Estrogen receptors and their downstream targets in cancer

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Summary. Estrogen has crucial roles in the proliferation of cancer cells in reproductive organs such as the breast and uterus. Estrogen-stimulated growth requires two estrogen receptors (ERα and ERβ) which are ligand-dependent transcription factors. High expression of ERs is observed in a large population of breast tumors. In addition, the positive expression of ERs correlates with well-differentiated tumors, a favorable prognosis, and responsiveness to an endocrine therapy with anti-estrogen drugs in patients with breast cancer. Transcription activities of ERs can be regulated by interacting proteins such as coactivators and kinases as well as ligand-binding. Moreover, ER isoforms lacking an ability to transactivate are involved in breast cancer. Downstream target genes of ERs have important roles in mediating the estrogen action in breast cancer. We have isolated and characterized several novel estrogen-responsive genes to clarify the molecular mechanism of the estrogen action in target cells. Among these genes, the estrogen-responsive finger protein (Efp) was found to be highly expressed in breast cancer. Efp as a ubiquitin ligase (E3) is involved in the proteasome-dependent degradation of the 14-3-3σ protein, one of cell cycle brakes, this degradation resulting in the promotion of breast cancer growth. A full understanding of the expression and function of ERs and their target genes could shed light on how estrogen stimulates the initiation and promotion of cancer, providing a new approach to diagnose and treat cancer.

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Background

Estrogen, a sex steroid hormone, exhibits important biological functions in the target tissues such as reproductive organs. Among these tissues, the growth of the mammary gland and uterine endometrium during pregnancy and the menstrual cycle is dependent on estrogen. In addition to proliferative effects on normal cells, estrogen is considered as a stimulant for the initiation and promotion of tumors in these organs. Epidemiological studies show that prolonged exposure to estrogen, i.e. early menarche, late menopause, and estrogen replacement therapy, can be a risk factor in breast and uterine cancers (Rose, 1996; Clemons and Goss, 2001). In vitro experiments indicate that cells derived from breast and uterine tumors are capable of growing in response to estrogen administration (Holinka et al., 1986; Foster et al., 2001). It is reasonable to assume that the stimulatory effects of estrogen on cell proliferation also contribute to malignant tumor growth. Following prolonged exposure to estrogen, an increase in cell proliferation would be expected to cause an increase in spontaneous DNA replication errors. When mutated in target cells of estrogen, it would enhance the replication of clones of cells carrying such genetic errors. It is, therefore, important to understand mechanisms by which estrogen increases cell proliferation in estrogen-associated cancer.

The estrogen-stimulated growth in tumor cells as well as in normal cells requires the estrogen receptor (ER). It has been shown that about two-thirds of human breast tumors express higher concentrations of ERs than normal breast tissues (Early Breast Cancer Trialists’ Collaborative Group, 1998). The ER expression status is related to a variety of histologic characteristics of breast cancer. Most tumors with low grades are ER-positive but, in contrast, tumors demonstrating histologic evidence of poor tumor differentiation are frequently ER-negative (Millis, 1980; Fisher et al., 1981). Breast tumors which lack any ER expression often reveal more aggressive phenotypes (Clarke et al., 1994).
Clinically, endocrine therapy with anti-estrogen drugs or aromatase inhibitors is utilized to treat hormone-related cancer (Howell, 2000; Ali and Combos, 2002). It is expected that tamoxifen, an anti-estrogen drug, binds to ER, making it nonfunctional, while aromatase inhibitors reduce estrogen levels. As discussed below, most breast tumors expressing ER are primarily able to respond to tamoxifen. Aromatase inhibitors such as anastrozole and letrozole are especially useful in patients who are or become resistant to tamoxifen. However, a substantial portion of patients with breast cancer eventually acquire resistance against these treatments. In addition, most of the ER-negative breast tumors can not respond to the anti-estrogen drug. Furthermore, several side effects by treatment with tamoxifen and aromatase inhibitors to ER positive cancer such as breast cancer, have been reported (Wiseman and Adkins, 1998; Buzdar and Hortobagyi, 2000; Howell, 2000; The ATAC Trialists’ Group, 2002).

It is thus important to uncover the precise mechanism of the estrogen action in breast cancer. In particular, the elucidation of regulatory mechanisms for the expression and function of ERs could provide useful information to predict the responsiveness to endocrine therapy and the prognosis. Moreover, it is important to reveal roles of downstream target genes for ERs, which mediate the effects of estrogen on the proliferation of cancer cells while these genes can be targeted to treat and diagnose estrogen-associated tumors.

**Estrogen receptors**

As stated above, ER has two subtypes, ERα and ERβ. They belong to the superfamily of nuclear receptors that share similar structures and modes of action (Nuclear Receptors Committee, 1999) (Fig. 1). Namely, estrogen-bound ERs bind as a homodimer or as a heterodimer with an estrogen-responsive element (ERE) with their DNA-binding domain and regulate the transcription of the target genes. ERs contain two independent transcriptional activation functions (AF): the N-terminal A/B domain possesses an autonomous

**Fig. 1.** Schematic representation of human ERα and ERβ. The ERα and ERβ are transcription factors whose activities are regulated by their ligand binding. ERs are members of a nuclear receptor superfamily comprised of six regions (A–F). The ligand-binding domain (LBD) in region E also contains an estrogen-inducible transcription-activating function called AF-2. A constitutively active transcription-activating function (AF-1) is located in the A/B region. Percentages of amino acid identities between the corresponding regions are represented.

**Fig. 2.** A model for the regulation of estrogen receptor (ER)-mediated transcription of estrogen-responsive genes. Liganded ERα and ERβ bind as a homodimer or as a heterodimer with an estrogen-responsive element and regulate the target gene transcription. Coactivators are required to mediate ligand-activated transcription by enhancing nuclear receptor transactivation through contacts with the basal transcriptional machinery. Phosphorylation of the ER also modulates the transcription activity.
AF-1, while the E-domain possesses a ligand-dependent AF-2. Biological activities of ERs could be controlled by a number of interacting proteins. The ligand-dependent transactivation of ERs requires the recruitment of coactivators such as TIF2 and SRC-1 (Glass and Rosenfeld, 2000). Transcription activities of ERs are also regulated by phosphorylation. In particular, the serine residue at 118 within the A/B domain of human ERα is a major target site of phosphorylation by MAPK in the presence of growth factors (Kato et al., 1995) and by Cdk7 in a ligand-dependent manner (Chen et al., 2000). Recently, we demonstrated that this serine residue is opposingly dephosphorylated by protein phosphatase 5 (Ikeda et al., 2004) (Fig. 2). The protein level of ERα is regulated by the ubiquitin-mediated proteasomal degradation (Nawaz et al., 1999; Tateishi et al., 2004). In addition, some elements in the promoter region have been shown to be responsible for a high expression of ER α in breast cancer cells (Hayashi et al., 1997; Tanimoto et al., 1999). Collectively, it is reasonable to assume that these regulatory mechanisms of ERs are closely associated with oncogenesis and tumor growth. Moreover, it is also indispensable for the diagnosis and treatment of estrogen-associated cancer to reveal the regulatory mechanisms for expression levels of the ER mRNA and protein.

The expression of ERβ has been detected in various tumors including breast cancer (Omoto et al., 2002), uterus

![Fig. 3. Immunohistochemical staining of COX7RP (A and C) and ERα (B and D) in human endometrium in the proliferative phase of the menstrual cycle. ERα and COX7RP immunoreactivities were detected in the nucleus and cytoplasm, respectively. Strong immunoreactivities of ERα and COX7RP were detected in the glandular epithelia. Scale bar=100 μm (A, B); 10 μm (C, D).]
cancer (Sasano et al., 1999), and prostate cancer (Fujimura et al., 2001). In breast cancer, ERβ shows a tendency to be expressed in ERα-positive carcinomas, while ERα and ERβ double positive cells are also detected. ERβ, as well as ERα, serves as an indicator of a good prognosis in breast cancer (Omoto et al., 2002). It has been found that several variants of ERβ are expressed in breast cancer cells (Leygue et al., 1999). We originally isolated an ERβ isoform, ERβcx (Ogawa et al., 1998), which lacks the last 61 C-terminal amino acids and has an alternative 26 unique amino acids. The ERβcx isoform shows no ligand binding ability and has no capacity to activate transcription in response to estrogen (Ogawa et al., 1998). Moreover, ERβcx shows preferential heterodimerization with ERα rather than with ERβ, inhibiting ERα DNA binding and transactivation. In ERα positive breast cancer, the presence of ERβcx is significantly correlated with the absence of a progesterone receptor (PR) which is a downstream target of activated ER, indicating that ERβcx is a dominant repressor of the ER function in breast cancer. (Saji et al., 2002). These lines of evidence suggest that ERβ isoforms are important functional modulators of estrogen-signaling pathways in breast cancer cells and may affect the clinical outcome of patients with breast cancer.

**Estrogen-responsive genes in cancer**

Estrogen modulates transcription of downstream target genes through ERs. It is thus fundamentally important to identify genes whose expression is regulated by estrogen and to reveal the functions of their protein products. Although a list of ER-target genes has been accumulating, the entire mechanism by which ER enhances the proliferation and progression of tumors remains unknown. In particular, only a few genes are known to be directly regulated by ER through EREs. In order to isolate estrogen-responsive genes having EREs in their transcription regulatory region, we have developed the genomic binding-site cloning (GBSC) method (Inoue et al., 1991, 1999). Using this method, several genomic sequences containing EREs were successfully isolated. Subsequently, novel estrogen-responsive genes were identified nearby the EREs (Inoue et al., 1993; Watanabe et al., 1998). Protein products of these genes include the estrogen-responsive finger protein (Efp), the cytochrome c oxidase subunit VIIa-related polypeptide (COX7RP), and the estrogen receptor-binding fragment-associated antigen 9 (EBAG9).

COX7RP has a well conserved region with cytochrome c oxidase subunit VIIa (Watanabe et al., 1998). Expression of the COX7RP mRNA was up-regulated by estrogen in
MCF7 cells. The perfect palindromic ERE found in the first intron possesses an estrogen-dependent enhancer activity in these cells. In addition, an immunohistochemical study demonstrated that the COX7RP protein is co-expressed with the ERα protein in the endometrial glandular epithelium of the human uterus (Fig. 3). We speculate that COX7RP is involved in the regulation of energy production in target cells by estrogen.

Molecular mechanism of Efp function in breast cancer

Among ER-downstream molecules isolated by the GBSC method, we have clarified the molecular mechanism of Efp, which possesses a RING finger motif, two B-boxes, a-helical coiled-coil domains, and a C-terminal SPRY domain (Inoue et al., 1993) (Fig. 4). The RING finger motif is comprised of a unique linear series of conserved cysteine and histidine residues that features a ‘cross-brace’ arrangement with two zinc ions (Pickart, 2001). Members of the RING finger family grow enormously; some of them have been shown to be responsible for malignant tumors. For instance, PML is responsible for acute premyelocytic leukemia when it forms a fusion protein with the retinoic acid receptor (RAR)α by chromosomal translocation (Jensen et al., 2001). Loss of the tumor suppressor BRCA1 results in chromosomal instability leading to the development of familial breast and ovarian tumors (Ruffner et al., 2001). Efp is predominantly expressed in estrogen target tissues and cells including the mammary gland and uterine epithelial cells (Orimo et al., 1995). Efp is also highly expressed in breast tumors (Ikeda et al., 2000). Expression of the Efp mRNA was shown to be elevated after estrogen treatment in MCF7 cells (Fig. 5). Thus, Efp could function as an estrogen-responsive gene that mediates the estrogen action in cancer. The estrogen-responsive proliferation of the uterine endometrium which expresses abundant ERα was
shown to be impaired in Efp knockout mice, suggesting that Efp is a mediator of estrogen-dependent cell growth (Orimo et al., 1999).

To investigate the role of Efp in breast tumor growth, we examined the effects of Efp antisense oligonucleotides on tumor formation in female nude mice inoculated with MCF7 cells (Urano et al., 2002). These mice were ovariectomized or administrated with antisense/sense Efp oligonucleotides. We revealed that the Efp antisense oligonucleotide effectively inhibits the tumor growth generated by MCF7 cells in the recipient mice. MCF7 cells stably expressing Efp (Efp-MCF7) could proliferate even in estrogen-deprived ovariectomized mice. The Efp-MCF7 cells have lower concentrations of the 14-3-3σ protein, which is a negative regulator of the cell cycle progression. The 14-3-3σ protein is important for maintaining G2 arrest by sequestering phosphorylated Cdc2-cyclin B1 from the nucleus into the cytosol (Chan et al., 1999). Interestingly, the expression level of this protein is significantly low in breast tumors (Vercoutter-Edouart et al., 2001; Umbrich et al., 2001). We found that Efp associates with the 14-3-3σ protein. We then demonstrated that Efp functions as a ubiquitin ligase, E3, that ubiquitinates the 14-3-3σ protein, this ubiquitination resulting in the cell cycle progression via the proteasome-dependent degradation of the 14-3-3σ protein (Urano et al., 2002) (Fig. 6).

**Perspective**

A better understanding of the molecular mechanisms by which estrogen stimulates cell growth can provide new insights into diagnosis, treatment and prevention in estrogen-associated tumors. For this reason, it is indispensable to reveal the expressional and functional regulation of ERs and their target genes. Especially, the identification of estrogen-responsive genes which are closely related to the cancer biology could provide us new approaches for these fields.
Efp, an estrogen-responsive gene, would contribute to the deregulated proliferation of breast cancer cells by the accelerated destruction of a cell cycle regulator, 14-3-3σ. We speculate that Efp could promote tumor growth even in the absence of estrogen and, therefore, the high expression of Efp might be one of reason for acquiring the ability to proliferate independently of estrogen. The future investigation of the relationship between Efp expression and clinical or pathological features could indicate its usefulness as a potential prognostic factor. These trials may lead to the utilization of Efp as a prognostic marker and a therapeutic target in breast cancer. Thus, the accumulation of experimental evidence concerning the estrogen-responsive genes such as Efp can allow us to develop novel cancer treatments separately targeted for each downstream molecule that directly mediates the estrogen action.

References


