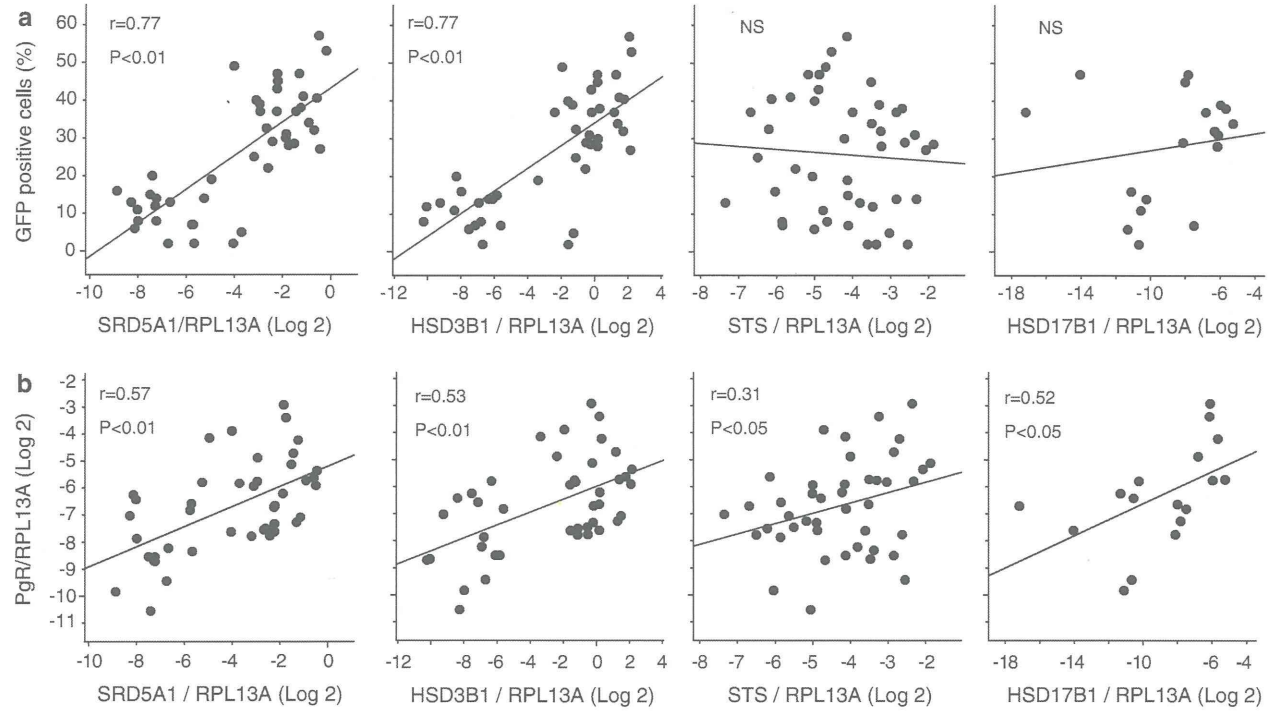

Fig. 2 Relationships between the expression of non-aromatase steroid-metabolizing enzymes and ER activity in breast cancer tissue. **a** and **b** Expression levels of SRD5A1, HSD3B1, STS, and HSD17B1 mRNA were assessed using real-time PCR. The relationships between these steroid-metabolizing enzymes, and ER activities ex vivo (**a**) and in vivo (**b**) determined by ERE-GFP assay [GFP-positive cells (%)], and PgR mRNA expression levels [PgR/PRL13A(log2)], respectively, were analyzed by Pearson's correlation. Pearson's correlation coefficients (r), and p values are indicated

respectively. Of note, one case with undetectable HSD3B1 expression was classified in the low-expression group. HSD17B1 mRNA expression levels were divided into a negative-expression group (undetectable) and a positive-expression group (detectable). The ER activities in the low- and high-expression groups were also compared according to their menopausal status (pre or post).

High HSD3B1 and SRD5A1 expression levels were associated with greater ex vivo ER activity compared with the low-expression groups, regardless of menopausal status (Fig. 3a, b). However, for STS and HSD17B1, there were no significant differences in ex vivo ER activity between the high- and low-expression groups (Fig. 3c, d).

There were no significant differences in in vivo ER activity between the low- and high-expression groups with respect to any of the steroid-metabolizing enzymes in premenopausal cases. In contrast, high-expression levels tended to be associated with higher in vivo ER activity with respect to all the enzymes studied in postmenopausal cases, though the differences were not significant for STS and HSD17B1 (Fig. 4a, d).

Although the results of ex vivo and in vivo assays were inconsistent, they still suggest that non-aromatase steroid-metabolizing enzymes are involved in the activation of the ER in vivo, especially in the postmenopausal state.

Steroid-metabolizing enzymes could function in pathways

We analyzed the relationships among the expression levels of non-aromatase steroid-metabolizing enzymes using Pearson's correlation (Fig. 5). Expression levels of SRD5A1 and HSD3B1, and of STS and HSD17B1, were positively correlated. Conversely, no correlations were observed for other combinations. These results suggest that these non-aromatase steroid-metabolizing enzymes could function in pathways producing estrogenic steroids in an aromatase-independent manner (Fig. 6).

Discussion

The results of this study suggest that steroid-metabolizing enzymes, such as 5α-reductase type 1, 3β-HSD type 1, STS, and 17β-HSD type 1, could contribute to ER activation, especially in the postmenopausal state. Furthermore, the tendencies of SRD5A1 and HSD3B1, and STS and HSD17B1 to be co-expressed with each other suggest that these enzymes might function together in pathways responsible for producing estrogenic steroids in an aromatase-independent manner.

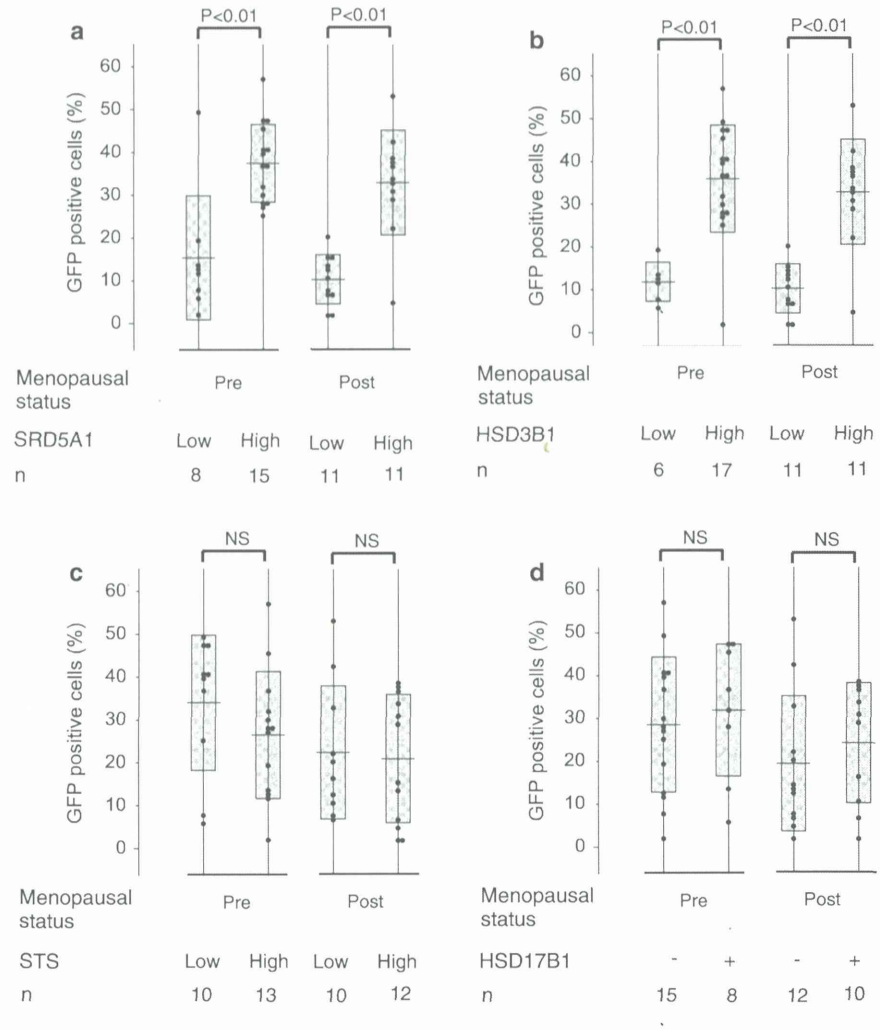

Fig. 3 Relationships between non-aromatase steroid-metabolizing enzymes and ex vivo ER activity before and after menopause. (**a**–**d**) mRNA expression levels of the non-aromatase steroid-metabolizing enzymes, SRD5A1 (**a**), HDS3B1 (**b**), STS (**c**) and HSD17B1 (**d**), were analyzed by real-time PCR in 45 breast cancer cases from either pre (Pre)- or post (Post)-menopausal patients. Samples were further divided into two groups of below (Low)- and above (High)-average expression levels of the steroid-metabolizing enzymes SDR5A1 (**a**), HSD3B1 (**b**) and STS (**c**). For HSD17B1 (**d**), mRNA expression levels were divided into two groups with undetectable (−) and detectable (+) expression levels. Ex vivo ER activities, measured by ERE-GFP assay [GFP-positive cells (%)], in low/− and high/+ expression groups were compared. Unpaired two-tailed *t* tests were performed to assess differences between two groups. *p* Values and sample numbers (*n*) are indicated; not significant (NS)

Despite a prominent reduction in serum E2 concentrations associated with the menopause, concentrations of estrogens in breast tissues of pre and postmenopausal women are known to be comparable [37]. This reflects the local biosynthesis of estrogens, mainly by aromatase [37], and is consistent with our observation that in vivo ER activity in breast cancer tissue was not solely dependent on the concentrations of circulating E2. The ER-activating abilities of non-estradiol steroids have been reported [17, 18], and these steroids and E2 were shown to be generated by multiple aromatase-independent pathways [1, 14, 15, 19, 21, 24]. We therefore focused on the ER activity and aromatase-independent metabolic pathways that produce estrogenic steroids, including E2.

In the adenovirus ERE-GFP assay system, GFP expression is fully dependent on ER activity because of the consensus ERE used in the reporter sequence [29], suggesting that GFP expression reflects highly specific ER activity; however, this does not represent normal physiological conditions. In contrast, the expression levels of PgR, which is a well-known ER target gene [32], are considered to reflect ER activity in a more physiological environment; however, the PgR gene has other regulatory regions, in addition to the half ERE in its promoter region [32]. Because these two methods have different advantages and disadvantages, we used both methods to assess ER activity. In contrast with the in vivo results, expression levels of STS and HSD17B1 were not correlated with ex vivo ER activity. This discrepancy between the ex vivo and in vivo assays can be explained as follows. Steroid sulfate conjugate might not be contained in the ex vivo assay system. Because steroid-depleted medium was used in these assays, the steroid included in the ex vivo system was considered to be derived from the cancer cells or stromal cells. Steroid sulfate conjugates, which are metabolized by the STS-HSD17B1 pathway, might rarely be included in these cells. There were no significant differences in in vivo ER activity between the low- and high-expression groups in premenopausal cases, in respect of any of the steroid-metabolizing enzymes. ER activity in

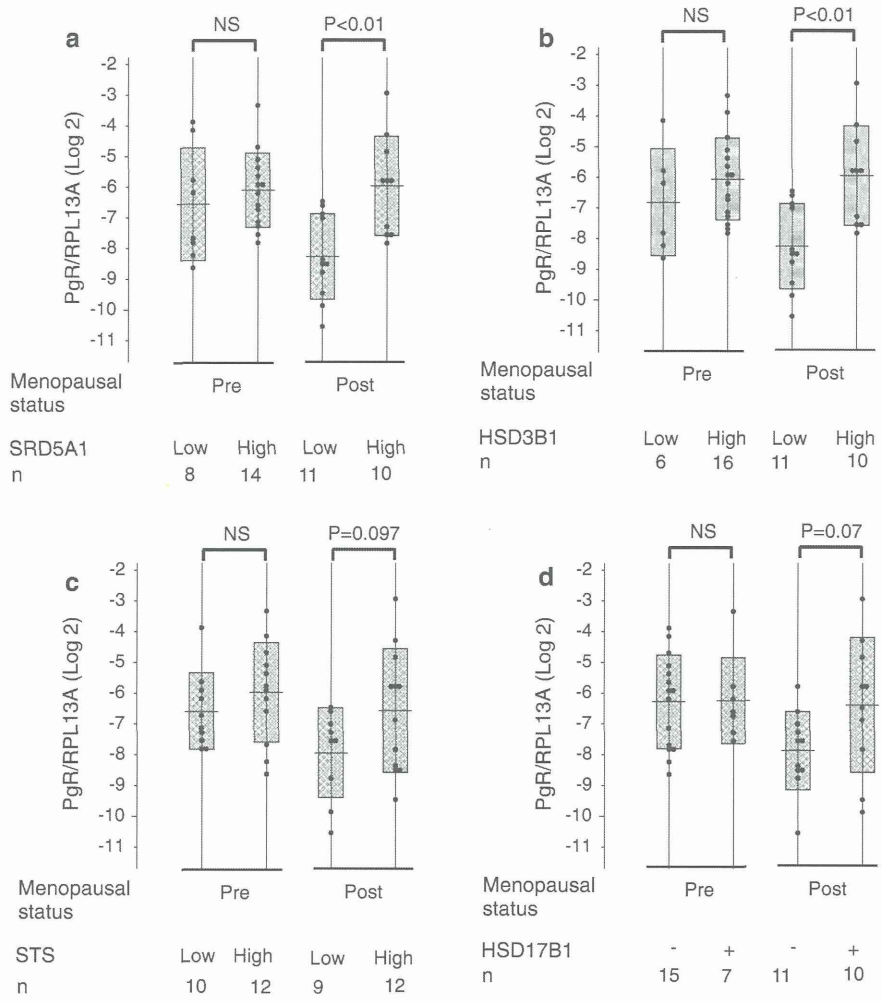

Fig. 4 Relationships between non-aromatase steroid-metabolizing enzymes and in vivo ER activity before and after menopause. (**a**–**d**) mRNA expression levels of the non-aromatase steroid-metabolizing enzymes, SRD5A1 (**a**), HDS3B1 (**b**), STS (**c**), and HSD17B1 (**d**), were analyzed by real-time PCR in 45 breast cancer cases from either pre (Pre)- or post (Post)-menopausal patients. Samples were further divided into two groups of below (Low) and above (High) average expression levels for the steroid-metabolizing enzymes SRD5A1 (**a**), HSD3B1 (**b**), and STS (**c**). For HSD17B1 (**d**), mRNA expression levels were divided into two groups with undetectable (−) and detectable (+) expression levels. In vivo ER activities determined by PgR mRNA expression, in low/− and high/+ expression groups were compared. Unpaired two-tailed *t* tests were performed to assess differences between two groups. p Values and sample sizes (*n*) are indicated; not significant (NS)

premenopausal patients was considered to be independent of steroid-metabolizing enzymes in cancer tissues because of their high circulating E2 concentrations. In contrast, patients with high HSD3B1 or SRD5A1 expression levels showed higher ex vivo ER activity compared with the low-expression groups, regardless of menopausal status. SRD5A1-HSD3B1-pathway-dependent ER activity might have been unmasked in the ex vivo system compared with the in vivo system because of the lower levels of E2. Nevertheless, we consider that the results of the ex vivo assay are supplementary of those of the in vivo assay.

DHT is synthesized from TS in an irreversible reaction catalyzed by 5α-reductase, and is a highly potent androgen with inhibitory effects in hormone-responsive breast cancer cells [38–40]. Thus, high expression of 5α-reductase type 1 alone is considered to have an adverse effect on the survival of breast cancer cells. However, DHT can be further metabolized by 3β-HSDs to 3β-diol, which has substantial estrogenic activity [17, 18, 24, 25]. Although evidence for the function of HSD3B1 in human breast cancer is limited, we recently found that ectopic expression of HSD3B1 induced DHT-dependent ER activation and cell proliferation in E10 cell lines derived from MCF-7 breast cancer cells [28]. Together, these data suggest that breast cancer cell survival might rely on 3β-HSD type 1 as a means of reducing the inhibitory effect of DHT. In support of this hypothesis, we demonstrated co-expression of SRD5A1 and HSD3B1, which was also in agreement with the results of a previous report [23].

In the present study, we demonstrated that ER activity in clinical breast cancers was positively correlated with the expression levels of steroid-metabolizing enzymes involved in the production of 3β-diol. This finding is consistent with other studies that demonstrated the estrogenic activity of 3β-diol in in vitro models [14]. Intratumoral DHT concentrations were also shown to be significantly higher in breast cancer tissues following AI treatment [22], suggesting that these alternative metabolic pathways which produce estrogenic androgen from androgens can potentially function as escape routes from

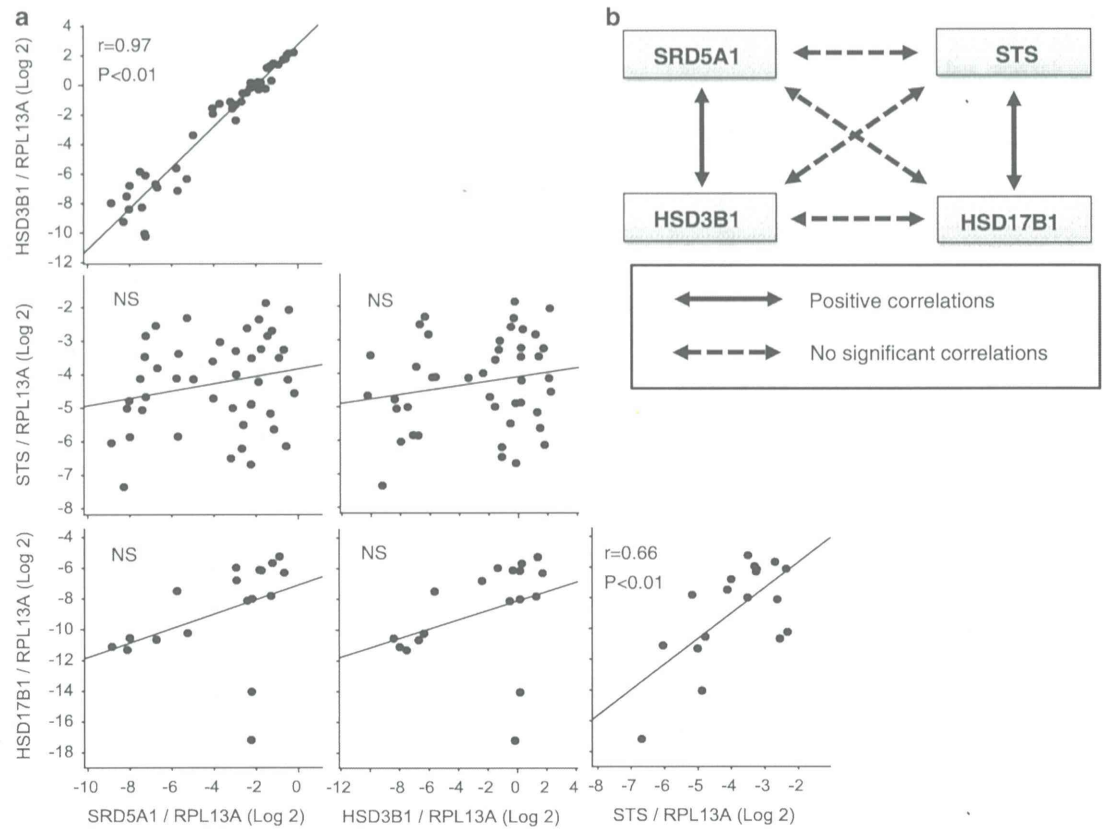

Fig. 5 Relationships between expression levels of non-aromatase steroid-metabolizing enzymes. **a** Scatterplots of relationships between mRNA expression levels of non-aromatase steroid-metabolizing enzymes, SRD5A1, HSD3B1, STS, and HSD17B1. The mRNA expression level of each enzyme is presented as a ratio of the control gene RPL13A (log2). Relationships between the different non-aromatase steroid-metabolizing enzymes were examined using Pearson's correlation. Pearson's correlation coefficients (r) and p values are indicated; not significant (NS). **b** Schematic representation of the relationships between expression levels of non-aromatase steroid-metabolizing enzymes. *Solid arrows* represent positive correlations; *dashed arrows* represent non-significant correlations

AI treatment. It is therefore interesting to speculate that 3β-HSD type 1 could represent a novel therapeutic target. Trilostane has been reported to act as a specific inhibitor of 3β-HSD type 1 [41]. A previous cross-over study of aminoglutethimide and trilostane in advanced postmenopausal breast cancer [42] found no differences in response rates of either drug, or in the average time of disease progression. Our results suggest that inhibition of 3β-HSD type 1 might contribute to reduced 3β-diol-mediated ER activation in breast cancer tissue, suggesting that the clinical efficacy of trilostane should be reconsidered.

Steroid sulfates such as E1S and DHEAS are metabolized to E1 and DHEA, respectively, by STS. E1 and DHEA are further metabolized to E2 and A-diol, respectively [2, 21, 27]. A-diol has been shown to have potent estrogenic activity and to stimulate the proliferation of breast cancer cells in vitro [17–20]. Although MCF-7 breast cancer cells are insensitive to sulfated estrogen because of their low endogenous STS level [43], overexpression of STS in MCF-7 cells resulted in increased cell proliferation in response to E1S or DHEAS [44], suggesting that the STS pathway could function as an estrogenic steroid-producing pathway in vitro.

Based on their high concentrations [45] and long half-lives [46] in blood, steroid sulfate conjugates such as DHEAS and E1S are thought to act as a central reservoir for the formation of biologically active estrogens, although they themselves are biologically inactive [21]. However, contrary to the results in vivo, expression levels of STS, or HSD17B1 were not correlated with ex vivo ER activity, suggesting that the continuous uptake of steroid sulfates from the reservoir (i.e., blood vessels) might be important for the production of estrogenic steroids by 17β-HSD type 1 and STS. The STS pathway has been noted as a therapeutic target, and its clinical application is already underway [47, 48]. However, there is currently insufficient evidence to support a role for the STS pathway in the regulation of ER activity through the production of estrogenic steroids in clinical breast cancer. Our results indicating that expression levels of STS and HSD17B1 tended

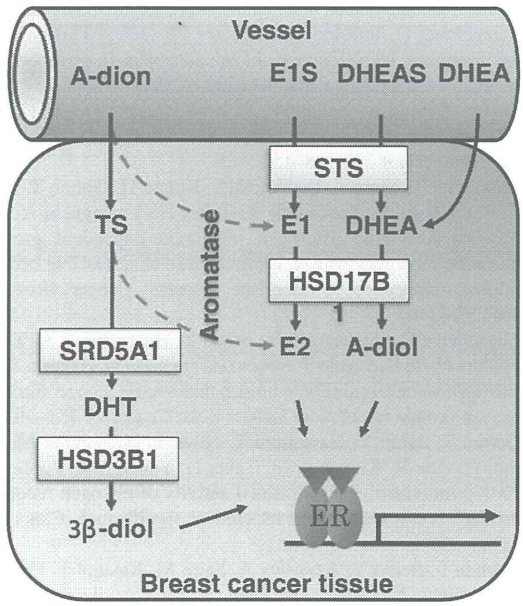

Fig. 6 Hypothesized mechanisms of local steroid metabolism to produce estrogenic steroids in an aromatase-independent manner in breast cancer. 5α-reductase type 1 (SRD5A1) and 3β-HSD type 1 (HSD3B1) produce 3β-diol from androgens. Steroid sulfatase (STS) and 17β-HSD type 1 (HSD17B1) produce E2 or A-diol from E1S, or dehydroepiandrosterone sulfate. These steroid-metabolizing enzymes might function together in pathways to produce estrogenic steroids such as 3β-diol, A-diol as well as E2 in an aromatase-independent manner

to correlate with in vivo ER activity are therefore important, eventhough the result was not statistically significant. We suggest that this finding should form the basis for future research in this field. STS and 17β-HSD type 1 expression levels were recently shown to increase following AI neoadjuvant therapy in postmenopausal ER-positive breast carcinoma patients [49]. All these data indicate that the STS pathway might contribute to the escape of breast cancer from AI therapy.

Our results concerning the correlations between enzyme expression levels and in vivo ER activities in postmenopausal patients suggested that the contribution of the 3β-diol-producing pathway to ER activity was greater than that of the STS pathway, which produces E2. This finding was inconsistent with the previous studies that found lower binding affinity of 3β-diol compared with E2 [17, 18]. Although mRNA expression levels are considered to reflect protein expression levels, they do not necessarily reflect actual protein expression levels exactly. We should therefore avoid making simple assumptions about the contributions of each pathway to ER activities. In this regard, further IHC evaluations of the enzymes are needed to validate the results.

In conclusion, the aim of present study was to verify the functions of non-aromatase steroid-metabolizing enzymes such asSRD5A1, HSD3B1, HSD17B1, and STS in untreated breast cancer. This was the first study to suggest that these enzymes function in together and contribute to ER activation especially in postmenopausal women. This was a small observational study utilizing clinical samples and further investigation is needed to provide the mechanistic insight about role of the SRD5A1-HSD3B1 and STS-HSD17B pathways, and to verify whether these pathways are actively involved in the AI-resistance mechanisms in the future. However, our study provides novel findings into the possible role of the SRD5A1-HSD3B1 and STS-HSD17B pathways as an alternative estrogenic steroid-producing, aromatase-independent pathways.

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

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

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ORIGINAL PAPER

Detection of Estrogen-Independent Growth-Stimulating Activity in Breast Cancer Tissues: Implication for Tumor Aggressiveness

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Abstract Estrogen and various growth factors affecting tumor behavior are present in the breast cancer microenvironment, but their comprehensive effects and signal crosstalks are different in each case. However, there is no system to evaluate the factors, detected in individual breast cancer cases, that regulate ER activity and tumor progression. In this study, we analyzed the effects of individual breast cancer extracts by our original system using an estrogen-signal reporter cell line, MCF-7-E10, which we previously established. MCF-7-E10 cell line is stably transfected by an estrogen response element (ERE)-green fluorescent protein (GFP) gene; it expresses GFP when estrogen receptors (ERs) are activated by estrogen or growth factor signal-mediated ER phosphorylation. Using this cell line, we analyzed the comprehensive effects of factors derived from breast cancer tissues on ER activity and growth of MCF-7-E10 cells for each case. We also analyzed relationships between these activities and clinicopathologic characteristics of patients who provided cancer specimens. The breast cancer extracts, which reflect the combined activities of growth factors present in individual cases, stimulated MCF-7-E10 cell growth in an estrogen-independent manner, and specifically stimulated growth of other breast cancer cell lines, regardless of ER expression. High growth-promoting activities were seen in tumor regions of specimens with tumors > 10 mm in size, HER2 intrinsic subtype, and scirrhous and solid-tubular carcinoma histological subtypes. Anti-human hepatocyte growth factor (HGF) antibody and an inhibitor for insulin-like growth factor-1 (IGF-1) receptor inhibited MCF-7-E10 cell growth by the breast cancer extracts, indicating that signal pathways via HGF or IGF-1 receptor significantly affect breast cancer. These data suggest that growth factors other than estrogen in the tumor extract significantly affect breast cancer aggressiveness in an estrogen-independent manner, and could be useful therapeutic targets.

Keywords Tumor microenvironment · Estrogen-independent · HGF · IGF1-R · Breast cancer

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Background

The tumor microenvironment is enriched in factors such as growth factors, cytokines and chemokines, and critically affects initiation and progression of various tumor types [1–5]. For postmenopausal women with low levels of plasma estrogen, breast cancer growth and progression are mainly caused by estrogen produced locally in the tumor microenvironment [6–8]. Intratumoral production of estrogen is induced by aromatase, a key enzyme in estrogen biosynthesis, which is expressed by carcinoma-associated stromal fibroblasts [7–10]. Aromatase is a target of endocrine therapy for breast

cancers; aromatase inhibitors attenuate estrogen biosynthesis in treating hormone-responsive breast cancer [11, 12]. Estrogen stimulates breast cancer growth via expression of a diverse set of growth-related genes in tumor cells, and through activation of estrogen receptor (ER), a transcription factor [13, 14]. ERα is a primary predictive marker for hormonal therapy in breast cancer, but approximately one-third of ER + patients do not respond to this therapy, suggesting that ERα is not a perfect predictor for hormonal therapy. To shed light on these issues, and to study the molecular basis for breast cancer, we first focused on analysis of estrogen signals by development of a custom-made cDNA microarray, and provided novel diagnostic and prognostic estrogen-induced genes [15–17].

In addition to the genomic pathway, estrogen induces non-genomic pathways by interacting with signal cascades for growth factors [4, 13], such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1), which activate ERα in an estrogen-independent manner by phosphorylating several ERα sites using their downstream signal kinases, including MAPK and PI3K [4, 5, 18–20]. Growth factors are produced by malignant cells themselves, adjacent tumor stromal fibroblasts and inflammatory cells in the microenvironment.

To analyze the carcinoma-associated fibroblasts-induced ER activation in individual breast cancers, we established an estrogen response element (ERE)-green fluorescent protein (GFP) assay system. It allows us to detect estrogen- and phosphorylation-dependent ER-activating ability of stromal fibroblasts adjacent to tumor cells under coculture with MCF-7-E10 cells, a clone of MCF-7 stably transfected with the *ERE-GFP* gene [20]. Using this system, we examined relationships between ER-activating ability of stromal fibroblasts and clinicopathological characteristics. We found that, although ER-activating abilities of stromal fibroblasts vary among breast cancers, they are higher in breast cancers from postmenopausal patients than in those from premenopausal patients [20]. This is in accordance with the fact that intratumoral estrogen production causes progression of postmenopausal breast cancers [6–8]. ER-activating abilities of fibroblasts in grade 3 breast cancers are lower than in grade 1 breast cancers, suggesting that the grade 3 microenvironment stimulates proliferation of breast cancer cells via an estrogen-independent pathway [20].

In the breast cancer microenvironment, various growth factors and cytokines reportedly interact to control tumor growth, as described above. However, their significance in tumor growth in vivo and in response to hormonal therapy are unclear. To investigate the role of breast cancer-derived factors on breast cancer growth, we studied effects of the supernatants of minced breast cancer tissues on MCF-7-E10 cell growth. Tissue supernatant, unlike tissue extract or conditioned medium of tissue prepared after culture for several days in vitro, reflects the comprehensive effects of factors detected in the tumor in vivo. In addition to estrogen-related signals, we found that the breast cancer-derived factors effectively stimulate MCF-7-E10 cell growth via an estrogen-independent pathway.

Methods

Cells & Cell Culture

Cell lines used in this study were cultured in RPMI1640 medium (GIBCO) supplemented with 10 % FCS (Tissue Culture Biologicals) at 37 °C in a humidified atmosphere of 5 % CO_2. We previously established the estrogen-signal reporter cell line MCF-7-E10 derived from MCF-7 cells by stable transfection with an *ERE-GFP* reporter plasmid [20]. To analyze the effect of breast cancer tissue supernatant (BCTS) on ER activity in MCF-7-E10 cells, cells were precultured in estrogen-deprived medium (phenol red-free RPMI1640 medium supplemented with 10 % dextran-coated, charcoal-treated FCS) for 3 days.

Preparation of BCTS

Breast cancer specimens were processed within 1 h after surgical resection. After being weighed, specimens were transferred to tubes containing phenol red- and serum-free RPMI 1640 medium at 100 mg/ml, and minced to particles $< \sim 1\ mm^3$ in size. The suspension was centrifuged (600 × *g*, 10 min, 4 °C) and the supernatant was further centrifuged (12, 000 × *g*, 10 min, 4 °C) to obtain BCTS. The protein concentration of each sample was determined using BCA Protein Assay Reagent (PIERCE).

Human breast cancer tissues were obtained by surgery at the Saitama Cancer Center Hospital (Saitama, Japan) after informed consent had been obtained from the patients. The Saitama Cancer Center Ethics Committee approved this study. In the clinicopathological classifications of the patients (Table 1), ER and progesterone receptor (PgR) status was determined using monoclonal anti-ERα antibody 1D5 (Dako, Glostrup, Denmark) and monoclonal anti-PgR antibody PgR636 (Dako), and evaluated on the basis of Allred scoring [21]. HER2 protein expression was scored as 0, 1+, 2+ or 3+ using the HercepTest™ (Dako); *HER2* genome status was evaluated by fluorescent in situ hybridization (FISH) using PathVysion HER-2 DNA Probe Kit (Abbott Laboratories, Abbott Park, IL, USA). According to the ASCO/CAP guidelines [22], absolute *HER2* gene/chromosome 17 copy number ratios

Table 1 Clinical characteristics of patients

	No. of patients[a]
Age (y)	
< 51	26
≥ 50	65
Unknown	2
Menopausal status	
Pre	28
Post	64
Male	1
Tumor diameter (cm)	
≤ 1	15
> 1	75
Unknown	3
ER	
Positive	69
Negative	24
PgR	
Positive	52
Negative	41
HER2	
0	37
1	31
2	10
3	13
Unknown	2
Stage	
0	1
I	37
II	42
III	11
Unknown	2
Histology	
Scirrhous	41
Solidtubular	23
Papillotubular	13
Mucinous	7
Apocrine	2
DCIS	3
Unknown	4
Grade	
1	18
2	17
3	47
Unknown	11
Nodal status	
Negative	38
Positive	53
Unknown	2

[a] Total number of patients was 93

greater than 2.2 and less than 1.8 indicated *HER2* amplification (positive) and *HER2* non-amplification (negative), respectively. Histologic grading was evaluated according to the Elston and Ellis grading scheme [23].

Cell Growth Assay

After 3 days of culture in estrogen-deprived medium, cells were seeded at 1×10^3/150 μl in a 96-well multi-dish culture plate, or at 1×10^4/1 ml in a 24-well plate, with or without BCTS at indicated protein concentrations for 4 days. Viable cells were examined using a Cell Counting Kit-8 assay according to manufacturer's instructions (Dojindo Laboratories, Japan).

Evaluation of ER Activity

ER activities in MCF-7-E10 cells, which had been transfected with *ERE-GFP*, after incubation with BCTS or E2, were monitored through GFP expression [20]. To quantify GFP expression, cells expressing GFP were counted under a fluorescence microscope after the cells were harvested by treatment with trypsin. Data are presented as percentage of cells expressing GFP.

Quantification of Growth Factors in BCTS by ELISA

Human EGF and IGF-1 levels in BCTS were quantified by ELISA using Quantikine (R&D Systems, MN, USA) specific for each growth factor.

Materials

Unless otherwise stated, all other materials were from Sigma-Aldrich Inc. (St. Louis, MO, USA). Inhibitors for EGF receptor and IGF receptor, and normal mouse IgG were from Calbiochem. Mouse anti-human HGF monoclonal antibody was from the Institute of Immunology (Tokyo, Japan). Mouse IgG1 antibody (Chemicon International, CA, USA) was used as an isotype control. IGF-1 receptor inhibitor, AG1024, and EGF receptor inhibitor, AG1478, were from Chemicon International.

Statistical Analysis

Statistical analyses were performed using the Stat Flex version 6.0 software program (Artech Co., Ltd., Osaka, Japan). In comparisons among groups, ANOVA and two-sample *t*-tests were used to assess the statistical significance of differences.