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Estrogen-related receptor γ modulates cell proliferation and estrogen signaling in breast cancer

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ABSTRACT

Breast cancer is primarily a hormone-dependent tumor that can be regulated by status of steroid hormones including estrogen and progesterone. Estrogen-related receptors (ERRs) are orphan nuclear receptors most closely related to estrogen receptor (ER) and much attention has been recently paid to the functions of ERRs in breast cancer in terms of the interactions with ER. In the present study, we investigated the expression of ERRγ in human invasive breast cancers by immunohistochemical analysis ($n = 110$) obtained by radical mastectomy. Nuclear immunoreactivity of ERRγ was detected in 87 cases (79%) and tended to correlate with the lymph node status. No significant associations were observed with other clinicopathological characteristics, including the expression levels of both estrogen and progesterone receptors. In MCF-7 breast cancer cells, we demonstrated that ERRγ mRNA was up-regulated dose-dependently by estrogen, and that this up-regulation of ERRγ mRNA by estrogen was abolished by ICI 182,780 treatment. We also demonstrated that exogenously transfected ERRγ increased MCF-7 cell proliferation. Furthermore, ERRγ enhanced estrogen response element (ERE)-driven transcription in MCF-7 cells. In 293T cells, ERRγ could also stimulate ERE-mediated transcription with or without ERα. These results suggest that ERRγ plays an important role as a modulator of estrogen signaling in breast cancer cells.

1. Introduction

Estrogen-signaling pathways are involved in the growth and development of breast tumors through the activation of estrogen receptor α (ERα) [1]. The cells of most breast cancers express high levels of ERα and exhibit estrogen-dependent proliferation. ERs are the members of the nuclear receptor superfamily and regulate various cellular events, including cell growth and apoptosis, by acting as transcription factors activating the expression of target genes. Therefore, comprehensive understanding of estrogen-signaling pathways in breast cancer is required for both treatment and diagnosis of the disease.

Recently, several researchers have focused on estrogen-related receptors (ERRs) as modulating factors for estrogen-signaling pathways [2,3]. ERRs (ERRα, ERRβ, and ERRγ) are orphan nuclear receptors that possess certain homologies to ER but cannot bind estrogen. In ERR-mediated transcriptional activation, coactivators are required in the interaction between the receptors and basal transcriptional machinery. Among such coactivators, peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) and PGC-1β have been revealed to play important roles in ERR-mediated transcription [4–6]. In addition to PGC-1α/β, other coactivators have been shown to associate with both ERRs and ERs, suggesting that transcriptional cofactors are partially shared between ERs and ERRs. Furthermore, ERRs can bind to estrogen response elements (EREs) as well as ERR response elements (ERREs), suggesting that ERRs can affect ER-mediated signaling.

In vivo functions of ERRα and ERRγ were partly revealed using knockout mice. ERRα-knockout mice are viable but exhibit a phenotype characterized by reduced body weight, peripheral fat deposits, and resistance to high-fat diet-induced obesity [7]. On the

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other hand, ERRγ-null mice die during the early postnatal period as a result of abnormal heart function; these mice fail to make the transition at birth from the utilization of glucose as a fetal energy substrate to mitochondrial fatty-acid oxidation (FAO) [8]. The expression of ERRα is more abundant than that of the other 2 ERR subtypes and is detected in tissues with high metabolism, such as the heart, kidney, intestinal tract, skeletal muscle, and brown adipose tissue [9]. The expression patterns of ERRβ and ERRγ are more restricted, but these receptors are abundantly expressed in the heart and kidneys [9,10]. We have previously shown that ERRα and ERRγ are upregulated in preadipocyte cells and pluripotent mesenchymal cells under adipogenic conditions and that they positively regulate lipid accumulation in preadipocyte cells [11,12].

On the other hand, ERR is thought to be involved in the development of human cancer [13,14]. Expressions of ERRα and ERRγ mRNAs are associated with an unfavorable and favorable prognosis of breast cancer, respectively [15]. Expression of ERRα protein in breast tumors correlates with an increased risk of recurrence and a poor prognosis [13]. In human prostate cancer, patients with high ERRα and low ERRγ immunoreactivities show poor cancer-specific survival [16]. However, there has been no study investigating the association of ERRγ protein expression with breast cancer, and its role is yet to be elucidated.

In the present study, we evaluated the expression of ERRγ in human breast cancers by using immunohistochemistry; we then investigated the correlation between the ERRγ expression levels and clinicopathophysiological findings. Furthermore, we showed the estrogen-induced expression of ERRγ in human breast cancer MCF-7 cells and the stimulating effects of ERRγ on proliferation of MCF-7 cells. Finally, we revealed that ERRγ elevates ER-mediated transcription.

Table 1
Relationship between immunoreactivity of ERRγ and clinicopathological findings in invasive breast cancer ($n = 110$).

Clinical findings	Immunoreactive score of ERRγ[a]		*P* value
	High ($n = 87$)	Low ($n = 23$)	
Age	59.0 ± 14.7	59.8 ± 14.7	0.83
≤50	24	7	0.99
>50	63	16	
Menopause			
Pre	25	6	0.99
Post	62	17	
pT			
≤20 mm	41	13	0.61
>20 mm	29	6	
Stage			
I, II	81	22	0.97
III, IV	6	1	
Grade			
I	32	8	0.95
II, III	32	9	
ER			
Positive (PS ≥ 3)	57	13	0.43
Negative (PS ≤ 2)	30	10	
PgR			
Positive (PS ≥ 3)	35	8	0.78
Negative (PS ≤ 2)	51	15	
HER2			
Positive	19	7	0.28
Negative	58	10	
Lymph node			
Positive ($n \geq 4$)	12	0	0.06
Negative ($n \leq 3$)	51	16	

ER, estrogen receptor; ERR, estrogen-related receptor; PgR, progesterone receptor; TS, total score; PS, proportion score.
[a] ERRγ immunoreactive scores of 0–3 and 4–8 were defined as low and high immunoreactivity, respectively.

2. Materials and methods

2.1. Tissue selections and patient characteristics

Between January 2005 and March 2006, 110 consecutive patients were diagnosed with invasive breast cancer using a vacuum-assisted biopsy device (Mammotome®, Ethicon Endo-surgery, Inc., Cincinnati, OH) at Saitama Medical University Hospital. Formalin-fixed, paraffin-embedded sections obtained by biopsy or surgery were used in this study. The study was approved by the institutional review board at Saitama Medical University, and informed consent was obtained from all patients. Patient age ranged from 32 to 89 years (mean, 59.5). The clinicopathological characteristics of the series are presented in Table 1.

2.2. Antibodies

Anti-Flag M2 and anti-β-actin antibodies were purchased from Sigma–Aldrich (St. Louis, MO). Anti-ERRγ antibody was generated from rabbit serum using a glutathione S-transferase (GST) fusion protein with amino acids 2–51 of human ERRγ protein as an antigen. The antiserum was then purified using an affinity column filled with GST protein-coupled Affi-Gel 10 (Bio-Rad, Hercules, CA) to remove anti-GST antibody. The characterization of the antibody was previously confirmed by western blot analysis in pcDNA3-Flag-hERRγ-transfected 293T cells [16].

2.3. Immunohistochemistry

Immunohistochemical analysis for ERRγ was performed using an EnVision+ visualization kit (Dako, Carpinteria, CA), as previously described [16]. Tissue sections (6 μm) were deparaffinized, rehydrated through graded ethanol, and rinsed in Tris-buffered saline with 0.05% Tween-20 (TBST). To retrieve the antigens, the sections were heated in an autoclave at 121 °C for 10 min in 10 mM sodium citrate buffer (pH 6.0). The sections were blocked with endogenous peroxidase using 0.3% H_2O_2 and incubated in 10% bovine serum for 30 min. The primary antibody, a polyclonal antibody for ERRγ (1:1000 dilution), was applied and incubated at 4 °C overnight. The sections were rinsed in TBST and incubated with EnVision+ and anti-rabbit antibody for 1 h at room temperature. The antigen-antibody complex was visualized with 3, 3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer [pH 7.6], and 0.006% H_2O_2). As a positive control, a section of human kidney tissue was immunostained with the anti-ERRγ antibody in the same manner as described above. Rabbit IgG was used in place of the primary antibody as a negative control.

2.4. Immunohistochemical assessment

Slides were evaluated for the proportion (proportion score [PS]: (0) none; (1) <1/100; (2) 1/100-1/10; (3) 1/10-1/3; (4) 1/3-2/3; and (5) >2/3) and staining intensity (intensity score [IS]: (0) none; (1) weak; (2) moderate; and (3) strong) of positively stained cells. The total immunoreactivity score (TS: 0, 2–8) was determined as the sum of the proportion and intensity scores [17]. Two investigators (H.T. and A.O.) evaluated the tissue sections independently. If the immunoreactivity score differed between the 2 investigators, a third investigator (T.S.) evaluated the tissue sections, and the average immunoreactivity score was used. When the 2 investigators found it difficult to evaluate the TS of the heterogeneous cancerous lesions, the third investigator estimated the latter and decided the immunoreactivity score. We defined a TS of 3 as the cut-off for high ERRγ immunoreactivity to identify a potential correlation between ERRγ expression in the malignant epithelium and clinicopathological characteristics.

2.5. Statistical analyses

The correlation between the immunoreactivity score and clinicopathological characteristics was evaluated with the chi-square test. P values < 0.05 were regarded as statistically significant. Differences between the 2 groups in luciferase and cell proliferation assays were analyzed using a 2-sample, 2-tailed Student's t test. A P value < 0.05 was considered to be significant. All data are presented in the text and figures as the mean (standard deviation (SD)).

2.6. Plasmid construction

Human ERα (hERα, amino acids 2-595) and ERRγ (hERRγ, amino acids 2-458) were N-terminally tagged with Flag and subcloned into pcDNA3 vector (pcDNA3-Flag-hERα and pcDNA3-Flag-hERRγ, respectively).

2.7. Cell culture and transfection

The 293T and MCF-7 cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. 17β-estradiol and ICI 182,780 were purchased from Sigma and Tocris Bioscience, respectively. Transfection of hERRγ was performed using 2 μg of pcDNA3-Flag-hERRγ and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 48 h, cell extracts were analyzed by western blot analysis.

2.8. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA extraction, first-strand cDNA synthesis, quantitative PCR, and primer sequences have been described elsewhere [11,18,19]. Fold induction of mRNA expression levels was determined by comparing the mRNA levels of the estrogen-treated samples with those of the vehicle-treated control.

2.9. Luciferase assay

MCF-7 and 293T cells were plated in 24-well culture plates at a density of 10,000 cells/well in phenol red-free medium containing 5% charcoal-stripped serum and transfected with 0.1 μg of ERE-tk-luc [20], together with 0.02 μg of pRL-cytomegalovirus (CMV; Promega, Madison, WI) using a Lipofectamine 2000 reagent (Invitrogen). Twelve hours after transfection, cells were treated with 100 nM 17β-estradiol or vehicle (ethanol) for 24 h, and luciferase activities were determined by a MicroLumatPlus microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) using a Dual-Luciferase Assay System (Promega). Data are expressed as mean (SD) of 3 independent experiments performed in triplicate.

2.10. Cell proliferation assay

MCF-7 cells were seeded in 96-well plates at a density of 5000 cells/well in phenol red-free medium containing 5% charcoal-stripped serum for 24 h. Then, pcDNA3-Flag or pcDNA3-Flag-hERRγ was transfected for 12 h and incubated with 100 nM estradiol or vehicle for 72 h. Cell proliferation was examined by a tetrazolium salt (WST-8) assay kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol.

3. Results

3.1. Correlation of ERRγ protein expression with clinicopathological values of invasive breast cancer

To investigate the expression levels of ERRγ protein in breast cancer, immunohistochemical analysis was performed using 110 invasive breast cancers (Fig. 1). Strong nuclear immunoreactivity of ERRγ was detected in 79% of breast cancer specimens (Fig. 1A and C). A human kidney tissue was immunostained with the ERRγ antibody as a positive control, and ERRγ immunoreactivity was also observed in the nuclei of kidney tubule cells (Fig. 1E). Statistical analysis showed that the nuclear immunoreactivity of ERRγ tended to correlate with lymph node status (P = 0.06) while no significant associations were found with other clinicopathological characteristics (Table 1). In the DCIS component of the ERRγ-positive invasive carcinomas, ERRγ immunoreactivity was detected in the nucleus. ERRγ immunoreactivity was also detected in the nuclei of normal mammary epithelium and intratumoral stromal cells (data not shown).

3.2. Upregulation of ERRγ in estrogen-treated MCF-7 cells

Next, we examined the expressional regulation of 3 ERR genes (ERRα, ERRβ, and ERRγ) by estrogen in an ERα-positive breast cancer cell line (MCF-7) using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 2A–C). ERRγ mRNA level was significantly up-regulated by 6-fold at 3 h after estrogen stimulation. ERRα mRNA was also slightly up-regulated by 2.5-fold in a time-dependent manner while ERRβ mRNA was not largely influenced by estrogen. In addition, ERRγ mRNA was up-regulated dose-dependently by estrogen (Fig. 2D). This estrogen-dependent up-regulation of ERRγ mRNA was abolished by ICI 182,780 treatment, while ICI 182,780 itself did not up-regulate ERRγ mRNA expression (Fig. 2D). These results suggested that ERRγ expression is regulated by estrogen.

3.3. ERRγ contributes to estrogen-dependent and estrogen-independent proliferation in breast cancer cells

To further assess the role of ERRγ in breast cancer, we performed a gain-of-function study for ERRγ. Under an estrogen-deprived culture condition, ERRγ-overexpressing MCF-7 cells exhibited a significantly higher growth rate compared with control cells expressing empty vector at days 3 and 4 (Fig. 3A). Furthermore, growth of ERRγ-overexpressing MCF-7 cells was also stimulated in the presence of 100 nM estrogen (Fig. 3B). We confirmed that the ERRγ protein was overexpressed in MCF-7 cells after transient transfection with ERRγ expression plasmid by immunoblotting (Fig. 3C). These results indicate that ERRγ promotes proliferation of breast cancer cells regardless of the presence or absence of estrogen.

3.4. ERRγ promotes ER-mediated transcription

To examine whether ERRγ influences ER-ERE-mediated transcription, a luciferase reporter vector containing an ERE (ERE-tk-luc) was introduced into 293T cells with or without ERRγ expression vector (Fig. 4A). The result showed that ERRγ significantly stimulated ER-ERE-mediated transactivation in 293T cells when the cells were transfected with ERα and treated with estrogen. We also observed that the estrogen-dependent transactivation was elevated depending on the increasing amount of ERRγ in MCF-7 cells (Fig. 4B). These results indicate that ERRγ stimulates ER transcription activity in response to estrogen. Next, we examined the effect of ERRγ on ERE-mediated transcription under the

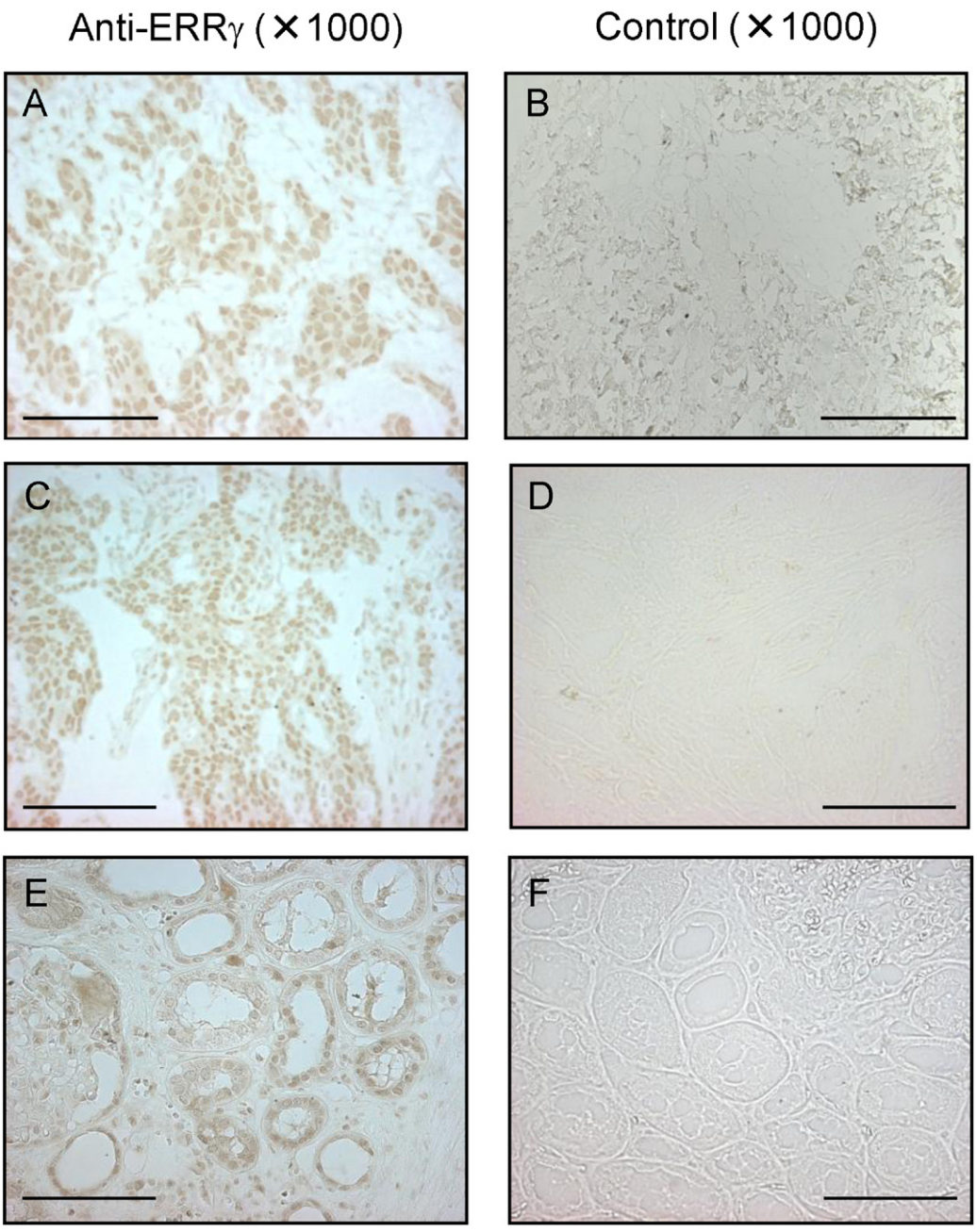

Fig. 1. Immunohistochemistry of estrogen-related receptor (ERR) γ in breast cancer. Representative immunohistochemical staining of breast cancer tissues (A–D) and normal kidney tissue (E and F) with anti-ERRγ (A, C, and E) and rabbit IgG (B, D, and F). Positive staining for ERRγ was observed in the nuclei of breast cancer cells, as well as of kidney tubule cells. Bar, 100 μm.

estrogen-free condition. Intriguingly, the transcriptional activity of the ERE-luciferase reporter construct was dose-dependently stimulated by ERRγ in an estrogen-independent manner (Fig. 4C).

4. Discussion

In the present study, ERRγ immunoreactivity was detected in 79% of invasive breast cancers ($n = 110$) by immunohistochemistry and tended to correlate with the lymph node status ($P = 0.06$). Generally the ER-positive breast cancer has a better prognosis than ER-negative one. Consistently with this observation, immunostainings of some estrogen responsive genes, such as progesterone receptor [21], were known to be correlated with a good prognosis. On the other hand, immunostainings of some estrogen responsive genes, such as cathepsin D [22], ERRα [13,23] and Efp [24], were shown to be as poor prognostic factors. Indeed, the immunoreactivities of Efp and ERRα in breast cancer specimens were reported to be positively correlated with lymph node status. Besides, as demonstrated in the present study, ERRγ could function as an estrogen responsive gene and facilitate the proliferation of MCF-7 cells. Notably, ERRγ could also stimulate the ERE-mediated transcription by itself. These findings may explain that the immunoreactivity of ERRγ tends to be correlated with the lymph node status in breast cancer. In our immunohistochemical analysis of invasive breast carcinomas, no significant association between ERRγ and ERα expressions was observed. Although the present study is the first to evaluate the expression of ERRγ protein in human breast cancers by using immunohistochemistry, a previous association study reported that overexpression of ERRγ mRNA is associated with ER-positive and PgR-positive status in primary breast tumors

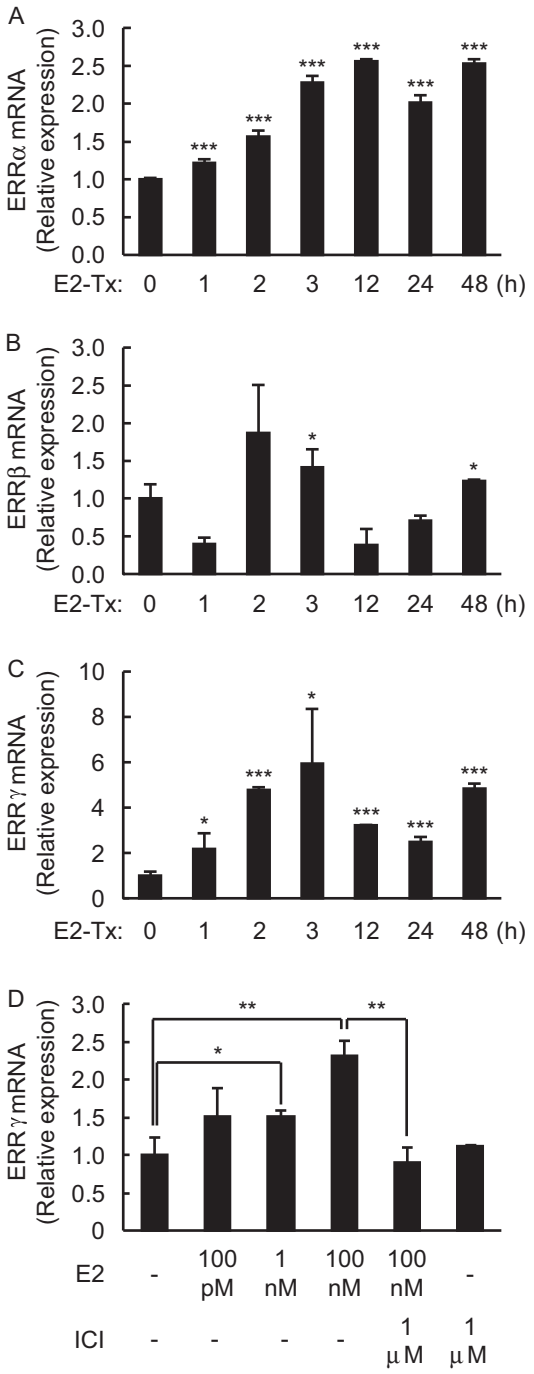

Fig. 2. Regulation of ERRs in MCF-7 cells by estrogen stimulation. (A–C) MCF-7 cells were treated with 100 nM 17β-estradiol for 48 h. ERRα (A), ERRβ (B), and ERRγ (C) mRNA levels were examined at indicated time points by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), and the results are shown as fold change over the expression level at 0 h. $^{*}P < 0.05$ compared with 0 h; $^{***}P < 0.001$ compared with 0 h (by Student's *t* test). (D) MCF-7 cells were treated with 17β-estradiol (100 pM, 1 nM, or 100 nM) and/or ICI 182,780 (1 μM), or vehicle for 2 h. ERRγ mRNA levels were examined by qRT-PCR, and the results are shown as fold change over the expression level with vehicle treatment. $^{*}P < 0.05$ compared with vehicle treatment; $^{**}P < 0.01$ compared with vehicle treatment or 100 nM E2 treatment (by Student's *t* test).

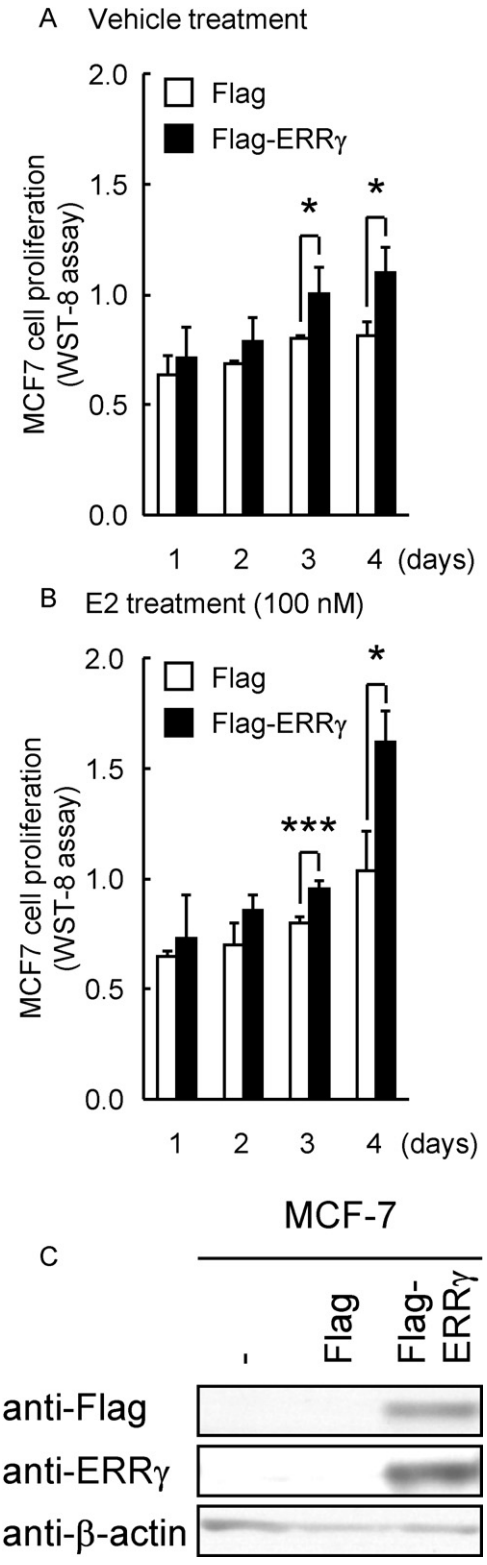

Fig. 3. ERRγ overexpression promotes estrogen-dependent and estrogen-independent proliferation of MCF-7 cells. (A and B) MCF-7 cells were transfected with pcDNA3-Flag-hERRγ for 24 h and then treated with vehicle (A) or 100 nM 17β-estradiol (E2) (B) for 4 days. Cell proliferation was examined using a tetrazolium salt (WST-8) assay kit. $^{*}P < 0.05$ compared with vehicle; $^{***}P < 0.001$ compared with vehicle (by Student's *t* test). (C) Total cell lysates from the parental MCF-7 cells (−) or the MCF-7 cells transfected with pcDNA3-Flag (Flag) or pcDNA3-Flag-hERRγ (Flag-ERRγ) for 48 h were immunoblotted by anti-Flag, -ERR, or -β-actin antibodies.

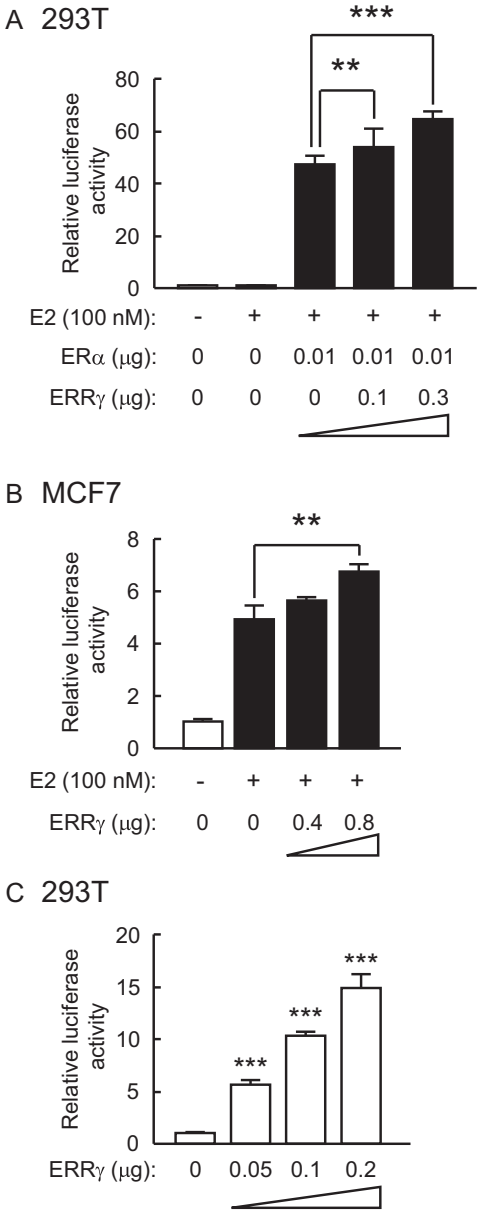

Fig. 4. Effect of ERRγ overexpression on ERE-mediated transcription activity in 293T (A and C) and MCF-7 (B) cells. (A) The 293T cells were transfected with a DNA mixture of 100 ng of estrogen response element (ERE)-tk-Luc, 0.02 μg of pRL-CMV with or without 0.01 μg of pcDNA3-Flag-ERα, and increasing amounts of pcDNA3-Flag-hERRγ. After a 12-h incubation, cells were treated with 17β-estradiol (100 nM, E2) or vehicle (EtOH) for 24 h. (B) MCF-7 cells were transfected with a DNA mixture of 100 ng of ERE-tk-Luc, 0.02 μg of pRL-CMV, and increasing amounts of pcDNA3-Flag-hERRγ. After a 12-h incubation, cells were treated with 17β-estradiol (100 nM) or vehicle for 24 h. (C) The 293T cells were transfected with a DNA mixture of 100 ng of ERE-tk-Luc, 0.02 μg of pRL-CMV, and increasing amounts of pcDNA3-Flag-hERRγ, and incubated for 36 h. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with corresponding conditions with no ERRγ transfection (by Student's *t* test).

($n = 38$) [15]. One possible explanation for this discrepancy is that the expression level of ERRγ protein did not necessarily correlated with that of ERRγ mRNA in breast cancer cells. Alternatively, this may have resulted from the differences in numbers or types of breast tumor specimens. One third of the clinical cases of ER-positive/PR-positive breast tumor patients treated with tamoxifen do not respond to initial treatment, and the remaining 70% are still at risk for relapse in the future. Riggins et al. recently reported that ERRγ expression was increased in a tamoxifen-resistant invasive lobular carcinoma (ILC) cell model [25]. In line with this notion, we observed that the ERRγ mRNA level in ER-negative breast cancer MDA-MB-231 cells was higher than that in MCF-7 cells (data not shown), suggesting that ERRγ could be involved in tamoxifen resistance of breast cancer cells.

In the present study, we demonstrated that ERRγ expression is stimulated dose-dependently by estrogen in MCF-7 breast cancer cells, while this stimulation was abolished by ICI 182,780 (Fig. 2C and D). Supporting our results, several estrogen receptor-binding sites (ERBSs) have been found within the second intron of the human ERRγ gene by genome-wide chromatin immunoprecipitation (ChIP)-on-chip analysis using MCF-7 cells [26]. Thus, it is possible that ERRγ expression is regulated by ER through these ERBSs. In addition, we found that ERRγ overexpression promotes the growth of MCF-7 cells. Moreover, a transcriptional reporter assay revealed that ERRγ enhances ER-mediated transcription in MCF-7 cells. ERR recognizes not only the consensus sequence TCAAGGTA, referred to as ERRE, but also the ERE that is bound to ER [10]. Thus, ERR control of the transcription of target genes partly overlaps with ERα [2,10,27]. It has also been reported that ERR associates with ER and modulates ER-mediated transcription [28,29]. Besides, ERRγ itself could stimulate the ERE-mediated transcription in ER-negative 293T cells in an estrogen-independent manner. These findings together with our data suggest that ERRγ, a downstream target of ERα itself, could stimulate the growth of breast cancer cells by modulating estrogen-signaling pathways or transcriptional activity of ERα.

Our data and a previous report indicate that, as in the case of ERRγ mRNA, expression of ERRα mRNA is induced by estrogen in MCF-7 cells [23]. ERRα immunoreactivity was also noted to be significantly associated with an increased risk of recurrence and adverse clinical outcome in breast cancer, but it does not correlate with ERα immunoreactivity [13]. Although ERR possesses characteristics that are structurally and functionally similar to ER, ERR has no natural ligand and regulates expression of target genes that are distinct from those of ER, except for coregulated genes. Therefore, ERR is assumed to have ER-independent functions in breast cancer. For example, ERRs are implicated in the transcriptional response to hypoxia and the growth of solid tumors. The development of intratumoral hypoxia is a universal hallmark of rapidly growing solid tumors, and the adaptive response to hypoxia is mediated primarily through the hypoxia-inducible factor (HIF)-dependent transcriptional program. HIF regulates gene networks involved in glucose uptake and metabolism in tumor angiogenesis. ERRγ, as well as ERRα and ERRβ, can physically interact with HIF1α, HIF2α, and HIF1β both *in vitro* and *in vivo* to enhance HIF-mediated gene transcription, suggesting that ERRs may be required for HIF function [30].

In summary, our results suggest that ERRγ expression is induced by estrogen in breast cancer cells, and expression of this receptor promotes cancer cell proliferation by enhancing ERE-mediated transcription. These results further suggest that pharmacological modulation of ERRγ activity may be clinically useful to prevent and/or treat breast cancer.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

[1] N. Platet, A.M. Cathiard, M. Gleizes, M. Garcia, Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion, Crit. Rev. Oncol. Hematol. 51 (1) (2004) 55–67.

[2] V. Giguere, To ERR in the estrogen pathway, Trends Endocrinol. Metab. 13 (5) (2002) 220–225.

[3] N. Yang, H. Shigeta, H. Shi, C.T. Teng, Estrogen-related receptor, hERR1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter, J. Bio. Chem. 271 (10) (1996) 5795–5804.

[4] D. Knutti, A. Kralli, PGC-1, a versatile coactivator, Trends Endocrinol. Metab. 12 (8) (2001) 360–365.

[5] P. Puigserver, B.M. Spiegelman, Peroxisome proliferator-activated receptor-g coactivator 1a (PGC-1a): transcriptional coactivator and metabolic regulator, Endocr. Rev. 24 (1) (2003) 78–90.

[6] J. Lin, P.T. Tarr, R. Yang, J. Rhee, P. Puigserver, C.B. Newgard, B.M. Spiegelman, PGC-1b in the regulation of hepatic glucose and energy metabolism, J. Biol. Chem. 278 (33) (2003) 30843–30848.

[7] J. Luo, R. Sladek, J. Carrier, J.A. Bader, D. Richard, V. Giguère, Reduced fat mass in mice lacking orphan nuclear receptor estrogen-related receptor α, Mol. Cell. Biol. 23 (22) (2003) 7947–7956.

[8] W.A. Alaynick, R.P. Kondo, W. Xie, W. He, C.R. Dufour, M. Downes, J.W. Jonker, W. Giles, R.K. Naviaux, V. Giguère, R.M. Evans, ERRg directs and maintains the transition to oxidative metabolism in the postnatal heart, Cell Metab. 6 (1) (2007) 13–24.

[9] A.L. Bookout, Y. Jeong, M. Downes, R.T. Yu, R.M. Evans, D.J. Mangelsdorf, Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network, Cell 126 (4) (2006) 789–799.

[10] V. Giguere, Transcriptional control of energy homeostasis by the estrogen related receptors, Endocr. Rev. 29 (6) (2008) 677–696.

[11] N. Ijichi, K. Ikeda, K. Horie-Inoue, K. Yagi, Y. Okazaki, S. Inoue, Estrogen-related receptor a modulates the expression of adipogenesis-related genes during adipocyte differentiation, Biochem. Biophys. Res. Commun. 358 (3) (2007) 813–818.

[12] M. Kubo, N. Ijichi, K. Ikeda, K. Horie-Inoue, S. Takeda, S. Inoue, Modulation of adipogenesis-related gene expression by estrogen-related receptor g during adipocytic differentiation, Biochim. Biophys. Acta 1789 (2) (2009) 71–77.

[13] T. Suzuki, Y. Miki, T. Moriya, N. Shimada, T. Ishida, H. Hirakawa, N. Ohuchi, H. Sasano, Estrogen-related receptor a in human breast carcinoma as a potent prognostic factor, Cancer Res. 64 (13) (2004) 4670–4676.

[14] R.J. Kraus, E.A. Ariazi, M.L. Farrell, J.E. Mertz, Estrogen-related receptor a1 actively antagonizes estrogen receptor-regulated transcription in MCF-7 mammary cells, J. Bio. Chem. 277 (27) (2002) 24826–24834.

[15] E.A. Ariazi, G.M. Clark, J.E. Mertz, Estrogen-related receptor a and estrogen related receptor g associated with unfavorable and favorable biomarkers, respectively, in human breast cancer, Cancer Res. 62 (22) (2002) 6510–6518.

[16] T. Fujimura, S. Takahashi, T. Urano, N. Ijichi, K. Ikeda, J. Kumagai, T. Murata, K. Takayama, K. Horie-Inoue, Y. Ouchi, M. Muramatsu, Y. Homma, S. Inoue, Differential expression of estrogen-related receptors b and g (ERRb and ERRg) and their clinical significance in human prostate cancer, Cancer Sci. 101 (3) (2010) 646–651.

[17] D.C. Allred, G.M. Clark, R. Elledge, S.A. Fuqua, R.W. Brown, G.C. Chamness, C.K. Osborne, W.L. McGuire, Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer, J. Natl. Cancer Inst. 85 (3) (1993) 200–206.

[18] K. Horie-Inoue, K. Takayama, H.U. Bono, Y. Ouchi, Y. Okazaki, S. Inoue, Identification of novel steroid target genes through the combination of bioinformatics and functional analysis of hormone response elements, Biochem. Biophys. Res. Commun. 339 (1) (2006) 99–106.

[19] K. Takayama, K. Kaneshiro, S. Tsutsumi, K. Horie-Inoue, K. Ikeda, T. Urano, N. Ijichi, Y. Ouchi, K. Shirahige, H. Aburatani, S. Inoue, Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis, Oncogene 26 (30) (2007) 4453–4463.

[20] K. Ikeda, S. Ogawa, T. Tsukui, K. Horie-Inoue, Y. Ouchi, S. Kato, M. Muramatsu, S. Inoue, Protein phosphatase 5 is a negative regulator of estrogen receptor-mediated transcription, Mol. Endocrinol. 18 (5) (2004) 1131–1143.

[21] B.H. Mason, I.M. Holdaway, P.R. Mullins, L.H. Yee, R.G. Kay, Progesterone and estrogen receptors as prognostic variables in breast cancer, Cancer Res. 43 (6) (1983) 2985–2990.

[22] A.K. Tandon, G.M. Clark, G.C. Chamness, J.M. Chirgwin, W.L. McGuire, Cathepsin D and prognosis in breast cancer, N. Engl. J. Med. 322 (5) (1990) 297–302.

[23] D. Liu, Z. Zhang, W. Gladwell, C.T. Teng, Estrogen stimulates estrogen-related receptor a gene expression through conserved hormone response elements, Endocrinology 144 (11) (2003) 4894–4904.

[24] T. Suzuki, T. Urano, T. Tsukui, K. Horie-Inoue, T. Moriya, T. Ishida, M. Muramatsu, Y. Ouchi, H. Sasano, S. Inoue, Estrogen-responsive finger protein as a new potential biomarker for breast cancer, Clin. Cancer Res. 11 (17) (2005) 6148–6154.

[25] R.B. Riggins, J.P. Lan, Y. Zhu, U. Klimach, A. Zwart, L.R. Cavalli, B.R. Haddad, L. Chen, T. Gong, J. Xuan, S.P. Ethier, R. Clarke, ERRg mediates tamoxifen resistance in novel models of invasive lobular breast cancer, Cancer Res. 68 (21) (2008) 8908–8917.

[26] J.S. Carroll, C.A. Meyer, J. Song, W. Li, T.R. Geistlinger, J. Eeckhoute, A.S. Brodsky, E.K. Keeton, K.C. Fertuck, G.F. Hall, Q. Wang, S. Bekiranov, V. Sementchenko, E.A. Fox, P.A. Silver, T.R. Gingeras, X.S. Liu, M. Brown, Genome-wide analysis of estrogen receptor binding sites, Nat. Genet. 38 (11) (2006) 1289–1297.

[27] G. Deblois, J.A. Hall, M.C. Perry, J. Laganière, M. Ghahremani, M. Park, M. Hallett, V. Giguère, Genome-wide identification of direct target genes implicates estrogen-related receptor a as a determinant of breast cancer heterogeneity, Cancer Res. 69 (15) (2009) 6149–6157.

[28] N. Yang, H. Shigeta, H. Shi, C.T. Teng, Estrogen-related receptor, hERR1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter, J. Biol. Chem. 271 (10) (1996) 5795–5804.

[29] V. Bombail, F. Collins, P. Brown, P.T. Saunders, Modulation of ERa transcriptional activity by the orphan nuclear receptor ERRb and evidence for differential effects of long- and short-form splice variants, Mol. Cell. Endocrinol. 314 (1) (2010) 53–61.

[30] A. Ao, H. Wang, S. Kamarajugadda, J. Lu, Involvement of estrogen-related receptors in transcriptional response to hypoxia and growth of solid tumors, Proc. Natl. Acad. Sci. U.S.A. 105 (22) (2008) 7821–7826.