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Estrogen and growth factor signaling pathway: basic approaches for clinical application☆

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Abstract

Estrogen and its receptor play important roles in genesis and malignant progression of estrogen-dependent cancers, together with various growth factors. Functional cross-talk between estrogen-signaling and growth factor-mediated signaling pathways has been reported. Firstly, we show an example of the cross-talk that may alter the effect of antagonist on the breast and endometrial cancer cell growth. Our observations suggest that the constitutively activated MAP kinase-signaling pathway in endometrial cancer cells might enhance the transcriptional activity of ERα via phosphorylation of AF-1 domain. This mechanism may cause the growth stimulative effect of tamoxifen on the endometrium. Secondly, we show our recent study for comprehensive understanding of estrogen-signaling pathway using cDNA microarray. According to the results of the expression profiling of estrogen-responsive genes in ER-positive breast cancer cells using large-scale cDNA microarray, the custom-made cDNA microarray, on which only estrogen-responsive genes were loaded, was produced. Using this microarray consisting of the narrowed gene subset, we analyzed estrogen responsiveness of various cell lines and effect of estrogen antagonists. Aim of this study is not only to address the molecular mechanisms of estrogen-dependent growth of breast cancer, but also to develop the new diagnostic tools for responsiveness to hormone therapy of primary breast cancer patients. Finally, in order to understand the local tumor biology including stroma–cancer interaction, we recently developed the new analytical system using ERE-GFP introduced into breast cancer cells. Several observations indicated that these reporter cells were useful for assessment of stimulative effects of stroma cells adjacent to breast cancer on the estrogen-signaling pathway.

These studies may provide not only new clues for elucidation of the molecular mechanisms of estrogen-dependent growth of breast cancer, but also assessment of anti-estrogen responses of individual breast cancer for patient-tailored hormone therapy.
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1. Introduction

A number of basic and clinical studies have revealed various factors involved in the genesis and malignant progression of hormone-dependent tumors, although the molecular mechanisms remain unclear. Among the various biological factors, estrogen and its receptor play important roles in these processes, together with various growth factors. Furthermore, the functional cross-talk between estrogen-signaling and growth factor-signaling pathways has been reported (Fig. 1). Here we show an example of cross-talk between estrogen and growth factor-mediated signaling, which affects the clinical response to tamoxifen. Tamoxifen is an anti-estrogen drug that has been used extensively for adjuvant therapy of breast cancer [1]. Although tamoxifen is a beneficial treatment for ERα-positive breast cancer, it increases the risk of endometrial cancer [1–3]. This increased risk was also reported in a large randomized clinical trial of tamoxifen for breast cancer prevention [4]. Clinical findings that tamoxifen increases the risk of endometrial cancer are also supported by in vitro studies. Tamoxifen stimulates the growth of endometrial cancer cells, but not breast cancer cells, in cell culture [5,6] as well as when transplanted into athymic mice [7]. However, the mechanism responsible for the differential effects of tamoxifen in endometrial cancer and breast cancer has not yet been elucidated. Here we examined the effects of tamoxifen on endometrial cancer cells using a subclone of Ishikawa cells, Ishikawa 3H-12, which expresses functional ERα protein.

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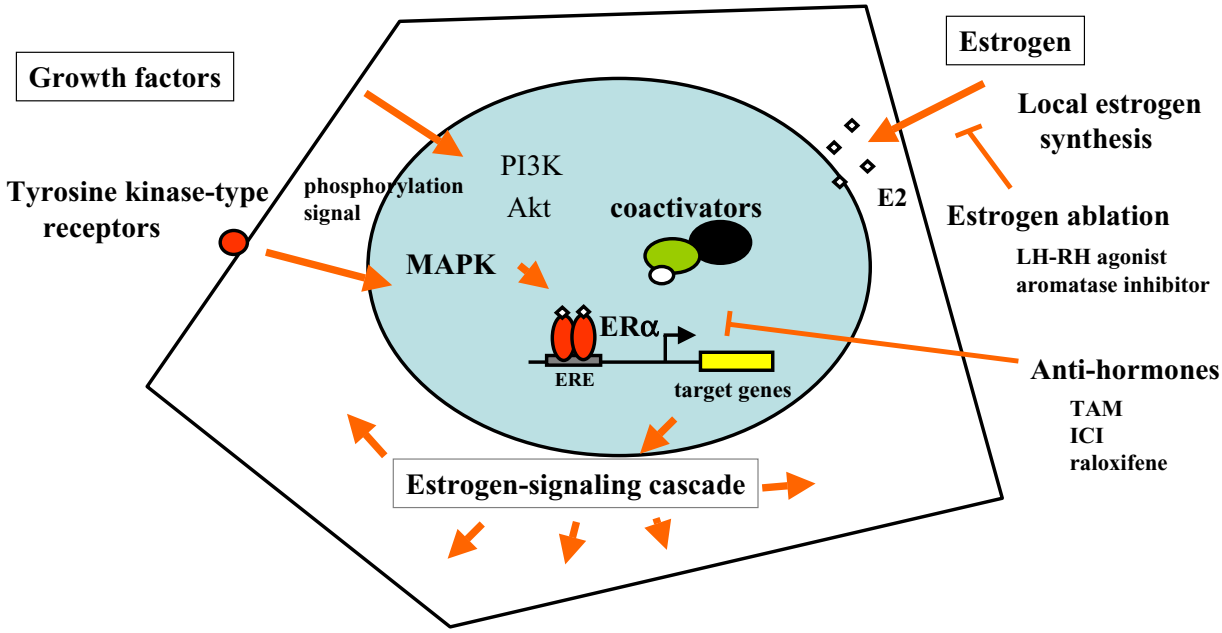

Fig. 1. Cross-talk of estrogen and growth factor signaling in cancer microenvironment.

We found that the growth-stimulatory effect of tamoxifen in endometrial cancer cells may be at least partially caused by ERα transactivation through phospholylation by MAP kinase.

Next, we analyzed the expression profile of estrogen-responsive genes using DNA microarray for understanding estrogen-signaling pathway. The expression status of ERα is a primary determinant in the anti-hormone therapy of breast cancer using antagonists to ERα such as tamoxifen [8,9]. However, the assay of ERα status is not at present completely predictive for responsiveness of the tumors to anti-estrogens; not all tumors of the patients diagnosed as ER-positive respond to anti-hormones. Therefore, there is a need for novel prediction method for hormone therapy. Although there are many reports concerning the target genes transcriptionally activated by ERα such as *pS2* [10] and cathepsin D [11], 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 [12–15] and a novel gene whose expression status was highly correlated with prognosis of patients was identified [16]. 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 anti-hormone therapy. In the present report we first analyzed the expression profiles of ~9000 genes in human MCF-7 breast cancer cells in response to estrogen. Based on the results, we selected the estrogen-responsive genes and developed custom-made microarray systems. The results obtained in this study 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.

After menopause the plasma levels of estradiol fall but the incidence of breast cancer is higher among postmenopausal patients than premenopausal patients. Breast tumors from postmenopausal patients maintain a high estrogen content, suggesting that in situ estrogen formation plays an important role in breast cancer development [17,18]. Several studies have demonstrated that conversion of androgen to estrogen by aromatase is the rate-limiting step for estrogen synthesis in breast tumors and that the expression of aromatase is detected at high levels in stromal tissues localized near actively replicating tumor cells [19,20]. These results suggest that tumor–stromal interactions play a significant role in progression of breast cancer. Several compounds that selectively inhibit aromatase are now available for the treatment of breast cancer [21]. However, the system to predict their efficacy for an individual patient and to analyze the tumor–stromal interactions has not been developed. In these aspects, we developed a new system by using human breast cancer MCF-7 cells stably transfected with ERE-GFP gene and then analyzed the overall effects of tumor–stromal interactions on ERα activity.

These studies analyzing growth factor 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.

2. Materials and methods

2.1. Cells and culture

Human breast cancer cell lines, MCF-7, was maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal calf serum (FCS; Tissue Culture Biologicals, Turale, CA, USA). Human endometrium cancer Ishikawa-3H12 cells were cultured in Eagle's Minimum Essential Medium (Nissui Pharmaceutical, Tokyo) supplemented with 10% FCS. For treatment of the cells with estrogen or estrogen antagonists, each medium was replaced with phenol red-free RPMI 1640 medium (Sigma, St. Louis, MO, USA) or phenol red-free EMEM (Sigma, St. Louis, MO, USA) containing FCS treated with dextran-coated charcoal (DCC-FCS). All the culture media contained 2 mM L-glutamine (Sigma, St. Louis, MO, USA) and 40 μg/ml gentacin (Schering-Plough, Osaka, Japan). All cells were incubated at 37 °C in humidified air containing 5% CO_2.

2.2. Colony formation assay

Colony formation assays were performed as previously described [22] with slight modifications. 1×10^4 cells were seeded in DMEM supplemented with 10% FCS and agar (0.3% agar for the top layer and 0.5% agar for the base layer). Cells were grown in 60-mm dishes in the presence or absence of 10^{-8} M of 17β-estradiol (E2) or 10^{-5} M of 4-hydroxytamoxifen (4-OHT). After 14 days, colonies larger than 100 μm in diameter were counted under a microscope in triplicate. Fisher's protected least significant difference test (PLSD test) was used for the statistical analysis. Differences were considered statistically significant when a P-value < 0.05 was obtained.

2.3. Luciferase assay

Estrogen-responsive reporter plasmid ptk-ERE-Luc containing Xenopus vitellogenin A2 ERE was previously described [23]. The pRL-SV40 control vector (Toyo Ink Mfg. Co., Tokyo, Japan) was used as an internal control for transfection efficiency in assays measuring the transcriptional activity of endogenous ER. Transcriptional activity of endogenous ER or ERα derivatives was measured using luciferase assays as described previously [23] with slight modifications. Two μg of ptk-ERE-Luc plasmid and 0.04 μg of pRL-SV40 control plasmid were used to measure the transcriptional activity of endogenous ER. Cells were plated on plastic culture 60-mm dishes to 30–50% confluency, and transient transfections were carried out using lipofectamine (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA). After incubation of the cells in growth medium supplemented with 10% FCS for 24 h under diverse conditions, the luciferase activity of lysates was measured using the Picagene Dual Seapansy Luminescence Kit (Toyo Ink Mfg. Co.) and Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany). The transfection efficiency was normalized against Renilla luciferase activity using pRL-SV40 control vector in assays measuring the transcriptional activity of endogenous ER.

2.4. Immunoblotting

Western blots were performed as previously described [22] with slight modifications. Cells were incubated in growth medium supplemented with 10% FCS for 16 h in the presence or absence of 10^{-8} M E2 (Sigma Chemical Co., St. Louis, MO, USA) or 10^{-5} M 4-OHT (Sigma Chemical Co.). To detect activated and state-independent total MAP kinase, one hundred μg of total cell proteins prepared with ice-cold lysis buffer (20 mM Tris–HCl pH8.0, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 1 mM PMSF, 1 μg/ml leupeptin, and 10 μg/ml aprotinin) were subjected to Western blot analysis using 1:5000 diluted anti-active MAP kinase rabbit polyclonal antibody (Promega Co., Madison, WI, USA) or 1:5000 diluted anti-ERK1/2 rabbit polyclonal antibody (Promega Co.), respectively. As a positive control, cells were incubated in growth medium supplemented with 1% FCS for 16 h, then treated with 50 ng/ml epidermal growth factor (EGF) for 10 min.

2.5. Large-scale cDNA microarray analysis

Estrogen-responsive gene expression profiles were analyzed with a Human UniGEM^{TM} V 2.0 microarray system (IncyteGenomics, CA, USA) consisting of 9,128 human cDNA clones covering 8,502 unique gene/EST clusters. MCF-7 cells were cultured in the indicated phenol red-free medium with 10% DCC-FCS for 5 days and treated with 10 nM E2 for 72 h. From the cells, mRNA was prepared using a PolyATtract mRNA Isolation System III (Promega, Madison, WI, USA), following total mRNA isolation using an RNeasy kit (Qiagen, Tokyo), according to the manufacturer's instructions. Preparation of Cy3 or Cy5-labeled cDNA, hybridization, quantification of Cy3 and Cy5 signal intensities, and data analysis were carried out with IncyteGenomics.

2.6. Production of custom-made cDNA microarrays

To produce a prototype of the custom-made cDNA microarray, a total of 148 genes were selected from the gene set included in Human UniGEM^{TM} V 2.0, according to the results obtained using this large-scale microarray system. The selected gene subset consisted of 138 genes which showed up- or down-regulation by estrogen and 10 genes which

showed no response to estrogen (for internal control) in the large-scale microarray analysis. For production of a second version of the custom-made cDNA microarray (InfoArray; InfoGenes Co. Ltd., Tsukuba, Japan), new genes were added, resulting in a total of 204 genes including 27 genes for internal control. For each gene, PCR-amplified cDNA fragments were obtained and spotted in duplicate on each glass slide.

2.7. Custom-made cDNA microarray hybridization

For estrogen-responsive gene expression profiling, human cancer cells were grown in the indicated phenol red-free medium with 5 or 10% DCC-FCS for 5 days and treated with 10 nM E2 for 72 h. For time-course analysis of estrogen-responsive gene expression profiles in MCF-7 cells, the durations of estrogen treatment were 6, 12, 24, and 72 h. As the sample at 0 h, the cells were also similarly cultured in estrogen-deprived medium but without any treatment. From the cells treated with those reagents, mRNA was isolated using PolyATtract system 1000 (Promega, Madison, WI, USA). The preparation of Cy3- or Cy5-labeled cDNA and hybridization procedure were performed according to a previous report [24].

2.8. Scanning and data analysis

The fluorescent signals on the slides were scanned by ChipReader (Virtek, Ontario, Canada) and quantitative values for the signals were calculated using IPLab (Scanalytics, Fairfax, VA, USA) according to the manufacturer's instructions. Further data processing was done using Microsoft Excel software. For each spot, the ratio of Cy3 and Cy5 signal intensities (Cy3/Cy5) was calculated and $\log_2$-transformed. Each $\log_2$(Cy3/Cy5) value was normalized by subtracting the average of $\log_2$(Cy3/Cy5) values for internal control genes and the duplicated $\log_2$(Cy3/Cy5) values for each gene were averaged. In the case of the analysis using InfoArray, the data for the spots with poor hybridization (signal areas of either Cy3 or Cy5 were below 100) were removed from the data processing described above. This improved the correlation coefficients between duplicated sets of $\log_2$(Cy3/Cy5) values. Average-linkage hierarchical clustering was applied using the CLUSTER program and the results were displayed using the TREEVIEW program (Both programs were developed by Eisen et al. [25]).

2.9. Isolation of a MCF-7 clone expressing ERE-GFP

MCF-7 cells were transfected with d2E-GFP vector alone (Clontech, Palo Alto, CA, USA) or carrying ptk-ERE insert using *Trans* IT (PanVera, Madison, WI, USA). After 24 h, the cells were subjected to selection in growth medium containing geneticin (1 mg/ml). Transfection efficiency was monitored by fluorescence microscopy.

2.10. Cocultre of MCF-7 cells with primary stromal cells

Stromal cells were prepared from human breast cancer tissues on surgery at Saitama Cancer Center Hospital after informed consent was obtained from the patients. The stromal cell isolation procedure is similar to that described by Ackerman et al. [26]. Breifly, 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-7 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.

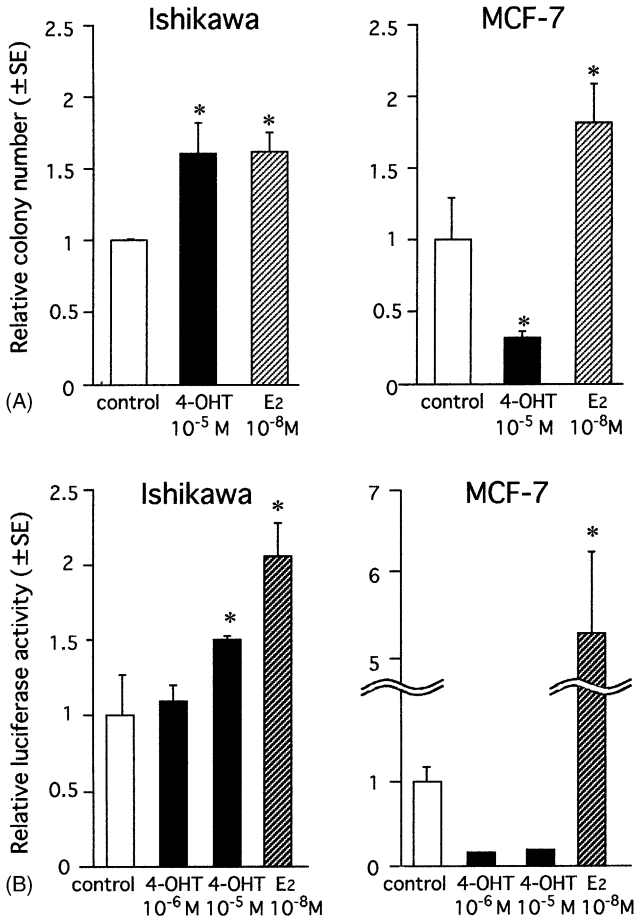

Fig. 2. The effects of E2 and OHT on cell growth (A) and endogenous ER transcriptional activities (B) in Ishikawa and MCF-7 cells. Cells were transiently transfected with ptk-ERE-Luc plasmid and pRL-SV40 control plasmid. After incubation of the cells in growth medium supplemented with 10% FCS for 24 h in the presence or absence of 10^{-8} M E2 or 10^{-5} M OHT, lysates were assayed for luciferase activity. The transfection efficiency was normalized against Renilla luciferase activity using pRL-SV40 control vector. Results are representative of at least three independent experiments. Data are expressed as the ratio of luciferase activity to control luciferase activity. Columns, means; Bars, standard error, $^{*}P < 0.05$, Fisher's PLSD vs. control.

3. Results and discussion

3.1. Effect of tamoxifen on endometrial cancer

To investigate the mechanism mediating the stimulatory effect of tamoxifen on endometrial cancer, we addressed several approaches using Ishikawa 3H-12, human endometrial cancer cell line, which expresses functional ERα. Cell-growth and ERE-reporter activity of the cells were stimulated by 4-hydroxytamoxifen (OHT) as well as 17β-estradiol (E2), while those of MCF-7 cells were inhibited by addition of OHT (Fig. 2). It is known that ERα is stimulated by two distinct activation regions, activation function (AF) 1 and AF2. AF1, which is located in the N-terminal A/B domain, is constitutively activated in a cell- and promoter-specific manner [27] and it is responsible for the partial agonist activity of tamoxifen [28–30]. AF2 is located in the C-terminal hormone binding domain and exerts ligand-dependent transcriptional activity [27]. AF1 and AF2 activate transcription independently and synergistically and act in a promoter-specific and cell-specific manner [30]. A possible explanation for the different effects of tamoxifen may include different activity levels of AF1 and AF2 in these cells.

Then, we next assessed the effects of E2 and tamoxifen on AF1- or AF2-dependent transcription activity in Ishikawa cells using expression plasmid containing either AF1 or AF2 domain fused to GAL4 DNA-binding domain. Although AF2-dependent transcriptional activity was enhanced by E2 and reduced by OHT, E2 and OHT did not affect the AF1-dependent transcriptional activity. Interestingly, the relative transcriptional activity of AF1 compared with that of the AF2 domain was four-fold higher in Ishikawa cells than in MCF-7 cells (data not shown). Many studies have reported the cross-talk between ERα-mediated signals and growth factor-mediated signals. For example, mitogen-activated protein (MAP) kinase enhances the AF1 transcriptional activity of ERα via phosphorylation of Ser^{118} of ERα [31–33]. Then, to investigate the possibility that a MAP kinase is involved in the activation of AF1 in Ishikawa cells, we analyzed MAP kinase activity in Ishikawa and MCF-7 cells using Western blot analysis. The constitutive activation of ERK2 was observed only in Ishikawa cells, regardless of the presence or absence of E2 or OHT, while no activation was observed in MCF-7 cells (Fig. 3). These observations suggest that constitutively activated MAP kinase-signaling pathway in Ishikawa cells enhances the transcriptional activity of ERα through the AF1 domain, and this activation pathway may be involved in the stimulatory effect of tamoxifen on the development and/or progression of endometrial cancer (Fig. 4). MAP kinase also stimulates ERα transcriptional activity via phosphorylation of SRC-1 [34] and AIB1 [35] coactivators. Therefore, another possibility of the mechanisms of tamoxifen effect on endometrial cancer might be considered.

3.2. cDNA microarray for expression profiling of estrogen-responsive genes

In order to understand the intracellar estrogen-signaling and develop a new tool for diagnosis of estrogen-dependent cancer, we have been promoting a strategy using cDNA microarray technique as shown in Fig. 5. We first analyzed the estrogen-responsive gene expression profiles in human MCF-7 breast cancer cells using a Human UniGEM^{TM} V 2.0 microarray system (IncyteGenomics, CA, USA) consisting of 9128 human cDNA clones covering 8502 unique gene/EST clusters. One of the purposes of this study was to find the molecular markers which reflect the physiological status of ER-positive breast tumors supplied with

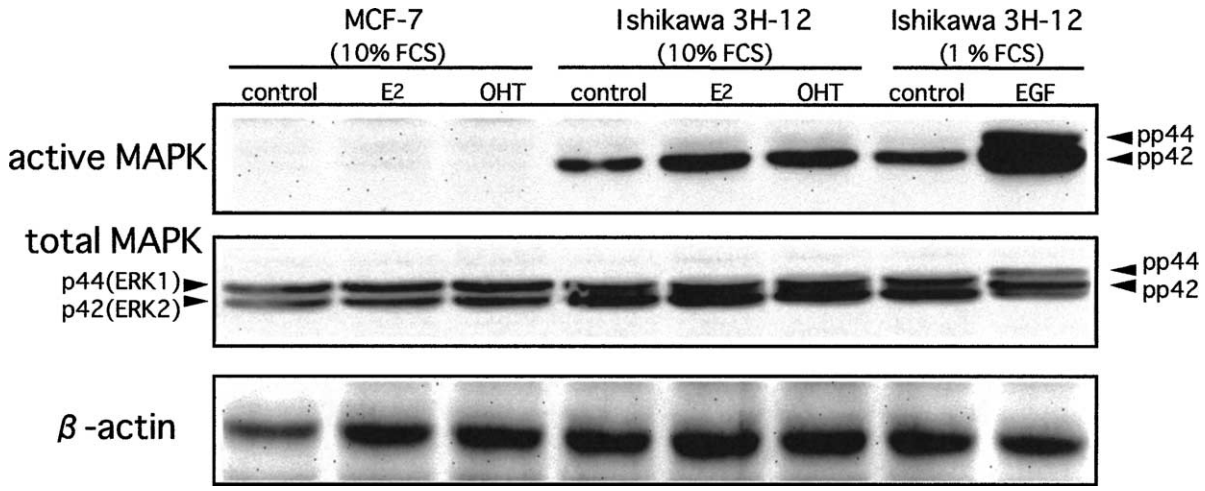

Fig. 3. Activities of MAP kinases, ERK1 and ERK2, in MCF-7 cells and Ishikawa cells. After cells were incubated in growth medium supplemented with 10% FCS for 16 h in the presence or absence of 10^{-8} M E2 or 10^{-5} M OHT, 100 μg of cell extract was subjected to Western blot analysis using anti-active MAP kinase rabbit polyclonal antibody or anti-ERK1/2 rabbit polyclonal antibody. As a positive control, cells were incubated in growth medium supplemented with 1% FCS for 16 h, then treated with 50 ng/ml EGF for 10 min.

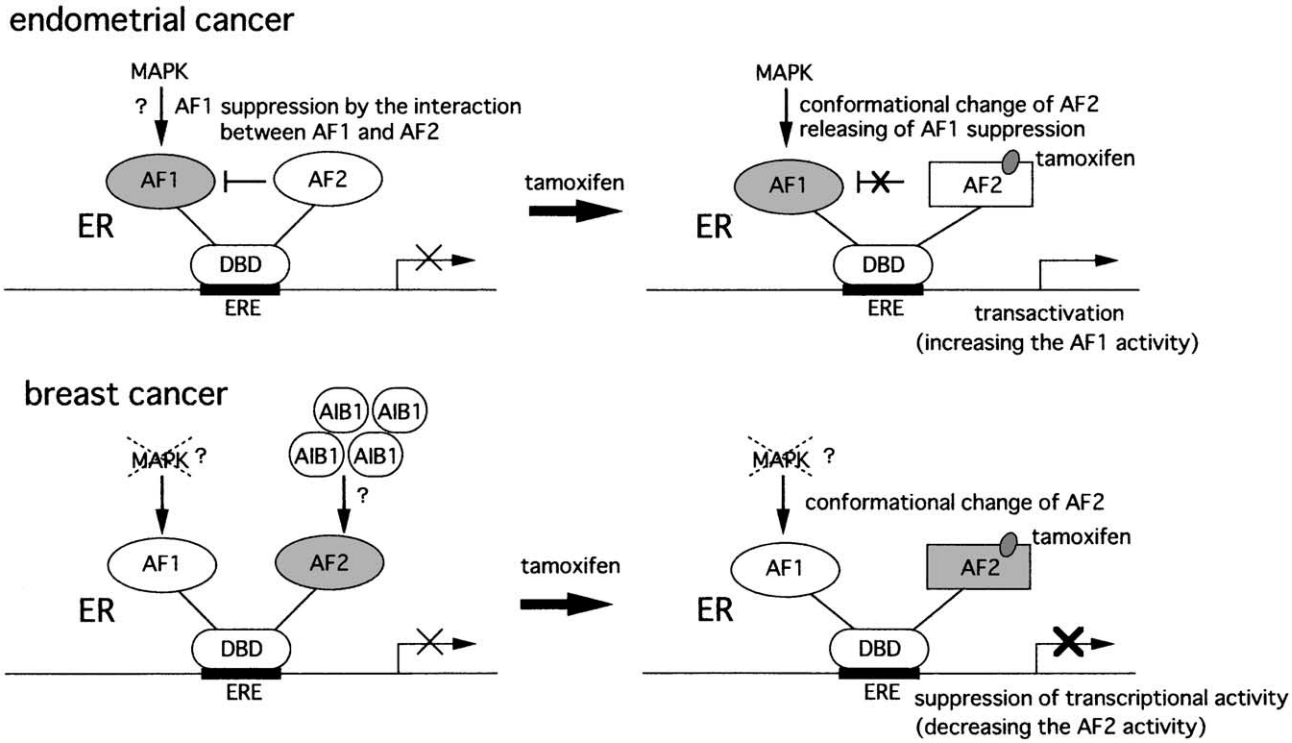

Fig. 4. Possible mechanism for the stimulatory effects of tamoxifen in endometrial cancers. MAP kinase is constitutively activated in endometrial cancer cells. In the absence of ligands, AF1 activity is suppressed by the interaction between AF1 and AF2. When tamoxifen binds to AF2, a conformational change is induced in AF2, releasing the suppression of AF1 transcriptional activity caused by the AF1–AF2 interaction. In breast cancer cells, AF2 transcriptional activity is dominant, which may be at least partially caused by AIB1 overexpression. Therefore, the antagonist activity of tamoxifen in breast cancer cells is caused by the repression of AF2-dependent transcriptional activity.

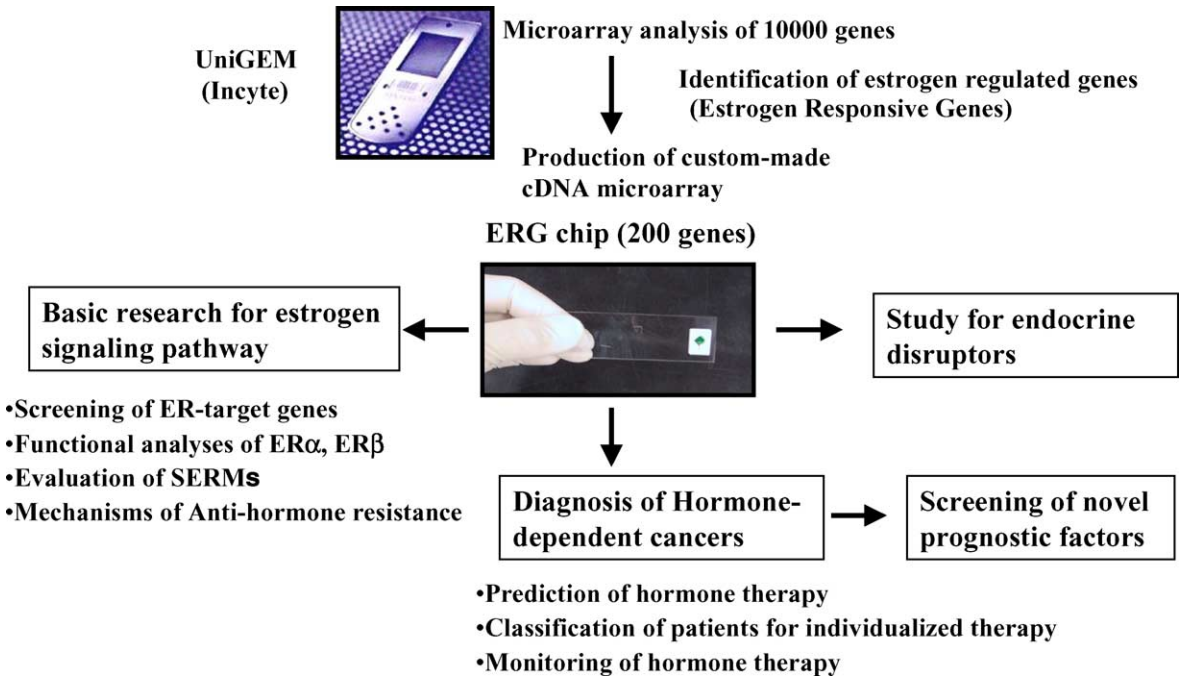

Fig. 5. Development and application of estrogen-responsive cDNA microarray.

a large amount of estrogen in situ or surrounding stromal cells for a long period. If such markers actually exist, these expression patterns should be quite valuable information for clinical application such as the diagnosis of estrogen- and anti-estrogen-responsiveness of mammary tumors. As a model cell line, we chose the MCF-7 cell line because it is ERα rich, highly responsive to estrogen, and therefore, has been extensively investigated as one of the standard models of ER-positive breast cancer.

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, [36]), PDZK1 [37], insulin-like growth factor-binding protein 4 [38] and nuclear receptor interacting protein 1 [39], 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 at first analyzed the time course of the estrogen-responsive gene expression profiles in MCF-7, resulting in subdivision of the genes up-regulated by estrogen into early-responsive and late-responsive genes (Fig. 6). The expression patterns of several genes were confirmed by Northern blot analysis. We also analyzed the effects of estrogen antagonists ICI 182,780 and 4-OHT on the estrogen-responsive gene expression profiles in MCF-7. While the regulation of most of the genes by estrogen was completely abolished by ICI 182,780, some genes were partially regulated by estrogen even in

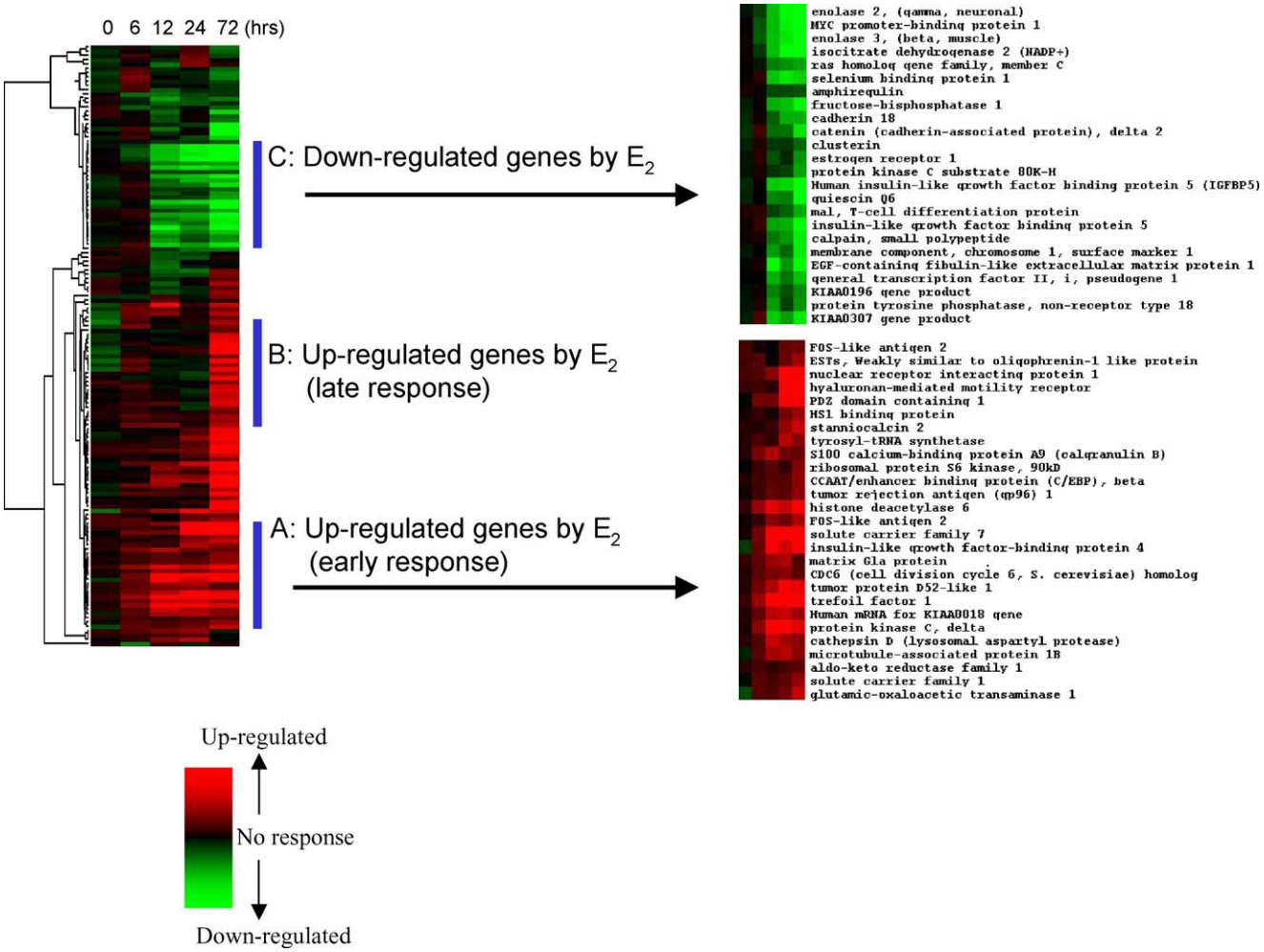

Fig. 6. Gene expression patterns in MCF-7 cells at different times after estrogen stimulation. MCF-7 cells incubated in estrogen-deprived medium were treated with 10 nM 17β-estradiol (E_2^+) or ethanol (E_2^-) for 6, 12, 24 and 72 h, then mRNA was isolated from each cell culture and the cells without any treatment (shown as 0 h), then used for prototype customized cDNA microarray analysis as described in Section 2. The expression patterns of 148 genes were analyzed by hierarchical clustering and represented as a pseudo-color visualization matrix using CLUSTER and TREEVIEW [25]. In the matrix, red and green color shows up-regulation and down-regulation respectively, and color saturation indicates ratio of differential expression. Gene clusters denoted by the bars and letters A–C are the groups containing early-responsive estrogen-induced genes, late-responsive estrogen-induced genes and estrogen-repressed genes, respectively. The gene groups A and C are zoomed and the expression patterns with gene names are shown on the right panel.

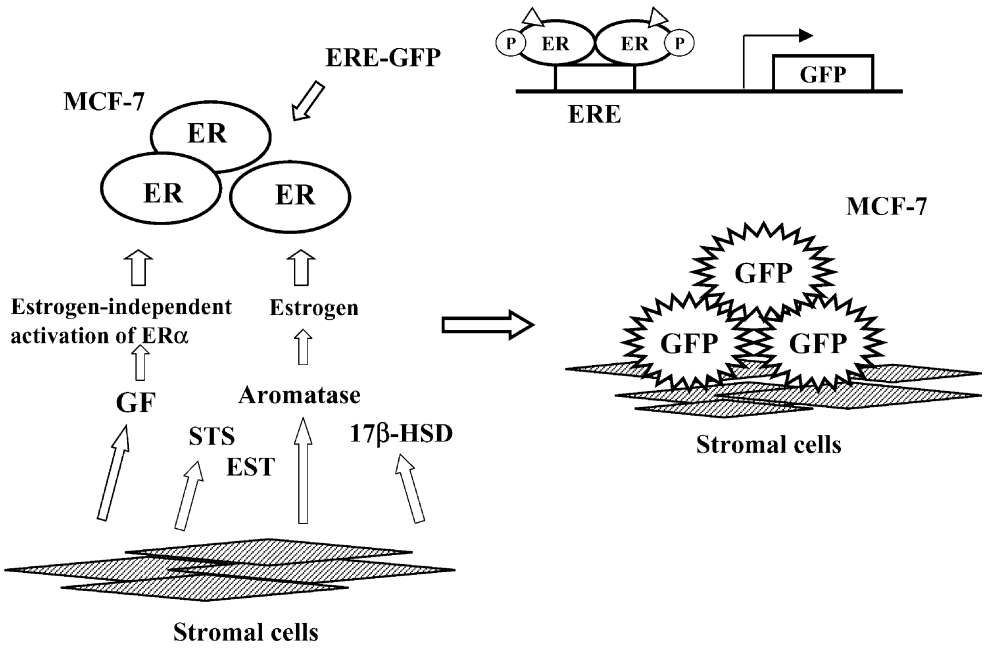

Fig. 7. System for analysis of tumor–stromal interactions in breast cancer by visualization of estrogen-signaling pathway. MCF-7 cells were stably transfected with ERE-GFP gene, and stromal cells were isolated from breast cancer tissues. In coclture of MCF-7 with stromal cells, ERα was activated in an estrogen-dependent and -independent manner. Expression of GFP reflects total ERα activity produced in this system.

the presence of OHT. Furthermore, the estrogen-responsive gene expression profiles of twelve cancer cell lines derived from the breast, ovary or stomach *etc*. were obtained and analyzed by hierarchical clustering including the profiles in MCF-7 cells. Several genes also showed up-regulation or down-regulation by estrogen in cell lines other than MCF-7 cells.

Here we showed our on-going cDNA microarray analyses to specify estrogen-responsive genes, some of which would be critical for the development of breast cancer and also for the prediction of responses to anti-hormone therapy. This down-sized microarray will be especially useful clinically for assessment of individual response to this therapy including neoadjuvant treatment of tamoxifen or aromatase inhibitors. Furthermore, it has been shown that this gene subset is also useful for studying the ER-mediated estrogen-signaling pathway.

3.3. Analysis of tumor–stroma interactions in breast cancer by visualization of estrogen-signaling pathway

In breast tumors in postmenopausal women, production of estrogen in stromal tissues acts locally to promote the growth of tumor. Aromatase is the key enzyme of estrogen synthesis and it mainly expressed in stromal tissues of breast tumor, which is considered to cause an increase of local estrogen levels [17,18]. In stromal adipose tissues, aromatase expression is also stimulated by cytokines such as IL-6, IL-11 and TNFα [40]. In addition to aromatase, other estrogen-metabolizing enzymes such as 17β-hydroxysteroid dehydrogenase, estrone sulfatase and estrogen sulfotransferase also regulate the in situ estrogen levels [41]. On the other hand, estrogen is also known to induce the production of growth factors such as EGF, IGF-1 and TGFα, which in turn stimulate the proliferation of tumor cells, and the cross-talk between ERα-mediated signals and these growth factor-mediated signals has been reported [31,42]. EGF and IGF-1 induce phosphorylation of ERα via MAPK kinase and cause an increase in ERα transactivation function [31]. Recently, phosphatidylinositol 3-kinase/AKT is reported to activate ERα by phosphorylation in the absence of estrogen [43]. Therefore, ERα in breast tumor could be activated by local production of estrogen and growth factors, and this process is totally regulated by tumor–stromal interactions (Fig. 7). To clarify the mechanisms of local ERα activation and in future to predict efficacy of hormone therapy for an individual breast cancer patient, we developed a system to detect ERα activity based on tumor–stromal interactions. We used MCF-7 cells that are stably transfected with ERE-GFP as reporter cells for ERα activity. We isolated several clones (MCF-7-E10, -E5, E-20), and found that MCF-7-E10 cells highly expressed GFP on treatment with estradiol. Stromal cells were isolated by treatment with collagenase from tissues of breast cancer following surgery. When MCF-7-E10 cells are cocultured with these primary stromal cells, the expression of GFP protein is specifically induced in MCF-7-E10 cells (Fig. 8). The expression of GFP reflects ERα activation regulated by tumor–stromal interactions. Using this coculture system, it is possible to detect ERα activity induced in ligand-dependent and -independent

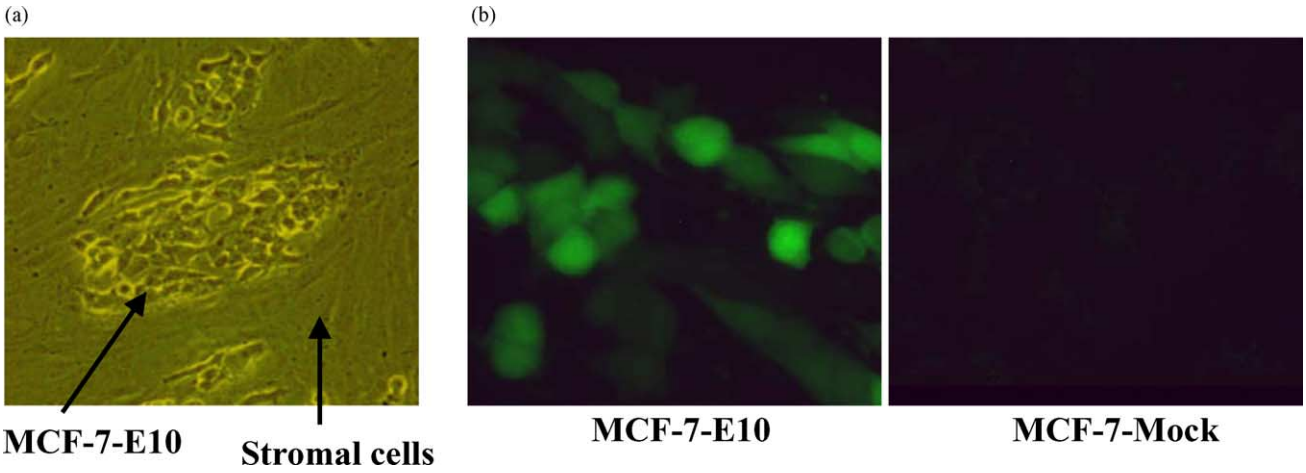

Fig. 8. Visualization of ERα activity in MCF-7 cells stably expressing ERE-GFP (MCF-7-E10 cells) after coculture with breast tumor-derived stromal cells. (a) MCF-7 cells were plated onto subconfluent primary stromal cells isolated from breast tumor tissues as described in Section 2. (b) After 24 h, the expression of GFP in MCF-7-E10 cells was observed by fluorescence microscopy. MCF-7-Mock cells were transfected with control vector.

manner under tumor–stromal interactions. Moreover, this system might be useful for assessment of factors regulating aromatase activity, including aromatase inhibitors.

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