FE65 in breast cancer and its clinicopathological significance

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Abstract

Background Transcription coregulator adapter protein FE65 is well known to play pivotal roles in pathogenesis of Alzheimer's disease by regulating amyloid precursor protein (APP) expression and processing. APP was recently reported to be also involved in development of human malignancies. Therefore, in this study, we studied FE65 status in different subtypes of human breast cancer and correlated the results with cell proliferation and migration of carcinoma cells and clinicopathological features of breast cancer patients to explore its biological and clinical significance in breast cancer.

Methods We first immunolocalized FE65 and APP in 138 breast cancer patients and correlated the results with their tumor grade. Then, we did further exploration by proximity ligation assay, WST-8, and wound-healing assay.

Results FE65 immunoreactivity in carcinoma cells was significantly associated with lymph-node metastasis, ERα, and high pathological N factor. APP immunoreactivity was significantly positively correlated with high pathological N factor. FE65, APP, and p-APP were all significantly correlated with shorter disease-free survival of breast cancer patients. In addition, the status of FE65 was significantly associated with overall survival. Results of in vitro analysis revealed that FE65 promoted the migration and proliferation of T-47D and ZR-75–1 breast carcinoma cells. In situ proximity ligation assay revealed that FE65 could bind to APP in the cytoplasm. FE65 was also associated with APP and ERα in carcinoma cells, suggesting their cooperativity in promoting carcinoma cell proliferation and migration. APP was also significantly associated with adverse clinical outcome of the patients.

Conclusions This is the first study to explore the clinical significance of FE65 in human breast cancer. The significant positive correlation of FE65 with poor clinical outcome, direct binding to APP, and promotion of carcinoma cell proliferation and migration indicated that FE65–APP pathway could serve as the potential candidate of therapeutic intervention in breast cancer patients.

Keywords FE65 · APP · p-APP · Breast cancer · ERα

Introduction

Breast cancer is one of the leading causes of cancer death among women [1]. Metastasis is the main cause of poor prognosis and demise of the patients [2]. Therefore,

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elucidating the molecular mechanisms of carcinoma cell metastasis could provide new treatment directions. The transcription coregulator adapter protein FE65, APP-binding family B member 1 (APBB1), is well known to form a complex molecular structure with three different protein-binding domains, a WW domain and two consecutive PTB domains [3–5]. It is abundantly expressed in neurons throughout life, suggesting important functions in neuronal migration [6], synapse formation [7], and learning and memory [8]. More than 20 FE65-interacting proteins have been identified, but the most well-characterized one is amyloid precursor protein (APP) [9]. FE65 binds to APP through an interaction between its COOH-terminal PID interacting and the YENPTY motif in the cytoplasmic domain of APP, and this interaction could contribute to the pathogenesis of Alzheimer's disease [6, 10]. Membrane-bound APP can be

enzymatically processed to produce pathogenic extracellular amyloid-β (Aβ) and the APP intracellular domain (AICD) in cytoplasm [6, 10]. The phosphorylation of AICD at Thr668 is required for its unbinding to FE65 and ensuing nuclear translocation of FE65 [6, 10]. Within the nuclei, FE65 modulated gene transcription mediated by the transcription factors CP2 (also termed CP2/LSF/LBP1) and Tip60 via binding of the WW domain to the nucleosome assembly factor SET [11]. Proteolytic processing of APP was known to be increased by FE65 overexpression [12], resulting in a shift in APP subcellular localization to the cell surface. Thus, FE65 binding to the YENPTY motif could regulate the biological function of APP by adjusting its amounts on the cell surface [13]. APP was also reported to regulate the cell proliferation, migration, and invasion of many human malignancies, including acute myeloid leukemia and carcinoma of colon, thyroid, pancreas, lung, and breast [14]. For instance, APP upregulation was reported to be associated with lymph-node metastasis in breast cancer patients [15]. In addition, APP regulated the proliferation and progression of estrogen receptor (ER) α-positive breast carcinoma cells and its elevated expression was reported to predict the adverse clinical outcome among breast cancer patients [16]. For instance, non-luminal breast cancer patients with APP abundance tended to have poor prognosis [15]. Furthermore, the presence of APP molecule phosphorylated at Thr668 (phospho-APP or p-APP) was associated with adverse clinical outcome of non-small cell lung carcinoma patients [17]. However, p-APP has not been studied in breast cancer and its significance has remained largely unknown.

Considering rather close association between APP and FE65 in nerve cells above, FE65 is reasonably postulated to regulate biological behavior of breast cancer by interacting with APP. In fact, recent in vitro studies did suggest that FE65 could play a dual role in the development of breast tumors [18], stimulating the growth of ERα-positive tumors by acting as an ERα co-activator and inhibiting metastasis of ERα-negative tumors by Tip60-mediated cortactin acetylation [18]. However, these complex functions of FE65 and various associations with clinicopathological parameters have remained unknown in breast cancer. Therefore, in this study, we first immunolocalized FE65, APP, and p-APP in human breast cancer cases and compared the results with clinicopathologic factors of individual patients. We then performed in vitro studies to explore the biological significance of APP–FE65 binding and the influence of FE65 on cell proliferation and migration of breast carcinoma cells.

Materials and methods

Breast cancer specimens

138 breast cancer specimens were retrieved from Japanese female patients (age 30–83 years) who underwent surgical resections from 1998 to 2013 at Tohoku University Hospital (Sendia, Japan). All the specimens had been fixed in 10% neutral formalin and embedded in paraffin. We obtained the consent from each patient after full explanation of the purpose and nature of all procedures used. The ethics committee of Tohoku University School of Medicine approved this research protocol (2020–1-549).

Immunohistochemistry (IHC)

10% formalin fixed and paraffin-embedded (FFPE) blocks were cut into 3-μm slices and immunostained using the labeled streptavidin–biotin (LSAB) method (Histofine kit, Nichirei Biosciences, Tokyo, Japan). Slides were soaked in phosphate-buffer saline (PBS) heated to 121 °C for 5 min in an autoclave for antigen retrieval, blocked in 10% normal rabbit or goat serum for 30 min, and treated with the indicated primary antibodies at 4 °C overnight. Characteristics of the primary antibodies are summarized in Table 1. The following day, sections were incubated with secondary antibody for 30 min and the antigen–antibody complexes visualized by 3,3-diaminobenzidine staining at room temperature. The reacted slides were then counterstained with hematoxylin. Human cerebral tissue from an Alzheimer's disease patient obtained at autopsy was used as a positive control for APP, p-APP, and FE65 immunohistochemistry [17, 19]. Absorption tests in which the antibodies were treated with corresponding antigens were performed as negative controls (and all yielded negative immunoreactivity). APP and p-APP

Table 1 Antibodies in this study

Antigen	Clone	Species	Description	Reference	Application
App	Polyclonal	Rabbit	Polyclonal	Cell Signaling Technologies (Danvers, MA, USA)	IHC, PLA
p-app	Polyclonal	Rabbit	Polyclonal	Cell Signaling Technologies (Danvers, MA, USA)	IHC, PLA
FE65	Polyclonal	Goat	Polyclonal	Abcam (Cambridge, United Kingdom)	IHC, PLA, ICC
Ki-67	MIB1	Mouse	Monoclonal	Dako	IHC
ERα	6F11	Mouse	Monoclonal	Leica	IHC
HER-2	Polyclonal	Rabbit	Polyclonal	Dako	IHC

immunoreactivity in the cytoplasm of carcinoma cells were evaluated using a semi-quantitative score (0—completely negative, 1—weakly positive, 2—moderately positive, and 3—markedly positive) according to the previously published reports [16]. FE65 was immunolocalized in the nuclei of carcinoma cells and results were similarly evaluated semi-quantitatively (0—completely negative, 1—weakly positive, 2—moderately positive, and 3—markedly positive). Expression levels were then stratified as negative or positive according to scores of 0 or 1 and 2 or 3, respectively. Ki67 immunoreactivity in the nuclei of breast carcinoma cells was determined as a labeling index (LI) by counting more than 500 carcinoma cells. The LI more than 10% was considered positive according to previous reports [16].

Cell lines and culture

The human breast carcinoma cell lines ZR-75–1 and T-47D were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All these cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (FBS; Biosera, France) and 100 mg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA).

In situ proximity ligation assay (in situ PLA)

The ZR75-1 and T-47D cell lines were seed in 8-well plates (Millicell® EZ slide, Merk, USA) at a density of 5×10^4 cells/ml, cultured for 24 h, and then fixed in a 4% paraformaldehyde–PBS solution containing 0.1% Triton-X-100. ER-positive breast cancer tissue had also been fixed in 10% neutral formalin and embedded in paraffin. The assay was performed using the Duolink in situ PLA kit (Sigma, St. Louis, MO, USA). Cells and tissue were incubated overnight at 4 °C in blocking solution with primary antibodies (Table 1), at 37 °C for 60 min with goat and rabbit probes in Ligation-Ligase solution, and finally in amplification-polymerase solution for 100 min at 37 °C. The reacted slides were then counterstained with DAPI and subsequently imaged under a fluorescence microscope to detect cross-linking of the target proteins.

Interfering RNA transfection

The ZR-75–1 and T-47D cell lines were transfected with a small interfering RNA (siRNA) targeting FE65 (siFE65) or a negative control (siCTRL) to examine the effects of FE65 expression level on cell proliferation rate and metastatic capacity. Briefly, ZR75-1 and T-47D cells were seeded in 6-well plates at 5×10^4 cells/ml, and 5 nM FE65 siRNA1 (Merck), 5 nM FE65 siRNA2, or 5 nM siCTRL transfected

using Lipofectamine® RNAiMAX Reagent (Life Technologies, Carlsbad, CA, USA).

Real-time PCR

Total RNA was extracted from ZR-75–1 and T-47D cells using TRIzol reagent and reverse transcribed to cDNA using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). Real-time PCR was then conducted using the LightCycler System, FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany), and the following PCR primer sequences for FE65 (target) and ribosomal protein L13A (RPL13A, internal control) FE65 forward 5'-TCCCCAGAGGACACAGATTC-3' and reverse 5'-GTG AGCTGGGACTCCTCTTG-3', RPL13A forward 5'-CCT GGAGGAGAAGAGGAAAG-3' and reverse 5'-TTGAGG ACCTCTGTGTATTT-3'. FE65 mRNA expression level was normalized to RPL13A expression.

Immunocytochemistry (ICC)

Cells treated as indicated were harvested, centrifuged in PBS at 1500 rpm for 3 min, fixed for 30 min in 10% neutral-buffered formalin, centrifuged at 1500 rpm for 3 min, washed 3 times with PBS, and embedded in gel using the iPGell kit (Genostaff, Tokyo, Japan) according to the manufacturer's protocol. The gel-embedded cell suspension was transferred into tissue cassettes and immersed in paraffin containing ethanol and xylene. Finally, the paraffinized blocks were cut into 3 μm slices for immunocytochemistry as described above. Characteristics of the primary antibodies employed in this study are summarized in Table 1.

Cell proliferation assay

Following transfection, siRNA knockdown and control ZR-75–1 and T-47D cells were seeded in 96-well plates at 5000 cells/well. After 24, 48, and 72 h, cell numbers were measured by the WST-8 [2-(2-methoxy- 4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] method using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

Wound-healing assay

Transfected ZR-75–1 and T-47D cells were seeded into 6-well plates at 5×10^4 cells/ml. After 48 h, the cells were reseeded on Culture-Insert plates (Ibidi, GmbH, Munich, Germany) at 3×10^5 cells/ml. After 48 h, the Culture-Insert was removed, three pairs of horizontal points were selected in cell-free gaps, and the distance between the points was measured after 6, 24, 36, and 48 h to calculate migration distance and speed, respectively.

Statistical analysis

All statistical analyses were conducted using JMP 14.0.0 software (SAS Institute Japan, Tokyo, Japan). Clinicopathological factors were compared by Mann–Whitney's test, Fisher exact test, or log-rank test as indicated. $p < 0.05$ (two-tailed) was considered statistically significant for all tests.

Results

FE65, APP, and p-APP in breast cancer and correlation with clinicopathological factors of breast cancer patients

FE65 immunoreactivity was mainly detected in the nuclei of carcinoma cells, while both APP and p-APP mainly in the cytoplasm (Fig. 1). Table 2 summarizes the associations among FE65, APP, and p-APP status (positive or negative) and individual clinicopathological factors of breast cancer patients examined in this study. Positive FE65 immunoreactivity was significantly associated with high lymph-node metastasis ($p = 0.003$), pathological N factor ($p = 0.039$), and high ERα status of carcinoma cells ($p = 0.007$). APP was positively significantly associated with high pathological N factor ($p = 0.031$) and high human epidermal growth factor receptor 2 (HER-2) status ($p = 0.018$). In addition, FE65,

APP, and p-APP were significantly associated with shorter disease-free survival of the patients ($p = 0.031$, $p = 0.003$ and $p = 0.014$, respectively). In addition, overall survival of the patients was significantly associated with FE65 expression ($p = 0.037$). APP immunoreactivity was positively associated with p-APP ($p = 0.002$), while no associations between FE65 and either APP or p-APP detected. Table 3 summarizes the correlation of results with clinicopathological factors of all ER-positive patients (Number = 91). FE65 was positively correlated with the status of lymph-node metastasis ($p = 0.005$) (Fig. 2).

FE65, APP, and p-APP interactions in breast cancer cells and tissue

In situ PLA analysis demonstrated FE65–APP binding in the cytoplasm (red dots, Fig. 3), while no FE65–p-APP or APP–p-APP conjugation was detected in the cytoplasm.

Effects of FE65 on the cell proliferation rates of breast carcinoma cell lines

In vitro experiments were then conducted to examine the association between FE65 expression and proliferation of T-47D and ZR-75–1 cell lines by knockdown of FE65. These cells were selected due to their high ERα and low HER-2 expression (Fig. 4). Both siRNA 1 and siRNA 2 effectively
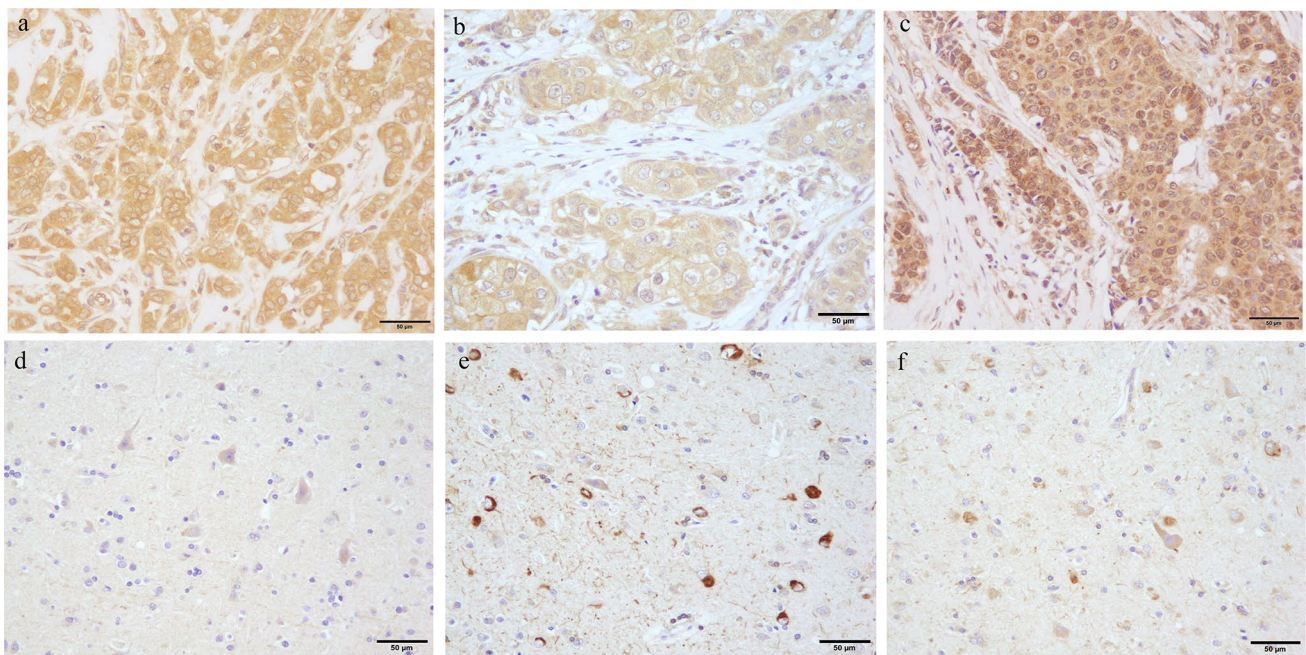
Fig. 1 Subcellular localization of APP, p-APP, and FE65 in human breast cancer specimens as revealed by immunohistochemistry. **a–c** Immunolocalization of APP (**a**), p-APP (**b**), and FE65 (**c**) in human breast carcinoma cells. APP and p-APP immunoreactivity were detected in the cytoplasm of breast carcinoma cells, while FE65 was mainly detected in the nuclei. **d–f** Positive controls for APP (**d**), p-APP (**e**), and FE65 (**f**) immunostaining in human cerebral tissue from a patient with Alzheimer's disease. Scale bar = 50 μm

Table 2 APP, p-APP, and FE65 expression with clinicopathological factors

Characteristic	APP expression			p-app expression			FE65 expression		
	Negative	Positive	p value	Negative	Positive	p value	Negative	Positive	p value
Age (years)	55.69±12.52	56.38±12.64	0.778	56.08±13.16	56.14±11.26	0.764	56.17±13.2	55.55±11.83	0.551
Lymph-node metastasis									
Negative	20	38	0.067	40	28	0.896	29	58	0.003
Positive	15	12		19	8		5	43	
Pathological stage									
I	16	31	0.257	18	29	0.270	21	56	0.442
II	14	12		15	11		11	29	
III	4	6		5	5		2	14	
Histological grade									
1 (Well)	3	6	0.778	1	8	0.113	4	24	0.076
2 (Moderate)	15	26		19	22		14	52	
3 (Poor)	16	21		18	19		15	26	
Pathological *N* factor (pN)									
pN0	19	39	0.031	24	34	0.366	28	64	0.039
pN1-3	16	12		14	13		6	37	
Pathological T factor (pT)									
pT1	24	36	0.449	23	37	0.257	25	71	0.745
pT2-3	10	17		14	13		9	32	
pT4	1	0		1	0		0	1	
PR expression									
Negative	25	44	0.195	27	42	0.143	22	55	0.228
Positive	10	9		11	8		12	49	
HER-2 expression									
0	22	17	0.018	19	20	0.719	20	56	0.838
1	1	6		3	4		3	15	
2	2	11		4	9		4	10	
3	10	19		12	17		7	22	
ER expression									
Negative	17	30	0.460	19	28	0.576	18	29	0.007
Positive	18	23		19	22		16	75	
Ki-67 LI (%)									
<10%	9	13	0.899	7	15	0.214	11	48	0.112
≧10%	26	40		31	35		23	52	
FE65 expression									
Negative	10	17	0.727	13	13	0.403			
Positive	25	36		25	37				
p-app expression									
Negative	22	16	0.002						
Positive	13	37							

LI labeling index, *PR* progesterone receptor, *ER* estrogen receptor, *HER-2* human epidermal growth factor receptor 2

reduced the transcription of FE65 in T-47D and ZR-75–1 cells (Fig. 5) and significantly inhibited proliferation rates as measured by CCK-8 assays at 24, 48, and 72 h post-seeding (Fig. 6). These results did suggest that elevated FE65 expression enhanced tumor growth of these two cell lines above.

Effects of FE65 on the migration of breast carcinoma cell lines

Wound-healing assays revealed that FE65 knockdown by siRNA 1 and 2 significantly reduced the migration rate of

Table 3 FE65 expression with clinicopathological factors in ER+ breast cancer patients

Characteristic	FE65 expression		
	Negative	Positive	*p* value
Age (years)	56.18±10.78	54.74±11.15	0.642
Lymph-node metastasis			
Negative	15	43	0.005
Positive	1	32	
Pathological stage			
I	11	43	0.371
II	5	23	
III	0	8	
Histological grade			
1 (well)	4	22	0.528
2 (moderate)	8	41	
3 (poor)	4	10	
Pathological N factor (pN)			
pN0	14	48	0.076
pN1-3	2	26	
Pathological T factor (pT)			
pT1	12	55	0.896
pT2-3	4	19	
pT4	0	1	
PR expression			
Negative	4	26	0.455
Positive	12	49	
Ki-67 LI (%)			
<10%	10	40	0.652
≧10%	6	31	

LI labeling index, *PR* progesterone receptor

T-47D and ZR-75–1 cells compared to cells transfected with control siRNA at 24, 48, and 72 h (Fig. 7), suggesting that elevated FE65 expression enhanced metastasis.

Discussion

Various functions of FE65 have been extensively studied in Alzheimer's disease but not in human malignancies, especially breast cancer despite well-known roles of its binding partner APP. In addition, microarray studies did reveal FE65 abundance in breast cancer tissues, but its clinicopathological significance has remained virtually unknown [20]. Therefore, this is the first study to explore the possible association of FE65 with clinicopathological parameters of human breast cancer patients. To this end, we immunolocalized FE65, APP, and p-APP and correlated the findings with clinicopathological factors of the patients including their clinical outcome. We then used proximity ligation assays to further assess molecular interactions among these three proteins above and subsequently performed in vitro analysis including cell proliferation assays to study their potential effects on tumor growth and cell migration assays to explore their influence on tumor invasion and metastasis. Results of our present study did first demonstrate an involvement of FE65 in biological behavior of breast cancer. FE65 was tightly bound to APP in cytoplasm and FE65 knockdown suppressed both proliferation and migration of breast carcinoma cell lines. Of particular interest, FE65 was significantly correlated with ERα expression.

APP was significantly positively connected with high pathological N factor. APP expression was reported to be relative to lymph-node metastasis in ERα-positive breast cancer patients [16], and results of our present study did confirm that APP expression significantly had much to do with lymph-node metastasis and clinical outcome even in ERα-negative breast cancer patients. In addition, the levels of FE65, APP, and p-APP were associated with shorter disease-free survival, and FE65 expression was significantly associated with overall survival of the patients, suggesting that FE65, APP and p-APP could represent valuable prognostic markers for breast cancer patients regardless of the presence or absence of ERα in carcinoma cells.

We also first demonstrated the potential roles of phosphorylated APP in breast cancer patients. We previously reported that APP and p-APP exerted different effects in non-small cell lung cancer patients, but its mechanisms have remained virtually unknown [17] Therefore, in this study, we particularly focused on FE65, which was reported to modify APP and p-APP functions through protein–protein interactions. Proximity ligation assay demonstrated binding of APP to FE65 in the cytoplasm of ZR-75–1, T47D cells, and paraffin sections, while no binding of p-APP to FE65 was detected. These results did indicate that the biological effects of FE65 could be related to the function of APP as in human neuronal cells [4] and that APP–FE65 binding could be disrupted by APP phosphorylation. Results of our present study thus indicated that APP could serve as an important mediator of FE65 effects on carcinoma cell metastasis and proliferation in breast cancer. APP was reported to promote the migration of prostate carcinoma cells and the hematogenous metastasis of melanoma and lung carcinoma cells [21]. In addition, overexpression of APP in acute myeloid leukemia cells was reported to induce extramedullary infiltration [22] and that APP was proteolytically cleaved in breast carcinoma cells, resulting in the release of soluble APPα (sAPPα), which in turn could promote breast carcinoma cell migration and proliferation [23]. Moreover, APP significantly increased the proliferation of breast carcinoma cells and APP status was significantly appeared simultaneously with high Ki-67 labeling index in ER-positive
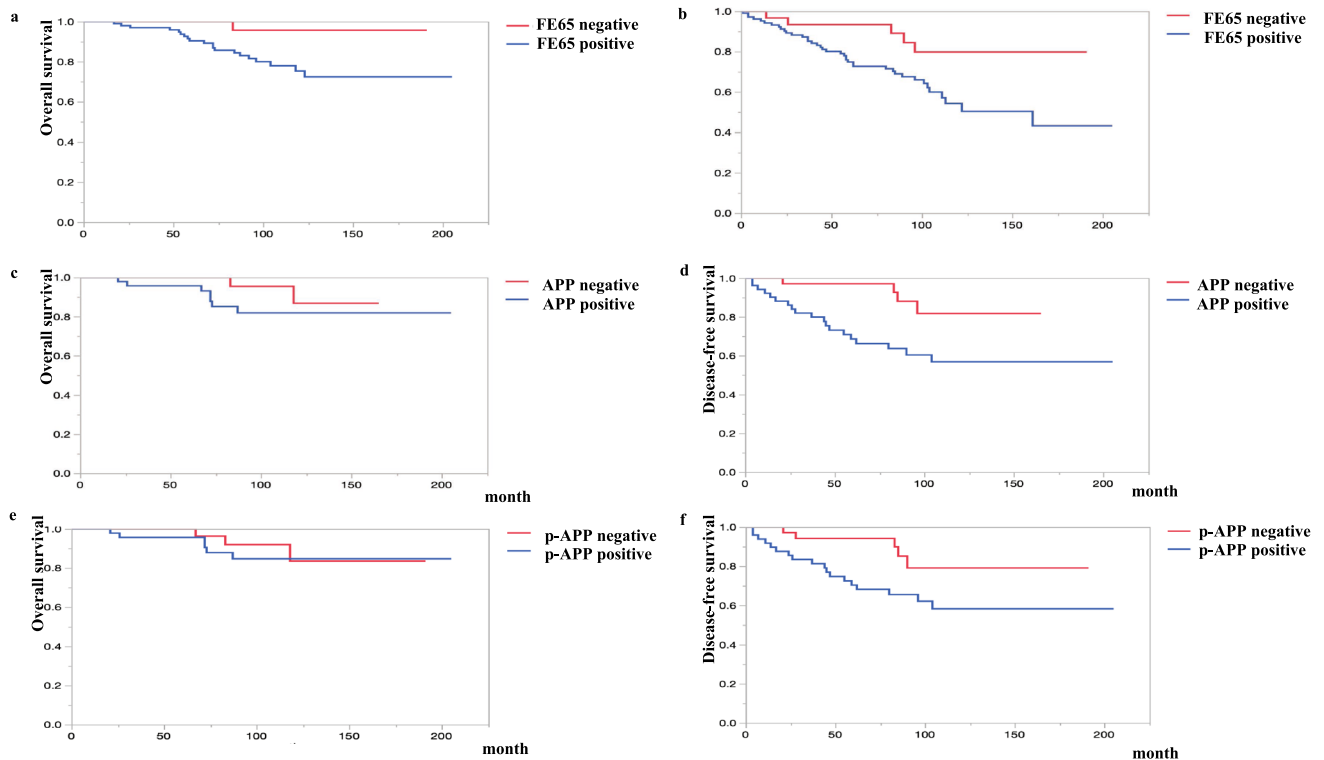
Fig. 2 Associations of FE65, APP, and p-APP expression levels (positive vs. negative) with overall survival and disease-free survival. There were no associations APP ($p=0.113$) and p-APP ($p=0.435$) levels with overall survival of the patients. However, there were sig-nificant association of APP ($p=0.003$), p-APP ($p=0.014$), and FE65 ($p=0.031$) with disease-free survival. Moreover, FE65 status was sig-nificantly associated with overall survival of the patients ($p=0.037$)
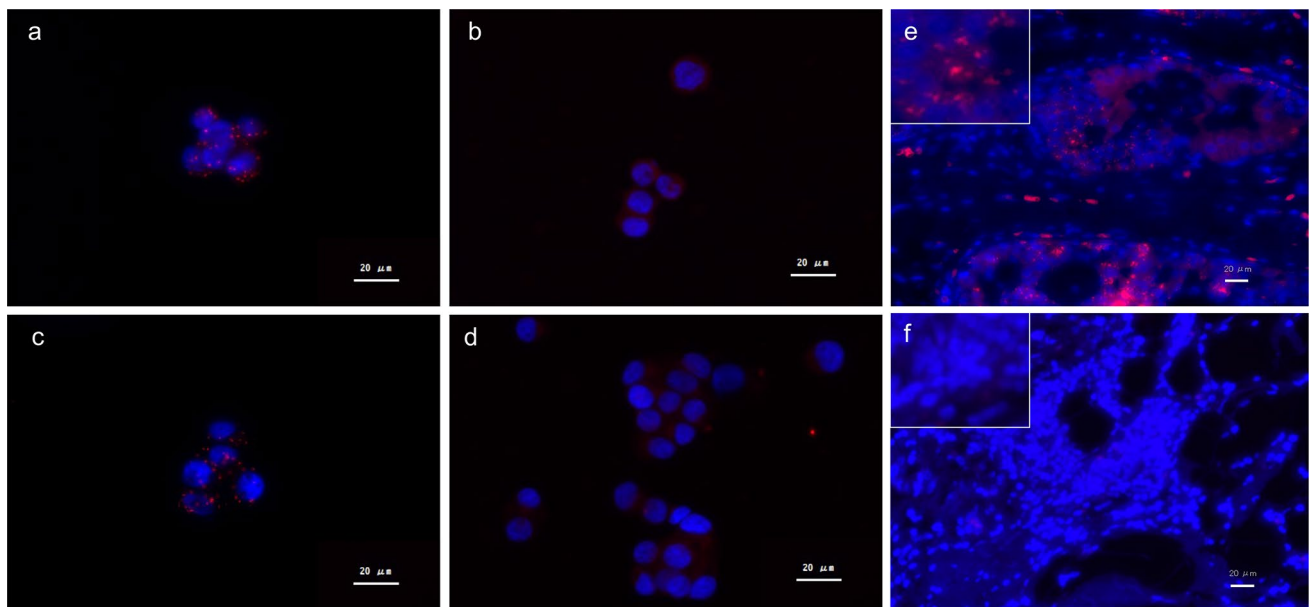
Fig. 3 Direct molecular interactions between FE65 and APP. **a–f** Molecular interactions of FE65 with APP (**a**) and p-APP (**b**) in T-47D cells and molecular interactions of FE65 with APP (**c**) and p-APP (**d**) in ZR-75–1 cells. Molecular interactions of FE65 with APP (**e**) and p-APP (**f**) in FFPE tissues were assessed by in situ prox-imity ligation assays. Interactions were demonstrated by the presence of Texas red emission (red dots). Nuclei were labeled blue by DAPI staining. Scale bar = 20 μm
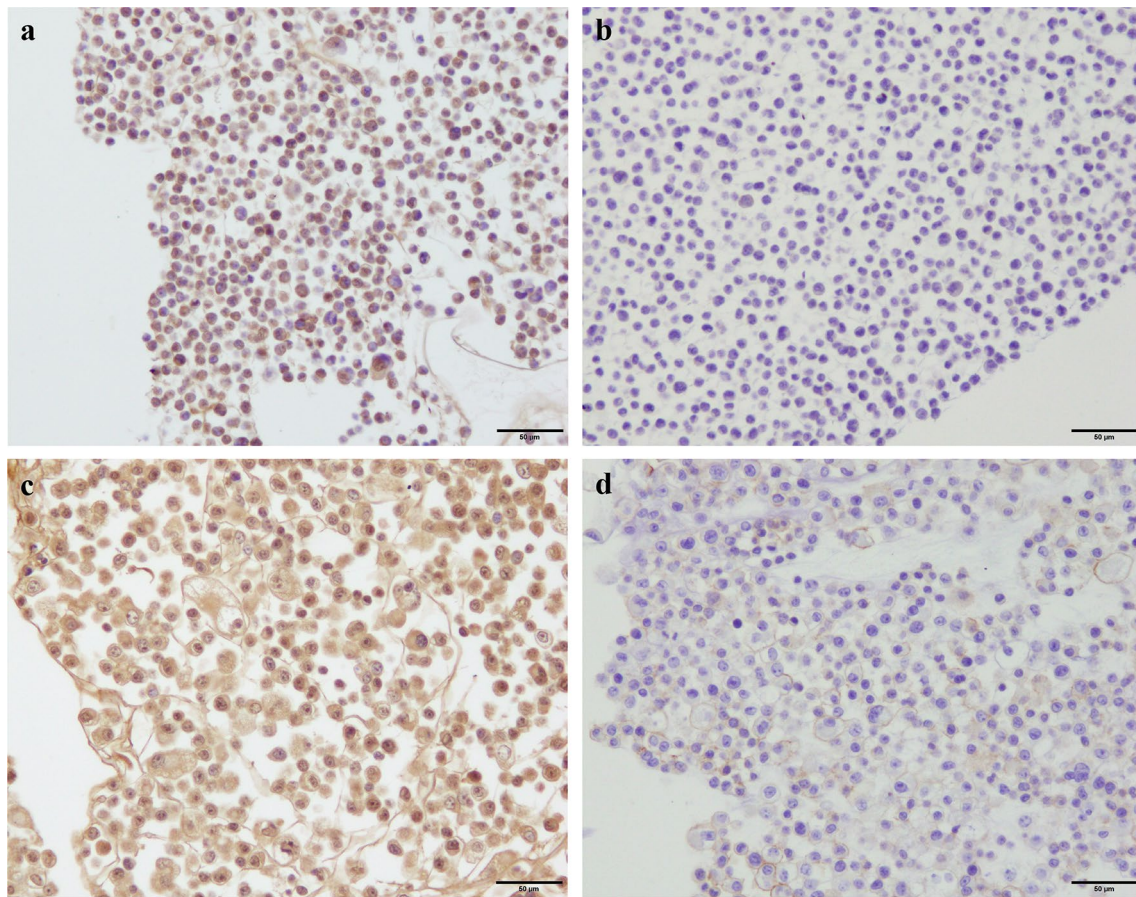
Fig. 4 ER and HER2 expression by T-47D and ZR-75–1 cells. **a–d** ER (**a**) and HER2 (**b**) expression by T-47D, ER (**c**) and HER2 (**d**) expression by ZR-75–1 cells. Scale bar = 50 μm

breast cancer in our present study. APP was reported to be predictive of disease-free survival among breast cancer patients [16], which is consistent with results of our present study. Consequently, increased APP expression was considered to upregulate FE65 expression and FE65 entry into the nuclei to influence the expression of oncogenic genes. This signaling pathway could be associated with ER expression, because results of our present study demonstrated an association between FE65 and ERα in breast cancer patients. Accordingly, effects of FE65 on breast carcinoma cells could be related to estrogenic signaling in

carcinoma cells. The estrogen gene promoter in breast carcinoma cells was well known to bind to FE65, and FE65 enhanced the recruitment of ERα and its co-activator to the promoter. Furthermore, FE65 can increase the agonistic activity of 17β-estradiol [20]. In addition, estrogens were reported to promote the non-amyloid processing of APP through the MAPK/ERK pathway [24]. APP activated MAPK signaling pathway-related proteins to regulate the expression of epithelial–mesenchymal transition (EMT)-related genes in breast carcinoma cells [25, 26]. An activation of these factors thus could promote the EMT
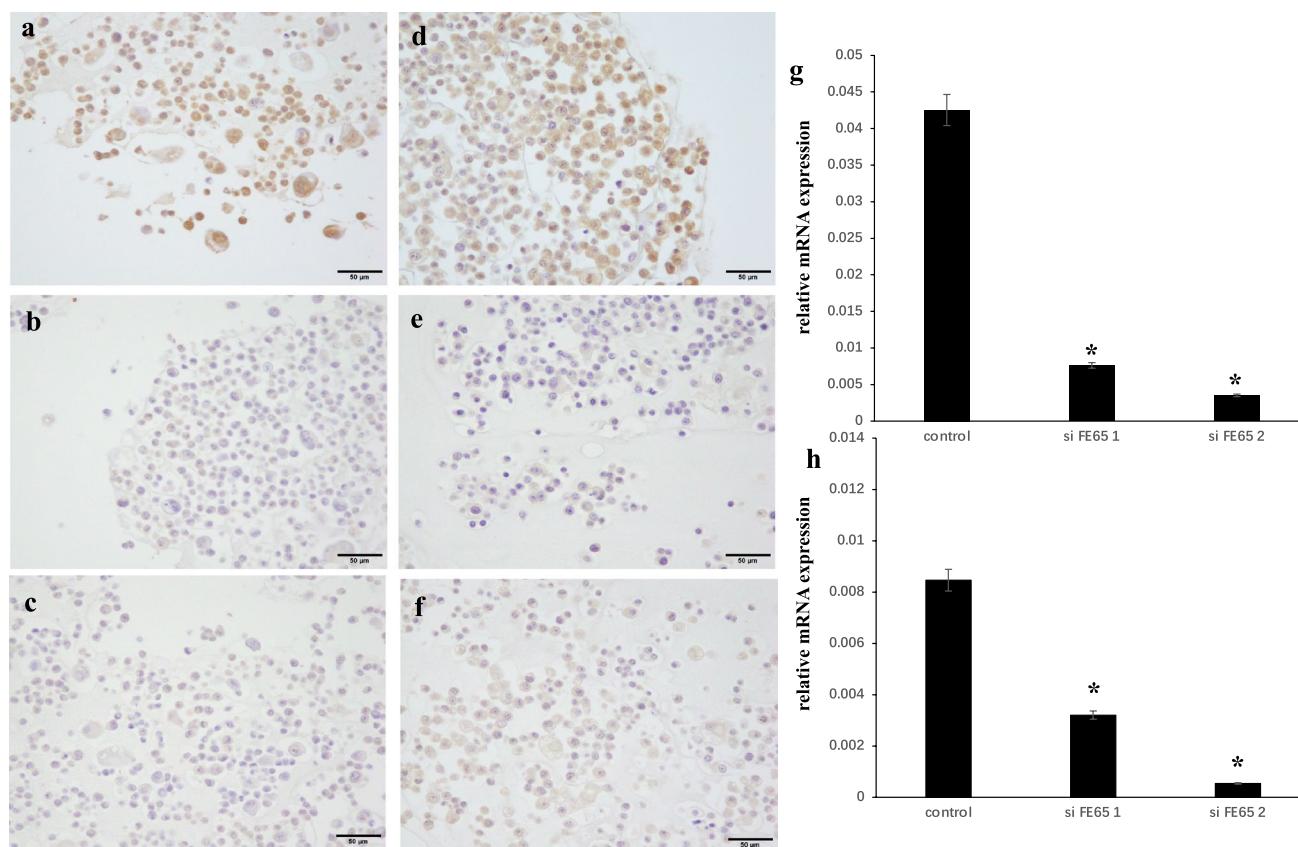
Fig. 5 Knockdown efficiency of FE65-targeted siRNAs in T-47D and ZR-75–1 breast cancer cells. **a–h** Both siRNA 1 and siRNA 2 effectively reduced the expression of FE65 at mRNA and protein levels as revealed by real-time PCR (**g** T-47D, **h** ZR-75–1) and immunocytochemistry, respectively. **a–c** FE65 mRNA and protein expression levels in T-47D cells transfected with **a** negative control siRNA (siCTRL), **b** siFE65-1, or **c** siFE65-2 (siRNA 1 $p = 0.049$, siRNA 2: $p = 0.049$ vs. negative control). **d–f** Expression levels of FE65 in ZR75-1 cells transfected with **d** negative control siRNA (siCTRL), **e** siFE65-1, or **f** siFE65-2 (siRNA 1: $p = 0.049$, siRNA 2, $p = 0.049$ vs. negative control). *$p < 0.05$

of breast cancer cells, thereby increasing migration and invasion [25, 26].

In summary, this is the first study to explore the clinical significance of FE65 in human breast cancer. Results of our present study demonstrated a significant positive correlation of FE65 with tumor grade, direct binding to APP, and promotion of carcinoma cell proliferation and migration. Following hypothesis was proposed from results of our present study as well as previously reported investigations. Phosphorylation of cytoplasmic APP released FE65, which was then translocated to the nucleus resulting in the induction of various oncogenic genes, including ERα. In turn, elevated ERα could promote cell migration and proliferation, resulting in tumor growth and lymph-node metastasis. Therefore, elevated APP or p-APP in carcinoma cells could predict adverse clinical outcome of breast cancer patients. FE65 was considered equally pivotal as a valuable prognostic biomarker of breast cancer patients.
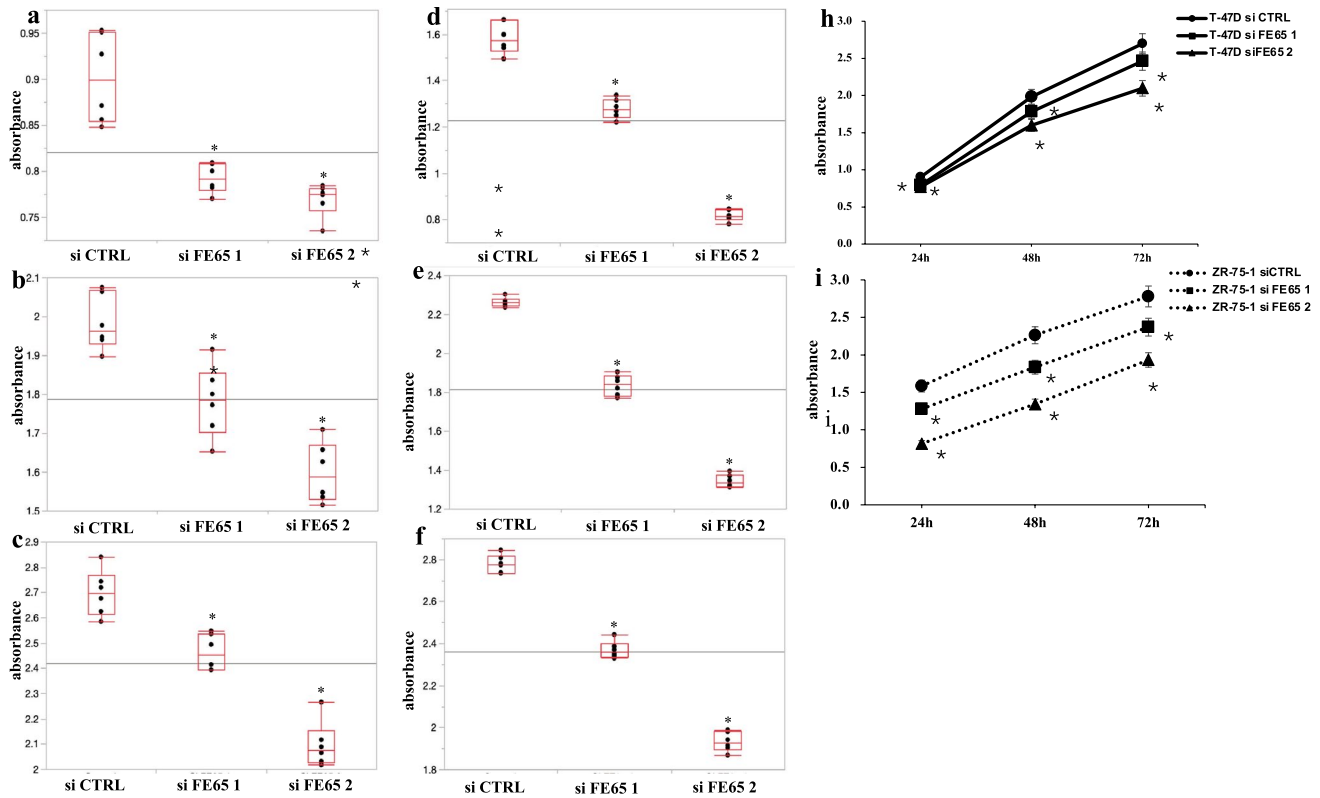
Fig. 6 Knockdown of FE65 reduced the proliferation of T-47D and ZR-75–1 cells. **a–c** Proliferation of T-47D cells transfected with siRNA (siFE65-1 and siFE65-2) or negative control siRNA at **a** 24 h, **b** 48 h, and **c** 72 h after seeding. *$p < 0.05$ vs. negative control. **d–f** Proliferation of ZR-75–1 cells transfected with siRNA (siFE65-1 and siFE65-2) or negative control siRNA at **d** 24 h, **e** 48 h, and **f** 72 h after seeding. *$p < 0.05$ vs. negative control. **h**, **i** Proliferation of T-47D cells (**h**) and ZR-75–1 cells (**i**) was significantly inhibited by

siRNA 1 and RNA 2 at 24 h (siRNA 1 in T-47D $p = 0.003$, siRNA 2 in T-47D $p = 0.003$, siRNA 1 in ZR-75–1 $p = 0.003$, siRNA 2 in ZR-75–1 $p = 0.003$), 48 h (siRNA 1 in T-47D $p = 0.003$, siRNA 2 in T-47D $p = 0.0003$, siRNA 1 in ZR-75–1 $p = 0.003$, siRNA 2 in ZR-75–1 $p = 0.003$), and 72 h (siRNA 1 in T-47D: $p = 0.003$, siRNA 2 in T-47D $p = 0.0003$, siRNA 1 in ZR-75–1 $p = 0.003$, siRNA 2 in ZR-75–1 $p = 0.003$)
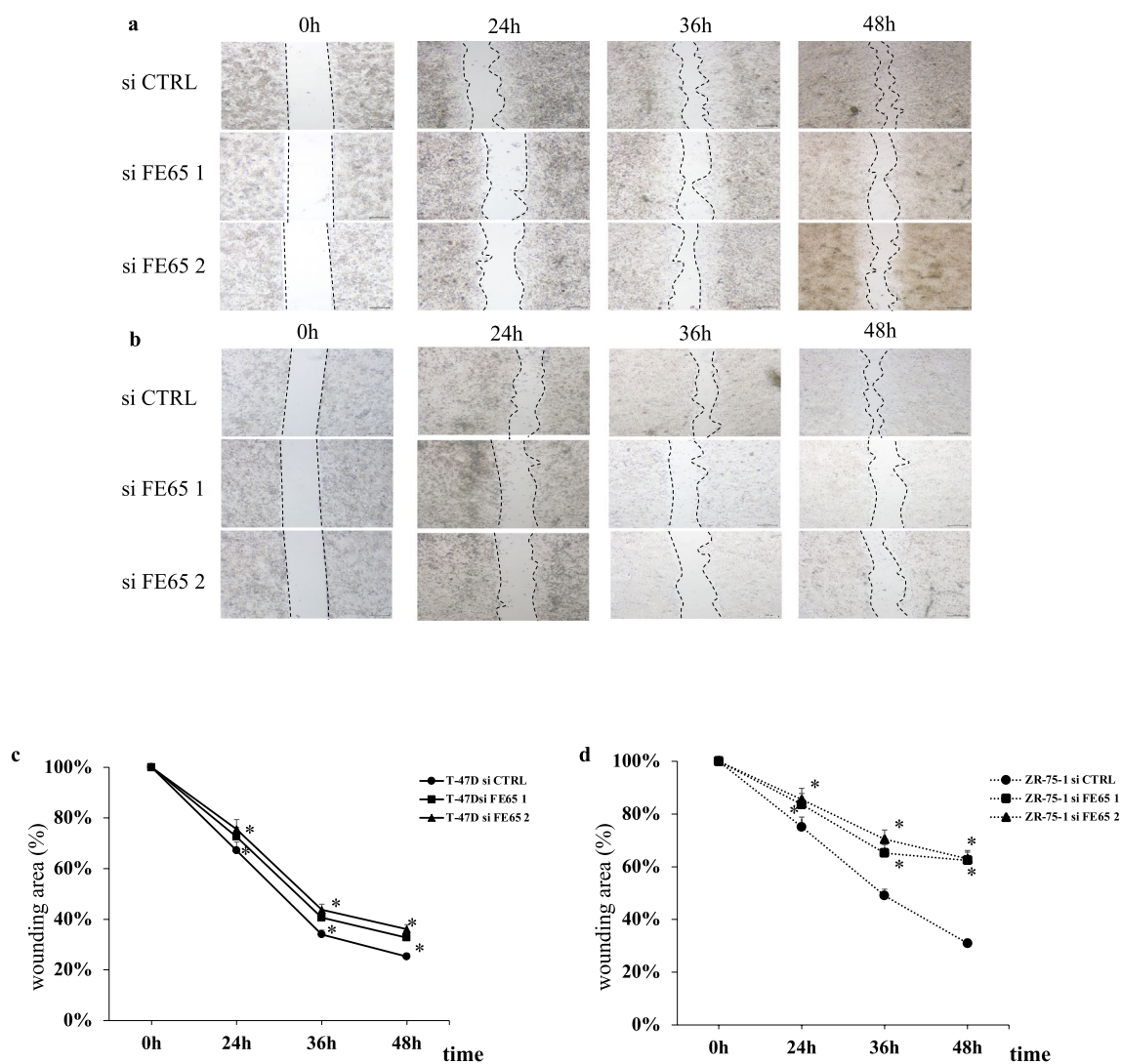
Fig. 7 Migration of T-47D and ZR-75–1. **a–d a** T-47D cells at 24, 36, and 48 h after defining cell gaps. Scale bar = 50 μm. **b** ZR-75–1 cells at 24, 36, and 48 h after defining cell gaps. Scale bar = 50 μm. **c**, **d** T-47D cells **c** and ZR-75–1 cells **d** cells transfected with siRNA (siFE65-1 and siFE65-2) or negative control siRNA (siCTRL) at 24, 36, and 48 h after defining cell gaps. *$p < 0.05$ vs. control. 24 h siRNA 1 in T-47D ($p = 0.049$), siRNA 2 in T-47D ($p = 0.049$), siRNA 1 in ZR-75–1 ($p = 0.049$), siRNA 2 in ZR-75–1 ($p = 0.049$). 36 h siRNA 1 in T-47D ($p = 0.049$), siRNA 2 in T-47D ($p = 0.049$), siRNA 1 in ZR-75–1 ($p = 0.049$), siRNA 2 in ZR-75–1 ($p = 0.049$). 48 h siRNA 1 in T-47D ($p = 0.049$), siRNA 2 in T-47D $p = 0.0495$, siRNA 1 in ZR-75–1 ($p = 0.049$, siRNA 2 in ZR-75–1 ($p = 0.049$)

Author contribution JX performed and oversaw the study, analyzed data, and wrote the manuscript. YM designed this study. JX and EI contributed to staining and evaluation of IHC sections. TI, EI, and AK contributed to collection of clinical data from Tohoku University Hospital. EI, YM, and HS supervised the study.

Declarations

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