Biologicals Inc., Tulare, CA, USA) as the regular growth medium at 37 °C in a humidified atmosphere of 5 % CO_2 in air. Phenol red-free RPMI1640 (PRF-RPMI) (Gibco Brl, Grand Island, NY, USA) supplemented with 10 % dextran-coated charcoal-treated FCS was used as steroid-depleted medium for each experiment.

Screening of variant cell lines

A schema of the screening method for the variant cell lines is shown in Fig. S1B (Online Resource 1). E10 cells were cultured for 3 months in steroid-depleted medium with 100 nM TS. Five colonies showing luminescence by GFP expression were selected and seeded separately. Further screening was carried out by assessing ER activities under the following treatments. The ER activity of each clone was measured based on the proportion of GFP-positive cells in culture (ERE-GFP assay). ER activity in steroid-depleted TS-supplemented cultures was initially measured (a), and the measurement was repeated after 3 days of culture in steroid-depleted medium (b), to exclude GFP-positive clones with constitutively-activated ER. TS 100 nM and 3β-diol 100 nM were added continuously to the culture medium and ER activity was confirmed at 24 h (c), to select the clones with a high affinity for the androgen metabolite. Finally, clones that retained ER activity even after the addition of 100 nM letrozole (d), were established, and named V1 and V2 cell lines. Simultaneously, ER activities in these cell lines were confirmed to be inhibited by 1 μM fulvestrant (e). These variant cell lines were maintained in steroid-depleted medium supplemented with 100 nM TS.

Cell growth assay

For assays under defined steroid hormone conditions, cells were washed and grown in steroid-depleted medium for 3 days, then plated in 24-well culture plates at a density of 10,000 cells/well in steroid-depleted medium. The cells were incubated for 4 days in the absence or presence of the tested drugs and hormones. The cells in each well were washed and harvested, and then counted using a Sysmex CDA-500 automated cell counter (Sysmex Corporation, Kobe, Japan).

ERE-luciferase reporter assays

ERE activity in each cell line was measured using the Dual-luciferase reporter assay system (Promega, Madison, WI, USA). The estrogen reporter plasmid used, ERE-tk-Luci, has been described previously [19]. The vector pRL-TK (Promega) was used as an internal control of transfection efficiency in reporter assays. Transient transfection was performed as described previously [19]. After culturing the cells for a further 24 h in the absence or presence of the tested drugs and hormones, luciferase activity was measured using the Dual-luciferase reporter assay system, according to the manufacturer's instructions.

Real-time polymerase chain reaction in cell lines

Total RNA was extracted from each cell line cultured in the indicated growth medium using Isogen (Nippon Gene Co., Ltd., Toyama, Japan), according to the manufacturer's instructions. The extracted RNA (1 μg) was converted to first-stranded cDNA primed with a random 9-mers in a 10-μL reaction volume using a Takara RNA PCR kit (AMV) Ver. 3.0 (Takara Bio Inc. Otsu, Japan). A 2-μL aliquot was used as a template for real-time polymerase chain reaction (PCR).

Real-time PCR to detect expression of the indicated mRNA was carried out according to the manufacturer's standard protocol using an Applied Biosystems StepOne real-time PCR system (Life Technologies Japan, Tokyo, Japan). The expression of the target gene relative to RPL13A internal control was calculated. All PCRs were performed in duplicate, and the specificity of the reaction was determined by melting-curve analysis at the dissociation stage. Primer data and GenBank accession numbers of reference sequences are shown in Table S1 (Online Resource 2).

Establishment of HSD3B1 over-expressing E10 cells

Construction of the HSD3B1 expression vector and control vector was carried out as follows. The SV40 early promoter, blasticidin deaminase and SV40 poly(A) gene cassette were spliced out from the pMAM2-BSD vector (Kaken Chemicals Co., Tokyo, Japan) and inserted into the BamHI site of the pRL-CMV vector (Promega). The *Renilla* luciferase (Rluc) gene in the generated vector was then replaced with full-length HSD3B1 cDNA (HSD3B1 expression vector) or cut-off (control vector). The HSD3B1 expression or control vector was then transfected into E10 cells using TransIT LT-1 reagent (Mirus Co., Madison, WI, USA), following the manufacturer's protocol. Cells transfected with the HSD3B1 expression or control vector were selected using 10 mg/mL blasticidin. The expression levels of HSD3B1 were analyzed by real-time PCR (Online Resource 3, Fig. S2).

Cell growth and ERE-GFP reporter assays in coculture system

We were unable to assess the inhibitory effect of AIs in the E10 or variant cell lines because of their low aromatase expression. We therefore used cocultures, in which cancer and stromal cells were grown separately by a membrane, but interacted via soluble factors and shared the same microenvironment, including local estrogen synthesis from androgens by aromatase in stromal cells (Online Resource 4, Fig. S3).

All the cell lines were washed and grown in steroid-depleted medium for 3 days. Cancer cell lines were plated in the bottom well of 24-well culture plates at a density of 10,000 cells/well in steroid-depleted medium. Stromal cells were plated in the insert layer (0.4 μm pore cell-culture insert; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at a density of 30,000 cells/well in steroid-depleted medium supplemented with 1 μM dexamethasone to enhance aromatase gene expression [6, 34]. The cells were then incubated for 4 days in the absence or presence of the tested drugs and hormones before being subjected to cell-growth and ERE-GFP reporter assays. Cancer cells in each bottom well were washed and harvested for cell counting and ERE-GFP assay.

Statistical analyses

Analyses of cell growth, and ERE-luciferase and ERE-GFP reporter assays were performed in triplicate. Data are presented as mean ± standard deviation (mean ± SD) except for the GFP assay for the establishment of variant cell lines. The Kruskal–Wallis test was used to compare three or more independent groups. Dose–response curves for growth of each cell line induced by various steroids were compared using two way ANOVA test. The level of significance was set at $P < 0.05$.

Results

Selection of two variant cell lines showing androgen metabolite-dependent ER activity

After 3 months of culture in TS-supplemented steroid-depleted medium, five clones that retained ER activity were

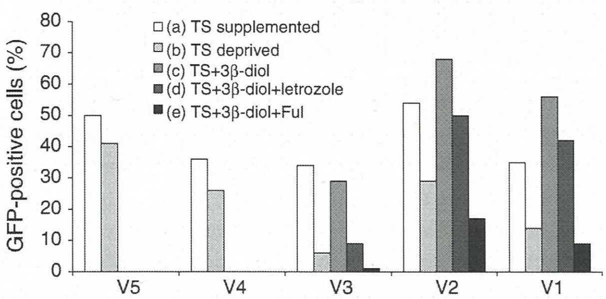

Fig. 1 Screening of variant cells. After 3 months of culture in TS-supplemented steroid-depleted medium, five clones expressing GFP were selected. ER activities were assessed in a single experiment as the proportion of GFP-positive cells at each step

selected. The ER activities of these clones at each step are summarized in Fig. S1B and Fig. 1. Clones V4 and V5 retained ER activity in steroid-depleted conditions and were therefore considered to have constitutively-activated ER, by the growth factor receptor pathway or some other mechanism. ER activity in the V3 cell line was induced by TS and 3β-diol and completely suppressed by letrozole, suggesting that this ER activity depended on estrogens supplied by aromatase from TS, rather than 3β-diol. TS-induced ER activities in the V1 and V2 cell lines were inhibited by steroid depletion, and ER activities induced by TS and 3β-diol were higher than that induced by TS alone, and were not sufficiently inhibited by letrozole. These results suggested that ER activity of V1 and V2 cell lines mainly depends on androgen metabolites which are generated by aromatase-independent pathway and that V1 and V2 cell lines show high dependence on the androgen metabolite. V1 and V2 were therefore defined as variant cell lines.

Androgens and 3β-diol showed estrogenic rather than androgenic function in variant cell lines

Dose–response curves for growth induced by TS, DHT, 3β-diol, and E2 are shown in Fig. 2a. Growth was stimulated in a dose-dependent manner by E2 in all cell lines, while all cell lines showed growth inhibition by TS and low-dose of DHT (1 and 10 nM). Growth inhibition by 100 nM TS and 10 nM DHT in variant cell lines was significantly weaker than in the parental E10 cell line. In contrast to the parental E10 cell line, growth in the variant cell lines was stimulated by 100 nM DHT. All cell lines were stimulated by 3β-diol in a dose-dependent manner, but low-dose 3β-diol (1 or 10 nM) stimulated growth in the variant cell lines significantly more than in the parental E10 cell line.

The results of the ERE-luciferase reporter assay are shown in Fig. 2b. DHT 100 nM induced ER activity in variant, but not parental E10 cell lines. In addition, 100 nM 3β-diol-induced ER activities were two-fold higher in variant cell lines compared with the parental E10 cell lines. ER activity induced by DHT or 3β-diol was inhibited by 1 μM fulvestrant, but not by 100 nM letrozole.

The induction of estrogen- and androgen-responsive genes by TS, DHT, and 3β-diol was analyzed using real-time PCR (Online Resource 5, Fig. S4). Variant cell lines showed increased induction of the estrogen-responsive genes for progesterone receptor, the transcription factor EGR3 [7] and Bcl2 by TS, DHT, and 3β-diol, compared with the parental E10 cell line. In contrast, the androgen-responsive gene KLK3 [12] was induced by TS, DHT, and 3β-diol in parental E10 cells, but not in variant cell lines.

These results indicate that the variant cell lines were adapted to androgen-abundant conditions and were hypersensitive to 3β-diol. It seems likely that this was the result

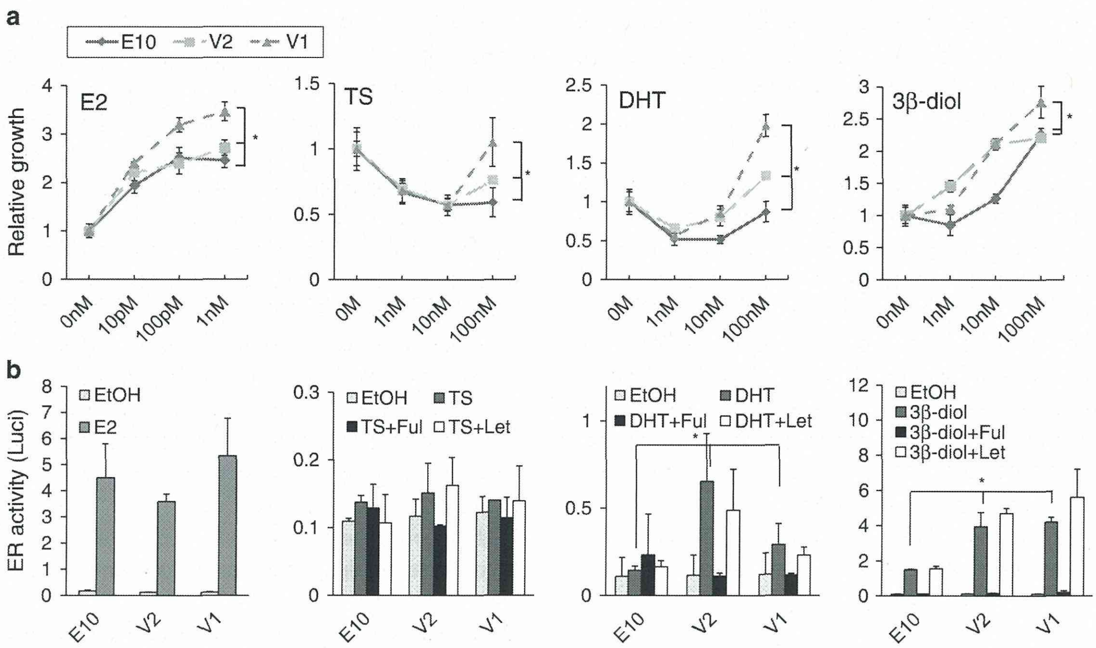

Fig. 2 Steroid-induced growth and ER activity of cell lines. **a** Steroid-induced growth of E10 and variant cell lines. Cells were plated in 24-well culture plates at a density of 10,000 cells/well with steroid-depleted medium, after 3 days of incubation in steroid-depleted medium. The indicated concentrations of E2, TS, DHT, 3β-diol or vehicle control (EtOH) were added to each well for 4 days. Cells from each well were then harvested and counted. Value relative to the vehicle control is shown. All data are shown as mean ± SD of three independent experiments (*$P < 0.05$). **b** ER activity of variant cell lines induced by androgen or 3β-diol. After 3 days of incubation in steroid-depleted medium, each cell line was plated on culture plates in the same medium and incubated for 48 h. Each cell line was then cotransfected with the ERE-luciferase reporter and the pRL-luciferase plasmid as a control. Luciferase activities were assayed after culturing for a further 24 h in the presence of 100 nM TS, DHT, 3β-diol, 10 nM E2, with or without 100 nM letrozole or 1 μM fulvestrant. All data shown are mean ± SD of three independent experiments (*$P < 0.05$)

of increased estrogenic, rather than androgenic functions of androgen and 3β-diol. These phenomena were also independent of aromatase activity.

Increased DHT metabolism and AR signal reduction might provide adaptability to androgen-abundant conditions

mRNA levels of androgen-producing and -metabolizing enzymes and hormone receptors in the variant and parental cells lines are shown in Fig. 3. Both variant lines (V1, V2) showed increased mRNA expression of HSD3B1 and reduced 5α-reductase type 1 and AR expression. AKR1C3 and ER were up-regulated in V2 cells. Aromatase (CYP19) and 17β-hydroxysteroid dehydrogenase type 2 were not detectable in any of the cell lines (data not shown). These results suggest that androgens may be metabolized more efficiently to 3β-diol (Online Resource 6, Fig. S5), which may exert estrogenic, rather than androgenic actions in variant cell lines, because of reduced androgen signal transduction.

In line with these hypotheses, HSD3B1-overexpressing E10 cells showed increased ER activity and growth stimulation by high-dose DHT (100 nM), in contrast to control E10 cells (Fig. 4a). Furthermore, the AR inhibitor bicalutamide reduced the inhibitory effect of DHT in E10 cells, and up-regulated their sensitivity to 3β-diol (Fig. 4b).

These results suggest that increased expression of HSD3B1 and decreased expression of AR may contribute to cell adaptation to androgen-abundant conditions, as demonstrated by the increased conversion of DHT to 3β-diol by HSD3B1 and AR signal reduction.

Variant cell lines were less sensitive to letrozole

We examined AI resistance in the variant cell lines using a coculture system to mimic local estrogen production from androgen in cancer tissues (Online Resource 4, Fig. S3). ER activities and cell growth were assessed under various conditions using this coculture system (Fig. 5). In coculture with stromal cells, 100 nM TS supplementation resulted in ER activation and growth stimulation in all cell lines, compared with untreated controls. ER activity in E10 cells induced by 100 nM TS was inhibited to untreated-control levels by 100 nM letrozole, while ER activity in variant cell lines was not completely inhibited. Cell growth of E10

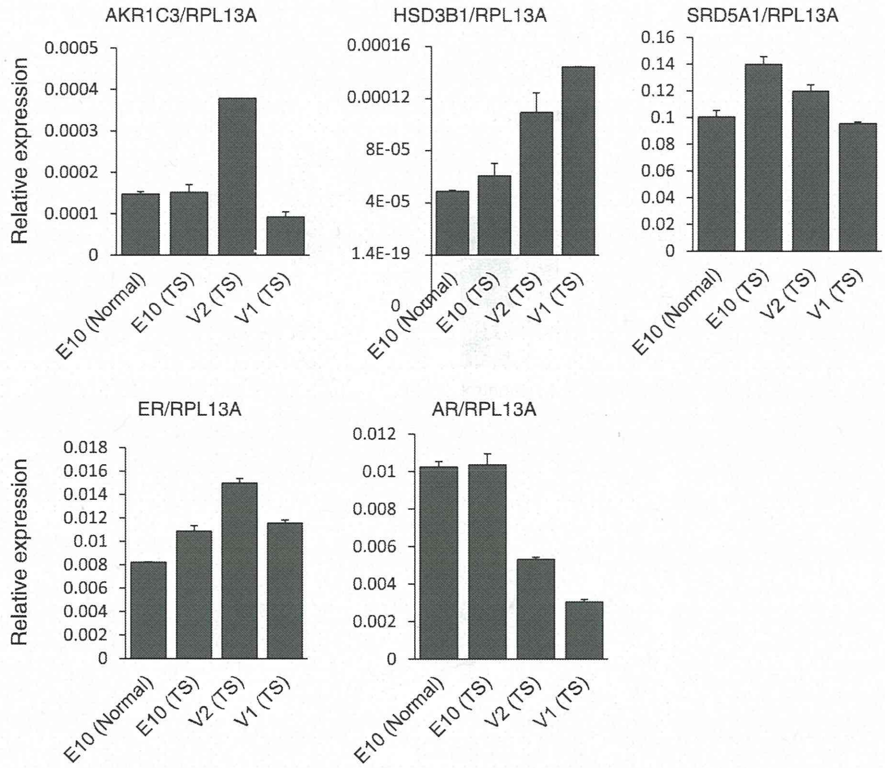

Fig. 3 mRNA expression of androgen-metabolizing enzymes and steroid receptors in E10 and variant cell lines. Total RNA was extracted from each cell line cultured in regular growth medium (Normal) or steroid-depleted medium supplemented with 100 nM TS for at least 3 days. All PCRs were performed in duplicate, and the expression of the target gene relative to RPL13A as an internal control is shown (mean ± SD)

cell lines induced by 100 nM TS supplementation was inhibited to less than that of untreated controls by 100 nM letrozole, while cell growth of the variant cell lines was unaffected by 100 nM TS- or 100 nM letrozole-supplementation. The variant cell lines were therefore considered to be less sensitive to letrozole than the parental lines.

Discussion

In the present study, we successfully cloned two stable variant cell lines with androgen metabolite-dependent ER activity. Investigation of the process of adaptation to androgen-abundant conditions in these cell lines suggested that increased expression of HSD3B1 and reduced expression of AR might reduce the sensitivity of cells to AIs, as demonstrated by enhancement of androgen metabolite-induced ER activation and cell growth. This is the first study that has established stable variant cell lines as AI-resistant models which have androgen metabolite-induced ER activation and cell growth mechanism.

Although this model system had some limitations in replicating the endocrinology of postmenopausal women, the androgen-abundant and estrogen-depleted culture conditions reflected AI treatment conditions, rather than simple estrogen-depleted conditions. Even without AIs, these conditions were similar to AI treatment conditions in MCF-7 cells because of their low aromatase expression [26, 27]. We also confirmed that aromatase mRNA expression (CYP19) was not detectable in our E10 cells (data not shown).

As noted above, we aimed to establish clones based on a single mechanism, namely ER activation by androgen metabolites. We therefore used the E10 cell line to assess the ER activity of living cells and to select suitable clones. We subsequently obtained ER-dependent clones by assessing the ER activity of individual cells, and performed further screening to select clones that showed ER activity in an aromatase-independent and TS-dependent manner, excluding clones with constitutive ER activity, or clones in which ER activity was dependent on estrogens supplied by aromatase from TS. However, the following two points should be noted. The V2 cell line showed relatively high ER activity in TS-depleted conditions, suggesting the possibility of ligand-independent ER activity. ER activities of V1 and V2 were marginally inhibited by letrozole suggesting that ER activity of these cell lines may partly depends on aromatase.

Analysis of cell growth and ER activity induced by androgen or 3β-diol showed that variant cell lines were adapted for androgen-abundant conditions. Quantitative analysis of mRNA expression suggested that androgen or 3β-diol exerted estrogenic behavior independently of aromatase, as demonstrated by the increased conversion of DHT into 3β-diol and the reduced androgen signal in variant cell lines. These hypotheses support the idea that

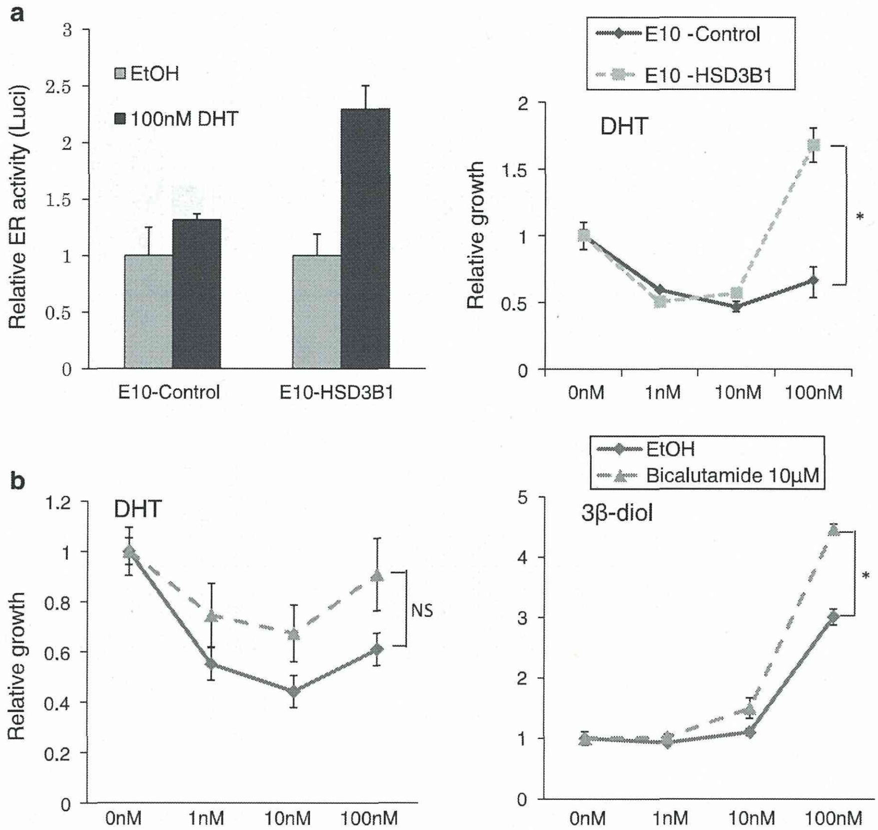

Fig. 4 Effects of HSD3B1 over-expression and androgen receptor inhibitor in E10 cells. **a** Steroid-induced growth of E10 cells transfected with HSD3B1 expression vector. Cells that were transfected with HSD3B1 expression vector (E10-HSD3B1) or control vector (E10-Control) were generated as described in Materials and methods. After 3 days of incubation in steroid-depleted medium, each cell line was plated on culture plates in the same medium and incubated for 48 h. Each cell line was then cotransfected with the ERE-luciferase reporter and the pRL-luciferase plasmid as a control. Luciferase activities were assayed after culturing for a further 24 h in the presence of 100 nM DHT or vehicle control (EtOH). The value relative to the vehicle control is shown. All data are shown as mean ± SD of three independent experiments (*left graph*). After 3 days of culture in steroid-depleted medium, these cells were plated in 24-well culture plates at a density of 10,000 cells/well with steroid-depleted medium. The indicated concentrations of DHT or the vehicle control (EtOH) were added to each well for 4 days. Cells from each well were then harvested and counted. Values relative to the vehicle control are shown (*right graph*). All data shown are mean ± SD of three independent experiments. **b** Effect of androgen receptor inhibitor bicalutamide in parental E10 cell line. After 3 days of culture in steroid-depleted medium, E10 cells were plated in 24-well culture plates at a density of 10,000 cells/well in steroid-depleted medium. The indicated concentrations of DHT, 3β-diol or vehicle control (EtOH), with or without bicalutamide (10 μM), were added to each well for 4 days. Cells from each well were then harvested and counted. Values relative to the vehicle control are shown. All data shown are mean ± SD of three independent experiments

overexpression of HSD3B1 and inhibition of AR in E10 cells resulted in adaptation to estrogen-deprived and androgen-abundant conditions. A previous report showed that AR and ERα can interact directly and inhibit each other's transcriptional activity [21, 22]. Reduced AR expression may thus be one of the factors responsible for interrupting androgen signal transduction in variant cell lines.

Aromatase is highly expressed in the adipose stromal cells adjacent to the tumor in breast tumors [18, 24]. We previously reported that ERs were activated by coculture with adipose stromal cells isolated from breast tumor tissues in the presence of TS, as a substrate for aromatase [34]. The addition of TS alone had no effect on ER activity and an inhibitory effect on E10 cell growth, but TS induced ER activity and growth of E10 cells in a dose-dependent manner when cocultured with stromal cells, which effects were inhibited by 100 nM letrozole (data not shown). We used this coculture system to assess the inhibitory effect of AIs. AIs inhibit breast cancer cells by at least two separate mechanisms (Fig. 6a). They eliminate the growth-stimulating effect of estrogens by blocking estrogen production. ER activity induced by 100 nM TS in variant cell lines was not completely inhibited in this coculture system, suggesting that they might be the result of ER activation by androgen metabolites, including 3β-diol, produced in an aromatase-

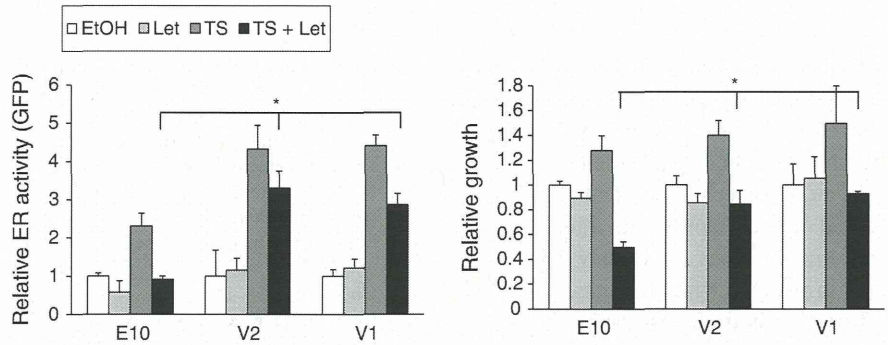

Fig. 5 Cell growth and ERE-GFP reporter assays in coculture with primary stromal cells. After 3 days of incubation in steroid-depleted medium, cancer cell lines and stromal cells were plated in the bottom of wells of a 24-well culture plate with an insert layer as described in Materials and methods. The cells were then incubated for 4 days with or without 100 nM TS and 100 nM letrozole before cell growth and ERE-GFP reporter assays. Cancer cells at the bottom of each well were washed, harvested, and counted. ER activity of cancer cells in coculture was also assessed by counting the proportion of GFP-positive cells in the well bottom. The values relative to that of the vehicle control are shown. All data shown are mean ± SD of three independent experiments (*$P < 0.05$). *Let* letrozole

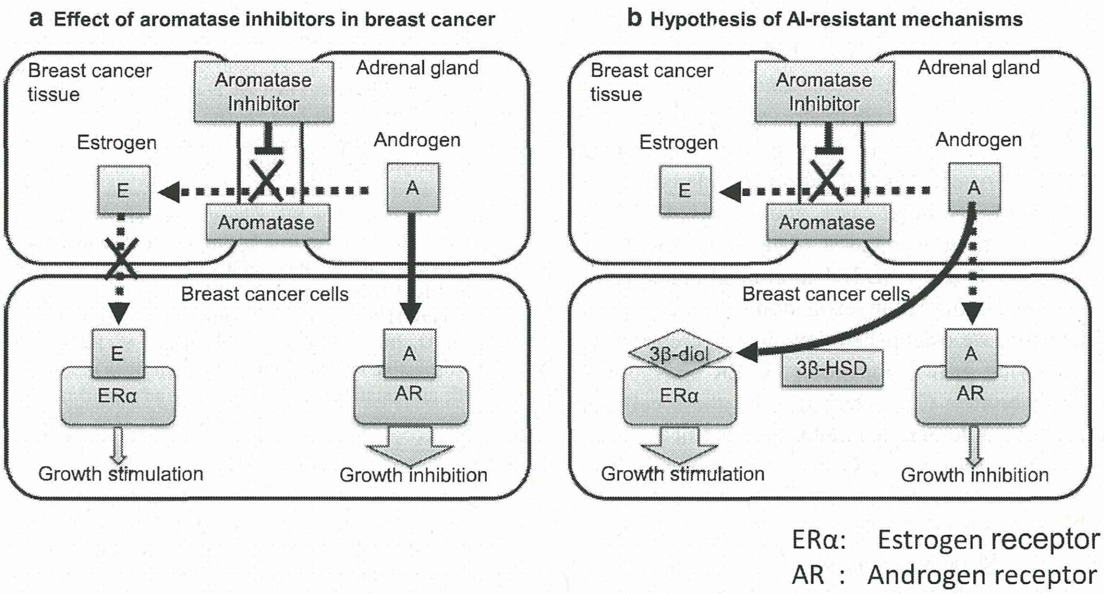

Fig. 6 Effect of aromatase inhibitors in breast cancer and hypothesis of AI-resistant mechanisms. **a** AIs inhibit breast cancer cells by at least two separate mechanisms. They eliminate the growth-stimulating effect of estrogens by blocking estrogen production. The growth-inhibitory effect of androgens represents another possible mechanism. **b** This figure shows the hypothesis of AI-resistance mechanisms that we propose from the above results. AR signal reduction and ER activation by androgen metabolites produced in an aromatase-independent manner may function as an AI-resistance mechanism

independent manner. The growth-inhibitory effect of androgens represents another possible mechanism. Androgens have been reported to have inhibitory effects in hormone-dependent breast cancer cells [1, 11, 20]. Luciana et al. [14] found that cell growth was suppressed by low androgen levels in cells not supplemented by androgens and in conditions under which androgen cannot be converted to estrogen. Letrozole therefore exerted an extra effect beyond that produced by androgen supplementation in parental E10 cells. In contrast, established variant cell lines showed letrozole-resistant ER activity and maintained their growth under TS- and letrozole-supplemented conditions in coculture with stromal cells. These results suggest that these cell lines were less sensitive to letrozole, because they showed both AR signal reduction and ER activation by androgen metabolites produced in an aromatase-independent manner. Fig. 6b shows the hypothesis of AI-resistance mechanisms that we propose from the above results.

It has been suggested that androgen metabolite-dependent ER activation and cell growth may play some roles in the mechanism of AI resistance. It is therefore necessary to investigate treatment strategies that will be effective against androgen metabolite-dependent ER activation and cell growth. 3β-Diol-induced growth in variant cell lines was inhibited by OHT, toremifene or fulvestrant, but not by AIs, suggesting a promising effect of SERMs on androgen metabolite-dependent growth mechanism (Online resource 7; Fig. S6). Sequential use of tamoxifen, toremifene or fulvestrant after first-line AIs has previously been suggested to be effective in patients with aromatase inhibitor-refractory advanced or metastatic breast cancer [2, 9, 32, 35]. These findings might partly account for the efficacy of sequential use of SERMs or fulvestrant after first-line AIs.

Our in vitro data suggest that androgen metabolite-induced ER activation and androgen signal reduction may play important roles in the mechanism behind AI resistance. Further investigation can contribute to the development of new therapeutic strategies and to the search for new therapeutic targets and agents against the AI resistant breast cancer. Therefore, we have initiated a clinical study to determine if androgen metabolite-dependent growth mechanisms are involved in AI-resistance in clinical breast cancer cases. Immunohistochemistry and real-time PCR analyses of nine pairs of primary and recurrent tissue samples from AI-resistant breast cancer revealed decreased AR protein expression in all cases, and increased HSD3B1 mRNA expression in five cases (data not shown). The significances of AR and HSD3B1 in clinical breast cancer are currently under further investigation.

In conclusion, we established new human breast cancer cell lines showing growth induction via ER activation by androgen metabolites. Characterization of these cell lines suggests that the androgen metabolite-dependent growth of hormone receptor-positive breast cancer may play a role in the mechanism of AI resistance. These cell lines are useful for further studying the mechanisms behind AI resistance in vitro, and could be valuable for developing diagnostic markers or novel therapeutic strategies for AI-resistant breast cancer.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard All experiments complied with the current laws of Japan.

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Estrogen Response element-GFP (ERE-GFP) introduced MCF-7 cells demonstrated the coexistence of multiple estrogen-deprivation resistant mechanisms

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ABSTRACT

The acquisition of estrogen-deprivation resistance and estrogen receptor (ER) signal-independence in ER-positive breast cancer is one of the crucial steps in advancing the aggressiveness of breast cancer; however, this has not yet been elucidated in detail. To address this issue, we established several estrogen-deprivation-resistant (EDR) breast cancer cell lines from our unique MCF-7 cells, which had been stably transfected with an ERE-GFP reporter plasmid. Three cell lines with high ER activity and another 3 cell lines with no ER activity were established from cell cloning by monitoring GFP expression in living cells. The former three ERE-GFP-positive EDR cell lines showed the overexpression of ER and high expression of several ER-target genes. Further analysis of intracellular signaling factors revealed a marked change in the phosphorylation status of ERα on Ser167 and Akt on Thr308 by similar mechanisms reported previously; however, we could not find any changes in MAP-kinase factors. Comprehensive phosphoproteomic analysis also indicated the possible contribution of the Akt pathway to the phosphorylation of ERα.

On the other hand, constitutive activation of c-Jun N-terminal kinase (JNK) was observed in ERE-GFP-negative EDR cells, and the growth of these cells was inhibited by a JNK inhibitor. An IGF1R-specific inhibitor diminished the phosphorylation of JNK, which suggested that a novel signaling pathway, IGF1R-JNK, may be important for the proliferation of ER-independent MCF-7 cells. These results indicate that ER-positive breast cancer cells can acquire resistance by more than two mechanisms at a time, which suggests that multiple mechanisms may occur simultaneously. This finding also implies that breast cancers with different resistance mechanisms can concomitantly occur and mingle in an individual patient, and may be a cause of the recurrence of cancer.

1. Introduction

Estrogen plays a pivotal role in the development and progression of breast cancers. It exerts its effects by binding to estrogen receptor α (ERα). This complex then interacts with estrogen response element (ERE) and regulates the transcription of target genes controlling proliferation and cell survival. Therefore, blockade of the estrogen signal is an important strategy for ERα-positive breast cancers. Aromatase inhibitors (AI) that inhibit the biosynthesis of estrogen and antiestrogens that compete for binding to ER have improved the prognosis of patients. Approximately 70% of breast cancer cases have ERα-expressing tumors; therefore, they can be candidates for hormonal therapy targeting ERα. Since hormonal therapy is very effective for ER-positive breast cancer patients without severe adverse events, it is widely used not only in advanced cancer, but also in an adjuvant setting [1]. However, more than a few patients relapse. Therefore, numerous studies on hormonal therapy resistance have been undertaken, especially on tamoxifen

Abbreviations: EDR, estrogen-deprivation-resistant; LTED, long-term estrogen-depleted.

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resistance [2]. Aromatase inhibitors have recently replaced antiestrogens as a more appropriate hormonal therapy for advanced breast cancer and also in an adjuvant setting [3]. Although estrogen ablation is another effective strategy for ER-positive breast cancer, the acquisition of estrogen-deprivation resistance is one of the crucial steps in the progression to hormonal therapy resistance and more aggressive tumors in ERα-expressing breast cancer. The mechanisms for the estrogen-deprivation resistance of ERα-expressing breast cancer cells have also been explored by several laboratories [4–6] using whole cells cultured for a long-term period in estrogen-deprivation medium. These reports suggested that resistant cells acquired estrogen hypersensitivity by crosstalk with the MAP-kinase or PI3K-Akt pathway and the involvement of membrane-associated ER [7]. However, the precise mechanisms are not fully understood, and several questions remain unanswered in terms of whether any other mechanisms are associated with this resistance. To address these issues, we here established several MCF-7 cell sub-lines by isolating single colonies under long-term estrogen-deprivation conditions. MCF-7 cells stably transfected with the ERE-GFP reporter gene were used as parental cells for this cell cloning. These cells expressed GFP on ER activation, and ER activity was assessed in living cells by fluorescence [8,9]. Using this system, we eventually established the two types of clones as long-term estrogen depletion resistant (EDR) cell lines and characterized them. Our study indicated that more than two clearly distinct mechanisms exist in estrogen-deprivation resistance.

2. Materials and methods

2.1. Reagents

Estradiol (E2) and 4-hydroxytamoxifen were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fulvestrant (Ful) and toremifene (TOR) were kindly provided by AstraZeneca Pharmaceuticals (London, UK) and Nippon Kayaku Co. Ltd. (Tokyo, Japan), respectively. The sources of antibodies for Western blotting were as follows: total ERα (H-184) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), HER2 from DakoCytomation (Glostrup, Denmark), EGFR, phospho-ERα (Ser167), phospho-ERα (Ser118), total and phospho-p44/42 MAPK (Erk1/2), total and phospho-Akt (Thr308), total and phospho-JNK, β-tubulin, and the PI3K inhibitor, LY294002 from Cell Signaling Technology Inc. (Danvers, MA, USA). Secondary antibodies conjugated with alkaline phosphatase were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). The JNK inhibitor, SP600125, IGF-1R inhibitor, AG1024, and EGFR inhibitor, AG1478, were purchased from Cayman Chemical Company (Ann Arbor, MI, USA) or EMD Biosciences, Inc. (La Jolla, CA, USA).

2.2. Cells and cell culture

MCF-7-E10 cells were established from human breast cancer MCF-7 cells, into which the ERE-GFP reporter gene had been stably introduced. These cells expressed GFP in the presence of estrogen under fluorescence. MCF-7-E10 cells were routinely cultured in RPMI1640 medium (Sigma–Aldrich) containing 10% fetal calf serum (FCS; Tissue Culture Biologicals, Turale, CA, USA) and 1% penicillin/streptomycin (Sigma–Aldrich).

MCF-7-E10 cells were cultured in estrogen-deprived medium for 3 months. Among the surviving cells, ERE-GFP-expressing colonies and ERE-GFP-negative colonies were picked up separately and established as long-term estrogen-deprivation-resistant (EDR) cell lines. EDR cells were maintained in phenol red-free RPMI1640 medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% dextran-coated charcoal-treated FCS (DCC-FCS) and 1% penicillin/streptomycin. All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The characteristics of these cells did not change with the number of passages and cells that had gone through as low a number of passages as possible were used in the experiments of this study.

2.3. ERE-GFP assay

We assessed ERE activation by estimating GFP expression levels as reported previously [8–10]. Briefly, the number of cells expressing GFP was counted under fluorescence microscopy after the cells had been harvested by treatment with trypsin. Cells expressing strong levels of GFP were counted. ERE activity were expressed as the percentage of cells expressing GFP.

2.4. Cell growth assay

MCF-7-E10 cells (which had previously been stripped of steroids for 3 days by DCC-FCS-containing medium) and EDR cells were seeded at a density of 5×10^3 cells per well in a 24-well culture plate treated with the indicated concentrations of E2 for 4 days. Cells were counted using a CDA-500 Sysmex automated cell counter (Sysmex Corp., Kobe, Japan). Data are indicated as values relative to the cell numbers of the vehicle-treated control.

2.5. Real-time PCR

Total RNA was extracted from whole cells using Isogen (Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's instructions. Extracted RNA (1 μg) was converted to first-strand cDNA primed with a random hexamer in a 10 μl reaction volume using an RNA PCR kit (Takara Bio Inc., Otsu, Japan) and a 2 μl aliquot was used as a template for real-time PCR. All RNA quantification was carried out according to the standard protocol on an Applied Biosystems Step One real-time PCR system (Applied Biosystems Inc., Foster City, CA, USA). Target gene expression was normalized to *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*. All PCRs were performed at least twice and the results shown were from samples analyzed in triplicate in one experiment. These results confirmed the reproducibility of the data obtained. The sequences of the primer sets were as follows: *EGR3*-forward, 5′-GAG CAG TTT GCT AAA CCA AC-3′; reverse, 5′-AGA CCG ATG TCC ATT ACA TT-3′; *pS2*-forward, 5′-TCC CCT GGT GCT TCT ATC CTA A-3′; reverse, 5′-ACT AAT CAC CGT GCT GGG GA-3′; *PgR*-forward, 5′-AGC TCA CAG CGT TTC TAT CA-3′; reverse, 5′-CGG GAC TGG ATA AAT GTA TTC-3′; *Bcl-2*-forward, 5′-GTG GAT GAC TGA GTA CCT GAA C-3′; reverse, 5′-GCC AGG AGA AAT CAA ACA-3′; *CyclinD1*-forward, 5′-GGA GCC CGT GAA AAA GAG-3′; reverse, 5′-CAG GTT CCA CTT GAG CTT GT-3′; *GAPDH*-forward, 5′–ACA TCG CTC AGA CAC CAT GG-3′; reverse, 5′–GTA GTT GAG GTC AAT GAA GGG-3′.

2.6. Western blot analysis

Cell lysates were prepared using Lysis-M Reagent (Roche Diagnostics GmbH, Mannheim, Germany) supplemented with phosphatase inhibitor cocktails, Phos STOP (Roche Diagnostics), according to the manufacturer's instructions. Total proteins were run on SDS-PAGE using 10% acrylamide gels (SuperSep™ ace; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and proteins were transferred to PVDF, Amersham Hybond™-P (GE Healthcare UK, Ltd., UK). The expression of proteins was determined by Western blotting with specific antibodies listed in *Reagents*, and expression signals using Immun-star AP substrate (Bio-Rad) were obtained by enhanced chemiluminescence. Densitometry was performed on three blots (two blots only in Fig. 4A) and these results