Michigan State
University

This is to certify that the

dissertation entitled

THE ROLE OF RETINOBLASTOMA PROTEIN AND INSULIN LIKE GROWTH FACTOR SIGNALING IN ANTIESTROGEN RESISTANCE

presented by

Hemant Varma

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry
and Molecular Biology

Susan J. Conn
Major professor

Date 12/13/2001

O-12771

THE ROLE OF RETINOBLASTOMA PROTEIN AND INSULIN LIKE GROWTH FACTOR SIGNALING IN ANTIESTROGEN RESISTANCE

By

Hemant Varma

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Molecular Biology

2001

ABSTRACT

THE ROLE OF RETINOBLASTOMA PROTEIN AND INSULIN LIKE GROWTH FACTOR SIGNALING IN ANTIESTROGEN RESISTANCE

By

Hemant Varma

Approximately 40% of all human breast cancers are estrogen dependent for proliferation, making them responsive to antiestrogen therapy. However during the disease course, these tumors often progress to an antiestrogen resistant phenotype, resulting in high mortality and underscoring an urgent need to understand the basis of antiestrogen resistance. Members of the retinoblastoma family proteins are key negative regulators of cell cycle progression and are frequently mutated in a variety of tumors including breast tumors. MCF-7 is a human breast cancer cell line, that proliferates in response to estrogen treatment and is growth inhibited upon treatment with antiestrogens. The results presented in this thesis demonstrate that inactivation of the tumor suppressors of the retinoblastoma family using DNA tumor virus large T antigens enables MCF-7 cells to proliferate in the presence of antiestrogens. Using a number of SV40 large T mutants, we further demonstrate that the inactivation of pRb family members by large T is absolutely required for and may be sufficient for MCF-7 cells to acquire antiestrogen resistance. In addition, we have established MCF-7 derivatives that inducibly express Polyoma large T and find that expression of large T in antiestrogen treated MCF-7 cells

causes an induction of cyclin A, cdk2 and cdk4 activities, key regulators of cell cycle progression that are inhibited by antiestrogens.

In a related study, we tested whether the upregulation of Insulin Like Growth Factor-1 (IGF-1) signaling could cause antiestrogen resistance. The impetus for this study was the observation that IGF-1 is a potent mitogen of breast cancer cells and antiestrogens have been reported to inhibit IGF-1 stimulated proliferation. Numerous studies support the view that antiestrogens regulate breast cancer proliferation indirectly by downregulating the IGF-1 signaling pathway. Our results point to the contrary, since antiestrogen treatment did not prevent or decrease the signaling events induced by IGF-1 including the activation of MAPK and PI3K, key downstream effectors of IGF-1, as well as some of the cell cycle changes induced by IGF-1. Further, activation of intermediates in IGF-1 signaling pathway did not confer antiestrogen resistance to MCF-7 cells. These results suggest that activation of the IGF-1 signaling cascade alone is not sufficient to cause antiestrogen resistance.

In summary, the results of this study demonstrate that estrogen mediates its proliferative effects in MCF-7 cells by inactivating the pRb family members and suggest that loss of function or deregulation of the pRb pathway can potentially lead to antiestrogen resistance in breast cancer patients. Additionally, estrogen affects breast cancer proliferation without regulating growth factor (IGF-1) signaling but by impinging on the cell cycle components. These results suggest that deregulation of IGF-1 signaling alone does not confer antiestrogen resistance in breast cancer patients.

ACKNOWLEDGEMENTS

I would like to thank my mentor Susan Conrad for her advice, support and encouragement throughout these years. I would also like to thank my guidance committee members, Zachary Burton, Donald Jump, Richard Miksicek and Steve Triezenberg for their helpful advice and discussions. I would like to thank Michelle Fluck for her encouragement through these years and to Ron Patterson for teaching me how to play the "game". A big thanks to the current members of Conrad lab, Shibani Mukherjee, Andy Skildum and Yu Ping Tang for their help and their patience with high entropy.

Also thanks to all the past members of our lab and the Fluck lab, Feng Han, Heather Hirsch, Christopher Ontiveros, Maria Pino and Cunjie Zhang for their help and friendship over the years. I would also like to thank Sridhar Venkataraman and Dipnath Baidyaroy for their friendship, encouragement and for sharing their enthusiasm for science.

TABLE OF CONTENTS

CHAPTER 4

Antiestrogen ICI 182,780 Decreases Proliferation of IGF-1 Treated MCF-7 Cells Without Inhibiting the PI3K/AKT Pathway.

CHAPTER 5

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Mammary Gland Development

Mammalian newborns are dependent on maternal nutrition in the form of milk for survival during early infancy. To nurse their young ones mammals have evolved specialized organs called mammary glands that produce and secrete milk. The mammary glands remain rudimentary until puberty; they then develop in preparation for reproduction and eventual nursing of the newborn. At puberty, breast ducts grow and show increased branching, in part due to epithelial cell proliferation in response to complex hormonal and growth factor stimulation (1). This sets the stage for later development of lobuloalveoli, the milk producing tissue at the ends of ducts, which occurs during pregnancy.

During the menstrual cycle, cyclic changes in the levels of hormones cause alterations in breast epithelial cell proliferation (2). These regular alterations in the proliferative status of breast epithelial tissue during the long reproductive span (30-40 years) of women, makes breast epithelial tissue highly susceptible to tumor formation. At least one in eight women in the U.S. will develop breast cancer during her lifetime making it the most common cancer among women with 90 percent of these tumors being epithelial in origin. Despite current therapies, 50 percent of women diagnosed with breast cancer eventually die of the disease, making it the second leading cause of cancer death

among women in the U.S.A. The high incidence and mortality associated with breast cancer underscores the need for improved therapy for this disease.

Breast Cancer Genetics

Aberrant gene expression has been implicated in the development of breast cancer with the identification of genes whose deregulation can cause increased susceptibility to the genesis of breast cancer. Many insights have arisen from transgenic mouse models, where specific gene products are overexpressed in the mammary gland using mammary specific promoters. This has led to the identification of a number of genes including erbB2/Neu, c-myc, Wnt-1 and cyclin D1 whose overexpression can potentially lead to the development of breast cancers (3-7). On the other hand, familial cases of breast cancer have led to the identification of two genes, BRCA1 and BRCA2, that act as tumor suppressors and whose loss leads to the development of breast cancer at a very high frequency (8). Mutations in BRCA1 and BRCA2 account for only 5-10 percent of breast tumors (9).

Estrogen /Antiestrogens and Breast Cancer

Despite the identification of genes responsible for breast cancer in a small subset of patients, current knowledge regarding the genetic alterations in breast cancer is insufficient to target specific therapy for a large majority of patients. Furthermore, the underlying genetic changes leading to breast cancer are likely to vary widely between patients, preventing any generalized therapy. However, the proliferation of a large percentage of breast tumor cells remains responsive to estrogens, and the role of estrogen

in breast cancer growth is highlighted by the fact that 40% of breast cancers require estrogen for proliferation (10). The first insight into the regulation of breast cancer proliferation by estrogen was provided by Beatson's observation in the 1890's that surgical removal of the ovaries of breast cancer patients caused tumor regression (11). It was later discovered that estrogens were key ovarian hormones involved in both normal and breast tumor proliferation. Estrogens are steroidal compounds that play a role in regulating the development, differentiation and proliferation of reproductive tissues. 17β-estradiol, esterone and estriol are the major estrogenic compounds secreted by ovaries, and of these 17β-estradiol is the most potent estrogen. The circulating levels of these hormones increase dramatically at puberty, oscillate at these elevated levels during the menstrual cycle throughout the reproductive span of females, and finally decrease at menopause to prepubertal levels. These changes in the levels of estrogens correlate well with breast epithelial proliferation, as demonstrated by studies that ovariectomy in the prepubertal stage in mice prevents breast development and this can be reversed by estrogen supplementation (12). Furthermore, postmenopausal women show a decrease in ductal branching and atrophy of ducts due to decreased ovarian function.

The recognition of a key role for estrogens in breast cancer proliferation provided the rationale for the development of antiestrogenic compounds for the treatment of breast cancer patients. Thus, one of the major therapies for breast cancer consists of estrogen antagonists such as Tamoxifen and ICI 182,780. Tamoxifen, a non-steroidal analogue of estradiol is the most widely used antiestrogen therapy and has decreased breast cancer morbidity greatly, while having minimal side effects. Despite tumor regression and increased disease free survival, tumors initially sensitive to Tamoxifen eventually

develop antiestrogen resistance, and continue to grow upon Tamoxifen treatment (13). In order to develop improved therapeutics, it is essential to understand the mechanism(s) by which estrogens regulate the proliferation of breast tumors, and how tumors evolve to an antiestrogen resistant phenotype.

Estrogen Receptor: Mechanism of action of estrogen

Estrogens mediate their cellular effects by binding to specific receptor proteins, estrogen receptors (ERs) that are members of a family of ligand activated nuclear transcription factors. These are evolutionarily conserved proteins that bear homology across vertebrates -from fish to mammals (14). Presently, two genes for ER have been identified, ERα and ERβ. Both genes encode proteins with a high degree of homology, but with differences in tissue expression and function (15). ERα is expressed in a broad range of tissues, whereas ERβ is expressed largely in ovaries, prostate, epididymis, lung, and hypothalamus (16). The phenotypes of ER knockout mice suggest that ERα is involved in breast development and proliferation, since ERα knockout mice fail to develop breast tissue at puberty and breast tissue remains immature (17). In contrast, ERβ mice are fertile and their breast development is not impaired, suggesting that this receptor may not play a major role in breast proliferation (18).

ERα is a multidomain nuclear phosphoprotein that regulates transcription of cellular genes by binding to specific DNA sequences called enhancer elements present in target genes. It is composed of a DNA binding domain, a ligand binding domain, two transcription activation domains, one at the N-terminus (AF-1) and the other at the C-terminus (AF-2) and a receptor dimerization domain. At least two different modes of ER

activ

indep

calle

from

activ

prot

regu

addi

inde

(ser

tran

coa

A

tran

cha

fan

ass

lea

(26

fun

anc

gla

activation are known, one involving ligand binding and the other being ligand independent. Ligand binding induces ER dimerization and binding to enhancer elements called estrogen response elements (EREs) in the target genes, and induces transcription from target genes. The C-terminal AF-2 is largely involved in this ligand induced ER activation (19). Alternatively, the ligand bound ER may activate certain genes by protein-protein interactions with members of the activator protein-1 (AP-1) family, and thereby regulate the transcription of genes containing the AP-1 DNA binding site (20). In addition, ER has been reported to enhance transcription of target genes via a ligand independent mechanism. This involves phosphorylation of certain serine residues (ser118) in the AF-1 region by growth factor signaling cascades, which enhance ER's transcriptional activity on ERE containing genes (21).

Ultimately, the regulation of gene transcription by ER involves recruitment of coactivators that activate the transcriptional machinery on the promoters of target genes. A number of coactivators have been discovered, and their roles in ER mediated transcription have been somewhat defined. Upon ligand binding to ER, a conformational change is induced in the AF-2 domain (22). This results in the recruitment of the p160 family of coactivators, that include SRC-1, TIF-2 or Grip1 and AIB (23-25) and associated factors involved in chromatin remodeling to the promoters. These changes lead to the activation of RNA polymerase mediated transcription from the target genes (26). The relevance of coactivators to ER function, as well as the overlap in coactivator function, is demonstrated by the phenotype of SRC-1 knockout mice, which are viable and fertile, but partially resistant to the effects of estrogen and show decreased mammary gland proliferation (27). Since coactivators are involved in ER's actions at the

transcriptional level, alterations of these cofactors could potentially be involved in the deregulation of ER mediated gene expression and proliferation. Indeed, the SRC family member AIB-1/SRC-3 is overexpressed in 60% of primary breast cancers, and is amplified in 10% of breast tumors (24, 28).

Eukaryotic Cell Cycle

In order to increase the proliferation of cells, mitogens including estrogen have to impinge on the machinery controlling cell proliferation. Over the past two decades, a better understanding of the players involved in cell cycle progression and their roles in proliferation has been gained. Cell proliferation involves the doubling of the DNA content of a cell by the process of DNA replication, followed by equal distribution of the DNA to daughter cells during cell division. Most somatic cells proliferate via a highly ordered sequence of events that can be divided into four different phases. Initially cells with a 2N content of DNA go through a growth phase called gap1 or G1. During this phase the cell integrates various internal and environmental signals before committing to cell division. This phase is mitogen responsive, and at a point late in G1 called the restriction point, a cell commits to cell division. Once the decision to divide is made, the cells are mitogen insensitive and progress into S phase where DNA replication occurs. This is followed by a gap2 or G2 phase where cells prepare for mitosis, and finally cell division takes place by mitosis followed by cytokinesis in the M phase (See Figure 1.1).

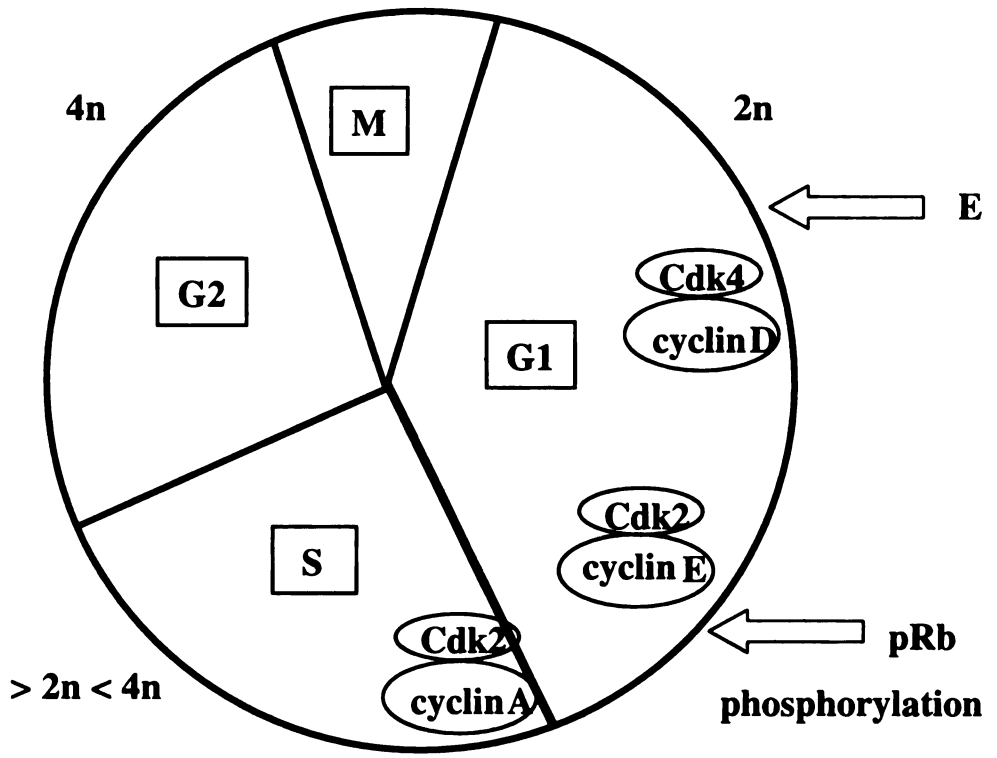


Figure 1.1. Simplified model of the eukaryotic cell cycle. Cells progress through a highly ordered sequence of events called the cell cycle they replicate their DNA and undergo cell division. Cells in G1 are stimulated by mitogens such as estrogen (E), which enhance progression through this phase of the cell cycle. Mitogens enhance cell cycle progression through the G1 phase by activating serine threonine kinases called Cdks of which there are two types, Cdk4 and Cdk2. Cdk4 is activated upon binding to cyclin D in early G1 while Cdk2 is activated as complexes with both cyclin E or cyclin A in late G1. These kinases phosphorlylate and inactivate the pRb family of tumor suppressors during mid to late G1 that results in the release of transcription factors that regulate genes required for DNA replication. Thereafter the cells progress into S phase where they duplicate their DNA (from 2n to 4n). This is followed by a gap phase G2 that precedes cell division in M Phase.

Tumor Suppressors of the pRb family

The decision to divide is based on a balance between negative and positive regulators of cell division. At the center of the negative regulation of the cell cycle is a family of tumor suppressors called the retinoblastoma family of proteins, which consists of pRb and the related family members p107 and p130. The role of pRb as a tumor suppressor was established by the fact that individuals lacking one copy of the pRb gene developed a rare form of retinal tumor called retinoblastoma (29). Further studies demonstrated that pRb serves a general role as a regulator of cell cycle progression (See Figure 1.2) (30) and have better defined the role of pRb in cell cycle progression. pRb is a 928 amino acid nuclear phosphoprotein that has at least 16 potential serine/threonine phosphorylation sites (31, 32). In its under/hypophosphorylated state, pRb binds to and inhibits the E2F family of transcription factors (33) that regulate transcription of genes that are important for DNA synthesis (34). Upon phosphorylation, pRb is inactivated and is no longer able to bind to the E2F family proteins, making them free to activate transcription (35, 36). Since pRb phosphorylation occurs during mid to late G1, it has been suggested that it may represent the "restriction point" that commits cells to progress to S phase, and that once pRb is inactivated cells may be mitogen independent. The importance of pRb inactivation in cell cycle progression is highlighted by the fact that mutations in pRb are reported in a variety of tumors, including breast tumors (30). Additionally, various DNA tumor viruses that usurp the cellular DNA replication machinery to replicate their DNA, encode viral proteins that specifically target and inactivate the pRb family of proteins, thus inducing resting cells to initiate DNA replication (37). Large Tumor (LT) antigens encoded by the SV40 and polyoma viruses,

adenoviral E1A protein and E7 protein of human papillomaviruses, all bind to and inactivate hypophosphorylated pRb family members (38, 39). The role of the other pRb family members in cell cycle progression is less well defined. Although overexpression of p107 or p130 causes growth arrest in cells, they are only rarely mutated in tumors. Both p107 and p130 are phosphorylated in a manner similar to pRb and bind to members of the E2F family of transcription factors. The phenotype of knockout mice for the various family members demonstrate that pRb is essential for murine development, as Rb null mice die in utero due to defects in hematogenesis and neurogenesis (40-42). In contrast single p107 and p130 knockout mice are viable, and do not show any apparent phenotype (43, 44) whereas p107/p130 double knockout die shortly after birth (44). These studies highlight the essential but distinct functions of pRb and p107/p130 proteins. Further, these results suggest that p107/p130 proteins have overlapping function(s) that are distinct from pRb (43, 45). Studies using mouse embryo fibroblasts derived from Rb knockout mice show a distinct decrease in the duration of G1, and upregulation of E2F target genes, though cells are not transformed (46). Recently, triple knockout mice have been generated in which all three pRb members are deleted, and fibroblasts derived from these embryos show a faster population doubling time compared to single or double knockout mice with any combination of pRb family members inactivated. Further, these cells are not contact inhibited, are immortalized in culture, and show features of transformed cells. This provides strong evidence that the loss of all three members contributes to transformation, and suggests some degree of functional overlap between the pRb family members (47).

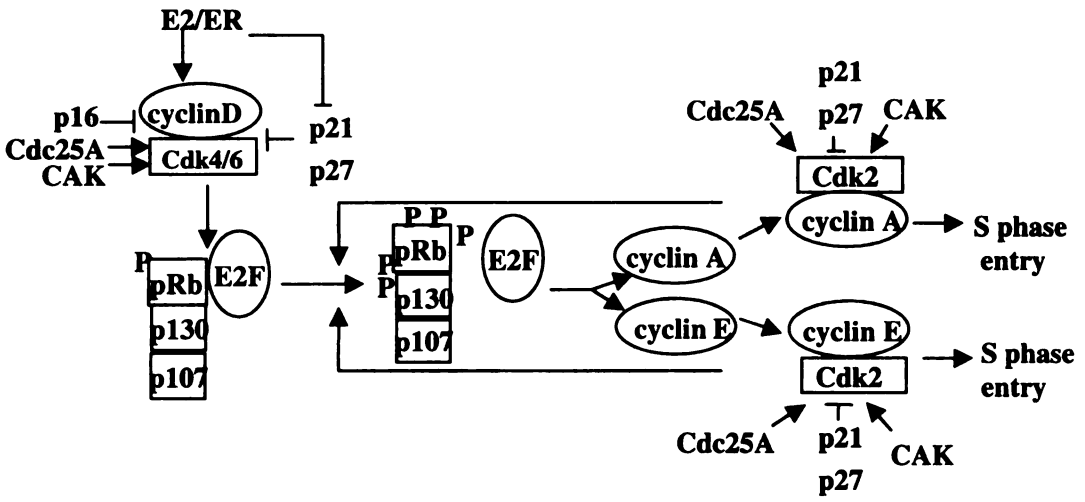


Figure 1.2. Model of the cyclin/ Cdk pathway. (adapted from Sherr C.J., Science 274: 1674 Fig.2). In this model cyclin D1/Cdk4 complexes phosphorylate the pRb family thereby releasing the E2F family of transcription factors. E2F regulated genes such as cyclin E and cyclin A get induced and activate Cdk2 to promote S phase entry. A positive feedback loop exists wherein the activated Cdk2 complexes can also phosphorylate pRb. Cyclin-Cdk activity is also controlled by interaction with two families of Cdk inhibitors: the INK4 family which includes $p16^{INK4A}$, and the $p21^{Waf1}/p27^{KIP}$ family. An additional level of regulation is provided by Cdc25A phoshatase, which removes inhibitory phosphorylation on Cdk's, and by the Cdk activating kinases (CAKs), which carry out phosphorylation/dephosphorylation of specific conserved residues on the Cdks. Some of the known targets of estrogen are indicated.

Kinase

kinase

throug

name

of w

Cdk-

prog

dem

of f

the

kn

oth

T

C

C

f

Kinase Inhibitory Proteins: KIP and INK4 Families

A second class of tumor suppressors consists of two families of cyclin dependent kinase inhibitors, the INK4 and the KIP families, which negatively regulate progression through the cell cycle. The INK4 family of kinase inhibitors consists of four proteins named for their apparent molecular weight of p15, p16, p18 and p19 (48). These proteins, of which $p16^{INK4A}$ was initially discovered, bind to and inactivate a subset of kinases, Cdk4/6, that phosphorylate pRb (49). The members of the INK4 family prevent cell cycle progression by inhibiting the activity of kinases involved in pRb phosphorylation, as demonstrated by their ability to inhibit proliferation in the presence but not in the absence of functional Rb (50). The importance of $p16^{INK4A}$ in cell proliferation is highlighted by the observation that $p16^{INK4A}$ is frequently mutated in tumors and the fact that $p16^{INK4A}$ knockout mice though viable, have an increased frequency of tumors (51). The role of the other family members in vivo appears to be similar to that of $p16^{INK4A}$.

The second family consists of three kinase inhibitors, $p21^{Waf1}$, $p27^{Kip1}$ and $p57^{Kip2}$. These kinase inhibitors bind to and inhibit Cdk2 activity (52). Their role as inhibitors of Cdk4/6 is more controversial, since some reports suggest that they are inhibitors of Cdk4/6 (52) while others have reported that these are necessary for Cdk4/6 complex formation (53). The levels of these kinase inhibitors are regulated by mitogens and other growth stimuli, with mitogens downregulating and anti-mitogens increasing their levels. This is demonstrated by contact and TGFβ mediated arrest of mink lung fibroblasts, which is caused by increases in $p27^{Kip1}$ protein levels and resulting decreases in Cdk2 activity (54). Consistent with its role as a tumor suppressor, $p21^{Waf1}$ knockout mice have

defectiv

knocko

56. 5

sugges

doubl

differ

alone

deper

Cdk

that

(60

type

con

to

pho

dep

tig

sp

th

D

defective G1 checkpoint controls (55). A similar phenotype was noted for $p27^{Kip1}$ knockout mice, with increased body weight, tumor formation and increased Cdk2 activity (56, 57). The $p57^{Kip2}$ knockout mice have severe developmental abnormalities (58), suggesting functional differences between the various Kip family members. Interestingly, double knockout mice generated by knocking out individual members from the two different families, Kip and INK4, show enhanced tumor formation compared to either alone, suggesting functional collaboration between the different families of cyclin-dependent kinase inhibitors (59).

Cdks, Cyclins , and Cdc25 phosphatases

The positive regulators of the cell cycle are a group of serine/threonine kinases that in the active state exist as binary complexes of a catalytic and a regulatory subunit (60). The catalytic unit is a cyclin dependent kinase (Cdk) of which there are two major types Cdk4/6 and Cdk2 in higher eukaryotes. The levels of expression of the Cdks are constitutive, but their activity is regulated by multiple mechanisms including by binding to specific cyclin partners, interaction with two families of Cdk inhibitors, phosphorylation on certain conserved residues by Cdk activating enzymes and dephosphorylation by phosphatases of the Cdc25A family. The levels of cyclins are tightly regulated by mitogenic stimuli and the cell cycle phase. By regulating the levels of specific cyclins, mitogens and antimitogens regulate the activities of specific Cdks during the different phases of the cell cycle.

Cyclin D is a major group of G1 cyclins, consisting of three different proteins D1, D2 and D3 with overlapping expression in proliferating cells. These proteins are

expressed in response to mitogen stimulation, and activate the Cdk4/6 group of cyclin dependent kinases (61). The only known targets for cyclin D-Cdk4/6 complexes are the members of the pRb protein family (62). Cyclin D1 is better studied compared to the other members of the cyclin D family and appears to play an important role in the proliferation of both normal and tumor breast tissue. The role of cyclin D1 protein in mediating mitogenic responses is highlighted by the fact that overexpression of cyclin D1 reduces serum dependence for proliferation and accelerates G1 progression (63-65). Further, overexpression of cyclin D1 is found in a large percentage of tumors including breast cancers (30, 66, 67). Transgenic mice overexpressing cyclin D1 under a mammary specific promoter develop mammary carcinomas (7). Conversely, cyclin D1 knockout mice are smaller in size than wild type mice, show defects in mammary gland development, and are resistant to tumor formation in response to overexpression of certain oncogenes showing an important and somewhat specific role for cyclin D1 in mammary tissue proliferation (68). Recently, a role for cyclin D1-Cdk4 complexes in sequestering $p21^{Waf1}$ and $p27^{Kip1}$ from the Cdk2 complexes has been proposed (69). This model is supported by *in vivo* genetic evidence, demonstrating that the phenotype of cyclin D1 knockout mice is restored in $p27^{Kip1}$/cyclin D1 double knockout mice (70).

Cyclin E, the other major G1 cyclin, forms complexes with and activates Cdk2 and plays an essential role in G1 and S phase progression (71-73). Cyclin E is upregulated in response to mitogenic stimuli and has E2F responsive elements in its promoter, making it a downstream target of pRb (74). One of the targets of cyclin E/Cdk2 complexes are the pRb family members, suggesting the presence of a feedback loop that enhances the inactivation of pRb and accumulation of active cyclin E-Cdk2 kinase

comp

Cdk

Cycl

tissu

acti

p16

acti

oth

neg

nov

tran

DN

pro

cle

a p

ph

thr

re

de

C

de

C

complexes (Figure 1.2) (74). Evidence in the past few years has suggested that cyclin E-Cdk2 may be the ultimate activity required for G1 progression and entry into S phase. Cyclin D1 knockout mice recover their phenotype upon expression of cyclin E in target tissues (75). Further, growth arrest mediated via activation of pRb using a constitutively active pRb that is non-phosphorylatable (76) or by inhibition of Cdk4/6 activities by $p16^{INK4A}$ can be overcome by cyclin E overexpression, placing cyclin E-Cdk2 kinase activity downstream of cyclin D1-Cdk4 and pRb (77). The cyclin E-Cdk2 has targets other than pRb family members, as inferred by its requirement for proliferation in pRb negative cells, unlike cyclin D1 which is dispensable in such cells (73). Recently, several novel substrates of cyclin E-Cdk2 have been identified, including NPAT, a transcriptional activator of histone genes. Histones are essential for the packaging of DNA into nucleosomes, partly explaining the importance of cyclin E-Cdk2 for proliferation (78). Although all the targets of cyclin E-Cdk2 are yet to be identified, it is clear that its kinase activity is essential for G1 progression and S phase entry, probably by a pathway parallel to or downstream of pRb.

An additional layer of regulation of Cdks occurs at the post-translational level by phosphorylation and dephosphorylation events. Cdks are phosphorylated at a conserved threonine residue by Cdk activating kinase (CAK), with phosphorylation at this site being required for Cdk activation (60). Cdk2 activation also involves the removal of a deactivating phosphorylation that is accomplished by a phosphatase called Cdc25A. Cdc25A is a member of Cdc25 family of dual-specificity phosphatases that dephosphorylates the negative regulatory phosphates at threonine-14 and tyrosine-15 in Cdks, leading to their activation (79). The Cdc25 family of phosphatases consists of three

different enzymes Cdc25A, B and C, encoded by 3 different genes. Of these, Cdc25A phosphatase is involved in the G1-S transition, since antisense to Cdc25A arrests cells in the G1 phase of the cell cycle (80) and overexpression leads to premature activation of Cdk2 activity, and decreased duration of G1 (81). Moreover, overexpression of Cdc25A has been found in variety of tumors implicating it as an oncogene (82). Interestingly, Cdc25A activity is regulated by phosphorylation that is accomplished by cyclin E-Cdk2 kinase (83) suggesting a positive feedback loop whereby the cyclin E-Cdk2 activates Cdc25A that in turn activates cyclin E-Cdk2 by dephosphorylation of Cdk2. In addition, reports suggest that Cdk4 activity may be activated by Cdc25A mediated de-phosphorylation under certain circumstances (84).

Once cells have committed to DNA replication in late G1 phase, they enter a phase of duplication of the DNA content called S phase. Entry into and progression through this phase requires cyclin A-Cdk2 activity that is induced at the G1-S phase boundary and is highly expressed throughout S phase. Inhibition of cyclin A by microinjecting antibodies to cyclin A results in an arrest at the G1-S boundary (85). The cyclin A gene is positively regulated by E2F transcription factors, and is induced as a consequence of pRb inactivation.

After DNA replication in S phase, cells pass through a G2 or growth phase where cells prepare for cell division in which the replicated DNA is equally divided between the two daughter cells. The relative duration of these phases are usually cell type specific and invariant for a certain cell type. The actual division of the genetic material and cell division occur during mitosis or M phase. During this phase pRb is dephosphorylated, by as yet unidentified phosphatases to return the cells into the G1 phase.

Estrogen and Cell Cycle regulation

With the identification of the events and the players controlling the cell division cycle, the identity of the specific targets that estradiol regulates in order to induce proliferation of breast tumor cells was addressed by a number of laboratories including ours. The models to study breast cancer proliferation induced by estradiol were developed in the 1970's. These models consist of tumor derived cell lines from patients with advanced breast cancer. One of the earliest successful attempts at this approach resulted in the development of an estrogen receptor positive breast cancer cell line at the Michigan Cancer Foundation by H.D. Soule, which was designated the MCF-7 cell line (86, 87). This cell line grows in response to estrogen in tissue culture media, as well as when implanted into nude mice. Furthermore, tumors derived from MCF-7 cells in nude mice are absolutely dependent upon estrogen for growth, as ovariectomized mice do not form tumors without estrogen supplements (88). MCF-7 cells are very widely studied as a model of estrogen dependent human breast cancers. Other human breast cancer cell lines that are estrogen responsive include T47D, ZR-75-1 and CAMA-1 (89-91). Studies using these cell lines have identified targets of estrogen that are involved in proliferation, and are building a comprehensive picture of how estrogens cause proliferation of breast cancer cells. Treatment of estrogen responsive cells with estrogen enhances progression of cells through the G1 phase of the cell cycle, and thereby increases the percentage of cells that synthesize DNA. The duration of the other cell cycle phases is unaffected.

The protooncogenes c-fos, c-jun and c-myc are one of the first groups of genes induced by estrogen (92). These protooncogenes play an essential role in cell cycle progression. c-Myc is a transcription factor that is rapidly induced upon estrogen

treatment of estrogen responsive cells (93). The c-Myc protein in turn induces transcription of a number of cell cycle regulatory proteins including cyclin E, Cdc25A, and decreases transcription of the $p21^{Waf1}$ kinase inhibitor in different cell types (94). c-Myc's essential role in estrogen-induced proliferation is highlighted by the fact that c-myc antisense inhibits estrogen-induced proliferation (95). Furthermore, overexpression of c-Myc can cause estrogen independent proliferation of MCF-7 cells (96). c-Jun appears to play an important role in estrogen induced proliferation, as overexpression of this protein in MCF-7 cells leads to proliferation in the absence of estrogen (97). c-Jun is implicated in the regulation of cyclin D1 expression. Estrogen induced transcription of cyclin D1 has been shown to be regulated by a CRE element that binds to Jun-ATF-2 heterodimers (98). c-Fos also apparently plays an important role in estrogen-induced proliferation since downregulation of c-Fos levels by an antisense oligonucleotide strategy inhibits estrogen induced proliferation (99). Thus the protooncogenes regulated by estrogen connect estrogen signaling to the cell cycle machinery, although the exact mechanisms involved and targets in the cell cycle machinery of some of these immediate early genes still remain to be determined.

The regulation of components of the cell cycle machinery by estrogen has been extensively studied. Upon estrogen treatment of estrogen starved or antiestrogen treated cells, an increase in Cdk4 and cyclin E-Cdk2 activities are observed and are accompanied by increases in pRb phosphorylation. An increase in the levels of cyclin D1 protein is one of the earliest changes in the cell cycle regulatory machinery, occurring between 2-4 hours following estrogen treatment and has been proposed to account for the increase in cyclin D1 associated Cdk4 activity. In MCF-7 cells the levels of cyclin E are constitutive

and

level

cycli

kina

activ

may

supp

cell

cyc

acti

me

me

pro

cyc

or

dis

Th

in

ind

cy

es

ex

and increases in cyclin E-Cdk2 activity cannot be explained by changes in cyclin E levels. The increase in cyclin D1 has also been implicated in relieving inhibition of Cdk2-cyclin E complexes by titrating the kinase inhibitor $p21^{Waf1}$ from the cyclin E-Cdk2 kinase complexes to cyclin D1-Cdk4 complexes (100, 101). Because cyclin E-Cdk2 activity is essential for proliferation, it has been suggested that upregulation of cyclin D1 may thus mediate estrogen's effect on cell cycle progression. This interpretation is supported by the fact that overexpression of cyclin D1 can cause proliferation of MCF-7 cells arrested in G1 by antiestrogen treatment (96). The increase in proliferation upon cyclin D1 overexpression is accompanied by an induction of Cdk4 and cyclin E-Cdk2 activities and pRb phosphorylation and is consistent with increases in cyclin D1 mediating estrogen's effects.

However, other studies have demonstrated a role for additional pathways in mediating the proliferative effects of estrogens. c-Myc overexpression induces proliferation in antiestrogen treated MCF-7 cells, and is accompanied by an increase in cyclin E-Cdk2 activity and pRb phosphorylation, but without an induction of cyclin D1 or Cdk4 activity (96). Thus, it appears that both cyclin D1 and c-Myc expression are distinct targets and are independently required for the effects on estrogen on proliferation. This model is supported by a recent study which showed that inhibition of Cdk4 activity in MCF-7 cells, by infection with a $p16^{INK4A}$ encoding adenovirus, did not prevent the induction of c-Myc or Cdc25A and downregulation of $p21^{Waf1}$ by estrogen, but inhibited cyclin E-Cdk2 activation, pRb phosphorylation and proliferation (102). The fact that estrogen could not induce proliferation in $p16^{INK4A}$ overexpressing cells despite c-Myc expression indicates the levels of c-Myc induced by estrogen are not sufficient to activate

cyclin

regul

regul

of es

upon

the l

7 ce

expr

loss

inhi

mul

mec

act

qua

act

tha

on

be

G

F

cyclin E-Cdk2 kinase activity and proliferation. It also suggests that estrogen is regulating at least two distinct pathways affecting the cell cycle. In addition, a role for the regulation of the Cdk inhibitors $p21^{Waf1}$ and $p27^{Kip1}$ in mediating the proliferative effects of estrogen is suggested by the observations that both these proteins are downregulated upon treatment of MCF-7 cells with estrogen (100, 101, 103). Furthermore, decreasing the levels of these proteins using antisense oligonucleotides causes proliferation of MCF-7 cells arrested in G1 by antiestrogen treatment (104). Significantly, a loss of $p27^{Kip1}$ expression has been correlated with poor prognosis in breast cancer patients (105). The loss of these kinase inhibitors would be expected to release the cyclin E-Cdk2 from inhibition and thus induce proliferation. These studies bring into focus not only the multiple targets that may be involved in estrogen mediated proliferation but also mechanisms that may lead to cells becoming antiestrogen resistant.

Since the changes in cell cycle components induced by estrogen result in the activation of Cdks, and pRb inactivation may require two distinct Cdks (32, 106), a question that is still unanswered is whether the ultimate effect of Cdk4 and Cdk2 activation by estrogen is to inactivate pRb, or if there are additional targets of estrogen that are required for its proliferative effects. The answer to this question is important not only for understanding the pathways involved in estrogen mediated proliferation, but also because of the implications this may have for acquired antiestrogen resistance.

Growth Factors and Breast Cancer

In addition to estrogens, a variety of growth factors including Insulin Like Growth Factor-1 and 2 (IGF-1 and IGF-2), Epidermal Growth Factor (EGF), Transforming

Growth Factor α (TGFα) and Fibroblast Growth Factor (FGF) have been identified as potent mitogens for breast tumor proliferation (107-109). A number of studies have suggested that estrogens affect breast tissue proliferation in an indirect manner by inducing secretion of growth factors (110, 111). The secretion of both EGF and IGF-1 have been reported to increase upon estrogen treatment of breast cancer cell lines *in vitro* (112, 113), and estrogen induced growth factors could partially replace estrogen to promote tumor growth (114). A paracrine and/or autocrine role for growth factors in mediating estrogen's effects on mammary epithelial growth *in vivo* is supported by the observation that proliferating breast epithelial cells *in vivo* are not ER positive (115). In addition, breast epithelium from ER knockout mice transplanted into the fat pads of wild type mice is capable of proliferation in response to estrogens. However, in the reverse experiment, breast epithelial cells failed to proliferate, suggesting a role for paracrine factors in the stroma, in the induction of breast tissue proliferation (116). However, the role of paracrine factors induced by estrogens in breast cancer proliferation is still not well defined. ER may exert a more direct effect on breast cancer proliferation since 70% of breast cancers that are ER positive are responsive to the proliferative effects of estrogen while ER negative tumors are rarely so. In addition, all estrogen responsive breast cancer cell lines are ER positive and are responsive to estrogen *in vitro* in the absence of stromal components. However, a role for autocrine factors secreted by estrogen cannot be ruled out.

IGF-1 is a potent mitogen for breast cancer cells (117). Alterations in the IGF-1 signaling pathway have been implicated in breast tumor progression (118), and have been correlated with decreased disease free survival in ER positive breast cancer patients

(11

IGF

mic

est

pro

str

IGF

het

pri

unc

adj

act

pat

is

pat

int

pro

mo

inc

act

to

the

(119). The role of IGF-1 in increasing breast cancer proliferation is not surprising since IGF-1 is required for the proliferation of normal breast tissue *in vivo*. IGF-1 knockout mice fail to develop breast tissue beyond the prepubertal stage despite the presence of estrogen, suggesting a critical role for IGF-1 in breast tissue development and proliferation (120).

IGF-1 is a 70 amino acid protein secreted largely by the liver and the breast stroma, (121) consistent with its postulated paracrine role in breast proliferation (122). IGF-1 binds to its cognate receptor, the IGF-1 receptor (IGF-1R), which is a heterotetrameric transmembrane protein tyrosine kinase expressed on the surface of primary breast tumor cells (123). Upon activation by IGF-1 binding, the IGF-1R undergoes autophosphorylation on tyrosine residues. This stimulates the recruitment of adapter proteins like Insulin receptor substrate-1 (IRS-1) (124), which in turn recruit and activate signaling molecules resulting in the activation of the MAPK and PI3K signaling pathway and increased proliferation (125, 126). Interestingly, IGF-1 induced proliferation is inhibited by antiestrogen treatment *in vitro*, suggesting that these two signaling pathways interact (127, 128). A number of studies have reported regulation of intermediates in the IGF-1 signaling pathway, including the levels of IGF-1R and IRS-1 protein and the phosphorylation of IRS-1, PI3K and AKT, the downstream signaling molecules in the IGF-1 signaling pathway by estrogens and antiestrogens. These data indicate an indirect effect of estrogens on proliferation (129-133). Similarly, IGF-1 activates the MAPK signaling cascade in MCF-7 cells, and estrogens have been reported to regulate the MAPK signaling pathway (134, 135), although there are some reports to the contrary. Despite the above mentioned data, it is still unclear if antiestrogens inhibit

growth factor induced proliferation by downregulating components of growth factor signaling, or if both growth factor and ER signaling are independently required for proliferation. In the former case an upregulation of growth factor signaling components could result in antiestrogen resistance, whereas in the latter case this would not be true.

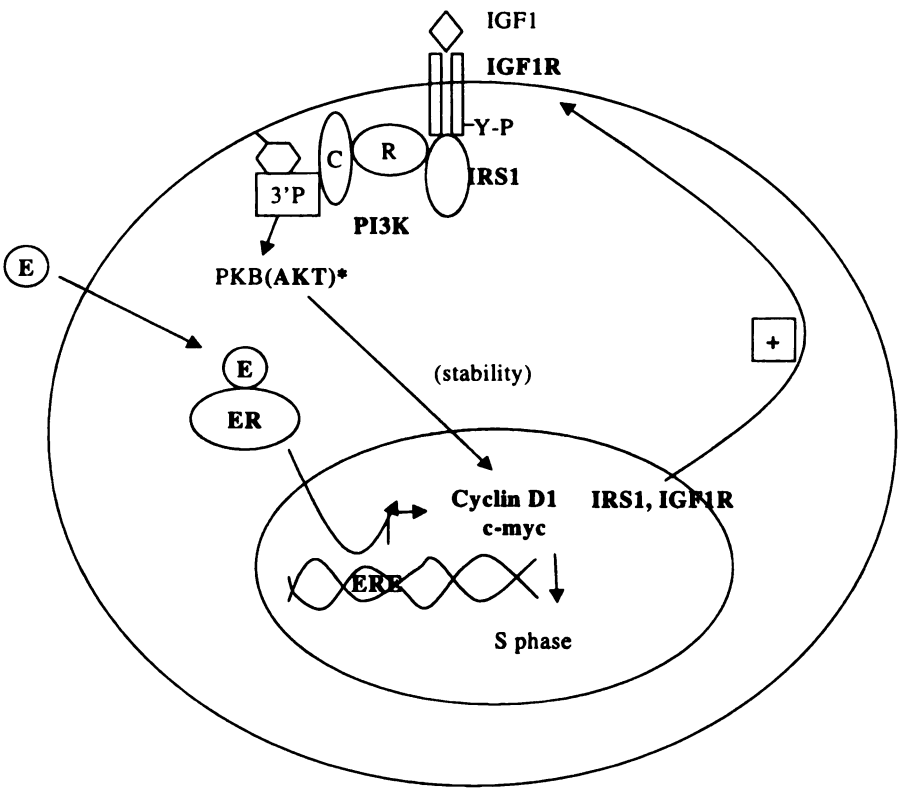


Figure 1.3. Interaction of IGF-1 and estrogen signaling. IGF-1 signaling and signaling via estrogen receptor can potentially interact at multiple levels to regulate the proliferation of breast cancer cells. IGF-1 binds to its membrane bound receptor (IGF-1R) and leads to autophosphorylation of the receptor. Phosphorylated IGF-1R then recruits IRS-1, which causes membrane localization of PI3K, a lipid inositol kinase. This further recruits enzymes that lead to a cascade of signaling events including AKT activation. Activated AKT causes increased cyclin D1 stability and is one of the ways that IGF-1 signaling upregulates cell cycle components. Estrogen by activating the estrogen receptor can also regulate the transcription of cyclin D1. Thus, both IGF-1 and estrogen could be independently regulating the same cell cycle components However, both IGF-1R and IRS-1 genes are transcriptionally upregulated by estrogen, and an increase in IGF-1 signaling could explain estrogen's proliferative effects on breast cancer cells.

Antie

devel

devel

is the

led to

and

Tam

tissu

latte

carc

the

can

cor

dif

cor

Ta

or

re

in

of

dc

Antiestrogen therapy and antiestrogen resistance

Once the role of estrogen in breast cancer proliferation was recognized, efforts to develop a rational therapy that would antagonize the effects of estrogen led to the development of antiestrogenic compounds. Tamoxifen is a nonsteroidal antiestrogen and is the most widely used therapy to treat estrogen responsive breast tumors. Later research led to the development of steroidal antagonists of estradiol, such as ICI 182,780 (136).

Tamoxifen is a partial estrogen antagonist. In certain tissues it acts as an agonist and activates estrogen responsive genes, while in others it antagonizes estrogen's effects. Tamoxifen acts as an antagonist in breast tissue, while it is an agonist in some other tissues such as the uterine endometrium (137), cardiovascular and skeletal tissues. In the latter two tissues, the effects of estrogens are thought to be protective against cardiovascular disease and osteoporosis (138, 139). The protective effects of estrogen on the above tissues are not inhibited by Tamoxifen, making it a specific treatment for breast cancer with minimal adverse effects. Although both steroidal and nonsteroidal compounds are competitive inhibitors of estrogen binding to the ER, they have somewhat different actions. Tamoxifen has a 100 fold lower binding affinity to the estrogen receptor compared to estradiol and the other steroidal antagonists such as ICI 182,780. Binding of Tamoxifen to the ligand binding domain of estrogen receptor does not affect dimerization or DNA binding of ER but causes changes in ER conformation (22) that result in recruitment of corepressors instead of coactivators to the promoter (26), and concurrent inhibition of transcription of genes important for proliferation. Interestingly, recruitment of a coactivator to the Tamoxifen bound ER, by substituting its nuclear receptor binding domain with that of a corepressor, induces transcription from target genes by Tamoxifen

bound ER (26). The partial agonist activity of Tamoxifen in some tissues is yet to be explained, but may be due to differential expression or binding of coactivator/corepressors in different tissues. In contrast, the steroidal antagonists such as ICI 182,780 have no agonist effects, in part explained by the degradation of the ER induced by these compounds and leads to adverse effects in other tissues where estrogen is protective.

Numerous studies have shown that cells that are initially sensitive to Tamoxifen eventually stop responding to Tamoxifen over the course of therapy (140). This acquired resistance is recapitulated in both *in vivo* and *in vitro* models using MCF-7 cells. Work by K. Osborne has shown that MCF-7 cells implanted in nude mice initially do not grow in the presence of Tamoxifen (141). However, over the course of 3-4 months, these tumors develop to a state of antiestrogen resistance (142). Some of these Tamoxifen resistant tumors are responsive to ICI 182,780, suggesting that the ER is still required for their proliferation (143). However, the adverse effects of steroidal antagonists, such as exacerbated bone loss, limit the use of these drugs as second lines of treatment. There is still limited clinical information on the development of resistance to steroidal antagonists such as ICI 182,780, because this compound has been used in clinical trials only recently (143). However, resistance to ICI 182,780 can potentially occur, as some Tamoxifen resistant tumors do not respond to steroidal antiestrogens (144) and ICI resistant clones can be selected *in vitro* (145) and in mice (146) and this can occur without a loss of ER (147-149).

Because antiestrogen resistance is a major cause of treatment failure and mortality in breast cancer, understanding the mechanisms of this resistance is critical to the

develo

can e

metab

resist

therap

antie

antie

estro

evide

expr

gene

cont

Rece

in es

cell

(15

for

acti

stu

pat

tha

red

red

development of improved therapeutics for these patients. It is apparent that breast cancers can employ a variety of mechanisms to acquire antiestrogen resistance (150). Altered metabolism of antiestrogens by tumor cells could potentially contribute to antiestrogen resistance, as could increased production of estrogen by patients undergoing prolonged therapy with Tamoxifen (13). The loss of estrogen receptor (ER) would result in antiestrogen resistance, with a subset of tumors showing receptor loss, though most antiestrogen resistant tumors retain ER expression (151). Expression of variant forms of estrogen receptor may be involved in the development of antiestrogen resistance, but the evidence for such a mechanism is still not definitive (152). Additionally, changes in the expression of coactivators/corepressors are another potential mechanism for the generation of resistance. Changes in growth factor signaling have also been reported to contribute to antiestrogen resistance. Epidermal growth factor activates the EGF-Receptor (EGF-R), a membrane bound tyrosine kinase receptor that leads to proliferation in estrogen dependent and independent breast cancer cells and of normal breast epithelial cells (114, 153). Expression of the EGF-R has been correlated with Tamoxifen resistance (154),(155) and the related receptor erbB2 has been shown to decrease the requirement for estrogen for growth (156). Also, changes in the expression of EGF/HER2 ligands that activate the EGF-R have been correlated with antiestrogen resistance (157). Similarly, studies have suggested that overexpression of components of the IGF-1 signaling pathways may contribute to a loss of estrogen dependence for growth. MCF-7 cell lines that overexpress IGF-1 receptor (IGF-1R) or the IGF-1R downstream target IRS-1, have reduced requirements for estrogen for proliferation. Also cells expressing FGFα have reduced requirements for estrogens for proliferation, and are able to proliferate in the

pres

over

(161

Tam

deve

pote

mod

ove

cyc

ove

a fa

not

dec

by

res

ant

cau

sig

IGI

hav

IGI

presence of Tamoxifen and ICI 182,780 (144, 158-160). Similarly, MCF-7 cells overexpressing TGFα show an estrogen independent and Tamoxifen resistant phenotype (161). In summary, a variety of events, including a loss of ER, altered metabolism of Tamoxifen and upregulation of growth factor signaling pathways can lead to the development of antiestrogen resistance.

Alterations in the cell cycle components regulated by estrogen could also potentially lead to the development of antiestrogen resistance. Studies using *in vitro* models have suggested that alterations in a number of cell cycle components including overexpression of c-Myc, c-Jun, cyclin D1 and loss of the negative regulators of cell cycle $p21^{Waf1}$ and $p27^{Kip1}$ can result in antiestrogen resistance (96, 97, 104). Though overexpression of cyclin D1 and c-myc as well as loss of $p27^{Kip1}$ has been documented in a fair percentage of breast cancers, the clinical relevance of these findings at present is not fully defined, nor are the levels of these proteins used for diagnostic or therapeutic decisions at present.

The goal of this study was to define the events in the cell cycle that are regulated by estrogens and whose deregulation may lead to the development of antiestrogen resistance. In particular, we have focused on studying the effects of pRb inactivation on antiestrogen resistance, since estrogen appears to mediate its proliferative effect by causing pRb inactivation and alterations in the pRb pathway have been reported in a significant percentage of breast cancer patients. In addition, the effects of activating the IGF-1 signaling pathway on antiestrogen resistance were assessed, as numerous studies have suggested that estrogen regulates breast cancer proliferation indirectly by regulating IGF-1 signaling. Thus activation of IGF-1 signaling could potentially lead to antiestrogen

resistance. We therefore tested the hypothesis that antiestrogens downregulated IGF-1 induced signaling events in MCF-7 cells and further if activation of IGF-1 signaling could confer antiestrogen resistance.

REFERENCES

1. Fendrick, J. L., Raafat, A. M., and Haslam, S. Z. Mammary gland growth and development from the postnatal period to postmenopause: ovarian steroid receptor ontogeny and regulation in the mouse, J Mammary Gland Biol Neoplasia. *3:* 7-22., 1998.

2. Soderqvist, G., Isaksson, E., von Schoultz, B., Carlstrom, K., Tani, E., and Skoog, L. Proliferation of breast epithelial cells in healthy women during the menstrual cycle, Am J Obstet Gynecol. *176:* 123-8., 1997.

3. Andrechek, E. R., Hardy, W. R., Siegel, P. M., Rudnicki, M. A., Cardiff, R. D., and Muller, W. J. Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis, Proc Natl Acad Sci U S A. *97:* 3444-9., 2000.

4. Leder, A., Pattengale, P. K., Kuo, A., Stewart, T. A., and Leder, P. Consequences of widespread deregulation of the c-myc gene in transgenic mice: multiple neoplasms and normal development, Cell. *45:* 485-95., 1986.

5. D'Cruz, C. M., Gunther, E. J., Boxer, R. B., Hartman, J. L., Sintasath, L., Moody, S. E., Cox, J. D., Ha, S. I., Belka, G. K., Golant, A., Cardiff, R. D., and Chodosh, L. A. c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations, Nat Med. *7:* 235-9., 2001.

6. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T., and Varmus, H. E. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice, Cell. *55:* 619-25., 1988.

7. Wang, T. C., Cardiff, R. D., Zukerberg, L., Lees, E., Arnold, A., and Schmidt, E. V. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice, Nature. *369:* 669-71., 1994.

8. Welcsh, P. L. and King, M. C. BRCA1 and BRCA2 and the genetics of breast and ovarian cancer, Hum Mol Genet. *10:* 705-13., 2001.

9. Claus, E. B., Schildkraut, J. M., Thompson, W. D., and Risch, N. J. The genetic attributable risk of breast and ovarian cancer, .

10. Melora, D. B., Allred, C. C., and O'Conell, P. Principles of Molecular Medicine, 1st edition, p. 625-632: Humana Press, 1998.

11. Beatson, G. T. On the treatment of inoperable cases of carcinoma of the mamma: Suggestions from a new method of treatment, with illustrative cases, Lancet. *ii:* 104-107, 1896.

12. Mixner, J. a. C. T. Role of estrogen in the stimulation of lobule-alveolar growth by progesterone and by the mammogenic lobule-alveolar growth factor of the anterior pituitary., Endocrinology. *30:* 591-597, 1942.

13. Clarke, R., Leonessa, F., Welch, J. N., and Skaar, T. C. Cellular and molecular pharmacology of antiestrogen action and resistance, Pharmacol Rev. *53:* 25-71., 2001.

14. Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J. A. Mechanisms of estrogen action, Physiol Rev. *81:* 1535-65., 2001.

15. Hall, J. M., Couse, J. F., and Korach, K. S. The multifaceted mechanisms of estradiol and estrogen receptor signaling, J Biol Chem. *276:* 36869-72., 2001.

16. Couse, J. F., Lindzey, J., Grandien, K., Gustafsson, J. A., and Korach, K. S. Tissue distribution and quantitative analysis of estrogen receptor- alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse, Endocrinology. *138:* 4613-21., 1997.

17. Couse, J. F. and Korach, K. S. Estrogen receptor null mice: what have we learned and where will they lead us?, Endocr Rev. *20:* 358-417., 1999.

18. Krege, J. H., Hodgin, J. B., Couse, J. F., Enmark, E., Warner, M., Mahler, J. F., Sar, M., Korach, K. S., Gustafsson, J. A., and Smithies, O. Generation and reproductive phenotypes of mice lacking estrogen receptor beta, Proc Natl Acad Sci U S A. *95:* 15677-82., 1998.

19. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. The human estrogen receptor has two independent nonacidic transcriptional activation functions, Cell. *59:* 477-87., 1989.

20. Kushner, P. J., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiau, A. K., Uht, R. M., and Webb, P. Estrogen receptor pathways to AP-1, J Steroid Biochem Mol Biol. *74:* 311-7., 2000.

21. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., and et al. Activation of the estrogen receptor through phosphorylation by mitogen- activated protein kinase, Science. *270:* 1491-4., 1995.

22. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. Molecular

basis of agonism and antagonism in the oestrogen receptor, Nature. *389:* 753-8., 1997.

23. Voegel, J. J., Heine, M. J., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways, Embo J. *17:* 507-19., 1998.

24. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer, Science. *277:* 965-8., 1997.

25. Norris, J. D., Fan, D., Stallcup, M. R., and McDonnell, D. P. Enhancement of estrogen receptor transcriptional activity by the coactivator GRIP-1 highlights the role of activation function 2 in determining estrogen receptor pharmacology, J Biol Chem. *273:* 6679-88., 1998.

26. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A., and Brown, M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription, Cell. *103:* 843-52., 2000.

27. Xu, J., Qiu, Y., DeMayo, F. J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene, Science. *279:* 1922-5., 1998.

28. Bautista, S., Valles, H., Walker, R. L., Anzick, S., Zeillinger, R., Meltzer, P., and Theillet, C. In breast cancer, amplification of the steroid receptor coactivator gene

AIB1 is correlated with estrogen and progesterone receptor positivity, Clin Cancer Res. *4:* 2925-9., 1998.

29. Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma, Nature. *323:* 643-6., 1986.

30. Sherr, C. J. Cancer cell cycles, Science. *274:* 1672-7., 1996.

31. Knudsen, E. S. and Wang, J. Y. Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites, J Biol Chem. *271:* 8313-20., 1996.

32. Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., and Dean, D. C. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1, Cell. *98:* 859-69., 1999.

33. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. The E2F transcription factor is a cellular target for the RB protein, Cell. *65:* 1053-61., 1991.

34. DeGregori, J., Kowalik, T., and Nevins, J. R. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes, Mol.Cell.Biol. *15:* 4215-4224, 1995.

35. Dyson, N. The regulation of E2F by pRB-family proteins, Genes Dev. *12:* 2245-62., 1998.

36. Chittenden, T., Livingston, D. M., and DeCaprio, J. A. Cell cycle analysis of E2F in primary human T cells reveals novel E2F complexes and biochemically distinct forms of free E2F, Mol Cell Biol. *13:* 3975-83., 1993.

37.

38.

39.

40.

41.

42.

43.

37. Dyson, N., Bernards, R., Friend, S. H., Gooding, L. R., Hassell, J. A., Major, E. O., Pipas, J. M., Vandyke, T., and Harlow, E. Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein, J Virol. *64:* 1353-6., 1990.

38. Ludlow, J. W., DeCaprio, J. A., Huang, C. M., Lee, W. H., Paucha, E., and Livingston, D. M. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family, Cell. *56:* 57-65, 1989.

39. Ludlow, J. W. and Skuse, G. R. Viral oncoprotein binding to pRB, p107, p130, and p300, Virus Res. *35:* 113-21, 1995.

40. Clarke, A. R., Maandag, E. R., van Roon, M., van der Lugt, N. M., van der Valk, M., Hooper, M. L., Berns, A., and te Riele, H. Requirement for a functional Rb-1 gene in murine development, Nature. *359:* 328-30., 1992.

41. Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. Effects of an Rb mutation in the mouse, Nature. *359:* 295-300., 1992.

42. Lee, E. Y., Chang, C. Y., Hu, N., Wang, Y. C., Lai, C. C., Herrup, K., Lee, W. H., and Bradley, A. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis, Nature. *359:* 288-94., 1992.

43. Lee, M. H., Williams, B. O., Mulligan, G., Mukai, S., Bronson, R. T., Dyson, N., Harlow, E., and Jacks, T. Targeted disruption of p107: functional overlap between p107 and Rb, Genes Dev. *10:* 1621-32., 1996.

44.

45.

46.

47.

48.

49.

50

51

44. Cobrinik, D., Lee, M. H., Hannon, G., Mulligan, G., Bronson, R. T., Dyson, N., Harlow, E., Beach, D., Weinberg, R. A., and Jacks, T. Shared role of the pRB-related p130 and p107 proteins in limb development, Genes Dev. *10:* 1633-44., 1996.

45. Chen, G., Guy, C. T., Chen, H. W., Hu, N., Lee, E. Y., and Lee, W. H. Molecular cloning and developmental expression of mouse p130, a member of the retinoblastoma gene family, J Biol Chem. *271:* 9567-72., 1996.

46. Herrera, R. E., Sah, V. P., Williams, B. O., Makela, T. P., Weinberg, R. A., and Jacks, T. Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in Rb-deficient fibroblasts, Mol.Cell.Biol. *16:* 2402-2407, 1996.

47. Sage, J., Mulligan, G. J., Attardi, L. D., Miller, A., Chen, S., Williams, B., Theodorou, E., and Jacks, T. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization, Genes Dev. *14:* 3037-50., 2000.

48. Sherr, C. J. and Roberts, J. M. CDK inhibitors: positive and negative regulators of G1-phase progression, Genes Dev. *13:* 1501-12, 1999.

49. Serrano, M., Hannon, G. J., and Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4, Nature. *366:* 704-7., 1993.

50. Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G., and Bartek, J. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16, Nature. *375:* 503-6., 1995.

51. Sharpless, N. E., Bardeesy, N., Lee, K. H., Carrasco, D., Castrillon, D. H., Aguirre, A. J., Wu, E. A., Horner, J. W., and DePinho, R. A. Loss of p16Ink4a

with retention of p19Arf predisposes mice to tumorigenesis, Nature. *413:* 86-91., 2001.

52. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. p21 is a universal inhibitor of cyclin kinases, Nature. *366:* 701-4., 1993.

53. LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. New functional activities for the p21 family of CDK inhibitors, Genes Dev. *11:* 847-62., 1997.

54. Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M., and Koff, A. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest, Genes Dev. *8:* 9-22., 1994.

55. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control, Cell. *82:* 675-84., 1995.

56. Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., and Loh, D. Y. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors, Cell. *85:* 707-20., 1996.

57. Nakayama, K. Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development, Bioessays. *20:* 1020-9., 1998.

58. Yan, Y., Frisen, J., Lee, M. H., Massague, J., and Barbacid, M. Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development, Genes Dev. *11:* 973-83., 1997.

59.

60.

61.

62.

63

6

6

6

59. Franklin, D. S., Godfrey, V. L., O'Brien, D. A., Deng, C., and Xiong, Y. Functional collaboration between different cyclin-dependent kinase inhibitors suppresses tumor growth with distinct tissue specificity, Mol Cell Biol. *20:* 6147-58., 2000.

60. Morgan, D. O. Principles of CDK regulation, Nature. *374:* 131-4., 1995.

61. Sherr, C. J. D-type cyclins, Trends Biochem Sci. *20:* 187-90., 1995.

62. Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4, Genes Dev. *7:* 331-42., 1993.

63. Zwijsen, R. M., Klompmaker, R., Wientjens, E. B., Kristel, P. M., van der Burg, B., and Michalides, R. J. Cyclin D1 triggers autonomous growth of breast cancer cells by governing cell cycle exit, Mol Cell Biol. *16:* 2554-60., 1996.

64. Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J. Y., Bar-Sagi, D., Roussel, M. F., and Sherr, C. J. Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts, Genes Dev. *7:* 1559-71., 1993.

65. Roussel, M. F., Theodoras, A. M., Pagano, M., and Sherr, C. J. Rescue of defective mitogenic signaling by D-type cyclins, Proc Natl Acad Sci U S A. *92:* 6837-41., 1995.

66. Bartkova, J., Lukas, J., Muller, H., Lutzhoft, D., Strauss, M., and Bartek, J. Cyclin D1 protein expression and function in human breast cancer, Int J Cancer. *57:* 353-61., 1994.

67. Leach, F. S., Elledge, S. J., Sherr, C. J., Willson, J. K., Markowitz, S., Kinzler, K. W., and Vogelstein, B. Amplification of cyclin genes in colorectal carcinomas, Cancer Res. *53:* 1986-9., 1993.

68. Yu, Q., Geng, Y., and Sicinski, P. Specific protection against breast cancers by cyclin D1 ablation, Nature. *411:* 1017-21., 2001.

69. Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M., and Sherr, C. J. The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts, EMBO J. *18:* 1571-83, 1999.

70. Geng, Y., Yu, Q., Sicinska, E., Das, M., Bronson, R. T., and Sicinski, P. Deletion of the p27Kip1 gene restores normal development in cyclin D1- deficient mice, Proc Natl Acad Sci U S A. *98:* 194-9., 2001.

71. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. O., Franza, B. R., and Roberts, J. M. Formation and activation of a cyclin E-Cdk2 complex during the G1 phase of the human cell cycle, Science. *257:* 1689-94., 1992.

72. Dulic, V., Lees, E., and Reed, S. I. Association of human cyclin E with a periodic G1-S phase protein kinase, Science. *257:* 1958-61., 1992.

73. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition, Mol Cell Biol. *15:* 2612-24., 1995.

74. Geng, Y., Eaton, E. N., Picon, M., Roberts, J. M., Lundberg, A. S., Gifford, A., Sardet, C., and Weinberg, R. A. Regulation of cyclin E transcription by E2Fs and retinoblastoma protein, Oncogene. *12:* 1173-80., 1996.

75. Geng, Y., Whoriskey, W., Park, M. Y., Bronson, R. T., Medema, R. H., Li, T., Weinberg, R. A., and Sicinski, P. Rescue of cyclin D1 deficiency by knockin cyclin E, Cell. *97:* 767-77., 1999.

76. Santoni-Rugiu, E., Falck, J., Mailand, N., Bartek, J., and Lukas, J. Involvement of Myc activity in a G(1)/S-promoting mechanism parallel to the pRb/E2F pathway, Mol Cell Biol. *20:* 3497-509., 2000.

77. Alevizopoulos, K., Vlach, J., Hennecke, S., and Amati, B. Cyclin E and c-Myc promote cell proliferation in the presence of p16INK4a and of hypophosphorylated retinoblastoma family proteins, Embo J. *16:* 5322-33., 1997.

78. Wang, C., Fu, M., Mani, S., Wadler, S., Senderowicz, A. M., and Pestell, R. G. Histone acetylation and the cell-cycle in cancer, Front Biosci. *6:* D610-29., 2001.

79. Hunter, T. Oncoprotein networks, Cell. *88:* 333-46., 1997.

80. Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H., and Okayama, H. Cdc25A is a novel phosphatase functioning early in the cell cycle, Embo J. *13:* 1549-56., 1994.

81. Blomberg, I. and Hoffmann, I. Ectopic expression of Cdc25A accelerates the G(1)/S transition and leads to premature activation of cyclin E- and cyclin A-dependent kinases, Mol Cell Biol. *19:* 6183-94., 1999.

82. Galaktionov, K., Lee, A. K., Eckstein, J., Draetta, G., Meckler, J., Loda, M., and Beach, D. CDC25 phosphatases as potential human oncogenes, Science. *269:* 1575-7., 1995.

83. Hoffmann, I., Draetta, G., and Karsenti, E. Activation of the phosphatase activity of human cdc25A by a Cdk2-cyclin E dependent phosphorylation at the G1/S transition, Embo J. *13:* 4302-10., 1994.

84. Iavarone, A. and Massague, J. Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15, Nature. *387:* 417-22., 1997.

85. Girard, F., Strausfeld, U., Fernandez, A., and Lamb, N. J. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts, Cell. *67:* 1169-79., 1991.

86. Brooks, S. C., Locke, E. R., and Soule, H. D. Estrogen receptor in a human cell line (MCF-7) from breast carcinoma, J Biol Chem. *248:* 6251-3., 1973.

87. Soule, H. D., Vazguez, J., Long, A., Albert, S., and Brennan, M. A human cell line from a pleural effusion derived from a breast carcinoma, J Natl Cancer Inst. *51:* 1409-16., 1973.

88. Brunner, N., Bronzert, D., Vindelov, L. L., Rygaard, K., Spang-Thomsen, M., and Lippman, M. E. Effect on growth and cell cycle kinetics of estradiol and tamoxifen on MCF-7 human breast cancer cells grown in vitro and in nude mice, Cancer Res. *49:* 1515-20., 1989.

89. Lippman, M., Bolan, G., and Huff, K. The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture, Cancer Res. *36:* 4595-601., 1976.

90. Engel, L. W., Young, N. A., Tralka, T. S., Lippman, M. E., O'Brien, S. J., and Joyce, M. J. Establishment and characterization of three new continuous cell lines derived from human breast carcinomas, Cancer Res. *38:* 3352-64., 1978.

91.

92.

93.

94.

95.

96.

97

98

91. Darbre, P. D., Curtis, S., and King, R. J. Effects of estradiol and tamoxifen on human breast cancer cells in serum-free culture, Cancer Res. *44:* 2790-3., 1984.

92. Weisz, A. and Bresciani, F. Estrogen regulation of proto-oncogenes coding for nuclear proteins, Crit Rev Oncog. *4:* 361-88, 1993.

93. Liao, D. J. and Dickson, R. B. c-Myc in breast cancer, Endocr Relat Cancer. *7:* 143-64., 2000.

94. Nasi, S., Ciarapica, R., Jucker, R., Rosati, J., and Soucek, L. Making decisions through Myc, FEBS Lett. *490:* 153-62., 2001.

95. Watson, P. H., Pon, R. T., and Shiu, R. P. Inhibition of c-myc expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer, Cancer Res. *51:* 3996-4000., 1991.

96. Prall, O. W., Rogan, E. M., Musgrove, E. A., Watts, C. K., and Sutherland, R. L. c-Myc or cyclin D1 mimics estrogen effects on cyclin E-Cdk2 activation and cell cycle reentry, Mol Cell Biol. *18:* 4499-508, 1998.

97. Smith, L. M., Wise, S. C., Hendricks, D. T., Sabichi, A. L., Bos, T., Reddy, P., Brown, P. H., and Birrer, M. J. cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype, Oncogene. *18:* 6063-70., 1999.

98. Sabbah, M., Courilleau, D., Mester, J., and Redeuilh, G. Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element, Proc Natl Acad Sci U S A. *96:* 11217-22., 1999.

99.

100

101

102

103

10-

99. Arteaga, C. L. and Holt, J. T. Tissue-targeted antisense c-fos retroviral vector inhibits established breast cancer xenografts in nude mice, Cancer Res. *56:* 1098-1103., 1996.

100. Planas-Silva, M. D. and Weinberg, R. A. Estrogen-dependent cyclin E-Cdk2 activation through p21 redistribution, Mol Cell Biol. *17:* 4059-69., 1997.

101. Prall, O. W., Sarcevic, B., Musgrove, E. A., Watts, C. K., and Sutherland, R. L. Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E- Cdk2, J Biol Chem. *272:* 10882-94., 1997.

102. Foster, J. S., Henley, D. C., Bukovsky, A., Seth, P., and Wimalasena, J. Multifaceted regulation of cell cycle progression by estrogen: regulation of Cdk inhibitors and Cdc25A independent of cyclin D1-Cdk4 function, Mol Cell Biol. *21:* 794-810., 2001.

103. Foster, J. S. and Wimalasena, J. Estrogen regulates activity of cyclin-dependent kinases and retinoblastoma protein phosphorylation in breast cancer cells, Mol Endocrinol. *10:* 488-98., 1996.

104. Cariou, S., Donovan, J. C., Flanagan, W. M., Milic, A., Bhattacharya, N., and Slingerland, J. M. Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen- mediated cell cycle arrest in human breast cancer cells, Proc Natl Acad Sci U S A. *97:* 9042-6., 2000.

105. Tsihlias, J., Kapusta, L., and Slingerland, J. The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer, Annu Rev Med. *50:* 401-23, 1999.

106. Lundberg, A. S. and Weinberg, R. A. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-Cdk complexes, Mol Cell Biol. *18:* 753-61., 1998.

107. Furlanetto, R. W. and DiCarlo, J. N. Somatomedin-C receptors and growth effects in human breast cells maintained in long-term tissue culture, Cancer Res. *44:* 2122-8., 1984.

108. Osborne, C. K., Hamilton, B., Titus, G., and Livingston, R. B. Epidermal growth factor stimulation of human breast cancer cells in culture, Cancer Res. *40:* 2361-6., 1980.

109. Karey, K. P. and Sirbasku, D. A. Differential responsiveness of human breast cancer cell lines MCF-7 and T47D to growth factors and 17 beta-estradiol, Cancer Res. *48:* 4083-92., 1988.

110. Dickson, R. B., Kasid, A., Huff, K. K., Bates, S. E., Knabbe, C., Bronzert, D., Gelmann, E. P., and Lippman, M. E. Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17 beta-estradiol or v-Ha-ras oncogene, Proc Natl Acad Sci U S A. *84:* 837-41., 1987.

111. Kasid, A., Knabbe, C., and Lippman, M. E. Effect of v-rasH oncogene transfection on estrogen-independent tumorigenicity of estrogen-dependent human breast cancer cells, Cancer Res. *47:* 5733-8., 1987.

112. Huff, K. K., Kaufman, D., Gabbay, K. H., Spencer, E. M., Lippman, M. E., and Dickson, R. B. Secretion of an insulin-like growth factor-I-related protein by human breast cancer cells, Cancer Res. *46:* 4613-9., 1986.

113. Dickson, R. B., Huff, K. K., Spencer, E. M., and Lippman, M. E. Induction of epidermal growth factor-related polypeptides by 17 beta- estradiol in MCF-7 human breast cancer cells, Endocrinology. *118:* 138-42., 1986.

114. Dickson, R. B., McManaway, M. E., and Lippman, M. E. Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth, Science. *232:* 1540-3., 1986.

115. Daniel, C. W., Silberstein, G. B., and Strickland, P. Direct action of 17 beta-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autoradiography, Cancer Res. *47:* 6052-7., 1987.

116. Cunha, G. R., Young, P., Hom, Y. K., Cooke, P. S., Taylor, J. A., and Lubahn, D. B. Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants, J Mammary Gland Biol Neoplasia. *2:* 393-402., 1997.

117. Osborne, C. K., Clemmons, D. R., and Arteaga, C. L. Regulation of breast cancer growth by insulin-like growth factors, J Steroid Biochem Mol Biol. *37:* 805-9., 1990.

118. Sachdev, D. and Yee, D. The IGF system and breast cancer, Endocr Relat Cancer. *8:* 197-209., 2001.

119. Lee, A. V., Jackson, J. G., Gooch, J. L., Hilsenbeck, S. G., Coronado-Heinsohn, E., Osborne, C. K., and Yee, D. Enhancement of insulin-like growth factor

signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo, Molecular Endocrinology. *10:* 787-796, 1999.

120. Ruan, W. and Kleinberg, D. L. Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development, Endocrinology. *140:* 5075-81., 1999.

121. Jones, J. I. and Clemmons, D. R. Insulin-like growth factors and their binding proteins: biological actions, Endocr Rev. *16:* 3-34., 1995.

122. Yee, D., Paik, S., Lebovic, G. S., Marcus, R. R., Favoni, R. E., Cullen, K. J., Lippman, M. E., and Rosen, N. Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer, Mol Endocrinol. *3:* 509-17., 1989.

123. Pollak, M. N., Perdue, J. F., Margolese, R. G., Baer, K., and Richard, M. Presence of somatomedin receptors on primary human breast and colon carcinomas, Cancer Lett. *38:* 223-30., 1987.

124. Jackson, J. G., White, M. F., and Yee, D. Insulin receptor substrate-1 is the predominant signaling molecule activated by insulin-like growth factor-I, insulin, and interleukin-4 in estrogen receptor-positive human breast cancer cells, J Biol Chem. *273:* 9994-10003., 1998.

125. Skolnik, E. Y., Lee, C. H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., Myers, M. J., Jr., Backer, J. M., Ullrich, A., White, M. F., and et al. The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine- phosphorylated IRS1

and Shc: implications for insulin control of ras signalling, Embo J. *12:* 1929-36., 1993.

126. Tartare-Deckert, S., Murdaca, J., Sawka-Verhelle, D., Holt, K. H., Pessin, J. E., and Van Obberghen, E. Interaction of the molecular weight 85K regulatory subunit of the phosphatidylinositol 3-kinase with the insulin receptor and the insulin- like growth factor-1 (IGF- I) receptor: comparative study using the yeast two-hybrid system, Endocrinology. *137:* 1019-24., 1996.

127. Vignon, F., Bouton, M. M., and Rochefort, H. Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens, Biochem Biophys Res Commun. *146:* 1502-8., 1987.

128. Freiss, G., Prebois, C., Rochefort, H., and Vignon, F. Anti-steroidal and anti-growth factor activities of anti-estrogens, J Steroid Biochem Mol Biol. *37:* 777-81., 1990.

129. Huynh, H., Nickerson, T., Pollak, M., and Yang, X. Regulation of insulin-like growth factor I receptor expression by the pure antiestrogen ICI 182780, Clin Cancer Res. *2:* 2037-42., 1996.

130. Molloy, C. A., May, F. E., and Westley, B. R. Insulin receptor substrate-1 expression is regulated by estrogen in the MCF-7 human breast cancer cell line, J Biol Chem. *275:* 12565-71., 2000.

131. Ahmad, S., Singh, N., and Glazer, R. I. Role of AKT1 in 17beta-estradiol- and insulin-like growth factor I (IGF- I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells, Biochem Pharmacol. *58:* 425-30., 1999.

132. Guvakova, M. A. and Surmacz, E. Tamoxifen interferes with the insulin-like growth factor I receptor (IGF-IR) signaling pathway in breast cancer cells, Cancer Res. *57:* 2606-10., 1997.

133. Lee, A. V., Jackson, J. G., Gooch, J. L., Hilsenbeck, S. G., Coronado-Heinsohn, E., Osborne, C. K., and Yee, D. Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo, Mol Endocrinol. *13:* 787-96., 1999.

134. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol- receptor complex in MCF-7 cells, Embo J. *15:* 1292-300., 1996.

135. Castoria, G., Barone, M. V., Di Domenico, M., Bilancio, A., Ametrano, D., Migliaccio, A., and Auricchio, F. Non-transcriptional action of oestradiol and progestin triggers DNA synthesis, Embo J. *18:* 2500-10., 1999.

136. Wakeling, A. E., Dukes, M., and Bowler, J. A potent specific pure antiestrogen with clinical potential, Cancer Res. *51:* 3867-73., 1991.

137. Kedar, R. P., Bourne, T. H., Powles, T. J., Collins, W. P., Ashley, S. E., Cosgrove, D. O., and Campbell, S. Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial, Lancet. *343:* 1318-21., 1994.

138. Love, R. R., Wiebe, D. A., Newcomb, P. A., Cameron, L., Leventhal, H., Jordan, V. C., Feyzi, J., and DeMets, D. L. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women, Ann Intern Med. *115:* 860-4., 1991.

139. Love, R. R., Barden, H. S., Mazess, R. B., Epstein, S., and Chappell, R. J. Effect of tamoxifen on lumbar spine bone mineral density in postmenopausal women after 5 years, Arch Intern Med. *154:* 2585-8., 1994.

140. Wiebe, V. J., Osborne, C. K., Fuqua, S. A., and DeGregorio, M. W. Tamoxifen resistance in breast cancer, Crit Rev Oncol Hematol. *14:* 173-88., 1993.

141. Osborne, C. K., Hobbs, K., and Clark, G. M. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice, Cancer Res. *45:* 584-90., 1985.

142. Osborne, C. K., Coronado, E. B., and Robinson, J. P. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy, Eur J Cancer Clin Oncol. *23:* 1189-96., 1987.

143. Howell, A., Osborne, C. K., Morris, C., and Wakeling, A. E. ICI 182,780 (Faslodex): development of a novel, "pure" antiestrogen, Cancer. *89:* 817-25., 2000.

144. McLeskey, S. W., Zhang, L., El-Ashry, D., Trock, B. J., Lopez, C. A., Kharbanda, S., Tobias, C. A., Lorant, L. A., Hannum, R. S., Dickson, R. B., and Kern, F. G. Tamoxifen-resistant fibroblast growth factor-transfected MCF-7 cells are cross-resistant in vivo to the antiestrogen ICI 182,780 and two aromatase inhibitors, Clin Cancer Res. *4:* 697-711., 1998.

145. Lykkesfeldt, A. E., Larsen, S. S., and Briand, P. Human breast cancer cell lines resistant to pure anti-estrogens are sensitive to tamoxifen treatment, Int J Cancer. *61:* 529-34., 1995.

146.

147.

148.

149

150

15

146. Osborne, C. K., Coronado-Heinsohn, E. B., Hilsenbeck, S. G., McCue, B. L., Wakeling, A. E., McClelland, R. A., Manning, D. L., and Nicholson, R. I. Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer, J Natl Cancer Inst. *87:* 746-50., 1995.

147. Larsen, S. S., Madsen, M. W., Jensen, B. L., and Lykkesfeldt, A. E. Resistance of human breast-cancer cells to the pure steroidal anti- estrogen ICI 182,780 is not associated with a general loss of estrogen- receptor expression or lack of estrogen responsiveness, Int J Cancer. *72:* 1129-36., 1997.

148. Brunner, N., Boysen, B., Jirus, S., Skaar, T. C., Holst-Hansen, C., Lippman, J., Frandsen, T., Spang-Thomsen, M., Fuqua, S. A., and Clarke, R. MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen, Cancer Res. *57:* 3486-93., 1997.

149. Dumont, J. A., Bitonti, A. J., Wallace, C. D., Baumann, R. J., Cashman, E. A., and Cross-Doersen, D. E. Progression of MCF-7 breast cancer cells to antiestrogen-resistant phenotype is accompanied by elevated levels of AP-1 DNA-binding activity, Cell Growth Differ. *7:* 351-9., 1996.

150. Osborne, C. K. Mechanisms for tamoxifen resistance in breast cancer: possible role of tamoxifen metabolism, J Steroid Biochem Mol Biol. *47:* 83-9., 1993.

151. Johnston, S. R., Saccani-Jotti, G., Smith, I. E., Salter, J., Newby, J., Coppen, M., Ebbs, S. R., and Dowsett, M. Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer, Cancer Res. *55:* 3331-8., 1995.

152. Murphy, L. C., Dotzlaw, H., Leygue, E., Douglas, D., Coutts, A., and Watson, P. H. Estrogen receptor variants and mutations, J Steroid Biochem Mol Biol. *62:* 363-72., 1997.

153. Gabelman, B. M. and Emerman, J. T. Effects of estrogen, epidermal growth factor, and transforming growth factor-alpha on the growth of human breast epithelial cells in primary culture, Exp Cell Res. *201:* 113-8., 1992.

154. Davidson, N. E., Gelmann, E. P., Lippman, M. E., and Dickson, R. B. Epidermal growth factor receptor gene expression in estrogen receptor- positive and negative human breast cancer cell lines, Mol Endocrinol. *1:* 216-23., 1987.

155. Nicholson, R. I., McClelland, R. A., Gee, J. M., Manning, D. L., Cannon, P., Robertson, J. F., Ellis, I. O., and Blamey, R. W. Epidermal growth factor receptor expression in breast cancer: association with response to endocrine therapy, Breast Cancer Res Treat. *29:* 117-25., 1994.

156. Pietras, R. J., Arboleda, J., Reese, D. M., Wongvipat, N., Pegram, M. D., Ramos, L., Gorman, C. M., Parker, M. G., Sliwkowski, M. X., and Slamon, D. J. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells, Oncogene. *10:* 2435-46., 1995.

157. Benz, C. C., Scott, G. K., Sarup, J. C., Johnson, R. M., Tripathy, D., Coronado, E., Shepard, H. M., and Osborne, C. K. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu, Breast Cancer Res Treat. *24:* 85-95, 1993.

158. Hajitou, A., Deroanne, C., Noel, A., Collette, J., Nusgens, B., Foidart, J. M., and Calberg-Bacq, C. M. Progression in MCF-7 breast cancer cell tumorigenicity: compared effect of FGF-3 and FGF-4, Breast Cancer Res Treat. *60:* 15-28., 2000.

159. Zhang, L., Kharbanda, S., Hanfelt, J., and Kern, F. G. Both autocrine and paracrine effects of transfected acidic fibroblast growth factor are involved in the estrogen-independent and antiestrogen- resistant growth of MCF-7 breast cancer cells, Cancer Res. *58:* 352-61., 1998.

160. Zhang, L., Kharbanda, S., Chen, D., Bullocks, J., Miller, D. L., Ding, I. Y., Hanfelt, J., McLeskey, S. W., and Kern, F. G. MCF-7 breast carcinoma cells overexpressing FGF-1 form vascularized, metastatic tumors in ovariectomized or tamoxifen-treated nude mice, Oncogene. *15:* 2093-108., 1997.

161. Arteaga, C. L., Carty-Dugger, T., Moses, H. L., Hurd, S. D., and Pietenpol, J. A. Transforming growth factor beta 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice, Cell Growth Differ. *4:* 193-201., 1993.

CHAPTER 2

Reversal of an Antiestrogen-Mediated Cell Cycle Arrest of MCF-7 Cells by Viral Tumor Antigens Requires the Retinoblastoma Protein-Binding Domain

ABSTRACT

Proliferation of MCF-7 cells is estrogen dependent and antiestrogen sensitive. In the absence of estrogens or presence of antiestrogens MCF-7 cells arrest in the G1 phase of the cell cycle, and this arrest is associated with an accumulation of the active, hypophosphorylated form of the retinoblastoma protein (pRb). Because active pRb negatively regulates passage from G1 to S phase, this suggests that pRb is a crucial target of estrogen action, and that its inactivation might lead to antiestrogen resistance. We tested this hypothesis by expressing viral tumor antigens (T antigens), which bind and inactivate pRb, in MCF-7 cells, and determining the effects on cell proliferation in the presence of antiestrogens. The results of these experiments demonstrate that T antigen expression confers antiestrogen resistance to MCF-7 cells. Using a panel of mutant T antigens, we further demonstrate that the pRb-binding, but not the p53 binding domain is required to confer antiestrogen resistance. Thus, pRb is an important target of estrogen action, and its inactivation can contribute to the development of antiestrogen resistance.

Note. The content of this chapter has been published as Varma, H., and Conrad, S.E. 2000. Reversal of an Antiestrogen-Mediated Cell Cycle Arrest of MCF-7 Cells by Viral Tumor Antigens Requires the Retinoblastoma Protein-Binding Domain. Oncogene *19*: 4746-4753.

estra

proli

cell

used

initi

cell

Und

pass

there

treat

a fa

regu

cycl

targ

hyp

tran

Upo

gen

prin

hur

ant

INTRODUCTION

Approximately 40% of human breast cancers require estrogens such as 17β-estradiol for growth (1). In the absence of estrogens or the presence of antiestrogens, proliferation of these tumor cells is inhibited and they accumulate in the G1 phase of the cell cycle (2, 3). Because of their ability to inhibit proliferation, antiestrogens are often used to treat breast cancer patients. However, a significant proportion of tumors that initially respond to antiestrogen therapy eventually progress to a resistant state in which cell proliferation is no longer dependent on estrogens or inhibited by antiestrogens. Understanding the molecular mechanisms by which estrogens and antiestrogens regulate passage from G1 to S phase, and those by which cells become antiestrogen resistant, are therefore important goals that may lead to improvements in breast cancer diagnosis and treatment.

Transit through G1 into S phase is regulated by cyclin dependent kinases (Cdks), a family of protein kinases that are activated by association with cyclins. The Cdks that regulate passage through G1 include Cdk4 and Cdk6, which are activated by the D-type cyclins, and Cdk2, which is activated by both cyclin E and cyclin A (4, 5). One important target of G1 cyclin/Cdk complexes is the tumor suppressor protein pRb (6). In its hypophosphorylated form, pRb associates with members of the E2F family of transcription factors, and prevents transcription of genes required for entry into S phase. Upon phosphorylation of pRb, E2F transcription factors are released and activate target genes that promote S phase entry (Figure 2.1A) (7, 8).

The effects of estrogen and antiestrogen treatments on cell cycle progression have primarily been investigated in the MCF-7 cell line, which is an estrogen receptor positive human breast cancer cell line. Proliferation of MCF-7 cells is estrogen-dependent and antiestrogen sensitive both *in vitro* and *in vivo* (2, 9, 10), and in the presence of

antiestrogens these cells accumulate in G1. Estrogen treatment of G1 -arrested MCF-7 cells induces S phase entry, and this induction is associated with increases in cyclin D1 protein levels, cyclin D1/Cdk4 kinase activity, Cdk2 kinase activity, and pRb phosphorylation (11-14). In addition, overexpression of cyclin D1 in the presence of antiestrogens leads to pRb phosphorylation, and is sufficient to promote proliferation for at least one cell cycle (15, 16).

One explanation for the findings outlined above is that pRb is the ultimate target of the proliferative effects of estrogen, and that overexpression of cyclin D1 promotes proliferation in the presence of antiestrogens by increasing pRb phosphorylation via the activation of cyclinD1-Cdk4 complexes. However, it is also possible that functions of cyclin D1 other than activating Cdk4 contribute to or are responsible for antiestrogen resistance. For example, regulation of cyclin E-Cdk2 kinase activity by estrogen and antiestrogens occurs in the absence of changes in cyclin E or Cdk2 protein levels. To account for this observation, it has been proposed that induced or overexpressed cyclin D1 protein binds and sequesters the Cdk inhibitor $p21^{Waf1}$, leading to activation of cyclin E-Cdk2 complexes (12, 13). Another potential mechanism of cyclin D1 action is via its interactions with cellular transcription factors. Cyclin D1 binds to and modulates the activity of several transcription factors, including the estrogen receptor (17, 18) and a myb-like transcription factor DMP1 (19, 20), and either of these activities could have profound effects on cell proliferation.

If pRb is the ultimate target of the proliferative effects of estrogen, one would predict that inactivation of this tumor suppressor protein would be sufficient to confer antiestrogen resistance to MCF-7 cells. Alternatively, if functions of cyclin D1 other than promoting pRb phosphorylation are critical for its effects on estrogen dependence, then inactivation of pRb would not be sufficient to promote antiestrogen resistance. To directly test the effect of pRb inactivation on antiestrogen sensitivity, we have expressed

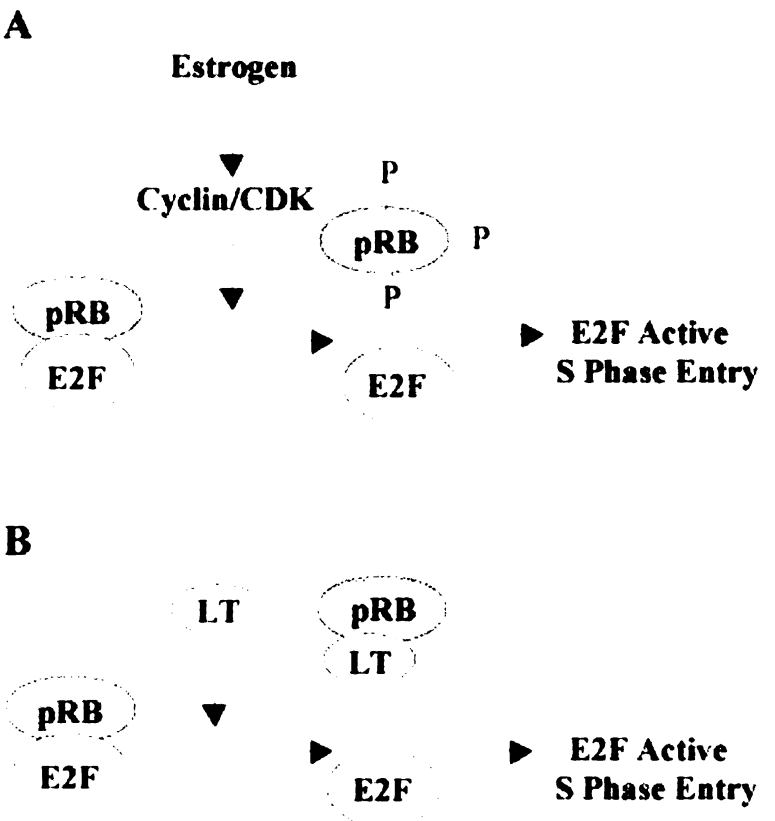


Figure 2.1. Model for pRb inactivation by (A) estrogen treatment and (B) LT expression in MCF-7 cells.

the simian virus 40 (SV) or polyoma virus (Py) large T antigens (LTs) in MCF-7 cells. Both SVLT and PyLT bind to and inactivate pRb (21-24), thereby allowing E2F to activate transcription and promote S phase entry (Figure 2.1B). SVLT has several other activities in addition to pRb-binding, including the ability to inactivate the tumor suppressor protein p53 (25, 26). PyLT shares most of these properties, but differs from SVLT in that it does not bind to p53 (21, 27). Using wild type and mutant forms of the viral proteins, we demonstrate that expression of either SV40 or polyoma virus LT is sufficient to confer antiestrogen resistance to MCF-7 cells. This effect is dependent upon an intact pRb binding domain, but does not require a p53 binding domain. These data establish that pRb or a related protein is an important functional target of estrogen, and suggest that inactivation of normal pRb function could play a role in the development of antiestrogen resistance during tumor progression *in vivo*.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells were obtained from Dr. Michael Johnson (Lombardi Cancer Center) and were maintained in IMEM media (Biofluids Inc., Rockville, MD) supplemented with 5% fetal bovine serum (Hyclone), penicillin (100 units/ml) and streptomycin (100 μg/ml). To study the effects of estrogen and antiestrogens, cells were cultured in phenol red free IMEM supplemented with 5% charcoal stripped fetal bovine serum (Hyclone), with or without the addition of 0.2 nM 17β-estradiol (Sigma), 1 μM 4-hydroxytamoxifen (Sigma), or 100 nM ICI 182,780 (Zeneca Pharmaceuticals)

Antibodies and Plasmids. The antibody to SVLT was a supernatant of the L19

hybr

Ab-

poly

Mic

mou

shee

rat.

Bio

Jacl

Cha

at t

trur

enc

rep

enc

dH

(2:

Tr

tis

P

pr

5

d

ir

p

hybridoma cell line, which was a gift of Dr. Ed Harlow (28). A rat monoclonal antibody, Ab-1 (Oncogene Science) was used to detect PyLT by indirect immunofluorescence. A polyclonal rat antiserum was used for PyLT immunoblotting, and was a gift of Dr. Michele Fluck. Antibodies for pRb, cyclin A, and p53 were from Pharmingen. The mouse α-BrdU monoclonal antibody was obtained from Boehringer Mannheim, and sheep α-BrdU was from Fitzgerald Industries, Concord, MA. FITC conjugated goat α-rat, donkey α-sheep, and goat α-mouse antibodies were obtained from Sigma Biochemicals. Rhodamine Red-X conjugated donkey α-mouse antibody was from Jackson Immunoresearch.

The plasmids encoding wild type SVLT, PVU1, and 2809 were obtained from Dr. Charles Cole (29, 30). The SV40 genome in all of these plasmids contains a 6 bp deletion at the viral origin of replication that renders them replication incompetent (30-32). The truncation mutant (T1-259) was provided by Dr. Ellen Fanning (33). The T antigen encoded by this mutant lacks helicase and ATPase activity and will therefore not promote replication from the viral origin (34-36). The plasmid pCMVLT, which contains a cDNA encoding PyLT, was obtained from Dr. Brian Schaffhausen (37). The pneoLT1 and dl141-encoding vectors used for stable transfections were provided by Dr. Michael Bastin (23, 38). The p53 dominant negative mutant (del239) was a gift of Dr. John Minna (39).

Transfections. For transient transfections, $5x10^5$ MCF-7 cells were plated in 60 mm tissue culture dishes containing 2-18 mm cover slips and incubated at 37°C overnight. Plasmid DNA was added to the cells using the Superfect reagent (Qiagen) following the protocol suggested by the manufacturer. SV40 LT transfections were done using 2.5 and 5 μg of DNA per dish, and pCMV-LT transfections were done using 0.3 μg DNA per dish. These amounts were chosen because they gave the highest transfection efficiencies in pilot experiments. After 3 hr, the transfection medium was removed and replaced with phenol red free IMEM supplemented with 5% charcoal stripped serum and 100 nM ICI

182.
deox
med
For
cells
in n
expr

Indi
were
buff
PBS
cells
then
temp
etha
4 N
was
anti-
and
don
was
(Oly
or b
nega
take
com

182,780. Cells were incubated for 43 hr and then labeled with 25 μM 5-bromo-2'-deoxyuridine (BrdU) for 5 hr. For control proliferating cultures, cells were grown in medium containing charcoal stripped serum supplemented with 0.2 nM 17β-estradiol. For stable transfections, 1 μg of DNA (pneoLT1 or dl141) was introduced into MCF-7 cells using Lipofectin reagent (Gibco BRL). Colonies were selected for G418 resistance in medium containing 400 μg/ml G418 and expanded into clonal cell lines. PyLT expression in these cell lines was assayed by Western blotting.

Indirect Immunofluorescence. Forty-eight hours after transfection, cells on coverslips were fixed with ice cold acetone/methanol (50:50v/v), and washed with phosphate buffered saline (PBS). The following procedures were used for antibody staining, with PBS washes (3X) being conducted between each treatment. Coverslips containing fixed cells were incubated with a primary antibody against SVLT or PyLT for 45 min at 37°C, then with the appropriate fluorochrome-conjugated secondary antibody for 1 hr at room temperature. Following this procedure, cells were re-fixed in a 50mM glycine, 50% ethanol solution (pH 2.1) for 45 min at room temperature. Samples were then treated with 4 N HCl for 15 min to denature the DNA. The acid was aspirated, and coverslips were washed in PBS to neutral pH (7.0). Samples were then incubated with an appropriate anti-BrdU antibody (sheep for SVLT and mouse for PyLT) for 45 min at 37°C, washed, and incubated with a second fluorescent antibody (donkey anti-sheep FITC for SVLT and donkey anti-mouse rhodamine Red X for PyLT). Finally, the coverslips were extensively washed with PBS, mounted on glass slides, and viewed under a flourescence microscope (Olympus). The percentage of cells that were positive for LT (or p53) alone, BrdU alone, or both, were counted in each transfection. More than 100 LT-positive and 100 LT-negative cells were counted in each experiment. The images shown in Figure 2.3 were taken on a Meridian Instruments confocal microscope, and processed with Insight IQ computer software to generate single color and overlay images.

RESULTS

Inhibition of DNA synthesis by antiestrogens in MCF-7 cells. Antiestrogens inhibit proliferation of MCF-7 cells. This is demonstrated in Figure 2.2A, in which the percentages of cells incorporating BrdU into DNA are compared in cultures incubated in basal medium containing 5% charcoal stripped serum (CSS), and in this medium supplemented with 17β-estradiol (E), 4-hydroxytamoxifen (TAM) or ICI 182,780 (ICI). As previously reported by others, the dependence of MCF-7 cell proliferation on exogenous E in cell culture is not absolute (2, 40, 41). E stimulated proliferation, but there was considerable BrdU incorporation in the basal medium in these assays. TAM is a non-steroidal antiestrogen with both agonist and antagonist activities. It acted as an antagonist in these assays, and decreased proliferation below the level observed in CSS alone. ICI is a steroidal antiestrogen with no agonist activity. As previously reported, it was a more potent inhibitor of MCF-7 cell proliferation than TAM (41), and decreased the percentage of cells incorporating BrdU to approximately 3-5%. In addition, MCF-7 cell derivatives that are resistant to ICI are cross-resistant to TAM, while TAM^r cells are often sensitive to ICI (42, 43). The ability of a treatment to confer ICI resistance is therefore likely to also confer TAM resistance, while the converse is not true. For these reasons, ICI was the antiestrogen used in the remainder of our experiments.

Expression of SV40 large T antigen confers antiestrogen resistance to MCF-7 cells, and the pRb binding function is required for this effect. To determine the effect of SVLT expression on antiestrogen sensitivity, plasmids encoding either wild type or mutant SVLT proteins were introduced into MCF-7 cells, and the ability of the transfected cells to incorporate BrdU into DNA in the presence of ICI was determined as described in Materials and Methods.

Table 2.1. Characteristics of wild type and mutant viral T-antigens used in study.

LT Protein	pRb binding	p53 binding	Mutation	Reference
PVU1 (SV)	-	+	del aa-107/112	(56)
2809 (SV)	+	-	ins 409 (4aa)	(29)
T1-259 (SV)	+	-	truncation	(33)
PyLT	+	-	none	(23, 37)
dl141 (Py)	-	-	del aa 141/146	(38)

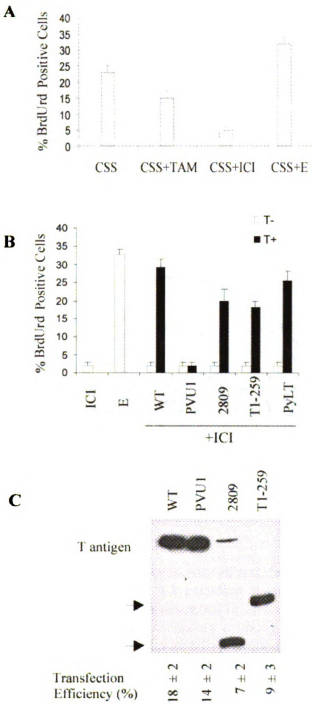
SVLT is a multifunctional protein that interacts with a number of cellular proteins, including the pRb family of proteins (pRb, p107, p130), p53, p300 and TBP (21, 27). The mutants used in these experiments (Table 2.1) were defective in one or more of these activities that seemed likely to play a role in promoting antiestrogen resistance. As described in Materials and Methods, none of the constructs encoding SVLT were capable of replicating extrachromosomally, and any BrdU incorporation detected therefore represented cellular DNA replication.

Representative micrographs of cells expressing SVLT and/or incorporating BrUrd are shown in Figure 2.3. The percentages of SVLT positive and SVLT negative cells in each transfected culture that were also BrdU positive was determined, and the results of this analysis are shown in Figure 2.2B. The percentage of SVLT negative cells that incorporated BrdU in the transfected cultures (3-5%) was similar to untransfected, ICI treated controls (Figures 2.2A and 2.2B), indicating that the transfection protocol did not cause proliferation. In contrast, cells expressing wild type SVLT incorporated BrdU at a level comparable to the cycling (E) population. These results indicate that SVLT is able to completely overcome an ICI-mediated cell cycle arrest in this assay.

The PVU1 mutant is defective in binding to pRb family members. In the presence of ICI, cells expressing PVU1 protein incorporated BrdU to the same low extent as untransfected cells (Figures 2.2B and 2.3). Both the transfection efficiency of the PVU1 construct, and the level of expression of PVU1 protein in transfected cells were equivalent to the wild type (Figure 2C), indicating that the defect in this mutant was not due to a failure of the protein to be expressed. We therefore conclude that the pRb-binding domain of SVLT is absolutely required to overcome an ICI induced cell cycle arrest.

Two SVLT mutants defective in p53 binding were also examined. One (2809) contains an insertion of 4 amino acids at position aa409, and the second (T1-259) is a

Figure 2.2. Ability of wild type and mutant LT proteins to promote antiestrogen resistance. MCF-7 cells were transfected with LT-encoding plasmids, labeled with BrdU, and stained for LT expression and BrdU incorporation as described in Materials and Methods. (A) The percentage of cells in untransfected cultures that incorporated BrdUrd during a 5 hr labeling in the presence of CSS, CSS+E, CSS+TAM or CSS+ ICI. The results represent the mean +/- SE of an experiment done in triplicate. (B) The percentages of LT positive (filled bars) and LT negative (open bars) cells that also incorporated BrdU in the presence of ICI in transfected cultures. The percentage of cells that incorporated BrdU during a 5 hr labeling in the presence of E or ICI in untransfected cultures are shown on the left side of the figure The results represent the mean $\pm$ SE of at least three independent experiments. (C) A representative western blot showing expression levels of wild type and mutant SVLTs. Arrows point to the T1-259 truncation mutant, and to a smaller (20-25 kDa) form of LT detected in cells transfected with the 2809 mutant. Values for the transfection efficiency (percentage) of each construct are represented as the mean $\pm$ SE for at least three independent experiments.


A
% BrdUrd Positive Cells
40
35
30
25
20
15
10
5
0
CSS
CSS+TAM
CSS+ICI
CSS+E
B
% BrdUrd Positive Cells
T-
T+
ICI
E
WT
PVU1
2809
T1-259
PyLT
+ICI
C
WT
PVU1
2809
T1-259
T antigen
Transfection Efficiency (%)
18 ± 2
14 ± 2
7 ± 2
9 ± 3

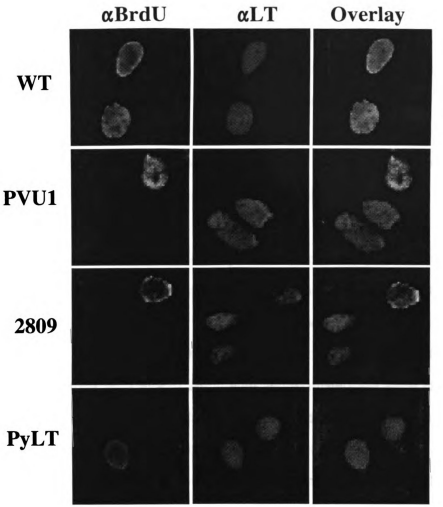


Figure 2.3. Double indirect immunofluorescence of transfected cells. MCF-7 cells were transfected with wild type or mutant LT-encoding plasmids, incubated in medium containing ICI, and labeled with BrdU as described in Materials and Methods. SVLT was detected using a rhodamine-conjugated secondary antibody (red fluorescence), and BrdU incorporation with an FITC-conjugated secondary antibody (green fluoresence). PyLT was detected using an FITC-conjugated secondary antibody, and in this case BrdU incorporation was detected with a rhodamine-conjugated secondary antibody. Overlay images were generated to show nuclei that were positive for both LT and BrdU, which appear yellow in the photograph.

truncat
T1-25
the pr
reproc
both t
encod
2.2C.
cultu
staini
agree
were
Inter
LT p
T1-2
leve
kDa
SV
bin
pro
pro
de
H
ty
d
a
e

truncation mutant encoding the first 259 amino acids of the protein. Both the 2809 and T1-259 mutants were 60-70% as effective as wild type SVLT at inducing proliferation in the presence of ICI (Figure 2.2B). To investigate potential reasons for this small, but reproducible decrease in the ability of these mutants to overcome an ICI-induced arrest, both the transfection efficiency of the DNA constructs and the level of expression of the encoded proteins in transfected cell populations were examined. As shown in Figure 2.2C, the percentage of cells expressing detectable SVLT in 2809 and T1-259 transfected cultures was approximately 2-fold lower than with the wild type, although the intensity of staining in LT positive cells was not detectably different between the three constructs. In agreement with these observations, overall T1-259 protein levels in transfected cultures were approximately 2-fold lower than wild type as determined by western blots. Interestingly, cultures containing the 2809 mutant expressed very low levels of full length LT protein, although they incorporated BrdU with approximately the same efficiency as T1-259. However, it was noted that 2809-transfected cells expressed relatively high levels (equal to that of the T1-259 mutant) of a small protein of approximately 20-25 kDa. Since the antibody used in these western blots recognizes an N-terminal epitope of SV- LT (28), the 20-25 kDa peptide may be an N-terminal fragment that retains the pRb binding domain and has activity in proliferation assays. Alternatively, all of the proliferative activity of the 2809 mutant may be due to the small amount of full-length protein present in the transfected cells. In summary, two different SVLT mutants that are defective for p53 binding were able to induce proliferation in ICI treated MCF-7 cells. However, the magnitude of the effect was significantly less than that seen with the wild type protein. Due to differences in the levels of expression of the encoded proteins, the data obtained did not allow us to distinguish whether the decreased ability of the 2809 and T1-259 mutants to overcome an antiestrogen-induced arrest was due to decreased expression of these proteins, or if they were truly less active than wild type SVLT.

Inact

possil

resist

MCF

encoc

was

micr

Figu

than

there

med

effe

con

inac

plas

MC

see

the

tha

re

M

P

th

sl

a

Inactivation of p53 does not confer antiestrogen resistance. To further investigate the possibility that p53 inactivation played a role in SVLT's ability to confer antiestrogen resistance, we examined the ability of PyLT to promote proliferation of ICI-arrested MCF-7 cells. PyLT binds to pRb, but does not bind or inactivate p53 (21, 27). A plasmid encoding wild type PyLT (pCMVLT) was transfected into MCF-7 cells, and proliferation was assayed in the presence of ICI as described above. Representative fluorescence micrographs are shown in Figure 2.3, and summaries of the experiments are shown in Figures 2.2B and 2.4. Although the average induction of proliferation was slightly lower than seen with SVLT, the difference was not statistically significant. These results therefore indicate that PyLT is as efficient as SVLT in overcoming an antiestrogen-mediated arrest of MCF-7 cells, and establish that p53 inactivation is not required for this effect.

The results presented above indicated that p53 inactivation was not required to confer antiestrogen resistance to cells under conditions where pRb family members were inactivated. To examine the effects of p53 inactivation alone on antiestrogen sensitivity, a plasmid encoding del239, a dominant negative (dn) p53 mutant (44), was transfected into MCF-7 cells. Expression of this protein did not increase BrdU incorporation above that seen in untransfected cells (Figure 2.4). In addition, del239 expression did not enhance the level of proliferation induced by PyLT alone in co-transfection experiments. We therefore conclude that p53 inactivation alone is not sufficient to confer antiestrogen resistance, and that it does not enhance the resistance conferred by pRb inactivation, in MCF-7 cells.

PyLT confers antiestrogen resistance in long term assays. It was previously reported that over-expression of cyclin D1 conferred antiestrogen resistance to MCF-7 cells in short term assays, but was not sufficient to promote long term growth in the presence of antiestrogen (15). We therefore wanted to determine if LT expression was sufficient to

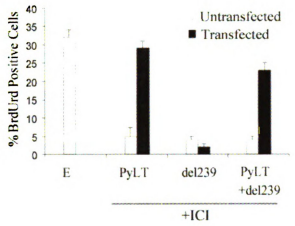


Figure 2.4. Effects of PyLT and dominant negative p53 expression on ICI sensitivity in MCF-7 cells. MCF7 cells were transfected with plasmids encoding PyLT, a dn p53 (del239), or both. BrdU incorporation in the presence of ICI was determined in PyLT or del239 positive cells (filled bars) and PyLT or del239 negative cells (open bars), and is expressed as a percentage of the total cells. BrdU incorporation was also measured in cycling (E) cells as a positive control. Results represent the mean +/- SE of three independent experiments.

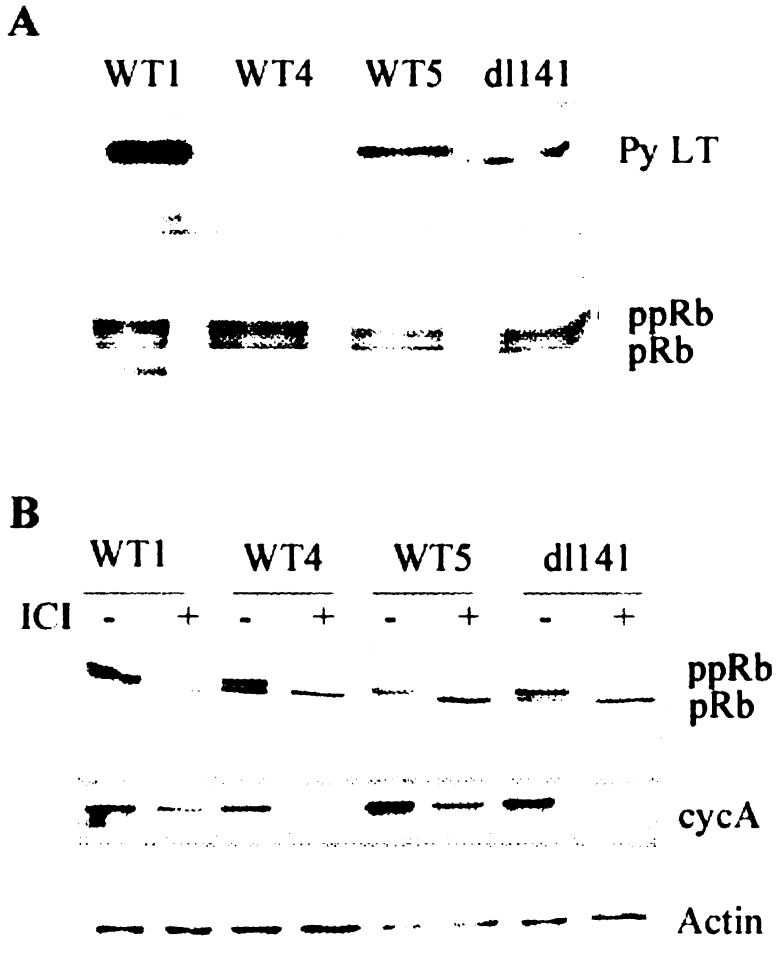


Figure 2.5. Effects of PyLT expression on ICI sensitivity in stably transfected cells. MCF-7 cells were transfected with vectors encoding PyLT, and G418^r colonies were selected and propagated as described in Materials and Methods. A) G418^r cell lines were assayed for PyLT expression and pRb phosphorylation by western blotting. Cell lines WT1 and WT5 expressed wild type PyLT. dl-141 cells expressed a pRb binding mutant of PyLT. WT4 was a G418^r clone that did not express detectable PyLT, and served as a negative control. Hyperphosphorylated pRb (ppRb) migrates more slowly than the hypophosphorylated form (pRb) on these gels. B) Cell lines were plated in medium containing 5% FBS, treated with either ICI (+) or vehicle (-) for 48 hrs, and cell extracts were prepared and analyzed for pRb phosphorylation and cyclin A expression by western blotting. The blots were also probed for actin as a control for protein loading.

cont

Con

intr

sele

into

line

bin

line

con

sim

(Fi

eva

we

sho

tre

hy

co

wi

the

sir

rec

confer antiestrogen resistance to MCF-7 cells in long term proliferation assays. Constructs encoding either a wild type or pRb binding mutant (dl141) of PyLT were introduced into MCF-7 cells using the pneo-LT1 vector, which contains a G418^r gene for selection of stably transfected cells. Individual G418^r colonies were selected, expanded into cell lines, and tested for expression of PyLT protein by western blotting. Two cell lines expressing the wild type protein (WT1 and WT5) and one expressing the pRb binding mutant (dl 141) were chosen for further analysis (Figure 2.5 A). One G418^r cell line that did not express detectable levels of PyLT protein (WT4), was used as a negative control. All of these cell lines grew well in the presence of 5% FBS, and contained similar amounts and ratios of hyper- and hypo-phosphorylated pRb in this medium (Figure 2.5 A).

The effects of ICI treatment on proliferation of the PyLT expressing cells were evaluated in several ways. In Figure 2.5B, pRb phosphorylation and cyclin A expression were examined by western blotting after 2 days of ICI treatment. Previous studies have shown that both pRb phosphorylation and cyclin A expression are inhibited by ICI treatment of the parental MCF-7 cell line (14). In agreement with this, hyperphosphorylated pRb and cyclin A protein were undetectable in ICI-treated WT4 control cells, which do not express PyLT. In contrast, WT1 and WT5 cells, which express wild type PyLT, contained both hyperphosphorylated pRb and cyclin A when grown in the presence of ICI. Cells expressing dl141, the pRb binding mutant of PyLT, had a similar phenotype to WT4 cells, confirming that the pRb-binding domain is absolutely required for viral tumor antigens to promote proliferation in the presence of ICI. The

effect

by c

incor

thos

resis

pho

ina

test

the

lar

ant

vir

Th

its

pr

ro

pr

a

effects of ICI treatment on pRb phosphorylation and cyclin A expression were paralleled by changes in cell proliferation, as measured by either ^{3}H-thymidine or BrdU incorporation (data not shown). Taken together, the results in stable transfectants confirm those seen in transient assays, and indicate that expression of PyLT confers antiestrogen resistance to MCF-7 cells

DISCUSSION

Previous evidence linking estrogen and antiestrogen treatments to pRb phosphorylation suggested that pRb might be a crucial target of estrogen, and that inactivation of this tumor suppressor protein might lead to antiestrogen resistance. We tested this possibility by expressing viral T antigens in MCF-7 cells, and determining their effects on antiestrogen sensitivity. Expression of either the SV40 or polyoma virus large T antigen promotes MCF-7 cell proliferation in the presence of the pure antiestrogen ICI 182,780. Furthermore, this effect is dependent upon the ability of the viral proteins to bind and inactivate pRb, but is independent of their ability to bind p53. Thus, pRb is an important target of estrogens in regulating MCF-7 cell proliferation, and its inactivation can contribute to the development of antiestrogen resistance.

The fact that both pRb inactivation and overexpression of cyclin D1 (15, 16) promote MCF-7 cell proliferation in the presence of antiestrogens confirms an important role for the cyclin D/pRb pathway in estrogen dependence. It also suggests that the primary mechanism by which cyclin D1 overcomes an antiestrogen-mediated arrest is by activating Cdk 4/6, which in turn phosphorylates pRb. As discussed previously, Cdk2

activity is inhibited in antiestrogen treated MCF-7 cells, and it has been proposed that a second role for cyclin D1 in promoting antiestrogen resistance is to sequester $p21^{Waf1}$, thereby leading to Cdk2 activation. The results reported here suggest that either antiestrogen treatment fails to inhibit Cdk2 activity in MCF-7 cells expressing T antigens, or that the requirement for Cdk2 activity is bypassed in these cells. Since $p21^{Waf1}$ transcription is induced by p53 (45), one mechanism by which SVLT could lead to Cdk2 activation would be by binding and inactivating p53, leading to decreases in $p21^{Waf1}$ levels. However, this does not appear to play a role in promoting antiestrogen resistance, since LTs that do not inactivate p53 induce proliferation in the presence of ICI, and since a dn p53 mutant does not induce proliferation in the presence of ICI. The mechanisms by which LTs overcome the inhibition of Cdk2 activity by antiestrogens therefore remain to be elucidated.

Although our results clearly demonstrate that an intact pRb -binding domain is required, they do not establish if pRb inactivation alone is sufficient to confer antiestrogen resistance to MCF-7 cells. The Rb family of proteins also contains the related proteins p107 and p130. We cannot eliminate a role for p107 and/or p130 inactivation in conferring antiestrogen resistance, since both SVLT and PyLT bind these proteins, and since the PVU1 and dl141 mutations eliminate binding to all members of the pRb family (21, 27). Since pRb is known to be phosphorylated in response to estrogen treatment, and since its role in regulating cell proliferation is well established, it is likely to be the major target of estrogen action. However, it would be interesting to determine if other pRb family members also contribute to estrogen/antiestrogen sensitivity in breast cancer cells.

SVLT possesses additional functions that might contribute to antiestrogen resistance, including the ability to bind p300 and components of the basal transcription

apparatus (21, 27). It is possible that LT binding to components of the basal transcription apparatus plays a role in promoting antiestrogen resistance, since this function maps to N-terminal domains of SVLT that are maintained in the T1-259 mutant (46, 47). We are not aware of any reports that PyLT also binds to basal transcription factors, but it may do so. In contrast, the p300 binding activity of LT is unlikely to be involved in promoting antiestrogen resistance, since several reports indicate that C-terminal sequences that are missing in the T1-259 mutant are required for p300 binding by both SVLT and PyLT (48, 49). As discussed previously, the p53 binding/inactivation function of SVLT is not required to confer antiestrogen resistance, since it is missing in both the T1-259 and 2809 mutants, and in PyLT.

It is interesting to compare the LT domains required to overcome cell cycle arrests in different systems. Expression of SVLT can overcome a TGF-β1 mediated G1 arrest in keratinocytes (50) and mink lung epithelial cells (51), and this effect is dependent upon the pRb-binding domain. It is also probably independent of p53 binding, since the HPV E7 protein, which does not bind p53, has a similar effect (51). However, the absolute requirement for a pRb-binding domain, and the lack of a role for p53 binding, in promoting proliferation is not universal. For example, at least four independent functions of SVLT, including pRb binding, p53 binding, and an unidentified function abolished by the S189N mutation, contribute to its ability to promote proliferation in serum starved CV1P cells (33, 52). Mutations in any one of these functions, including pRb binding, had little effect on LT's ability to promote proliferation in this system. In contrast, double mutants, including pRb binding/p53 binding double mutants, showed significant defects. This variability in the requirements for pRb and/or p53 inactivation in overcoming cell cycle arrests may be due to differences in the mechanisms by which treatments such as ICI, TGF-β1 and serum starvation induce cell cycle arrests, or may represent differences in the requirements for pRb and/or p53 inactivation between cell lines.

We have also considered the possibility that small T antigen (ST) plays a role in conferring antiestrogen resistance, since the SVLT encoding vectors used in these experiments contain the entire early region of the viral genome, and therefore also encode ST. The PVU1 mutant encodes a wild type ST, yet it is completely defective in promoting proliferation in the presence of ICI. Thus, ST expression is not sufficient to confer antiestrogen resistance. In addition, the CMVLT vector used to express PyLT in transient transfections contains a LT cDNA, and does not encode ST. This plasmid confers ICI resistance to MCF-7 cells with the same efficiency as SVLT, indicating that ST expression is not required.

We attempted to determine whether LT expression confers antiestrogen resistance to MCF-7 cells in long term proliferation assays. Although overexpression of cyclin D1 conferred antiestrogen resistance to MCF-7 cells in short term assays, it was not sufficient to promote the long-term growth of these cells in the presence of antiestrogen (15). Stably transfected cells expressing PyLT were resistant to ICI treatment, as demonstrated by the facts that they contained cyclin A and hyperphosphorylated pRb, and synthesized DNA in the presence of ICI. However, this resistance was not complete. As shown in Figure 2.5B, both pRb phosphorylation and cyclin A protein levels were reduced somewhat by ICI treatment of WT1 and WT5 cells, although to a lesser extent than in the control WT4 or dl141 cells. Similar effects were seen on BrdU and ^{3}H-thymidine incorporation (data not shown), suggesting that proliferation of PyLT expressing MCF-7 cells is not completely resistant to antiestrogen treatment. However, when the stably transfected cell lines were examined for PyLT expression by immunofluorescence, only a subset of cells had detectable levels of LT, even during selective growth in G418 (data not shown). The apparent partial ICI-sensitivity seen in these experiments may therefore be due to variable expression of PyLT in the transfected cells, rather than to an inability of LT to confer long-term antiestrogen resistance.

Finally, the fact that both pRb inactivation and cyclin D1 overexpression promote

antiestro

might o

pieces o

examine

to date

a signi

failure

depend

pRb st

resista

antiestrogen resistance in MCF-7 cells suggests that deregulation of the pRb pathway might occur during the development of antiestrogen resistance *in vivo*. Several indirect pieces of evidence support this suggestion. Although 20-25% of breast cancer cell lines examined lack functional pRb, all estrogen-responsive breast cancer cell lines identified to date contain wild type pRb (53, 54). In addition, in one study of primary breast cancers a significant correlation was observed between the absence of pRb expression and a failure to respond to antiestrogen therapy (55). Together, these data suggest that hormone dependence cannot be maintained in the absence of pRb function, and that changes in pRb status or regulation could be involved in the progression of tumors to antiestrogen resistance.

REFERENCES

1. Melora, D. B., Allred, C. C., and O'Conell, P. Principles of Molecular Medicine, 1st edition, p. 625-632: Humana Press, 1998.
2. Sutherland, R. L., Green, M. D., Hall, R. E., Reddel, R. R., and Taylor, I. W. Tamoxifen induces accumulation of MCF 7 human mammary carcinoma cells in the G0/G1 phase of the cell cycle, Eur J Cancer Clin Oncol. *19:* 615-21., 1983.
3. Sutherland, R. L., Reddel, R. R., and Green, M. D. Effects of oestrogens on cell proliferation and cell cycle kinetics. A hypothesis on the cell cycle effects of antioestrogens, Eur J Cancer Clin Oncol. *19:* 307-18., 1983.
4. Sherr, C. J. Mammalian G1 cyclins, Cell *73:* 1059-65., 1993.
5. Sherr, C. J. G1 phase progression: cycling on cue, Cell. *79:* 551-5., 1994.
6. Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4, Genes Dev. *7:* 331-42., 1993.
7. Hatakeyama, M. and Weinberg, R. A. The role of RB in cell cycle control, Prog Cell Cycle Res. *1:* 9-19, 1995.
8. Nevins, J. R., Leone, G., DeGregori, J., and Jakoi, L. Role of the Rb/E2F pathway in cell growth control, J Cell Physiol. *173:* 233-6., 1997.
9. Osborne, C. K., Hobbs, K., and Clark, G. M. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice, Cancer Res. *45:* 584-90., 1985.
10. Soule, H. D. and McGrath, C. M. Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice, Cancer Lett. *10:* 177-89., 1980.
11. Foster, J. S. and Wimalasena, J. Estrogen regulates activity of cyclin-dependent

kinases and retinoblastoma protein phosphorylation in breast cancer cells, Mol Endocrinol. *10:* 488-98., 1996.

12. Planas-Silva, M. D. and Weinberg, R. A. Estrogen-dependent cyclin E-Cdk2 activation through p21 redistribution, Mol Cell Biol. *17:* 4059-69., 1997.

13. Prall, O. W., Sarcevic, B., Musgrove, E. A., Watts, C. K., and Sutherland, R. L. Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E- Cdk2, J Biol Chem. *272:* 10882-94., 1997.

14. Watts, C. K., Brady, A., Sarcevic, B., deFazio, A., Musgrove, E. A., and Sutherland, R. L. Antiestrogen inhibition of cell cycle progression in breast cancer cells in associated with inhibition of cyclin-dependent kinase activity and decreased retinoblastoma protein phosphorylation, Mol Endocrinol. *9:* 1804-13., 1995.

15. Pacilio, C., Germano, D., Addeo, R., Altucci, L., Petrizzi, V. B., Cancemi, M., Cicatiello, L., Salzano, S., Lallemand, F., Michalides, R. J., Bresciani, F., and Weisz, A. Constitutive overexpression of cyclin D1 does not prevent inhibition of hormone-responsive human breast cancer cell growth by antiestrogens, Cancer Res. *58:* 871-6., 1998.

16. Prall, O. W., Rogan, E. M., Musgrove, E. A., Watts, C. K., and Sutherland, R. L. c-Myc or cyclin D1 mimics estrogen effects on cyclin E-Cdk2 activation and cell cycle reentry, Mol Cell Biol. *18:* 4499-508., 1998.

17. Neuman, E., Ladha, M. H., Lin, N., Upton, T. M., Miller, S. J., DiRenzo, J., Pestell, R. G., Hinds, P. W., Dowdy, S. F., Brown, M., and Ewen, M. E. Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of Cdk4, Mol Cell Biol. *17:* 5338-47., 1997.

18. Zwijsen, R. M., Wientjens, E., Klompmaker, R., van der Sman, J., Bernards, R.,

and Michalides, R. J. CDK-independent activation of estrogen receptor by cyclin D1, Cell *88:* 405-15., 1997.

19. Hirai, H. and Sherr, C. J. Interaction of D-type cyclins with a novel myb-like transcription factor, DMP1, Mol Cell Biol. *16:* 6457-67., 1996.

20. Inoue, K. and Sherr, C. J. Gene expression and cell cycle arrest mediated by transcription factor DMP1 is antagonized by D-type cyclins through a cyclin-dependent-kinase- independent mechanism, Mol Cell Biol. *18:* 1590-600., 1998.

21. Brodsky, J. L. and Pipas, J. M. Polyomavirus T antigens: molecular chaperones for multiprotein complexes, J Virol. *72:* 5329-34., 1998.

22. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E., and Livingston, D. M. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene, Cell *54:* 275-83., 1988.

23. Larose, A., St-Onge, L., and Bastin, M. Mutations in polyomavirus large T affecting immortalization of primary rat embryo fibroblasts, Virology *176:* 98-105., 1990.

24. Ludlow, J. W., Shon, J., Pipas, J. M., Livingston, D. M., and DeCaprio, J. A. The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T, Cell *60:* 387-96., 1990.

25. Lane, D. P. and Crawford, L. V. T antigen is bound to a host protein in SV40-transformed cells, Nature *278:* 261-3., 1979.

26. Linzer, D. I. and Levine, A. J. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells, Cell *17:* 43-52., 1979.

27. Cole, C.N. Fundamental Virology, Lippincott-Raven, 917-945, 1996.

28. Harlow, E., Crawford, L. V., Pim, D. C., and Williamson, N. M. Monoclonal

antibodies specific for simian virus 40 tumor antigens, J Virol. *39:* 861-9., 1981.

29. Zhu, J. Y. and Cole, C. N. Linker insertion mutants of simian virus 40 large T antigen that show trans-dominant interference with wild-type large T antigen map to multiple sites within the T-antigen gene, J Virol. *63:* 4777-86., 1989.

30. Zhu, J. Y., Rice, P. W., Chamberlain, M., and Cole, C. N. Mapping the transcriptional transactivation function of simian virus 40 large T antigen, J Virol. *65:* 2778-90., 1991.

31. Gluzman, Y., Frisque, R. J., and Sambrook, J. Origin-defective mutants of SV40, Cold Spring Harb Symp Quant Biol. *44:* 293-300., 1980.

32. Keller, J. M. and Alwine, J. C. Activation of the SV40 late promoter: direct effects of T antigen in the absence of viral DNA replication, Cell *36:* 381-9., 1984.

33. Dobbelstein, M., Arthur, A. K., Dehde, S., van Zee, K., Dickmanns, A., and Fanning, E. Intracistronic complementation reveals a new function of SV40 T antigen that co-operates with Rb and p53 binding to stimulate DNA synthesis in quiescent cells, Oncogene *7:* 837-47., 1992.

34. Clark, R., Peden, K., Pipas, J. M., Nathans, D., and Tjian, R. Biochemical activities of T-antigen proteins encoded by simian virus 40 A gene deletion mutants, Mol Cell Biol. *3:* 220-8., 1983.

35. Cole, C. N., Tornow, J., Clark, R., and Tjian, R. Properties of the simian virus 40 (SV40) large T antigens encoded by SV40 mutants with deletions in gene A, J Virol. *57:* 539-46., 1986.

36. Pipas, J. M., Peden, K. W., and Nathans, D. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T-antigen gene, Mol Cell Biol. *3:* 203-13., 1983.

37. Howes, S. H., Bockus, B. J., and Schaffhausen, B. S. Genetic analysis of polyomavirus large T nuclear localization: nuclear localization is required for

productive association with pRb family members, J Virol. *70:* 3581-8., 1996.

38. Larose, A., Dyson, N., Sullivan, M., Harlow, E., and Bastin, M. Polyomavirus large T mutants affected in retinoblastoma protein binding are defective in immortalization, J Virol. *65:* 2308-13., 1991.

39. Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. p53: a frequent target for genetic abnormalities in lung cancer, Science *246:* 491-4., 1989.

40. Lippman, M., Bolan, G., and Huff, K. The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture, Cancer Res. *36:* 4595-601., 1976.

41. Wakeling, A. E., Dukes, M., and Bowler, J. A potent specific pure antiestrogen with clinical potential, Cancer Res. *51:* 3867-73., 1991.

42. Brunner, N., Frandsen, T. L., Holst-Hansen, C., Bei, M., Thompson, E. W., Wakeling, A. E., Lippman, M. E., and Clarke, R. MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780, Cancer Res. *53:* 3229-32., 1993.

43. Brunner, N., Boysen, B., Jirus, S., Skaar, T. C., Holst-Hansen, C., Lippman, J., Frandsen, T., Spang-Thomsen, M., Fuqua, S. A., and Clarke, R. MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen, Cancer Res. *57:* 3486-93., 1997.

44. Gao, Q., Hauser, S. H., Liu, X. L., Wazer, D. E., Madoc-Jones, H., and Band, V. Mutant p53-induced immortalization of primary human mammary epithelial cells, Cancer Res. *56:* 3129-33., 1996.

45. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression, Cell *75:* 817-25., 1993.

46. Damania, B. and Alwine, J. C. TAF-like function of SV40 large T antigen, Genes Dev. *10:* 1369-81., 1996.

47. Johnston, S. D., Yu, X. M., and Mertz, J. E. The major transcriptional transactivation domain of simian virus 40 large T antigen associates nonconcurrently with multiple components of the transcriptional preinitiation complex, J Virol. *70:* 1191-202., 1996.

48. Lill, N. L., Tevethia, M. J., Eckner, R., Livingston, D. M., and Modjtahedi, N. p300 family members associate with the carboxyl terminus of simian virus 40 large tumor antigen, J Virol. *71:* 129-37., 1997.

49. Nemethova, M. and Wintersberger, E. Polyomavirus large T antigen binds the transcriptional coactivator protein p300, J Virol. *73:* 1734-9., 1999.

50. Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, M. R., Munger, K., Howley, P. M., and Moses, H. L. TGF-beta 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains, Cell *61:* 777-85., 1990.

51. Laiho, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M., and Massague, J. Growth inhibition by TGF-beta linked to suppression of retinoblastoma protein phosphorylation, Cell *62:* 175-85., 1990.

52. Dickmanns, A., Zeitvogel, A., Simmersbach, F., Weber, R., Arthur, A. K., Dehde, S., Wildeman, A. G., and Fanning, E. The kinetics of simian virus 40-induced progression of quiescent cells into S phase depend on four independent functions of large T antigen, J Virol. *68:* 5496-508., 1994.

53. Lee, E. Y., To, H., Shew, J. Y., Bookstein, R., Scully, P., and Lee, W. H. Inactivation of the retinoblastoma susceptibility gene in human breast cancers, Science *241:* 218-21., 1988.

54. T'Ang, A., Varley, J. M., Chakraborty, S., Murphree, A. L., and Fung, Y. K. Structural rearrangement of the retinoblastoma gene in human breast carcinoma,

Science *242:* 263-6., 1988.

55. Anderson, J. J., Tiniakos, D. G., McIntosh, G. G., Autzen, P., Henry, J. A., Thomas, M. D., Reed, J., Horne, G. M., Lennard, T. W., Angus, B., and Horne, C. H. Retinoblastoma protein in human breast carcinoma: immunohistochemical study using a new monoclonal antibody effective on routinely processed tissues, J Pathol. *180:* 65-70., 1996.

56. Kalderon, D. and Smith, A. E. In vitro mutagenesis of a putative DNA binding domain of SV40 large-T, Virology. *139:* 109-37., 1984.

CHAPTER 3

Abrogation of Polyoma LT Induced Proliferation of Antiestrogen Treated MCF-7 Cells by $p21^{Waf1}$ but not by $p16^{INK4A}$

ABSTRACT

Estrogens increase the proliferation of MCF-7 cells, a human breast cancer cell line, by activating Cdk4 and Cdk2 and this is accompanied by pRb phosphorylation. pRb is a substrate for these kinases, and its phosphorylation de-represses the transcription of cyclin E and cyclin A, both positive regulators of Cdk2. It is not known if estrogens regulate Cdk2 activity via pRb inactivation or in a pRb independent manner. Previously, we had demonstrated that inactivation of pRb using viral large tumor antigens caused proliferation of antiestrogen-treated MCF-7 cells. Using $p16^{INK4A}$ and $p21^{Waf1}$, inhibitors of Cdk4 and Cdk2 respectively, we demonstrate here that Cdk2 but not Cdk4 is required for proliferation in cells where pRb is inactivated by Polyoma LT. Furthermore, we demonstrate that expression of PyLT induces Cdk2 activity in antiestrogen treated MCF-7 cells. These results support a model in which estrogen increases Cdk2 activity by regulating pRb phosphorylation.

INTRODUCTION

Approximately 40% of all human breast cancers are estrogen dependent for proliferation (1). MCF-7 cells are widely studied as a model of hormone dependent human breast cancers. Their proliferation is stimulated by estrogens and is inhibited by antiestrogens such as tamoxifen and ICI 182,780, *in vivo* as well *in vitro* (2-4). Estrogens stimulate MCF-7 proliferation by regulating multiple components of the cell cycle regulatory machinery that lead to activation of cyclin dependent kinases (Cdks), Cdk4 and Cdk2. These Cdks phosphorylate and thereby inactivate tumor suppressors of the retinoblastoma family (5). The pRb family of proteins negatively regulate the transition from G1 to S phase by sequestering the E2F family of transcription factors (6). MCF-7 cells have wild type pRb (7), and estrogen treatment of these cells arrested in G1 by estrogen deprivation or antiestrogen treatment leads to phosphorylation and inactivation of the pRb family of tumor suppressors, E2F release and progress into S phase (8, 9).

We have previously demonstrated that inactivation of pRb family members by SV40 large tumor (LT) and Polyoma LT virus (LT) antigens causes proliferation of antiestrogen arrested MCF-7 cells (10). This ability of LTs to induce proliferation of antiestrogen treated cells was dependent upon an intact pRb binding domain. Furthermore, the ability of SV40 LT to overcome the cell cycle arrest resided within the N terminal 136 amino acids of the T antigen (unpublished results), thereby excluding a role for p53 binding or a host of other functions in this large multifunctional protein, in overcoming an ICI arrest. Together, these results suggest that inactivation of pRb family

members is required for and may be sufficient to overcome the antiestrogen mediated arrest.

Both Cdk4 and Cdk2 can phosphorylate pRb, and both of their activities are regulated by estrogen and antiestrogens in MCF-7 cells (11). However, it is unclear whether both are upstream of pRb, or whether Cdk2 is activated in a pRb dependent manner (See Figure 3.1.). In order to distinguish between these two models, we tested whether Cdk4 and Cdk2 activities were required in cells in which pRb family members were inactivated by LT. Further, we tested if the activities of these kinases were still regulated by antiestrogens. The results indicated that Cdk4 activity is not required for proliferation of MCF-7 cells in which pRb has been inactivated by LT expression, but is required in cells with intact pRb. In contrast, Cdk2 activity is required for proliferation irrespective of the pRb status of cells. These results indicate that Cdk4 is mainly required for in pRb inactivation while Cdk2 has targets in addition to pRb that are required for inducing proliferation. Additionally, using a stable cell line that inducibly expresses LT, we directly demonstrate that Cdk2 is activated upon LT expression in ICI treated MCF-7 cells. These results indicate that in the absence of pRb function, Cdk2 activity is not fully inhibited by antiestrogens in MCF-7 cells.

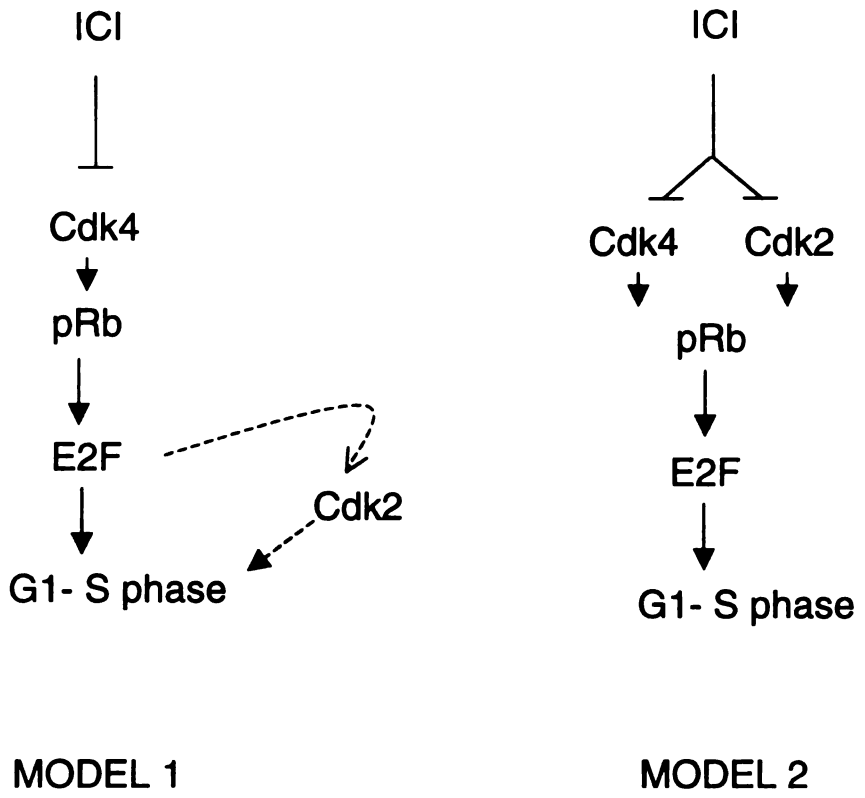


Figure 3.1. Models for the regulation of Cdk4 and Cdk2 activities by antiestrogens. In Model 1, ICI regulates Cdk4 and thereby indirectly regulates Cdk2 activity in a pRb dependent manner. The predictions of this model are that Cdk4 would be redundant in cells where pRb is inactivated while Cdk2 would be still required. In addition, Cdk2 activity would not be inhibited by antiestrogens in cells where pRb is inactivated. In Model 2, both Cdk4 and Cdk2 activities are independently regulated by antiestrogens via a pRb independent mechanism. In this case, both would be redundant and not required for proliferation in cells in which pRb is inactivated.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells were obtained from Michael Johnson at Lombardi Cancer Center and were routinely maintained in IMEM medium (Biofluids) containing 5% fetal bovine serum (FBS) (HyClone), Penicillin (100 units/ml), Streptomycin (100 μg/ml). The PyLT inducible cell lines were routinely maintained in the selective media containing Hygromycin 10 μg/ml plus G418 (50 μg/ml).

Cell Cycle Analysis. Cells were trypsinized, washed in phosphate buffered saline (PBS), suspended in PBS+10% FBS, fixed with 80% ice cold ethanol and stored at –20□ C. Prior to analysis, cells were washed twice with PBS, then suspended in PBS + 1 mg/ml RNaseA, 0.2 mg/ml propidium iodide, 0.5 mM EDTA and 0.1% Triton-X-100. Cells were then analyzed for red fluorescence on a FACS Vantage flow cytometer; cell cycle distribution was determined using WCycle software.

Plasmids and Cloning. Plasmids encoding the wild type LT and pRb binding mutant (Rb-LT) were obtained from Dr. Brian Schaffhausen and are described previously (12). The pGFP CMV vectors encoding the $p16^{INK4A}$ and $p21^{Waf1}$ as GFP fusion proteins were obtained from Dr. Steve Weintraub, Washington University, Saint Louis, MO. LT cDNA was cloned into the pLH-Z12-I vector in a two-step ligation. The LT cDNA was excised from the pCMV LT vector using *Sal*I and *Bam*HI and ligated into *Sal*I and *Bam*HI digested pIC19H vector to generate the pIC19H-LT vector. In the second step, the LT cDNA was excised from the pIC19H-LT using *Sal*I (site was filled in with nucleotides

using T4 DNA polymerase to generate blunt ends) and *Cla*I, and ligated into the polylinker region of pLH-Z12-I vector that was digested with *EcoR*I (site filled in with nucleotides using T4 DNA polymerase to generate blunt ends) and *Cla*I, to generate the pLH-Z12-LT.

Construction of stably transfected cell lines expressing LT from an inducible promoter. A novel gene regulation system based on small molecule regulated protein dimerization, consisting of two plasmids and a dimerizer AP1510 was used (ARIAD pharmaceuticals) (13). The first plasmid (pCEN-F3p65/Z1F3/Neo) contains a neomycin resistance gene and encodes two distinct fusion proteins, one containing a DNA–binding domain and the other a transcriptional activation domain. Each of these domains is fused to a cellular protein (FKBP) that interacts with a small molecule dimerizer (AP1510). In the absence of the dimerizer, the two fusion proteins have no affinity for one another, but in the presence of the dimerizer they interact to form an active transcription factor. A cell line, MCF-7-6, that was stably transfected with pCEN-F3p65/Z1F3/Neo was established in our lab (C. J. Zhang unpublished results). The second plasmid (pLH-Z12-I) contains a hygromycin resistance gene, and a polylinker downstream of a promoter that contains DNA elements that are recognized by the DNA binding domain encoded by pCEN-F3p65/Z1F3/Neo. The pLH-Z12-I-LT vector was stably transfected into the MCF-7-6 cell line using the Lipofectin reagent (Gibco BRL) and hygromycin resistant clones were selected (30 μg/ml) and then screened by immunoblotting and immunofluorescence.

Chemicals and Antibodies. ICI 182,780 was a gift of A. Wakeling (Zeneca Pharmaceuticals) and 17β-estradiol (E) was purchased from Sigma. 5' Bromodeoxyuridine was obtained from Boehringer Mannheim. AP1510 was kindly provided by ARIAD Pharmaceuticals (Cambridge, MA). LT antibody was a rat polyclonal and was a kind gift of Dr. Michelle Fluck. Cdk4 antibody was a goat H-22 antibody from Santa Cruz Biotechnology, whereas the Cdk2 antibody was a rabbit polyclonal gifted by Charles J. Sherr. Antibodies to cyclins A, D1, E, the kinase inhibitors $p21^{Waf1}$ and $p27^{Kip1}$ and actin are described in the following chapter.

Immunofluorescence. MCF-7-6 cells were plated on glass coverslips and transiently transfected with either the LT plasmid alone or in combination with the various pGFP plasmids using the Superfect transfection reagent (Qiagen). After transfection, cells were treated with medium containing 5% charcoal stripped serum and ICI (100 nM) and were labeled with 25 μM 5-Bromo-2'-deoxyuridine (BrdU) for 5 hours before fixation at 48 hours post transfection. The double immunofluorescence procedure for detecting transfected proteins and BrdU has been described previously (10). BrdU incorporation was detected using the appropriate α-BrdU antibody (mouse α-BrdU). Cells were viewed under a fluorescent microscope (Olympus), and the percentage of LT expressing cells that were positive for BrdU, and of cells that were positive for BrdU alone was determined. At least one hundred LT positive and negative cells were counted in each experiment. For the detection of LT expression in the stable inducible cell lines, cells were plated on glass coverslips and were treated with AP1510 or untreated and harvested 24 hr after treatment. Imunofluorescence for LT was done as described above and cells

were viewed under a fluorescent microscope. The images shown in Figure 3.3B. were taken on the Olympus fluorescent microscope, scanned and converted to grayscale using the Adobe Photoshop software program.

Long Term Growth Assays. Cells were plated at 20,000/well on a 24 well tissue culture plate and after overnight incubation in FBS containing media, they were kept in CSS containing media with 100nM ICI for 48 hr to induce a G0/G1 arrest. The media was then changed to either CSS, CSS plus ICI or CSS plus E alone or to these media plus AP1510 (300nM). Cells were harvested every 2 or 3 days and fresh media with the appropriate treatments added every 2 or 3 days for the duration of the experiment. All samples were harvested in triplicate and the DNA content determined by a fluorometric assay described previously (14).

Immunoprecipitations and Kinase Assays. Immunoprecipitations (IP) and Cdk4 activity measurements were performed using a modification of a published method (15). Unless indicated otherwise, all manipulations were carried out on ice. Cells were washed twice in ice cold PBS, harvested by scraping in PBS and pelleted; the pellets were frozen and stored in liquid nitrogen until the day of analysis. Cells were resuspended and lysed by sonication in IP buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20, 10% glycerol, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF, 1mM $NaVO_4$, 0.1 mM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). The lysates were cleared by centrifugation, and protein concentrations were quantitated using BioRad's protein assay reagent. For IPs, 75 μg of total protein was diluted to 500 μl with

IP buffer, then incubated for 60 minutes (m) rocking at 4° C with 1.5 μg antibody (anti-Cdk4 H-22-G or normal goat IgG, Santa Cruz or anti-Cdk2 antiserum) bound to 7.5 μl protein G agarose beads (Boehringer Mannheim). Beads were pelleted and washed four times with 100 μl IP buffer and twice with 100 μl kinase buffer (50 mM HEPES pH7.5, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM $NaVO_4$, and 1 mM NaF). Pellets were then suspended in 40 μl kinase buffer containing 10 μCi γ-(32P) ATP, 20 μM cold ATP, and 1.0 μl glutathione sepharose 4B (Amersham Pharmacia) to which approximately 2.0 μg of a fusion protein between glutathione-S-transferase (GST) and amino acids 792 to 928 of human pRb were bound (GST-Rb bacterial expression vector was kindly provided by Dr. William Kaelin of the Dana Farber Cancer Institute) or Histone H1 (HH1) was used for Cdk2 assays. The reactions were incubated at 30° C for 30 m with occasional mixing, after which they were boiled in SDS loading buffer containing mercaptoethanol. Beads were pelleted and the supernatants transferred to clean tubes. Aliquots of the reaction products were resolved on 10% SDS polyacrylamide gels, which were then dried and exposed to autoradiography film. Phosphorylated GST-Rb or HH1 bands were quantitated by phosphorimaging with a Storm phosphorimager (Molecular Dynamics) using ImageQuant software.

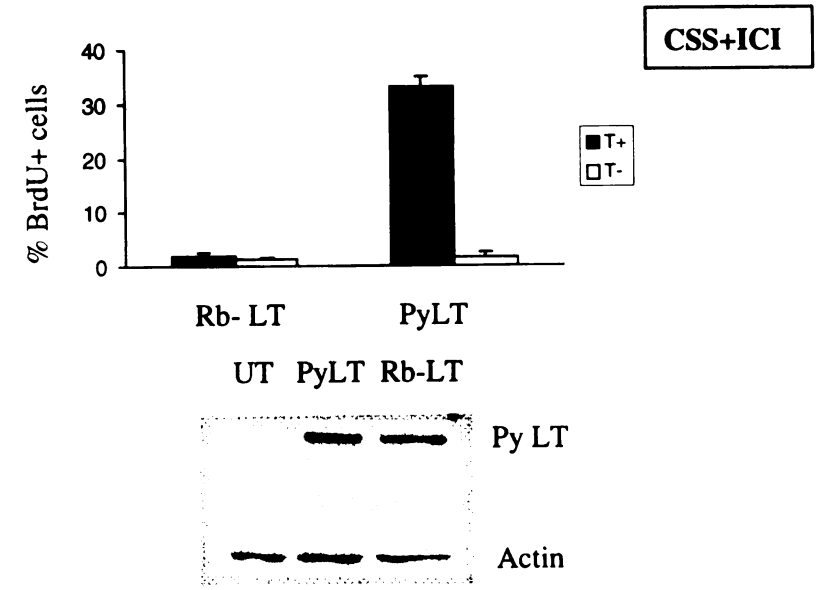
RESULTS

LT induced proliferation is sensitive to $p21^{Waf1}$ but resistant to $p16^{INK4A}$ Cdk inhibitor. A requirement for the pRb binding domain of PyLT antigen for conferring antiestrogen resistance was confirmed in initial experiments. Plasmids encoding wild type or a pRb binding mutant (Rb- LT) of PyLT were transiently transfected into MCF-7 cells and the cells were treated with antiestrogen ICI 182,780 for 43 hr and then BrdU (25 μM) labeled for an additional 5hr before fixation. The fixed cells were analyzed for PyLT expression and BrdU incorporation by indirect immunofluorescence. The percentage of cells that were actively synthesizing DNA was determined in PyLT expressing cells and in untransfected cells (Figure 3.2A upper panel). The results of this experiment demonstrated that expression of PyLT caused antiestrogen resistance. The pRb binding domain of wild type PyLT was absolutely required for this effect, as shown by the inability of the Rb-LT mutant to increase proliferation above the levels observed in untransfected cells despite similar expression levels of both proteins (Figure 3.2A, lower panel). These results confirmed previous results obtained with SV40 LT (10).

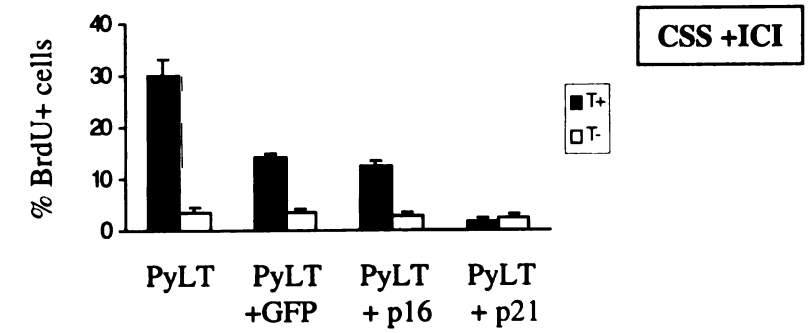
To determine if cells in which pRb family members were inactivated by LT required Cdk4 and Cdk2 activities for proliferation, MCF-7 cells were transfected with PyLT alone, or together with a vector expressing either Green Fluorescent protein (GFP), $p16^{INK4A}$-GFP or $p21^{Waf1}$-GFP. $p16^{INK4A}$ is a specific inhibitor of Cdk4 (6) whereas $p21^{Waf1}$ inhibits both Cdk2 and Cdk4, although recent reports suggest that it may also facilitate active cyclin D-Cdk4 complex formation (16). These inhibitors were expressed

Figure 3.2. MCF-7 cells expressing PyLT are sensitive to $p21^{Waf1}$ but refractory to $p16^{INK4A}$ arrest. A) MCF-7 cells were transfected with wild type PyLT or a Rb binding mutant (Rb-) of PyLT, treated with ICI (100nM), and proliferation was assayed by double indirect immunofluorescence for PyLT and BrdU at 48 hr post transfection as described in Materials and Methods. The percentage of in PyLT positive and negative cells that were aslo BrdU postive cells is shown as mean +/- S.E. of at least three independent experiments. At least 100 LT positive and negative cells were counted in each experiment. In a parallel experiment, wild type and mutant PyLT expression was assessed by Western blotting (lower panel, UT-untransfected control). B) MCF-7 cells were cotransfected with PyLT plus either pGFP (vector), p16-GFP or p21-GFP encoding plasmids. Proliferation in the presence of ICI was assayed by double indirect immunofluorescence for LT and BrdU(■ LT Positive cells, □ LT negative cells) C) The Rb binding mutant (Rb-LT) of PyLT was cotransfected with the above GFP constructs in cycling MCF-7 cells, and proliferation was assayed as described above.

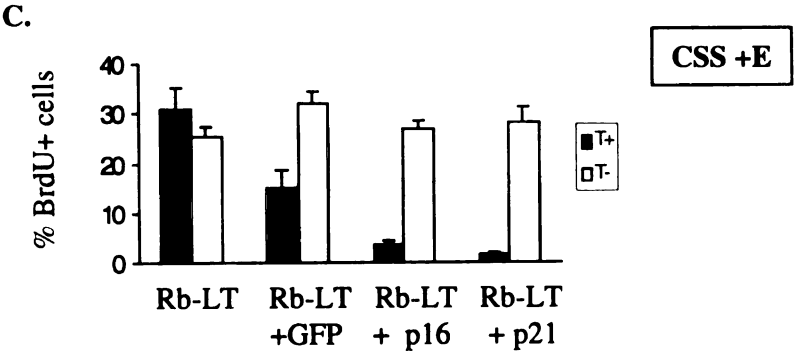
A.


CSS+ICI
% BrdU+ cells
40
30
20
10
0
T+
T-
Rb- LT
PyLT
UT
PyLT
Rb-LT
Py LT
Actin

B.


CSS +ICI
% BrdU+ cells
40
30
20
10
0
T+
T-
PyLT
PyLT +GFP
PyLT + p16
PyLT + p21

C.


CSS +E
% BrdU+ cells
40
30
20
10
0
T+
T-
Rb-LT
Rb-LT +GFP
Rb-LT + p16
Rb-LT + p21

as fusion proteins with GFP to facilitate their detection by visual inspection under a fluorescent microscope. In these cotransfection experiments, a higher percentage of cells expressed the GFP fusion proteins (15-20%) compared to PyLT (~5%). Therefore, it was likely that cells expressing PyLT would also express the GFP fusion proteins. The ability of these proteins to prevent PyLT mediated proliferation of ICI treated cells was assessed by double indirect immunofluorescence for PyLT and BrdU as described above for transfections with PyLT alone. In control experiments, Rb-LT was cotransfected with pGFP, $p16^{INK4A}$-GFP or $p21^{Waf1}$-GFP in 17β-estradiol (E) treated MCF-7 cells to determine if $p16^{INK4A}$ or $p21^{Waf1}$ inhibited estrogen induced proliferation of MCF-7 cells where Rb was functional. The results shown in Figure 3.2B and 3.2C demonstrate that cells expressing the pGFP vector exhibited a twofold decrease in proliferation relative to untransfected cells treated with estrogen or to those transfected with PyLT alone. Thus, GFP alone inhibits proliferation, but not down to levels observed in cells treated with ICI. The level of proliferation in GFP cotransfected cells therefore served as a baseline to assess the effects of $p16^{INK4A}$ and $p21^{Waf1}$ on the inhibition PyLT induced proliferation. The Cdk2 inhibitor, $p21^{Waf1}$ inhibited proliferation in PyLT expressing cells while the Cdk4 inhibitor, $p16^{INK4A}$ did not (Figure 3.2B). However, in control transfections with Rb-LT, $p16^{INK4A}$ inhibited proliferation as efficiently as $p21^{Waf1}$ (Figure 3.2C). These results demonstrate that Cdk4 activity is not required in cells in which pRb has been inactivated, but is required in cells with intact pRb. However, Cdk2 activity is required regardless of the pRb status of cells. These results also indicate that Cdk4 is required for pRb inactivation, while the Cdk2 has targets in addition to pRb. Finally, they suggest that antiestrogens do not. inhibit Cdk2 activity if pRb family members have been inactivated

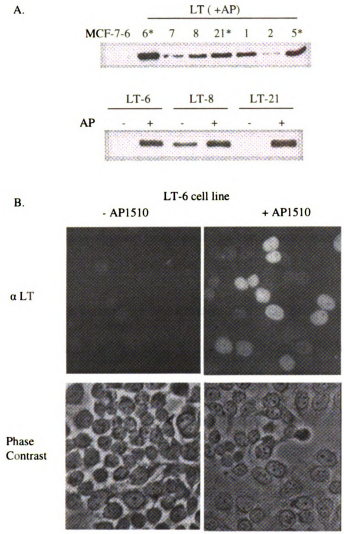


Figure 3.3. Selection of stable cell lines that express PyLT in an inducible manner. A) The MCF-7 derivative (7-6) was transfected with the plasmid expressing PyLT from an inducible promoter (pLH-Z12-I-LT), and clones were selected in Hygromycin (30 μg/ml), and screened for PyLT expression by Western blotting after 24 hrs of AP1510 (300nM) (+AP) treatment (top panel). Three clones exhibiting high expression were then tested for expression in the absence and presence of AP1510 (bottom panel). B) One clonal cell line, LT-6, was analyzed for PyLT expression by indirect immunofluorescence. The figure shows immunofluorescence for PyLT in the absence (-AP1510) and presence (+AP1510) of AP1510, along with a phase contrast picture of the same fields. The Fluorescent images were converted to grayscale using the Adobe Photoshop software program.

Screening PyLT inducible cell lines. To test whether Cdk2 activity is induced by PyLT expression, and to address the mechanism of its induction, we constructed stable inducible cell lines in which the expression of PyLT was regulated by the addition of a small molecule dimerizer, AP1510. The PyLT encoding cDNA was cloned into a vector downstream of an AP1510 inducible promoter. The description of the inducible plasmid system and the selection of stable cell lines is described in Materials and Methods. Briefly, MCF-7-6 cells were transfected with a vector expressing PyLT from the inducible promoter, and hygromycin resistant clones were selected and expanded into cell lines.

These clones were screened by Western blotting (Figure 3.3A), and several with low basal and high induced levels of PyLT induction upon the addition of AP1510 were chosen for further characterization. LT-5, LT-6 and LT-21 were three clonal cell lines used for further studies (Figure 3.3A lower panel and data not shown). The expression of PyLT in the inducible cell lines was also examined by immunofluorescence. Significant variability in the expression of PyLT was observed upon AP1510 treatment (Figure 3.3B), ranging from undetectable to very high levels. This variability in expression was observed for all three clonal cell lines tested. Approximately 50-60 % of the cells expressed detectable PyLT even in the continued presence of the selective agent, hygromycin (10 μg/ml).

PyLT induces proliferation of ICI treated cells. Initially, the effects of PyLT induction on cell cycle progression of cells arrested in G0/G1 by ICI treatment were assessed in LT-6, LT-5 and LT-21 cell lines. The results from one experiment in LT-5

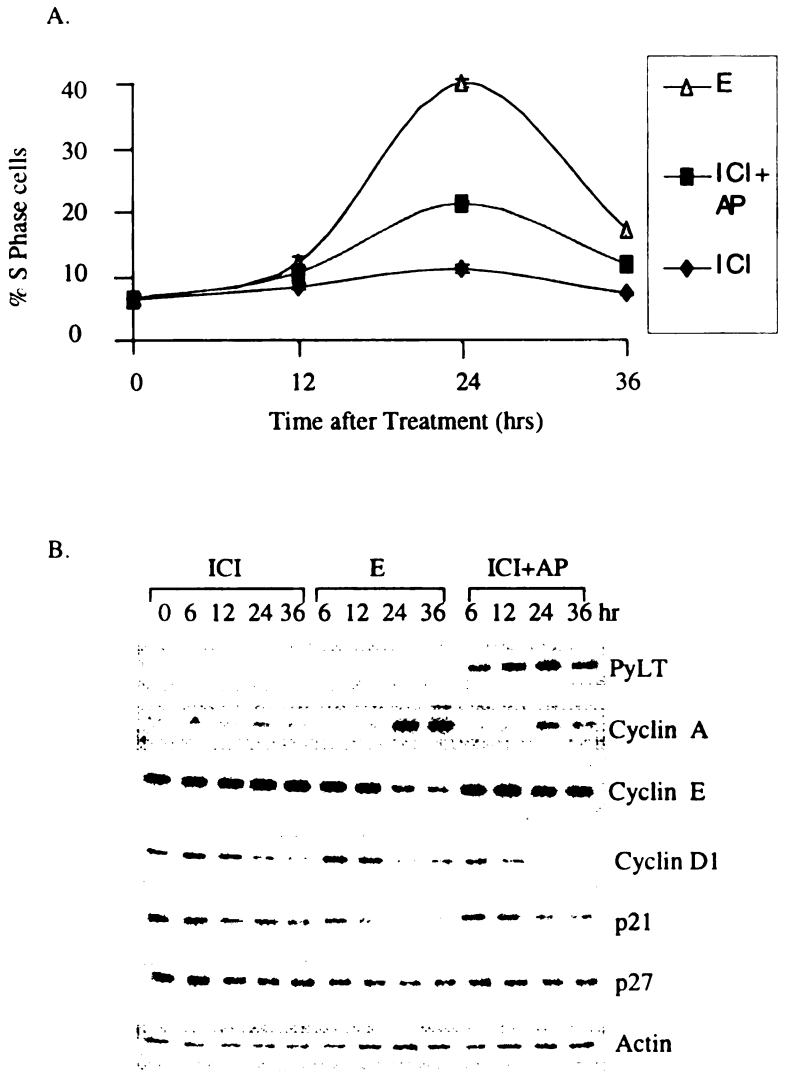


Figure 3.4. PyLT induces S phase entry in ICI treated MCF-7 cells in short term assays. A) LT-5 cells were growth arrested in ICI for 48 hours, treated with CSS+ICI, CSS+E or CSS+ICI+AP, then harvested at 12 hr intervals and the cell cycle profile was analyzed by flow cytometry. The percentage of cells in S phase at the various time points after the indicated treatments is shown. Results represent the average +/- S.E. of an experiment done in triplicate. B) In a parallel experiment, LT-5 cells were treated as in (A) above. They were harvested at the time points indicated, and cell extracts were analyzed by western blotting for PyLT, cyclin A, cyclin E and cyclin D1, and the Cdk inhibitors $p21^{Waf1}$ and $p27^{Kip1}$. Actin was probed as a loading control

cells is shown in Figure 3.4A. LT-5 cells were pre-arrested with ICI (10nM) for 48 hrs, and were then treated in one of the following ways. PyLT expression was induced by the addition of AP1510 (300nM) in the continued presence of ICI, or cells were treated with ICI alone or with E (10 nM) to serve as negative and positive controls respectively. Cells were harvested at 12 hr intervals after treatment, and analyzed by flow cytometry to determine their cell cycle phase distribution. Expression of PyLT caused an increase in the percentage of cells in S phase in ICI treated cells between 12 and 24 hours, from 6-7 % in S phase at the 0 hr time point to 20 % cells in S phase by 24 hr (Figure 3.4A). Similar results were obtained for the LT-6 and LT-21 cell lines (data not shown). In comparison, cells treated with E showed a much higher percentage of cells in S phase (upto 40 %) at the 24 hr time point. These results demonstrate that induction of PyLT expression increased proliferation of ICI treated cells though not to as high a level as E treatment and qualitatively confirms the results obtained in the transient transfection experiments.

To investigate the effects of PyLT expression on cell cycle components that might contribute to antiestrogen resistance, cells were harvested at the given time points after AP1510 treatment in an experiment performed in parallel to the one shown in Figure 3.4A. They were analyzed by Western blotting for the expression of PyLT and cell cycle regulatory proteins including cyclins A, E and D1 and the kinase inhibitory proteins, $p21^{Waf1}$ and $p27^{Kip1}$ (Figure 3.4B). Actin was used as a loading control. A clear induction of PyLT protein was observed by 6 hours after AP1510 addition, and high levels were maintained for the duration of the experiment. Cyclin A protein levels increase at the G1-S phase transition making this protein a marker for S phase progression. An increase in

cyclin A protein levels was observed by 24 hrs after AP1510 treatment. However, the levels of cyclin A induced were lower than in cells treated with E at the corresponding time points, which correlated with the cell cycle data. Cyclin D1 and cyclin E did not show any obvious increase at the 6 or 12 hrs time point upon LT induction. However, at the later time points, E treated cells showed a decline in cyclin E. Since cyclin E is a G1 cyclin that is degraded during late S phase (17), this decrease is likely a consequence of cell cycle progression. A similar decline in cyclin D1 levels was noted in all treatments at the 24 and 36 hr time points. One possible explanation for this result in E treated cells is that the levels of cyclin D1 are downregulated in S phase (6). Treatment with ICI has also been reported to decrease the levels of cyclin D1 and could account for the decreases seen in cells treated with ICI alone (18). The reasons for the decline in cyclin D1 levels in cells treated with ICI plus AP are less clear, and either mechanism could be responsible. In any case, the levels of cyclin D1 or cyclin E were not elevated by AP treatment, and thus could not explain the increase in the percentage of cells in S phase in PyLT expressing cells. The levels of the kinase inhibitor, $p21^{Waf1}$, were lower in PyLT expressing cells compared to cells treated with ICI alone, though but not as low as seen in E treated cells, suggesting that changes in the levels of $p21^{Waf1}$ might be contributing to the S phase progression upon PyLT induction. The levels of $p27^{Kip1}$ did not change significantly upon PyLT induction.

PyLT induces both Cdk2 and Cdk4 activities in ICI treated MCF-7 cells. In order to determine the mechanism(s) by which PyLT expression induced proliferation of ICI

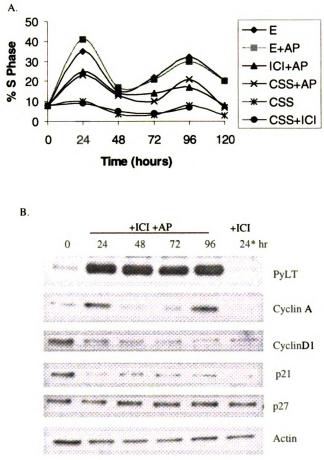


Figure 3.5. PyLT induces proliferation of ICI treated cells over 2 cell cycles. A) LT-6 cells were growth arrested in ICI for 48 hours, then treated with CSS, CSS+ICI, CSS+AP, CSS+E, CSS+AP+E or CSS+ICI+AP. Cells were then harvested at 24 hour intervals and the cell cycle profile was analyzed by flow cytometry. The percentages of cells in S phase at various time points after the indicated treatments are shown. B) LT-6 cells arrested as described in (A), were treated with AP1510; then analyzed for PyLT, cyclin A, cyclin D1 and $p21^{Waf1}$ and $p27^{Kip1}$ levels by immunoblotting. A time matched control (24 hr*) of ICI treated cells, was included. Actin blots were done to confirm equal loading.

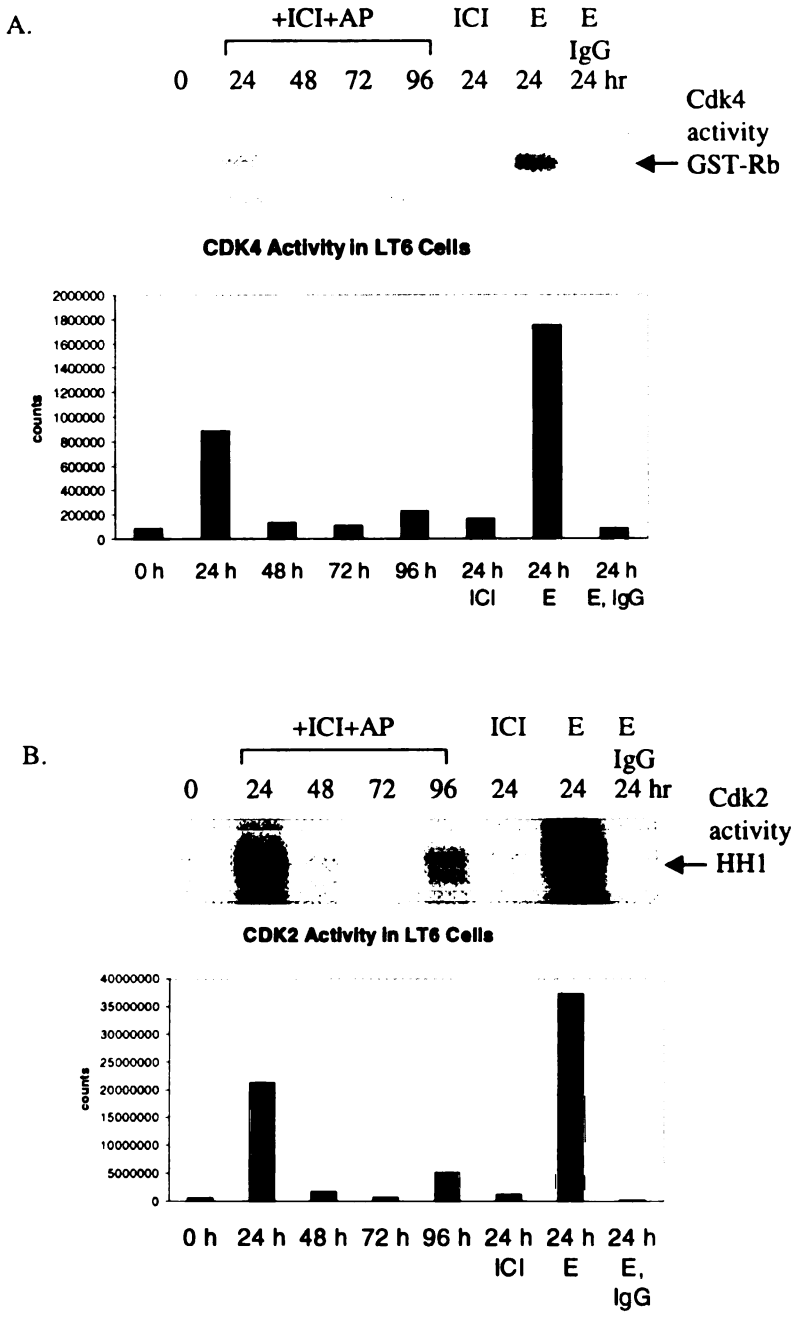


Figure 3.6. PyLT induces Cdk2 and Cdk4 activities in ICI treated MCF-7 cells. LT-6 cells were growth arrested in CSS+ICI (10 nM) containing media for 48 hrs. After 48 hrs, PyLT was induced by AP1510 (300 nM) treatment of cells in ICI containing media. Time matched controls (24 hr) where only ICI was added, and one with E (10 nM), were included as negative and positive controls, respectively. Cell extracts were prepared at 24 hr intervals and were analyzed for A) Cdk4 (GST-Rb as substrate) and B) Cdk2 (Histone H1 as substrate) in vitro kinase activity as described in Materials and Methods. Normal pre-immune serum was used as negative control for the kinase assays (IgG). The kinase activity was quantitated by phosphorimager scanning and is shown graphically below the respective autoradiographs. These results are representative of two independent experiments.

treated cells, LT-6 cells were prearrested with ICI (10 nM) and then either treated with ICI, E or CSS individually or in combination with AP1510. Cells were harvested at 24 hr intervals for 4 to 5 days and were analyzed for cell cycle distribution. Results are shown in Figure 3.5A. Py LT expression caused an increase in the percentage of cells in S phase to a similar extent both in the absence of E and the presence of ICI. However, the percentage of S phase observed in the E or E plus AP treated cells was approximately two fold higher than in PyLT expressing cells and is similar to the results described in the previous section for the LT-5 cell line. The fact that E and E+AP treated cells showed similar levels of proliferation indicated that PyLT expression did not interfere with E stimulated proliferation and was not toxic to these cells.

In a parallel experiment, ICI arrested cells were induced with AP1510, harvested at every 24 hr intervals for 4 days and were subjected to Western blotting and *in vitro* kinase assays for Cdk4 and Cdk2 activity. ICI and E treated cells were harvested 24 hrs after treatment and served as negative and positive controls, respectively. As noted previously, the increase in cyclin A protein at the 24 and 96 hr time points corresponded with the increase in the percentage of cells in S phase. Cdk2 activity was clearly induced at 24 hr after AP1510 treatment of ICI treated (Figure 3.6B). However, E treated cells showed approximately two-fold higher Cdk2 activity at the 24 hr time point, which correlated with the increased percentage of cells in S phase in the E treated cells. Thus, PyLT expression causes activation of total Cdk2 activity that closely parallels the induction of cyclin A protein and S phase entry in ICI treated MCF-7 cells. Surprisingly, Cdk4 activity was also induced at the 24 hr time point after PyLT induction (Figure 3.6A).

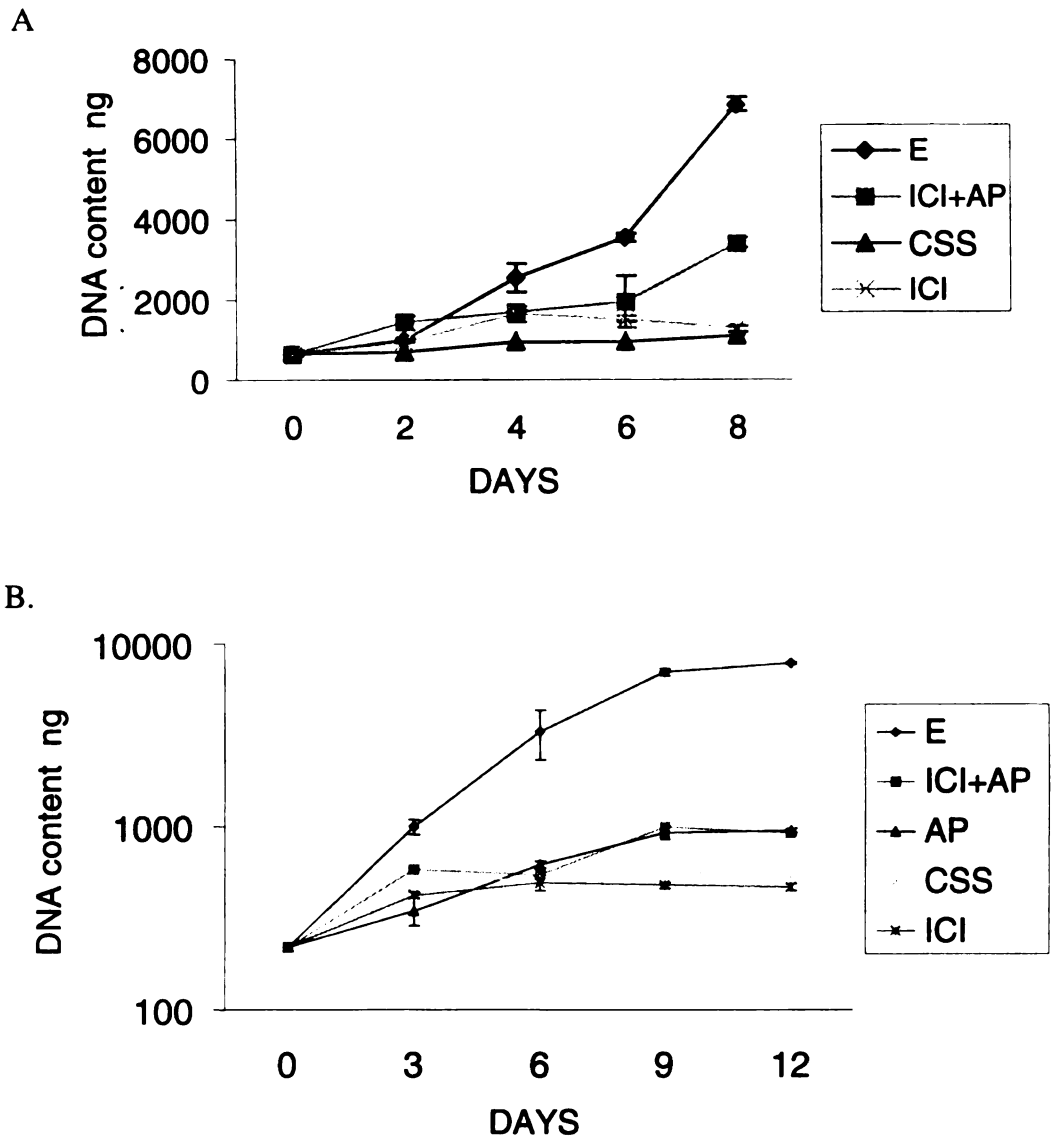


Figure 3.7. PyLT confers partial antiestrogen resistance in long-term assays. PyLT inducible cells (LT-6) were growth arrested in CSS+ICI containing media for 48 hrs. The media was then changed to CSS+ICI, CSS alone or CSS+E or to these media plus AP1510 (300nM). A) Cells were harvested every 2 or B) 3 days. Fresh media with the appropriate treatments were added every 3 days for the duration of the experiment. All samples were harvested in triplicate and the DNA content per well in nanograms (ng) was determined by a fluorometric assay and is represented as mean +/- S.E. The results from two independent experiments are shown. Note that graph B. is plotted on a logarithmic scale to show the small increase in proliferation upon PyLT expression. This increase is difficult to see on a linear scale due to the large differences in proliferation between E and ICI treated cells in this experiment.

These results demonstrate that ICI cannot completely inhibit Cdk4 or Cdk2 activities in MCF-7 cells in which pRb function is abrogated by LT.

Effect of LT induction on Long Term Growth of ICI treated MCF-7 cells. To assess the effects of pRb inactivation on the long-term growth of MCF-7 cells, proliferation assays were carried out. LT-6 cells were plated on 24 well plates, prearrested with ICI and treated as described in Materials and Methods. Cells were harvested every 2-3 days over a period of 8 to 12 days and the DNA content measured by a fluorometric assay. The results from two independent experiments are shown graphically in Figure 3.7. They demonstrate that expression of PyLT enhanced the proliferation of cells treated with ICI above the levels seen in cells treated with ICI or depleted of E in the long term and are qualitatively similar to the short-term results. Thus inactivation of pRb leads to at least partial antiestrogen resistance in the long term However, in the long-term, the proliferation upon PyLT expression was much lower than that seen in the short-term assays suggesting that events in addition to pRb inactivation may be required to confer complete antiestrogen resistance in the long term. The possible reasons for this are discussed below.

DISCUSSION

The Rb gene is mutated in a significant percentage of breast cancers (19, 20) and a much larger percentage of breast cancer patients show alterations in the pRb pathway resulting from overexpression of cyclin D1 or loss of $p16^{INK4}$. Significantly, estrogens

regulate proliferation of estrogen responsive human breast cancer cells by activating Cdk4 and Cdk2, which phosphorylate and thereby inactivate pRb (8, 9). However, the contribution of pRb inactivation to the development of antiestrogen resistance, a significant problem in breast cancer therapy, is not understood. We previously demonstrated that expression of SV40 and polyoma viral LT antigens caused antiestrogen resistance in MCF-7 cells, and that the pRb interaction domain of these T antigens was required for conferring antiestrogen resistance (10).

Since both Cdk4 and Cdk2 can contribute to pRb inactivation, and their activities are inhibited by ICI, it was unclear if antiestrogens regulate Cdk2 activity via a pRb dependent or independent mechanism (See Figure 3.1). This is important to determine, because if ICI regulates the activities of both Cdk4 and Cdk2 independently, deregulation of both would be required for tumors to acquire antiestrogen resistance. We initially determined if both Cdk4 and Cdk2 were required for proliferation in cells where pRb was inactivated and Cdk2 activity was regulated by ICI under these conditions. Using inhibitors of Cdk4 and Cdk2, we demonstrate that Cdk2 but not Cdk4 kinase activity is required for proliferation in cells where pRb family members are inactivated. In addition, using stable inducible cell lines, we showed that both Cdk4 and Cdk2 activities are induced by LT expression in the presence of the antiestrogen, ICI. Further, we confirm that PyLT expressing cell lines proliferate in the presence of ICI, at least in the short term.

Reports have suggested that estrogen activates cyclin E-Cdk2 complexes by inducing cyclin D1 protein expression, resulting in the redistribution of $p21^{Waf1}$, from these complexes to cyclin D1-Cdk4 complexes, and additionally by decreasing the levels

of $p21^{Waf1}$ (8, 9, 21). The results presented here indicate that total Cdk2 kinase activity is induced upon PyLT expression in ICI treated cells without any increase in cyclin D1 levels. Although we have not specifically tested whether cyclin E-Cdk2 activity is induced upon PyLT induction, our results demonstrate that total Cdk2 activity is partially regulated by ICI in a pRb dependent manner. The observed increase in Cdk2 activity in ICI treated cells upon PyLT expression could be explained by the increase in cyclin A protein levels (Figure 3.5B). Since the cyclin A promoter has been reported to be regulated in a E2F dependent manner, and cyclin A-Cdk2 activity is essential for G1 to S phase transition (22, 23), release of E2F upon pRb inactivation could explain the upregulation of cyclin A, the increase in Cdk2 activity and proliferation. Alternatively, cyclin A-Cdk2 complexes could titrate $p21^{Waf1}$ and lead to cyclin E-Cdk2 activation.

The mechanism of activation and the downstream targets of Cdk4 and Cdk2 have been under study for some time. pRb has been identified as the major physiological target of Cdk4 (15). Cdk4 activity is redundant in cells where pRb has been inactivated, suggesting that the only role for Cdk4 is the inactivation of pRb (24). This is consistent with our result that the specific Cdk4 inhibitor, $p16^{INK4A}$, did not prevent the proliferation of cells transfected with PyLT, while it did prevent proliferation of cells transfected with the pRb binding mutant of PyLT. Although Cdk4 activity was not required for proliferation, surprisingly, it was induced by PyLT. To our knowledge, this is the first observed regulation of Cdk4 activity by PyLT, and the mechanism of this activation needs to be further investigated.

An interesting question raised by our studies arises from the observation that expression of PyLT increased proliferation above the levels seen in ICI treated cells, but

to levels approximately two fold lower than those observed in E treated cells in the short term assays (Figure 3.4A and 3.5A). Similar results were observed for three independent PyLT inducible cell lines LT-5, LT-6 and LT-21. Furthermore, this correlated with 2 fold lower levels of Cdk2 and Cdk4 activities in PyLT expressing compared to E treated cells. In long-term proliferation assays, PyLT induction increased proliferation of ICI treated cells, but to much lower levels than that observed in E treated cells. One interpretation of these results is that pRb inactivation is not as efficient as estrogen in causing proliferation, suggesting that there are other targets of estrogen that mediate its proliferative effects. Estrogen induces multiple components of the cell cycle machinery in MCF-7 cells, including cyclin D1, c-Myc and Cdc25A and decreases the levels of $p21^{Waf1}$ and $p27^{Kip1}$ kinase inhibitors (5). Of these effects, c-Myc is known to promote proliferation via a pRb independent mechanism that involves activation of cyclin E-Cdk2 activity (25). Since we have measured total Cdk2 activity, it is possible that cyclin E associated kinase activity remained low in PyLT expressing cells resulting in decreased total Cdk2 activity and proliferation compared to E treated cells. However, cyclin E-Cdk2 activity has been shown to be required for G1-S phase transition regardless of the pRb status of cells (26). Therefore, it would be interesting to determine if this is true in PyLT expressing cells.

Another possible explanation for decreased proliferation upon PyLT expression is that only cells that express PyLT above a certain threshold level are able to overcome an ICI mediated arrest. This is consistent with the observation that transfected cell lines with lower levels of PyLT expression, exhibited no detectable increase in the proliferation of ICI treated cells upon induction of PyLT (data not shown). Since there appears to be

variable expression of PyLT as assessed by immunofluorescence (Figure 3.3B and data not shown) with approximately 50-60 percent cells showing detectable PyLT expression, it is possible that only cells expressing "high" levels of PyLT in the population will proliferate in the presence of ICI.

In conclusion, the results presented in this chapter confirm that inactivation of pRb and related family members in MCF-7 cells causes partial antiestrogen resistance, at least in the short term. This is accompanied by an induction of cyclin A protein, and total Cdk2 activity. They confirm that the role of Cdk4 in proliferation is largely to inactivate pRb or a related family member, whereas Cdk2 has other functions that are necessary for proliferation. Interestingly, at least a part of the regulation of Cdk2 activity by estrogen appears to be via pRb inactivation. These results support a model for estrogen mediated proliferation in which estrogen activates Cdk4, and this leads to Cdk2 activation indirectly by inhibiting pRb function (For model see Figure 3.8).

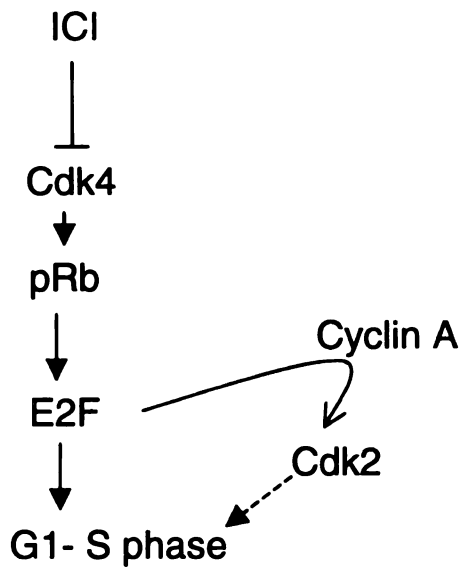


Figure 3.8. Model for the regulation of Cdk4 and Cdk2 activities by antiestrogen. According to this model, ICI indirectly regulates Cdk2 activity in a pRb dpendent manner. ICI regulates the activity of Cdk4 that causes pRb phosphorylation and inactivation. Inactivation of pRb releases the E2F transcription factor that increases the transcription of cyclin A protein levels and thereby results in Cdk2 activation and contributes to G1-S phase transition.

REFERENCES

1. Melora, D. B., Allred, C. C., and O'Conell, P. Principles of Molecular Medicine, 1st edition, p. 625-632: Humana Press, 1998.
2. Lippman, M., Bolan, G., and Huff, K. The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture, Cancer Res. *36:* 4595-601., 1976.
3. Gibson, D. F., Gottardis, M. M., and Jordan, V. C. Sensitivity and insensitivity of breast cancer to tamoxifen, J Steroid Biochem Mol Biol. *37:* 765-70., 1990.
4. Brunner, N., Bronzert, D., Vindelov, L. L., Rygaard, K., Spang-Thomsen, M., and Lippman, M. E. Effect on growth and cell cycle kinetics of estradiol and tamoxifen on MCF-7 human breast cancer cells grown in vitro and in nude mice, Cancer Res. *49:* 1515-20., 1989.
5. Foster, J. S., Henley, D. C., Bukovsky, A., Seth, P., and Wimalasena, J. MuLTifaceted regulation of cell cycle progression by estrogen: regulation of Cdk inhibitors and Cdc25A independent of cyclin D1-Cdk4 function, Mol Cell Biol. *21:* 794-810., 2001.
6. Sherr, C. J. Cancer cell cycles, Science *274:* 1672-7., 1996.
7. T'Ang, A., Varley, J. M., Chakraborty, S., Murphree, A. L., and Fung, Y. K. Structural rearrangement of the retinoblastoma gene in human breast carcinoma, Science *242:* 263-6., 1988.

8. Planas-Silva, M. D. and Weinberg, R. A. Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution, Mol Cell Biol. *17:* 4059-69., 1997.

9. Prall, O. W., Sarcevic, B., Musgrove, E. A., Watts, C. K., and Sutherland, R. L. Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E- Cdk2, J Biol Chem. *272:* 10882-94., 1997.

10. Varma, H. and Conrad, S. E. Reversal of an antiestrogen-mediated cell cycle arrest of MCF-7 cells by viral tumor antigens requires the retinoblastoma protein-binding domain, Oncogene *19:* 4746-53., 2000.

11. Foster, J. S., Henley, D. C., Ahamed, S., and Wimalasena, J. Estrogens and cell-cycle regulation in breast cancer, Trends Endocrinol Metab. *12:* 320-7., 2001.

12. Howes, S. H., Bockus, B. J., and Schaffhausen, B. S. Genetic analysis of polyomavirus large T nuclear localization: nuclear localization is required for productive association with pRb family members, J Virol. *70:* 3581-8., 1996.

13. Amara, J. F., Clackson, T., Rivera, V. M., Guo, T., Keenan, T., Natesan, S., Pollock, R., Yang, W., Courage, N. L., HoLT, D. A., and Gilman, M. A versatile synthetic dimerizer for the regulation of protein-protein interactions, Proc Natl Acad Sci U S A. *94:* 10618-23., 1997.

14. Labarca, C. and Paigen, K. A simple, rapid, and sensitive DNA assay procedure, Anal Biochem. *102:* 344-52., 1980.

15. Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J., and Kato, J. Y. D-type cyclin-dependent kinase activity in mammalian cells, Mol Cell Biol. *14:* 2066-76., 1994.

16. Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M., and Sherr, C. J. The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts, Embo J. *18:* 1571-83., 1999.

17. Dulic, V., Lees, E., and Reed, S. I. Association of human cyclin E with a periodic G1-S phase protein kinase, Science *257:* 1958-61., 1992.

18. Watts, C. K., Sweeney, K. J., WarLTers, A., Musgrove, E. A., and Sutherland, R. L. Antiestrogen regulation of cell cycle progression and cyclin D1 gene expression in MCF-7 human breast cancer cells, Breast Cancer Res Treat. *31:* 95-105, 1994.

19. Jares, P., Rey, M. J., Fernandez, P. L., Campo, E., Nadal, A., Munoz, M., Mallofre, C., Muntane, J., Nayach, I., Estape, J., and Cardesa, A. Cyclin D1 and retinoblastoma gene expression in human breast carcinoma: correlation with tumour proliferation and oestrogen receptor status, J Pathol. *182:* 160-6., 1997.

20. Dublin, E. A., Patel, N. K., Gillett, C. E., Smith, P., Peters, G., and Barnes, D. M. Retinoblastoma and p16 proteins in mammary carcinoma: their relationship to cyclin D1 and histopathological parameters, Int J Cancer. *79:* 71-5., 1998.

21. Cariou, S., Donovan, J. C., Flanagan, W. M., Milic, A., Bhattacharya, N., and Slingerland, J. M. Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen- mediated cell cycle arrest in human breast cancer cells, Proc Natl Acad Sci U S A. *97:* 9042-6., 2000.

22. Schulze, A., Zerfass, K., Spitkovsky, D., Middendorp, S., Berges, J., Helin, K., Jansen-Durr, P., and Henglein, B. Cell cycle regulation of the cyclin A gene promoter is mediated by a variant E2F site, Proc Natl Acad Sci U S A. *92:* 11264-8., 1995.

23. Liu, N., Lucibello, F. C., Engeland, K., and Muller, R. A new model of cell cycle-regulated transcription: repression of the cyclin A promoter by CDF-1 and anti-repression by E2F, Oncogene *16:* 2957-63., 1998.

24. Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G., and Bartek, J. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16, Nature *375:* 503-6., 1995.

25. Alevizopoulos, K., Vlach, J., Hennecke, S., and Amati, B. Cyclin E and c-Myc promote cell proliferation in the presence of p16INK4a and of hypophosphorylated retinoblastoma family proteins, Embo J. *16:* 5322-33., 1997.

26. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition, Mol Cell Biol. *15:* 2612-24., 1995.

CHAPTER 4

Antiestrogen ICI 182,780 Decreases Proliferation of IGF-1 Treated MCF-7 Cells Without Inhibiting the PI3K/AKT Pathway.

ABSTRACT

Both estrogens and growth factors such as Insulin like growth factor-1 (IGF-1) are potent mitogens for human breast cancer cells. Antiestrogens, including ICI 182,780 (ICI) and tamoxifen, inhibit both estrogen and IGF-1 induced proliferation of estrogen responsive breast cancer cells. The mechanism(s) by which ICI inhibits IGF-1 mediated proliferation of MCF-7 cells, an estrogen receptor (ER) positive, estrogen responsive human breast cancer cell line, were addressed in this study. IGF-1 induces proliferation in MCF-7 cells by activating phosphoinositol 3(OH) kinase (PI3K) and its downstream target AKT, which regulates components of the cell cycle machinery including cyclin D1 protein. Numerous studies have suggested that antiestrogens regulate MCF-7 cell proliferation by affecting the components of the IGF-1 signaling pathway including IGF-1 Receptor protein levels, Insulin receptor substrate-1 (IRS-1) protein levels and PI3K/AKT activity. The results presented here demonstrate that ICI does not abrogate the ability of IGF-1 to induce proliferation or cyclin D1 expression. In addition IGF-1 stimulated phosphorylation of AKT is unaffected by ICI treatment, and transient expression of constitutively active PI3K or AKT was unable to induce proliferation in ICI

treated MCF-7 cells. Together, these results demonstrate that ICI does not block IGF-1 signaling, and suggest a model wherein ICI inhibits IGF-1 mediated proliferation by mechanisms other than downregulating the PI3K/AKT signaling pathway.

INTRODUCTION

Both growth factors and the sex steroid, estrogen, regulate proliferation of human breast cancer cells. Growth factors bind to membrane bound tyrosine kinase receptors that activate signaling cascades affecting cell cycle progression. Insulin like growth factor-1 (IGF-1) stimulates proliferation of the MCF-7 human breast cancer cell line by activating the membrane bound tyrosine kinase receptor, IGF1-R (1, 2). Activated IGF1-R recruits and phosphorylates the insulin receptor substrate-1 (IRS-1), which in turn activates phosphoinositol 3(OH) kinase (PI3K), a major mediator of the IGF-1 signaling pathway (3). PI3K activity is essential for the proliferation of MCF-7 cells, as chemical inhibitors of PI3K prevent MCF-7 proliferation (4). PI3K activates downstream kinases including AKT, p70S6 kinase and glycogen synthetase 3 kinase (5). One of the key downstream targets of these kinases is cyclin D1, an important regulator of cell cycle progression (6).

Estrogens activate the estrogen receptors alpha (ERα) and beta (ERβ), ligand activated nuclear transcription factors (7) that regulate transcription of target genes. ERα plays a critical role in breast tissue proliferation, as ERα knockout mice do not develop breast tissue at puberty. ERβ does not appear to be important for breast epithelial proliferation, since ERβ knockout mice show normal breast development (8). MCF-7

cells are ERα positive (9), and their proliferation is regulated by estrogens and antiestrogens (10-12). Evidence obtained in the past several years has led to the development of a model to explain estradiol (E) induced proliferation of MCF-7 cells. In this model, E induces cyclin D1 protein expression, which then activates the cyclin dependent kinases Cdk4 and cyclin E-Cdk2. Cdk4 is activated by direct binding of cyclin D1, whereas activation of Cdk2 involves redistribution of the kinase inhibitor, $p21^{Waf1}$, from cyclin E-Cdk2 to cyclin D1-Cdk4 complexes, thereby relieving inhibition of cyclin E-Cdk2 kinase (13, 14). Additionally, downregulation of $p21^{Waf1}$ and $p27^{Kip1}$ and activation of Cdc25A phosphatase by E, have been proposed to contribute to E stimulated activation of Cdk2 complexes (15-17). Active Cdk4 and Cdk2 phosphorylate and inactivate pRb, a negative regulator of cell cycle progression, releasing the E2F family of transcription factors that turn on genes important for cell cycle progression (18). Antiestrogens inhibit proliferation of MCF-7 cells, and reverse the above mentioned cell cycle changes induced by estrogen treatment (13, 14).

In addition to reversing the proliferative effects of estrogens, antiestrogens such as tamoxifen and ICI 182,780 (ICI) inhibit growth factor induced proliferation of MCF-7 cells (19-21), but the mechanism(s) by which they do so are unclear. Defining them may contribute to the understanding of antiestrogen resistance, because deregulation of growth factor signaling pathways has been implicated in the development of antiestrogen resistance in breast cancer patients (22, 23). Considerable evidence indicates that antiestrogens and estrogens regulate intermediates in the IGF-1 signaling pathway, including IGF1-R (24, 25) and IRS-1 protein levels (26), and IRS-1 phosphorylation (27), suggesting that antiestrogens might inhibit proliferation by inhibiting IGF-1 signaling.

This study was undertaken to define the molecular mechanism(s) by which ICI inhibits IGF-1 induced proliferation in MCF-7 cells. To approach this, we investigated the effects of IGF-1 on cell proliferation and the expression of cell cycle regulatory molecules in the presence and absence of ICI. Our results demonstrate that while ICI decreases the absolute levels of proliferation and the cell cycle regulatory molecules including cyclin D1, it does not eliminate the effects of the effects of IGF-1 on these targets. In addition, IGF-1 induced phosphorylation of AKT, a key regulator of cyclin D1 protein levels (28) was not inhibited by ICI. Finally, the expression of constitutively active PI3K or AKT was insufficient to overcome an antiestrogen mediated growth arrest of MCF-7 cells. Together, these results suggest a model in which ICI decreases proliferation of IGF-1 treated cells by affecting pathways other than the PI3K/AKT pathway.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells were obtained from the Dr. Michael Johnson at the Lombardi Cancer Center and were routinely maintained in IMEM medium (Biofluids) containing 5% fetal bovine serum (FBS) (HyClone), penicillin (100 units/ml) and streptomycin (100 μg/ml). For serum free experiments, MCF-7 cells were trypsinized, resuspended in medium containing 5% FBS and centrifuged. The cell pellets were washed twice in PBS to remove any residual serum, and the washed cells were then plated at 10^6 cells /100 mm plate or 50,000 cells/well of a 24 well plate in phenol red free DMEM F12 serum free medium (Sigma) on collagen 1 (4 μg/cm2) coated tissue culture plates. After overnight

incubation, cells were either treated with ICI 182,780 (100 nM), E (1 nM), IGF-1 (10 ng/ml), ICI plus E, IGF-1 plus E or IGF-1 plus ICI. For ICI pretreatment, ICI was added for 24 hr before IGF-1 addition.

Plasmids. Heamagglutinin tagged AKT (pCEFL-myr-HA-AKT) contains a myristylation signal that causes membrane localization and has been described earlier (29). The plasmid was a gift of Dr. Yi Li and J.S. Gutkind. The constitutively activate myc tagged PI3K (p110*) and the kinase dead version (ΔKin) have been described previously (30), and were provided by Dr. Anke Klippel. The SRE-luciferase and the CMV β-galactosidase plasmid were obtained from Clontech. The cyclin D1 cDNA containing plasmid that was used to prepare the probe for Northern Blotting was provided by Dr. Steven Reed. A plasmid containing the 18S rRNA gene was obtained from Dr. Laura. McCabe (Department of Physiology, Michigan State University).

Chemicals, Growth factor and Antibodies. ICI 182,780 was a gift of A. Wakeling (Zeneca Pharmaceuticals) and 17β-estradiol was purchased from Sigma. IGF-1 was obtained from GroPep Pty. Ltd. (Adelaide, Australia) and the PI3K inhibitor, LY294002, was obtained from Calbiochem. Rat tail collagen 1 was purchased from Collaborative Biomedical Products (Bedford, MA). ^{3}H-thymidine (50 Ci/mmol) was purchased from ICN Biomedicals, Inc. Cyclin D1 antibody was a rabbit polyclonal from Upstate Biotechnology. Antibodies to cyclin E, cyclin A and the pRb protein were purchased from Pharmingen. ERα antibody was a monoclonal (Mab17) antibody (31) and was a gift of Dr. Richard Miksicek (Department of Physiology, Michigan State University).

Phosphoserine 473 AKT antibody and the AKT antibody were rabbit polyclonal antibodies from New England Biolabs. Rat anti-HA and mouse anti-myc (clone 9E10.3) antibodies were from Boehringer Mannheim and Neomarkers respectively. Sheep anti-BrdU and mouse anti- BrdU were from Fitzgerald Industries and Boehringer Mannheim respectively. FITC conjugated donkey anti-sheep antibody, goat anti-rat antibody were from Sigma, while the rhodamine conjugated goat anti- mouse secondary was from Boehringer Mannheim. The anti β-actin antibody was purchased from Sigma.

Transfections and Luciferase Assays. MCF-7 cells were plated at $5x10^5$ cells/60mm plate and transfected with plasmid DNA using the Superfect reagent following the protocol provided by the manufacturer (Qiagen). 1 μg of SRE-luc plasmid was cotransfected with either 1 μg vector, p110* or ΔKIN along with 0.2 μg of pCMV β-galactosidase as a control for transfection efficiency. Cells were kept in 5% charcoal dextran stripped serum (CSS) containing medium for 24 hours followed by serum free media for an additional 24 hours. Cells were harvested 48 h post-transfection and both the luciferase and β-galactosidase activities were measured using the detection reagent and protocol provided by the manufacturer (Clontech) on a Turner TD 20E luminometer (Turner Designs). Each transfection was done in triplicate, and the luciferase activity in each transfection was normalized to β-galactosidase activity.

^{3}H Thymidine Incorporation. Cells were plated at 50,000 cells/well on collagen 1 coated 24 well plates and treated as described for the respective experiments. 1 μCi of tritiated thymidine was added to each well and the cells were harvested in 0.5% NaOH,

0.1% Triton-X 100 after 24 hours of labeling. The cell lysates were precipitated with trichloroacetic acid (10 %) and the acid precipitable counts were measured using liquid scintillation counting. Six wells were harvested for each treatment with 3 wells being used for thymidine incorporation and the remaining 3 wells used to determine the DNA content by a fluorometric assay described previously (32). The ^{3}H thymidine incorporation per well was normalized to DNA content.

Immunoblotting. MCF-7 cells were lysed in Sherr lysis buffer (33) and the total protein was quantitated using the Bradford protein assay (Biorad). Twenty micrograms of total protein were subjected to SDS-Polyacrylamide gel electrophoresis (12% or 7.5%), transferred to a PVDF membrane and probed for the various proteins using appropriate primary antibodies followed by horseradish peroxidase conjugated secondary antibodies. The bands were visualized using the ECL chemiluminescent reagent (Pierce). The cyclin D1 levels on immunoblots for the time course experiment (Figure 4.2B) were quantitated by densitometric scanning.

Northern Blotting. Cells were lysed using the Trizol reagent (GIBCO BRL) and total RNA was extracted as described in the instructions provided by the manufacturer. Total RNA was quantitated by measuring optical density at 260 nm, and 20 micrograms of RNA was loaded on 1% formaldehyde gels, transferred to a nitrocellulose membrane and hybridized with a ^{32}P labeled cDNA probe for cyclin D1. The membrane was stripped and reprobed with an 18S rRNA probe as a loading control. The specific RNA was

quantitated by phosphoimager scanning (Molecular Dynamics) and the cyclin D1 mRNA levels were normalized to 18S rRNA.

Indirect Immunofluorescence. MCF-7 cells were plated on glass coverslips and transiently transfected with either the HA-tagged AKT plasmid or the myc-tagged PI3K (p110*) plasmid using the Superfect transfection reagent (Qiagen). After transfection, cells were treated with medium containing 5% fetal bovine serum and ICI (100 nM) and were labeled with 25 μM 5-Bromo-2'-deoxyuridine (BrdU) for 5 hours before fixation at 48 hours post transfection. The double immunofluorescence procedure for detecting transfected proteins and BrdU has been described previously (34). BrdU incorporation was detected using the appropriate α-BrdU antibody (mouse α-BrdU for HA-AKT and sheep α-BrdU for myc-PI3K). Cells were viewed under a fluorescent microscope (Olympus) and the percentage of HA (or myc) expressing cells that were also positive for BrdU, and of cells that were positive for BrdU alone was determined. At least one hundred HA/myc positive and negative cells were counted in each experiment. The images shown in Figure 4.6A were taken on the Olympus microscope, scanned and converted to grayscale using the Adobe Photoshop software program.

RESULTS

ICI decreases the proliferation of IGF-1 treated cells To assess the effects of ICI, E and IGF-1 on MCF-7 cell proliferation, cells were cultured in serum free medium (SFM) and treated with ICI, E or IGF-1 alone or with a combination of ICI plus E, IGF-1 plus E or IGF-1 plus ICI. Untreated cells in SFM served as controls. Proliferation was assayed by measuring ^{3}H-thymidine incorporation over a 24 h labeling period beginning 24 hours after plating. Results from one representative experiment are shown in Figure 4.1A. MCF-7 cells in SFM showed basal levels of ^{3}H thymidine incorporation that decreased 4-5 fold upon treatment with ICI. This result suggested that a ligand independent activity of ER was inhibited by ICI. The absence of E in the phenol red free SFM conditions was confirmed by analyzing protein levels of ERα and progesterone receptor (Figure 4.1C and data not shown). The levels of ERα were decreased in both ICI and E treated cells compared to cells in SFM or treated with IGF-1. ICI has been previously shown to decrease ERα levels by causing receptor degradation (35), while E causes downregulation of ERα by decreasing transcription and also by decreasing ERα mRNA and protein stability of (36, 37). In addition, the levels of progesterone receptor protein, a known transcriptional target of ER (38), were increased by E treatment of cells in SFM (data not shown). These results confirmed that the concentration of E in SFM was below the levels required to activate transcription from E target genes. E alone induced a 2-fold increase in proliferation that was completely reversed by ICI. Treatment with IGF-1 alone increased proliferation 3-4 fold over control levels, and E had an additive effect

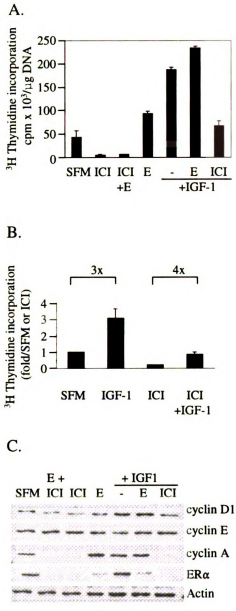


Figure 4.1. ICI decreases proliferation in the presence of IGF-1. MCF-7 cells were plated in SFM, and after overnight culture were treated with ICI (100nM), E (1nM) or IGF-1 (10 ng/ml) or various combinations thereof as described in Materials and Methods. Untreated cells in SFM served as controls. A) ^{3}H thymidine (1 µCurie) was added to each well after 24 hr treatment and cells were harvested after 24 hr labeling. Acid precipitable counts were measured, and normalized to DNA content. Results from one experiment are shown as the average +/- S.E. from triplicate wells. B) ^{3}H thymidine incorporation from three independent experiments is shown as the average +/- S.E. Incorporation is expressed relative to the amount in SFM, which was defined as 1. The fold induction of proliferation by IGF-1 over SFM or ICI treated cells is shown above the respective histograms. C) In experiments carried out in parallel to the thymidine incorporation studies shown in (A), cells were harvested after 2 days of treatment and lysates were subjected to immunoblotting for cyclin D1, cyclin E, cyclin A and ERα. Actin served as a loading control.

with IGF-1 In agreement with published results (19, 20), ICI treatment decreased proliferation of IGF-1 treated cells, but not to the level seen in cells treated with ICI alone. In contrast, E induced proliferation was completely abrogated by ICI. We determined the extent to which IGF-1 stimulated proliferation in the presence and absence of ICI and the results are shown in Figure 4.1B. Although the absolute levels of proliferation were lower in ICI treated cells, IGF-1 increased ^{3}H thymidine incorporation 3-4 fold over basal levels in both untreated and ICI treated cells.

Both ICI and IGF-1 regulate cyclin D1 protein levels. To investigate the mechanism by which ICI decreased proliferation, we examined the effects of ICI, E and IGF-1 on the expression of cyclins, key regulators of the G1-S phase transition. MCF-7 cells were treated as described above, and after 2 days of treatment, protein extracts were prepared and subjected to Western blot analysis for cyclins D1, E and A. Results from one representative experiment are shown in Figure 4.1C. Cells in SFM expressed basal levels of cyclin D1 that were decreased upon treatment with ICI. Treatment of cells with IGF-1 or with IGF-1 plus E increased the levels of cyclin D1 compared to SFM controls. Interestingly, cells co-treated with ICI and IGF-1 had lower levels of cyclin D1 than cells treated with IGF-1 alone. These results indicated that ICI decreased both basal and IGF-1 induced cyclin D1 protein levels. The levels of cyclin E protein did not show any changes with the various treatments. Cyclin A protein is induced at the G1-S transition, and serves as a marker of S phase entry. Cells treated with ICI alone, or in combination with E or IGF-1, showed decreased levels of cyclin A, correlating with the inhibition of proliferation shown in Figure 4.1A and 4.1B.

ICI pretreatment does not abrogate IGF-1's ability to induce cyclin D1 expression or proliferation. A number of studies have suggested that antiestrogens inhibit proliferation by downregulating the IGF-1 signaling pathway (24-27). Since in the experiments described above, cells were treated simultaneously with both IGF-1 and ICI, it was possible that ICI had insufficient time to block IGF-1 signaling and thus could not inhibit its effect upon cyclin D1 expression and proliferation. To determine if ICI pretreatment could abrogate IGF-1's effects, cells were plated in SFM as described above and were either untreated or preincubated with ICI for 24 hours. They were then stimulated with IGF-1, harvested at various time points and analyzed by Western blotting for cyclin D1, cyclin A, the Cdk inhibitory proteins, $p21^{Waf1}$ and $p27^{Kip1}$, and pRb. Results of one representative experiment are shown in Figure 4.2A. ICI pretreatment decreased the levels of cyclin D1 compared to cells in SFM at the zero hour time point. However, in both ICI pretreated and control cells; IGF-1 induced cyclin D1 protein by 6 hours, with maximal levels being observed by 12 hours. The maximal levels of cyclin D1 expressed in ICI pretreated cells were approximately 2 fold lower than in cells treated with IGF-1 alone as determined by quantitation based on densitometric scanning. The levels of cyclin D1 stayed constant in ICI plus IGF-1 treated cells until 36 hours, but showed a decline in cells treated with IGF-1 alone between 24 and 36 hours. This decrease correlated with the entry of cells into S phase, indicated by cyclin A induction and pRb phosphorylation. Since cyclin D1 protein levels vary in the different phases of the cell cycle, with high levels in mid to late G1 and decreased levels in S phase, the decline in cyclin D1 levels is likely a consequence of cell cycle progression (6).

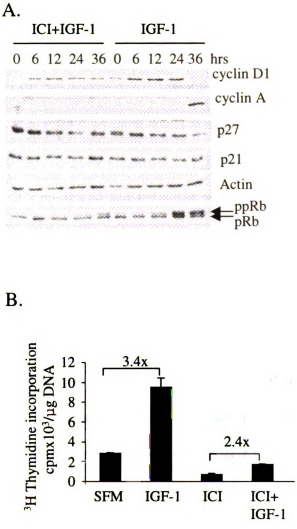


Figure 4. 2. ICI pre-treatment does not abrogate IGF-1 induced expression of cyclin D1 or proliferation. MCF-7 cells incubated in SFM or pretreated with ICI for 24 hours were stimulated with IGF-1. A) Cell lysates were harvested at the time points indicated and subjected to western blotting for cyclin D1, cyclin A, $p21^{Waf1}$, $p27^{kip1}$, pRb, or β-actin. B) In a parallel experiment, ^{3}H thymidine incorporation was determined during the 24 hr period after IGF-1 treatment. Results represent the average +/- S.E. of triplicate wells normalized to DNA content. Fold induction of ^{3}H thymidine incorporation by IGF-1 treatment in either SFM or ICI treated cells is shown above the bars for the respective treatments. The results shown are representative of three independent experiments.

In addition to decreasing cyclin D1 protein levels, ICI reportedly increases the levels of the kinase inhibitors $p27^{Kip1}$ and $p21^{Waf1}$ and these contribute to the inhibition of proliferation (17). Since IGF-1 decreases $p27^{Kip1}$ protein levels and may increase $p21^{Waf1}$ levels in MCF-7 cells (39), we assessed if ICI treatment could prevent the effects of IGF-1 on these proteins. The $p27^{Kip1}$ protein levels were higher in ICI treated cells than untreated cells at the 0 hr time point (Figure 4.2A). However, IGF-1 treatment decreased $p27^{Kip1}$ levels both in ICI pretreated and untreated cells. The levels of $p21^{Waf1}$ were not reproducibly altered by IGF-1 treatment in the presence or absence of ICI in these experiments. These results indicated that ICI pretreatment could not prevent the effects of IGF-1 on both positive and negative regulators of proliferation, as exemplified by the regulation of cyclin D1 and $p27^{Kip1}$ protein levels.

The effects of ICI pretreatment on proliferation were also assessed. As shown in Figure 4.2A, ICI pretreated cells showed small increases in cyclin A expression and pRb phosphorylation at the 36 hr time point. This was reflected in increases in levels of ^{3}H thymidine incorporation upon IGF-1 treatment of ICI pretreated cells. Even in the presence of ICI, IGF-1 increased proliferation 2.4 fold over untreated cells (Figure 4.2B)

Cyclin D1 mRNA and protein levels are coordinately regulated by ICI and IGF-1.

Mitogens can lead to the stabilization of cyclin D1 protein via AKT activation (28). Since AKT is a downstream target of IGF-1 signaling, IGF-1 treatment could lead to increases in cyclin D1 protein without affecting cyclin D1 mRNA levels. If the levels of cyclin D1 mRNA induced by IGF-1 in the presence and absence of ICI were similar, it would support such a mechanism. To test this possibilty, total cellular RNA was extracted at

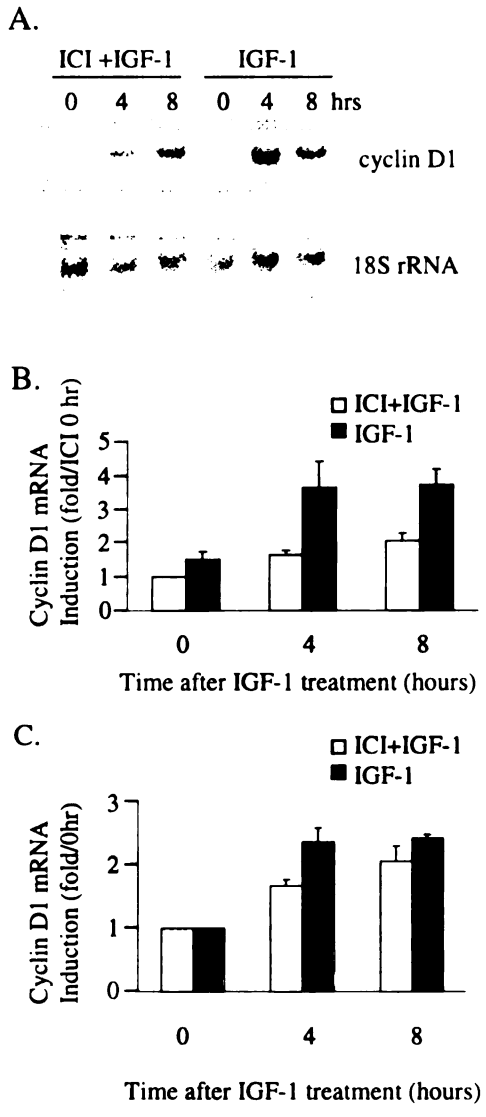


Figure 4. 3. ICI pre-treatment does not prevent induction of cyclin D1 mRNA by IGF-1. A) MCF-7 cells were incubated in SFM or SFM+ICI for 24 hours, and then stimulated with IGF-1. At the times indicated after IGF-1 treatment, total RNA was isolated and analyzed for cyclin D1 mRNA by Northern blotting. B) Cyclin D1 mRNA levels were normalized to 18S rRNA and are represented as fold induction over the 0 hr ICI treated time point. C) Cyclin D1 mRNA induction by IGF-1 in the presence or absence of ICI is represented as fold induction over the respective 0 hr time points. The results are the mean +/- S.E. of three independent experiments.

various time points after IGF-1 stimulation of ICI pretreated or control cells, and Northern blotting was carried out as described in Materials and Methods. Results from one representative experiment are shown in Figure 4.3A and the quantitation of cyclin D1 mRNA induction in the presence and absence of ICI from three independent experiments is shown in Figures 4.3B and 4.3C. IGF-1 treatment increased cyclin D1 mRNA 2-3 fold in both ICI treated and untreated cells. In Figure 4.3B, the amount of cyclin D1 mRNA at the 0 hr time point in the presence of ICI was defined as 1 in each experiment, and other values were expressed relative to this level. Consistent with the protein data, the absolute levels of cyclin D1 mRNA attained are approximately 2 fold higher in the absence of ICI. However as shown in Figure 4.3C, when the amount of mRNA at the 0 hr time point for each treatment is defined as 1, the fold induction by IGF-1 is similar (2-2.5 fold) in the presence and absence of ICI. These results demonstrate that cyclin D1 mRNA levels parallel protein levels in these experiments, and suggest that regulation of translation or protein stability are unlikely mechanisms of cyclin D1 regulation by IGF-1. They also demonstrate that both IGF-1 and ER signaling pathways regulate cyclin D1 mRNA levels.

ICI treatment does not inhibit activation of AKT by IGF-1. It has been reported that activation of PI3K by IGF-1 is required to induce cyclin D1 protein and proliferation of MCF-7 cells (4). We confirmed this requirement using LY 294002, a specific PI3K inhibitor (40). Treatment with this inhibitor prevented the induction of cyclin D1 protein (Figure 4.4A), mRNA and proliferation (data not shown) seen upon IGF-1 treatment, confirming a critical role for PI3K activity in IGF-1 induced cyclin D1 expression and

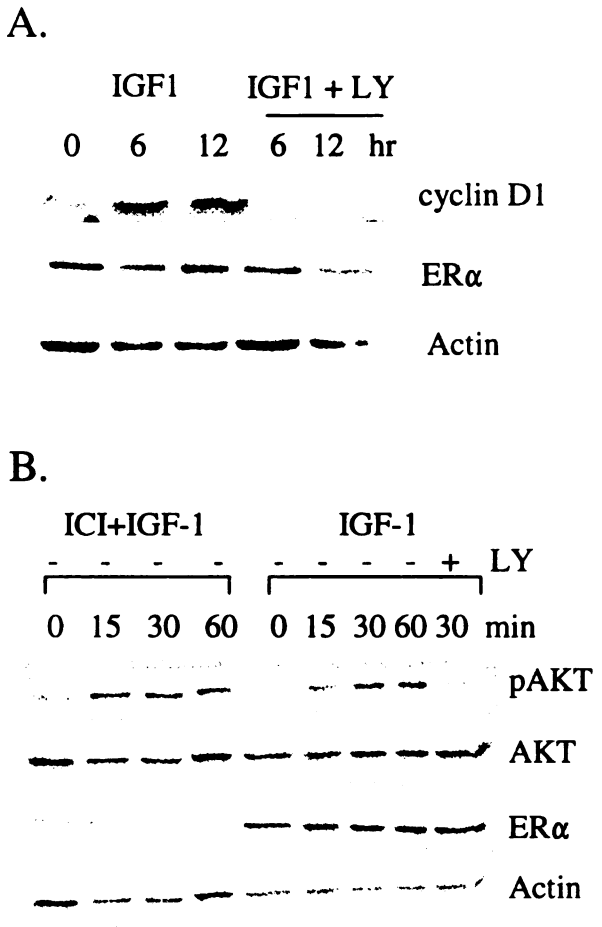


Figure 4.4. IGF-1 induced PI3K activation is required for cyclin D1 expression, and is inhibited by LY294002 but not by ICI. A) MCF-7 cells were incubated in SFM or SFM+LY294002 (25 μM) for 30 min, treated with IGF-1 (10 ng/ml) and lysates prepared at the time points indicated. The lysates were analyzed by Western blotting for cyclin D1 and ERα protein. Actin was used as a loading control. B) MCF-7 cells were untreated, pretreated with ICI for 24 hours, or pretreated with LY294002 (25 μM) for 30 minutes. Cells were then stimulated with IGF-1, and lysates were prepared at the time points indicated and analyzed by immunoblotting with an antibody specific for phosphorylated Ser473 AKT. The blots were also probed with antibodies to total AKT, ERα and Actin.

proliferation of MCF-7 cells.

An important downstream target of PI3K is AKT (41). We reasoned that if ICI inhibited IGF-1 signaling upstream of PI3K, then it would prevent activation of AKT by IGF-1. To test this possibility, MCF-7 cells were plated in SFM and incubated in the presence or absence of ICI for 24 hours. IGF-1 was then added and cell extracts were prepared at various time points. Activation of AKT was assessed by immunoblotting with a phosphospecific antibody that detects AKT phosphorylated on Ser 473 (42). The results of one representative experiment are shown in Figure 4.4B. IGF-1 treatment induced a rapid phosphorylation of Ser473, with the levels of phosphorylated AKT being maintained for 1 hour. This induction was not inhibited by ICI pretreatment, which downregulated ERα protein levels. However, it was inhibited by treatment with the PI3K inhibitor LY294002, indicating that induction of Ser473 phosphorylation by IGF-1 does not require ERα activity but does require PI3K activation.

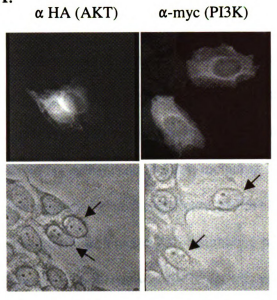
Constitutively active PI3K or AKT does not confer ICI resistance. The above results indicated that ICI does not inhibit the IGF-1 signaling pathway leading to AKT activation. Because PI3K is upstream of AKT, they also suggest that PI3K activity is not regulated by ICI. To determine if activating PI3K could overcome the antiproliferative effects of ICI, constitutively activated forms of a myc-tagged PI3K (p110*) or HA-tagged AKT were transiently transfected into MCF-7 cells. The cells were labeled with BrdU for 5 hr, then fixed and examined for AKT/PI3K expression as described in Materials and Methods. As shown in Figure 4.5A, the tagged versions of both PI3K and AKT were detectable in the cytoplasm and possibly the membrane of transfected cells. Proliferation was assayed by determining the percentages of transfected and

untransfected cells incorporating BrdU by double indirect immunoflourescence for BrdU and either PI3K or AKT as described in Materials and Methods. The results indicate that in the presence of ICI neither activated PI3K nor AKT was able to increase proliferation in the presence of ICI above the level seen in untransfected cells (Figure 4.5B).

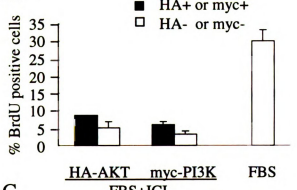
Because constitutively active PI3K (p110*) was unable to overcome an ICI induced growth arrest, we confirmed the activity of this construct in MCF-7 cells by assaying its ability to activate transcription from a serum response element (SRE) promoter luciferase reporter. The SRE is a part of the complex promoters of genes such as c-fos, and is activated by PI3K (43, 44). As shown in Figure 4.5C, the constitutively active PI3K (p110*), but not a kinase dead mutant (ΔKIN) of PI3K, induced a tenfold increase in of SRE-luciferase promoter activity over vector alone, confirming that it is active in transfected MCF-7 cells These results suggest that signaling via the PI3K pathway is not sufficient to support proliferation of MCF-7 cells in the presence of ICI.

Figure. 4. 5. Constitutively active PI3K or AKT are insufficient to overcome an ICI mediated cell cycle arrest. MCF-7 cells were transiently transfected with epitope tagged constitutively activated AKT (HA-tag) or PI3K (myc-tag). After transfection, cells were incubated for 48 hr in medium containing 5% FBS + ICI, and BrdU (25 μM) was added for the final 5 hr before fixation. Cells in medium containing 5% FBS served as a control population of cycling cells. Expression of the transfected proteins and BrdU incorporation were detected by double indirect immunofluorescence as described in Materials and Methods. A) Representative micrographs showing expression of the tagged proteins. B) The percentage of PI3K or AKT expressing cells that were also BrdU positive is represented by filled bars. Open bars represent the percentage of untransfected cells that were BrdU positive in the same cultures. The results are the average +/- S.E. of three independent experiments. C) MCF-7 cells were transiently co-transfected with a SRE-luc plasmid and either vector alone (V), constitutively active PI3K (p110*) or a kinase dead version of PI3K (ΔKIN). All transfections also included a CMV promoter driven β-galactosidase plasmid as a control for transfection efficiency. The luciferase activity was determined and normalized to β-galactosidase activity. The results represent the fold luciferase induction over vector alone control, and are shown as the average +/- S.E. of three independent experiments, each done in triplicate.

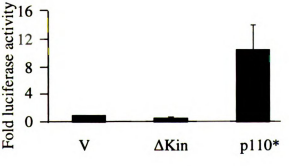
A.


α HA (AKT)
α-myc (PI3K)

B.


HA+ or myc+
HA- or myc-
% BrdU positive cells
35
30
25
20
15
10
5
0
HA-AKT
myc-PI3K
FBS
FBS+ICI

C.


Fold luciferase activity
16
12
8
4
0
V
ΔKin
p110*

DISCUSSION

There is considerable evidence for cross talk between the IGF-1 and estrogen signaling pathways in the regulation of breast cancer cell proliferation (45). Initial work (19, 20) demonstrated that the antiestrogen tamoxifen inhibits insulin/IGF-1 induced proliferation of ER positive breast cancer cell lines, including MCF-7 cells. Results from several laboratories have suggested that the decrease in IGF-1 induced proliferation by antiestrogens is the result of inhibition of the IGF-1 signaling pathway, and that estrogens and antiestrogens mediate their proliferative effects via this pathway. Both IGF-1R and IRS-1 protein levels have been reported to be down regulated by ICI (25, 26, 46), as have PI3K (27) and AKT (47) activities. Additional studies have indicated that E can enhance both PI3K and AKT activation by IGF-1 (39), and a recent study has reported rapid and transient activation of PI3K activity by E in MCF-7 cells (48). All of these findings support the idea that estrogens and antiestrogens regulate the IGF-1 signaling pathway. However, evidence to the contrary also exists. Several studies have reported that E alone does not affect either PI3K or AKT activity (39, 49). In addition, overexpression of IGF-1R (50), IRS-1 (51) or constitutively active AKT (52) in MCF-7 cells does not lead to ICI resistance, indicating that there are additional targets of ICI that mediate its anti-proliferative effects.

In this report, we have investigated the effects of ICI on several targets of IGF-1 signaling. As previously reported, ICI treatment lowered the absolute level of proliferation attained in IGF-1 treated cells. However, ICI could not completely inhibit proliferation in the presence of IGF-1. This can be explained by the fact that although ICI

treatment decreased basal proliferation in SFM, IGF-1 could still induce proliferation (2-4 fold) in the presence of ICI (Figures 4.1C and 4.2B). These results suggest that the proliferative effects of IGF-1 are not subject to inhibition by ICI. Similar results were obtained with regard to the induction of cyclin D1, an important cell cycle regulatory protein, by IGF-1. While the absolute levels attained were lower in the presence of ICI, the extent of both mRNA and protein induction by IGF-1 were similar in the presence and absence of ICI (Figures 4.2A and 4.3C). These results are in agreement with a recent report (16) showing that ICI pretreatment of MCF-7 cells did not prevent cyclin D1 induction by insulin, which, at the concentrations used in that study, acts via the IGF-1R (53). Similarly, IGF-1 could down regulate the levels of $p27^{Kip1}$ in the presence of ICI, demonstrating that ICI could not prevent the effects of IGF-1 on multiple cell cycle components.

An interesting question raised by these results is whether the lower levels of proliferation in IGF-1 plus ICI treated cells relative to those treated with IGF-1 alone is due to lower cyclin D1 levels, or whether there are additional targets of ICI that contribute to its effects. As shown in Figure 4.2B, although cyclin D1 is induced in the presence of ICI, and accumulates to approximately 50% of the levels seen in the absence of ICI, there is little hyperphosphorylated pRb or cyclin A expression in the presence of ICI. Since cyclin D1 can activate Cdk4 directly, and has been proposed to activate Cdk2 indirectly by titrating the Cdk inhibitor $p21^{Waf1}$ away from cyclin E-Cdk2 complexes into cyclin D1-Cdk4 complexes (13, 14), a certain threshold level of cyclin D1 expression may be required to fulfill both of these functions. This possibility is supported by the fact that a reduction in cyclin D1 levels by 50% using antisense oligonucleotides can result in

an almost complete inhibition of MCF-7 proliferation and cyclin E-Cdk2 activity (54). However, E and ICI have also been reported to affect other cell cycle regulators including c-myc, the Cdk inhibitors $p21^{Waf1}$ and $p27^{Kip1}$, and the Cdk activating phosphatase Cdc25A (13, 15, 17), and a combination of several of these effects is likely to be responsible for ICI's ability to decrease proliferation in the presence of IGF-1.

IGF-1 activates several different intracellular pathways, including the MAPK and PI3K signaling pathways (55, 56). Conflicting data has been presented regarding the role of MAPK in E induced proliferation, with some studies showing no effects of E on MAPK activity (57, 58) and others showing rapid and transient activation (48, 59). Under our experimental conditions, MAPK activation by IGF-1 was not regulated by ICI treatment (data not shown). Numerous studies have indicated that the proliferative effects of IGF-1 in MCF-7 cells are mediated by PI3K. IGF-1 treatment activates PI3K in MCF-7 cells (3), and inhibition of PI3K activity with a specific chemical inhibitor LY 294002 (4), or by constitutive expression of PTEN, a phosphotidylinositol 3-phosphatase that inhibits the PI3K pathway, causes a G1 arrest in these cells (60). To directly determine if ICI inhibits or attenuates the PI3K pathway, we examined its effects on AKT activation. AKT is implicated in both cyclin D1 induction and proliferation, and its phosphorylation and activation are induced by PI3K (5, 61). Our results show that phosphorylation of AKT on Ser 473 is induced by IGF-1 in MCF-7 cells. This phosphorylation was inhibited by LY 294002, but was unaffected by ICI treatment, indicating that the IGF-1 signaling pathway upstream of AKT is intact in ICI treated cells. Together with our findings that neither constitutively active AKT nor PI3K were able to promote proliferation in the

presence of ICI, these results establish that ICI does not block proliferation by inhibiting the IGF-1/PI3K/AKT or the MAPK signaling pathways in MCF-7 cells.

There are several other possible mechanisms by which ICI treatment might lower the absolute level of cyclin D1 expression and proliferation in the presence of IGF-1 including the following: 1) Both IGF-1 and ER signaling may independently regulate cell cycle components, and both may be necessary to promote full proliferation. 2) Part of the effect of IGF-1 in MCF-7 cells may be due to its ability to activate the ER in a ligand independent manner, and this effect would be neutralized in the presence of ICI since it causes degradation of the ER. Additional work is required to distinguish between these possibilities.

In summary, our results argue against a model in which ICI inhibits MCF-7 cell proliferation by preventing the early signaling events induced by IGF-1 including PI3K/AKT or MAPK activities (See Figure 4.6 for model). Rather, they suggest that the ER and IGF-1 signaling pathways converge to regulate cell cycle components including cyclin D1 and $p27^{Kip1}$ protein levels, and possibly others. Both pathways appear to be required in order to obtain maximal proliferation, suggesting that deregulation of the IGF-1 signaling pathway in ER positive breast tumors would be insufficient to convert them to an antiestrogen resistant phenotype.

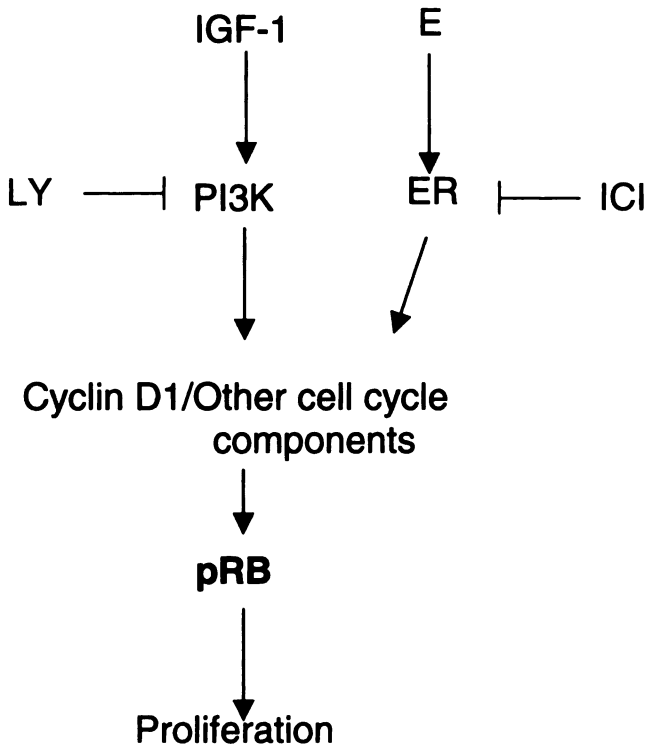


Figure 4.6. Model of IGF-1 and ER signaling interaction. IGF-1, by activating PI3K signaling regulates cyclin D1 and possibly other cell cycle components that lead to Cdk activation. This in turn leads to pRb phosphorylation and proliferation. Inhibition of the PI3K pathway with a specific PI3K inhibitor, LY 294002, prevents the induction of cyclin D1 and proliferation, indicating that this pathway is essential for IGF-1 induced proliferation. ER signaling does not interact with the IGF-1 signaling via the PI3K pathway but appears to converge on downstream cell cycle components including cyclin D1. Treatment of cells with ICI does not inhibit IGF-1 signaling. However, it decreases pRb phosphorylation and proliferation possibly by decreasing the absolute amount of cyclin D1 induced and also by regulating other cell cycle components. According to this model, activation of the IGF-1 signaling intermediates would be insufficient to confer antiestrogen resistance, whereas the inactivation of pRb, which is downstream of IGF-1 and ER signaling, would result in antiestrogen resistance.

REFERENCES

1. Surmacz, E. and Burgaud, J. L. Overexpression of insulin receptor substrate 1 (IRS-1) in the human breast cancer cell line MCF-7 induces loss of estrogen requirements for growth and transformation, Clin Cancer Res. *1:* 1429-36., 1995.
2. Osborne, C. K., Clemmons, D. R., and Arteaga, C. L. Regulation of breast cancer growth by insulin-like growth factors, J Steroid Biochem Mol Biol. *37:* 805-9., 1990.
3. Jackson, J. G., White, M. F., and Yee, D. Insulin receptor substrate-1 is the predominant signaling molecule activated by insulin-like growth factor-I, insulin, and interleukin-4 in estrogen receptor-positive human breast cancer cells, J Biol Chem. *273:* 9994-10003., 1998.
4. Dufourny, B., Alblas, J., van Teeffelen, H. A., van Schaik, F. M., van der Burg, B., Steenbergh, P. H., and Sussenbach, J. S. Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase, J Biol Chem. *272:* 31163-71., 1997.
5. Toker, A. Protein kinases as mediators of phosphoinositide 3-kinase signaling, Mol Pharmacol. *57:* 652-8., 2000.
6. Sherr, C. J. D-type cyclins, Trends Biochem Sci. *20:* 187-90., 1995.
7. Hall, J. M., Couse, J. F., and Korach, K. S. The multifaceted mechanisms of estradiol and estrogen receptor signaling, J Biol Chem. *276:* 36869-72., 2001.

8. Couse, J. F. and Korach, K. S. Estrogen receptor null mice: what have we learned and where will they lead us?, Endocr Rev. *20:* 358-417., 1999.

9. Brooks, S. C., Locke, E. R., and Soule, H. D. Estrogen receptor in a human cell line (MCF-7) from breast carcinoma, J Biol Chem. *248:* 6251-3., 1973.

10. Soule, H. D. and McGrath, C. M. Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice, Cancer Lett. *10:* 177-89., 1980.

11. Lippman, M., Monaco, M. E., and Bolan, G. Effects of estrone, estradiol, and estriol on hormone-responsive human breast cancer in long-term tissue culture, Cancer Res. *37:* 1901-7., 1977.

12. Lippman, M., Bolan, G., and Huff, K. The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture, Cancer Res. *36:* 4595-601., 1976.

13. Planas-Silva, M. D. and Weinberg, R. A. Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution, Mol Cell Biol. *17:* 4059-69., 1997.

14. Prall, O. W., Sarcevic, B., Musgrove, E. A., Watts, C. K., and Sutherland, R. L. Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E- Cdk2, J Biol Chem. *272:* 10882-94., 1997.

15. Foster, J. S., Henley, D. C., Bukovsky, A., Seth, P., and Wimalasena, J. Multifaceted regulation of cell cycle progression by estrogen: regulation of Cdk inhibitors and Cdc25A independent of cyclin D1-Cdk4 function, Mol Cell Biol. *21:* 794-810., 2001.

16. Lai, A., Sarcevic, B., Prall, O. W., and Sutherland, R. L. Insulin/insulin-like growth factor-I and estrogen cooperate to stimulate cyclin E-Cdk2 activation and cell cycle progression in MCF-7 breast cancer cells through differential regulation of cyclin E and p21(WAF1/Cip1), J Biol Chem. *276:* 25823-33., 2001.

17. Cariou, S., Donovan, J. C., Flanagan, W. M., Milic, A., Bhattacharya, N., and Slingerland, J. M. Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen- mediated cell cycle arrest in human breast cancer cells, Proc Natl Acad Sci U S A. *97:* 9042-6., 2000.

18. Nevins, J. R. The Rb/E2F pathway and cancer, Hum Mol Genet. *10:* 699-703., 2001.

19. Vignon, F., Bouton, M. M., and Rochefort, H. Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens, Biochem Biophys Res Commun. *146:* 1502-8., 1987.

20. Wosikowski, K., Kung, W., Hasmann, M., Loser, R., and Eppenberger, U. Inhibition of growth-factor-activated proliferation by anti-estrogens and effects on early gene expression of MCF-7 cells, Int J Cancer. *53:* 290-7., 1993.

21. Freiss, G., Prebois, C., Rochefort, H., and Vignon, F. Anti-steroidal and anti-growth factor activities of anti-estrogens, J Steroid Biochem Mol Biol. *37:* 777-81., 1990.

22. Wiebe, V. J., Osborne, C. K., Fuqua, S. A., and DeGregorio, M. W. Tamoxifen resistance in breast cancer, Crit Rev Oncol Hematol. *14:* 173-88., 1993.

23. Pietras, R. J., Arboleda, J., Reese, D. M., Wongvipat, N., Pegram, M. D., Ramos, L., Gorman, C. M., Parker, M. G., Sliwkowski, M. X., and Slamon, D. J. HER-2

tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells, Oncogene *10:* 2435-46., 1995.

24. Stewart, A. J., Johnson, M. D., May, F. E., and Westley, B. R. Role of insulin-like growth factors and the type I insulin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells, J Biol Chem. *265:* 21172-8., 1990.

25. Huynh, H., Nickerson, T., Pollak, M., and Yang, X. Regulation of insulin-like growth factor I receptor expression by the pure antiestrogen ICI 182780, Clin Cancer Res. *2:* 2037-42., 1996.

26. Molloy, C. A., May, F. E., and Westley, B. R. Insulin receptor substrate-1 expression is regulated by estrogen in the MCF-7 human breast cancer cell line, J Biol Chem. *275:* 12565-71., 2000.

27. Guvakova, M. A. and Surmacz, E. Tamoxifen interferes with the insulin-like growth factor I receptor (IGF-IR) signaling pathway in breast cancer cells, Cancer Res. *57:* 2606-10., 1997.

28. Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization, Genes Dev. *12:* 3499-511., 1998.

29. Murga, C., Laguinge, L., Wetzker, R., Cuadrado, A., and Gutkind, J. S. Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinasegamma, J Biol Chem. *273:* 19080-5., 1998.

30. Hu, Q., Klippel, A., Muslin, A. J., Fantl, W. J., and Williams, L. T. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase, Science *268:* 100-2., 1995.

31. Neff, S., Sadowski, C., and Miksicek, R. J. Mutational analysis of cysteine residues within the hormone-binding domain of the human estrogen receptor identifies mutants that are defective in both DNA-binding and subcellular distribution, Mol Endocrinol. *8:* 1215-23., 1994.

32. Labarca, C. and Paigen, K. A simple, rapid, and sensitive DNA assay procedure, Anal Biochem. *102:* 344-52., 1980.

33. Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J., and Kato, J. Y. D-type cyclin-dependent kinase activity in mammalian cells, Mol Cell Biol. *14:* 2066-76., 1994.

34. Varma, H. and Conrad, S. E. Reversal of an antiestrogen-mediated cell cycle arrest of MCF-7 cells by viral tumor antigens requires the retinoblastoma protein-binding domain, Oncogene *19:* 4746-53., 2000.

35. Dauvois, S., Danielian, P. S., White, R., and Parker, M. G. Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover, Proc Natl Acad Sci U S A. *89:* 4037-41., 1992.

36. Saceda, M., Lippman, M. E., Chambon, P., Lindsey, R. L., Ponglikitmongkol, M., Puente, M., and Martin, M. B. Regulation of the estrogen receptor in MCF-7 cells by estradiol, Mol Endocrinol. *2:* 1157-62., 1988.

37. Saceda, M., Lindsey, R. K., Solomon, H., Angeloni, S. V., and Martin, M. B. Estradiol regulates estrogen receptor mRNA stability, J Steroid Biochem Mol Biol. *66:* 113-20., 1998.

38. Bouchard, P. Progesterone and the progesterone receptor, J Reprod Med. *44:* 153-7., 1999.

39. Dupont, J., Karas, M., and LeRoith, D. The potentiation of estrogen on insulin-like growth factor I action in MCF-7 human breast cancer cells includes cell cycle components, J Biol Chem. *275:* 35893-901., 2000.

40. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4- morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), J Biol Chem. *269:* 5241-8., 1994.

41. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase, Cell *81:* 727-36., 1995.

42. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. Akt activation by growth factors is a multiple-step process: the role of the PH domain, Oncogene *17:* 313-25., 1998.

43. Wang, Y., Falasca, M., Schlessinger, J., Malstrom, S., Tsichlis, P., Settleman, J., Hu, W., Lim, B., and Prywes, R. Activation of the c-fos serum response element by phosphatidyl inositol 3-kinase and rho pathways in HeLa cells, Cell Growth Differ. *9:* 513-22., 1998.

44. Poser, S., Impey, S., Trinh, K., Xia, Z., and Storm, D. R. SRF-dependent gene expression is required for PI3-kinase-regulated cell proliferation, Embo J. *19:* 4955-66., 2000.

45. Smith, C. L. Cross-talk between peptide growth factor and estrogen receptor signaling pathways, Biol Reprod. *58:* 627-32., 1998.

46. Lee, A. V., Jackson, J. G., Gooch, J. L., Hilsenbeck, S. G., Coronado-Heinsohn, E., Osborne, C. K., and Yee, D. Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo, Mol Endocrinol. *13:* 787-96., 1999.

47. Ahmad, S., Singh, N., and Glazer, R. I. Role of AKT1 in 17beta-estradiol- and insulin-like growth factor I (IGF- I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells, Biochem Pharmacol. *58:* 425-30., 1999.

48. Castoria, G., Barone, M. V., Di Domenico, M., Bilancio, A., Ametrano, D., Migliaccio, A., and Auricchio, F. Non-transcriptional action of oestradiol and progestin triggers DNA synthesis, Embo J. *18:* 2500-10., 1999.

49. Lobenhofer, E. K. and Marks, J. R. Estrogen-induced mitogenesis of MCF-7 cells does not require the induction of mitogen-activated protein kinase activity, J Steroid Biochem Mol Biol. *75:* 11-20., 2000.

50. Daws, M. R., Westley, B. R., and May, F. E. Paradoxical effects of overexpression of the type I insulin-like growth factor (IGF) receptor on the responsiveness of human breast cancer cells to IGFs and estradiol, Endocrinology *137:* 1177-86., 1996.

51. Salerno, M., Sisci, D., Mauro, L., Guvakova, M. A., Ando, S., and Surmacz, E. Insulin receptor substrate 1 is a target for the pure antiestrogen ICI 182,780 in breast cancer cells, Int J Cancer. *81:* 299-304., 1999.

52. Campbell, R. A., Bhat-Nakshatri, P., Patel, N. M., Constantinidou, D., Ali, S., and Nakshatri, H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance, J Biol Chem. *276:* 9817-24., 2001.

53. Milazzo, G., Giorgino, F., Damante, G., Sung, C., Stampfer, M. R., Vigneri, R., Goldfine, I. D., and Belfiore, A. Insulin receptor expression and function in human breast cancer cell lines, Cancer Res. *52:* 3924-30., 1992.

54. Carroll, J. S., Prall, O. W., Musgrove, E. A., and Sutherland, R. L. A pure estrogen antagonist inhibits cyclin E-Cdk2 activity in MCF-7 breast cancer cells and induces accumulation of p130-E2F4 complexes characteristic of quiescence, J Biol Chem. *275:* 38221-9., 2000.

55. Tartare-Deckert, S., Murdaca, J., Sawka-Verhelle, D., Holt, K. H., Pessin, J. E., and Van Obberghen, E. Interaction of the molecular weight 85K regulatory subunit of the phosphatidylinositol 3-kinase with the insulin receptor and the insulin- like growth factor-1 (IGF- I) receptor: comparative study using the yeast two-hybrid system, Endocrinology *137:* 1019-24., 1996.

56. Skolnik, E. Y., Lee, C. H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., Myers, M. J., Jr., Backer, J. M., Ullrich, A., White, M. F., and et al. The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine- phosphorylated IRS1

and Shc: implications for insulin control of ras signalling, Embo J. *12:* 1929-36., 1993.

57. Bonapace, I. M., Addeo, R., Altucci, L., Cicatiello, L., Bifulco, M., Laezza, C., Salzano, S., Sica, V., Bresciani, F., and Weisz, A. 17 beta-Estradiol overcomes a G1 block induced by HMG-CoA reductase inhibitors and fosters cell cycle progression without inducing ERK-1 and -2 MAP kinases activation, Oncogene *12:* 753-63., 1996.

58. Caristi, S., Galera, J. L., Matarese, F., Imai, M., Caporali, S., Cancemi, M., Altucci, L., Cicatiello, L., Teti, D., Bresciani, F., and Weisz, A. Estrogens Do Not Modify MAP Kinase-dependent Nuclear Signaling during Stimulation of Early G(1) Progression in Human Breast Cancer Cells, Cancer Res. *61:* 6360-6366., 2001.

59. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol- receptor complex in MCF-7 cells, Embo J. *15:* 1292-300., 1996.

60. Weng, L. P., Smith, W. M., Dahia, P. L., Ziebold, U., Gil, E., Lees, J. A., and Eng, C. PTEN suppresses breast cancer cell growth by phosphatase activity-dependent G1 arrest followed by cell death, Cancer Research. *59:* 5808-5814, 1999.

61. Muise-Helmericks, R. C., Grimes, H. L., Bellacosa, A., Malstrom, S. E., Tsichlis, P. N., and Rosen, N. Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway, J Biol Chem. *273:* 29864-72., 1998.

CHAPTER 5

SUMMARY AND FUTURE EXPERIMENTS

A majority of breast cancers are estrogen dependent for proliferation making them amenable to antiestrogen therapy. However, over the course of the disease, these tumors progress to an antiestrogen resistant state resulting in treatment failure and contributing to the high mortality associated with this disease. The focus of the studies described in this thesis was to investigate the mechanisms by which breast cancer cells progress from an antiestrogen sensitive to a resistant phenotype. The studies described have addressed two distinct mechanisms that have been proposed to contibute to antiestrogen resistance.

Retinoblastoma protein (pRb) is a negative regulator of cell cycle progression whose inhibitory effect on proliferation is inhibited by phosphorylation by cyclin dependent kinases (Cdks) Cdk4 and Cdk2. Estrogen treatment of MCF-7 cells, an estrogen responsive human breast cancer cell line, leads to activation of Cdk4 and Cdk2 and increased pRb phosphorylation. To establish whether pRb is an important functional target of estrogens and antiestrogens, we asked whether functional inactivation of pRb would allow MCF-7 cells to proliferate in the presence of antiestrogens. Using a panel of SV40 and Polyoma virus large T antigens, we demonstrated that inactivation of pRb is required and may be sufficient for proliferation in the presence of antiestrogens. Further experiments using inhibitors of Cdk4 and Cdk2 demonstrated that cells in which pRb has been inactivated still require Cdk2 activity for proliferation but no longer require Cdk4 activity. This suggests that Cdk4's primary role in MCF-7 cells is to inactivate pRb,

whereas Cdk2 has additional targets important for promoting proliferation. The facts that antiestrogens inhibit Cdk2 activity and that Cdk2 activity is required for proliferation upon pRb inactivation in antiestrogen treated cells, suggests that Cdk2 activity must be induced upon pRb inactivation in the presence of antiestrogens. Further these results suggest that estrogens regulate Cdk2 activity at least partially via a pRb dependent mechanism. Using stably transfected cell lines, we confirmed that expression of Polyoma LT in ICI treated cells leads to Cdk2 activation. The mechanism of this activation has not been characterized but it correlates with increases in cyclin A protein levels and may therefore be due to formation of active cyclin A-Cdk2 complexes.

In summary, our experiments suggest a model for estrogen mediated cell cycle progression, in which estrogen leads to Cdk4 activation, which then phosphorylates pRb and results in Cdk2 activation and proliferation of cells. Furthermore, the fact that pRb inactivation can promote proliferation in the presence of antiestrogens suggest that inactivation of the pRb pathway is a potential cause of antiestrogen resistance in breast cancer patients.

Our experiments using viral T antigens also raised some interesting questions that remain to be addressed. First, it is not known if pRb inactivation by PyLT causes activation of both cyclin E and/or cyclin A associated Cdk2. Since cyclin E-Cdk2 is essential for progression to S phase, one would predict that its activity would be increased upon PyLT induction. However, cyclin E levels are constitutive during G1 in MCF-7 cells. During estrogen-induced proliferation, increases in cyclin D1-Cdk4 complexes have been proposed to sequester inhibitors from cyclin E-Cdk2 complexes, thus activating cyclin E-Cdk2. Since we have not observed an increase in cyclin D1 or

cyclin E protein levels upon PyLT expression, the above cannot occur. It is also possible that an increase in cyclin A-Cdk2 complexes leads to some inhibitor free active Cdk2 complexes. A second issue is that in stable cell lines, PyLT induced proliferation was two fold lower than that seen in estrogen treated cells. This might be due to the fact that approximately half the cells in the population did not express detectable PyLT. This possibility can be tested by assaying BrdU incorporation in the presence of ICI in PyLT positive and PyLT negative cells using double indirect immunofluorescence. Additionally, in long term (8-12 days) assays, proliferation of PyLT expressing cells in the presence of ICI was much lower than estrogen treated cells. The reason(s) for this are unclear, but may involve one of several explanations. The inactivation of pRb may not be as efficient as estrogen treatment suggesting that estrogen has targets in addition to pRb that promote proliferation. If cyclin E-Cdk2 is not efficiently activated upon PyLT expression, it would provide an explanation for decreased proliferation of PyLT expressing cells in the presence of ICI in the long term. Finally, PyLT may be causing growth inhibition or apoptosis in the long term, though in the short term this does not appear to be the case.

The role of increased Insulin like growth factor-1 (IGF-1) signaling as a second potential mechanism of antiestrogen resistance was addressed in these studies. Antiestrogens decrease proliferation of IGF-1 treated cells and it has been proposed that they do so by downregulating IGF-1 signaling. Our results suggest that this hypothesis is not true. Cells treated with ICI did not show an inhibition of the IGF-1 signaling pathway, as determined by measuring activation of Akt or the induction of cyclin D1 by IGF-1. Furthermore, activation of either PI3K or Akt, intermediates in the IGF-1

signaling pathway that mediate IGF-1's proliferative effects, was unable to induce proliferation in the presence of ICI. Treatment of cells with ICI did cause a twofold decrease in the absolute levels of cyclin D1 expression in IGF-1 treated cells, and this decrease correlated with decreased cyclin A protein levels, pRb phosphorylation and proliferation. These results suggest that both IGF-1 and ER signaling are required to promote proliferation in MCF-7 cells, and these two signaling pathways converge on cell cycle components. They also predict that activation of the IGF-1 signaling pathway alone would be insufficient to confer antiestrogen resistance.