Silencing the *COPB2* gene decreases the proliferation, migration and invasion of human triple-negative breast cancer cells

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Abstract. Triple-negative breast cancer (TNBC) is highly invasive, has a high rate of recurrence and is associated with a poor clinical outcome when compared with non-TNBC due to a lack of effective and targeted treatments. The coatomer protein complex subunit β2 (COPB2) is upregulated in various types of malignant cancer. The present study demonstrated that COPB2 expression levels were significantly upregulated in breast carcinoma HS-578T cells (clonal cells originating from TNBC) when compared with non-TNBC MCF-7 cells. HS-578T cells also exhibited higher rates of proliferation, invasion and transendothelial migration when compared with MCF-7 cells. Moreover, it was identified that genetically silencing the *COPB2* gene using a lentivirus-short hairpin RNA inhibited the proliferative, colony formation, migratory and invasive properties of the TNBC HS-578T cells. Mediation of the *COPB2* silencing effect may be associated with regulating the phosphorylation of serine/threonine kinase AKT in the PI3K/AKT signaling pathway. These results suggested the importance of COPB2 in promoting the proliferation of TNBC cells and identified COPB2 as a potential novel therapeutic target.

Introduction

Breast cancer (BC) is a common malignancy in females, with an estimated 2.1 million new cases (11.6%) and 626,679 deaths (6.6%) in 2018 (1,2). Triple-negative BC (TNBC) often develops in young women and is phenotypically defined as the lack of receptors for estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER-2) (3). Cells from TNBC are more invasive, prone to lymph node metastasis and are associated with poor clinical outcomes, when compared with cells from non-TNBC (4-6). TNBC cells respond poorly to endocrine and anti-HER-2 therapeutic strategies, as they lack the receptors targeted by these therapies and, thus, surgical treatment is preferred, followed by postoperative chemotherapy or radiotherapy (2). Ongoing clinical studies are evaluating the efficacy of targeting PI3K and inhibiting EGFR (7), but their efficacies remain unknown.

BC cells invade and migrate to surrounding or distant tissues via the process of epithelial-mesenchymal transition (EMT) (8), which converts cancerous epithelial cells to mesenchymal cells under defined physiologic or pathological conditions (9). EMT is characterized by the decreased expression of epithelial markers, such as E-cadherin (E-Ca) and increased expression of mesenchymal markers, such as N-cadherin (N-Ca) and vimentin (10,11). While being homologous and belonging to the same cadherin superfamily, E-Ca is primarily expressed on epithelial cells and is reported to inhibit cancer cell invasion (12,13), while N-Ca is one of the major cadherins expressed on mesenchymal cells. Therefore, switching expression from E-Ca to N-Ca is a key cellular signature of EMT. Thus, suppressing the expression of N-Ca could potentially decrease the invasion and migration of cancer cells (14). Vimentin is one of the most widely expressed and highly conserved proteins in the type III intermediate filament protein family (15), and is known to promote the invasion and migration of cancer cells. Furthermore, EMT markers are associated with the biological features of tumors, such as the characteristics of the tumor, and its invasive and migratory abilities. Detection of changes in the expression levels of EMT markers could assist in investigating the differences in the biological features and the changes in protein expression levels. This method has been used to evaluate the prognosis of patients with hepatocellular carcinoma, prostate cancer and BC (8,16,17).

The coatomer protein complex subunit β2 *(COPB2)* gene located in chromosome 3q2.3 encodes the 102-kDa nucleoprotein COPB2, which is a member of the 7 protein

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Golgi coatomer complex. COPB2 is essential for budding and vesicular trafficking between the endoplasmic reticulum and Golgi membrane. It is therefore essential in maintaining cellular homeostasis, including the transcriptional regulation and signal transduction of cells (18-22).

Previous studies have revealed that the expression of COPB2 is significantly upregulated in prostate cancer, cholangiocarcinoma, lung cancer and colon cancer cells, and that it enhances proliferation (23-26). Furthermore, COPB2 regulates the proliferation of colon cancer cells via the JNK/c-Jun signaling pathway (26). Moreover, silencing the *COPB2* gene decreases the expression levels of proteins associated with the receptor-tyrosine kinase (RTK) signaling pathway in gastric cancer cells, such as EGFR, HER-2, fms related RTK 3 and phosphorylated (p)-AKT (27).

The role of COPB2 in the pathogenesis of TNBC remains poorly understood. However, circumstantial evidence reported in the abovementioned literature suggests that COPB2 may be a key contributor to the phenotype of TNBC cells and that its action may be mediated via the AKT signaling pathway. The present study hypothesized that the increased expression level of COPB2 in TNBC cells may contribute to the occurrence of EMT, which could promote the migration and invasion capacities of TNBC cells *in vitro*. This effect may be mediated via the AKT signaling pathway.

Materials and methods

Cell lines and culture conditions. The clonal TNBC HS-578T and non-TNBC MCF-7 cell lines (The Type Culture Collection of The Chinese Academy of Sciences) were cultured at 37°C with 5% CO_2 in DMEM (Hyclone; Cytiva) supplemented with 10 and 15% FBS (Biological Industries).

Lentiviral transduction of HS-578T cells. HS-578T cells were seeded in 6-well culture plates ($4x10^4$ cells/well) and incubated at 37°C until 30% confluent. Cells were then divided into three groups: Infection with lentiviral vector (Lv)-short hairpin (sh)RNA COPB2 with a green fluorescent protein (GFP) tag (sh-COPB2 from Shanghai GenePharma Co., Ltd.); infection with empty lentiviruses with a GFP tag (sh-Control; Shanghai GenePharma Co., Ltd.); or no infection. sh-COPB2 (AGA TTAGAGTGTTCAATTA) was inserted into Gv248 lentiviral vectors (Shanghai GenePharma Co., Ltd.) and the cells were infected as previously described (26). Then, 96 h after the final infection, the cells were analyzed for infection efficiency by counting the number of GFP-positive cells under a fluorescence microscope (ECLIPSE 80i; Nikon Corporation).

Immunoblots. HS-578T (three experimental groups) and MCF-7 cells were lysed in a RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing 1 mM PMSF (Applygen Technologies, Inc.). The Cell lysates (60 μg total proteins/lane) were separated via 10% SDS-PAGE and transferred to a PVDF membrane (EMD Millipore). The membrane was blocked with 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 h and then probed with antibodies (1:1,000 dilution) against COPB2 (cat. no. HPA036867; MilliporeSigma),E-Ca(cat.no.3195S;CellSignalingTechnology, Inc.), N-Ca (cat. no. 13116S; Cell Signaling Technology, Inc.),

vimentin (cat. no. 5741S; Cell Signaling Technology, Inc.), p-AKT (cat. no. 4060T; Cell Signaling Technology, Inc.), AKT (cat. no. 4685S; Cell Signaling Technology, Inc.) and AKT agonist SC79 (cat. no. HY-18749; MedChemExpress). GAPDH (cat. no. YM3040; ImmunoWay Biotechnology Company) served as the protein loading control and was probed using a monoclonal antibody. ImageJ 1.51j8 software (National Institutes of Health) was used to analyze the density of the bands.

MTT cell proliferation assay. HS-578T and MCF-7 cells were seeded at a cell density of $2x10^3$ cells/well in 96-well plates and cultured at 37°C with 5% CO_2. Cell densities were analyzed on day 1, 2, 3, 4 and 5 after seeding. At each time point, the cells were incubated with 20 μl MTT solution for 4 h at 37°C, and then cells were treated with 150 μl DMSO with constant agitation. After a 10-min incubation at room temperature, the supernatant was collected and MTT signals at an optical density (OD) of 490 nm were quantitatively detected using a microplate reader (Bio-Rad Laboratories, Inc.).

Colony formation assay. HS-578T and MCF-7 cells were seeded in 6-well plates at a density of $1x10^3$ cells/well and cultured at 37°C with 5% CO_2 for 10 days. Cells were then stained for 10 min with crystal violet and fixed with 4% paraformaldehyde for 30 min at room temperature. The number of cell colonies was counted, and images were captured under a light microscope.

Cellular migration and invasion assay. Cell migration and invasion assays were performed using a 24-well Transwell chamber (pore size, 8 μm; Corning, Inc.) as previously described (26). For the invasion assay, the upper chambers were coated with 40 μl Matrigel (BD Biosciences) diluted in DMEM for 30 min at 37°C. HS-578T and MCF-7 cells were cultured in the upper chamber at a density of $2x10^5$ cells/ml (200 or 160 μl/well for migration and invasion assays, respectively). The upper chambers were submerged into the lower chamber containing 15% FBS-supplemented medium (500 μl). After 24 and 48 h in culture at 37°C (5% CO_2), non-migrated cells that remained in the upper chambers were removed using a cotton swab. The migrated or invaded cells on the opposite side of the membrane were stained with 0.1% crystal violet for 10 min at room temperature, and counted under an ECLIPSE 80i fluorescence microscope.

Statistical analysis. The dataset regarding *COPB2* gene expression and histological grade of BC was obtained from The Cancer Genome Atlas (TCGA) database. The differential expression analysis was performed using the R package, DESeq2 (https://www.bioconductor.org/packages/devel/bioc/html/DESeq2.html). Quantitative data from multiple independent experiments were expressed as the means ± SD. The quantitative data were analyzed using Student's unpaired t-test to compare between two groups. To compare the data of three or more groups, one-way ANOVA and Tukey's post hoc test was used. All statistical analysis was performed using GraphPad Prism 8.0 statistical software (GraphPad Software, Inc.) and SPSS 19.0 software (IBM Corp.) and $P<0.05$ was considered to indicate a statistically significant difference.
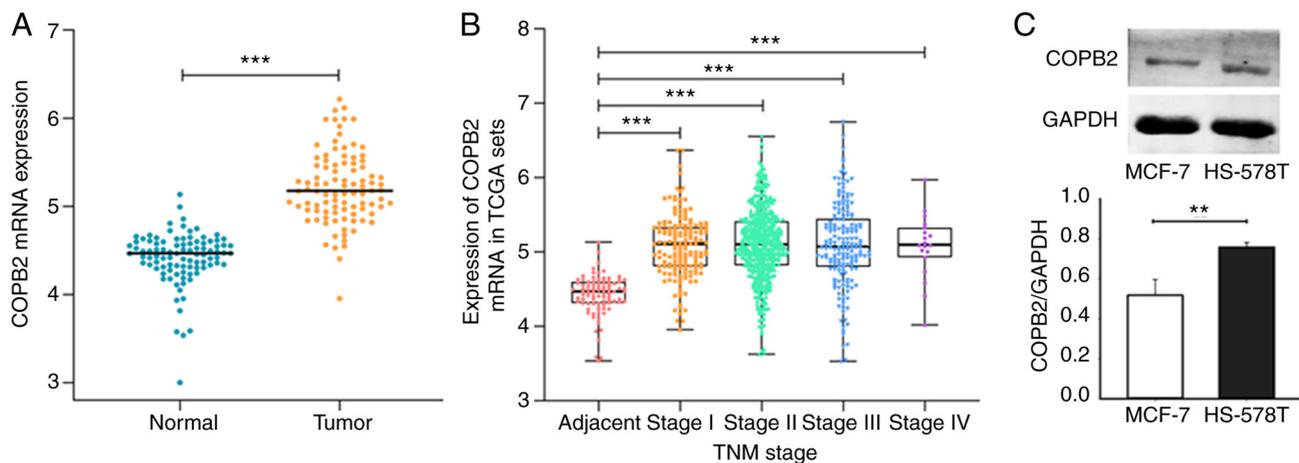
Figure 1. COPB2 expression in breast cancer. COPB2 mRNA expression between (A) breast cancer and paracancerous tissue samples (n=110) and among (B) breast cancer at different pathological stages (n=446) using data from TCGA database. ***P<0.001. (C) COPB2 protein expression was detected in TNBC HS-578T and non-TNBC MCF-7 cells using immunoblots and was semi-quantified using densitometry (n=3). **P<0.01. TCGA, The Cancer Genome Atlas; COPB2, coatomer protein complex subunit β2; TNBC, triple-negative breast cancer.

Results

COPB2 mRNA is upregulated in TNBC. COPB2 mRNA expression was evaluated using the data of 110 patients with BC obtained from TCGA database. It was found that mRNA expression levels of *COPB2* in BC tissue were significantly higher when compared with those in paracancerous tissue (Fig. 1A). Furthermore, the *COPB2* mRNA expression level progressively increased in line with the pathological stage of the cancer (Fig. 1B). As the TNBC status was not provided for patients in TCGA database, COPB2 expression was analyzed in the cultured TNBC-derived HS-578T cells and the non-TNBC-derived MCF-cells. The HS-578T cells expressed more COPB2 protein when compared with the non-TNBC MCF-7 cells (Fig. 1C). These results suggest that COPB2 expression was increased in all BC cells, but the increase was significantly greater in TNBC cells. Thus, differential COPB2 expression may contribute to the distinct phenotypes between TNBC and non-TNBC cells.

HS-578T and MCF-7 cells exhibit differential proliferative rates. HS-578T cells demonstrated a greater rate of proliferation in culture when compared with MCF-7 cells, as detected by the MTT assay (Fig. 2A). Moreover, HS-578T cells formed significantly more cellular colonies (Fig. 2B). Using the Transwell chamber system as previously described (26), it was identified that HS-578T cells invaded and transmigrated through the extracellular matrix of the Matrigel significantly faster than the MCF-7 cells (Fig. 2C and D).

To investigate the regulatory pathways responsible for the distinct phenotypes of HS-578T and MCF-7 cells, the expression levels of factors involved in EMT were detected. The results demonstrated that epithelial E-Ca was predominantly expressed in MCF-7 cells, whereas the homologous mesenchymal N-Ca was mostly detected in HS-578T cells (Fig. 2E). The cytoskeletal protein vimentin, another EMT marker, was also increased in HS-578T cells. These data indicated that the TNBC HS-578T and non-TNBC MCF-7 cells had different proliferative potentials that were likely determined by their EMT states.

Silencing COPB2 decreases the proliferation of HS-578T cells. As high COPB2 expression was detected in tissue samples collected from patients with BC, and differential expressions of COPB2 was observed in the clonal TNBC HS-578T and non-TNBC MCF-7 cells (Fig. 1); the role of COPB2 in the formation of TNBC was subsequently investigated. The present study examined the phenotypic changes of the TNBC HS-578T cells after the *COPB2* gene was silenced using a lentivirus as the carrier, which infected >80% of cells (Fig. 3A; upper panel) without inducing significant cell death or detachment (Fig. 3A; lower panel). When infected with the Lv-sh-COPB2 lentivirus, COPB2 expression was decreased by 90%, whereas the control lentivirus achieved a similar infection efficiency, but did not downregulate COPB2 expression (Fig. 3B).

Using these techniques, it was found that HS-578T cells infected with the Lv-sh-COPB2 lentivirus had a significantly decreased rate of proliferation when compared with parental cells and the cells infected with the sh-Control lentivirus (Fig. 3C). The inhibition of proliferation was detected primarily on days 3, 4 and 5 after infection. Consistent with the results from the MTT assay, colony formation was also reduced in the HS-578T cells infected with Lv-sh-COPB2 lentivirus (Fig. 3D).

The ability of HS-578T cells to invade and migrate via the subendothelial matrix was reduced after infection with Lv-sh-COPB2, but this was not observed in the control lentivirus group (Fig. 4A and B). These data suggest that silencing COPB2 expression decreased the proliferation of the TNBC HS-578T cells. Moreover, the expression levels of vimentin and N-Ca were decreased in HS-578T cells when the *COPB2* gene was silenced, while E-Ca expression (which was low in HS-578T cells, as shown in Fig. 2E) and the quantitative analysis remained unchanged (with the parental cell serving as a baseline; Fig. 4C). These results suggest that silencing COPB2 altered the EMT status of HS-578T cells, leading to changes in the proliferative characteristics of these cells.

COPB2 silencing alters AKT signaling in HS-578T cells. The AKT signaling pathway is a major pathway that regulates the proliferation, migration and invasion of cancer cells (28). It
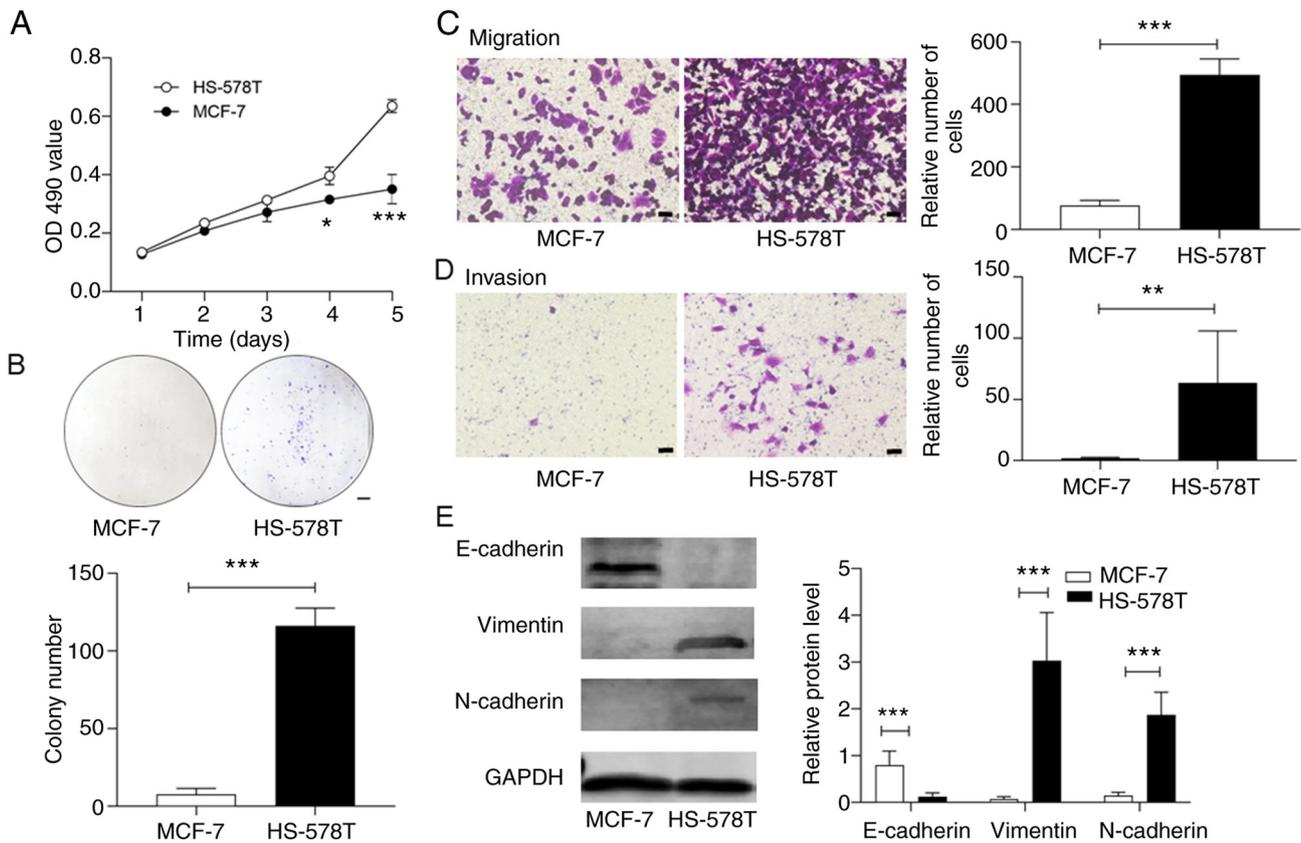
Figure 2. Proliferation, migration and invasion of MCF-7 and HS-578T cells. (A) Proliferation of HS-578T and MCF-7 cells was detected using an MTT assay (n=3). ***P<0.001 and *P<0.05 vs. HS-578T. (B) Colony formation of HS-578T and MCF-7 cells was quantified by counting the number of colonies in five randomly selected fields of view (magnification, x10; n=5). ***P<0.001. (C) Migration and (D) invasion of HS-578T and MCF-7 cells were detected using a Transwell chamber assay. Representative images of cells stained with crystal violet (scale bar, 20 μm). The graphs demonstrate the numbers of transmigrated and invaded cells (n=3). ***P<0.001 and **P<0.01. (E) Protein expression levels of E-Ca, vimentin and N-Ca in HS-578T and MCF-7 cells, as determined by immunoblots and semi-quantification using densitometry (n=3). ***P<0.001. N-Ca, N-cadherin; E-Ca, E-cadherin.

was identified that silencing the *COPB2* gene significantly decreased the phosphorylation of AKT (Fig. 5A). The AKT agonist, SC79 (5 μg/ml) served as a control, and increased the rate of AKT phosphorylation (Fig. 5B) without significantly changing the expression of COPB2 (Fig. 5C). SC79 also promoted the invasion and migration of HS-578T cells, as detected in the Transwell assay (Fig. 5D and E). These results indicated that COPB2 may regulate AKT signaling to alter the proliferative rate of HS-578T cells.

Discussion

Tumor targeting therapy is increasingly recognized as an effective method of improving the efficacy and reducing the cytotoxicity of anti-cancer drugs (2). As TNBC cells lack receptors targeted by anti-HER-2 receptor, and anti-ER and anti-PR drugs, patients with TNBC have fewer therapeutic options than those with non-TNBC. Therefore, developing novel targeted therapeutic strategies for TNBC is highly desirable. The present study identified a key role of COPB2 in the EMT transition of TNBC cells.

In the present study, it was found that BC tissue samples from patients and clonal cells in culture expressed high levels of COPB2. These findings differ from a previous study, which observed that the expression level

of COPB2 was decreased in cervical cancer cells (29). However, the current findings were similar to a study by Bhandari *et al* (30) who identified that COPB2 was upregulated in BC. Thus, it was suggested that the expression of COPB2 in BC is an important prognostic (30). The present study also found that the TNBC cells expressed COPB2 at a level significantly higher than the non-TNBC MCF-7 cells, indicating that COPB2 expression was associated with TNBC. Notably, high COPB2 expression levels resulted in greater rates of proliferation, migration and invasion of the HS-578T cells in culture, when compared with the MCF-7 cells, and silencing COPB2 decreased the rate of EMT transformation. The current findings are consistent with the EMT state of HS-578T cells, showing a transition from expressing epithelial E-Ca to expressing mesenchymal N-Ca and vimentin. Moreover, these findings are consistent with a previous study, that HS-578T cells are deficient in E-Ca expression and that MCF-7 cells lack vimentin and N-Ca expression (31).

The present study used lentiviruses carrying a COPB2 inhibitory RNA sequence to decrease COPB2 transcription. Using this approach, the present study was able to silence COPB2 expression by >90% without affecting cell survival. Furthermore, silencing the *COPB2* gene decreased the proliferative, invasive and migratory rates of HS-578T cells
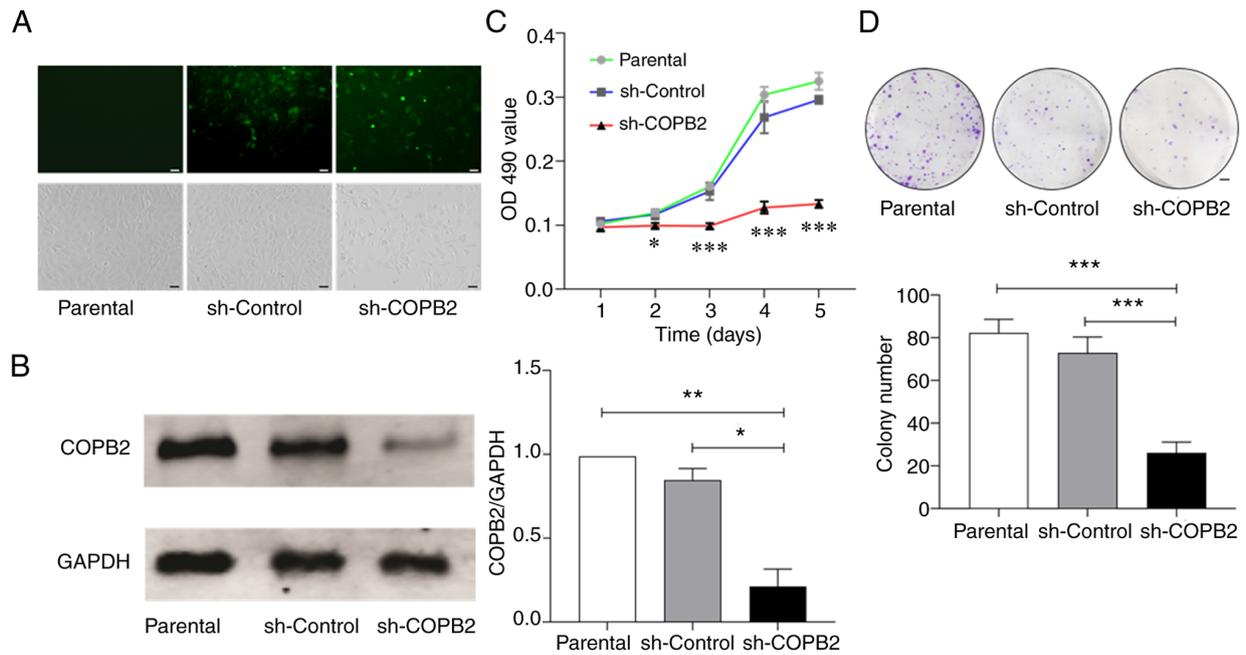
Figure 3. Impact of silencing the *COPB2* gene. (A) Representative images of HS-578T cells that were not infected (parental) or were infected with either control (sh-Control) or shRNA COPB2 lentivirus (sh-COPB2). Fluorescence due to infection is presented in the upper panel and phase contrast images of the cells are presented in the lower panel (scale bar, 20 μm). (B) Protein expressions levels of COPB2 and GAPDH (control) in HS-578T cells that were not infected (parental) or were infected with either control (sh-Control) or shRNA COPB2 lentivirus (sh-COPB2). Semi-quantitative data of the protein expression levels from three independent experiments. ***$P<0.001$, **$P<0.01$ and *$P<0.05$. (C) Proliferation of HS-578T cells after silencing the *COPB2* gene was detected using an MTT assay (n=3). ***$P<0.001$ and *$P<0.05$ vs. parental cells. (D) Colony formation of HS-578T cells after silencing the *COPB2* gene as demonstrated by representative images of the assay and quantitative data from three independent experiments. ***$P<0.001$. COPB2, coatomer protein complex subunit β2; sh, short hairpin RNA.
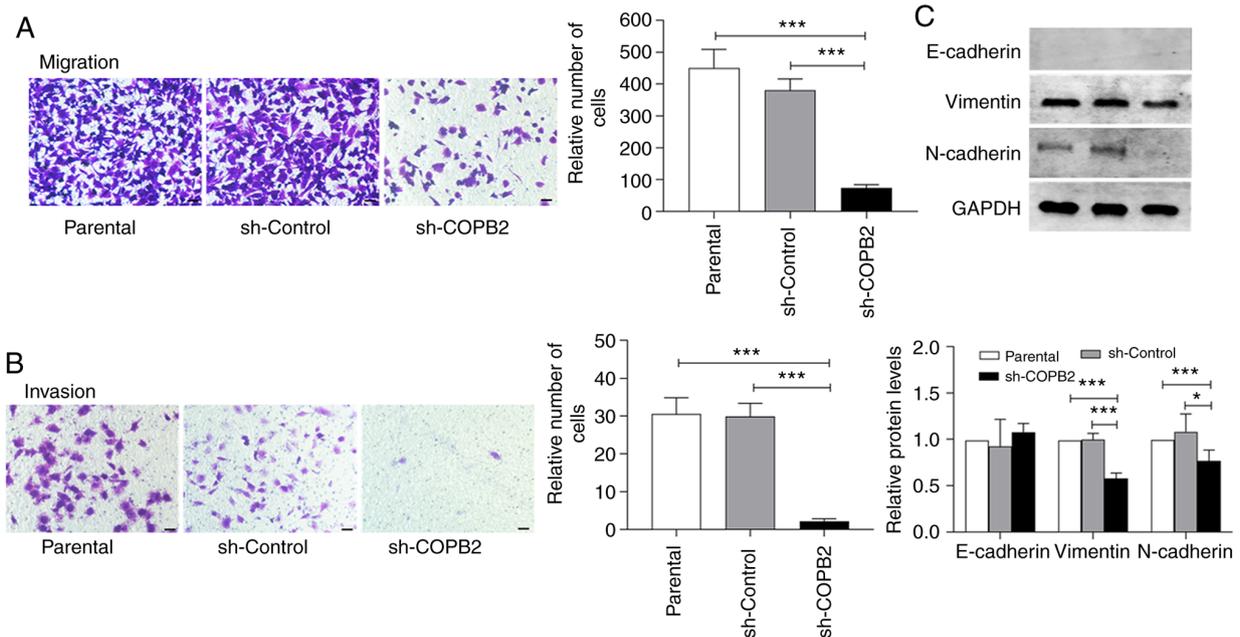

Figure 4. Effects of silencing the *COPB2* gene on cell migration. Representative images (scale bar, 20 μm) and quantitative data from three independent experiments of (A) cell migration and (B) cell invasion as detected by Transwell chamber assay with cells stained using crystal violet. ***$P<0.001$. (C) Expression levels of E-Ca, vimentin and N-Ca of HS-578T cells after silencing the *COPB2* gene were determined via immunoblots. The four panels are representative images of the immunoblots and semi-quantitative densitometry data was obtained from three independent experiments. The protein expression of the parental cells was set as the baseline (set to 1). ***$P<0.001$ and *$P<0.05$. COPB2, coatomer protein complex subunit β2; N-Ca, N-cadherin; E-Ca, E-cadherin.

in vitro. These functional effects of COPB2 silencing appeared to be mediated via downregulation of the EMT proteins, N-Ca and vimentin, with a minimal impact on E-Ca expression.

These *in vitro* findings justify the requirement for further investigation into the potential of gene therapies targeting COPB2.
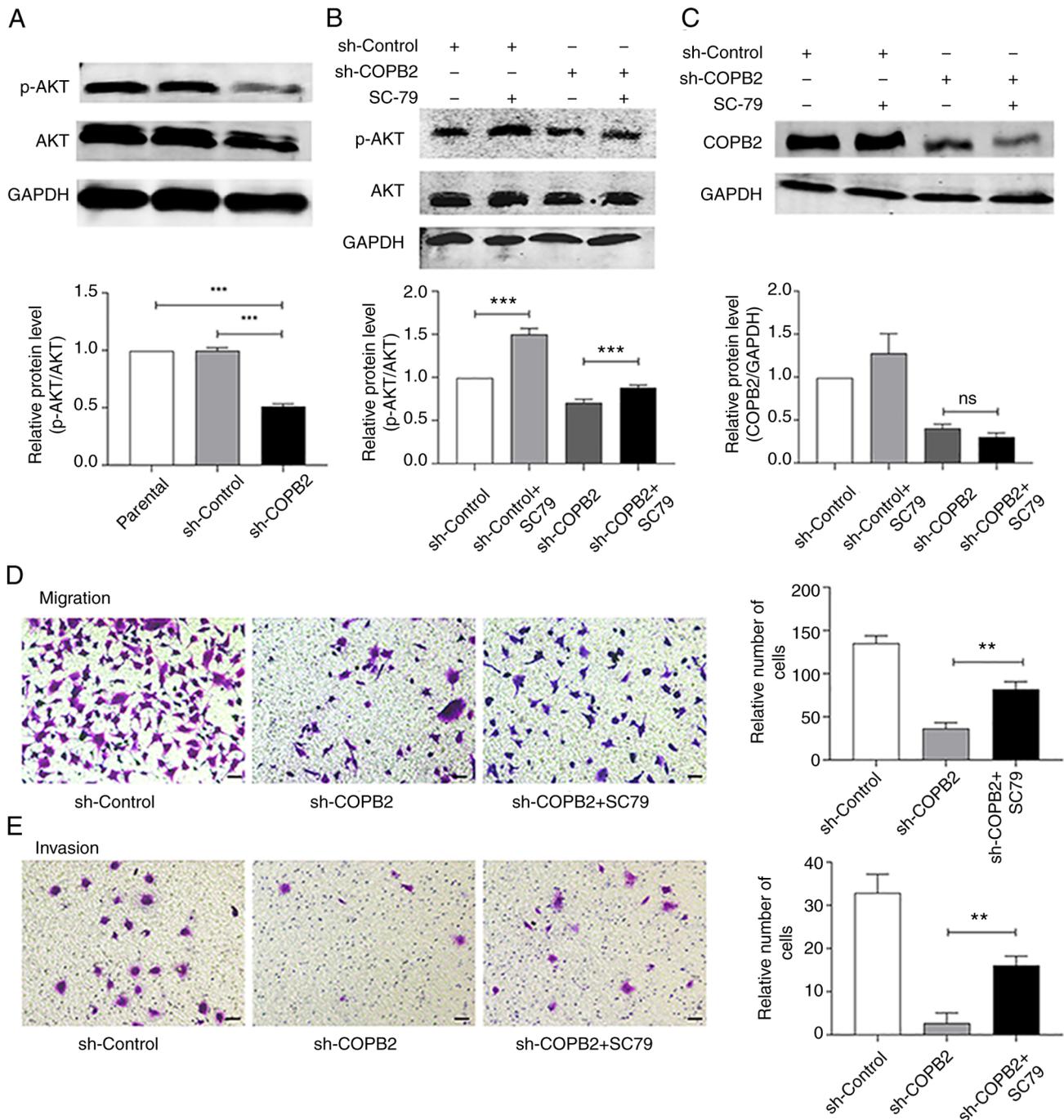
Figure 5. Effect of COPB2 on AKT. (A) Representative images and semi-quantitative densitometry data from three independent experiments of AKT phosphorylation in HS-578T cells after silencing the *COPB2* gene. ***P<0.001. (B) Representative images and quantitative densitometry from three independent experiments of AKT phosphorylation after HS-578T cells were treated with 5 µg/ml SC79. ***P<0.001. (C) Representative blots and quantitative densitometry from three independent experiments to demonstrate COPB2 expression in HS-578T cells stimulated with 5 µg/ml SC79. (D) Migration and (E) invasion of HS-578T cells infected with inhibitory lentiviruses and stimulated with SC79, as shown by staining with crystal violet (scale bar, 20 µm; n=3) with results presented from three independent experiments. **P<0.01. COPB2, coatomer protein complex subunit β2; p, phosphorylated; ns, not significant.

The present study demonstrated that the levels of AKT phosphorylation were elevated in HS-578T cells and were reduced by COPB2 silencing. In reciprocal experiments, the AKT agonist, SC79 increased the migration and invasion of HS-578T cells. These observations are consistent with the proposal that the AKT signaling pathway promotes EMT transition and the proliferation of cancer cells (32,33). The present results are also consistent with those of various previous studies (34-36). For example, AKT phosphorylation

is downregulated after silencing COPB2 in gastric cancer cells (27). The AKT agonist, SC79 only partially increased the migration and invasion of HS-578T cells to a level similar to that before silencing, suggesting that COPB2 may also regulate cell invasion and migration via other signaling pathways, such as the RTK signaling pathway and the inflammatory immune-related pathway (27,37). Furthermore, SC79 increased AKT phosphorylation, but did not change the expression of COPB2, indicating that AKT phosphorylation is a downstream

event that COPB2 regulates. However, this experimental study was only validated in triple-negative breast cancer HS-578T cells, therefore subsequent experiments are required to further verify this in the pathogenesis of BC. Additionally, the correlation between the effect of COPB2 and the AKT signaling pathway requires further investigation in the future.

Thus, the present study demonstrated that COPB2 expression was upregulated in BC cells and that the increase was greater in TNBC cells when compared with non-TNBC cells. Furthermore, higher levels of COPB2 expression enhance the proliferation of TNBC cells. However, when COPB2 was silenced, EMT transition was blocked and the proliferation of TNBC cells was decreased. These findings suggested that COPB2 may be involved in the clinical progression of TNBC and its underlying mechanism may be associated with upregulation of the AKT signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ML and FZ participated in conceiving the study, the design of the experiments, interpretation of the results and drafting of the manuscript. ML and FZ confirmed the authenticity of all the raw data. WW performed the experiment, data analysis and wrote part of the manuscript. CW contributed to data analysis, wrote part of the manuscript and prepared the figures. FW, YW, YJ, JL, MW, CZ and SW collected the majority of the data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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