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Estrogen signaling in cancer microenvironment and prediction of response to hormonal therapy☆

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ABSTRACT

Estrogen plays an essential role in growth and progression of human breast cancer. Particularly, local estrogen biosynthesis must be important for etiology of this disease. Since estrogen signaling is also activated by the growth factor-mediating phosphorylation signal, breast cancer strongly depends upon local cancer microenvironment. Then, to analyze the estrogen-related cancer microenvironment of individual breast cancer tissues, we established new reporter cell system, which was stably transfected GFP reporter DNA inserted estrogen response element in MCF-7 cells. It enables to analyze ERα-activation activity of stromal cells in individual cancer patients. We found that ERα-activation activity and effect of aromatase inhibitors varied among the individual cases but correlated with histological grade, indicating that the ability of stromal cells in adjacent to cancer cells must be unique and important. Furthermore, these ERα-activation signals in the microenvironment stimulate following intracellular estrogen-signal transduction in cancer cells. Our estrogen-responsive microarray analysis, real-time RT-PCR, and immunohistochemical technique revealed several new target genes which correlate with prognosis of breast cancer and play an important role in cancer development. For example, we found that transcription factor EGR3 was the bona fide target gene for ERα and might involve with invasive property in breast cancer. Furthermore, the expression of another downstream gene HDAC6 significantly correlated with survival of breast cancer patients. In vitro study revealed that the HDAC6 caused the deacetylation of α-tubulin in cytosol and induced cell motility in ERα-positive breast cancer cells. We hope that these approaches could provide not only new clues for elucidation of the mechanisms of estrogen-dependent growth and development of breast cancer, but also clinical benefits to patients by assessment of individual response to hormonal therapy.

1. Introduction

Estrogen and its receptor play important roles in genesis and malignant progression of estrogen-dependent cancers, together with various growth factors. To understand the whole figure of estrogen signaling is very important to clarify the biology of breast cancer. On the other hand, the hormonal therapy for breast cancer targeting this estrogen-signaling has recently been progressing dramatically, using new drugs such as new selective estrogen receptor modulators (SERMs), LH–RH agonist, and the third generation of aromatase inhibitors. Then, the prediction of individual response to these hormonal therapies is becoming very important for treatment of breast cancer patients. In recent several years, to address these basic and clinical issues, we are focusing our research to develop new tools such as ERE-GFP reporter cells and estrogen-responsive microarray, which enable to characterize the estrogen-responsive genes in breast cancer cells and estrogen signal-sensitivity in individual breast cancer.

2. Materials and methods

2.1. Cells and culture

The human breast cancer cell lines MCF-7 was cultured in RPMI1640 medium (Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal calf serum (FCS; Tissue Culture Biologicals, Turale, CA, USA) at 37 °C in a humidified atmosphere of 5% CO_2 in air. For the experiments designed to evaluate the effect of 17β-estradiol (E2) (Sigma, St. Louis, MO, USA) or stromal cells, phenol red-free RPMI1640 (PRF-RPMI) (Sigma, St. Louis, MO, USA) and dextran-coated charcoal-treated FCS (DCC-FCS) were substituted for RPMI1640 and FCS, respectively. Anastrozole, letrozole and

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2.2. ERE-GFP transfected cell line MCF-7E10

MCF-7 cells were transfected with the d2E-GFP vector alone (Clontech, Palo Alto, CA, USA) or carrying the ptk-ERE insert using Trans IT LT-1 reagent (Takara Shuzo Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. After 24 h, the cells were subjected to selection in growth medium containing geneticin (1 mg/ml). 35 clones were isolated and the transfection efficiency was monitored under fluorescence microscopy after treatment with estrogen. The ERE-GFP-MCF-7 clones that expressed high levels of GFP in the presence of estrogen, and did not in the absence of estrogen, were selected. Expression of GFP was also analyzed on an Epics XL flow cytometer (Beckman Coulter). To quantify the GFP expression level, the number of cells expressing GFP was counted under fluorescence microscopy after the cells were harvested by treatment with trypsin. The cells expressing high levels of GFP were counted. Data are expressed as the percent of cells expressing GFP.

2.3. Coculture of MCF-7E10 cells with primary stromal cells

Stromal cells were prepared from human breast cancer tissues. The stromal cell isolation procedure is similar to that described by Ackerman et al. [1]. Briefly, tissue pieces were minced and digested with collagenase. Stromal cells were filtered through nylon mesh, recovered by centrifugation and washed several times with Hanks' balanced salt solution. The cells were suspended in minimum essential medium alpha containing 10% FCS and cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air. Both MCF-7E10 cells and stromal cells were precultured in phenol red-free medium containing 10% DCC-FCS for 48 h and then used for coculture. Testosterone (10^{-7} M) was added as substrate for aromatase.

After being cocultured for 4 days, the cells were collected by mild trypsinization, and the GFP expression was analyzed. Both cells were able to be easily discriminated by their morphology. For the experiment that examined the effect of cell–cell interactions, the cells were separately cultured in Transwell-Clear culture dishes (Costar, Cambridge, MA, USA).

2.4. Tumor samples

Human breast cancer tissues were obtained by surgery at the Saitama Cancer Center Hospital after informed consent was obtained from the patients. The Saitama Cancer Center Ethics Committee approved this study. The clinicopathological characteristics are summarized in Table 1. Histological grading was evaluated according to the modified and simplified Bloom–Richardson grading scheme [2]. The ER and PgR status were evaluated by Allred scoring [3] and tumors with more than a score of 3 were identified as ER- or PgR-positive. Fixation, serial sectioning, paraffin embedding, H&E staining and immunohistochemical staining were performed as previously described [4].

2.5. Immunohistochemistry for HDAC6

Formaldehyde-fixed, paraffin-embedded samples were sequentially cut into 4 μm sections for staining. Antigens were retrieved by boiling sections in 0.01 M citric buffer (pH 6.0) for 30 min. The primary antibody was H-300 for HDAC6 (1/50, Santa Cruz Biotech, Santa Cruz, CA). Since the antigen peptide for HDAC6 antibody was not available, normal rabbit serum was used as a control antibody for the HDAC6 study. Since HDAC6 signals were generally observed in entire portions of the malignant ductal component, we evaluated the most intense signals in the whole slide as the intensity score (IS) 0–3. After careful evaluation of each cut-off point in comparison with clinical data, we determined that IS rated at 1 or more was considered positive for HDAC6 staining.

Table 1
Relationship between clinicopathological parameter and high ER stimulating ability of stromal cells

Total	No.	Active case no. (%)[a]		
Age				
<51	17	2 (11.8)		
≥50	50	11 (22.0)		
Menopausal Status				
Pre	25	2 (8.0)	$P = 0.045$[b]	
Post	42	12 (28.6)		
Tumor diameter				
<2 cm	32	7 (21.9)		
≥2 cm	35	7 (20.0)		
ER				
Positive	51	12 (23.5)	$P = 0.3438$	
Negative	16	2 (12.5)		
PgR				
Positive	42	9 (21.4)		
Negative	24	5 (20.8)		
Stage				
I	13	2 (15.4)		
II	44	10 (23.0)		
III	7	2 (29.0)		
Unknown	3			
Histology				
Invasive ductal	61	14 (23.0)		
Special	3	0		
Non-invasive	3	0		
Grade				$P = 0.0025$
1	13	6 (46.0)		
2	6	4 (66.7)	$P = 0.004$	
3	36	3 (8.3)		
Nodal status				
Negative	35	7 (20.0)		
Positive	31	8 (25.8)		
Unknown	1			

[a] The numbers of the cases in which the stromal cells induced GFP expression in >30% of E10 cells. The percentages of the active cases are shown in the parentheses.
[b] Comparison of two proportions.

2.6. Statistical analysis

Depending on the tumor characteristics of each group, comparisons were made using the χ^2-test or Fisher's probability test. The distributions of relapse-free survival and overall survival were calculated using the Kaplan–Meier method. The significance of the differences between the survival curves was tested by the log-rank test. These evaluations and multivariate analyses with the Cox proportional hazard model were performed with StatView software (SAS Institute, SAS Campus Drive, Cary, NC). $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Assessment of estrogen signal-related tumor–stromal interactions

It is true that a majority of breast cancers arise after menopause even ceased estrogen production. Miller et al. [5] have shown that tissue concentrations of estrogen were more than several times higher in breast cancer than in plasma, suggesting in situ production of estrogen within the tumor. The adipose stromal cells adjacent to tumors express aromatase, a key enzyme of estrogen synthesis, provide intratumoral estrogen production

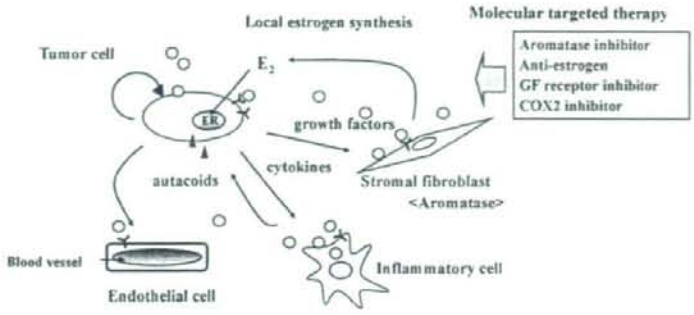

Fig. 1. Cooperation of estrogen and other signaling factors in breast cancer microenvironment. Local estrogen biosynthesis by aromatase or other enzymes in the stromal fibroblast supplies growth stimulation to estrogen-dependent cancer. Furthermore, other factors such as cytokines, growth factors, and autacoids also stimulate or support the survival of breast cancer cells. These intercellular communications between cancer cells and adjacent stroma cells are very important for the maintenance and development of breast cancer. The blockade of the intercellular-communication is the most effective strategy for molecular targeted therapies, including aromatase inhibitor.

and cause tumor growth [6–9]. In addition to aromatase, other estrogen metabolizing enzymes including 17-β-hydroxysteroid dehydrogenase-1,-2, (17β-HSD1,2), steroid sulfatase (STS), and estrogen sulfotransferase (EST), which are primarily provided by stromal cells, also affect intratumoral estrogen levels [10]. Therefore, the local estrogen biosynthesis must be important for etiology of this disease. Furthermore, estrogen signaling is not only activated by ligands but also by the growth factor-mediating phosphorylation signal cascade. Tumor–stromal interactions also provide growth factors such as EGF and IGF-1 that activate ER via phosphorylation in the absence or in the presence of a low concentration of estrogen [11]. For example, mitogen-activated protein (MAP) kinase enhances the AF-1 transcriptional activity of ERα via phosphorylation of Ser118 of ERα [12,13]. Growth factors and its activated receptors evoke ligand-independent activation of ERα and might be involved with antiestrogen resistant [14,15]. These observations indicate that breast cancer strongly depend upon local cancer microenvironment (Fig. 1).

Then, to analyze the estrogen-related cancer microenvironment of individual breast cancer tissues, we developed a comprehensive evaluation system to visualize the ER-activating ability of adipose stromal cells based on tumor–stromal interactions [16] (Fig. 2A). We constructed GFP reporter plasmid which was inserted estrogen-responsive element (ERE) into 5′-flanking region of TK-promoter, and transfected it in ERα-positive breast cancer cells, MCF-7, and established a reporter cell line (MCF-7E10). The ERα-activations in these cells were analyzed by coculture with stromal cells obtained from individual cancer patients. To quantify GFP expression, the numbers of E10 cells expressing GFP were counted. Fig. 2B shows the effect of coculture with stromal cells on the induction of GFP in E10 cells. In the presence of testosterone, a substrate for aromatase, GFP was specifically induced in E10 cells cocultured with stromal cells while no GFP was observed without testosterone. GFP expression in E10 cells increased when stromal cells were treated with dexamethasone, a stimulator of aromatase gene expression, indicating that GFP expression reflects the intensity of ER-activating signals produced in the coculture (data not shown).

3.2. Characterization of individual adipose stromal cells in breast cancer

Using this system, we characterized the adipose stromal cells of individual human breast cancers, and Fig. 2B shows the rep-

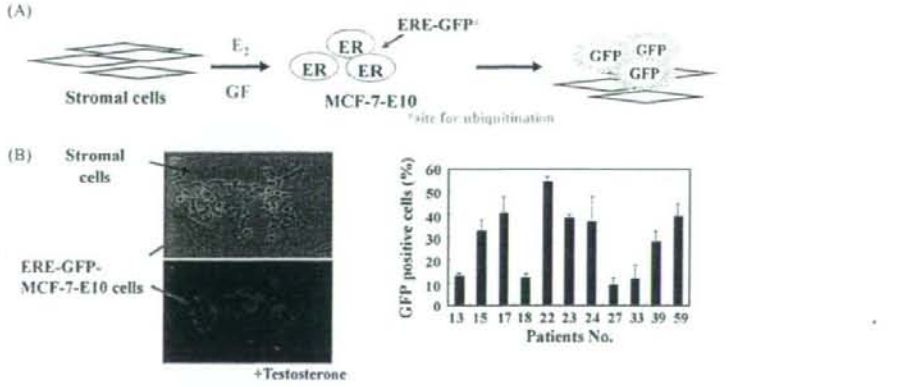

Fig. 2. System for analysis of tumor–stromal interactions in breast cancer by visualization of estrogen signaling pathway. MCF-7 cells were stably transfected with ERE-GFP gene, and stromal cells were isolated from breast cancer tissues. In coculture of MCF-7 with stromal cells, ERα was activated in an estrogen-dependent and -independent manner. Expression of GFP reflects total ERα activity produced in this system. (A) MCF-7 cells were plated onto subconfluent primary stromal cells isolated from breast tumor tissues as described in Section 2. (B) After 72 h, the expression of GFP in MCF-7-E10 cells was observed by fluorescence microscopy. MCF-7-Mock cells were transfected with control vector.

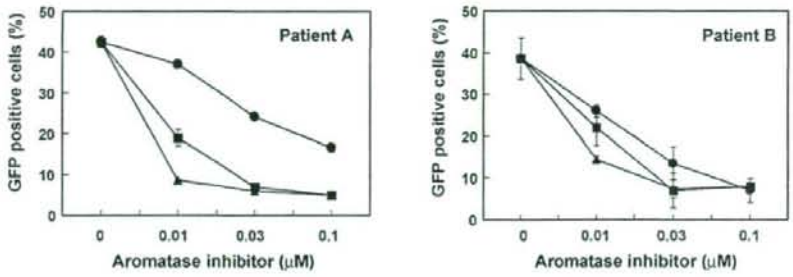

Fig. 3. Aromatase inhibitors effectively inhibited induction of GFP expression in MCF-7-E10 cells cocultured with stromal cells. MCF-7-E10 cells were cocultured with stromal cells as described in Section 2 in the presence of various concentrations of anastrozole (●), letrozole (▲), or exemestane (■), and testosterone (1×10^{-7} M) for 4 days. The numbers of MCF-7-E10 cells expressing GFP were counted, and the data are expressed as percentages of GFP-expressing cells. Points, average, bars, and S.D.

resentative results that in some cases, no significant induction of GFP was observed, and in other cases, the coculture induced high GFP expression levels. Thus, stromal cells in breast cancer tissues have their own properties with respect to the activation of estrogen signals.

We characterized stromal cells for 67 breast cancers, and analyzed the relationship between the ER-activating ability of stromal cells and the clinicopathologic characteristics of the specimens (Table 1). Although in hormonal therapy of breast cancer the expression status of ERα is a primary determinant for the prediction of its efficacy, no significant relationship between GFP expression and ER status was observed. However, the ratio of the cases with a high ER-activating ability was significantly higher in the postmenopausal patients than in the premenopausal ones. These data are consistent with reports that local estrogen signals play an important role in the progression of postmenopausal breast cancers. Grade 3 cases showed significantly lower GFP expression levels than grade 1 or 2 cases (Table 1), indicating that tumor cells in the grade 3 might grow independently of ER-activating signals supplied from the surrounding stromal cells.

We next examined the effect of aromatase inhibitors which have been approved to be used as a first-line treatment in hormonal therapy of breast cancer. They markedly inhibited the induction of GFP expression in the coculture, but the sensitivities to the drugs differed for each case (Fig. 3). Aromatase gene expression levels in adipose stromal cells did not simply correlate with their ability to induce GFP. These results suggest that the analysis of adipose stromal cells for individual breast cancers is essential for the prediction of hormonal therapy efficacy.

3.3. Assessment of estrogen responsive genes by DNA microarray

The extra-cellular ERα-activation signals in the microenvironment stimulate intracellular estrogen-signal transduction in cancer cells. In recent several years, we analyzed the expression profile of estrogen-responsive genes using DNA microarray for understanding estrogen-signaling pathway in ERα-positive breast cancer cells. The expression status of ERα is a primary determinant in the anti-hormone therapy of breast cancer using antagonists to ERα such as tamoxifen [17,18]. However, the assay of ERα status is not at present completely predictive for responsiveness of the tumors to antiestrogens; not all tumors of the patients diagnosed as ERα-positive respond to anti-hormones. Furthermore, recent progress of hormonal therapies produced various new drugs such as aromatase inhibitors. Therefore, there is a need for novel prediction method for hormonal therapy. Although there are many reports concerning the target genes transcriptionally activated by ERα such as *pS2* [19] and cathepsin D [20], the entire mechanism of the pathway from ERα leading to the proliferation and progression of mammary tumors is far from being completely clarified. For elucidation of the scheme of estrogen-signaling and improvement of clinical decisions, expression profiling analysis using cDNA microarray technology should be one of the most effective procedures. Several laboratories have carried out cDNA microarray analysis of breast tumors from patients [21–24] and a novel gene whose expression status was highly correlated with prognosis of patients was identified [25]. Nonetheless, there is little information on how many markers are sufficient and which markers are suitable for accurate prognosis and diagnosis of breast tumors, especially regarding sensitivity to hormonal therapy.

In order to understand the intracellular estrogen-signaling and develop a new tool for diagnosis of estrogen-dependent cancer, we promoted a strategy using cDNA microarray technique. We first analyzed the estrogen-responsive gene expression profiles in human MCF-7 breast cancer cells using a Human UniGEM™ V 2.0 microarray system (IncyteGenomics, CA) consisting of 9128 human cDNA clones covering 8502 unique gene/EST clusters [26]. Among a total of 9128 clones, 181 genes showed differential expression ratios equal or more than 2.0 and 105 genes showed differential expression ratios equal or less than 0.5; the remaining 96% of the genes revealed no significant differences in their expression levels. In the total of 286 genes which proved to be potentially estrogen-responsive genes by this analysis, there were some genes which had previously been reported to be induced by estrogen such as *pS2* (trefoil factor 1 [27]), PDZK1 [28], insulin-like growth factor-binding protein 4 [29] and nuclear receptor interacting protein 1 [30], indicating the reliability of this analysis.

Based on the results above, estrogen-responsive genes were selected for production of a custom-made cDNA microarray. Using a microarray consisting of the narrowed-down gene subset (about 200 genes), we further analyzed the time course of the estrogen-responsive gene expression profiles, effects of estrogen antagonists, the estrogen-responsive gene expression profiles in cell lines derived from other organs such as uterus, ovary and stomach, etc. The results obtained from these studies indicated that this custom-microarray was useful not only for understanding of the mechanism of estrogen signaling to clarify the estrogen-dependent cancer biology, but also for clinical diagnosis.

3.4. New estrogen-responsive genes which have clinical impacts

Our estrogen-responsive microarray analysis, real-time RT-PCR, and immunohistochemical technique revealed several new target genes which correlate with prognosis of breast cancer and play an important role in cancer development [31]. For example, we found that transcription factor EGR3 is the bona fide target gene for ERα and mediates the estrogen-signal to downstream genes [32], and

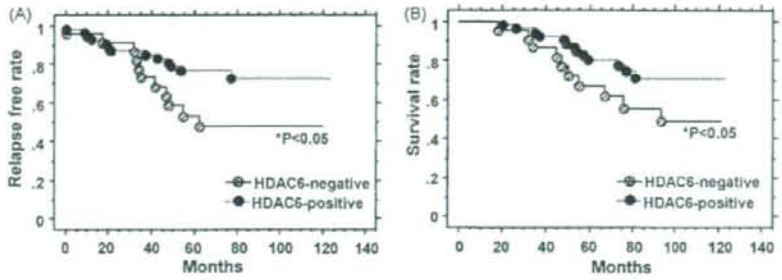

Fig. 4. Kaplan–Maier analysis of relapse free and overall survival of ERα-positive breast cancer patients based on the expression of HDAC6. ERα-positive patients were separated to two groups according to the level of HDAC6 expression. Survival curves were tested by the log-rank test and *P* values are indicated.

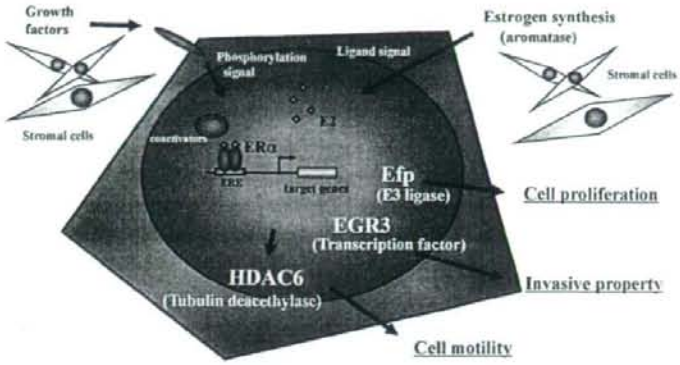

Fig. 5. Estrogen signaling pathway in breast cancer microenvironment. Extra-cellular signals in the breast cancer microenvironment stimulate intracellular estrogen-signal transduction. Their several downstream genes including HDAC6, EGR3 and others induce not only cell proliferation but also cell motility and invasive property. They might contribute to estrogen-dependent development of breast cancer.

recently revealed that it might involve with invasive property in breast cancer. Furthermore, the expression of another downstream gene HDAC6 significantly correlated with survival of breast cancer patients [33]. As shown in Fig. 4, relapse-free survival and overall survival of HDAC6-positive group showed better prognosis in ERα-positive patients, particularly tamoxifen-treated ones. The same observation was also reported by Zhang et al. [34]. These results suggested that HDAC6-positive patients might benefit more from antiestrogen treatment. In vitro study revealed that the HDAC6 caused the deacetylation of α-tubulin in cytosol and induced cell motility in ER-positive breast cancer cells [33] (Fig. 5).

These studies analyzing cancer microenvironment and estrogen-mediated signaling from several approaches could provide not only perspectives for progression of diagnosis and therapy of hormone-dependent cancers, but also new insight for mechanisms of hormone-dependent carcinogenesis and development of cancer.

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Adaptation to Estradiol Deprivation Causes Up-Regulation of Growth Factor Pathways and Hypersensitivity to Estradiol in Breast Cancer Cells

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Abstract

Deprivation of estrogen causes breast tumors in women to adapt and develop enhanced sensitivity to this steroid. Accordingly, women relapsing after treatment with oophorectomy, which substantially lowers estradiol for a prolonged period, respond secondarily to aromatase inhibitors with tumor regression. We have utilized in vitro and in vivo model systems to examine the biologic processes whereby Long Term Estradiol Deprivation (LTED) causes cells to adapt and develop hypersensitivity to estradiol. Several mechanisms are associated with this response including up-regulation of ERα and the MAP kinase, PI-3-kinase and mTOR growth factor pathways. ERα is 4–10 fold up-regulated as a result of demethylation of its C promoter, is nuclear receptor then co-opts a classical growth factor pathway using SHC, Grb-2 and Sos. is induces rapid nongenomic effects which are enhanced in LTED cells.

The molecules involved in the nongenomic signaling process have been identified. Estradiol binds to cell membrane-associated ERα which physically associates with the adaptor protein SHC and induces its phosphorylation. In turn, SHC binds Grb-2 and Sos which results in the rapid activation of MAP kinase. These nongenomic effects of estradiol produce biologic effects as evidenced by Elk-1 activation and by morphologic changes in cell membranes. Additional effects include activation of the PI-3-kinase and mTOR pathways through estradiol-induced binding of ERα to the IGF-1 and EGF receptors.

A major question is how ERα locates in the plasma membrane since it does not contain an inherent membrane localization signal. We have provided evidence that the IGF-1 receptor serves as an anchor for ERα in the plasma membrane. Estradiol causes phosphorylation of the adaptor protein, SHC and the IGF-1 receptor itself. SHC, after binding to ERα, serves as the "glue" which tethers ERα to SHC binding sites on the activated IFG-1 receptors. Use of siRNA methodology to knock down SHC allows the conclusion that SHC is needed for ERα to localize in the plasma membrane.

In order to abrogate growth factor induced hypersensitivity, we have utilized a drug, farnesylthiosalicylic acid, which blocks the binding of GTP-Ras to its membrane acceptor protein, galectin 1 and reduces the activation of MAP kinase. We have shown that this drug is a potent inhibitor of mTOR and this provides the major means for inhibition of cell proliferation. The concept of "adaptive hypersensitivity" and the mechanisms responsible for this phenomenon have important clinical implications. The efficacy of aromatase inhibitors in patients relapsing on tamoxifen could be explained by this mechanism and inhibitors of growth factor pathways should reverse the hypersensitivity phenomenon and result in prolongation of the efficacy of hormonal therapy for breast cancer.

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Introduction

Cancer cells adapt in response to the pressure exerted upon them by various hormonal treatments. Ultimately, this process of adaptation renders them insensitive to hormonal therapy. In patients, clinical observations suggest that long term deprivation of estradiol causes breast cancer cells to develop enhanced sensitivity to the proliferative effects of estrogen. Premenopausal women with advanced hormone dependent breast cancer experience objective tumor regressions in response to surgical oophorectomy which lowers estradiol levels from mean levels of approximately 200 pg/ml to 10 pg/ml.[1] After 12–18 months on average, tumors begin to regrow even though estradiol levels remain at 10 pg/ml. Notably tumors again regress upon secondary therapy with aromatase inhibitors which lower estradiol levels to 1–2 pg/ml. These observations suggest that tumors develop hypersensitivity to estradiol as demonstrated by the fact that untreated tumors require 200 pg/ml of estradiol to grow whereas tumors regrowing after oophorectomy require only 10 pg/ml. We have shown in prior studies that up-regulation of growth factor pathways contributes to the phenomenon of hypersensitivity.[2–10] Ultimately these tumors adapt further and grow exclusively in response to growth factor pathways and do not require estrogens for growth.

In order to provide direct proof that hypersensitivity does develop and to study the mechanisms involved, we have utilized cell culture and xenograft models of breast cancer as experimental tools.[5,8,9,11–13]

Phenomenon of Hypersensitivity: Mechanisms and Pathways

To induce hypersensitivity, wild type MCF-7 cells require culturing over a 6–24 month period in estrogen-free media to mimic the effects of ablative endocrine therapy such as induced by surgical oophorectomy or aromatase inhibitors.[11,12] is process involves Long Term Estradiol Deprivation and the adapted cells are called by the acronym, LTED cells. As evidence of hypersensitivity, a three log lower concentration of estradiol can stimulate proliferation of LTED cells compared to wild type MCF-7 cells (Fig. 1A).[7] We reasoned that the development of hypersensitivity could involve modulation of the genomic effects of estradiol acting on transcription, nongenomic actions involving plasma membrane related receptors, cross talk between growth factor and steroid hormone stimulated pathways, or interactions among these various effects.[5,7–9,11–13]

We initially postulated that enhanced receptor mediated transcription of genes related to cell proliferation might be involved. Indeed, the levels of ERα increased 4–10 fold during long term estradiol deprivation.[11] The up-regulation of ER alpha results from demethylation of promoter A and C of the estrogen receptor (Fig. 1B and 1C). The transcripts stimulated by this promoter increase by 149 fold and the DNA of this segment exhibits a marked increase in demethylation. [13A]We initially reasoned that the up-regulation of ERα would directly result in hypersensitivity to estradiol (E_2). Accordingly, to directly examine whether enhanced sensitivity to E_2 in LTED cells occurred at the level of ER mediated transcription, we quantitated the effects of estradiol on transcription in LTED and in wild type MCF-7 cells. As transcriptional readouts, we measured the effect of E_2 on progesterone receptor (PgR) and pS2 protein concentrations and on ERE-CAT reporter activity (Fig. 2A–F).[9,13] We observed no shift to the left in estradiol dose response curves (the end point utilized to detect hypersensitivity) for any of these responses (i.e., PgR, pS2, CAT activity) when comparing LTED with wild type MCF-7 cells. On the other hand, basal levels (i.e., no estrogen added) of transcription of three ER/ERE related reporter genes were greater in LTED than in wild type MCF-7 cells (Fig. 2D–F).[13]

To interpret these data, we used the classic definition for hypersensitivity, namely a significant shift to the left in the dose causing 50% of maximal stimulation. Accordingly, these data suggest

that hypersensitivity of LTED cells to the proliferative effects of estradiol does not occur primarily at the level of ER-mediated gene transcription (Fig. 2A–C) but may be influenced by the higher rates of maximal transcription (Fig. 2D–F).

We next considered that adaptation might involve dynamic interactions between pathways utilizing steroid hormones and those involving MAP kinase and PI-3-kinase for growth factor signaling (Fig. 3A).[5,7–9,11–16] Our initial approach demonstrated that basal levels of MAP kinase were elevated in LTED cells in vitro (Fig. 2B, top panel) and in xenografts (data not shown) and were inhibited by the pure antiestrogen, fulvestrant.[8,11]

We further demonstrated that activated MAP kinase is implicated in the enhanced growth of LTED cells since inhibitors of MAP kinase such as PD98059 or U-0126 block the incorporation of tritiated thymidine into DNA.[7] To demonstrate proof of the principle of MAP kinase participation, we stimulated activation of MAP kinase in wild type MCF-7 cells by administering TGFα (data not shown). Administration of TGFα caused a two log shift to the left in the ability of estradiol to stimulate the growth of wild type MCF-7 cells. To demonstrate that this effect was related specifically to MAP kinase and not to a nonMAP kinase mediated effect of TGF alpha, we co-administered PD 98059. Under these circumstances, the two log left shift in estradiol dose response, returned back to the baseline dose response curve.[7] As further evidence of the role of MAP kinase, we administered U-0126 to LTED cells and examined its effect on level of sensitivity to estradiol. is agent partially shifted dose response curves to the right by approximately one-half log (data not shown).

While an important component, MAP kinase did not appear to be solely responsible for hypersensitivity to estradiol. Blockade of this enzyme did not completely abrogate hypersensitivity. Accordingly, we examined the PI-3-kinase pathway to determine if it was up-regulated in LTED cells as well (Fig. 3B) and examined several signaling molecules downstream from this regulatory kinase.[16] We determined that LTED cells exhibit an enhanced activation of AKT (Fig. 3B, second panel), P70 S6 kinase (Fig. 3B, third panel) and PHAS-1/4E BP-1 (Fig. 3B, fourth panel; see also below).[16] Dual inhibition of PI-3-kinase with Ly 294002 (specific PI-3-kinase inhibitor) and MAP kinase with U-0126 shifted the level of sensitivity to estradiol more dramatically: more than two logs to the right (Fig. 3C).[7]

One possible mechanism to explain the activation of MAP kinase would be through nongenomic effects of estrogen acting via ERα located in or near the cell membrane.[17–19] We postulated that membrane associated ERα might utilize a classical growth factor pathway to transduce its effects in LTED cells. The adaptor protein SHC represents a key modulator of tyrosine kinase activated peptide hormone receptors.[14–15,20] Upon receptor activation and auto-phosphorylation, SHC binds rapidly to specific phosphotyrosine residues of receptors through its PTB or SH2 domain and becomes phosphorylated itself on tyrosine residues of the CH domain.[14,15] The phosphorylated tyrosine residues on the CH domain provide the docking sites for the binding of the SH2 domain of Grb2 and hence recruit SOS, a guanine nucleotide exchange protein. Formation of this adapter complex allows Ras activation via SOS, leading to the activation of the MAPK pathway.[20]

We postulated that estrogen deprivation might trigger activation of a nongenomic, estrogen-regulated, MAP kinase pathway which utilizes SHC.[14–15,20–22] We employed MAP kinase activation as an endpoint with which to demonstrate rapid nongenomic effects of estradiol (Fig. 4A). The addition of E_2 stimulated MAP kinase phosphorylation in LTED cells within minutes. The increased MAP kinase phosphorylation by E_2 was time and dose-dependent, being greatly stimulated at 15 min and remaining elevated for at least 30 min. Maximal stimulation of MAP kinase phosphorylation was at 10^{-10} M of E_2.

We then examined the role of peptides known to be involved in growth factor signaling pathways that activate MAP kinase. SHC proteins are known to couple tyrosine kinase receptors to the MAPK pathway and activation of SHC involves the phosphorylation of SHC itself.[20–22] To investigate if the SHC pathway was involved in the rapid action of estradiol in LTED cells, we immunoprecipitated tyrosine phosphorylated proteins and tested for the presence of SHC under E_2 treatment. E_2 rapidly stimulated SHC tyrosine phosphorylation in a dose and time dependent fashion with a peak at 3 minutes.[20] The pure estrogen receptor antagonist, fulvestrant, blocked E_2-induced SHC and MAPK phosphorylation at 3 min and 15 min respectively. To demonstrate that the classical ER alpha mediated this response, we transfected a siRNA against ER alpha and showed down-regulation of this receptor and also abrogated the effect of estradiol to rapidly enhance MAP kinase activation. The time frame suggests that SHC is an upstream component in E_2-induced MAPK activation.

We reasoned that the adapter protein SHC may directly or indirectly associate with ERα in LTED cells and thereby mediate E_2-induced activation of MAP kinase. We considered this likely in light of recent evidence regarding ERα membrane localization.[23–25] To test this hypothesis, we immunoprecipitated SHC from nonstimulated and E_2-stimulated LTED cells and then probed immunoblots with anti-ERα antibodies. Our data showed that the ERα/SHC complex pre-existed before E_2 treatment and E_2 time-dependently increased this association.[20] In parallel with SHC phosphorylation, we observed a maximally induced association between ERα and SHC at 3 min (data not shown). MAP kinase pathway activation by SHC requires SHC association with the adapter protein Grb2 and then further association with SOS. By immunoprecipitation of Grb2 and detection of both SHC and SOS, we demonstrated that the SHC-Grb2-SOS complex constitutively existed at relatively low levels in LTED cells, but was greatly increased by treatment of cells with 10^{-10} M E_2 for 3 min.[20]

After the demonstration of protein-protein interactions, we wished to provide evidence that these biochemical steps resulted in biologic effects. Accordingly, we evaluated the role of estrogen activated MAP kinase on the function of the transcription factor, Elk-1. When activated, Elk-1 serves as a down stream mediator of cell proliferation. The phosphorylation of Elk1 by MAPK can up-regulate its transcriptional activity through phosphorylation. By cotransfection of LTED cells with both GAL4-Elk and its reporter gene GAL4-luc,[26,27] we were able to show that E_2 dose-dependently increased Elk-1 activation at 6 hours as shown by luciferase assay (Fig. 4B).[20]

We also wished to demonstrate biologic effects on cell morphology. To examine E_2 effects on reorganization of the actin cytoskeleton, we visualized the distribution of F-actin by phalloidin staining and also redistribution of the ERα localization in LTED and MCF-7 cells (data not shown).[20] Untreated MCF-7 cells expressed low actin polymerization and a few focal adhesion points. After E_2 stimulation, in contrast, the cytoskeleton underwent remodeling associated with formation of cellular ruffles, lamellipodia and leading edges, alterations of cell shape and loss of mature focal adhesion points. A sub-cellular redistribution of ERα to these dynamic membranes upon E_2 stimulation represented another important feature. The ER antagonist ICI 182 780 at 10^{-9} M blocked E_2-induced ruffle formation as well as redistribution of ERα to the membrane with little effect by itself. Therefore, these studies further demonstrated the rapid action of E2 with respect to dynamic membrane alterations in LTED cells.

A key unanswered question was how the ER could localize in the plasma membrane when it does not contain membrane localization motifs. We postulated that the IGF-1-receptor and SHC might be involved in this process (Fig. 5A).[28] A series of studies by other investigators suggested that ERα and the IGF-1 receptor might interact.[28] We tested the model that estradiol caused binding of SHC to ERα but also caused phosphorylation of the IGF-1 receptor. In this way, SHCwould serve as the "glue" which would tether ER alpha to the plasma membrane

where it would bind to the SHC acceptor site. To assess this possibility, we immunoprecipitated IGF-1 receptors before and after addition of estradiol. is caused SHC to bind to the IFG-1 receptor (Fig. 5C) and caused the IGF-1 receptor to become phosphorylated (Fig. 5B,C). In order to prove a causal effect for this role of SHC, we utilized an siRNA methodology to knock down SHC and showed that this prevented ERα from binding to the IGF-1 receptor.[28] As further evidence, we conducted confocal microscopy experiments to show that knockdown of SHC prevented ERα from localizing in the plasma membrane (data not shown).[29]

Ellis Levin and colleagues recently showed that ER alpha must be palmitylated before it can localize in the plasma membrane.[29A] Although speculative, we postulate that ER alpha requires palmitylation to travel to the plasma membrane but activated SHC serves to tether it to the membrane via IGF-1-R. In contrast to our previous concept that SHC serves as the "bus" to carry ER alpha to the membrane, we now postulate that SHC is the "glue" that tethers ER alpha there after binding to the IGF-1-R. Further studies will be necessary to dissect out each component of these interactions and their biologic relevance.

From the data reviewed, we conclude that membrane related ERα plays a role in cell proliferation and in activation of MAP kinase. It appeared likely then that LTED cells might exhibit enhanced functionality of the membrane ERα system. As evidence of this, we examined the ability of estradiol to cause the phosphorylation of SHC in wild type and MCF-7 cells and also to cause association of SHC with the membrane ERα. We demonstrated a marked enhancement of both of these processes in LTED as opposed to wild type cells. Considering all of these data together, it is still not clear at the present time what is responsible for enhancement of the nongenomic ERα mediated process.

If adaptive hypersensitivity results from the up-regulation of growth factor pathways, an inhibitor of MAP kinase and downstream PI-3-kinase pathways could be important in abolishing hypersensitivity and in inhibiting cell proliferation. We had been studying the effects of a MAP kinase inhibitor, farnesylthiosalicylic acid (FTS), which has been shown to block proliferation of LTED cells. is agent interferes with the binding of GTP-Ras to its acceptor site in the plasma membrane, a protein called galectin 1.[30] While examining its downstream effects, we have shown that this agent is also a potent inhibitor of phosphoinositol-3-kinase (PI-3-kinase). We postulated that an agent which blocks not only the MAP kinase pathway but also downstream actions of the PI-3-kinase pathway might be ideal to inhibit hypersensitivity. Accordingly, we have intensively studied the effects of FTS on mTOR.

The mammalian target of rapamycin, mTOR, is a Ser/r protein kinase involved in the control of cell growth and proliferation.[31] One of the best characterized substrates of mTOR is PHAS-1 (also called 4E-BP1).[32,33] PHAS-I/4E-BP1 binds to eIF4E and represses cap-dependent translation by preventing eIF4E from binding to eIF4G.[32,33] When phosphorylated by mTOR, PHAS-I/4E-BP1 dissociates from eIF4E, allowing eIF4E to engage eIF4G, thus increasing the formation of the eIF4F complex needed for the proper positioning of the 40S ribosomal subunit and for efficient scanning of the 5'-UTR.[31] In cells, mTOR is found in mTORC1, a complex also containing raptor, a newly discovered protein of 150kDa. It has been proposed that raptor functions in TORC1 as a substrate-binding subunit which presents PHAS-I/4E-BP1 to mTOR for phosphorylation.[31,32] Our results suggest that FTS inhibits phosphorylation of the mTOR effectors, PHAS-I/4E-BP1 and S6K1, in response to estrogen stimulation of breast cancer cells.[2]

To investigate the effects of FTS on mTOR function, we utilized 293T cells and monitored changes in the phosphorylation of PHAS-I/4E-BP1.[2] Incubating cells with increasing concentrations of FTS decreased the phosphorylation of PHAS-I/4E-BP1, as evidenced by a

decrease in the electrophoretic mobility. To determine whether FTS also promoted dephosphorylation of r36 and r45, the preferred sites for phosphorylation by mTOR[31], an immunoblot was prepared with P r36/45 antibodies. Increasing FTS markedly decreased the reactivity of PHAS-I/4E-BP1 with the phosphospecific antibodies (Fig. 6A and B).

To investigate further the inhibitory effects of FTS on mTOR signaling, we determined the effect of the drug on the association of mTOR, raptor and mLST8 (Fig. 6A and B). AU1-mTOR and HA-tagged forms of raptor and mLST8 were overexpressed in 293T-cells, which were then incubated with increasing concentrations of FTS before AU1-mTOR was immunoprecipitated with anti-AU1 antibodies. Immunoblots were prepared with anti-HA antibodies to assess the relative amounts of HA-raptor and HA-mLST8 that co-immunoprecipitated with AU1-mTOR. Both HA-tagged proteins were readily detectable in immune complexes from cells incubated in the absence of FTS, indicating that mTOR, raptor and mLST8 form a complex in 293T cells. FTS did not change the amount AU1-mTOR that immunoprecipitated; however, increasing concentrations of FTS produced a progressive decrease in the amount of HA-raptor that co-immunoprecipitated. The half maximal effect on raptor dissociation from mTOR was observed at approximately 30 μM FTS (Fig. 6A, B). Results obtained with over-expressed proteins are not necessarily representative of responses of endogenous proteins. Therefore, experiments were conducted to investigate the effect FTS on the endogenous TORC1 in nontransfected cells. Similar results were found indicating the FTS blocks the association of raptor from mTOR.[2]

Incubating cells with FTS produced a stable decrease in mTOR activity that persisted even when mTOR was immunoprecipitated. The dose response curves for FTS-mediated inhibition of AU1-mTOR activity (Fig. 6C, D) and dissociation of AU1-mTOR and HA-raptor were very similar, with half maximal effects occurring between 20–30 μM. These results indicate that FTS inhibits mTOR in cells by promoting dissociation of raptor from mTORC1.

These studies provide direct evidence that FTS inhibits mTOR activity. The finding that the inhibition of mTOR activity by increasing concentrations of FTS correlated closely with the dissociation of the mTOR-raptor complex, both in cells and in vitro (Fig. 6), supports the conclusion that FTS acts by promoting dissociation of raptor from mTORC1.

Since FTS blocks both MAP kinase and mTOR, it was reasonable to conclude that it could block cell proliferation. For that reason, we conducted extensive studies to demonstrate that FTS blocks the growth of LTED cells. As shown in Figure 7A, B, FTS blocks the growth on LTED cells both in vitro and in vivo.

Our studies to date have predominantly concentrated on long term estradiol deprivation as a mode of development of resistance to aromatase inhbitors. More recently, we have examined the effect of long term tamoxifen treatment (LTTT) on MCF-7 cells. Interestingly, this maneuver also causes enhanced sensitivity to estradiol, both in vitro and in vivo.[34,35] While the up-regulation of MAP kinase is only transitory for a period of 2–3 months, these cells become hypersensitive to EGF-R mediated pathways. At the same time, we have demonstrated increased complex formation between ER alpha and the EGF-R and between ER alpha and cSRC. These studies also demonstrate that the tamoxifen resistant cells become hypersensitive to the inhibitory properties of the EGF-R tyrosine kinase inhibitor, AG 1478.

Significance of Our Findings to Development of Further Therapies

Our data suggest that cells adapt to hormonal therapy by up-regulation of growth factor pathways and ultimately become resistant to that therapy. Blockade of the pathways involved might then allow enhancement of the duration of responsiveness to various hormonal agents. Studies by Osborne and Schiff etal [36,37] and by Nicholson and his group[38,39] have

demonstrated this phenomenon both in vitro and in vivo. For example, Schiff and Osborne have treated HER-2/neu transfected MCF-7 cells with a cocktail of three kinase inhibitors: pertuzamab, gefitamab and traztuzamab as well as tamoxifen.[40] Each sequential growth factor inhibitor caused a further delay in development of resistance. Only 2/20 tumors began to regrow as a reflection of resistance when the four agents were used in combination (i.e., tamoxifen, pertuzamab, gefitimab and traztuzamab).

There are multiple agents currently in development to block growth factor pathways. Agents are available to block HER-1, 2, 3 and 4; EGF-R, IGF-R, mTOR, MAP kinase, Raf and MEK. Each of these agents might potentially be used in combination with an endocrine therapy. At the present time, this strategy is being used in several studies. A recent presentation demonstrated proof of the principle of this concept. Women with metastatic breast cancer selected to be ERα and HER-2 positive were treated either with an aromatase inhibitor alone or in combination with Herceptin. The percent of patients achieving clinical benefit (i.e., complete objective tumor regression, partial regression or stable disease for > 6 months was 27.9% percent in the aromatase inhibitor alone group and 42.9% in the combined group, a statistically significant ($p = 0.026$) finding.[41] Further studies will be necessary to determine the optimal combinations of growth factor and aromatase inhibitors in the future. However, based upon the Tandem study (examining the efficacy of aromatase inhibitor plus herceptin), this approach appears to be promising.

Synthesis of Our Current inking

Our current working model to explain adaptive hypersensitivity can be summarized as follows. Long term estradiol deprivation causes a four to ten fold up-regulation of the amount of ERα present in cell extracts and an increase in basal level of transcription of several estradiol stimulated genes. The up-regulation of the ER results from demethylation of promoter C of the ER. The lack of shift to the left in the dose response curves of these transcriptional endpoints suggested that hypersensitivity is not mediated primarily at the transcriptional level (Fig. 1 and 2). On the other hand, rapid, nongenomic effects of estradiol such as the phosphorylation of SHC and binding of SHC to ERα are easily demonstrable and appear enhanced in the LTED cells. Taken together, these observations suggest that adaptive hypersensitivity is associated with an increased utilization of nongenomic, plasma membrane mediated pathways. is results in an increased level of activation of the MAP kinase as well as the PI-3-kinase and mTOR pathways. All of these signals converge on downstream effectors which are directly involved in cell cycle functionality and which probably exert synergistic effects at that level. As a reflection of this synergy, E2F1, an integrator of cell cycle stimulatory and inhibitory events, is hypersensitive to the effects of estradiol in LTED cells.[7] us, our working hypothesis at present is that hypersensitivity reflects upstream nongenomic ERα events as well as downstream synergistic interactions of several pathways converging at the level of the cell cycle.

It is clear that primary endocrine therapies can exert pressure on breast cancer cells that causes them to adapt as a reflection of their inherent plasticity. Based upon this concept, we postulate that certain patients may become resistant to tamoxifen as a result of developing hypersensitivity to the estrogenic properties of tamoxifen. Up-regulation of growth factor pathways involving erb-B-2, IGF-1 receptor and the EGF receptor are associated with this process.[2] The estrogen agonistic properties of tamoxifen under these circumstances might explain the superiority of clinical responses in patients receiving aromatase inhibitors as opposed to tamoxifen. It is possible to counteract the effects of the adaptive processes leading to growth factor up-regulation. If breast cancer cells are exceedingly sensitive to small amounts of estradiol or to the estrogenic properties of tamoxifen, one therefore needs highly potent aromatase inhibitors to block estrogen synthesis or pure antiestrogens such as fulvestrant.

Blockade of the downstream effects of the IGF-1-R, EGF-R and erb-B-2 pathways would also be beneficial and allow continuing responsiveness to aromatase inhibitors or tamoxifen.

Disruption of each of several key steps could reduce the level of sensitivity to estradiol and block cell growth. Figure 8 illustrates the potential sites for disruption of adaptive hypersensitivity. An agent that blocks the nodal points through which several growth factor pathways must pass might be a more suitable therapy than combination of several growth factor blocking agents. Our preliminary data suggest that FTS blocks two nodal points, the functionality of Ras and the activity of mTOR. FTS also effectively inhibits the proliferation of MCF-7 breast cancer cells in culture. Since this agent blocks MAP kinase as well as mTOR, it may be ideal for the prevention of adaptive hypersensitivity and prolongation of the effects of hormonal therapy in breast cancer. We are currently conducting further studies in xenograft models to demonstrate its efficacy. We envision the possibility that women with breast cancer will receive a combination of aromatase inhibitors plus FTS. In this way, the beneficial effects of the aromatase inhibitor may be prolonged and relapses due to growth factor over-expression might be prevented or retarded.

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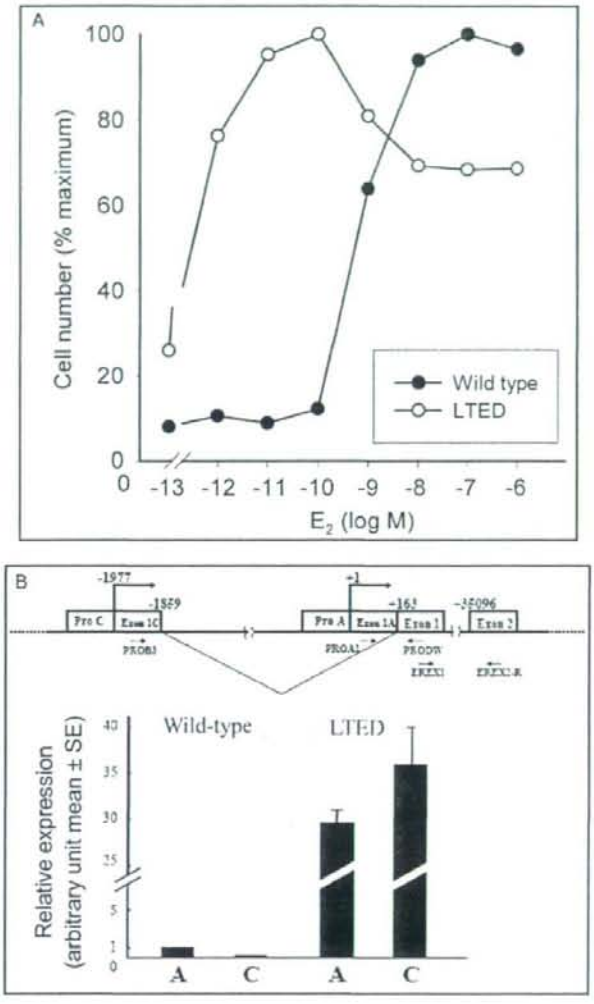

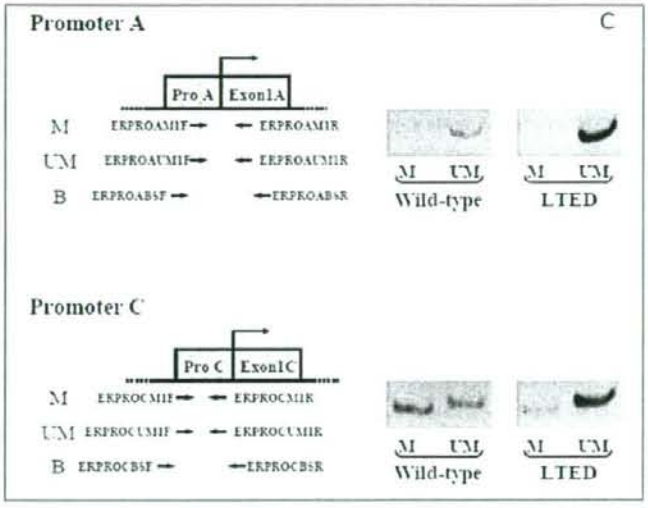

Figure 1.

A) E_2-induced cell proliferation. Wild type MCF-7 and LTED cells were plated in 6 well plates at a density of 60,000 cells/well. After 2 days the cells were refed with phenol-red and serum free IMEM (improved modified Eagles medium) and cultured in this medium for another 2 days before treatment with various concentrations of E_2 in the presence of ICI 182,780 (fulvestrant) at a 1 nmol concentration to abrogate the effects of any residual estradiol in the medium. Cell number was counted 5 days after treatment.[7,9] From: Yue W et al. Endocrinology 2002; 143(9):3221-9;[9] with permission of The Endocrine Society. B) Schematic representation of a part of ER alpha gene organization is shown. The transcription start site of Promoter A is defined as +1. Relative expression of ER alpha mRNA from promoters A and C in wild type and LTED cells is shown. Expression levels of ER alpha mRNA from promoters A and C were quantified by RT-PCR. C) COBRA assay for gene promoter C of ER α in wild type and LTED cells: an image of the polyacrilamide gel showing the methylated (M) and unmethylated (UM) products. B,C) From: Sogon T et al. J Steroid Biochem Mol Biol 2007; 105(1–5):106–14;[13a] with permission of Elsevier.

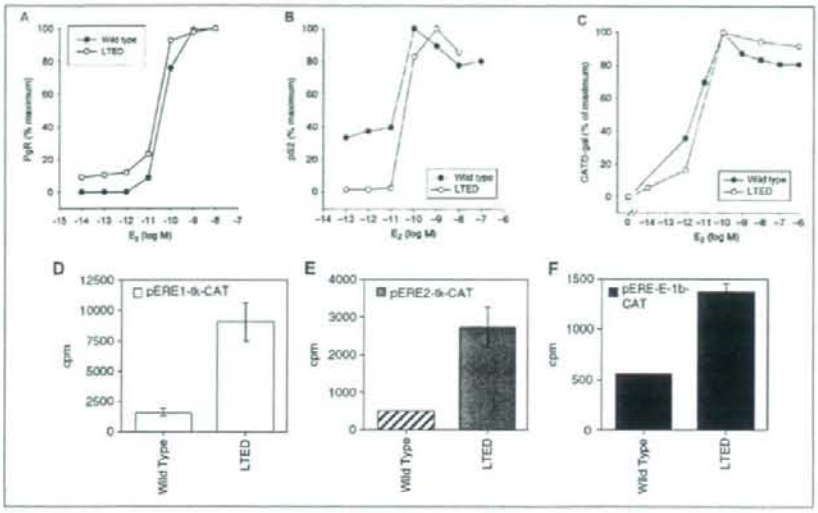

Figure 2.

A–C) Wild–type MCF-7 and LTED cells, deprived of E_2, were treated with different concentrations of E_2. Cytosols were measured for PgR (A), pS2 protein (B) and ERE-TK-CAT activity (C) 48 h after E_2 treatment. A–C) From: Yue W et al. Endocrinology 2002; 143(9): 3221-9;[9] with permission of The Endocrine Society. D–F) ER trans-activation function in wild-type MCF-7 and LTED cells under basal conditions. Wild type and LTED cells were deprived of estrogen and transfected with ERE-TK-CAT (D), pERE-2-TK-CAT E) or pERE-E1b-CAT (F) reporter plasmids in conjunction with pCMV-beta Gal plasmid as internal control. Two days later, cell cytosols were collected and assayed for CAT activities using the same amount of beta-galactosidase units.[9,11,13] D–F) From: Jeng MH et al. Endocrinology 1998; 139(10): 4164–74;[13] with permission of The Endocrine Society.

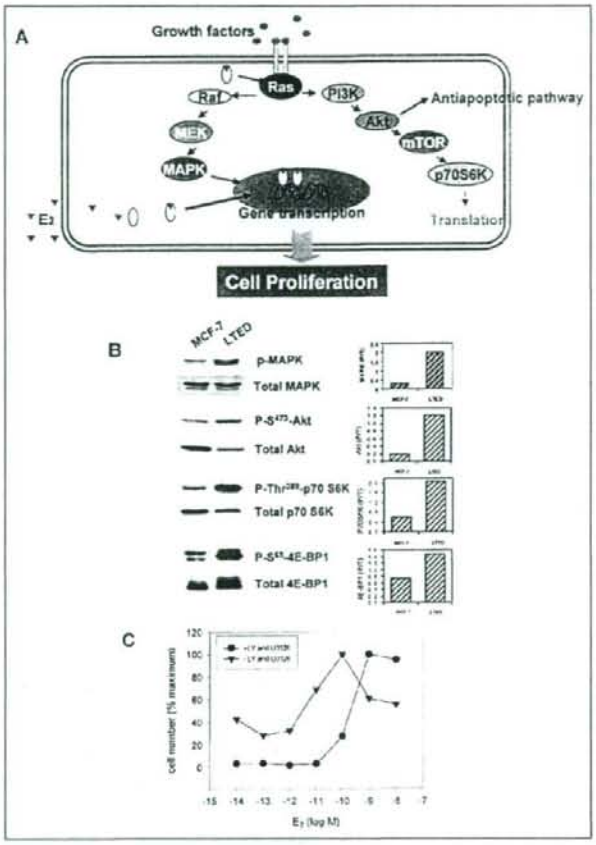

Figure 3.

A) Diagrammatic representation of the MAP kinase and PI-3-kinase signaling pathways activated when growth factors bind to their trans-membrane receptors. After auto-phosphorylation of the receptor, a series of events occurs which results in the activation of Ras. Downstream from Ras is the activation of the MAP kinase pathway with its components Raf and Mek and the activation of the PI-3-kinase pathway with its downstream components Akt, mTOR and p70S6K. At the same time, estradiol binds to the estrogen receptor and initiates transcription in the nucleus. B, top) Comparison of total and activated MAP kinase, detected with a phosphospecific antibody directed against activated MAP kinase and an antibody directed against total MAP kinase, in WT (wild-type MCF-7) and LTED cells. The right portion of the panel is a quantitation of the ratio of activated to total MAP kinase in WT and LTED cells.[16] B, second, third and fourth panels) Use of phosphospecific antibodies to quantitate the levels of activated Akt (second panel), p70S6 kinase (third panel) and 4E-BP1 (fourth panel) in wild type MCF-7 and LTEDS cells.[16] C) Treatment of LTED cells with an inhibitor of MAP kinase (U-0126) and PI-3- kinase (LY 292004) to demonstrate a shift to the right of LTED cells to a normal level of sensitivity to estradiol.[7,9] From: Yue W et al. J Steroid Biochem Mol Biol 2003; 86(3–5):265–74;[8] ©2003 with permission from Elsevier.