A Mechanistic Study of Doxorubicin/Adriamycin's Effect on the Estrogen Response in a Breast Cancer Model

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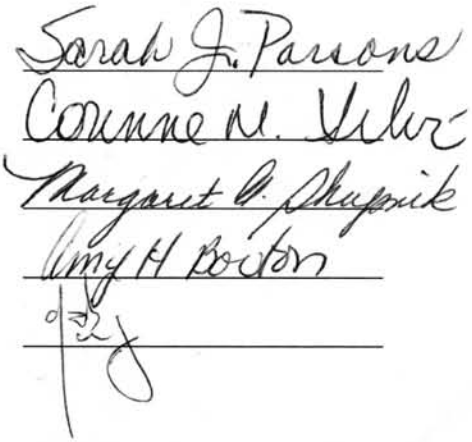

Abstract

Doxorubicin is a common chemotherapeutic treatment for ER+ breast tumors despite clinical reports demonstrating that ER+ patients respond less well to chemotherapy than ER- patients. Studies using ER+ breast cancer cell lines have shown that estrogen treatment counters chemotherapeutic effects, suggesting estrogen-induced signaling may confer chemotherapeutic resistance. However, little is known about whether and how chemotherapy affects estrogen/ER signaling, the effectors mediating chemotherapy's effects, and whether these signaling molecules promote chemotherapeutic resistance. Mimicking the prolonged estrogen and doxorubicin exposure seen clinically, this study used an ER+ breast cancer cell model to identify estrogen effectors that could serve as therapeutic targets to enhance doxorubicin's efficacy. Alterations in protein and phosphorylation levels of known estrogen signaling proteins (ER-α, c-Src, ERK, PI3K, AR, and EGFR) induced by doxorubicin or estrogen treatment individually were compared to untreated controls or combined treatment. Prolonged estrogen treatment alone modulated levels and phosphorylation of specific signaling molecules much like that seen previously with shorter estrogen treatment. Doxorubicin further upregulated some hormone and growth factor signaling molecules that were downregulated by estrogen alone, suggesting a mechanism by which doxorubicin could molecularly counteract the estrogen's biological effects. Co-treatment with estrogen and doxorubicin modestly enhanced changes induced by estrogen alone and markedly enhanced pro-growth alterations when compared to doxorubicin alone. Thus, the net effect of doxorubicin/estrogen co-treatment is pro-proliferative and pro-survival, suggesting a molecular mechanism for the poorer chemotherapeutic responses of ER+ breast tumors.

We show that inhibitors of MEK and metalloprotienases cooperated with doxorubicin to reduce cell proliferation, while inhibitors of the ER, SFKs, EGFR, and PI3K functioned independently of doxorubicin. Results indicate that both classes of inhibitors are potential candidates for overcoming doxorubicin resistance in ER+ breast cancers.

Additionally, the AR upregulation seen with estrogen and/or doxorubicin treatment suggested that AR may mediate the biological responses of ER+ breast cancer cells to these agents. Studies in the aforementioned conditions tested the contribution of AR to proliferation, survival, migration, and invasion. Preliminary results suggest that the AR contributes little to proliferation or survival, but might regulate migration and invasion in the absence or presence of estrogen and/or doxorubicin.

Dedication

This dissertation is dedicated to my God, who sustains me and gives me purpose,

"LORD, you are my God;

I will exalt you and praise your name,

for in perfect faithfulness

you have done wonderful things,

things planned long ago."

Isaiah 25:1

and also to Laurie Curtain, Bev Sutter, and Mary Vazquez-Amaral,

whose battles with breast cancer reminded me of the importance of this work.

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I would like to thank my parents, Alan and Judy Pritchard, for never questioning my ability or resolve when I proclaimed my intent to "get my Ph.D in biology" at the age of 14 or in the several years that lapsed between then and now. I am grateful to both parents and my extended family for their love, support, and prayers, as well as understanding when I rarely found time to call or visit. My Mamaw (Hazel Pritchard) and Aunt Sue (Sue Hatcher) provided me with many care packages throughout this process and my Aunt Ruthie (Ruthanne Risser) heard the cries of my heart. My cousin and fellow

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Finally, I am grateful to many friends who have shown me great kindness and love over the years and without whom this would have been a far more difficult endeavor. They are too many to list here but I feel I must at least mention Laura Veverka, Jenny Molaison, Gayle Hagler, Amy Vinson, Jill Mahler, Sarah Miller, Katie Hulse, Maria Selig, Sarah Malpass, Dave and Jenny Moore, Nathaniel Madura, Wesley Herron, the past and present members of the Christ Community Church Mosby Mountain Home Group, and the Board of Vwazen Nou.

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3D: three-dimensional

A.A.: amino acid

AF: activation function

AI: aromatase inhibitor

ANOVA: ANalysis Of Variance

AR: androgen receptor

ARE: androgen response element

Bic: bicalutamide, AR-targeted drug

BSA: bovine serum albumin

C/EBPß: CCAAT/enhancer binding protein ß

CGH: Clone: Globular Head

CML: chronic myelogenous leukemia

Complex I: NADH-dehydrogenase

Complex II: succinate dehydrogenase

Complex III: cytochrome c reductase or bc_1 complex

Complex IV: cytochrome c oxidase

Complex V: ATP synthase

Cox II: cytochrome c oxidase subunit II

Cox: cytochrome c oxidase

CSSM: charcoal-stripped serum medium

Cyt c: cytochrome c

DBD: DNA binding domain

DCIS: ductal carcinoma *in situ*

DHT: α-dihydrotestosterone

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethyl sulfoxide

Dox: doxorubicin

E: estrogen

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

EGFR: epidermal growth factor receptor

EMT: epithelial-mesenchymal transition

ER: estrogen receptor

ERE: estrogen response element

ERK: exracellular regulated kinase

ETC: electron transport chain

ETV1: Ets variant gene 1

FAK: focal adhesion kinase

FBS: fetal bovine serum

Fdx: Faslodex, ER inhibitor

FlnA: filamin A

Gef: gefitinib, EGFR inhibitor

GM: GM 6001, MMP inhibitor

GPCR: G protein-coupled receptor

GPR30: G-protein coupled receptor 30

HB-EGF: heparin-binding EGF

HER: human EGFR

HR: hormone receptor

IDC: infiltrating ductal carcinoma

IGF-1: insulin-like growth factor-1

IPTG: isopropyl-beta-D-thiogalactopyranoside

LBD: ligand binding domain

LY: LY 294002, PI3K inhibitor

MAPK: mitogen-activated protein kinase

MAPK: mitogen-activated protein kinase

MEK: mitogen-activated ERK-activating kinase

MMP: matrix metalloproteinase

MNAR: modular of nongenomic actions of the estrogen peceptor

NES: nuclear export sequence

NLS: nuclear localization sequence

NMR: nuclear magnetic resonance

NS: non-specific

NSCLC: non-small cell lung carcinoma

PARP: poly ADP-ribose polymerase

PBS: phosphate-buffered saline

PD: PD-98059, MEK inhibitor

PDK1: phosphoinositide-dependent protein kinase-1

PI3K: phosphoinositide 3-kinase

PIP_2: phosphotidylinositol (4,5)-phosphate

PIP_3: phosphotidylinositol (3,4,5)-phosphate

PR: progesterone receptor

pS: phosphoserine

pT: phosphothreonine

pY: phosphotyrosine

SARM: selective AR modulator

SCCHN: squamous cell carcinoma of the head and neck

SEM: standard error of the mean

SERD: selective estrogen receptor downregulator

SERM: selective estrogen receptor modulator

SFK: c-Src family kinase

SH2: Src homology 2

shRNA: short hairpin RNA

siRNA: small interfering RNA

SU: SU6656, SFK inhibitor

TBST: Tris-buffered saline Tween-20 buffer

TFF3: trefoil factor 3

TGF-α: transforming growth factor-α

TKI: tyrosine kinase inhibitor

TM4SF-1: transmembrane 4 L six family member 1

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

Chapter 1. Introduction

Breast cancer is the most commonly diagnosed and the second most deadly cancer in American women (NCI, 2011c). Breast tumors are typically classified according to their primary site, the extent of their spread (stage), pathology (grade), and expression of molecular markers, such as HER2, the progesterone receptor (PR) and/or the estrogen receptor (ER). ER+ breast cancers are three times more prevalent than ER- tumors (Kocic et al., 2010) and this, along with several of the risk factors for breast cancer, suggest that estrogen plays an important role in the development of the disease. These risk factors that suggest a causative role for estrogen include early onset of menses, late menopause (MacMahon, 2006), nulliparity, high alcohol consumption (which increases sex hormone levels and ER expression) (Chen and Colditz, 2007), obesity (as fat tissue is a site of estrogen production), and higher-than-normal circulating estrogen levels post-menopause (Cleary and Grossmann, 2009). As early as 1896, physicians observed that removal of the ovaries, the primary site of estrogen production in premenopausal woman, reduced breast cancer incidence, further supporting a role for estrogen in breast cancer (MacMahon, 2006). More direct evidence of estrogen/ER involvement in breast cancer development and progression comes from studies that have shown that hormonal therapies targeting estrogen production and/or the ER are effective preventative and therapeutic treatments for the disease (Yager and Davidson, 2006).

Breast cancer patients with ER+ tumors are often treated with surgical resection, hormonal therapies to reduce estrogen responses within the tumor, molecularly-targeted therapies, and/or chemotherapy. Though ER+ patients do often respond to

chemotherapy, several clinical reports have demonstrated that ER+ patients derive less benefit than their ER- counterparts (Berry et al., 2006; Bonilla et al., 2010; Conforti et al., 2007; Kuerer et al., 1999; Miles et al., 1999; Sertoli et al., 1995). Laboratory studies using ER+ breast cancer cell lines have also demonstrated that estrogen treatment counters the effects of chemotherapy, which may be the basis for clinical observations in the estrogen-responsive tumors (Huang et al., 1997; Leung and Wang, 1999; Razandi et al., 2000; Teixeira et al., 1995; Vasconsuelo et al., 2008).

Chemotherapeutics for the Treatment of Breast Cancer

The standard treatment administered to a(n) ER+ and/or PR+ (hormone receptor-positive, HR+) patient is dependent upon the characteristics of her tumor. Patients presenting with ductal carcinoma *in situ* (DCIS) typically receive only resection and radiation, whereas those with disease stages I or higher often receive a combination of surgery, radiation, endocrine therapy, and chemotherapy, based on the patient's treatment goals. With the exception of DCIS, trastuzumab (a HER2-targeted therapy) is added to the adjuvant therapy of HER2+ patients. Chemotherapeutic treatment is considered the standard of care for lymph node-positive breast cancers with primary tumors larger than 1 cm (Maughan et al., 2010).

A variety of chemotherapeutic agents are employed in the treatment of breast cancer. These include anthracyclines and anthraquinones (such as doxorubicin and epirubicin), taxanes (such as docetaxel and paclitaxel), anti-metabolites (such as 5-fluorouracil, capecitabine, gemcitabine, and methotrexate), vinca alkaloids (such as vinblastine), alkylating agents (such as cyclophosphamide, mitomycin, and the platinum analogs,

cisplatin and carboplatin), etoposide, and irinotecan (Mayer and Burstein, 2007). Treatment guidelines have not promoted one group of chemotherapy as preferable for a first, second, or third-line treatment of metastatic breast cancer (Mayer and Burstein, 2007). The National Institutes of Health recommends administration of multiple chemotherapeutic agents over treatment with one and recognizes that four to six rounds of chemotherapy (lasting for 3-6 months total) provides optimal benefit, while minimizing toxicity (NIH, 2001). In the time since the last recommendations were issued, many trials have studied various chemotherapeutic combinations and dosing schedules, though no one regimen has become the standard of care. Additionally, the most recent guidelines from the NIH do not support using biological markers (e.g. ER status) for any the choice of any specific chemotherapeutic regimen (NIH, 2001). Taxanes (docetaxel and paclitaxel) are often used to treat metastatic breast cancer, as they have been shown to be especially effective treatments within that setting. However, they are not recommended for the treatment of lymph node-negative breast tumors (NIH, 2001). Anthracyclines, such as doxorubicin, are a common treatment for both early-stage and advanced breast cancer (Mayer and Burstein, 2007) and data suggest that anthracycline-based chemotherapeutic regimens confer a small but statistically-significant increase in the survival of breast cancer patients when compared to those who received a non-anthracycline-based treatment (NIH, 2001). Because anthracycline and cyclophosphamide have been shown in clinical trials to be two of the more beneficial chemotherapeutic drugs for breast cancer treatment, these are often co-administered (NIH, 2001).

Doxorubicin

As discussed above, a common chemotherapeutic agent used to treat ER+ breast tumors is doxorubicin hydrochloride (trade names: Adriamycin, Rubex), an anthracycline antibiotic that was isolated from *Streptomyces peucetius var. caesius*. It inhibits DNA and RNA synthesis through its intercalation into double-stranded DNA (Barranco, 1984), binds to and alters phospholipid structure (Goormaghtigh et al., 1980; Parker et al., 2001), changes membrane fluidity and membrane protein aggregation by intercalating between membrane lipids, and its metabolites interfere with mitochondrial energy pathways, resulting in free radical release. These actions affect all stages of the cell cycle (Barranco, 1984).

Doxorubicin has been shown to negatively impact the biological responses of several cancers, including ER+ breast cancer. Following three to four days of doxorubicin treatment, leukemia (McCubrey et al., 2008), hepatocellular carcinoma (Choi et al., 2008), human epidermoid carcinoma (Brantley-Finley et al., 2003), Ewing's sarcoma (Mitsiades et al., 2001), and ER+ breast cancer (Fornari et al., 1994; Turton et al., 2001) cell lines demonstrated reduced viability/metabolic activity, as measured by the MTT assay. This reduction in viability is consistent with a doxorubicin-induced enhancement of killing in both dividing and non-dividing cells, though cells in S phase are the most sensitive (Barranco, 1984). Increased cell death has been shown in ER+ breast cancer (Sharma et al., 2004) and hepatocellular carcinoma (Choi et al., 2008) cell lines following 48 hrs of doxorubicin treatment, and work in rat neuronal cultures suggests that doxorubicin-induced apoptosis may be the result of Fas signaling (Wetzel et al., 2003). Additionally, studies in ER- breast carcinoma, melanoma, and fibrosarcoma models have shown that doxorubicin can impair migration and invasion, and this may occur through

the negative regulation of FAK and Rho activity (Fourre et al., 2008; Millerot-Serrurot et al., 2010; Pichot et al., 2009; Repesh et al., 1993). Interestingly, an *in vitro* study in murine neuroblastoma revealed that doxorubicin induced the partial differentiation of cancer cells in that system (Schengrund and Sheffler, 1982). The negative impact of doxorubicin on ER+ breast cancer cell lines, such as decreased cell viability (Fornari et al., 1994), apoptotic induction (Sharma et al., 2004), and cell cycle arrest (Janicke et al., 2001; Rusetskaya et al., 2009), contrast with the positive influences of estrogen on these biological processes. Therefore, estrogen and doxorubicin may employ some of the same signaling molecules and/or pathways to modulate biological outcomes in opposite ways. However, little is known about whether and how doxorubicin affects estrogen/estrogen receptor signaling and the cellular components that mediate this signaling, and whether estrogen signaling molecules may mediate resistance to doxorubicin.

Mechanisms of Estrogen Action

Estrogen regulates the cellular proliferation, survival, differentiation, function, and homeostasis of many tissues, including the male and female reproductive systems (Akingbemi, 2005; Brisken and O'Malley, 2010; Critchley and Saunders, 2009; McPherson et al., 2008), the cardiovascular system (Ling et al., 2006; Simoncini et al., 2006), auditory system (Charitidi et al., 2009), skeletal and smooth muscle (Boland et al., 2008; Ropero et al., 2008), adipose tissue (Ropero et al., 2008), brain (Belcher, 2008; Ropero et al., 2008), bone (Khosla, 2010), liver (Alvaro et al., 2006; Ropero et al., 2008), pancreas (Ropero et al., 2008), and skin (Verdier-Sevrain et al., 2006). Estrogen is synthesized from androgens by the aromatase enzyme complex (Cleary and Grossmann,

2009) and is predominately produced by the ovaries in premenopausal women, while in men and pre- and postmenopausal women it is made from adrenal androgens at low levels by adipose tissue, skin, bone, and brain (Simpson et al., 1999). In obese postmenopausal women, adipose tissue is the main site for estrogen production, which may explain the inverse correlation between obesity and survival in breast cancer patients (Cleary and Grossmann, 2009). Moreover, breast tumors are capable of secreting factors, such as EGF and TGF-α, that increase aromatase activity in adipose fibroblasts (Suzuki et al., 2010), offering an explanation for reports of estrogen levels at breast tumor sites ten-fold higher than circulating levels (Cleary and Grossmann, 2009). In ER+ breast cancers, estrogen stimulates proliferation, survival and anchorage-independent growth (Stoica et al., 2003); these biological responses are all hallmarks of cancer when unchecked.

Canonical Estrogen Receptor Structure

Estrogen can act by binding to its canonical receptors, ER-α and/or ER-β, which form homo- and heterodimers that, in turn, activate transcriptional and rapid signaling cascades. Though their expression pattern varies by tissue, many early model breast cancer cell lines, such as MCF-7 and T47D, express both isoforms (Vladusic et al., 2000). Both isoforms of the estrogen receptor are highly homologous transcription factors that share the same overall structure (Fig. 1). The ER is divided into functional domains: the N-terminal A/B region, domain C, and the C-terminal D/E/F region. The A/B domain mediates protein-protein interactions and ligand-independent target gene expression. The

Figure 1. Canonical estrogen and androgen receptor structures.

The ERs and ARs both belong to the steroid receptor family and, therefore, have similar structures. A. The ER isoforms ER-α and ER-β are composed of six domains: the A domain (cyan), B domain (red), C domain or DBD (green), D domain or hinge region (purple), E domain or LBD (orange), and F domain (indigo). The B domain contains the AF-1, whereas the E domain contains the AF-2. Serine (yellow), threonine (pink), and tyrosine (light blue) circles depict phosphorylation sites. Reported homologies between the ER isoform domains B-F are listed between the schematics of ER-α and ER-β and color-coded according to their respective domains. B. AR-A and AR-B are each composed of a NTD (red), DBD (green), hinge region (purple), and LBD (orange). The NTD contains the AF-1, comprised of the Tau-1 and Tau-5 regions (dark red), and the LBD contains the AF-2 (light brown). The first 187 amino acids of the NTD of AR-A are deleted in the AR-B isoform but it is otherwise identical to the AR-A isoform. Phosphorylations are depicted as in Panel A, and homologies between the ERs and AR-A are shown between Panel A and Panel B. The figure is adapted from (Gao et al., 2005; Klinge, 2000; Kong et al., 2003; Koochekpour, 2010; Li and Al-Azzawi, 2009; Ward and Weigel, 2009).

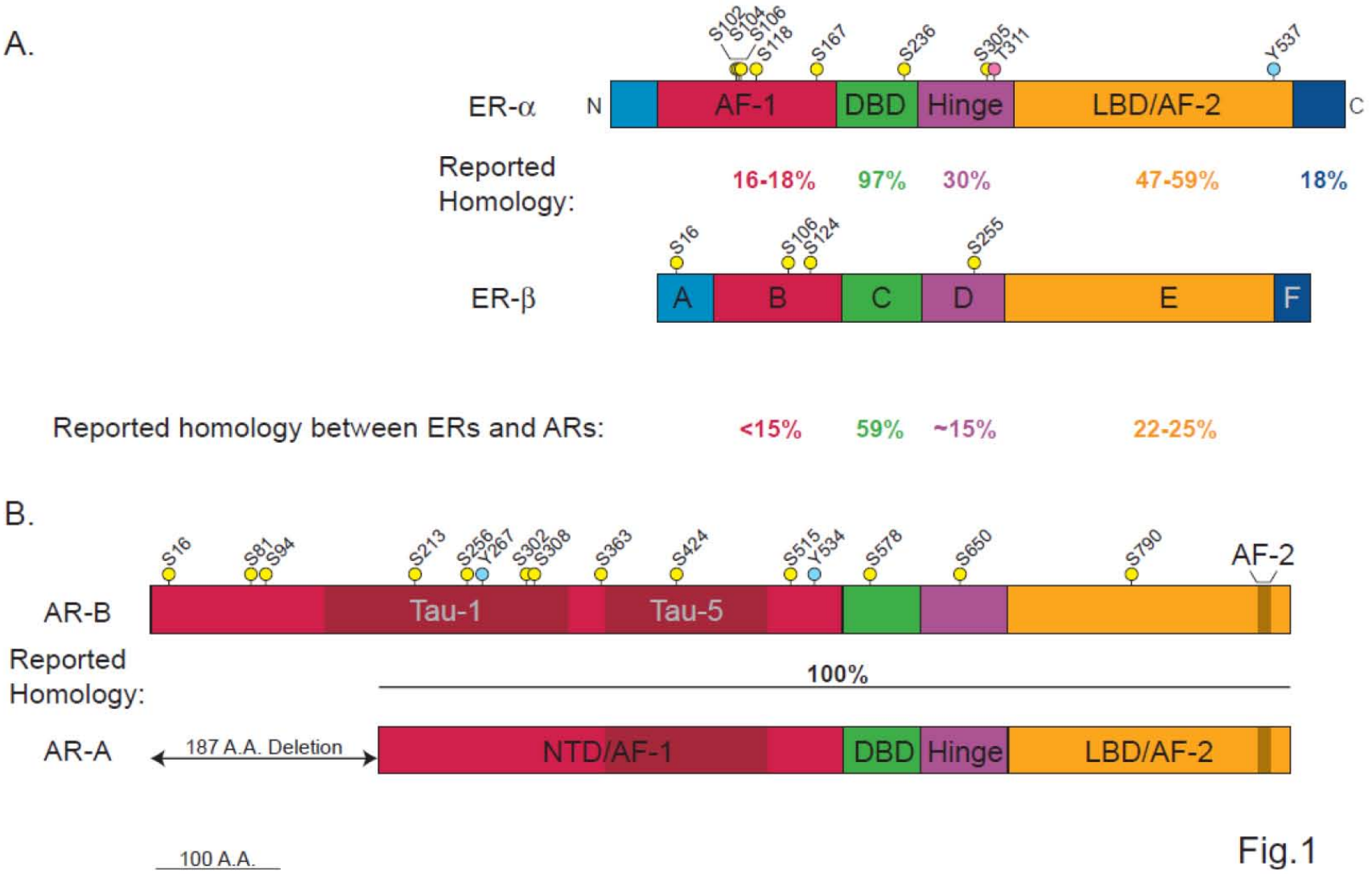

A.
S102
S104
S106
S118
S167
S236
S305
T311
Y537
ER-α
N
AF-1
DBD
Hinge
LBD/AF-2
C
Reported Homology:
16-18%
97%
30%
47-59%
18%
S16
S106
S124
S255
ER-β
A
B
C
D
E
F
Reported homology between ERs and ARs:
<15%
59%
~15%
22-25%
B.
S16
S81
S94
S213
S256
Y267
S302
S308
S363
S424
S515
Y534
S578
S650
S790
AF-2
AR-B
Tau-1
Tau-5
Reported Homology:
100%
AR-A
187 A.A. Deletion
NTD/AF-1
DBD
Hinge
LBD/AF-2
100 A.A.

Fig.1

ligand-independent activation function-1 (AF-1) located in this region is very active in ER-α but has negligible activity under the same conditions in ER-β (Nilsson et al., 2001), a difference reflected by only a 16-18% homology for this region between the two isoforms (Klinge, 2000; Kong et al., 2003; Yager and Davidson, 2006). The AF-1 region also contains several serines that are phosphorylated in response to estrogen-dependent and estrogen-independent signaling, including S118 and S167. Phosphorylation of AF-1 domain serines tends to promote co-activator recruitment and ER-mediated transcription (Lannigan, 2003), such as occurs with ER-α S167, which has been shown to mediate both ligand-dependent and -independent transcription (Ward and Weigel, 2009). However, there is some debate in the literature as to the role of pS118 in transcription as mutation of this residue to alanine yields varied results (Lannigan, 2003). Domain C serves as the DNA binding domain (DBD) and is 96-97% homologous between both isoforms (Klinge, 2000; Kong et al., 2003; Yager and Davidson, 2006), resulting in similar specificities and affinities for estrogen response elements (EREs) in the promoters and enhancers of estrogen-responsive genes. This domain contains two zinc fingers that contribute to receptor dimerization and binding to specific DNA sequences. Domains D, E, and F contain the hinge (domain D) region and ligand binding domain (LBD, domains E/F). These domains facilitate ligand binding, receptor dimerization, nuclear translocation, and target gene expression (Gao et al., 2005; Klinge, 2000; Kong et al., 2003; Koochekpour, 2010; Li and Al-Azzawi, 2009; Ward and Weigel, 2009). The ligand-dependent AF-2 is associated with the LBD and is altered structurally and functionally upon ligand binding. Agonists interact with this region to promote coactivator binding, whereas antagonists (such as anti-estrogens) block the coactivator

interaction surface and prevent transcription (Nilsson et al., 2001). Homology between isoforms in the LBD is about 50%, lending to some variability in agonist and antagonist binding and responses (Nilsson et al., 2001). ER-α's AF-2 also contains a tyrosine phosphorylation site (Y537) that has been shown to regulate downstream estrogen signaling, ER-mediated transcription, and possibly, ligand binding (Klinge, 2000; Kong et al., 2003; Yager and Davidson, 2006). The various estrogen-induced signaling and transcriptional actions of the ER promote proliferation, differentiation, survival, and angiogenesis in tissues responsive to estrogen.

ER-mediated Transcription

Classical ER-mediated Transcription

Estrogen receptors influence the cell by two distinct pathways, known as classical (genomic) and rapid (non-genomic) signaling. Apo-ERs are properly folded and undergo maturation that facilitates ligand-binding in the cytoplasm through their interaction with a multimeric chaperone complex (Arnold et al., 1995b; Ballare et al., 2003; Migliaccio et al., 1998; Ward and Weigel, 2009; Yudt et al., 1999) that includes Hsp90, Hsp70, p23, immunophilins (FKBP51/52), cyclophilins (Cyp40), Bag1 and TPR domain adaptor proteins (such as Hip and Hop) (Kimmins and MacRae, 2000). Classical transcription is stimulated when estrogen binds the ER, which induces a conformational change that facilitates the release of chaperone proteins and promotes dimerization. The dimer then translocates to the nucleus where the estrogen-ER dimer binds to EREs, located in the promoters and enhancers of estrogen-responsive genes (Fig. 2, #1) (Hager et al., 2004). As many as 70,000 EREs have been found in the human genome, though only a small

Figure 2. Estrogen activates gene expression and cytoplasmic signaling by a variety of mechanisms.

1. Classical estrogen action occurs when estrogen-bound ER dimers bind to ERE sites in the promoters and enhances of estrogen-regulated genes and promote transcription (purple arrow). 2. Estrogen signaling through c-Src, ERK, and Akt promote the phosphorylation and activation of non-ER transcription factors (orange arrows). 3. Estrogen-bound ER indirectly influences transcription through association with transcription factors bound to non-ERE sites (dark green arrow). 4. Estrogen increases MNAR occupancy with ER-α and c-Src, resulting in enhanced c-Src, ERK, and PI3K phosphorylation. Both c-Src and ERK phosphorylate ER-α in response to estrogen, and Src-mediated ER-α phosphorylation enhances transcription at ERE-containing genes (light blue arrows). 5. Estrogen stimulates ER-α binding to the p85 subunit of PI3K, which causes PI3K and Akt activation. ER-α is a substrate for Akt-mediated phosphorylation (light green arrows). 6. Estrogen promotes ER-α and AR association (yellow arrow). 7. Growth factors, such as EGF, stimulate unliganded ER to bind directly to DNA and promote transcription (red arrows). 8. Estrogen binds to the non-canonical estrogen receptor, GPR30, and stimulates EGFR activation through a c-Src and HB-EGF-dependent mechanism (blue arrows). 9. Estrogen upregulates IGF-1 through an unknown, ER-dependent mechanism. IGF-1 binds its receptor, IGF-1R, to promote MMP activation and HB-EGF release, resulting in EGFR activation and signaling (magenta arrows). Pathways illustrated in this figure are over-simplified and incomplete. Please see the text for a more detailed explanation.

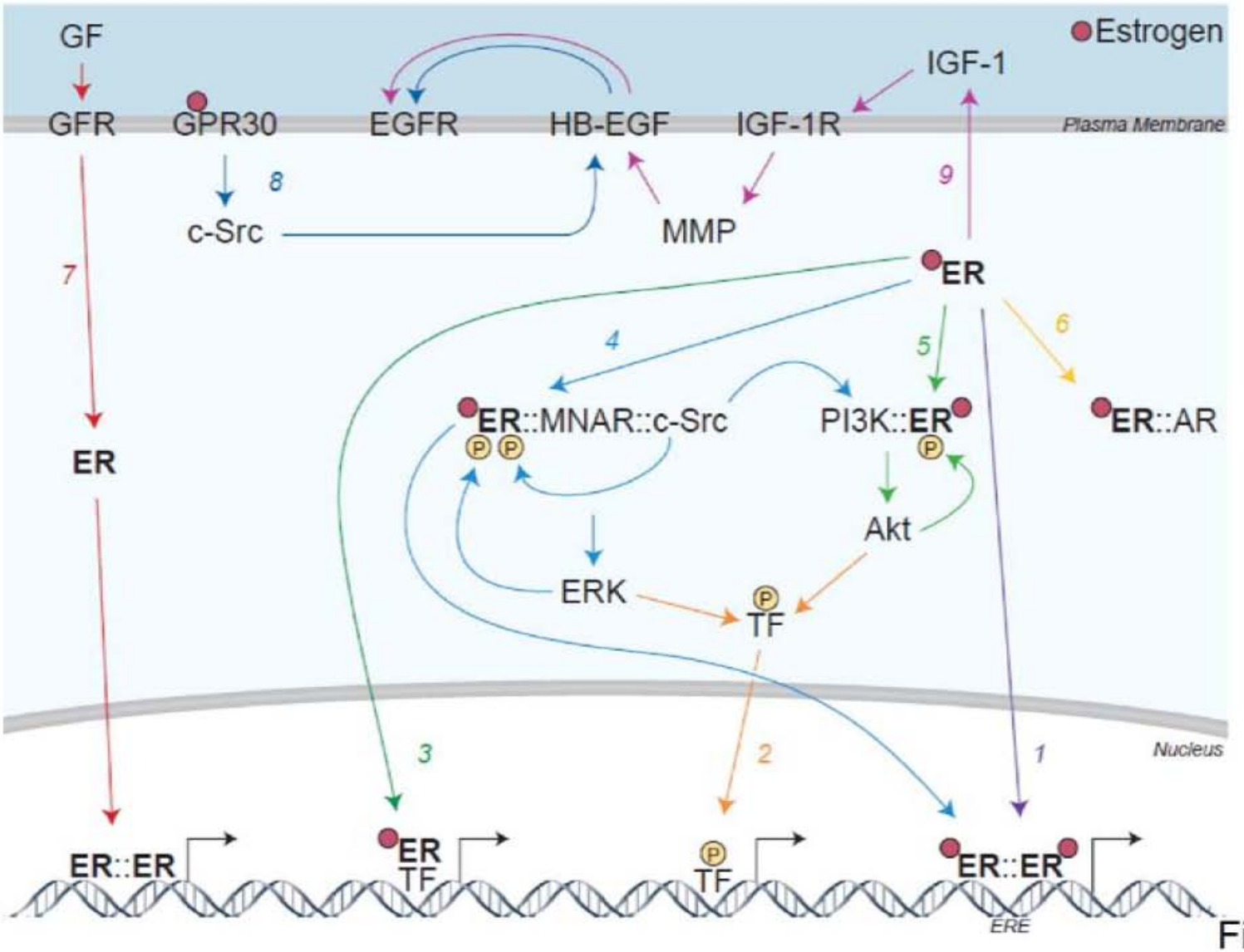

GF
Estrogen
IGF-1
GFR
GPR30
EGFR
HB-EGF
IGF-1R
Plasma Membrane
8
9
c-Src
MMP
7
ER
4
5
6
ER::MNAR::c-Src
PI3K::ER
ER::AR
ER
Akt
ERK
TF
Nucleus
3
2
1
ER::ER
ER
TF
TF
ER::ER
ERE

Fig.2

fraction have been found to mediate the 100-500 known estrogen-regulated genes (Nicholson et al., 1999; Welboren et al., 2009). Following ERE binding, coactivators that promote ER-mediated transcription through direct physical interaction with the transcription factor and RNA polymerase II, covalent histone modification, and chromatin remodeling (Welboren et al., 2009), form a complex with the estrogen-bound ER dimer, and transcription is initiated. Gene array experiments using cyclohexamide (a protein synthesis inhibitor) and ICI 182,780 (an ER-α and ER-β inhibitor) demonstrated that only about 23% of estrogen-regulated genes are direct ER targets. Genes that encode GREB1 (Li and Al-Azzawi, 2009), STAT-5a (Chakravarty et al., 2010a), and insulin-like growth factor-1 (IGF-1) (Welboren et al., 2009) are regulated through classical ER-mediated transcription. These actions are typically detected within hours of estrogen-stimulation (Hewitt et al., 2010).

Non-classical ER-mediated Transcription

In addition to its function in classical estrogen-induced transcription, the ER can participate in gene transcription through non-classical mechanisms, as evidenced by the expression of estrogen-regulated genes that do not contain EREs (Castellano et al., 2010; Gonzalez et al., 2006). This can occur through estrogen-bound ER indirectly binding DNA through association with another transcription factor (such as Sp1, AP-1, or NF-κB) (Fig. 2, #3 and next paragraph) or through an estrogen-bound ER initiating a cytoplasmic cascade culminating in phosphorylation and transcriptional activation of a non-ER transcription factor, as has been shown for STAT3 and STAT5 (Fig. 2, #2) (Bjornstrom and Sjoberg, 2005). In addition to estrogen-induced mechanisms, the ER

can also mediate transcription in the absence of hormone. In response to growth factor signaling, unliganded ER can directly (and indirectly) bind to DNA and promote gene expression (Fig. 2, #7) (Bjornstrom and Sjoberg, 2002; Fox et al., 2008; McDevitt et al., 2008; Welboren et al., 2009). Targets genes of non-classical ER-mediated transcription include the progesterone receptor, bcl-2, and cyclin D1 (Lupien et al., 2010; McDevitt et al., 2008), as well as several molecules that participate in proliferation, survival, differentiation, development, inflammation, and angiogenesis programs (Bjornstrom and Sjoberg, 2005; McDevitt et al., 2008).

Upon estrogen stimulation, ER can modulate transcription by interacting with DNA-bound transcription factors and functioning as their co-regulator (Fig. 2, #3). When the Jun and Fos heterodimer binds to AP-1 sites, the ER acts as a co-activator of Jun/Fos at those sites despite its inability to directly bind the AP-1 sequence (Welboren et al., 2009). The estrogen-induced interaction between the Jun/Fos heterodimer and ER-α requires its intact AF-1 and AF-2 but not DBD, as demonstrated through gene expression assays in the presence of estrogen using ER-α deletion and inactivation mutants. Binding may occur indirectly through association with CBP/p300 coactivators, which are also necessary for increased ER-dependent activity (Kushner et al., 2000). In some instances of ER-β-modulated ERE-independent gene transcription, certain residues of its DBD have been shown to mediate its co-regulatory function, though the DBD was not necessary for binding a DNA sequence. Genes that are activated through estrogen-induced ER interaction with Fos and Jun at AP-1 sites include ovalbumin, IGF-I, collagenase, and cyclin D1. This same estrogen-induced association can also suppress expression of AP-1 site-containing genes, such as occurs at the choline acetyltransferase

gene. Interactions between ER and Sp1 similarly regulate genes with GC-rich sequences in their promoters, such as the low-density lipoprotein receptor, c-*fos*, and cyclin D1 genes. Additionally, ER interaction with NF-κB and CCAAT/enhancer binding protein ß (C/EBPß) is critical to estrogen-induced downregulation of IL-6 gene expression (Bjornstrom and Sjoberg, 2005; Kushner et al., 2000). The expression of some genes, such as retinoic acid receptor α, TGF α, and progesterone receptor, is regulated through both ERE half-sites and Sp1 sites and are, therefore, subject to both classical and non-classical ER-mediated mechanisms (Bjornstrom and Sjoberg, 2005).

Another mechanism by which the ERs can indirectly facilitate estrogen-induced transcription is by promoting signaling that leads to the phosphorylation of other transcription factors and culminates in expression of their target genes (Fig. 2, #2). Estrogen has been shown to require both ER and ERK to induce the phosphorylation of Elk-1, a known ERK substrate, and promote transcription from serum response elements, suggesting a role for serum response factor, as well (Bjornstrom and Sjoberg, 2005). Additionally, estrogen stimulates ERK to phosphorylate CREB, and this event induces expression of genes containing the cAMP response element. Similar results have also been seen for estrogen-induced regulation of AP-1 expression through ERK-mediated phosphorylation (Bjornstrom and Sjoberg, 2005). There have also been reports demonstrating STAT3, STAT5a, and STAT5b transcription factor-dependent, estrogen-induced gene expression requires Src family kinase (SFK) activity and the ER(s) (Bjornstrom and Sjoberg, 2005); it appears that this process occurs independently of ER-α's AF-1 or ability to bind DNA but requires an intact AF-2 (Bjornstrom and Sjoberg, 2002; Fox et al., 2008) and may be necessary for estrogen-induced proliferation

(Bjornstrom and Sjoberg, 2002). Genes regulated by this mechanism include the genes for β-casein, cyclin D1, c-Myc (Fox et al., 2008).

The canonical ERs have also been shown to participate in growth factor-stimulated transcription in the absence of estrogen and to regulate the expression of genes encoding WISP-2 (Bjornstrom and Sjoberg, 2002; Fox et al., 2008), LIV-1, and pS2 (Banerjee et al., 2005). EGF, IGF-1, and heregulin have all been shown to support expression from an ERE-containing promoter *in vitro*, suggesting that the ER is activated in the absence of hormone (El-Tanani and Green, 1997); this was shown to occur *in vivo* through the use of a transgenic mouse model expressing the luciferase gene downstream of an ERE-containing promoter. When the animal was stimulated with IGF-1, transcription from the ERE-containing promoter occurred in an ER-dependent manner, as gene expression was blocked by use of an ER inhibitor (Garcia-Segura et al., 2010; Weigel and Zhang, 1998). In addition to ER-mediated transcription at EREs, it appears that growth factor signaling can also promote ER-mediated transcription at non-estrogen-stimulated genes and independently of EREs. A recent report in ER+ MCF-7 breast cancer cells showed that of the 302 genes found to be upregulated following EGF stimulation, 109 were dependent upon ER-α, and only eight of those overlapped with genes that were upregulated by estrogen treatment. Similarly, EGF repressed the expression of 211 genes, 47 of which were ER-α dependent and four of which were both ER-α-dependent and regulated by estrogen. Unique EGF-regulated genes to which ER-α bound, as determined through genome-wide chromatin immunoprecipitation (ChIP on a chip), were enriched in forkhead and AP-1 binding sites, as opposed to ERE motifs (Klotz et al., 2002). This finding suggests that one mechanism by which ER-α regulates EGF-induced gene

transcription may occur through indirect DNA binding; indeed, ER-α and AP-1 were shown to be co-recruited to identified EGF-induced ER-α binding sites (Lupien et al., 2010). Perhaps because of this variety of gene targets, the growth factor downstream signaling mechanisms that support ER-mediated transcription appear to be diverse; this is supported by evidence that EGF requires ER-α S118 but not its AF-2, whereas insulin utilizes the C-terminal portion of the ER for gene expression (Lupien et al., 2010). EGF also stimulates transient, MEK-dependent ER-α phosphorylation at S118 (Weigel and Zhang, 1998) and Rsk-mediated phosphorylation at S167 (Banerjee et al., 2005; Chen et al., 2002), sites that may promote gene expression (Joel et al., 1998). Additionally, ovariectomized rats into which IGF-1 was injected demonstrated association of IGF-1R and ER-α, in contrast to their unstimulated counterparts (Lannigan, 2003). Though many questions remain as to how the ER participates in growth factor-induced gene expression, that this is an important aspect of the cellular response is underscored by the observation that the ER is critical for the full EGF-induced proliferation of MCF-7 cells (Mendez et al., 2003).

ER Rapid Action

Estrogen induces the ER to participate in signaling events as well as transcriptional activities. These "rapid estrogen actions" originate in the cytoplasm and culminate in proliferative and survival responses. These actions are so called because they can be detected within minutes of estrogen exposure through the increased phosphorylation of many signaling molecules including ER-α, ERK 1/2, c-Src, and PI3-Kinase (PI3K) (Lupien et al., 2010). Short stimulations with estrogen also induce the formation of

complexes between ER-α, c-Src, PI3K, and/or MNAR (a scaffolding protein also known as PELP-1) ,as well as between the androgen receptor (AR) and ER-α or c-Src (Fig. 2, #4-6). Formation of some of these complexes have been shown to result in post-translational modifications that enhance downstream signaling and ultimately gene transcription (Fox et al., 2009; Lannigan, 2003). (These complexes will be discussed in more detail later.) Phosphorylation of ER-α can alter its participation in transcriptional activities, while phosphorylation of ERK 1/2, c-Src and PI3K can result in the activation of signaling cascades that regulate proliferation, survival, adhesion, and migration independently of and together with ER (Barletta et al., 2004; Cheskis et al., 2008; Chieffi et al., 2003; Migliaccio et al., 2007; Wong et al., 2002). Because of their involvement in normal estrogen/ER actions that support growth and survival, effector molecules involved in estrogen signaling are prime candidates for mediating resistance of ER+ breast cancer cells to chemotherapy.

Protein Effectors of Rapid Estrogen Action

Androgen Receptor

AR Structure and Function

Because the AR and canonical ERs are both members of the steroid receptor family (Lannigan, 2003; Thomas and Brugge, 1997), they share similar structures and functions. The AR isoforms are full-length AR-B and truncated AR-A, which lacks the N-terminal 187 amino acids of AR-B. Both isoforms are composed of an N-terminal domain (NTD), DBD, hinge region, and LBD (Fig. 1). The NTD regulates AR-mediated transcription through binding co-regulator proteins, and its structure changes upon binding to proteins

or DNA (Gao et al., 2005). This domain contains the AF-1, which is further divided into the Tau-1 and Tau-5 regions (Fig. 1) (Li and Al-Azzawi, 2009), these regions are alternately denoted as AF-1 and AF-5, creating some confusion within the literature. Tau-1 strongly contributes to transcriptional activation and has some dependency upon the LBD to achieve this; it is thought that Tau-1 binds coactivators, though these have not yet been defined. This region additionally contains a motif that is necessary for interaction with the E3 ligase, CHIP, which has been shown to downregulate AR levels (Claessens et al., 2008; Jenster et al., 1995). Four mutations in this region (K179R, A197G, A234T, and E236G) have been found in prostate cancer through analysis of tumor samples or the TRAMP mouse model, and *in vitro* studies have shown that these changes result in a more transcriptionally active AR (Claessens et al., 2008). In addition, the E236G mutation has been shown to promote metastasis in a xenograft prostate cancer model. Tau-5 has also been shown to facilitate AR transcriptional activity, and it does this independently of the LBD, in contrast to Tau-1. Tau-5 has been shown to interact with the SRC/p160 coactivators and to contain sumoylation sites, though the implications of these sites are unclear (Callewaert et al., 2006; Claessens et al., 2008). Both the Tau-1 and Tau-5 regions of AF-1 are necessary and sufficient for the function of the NTD and the full activity of the AR, as their deletion in the context of full-length AR-A has been shown to impair classical AR transcription (Claessens et al., 2008). Like the ER, the AF-2 is located in the LBD and mediates transcriptional activity. It assumes a functional conformation upon ligand binding, which is required for co-activator recruitment (Claessens et al., 2008; Jenster et al., 1995). Though the LBD of the AR has low sequence homology to steroid receptor family members, all members demonstrate a

conserved three-dimensional structure (Gao et al., 2005; Li and Al-Azzawi, 2009). The AR's highest-affinity ligand is α-dihydrotestosterone (DHT), though the LBD can bind to other androgens and selective AR modulators (AR antagonists) (Gao et al., 2005). Apo-AR is localized to the cytoplasm and complexes with heat shock proteins via interactions with its LBD much as has been described for the ER (Suzuki et al., 2010), and in response to androgen, the AR sheds these chaperones and mediates signaling and transcription at androgen response elements (AREs) in a manner analogous to the ERs.

Much like the ER proteins, the AR can also participate in signaling in the absence of its cognate ligand. Interaction with signaling proteins can result in phosphorylation at several sites on the AR, including S213 and Y534, both located in the AF-1. AR S213 is an Akt consensus site that is phosphorylated in response to DHT, testosterone, R1881 (a synthetic androgen), EGF, and IGF-1 and has been shown to modulate AR's transcriptional activities (Gao et al., 2005). Though it has not been shown to be a direct Akt target, this assumption is supported by inhibition of S213 phosphorylation upon inactivation of PI3K, a lipid kinase upstream of Akt (Bennett et al., 2010; Taneja et al., 2005; Wen et al., 2000). In contrast, Y534 has been shown to be phosphorylated by c-Src through an *in vitro* kinase assay with recombinant proteins, indicating that this site is a direct target of that kinase (Taneja et al., 2005). Work in LNCaP prostate cancer cell lines indicates that c-Src phosphorylates AR Y534 in response to EGF, IL-6, PTHrP, and bombesin (Kraus et al., 2006) and that this site is necessary for nuclear translocation, prostate cancer growth under androgen-deprivation conditions (*in vitro* and *in vivo*) (DaSilva et al., 2009; Liu et al., 2010), and EGF- and DHT- (50-500 pM) stimulated AR-mediated gene expression (Ward and Weigel, 2009). Interestingly, concentrations of

DHT 1 nM or higher do not require this site for AR transcriptional activation (Guo et al., 2006), suggesting that DHT-induced gene expression is mediated by multiple mechanisms. The AR is expressed in male and female reproductive tissues, brain, skin, kidney, intestine, thymus, vasculature, adipose tissue, skeletal muscle and bone, and the results of its participation in transcriptional and signaling programs is modulation of cell proliferation, differentiation, apoptosis, and metabolism, development of many tissues and organs in both males and females, and homeostasis and proper function of tissues and organs (Guo et al., 2006). Additionally, androgens inhibit estrogen-stimulated proliferation in breast cancer cell lines, presumably through the AR (Bennett et al., 2010; Li and Al-Azzawi, 2009).

The AR Participates in Estrogen Signaling

The AR has been shown to participate in estrogen signaling in both breast and prostate cancer cells. Panet-Raymond and colleagues showed that full-length AR and the ER-α LBD interact in a yeast 2-hybrid assay (Suzuki et al., 2010); this association was more recently confirmed in EPN normal prostate cells following 5 min of estrogen stimulation (Fig. 2, #6) (Panet-Raymond et al., 2000). Estrogen also induces AR association with c-Src through a proline-rich region in the NTD of the AR and (presumably) c-Src's SH3 domain. This interaction is necessary for estrogen-induced DNA synthesis (as measured by bromodeoxyuridine incorporation) but not transcription from an ERE, as demonstrated through use of a peptide inhibitor of the protein-protein interaction (Chieffi et al., 2003). A study in LNCaP prostate cancer cells showed the AR to be necessary for estrogen-stimulated viability; however, estrogen-induced viability in ER+ T47-D breast cancer

cells was inhibited by a transcriptionally-active AR mutant lacking the AF-2 domain (Migliaccio et al., 2007). There are several possible explanations for this apparent paradox, including cell type differences, a requirement for the AR AF-2 to mediate viability, the AR containing both pro-growth signaling and anti-growth transcription functions, and/or that the transcriptionally-active mutant can sequester the AR away from pro-growth cytoplasmic interaction partners. Finally, several days of continuous estrogen stimulation leads to AR protein upregulation (Arnold et al., 2007; Peters et al., 2009). Together, these data demonstrate that the AR is modulated by and participates in estrogen signaling.

The AR in Breast Cancer

Since the AR is an estrogen effector, it is not surprising that the AR is detectable in 70-90% of breast tumors and coexpresses with the ER in 80-90% of infiltrating ductal carcinomas (IDCs) (Apparao et al., 2002). Almost all AR+ breast cancers are positive for two androgen-dependent transcription products, PSA (98%) and GDDFP-15 (92%), suggesting that the AR in breast cancer is transcriptionally active (Peters et al., 2009; Suzuki et al., 2010). That transcriptionally-active AR suppresses estrogen-induced viability in ER+ breast cancer cells may explain why AR expression is a positive prognostic factor for ER+ breast cancer (Suzuki et al., 2010). Additionally, ER+ breast cancer patients that express the AR and are treated with chemo- and endocrine therapies have better survival outcomes (Peters et al., 2009). However, the literature is unresolved as to the relationship between breast cancer incidence/aggression and the polymorphic length of poly-glutamine $(CAG)_n$ repeat in the NTD of the AR (its length inversely

correlates with activity) (Castellano et al., 2010), suggesting that the AR may either positively or negatively influence outcomes in ER+ breast cancer, depending on its form.

EGFR and HER2

EGFR and HER2 Structure and Activity

The epidermal growth factor (EGF) receptor (EGFR) is the prototypical member of the human EGFR (HER) family of transmembrane receptor tyrosine kinases that contains four members: HER1 (EGFR), HER2, HER3, and HER4. The extracellular domain mediates ligand binding and receptor dimerization, while its intracellular portion contains the catalytic domain and phosphorylation sites (Diaz-Chico et al., 2007; Gao et al., 2005; Hao et al., 2010). HER2 is not activated by any known ligand but participates in signaling predominately through heterodimerization with ligand-activated HER family members (Wells, 1999). In fact, EGFR and HER2 are the preferred dimer pair for mitogenic activation by EGF, heparin-binding EGF (HB-EGF), and epiregulin (Harari and Yarden, 2000; Nicholson et al., 2001). Signaling downstream of EGF has been shown to mediate development in mice, proliferation, migration, invasion, survival, and angiogenesis (Harari and Yarden, 2000).

Known ligands for EGFR include epidermal growth factor EGF, HB-EGF, EGF-like growth factor, transforming growth factor-alpha (TGF-α), epiregulin, and beta-cellulin (Jorissen et al., 2003; Kim et al., 2001). Upon ligand binding and homo- or heterodimerization with HER family members the receptor undergoes trans-(auto)-phosphorylation on tyrosine residues in the C-terminal tail. These residues provide docking sites for adaptor proteins and phosphorylation substrates with Src homology 2

(SH2) domains, which in turn mediate downstream signaling. In addition to activation by its own ligands, cytokines, sex hormones, and G protein-coupled receptor (GPCR) ligands have been shown to signal through the activated EGFR (Bogdan and Klambt, 2001), and this EGFR activation has been shown to promote MAPK, PI3K, c-Src, and STAT signaling (Biscardi et al., 2000).

EGFR and HER2 in Breast Cancer

The EGFR is overexpressed in approximately one-third of epithelial cancers (Jorissen et al., 2003), including carcinomas of the head and neck, ovary, bladder and breast. In breast cancer, EGFR overexpression correlates strongly with a loss of differentiation, advanced clinical stage, enhanced tumor proliferation, resistance to endocrine therapy, and poor prognosis (Mendelsohn, 2002), suggesting that the EGFR is an important player in breast cancer progression. Additionally, overexpression of EGFR in cell culture models can result in morphological transformation and *in vivo* tumorigenesis (Mendelsohn, 2002; Nicholson et al., 2001), and targeted overexpression of TGF-α in the mouse mammary gland causes hyperplasia and adenomas following multiple pregnancies and lactation (Velu et al., 1987). Aberrant EGFR activation results from gene amplification, transcriptional overexpression, and/or autocrine stimulation of the receptor (Jorissen et al., 2003). Several tyrosine kinase inhibitors (such as gefitinib and erlotinib) and monoclonal antibodies (such as cetuximab and panitumumab) have been developed to target the EGFR therapeutically (Nicholson et al., 2001). Though EGFR inhibitors have shown mostly promising results in ER+ breast cancer cell lines (Ciardiello and Tortora, 2008; Rocha-Lima et al., 2007), clinical trials have indicated that the

therapeutics work poorly as single agents in ER+ breast tumors (Dai et al., 2005; Lv et al., 2010; Okubo et al., 2004; Sonne-Hansen et al., 2010). However, a small study including both ER+ and ER- locally advanced breast cancer patients treated with doxorubicin-based chemotherapy showed that those with EGFR+ tumors had reduced disease-free and overall survival (Creighton et al., 2008; Finn et al., 2009), suggesting that the EGFR may have mediated resistance to chemotherapy in this population. Studies in fibrosarcoma, rhabdomyosarcoma, leiomyosarcoma, and thyroid cancer cell lines support the hypothesis that the EGFR can mediate doxorubicin resistance by demonstrating that an EGFR kinase inhibitor could restore sensitivity to the drug (Buchholz et al., 2005). These studies indicate that the EGFR is a breast cancer proto-oncogene that may be an effective target for improving chemotherapeutic outcomes in tumors overexpressing the protein.

HER2 is amplified in 20-30% of all breast tumors; however, its expression is often inversely correlated with that of the ER (Lopez et al., 2007; Ren et al., 2008). HER2 expression is a poor prognostic marker for bladder, breast, cervical, colorectal, endometrial, esophageal, head and neck, gastric, non-small cell lung cancer, and ovarian tumors (Harari and Yarden, 2000). Ectopic overexpression of HER2 in several cancer cell models, including breast cancer, enhances tumorigenicity, possibly through the basal autophosphorylation of HER2 (Nicholson et al., 2001). HER2's oncogenic role was additionally demonstrated by a NIH-3T3 (fibroblast) tet-off system in which HER2 supported tumor growth and loss of HER2 caused tumor regression through increased apoptosis (Harari and Yarden, 2000). Trastuzumab, a HER2-targeted monoclonal antibody, has been used successfully in the treatment of HER2-positive advanced and

metastatic breast cancers; early trials showed that this effect was independent of the ER status of the tumor (Schiffer et al., 2003). Of additional interest is the observation that breast cancer patients treated with chemotherapy that had high/amplified HER2 expression demonstrated worse clinical outcomes (Piccart-Gebhart et al., 2005; Vogel et al., 2002). However, when patients were additionally stratified by their HR status, the results were very different: HR+ breast cancer patients with HER2+ tumors had better responses (Del Mastro et al., 2005; Gilcrease et al., 2009; Pritchard et al., 2006; Tiezzi et al., 2007; Tubbs et al., 2009), suggesting that HER2 sensitized ER+ breast cancer to chemotherapy. HER2 has clearly been shown to promote breast cancer, but it appears to have some divergent functions in ER+ and ER- tumors.

Crosstalk between ER and EGFR/HER2 Signaling

While cancerous cells within a breast tumor demonstrate an inverse correlation between ER and EGFR / HER2 expression, the adjacent normal cells often express both ER and EGFR. This expression pattern suggests that cross-regulation of the estrogen and EGF signaling axis is an important characteristic of breast cancer (Andre et al., 2008; Darb-Esfahani et al., 2009; Huober et al., 2010; Petit et al., 2010). Inhibitor and overexpression studies have demonstrated that signaling from either the EGFR and/or HER2 suppresses ER-α expression in ER+ breast cancer (Harari and Yarden, 2000; Lichtner, 2003). Conversely, estrogen causes the downregulation of both EGFR and HER2 mRNA in ER+ breast cancer cell lines, and evidence suggests that this occurs through an ER-mediated mechanism (Oh et al., 2001; Stoica et al., 2003). Increases in EGFR/HER2 protein levels correlates with decreases in estrogen-responsiveness, as

MCF-7 cells exogenously overexpressing EGFR or HER2 are more hormone-insensitive, as compared to their parental cell lines (Hurtado et al., 2008; Yarden et al., 2001). Additionally, treatment of MCF-7 cells with a HER2 tyrosine kinase inhibitor (TKI) blocks estrogen-induced anchorage-dependent growth, indicating that it is the enzymatic activity of HER2 that supports estrogen-resistance (Lichtner, 2003; Pietras et al., 1995; Yang et al., 2004).

In spite of reciprocal, negative cross-talk between EGFR/HER2 and ER, EGF downstream signaling has been shown to regulate several aspects of ER function. EGF can stimulate the direct interaction of ER-α and -β with the EGFR, and the co-expression of ER-α and EGFR in COS-7 cells enhances EGF-induced proliferation and decreases apoptosis over that seen when either protein is expressed alone (Stoica et al., 2003). Additionally, EGF stimulates phosphorylation of ER-α serine and tyrosine residues (Marquez et al., 2001); indeed, ER-α pY537 is necessary for full EGF-induced growth and survival (Bunone et al., 1996; Kato et al., 1995; Marquez et al., 2001). The necessity of ER-α for EGF signaling is further supported by the inability of ER-α null (ERKO) mice to support EGF-stimulated (via an EGF-pellet) uterine growth (Marquez et al., 2001). Observations that anti-estrogens ablate EGF-induced DNA synthesis in MCF-7 cells (Curtis et al., 1996) and significantly reduce EGF-stimulated DNA synthesis and lipid metabolism in the uterine tissue of ovariectomized mice (Vignon et al., 1987) additionally indicate that the ER mediates important biological outcomes of growth factor signaling.

Conversely, estrogen signaling can utilize EGFR transactivation to achieve effector-molecule activation and biological responses. Estrogen signaling through the ER leads to

increased IGF-1 levels and IGF-1R-, MMP-, and HB-EGF-dependent EGFR activation in MCF-7 cells (Fig. 2, #9) (Ignar-Trowbridge et al., 1992). This pathway is necessary for estrogen-induced ERK 1/2 phosphorylation, net growth, and decreased apoptosis (Santen et al., 2009; Song et al., 2010; Song et al., 2007). It is unclear whether elevated IGF-1 is the direct result of ER-mediated transcription or some other mechanism, though the length of estrogen-stimulation would allow for the former and the IGF-1 gene is a target of classical ER-α transcription (Santen et al., 2009).

In contrast to this ER-dependent mechanism, studies in ER-α-negative cell lines have shown that the non-canonical estrogen receptor, GPR30, also signals through the EGFR (Fig. 2, #8). GPR30 is a GPCR that stimulates HB-EGF cleavage through a c-Src-dependent mechanism, thereby stimulating EGFR-mediated ERK and PI3K activation to support estrogen-stimulated DNA synthesis and survival (Hewitt et al., 2010). Interestingly, GPR30 was shown to associate with ER-α in an EGFR-independent manner following 5 min of estrogen stimulation in ER+ Ishikawa endometrial cancer cells (Filardo, 2002; Filardo et al., 2000); however, the implications of this association have yet to be demonstrated. Though HER2 has not been explicitly implicated in estrogen-induced EGFR signaling, its ability to heterodimerize with EGFR and modulate estrogen responses suggest that it may also participate in downstream estrogen signaling (Vivacqua et al., 2009). Together, these data suggest that estrogen/ER and EGFR/HER2 signaling crosstalk to mediate biological outcomes in normal and cancerous tissues.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent endopeptidases whose actions promote wound healing, proliferation, organogenesis, and reproductive organ function (Buzdar, 2009). MMP proteins have been shown to both promote and inhibit cancer progression, and some family members are also overexpressed on both cancer cells, tumor stromal cells, and local immune cells through the paracrine regulation of their expression (Gialeli et al., 2011). Though not directly shown in breast cancer models, work in Ewing' sarcoma and rat cerebral cortical cultures suggest that some MMPs, such as MMP-3 and MMP-7, suppress doxorubicin-induced death and that treatment with an MMP inhibitor may sensitize cells to chemotherapy (Gialeli et al., 2011; Hulboy et al., 1997). MMP-2 and MMP-9 are poor prognostic indicators in DCIS and IDC, and their co-expression positively correlates with aromatase expression by immunohistochemistry in ER+ and/or PR+ breast cancers (Mitsiades et al., 1999; Mitsiades et al., 2001; Wetzel et al., 2003), suggesting that these detrimental proteins are most common in an estrogenic environment. Paradoxically, experiments in MCF-7 and T47D cells showed that estrogen not only suppresses the activity of MMP-2 and MMP-9 in a time- and dose-dependent manner but also downregulates the expression of these MMPs (Bagnoli et al., 2010; Gialeli et al., 2011). It is unclear what accounts for these differences, but one possible explanation is that cancer cell growth in a tumor involves growth in three dimensions and influences from many cell types, whereas the laboratory models use a two-dimensional plastic substrate in the absence of other cell types or growth factors. Whereas MMP-2 and MMP-9 are poor prognostic markers in breast cancer, MMP-26 expression in DCIS correlates with prolonged survival (Nilsson et al., 2007; Philips and McFadden, 2004). This may be related to MMP-26's role in the

cleavage of ER-β, which has been shown *in vitro* and *in vivo*; indeed, expression of these two proteins is inversely correlated in breast tumors (Savinov et al., 2006). Taken as a whole, these results indicate that MMP family members function in a variety of ways to influence breast cancer outcomes.

MMPs are best known for remodeling the extracellular matrix (ECM) surrounding cancer cells during cancer progression (Savinov et al., 2006); this has both direct and indirect effects on the biological responses of the tumor. Degradation of the ECM removes physical barriers, releases growth factors (such as TGF-β), and exposes adhesion proteins (such as integrins) that promote epithelial-mesenchymal transition (EMT), migration, and invasion. Tumor vasculature is regulated by MMPs through the break-down of several collagen isoforms, which releases both pro- and anti-angiogenic factors (Gialeli et al., 2011). Additionally, proliferation and apoptosis are modulated through MMP-mediated cleavage and/or shedding of membrane-bound proteins. MMP-3, MMP-7, ADAM10, and ADAM17 have been shown to release membrane-bound EGFR ligands, such as HB-EGF, to stimulate DNA synthesis (Gialeli et al., 2011); MMPs similarly regulate IGFR ligands to cause growth and survival (Gialeli et al., 2011; Suzuki et al., 1997). These observations provide a potential mechanism for MMP-3- and MMP-7-mediated chemotherapeutic resistance. MMPs can also inhibit apoptosis through cleaving Fas ligand and shedding MHC-1 tumor-associated proteins from the cell surface, actions which have been shown to suppress immune surveillance and tumor recognition by cytotoxic effector cells (Gialeli et al., 2011). Thus, through several mechanisms, MMPs are capable of regulating breast cancer cell responses.

MMPs are regulated by the tissue inhibitors of metalloproteinases (TIMP) family, which is composed of four endogenous inhibitors (TIMP1-4) that act by inserting into the MMP active site in such a way as to prevent catalysis and substrate recognition. The TIMPs that have slightly different target specificities, TIMPS-2, -3, and -4, regulate all or most members of the MMP family and through their broad-spectrum regulation of metalloproteinases modulate cell proliferation, angiogenesis, and survival (Gialeli et al., 2011), processes that have been shown to modulate tumor growth. TIMP-1 can both promote and inhibit cancer growth in various experimental systems and in one study with mammary epithelial cells TIMP-1 inhibited apoptosis through interaction with its receptor, CD63, and β_1 integrin at the cell surface (Brew and Nagase, 2010). TIMP-1 levels are elevated in and a negative prognostic factor for breast and colorectal cancers, in contrast to TIMP-2, which is suppressed in many cancers, a characteristic associated with poorer outcomes (Bourboulia and Stetler-Stevenson, 2010). Additional evidence that TIMPs are relevant to cancer is provided by a study on urine sediment DNA from bladder cancer patients, which demonstrated that those with hypermethylated TIMP-3 promoters had poorer overall survival; methylation was especially prognostic for those with stage 3 and 4 tumors (Stetler-Stevenson, 2008). Together, these data indicate that the TIMP proteins mediate cancer outcomes, presumably through their regulation of metalloproteinases.

C-Src

C-Src Structure and Activity

C-Src is an intracellular, membrane-localized non-receptor tyrosine kinase that induces cell cycle progression, survival, and gene transcription, enhances cell spreading through focal adhesion formation, induces lamellipodia formation, promotes migration and angiogenesis, and inhibits differentiation in some model systems (Hoque et al., 2008). C-Src is one of nine members of the Src family kinases and is composed of five domains: the Src homology 4 (SH4) domain, unique domain, SH3 domain, SH2 domain, and the kinase domain. The N-terminal SH4 domain is responsible for membrane localization, which is critical to c-Src's function (Summy and Gallick, 2003; Thomas and Brugge, 1997), and the unique domain is that portion of the protein that is unlike other Src family members and is thought to confer a level of substrate specificity. The SH3 portion binds intra- and intermolecular proline-rich sequences with core residues of PXXP (Brown and Cooper, 1996). In addition to substrate recognition, this domain is involved in negative regulation of kinase activity and cytoskeletal localization. The SH2 domain binds phospholipids and phosphotyrosines (with a preference for pYEEI) and participates in substrate recognition and negative regulation. Proteins that bind c-Src can bind either or both of the substrate-recognition domains. Flexible linkers connect the SH3 domain to the SH2 and kinase domains. The kinase domain is a highly-conserved, tyrosine-specific catalytic domain in the Src kinase family whose activity promotes tumorigenic growth by amplifying signals downstream of receptors, regulating receptor endocytosis and phosphorylating proteins that remodel the actin cytoskeleton (Thomas and Brugge, 1997). Tyrosine 418 has been shown to be a major autophosphorylation site located in the activation loop of the domain that it is pivotal to the regulation of c-Src's activity. The C-terminal tail of the protein contains Y527, whose phosphorylation

negatively regulates the enzyme by intramolecular interaction with the SH2 domain. When this occurs and the SH3 domain associates with the linker region between SH2 and SH3, Y418 becomes inaccessible and the protein is catalytically inactivated (Biscardi et al., 2000)). Binding of the SH3 and SH2 domains to cellular proteins alters the conformation so as to relieve this inhibition, resulting in activation of the kinase, dephosphorylation of Y527, and autophosphorylation of Y418 (Brown and Cooper, 1996; Thomas and Brugge, 1997). C-Src substrates include many known estrogen effector proteins, including ER-α (Thomas and Brugge, 1997), PI3K (Arnold et al., 1995a), AR (Castoria et al., 2001) and EGFR (Kraus et al., 2006), and it has also been shown to regulate the expression of MMP-9 (Biscardi et al., 1998; Kansra et al., 2005; Knowlden et al., 2005; Stover et al., 1995).

C-Src in Cancer

C-Src has been implicated in colorectal, breast, melanoma, ovarian, gastric, head and neck, pancreatic, lung, brain, and blood cancer development/progression (Cheng et al., 2010; Lee et al., 2007; Wu et al., 2008), and its expression has been shown to be elevated in up to 70% of breast cancers tumors (Summy and Gallick, 2003). C-Src alone is poorly oncogenic, as demonstrated by experiments done with a constitutively-active c-Src, whose expression was limited to the mammary glands of mice: females often formed epithelial hyperplasias that infrequently progressed to neoplasias (Biscardi et al., 2000). However, mice expressing endogenous c-Src and the polyomavirus middle T antigen transgene under the control of a mammary-specific promoter formed more tumors at a faster rate than those expressing polyomavirus middle T on a c-Src null background

(Summy and Gallick, 2003), indicating that c-Src cooperates with other oncogenic factors to enhance tumorigenicity.

C-Src Participates in Estrogen Signaling

C-Src's oncogenic role in breast cancer is consistent with its function as an estrogen effector. Perhaps the most direct evidence supporting this role is that both c-Src and Lck, another SFK, can phosphorylate recombinant, human ER-α at Y537 (Summy and Gallick, 2003). In MCF-7 cells, estrogen rapidly enhances c-Src activity and ER-α Y537 phosphorylation (as measured by *in vitro* kinase assays with both purified components and cell extracts) (Migliaccio et al., 1996). C-Src's SH2 domain binds ER-α pY537 even as MNAR, a scaffolding protein, interacts with both the SH3 of c-Src and A/B domain of ER-α to stabilize the complex (Fig. 2, #4) (Arnold et al., 1995a; Migliaccio et al., 2002a; Migliaccio et al., 1996). Phosphorylation of ER-α Y537 by c-Src enhances ER-α binding to EREs *in vitro* and *in vivo* (Barletta et al., 2004; Migliaccio et al., 1998) (Fig. 2, #4). In contrast, ER+ BT-474 cells grown in fetal bovine serum showed that a SFK-targeted TKI increased ER-α protein levels and ER-mediated transcription (Arnold et al., 1995b; Yudt et al., 1999). Together, these results suggest that SFKs can stimulate or inhibit ERE binding and transcription, depending upon the cell type and treatment. Additionally, estrogen stimulated a c-Src-mediated cascade involving PI3K and Akt activation that culminated in cyclin D1 expression and cell cycle progression (Fig. 2, #4) (Chen et al., 2010; Fox et al., 2008); in fact, c-Src kinase activity was required for estrogen-stimulated proliferation in some systems (Castoria et al., 2001; Summy and Gallick, 2003). As mentioned in the ER-EGFR transactivation section, c-Src also

mediated estrogen-induced, GPR30-mediated activation of the EGFR and its downstream signaling (Fig. 2, #8) (Migliaccio et al., 2002a). Finally, SFK TKI-treatment of MCF-7 cells increased doxorubicin-induced death, implicating SFK proteins in resistance to this drug (Filardo, 2002). These data demonstrate that, not only is c-Src an important mediator of many biological responses that support breast cancer progression, it can do so through its participation in estrogen signaling.

PI3-Kinase

PI3K Structure and Activity

The PI3K family is composed of three classes of lipid kinases. Class I PI3Ks convert phosphotidylinositol (4,5)-phosphate (PIP_2) to phosphotidylinositol (3,4,5)-phosphate (PIP_3) and is comprised of subgroup IA, which are activated by receptor tyrosine kinases and are heterodimers of a p85 (α, β, or γ) regulatory subunit and a p110 (α, β, or δ) catalytic subunit, and subgroup IB, which is activated downstream of GPCRs and composed of the same p85 subunits and p110γ. Class II and III proteins convert PI to PI-3-P. Class II proteins bind to clathrin in coated pits, suggesting a role for them in membrane trafficking, whereas Class III is represented by one protein in mammals, VPS34, which senses the availability of amino acids and signals to regulate cell growth and autophagy. As Class IA proteins are the only PI3Ks implicated thus far in cancer (Ta et al., 2008), it will be the focus of this discussion and will be referred to simply as "PI3K," henceforth.

As mentioned, Class IA PI3K is composed of two subunits, p85 and p110. The p110 subunit contains the kinase and Ras-binding domains and is encoded by the *PIK3CA*

gene, whereas p85 is the SH2-containing regulatory subunit that, in its basal state, inhibits p110. When p85 is tyrosine phosphorylated by kinases, such as EGFR or c-Src, the inhibition is relieved and p110 is recruited to the plasma membrane through the SH2 domain(s) of the p85 subunit and p110 is activated (Chalhoub and Baker, 2009). P110 converts PIP_2 in the membrane to PIP_3 (Castaneda et al., 2010; Chalhoub and Baker, 2009; Chang et al., 2003), a reaction that is negatively regulated by the tumor-suppressor, PTEN, a phosphatase that dephosphorylates PIP_3 (Chang et al., 2003). PIP_3 functions as a second messenger through its recruitment of Pleckstrin homology domain-containing proteins to the membrane, such as the serine/threonine kinases 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt/PKB (Chalhoub and Baker, 2009). Akt is activated by phosphorylation at T308 by PDK1 and/or S473 by mTORC2, and activated Akt mediates much of the signaling downstream of PI3K. Akt inhibits apoptosis through activation of MDM2 and NF-κB and inhibition of FOXO and Bad as well as promotes cell cycle progression through its inhibition of FOXO and GSK3. Akt signaling has been shown to suppress nutrient deprivation-induced cell cycle arrest and autophagy through its phosphorylation and inhibition of TSC1/2 (Castaneda et al., 2010; Chang et al., 2003; Osaki et al., 2004). PI3K signaling frequently crosstalks with other pathways through protein-protein interactions and activation, such as occurs with the c-Src and MAPK pathways (Chalhoub and Baker, 2009) through PI3K's association with Ras and phosphorylated c-Src via its p110 subunit and p85 SH2 domain, respectively. A mouse knock-in model with a mutation to the p110α Ras-binding domain that preserved kinase activity but was unable to bind Ras showed that the p110::Ras interaction was critical for proper development and for the formation of Ras-induced lung and skin cancers (Castoria

et al., 2001; Chang et al., 2003; Cosentino et al., 2007). Data suggest that PI3K can additionally regulate MAPK signaling through Akt-mediated inactivation of Raf-1 (Chalhoub and Baker, 2009). Moreover, the PI3K pathway involves some of the same proteins as the c-Src and/or MAPK cascades, such as Raf, Bad, CREB, TSC1/2 and ER-α (Chang et al., 2003). PI3K downstream signaling has been shown to regulate gene transcription, cell cycle entry, cell proliferation, metabolism, survival, angiogenesis, EMT, invasion, and DNA repair (Castaneda et al., 2010; Chang et al., 2003; Lannigan, 2003).

PI3K in Breast Cancer

Signaling through the PI3K pathway has been shown to promote several types of cancer, including prostate, lung, melanoma, leukemia, and breast (Castaneda et al., 2010; Chang et al., 2003). As many as 70% of breast tumors elicit aberrant activation of this pathway. This can occur through loss of PTEN, an activating mutation in *PIK3CA*, or mutation of the gene encoding Akt; the last two methods have been shown to correlate with hormone receptor positivity. Activation of this pathway correlates with endocrine resistance in preclinical studies, and ER+ breast cancer patients with Akt activation or PTEN loss are more resistant to endocrine therapy (Chang et al., 2003). Blockade of PI3K activity by an inhibitor in ER+ breast cancer cell lines suppresses growth but promotes transcription of estrogen-regulated target genes (Castaneda et al., 2010) as well as increases susceptibility to doxorubicin-induced apoptosis (Creighton et al., 2010), providing evidence that PI3K promotes ER+ breast cancer cell function and chemotherapeutic resistance.

PI3K Participates in Estrogen Signaling

Several groups have shown that PI3K is an effector of estrogen signaling. The p85α subunit of PI3K can associate with ER-α in as little as three minutes of estrogen stimulation, and this association correlates with increased PIP_3 levels and Akt phosphorylation, indicating PI3K activation (Fig. 2, #5) (Clark et al., 2002). The domains involved in this interaction have not been fully defined, though it has been shown that the PI3K phosphorylation site, S83 of the p85 subunit, and methylation of ER-α at R260 are required (Castoria et al., 2001; Cheskis et al., 2008; Cosentino et al., 2007; Mannella and Brinton, 2006; Sun et al., 2001) and that c-Src enhances the interaction; however, the N-terminus of ER-α is not necessary for association with PI3K (Cosentino et al., 2007; Le Romancer et al., 2008). Longer courses of estrogen (24-72 hrs) increase PI3K p85 and PIP_3 levels and sustain Akt activation (Castoria et al., 2001), suggesting that the abundance of p85 correlates with active downstream signaling. Moreover, Akt and Rsk (a protein downstream of PDK1) have been shown to phosphorylate ER-α S167 *in vitro* and *in vivo* (Stoica et al., 2003). Use of PI3K inhibitors and/or kinase-defective mutants have also shown that estrogen-induced down-regulation of ER-α and stimulation of DNA synthesis both require PI3K kinase activity (Joel et al., 1998; Lannigan, 2003; Sun et al., 2001). These results support the hypothesis that estrogen utilizes PI3K downstream signaling in ER+ breast cancer to bring about its biological outcomes and also as part of a feedback loop to regulate the estrogen response.

MNAR

MNAR Function and Participation in Estrogen Signaling

As its name suggests, modulator of non-genomic actions of the estrogen receptor (MNAR, or PELP1) functions to mediate transcriptional and rapid estrogen actions through interaction with many estrogen effector proteins via its LXXLL motif and PERD domain (Castoria et al., 2001; Stoica et al., 2003). This cytoplasmic and nuclear scaffolding protein binds to and modulates actions of several nuclear receptors and transcriptional activators including ER-α, ER-β, AR, PR, estrogen-related receptor, glucocorticoid receptor, retinoid X receptor, AP-1, and STAT3 (Barletta et al., 2004; Haas et al., 2005). MNAR also directly associates with cytosolic signaling molecules, such as PI3K and c-Src (Chakravarty et al., 2010b; Wong et al., 2002), and is serine and tyrosine phosphorylated by several kinases, including HER2 and c-Src, in response to estrogen and growth factors (Barletta et al., 2004). Within 5 min of estrogen stimulation in MCF-7 cells, MNAR and c-Src associate, and this association correlates with an increase in c-Src, ERK, and Akt phosphorylation (Fig. 2, #4) (Chakravarty et al., 2010b); prior work had shown that MNAR is necessary for full estrogen-induced c-Src activation (Chakravarty et al., 2010a). The importance of this scaffolding protein in mediating estrogen-induced transcription and signaling is underscored by the observation that MNAR is required for estrogen-stimulated cell proliferation in Ishikawa cells (Wong et al., 2002) and promotes estrogen-induced cell cycle progression in MCF-7 cells (Vadlamudi et al., 2004).

MNAR functions both as a transcriptional co-activator and as a regulator of cytoskeletal remodeling. Overexpression studies in ER+ breast cancer cell lines showed that MNAR enhances transcription at EREs (Fig. 2, #4), progesterone response elements,

and glucocorticoid response elements following hormone stimulation, potentially through MNAR association with the coactivators, CBP and P300 (Balasenthil and Vadlamudi, 2003), its modulation of histone acetylation, and/or its interaction with methylases and demethylases (Vadlamudi et al., 2001). MNAR has also been shown to associate with proteins that mediate cytoskeletal remodeling; in fact, overexpression of MNAR results in increased motility as well as estrogen-induced membrane ruffling, filipodia-like structure formation, migration, and anchorage-independent growth, whereas its silencing reduces anchorage-independent growth (Chakravarty et al., 2010b).

MNAR in Cancer

MNAR is expressed throughout the body and in many estrogen-responsive tissues, including the male and female reproductive systems, brain, heart, skeletal muscle, and liver, with one of the sites of its highest expression being the mammary gland (Chakravarty et al., 2008; Chakravarty et al., 2010b; Rajhans et al., 2007). Interestingly, MNAR is deregulated in breast, endometrial, ovarian, prostate, salivary, colon, and lung tumors (Vadlamudi et al., 2001; Wong et al., 2002). Its expression is elevated in breast cancer, as compared to normal tissue, and it has been detected in several ER+ breast cancer cell lines, including MCF-7, T47D, and ZR75-1 cells (Chakravarty et al., 2010b). Studies in human fibroblasts and rat kidney epithelial cells demonstrated that MNAR overexpression is sufficient to induce transformation (Vadlamudi et al., 2001), suggesting that MNAR may function as a proto-oncogene. Supporting evidence for this claim is provided by a study in which ER+ breast cancer cells (ZR-75-1) stably overexpressing MNAR caused increased tumor incidence following cardiac injection into nude mice and

more metastatic lesions in the lung and liver following tail vein injection than their non-overexpressing counterparts (Rajhans et al., 2007). Additionally, MNAR silencing in an ovarian cancer xenograft model reduces tumor volume and weight (Chakravarty et al., 2010a). A single report suggests MNAR may also promote breast cancer progression through its participation in a transcriptional complex at the aromatase promoter that stimulates ER-α-independent aromatase expression and results in increased aromatase activity in MNAR-overexpressing cells (Chakravarty et al., 2008), which could potentially elevate estrogen levels in the tumor vicinity. MNAR expression in ER+ breast cancers inversely correlates with overall and disease-free survival (Rajhans et al., 2008), and metastatic breast cancers have been shown to express more MNAR than node-negative tumors (Habashy et al., 2010). Also, cytoplasmic MNAR localization as shown by immunohistochemistry has been associated with tamoxifen-resistance in breast cancer (Rajhans et al., 2007), suggesting that MNAR's role in cytoplasmic signaling may result in worse outcomes for ER+ breast cancer patients.

MEK and ERK

MEK and ERK are members of the cytoplasmic mitogen-activated protein kinase (MAPK) signaling cascade that is triggered by growth factors, cytokines, and hormones. Upon stimulation, membrane-anchored, GTP-bound Ras GTPase recruits Raf to the plasma membrane where Ras induces a conformational change in Raf which stimulates Raf to phosphorylate and activate MEK1 and MEK2 (Chakravarty et al., 2010b), two specific kinases for and activators of ERK1 and ERK2 (Roskoski, 2010; Terai and Matsuda, 2005; Wellbrock et al., 2004). ERK1 and ERK2 are highly homologous

serine/threonine kinases that are generally considered to be functionally redundant, though some differences in substrate specificity have been reported (Seger and Krebs, 1995; Whyte et al., 2009). ERK phosphorylates a wide spectrum of substrates, including transcriptional regulators, apoptotic regulators, and hormone receptors (Seger and Krebs, 1995). ERK has also been shown to regulate growth factor receptors and cytoskeletal proteins, crosstalk with the PI3K/Akt pathway, and downregulate its own activation through negative feedback loops. The net result of MEK/ERK signaling on the cell is proliferation, differentiation, angiogenesis, EMT, motility, invasion, regulation of cytoskeletal rearrangement, and either apoptosis or survival (depending upon the context) (Whyte et al., 2009).

MEK and ERK Participate in Estrogen Signaling

MEK and ERK have both been implicated in estrogen signaling. Estrogen stimulation of LNCaP cells requires MEK activity for proliferation (Seger and Krebs, 1995; Whyte et al., 2009), and in MCF-7 cells ERK is phosphorylated within 5 min of estrogen exposure (Migliaccio et al., 2002a). ERK activation can be achieved through estrogen-stimulation of the ER-α::MNAR::c-Src complex (Fig. 2, #4) (Yang et al., 2004) and by GPR30-mediated EGFR transactivation (Chakravarty et al., 2010a). ERK activation regulates ER-α by phosphorylating ER-α on S118 and, to a lesser extent, on S167 (Fig. 2, #4) (Yang et al., 2004), sites which contribute to the transcriptional activity of the receptor (Seger and Krebs, 1995). Rsk, a downstream target of ERK, has also been shown to phosphorylate ER-α S167 (Murphy et al., 2006).

MEK and ERK in Breast Cancer

About 30% of breast cancer tumors have a mutation in the Ras/Raf/MEK/ERK cascade, which may explain why ERK is hyper-activated in many breast cancers, especially those that are metastatic (Lannigan, 2003). Overexpression of MEK1 in normal mammary epithelial cells is sufficient for transformation (Whyte et al., 2009), suggesting that over-activation of this pathway mediates cancer progression. This hypothesis was further supported by studies with estrogen-induced breast cancer cell lines that demonstrated that ERK activation correlates with cell proliferation, survival, and aggressive tumor behavior (Whyte et al., 2009). ERK may also indirectly influence the clinical course of ER+ breast cancers through its direct phosphorylation of ER-α at S118 (its preferred site) and S167, as well as its indirect phosphorylation of S167 via regulation of the ERK effector, Rsk (Levin, 2003). Increased levels of ER-α pS167 correspond to a better prognosis and hormonal therapy response (Anjum and Blenis, 2008; Lannigan, 2003) whereas elevation of ER-α pS118 in breast tumors has been reported as being both a positive and negative prognostic factor in women treated with endocrine therapy (Jiang et al., 2007; Yamashita et al., 2005; Yamashita et al., 2008). Additionally, studies in hematopoietic, hepatocellular carcinoma, and epidermoid carcinoma cell lines indicated that ERK expression correlates with doxorubicin- and paclitaxel-resistance and that MEK kinase activity is necessary for doxorubicin insensitivity (Murphy et al., 2004; Murphy et al., 2009; Yamashita et al., 2008). However, MEK1 protects against 5-fluorouracil but is critical for doxorubicin- and gemcitabine-induced apoptosis in chemo-resistant pancreatic cancer cells, indicating that the function of MEK1 varies by cancer and treatment (Brantley-Finley et al., 2003; Choi

et al., 2008; McCubrey et al., 2007). Though a critical role in chemotherapeutic insensitivity for MEK and ERK has not yet been tested in ER+ breast cancers, these findings suggest that the MEK/ERK pathway could promote resistance in breast cancers. Together these data suggest that MEK and ERK participate in a broad array of signaling programs, including estrogen-signaling, to influence the biological outcomes of ER+ breast cancer patients.

Estrogen Effector-Targeted Drugs as Cancer Therapeutics

The following is a survey of drugs in various stages of clinical development that inhibit molecules shown to participate in estrogen signaling.

ER-Targeted Drugs

Estrogen-targeted inhibitors fall into three main classes: selective estrogen receptor modulators (SERMs, such as tamoxifen and raloxifen), aromatase inhibitors (AIs, such as anastrozole, letrozole, and exemestane), and selective estrogen receptor downregulators (SERDs, such as fulvestrant). SERMs have mixed antagonist/agonist activity on the ER-α and –β isoforms, whereas SERDs act as pure estrogen antagonists of the ERs. Unlike the SERMs and SERDs, AIs do not directly interact with ERs but rather suppress their activity indirectly through the inhibition of aromatase, the enzyme responsible for the conversion of androgens to estrogen (Zhao et al., 2006).

Selective Estrogen Receptor Modulators

Tamoxifen citrate (Nolvadex) is the best characterized member of the SERMs. This drug has been the standard of care for early and advanced breast cancer and, because of its action on the ERs, it is considered the first molecularly targeted therapy used to treat breast cancer. It is a competitive inhibitor of estrogen and inhibits ER-α AF-2 but not its AF-1, resulting in a partial inhibition of the receptor (Orlando et al., 2010). Tamoxifen largely acts as an antagonist of ER-α but an agonist of ER-β, leading to varied responses in tissues depending on the predominant isoform of ER expressed, cellular environment, and whether target genes are directly or indirectly regulated by the ER(s). This agonist activity on ER-β is thought to be the cause of tamoxifen-associated thromboembolitic events and endometriosis/endometrial cancer, as ER-β is more highly expressed in the vasculature and endometrium (Orlando et al., 2010). Standard tamoxifen treatment for the treatment of HR+ breast cancer is a five year course of 20 mg/day (Lin et al., 2010), and its use as an adjuvant treatment for both premenopausal and postmenopausal women with ER+ breast cancer significantly decreases risk of recurrence and death as well as causes an objective response in 25-50% of metastatic patients. Raloxifene (Evista) is structurally similar to tamoxifen and has been shown to be as effective as tamoxifen for the prevention of invasive and non-invasive breast cancer in postmenopausal women but has fewer associated thromboembolitic events and endometriosis/endometrial cancer than tamoxifen. (Lin et al., 2010; Orlando et al., 2010). However, raloxifene's predominate clinical application is as a treatment for osteoporosis as it inhibits bone resorption, much like estrogen (Orlando et al., 2010).

Selective Estrogen Receptor Downregulators

Fulvestrant (Faslodex) is a SERD that acts as a pure estrogen antagonist, causing a reduction in ER-mediated gene transcription and downregulation of the receptor. Fulvestrant inhibits the growth of both tamoxifen-sensitive and -resistant breast cancer cell lines; this is consistent with clinical observations that the drug is effective treatment for postmenopausal women with advanced breast cancer who have failed previous endocrine therapy. As a first line therapy for HR+ breast cancer, it was shown to be as effective as tamoxifen with respect to time to progression and more effective than tamoxifen in terms of objective response (Bryant, 2001). A phase II trial on HR+ postmenopausal women with advanced breast cancer demonstrated that first-line treatment with a high dose of fulvestrant (500 mg) prolonged time to progression as well as duration of response and clinical benefit among responders, as compared to the population receiving anastrozole (Orlando et al., 2010). There are currently twenty ongoing stage II or III clinical trials with fulvestrant being carried out in breast cancer; of these, three are investigating its efficacy as a monotherapy and twelve are testing it with or against AI drugs (Robertson et al., 2009).

Aromatase Inhibitors

Current aromatase inhibitors are third generation and classified as steroidal or non-steroidal, according to their chemical structure. Anastrozole (Arimidex) and letrozole (Femara) are non-steroidal AIs that bind to and inhibit the cytochrome P450 site of the aromatase complex, whereas exemestane (Aromasin) is a steroidal AI that binds to and blocks the aromatase substrate-binding site (NCI, 2011b). Anastrozole, letrozole, and exemestane have all shown longer times to progression than tamoxifen when given as

first-line treatments, though their superiority to tamoxifen in terms of survival has not been as clear. These AIs have also shown improved outcomes when given as adjuvant therapy to HR+, postmenopausal women with breast cancer. Additionally, clinical trials suggest there is some clinical benefit to patients following tamoxifen treatment with AI therapy (Lin et al., 2010). Clinical guidelines issued last year by the American Society of Clinical Oncology for the treatment of HR+ breast cancer in postmenopausal women recommend using an AI as a first-line monotherapy or following a course of tamoxifen treatment to reduce the risk of recurrence, as AIs have been shown to be more efficacious than a 5-year course with tamoxifen alone (Orlando et al., 2010).

AR-Targeted Drugs

Anti-androgens

Anti-androgens are small molecules that antagonize the AR by competing with its agonists for binding sites and have therapeutic potential for treatment of prostate cancer, benign prostatic hyperplasia, acne, virilization in women, and as male contraceptives. Anti-androgens with steroidal structures have had limited clinical application due to their poor oral bioavailability, hepatotoxicity risk, poor tissue selectivity, and the occasional cross-reactivity with other steroid receptors. Better success has been seen with non-steriodal anti-androgens; however, therapeutic doses of these block the AR in both the prostate and pituitary gland, resulting in a surge in luteinizing hormone release that raises circulating testosterone levels. Therefore, nonsteriodal anti-androgens are typically co-administered with a gonadotropin releasing hormone analog to suppress testicular testosterone production, resulting in “chemical castration.” Flutamide (Eulexin) is a non-

steroidal anti-androgen that has little agonist activity with the wild-type AR and little cross-reactivity with other steroid receptors. Like most non-steroidal anti-androgens, its main clinical application has been the treatment of androgen-sensitive prostate cancer or benign prostatic hyperplasia. The main metabolite of flutamide has a higher AR affinity and is a more powerful antagonist than flutamide. However, the hepatoxicity associated with this drug has limited its long-term use. Bicalutamide (Casodex) is the preferred non-steroidal anti-androgen used for the clinical treatment of prostate cancer as it is associated with less hepatoxicity and has a longer half-life than flutamide. Some evidence suggests that it functions by recruiting the co-repressor, NCoR, to DNA-bound AR. Other non-steroidal anti-androgens that are in the early stages of use for prostate cancer or are in preclinical development include DIMP, BMS-1, BMS-15, and BMS-501949 (Burstein et al., 2010).

Selective Androgen Receptor Modulators

Selective AR modulators (SARMs) act as antagonists or weak agonists in the prostate but as agonists in the pituitary gland, muscle, and bone. They are designed to be orally bioavailable and have low hepatotoxicity. Many of these are in the early stages of clinical development. S1 and S4 are lead compounds that have been validated in intact male mice as potential treatments for benign prostatic hyperplasia and as male contraceptives. BMS-564929 is a high affinity, highly specific SARM that demonstrates tissue selectivity in castrated rats (i.e. prostate is less sensitive to its agonist effects than muscle) and is orally available in humans. It suppresses luteinizing hormone but may function as an androgen replacement therapy due to its demonstrated agonist actions

outside the prostate in mouse models, though its effects on bone and muscle have not been investigated in humans (Gao et al., 2005).

Though the AR has been implicated in estrogen signaling and breast cancer (see the "Androgen Receptor" section), many of these compounds have not been tested in ER+ breast cancers in the clinic. However, abiraterone acetate (CB7630) is currently in a phase I/II trial for postmenopausal women with advanced or metastatic BC. This orally-available drug is functionally analogous to an AI in that it blocks androgen production, which should serve to reduce estrogen production from androgen precursors (Gao et al., 2005).

EGFR and HER2 Inhibitors

Several small-molecule and monoclonal antibody inhibitors of EGFR and/or HER2 have been developed and shown success in the clinic for the treatment of colon, gastric/gastroesophageal, breast, pancreatic, and non-small cell lung cancers.

EGFR Inhibitors

SMALL MOLECULE INHIBITORS

Perhaps the best known EGFR inhibitor is gefitinib (Iressa, ZD1839), an orally-active, reversible ATP-competitive TKI (NCI, 2011b), approved for locally advanced or metastatic non-small cell lung cancer (NSCLC) that is refractory to chemotherapy (Moon et al., 2010). Studies indicate that gefitinib and endocrine therapy co-treatment of endocrine-resistant cell lines restores sensitivity to the hormonal treatment (NCI, 2011a), and results from a phase II trial show that gefitinib is a promising treatment for

tamoxifen-resistant, hormone-insensitive breast tumors (Buzdar, 2009). However, in a phase II trial testing the efficacy of gefitinib over placebo when given with tamoxifen to 290 HR+ metastatic breast cancer patients, little to no survival benefit was seen over the placebo + tamoxifen group (Lin et al., 2010). This trial showed a statistically insignificant increase in progression-free survival in the gefitinib arm; the greatest gefitinib benefit, though statistically insignificant, was seen in endocrine-naïve patients (median progression-free survival of 10.9 mos. with gefitinib + tamoxifen vs. 8.8 mos. with placebo + tamoxifen). Additionally, a phase II trial in 93 HR+, endocrine-naïve postmenopausal women with metastatic breast cancer treated with anastrozole and gefitinib or placebo showed an increase in progression-free survival in the gefitinib arm (14.5 months vs. 8.2 months) (Osborne et al., 2011). The differences between the relative activities of the gefitinib in these two trials that stratified endocrine-naïve patients probably arises from the comparison to tamoxifen alone or a placebo. These data suggest that while gefitinib may be advantageous to some HR+ breast cancer populations, those subsets need to be more clearly defined. To this end, several phase II trials assessing its use as a monotherapy or in conjunction with ER-targeted drugs are currently being carried out in metastatic HR+ breast cancer patients (Cleator et al., 2009).

Erlotinib (Tarceva) is another orally active, reversible EGFR TKI (NCI, 2011b); it has been approved both for the treatment of locally advanced or metastatic non-small cell lung cancers (NSCLC) that have failed chemotherapy and as co-treatment with gemcitabine to treat irresectable or metastatic pancreatic cancer (Moon et al., 2010). This drug is currently being evaluated in phase II trials in HR+ breast cancer. One trial on postmenopausal women with HR+ breast cancer is evaluating the efficacy of erlotinib in

conjuction with letrozole, whereas another is measuring the time to progression on fulvestrant with erlotinib or a placebo in HR+, metastatic breast cancer patients that have progressed on first-line hormonal therapy (NCI, 2011a).

Monoclonal Antibodies

Cetuximab (Eribitux) and panitumumab (Vectibix) are monoclonal antibodies targeting the EGFR extracellular LBD that function as competitive inhibitors of EGFR ligands (NCI, 2011b). Preclinical work showed that Cetuximab can downregulate the EGFR, reduce angiogenesis in xenograft models, and reduce cell proliferation *in vitro*. Following studies of squamous cell carcinoma of the head and neck (SCCHN) in xenograft models and clinical trials that demonstrated its function as a radio- and chemo-sensitizer (Moon et al., 2010), cetuximab was approved for the treatment of SCCHN that has recurred or metastasized after chemotherapy as a monotherapy, as a first-line treatment for advanced SCCHN, and as a radiosensitizer for this cancer. It is also an approved therapy for colon cancer that is metastatic and has failed to respond to chemotherapy (Moon et al., 2010). Like cetuximab, the fully humanized panitumumab has been investigated in SCCHN. A phase I trial with 19 treatment-naïve stage III and IV SCCHN patients given panitumumab, carboplatinum, and radiotherapy showed impressive results: 87% of the patients receiving this cocktail demonstrated a complete response. Phase II and III trials in this cancer are currently ongoing (NCI, 2011a). Panitumumab is approved as a monotherapy for the treatment of EGFR+, metastatic colon cancer that has progressed on chemotherapy but has only shown efficacy against those tumors expressing wild-type *KRAS* (Moon et al., 2010). Neither antibody is

currently being tested in clinical trials for HR+ breast cancer (Moon et al., 2010; NCI, 2011a).

HER2 Inhibitors

Trastuzumab (Herceptin) is a humanized monoclonal antibody that targets the HER2 extracellular domain (NCI, 2011b) and is approved for the treatment of HER2+ breast cancer as a monotherapy or in conjunction with other therapies and for the treatment of metastatic gastric and gastroesophageal junction adenocarcinoma, as one of multiple drugs (Lin et al., 2010). Trastuzumab improves survival in HER2+ breast cancer patients with resected tumors (NCI, 2011a), and at least two large trials have shown that administration of trastuzumab with chemotherapy improves the survival of patients with early breast cancer, as compared to those who received chemotherapy alone, regardless of HR status. There is currently an ongoing phase III trial in women with stage I-III HER2+ breast cancer comparing two regimens of chemotherapy given with trastuzumab (Lin et al., 2010) to determine the best cocktail of chemotherapy and trastuzumab. In preclinical studies, treatment of a patient-derived letrozole-resistant cell line with this antibody inhibited its growth, and pretreatment with the antibody restored letrozole sensitivity (NCI, 2011b). These findings were extended to patients in a phase II trial in which 207 postmenopausal, HER2+, HR+ breast cancer patients were treated with anastrozole with or without trastuzumab. The results demonstrated that the combination significantly improved disease-free survival, response rate, time to progression, and clinical benefit as compared to the AI alone (Buzdar, 2009). Given the efficacy of trastuzumab, a

monoclonal antibody that inhibits HER2 and HER3 dimerization, pertuzumab, has been developed and is being evaluated in phase III clinical trials (Buzdar, 2009).

Dual EGFR/HER2 Inhibitors

Lapatinib ditosylate (Tykerb) is an orally-active, reversible, TKI targeting EGFR and HER2 (Lin et al., 2010) approved for use with capecitabine or letrozole in certain populations of HER2+ advanced or metastatic breast cancer (Lin et al., 2010; Moon et al., 2010). A phase I trial in which 1286 HR+ patients, 219 of which had HER2-overexpressing disease, were treated with letrozole plus lapatinib or placebo. HER2+ patients showed a statistically-significant increase in their response rate and progression-free survival with letrozole and lapatinib treatment, as compared to those who received letrozole and placebo. However, treatment with lapatinib was of no additional benefit to the HER2-negative population (NCI, 2011a). Following this logic, a phase II study is currently underway testing whether treatment with lapatinib and tamoxifen improves the outcomes of patients with locally advanced or metastatic breast cancer that had previously failed tamoxifen treatment, and a phase III trial in postmenopausal women with stage III or IV HR+ breast cancer is currently comparing outcomes of patients treated with fulvestrant and lapatinib or fulvestrant and a placebo. Additionally, lapatinib is being evaluated as a monotherapy as a treatment for metastatic breast cancer resistant to hormone therapy in a phase II study (Cleator et al., 2009).

MMP Inhibitors

Dysregulated MMP expression has been shown in several diseases, including those of the cardiovascular system, arthritis, asthma, and cancer (NCI, 2011b). While some MMPs have been shown to promote these conditions, murine knock-out models have demonstrated that some MMPs, such as MMP-3, -8, and -9, provide some protection from tumor development and/or aggressiveness (Fingleton, 2008), suggesting that inhibition of these enzymes could be detrimental to cancer patients. Sparing these MMPs while maintaining a broad specificity for the other family members has been one of the challenges in the design of third generation inhibitors currently in development, as many of these enzymes have similar active sites (Fingleton, 2008).

First generation MMP inhibitors targeted a ubiquitous functional group in the catalytic site of MMPs and were potent, competitive, reversible inhibitors of enzyme activity (Overall and Kleifeld, 2006), resulting in a broad specificity across the MMP family. Many of these first generation peptidomimetic compounds, such as batimastat (BB94) and ilomostat (GM-6001), were poorly orally bioavailable and therefore limited to laboratory experiments (Zucker et al., 2000). Preclinical studies in a mouse model of intestinal cancer showed that either MMP-7 silencing or treatment with the broad-range MMP inhibitor, batimastat, reduced polyps 50-60% (Overall and Kleifeld, 2006). Additionally, mice treated with the MMP inhibitor, BMS-275291, developed fewer metastatic lung lesions following tail-vein injection of a melanoma cell line (Fingleton, 2008). However, a study in a pancreatic cancer mouse model demonstrated that while batimastat was an efficacious preventative or treatment for small tumors, it was unable to cause the regression of large tumors (Naglich et al., 2001). These data suggested that MMP inhibition may prove therapeutic in some cancers.

Clinical trials with second generation MMP inhibitors, such as the broad-range inhibitors, marimastat (BB2516, TA2516, Usan), prinomastat (AG3340), and tanomastat (BAY 12-9566), have largely failed (Zucker et al., 2000), in part due to the frequent occurrence of a painful musculoskeletal syndrome that resulted in many patients enrolled in clinical trials to withdraw or reduce doses to levels of questionable efficacy (Overall and Kleifeld, 2006). Though co-treatment of several pre-clinical cancer mouse models with prinomastat and chemotherapy demonstrated that the combination was more efficacious than either therapy alone, phase III trials testing marimastat or tanomastat in unresectable pancreatic cancer showed that the drug was not superior to treatment with gemcitabine. Likewise, a phase III trial on glioblastoma showed marimastat lacked efficacy as a single agent. Clinical trials using BAY 12-9566 were suspended in September 1999 following the release of data demonstrating advanced small cell lung cancer patients receiving the inhibitor had reduced survival, as compared to controls given placebo. Trials using prinomastat were closed when interim data from trials on advanced small cell lung cancer and hormonal therapy-refractory prostate cancer failed to improve outcomes. Conversely, patients with inoperable gastric cancer who had progressed on chemotherapy demonstrated prolonged progression-free survival and a trend towards increased length of median survival (Fingleton, 2008). It is hoped with the advent of more selective third-generation inhibitors, MMPs that promote cancer will be targeted while those that suppress progression can be spared, resulting in better outcomes and fewer adverse events.

SFK Inhibitors

Dasatinib

To date, no specific TKI of c-Src has been developed for laboratory or clinical use due, in part to the similarity between the ATP-binding pocket of c-Src and its family members. Additionally, this structural similarity has resulted in many of the ATP-competitive inhibitors of SFKs also inhibiting the kinase, Abl (Zucker et al., 2000). About 90% of chronic myelogenous leukemia (CML) patients have the Philadelphia chromosomal fusion that results in constitutively active, oncogenic Bcl-Abl (Boschelli et al., 2010). For this reason, several SFK/Abl TKIs have been predominately used in the treatment of Bcr-Abl-expressing cancers. One such drug is dasatinib (BMS-354825, Sprycel), which has been shown to act as an ATP-competitive inhibitor (Araujo and Logothetis, 2010; Boschelli et al., 2010) of SFKs, Bcr-Abl, c-Kit, PDGFR, c-FMS, EphA2 (Boschelli et al., 2010). Dasatinib is approved for the treatment of imatinib (an Abl inhibitor)-resistant or –intolerant CML and Philadelphia chromosome-positive acute lymphoblastic leukemia patients and is in phase III trials as a first-line CML treatment (Araujo and Logothetis, 2010). It has also been evaluated in preclinical models of prostate, breast, glioblastoma, NSCLC, colorectal, pancreatic, melanoma, and head and neck squamous cell carcinomas. Dasatinib was shown to reduce tumor size and the number of metastases in prostate cancer xenograft models as well as glioblastoma tumor growth in a xenograft mouse model, as compared to untreated controls. In experiments using a panel of 39 breast cancer cell lines, dasatinib was shown to be most effective against ER, PR, and HER2-negative breast cancer cells and was able to inhibit cell lines that overexpressed EGFR. Additionally, preclinical studies show promise in NSCLC and pancreatic cancer, in contrast to the poor results observed in preclinical models of colon

cancer and melanoma. Across cancer types, this inhibitor has shown variable results in terms of proliferation and apoptosis but has fairly consistently inhibited the migration, invasion and adhesion of cancer cells (Araujo and Logothetis, 2010; Boschelli et al., 2010).

The efficacy of dasatinib has been and is currently being tested in clinical trials for breast cancer. Early results from a phase I trials evaluating dasatinib and capecitabine, a chemotherapeutic agent, in forty advanced breast cancer patients demonstrated a 22% partial response rate and 33% who had stable disease (Araujo and Logothetis, 2010). Poorer responses were seen in two phase II trials evaluating it as a monotherapy for advanced HR- and HER2-negative breast cancer and for advanced HR+ or HER2+ breast cancer. Preliminary results from these studies showed partial response rates of 4-5% and stable disease lasting sixteen or more weeks in 5-9% of the patients (Somlo et al., 2009). Additionally, ongoing trials are testing the efficacy of dasatinib or a placebo with exemestane in postmenopausal HR+ breast cancer patients and fulvestrant with or without dasatinib in men and postmenopausal women with HR+ BC previously treated with an AI (Araujo and Logothetis, 2010; Mayer et al., 2009).

Saracatinib

Saracatinib (ADZ0530) is a selective, potent, orally-available competitive ATP-inhibitor that targets both SFKs and Abl and has demonstrated inhibition of the *in vitro* proliferation of Philadelphia chromosome-positive leukemia, prostate, colon, ovarian, breast (both ER- and ER+), and NSCLC cell lines. It also inhibits migration and invasion of NSCLC cell lines and is effective against xenografts of human NSCLC and ER-

negative breast cancer cell lines, as compared to untreated controls. However, saracatinib did suppress xenograft tumor growth of human colon or oral cancer cell lines and showed mixed results in pancreas and ER+ breast cancer xenografts (NCI, 2011b). This inhibitor is being evaluated in an ongoing phase I/II trial in postmenopausal advanced/metastatic HR+ breast cancer patients being treated with saracatinib or placebo with anastrozole (Green et al., 2009).

Bosutinib

Bosutinib (SKI-606) is an ATP-competitive inhibitor that inhibits SFKs, the kinase Csk (which phosphorylates the inhibitory tyrosine of c-Src), Abl, Eph receptors, Sterile 20 kinases, Trk family, Tec family and Axl family kinases, and FAK. Interestingly, this drug appears to be a poor substrate for multidrug-resistance transporters and even to inhibit them at high concentrations, which may explain some reports of increased accumulation of bosutinib in some tumor tissues. *In vitro* experiments showed it to be efficacious against Philadelphia chromosome-positive but not -negative leukemia cell lines and to have mixed efficacy against imatinib-resistant CML xenografts. Bosutinib is in phase III trials as a first-line treatment for CML (NCI, 2011b).

PI3K/Akt Inhibitors

PI3K class IA proteins have been widely recognized as critical to the tumorigenic properties of many cancers and because of this, several inhibitors are currently being assessed in preclinical and early clinical studies. GDC-0941, SF1126, and BKM120 are broad-range class I inhibitors undergoing phase I studies; preliminary results from the

BKM120 trial show some benefit in breast cancer patients, of which there are eight (Boschelli et al., 2010). PI-39 is a selective p110δ inhibitor being tested in a phase I trial on patients with solid tumors, and TG100-713, a PI3Kγ/PI3Kδ inhibitor, is being evaluated in phase I and II trials for benefit in patients who have had myocardial infarctions (Castaneda et al., 2010; Marone et al., 2008). BEZ235 inhibits wild-type and mutant forms of the p110α subunit of PI3K (Marone et al., 2008) as well as mTOR, which functions to prevent positive feedback to PI3K (Porta and Figlin, 2009). This drug causes G1 arrest in several cancer cell lines (Castaneda et al., 2010), and results from a small phase I trial that includes thirteen breast cancer patients show encouraging results (Porta and Figlin, 2009).

Of all the PI3K and Akt drugs in development, perifosine (KRX-0401) is in the most advanced stage (Castaneda et al., 2010). This compound is an orally-available, membrane-permeable, phosphatidylinositol analog inhibitor that prevents Akt phosphorylation by a poorly-characterized mechanism (Porta and Figlin, 2009). A recent study also showed that perifosine induces the degradation of mTOR signaling pathway components, a cascade which has been shown to regulate autophagy (Nelson et al., 2007; Porta and Figlin, 2009). *In vivo* and *in vitro* studies in cancer cells showed that this inhibitor promotes cell cycle arrest, autophagy, and apoptosis, reduces angiogenesis and invasion, and synergizes with histone deacetylase inhibitors and etoposide to enhance cell death (Sun, 2010). A phase I trial in 42 patients with advanced solid tumors demonstrated one partial response and several disease stabilizations; patients in the study with renal cell carcinoma treated with this monotherapy especially benefited. Following these encouraging findings, perifosine was or is being tested in phase II studies for

efficacy in renal cell, pancreatic, prostate, and hepatocellular carcinomas, as well as sarcoma and Waldenström's macroglobulinemia (Nelson et al., 2007; Porta and Figlin, 2009; Sun, 2010). Phase II trials in recurrent or metastatic prostate cancer demonstrated slight decreases in PSA levels and PSA stabilization, respectively, in about 20-25% of the participants (Porta and Figlin, 2009). Additionally, phase I trials are evaluating this drug in combination with several chemotherapeutic agents (Nelson et al., 2007). However, neither this drug nor any PI3K inhibitor is currently being tested in the HR+ breast cancer population (Nelson et al., 2007).

MEK/ERK Inhibitors

Because of the selectivity of Ras, Raf, and MEK, this pathway, culminating in ERK, can be targeted through the inhibition of several proteins. However, this section will focus on MEK inhibitors that have been or are currently in clinical studies. The large number of MEK1/2 inhibitors, including GDC-0973, RDEA119, GSK1120212, AZD8330, RO5126766, RO4987655, AS703026, and TAK-733 (NCI, 2011b), that are undergoing stages I and II clinical trials for a variety of cancers indicate that there is a strong rationale for the targeting of this pathway. Based on preclinical work, the patients who are expected to most benefit from these drugs are those with the V600E B-Raf mutation, which sensitizes cells to MEK inhibition (Fremin and Meloche, 2010).

The first MEK1/2 inhibitor to be tested in clinical trials was CI-1040. This compound is a potent and selective inhibitor that does not compete with ATP- or ERK-binding but instead binds to an allosteric site, catalytically inactivating MEK. In preclinical mouse xenograft studies of colon cancer the drug reduced tumor growth up to 80%, and a phase

I trial showed promising results in solid tumors. However, a phase II study on solid tumors, including breast cancer, showed no complete or partial responses and little stabilization of disease, leading to its discontinuation in further trials. PD0325901, a CI-1040 analog, demonstrated better outcomes in a phase I/II trial than CI-1040 but more severe toxicities, also leading to its discontinuation (Duffy and Kummar, 2009; Solit et al., 2006).

Selumetinib (AZD6244) is a potent MEK1/2 inhibitor that does not compete with ATP and has shown anti-tumor activity in preclinical xenograft models of colorectal, pancreatic, liver, skin, and lung cancers. In a phase I trial of advanced cancer patients, nine of fifty-seven treated with the monotherapy had stable disease for at least five months (Fremin and Meloche, 2010). Selumetinib has been or is in phase II trials for NSCLC, hepatocellular carcinoma, melanoma, and biliary cancer, with mixed results. By far, the best result was seen in biliary cancer; of the twenty-eight patients treated with this drug, there was one complete response, two partial responses, and seventeen patients who had stable disease. This drug also demonstrated antitumor activity in NSCLC and melanoma patients but was not an improvement over standard treatments, and in the melanoma trial, only patients with a B-Raf mutation benefited. However, the hepatocellular carcinoma trial was halted because of a lack of any objective response (Fremin and Meloche, 2010). A phase II trial is currently underway to test selumetinib, a in patients with advanced HR+ breast cancer that progressed after AI therapy (Duffy and Kummar, 2009; Fremin and Meloche, 2010).

Implications of Estrogen Effector Inhibition on the Treatment of Breast Cancer

Inhibitors of all the estrogen signaling effectors described in this section and investigated in this dissertation have been or are in clinical development as anti-cancer drugs. Additionally, ER, AR, EGFR, HER2, SFKs, PI3K, and MEK are currently being used in or evaluated as breast cancer treatments, some in combination with chemotherapeutic regimens. Because of the wide-spread use of doxorubicin as a treatment for ER+ breast cancer, there is a need to understand the mechanistic implications of targeting these estrogen effectors in doxorubicin-treated tumors. However, there is little known about these proteins' regulation in the presence of doxorubicin or whether their inhibition improves the cytotoxicity of this chemotherapeutic agent in ER+ breast cancer cells.

Outstanding Questions and Scope of Study

Clinical observations and laboratory studies suggest that estrogen confers chemotherapeutic resistance in ER+ breast tumors. Doxorubicin and estrogen typically confer opposing biological outcomes in ER+ breast cancer cell models, and inhibition of several known estrogen effectors has been shown to increase sensitivity to chemotherapy, suggesting that estrogen and doxorubicin may use some of the same proteins to bring about their biological outcomes. However, little is known about whether and how the chemotherapy affects estrogen/estrogen receptor signaling, the components of these pathways mediating chemotherapy's effects, and whether these signaling molecules promote resistance to chemotherapy.

This dissertation focuses on the interplay between estrogen and non-cytotoxic concentrations of doxorubicin in an ER+ breast cancer cell model in order to identify

components of estrogen signaling pathways that could be targeted therapeutically to enhance the actions of doxorubicin. Our model was designed to mimic the prolonged estrogen treatment preceding doxorubicin exposure seen clinically. The results show that prolonged estrogen treatment alone modulated levels and phosphorylation of specific signaling molecules much in the same way as shorter estrogen treatments have been reported to do. Though the doxorubicin concentration used in these experiments (25 nM) was much lower than patient serum levels in the 48 hrs following drug administration (34.5 nM - 3.4 μM) (NCI, 2011b), it reflected ER+ breast cancer patient outcomes through its partial inhibition of estrogen-induced proliferation. Doxorubicin given alone modestly upregulated levels of several hormone and growth factor signaling molecules that were downregulated by estrogen alone, suggesting a mechanism by which it could counteract the effects of estrogen. Surprisingly, treatment with both estrogen and doxorubicin modestly enhanced changes stimulated by estrogen alone and markedly induced pro-growth alterations when compared to doxorubicin alone, providing molecular evidence for the poorer responses of ER+ tumors to doxorubicin. Various inhibitors of proteins involved in estrogen signaling were also tested to identify molecules whose inhibition would augment the effects of doxorubicin; results showed that inhibitors of the MAPK cascade (MEK) and MMP proteins cooperate with doxorubicin to reduce cell proliferation, while inhibitors of ER(s), Src-family kinase (SFK) proteins, AR, and PI3K function independently of doxorubicin. Both classes of inhibitors, however, are potential candidates for overcoming doxorubicin resistance in ER+ breast cancers. We additionally show that although AR levels were upregulated in response to estrogen and doxorubicin, the AR had little to no effect on the proliferation or

survival of an ER+ breast cancer cell line (MCF-7), but it did participate in migration and invasion responses to estrogen, doxorubicin, and their combination.

Chapter 2. Materials and Methods

Reagents

17-β estradiol was purchased from Sigma-Aldrich Corp. (Saint Louis, MO) and resuspended in ethanol for storage. The final media concentration used in experiments (10 nM) contained no more than 0.001% ethanol. Doxorubicin hydrochloride was obtained from Calbiochem (San Diego, CA). For inhibitor experiments PD-98059 (final concentration, 50 μM) was obtained from Biomol International (Enzo Life Sciences, Plymouth Meeting, PA), fulvestrant (ICI, 1 μM) from AstraZeneca (Wilmington, DE), GM 6001 (10 μM) from Biomol International, gefitinib (10 μM) from LC Laboratories (Woburn, MA), SU6656 (1 μM) from Calbiochem, and LY 295002 (25 μM) from Cayman Chemical (Ann Arbor, MI). All inhibitors were suspended in dimethyl sulfoxide (DMSO; Fisher Scientific) except fulvestrant, which was mixed with ethanol. The final concentration of inhibitors contained no more than 1% DMSO or ethanol. Unless otherwise specified, all other chemical reagents were purchased from Fisher Scientific (Waltham, MA).

Cell Lines

The human ER+ breast cancer cell lines, T47-D and MCF-7, were acquired from ATCC (Manassas, VA) and the A. Bouton lab (Univ. of Va.) and maintained at 37°C in a humidified 5% CO_2 environment. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, USA origin), 1 mM sodium pyruvate, and 1% penicillin/streptomycin. For hormone-starvation, cells

were cultured in phenol red-free, low glucose DMEM supplemented with 5% charcoal-stripped FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.76% D-(+)-glucose (45% w/v, Sigma), designated "CSSM." Except where noted, all liquid cell culture reagents were from Gibco (Billings, MT).

Growth Assay

For each condition tested, 5 x 10^4 MCF-7 or 10^5 T47-D cells were seeded per well in a 6-well dish (Corning, Corning, NY) and incubated overnight. The next day, wells were washed three times with Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco) and replenished with fresh CSSM. Inhibitors were added to the appropriate wells after 23 hrs, and 10 nM estrogen was added 1 hr later. In the absence of inhibitor, groups were incubated 24 hrs prior to estrogen addition. Cells were incubated an additional 24 hrs, and then the media was changed to fresh CSSM with or without doxorubicin. Estrogen and inhibitors were added again to the appropriate groups, and cells were incubated another 48 hrs, trypsinized, and counted on a hemocytometer.

Cell Cycle Analysis

MCF-7 treatment groups of CSSM alone, CSSM + estrogen (E), CSSM + doxorubicin (Dox), and CSSM + estrogen + doxorubicin (Dox + E) were seeded in CSSM in triplicate at a density of 2 x 10^6 cells per 10 cm dish (Greiner bio-one, Frichenhausen, Germany), incubated overnight, and cultured as described for the growth assay. Following a PBS (1.4 M NaCl, 26.8 mM KCl, 40.6 mM $Na_2HPO_4 \cdot 7H_2O$) wash cells were detached with 5 ml/dish Accutase (Innovative Cell Technologies, San Diego, CA), and triplicate plates

combined. All subsequent steps were at 4°C and utilized polypropylene tubes (Becton Dickenson, Franklin Lakes, NJ). Cell clumps were broken apart by pipetting repeatedly, centrifuged for 6 min at 200 x g, and resuspended in 0.5 ml PBS. Cells were then fixed in 4.5 ml cold 70% ethanol (Fisher Scientific) in ddH_2O and stored at -20°C until analyzed. Just prior to analysis, ethanol-suspended cells were centrifuged 5 min at 200 × g, suspended in 1 ml cold PBS and counted by hemacytometer. One million cells were washed in cold PBS, pelleted, resuspended in 1 ml DAPI/Triton X-100 staining solution (0.1% w/v Triton-X-100 [LabChem Inc., Pittsburg, PA] and 1 μg/ml DAPI [Sigma-Aldrich] in ddH_2O) for at least 30 min in the dark. DAPI fluorescence was detected by a CyAn™ ADP LX 9 Color flow cytometer (Beckman Coulter, Brea, CA). The pulse width–pulse area signal was used to discriminate between G_2 cells and cell doublets and gate out the latter. Data were analyzed with the ModFit LT program, version 3.2.1 (Verity Software House, Topsham, ME).

Immunoprecipitation and Western Blot Analysis

For each of the four treatment groups, cells were scraped, pelleted, and lysed in NP-40 lysis buffer (1% (v/v) NP-40, 20 mM Tris HCl pH 8.0, 137 mM NaCl, 10% (v/v) glycerol, 2 mM EDTA) supplemented with Protease Inhibitor Cocktail Set I (Calbiochem) and 100 μM Na_3VO_4, and clarified by centrifugation (9300 x g, 5 min). The protein concentration of the lysates was quantitated with BioRad Protein Assay reagent (Hercules, CA) and the Multiskan MCC plate reader (Fisher Scientific).

Protein G beads (Millipore, Temecula, CA) used for immunoprecipitation were prepared one day prior to lysis by gently agitating together 40 μl beads in 500 μl PBS

with the immunoprecipitating antibody or isotype-specific IgG control antibody for 30 min at 4°C. Immunoprecipitations of ER-α were carried out with anti-ER-α, clone 1D5 (Vector Labs, Burlingame, CA) at 1 μg antibody/mg lysate, of AR with PG-21 (Millipore) at 0.5 μg/mg, and of c-Src with 2-17 (S. Parsons lab) at 1 μg/mg. Isotype-specific IgG control antibodies were used at the same concentration as the precipitating antibodies. Beads were pelleted and resuspended in 500 μl of a 1% BSA (Roche Diagnostics GmbH, Mannheim, Germany) solution in PBS and incubated overnight at 4°C with gentle agitation. The next day beads were washed once in NP-40 lysis buffer and incubated with lysate overnight at 4°C with gentle agitation. Beads were washed three times in NP-40 buffer, resuspended in sample buffer, and boiled for 5 min to denature proteins. Because multiple co-precipitating proteins were analyzed per immunoprecipitation, 6 mg lysate was immunoprecipitated per treatment group and this was subdivided into three portions for SDS-PAGE analysis.

In addition to immunoprecipitations, whole cell lysates were analyzed by Western blot (100 μg/lane). Proteins were separated on 8% SDS-PAGE gels and transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were blocked in 5% milk or BSA w/v in Tris-buffered saline Tween-20 buffer (TBST; 100 μM Tris base, 1.5 mM NaCl, 1% (v/v) Tween-20) for 1-2 hrs at room temperature and then incubated in a primary antibody solution (identical to blocking buffer) overnight at 4°C (see Table 1 for details (Micromedex, 2010; Robert et al., 1985)). When blotting immunoprecipitates, cross-species antibodies were for immunoblotting, and dilutions were adjusted to optimize detection. Membranes were then thoroughly washed in TBST and incubated at room temperature with HRP-conjugated secondary antibody (goat anti-mouse or sheep anti-

Table 1. Conditions used in the Western blot protocol.

Protein Target	Block & Ab Solution	Primary Ab Source, Clone/Catalog Number	Primary Ab Dilution	Primary Ab Host Species
AR	5% milk	M. Weber lab (Univ. of VA), AR-21	1:10,000	rabbit
AR pS210/S213	5% BSA	Imgenex (San Diego), 156C125.2	1:1000	mouse
AR pY534	5% BSA	(Waller, *et al.*, 2000)	1:1000	rabbit
β-actin	5% milk	Sigma, AC-15	1:10,000	mouse
c-Src	5% milk	Cell Signaling, #2108	1:1000	rabbit
c-Src	5% milk	S. Parsons lab, 2-17	1:1000	mouse
c-Src pY418	5% BSA	Biosource (Camarillo, TX), #44-660G	1:5000	rabbit
EGFR	5% BSA	Cell Signaling, #2232	1:1000	rabbit
ER-α	5% milk	Vector Labs, 1D5	1:1000	mouse
ER-α	5% milk	Santa Cruz, HC-20	1:1000	rabbit
ER-α pS118	5% BSA	Cell Signaling, #2515	1:2000	rabbit
ER-α pS167	5% BSA	Cell Signaling, #2514	1:1000	rabbit
ER-α pY537	5% BSA	Abcam (Cambridge, MA), ab59177	1:1000	rabbit
ER-β	5% milk	Millipore, 68-4	1:1000	rabbit
ER-β	5% milk	Abcam, 9.88	1:1000	mouse
ERK 1/2	5% milk	B3B9 (Reuter, *et al.*, 1995)	1:1000	mouse
ERK pT183/Y185	5% BSA	Sigma, MAPK-YT	1:10,000	mouse
GAPDH	5% milk	Millipore, 6C5	1:10,000	mouse
HER2	5% milk	Santa Cruz, C-18	1:1000	rabbit
HER2 pY877	5% BSA	Cell Signaling, #2241	1:1000	rabbit
MNAR	5% milk	Bethyl (Montgomery, TX), #A300-180A-1	1:5000	rabbit
PARP*	5% milk	Calbiochem, Ab-2	1:500-1:1000	mouse
PI3K p85	5% milk	Millipore, AB6	1:125	mouse

*Lysates require sheering through a needle

rabbit, GE Healthcare, Waukesha, WI, both 1:2000) for 1 hr. Membranes were again thoroughly washed and incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermoscientific, Waltham, MA) for 2 min in the dark. Membranes were exposed to film (Blue Lite Autorad film, ISC Bioexpress, Kaysville, UT) and processed.

Stress Granule Analysis

Eight groups of MCF-7 cells (growth medium, growth medium + 500 μM NaArs, CSSM, CSSM + 25 nM Dox, CSSM + 10 nM E, CSSM + 25 nM Dox + E, CSSM + 100 nM Dox, and CSSM + 1 μM Dox) were seeded in triplicate in growth medium at a density of 2 x 10^4 cells per well in 6-well dishes to which glass coverslips (Fisher Scientific) had been added and incubated overnight. The next day, wells were washed two times with PBS, fresh growth medium or CSSM was added, and cells were incubated 24 hrs. Estrogen was then added to the appropriate groups which were incubated an additional 24 hrs, after which media was changed to fresh growth medium, CSSM, or CSSM + Dox. Following 48 hrs of incubation, the positive control received sodium arsente for 1 hr while the other groups were incubated in its absence.

For staining, coverslips were washed twice with room temperature PBS, fixed in 4% paraformaldehyde (Formalde-Fresh, Fisher Scientific) for 15 min, and again washed twice with PBS. Cells were permeabilized with 0.5% Triton-X-100 in PBS for 15 min, blocked in 10% BSA in PBS for 2 hrs, and incubated with anti-TIA-1 (1:100 in 3% BSA/PBS solution; Santa Cruz, goat) overnight at 4°C. Coverslips were washed three times in 3% BSA with gentle agitation and incubated with FITC-conjugated anti-goat in 3% BSA for 1 hr. Cells were then washed once in PBS, and nuclei were stained with

DAPI, followed by another PBS wash. Coverslips were affixed to glass slides and viewed through a Leica fluorescence microscope.

LNCaP Treatment with R1881 and Bicalutamide

The human prostate cancer cell line, LNCaP, was passaged and seeded in growth medium: T medium supplemented with 5% FBS, 2 mM L-glutamine solution, and 1% penicillin/streptomycin solution. For assaying, 2.5 x 10^6 cells were seeded per 10 cm dish for each group and incubated overnight. The next day plates were washed three times with DPBS and depleted of hormones by addition of starvation medium (phenol-red-free RPMI, 5% charcoal-stripped FBS). After 24 hrs, DMSO or 1 nM R1881 (PerkinElmer, Waltham, MA) was added to half the plates, and all were incubated overnight. Thirty micromolar bicalutamide was then added and incubated 0.5, 1, 2, 4, or 6 hrs. Controls untreated with bicalutamide for 6 hrs were also included (0 time point). Cells were lysed, and proteins were separated and analyzed by Western blotting as described.

Sliencing of Androgen Receptor

For the mock control, non-specific (NS) siRNA control, and AR siRNA transfected groups, siRNA resuspension buffer (Dharmacon 5x siRNA Buffer, ThermoScientific), 100 μM NS siRNA stock (Reuter et al., 1995; Waller et al., 2000), or 100 μM AR siRNA stock of Thermoscientific On-Target Plus Smartpool human AR siRNA (Thermoscientific) was diluted to 1 pmol/ml final volume in Opti-MEM I Reduced-Serum Medium (250 μl/ml final volume or 1/3 cell suspension volume, Gibco).

RNAiMAX (Invitrogen, Paisley, PA) was then added to the siRNA solution at a concentration of 0.625 μl/ml final volume and incubated 20 min at room temperature. Cells were detached with trypsin and diluted in antibiotic-free growth medium (DMEM supplemented with 10% fetal bovine serum and 1% 100 mM sodium pyruvate solution) to a density of 2.5×10^4 cells/ml. Cells were then added to the siRNA suspensions and immediately aliquoted into tissue culture dishes, which were incubated overnight at changing media. The final volume of the transfection-cell mixture was calculated by dividing the total cell number by 1.875×10^4.

Trypan-Blue Assay

MCF-7 cells were either mock transfected or transfected with AR siRNA as described above, treated with 0, 0.025 μM, 0.250 μM, or 2.5 μM doxorubicin, and subjected to a trypan blue assay according to the following protocol. Following transfection, cells were seeded in duplicate at a density of 3.75×10^4 per well of a 6-well dish and incubated overnight. Cells were then washed three times with PBS and deprived of serum for 48 hrs at 37°C in phenol red-free, serum-free DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.76% D-(+)-glucose (45% w/v) (PRF SFM), and incubated 48 hrs. Media was then replaced with PRF SFM into which an appropriate dilution of doxorubicin had been added, and cells were again incubated 48 hrs longer.

For the trypan blue assay, cells from spent media, from a PBS wash of the monolayer, and from trypsin-detachment were combined, pelleted, and resuspended in PRF SFM. Just prior to counting, an equal volume of 0.4% trypan blue solution (Sigma-Aldrich) was

added to the resuspended cells, cell clumps were gently broken apart by pipetting, and both non-viable (blue) and total cells were manually counted on a hemocytometer.

Migration and Invasion Assays

MCF-7 cells were either mock transfected or transfected with AR siRNA and treated with or without estrogen and/or doxorubicin as described for the cell growth assay. Following treatment, adherent and non-adherent cells were collected, pelleted, and reconstituted in PRF SFM at a density of 2.5 x 10^5 cells/ml (invasion assay) or 10^6 cells/ml (migration assay). A 500 μl aliquot of suspended cells was placed into each top well of a Boyden chamber fitted into a 24 well plate (for migration: BIOCOAT Cell Culture Inserts Control Insert [No ECM], BD Biosciences; for invasion: BD BioCoat Matrigel Invasion Chamber rehydrated with PRF SFM for 2 hrs at 37°C, BD Biosciences). Seven-hundred, fifty microliters of fresh growth medium (experimental groups) or PRF SFM (random motion control) was added to the bottom chambers, and plates were incubated at 37°C for 6 hrs (migration assay) or 24 hrs (invasion assay). After swabbing the tops of the membranes to remove non-migratory or non-invasive cells, membranes were washed twice with PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, washed again twice with PBS, and then incubated in 0.1% crystal violet (Sigma-Aldrich) in 20% ethanol for 10 min at room temperature to stain migratory or invasive cells. Membranes were then washed twice with PBS and once with dH_2O, and air-dried overnight. The following morning, cells on the bottoms of membranes were counted through an Olympus light microscope (Tokyo, Japan).

Quantitation and Statistical Analysis

AlphaEaseFC version 3.1.2 (Alpha Innotech Corp., San Leandro, CA) was used to quantitate scanned protein bands on film. All total protein levels were normalized to the loading control. Specific phosphorylations were calculated by first normalizing the phospho- and total protein bands to the loading control and then dividing the normalized phosphorylated band by the normalized total protein. Relative associations of immunoprecipitated proteins reflect the proportion of a protein within a cell associating with another protein. This was calculated by first dividing the immunoprecipitated and associated proteins by their respective normalized protein levels in the whole cell lysate. The normalized associated protein was then divided by the normalized immunoprecipitated protein. All conditions were divided by the value for the untreated control and expressed as the fold change of the control. Comparisons between treatment groups were carried out using Student's t-test or ANOVA corresponding to factorial experiments done in randomized blocks after transforming values to the log scale (to account for experiment-to-experiment variation in growth). All error bars indicate standard errors of the mean.

Chapter 3. Results: A Mechanistic Study of Doxorubicin/Adriamycin's Effect on the Estrogen Response in a Breast Cancer Model

Introduction

Doxorubicin is a common chemotherapeutic treatment for estrogen receptor-positive (ER+) breast tumors despite clinical reports demonstrating that ER+ patients derive less benefit from chemotherapy than their ER- counterparts (Gioeli et al., 2006). Studies using ER+ breast cancer cell lines have shown that estrogen treatment counters the effects of chemotherapy (Berry et al., 2006; Conforti et al., 2007; Kuerer et al., 1999; Miles et al., 1999; Sertoli et al., 1995), implying that estrogen-induced signaling may confer chemotherapeutic resistance. Additionally, estrogen-induced signaling has been shown to support proliferation, survival, differentiation, development, inflammation, angiogenesis, EMT, migration, and invasion in various systems (Huang et al., 1997; Leung and Wang, 1999; Razandi et al., 2000; Teixeira et al., 1995; Vasconsuelo et al., 2008), and several of these same processes have been shown to be inhibited by doxorubicin (Giretti et al., 2008; Malek et al., 2006; Planas-Silva and Waltz, 2007; Welboren et al., 2009). This suggests that some of the same molecules that mediate estrogen signaling may also mediate doxorubicin effects. However, little is known about whether and how the chemotherapy affects estrogen/estrogen receptor signaling, the components of these pathways mediating chemotherapy's effects, and whether these signaling molecules promote resistance to chemotherapy. In this chapter, the interplay between estrogen and non-cytotoxic concentrations of doxorubicin in an ER+ breast

cancer cell model will be discussed, and components of estrogen signaling pathways that are potential clinical targets will be identified.

Results

Doxorubicin Impairs Estrogen-Induced Proliferation.

Doxorubicin is a well-characterized cytotoxic drug that is frequently used to treat ER+ breast tumors. However, ER+ tumors respond less well to this drug than ER- tumors, an outcome that is not well understood at a molecular level. Patients receiving chemotherapy (including doxorubicin) have been endogeneously exposed to estrogen prior to and during treatment (in the absence of additional endocrine therapy), consistent with the hypothesis that continued estrogen signaling itself may counteract the effects of chemotherapy. To develop a cell-culture system that would reflect the clinical treatment paradigm and allow us to address these biological and molecular questions, two ER+ breast cancer cell lines (MCF-7 and T47-D) were treated as depicted in Figure 3. Cells were hormone-starved and exposed to 10 nM estrogen alone, doxorubicin alone, estrogen and doxorubicin together, or maintained in hormone depleted conditions for a five day period. Following treatment, cells were assessed for protein levels and phosphorylation status of molecules involved in estrogen signaling by immunoprecipitation/Western blot or for biological responses, such as proliferation, cell death, and cell cycle progression. Figures 4-11 depict representative results of these studies and are described in more detail below.

Previous studies have shown that ER+ breast cancer cells treated with less than 1 μM doxorubicin can survive in the presence of estrogen, suggesting that the poor clinical

Figure 3. Schematic depiction of the cell culture treatment protocol.

ER+ breast cancer cells were treated with estrogen (E) alone, doxorubicin (Dox) alone, or co-treated as depicted. Cells were seeded in growth medium, incubated overnight, and then hormone-starved. A day later, 10 nM estrogen was added to the appropriate groups, and cells were incubated for an additional 24 hrs. At that time, spent media was replaced with fresh starvation media supplemented with or without doxorubicin and estrogen. Forty-eight hours later, cells were lysed for biochemical analysis by immunoprecipitation or Western blotting, counted, or fixed and stained for cell cycle analysis by flow cytometry.

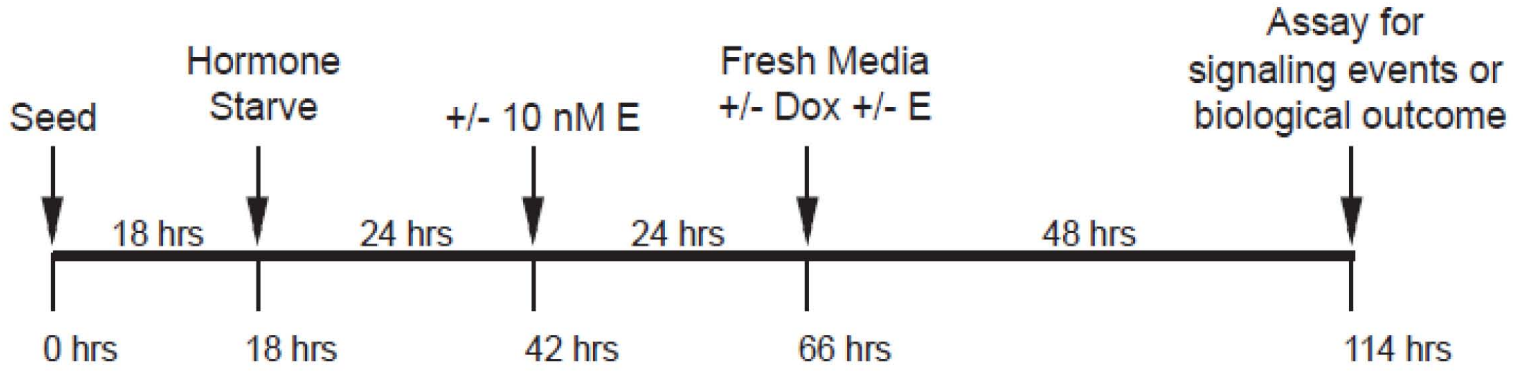

Seed
Hormone Starve
+/- 10 nM E
Fresh Media +/- Dox +/- E
Assay for signaling events or biological outcome
18 hrs
24 hrs
24 hrs
48 hrs
0 hrs
18 hrs
42 hrs
66 hrs
114 hrs

Fig. 3

responses of ER+ breast cancers to chemotherapy may be due to low intratumoral chemotherapy concentrations (Leung and Wang, 1999; Teixeira et al., 1995). Given these results, we sought to establish the biological effect of nanomolar concentrations of doxorubicin in our system. MCF-7 and T47-D cells were exposed to doxorubicin in the presence or absence of estrogen (according to the scheme in Fig. 3) and counted after five days as a measure of net growth and survival. Figure 4 shows that estrogen-induced proliferation was diminished in both cell lines with increasing doxorubicin concentrations, with an approximate 50% decrease at 50 nM in MCF-7 cells. Since the estrogen response in MCF-7 cells in the absence of doxorubicin was more robust than in T47-D cells, and the 25 nM drug concentration permitted an analysis of both estrogen and doxorubicin effects, this doxorubicin concentration and the MCF-7 cell model were chosen for all subsequent experiments. Within the doxorubicin range tested (0 – 1 μM) and in the absence of estrogen, little change in cell number was observed, consistent with the findings that doxorubicin is less cytotoxic to non-proliferating cells, and ER+ cells require estrogen for proliferation (Barranco, 1984; Migliaccio et al., 2007).

Because studies in various cell lines had demonstrated detrimental effects of a wide range of doxorubicin concentrations, we desired to know if low concentrations of doxorubicin induced cytotoxicity in our MCF-7 model, either in the presence or absence of estrogen. This was assessed through the measurement of apoptotic markers in cells treated with a range of doxorubicin concentrations. MCF-7 cells failed to induce poly ADP-Ribose polymerase (PARP) cleavage, a hallmark of apoptotic signaling, following treatment with 0 – 1 μM doxorubicin in the absence or presence of estrogen (Fig. 5). However, PARP cleavage was evident with 10 μM doxorubicin treatment, and estrogen

Figure 4. Doxorubicin impairs estrogen-induced proliferation.

Cells were treated as described in Figure 3 and analyzed in a cell count assay. MCF-7 (left panel) and T47-D (right panel) cells were treated with 0, 25, 50, 75, 100, or 1000 nM doxorubicin in the presence (dashed line) or absence (solid line) of 10 nM estrogen as described in Materials and Methods. The arrow indicates 25 nM doxorubicin in MCF-7 cells. *Indicates statistical significance between groups treated with and without estrogen ($p \leq 0.05$). The results are expressed as the mean fold change in cell number ± SEM for five experiments.

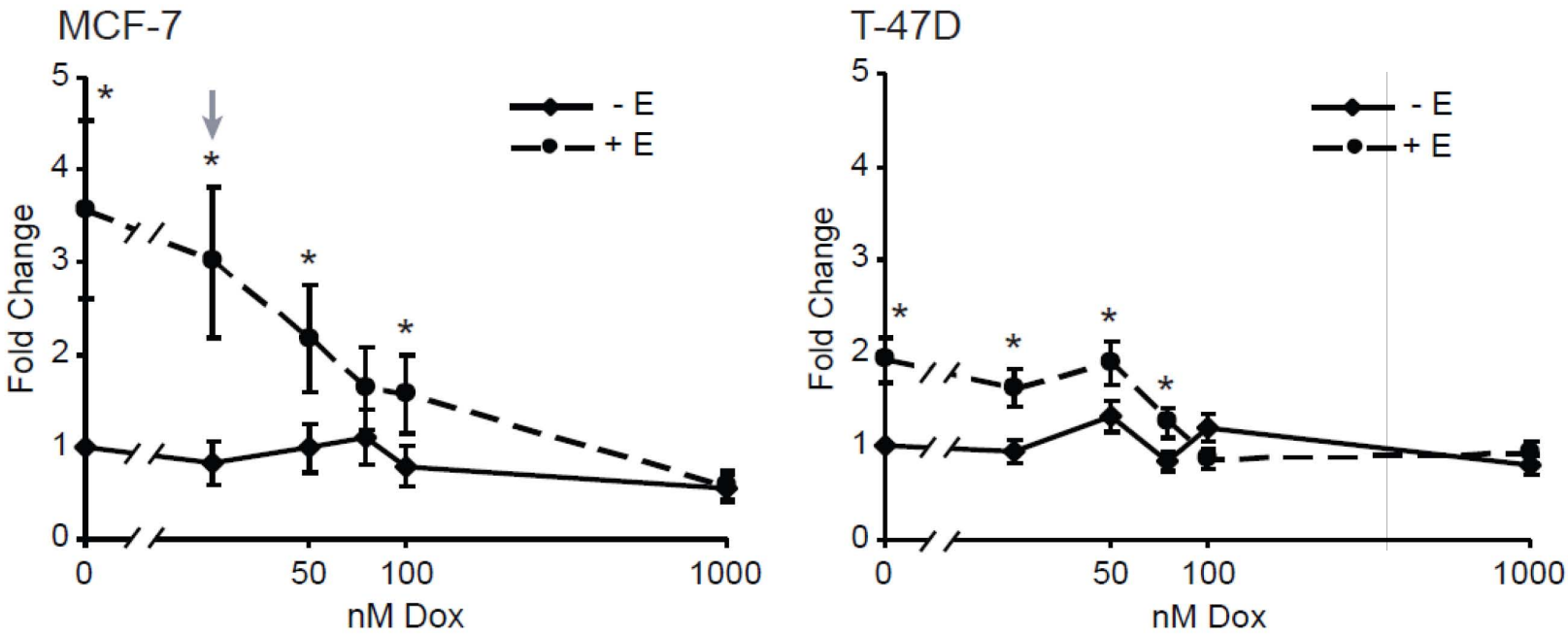


Fig. 4

Figure 5. Doxorubicin concentrations from 0-1 μM do not induce PARP cleavage. MCF-7 cells were treated as depicted in Figure 3, lysed, and probed for PARP and β-actin (loading control) by Western blotting as described in Materials and Methods. Cleavage was only induced by 10 μM doxorubicin.

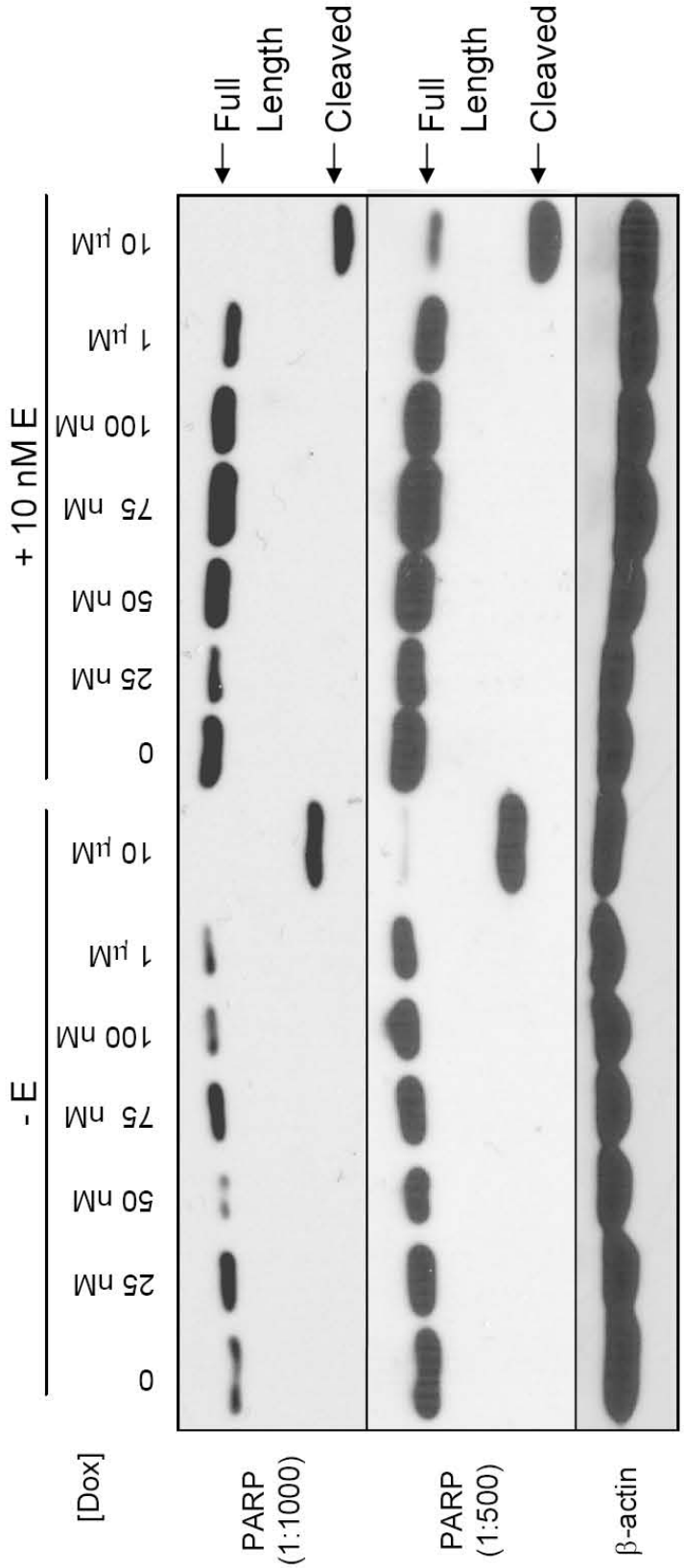

[Dox]
- E
0
25 nM
50 nM
75 nM
100 nM
1 μM
10 μM
+ 10 nM E
0
25 nM
50 nM
75 nM
100 nM
1 μM
10 μM
PARP (1:1000)
PARP (1:500)
β-actin
Full Length
Cleaved
Full Length
Cleaved

Fig. 5

had only a small inhibitory effect at this concentration. Results from deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and trypan blue assays provided further evidence that 0 – 1 μM doxorubicin and/or estrogen did not induce cell death (data not shown).

Stress granules, which are transient, phase-dense structures resulting from sudden translational arrest, form in response to many environmental insults (Kedersha and Anderson, 2007). To determine if doxorubicin treatment induced stress granule formation, MCF-7 cells were hormone-starved and exposed to increasing concentrations of doxorubicin in the presence or absence of estrogen and stained for the presence of TIA-1, a protein component of stress granules (Kedersha and Anderson, 2007). Treatment with sodium arsenite was used as a positive control and growth medium as a negative control. MCF-7 cells that were hormone-starved and treated with 0 - 10 μM doxorubicin did not form stress granules, and estrogen had no affect on stress granule formation alone or in the presence of 25 nM doxorubicin (Fig. 6 and data not shown). Together these data indicated that neither doxorubicin concentrations between 25 nM and 10 μM nor estrogen promoted measurable changes to apoptosis or stress granule formation (suggesting no impact on translation) in MCF-7 cells.

The observation that doxorubicin decreased estrogen-stimulated proliferation in the absence of apoptosis raised the question of what effect, if any, doxorubicin might have on cell cycle progression, in both the absence and presence of estrogen. To address this question, MCF-7 cells were either left untreated or treated with 25 nM doxorubicin alone or together with estrogen for five days and subjected to cell cycle analysis by flow cytometry. Figure 7A shows that doxorubicin alone-treated cells arrested in G1, and

Figure 6. Doxorubicin concentrations from 1-10 μM do not induce stress granule formation.

MCF-7 cells were treated with growth medium, CSSM, CSSM + indicated concentration of doxorubicin (Dox), CSSM + estrogen (E), or CSSM + Dox + E according to the scheme depicted in Figure 3. Following 48 hrs of incubation with or without doxorubicin, the growth medium-treated positive control received 500 μM sodium arsenite for 1 hr while the other groups were incubated in its absence. Growth medium alone served as a negative control. Cells were stained with anti-TIA-1 to detect stress granule and with DAPI to visualize DNA as described in Materials and Methods. Cells were analyzed with a fluorescent microscope. Representative images are shown. CSSM and estrogen-treated controls were negative.

Fig. 6

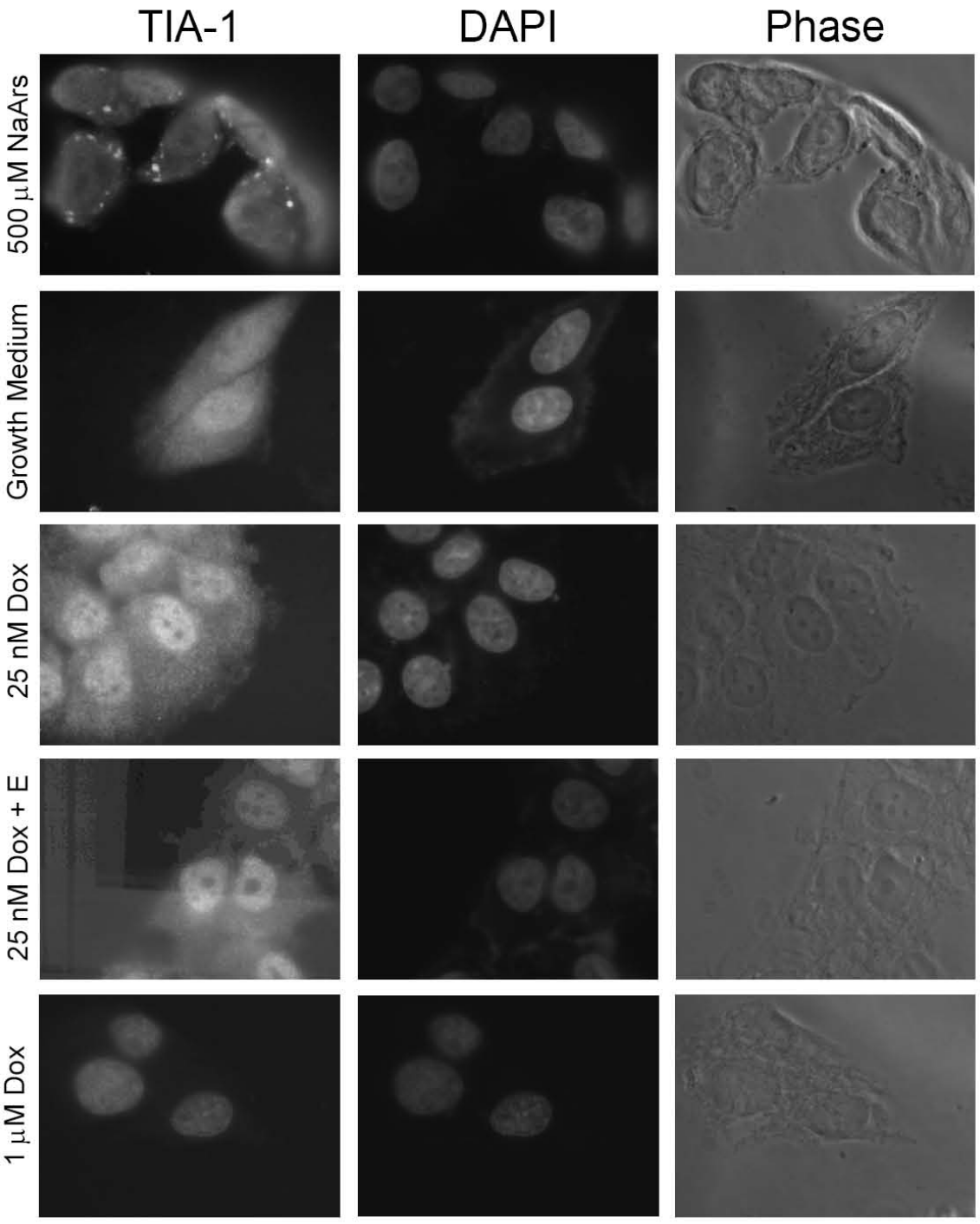

TIA-1
DAPI
Phase
500 μM NaAs
Growth Medium
25 nM Dox
25 nM Dox + E
1 μM Dox

Figure 7. Doxorubicin induces cell cycle changes.

Cells were treated as described in Figure 3 and analyzed in for cell cycle distribution. A. MCF-7 cells treated with or without 25 nM doxorubicin were stained with DAPI and subjected to cell cycle analysis, as described in Materials and Methods. Results are expressed as the mean percent cells in G1 (black bars), S (grey bars), and G2 (white bars) ± SEM for ten experiments. *Indicates statistical significance between groups treated with and without doxorubicin ($p \leq 0.05$). B. MCF-7 cells treated with 10 nM estrogen were left untreated or exposed to 25 nM doxorubicin and analyzed as in 2B. *Indicates statistical significance between groups treated with and without estrogen ($p \leq 0.05$).

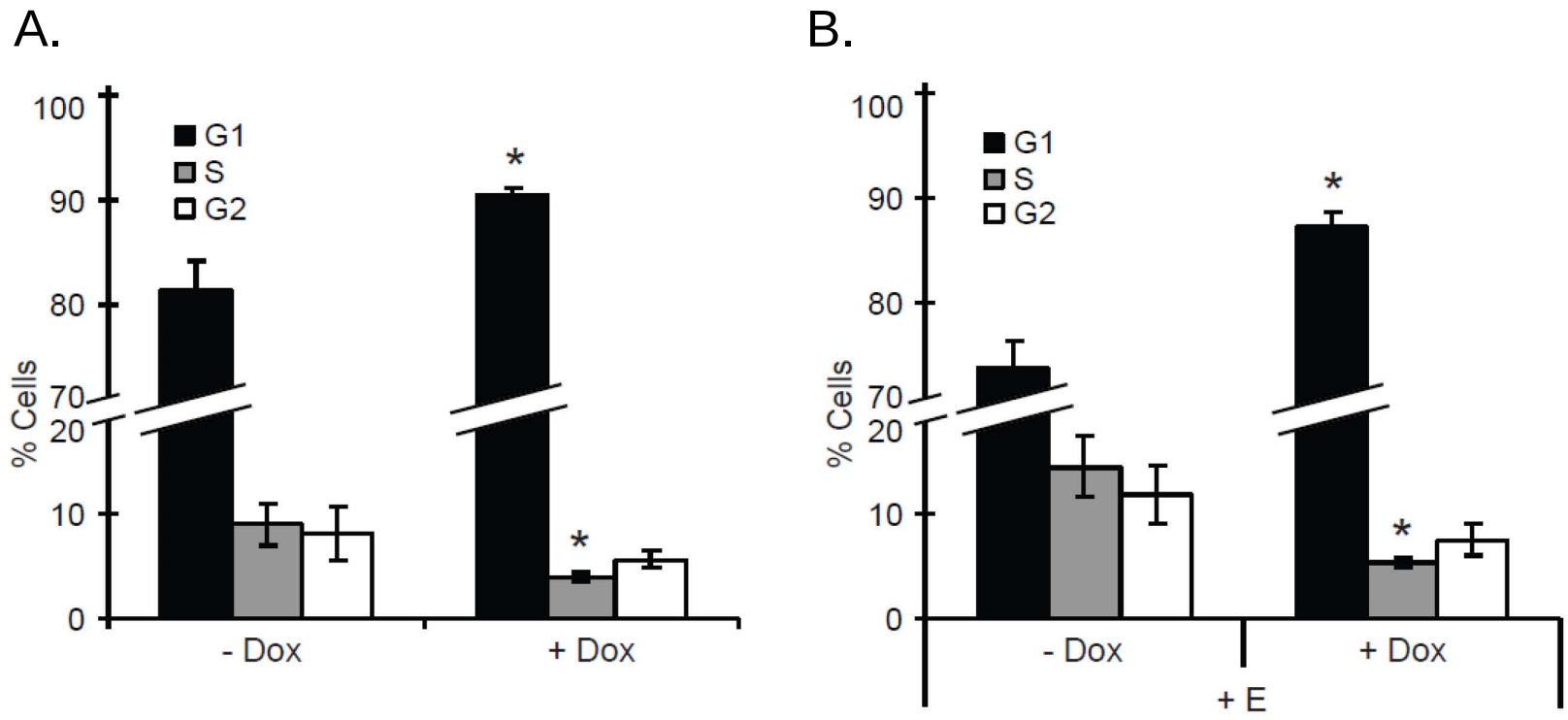


Fig. 7

fewer progressed to S phase compared to non-treated cells, consistent with prior reports and further affirming the validity of our model system (Rusetskaya et al., 2009; Yun et al., 2000). Additionally, prolonged estrogen treatment alone significantly decreased the proportion of cells in G1 phase and increased those in S phase. Cells treated with doxorubicin and estrogen together exhibited a similar G1 accumulation and decrease in S phase entry as occurred with doxorubicin treatment alone (Fig. 7B). These data indicate that a sub-apoptotic doxorubicin concentration can cause cell cycle blocks that estrogen cannot fully overcome.

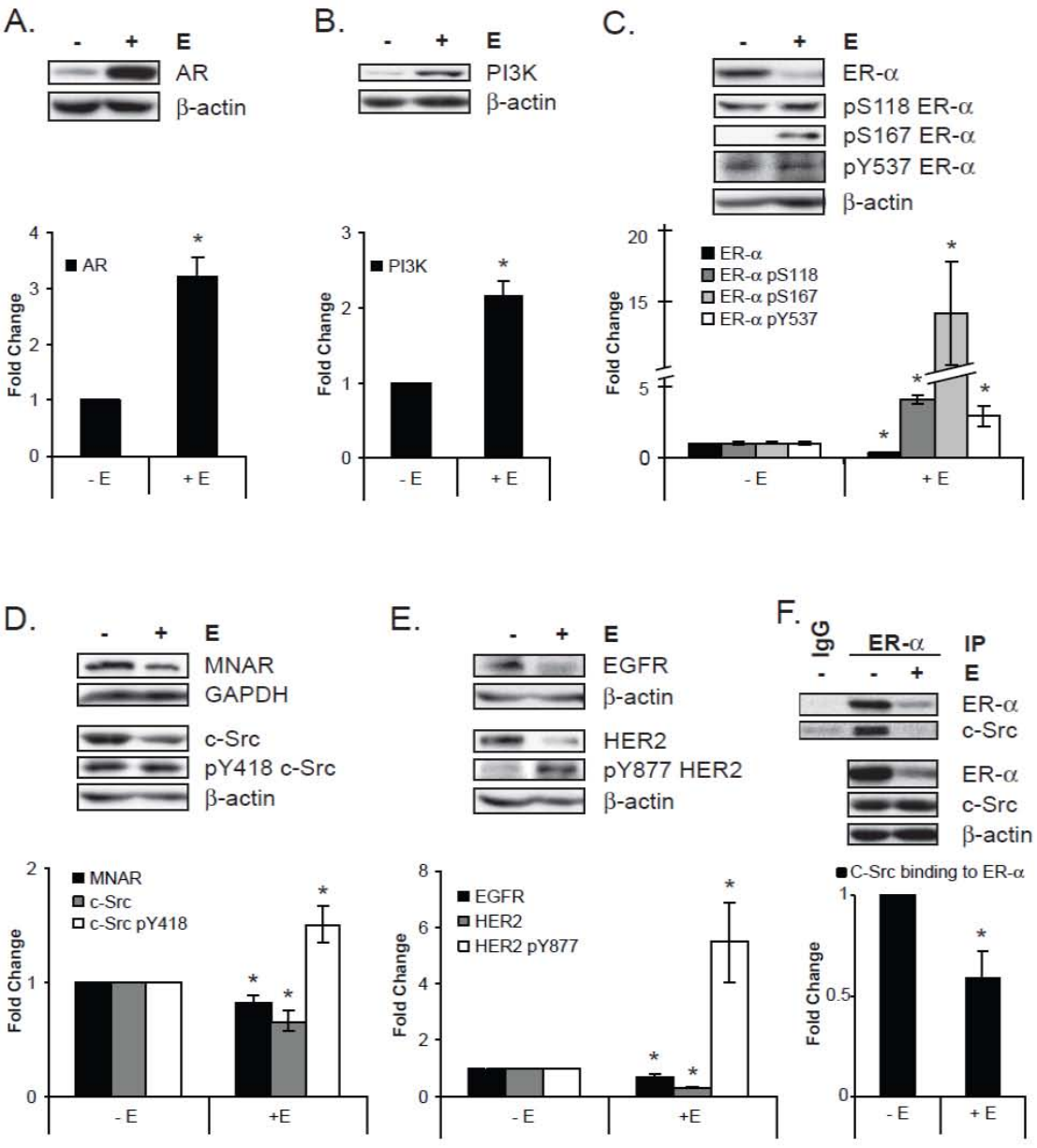
Prolonged Estrogen and/or Doxorubicin Treatment Cause Changes in Levels and/or Phosphorylation Status of Estrogen Signaling Molecules.

To gain insight into the effects of the various doxorubicin/estrogen treatments on molecules known to be involved in estrogen signaling, lysates from treated cells were analyzed by Western blotting for total protein levels or specific phosphorylations. Our first analyses were carried out on cells stimulated long-term (72 hrs) with estrogen alone and compared to those maintained under hormone-deprived, or non-treated, conditions. Figure 8 shows that when compared to hormone-deprived controls, prolonged estrogen treatment alone caused accumulation of AR (Fig. 8A) and PI3K (Fig. 8B) proteins, both of which have been shown to mediate estrogen-induced proliferation and/or survival (Rusetskaya et al., 2009; Yun et al., 2000). Reduction of ER-α (Fig. 8C), MNAR (Fig. 8D), c-Src (Fig. 8D), EGFR (Fig. 8E), and HER2 (Fig. 8E) protein levels was also observed. Changes to these levels may be the result of rapid protein turnover, a hallmark

Figure 8. Prolonged levels of estrogen alter the protein levels and increase specific phosphorylation of estrogen signaling molecules.

MCF-7 cells, cultured for 72 hrs in the presence or absence of estrogen, were lysed and analyzed by immunoprecipitation and/or Western blotting. Within each panel, the Western blots are representative of nine or more experiments that were quantitated and shown in the graph. For each graph, the untreated control was set to 1, and the estrogen-treated group was expressed as the mean fold change of the untreated control ± SEM, as described in Materials and Methods. All phosphorylations have been normalized to both the total protein levels and the loading control. *Indicates statistical significance between groups treated with and without estrogen ($p \leq 0.05$). A. Androgen receptor protein levels increased with estrogen treatment. B. Estrogen enhanced PI3K protein levels. C. ER-α levels (black bars) were reduced by estrogen treatment, even as specific phosphorylations at ER-α S118 (dark grey bars), ER-α S167 (light grey bars), and ER-α Y537 (white bars) were elevated. D. MNAR (black bars) and c-Src (grey bars) protein levels decreased with estrogen treatment, but the specific phosphorylation of c-Src's activating tyrosine (c-Src pY418, white bars) was increased. E. EGFR (black bars) and HER2 (grey bars) levels were reduced, but HER2 specific phosphorylation at Y877 (white bars) was enhanced with estrogen exposure. F. The relative association of c-Src with immunoprecipitated ER-α decreased with prolonged estrogen treatment.

Fig. 8


A.
- + E
AR
β-actin
Fold Change
AR
- E
+ E
B.
- + E
PI3K
β-actin
Fold Change
PI3K
- E
+ E
C.
- + E
ER-α
pS118 ER-α
pS167 ER-α
pY537 ER-α
β-actin
Fold Change
ER-α
ER-α pS118
ER-α pS167
ER-α pY537
- E
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D.
- + E
MNAR
GAPDH
c-Src
pY418 c-Src
β-actin
Fold Change
MNAR
c-Src
c-Src pY418
- E
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E.
- + E
EGFR
β-actin
HER2
pY877 HER2
β-actin
Fold Change
EGFR
HER2
HER2 pY877
- E
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F.
IgG
ER-α
IP
- - + E
ER-α
c-Src
ER-α
c-Src
β-actin
C-Src binding to ER-α
Fold Change
- E
+ E

of active signaling, and/or reduced gene expression (Castoria et al., 2001; Lobenhofer et al., 2000). In addition, specific phosphorylations, associated with increased activity, on ER-α (Fig. 8C), c-Src (Fig. 8D), and HER2 (Fig. 8E) were elevated, similar to that seen with acute estrogen action (Bourdeau et al., 2004; Read et al., 1990). Table 2 (-Dox columns) provides the quantification of these and other effects on ER-β and ERK 1/2 proteins and on AR and ERK 1/2 phosphorylations, which were modestly or insignificantly altered by prolonged estrogen treatment.

ER-α has been shown to increase complex formation with various signaling molecules, including MNAR, c-Src, AR, and PI3K, within minutes of estrogen stimulation; these complexes have been shown to support protein activation of their component proteins (Antoniotti et al., 1994; Arnold et al., 1994; Arnold et al., 1995b; Murphy et al., 2006). In our current study, examination of ER-α association following prolonged estrogen treatment revealed that in contrast to the increased association between ER-α and c-Src reported in MCF-7 cells following 5 min estrogen treatment (Castoria et al., 2001; Chieffi et al., 2003; Wong et al., 2002), several days of estrogen exposure modestly but significantly decreased the relative association of c-Src with immunoprecipitated ER-α when compared to a hormone-deprived control (Fig. 8F). Additional interactions between the ER-α, AR, MNAR, and c-Src were also analyzed but, with the aforementioned exception, no significant changes to relative associations were seen between estrogen-treated and –untreated groups (data not shown). Together, these results indicate that prolonged estrogen exposure molecularly resembles that of shorter estrogen courses with respect to protein levels and phosphorylations but that a key

Table 2. Changes in levels and/or phosphorylation of estrogen signaling molecules after prolonged estrogen and/or doxorubicin treatment in MCF-7 cells.

	- Dox		+ Dox	
	- E	+ E	- E	+ E
AR	1	**3.21 +/- 0.33**	*1.57 +/- 0.12*	***4.87 +/- 0.59***
pS210/S213	1	0.99 +/- 0.22	0.79 +/- 0.11	0.86 +/- 0.25
pY534	1	1.57 +/- 0.44	0.91 +/- 0.10	1.20 +/- 0.18
EGFR	1	**0.69 +/- 0.09**	*2.19 +/- 0.39*	**0.96 +/- 0.19**
ER-α	1	**0.31 +/- 0.03**	*1.47 +/- 0.13*	**0.38 +/- 0.06**
pS118	1	**4.06 +/- 0.43**	1.01 +/- 0.09	**4.26 +/- 0.60**
pS167	1	**14.15 +/- 2.23**	*0.75 +/- 0.06*	**16.59 +/- 2.61**
pY537	1	**2.91 +/- 0.35**	1.02 +/- 0.10	**3.99 +/- 0.68**
ER-β	1	1.02 +/- 0.35	1.04 +/- 0.17	0.83 +/- 0.26
HER2	1	**0.30 +/- 0.04**	*1.84 +/- 0.23*	**0.38 +/- 0.05**
pY877	1	**5.48 +/- 1.43**	1.39 +/- 0.36	***6.79 +/- 1.59***
ERK 1/2	1	1.10 +/- 0.10	1.08 +/- 0.13	1.16+/- 0.22
pT183/Y185	1	**0.62 +/- 0.15**	1.07 +/- 0.19	0.92 +/- 0.19
MNAR	1	**0.82 +/- 0.07**	1.07 +/- 0.09	***0.64 +/- 0.06***
PI3K	1	**2.16 +/- 0.21**	1.11 +/- 0.11	*1.64 +/- 0.24*
c-Src	1	**0.66 +/- 0.09**	1.08 +/- 0.12	***0.51 +/- 0.09***
pY418	1	**1.51 +/- 0.15**	1.16 +/- 0.12	***2.07 +/- 0.26***

All values are obtained from analyses of data depicted in Figs. 3-6 and expressed as the mean fold change in relationship to the untreated (- Dox, - E) control.

Bold denotes $p < 0.05$ when compared to - E counterpart

Highlighted italics denote $p < 0.05$ when compared to - Dox counterpart

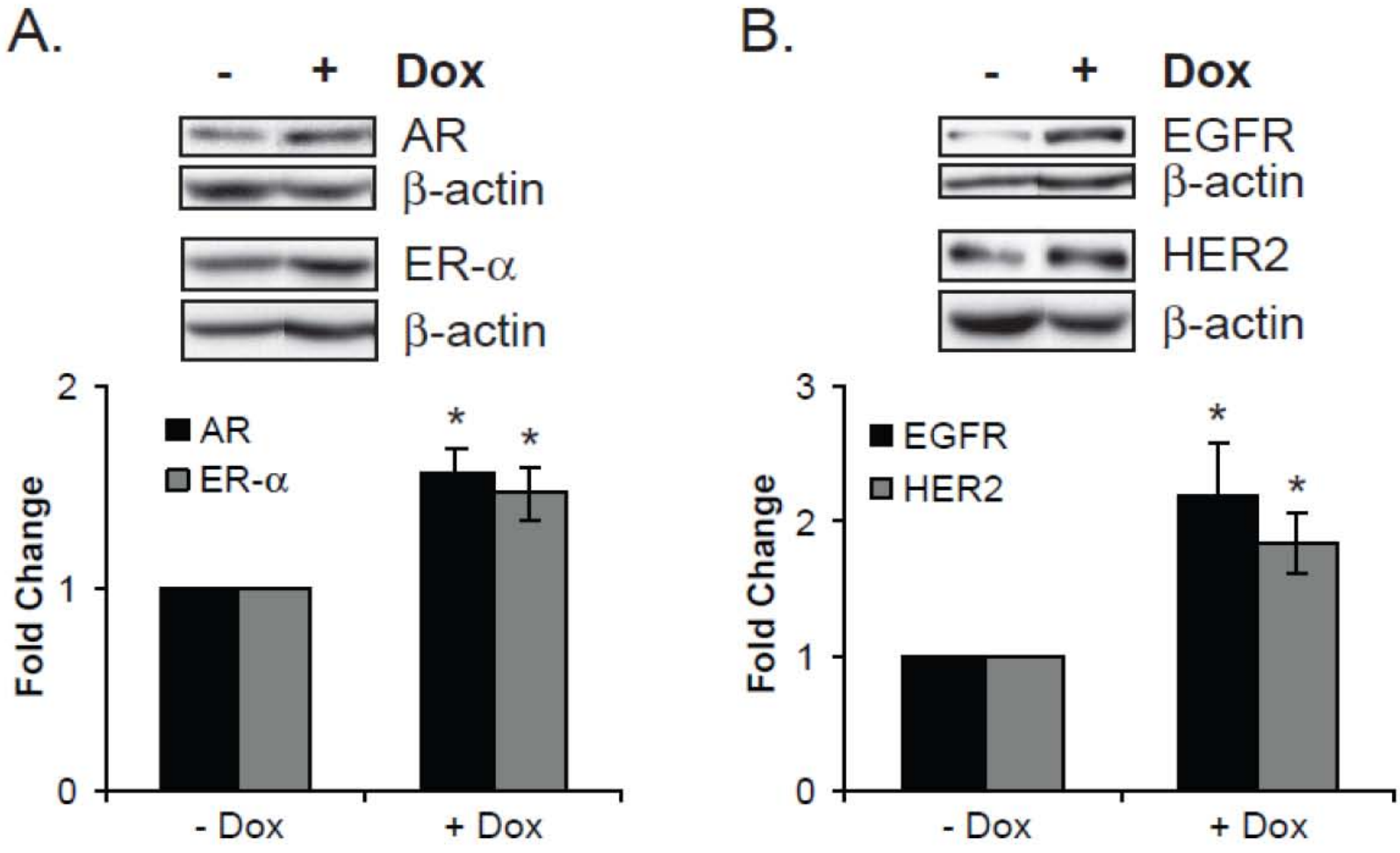
signaling protein-protein interaction that forms within minutes of stimulation is altered by sustained estrogen exposure.

The finding that 25 nM doxorubicin could induce a G1 block in the presence of estrogen suggested that extended doxorubicin treatment could affect estrogen downstream signaling (Fig. 7B). Given that low levels of doxorubicin alone also caused cell cycle arrest (Fig. 7A), we asked whether molecules involved in estrogen signaling were affected by doxorubicin treatment in the absence of estrogen. Cell lysates were analyzed by Western blotting following treatment with 25 nM doxorubicin to study these molecular effects. Figure 9 shows that in the absence of estrogen, prolonged doxorubicin treatment resulted in increases in AR (Fig. 9A), ER-α (Fig. 9A), EGFR (Fig. 9B), and HER2 (Fig. 9B) protein levels, an effect that is opposite that of prolonged estrogen alone treatment (with the exception of AR, whose protein levels were elevated by both treatments [Fig. 8]). See also Table 2 (-E, compare –Dox to +Dox), which in addition indicates a small but significant reduction in ER-α pS167 levels. These results indicate that doxorubicin alone can have effects on proteins involved in estrogen signaling that are antagonistic to the effects of estrogen alone.

Results depicted in Figures 8 and 9 indicate that estrogen and doxorubicin individually affect estrogen signaling molecules, but little is known about the signaling changes that occur in an estrogen-responsive tumor treated with doxorubicin. To address this question, levels and phosphorylation states of proteins from estrogen-exposed cells were compared to those treated with both estrogen and doxorubicin. Surprisingly, doxorubicin and estrogen co-treatment augmented several estrogen-alone responses (Figs. 8 and 10). For example, doxorubicin plus estrogen modestly but significantly

Figure 9. Doxorubicin treatment enhances the levels of AR, ER-α, EGFR, and HER2.

MCF-7 cells were treated as depicted in Figure 3, lysed, and analyzed by Western blotting. Within each panel, the Western blots are representative of the ten or more experiments quantitated in the graph. For each graph the untreated control was set to 1, and the doxorubicin-treated group is expressed as the mean fold change of the untreated control ± SEM, as described in Materials and Methods. *Indicates statistical significance between groups treated with and without doxorubicin ($p \leq 0.05$). A. Doxorubicin modestly increased the protein levels of the hormone receptors AR (black bar) and ER-α (grey bar). B. Levels of the growth factor receptor signaling molecules EGFR (black bar) and HER2 (grey bar) were also elevated with doxorubicin treatment.


A.
- + Dox
AR
β-actin
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Fold Change
2
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AR
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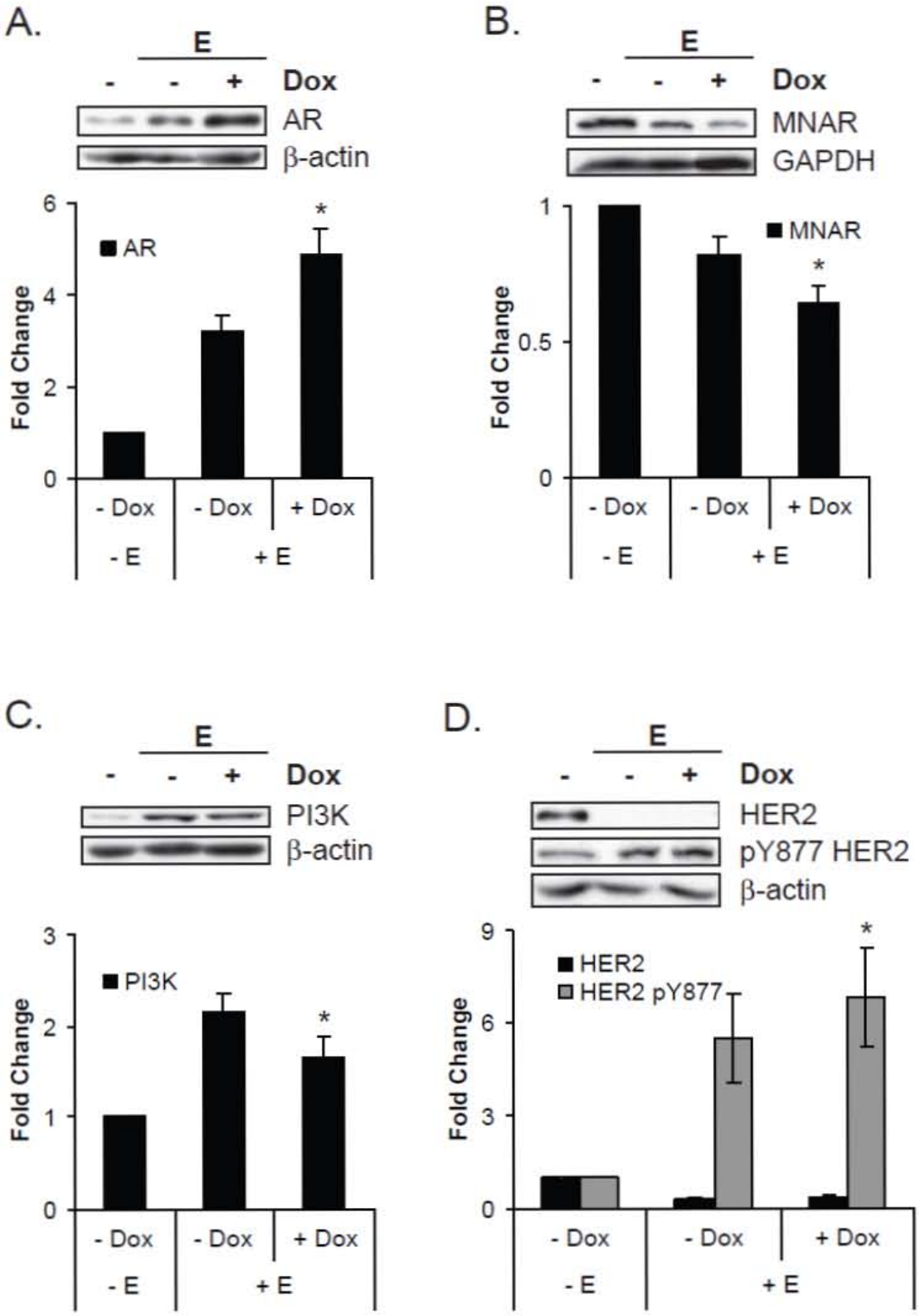
B.
- + Dox
EGFR
β-actin
HER2
β-actin
Fold Change
3
2
1
0
EGFR
HER2
*
*
- Dox
+ Dox

Fig. 9

Figure 10. A low concentration of doxorubicin has both positive and negative effects on estrogen signaling.

MCF-7 cells were treated as depicted in Figure 3, lysed, and analyzed by Western blotting. Within each panel, the Western blots are representative of the nine or more experiments that were quantitated in the graph. Estrogen-treated groups are expressed as the mean fold change of the untreated control ± SEM, as described in Materials and Methods. All phosphorylations have been normalized to both the total protein levels and the loading control. *Indicates statistical significance between groups treated with estrogen and with or without doxorubicin ($p \leq 0.05$). A. The addition of doxorubicin to estrogen treatment further increased estrogen-induced AR protein levels. B. MNAR levels were further decreased when doxorubicin was added to estrogen. C. Estrogen-induced elevation of PI3K protein levels was partially reversed by the addition of doxorubicin. D. Though the co-treatment of doxorubicin and estrogen did not significantly change HER2 levels (black bars) from estrogen-alone levels, it caused a slight increase in HER2 specific phosphorylation at Y877 (grey bars).

Fig. 10


A.
E
- - + Dox
AR
β-actin
Fold Change
AR
- Dox
- Dox
+ Dox
- E
+ E
B.
E
- - + Dox
MNAR
GAPDH
Fold Change
MNAR
- Dox
- Dox
+ Dox
- E
+ E
C.
E
- - + Dox
PI3K
β-actin
Fold Change
PI3K
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- E
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D.
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HER2
pY877 HER2
β-actin
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HER2
HER2 pY877
- Dox
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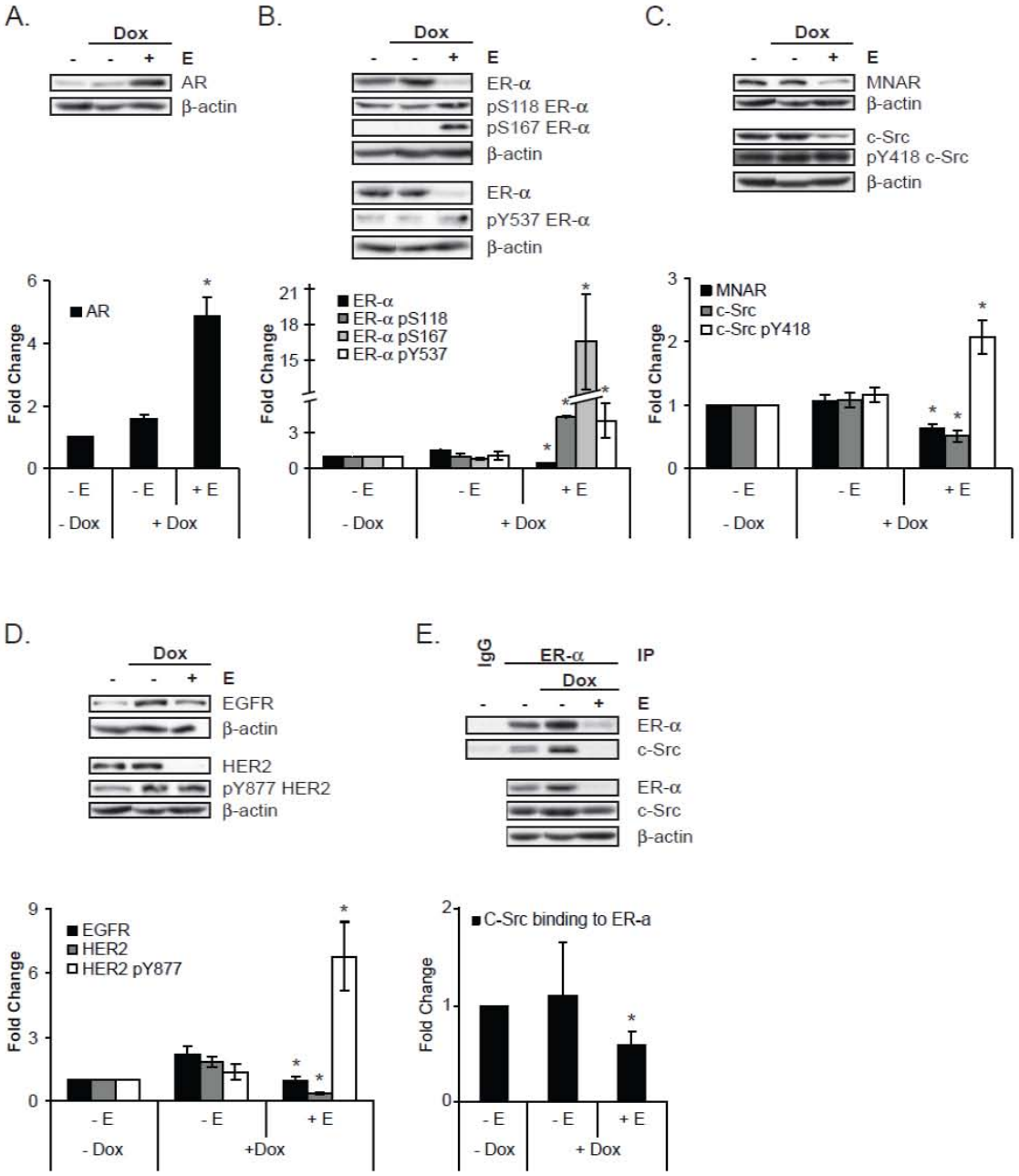
increased AR protein levels (Fig. 10A), increased HER2 specific phosphorylation (Fig. 10D), and reduced MNAR protein (Fig. 10B) to a greater extent than estrogen alone. In contrast, dual doxorubicin/estrogen treatment modestly reduced estrogen alone-induced increases in PI3K levels (Fig. 10C). However, doxorubicin did not affect many estrogen alone-stimulated changes, including those to ER-α, c-Src and EGFR protein levels and ER-α and c-Src phosphorylations. (See Table 2 for quantitative comparisons.) Contrary to expectations, these results suggest that although doxorubicin had a slight detrimental effect on estrogen signaling (on PI3K, Fig. 10C), it also left unaffected and even enhanced other aspects of the estrogen signaling pathway.

In addition to chemotherapy, some ER+ patients are candidates for adjuvant estrogen-ablation therapy. To better understand the molecular changes that occur when ER+ breast tumors are treated with chemotherapy in the presence or absence of estrogen modulators, we compared cells exposed to doxorubicin with and without estrogen treatment. Figure 11 and Table 2 show that compared to doxorubicin alone, co-treatment with estrogen resulted in increased AR (Fig. 11A) and decreased ER-α (Fig. 11B), MNAR (Fig. 11C), c-Src (Fig. 11C), EGFR (Fig. 11D) and HER2 (Fig. 11D) protein levels, consistent with changes seen with estrogen alone (Fig. 8). We also observed increased specific phosphorylation at residues correlated with ER-α (Fig. 11B), c-Src (Fig. 11C), and HER2 (Fig. 11D) activation, indicating active estrogen signaling in the presence of doxorubicin. Moreover, Figure 11E shows that the relative association of c-Src with immunoprecipitated ER-α was reduced with the combination treatment. This protein-protein interaction, seen when doxorubicin alone was compared to co-treatment, reflected results of comparing no treatment to estrogen treatment (Fig. 11E vs. 8F).

Figure 11. Estrogen signaling persists in the presence of doxorubicin.

MCF-7 cells were treated according to the scheme depicted in Figure 3. Following lysis, proteins were analyzed by immunoprecipitation and/or Western blotting, as represented in the upper portion of each panel. Graphs depict the quantitations of the eight or more experiments. The doxorubicin-treated groups with and without estrogen were expressed as the mean fold change of the untreated control ± SEM, as described in Materials and Methods. All phosphorylations have been normalized to both the total protein levels and the loading control. *Indicate statistical significance between the doxorubicin-treated groups with and without estrogen ($p \leq 0.05$). A. As compared to doxorubicin alone, co-treatment with estrogen and doxorubicin increased AR levels. B. The addition of estrogen to doxorubicin decreased ER-α protein (black bars) but increased specific phosphorylation at S118 (dark grey bars), S167 (light grey bars), and Y537 (white bars). C. MNAR (black bars) and c-Src (grey bars) protein levels were decreased, while specific phosphorylation at c-Src Y418 (white bars) was increased from doxorubicin alone by co-treatment with doxorubicin and estrogen. D. As compared to doxorubicin alone, EGFR (black bars) and HER2 (grey bars) protein levels were reduced, but specific phosphorylation of HER2 Y877 (white bars) was increased by the addition of estrogen to doxorubicin treatment. E. In the presence of estrogen, doxorubicin reduced the relative association of c-Src with immunoprecipitated ER-α from levels seen with doxorubicin alone.

Fig. 11


A.
Dox
- - + E
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AR
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B.
Dox
- - + E
ER-α
pS118 ER-α
pS167 ER-α
β-actin
ER-α
pY537 ER-α
β-actin
Fold Change
ER-α
ER-α pS118
ER-α pS167
ER-α pY537
C.
Dox
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MNAR
β-actin
c-Src
pY418 c-Src
β-actin
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MNAR
c-Src
c-Src pY418
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EGFR
β-actin
HER2
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β-actin
Fold Change
EGFR
HER2
HER2 pY877
+Dox
E.
IgG
ER-α
IP
Dox
- - - + E
ER-α
c-Src
ER-α
c-Src
β-actin
Fold Change
C-Src binding to ER-a

Together, these data indicated that a low dose of doxorubicin had little to no effect on estrogen signaling in estrogen-responsive breast cancer cells.

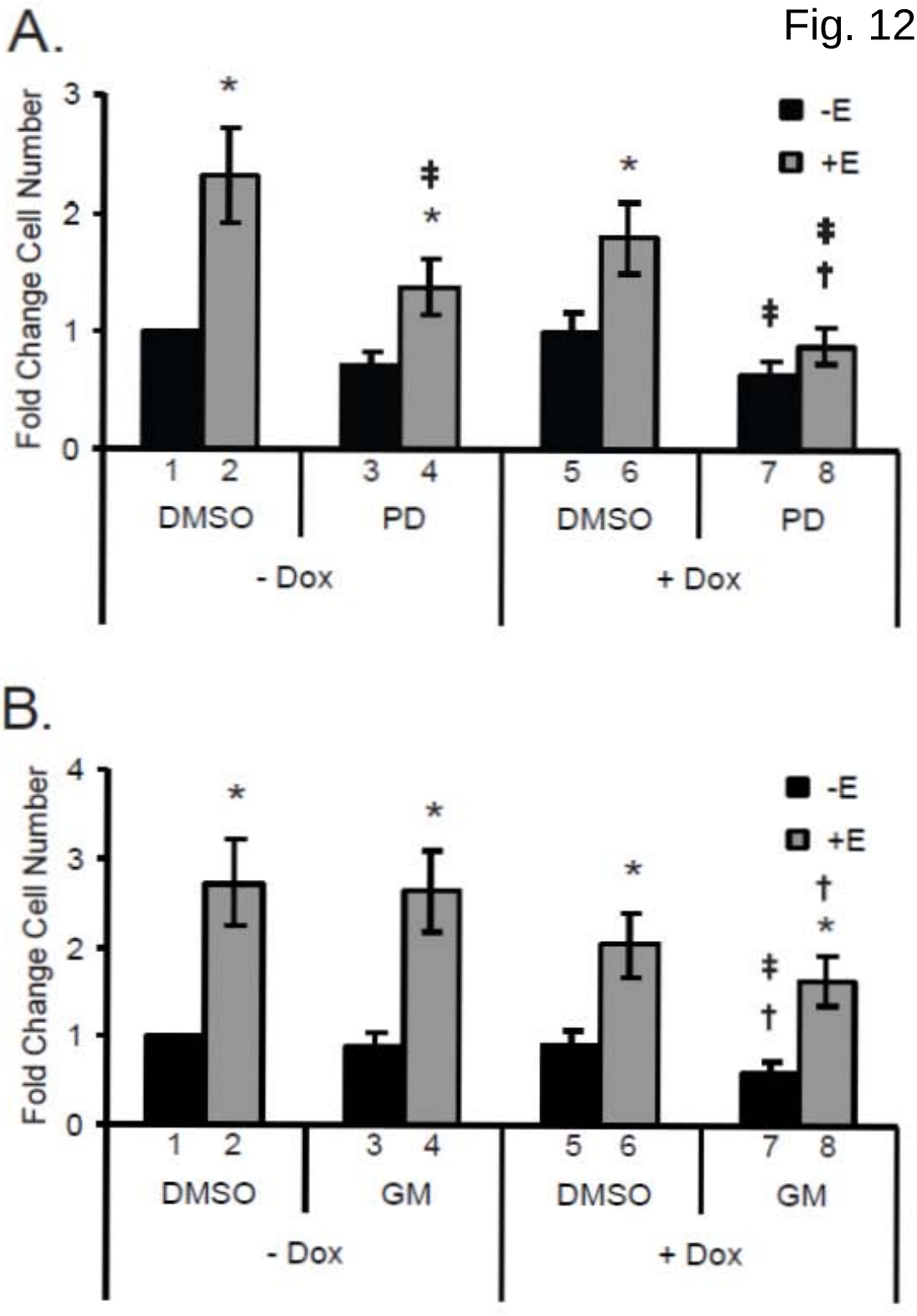
Inhibitors of Estrogen Signaling Molecules Reduce MCF-7 Cell Growth in Doxorubicin-Dependent and –Independent Manners.

The biochemical studies depicted in Figs. 8-11 and summarized in Table 2 demonstrated that doxorubicin treatment resulted in changes to proteins involved in estrogen signaling that, when administered alone, were opposite the biochemical effects of estrogen treatment or, when administered with estrogen, either augmented or had little to no affect. Together these results suggest that components of the estrogen signaling pathway could mediate resistance to doxorubicin. Given these alterations, we sought to determine if targeted inhibitors of estrogen-induced signaling molecules would improve the cytotoxic effects of doxorubicin in the presence of estrogen. To this end, various combinations of doxorubicin, estrogen, and inhibitors of the ER(s), SFK family, EGFR, PI3K, MEK, AR, and MMP proteins were tested for their net effects on MCF-7 cell proliferation. Figure 12 shows that neither PD-98059, a MEK inhibitor, nor GM 6001/Galardin, a pan-MMP inhibitor, significantly affected cell number in the absence of estrogen whether doxorubicin was present or not (Figs. 12A & B, bars 1 vs. 3 and 5). However, in the presence of estrogen, the MEK inhibitor alone reduced cell number (Fig. 12A, bar 2 vs. 4), an effect that was significantly augmented by doxorubicin (Fig. 12A, bar 4 vs. 8). The MMP inhibitor, on the other hand, had little effect on cell number in the absence of doxorubicin and presence of estrogen (Fig. 12B, bar 2 vs. 4) but significantly (but modestly) enhanced the cytotoxic effect of doxorubicin when estrogen was present

Figure 12. MEK and MMP inhibitors cooperate with doxorubicin to inhibit growth of MCF-7 cells.

MCF-7 cells treated according to the protocol depicted in Figure 3 were also treated with targeted inhibitor or DMSO 1 hr prior to estrogen addition. The inhibitor or DMSO remained present in the media until cells were counted. Graphs depict the results of three or more experiments as the mean fold change in cell number ± SEM for each treatment group compared to the - Dox, - E, DMSO control (bars 1), which was set to 1. Black bars represent groups treated in the absence of estrogen whereas grey bars indicate estrogen-stimulated groups. *Indicates that the addition of estrogen has significantly altered ($p \leq 0.05$) a level from that of its non-estrogen treated counterpart (e.g. DMSO - Dox - E vs. DMSO - Dox + E), †denotes a significant change with doxorubicin-treatment from the level of its non-doxorubicin treated counterpart (e.g. DMSO - Dox - E vs. DMSO + Dox - E), and ‡ signifies a difference between groups treated with DMSO and an inhibitor (e.g. DMSO - Dox + E vs. inhibitor - Dox + E). A. The MEK inhibitor, PD-98059 (PD, 50 μM), impaired estrogen-induced proliferation and cooperated with doxorubicin in the presence of estrogen to further reduce cell numbers to basal levels. B. GM 6001 (GM, 10 μM), a pan-MMP inhibitor, demonstrated doxorubicin-dependent decreases in cell number.

Fig. 12



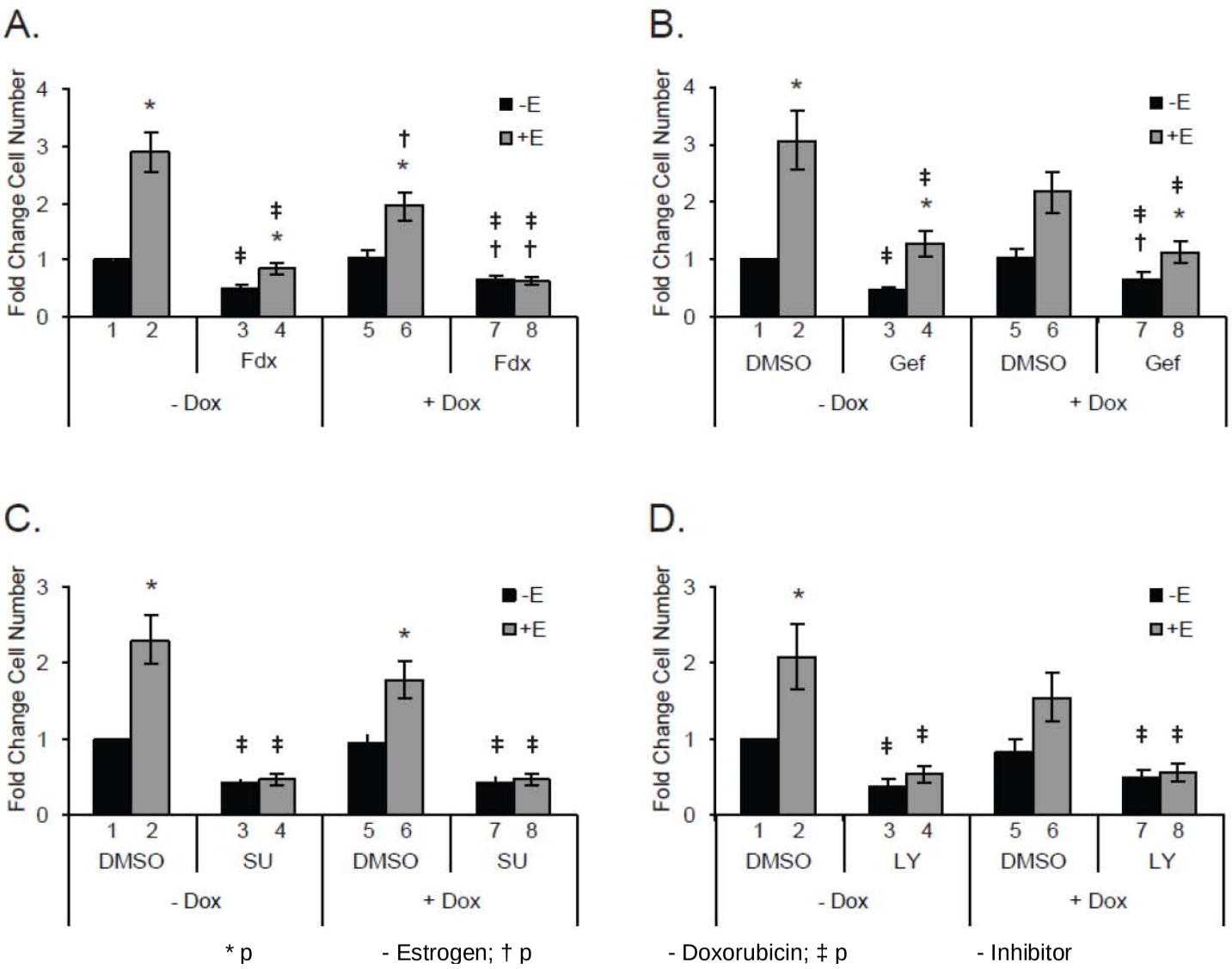
* p ≤ 0.05 for +/- Estrogen; † p ≤ 0.05 for +/- Doxorubicin; ‡ p ≤ 0.05 for +/- Inhibitor

(Fig. 12B, bar 6 vs. 8) or not (Fig. 12B, bar 5 vs. 7). Of all the tested inhibitors, only PD-98059 and GM 6001 exhibited this cooperativity with doxorubicin to reduce cell growth. These results indicated that MEK and MMPs played key roles in limiting the effectiveness of the actions of doxorubicin in MCF-7 cells, suggesting that therapeutically targeting these molecules in doxorubicin-resistant ER+ breast cancers is worthy of further investigation.

In contrast to the MEK and MMP inhibitors, the remainder of the tested targeted drugs acted largely independently of doxorubicin. Figure 13 shows that fulvestrant/Faslodex (an ER-α and -β targeted-drug, Fig. 13A), gefitinib/Iressa (an EGFR inhibitor, Fig. 13B), SU6656 (a SFK inhibitor, Fig. 13C), and LY 294002 (a PI3K inhibitor, Fig. 13D) inhibited estrogen responses to nearly the same extent in the presence or absence of doxorubicin (Bars 4 vs. 8 in each panel). Though it was known that these targets mediated estrogen-induced proliferation/survival of ER+ breast cancer cells (Arnold et al., 2007; Castoria et al., 1999; Castoria et al., 2001; Lobenhofer et al., 2000; Migliaccio et al., 2002b; Migliaccio et al., 2005; Migliaccio et al., 2007; Song et al., 2010; Stabile et al., 2005), the efficacy of their inhibitors in the presence of doxorubicin and estrogen had not previously been tested. The strongest inhibition of estrogen-responses was seen with fulvestrant, SU6656, and LY 294002. The ER protein(s), SFK protein(s), and PI3K are critical to tumor cell growth in the presence or absence of doxorubicin and could be exploited as therapeutic targets in treating doxorubicin-resistant breast cancer cells.

Figure 13. ER, EGFR, SFK, PI3K, and AR-targeted drugs are effective inhibitors of estrogen-induced MCF-7 cell growth that act independently of doxorubicin.

MCF-7 cells were treated and analyzed as in Figure 12. *Indicates that the addition of estrogen has significantly altered ($p \leq 0.05$) a level from that of its non-estrogen treated counterpart (e.g. DMSO - Dox - E vs. DMSO - Dox + E), †denotes a significant change with doxorubicin-treatment from the level of its non-doxorubicin treated counterpart (e.g. DMSO - Dox - E vs. DMSO + Dox - E), and ‡ signifies a difference between groups treated with DMSO and an inhibitor (e.g. DMSO - Dox + E vs. inhibitor - Dox + E). $n \geq 3$. A. Cells treated with 1 μM fulvestrant (Fdx, an ER-targeted drug); B. 10 μM gefitinib (Gef, an EGFR inhibitor); C. 1 μM SU6656 (SU, a SFK inhibitor); or D. 25 μM LY 294002 (LY, a PI3K inhibitor) demonstrated inhibitor-dependent decreases in estrogen-induced proliferation in both the absence and presence of doxorubicin.



* p - Estrogen; † p - Doxorubicin; ‡ p - Inhibitor

Fig. 13

Discussion

The majority of breast tumors can be classified as ER+ by immunohistochemistry and, of these, most are treated with a course of chemotherapy, which includes doxorubicin (Teixeira et al., 1995). However, several studies have demonstrated that chemotherapy is less effective in ER+ than ER- patients (Berry et al., 2006). This finding suggested that the tumor's response to estrogen is stronger than the negative growth and survival effects of chemotherapy. However, little work has been done to study the molecular mechanisms underlying the antagonistic relationship between estrogen and chemotherapy in ER+ breast cancer. Therefore, we sought to define proteins whose levels or activation state were altered by doxorubicin with the goal of identifying druggable targets for the improvement of chemotherapeutic outcomes of ER+ patients. We found that sub-apoptotic levels of doxorubicin enhanced estrogen-induced changes in a subset of estrogen signaling molecules and that many of these same molecules were critical for proliferation in the presence of doxorubicin and estrogen. These findings suggest that several mediators of estrogen signaling, including ER, c-Src, PI3K, MEK, and MMP proteins, are promising therapeutic targets when combined with doxorubicin.

Although high levels of doxorubicin are administered to patients, the intratumoral concentration of the drug has not been reported. We hypothesized that the intratumoral concentrations of the drug are quite low given the poor apoptotic responses of doxorubicin-treated ER+ breast tumors. Our data demonstrated that in ER+ cell lines estrogen-induced proliferation is evident following a 48 hrs treatment of doxorubicin at concentrations of 100 nM or less (Fig. 4 and data not shown). Within this range, doxorubicin inhibited the proliferative effect of estrogen by causing G1 accumulation and

reduced S phase entry but did not induce apoptosis or stress granule formation (Figs. 5, 6 and 7). Therefore, a very low intratumoral concentration of chemotherapy coupled with an estrogenic environment may explain the poor outcomes of ER+ patients treated with chemotherapy alone.

In order to model the physiological situation of an ER+ breast tumor treated with chemotherapy, ER+ MCF-7 breast cancer cells were exposed to a prolonged course of estrogen treatment prior to and during low-level doxorubicin exposure (Fig. 3). The results, shown in Figure 8 and Table 2, demonstrated that ER+ cancer cells subjected to prolonged estrogen elicited similar molecular responses to those described in the literature for cells exposed to brief estrogen treatments (Arnold et al., 1994; Arnold et al., 1995a; Arnold et al., 1995b; Chen et al., 2010; Crowder et al., 2009; Hitosugi et al., 2007; Lannigan, 2003; Migliaccio et al., 2002b; Read et al., 1990). Increased specific phosphorylation of ER-α, c-Src, and HER2 indicated active signaling much like that which occurs within minutes of estrogen stimulation. Though we chose to focus on changes in members of the rapid estrogen signaling pathways in this study, transcriptional changes have also been reported to occur within hours of estrogen stimulation (Hewitt et al., 2010; Sun et al., 2007). Indeed, ER-α, AR, EGFR, and HER2 protein level fluctuations that we observed are consistent with estrogen-regulated, ER-α–mediated transcriptional mechanisms that have been described previously following several days of estrogen exposure, further validating our model (Apparao et al., 2002; Bourdeau et al., 2004; Lee et al., 2005b; Read et al., 1990; Yarden et al., 2001). We speculate that the decrease in relative association of c-Src with immunoprecipitated ER-α may be due to altered turn-over rates of these proteins and/or increased ER-

α participation in transcription compared to that which occurs with either in the absence of hormone or with very short estrogen stimulations. Overall, our data indicated that prolonged estrogen exposure mirrored pro-proliferation and pro-survival responses stimulated by shorter courses of estrogen, with the notable exception of one protein-protein interaction, and that this correlated with enhanced growth and survival (Fig. 14A).

Interestingly, our data suggested that doxorubicin suppresses estrogen signaling pathways when administered alone (Fig. 9). The upregulation of ER-α, EGFR, and HER2 that occurred in the presence of doxorubicin was opposite of estrogen's effect on these molecules, indicating that active signaling was not occurring through the estrogen and/or growth factor pathways. In fact, the increased AR levels may mediate doxorubicin sensitivity, as ER+ breast cancer patients that express the AR and are treated with chemo- and endocrine therapies have better survival outcomes (Castellano et al., 2010). We showed that doxorubicin-induced upregulation of ER-α and AR correlated with cell cycle arrest (Figs. 7B and 9A), suggesting that the upregulation of the AR may have facilitated a doxorubicin response in our model, as well.

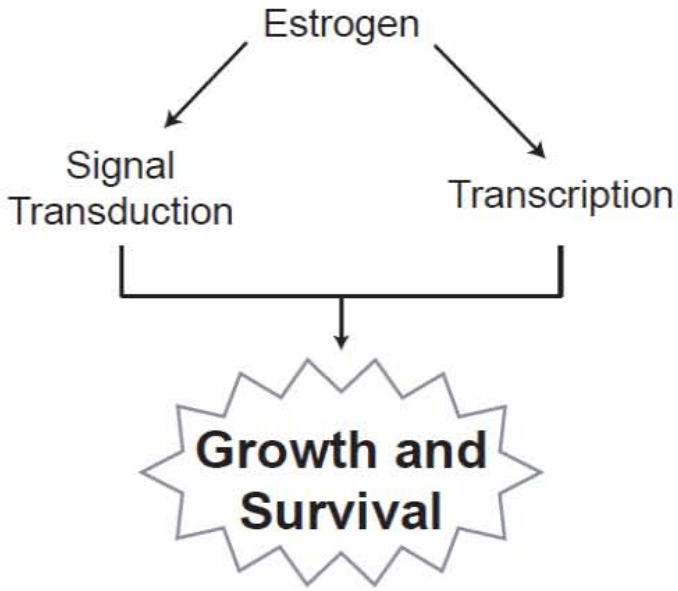
Doxorubicin-induced changes, however, were counteracted by the addition of estrogen. As shown by the increased phosphorylations and protein level modulations in Figure 11, doxorubicin was unable to prevent estrogen signaling, which was even enhanced in the presence of doxorubicin (Figs. 10 and 11). We also showed that associations between c-Src, and ER-α that have been described as supporting estrogen signaling within minutes of stimulation (Barletta et al., 2004; Cheskis et al., 2008; Wong et al., 2002) were reduced with persistent estrogen and doxorubicin treatment, much as

Figure 14. Model of doxorubicin action on an estrogen-stimulated ER+ breast cancer cell.

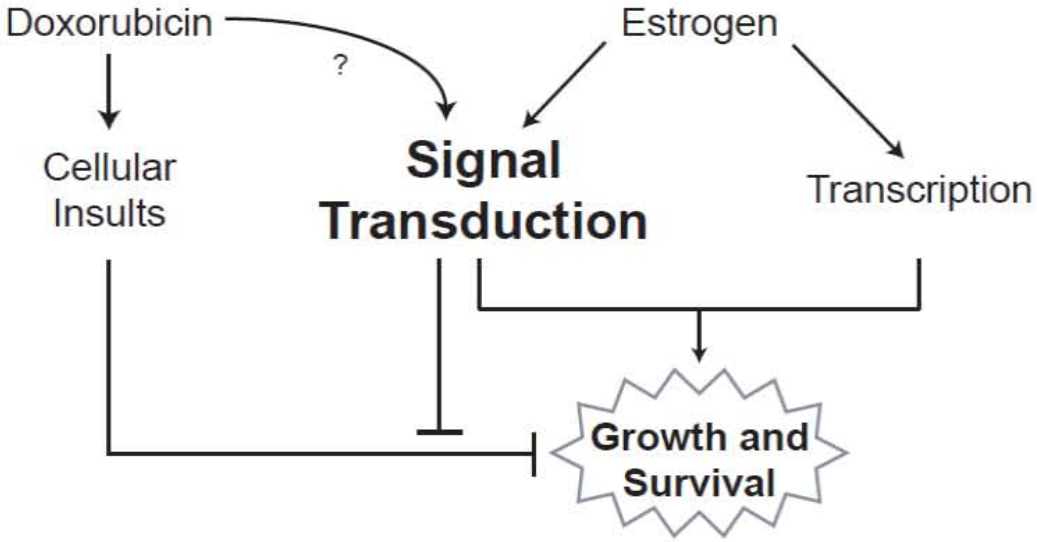
A. Estrogen increases growth and survival through intracellular signaling and transcriptional mechanisms. B. Doxorubicin induces cellular insults that impair estrogen-stimulated growth and survival responses. Through an unknown mechanism, doxorubicin also alters the protein levels and phosphorylation states of various ER effector molecules in the presence of estrogen in ways that are consistent with more robust estrogen signaling. Additionally, some estrogen signaling molecules (such as MEK and MMP proteins) counteract doxorubicin's impairment of growth and survival, thereby lessening the cytotoxic effects of doxorubicin. The net effect of doxorubicin action in the presence of estrogen is reduced but persistent growth and survival of ER+ breast cancer cells.

Fig. 14

A.


Estrogen
Signal Transduction
Transcription
Growth and Survival

B.


Doxorubicin
?
Estrogen
Cellular Insults
Signal Transduction
Transcription
Growth and Survival

was seen with prolonged estrogen treatment (Fig. 8). Together with the cell count proliferation assays (Figs. 4, 12, 13), these data demonstrated that low levels of doxorubicin do not completely ablate estrogen signaling.

Contrary to expectations, when compared to estrogen-alone, doxorubicin plus estrogen modestly enhanced AR and MNAR protein levels and HER2 specific phosphorylations, while reducing increases in PI3K levels (Fig. 10). Despite the negative impact of doxorubicin on proliferation (as seen in Figs. 7A, 7B, 12, and 13), most specific phosphorylation levels and pro-proliferative protein-protein interactions of estrogen signaling effectors were maintained at estrogen-alone levels (Table 2 and data not shown). These findings suggested that the anti-proliferative intracellular insults of doxorubicin resulted in compensatory estrogen signaling in the presence of estrogen (Fig. 14B).

Previous small molecule inhibitor studies had identified mediators of growth and survival in ER+ breast cancer cells but had not considered their effects in the context of estrogen and chemotherapy together (Castoria et al., 2001; Creighton et al., 2010; Lobenhofer et al., 2000; Okubo et al., 2004; Reddy and Glaros, 2007). Our findings (Figs. 12 and 13) provide rationale for targeting the canonical ER proteins themselves, the SFK proteins, PI3K, MEK, and/or EGFR in patients treated with doxorubicin. In our study the most robust inhibitions of proliferation and survival were demonstrated by fulvestrant, SU6656, and LY 294002, respective inhibitors of ER(s), SFK proteins, and PI3K, independently of doxorubicin. These inhibitors have all shown efficacy as single agents in inhibiting growth and survival in ER+ cell lines (Chen et al., 2010; Creighton et al., 2010; Crowder et al., 2009; Okubo et al., 2004; Reddy and Glaros, 2007); however,

monotherapies targeting several of these pathways have failed in preclinical xenograft models or clinical trials for ER+ tumors (Creighton et al., 2008; Perrault et al., 1988; Regan and Gelber, 2007; Untch et al., 2008; Zhao and He, 1988), the major exception being ER-targeted therapeutics (Gibson et al., 2009; Group, 2001). For this reason, outcomes may be improved with combinatorial treatment of targeted therapies with one another or with chemotherapeutic agents. Fulvestrant, gefitinib, LY 294002 and dasatinib, a kinase inhibitor that targets the SFK proteins, have all shown promising results when combined with doxorubicin in ER+ breast cancer cell culture and/or xenograft studies, suggesting that these inhibitors may improve clinical outcomes when combined with chemotherapy (Pichot et al., 2009; Ren et al., 2008; Teixeira et al., 1995; Wang et al., 2008).

In contrast to the ER, SFK, PI3K, and EGFR, inhibition of MEK and MMP proteins in our study enhanced the cytotoxic effect of doxorubicin (Fig. 12), suggesting that these enzymes play important roles in MCF-7 cell resistance to doxorubicin. Work in sarcoma and rat neuronal cells showed that MMP-7 and MMP-3 inhibit doxorubicin action by negatively regulating Fas action; perhaps these family members function similarly in ER+ breast cancer, as well (Mitsiades et al., 2001; Wetzel et al., 2003). Previous studies utilizing mutant forms or inhibitors of MEK in hepatocellular carcinoma, leukemia, and human epidermoid carcinoma cell lines have found that MEK functions similarly as in MCF-7 cells (Brantley-Finley et al., 2003; Choi et al., 2008; McCubrey et al., 2008). However, in chemo-resistant pancreatic cancer cells, MEK1 protected against 5-fluorouracil but was critical for doxorubicin- and gemcitabine-induced apoptosis,

indicating that the function of MEK1 can vary by cancer and treatment (Zhao et al., 2006).

In summary, this study demonstrated the surprising finding that ER+ breast cancer cells responded to sub-apoptotic levels of doxorubicin (which may occur intratumorally) by enhancing estrogen-stimulated changes to phosphorylation and protein levels of a subset of estrogen signaling molecules. It also showed that the negative effects of doxorubicin alone correlated with suppression of the estrogen signaling pathway (Fig. 14). These data provide further rationale for targeting estrogen/ER effector proteins to provide better outcomes for ER+ patients receiving chemotherapy. Only a few clinical trials have been conducted that combined conventional chemotherapy with targeted therapy to estrogen signaling molecules in ER+ breast cancer patients, with the exception of HER2 and the canonical ER proteins (Buzdar, 2009; NCI, 2011b). Our study indicates that the addition of drugs targeting MEK, the ER proteins, EGFR, the SFKs, PI3K, and/or the MMP proteins to a conventional chemotherapy regimen may improve the survival of ER+ breast cancer patients.

Chapter 4. Results: The Role of the AR in Estrogen-Regulated Biological Processes of ER+ Breast Cancer Cells

Introduction

The AR has been shown to be critical for the proliferation and survival of androgen-responsive prostate cancers (Balk and Knudsen, 2008; Zhu and Kyprianou, 2008). Recently, *in vivo* AR silencing in a xenograft bladder tumor model as well as diethylnitrosamine-induced hepatocellular carcinoma in a liver-specific AR knock-out mouse model both demonstrated that loss of the AR resulted in development of less aggressive bladder and hepatocellular carcinomas compared to those of control tumors/mice expressing the AR. These results suggest that the AR can promote androgen-independent cancers, as well (Ma et al., 2008; Wu et al., 2010). Experiments in ER+ breast cancer cell lines have shown that an AR-inhibitory-drug or a peptide-inhibitor of AR association with c-Src reduces estrogen-mediated viability and DNA synthesis, respectively (Migliaccio et al., 2007; Yeh et al., 2003). As described in Chapter 3, prolonged treatment of ER+ MCF-7 breast cancer cells with estrogen, doxorubicin, or both agents upregulated protein levels of the AR. The AR consistently remained phosphorylated and associated with ER-α and c-Src throughout these treatments. These data suggested that the AR may also regulate the biological outcomes of ER+ breast cancer cells in the prolonged presence of these agents. In this chapter, experimental results that further test this hypothesis will be discussed.

Results

The Function of the AR in Proliferation and Survival

Based on the published studies described above and our biochemical observations on the AR's role in estrogen signaling (Ch. 3, Fig. 11), we hypothesized that the AR would be critical for the partial estrogen response that occurs in the presence of doxorubicin. To test this hypothesis, cells were treated with an AR-targeted drug (bicalutamide) or AR siRNA, cultured as in Chapter 3, and assayed for changes in cell number or cell death.

Bicalutamide is a common treatment for androgen-responsive prostate cancer and has been shown to reduce AR protein levels, androgen-induced phosphorylation of signaling molecules, and AR-mediated transcription in LNCaP and CWR22-R3 cells (prostate cancer cell lines), as well as in HeLa cells transfected with wild-type AR or AR containing the LNCaP mutation (Bai et al., 2005; Feng et al., 2009; Masiello et al., 2002; Unni et al., 2004; Waller et al., 2000; Wang et al., 1998; Yuan et al., 2006). Because of bicalutamide's described anti-androgenic activity on the AR and the pro-proliferative role of this receptor in androgen-responsive prostate cancer (Balk and Knudsen, 2008; Zhu and Kyprianou, 2008), we used this drug to determine if the AR's upregulation by estrogen and doxorubicin treatment corresponded to a function for it in estrogen and/or doxorubin-mediated biological action in ER+ breast cancer cells. Following hormone-starvation and treatment with DMSO (the bicalutamide solvent), 3 μM bicalutamide, 10 nM estrogen, and/or 25 nM doxorubicin, MCF-7 cells were counted. Results indicated that the bicalutamide significantly reduced estrogen-induced proliferation independently of doxorubicin (Fig. 15A, bars 2 vs. 4 and 6 vs. 8) and had no effect on doxorubicin action in the absence of estrogen (Fig. 15B, bars 5 vs. 7). To control for the efficacy of

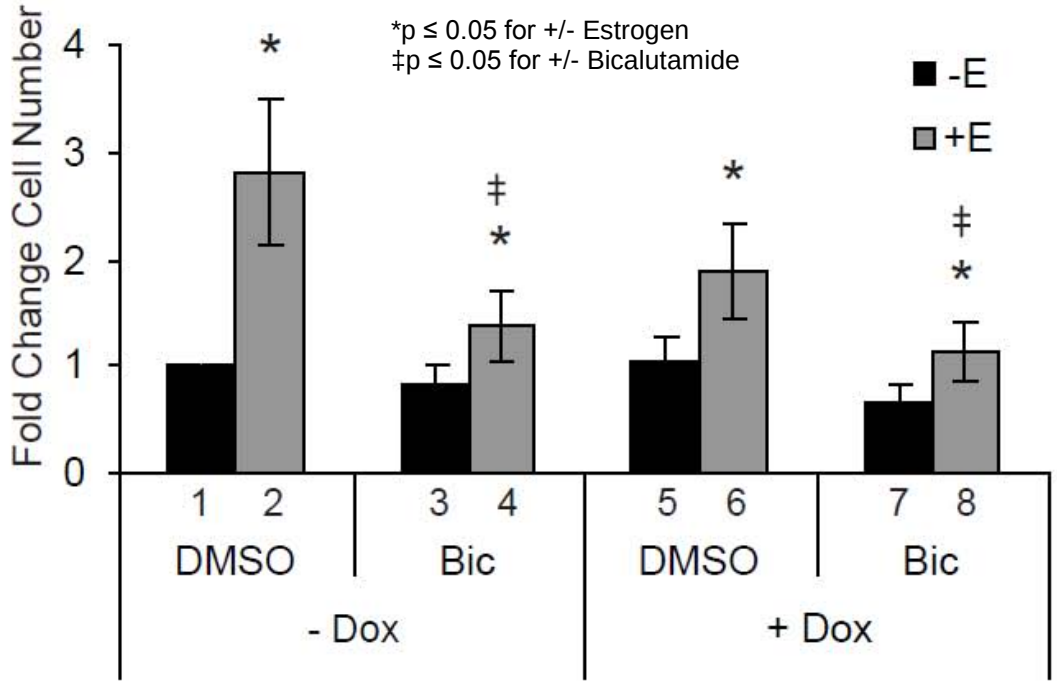
Figure 15. Bicalutamide indicates a role for the AR in the estrogen-induced proliferation of MCF-7 cells.

A. MCF-7 breast cancer cells treated with bicalutamide demonstrated inhibitor-dependent decreases in estrogen-induced proliferation in both the absence and presence of doxorubicin. MCF-7 cells treated with or without estrogen and/or doxorubicin (according to the protocol depicted in Figure 3) were also treated with DMSO or bicalutamide 1 hr prior to estrogen addition. DMSO or 3 μM bicalutamide remained present in the media until cells were counted. Graphs depict the results of four experiments as the average cell number ± SEM for each treatment group. All values are expressed as the fold change of the DMSO control (- Dox – E, bar 1), which was set to 1. Black bars represent groups not treated with estrogen, whereas grey bars indicate estrogen-stimulated groups. Significance was determined by log ANOVA. An asterisk indicates that the addition of estrogen has significantly altered ($p \leq 0.05$) a level from that of its non-estrogen treated counterpart (i.e. bars 1 vs. 2, 3 vs. 4, 5 vs. 6, and 7 vs. 8) and a double-dagger signifies a difference between groups treated with DMSO and bicalutamide (i.e. bars 2 vs. 4 and 6 vs. 8). B. Bicalutamide treatment downregulates the AR in LNCaP prostate cancer cells independently of the synthetic androgen, R1881. LNCaP cells were hormone-starved, cultured with 1 nM R1881 or vehicle, treated with 30 μM bicalutamide for the indicated times, lysed, and analyzed by Western blotting as described in Materials and Methods. Controls untreated with bicalutamide were also included (0 time point). Data are representative of two experiments. C. In contrast to LNCaP cells, bicalutamide increased AR levels and phosphorylation in the absence of

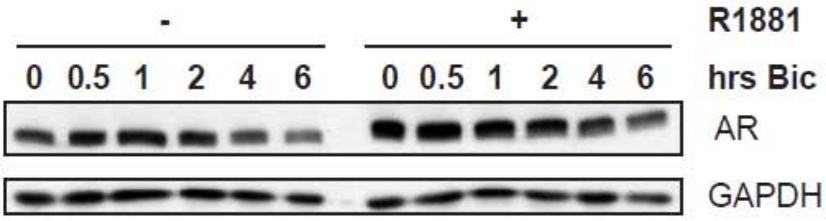
estrogen in MCF-7 cells and had no effect on these levels in the presence of estrogen. MCF-7 cells were cultured with or without estrogen and/or doxorubicin as described in Figure 3. One hour prior to estrogen addition vehicle or escalating doses of bicalutamide (0, 3, 30 μM) were added, and DMSO or bicalutamide remained present for all subsequent manipulations. Extracts were prepared and subjected to SDS-PAGE and immunoblotting as described in Materials and Methods. Results are representative of four independent experiments.

Fig. 15

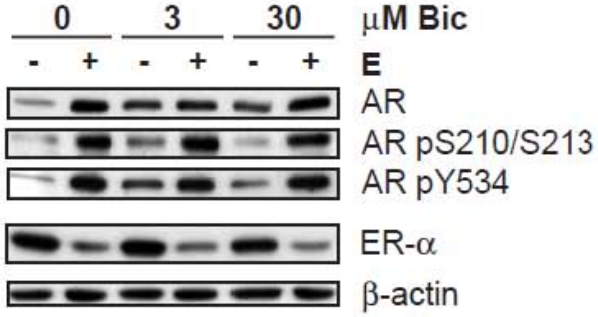
A. MCF-7



B. LNCaP



C. MCF-7



this drug used in the cell growth assay, we tested the bicalutamide in an androgen-responsive prostate cancer cell line. LNCaP cells were treated with 30 μM bicalutamide for up to 6 hrs in the presence or absence of R1881, a synthetic androgen that stabilizes the AR, and extracts were examined by Western blotting for effect of the drug on AR levels. Figure 15B demonstrates that bicalutamide treatment of LNCaP cells down-regulated AR levels independently of R1881 treatment, confirming that the bicalutamide functioned in LNCaP cells as the literature described. However, in contrast to the prostate cancer cell line, when bicalutamide-treated, hormone-starved MCF-7 cells (cultured as in the cell count assay) were lysed and analyzed by Western blotting, AR levels and phosphorylation were found to be elevated over those in bicalutamide untreated cells (DMSO controls) (Fig. 15C). Similarly, MCF-7 cells that also received estrogen showed an estrogen-induced increase in AR protein levels and phosphorylation that was unaffected by bicalutamide. These results were also confirmed in T47-D cells (data not shown), indicating that our observations were not breast cancer cell line-specific and that ER+ breast cancer cells respond differently to this drug than prostate cancer cells. As bicalutamide was unable to reduce expression or phosphorylation of the AR and its impact on AR-mediated transcription is unknown in MCF-7 cells, it was unclear whether the AR had a positive or negative role in estrogen-induced proliferation in the presence and absence of doxorubicin.

Because the requirement for the AR in estrogen-induced proliferation could not easily be determined from the use of bicalutamide, we employed a gene silencing strategy to reduce AR levels within MCF-7 cells. Cells were either mock-transfected or transfected with non-specific (NS) or AR siRNA oligomers just prior to seeding, hormone-starved,

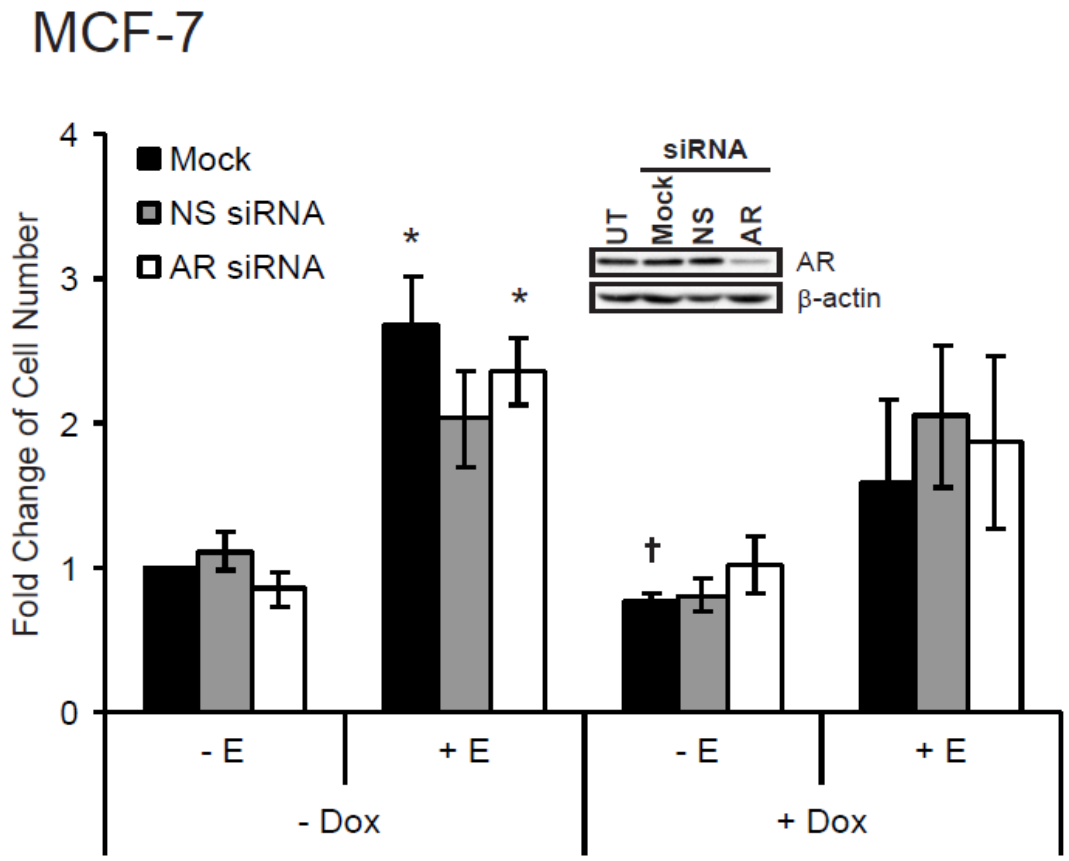
cultured with or without estrogen and/or doxorubicin, and counted manually to assess net cell growth. Figure 16 shows that AR silencing had no significant effect on the responses to estrogen or doxorubicin treatments when compared to mock or non-specific controls. Lysates analyzed by Western blotting demonstrated that AR siRNA reduced total AR levels about 90% from untreated, mock- and NS siRNA-transfected controls (Fig. 16, inset). These results suggest that the AR does not play a role in estrogen-induced proliferation or survival in MCF-7 cell treated with or without doxorubicin. These data contrast with the results of the bicalutamide cell growth experiment (Fig. 15B), implying that the reductions seen with bicalutamide were due to its off-target effects or that it completely inhibited some critical aspect of AR function that was not reduced sufficiently through AR silencing (see Discussion).

As AR levels were regulated by estrogen and doxorubicin in our studies, both of which influence survival, and compelling evidence that indicated that the AR protected from cell death in prostate, bladder, and hepatocellular carcinomas (Balk and Knudsen, 2008; Ma et al., 2008; Wu et al., 2010; Zhu and Kyprianou, 2008), we posited that the AR facilitated this biological response. To test this question, mock or AR siRNA transfected cells were analyzed for cell death by trypan blue assays. Initial experiments that were designed as illustrated in Figure 3 resulted in extremely low levels of cell death in all treatment groups and did not indicate a role for the AR in cell survival (data not shown). Concerned that remnant growth factors present in the 5% charcoal-stripped serum were protecting cells from death and masking the influence of the AR, experiments were repeated with 0.1% charcoal-stripped serum. Again, death was independent of the AR (data not shown). Having evidence that the AR was not necessary for survival even

Figure 16. AR silencing in MCF-7 cells had no effect on estrogen-induced proliferation in the absence or presence of doxorubicin.

MCF-7 cells were mock-transfected (black bars) or transfected with either a non-specific (NS) siRNA (grey bars) or AR siRNA (white bars), seeded into 6-well dishes, hormone-starved, and treated with estrogen and/or doxorubicin according to the scheme depicted in Figure 3. Cells were counted for net effects of the various treatments on growth. Results are expressed as the mean fold change relative to the untreated mock control, which was set to 1, ± SEM for each treatment group. Significance was determined by Student's t-test. n = 3; *p ≤ 0.05 comparing estrogen- and non-estrogen-treated counterparts; † p ≤ 0.05 comparing doxorubicin- and non-doxorubicin treated counterparts. The inset Western blot demonstrates specific knock-down with an AR siRNA. The UT group was an untransfected control.

Fig. 16


MCF-7
Mock
NS siRNA
AR siRNA
Fold Change of Cell Number
4
3
2
1
0
*
*
†
siRNA
UT
Mock
NS
AR
AR
β-actin
- E
+ E
- E
+ E
- Dox
+ Dox

in low concentrations of charcoal-stripped serum, we asked if it had a role in protection from cell death at a known apoptotic concentration of doxorubicin (2.5 μM) in the absence of serum. To test this question, mock or AR siRNA-transfected cells were serum-starved in phenol red-free medium and exposed to 0, 0.025, 0.25 or 2.5 μM doxorubicin for 48 hrs prior to a trypan blue assay. Figure 17 shows that cell death increased with higher concentrations of doxorubicin. Cells lacking the AR demonstrated a slight but statistically insignificant escalation in cell death, suggesting that the AR does not significantly influence cell survival within the conditions tested.

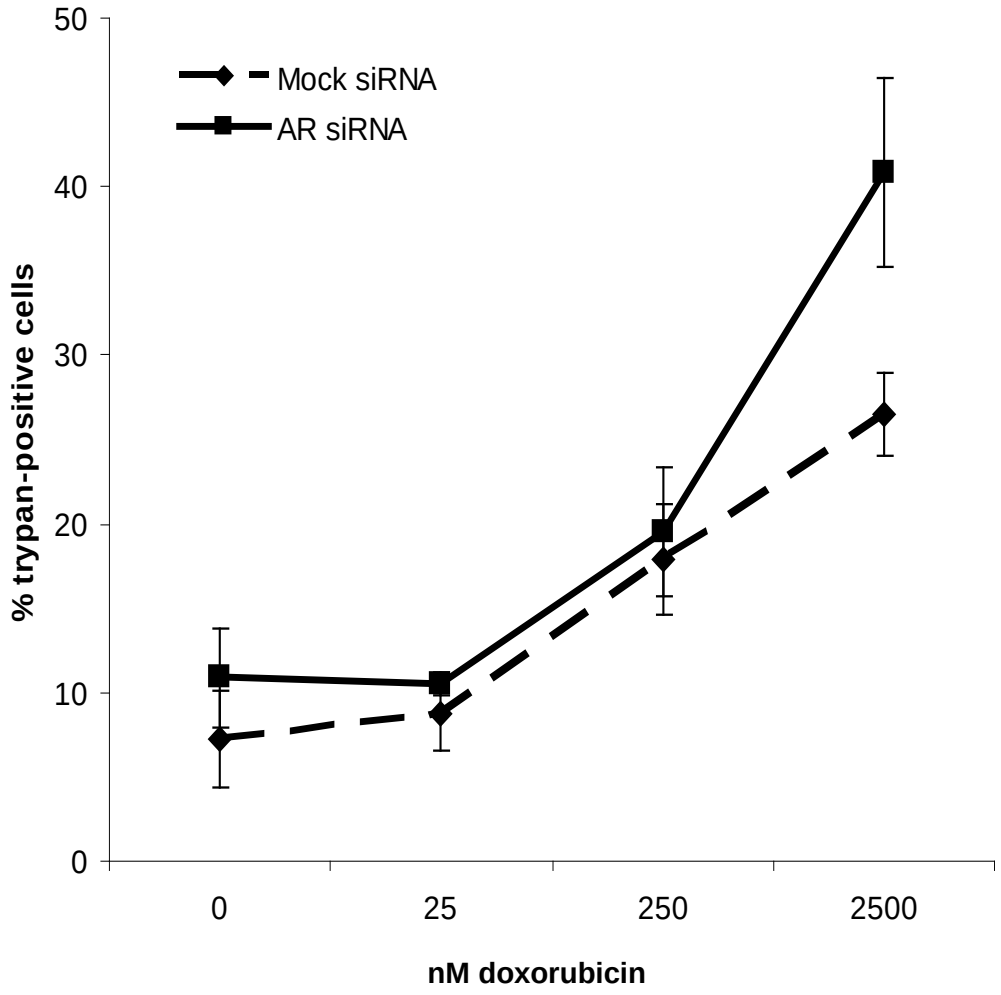
The Function of the AR in Migration and Invasion

Both estrogen and doxorubicin have been shown to independently modulate migration and invasion of cancer cells. Estrogen exposure at the time of assaying MCF-7 and T47-D cell migration towards fetal bovine serum (FBS) demonstrated an ER-dependent migration and invasion following a short stimulation with estrogen (Giretti et al., 2008; Malek et al., 2006). In contrast, doxorubicin concentrations in the nanomolar range have been shown to reduce migration and/or invasion of melanoma cells, sarcoma cells, and the ER-negative MDA-MB-231 breast cancer cell line (Fourre et al., 2008; Pichot et al., 2009; Repesh et al., 1993). Interestingly, 50 nM doxorubicin was reported to have no effect on the invasion of MCF-7 cells towards serum (Woodward et al., 2005). However, little has been done to test the effects of estrogen or doxorubicin on migration and invasion following more clinically relevant prolonged pre-treatments with these agents or to test them in combination with one another in an ER+ breast cancer line.

Figure 17. The AR has little, if any, effect on MCF-7 cell survival.

Cells were mock-transfected (solid lines) or transfected with AR siRNA (dashed lines), cultured in PRF SFM, exposed to increasing concentrations of doxorubicin (as indicated) for 48 hrs, and subjected to a trypan blue assay as described in Materials and Methods to assess cell survival. The graph depicts the average cell number ± SEM for each treatment group and represents three experiments. A slight increase in cell death was observed at the highest doxorubicin concentration with the AR siRNA, but it was not significantly different from mock treatment as determined by Student's t-test. The efficacy of the siRNA treatment was confirmed by Western blotting (as in Fig. 16).

Fig. 17

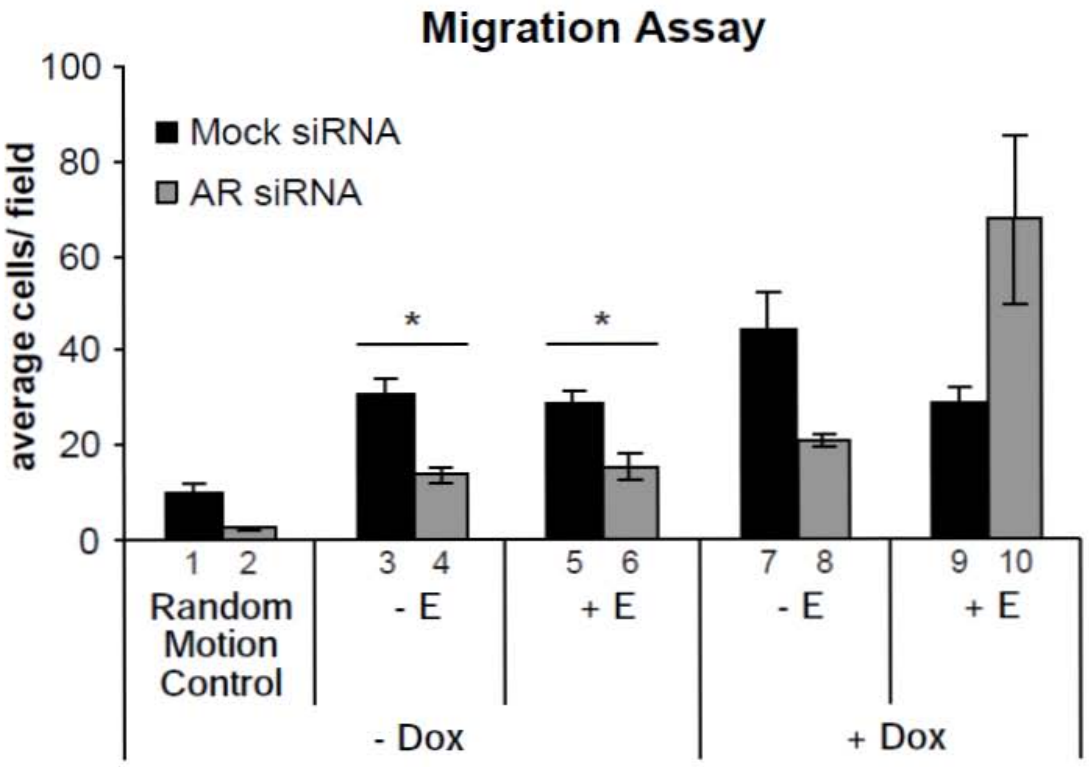

50
40
30
20
10
0
Mock siRNA
AR siRNA
% trypan-positive cells
0
25
250
2500
nM doxorubicin

The AR has been shown to mediate migration in bladder cancer and several prostate cancer cell lines (Kang et al., 2009; Wu et al., 2010). However, there are few studies in ER+ breast cancer cells on AR's participation in estrogen-induced migration. To address this question, as well as the effects of estrogen and doxorubicin individually and jointly on MCF-7 cell migration, cells were mock-transfected or transfected with AR siRNA, cultured as described in Figure 3, and assayed in a Boyden chamber for migration through pores in the membrane. Cells that migrated from the hormone-free, serum-free medium to growth medium containing 10% FBS were counted. Preliminary results shown in figure 18 demonstrate that neither estrogen nor doxorubicin alone or in combination significantly altered the migratory response of MCF-7 cells from untreated levels (compare bar 3 with bars 5, 7, and 9), results that contrast with the aforementioned reports using shorter treatments with either of these agents. Our findings suggest that prolonged exposures may reduce effects of estrogen and doxorubicin on migration. In contrast, the AR may modulate migratory motion. When the random motion of MCF-7 cells was measured, those in which the AR had been silenced showed a statistically-insignificant trend towards less chemokinetic motion (Fig. 18, bar 1 vs. 2). AR silencing significantly reduced migration in the untreated condition (Fig. 18, bar 3 vs. 4) and in the presence of estrogen (Fig. 18, bar 5 vs. 6), and also exhibited the same inhibitory trend with doxorubicin alone, though statistically insignificant (Fig. 18, bar 7 vs. 8). One explanation for the changes seen in the migratory behavior of AR-silenced cells treated with or without estrogen or doxorubicin may be the reduction in random migration seen with AR silencing. When cells were treated with both doxorubicin and estrogen, AR silencing showed enhanced migration (albeit statistically insignificant) (Fig. 18, bar 9 vs.

Figure 18. The AR has both pro- and anti-migratory functions in MCF-7 cells treated with doxorubicin.

MCF-7 cells were mock-transfected (black bars) or transfected with AR siRNA (grey bars) and treated as described in Figure 3. Cells were resuspended in PRF SFM and added to the upper chamber of Boyden trans-well plates. The bottom wells contained growth medium with serum for all but the random motion control, which contained PRF SFM. Following 6 hrs of incubation, cells were removed, membranes were fixed and stained, and migratory cells were counted. Results are expressed as the mean cells per field ± SEM for each treatment group. Significance was determined by Student's t-test. n = 3; *p ≤ 0.05 The efficacy of the siRNA treatment was confirmed by Western blotting (as in Fig. 16).

Fig. 18


Migration Assay
average cells/ field
100
80
60
40
20
0
Mock siRNA
AR siRNA
*
*
1 2
3 4
5 6
7 8
9 10
Random Motion Control
- E
+ E
- E
+ E
- Dox
+ Dox

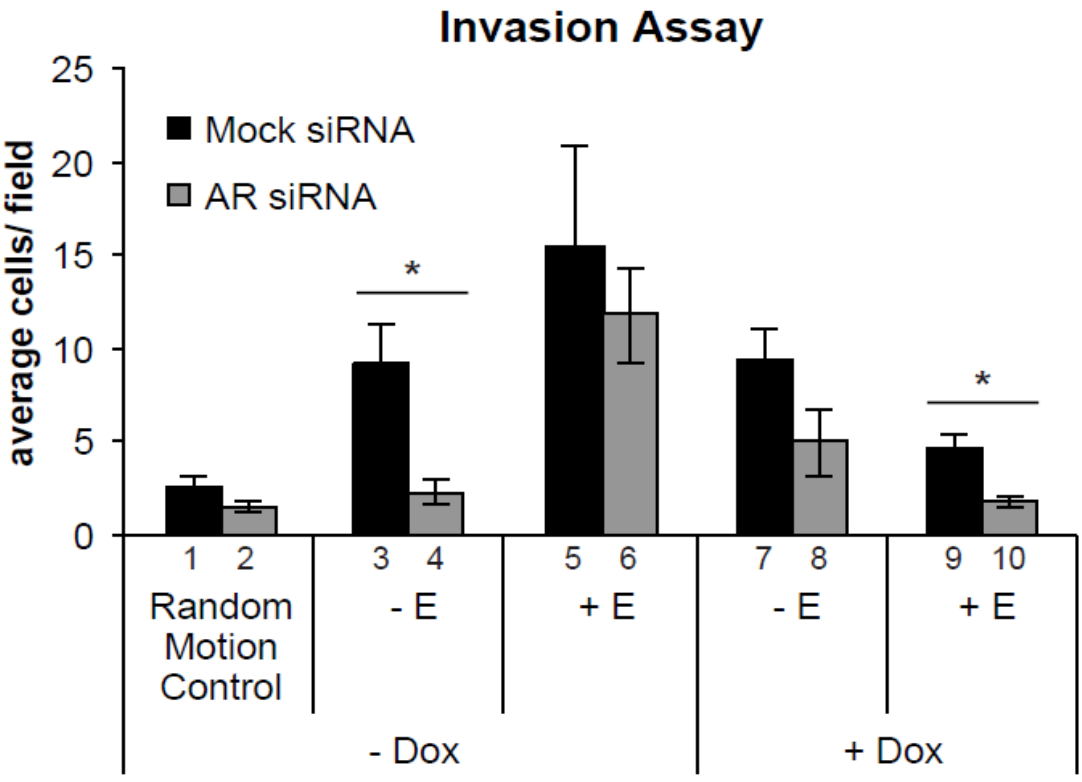
10), suggesting that the mechanism supporting migration in this condition may differ from that for basal, estrogen alone, or doxorubicin alone and that AR may play an anti-migratory role in this setting, though more experiments need to be done to verify this.

Hara and colleagues showed that the invasiveness of prostate cancer cells towards FBS directly correlated with AR expression and that invasion was AR-dependent (Hara et al., 2008). However, the role of the AR in ER+ breast cancer is poorly characterized. To study the effects of the AR, estrogen and/or doxorubicin treatment on invasive properties of MCF-7 cells, cells were prepared as described for the migration assay and allowed to invade through Matrigel towards serum. The preliminary results in Figure 19 indicate that neither prolonged estrogen nor doxorubicin treatment alone significantly altered invasion from the untreated control level (bar 3 vs. 5 and 7) but that their combination showed a trend towards reduced invasion from estrogen-alone levels (bar 5 vs. 9). These results imply that doxorubicin and estrogen may promote invasion by unique signaling pathways that interfere when activated by co-treatment, resulting in reduced invasion. Though AR silencing did not significantly alter random invasion (Fig. 19, bar 1 vs. 2), it reduced invasion in hormone-starved conditions (Fig. 19, bar 3 vs. 4) and showed a statistically-insignificant trend towards lower invasion with doxorubicin treatment (Fig. 19, bar 7 vs. 8), suggesting a potential pro-invasive role for the AR in these conditions. AR silencing appeared to have no effect on invasion in the presence of estrogen (Fig. 19, bar 5 vs. 6), but co-treatment with doxorubicin resulted in lower invasion in the AR-deficient group (Fig. 19, bar 9 vs. 10). These data suggest that AR may play a role in the invasive capacity of MCF-7 cells in the presence of both doxorubicin and estrogen, but this conclusion requires further investigation. Our findings also support a potential

Figure 19. The AR has a pro-invasive function in MCF-7 cells.

MCF-7 cells were mock-transfected (black bars) or transfected with AR siRNA (grey bars) and treated as described in Figure 3. Cells were resuspended in PRF SFM and added to the upper chamber of Matrigel trans-well plates. The bottom wells contained growth medium with serum for all but the random motion control, which contained PRF SFM. Following 24 hrs of incubation, cells were removed, membranes were fixed and stained, and invasive cells were counted. Results are expressed as the mean cells per field ± SEM for each treatment group. Significance was determined by Student's t-test. n = 3; *$p \leq 0.05$. The efficacy of the siRNA treatment was confirmed by Western blotting (as in Fig. 16).

Fig. 19


Invasion Assay
average cells/ field
25
20
15
10
5
0
Mock siRNA
AR siRNA
*
*
1 2
3 4
5 6
7 8
9 10
Random Motion Control
- E
+ E
- E
+ E
- Dox
+ Dox

mechanism for invasion in the presence of estrogen or doxorubicin that is distinct from the AR-dependent invasion mechanism of estrogen and doxorubicin co-treatment (Fig. 19, bar 6 vs. 10).

Summary and Discussion

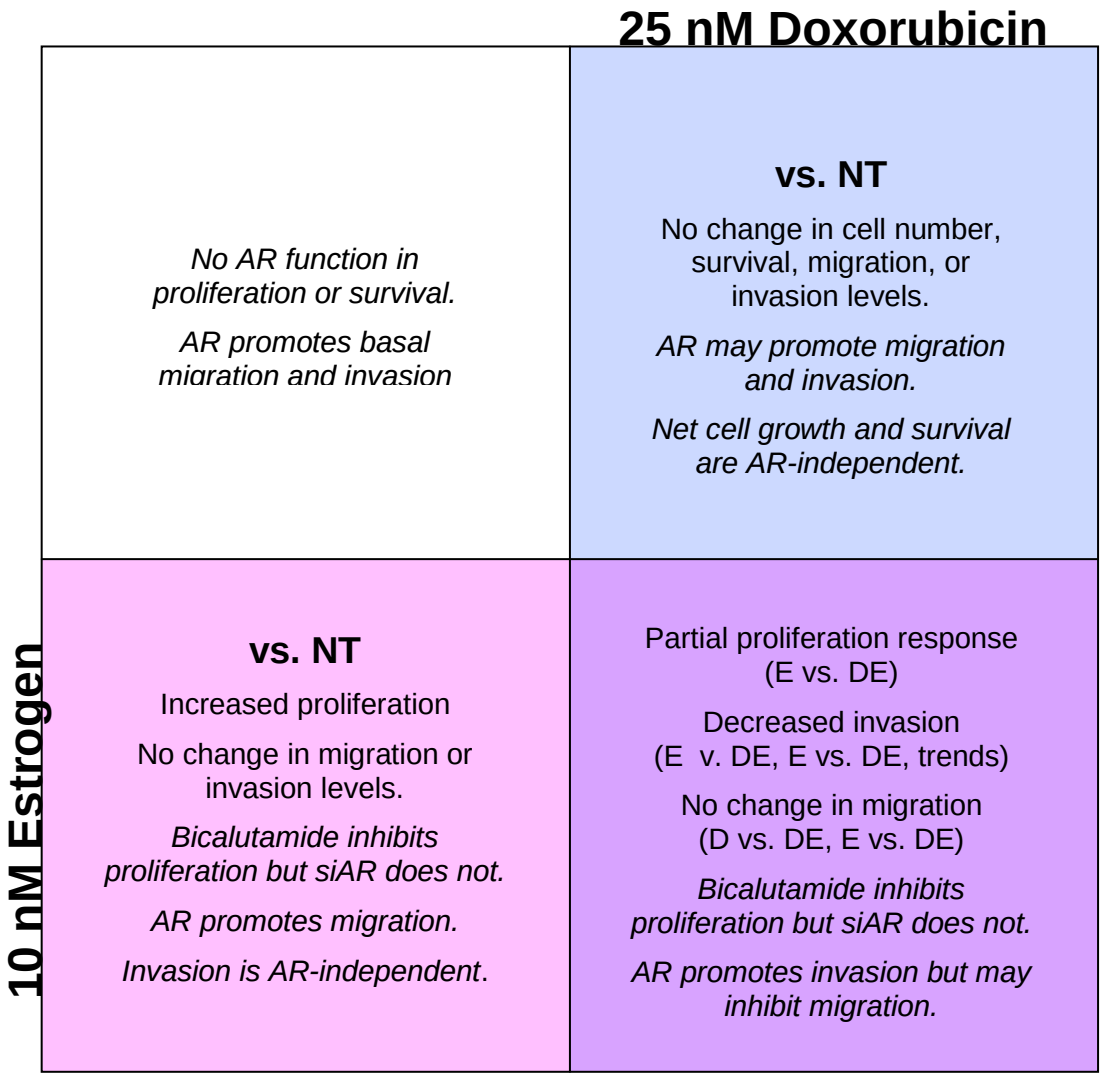
As summarized in Figure 20, the preliminary data described in this chapter suggest that the AR did little to mediate proliferation and survival but may play a larger role in modulating the migration and invasion of MCF-7 cells. In untreated conditions (- Dox - E), the inhibition or silencing of the AR had little effect on proliferation, survival, migration or invasion. Following prolonged estrogen treatment, the AR appeared to promote migration, potentially as a function of its support of random migratory motion, but not invasion. Its role in proliferation was less clear, as bicalutamide treatment reduced cell numbers, but AR silencing did not. Migration and invasion in the presence of 25 nM doxorubicin may also be dependent upon the AR, in contrast to survival and proliferation, which were AR-independent processes. Finally, the data suggest that AR promoted invasion upon co-treatment with estrogen and doxorubicin but inhibited migration. As with estrogen treatment alone, bicalutamide treatment but not AR silencing inhibited proliferation of cells co-treated with estrogen and doxorubicin.

Multiple lines of evidence show AR involvement in the proliferation and survival of several different cancers (Balk and Knudsen, 2008; Ma et al., 2008; Wu et al., 2010; Zhu and Kyprianou, 2008); however, to this point the involvement of the AR in estrogen- or doxorubicin-treated breast cancer cells has not been studied. Our results showed an increase in AR protein levels and sustained AR phosphorylation (Ch. 3) in an ER+ breast

Figure 20. Summary of the role of the AR in estrogen- and/or doxorubicin-induced biological responses.

This chart details the impact of the AR (mock vs. siAR, in italics) on proliferation, survival, migration, and invasion basally (white box), in response to 10 nM estrogen alone (pink box), in response to 25 nM doxorubicin alone (blue box), or as the result of co-treatment with estrogen and doxorubicin (purple box). The overall changes that occur between treatment groups are denoted in regular font (i.e. conclusions from mock or DMSO conditions). Conclusions are drawn from Figures 15-19.

Fig. 20


25 nM Doxorubicin
10 nM Estrogen
No AR function in proliferation or survival.
AR promotes basal migration and invasion
vs. NT
No change in cell number, survival, migration, or invasion levels.
AR may promote migration and invasion.
Net cell growth and survival are AR-independent.
vs. NT
Increased proliferation
No change in migration or invasion levels.
Bicalutamide inhibits proliferation but siAR does not.
AR promotes migration.
Invasion is AR-independent.
Partial proliferation response (E vs. DE)
Decreased invasion (E v. DE, E vs. DE, trends)
No change in migration (D vs. DE, E vs. DE)
Bicalutamide inhibits proliferation but siAR does not.
AR promotes invasion but may inhibit migration.

cancer cell line upon treatment with estrogen and/or doxorubicin, leading us to hypothesize that the upregulation of the AR had biological significance in proliferation and/or survival. Results in this chapter indicate that the AR does not have a clear role in proliferation or survival responses in MCF-7 cells treated with doxorubicin and/or estrogen. In contrast, silencing or inhibition of the AR appeared to have effects on both invasion and migration that varied with treatment.

Studies described in this chapter demonstrated different biological outcomes in cells treated with bicalutamide and AR siRNA (Fig. 15A v. 16). Several possible explanations can be offered to account for this divergence. First, the data suggest that the difference cannot be explained by bicalutamide action on AR levels and/or phosphorylation. Were bicalutamide-induced changes to AR protein and/or phosphorylation levels necessary for its biological activity, it would be expected that the drug's biological impact would correlate with the degree to which it altered protein levels/or phosphorylation. However, in MCF-7 cells the largest bicalutamide-induced changes (AR upregulation and phosphorylation) occurred in the absence of estrogen, where the drug had no effect on proliferation. Bicalutamide inhibited proliferation only when cells were in the presence of estrogen, and it failed to modulate AR protein or phosphorylation levels.

One reason for the dichotomy between the effects of bicalutamide and AR silencing may be potential off-target effects of either of these treatments. Bicalutamide has not been well characterized with regard to its impact on proteins other than the AR, especially in the context of breast cancer and estrogen-treatment. The changes seen with bicalutamide treatment may be due to the drug altering the levels, modifications, and/or activity of other proteins. Similarly, the oligonucleotides used for AR silencing could be

silencing other proteins in addition to the AR we observed no effects on c-Src or ER-α (data not shown).

Another mechanism that could explain the variation between bicalutamide and AR siRNA could be different impacts of these treatments on AR-mediated gene transcription. If AR-mediated transcription is necessary and sufficient for estrogen-induced proliferation, the near-total AR silencing achieved in our experiments might still permit low levels of gene expression by residual AR, while bicalutamide may completely inhibit AR-mediated transcription. In this situation, incomplete AR silencing by AR siRNA could permit proliferation, in contrast to bicalutamide.

Cancer is a complex biological state characterized by persistent proliferative signaling, evasion of growth suppressors, eluding death, immortality, angiogenic induction, invasion/metastasis, altered energy production, and evasion of the immune response (Hanahan and Weinberg, 2011). In ER+ breast cancer, clinical studies suggest that the biological processes that most contribute to patient outcome are estrogen regulated, as estrogen ablation therapy improves survival (Gibson et al., 2009). Our results show that estrogen enhanced the net proliferation of ER+ breast cancer cells (Figs. 15A and 16 and Ch. 3, Fig. 4), indicating that cell proliferation may be an important contributor to cancer-related deaths. Conversely, neither migration (Fig. 18) nor invasion (Fig. 19) demonstrated an estrogen response in our model, suggesting that in the absence of other biological responses (such as proliferation), cell motility may be insufficient to jeopardize patient survival. However, patient outcomes are linked to the suite of biological responses in a cancer cell (e.g. the cell must both proliferate and survive to increase tumor burden). Therefore, biological processes must be considered together for clinical

relevance, though it is beneficial to study them independently in the laboratory. So far, our preliminary results would support the conclusion that in the presence of estrogen alone (as would occur in the breast of an untreated cancer patient), the AR promotes migration (Fig. 18), but does not mediate invasion (Fig. 19), and has an undetermined role in proliferation (Figs. 15 and 16), suggesting that inhibition or loss of the AR could potentially reduce metastases but may be insufficient to improve patient survival apart from a treatment that decreased net proliferation. In the presence of estrogen and doxorubicin (as would occur if a patient was treated with anthracycline-based chemotherapy), the AR promotes invasion (Fig. 19), may inhibit migration (Fig. 18), and continues to have an unclear contribution to proliferation (Figs. 15 and 16). In this situation, adding an AR-inhibitory drug to chemotherapy, which reduces net proliferation, might be inadvisable, as the AR has opposing effects on migration and invasion, leaving this treatment with the potential to increase metastasis. Should this occur and the patient maintain low-level cancer cell proliferation or develop resistance to chemotherapy (by AR-independent mechanisms), we speculate that the growth of these metastases could reduce her life-span as compared to those who did not receive an AR-targeted therapy.

Our observations raise questions about the safety and efficacy of using a bicalutamide analog to treat ER+ breast cancers in the clinic. Though this is not a standard treatment, clinical trials of AR-targeted drugs have begun in ER+ patients (NCI, 2011b). Bicalutamide inhibition of estrogen-stimulated proliferation (Fig. 15A) suggests clinical benefit. However, as the compound upregulates the AR and sustains phosphorylation in ER+ breast cancer cells (Fig. 15C), it could also lead to unintended side-effects resulting

from AR-mediated protein-protein interactions and signaling. Moreover, if bicalutamide mimics AR silencing by inhibiting critical actions of the AR for regulation of migration and invasion, then this drug could release AR-mediated repression of migration that occurs with doxorubicin and estrogen co-treatment, even as it reduces invasion. The opposite effect of the AR on these two processes leaves unresolved the net effect of an AR inhibitor on metastasis. Given the results of this chapter, it is reasonable to suggest that any AR-targeted drug is thoroughly studied in the context of both estrogen and chemotherapy prior to its introduction into humans. The outcomes of current clinical trials should lend some insight into potential problems associated with such therapy.

Chapter 5. Summary and Perspectives

Summary

Breast cancer is the most frequently diagnosed form of cancer in women, and it is the second most fatal (NCI, 2011c). Of those women with breast cancer, the majority will present with ER+ tumors (Kocic et al., 2010), and are likely to be less responsive to chemotherapeutic treatments than their ER-negative counterparts (Berry et al., 2006; Bonilla et al., 2010; Conforti et al., 2007; Kuerer et al., 1999; Miles et al., 1999; Sertoli et al., 1995). It has been shown in laboratory ER+ breast cancer cell lines that estrogen is protective against chemotherapy's cytotoxic effects (Huang et al., 1997; Leung and Wang, 1999; Razandi et al., 2000; Teixeira et al., 1995; Vasconsuelo et al., 2008), suggesting that ability of estrogen and doxorubicin to antagonize one another's biological impacts may occur through perturbations of common proteins. This hypothesis is further supported by preclinical and clinical studies that have shown that inhibition or silencing of estrogen signaling molecules, such as ER, AR, EGFR, HER2, c-Src, MMPs, PI3K, and ERK, can sensitize cancer cells to chemotherapy (Alvaro et al., 2006; Araujo and Logothetis, 2010; Buchholz et al., 2005; Creighton et al., 2010; Gialeli et al., 2011; Hulboy et al., 1997; Lin et al., 2010; Murphy et al., 2004; Murphy et al., 2009; Pichot et al., 2009; Sirotnak et al., 2002; Teixeira et al., 1995; Wang et al., 2008; Yamashita et al., 2008). However, the biochemical impacts of doxorubicin on estrogen effectors has not been investigated as a potential mechanism for the biological antagonism between estrogen and doxorubicin. We hypothesized that components of the estrogen receptor signaling pathway may mediate the biological outcomes of doxorubicin in the presence or

absence of estrogen. The studies described in this dissertation have aimed to address the effects of doxorubicin on the estrogen/estrogen receptor signaling molecules, to identify components of the pathway that mediate doxorubicin signaling, and determine if estrogen effector molecules reduce sensitivity to doxorubicin in the context of ER+ breast cancer. The ultimate goal of these studies was to identify and recommend, through biochemical and biological analyses, estrogen signaling pathways that could be targeted therapeutically to enhance the actions of doxorubicin in ER+ breast cancer.

In the course of these studies, it became evident that the AR was upregulated 1.5-5.5 fold by several days of treatment with doxorubicin, estrogen, or their combination. This finding raised the question of whether the AR was necessary for biological outcomes resulting from these agents. To test this, the role of the AR in the proliferation, survival, migration, and invasion of ER+ breast cancer cells was characterized in response to estrogen and/or doxorubicin.

This chapter aims to highlight the major findings of this dissertation, to place them in the context of the clinic, and to raise questions stemming from our experimental results.

Perspectives and Future Directions

Estrogen signaling effectors are modulated by doxorubicin.

Work in Chapter 3 of this dissertation demonstrates that the protein levels and phosphorylation states of estrogen effector molecules are altered by doxorubicin treatment of the ER+ MCF-7 breast cancer cell line. Proteins that have been described in the literature as being involved in estrogen-induced signaling, such as ER-α, AR, EGFR, HER2, MNAR, and c-Src, were found in our model to be regulated by prolonged

estrogen treatment. Doxorubicin treatment alone modulated ER-α, AR, EGFR, and HER2 levels in ways opposite to that seen with estrogen treatment, and this correlated with cell cycle arrest in G1, suggesting that these protein changes in the presence of doxorubicin may be linked to biological outcomes. Co-treatment of MCF-7 cells with estrogen and doxorubicin showed the surprising result that rather than counteracting estrogen signaling, doxorubicin slightly enhanced the pathway, even as proliferation was reduced from estrogen alone levels and the proportion of cells in G1 increased. It is unclear whether the changes to estrogen effectors with co-treatment are due to direct effects of doxorubicin or are part of a compensatory mechanism by which the cell maintains proliferation despite the insult of chemotherapy. Overall, these doxorubicin-induced alterations to estrogen effector levels and phosphorylations suggest that the drug affects the activity of signaling pathways downstream of the ER and may be a way in which estrogen-responsive breast cancer cells respond to the otherwise detrimental effects of doxorubicin treatment.

Are estrogen effectors regulated by doxorubicin through altered gene expression, protein synthesis, and/or stability?

While it is evident from these data that doxorubicin perturbs estrogen effector proteins, the mechanisms by which these changes are achieved remain unresolved. Possible explanations for the increase in protein levels of ER-α, AR, EGFR, and HER2 in the presence of doxorubicin alone include an increase in gene expression, enhanced protein synthesis, or a reduction in the degradation of these proteins (or a combinations of these three mechanisms). Doxorubicin has been shown to inhibit both DNA and RNA

synthesis through its intercalation into double-stranded DNA (Barranco, 1984), suggesting that this drug would reduce, rather than enhance gene expression. Since this is only one of several mechanisms by which doxorubicin acts on the cell, it is possible that one or more of its other effects could enhance expression of these genes. Thus, it is important to test whether the increased levels of the aforementioned proteins in the presence of doxorubicin are the result of altered gene expression. Measurement of the mRNA transcript levels of the ER-α, AR, EGFR, and HER2 genes would allow evaluation of the effect of doxorubicin on gene expression. Approaches to do this would include the isolation of mRNA from cells cultured in the absence or presence of doxorubicin and comparison of their levels through Northern blotting or amplification by RT-PCR and quantification using fluorescent probes. Increased mRNA levels of one or more of these genes in the doxorubicin-treated group, as compared to the hormone-starved cells, would suggest that doxorubicin enhances protein levels through elevated gene expression.

Use of cycloheximide, a protein synthesis inhibitor, would aid in determining whether increased protein levels result from enhanced mRNA translation. In a time-course experiment, cycloheximide or its vehicle would be added to MCF-7 cells cultures with or without doxorubicin for 48 hrs (according to the schema depicted in Fig. 3), cells would be lysed in intervals for up to 24 hrs following cycloheximide addition, and lysates would be analyzed by Western blotting for protein levels. MCF-7 cells lysed at the 0 hr time point would serve as a control for basal protein levels, and the vehicle-treated cells would control for changes in protein levels unrelated to protein synthesis. A decrease in the net protein levels of doxorubicin-treated cells relative to hormone-starved cells would

indicate that mRNA translation may be a mechanism by which the cell increases ER-α, AR, EGFR, and/or HER2 protein levels in response to doxorubicin treatment alone. This would be further supported if mRNA transcripts of the genes encoding ER-α, AR, EGFR, and HER2 were higher in doxorubicin-treated than hormone-starved cells.

As mentioned above, another way in which doxorubicin could increase protein levels is through enhancing protein stability. Mechanisms involved in increasing protein stability include reduction of poly-ubiquitination and decreased proteasomal degradation of proteins in response to doxorubicin treatment, as compared to those that have not been treated with the chemotherapeutic agent. To test for differences in ubiquitination, cells treated with or without doxorubicin could be treated briefly with MG-132 (a proteasomal inhibitor) prior to lysis, and immunoprecipitated ER-α, AR, EGFR, and HER2 analyzed for ubiquitination by Western blotting. A decrease in the ubiquitination of proteins treated with doxorubicin from ubiquitin levels seen in the absence of doxorubicin would suggest that increased protein levels are achieved, at least in part, through the reduction of ubiquitin-mediated protein degradation. This reduction could occur through decreased inhibition of ubiquitinating proteins or increased activation of deubiquitinating enzymes. ER-α, AR, EGFR, and HER2 have been shown to be regulated by many ubiquitinating enzymes, but relatively few deubiquitinases. For example, UBPy targets EGFR and HER3 for deubiquitination (Niendorf et al., 2007) (and is a reasonable candidate for HER2), ER-α is a substrate of the deubiquitinase, OTUB1 (Stanisic et al., 2009), and AR is deubiquitinated by USP26 (Dirac and Bernards, 2010). While studying ubiquitinating enzymes in the context of doxorubicin treatment would be informative, our initial focus would be on the contribution of these deubiquitinating enzymes because of their

relatively small number, as compared to the ubiquitinases. In the case of reduced ubiquitination with doxorubicin treatment, gene silencing could determine if these deubiquitination enzymes are necessary for the drug to enhance the stability of ER-α, AR, EGFR, and HER2.

As described in this dissertation, co-treatment of MCF-7 cells with doxorubicin and estrogen alters the protein levels of several proteins from those levels seen with estrogen or doxorubicin alone, and these changes may be the result of changes to the proteins' stability or expression. In addition to employing the experiments described above as tests for the co-treatment condition, proteins that are downregulated with co-treatment, such as MNAR, could also be analyzed to see if their reduction is due to proteasomal degradation. MG-132 could again be used to test this possibility. A proteasomal mechanism of regulation of MNAR levels would be suggested if the MNAR protein levels in the MG-132-treated group remained stable or increased, as compared to the untreated control. Implication of a proteasomal mechanism to reduce protein levels would be expected to correlate with enhanced ubiquitination of the protein, which could be followed up with site-directed mutagenesis experiments to identify key lysines employed in its ubiquitination and regulation by doxorubicin and estrogen co-treatment.

<u>Do the cytoplasmic and/or nuclear actions of ER-α modulate doxorubicin-induced alterations?</u>

In addition to the transcriptional, translational, and degradative mechanisms possible for the doxorubicin-induced regulation of protein levels, it would be informative to study the pathway by which ER-α regulates the changes in protein levels, phosphorylations,

and biological outcomes so as to gain a better understanding of the role of the ER in mediating doxorubicin effects. As detailed in the introduction to this dissertation (Ch. 1), the ER can participate in both transcriptional and cytoplasmic signaling activities upon stimulation with estrogen; one or both of these functions of the ER may occur upon doxorubicin treatment, though this has not been tested directly. One way to address the relative contribution of ER's cytoplasmic and transcriptional actions on changes to protein levels and phosphorylations of other estrogen signaling proteins in response to doxorubicin treatment would be through the use of ER mutants that are restricted to either the nuclear or cytoplasmic compartments. A recent report by Burns and colleagues described two human ER-α mutants, one with a mutated nuclear localization sequence (NLS, A.A. 260-279) and another with a mutated nuclear export sequence (NES, R276/279L) that were constitutively localized to the cytoplasm or nucleus, respectively (Burns et al., 2011). In contrast to the cytoplasmically-localized mutant, the nuclearly-localized mutant was transcriptionally responsive to estrogen and supported expression from both ERE and non-classically regulated, estrogen-responsive genes (such as those regulated through ER interaction with Sp1 or AP-1). The cytoplasmically-localized mutant was able to support rapid ERK phosphorylation in response to estrogen to the same degree as the wild-type ER-α, indicating its participation in active signaling. (This was not tested for the nuclearly-localized mutant). However, one caveat to using these constructs is that transcription resulting from the phosphorylation of transcription factors downstream of ER-α's signaling could still take place with the cytoplasmically-localized mutant. Nonetheless, these mutants restricted to the cytoplasmic or nuclear

compartments would allow for the delineation of biochemical changes resulting from ER-α participation in nuclear transcription complexes and ER-α cytoplasmic signaling.

The effects of the nuclearly- and cytoplasmically-localized ER mutants could be tested in human, ER+ MCF-7 breast cancer cells through the silencing of endogenous ER-α and reconstitution with silencing-resistant mutant ER-α homologs. The NLS and NES are conserved between the mouse and human ER-α homologs, and though the mouse and human forms are highly conserved, there are variations in the N- and C-termini that may be exploited to create a silencing-resistant murine Esr1 mutant (as determined through protein sequence comparison of human ER-α and its murine homolog, Esr1). These Esr1 mutants would require verification of proper localization as well as appropriate transcriptional and cytoplasmic signaling activities (as described for the human ER-α mutants in the report by Burns and colleagues and summarized above). In experiments testing the effect of ER-α localization on doxorubicin-induced outcomes, MCF-7 cells in which endogenous ER-α was silenced would be transiently transfected with wild-type Esr1, a cytoplasmically-localized Esr1 mutant, a nuclearly-localized Esr1 mutant, or vector control, treated with or without estrogen and/or doxorubicin as previously described, and either lysed for biochemical analysis, counted for cell growth assays, or subjected to cell cycle analysis. It would be expected for the cytoplasmically-localized mutant to facilitate much of the phosphorylation seen with co-treatment of estrogen and doxorubicin. Apart from this, we conjecture that both ER-α's cytoplasmic and nuclear actions participate in the other doxorubicin-induced biochemical and biological outcomes.

Some estrogen signaling effectors reduce sensitivity to doxorubicin.

Results in this dissertation suggest that the estrogen effectors, MEK1/2 and MMP(s), suppress the cytotoxic effects of doxorubicin when in the presence of estrogen. Data indicate that treatment of MCF-7 cells with MEK or pan-MMP inhibitors suppresses estrogen-induced proliferation in a doxorubicin-dependent manner. MEK- or MMP-induced suppression of doxorubicin's effects could allow proliferation and survival of ER+ breast cancer in presence of doxorubicin, and this effect suggests that inhibition of these molecules has the potential to improve patient outcomes when administered along with anthracycline-based chemotherapies.

Which MMP proteins protect against doxorubicin?

GM6001 is the broad-range MMP inhibitor that was used in the cell growth assays described in Chapter 3. Because of its lack of specificity, the MMP(s) involved in protection from doxorubicin has/have not been defined by these experiments. Additionally, though MMP expression profiles have been defined in breast cancer patients and studied in response to estrogen in ER+ breast cell lines (see Ch. 1), MMPs that protect against chemotherapeutic agents have not been defined in ER+ breast cancer. However, MMP-3 and MMP-7 have been described as protective against doxorubicin in Ewing' sarcoma and rat cerebral cortical cultures (Mitsiades et al., 1999; Mitsiades et al., 2001; Wetzel et al., 2003) and are good candidates to test in our MCF-7 model. In-gel zymography assays (which measure MMPs' enzymatic activity) following culturing of cells in the presence or absence of doxorubicin and/or estrogen would reveal whether

these proteins are activated by doxorubicin. Additionally, MMP-3 or -7 could be silenced or overexpressed during treatment with doxorubicin and/or estrogen and cells analyzed in the cell growth assay. One would expect if these MMPs are involved in the suppression of doxorubicin's cytotoxic effects that silencing would reduce cell numbers in the presence of doxorubicin, whereas overexpression would provide additional protection and maintain cell numbers at levels comparable to the groups not treated with doxorubicin. Alternatively, protection by these MMPs would be demonstrated if cells in which they were overexpressed proliferated to a greater extent than their vector-control counterparts when in the presence of estrogen and challenged with a range of doxorubicin concentrations. By better defining the MMPs mediating protection from doxorubicin, it is possible that the more selective MMP inhibitors currently in development could be used to improve outcomes by targeting only those proteins shown to subvert chemotherapeutic responses in patients with ER+ breast cancer.

Are MEK and MMP inhibitors effective sensitizers to doxorubicin *in vivo*?

Another key question raised by the results of the MEK and MMP inhibitor studies is whether these drugs could be used individually or in combination to improve sensitivity to doxorubicin *in vivo*. The hypothesis that these would improve ER+ breast tumor responses could be tested in a xenograft mouse model. Athymic, ovariectomized, female nude mice into which estrogen pellets had been implanted could be orthotopically injected with MCF-7 cells into mammary fat pad and, following tumor establishment, treated with a MEK inhibitor, MMP inhibitor, or vehicle in the presence or absence of doxorubicin. Through regular caliper measurements of the tumor size over time as well

as tumor size and weight upon sacrifice, results would reveal if these inhibitors reduced tumor burden alone or improved doxorubicin sensitivity, depending on their treatment group. A similar trial testing the combination of MMP and MEK inhibitors in the presence or absence of doxorubicin would also be interesting and clinically relevant. Additionally, this *in vivo* methodology could be used to evaluate the efficacy of combining doxorubicin treatment with inhibitors of the ERs, SFKs, PI3K, and EGFR. Though our *in vitro* results did indicate inhibition of these proteins cooperated with doxorubicin, their targeting reduced net cell proliferation and these data provides rationale for their combination with doxorubicin *in vivo*.

The AR mediates the motility of MCF-7 cells.

Work detailed in this dissertation indicated that though the AR lacks a clear role in the survival or proliferation of MCF-7 cells, it appears to regulate their migration and invasion. As migration and invasion support metastasis, these data suggest that the AR may contribute less to the primary ER+ breast tumor burden and more to its invasion and metastasis. Through preliminary AR silencing experiments, we demonstrated that the AR may facilitate migration and invasion following hormone-starvation or doxorubicin-treatment. Results showed that the AR participated in MCF-7 cell migration in the presence of estrogen, in contrast to what was seen with invasion, which was AR-independent. When co-treated with estrogen and doxorubicin, the AR promoted invasion but may have suppressed migration, raising questions as to the net contribution of the AR in the metastatic process and the safety of AR-targeted drugs in the clinical treatment of ER+ breast cancer, particularly when administered in conjunction with doxorubicin.

What effect does the opposing regulation of migration and invasion by the AR have on metastasis?

When MCF-7 cells were co-treated with estrogen and doxorubicin, the AR appeared to be pro-invasion but anti-migratory. Determining the net effect of the AR on metastasis, which is facilitated by both these processes, is important as it could mean the difference between an effective AR-targeted therapeutic for the prevention of metastases or a detrimental one that reduces patient survival. To this end, I propose testing the effect of AR silencing on the metastasis of ER+ breast cancer *in vivo*.

Experience has taught us that ER+ breast cancer cell lines are poorly metastatic in xenograft models; however, that metastasis occurs in ER+ breast cancer patients suggests that estrogen-sensitive cancer cells are capable of metastasis. Harrell and colleagues have described a MCF-7 cell line stably expressing a coral reef fluorescence protein that allows for the sensitive detection of metastasis (through fluorescent, whole-body imaging) when injected into the mammary glands of ovariectomized, athymic female nude mice implanted with estrogen pellets (Harrell et al., 2006). Though distant metastases to the lungs are rare in this model, micro-metastases in the lymph nodes and lymphatic vessels are detectable shortly following implantation. These cells provide a valuable tool in which the contribution of the AR to tumor dissemination could be assessed.

In order to test whether AR inhibition at the time of doxorubicin administration (as would occur in the clinic if an AR-targeted drug were used to supplement chemotherapy treatment) would increase or decrease tumor metastases, it would be advantageous to

establish an inducible model of AR-silencing in the fluorescent MCF-7 cells. AR expression could be regulated in these cells through the stable integration of a Tet-on plasmid expressing a shRNA targeting the AR (or a non-specific sequence as a control). Following a short period to establish tumors, mice would be treated with tetracycline (to induce silencing of the AR) and doxorubicin or vehicle. Whole body scans taken at frequent intervals and inspection upon sacrifice would allow for the quantitation of tumor dissemination. Within the doxorubicin-treated and -untreated groups, comparison of the mice bearing AR-expressing and AR-silenced tumors would indicate whether the net effect of the AR is pro- or anti-metastatic and would suggest whether targeting this protein clinically would be of therapeutic benefit.

Is AR-mediated gene transcription necessary for the regulation the motility of MCF-7 cells?

The AR has been shown to mediate the migration and invasion of prostate cancer cell lines in response to androgen stimulation (Castoria et al., 2011; Chuan et al., 2006; Hara et al., 2008; Kang et al., 2009), but this has not been reported to occur in ER+ breast cancer cell lines or for these processes to be AR-regulated in response to estrogen and doxorubicin. As such, many questions remain concerning the mechanism by which the AR regulates these processes in MCF-7 cells. One mechanism in prostate cancer cells that may take place in MCF-7 cells is AR-mediated transcriptional regulation of pro-migratory and pro-invasive molecules, such as transmembrane 4 L six family member 1 (TM4SF-1) (Allioli et al., 2011), trefoil factor 3 (TFF3) (Rickman et al., 2010), Ets variant gene 1 (ETV1) (Chuan et al., 2006), and MMP-2 (Liao et al., 2003).

Transcriptional regulation of these genes in MCF-7 cells could be tested through the use of endogenous AR silencing (and a NS control) and reconstitution with a silencing-resistant murine wild-type AR, an AR DBD mutant that cannot activate gene transcription, or a vector control. (As with the ER, human and murine ARs are highly homologous but contain regions of dissimilarity.) In these experiments, transfected cells would be treated as described in Figure 3 and subjected to migration or invasion assays. It would be expected that the AR-silenced group without reconstitution would exhibit migration and invasion results comparable to the "AR siRNA" groups in Figures 18 and 19 and that the cells treated with NS siRNA and the AR vector control would serve as the baseline control (comparable to mock treatments in Figs. 18 and 19). Reconstitution with murine, wild-type AR would be expected to rescue the effect seen with AR silencing. The inability of the reconstituted AR DBD mutant to rescue the effects of silencing on migration and/or invasion would implicate a transcriptional mechanism of the AR in this/these biological process(es), whereas restoration of migration and invasion to levels seen with the endogenous receptor would suggest the opposite conclusion. These findings could also be correlated with mRNA levels of TM4SF-1, TFF3, ETV-1, and MMP2. Prostate cancer cells do not express the aforementioned genes in charcoal-stripped serum but do transcribe them when stimulated with androgen, indicating that AR-mediated gene expression of these motility-promoting proteins can be altered by treatment conditions. In the absence of androgen but presence of estrogen and/or doxorubicin, it is possible that the AR could participate in (a) non-classical transcriptional mechanism(s) that would affect motility to varying degrees in MCF-7 cells, as well.

Does the AR interaction with filamin A regulate motility?

Few cytoplasmic interactions of the AR have been shown to regulate motility. However, a recent report by Castoria and colleagues described a cytoplasmic mechanism by which the AR associates with and regulates the activity of several proteins involved in adhesion and motility (Castoria et al., 2011). This study demonstrated that androgen-induced AR interaction with filamin A (FlnA) and integrin-β1 correlated with the phosphorylation of focal adhesion kinase (FAK) and paxillin, as well as affirmed that R1881-induced migration required the AR, suggesting that the pro-migratory abilities of the AR may be linked to its interaction with FlnA and integrin-β1. A previous study demonstrating that FlnA binds to the AR and negatively regulates its transcriptional activation additionally described an AR mutant incapable of binding FlnA (Loy et al., 2003). Silencing and reconstitution experiments similar to those outlined in the previous section could exploit this interaction mutant to determine if interaction with filamin A (either at the membrane or in the nucleus) facilitates AR's effects on migration and/or invasion. Failure of a silencing-resistant version of this mutant to rescue loss of migration or invasion due to endogenous AR silencing would indicate that the association of the AR and FlnA is necessary for full migration or invasion. Should this be the case, Western blotting of lysates for FAK and paxillin phosphorylation would indicate if the same mechanism described downstream of the interaction in prostate cancer cells also occurs in MCF-7 cells. Much like AR-mediated transcription, different culture conditions may invoke this mechanism to varying extents or not at all in the regulation of motility.

Clinical Implications and Concluding Remarks

Current guidelines for the adjuvant treatment of breast cancer rely heavily on chemotherapy, tamoxifen for HR+ tumors, and/or trastuzumab for HER2+ cancers. The most common chemotherapeutic regimen is co-treatment with an anthracycline, such as doxorubicin or epirubicin, and capecitabine, followed by paclitaxel; it is recommended that chemotherapeutic treatment commence within 4-6 weeks following surgical resection. Within the United States, the convention is to treat lymph node-positive, HR+ patients with both chemo- and endocrine therapies, though internationally, patients tend to receive endocrine therapy alone. The treatment of node-negative, HR+ patients is quite controversial and physicians may treat only those with a high risk of recurrence with both therapies (Burnstein, 2011b). Because two large clinical trials have shown that disease-free and overall survival are superior when tamoxifen is given serially rather than concurrently with chemotherapy, tamoxifen is typically administered alone following chemotherapy and radiation. However, there is little available data or consensus on treatment with AIs or fulvestrant with regard to their timing with chemotherapy or the optimal duration of their administration. Because of the precedence with tamoxifen, clinical practice often substitutes AIs or fulvestrant for tamoxifen in protocols for adjuvant treatment with chemotherapy (Pritchard, 2011).

The treatment of HER2+ breast tumors typically involves both chemotherapy and trastuzumab. The standard of care for these patients is simultaneous treatment with an anthracycline (typically doxorubicin) and capecitabine, followed by co-treatment with paclitaxel and trastuzumab (Burnstein, 2011a). It is unresolved in the literature whether trastuzumab should be given concurrently with or following paclitaxel treatment;

however, the standard practice is to administer these concurrently and then to continue trastuzumab for a total of one year. Trastuzumab is also approved for combination with docetaxel and carboplatin, but the efficacy of these compared to paclitaxel is unresolved and the addition of other chemotherapeutic agents to paclitaxel has not demonstrated additional benefit (Burnstein, 2011a; Hayes, 2011). In contrast, concurrent treatment with anthracycline-based chemotherapy and trastuzumab has been shown to increase the risk of cardiomyopathy and is contraindicated. There are several ongoing trials in HER2+ breast cancer examining the safety or efficacy of trastuzumab in various forms of breast cancer, in tumors of variable HER2 expression, in its timing of administration (e.g. pre- or post-surgery), in comparison to or combination with other HER2-targeted inhibitors, and in combination with different chemotherapeutic agents. Most trials adhere to the convention of trastuzumab concurrent administration with paclitaxel, following treatment with a cocktail of capecitabine and doxorubicin or epirubicin. The few that are testing this targeted therapy in combination with other chemotherapeutic agents tend to maintain trastuzumab co-treatment with paclitaxel and vary the chemotherapeutic drugs given before or after (NCI, 2011b). In addition to trastuzumab, the dual EGFR/HER2 inhibitor, lapitinib, is also approved for use with capecitabine in HER2+ metastatic or advanced breast cancer (Lin et al., 2010; Moon et al., 2010) and has been combined with letrozole and capecitabine or trastuzumab for the treatment of HER2+ breast cancer that has progressed on trastuzumab alone (Hayes, 2011).

The data discussed in this dissertation provide rationale for the use of estrogen effectors as clinical targets. However, outside of endocrine therapies, trastuzumab, and

lapatinib, few therapeutics targeting this pathway are used in the clinic, and none are routinely combined with anthracycline-based therapy. From our inhibitor studies, we conclude that MEK and MMP inhibitors given concurrently with doxorubicin may improve the cytotoxic responses of ER+ breast cancers and potentially improve patient outcomes. Though our data showed that a MEK inhibitor was able to partially suppress the estrogen-induced proliferation of an ER+ breast cancer cell line, it was in combination with doxorubicin that the inhibition of estrogen-induced growth was greatest. The inability of a pan-MMP inhibitor to reduce estrogen-induced proliferation in ER+ breast cancer cells supports clinical observations that MMP inhibitors have poor efficacy as monotherapies. Despite this, when combined with doxorubicin, the MMP inhibitor was able to significantly suppress basal and estrogen-induced proliferation, suggesting that MMP inhibitors may be most useful when combined with anthracycline-based therapies. However, there are no current trials that include ER+ breast cancer patients on the combination of doxorubicin with MEK or MMP inhibitors (NCI, 2011b).

As the ER, EGFR, SFK, and PI3K inhibitors tested in this study demonstrated robust inhibition of cell numbers both in the absence and presence of estrogen and independently of doxorubicin, it is my recommendation that these be given as monotherapies or serially with chemotherapy, as opposed to concurrently with doxorubicin. Indeed, this is the current practice both in the majority of clinical trials and for the approved endocrine therapies used in the clinic. The rationale for this is three-fold: the combination of doxorubicin and these inhibitors did not confer any additional benefit over the inhibitors alone, the temporal separation of a targeted inhibitor from chemotherapy may lessen toxicity (as compared to co-treatment), and chemotherapy is

most effective against actively dividing cells but the ER, EGFR, SFK, and PI3K inhibitors suppressed cell growth. A phase III trial on 211 breast cancer patients compared the anthracycline and doxorubicin-derivative, epirubicin, alone to its combination with tamoxifen during administration, followed by a five-year course of tamoxifen for both groups. The tumors of the ER+ cohort receiving tamoxifen at the time of epirubicin administration had significantly lower Ki67 scores (indicating less cell proliferation), though neither ER+ nor ER- patients demonstrated any difference in clinical responses between the two treatment groups (Bottini et al., 2005). In addition, a stage II trial in HR+ breast cancer demonstrated no difference in the disease-free survival of patients treated with tamoxifen alone for two years and those treated with tamoxifen and a low-dose cocktail of doxorubicin, vincristine, cyclophosphamide, methotrexate, and fluorouracil (Jakesz et al., 1999), suggesting that the concurrent treatment of ER+ tumors with ER-targeted drugs and anthracycline-based chemotherapy may be an ineffective strategy. Two trials are currently underway in metastatic breast cancer that are testing the safety and efficacy of the concurrent administration of doxorubicin and lapatinib, an EGFR/HER2 dual kinase inhibitor. Unfortunately, neither trial is stratifying by estrogen receptor status (NCI, 2011b), limiting its use as a test of my hypothesis. There are no current trials testing doxorubicin-based chemotherapy in combination with other ER, EGFR, SFK, and PI3K inhibitors (either serially or concurrently) (Bagnoli et al., 2010; NCI, 2011b).

In summary, this dissertation has characterized the interplay of estrogen and doxorubicin in an ER+ breast cancer model through biochemical and biological assays. These studies have demonstrated that doxorubicin perturbs estrogen signaling molecules

and that some of these proteins act to suppress the effects of doxorubicin's cytotoxic effects on the cell when in the presence of estrogen. We have also shown that the AR may mediate migration and invasion of MCF-7 cells, though it does not appear to regulate proliferation or survival. The mechanisms by which these biochemical changes, protection from doxorubicin and AR-mediated motility, are achieved have yet to be defined. Additionally, these data suggest that MEK and MMP inhibition may improve the efficacy of anthracycline-based chemotherapy and raise questions regarding the value of AR inhibition, highlighting the importance of and need for further studies on doxorubicin in estrogen-responsive breast cancer. Based on our findings, we additionally recommend that inhibitors of the ER, EGFR, SFKs, and PI3K may be most useful in the treatment of ER+ breast cancer as monotherapies or when given serially with anthracycline-based chemotherapy. Together, the results of this study suggest that the mechanism(s) by which estrogen and doxorubicin antagonize one another biologically in ER+ breast cancer may be through changes to estrogen signaling molecules. These proteins had not previously been defined in relationship to doxorubicin treatment and our studies provide additional rationale for their clinical inhibition in conjunction with doxorubicin treatment, with the possible exception of the AR.

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Addendum. Cox II interaction with the EGFR

Introduction

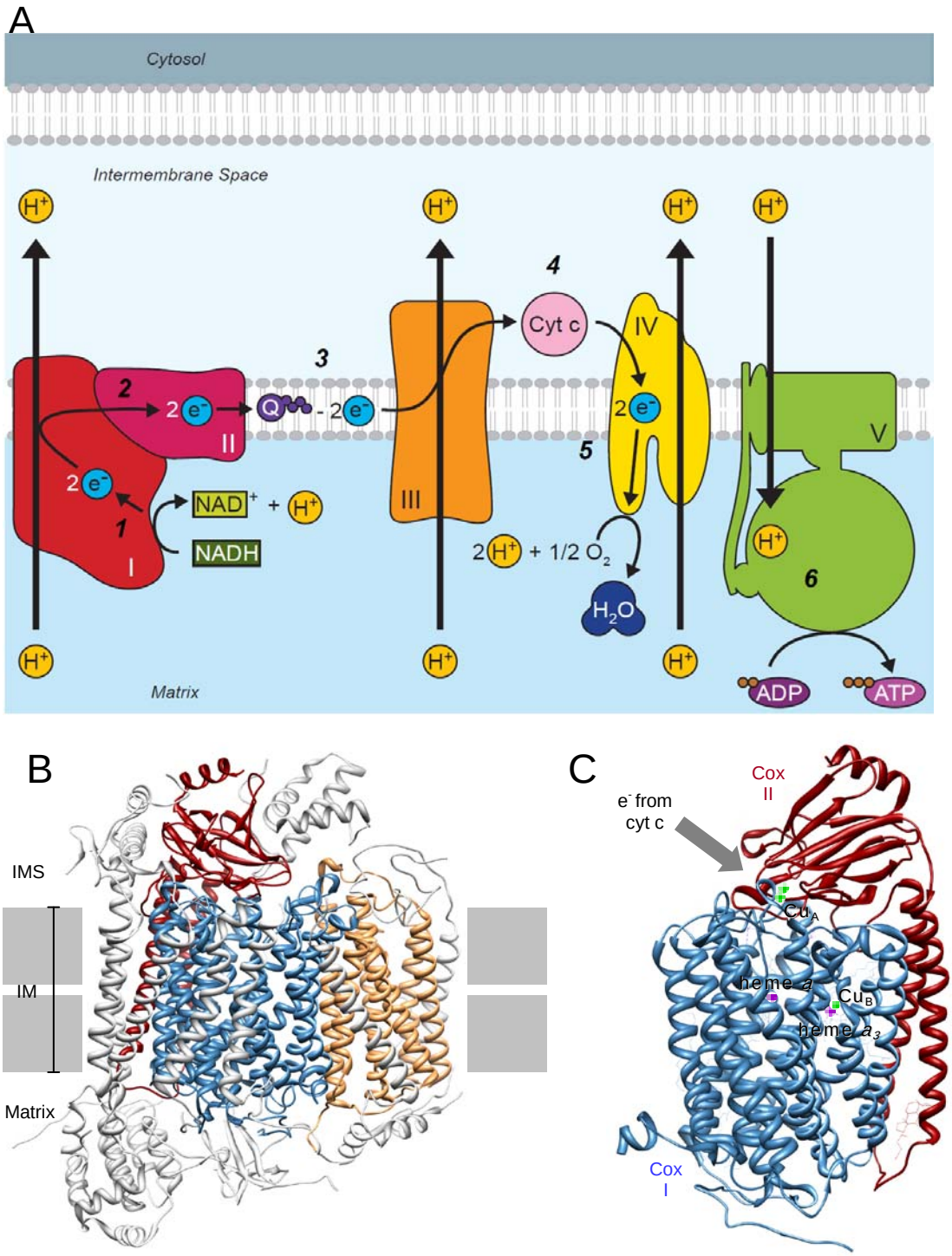
The mitochondrion is composed of an outer membrane (OM), intermembrane space (IMS), inner membrane (IM), and matrix; this structure allows the organelle to efficiently produce ATP for cellular energy needs (Loy et al., 2003). In addition to its contribution to the cell's bioenergetics, the mitochondrion also synthesizes amino acids, vitamin cofactors, fatty acids, and iron-sulfur clusters (Alberts et al., 2002). Interestingly, this organelle can also synthesize its own proteins from a circular mitochondrial genome. In humans, the thirteen mitochondrial genes encode proteins essential for energy production. However, most mitochondrial proteins are nuclearly expressed and imported into the organelle. Several of these imported proteins are critical for the regulation of apoptosis, another important function of the mitochondrion (Logan, 2006).

Oxidative phosphorylation couples ATP production to an electrochemical gradient that is generated by electron transport chain (ETC) components pumping protons across the IM (Fig. 21A) (Alberts et al., 2002). The ETC is composed of five complexes: NADH-dehydrogenase (Complex I), succinate dehydrogenase (Complex II), cytochrome c reductase or bc_1 complex (Complex III), cytochrome c oxidase (Complex IV), and ATP synthase (Complex V) (Alberts et al., 2002). Electrons from NADH are transported in a step-wise fashion from Complexes I to II to III by coenzyme Q and then transferred from Complex III to cytochrome c (cyt c). Cyt c donates the electrons to Complex IV, which converts two molecular oxygens to two water molecules. Throughout this process, Complexes I – IV pump protons across the IM, producing an electrochemical gradient

Figure 21. The components of electron transfer in respiration.

A. The path of protons and electrons through the electron transport chain. Electrons from NADH enter Complex I (1) and are transferred to Complex II via coenzyme Q (Q) (2), which then donates electrons to Complex III (3). Cytochrome c (Cyt c) carries electrons from Complex III to Complex IV (4), which catalyzes the reaction of electrons, protons and molecular oxygen to produce water (5). Throughout this process, Complexes I, III, and IV pump protons across the inner membrane, creating an electrochemical gradient. Energy is produced when protons transverse the membrane through Complex V (ATP synthase), allowing the conversion of ADP to ATP (6). This illustration was adapted from Cooper (2000). B. The macromolecular structure of Complex IV (Cox). Cox is composed of 13 unique subunits and is situated in the inner mitochondrial membrane. The blue (Cox I), red (Cox II), and yellow (Cox III) subunits are the only components of this complex encoded on the mitochondrial genome, and they form the functional core of the enzyme. C. Cox I and II contain the copper (green) and heme (iron, purple) groups that mediate electron transfer from cyt c and the conversion of protons, electrons, and oxygen to water. Illustrations in panels B and C are adapted from Alberts, et al. (2002).

Fig. 21


A
Cytosol
Intermembrane Space
Matrix
H+
2 e-
Q
- 2 e-
NAD+ + H+
NADH
Cyt c
2 H+ + 1/2 O2
H2O
ADP
ATP
I
II
III
IV
V
1
2
3
4
5
6
B
IMS
IM
Matrix
C
Cox II
e- from cyt c
CuA
heme a
CuB
heme a3
Cox I

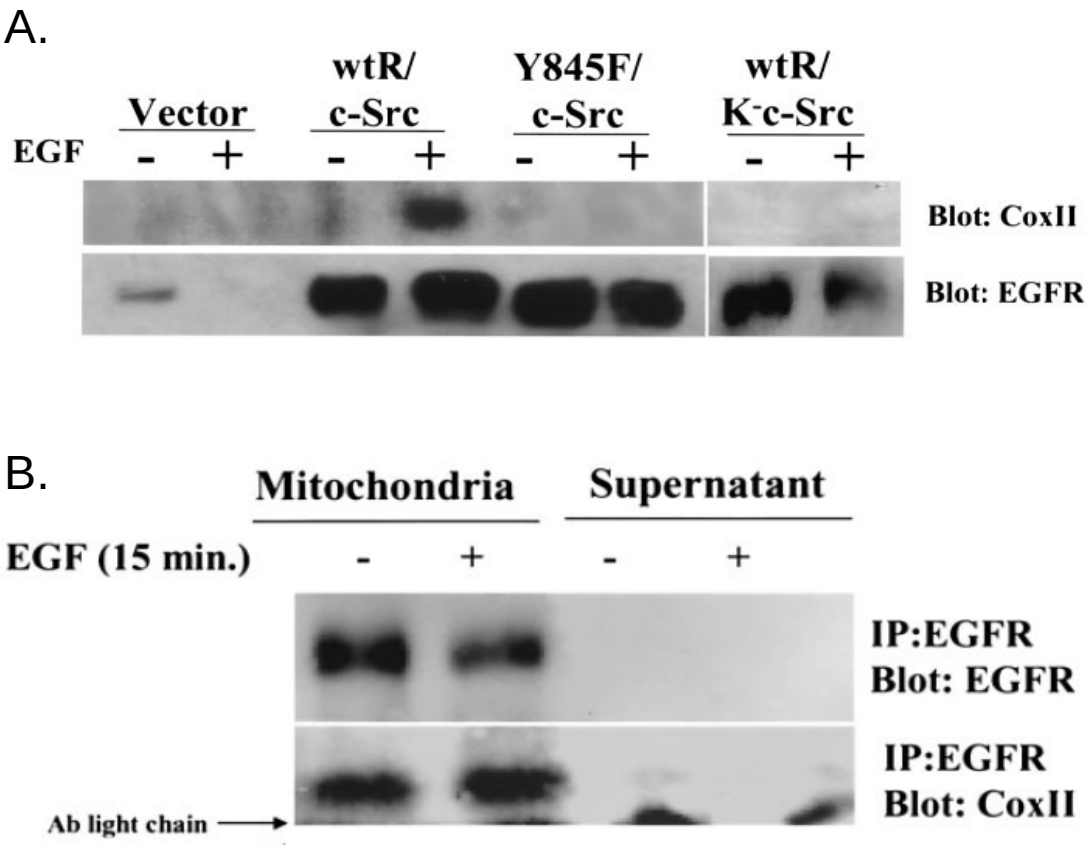
and membrane potential. ATP synthase harnesses energy of the proton gradient to convert ADP to ATP (Hosler et al., 2006).

Complex IV or cytochrome c oxidase (Cox) is composed of thirteen subunits (Fig. 21B). Of these, the three mitochondrially encoded subunits form the functional core of the complex. Cox subunits I and II (Cox I and Cox II) are adjacent to one another and contain the metallic binding sites essential for electron transfer (Fig. 21C) (Cooper, 2000). Interestingly, Cox II was identified as a potential binding partner of EGFR pY845 in a phage-display screen using a human breast cancer library as prey and an EGFR pY845 peptide as bait (Alberts et al., 2002). Boerner and colleagues (2004) demonstrated that EGF induced EGFR association with Cox II in an EGFR Y845- and c-Src kinase-dependent manner (Fig. 22A) and that the EGFR and Cox II co-localized with one another and mitochondria following EGF-stimulation. EGFR was shown to be basally present and phosphorylated at Y845 within mitochondria of breast cancer cells while EGF treatment increased both the presence and phosphorylation of EGFR in this organelle. Additionally, Cox II and EGFR were shown to associate within the mitochondria of breast cancer cells, independently of EGF (Fig. 22B). Cox II is known to facilitate the release of cyt c under cellular stress, which in turn activates the pro-apoptotic caspase cascade. When MDA-MB-231 breast cancer cells were treated with an apoptotic concentration of doxorubicin, EGFR Y845 was found to be necessary for EGF-induced protection from caspase 3 activity (Fig. 22C). One explanation for this observation is that EGFR Y845 was necessary for interaction with Cox II and this association had anti-apoptotic consequences.

Figure 22. Cox II association with the EGFR may protect against apoptosis.

A. Cox II association with EGFR requires EGFR Y845. Cos-7 cells were transfected with vector only or plasmids encoding c-Src, K^- c-Src (kinase defective), the wt EGFR, or the mutant Y845F-EGFR, serum starved for 24 hr, left unstimulated, or stimulated with 50 ng of EGF/ml for 20 min, and lysed. A total of 500 μg of cell lysate was immunoprecipitated with EGFR-specific MAb 108, and precipitated proteins were immunoblotted with CoxII or EGFR (Neomarkers) antibodies. B. The EGFR and Cox II coimmunoprecipitate from the mitochondrial fraction. MDA-MB-231 breast cancer cells were cultured in serum-free medium for 24 hr, left unstimulated, or stimulated with 50 ng of EGF/ml for 20 min and then subjected to biochemical fractionation. The indicated fractions were immunoprecipitated with EGFR-specific MAb 108, and precipitated proteins were immunoblotted with EGFR (top panel; Neomarkers) or CoxII (bottom panel) antibodies. C. EGFR pY845 mediates EGF-induced protection from caspase 3 cleavage. Electroporated MDA-MB-231 cells expressing wt (white bars) or Y845F EGFR (grey bars) were treated with doxorubicin for 1 hr prior to a 24 hr incubation in SF DMEM or 50 ng/ml EGF and assessment of caspase 3 activation. The results from three experiments are graphed as the percentage of cells expressing WT or Y845F EGFR that were positive for active caspase 3. Significantly greater numbers of positive cells were observed in the mutant EGFR-expressing population than in the WT EGFR population in response to EGF. *, $P < 0.03$. D. Representative immunoblot demonstrating similar amounts of EGFR and Y845F-EGFR protein expressed after transfection. (Miyazaki et al., 2003)

Fig. 22



Boerner, J. L., M. L. Demory, et al. (2004). "Phosphorylation of Y845 on the epidermal growth factor receptor mediates binding to the mitochondrial protein cytochrome c oxidase subunit II." Mol Cell Biol **24**(16): 7059-71.

Though it was known that the EGFR and Cox II associated, it was unclear whether this interaction was direct and, if so, which residues were necessary for binding. The study described in this chapter set out to address the nature of the EGFR and Cox II interaction through a variety of methods (Fig. 23). A truncated Cox II construct was designed and generated using data from clones identified in the phage display screen, and its transient co-overexpression with EGFR demonstrated that a portion of the Cox II globular head was necessary for association. This Cox II truncation mutant was also bacterially expressed for future use in pull-down assays to test for direct interaction with the EGFR and for nuclear magnetic resonance (NMR) imaging to identify Cox II residues that specifically interacted with EGFR pY845. A better understanding of the association between these proteins could lead to the development of a protein-protein interaction inhibitor potentially capable of overcoming a cancer cell's anti-apoptotic signaling.

Materials and Methods

Cell Lines

The MCF-7 cells from which mitochondrial DNA was isolated were purchased directly from ATCC and grown in DMEM supplemented with 10% FBS, 1.5 g/L sodium bicarbonate (Sigma-Aldrich), and 0.01 mg/ml insulin (from bovine pancreas, Sigma-Aldrich). HEK293t cells were passaged in DMEM with 10% FBS.

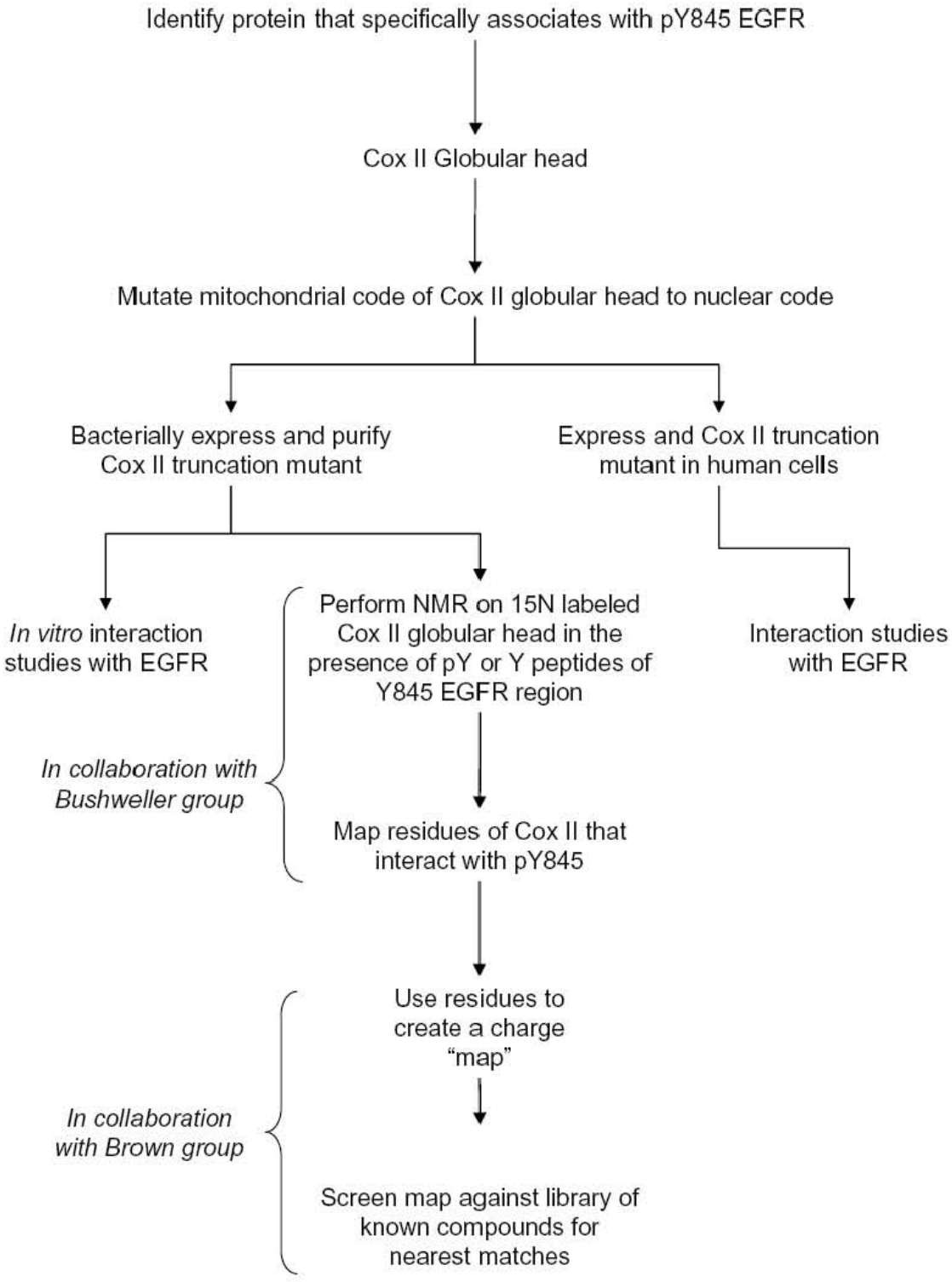
Isolation and Purification of Mitochondrial DNA

In order to study the interaction of Cox II with the EGFR, we endeavored to isolate the mitochondrial gene for Cox II, clone a portion hypothesized to interact with EGFR

Figure 23. Flow chart depicting goals of this study.

A phage display that screened a breast cancer library with an EGFR pY845 peptide identified the Cox II globular head as interacting with EGFR pY845. This led to the construction and expression of a Cox II truncation mutant in bacteria and mammalian cells for interaction studies with the EGFR and residue mapping by NMR.

Fig. 23


Identify protein that specifically associates with pY845 EGFR
Cox II Globular head
Mutate mitochondrial code of Cox II globular head to nuclear code
Bacterially express and purify Cox II truncation mutant
Express and Cox II truncation mutant in human cells
In vitro interaction studies with EGFR
Perform NMR on 15N labeled Cox II globular head in the presence of pY or Y peptides of Y845 EGFR region
Interaction studies with EGFR
In collaboration with Bushweller group
Map residues of Cox II that interact with pY845
Use residues to create a charge "map"
In collaboration with Brown group
Screen map against library of known compounds for nearest matches

pY845 based on data from a phage-display screen, mutate that region to the nuclear code, and express the Cox II truncation mutant in bacteria and mammalian cells. We here describe isolation of the mitochondrial genome. Mitochondria were isolated from MCF-7 cells using the Mitochondrial Isolation Kit for Cultured Cells (Pierce, Rockford, IL) according to manufacturer's instructions. Mitochondrial DNA was extracted and purified using the phenol-chloroform-isoamylalcohol method. A solution of phenol, chloroform, and isoamylalcohol (PCI solution) was prepared by mixing 25 ml lower phase of Tris-phenol (Gibco), 24 ml chloroform (Fisher Scientific), and 1 ml isoamylalcohol (Sigma-Aldrich). Following an hour incubation at 4°C, 200 µl of the lower phase was combined with a total volume of 200 µl isolated mitochondria in TE buffer (10 mM Tris base [Fisher Scientific], 1 mM Na_2EDTA [Fisher Scientific], pH 8.0) to extract mitochondrial DNA. This solution was centrifuged at 9300 x g for 10 min at room temperature. The upper phase was removed and mixed with 80 µl 3M sodium acetate (Fisher Scientific) and 800 µl ethanol (Fisher Scientific) and incubated overnight at -20°C to precipitate DNA. The next day the sample was centrifuged for 10 min and the supernatant discarded. The DNA was washed with 400 µl 70% ethanol, pelleted for 5 min at 4°C, air-dried, and then resuspended in TE buffer.

Mutation and Construction of Nuclearly Encoded Cox II Clone: Globular Head Construct

To generate a mitochondrial DNA fragment encoding Cox II, mitochondrial genomic DNA was PCR amplified using the JP_CoxII_C_forward and JP_CoxII_GlobularHead_reverse primers in a PCR reaction (see "DNA Amplification by

PCR" for details). The reaction products were separated by electrophoresis on an agarose gel and purified using GenElute Agarose Spin Columns (Sigma-Aldrich), according to manufacturer's instructions. The Clone: Globular Head (CGH) portion was isolated from the larger Cox II fragment by PCR using the JP_CoxII_C_forward and JP_CoxII_A540G_reverse primers (see Table 3) and products were in-gel purified as described.

Table 3. Primers Used in the Cloning of Cox II Constructs for Nuclear Transcription

Name	***Mutation***	***Sequence (5' → 3')***
JP_Mito7287_forward		CCGGAATTCTCTAACAGCAGTAATATTAAT
JP_CoxII_start_forward		CCGGAATTCATGGCACATGCAGCGCAAGTA
JP_CoxII_GlobularHead_reverse		CCGCTCGAGCTATAGGGTAAATACGGGCCCTA
JP_CoxII_C_forward	A135G	CCGGAATTCATGACAGACGAGGTCAACGA
JP_CoxII_A195G_forward	A195G	AATGGTACTGGACCTACGAGTACACCGACTA
JP_CoxII_A195G_reverse	A195G	ACTCGTAGGTCCAGTACCATTGGTGGCCAATT
JP_CoxII_A244G_forward	A244G	ACTCCTACATGCTTCCCCCATTATTCCTAGA
JP_CoxII_A244G_reverse	A244G	ATGGGGGAAGCATGTAGGAGTTGAAGATTAG
JP_CoxII_A333/336G_forward	A333/336G	CCATTCGTATGATGATTACATCACAAGACGTCTT
JP_CoxII_A333/336G_reverse	A333/336G	GTGATGTAATCATCATACGAATGGGGGCTTCAAT CG
JP_CoxII_A366G_forward	A366G	TGCACTCATGGGCTGTCCCCACATTAGGCTT
JP_CoxII_A366G_reverse	A366G	TGGGGACAGCCCATGAGTGCAAGACGTCTTG
JP_CoxII_A540G_reverse	A540G	CCGCTCGAGCTATAGGGTAAATACCCCGGGCATT TCAAAGATTTTTAGGGG

All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Because of the differences between the codons encoding methonine (Table 4, highlighted) in the mitochondrial and nuclear codes, mutations to the mitochondrial Cox II gene CGH fragment were made for proper nuclear expression. The underlined adenines in the Cox II gene shown in Table 4 were changed to guanines. Bolded bases comprise Cox II CGH.

Table 4. Cox II Mitochondrial Gene Sequence

ATG GCA CAT GCA GCG CAA GTA GGT CTA CAA GAC GCT ACT TCC CCT ATC ATA GAA

GAG CTT ATC ACC TTT CAT GAT CAC GCC CTC ATA ATC ATT TTC CTT ATC TGC TTC

CTA GTC CTG TAT GCC CTT TTC CTA ACA CTC ACA ACA AAA CTA ACT AAT ACT AAC

ATC TCA GAC GCT CAG GAA ATA GAA ACC GTC TGA ACT ATC CTG CCC GCC ATC ATC

CTA GTC CTC ATC GCC CTC CCA TCC CTA CGC ATC CTT TAC **ATA ACA GAC GAG GTC**

AAC GAT CCC TCC CTT ACC ATC AAA TCA ATT GGC CAC CAA TGG TAC TGA ACC TAC

GAG TAC ACC GAC TAC GGC GGA CTA ATC TTC AAC TCC TAC ATA CTT CCC CCA TTA

TTC CTA GAA CCA GGC GAC CTG CGA CTC CTT GAC GTT GAC AAT CGA GTA GTA CTC

CCG ATT GAA GCC CCC ATT CGT ATA ATA ATT ACA TCA CAA GAC GTC TTG CAC TCA

TGA GCT GTC CCC ACA TTA GGC TTA AAA ACA GAT GCA ATT CCC GGA CGT CTA AAC

CAA ACC ACT TTC ACC GCT ACA CGA CCG GGG GTA TAC TAC GGT CAA TGC TCT GAA

ATC TGT GGA GCA AAC CAC AGT TTC ATG CCC ATC GTC CTA GAA TTA ATT CCC CTA

AAA ATC TTT GAA ATA GGG CCC GTA TTT ACC CTA TAG

Mutations were made serially through PCR, and products were in-gel purified as described and the final product was confirmed by sequencing. For each of the mutations (see Table 4), the DNA template was amplified in separate reactions using the primer pair JP_CoxII_C_forward and JP_CoxII_(mutation)_reverse or JP_CoxII_(mutation)_forward and JP_CoxII_ A540G _reverse. Following in-gel purification, these fragments were ligated using JP_CoxII_C_forward and JP_CoxII_ A540G _reverse. The ligated fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) prior to introduction of another mutation. After all mutations were introduced, the CGH DNA was inserted into the pBluescriptIIKS+ (pBSKII, Stratagene, La Jolla, CA) cloning vector via Eco RI and Xho I restriction sites using enzymes from New England

Biolabs (Ipswich, MA). Products were in-gel purified and inserted into the cloning vector using the Rapid Ligation Kit (Roche) according the manufacturer's protocol. To amplify the Bluescript CGH vector, *E. coli* strain DH5α was transformed with the construct by heat shock and grown overnight on Luria Broth-Amp plates (10 g/L tryptone [Becton, Dickinson and Co., Sparks, MD], 5 g/L yeast extract [Becton, Dickinson and Co.], 10 g/L NaCl [Fisher Scientific], 15 g/L agarose [Becton, Dickinson and Co.], pH 7.5, 50 μg/ml ampicillin) supplemented with 100 μl 2% X-gal (Sigma-Aldrich) in dimethylformamide per plate. White colonies were selected and grown in Luria broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5) with the appropriate antibiotic overnight. The next day, cultures were miniprepped with the QIAprep Spin Miniprep Kit (Qiagen) and sent for DNA sequencing (UVA Sequencing Facility). Upon sequence confirmation, the CGH insert was isolated from its cloning vector (pBSK-COXII CGH clone 221U-1) and introduced into pET 28a (bacterial expression; Novagen, Darmstadt, Germany), pET 32a (bacterial expression, Novagen), and pc3-DNA (mammalian expression, Invitrogen) vectors at the Eco RI and Xho I sites. These plasmids were screened for ampicillin resistance except for pET28a, which was kanamycin-resistant, and confirmed by DNA sequencing.

DNA Amplification by PCR

The iCycler Thermal Cycler (BioRad) was used to amplify DNA. Briefly, the start cycle was 1-2 min at 95°C followed by 25-30 amplification cycles of 95°C for 30-45 sec, 56-57°C for 1 min, and 72°C for 30 sec - 1 min. This was followed by 7 min at 72°C. Reaction mixtures contained 0.5 μl forward primer (100 μM stock), 0.5 μl reverse primer

(100 μM stock), 0.5 μl template DNA (diluted 1:10-1:100) and either the Invitrogen PCR Selection kit containing high fidelity and Accuprime Pfx DNA polymerase or a mixture of 5 μl 10X buffer (Roche), 1 μl dNTP (Roche) and 0.3 μl Taq polymerase (Roche) in a total volume of 50 μl.

Verification of Bacterial Cox II CGH Expression

The *E. coli* strain BL21 (BL21star [DE3], Invitrogen) was transformed by heat shock with a bacterial expression vector containing the CGH cassette, pET28a-CGH or pET32a-CGH. After selection on LB plates containing the proper antibiotic, clones were selected and grown in antibiotic-containing LB. Once an optical density of 0.6 at 600 nm was reached, isopropyl-beta-D-thiogalactopyranoside (IPTG, 80 mM final concentration; Sigma-Alrich) was added to the culture to stimulate transcription from the *lac* promoter, and cultures were incubated for 6 hrs with shaking. Cells were pelleted and sonicated, and proteins were separated on a 15% SDS-PAGE gel, which was Coomassie-stained to detect proteins.

Transient Transfection of Mammalian Cells

HEK293t cells were seeded in DMEM supplemented with 10% FBS. Once 50% confluent, cells were transfected with one of the following plasmids using Lipofectamine 2000 (Invitrogen) in OptiMEM medium (Gibco) according to manufacturer's instructions: pc3DNA (vector control for Cox II and full-length EGFR constructs), pCMV-Myc (vector control for EGFR kinase domain constructs), pc3-3HA-CGH (Cox II CGH construct), pTM860 (WT EGFR kinase domain), pTM861 (Y845F EGFR kinase

domain), pc3-WT EGFR (full-length WT EGFR), and/or pc3-YF EGFR (full-length Y845F EGFR). Cells were cultured 24 hrs post-transfection and then lysed. EGFR kinase domain constructs were made by T. Miyake, full length EGFR constructs were provided by J. Boerner, and the Cox II CGH construct was the work of J. Pritchard.

Immunoprecipitation and Western Blotting

HEK293t cells were grown in DMEM + 10% FBS and transfected with the indicated plasmid using Lipofectamine 2000 when 50% confluent. After 24 hrs, cells were lysed in CHAPS buffer (10.6 mM CHAPS [Sigma-Aldrich], 25 mM Tris base, pH 8.0, 150 mM NaCl, 2 mM Na_2EDTA) supplemented with Protease Inhibitor Cocktail Set I (Calbiochem) and100 μM Na_3VO_4. Lysates were clarified and quantitated using BioRad Protein Assay reagent. The immunoprecipitating antibody was added to lysate (mouse HA.11 [Covance] for CGH, mouse anti-Myc 9B11 [Cell Signaling] for EGFR kinase domain, or mouse IgG [ChromPure, whole molecule; Jackson Immunoresearch Laboratories, Inc., West Grove, PA]) and incubated 1 hr at 4°C with gentle agitation. Protein G beads (Millipore) were added to capture immune complexes and incubated overnight at 4°C with gentle agitation. Beads were washed three times with CHAPS buffer prior to the addition of sample buffer. Alternately, CGH was immunoprecipitated with Sigma-Aldrich EZView monoclonal anti-HA agarose conjugate (clone HA-7). In these instances the conjugated beads were washed in NP-40 buffer, lysate was added, and the immunoprecipitation incubated overnight at 4°C with gentle agitation. Beads were pelleted and washed in NP-40 buffer prior to sample buffer addition. Samples were boiled prior to separation on a 12% SDS-PAGE gel and transferred onto PVDF

membranes which were then blocked in 5% milk in TBST for 1 hr at room temperature. The membrane was probed for Cox II using anti-HA (Covance HA.11, mouse, 1:1000), for EGFR kinase domain constructs using anti-Myc (Cell Signaling anti-Myc 71D10, rabbit, 1:1000), for full-length EGFR (Cell Signaling, rabbit, 1:500), or for β-actin (Sigma-Aldrich, mouse, 1:10,000) with gentle agitation for 1 hr at room temperature or overnight at 4°C. Membranes were extensively washed in TBST and incubated at room temperature with HRP-conjugated secondary antibody (goat anti-mouse or sheep anti-rabbit, GE Healthcare, both 1:2000) for 1 hr. Membranes were again washed, incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermoscientific, Waltham, MA) for 2 min in the dark, and exposed to film (Blue Lite Autorad film, ISC Bioexpress, Kaysville, UT) and processed.

Computer Modeling of Cox II

The amino acid sequences of human (cox2_human) and bovine (cox2_bovin) Cox II were obtained from the NCBI protein database (Boerner et al., 2004). These were aligned using NCBI's BLAST and ProteinPredict functions, and sequence variation was determined using the MAXHOM alignment. The constraint-based multiple alignment tool, Cobalt (NCBI, Bethesda, MD), was used to compare the Cox II sequence of *Paracoccus denitificans* (GenBank: ABL71766.1) to that of human for the purpose of identifying human homologs to the cyt c-binding residues in that bacterium (NCBI, 2006b). The complete three-dimensional (3D) structure of oxidized bovine Cox (1V54) was obtained from the NCBI structure database (Witt et al., 1998). UCSF Chimera version 1.5.2 (San Francisco, CA) was used to manipulate and view the structure of Cox

II. Tyrosine and cyt c binding sites from the human Cox II sequence were superimposed on the bovine molecule. The TMHMM (Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark), TMpred (European Molecular Biology Netword, Swiss Node, Lausanna, Switzerland), and DAS (Stockholm University, Stockholm, Sweden) transmembrane prediction programs were used to identify regions of human Cox II with transmembrane topology and hydrophobicity.

Results

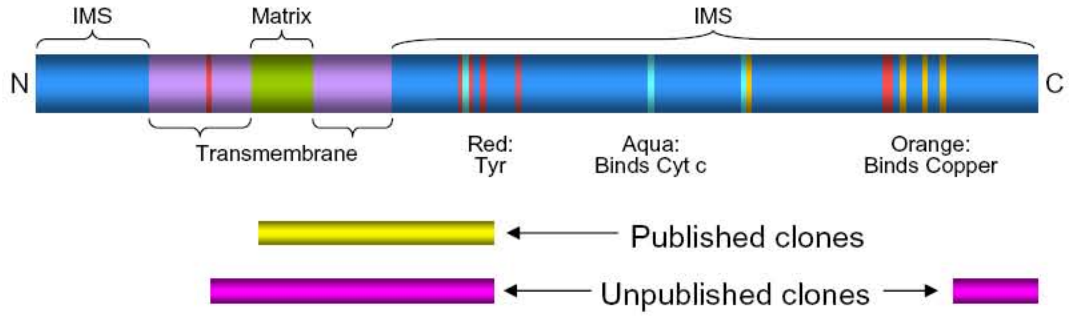
Cox II is a double-pass transmembrane protein that has a small N-terminal tail in the IMS, a short loop in the matrix, and a large, globular head in the IMS (Fig. 24). The globular head contains residues that bind copper atoms, which are necessary for electron transfer and proper function of the enzyme, specific acidic acids that participate in cyt c binding, and most of the protein's tyrosines (Fig. 24A). As mentioned in the introduction, a phage-display screen for binding partners to EGFR pY845 identified a clone of Cox II that spanned the matrix loop, a transmembrane domain, and a portion of the globular head that contained several tyrosines (NCBI, 2006a). Upon further inspection, T. Miyake found two additional Cox II clones, one of which encompassed that described by Boerner and colleagues; the other consisted of the C-terminal portion of the globular head (Fig. 24A). Though these clones were distant from one another on the linear amino acid sequence, they are quite near in the 3D macromolecular structure of Cox II (Fig. 25). The clone-identified regions lie on the exterior of the globular head and potentially form a docking site for the EGFR in proximity to Cox II's tyrosines and cyt c binding sites. Additionally, the spatial arrangement of the tyrosines and cyt c binding

Figure 24. The CGH truncation mutant incorporates the majority of the amino acids in the globular head of Cox II.

A. Structure of Cox II protein. The Cox II protein is composed of 225 amino acids. Amino acids located in the mitochondrial intermembrane space (IMS) are depicted in blue, those spanning the membrane are purple, and those in the mitochondrial matrix are green. Tyrosines are red, acidic acids that bind cytochrome c (cyt c) are aqua, and amino acids that bind copper are orange. Below the Cox II structure are the aligned depictions of the published (yellow) (Boerner et al., 2004) and unpublished (pink) phage display clones shown to interact with EGFR pY845 that were identified by J. Boerner and T. Miyake (Boerner et al., 2004). B. Cox II amino acid sequence. Highlighted yellow and text in pink indicate residues that correspond to the unpublished and published clones, respectively. The CGH truncation mutant comprises a small portion of the transmembrane residues and most of the globular head of Cox II. C. Cartoon of full length Cox II and the CGH mutant oriented in the mitochondrial membrane. CGH expression vectors and their tags are listed to the right of the illustration.

Fig. 24

A.



B. **Cox II Amino Acid Sequence:**

Transmembrane

MAHAAQVGLQDATSPIMEELITFHDHALMIIFLICFLVLYALFLTL

Transmembrane *Transmembrane* *CGH start*

TTKLTNTNISDAQEMETVWTILPAIILVLIALPSLRILYMTDEVND

PSLTIKSIGHQWYWTYEYTDYGGLIFNSYMLPPLFLEPGDLRLL

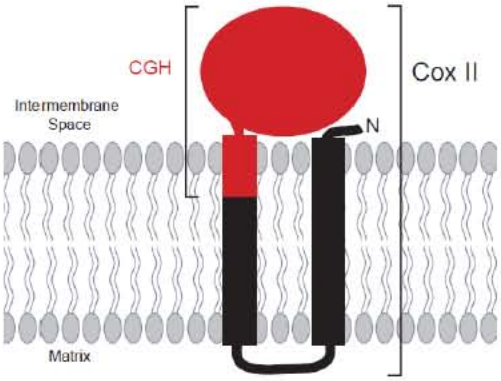
DVDNRVVLPIEAPIRMMITSQDVLHSWAVPTLGLKTDAIPGRLN

CGH end

QTTFTATRPGVYYGQCSEICGANHSFMPIVLELIPLKIFEMGPV

FTL

C.



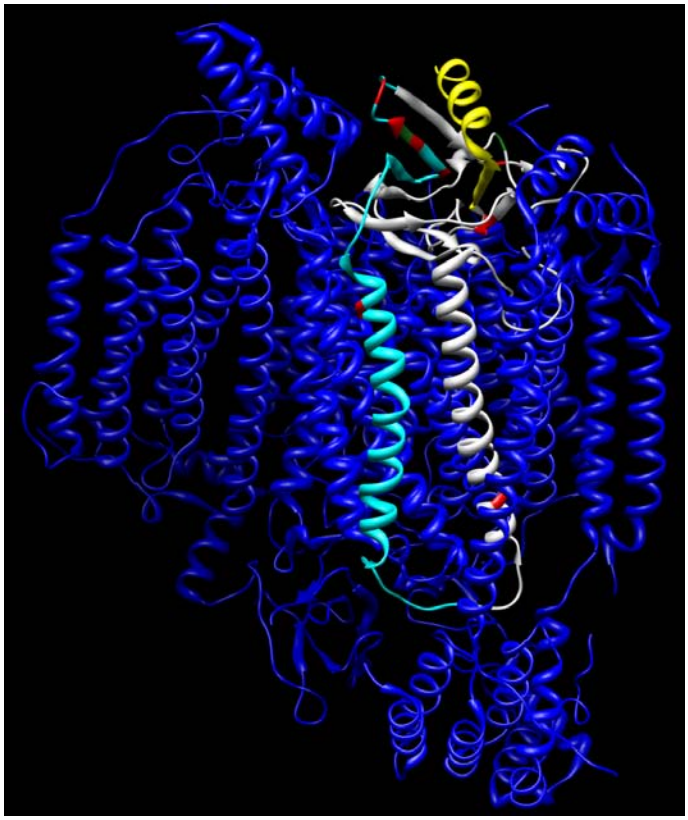
Vector	Tag(s)
Bacterial:	
pET28a-CGH	2 His, S, Trx
pET32a-CGH	2 His, T7
Mammalian:	
pc3-3HA-CGH	3 HA

Figure 25. The phage display fragments are in close proximity to one another and contain tyrosines and a cyt c binding site.

The front (A), back (B), and top (C) views of the Cox II 3D macromolecular structure are shown in the context of Cox I, III, and IV (blue). Panels B and C are close-ups of the Cox II globular head. The N-terminal and C-terminal unpublished phage display fragments are depicted in aqua and yellow, respectively. Tyrosines are red and acidic acids binding cyt c are green. All other residues of Cox II are white.

Fig. 25

A. Front View



Blue: Cox I, III, IV

Aqua: N-terminal Cox II phage display fragment

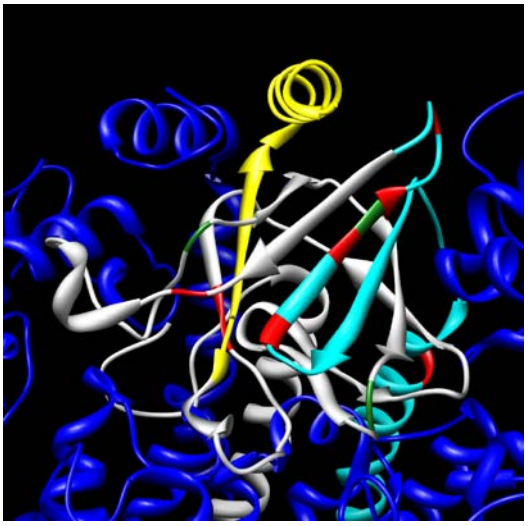
Yellow: C-terminal Cox II phage display fragment

White: Cox II portions not identified by phage display

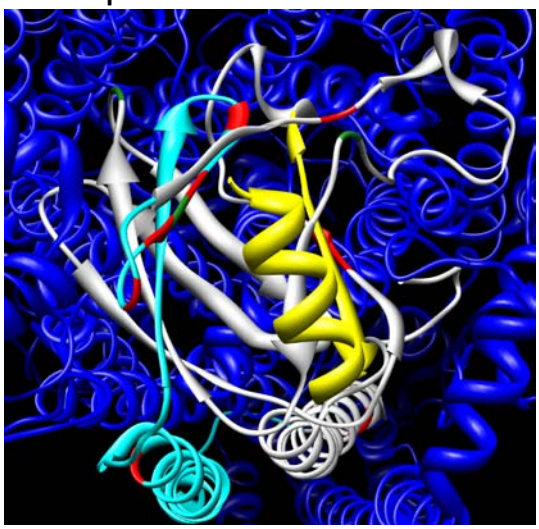
Red: Tyrosines in Cox II

Green: Cytochrome c binding sites

B. Back View: Globular Head



C. Top View: Globular Head



sites suggests that phosphorylation of Cox II by EGFR could regulate cyt c docking and Cox II function.

To better study the nature of Cox II and EGFR interaction, we designed a Cox II truncation mutant based on data from the phage-display. This mutant excluded most of the hydrophobic, transmembrane portion of the N-terminal clones but comprised the majority of the globular head so as to include portions of both the N- and C-terminal clones (Fig. 24B, 24C). In order to effectively express the Cox II truncation mutant (CGH) exogenously from a plasmid, the mitochondrial gene required seven mutations to adjust for differences between the methonine-encoding codons in the mitochondrial and nuclear codes (see Materials and Methods for details). Following sequence confirmation, the truncated Cox II mutant was inserted into tagged plasmids for bacterial and mammalian expression.

Two tagged, bacterial expression plasmids, pET-32a and pET-28a, were chosen to carry the truncated Cox II mutant construct, each for a different purpose. For use *in vitro* nickel-column pull-down assays between purified EGFR and truncated Cox II, His-tagged pET28a was used. Because NMR studies to identify Cox II residues interacting with EGFR pY845 would require high yields of very pure truncated Cox II from which the tags have been removed, pET32a was selected. This plasmid is ideal for such yields and purification as it has both the His and S tags to aid in the initial isolation (His tag) and attachment to a column (S tag) prior to cleavage. Figure 26A and B shows that the tagged, truncated Cox II constructs were successfully expressed from both plasmids in *E. coli* induced with IPTG.

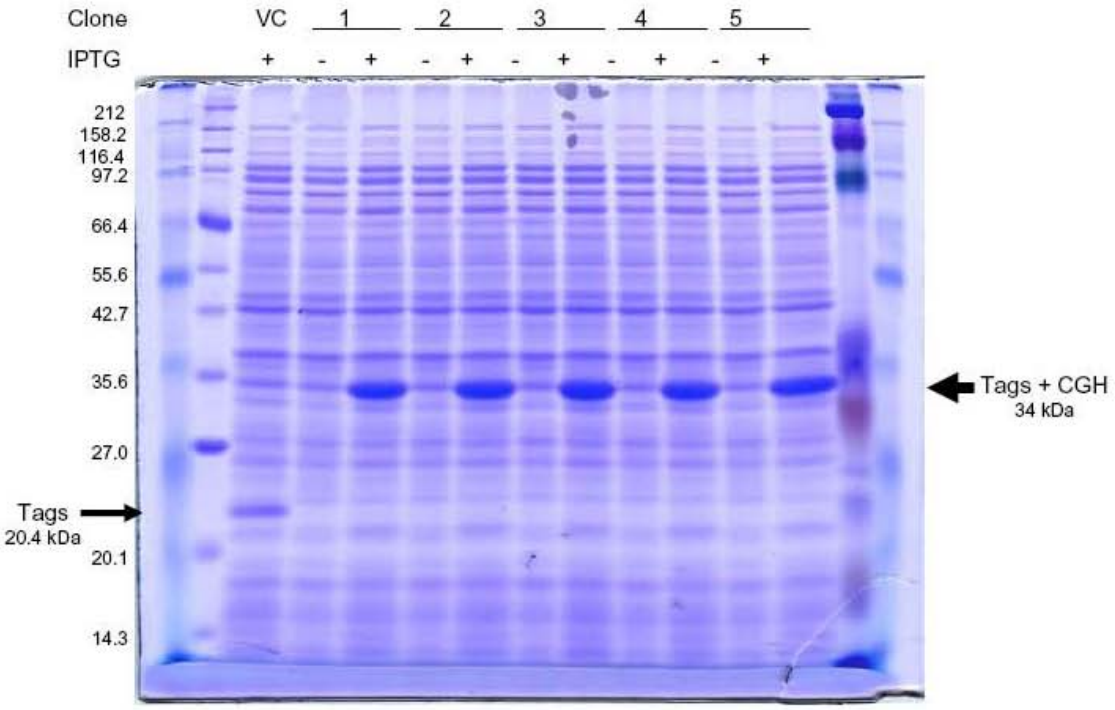
To test the ability of the truncated Cox II mutant to interact with the EGFR

Figure 26. The Cox II CGH truncation mutant was bacterially expressed.

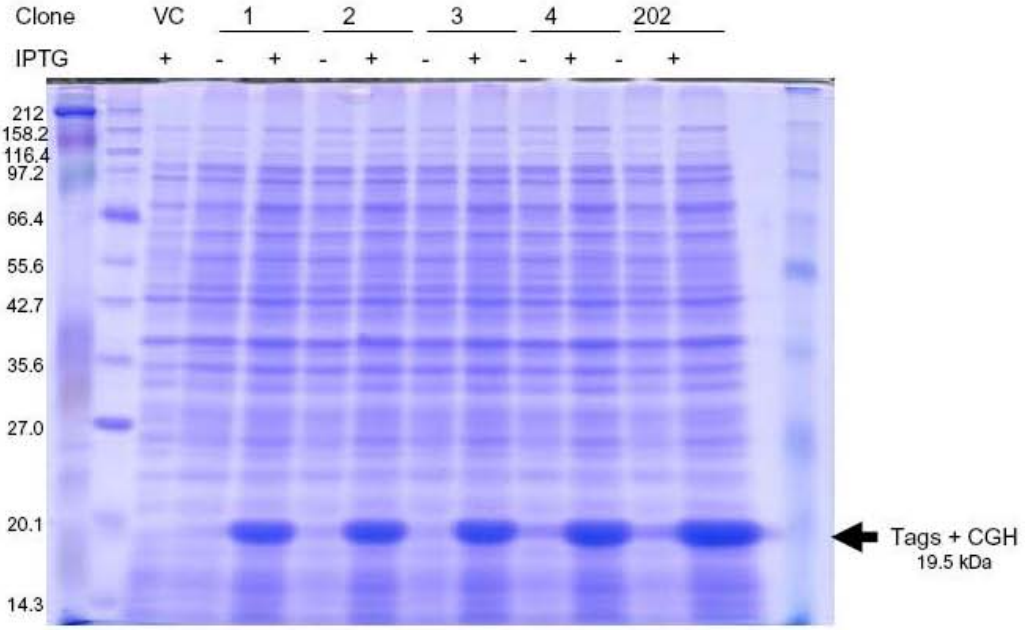
Tagged CGH was expressed from the bacterial vector, pET32a-Cox II CGH, in *E. coli* strain BL21 following 6 hrs IPTG treatment, as described in Materials and Methods. The tags expressed from the empty vector control were 20.4 kDa whereas tagged CGH was 34 kDa. B. Tagged CGH was expressed from pET28a-Cox II CGH n BL21 cells as described. Tagged CGH was 19.5 kDa where as the tags expressed from the empty vector were 2.5 kDa.

Fig. 26

A. Bacterial Vector pET32a-Cox II CGH



B. Bacterial Vector pET28a-Cox II CGH



receptor, human, transformed epithelial HEK293t cells were first transiently transfected with the triple-HA-tagged Cox II CGH construct and assayed for their ability to express the protein fragment. Figure 27 demonstrates protein expression of the mutant in these cells. Having confirmed mammalian expression of the Cox II truncation mutant, we next studied the ability of the mutant to interact with the EGFR kinase domain, which contains Y845, and the role this residue may play in any association. HEK293t cells were transfected with the truncated HA-tagged Cox II construct, a wild-type (WT) or myc-tagged Y845F EGFR kinase domain, or both EGFR and Cox II plasmids. Immunoprecipitation of the EGFR kinase domain revealed that truncated Cox II associated with WT EGFR kinase domain, indicating that this portion of the globular head is sufficient for interaction with the EGFR kinase domain in HEK293t cells (Fig. 28A). Interestingly, the Cox II fragment was also capable of association with Y845F EGFR. This was counter to our expectation given that the phage-display negatively selected against unphosphorylated Y845 EGFR and published results have shown that endogenous Cox II does not associate with full-length Y845 EGFR (Boerner et al., 2004). It may be that the association seen between the Y845F EGFR mutant and Cox II fragment was an artifact of overexpression or due to a loss of steric hindrance normally imposed by their full-length, membrane bound counterparts. To confirm that truncated Cox II could interact with the full-length WT EGFR, transient transfections were carried out HEK293t cells and the Cox II construct was immunoprecipitated. Figure 28B shows that the EGFR and truncated Cox II globular head are able to associate in cellular overexpression system, confirming the sufficiency of the fragment for interaction.

Figure 27. The Cox II CGH truncation mutant expresses in mammalian HEK 293t cells.

HEK 293t cells were mock transfected or transfected with 8 μg vector control or 2, 4, or 8 μg pc3-3HA-CGH, a mammalian plasmid expressing the triple HA-tagged Cox II CGH construct. Twenty-four hours later cells were lysed and analyzed by Western blotting for HA or β-actin (loading control), as described in Materials and Methods.

Fig. 27

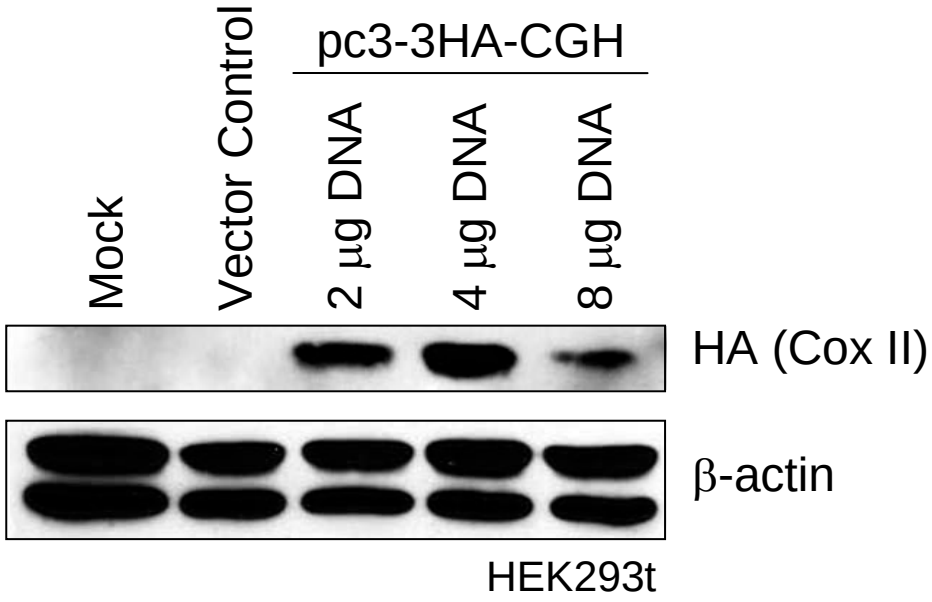
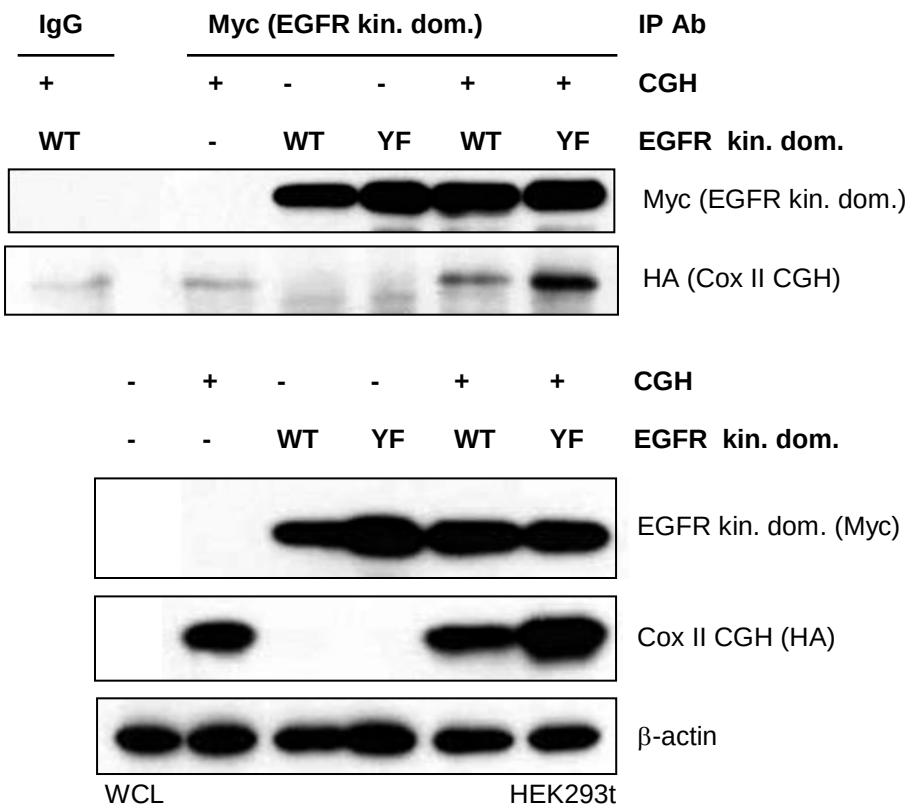

pc3-3HA-CGH
Mock
Vector Control
2 μg DNA
4 μg DNA
8 μg DNA
HA (Cox II)
β-actin
HEK293t

Figure 28. Cox II CGH associates with EGFR.

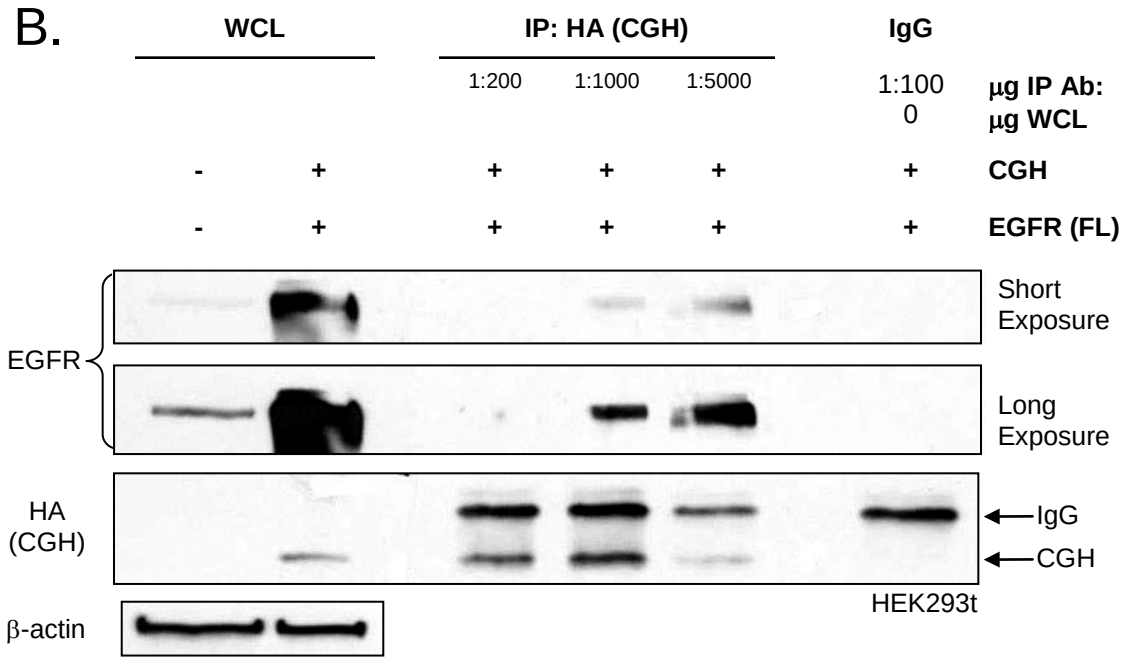
A. The Cox II CGH construct associates with the EGFR kinase domain (kin. dom.). HEK293t Cells were transfected with Cox II CGH, WT EGFR kinase domain, and/or Y845F (YF) EGFR kinase domain expressing plasmids and 24 hrs later lysed as described in Fig. 27. The EGFR kinase domain was immunoprecipitated by anti-Myc, and the immunoprecipitations and whole cell lysates (WCL) were immunoblotted for Myc, HA (Cox II CGH), and/or β-actin. B. The Cox II CGH construct binds full-length EGFR. Full-length WT EGFR and Cox II CGH were coexpressed in HEK 293t, and lysates were immunoprecipitated with anti-HA (Cox II CGH). Immunoprecipitates and WCL were immunoblotted for EGFR, HA (Cox II CGH), and/or β-actin.

Fig. 28

A.


IgG
Myc (EGFR kin. dom.)
IP Ab
+ + - - + + CGH
WT - WT YF WT YF EGFR kin. dom.
Myc (EGFR kin. dom.)
HA (Cox II CGH)
- + - - + + CGH
- - WT YF WT YF EGFR kin. dom.
EGFR kin. dom. (Myc)
Cox II CGH (HA)
β-actin
WCL
HEK293t

B.


WCL
IP: HA (CGH)
IgG
1:200 1:1000 1:5000
1:100
0
µg IP Ab:
µg WCL
- + + + + + CGH
- + + + + + EGFR (FL)
EGFR
Short Exposure
Long Exposure
HA (CGH)
IgG
CGH
HEK293t
β-actin

Discussion

This study demonstrated that a portion of the Cox II globular head is sufficient for association with the EGFR in an *in vitro* overexpression system. Though we did not identify specific residues of the Cox II globular head that mediated this interaction, super-imposition of the phage-display clones on the Cox II 3D structure suggests that interaction with the Y845 EGFR peptide (from a portion of the kinase domain) occurs in a region of Cox II containing several tyrosines that are exposed to intermembrane space. Given the accessibility of these tyrosines and EGFR association with full-length Cox II (Boerner et al., 2004), it would not be surprising if the EGFR phosphorylated the Cox II globular head.

Many ETC components are phosphorylated and, in some instances, this modification has been shown to regulate function (Augereau et al., 2005; Bender and Kadenbach, 2000; Helling et al., 2008; Lee et al., 2005a; Miyazaki et al., 2003; Samavati et al., 2008; Steenaart and Shore, 1997). Our laboratory demonstrated that tyrosine phosphorylation of endogenous Cox II increased following 20 min of EGF stimulation, a time point that corresponded with increased EGFR localization to the mitochondria, suggesting that the EGFR may participate in the phosphorylation of endogenous Cox II (Demory et al., 2009). It was also shown that the EGFR directly phosphorylated a Cox II fragment that was based on the published phage-display clone by Boerner and colleagues (Boerner et al., 2004; Demory et al., 2009). This construct contained four tyrosines, all of which were located in the N-terminal region of the globular head and in spatial proximity to the cyt c binding residues. Given that our data demonstrated association of a portion of the Cox II globular head with the EGFR and that the direct phosphorylation of a Cox II

phage-display fragment by EGFR suggested interaction, we hypothesize that the overlapping 30 amino acids between the two constructs (in the N-terminus of the CGH clone, see Fig. 29) are necessary for interaction with the EGFR, though this association may be indirect.

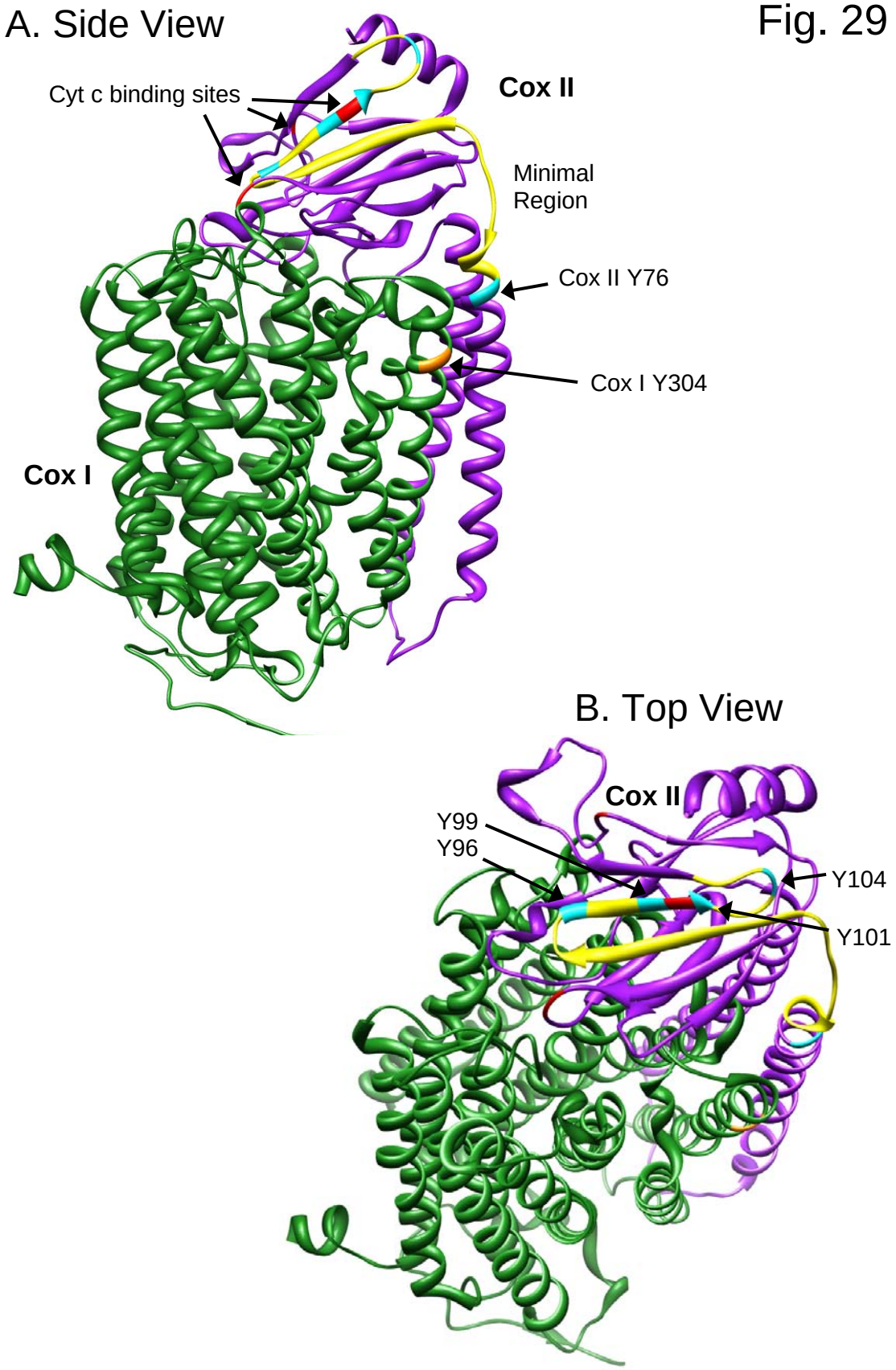
A possible mechanism by which EGFR-mediated tyrosine phosphorylation could alter Cox activity is through phosphorylation of Cox II Y76 (Fig. 29A). This residue is located near the transmembrane domain in the Cox II clone used by Demory and colleagues, is just below the minimal region described above, and is in the proximity of Cox I Y304 (human sequence). Two groups have shown that phosphorylation of bovine Cox I Y304 downregulates Cox activity (Lee et al., 2006; Samavati et al., 2008), which is consistent with the observations by Demory and colleagues following EGFR translocation to the mitochondria and Cox II tyrosine phosphorylation (Demory et al., 2009). The mechanism of Cox I pY304 modulation of Cox activity is not known, but if it is due to a structural change that alters the association/orientation of Cox I and II, then phosphorylation of Cox II Y76 may function similarly to provide an additional point of regulation for cellular bioenergetics.

Another potential consequence of EGFR association with and phosphorylation of the Cox II globular head could be regulation of cyt c binding to Cox II. The cyt c-binding residues are surrounded by several tyrosines (Figs. 25 and 29B), including Y96, Y99, Y101, and Y104, located in the hypothetical minimal region. Phosphorylation of these tyrosines would increase the negative charges on the surface of the Cox II globular head and could potentially aid in the orientation of cyt c's positively charged iron atoms for improved docking on Cox II. This may occur in a manner similar to the two-step

Figure 29. A 30 amino acid region of Cox II may facilitate its interaction with the EGFR.

The crystallized structures of Cox I (green) and Cox II (purple and yellow) are shown in association. The yellow portion of Cox II indicates the 30 amino acids that overlap between CGH clone presented in this chapter and the truncation mutant described by Demory and colleagues (Boerner et al., 2004). Tyrosines are indicated in cyan, with Cox I Y304 denoted by orange. Residues in Cox II that participate in cyt c binding are red. A. Side view B. Top view

Fig. 29


A. Side View
Cyt c binding sites
Cox II
Minimal
Region
Cox II Y76
Cox I Y304
Cox I
B. Top View
Cox II
Y99
Y96
Y104
Y101

docking process postulated for cyt c docking on the oxidase complex (Maneg et al., 2004). In this scenario, electrostatic forces pre-orient cyt c and Cox II, and then salt bridges rearrange both proteins for more productive electron transfer, allowing for an efficient and transient interaction between these molecules. If EGFR phosphorylation of Cox II's tyrosines altered cyt c docking, this could affect both bioenergetics and apoptotic signaling involving cyt c release and would be consistent with published increases in Cox activity following c-Src tyrosine phosphorylation of Cox II (Miyazaki et al., 2003).

This work lays a foundation for future studies on the interaction of EGFR and Cox II. As posited above, a 30 amino acid minimal region for interaction was suggested by the nexus of these studies with those of Demory and colleagues; this hypothesis, however, remains to be tested. Pull-down of the EGFR by a minimal mutant or the truncated Cox II globular head described in this addendum would be useful in defining a region of Cox II involved in the direct association of these proteins. Additionally, expression, ^{14}N labeling, and purification of the truncated Cox II globular head for NMR analysis with an EGFR pY845 peptide could identify critical residues of Cox II for interaction. These NMR data could be used for the rational drug design of a protein-protein interaction inhibitor for an improved definition of the biological effects of the association between EGFR and Cox II and, potentially, for the treatment of cancers overexpressing EGFR, such as glioblastoma, lung, and breast cancers.