Estrogen signaling ability in human endometrial cancer through the cancer–stromal interaction

Mitsuyo Matsumoto1,2, Yuri Yamaguchi4, Yuko Seino4, Atsushi Hatakeyama2, Hiroyuki Takei5, Hitoshi Niikura2, Kiyoshi Ito2, Takashi Suzuki3, Hironobu Sasano3, Nobuo Yaegashi2 and Shin-ichi Hayashi1

Departments of1Medical Technology, 2Obstetrics and Gynecology and 3Pathology, Tohoku University School of Medicine, 2-1, Seiryo-machi, Aoba-ku, Sendai 981-0872, Japan
4Research Institute for Clinical Oncology and 5Division of Breast Surgery, Saitama Cancer Center, Saitama 362-0806, Japan

(Correspondence should be addressed to M Matsumoto, Department of Obstetrics and Gynecology, Tohoku University School of Medicine, 1-1, Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan; Email: m-matsumoto@mail.tains.tohoku.ac.jp)

Abstract
The estrogen pathway plays an important role in the etiology of human endometrial carcinoma (EC). We examined whether estrogen biosynthesis in the tumor microenvironment promotes endometrial cancer. To examine the contribution of stromal cells to estrogen signaling in EC, we used reporter cells stably transfected with the estrogen response element (ERE) fused to the destabilized green fluorescent protein (GFP) gene. In this system, the endometrial cancer stromal cells from several patients activated the ERE of cancer cells to a variable extent. The GFP expression level increased when testosterone, a substrate for aromatase, was added. The effect was variably inhibited by aromatase inhibitors (AIs), although the response to AIs varied among patients. These results suggest that GFP expression is driven by estrogen synthesized by aromatase in the endometrial cancer stromal cells. In a second experiment, we constructed an adenovirus reporter vector containing the same construct as the reporter cells described above, and visualized endogenous ERE activity in primary culture cancer cells from 15 EC specimens. The GFP expression levels varied among the cases, and in most primary tissues, ERE activities were strongly inhibited by a pure anti-estrogen, fulvestrant. Interestingly, a minority of primary tissues in endometrial cancer showed ERE activity independent of the estrogen-ER pathway. These results suggest that AI may have some therapeutic value in EC; however, the hormonal microenvironment must be assessed prior to initiating therapy.

Endocrine-Related Cancer (2008) 15 451–463

Introduction
Endometrial carcinoma (EC) is one of the most common gynecological cancers found in women worldwide (Landis et al. 1999). Estrogen contributes to endometrial carcinogenesis (Hecht & Mutter 2006, Ito 2007) and malignant transformation. Estrogen influences the activation and/or expression of growth factors such as insulin-like growth factor (IGF-I; Rutanen et al. 1993, O’Toole et al. 2005) and vascular endothelial growth factor (O’Toole et al. 2005). These growth factors play important roles in the development and progression of EC (Mochizuki et al. 2006).

Recent studies reveal that estrogen receptors (ERs) are activated not only by estrogen but also by protein phosphorylation by kinases such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt (Campbell et al. 2001, Stoica et al. 2003). Activated ER contributes to the proliferation, anti-apoptosis, and metastasis of tumor cells. This effect is the result of the induction of its downstream genes whose promoter regions contain the estrogen response element (ERE). The molecular mechanisms of the regulation of transcriptional activity by ER have been well investigated in breast cancer cells. Although both EC and breast cancer are
considered as estrogen-dependent carcinomas, they differ in their responses to anti-estrogens, particularly tamoxifen (TAM; Ito 2007). Thus, the actions of estrogen in EC might be different from those in breast carcinoma. The molecular mechanisms of the actions of estrogen, the target genes of the ER, and the pathway of estrogen signaling in EC have not been elucidated.

Cancer–stromal interactions play important roles in the genesis and progression of malignancies. We reported previously that stromal cells obtained from individual primary breast cancer patients activate the estrogen signaling pathway in breast cancer cells through tumor–stromal interaction (Yamaguchi et al. 2005). ER-activating activity is correlated with menopausal status and histological grade.

The estrogen level in EC tissue is higher than that in the endometrial tissue of healthy women (Naitoh et al. 1989, Berstein et al. 2003). Furthermore, estrogen-metabolizing enzymes such as aromatase, sulfatase, sulfotransferase, and 17β-hydroxysteroid dehydrogenases (17β-HSDs) exist in stromal cells adjacent to the tumor. These convert androgens and inactive estrogens (estrone (E1), estrone sulfate) into active estrogen (17β-estradiol, E2; Pasqualini & Chetrite 2006, Takase et al. 2006).

Aromatase is a key enzyme that catalyzes the conversion of androgens to estrogen (Pasqualini & Chetrite 2006, Takase et al. 2006). Aromatase mRNA is expressed in various tissues, such as adipose tissue, bone, brain, skin, and breast cancer (Bulun et al. 2005). The promoter driving the expression of aromatase mRNA is specifically activated in tissues where the gene is normally expressed (Bulun et al. 2005). The mRNA and protein of aromatase are detectable in EC but are absent in disease-free endometrium (Bulun et al. 1994, Watanabe et al. 1995). Furthermore, the endometrial cancer patients with aromatase-positive stromal cells have poor survival prognosis compared with patients with aromatase-negative stromal cells (Segawa et al. 2005). This evidence also suggests that aromatase is a key enzyme in the etiology of EC; however, the participation of local estrogen in the activation of ER in individual EC tumor cells has not been evaluated. Hormonal therapies such as anti-estrogens and aromatase inhibitors (AIs) are widely used in breast cancer patients, particularly ER-positive cases. However, with the exception of medroxyprogesterone acetate (MPA) in advanced disease, the use of hormonal therapies in endometrial cancer is not prevalent.

In this study, we analyzed tumor–stromal interactions in EC and examined whether estrogen biosynthesis functions importantly in the local environment of tumor tissue. We also succeeded in measuring estrogen-mediated ER activation of primary tumor cells obtained from individual EC patients. Our results identify AIs as another potential hormonal treatment for endometrial cancer. Our method of measuring estrogen activity may be used as a diagnostic tool for identifying estrogen-dependent endometrial cancer. Finally, our results underscore the importance of tailoring therapy to individual patients, and our assay provides a way to accomplish this.

Materials and methods

Cells and cultures

ER-Etk-green fluorescent protein (GFP)-MCF-7 cells (E10 cells) were established from a human breast cancer cell line, MCF-7, by the introduction of a plasmid carrying the ERE fused with the ERE-GFP gene, as described previously (Yamaguchi et al. 2005). MCF-7 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The human EC cell lines, Ishikawa, was provided by Dr Masato Nishida, Kasumigaura National Hospital, Japan; Sawano (RCB1152), HHUA (RCB0658), A431 (RCB0202), JHUEM2 (RCB1551), and JHUAS1 (RCB1544) were purchased from RIKEN (Ibaki, Japan); and Hec1A (HTB-112) and RL95-2 (CRL-1671) were purchased from ATCC. MCF-7 cells and the human EC cell lines were cultured in RPMI 1640 (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Tissue Culture Biologicals, Turale, CA, USA). For co-culturing with stromal cells or treatment with AIs, the cells were cultured in phenol red-free (PRF) RPMI 1640 supplemented with 10% dextran-coated charcoal-treated FCS (DCC-FCS). The culture media contained 0.1% penicillin/streptomycin (GIBCO BRL). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air. 293A cells, a line of human kidney cells, were purchased from Invitrogen (Carlsbad, CA, USA), cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), and used to propagate the adenovirus.

Drugs

Anastrozole (aromatase inhibitor) and ICI 182 780 (fulvestrant, pure anti-estrogen) were kindly provided by AstraZeneca Pharmaceuticals; letrozole (aromatase inhibitor) was from Novartis Pharmaceuticals (Basel, Switzerland); and exemestane (aromatase inhibitor) from Pharmacia Co. (Bridgewater, NJ, USA; currently Pfizer Inc., New York, NY, USA). Testosterone and E2 were purchased from Sigma.
Tumor samples

Specimens of human EC tissues were obtained from female patients who underwent hysterectomy from 2004 to 2006 in the Department of Gynecology at Tohoku University Hospital (Miyagi, Japan). Informed consent was obtained from all 18 patients prior to their surgery and examination of the specimens (Table 1). Human breast cancer tissues were obtained from surgical specimens at the Saitama Cancer Center Hospital (Saitama, Japan) after obtaining informed consent from the patients. The Tohoku University Ethics Committee and the Saitama Cancer Center Ethics Committee approved this study.

Isolation of primary stromal cells from cancer tissue

The isolation procedure of stromal cells was as described previously by Ackerman et al. (1981) with slight modifications. Briefly, tissue specimens were minced to \( \sim 1 \text{mm}^3 \) in size after being rinsed with PBS and digested with collagenase solution (2.5 mg/ml collagenase, 40 mg/ml BSA, 2 mg/ml glucose, 1× antibiotic–antimycotic, and 50 μg/ml gentamicin in HBSS) for 20–30 min at 37 °C. The cells, including stromal cells, were washed several times with PBS after centrifugation and cultured at 37 °C in 5% CO\(_2\)–95% air following suspension in PRF-RPMI 1640 containing 10% FCS. Outgrowth of cells was observed after 5–10 days, and the medium was renewed twice weekly.

Co-culture of MCF-7 cells with primary stromal cells

Co-culturing of E10 cells plus stromal cells was done as described previously (Yamaguchi et al. 2005). Briefly, \( 5 \times 10^4 \) stromal cells were seeded onto 24-well plates following pre-culturing in PRF-RPMI 1640 containing 10% DCC-FCS for 96 h. After 2 h, \( 5 \times 10^4 \) E10 cells were seeded on top of the stromal cells in media containing testosterone at \( 1 \times 10^{-7} \text{ mol/l} \) as a substrate for aromatase. After further culturing for 4 days, the co-cultured cells were collected by centrifugation after 0.05% trypsinization, and the GFP-expressed E10 cells were counted on glass slides using fluorescence microscopy. Regardless of the fluorescence intensity, all GFP-expressed E10 cells were identified as GFP-positive cell to avoid wrong evaluation. E10 and stromal cells were easily discriminated by their morphology. To avoid the effects of aging, stromal cells were used within ten passages.

Quantitative reverse transcription-PCR for aromatase, 17β-HSD-type 2 and RPL13A

Total RNA of stromal cells was prepared using ISOGEN (Nippon Gene Co., LTD, Toyama, Japan) by the method of Chomczynski & Sacchi (1987). Reverse transcription and quantitative PCR were performed using SuperScript III RT (Invitrogen) and LightCycler FastStart DNA Master SYBR Green I with LightCycler DX400 (Roche Diagnostics AG, Rotkreuz, Switzerland) respectively. The oligonucleotides used in quantitative PCR were as follows: 5'-CTT CGT CGT GTC ATG CT-3' and 5'-GGA GAG CTT GCC ATG CAT CAA-3' for aromatase; 5'-CAA AGG GAG GCT GTT GAA T-3' and 5'-TCA CTG GTG CCT GCC ATA-3' for 17β-HSD type 2; and 5'-CTT GGA GAA GAG CAG TTT GTT G-3' and 5'-TTG AGG ACC TCT GTG TAT TTG TCA A-3' for ribosomal protein L13a (RPL13A), internal control (Vandesompele et al. 2002).

Table 1 Clinicopathologic variables of cancer patients

<table>
<thead>
<tr>
<th>Total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
</tr>
<tr>
<td>&lt;50</td>
</tr>
<tr>
<td>≥50</td>
</tr>
<tr>
<td>Menopausal</td>
</tr>
<tr>
<td>Pre</td>
</tr>
<tr>
<td>Post</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td>Grade</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td>Stage</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>Metastasis</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Histology</td>
</tr>
<tr>
<td>Endometriod</td>
</tr>
<tr>
<td>Serous</td>
</tr>
<tr>
<td>Carcinosarcoma</td>
</tr>
<tr>
<td>Muscular invasion</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>≤1/2</td>
</tr>
<tr>
<td>≥1/2</td>
</tr>
<tr>
<td>Vascular invasion</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
</tr>
</tbody>
</table>

www.endocrinology-journals.org
Luciferase assays

ERE activity in tumor cell lines was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The estrogen reporter plasmid ERE-tk-Luci was described previously (Omoto et al. 2001). The control vector pRL-TK (Promega) was used as an internal control of transfection efficiency in reporter assays. Transient transfection was performed using the method described previously by Omoto et al. Briefly, after 4 days of culture in PRF-RPMI 1640 with 10% DCC-FCS, 5×10^5 cells were seeded per well onto a 6 well plate in the same medium and incubated for 24 h. One microgram of pERE-tk-Luci plasmid and 0.1 μg pRL-TK were mixed with 5 μl TransIT LT-1 reagent (Mirus Co., Madison, WI, USA) in 300 μl serum-free medium and subjected to transfection according to the manufacturer’s instructions. Plasmid-transfected cells were cultured with/without 1×10^{-8} mol/l E2 for 24 h, and luciferase activity (i.e., ERE activity) was measured according to the manufacturer’s instructions using the Dual-Luciferase Reporter Assay System.

Reverse transcription-PCR for ERα and β-actin

Total RNA of the endometrial cancer cell lines, Ishikawa, Sawano, HHUA, A431, Hec1A, RL95-2, JHUEM2, JHUAS1, and a breast cancer cell line, MCF-7, was prepared using ISOGEN. Reverse transcription and PCR were performed using SuperScript III RT and ExTaq (Takara, Tokyo, Japan) respectively. Oligonucleotides used for PCR were as follows: 5′-CAT GAT CAA CTG GGC GAA GA-3′ and 5′-ACC GAG ATG ATG TAG CCA GC-3′ for ERα; 5′-CCA ACC GCG AGA AGA TGA C-3′ and 5′-GGA AGG GCT GGA AGA GT-3′ for β-actin as a control.

Construction of Ad-ERE-tk-GFP and Ad-cytomegalovirus (CMV)-DsRed

The consensus estrogen-responsive element and TK promoter gene cassette (ERE-tk) was spliced out from pRC-ERE-tk-Luci (Omoto et al. 2001) and was inserted into the multi-cloning site (MCS) in front of the GFP cDNA of pEGFP-1 (pEGFP-1-ERE-tk; Clontech Laboratories Inc). After the ERE-tk-GFP cassette was spliced out from pEGFP-1-ERE-tk, it was inserted into the MCS of the pENTER 1A vector (pENTER-ERE-tk-GFP; Invitrogen). The ERE-tk-GFP cassette was inserted into the adenovirus vector (pAd/PL-DEST; Invitrogen) by homologous recombination using the pENTER 1A vector, and the resultant vector was named pAd-ERE-tk-GFP. The ampicillin and pUC ori region was removed from pAd-ERE-tk-GFP, and the resultant vector was transfected into human kidney 293A cells using TransIT (Takara). After a few days, the virus Ad-ERE-tk-GFP was recovered in the medium from the 293A cells. The Ad-ERE-tk-GFP used in the experiments was propagated by culturing in PRF-RPMI 1640 supplemented with 10% DCC-FCS at 37 °C in 5% CO₂–95% air.

Ad-CMV-DsRed was constructed to assess the infectivity of the adenovirus in primary tumor cells as a control for transfection of Ad-ERE-tk-GFP. The immediate early promoter of CMV and the DsRed gene of red fluorescent protein were spliced out from pCMV-DsRed-Express (BD Biosciences, Palo Alto, CA, USA). Thereafter, they were inserted into the pENTER 1A vector (pENTER-CMV-DsRed), and pENTER-CMV-DsRed was transfected together with the adenovirus vector and recovered as Ad-CMV-DsRed virions using the same strategy described above for Ad-ERE-tk-GFP.

Assay of ERE activity in primary tumor cells

To assess ERE activation in primary tumor cells, we used Ad-ERE-tk-GFP. Cancer tissue specimens were minced to ~1 mm³ after rinsing with PBS and digested with collagenase solution for 20–30 min at 37 °C. The cells, including tumor cells, were washed several times with PBS after being recovered by centrifugation, and incubated in 24-well plates using 400 μl PRF-RPMI 1640 supplemented with 10% DCC-FCS. The cells were then promptly or 1 day later infected with 2×10^9 PFU (in 293A cells) Ad-ERE-tk-GFP, and incubated for 3 days at 37 °C in 5% CO₂–95% air. To examine the infectivity of the adenovirus in primary tumor cells, the primary cells were infected with 2×10^9 PFU Ad-CMV-DsRed or Ad-ERE-tk-GFP. GFP- or DsRed-expressing cells were counted by fluorescence microscopy after incubation for 3 days at 37 °C in 5% CO₂–95% air.

Immunohistochemistry of the ER

ER expression in individual EC patients was assayed with immunohistochemistry. To activate paraffin sections, the slides were heated at 120 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)) by autoclaving. Analysis was performed using the streptavidin–biotin amplification method using a Histofine kit (Nichirei, Tokyo, Japan). The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6), and 0.006% H₂O₂), and counterstaining was performed using hematoxylin. Monoclonal antibodies for the ER (ER1D5) were purchased from Immunotech
(Marseille, France) and used at a dilution of 1:50. For evaluation of ERα immunoreactivity, more than 1000 tumor cells from three different representative fields per case were counted, and the percentage immunoreactivity (i.e., labeling index (LI)) was determined.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney U test for comparison of two independent groups using the StatView 5.0 software program (SAS Institute Inc., Cary, NC, USA). For comparison among three or more groups, the Kruskal–Wallis test was used to assess the differences. Data were expressed as mean ± S.D. P < 0.05 was considered statistically significant.

Results

Detection of ER-activating ability of stromal cells in endometrial cancers

E10, an ER activity reporter cell line, was previously established from a clone of the human breast cancer cell line MCF-7 by stable transfection with the ERE-tk-GFP gene. E10 cells showed a high ERα expression level, and specifically expressed GFP upon treatment with E2 (Fig. 1A). Testosterone alone had no effect on the induction of GFP expression in E10 cells (Fig. 1A). Using E10 cells, we developed a system to visualize the ERE-activating ability of stromal cells in breast cancers based on tumor–stromal interactions (Yamaguchi et al. 2005).

Figure 1 GFP expression in E10 cells co-cultured with endometrial cancer stromal cells. (A) 17β-Estradiol (E2), but not testosterone, induced GFP expression in E10 cells. After 3 days of culture, E10 cells were cultured in the presence of testosterone, E2, or ethanol (control) for 4 days. The E10 cells expressing GFP were then counted. (B) Detection of GFP expression in E10 cells co-cultured with stromal cells. After 3 days of culture, E10 cells were cultured with stromal cells in the presence of testosterone or ethanol (control) for 4 days ((a) bright field, (b) dark field). The E10 cells expressing GFP were counted after treatment with trypsin–EDTA ((c) bright field, (d) dark field). Solid and open arrows show stromal cells of the endometrial tissue and E10 cells respectively. (C) GFP expression in E10 cells co-cultured with stromal cells obtained from individual endometrial cancers. After 3 days of culture, E10 and stromal cells were co-cultured in the presence or absence of testosterone for 4 days. E10 cells expressing GFP were counted, and the data are shown as percentages of GFP-expressing cells. All experiments were done in triplicate. Bars, average; error bars, s.d.
In this study, we examined the ability of stromal cells obtained from EC to activate the ERE. Figure 1B shows a case in which E10 cells co-cultured with stromal cells from EC expressed GFP in the presence of testosterone, a substrate of aromatase. GFP expression in E10 cells was usually analyzed after mild trypsinization, which enabled easy discrimination of E10 cells from stromal cells based on their morphology (Fig. 1B). Using this system, we analyzed whether the stromal cells obtained from various ECs activated estrogen signaling (Fig. 1C). The induction of GFP-positive cells was observed in most cases, though the percentage of GFP-positive cells was variable. In some cases, the presence of testosterone significantly increased GFP expression by 10- to 15-fold. This suggests that stromal cells in EC convert testosterone to E2 and activate estrogen signaling in tumor cells expressing ER. Interestingly, in one case (patient 13), the GFP-positive levels were high in the absence and low in the presence of testosterone. When the percentages of GFP-positive cells were analyzed with respect to clinicopathological variables, no statistically significant differences were found.

Expression of aromatase and 17β-HSD type 2 mRNA in primary endometrial cancer stromal cells

The concentration of intratumoral E2 is higher in the endometrial cancer tissues than in normal endometrium (Naitoh et al. 1989, Berstein et al. 2003), and is regulated by estrogen-metabolizing enzymes such as aromatase, 17β-HSD types 2 and 5 (Ito et al. 2006; Fig. 2C). Aromatase and 17β-HSD type 5 increase local estrogen production, whereas 17β-HSD type 2 decreases (Fig. 2C). To analyze the relationship between these enzymes and ER-activating ability in stromal cells, we examined the expression levels of aromatase and 17β-HSD type 2 genes by real-time PCR (Fig. 2A and B). We did not analyze 17β-HSD type 5 because our

Figure 2 Local estrogen biosynthesis in stromal cells of endometrial cancers. (A) Influence of aromatase and 17β-HSD type 2 gene expressions in stromal cells. The ratios of GFP-expressing cells increased following the addition of testosterone. Aromatase (○) and 17β-HSD type 2 (●) gene expressions in stromal cells were analyzed by real-time PCR. The expression levels were compared with the increase in the ratios of GFP-expressing cells following the addition of testosterone to E10 cells co-cultured with stromal cells. (B) Six representative samples showing aromatase and 17β-HSD type 2 gene expressions in stromal cells and GFP expression in the co-culture system. (C) Estrogen biosynthesis pathways in the microenvironment of endometrial cancer.
co-culture system contained testosterone, a reaction product of 17β-HSD type 5. The GFP expression level (the fold increase in estrogen signaling by the addition of testosterone) was not correlated \( R = 0.19 \) with aromatase, but was inversely correlated \( R = -0.51 \) with 17β-HSD type 2 mRNA expression levels (Fig. 2A). The data from individual patients suggested that ER activation in EC was greatly affected by estrogen biosynthesis, mediated by aromatase in the stromal cells, as shown in Fig. 2B. However, intratumoral E2 levels may also be regulated by other estrogen-metabolizing enzymes. The percentage of GFP-positive cells was low in cases such as patient 9. This case had a low expression level of aromatase and a high expression level of 17β-HSD type 2. This may have resulted in low synthesis of E2 in the local cells (Fig. 2B). In patient 14, stromal cells showed high ERE-activating ability, although they had a low level of aromatase gene expression. This might have been due to the lack of 17β-HSD type 2 expression.

**Effects of AIs on the induction of GFP in the co-culture system**

Our results suggest that aromatase in stromal cells plays a significant role in the regulation of local estrogen signaling; therefore, we examined the effects of AIs on ER activation in a co-culture system with endometrial cancers. AIs widely applied in breast cancers, including anastrozole, exemestane, and letrozole (Brueggemeier et al. 2005), were tested in 18 cases of EC. Figure 3A shows that all of these inhibitors significantly inhibit the induction of GFP expression (Kruskal–Wallis test: \( P < 0.01 \)). As in the case of breast cancers (Yamaguchi et al. 2005), the sensitivity of stromal cells to the drugs was variable among the cases (Fig. 3B). These results suggest that endocrine therapy using AIs might be effective for EC, especially those who have high sensitivity to AI; however, a method to select the most suitable drug for an individual patient needs to be developed first.

**Establishment of a new reporter system to analyze ERE activities in endometrial cancer**

The results obtained with the co-culture system indicate that stromal cells in EC locally produce estrogen signals. The significance of ER signaling in endometrial tumor cells has not been studied to date. We analyzed ERE activity in various endometrial cell lines (Ishikawa, Sawano, A431, RL95-2, JHUEM2, JHUAS1) and a breast cancer cell line (MCF-7) as a control, using a luciferase reporter assay. Somewhat unexpectedly, the ERE activities of the endometrial cancer cells were much lower than that in MCF-7 cells (Fig. 4).
Given the relatively lower ERE expression in the EC lines, we realized that the efficiency of the luciferase assay was not sufficient to measure the ERE activity in primary endometrial tissues containing a mixture of cells. Therefore, we developed a new reporter system in which the ERE-tk-GFP gene was transfected into tumor cells using an adenovirus vector. This system enabled us to directly detect the ER activity in individual endometrial cancers by monitoring GFP expression. We first examined the validity of this system using MCF-7 cells (Fig. 5). When MCF-7 cells were infected with Ad-ERE-tk-GFP, GFP expression was induced by E2 in a dose-dependent manner. GFP expression was detected at 3 pmol/l, and reached a maximal level at 100 pmol/l (Fig. 5A). GFP expression increased for 72 h and then decreased. Fulvestrant, an ER antagonist, inhibited the GFP expression, indicating that the expression was induced in an estrogen-specific manner (Fig. 5A and B). Adenovirus infectivity in MCF-7 cells was examined using Ad-CMV-DsRed, and a minimum of 95% of cells were infected (Fig. 5A and B). We then analyzed estrogen signals in primary endometrial cancer cells with this new reporter assay system.

### Detection of estrogen signal activity in primary endometrial cancer cells

Using the above adenovirus system, we characterized the ERE activity in primary tumor cells obtained from EC after treatment with collagenase (Fig. 6A). Three days after infection with Ad-ERE-tk-GFP, the GFP expression was observed (Fig. 6A). Primary tumor cells significantly expressed GFP, but the percentage of GFP-positive cells varied among individual cases.

The adenovirus infectivity of primary tumor cells, as estimated by infection with Ad-CMV-DsRed, was not less than 75% (data not shown). In some cases, primary EC tumor cells showed high percentages of GFP-positive cells comparable with the percentages seen in breast cancer tumor cells. ER expression in cancer cells was detected by immunohistochemical analysis and compared with the percentage of GFP-positive cells. The GFP expression levels of endometrial tumor cells were related to their ER expression levels, suggesting that estrogen signaling was conducted via the ER in endometrial cancers (Fig. 6B).

We then examined whether the ERE activities in the endometrial cancer cells were linked to estrogen/testosterone activation of the ER. Cancer tissues were prepared from four patients for Ad-ERE-tk-GFP infection. The infection was performed immediately after collagenase treatment or performed after overnight culture in estrogen-depleted medium. In three of four specimens, the GFP expression levels decreased in the cells infected immediately after the collagenase treatment compared with the cells infected after overnight culture (Fig. 7, gray bars). This might have been due to the decrease in intracellular estrogen during culture, since the addition of estrogen induced high GFP expression (Fig. 7, hatched bars). GFP expression was also induced by the addition of testosterone in the cell infected after overnight culture, and this induction was inhibited by fulvestrant. These results indicate that stromal cells, present in primary cell cultures, could supply estrogen via their expression of aromatase. In one specimen (patient 35), the GFP expression levels did not differ between the cells infected immediately after the collagenase treatment and the cells infected after overnight culture, even after the addition of estrogen, testosterone, and fulvestrant. In this specimen, the ERE activity was independent of the estrogen-ER signal pathway. Overall, our adenovirus vector system enabled us, for the first time, to detect the ER activity in primary endometrial cancer cells. This confirms that endometrial tumor cells can respond to estrogen signals.

### Discussion

Interaction among tumor and stromal cells has recently been shown to influence carcinogenesis or malignant transformation of cancer cells. Estrogen is one of the most important mediators of tumor–stromal interaction.
in breast cancer (Yamaguchi et al. 2005) and endometrial cancer (Ito 2007). Immunohistochemical studies of the biosynthesis and metabolism of estrogen in EC confirm that estrogen-metabolizing enzymes, such as 17β-HSD types 2 and 5, are present in the cytoplasm of tumor cells but not stromal cells (Ito et al. 2006). By contrast, aromatase is more highly expressed in stromal than tumor cells (Watanabe et al. 1995, Sasano et al. 1996), and its expression is correlated with poor survival (Segawa et al. 2005). To date, there has been no direct confirmation of stromal–tumor interactions mediated by estrogen signaling and the transactivation of ERE by endometrial stromal cells.

In this study, we demonstrated for the first time, the ability of stromal cells obtained from individual EC patients to stimulate the estrogen-signaling pathway in previously established ER-positive reporter cells (Yamaguchi et al. 2005). Furthermore, we evaluated the estrogen signaling sensitivity of primary tumor cells from individual patients using a unique system developed to visualize ER activity.

The ability of stromal cells to activate the ER varied among patients. In all cases except one, the expression of GFP was induced by the addition of testosterone, which was utilized as a substrate of aromatase (Fig. 1C). Aromatase is a key enzyme for estrogen
synthesis. The estrogen activity in tissues, however, is regulated not only by aromatase but also by various estrogen-metabolizing enzymes, as shown in Fig. 2C. From our data, the local estrogen level in cancer tissues seemed to be determined by the balance of all of these estrogen-metabolizing enzymes. However, the expression of GFP significantly decreased when AIs were added to the co-culture of stromal and E10 cells treated with testosterone (Fig. 3A). This indicates that the provision of estrogen from endometrial stromal cells is strongly dependent on aromatase activity.

Testosterone levels in the ovarian vein are higher in patients with endometrioid EC than those in healthy subjects (Nagamani et al. 1986), and the testosterone levels in tumor tissues are 10–40 times higher than those in serum (Ito 2007). Therefore, it is conceivable that conversion in the local environment may increase the local concentration of estrogen in the EC tissues. Interestingly, the expression level of GFP in patient 13 was high even without the addition of testosterone (Fig. 1C). In addition, the expression level of GFP was not decreased by the addition of AIs in this case (data not shown). We did not identify similar outliers in our previous study of breast cancer patients (Yamaguchi et al. 2005). In addition to estrogen, growth factors such as epidermal growth factor and IGF-I, which activate downstream kinases including MAPK and PI3K/Akt,
activate ER via its phosphorylation (Kato et al. 1995, Bunone et al. 1996, Ignar-Trowbridge et al. 1996, Lian et al. 2006). MAPK also stimulates the activity of ER via the phosphorylation of the ER-associating coactivators, amplified in breast cancer 1 (AIB1; Font de Mora & Brown 2000) and human steroid receptor coactivator 1 (SRC-1; Rowan et al. 2000). In the mouse endometrium, loss of phosphate and tensin homologue deleted on chromosome 10 (PTEN) activates the PI3K/Akt pathway and results in the activation of the ER (Dickson & Lippman 1995). In patient 13 (Fig. 1C), ER activation may also have depended on ligand-independent activation. The stimulating factors supplied from adjacent stromal cells may be important for this estrogen independence. Our system has the advantage of being able to evaluate overall estrogen signaling in primary cells of individual cases.

We developed a unique system that was able to assess the ER-activating ability of individual stromal cells using E10 cells. Here, we showed that all three AIs tested, anastrozole, letrozole, and exemestane, significantly suppressed the ER-activating ability of stromal cells in EC (Fig. 3A). Furthermore, the sensitivity to each aromatase inhibitor differed among individual EC patients (Fig. 3B). The individual variation of AI sensitivity may be attributed to individual differences in ligand-independent ER activation. Moreover, the differing sensitivity to each AI may be a reflection of individual differences in metabolizing enzymes such as cytochrome P450s (Grimm & Dyroff 1997). Thus, a system to predict the effectiveness of therapy is necessary for individual patients with estrogen-dependent cancers.

This study was the first to document ER activity in the EC cells from individual surgical specimens (Fig. 6A). In order to examine the state of ER activity in EC in vivo, a novel assay system using Ad-ERE-tk-GFP, which has an ERE-tk-GFP reporter gene in an adenovirus expression vector, was constructed and used to infect primary culture cells. Although most of the established, widely distributed, the EC cell lines showed extremely low ER activity in comparison with the ER-positive breast cancer cell line (Fig. 4), the cells isolated from individual ECs showed expression levels of GFP equivalent to those in breast cancers. Furthermore, as in breast cancer, the levels varied in individual patients (Fig. 6A). The levels seemed to be related to the expression levels of ER in the tumor cells (Fig. 6B). In most cases, except patient 35, tumor cells infected with Ad-ERE-tk-GFP after one night of precultivation showed reduced GFP expression compared with cells infected immediately after collagenase treatment. When estrogen or testosterone was added to the medium, the GFP expression was restored. The induction by testosterone was decreased by fulvestrant. These observations suggest that these EC cells are estrogen responsive, and probably depended on endogenous ER and local estrogen biosynthesis.

Interestingly, one case (patient 35) did not show sensitivity to estrogen, although the GFP expression was high. A similar phenomenon was also observed in patient 13, as shown in Fig. 1C. An explanation might be other factors that activate transcription through ERE in a ligand-independent fashion in EC. Therefore, our system may be useful for predicting the effectiveness of hormone therapy for estrogen-dependent cancers.

In this study, we demonstrated that a tumor–stromal interaction through local estrogen biosynthesis is active and important in endometrial cancer. Presently, there are limited options for endocrine therapy of EC. High-dose progesterin therapy using MPA is currently applied in advanced cancer. TAM, an anti-estrogen, which is widely used for breast cancer treatment, however, increases the risk of EC (Fisher et al. 1994, Grilli 2006). By contrast, AIs do not increase the risk of EC carcinogenesis in breast cancer patients, and have an excellent safety profile in postmenopausal women with breast cancer (Duffy & Greenwood 2003). In our study, AIs decreased estrogen production by stromal cells and suppressed ER activation in tumor cells (Fig. 3A). Although efficacy is variable based on a few case studies (Rose et al. 2000, Berstein et al. 2002, Burnett et al. 2004, Leunen et al. 2004), AIs do show promise as an alternative endocrine therapy for EC especially who has high sensitivity to AI. Our system suggests that AIs may be effective in a subset of carefully screened patients with estrogen-dependent EC.

**Acknowledgements**

This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas; a Grant-in-Aid for Scientific Research (B) and (C); a Grant-in-Aid for Young Scientists (B); Grant-in-Aid for Exploratory Research, from the Ministry of Education, Science, Sports and Culture, Japan; a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan; the 21st Century COE Program Special Research Grant (Tohoku University) from the Ministry of Education Science, Sports and Culture, Japan; a Grant-in-aid from the Kurokawa Cancer Research Foundation; and the Uehara Memorial Foundation. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.
References


Duffy S & Greenwood M 2003 The endometrial cancer data from the ATAC (arimidex, tamoxifen, alone or in combination) trial indicates a protective effect on anastrozole (arimidex) upon the endometrium. *Breast Cancer Research and Treatment* 82 (Supplement 1) S29.


Font de Mora J & Brown M 2000 AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Molecular and Cellular Biology* 20 5041–5047.


Leunen M, Breugelmans M, De Sutter Ph, Bourgain C & Amy JJ 2004 Low-grade endometrial stromal sarcoma treated with the aromatase inhibitor letrozole. *Gynecologic Oncology* 95 769–771.


