Original paper

# Knockdown of TRIM66 in MDA-MB-468 triple negative breast cancer cell line suppresses proliferation and promotes apoptosis through EGFR signaling

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Triple negative breast cancer (TNBC) is one of the most common malignant tumors in women and the tripartite motif 66 (TRIM66) is closely associated with the behaviors of wide variety of cancer. Thus, in this study, we aimed to explore the effect of TRIM66 in MDA-MB-468 TNBC cell line. Western blot and RT-PCR assays were used to detect the expression level of TRIM66 in human normal mammary cells and human breast cancer cell lines. We silenced its expression in MDA-MB-468 cells by transient siRNA transfection to ascertain the function of TRIM66 in MDA-MB-468 cells. CCK8 and clonogenicity assays were used to evaluate the ability of cell proliferation. Wound healing and Transwell were used to detect cell invasion and migration. TUNEL was applied to detect the apoptosis level. Western blotting was used to detect the expression of related proteins. TRIM66 was observed markedly elevated in breast cancer cell line. Knockdown of TRIM66 inhibited the expression of EGFR, P-Jak2, P-STAT3, JAK2, and STAT3 in MDA-MB-468 cells through regulating the epidermal growth factor receptor (EGFR) signaling. Knockdown of TRIM66 suppresses proliferation, invasion and migration of MDA-MB-468 cell line and promotes apoptosis in these cells through EGFR signaling.

**Key words:** triple negative breast cancer, TRIM66, epidermal growth factor receptor, apoptosis.

## Introduction

Breast cancer is recognized as one of the most common malignant tumors in women, and its morbidity and mortality are increasing year by year [1]. Triple negative breast cancer (TNBC) is a subtype that accounts for about 10-15% of breast cancers and is difficult to treat [2]. Unlike most other breast cancers, TNBCs lack estrogen and progesterone receptors and lack expression of HER2 growth factor in immunohistochemical studies. As a result, TNBC has a high risk of early metastatic recurrence, no separate treatment target, and a particularly poor prognosis [3, 4]. Therefore, it is of great significance to elucidate the molecular mechanism of TNBCs to improve the therapeutic effect and clinical prognosis. The mechanism may be explored in studies based on TNBC cell lines. Currently known TNBC cell lines include MDA-MB-468, BT20, HCC-1937, MDA-MB-231, and MDA-MB-436. Of these, MDA-MB-468 cells were selected in this study [5].

The tripartite motif 66 (TRIM66, also known as transcriptional interfactor 1, TIF1) is a member of the tri-motif protein family [6]. Studies have shown

that altered expression of TRIM family members is associated with cancer and cancer-related diseases [7, 8, 9, 10]. Little is known about the expression pattern and biological function of TRIM66 in cancer [6, 11, 12]. Studies have shown that TRIM66 promotes the expression of prostate cancer by activating JAK/STAT, and interferes with its inhibition of the JAK/STAT signal to inhibit the malignant progression of colorectal cancer [13, 14]. The role of TRIM66 in breast cancer has not been studied.

Abnormal activation of the epidermal growth factor receptor (EGFR) has been shown to be significantly involved in the occurrence of tumors in invasive TNBC by promoting cell proliferation, migration and angiogenesis [15]. EGFR can induce the downstream PI3K/Akt and JNK/STAT signaling pathways to participate in the malignant progression of tumor cells [16, 17]. Patients with TNBC may benefit from anti-EGFR therapy because EGFR overexpression was found in about 44% of BRCA1-associated breast cancers, most of which are triple negative and clinical trials are currently underway [18, 19].

The aim of the study is to unveil the role of TRIM66 in MDA-MB-468 TNBC cell line.

## Materials and methods

### Cell culture and cell transfection

Human normal mammary cells MCF-10A and human breast cancer cell lines MDA-MB-468 and BT20 were purchased from BeNa Culture Collection (BNCC, Beijing, China) and cultured in the DMEM medium (Gibco; Thermo Fisher Scientific) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific) under constant conditions at 37°C in a humidified atmosphere with 5% $CO_2$.

siRNAs against TRIM66 (TRIM66-siRNA-1 and TRIM66-siRNA-2), scrambled siRNA group (transfected with the empty vector), the adenovirus overexpressing EGFR (OV-EGFR) and the control (OV-NC) were constructed by Hanbio company (Shanghai, China). Cell transfection was performed according to manufacturer's instruction of Lipofectamine™ 3000 (Invitrogen).

### Cell proliferation assay

CCK8 and clonogenicity assays were used to evaluate the ability of cell proliferation, as previously described [20]. In brief, MDA-MB-468 cells ($1 \times 10^3$ cells/per well) were seeded in 96-well plates and cultured for 24 h. At 24, 48 and 72 h after incubation, CCK8 solution (Sigma, St. Louis, Missouri, USA) was added to each well. The cells were incubated for an additional 1h to determine the cell viability. The optical density was determined at 450 nm using a microtiter plate reader.

Transfected MDA-MB-468 cells were seeded into 6-well plates. After an incubation period of 14 days at 37°C, the colonies were fixed with 100% methanol and stained with 0.1% crystal violet in absolute ethanol for 15 min. Visible colonies of more than 50 cells were counted and analyzed under an inverted microscope.

### Wound healing and Transwell assays

For the wound-healing assay, transfected MDA-MB-468 cells were cultured in six-well plates to 70-80% confluency and were wounded with a 200-$\mu$l sterile pipette tip. After washing with PBS, the cells were cultured in serum-free medium. Images were acquired at each time point (0 and 24 h).

For Transwell assay, the cell invasive ability was evaluated in Transwell chambers coated with Matrigel (BD Biosciences, San Diego, CA, USA) as previously described [21]. In brief, MDA-MB-468 cells (a cell density of $3 \times 10^4$ cells) were plated in the plasma-free medium. Upper chamber was pre-coated with Matrigel (Sigma-Aldrich) and cells were then cultivated in the upper chamber with 0.1 ml cell suspension in each well, and the lower chamber was filled with cell culture medium containing 20% FBS. Cells were cultivated for 24 h. After that, the upper chamber was collected and cleaned, followed by 0.5% Crystal Violet (Sigma-Aldrich) staining at room temperature for 10 min. Stained cells were counted under an optical microscope.

### Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay

MDA-MB-468 cells were fixed in 4% formaldehyde at room temperature for 10 min and washed with PBS buffer solution 2 times. The apoptosis of MDA-MB-468 cells were investigated using a TUNEL assay kit (cat. no. ab206386; Abcam), according to the manufacturer's protocol. TUNEL-positive cells appeared with green nuclei under the fluorescence microscope (Olympus FV500; Olympus Corporation).

### Western blot

Total proteins were extracted from MDA-MB-468 cells using RIPA buffer and quantified by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). After denaturing, electrophoresis was performed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Following gel transfer onto polyvinylidene difluoride (PVDF) membranes, membranes were blocked in 5% fat-free milk for 2 h at room temperature. After washing, membranes were incubated with primary antibodies at 4°C overnight and subsequently incubated with IgG-HRP-conjugated goat anti-rabbit secondary

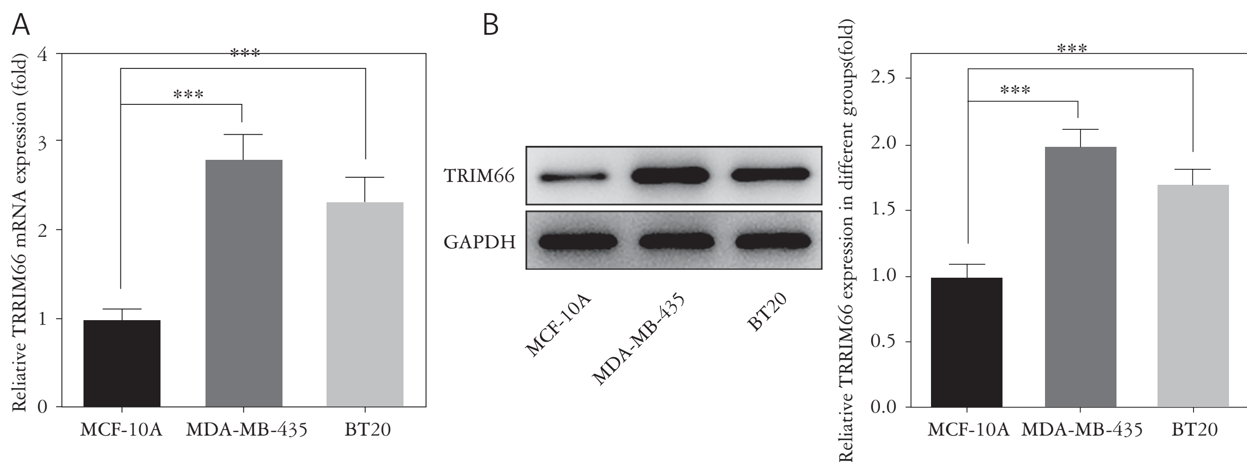


**Fig. 1.** TRIM66 was upregulated in human breast cancer cells. RT-qPCR (A) and western blot (B) was performed to detect TRIM66 expression human normal mammary cells MCF-10A and human breast cancer cell lines MDA-MB-468 and BT20 cell lines. ***P < 0.001 vs. MCF-10A.

antibody for 1h at room temperature. Proteins were then detected using the ECL reagent (Millipore). Data normalizations were performed using Image J v1.46 software.

### Statistical analysis

All experiments were repeated at least 3 times independently and results are expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS 19.0 software (SPSS, Chicago, IL, USA). Student's t-test and one-way ANOVA followed by Tukey test were used to evaluate the statistical significance. $P < 0.05$ was considered to be statistically significant.

## Results

### TRIM66 was upregulated in human breast cancer cells lines

Firstly, the expression of TRIM66 in MDA-MB-468 and BT20 was measured by qRT-PCR and western blot. As shown in Fig. 1A and B, RT-qPCR assay and western blot revealed that TRIM66 was evidently increased in human breast cancer cell lines compared with MCF-10A cells. Considering MDA-MB-468 cells had higher TRIM66 expression among human breast cancer cell lines, we chose MDA-MB-468 cells for further study.

### TRIM66 knockdown suppressed the expression of p-JAK2 and p-STAT3 by regulating EGFR

To investigate the function of TRIM66 in MDA-MB-468 cells, we used siRNA-mediated knockdown of TRIM66 in MDA-MB-468 cells. As presented in Fig. 2A, the transfection efficiency of TRIM66 siRNA-1 and TRIM66 siRNA-2 in MDA-MB-468 cells was verified using RT-qPCR. We observed that TRIM66 was obviously lower in TRIM66 siRNA-transfected MDA-MB-468 cells than in si-NC group, as well as TRIM66 siRNA-1 transfection resulted in a higher knockdown efficiency than TRIM66 siRNA-2 in MDA-MB-468 cells. Therefore, TRIM66 siRNA-1 was chosen for further experiments. Herein, as shown in Fig. 2B, the expression of EGFR in overexpression-EGFR (ov-EGFR) group was significantly higher than that in the control group. Compared with the control group, TRIM66 knockdown significantly decreased the expression of EGFR, JAK2 and STAT3. These above changes could be partially reversed by overexpression of EGFR.

### TRIM66 knockdown suppressed the cell proliferation, invasion and migration of breast cancer cells by regulating EGFR

We further confirmed whether TRIM66 knockdown exerted its function in breast cancer cells by downregulating EGFR. CCK8 assay suggested that TRIM66 knockdown resulted in the proliferation inhibition in MDA-MB-468 cells, while the inhibitory effect of TRIM66 knockdown was abolished when EGFR expression was induced (Fig. 3A). In addition, colony formation assay results also demonstrated that TRIM66 knockdown suppressed the cell proliferation of breast cancer cells by downregulating EGFR.

As shown in Fig. 3C, D, TRIM66 knockdown led to a marked repression of cell migration and invasion of MDA-MB-468 cells while ov-EGFR significantly abolished these effects. Furthermore, TRIM66-depleted MDA-MB-468 cells exhibited significant downregulation of MMP2 and MMP9, whereas ov-EGFR dramatically abrogated these effects, as evidenced by western blotting (Fig. 3E).

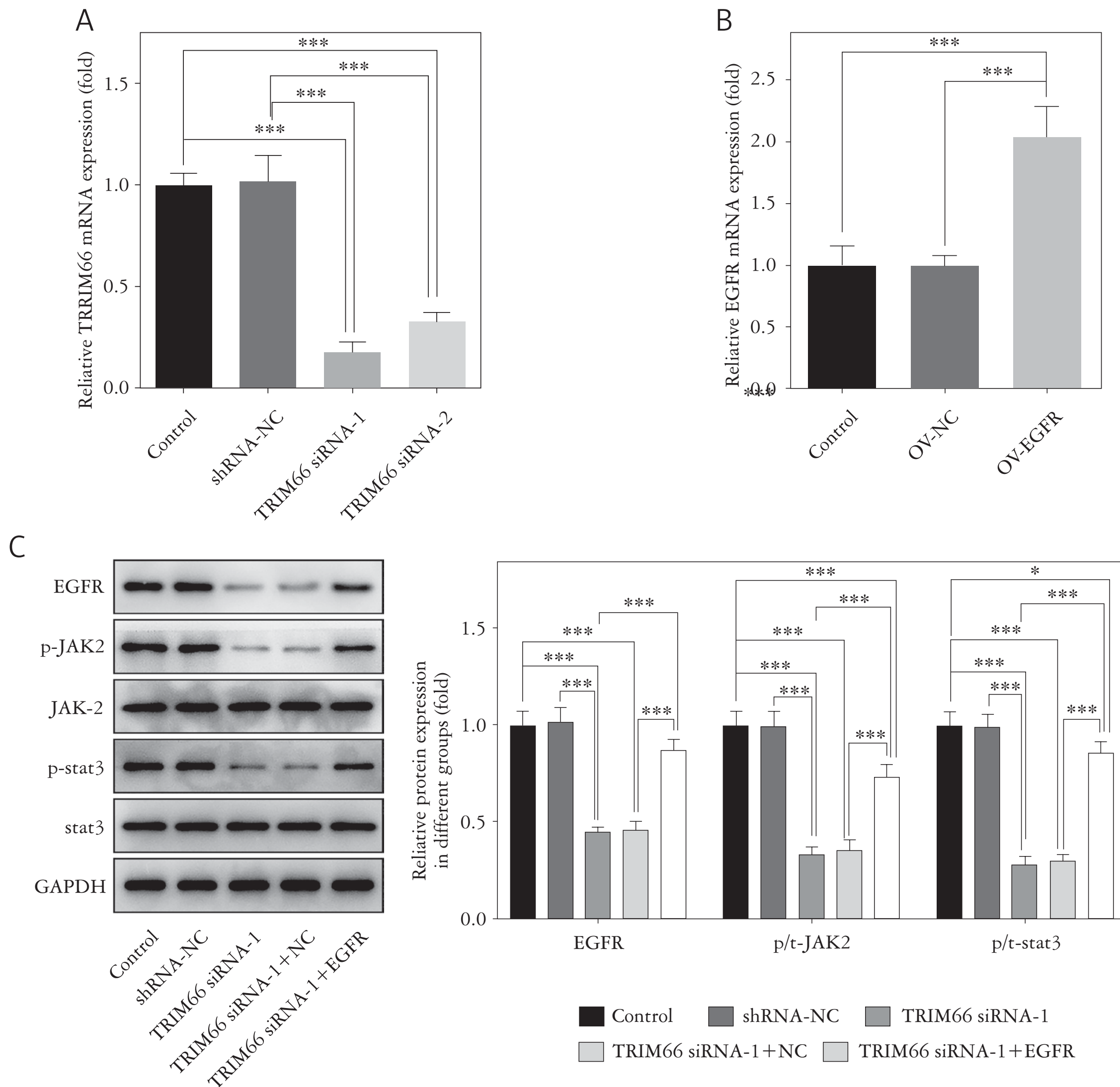


**Fig. 2.** TRIM66 knockdown suppressed the expression of p-JAK2 and p-STAT3 by regulating EGFR. A) MDA-MB-468 cells were transfected with si-NC or TRIM66 siRNA (TRIM66 siRNA-1 and TRIM66 siRNA-2). The level of TRIM66 was detected by RT-qPCR in MDA-MB-468 cells. ***$P < 0.001$ vs. control or si-NC. B) MDA-MB-468 cells were transfected with overexpression-EGFR (ov-EGFR) and overexpression-NC (ov-NC). ***$P < 0.001$ vs. control or ov-NC. C) Protein expression levels of EGFR, JAK2 and STAT3 in MDA-MB-468 cells transfected were evaluated by western blot. *$P < 0.05$ and ***$P < 0.001$ vs. control or si-NC or TRIM66 siRNA-1+NC

All these results demonstrated that TRIM66 knockdown suppressed the cell proliferation, invasion and migration of breast cancer cells by regulating EGFR.

### TRIM66 knockdown promotes the cell apoptosis of breast cancer cells by regulating EGFR

To verify the effect of TRIM66 on the apoptosis of MDA-MB-468 cells, TUNEL staining was performed. We found that the ratio of apoptosis (green represents apoptotic cells) in the TRIM66 siRNA-1 group was higher than that in the si-NC and control group, whereas ov-EGFR reduced cell apoptosis obviously (Fig. 4A). These results were further confirmed by detecting the expression of apoptosis-related proteins. As shown in Fig. 4B, TRIM66 siRNA-1 could upregulate Bax and cleaved-caspase3/caspase3 expressions in the MDA-MB-468 cells, but suppressed the expression of anti-apoptosis protein Bcl-2, these above changes could be partially reversed by overexpression of EGFR. These findings demonstrated that TRIM66 knockdown promotes the cell apoptosis of breast cancer cells by regulating EGFR.

## Discussion

Emerging evidence suggested that the TRIM family is involved in tumorigenesis and tumor development [20, 21, 22, 23]. TRIM66 is an important

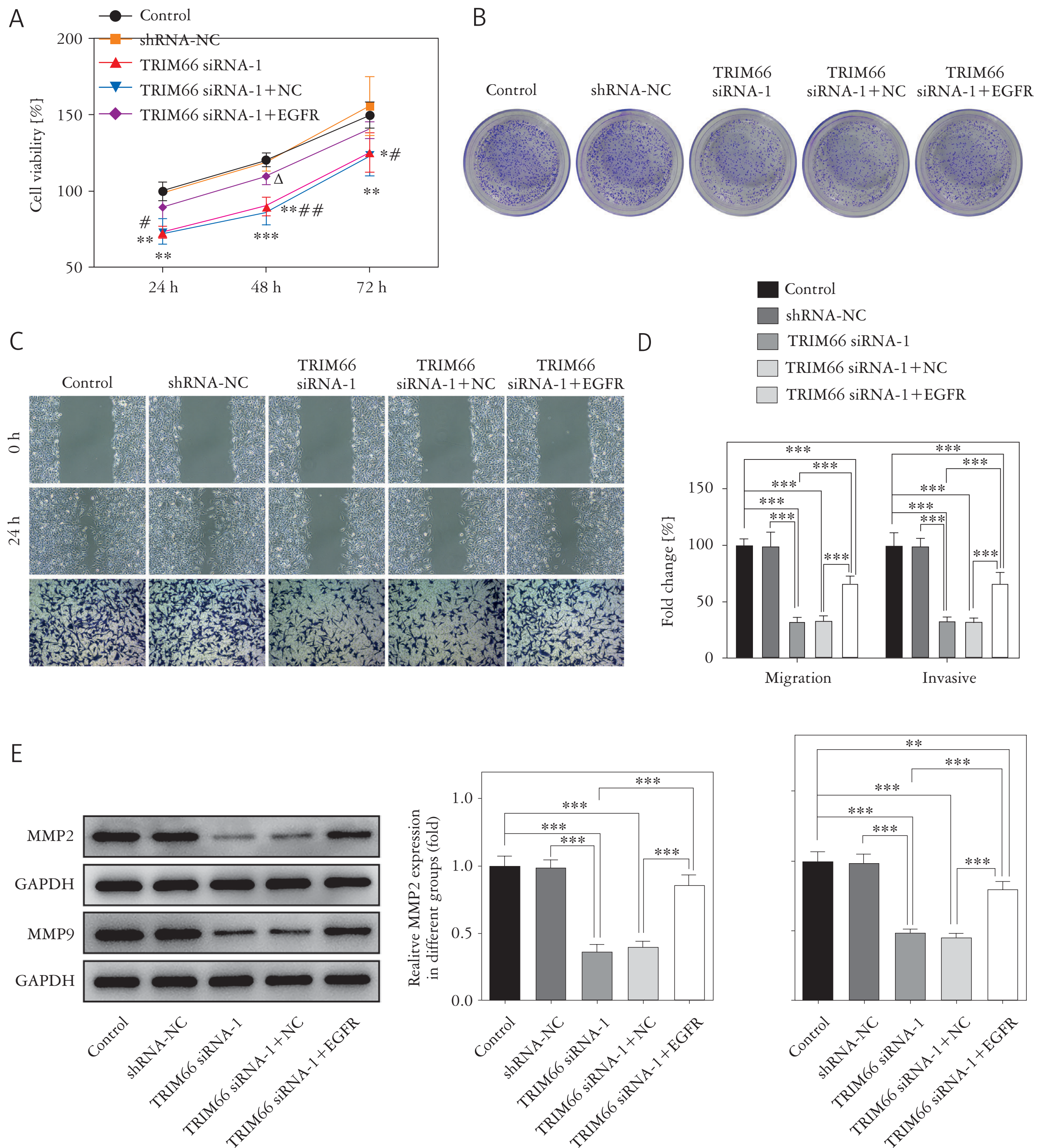


**Fig. 3.** TRIM66 knockdown suppressed the cell proliferation, invasion and migration of breast cancer cells by regulating EGFR. A) CCK8 assay was performed to detect the cell proliferation at 24, 48 and 72 h after transfection in MDA-MB-468 cells. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control; *P < 0.05, **P < 0.01 vs. si-NC; P < 0.05 vs. TRIM66 siRNA-1+NC. B) Colony formation assay was used to measure colony survival rate. C-D) Representative images of Transwell invasion assay of MDA-MB-468 cells transfected with si-NC or TRIM66 siRNA or ov-EGFR or overexpression-NC. ***P < 0.001 vs. control or si-NC or TRIM66 siRNA-1+NC. E) Protein expression levels of MMP2 and MMP9 were evaluated by western blot. **P < 0.01 and ***P < 0.001 vs. control or si-NC or TRIM66 siRNA-1+NC

member of this population and has obvious tissue and cell specificity [11, 12]. TRIM66 is highly expressed in prostate cancer [13]. However, the functions of TRIM66 in TNBC, as well as its involvement in other human diseases are unknown. In the current study, we showed that TRIM66 was overexpressed in human breast cancer cell lines.

Studies have shown that TRIM66 plays an oncogene role in the process of osteosarcoma carcinogenesis by inhibiting apoptosis pathway and promoting TGF-β

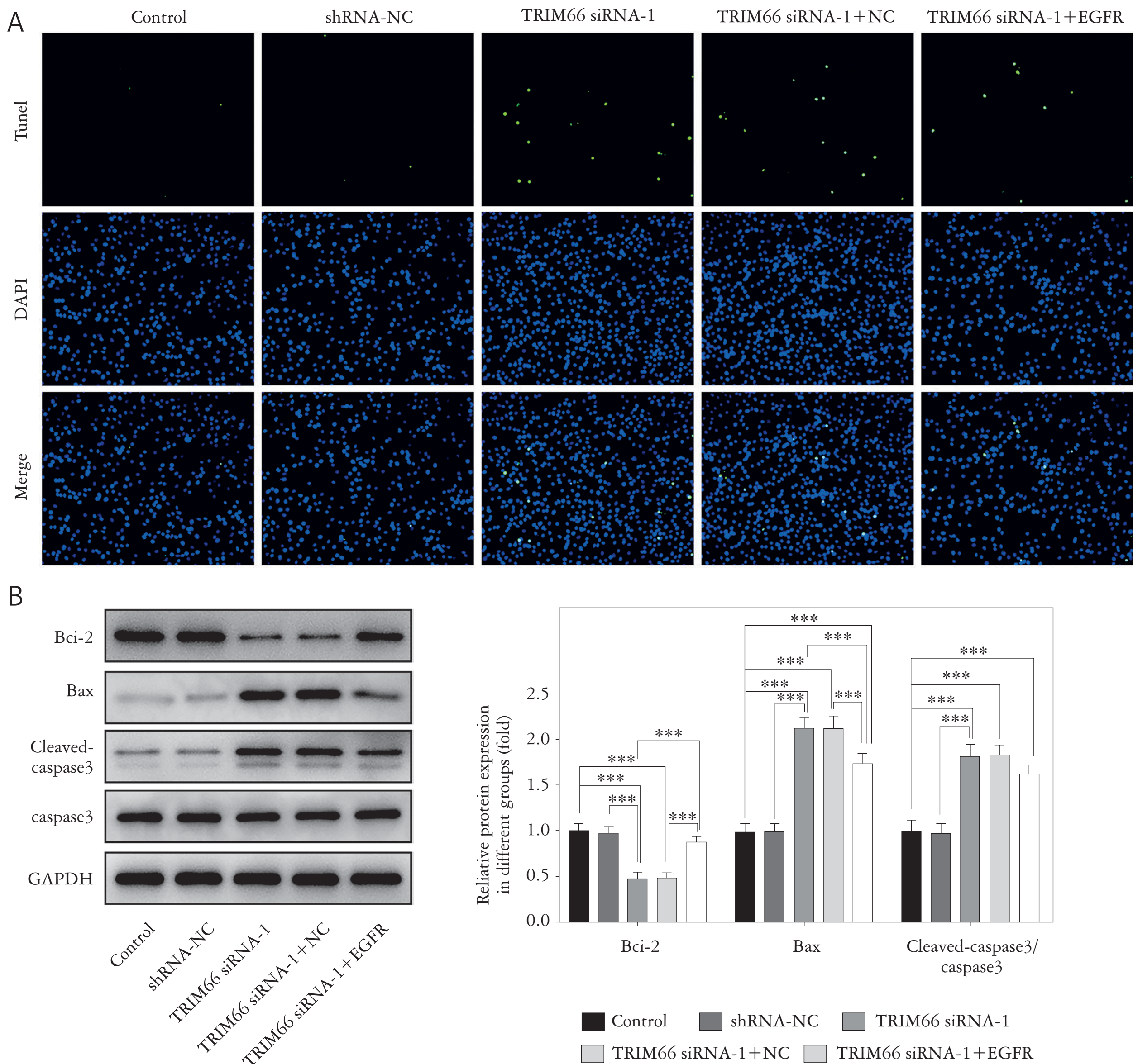


**Fig. 4.** TRIM66 knockdown promotes the cell apoptosis of breast cancer cells by regulating EGFR. A) TUNEL staining was performed the ratio of apoptosis (green represents apoptotic cells). B) Protein expression levels of Bcl-2, Bax and cleaved-caspase3/caspase3 were evaluated by western blot. ***P < 0.001 vs. control or si-NC or TRIM66 siRNA-1+NC

signaling pathway. TRIM66 may be a prognostic factor and a potential therapeutic target for osteosarcoma [11]. In addition, TRIM66 significantly promoted the invasion and migration of hepatocellular carcinoma cells through the EMT pathway, and TRIM66 inhibited E-cadherin through the EMT pathway, promoting the malignant progression of hepatocellular carcinoma [24]. At the same time, other studies demonstrated the oncogenic effect of TRIM66 in prostate cancer cells through the positive regulation of JAK/STAT signals [13]. For colorectal cancer, knockout of TRIM66 can demonstrate anti-tumor activity by inhibiting the JAK2/STAT3 signaling pathway [14]. In the present study, we found that knockdown of TRIM66 inhibited the expression of EGFR, P-Jak2, P-STAT3, JAK2, and STAT3 in MDA-MB-468 cells through regulating the epidermal growth factor receptor (EGFR) signaling.

EGF stimulate EGFR to promote cell migration through the PI3K intracellular pathway [25, 26, 27]. As a downstream signaling molecule of PI3K, Akt can phosphorylate a large number of substrates or regulate gene expression to regulate cell migration, proliferation and apoptosis [28]. Several studies have shown that the PI3K-Akt signaling pathway mediates the migration of cancer cells through the activation of EGF/EGFR or other growth factors/receptors [29]. In the study of the retinal pigment epithelial cell line, it was found that the migration and invasion ability of cells enhanced EGF in a dose-dependent manner, while pretreatment with EGFR, PI3K or AKT

inhibitors inhibited this phenomenon. Studies have shown that EGF/EGFR - PI3K-AKT signaling pathway mediates cell migration [30]. Zhao *et al.* studied the effect on the migration ability of TNBC cells. The results showed that gefitinib could effectively inhibit the phosphorylation of EGFR and its downstream key proteins in a dose-response relationship. At the same time, gefitinib significantly prolonged the healing time of cell scratches, reduced the number of cells invaded by Transwell and inhibited the formation of lamellar liposomes, cytoskeletal remodeling and cytoskeletal polarization. In the present study, knockdown of TRIM66 inhibited proliferation, migration, invasion and promoted apoptosis of breast cancer cells by downregulating EGFR.

## Conclusion

In summary, our findings suggested that TRIM66 was overexpressed in the MDA-MB-468 cell line. Moreover, knockdown of TRIM66 inhibited proliferation, migration, invasion and promoted apoptosis of MDA-MB-468 cancer cells by downregulating EGFR. Our results demonstrated that TRIM66/EGFR may be useful in future studies concerning therapeutic strategies in patients with TNBC.

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*The authors declare no conflict.*

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