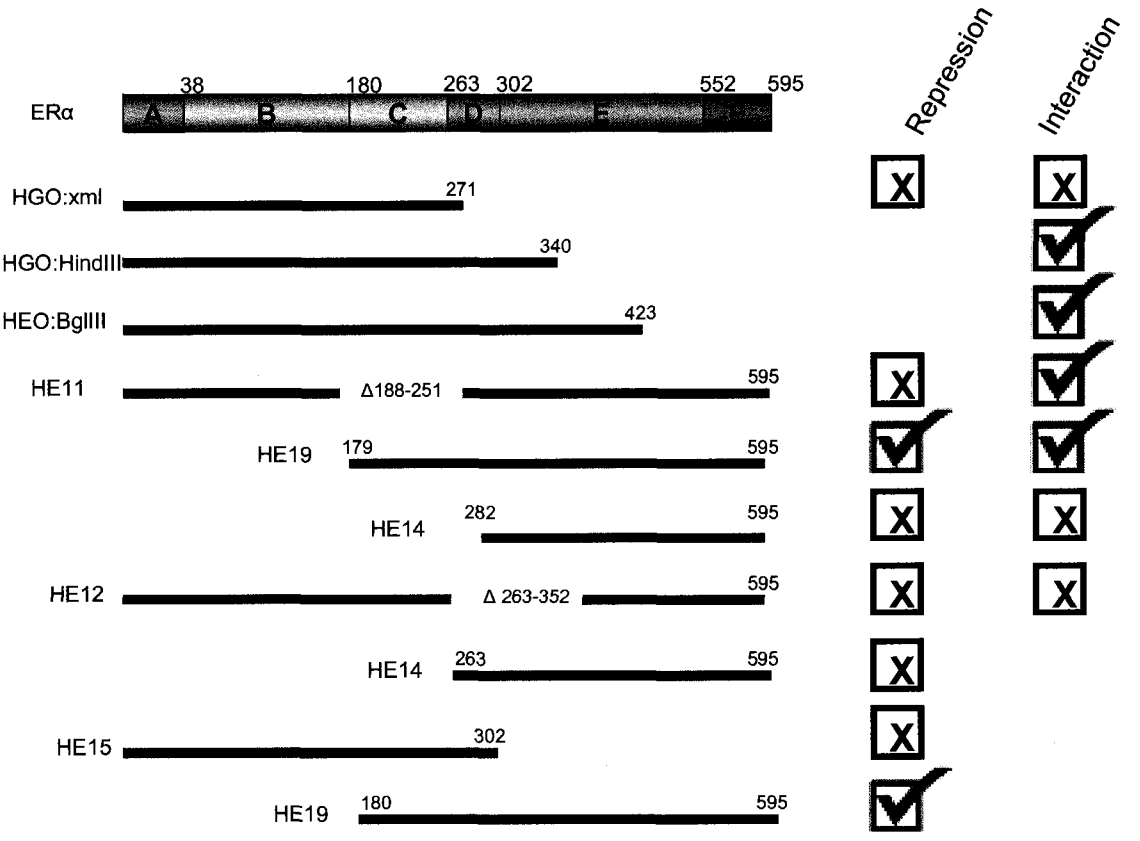



**Figure 4. Regions of ERα required for interaction with and repression of NF-κB.**

ERα deletion mutants lacking the C domain, D domain and part of the E domain are unable to repress NF-κB activity. ERα deletion mutants lacking the C domain and part of the E domain are capable of interacting with NF-κB. Deletion of the D domain abolishes ERα/ NF-κB interaction.

## 1.5 Statement of the Problem

Significant progress has been made in elucidating the roles of both NF-κB and ER in breast cancer. There is an increasing amount of evidence linking NF-κB activity and the acquisition of an estrogen-independent phenotype in breast cancer cells. Furthermore, treatment with E2 has been shown to decrease NF-κB-mediated transcription activation and a direct physical interaction between the two transcription factors has been shown. In our initial studies we found that p50 is capable of forming a complex with ligand-bound ERα on the *ERE in vitro*. However, no interaction of the ERα with the NF-κB enhancer element was found, suggesting sequestration of p50 by the ligand-bound ERα. Consequently, we hypothesize that NF-κB is activated in both ER-positive and ER-negative breast cancer cells and that it is attenuated by liganded ERα in ER-positive cells by regulating the formation of p50 and p65 complexes. It is possible that mutations in the ER alter its ability to bind and regulate NF-κB activity, which allows constitutive activation of NF-κB and confers estrogen-independence to these cells.

**OBJECTIVES**

In order to further investigate the mechanisms of estrogen/ER-dependent regulation of NF-κB and interactions between the ER and NF-κB we sought to satisfy the following three objectives:

I. Determine if NF-κB forms a complex on the ERE.

1. *Confirm if there is differential binding pattern of ERα:p50:ERE in MCF-7 ($ER^{wt}$)and LCC1($ER^{mutant}$) cell lines.*
2. *Determine the effect that wild type ER has on NF-kB activity in ER-positive and ER-negative breast cancer cells.*
3. *Determine the ability of E2/ER to recruit NF-κB proteins to an ERE in vivo.*
4. *Confirm binding of ER to p65 and p50 in vitro.*

II. Determine what effect 4-OH-Tamoxifen has on NF-κB activity.

III. Determine what effect inhibition of NF-κB has on the expression of E2 regulated proteins.

# *CHAPTER 2*

# MATERIALS AND METHODS

## 2.1 CELL CULTURE

MCF-7 and LCC1 breast cancer cells were maintained in high glucose Dulbecco's modified Eagle Medium (DMEM) (Invitrogen) containing phenol red and supplemented with 5% v/v heat inactivated fetal bovine serum (FBS) (Wisent), 1% non-essential amino acids, and 10μg/ml gentamicin (Invitrogen). Stable MCF-7 clones expressing an IκBα super repressor ($IκB^{sr}$) (generated in our lab) and pcDNA3 controls generated in our lab) were maintained in high glucose DMEM supplemented as above with the addition of 2mg/μl puromycin (Sigma). MDA-231 cells were maintained in low glucose DMEM supplemented as above. SK-BR-3 breast cancer cells were maintained in the same conditions as above except that the DMEM contained 10% FBS rather than 5%. Cells were incubated at 37 °C in 5% $CO_2$. For passaging cells were washed with phosphate buffered saline (PBS) (137 mM sodium chloride [NaCl], 2.7 mM potassium chloride [KCl], 10mM disodium hydrogen orthophosphate, 1.76 mM potassium dihydrogen orthophosphate [pH 7.0 – 7.4]) prior to trypsinization. PBS was removed by aspiration and 1ml trypsinizing solution (0.25% trypsin [Invitrogen], 1mM EDTA in PBS) was added per 100 mm plate. The cells were incubated for 3 min at 37 °C, dispersed by vigorous pipetting then added to fresh media containing serum to inactivate the trypsin. The cells were then plated at the

required density or isolated by centrifugation. For experiments requiring estrogen (E2) depletion, cells were pre-cultured for 4 - 7 days in phenol red-free (PRF) DMEM (Invitrogen) containing 5% FBS stripped of steroids by absorption to dextran-coated charcoal (DCC) (see below). E2 (17-β-estradiol) (Sigma – E2758) was added for the indicated times at a final concentration of $10^{-8}$ M from a $10^{-5}$M stock solution in ethanol for western blot and EMSA analysis and at a final concentration of $10^{-5}$ M (100nM) for ChIP assays. In some cases cells were treated with 4-hydroxytamoxifen (Sigma – H7904) for the indicated times at a final concentration of $10^{-7}$ M from a $10^{-3}$ M stock.

## 2.2 Preparation of Dextran-Coated Charcoal (DCC) Stripped Serum

FBS was stripped of endogenous steroid hormones by treatment with DCC (0.25% Norit A, 0.0025% dextran in 0.01M Tris-HCl pH 8.0). The serum was incubated with the DCC for 30 min at 45°C, and the charcoal, along with steroid hormones, was removed by centrifugation at 3000rpm for 10 min at 4°C.

## 2.3 EMSA Analysis

Electrophoretic mobility shift assays (EMSA) were performed with nuclear extracts from cultured cells as described by Fried (1989) (Fried 1989). NF-κB oligonucleotides, obtained from Promega (E3291), and the ERE oligonucleotides were end labeled with T4 polynucleotide kinase using [γ-$^{32}$P] ATP (Amersham). 5μg of nuclear extract was mixed with 5μl of DNA binding buffer (20mM HEPES

[pH 7.9], 0.2 mM EDTA, 0.2nM EGTA, and 2mM DTT in 50% glycerol), 5μg of poly(dI-dC), and 0.2ng of labeled probe in a final volume of 20μl and then incubated at room temperature for 25 min. For supershift experiments, 2 μg of each antibody was added to extracts and left for 1 hr prior to addition of the labeled probe. Equivalence of extract loading was demonstrated by EMSA with a DNA fragment containing the consensus Sp1 binding site (Promega). Samples were loaded on a 5% native polyacrylamide gel and run in nondenaturing Tris-glycine buffer.

## 2.4 ENUMERATION OF VIABLE CELLS

Cells were washed once with 1X PBS and isolated by trypsinization and centrifugation as described above. Cells were stained with 0.2% trypan blue (GIBCO) and viable cells enumerated using a hemocytometer as determined by their ability to exclude trypan blue. At least 3 samples were counted from each plate, and 3 different plates were analyzed for each parameter.

## 2.5 WESTERN BLOT ANALYSIS

Cell monolayers were washed twice with PBS and lysed in 200 μl of radioimmunoprecipitation assay buffer (RIPA) (1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS) in PBS plus 10μl/ml PMSF [Sigma], 1mM sodium orthovanadate [Sigma], 30μl/ml aprotinin [Sigma]). The cell lysate was incubated for 10 min on ice. Insoluble material was removed following centrifugation at 12,000 x *g* for 20 min at 4 °C, and soluble protein concentrations

were determined with a Biorad Dc Protein Assay kit. Proteins (15 - 25 μg) were separated on 7%, 10% or 12.5% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) transfer membranes (Perkin Elmer). The membranes were blocked for one hr in 5% skim milk powder dissolved in Tris-buffered saline (20mM Tris-HCl [pH 7.6], 137 mM NaCl) containing 0.1% Tween-20 (TBS-T), and then incubated with the appropriate primary antibody dissolved in blocking solution plus 0.02% sodium azide for 1 hr at room temperature. The blots were then washed for 15 min and once for 5 min in TBS-T prior to a one-hr incubation with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody, diluted to 1:5000 in blocking solution. The blots were again washed in TBS-T as above and the bands were detected using the Enhanced Chemiluminescence (ECL) Analysis System (Amersham Biosciences) according to the manufacturer's protocol.

## 2.6 Plasmid Isolation

Plasmids were isolated from overnight DH5α bacterial cultures grown in Luria-Bertani (LB) broth (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl containing 50ug/ml Ampicillin (Amp) A (Sambrook and Russel 2001). Competent cells were transformed with plasmid DNA by incubation on ice for 45 min, followed by a 45 second heat shock period at 42 °C and a 1 hr incubation period at 37 °C with shaking. Colonies were grown overnight on LB agar plates (Sambrook and Russel 2001) containing 50 μg/ml Amp (VWR). Single colonies were picked with a sterile pipette tip and grown overnight in LB containing100

μg/ml Amp. Plasmid DNA was isolated from the overnight culture using either the QIAGEN or Sigma Maxi Kit or Mini prep kit as per the manufacturer's protocol.

## 2.7 ADENOVIRUS INFECTION

Cells were plated at desired confluence in half of the final volume serum free media and incubated at 37 °C for 1 hr. Adenovirus was diluted to 2.5 X $10^5$ plaque forming units (PFU) in serum free media and the appropriate volume was added to the cell culture depending on the desired multiplicity of infection (MOI). Following infection the cells were then incubated for an additional 4 hrs after which media containing 2 times the amount of serum was added to the final volume. Cells were incubated for 3 to 4 days before harvesting.

## 2.8 TRANSIENT TRANSFECTIONS

Cells were transfected in 60mm or 100mm dishes using the Fugene 6 Transfection Reagent (Roche Molecular Biochemicals) as per the manufacturer's protocol. Cells were seeded at 2.5-3.0 x $10^5$ cells/60mm plate or 7.5 x $10^5$ cells/100mm plate. For κB reporter assays cells were transfected with an expression vector containing the human estrogen receptor (hER) or empty vector (pcDNA3) and co-transfected with either a NF-κB -luciferase reporter gene or mutant kB-luciferase reporter gene (control vector). For the first ERα reporter assay cells were transfected with either a vector containing two copies of the Bcl-2 promoter containing EREs (Dong et al. 1999) driving luciferase expression (Bcl-2-Bcl-2-luciferase )(generated in our lab), empty vector (pGL3-basic) as a negative control or pGL3-control as a positive control. For the second ERα

reporter assay cells were transfected with either a vector containing the vitellogenin ERE (Klein-Hitpass et al. 1988) driving the expression of chloramphenicol acetyltransferase (CAT) or empty vector (pcDNA3) as a negative control. Cells were incubated with the fugene/DNA mixture in DMEM containing 5% FBS as well as other appropriate supplements (see cell culture). In some cases cells were grown in PRF DMEM containing charcoal-treated FBS and were treated with either vehicle (0.1% ETOH), E2 ($10^{-8}$M) or 4-hydroxytamoxifen ($10^{-7}$M).

## 2.9 Chloramphenicol AcetylTransferase (CAT) Assay

Cell monolayers were washed twice with PBS and harvested in 200µl of cold 0.25M Tris pH 7.8 then lysed by two freeze thaw cycles with vortexing in between. Cell extracts were then heated to 65 °C for 10 min. 40µl of cell lysate was then added to CAT assay buffer {[$^3$H]Acetyl-coenzyme A (CAT assay grade) (Amersham - TRK688) 0.375µCi or 1.5 µl at 250µCi/ml)}, 169mM Tris [pH 7.8], 2mM chloramphenicol) and incubated 1 hr at 37°C. After incubation the cell extract was diluted with 200µl of ice cold ethyl acetate, vortexed for 1 minute and centrifuged for 3 min at 12 000 x g. After centrifugation, 180µl of the upper aqueous phase containing the n-butyryl-$^3$H-labelled chloramphenincol was transferred to a scintillation vial for the liquid scintillation counting (LSC) of the radioactive product.

## 2.10 LUCIFERASE ASSAY

Cells were washed twice with PBS buffer and harvested in 200µl of 1X Reporter Lysis Buffer (Promega). Room temperature extract was mixed with 100µl of room temperature Luciferase Assay Reagent (Promega) and immediately placed in a Lumat LB 9507 (Berthold Technology) luminometer where the light intesity was measured.

## 2.11 CHROMATIN IMMUNOPRECIPITATION ASSAY (ChIP)

The ChIP assays were performed by using a ChIP assay kit (Upstate, 17-295) as suggested by the manufacturer with some modifications. MCF-7 cells were grown to 80% confluence in PRF DMEM supplemented with 5% charcoal-dextran-stripped FBS. Cells were treated with E2 (100nM) for 45 min after which cells were washed twice with PBS and chromatin was cross-linked with 1% formaldehyde at room temperature for 10 min. Cells then were rinsed twice with ice-cold PBS containing protease inhibitors (1mM PMSF, 1µg/ml aprotinin and 1µg/ml pepstatin A), collected into PBS containing protease inhibitors (1mM PMSF, 1µg/ml aprotinin and 1µg/ml pepstatin A) and centrifuged for 5 min at 2000 x g. Cells were then resuspended in 200µl of Lysis buffer per 1 X $10^6$ cells, supplemented with protease inhibitors (1mM PMSF, 1µg/ml aprotinin and 1 µg/ml pepstatin A), and incubated on ice for 10 min. Extracts were sonicated to shear chromatin. After centrifugation, supernatants were diluted 10-fold in Dilution Buffer (supplemented with protease inhibitors as above) and precleared at 4°C

for 30 min with salmon sperm DNA-protein A-Sepharose and immunoprecipitated with anti-Erα, anti-p50 or anti-p65 antibody overnight at 4°C. Immunoprecipitation with normal rabbit immunoglobulin G (IgG) was performed to evaluate the presence of nonspecific interactions, and aliquots of genomic DNA were analyzed by PCR to normalize for DNA input. Immunocomplexes were collected with 60 µl of salmon sperm DNA-protein A-Sepharose for 1 hr at 4°C. Pellets were washed for 3-5 min on a rotating platform with 1ml of each of the following buffers: i) Low Salt Immune Complex Wash Buffer; ii) High Salt Immune Complex Wash Buffer; iii) LiCl Immune Complex Wash Buffer. Precipitates were then washed two times with TE buffer and extracted by adding 100µl of elution buffer (1% SDS, 0.1 M NaHCO3, 0.2 M NaCl) and incubated overnight at 65 °C. DNA fragments were purified with a QIAquick Spin Kit (Qiagen). The primers used for the ChIP assays were as follows: PS2 forward primer, 5'-CAG GCC TAC AAT TTC ATT AT - 3'; PS2 reverse primer, 5'-GGG GTG CCA CCG TGA CCT TCG AG-3'. For PCR, 1-5 µl from a 30 µl DNA extraction and 20-30 cycles of amplification were used.

## 2.12 *IN VITRO* TRANSLATION OF P50 AND P65

Full length p50 and p65 were *in vitro* transcribed and translated using the TNT T7/T3 coupled reticulocyte lysate system (Promega) as recommended by the supplier. Radioactive translation was performed in parallel and the products

subjected to migration in 12% SDS–PAGE. The gel was dried and autoradiographed on Hyperfilm MP (Amersham).

## 2.13 GST PURIFICATION

GST-ER deletion mutants were first grown by transforming BL21 competent bacteria and incubating 50 ml cultures overnight. After overnight incubation cultures were diluted 1:10 in LB and incubated at 37°C for an additional 3 to 4 hrs until the $OD_{600}$ reached between 0.5 and 1.0. Production of the recombinant GST fusion protein was then induced by adding 1mM isopropylthioglyycoside (IPTG) at 37 °C for 4 hrs. Cells were recovered by centrifugation and lysed in 25 ml 1 X PBS containing 5mM EDTA, 0.1mg/ml PMSF, 1mg/ml lysosyme, 1% Triton X, and one Complete Mini, EDTA-free protease inhibitor cocktail tablet (Roche). The lysate was sonicated 6 times using a Sonifier 400 (Branson) at a constant duty cycle and an output level of 2 for 30 seconds each time. Samples were then clarified by centrifugation at 10000 xg. The supernatant was incubated for 1 hr on a rotating platform at 4°C with 750 µl of a 50% slurry of glutathione sepharose 4B (Amersham) that had been equilibrated with 1 X PBS containing 1mM DTT. Beads were washed 4 times with 1X PBS. The purified GST fusion proteins were analyzed by SDS-PAGE.

## 2.14 GST PULLDOWN

100µl of Glutathione-Sepharose 4B bead-GST fusion protein mix was combined with 5 µl of a $^{35}$S-labeled *in vitro* transcribed and translated p50 or p65 in a final volume of 200 µl of LSBT (20 mM HEPES-NaOH (pH 7.9)-25 mM NaCl-2.5 mM MgCl2-0.1 mM EDTA-0.05% Nonidet P-40-1% Triton X-100 ) containing 1 mM DTT and 1 mM PMSF. Samples were incubated on a rotary shaker overnight at 4 °C. The beads were then washed four times with LSBT and once with 50 mM Tris (pH 6.8). The bound proteins were then mixed with an equal volume of 2X-SDS PAGE gel-loading buffer. Samples were boiled for 10 min and then analyzed by SDS-PAGE.

## 2.15 ANTIBODIES

The following antibodies were used for western blot analysis: anti-ERα (HC-20) (Santa Cruz), anti-NF-κB p50 (H-110) (Santa Cruz), anti-ErbB2 (C-18) (Santa Cruz – sc-284), anti-HIAP-1 (E40) (Epitomics), anti-actin (Sigma A-2066), anti-cyclin D1 (HD-11) (Santa Cruz), anti-GADD45α (C-4) (Santa Cruz) and anti-pS2 (FL-84) (Santa Cruz).

The following secondary antibodies were used for western blot analysis: peroxidase-conjugated AffiniPuro goat anti-rabbit IgG (H + L) (Jackson Research Laboratories Inc.); peroxidase-conjugated AffiniPuro goat anti-mouse IgG (H + L) (Jackson Research Laboratories Inc.).

The following antibodies were used for supershift in electrophoretic mobility shift assays: Anti-NF-κB p50 (H-119) (Santa Cruz), anti-NF-κB p65 (Santa Cruz), anti-ERα (Santa Cruz).

The following antibodies were used in chromatin immunoprecipitation assays: NF-κB –50 (Upstate), Anti-NF-κB p50 (Rockland), NF-κB p65 (Santa Cruz), anti-ERα (HC-20) (Santa Cruz) and normal Rabbit IgG (Santa Cruz).

# *CHAPTER 3*

# RESULTS

## 3.1 INCREASED ERα EXPRESSION REDUCES VIABLE CELL NUMBER IN MDA-231, SKBR-3, LCC1 AND MCF-7 CELLS

Previous studies have shown that estrogen inhibits the growth of longterm tamoxifen-treated breast tumors (Liu et al. 2003). Antitumor activity of E2 has also been reported for estrogen-deprived breast cancer cells *in vitro* (Fernandez et al. 1998, Song et al. 2001) and tamoxifen-stimulated breast tumors *in vivo* (Chisamore et al. 2001). Moreover, these findings have been extended to the clinical setting, where estrogen has been shown to prevent the growth of breast cancers that have developed resistance to successive antiestrogenic therapies (Lonning et al. 2001). We therefore wanted to determine if re-expression of ERα in ER-negative cells and overexpression in ER-positive cells would result in similar growth inhibitory effects. In order to determine the effect wild type ERα would have on cell viability and morphology we infected two ER-negative breast cancer cell lines, MDA-MB-231 and SKBr-3, and two ER-positive cell lines, the estrogen-independent LCC1 cells and estrogen-dependent MCF-7cells, with an adenovirus carrying the human ERα gene. Cells were grown in DMEM media

without added estrogen. All experiments were done in triplicate and mean values were calculated.

Four days after infection, the ERα-expressing MDA-231 cells began to show morphological differences (Figure 5). Cells expressing ERα had smaller, flattened cell bodies and began to develop processes. In this same time period, only 34 % of ERα-expressing cells were viable relative to a Lac Z control population (Figure 6A). Consistently, after four days of infection with ERα only 62% of SKBr3 cells were viable compared to the Lac Z control population (Figure 6B). Similar to MDA-231 cells, ERα-expressing SKBr-3 had smaller cell bodies and began to develop processes (Figure 5).

LCC1 cells are derivatives of ER-positive/E2-dependent MCF-7 breast cancer cells, however, LCC1 cells display E2-independent growth and tumorigenicity (Brunner et al. 1993), a process correlated with constitutive NF-κB DNA binding activity (Pratt et al. 2003). In addition LCC1 cells have been shown to express a mutated ERα lacking exon 3 (Han et al. 2004). We therefore wanted to determine what effect re-expression of wild type ERα would have on cell viability and morphology in LCC1 cells. ERα-infected cells appeared to be swollen and flat after four days and began to take on morphological features similar to their parental MCF-7 cell line (Figure 5). Four days after infection, only 62% of wild type ERα-expressing cells were viable relative to a Lac Z control population (Figure 6C).

We also wanted to determine if overexpression of ERα would have a similar effect on MCF-7 cells which express high levels of wild type ERα. We

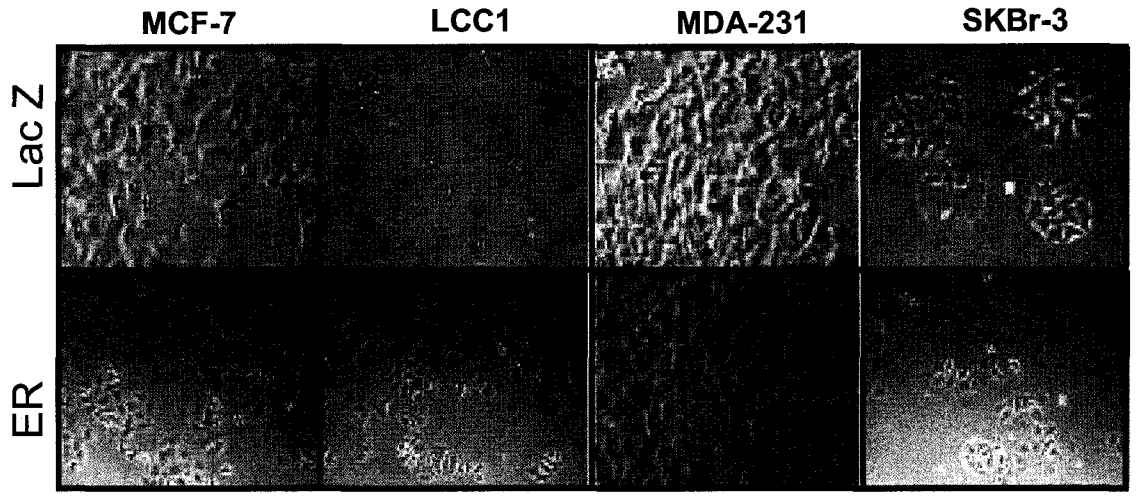
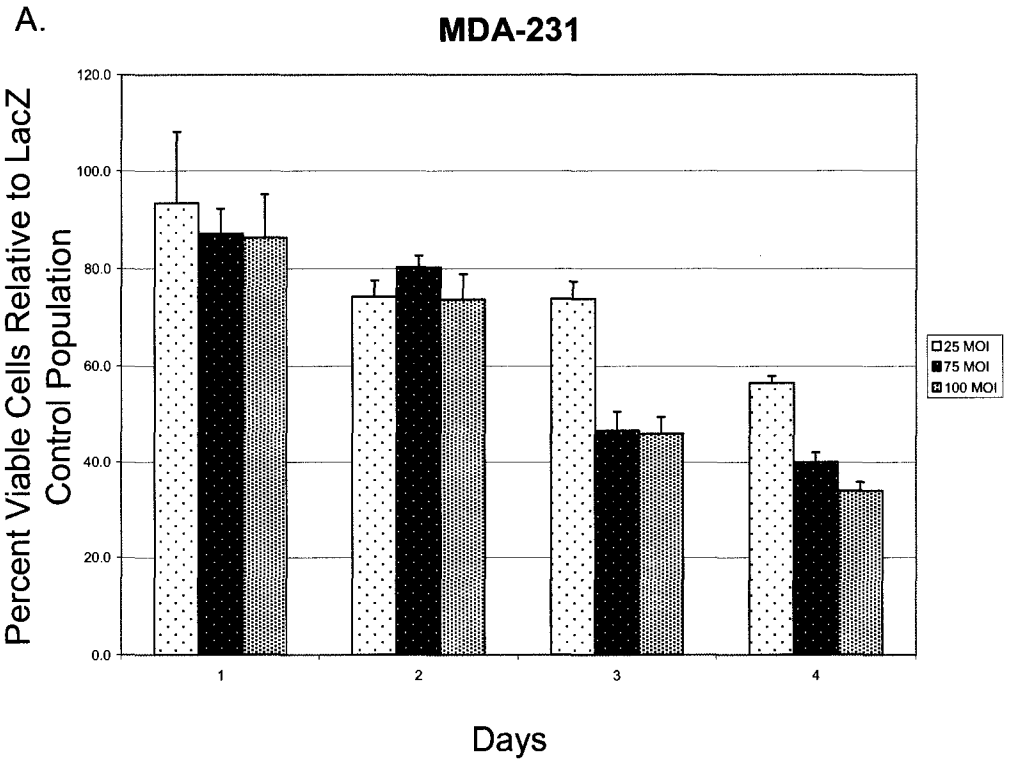
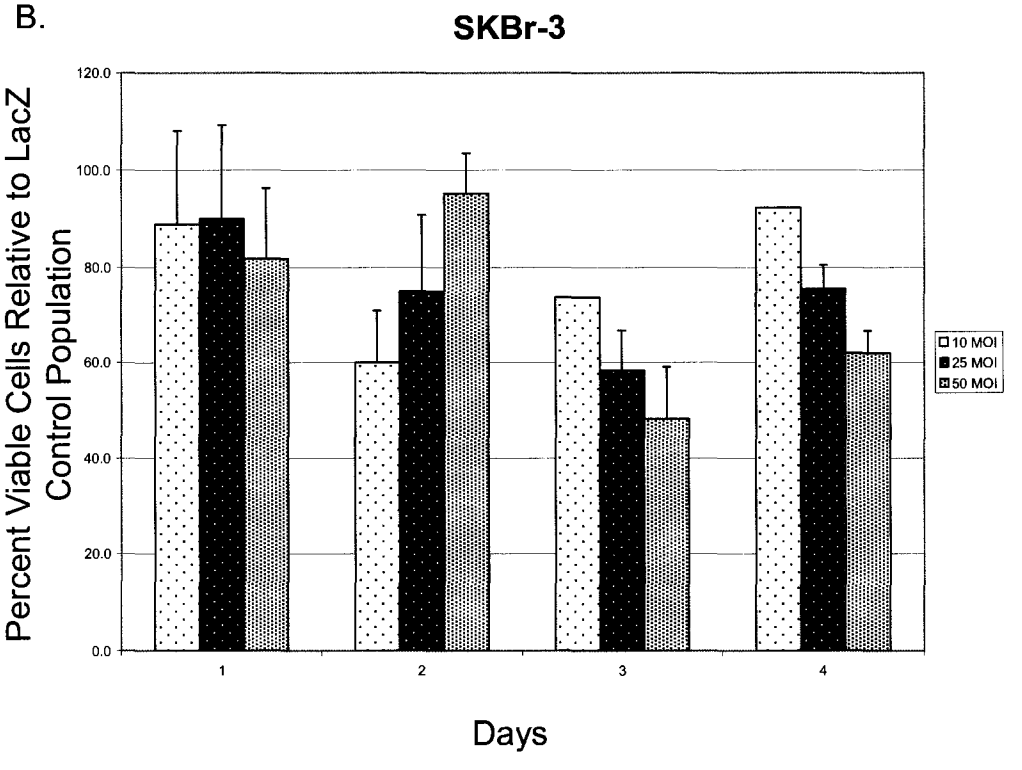



**Figure 5. Effect of ERα expression on morphology.**
Two ER-positive cell lines, the estrogen-independent LCC1 cells and estrogen-dependent MCF-7 cells, and two ER-negative breast cancer cell lines, MDA-MB-231 and SKBr-3, were infected with an adenovirus expressing the human ERα gene or Lac Z control. Changes in cellular morphology were noted four days after infection. Because of resistance to infection different MOIs were used in order to produce similar levels of ERα expression; MCF-7, LCC1 and SKBr3 cells were infected at 50 MOI, whereas MDA-231 cells were infected at 150 MOI.

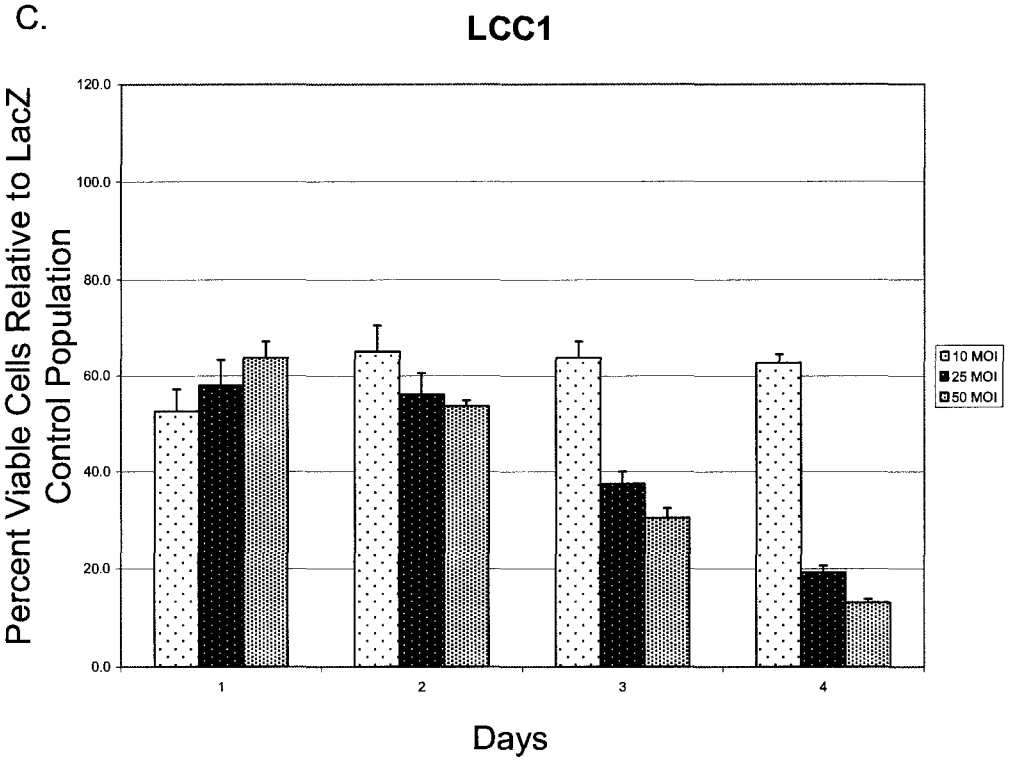
A.


MDA-231
Percent Viable Cells Relative to LacZ Control Population
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
Days
25 MOI
75 MOI
100 MOI

B.


SKBr-3
Percent Viable Cells Relative to LacZ Control Population
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
Days
10 MOI
25 MOI
50 MOI

C.


LCC1
Percent Viable Cells Relative to LacZ Control Population
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
Days
10 MOI
25 MOI
50 MOI

D.

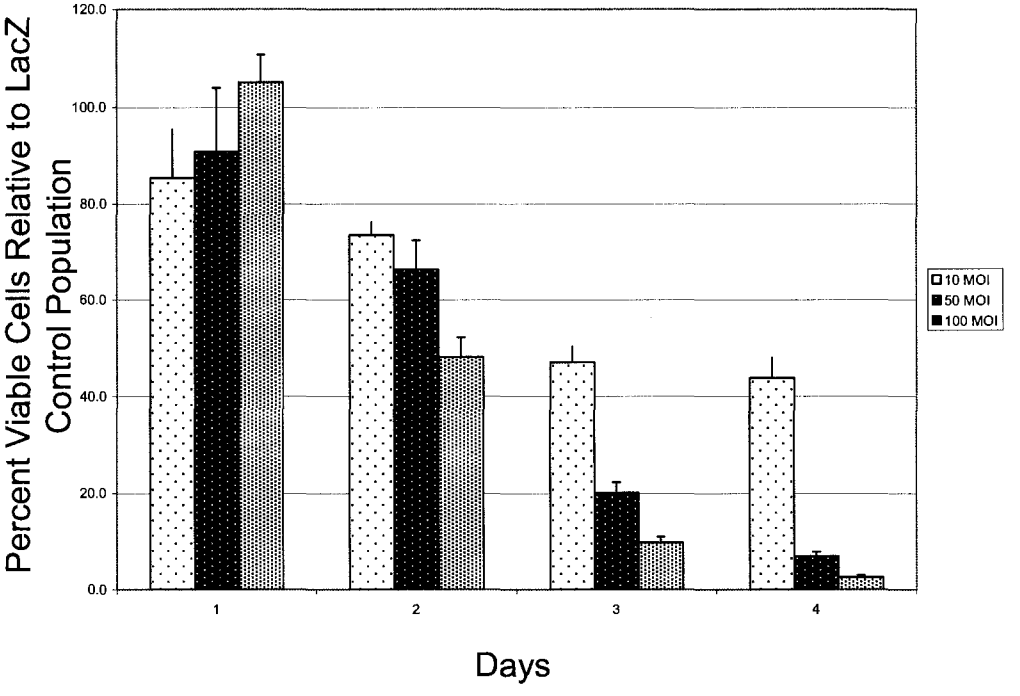

MCF-7
Percent Viable Cells Relative to LacZ Control Population
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
Days
10 MOI
50 MOI
100 MOI

**Figure 6. ERα expression results in decreased cell viability.**
Two ER-negative breast cancer cell lines; MDA-MB-231(A) and SKBr-3 (B), and two ER-positive cell lines; the estrogen-independent LCC1 cells (C) and estrogen-dependent MCF-7cells (D) were infected with an adenovirus expressing the human ERα gene or a Lac Z control. All experiments were done in triplicate. Viable cells were counted every day for four days and mean values were plotted relative to the Adeno-LacZ infected control population.

found that overexpression of ER had no effect on cell morphology; however, overexpression of ERα did reduce the number of viable cells. Four days after infection only 44% of the ERα-overexpressing (10 MOI) cells were viable compared to a Lac Z control population (Figure 6D).

These results suggest that re-expression of ERα alone reduces cell viability in breast cancer cells that no longer rely on the estrogen pathway for growth and survival.

## 3.2 Increased Expression of ERα Decreases NF-κB DNA Binding Activity

ER-positive breast cancer cell lines display lower levels of NF-κB activity in comparison to ER-negative cell lines, which display high constitutive NF-κB activity (Pratt et al. 2003). In addition, previous studies have reported inhibition of NF-κB activity by ligand bound ERα (Stein and Yang 1995, Galien and Garcia 1997, Ray et al. 1997). We have shown that increased expression of ERα reduced cell viability and therefore sought to determine whether re-expression of ERα in ER-negative and ER-positive/E2-independent cells would inhibit NF-κB DNA binding. Nuclear extracts from MDA-231, LCC1 and SkBR-3 cells grown in DMEM media in the absence of added E2 were infected with an adenovirus expressing the human ERα gene or a Lac Z control were subjected to Electrophoretic Mobility Shift Assay (EMSA) (Figure 7). We found that expression of ERα in ER-negative cells reduced NF-κB DNA binding relative to

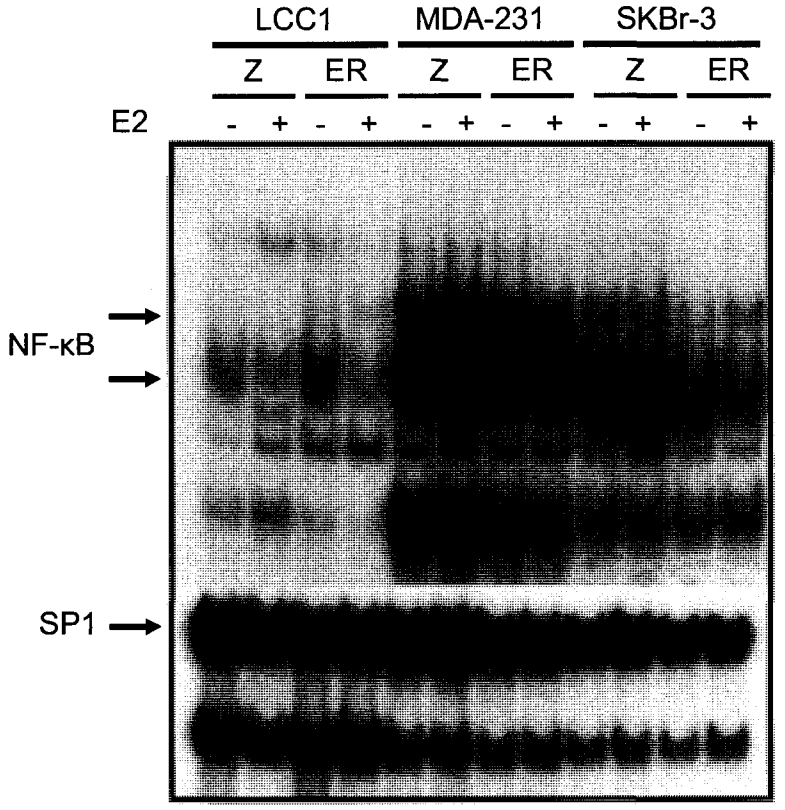


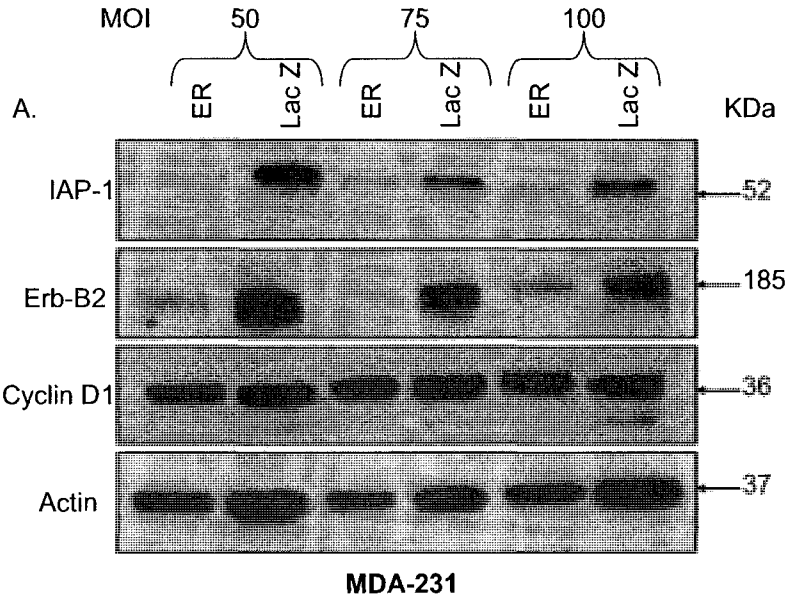

**Figure 7. ERα expression decreases NF-κB DNA binding.**
ER-positive/ estrogen-independent LCC1 cells and two ER-negative breast cancer cell lines, MDA-MB-231 and SKBr-3, were infected with an adenovirus carrying the human ERα gene or a Lac Z control (Z). Cells were grown in PRF media for 4 days then treated with E2 or vehicle for 4 days. Nuclear extracts were collected and subjected to Electrophoretic Mobility Shift Assay (EMSA) as described in the materials and methods section, using an oligonucleotide containing the consensus κB-response element. Arrows indicate fast (p50/p50 or p52) and slow (p50/p65)migrating NF- κB-complexes (Pratt et al 2003). Five migrograms of the same nuclear extract was subjected to EMSA using the consensus Sp1 enhancer element as a loading control.

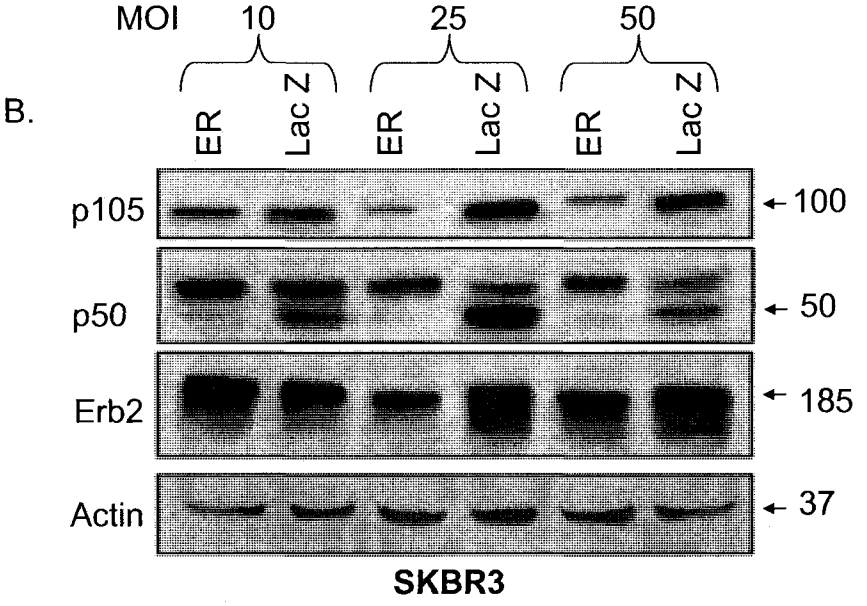
the Lac Z control population. Furthermore, treatment of ERα-expressing SKBr-3 cells with E2 further reduced NF-κB DNA binding. Interestingly, ERα-expressing LCC1 cells also showed decreased in NF-κB binding in response to treatment with E2. These results suggest that re-expression of ERα in the ER-negative and ER-positive/E2-independent cells decreases NF-κB DNA binding.

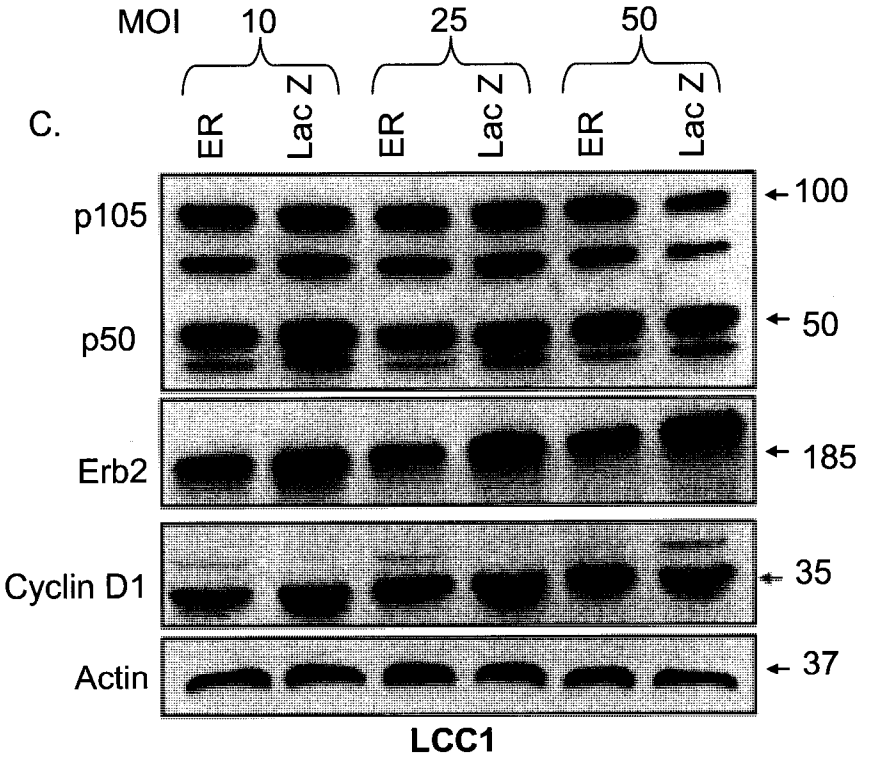
## 3.3 Increased Expression of ERα Decreases the Expression of NF-κB Regulated Proteins

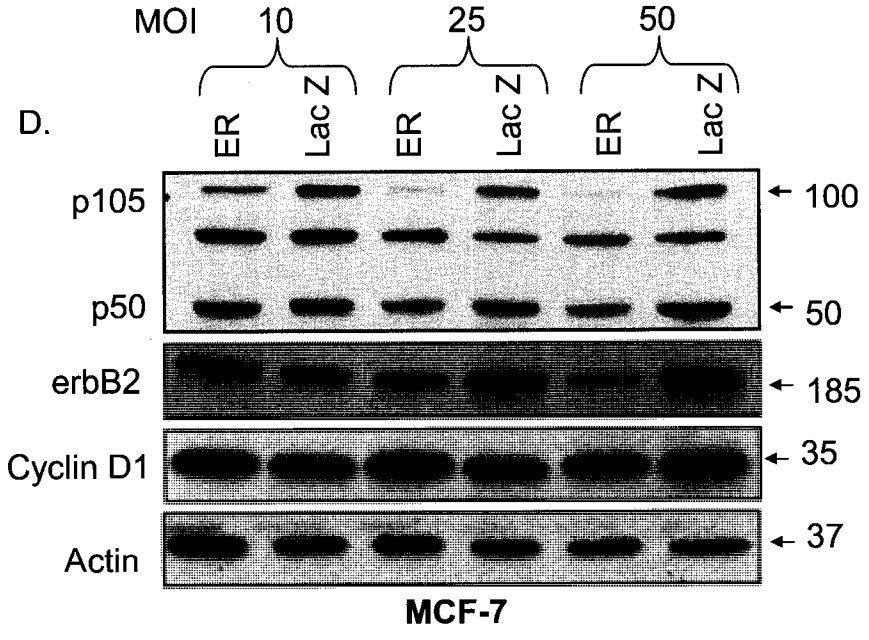
We next wanted to determine the impact of re-expression of ERα on the levels of known NF-κB regulated proteins. These experiments were done in ER-negative MDA-231, SKBr3 and ER-positive MCF-7 and LCC1 breast cancer cells. Using cell extracts from ERα or Lac Z adenovirus infected cells, western blots were performed for either p105/p50, h-IAP-1, ErbB2 and/or cyclin D1, four proteins known to be transcriptionally regulated by NF-κB (Paya et al. 1992, Chu et al. 1997, Hinz et al. 1999, Kitamura et al. 2005).

ER-negative MDA-231 and SKBr-3 cells, and ER-positive MCF-7 and LCC1 cells were infected with an adenovirus expressing either ERα or LacZ as a control. Cells were infected at different multiplicities of infection (MOIs) in order to produce similar expression levels between cell lines. MDA-231 cells were infected at 25, 75 and 100 MOI, SKBr-3 and LCC1 cells were infected at 10, 25, and 50 MOI, and finally MCF-7 cells were infected at 10, 50 and 100 MOI. Four days after infection, whole cell extracts were prepared and subject to immunoblot analysis. The re-expression of ERα in MDA-MB-231 correlated with a decrease


MOI
50
75
100
A.
ER
Lac Z
ER
Lac Z
ER
Lac Z
KDa
IAP-1
52
Erb-B2
185
Cyclin D1
36
Actin
37
MDA-231


MOI
10
25
50
B.
ER
Lac Z
ER
Lac Z
ER
Lac Z
p105
100
p50
50
Erb2
185
Actin
37
SKBR3


C.
MOI
10
25
50
ER
Lac Z
ER
Lac Z
ER
Lac Z
p105
p50
Erb2
Cyclin D1
Actin
100
50
185
35
37
LCC1


D.
MOI
10
25
50
ER
Lac Z
ER
Lac Z
ER
Lac Z
p105
p50
erbB2
Cyclin D1
Actin
100
50
185
35
37
MCF-7

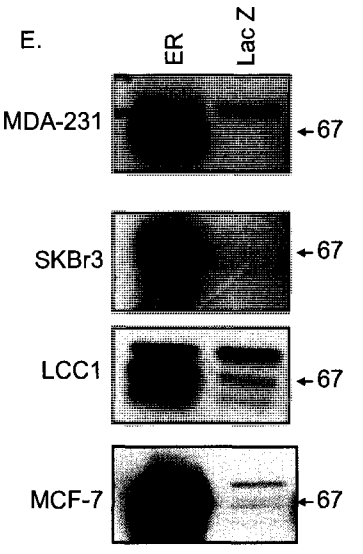



**Figure 8. ERα expression decreases levels of NF-κB regulated proteins.** ER-negative, MDA-231 (A) and SKBr-3 (B), cells and two ER-positive, LCC1 (C) and MCF-7 (D) cells were infected with an adenovirus carrying the human ERα gene or a Lac Z control. Fifteen micrograms of whole-cell protein extract was subjected to immunoblot analysis of NF-κB regulated proteins. To verify infection efficiency immunoblot analysis of ERα was performed in all 4 cells lines (E). The expression of actin was used as an internal loading control. The numbers on the right indicate the molecular masses in kilodaltons.

in h-IAP-1 and ErbB2 protein levels, as determined by immunoblot (Figure 8A). We found similar results using SKBr3 cells extracts, ERα expression resulted in a marked decrease in p105, p50 levels and ErbB2 levels (Figure 8B).

Re-expression of wild-type ERα in LCC1 resulted in reduced ErbB2, p50 and to a lesser degree p105 levels (Figure 8C). Overexpression of ERα in MCF-7 cells also correlated with a decrease in p105, p50 and ErbB2 protein levels (Figure 8D). We saw no change in cyclin D1 protein levels, in any of the cells (Figure 8). This result was not unexpected since ERα has also been shown to positively regulate cyclin D1 expression (Altucci et al. 1996). These results suggest that expression of wild type ERα alone is sufficient to reduce levels of NF-κB regulated proteins.

## 3.4 Overexpression of ERα in ER-positive Breast Cancer Cells Decreases NF-κB Transcriptional Activity

We next wanted to confirm that the decrease in NF-κB regulated protein levels in ER-infected cells was in fact due to an inhibition of NF-κB transcriptional activation. MCF-7 cells were therefore transiently transfected with either a κB-luciferase or mutant-κB-luciferase reporter gene and co-transfected with either hERα or empty vector (pcDNA3). This experiment was done in triplicate and we found that co-transfection of hERα reduced luciferase activity by an average of 44% ($p= 0.0094$) (Figure 9). These results suggest that the ER reduces the levels of NF-κB regulated proteins by directly inhibiting NF-κB activity.

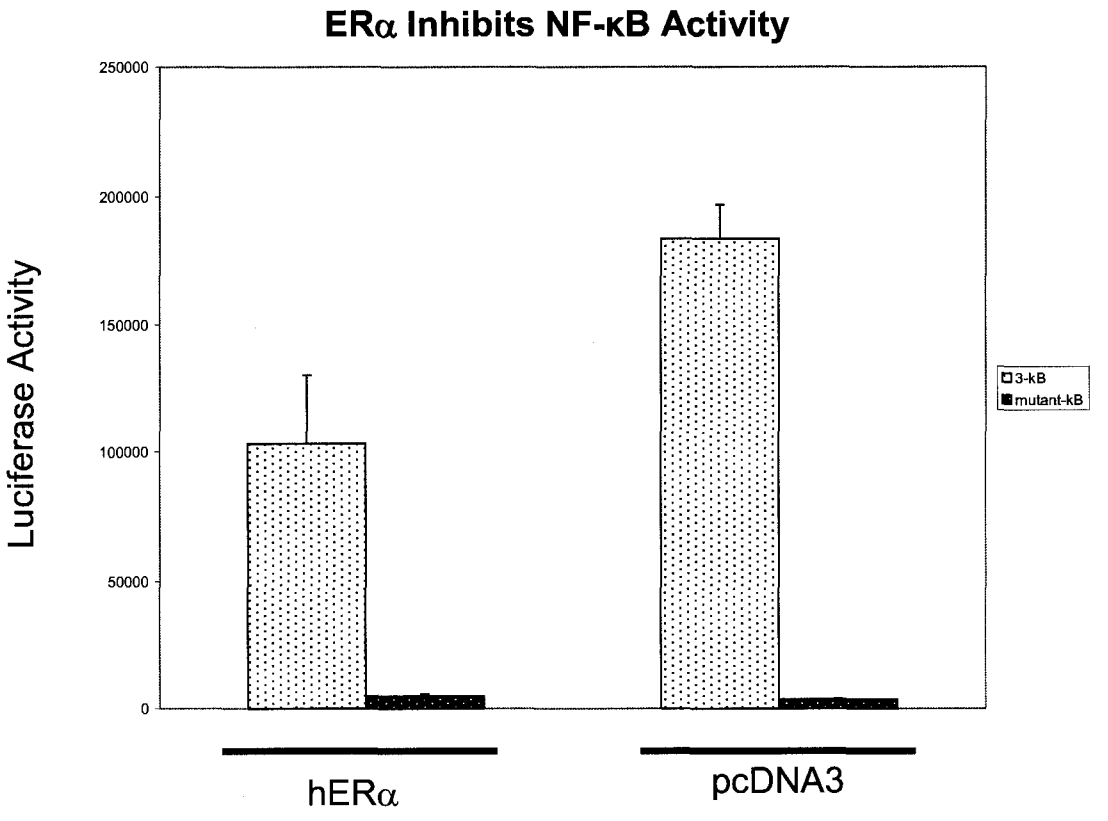



**Figure 9. Overexpression of ERα inhibits NF-κB activity.**
MCF-7 cells were transiently transfected with a κB-luciferase or mutant-κB-luciferase reporter gene and co-transfected with either hERα or empty vector (pcDNA3). Cells were harvested 2 days after transfection and luciferase activity was measured.

## 3.5 *IN VITRO* ASSOCIATION OF ERα WITH NF-κB SUBUNITS P50 AND P65

Thus far, we have shown that expression of ERα can reduce NF-κB DNA binding. Moreover, the reduced DNA binding is associated with reduced NF-κB activity and lower levels of NF-κB regulated proteins. We next sought to further define the mechanism of ER-mediated inhibition of NF-κB. Previous studies have reported direct physical interaction between transcription factors, such as GR and AP-1 as well as NF-κB and PR, to be essential for the repression (Schule et al. 1990, Yang-Yen et al. 1990). Studies using GST-pulldown and co-immunoprecipitation analysis show that ER is capable of interacting with NF-κB subunits p50 and p65 *in vitro* (Stein and Yang 1995, Ray et al. 1997). The results from these studies also indicate a dependence on the hormone-binding domain and the D domain (hinge region) of the ERα for ER/NF-κB complex formation (Stein and Yang 1995). In addition, the interaction was found to be independent of E2 (Stein and Yang 1995). As a first step towards investigating the mechanism of NF-κB inhibition we wanted to confirm a direct interaction between ERα and NF-κB *in vitro*. For these studies, expression vectors containing a series of ERα deletion mutants (Figure 10) were expressed in bacteria using the pGex expression vector system (Figure 11C). [35S]-Met labeled wild type p50 and p65 were *in vitro* transcribed and translated, and a GST-pulldown assay was performed in the absence of E2. This experiment was repeated 3 times and we were able to demonstrate the specific binding of GST-ER (CDEF) fusion-protein to both p65 and p50 in all three experiments (Figure

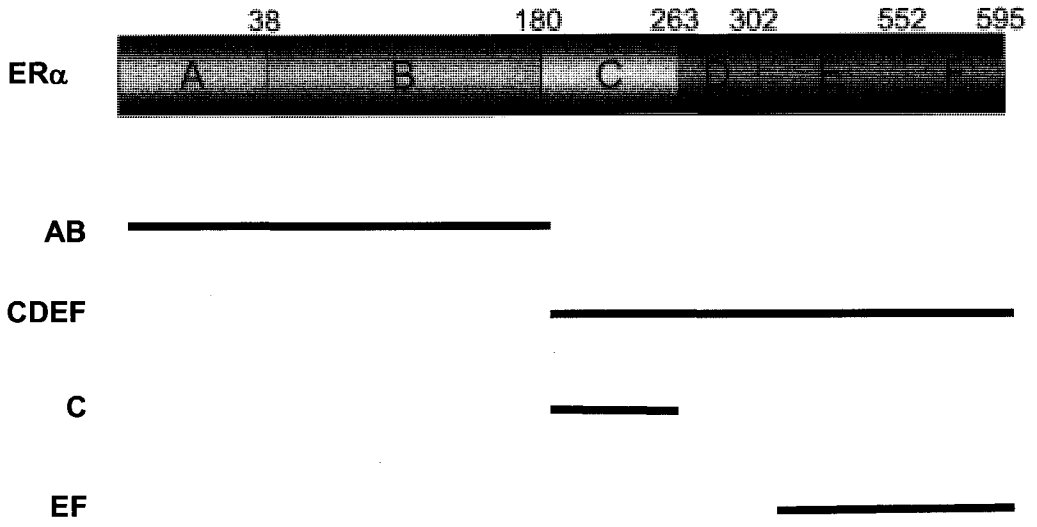
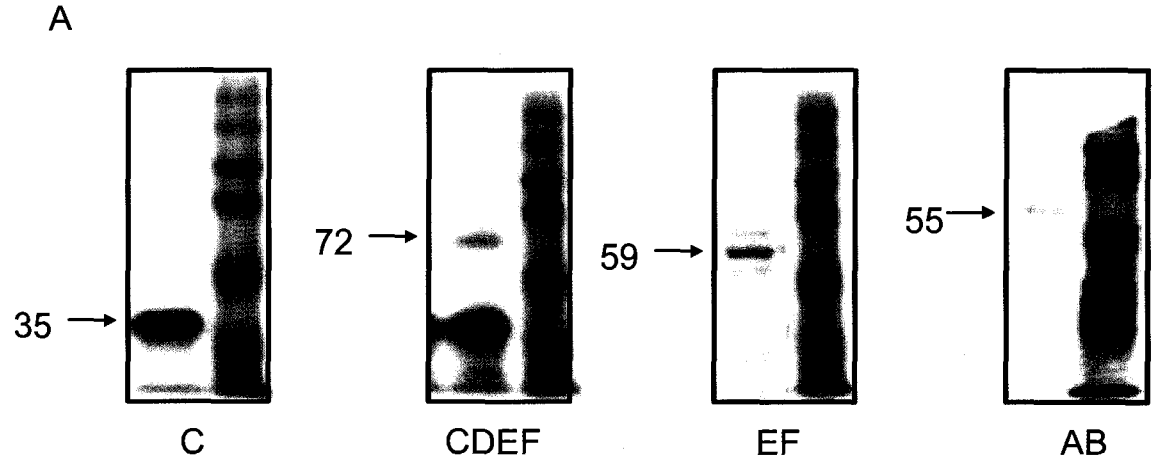


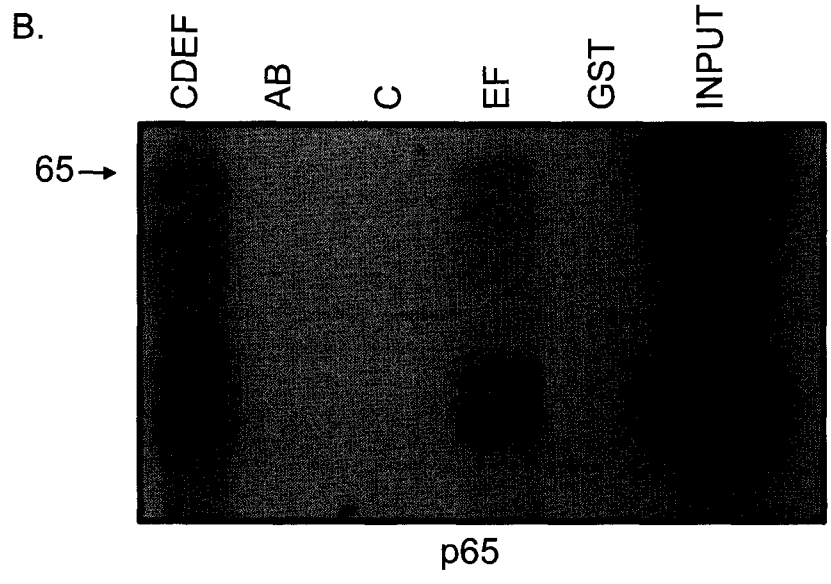

**Figure 10. ERα GST-fusion proteins.**
GST-ER-AB contains amino acids 1 through 180 corresponding to the A and B domains. GST-ER-CDEF contains amino acids 180 through 595 corresponding to the C,D,E and F domains. GST-ER-C contains amino acids 180 through 263 corresponding to the C domain. GST-ER-EF contains amino acids 302 to 595 corresponding to the E and F domains.

11). Shorter deletion mutants of ERα encompassing only the AB domains or C domain were unable to bind p65 or p50. There was a slight interaction of an ER deletion mutant containing only the E and F domains with p65 in two of three experiments (Figure 11B). There was no interaction of the ER deletion mutant containing only the E and F domains with p50 (Figure 11B). These results confirm that ERα can interact with p50 and p65 *in vitro* in the absence of DNA and that the interaction does not involve the A or B domains. In addition, the C, D, E and F domains appear to be involved in the interaction, although the C domain alone is insufficient. Furthermore, it is possible that the E and F domains of ERα are sufficient for interaction with p65 although different binding sites are required for interaction with p50. These observations are consistent with previous reports that the DNA binding (C) domain, the hinge region (D) as well as the hormone binding domain (EF) may be required for E2 mediated repression of NF-κB (Stein and Yang 1995, Ray et al. 1997).

## 3.6 ESTROGEN RECRUITS NF-κB PROTEINS TO AN ERE *IN VIVO*

To investigate the assembly of an ERα/NF-κB protein complex *in vivo*, we examined the recruitment of ERα and NF-κB subunits p50 and p65 to the promoter of an endogenous estrogen responsive target gene following treatment with E2. The status of the endogenous complex present on the estrogen responsive region of the pS2 gene was determined using chromatin immunoprecipitation (ChIP) assay. MCF-7 cells were grown in phenol red-free


A
35
72
59
55
C
CDEF
EF
AB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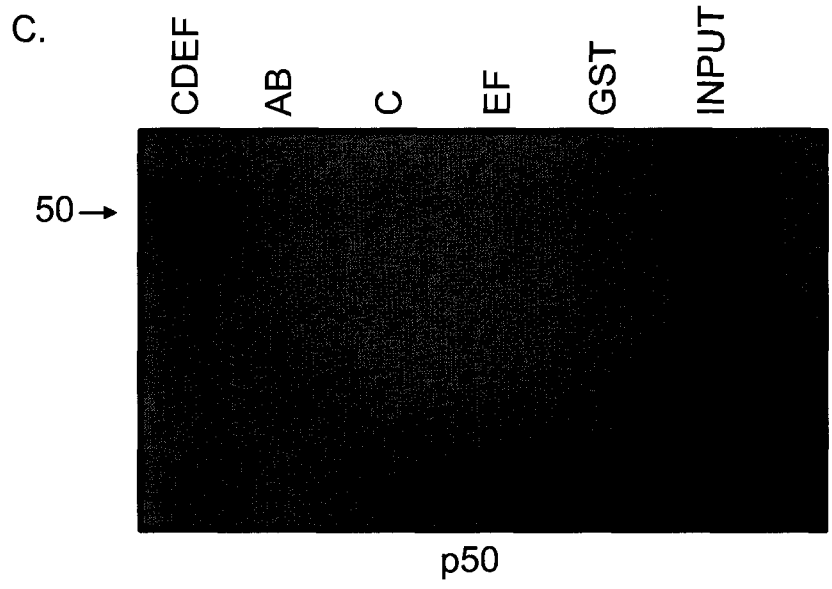



**Figure 11. ERα interacts with NF-κB in vitro.**
Expression vectors containing a series of ERα deletion mutants were expressed in bacteria by use of the pGex expression vector system. The indicated GST-fusion proteins were purified as described in the methods and subjected to SDS-PAGE then Coomassie stained to visualize proteins (A). [35S]-met labeled wild type p65 (B) and p50 (C) were *in vitro* transcribed and translated and a GST-pulldown assay was performed in the absence of E2. 100µl of GST-fusion protein was added to 5µl of $^{35}$S-Met labeled p50 or p65. The experiment was repeated three times successfully. The number on the left indicates the relative molecular weight in kilodaltons.

(PRF) media for 4 days, to deprive cells of estrogen, and treated with either E2 ($10^{-8}$M) or vehicle for 45 min. Chromatin was then crosslinked and samples were sonicated to shear DNA. Thereafter, using anti-ERα, anti-p50 or anti-65 antibody, we immunoprecipitated the complexes and the binding of p50, p65 and ERα to the ERE within the pS2 promoter was revealed by PCR. In order to verify interaction of ERα with p50 and p65 DNA, 3µl of ChIP sample was used for PCR reactions (Figure 12A). We found that both p50 and p65 are capable of interacting with the ERα on the ERE of the pS2 promoter, and that treatment with E2 increased the recruitment of p50 and p65 to this ERE (Figure 12A). These results indicate that the ERα is capable of forming a complex with NF-κB subunits p50 and p65 on the ERE *in vivo*. In order to determine loading differences, 3µl of input fraction from cells treated with E2 and with vehicle were compared by PCR. We found that input levels were similar between samples (Figure 12B). Finally, to verify specificity of the antibodies, we used negative control primers selected to amplify a region of the ERα coding sequence between bases 348 and 554. These primers were able to amplify DNA from the input fractions and produced bands of similar intensities. In contrast, there was no amplification of DNA in the immunoprecipitation samples with the negative control antibodies, confirming the specificity of the ERα, p50 and p65 antibodies (Figure 12C). Taken together, these results suggest that immunoprecipitation with the ERα, p50 and p65 antibodies enriched pS2 promoter DNA, and that this enrichment was not the result of non-specific binding of the antibodies to the chromatin.

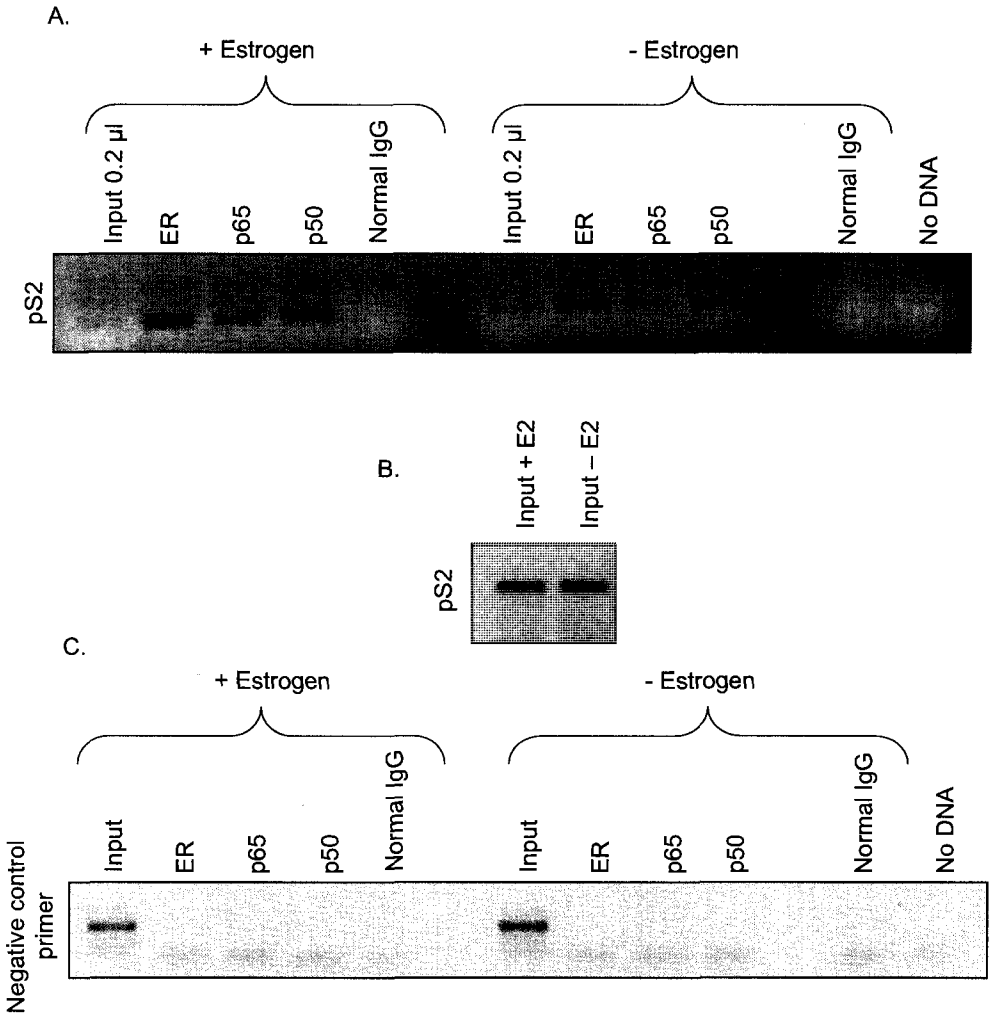



**Figure 12. ER/NF-κB complex formation in vivo on the pS2 promoter.** The status of the endogenous complex present on the estrogen responsive regions was determined using chromatin immunoprecipitation (ChIP). MCF-7 cells were grown in phenol red free media for 4 days and treated with either E2 ($10^{-8}$M) or vehicle for 45 min. Chromatin was then crosslinked and sonicated to shear DNA followed by immunoprecipitation with anti-ER, anti-p50 or anti-65 antibody. Following immunoprecipitation the crosslinks were reversed, the DNA was extracted and interactions with ERα, p50 and p65 were analyzed by PCR using a specific pair of primers spanning the ERE of the pS2 promoter. 2µl of sample was used to amplify DNA immunoprecipitated with ER, p50 and p65 antibody, and 0.2µl of input DNA was used (A). In order to verify that input between samples was consistent, 3µl of input fraction from cells treated with E2 and from cells treated with vehicle were compared by PCR (B). In order to confirm ERα, p50 and p65 antibody specificity negative control primers spanning a sequence within the estrogen receptor coding sequence were used as a negative control (C)

## 3.7 Estrogen-Independent LCC1 Cells Show Reduced ER:p50 Association on the ERE Compared to Estrogen-Dependent MCF-7 cells.

LCC1 cells have been shown to express a mutant ERα (Han et al. 2004) and display constitutive NF-κB activation (Pratt et al. 2003). Our previous results have shown that re-expression of wild type ERα in LCC1 cells reduces NF-κB activity. We hypothesized that the ERα may be inhibiting NF-κB by physically interacting with p50 and/or p65 and sequestering it, preventing NF-κB from binding the κB response element and activating gene transcription. We also proposed that, in E2-independent cells, the ERα is no longer capable of binding and inhibiting NF-κB activity. In order to examine this possibility, we compared the interaction of ER:p50 and ER:p65 to the ERE in both the E2-dependent MCF-7 cells and E2-independent LCC1 cells. We used nuclear extracts from both MCF-7 and LCC1 breast cancer cells grown in PRF media for 7 to 10 days. Cells were treated with either E2 or vehicle for 4 days and nuclear extracts were isolated. Using an EMSA, we show that the binding pattern of the LCC1 ER to the ERE is distinctly different when compared to that of MCF-7 cells. More specifically, we demonstrate that there is significantly weaker ERα/p50 binding to the ERE in LCC1 cells than in MCF-7 cells (Figure 13). We saw no interaction of p65 with the ERE in MCF-7 or LCC1 cells. These results suggest that the E2-independent cells have lost the ability to bind and sequester p50, therefore inhibiting NF-κB DNA binding in the presence of E2.

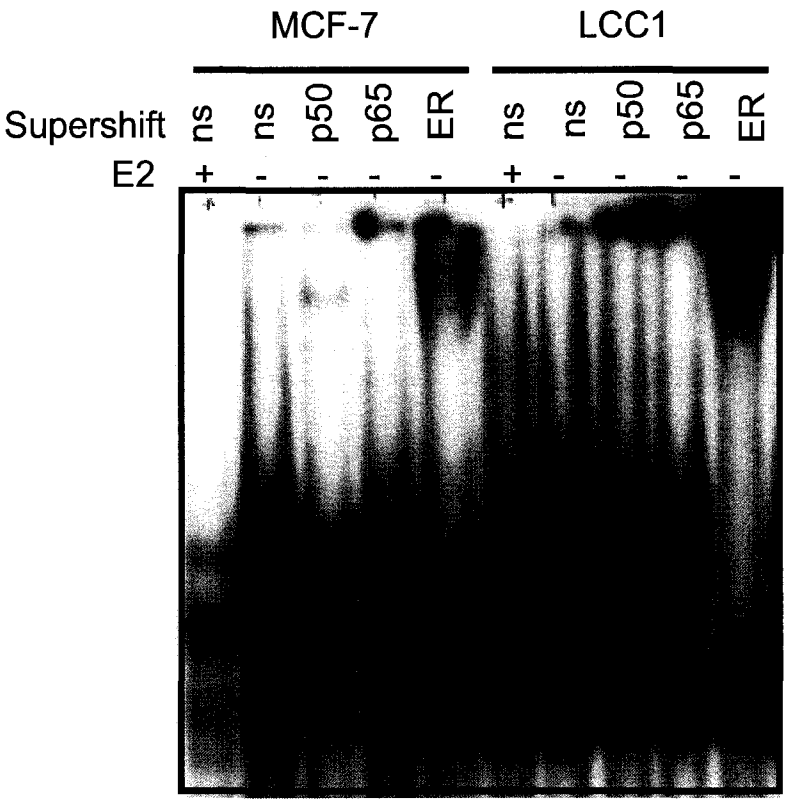



**Figure 13. LCC1 cells show reduced ERα:p50 association on an ERE.** ER-positive/ estrogen-independent LCC1 cells and ER-positive/estrogen-dependent MCF-7 cells were grown in PRF media for 4 days followed by treatment with either E2 (+) or vehicle(-) for 4 days . Five micrograms of nuclear extracts were subjected to Electrophoretic Mobility Shift Assay (EMSA) as described in the materials and methods section, using an oligonucleotide containing the consensus estrogen response element. Complexes were supershifted with anti-p50, p65 or ERα antibody.

## 3.8 TAMOXIFEN HAS NO EFFECT ON NF-κB ACTIVITY

The ability of ER to activate transcription is profoundly affected by tamoxifen binding. In fact, tamoxifen induces conformational changes in the ER that prevents binding of co-activators and therefore blocks AF-2-induced (ligand-dependent) transcription (Shiau et al. 1998). Furthermore, we have shown the E2 inhibits NF-κB activity. NF-κB is involved in cell cycle progression, anti-apoptosis and tumorigenesis. We therefore sought to determine if the growth inhibitory effects of tamoxifen could be, in part, due to an inhibition of NF-κB acitivty. We used MCF-7 cells, which display low NF-κB activity, and MDA-231 cells, which display high NF-κB activity, infected with an adenovirus expressing the ERα gene. Cells were grown in DMEM media and treated with $10^{-9}$M 4-OH-tamoxifen or vehicle. Immunoblot analysis was then used in order to assay NF-κB regulated proteins. As a control, we also assayed for E2 regulated proteins. As expected, tamoxifen inhibited the expression of the E2 regulated proteins, Bcl-2 and cyclin D1, in MCF-7 cells (Figure 14A). ERα-expressing MDA-231 cells showed lower Bcl-2 protein levels when compared to Lac Z controls. NF-κB has been shown to regulate Bcl-2 expression (Catz and Johnson 2001), it is therefore possible that the decrease of Bcl-2 levels is due to an inhibition of NF-κB activity by ERα expression. We saw no further decrease in Bcl-2 levels after treatment of the ERα-expressing MDA-231 cells with tamoxifen (Figure 14B). In addition, tamoxifen treatment did not have any significant effect on ErbB2, p50 or cIAP levels in MCF-7 or ERα-expressing MDA-231 cells. (Figure 14).

## 3.9 Inhibition of NF-κB Increases the Expression of E2-Regulated Proteins.

Our previous studies have shown that in ER-negative cell lines there is constitutive NF-κB activation (Pratt et al. 2003). Furthermore, treatment of ER-α-expressing MDA-231 cells with E2 had opposite effects on some E2-regulated proteins when compared to treatment of MCF-7 cells with E2. For example, *c-myc* gene expression was strongly inhibited by E2 in ER-expressing MDA-231 cells, however, *c-myc* expression is strongly correlated with E2 treatment in MCF-7 cells. In addition, E2 treatment of MCF-7 cells increases BRCA1 and BRCA2 expression, however, treatment of ER-expressing MDA-231 cells resulted in reduced BRCA1 and BRCA2 expression (Lazennec and Katzenellenbogen 1999). It is therefore possible that high NF-κB activity in ER-negative cells is responsible for the inhibition of ERα when re-expressed in these cells and prevents E2 signaling. Mutual repression of transcription factors has previously been demonstrated between NF-κB and the progesterone receptor. Activation of NF-κB by tumor necrosis factor-α (TNF-α) results in repression of PR, while hormone-induced PR is able to repress TNF-α-induced NF-κB activity (Kalkhoven et al. 1996). We therefore sought to determine if there was a mutual repression occurring between ERα and NF-κB. Normally NF-κB is maintained in the cytoplasm through an interaction with its inhibitor IκBα. Mutation of IκBα at serines 32 and 36 prevents both phosphorylation and degradation of the inhibitor. Transfected mutated IκBα competes with endogenous IκBα molecules to block

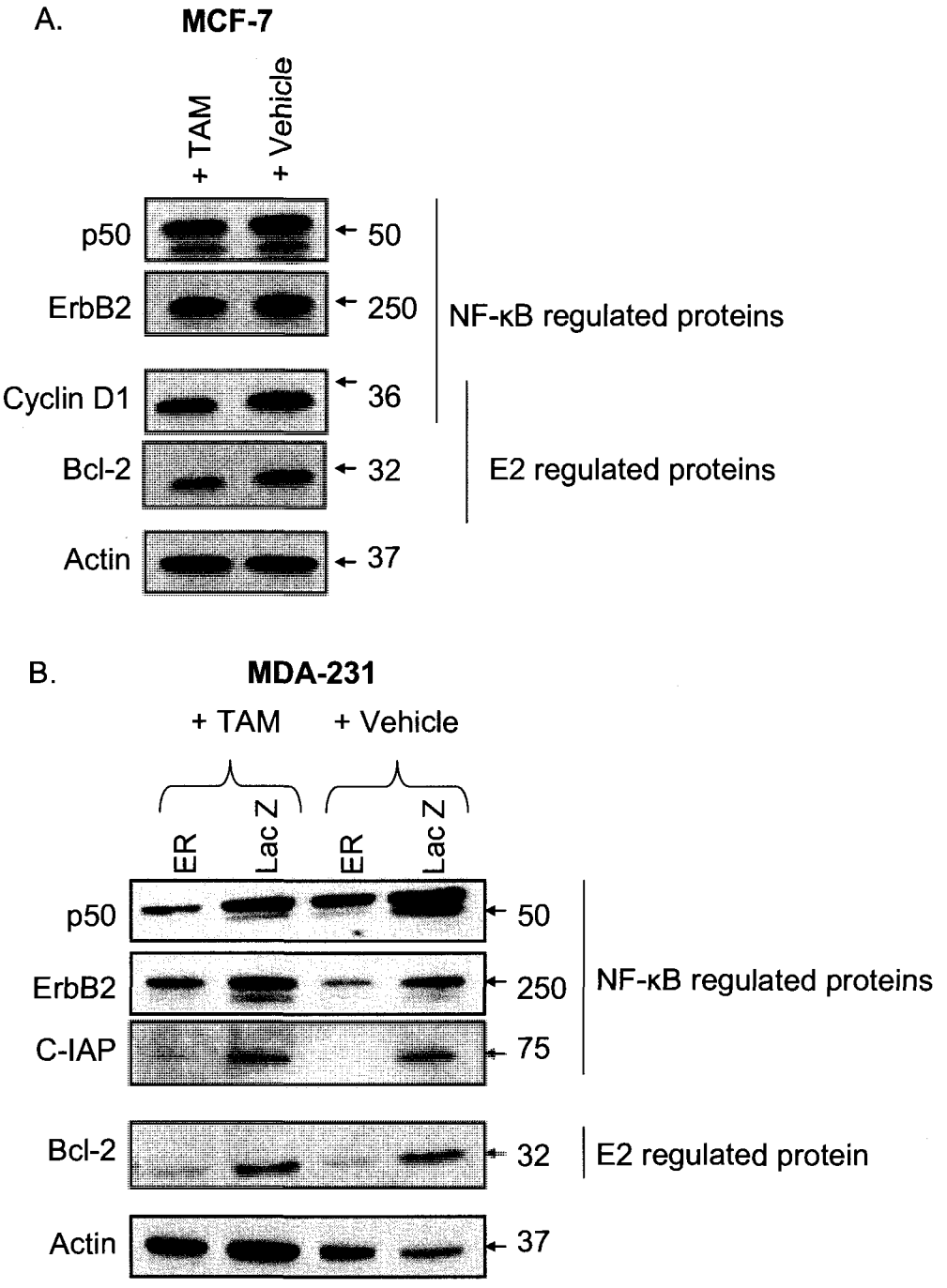



**Figure 14. Tamoxifen has no effect on NF-κB regulated proteins.**
Fifteen micrograms of whole-cell extract from MCF-7 cells (A) or MDA-231 cells infected with an adenovirus carrying the ERα gene (B) were subjected to immunoblot analysis of NFκB-regulated proteins. The cells were grown in DMEM media and treated with $10^{-9}$M 4-OH-tamoxifen for 4 days. The expression of actin was used as an internal loading control. The numbers on the right indicate the molecular masses in kilodaltons.

NF-κB activation (Mayo and Baldwin 2000, Biswas et al. 2005).

In order to determine whether inhibition of NF-κB would allow increased expression of E2 regulated proteins we used MCF-7 cells that were stably expressing a mutant IκBα super repressor ($IκBα^{SR}$). The cells were maintained in phenol red free media for at least 4 days and treated with E2 ($10^{-9}$M) or vehicle for 4 days after which western blot analysis was performed in order to verify expression levels of E2-regulated genes. Stable MCF-7 clones transfected with empty pcDNA3 vector were used as a control. In order to verify inhibition of NF-κB we blotted for GADD45α. NF-κB inhibition due to IKKβ deficiency has been shown to enhance the stability of GADD45α mRNA resulting in increased protein levels (Zheng et al. 2005). Stable clones expressing $IκBα^{SR}$ were shown to have increased GADD45α protein levels when compared to Lac Z control cells (Figure 15). In addition, treatment with E2 further increased the protein levels of GADD45α, confirming the inhibitory effects of E2 on NF-κB activity (Figure 15). In order to determine what effect NF-κB inhibition had on E2-regulated genes, we blotted for two E2-regulated proteins: Bcl-2 and pS2. We found that the expression of $IκBα^{SR}$ correlated with an increase in Bcl-2 and pS2 protein levels. In addition, treatment with E2 further increased protein levels of these two proteins (Figure.15). These results suggest that inhibition of NF-κB increases the levels of E2 regulated proteins, possibly by alleviating inhibition of ERα transcriptional activation by NF-κB.

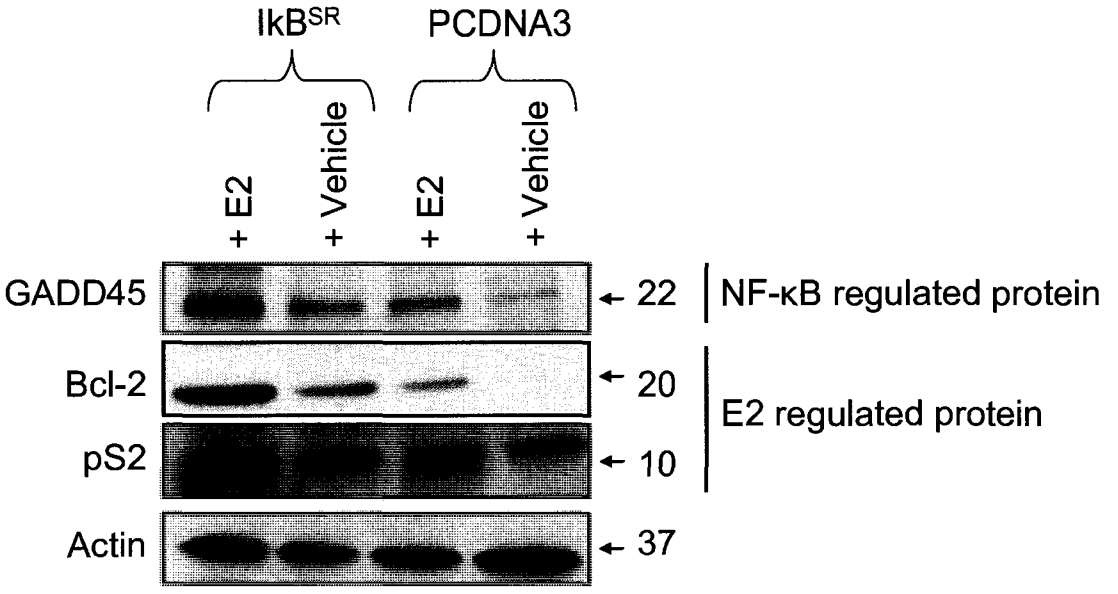



**Figure 15. Inhibition of NF-κB increases the expression of E2 regulated proteins.**

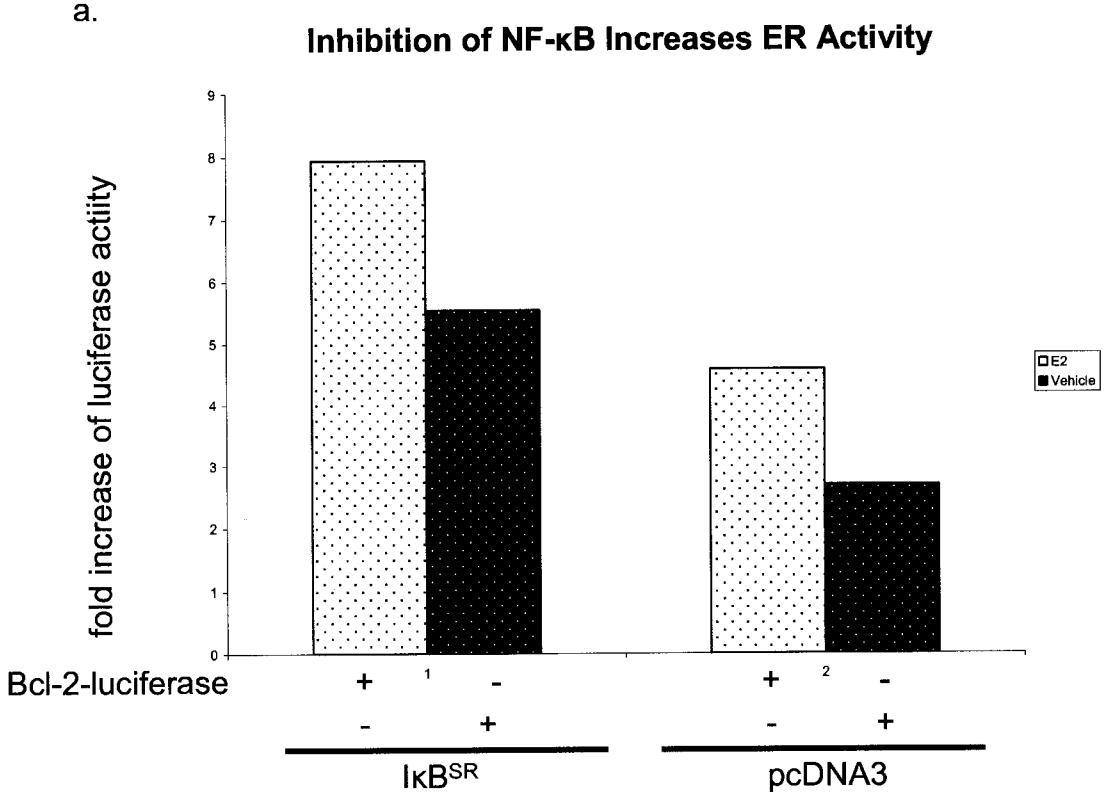
MCF-7 cells that were stably expressing a mutant IκBα super repressor (IκBα$^{SR}$) were maintained in phenol red free media for at least 4 days and treated with E2 ($10^{-9}$M) or vehicle for 4 days. Stable MCF-7 clones transfected with empty pcDNA3 vector were used as a control. Fifteen micrograms of whole-cell protein extract was subjected to immunoblot analysis of E2 regulated proteins. The expression of GADD45α was used to verify inhibition of NF-κB. The expression of actin was used as an internal loading control. The numbers on the right indicate the molecular masses in kilodaltons.

## 3.10 Inhibition of NF-κB Increases ERα Activity

We next wanted to confirm that the increase in E2 regulated protein levels was in fact due to an inhibition of ER transcriptional activation. In order to do so MCF-7 cells stably expressing an IκBα$^{SR}$ were transiently transfected with an reporter construct containing the two motifs of the Bcl-2 ERE driving luciferase expression (Bcl-2-luciferase) or empty vector (pGL3-basic) (Bynoe et al. 2000). We found that expression of IκBα$^{SR}$ correlated with a 1.7 fold increase in luciferase activity ($p=1.96 \times 10^{-4}$) (Figure 16A).

In a second experiment, MCF-7 cells stably expressing IκBα$^{SR}$ were transiently transfected with a reporter construct containing the Vitellogenin gene ERE (Klein-Hitpass et al. 1988) driving the expression of chloramphenicol acetyl transferase (CAT) (Vitellogenin-CAT) or control vector (pcDNA3). Similar to the Bcl-2 ERE transfected cells, we found that expression of IκBα$^{SR}$ correlated with a 4-fold increase in CAT activity ($p=5.58 \times 10^{-7}$) relative to cells transfected with the empty vector (pcDNA3) (Figure 16B). In order to control for transfection efficiencies across cell lines, both stable clones were co-transfected with CMV-luciferase and luciferase activity was measured. All experiments were done in triplicate and the (Putti et al. 2005) mean values were normalized to the positive control (CMV-luciferase). Taken together with the studies described above, these results suggest that inhibition of NF-κB increases ER transcriptional activity in a ligand-independent manner.

a.


Inhibition of NF-κB Increases ER Activity
fold increase of luciferase actiity
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3
4
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Vehicle
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-
1
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+
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2
-
+
IκB^SR
pcDNA3

B.

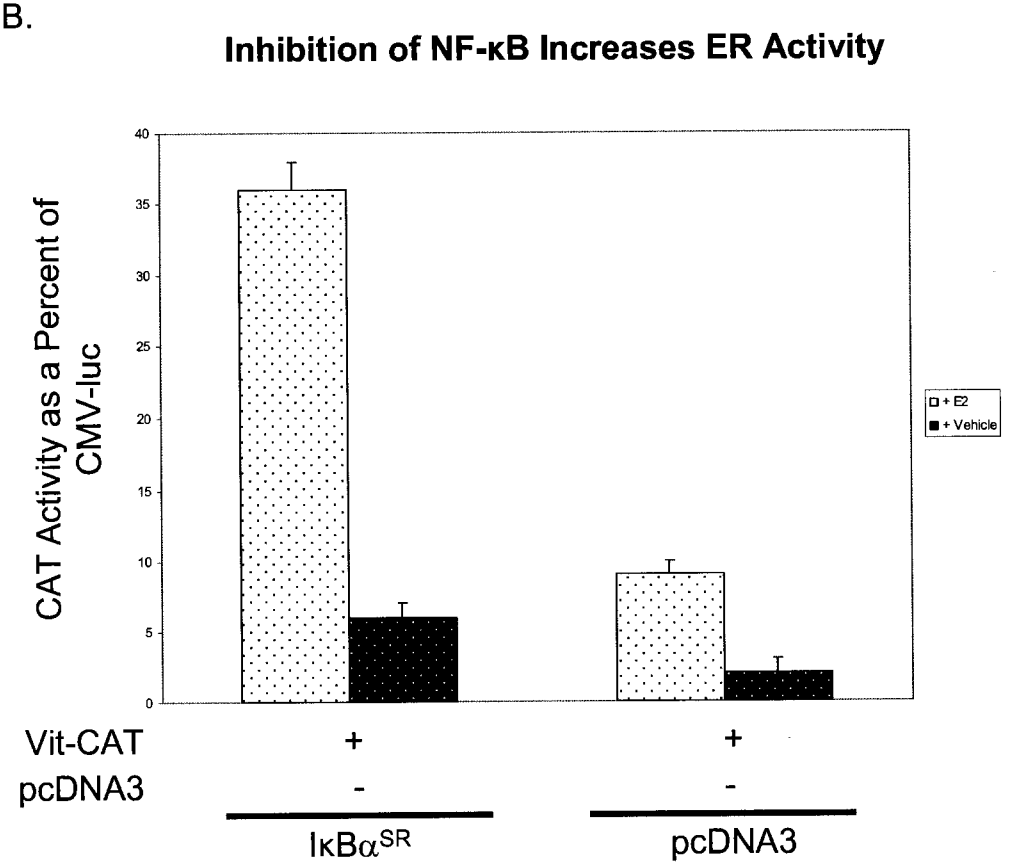

Inhibition of NF-κB Increases ER Activity
CAT Activity as a Percent of CMV-luc
0
5
10
15
20
25
30
35
40
+ E2
+ Vehicle
Vit-CAT
+
+
pcDNA3
-
-
IκBα^SR
pcDNA3

**Figure 16. Inhibition of NF-κB Increases ERα Activity**
(A.) MCF-7 cells stably expressing and IκBα$^{SR}$ were transiently transfected with an expression vector containing two copies the Bcl-2 ERE driving luciferase expression (Bcl-2-luciferase) or empty vector (pGL3-basic). (B) MCF-7 cells stably expressing and IκBα$^{SR}$ were transiently transfected with an expression vector containing the Vitellegenin ERE driving the expression of Chloramphenicol acetyl transferase (CAT) (Vitellogenin-CAT) or control vector (pcDNA3). As a control we used MCF-7 cells stably transfected with empty vector (pcDNA3). In order to control for transfection efficiencies across cell lines both stable clones were also transfected with CMV-luciferase and luciferase activity was measured. All experiments were done in triplicate and mean values were calculated as a percentage of the positive control (CMV-luciferase) and plotted as a fold increase of the negative control (pcDNA3).

# *CHAPTER 4*

# *DISCUSSION*

Breast cancer is a disease in which the NF-κB and ER pathways intersect. Two thirds of breast cancers are ER-positive (Putti et al. 2005) and the principle role of estrogen is to stimulate cell growth and survival. Many of the current treatments for breast cancer involve blocking the mitogenic effects of estrogen. Nevertheless, many tumors that are initially dependent on E2 for growth and tumorigenicity eventually acquire the ability to grow in the absence of estrogen. However, the mechanism associated with the development of estrogen-independence is poorly understood. Studies have shown that there is a correlation between the loss of responsiveness to E2 and an increase in NF-κB activity (Pratt et al. 2003). There is increased NF-κB DNA binding in the majority of ER-negative tumors, whereas NF-κB DNA binding was almost absent in ER-positive tumor samples (Nakshatri et al. 1997, Pratt et al. 2003, Biswas et al. 2004). Studies have also shown that ER-positive E2-independent LCC1 breast cancer cells show constitutive NF-κB DNA binding when compared to their parental E2-dependent cell line, MCF-7 (Pratt et al. 2003). Furthermore, inhibition of NF-κB in E2-independent LCC1 cells reverts them back to an E2-dependent tumor phenotype (Pratt et al. 2003). ERα has also been shown to inhibit NF-κB activity and a direct physical interaction between the two proteins has been demonstrated *in vitro* (Stein and Yang 1995, Ray et al. 1997).

Together, these observations implicate an interaction between ERα and NF-κB in having a vital role in the development of breast cancer and estrogen independence.

Thus it appears that that NF-κB is activated in both ER-positive and ER-negative breast cancer cells and that it is attenuated by liganded ERα in ER-positive cells by regulating the formation of p50 and p65 complexes. We therefore proposed to determine i) the effects estrogen and the ERα have on NF-κB activity and cell viability; ii) the effect 4-OH-Tamoxifen has on NF-κB activity; iii) the effect that inhibition of NF-κB has on ER activity.

Collectively, our results suggest that there is mutual repression between NF-κB and ERα and that in the presence of E2, ERα is capable of binding and sequestering p50 and p65 to an endogenous ERE, a phenomenon accompanied by inhibition of NF-κB transcriptional activation. We also show that the overexpression of ERα has catastrophic effects on cell viability. Lastly, we show that tamoxifen does not interfere with NF-κB activity.

## 4.1 ERα Expression Reduces Cell Viability

Our data demonstrates that expression of ERα in ER-negative cells and overexpression of ERα in ER-positive breast cancer cells results in reduced cell viability. Estrogen, through its cognate ERα, is known to be involved in normal development and differentiation of mammary tissues and has been implicated in the progression of breast cancer. The principle role of ERα is to promote cell growth and survival. However, our results show that an increase in ERα

expression is associated with decreased cell survival. Interestingly, it has been shown that estrogen inhibits the growth of longterm tamoxifen-treated breast tumors (Liu et al. 2003). Antitumor activity of E2 has also been reported for estrogen-deprived breast cancer cells *in vitro* (Fernandez et al. 1998, Song et al. 2001) and tamoxifen-stimulated breast tumors *in vivo* (Chisamore et al. 2001). Moreover, high dose estrogen has been used as an effective treatment for breast cancers that have developed resistance to successive antiestrogenic therapies (Lonning et al. 2001). The mechanism by which E2 eventually loses its ability to promote cell survival and becomes antitumorigenic is currently unknown. Our results demonstrate that ERα is capable of inhibiting NF-κB activity. NF-κB is involved in the inhibition of cell apoptosis (Bours et al. 2000), cell-cycle progression (Karin et al. 2002), and has been implicated in oncogenesis (Chen et al. 2001). Therefore, it is possible that after longterm growth in the absence of E2, or with tamoxifen treatment, epigenetic changes may alter the ERα signaling pathway rendering it dysfunctional. However, ER retains its ability to inhibit the anti-apoptotic effects mediated by NF-κB, and that this may be the basis for the antitumorigenic effects of E2 on longterm estrogen deprived or tamoxifen treated breast cancer cells (Figure 17).

## 4.2 ERα Inhibits NF-κB Activity

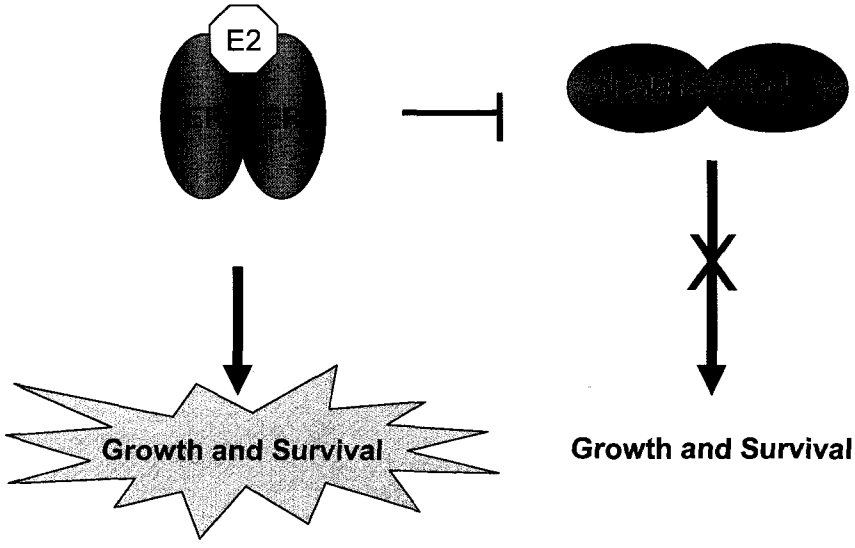
### *4.2.1 ERα Inhibits NF-κB DNA binding*

In order to examine if the expression of ERα is capable of inhibiting NF-κB activity, we first analyzed it's effect on DNA binding by NF-κB. We found that expression of ERα in ER-negative breast cancer cells resulted in a reduction in NF-κB-DNA binding. Moreover, treatment with E2 further reduced binding of NF-κB to DNA in ER-negative SKBr-3 cells.

LCC1 cells have been shown to express a 61kDa variant of ER, corresponding to an exon 3 deletion. It is believed that the exon 3 deletion is a result of aberrant mRNA splicing, therefore wild type ERα is also expressed. Exon 3 encodes the second zinc finger of the DNA binding domain (C domain). Therefore, the ERΔ3 protein is not able to bind EREs and does not activate transcription by directly binding DNA (Wang and Miksicek 1991). Using GST-pulldown assays we show that the C domain appears to be required for interaction with p50 and to a lesser degree p65. In addition, studies have shown that mutants devoid of the DNA binding domain are unable to inhibit NF-κB activity (Ray et al. 1997). LCC1 cells have previously been shown to display constitutive NF-κB activation. We found that treatment of wt ERα-expressing LCC1 cells led to a decrease in NF-κB DNA binding after treatment with E2. It is therefore possible that the exon 3 deletion mutant expressed in LCC1 cells is unable to bind p50 or p65, and this allows constitutive NF-κB activation.

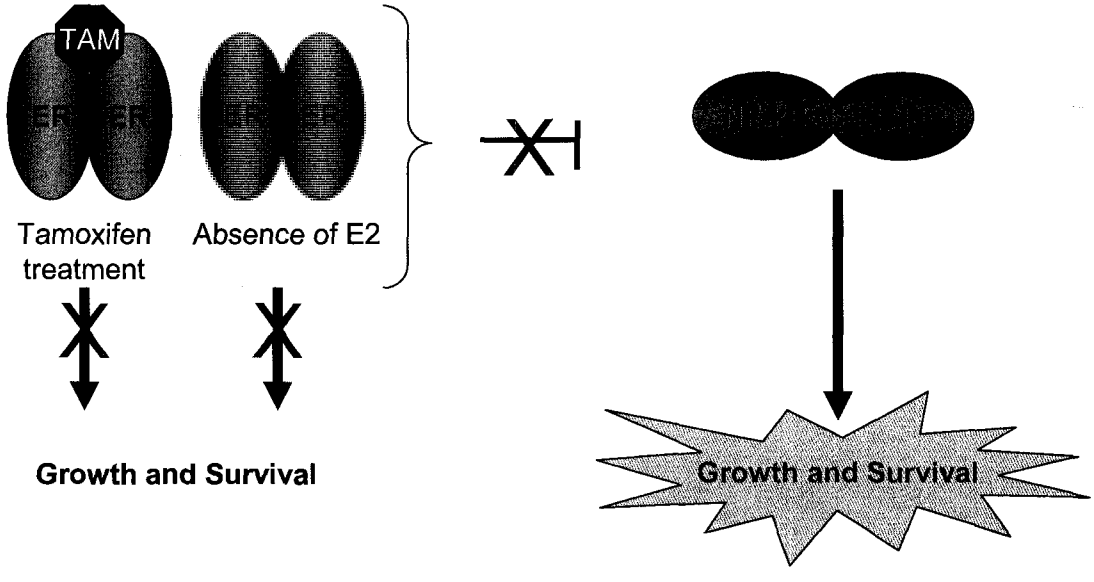
**Growth in the presence of E2**

A




**Longterm growth in the absence of E2 or tamoxifen treatment**

B



C

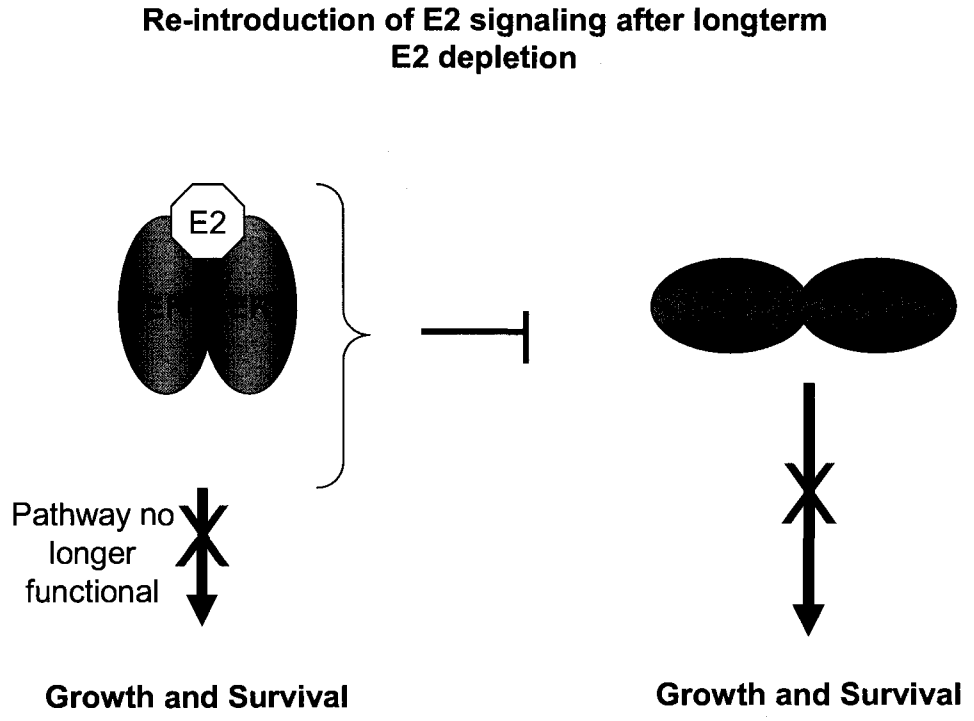



**Figure 17. Model of Apoptotic effect of E2.**
In hormone dependent breast cancer cells grown in the presence of E2 the ER provides growth and survival signals to the cells and inhibits NF-κB activity (A). After longterm growth in the absence of E2 or with tamoxifen treatment, the ERα signaling pathway becomes dysfunctional and cells survive from the growth and survival signals provided by NF-κB activity (B). Re-introduction of E2 signaling leads to reduced cell viability because the ERα retains its ability to inhibit the anti-apoptotic effects mediated by NF-κB in the presence of E2 (C). This may be the basis for the antitumorigenic effects of E2 on longterm estrogen deprived or tamoxifen-treated breast cancer cells.

### 4.2.2 *ERα Inhibits NF-κB Activity*

We demonstrate that the expression of ERα was also associated with a reduction in NF-κB activity. Western blot analysis shows that the expression of ERα correlated with a reduction in levels of NF-κB-regulated proteins in both ER-positive and ER-negative cell lines. Furthermore, using a κB-reporter assay, we demonstrate that increased ERα expression leads to a decrease in NF-κB activity.

These results show that under the conditions of these experiments ERα is capable of inhibiting NF-κB activity independent of E2. It is possible that the inhibition of NF-κB is independent of E2 in these experiments because the ERα is being expressed at much higher levels than normally present in breast cancer cells. In the absence of E2, the ERα is normally part of a protein complex consisting of a dimer of hsp90 and hsp70 (Chambraud et al. 1990). Ligand binding induces conformational changes in the ER, which causes dissociation of chaperones such as hsp70 and hsp90 (Chambraud et al. 1990), and association of co-regulatory proteins. In addition, in the absence of E2, the ERα can also interact with the nuclear receptor corepressors SMRT and NcoR (Smith et al. 1997, Lavinsky et al. 1998, McKenna et al. 1999) to repress transcription. It is possible that complex formation with either hsp90 or hsp70 or with co-repressor proteins prevents association of ERα with NF-κB. However, the ERα is highly overexpressed in this system and it is possible that there are not enough chaperone or co-repressor proteins to prevent association with NF-κB. It is also possible that ERα is capable of interacting with NF-κB both in the presence and absence of E2, and that there is a higher affinity interaction in the presence of E2.

However, the overabundance of ERα in this system favors the lower affinity interaction, leading to the repression of NF-κB in low E2 conditions.

### *4.2.3 ERα Interacts with NF-κB*

We hypothesized that in the presence of E2, ERα is capable of interacting with NF-κB and inhibiting transactivation. We propose that the inhibition is due to a sequestration of NF-κB by the ERα to the ERE. Our data demonstrates direct association of an ERα deletion mutant, containing the C,D,E and F domains, with p65 *in vitro*, indicating that the A and B domains are not required for the interaction with p65. We further show that a mutant containing only the E and F domains of ERα is also capable of interacting with p65, although the interaction is much weaker than that of the mutant containing the C,D,E and F domains. These studies are consistent with previous reports indicating that the A, B and C domains of ERα are not required for interaction with p65 and we show that, although the interaction is weaker, the D domain is also not necessary for the interaction (Stein and Yang 1995).

Studies have also shown that ERα is capable of interacting with p50 but have not investigated the domains of ERα involved in the interaction (Stein and Yang 1995). We show that the ERα deletion mutant containing the C, D, E and F regions can interact with p50 and that deletion of the C and D domains abolishes the interaction. This data suggests that the A and B domains are not required and, unlike p65, the C and D, as well as the E and F domains are required for interaction with p50. Our results indicate a dependence on the hormone-binding

domain (EF), the D domain (hinge region) and the C domain (DNA binding domain) for ER/p50 complex formation. These studies confirm that ERα is capable of interacting with both p65 and p50 and that the interaction with these two proteins may require different domains of ERα.

The mechanism of NF-κB inhibition by ERα is unknown and there are several possible explanations. The first possibility is that NF-κB and ERα could compete for common co-activators or transcription intermediary factors, a process referred to as squelching. However, overexpression of p160 and cAMP-response element-binding protein-binding protein/p300, co-activators shared by both NF-κB and ER, failed to relieve repression of p65, suggesting that the inhibition of p65 can occur independently of these co-activators (Speir et al. 2000). Furthermore, our results show that ER is capable of inhibiting NF-κB activity even in the absence of E2. Since the recruitment of coactivators is ligand-dependent then in the absence of E2 there should be little ER:coactivator complex formation. A second possibility is that ERα inhibits IKKα-mediated activation and nuclear translocation of NF-κB. Normally, IKKα phosphorylates the IκB-like C-terminal domain of p100 and p105, resulting in the translocation of p52 and p50 dimers to the nucleus. Notably, IKKα has also been shown to associate with ERα on the ERE in response to treatment with E2 (Park et al. 2005). It is therefore possible that in the presence of E2, ERα recruits IKKα preventing it from phosphorylating and allowing NF-κB translocation to the nucleus. However, previous studies have shown that there is no change in p50 or p65 intracellular location in response to treatment with E2 (Ghisletti et al.

2005). A third possibility is that a direct interaction between the two proteins could account for the repression. Such a complex could either (i) be unable to bind DNA or (ii) result in formation of inactive complexes on the DNA by preventing the interaction of essential co-factors or the basal transcription machinery.

Using a chromatin immunoprecipitation assay, we demonstrate that ERα forms a complex on an endogenous ERE with NF-κB subunits p50 and p65 and that this association is amplified in the presence of E2. EMSA studies performed in our lab have also demonstrated that there is no interaction between ERα and the κB-response element indicating that when bound to ERα NF-κB can no longer bind the κB-response element. This evidence is consistent with our hypothesis suggesting sequestration of NF-κB to the ERE in the presence of E2. Therefore, our data supports the third possibility that in ER-positive breast cancer cells, E2-bound ERα is capable of binding and sequestering NF-κB to the ERE, thereby preventing NF-κB from binding its own response element and activating transcription.

Our results suggest that the expression of ERα results in reduced NF-κB transactivation and the inhibition of NF-κB by ERα could possibly play a role in the antitumorigenic effects of E2 on longterm estrogen-deprived or tamoxifen-treated breast cancer. Furthermore, it is possible that loss of NF-κB inhibition due to growth in the absence of E2, loss of ER expression or ER mutation might send proliferative signals to breast cancer cells allowing their survival in the absence of E2 (Figure 18).

## 4.3 MUTUAL REPRESSION OF ERα AND NF-κB

In order to examine the effect of NF-κB inhibition on ER activity, we used stable IκBα$^{SR}$ expressing clones. We are the first to show that inhibition of NF-κB resulted in increased levels of E2-regulated proteins. Furthermore, E2-reporter assays verified that there was an increase in ER activity. These data demonstrate that a mutual repression exists between ERα and NF-κB. In support of this notion, other studies have shown the existence of this type of interaction between NF-κB and the progesterone receptor (PR). Activation of NF-κB by tumor necrosis factor-α (TNF-α) results in repression of PR, while hormone-induced PR is able to repress TNF-α-induced NF-κB activity (Kalkhoven et al. 1996). There are several possibilities for the mechanism of NF-κB-mediated repression of ERα. The first is that NF-κB and ER could compete for common co-activators. A second possibility is that a direct interaction between the two proteins could account for the repression by inhibiting binding of ERα to DNA or by the formation of inactive complexes on DNA by preventing the interaction of essential co-factors or the basal transcription machinery. Our results show that ERα is capable of forming a complex on the ERE with NF-κB subunits (Figure 12). Therefore, this suggests that NF-κB does not inhibit DNA binding by ERα. In order to determine which of the other two mechanisms underlies the NF-κB mediated inhibition of ERα, further investigation is required. For example, one could overexpress the co-activator proteins shared by NF-κB and ERα, to determine if this would prevent inhibition.

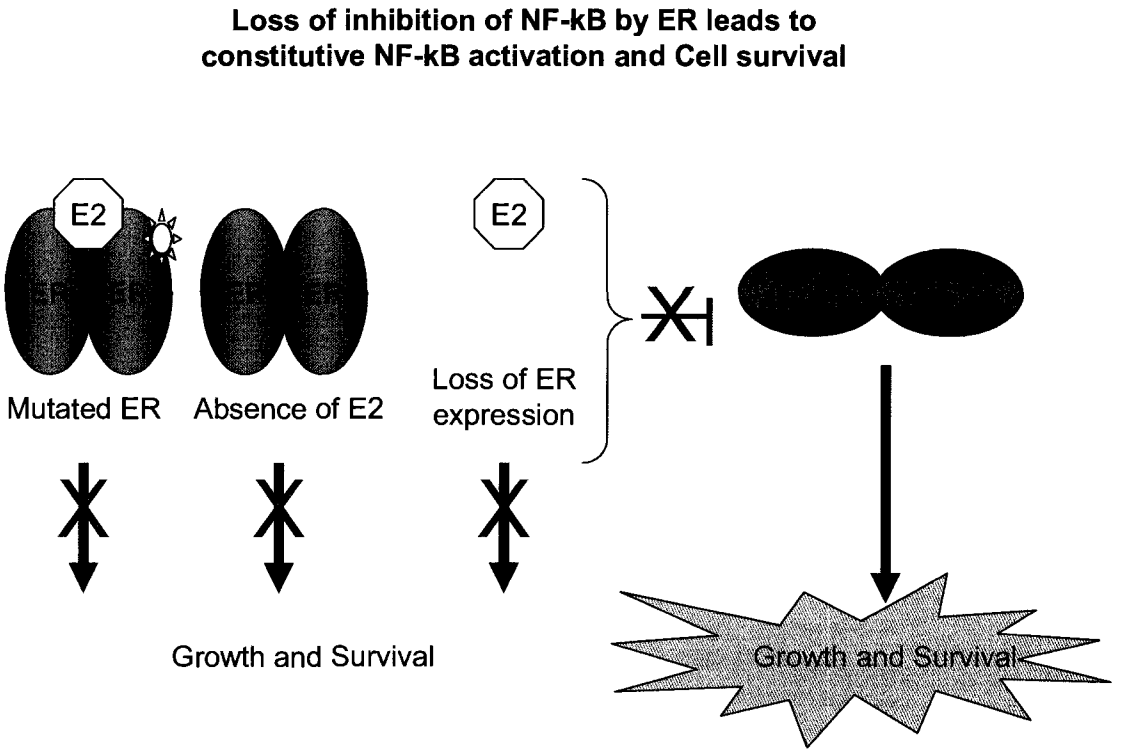



**Figure 18. Model of development of E2-independence.**
Loss of E2 signaling, ER expression or mutation of the ER leads to loss cell growth and survival signals from the E2 dependent pathway and also leads to loss of inhibition of NF-kB. This leads to constitutive NF-kB activation which provides proliferative and anti-apoptotic signals to breast cancer cells allowing their survival in the absence of E2.

The cell proliferative actions of both estrogen and active NF-κB are mediated in part by increased expression of cyclin D1, driving S phase and cell cycle progression (Biswas et al. 2005). Thus the regulation of cyclin D1 is a point at which the paths of estrogen and NF-κB signaling merge (Biswas et al. 2005) and promote cell survival. Interestingly, cyclin D1 is elevated in 50% of breast cancers and has been suggested to be an essential NF-κB target gene in breast tumorigenesis (Joyce et al. 2001). We also demonstrate here that although expression of ERα reduced levels of some NF-κB regulated proteins, there was no decrease in cyclin D1 levels. It is possible that because cyclin D1 is regulated by both ERα and NF-κB, the inhibition of NF-κB-mediated expression of cyclin D1 is counterbalanced by increased Erα-mediated expression of cyclin D1, therefore there is no net change in cyclin D1 protein levels. Thus the mutual repression of ERα and NF-κB prevents overexpression of cyclin D1. Loss of Erα-mediated repression of NF-κB could lead to increased levels of cyclin D1, uncontrolled cell growth and tumorigenesis. On the other hand, overexpression of some genes involved in cell growth can promote rather than suppress apoptosis. For example, in normal cells *c-myc* drives proliferation and prevents cell-cycle arrest upon serum withdrawal. However, while *c-myc* expressing cells continue to proliferate in low serum, cells do not accumulate because they die by apoptosis (Evan et al. 1992). Therefore, the pro-apoptotic activities of *c-myc* appear to be coupled to its hyperproliferative activity, implying that apoptosis acts as a protective mechanism against oncogenic transformation. It is therefore possible that overexpression of cyclin D1 could also lead to cell death in the absence of

other anti-apoptotic signals. Interestingly overexpression of cyclin D1 makes MCF-7 cells highly susceptible to apoptosis induced by retinoic acid (Teixeira and Pratt 1997).

## 4.4 TAMOXIFEN HAS NO EFFECT ON NF-κB ACTIVITY

Tamoxifen has been shown to have both agonistic and antagonistic effects on ERα activity. Tamoxifen induces a conformational change in the ER that prevents binding of co-activators and therefore blocks AF-2-induced (ligand-dependent) transcription (Shiau et al. 1998). Therefore, tamoxifen blocks the transcription of genes that depend essentially on AF-2 for gene expression. However, for AF-1-dependent genes, tamoxifen can function as an agonist (Tzukerman et al. 1994). In addition, it has been shown that tamoxifen interacts with co-repressors when bound to ER, and that this mechanism is involved in the suppression of transcription (Schiff et al. 2003). Therefore, the availability of co-regulators may be the cause of the tissue-dependent effects of tamoxifen. Since tamoxifen interacts with ER we sought to determine if the growth inhibitory effects of tamoxifen could be, in part, due to an inhibition of NF-κB acitivty. We found that although tamoxifen inhibited the expression of E2-regulated proteins, it did not have any effect on the expression of NF-κB-regulated proteins ErbB2, p50 or cIAP. It is therefore possible that resistance to SERMs could in part be due to an inability to inhibit NF-κB activity. Therefore, once activated NF-κB could then replace the growth signals normally provided by E2.

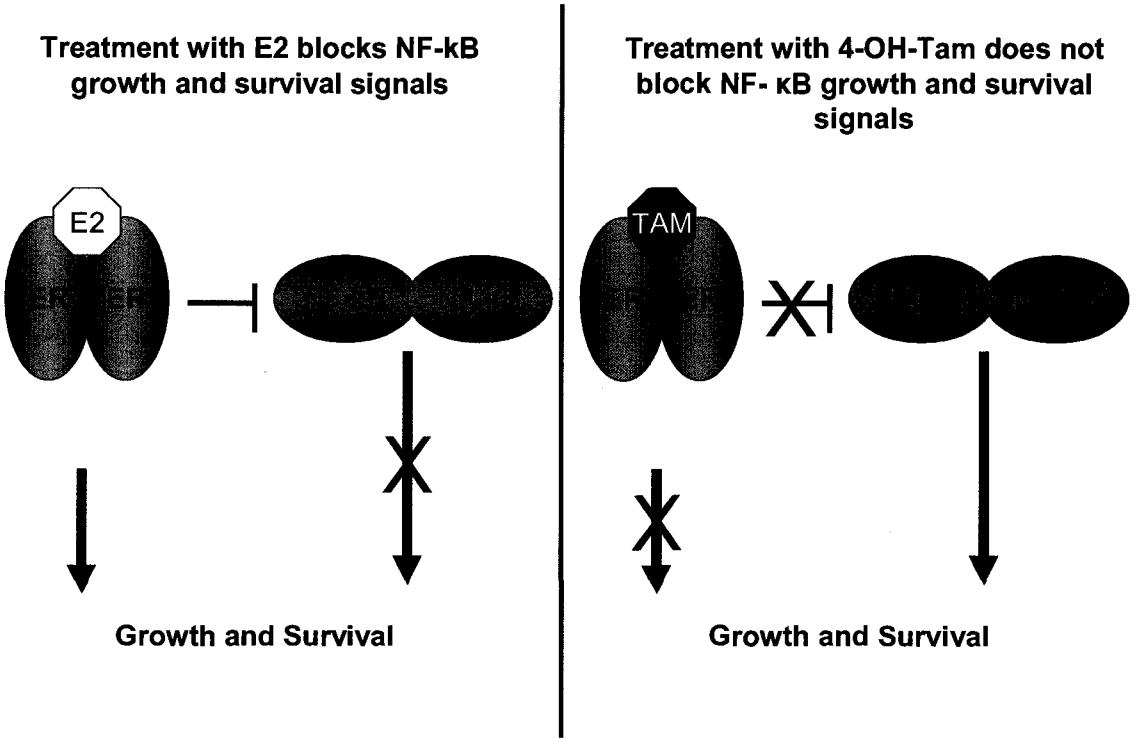



**Figure 19. Model of NF-κB activation and cell survival in response to treatment with tamoxifen.**
Treatment with E2 activates the ERα signaling pathway leading growth and survival signals in breast cancer cells and also inhibition of NF-κB mediated growth and survival. Replacement of E2 with tamoxifen inhibits the ERα pathway, however, tamoxifen does not induce inhibition of NF-κB by ERα . Therefore NF-κB is free to activate the transcription of genes involved in growth and survival, replacing the growth signals normally provided by E2 with its own.

In order to confirm that tamoxifen has no effect on NF-κB activity one could perform an EMSA analysis to verify that tamoxifen does not inhibit NF-κB DNA binding. One could also perform a ChIP to verify that tamoxifen treatment does not increase p50 or p65 complex formation on the ERE.

## 4.5 Implications in Breast Cancer

Following acquisition of E2-independent growth, breast cancer cells soon develop a resistance to antiestrogen treatments. Resistance to antiestrogens such as SERMs is believed to result from a complex series of changes that prevent apoptosis and thereby enhance cell proliferation and survival. Alterations in several signal transduction pathways have been described for tamoxifen resistance, including enhanced activity of the activation protein 1 (AP-1) (Schiff et al. 2000), and increased expression of protein kinase ErbB2 (Chung et al. 2002), and insulin like growth factor 1 (Parisot et al. 1999); all of these events have been linked to the activation of NF-κB (Parisot et al. 1999, Vertegaal et al. 2000, Zhou et al. 2000, Bhat-Nakshatri et al. 2002). We show that inhibition of NF-κB activity also increased levels of GADD45α, which suppresses tumor cell growth and colony formation (Ying et al. 2005). In our studies, we also show that ERα expression reduces levels of ErbB2. Increased expression of ErbB2 due to increased copy number is found in 20% of breast cancers (Frank et al. 2005). As previously mentioned, NF-κB positively regulates the expression of ErbB2 (Kitamura et al. 2005). Therefore, loss of inhibition of NF-κB would also lead to increased ErbB2 levels. Increased levels of ErbB2 enhances the estrogen

agonistic properties of tamoxifen *in vitro* (Smith et al. 1997) and patients whose tumors expressed high levels of both steroid coactivator 3 (SRC-3) and ErbB2 had worse outcomes with tamoxifen therapy than all other patients combined (Osborne and Fuqua 1994). ErbB2 also activates downstream signals such as the MAPK pathway and the AKT pathway. Activation of these pathways lead to cell division and migration (both associated with tumorigenesis), as well as cell-adhesion, differentiation and apoptosis (Yarden and Sliwkowski 2001). The MAPK pathway is also involved in ligand-independent activation of ERα. Therefore it is possible that loss of inhibition of NF-κB by ERα leads to increased expression of ErbB2, and ultimately leads to the ligand-independent activation of ERα through the MAPK pathway.

Studies have previously shown that ER-negative and E2-independent cells show elevated NF-κB activity (Nakshatri et al. 1997, Pratt et al. 2003). We have demonstrated that there is a mutual repression between ERα and NF-κB. We further show that the ERα and NF-κB are capable of physically interacting on the ERE. We propose that in E2-dependent breast cancer cells, E2-bound ERα inhibits NF-κB activity. Therefore, in E2-dependent cells, growth and survival signals are mediated by ERα and NF-κB survival signals are blocked. However, loss of inhibition of NF-κB by ERα due to loss of ERα expression, mutation or growth in the absence of E2, leads to constitutive NF-κB activation. NF-κB regulated proteins are involved in cell cycle progression, anti-apoptosis, cell survival, hormone-independence and tamoxifen resistance (Figure 20).

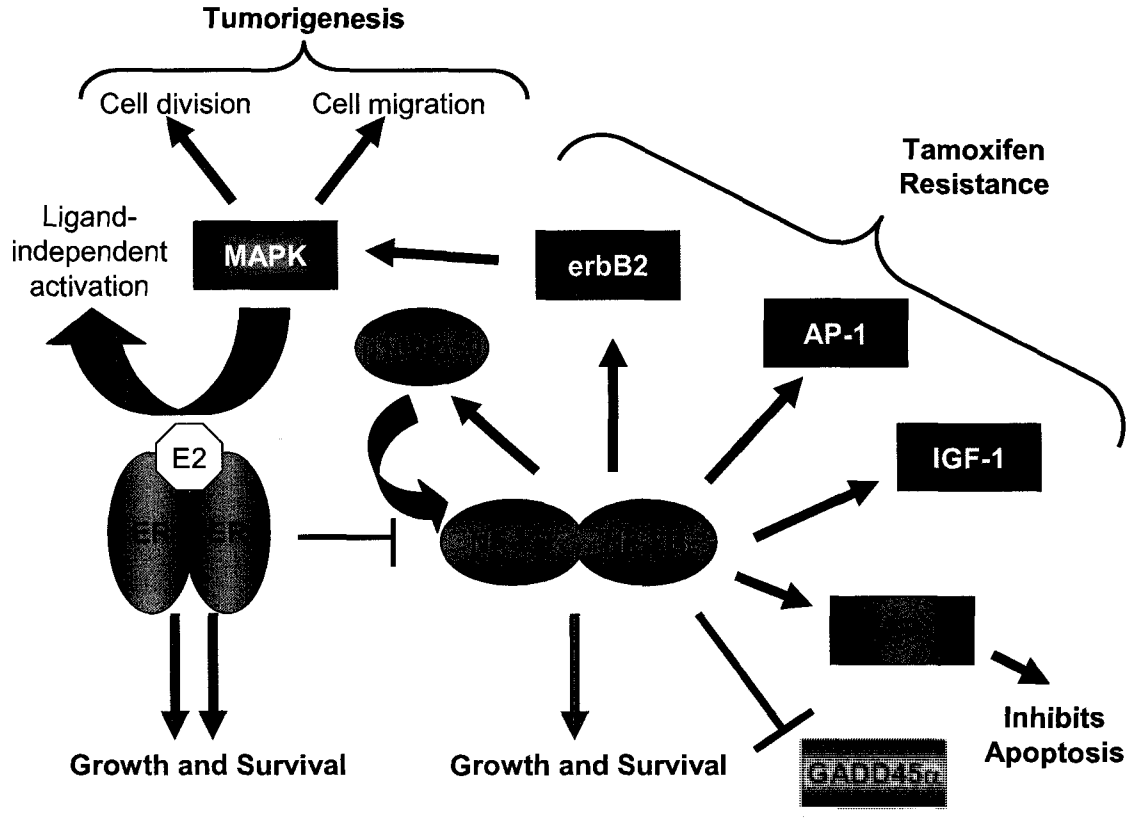



**Figure 20. Model of role of ERα and NF-κB in breast cancer development and hormone-independence.**

Loss of inhibition of NF-κB by ERα leads to an increase in the expression of NF-κB regulated proteins involved in cell growth and survival. Among these proteins are AP-1, IGF-1 and erbB2, all of which are associated with tamoxifen resistance. Furthermore, erbB2 regulates the MAPK pathway which is involved in ligand-independent activation of ERα and also promotes cell division and migration, both associated with tumorigenesis. These cells are now receiving growth and survival signals from the NF-κB pathway and are no longer dependent on E2. (Red arrows indicate consequence of loss of repression of NF-κB by ERα.)

Crosstalk between transcription factors of distinct families is an important phenomenon in regulating gene transcription and has recently become the subject of intensive investigation. The existence of crosstalk between estrogen and NF-κB signaling traffic could provide rationale for combined targeting of both ER and NF-κB in ER-positive/E2-dependent breast cancer to prevent acquisition of hormone independence. There are compounds that are capable of interacting with ER and inducing the repression-specific effects, including repression of NF-κB activity. However, they would not induce the expression of proliferative genes normally induced by E2. Therefore these drugs could kill two birds with one stone, first by blocking the tumor survival signals of E2 while simultaneously repressing NF-κB activity. Such drugs could prove to be most beneficial in the treatment of both E2-dependent and E2-independent breast cancers. One such drug has since been identified and is under investigation. The nonsteroidal compound WAY-169916 has been shown to block proinflammatory signals mediated by NF-κB in rat liver and also in human aorta endothelial cell-1 (HAEC-1) cells. Inhibition of NF-κB signaling requires binding of the selective ligand WAY-169916 to either of the two forms of ER (ERα or ERβ). Furthermore, WAY169916 failed to induce effects of estrogen, mediated by its classical ligand-dependent function in promoting gene expression (Chadwick et al. 2005). Thus, WAY-169916 appears to be an agent that can inhibit NF-κB signaling through the ER but without inducing the classical estrogen effects (Chadwick et al. 2005). Further studies are required to determine what effect this compound might have on breast cancer cells, both E2-dependent and independent.

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