ROLE OF ESTROGEN RECEPTOR IN GROWTH AND GENE EXPRESSION IN OVARIAN AND BREAST CARCINOMAS

by

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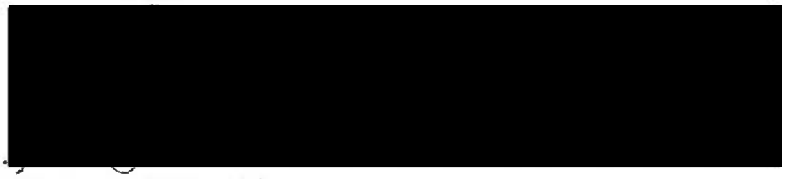

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Abstract

Estrogen is known to be important in growth and development of steroid responsive tissue, and is believed to play a critical role in the oncogenic behavior of breast tumors. Although estrogen receptor (ER) is expressed in ovarian cancer, the role of estrogen in this disease is not known. Unlike most breast cancer that is ER^+ and estrogen growth responsive, ovarian cancer is commonly ER^+ but estrogen independent for growth. In the current study, we described the molecular functions of estrogen on growth and gene regulation in SKOV3 ovarian carcinoma cells as a model for estrogen growth independent ovarian cancer. In comparison, we used the well-characterized estrogen growth responsive T47D breast carcinoma cells. Although the growth of SKOV3 cells was not affected by estradiol (E_2), ER exhibited apparent normal functions in ligand binding and nuclear association in response to E_2. However, the transcriptional regulation by estrogen was abnormal in SKOV3 cells. While E_2 did not induce expression of PR or of a stably transfected estrogen response element (ERE) driven reporter gene that required direct DNA binding of ER to the estrogen response element, the expression of the early growth response genes, c-*myc* and c-*fos* was induced. Thus, ER in SKOV3 cells did not function in ERE driven transcriptional activation, suggesting a defect in function of the DNA binding domain, but did function in transactivation of c-*myc* and c-*fos* expression, indicating that separate domains of ER were involved in estrogen mediated gene regulation. Early growth

response genes are critical in growth and differentiation of cells. However, in our study, we could not distinguish E_2 growth independent SKOV3 cells from E_2 growth responsive PEO4 cells by the extent of E_2 induction of c-*myc* and c-*fos*. This result suggested that induction of c-*myc* and c-*fos* was not sufficient to achieve mitogenic response in SKOV3 cells.

HER-2/neu is another critical factor in the oncogenesis of breast and ovarian carcinomas. In human breast and ovarian cancer, both HER-2/neu overexpression and loss of ER correlate with poor prognosis. These two factors may be mechanistically related, since E_2 treatment results in repressed HER-2/neu expression in estrogen responsive breast carcinoma cells and this repression is originally thought to be exclusively at mRNA level. However, in our studies that aimed at examining the mechanism of E_2 regulation of HER-2/neu in T47D breast carcinoma cells, we found that the repression of HER-2/neu at the protein synthesis level was significantly greater than the extent of repression at the mRNA level. In addition, repression of $p185^{HER\text{-}2/neu}$ was specific for E_2, because (a) this repression occurred in serum free medium; (b) another female steroid hormone, progesterone, had no effect on $p185^{HER\text{-}2/neu}$ expression; and (c) EGF receptor, a tyrosine kinase receptor that has a negative association with ER status in breast tumors was not modulated. Furthermore, modulation of $p185^{HER\text{-}2/neu}$ was cell type specific and was not observed in two ER^+ ovarian carcinoma cell lines. Thus the repression of $p185^{HER\text{-}2/neu}$ by E_2 was specific and was through different estrogen regulation mechanisms.

Introduction

I. Key players in growth and development of breast and ovarian cancers

Breast and ovarian carcinoma are both malignant tumors of female steroid responsive tissue. Breast carcinoma is the most frequent malignant tumor to occur in women, and one of the leading causes of death from cancer among women. Ovarian carcinoma on the other hand, are the most malignant tumors in women, which accounts for 4% of cancers and 6% of cancer deaths each year (1). Female steroid hormones, estrogen and progesterone, are two important factors believed to play a role in the growth and malignant progress of breast and ovarian cancer. Their receptors, estrogen receptor (ER) and progesterone receptor (PR) are detected in most breast and ovarian cancer (2-4), and are known as nuclear receptors that regulate the expression of a variety of genes, including growth regulatory proto-oncogenes. Estrogen has been shown to stimulate the growth of some ovarian and breast cancer cells cultured *in vitro* (5-11). In contrast, tamoxifen, an anti-estrogen, is used in endocrine therapy of some breast cancer patients but is ineffective in ovarian cancer patients (4,12).

In order to improve the treatment and prognosis of patients, a tremendous amount of effort has been made in the past decade to understand the key players and molecular mechanisms behind the development of breast cancer. Though little work has been conducted in ovarian cancer research, findings about breast cancer add very important information to the understanding of the possible role of

E_2 in the tumorigenesis of female steroid responsive tissue. Besides steroid hormone receptors, oncogenes and anti-oncogenes also play very important roles in the development of tumors. Proto-oncogenes are growth regulatory genes that may become oncogenes by point mutations, translocations, amplifications, and mechanisms that simply result in overexpression of their protein products. Such abnormalities in oncogenes are often found in cancer cells and experimental studies have implicated many oncogene abnormalities in carcinogenesis. (a) Growth factor *int*-2 and *hst*, which have homology with fibroblast growth factor (FGF), are amplified in some breast carcinomas (13). The expression level of other peptide growth factors, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) is also stimulated upon estrogen treatment in some breast cancer cells. These overexpressed growth factors are suggested to function as autocrine or paracrine factors in promoting tumor growth (14). (b) Overexpression of growth factor receptors, epidermal growth factor receptor (EGFR) and $p185^{HER\text{-}2/neu}$ that are members of the receptor tyrosine kinase family, are also detected in some breast and ovarian cancer, and are believed to be related to the hormone independence of tumors and to poor prognosis of patients (13,15-18). (c) Cytoplasmic signal transduction proto-oncogene Ha-*ras* and Ka-*ras* encode p21*ras*. Upon activation, p21*ras* delivers a signal to a yet unknown second messenger. Mutations and overexpression of the Ha-*ras*, and Ka-*ras* oncogenes have been identified in a number of human breast and ovarian cancers (19-22). (d) Nuclear immediate early genes are DNA binding

transcription regulators and are important factors in cellular growth control. Among them, c-*myc* is overexpressed in some breast cancers, but not in normal breast tissue (23,24). High levels of c-*myc* expression correlate with metastasis of breast tumors to lymph nodes, and predict poor prognosis of patients (24). Thus, abnormal functions of growth regulatory oncogenes at every step in signal transduction seem to be involved in the oncogenesis of malignant growth of breast cancer.

Besides oncogenes, mutations of tumor suppressor genes are other contributors in oncogenesis. Tumor suppressor genes represent part of the homeostatic balance in cellular growth control, offsetting or inhibiting the growth potentiating effects of oncogenes. Among them, the retinoblastoma (RB1) gene is located on the long arm of chromosome 13 (13q14), and p53 gene is located on the short arm of chromosome 17 (17p13) (25). In addition, the long arm of chromosome 17 (17q21) also appears to carry a breast tumor susceptibility gene that contributes to familial breast carcinoma and precancerous proliferative breast disease in young women (26,27). Furthermore, mutations of the tumor suppressor gene and frequent allelic loss on chromosome 17p and 13q in breast and ovarian cancer also suggest that tumor suppressor genes may play a role in the development of at least some ovarian cancer (28-32).

II. Relationship of ER and PR status in clinical findings of breast and ovarian cancer

A. Breast carcinoma

There are two general categories of breast cancer according to ER status: ER^+ and ER^-. ER is expressed in more than 60% of breast cancer (2,3). Among the ER^+ tumors, some are ER^+/PR^+, while others are ER^+/PR^- (3) and more than 60% of the ER^+ tumors respond to the anti-estrogen, tamoxifen. Statistics shows that cancer patients with ER^+, especially ER^+/PR^+ status generally have a better prognosis (3,33). However, patients with tumors that are ER^- rarely respond to anti-estrogen therapy (2,3,33,34) and their prognosis is less favorable than patients with tumors that are ER^+ (3,34).

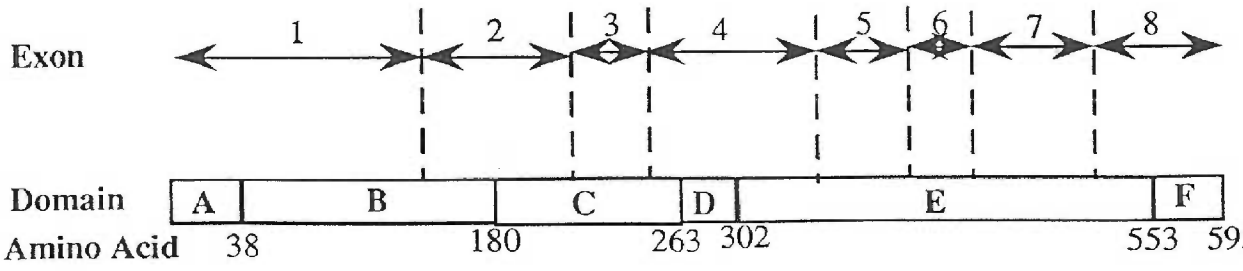
B. Ovarian carcinoma

ER and PR are present in normal ovaries, as well as in benign ovarian tumors. However in malignant ovarian tumors, the expression level of ER is generally higher and PR is generally lower as compared to that in benign tumors or in normal ovaries (35). Rao and Slotman summarize 21 separate studies of 1360 ovarian cancer patients and find that 62% of tumors are ER^+. Among the ER^+ tumors, 58% are ER^+/PR^+ (4). Another study conducted by Kauppila et al. shows that tumors in early stages (stage I & II) have higher levels of ER or PR than tumors in advanced stages (stage III & IV) (36). When the steroid hormone status and the proliferation rate of ovarian cancer are correlated, tumors that are ER^+ and/or PR^+ grow significantly slower than tumors that are ER^-/PR^- (37). The result of this study is supported by the observation that patients with ovarian cancer that is ER^+ especially ER^+/PR^+ have better prognosis than those that are

ER^- (38,39). Similar to the treatment of breast cancer, tamoxifen has also been used as one of the endocrine therapeutic drugs in ovarian cancer. However, unlike breast cancer that is ER^+, only about 15% of the ER^+ ovarian cancer patients respond to tamoxifen (40,41).

III. Domain Structure and Function of ER

Estrogen receptor belongs to a superfamily of related nuclear proteins, including receptors for glucocorticoid and progesterone, that act as ligand inducible transcription factors (42,43). The human ER (hER) gene has been cloned (44-46). The gene contains 8 exons that code for an mRNA of 6.6 kb, an a protein of 66 kDa (44). The hER protein can be divided into six structural domains (A-F), in a N- to C- direction, on the basis of sequence homology (43,47 with other receptors in the superfamily. hER consists of functionally independen domains: (a) Two transactivation domains that consist of A/B and E structural domains. (b) A DNA binding C domain. (c) A hinge domain containing domain D. (d) A hormone binding E domain that also functions in hormone induced dimerization with the participation of the F domain. (e) Multiple nuclear localization signals that distribute at C/D border and in the D, E domains (43,48,49).



A. Ligand binding domain (domain E)

The ligand binding domain of hER is coded by the part of hER gene that spans the 3' half of exon 4 through the 5' half of exon 8 (50). This domain is a complex region containing the hormone binding site (E domain), and a region required for a 'strong' ligand inducible homo-dimerization of the receptor (E/F domains)(51-53). In addition, this region contains a transcriptional activation function that is hormone inducible and synergizes with the transactivation activity of A/B region depending on the promoter context of target genes (54,55).

B. DNA binding domain (Domain C)

The DNA binding domain corresponds to exon 3 and part of both exon 2 and 4 of hER DNA (50,51). Structurally, this region contains two cysteine rich 'zinc fingers' which are involved in the specific binding of ER to DNA (56,57). It has been shown that ER modulates transcription of most of its target genes by binding with specific estrogen response elements (EREs). These EREs are perfect or imperfect palindromes of 5'-AGGTCAnnnTGACCT -3' that usually locate in the promoter region of the estrogen responsive genes (58). Among the EREs, the *Xenopus* vitellogenin ERE, which is a perfect palindrome of 5'-TCAGGTCACTGTGACCTGA-3' binds ER with the highest affinity (59,60). Besides ligand induced DNA binding, there also exists a 'weak' constitutive receptor dimerization function in the DNA binding domain (52,61) that is involved in ligand independent DNA binding (52,62). Furthermore, the DNA binding domain of ER is also suggested to be involved in protein-protein

interaction of ER with other transcriptional regulators (63).

C. Hinge domain (domain D)

The short hinge region of hER, located between the DNA and ligand binding domains, is coded by part of the exon 4 of the ER gene. Besides the hinge function, evidence indicates that this region may also play a role in the protein-protein interaction of ER with other transcription regulators as in the regulation of prolactin gene expression by estrogen (64).

D. Nuclear Localization signal

The unbound receptor is originally thought to be localized in the cytoplasm and translocate to the cell nucleus upon ligand binding (65). However, this classical concept is modified when intranuclear localization of the unbound hER is demonstrated (66,67). Three constitutive and multiple estrogen-inducible hER nuclear targeting sequences, proto-nuclear localization sequences (p-NLSs), are found in the receptor. The three constitutive p-NLSs are short lysine/arginine-rich motifs located at the C/D border and D domain, whereas the ligand-inducible p-NLSs are found in the hormone binding domain. As none of these p-NLSs individually is sufficient for nuclear targeting of the hER, the cooperative action of the multiple p-NLSs is important for nuclear localization (49). Thus, it is believed that the unbound ER is loosely associated with nuclei, and binding of its ligand leads to a tighter nuclear binding of the receptor (66,67)

E. Transactivation domains (domain A/B and E)

A transactivation domain is defined as a portion of the protein which when

combined with DNA binding activity, produces a change in transcription of the target gene. In hER, there are two regions that serve the transactivation function. The hormone dependent transactivation activity is located in the ligand binding domain (52,55,68). The hormone independent transactivation activity is located at the A/B region of the receptor which is coded by exon 1 and part of exon 2 of the hER gene (50). It seems that the function of the hormone independent transactivation activity can be inhibited by the hormone binding domain (HBD), since in the cases of glucocorticoid and progesterone receptors deletion of the HBD results in constitutive gene activation *in vivo* (69).

IV. Mechanisms of gene regulation by ER

A. Transcriptional and translational regulation by estrogen

ER is generally considered to exert its control of gene expression at the transcriptional level. ER is inactive *in vivo* in the absence of ligand under normal physiological conditions. However addition of hormone to cells results in a rapid transformation of the inactive receptor to an active state. This process, referred to as receptor activation, includes ligand induced receptor dimerization and increased DNA binding affinity (52,68,70). ER, once positioned on the correct promoter, functions as a transcriptional regulator through a yet not completely understood mechanism (71-73). Besides transcriptional regulation, a number of studies indicate that steroids may also regulate gene expression post-transcriptionally. Estrogen treatment decreases the translation rate from ER

mRNA and increases the translation rate from myelin basic protein mRNA in a cell free translation system (74). How estrogen regulates the translation of mRNA is not known. However, in a parallel experiment by using hydrocortisone, the effect on translational regulation by hydrocortisone can not be blocked by the glucocorticoid receptor (GR) antagonist RU38486. This indicates that post-transcriptional regulation by hydrocortisone may not involve the participation of classic GR (74).

B. Complexity of transcriptional regulation by ER

1. Receptors compete for common transcription factors

It is reported that introducing ER into Hela cells can inhibit PR induced transcription from a reporter gene in an ER dose- and estrogen- dependent manner, and does not appear to involve direct interaction between ER and either reporter gene or PR. In contrast, overexpression of PR has a similar inhibitory effect on ER induced transcription. Similar transcriptional interference of the transactivation function of transfected ER reporter gene occurs in T47D and MCF-7 cells by endogenous PR and ER (75). However, no ER-PR heterodimer has been detected. Interestingly, in an *in vitro* binding study, both ER and PR are reported to bind with transcription factor II-B (TFIIB)(76). Thus, the transcriptional activation by steroid receptors may also involve interaction with other general transcription factor(s). If this common transcription factor is limiting, a much higher concentration of one receptor would effectively compete with the other receptor.

2. Regulation of ER activity upon receptor phosphorylation

The best studied posttranslational modifications involved in regulating nuclear receptor activity are phosphorylation and dephosphorylation. Evidence has shown that an estrogen-dependent phosphorylation of serine residues of ER enhances its DNA binding and transcription regulation activity (77-79). Thus, phosphorylation and dephosphorylation of ER present another form of transcription regulation.

3. Communication with kinase pathways

ER is also involved in various signal transduction pathways. The growth factor, EGF, is reported to increase the binding of ER to vit-ERE sequence on a gel retardation assay. The EGF Stimulated DNA synthesis in mice can also be inhibited by anti-estrogen ICI164,384 (80). These results indicate that EGF can imitate the effect of estrogen in ER activation. In addition, estrogen synergistically regulates cell growth and gene expression with peptide growth factors or protein kinase activators (80-83). All these findings suggest that ER is involved in a complicated signal transduction system.

One mechanism of cross-talk involves protein-protein interaction that allows hormone-dependent repression, or activation of gene transcription through interaction of nuclear receptors and the transcription factor AP-1 (84). AP-1 is a protein complex mainly composed of c-*jun* and c-*fos* (85). Its activity is modulated by growth factors, cytokines, oncogenes, and tumor promoters that activate protein kinase C. AP-1 regulates transcription through interaction with a

specific DNA recognition sequence, the 12-O tetradecanoyl-phorbol-13 acetate (TPA) responsive element (TRE). The AP-1 binding site is recognized by *fos-jun* heterodimer, or *jun-jun* homodimer (86). It is reported that interaction between glucocorticoid receptor (GR) and *Fos/Jun* complex inhibits the basal and TPA induced, AP-1 mediated collagenase expression in HeLa cells. Conversely, overexpression of c-*Jun* prevents the GR induction of genes that carry the functional glucocorticoid response element (GRE) (63,87,88). Analysis with deletion mutants of GR indicates that the DNA binding domain appears to be the primary mediator of repression. However, DNA binding *per se* is probably not required because G422 mutant of GR, which binds DNA, does not repress gene expression (87). Besides functioning as an antagonist of AP-1 activity, steroid receptors are also reported to cooperate with AP-1 and increase AP-1 induced gene activity as in the case of ER and c-erbA (81,89,90).

V. Mechanism of ER in oncogenesis of breast and ovarian cancer cells.

A. Different growth response to estrogen

Evidence indicates that estrogen is important for the growth and development of the normal mammary gland (91), as well as most ER^+ mammary carcinomas (71). E_2 stimulates the growth of most of the ER^+ breast cell lines including MCF-7, T47D, and ZR-75-1. Growth response of these cells to E_2 is lost, however, after long term culturing under condition of steroid deprivation (8,9,92,93). Similarly, in ER^+ ovarian cancer cell lines, E_2 has been found to

regulate the growth of BG-1 and PEO4 cells *in vitro* (5,6,10,11), and to promote tumor growth in nude mice injected with OVA-5 ovarian carcinoma cells (7). However, SKOV3 cells are ER^+ as shown in my studies.

The ER^- breast cancer cell lines, Hs578T and MDA-MB-231 can form rapidly growing, estrogen-independent tumors in nude mice (94). However, the estrogen growth response in MDA-MB-231 cells is restored upon introducing the ER gene into cells. Surprisingly, estrogen inhibits the growth of these ER transfected MDA-MB-231 cells *in vitro*, as well as decreases the metastatic ability of these cells in nude mice (95;96). The above result suggests that ER^+ breast cancer cells can tolerate a higher constitutive level of ER expression than ER^- cells. Similar negative growth regulatory effect of estrogen is also observed in ER transfected, normal ER^- human mammary epithelial cells (HMEC) (97). Since ER is only detected in 6-10% of epithelial cells in the normal mammary gland (98), but in approximately 60% of breast carcinomas (2,3), high level expression of ER itself may be important in the carcinogenic process of breast cancer.

B. Mechanisms of growth regulation of breast cancer cells by estrogen

ER functions as a critical gene regulator of multiple genes in growth regulatory pathways. Balanced regulation of this complicated system is important for maintaining normal cell growth. In contrast, abnormal regulation of growth regulatory genes may lead to the malignant growth of breast cancer.

Efforts have been made in breast cancer research to understand the mechanisms behind the growth regulation by estrogen. In well studied MCF-7

cells, estrogen induces the expression of a large number of enzymes involved in nucleic acid synthesis, including DNA polymerase, thymidine and uridine kinase, and dihydrofolate reductase (99-102). Estrogen stimulates the phosphatidyl inositol (PI) turn over in MCF-7, and thus regulates the level of 'second messenger' in the signal transduction pathway (103).

Estrogen also increases the expression of immediate early genes, c-*myc*, c-*fos* and c-*jun* (104). Immediate early genes are growth regulatory proto-oncogenes that bind DNA and regulate multiple gene expressions (105,106). Among them, *jun-jun* homodimer and *fos-jun* protein heterodimer are major components of the transcription factor AP-1 (106,107), and regulate gene expression through binding with TRE (106,108). ERE and AP-1 binding sites have both been located in 5' promoter of human c-*fos* (109). However, though E_2 induces c-*myc* promoter driven reporter gene expression, no consensus sequence resembling ERE has been found (110,111). In breast carcinomas, estrogen is consistently reported to increase c-*myc* expression at the mRNA level (72,111,112). Treatment of MCF-7 cells with antisense-*myc* oligonucleotide inhibits estrogen stimulated cell growth. This result indicates a critical role for c-*myc* in the growth of breast cancer cells (113). However, studies on the estrogen regulation of c-*fos* and c-*jun* expression in breast cancer are rather controversial, some show little (89,114) and some show significant stimulatory effect (72,115). At least in MCF-7 cells, estrogen and IGF-1 synergistically stimulate the transcriptional activity from TRE-CAT reporter gene, with (72) or without (89) the induction of c-*fos* and c-*jun* expression. These

result indicates that by interacting and inducing the function of AP-1, estrogen receptor directly impacts nuclear events that are also induced by polypeptide growth factors and thus forms a critical step in the induction of cellular proliferation (116).

In addition to the regulation of growth-controlling genes, others have identified E_2 induction of progesterone receptor (PR) in MCF-7 and T47D cells (8,9,117-119). Although progesterone does not directly modulate growth of human breast cancer at physiological concentrations, the expression of PR does appear to be coupled to functional growth regulation by estrogen (117,119). Thus PR content of breast tumors is used (along with ER) as a marker for anti-estrogen responsiveness of breast and ovarian tumors in clinical therapy (3,4), and estrogen stimulation of PR expression is used as a classical marker for a functioning ER.

The expression level of peptide growth factors and their receptors are also under the regulation of estrogen in breast carcinoma cells. The secretion levels of EGF, TGF-α, and IGF-I growth factors are increased upon estrogen treatment of MCF-7, and T47D cells (94,120). It has been reported that the conditioned media from estrogen treated MCF-7 cells is sufficient to stimulate MCF-7 tumor growth in ovariectomized athymic mice. Thus, these growth factors may act as estrogen induced 'second messengers' in estrogen responsive growth of human breast cancer (94). Estrogen is also shown to inhibit $p185^{HER\text{-}2/neu}$ expression (121-124). The changes in the expression level of various growth regulatory factors may also

serve as secondary regulators in the estrogen mediated malignant growth of cells.

C. Mechanisms of development of estrogen resistance of breast cancer

Though not fully elucidated, ER^+ breast cancer escapes estrogen mediated growth regulation through different mechanisms. Constitutive secretion of autocrine-paracrine growth factors is suggested as one mechanism (71), because high levels of TGF-α are secreted in some estrogen-independent breast cancer cells (94). Variant ERs that are formed by alternative splicing are suggested as another mechanism, since various mutations of ER have been found in breast cancer and have been reported to have altered hormone binding, DNA binding and transactivation activities.

1. Constitutive production of autocrine-paracrine growth factors:

When estrogen growth responsive cells MCF-7 and T47D are cultured under long term steroid deprivation, new cell lines are derived from parental cells that acquire estrogen growth independence regardless of ER^+ status (8,9,93). These ER^+ estrogen growth resistant cell lines provide a unique system for studying the progression to hormone independence and the changes that accompanies this progression. It has been shown that in some of these estrogen growth independent cell lines, the basal growth rate is increased in comparison to their estrogen growth responsive parental lines. Since ER in these cells is functioning to regulate gene expression (8,9), defective ER does not seem to be the cause of estrogen growth resistance. However secretion of TGF-α and TGF-β was increased, which supports the autocrine-paracrine regulation theory (8). It is

possible that once maximum growth capacity of the breast cancer cells is reached through autocrine-paracrine growth factors, estrogen no longer stimulates cell growth.

2. ER mutations

Mutation of ER is suggested as another mechanism for estrogen resistance in growth of breast cancer. Various ER mutants have been discovered in breast cancer biopsy samples. Tumors that express ER in the absence of PR have been postulated to contain abnormally functioning ER, since estrogen induction of PR is a normal function in steroid responsive tissue (117,125,126). (a) Mutations of the DNA-binding C domain are reported in some ER^+/PR^- breast cancer. Two truncated ER cDNA clones have been reported by Murphy & Dotzlaw (127,128). One is identical to wild-type ER up to exon 3/intron border, then diverges into a sequence that is not related to ER mRNA; the other shows a missing exon 2 as well as a divergent sequence 3' down stream from the exon 3/intron border. The fact that these variants usually involve changes at exon/intron borders suggests that mRNA splicing errors may cause these mutant ERs (128,129). The ER^+/PR^- phenotype can be explained by ER that lacks DNA binding ability prohibiting PR expression, but maintains its hormone binding capacity. (b) Some ER variants do not have transcription activation functions and prevent action of the normal receptor. This is the case for an ER variant lacking exon 7 that has been detected in ER^+/PR^- breast tumors (3,130). Since exon 7 encompasses part of the hormone binding domain, the variant itself presumably does not bind

hormone. However, when this variant is introduced into yeast cells with wild type ER, the mutant ER shows a 'dominant negative' inhibition effect on transactivation of a reporter gene by the wild type ER (3). Some ER variants prevent the binding of wild-type ER to its cognate response element (131). Many breast cancers show a heterogenous population of mutant and wild type ER (131). While the wild type ER binds ligand, the 'dominant-negative' mutant will block PR induction, thus explaining the ER^+/PR^- phenotype in these breast cancers.

(c) About 2% of all breast tumors are ER^-/PR^+. This phenotype could result from a variant ER that is unable to bind hormone and thus appears negative in a ligand-binding assay, but is still functional in stimulating the PR expression. This may be a potential mechanism for the escape of tumors from hormone control. In some ER^-/PR^+ breast cancer, ER forms have been found that are completely missing exon 5 corresponding to the hormone binding E domain. This results in a truncated protein of approximately 40 kDa (131). When functional analysis is conducted, this cloned 40 kDa variant is reported to constitutively stimulate estrogen-responsive genes in a yeast reporter system (131). It is suggested that HBD of steroid receptors has an inhibitory effect on A/B domain associated constitutive transactivation function (69). Therefore, deletion of the HBD domain of ER would generate a constitutively active, dominant-positive receptor, and thus may account for the PR expression in these ER^-/PR^+ breast cancers.

Since most ovarian cancer patients with ER^+ tumors seldom respond to anti-estrogen treatment (40,41), escape of E_2 regulation is also an important

phenomenon in ovarian tumorigenesis. However, what accounts for hormone independence in ovarian cancer is not known. Ovarian cancer may be tamoxifen resistant because the tumor cells have progressed to hormone independence when the disease is detected. Thus, study of the molecular role of E_2 in ovarian cancer is very important. The existence of a large proportion of ER^+ tumors that do not express PR (ER^+/PR^-) (4,39) may point to abnormal functions of ER in many ovarian cancers.

VI. Role of HER-2/neu in malignant growth of breast and ovarian tumors

HER-2/neu encodes a tyrosine kinase of 185 kDa with structural features analogous to epidermal growth factor receptor (132). Like other tyrosine kinase growth factor receptors, $p185^{HER\text{-}2/neu}$ has a cysteine rich and glycosylated, extracellular domain, a transmembrane domain, and a cytoplasmic domain that contains tyrosine kinase activity and autophosphorylation sites (133). Activation of $p185^{HER\text{-}2/neu}$ is believed to occur by dimerization of receptors at the cell surface, a process that may occur in the absence of ligand in some carcinoma cells (134). Recent reports have characterized several ligands that bind and activate $p185^{HER\text{-}2/neu}$ (135,136).

A. HER-2/neu status in clinical findings of breast and ovarian cancers

One of the most common abnormalities observed in breast carcinoma is the overexpression of HER-2/neu gene which occurs in 20-30% of the cases (34,137). $p185^{HER\text{-}2/neu}$ is overexpressed more often in advanced tumors (138-141).

Overexpression of $p185^{HER\text{-}2/neu}$ correlates with less differentiated and faster growing breast cancer (125,139,142), and negatively associates with the expression of ER and/or PR in breast cancer (31,140,142). While a few reports indicate no significant prognostic effect of overexpression of $p185^{HER\text{-}2/neu}$ (143-145), it is generally accepted that there is a correlation of $p185^{HER\text{-}2/neu}$ overexpression with recurrent disease and poor prognosis in breast cancer patients, both in auxiliary lymph node positive and node negative cases [18,34,137,140,146-148).

Overexpression of the $p185^{HER\text{-}2/neu}$ occurs in about 20% of ovarian cancer patients and is the most frequently observed oncogene abnormality in ovarian tumors (16,19,34). Though one study by Haldane, et al. did not find a relationship of $p185^{HER\text{-}2/neu}$ overexpression and the prognosis of ovarian cancer, most studies suggest that overexpression of $p185^{HER\text{-}2/neu}$ is associated with poor prognosis (16,34,149,150). Thus, $p185^{HER\text{-}2/neu}$ is another key player in oncogenesis of breast and ovarian cancer. Therefore, understanding the regulation of HER-2/neu expression by estrogen may be important for revealing mechanisms controlling differentiation, growth, and malignant progression of breast and ovarian epithelial tissue.

B. Mechanism of HER-2/neu induced tumorigenesis

Overexpression of the normal cellular HER-2/neu gene appears to be the most common proto-oncogene overexpression in human carcinomas (151-153). It has been reported that allelic loss of chromosome 17p and 17q exists in some breast cancer. Since both tumor suppressor gene and HER-2/neu are located on

chromosome 17, loss of tumor suppressor gene could be another cause of HER-2/neu overexpression (31,32). In the human ovarian carcinoma cell line SKOV3, it has been found that enhanced HER-2/neu expression induces larger tumors, and results in shorter survival of nude mice (151). Overexpression of $p185^{HER\text{-}2/neu}$ causes transformation of 3T3 cells and tumor formation in nude mice (154,155). In addition, single step induction of HER-2/neu expression by a MMTV promoter can induce mammary carcinoma in transgenic mice (156). This evidence indicates that activation and overexpression of $p185^{HER\text{-}2/neu}$ lead to malignant growth of cells.

C. Regulation of HER-2/neu expression by ER

Clinical observation of a reciprocal relationship of expression of HER-2/neu and ER suggests that there is crosstalk between ER and $p185^{HER\text{-}2/neu}$. In breast cancer carcinoma cell lines including MCF-7, T47D, and ZR-75-1 cells, E_2 have been found to regulate expression of the HER-2/neu proto-oncogene (121-124,157). Because a reduction in HER-2/neu mRNA levels has been observed in all cases where the protein product is reduced, it has been suggested that repression of HER-2/neu mRNA accounts for the reduction in the amount of $p185^{HER\text{-}2/neu}$ protein product (121-124,157). The HER-2/neu promoter has been cloned. Although a classical ERE has not been found, functional analysis indicates that there is a estrogen responsive region in the HER-2/neu promoter, since E_2 inhibits the expression of a HER-2/neu driven reporter gene (157). Gel-shift assays further show that estrogen treatment of ZR-75-1 breast carcinoma

cells induces binding of a protein complex with an oligonucleotide from the HER-2/neu promoter. This observation indicates that ER either binds the HER-2/neu promoter, or induces other transcription regulators that bind the HER-2/neu promoter.

VI. Thesis rationale

Although much progress has been made in elucidating the function of oncogenes and anti-oncogenes in regulation of cellular processes, the steps that lead to malignant cell growth is still not understood. In contrast to breast cancer, little research has been conducted on growth and gene regulation in ovarian carcinomas. Thus, the primary goal of the work in my first manuscript was to investigate the effect of estrogen on the growth regulatory genes, in order to shed light on how cells escape estrogen regulated growth in ovarian carcinomas. Because most of the ER^+ ovarian carcinomas are hormone independent, I chose SKOV3 ovarian carcinoma cells, which are estrogen growth independent, as a model. Western and Northern blotting analyses revealed that although ER was expressed in these cells, PR was not induced by E_2. Thus SKOV3 cells are an example of ER^+/PR^- ovarian cancer. Since it had been reported that breast cancer exhibiting the ER^+/PR^- phenotype may be caused by mutations in ER, I investigated the function of E_2 in SKOV3 cells. The functional studies included, estrogen binding, nuclear localization, and gene regulation. The results indicated abnormal transactivation of gene expression by ER in this ovarian cancer cell line.

It is well known that HER-2/neu overexpression is a malignant marker in breast and ovarian cancer prognosis. Although estrogen has been shown to inhibit HER-2/neu expression at the mRNA level, whether transcriptional inhibition accounts for the level of repression of $p185^{HER\text{-}2/neu}$ protein is not known. In manuscript #2, I investigated the regulation of HER-2/neu expression by E_2 both at the mRNA and protein synthesis level in T47D breast carcinoma cells. My results suggested that in addition to the inhibition at the mRNA level, estrogen exerted its major inhibitory effect on the rate of $p185^{HER\text{-}2/neu}$ protein synthesis in these cells.

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[1]The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; E_2 estradiol; ER, estrogen receptor; ERE, estrogen response element; FGF, fibroblast growth factor; GR, glucocorticoid receptor; PR, progesterone receptor; GRE, glucocorticoid response element; TRE, TPA response element; vit, vitellogenin.

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Abnormal Transcriptional Regulation by Estrogen in Hormone Resistant Ovarian Carcinoma Cells

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Abstract

Ovarian cancers are commonly estrogen receptor positive / progesterone receptor negative (ER^+/PR^-) and steroid hormone independent for growth. In the current study, we describe the molecular functions of ER in the SKOV3 ovarian carcinoma cell line as a model for estrogen independent ovarian cancer, in comparison with the well-characterized estrogen responsive T47D breast carcinoma cells. For SKOV3 cells, we found: (1) Cell growth was not affected by estradiol (E_2) treatment. (2) ER had a normal apparent Kd for binding with E_2 and exhibited normal nuclear translocation in response to E_2. (3) E_2 did not induce expression of PR or expression of a stably transfected estrogen response element (ERE) driven reporter gene. (4) E_2 stimulated expression of the early growth response genes c-*myc*, and c-*fos* in SKOV3 cells to similar levels as in the estrogen responsive PEO4 ovarian carcinoma cells. Thus ER in SKOV3 cells did not function in ERE driven transcriptional activation but did function in transactivation of c-*myc* and c-*fos* expression. This finding suggests that different regulatory mechanisms were involved in the E_2 regulation of these genes.

Introduction

Ovarian carcinomas are the most lethal tumors of the female genital tract (1-3). Typically, the disease remains clinically silent until it is advanced. Improved treatment strategies and early diagnosis will depend on a better understanding of the molecular defects involved in this disease.

Similar to breast cancer, female sex steroid hormones are believed to play a role in the growth and malignant progression of ovarian cancer (3). Estrogen receptor (ER) is expressed in more than 60% of ovarian and breast cancers (4-6). In addition, estrogen has been found to regulate the growth of some ovarian carcinoma cell lines *in vitro* (7-11) and to promote tumor growth in nude mice injected with OVA-5 ovarian carcinoma cells (9).

Estrogen regulated growth is believed to occur by altered expression of growth regulatory genes that are under transcriptional control by ER (12-14). Although the molecular role of estrogen in growth and gene expression has been studied extensively in breast carcinoma cells, the mechanism by which estrogen regulates growth has not been elucidated. In contrast to breast cancer, there have been few studies conducted on the molecular role of estrogen in ovarian cancer.

While most ER^+ breast cancers respond to the antiestrogen, tamoxifen (15,16), ovarian cancer patients with ER^+ tumors seldom respond to anti-estrogen treatment (17, 18). Ovarian cancer may be hormone resistant because the tumor cells have progressed to hormone independence before the disease is detected. Indeed, ER^+ breast cancers that initially respond to antiestrogen therapy usually

progress to hormone independence (19). Progression to hormone independence has also been shown to occur *in vitro* in T47D and MCF-7 breast carcinoma cells cultured continuously in estrogen depleted medium (20-22). Generation of defective or abnormal ER has been suggested to be a mechanism behind progression to hormone independence (23-25).

Tumors that express ER in the absence of progesterone receptor (PR) have been postulated to contain abnormally functioning ER, since estrogen induction of PR is a normal function in steroid responsive tissue (26-28). Indeed, variant ER have been identified in ER^+/PR^- breast cancers (19,23-25), which have a less favorable prognosis than ER^+/PR^+ cancer (29,30). Although the prognostic significance of ER and PR status in ovarian cancer is controversial (3-6,31,32), the existence of a large proportion of ER^+ tumors that do not express PR (ER^+/PR^-) (3,33) points to abnormal function of ER in many ovarian cancers.

In the current study, we describe the molecular expression of ER in several ovarian carcinoma cell lines. We characterize an ovarian carcinoma cell line, SKOV3, as a model for ER^+/PR^-, hormone independent ovarian cancer. The function of E_2 in cell growth, nuclear association, regulation of gene expression, and expression of transfected ERE driven reporter gene is compared in the SKOV3 cells with the well-characterized estrogen responsive T47D breast carcinoma cells. Further we compare the estrogen induction of the early growth response, cell cycle control genes, c-*myc*, c-*fos*, and c-*jun* in the SKOV3 ovarian carcinoma cells that are estrogen independent with the T47D breast carcinoma

cells and PEO4 ovarian carcinoma cells that are estrogen responsive for growth.

Materials and Methods

Chemicals

Chemicals and reagents were obtained from Sigma (St. Louis, MO) unless specified. Reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were obtained from Bio-Rad (Richmond, CA).

Cell Lines

The T47D breast carcinoma cell line (34), and the three ovarian carcinoma cell lines: CAOV3, NIH.OVCAR-3, and SKOV3 were all obtained from American Type Culture Collection (A.T.C.C., Rockville, MA). CAOV3 cells were originally isolated from an ovarian tumor by J. Fogh in 1976. The NIH.OVCAR-3 (35) and SKOV3 cells (36,37) were both isolated by cultivation of ascitic fluid from patients with ovarian cancer. PEO4 ovarian carcinoma cells, a gift of Dr. Thomas C. Hamilton, were derived from the malignant ascites of an ovarian cancer patient with a recurrent mucinous ovarian adenocarcinoma (7,8). The normal ovarian epithelial cell line of limited passage, IOSE.VAN (38), was obtained from Dr. Nelly Auersperg.

T47D and PEO4 cells were routinely maintained in RPMI 1640 (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (FBS) (Hyclone Lab. INC., Logan, UT), 10 μg/ml insulin, and 0.5% gentamicin (GIBCOBRL, Gaithersburg, MD). SKOV3, CAOV3 and NIH.OVCAR-3 cells were maintained in DMEM (JRH Biosciences) supplemented with 10% FBS and 0.5% gentamicin. IOSE.VAN Cells were cultured in 50% Medium 199 (Sigma,

St. Louis, MO) and 50% MCDB 105 supplemented with 10% FBS and 0.5% gentamicin. All cells were incubated at 37°C in a humidified incubator with 5% CO_2.

cDNA Probes

The 1.96 kb human estrogen receptor (hER) full length cDNA was generated from pER7 plasmid (39a,39b). The 2.8 kb full length human progesterone receptor cDNA was a gift from Dr. David Brandon, and was generated by EcoRI digestion of hPR-pGEM-4 plasmid (40). The 1 kb AvaI fragment of rat c-*fos* cDNA was recovered from pSP65-c-*fos*-Rat plasmid (41), obtained from Dr. Donna Cohen. The 1.5 kb sstI fragment of exon 2 from the human c-*myc* gene (42) was generated from pCmycsst1.5uc plasmid, a gift from Dr. Grover Bagby. The 800 base pair HindIII-PstI fragment of human c-*jun* was recovered from the RSV-cJ plasmid (43) originally from Dr. Peter Angel.

Estrogen Effects

To test effects of estrogen, cells were first depleted of steroids. The cells were plated in complete growth medium overnight and then washed three times with phosphate buffered saline (PBS) (3 mM KCL, 1.5 mM KH_2PO_4, and 0.14 M NaCl, pH 7.4). The medium was then replaced with phenol-red free DMEM supplemented with 5% dextran charcoal treated FBS (DCFBS) (stripped medium). To prepare DCFBS, 1% Norit A and 0.01% Dextran T-40 were incubated in a buffer containing 0.25 M Sucrose, 1.5 mM $MgCl_2$, 10 mM HEPES pH 7.4 at 4°C overnight. The dextran charcoal was then mixed with FBS, and the

mixture was heated at 55°C for 2 h, and then incubated at 4°C overnight. The charcoal dextran was removed by centrifugation at 3,000 rpm in a model TJ-6 Beckman table top centrifuge for 15 min, and by filtering the serum through a 0.2 μm filter (Becton Dickinson Labware, Lincoln Park, N.J.). Following cultivation in stripped medium, 10 nM of 17-β estradiol (E_2) or the ethanol vehicle was added. The maximum concentration of ethanol in cultured cells was 0.5%. To test E_2 effects on expression of early growth response genes, cells were grown in stripped medium for 5 days, washed three times with PBS, and incubated for 48 h in 1% dialyzed DCFBS prior to administration of E_2.

Nuclear and Cytosolic Fractionation

Cell fractionation was conducted using a modification of previously described procedures (44,45). 1 x 10^7 cells were incubated in PBS containing 1 mM EDTA, pH 7.4 for 10 min at room temperature, and collected by centrifugation. The cells were suspended in 2 ml TETG buffer {10 mM Tris. pH 7.4, 1.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 10% glycerol, 1% aprotinin, and 2 mM phenylmethyl-sulfonyl fluoride (PMSF)}, and disrupted on ice using a Dounce homogenizer (Wheaton Scientific, Millville, NJ) with about 100 strokes of a pestle-B until 95% of cells were lysed as observed by microscopy. The homogenized cells were centrifuged at 800 x g for 15 min at 4°C, and the crude nuclear pellet was collected and washed twice more with 0.2 ml of TETG buffer. The cytosol obtained from the combined supernatant fractions was centrifuged at 12,000 x g for 30 min at 4°C and the pellet was discarded. The nuclear fraction

was obtained by incubating the crude nuclear pellet with 200 μl of TETG-0.6 M KCl on ice for 1 h with resuspension every 15 min, and then centrifugation at 12,000 x g for 30 min at 4°C. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

Western Blot Analysis

SDS-PAGE and Western blot analysis was conducted as previously described (46). Protein, resolved by SDS-PAGE in a 6% polyacrylamide gel, was transferred onto nitrocellulose membranes (Costar, Cambridge, MA), which were then blocked with 5% nonfat dried milk in water at 24°C for 1 h. After incubating blots with anti-ER or anti-PR monoclonal antibodies, horseradish peroxidase conjugated goat anti-rat IgG (GIBCOBRL, Gaithersburg, MD) was used as the second antibody. Blots were developed by Enhanced Chemiluminescence reagent (Amersham Life Science, Arlington Heights, IL) and exposed to X-OMAT K film (Eastman Kodak Co., Rochester, NY).

Kd Determination of E_2 for ER

Hormone binding was conducted as described (47). Aliquots of cytosol (100 μl) containing 5 mg/ml protein were incubated overnight at 4°C with 100 μl [^{3}H]-E_2 (New England Nuclear, Boston, MA) at concentrations between 0.2 nM - 4 nM, or with 200-fold excess of unlabeled diethylstilbestrol (DES) to eliminate non-specific binding. The amount of unbound [^{3}H]-E_2 was determined in an aliquot that was treated with dextran charcoal. The amount of bound E_2 was

calculated by subtracting the unbound from total amount of [^{3}H]-E_2 determined from a replicate sample that was not treated with charcoal-dextran. The data were analyzed by Scatchard analysis plotting the value of Bound E_2 as X-axis, and Bound/Free as Y-axis, and the dissociation constant (Kd) was calculated as the slope of the line.

Isolation of Poly(A) RNA

Isolation of mRNA was performed according to a procedure described by Schwab,M. et al. (48). 3 x 10^7 cells were lysed in 7.5 ml of 10 mM Tris - HCl pH 7.4, 0.1 M NaCl, 2 mM EDTA, and 1% SDS. DNA was sheared by passing the lysate through a 21 gauge needle. The lysate was digested with 100 μg/ml of proteinase K for 30 min at 37°C, and NaCl was added to a final concentration of 0.4 M. The lysate was then mixed overnight at 24°C with 160 mg of pre-washed Oligo - dT cellulose (Collaborative Research, Bedford, MA), collected by centrifugation, and washed once in 10 ml high salt buffer (10 mM Tris - HCl pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.2% SDS). Oligo - dT, resuspended in 10 ml of high salt buffer, was poured into a polyprep column (Bio Rad, Richmond, CA), and the column was washed once with 10 ml of high salt, and once with 1.75 ml of low salt buffer with 0.1 M NaCl. mRNA was eluted from the column with 3.5 ml of no salt buffer. The eluate was adjusted to 0.2 M sodium acetate and 67% ethanol and the mRNA was precipitated overnight at -20°C. The mRNA was recovered by centrifugation at 7,000 rpm in an SS34 Beckman rotor for 45 min at 4°C and air dried. mRNA was resuspended in TE buffer (1 mM EDTA, 10 mM

Tris - HCl pH 7.4) and stored at -70°C.

Northern Blot Analysis

5 μg of poly(A) RNA was separated on a formaldehyde-denaturing gel containing 1% agarose and was then transferred onto a nytran membrane (Schleicher & Schuell, Keene, NH) by capillary action as described (49). RNA was fixed on the membrane by UV crosslinking and membranes were prehybridized for 4 h at 42°C in a hybridization buffer containing 50% formamide, 5 x SSC (1 x SSC = 0.15 M NaCl and 15 mM Sodium Citrate, pH 7.0), 0.1% SDS, 250 μg/ml herring sperm DNA, and 1 x Denhardt's solution to block non-specific cDNA binding. cDNA probes were radiolabeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA), using random primer DNA labelling kit (Boehringer Mannheim, Indianapolis, IN), and hybridization was carried out overnight in hybridization buffer containing 0.5-1 x 10^6 cpm/ml of [α-^{32}P]dCTP-labeled cDNA probe. Membranes were washed 20 min at room temperature and 65°C for 5 min in 2 x SSC containing 1% SDS and for 60 min in 1 x SSC containing 1% SDS with three changes. The membranes were exposed to X-ray film and autoradiographs were analyzed by a laser densitometer (Bio-Rad, Hercules, CA). The membranes were then stripped of probe by boiling in two changes of 0.1 x SSC for 5 min and were then used for hybridization with additional probes.

Stable Transfection and Luciferase Assay

pGeoffERE (also called ERE-Luciferase) expression plasmid obtained from Dr. Geoff Rosenfeld was constructed by insertion of a perfect palindrome

the same as the *xenopus* vitellogenin estrogen response element (ERE): 5'-TCAGGTCACTGTGACCTGA- 3' into the BamHI site of the polylinker region of pT8Luc plasmid (A.T.C.C., Rockville, MA). Stable transfection marker plasmid pSV2-neo was obtained from A.T.C.C. (Rockville, MA). Another marker plasmid pSV2-Hygro3 was constructed by Dr. Eric Barklis by inserting 1.1 kb hygromycin cDNA (50) into SV2gpt plasmid (51). For transfection, 3 x 10^6 cells in a 10 cm diameter culture dish were incubated in complete medium for 24 h with addition of fresh medium 2 h before transfection. Cells were then transfected by the calcium-phosphate transfection kit (GIBCOBRL, Gaithersburg, MD) as described (49). 20 μg pGeoffERE plasmid, and 0.5 μg pSV2neo selectable marker were cotransfected into T47D cells. For SKOV3 cells, 0.5 μg pSV2-Hygro3 plasmid was used as the selectable marker. 800 μg Geneticin (G418)/ml for T47D, or 300 μg hygromycin/ml for SKOV3 cells was then added to transfected cells. 1-2 weeks later, colonies were isolated and transferred into 24 well plates, and passaged 3 times. Transfected cell lines were maintained in media containing 400 μg/ml G418 or 150 μg/ml hygromycin.

To assess expression of the luciferase reporter gene, the luciferase assay was conducted as described (52). 3 x 10^6 washed cells were scraped from the culture dishes, and resuspended in 100 μl ice cold 100 mM KH_2PO_4, 1mM DTT. Cells were disrupted by three freeze/thaw cycles in dry ice-ethanol/37°C, and the insoluble material was removed by centrifugation. 5 μl of cell lysate was mixed with 350 μl 5 mM ATP, 25 mM glycylglycine, pH 7.8, and 15 mM $MgSO_4$,

followed by direct addition of 100 μl 0.1mM luciferin into the sample in the Packard Picolite luminometer (United Technologies Packard, Downers Grove, IL). The light output was measured for 10 sec at 25°C.

Results

Expression of Estrogen Receptor mRNA and Protein in Ovarian Cells

To investigate the role of estrogen and ER in ovarian cancer, model cell lines in which the hormonal environment can be experimentally controlled are crucial. Because ovarian carcinoma cells have not been examined for molecular expression and transcriptional function associated with ER, we characterized four cell lines, CAOV3 (37), NIH.OVCAR-3 (35), PEO4 (7,8), and SKOV3 (36,37), as well as normal ovarian epithelial cells of limited life span, IOSE.VAN (38) for expression of ER mRNA and protein levels. For comparison, we used the well characterized breast carcinoma cells, T47D, which are estrogen responsive for growth (53). Northern blot analysis was conducted on poly (A) RNA extracted from each of these cell lines using [α-^{32}P]dCTP labeled ER cDNA probe. As expected, the T47D cells contained mRNA that hybridized with the ER cDNA probe (Fig. 1*B*). It was also anticipated that ER mRNA would be in PEO4 cells since they have been reported to contain ER based on estrogen binding studies (7,54). Indeed mRNA of about 6.6 kb was observed (Fig. 1*B*). ER mRNA was also detected in the SKOV3 ovarian cancer cells, but not in NIH.OVCAR-3, CAOV3, or in the IOSE.VAN cells (Fig. 1*B*). While SKOV3 and T47D cells expressed similar levels of ER mRNA, the PEO4 cells contained about 5 fold higher levels when standardized to the amount of β-actin mRNA (Fig. 1*B*). To assess whether the mRNA levels reflected the amount of the ER protein, extracts from each cell line were analyzed by Western blotting using anti-ER monoclonal

antibody D75 (55). The ER protein standardized to total cell protein was similar in T47D and SKOV3 cells (Fig. 1*A*). Therefore, PEO4 and SKOV3 ovarian carcinoma cells expressed ER mRNA and protein with no evidence of variant ER. The ratio of ER protein to mRNA appeared to be 2-3 times lower in PEO4 cells.

Growth Regulation by Estrogen

While PEO4 cells (7,8, and our unpublished results) as well as T47D cells (53) are estrogen responsive for growth, SKOV3 cells had not previously been examined for E_2 response. We therefore tested E_2 for effects on growth of SKOV3 cells and, for comparison, on T47D cells. The cells were first depleted of estrogen in stripped medium for 4 days and then 10 nM E_2 was added for an additional 7 days and cell numbers were determined in triplicate cultures. In T47D cells, 10 nM E_2 increased the number of cells about 4 fold relative to the ethanol vehicle-treated control (Fig. 2*A*). However, in SKOV3 cells, there was no significant mitogenic effect of E_2 when tested at three different concentrations (Fig. 2*B*). It was possible that the SKOV3 cells were at their maximum growth rate thereby obscuring any E_2 effects on growth. We therefore cultured SKOV3 cells in serum free, phenol-red free medium supplemented with insulin, transferrin and selenium (ITS) in which the growth rate of the SKOV3 cells was greatly reduced. Under these conditions of dampened growth rate, there was no detectable growth response to 10 nM E_2 in triplicate cell cultures counted at 6 and 12 days of E_2 treatment (data not shown). Although the SKOV3 cells contain ER mRNA and protein, these ovarian carcinoma cells were estrogen independent

for growth.

Examination of the Cellular Localization of ER

In response to ligand treatment, ER becomes tightly associated with the nucleus where it functions as a transcriptional regulator (56,57,58). Association with the nucleus is a function that has been assigned to the nuclear localization sequence located at the C-terminal extremity of the DNA binding domain, the hinge domain, and the hormone binding domain of ER (59). A defect in binding to the nucleus may be a functional abnormality of ER in hormone resistant breast cancer (23). To evaluate location, ER from whole cell extracts (Fig. 3*A*), or from cells that were homogenized and fractionated into cytosolic (Fig. 3*B*) and nuclear fractions (Fig 3*C*) were examined for ER by Western blotting. In response to E_2 treatment, the amount of ER in whole cell extracts was decreased by 2 fold in SKOV3 and T47D cells (Fig. 3*A*). When cytosolic ER was examined, the levels were markedly reduced in both cell lines in response to E_2 (Fig. 3*B*). In SKOV3 cells, the E_2-dependent reduction in cytosolic ER corresponded to a striking increase in the proportion of ER associated with the nuclear fraction, consistent with hormone-dependent, stable nuclear association (Fig. 3*C*). Interestingly, in the T47D cells, most of the ER was tightly associated with the nuclear fraction in the absence of hormone suggesting that ER may be functioning as a ligand independent transcriptional regulator. Therefore, ER from SKOV3 cells exhibited down regulation and tight association with the nucleus in response to E_2.

Determination of the Affinity of ER for E_2

To examined the function of hormone binding domain of ER, we determined the affinity of E_2 binding to ER. Cells were cultured in stripped medium for 7 days, the cell extracts were then used to measure E_2 binding and to calculate the dissociation constant (Kd) from the Scatchard plot (see appendix). The dissociation constant, Kd, in T47D cells was 1.8×10^{-10} M with a - 0.96 correlation coefficient, and the Kd in SKOV3 cells was 2.0×10^{-10} M with a - 0.84 correlation coefficient. In a duplicate experiment the Kd was 1.04×10^{-10} M, for ER in SKOV3 cells. Therefore, ER from SKOV3 cells appeared normal in size, E_2 binding affinity, and nuclear translocation.

Examination of the Transcriptional Activation Function of ER in Induction of PR Expression

A classic function of E_2 in responsive breast carcinoma cells (26,27) and normal rat uterus (60) is induction of PR expression through an estrogen response element (ERE) in the promoter region of the PR gene (61). To further assess ER function in the ovarian carcinoma cells, the effect of E_2 on PR expression was analyzed. As previously reported (27), E_2 induced both A and B forms of PR by about 10 fold in T47D cells (Fig. 4*A*). However, PR was not induced by E_2 in SKOV3 cells and was undetectable even after the blot was over-developed revealing background bands (Fig. 4*A*). Moreover, no PR mRNA was detected in SKOV3 after E_2 treatment for 1, 3, 6, or 12 h, while 4 to 5 fold induction of 11.4 kb, and 6.1 kb species of PR mRNA (62) was observed by 1 h of hormone treatment of T47D cells (Fig. 4*B*). Expression of significant levels of PR mRNA

in the absence of added E_2 may be due to the ligand-independent nuclear associated ER in T47D cells (see Fig. 3).

Evaluation of the Transcriptional Activation Function of ER by Expression of a Transfected ERE-luciferase Reporter Plasmid

A defect in E_2 induction of PR suggested an abnormality in the transcriptional activator function of ER in SKOV3 cells. To further evaluate the DNA binding and transcriptional activation function of ER, SKOV3, and for comparison, T47D cells were stably transfected with the ERE from the vitellogenin gene (63,64) linked to the luciferase reporter gene (pGoffERE). The stably transfected cell lines were analyzed by Western blotting to verify that ER levels were maintained and then were cultivated in complete medium or in stripped medium with E_2 or the ethanol vehicle. Luciferase activity was then determined in triplicate cultures. In the stably transfected EN1 clone of T47D cells (T47D-EN1), there was a clear E_2-dependent induction of ERE-luciferase. Fig. 5*A* illustrates an approximate four fold decrease in luciferase activity in stripped medium, while luciferase activity was stimulated by E_2 addition. In contrast, removal or administration of E_2 did not significantly affect luciferase activity in a stably transfected clone of SKOV3 cells (SKOV3-EH4) (Fig. 5*B*). A second transfected clone of SKOV3 cells, SKOV3-EH3, also illustrated no E_2 effect on expression of ERE-luciferase (data not shown). Therefore, ER from SKOV3 cells appeared to have a defect in transcriptional activation mediated by an ERE.

Examination of ER Function in Induction of Early Growth Response Genes c-myc, c-fos, and c-jun

Early growth response proto-oncogenes are induced by a variety of mitogenic agents and these genes are believed to be instrumental in cell proliferation and differentiation (14, 65-68). The early growth response gene most strongly associated with estrogen induced growth of breast carcinoma cells is c-*myc* (66,67), where a functional role in proliferation of MCF-7 breast cancer cells has been suggested using c-*myc* specific antisense oligonucleotides (66). While c-*fos* is induced by estrogen in rat uterus (69) and in human endometrial carcinoma cells (70), neither c-*fos* nor c-*jun* have been found to be consistently induced in breast carcinoma cells (71, 72). Because expression of nuclear proto-oncogenes has not been studied in ovarian carcinoma cells, we investigated the estrogen-independent SKOV3 and the estrogen responsive PEO4 cells. The cells were cultivated in low serum (1% dialyzed, stripped serum) for 48 h to achieve quiescence, E_2 was added for 60 min in the presence of 5% or 10% stripped serum, and the RNA was extracted and subjected to Northern blot analysis. C-*myc* mRNA levels were elevated 3-4 fold relative to β-actin mRNA in response to E_2 treatment of T47D cells and the level of c-*myc* mRNA was not greatly affected whether E_2 was administered in 5% or 10% stripped serum (Fig. 6*A*). Although the basal level of c-*myc* was higher in PEO4 cells, the extent of E_2 induction of c-*myc* mRNA was similar (3-4 fold) in the PEO4 and SKOV3 cells, even though the SKOV3 cells expressed lower levels of ER and did not exhibit a mitogenic

response to E_2. When the Northern blots were stripped, tested for removal of isotope by film exposure, and then reprobed with the c-*fos* cDNA probe, the level of E_2 induction was similar in T47D cells and in ovarian carcinoma cells for less than three fold (Fig. 6*B*). Again, the SKOV3 cells could not be distinguished from the estrogen responsive PEO4 cells by the extent of E_2 induction of c-*fos*. In the case of c-*jun*, as previously reported (72), there was little or no induction in T47D cells and no E_2 effect on c-*jun* expression was observed in the ovarian carcinoma cells (Fig. 6*C*).

It was surprising that SKOV3 cells exhibited E_2 induction of c-*myc*, and to a lesser extent, c-*fos*, since these cells do not respond mitogenically to E_2 and ER appears to be defective in transcriptional activation of PR and of ERE-luciferase. Therefore we further explored E_2-dependent expression of c-*myc* and c-*fos* at different times following administration of E_2. Increased c-*myc* mRNA levels were detected by 30 min with an approximate 3 to 4 fold increase by 1 h (Fig. 7*A*). Enhanced c-*myc* levels were observed for up to 12 h of E_2 treatment (Fig. 7*A*). This time course is consistent with previously reported results in which c-*myc* mRNA was induced by 30 min in breast carcinoma cells and the effect gradually declined by about 12 h (73,74). C-*fos* mRNA was found to be strongly induced by serum and the levels rapidly declined by 1 h (Fig. 7*B*), consistent with previous observations (75). An approximate two fold increase in c-*fos* mRNA was observed at 30 min of E_2 treatment and this induction was maintained up to 1 h and then declined in SKOV3 cells (Fig. 7*B*). C-*jun* mRNA levels were

stimulated 2-3 fold by serum, but did not appear to respond to E_2 treatment (Fig. 7*C*). In summary, the effects of E_2 on c-*myc* and c-*fos* mRNA levels in SKOV3 cells were detected early, by 30 min to 1 h, and were reproducible.

Discussion

With the goal of understanding the function of estrogen receptor in ovarian cancer, we have analyzed the molecular expression and E_2-mediated functions of ER in ovarian carcinoma cell lines. ER mRNA and protein of apparently normal size were expressed in SKOV3 and PEO4 ovarian carcinoma cell lines, with no evidence of variant forms. No ER expression was detected in NIH.OVCAR-3, CAOV3, or in the IOSE.VAN cell line derived from normal ovarian surface epithelial cells (38), nor did any of these cells exhibit E_2 induction of PR (Hua and Clinton, unpublished observations). However, previous studies of NIH.OVCAR-3 cells indicated E_2 binding and induction of PR (35). This discrepancy may be explained by differences in cell lines. The ovarian carcinoma cell lines PEO4 (7,8) and BG-1 (10,11) have previously been found to be growth responsive to E_2 indicating that estrogen may be mitogenic in ovarian carcinoma cells. However, we saw no E_2-dependent effect on growth in SKOV3 cells, suggesting that the ER expressed in SKOV3 cells does not function in mitogenesis. While SKOV3 cells did not exhibit a mitogenic response to estrogen, ER in these cells exhibited some apparently normal functions in response to E_2. E_2 addition to SKOV3 cells resulted in decreased ER expression and translocation of ER to the nucleus indicating a normal nuclear translocation signal.

We also observed an E_2 dependent decrease in ER amounts in SKOV3 and T47D cells. Down regulation of ER by E_2 has not been definitively established. In MCF-7 breast carcinoma cells (76,77) E_2 treatment leads to down

regulation of ER, while in T47D cells E_2 has no effect, or may even up regulated the expression of ER (76,77). Thus, E_2 dependent decrease of ER in SKOV3 whole cell extract may be caused by down regulation of the receptor, or by E_2 induced tight nuclear association of ER. ER may not have been efficiently extracted from the nuclei in the E_2 treated SKOV3 whole cell lysate. When the Scatchard analyses were conducted in SKOV3 cells, the affinity of ER for E_2 was in the normal range, suggesting that ligand binding domain was not defective.

A defect in the transcriptional activation function of ER in SKOV3 cells was suggested by studies of cells transfected with ERE linked to the reporter gene, luciferase. Two different clones of transfected SKOV3 cells were refractory to E_2 effects on ERE-luciferase expression while transfected T47D cells exhibited E_2 induction of ERE-luciferase activity. Additional evidence for defective transcription activation function was that PR expression was not induced by E_2 in SKOV3 cells. There does not appear to be an ovarian tissue-specific restriction to E_2 induction of PR, since other ovarian carcinoma cells, including BG-1 cells, appear to have PR coupled to ER expression (78). Induction of PR is a classic marker for ER function and requires the ER DNA binding domain (26,27). PR induction occurs by interaction of ER with a half palindromic ERE in the PR gene (61,79). ER binding with DNA is also required for E_2 induction of *Xenopus* vitellogenin ERE reporter gene (63,64). Thus the absence of E_2 induction of PR and vitERE suggested a defect in DNA binding function of ER, or the existence of cell specific factors that blocked the DNA binding and/or transactivation

function of ER.

ER^+/PR^- breast tumors have been described and have been suggested to have ER with a defective DNA binding domain (19). Studies of some ER^+/PR^- breast tumors as well as T47D breast carcinoma cells have revealed the presence of truncated ER variants (19,23,25,80). Some variant ER forms may inhibit the transcriptional function of ER by blocking binding of wild-type ER to ERE in a dominant negative manner (19,23,25,81,82). However, several fold excess amounts of variant over wildtype ER are required to achieve significant inhibition of wildtype function (81,82). If variant ERs are expressed in SKOV3 cells, either they could not be distinguished by size in Western or Northern blot analyses, or they were at much lower levels than wildtype ER.

While ER in the SKOV3 cells was defective in transcriptional activation of PR and an ERE reporter gene, there was an E_2 induction of c-*myc* and c-*fos* in these cells. Therefore, there may be distinct transactivation functions of ER. One transactivation function may require direct binding to an ERE. A second function may be involved in E_2 induction of c-*myc* and c-*fos*. While DNA binding domain of ER is required for E_2 induction of c-*myc* promoter driven reporter gene expression, there is no evidence for the presence of an ERE (83). There are however, other transcription regulator binding sites. For example, TPA response element (TRE) (84) and Sp1 binding site are found in the c-*myc* promoter region (83). In addition, the 5' promoter region of the c-*fos* gene has been reported to contain two imperfect palindromic EREs and tumor promoter response elements

(TREs) (85). It is possible that ER regulates c-*myc* and c-*fos* expression by protein-protein interaction with other transcription regulators. ER is known to complex with other transcription regulators for example activator protein -1 (AP-1) that binds TRE. ER and AP-1 has been shown cooperatively increase the expression of ovalbumin promoter without binding of ER to the ovalbumin promoter (86). Thus, based on our findings that ER was defective in inducing gene expression through ERE, but functioned in induction of c-*myc* and c-*fos* in SKOV3 cells, we propose that ER may have defect in transactivation mediated by binding to ERE, but may have normal transactivation mediated through protein-protein interaction with other transcription regulators.

The SKOV3 cells, which did not exhibit a mitogenic response to E_2, could not be distinguished from the estrogen responsive PEO4 ovarian carcinoma cells by the extent of E_2 induction of early growth response proto-oncogenes, c-*myc*, c-*fos*, and c-*jun*. Both cell lines, after serum deprivation, exhibited about 2 fold induction of c-*fos* mRNA, 3-4 fold induction of c-*myc* mRNA, and no detectable modulation of c-*jun* mRNA in response to E_2 administered in 5% stripped serum. These studies suggest that induction of c-*myc*, and c-*fos* is not sufficient to achieve a mitogenic response in SKOV3 ovarian carcinoma cells. It is also possible that the overexpression of HER-2/neu observed in the SKOV3 cells (87, and Hua and Clinton, unpublished observations) has caused saturation of some growth signaling pathways.

2

[2]Abbreviations used are: AP-1, activator protein -1; DES, diethylstilbestrol; DTT, dithiothreitol; ER, estrogen receptor; E_2, estradiol; ERE, estrogen response element; FBS, fetal bovine serum; DCFBS, dextran charcoal treated FBS; Kd, dissociation constant; PR, progesterone receptor; PBS, phosphate buffered saline; PMSF, phenylmethyl-sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; TRE, tumor promoter response elements.

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Figures and Legends

Figure 1. Expression of ER mRNA and protein

A. 400 μg of protein from each cell line was analyzed by Western blotting using anti-ER monoclonal antibody D75 at 1 μg/ml (55). *B*. Poly(A) RNA from each cell line was extracted and about 3 μg were analyzed by Northern blotting as described in Materials and Methods. The Northern blot was hybridized with [α-^{32}P]dCTP labeled random primed human ER cDNA probe. The blots were stripped and hybridized with ^{32}P-labeled β-actin (bottom panel) to standardize for amount of mRNA.

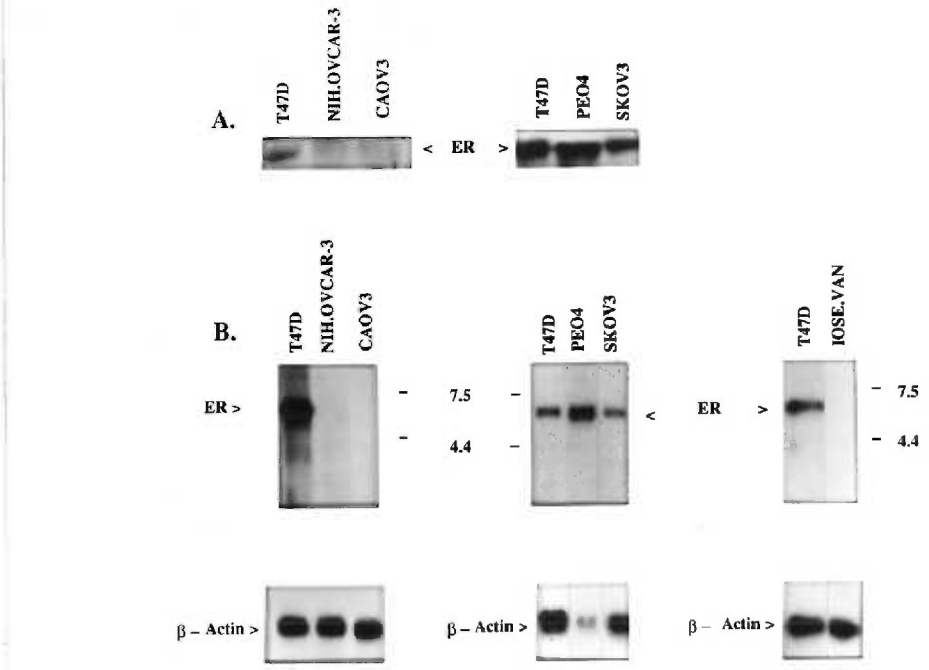

A.
T47D
NIH.OVCAR-3
CAOV3
< ER >
T47D
PEO4
SKOV3
B.
T47D
NIH.OVCAR-3
CAOV3
ER >
7.5
4.4
β – Actin >
T47D
PEO4
SKOV3
<
β – Actin >
T47D
IOSE.VAN
ER >
7.5
4.4
β – Actin >

Figure 2. Effects of E_2 on growth of SKOV3 and T47D cells

5,000 cells/cm^2 were cultured in 6 well plates in phenol red free DMEM supplemented with 5% DCFBS (stripped medium). On day 5, E_2 or the ethanol vehicle were added daily in fresh medium for another 7 days. Cells in triplicate wells were trypsinized and counted using a cytometer. *A*. T47D cell, with stripped medium containing the ethanol vehicle (SM) or with 10 nM E_2. *B*. SKOV3 cells in stripped medium with ethanol vehicle (SM), or with 0.1 nM, 1 nM, and 10 nM of E_2. Each bar represents the mean and standard deviation of the experiments conducted in triplicate.

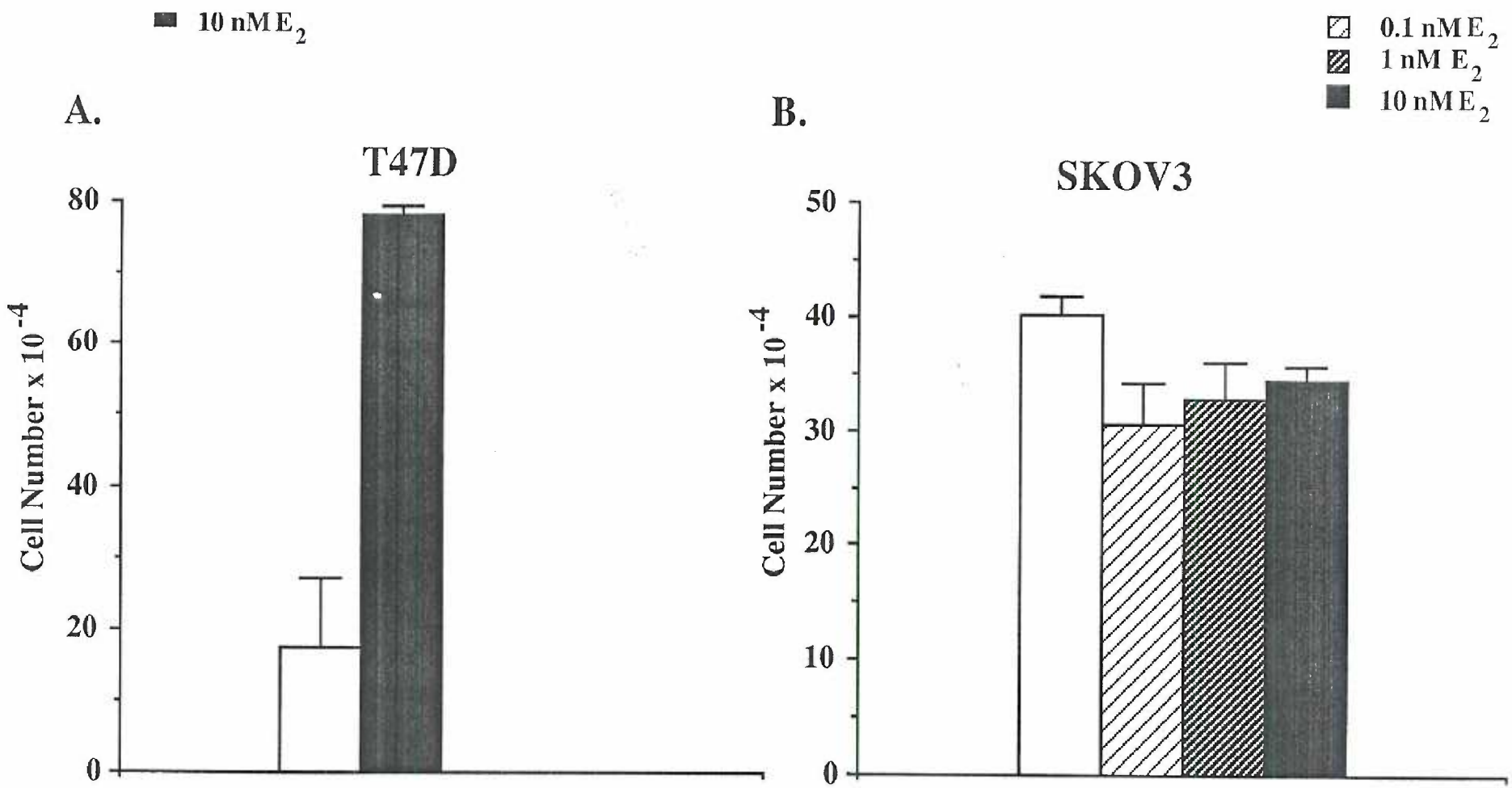

10 nM E2
A.
T47D
Cell Number x 10-4
80
60
40
20
0
0.1 nM E2
1 nM E2
10 nM E2
B.
SKOV3
Cell Number x 10-4
50
40
30
20
10
0

Figure 3. Effect of E_2 on distribution of ER

Cells were cultured in stripped media for 4 days and then treated with E_2 or ethanol vehicle for 4 days. Whole cells were lysed in M-RIPA buffer directly, or fractionated into nuclear and cytosolic fractions as described in Materials and Methods. 400 μg protein from whole cells, 400 μg cytosolic protein, and an equivalent aliquot of the nuclear fraction were analyzed by Western blotting using anti-ER monoclonal antibody D75. *A*. whole cell extracts. *B*. cytosolic fraction. *C*. nuclear fraction.

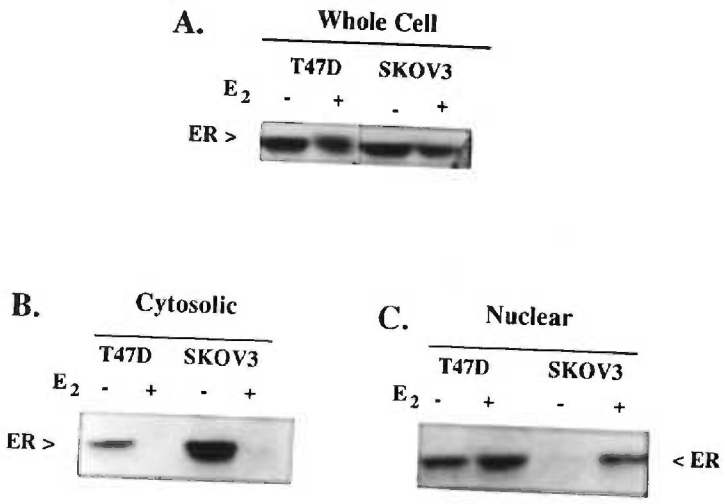

A.
Whole Cell
T47D
SKOV3
E2
-
+
-
+
ER >
B.
Cytosolic
T47D
SKOV3
E2
-
+
-
+
ER >
C.
Nuclear
T47D
SKOV3
E2
-
+
-
+
< ER

Figure 4. E_2 effects on PR expression

A. T47D and SKOV3 cells were cultured in stripped media for 4 days and then in stripped media with ethanol vehicle (*lane 1*), or 10 nM E_2 (*lane 2*) of each panel for 5 days. 400 μg protein from each sample was resolved by SDS-PAGE and immunobloted with 0.01 μg/ml monoclonal anti-PR antibody JZB39 (88). *B*. For PR mRNA detection, T47D and SKOV3 cells were cultured in stripped media for 5 days, and with ethanol vehicle or 10 nM E_2 in media supplemented with 5% DCFBS for the indicated times. Poly(A) RNA was extracted and 5μg was Northern blotted and probed with ^{32}P-labeled human PR cDNA probe. The blots were stripped and rehybridized with ^{32}P-labeled β-actin to standardize for amount of mRNA.

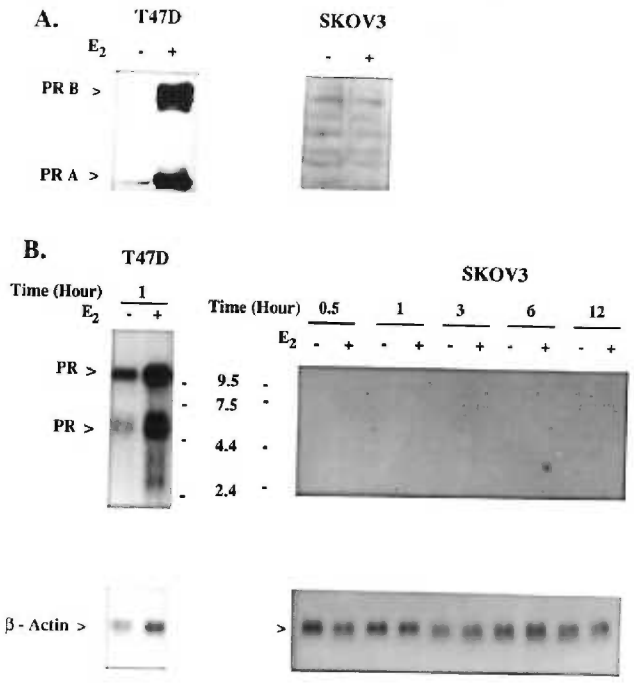

A.
T47D
SKOV3
E2 - +
- +
PR B >
PR A >
B.
T47D
SKOV3
Time (Hour) 1
E2 - +
Time (Hour) 0.5 1 3 6 12
E2 - + - + - + - + - +
PR >
PR >
9.5
7.5
4.4
2.4
β - Actin >
>

Figure 5. E_2 effect on stable transfected ERE-luciferase reporter activity

To test the effect of E_2 on the expression of ERE-Luciferase reporter plasmid, T47D-EN1 (5*A*) and SKOV3-EH4 (5*B*) cells were grown in complete medium (CM), or stripped medium (SM) for 5 days and then were treated with ethanol vehicle (SM and CM), or 10 nM E_2 for 12 h. Luciferase assays were conducted in triplicate experiments as described in Materials and Methods. The error bar indicates the standard deviation.

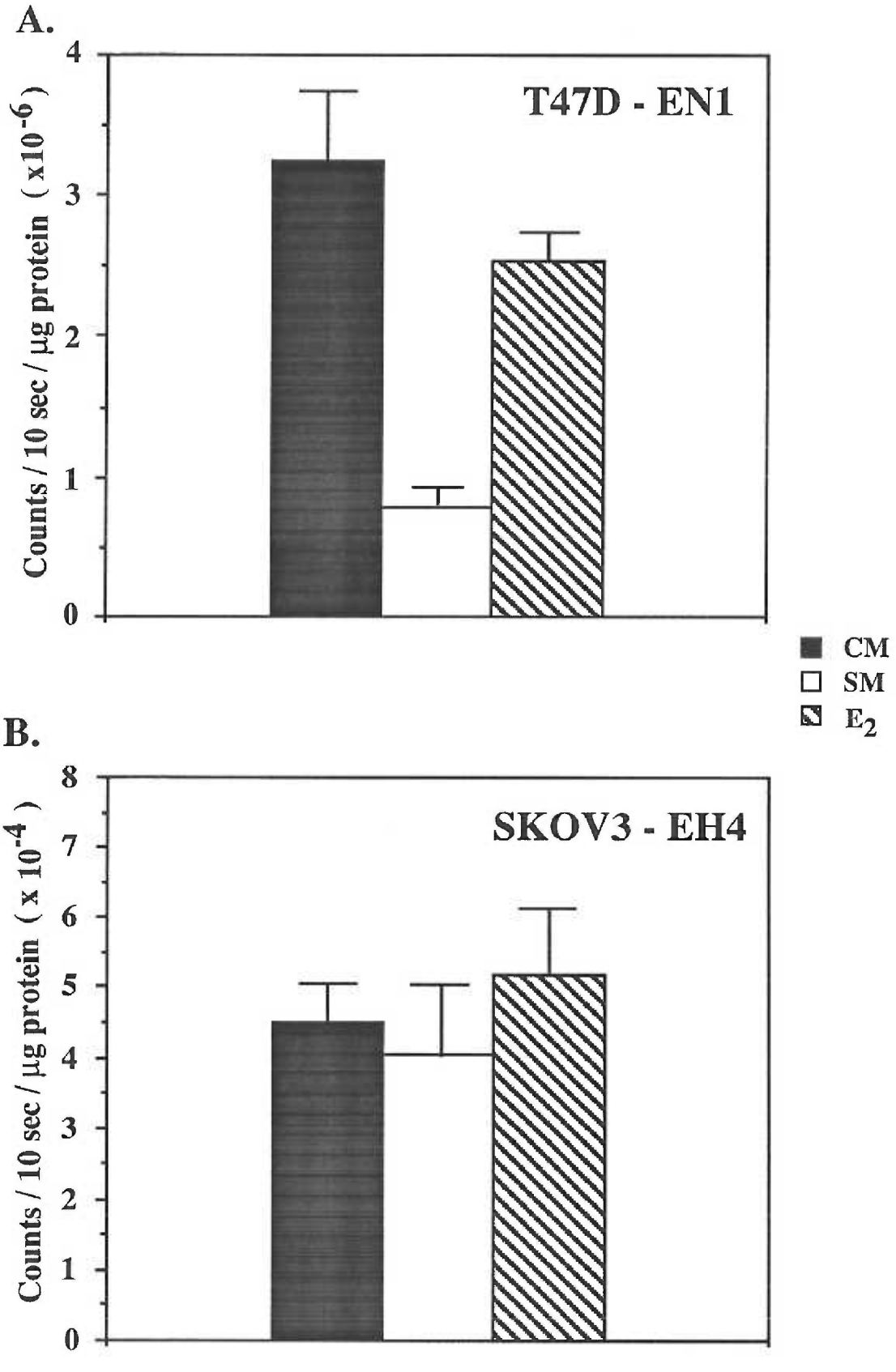

A.
T47D - EN1
Counts / 10 sec / µg protein (x10-6)
0
1
2
3
4
CM
SM
E2
B.
SKOV3 - EH4
Counts / 10 sec / µg protein (x 10-4)
0
1
2
3
4
5
6
7
8

Figure 6. E_2 regulation of c-myc, c-fos, and c-jun expression T47D, SKOV3 and PEO4 cells were cultured first in stripped media with 5% DCFBS for 5 days, in stripped media with 1% dialyzed DCFBS for 48 h, and then treated with ethanol vehicle or 10 nM E_2 in media supplemented with 1%, 5% or 10% DCFBS for 1 h. Poly(A) RNA was extracted from about 3 x 10^7 cells, and 5μg of RNA were analyzed by Northern blotting. The Northern blot was hybridized with [α-^{32}P]dCTP labeled random primed cDNA probes. Blots were hybridized with ^{32}P-labeled c-*myc*, c-*fos*, c-*jun*, and with β-Actin to standardize for amount of RNA.

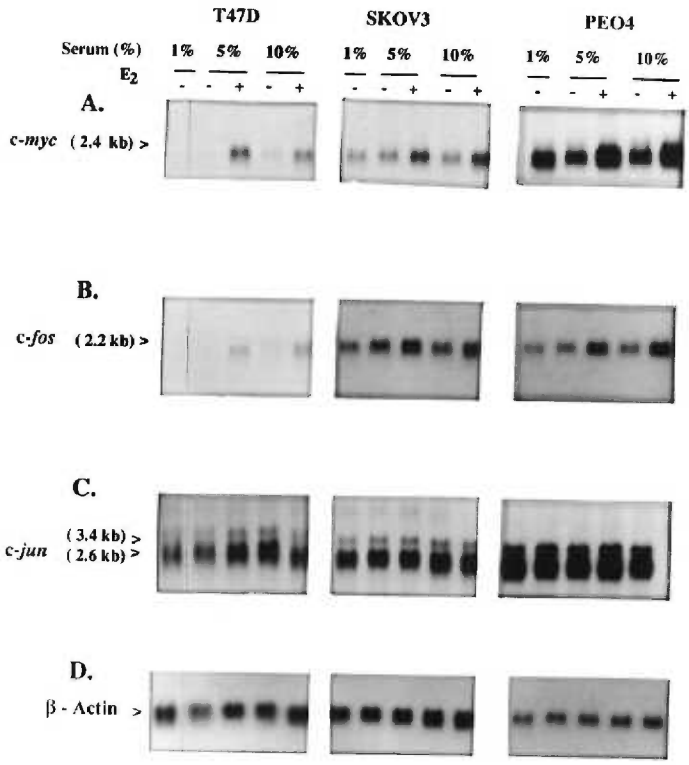
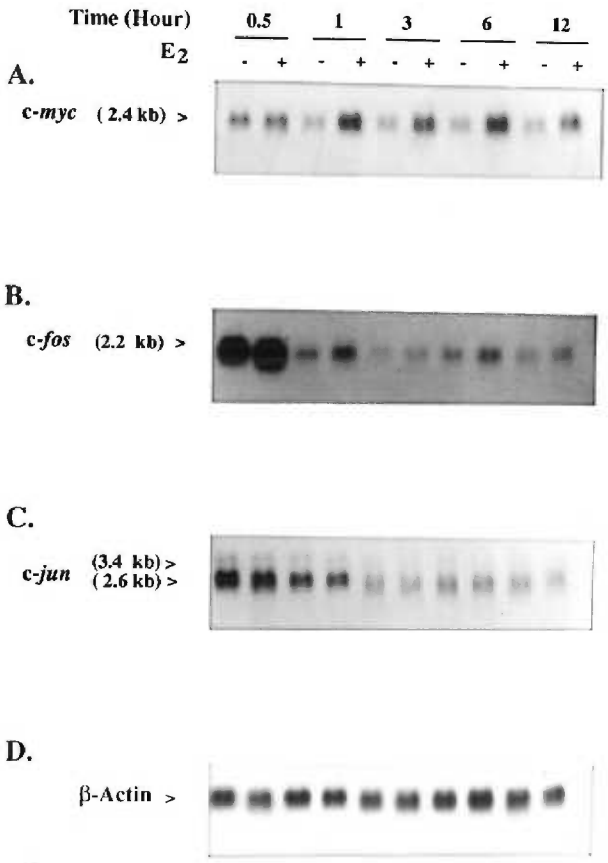

T47D
SKOV3
PEO4
Serum (%)
1% 5% 10%
1% 5% 10%
1% 5% 10%
E2
- - + - +
- - + - +
- - + - +
A.
c-myc (2.4 kb) >
B.
c-fos (2.2 kb) >
C.
c-jun (3.4 kb) >
(2.6 kb) >
D.
β - Actin >

Figure 7. E_2 regulation of c-myc, c-fos, and c-jun expression in SKOV3 cells with time

SKOV3 Cells were cultured and synchronized as in Fig. 6. Cells were treated with ethanol vehicle or 10 nM E_2 in stripped medium with 5% DCFBS for 0.5, 1, 3, 6, and 12 h. Poly(A) RNA was extracted and 5μg of RNA was analyzed by Northern blotting. The Northern blot was hybridized, stripped and rehybridized with different [α-^{32}P]dCTP labeled random primed cDNA probes as in Fig. 6. *A*. c-*myc*, *B*. c-*fos*, *C*. c-*jun*, and *D*. β-Actin.


Time (Hour)
0.5
1
3
6
12
E2
- + - + - + - + - +
A.
c-myc (2.4 kb) >
B.
c-fos (2.2 kb) >
C.
c-jun (3.4 kb) >
(2.6 kb) >
D.
β-Actin >

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Estrogen Represses the rate of p185$^{HER-2/neu}$ Protein Synthesis in T47D Breast Carcinoma Cells

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Abstract

In human breast cancer, both HER-2/neu overexpression and loss of estrogen receptor (ER) correlate with poor prognosis. These two factors may be mechanistically related since 17-β-estradiol (E_2) treatment results in repressed HER-2/neu expression in hormone responsive breast carcinoma cell lines. In studies aimed at examining mechanisms of E_2 regulation of HER-2/neu expression, we found: (a) A 6-12 fold (N=8) repression in the rate of synthesis of the HER-2/neu protein product, $p185^{HER\text{-}2/neu}$, while the extent of repression of HER-2/neu mRNA was 2-2.5 fold (N=6) at 4 to 5 days of E_2 treatment. (b) Modulation of $p185^{HER\text{-}2/neu}$ by E_2 was cell type specific and was not observed in two ER^+ ovarian carcinoma cell lines. (c) Repression of $p185^{HER\text{-}2/neu}$ was specific for E_2 since it occurred in serum-free medium and the sex steroid hormone, progesterone, had no effect. (d) The EGF receptor, a tyrosine kinase receptor that has a negative association with ER status in breast tumors, was not modulated by E_2.

Introduction

Estrogen is known to play a prominent role in breast cancer (1). The receptor for estrogen (ER), is present in most breast tumors, and about 60% of patients with ER^+ tumors have disease that responds to the anti-estrogen, tamoxifen. Patients with tumors that are ER^- rarely respond to anti-estrogen therapy (2-5) and their prognosis is less favorable than patients with tumors that are ER^+ (3,5).

The mechanisms by which estrogen participates in malignant growth have not been elucidated. ER is a DNA binding protein that functions as a transcriptional regulator (1,6,7) and is believed to participate in malignant growth via modulation of expression of oncogenes and other growth regulatory genes (8-16). Steroids exert their control of gene expression at the transcriptional level by well-described mechanisms that involve receptor binding to steroid response elements in the target gene (7,17,18). However, a number of studies indicate that steroids may also regulate gene expression post-transcriptionally. Post-transcriptional regulation by steroids can occur by altering the stability of mRNAs as has been reported in estrogen induction of c-*myc* expression in MCF-7 breast carcinoma cells (9). Examples of steroid modulation of the rate of translation of responsive mRNAs have also been reported (19-22). For example, steroids have been shown to directly alter the translation rates of myelin basic protein and ER mRNAs (23,24). Therefore, estrogen may mediate the expression of growth regulatory genes in breast carcinomas by multiple mechanisms.

One of the most common oncogene abnormalities observed in breast carcinomas is the over-expression of the HER-2/neu gene which occurs in 20-30% of the cases (3,25). Overexpression of the protein product of HER-2/neu either in the presence or absence of gene amplification has been associated with more aggressive disease (3,26-31), loss of differentiation (30-33), and ER^- status in breast carcinomas (27,28,30). Therefore, understanding regulation of HER-2/neu expression by estrogen may be important for revealing mechanisms controlling differentiation, growth, and malignant progression of breast epithelial tissue.

Studies conducted on several estrogen responsive breast carcinoma cell lines including MCF-7, T47D, and ZR-75-1 have revealed that estrogen regulates expression of the HER-2/neu proto-oncogene (13,14,34-36), suggesting that this may be a common function of ER in hormone responsive breast carcinoma cells. Repression of HER-2/neu mRNA levels has been suggested to account for reduction in the amount of the $p185^{HER\text{-}2/neu}$ protein product (13,14,34-36).

We report here that there is a significantly greater effect of estrogen on the rate of protein synthesis as well as steady state $p185^{HER\text{-}2/neu}$ protein levels than on HER-2/neu mRNA levels in T47D cells. Therefore, in the breast carcinoma cells estrogen may affect the rate of translation of HER-2/neu mRNA. In addition, we report that repression of $p185^{HER\text{-}2/neu}$ by estrogen is cell type specific and is not observed in two ER^+ ovarian carcinoma cell lines.

Materials and Methods

Materials

Chemicals and reagents were obtained from Sigma (St. Louis, MO) unless specified. Reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoreses (PAGE) were obtained from Bio-Rad (Hercules, CA).

Cell lines and cell culture

T47D (Breast carcinoma) (37) and SKOV3 (ovarian carcinoma) (38,39) cell lines were obtained from American Type Culture Collection (A.T.C.C.). PEO4 (ovarian carcinoma) cells were provided by Dr. Thomas C. Hamilton (40). T47D and PEO4 cells were routinely maintained in RPMI 1640 (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (FBS) (Hyclone Lab. Inc., Logan, UT) 10 μg/ml insulin, and 0.5% gentamicin. SKOV3 cells were maintained in DMEM (JRH Biosciences) supplemented with 10% FBS and 0.5% gentamicin. All cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Treatment of Cells with Estrogen

Cells were plated in complete growth medium overnight and washed three times with phosphate buffered saline (PBS) (2.7 mM KCL, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4 and 0.14 M NaCl, pH 7.4). The medium was then replaced with phenol-red free DMEM (JRH Biosciences, Lenexa, KS) supplemented with 5% dextran charcoal treated FBS (DCFBS) (stripped media). To prepare DCFBS, 1% Norit A and 0.1% Dextran T-40 were incubated in a buffer containing 0.25 M

Sucrose, 1.5 mM $MgCl_2$, 10 mM HEPES pH 7.4 at 4°C overnight. The dextran charcoal was then mixed with FBS, and the mixture was heated at 55°C for 2 h and then incubated at 4°C overnight. The dextran charcoal was removed by centrifugation at 3,000 rpm in a TJ-6 Beckman table top centrifuge for 15 min, and by filtering the serum through a 0.2 μm filter (Becton Dickinson Labware, Lincoln Park, N.J.). After the cells were incubated in stripped media for 4 days with daily changes of media, they were then treated with, 10 nM of 17-β Estradiol (E_2) or with ethanol vehicle.

Antibodies

Monoclonal anti-PR antibody JZB39 was a generous gift from Dr. Geoffrey L. Greene (41). Polyclonal anti-HER-2 antibody was produced as described (42). Antibody to EGFR was an antipeptide antibody against a sequence in the C-terminus of the protein (43).

[^{35}S]met/cys metabolic labeling of cells

3 x 10^6 cells in a 6 cm culture plate were washed 3 times with HANK's balanced salt solution and starved for methionine and cysteine by incubation in HANK's for 1 h. The media was removed and the cells were labeled with [^{35}S]met/cys in 1.2 ml of HANK's. Cells were then washed with PBS 3 times and solubilized in M-RIPA buffer {50 mM Tris-HCl pH 7.4, 1% NP40, 0.1% Deoxycholate Acid, 0.15 M NaCl, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 10 mM Na Pyrophosphate, 10 mM NaF, 4 mM EDTA, 2 mM Na_3VO_2, 1% Aprotinin and 10 μg/ml Leupeptin}. Cell lysates were centrifuged in a

microcentrifuge at maximal speed for 10 minutes to remove insoluble material. Protein concentration in the supernatant was determined using a Bio Rad protein assay kit (Bio-Rad, Hercules, CA).

To determine incorporation of [^{35}S]met/cys into total cell protein, 5 μl of radiolabeled cell extract was mixed with 5 μl of 10 mg/ml bovine serum albumin and 200 μl of 10% trichloracetic acid (TCA) and the mixture was incubated at 4°C for 10 min. Precipitated protein was collected by centrifugation in a microcentrifuge at 12,000g for 1 min, dispersed and washed twice with 500 μl of 10% TCA, and dissolved in 100 μl of water. The protein solution was mixed with 10 ml Scintiverse BD cocktail (Fisher Scientific, Fair Lawn, NJ) and incorporated isotope was quantitated using a Beckmann LS1800 scintillation counter.

Immunoprecipitation

Immunoprecipitation of ^{35}S-labeled cell extract was conducted as described (42), The extract was precleared by incubation for 30 min with 60 μl of a 50% suspension of sepharose-6B. The cleared extract was immunoprecipitated using 6 μl of anti-HER-2/neu antibody bound to protein A sepharose. Samples were resolved by SDS-PAGE containing 7.5% acrylamide. The gel was enhanced in Fluoro-Hance autoradiography enhancer (Research Products International Corp., Mount Prospect, IL) for 30 min, and the dried gel was autoradiographed at -70°C with an intensifying screen (X-ray Inc., Portland, OR).

Western Blot Analysis

Western blot analysis was conducted as described (42). When polyclonal

anti-HER-2, or anti-EGFR was the first antibody, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Bio Rad, Hercules, CA) was used as the second antibody. When monoclonal anti-PR antibody was the first antibody, rabbit-anti-rat IgG (DAKO Corporation, Carpinteria, CA) was used as bridging antibody.

To strip the blot, the membrane was incubated in 62.5 mM Tris.HCl, pH 6.7, 2% SDS and 100 μM β-mercaptoethanol (stripping buffer) for 30 minutes at 50°C, rinsed with saline, and was then blocked with 5% nonfat milk for 1 h at 22-24°C. The membrane was then ready to react with a different antibody.

Isolation of Poly(A) RNA

Isolation of mRNA was performed according to a procedure described by Schwab,M. et al. (44). 3 x 10^7 cells were lysed in 7.5 ml of 10 mM Tris - HCl pH 7.4, 0.1 M NaCl, 2 mM EDTA, and 1% SDS. DNA was sheared by passing the lysate through a 21 gauge needle. The lysate was digested with 100 μg/ml of proteinase K for 30 min at 37°C, and NaCl was added to a final concentration of 0.4 M. The lysate was then mixed overnight at 24°C with 160 mg of pre-washed Oligo-dT cellulose (Collaborative Research, Bedford, MA), collected by centrifugation, and washed once in 10 ml high salt buffer (10 mM Tris - HCl pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.2% SDS). Oligo-dT, resuspended in 10 ml of high salt buffer, was poured into a polyprep column (Bio Rad, Hercules, CA), and the column was washed once with 10 ml of high salt, and once with 1.75 ml of low salt buffer with 0.1 M NaCl. mRNA was eluted from the column with 3.5 ml of buffer containing no salt. The eluate was adjusted to 0.2 M sodium acetate and

67% ethanol and the mRNA was precipitated overnight at -20°C. The mRNA was recovered by centrifugation at 7,000 rpm in an SS34 Beckman rotor for 45 min at 4°C and air dried. mRNA was resuspended in TE buffer (1 mM EDTA, 10 mM Tris - HCl pH 7.4) and stored at -70°C.

Northern Blot Analysis

5 μg of poly(A) RNA was separated on a formaldehyde-denaturing gel containing 1% agarose and was then transferred onto a nytran membrane (Schleicher & Schuell, Keene, NH) by capillary action as described (45). RNA was fixed on the membrane by UV crosslinking and membranes were prehybridized for 4 h at 42°C in a hybridization buffer containing 50% formamide, 5 x SSC (1 x SSC = 0.15 M NaCl and 15 mM Sodium Citrate, pH 7.0), 0.1% SDS, 250 μg/ml herring sperm DNA, and 1 x Denhardt's solution to block non-specific cDNA binding. cDNA probes were radiolabeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA), using random primer DNA labeling kit (Boehringer Mannheim, Indianapolis, IN), and hybridization was carried out overnight in hybridization buffer containing 0.5-1 x 10^6 cpm/ml of [α-^{32}P]dCTP-labeled cDNA probe. Membranes were washed 20 min at room temperature followed by 5 min at 65°C in 2 x SSC containing 1% SDS and then at room temperature for 60 min in three changes of 1 x SSC containing 1% SDS. The membranes were exposed to X-ray film and autoradiographs were analyzed by a laser densitometer (Bio Rad, Hercules, CA). For hybridization with additional probes, the membranes were first stripped by boiling in two changes of 0.1 x SSC for 5 min.

Results

Effect of E_2 on HER-2/neu mRNA levels

E_2 treatment of breast carcinoma cell lines has been reported to result in repression of HER-2/neu expression (13,14,34-36). It is unclear, however, if the reduction in mRNA accounts for the extent of repression of the $p185^{HER\text{-}2/neu}$ protein product. To further investigate mechanisms of regulation by E_2, HER-2/neu mRNA levels were assessed by Northern blot analyses of cells treated with E_2 for different periods of time up to 5 days. A reduction of HER-2/neu mRNA levels relative to β-actin mRNA was detected as early as 6 h following administration of E_2 (Fig. 1A) with an approximate 50% reduction observed by 24 h. A 2-2.5 fold repression of HER-2/neu mRNA was observed at 5 days of E_2 treatment in six separate experiments. On the other hand, β-actin mRNA levels were not significantly altered for up to 5 days of E_2 treatment (Fig 1B).

Effects of E_2 on $p185^{HER\text{-}2/neu}$ synthesis

To determine whether the reduction in mRNA levels was reflected in $p185^{HER\text{-}2/neu}$ protein levels, we conducted Western blot analysis of cells treated with or without E_2 for different times. A small reduction in steady state levels was detected as early as 1 day of hormone treatment (data not shown), and an average 10 fold (N = 15) reduction in $p185^{HER\text{-}2/neu}$ protein levels was observed by 3-7 days of E_2 treatment (see Fig. 4A and 6A). Therefore, the extent of repression of steady state levels of $p185^{HER\text{-}2/neu}$ was substantially greater than that of HER-2/neu mRNA levels.

To assess whether E_2 affected the rate of protein synthesis, we determined the amount of [^{35}S]met/cys incorporated into total cell protein and specifically into p185$^{HER\text{-}2/neu}$ during a 1 hr pulse labeling period. In comparison to an approximate 25% depression in mRNA levels (Fig. 1A), there was about a 3 fold reduction in isotope incorporation into p185$^{HER\text{-}2/neu}$ at 6 h of E_2 treatment (Fig. 2B). E_2 caused a specific reduction in radio-labeled precursor incorporation into p185$^{HER\text{-}2/neu}$ rather than affecting incorporation into total protein, since the amount of [^{35}S]met/cys per μg of total protein was not significantly altered by E_2 treatment (Fig. 2B). Therefore, E_2 appeared to affect the synthetic rate of p185$^{HER\text{-}2/neu}$. At 5 days of E_2 treatment, there was an 8 fold reduction in the rate of incorporation of [^{35}S]met/cys into p185$^{HER\text{-}2/neu}$ (Fig. 3). In separate experiments (N=8), E_2 effects on synthetic rate varied from 6-12 fold. These studies indicate that E_2 treatment reduced the rate of p185$^{HER\text{-}2/neu}$ protein synthesis to a similar extent as the steady state levels of p185$^{HER\text{-}2/neu}$ protein product. The extent of repression at the protein level was consistently and reproducibly greater than the repression observed in the mRNA levels.

Effects of E_2 on levels of p185$^{HER\text{-}2/neu}$ in serum-free medium

Studies of E_2 effects on p185$^{HER\text{-}2/neu}$ have been conducted by culturing cells in charcoal stripped serum (CSS) for 3 to 6 days to deplete steroid levels, followed by addition of E_2 (13,14,36) However, stripping serum with charcoal/dextran depletes components other than estrogen. To test whether E_2-repression of p185$^{HER\text{-}2/neu}$ depended on serum factors, T47D cells were cultured

in serum free, phenol red-free medium that was supplemented with insulin, transferrin, and selenium (ITS) for 4 days before E_2 or ethanol vehicle was added to cells. A decrease of about 8 fold in $p185^{HER\text{-}2/neu}$ levels was observed by as early as 3 days of treatment with E_2 (Fig. 4A). Repressed levels of $p185^{HER\text{-}2/neu}$ were maintained for at least 7 days in the presence of E_2. This result suggests that E_2 functions in the absence of serum to down-regulate $p185^{HER\text{-}2/neu}$ in T47D breast carcinoma cells.

Estrogen is known to stimulate production of progesterone receptor (PR) (8), a transcriptional regulator (46-48) with central importance in controlling the growth and function of female reproductive tissue (49). To contrast the effects of E_2 on PR and $p185^{HER\text{-}2/neu}$, the blot illustrated in Figure 4A was stripped and reprobed with monoclonal antibody against PR. Corresponding to inhibition of $p185^{HER\text{-}2/neu}$, there was an increase in the A and B forms of PR (Fig. 4B). However, the effect of E_2 on PR was less with a 2 fold increase observed at 3 days and a maximum increase of 4 fold at 7 days.

Repression of $p185^{HER\text{-}2/neu}$ is specific for E_2

Because PR regulates gene expression and is induced by estrogen, we considered the possibility that progesterone may participate in the repression of $p185^{HER\text{-}2/neu}$. We therefore tested the effects of progesterone (P_4) on $p185^{HER\text{-}2/neu}$ levels in T47D cells. The cells were grown in stripped medium for 4 days to deplete P_4 pools and were then treated with 100 nM or 1 μM P_4 for 7 days. As expected, incubation of cells in stripped medium greatly enhanced $p185^{HER\text{-}2/neu}$

levels. However, there was no detectable effect of P_4 on $p185^{HER\text{-}2/neu}$ levels (Fig. 5).

E_2 effects on $p185^{HER\text{-}2/neu}$ levels in ovarian carcinoma cells

E_2 has been found to down regulate HER-2/neu in three hormone responsive breast carcinoma cell lines T47D, MCF-7 and ZR-75-1 (13,14,35). Similar to breast cancer, about 60% of ovarian cancers express ER (50-52), some ovarian carcinoma cell lines are estrogen responsive for growth (40,53-56), and HER-2/neu is overexpressed in ovarian cancers (3,57,58). To determine whether ER may also regulate HER-2/neu levels in ovarian carcinomas, we examined two ovarian carcinoma cell lines that express ER. SKOV3 cells that are E_2 independent, and PEO4 cells that are E_2 responsive for growth (40,53) (Hua, Christianson, and Clinton submitted). For comparison, T47D cells were examined in parallel. There was little or no effect of stripped medium or E_2 treatment on $p185^{HER\text{-}2/neu}$ levels in either SKOV3 cells (Fig. 6B) or PEO4 cells (Fig. 6C). In marked contrast, $p185^{HER\text{-}2/neu}$ levels were elevated about 20 fold by cultivation of T47D cells in stripped media and were reduced about 15 fold by E_2 addition to the stripped medium (Fig. 6A). These results indicate a cell type specificity in E_2 down regulation of $p185^{HER\text{-}2/neu}$ in breast but not in two ovarian carcinoma cell lines.

Since clinical observations suggest a negative correlation between ER and epidermal growth factor receptor (EGFR) expression (59,60), we tested whether EGFR levels were affected by E_2 treatment of the T47D cells. The immunoblot

shown in Figure 6A was stripped and probed with antibodies against EGFR. In marked contrast to $p185^{HER\text{-}2/neu}$, E_2 had no effect on the expression of EGFR (Fig. 6D).

Discussion

We conclude that the E_2-dependent reduction of steady state levels of $p185^{HER\text{-}2/neu}$ was consistently greater than the extent of repression of HER-2/neu mRNA levels. While mRNA levels were reduced 2.5 fold at the most (N=6) as a result of E_2 treatment the reduction of $p185^{HER\text{-}2/neu}$ levels averaged about 10 fold (N=15).

To evaluate the effect of E_2 on the rate of $p185^{HER\text{-}2/neu}$ synthesis, we utilized short pulse labeling with the radiolabeled precursor [^{35}S]met/cys. The rate of incorporation into immunoprecipitated $p185^{HER\text{-}2/neu}$ was linear for up to 60 min in the presence or absence of E_2. Therefore a pulse of 60 min or less reflected rate of incorporation without the complication of protein turnover. Further, since E_2 did not significantly affect rate of incorporation of ^{35}S into total protein, it appeared that E_2 effects on $p185^{HER\text{-}2/neu}$ reflected the synthetic rate of this protein. The effect of E_2 on steady state levels and on the rate of synthesis of $p185^{HER\text{-}2/neu}$ were similar (about 10 fold) providing further evidence that the pulse labeling experiments accurately reflected E_2 effects on rate of protein synthesis. A 15 min pulse label followed by a chase with excess unlabeled met/cys did not reveal an effect of E_2 on $p185^{HER\text{-}2/neu}$ turnover rate (Hua and Clinton, unpublished observations). Together these results all suggest that E_2 down regulates HER-2/neu expression at the level of protein synthesis in T47D cells.

While we observed the main effect of E_2 on HER-2/neu expression to be

at the level of protein rather than mRNA, others have reported effects to be at the mRNA level (13,14,35,36). There are several possible explanation for this discrepancy. There appears to be a wide variation in the extent of E_2 repression of $p185^{HER\text{-}2/neu}$ levels in separate isolates of the same cell type. For example Read & Katzenellebogen reported no E_2 effect on HER-2/neu expression in T47D cells, while Dati et al. (14) reported a strong effect in T47D cells. For the T47D cells used in our study, the repression in steady state levels of $p185^{HER\text{-}2/neu}$ by E_2 measured in separate experiments ranged widely from 5 to 20 fold. On the other hand, E_2 dependent repression of HER-2/neu mRNA levels was constant ranging only from 2 to 2.5 fold (N=6). Therefore, it appears that environmental as well as cell specific factors influence the extent of E_2 down regulation of HER-2/neu protein product to a greater extent than HER-2/neu mRNA levels.

While most estrogen responsive breast carcinoma cells exhibit E_2 down-regulation of HER-2/neu, the HER-2/neu from ovarian carcinoma cell lines SKOV3 and PEO4 was not affected by estrogen. This could not be explained by differences in growth response, since SKOV3 are estrogen independent (Hua, Christianson and Clinton, submitted), while PEO4 cells are estrogen responsive for growth (40,53). The function of E_2 in down regulation of HER-2/neu may be different in ovarian tissue. It is also possible that the function of ER is altered in these two ovarian carcinoma cell lines. Neither the PEO4 cells nor the SKOV3 cells exhibit E_2 induction of PR (Hua, Christianson, and Clinton, submitted). It is possible that the same functional region of ER is responsible for PR induction

and for HER-2/neu down-regulation explaining the results obtained in the ovarian carcinoma cells.

1

[1]Abbreviations used are: EGFR, epidermal growth factor receptor; ER, estrogen receptor; E_2, 17-β-estradiol; ERE, estrogen response element; FBS, fetal bovine serum; DCFBS, dextran charcoal treated FBS; HRP, horse radish peroxidase; PR, progesterone receptor; P_4, progestin; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoreses; TCA, trichloroacetic acid.

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Figures and Legends

Figure 1. Effect of E_2 on HER-2/neu mRNA

T47D cells were deprived of E_2 in stripped medium for 4 days *A*. Cells were treated for 0, 6, 12, 24 and 48 h with 10 nM E_2, and in *B*. replicate cultures were treated with 10 nM E_2 (lanes 2 and 4) or ethanol vehicle (lanes 1 and 3) for 5 days. 5 μg of Poly (A) mRNA was analyzed by Northern blotting. The Northern blot was hybridized with [α-^{32}P]dCTP random primed 420 bp rat-neu cDNA (61). Blots were then hybridized with ^{32}P-labeled β-actin to standardize for amount of RNA. *A*. HER-2/neu mRNA relative to β-actin mRNA level was determined by densitometer scanning (Bio Rad, Hercules, CA) of the autoradiograph.

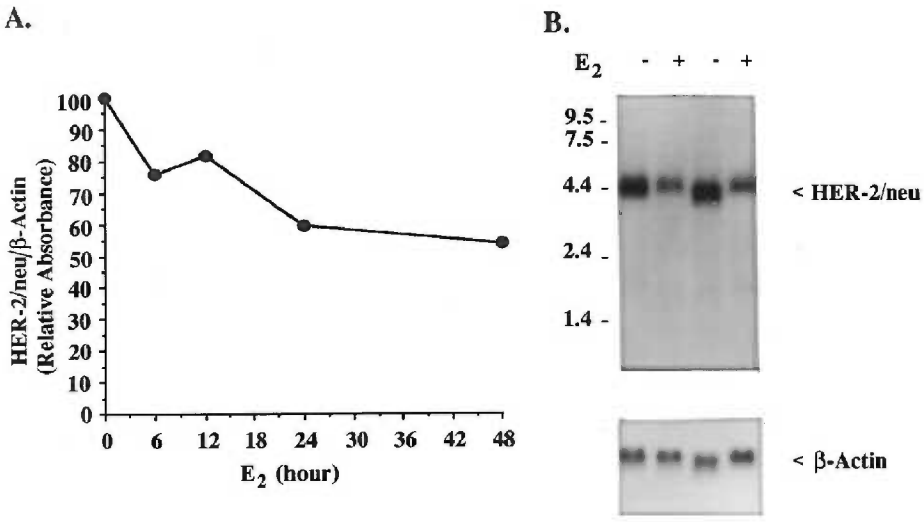

A.
HER-2/neu/β-Actin
(Relative Absorbance)
100
90
80
70
60
50
40
30
20
10
0
0 6 12 18 24 30 36 42 48
E2 (hour)
B.
E2 - + - +
9.5
7.5
4.4
2.4
1.4
< HER-2/neu
< β-Actin

Figure 2. E_2 regulation of p185$^{HER-2/neu}$ protein synthesis.

A. T47D cells were cultivated in 'stripped medium' for 4 days and then treated with ethanol vehicle (lane 1) or 10 nM E_2 (lanes 2-5) for the indicated time. During the last 2 h treatment cells were incubated in HANK's salts solution for 1 h and pulse labeled for 1 h with 200 μCi/ml [^{35}S]Met/Cys in the presence of ethanol vehicle or E_2. p185$^{HER-2/neu}$ was immunoprecipitated from radio-labeled cell extract with 6 μl anti-HER-2/neu antibody. *B*. Quantitation of p185$^{HER-2/neu}$ levels by densitometric scanning of film shown in *A*. To determine amount of isotope incorporated into total protein, 5μl aliquots of radiolabeled cell extract was precipitated with 10% TCA, washed, and counted by scintillation spectroscopy. Incorporation was standardized to amount of protein in each sample.

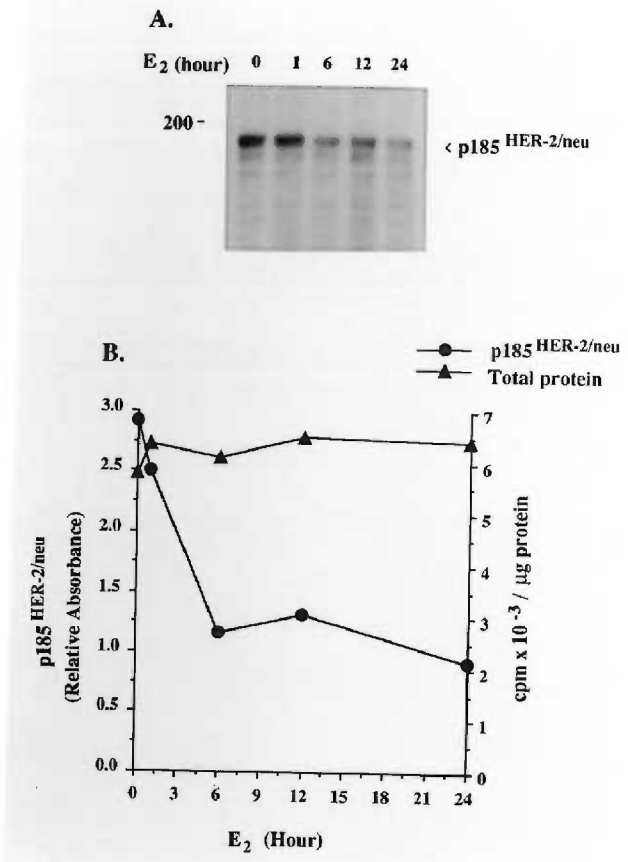

A.
E2 (hour) 0 1 6 12 24
200 -
‹ p185 HER-2/neu
B.
p185 HER-2/neu
Total protein
p185 HER-2/neu (Relative Absorbance)
cpm x 10 -3 / µg protein
E2 (Hour)

Figure 3. Rate of synthesis of p185$^{\underline{\text{HER-2/neu}}}$ at 5 days of $E_{\underline{2}}$ treatment

T47D cells were cultured in 'stripped media' for 4 days, treated with ethanol vehicle, or 10 nM E_2 for 5 days, then labeled with 300 μCi/ml [^{35}S]Met/Cys for 15, 30, 60 min. *A*. immunoprecipitation of p185$^{\text{HER-2/neu}}$. *B*. The amount of p185$^{\text{HER-2/neu}}$ was plotted according to densitometric scanning of p185$^{\text{HER-2/neu}}$ amount in *A*.

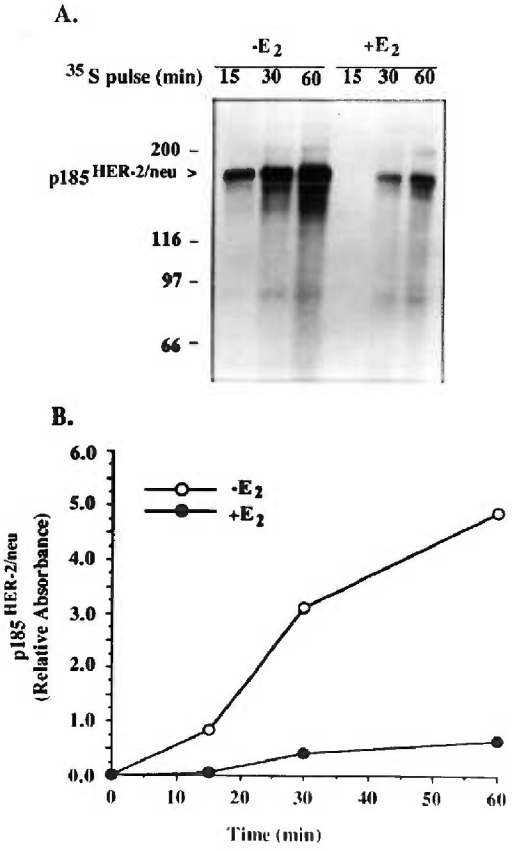

A.
-E2
+E2
35S pulse (min)
15 30 60 15 30 60
200 -
p185HER-2/neu >
116 -
97 -
66 -
B.
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-E2
+E2
p185HER-2/neu (Relative Absorbance)
0 10 20 30 40 50 60
Time (min)

Figure 4. Effects of E_2 on p185$^{HER\text{-}2/neu}$ and PR levels in serum free media

A: Cells maintained for 4 days in serum free, phenol red-free DMEM, and supplemented with 5 μg/l insulin, 5 μg/l transferrin, and 5 ng/l selenium (ITS) (Collaborative research Inc., Bedford, MA) were then treated with ethanol vehicle for 3 days and 7 days, or with 10 nM E_2 for 3 days, and 7 days. 200 μg of protein extract was subjected to Western blot analysis using anti-HER-2 antibody. *B*: The blot in *A* was stripped and incubated with 0.01 μg/ml anti-PR antibody JZB39.

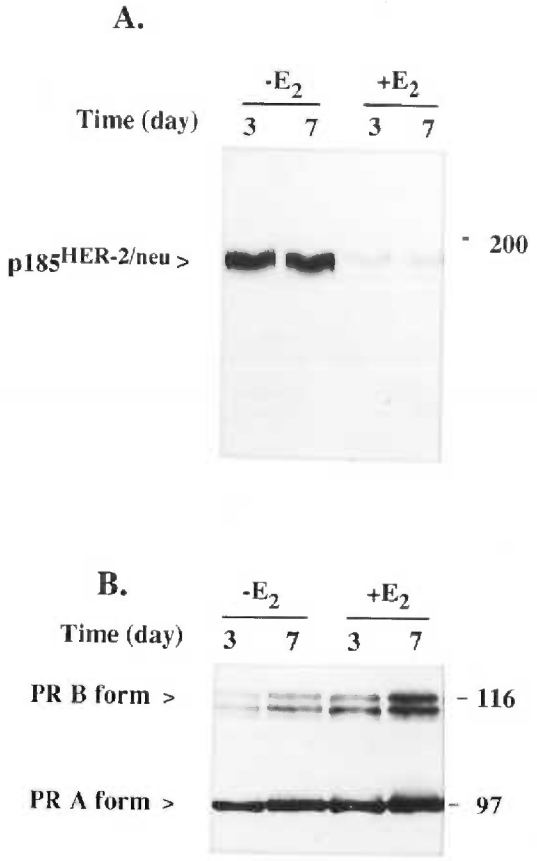

A.
-E2
+E2
Time (day)
3
7
3
7
p185HER-2/neu >
200
B.
-E2
+E2
Time (day)
3
7
3
7
PR B form >
116
PR A form >
97

Figure 5. Effect of P_4 on $p185^{HER\text{-}2/neu}$ expression

T47D cells were cultured in 'complete medium' (lane 1), or 'stripped medium' (lanes 2-4) for 4 days and then were treated with ethanol vehicle (CM and SM), or with either 100 nM or 1 μM of P_4 for 7 days. $p185^{HER\text{-}2/neu}$ was detected by Western blotting.

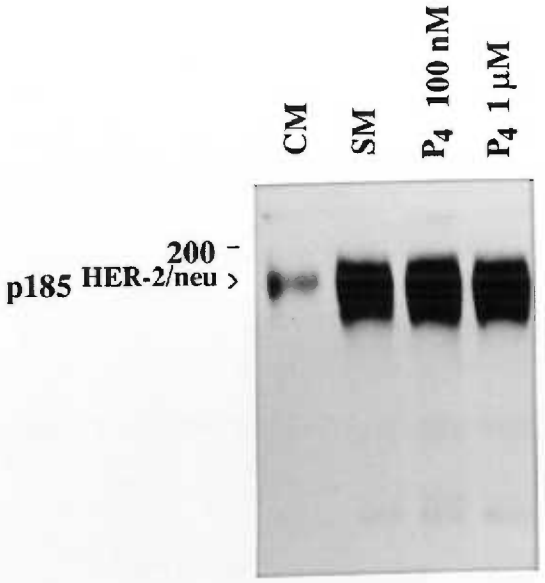
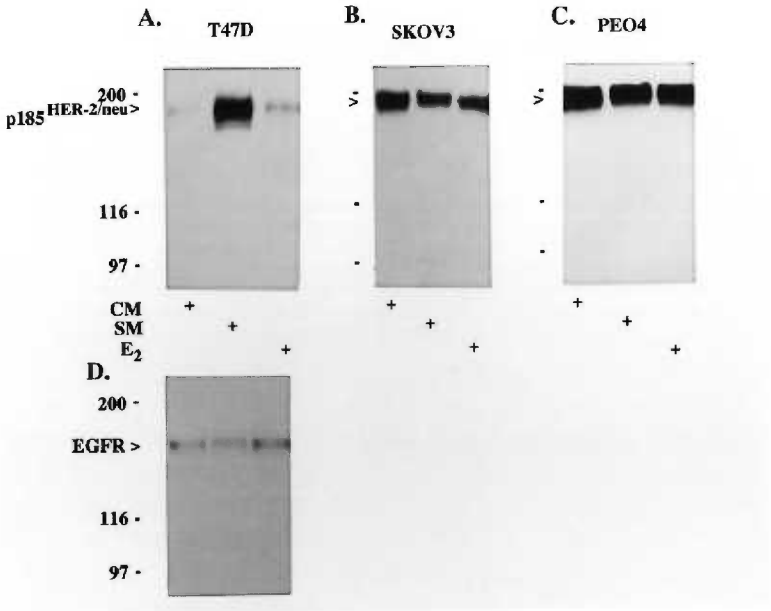

CM
SM
P4 100 nM
P4 1 μM
200
p185 HER-2/neu

Figure 6. Cell type specificity in regulation of p185$^{HER-2/neu}$ expression by E_2

T47D (*A*), SKOV3 (*B*), or PEO4 (*C*) cells were maintained in 'complete media' (lane 1), or 'stripped media' (lanes 2 and 3) for 4 days and then were treated with ethanol vehicle (CM and SM), or 10 nM E_2 (E) for 7 days. 200 μg of protein was subjected to Western blot analysis using anti-HER-2 antibody. *D*. The blot in *A* was stripped and reprobed with 1:5,000 dilution of anti-EGFR antibody.


A.
T47D
B.
SKOV3
C.
PEO4
200 -
p185HER-2/neu>
116 -
97 -
CM
SM
E2
+
D.
200 -
EGFR >
116 -
97 -

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Conclusions and Discussions

Estrogen is known to play a critical role in breast cancer. Although ER is expressed in most ovarian cancer, the role of estrogen in this disease is not known. In the current study, I describe molecular effects of estrogen on growth and gene regulation in ovarian and breast carcinoma cells.

I. Molecular effects of E_2 in ovarian cancer cells

With the goal of understanding the function of ER in ovarian cancer, the molecular expression and E_2-mediated functions of ER were analyzed. Much of these studies were focussed on SKOV3 cells that are ER^+ but E_2 growth independent, which is a model for hormone independent ovarian cancer.

A. E_2 Response of ER in SKOV3 cells

Although E_2 did not induce growth of SKOV3 cells, ER exhibited several normal functions. ER in SKOV3 cells bound ligand with normal affinity indicating that the hormone binding domain (HBD) functioned normally. E_2 induced nuclear localization of ER in SKOV3 cells, indicating normal functioning nuclear localization signals. Constitutive nuclear localization signals have been located at the C/D border and in the D domain, corresponding to the C-terminus of the DNA binding domain and hinge domain of ER (1). However, the hormone binding domain is required in addition to these nuclear localization signals in order to achieve hormone induced nuclear localization of ER (1). By using

immunohistochemistry techniques, Ylikomi et al. (1) showed ligand induced nuclear accumulation of an ER mutant with deletions in both the A/B domain and the zinc finger in the DNA binding C domain. Therefore, the nuclear localization signal in ER from SKOV3 cells appears to be normal. Tight nuclear association of ER likely occurs by interaction of ER with nuclear proteins, since a functional DNA binding domain is not required.

B. ER is defective in transcriptional regulation in SKOV3 cells.

When the transactivation function of ER was studied, we found that in SKOV3 cells, E_2 was unable to induce the expression of PR and a vitellogenin ERE (vitERE) driven reporter gene when directly compared to T47D breast carcinoma cells.

Induction of PR is a classical marker for ER function (2,3). A half palindrome ERE is found in hPR, and it is believed that ER induces the expression of PR through binding with this ERE (4,5). ER deletion mutants that lack a DNA binding domain (DBD), or 'dominant negative' mutations that interfere with the DNA binding of wild type ER do not induce PR (6,7,8). ER binding with DNA is also required for E_2 induction of vit ERE reporter gene (9,10), which contains a perfect palindrome ERE that binds ER with high affinity (10).

In summary, the absence of E_2 induction of PR and vitERE suggests a defect in DNA binding function of ER. One possibility may be a defect in the DNA binding domain of ER that abolishes DNA binding ability. Another

possibility may be the presence of a 'dominant negative' mutation that interferes with the function of wild ER in SKOV3 cells. However, for efficient interference of wild type ER function by 'dominant negative' mutations, the mutant ER has to be 10-20 times more abundant than the wild type ER (8). Dominant mutations that have been reported in breast cancers are deletion mutations that are derived from alternative splicing of ER mRNA (8,11). We detected ER of normal size in SKOV3 cells and did not detect alternative forms or variant ERs. It is possible that there were point mutations, or very small deletions of ER that could not be detected in the Northern or Western blot analysis, or variant ER was in such a small amount that it was below the sensitivity limit (less than 1/10 of the ER mRNA and 1/20 of ER protein detected in SKOV3 cells) of Northern and Western analysis. These results may also be explained by the existence of cell specific inhibitors or the absence of activators that may affect binding of ER with ERE, or may interfere with the transactivating activity of ER. Furthermore ER could also exibit a defect in a transactivation domains that specifically blocks expression of genes that contain an ERE.

C. ER is a transactivator of early growth regulatory genes in SKOV3 cells

Although PR and vitERE were not induced, E_2 stimulated expression of c-*myc* and c-*fos* in SKOV3 cells. This result indicated that ER functioned as a transcription activator in regulation of these two immediate early genes in SKOV3 cells. Interestingly, the c-*myc* promoter does not contain an ERE (12). Thus it seems that ER has separate functions in transactivation of genes through ERE

binding on one hand, and transactivation of the c-*myc* promoter on the other hand.

D. Hypothesis to explain abnormal transcription regulation by E_2 in SKOV3 cells

Based on our finding that ER was defective in PR and vitERE-reporter gene induction but functioned in induction of c-*myc* and c-*fos* in SKOV3 cells, I propose that either ER has a defect in gene regulation mediated by binding to ERE but may function normally in gene regulation through protein-protein interaction with other transcription regulators involved in expression of a separate class of genes such as c-*myc*, or ER may bind ERE but may be unable to mediate transactivation through ERE binding because of cell specific factors.

Protein-protein interaction of ER with other transcription regulators has been reported. For example, AP-1 (a transcription complex that contains *fos-jun*, and *jun-jun* dimers) and ER cooperatively increase expression of ovalbumin reporter plasmid which contains a consensus sequence that binds both ER and AP-1 (13). Evidence has shown that direct ER binding to ERE is not required in this activation, because ER with deletion of the DNA binding domain still functioned (13). Another example is E_2 inhibition of prolactin promoter driven reporter plasmid that contains pit-1 binding sites. In this system the hinge domain, but not the DNA binding domain of ER is needed for ER mediated transcription (14).

In the case of E_2 regulation of c-*myc* expression, the DNA binding domain of ER is required for induction of expression from a c-*myc* promoter driven

reporter gene (12). A TRE sequence that binds AP-1 complex, is also located in this c-*myc* promoter region (12). One possibility is that interaction between a region in the DNA binding domain of ER with the AP-1 complex that binds TRE may be important for E_2 induction of c-*myc* expression. This region of the DNA binding domain of ER would be different from the zinc finger region that is known to directly bind ERE. Therefore, in SKOV3 cells, ER which has defective DNA binding function to an ERE may induce c-*myc* expression through interacting with AP-1 or other transcription factors.

The c-*fos* promoter has both ERE and TRE sequences (15). ER may regulate c-*fos* expression by binding with DNA through ERE, or by interacting with AP-1. Thus in SKOV3 cells that have a defect in ER binding with ERE, ER may induce c-*fos* expression by interacting with AP-1 or with other transcription complexes.

Future experiments need to be conducted to test this hypothesis. Since our hypothesis states that ER in SKOV3 cells may have deficiency in DNA binding, we may study whether ER binds ERE in a gel shift assay. If there is a DNA binding defect, we may look for ER mutation by sequencing the ER cDNA of DNA binding domain. We may find mutation(s) that directly inhibits the DNA binding of ER to ERE, or 'dominant negative' mutation that coexists in the cells with wild type ER but block the DNA binding ability of wild type ER. We may also find a defect in E_2 induced phosphorylation which may cause defective DNA binding. ER is reported to be serine phosphorylated upon E_2 binding, and the

ligand induced phosphorylation has been shown to be important for DNA binding activity of ER (16,17). Defective phosphorylation could be caused by mutation at or near phosphorylation sites of ER, or there could be deficient kinase or phosphatase in these cells. To test this possibility, we may study the protein phosphorylation level of ER in the presence or absence of estrogen. If ER shows abnormal E_2 induced serine phosphorylation, we may then look into the cause. In general, if mutant ER exists in SKOV3 cells, introducing wild type ER into SKOV3 cells is expected to restore estrogen induction of PR and vit-ERE reporter expression. The introduced wild type ER is also expected to compete with the 'dominant negative' mutation in restoring normal function.

If DNA binding of ER appears normal in a gel shift assay, the lack of transactivation of PR and ERE driven reporter plasmid may be caused by the following: (A) There may be specific inhibitors in SKOV3 cells that block the binding of ER to ERE *in vivo*. To study this possibility, we may conduct another gel shift assay and test whether SKOV3 cell extract (that has ER removed) has any inhibitory effect on the binding of wild type ER with ERE. (B) Inhibitors may specifically interfere with the transactivating activity of ER to regulate gene expression through ERE binding as is observed in the regulation of PR and ERE reporter expression. If this is the reason, introducing the ER gene from SKOV3 cells into an ER^- ovarian cancer cell line should restore the PR and ERE-reporter transactivation activity. (C) Mutations in the transactivation domains may specifically block E_2 regulation of gene expression through ERE binding, but may

have no effect on E_2 induction of c-*myc* and c-*fos*. Cloning and sequencing of the A/B or E transactivation domains of ER will give us the answer.

E. Possible significance of ER regulation of early growth response genes in ovarian tumorigenesis

Immediate early genes are critical in growth and differentiation of cells. It is reported that continuous expression of c-*fos* precedes programmed cell death in transgenic mice and increased c-*myc* expression induces G0/G1 transition (18). In our study, E_2 induced the expression of c-*myc* and c-*fos* mRNA in both SKOV3 and PEO4 ovarian carcinoma cells. The level of E_2 induction of c-*myc* and c-*fos* was indistinguishable in SKOV3 cells which are hormone independent and PEO4 cells which are E_2 growth responsive, indicating induction of these immediate early genes was insufficient to cause E_2 induced growth. However, E_2 induction of c-*myc* and c-*fos* may play an important role in signal transduction or oncogenic behavior of ovarian cancer cells. The presence of defective ER that regulates expression of immediate early genes c-*myc* and c-*fos*, but do not regulate ERE containing genes such as of PR have potentially important and unexplored consequences on the behavior of ovarian cancer cells.

In future research it will be of interest to study: Does the uncoupling of E_2 regulation of c-*myc* and c-*fos* expression from HER-2/neu expression and cell growth exists in other ovarian carcinoma cells? Why are ER^+/PR^- ovarian tumors more aggressive than ER^+/PR^+ tumors? Do mutant ER directly affect the malignant behavior? Experiments could be designed to introduce defective ER

gene, for example from SKOV3 cells or wild type ER into ER^- ovarian tumors cells to test effects on growth and gene expression, on tumor formation in nude mice, and on tumor metastasis.

II. E_2 Regulation of HER-2/neu Expression in Breast and Ovarian Cancer

A. Mechanism of HER-2/neu regulation by E_2 in T47D cells

We observed that repression of HER-2/neu expression by E_2 was at the mRNA level and on the rate of $p185^{HER\text{-}2/neu}$ synthesis. E_2 repression of HER-2/neu mRNA expression has been reported in T47D, MCF-7, and ZR-75-1 cells. Russell and Hung reported that E_2 represses the expression of HER-2/neu promoter driven reporter, but the effects were small, about 2 fold (19). They suggest that E_2 regulates HER-2/neu at the mRNA transcription level. However, our data suggests that besides repression of HER-2/neu at the mRNA level, repression of the rate of $p185^{HER\text{-}2/neu}$ protein synthesis may be another mechanism by which E_2 regulates HER-2/neu expression.

E_2 specifically inhibits the rate of $p185^{HER\text{-}2/neu}$ synthesis but not the overall protein synthesis rate in T47D cells. Therefore, E_2 is not likely to alter the activity of general translation initiation or elongation factors in these cells. Control of specific mRNA translation in mammalian cells has been extensively studied in iron regulation of ferritin translation. A 35 nucleotide iron response element (IRE) is located near the CAP site of ferritin mRNA (20). In the absence of iron, a repressor protein binds IRE and blocks the translation of ferritin. As iron levels increase, the binding of the repressor protein to IRE is

weakened and the translation of ferritin is increased (For review, see 21,22). A similar mechanism is also observed in stimulation of myelin basic protein mRNA translation by hydrocortisone (23). An 8 bp steroid modulatory element for translation is localized in the 5' untranslated region of MBP mRNA. This sequence is critical in mediating the steroid effect, because an antisense oligo of the steroid modulatory element inhibits the stimulatory effect of hydrocortisone. Although estrogen increases myelin basic mRNA and represses ER mRNA translation in an *in vitro* translation system (23), the mechanism behind regulation of mRNA translation is not known. Even though ER is considered to be mainly a nuclear receptor, about 20% of ER has been detected in the cytosol in the absence of ligand binding (1,24). In addition, the estrogen binding component has also been found in ribosomes isolated from rat uterus (25). Thus it is possible that estrogen may interact with protein translation machinery in the cytosol and effect $p185^{HER\text{-}2/neu}$ synthesis in T47D cells. It will be interesting to look for the existence of an estrogen modulation element in the 5' region of HER-2/neu mRNA. ER may inhibit the translation of $p185^{HER\text{-}2/neu}$ by directly binding the estrogen modulation element, by decreasing the affinity of an enhancer or increasing the affinity of an inhibitor that binds this response element.

Future research could be conducted towards understanding the mechanism of E_2 translational regulation of $p185^{HER\text{-}2/neu}$. To support our finding of E_2 inhibition of the rate of $p185^{HER\text{-}2/neu}$ protein translation, we may test for E_2 effect on translation of HER-2/neu mRNA in a cell free translation system. (A) A

result of E_2 repression of $p185^{HER\text{-}2/neu}$ translation in this cell free system will indicate that interaction of E_2 with the translation machinery mediates the E_2 effect. However, if we do not observe E_2 repression of $p185^{HER\text{-}2/neu}$ translation in this cell free system *in vitro*, then our observation of E_2 repression of $p185^{HER\text{-}2/neu}$ translation *in vivo* in T47D cells may be a secondary effect of E_2 based on E_2 regulation of the level of specific translation modulators of $p185^{HER\text{-}2/neu}$. (B)If E_2 inhibits $p185^{HER\text{-}2/neu}$ translation, we may search for the estrogen modulation element by conducting *in vitro* translation of different deletion mutant of HER-2/neu mRNA. Once the estrogen modulation element is localized in HER-2/neu mRNA, we may sequence the oligonucleotide and compare it with the hydrocortisone response element that is found in myelin basic protein. (C) E_2 may inhibit protein synthesis by binding with ER, or by binding with a novel estrogen binding protein, since evidence has shown that the sedimentation coefficient and estrogen binding affinity of the ribosomal estrogen binding component in rat uterus are different from those of ER (25). To study whether ER is involved in translation repression of $p185^{HER\text{-}2/neu}$ by E_2, we may test the effect of anti-estrogens, ICI164,384 or tamoxifen on E_2 regulation of $p185^{HER\text{-}2/neu}$ translation. If ICI164,384 or tamoxifen block the E_2 repression of $p185^{HER\text{-}2/neu}$ translation, ER is very likely to be involved in $p185^{HER\text{-}2/neu}$ translation regulation by E_2. Otherwise, a novel estrogen binding protein may be involved in this process.

B. E_2 did not Regulate p185$^{HER-2/neu}$ Levels in SKOV3 and PEO4 Ovarian Carcinoma Cells

Although E_2 has been shown to repress HER-2/neu mRNA expression in MCF-7, T47D and ZR-75-1 breast carcinoma cells (26-28), we did not detect any E_2 effect on p185$^{HER-2/neu}$ expression in SKOV3 and another ovarian carcinoma cell line, PEO4 cells. Even though SKOV3 contains overexpressed levels of p185$^{HER-2/neu}$ (29). Our results may indicate a cell type specificity in E_2 down regulation of p185$^{HER-2/neu}$ in breast but not in two ovarian carcinoma cell lines, or indicate defective ER in ovarian carcinoma cells that interfere translational regulation by ER.

In summary, our findings contribute one more dimension in regulation of p185$^{HER-2/neu}$ expression by estrogen. A better understanding of the mechanism of estrogen regulation of HER-2/neu expression may help future treatment of breast and ovarian carcinomas, because control of HER-2/neu expression level may be critical in growth and differentiation of these tumor cells.

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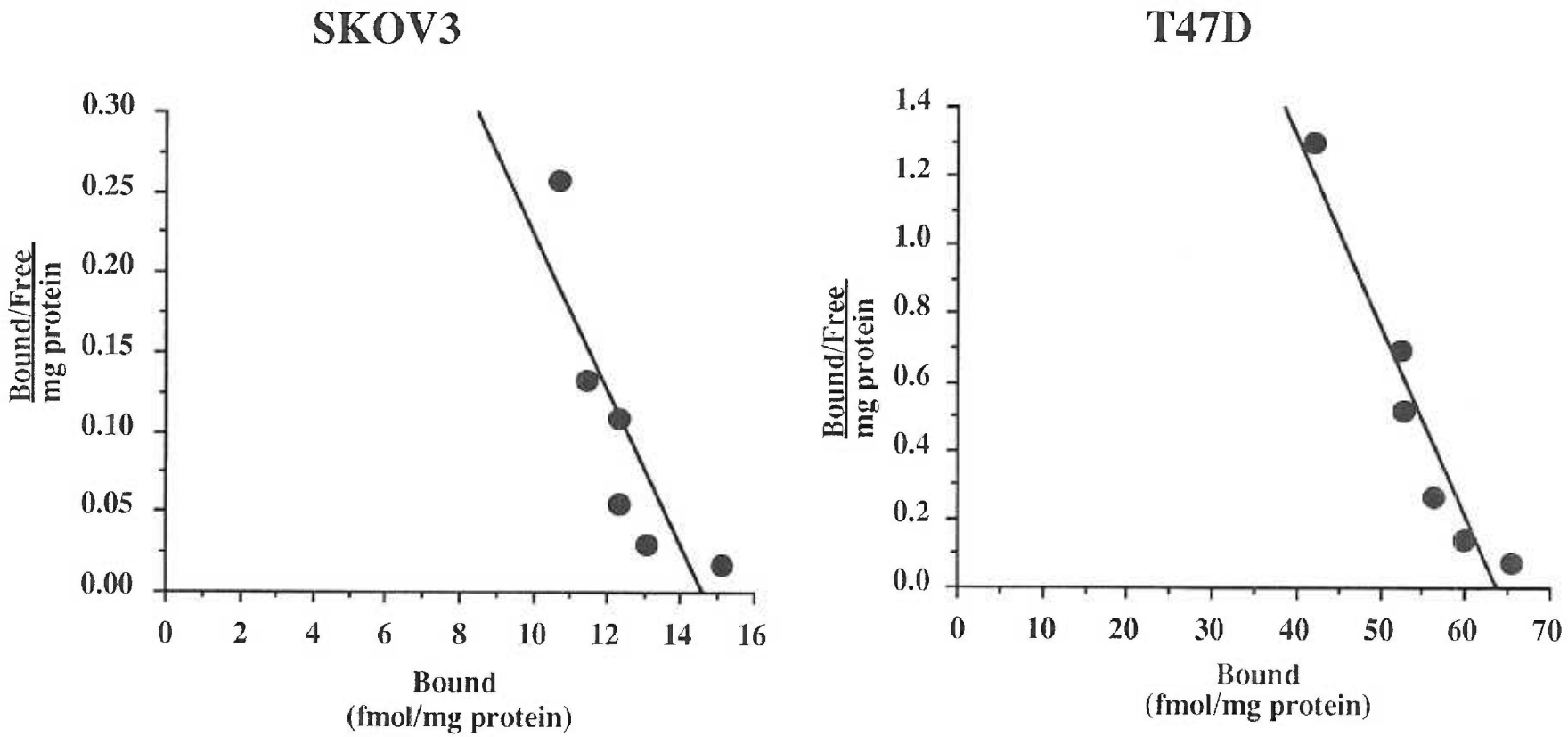
Appendix

Scatchard analysis of [^{3}H]-E_2 binding in SKOV3 and T47D cells

Aliquots of cytosol containing 5 mg/ml protein were incubated overnight at 4°C with [^{3}H]-E_2 at concentrations between 0.2 nM - 4 nM, or with 200- fold excess of unlabeled diethylstilbestrol (DES) to eliminate non-specific binding. The amount of unbound [^{3}H]-E_2 was determined in an aliquot that was treated with dextran charcoal. The amount of bound E_2 was calculated by subtracting the unbound from total amount of [^{3}H]-E_2 determined from a replicate sample that was not treated with charcoal-dextran.


SKOV3
Bound/Free
mg protein
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
10
12
14
16
Bound
(fmol/mg protein)
T47D
Bound/Free
mg protein
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
Bound
(fmol/mg protein)