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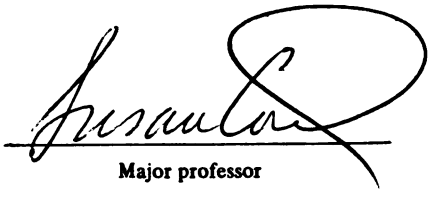

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IDENTIFICATION OF AN ESTROGEN RECEPTOR VARIANT IN ESTROGEN INDEPENDENT AND/OR TAMOXIFEN RESISTANT HUMAN BREAST CANCER CELL LINES

By

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ABSTRACT

IDENTIFICATION OF AN ESTROGEN RECEPTOR VARIANT IN ESTROGEN INDEPENDENT AND/OR TAMOXIFEN RESISTANT HUMAN BREAST CANCER CELL LINES

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Estrogen receptor is important in the development of breast cancer, but a role for it in the development of antiestrogen resistance has not been established. In this study, an ER variant, ERΔE3, was observed in two naturally selected cell lines that are estrogen independent and/or tamoxifen resistant. The presence of ERΔE3 decreased overall ER function, as indicated by a 3-5 fold reduction of ERE activity in these cells. ERΔE3 was ectopically expressed in MCF-7 and LCC1 cells, and the phenotype of transfectants was examined by anchorage independent proliferation assays. It was observed that expression of ERΔE3 inhibited proliferation and was not sustainable in MCF-7 cells. In contrast, it could be maintained for at least 15 passages in LCC1 cells, where it did not detectably inhibit proliferation. Expression of ERΔE3 did not alter tamoxifen sensitivity in either cell line. These results suggest that ERΔE3 competitively inhibits wild-type ER activity and negatively regulates cell proliferation. They further suggest that a change has occurred in LCC1 cells that both permits the development of estrogen independence and allows the sustained expression of ERΔE3, and that additional changes may lead to tamoxifen resistance.

To Joy

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TABLE OF CONTENTS

CHAPTER 2

LIST OF FIGURES

CHAPTER 1

Literature Review

1. Introduction

1.1. Breast cancer and estrogens

Breast cancer is the most common type of cancer among women with an annual worldwide incidence of one million. In western countries, the rate is especially high. One out of every eight women will develop breast cancer in her lifetime.

Estrogen plays several important roles in the development of the reproductive tract and secondary sex organs. These roles are reflected in the development of mammary gland, regulation of the estrus cycle, and control of lactation. Estrogen also exerts effects on the bone, liver, and cardiovascular systems.

Estrogen also plays important roles in the development and progression of breast cancer. In the late 1890's, it was observed that ovariectomy in cases of premenopausal breast cancer led to tumor regression. In athymic nude mice xenografted with human breast cancer cells, tumor formation and growth require the presence of estrogen(1). Through experimental models of carcinogen-induced mammary carcinoma, studies have shown that estrogen is essential for the initiation and progression of breast tumors(2). Based on this pivotal role of estrogen in the initiation and development of breast cancer, endocrine therapy has been used in the treatment of breast cancer.

1.2. Endocrine therapy, estrogen independence, and antiestrogen resistance

Current primary therapies for breast cancer include excision surgery, cytotoxic chemotherapy, and radiation therapy. After these treatments, endocrine therapy has been developed as the major adjuvant treatment for breast cancers that are responsive to estrogen. The general principle of endocrine therapy is to inhibit the mitogenic stimulus generated from estrogens. Antiestrogens have been developed for this purpose. Having similar structures to estrogen, antiestrogens act through their competitive antagonism of estrogen. Currently, several types of antiestrogens have been developed, including nonsteroidal and steroidal. Among these, tamoxifen (a non-steroidal antiestrogen) has been the most widely used and clinically proven effective(3).

Following endocrine therapy, most breast tumors undergo remission. However, many breast tumors that initially respond to endocrine therapy eventually become estrogen independent, and the patient relapses with acquired resistance to antiestrogens. In fact, the development of multiple drug resistance in advanced breast cancer is the primary reason for failures of current breast cancer therapies(3).

1.3. Tamoxifen resistance

Tamoxifen treatment is the most commonly used endocrine therapy in hormone-responsive breast cancer, especially as adjuvant therapy after removal of the primary tumor(4). In fact, the application of tamoxifen clinically has been very effective in decreasing both disease progression and the mortality rate. Worldwide clinical trials indicate that the 5- and 10-year mortality rates of breast cancer patients can be reduced 20-25% by tamoxifen treatment(5). Nevertheless, the selective growth pressure by

tamoxifen often gives rise to tamoxifen resistance in many patients under tamoxifen treatment. Tamoxifen resistance is the most significant problem of endocrine therapy.

2. Molecular biology of estrogen receptor

2.1. The role of estrogen receptor in breast cancer

Estrogen functions by binding to the estrogen receptor (ER). ER belongs to the nuclear receptor superfamily of ligand-inducible transcription factors. ER has important effects on the differentiation and maintenance of diverse tissues, including neural, skeletal, cardiovascular, and reproductive(6, 7).

Based on the close correlation between estrogen and breast cancer, the importance of estrogen receptor in the development of breast cancer has been established. Studies have shown that nearly 70% of primary breast cancers have measurable ER expression while only 7% of the epithelial cells in the normal breast tissue have the same level of ER expression(8).

2.2. Isoforms of estrogen receptor

There are two isoforms of ER, ER_α and ER_β. The two isoforms of ER share many similarities. Both ER_α and ER_β respond to estrogen and stimulate transcription from promoters containing estrogen responsive element (ERE) (9). Their functions are also regulated by coregulators (10). While both ER_α and ER_β have the same binding affinity for estrogen, they are regulated differently by estrogen and may play different roles in gene regulation. For example, depending upon cell types and promoter context, estrogen

can activate transcription from an AP-1 site with ER_α; while it inhibits AP-1 activity with ER_β. In addition, compounds that act as antiestrogens with ER_α, including tamoxifen, raloxifene, and ICI are potential activators with ER_β at an AP-1 site(11). The current understanding of ER_β function is mostly limited to brain and ovary tissues(12-14). In breast tissues, most work has been done towards clarifying the mechanisms by which ER_α mediates the proliferation effects of estrogen. In the following discussions, ER refers to ER_α unless specified otherwise.

2.3. Structure of estrogen receptor

ER has a structure typical of nuclear receptors. It is composed of six structural domains, A-F (Figure 1). Its functional domains include two transactivation domains (AF-1 and AF-2), a DNA binding domain, a ligand binding domain (LBD), and a hinge region. AF-1 is located at the N-terminus and AF-2 is within the LBD. The LBD domain is located in the C-terminal region of ER_α. The structure of ER is important for its binding to estrogens and antiestrogens, and its subsequent interactions with co-regulators, which determines its effects on gene transcription.

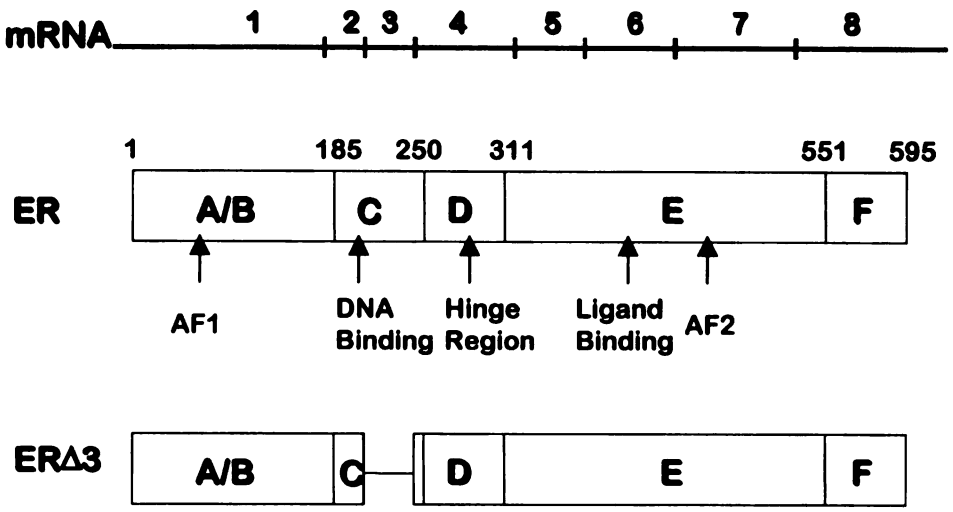

Figure 1.1 Structure of the estrogen receptor and the estrogen receptor variant $ER_{\Delta}E3$.

2.4. Molecular mechanism of transcription activation by estrogen

Currently, there are two known modes of ER function. Without the presence of estrogen, ER exists in the form of complex with several heat shock proteins in the nucleus. When bound by estrogen, ER undergoes an activating conformational change and dissociates from the complex(15). In the "classical mode", estrogen binds to the ligand-binding domain and induces ER dimerization. Dimerized ER binds to estrogen response elements (EREs) in the 5' promoter region of target genes and activates transcription of these genes(16). The second mode does not require ER dimerization or DNA binding by ER. After estrogen binds to ER, the complex can activate transcription by protein-protein interaction with other transcription factors like c-Fos and c-Jun at AP-1 sites(11).

ER mediates transcription activation through its two transactivation domains, AF-1 and AF-2. AF-1 is activated by growth factors through the MAP kinase pathway(17); while AF-2 responds to ligand binding (18). Transcriptional activation by both AF-1 and AF-2 domains relies on specific promoter sequences, and may be tissue specific. AF-1 and AF-2 can function independently from each other; but optimum activity requires the activation of both domains(19).

Estrogen binds to ER through LBD (ligand binding domain). The crystal structure of the human ER LBD, and how ligand binding affects this structure have been established(20). After ligand binds to the LBD, it induces complex conformational changes in and around the ligand-binding pocket, resulting in a repositioning of Helix 12, the most C-terminal helix of ER. The repositioning of Helix 12 plays an essential role in the activation of AF-2 and the subsequent recruitment of co-regulators(21-24). When ER LBD is bound by agonists such as estrogen, the conformation of the repositioned Helix 12 allows coactivators such as GRIP1 to bind. When ER LBD is bound by antagonists, different conformational changes occur, and coactivators can not bind to Helix 12. In turn, transcription cannot be activated(25, 26). Therefore, the conformation of ER resulted from ligand binding is essential for its function in transcriptional activation.

2.5. Coregulators of estrogen receptor

ER transcriptional activity is regulated by various coactivators and corepressors. The result of the interactions between ER and its coregulators is dependent upon the specific ligand bound to ER. Different ligands result in different conformations of ER, resulting in

the recruitment of different co-regulators, which could in turn regulate different subsets of estrogen-responsive genes.

Coactivators play an important role in the ligand-dependent activation of transcription by estrogen receptor. Agonists promote coactivator binding, while antagonists inhibit coactivator binding(20). Transcriptional coactivators are a group of proteins that can enhance ligand-dependent transcriptional activation by nuclear receptors(27, 28). After binding to ligand-bound nuclear receptors, coactivators facilitate the recruitment of the pre-initiation transcription complex and the activation of gene transcription. Coactivators recognize agonist-bound nuclear receptor LBDs through the NR box, a short sequence motif, LXXLL (L represents leucine and X represents any amino acid). These coactivators include GRIP1/TIF-2/N-CoA2(29-31), SRC-1/N-CoA1(25, 32, 33), p/CIP/RAC3/ACTR/AIB1(31, 34, 35), and CBP/p300(36). Mutations at the NR box could lead to the loss of affinity of coactivators for nuclear receptor LBDs(37). After coactivators bind to estrogen-ER complex, transcriptional activation of estrogen-responsive genes can be further stimulated. It has been show that SRC-1's binding to estrogen-ER complex could increase histone acetyltransferase activity*(38)*, which functions to relax promoter DNA structure and facilitate the initiation of transcription.

ER can also recruit many corepressors including SMRT and N-CoR and negatively regulate transcription(39, 40). ER-ligand complexes containing corepressors such as N-CoR can increase histone deacetylase activity(38), which would be expected to inhibit transcription (41).

The effects of coregulators on ER-mediated transcription can be very complex. For example, SRC-1 enhances the agonist activity of 4-OH TAM, and also interacts with

other coactivators synergistically to stimulate ER-dependent gene transcription(40, 42). While N-CoR inhibits the agonist activity of 4-OH TAM, it cannot bind to the ICI 182,780-ER complex(43). The ultimate effects of coregulators on ER-mediated transcriptional activation may depend on specific ligands bound by ER, various promoter contexts, and different cell types.

3. Estrogens, antiestrogens, and selective estrogen receptor modulators

3.1. Categorization of compounds bound to estrogen receptor

The ER LBD can recognize compounds of different sizes, shapes, and chemical properties. This ability enables a variety of compounds to be developed in order to modulate ER transcriptional activity. These compounds, termed estrogen receptor modulators, have been used to treat breast cancer, osteoporosis, and cardiovascular disease. Based on their different behavior in transcriptional activation, they can be divided into three categories- pure agonists, such as the endogenous estrogen 17β-estradiol and the synthetic nonsteroidal estrogen diethylstilbestrol (DES); pure antagonists, such as ICI 164,384; and selective estrogen receptor modulators (SERMs) including tamoxifen and raloxifene(RAL).

While pure agonists stimulate and pure antagonists inhibit ER activity, SERMs can act as either agonists or antagonists depending on specific tissue and promoter contexts(44). For example, tamoxifen has tissue-specific effects. It is known to antagonize the effects of estrogen on mammary tissue, but acts as an estrogen agonist in bone and the cardiovascular system. While tamoxifen reduces breast cancer incidence by 45% in high-risk women(45), it also increases the incidence of endometrial cancer (45,

46). The increased risk of endometrial cancer for patients treated with tamoxifen is a result of tamoxifen's effect as an agonist in the uterus. Another SERM, raloxifene, functions entirely as an antagonist in the same tissue(46).

The mechanism of tissue specific actions of SERMs is not clear. One possible explanation is that specific conformations of the ER-ligand complex may be determined by the molecular structures of different ER modulators. The resulting difference in the conformation of the ER-ligand complex might determine different transactivation properties, which eventually results in tissue selectivity (15, 16, 44).

3.2. Chemical structures of estrogens, antiestrogens, and selective estrogen receptor modulators

The chemical structures of commonly seen estrogens, antiestrogens, and SERMs are listed in Fig. 2.

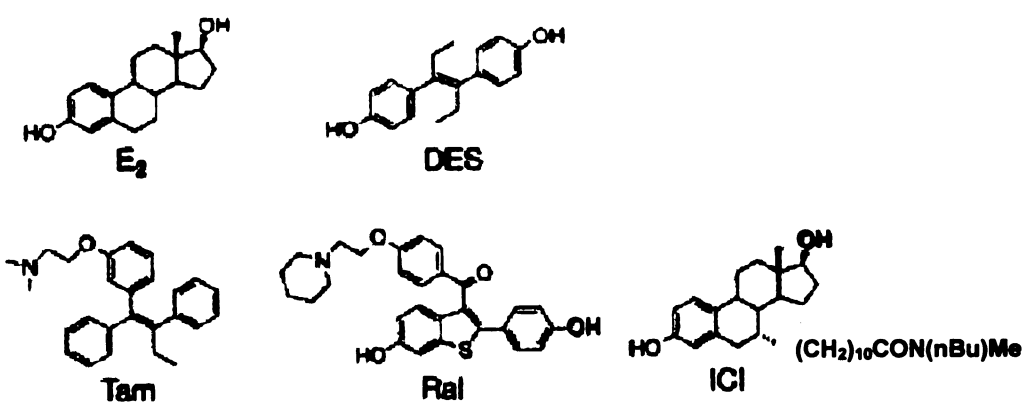

Figure 1.2 Chemical structures of estrogens and antiestrogens.

3.3. Molecular basis of antiestrogen action

Antiestrogens inhibit cell proliferation primarily by acting as antagonists to estrogen. For example, Tamoxifen is a nonsteroidal triphenyl ethylene and has a similar structure as estrogen. It acts as a competitive inhibitor of estrogen by binding to ER. When bound by tamoxifen, ER can still dimerize, but cannot bind to EREs and hence gene transcription cannot be activated. However, different antiestrogens might exert their antagonism differently. For example, ICI has a higher ER binding affinity than tamoxifen. When bound by ICI, ER cannot dimerize and therefore DNA binding and transcriptional activation cannot be initiated. Furthermore, in addition to its inhibitory effect through competitive binding to ER, ICI also significantly reduces the stability of ER, leading to a rapid decrease in ER protein level and transcriptional activity(47). It is also possible that different antiestrogens regulate different subsets of genes. It was demonstrated that different antiestrogens, including 4-OH TAM, idoxifene, raloxifene, GW7604, and ICI 182,780 induce distinct ERα-ligand conformations. These distinct conformations could be recognized differentially by the transcription machinery, resulting in different down-stream signaling(48).

4. Estrogen Receptor Variants

4.1. The presence of estrogen receptor variants in breast cancer

The ER gene has eight coding exons which generate the 6.2 kb wild-type ER mRNA. Abberant mRNA splicing can produce different forms of ER mRNA with single or multiple exon deletions from exon 2 to exon 7. The presence of these ER variant

mRNAs have been widely detected in many breast cancers varying widely in terms of their proportion to wild type ER mRNA and in different studies(49-57). The significance of these ER variants has been studied both for diagnostic purposes and for understanding antiestrogen resistance. Among these ER variants, the most common and extensively studied ones include exon 3 deletion (ERΔE3) and exon 5 deletion (ERΔE5).

Exon 3 codes for the second zinc finger of the DNA binding domain. The ERΔE3 therefore does not have a functional DNA binding domain. Consequently, the ERΔE3 protein cannot bind to EREs and therefore cannot activate ERE activity in transient transfection assays. Studies also confirmed this. When mixed with wild type ER at a 1:1 ratio, ERE activity was inhibited by 30%, suggesting that ERΔE3 functions as a dominant negative ER variant (51). Furthermore, ectopic expression of ERΔE3 in estrogen responsive human breast cancer cells suppressed their *in vivo* invasiveness(58). Exon 5 encodes for part of the ligand binding domain. The ERΔE5 lacks both a functional LBD and the second transactivation domain AF-2. Unlike ERΔE3, ERΔE5 cannot dimmerize with both wild type ER and ER variants. It has been observed that increased levels of ERΔ5 mRNAs are present in some breast tumor tissues(49).

4.2. Paradoxical roles of estrogen receptor variants in the development of breast cancer and antiestrogen resistance

The role of ER variants in the development of breast cancer and antiestrogen resistance has been widely studied and controversial. Using sensitive techniques such as RT-PCR, many variant ER mRNAs have been detected in normal breast tissues, breast cancer cell lines, and clinical breast tumor samples. Some of these ER variants were

shown to arise from abberant mRNA splicing. However, the origin and consequence of these variants are not clear. Most breast tumors express wild-type ER at the same time as they express mutant ER. Some researchers showed the presence of ER variant mRNAs in more than 30% of breast tumors and the rate increased with the emergence of tamoxifen resistance, while others failed to observe any evidence of ER variants in in vitro derived tamoxifen resistant cell lines(59-61). Furthermore, overexpression of ERΔE3 or ERΔE5 is not sufficient to induce tamoxifen resistant phenotype in MCF-7 cells(57). Although there were small proportion of ER variants with protein produced, whether the majority of the ER variants are expressed and translated into a large amount of proteins that result in different activities on antiestrogen resistance is not known (62).

The effects of different ER variants on ER-regulated gene transcription are likely to be complex. Some variant ERs such as ERΔE3 have been shown to be dominant negative ER mutants that substantially inhibit transcription at EREs, while others such as ERΔE5 can constitutively activate transcription at an ERE site in yeast (49). Even for ERΔE3, there is evidence that it can activate transcription at AP-1 site in transient transfection assays(63). While ER variants with exon deletions have been widely detected, other types of mutant ERs have also been observed. An ER mutant can use tamoxifen as an agonist in some MCF-7 cell derivatives (64). While most ER variants show decreased responses to estrogen, a mutation affecting the border of the hinge and LBD enabled increased sensitivity to estrogen compared to wild-type ERα. The ectopic expression of this ER variant increased cell proliferation in stably transfected breast cancer cells and enhanced TIF-2 binding at low levels of estrogen. In addition, a significant proportion (34%) of hyperplasias, a type of early premalignant lesion, had such mutations(65). Because most

cancers arise from premalignant lesions, the existence of such ER variants in those premalignant lesions indicates a possible importance of ER variants in the development of breast cancer. While some ER variants exhibit some functional properties in breast cancer cell lines, it is not known that whether these effects would occur in a significant proportion of breast cancer patients.

5. Estrogen receptor downstream signaling

5.1. Oncogenes and tumor suppressor genes regulated by estrogen

Because of the importance of oncogenes in carcinogenesis, the relationship between estrogen and oncogenes has been extensively studied. Oncogenes that are regulated by estrogens and antiestrogens include c-*myc, c-fos, and c-jun.* It was found that the transcription of c-myc and c-fos was directly regulated by estrogen in breast cancer cell lines(66). When antisense c-myc oligonucleotides were introduced into MCF-7 cells, the proliferation of the cells in the presence of estrogen was inhibited(67). Through ER, c-myc expression is regulated by estrogen and is also required for estrogen-induced proliferation.

5.2. Growth factors and growth factor receptors regulated by estrogen

Many growth factors and growth factor receptors have been identified to be involved in breast cancer initiation and development (68, 69). For example, Overexpression of the c-erb-B2 gene is detected in approximately 25% of human breast tumors (70). Increased IGF-II mRNA or protein has also been observed in breast cancers (71). In addition,

overexpression of EGF can induce MCF-7 cells to form tumors in ovariectomized nude mice. Although overexpression of growth factors is not essential to breast cancer development, antisense to EGF-R reduces the tumorigenicity of three breast tumor models (72).

It has been demonstrated that the expression of many growth factors and their receptors are regulated by estrogen. These estrogen-responsive growth factors include Epidermal Growth Factor (EGF), Transforming Growth Factors (TGFα, TGFβ), c-erb-B2, and Insulin-Like Growth Factors (IGF-I and IGF-II). The subsequent effects of these growth factors on cell proliferation are associated with growth factor receptors such as EGF receptors (EGFR) and IGF receptor (IGF-IR).

Presumably through ER, studies have shown that estrogen increases expression of growth factors. Increased levels of growth factors and/or decreased levels of growth factor inhibitors were found in estrogen treated breast cancer cells (73-77). Estrogen increases EGF-R expression in hormone responsive tissues*(78)*. In some cells, IGF-II expression can be induced by estrogen(79). The stimulatory effect of EGF on proliferation can be inhibited by tamoxifen (80, 81), suggesting the up-regulation of growth factors by estrogen is mediated through ER and could be reversible. In addition, cross talk exists between growth factors and estrogen-regulated genes. It has been shown that pS2, an estrogen-responsive gene, is up-regulated by TGF.

In conclusion, some of growth factors are up-regulated by estrogen. The interactions between those growth factors and other estrogen regulated genes are likely to be complex.

6. Possible cellular and molecular mechanisms of tamoxifen resistance

Understanding the mechanism of tamoxifen resistance is critical to the development of new drugs and to improving the treatment of breast cancer patients. Several possible mechanisms have been proposed to account for antiestrogen resistance including: changes in pharmacokinetics, abnormal ER functions, overexpression of growth factors or their receptors (82-84). This review will address recent studies on ER signaling and overexpression of growth factors.

6.1. Estrogen receptor and its variants

Many breast tumors that initially respond to tamoxifen eventually recur with acquired resistance. However, most tamoxifen resistant patients are still sensitive to ICI treatment, an alternative form of endocrine therapy. This indicates that the ER is still involved in promoting tumor growth(83). By immunohistochemical assay, it was found that ER expression was reduced, but still maintained, in tamoxifen resistant tumors(85).

ER can interact with the activator protein-1 (AP-1) transcription factor complex through protein-protein interactions that are independent of ER DNA binding and, in certain ER-positive cells, this may allow TAM to exert an agonist activity on AP-1-regulated genes. One AP-1 activating enzyme is c-Jun NH2-terminal kinase (JNK). In human breast tumors with acquired tamoxifen resistance, increased AP-1 DNA binding and JNK activity was observed(86).

Changes in ER function and subsequent signaling could also be mediated by ER mutants or variants, since such variants could affect the ability of tamoxifen to form

complexes and/or regulate the activity of ER. Various ER variants have been observed in cell lines selected in the presence of antiestrogens *in vitro*, as well as in tamoxifen resistant tumor samples(87). While some studies found that ER variants such as ERΔE3 and ERΔE5 were present at higher levels in tamoxifen resistant breast tumors, the ectopic expression of those variants alone failed to confer estrogen independence and/or tamoxifen resistance to estrogen-responsive breast cancer cells(57, 58).

It has also been speculated that the interaction between ERα and ERβ plays a role in tamoxifen resistance. ERα and ERβ can form heterodimers, and 4-OH TAM can activate transcription through these heterodimers in the context of specific promoters and cell types(88, 89). This suggests that with both ERβ and ERα present in breast cancer cells, 4-OH TAM could act as an agonist instead of an antagonist in terms of cell proliferation.

Interestingly, some estrogen independent and/or antiestrogen resistant cell lines still express wild-type ER at significant levels(64). In such cases, it was suggested that other factors, including ER coregulators and growth factors, might be involved in causing estrogen independence and antiestrogen resistance.

6.2. Estrogen receptor coregulators

The importance of coregulators in ER-mediated transcription suggests a hypothesis that changes in the expression or recruitment of coregulators could lead cells to tamoxifen resistance (40, 90). While the estrogenic activity of 4-OH TAM can be increased by coactivators such as L7/SPA (43), it can be inhibited by corepressors such as SMRT (40). In tamoxifen resistant MCF-7 xenografts in nude mice, the expression of N-CoR, an ER corepressor, was reduced(91). These findings suggest that both the

elimination of corepressors and recruitment of coactivators could confer tamoxifen resistance to cells. However, there is also evidence that is contradictory to this hypothesis. Studies have shown that overexpression of SRC-1 in MCF-7 cells does not induce changes in their response to 4-OH TAM(92). A close examination of several tamoxifen resistant cell lines and a number of tamoxifen resistant human breast tumors revealed no change in the expressions of TIF-1, RIP 140, or SMRT (93). The effect of ER coregulators on tamoxifen resistance therefore still needs to be further studied.

6.3. Growth factors

Changes in the signaling pathways that regulate cell proliferation and apoptosis could also influence ER-regulated gene networks and confer tamoxifen resistance on cells. As discussed previously, growth factors can affect ER function and participate in the ER-regulated gene network. The changes in growth factor expression and signaling can reduce the requirement for estrogen stimulation of cells to proliferate, giving cells phenotypes such as estrogen independence and/or tamoxifen resistance. In such cases, estrogenic signaling pathways from the ER could still remain intact in those cells.

There is abundant evidence that growth factors are involved in the development of estrogen independence and tamoxifen resistance. The initial evidence of growth factor involvement in tamoxifen resistance came in the 1980's. In several hormone-independent breast cancer cell lines, a number of growth factors were expressed at high levels and were no longer regulated by hormones(69, 76). Subsequently, a variety of growth factors have been examined in tamoxifen resistant cell lines and tumors. TGF-α was found to be constitutively expressed in many estrogen-independent cells (76, 94).

Estrogen-independent breast cancer cell lines also expressed high levels of EGF-R relative to hormone-dependent cells (95, 96). IGF-I, TGF-α, and PDGF were found to be secreted constitutively by hormone- independent human breast cancer cells and their activities can induce the formation of estrogen-independent tumors in MCF-7 cell-xeografted ovariectomized athymic nude mice (80). Some researchers have reported an increase in the levels of IGF-II in tamoxifen treated patients(97, 98).

Studies on the effects of growth factors on tamoxifen resistance have either overexpressed growth factors or inhibited growth factor functions. While overexpression of some growth factors or growth factor receptors confers estrogen independence and/or tamoxifen resistance, others fail to change the phenotype of the cells. Overexpression of TGF-β has been found in human breast tumors resistant to tamoxifen. By introducing TGF-β antibodies into tamoxifen resistant breast cancer cells, it was found that the cells lost their resistance to tamoxifen when inoculated into nude mice(99). Overexpression of EGF and IGF-I partially reverse the growth inhibitory effects of antiestrogens (100). Overexpression of IGF-I receptor moderately enhanced the growth responsiveness to estrogen and reduced the estrogen growth requirement in MCF-7 cells(101). In addition, overexpression of *c-erb-B2* enabled estrogen responsive breast cancer cells to acquire estrogen-independent growth and reduced responsiveness to tamoxifen (102-104). On the other hand, the ectopic expression of TGF-α failed to induce hormone-independence in MCF-7 cells(69). However, this does not exclude the possible roles of these growth factors in the development of estrogen independence. Other recent studies provide more evidence supporting this statement. It has been shown that by introducing antisense TGF-α oligonucleotide into estrogen-responsive breast cancer cells, the estrogen

responses in those cells were reduced (105, 106). Overexpression of TGF-α was observed in several tamoxifen resistant cell lines (99, 107), but introducing TGF antibodies into those cells could not restore their tamoxifen responsiveness (99).

7. In vitro models of tamoxifen resistance—The LCC series

The development of estrogen-independent cell lines has been widely used as laboratory model to study the problem of clinically antiestrogen resistance clinically. For estrogen-responsive cell lines, MCF-7, ZR-75-1 and T47D are commonly used. MDA-MB-231 and MDA-MB-435 are ER negative cell lines. Studies using these cell lines have provided much understanding of the mechanisms of how hormones regulate cell proliferation.

My research utilized the MCF-7, LCC1, and LCC2 cell lines. MCF-7 cells were originally isolated from a malignant pleural effusion in a postmenopausal breast cancer patient, and have been widely used in breast cancer research. MCF-7 cells are estrogen dependent and antiestrogen sensitive. Dr. Robert Clarke at the Vincent T. Lombardi Cancer Center developed a series of MCF-7 derivatives which mimic the development of estrogen-independent and antiestrogen resistant tumor growth observed in human breast cancer patients. MCF-7 cells were inoculated into ovariectomized athymic nude mice and selected *in vivo* for estrogen-independent growth. The circulating steroid hormone profile of these mice is similar to that seen in postmenopausal women. LCC1 cells were isolated and characterized as estrogen independent but antiestrogen sensitive. LCC1 cells mimic some of the critical aspects of the early progression to a more aggressive tumor type--

Estrogen and progesterone receptor positive, steroid non-responsive, and increased metastatic potential(108). LCC1 cells were further selected in vitro for growth in the presence of triphenylethlene tamoxifen or ICI 182,780. LCC2 cells were isolated as tamoxifen resistant but ICI sensitive(109), and LCC9 cells as both ICI and tamoxifen resistant(110). The characteristics of LCC1, LCC2, and LCC9 cells were assessed, including their *in vitro* proliferative capacity, *in vivo* tumorgenicity, and competitive binding activities of steroid hormone receptors (109, 111). LCC1 cells proliferate well in the absence of estrogen, but still sensitive to tamoxifen. LCC2 cells can proliferate in an anchorage-independent manner and have significantly increased ability to form tumors in nude mice in the presence of tamoxifen. While the proliferation of LCC2 cells is inhibited by ICI, there is no inhibitory effect of ICI on the proliferation of LCC9 cells. Both ER and progesterone receptor are present and are regulated by estrogen in MCF-7, LCC1, LCC2, and LCC9 cells. LCC9 cells have a significantly elevated PR level compared to other cell lines. The fact that the growth of LCC1 and LCC2 cells can still be further stimulated by estrogen and estrogen-regulated genes such as pS2 are still regulated by estrogens and antiestrogens suggests the existence of functional ER in these cells. The establishment of LCC1 and LCC2 cells is the first case of tumor formation from MCF-7 cells grown *in vivo* without estrogen (112). Especially, the phenotype of LCC2 cells (TAM resistant, but ICI senstitive) mimics a clinical pattern of tamoxifen resistant patients that have undergone endocrine therapy. The pattern of metastasis exhibited by these cells is very similar to that observed in breast cancer patients, and hence these cells can be used as a model for the study of antiestrogen resistance.

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CHAPTER 2

Impaired ER Function in Estrogen Independent and/or Tamoxifen Resistant Human Breast Cancer Cell Lines

Introduction

Breast cancer is the most common type of cancer in women, with an annual worldwide incidence of one million new cases. Studies have shown that estrogen is essential for the initiation and progression of breast tumors (1, 2). Based on the pivotal role of estrogen, antiestrogens have been developed as major drugs for the treatment of breast cancer patients. Among them, tamoxifen is the most commonly used endocrine therapy in hormone-responsive breast cancer, especially as adjuvant therapy after removal of the primary tumor(3). The application of tamoxifen clinically has proved very effective in both decreasing disease progression and reducing the mortality rate of breast cancer. Worldwide clinical trials indicate that the 5- and 10-year mortality rates of breast cancer patients can be reduced 20-25% by tamoxifen treatment (4). Nevertheless, many breast tumors that initially respond to endocrine therapy become estrogen independent. In addition, selective growth pressure gives rise to tamoxifen resistance in many patients under tamoxifen treatment. In fact, the development of tamoxifen resistance in advanced breast cancer is a primary reason for the failure of current breast cancer therapies.

Estrogen functions by binding to the estrogen receptor (ER), which has been shown to play an important role in the development of breast cancer. Studies have shown that nearly 70% of primary breast cancers have measurable ER expression, while only 7% of the epithelial cells in normal breast tissue have detectable ER expression (5). There are

two isoforms of ER, ERα and ERβ (6). In breast tissue, most work has been done towards clarifying the mechanisms by which ERα mediates estrogen's growth stimulatory effects. In the absence of estrogen, ER exists in a complex with several heat shock proteins in the nucleus. When bound to estrogen, ER undergoes an activating conformational change, and dissociates from this complex (7). Currently, there are two known modes of ER function. In the "classical mode", estrogen binds to the ligand-binding domain and induces ER dimerization. Dimerized ER binds to estrogen response elements (EREs) in the 5' promoter region of target genes, and activates transcription of these genes (8). The second mode does not require ER dimerization or DNA binding by ER. After estrogen binds to ER, the complex can activate transcription by protein-protein interaction with other transcription factors like c-Fos and c-Jun at AP-1 sites (9).

The ER gene has eight coding exons, which generate the 6.2 kb wild-type ER mRNA. Aberrant mRNA splicing can produce ER variant mRNAs with various exon deletions(10-18). The significance of these ER variants has been investigated for both diagnostic purposes and for understanding antiestrogen resistance. The role of ER variants in the development of breast cancer or antiestrogen resistance has been controversial. By RT-PCR, variant ER mRNAs have been detected in normal breast tissue, breast cancer cell lines, and clinical tumor samples. Although there were a small proportion of samples where ER variant proteins were produced, whether the majority of the ER variants are expressed and translated into protein is not known (15). Some researchers showed the presence of ER splice variant mRNAs in more than 30% of breast tumors and the prevalence of certain variants increased with the emergence of tamoxifen resistance, while others failed to observe any evidence for ER variants in *in vitro* derived

tamoxifen resistant cell lines (18-20). Among the ER variants detected, one of the most commonly seen is an exon 3 deletion (ERΔE3). Exon 3 encodes the second zinc finger of the DNA binding domain. The ERΔE3 protein cannot bind to EREs and therefore cannot activate ERE activity in transient transfection assays. When ERΔE3 was mixed with wild type ER at a 1:1 ratio, ERE activity was inhibited by 30%, suggesting that ERΔ3 functions as a dominant negative ER variant (17). Furthermore, ectopic expression of ERΔE3 in estrogen responsive human breast cancer cells suppressed their *in vivo* invasiveness (21).

Understanding the mechanism of tamoxifen resistance is critical to the development of new drugs and to improvements in the treatment of breast cancer patients. Tamoxifen acts as a competitive inhibitor of estrogen by binding to ER. Several possible mechanisms have been proposed to account for antiestrogen resistance including: loss of ER, loss of ER cofactor(s), abnormal metabolism of tamoxifen, mutant ER, and overexpression of growth factor receptors (22-24). Various ER mutations have been observed in some of the cell lines selected *in vitro* for resistance to antiestrogens, as well as in tumor samples (25, 26). However, many estrogen independent and/or antiestrogen resistant cell lines still express wild-type ER at significant levels (27), suggesting that other mechanisms of resistance might exist.

The development of estrogen-independent cell lines has been used as a laboratory model to study the problem of antiestrogen resistance. This study will utilize the MCF-7, LCC1, and LCC2 cell lines. MCF-7 cells were originally isolated from a malignant pleural effusion in a postmenopausal breast cancer patient, and have been widely used in breast cancer research. MCF-7 cells are estrogen dependent and antiestrogen sensitive.

Dr. R Clarke et al at the Vincent T. Lombardi Cancer Center developed a series of MCF-7 derivatives, which mimic the development of estrogen-independent and antiestrogen resistant tumor growth observed in human breast cancer patients (28-31). MCF-7 cells were inoculated into ovariectomized athymic nude mice and selected *in vivo* for estrogen-independent growth. LCC1 cells were isolated and characterized as estrogen independent but antiestrogen sensitive. Then, LCC1 cells were selected in vitro for growth in the presence of tamoxifen, giving rise to LCC2 cells. LCC2 cells are tamoxifen resistant, but still sensitive to the steroidal antiestrogens such as ICI 182,780. Among these MCF-7 derivatives, we are particularly interested in LCC2 cells because their phenotype is similar to those of breast cancer patients who develop tamoxifen resistance during endocrine therapy. This study was aimed at identifying the biochemical changes occurring during the development of estrogen independence and tamoxifen resistance in these cell lines.

Materials and Methods

Cell culture

MCF-7 cells were obtained from Dr. Michael Johnson. LCC1 and LCC2 breast cancer cells were obtained from Dr. Robert Clarke (Lombardi Cancer Center) and were maintained in IMEM/FBS, i.e. IMEM media with 2mM glutamine (Biofluids, Inc., Rockville, MD) supplemented with 5% fetal bovine serum (Hyclone), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Life Technologies). To study the effects of estrogen and antiestrogens, cells were depleted of estrogen by plating in IMEM/CSS, i.e. IMEM without phenol red and supplemented with 5% of charcoal-stripped fetal bovine serum (Hyclone) (32).

Reagents and plasmids

17ß-estradiol (E) and 4-hydroxytamoxifen (4-OH TAM) were purchased from Sigma. ICI 182,780 (ICI) was purchased from AstraZeneca. ERΔE3 and wild-type ER expression vectors (pCDNA3-ERΔE3 and pCDNA3-wtERα) were constructed by insertion of ERΔ3 and wild type ERα cDNAs into a G418 selectable plasmid vector containing CMV promoter, pCDNA3 (Invitrogen). pßgal-Basic was purchased from Clontech Laboratories. Lipofectin were purchased from Life Technologies. The ERE-luciferase reporter construct, ERE2-tk109-luc, was obtained from Dr. Gehm at Northwestern University Medical School (33).

Primers and PCR conditions

The primers used for RT-PCR were (5'-CTGCCAAGGAGACTCGCTAC-3') as the upstream primer and (5'-AAGGCACTGACCATCTGGTC-3') as the downstream

primer. The primers used for genomic PCR were (5'-CGCTCGAGTGGGGTGCAACGTAGTAAGA-3') as the upstream primer and (5'-GCGAATTCCAATGGGTAGAGCCAG-3') as the downstream primer. PCR reactions were performed under the following conditions. Five μg of total RNA and 10 pmol of the downstream primer were denatured by incubation for 10 min at 65°C. Twenty μl of reverse transcription mixture (50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM MgCl2, 1 mM DTT, 50 μM dNTPs, 200 units of MMLV-Reverse Transcriptase (Life Technologies)) were incubated for 1 h at 37°C. Four μl of reverse transcription mixture (or 1 μg of genomic DNA) was amplified in a final volume of 100 μl containing 250 nM of each primer, 200 μM dNTPs, 1.5mM $MgCl_2$, and 2.5 units of TAQ DNA polymerase (Life Technologies). Each RT-PCR consisted of 35 cycles (60 s at 62°C, 60 s at 74°C, and 30 s at 94°C). Each genomic PCR consisted of 40 cycles (60 s at 58°C, 60 s at 74°C, and 60 s at 94°C). PCR products were visualized on agarose gels stained with ethidium bromide. The DNA fragments of interest were then recovered and purified using GenElute Agarose Spin Column (Sigma), and sequenced at the Michigan State University Sequencing Center.

Western blotting

Cell extracts were prepared as follows. Cell monolayers (80% confluent) were washed twice in ice-cold PBS and collected by scraping and centrifugation. Cell pellets were resuspended in ice-cold Lysis Buffer (50 mM HEPES, 150 mM NaCl, pH 7.5, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 10 mM β-glycerophosphate, and 1 mM NaF) and

lysed by sonication on ice (Duty cycle 80%, output 6, 10 s) using a Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT). The cellular debris was removed by centrifugation (12,000g, 2 min, 4°C). The total protein concentration in the cell lysate was measured using a Bradford Assay (Bio-Rad, Inc). Twenty μg of total protein from each sample was separated on a 12% polyacrylamide gel by SDS-PAGE and then transferred to PVDF membrane (NEN Life Science Products) in transfer buffer (25 mM Tris, 192 mM Glycine, and 15% methanol) using the XCELL gel transfer system (Novex Experimental Technology). Western analysis of ER utilized a mouse monoclonal antibody (Mab-17) that was raised against recombinant ERα protein and which recognizes an amino-terminal epitope present in both wild-type ER and ERΔE3. The membrane was blocked in 5% dry milk in PBST (PBS + 0.1% Tween 20) and incubated with a 1:2 dilution of the ER antibody in PBS at 4°C overnight. The antibody was removed and the membrane was washed with PBST three times for 10 min each. A peroxidase-labeled secondary antibody (American Qualex, San clemente, CA) was diluted 1:2000 in 5% dry milk in PBST and incubated with the membrane for 1 h at room temperature. Proteins were then visualized using Supersignal West Pico Chemiluminescent Substrate (Pierce).

Transient transfection and luciferase assay

Cells were plated in IMEM/CSS at 4×10^5 cells per 60 mm tissue culture plate, and incubated for two days to deplete them of estrogen. Five μg of ERE2-tk109-luc and 1 μg of pßgal-Basic were then co-transfected using Lipofectin (Life Technologies) as the transfection agent. Cells were incubated with Lipofectin-DNA complexes in phenol red-

free IMEM media for 6 h. Media containing Lipofectin and DNA was removed, and cells were washed twice in PBS and then incubated in IMEM supplemented with 5% CSS with or without E (10^{-9}M), 4-OH TAM (10^{-6}M), or ICI (10^{-9}M). Treatments were added as stock solutions in absolute ethanol, and ethanol was added to control media to the same final concentration (0.1%) in all plates. Eight h after treatment, cells were washed twice with PBS and harvested by scraping and centrifugation. Cell pellets were resuspended in 200 µl of Reporter Lysis Buffer (Promega Corp., Madison, WI) and lysed by freeze-thawing. The protein concentrations in the extracts were determined using the Modified DC Assay (Bio-Rad). Aliquots of the lysate were used to assay ß-galactosidase activity according to manufacturer's manual (34). Briefly, cell lysate was incubated with Reaction Buffer Mixture (Clontech Laboratories) at room temperature for 1 h, and the ß-galactosidase activity was determined by measuring the light emitted from the mixture using a Turner TD-20e luminometer. The luciferase assay was carried out with 20 µl of lysate and 100 µl of Luciferase Assay Reagent (Promega). The light emitted was measured for 10 s using a Turner TD-20e luminometer. The final luciferase activity was expressed relative to the β-galactosidase activity in the same extract, and is shown as means ± SD of three independent transfection experiments done in triplicate.

Stable transfections

Stable cell lines were obtained by transfecting MCF-7 and LCC1 cells with pCDNA3-ERΔ3 and pCDNA3-wtERα by Lipofectin. Cell lines were isolated in medium containing 400 µg/ml G418. Single colonies were isolated by ring cloning and serial dilution in 96-well dishes. Cells were subsequently maintained in medium containing 40

ug/ml G418. Clones expressing ERΔE3 or wild-type ER were identified by Western blotting of cell extracts.

Cell growth assay (soft agar assay)

Cells were plated in phenol red-free IMEM with 5% CSS for two days and then re-plated at a density of $5X10^4$ cells/well in soft agar in 6-well dishes. Briefly, cells were suspended in IMEM with 5% CSS containing various treatments and 0.3% Agar Nobel (Difco Laboratories, Detroit, MI), and plated on the top of IMEM with 5% CSS containing treatments and 0.6% Agar Nobel. Cells were grown for 21 days, with feeding every 5 days. Colonies were then stained with 0.1% neutral red and analyzed by microscopy. Colonies larger than 60 μm (more than 50 cells) were counted, and six independent wells per treatment per sample were averaged. The results were expressed as mean +/- standard deviations.

RNA extraction and northern blot analysis

RNA was isolated using Trizol reagent and quantified by spectrometry. Twenty μg of total RNA was separated on 1.0% agarose/formaldehyde gels and transferred overnight to nitrocellulose membrane (Schleicher & Schuell). Membranes were prehybridized in prehybridization buffer (50% formamide, 5% dextran sulfate, 50 mM $NaPO_4$, pH 6.8, 0.1% SDS, 3×SSC, and 5×Denhardt's Solution) with 100 μg/ml sheared salmon sperm DNA at 42°C for 2 h. Membranes were then hybridized to 10^7 dpm of ^{32}P-labeled c-*myc* cDNA probe. Blots were hybridized at 42°C for 16–24 h. After hybridization, blots were subjected to two washes (0.2 x SSC, 0.1% SDS) at 42°C for 10 min each. Signal

intensities were measured using the PhosphorImager system (Molecular Dynamics), and blots were exposed to X-ray film at -80°C overnight. To normalize, blots were stripped and probed with labeled human GAPDH.

Results

ER activities in MCF7, LCC1 and LCC2 cells

To identify the biochemical changes that are responsible for the estrogen independence and/or tamoxifen resistance of LCC1 and LCC2 cells, their ER activities were compared to that in MCF7 cells (Figure 2.1). MCF-7, LCC1, and LCC2 cells were plated in medium containing 5% charcoal stripped serum (CSS) for two days to deplete cells of estrogen. An ERE-luciferase plasmid construct was then transiently transfected into cells along with a control β-galactosidase construct. After transfection, cells were treated with E, 4-OH TAM, or ICI for 6 h, harvested and analyzed for luciferase activity as described in Materials and Methods. The luciferase activities, normalized to β-galactosidase activity, are shown in Figure 1. In all three cell lines, ER activity was up-regulated by E and down-regulated by 4-OH TAM and ICI, suggesting that the estrogen independence of LCC1 cells and the tamoxifen resistance of LCC2 cells are not due to changes in ER function. Interestingly, although ER was still responsive to E, 4-OH TAM, and ICI, the maximum ER activity in LCC2 cells was 4-5 fold lower than that in MCF-7 and 3-4 fold lower than that in LCC1 cells, suggesting some defect in ER levels or function.

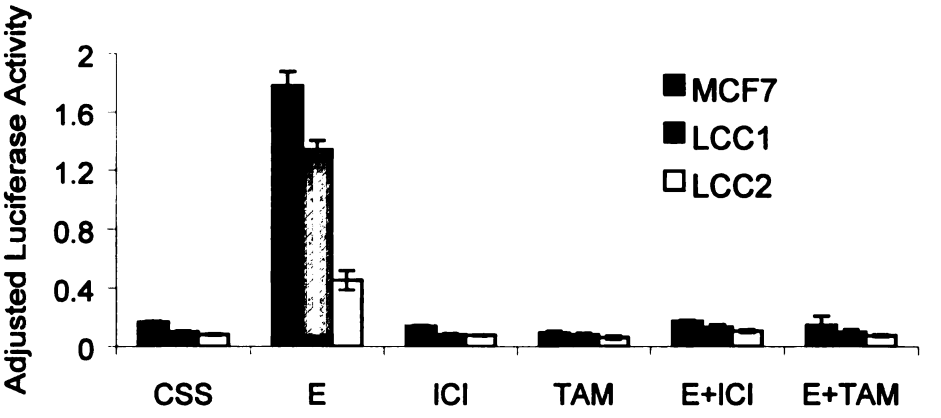


Figure 2.1 Decreased ER function in LCC1 and LCC2 cells. ER function was assessed by transient transfection of an ERE-luciferase construct into MCF-7 and LCC2 cells along with pßgal-Basic as a control. Cells were treated with E (10^{-9}M), 4-OH TAM (10^{-6}M), or ICI (10^{-9}M). Six h later, cells were harvested and luciferase and β-galactosidase activities were then measured. The Adjusted Luciferase Activity was luciferase activity divided by β-galactosidase activity.

Identification of ERΔE3 in LCC1 and LCC2 cells

To investigate the reason for the decreased ER activity in LCC2 cells, MCF-7, LCC1 and LCC2 cells were plated in IMEM/CSS for two days and then treated with E, 4-OH TAM, or ICI. After forty-eight h, cells were harvested and cell lysate was analyzed by western blotting using an antibody against an N-terminal epitope of wild-type ER (Figure 2.2). The regulation of ER expression in the three cell lines followed the same pattern; it was up-regulated in the absence of E or presence of 4-OH TAM, and down-regulated in the presence of E or ICI. Notably, an ER variant of approximately 61 kD was detected at significant levels in LCC1 and LCC2 cells. In LCC2 cells, the protein level of the 61 kD ER variant was comparable to that of the full length wild-type ER. The expression of the 61 kD ER variant was regulated by E, 4-OH TAM and ICI in the same way as that of wild type ER.

The existence of the ER variant in LCC1 and LCC2 cells posed the interesting question of whether the increase in its amount had anything to do with the phenotypes of these cells, estrogen independence and tamoxifen resistance, respectively. To further investigate the function of the ER variant in LCC1 and LCC2 cells, it was first characterized molecularly. Based on the size of the protein, it was suspected to arise from a deletion of exon 3. To test this hypothesis, a primer set was designed with the upstream primer located in exon 2 and the downstream in exon 4. Total RNA was isolated from MCF-7 and LCC2 cells, and RT-PCR was performed. In parallel, PCR reactions were carried out with cDNA controls for both wild-type and Δ3 ERs (Figure 2.3A). In addition to the 420 bp fragment predicted from wild type ER mRNA, a 320 bp fragment was amplified from LCC2 mRNA. Both fragments were present in roughly equal amounts.

The 320bp fragment was then purified and sequenced. The sequencing results confirmed the hypothesis that the 61 kD variant present in LCC1 and LCC2 cells was an ER exon 3 deletion, ERΔE3 (Figure. 2.3B). One possible mechanism for the generation of the ERΔE3 mRNA found in LCC2 cells would be a splice site mutation in the genomic DNA. In order to test for such a mutation, genomic PCR was carried out in MCF-7 and LCC2 cells using a primer set with the first strand primer located in intron 2 and the second in intron 4. The resulting 313 bp fragment was then purified and sequenced. No genomic DNA mutations were observed within 122 bp upstream or 77 bp downstream of the region encoding exon 3 in either MCF-7 or LCC2 cells (Data not shown).

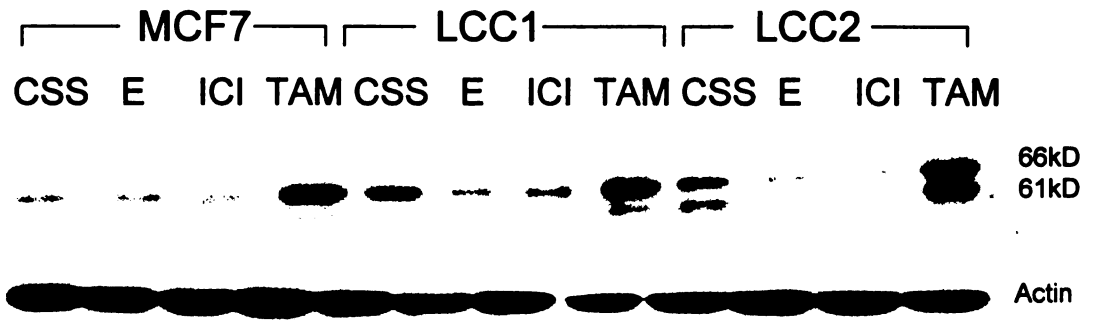


Figure 2.2 Expression of ER in MCF-7 derivatives and identification of a 61 KD ER variant in LCC2 cells. MCF-7, LCC1, and LCC2 cells were treated with E (10^{-9}M), 4-OH TAM (10^{-6}M), and ICI (10^{-9}M) for 24 hours. Expression of ER was analyzed by Western blotting using an ER antibody.

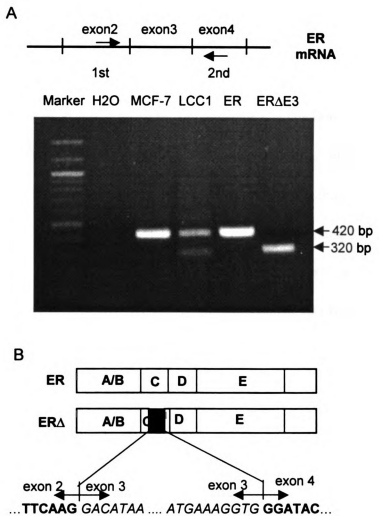


Figure 2.3 Identification of ERΔ3 expression in LCC2 cells. A. Total mRNA from MCF-7 and LCC2 cells were purified. RT-PCR using primers as indicated was performed with ER and ER Δ3 cDNAs as controls. B. PCR products (420bp and 320bp fragments) were purified and sequenced. The results were illustrated using a diagram.

Ectopic expression of ERΔE3 in stably transfected MCF-7 and LCC1 cells

The increased amount of ERΔE3 in LCC1 and LCC2 cells led us to investigate whether this variant might play a role in the phenotype of the two cell lines, estrogen independence and tamoxifen resistance respectively. The ERΔ3 cDNA was inserted into a pCDNA3 vector, and pCDNA3- ERΔE3 was obtained. The ability of this construct to express ERΔE3 was confirmed by transient transfection into COS-7 cells followed by western analysis of the cell lysate (Data not shown). pCDNA3- ERΔE3 or pCDNA3-wtERα were then transfected into MCF-7 and LCC1 cells, and stable transfectants were selected in G418. Stably transfected cell clones expressing ERΔE3 or wild type ER were identified using RT-PCR and western blotting. Early passages of three independent clones of MCF-7 and LCC1 expressing ERΔE3 are shown in Figure 2.4A. To determine if these transfected MCF-7 and LCC1 cell lines maintained expression of ERΔE3 in the long term, they were cultured in the presence of G418 for more than 15 passages. Cells were then harvested, and cell lysates were analyzed for ER expression by western blotting (Figure 2.4B). While the transfected MCF-7 cells lost ERΔE3 expression by passage 10, the transfected LCC1 cells maintained expression of ERΔE3 for at least 17 passages. This indicates that there is a difference in the ability of MCF-7 and LCC1 cells to tolerate expression of ERΔE3.

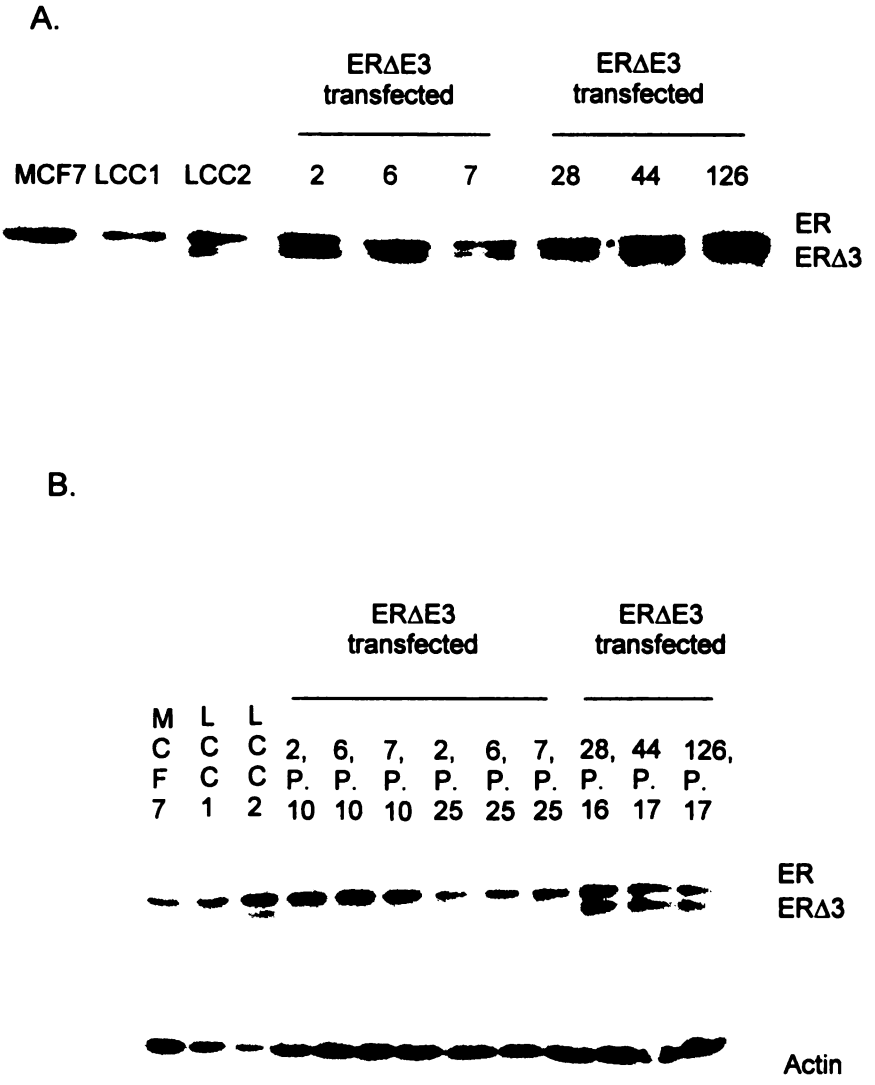


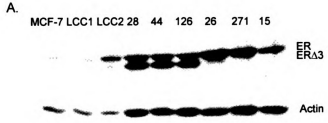
Figure 2.4 A.Verification of ERΔE3 expression in pCDNA3-ERΔE3 transfected MCF-7 and LCC1 cells. B. Loss of ERΔE3 expression in stably transfected MCF-7 cells and sustained expression of ERΔE3 in stably transfected LCC1 cells. Cells were grown in IMEM/FBS. ERΔE3 expression was analyzed by Western blotting using an ER antibody. 2, 6, 7 were three independent pCDNA3-ERΔE3 transfected MCF-7 cell clones. 28, 44, 126 were three independent pCDNA3-ERΔE3transfected LCC1 cell clones. Their passages were indicated above.

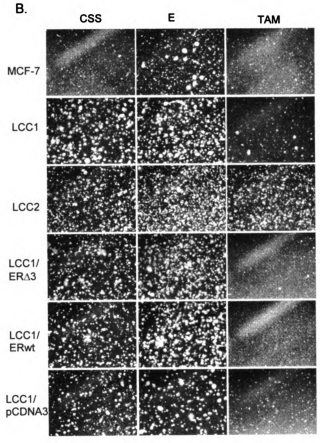
Anchorage-independent proliferation of LCC1 cells expressing ERΔE3

To investigate whether ectopic expression of ERΔE3 would lead to tamoxifen resistance in LCC1 cells, the ability of the stably-transfected cells to proliferate in the presence of tamoxifen was examined. Control cells transfected with a wild-type ER construct were also examined, as were untransfected MCF-7, LCC1, and LCC2 cells. The expression of both ERΔE3 and wild-type ER were confirmed by western blotting of cell lysates (Figure 2.5A). Due to the fact that the phenotypes of MCF-7, LCC1, and LCC2 cells are not obvious on plastic, soft agar assays were used to assess the ability of the transfectants to show anchorage-independent proliferation. In these assays, cells were plated in IMEM/CSS for two days, and then suspended in 0.3% agar containing 1×DMEM/F12 and 5% CSS with or without E or 4-OH TAM. The cells were then incubated for 21 days, during which time they were fed every 5 days. The ability of cells to form colonies in soft agar was interpreted by microscopy (Figure 2.5B) and direct counting of colonies (Figure 2.5c).

As shown in Figure 2.5, MCF-7 cells only formed large colonies in the presence of E. LCC1 cells formed colonies both with and without E, but not in the presence of 4-OH TAM. LCC2 cells formed colonies in the absence of E and the presence of 4-OH TAM. The ability of these cells to form colonies in soft agar clearly correlated with their reported phenotypes. The phenotype of LCC1 cells transfected with either pCDNA3-wtERα or pCDNA3- ERΔE3 was unchanged; that is, they formed colonies in the absence of E, but not in the presence of 4-OH TAM. These results indicate that ectopic expression of ERΔE3 is not sufficient to confer tamoxifen resistance to LCC1 cells.

Although MCF-7 cells were not able to sustain long-term expression of ERΔE3, the effect of ERΔE3 expression on the anchorage-independent proliferation of early passage transfectants was examined. The initial results of these soft agar assays suggested that ectopic expression of ERΔE3 abolished the ability of MCF-7 cells to proliferate in soft agar in the presence of E (data not shown). However, this experiment could not be repeated because expression of ERΔE3 was not sustained during cell passage. However, they suggest that the expression of ERΔE3 had a negative impact on the proliferation of MCF-7 cells, and therefore exerted a selective pressure towards its own elimination.


A.
MCF-7 LCC1 LCC2 28 44 126 26 271 15
ER
ERΔ3
Actin


B.
CSS
E
TAM
MCF-7
LCC1
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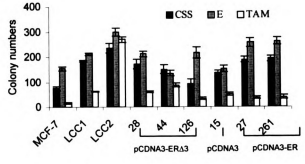
LCC1/ ERwt
LCC1/ pCDNA3


C.
400
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Colony numbers
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MCF-7
LCC1
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28
44
126
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261
pCDNA3-ERΔ3
pCDNA3
pCDNA3-ER

Figure. 2.5 A. Verification of ER and ERΔE3 expression in stably transfected LCC1 cells. Cells were grown in IMEM/FBS. Expression of ER and ERΔE3 was analyzed by Western blotting using an ER antibody. 28, 44, and 126 were three independent pCDNA3-ERΔE3 transfected LCC1 cell clones. 26 and 271 were two independent pCDNA3-wtERα transfected LCC1 cell clones. 15 was pCDNA3 transfected LCC1 cell clone. B, C. Effect of ER and ERΔE3 expressions on the anchorage independent proliferation of LCC1 cells. LCC1/ERΔE3 represents data from Clone 28, which is similar to Clone 44 and Clone 126. LCC1/ERwt represents data from Clone 26, which is similar to Clone 271. LCC1/pCDNA3 represents Clone 15's data. Cells were plated in IMEM/CSS for two days and then grown in 0.3% soft agar with or without E (10^{-9}M) and 4-OH TAM (10^{-6}M) for 21 days. Colonies larger than 60 μm (more than 50 cells) were analyzed using microscopy and counted, and six independent wells per treatment per sample were averaged. The results were

Discussion

In this study, we have identified the presence of an ER variant, ERΔE3, in a series of MCF-7 derivatives (LCC1 and LCC2 cells) that are estrogen independent and/or tamoxifen resistant. Although there have been reports of the presence of ER variant mRNAs in breast cancer cells, this is the first evidence that a significant amount of an ER variant is expressed at the protein level. Previously, the amount of ER variants in both breast tumor tissues and tamoxifen resistant cell lines has been limited to a level that could only be detected by sensitive techniques such as RT-PCR (10-18). This study demonstrates that an ER variant can be present in amounts comparable to wild-type ER in a tamoxifen resistant breast cancer cell line, i.e. LCC2 cells.

Previous studies have shown that a number of different ER variant mRNAs can co-exist both in normal and transformed estrogen target cells (12). This suggests that the existence of these ER variants generally does not result from mRNA splice site mutations. We have also shown here that the presence of the ERΔE3 in LCC1 and LCC2 cells is not a result of a simple splice site mutation. Our analysis does not, however, rule out the complete loss of exon 3 and surrounding sequences from one allele of the ER gene in LCC2 cells. Early evidence suggests that exon skipping is one of the likely causes of the presence of ER variant mRNAs in breast cancer cells (14), and our observations are consistent with that suggestion. In addition, the increasing amount of ERΔ3 from MCF-7 to LCC1 and LCC2 cells suggests that exon skipping could somewhat be regulated. How it is regulated and whether its regulation relates to estrogen independence and/or tamoxifen resistance still need to be investigated.

A second interesting observation is that the increasing amount of ERΔE3 from LCC1 to LCC2 cells correlates with decreased ERE responses to estrogen in these cells. ERΔ3 seems to act as a dominant negative ER in both LCC1 and LCC2 cells. This is consistent with previous results(17, 21, 35). Furthermore, since it cannot be maintained in stably transfected MCF-7 cells, ERΔE3 also seems to inhibit estrogen-dependent proliferation in these cells. This observation is also consistent with earlier studies (21). In contrast, ERΔE3 expression can be maintained in LCC1 cells. This suggests that a change has occurred in LCC1 cells that allows ERΔE3 expression and it is possibly the same change that confers estrogen independence. Such changes are likely to include genes that are involved in estrogen regulation. Some possible candidates could be ER coregulators and growth factors (24). The tolerance of ERΔE3 expression in LCC1 cells further indicates that ER function can be inhibited without interfering with proliferation at the same time. Surprisingly, LCC1 cells are still sensitive to tamoxifen, suggesting that they are not completely independent of ER function. A possible explanation could be that tamoxifen-bound ER has unique effects on regulation of genes responsible for cell proliferation. It could either suppress genes required for proliferation or activate genes that inhibit proliferation.

The ectopic expression of ERΔE3 alone does not confer tamoxifen resistance to either MCF-7 or LCC1 cells. It suggests that ERΔE3 alone cannot directly confer tamoxifen resistance to estrogen-responsive breast cancer cells. This is consistent with the results of other similar studies (21). However, the possibility of an indirect or contributory effect of increased ERΔE3 expression on causing tamoxifen resistance cannot be excluded. Perhaps the continued presence of ERΔE3 imposes a selective pressure on cells (even in

LCC1 cells) to become less dependent of ER function. Therefore, over time, cells that are tamoxifen resistant are favored.

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