

Association of Double-Positive FOXA1 and FOXP1 Immunoreactivities with Favorable Prognosis of Tamoxifen-Treated Breast Cancer Patients

Nobuhiro Ijichi · Takashi Shigekawa · Kazuhiro Ikeda ·
Kuniko Horie-Inoue · Chikako Shimizu ·
Shigehira Saji · Kenjiro Aogi · Hitoshi Tsuda ·
Akihiko Osaki · Toshiaki Saeki · Satoshi Inoue

Received: 6 January 2012 / Accepted: 16 March 2012 / Published online: 3 April 2012
© Springer Science+Business Media, LLC 2012

Abstract Breast cancer is primarily a hormone-dependent tumor that can be regulated by the status of the steroid hormones estrogen and progesterone. Forkhead box A1 (FOXA1) is a member of the forkhead box transcription factor family and functions as a pioneer factor of the estrogen receptor (ER) in breast cancer. In the present study, we demonstrate that FOXA1 mRNA was upregulated by estrogen and that estrogen receptor- α (ER α) recruitment to ER-binding sites in the vicinity of the FOXA1 gene was increased by estrogen in ER α -positive MCF-7 breast cancer cells. The estrogen-induced FOXA1 upregulation was repressed by 4-hydroxytamoxifen treatment. We also demonstrated that the

proliferation and the migration of MCF-7 cells were decreased by FOXA1-specific small interfering RNA (siRNA; siFOXA1). Furthermore, siFOXA1 decreased the estrogen response element-driven transcription and the estrogen-dependent upregulation of ER α target genes in MCF-7 cells. Next, the immunohistochemical analyses of FOXA1 were performed using two groups of breast cancer specimens. The nuclear immunoreactivity of FOXA1 was detected in 80 (74 %) of 108 human invasive breast cancers and was negatively correlated with tumor grade and positively correlated with hormone receptor status, including ER α and progesterone receptor, pathological tumor size, and immunoreactivity

N. Ijichi · T. Shigekawa · K. Ikeda · K. Horie-Inoue · S. Inoue
Division of Gene Regulation and Signal Transduction, Research
Center for Genomic Medicine, Saitama Medical University,
Saitama, Japan

T. Shigekawa · A. Osaki · T. Saeki
Department of Breast Oncology, International Medical Center,
Saitama Medical University,
Saitama, Japan

C. Shimizu
Division of Breast and Medical Oncology,
National Cancer Center Hospital,
Tokyo, Japan

T. Shigekawa · S. Saji
Division of Clinical Trials and Research
and Department of Surgery, Tokyo Metropolitan Cancer
and Infectious Disease Center, Komagome Hospital,
Tokyo, Japan

S. Saji
Department of Target Therapy Oncology,
Graduate School of Medicine, Kyoto University,
Kyoto, Japan

H. Tsuda
Department of Pathology and Clinical Laboratories,
National Cancer Center Hospital,
Tokyo, Japan

K. Aogi
Department of Surgery, National Shikoku Cancer Center,
Ehime, Japan

S. Inoue
Department of Anti-Aging Medicine,
Graduate School of Medicine, The University of Tokyo,
Tokyo, Japan

S. Inoue (✉)
Department of Geriatric Medicine,
Graduate School of Medicine, The University of Tokyo,
7-3-1 Hongo,
Bunkyo-ku, Tokyo 113-8655, Japan
e-mail: INOUE-GER@h.u-tokyo.ac.jp

of FOXP1, another FOX family transcription factor. FOXA1 immunoreactivity was significantly elevated in the relapse-free breast cancer patients treated with tamoxifen. Notably, the double-positive immunoreactivities of FOXA1 and FOXP1 were significantly associated with a favorable prognosis for the relapse-free and overall survival of patients with tamoxifen-treated breast cancer, with lower *P* values compared with FOXA1 or FOXP1 immunoreactivity alone. These results suggest that FOXA1 plays an important role in the proliferation and migration of breast cancer cells by modulating estrogen signaling and that the double-positive immunoreactivities of FOXA1 and FOXP1 are associated with a favorable prognosis of tamoxifen-treated breast cancer.

Abbreviations

FOXA1	Forkhead box A1
FOXP1	Forkhead box P1
ER α	Estrogen receptor alpha
PgR	Progesterone receptor
GREB1	Growth regulation by estrogen in breast cancer 1
TFF1	Trefoil factor 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ERE	Estrogen response element
siRNA	Small interfering RNA
qRT-PCR	Quantitative real-time reverse transcriptase polymerase chain reaction

Introduction

Estrogen signaling pathways regulate various cellular events, including cell growth and apoptosis, through the activation of estrogen receptor alpha (ER α) [1]. ER α functions as a transcription factor to activate the target gene expression. Clinically, ER α is noted as the defining feature of luminal breast cancers, which comprise most breast cancers. Luminal breast cancers are generally treated with endocrine therapies, including the antiestrogen tamoxifen which acts as an antagonist for the estrogen receptor (ER) in breast cancer cells. Due to the sensitivity to endocrine therapy, ER-positive luminal breast cancers are considered to have better prognosis than ER-negative breast cancers. However, resistance for antiestrogen therapies is acquired in a substantial fraction of recurrent breast cancers. Identification of the factors involved in drug resistance, recurrence, or poor prognosis of breast cancer would be useful for the treatment and the diagnosis of the disease.

The transcriptional activity of ER α is regulated by various co-activators and co-repressors [2], as well as by interactions with other transcription factors, including the

forkhead box (FOX) family. FOX family transcription factors influence ER α -regulated transcription by interacting with the ER α protein, as exemplified by FOXA1 [3]. Recent global gene expression studies of breast cancer revealed that a high FOXA1 expression was positively correlated with ER α and PR, but negatively correlated with histological grade and proliferation markers [4–6]. In addition, FOXA1 expression was associated with better cancer-specific survival, indicating that it is a better predictor of survival in breast cancer [4–6]. Recent genome-wide studies aimed at identifying ER α and androgen receptor (AR)-binding sites have shown that FOXA1 plays a role in regulating both of these nuclear receptor networks [7,8]. FOXA1 is recognized as a pioneer transcription factor because chromatin binding by this protein can enable subsequent binding by the ER and AR [8,9]. More recently, genome-wide mapping of ER α -, AR-, and FOXA1-binding events in breast and prostate cancer cells by the use of high-throughput sequencing has uncovered additional details of transcriptional control mechanisms in the nuclear receptor-mediated transcription involved in several collaborative factors, including TLE1 and AP-2 γ [10,11].

Gene expression profiling studies have classified breast cancer into five intrinsic subtypes with unique molecular characteristics and prognostic significance [12,13]: the luminal A and B, HER2+/ER-, basal-like, and normal-like subtypes. Luminal subtypes A and B are ER α -positive breast cancers, with subtype A expressing higher levels of ER α and having a better prognosis than subtype B [13]. FOXA1 expression correlates with luminal subtype A breast cancer, and the immunoreactivity of FOXA1 is shown as a significant predictor of cancer-specific survival in patients with ER-positive tumors [5]. The prognostic ability of FOXA1 in these low-risk breast cancers may prove to be useful in clinical treatment decisions [5,6].

The altered expression of FOXP1, another FOX family member, is associated with various types of tumors, including breast cancer [14–18]. We recently demonstrated that FOXP1 plays an important role in the proliferation of breast cancer cells by modulating estrogen signaling, and FOXP1 immunoreactivity could be associated with the immunoreactivity of ER α and PR in breast cancer, which may predict favorable prognosis in patients treated with tamoxifen [19]. However, the clinical significance of FOXA1 on tamoxifen resistance in breast cancer and the correlation between FOXA1 and FOXP1 remain to be elucidated.

In the present study, we show that FOXA1 mRNA expression is induced by estrogen and that ER α recruitment to the ER-binding sites in the FOXA1 gene region is elevated by estrogen in ER α -positive MCF-7 human breast cancer cells. Functional analyses reveal that the knockdown of FOXA1 impairs the proliferation and migration of MCF-7 cells as well as ER-mediated transcription and estrogen-

dependent upregulation of ER α target genes in MCF-7 cells. Furthermore, we evaluated the FOXA1 expression in human breast cancers using immunohistochemistry and investigated the correlations between FOXA1 expression levels and clinicopathophysiological findings. Finally, FOXA1 immunoreactivity is negatively associated with the recurrence of tamoxifen-treated breast cancer. Together with our previous report on FOXP1 [19], the double-positive immunoreactivities of FOXA1 and FOXP1 are significantly associated with longer survival in patients with tamoxifen-treated breast cancer. Our study provides new insight into the association between FOXA1 and FOXP1 in estrogen signaling and the prognosis of tamoxifen-treated breast cancer.

Materials and Methods

Tissue Selection and Patient Characteristics

Between January 2005 and March 2006, 108 consecutive patients with invasive breast cancer diagnosed using a vacuum-assisted biopsy device (Mammotome, Ethicon Endo-surgery Inc., Cincinnati, OH) at the Saitama Medical University Hospital were included in a cohort study.

For a nested control study of the therapeutic effect of tamoxifen on recurrent breast cancer, 162 patients with breast cancer diagnosed between 1989 and 1998 with or without distant metastases during or after tamoxifen therapy were identified from three institutions (National Hospital Organization Shikoku Cancer Center, Matsuyama, Japan; National Cancer Center Hospital, Tokyo, Japan; and Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo, Japan). Patients experiencing relapse were defined as those with distant metastases within 5 years after surgery followed by tamoxifen treatment, whereas those who were considered relapse-free were those without distant metastases. A total of 113 patients met our protocol criteria and were included in this study.

Formalin-fixed paraffin-embedded tissue sections obtained by biopsy or surgery were used in these studies. These studies were approved by the institutional review board of Saitama Medical University, and informed consent was obtained from all the patients. The clinicopathological characteristics of the series are presented in Tables 1 and 2.

Antibodies

Mouse monoclonal anti-FOXA1 antibody was purchased from Abcam (Cambridge, UK). Affinity-purified rabbit polyclonal anti-FOXP1 antibody (anti-GX5050) was generated by the Genome Network Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan (<http://genomenetwork.nig.ac.jp>), from serum derived from

rabbits immunized with a peptide epitope consisting of amino acids 519–677 of the human FOXP1 protein.

Immunohistochemistry

The immunohistochemical analyses for FOXA1 and FOXP1 were performed using an EnVision+ visualization kit (Dako, Carpinteria, CA) as previously described [20]. The tissue sections (6 μ m) were deparaffinized, rehydrated through graded ethanol, and rinsed in Tris-buffered saline with 0.05 % Tween-20 (TBST). For antigen retrieval, the sections were heated in an autoclave at 121 °C for 5 min in 10 mM sodium citrate buffer (pH 6.0). The sections were blocked with endogenous peroxidase (0.3 % H₂O₂) and incubated in 10 % fetal bovine serum for 30 min. The primary antibody, a monoclonal antibody for FOXA1 (1:2,000 dilution) or a polyclonal antibody for FOXP1 (1:1,000 dilution), was applied, and the samples were incubated overnight at 4 °C. The sections were rinsed in TBST and incubated with EnVision+ horseradish peroxidase-labeled polymer for 1 h at room temperature. The antigen–antibody complex was visualized using the 3,3'-diaminobenzidine substrate kit for peroxidase (Vector Laboratories, Burlingame, CA). Mouse or rabbit immunoglobulin G was used in place of the primary antibody as a negative control.

Immunohistochemical Assessment

The 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 the 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 [21]. Two investigators (H.T. and A.O.) evaluated the tissue sections independently. If the immunoreactivity score differed between the two investigators, a third investigator (T.S.) evaluated the tissue sections and the mean immunoreactivity score was used. When the two investigators found it difficult to evaluate the TS of heterogeneous cancerous lesions, the third investigator made the deciding estimate. To identify potential correlations between the FOXA1 or FOXP1 expression in the malignant epithelium and clinicopathological characteristics, immunoreactivity scores of 0 or 2 and 3–8 were defined as negative and positive immunoreactivity, respectively.

Small Interfering RNA

Synthetic small interfering RNA (siRNA) duplexes targeting the human FOXA1 gene (Silencer Select Pre-designed siRNA) and the luciferase reporter plasmid pGL2

Table 1 Relationship between the immunoreactivity of FOXA1 and clinicopathological findings in invasive breast cancer ($n=108$)

Clinical findings		Immunoreactivity score of FOXA1 ^a		
		Positive ($n=80$)	Negative ($n=28$)	<i>P</i> value
Age ^b (mean±SD)		59.9±14.8	57.1±14.2	
	≤50	23 (21.3 %)	9 (8.3 %)	0.92
	>50	57 (52.8 %)	19 (17.6 %)	
pT	≤20 mm	44 (48.9 %)	9 (10.0 %)	0.047
	>20 mm	23 (25.6 %)	14 (15.5 %)	
Stage	I, II	73 (68.2 %)	28 (26.2 %)	0.31
	III, IV	6 (5.6 %)	0 (0.0 %)	
Grade	I, II	58 (70.7 %)	16 (19.5 %)	0.024
	III	3 (3.7 %)	5 (6.1 %)	
ER	Positive (PS≥3)	56 (51.9 %)	13 (12.0 %)	0.025
	Negative (PS≤2)	24 (22.2 %)	15 (13.9 %)	
PgR	Positive (PS≥3)	36 (33.3 %)	5 (4.6 %)	0.020
	Negative (PS≤2)	44 (40.8 %)	23 (21.3 %)	
HER2	Positive	17 (18.5 %)	9 (9.8 %)	0.37
	Negative	51 (55.4 %)	15 (16.3 %)	
Lymph node	Positive	28 (31.5 %)	9 (10.1 %)	0.92
	Negative	41 (46.1 %)	11 (12.3 %)	
FOXP1 ^a	Positive	58 (55.2 %)	13 (12.4 %)	0.027
	Negative	21 (20.0 %)	13 (12.4 %)	

All other values represent the number and proportion of cases
ER estrogen receptor, *PgR* progesterone receptor, *TS* total score, *PS* proportion score
^aFOXA1 and FOXP1 immunoreactivity scores of 0, 2, and 3–8 were defined as negative and positive immunoreactivity, respectively

^bData are presented as the mean ±SD

(Luciferase GL2 Duplex) used as a negative control were purchased from Applied Biosystems (Carlsbad, CA) and Dharmacon (Lafayette, CO), respectively.

Cell Culture and Transfection

MCF-7 cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (FBS) at 37 °C in 5 % CO₂. 17β-Estradiol (E₂) and 4-hydroxytamoxifen (4-OHT) were purchased

from Sigma-Aldrich (St. Louis, MO). The transfection of siRNA was performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA extraction, first-strand cDNA synthesis, and quantitative PCR (qPCR) protocols have been described elsewhere [20]. The primers for FOXA1, glyceraldehyde-3-phosphate

Table 2 Clinicopathological findings in adjuvant tamoxifen-treated invasive breast cancer patients followed up for 5 years after surgery ($n=113$)

Clinical findings		Relapse ($n=43$)	Relapse-free ($n=70$)	<i>P</i> value
Age ^a (mean±SD)		53.2±9.9	55.2±12.4	0.39
Age	≤50	20 (17.7 %)	28 (24.8 %)	0.50
	>50	23 (20.3 %)	42 (37.2 %)	
pT	≤30 mm	22 (19.5 %)	52 (46.0 %)	0.012
	>30 mm	21 (18.6 %)	18 (15.9 %)	
Lymph node	Positive ($n≥1$)	30 (26.5 %)	30 (26.5 %)	0.005
	Negative ($n=0$)	13 (11.5 %)	40 (35.4 %)	
ERα	Positive	39 (34.5 %)	64 (56.6 %)	0.84
	Negative	4 (3.6 %)	6 (5.3 %)	
PgR	Positive	36 (31.9 %)	60 (53.1 %)	0.99
	Negative	7 (6.2 %)	10 (8.8 %)	
FOXA1	Positive	19 (16.8 %)	51 (45.1 %)	0.002
	Negative	24 (21.2 %)	19 (16.8 %)	
FOXP1	Positive	22 (19.5 %)	57 (50.4 %)	<0.001
	Negative	21 (18.6 %)	13 (11.5 %)	

All other values represent the number and proportion of cases
pT pathological T stage, *ERα* estrogen receptor-α, *PgR* progesterone receptor

^aData are presented as the mean±SD

dehydrogenase (GAPDH), progesterone receptor (PgR), and growth regulation by estrogen in breast cancer 1 (GREB1) are as follows: FOXA1, 5'-AGGTGTGTATTCCAGACCCG-3' and 5'-TTGACGGTTTGGTTTGTGTG-3'; GAPDH, 5'-GGTGGTCTCCTCTGACTTCAACA-3' and 5'-GTGGTTCGTTGAGGGCAATG-3'; PgR, 5'-CGTGCCATCCTGCCTCTCA-3' and 5'-CCGCCGTCGTAACCTTTCG-3'; GREB1, 5'-CGACCATCGGCTTTAGGTATCTT-3' and 5'-CCACCTTTGTGGCGTTTT-3'. The fold induction of the mRNA expression levels was determined by comparing the estrogen-treated samples with the vehicle-treated controls.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay and the qPCR were performed as previously described [22,23]. After 72-h hormone depletion, MCF-7 cells were treated with 100 nM E₂ or vehicle (0.1 % ethanol) for 45 min. The cells were fixed in 1 % formaldehyde for 5 min at room temperature. Chromatin was sheared to a mean size of 500 bp by sonication using a Bioruptor ultrasonicator (Cosmo-Bio, Tokyo, Japan). The lysates were rotated overnight at 4 °C, with an ER α -specific antibody (sc-543, Santa Cruz Biotechnologies, Santa Cruz, CA). Protein A-agarose (Upstate Biotechnology, Lake Placid, NY) was added and incubated for 2 h. Precipitated DNA was used as a template for qPCR using an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA) based on SYBR Green I fluorescence. A genomic fragment containing ERE in the enhancer region of trefoil factor 1 (TFF1; 405/393 bp from the transcription start site) was used as a positive control for ER binding [24]. The sequences of PCR primers are as follows: TFF1_ERE, 5'-TGAGATTCA GAAAGTCCCTCTTTC-3' and 5'-TGGGCTTCAT GAGCTCCTT-3'; FOXA1_ER_8127, 5'-TGGGTCAG GAAGAAAACATCTGT-3' and 5'-TGTGGAAACAC CCAGACGAA-3'; FOXA1_ER_8128, 5'-GAG GACGGCCCGACTTG-3' and 5'-CACCGGTTTA TATCTTTATGACATAACTTT-3'; GAPDH, 5'-GGTGGTCTCCTCTGACTTCAACA-3' and 5'-GTGGTTCGTTGAGGGCAATG-3'; Myoglobin, 5'-AAGTTTGACAAGTTCAAGCACCTG-3' and 5'-TGGCACCATGCTTCTTTAAGTC-3'.

Luciferase Assay

The MCF-7 cells were plated in 24-well culture plates at a density of 10,000 cells per well in phenol red-free medium containing 10 % charcoal-stripped FBS and transfected with 0.1 μ g of a luciferase reporter vector containing ERE (ERE-TK-LUC) [25] and 0.02 μ g of pRL-CMV (Promega,

Madison, WI), together with siRNA for FOXA1 using the Lipofectamine 2000 reagent (Invitrogen). Twelve hours after the transfection, the cells were treated with 100 nM E₂ or vehicle (ethanol) for 24 h, and the luciferase activities were then determined using the MicroLumatPlus microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) and the Dual-Luciferase Assay System (Promega). The data are expressed as the mean \pm SD of three independent experiments performed in triplicate.

Cell Proliferation Assay

The MCF-7 cells were seeded in 96-well plates at a density of 5,000 cells per well in DMEM containing 10 % FBS for 24 h. Then, 20 pmol of the siRNA described above was transfected for 12 h and incubated for 96 h. Cell proliferation was examined using a (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; WST-8) assay kit (Nacalai Tesque, Kyoto, Japan), performed according to the manufacturer's protocol.

Cell Migration Assay

To measure the cell migration activity, migration assays were performed using the Cell Culture Inserts for 24-well plates (8.0- μ m pore; BD Falcon, BD Biosciences, Franklin Lakes, NJ). Before cell culturing, the inside of the insert membranes was coated with purified fibronectin (BD Falcon) in PBS at a concentration of 10 μ g/mL for 30 min at room temperature. After the transfection of siRNA for 48 h, the MCF-7 cells were collected and resuspended in DMEM containing 10 % FBS. The MCF-7 cells were subsequently added to the upper chamber at 3×10^5 cells per well. After the 24-h incubation, the cells on the upper surface of the membrane were completely wiped away with a cotton swab. The cells on the lower surfaces of the membrane were fixed with 100 % methanol, the insert membranes were cut and stained with Giemsa stain solution (Wako, Tokyo, Japan), and the permeating cells within five randomly selected areas were counted under an inverted microscope. At least three independent experiments were performed for all the conditions. The data are shown as the mean \pm SD.

Statistical Analyses

The differences between the two groups were analyzed using a two-sample, two-tailed Student's *t* test. Value of $P < 0.05$ was considered statistically significant. All data are presented in the text and the figures as the mean \pm SD. The correlation between the immunoreactivity score and the clinicopathological characteristics was evaluated using the chi-square test. Relapse-free and overall survival curves

were obtained using the Kaplan–Meier method and verified using the log-rank (Mantel–Cox) test. Univariate and multivariate analyses were performed using a logistic regression model with the JMP 9 software (SAS Institute, Cary, NC), and $P < 0.05$ was regarded as statistically significant.

Results

Estrogen Upregulates FOXA1 mRNA Expression and ER α Recruitment to the Putative ER-Binding Sites Within the FOXA1 Gene Region in MCF-7 Cells

We examined the transcriptional regulation of the FOXA1 gene by 17 β -estradiol (E_2) in the ER α -positive MCF-7 cells (Fig. 1a). The FOXA1 mRNA level was significantly upregulated twofold at 3 h and threefold at 48 h after E_2 stimulation, while E_2 -mediated upregulation of FOXA1 was repressed by 4-OHT (Fig. 1b). These results suggest that the mRNA expression of FOXA1 is transcriptionally regulated by estrogen in breast cancer cells owing to the relatively early response (by 1 h). In support of these results, the genome-wide ChIP-on-chip analysis using the MCF-7 cells reported by Carroll et al. [26] demonstrated the presence of two estrogen receptor-binding sites (ERBSs; ER_8127 and 8128) within a 3-kb distance from the start or end point of the gene (Fig. 1c). To determine whether these ERBSs are bona fide ERBSs in the MCF-7 cells, we performed a conventional ChIP assay using ER α -specific antibody in comparison with a known estrogen response element of TFF1 as a positive control and the coding region of myoglobin (MB) as a negative control (normalized by the coding region of GAPDH) [24]. As shown in Fig. 1d, basal estrogen-independent ER α binding on ER_8128 in the FOXA1 promoter region is larger than that on ER_8127 in the downstream region, and substantial E_2 -induced ER α recruitment is observed in both ER_8127 and ER_8128. These data suggest that both ERBSs contribute to the estrogen-dependent transcription of FOXA1.

FOXA1 Contributes to Breast Cancer Cell Proliferation and Migration

To assess the role of FOXA1 in breast cancer, we performed loss-of-function studies for it. Transient transfection of siFOXA1 in MCF-7 cells resulted in a downregulated FOXA1 expression compared with that of the control siRNA (siControl), as confirmed by qRT-PCR (Fig. 2a). The MCF-7 cell growth was suppressed by the siFOXA1 treatment (Fig. 2b). Furthermore, the siFOXA1 treatment resulted in a decreased MCF-7 cell migration (Fig. 2c, d). These results indicate that FOXA1 regulates breast cancer cell proliferation and migration.

FOXA1 Regulates ER-Mediated Transcription

To examine whether FOXA1 influences ER-ERE-mediated transcription, ERE-TK-LUC was introduced into the MCF-7 cells together with siFOXA1 or siControl (Fig. 2e). The results indicate that siFOXA1 reduced ER-ERE-mediated transactivation in the presence or absence of estrogen in MCF-7 cells. To further confirm the contribution of FOXA1 to ER-mediated transcriptional regulation, we evaluated the expressions of endogenous ER α target genes including PgR and GREB1 in the presence or absence of FOXA1 siRNA (Fig. 2f, g). We showed that E_2 -induced expressions of PgR and GREB1 were reduced by FOXA1 siRNA treatment, suggesting that FOXA1 affects E_2 -mediated target gene activation.

Correlation Between FOXA1 Immunoreactivity and the Clinicopathological Features of Invasive Breast Cancer

To investigate the expression levels of the FOXA1 protein in breast cancer, immunohistochemical analysis was performed using samples from 108 invasive breast cancers. Representative images of FOXA1 immunostaining are shown in Fig. 3. Positive nuclear immunoreactivity of FOXA1 was detected in 74 % of breast cancer specimens (Table 1). FOXA1 immunoreactivity in grade III breast cancer was significantly lower than that in grade I breast cancer. Namely, the mean Allred score of FOXA1 immunoreactivity in grade I was 5.83, whereas that in grade III was 2.38 ($P = 0.002$). The nuclear immunoreactivity of FOXA1 was significantly correlated with ER α immunoreactivity ($P = 0.025$, Table 1). The positive nuclear immunoreactivity of FOXA1 was also significantly correlated with pathological tumor size (pT), low histological grade, and positive immunoreactivity of PgR and FOXP1 ($P = 0.047$, 0.024, 0.020, and 0.027, respectively), whereas no significant association was found with other clinicopathological characteristics (Table 1).

Positive FOXA1 Immunoreactivity is Correlated with Good Prognosis for the Relapse-Free Survival in Patients with Tamoxifen-Treated Breast Cancer

To examine the correlation of FOXA1 with the recurrence of breast cancer after endocrine therapy, immunohistochemical analysis was performed using samples from 113 patients with tamoxifen-treated invasive breast cancers whose relapse-free survival could be followed. Within 5 years after the surgery, a relatively large fraction of the relapse-free patients showed positive FOXA1 immunoreactivity (Table 2). Furthermore, patients with positive immunoreactivity for FOXA1 showed good relapse-free and overall survival, with

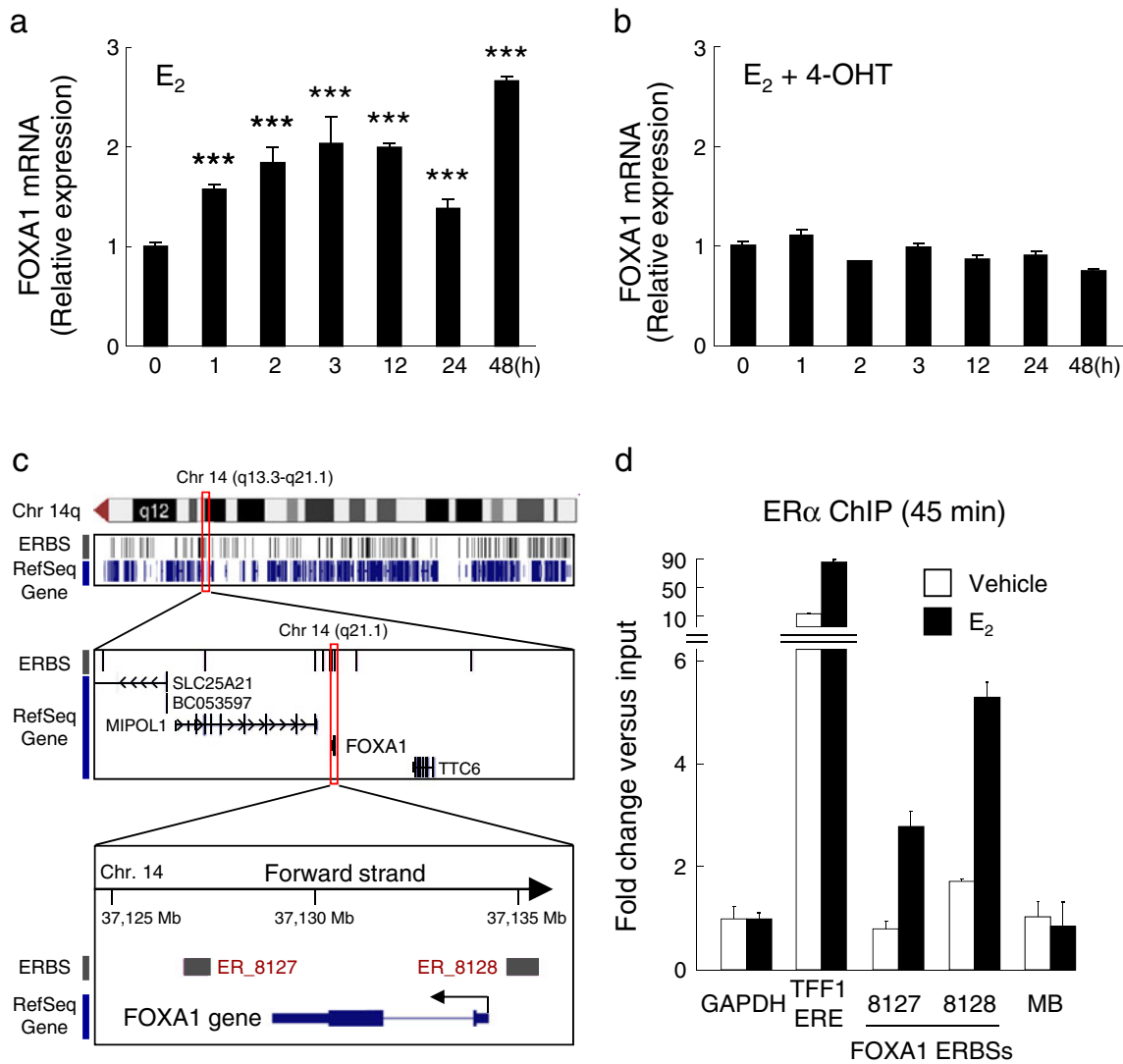


Fig. 1 Estrogen-induced expression of FOXA1 in the MCF-7 cells. **a**, **b** MCF-7 cells were treated with 100 nM 17β-estradiol (E₂) (**a**) or with 100 nM E₂ and 1 nM 4-OHT (**b**) for 48 h. The FOXA1 mRNA level was examined at indicated time points by qRT-PCR; the results are shown as a fold change over the expression level at 0 h. ****P*<0.001 compared with 0 h (by Student’s *t* test). **c** Schematic representation of estrogen receptor-binding sites (ERBSs) in the vicinity of FOXA1 gene in chromosome 14. Cytogenetic location of the FOXA1 gene in chromosome 14 and UCSC Genome Browser view customized with estrogen-dependent ERα binding sites (ERBSs) determined by ChIP-on-chip at the 14q region [26] are shown. Two significant ERBSs

(ER_8127 and 8128) at a cutoff value *P*<1e−3 are identified in the vicinity of FOXA1 gene region. **d** Estrogen-dependent recruitment of ERα to the FOXA1 ERBSs. The MCF-7 cells were treated with 100 nM of E₂ or vehicle for 45 min and then subjected to ChIP analysis with the ERα-specific antibody. Immunoprecipitated DNA was quantified by qPCR; the results were represented as the fold enrichment of ERα occupancy in the immunoprecipitated DNAs vs. input DNAs. The estrogen response element of the Trefoil factor 1 (*TFF1* gene (*TFF1* ERE) and non-ERBS within the myoglobin (*MB*) gene region were used as the positive and negative controls, respectively

significant differences compared with FOXA1-negative patients (Fig. 4a, b, respectively).

We recently reported that FOXP1, another FOX family member, was also correlated with the relapse-free survival of the patients with tamoxifen-treated breast cancer [19]. In this study, patients with positive immunoreactivity for FOXP1 also showed good relapse-free and overall survivals, with significant differences compared with the FOXP1-negative patients (Fig. 4c, d, respectively). Finally, we

divided the patients into two groups: those with double-positive FOXA1 and FOXP1 immunoreactivities and those with the FOXA1-positive/FOXP1-negative, FOXA1-negative/FOXP1-positive, and FOXA1-negative/FOXP1-negative immunoreactivities. It is notable that patients with double-positive FOXA1 and FOXP1 immunoreactivities showed good relapse-free and overall survival with significant differences compared with the FOXA1-positive/FOXP1-negative, FOXA1-negative/FOXP1-positive, and FOXA1-

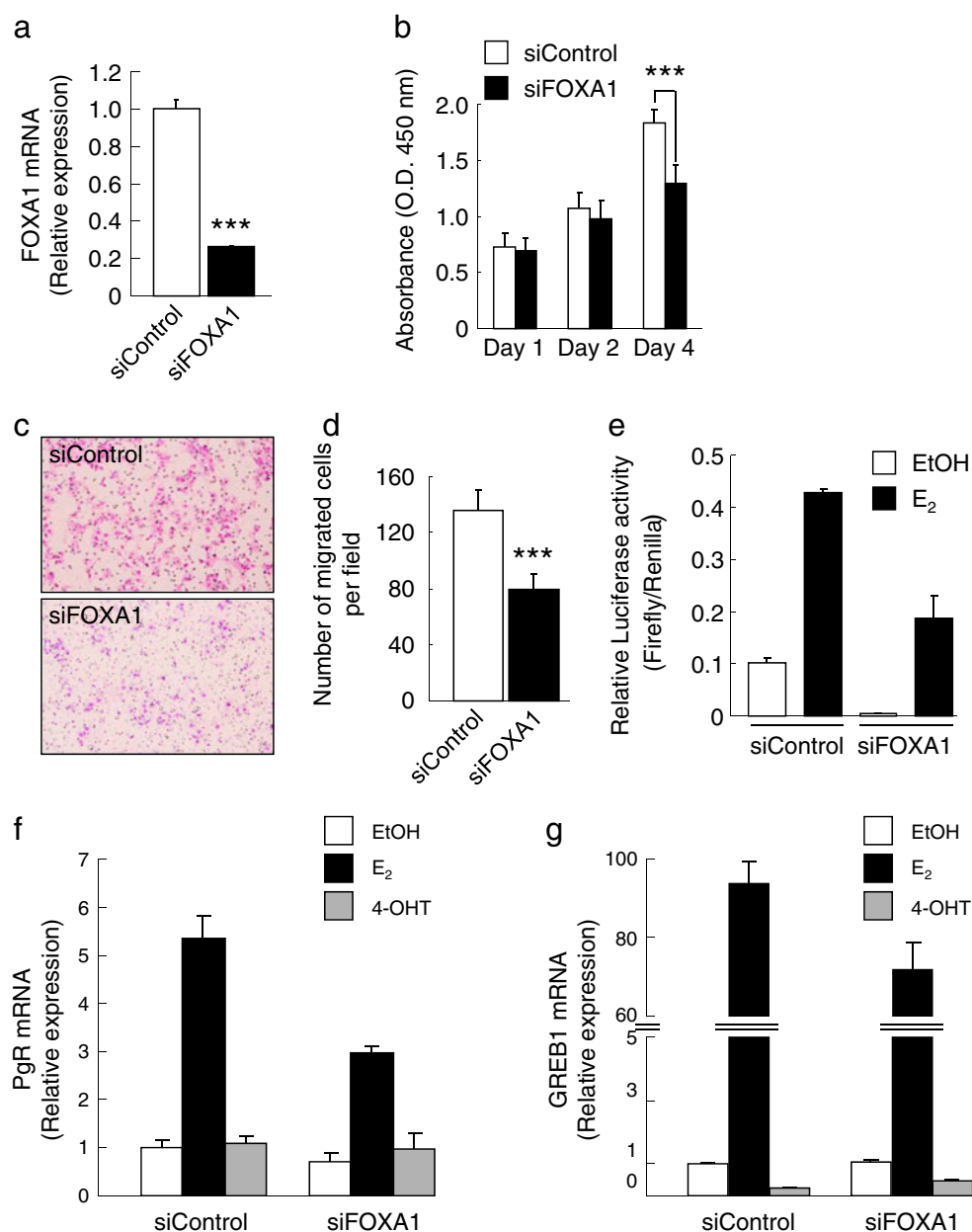


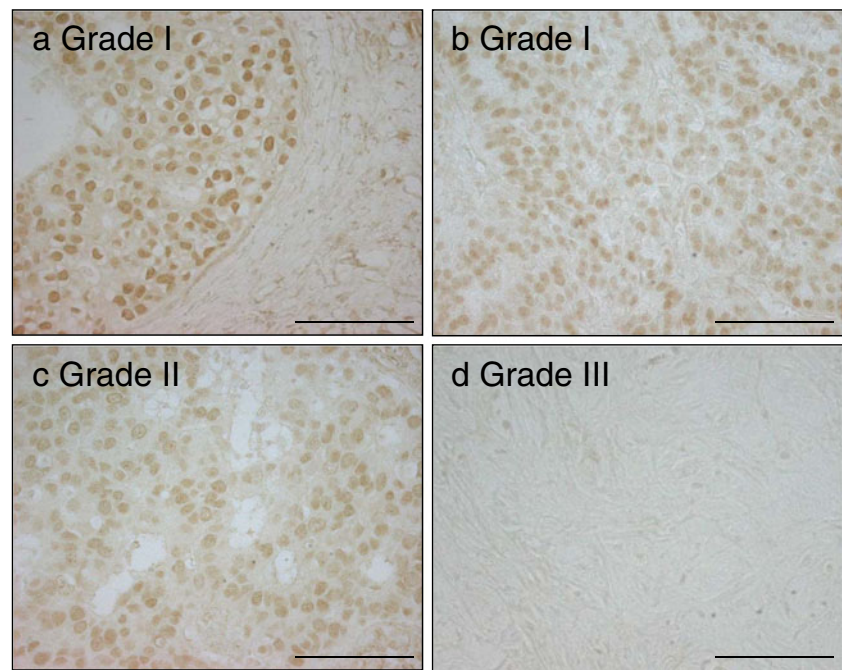
Fig. 2 Knockdown of FOXA1 suppresses cell proliferation and migration of the MCF-7 cells. **a** Total RNA was prepared from the MCF-7 cells transfected with 20 nM of siRNA specific for FOXA1 (*siFOX A1*) or control siRNA (*siControl*) for 48 h; the FOXA1 mRNA level was examined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). *** $P < 0.001$ compared with the siControl (Student's *t* test). **b** MCF-7 cells were transfected with 20 nM of siFOX A1 or siControl for 4 days in Dulbecco's modified Eagle medium (DMEM). The relative cell proliferation at indicated time points was examined using a WST-8 assay kit and determined photometrically as the absorbance at OD=450 nm. *** $P < 0.001$ compared with the siControl (Student's *t* test). **c**, **d** The MCF-7 cells were transfected with 20 nM of siFOX A1 or siControl for 48 h in DMEM, and then the cells were transferred into transwells for an additional 48 h. The representative

photographs (**c**) and numbers of migrated cells (**d**) are shown. **e** Effect of FOXA1 on estrogen response element (ERE)-mediated transcription in the MCF-7 cells. The MCF-7 cells were transfected with a DNA mixture of 0.1 μ g of ERE-TK-LUC, 0.02 μ g of pRL-CMV, and 20 nM of siFOX A1 or siControl. After 12 h, the cells were treated with 17 β -estradiol (100 nM) or vehicle (EtOH) for 24 h, and then the cell lysates were prepared for and used in the luciferase assay. **f**, **g** Effect of siFOX A1 on the expression of ER α target genes. MCF-7 cells were transfected with 20 nM of siFOX A1 or siControl. After 12 h, cells were treated with E₂, 4-OHT, or EtOH for 24 h. mRNA levels of progesterone receptor (*PgR*) (**f**) and growth regulation by estrogen in breast cancer 1 (*GREB1*) (**g**) were examined by qRT-PCR; the results are shown as a fold change over EtOH treatment

negative/FOXP1-negative immunoreactivities (Fig. 4e, f, respectively).

Table 3 shows the results of the univariate and multivariate proportional analyses of the relapse-free and overall

Fig. 3 Immunohistochemistry of FOXA1 in breast cancer. The representative images of the immunohistochemical staining of breast cancer tissues with the anti-FOXA1 antibody are shown. Positive staining for FOXA1 was observed in the nuclei of the breast cancer cells. The FOXA1 immunoreactivities in grade III breast cancer were significantly lower than those in grade I breast cancer (5.83 vs. 2.38, $P=0.002$). Bar, 100 μm



survival rates associated with FOXA1 and FOXP1 immunoreactivities and the patients' clinicopathological characteristics. For the relapse-free survival, either FOXP1 or FOXA1 immunoreactivity was found to be a significant prognostic predictor through the univariate analysis ($P=0.001$ and 0.002 , respectively), whereas only FOXP1 immunoreactivity was a significantly better prognostic predictor based on multivariate analysis ($P=0.026$, odds ratio=0.35, 95 % confident index=0.14–0.88; Table 3, relapse-free survival). On the other hand, neither FOXP1 nor FOXA1 was significantly associated with overall survival based on the multivariate analysis (Table 3, overall survival). It is an interesting finding that double-positive FOXP1 and FOXA1 immunoreactivities were significantly associated with favorable prognosis for the relapse-free and overall survival with lower P values compared with either FOXP1 or FOXA1 immunoreactivity based on the multivariate analyses ($P=0.002$ and 0.002 in Table 4, respectively). These results indicate that the double-positive FOXA1 and FOXP1 immunoreactivities are favorable prognostic predictors of the relapse-free and overall survivals in patients with tamoxifen-treated breast cancer.

Discussion

In the present study, the FOXA1 mRNA expression was found to be upregulated by estrogen in ER α -positive MCF-7 breast cancer cells. In addition, ER α recruitment to ERBSs within the FOXA1 gene region was significantly enriched by estrogen treatment in the ChIP assays using

the ER α antibody. In the reporter assay, knockdown of FOXA1 impaired ER α -ERE-mediated transcription in the MCF-7 cells. Together with these findings and our previous report on FOXP1 [19], it is possible that ER α receives positive feedback regulation from FOXA1 and FOXP1 in breast cancer cells.

FOXA1-binding sites were detected in 50 % of genes that are regulated by ER, and FOXA1 depletion partially attenuated the estrogen response in breast cancer cells [7,27,28] responsible for the transcriptional regulation of various ER α -target genes, including TFF1, GREB1, and cyclin D1. We showed previously that FOXP1 overexpression upregulates the transcription of ER α target genes including LRH-1 and SHP in breast cancer cells [19]. Notably, the binding site for ER α was identified to overlap with that for FOXA1 in the far upstream of the LRH-1 gene region [26]. These findings would suggest that FOXA1 and FOXP1 have a partially redundant function of modulating ER α -mediated transcription. It would be worthwhile to identify the genome-wide binding sites of FOXP1 to elucidate the functional correlation between FOXA1 and FOXP1 in ER α -mediated transcription in breast cancer cells.

In our previous data, FOXP1 immunoreactivity was significantly elevated in tamoxifen-treated breast cancer patients without relapse compared with those with relapse ($P<0.001$) [19]. FOXP1 immunoreactivity is also an independent prognostic factor for relapse-free survival by both univariate and multivariate analyses in the present study ($P=0.001$ and 0.026 , respectively, Table 3). With regard to FOXA1 immunoreactivity, a significant association with relapse-free survival was shown by univariate analysis ($P=0.002$, Table 3). Consistent with our observation in part,

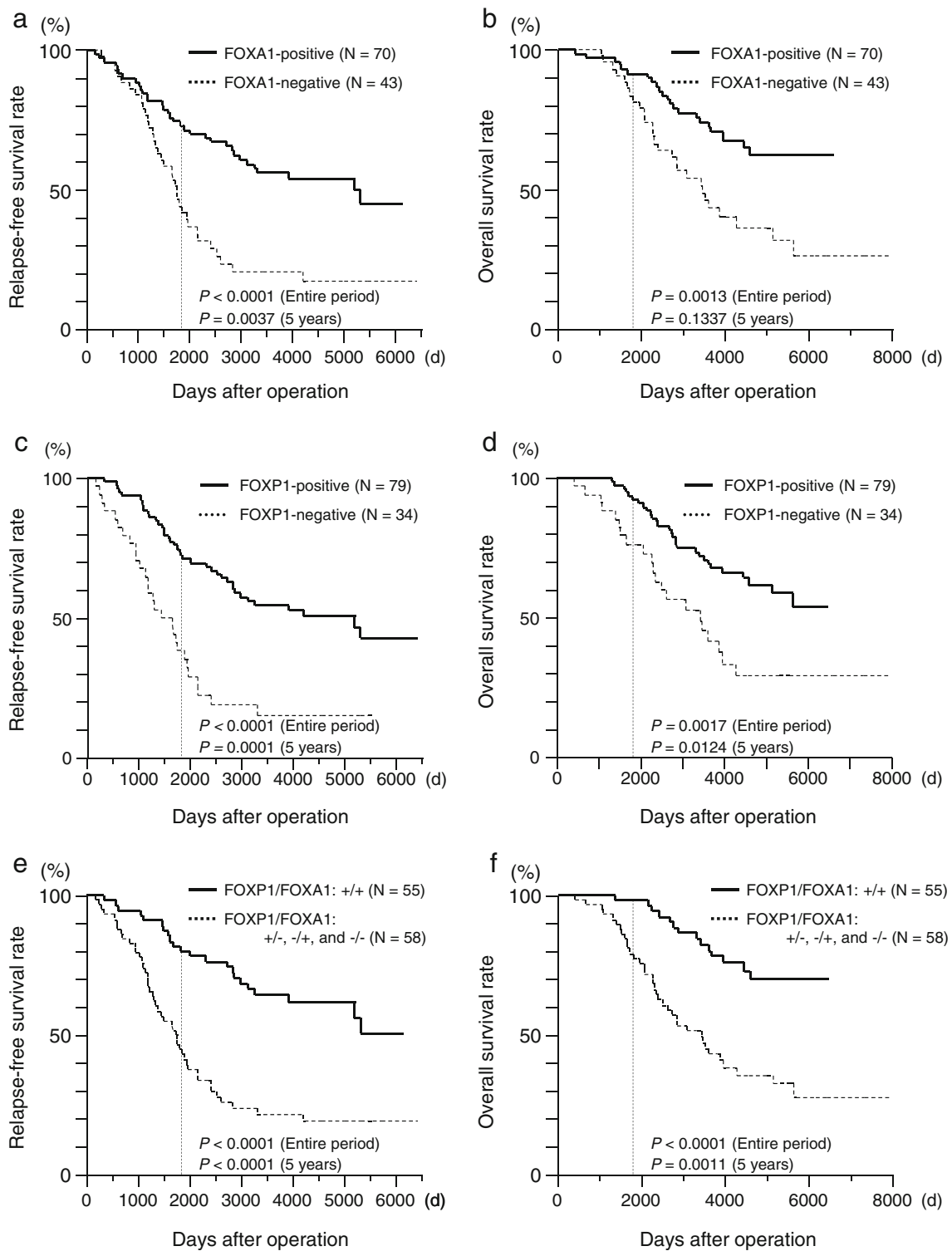


Fig. 4 Relapse-free survival and overall survival according to the FOXA1 and/or FOXP1 immunoreactivities in the patients with tamoxifen-treated breast cancer with or without distant metastasis within 5 years after surgery or entire follow-up period ($n=113$). The Kaplan–Meier survival curves were plotted using JMP 9 software, and the statistical significances were determined using the log-rank test. **a, b** Patients with the positive FOXA1 immunoreactivity showed good relapse-free survival and overall survival (**a** and **b**, respectively). **c, d**

Relapse-free survival and overall survival according to the FOXP1 immunoreactivity in the patients with tamoxifen-treated breast cancer with or without distant metastasis within 5 years after surgery or entire follow-up period. Patients with positive FOXP1 immunoreactivity showed good relapse-free survival and overall survival (**c** and **d**, respectively). **e, f** Patients with double-positive FOXA1 and FOXP1 immunoreactivities showed better relapse-free survival and overall survival (**e** and **f**, respectively)

Table 3 Univariate and multivariate analyses of relapse-free survival and overall survival in tamoxifen-treated patients with breast cancer ($n=113$)

Variable	Univariate		Multivariate	
	Odds ratio (95 % CI)	<i>P</i> value	Odds ratio (95 % CI)	<i>P</i> value
Relapse-free survival				
FOXA1 (positive vs. negative)	0.30 (0.13–0.65)	0.002 ^a	0.44 (0.18–1.07)	0.070
FOXP1 (positive vs. negative)	0.24 (0.10–0.55)	0.001 ^a	0.35 (0.14–0.88)	0.026
pT (≤ 30 vs. >30 mm)	0.36 (0.16–0.80)	0.013 ^a	0.59 (0.24–1.45)	0.25
Lymph node (0 vs. ≥ 1)	0.33 (0.14–0.72)	0.005 ^a	0.51 (0.21–1.23)	0.14
ER α (positive vs. negative)	0.91 (0.25–3.76)	0.90		
PgR (positive vs. negative)	0.86 (0.30–2.55)	0.77		
Age (≤ 50 vs. >50 years)	1.30 (0.60–2.82)	0.50		
Overall survival				
FOXA1 (positive vs. negative)	0.41 (0.13–1.27)	0.12		
FOXP1 (positive vs. negative)	0.27 (0.08–0.84)	0.024 ^a	0.34 (0.09–1.11)	0.072
pT (≤ 30 vs. >30 mm)	0.24 (0.07–0.76)	0.015 ^a	0.30 (0.08–0.97)	0.044
Lymph node (0 vs. ≥ 1)	0.41 (0.13–1.31)	0.14		
ER α (positive vs. negative)	0.53 (0.12–3.76)	0.47		
PgR (positive vs. negative)	2.51 (0.45–47.1)	0.34		
Age (≤ 50 vs. >50 years)	1.97 (0.64–6.39)	0.24		

CI confidence interval, ER α estrogen receptor- α , PgR progesterone receptor

^aData were considered significant in the univariate analyses and examined in the multivariate analyses

Mehta et al. [29] recently reported that FOXA1 is an independent prognostic marker for ER-positive breast cancer. Although significant correlations are not observed between overall survival and either FOXA1 or FOXP1 immunoreactivities, we found that double-positive staining of FOXA1 and FOXP1 is a powerful independent prognostic factor for both relapse-free and overall survival using univariate and multivariate analyses, independent of ER α status of the tumors in the tamoxifen-treated patients. These results suggest that the combined analyses of the FOXA1 and FOXP1

immunoreactivities provide powerful prognostic indicators for the tamoxifen-treated patients with ER α -positive breast cancers.

In the context of ER/FOXA1-driven signaling in breast cancer cells, it remains to be clarified whether the estrogen-mediated induction of FOXA1 contributes to the increased proliferation and whether the migration of cells or the estrogen-mediated proliferation leads to the enhancement of FOXA1 induction. In the present study, we showed that the estrogen-dependent upregulation of FOXA1 was

Table 4 Univariate and multivariate analyses including double-positive FOXA1 and FOXP1 immunoreactivities of relapse-free survival and overall survival in tamoxifen-treated patients with breast cancer ($n=113$)

Variable	Univariate		Multivariate	
	Odds ratio (95 % CI)	<i>P</i> value	Odds ratio (95 % CI)	<i>P</i> value
Relapse-free survival				
FOXA1/FOXP1 (+/+ vs. +/-, -/+, and -/-)	0.20 (0.09–0.46)	$<0.001^a$	0.26 (0.11–0.62)	0.002
pT (≤ 30 vs. >30 mm)	0.36 (0.16–0.80)	0.013 ^a	0.55 (0.23–1.34)	0.19
Lymph node (0 vs. ≥ 1)	0.33 (0.14–0.72)	0.005 ^a	0.46 (0.19–1.10)	0.081
ER α (positive vs. negative)	0.91 (0.25–3.76)	0.90		
PgR (positive vs. negative)	0.86 (0.30–2.55)	0.77		
Age (≤ 50 vs. >50 years)	1.30 (0.60–2.82)	0.50		
Overall survival				
FOXA1/FOXP1 (+/+ vs. +/-, -/+, and -/-)	0.06 (0.003–0.34)	$<0.001^a$	0.08 (0.004–0.44)	0.002
pT (≤ 30 vs. >30 mm)	0.24 (0.07–0.76)	0.015 ^a	0.36 (0.10–1.20)	0.096
Lymph node (0 vs. ≥ 1)	0.41 (0.13–1.31)	0.14		
ER α (positive vs. negative)	0.53 (0.12–3.76)	0.47		
PgR (positive vs. negative)	2.51 (0.45–47.1)	0.34		
Age (≤ 50 vs. >50 years)	1.97 (0.64–6.39)	0.23		

CI confidence interval, ER α estrogen receptor- α , PgR progesterone receptor

^aData were considered significant in the univariate analyses and examined in the multivariate analyses

repressed by 4-hydroxytamoxifen. Migration study also showed that the siRNA-mediated knockdown of FOXA1 leads to the reduction of cell migration, suggesting that ER-regulated FOXA1 plays a role in the pathophysiology of breast cancer cells. Our conventional ChIP assay data may also support our idea as the early response of ER α recruitment was observed in the ER-binding sites in the vicinity of FOXA1 gene region. In terms of FOXA1 protein expression, Laganière et al. [27] showed that the estrogen upregulates also FOXA1 protein level in MCF-7 cells 4–8 h after stimulation. Taken together, these data suggest that FOXA1 functions as an ER target in the MCF-7 cells, although it might also be possible that the estrogen-dependent proliferation at late phase will affect the expression profile of FOXA1.

Our clinical data revealed that positive FOXA1 immunoreactivity is correlated with good relapse-free and overall survival in patients with tamoxifen-treated breast cancer, which is consistent with the previous gene expression profiling study [13] as FOXA1 expression is correlated with luminal subtype A with higher levels of ER α expression and having a better prognosis than subtype B. Similar to FOXA1, another ER target cyclin D1 has been shown as a marker of good prognosis in previous studies [30,31], although this molecule is a positive cell cycle regulator and responsible for the estrogen-dependent proliferation in MCF-7 cells [32]. In contrast, Ross-Innes et al. [33] recently proposed that FOXA1 also plays a role in the differential ER-binding events in the tumors with poor outcome. Notably, it has been shown that ER α signals including ER α occupancy and estrogen-dependent cell proliferation are FOXA1-dependent in both tamoxifen-sensitive and tamoxifen-refractory MCF-7 cells by performing the siRNA-mediated knockdown study [28]. Further study will be required to answer the question whether ER/FOXA1-driven proliferation is associated with tumor recurrence in various disease stages.

Recent genome-wide study revealed that most of ER–chromatin interactions and gene expression changes depended on the presence of FOXA1 [28]. FOXA1 influenced genome-wide chromatin accessibility under different ligand conditions, including estrogen and tamoxifen in breast cancer cells [28]. Thus, it is suggested that FOXA1 is a major determinant of estrogen–ER activity and endocrine response in breast cancer cells. Taken together with our data showing that FOXA1 and FOXP1 have analogous functions on the ER-mediated transcription and clinicopathological significance in breast cancer, it can be speculated that FOXP1 may have an important role in the regulation of ER activity and tamoxifen response in conjunction with FOXA1 in breast cancer cells.

In summary, our results show that FOXA1 expression was induced by estrogen in breast cancer cells and that

FOXA1 promoted cancer cell proliferation and migration by enhancing ERE-mediated transcription. We further demonstrated that immunoreactivities for FOXA1 and FOXP1 were positively correlated with the relapse-free survival for tamoxifen resistance in breast cancer. These results suggest that pharmacological modulation of FOXA1 and FOXP1 activities may be clinically useful in the prevention and/or treatment of breast cancer and that the combined evaluation of FOXA1 and FOXP1 immunoreactivities may predict the prognosis of tamoxifen-treated patients with breast cancer.

Acknowledgments We thank Ms. Saori Miyoshi, Ms. Kayoko Kanegae, and Mr. Wataru Sato for their technical assistance. We also thank Dr. Shin-ichiro Horiguchi, Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital, for the preparation of specimens. This work was supported in part by Grants-in-Aid for Cancer Research (21-4) from the Ministry of Health, Labor and Welfare; the Program for Promotion of Fundamental Studies in Health Science of the NIBIO; grants from JSPS, grants of the Cell Innovation Program and the Support Project of Strategic Research Center in Private Universities from the MEXT; Institutional Grant from the Medical Research Center, Saitama Medical University (no. 23-1-1-09, NI).

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

References

1. Platet N, Cathiard AM, Gleizes M, Garcia M (2004) Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion. *Crit Rev Oncol Hematol* 51:55–67
2. Hall JM, McDonnell DP (2005) Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. *Mol Interv* 5:343–357
3. Carroll JS, Brown M (2006) Estrogen receptor target gene: an evolving concept. *Mol Endocrinol* 20:1707–1714
4. Habashy HO, Powe DG, Rakha EA, Ball G, Paish C, Gee J, Nicholson RI, Ellis IO (2008) Forkhead-box A1 (FOXA1) expression in breast cancer and its prognostic significance. *Eur J Cancer* 44:1541–1551
5. Badve S, Turbin D, Thorat MA, Morimiya A, Nielsen TO, Perou CM, Dunn S, Huntsman DG, Nakshatri H (2007) FOXA1 expression in breast cancer—correlation with luminal subtype A and survival. *Clin Cancer Res* 13:4415–4421
6. Thorat MA, Marchio C, Morimiya A, Savage K, Nakshatri H, Reis-Filho JS, Badve S (2008) Forkhead box A1 expression in breast cancer is associated with luminal subtype and good prognosis. *J Clin Pathol* 61:327–332
7. Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao W, Hestermann EV, Geisler TR, Fox EA, Silver PA, Brown M (2005) Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* 122:33–43
8. Lupien M, Eeckhoute J, Meyer CA, Wang Q, Zhang Y, Li W, Carroll JS, Liu XS, Brown M (2008) FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* 132:958–970
9. Grange T, Roux J, Rigaud G, Pictet R (1991) Cell-type specific activity of two glucocorticoid responsive units of rat tyrosine aminotransferase gene is associated with multiple binding sites

- for C/EBP and a novel liver-specific nuclear factor. *Nucleic Acids Res* 19:131–139
10. Holmes KA, Hurtado A, Brown GD, Launchbury R, Ross-Innes CS, Hadfield J, Odom DT, Carroll JS (2011) Transducin-like enhancer protein 1 mediates estrogen receptor binding and transcriptional activity in breast cancer cells. *Proc Natl Acad Sci U S A* 2011. doi:10.1073/pnas.1018863108
 11. Tan SK, Lin ZH, Chang CW, Varang V, Chng KR, Pan YF, Yong EL, Sung WK, Cheung E (2011) AP-2 γ regulates oestrogen receptor-mediated long-range chromatin interaction and gene transcription. *EMBO J* 30:2569–2581
 12. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lønning PE, Børresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. *Nature* 406:747–752
 13. Sørlie 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 Lønning P, Børresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98:10869–10874
 14. Banham AH, Beasley N, Campo E, Fernandez PL, Fidler C, Gatter K, Jones M, Mason DY, Prime JE, Trougouboff P, Wood K, Cordell JL (2001) The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p. *Cancer Res* 61:8820–8829
 15. Prown PJ, Ashe SL, Leich E, Burek C, Barrans S, Fenton JA, Jack AS, Pulford K, Rosenwald A, Banham AH (2008) Potentially oncogenic B-cell activation induced smaller isoforms of FOXP1 are highly expressed in the activated B cell-like subtype of DLBCL. *Blood* 111:2816–2824
 16. Fox SB, Brown P, Han C, Ashe S, Leek RD, Harris AL, Banham AH (2004) Expression of the forkhead transcription factor FOXP1 is associated with estrogen receptor α and improved survival in primary human breast carcinomas. *Clin Cancer Res* 10:3521–3527
 17. Banham AH, Boddy J, Launchbury R, Han C, Turley H, Malone PR, Harris AL, Fox SB (2007) Expression of the forkhead transcriptional factor FOXP1 is associated both with hypoxia inducible factors (HIFs) and the androgen receptor in prostate cancer but is not directly regulated by androgens or hypoxia. *Prostate* 67:1091–1098
 18. Takayama K, Horie-Inoue K, Ikeda K, Urano T, Murakami K, Hayashizaki Y, Ouchi Y, Inoue S (2008) FOXP1 is an androgen-responsive transcription factor that negatively regulates androgen receptor signaling in prostate cancer cells. *Biochem Biophys Res Commun* 374:388–393
 19. Shigekawa T, Ijichi N, Ikeda K, Horie-Inoue K, Shimizu C, Saji S, Aogi K, Tsuda H, Osaki A, Saeki T, Inoue S (2011) FOXP1, an estrogen-inducible transcription factor, modulates cell proliferation in breast cancer cells and 5-year recurrence-free survival of patients with tamoxifen-treated breast cancer. *Horm Cancer* 2:286–297
 20. Ijichi N, Shigekawa T, Ikeda K, Horie-Inoue K, Fujimura T, Tsuda H, Osaki A, Saeki T, Inoue S (2011) Estrogen-related receptor γ modulates cell proliferation and estrogen signaling in breast cancer. *J Steroid Biochem Mol Biol* 123:1–7
 21. Allred DC, Clark GM, Elledge R, Fuqua SA, Brown RW, Chamness GC, Osborne CK, McGuire WL (1993) Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. *J Natl Cancer Inst* 85:200–206
 22. Horie-Inoue K, Bono H, Okazaki Y, Inoue S (2004) Identification and functional analysis of consensus androgen response elements in human prostate cancer cells. *Biochem Biophys Res Commun* 325:1312–1317
 23. Horie-Inoue K, Takayama K, Bono HU, Ouchi Y, Okazaki Y, Inoue S (2006) Identification of novel steroid target genes through the combination of bioinformatics and functional analysis of hormone response elements. *Biochem Biophys Res Commun* 339:99–106
 24. Giamarchi C, Solanas M, Chailleux C, Augereau P, Vignon F, Rochefort H, Richard-Foy H (1999) Chromatin structure of the regulatory regions of pS2 and cathepsin D genes in hormone-dependent and -independent breast cancer cell lines. *Oncogene* 18:533–541
 25. Ikeda K, Ogawa S, Tsukui T, Horie-Inoue K, Ouchi Y, Kato S, Muramatsu M, Inoue S (2004) Protein phosphatase 5 is a negative regulator of estrogen receptor-mediated transcription. *Mol Endocrinol* 18:1131–1143
 26. Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoutte J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, Wang Q, Bekiranov S, Sementchenko V, Fox EA, Silver PA, Gingeras TR, Liu XS, Brown M (2006) Genome-wide analysis of estrogen receptor binding sites. *Nat Genet* 38:1289–1297
 27. Laganière J, Deblois G, Lefebvre C, Bataille AR, Robert F, Giguère V (2005) From the cover: location analysis of estrogen receptor α target promoters reveals that FOXA1 defines a domain of the estrogen response. *Proc Natl Acad Sci U S A* 102:11651–11656
 28. Hurtado A, Holmes KA, Ross-Innes CS, Schmidt D, Carroll JS (2011) FOXA1 is a key determinant of estrogen receptor function and endocrine response. *Nat Genet* 43:27–33
 29. Mehta RJ, Jain RK, Leung S, Choo J, Nielsen T, Huntsman D, Nakshatri H, Badve S (2012) FOXA1 is an independent prognostic marker for ER-positive breast cancer. *Breast Canc Res Treat* 131:881–890
 30. Hwang TS, Han HS, Hong YC, Lee HJ, Paik NS (2003) Prognostic value of combined analysis of cyclin D1 and estrogen receptor status in breast cancer patients. *Pathol Int* 53:74–80
 31. Boström P, Söderström M, Palokangas T, Vahlberg T, Collan Y, Carpen O, Hirsimäki P (2009) Analysis of cyclins A, B1, D1 and E in breast cancer in relation to tumour grade and other prognostic factors. *BMC Res Notes* 2:140
 32. Prall OW, Rogan EM, Musgrove EA, Watts CK, Sutherland RL (1998) c-Myc or cyclin D1 mimics estrogen effects on cyclin E-cdk2 activation and cell cycle reentry. *Mol Cell Biol* 18:4499–4508
 33. Ross-Innes CS, Stark R, Teschendorff AE, Holmes KA, Ali HR, Dunning MJ, Brown GD, Gojis O, Ellis IO, Green AR, Ali S, Chin SF, Palmieri C, Caldas C, Carroll JS (2012) Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. *Nature* 481:389–393