the time of infection. To quantify the GFP expression level, the number of cancer cells expressing GFP was counted under a fluorescence microscope after harvesting by treatment with trypsin. The pathologist checked that only cancer cells expressed GFP. All experiments were done in duplicate, and the ERE activity was determined by the percentage of cells expressing GFP.

Total RNA preparation and real-time reverse transcription PCR

RNA was extracted from 40 μm FFPE sections containing a large tumor site using RecoverAll™ Total Nucleic Acid Isolation (Ambion, Austin, TX) according to the manufacturer's instructions after paraffin removal with xylene. The RNA concentration from FFPE samples was determined using the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). Total RNA (0.5 or 1 μg) was converted to first-strand cDNA primed with a random hexamer in a 20 μL reaction volume using a TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Bio Inc., Otsu, Japan). An aliquot of this solution (2 or 4 μL) was used as a template for real-time reverse transcription PCR to quantify the mRNA expression levels of ER and several ER target genes that were identified in our previous study [15–18] (Table 2) using the StepOne™ Real-Time PCR System (Applied Biosystems Inc., Foster City, CA). The PCR thermal settings were as follows: initial denaturation at 95°C for 10 min followed by 40 amplification cycles of 95°C for 15 sec, and annealing and elongation at 60°C for 1 min. The primer sequences used in this study are listed in Table 2.

Table 2. Primers used for real-time PCR.

Gene	Sequence
RPL13A	F: 5′-CCT GGA GGA GAA GAG GAA AG-3′
	R: 5′-TTG AGG ACC TCT GTG TAT TT-3′
Bcl-2	F: 5′-GTG GAT GAC TGA GTA CCT GAA C-3′
	R: 5′-GCC AGG AGA ATT CAA ACA-3′
Efp	F: 5′-CAT CTC TCA AGG CCA AGG-3′
	R: 5′-GCT ACT GTA TAG CAC TCT GAG A-3′
EGR3	F: 5′-GAG CAG TTT GCT AAA CCA AC-3′
	R: 5′-AGA CCG ATG TCC ATT ACA TT-3′
ERα	F: 5′-CTC CCA CAT CAG GCA CAT-3′
	R: 5′-CTC CAG CAG CAG GTC ATA-3′
HDAC6	F: 5′-GTC TAC TGT GGT CGT TAC ATC-3′
	R: 5′-GGC CTG ACA GTA GTA ACA C-3′
IGFBP4	F: 5′-CCA CGA GGA CCT CTA CAT CAT AC-3′
	R: 5′-ACA CAC CAG CAC TTG CCA C-3′
IGFBP5	F: 5′-TCT CTG CAC CTG AGA TGA GA-3′
	R: 5′-GTC ACA ATT GGG CAG GTA-3′
Ki67	F: 5′-GTC TCT GGT AAT GCA CAC TC-3′
	R: 5′-TCC ACA TGG ATT TCT GAA C-3′
PgR	F: 5′-AGC TCA CAG CGT TTC TAT CA-3′
	R: 5′-CGG GAC TGG ATA AAT GTA TTC-3′

Statistical analysis

Statistical analysis for comparison of two independent groups was performed with the Mann–Whitney U test and the StatFlex 6.0 software program (Artech Co., Ltd., Osaka, Japan). For comparison among three groups or more, the Kruskal–Wallis test was used. Correlation coefficients were also calculated with StatFlex 6.0. Data are expressed as mean ± standard deviation. $P < 0.05$ was considered statistically significant.

Results

Human breast cancer clinical samples exhibit varying ERE transcriptional activity and drug sensitivity

We have previously established an adenovirus-mediated ERE-GFP assay, named Ad-ERE-GFP assay, which enables the quantitative evaluation of endogenous ER transcriptional activity in clinical specimens [12, 19, 20]. Using this assay system, we investigated the ERE transcriptional activity of breast cancer cells isolated from surgical specimens. These clinical samples showed various levels of GFP expression representative of ERE activity, which was not associated with the status of ER (Fig. 2A). The range of the GFP positivity measured for all samples was 0–57%, where the average and median were 23.8% and 20%, respectively. In the ER-positive group alone, the range of GFP positivity was 2–57% (0–55%), and the average and median were 26.2% (17.1%) and 28.5% (18%), respectively. In drug sensitivity tests (Fig. 2B), Tamoxifen (Tam) and Fulvestrant (Ful) treatments effectively reduced ERE transcriptional activity to 75% and 85% of ER-positive samples, respectively; however, some samples were insensitive to either one (representative samples 340, 341, and 453, Fig. 2B) or both drugs (representative samples 493, 467, and 379, Fig. 2B). Notably, some ER-negative samples showed high GFP positivity that was reduced by antiestrogen treatment (representative samples 363, 342, 361, and 385, Fig. 2B). Furthermore, local recurrence was reported for two patients: ER-positive 467 and ER-negative 385. While ER-positive 467 showed low drug sensitivity in our test, ER-negative 385 showed high drug sensitivity. These data reiterate that sensitivity to endocrine therapy is not solely dependent on the status of ER. Thus, these results suggest that IHC to determine the ER status combined with Ad-ERE-GFP assay as an auxiliary diagnostic might more accurately predict the sensitivity of breast cancers

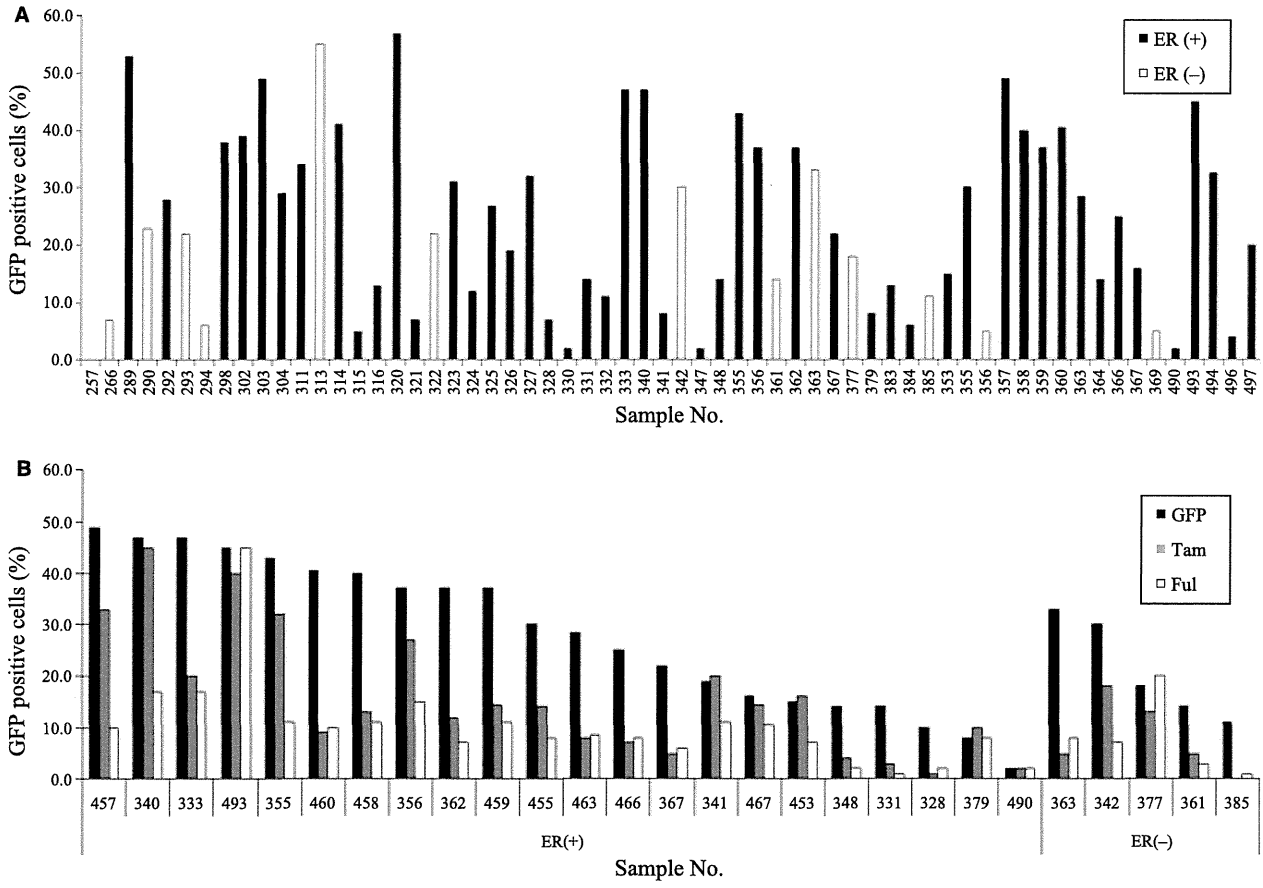

Figure 2. ERE transcriptional activity of primary breast tumor cells. (A) Primary breast tumor cells were infected with Ad-ERE-GFP and incubated for 3 days. Cells expressing GFP were then counted. Black bars represent ER-positive samples and white bars represent ER-negative samples. (B) Ad-ERE-GFP infected cells simultaneously received ethanol (EtOH; black bars), 4-hydroxytamoxifen (Tam; gray bars), and ICI 182,780 (Ful; white bars) at a final concentration of 1 μmol/L to determine drug sensitivity.

to hormonal therapy. Furthermore, some patients defined as ER negative may still be candidates for endocrine therapy.

ERE transcriptional activity significantly correlates with PgR protein expression

We next assessed the relationship between ERE transcriptional activity and clinicopathological information including ER, PgR, and HER2 protein expression as assessed by IHC (Fig. 3). ER protein expression appeared to correlate with ERE transcriptional activity, but this was not statistically significant (Fig. 3A). In contrast, ERE transcriptional activity was significantly correlated with the protein expression of PgR, an ER target gene (Fig. 3B). HER2 protein expression, on the other hand, did not correlate with ERE transcriptional activity (Fig. 3C). We also examined whether ERE transcriptional activity might be associated with other clinical information including age and tumor grade and whether patients were pre- or postmenopausal. In this analysis, ERE transcriptional activity was only correlated with postmenopausal status (Fig. 3D); age and tumor grade did not associate with ERE transcriptional activity. The malignant phenotype, however, such as tumor size or higher clinical stage, tended to show low-ERE transcriptional activity (data not shown). The positive correlation of ERE transcriptional activity with PgR protein suggests that our Ad-ERE-GFP assay reliably reflects ERE transcriptional activity and tumor malignancy as ER functional target. Additionally, because Ad-ERE-GFP uses only ERE as readout of ER-driven transcriptional activity, it is more specific than PgR, which is influenced by many transcriptional cofactors.

ER target gene expression does not correlate with ERE transcriptional activity

Next, we focused on the relationship between ER protein expression and ERE transcriptional activity. According to our previous studies [25, 26], samples with no less than

20% GFP positivity were designated as having high-ERE transcriptional activity. Using this threshold, samples were divided into two groups of high- and low-ERE transcriptional activity. We then compared ERE transcriptional activity, from the high and low groups, with mRNA expression levels of ER and three ER target genes, FOXA1, GATA3, and PgR, in ER-positive cases (Fig. 4). Statistical analysis uncovered significant intergroup differences in ER mRNA expression. ER mRNA expression was significantly higher in the low-ERE group than in the high-ERE group (Fig. 4A). Although PgR mRNA expression was not significantly different between low- and high-ERE groups, there was a tendency for mRNA expression to be higher in the high-ERE-activity group than in the low-ERE-activity group that was in agreement with protein expression analysis (Figs. 3B and 4D). For the other ER target genes examined (Efp, EGR3, HDAC6, IGFBP4, and IGFBP5), mRNA expression levels were not significantly different between low- and high-ERE transcriptional activity groups (data not shown). FOXA1 (Fig. 4B) and GATA3 (Fig. 4C), two genes recently proposed to be related to Luminal-type breast cancer [5–7], also showed no significant difference in mRNA expression regardless of the level of ERE transcriptional activity (FOXA1, $P = 0.786$; GATA3, $P = 0.689$). Therefore, our data suggest that ER target gene expression is not correlated with

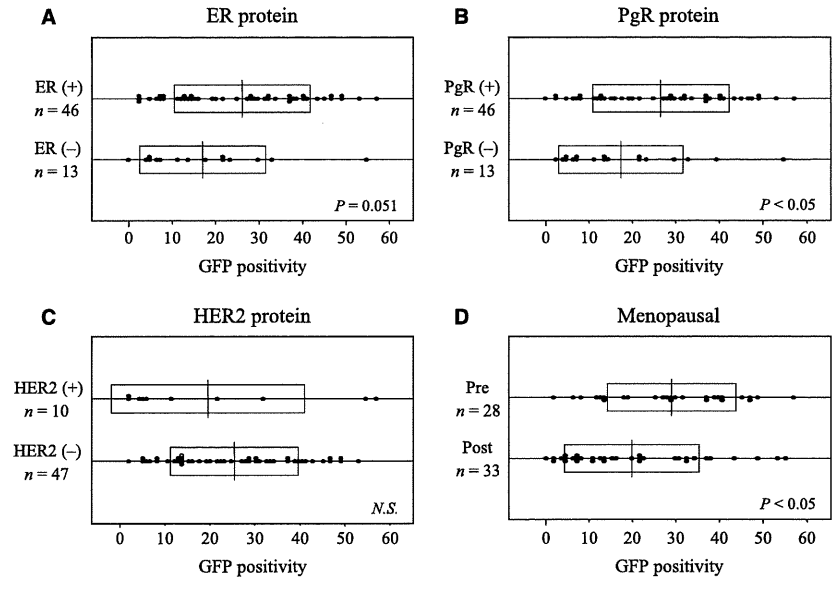

Figure 3. Comparative analysis of GFP positivity in 62 primary breast tumor samples by clinicopathological information. These box plots show the intergroup comparison of (A) ER protein expression, (B) PgR protein expression, (C) HER2 protein expression, and (D) menopausal status.

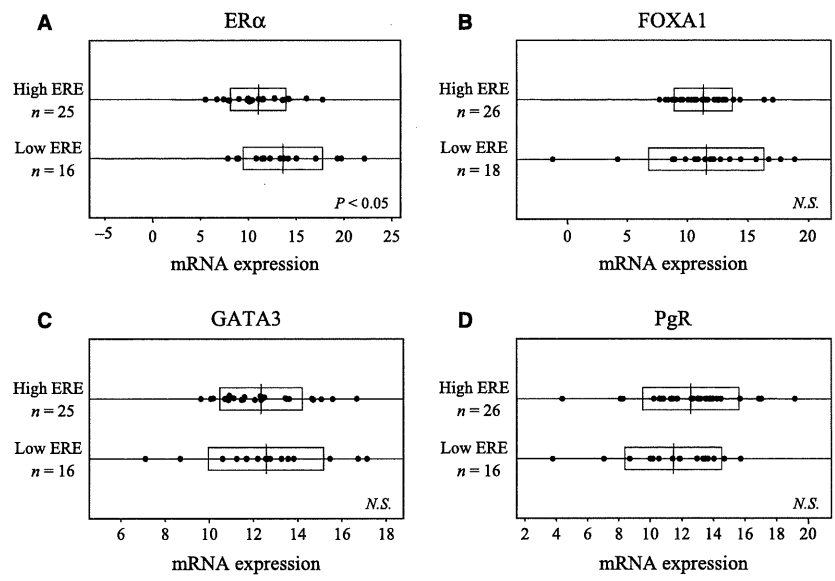

Figure 4. The intergroup difference of ERα and its related or target gene mRNA expression in 46 ER-positive breast tumor samples divided into high- or low-ERE transcriptional activity groups. These box plots show the intergroup differences of (A) ERα; (B and C) ER-related genes: (B) FOXA1, (C) GATA3; and (D) ER target gene: PgR.

ERE transcriptional activity. Thus, the regulation of ER target genes is likely not solely dependent on ER, but could instead involve the convergence of other signaling pathways.

ERE transcriptional activity suggests there are two distinct classes of Luminal A-type breast cancer

Because no significant difference in FOXA1 and GATA3 mRNA expression was observed in the ER-positive group, we decided to explore a more specific breast cancer subtype. Therefore, we conducted correlation analysis of ER and its target genes in Luminal A group breast cancer (Fig. 5). Analysis of this subset of ER-positive breast cancer specimens unveiled that ERα mRNA expression levels significantly correlated with Efp, IGFBP4, IGFBP5, FOXA1, and GATA3 in the high-ERE group, but not in the low-ERE-group, with the exception of GATA3. Moreover, FOXA1 and GATA3 mRNA levels correlated not only with ERα but also the other ER target genes: Efp, EGR3, HDAC6, IGFBP4, and IGFBP5, in the high-ERE group alone. On the other hand, some ER target genes, HDAC6, IGFBP4, and IGFBP5, significantly correlated with each other in the low-ERE group (data not shown). This result supports the hypothesis that some ER target genes are activated through signal pathways other than ER. These data also suggest that ERE activity can further distinguish Luminal-type breast cancer into two classes. Although there was large variation in the mRNA expression profiles of ER target genes between tumor cases, the determination of ERE transcriptional activity appears to be worthwhile for distinguishing ER function-dependent and -independent cases among Luminal A-type breast cancer.

Ki67 is strongly inversely correlated with ERE transcriptional activity

Ki67 [4] and Bcl-2 [27] have been reported to correlate with the malignancy of breast cancer. Therefore, we determined the correlation between ERE transcriptional activ-

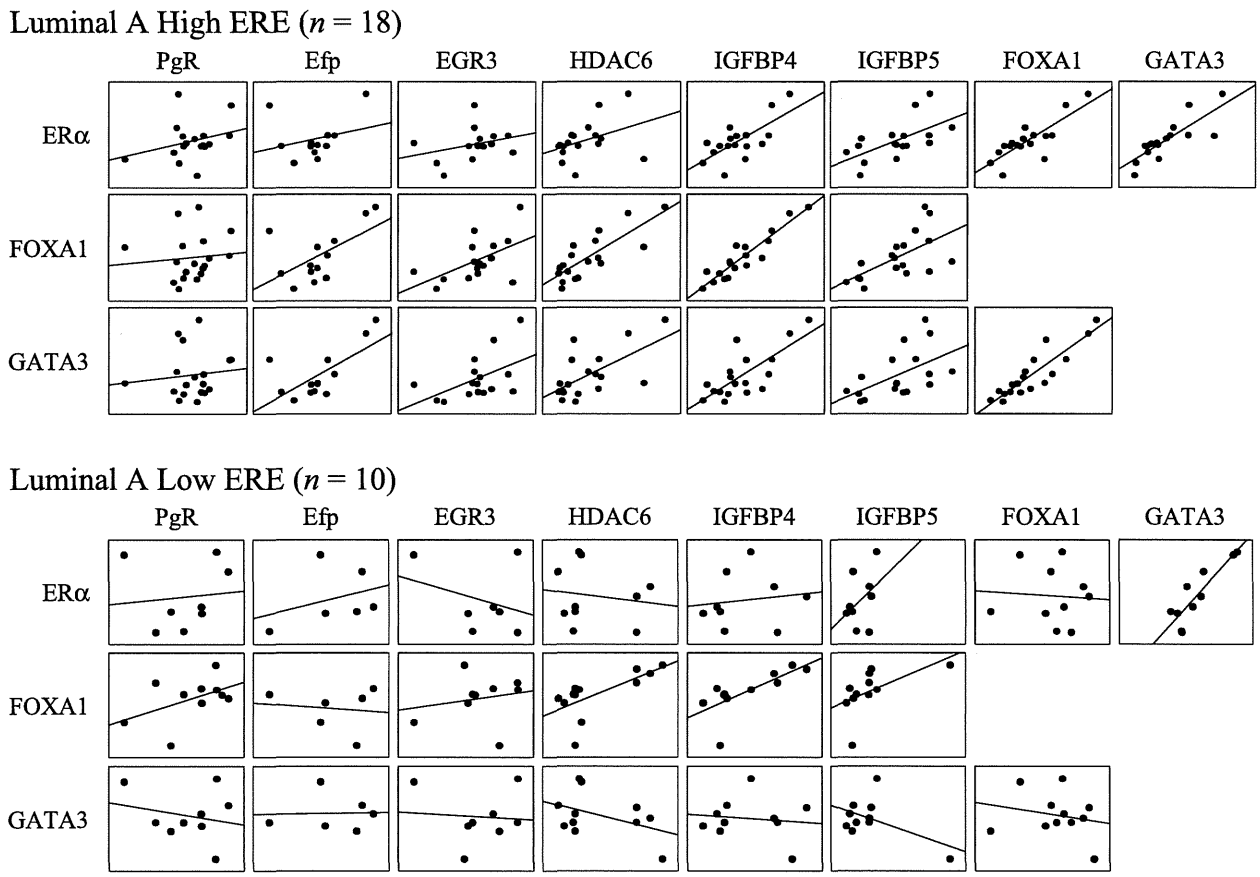

Figure 5. Correlation diagrams of ERα and ER target genes in 28 Luminal A-type breast tumor samples divided into high- or low-ERE transcriptional activity groups. The dots in each square represent the mRNA expression of each gene, and the straight lines show the correlation graphs. The gray squares represent significant correlation ($P < 0.05$), and the white squares reflect no significant correlation.

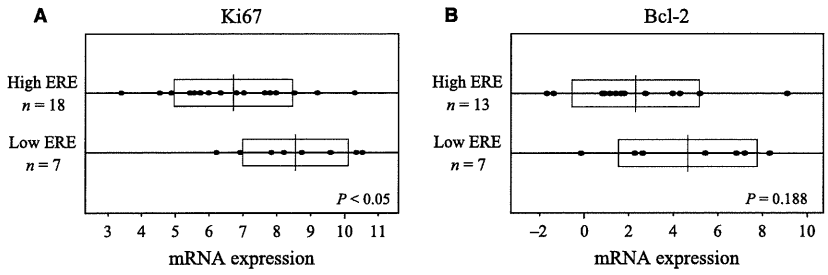

Figure 6. The intergroup difference of Ki67 and Bcl-2 mRNA expression in 28 Luminal A-type breast tumor samples divided into high- or low-ERE transcriptional activity groups. These box plots show the intergroup difference of (A) Ki67 and (B) Bcl-2 mRNA expression levels in each group.

ity and mRNA expression levels of Ki67 and Bcl-2 in Luminal A breast cancer samples. Interestingly, Ki67 mRNA expression was significantly higher in the low-ERE-activity group than in the high-ERE-activity group (Fig. 6A). Bcl-2 mRNA expression also tended to be higher in the low-ERE-group than in the high-ERE group (Fig. 6B). These genes are recognized as poor prognosis factors, but their mechanisms of action for breast cancer are not well defined. Therefore, further exploration of the relationship between ERE transcriptional activity, Ki67 and Bcl-2 may lead to mechanistic insights and explain why the latter two are higher in the group with low-ERE activity.

Discussion

ER is one of the most important transcription factors related to malignancy and proliferation in breast cancer. In this study, we focused on the function of ER as a transcription factor and analyzed human-derived breast cancer specimens according to three features: ER protein expression, mRNA expression profiles of ER target genes, and ERE transcriptional activity as an index for ER function. First, we analyzed ERE transcriptional activity in human breast cancer clinical samples by Ad-ERE-GFP assay. Ad-ERE-GFP assay is highly sensitive, even more than luciferase assays. In contrast to FACS, the Ad-ERE-GFP assay requires fewer cells and can measure the ERE activity of living cells in culture. Therefore, this assay is suitable for measuring transcriptional activity of heterogeneous clinical samples. Indeed, using the Ad-ERE-GFP assay, we demonstrated that primary breast cancer tumor cells exhibit various levels of ERE transcriptional activity in spite of ER positivity (Fig. 2A). The GFP fluorescence, an index of ERE transcriptional activity, was reduced by antiestrogen treatment with either Tamoxifen or Fulvestrant in almost all samples (Fig. 2B). However, several samples did not show drug sensitivity, especially to tamoxifen, suggesting that ER antagonism does not always correlate with inhibition of ER target gene transcription. ER genomic effects are activated not only by estrogen but also by its phosphorylation mediated by signaling pathways such as MAPK or PI3K/AKT pathway [12, 13]. The breast cancer cells in which GFP (ERE transcriptional activity) was not reduced in response to antiestrogenic drugs may have adopted these pathways.

Next, we compared ERE transcriptional activity with general clinicopathological information. These analyses revealed that ERE transcriptional activity had a tendency to correlate with ER protein expression levels (Fig. 3A) as well as menopausal status, but these data were not statistically significant. In contrast, a significant correlation was observed between ERE transcriptional activity and PgR protein expression levels (Fig. 3B). PgR protein expression has been clinically used for evaluating the function of ER activity [1], as confirmed by the present result with Ad-ERE-GFP assay. However, ERE transcriptional activity remains a better readout of ER function as PgR is just one many ER target genes and is regulated by many other transcription factors such as Sp1 or AP-1 [28, 29]. Additionally, the Ad-ERE-GFP assay excludes the influence of other transcription factors and therefore more directly reflects the function of the ER protein than PgR. Our results also demonstrated that ERE transcriptional activity does not correlate with ER protein expression. Together with the results of the drug sensitivity tests mentioned above, our data suggest that not only ER protein expression but also its functional evaluation should be determined to more accurately decide the treatment with most likely efficacy for ER-positive breast cancers.

To more fully investigate the relationship of ERE transcriptional activity to ER status and ER target gene expression, we classified ER-positive primary breast cancer samples into two groups of high- and low-ERE transcriptional activity as evaluated by Ad-ERE-GFP assay. Of note, the low-ERE-activity group had significantly higher ER mRNA expression levels than the high-ERE-activity group. In terms of expression levels of the six ER target genes examined, there were no significant intergroup dif-

ferences between high- and low-ERE-activity groups. These results suggest that there is a group in which ER does not effectively transmit estrogen signaling, in spite of high-ER protein expression. This may be because the ERE transcriptional activity is intercepted downstream, or different feedback mechanisms may exist for each target gene. Therefore, analyzing ERE transcriptional activity may help determine whether and how much the breast cancer depends on ER signaling.

Because many Luminal A-type breast cancers were contained in ER-positive samples, we extracted the Luminal A group from the ER-positive group and investigated its mRNA expression profiles (Fig. 5). FOXA1 and GATA3 have recently been reported to be associated with the Luminal type [5, 6, 26], and ER protein expression level clearly reflected their mRNA expression levels, especially for GATA3. Although the mRNA expression of both genes was not significantly different regardless of ERE transcriptional activity when all ER-protein-positive tumors were examined (Fig. 4B and C), subclassification of Luminal-type breast tumors into low- and high-ERE-activity revealed that these two groups had different correlation tendencies between ERα, FOXA1, and GATA3 mRNA expression levels and ER target genes. These results suggest that ERE activity can classify the Luminal A-type into two distinctions, whereby determination of ERE transcriptional activity may support the assessment of endocrine therapy efficacy. More interestingly, Ki67 and Bcl-2 tended to be higher in the low-ERE-activity group in ER-positive breast cancer (Fig. 6). Ki67 expression is a validated index of malignancy in breast cancer [3]. At the time of this research, local recurrence was found in two patients included in the Luminal A group. Both patients were also from the low-ERE-group, with measured GFP positivity of 7% and 16%, respectively. Although further work is required, the discrepancy in Ki67 and ERE transcriptional activity may help to explain the relationship between Ki67 and breast cancer.

It is widely known that there are individual differences in endocrine therapy efficacy despite ER positivity [2]. In this study, recategorization of breast cancer by ERE transcriptional activity suggests the possibility of distinguishing groups for whom endocrine therapy would be effective and ineffective. The range of treatment choices could also be expanded, especially in Luminal A-type breast cancer patients. We expect that ERE transcriptional activity could become an additional or surrogate marker for analysis of ER protein function and subsequently the improved treatment of breast cancer.

Acknowledgments

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Conflict of Interest

None declared.

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ER-activating ability of breast cancer stromal fibroblasts is regulated independently of alteration of *TP53* and *PTEN* tumor suppressor genes

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ABSTRACT

Carcinoma-associated fibroblasts (CAFs) are associated with tumor progression and metastasis, and are able to activate estrogen receptor (ER) in breast cancer. We established a stable transformant of a human breast cancer cell line to detect CAF-specific ER-activating ability, and found that this CAF ability varied among tumors. Some studies have reported a high frequency of alterations among tumor suppressor genes in stromal cells, but do not generally agree as to the frequency. Moreover, the activation mechanism of CAF-induced estrogen signals, including the effects of these gene aberrations, is not fully understood. We investigated the relevance of tumor suppressor gene aberrations and ER-activating ability in CAFs derived from 20 breast cancer patients. Although CAF-specific ER-activating abilities varied among individual cases, all CAFs maintained wild-type alleles for *TP53* and *PTEN*. Also, copy number aberrations in these genes were not observed in any CAFs. Our results suggest that the ER-activating ability of the CAFs is regulated independently of aberrations in these genes; and that other mechanisms of tumor–stromal interaction may affect activation of estrogen signals in breast cancer.

1. Introduction

Carcinoma-associated fibroblasts (CAFs) in proximity to epithelial tumor cells have been associated with tumor-promoting roles in various human carcinomas. In the human prostate cancer model, CAFs have been grown with initiated nontumorigenic epithelial cells, stimulated tumor growth and altered histology of epithelial cells [1]. Significant evidence also shows that CAFs regulate tumor angiogenesis in neuroblastoma and prostate cancer [2,3]. Secreted factors, cytokines and cell surface proteins of CAFs are also associated with metastasis in colon and other tumors [4–6]. However, the activation mechanism of CAFs during tumor development is not yet fully understood.

In breast cancer, CAFs are similarly associated with tumor growth, metastasis and poor clinical outcome, and enhance tumor angiogenesis in comingled breast cancer cells [7,8]. Over two thirds of breast cancers express estrogen receptors (ERs), which can be mediated by two distinct types of signaling, often referred to as the genomic pathway, and the non-genomic or non-genotropic pathways. Therefore ERα expression is predictive of response to endocrine therapy to reduce estrogen stimulation for proliferation. While selective ER modulators (SERMs), including tamoxifen, have been used as first-line hormonal therapy for postmenopausal patients for many years, aromatase inhibitors (AIs) including letrozole, anastrozole and exemestane have shown benefit by minimizing risk of early relapse in advanced disease; AIs are potent inhibitors of aromatase activity that locally converts androgens into estrogens in a variety of tissues including muscle, connective tissue, skin and liver [9,10]. The CAFs in the vicinity of breast cancer tissues are known to express aromatase [11,12], and are target of AIs, which have now largely replaced tamoxifen as first-line therapy in the postmenopausal breast cancer [13,14].

High mutation frequencies for *TP53* and/or *PTEN* were described in CAFs of breast cancer tissue [15,16]. In addition, loss of heterozygosity (LOH) in *TP53*, *PTEN* and other loci was reported in CAFs [15–17]; such mutations and CAF-specific LOH were associated with lymph node metastasis in sporadic breast cancer [16]. For an *in vivo* model of prostate cancer, Hill et al. found that the selective mutation of *p53* in reactive stroma accelerates spontaneous tumor progression [18]. Although these results suggest that

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stromal mutations affect tumor growth and progression, further research has revealed that such mutations are rare events in stromal fibroblasts; this question is still controversial [19–21].

Previously, we reported establishing a stable transformant of a human breast cancer cell line to detect CAF-specific ER-activating ability in the co-culture by transfection with the estrogen-responsive element–GFP. This system is a useful tool for analyzing local ER-related signals and tumor–stromal interactions [22] (Fig. 1). We reported that the ability of CAFs to activate ERs and sensitivity to AIs varied among tumors, and that the analysis of CAF characteristics in an individual breast cancer is essential to prediction of hormone therapy efficacy [22]. However, the mechanism underlying regulation of ER-activating ability in CAFs, including the effects of genomic instability, remains unknown. In this study, we focused on aberrations of the tumor suppressor genes *TP53* and *PTEN* in CAFs of breast cancer, and clarified their relevance to clinicopathological features and ER-activating ability in CAFs.

2. Materials and methods

2.1. Cells and culture conditions

The human breast cancer cell line ERE–GFP-E10, a MCF7 clone stably transfected with the d2E-green fluorescent protein (GFP) vector carrying the ptk-estrogen-responsive element (ERE) insert, was isolated and described previously [22]. ERE–GFP-E10 was maintained in RPMI-1640 medium (Sigma–Aldrich Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS).

Human breast cancer tissues were obtained by surgery at the Saitama Cancer Center Hospital (Saitama, Japan) after informed consent was obtained from the patients. The Saitama Cancer Center Ethics Committee approved this study. We have previously described the isolation procedure of intratumoral stromal cells and the characterization of CAFs obtained from individual breast cancer patients [22]. Isolated primary CAFs were maintained in modified minimum essential medium (MEM)-Alpha (Invitrogen, Carlsbad, CA) with 10% FBS. The ER-activating ability of CAFs was detected with GFP signals of ERE–GFP-E10 co-cultured with CAFs after pre-culture in phenol red-free RPMI 1640 with dextran-coated and charcoal-treated 10% FCS (DCC–FCS); ability to activate ER had been previously evaluated by the individual value of the ratio of GFP-positive cells for CAFs from 20 breast cancers [22].

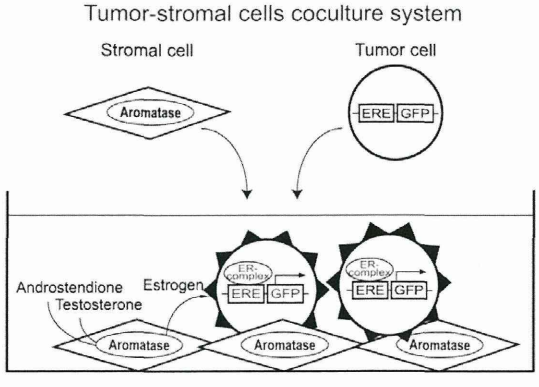

Fig. 1. Many breast cancer stromal fibroblasts can activate estrogen receptors (ER) and accelerate breast cancer proliferation and progression via the ER. Our established system detects ER-activating ability of CAFs by GFP signals of this human breast cancer cell line, and is useful for analysis of the local estrogen signaling pathway. ER: estrogen receptor; ERE: estrogen-responsive element.

2.2. Nucleic acid preparation

For genomic DNA isolation, a QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan) was used for CAFs from breast cancers and peripheral blood leukocytes from healthy volunteer, according to the manufacturer's instructions. DNA concentration and purity were determined by Nanodrop® ND-1000 Spectrophotometer (Laboratory & Medical Supplies, Tokyo, Japan), and then stored at −20 °C until analysis.

2.3. PCR amplification

The three fragments of *TP53* (corresponding to exon 4, exons 5–6 and exons 7–8) were amplified from genomic DNA extracted from CAFs for each case by polymerase chain reaction (PCR). The amplification conditions for exons 5–8 of *TP53* have been described in our previous report [23], with minor modifications. The primers for exon 4 were synthesized according to the IARC protocols of *TP53* direct sequencing (http://www-p53.iarc.fr/p53sequencing.html). Primer sequences of all sets are described in Additional file 1. The primers used for exons 1–9 of *PTEN* have been reported previously [24–26] (Additional file 1). PCR amplification was carried out in a total volume of 20 μl, consisting of 50 ng of DNA, 1× PrimeSTAR Buffer (Takara Bio Inc., Shiga, Japan), 200 μM dNTPs, 200 nM of each PCR primer, and 0.5 U of PrimeSTAR® HS DNA Polymerase (Takara Bio Inc.). PCR amplification was performed for 30–35 cycles of denaturation at 98 °C for 10 s, annealing at 55–63 °C for 5 s, and extension at 72 °C for 30 s. Annealing temperatures for each primer were shown in Additional file 1. Real-time RT-PCR was performed using a LightCycler® Carousel-Based System (Roche Diagnostics GmbH Mannheim, Germany) to analyze relative amounts of *CYP19A1* (Aromatae) mRNA; the averaged value of 12 samples in this standard curve method was used as cut-off value. The *CYP19A1* expression status of CAFs, including all 67 in our previous report [22], will be described elsewhere.

2.4. DNA sequence

Mutation analysis of *TP53* and *PTEN* was performed by direct sequencing. The purified PCR products were directly sequenced with upstream or downstream primers (Additional file 1) using Big Dye® Terminators v1.1 Cycle Sequencing Kit and ABI PRISM® 310 Genetic Analyzer (Life Technologies Corporation, Rockville, MD). The obtained nucleotide sequences were compared with the reference sequence of *TP53* and *PTEN* (GenBank accession number X54156 and AF067844, respectively).

2.5. Copy number analysis

TaqMan® Copy Number Assays (Life Technologies Corporation) were used to analyze loss of heterozygosity (LOH) of *TP53* and *PTEN* genes. PCR was performed using an Applied Biosystems 7300 Real-time PCR system (Life Technologies Corporation), and TaqMan® Copy Number Assays for *TP53* (Hs05516623_cn) and *PTEN* (Hs05177393_cn) were purchased from Life Technologies Corporation. PCR was performed with TaqMan® Genotyping Master Mix (Life Technologies Corporation) according to the manufacturer's instructions. PCR amplification was carried out in a total volume of 10 μl; the reaction mixture comprised 10 ng of DNA, 1× TaqMan® Copy Number Assay and 1× TaqMan® Copy Number Reference Assay RNase P. PCR amplification was performed using following conditions: 50 °C for 2 min, 95 °C for 10 min; and then 45 cycles of 95 °C for 15 s and 60 °C for 1 min. All PCR was performed in duplicate for each sample. Data analysis was carried out using the software CopyCaller v1.0 (Life Technologies

Table 1
Estrogen receptor (ER)-activating ability of CAFs and clinicopathological features of breast cancer patients ($n = 20$).

		ER-activating ability	
		<10% ($n = 7$)	⩾10% ($n = 13$)
ER, *n*	Positive	3	10
	Negative	4	3
PgR, *n*	Positive	1	4
	Negative	6	9
Histology, *n*	Papillotubular Ca.	0	2
	Solid-tubular Ca.	4	5
	Scirrhous Ca.	1	5
	Special types	1	1
	Invasive micropapillary Ca.	1	0
Tumor size, *n*	T1	4	9
	T2	2	4
	T3	1	0
Lymph node metastasis, *n*	N0	3	4
	N1	4	7
	N2	0	1
	Unknown	0	1
Menopausal status, *n*	Premenopausal	2	7
	Postmenopausal	5	6
Aromatase mRNA. *n*	High expression	3	2
	Low expression	3	4
	Unknown	1	7

ER and PgR status was determined using the Allred scoring system or enzyme immunoassay (EIA).
Tumor Allred scores ⩾3, or EIA ⩾15 fmol/mg were considered to be positive specimens in this study.

Corporation). Peripheral blood leukocyte DNA from a healthy volunteer was used to calibrate each experiment.

3. Results

3.1. Patient population and ER-activating ability of CAFs

The ER-activating ability of CAFs and clinicopathological features of the patients included in the study are listed in Tables 1 and 2. ER-activating ability of CAFs was evaluated by co-culture system with ERE–GFP-E10 cells in the presence of testosterone, an aromatase substrate. The ratio of GFP^+ cells was evaluated. Among the 20 breast cancer patients, 7 (35%) showed low ER-activating ability of CAFs (GFP^+ E10 cells <10%) and 13 (65%) were high ER-activating ability of CAFs (GFP^+ E10 cells ⩾10%). Levels similar to estrogen-induced ER-activation were detected in 3 out of 13 high ER-activating patients.

3.2. TP53 and PTEN sequence analysis of CAFs

Many CAFs activate ER in tumor cells by genomic or non-genomic pathways through the estrogen or growth factors, which are produced by the CAF itself. Because CAFs are reportedly associated with high frequency of genetic aberration, CAF-specific ER-activating ability may be affected by genomic instability. To clarify whether the aberrant tumor suppressor genes in CAFs affect the ER-activation, we performed the mutation analysis of *TP53* and *PTEN* gene. The CAF-derived DNA was analyzed for *TP53* mutations in exons 4–8 that corresponded to the sequence-specific DNA-binding domain, and for *PTEN* mutations in exons 1 and 3–9. As in the previous report [25], we could not detect exon 2 of *PTEN*, despite using two different primer sets. As shown in Table 2,

Table 2
Mutation analysis of *TP53* and *PTEN* in CAFs.

CAF No.	GFP positive rate (%)	*TP53*	*PTEN*
1	4.6	wt	wt
2	27.3	wt	wt
3	27.5	wt	wt
4	2.8	wt	wt
5	9.3	wt	wt
6	1.0	wt	wt
7	40.5	wt	wt
8	38.8	wt	wt
9	31.0	wt	wt
10	38.2	wt	wt
11	5.0	wt	wt
12	28.5	wt	wt
13	9.3	wt	wt
14	13.1	wt	wt
15	28.5	wt	wt
16	14.5	wt	wt
17	23.7	wt	wt
18	12.5	wt	wt
19	9.3	wt	wt
20	16.2	wt	wt

wt, wild-type.

mutations were not found among the regions of these genes in all 20 CAF samples. We also examined the genotypes of ten SNP sites to evaluate LOH using this sequence analysis. Seven out of 10 SNP sites (rs55950612, rs56196266, rs1642786, rs1642787, rs1642788, rs1794288, rs35979531) showed homozygous genotypes in all 20 CAF cells (data not shown), and 3 SNP sites showed a heterozygous genotype in at least one sample (Table 3). These results have shown that LOH had not occurred at least in 12 CAF samples for *TP53* and in 1 sample for *PTEN*.

3.3. Copy number analysis of TP53 and PTEN

The copy number of the *TP53* and *PTEN* gene was evaluated by quantitative real-time PCR-based Copy Number Assays for determining LOH. Copy number of *TP53* ranged from 1.73 to 2.39 copies; copy number aberrations were not observed in any CAFs (Fig. 2A). Although the calculated copy number of *PTEN* had extended more widely than that of *TP53*, LOH at these loci was not detected in any CAFs (Fig. 2B; range: 1.62–3.41). Four cases out of the 20 CAFs examined were predicted to show three copies of *PTEN*, with copy numbers of 2.84, 2.85, 3.05 and 3.41 (Fig. 2B). In these cases, no clear correlation was found between the ER-activating ability and *PTEN* gene copy number in CAFs. These results were true of both premenopausal and postmenopausal patients (Fig. 2). In addition, correlation between gene copy number and ER protein expression in tumor specimens were not found (data not shown).

4. Discussion

In the present study, we investigated genomic alterations of *TP53* and *PTEN* genes in breast cancer CAFs, and for the first time, examined their correlation with ER-activating ability of CAFs and clinicopathological features of tumors. Although various ER-activating abilities were detected in individual CAFs, all CAFs tested in this study maintained wild-type alleles for the genes. In addition, in contrast to previous reports [15,16,27], none of these breast-cancer CAFs showed any evidence of LOH in these genes. This genomically stable phenotype in all 20 CAFs agrees with two previous reports that aberration of these genes in CAFs is a rare event [19,21]. Therefore, our results suggest that the ER-activating ability of the CAFs is regulated independently of

Table 3
The genotypes of three SNP sites in CAFs.

CAF No.	*TP53*		*PTEN*
	rs.1042522	rs.12951053	rs.1799734
1	GCG	TTC	TTATC
2	GCG/GGG	TTC	TTATC
3	GCG/GGG	TTC/TGC	TTATC
4	GCG/GGG	TTC/TGC	TTATC
5	GCG/GGG	TTC/TGC	TTATC
6	GCG/GGG	TTC	TTATC/ –
7	GCG	TTC	– / –
8	GCG	TTC	TTATC
9	GCG/GGG	TTC	TTATC
10	GCG/GGG	TTC/TGC	TTATC
11	GGG	TGC	TTATC
12	GCG/GGG	TTC/TGC	TTATC
13	GCG	TTC	TTATC
14	GCG/GGG	TTC/TGC	TTATC
15	GCG	TTC	TTATC
16	GCG/GGG	TTC/TGC	TTATC
17	GCG	TTC	TTATC
18	GCG	TTC	TTATC
19	GCG/GGG	TTC/TGC	TTATC
20	GCG/GGG	TTC/TGC	TTATC

rs, reference SNP clusters number in NCBI's dbSNP database.

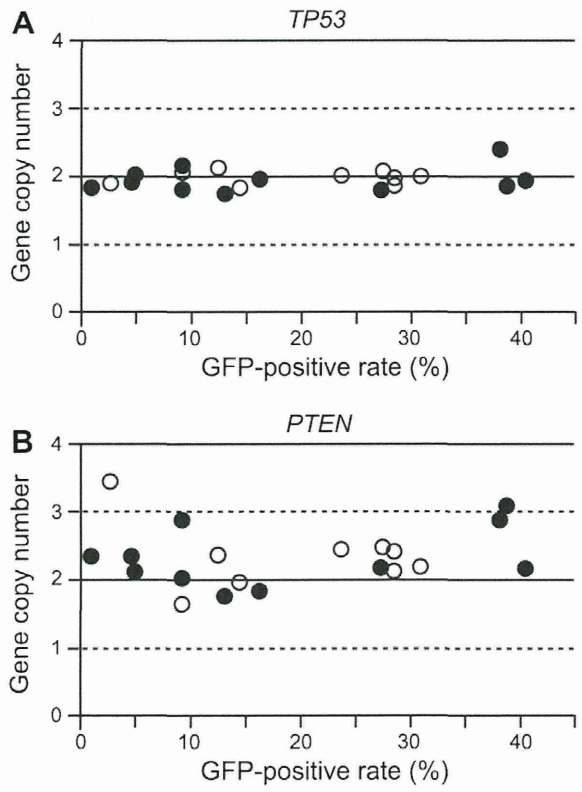

Fig. 2. Copy number analysis and GFP-inducing ability of CAFs. (A and B) Quantitative real-time PCR-based copy number assays for determining loss of heterozygosity (LOH) in CAFs show normal copy number variations of *TP53* (A) and *PTEN* (B). Open and closed circles indicated the menopausal status of premenopausal and postmenopausal patients, respectively.

aberrations in these tumor suppressor genes, and that the mutation or LOH of the genes is rare event.

High frequency mutations and LOH of stromal cells in the previous reports by Kurose et al. and by Patocs et al. have been investigated using DNA derived from microdissected formalin-fixed paraffin-embedded (FFPE) tissues [15,16]. Although obtaining LOH and mutation data from FFPE-derived DNA is a well-established method, low concentrations or low-yield template DNA for PCR might lead to false conclusions [28]. Consequently, the existence of frequent genetic alterations in CAFs is still controversial [19–21]. In this study, we investigated DNA from short-term cultured mammary fibroblasts, which were isolated from carcinoma-associated regions of tumor masses obtained from breast cancer patients. This isolation method can establish adipose stromal fibroblast cells from tumor masses [29], and avoids contamination by tumor cells and other effects.

In the present experiments, we showed that copy numbers were below the cut-off value at 4 copies, which indicates amplification, although the slight increase in copy number of *PTEN* gene was detected in four out of 20 CAFs. No relation was also found between the CAF-specific ER-activating ability and the gene copy number amplification. Somatic mutation and/or deletion of the *PTEN* tumor suppressor gene have been shown to play a crucial role in proliferation and cell survival [30,31]. While the copy number gain of *PTEN* gene in breast cancer does not significantly affect protein levels [32]. Therefore, it can be speculated that the PTEN in our CAFs is not the main regulating factor in activation of ER in tumor cells. However, two previous studies have reported genome-wide copy number analysis of breast CAF samples, and reported 2 CAFs to show genomic alterations in several loci of chromosomes [19,21]. Therefore, the effects of CAFs with low copy amplification of several genes remain to be elucidated.

As for the analysis of CAF-specific ER-activating ability, the detection system of the GFP-based ERE element had been established by our previous report (Fig. 1). We demonstrated that CAFs of postmenopausal patients did not always have high ER-activating ability. Furthermore, it was shown that GFP induction levels did not always correlate with expression of the aromatase gene in CAFs [22]. These results suggest that the ERE–GFP system is not only activated by the estrogen-dependent pathway, but also by estrogen-independent pathways, such as phosphorylation by growth factor-inducing signals. Nevertheless, our results demonstrated that *TP53* or *PTEN* mutation was not the main regulator in either pathway. In this study, we concluded that CAF-specific ER-activating ability is regulated independently from genetic aberrations; however, methylation patterns of several gene regions in tumor stroma have been shown to be distinctly different from normal breast tissue in one report [33]. Furthermore, other epigenetic modifiers of stromal fibroblasts, such as a microRNA that critically affects tumor suppressor function, have also been reported [34,35]. The functional contribution of ER-activation by CAFs in this microenvironment is still unclear; further studies are needed.

Authors' contributions

T.S. carried out the molecular biological studies and drafted the manuscript. H.O. measured aromatase transcripts with RT-PCR. H.O. and M.K. performed pathological analysis. H.T. collected and analyzed clinical data from breast cancer patients. S.H. reviewed all data, and contributed to the preparation of the manuscript. Y.Y. directed the overall project, and participated in the editing of final manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.035.

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