The American Journal of Pathology, Vol. 180, No. 3, March 2012

DOI: 10.1016/j.ajpath.2011.11.025

Tumorigenesis and Neoplastic Progression

Role of ERRF, a Novel ER-Related Nuclear Factor, in the Growth Control of ER-Positive Human Breast Cancer Cells

Dan Su,* Xiaoying Fu,† Songqing Fan,‡ Xiao Wu,* Xin-Xin Wang,§ Liya Fu,* Xue-Yuan Dong,† Jianping Jenny Ni,† Li Fu,§ Zhengmao Zhu,* and Jin-Tang Dong*†

From the Department of Genetics and Cell Biology, Nankai University College of Life Sciences, Tianjin, China; the Department of Hematology and Medical Oncology,† School of Medicine, Winship Cancer Institute, Emory University, Atlanta, Georgia; the Department of Pathology,‡ Second Xiangya Hospital, Central South University, Hunan, China; and the Key Laboratory of Breast Cancer Research,§ Department of Breast Cancer Pathology and Research Laboratory, Cancer Hospital of Tianjin Medical University, Tianjin, China*

Whereas estrogen–estrogen receptor α (ER) signaling plays an important role in breast cancer growth, it is also necessary for the differentiation of normal breast epithelial cells. How this functional conversion occurs, however, remains unknown. Based on a genome-wide sequencing study that identified mutations in several breast cancer genes, we examined some of the genes for mutations, expression levels, and functional effects on cell proliferation and tumorigenesis. We present the data for C1orf64 or ER-related factor (ERRF) from 31 cell lines and 367 primary breast cancer tumors. Whereas mutation of ERRF was infrequent (1 of 79 or 1.3%), its expression was up-regulated in breast cancer, and the up-regulation was more common in lower-stage tumors. In addition, increased ERRF expression was significantly associated with ER and/or progesterone receptor (PR) positivity, which was still valid in human epidermal growth factor receptor 2 (HER2)–negative tumors. In ER-positive tumors, ERRF expression was inversely correlated with HER2 status. Furthermore, higher ERRF protein expression was significantly associated with better disease-free survival and overall survival, particularly in ER- and/or PR-positive and HER2-negative tumors (luminal A subtype). Functionally, knockdown of ERRF in two ER-positive breast cancer cell lines, T-47D and MDA-MB-361, suppressed cell growth *in vitro* and tumorigenesis in xenograft models. These results suggest that ERRF plays a role in estrogen-ER–mediated growth of breast cancer cells and could, thus, be a potential therapeutic target. ***(Am J Pathol 2012, 180: 1189–1201; DOI: 10.1016/j.ajpath.2011.11.025)***

Breast cancer is a common malignancy that affects approximately one in eight women during their lifetime.[1] In the United States alone, an estimated 230,480 patients will be diagnosed as having breast cancer in 2011, and 39,520 patients will die of the disease.[1] At the time of diagnosis, most breast cancers are positive for estrogen receptor α (ER), and estrogen-ER signaling plays a necessary role in the proliferation of cancer cells,[2,3] which has laid the foundation for antiestrogen therapy using different approaches, including tamoxifen treatment. In normal luminal breast epithelial cells, however, estrogen-ER signaling does not seem to be proproliferative; it is, rather, necessary for the formation, maintenance, and homeostasis of luminal epithelial cells.[4] Normal ER-positive mammary epithelial cells are not proliferative, although they are often adjacent to proliferative epithelial cells in the breast.[5–8] Consistently, in cultured nontumorigenic yet ER-positive breast epithelial cells, estrogen inhibits cell proliferation.[9–11] Therefore, estrogen-ER sig-

Supported by the National Nature Science Foundation of China (30870980 and 30625032), the National "863" Program of China (2006AA02A249), the National "973" Program of China (2009CB521700), and the National Cancer Institute, NIH (R01CA085560).

Accepted for publication November 17, 2011.

D.S. and X.F. contributed equally to this work.

Supplemental material for this article can be found at *http://ajp.amjpathol.org* or at doi: 10.1016/j.ajpath.2011.11.025.

Address reprint requests to Jin-Tang Dong, Ph.D., Emory University, 1365 Clifton Rd., Atlanta, GA 30322; or Zhengmao Zhu, Ph.D., Department of Genetics and Cell Biology, Nankai University College of Life Sciences, 94 Weijin Rd., Tianjin, 300071 China or Li Fu, Ph.D., Key Laboratory of Breast Cancer Research, Department of Breast Cancer Pathology and Research Laboratory, Cancer Hospital of Tianjin Medical University, Tianjin, China. E-mail: jtdong@nankai.edu.cn, zhuzhengmao@nankai.edu.cn, or fuli@tijmu.edu.cn.

naling has different functions in cell proliferation between normal and cancer breast epithelial cells, yet the molecular basis for this difference is largely unknown. Furthermore, a substantial proportion of ER-positive breast cancers eventually fail to respond to antiestrogen therapies and lead to patient death,[12,13] and, thus, further understanding the conversion of ER function from prodifferentiation in normal cells to proproliferation in cancer cells should also help in improving antiestrogen therapy.

The putative uncharacterized *C1orf64* gene (chromosome 1 open reading frame 64), also designated as MGC24047 or RP11-5P18.4, was originally predicted from DNA sequencing and biological annotation of human chromosome 1 and was validated by the detection of a full-length cDNA sequence by the NIH Mammalian Gene Collection Program.[14,15] Based on the results described in this study, we named the gene *ERRF* for ER-related factor. The predicted *ERRF/C1orf64* gene encodes a protein of 169 amino acid residues that does not have known consensus domains. In a systematic analysis of well-annotated human protein-coding genes for mutations in 11 human breast cancers, Sjoblom et al[16] identified two somatic mutations of this gene which led to amino acid changes G52W and G100W, in a microdissected primary tumor and the HCC1395 breast cancer cell line. In addition, the chromosomal location of *ERRF/C1orf64*, 1p36, is a target of genetic instability in human breast cancer and melanoma.[17,18] These findings suggest that *ERRF/C1orf64* may play a role in human breast cancer.

To determine whether ERRF plays a role in breast cancer development, we examined its mutation frequency and expression at the RNA and protein levels in a large number of breast cancers and correlated the expression of ERRF with clinical and pathologic variables of breast cancer. We also analyzed the functional effect of ERRF on cell growth *in vitro* and on tumorigenesis in xenograft models. Although the mutation of ERRF is infrequent in human breast cancer, its expression is significantly associated with ER and/or progesterone receptor (PR) positivity and human epidermal growth factor receptor 2 (HER2) negativity (luminal A subtype) in breast cancer. In ER-positive breast cancer, higher expression of ERRF protein was significantly associated with better progression-free survival and overall survival (OS). Functionally, knockdown of ERRF expression dramatically retarded cell growth and tumorigenesis in the two ER-positive breast cancer cell lines tested.

Materials and Methods

Cell Lines, Primary Tumors, and Noncancerous Breast Tissues

Thirty-five breast epithelial cell lines were used in this study, including 31 breast cancer cell lines (BRF-71T, BT-20, BT-474, BT-549, BT-483, CAMA-1, DU4475, HCC1395, HCC1500, HCC1599, HCC1806, HCC1937, HCC202, HCC2218, HCC38, HCC70, Hs 578T, MCF7, MDA-MB-134, MDA-MB-157, MDA-MB-175, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-453, MDA-MB-468, SW527, T-47D, UACC893, ZR-75-1, ZR-75-30), 3 immortalized but nonneoplastic breast epithelial cell lines (184A1, 184B5, and BRF-97T), and 1 primary culture of human mammary epithelial cells (Cambrex Corp., East Rutherford, NJ). Except for BRF-97T and BRF-71T, which were from Biological Research Faculty & Facility Inc. (Ijamsville, MD), all the cell lines were purchased from American Type Culture Collection (Manassas, VA) and were propagated as described previously.[19] T-47D and MDA-MB-361, which were used in functional experiments, were shown to be mycoplasma free using the TaKaRa PCR mycoplasma detection kit (TaKaRa Biotechnology Co. Ltd., Dalian, China). In addition, short tandem repeat profiling was conducted at the Cancer Genomics Shared Resource of Emory Winship Cancer Institute to verify the identity of seven cell lines, including the two that were used for functional tests of ERRF (T-47D, MDA-MB-361, 184A1, MCF-10A, Hs 578T, MDA-MB-231, and ZR-75-1). Eight short tandem repeat markers were profiled, including CSF1PO, D13S317, D16S539, D5S818, D7S820, THO1, TPOX, and vWA. The short tandem repeat profile for each cell line was compared with its profile published by American Type Culture Collection, and the authenticity of all seven cell lines was confirmed.

A total of 367 breast cancer samples were used, including 285 consecutive formalin-fixed, paraffin-embedded primary tumor specimens used for protein expression analysis and 82 snap-frozen primary tumors and their matched noncancerous breast tissues used for RNA expression analysis ($n = 45$) or gene mutation analysis ($n = 48$). Clinicopathologic variables, including disease-free survival (DFS), were available for the 285 patients used for immunohistochemical (IHC) study. In addition, 54 formalin-fixed, paraffin-embedded noncancerous breast tissues, including 35 hyperplastic tissues from patients without breast cancer and 19 phenotypically normal tissues from patients with breast cancer, were also used for ERRF protein expression study. An RNA sample from a pool of normal breast tissues (Clontech, Mountain View, CA) was used as a control. HER2 status was determined by IHC staining using the c-erbB2 Ab-17 monoclonal antibody made by Neomarkers (clone designation: e2-4001 + 3B5; Lab Vision, Fremont, CA). Based on IHC staining signals, a scale of 0 to 3+ was used to define HER2 protein expression: 0, no staining; 1+, weak membrane staining in $<10\%$ of cells; 2+, moderate heterogeneous complete membrane staining in $\geq 10\%$ of cells; and 3+, uniform intense membrane staining in $>30\%$ of invasive tumor cells. Tumors with an IHC score of 0 or 1+ were considered HER2 negative, and those with a score of 2+ or 3+ were considered HER2 positive.

All tissue specimens were obtained from the surgical treatment of patients with breast cancer or breast hyperplasia at the Cancer Hospital of Tianjin Medical University. Use of the materials was approved by the hospital's ethics review committee. For the samples used for RNA analysis, resected tissues were cut into small pieces and then were snap frozen in liquid nitrogen immediately after

surgery. All the tumor tissues and paired normal tissues were histologically confirmed.

Genomic DNA and total RNA were extracted by using the DNeasy tissue kit (Qiagen, Shanghai, China) and the TRIzol reagent (Invitrogen, Beijing, China), respectively, according to the manufacturers' manuals.

Mutation Analysis

Mutation analysis of the *ERRF* gene was performed as described previously.[16,20] Briefly, PCR primers flanking the genomic DNA of each exon were synthesized based on previously designed primer sequences.[16,20] Forward and reverse PCR primers are located ≥50 bp to the exon-intron boundaries. A universal sequencing primer (M13 forward, 5′-GTAAAACGACGGCCAGT-3′) was appended to the 5′ end of one primer in a pair for sequencing. PCR products were purified and sequenced with the M13 forward sequencing primer. Sequences were aligned to the genomic reference sequences in the Entrez gene database (Build 37.1). Any sequence changes were considered as potential mutations, and the PCR sequencing process was repeated for confirmation.

Semiquantitative RT-PCR and Quantitative Real-Time PCR

Two micrograms of total RNA was reversely transcribed into cDNA using the M-MLV reverse transcriptase (Promega, Beijing, China) following the manufacturer's manual. Primer sequences for semiquantitative RT-PCR were 5′-GCTGAAGTAGCCGCATGG-3′ and 5′-CGGCCCTTCCAGCTAATC-3′ for *ERRF* and 5′-ATCACTGCCACCCAGAAGAC-3′ and 5′-ATGAGGTCCACCACCCTGTT-3′ for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). PCR amplification was performed in a volume of 20 μL containing 2 μL of cDNA, 0.25 μmol/L of each primer, 0.25 mmol/L of deoxyribonucleotide triphosphates, and 1 U of TaKaRa TaqHS polymerase (TaKaRa Biotechnology Co. Ltd.) for 30 cycles. PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide for visualization.

Quantitative real-time PCR was performed using the SYBR Premix ExTaq reagent (TaKaRa Biotechnology Co. Ltd.) in an iQ5 real-time PCR detection system (Bio-Rad Laboratories, Beijing, China). Primer sequences for real-time PCR were 5′-GAGCCAACCTCAAAGGC-3′ and 5′-CCGTGGGTGCAGTCAATA-3′ for *ERRF* and 5′-GGTGGTCTCCTCTGACTTCAACA-3′ and 5′-GTTGCTGTAGCCAAATTCGTTGT-3′ for *GAPDH*. Expression of the *ERRF* gene in each sample was defined by normalizing the ΔC_T ($C_T^{ERRF} - C_T^{GAPDH}$) for the sample against the ΔC_T for the pool of normal breast tissues from Clontech, assuming that the *ERRF* expression level in this pool of normal tissues presents its average expression level in normal breast.

IHC Staining

Tissue microarray slides containing 285 formalin-fixed, paraffin-embedded breast cancer tissues were used to detect ERRF protein expression by IHC staining. In addition, 54 formalin-fixed, paraffin-embedded noncancerous breast tissues were used to monitor ERRF expression in noncancerous breast cancer tissues. Briefly, after deparaffinization in xylene and rehydration in a series of alcohols (100 − 75%), slides were incubated in the dual endogenous enzyme block (Dako, Carpinteria, CA) for 15 minutes to inactivate endogenous peroxide activity and were treated in citrate buffer (pH 6.0) for 3 minutes in a pressure cooker for antigen retrieval. After cooling for 45 minutes at room temperature, slides were incubated with rabbit anti-ERRF antibody (HPA026676; Sigma-Aldrich, St. Louis, MO) at 1:600 dilution at 4°C overnight and with the secondary horseradish peroxidase–labeled polymer anti-rabbit Igs (Dako) for 30 minutes at room temperature. With diaminobenzidine tetrahydrochloride (Dako) as a chromogen, slides were counterstained with hematoxylin. Preimmune serum was used as the negative control for ERRF antibody. The specificity of ERRF antibody was evaluated by IHC staining of T-47D (ERRF-positive) and MCF-7 (ERRF-negative) breast cancer cell lines prepared in paraffin blocks (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).

Slides with IHC staining were examined independently by two investigators (X.F. and S.F.), and any discrepancy in the reading for a case was discussed and resolved. The intensity of nuclear staining (0 = negative, 1 = low, 2 = medium, and 3 = high) and the percentage of positively stained cells (0 − 100%) were recorded for each specimen, and ERRF expression was expressed as the multiplied score, which was calculated as intensity score × percentage for positive cells × 100. The average multiplied score for the 54 noncancerous breast tissues was ~15. In addition, the median of ERRF expression levels in primary tumors was 15. Therefore, the cutoff point of 15 was used to classify primary tumors into two groups. Those with a multiplied score <15 were defined as lower ERRF expression, and those with a multiplied score ≥15 were defined as higher ERRF expression. We also used the receiver operating characteristic curve to determine the optimal cutoff points for ERRF expression. In this analysis, the optimal cutoff point is defined when the Youden Index, the potential effectiveness of a biomarker, achieved the maximum.[21] With the Youden cutoff point, which varied among different variables, tumors were regrouped as ERRF higher or ERRF lower, and statistical analysis was performed again for each variable.

Cell Proliferation and Survival Assay in Vitro

Two different Stealth RNA interference (RNAi) small-interfering RNAs (siRNAs) for the *ERRF* gene were purchased from Invitrogen, and a nontarget control siRNA was purchased from Rui Bo (Guangzhou, China). Compared with traditional siRNA duplexes, Stealth RNAi siRNA has proprietary chemical modifications that make such siRNA

more stable in cell culture and *in vivo*, more potent for gene knockdown, and less stressful to cells. The target sequence of siM1 siRNA was 5′-GGCTGCGCCTGTGAGGTCTTCAACT-3′ and that of siM2 siRNA was 5′-GGGAACAGTCAAGGACTCACTGAAA-3′. T-47D and MDA-MB-361 cells were seeded into 24-well plates at a density of 5×10^4 per well. On the following day, siRNA transfection was conducted using the Lipofectamine RNAiMAX transfection reagent (Invitrogen). At set times after transfection, cells were fixed with 10% trichloroacetic acid, stained with 0.4% sulforhodamine-B, and washed by 1% acetic acid. The optical densities, which indicate cell numbers, were measured using an EMax Precision microplate reader (Molecular Devices, Shanghai, China) at a 490-nm wavelength. Each treatment was in triplicate.

In Vivo *Tumorigenesis Assay*

T-47D and MDA-MB-361 cells were transiently transfected with the same siRNAs against ERRF or control as described previously herein. Use of the Stealth RNAi siRNA could maintain the knockdown of ERRF for at least 7 days *in vitro*, so transiently transfected cells were used in the tumorigenesis assay. Forty-eight hours later, cells were injected subcutaneously into the right flank of 6-week-old female BALB/c nude mice at 5×10^6 cells per site. Some cells were used in real-time PCR assay to confirm the knockdown of ERRF in both cell lines. Three days before the injection, 17β-estradiol pellets (0.72 mg per pellet, 60-day release; Innovative Research of America, Sarasota, FL) were implanted into the left flanks of mice. Eight mice were used for each treatment in each group. Tumor volumes were measured weekly using a vernier caliper. Four or five weeks after injection, tumors were removed and tumor weights were measured.

Test of ERRF Antibody in Cells with Known ERRF Expression Status

T-47D (ER positive and ERRF positive) and MCF-7 (ER positive but ERRF negative) cells were collected in PBS, fixed in 10% buffered formalin for 2 hours at room temperature, and centrifuged and resuspended in 300 μL of PBS. After adding 600 μL of 3% low–melting point agarose in PBS, cell solution was chilled on ice, gelled pellet was removed and trimmed, and paraffin block was prepared. Sections were made and were subjected to IHC staining as with tissue sections.

Apoptosis Analysis

T-47D cells transfected with the siM1 siRNA against ERRF or the control siRNA for 2 days were subjected to apoptotic assay. Using the fluorescein isothiocyanate annexin V apoptosis detection kit from BD Pharmingen (Shanghai, China), cells were stained with annexin V–fluorescein isothiocyanate and then were flow sorted following a standard protocol. Briefly, cells floating in the medium and attached to the plate were collected, washed with cold PBS, and resuspended in 1× binding buffer [0.01 mmol/L HEPES (pH 7.4), 0.14 mol/L NaCl, and 2.5 mmol/L $CaCl_2$] at a concentration of 2×10^6 cells/mL. One hundred microliters of cell solution was transferred into a 5-mL culture tube, and 5 μL of annexin V and propidium iodide were added. After gentle mixing and incubation for 15 minutes at room temperature in the dark, 400 μL of the 1× binding buffer was added, and cells were analyzed by flow cytometry within 1 hour.

Cell-Cycle Analysis

T-47D cells transfected with the siM1 siRNA against ERRF or the control siRNA for 2 days were stained with propidium iodide and subjected to flow cytometry following a standard. Briefly, cells were collected and washed twice with cold PBS, fixed in 10 mL of 70% ethanol at −20°C overnight, washed again with PBS, stained with 0.5 mL of propidium iodide/RNase staining buffer for 15 minutes at room temperature, and analyzed by flow cytometry.

Statistical and Survival Analyses

The association between ERRF expression and clinicopathologic variables was examined using the χ^2 test or the Fisher exact test. Kaplan-Meier survival analysis was used to calculate DFS or OS rates in different groups of patients. Multivariate Cox regression analysis was used to determine whether ERRF expression was independent of other prognostic factors. Data from cell proliferation *in vitro* and tumorigenesis *in vivo* were analyzed by Student's *t*-test. All reported *P* values were 2-tailed. SPSS, version 15.0 (SPSS Inc, Chicago, IL) was used for statistical analysis.

Results

Infrequent Mutation of ERRF in Breast Cancer

In the 31 breast cancer cell lines and 48 primary breast tumors analyzed, only one mutation (*G366T*/G100W, homozygous) was detected in one sample (the HCC1395 breast cancer cell line). The same mutation in the same cell line was reported in a previous study.[16] Four single nucleotide polymorphisms (SNPs) were identified, each with different frequencies. SNP rs1763617 (*C86T*) showed a *C/T* heterozygosity in 4 breast cancer cell lines (BRF-71T, BT-20, HCC1806, and ZR-75-1) and in 15 primary tumors and a *T/T* homozygosity in five primary tumors but in no cell lines. SNP rs1763612 (*C308T*) was heterozygous in one cell line (HCC1806) and in two primary tumors; SNP rs34950166 (*C402T*) showed *T/T* in two cell lines (BT-483 and HCC202) but in no primary tumors; and SNP rs3738646 (*T416C*) was heterozygous in three cell lines (BRF-71T, BT-20, and ZR-75-1) and in 15 primary tumors and was homozygous *C/C* in five primary tumors but in no cell lines. No other cancer-specific sequence alterations were found in 79 breast cancers, suggesting that the *ERRF* mutation is relatively infrequent in breast cancer.

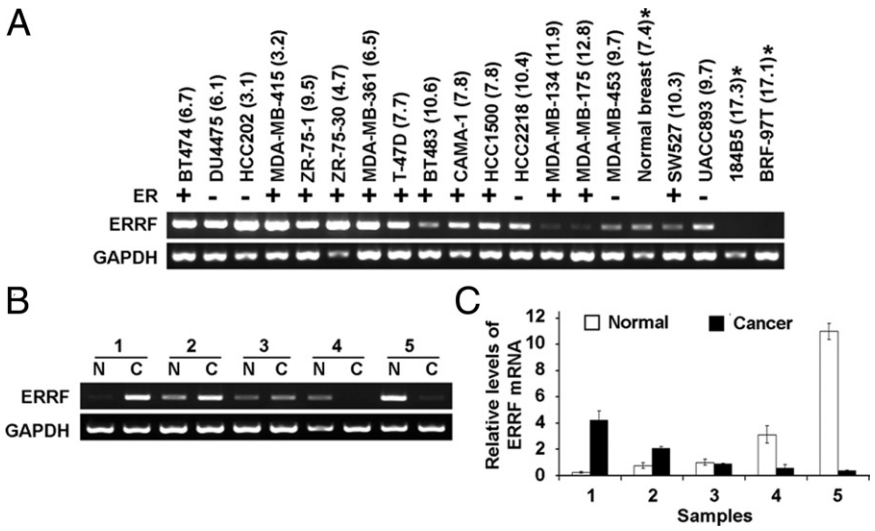

Figure 1. Expression of ERRF mRNA is associated with ER positivity in cell lines and primary tumors of breast cancer. **A:** Detection of ERRF mRNA in breast epithelial cell lines by regular and real-time RT-PCR. GAPDH was used as an internal control. The numbers in parentheses after each cell line are ΔC_T values detected by real-time PCR. **Asterisks** indicate normal breast tissues or immortalized non-tumorigenic epithelial cell lines. **B:** Representative results of ERRF mRNA expression in primary breast cancer samples (C) and adjacent noncancerous tissues (N). Samples 1, 2, and 3 are ER positive, whereas samples 4 and 5 are ER negative. **C:** Expression of ERRF mRNA, detected by real-time PCR, in the same samples as in **B**. Error bars represent SD.

Expression of ERRF mRNA Is Significantly Associated with ER Positivity in Cell Lines and Primary Tumors from Breast Cancer

We evaluated the expression of ERRF in normal and cancer cells of the breast. According to SAGEmap, a public gene expression resource based on the serial analysis of gene expression (SAGE) approach,[22] the expression of ERRF is restricted to a few normal tissues, including the brain, cortex, retina, and thyroid. It was not detected in the bladder, bone marrow, breast, cervix, colon, heart, kidney, liver, lung, lymph node, muscle, ovary, pancreas, placenta, prostate, salivary gland, skin, spinal cord, spleen, testis, or thymus. Breast and prostate cancers are the only two other tissues that showed detectable expression of ERRF in the SAGE database. We, therefore, examined the expression of ERRF mRNA by using PCR-based approaches in malignant and nonmalignant tissues and cell lines from the breast (Figure 1 and Table 1). Whereas ERRF mRNA was not detectable in cultured nonmalignant breast epithelial cells, including primary culture of human mammary epithelial cells and the immortalized breast epithelial cell lines 184A1, 184B5, and BRF-97T, it was clearly detected in a pool of

Table 1. Association of ERRF mRNA Expression with Clinicopathologic Variables in Normal and Tumor Cells of 45 Patients with Breast Cancer

		Normal			Tumor		
Variable	Total cases	ERRF lower	ERRF higher	*P* value*	ERRF lower	ERRF higher	*P* value*
Age (years)							
<50	14	11	3	0.006	6	8	0.344
≥50	31	11	20		18	13	
Stage							
I/II	23	11	12	0.817	10	13	0.526
III/IV	7	3	4		4	3	
Grade							
I	3	3	0	0.089	1	2	0.305
II	23	10	13		10	13	
III	10	4	6		7	3	
Lymph node							
+	17	8	9	0.850	8	9	0.663
−	26	13	13		14	12	
ER							
+	23	12	11	0.652	7	16	0.002
−	22	10	12		17	5	
PR							
+	28	14	14	0.848	12	16	0.071
−	17	8	9		12	5	
HER2							
+	20	12	8	0.182	10	10	0.688
−	25	10	15		14	11	

Data are given as number of patients. Higher or lower ERRF RNA expression was relative to the ERRF expression level in a pool of human normal breast tissues purchased from Clontech.

**P* values were determined using the χ^2 test.

normal breast tissues (Figure 1A). In adjacent noncancerous tissues from 45 breast cancers, ERRF mRNA expression showed a diverse range, with an average ΔC_T, which indicates expression levels in real-time PCR of ~7.4. The ΔC_T for the pool of normal breast tissues from Clontech was 7.42, which was almost identical to that for the 45 noncancerous samples, suggesting that ERRF expression in normal and noncancerous breast tissues is consistent among different samples. Therefore, the average ERRF expression level from the pool of normal breast tissues could serve as the cutoff point for the correlation analysis, and we defined the samples with a ΔC_T value $\leq$7.4 as ERRF higher and those with a ΔC_T value $>$7.4 as ERRF lower. ERRF RNA expression was positive in 23 of the 45 samples (51%) (Table 1). These results suggest that ERRF is transcribed in nonmalignant breast tissues but not in cultured breast epithelial cells.

In the 31 breast cancer cell lines examined, ERRF mRNA was detected in 17 (55%), including BT-474, BT-483, CAMA-1, DU4475, HCC1500, HCC202, HCC2218, MDA-MB-134, MDA-MB-175, MDA-MB-361, MDA-MB-415, MDA-MB-453, SW527, T-47D, UACC893, ZR-75-1, and ZR-75-30, although the expression in MDA-MB-134 and MDA-MB-175 was rather weak (Figure 1A). ERRF was not detectable in the remaining 14 cell lines (45%), including BRF-71T, BT-20, BT-549, HCC1395, HCC1599, HCC1806, HCC1937, HCC38, HCC70, Hs 578T, MCF7, MDA-MB-157, MDA-MB-231, and MDA-MB-468 (Figure 1A and data not shown). ERRF was highly expressed in 11 of the 12 ER-positive cell lines (92%)[23,24] but was undetectable in 13 of the 19 ER-negative lines (68%) (Figure 1A and data not shown). The Fisher exact test on ERRF expression and ER positivity in these cell lines indicated that ERRF mRNA expression is significantly associated with ER positivity ($P = 0.002$).

When the 31 cell lines were stratified by HER2 status, the association between ERRF expression and ER positivity became more obvious in HER2-negative breast cancers, as 8 of 9 ER-positive and HER2-negative cell lines (89%) but only 2 of 15 ER- and HER2-negative lines (13%) expressed ERRF ($P = 4.9 \times 10^{-4}$, Fisher exact test). In the HER2-positive group, three cell lines (BT474, MDA-MB-361, and ZR-75-30) were ER positive and four cell lines (HCC2218, HCC202, MDA-MB-453, and UACC893) were ER negative. ERRF expression was not significantly associated with ER expression in these HER2-positive breast cancer cell lines.

Of the 45 primary breast tumors, 21 (47%) showed higher ERRF RNA expression, and the remaining 24 were lower (Figure 1, B and C, and Table 1). Compared with matching noncancerous tissues, 17 of the 45 tumors showed down-regulation of ERRF, 14 showed no change, and 14 showed up-regulation. We then correlated ERRF mRNA expression with various characteristics of breast cancer, including age at diagnosis, tumor stage, tumor grade, lymph node status, and the status of ER, PR, or HER2 (Table 1). Only ER showed a significant correlation with ERRF expression, as 16 of 23 ER-positive tumors (70%) but only 5 of 22 ER-negative tumors (23%) were ERRF higher ($P = 0.002$). There also seemed to be an association between ERRF expression and PR positivity, but it did not reach a significant level ($P = 0.071$).

We also retrieved microarray-based expression data for breast cancer from the Oncomine database to further evaluate the association between ERRF mRNA expression and ER status. Oncomine is a cancer microarray database integrated with a data-mining platform.[25] There were eight independent data sets in which ER expression was either positive or negative in at least 10 cases. The expression of ERRF mRNA was significantly higher in ER-positive tumors than in ER-negative tumors in each of the eight data sets, further supporting an association between ERRF expression and ER positivity in breast cancer.

ERRF Protein Expression Significantly Correlates with ER or PR Positivity, Which Was also True in HER2-Negative Breast Cancers when Stratified with HER2 Status

To further evaluate the association of ERRF expression with ER status, we performed IHC staining with tissue microarray slides of 285 breast cancers and 54 noncancerous tissues. In phenotypically normal breast luminal cells, ERRF protein was clearly detected, and the staining was primarily located in the nucleus although also visible in the cytoplasm of some cells (Figure 2). In some noncancerous tissues, ERRF staining was stronger, but, in general, it was still weaker compared with tumors that stained high for ERRF. In breast cancer samples, ERRF protein was expressed at varying levels, with strong staining in some tumors but lower or even absent staining in other tumors (Figure 2 and Table 2). Compared with ER-negative tumors, ERRF expression was significantly

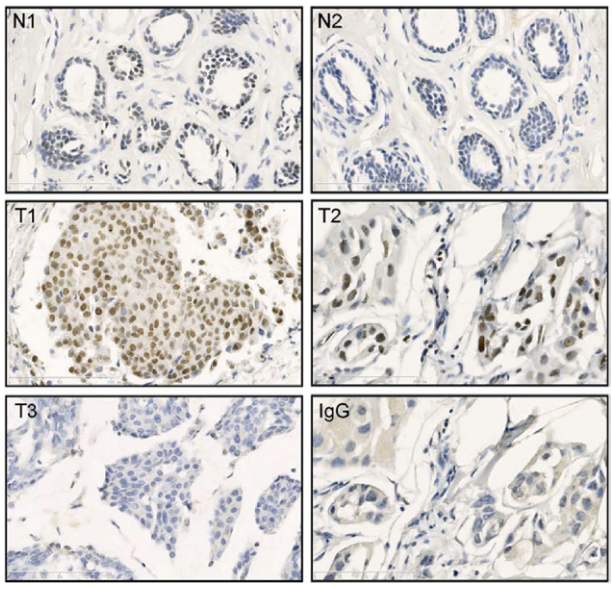

Figure 2. Detection of ERRF protein expression in breast tissues by IHC staining. N1 and N2, two representative noncancerous breast tissues; T1, T2, and T3, three representative breast cancer tissues showing different decreasing levels of ERRF expression; IgG, breast cancer tissue T2 stained with preimmune rabbit IgG (negative control).

Table 2. Association of ERRF Protein Expression with Clinical and Pathologic Variables in 285 Primary Tumors from Breast Cancer

Variable	Total cases	ERRF expression (M/Y)		*P* value (M/Y)*
		Lower	Higher	
Age (years)				
<50	108	59/51	49/57	0.171/0.046†
≥50	173	80/61	93/112	
Tumor size (cm)				
≤2	64	30/34	34/30	0.592/0.170
>2	217	110/136	107/81	
Stage				
I/II	209	96/96	113/113	0.031/0.031
III/IV	69	42/42	27/27	
Grade				
I/II	222	127/104	95/118	0.192/0.035†
III	49	28/36	21/13	
Lymph node				
+	153	76/142	77/11	0.855/0.048†
−	132	67/113	65/19	
ER				
+	170	70/65	100/105	$0.0002/5.34 \times 10^{-5}$
−	115	73/72	42/43	
PR				
+	166	74/93	92/73	0.026/0.002
−	119	69/88	50/31	
HER2				
+	140	94/117	46/23	$1.81 \times 10^{-8}/1.39 \times 10^{-9}$
−	145	49/72	96/73	

Data are given as number of tumors. Higher or lower ERRF expression was relative to the median (M) IHC staining score of 15 for noncancerous breast tissues or the optimal cutoff point determined by the Youden Index (Y) in tumors, which was 7.5 for age, 31.5 for tumor size, 14 for tumor stage, 34 for tumor grade, 132.5 for lymph node status, 11.5 for ER status, 38 for PR status, and 41.5 for HER2 status.

**P* values were determined by using the Pearson χ^2 test, and HER2 status was defined by IHC staining.

†The *P* value became smaller than 0.05 after the optimal cutoff point determined by the Youden Index was applied.

higher in ER-positive tumors, which is consistent with the RNA expression results.

When the tumors were stratified by HER2 status, the association between ERRF expression and ER positivity was detectable in HER2-negative tumors only, as 74 of 103 ER-positive tumors (72%) versus 22 of 42 ER-negative tumors (52%) express ERRF ($P = 0.025$; Table 3), which is consistent with the results from the 31 breast cancer cell lines. Also consistent with cell line results, ERRF expression showed a strong association with PR positivity in the HER2-negative tumors, as 68 of 90 PR-positive tumors (76%) versus 28 of 55 PR-negative tumors (51%) express ERRF ($P = 0.002$; Table 3). In HER2-positive tumors ($n = 140$), ERRF expression did not show an association with either ER or PR (Table 3).

Because the cross-talk between ER, PR, and HER2 signaling plays an important role in the development and progression of breast cancer,[26,27] breast cancers are categorized into four groups based on the expression status of ER, PR, and HER2: luminal A (ER positive and/or PR positive, HER2 negative), luminal B (ER positive and/or PR positive, HER2 positive), basal-like triple negative (ER negative, PR negative, and HER2 negative), and HER2 positive (ER negative, PR negative, and HER2 positive).[28,29] Consistent with a positive correlation between ERRF and ER or PR in HER2-negative tumors, we found that ERRF expression was significantly higher in luminal A tumors than in luminal B, triple-negative, or HER2-positive tumors (Table 4).

ERRF Expression Inversely Correlates with HER2 Positivity in ER-Positive Breast Cancer

In the 31 breast cancer cell lines, ERRF expression was detectable in 7 of the 7 HER2-positive lines but in only 10 of the 24 HER2-negative lines (42%) ($P = 0.009$, Fisher exact test; Table 3), suggesting a correlation between ERRF expression and HER2 positivity in breast cancer cell lines.

In primary tumors, however, ERRF expression showed an inverse correlation with HER2 positivity, as 96 of 145 HER2-negative tumors (66%) but only 46 of 140 HER2-positive tumors (33%) had ERRF expression ($P < 0.001$; Table 3). When we stratified tumors by ER status, the inverse correlation between ERRF and HER2 expression was still significant. In the ER-positive group, 74 of 103 HER2-negative tumors (72%) and 26 of 67 HER2-positive tumors (39%) had ERRF expression; in the ER-negative group, 22 of 42 HER2-negative tumors (52%) and 20 of 73 HER2-positive tumors (27%) had ERRF expression (Table 5).

Higher ERRF Expression Correlates with Older Age at Diagnosis, Lower Tumor Stage, Lower Tumor Grade, and Less Lymph Node Metastasis in Breast Cancer

When the median level of ERRF expression was used to group tumors into ERRF higher and ERRF lower, ERRF expression seemed to correlate with lower tumor stage,

Table 3. Positive Association of ERRF Expression with ER and PR Status Is Statistically Significant in Only HER2-Negative Primary Tumors (n = 145) and Cell Lines (n = 24)

Group	ER/PR	Cases	ERRF expression: Lower	ERRF expression: Higher	P value*
Primary tumors					
HER2−		145	49	96	0.038†
	ER+	103	29	74	0.025
	ER−	42	20	22	
	PR+	90	22	68	0.002
	PR−	55	27	28	
HER2+		140	94	46	0.001†
	ER+	67	41	26	0.151
	ER−	73	53	20	
	PR+	76	52	24	0.726
	PR−	64	42	22	
Cell lines					
HER2−		24	14	10	
	ER+	9	1	8	4.90×10^{-4}
	ER−	15	13	2	
	PR+	5	1	5	0.015
	PR−	19	13	5	
HER2+		7	0	7	
	ER+	3	0	3	
	ER−	4	0	4	
	PR+	2	0	2	
	PR−	5	0	5	

Data are given as number of tumors. ERRF expression in primary tumors was based on IHC staining, and that in cell lines was based on real-time PCR. The median of IHC staining scores from tumors was used as the cutoff point for protein expression, whereas the reading of real-time PCR from a pool of normal breast tissues was used as the cutoff point for cell lines.

*P values were determined using the χ^2 test.

†P values are for the comparison between primary breast cancer tumors and breast cancer cell lines.

as 113 of 209 stage I or II tumors (54%) were higher for ERRF expression but only 27 of 69 stage III or IV tumors (39%) were lower (Table 2; P = 0.031). There was no significant association between ERRF expression and age at diagnosis, tumor size, tumor grade, or lymph node status when the median ERRF expression level was used as the cutoff point (Tables 1 and 2). When the cutoff point of ERRF expression was defined by the Youden Index for each variable, however, higher ERRF expression showed a significant association with older age at diagnosis, lower tumor grade, and less lymph node metastasis (Table 2). Of the tumors that had higher levels of ERRF expression, 112 of 169 (66%) occurred in women 50 years or older and 57 of 169 (34%) occurred in women younger than 50 years (P = 0.046); 118 of 131 (90%) had a lower tumor grade, but only 13 of 131 (10%) had a higher tumor grade (P = 0.035); and 19 of 30 (63%) were negative for lymph node metastasis, whereas 11 of 30 (37%) were positive (P = 0.048) (Table 2).

Table 5. Inverse Correlation between ERRF Expression and HER2 Status Is Statistically Significant in Primary Tumors Regardless of ER Status, Although the Association Was Not Significant in Cell Lines

Group	HER2	Cases	ERRF expression: Lower	ERRF expression: Higher	P value*
Primary tumors					
ER+		170	70	100	0.051†
	+	67	41	26	1.9×10^{-5}
	−	103	29	74	
ER−		115	73	42	0.874†
	+	73	53	20	0.007
	−	42	20	22	
Cell Lines					
ER+		12	1	11	
	+	3	0	3	0.546
	−	9	1	8	
ER−		19	13	6	
	+	4	0	3	−
	−	15	13	2	

Data are given as number of tumors. ERRF expression in primary tumors was based on IHC staining of ERRF protein, and that in cell lines was based on real-time PCR of ERRF RNA.

*P values were determined using the χ^2 test.

†P values are for the comparison between primary breast cancer tumors and breast cancer cell lines.

Higher ERRF Expression Correlates with Better Prognosis in Breast Cancer

We then determined whether ERRF protein expression is associated with DFS or OS in the 285 patients with breast cancer stratified according to the expression status of ER, PR, or HER2 (data not shown). In univariate analysis, ERRF up-regulation showed an association with DFS and OS, along with tumor stage, tumor grade, and lymph node metastasis (Table 6). Patient age at diagnosis, tumor size, ER positivity, PR positivity, and HER2 positivity did not show a significant association with survival. Patients with higher ERRF expression (n = 142) had significantly better DFS than did those with lower ERRF expression (n = 143; P = 0.029; Figure 3A). Kaplan-Meier

Table 4. Differential Expression of ERRF Protein in Luminal A (LuA), Luminal B (LuB), Triple-Negative (TN), and HER2-Positive (HER2+) Subtypes of Breast Cancer

Type	Cases (no.)	ERRF expression (mean ± SD)	P value*: LuA	P value*: LuB	P value*: TN	Overall P value†
LuA	113	73.76 ± 7.16				5.8×10^{-10}
LuB	91	23.68 ± 3.93	1.8×10^{-8}			
TN	33	44.36 ± 10.57	0.053	0.299		
HER2+	48	18.71 ± 4.96	5.04×10^{-7}	0.964	0.208	

*P values were determined using the Tukey test.

†P value was determined using the F test with the general linear model.

Table 6. Identification of Survival Factors Using Cox Proportional Hazard Ratio Univariate and Multivariate Analyses Based on Backward Stepwise Logistic Regression

	DFS			OS		
Variable	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
Univariate Analysis						
ERRF (*n* = 283)	0.382	0.182 to 0.805	0.011	0.302	0.108 to 0.844	0.022
Age (*n* = 279)	1.235	0.596 to 2.562	0.570	1.612	0.575 to 4.522	0.364
Tumor size (*n* = 279)	1.115	0.484 to 2.569	0.798	1.171	0.388 to 3.532	0.780
Stage (*n* = 276)	2.230	1.109 to 4.485	0.024	2.561	1.022 to 6.413	0.045
Grade (*n* = 269)	2.186	1.034 to 4.619	0.040	3.136	1.210 to 8.128	0.019
Lymph node (*n* = 283)	2.103	1.001 to 4.418	0.050	3.410	1.131 to 10.283	0.029
ER (*n* = 283)	1.461	0.694 to 3.074	0.318	0.765	0.302 to 1.936	0.571
PR (*n* = 283)	1.088	0.541 to 2.189	0.813	0.925	0.370 to 2.311	0.867
HER2 (*n* = 283)	1.346	0.676 to 2.678	0.398	1.698	0.669 to 4.313	0.265
Multivariate Analysis*						
ERRF	0.361	0.167 to 0.778	0.009	0.358	0.124 to 1.029	0.057
Grade	1.993	0.912 to 4.353	0.084	2.397	0.898 to 6.402	0.081
Lymph node	1.976	0.897 to 4.353	0.091	3.835	1.092 to 13.472	0.036
ER	1.896	0.867 to 4.148	0.109			

*Based on backward stepwise logistic regression (*n* = 260).
CI, confidence interval.

survival analysis showed that patients with higher ERRF expression had significantly less chance of recurrence than did those with lower ERRF expression (*P* = 0.009; Figure 3B). Then we analyzed ERRF and DFS in ER-positive and ER-negative patients. Kaplan-Meier survival analysis further demonstrated that patients with higher ERRF expression had significantly less chance of recurrence in ER-positive patients (*P* = 0.002, data not shown) but not in ER-negative patients (data not shown), in PR-positive patients (*P* = 0.002, data not shown) but not in PR-negative patients (data not shown), and in HER2-negative patients (*P* = 0.008, data not shown) but not in HER2-positive patients (data not shown). As expected, patients with higher ERRF expression had less chance of recurrence in the luminal A subtype of breast cancer, which is defined as ER and/or PR positive and HER2 negative (*P* = 0.005; Figure 3C).[28,29]

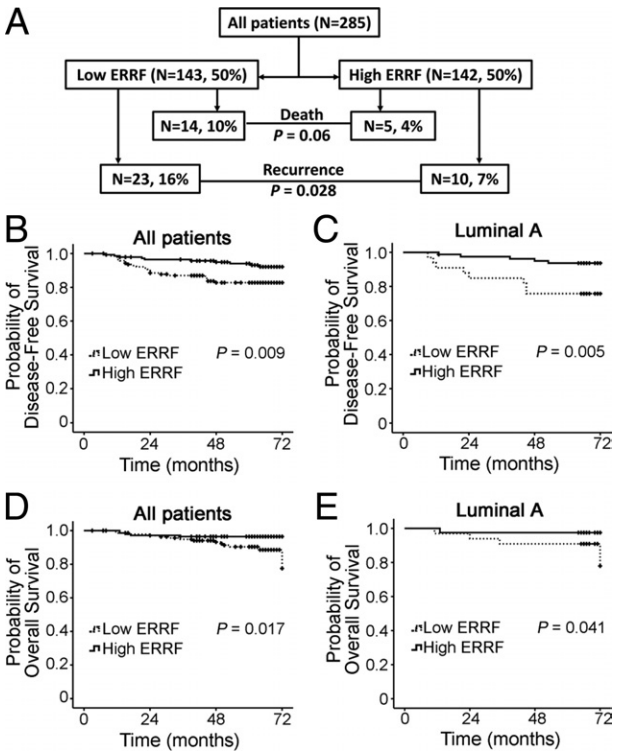

Figure 3. Association between ERRF expression and DFS or OS in patients with breast cancer. **A:** Number of patients with higher or lower ERRF expression, recurrence, and death. **B–E:** Kaplan-Meier analysis of ERRF expression and DFS (**B** and **C**) and OS (**D** and **E**) in all 285 patients (**B** and **D**) and in patients who had the luminal A subtype of breast cancer (**C** and **E**). *P* values were from the log-rank univariate test and are, thus, different from those derived from Cox regression tests.

Patients with higher ERRF expression (*n* = 142; Figure 3A) also showed better OS (*P* = 0.017; Figure 3D), and a significant association was detected in patients whose tumors were ER positive (*P* = 0.019), PR positive (*P* = 0.004), or luminal A subtype (*P* = 0.041; Figure 3E) but not in patients with ER- or PR-negative tumors (data not shown). There was also a trend toward association between OS and HER2 negativity, but it did not reach a significant level (*P* = 0.055).

Multivariate analysis of all patients based on backward stepwise logistic regression showed that the association of higher ERRF expression with better DFS was independent of known survival factors, such as lymph node metastasis and tumor grade (*P* = 0.009; Table 6). For OS, ERRF expression showed a trend toward association, but it did not reach a significant level (*P* = 0.057; Table 6), whereas lymph node metastasis showed a significant association.

Knockdown of ERRF by RNAi Inhibits the Growth of ER-Positive Breast Cancer Cells in Vitro

ER is required for the growth of most ER-positive breast cancers, and targeting ER by tamoxifen is an effective therapy for breast cancer.[13] Based on the significant association between ERRF expression and ER positivity described previously herein (Figures 1 and 2 and Tables

1 and 2), we tested whether ERRF plays a role in estrogen-mediated cell growth in ER-positive breast cancer cells. We chose the T-47D and MDA-MB-361 breast cancer cell lines for these experiments because they are ER positive and express higher levels of ERRF (Figure 1). When the expression of ERRF was knocked down by transfecting siRNAs into cells, as confirmed by RT-PCR for two different siRNAs, cell growth was significantly inhibited in the T-47D and MDA-MB-361 cell lines by both siRNAs, although the two siRNAs could have different potency (Figure 4). This experiment was repeated four times for the T-47D cell line and three times for the MDA-MB-361 cell line, and similar results were obtained. These results suggest that ERRF plays a role in the growth of ER-positive breast cancer cells.

To evaluate whether cell-cycle arrest or apoptosis is responsible for the decrease in cell numbers on the knockdown of ERRF, we conducted flow cytometry analysis of T-47D cells stained with annexin V–fluorescein isothiocyanate and propidium iodide. Whereas staurosporine, a potent apoptosis inducer, caused a significant level of cell death (9.7%) compared with the dimethyl sulfoxide solvent control (1%), neither the control siRNA nor the ERRF siRNA (siM1) had an obvious effect on cell death (see Supplemental Figure S2, A–D, at *http://ajp.amjpathol.org*). On the other hand, knockdown of ERRF significantly increased the number of cells in the G0/G1 phase and decreased the number of cells in the S and G2/M phases (see Supplemental Figure S2E at *http://ajp.amjpathol.org*). Therefore, siM1-mediated knockdown of ERRF causes cell-cycle arrest rather than cell death at least in T-47D cells.

Knockdown of ERRF Inhibits Tumor Growth in Xenograft Models

To further determine the effect of ERRF on the growth of ER-positive breast cancer cells, we also injected T-47D and MDA-MB-361 cells transfected with siRNAs against ERRF into female BALB/c nude mice and measured tumor volume and weight. Higher levels of estrogen were achieved by subcutaneously transplanting hormone pellets. Consistent with the *in vitro* results, knockdown of ERRF significantly suppressed tumor growth in both cell lines (Figure 5). These results further indicate that ERRF is involved in the growth of ER-positive breast cancer cells.

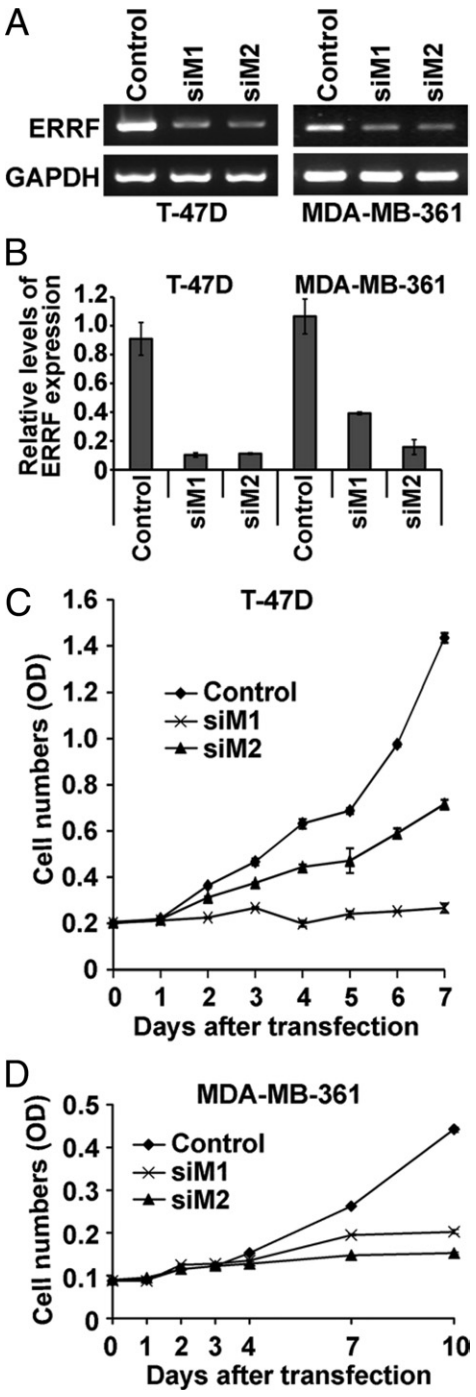

Figure 4. Knockdown of ERRF suppresses cell proliferation in T-47D and MDA-MB-361 breast cancer cell lines. **A** and **B:** Confirmation of ERRF knockdown in both cell lines by RT-PCR (**A**) and real-time PCR (**B**) in cells transfected with siRNA for 2 days. Control, control siRNA; siM1 and siM2, two different ERRF siRNAs from Invitrogen. Error bars represent SD. **C** and **D:** Growth curves of T-47D (**C**) and MDA-MB-361 (**D**) cells with ERRF knockdown for 1 to 10 days. OD, optical density of lysed cells.

Discussion

In this study, we examined a predicted gene that had two mutations in 11 breast cancers,[16] *C1orf64*, for its potential role in breast cancer. We examined its mutation, expression, and functional effect on cell and tumor growth in breast cancer cells. Except for a previously reported mutation in the HCC1395 breast cancer cell line,[16] no other mutations were detected in 78 breast cancers, although several SNPs were detected in these samples.

ERRF expression significantly correlates with ER and PR status in HER2-negative breast cancer. This conclusion is based on the analysis of ERRF RNA and protein in cell lines and primary tumors from breast cancer (Figures 1 and 2 and Tables 1–3). The association with ER status was significant in cell lines and primary tumors and at RNA and protein levels in primary tumors (Tables 1 and 2), which suggests that ERRF is transcriptionally up-reg-

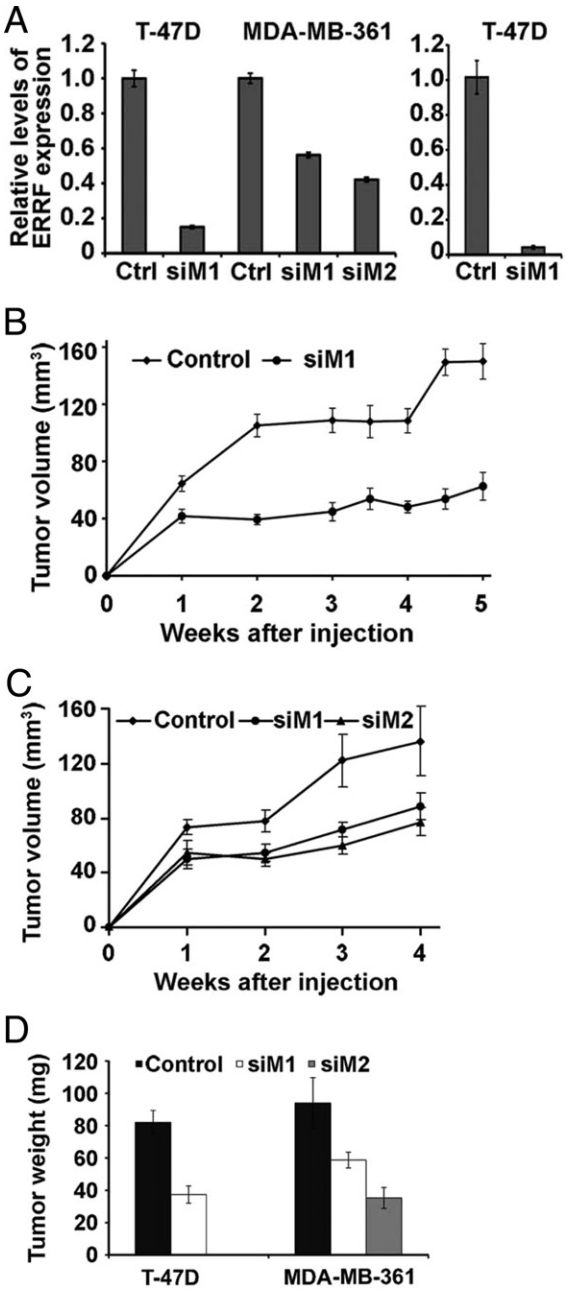

Figure 5. Knockdown of ERRF suppresses the tumorigenesis of T-47D and MDA-MB-361 breast cancer cell lines in nude mice. **A:** Confirmation of ERRF knockdown by RT-PCR in the two cell lines (T-47D and MDA-MB-361) transfected for 2 days (**left panel**) or in T-47D cells transfected for 7 days (**right panel**). Ctrl, control. **B** and **C:** Plot of tumor volume against time after tumor cells were injected into nude mice for T-47D (**B**) and MDA-MB-361 (**C**) cells. **D:** Tumor weight at the end of the xenograft experiments for T-47D and MDA-MB-361 cell lines with ERRF knockdown. Error bars represent SD.

ulated in ER-positive breast cancer. The association with PR status became more significant after the tumors were stratified by HER2 status (*P* value changed from 0.027 in all tumors to 0.002 in HER2-negative tumors; Table 3). Note that the association between ERRF and ER or PR did not exist in HER2-positive tumors (Table 3).

ERRF expression inversely correlated with HER2 status regardless of ER status in primary tumors (Tables 2 and 3). It has been demonstrated previously that ER expression also inversely correlates with HER2 in breast cancer,[30–32] which further suggests a functional interaction between ER and ERRF in breast epithelial cells. Opposite to this inverse correlation in primary tumors, all seven HER2-positive cell lines and only 10 of the 24 HER2-negative cell lines (42%) express ERRF compared with a frequency of 96 of 145 in HER2-negative primary tumors (66%) (Table 3). It is unknown whether this discrepancy between cell lines and primary tumors has biological significance as the number of cell lines available for analysis was rather small. In addition, it is well-known that cell lines are different from primary tumors in many aspects, and they can acquire or present additional or different features due to *in vitro* culture, homogeneity, selection for proproliferative cells, etc. One speculation is that ERRF is involved in the establishment and growth of HER2-positive breast cancer cell lines *in vitro*.

A positive correlation of ERRF with ER and PR and a negative correlation with HER2 suggest that ERRF is highly expressed in the luminal A subtype of breast cancer (ER and/or PR positive, HER2 negative). This is, indeed, the case, as luminal A tumors expressed significantly higher levels of ERRF than did luminal B or triple-negative tumors (Table 4). Patients with the luminal A subtype of breast cancer survive better than do those with luminal B or triple negative,[33,34] and as discussed later herein, ERRF expression is, indeed, associated with better patient survival.

Although ERRF expression is significantly increased in breast cancers, higher ERRF expression predicts better patient survival. For example, higher ERRF expression was associated with older age at diagnosis, lower tumor stage, lower tumor grade, and less lymph node metastasis (Table 2), which are established prognostic factors in breast cancer. Indeed, higher ERRF expression was significantly associated with better DFS and OS in the patients examined, along with lower tumor grade, lower tumor stage, and less lymph node metastasis (Table 6). The prognostic function of ERRF expression seems to be independent of the other three prognostic factors at least for DFS, as determined by the multivariate analysis (Table 6). The association of ERRF expression with patient survival became more significant in ER-positive tumors but disappeared in ER-negative patients, suggesting that ER positivity could drive the association between higher ERRF expression and better prognosis. It is possible that in ER-positive breast cancer, lower ERRF expression could promote tumor recurrence and metastasis.

ERRF is necessary for ER-positive breast cancer cells to proliferate. Estrogen-ER signaling regulates many genes in the mammary gland and breast cancer, and most of them are not directly involved in estrogen-modulated cell proliferation or tumorigenesis. There are some molecules that cooperate with ER through different mechanisms to promote tumor initiation and/or progression. Although our unpublished data suggest that estrogen-ER signaling does not induce ERRF and it is unknown whether ERRF regulates ER, our functional experiments revealed a necessary role of ERRF in the proliferation of ER-positive breast cancer cells, as knockdown of ERRF significantly impaired cell growth *in vitro* and tumorigen-

esis *in vivo* (Figures 4 and 5), and the mechanism for the suppression seems to be cell-cycle inhibition. These results, along with the significant correlation between ERRF expression and ER and PR positivity, suggest that ERRF is a novel ER-related factor that regulates the growth of breast cancer cells. We noticed that some ER-negative breast cancer cell lines also express higher levels of ERRF (Figure 1A). We are currently testing whether ERRF also plays a necessary role in the growth of these cells.

In ER-positive breast cancer, ER is expressed at higher levels, and estrogen-ER signaling is necessary for the proliferation of cancer cells.[2,3] In normal luminal epithelial cells of the mammary gland, ER is also expressed, although at lower levels, and estrogen-ER signaling is necessary for the formation, maintenance, and homeostasis of luminal epithelial cells.[4] ER-positive mammary epithelial cells do not seem to proliferate, although they are often adjacent to proliferative epithelial cells in the breast.[5–8] Consistently, in cultured nontumorigenic yet ER-positive breast epithelial cells, estrogen inhibits cell proliferation.[9–11] Taken together with our observations that ERRF expression associates with ER and PR expression and that ERRF is required by ER-positive cells to maintain proliferation, we hypothesize that ERRF plays a role in the conversion of ER function from prodifferentiation in the normal breast to proproliferation in breast cancer. We are currently testing this hypothesis in a series of functional experiments.

The function of ERRF in normal tissues could be related to the hormonal regulation of cellular behavior and/or function. In addition to the significant association between ERRF expression and the expression of ER and PR described in this study, all normal tissues that express ERRF are hormone related and/or hormone producing, including the brain, cortex, retina, and thyroid.[22] For example, retinal growth hormone is an antiapoptotic factor in embryonic retinal ganglion cell differentiation.[35] The cancer tissues with detectable ERRF expression in the SAGE database, ie, breast and prostate cancers, are highly regulated by the hormones estrogen and androgen, respectively. Assuming that ERRF is necessary for hormones to induce the proliferation of normal cells, one could speculate that the necessity of ERRF for the proliferation of ER-positive breast cancer cells could reflect ERRF's function in normal cells: proproliferative yet controlled and restricted, which could explain why patients with higher ERRF expression survive better.

The selective ER modulator tamoxifen not only is the most common endocrine agent used to treat all stages of ER-positive breast cancer but also is effective in the prevention of breast cancer in high-risk women.[36] Mechanistically, tamoxifen interferes with estrogen-mediated breast cancer growth by preventing estrogen from binding to ER. However, a substantial number of ER-positive breast cancers fail to respond to tamoxifen therapy by *de novo* or acquired resistance, which leads to disease progression and, ultimately, patient death.[12,13] ER is still maintained in most tamoxifen-resistant tumors and continues to regulate tumor growth.[12] Therefore, identification of ERRF as an ER-related factor that plays an important role in the growth of ER-positive breast cancer could provide a new opportunity for developing and/or improving therapeutic intervention in ER-positive breast cancer, especially for ER-positive but tamoxifen-resistant breast cancer. ERRF expression seems to be quite restrictive in normal tissues because so far it has been detected in only the brain, cortex, retina, and thyroid among at least 25 different normal tissues examined by the SAGE approach,[22] which could prove to be an advantage for targeting ERRF in therapeutic development.

In summary, we found that whereas the mutation of ERRF in breast cancer was rather infrequent (1 of 79 or 1.3%), the expression of ERRF was increased in breast cancer, and the increase was significantly associated with ER and PR expression in HER2-negative tumors. ERRF expression inversely correlated with HER2 status. Higher expression levels of ERRF in breast cancers were also significantly associated with older age at diagnosis, lower tumor grade, lower tumor stage, less lymph node metastasis, and better patient survival. Functionally, knockdown of ERRF in ER-positive breast cancer cell lines dramatically suppressed cell proliferation *in vitro* and in xenograft models. These results suggest that ERRF plays a role in the proproliferative function of estrogen-ER signaling in the luminal A subtype of breast cancer and that ERRF could be a potential therapeutic target for breast cancer treatment.

Acknowledgments

We thank Changsheng Xing, Mei Li, Zhiqian Zhang, Ang Luo, and Xin Li for their help in purifying DNA and RNA from breast tissues; Weidong Li and Jing Zhao for collecting some patient information; Yuan Liu for advice in the statistical analysis; and Peng Guo, Xiaodong Sun, Deepa Sikriwal, and Jianping Jenny Ni for their comments on preparation of the manuscript.

References

1. Siegel R, Ward E, Brawley O, Jemal A: Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin 2011, 61:212–236
2. Russo J, Russo IH: The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol 2006, 102:89–96
3. Yager JD, Davidson NE: Estrogen carcinogenesis in breast cancer. N Engl J Med 2006, 354:270–282
4. Matsuda M, Imaoka T, Vomachka AJ, Gudelsky GA, Hou Z, Mistry M, Bailey JP, Nieport KM, Walther DJ, Bader M, Horseman ND: Serotonin regulates mammary gland development via an autocrine-paracrine loop. Dev Cell 2004, 6:193–203
5. Anderson E, Clarke RB, Howell A: Estrogen responsiveness and control of normal human breast proliferation. J Mammary Gland Biol Neoplasia 1998, 3:23–35
6. Anderson E, Clarke RB: Steroid receptors and cell cycle in normal mammary epithelium. J Mammary Gland Biol Neoplasia 2004, 9:3–13
7. Polyak K: Breast cancer: origins and evolution. J Clin Invest 2007, 117:3155–3163
8. Russo J, Ao X, Grill C, Russo IH: Pattern of distribution of cells positive for estrogen receptor α and progesterone receptor in relation to proliferating cells in the mammary gland. Breast Cancer Res Treat 1999, 53:217–227
9. Zajchowski DA, Sager R, Webster L: Estrogen inhibits the growth of estrogen receptor-negative, but not estrogen receptor-positive, hu-

man mammary epithelial cells expressing a recombinant estrogen receptor. Cancer Res 1993, 53:5004–5011
10. Lundholt BK, Madsen MW, Lykkesfeldt AE, Petersen OW, Briand P: Characterization of a nontumorigenic human breast epithelial cell line stably transfected with the human estrogen receptor (ER) cDNA. Mol Cell Endocrinol 1996, 119:47–59
11. Pilat MJ, Christman JK, Brooks SC: Characterization of the estrogen receptor transfected MCF10A breast cell line 139B6. Breast Cancer Res Treat 1996, 37:253–266
12. Ali S, Coombes RC: Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer 2002, 2:101–112
13. Clarke R, Liu MC, Bouker KB, Gu Z, Lee RY, Zhu Y, Skaar TC, Gomez B, O'Brien K, Wang Y, Hilakivi-Clarke LA: Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. Oncogene 2003, 22:7316–7339
14. Bardeesy N, Cheng KH, Berger JH, Chu GC, Pahler J, Olson P, Hezel AF, Horner J, Lauwers GY, Hanahan D, DePinho RA: Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. Genes Dev 2006, 20: 3130–3146
15. Strausberg RL, Feingold EA, Grouse LH, et al: Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci U S A 2002, 99:16899–16903
16. Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE: The consensus coding sequences of human breast and colorectal cancers. Science 2006, 314:268–274
17. Ahomadegbe JC, Tourpin S, Kaghad M, Zelek L, Vayssade M, Mathieu MC, Rochard F, Spielmann M, Tursz T, Caput D, Riou G, Benard J: Loss of heterozygosity, allele silencing and decreased expression of p73 gene in breast cancers: prevalence of alterations in inflammatory breast cancers. Oncogene 2000, 19:5413–5418
18. Smedley D, Sidhar S, Birdsall S, Bennett D, Herlyn M, Cooper C, Shipley J: Characterization of chromosome 1 abnormalities in malignant melanomas. Genes Chromosomes Cancer 2000, 28:121–125
19. Dong XY, Guo P, Boyd J, Sun X, Li Q, Zhou W, Dong JT: Implication of snoRNA U50 in human breast cancer. J Genet Genomics 2009, 36:447–454
20. Dong XY, Rodriguez C, Guo P, Sun X, Talbot JT, Zhou W, Petros J, Li Q, Vessella RL, Kibel AS, Stevens VL, Calle EE, Dong JT: SnoRNA U50 is a candidate tumor-suppressor gene at 6q14.3 with a mutation associated with clinically significant prostate cancer. Hum Mol Genet 2008, 17:1031–1042
21. Ruopp MD, Perkins NJ, Whitcomb BW, Schisterman EF: Youden Index and optimal cut-point estimated from observations affected by a lower limit of detection. Biom J 2008, 50:419–430
22. Lash AE, Tolstoshev CM, Wagner L, Schuler GD, Strausberg RL, Riggins GJ, Altschul SF: SAGEmap: a public gene expression resource. Genome Res 2000, 10:1051–1060
23. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW: A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 2006, 10:515–527
24. Gazdar AF, Kurvari V, Virmani A, Gollahon L, Sakaguchi M, Westerfield M, Kodagoda D, Stasny V, Cunningham HT, Wistuba II, Tomlinson G, Tonk V, Ashfaq R, Leitch AM, Minna JD, Shay JW: Characterization of paired tumor and non-tumor cell lines established from patients with breast cancer. Int J Cancer 1998, 78:766–774
25. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A, Chinnaiyan AM: ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia 2004, 6:1–6
26. Arpino G, Wiechmann L, Osborne CK, Schiff R: Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. Endocr Rev 2008, 29:217–233
27. Collins LC, Schnitt SJ: HER2 protein overexpression in estrogen receptor-positive ductal carcinoma in situ of the breast: frequency and implications for tamoxifen therapy. Mod Pathol 2005, 18:615–620
28. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S, Deming SL, Geradts J, Cheang MC, Nielsen TO, Moorman PG, Earp HS, Millikan RC: Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA 2006, 295:2492–2502
29. Zhao J, Liu H, Wang M, Gu L, Guo X, Gu F, Fu L: Characteristics and prognosis for molecular breast cancer subtypes in Chinese women. J Surg Oncol 2009, 100:89–94
30. Balsari A, Casalini P, Tagliabue E, Greco M, Pilotti S, Agresti R, Giovanazzi R, Alasio L, Rumio C, Cascinelli N, Colnaghi MI, Menard S: Fluctuation of HER2 expression in breast carcinomas during the menstrual cycle. Am J Pathol 1999, 155:1543–1547
31. Huang HJ, Neven P, Drijkoningen M, Paridaens R, Wildiers H, Van Limbergen E, Berteloot P, Amant F, Vergote I, Christiaens MR: Hormone receptors do not predict the HER2/neu status in all age groups of women with an operable breast cancer. Ann Oncol 2005, 16:1755–1761
32. Schiff R, Massarweh SA, Shou J, Bharwani L, Arpino G, Rimawi M, Osborne CK: Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. Cancer Chemother Pharmacol 2005, 56(Suppl 1):10–20
33. Fadare O, Tavassoli FA: Clinical and pathologic aspects of basal-like breast cancers. Nat Clin Pract Oncol 2008, 5:149–159
34. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001, 98:10869–10874
35. Sanders EJ, Parker E, Aramburo C, Harvey S: Retinal growth hormone is an anti-apoptotic factor in embryonic retinal ganglion cell differentiation. Exp Eye Res 2005, 81:551–560
36. Keeton EK, Brown M: Coregulator expression and breast cancer: improving the predictive power of estrogen receptor α. Clin Cancer Res 2003, 9:1229–1230