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Estrogen-responsive RING finger protein controls breast cancer growth☆

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Abstract

Most of the breast cancers initially respond to endocrine therapy that reduces the levels of estrogens or competes with estrogen for binding to its receptor. Most of the patients, however, acquire resistance to endocrine therapy with tamoxifen and aromatase inhibitors later. We assumed that identification of estrogen-responsive genes those regulate the growth of breast cancer is indispensable to develop new strategies targeting the genes and overcome the resistance to current endocrine therapy. Estrogen-responsive finger protein (Efp) is one of the estrogen receptor (ER)-target genes we have cloned using genomic binding site cloning. Efp features a structure of the RING-finger B-box coiled-coil (RBCC) motif. We postulated that Efp is a critical factor in proliferation of breast tumors. In a model system using MCF7 cells grown in xenografts, we showed that inhibition of Efp expression by antisense oligonucleotide reduced the tumor growth. MCF7 cells overexpressing Efp formed tumors in xenografts even in estrogen deprivation environment. By yeast two-hybrid screen, we identified that Efp interacts with 14-3-3σ, which is known as a cell cycle brake that causes G2 arrest and expressed in normal mammary glands. In vitro studies have revealed that Efp functions as a ubiquitin-protein ligase (E3) that targets 14-3-3σ. These data suggest that Efp controls breast cancer growth through ubiquitin-dependent proteolysis of 14-3-3σ. Future studies may provide a new therapy to block breast tumor proliferation by targeting Efp.

Keywords: Breast cancer; Endocrine therapy; Estrogen; Efp; RING finger; RBCC motif; Ubiquitin-protein ligase (E3); 14-3-3σ

1. Introduction

Breast cancer is the most common type of cancer in female and continues to be a major cancer death among women in the western world. Although primary treatment of breast cancer is surgical removal of the tumor, patients treated by surgery alone are likely to be further suffered from recurrent and metastatic disease. More than 100 years ago, removal of ovaries had been found to be effective in remission of metastatic breast cancer [1]. The ovarian hormone estrogen was discovered to stimulate breast tumor growth. Efforts had been made thereafter to establish endocrine therapy to inhibit estrogen actions or estrogen synthesis [2](Fig. 1). The endocrine therapy since the 1940s, and tamoxifen in particular, has revolutionized the treatment of breast cancer.

The direct effect of estrogens on estrogen-responsive tissues are mediated via the estrogen receptors (ERs), namely ERα and ERβ, in low levels in normal mammary gland tissue and in higher concentrations in about two-third of human breast cancers [3]. It is known that most of the ER-positive breast cancers are primarily responsive to endocrine therapy. Tamoxifen, which is one of the selective estrogen receptor modulators or SERMs, was first used in the treatment of metastatic breast cancer, and now the first choice of adjuvant treatment after surgery [4].

In postmenopausal women, local estrogen synthesis is important in tumor progression because of cease of ovarian function. Aromatase in breast tissue is responsible for local estrogen synthesis [5]. Aromatase inhibitors such as anastrozole and letrozole are now being used as second- and third-line agents in endocrine therapy, once resistance to tamoxifen has developed [2].

In spite of all strategies of endocrine therapy, however, a substantial proportion of patients with breast cancers eventually acquire resistance against those treatment. Current antiestrogenic agents are not originally beneficial to patients with ER-negative breast tumors. Several critical side effects due to tamoxifen therapy are reported, including development of endometrial cancer or an increased incidence of venous thrombosis and strokes [4]. Aromatase inhibitors such as anastrozole have fewer thromboembolic and vaginal bleeding episodes than tamoxifen, yet have side effects including hot flashes, vaginal dryness, osteoporotic fractures, nausea, and gastrointestinal disturbances [6–8]. Thus, we need to

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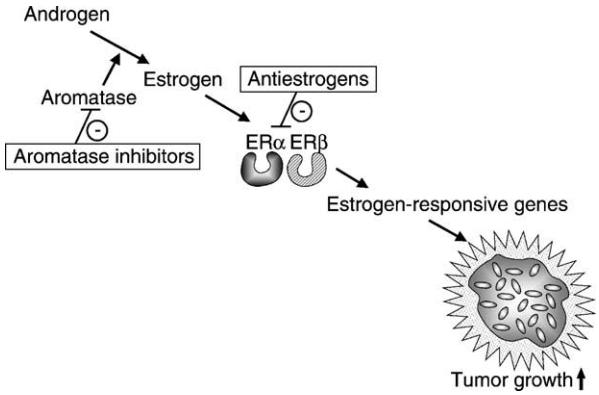

Fig. 1. Antiestrogenic drugs in cascade of estrogen action.

develop new agents that can overcome the resistance against today's endocrine therapy and have minimal side effects. Elucidation of precise estrogen/ER signaling pathway may give us some clues to develop future endocrine therapy.

2. Efp as an estrogen-responsive gene

Our group identified several downstream genes of ER that include estrogen-responsive elements in their promoter-enhancer regions, using genomic-binding site cloning technique that we previously designed [9,10]. We specially interested in one of the ER-downstream molecules, Efp or estrogen-responsive finger protein [11], containing a RING finger, two B-boxes, a α-helical coiled-coil domain, and C-terminal SPRY domain (Fig. 2A). The structure of Efp has the RING-finger B-box-coiled-coil motif or RBCC [9]. The RING finger features a set of cysteine and histidine residues that have a distinctive spacing owing to their roles as the ligands of two zinc ions that stabilize a characteristic globular conformation (Fig. 2B) [12]. It is notable that several RING finger proteins are known to be responsible for some malignant tumors (Fig. 3). For example, PML is responsible for acute premyelocytic leukemia when it forms a fusion protein with retinoic acid receptor (RAR) α [13], or loss of the tumor suppressor BRCA1 results in chromosomal instability leading to development of familial breast and ovarian cancers [14].

Efp is predominantly expressed in estrogen target tissues including mammary glands, uteri, and osteoblasts [15,16], and also in breast cancers [17]. Efp is essential for growth of female organs such as uteri, since mice deficient in Efp gene have underdeveloped uteri [18].

To investigate a role of Efp in breast tumor growth, we performed experiments to examine the effects of Efp antisense oligonucleotide on tumor formation in female nude mice inoculated with human breast cancer MCF7 cells [19]. When the tumor volume reached 300 mm^3, mice were treated with

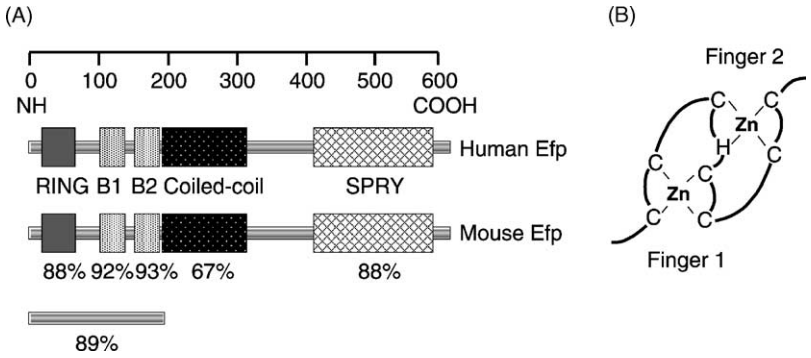

Fig. 2. Structure of Efp: (A) domain structure of human Efp and mouse Efp; (B) structure of RING finger motif.

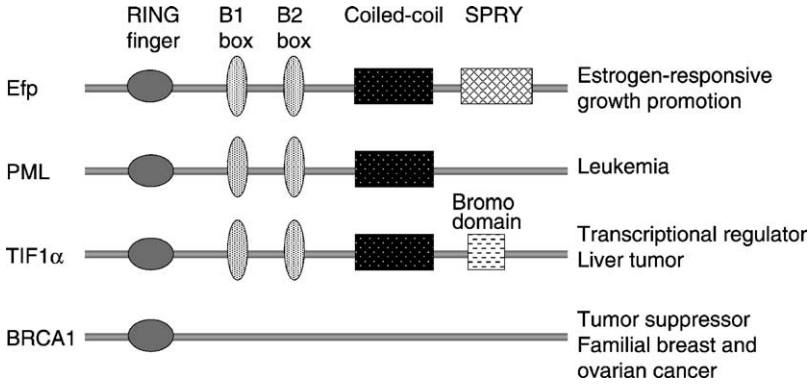

Fig. 3. Several members of RING finger family.

ovariectomy or with antisense/sense Efp oligonucleotides. Efp antisense oligonucleotide as well as ovariectomy efficiently reduced the size of tumor generated by MCF7 cells in the recipient mice. We postulated that Efp is an oncogenic factor in breast cancers.

MCF7 cells are originally ER-positive and can initially proliferate in the presence of estrogen. We next examined whether MCF7 cells can grow even in estrogen deprived environment by Efp overexpression. MCF7 cells stably expressing Efp (Efp-MCF7) could proliferate even in mice treated with ovariectomy. Cell cycle analysis revealed that a higher ratio of Efp-MCF7 cells were in the proliferating stage compared to control MCF7 cells transfected with vector alone (30–35% versus 10–15%). It is also notable that endogenous levels of negative regulators of cell cycle progression such as $p21^{Cip1}$ and 14-3-3σ were reduced in Efp-MCF7 cells as compared with control MCF7 cells. Those results give us a notion that elevated levels of Efp promote cell growth of breast cancer, indicating that Efp might directly regulate the cell cycle machinery.

3. Mechanism of Efp function in cell cycle progression

We next assessed the molecular mechanism of Efp in cell cycle progression. As a first step, we performed yeast two-hybrid screening from a mouse embryo cDNA library using Efp as a bait. These screens led to the identification of 14-3-3σ as an interacting clone with Efp [19]. 14-3-3σ is a cell cycle brake that causes G2 arrest by sequestrating cdc2 in the cytoplasm [20]. Although 14-3-3σ is well expressed in epithelial cells of normal mammary glands, reduced levels of 14-3-3σ seem to be related to breast malignancy, as downregulation of the protein [21] or hypermethylation of its promoter region [22] is reported in breast cancer.

Since we found that 14-3-3σ is an interacting clone of Efp and the amount of 14-3-3σ was reduced in Efp-MCF7 cells, we investigated whether Efp directly regulates the activity of 14-3-3σ. When we expressed Efp and 14-3-3σ in COS7 cells, the two proteins colocalized in the cytoplasm and the protein–protein interaction of those proteins was confirmed by immunoprecipitaion. Co-transfection of Efp and 14-3-3σ resulted in lower levels of 14-3-3σ protein compared with cells transfected with 14-3-3σ alone. We identified that the B-box coiled-coil domain in Efp is the motif that specially interacts with 14-3-3σ.

Recent advances of molecular research have revealed that a large number of RING finger proteins function as ubiquitin-protein ligases or E3s in the ubiquitination signaling pathway [13]. Ubiquitination regulates a variety of cellular functions, frequently by mediating the selective degradation of master regulatory proteins by proteasomes. The ubiquitin-dependent proteolysis is important to eliminate misfolded or abnormal proteins as well as to confer short half-lives on specific normal proteins such as mitotic cyclins whose critical concentrations must change promptly with alterations in the state of a cell. We hypothesized that Efp functions as an E3 that ubiquitinates 14-3-3σ (Fig. 4). By pulse and chase experiments, we confirmed that the degradation rate of 14-3-3σ protein was explicitly enhanced in Efp-MCF7 cells. The protein breakdown of 14-3-3σ is proteasome-dependent because a proteasome inhibitor MG132 increased the amount of 14-3-3σ protein binding to Efp. Finally, we confirmed that Efp directly degrades 14-3-3σ through a ubiquitin-dependent pathway in which Efp functions as an E3.

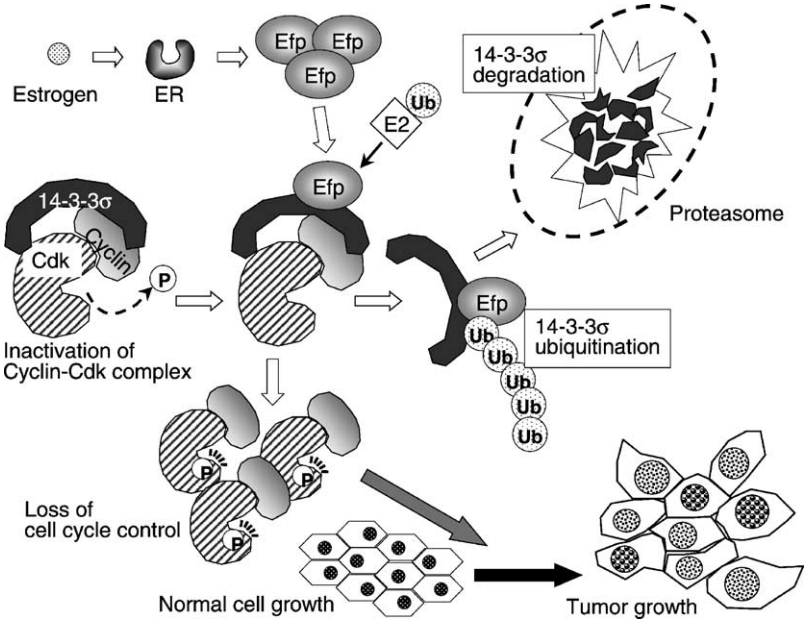

Fig. 4. Estrogen-responsive RING finger protein Efp targets 14-3-3σ for proteolysis as a ubiquitin ligase and stimulates tumor growth.

4. Perspective

Our experimental data suggest that Efp may provide unlimited proliferation of breast cancer cells by accelerated destruction of 14-3-3σ. It is intriguing that Efp might proliferate breast tumor in an estrogen deprivation environment although Efp has been originally identified as an estrogen-responsive gene. We speculate that overexpression of Efp might be one of the reasons for resistance to endocrine therapy. It remains to be determined whether Efp plays a similar critical role in human breast tumor progression. We anticipate that Efp could be used as a potential molecular target for clinical application that provides promising future direction of breast cancer treatment.

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