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General Review

Transfection of Human Estrogen Receptor (ER) cDNA into ER-negative Mammalian Cell Lines

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Estrogen responsiveness of breast tumors can be correlated with the presence or absence of the estrogen receptor (ER). Breast cancer cells that contain ER are, in general, responsive to stimulation by estrogen both *in vivo* and *in vitro*; therefore hormonal control is possible. Breast tumors that lose the ER, and become hormone-independent are refractory to the direct effect of estrogens and antiestrogens. It is therefore of interest to determine whether the re-expression of the ER will be sufficient to make ER-negative cells sensitive to the growth effect of estrogen. Transfection experiments with wild type and mutant ER cDNAs into different mammalian cell lines have been performed to re-establish hormonal control over hormone-independent cells. Paradoxically, introduction of exogenous ER into ER-negative cells and treatment with estrogen leads to growth inhibition rather than growth promotion. The activation of a number of estrogen-regulated genes has been examined in ER-transfectants but gene regulation is often variable. It is clear that the transfection of the ER gene into cells lacking this protein does not simply re-create the native ER-positive phenotype. Studies need to be extended to identify either the transcription factors that interact with ER to cause the negative effects of estrogen indirectly ("squelching") or the precise target genes that cause growth inhibition directly.

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INTRODUCTION

Steroid hormones play a central role in mammalian development by regulating the expression of a variety of target genes [1]. Estrogen, in particular, influences many developmental and physiological responses and has a profound effect on the proliferation and differentiation of estrogen-responsive tissues, such as uterus, the pituitary, and the mammary glands [2]. Regulatory functions of estrogen are mediated by the estrogen receptor (ER), a nuclear transcription factor. The estrogen receptor complex binds to specific DNA sequences, called estrogen-response elements (EREs), located in the promotor region of target genes, to modulate gene expression [3, 4]. The ligand–receptor complexes in addition to their primary role to stimulate or repress rates of specific gene transcription can also modulate gene transcription indirectly by interaction with other proteins that are components of the basal transcriptional apparatus [5, 6] and may also regulate post-transcriptional events [7]. Molecular cloning, functional analysis, and mutagenesis studies have revealed the existence of distinct functional domains (Fig. 1) in the receptor: the amino-terminal region, containing hormone-independent transcription function (TAF-1), a central domain responsible for DNA-specific recognition and binding, and a carboxy-terminal hormone-binding domain (HBD), containing its own hormone-dependent activation domain (TAF-2). The HBD appears to be the most complex region of the protein in both structure and function [8–10].

Despite extensive studies of ER domains, the precise molecular mechanism by which the receptor stimulates transcription of responsive genes in target cells and the molecular basis for cell-specific differences in the activity of the receptor, is still unknown. Identification of possible cell-specific proteins that may interact with the ER, and therefore regulate transcription of appropriate

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target genes, is a major challenge for the molecular biologist. Some of these estrogen-regulated genes are involved in growth regulation and developmental control and are of special interest to cancer research [7].

Estrogen responsiveness of different tumors can be correlated with the presence or absence of the ER. Breast cancer cells that contain ER are, in general, responsive to stimulation by estrogen both *in vivo* and *in vitro* [11–13]. Estrogen-induced tumor proliferation can be blocked by antiestrogens (e.g. tamoxifen), which forms the basis of their therapeutic use for the successful treatment of breast cancer [14–17]. However, 40–50% of ER-positive metastatic breast tumors fail to respond to antiestrogen therapy and only 10% of patients with ER-negative tumors respond [18]. What kind of progressive changes occur when growth control by tamoxifen is lost? One hypothesis is the loss of the functional receptor and the cells begin proliferating in a hormone-independent manner [19]. Another possibility would be the occurrence of mutations in the ER gene, that lead to either non-functional or constitutively active receptor [20]. A third reason could be the development of tamoxifen stimulated tumor growth [21].

Several laboratories have demonstrated the presence of altered ER mRNAs in breast tumor biopsies [22, 23] and breast cancer cell lines [19]. Different forms of ER (65 and 47 kDa) have been reported by Jozan *et al.* [24] in breast tumors and by Pink *et al.* [25] in the MCF-7 breast cancer cell line (65 and 80 kDa). Fuqua *et al.* [26, 27] have reported both constitutive active ER mutants, which can confer estrogen independence and a negatively acting mutant which while being transcriptionally inactive itself, inhibits the function of wild type ER. Recently we reported [28] the isolation of the first natural mutant ER which contains a point mutation at amino acid 351 where an aspartate is changed to a tyrosine. The mutant receptor forms at least 80% of the receptors observed in this tamoxifen(TAM)-stimulated breast tumor. Interestingly, the mutation is near a site where another mutation (VAL 400) has been shown to confer agonist activity to antiestrogenic ligands [29, 30]. Since only wild type ER was detected in three other TAM stimulated tumors [21], other mechanisms not requiring a mutant ER must also be capable of leading to TAM stimulated growth. Thus, although no clear association has been made between ER defects and estrogen and TAM resistance [31, 32] it is possible that mutant ERs may play a role in the etiology of resistance [19, 28, 33].

These arguments suggest two possible ways to re-establish hormonal control over proliferation of hormone-independent breast cancer tumor cell: (1) re-introduction of fully functional ER gene or (2) correction of a mutation that originally rendered the internal gene non-functional. Both ways may be feasible using the powerful tools of modern molecular biology as applied to therapeutic research.

Much has been learned about the action of estrogen since the human ER (hER) cDNA has been cloned from the human mammary carcinoma cell line MCF-7, and the DNA sequence of this gene has been determined [34–36]. The availability of the ER gene and its DNA target sequence [37] has greatly facilitated studies on the effects of deletions and mutations on ER activities [38–41] and the analysis of estrogen-mediated regulation of gene expression in different cell types [42–46]. These studies were performed either on estrogen-sensitive cells or on estrogen-insensitive cells into which ER was introduced *in vitro* by transfection. Since endogenous ER is a very rare protein it was important to have an expression system that yields a high level of active material for laboratory studies. Initially these kind of studies were done in yeast and insect systems [47, 48] but results were difficult to correlate with effects in mammalian cell system. Subsequently mammalian cell lines were established through stable or transient transfection protocols, and these cell lines are now widely used [45, 46, 49–55]. The choice between stably or transiently transfected

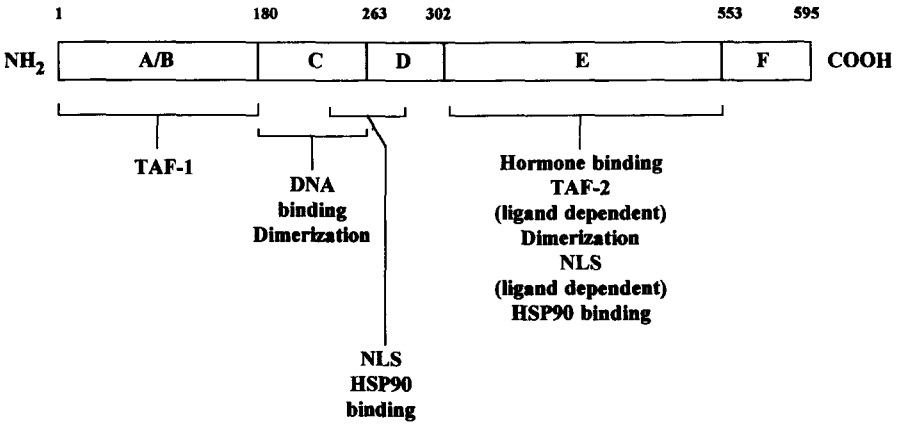


Fig. 1. Schematic representation of the structure and functional organization of ER. TAF, transcription activation function; NLS, nuclear localization signal.

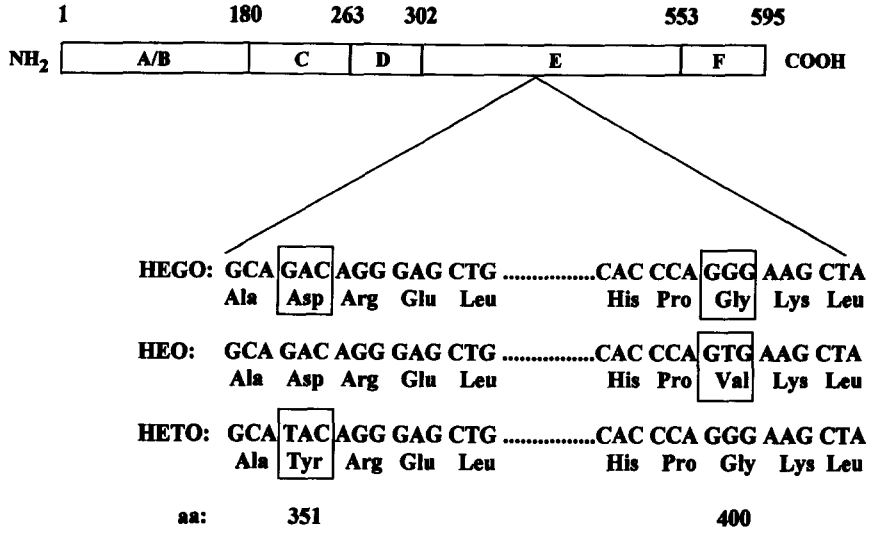


Fig. 2. Schematic representation of the structure of wild type hER cDNA (HEGO) and known single point mutations.

cell models depends on the aims of a particular study: stably transfected cell lines are absolutely necessary for proliferation assays [46, 49, 50, 55] but there is also a need for a transient transfection system that not only allows expression of high level of active receptor but also simplifies structure–activity studies on the introduced mutant proteins [52, 53, 56, 57].

At present it is not clear whether stable integration of transfected ER gene into chromosomal DNA might be important for the regulation of ER gene expression. Both stable and transient transfections are toxic to cells when the ER is artificially overexpressed. One possible explanation for the toxic effects of estrogen in stably transfected cells is that any overexpression of ER leads to ER-dependent binding to transcription factors ("squelching") which in turn causes activation of unknown lethal genes [51] or switches off important "house-keeping" genes. Alternatively, the toxic effect of estrogen on cells transiently transfected with ER gene may be due to the destabilizing influence of high levels of extrachromosomally replicating DNA [58].

To complicate matters further the ER gene originally cloned and used in many early studies contains a single point mutation, resulting in the Gly → Val replacement at amino acid position number 400 in the ligand-binding domain of the protein. The mutation is believed to be due to a cloning artifact [59]. At present, there are three standard or naturally occurring cDNAs for ER available for transfection studies: HEGO, HEO and HETO. The wild type ER cDNA is referred to as HEGO, the original cDNA contains valine at position 400 and is referred to as HEO. The cDNA of mutant ER obtained from a TAM-stimulated tumor which contains tyrosine at position 351 is referred to as HETO (Fig. 2). HEO has been reported to have reduced affinity for estradiol compared with the wild type receptor (HEGO) when tested at 25°C, although the affinity at 4°C is not affected [59]. The Val 400 mutation leads to enhanced estrogenic activity of antiestrogen 4-hydroxytamoxifen (4-OHT) [29] (Fig. 3) and of the steroidal antiestrogen RU 39411 [30] while the pharmacology of the pure antiestrogen ICI 164,384 is not altered by this mutation [46]. The mutation also causes changes in the characteristics of ER binding to the ERE [60] and possibly as a consequence, changes in the transfected cells. Overexpression of wild type ER is more toxic to the transfected cells than overexpression of receptor derived from HEO (wild type causes cell death at 100,000 receptors/cell compared to 6 million receptors/cell for the mutant ER) [61].

Analysis of transiently transfected cDNAs demonstrate that other point mutations and small deletions in the domains of ER may alter the function of the receptor. The Cys at amino acid 447 and 530 have been found to be important for the ability of ligand-binding [40] and the amino acids near C530 appear to be involved in receptor discrimination between estrogens and antiestrogenes [56].

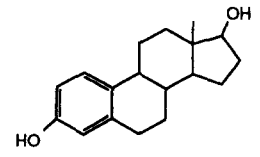
Although transient transfection allows the study of receptor–ligand transcription activities at a given ERE in appropriate reporter plasmids, the endpoint of major interest is the ability of ER complexes to regulate growth. We will focus upon the stably transfected hER gene into ER-negative cells to evaluate the effects of estrogen on cell proliferation.

EFFECTS OF ESTROGEN ON CELL PROLIFERATION IN CELLS TRANSFECTED WITH hER

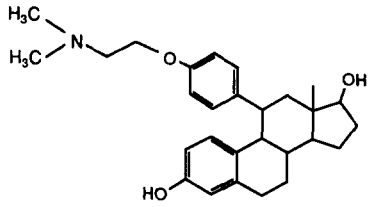
The mode of action of the estrogen in the regulation of growth is still a matter of controversy. In normal estrogen-dependent tissue, estrogen stimulation of growth and differentiation is carefully controlled. In

contrast proliferation of estrogen-dependent cancer cells is unchecked and unregulated. Estrogen (E2), upon binding to its high affinity receptor, triggers expression of multiple genes involved in the regulation of cell proliferation. It has been shown that expression of several proliferation-promoting genes (oncogenes c-*myc*, c-*fos*, c-*jun*) is affected by E2 [62–64] both *in vivo* and *in vitro*. In contrast there is a recent report [65] about the estrogen regulation of growth arrest specific (gas) genes.

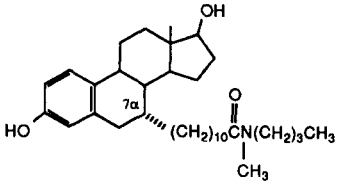
It has been of interest to determine whether the expression of the ER will be sufficient to make cells sensitive to the growth effect of E2. In order to examine the effect of E2 on proliferation of E2-unresponsive cells, mouse 3T3 fibroblasts were stably-transfected with hER cDNA (HEO) [45]. Contrary to the responses observed for the conventional E2 target tissues, E2 did not have a growth promoting effect in any of the sublines. In one subline with morphological and growth behavior characteristics of transformed cells, E2 was growth inhibitory and even toxic to the cell. Growth inhibitory and/or cytotoxic effects of E2 have been observed in several other cell lines originally lacking ER but expressing exogenous ER after transfection with either wild (HEGO) or mutant type (HEO) of ER gene. When the mutant ER cDNA (HEO) was transfected into HeLa cells (human adenocarcinoma cell line of cervical origin) E2 caused growth inhibition and this effect correlated with the ER content [49]. In contrast, Touiton *et al.* [44] did not observe any effects of E2 on cell proliferation of HeLa/ER+ (HEO) transfected clones. This controversy may



17β-ESTRADIOL



RU 39411



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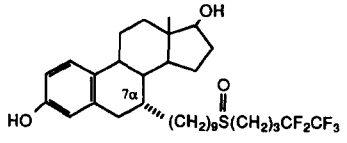

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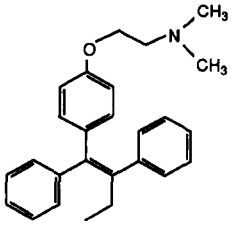
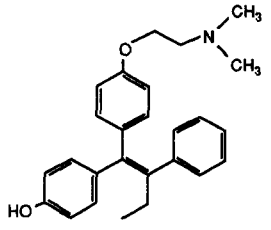
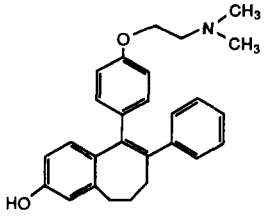


TAMOXIFEN



4-HYDROXYTAMOXIFEN



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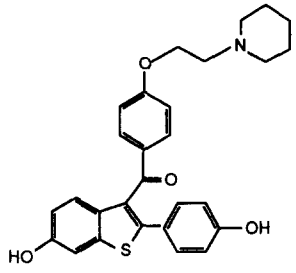

KEOXIFENE

Fig. 3. Structure of estrogenic and antiestrogenic compounds referred to in the text.

be due to much lower ER concentrations in this HeLa/ER+ clones (5–10 fmol/mg total protein) while Maminta *et al.* [49] reported ER concentrations of 223 fmol/mg protein. Similarly Kushner *et al.* [51] observed growth inhibition and toxic effect of E2 in Chinese hamster ovary (CHO) cell lines overexpressing hER (HEO).

In vitro studies with ER-positive osteosarcoma cell lines, expressing endogeneous ER, showed that E2 had growth-stimulatory effects [66–68]. However, when ER-negative human osteosarcoma cells HTB96 were transfected with hER (HEO) growth inhibition and not promotion was observed in response to E2 treatment [50, 69]. The same inhibitory effect of E2 was observed in rat osteoblastoma cell line (Ros 17/2.8) transfected with exogeneous hER [70].

In ER-positive breast cancer cell lines MCF-7 and T47D proliferation is greatly stimulated by E2 [71]. In accordance with all other data, introduction of exogeneous ER (either HEGO or HEO) into ER-negative cells MDA-MB-231 [46], MDA-MB-231 and 21T [54] or in immortalized normal breast cells [54, 72] leads to growth inhibition. Other investigators have also reported [55] E2-dependent growth inhibition and antimetastatic effects in MDA-MB-231 cells transfected with wild-type ER.

The effect of E2 and antiestrogens on cell proliferation in different ER-negative cell lines, transfected with hER cDNA, is shown in Table 1. The antiproliferative effect of estrogen is constant in all of these experiments and is not due to the presence of mutations in ER. Perhaps more importantly, as can be seen from Table 1, potent antiestrogens such as 4-OHT or RU 39411 act as agonist in most ER-transfectants which express mutant ER (HEO). Cells, expressing wild type ER (HEGO) react to these antiestrogens predominantly as an antiestrogens with very weak agonist activity [29, 30, 55]. Pure antiestrogens like ICI 164,384, or keoxifene [30] retain their antiestrogenic activity with both mutant and wild receptors. To explain the estrogenic activity of TAM in cells with mutant ER we have suggested that a point mutation of the ER may alter the folding of the receptor around an antiestrogen to produce an "E2-like" coupling [30]. It is possible that this kind of mechanism is responsible for a form of drug resistance in TAM-stimulated tumors. When mutant ER (351 TYR) (HETO) isolated from TAM-resistant tumor is stably transfected into MDA-MB-231 cells 4-OHT produce E2-like action similar to the artificial mutant HEO (see Table 1, [57]).

It would be especially interesting to determine the effect of the introduction of exogeneous ER into ER-positive breast tumor cells expressing endogeneous ER. Unfortunately, these kind of experiments have not provided unequivocal results. In work reported by Zajchowski *et al.* [54] ER-positive breast cancer cell lines MCF-7 and T47D were transfected with hER cDNA but neither an enhanced induction nor inhibition of cellular growth in response to E2 was observed. Interestingly recombinant ER mRNA was detected in only 40% of the cells but many of the ER transfectants did not express significantly greater levels of ER protein than the parental cells. It is well known that ER expression in cells is regulated by a variety of factors including cell density, growth rate, differentiation-inducing agents, tumor promoters, cytokines as well as estrogens themselves [73]. Uncontrolled expression of exogeneous ER in ER-positive cells may lead to down-regulation of endogeneous ER gene by a feed-back mechanism and therefore such cells will not exhibit any significant changes in E2-response. It would be of interest to compare expression of exo- and endogeneous ER genes at both the transcriptional and translational level.

ACTIVATION OF GENES BY ECTOPIC EXPRESSION OF ER IN DIFFERENT CELL LINES

To explain the mitogenic effect of E2 it is assumed that the hormone regulates the expression of genes whose products control the cell cycle [74]. E2 induces the expression of early-induced proto-oncogenes c-*fos* and c-*myc* [62, 63, 75, 76] and the expression of the c-*jun* gene [77, 78]. The direct effect of estrogen on c-*fos* and c-*myc* expression on cell proliferation in estrogen target cells has been reported [64].

Indirect growth stimulation by E2 involves the induction of a variety of effects. Most progress has come from the use of breast cancer cell lines *in vitro* [79]. E2 stimulation produces increased levels of many enzymes, such as thymidine kinase [80] and lysosomal protease cathepsin D [81]. The mechanism of E2 action could also involve regulation of different members of transforming growth factors (TGF) β family, which are growth inhibitors in breast cancer cell lines. These observations are reported not only for ER+ cells but also for ER− cells, although results were inconsistent [82–84]. In the paper by Jeng *et al.* [85] designed to evaluate the expression of three different types of TGFβ, it was found that TGFβ1 and TGFβ2 mRNAs were expressed both in ER+ (MCF-7) and ER− (MDA-MB-231) cells, whereas TGFβ3 mRNA was detected only in MCF-7 cells. E2 decreases mRNAs for TGFβ2 and TGFβ3 in MCF-7 cells. However, only MDA-MB-231 and early passages of MCF-7 cell are growth inhibited by TGFβs [85].

E2 is believed to regulate other endogenous E2-responsive genes, such as progesterone receptor (PgR), epidermal growth factor receptor (EGFR), prolactin (PRL), and the ovalbumin gene. Expression of these developmentally regulated E2-responsive genes is tissue-specific, and part of the differential effects from E2 can be attributed to the cell-specific expression of the ER [86, 87]. The tissue-specific differential regulation of E2 responsive genes indicates that other factors are necessary to bring about the complexity of E2 action *in vivo* [88].

Table 1. Effects of E2 and antiestrogens on cell proliferation in ER-transfectants

Cell type	cDNA	Growth response to ligand [M] alone: E2	Growth response to ligand [M] alone: Anti-estrogens	Activation activity against E2: Partial antiestrogens	Activation activity against E2: Pure antiestrogens	Ref.
3T3	HEO[a]	[10^{-9}] inhibition	TAM [10^{-6}] inhibition	agonist		45
		[10^{-7}] toxic	4-OHT [10^{-8}] inhibition	agonist		
CHO	HEO	[10^{-10}] inhibition	4-OHT: inhibition toxic			51
		[10^{-9}] toxic	ICI[1]: no effect nontoxic			
CHO	HEGO[b]	toxic even w/o E2				61
HeLa	HEO	[10^{-8}] inhibition	TAM and 4-OHT [10^{-6}] no effect	inactive		49
HeLa	HEO	[10^{-7}] no effect				44
HTB 96	HEO	[10^{-9}] inhibition	TAM and 4-OHT	weak antagonist		50
HTB 96	HEO	[10^{-8}] inhibition	TAM: part. inhibition	weak antagonist		69
MDA-MB-231 (S30)	HEGO	[10^{-10}] inhibition	4-OHT: [10^{-6}] part. inhibition	weak agonist		30
MDA-MB-231 (ML-α-2H)	HEO	[10^{-8}] inhibition	4-OHT: [10^{-6}] inhibition	agonist		
MDA-MB-231 (S30)	HEGO	[10^{-10}] inhibition	ICI[d]: [10^{-6}] no effect		antagonist	46
MDA-MB-231 (ML-α-2H)	HEO	[10^{-8}] inhibition	ICI[d]: [10^{-6}] no effect		antagonist	
MDA-MB-231	HEGO	[10^{-9}] inhibition	4-OHT and ICI: no effect	antagonist	antagonist	55
MDA-MB-231 (S30)	HEGO	[10^{-10}] inhibition	RU39411 [10^{-9}] part. inhibition	weak antagonist		31
			keoxifene [10^{-10}] no effect		antagonist	
MDA-MB-231 (ML-α-2H)	HEO	[10^{-8}] inhibition	RU39411 [10^{-10}] inhibition	agonist		
			keoxifene [10^{-10}] slight inhibition		antagonist	
MDA-MB-231 21T	HEO	[10^{-10}] inhibition	TAM: inhibition ICI: no effect		antagonist	54
	HEGO	inhibition	TAM. inhibition			
MDA-MB-231 (S30)	HEGO	[10^{-10}] inhibition	4-OHT[e]: [10^{-6}] part. inhibition	weak agonist	antagonist[f]	57
MDA-MB-231 (ML-α-2H)	HEO	[10^{-8}] inhibition	4-OHT: [10^{-6}] inhibition	agonist	antagonist	
MDA-MB-231 (BC-2)	HETO[c]	[10^{-10}] inhibition	4-OHT: [10^{-9}] inhibition	agonist	antagonist	

Growth response to ligand [M] alone is expressed in concentration required for shown growth effect. Antagonist activity against E2 indicates ability of antiestrogens to reverse the inhibitory effects of E2.

[a]VAL 400 hER cDNA; [b]the wild type hER cDNA; [c]TYR 351 hER cDNA; [d]ICI 164,384; [e]fixed ring 4-OHT; [f]ICI 182,780 (see Fig. 3 for the formulas of the compounds).

To try to understand the inhibitory effect of estrogen on the growth of transfected cells it is necessary to study the direct and indirect induction of genes involved in the regulation of cell proliferation. Several questions can be addressed:

(1) What are the effects of ectopic ER expression on cells that normally do not express ER?
(2) Is the loss of responsiveness of E2-regulated genes a reversible process? Of particular interest is the question whether the addition of functional receptor is sufficient to trigger the expression of genes which are normally E2 inducible in ER-positive breast cancer cell lines.
(3) What additional target genes are affected by E2 in ER-transfected cells?

The published results paint a complicated picture of E2 regulation of endogenous genes. Table 2 summarizes the available data.

Table 2. Effect of ectopic ER expression on endogenous genes known to be E2 regulated or ER-related

Cell type	cDNA	E2-regulated genes[a]	E2-non-regulated genes[b]	Ref.
3T3	HEO[c]		c-*myc* c-*jun*	45
RAT1	HEO	↑ PgR	EGFR PRL	88
HeLa	HEO	↑ c-*fos*	c-*myc*	74
HeLa CEF	HEO	↑ ovalbumin		89
HeLa	HEO	↑ cath.D	PgR pS2	44
HeLa	HEO	↓ c-*myc*	PgR pS2	49
HTB 96	HEO		PgR EGFR alkaline phosphatase	50
HMEC	HEO	↑ TGFα ↑ cath.D ↑ low pS2		92
HMEC	HEO	↓ c-*myc*	*erb*B2 EGFR	72
MDA-MB-231	HEGO[d] HEO	↑ low PgR ↑ very low PgR		46
MDA-MB-231	HEGO	↑ TGFα ↓ TGFβ2	TGFβ1 TGFβ3	91
MDA-MB-231	HEO	↑ IGFBP-4 ↑ pS2		93
MDA-MB-231	HEO	↑ RARα ↑ pS2		94

[a]↑ indicates increased levels of mRNAs following estrogen treatment; ↓ indicates decreased levels of mRNAs following estrogen treatment; [b]no effects were noted on the amounts of mRNAs following estrogen treatment; [c]VAL 400 hER cDNA; [d]the wild type hER cDNA.

Fibroblast and fibroblast-like cell lines are E2-independent and lack endogeneous ER. Studies on hER-transfected mouse 3T3 fibroblasts show that antimitogenic effects of E2 on these cells were not related to c-*myc* and c-*jun* mRNAs cellular contents [45]. However, activation of the silent PgR gene by ectopic expression of ER in rat embryonic fibroblast-like cells (Rat1) was reported [88]. In contrast, PRL, usually expressed in the pituitary and controlled by E2, is not expressed in these Rat1 cells transfected with ER gene in the presence or absence of E2. Another observation is that although EGFR is basally expressed in RAT1 transfected cells, the addition of E2 has no effect [88]. Touitou *et al.* [44] reported enhanced synthesis of endogenous cathepsin D in HeLa cells stably transfected with the ER gene, when cells were treated with E2, while two other genes PgR and pS2 known to be E2-inducible in breast cancer cell line were not expressed. Similarly, the transcription study of Maminta *et al.* [49] in HeLa cells with transduced ER gene showed that both PgR and pS2 were not induced upon addition of E2 while c-*myc* expression was decreased. Activation of some proto-oncogenes and the ovalbumin gene by the ER were demonstrated [74, 89].

Stable transfection of the ER gene into HTB 96 human osteosarcoma cells was performed to study the effect of exogenous ER expression upon genes known to be either E2 regulated or ER-related in bone and other E2-responsive tissues. Watts *et al.* [50] observed no E2 regulation of endogenous levels of PgR, EGFR and alkaline phosphatase activity in HTB 96 transfectants even though these genes are E2 regulated in ER-positive osteoblasts [90]. Interestingly, alkaline phosphatase activity was E2-induced in ROS17/2.8 rat osteoblasts transfected with mouse ER [70], although interpretation of these results is complicated by the presence of low levels of endogenous ER and E2-inducible alkaline phosphatase activity in the untransfected parent line [69].

The activation of a variety of endogenous genes has also been examined in breast cancer ER-negative cells transfected with the ER gene. Low level of PgR was measured in MDA-MB-231 cell line after transfection with ER [46]. Studies of TGFα and TGFβ mRNAs in MDA-MB-231 transfected with HEGO reveal that transfected cells regulate their growth factors in exactly the same way as MCF-7 cells, although the net result

is growth inhibition: E2 increases TGFα mRNA level and decreases TGFβ2 levels, whereas TGFβ1 and TGFβ3 levels are unaffected [91]. Similarly, the increased expression of TGFα mRNA levels is demonstrated in both immortal and tumorigenic HMEC ER-transfectants [92]. The authors also showed that E2 treatment increased expression of a 52 kDa cathepsin D mRNA levels in both transfectants as it does in ER-positive breast cancer cells; however induction of pS2 gene expression was not as dramatic as it was in ER-positive cells. Consistent with its growth inhibitory effects, E2 decreased the c-*myc* mRNA levels in HMEC transfectants, but no effects were noted on the amounts of *erb*B2 or EGFR mRNAs [72]. Expression of functional ERs in previously ER-negative MDA-MB-231 cells conferred the ability to enhance insulin-like growth factor binding protein-4 (IGFBP-4) mRNA levels suggesting direct relationship between the ER status and retinoic acid (RA)/estrogen modulation of (IGFBP-4) gene expression [93]. "Positive" results were obtained by these authors concerning the relationship between RA and estrogen regulation of HBC cell proliferation. They showed that the expression of functional ERs in MDA-MB-231 cells made them sensitive to RA-mediated growth inhibition by expressing constitutively higher level of retinoic acid receptors (RARs) mRNA than their parental counterparts [94].

SUMMARY AND CONCLUSIONS

Several general principles can be derived from the existing data, i.e. (1) the effect of E2 on cell proliferation is paradoxically opposite in most transfected cells when compared to cells expressing endogenous ER and (2) that E2-regulated endogenous genes are not always adequately responsive in transfected cells. We can conclude that transfection of the hER gene into cells lacking this protein does not simply re-create the native ER-positive phenotype. In agreement with our conclusion, other investigators proposed that the transfected ER gene is not necessarily functionally equivalent to endogenous ER [69].

Several possible explanations can be suggested to describe estrogenic regulation of both cell proliferation and gene expression in transfected cells: (1) perhaps antimitogenic activity of ER is a consequence of the induction of the expression of genes encoding growth inhibitory/cytotoxic proteins in the presence of E2 due to genetic re-arrangements consequent to transfection [45]. (2) It is possible that antimitogenic/cytotoxic properties of ER may be due to its unregulated constitutive overexpression, which artificially occurs in transfected cells [51, 61, 69]. As a consequence, the action of a large excess of free ER is no longer limited by the interaction of the receptor and ERE, but instead produces a transcriptional interference or "squelching", affecting genes which are not normally E2-responsive [50, 61, 95]. The principle of interference has already been shown for the ER and either glucocorticoid or PgR-mediated responses by ER transiently expressed in HeLa and HTB 96 cells [69, 96]. (3) Finally, cross-coupling mechanism may occur with other transcription factors as has been shown for ER interaction with AP1 [89]. Using ER with a mutated DNA binding domain, co-activation by ER and Fos was achieved without ER binding to the ERE [89]. Similarly, Zajchowski and Webster [97] reported inhibitory effects of E2 and TAM on HMEC cells stably expressing ER mutant that lacks the DNA binding domain, suggesting that there is possibility that the antiproliferative effects of E2 and TAM occur due to transcription of genes which do not require the binding of the ER to the ERE.

From the data one can therefore conclude, that the regulatory functions exerted by the ER are likely to be different in cells with endogeneous ER and transfected ER gene. In cells with endogeneous ER direct activation of appropriate genes is well documented and requires tight binding of the receptor to an ERE [3]. In ER-transfected cells it appears to be the indirect regulation of genes by exogenous ER interacting with essential growth regulators. ER can directly bind to other transcription factors to generate transcriptional activity through responsive elements for these transcription factors. Conversely ER can sequester unknown proteins necessary for the activation of growth; their removal therefore causes growth inhibition.

In conclusion, the models of ER response *in vitro* based on ER-transfected cells are probably not appropriate for the study of estrogen regulation of cell proliferation and gene expression, because ER triggers the opposite effect in ER-positive and ER-negative cell lines. However, these model systems provide a unique opportunity to study reporter gene regulations to determine responsive genes in different target cells and to learn about the molecular basis for cell-specific differences in the activity of the receptor. It has been found that the expression or activity of proteins–co-activators of ER are cell-specific [7]. Therefore, to understand the mechanism by which estrogens regulate cell growth and development we should focus attention on attempts to identify the unknown transcription factors which interfere with the activity of the ER in the regulation of genes in ER-transfectants. This approach might not only explain the paradoxical effects of E2 on cell proliferation but also provide a valuable key to the critical events that must be orchestrated to maintain cell replication.

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