

Tumor-Stromal Interaction through the Estrogen-Signaling Pathway in Human Breast Cancer

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Abstract

In postmenopausal breast cancers, locally produced estrogen by adipose stromal cells causes the progression of tumor growth. Although aromatase, a key enzyme of estrogen synthesis, is highly expressed in the adipose stromal cells, and aromatase inhibitors show greater efficacy in postmenopausal breast cancers, the mechanism of increasing aromatase activity in the stromal cells remains unclear. To analyze the estrogen signals and to detect the estrogen receptor (ER)-activating ability of adipose stromal cells for individual human breast cancers, we developed a new reporter cell system. To visualize the activation of ER, we first established a stable transformant, named E10, of human breast cancer MCF-7 cells by transfection with the estrogen-responsive element-green fluorescent protein (GFP) gene. E10 cells specifically express GFP when ER is activated by estrogen or by coculture with adipose stromal cells isolated from breast tumor tissues in the presence of testosterone, a substrate for aromatase. Treatment of adipose stromal cells with dexamethasone, a stimulator of aromatase gene expression, resulted in an increase in the expression of GFP in E10 cells in the coculture. Using this system, we characterized the adipose stromal cells of 67 human breast cancers and found that GFP expression levels vary among the cases, suggesting that the ability of adipose stromal cells to activate ERs is unique for individual breast cancers. High induction levels of GFP were observed more frequently in postmenopausal cases than in premenopausal cases, whereas they did not significantly correlate with the ER expression status. Aromatase inhibitors inhibited the induction of GFP expression in the coculture, but the sensitivities to the drugs varied among the individual cases. Aromatase gene expression levels in adipose stromal cells did not always correlate with their ability to induce GFP. These results suggest that this system to detect total ER activation based on the interaction with adipose stromal cells is a useful tool for analyzing local estrogen signals and for tumor-stromal interactions. (Cancer Res 2005; 65(11): 4653-62)

Introduction

Estrogen is well recognized to play a central role in the genesis and progression of breast cancers. However, the incidence of

breast cancer is high even in postmenopausal women when the ovaries have ceased to produce estrogen, which has been found to be partly due to the fact that estrogen synthesis is locally increased in the breast tumor (1, 2). Estrogen levels in the breast cancer tissue of postmenopausal women are ~10-fold higher than those in the plasma or in normal breast tissue (1, 3). Aromatase is a key enzyme that catalyzes the conversion of androgen to estrogen, and its levels in breast cancer tissue were significantly higher than those in benign breast lesions (4–6). It may be responsible for the increased local estrogen production, which in turn contributes to the estrogen-sensitive proliferation of breast cancer cells.

It has been shown that the aromatase gene is expressed in various cell types and is tissue specifically regulated through the alternative utilization of the multiple exons 1 and promoters (7, 8). In breast tumors, aromatase is highly expressed in the adipose stromal cells adjacent to the tumors (4, 6). Although the mechanism by which aromatase activity is increased in the adipose stromal cells remains unclear, studies have reported that aromatase expression is significantly affected by tumor-stromal interactions. Prostaglandin E₂ (PGE₂) produced by breast tumor cells is a potent stimulator of aromatase expression in adipose stromal cells (9, 10), which stimulates adenylyl cyclase, and the resultant increase in the concentration of intracellular cyclic AMP enhances aromatase activity (9). Furthermore, estrogen itself enhanced the expression of genes involved in PGE₂ production and signaling, which in turn induced aromatase expression and estrogen production (11). A recent study reported that breast cancer cell lines secrete factors inducing aromatase expression in adipose stromal cells although they have not been identified (12). Overall, the tumor cells produced various factors to further increase aromatase expression in the surrounding adipose stromal cells, and the growth of the breast tumor is promoted by the locally increased estrogen levels. Cytokines, such as tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and IL-11, which are mainly released by breast cancer cells and inflammatory cells that have invaded the tumors, also enhance aromatase expression (13, 14).

On the other hand, estrogen produced by locally increased aromatase is not the only factor that promotes the growth of breast cancer. Growth factors, such as epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), and transforming growth factor- α , are also able to activate estrogen receptor (ER) α and promote the growth and malignant behavior of breast cancers (15–17). Additionally, estrogen itself induces breast cancer cells to produce these growth factors (18–20), and the cross-talk between ER α -mediated and growth factor-mediated signals has been reported (17, 21, 22). Although the mechanisms of the cross-talk are not completely understood, EGF and IGF-I phosphorylate and then activate ER α in an estrogen-dependent or estrogen-independent

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manner via kinases, such as mitogen-activated protein kinase (17, 23) and phosphatidylinositol 3-kinase/Akt (21). ER α can also be phosphorylated at different sites by other kinases, which are involved in the regulation of the transcriptional activity of the receptor (24, 25). As described above, the ER-activating signals induced in breast cancers are regulated via several pathways, which in combination caused the progression of the tumor. Therefore, to analyze the mechanisms of the progression of breast cancer, a system to comprehensively detect these growth-stimulating signals is needed, and this is the aim of this study.

Estrogen deprivation therapy is an effective treatment for breast cancer, which has been achieved by ovarian ablation and recently by treatment with luteinizing hormone/releasing hormone agonists for premenopausal breast cancers (26). Anti-estrogen drugs that block the action of estrogen are also effective, and tamoxifen has been extensively used for postmenopausal patients for >25 years (27, 28). It inhibits the growth of breast tumors by the competitive antagonism of estrogen at its receptor site. Although it is a beneficial treatment for ER-positive breast cancers, its partial agonist effects resulted in increases in the risk of endometrial cancer (29). In addition, a tumor regression response to these endocrine therapies is observed for only two-thirds of ER-positive breast cancers, and resistance to these therapies has been commonly observed. Based on these aspects, aromatase inhibitors to block estrogen biosynthesis have been extensively developed and found to be valuable for the treatment of estrogen-dependent breast cancers (30, 31). The first aromatase inhibitor, aminoglutethimide, was developed in the 1970s but was subject to problems with specificity and potency. Numerous aromatase inhibitors were then developed, and in the early 1990s, the better tolerated, highly selective, and orally active aromatase inhibitors, such as anastrozole (Arimidex), letrozole (Femara), and exemestane (Aromasin), were developed. They effectively eliminate aromatase activity in the plasma of postmenopausal women and markedly inhibit intratumoral aromatase activity.

Recent large-scale clinical trials have reported that these aromatase inhibitors are suitable for clinical use as a first-line treatment for ER-positive advanced breast cancer in postmenopausal women and as a second-line treatment after tamoxifen failure (32–34). Furthermore, the results of the Arimidex, Tamoxifen Alone or in Combination Study have shown that adjuvant anastrozole is superior to tamoxifen in terms of disease-free survival, adverse effects, and prevention of contralateral breast cancer in postmenopausal women with ER-positive breast cancer (35, 36). In these therapies, the patients plan to receive a drug over 5 years, and the selection of the cases sensitive to aromatase inhibitors is critical to improve the clinical outcome. However, an appropriate system to predict the efficacy of aromatase inhibitors has not been available.

We have studied the molecular mechanisms of the genesis and progression of breast cancer in terms of the ER from several aspects (37–39). Recently, we focused on the analysis of estrogen signals by the development of a custom-made cDNA microarray consisting of ~200 estrogen-responsive genes and identified novel estrogen-induced genes (40–42). On the other hand, *in vivo* estrogen signals in the tumor cells are regulated by their interaction with stromal cells as mentioned above. Therefore, to address the mechanisms of the estrogen-dependent growth of breast cancer and to predict the efficacy of hormone therapy for breast cancer, a comprehensive evaluation system, which

Table 1. Relationship between the clinicopathologic variables and the ER-stimulating ability of stromal cells

	Total no.	Active case no. (%)	<i>P</i>	
Age (y)				
<51	17	2 (11.8)		
≥50	50	11 (22.0)		
Menopausal status				
Premenopausal	25	2 (8.0)	0.0452*	
Postmenopausal	42	12 (28.6)		
Tumor diameter (cm)				
≤2	33	7 (21.2)		
>2	34	7 (20.6)		
ER [†]				
Positive	51	12 (23.5)	0.348*	
Negative	16	2 (12.5)		
PgR [†]				
Positive	42	9 (21.4)		
Negative	24	5 (20.8)		
Stage				
I	13	2 (15.4)		
II	44	10 (23.0)		
III	7	2 (29.0)		
Unknown	3			
Histology				
Invasive ductal	61	14 (23.0)		
Special	3	0		
Noninvasive ductal	3	0		
Grade [‡]				
1	13	6 (46.0)	0.0025*	
2	6	4 (66.7)		0.0004*
3	36	3 (8.3)		
Nodal status				
Negative	35	7 (20.0)		
Positive	31	8 (25.8)		
Unknown	1			

NOTE: Clinicopathologic variables of breast cancers tested in this study and the numbers of the active cases in which the stromal cells induced GFP expression in >30% of E10 cells are shown. The percentages of these active cases are shown in the parentheses.

*Comparison of two proportions.

[†]ER and PgR status were evaluated by Allred scoring and tumors scoring more than score 3 were identified as ER or PgR positive.

[‡]Grades were unknown for 12 cases.

includes tumor-stromal interactions, should be considered. In this study, we developed a novel system to detect the overall estrogen signals based on tumor-stromal interactions by visualization.

Materials and Methods

Cells and cell culture. The human breast cancer cell line MCF-7 was cultured in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FCS (Tissue Culture Biologicals, Turale, CA) at 37°C in a humidified atmosphere of 5% CO₂ in air. For the experiments designed to evaluate the effect of 17 β -estradiol (E₂; Sigma, St. Louis, MO) or adipose stromal cells, phenol red-free RPMI 1640 (PRF-RPMI; Sigma) and dextran-coated, charcoal-treated FCS (DCC-FCS) were substituted for RPMI 1640 and FCS, respectively. Anastrozole, letrozole, and exemestane were kindly provided by AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom),

Novartis Pharmaceuticals (Basel, Switzerland), and Pharmacia Co. (Bridge-water, NJ; currently Pfizer, Inc., New York, NY), respectively.

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

Assay of cell growth. After 3 days of culture in PRF-RPMI with 10% DCC-FCS, the cells were seeded at 1×10^4 /mL in a 24-well multidish with or without E_2 for 3 days, and the viable cells were examined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MTT solution (100 μ L; 5 mg/mL in PBS) was added to each well. After incubation with MTT for 3 hours, the cells were centrifuged at $1,000 \times g$ for 10 minutes. The precipitates were dissolved in 1 mL DMSO and their absorbances at 560 nm were determined.

Tumor samples. Human breast cancer tissues were obtained by surgery at the Saitama Cancer Center Hospital (Saitama, Japan) after informed consent was obtained from the patients. The Saitama Cancer Center Ethics Committee approved this study. The clinicopathologic characteristics are summarized in Table 1. Histologic grading was evaluated according to the modified and simplified Bloom-Richardson grading scheme (43). The ER and progesterone receptor (PgR) status was evaluated by Allred scoring (44) and tumors with more than a score of 3 were identified as ER or PgR positive. Fixation, serial sectioning, paraffin embedding, H&E staining, and immunohistochemical staining were done as described previously (45).

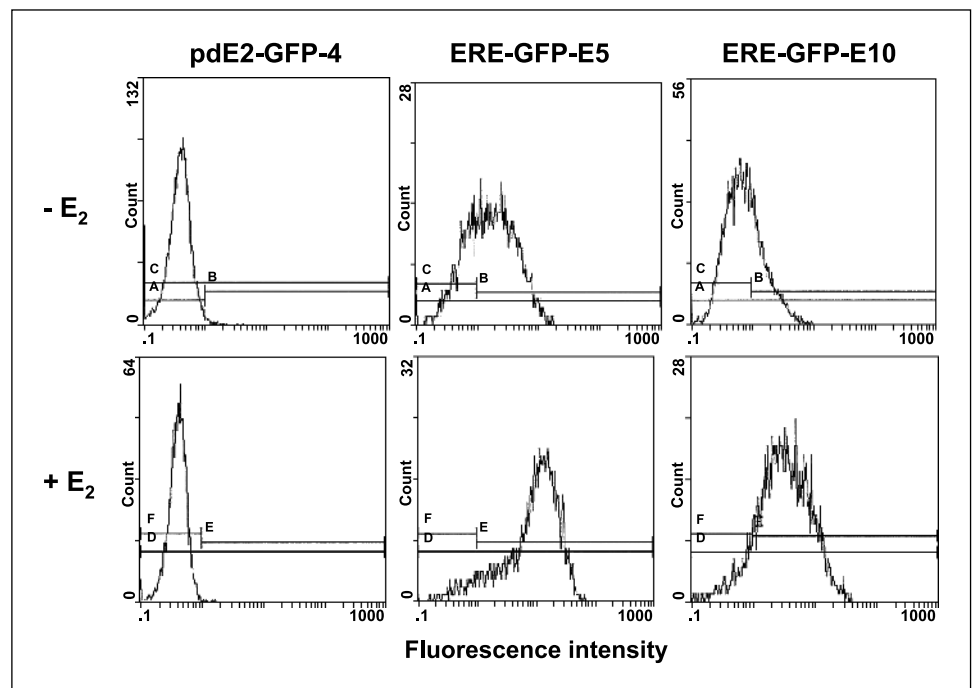
Isolation of primary stromal cells from tumors. The isolation procedure of stromal cells is similar to that described by Ackerman

et al. (46). Briefly, tissue specimens were rinsed with HBSS and minced to $\sim 1 \text{ mm}^3$ in size. They were digested with collagenase solution containing 1 mg/mL collagenase, 40 mg/mL bovine serum albumin, 2 mg/mL glucose, $1 \times$ antibiotic-antimycotic, and 50 μ g/mL gentamicin in HBSS for 2 to 3 hours at 37°C and then filtered through nylon mesh. The cells, including stromal cells, were recovered by centrifugation and washed several times with HBSS. The cells were suspended in MEM- α containing 10% FCS and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Outgrowth of cells was observed after 7 to 10 days and medium was renewed twice weekly thereafter.

Coculture of MCF-7 cells with primary stromal cells. Coculture experiments were carried out in PRF-RPMI containing 10% DCC-FCS and stromal cells were used within several passages to avoid effects induced by cell aging. The cells were precultured in PRF-RPMI containing 10% DCC-FCS for 72 hours. Stromal cells were seeded at 5×10^4 /mL in a 24-well multidish, and on the next day, 5×10^4 ERE-GFP-MCF-7 cells were seeded on the top of the adipose stromal cells with testosterone at 10^{-7} mol/L as a substrate for aromatase. After being cocultured for 4 days, the cells were collected by mild trypsinization, and the GFP expression was analyzed. Both cells could be easily discriminated by their morphology. For the experiment that examined the effect of cell-cell interactions, the cells were separately cultured in Transwell-Clear culture dishes (Costar, Cambridge, MA).

Analysis of aromatase mRNA by reverse transcription-PCR. Total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Total RNA (1 μ g) from the adipose stromal cells was converted to first-stranded cDNA primed with a random hexamer in a 20 μ L reaction volume using a RNA PCR kit (Takara Shuzo, Tokyo, Japan) and 4 μ L of this were used as a template in the PCR. The sense and antisense primers used in PCR amplification for aromatase were 5'-TACTACAACC GGGTATATGG-3' (the sequence in exon 3) and 5'-TGTTAGAGGTGCCAG-CATG-3' (the sequence in exon 5), respectively, as described previously (47). For the semiquantitative analysis, the PCR reactions consisted of 30, 33, and 36 cycles (94°C for 0.5 minute, 58°C for 0.5 minute, and 72°C for 0.5 minute), and linear conditions were determined. Amplification cycles were preceded by a denaturation step (94°C for 2 minutes) followed by an elongation step (72°C for 7 minutes). PCR products were analyzed on 2% polyacrylamide gels. The expression levels of individual mRNA bands were evaluated by Epilight UV, FA2000 (Aisin-Cosmo, Tokyo, Japan), and then normalized to

Figure 1. Induction of GFP expression by E_2 in MCF-7 clones stably transfected with ERE-GFP (*ERE-GFP-E5* and *ERE-GFP-E10*) or control vector (*pdE2-GFP-4*). The cells were cultured in PRF-RPMI with 10% DCC-FCS for 4 days and then incubated with 10 nmol/L E_2 for a further 2 days. Expression levels of GFP were analyzed by flow cytometry. Representative of three separate experiments, which gave similar results.



that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The sense and antisense primers for GAPDH were 5'-ACATCGCTCAGACAC-CATGG-3' and 5'-GTAGTTGAGGTCATGAAGGG-3', respectively, and PCR was done for 18 cycles.

Results

Isolation of a MCF-7 clone reporting estrogen receptor activity via green fluorescent protein expression. To address the analysis of the ER-activating ability of adipose stromal cells in breast cancer, we developed a new reporter cell system to visualize and quantify the activation of ER in breast cancer cells. First, we stably transfected MCF-7 cells with the ERE-GFP reporter plasmid, which has a ubiquitination site, so that it has a short half-life of ~2 hours. We isolated several clones that showed specific expression of GFP in the presence of estrogen, and the representative results of flow cytometry analysis of these clones are shown in Fig. 1. The clones transfected with ERE-GFP vectors, such as ERE-GFP-E5 and ERE-GFP-E10, specifically expressed GFP in an estrogen-dependent manner, whereas a mock-transfected MCF-7 clone GFP-4 showed no induction of GFP. We used a clone E10 in the following studies because the background level of GFP observed in the absence of estrogen is low.

Next, we examined the dose dependency and time course of the expression of GFP following treatment with estrogen. To quantify GFP expression, the cells were harvested after treatment with trypsin, and the numbers of E10 cells expressing GFP were counted. The results are shown as percentages of positive cells. This method enabled us to analyze GFP expression for individual cells and was better in a practical sense than flow cytometric analysis because the latter required many cells. In Fig. 2A, GFP was induced by estrogen in a dose-dependent manner and detected at 3 pmol/L, reaching a maximum level of 30 pmol/L estrogen. Estrogen has been reported to show its biological effects of a similar dose dependency. Stimulation of the growth of E10 cells by estrogen was also observed at these concentrations (Fig. 2A), indicating that GFP expression could reflect the activated status of ER in viable cells. The induction of GFP was observed after being incubated with estrogen for 1 day and reached a maximum level after a 2-day incubation (Fig. 2B).

Green fluorescent protein expression in E10 cells cocultured with adipose stromal cells of breast cancer tissues. Using E10 cells as reporter cells for ER activation, we next characterized the ability of adipose stromal cells isolated from breast cancer tissue to stimulate estrogen signals in breast cancer. *In vivo*, ERs in breast cancer cells can be activated by tumor-stromal interactions in both estrogen-dependent and estrogen-independent manners. As one of the estrogen-independent manners, it has been reported that growth factors, such as EGF and IGF-I, activate ERs via phosphorylation (17, 21, 23). Furthermore, in addition to aromatase, the estrogen content in the tumor is affected by several estrogen-metabolizing enzymes, which are primarily expressed in stromal cells (5). Thus, ER activation is regulated by multiple pathways based on the tumor-stromal interactions, and we developed a system to detect the overall activation of ERs via GFP expression in E10 cells cocultured with the tumor-derived adipose stromal cells. Figure 3 shows the effect of coculture with adipose stromal cells on the induction of GFP in E10 cells. In the presence of testosterone, a substrate for aromatase, GFP was specifically induced in E10 cells cocultured with adipose stromal cells, whereas no GFP was observed without testosterone. Addition of testosterone alone to E10 cells also did not show

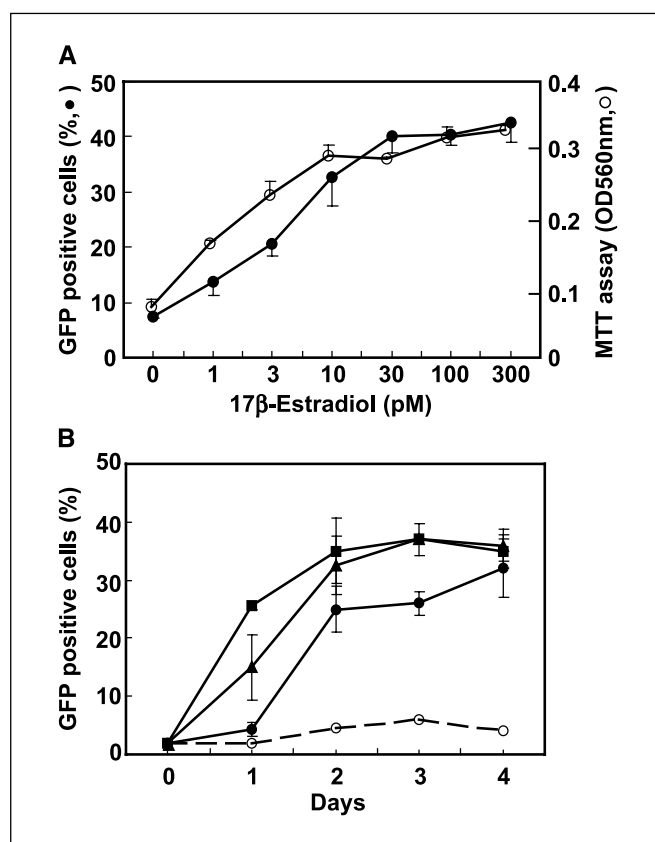


Figure 2. Effect of E₂ or adipose stromal cells isolated from breast cancer tissue on GFP expression and growth of E10 cells. *A*, after 3 days of culture in PRF-RPMI with 10% DCC-FCS, the cells were incubated with various concentrations of E₂ for 4 days. The numbers of cells expressing GFP (○) were counted, and data are expressed as percentages of GFP-expressing cells. The growth was assayed by MTT (●). *B*, after 3 days of culture in PRF-RPMI with 10% DCC-FCS, the cells were incubated with 3 pmol/L (▲) or 1 nmol/L (■) E₂ or cocultured with (●) or without (○) 32NT stromal cells in the presence of 1×10^{-7} mol/L testosterone for the indicated days. The numbers of E10 cells expressing GFP were counted, and data are expressed as percentages of GFP-expressing cells. All experiments were done in triplicate. Points, average; bars, SD. Two separate experiments gave similar results.

any induction of GFP expression (data not shown). No significant GFP expression was observed in a mock-transfected MCF-7 clone GFP-4 cocultured with adipose stromal cells even in the presence of testosterone (data not shown). Estrogen induced GFP expression after a 1-day treatment, whereas coculture with adipose stromal cells required 2 days to detect GFP expression in E10 cells (Fig. 2B). It is likely that the coculture required more time to produce ER-activating signals based on the tumor-stromal interactions. Finally, after 3 or 4 days of coculture, GFP expression reached a maximum level, which was comparable with that induced by estrogen (Fig. 2B). In the following experiments, GFP expression in the cocultures was analyzed after incubation for 4 days. The level of GFP expression increased as the number of adipose stromal cells plated increased (Fig. 3D).

Next, to show that GFP expression reflects the intensity of estrogen signals in the coculture, we examined the effect of dexamethasone (Fig. 4), which is known to stimulate the expression of the aromatase gene (8). Although treatment with dexamethasone had no effect on GFP expression in E10 cells cultured alone, it resulted in an increase of GFP expression in E10 cells cocultured with adipose stromal cells (Fig. 4, in the cases of 18T and 21T

stromal cells). It might have been caused by dexamethasone-induced estrogen production via an enhancement of aromatase gene expression. In the 17T adipose stromal cells, where the coculture maximally induced the expression of GFP in E10 cells, further treatment with dexamethasone induced a slight increase in GFP expression. These results suggest that GFP expression reveals the activated status of the ER and that this system could be useful to detect estrogen signals produced by adipose stromal cells. The result that tamoxifen at 1 $\mu\text{mol/L}$ blocked the expression of GFP under the coculture also indicates that GFP is induced through ER activation (data not shown).

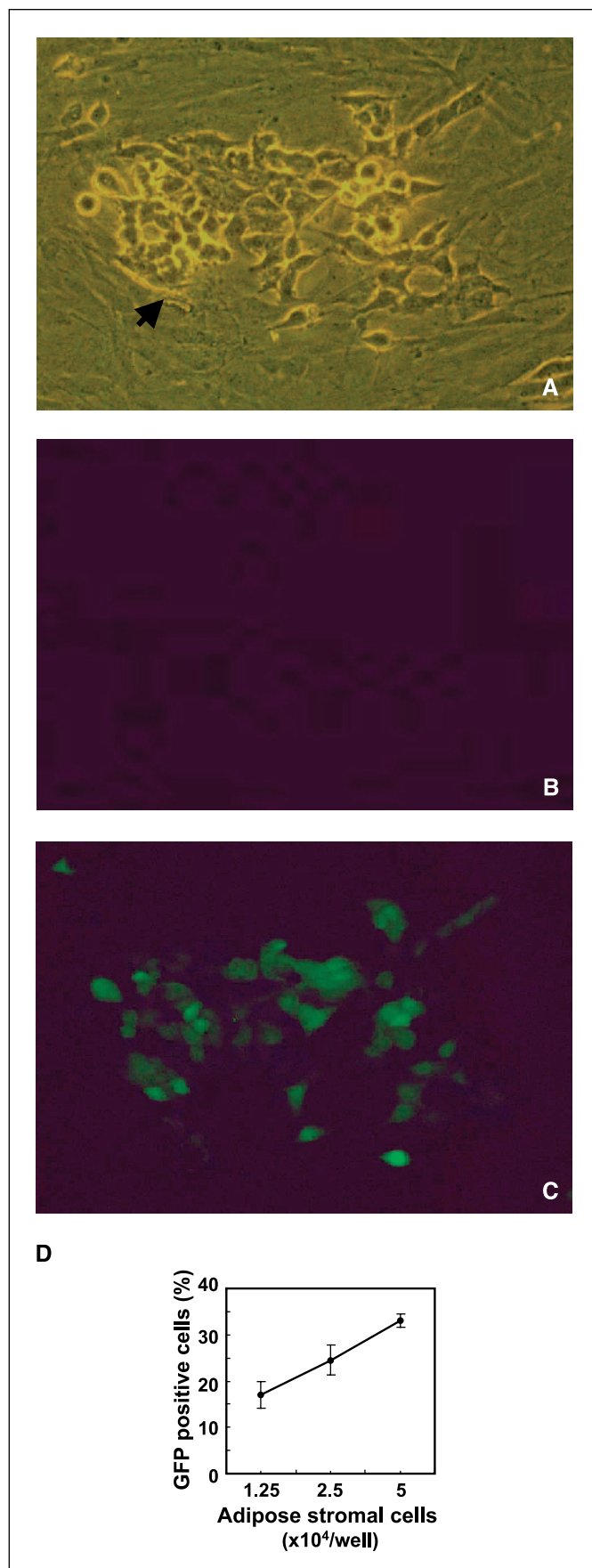
We also tested whether the induction of GFP expression requires cell-to-cell contact between E10 cells and adipose stromal cells using a Transwell culture dish in which both cells could be cultured separately by a microporous membrane. We tested several cases and found that the adipose stromal cells similarly induced GFP expression in these culture dishes (data not shown).

Characterization of adipose stromal cells obtained from individual patients. Using this system, we characterized the adipose stromal cells obtained from individual human breast cancer tissues in terms of their ability to stimulate estrogen signals. The clinicopathologic characteristics of the specimens are shown in Table 1. Figure 5 shows results representative of the fact that, in some cases, no significant induction of GFP was observed and, in other cases, the coculture induced GFP expression as effectively as by estrogen treatment. No significant induction of GFP was observed in the absence of testosterone for all cases tested (data not shown). Thus, the levels of GFP expression induced by the coculture varied among the cases, indicating that stromal cells in breast cancer tissues have their own properties with respect to the activation of estrogen signals. It may be closely related to the properties of individual breast cancers and in turn with their sensitivity to hormone therapy. This is the first report in which the function of adipose stromal cells in human breast cancer has been able to be evaluated for individual cases based on their ER-stimulating ability.

As it has been shown that aromatase is highly expressed in stromal adipose tissue proximal to the tumor (4, 6), the adipose stromal cells isolated from the region 2 cm distal to the tumor were also analyzed. There is no clear relationship between the regions where the adipose stromal cells were isolated and the GFP expression levels (Fig. 5).

In hormone therapy of breast cancer, the expression status of ER α is a primary determinant for the prediction of its efficacy, and the overexpression of ER α is frequently observed at an early stage in the progression of breast cancer. Then, we analyzed the relationship between the ability of adipose stromal cells to induce GFP expression and the status of ER α in breast cancer cells. In both ER-positive and ER-negative breast cancers, expression levels of GFP varied among the cases (Fig. 6A), and no significant relationship between GFP expression and ER status was observed.

Figure 3. Induction of GFP expression in E10 cells cocultured with stromal cells. After 3 days of culture in PRF-RPMI with 10% DCC-FCS, E10 cells (A, arrow) were cultured on stromal cells in the presence (C) or in the absence (B) of testosterone (10^{-7} mol/L) for 2 days. D, effect of the number of adipose stromal cells on GFP expression in E10 cells under the coculture. After 3 days of culture in PRF-RPMI with 10% DCC-FCS, E10 cells at 3×10^4 in a 24-well multidish were incubated with indicated numbers of 17T adipose stromal cells for 4 days. Then, the numbers of E10 cells expressing GFP were counted, and the data are expressed as percentages of GFP-expressing cells. Points, average; bars, SD.



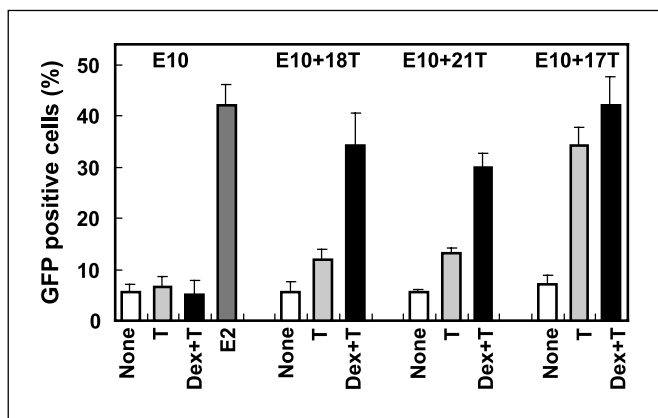


Figure 4. Dexamethasone enhanced GFP expression in E10 cells induced by coculture with stromal cells. After 3 days of culture in PRF-RPMI with 10% DCC-FCS, stromal cells (18T, 21T, and 17T) were incubated with or without dexamethasone (Dex; 2×10^{-6} mol/L) overnight and then cocultured with E10 cells in the presence or in the absence of testosterone (T; 1×10^{-7} mol/L) for 4 days. Then, the numbers of E10 cells expressing GFP were counted, and the data are expressed as percentages of GFP-expressing cells. All experiments were done in triplicate. Columns, average; bars, SD. Three separate experiments gave similar results.

The ratio of the cases with a high ER-stimulating ability that stromal cells induced GFP in >30% of E10 cells was also not significantly different between them (Table 1).

GFP expression levels also varied in both premenopausal and postmenopausal breast cancers (Fig. 6B), but the ratio of the cases with a high ER-activating ability was significantly higher in the postmenopausal patients than in the premenopausal ones (Table 1). These data are consistent with the reports that local estrogen signals play an important role in the progression of breast cancers in postmenopausal women (1–3). For the grade variables, the cases of grade 3 showed lower GFP expression levels than the cases of grade 1 or 2, although more samples are needed to determine its significance (Table 1). As for the other clinicopathologic variables, no significant difference was detected. We also examined the relationship between GFP and HER-2/*neu* or Ki-67 expression but did not detect any correlation (data not shown). The over-expression of HER-2 has been reported in advanced breast cancers, and trastuzumab, a humanized anti-HER-2 antibody, has been

specifically approved for HER-2-positive breast cancer. Ki-67 is a marker of cell proliferation.

Next, we examined the expression of the aromatase gene in adipose stromal cells by reverse transcription-PCR and found that the ability of adipose stromal cells to induce GFP expression did not always correlate with the aromatase expression levels (Fig. 7A and B). In some cases, such as 17T and 19T, both aromatase and GFP expression levels were high, and in other cases, such as 27T, both were low. However, although the adipose stromal cells from 17NT, 23NT, and 32NT induced high levels of GFP expression, they did not express high levels of the aromatase gene. A statistical correlation was not observed between them (Fig. 7C). These results indicate that the expression levels of the aromatase gene in adipose stromal cells could neither determine the intensity of ER activation nor predict the efficacy of aromatase inhibitors of breast cancers.

Effects of aromatase inhibitors on the induction of green fluorescent protein expression. Recent clinical data have shown that aromatase inhibitors are superior to the anti-estrogen agent, tamoxifen, in hormone therapy for breast cancer and that several potent aromatase inhibitors are clinically available (33, 34). These aromatase inhibitors have now been approved as a first-line treatment for hormone-dependent advanced breast cancer. However, a system to predict their efficacy for individual patients has not been developed. The ER status and the expression levels of the aromatase gene could not appropriately predict their efficacy as described above. The targets of the aromatase inhibitors are adipose stromal cells; thus, our coculture system to analyze the properties of adipose stromal cells for individual tumors may be useful to predict the efficacy of aromatase inhibitors.

We examined the effects of anastrozole, letrozole, and exemestane on the induction of GFP expression (Fig. 8). They effectively inhibited the induction of GFP expression in E10 cells cocultured with adipose stromal cells, but the sensitivity to the drugs varied according to the samples. Although these inhibitors inhibited GFP expression in similar dose dependencies in the 23T stromal cells, they showed different dose dependencies in the 32NT stromal cells. In the latter case, 1 μ mol/L anastrozole was needed to completely inhibit GFP expression (data not shown). Anastrozole has a tendency to require higher concentrations to inhibit GFP expression than the other drugs, which is consistent with the reports that letrozole is more potent than anastrozole by several

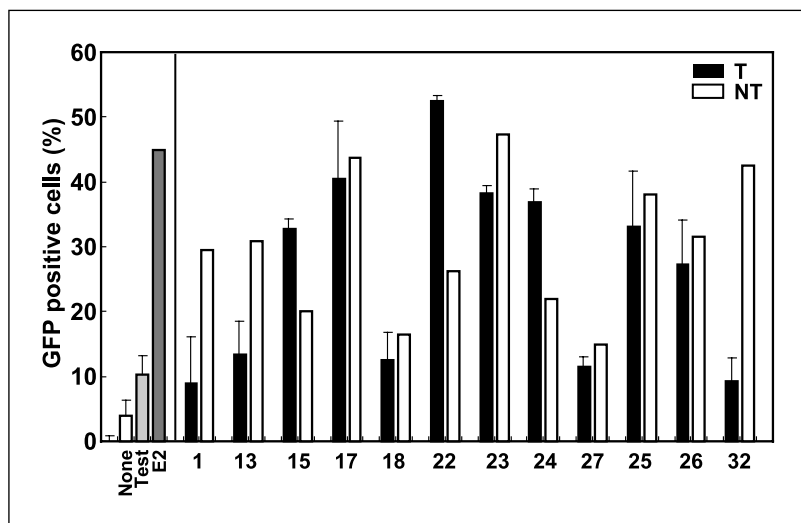


Figure 5. GFP expression in E10 cells cocultured with stromal cells obtained from individual breast cancer patients. After 3 days of culture in PRF-RPMI with 10% DCC-FCS, E10 cells were cultured with stromal cells obtained from breast cancer tissues for 4 days in the presence of testosterone (1×10^{-7} mol/L). The numbers of E10 cells expressing GFP were counted, and the data are expressed as percentages of GFP-expressing cells. X axis, case numbers. T, stromal cells obtained from tumor region; NT, stromal cells obtained from the region 2 cm distal to the tumor region. The cells were also analyzed after being cultured in the presence or absence (None) of testosterone (Test) or estrogen (E2) alone. All experiments were done in triplicate. Columns, average; bars, SD. Two separate experiments gave similar results.

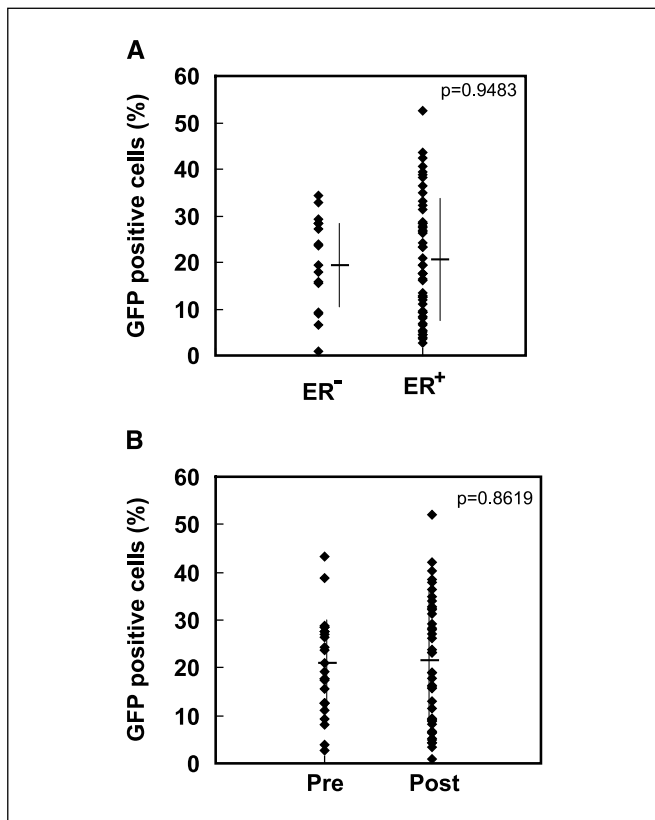


Figure 6. Relationship between ER α status (A) or menopausal status (B) and GFP-inducing ability of stromal cells. For 67 cases, the GFP-inducing ability of the stromal cells was analyzed based on their ER α expression status, which was determined by Allred score, or menopausal status. Filled diamonds, individual values of the ratio of GFP-positive cells; horizontal bar, median; vertical bar, SD. Two-sample *t* test.

variables, such as aromatization and its suppression of tumor growth in chemically induced rodent mammary tumors (30). Furthermore, the sensitivity to aromatase inhibitors did not correlate with the aromatase expression levels in stromal cells (Figs. 7 and 8).

Discussion

In this study, we have shown a novel system to analyze the regulation of estrogen signals in breast cancers based on the ER-activating ability of adipose stromal cells. The expression status of ER is a primary determinant in the hormone therapy of breast cancer, but it was not able to completely predict the responsiveness of the tumors to hormone therapy; not all ER-positive breast cancers respond to hormone therapy. Therefore, a new diagnostic marker or tool was required. Several studies have reported that the genesis and progression of breast cancer is regulated by its interactions with the stromal cells surrounding the tumor cells, which provide intratumoral estrogen signals and affect the efficacy of hormone therapy. In this regard, we have developed a system to estimate the function of adipose stromal cells for individual breast cancers.

First, to report ER activation, we established a clone of MCF-7, named E10, by stable transfection with the ERE-GFP gene. Expression of GFP in MCF-7-E10 cells is induced by treatment with E₂ in a specific and dose-dependent manner and was able to be induced at 3 pmol/L E₂ (Fig. 2A). The growth of E10 cells is also

induced by E₂ at a similar dose dependency (Fig. 2A). These results suggest that the induction of GFP expression in E10 cells is highly sensitive in its detection of estrogen-inducing signals. Using this clone, we examined the ability of adipose stromal cells isolated from breast cancer tissues to induce ER activation. A representative result in Fig. 3 showed that E10 cells specifically express GFP in the presence of testosterone when cocultured with adipose stromal cells. Because MCF-7 cells do not express the aromatase gene, which we also confirmed (data not shown), the addition of testosterone alone had no effect on the expression of GFP in E10 cells. Therefore, the induction of GFP expression indicates that estrogen and/or other ER-activating signals are supplied during the coculture of E10 cells with adipose stromal cells. In this system, we were able to visualize the ER activation for individual MCF-7-E10

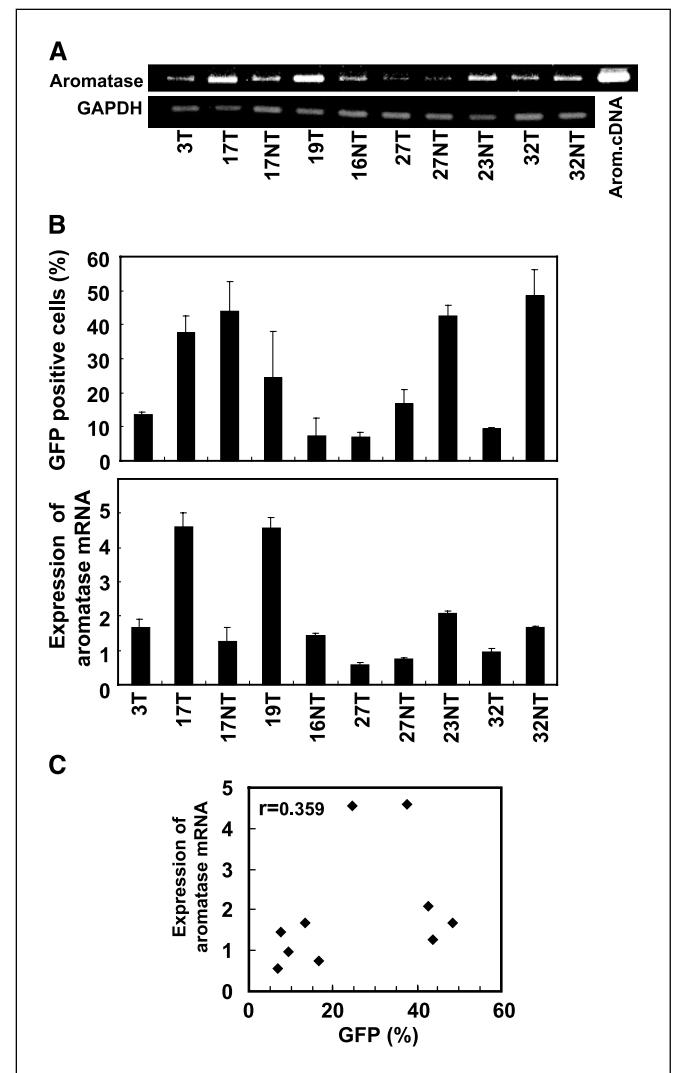


Figure 7. Aromatase gene expression in stromal cells and GFP expression in E10 cells cocultured with stromal cells. A, aromatase gene expression in stromal cells was analyzed by reverse transcription-PCR. Relative amounts of mRNA were determined as described in Materials and Methods. B, after 3 days of culture in PRF-RPMI with 10% DCC-FCS, E10 cells were cultured with stromal cells obtained from breast cancer tissues for 4 days in the presence of testosterone (1×10^{-7} mol/L). The numbers of E10 cells expressing GFP were counted, and the data are expressed as percentages of GFP-expressing cells. X axis, case numbers. All experiments were done in triplicate. Columns, average; bars, SD. Two separate experiments gave similar results. C, correlation of the expression between GFP and aromatase gene was analyzed.

cells, which enabled us to simultaneously analyze the expression of other proteins related to ER activation. The MTT assay was not sufficiently sensitive to analyze the effect of stromal cells on E10 cells due to the presence of stromal cells in the culture.

We reported here that although adipose stromal cells are similarly isolated their ability to activate the ER in E10 cells varied among the cases (Fig. 5). These results suggest that adipose stromal cells in the tumor have their own properties with respect to the activation of ERs. The adipose stromal cells activate ERs in breast cancer cells via the multiple pathways based on the tumor-stromal interactions as shown in Fig. 9. Aromatase is a key enzyme of estrogen biosynthesis and is mainly expressed in adipose stromal cells in breast cancers of postmenopausal patients. The expression of aromatase is stimulated by factors, including PGE₂, IL-6, IL-11, and TNF- α , which are provided from breast cancer cells and inflammatory cells. However, the induction level of GFP did not always correlate with the expression of the aromatase gene (Fig. 7). One reason for this is that the ER can be also activated by several growth factors, such as EGF and IGF-I, via phosphorylation in the absence or in the presence of a low concentration of E₂ (Fig. 9; refs. 17, 21–25). E₂ itself has been reported to induce production of these growth factors (18, 19, 20) and to also up-regulate the expression of IGF-I receptors and its signaling molecules (IRS-1 and IRS-2) in breast cancer cells (22). Furthermore, in addition to aromatase, the E₂ level in the coculture is also regulated by several estrogen-metabolizing enzymes, including 17 β -hydroxysteroid dehydrogenase (17 β -HSD)-1, 17 β -HSD-2, estrone sulfatase, and estrogen sulfotransferase (Fig. 9). Therefore, the GFP expression in the MCF-7 cells detected in our system showed that these overall ER-activating signals were provided by the interaction with adipose stromal cells.

On the other hand, GFP expression was not always associated with ER expression; GFP could be detected in some ER-negative cases and vice versa (Fig. 6A). These results suggest that the ER status alone cannot correctly predict the efficacy of aromatase inhibitors. As aromatase inhibitors mainly act on the adipose stromal cells, they may be ineffective in the case that GFP expression cannot be detected, although the ER status of the tumor tissue is

positive. In the case that the ER status is positive and GFP expression can be induced, two possibilities are suggested. One is that ERs in tumor cells are mainly activated by estrogen, and the other is that ERs are activated via an estrogen-independent pathway, such as phosphorylation by growth factor-inducing signals as described above. Aromatase inhibitors may be effective in the former case but not in the latter. The mechanisms of the estrogen signals and the prediction of hormone therapy in human breast cancers have been independently studied with respect to each variable, such as ER expression, E₂ content, or expression of the aromatase gene. On the contrary, our system analyzes the total effect of factors affecting estrogen signals, including these variables. Therefore, this system is useful to analyze the effect of aromatase inhibitors on estrogen signals based on the tumor-stromal interactions.

As for the other clinicopathologic characteristics, higher GFP expression is observed in postmenopausal cases than in premenopausal ones (Fig. 6B; Table 1). This is consistent with the previous reports that in postmenopausal breast cancers adjacent stromal cells play an important role in the intratumoral estrogen production (4, 14). In the cases with a high grade (grade 3), GFP levels were low, although the significance remains unknown. No significant correlation between GFP expression and other characteristics, such as histologic types, stages, or tumor sizes, was observed (Table 1).

Although adipose stromal cells within the early passages were used for the experiments, the properties of adipose stromal cells are relatively stable. The ability of adipose stromal cells to induce the expression of GFP in E10 cells was unchanged over increasing passages or after being frozen in liquid nitrogen (data not shown). These results suggest that the properties of adipose stromal cells are predetermined *in vivo* based on the interaction with tumor cells, which could be detected in our system.

Our previous study reported that we made a custom-made cDNA microarray consisting of estrogen-responsive genes using MCF-7 cells treated with E₂ and found a new predictive factor for hormone therapy in breast cancer and a new estrogen signal cascade (37, 40, 41). When breast cancer cells are affected by

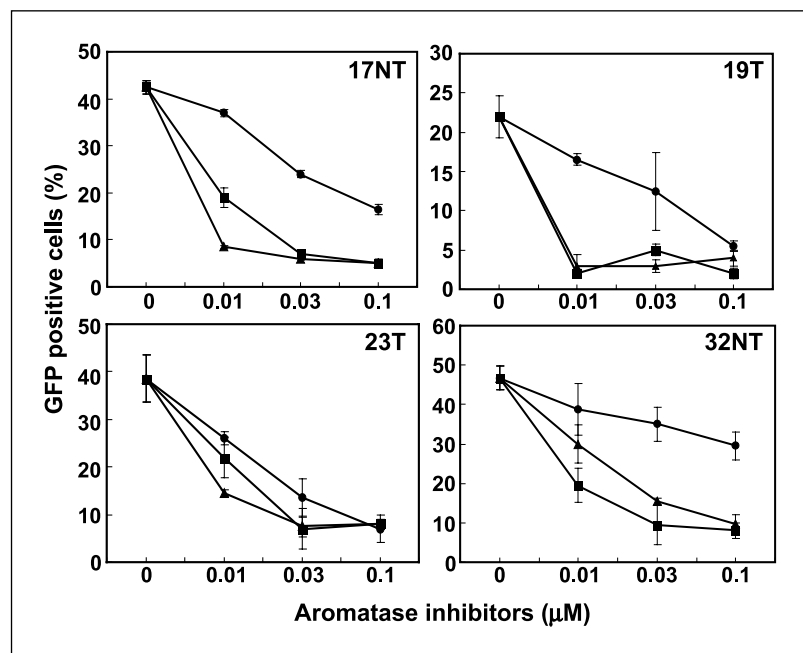
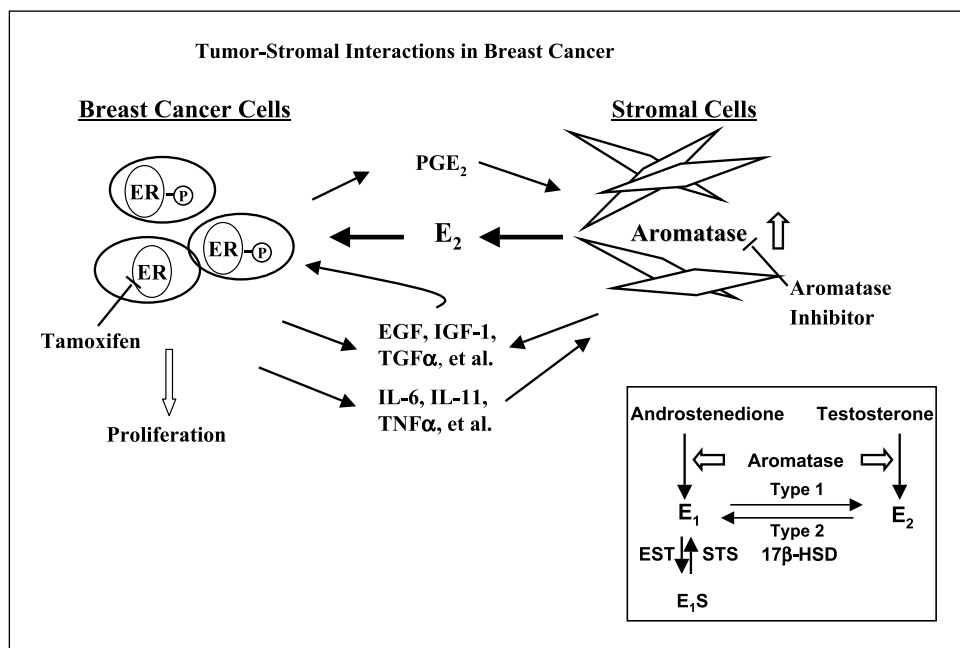


Figure 8. Effects of aromatase inhibitors on the induction of GFP expression in E10 cells cocultured with stromal cells. After 3 days of culture in PRF-RPMI with 10% DCC-FCS, E10 cells were cocultured with stromal cells in the presence of various concentrations of anastrozole (●), letrozole (▲), or exemestane (■) and testosterone (1×10^{-7} mol/L) for 4 days. The numbers of E10 cells expressing GFP were counted, and the data are expressed as percentages of GFP-expressing cells. All experiments were done in triplicate. Points, average; bars, SD.

Figure 9. Tumor-stromal interactions in breast cancer. ERs expressed in breast cancer cells are activated via several pathways under tumor-stromal interactions. Aromatase, a key enzyme of E_2 synthesis, is mainly expressed in stromal cells, which results in the supply of E_2 . The expression of aromatase itself is stimulated by several factors produced by breast cancer cells, such as PGE_2 and cytokines, including IL-6, IL-11, and $TNF-\alpha$. Other estrogen-metabolizing enzymes, such as estrogen sulfotransferase (*EST*), steroid sulfatase (*STS*), and 17β -HSD (shown in the square), which are expressed by stromal cells, also regulate E_2 production. Growth factors, including EGF, IGF-I, and transforming growth factor- α (*TGF α*), could activate ER via phosphorylation, and the cross-talk between ER-mediated and growth factor-mediated signals has been reported. These growth factors are supplied not only from stromal cells but also from breast cancer cells stimulated by E_2 .



various factors provided from the surrounding stromal cells, an additional set of genes may be induced. A study on the gene expression profiling of E10 cells cocultured with stromal cells is in progress, which will provide further information about the *in vivo* estrogen signals in breast cancer. Furthermore, a combination of gene expression profiling of estrogen-responsive genes in cancer cells and assessment of ER-activating ability by adjacent adipose stromal cells should provide a new tool for the analysis of the mechanisms of estrogen-dependent and estrogen-independent growth of breast cancer.

Recently, aromatase inhibitors have been reported to be effective for primary systemic therapy before breast cancer surgery. To apply our system for the prediction of the efficacy of aromatase inhibitors, a study to characterize adipose stromal cells isolated from core needle samples of breast cancer before primary systemic therapy is also in progress. In summary, using the system developed in this study, we were able to visualize the ability of adipose stromal cells to stimulate estrogen signals for individual

cases and found that the properties of adipose stromal cells and the sensitivity to aromatase inhibitors varies in the tumors. These results suggest that the tumor-stromal interactions totally control estrogen signals in the tumor and that the analysis of adipose stromal cells is essential for the prediction of efficacy of hormone therapy in breast cancers.

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