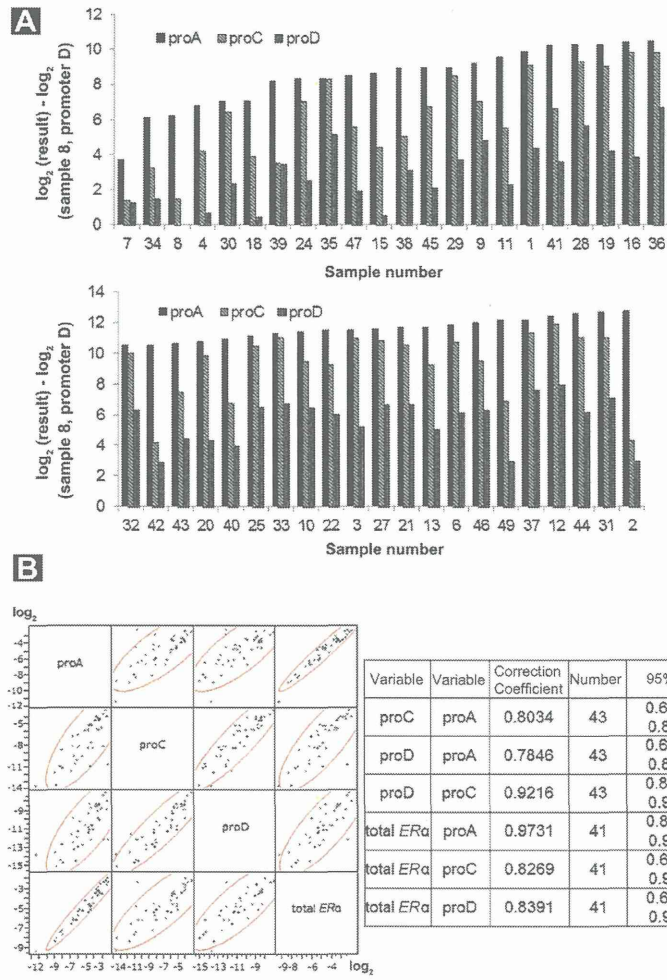
Figure 3 Real-Time Polymerase Chain Reaction of Messenger RNA in Individual Breast Cancers and Statistical Analyses. (A) Results of Real-Time Polymerase Chain Reaction (PCR) Assays of Individual Breast Cancers. The Vertical Axis Indicates the Quantity Obtained From This Formula "log2 (result) - log2 (sample 8, promoter D)". In Other Words, This Result From That Formula Indicates the Difference of the Result From the Smallest Quantity, Promoter D of Sample 8, in This Real-Time PCR Assay. The Results in That Formula Were Obtained in Real-Time PCR. The Result of Real-Time PCR Were Normalized to β-Actin and Were then Converted to Logarithmic Values (Base 2). These Result Were Listed From the Left End in the Increasing Order in the Result of Promoter A Obtained From That Formula. The Horizontal Axis Indicates Identification Number of Tissue Sample. (B) The Correlation Coefficient of Promoter-Specific *ERα* Messenger RNA (mRNA). A Matrix of Paired Correlation Coefficients With dot Maps is Presented. Oval: 95% of Examined Data Exist. Correlation Coefficients Were Estimated With $P < .05$ Significance. The Horizontal and Vertical Axes Indicate the Amount of Transcripts Specific to Each Promoter, Converted to Logarithm of Promoter-Specific mRNA Normalized to β-Actin (Base 2). (C) The Associations Among Promoter-Specific ERα mRNA and Clinicopathological Factors (age, Status of Menopause, ER Immunohistochemistry [IHC] and HER2 IHC). The Horizontal Axes Indicate age in Years, Menopausal State (Postmenopausal [post] and Premenopausal [pre]), Allred Score in ER IHC and HER2 Status in HER2 IHC. The Vertical Axes Indicate the Levels of Promoter-Specific mRNA Normalized to β-actin, Converted to Logarithmic Values (Base 2). Age was Tested by Single Regression Analysis and Regression Line is Indicated in This Figure. Menopause, ER IHC, and HER2 IHC Were Tested by the Student t Test and the Analysis of Variance (ANOVA). All Values Were Converted to the Logarithm (base 2) of Promoter-Specific mRNA Normalized to β-actin



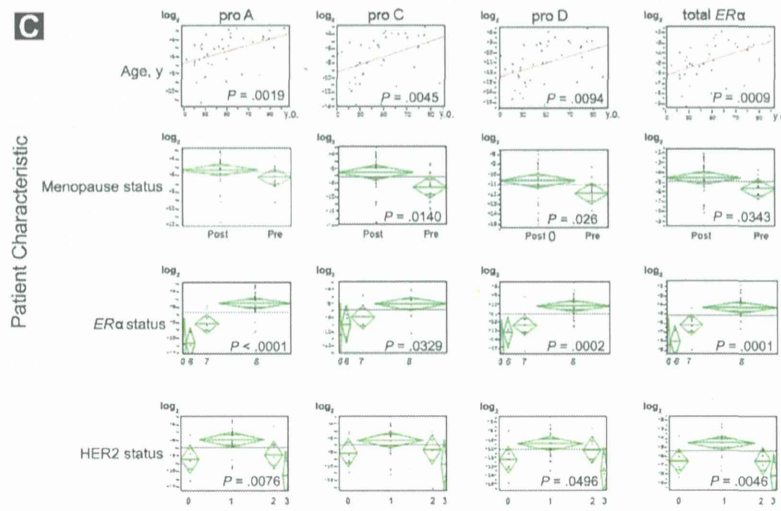
Variable	Variable	Correction Coefficient	Number	95% CI	*P* value
proC	proA	0.8034	43	0.6108-0.8693	<.0001
proD	proA	0.7846	43	0.6056-0.8672	<.0001
proD	proC	0.9216	43	0.8189-0.9440	<.0001
total *ERα*	proA	0.9731	41	0.8450-0.9843	<.0001
total *ERα*	proC	0.8269	41	0.6854-0.9006	<.0001
total *ERα*	proD	0.8391	41	0.6912-0.9026	<.0001

Abbreviations: proA = promoter A; proC = promoter C; proD = promoter D.

(Fig. 3B), which suggested that *ERα* transcripts in ERα$^+$ breast cancer tissues had the same promoter usage.

To investigate variations in *ERα* promoter choice by another method, we quantified promoter-specific transcripts and that of total ERα mRNA according to clinicopathological factor. The statistically significant ($P < .05$) result of this analysis came from 4 factors: patient's age, status of menopause, ER status, and human epidermal growth factor receptor 2 (HER2) status (Fig. 3C). ER

Figure 3 continued



and HER2 status were ascertained using immunohistochemistry. Transcripts of each promoter and total ERα increased similarly with age. Transcripts for promoters C and D and for total ERα were larger in postmenopausal breast cancers than in premenopausal cancers. Transcripts for each promoter and for total ERα were positively related with patients' ER-Allred scores,[23] but inversely related to HER2 scores except for score 0. These results showed that the association of each promoter with clinicopathological factors was the same as that of total ERα, which suggests that *ERα* transcripts in ERα$^+$ breast cancer have the same promoter usage.

Regression Analyses of ERα Transcription by 3 Promoters

As transcripts from 3 promoters were quantified, we used single and multiple regression analyses of associations among promoters and total *ERα* mRNA expression. Single regression analyses positively related transcripts from all 3 promoters to that of total ERα (Fig. 4A). Although our multiple regression analysis posited transcripts from the 3 promoters as independent variables, we considered that these variables examined for total ERα might influence each other, thus biasing this analysis. To overcome this problem, we calculated a variance inflation factor (VIF). For a VIF < 10, this influence could be generally excluded. As the VIF was < 10 for this study, we felt multiple regression analysis could account for total *ERα* mRNA. Only the coefficient of promoter A was significant ($P < .05$) in this analysis (Fig. 4B).

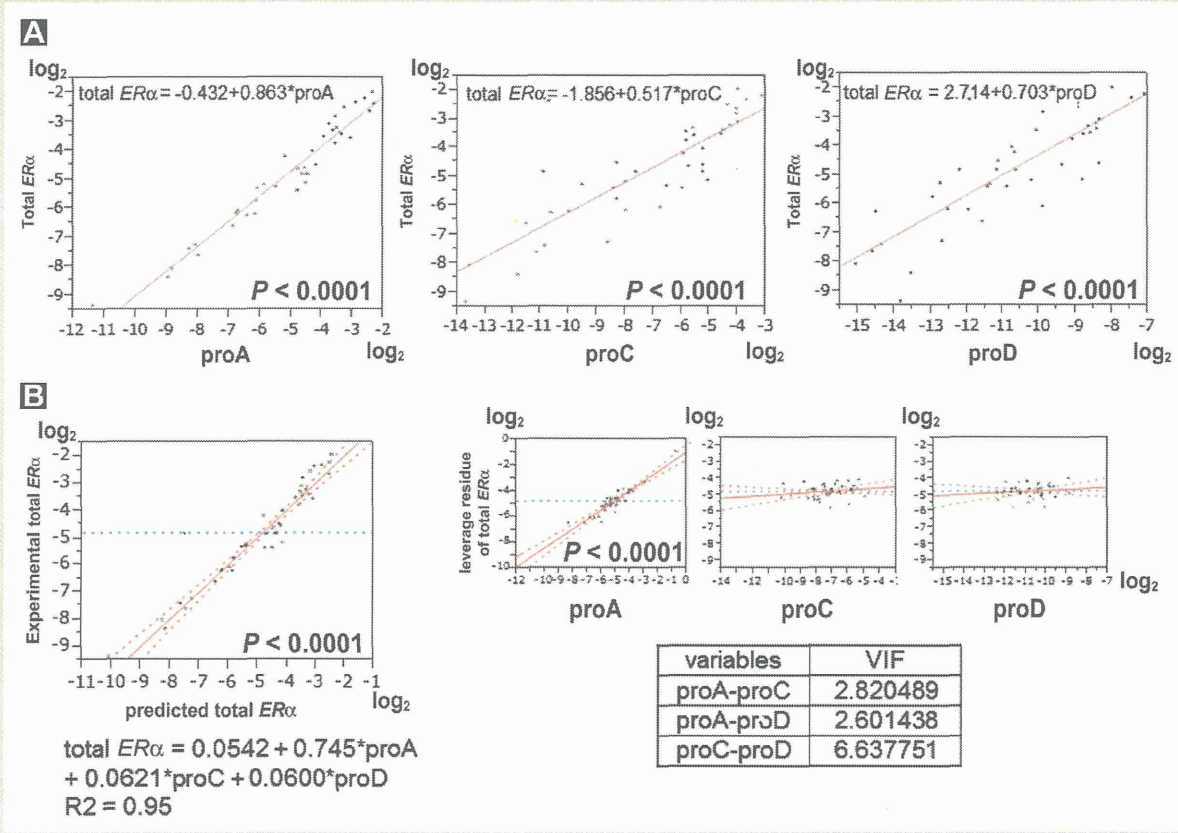
Discussion

In our previous study of an estrogen response element reporter gene assay for promoter-specific activity, a very high level of ER activity by promoter A and a moderate level of activity by promoter D were observed in ERα$^+$ breast cancer cell lines.[9] Though the results of this study differ from those of the previous report in the strict sense, they agree with the pattern of high luciferase activity for promoter A and moderate activity for promoter D in ERα$^+$ cell lines. Whereas promoter C luciferase activity was low in the previous study, promoter C transcripts significantly ($P < .05$) correlated with ER status assessed by EIA.[8] Promoter C transcripts have also been significantly ($P < .05$) associated with poor prognosis in breast cancer tissue.[22] Low luciferase activity for promoter C in the previous study might have been because the length of the sequence inserted to reporter plasmid was approximately 1.5k base pairs (bp) and the long insert might have included an unknown silencer for transcriptional activity in breast cancer cell lines.

Another of our previous studies reported that ER status in breast cancer tissues (per EIA) was significantly ($P < .05$) correlated with transcripts from promoter C rather than promoter A.[8] Results from this study also differed from those of our previous study about the correlation of promoter A transcripts, but this may have been affected by the stability of mRNA. The half-life of promoter A transcripts was much shorter than that of promoter C (promoter A: 2.85 h, promoter C: 7.42 h),[22] which implies that the instability of promoter A–specific mRNA might affect the associations of promoter A transcripts compared with those of total ERα in the previous study. Moreover, an RNA storage reagent was used to prevent total RNA degradation in this study, and efficiency of RNA collection in this study was thought to be improved over the previous study, allowing more precise measurement of promoter A transcript in this study. In any case, we are convinced that the result of this study did not negate the findings of our previous studies.

We analyzed *ERα* promoter usage by correlating promoter-specific transcripts with those of total *ERα*, and these transcripts with clinicopathological factors. These results suggest that ERα transcripts in ERα$^+$ breast cancer had the same usage of promoters. Alteration of promoter usage in *ERα* was reported previously in analyses of non–breast cancer cell lines[9,17] and normal human and rat tissues,[17,24-26] which suggested that tissue type drove the choice of promoters in *ERα* transcription. We therefore speculated that promoter usage was important to regulate expression of ERα in

Figure 4 Regression Analyses of Promoter-Specific Messenger RNA. (A) The Single Regression of Promoter-Specific Results for Total *ERα* Messenger RNA (mRNA), Shown With the *P* Value of the Analysis of Variance (ANOVA). The Horizontal and Vertical Axes Indicate the Amount of Transcripts Specific to Each Promoter and Total *ERα*. The Values of the Results Obtained in Real-Time Polymerase Chain Reaction Were Normalized to β-Actin and Were Converted to Logarithmic Values (Base 2). (B) The Multiple Regression Analysis of Promoter-Specific mRNA. Left: A dot Plot of Predicted Experimental Data; Regression Equation Shown With the ANOVA *P* Value. Right: Figures of Leverage Residue Plot Shown With the *P* Value and Variance Inflation Factor (VIF). Horizontal Dotted Line: Average Value. Solid Line: Approximate Line of Dots Intersected by Leverage of Promoter-Specific mRNA and Leverage Residue of Predicted Total *ERα*; Dotted Curves: 95% CI. The Horizontal Axis Indicates the Leverage Residues of Promoter-Specific Transcripts, and the Vertical Axis Indicates That of the Total *ERα* Transcripts. The Unit of the Vertical Axis is Logarithm of Promoter-Specific Total ERα mRNA Normalized to β-Actin



variables	VIF
proA-proC	2.820489
proA-proD	2.601438
proC-proD	6.637751

Abbreviations: proA = promoter A; proC = promoter C; proD = promoter D.

normal development and differentiation or carcinogenesis of breast cancer. Furthermore, the investigation of another cancer tissue with ERα expression (eg, endometrium) could confirm the biological significance of promoter choice.

These results also suggested that *ERα* transcription in breast cancer tissue mainly originated from the most proximal promoter and that more distal promoters were additionally utilized. However, the distance from the most proximal promoter to the most distal one is about 4 kbp; only the mechanism by which identical trans-factors were used in proportion to distance for initiation of *ERα* transcription from each promoter could not account for *ERα* transcription in ER^+ breast cancer tissues. Therefore, epigenetic dynamics might be associated with *ERα* transcription in breast cancer tissues. Because $ERα^+$ cell lines (MCF-7, T-47D, and ZR-75-1) showed very similar promoter choices for the ERα gene (Fig. 2), we analyzed the methylation status of CpG islands in regions from promoter A to promoter C in $ERα^+$ breast cancer cell lines, using the direct sequence method. The methylation status of CpG islands in these promoter regions was found to be different among these cell lines (data not shown), implying that methylation of CpG islands in the promoter regions of ERα gene could not fully account for the promoter use of *ERα*. Histone modulation might be associated with *ERα* transcription in breast cancer tissues, but this hypothesis needs further study.

ERα has at least 2 variants, the 46-kDa ERα (ERα46)[27] and the 36-kDa ERα (ERα36),[28] and these variants are prognostic factors.[29,30] We analyzed the transcripts of ERα46 because its mRNA had the same 5′-UTR of transcripts from promoter E and F and lacked only exon 1 among normal ERα exons (see Supplemental Fig. 1A in the online version at http://dx.doi.org/10.1016/j.clbc.2013.10.015). Our results indicated that the transcripts of ERα46, originating from both promoter E and F, were negligible in

Table 1 Patients' Clinicopathological Information

Age, years	Median	59.4	(40.2-87.2)
Menopause status	post	29	69.0%
	pre	13	31
	no data	1	
Cancer stage	I A	19	44.2%
	I B	1	2.3
	II A	13	30.2
	II B	8	18.6
	III A	2	4.7
ER IHC, Allred score	8	24	66.7%
	7	8	22.2
	6	3	8.3
	0	1	2.8
	no data	7	
PR (Allred)	8	11	30.6%
	7	8	22.2
	6	5	13.9
	5	6	16.7
	3	3	8.3
	0	3	8.3
	no data	7	
HER2 IHC, Allred score	3	2	5.6%
	2	6	16.7
	1	22	61
	0	6	16.7
	no data	7	
Lymph/vascular invasion	ly 2	6	13.9%
	1	18	41.9
	0	19	44.2
	v 2	1	2.3
	1	11	25.6
	0	31	72.1
Nuclear grade	3	18	41.9%
	2	13	30.2
	1	12	27.9
Nuclear Atypia	3	8	18.6%
	2	34	79.1
	1	1	2.3
Mitotic index	3	15	34.9%
	2	16	37.2
	1	12	27.9
Node metastasis	negative	29	69.0%
	positive	13	31
	no data	1	
Histology	papillotubular	11	25.6%
	solid-tubular	5	11.6
	scirrhous	18	41.9
	special type	9	20.9
E2 concentration in plasma, pg/mL	average	39.25	(22.8-208.9)

Estrogen receptor (ER) and progesterone receptor (PR) positivity of patients without ER and PR Allred scores were estimated as strong, moderate, weak, or none. Patients whose ER and PR scores could not be obtained were excluded.

the ERα-positive breast cancers (see Supplemental Fig. 1B in the online version at http://dx.doi.org/10.1016/j.clbc.2013.10.015). When the ERα46 transcripts were compared with those from promoter A, they were at most 1/400 in number of those originating from promoter A. In other words, cycle values exceeding threshhold for ERα46 transcripts were > 33, suggesting that the

amount of ERα46 transcripts was too little to evaluate the correlation with the clinicopathological factors of ERα+ breast cancer.

In this study, we found out that *ERα* transcription used the same promoter choice as promoter A, which was significantly ($P < .05$) associated with mRNA expression of the *ERα* gene in individual breast cancer.

Conclusion

We have investigated the transcriptional regulation of *ERα*, but the mechanism of the regulation remains to be discovered. In this article, we reinvestigated variations in the use of > 3 *ERα* promoters in breast cancer tissues and breast cancer cell lines with an eye toward using *ERα* promoter usage as a new biomarker, and found that the *ERα* promoter usage of ERα$^+$ breast cancer tissues and cell lines were similar, and the similarity was validated by examinations using correlation among transcripts from each promoter and that of total *ERα* and relation to clinicopathological factors. Although the likelihood of using *ERα* promoter usage in breast cancer tissues as a clinical biomarker was small, this article is meaningful in presenting the possibility that *ERα* promoter usage might be important for individual development, differentiation, or carcinogenesis, and that the biological meaning of *ERα* promoter usage could be discovered by comparison of the promoter usage in breast cancer cell lines with the promoter usage of other cancer tissues with ERα positivity.

Clinical Practice Points

- The *ERα* gene has at least 6 transcription start sites and 6 distinct first exons. It also probably has 6 promoters, which is unusual for functionally discovered nuclear receptors.
- Typical tissue promoter usages in cancer cell lines and normal tissues were found, using an ERE luciferase assay and quantification of promoter-specific mRNA of *ERα*.
- In this article, we investigated *ERα* promoter usage in individual breast cancer with an eye toward using *ERα* promoter usage as a new biomarker, using a real-time PCR method with primers and probes designed especially for this assay. We found that the *ERα* promoter usages of ERα$^+$ breast cancer tissues and cell lines were similar, and the similarity was validated by examinations using correlation among transcripts from each promoter and that of total *ERα* and relation to clinicopathological factors.
- Although the likelihood of using *ERα* promoter usage in breast cancer tissues as a clinical biomarker was small, this article is meaningful in presenting the possibility that *ERα* promoter usage might be important for individual development, differentiation, or carcinogenesis.

Disclosure

The authors have stated that they have no conflicts of interest.

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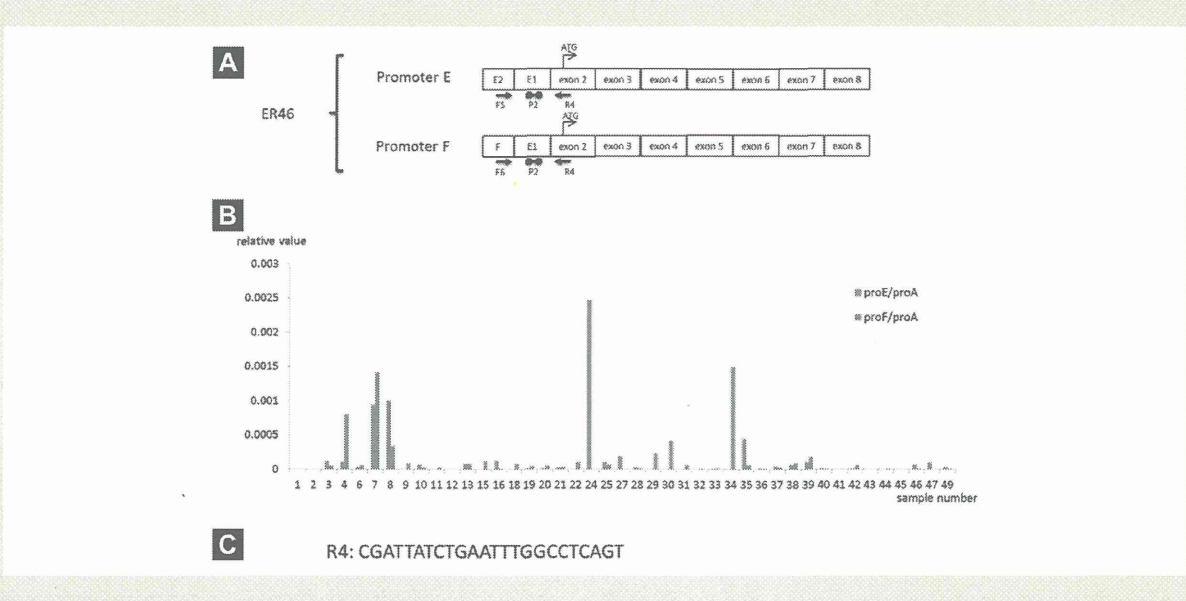
Supplemental Table 1 The Sequence of Primers and Probes Used in This Study

Primer List	Sequence (5'→3')
ERα Promoter A Forward Primer (F1)	CTGTGCTCTTTTTCCAGGTG
ERα Promoter B Forward Primer (F2)	CAGCGACGACAAGTAAAGTG
ERα Promoter C Forward Primer (F3)	GTTCTTGATCCAGCAGGGTG
ERα Promoter D Forward Primer (F4)	CACCTGAGAGAGCCAGTG
ERα Promoter Common Reverse Primer (R1)	AGGGTCATGGTCATGGTC
ERα Promoter E Forward Primer (F5)	ACCAATCCTTTTGATTGTGAA
ERα Promoter F Forward Primer (F6)	GCATAAGAAGACAGTCTCTGAGTGA
ERα Promoter Common Reverse Primer (R2)	GGCAGAAGGCTCAGAAACC
ERα Promoter Common Probe (P1)	CCGGTTTCTGAGCCTTCTGCCC
ERα Promoter Common Probe (P2)	ACATTCTCCGGGACTGCGGTACCA
Total ERα Exon7 Forward Primer (F7)	CTCCCACATCAGGCACAT
Total ERα Exon8 Reverse Primer (R3)	CTCCAGCAGCAGGTCATA

Supplemental Figure 1 Analyses of ER Alpha Variants With This Promoter-Specific Method. (A) Exon Structure of the ERα Variant ERα46. This Messenger RNA (mRNA) Originates From the Same Promoters, E and F, as Those of Normal *ERα* Gene. Two 5′-UTR Exons of This mRNA Were Directly Spliced to Exon 2, but not to Exon 1. (B) The Real-Time Polymerase Chain Reaction Analysis of *ERα46* Transcripts. The Amount of *ERα46* Transcripts Originating From Both Promoter E and F was Normalized to That of the Transcripts From Promoter A. The Horizontal Axis Indicates the Identification Number of Breast Cancer Samples and Vertical Axis Indicates the Levels of Transcripts Originating From Both Promoter E and F, Relative to Those Originating From Promoter A. (C) The Sequence of the Reverse Primer Designed for the Analysis of *ERα46*



Abbreviations: proA = promoter A; proE = promoter E; proF promoter F.

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Active Estrogen Synthesis and its Function in Prostate Cancer-derived Stromal Cells

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Abstract. *Background: It remains unclear whether estrogen is produced in prostate cancer (PCa) and how it functions in PCa. Materials and Methods: To examine the production of estrogen in PCa cells, the concentration of estrogen in the medium in which LNCaP cells and PCa-derived stromal cells (PCaSC) were co-cultured, was measured by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), while aromatase (CYP19) mRNA expression was confirmed by real-time polymerase chain reaction (RT-PCR) methods. To verify whether estrogen is synthesized from testosterone in PCaSC functions, PCaSC were co-cultured with breast cancer MCF-7-E10 cells, which were stably-transfected with ERE-GFP, in the presence of testosterone. GFP expression was detected when PCaSCs could synthesize estrogen. The proliferation of PC-3 cells in the presence of PCaSC was determined by cell count. Results: PCaSC metabolized excessive testosterone to estrogen, which activated estrogen receptor in breast cancer cells. Moreover, estrogen synthesized from testosterone in PCaSC regulated the proliferation of PC-3 cell via repression of some unknown growth factors that were secreted from PCaSC. Conclusion: A chimeric co-culture method between breast cancer cells and PCaSC revealed the production of active estrogen in PCaSC. High-dose testosterone therapy might introduce a new potential strategy to treat CRPC.*

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Key Words: Castration-resistant prostate cancer, estrogen, testosterone, prostate cancer-derived stromal cell, chimeric coculture.

Castration-naïve prostate cancer (PCa) develops into castration-resistant prostate cancer (CRPC) during androgen deprivation therapy (ADT) by various mechanisms. Especially, the androgen receptor (AR) signaling axis plays a key role in this specific development. PCa cells mainly adapt themselves to the environment of lower androgen concentrations and change into androgen-hypersensitive cells or androgen-independent cells. Androgens of adrenal origin and their metabolites synthesized in the microenvironment in an intracrine/paracrine fashion act on surviving PCa cells and secrete prostate specific antigen (PSA). Therefore, new recently developed medicines, abiraterone acetate that inhibits adrenal androgen synthesis enzyme (CYP17A1) and 2nd generation anti-androgen, enzalutamide that not only blocks androgen-AR interaction but also inhibits nuclear translocation of AR and DNA-binding, are clinically effective even after docetaxel-based chemotherapy (1, 2). However, CRPC further develops into a more malignant state after these treatments and finally causes death of patients. One of the mechanisms is that the AR splice variant in which the ligand-binding domain is deleted is constitutively activated without androgen promoting androgen depletion-resistant growth (3). Expression of AR splice variants in PCa bone metastases was associated with castration-resistance and short survival (4). Other mechanisms of development into more malignant CRPC is that CRPC also develops by clonal outgrowth of a small number of androgen-independent PCa cells that preexist or develop at a low frequency due to secondary genetic mutations (5).

Microenvironment, including stromal cells or cancer-associated fibroblasts, also affects PCa cell proliferation, invasion and metastatic progression (6-9). The activation of PCa cell growth through growth factor receptor expression resulted in the activity of androgen-independent stromal growth factor signals, such as fibroblast growth factor (FGF)-7 under conditions of androgen ablation (10). Such a

microenvironment may gradually cause clonal development from small number of androgen-independent PCa cells. Therefore, targeting the microenvironment surrounding PCa cells might become a new therapeutic strategy.

Estrogen-based therapies are also alternative ways for CRPC. Excess estrogen causes repression of luteinizing hormone-releasing hormone (LH-RH) secretion from the hypothalamus and subsequently blocks testosterone secretion from testes *via* repression of LH secretion from the pituitary, as well as LH-RH agonist. Therefore, estrogen-based therapies have been employed as initial hormonal therapies. Estrogen-based therapies are effective not only for castration-naïve PCa as initial treatment but also effective for CRPC (11, 12). However, the molecular mechanisms of effectiveness for CRPC are not fully elucidated yet. Moreover, excess testosterone treatment is effective for CRPC (13, 14). However, it remains unclear why excess testosterone treatment inhibited CRPC growth.

In the present study, stromal cells that co-exist in the microenvironment of PCa cells metabolized excessive testosterone to estrogen and repressed some unknown growth factors, which stimulated PCa cell proliferation.

Materials and Methods

Cell lines. LNCaP cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin (P/S; Invitrogen, Carlsbad, CA, USA) and 5% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO, USA). PC-3 cells (ATCC) were cultured in RPMI1640 supplemented with 1% P/S (Invitrogen) and 5% FBS. MCF-7-E10 cells were cultured in RPMI1640-10% FCS with 1% P/S. PCaSC which were previously established in our Institution, were cultured in RPMI1640-10% FBS (15).

Cell proliferation assay. Twenty-four hours after plating PC-3 cells at a density of 5×10^4 onto 12-well plates with DMEM-5% charcoal-stripped fetal calf serum (CCS; Thermo Scientific HyClone, City, UK), cells were cocultured with or without PCaSC-8 onto one layer. Both cells were treated with testosterone (T) or 17β- estradiol (E2) with or without 1 μM bicalutamide or 100 nM exemestane. The media were changed every two days and reagents were also added to the medium. In each experiment, cells were harvested and counted in triplicate using a hemocytometer. The data shown represent the means ± standard deviation (SD) of three replicates.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR). For RT-PCR, 24 h after plating at a density of 2×10^5 cells onto 6-well plates with DMEM-5% CCS, cells were harvested and total RNA was extracted. Total RNA was purified with the RNeasy mini kit (Qiagen, Valencia, CA, USA). cDNA was made by reverse transcription (RT) of 500 ng of each total RNA using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA). One μl aliquot was used as a template for real-time PCR using the CFX connect Real-time PCR system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. PCR for *AR*, aromatase (*CYP19*), estrogen receptor α (*ERα*), *ERβ* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was performed according to the manufacturer's instructions (Bio-Rad).

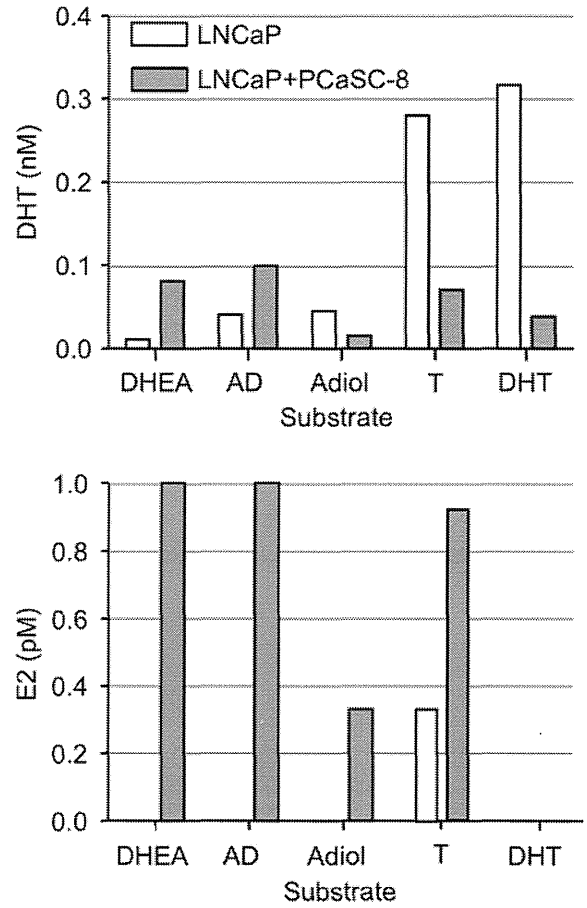

Figure 1. *DHT and E2 biosynthesis from androgens in LNCaP cells and PCaSC-8. The concentration of T and E2 in the medium used for co-culture with LNCaP and PCaSC after treating with 100 nM DHEA, 10 nM AD, Adiol, T and DHT is shown. Twelve hours after starting culture of 5×10^4 LNCaP cells with or without 5×10^4 PCaSC-8, 100 nM DHEA, 10 nM AD, Adiol, T or DHT were added to the medium. Then, aliquots of medium were collected after 24 h for measuring the concentration of DHT and E2 by LC–MS/MS. The lower limit of quantitation of DHT and E2 was 2.5 and 0.03 pg/assay, respectively.*

The primers were AR sense: 5'-TCCAAATCACCCCCCAGGAA-3' and antisense: 5'-GACATCTGAAAGGGGGCATG-3'; aromatase (*CYP19*) sense: 5'-GGAACACTAGAGAAGGCTGGTCAGT-3' and antisense: 5'-GCCTCGGGTCTTTATGGATACGGTT-3'; *ERα* sense: 5'-CACTGCGGGCTCTACTTCATCGCA-3' and antisense: 5'AAGAG CTACGGGAATCCTCACGCTT-3'; *ERβ* sense: 5'-CGGCTTTGT GGAGCTCAGCCTGTTC-3' and antisense: 5'-GCCGCTCTTGGCA ATCACCCAAACC-3'; *GAPDH* sense: 5'-CCACCCATGGCAAATT CCATGGCA-3' and antisense: 5'-TCTAGACGGCAGGTCAGGTCC ACC-3'. The annealing temperature of RT-PCR was 56°C for AR, and 65°C for aromatase (*CYP19*), *ERα*, *ERβ* and *GAPDH*.

Detection of estrogen receptor (ER)-activating ability of PCaSCs. ER-activating abilities of PCaSCs were detected by coculture with

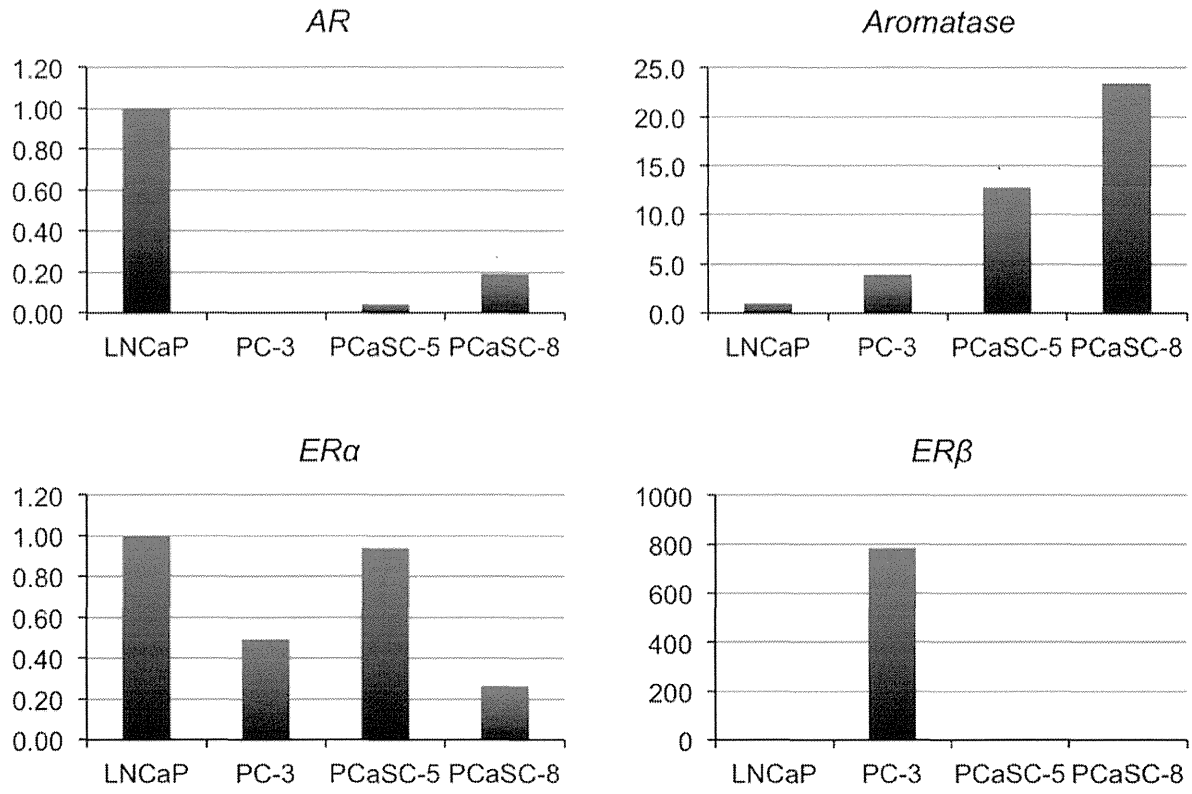

Figure 2. *Quantification of AR, aromatase (CYP19), ERα and ERβ mRNA expression level in LNCaP, PC-3, PCaSC-5 and PCaSC-8. The expression level of each PCR product was quantitated and normalized by the GAPDH expression level. The relative level of expression in each cell line was adjusted to the level of expression of LNCaP cells.*

MCF-7-E10 cells, ER activity reporter cells, as described in a previous study (16). MCF-7-E10 cells were established from human breast cancer MCF-7 cells by stable transfection with the estrogen response element (ERE)-green fluorescent protein (GFP) (ERE-GFP) reporter plasmid. These cells express GFP in the presence of estrogen. In the coculture of prostate cancer-CAFs (PCaSCs) and MCF-7-E10 cells, they were precultured in estrogen-deprived medium for 3 days. PCaSCs were seeded in a 24-well multi-well dish at 5×10^4 /ml, and on the next day the same number of MCF-7-E10 cells was seeded on the top of PCaSCs. Subsequently, the cellular mix was cultured for 4 days in the presence or absence of testosterone (100 nM) and/or fulvestrant (ICI 182780; 1 μM) or exemestane (100 nM). MCF-7-E10 cells expressing GFP were counted after collected with trypsinization. PCaSCs and MCF-7-E10 cells were easily discriminated by their morphology. Breast cancer-CAFs (#863, 870, 871,872 and 874) were used as a positive control.

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). After plating 5×10^4 LNCaP cells on 12-well plates in DMEM-5% CCS, LNCaP cells were co-cultured with PCaSC-8 for 24 h. Both cell lines were treated with 100 nM dehydroepiandrosterone (DHEA), 10 nM androstenedione (AD), 10 nM androstenediol (Adiol), 10 nM testosterone (T) or 10 nM dihydrotestosterone (DHT) for 24 h and then the media were collected. The concentration of estradiol (E2) in the media was measured by LC-MS/MS (Division of Pharmacological Research, Aska Pharma Medical Co. Ltd., Kawasaki, Japan).

Results

Synthesis of estrogen in PCa-derived stromal cells. We have previously shown that PCa-derived stromal cells (PCaSCs) expressed androgen synthesis enzymes and had the ability to synthesize testosterone and DHT from DHEA in PCa tissue (15). We here confirmed how DHEA, AD, -Adiol, T, DHT are metabolized to DHT and β-estradiol in the presence or absence of stromal cells. PCaSC-8 increased the concentration of testosterone and DHT when DHEA was added as substrate to the medium of LNCaP cells (Figure 1). Interestingly, PCaSC-8 reduced the concentration of testosterone and DHT when androstenediol, testosterone and DHT were added as substrates to the medium of LNCaP cells. Next we examined the expression of *AR, ERα, ERβ*, aromatase (*CYP19*) mRNA in LNCaP, PCaSC-5 and PCaSC-8 cells. Although *ERα* mRNA in well-expressed in all cells,