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Original Articles

A mutual activation loop between the Ca^{2+}-activated chloride channel TMEM16A and EGFR/STAT3 signaling promotes breast cancer tumorigenesis

Hui Wang[a,1], Fan Yao[b,1], Shuya Luo[a], Ke Ma[a], Mei Liu[a], Lichuan Bai[a], Si Chen[c], Chang Song[a], Tianyu Wang[a], Qiang Du[c], Huizhe Wu[d], Minjie Wei[d], Yue Fang[c], Qinghuan Xiao[a,*]

[a] Department of Ion Channel Pharmacology, School of Pharmacy, China Medical University, Shenyang, 110122, China
[b] Department of Breast Surgery and Surgical Oncology, Research Unit of General Surgery, The First Affiliated Hospital of China Medical University, Shenyang, 110001, China
[c] Department of Microbial and Biochemical Pharmacy, School of Pharmacy, China Medical University, Shenyang, 110122, China
[d] Department of Pharmacology, School of Pharmacy, China Medical University, Shenyang, 110122, China

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A B S T R A C T

The Ca^{2+}-activated chloride channel TMEM16A (anoctamin 1) is overexpressed in breast cancer. It remains unclear how TMEM16A overexpression plays a role in carcinogenesis in breast cancer. In this study, we found that high TMEM16A expression in combination with high EGFR or STAT3 expression was significantly associated with shorter overall survival in ER-positive breast cancer patients without tamoxifen treatment, and longer overall survival in patients with tamoxifen treatment. EGFR/STAT3 signaling activation by EGF promoted TMEM16A expression, and TMEM16A overexpression activated EGFR/STAT3 signaling in breast cancer cells. Both *in vitro* and in animal studies showed that TMEM16A overexpression promoted, and TMEM16A knockdown inhibited breast cancer cell proliferation and tumor growth. In addition, TMEM16A overexpression-induced cell proliferation was blocked by EGFR/STAT3 inhibitors, and TMEM16A knockdown reduced EGF-induced proliferation and tumorigenesis in breast cancer. Furthermore, inhibition of TMEM16A channel function effectively reduced breast cancer cell proliferation, especially in combination with EGFR inhibitors. Our findings identify a mutual activation loop between TMEM16A and EGFR/STAT3 signaling, which is important for breast cancer proliferation and growth. TMEM16A inhibition may represent a novel therapy for EGFR-expressing breast cancer.

1. Introduction

Breast cancer remains one of the leading causes of cancer death in women, although the mortality rate has declined over the past decades with the use of effective adjuvant medical treatments. Breast cancer is composed of distinct subtypes, which differ greatly with respect to cellular origin, genetic alteration, and clinical outcomes [1]. The heterogeneity posts a great challenge for the clinical management of breast cancer. Identifying new molecular targets is important for the individualization of breast cancer therapy.

TMEM16A (also known as anoctamin 1) is a newly identified Ca^{2+}-activated chloride channel that is overexpressed in many tumors, including breast cancer [2–4], head and neck squamous cell carcinoma (HNSCC) [5–7], colorectal cancer (CRC) [8], lung cancer [9], and gastric cancer [10]. The *TMEM16A* gene is located on chromosome 11q13, the most frequently amplified locus that contributes to tumorigenesis [11]. TMEM16A overexpression is associated with poor prognosis in breast cancer [2], HNSCC [6], gastric cancer [10], and CRC [12]. In addition, TMEM16A inhibition has been found to improve responses to EGFR/HER2-targeted therapy in HNSCC and breast cancer

Abbreviations: HNSCC, head and neck squamous cell carcinoma; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; ESCC, esophageal squamous cell carcinoma; CAMKII, calmodulin-dependent protein kinase II; IDC, invasive ductal carcinoma; IHC, immunohistochemical; shRNA, Small hairpin RNA; OS, overall survival

* Corresponding author. Department of Ion Channel Pharmacology, School of Pharmacy, China Medical University, No.77 Puhe Road, Shenyang North New Area, Shenyang, 110122, China.
E-mail address: qinghuanxiao12345@163.com (Q. Xiao).

[1] H.W. and F.Y. contributed equally to this work.

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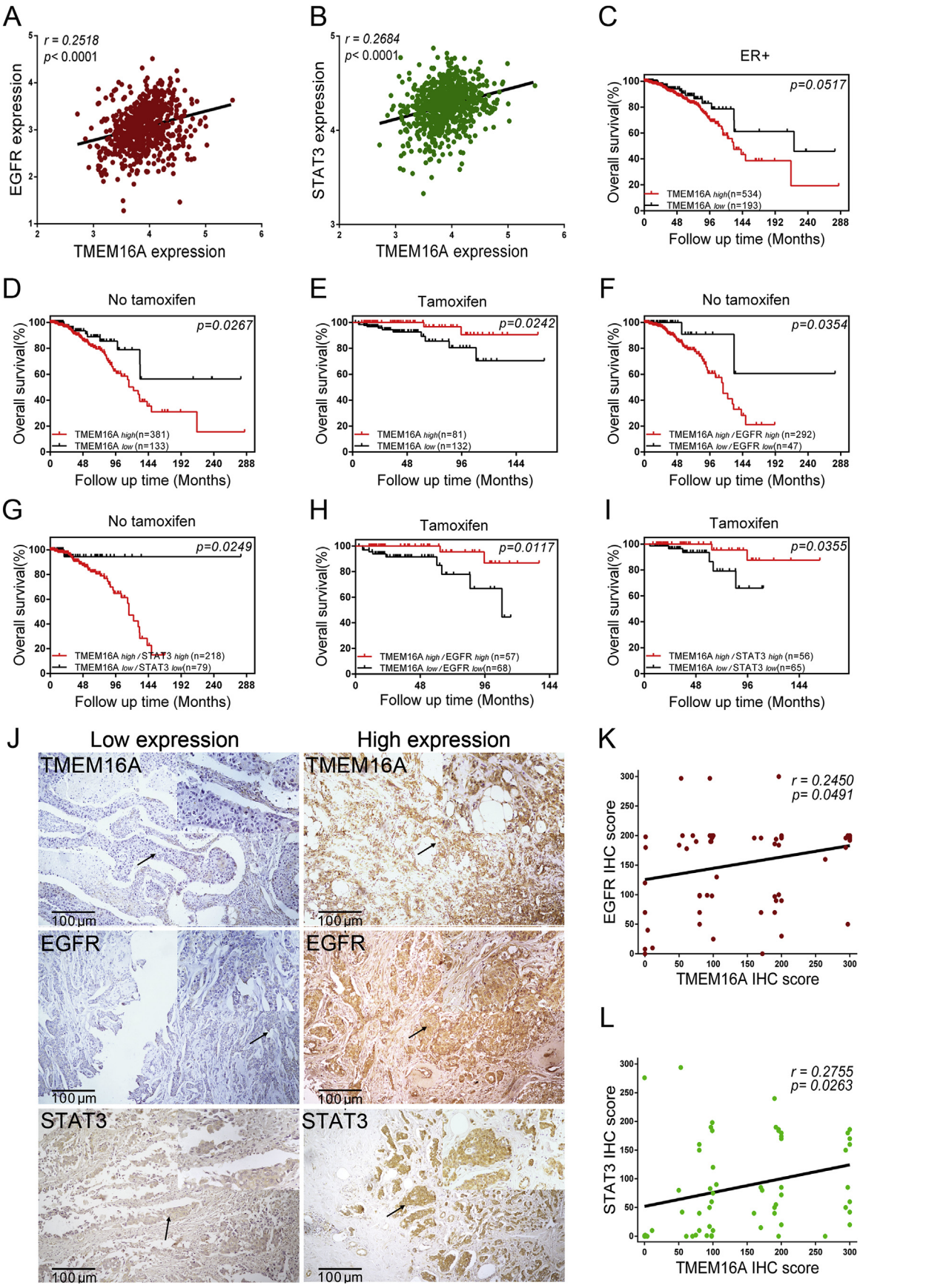

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Fig. 1. The expression of TMEM16A and EGFR/STAT3 in breast cancer patients. A, B. The correlation of TMEM16A mRNA expression with the expression of EGFR (A) and STAT3 (B) in human breast cancer tissues of ER-positive breast cancer patients in TCGA dataset. Data are presented as the z scores of log-transformed normalized read counts. The association was analyzed using spearman correlation. n = 727. C-E. Survival curves show the association of TMEM16A expression with the overall survival (OS) in ER-positive breast cancer (n = 727) (C), and those without tamoxifen treatment (n = 514) (D) and with tamoxifen treatment (n = 213) (E). F-I. Survival curves show the association of combined TMEM16A and EGFR expression (F, H) or combined TMEM16A and STAT3 expression (G, I) with the OS in ER-positive breast cancer patients without tamoxifen treatment (F,G) and with tamoxifen treatment (H, I). J. The representative immunohistochemical images for the low and high expression of TMEM16A, EGFR, and STAT3 in human breast cancer samples. Arrows indicate the magnified regions in the insert. Magnification: × 40. Scale Bars: 100 μm. K. L The correlation of TMEM16A protein expression with the expression of EGFR (K) and STAT3 (L) in 65 human breast cancer tissues. Data are expressed as the immunohistochemical (IHC) scores, and the association was analyzed using spearman correlations.

cells *in vitro* [13]. We have previously found that TMEM16A overexpression is associated with good prognosis in breast cancer patients following the treatment of tamoxifen [3], an ER modulator that also inhibits TMEM16A currents [14]. Therefore, TMEM16A may be a potential new biomarker for breast cancer therapy.

TMEM16A overexpression is caused by *TMEM16A* gene amplification in many cancers, including breast cancer [15]. However, only approximately 15% breast cancer patients carry 11q13 gene amplification [11], whereas TMEM16A overexpression is present in > 78% breast cancer samples [2,3]. Other mechanisms that regulate TMEM16A expression must exist in breast cancer. Mroz et al. found that EGF upregulated TMEM16A expression in intestinal epithelial cells [16]. In addition, Bill et al. reported that EGFR signaling activation increased TMEM16A expression in Tel1 cells stably expressing doxycycline-inducible expression constructs for EGFR [17]. He et al. reported that TMEM16A overexpression was correlated with the high expression of EGFR in tumor samples from patients with non-small cell lung cancer [18]. However, it remains unclear whether EGFR signaling activation contributes to TMEM16A overexpression in breast cancer patients.

TMEM16A promotes cell proliferation in many cancers such as breast cancer [2], HNSCC [5], CRC [8], esophageal squamous cell carcinoma (ESCC) [19], lung cancer [9], and prostate cancer [20]. TMEM16A appears to activate distinct signaling pathways in different cancers. For example, TMEM16A activates the Ras-Raf-MEK-ERK1/2 signaling pathway in HNSCC, the PI3K/AKT signaling pathway in ovarian cancer [21], the NFκB signaling pathway in glioma [22], and the p38 and ERK1/2 signaling pathways in hepatoma [23]. TMEM16A promotes cell proliferation by activating EGFR signaling in EGFR-expressing breast cancer [2] and HNSCC [13,17]. It appears that TMEM16A preferentially activates EGFR signaling in EGFR-expressing cancers.

In this study, we found positive correlation between TMEM16A expression and EGFR/STAT3 expression in breast cancer samples. TMEM16A and EGFR/STAT3 signaling mutually activated each other in breast cancer cells. This mutual activation loop was critical for breast cancer cell proliferation both *in vitro* and *in vivo*. In addition, inhibition of TMEM16A channel function enhanced the response of breast cancer cells to the EGFR inhibitor gefitinib. Our study suggests that TMEM16A inhibition may represent a promising novel therapy for EGFR-expressing breast cancer.

2. Materials and methods

2.1. Human breast cancer samples

The Medical Ethics Committee of China Medical University approved the clinical studies, and informed consent was obtained from the patients. Human breast tissue samples were obtained from 65 female patients with sporadic breast cancer, who underwent surgery at the Department of Breast Surgery at the First Affiliated Hospital of China Medical University between January 2011 and December 2011. The average age of these patients were 51 years (range, 24–78 years). All breast cancer patients were initially diagnosed and did not receive radiation therapy, chemotherapy, and hormonal therapy before surgery. Only breast cancer patients with invasive ductal carcinoma (IDC) were included in this study, and the diagnosis of IDC was confirmed by histopathological staining.

2.2. Immunohistochemistry

Formalin-fixed and paraffin-embedded sections (4 μm thick) of human breast cancer tissues were used for immunohistochemistry as previously described [3]. Sections were then incubated in primary antibodies against TMEM16A (1:400 dilution; Abcam Biotechnology, UK), EGFR (1:50 dilution; Abcam Biotechnology, UK), STAT3 (1:200 dilution; Abcam Biotechnology, UK) overnight at 4 °C. Then, sections were incubated with biotinylated secondary antibodies (1:3,000 dilution; UltraSensitiveTM-SP kit, Fuzhou Maixin, Fuzhou, China) for 20 min at room temperature followed by stain with 3, 3-diaminobenzidine (DAB). The intensity of immunoreactivity was scored as follows: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The immunohistochemical (IHC) score was determined by the multiplication of the intensity score (0–3) and the percentage (0–100%) of immunopositive cells, resulting in the final IHC score ranging from 0 to 300.

2.3. Cell culture

Breast cancer MCF-7 and T47D cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, HyClone) supplemented with 10% fetal bovine serum (Biological Industries, Israel) and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 humid incubator.

2.4. Transfection

CMV promoter-driven plasmids (pEGFP-N1) expressing TMEM16A was kindly provided by Dr. U. Oh (Seoul National University, Korea) [14]. Small hairpin RNA (shRNA) against TMEM16A in the pGPU6-EGFP vector was purchased from Shanghai GenePharma (Shanghai, China). The sequence was as follows: 5′-TTCTATAGATGATAACTCC-3'. A scramble shRNA was used as a negative control. MCF-7 or T47D cells were transfected with TMEM16A-expressing plasmids or TMEM16A-targeting shRNAs in culture medium without serum, using Lipofectamin 2000 (Invitrogen). After transfection for 48 h, the cells were used for the following experiments.

2.5. Western blot

MCF-7 and T47D cells were homogenized in ice-cold RIPA buffer containing protease inhibitor cocktail (Beyotime Biotechnology, China). Proteins were resolved by SDS–PAGE, and electrobloted onto polyvinylidene fluoride membranes. Membranes were incubated with primary antibodies against TMEM16A (1:3,000, Abcam Biotechnology, UK), EGFR (1:200, Abcam Biotechnology, UK), p-EGFR (1:500, Abcam Biotechnology, UK), STAT3 (1:2,000, Abcam Biotechnology, UK), and p-STAT3 (1:2,000, Abcam Biotechnology, UK) overnight at 4 °C. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (dilution 1:10,000, Abcam, USA) at room temperature for 1 h. Bands were visualized using an enhanced chemiluminescence detection system (BIO-RAD, USA).

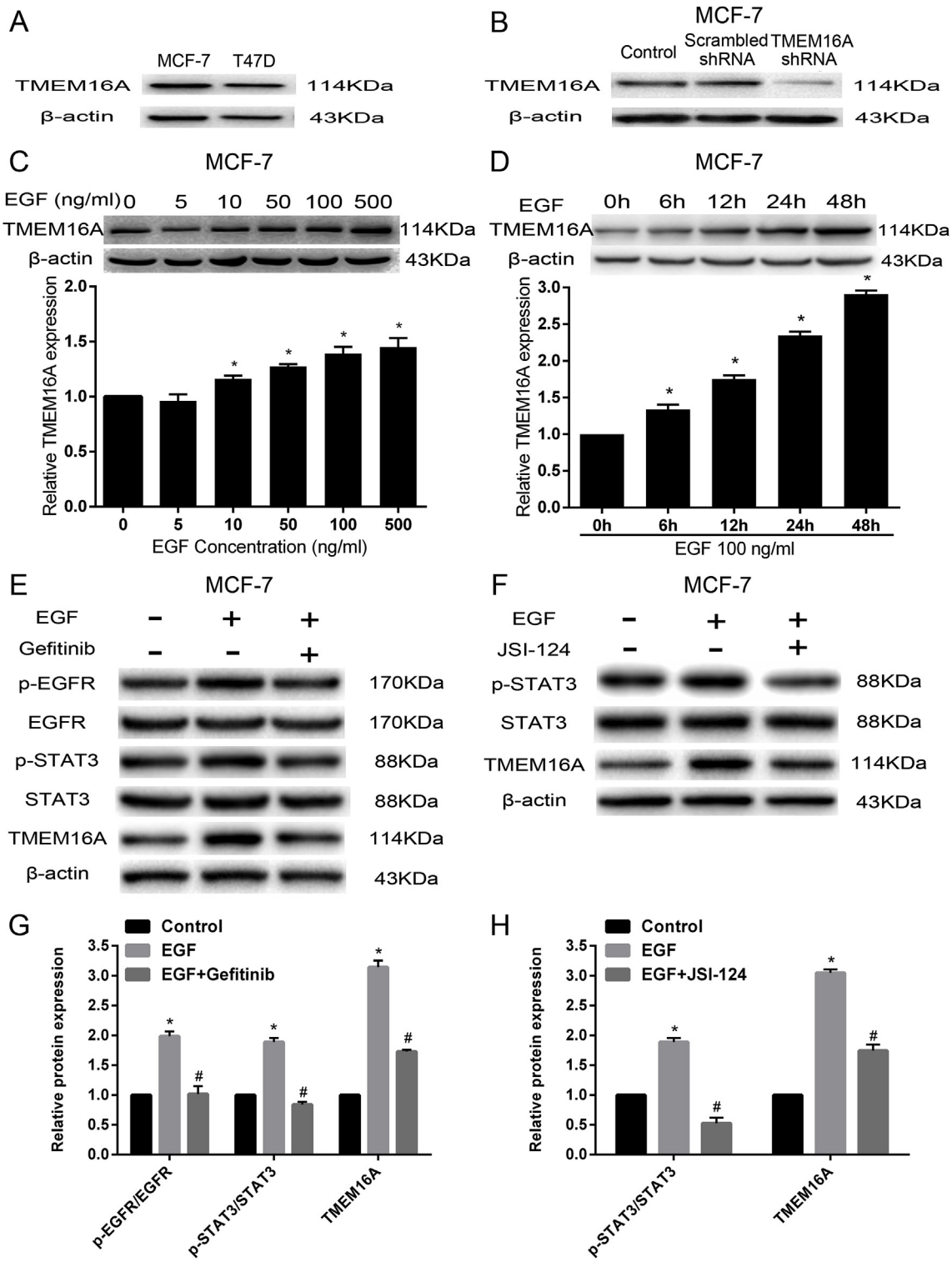

Fig. 2. EGF increased TMEM16A expression via the EGFR/STAT3 signaling pathway in MCF-7 cells. A. Western blot analysis showed TMEM16A expression in MCF-7 and T47D cells. B. Western blot analysis showed TMEM16A expression in MCF-7 cells treated with scrambled shRNAs or shRNAs against TMEM16A. C, D. Western blot analysis showed TMEM16A expression in MCF-7 cells treated with different concentrations of EGF (0–500 ng/ml) (C) for different times (0–48 h) (D). n = 3, $^{*}p < 0.05$ vs control. E, F. Western blot results showed the expression of p-EGFR, EGFR, p-STAT3, STAT3, and TMEM16A in MCF-7 cells treated with 100 ng/ml EGF for 24 h in the presence or absence of the EGFR inhibitor gefitinib (E) or the STAT3 inhibitor JSI-124 (F). G. H. Quantification results of the expression of p-EGFR/EGFR, p-STAT3/STAT3, and TMEM16A in MCF-7 cells treated with 100 ng/ml EGF for 24 h in the presence or absence of gefitinib (G) or JSI-124 (H). n = 3, $^{*}p < 0.05$ vs control, $\#p < 0.05$ vs EGF.

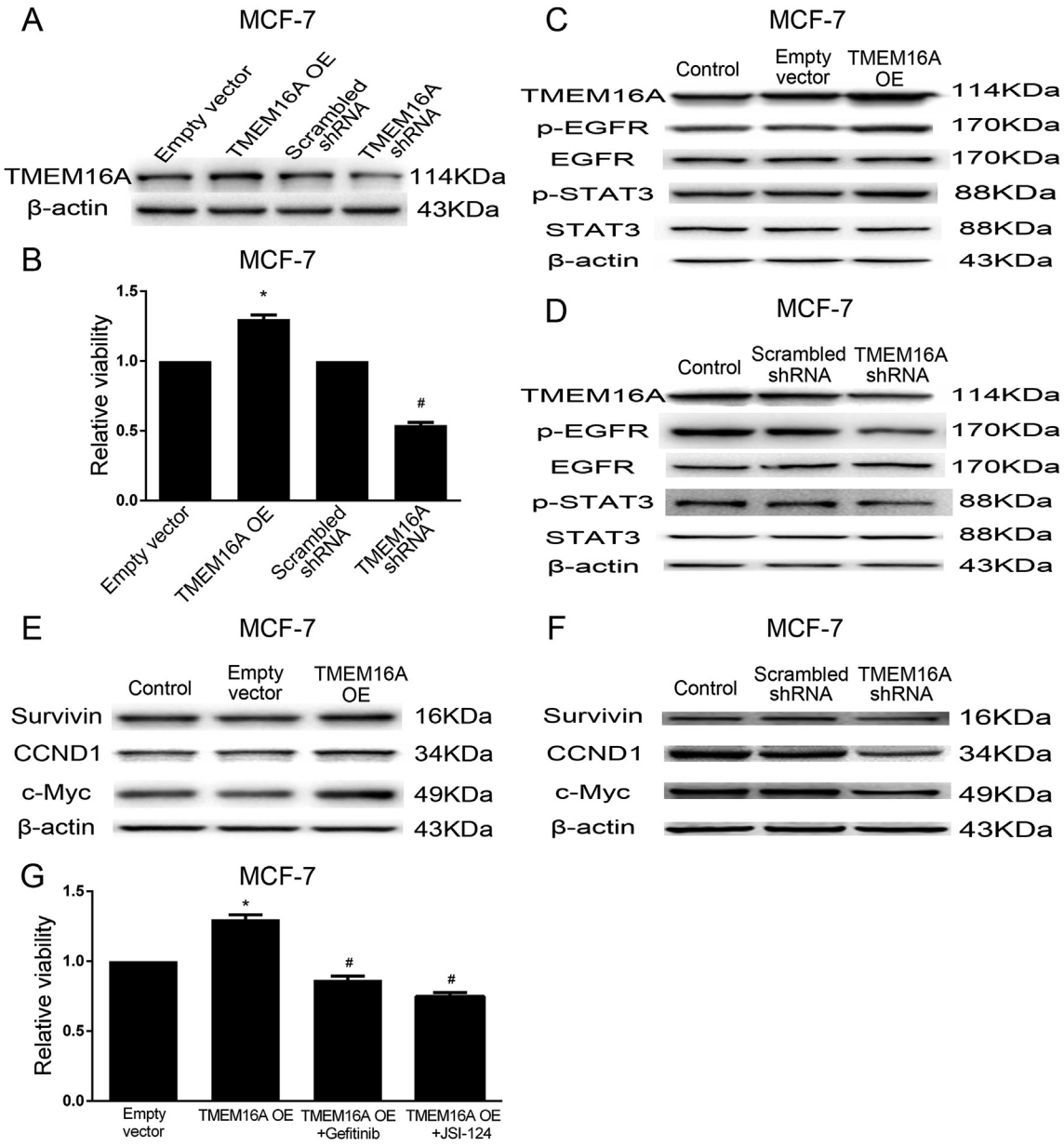

Fig. 3. TMEM16A promoted breast cancer cell proliferation via activation of the EGFR/STAT3 signaling pathway in MCF-7 cells. A. Western blot analysis showed TMEM16A expression in MCF-7 cells transfected with empty vector, TMEM16A-overexpressing plasmids, scrambled shRNAs, or TMEM16A-shRNAs. B. CCK8 assay showed cell viability in MCF-7 cells transfected with empty vector, TMEM16A-overexpressing plasmids, scrambled shRNAs, or TMEM16A-shRNAs. n = 3. $*p < 0.05$ vs vector; $\#p < 0.05$ vs scrambled shRNA. C-F. Western blot results showed the expression of TMEM16A, p-EGFR, EGFR, p-STAT3, and STAT3 (C, D) and Survivin, Cyclin D1 (CCND1) and c-Myc (E, F) in control cells (no transfection), MCF-7 cells transfected with empty vector, or TMEM16A-overexpressing (TMEM16A OE) plasmids (C, E), and MCF-7 cells transfected with scrambled shRNAs, or TMEM16A-shRNAs (D, F). G. CCK8 assays showed cell viability in MCF-7 cells transfected with empty vector or TMEM16A-overexpressing plasmids in the presence or absence of gefitinib and JSI-124 for 24 h n = 3. $*p < 0.05$ vs vector; $\#p < 0.05$ vs TMEM16A OE.

2.6. Cell counting Kit-8 (CCK-8) assay

Cell viability was measured by using CCK-8 assay (Biosharp). MCF-7 or T47D cells with indicated treatments were seeded onto 96-well plates at a density of 1×10^4 cells/well. After cells grew in the culture medium for 24 h, cells were incubated with CCK-8 solution for 2 h. Plates were read at 450 nm wavelength in a microplate reader (Molecular Devices).

2.7. Patch clamp recording

The electrophysiological recordings were performed in whole-cell configurations. Electrodes were pulled on a Sutter P97 puller (Sutter Instruments, CA), and had resistances of 2–4 mΩ. Data were recorded with Clampex 10 software on an Axopatch 200B amplifier via a Digidata 1322A data acquisition system (Molecular Device, CA, USA). Pipette solutions with different concentrations of free Ca^{2+} were made by mixing the "0" Ca^{2+} and "high" Ca^{2+} solutions as previously reported [24,25]. The "0" Ca^{2+} pipette solution contained (in mM): 146 CsCl, 2 $MgCl_2$, 5 EGTA and 8 HEPES, pH 7.3 adjusted with NMDG. The "high" Ca^{2+} pipette solution contained 5 mM Ca^{2+}-EGTA instead of EGTA, which supplied free Ca^{2+} of about 25 μM. The standard external solution contained (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.3). T47D cells were voltage clamped from a holding potential of 0 mV with 750-ms voltage steps from −100 to +100 mV.

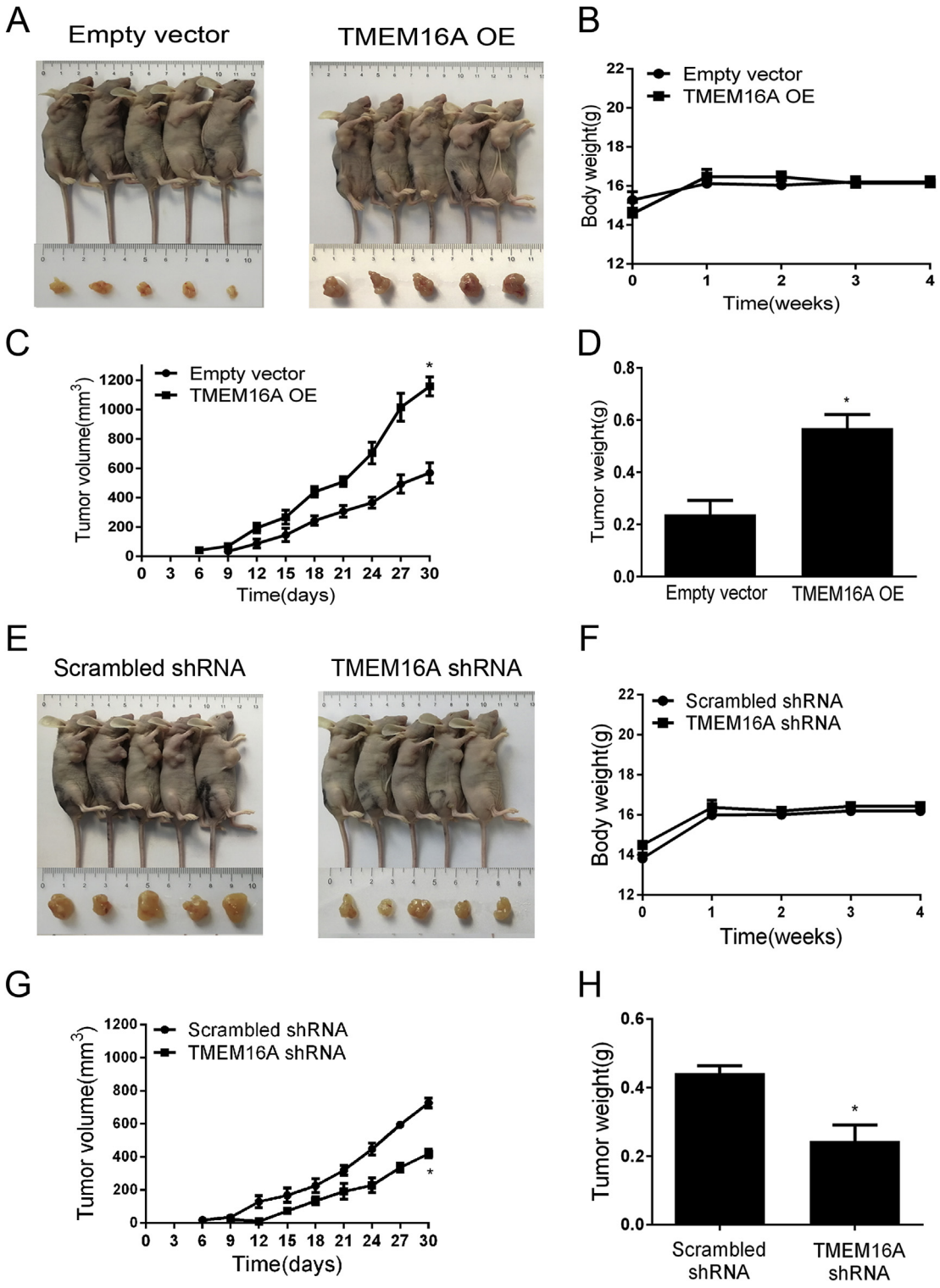

Fig. 4. TMEM16A promoted tumor growth *in vivo*. A. E. Images of mice bearing xenografted tumors removed at the 30th days after subcutaneously injected with MCF-7 cells transfected with empty vector or TMEM16A-overexpressing (TMEM16A OE) plasmids (A), and scrambled shRNAs or TMEM16A-shRNAs (E). B. F. The body weight of xenografted mice measured once a week. C. G. The tumor size of xenografted tumors in nude mice measured every 3 days. D. H. Tumor weight removed from mice after sacrifice. n = 5. *$p < 0.05$ vs control.

2.8. In vivo xenograft model of breast cancer

The animal study was approved by the Animal Ethics Committee of China Medical University, and all animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. BALB/c athymic nude mice (female, 4–6 weeks old, weighing 16–20 g) were bred in pathogen-free conditions at 25 °C with 12 h light/dark cycle. Animals received water and food *ad libitum*.

MCF-7 cells transfected with empty vector, TMEM16A-overexpressing plasmids, scrambled shRNA or TMEM16A-shRNA were used to investigate the effect of TMEM16A on breast cancer tumorigenesis.

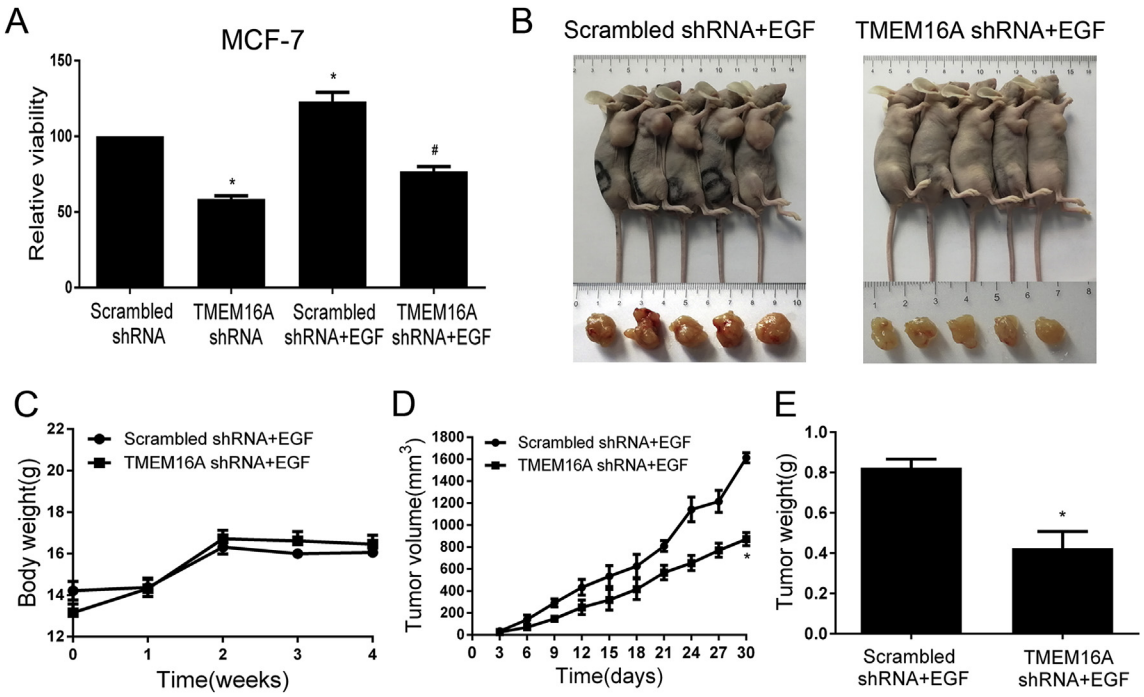

Fig. 5. TMEM16A mediated EGF-induced cell proliferation and tumorigenesis *in vitro* and *in vivo*. A. CCK8 assays showed cell viability in MCF-7 cells transfected with scrambled shRNAs or TMEM16A-shRNAs in the presence or absence of EGF (100 ng/ml) for 24 h n = 3. $^{*}p < 0.05$ vs scrambled shRNA; $^{\#}p < 0.05$ vs scrambled shRNA + EGF. B. Images of mice bearing xenografted tumors removed at the 30th days after subcutaneously injected with MCF-7 cells transfected with scrambled shRNAs or TMEM16A-shRNAs with EGF (100 ng/ml) treatment for 24 h. C. The body weight of xenografted mice measured once a week. D. The tumor size of xenografted tumors in nude mice measured every 3 days. E. Tumor weight removed from mice after sacrifice. n = 5. $^{*}p < 0.05$ vs scrambled shRNA + EGF.

To investigate the effect of TMEM16A on EGF-induced breast cancer tumorigenesis, MCF-7 cells transfected with scrambled shRNA or TMEM16A-shRNA were treated with or without 100 ng/ml EGF for 24 h. Then, MCF-7 cells were suspended in 200 μl PBS and subcutaneously injected into the right flank of nude mice. Tumor growth was measured using a caliper measurement. Tumor volume (V) was determined according to the following formula: $V = (L \times W^2) \times 0.5$, where L is the length, and W is the width of the tumor. Mice were sacrificed at 30 days after tumor transplantation, and tumors were removed and weighed.

2.9. Statistical analysis

The RNA expression data for TMEM16A, EGFR, and STAT3 were obtained from the Cancer Genome Atlas (TCGA), and z scores of log-transformed normalized read counts were calculated. The association of TMEM16A expression with EGFR expression and STAT3 expression was analyzed, using Spearman correlations. Survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test.

All numerical data are represented as mean ± standard deviation (SD). Student *t*-test or one-way analysis of variance (ANOVA) was used to compare the difference between two groups and among more than two groups, respectively. Statistical analyses were performed using SPSS 13.0. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. TMEM16A expression positively correlates with the expression of EGFR and STAT3 ad is associated with clinical outcomes in breast cancer patients

To identify the association between TMEM16A expression and EGFR/STAT3 expression in human breast cancer samples, we screened the mRNA expression data from the TCGA dataset, using the cBioportal tool (www.cbioportal.org). In ER-positive breast cancer (n = 727), TMEM16A expression was positively correlated with the expression of EGFR (r = 0.2518, p < 0.0001) and STAT3 (r = 0.2684, p < 0.0001) (Fig. 1A and B). High TMEM16A expression exhibited a tendency toward shorter overall survival (OS) in ER-positive breast cancer (n = 727, $p = 0.0517$, Fig. 1C). Since tamoxifen inhibits TMEM16A currents [14], we further evaluated the association of TMEM16A expression with OS in breast cancer patients with or without tamoxifen treatment. High TMEM16A expression was significantly associated with shorter OS in patients without tamoxifen treatment (n = 514; $p = 0.0267$, Fig. 1D), but longer OS in patients with tamoxifen treatment (n = 213; $p = 0.0242$, Fig. 1E). Furthermore, high TMEM16A/EGFR expression and high TMEM16A/STAT3 expression were significantly associated with shorter OS in patients without tamoxifen treatment ($p = 0.0354$ for TMEM16A/EGFR, Fig. 1F; $p = 0.0249$ for TMEM16A/STAT3, Fig. 1G), but longer OS in patients with tamoxifen treatment ($p = 0.0117$ for TMEM16A/EGFR, Fig. 1H; $p = 0.0355$ for TMEM16A/STAT3, Fig. 1I). These results suggested that TMEM16A overexpression promoted ER-positive breast cancer progression and TMEM16A inhibition by tamoxifen improved clinical outcomes in breast cancer patients. Furthermore, the protein expression of TMEM16A was positively correlated with the expression of EGFR and STAT3 in 65 human breast cancer samples (r = 0.2450, p = 0.0491 for EGFR; and r = 0.2755, p = 0.0263 for STAT3) (Fig. 1J-L).

3.2. EGF increases TMEM16A expression via the EGFR/STAT3 signaling pathway in MCF-7 breast cancer cells

Western blot results showed TMEM16A proteins were highly expressed in MCF-7 and T47D cells (Fig. 2A). TMEM16A-shRNAs effectively reduced TMEM16A expression in MCF-7 cells (Fig. 2B), further confirming that MCF-7 cells expressed TMEM16A. EGF (5–500 ng/ml) increased TMEM16A expression in a dose-dependent manner (Fig. 2C) and in a time-dependent manner (Fig. 2D). Furthermore, EGF treatment significantly increased phosphorylated EGFR and STAT3 expression in

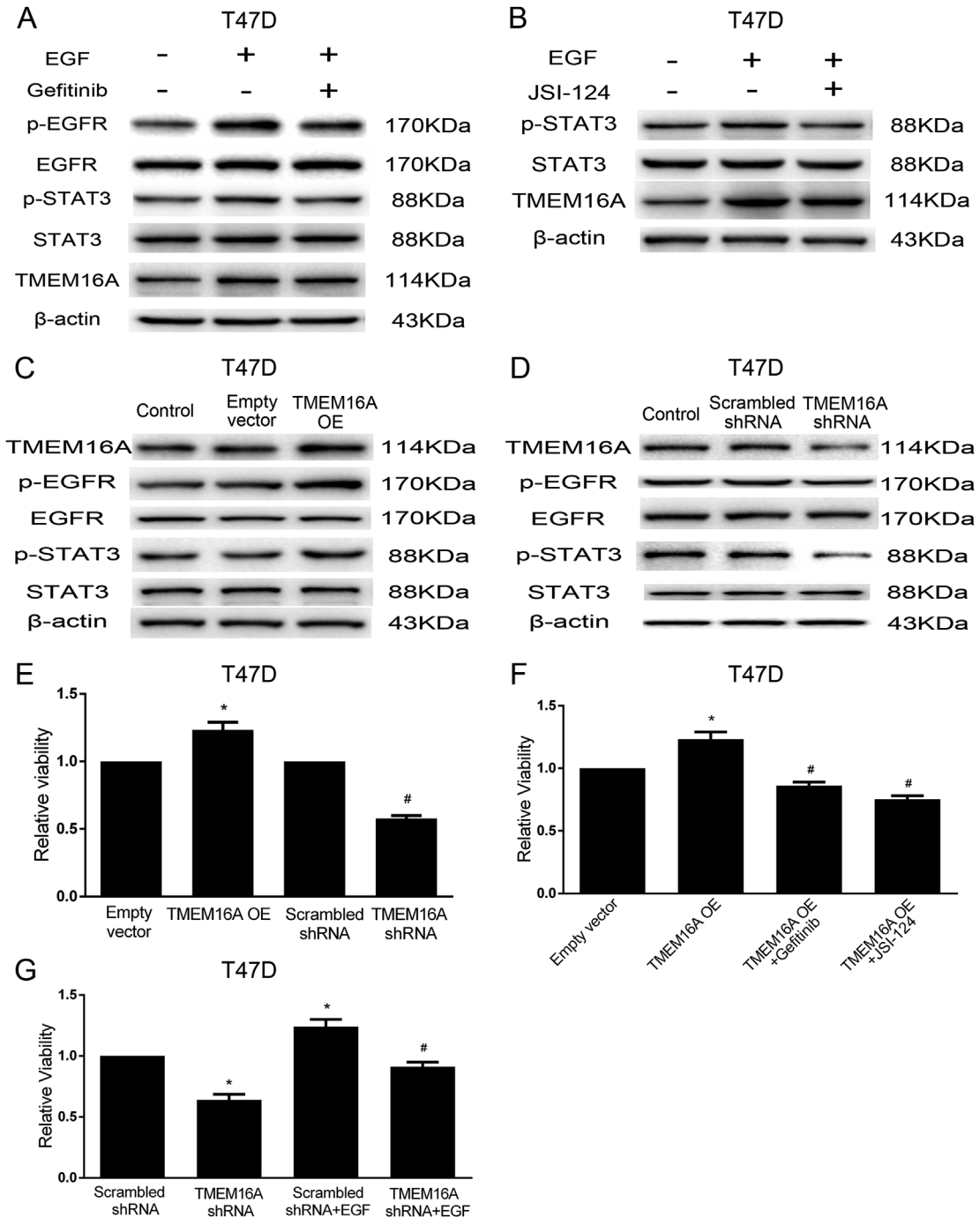

Fig. 6. Mutual activation between TMEM16A and EGFR/STAT3 signaling promoted cell proliferation in T47D cells. A, B. Western blot results showed the expression of p-EGFR, EGFR, p-STAT3, STAT3, and TMEM16A in T47D cells treated with 100 ng/ml EGF for 24 h in the presence or absence of the EGFR inhibitor gefitinib (A) or the STAT3 inhibitor JSI-124 (B). C, D. Western blot results showed the expression of TMEM16A, p-EGFR, EGFR, p-STAT3, and STAT3 in control cells (no transfection), T47D cells transfected with empty vector, or TMEM16A-overexpressing (TMEM16A OE) plasmids (C), and T47D cells transfected with scrambled shRNAs, or TMEM16A-shRNAs (D). E. CCK8 assay showed cell viability in T47D cells transfected with empty vector, TMEM16A-overexpressing plasmids, scrambled shRNAs, or TMEM16A-shRNAs. n = 3. *$p < 0.05$ vs vector; #$p < 0.05$ vs scrambled shRNA. F. CCK8 assays showed cell viability in T47D cells transfected with empty vector or TMEM16A-overexpressing plasmids in the presence or absence of gefitinib and JSI-124 for 24 h n = 3. *$p < 0.05$ vs vector; #$p < 0.05$ vs TMEM16A OE. G. CCK8 assays showed cell viability in T47D cells transfected with scrambled shRNAs or TMEM16A-shRNAs in the presence or absence of EGF (100 ng/ml) for 24 h n = 3. *$p < 0.05$ vs scrambled shRNA; #$p < 0.05$ vs scrambled shRNA + EGF.

MCF-7 cells, suggesting that EGF activated the EGFR/STAT3 signaling pathway. EGF-induced increase in TMEM16A expression was significantly inhibited by the EGFR inhibitor gefitinib and the STAT3 inhibitor JSI-124 (Fig. 2E–H), suggesting that EGF increased TMEM16A expression via the EGFR/STAT3 signaling pathway.

3.3. TMEM16A promotes breast cancer proliferation via activation of the EGFR/STAT3 signaling pathway

Western blot results confirmed that TMEM16A expression was increased in cells overexpressing TMEM16A, and decreased in cells treated with TMEM16A-shRNAs (Fig. 3A). TMEM16A overexpression

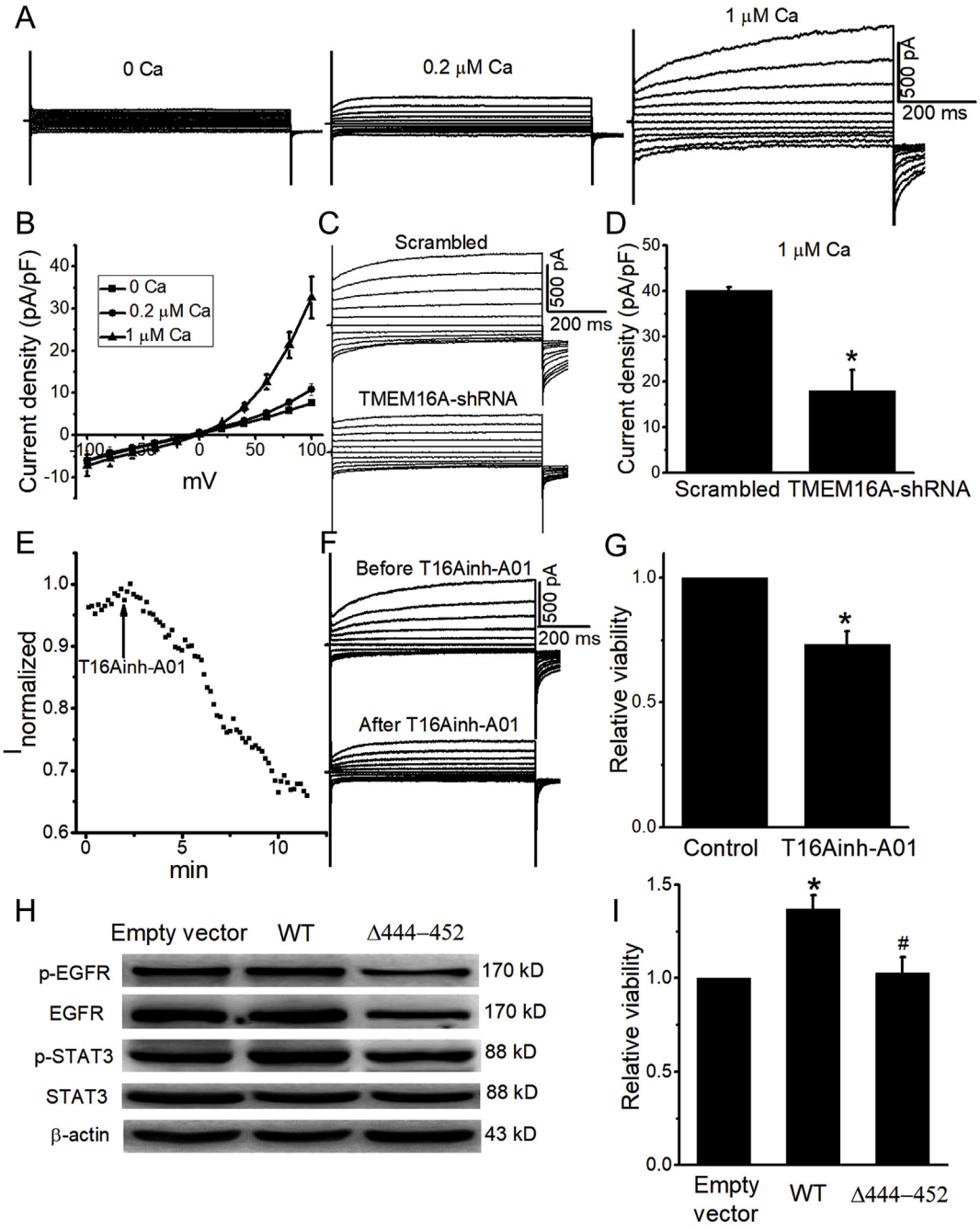

Fig. 7. TMEM16A channel function was important for cell proliferation in T47D cells. A. Representative whole-cell currents activated by 0, 0.2 and 1 μM Ca^{2+} in T47D cells. Cells were voltage clamped at a holding potential of 0 mV to potentials between −100 mV and +100 mV in 20 mV increments for 750 ms, followed by a 100-ms step to −100 mV. B. Steady-state current-voltage relationships for traces in (A). C. Representative whole-cell currents activated by 1 μM Ca^{2+} in T47D cells treated with scrambled shRNA (top) or TMEM16A-shRNA (bottom). n = 4; p < 0.05 vs scrambled. D. Mean current densities at +100 mV for cells treated with scrambled shRNA or TMEM16A-shRNA in (C). E. The time course of whole-cell currents activated by 1 μM Ca^{2+} in T47D cells treated with the TMEM16A inhibitor T16Ainh-A01 (20 μM). Voltage ramps from −100 mV to +100 mV with a duration of 750 ms were given at a holding potential of 0 mV at 10 s intervals. The arrow indicates application of T16Ainh-A01. F. Representative whole-cell currents activated by 1 μM Ca^{2+} before (top) and after (bottom) T16Ainh-A01 treatment in (E). G. CCK8 assays showed cell viability in T47D cells with or without T16Ainh-A01 treatment (20 μM) for 24 h n = 3. *p < 0.05 vs control. H. Representative Western blot analysis showed the expression of p-EGFR, EGFR, p-STAT3, and STAT3 in control T47D cells transfected with empty vector or plasmids containing wild type (WT) and Δ_{444}EEEEEAVKD$_{452}$ (Δ444-452) TMEM16A. n = 3. I. CCK8 assays showed cell viability in T47D cells transfected with empty vector or plasmids containing WT and Δ_{444}EEEEEAVKD$_{452}$ TMEM16A. n = 3. *p < 0.05 vs empty vector; #p < 0.05 vs WT-TMEM16A.

significantly promoted cell proliferation, and TMEM16A knockdown significantly reduced cell proliferation in MCF-7 cells (Fig. 3B). In addition, TMEM16A overexpression increased, and TMEM16A knockdown reduced phosphorylated EGFR and STAT3 expression in MCF-7 cells (Fig. 3C and D). The expression of Survivin, Cyclin D1, and c-Myc, which are known to be regulated by STAT3 [26,27], were increased in MCF-7 cells overexpressing TMEM16A, and was decreased in cells treated with TMEM16A-shRNAs (Fig. 3E and F). Furthermore, the EGFR inhibitor gefitinib and the STAT3 inhibitor JSI-124 inhibited TMEM16A overexpression-induced cell proliferation in MCF-7 cells (Fig. 3G). These data suggested that TMEM16A promoted cell proliferation by activating the EGFR/STAT3 signaling pathway in MCF-

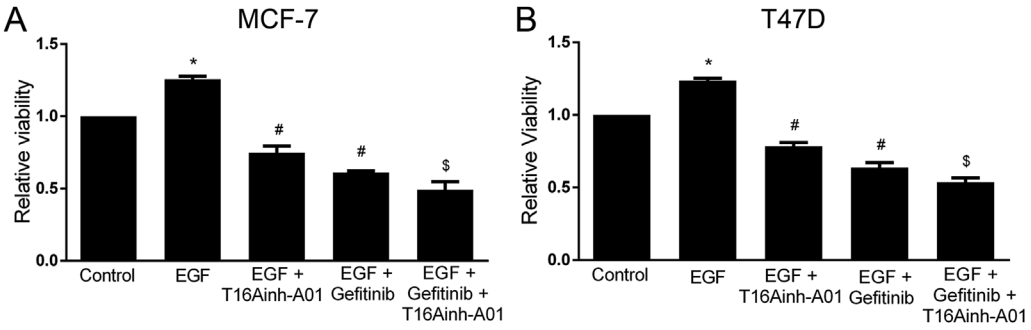

Fig. 8. TMEM16A inhibitors in combined with EGFR inhibitor inhibited EGF-mediated proliferation in MCF-7 and T47D cells. A. B, CCK8 assays showed cell viability in MCF-7 (A) and T47D (B) cells in the presence of EGF (100 ng/ml) for 24 h with gefitinib (10 μM) or T16Ainh-A01 (20 μM) alone or in combination. $^{*}p < 0.05$ vs control; $^{\#}p < 0.05$ vs EGF; $^{S}p < 0.05$ vs gefitinib or T16A-inhA01 alone.

7 cells.

In nude mice xenografted with MCF-7 cells, TMEM16A overexpression did not result in significant changes in the body weight of (Fig. 4A and B). TMEM16A overexpression promoted xenografted tumor growth, and the tumor was bigger in mice xenografted with TMEM16A-overexpressing cells (Fig. 4C and D). In contrast, TMEM16A knockdown inhibited xenografted tumor growth, and the tumor was smaller in mice xenografted with cells treated with TMEM16A-shRNAs (Fig. 4E–H).

3.4. TMEM16A knockdown inhibits EGF-induced proliferation and tumorigenesis in breast cancer

EGF increased cell proliferation in MCF-7 cells. TMEM16A knockdown by shRNAs significantly reduced EGF-induced cell proliferation (Fig. 5A). In mice xenografted with MCF-7 cells, TMEM16A knockdown inhibited EGF-induced tumor growth, and resulted in smaller tumors in mice xenografted with EGF-treated cells (Fig. 5B–E). These findings suggested that TMEM16A was important for EGF-induced breast cancer proliferation and tumorigenesis both *in vitro* and *in vivo*.

3.5. Mutual activation between TMEM16A and EGFR/STAT3 signaling promotes cell proliferation in T47D cells

Similar to MCF-7 cells, EGF treatment increased TMEM16A expression in T47D cells, and this effect was blocked by gefitinib and JSI-124 (Fig. 6A and B), suggesting that EGF promoted TMEM16A expression via activation of the EGFR/STAT3 signaling pathway. TMEM16A overexpression increased, and TMEM16A knockdown decreased phosphorylated EGFR and STAT3 expression in T47D cells (Fig. 6C and D). Furthermore, TMEM16A overexpression significantly promoted cell proliferation, and TMEM16A knockdown significantly reduced cell proliferation in T47D cells (Fig. 6E). TMEM16A overexpression-induced proliferation was blocked by gefitinib or JSI-124 in T47D cells (Fig. 6F). Furthermore, EGF-induced cell proliferation was inhibited by TMEM16A-shRNAs in T47D cells (Fig. 6G). Taken together, these results suggested that mutual activation loop between TMEM16A and EGFR/STAT3 signaling was also important for cell proliferation in T47D cells.

3.6. Inhibition of TMEM16A channel function improves the response of breast cancer cells to EGFR inhibitor gefitinib

Whole-cell patch clamp was performed to record Ca^{2+}-activated Cl^- currents (CaCCs) in T47D cells. The currents activated by Ca^{2+} exhibited strong outward rectification, and slowly activated with time upon depolarization with deactivating tail currents upon repolarization (Fig. 7A and B), similar to the CaCCs recorded in HEK293 cells overexpressing TMEM16A plasmids [25]. The CaCCs in T47D cells were inhibited by TMEM16A-shRNAs (Fig. 7C and D), confirming that TMEM16A mediated the CaCCs in T47D cells. The TMEM16A inhibitor T16Ainh-A01 (20 μM) inhibited CaCCs (Fig. 7E and F), and cell proliferation in T47D cells (Fig. 7G). Furthermore, overexpression of Δ_{444}EEEEEAVKD$_{452}$ mutants (deleting $_{444}$EEEEEAVKD$_{452}$ in the first intracellular loop of TMEM16A) with reduced Cl^- channel activities [24], reduced phosphorylated EGFR and STAT3 expression (Fig. 7H), and inhibited cell proliferation in T47D cells (Fig. 7I). These findings supported that TMEM16A channel function was critical for TMEM16A-induced proliferation in breast cancer cells.

We further investigated whether TMEM16A inhibition enhanced the response of breast cancer cells to the EGFR inhibitor gefitinib in MCF-7 and T47D cells. EGF-induced increase in cell proliferation was inhibited by gefitinib or T16Ainh-A01 alone (Fig. 8A and B). A combination of T16Ainh-A01 and gefitinib significantly reduced EGF-induced proliferation compared with gefitinib or T16Ainh-A01 alone (Fig. 8A and B). These findings suggested that combined inhibition of TMEM16A and EGFR more effectively inhibited breast cancer cell proliferation.

4. Discussion

TMEM16A promotes cell proliferation and tumor growth in EGFR-expressing cancers including breast cancer and HNSCC [2,17]. Therefore, EGFR signaling activation may be critical for the proliferation-promoting effect of TMEM16A in cancer. In the present study, we found that EGF upregulated TMEM16A expression in breast cancer via the EGFR/STAT3 signaling pathway, and TMEM16A overexpression activated the EGFR/STAT3 signaling in MCF-7 and T47D breast cancer cells. This mutual activation loop was important for cell proliferation in breast cancer cells both *in vitro* and *in vivo*. Furthermore, we found that combined inhibition of both TMEM16A and EGFR effectively reduced breast cancer cell proliferation in these EGFR-expressing breast cancer cells. Our findings establish a rationale for TMEM16A inhibition for breast cancer therapy, especially in EGFR-positive breast cancer.

TMEM16A overexpression in cancer is caused by many mechanisms such as *TMEM16A* gene amplification, transcriptional regulation, epigenetic regulation and microRNAs [15]. The presence of many initiator elements and/or transcriptional start sites in the promoter of the *TMEM16A* gene suggests that the transcriptional regulation of TMEM16A expression by diverse transcription factors may be important for TMEM16A expression [28]. IL-4 and IL-13 can induce TMEM16A upregulation by activating STAT6 in human airway epithelial cells [28,29]. Testosterone increases TMEM16A expression by binding to the androgen receptor in prostate cancer cells [30]. Here, we found that EGFR activation by EGF induced TMEM16A expression by activating STAT3 in breast cancer cells, suggesting that EGFR/STAT3 signaling activation promotes TMEM16A overexpression in breast cancer. Furthermore, TMEM16A overexpression activated the EGFR/STAT3 signaling pathway in breast cancer cells. These findings suggest that the mutual activation loop between TMEM16A and EGFR/STAT3 signaling may be important for constitutive TMEM16A overexpression and EGFR/STAT3 signaling activation in breast cancer. In addition, high TMEM16A/EGFR expression and high TMEM16A/STAT3 expression were significantly associated with poor OS in breast cancer patients, suggesting that the mutual activation between TMEM16A and EGFR/

STAT3 signaling may promote breast cancer progression.

TMEM16A overexpression promotes cell proliferation in many cancers by activating various signaling pathways such as the EGFR signaling pathway [2,17] Ras-Raf-MEK-ERK1/2 signaling pathway [5], and the NFκB signaling pathway [22]. In breast cancer, TMEM16A knockdown reduced EGFR phosphorylation, and subsequently resulted in inhibition of the downstream AKT, SRC, and ERK signaling pathways in breast cancer cells [2]. TMEM16A can activate EGFR by directly binding to EGFR and subsequently promoting EGFR phosphorylation [2,17]. We found that TMEM16A overexpression promoted EGFR phosphorylation in breast cancer cells. EGFR activates many downstream signaling pathways including the PI3K/AKT/mTOR pathway, the RAS/RAF/MEK/ERK pathway, and the STAT3 signaling pathways [31,32]. Here, we found that TMEM16A overexpression activated EGFR/STAT3 signaling, and its downstream proliferation-associated proteins such as Survivin, Cyclin D1, and c-Myc. Since TMEM16A activated the EGFR/STAT3 signaling pathway in the absence of EGF, and EGFR/STAT3 signaling activation upregulated TMEM16A expression, it appears that TMEM16A functions as a positive feedback regulator that is important for EGFR/STAT3 signaling activation in breast cancer proliferation. This idea is supported by our findings showing that TMEM16A inhibition by shRNAs or inhibitors reduced EGF-induced tumor proliferation both *in vitro* and *in vivo*.

It is unclear whether the proliferation-promoting effect of TMEM16A is caused by changes in protein expression levels or channel activities, since most studies have investigated the role of TMEM16A in cancer by knockdown or overexpression of TMEM16A, which can not distinguish protein expression levels and channel activities. Some studies have demonstrated that TMEM16A channel function is important for cancer cell proliferation, since overexpression of TMEM16A mutants with reduced channel activities inhibited cancer cell proliferation induced by WT TMEM16A overexpression [2,5]. Kulkarni et al. reported that TMEM16A Cl^- channel function was critical for EGFR activation and contributed to cell proliferation in breast cancer and HNSCC [13]. We have previously reported that the Δ_{444}EEEEEAVKD$_{452}$ mutant, which likely stabilizes in a closed conformational state, exhibits reduced channel activities [24]. We found that overexpression of WT TMEM16A, but not the $_{444}$EEEEEAVKD$_{452}$ mutant with reduced channel function, promoted cell proliferation in T47D cells. T16Ainh-A01 inhibited TMEM16A channel currents, and reduced cell proliferation in T47D cells. These results support that TMEM16A channel function is critical for breast cancer cell proliferation.

We have previously reported that TMEM16A overexpression is associated with good prognosis in breast cancer patients following the treatment of tamoxifen [3], the ER modulator that inhibits TMEM16A currents [14]. Here, we found that high TMEM16A expression alone and in combination with high EGFR and STAT3 expression was significantly associated with good clinical outcomes in breast cancer patients with tamoxifen treatment, suggesting that TMEM16A inhibition may be beneficial for breast cancer with high EGFR expression. Furthermore, we found that combined inhibition of TMEM16A channel by T16Ainh-A01 and EGFR by gefitinib more effectively inhibited breast cancer cell proliferation than these agents alone, suggesting that inhibition of both TMEM16A and EGFR may be a promising therapy for the treatment of EGFR-expressing breast cancer.

In summary, we found that a mutual activation loop between TMEM16A and EGFR/STAT3 signaling pathway was critical for breast cancer proliferation and tumor growth. In addition, combined inhibition of TMEM16A channels and EGFR effectively inhibited breast cancer cell proliferation. These findings suggest that breast cancer with high EGFR/TMEM16A expression may be suitable for the combined treatment with EGFR inhibitors and TMEM16A inhibitors.

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Authors' contributions

Q. Xiao, M. Wei, Y. Fang, and H. Wang conceived and designed this research. H. Wang, F. Yao, S. Luo, K. Ma, M. Liu, L. Bai, C. Song, S. Chen, T. Wang, Q. Du, and H. Wu conducted the experiments, and collected and analyzed data. Q. Xiao, M. Wei, Y. Fang, H. Wang, and F. Yao interpreted the data, and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Breast cancer tissues were obtained from patients with sporadic breast cancer, who underwent surgery at the Department of Breast Surgery at the First Affiliated Hospital of China Medical University. Informed consent was obtained from the patients for research use of the tissue. The Medical Ethics Committee of China Medical University approved the clinical studies, and informed consent was obtained from the patients. The animal study was approved by the Animal Ethics Committee of China Medical University, and all animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

Not applicable.

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