Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Ambra1 Deficiency Inhibits the Proliferation of Breast Cancer Cells Through the Akt-FoxO1-p27 Pathway

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Purpose: The unlimited proliferation of breast cancer (BC) cells is the basis for recurrence and metastasis. Ambra1 is involved in the regulation of cell proliferation, but its role may be cancer type-dependent, and the underlying mechanisms need further exploration. In addition, it remains unclear whether Ambra1 is involved in regulating the proliferation of BC cells. This study aims to explore the regulatory effect of Ambra1 on the proliferation of BC cells, as well as the underlying mechanisms.

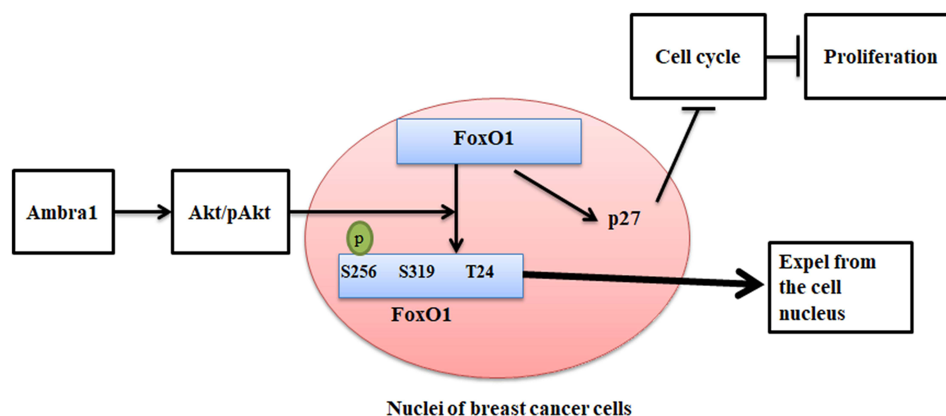
Methods: The effects of Ambra1 on cell proliferation were detected in MCF-7 and MDA-MB-231 cells using CCK-8, EdU, and colony formation assays. The role of Ambra1 in regulating p27 via the Akt-FoxO1 pathway was determined in MCF-7, MDA-MB-231, and 293T cells through Western blotting, qRT-PCR, and co-immunoprecipitation. Subsequently, the role of p27 in Ambra1-mediated regulation of cell proliferation was validated in cell models and xenograft mouse models.

Results: Ambra1 deficiency significantly inhibited the proliferation of BC cells. p27 played a crucial role in this process. Furthermore, Ambra1 regulates the phosphorylation of the Ser256 residue of FoxO1 through Akt, thereby altering the nuclear distribution of FoxO1 and the transcription of p27.

Conclusion: Ambra1 can control the proliferation of BC cells by regulating the Akt-FoxO1-p27 signaling pathway. Therefore, this protein is a potential therapeutic target for BC.

Keywords: breast cancer, Ambra1, proliferation, p27, FoxO1, Akt

Graphical Abstract



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Introduction

Breast cancer (BC) remains the most frequently diagnosed cancer and the leading cause of cancer-related mortality among women worldwide, presenting a substantial risk to women's health.[1] Approximately 3% to 10% of newly diagnosed patients already have distant metastases at the time of diagnosis.[2] Even among patients initially diagnosed with early-stage BC, approximately 30% experience recurrence and metastasis after receiving treatment.[2] Recurrence and metastasis are the main causes of treatment failure in patients with BC. Indeed, the continuous proliferative capacity of cancer cells is a critical factor contributing to cancer recurrence and metastasis.[3–5]

Autophagy and Beclin 1 Regulator 1 (Ambra1) was originally identified as an autophagy protein essential for autophagosome formation.[6] In addition to its role in autophagy, Ambra1 functions as a versatile scaffold protein, characterized by intrinsically disordered regions that enable it to interact with various cellular factors and participate in multiple cellular processes and pathological processes of cancer, such as apoptosis, cell cycle, cell proliferation, epithelial-mesenchymal transition (EMT), regulatory T-cell differentiation, and the tumor immune microenvironment.[7–19] Recently, it has been discovered that Ambra1 is a key factor in the degradation of D-type cyclins (cyclin D1, cyclin D2, cyclin D3) in cells.[13–17] Cyclin Ds are core regulatory factor in the cell cycle process, which can directly regulate the activity of the cyclin D-cyclin dependent kinase 4/6 (CDK4/6)-retinoblastoma protein (Rb) axis and control the transition of the cell cycle from G1 phase to S phase.[20,21] In the absence of Ambra1, cyclin Ds accumulate in cells, accelerating the cell cycle process and promoting cell proliferation.[13–15] In addition, Ambra1 can also inhibit the proliferation of mouse embryonic fibroblasts (MEFs) by promoting the dephosphorylation and degradation of the c-Myc protein.[22] However, its function differs across various types of cancer cells. In gastric adenocarcinoma cells, the deficiency of Ambra1 leads to a decrease in the expression of cyclin D1, accompanied by the inhibition of cell proliferation.[23] This suggests that the function of Ambra1 may vary across different types of cancer cells. In addition, it is still unclear whether Ambra1 is involved in the regulation of the proliferation of BC cells.

p27, encoded by the *CDKN1B* gene, plays a crucial role in controlling cell cycle progression and cell proliferation.[24–29] It is a target gene of FoxO1.[30–33] We previously confirmed that Ambra1 is highly expressed in breast cancer and regulates the transcriptional activity of FoxO1 through the Akt signaling pathway.[34,35] Thus, Ambra1 may regulate the expression of p27 through the regulation of FoxO1, thereby controlling the proliferation of BC cells. To verify this hypothesis, we used the BC cell lines MCF-7 and MDA-MB-231 and 293T cells as models to explore the interrelationships among Ambra1, Akt, FoxO1, and p27, as well as their effects on cell proliferation, and verified these findings in a xenograft tumor model in nude mice. We confirmed that Ambra1 controls the proliferation of BC cells by regulating the Akt-FoxO1-p27 signaling pathway.

Materials and Methods

Cell Lines and Culture

The human BC cell lines MCF-7 (TCHu74) and MDA-MB-231 (TCHu227) and 293T (SCSP-502) cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). MCF-7 and 293T cells were cultured in MEM (Thermo Fisher, Waltham, MA, USA), and MDA-MB-231 cells were cultured in L15 media (Thermo Fisher, Waltham, MA, USA). All culture media were supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher, Waltham, MA, USA) and 1% penicillin/streptomycin. The cell lines were maintained at 37°C in a humidified incubator with 5% CO_2. Before the study, the cells were passaged for 6 generations. The identity of the cell lines was confirmed by short tandem repeat (STR) profiling.

Agents, Assay Kits and Antibodies

GSK-690693 (HY-10249, GSK, used at a concentration of 1 nM) and cycloheximide (HY-12320, CHX, used at a concentration of 50 μg/mL) were purchased from MedChem Express (MCE, Princeton, NJ, USA). The cell counting kit-8 (CCK-8, C0038) and BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488 (EdU, C0071S) were obtained from the Beyotime Institute of Biotechnology (Shanghai, China). Anti-Ambra1 (ab223351), anti-p27 (ab32034), and

anti-Histone H3 (ab4441) antibodies were purchased from Abcam (Hong Kong, China). Anti-FoxO1 (2880), anti-pFoxO1^{S256} (9461), anti-pFoxO1^{T24} (9464) and anti-pFoxO1^{S319} (2486) antibodies were purchased from Cell Signaling Technology (Danvers, USA). The anti-FLAG antibody (F1804) was purchased from Sigma. Anti-GAPDH antibody (AP0063) was purchased from Bioworld Technology, Inc. (Bloomington, USA).

Lentiviral Vector and siRNA Construction and Transfection

A lentiviral vector-*AMBRA1* transfected with full-length human *AMBRA1* cDNA (LV-*AMBRA1*) and an empty vector (Empty, as a control) were constructed by GeneChem (Shanghai, China) using the HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper, R212-01), with RNA dosages of 500 ng. The 3 × Flag and 3 × Flag-*AMBRA1* lentiviral vectors were also constructed by GeneChem. Three target-specific *AMBRA1* shRNAs (sh*AMBRA1*-1, sh*AMBRA1*-2 and sh*AMBRA1*-3), a target-specific *CDKN1B* shRNA and a control scrambled shRNA (scramble) were synthesized by GenePharma (Shanghai, China). A target-specific *CDKN1B* siRNA, a target-specific *FOXO1* siRNA and control scrambled siRNA (siCtrl), were synthesized by GenePharma (Shanghai, China). The sequences of sh*AMBRA1*-1, sh*AMBRA1*-2 and sh*AMBRA1*-3 were GTC CAC GCT CTA CCT TCT TAT, CCA TAG ATG GAA CAG AAT TAT, and AGG CCA CTG GGA AAG AAT TTA, respectively. The sequence of sh*CDKN1B* was CAG AAG ACG UCA AAC GUA AAC, and the sequence of the scramble sequence was TTC TCC GAA CGT GTC ACG T. The sequence of si*CDKN1B* was the same as that of sh*CDKN1B*. The sequence of si*FOXO1* was ATG GTT CTA ATT TCC AGA TAA and the sequence of siCtrl was TTC TCC GAA CGT GTC ACG T. The siRNAs were transfected into cells using Lipofectamine™ 2000 (Invitrogen, California, USA) according to the manufacturer's instructions. Subsequent experiments were conducted 48 h after the transient transfection with shRNAs or siRNAs.

Real-Time Quantitative PCR (qRT–PCR)

Total RNA was extracted via TRIzol reagent (Generay Biotech, China) as instructed by the supplier. The extracted RNA was then reverse-transcribed into cDNA with a reverse transcription kit (Vazyme Biotech, China) according to the manufacturer's recommendations. cDNA expression levels were quantitatively assessed via real-time qPCR on an ABI 7500 system (Applied Biosystems, USA) using the ChamQ SYBR Color qPCR Master Mix (Vazyme Biotech, China). The sequences of primers used for amplification were as follows: *AMBRA1*-Forward: ACC CAG ACC CAG CGA GAT TA; *AMBRA1*-Reverse: TCT GTT GGT AGC GCA TGG AG; *CDKN1B*-Forward: AAC GTG CGA GTG TCTA AC GG; *CDKN1B*-Reverse: CCC TCT AGG GGT TTG TGA TTC T; *GAPDH*-Forward: CTG ACT TCA ACA GCG ACA CC; and *GAPDH*-Reverse: GTG GTC CAG GGG TCT TAC TC. The data were normalized to *GAPDH* expression.

Western Blotting

For Western blotting, cells were seeded in 25 cm^2 tissue culture flasks and were allowed to reach approximately 80% confluency in fresh medium before treatment with the agents. After treatment, detached and attached cells were collected by centrifugation. Subsequently, whole-cell lysates were obtained using a lysis buffer (1× Phosphate-Buffered Saline, pH 7.6, containing 1% NP-40, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate, with the addition of inhibitor cocktails); alternatively, nuclear proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology Inc., P0027) according to the manufacturer's instructions. Approximately 30~50 μg of total protein from each group was electrophoretically separated on 12% or 15% SDS–PAGE gels and electrotransferred to polyvinylidene fluoride membranes (PVDF) membranes (Pierce, Rockford, USA). The PVDF membranes were blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 20 (TBST, pH 7.6) for 1 hour at room temperature, incubated with primary antibodies diluted in 5% nonfat dry milk in TBST with light agitation overnight at 4 °C, washed with TBST 3 times, and incubated with the secondary antibodies diluted in 5% nonfat dry milk in TBST with light agitation for 1 hour at room temperature. The proteins were then detected with electro-chemiluminescence (Bio-Rad, Hercules, CA, USA).

CCK-8

For the CCK-8 assay, cells were seeded at 8×10^3 cells per well in 96-well flat-bottom plates and were allowed to attach overnight at 37°C. Then, medium containing the assay agents was added to each well, and the cells were further cultured at 37°C for the indicated times. The cell proliferation ability was estimated using a CCK-8 assay, and the absorbance was measured at 450 nm using a microplate reader.

EdU Assay

For the EdU assay, cells were seeded into 96-well plates at a density of 5×10^3 cells per well. After being cultured for the indicated times, the cells were incubated with 10 μM EdU for 2 hours. After EdU labeling, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and treated with 2 mg/mL glycine for 5 minutes. The cells were subsequently incubated with Apollo reagent in the dark for 30 minutes to detect EdU incorporation. Nuclei were counterstained with Hoechst 33342 in the dark for 15 minutes. The cells were then observed by fluorescence microscopy (Olympus, Tokyo, Japan).

Cell Cycle

To detect the cell cycle, the cells were collected and fixed in 70% ethanol overnight at 4 °C. The fixed cells were then washed twice with cold PBS and incubated with RNase A and propidium iodide (PI) at 37°C for 30 min in the dark. Afterward, quantitative analysis of the DNA content was performed by flow cytometry. The data were analyzed using FlowJo software to gate for the G0/1, S, and G2 phases.

Colony Formation

The cells were seeded into 6-well plates at a density of 500 cells per well to assess their colony-forming potential. After 14 days of incubation at 37°C, the cells were fixed with 4% paraformaldehyde at room temperature for 15 min, stained with 0.1% crystal violet for 30 min, photographed, and then analyzed via ImageJ software.

Luciferase Reporter Assay

First, the human *CDKN1B* promoter was cloned into the pGL3-basic vector carrying the firefly luciferase gene to construct the pGL3-*CDKN1B* plasmid, and the PGL4.51 Renilla luciferase plasmid was used as the control plasmid.

The cells were inoculated into 96-well plates at a density of 50%~70%. After 24 h, the cells were transfected using Lipofectamine™ 2000. Briefly, pGL3-*CDKN1B* or scramble and PGL4.51 Renilla luciferase (1 ng) plasmids were cotransfected into the cells. The cell extracts were prepared after 48 h of transfection, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Coimmunoprecipitation (Co-IP)

Coimmunoprecipitation (co-IP) was performed using Protein A/G PLUS-Agarose (Santa Cruz, Texas, USA, sc-2003) according to the manufacturer's instructions. The cells were treated with agents and then lysed in RIPA buffer (Beyotime, Shanghai, China, P0013B) supplemented with protease inhibitors. The lysates were incubated overnight on a carousal at 4 °C with the primary antibody. After the addition of beads, the mixture was incubated for another 90 min. The immunoprecipitates were extensively washed 3 times with washing buffer, eluted in SDS sample buffer, and boiled for 10 min at 70 °C. The samples were subsequently subjected to WB analysis.

Immunohistochemistry

Immunohistochemical staining was performed using primary antibodies against Ambra1 (1:150) and p27 (1:200). After deparaffinization of the sections, antigen retrieval was performed by microwaving in 10 mmol/L citrate buffer, pH 6.0. After blocking endogenous peroxidase, the sections were incubated overnight at 4 °C with individual primary antibodies. The sections were then incubated with secondary antibodies conjugated to peroxidase-labeled polymers. Color development was performed using diaminobenzidine, and the sections were counterstained with hematoxylin. Control sections were evaluated by substitution of the primary antibodies with nonimmunized serum, resulting in no signal detection.

Staining intensities were classified as negative (0), weak (1+), moderate (2+), or strong (3+) staining, and the percentage area of positive staining was scored as 0 (0%), 1 (1% ~ 25%), 2 (26% ~ 50%), 3 (51% ~ 75%), or 4 (76% ~ 100%). A composite score was calculated by adding the staining intensity and the percentage score of positive cells. Composite scores of 0 to 3 were defined as low expression, and scores of 4 to 7 were considered as high expression. The correlation between Ambra1 and p27 was analyzed based on their composite scores.

Mouse Xenograft Models

To generate murine subcutaneous tumors, 5×10^6 MDA-MB-231 cells transfected with scramble, sh*AMBRA1*, or sh*AMBRA1* + sh*CDKN1B* were injected subcutaneously into the right forelimb armpits of BALB/c nude mice (Shanghai SLAC Laboratory Animal, Shanghai, China). After the cells were inoculated for the indicated times, the subcutaneous tumors were removed, and the weights and volumes of the tumors were measured. The tumor volumes were calculated using the following formula: length × width^2 × Π/6.

Hematoxylin & Eosin Staining (HE)

The histological characteristics of the xenograft tumors were evaluated by HE staining. The tumors were paraffin-embedded for tissue sectioning. Then, the tissue sections were subjected to normal histology methods, which involved deparaffinization using xylene and staining with hematoxylin and eosin.

Statistical Analyses

Statistical comparison of the mean values was performed using IBM SPSS Statistics Version 26.0 to conduct a 2-tailed Student's *t* test. The data are shown as the means ± SDs of three independent experiments. A *p*-value less than 0.05 was considered statistically significant.

Results

Knockdown of Ambra1 Inhibits the Proliferation of BC Cells by Inducing G1 Phase Arrest

To determine the role of Ambra1 in the proliferation of BC cells, we used sh*AMBRA1*s to knockdown the expression of Ambra1 in the MCF-7 and MDA-MB-231 cells. As shown in Figure 1A (* $p < 0.05$, ns $p > 0.05$), sh*AMBRA1*-1 and sh*AMBRA1*-2 significantly inhibited the expression of both *AMBRA1* mRNA and protein in MCF-7 and MDA-MB-231 cells, whereas sh*AMBRA1*-3 was only effective in MDA-MB-231 cells. Therefore, sh*AMBRA1*-1 and sh*AMBRA1*-2 were used in subsequent studies. Next, a CCK-8 assay was used to detect the effect of Ambra1 on cell proliferation. The results revealed that the downregulation of Ambra1 significantly inhibited the proliferation of MCF-7 and MDA-MB-231 cells (Figure 1B, * $p < 0.05$, ns $p > 0.05$). This finding was also confirmed by the EdU assay (Figure 1C, * $p < 0.05$, ns $p > 0.05$). We subsequently explored the potential mechanisms by which Ambra1 regulates the proliferation of BC cells. Flow cytometry analysis revealed that fewer MCF-7 and MDA-MB-231 cells with Ambra1 knockdown were in the S phase and that more cells were in the G1 phase, whereas fewer cells were in the G2/M phase, indicating arrest of the cell cycle at the G1 phase (Figure 1D, * $p < 0.05$, ns $p > 0.05$).

Therefore, the knockdown of Ambra1 effectively induced cell cycle arrest and inhibited the proliferation of BC cells.

p27 is Essential for the Ability of Ambra1 to Regulate the Proliferation of BC Cells

p27 controls the cell cycle transition from the G1 phase to the S phase.[24–29] Given that the knockdown of Ambra1 led to arrest of the cell cycle at the G1 phase in BC cells, we explored whether p27 plays an important role in the regulation of BC cell proliferation by Ambra1. First, the regulatory effect of Ambra1 on p27 expression was assayed. MCF-7 and MDA-MB-231 cells were transfected with sh*AMBRA1*-1, sh*AMBRA1*-2 or scramble for 48 h. The results revealed that the knockdown of Ambra1 led to a significant increase in both the mRNA and protein levels of p27 (Figure 2A, * $p <$ 0.05, ns $p > 0.05$). To further clarify the correlation between Ambra1 and p27, we detected the expression of Ambra1 and

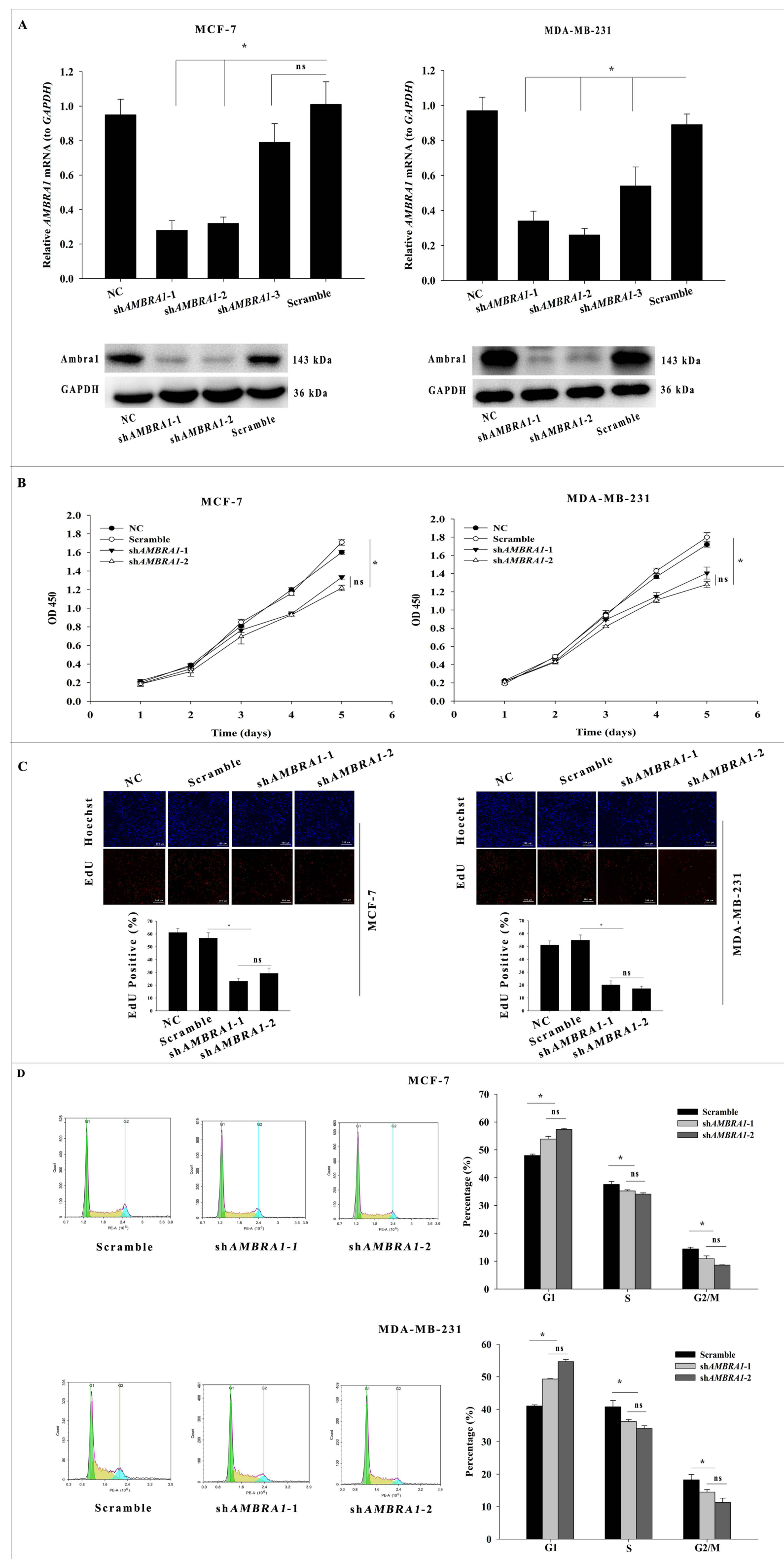

Figure 1 Knockdown of Ambra1 inhibits the proliferation of BC cells by inducing G1 phase arrest (**A**) MCF-7 and MDA-MB-231 cells were transfected with scramble, sh*AMBRA1*-1, sh*AMBRA1*-2 or sh*AMBRA1*-3 for 48 h, and then the mRNA and protein levels of Ambra1 were tested via qRT–PCR or Western blotting, respectively. MCF-7 and MDA-MB-231 cells were transfected with scramble, sh*AMBRA1*-1 or sh*AMBRA1*-2 for 48 h, the proliferation of the cells was detected by (**B**) the CCK-8 assay; (**C**) EdU (100 ×); (**D**) the cell cycle distribution was detected by flow cytometry. The results (means ± SDs) were derived from three independent experiments (*$p < 0.05$, ns $p > 0.05$).

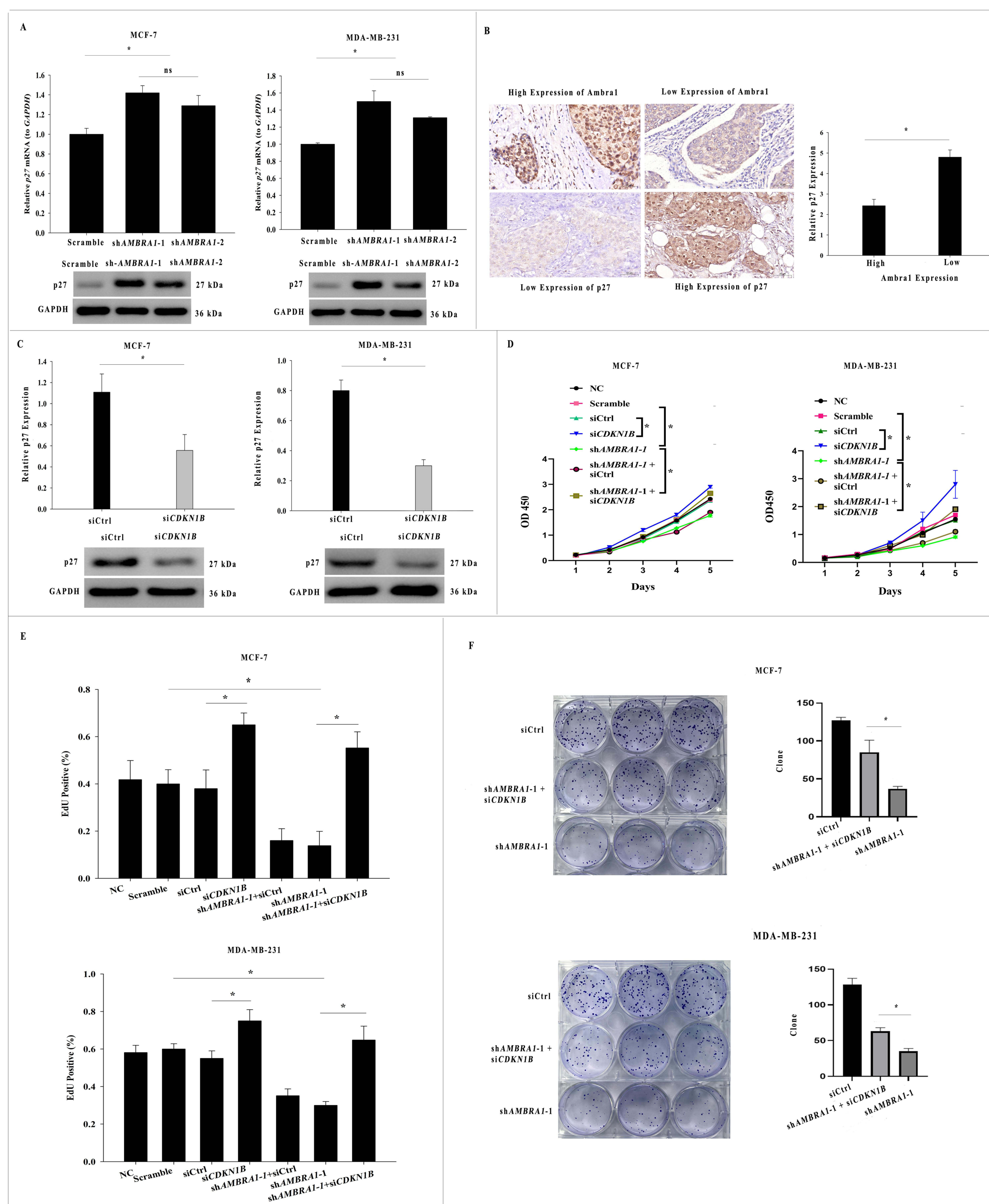

Figure 2 P27 is essential for the ability of Ambra1 to regulate the proliferation of BC cells (**A**) MCF-7 and MDA-MB-231 cells were transfected with scramble, sh*AMBRA1*-1 or sh*AMBRA1*-2 for 48 h, after which the mRNA and protein levels of p27 were measured via qRT–PCR and Western blotting, respectively. (**B**) Ambra1 and p27 expression in breast cancer tissues was detected by immunohistochemistry (600 ×). (**C**) MCF-7 and MDA-MB-231 cells were transfected with siCtrl or si*CDKN1B* for 48 h, and then the expression of p27 was tested by Western blotting. MCF-7 and MDA-MB-231 cells were transfected with scramble, siCtrl, sh*AMBRA1*-1, si*CDKN1B*, sh*AMBRA1*-1 + siCtrl or sh*AMBRA1*-1 + si*CDKN1B* for 48 h, and then the proliferation of the cells was detected by (**D**) the CCK-8 assay and (**E**) EdU. (**F**) MCF-7 and MDA-MB-231 cells were transfected with siCtrl, sh*AMBRA1*-1 or sh*AMBRA1*-1 + si*CDKN1B* for 48 h, and then the proliferation of the cells was detected by colony formation. The results (means ± SDs) were derived from three independent experiments (*p < 0.05, ns p > 0.05).

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p27 in 50 patients with breast cancer by immunohistochemistry. The results reveled that Ambra1 is negatively correlated with p27 in BC (Figure 2B, * $p < 0.05$).

To determine whether the regulation of BC cell proliferation by Ambra1 is dependent on p27, we used si*CDKN1B* to knock down p27 expression in MCF-7 and MDA-MB-231 cells (Figure 2C, * $p < 0.05$). Si*CDKN1B*, scramble or siCtrl was cotransfected with sh*AMBRA1*-1 into MCF-7 or MDA-MB-231 cells for 48 h, and cell proliferation was detected by a CCK-8 assay. The results showed that the cell proliferation rate in the sh*AMBRA1*-1 group was significantly slower than that in the scramble group, while the cell proliferation rate in the si*CDKN1B* group was significantly faster than that in the siCtrl group. Additionally, the cell proliferation rate in the sh*AMBRA1*-1 + si*CDKN1B* group was significantly faster than that in the sh*AMBRA1*-1 group (Figure 2D, * $p < 0.05$). These findings indicate that the downregulation of Ambra1 inhibits the proliferation of breast cancer cells, while the silencing of p27 promotes cell proliferation; in addition, the silencing of the p27 gene can reverse the inhibitory effect of decreased Ambra1 expression on the proliferation of breast cancer cells.

To further demonstrate the role of p27 in the regulation of BC cell proliferation by Ambra1, we also conducted EdU and colony formation assays. The results were all similar to those of the CCK-8 assay (Figure 2E and F, * $p < 0.05$).

In conclusion, the above data indicate that silencing p27 can effectively counteract the proliferation-inhibiting effect caused by the knockdown of Ambra1, suggesting that p27 plays a crucial role in the regulation of BC cell proliferation by Ambra1.

Knockdown of Ambra1 Promotes the Transcription of p27

To determine the mechanism by which Ambra1 regulates p27 expression, we first tested whether Ambra1 interacts with p27 using a coimmunoprecipitation (co-IP) assay in MDA-MB-231 cells. 3 × Flag and 3 × Flag-*AMBRA1* lentiviral vectors were transfected into MDA-MB-231 cells for 48 h. Then, a co-IP assay was performed. As shown in Figure 3A, there was no interaction between these two proteins. Next, we analyzed whether Ambra1 affects p27 degradation. MDA-MB-231 cells were treated with 50 μg/mL CHX with or without sh*AMBRA1*-1 for 0, 2, 4 or 6 h, after which the expression of p27 was evaluated by Western blotting. The results revealed that the knockdown of Ambra1 did not significantly affect the degradation of the p27 protein (Figure 3B).

To investigate whether p27 regulated at the transcriptional level, we cloned the *CDKN1B* promoter into the pGL3-basic vector and measured its activity by the luciferase signal in 293T cells. In 293T cells, sh*AMBRA1*-1 also significantly reduced the expression of Ambra1 (Figure 3C). As shown in Figure 3D, *CDKN1B* promoter activity was greatly increased in Ambra1-knockdown cells (*$p < 0.05$). Thus, the downregulation of Ambra1 promotes p27 transcription.

FoxO1 Mediates the Regulation of p27 Transcription by Ambra1

p27 is a target gene of the transcription factor FoxO1.[30–33] However, it remains unclear whether the regulation of p27 transcription by Ambra1 is mediated by FoxO1. Therefore, we subsequently investigated the role of FoxO1 in this process. To this end, we used si*FOXO1* to silence FoxO1 in MCF-7 and MDA-MB-231 cells (Figure 4A, * $p < 0.05$). After the cells were transfected with siCtrl, si*FOXO1*, sh*AMBRA1*-1, sh*AMBRA1*-1 + siCtrl or sh*AMBRA1*-1 + si*FOXO1* for 48 h, the expression of p27 was detected by Western blotting. The results revealed that in MCF-7 and MDA-MB-231 cells, compared with the siCtrl group, the expression of p27 in the si*FOXO1* group was significantly decreased, while that in the sh*AMBRA1*-1 group was significantly increased, and the expression of p27 in the sh*AMBRA1*-1 + si*FOXO1* group was significantly lower than that in the sh*AMBRA1*-1 group (Figure 4B, * $p < 0.05$).

To further confirm that Ambra1 regulates the transcription of p27 through FoxO1, we determined the activity of the *CDKN1B* promoter in 293T cells using the luciferase reporter assay. After the 293T cells were transfected with siCtrl, si*FOXO1*, sh*AMBRA1*-1, sh*AMBRA1*-1 + siCtrl or sh*AMBRA1*-1 + si*FOXO1* for 48 h, the activity of the *CDKN1B* promoter was detected. The results showed that compared with the siCtrl group, the activity of the *CDKN1B* promoter in the si*FOXO1* group was significantly decreased, while that in the sh*AMBRA1*-1 group was significantly increased; moreover, the activity of the *CDKN1B* promoter in the sh*AMBRA1*-1 group was significantly higher than that in the sh*AMBRA1*-1 + si*FOXO1* group (Figure 4C, * $p < 0.05$). Therefore, FoxO1 plays a crucial role in the regulation of p27 transcription and expression by Ambra1.

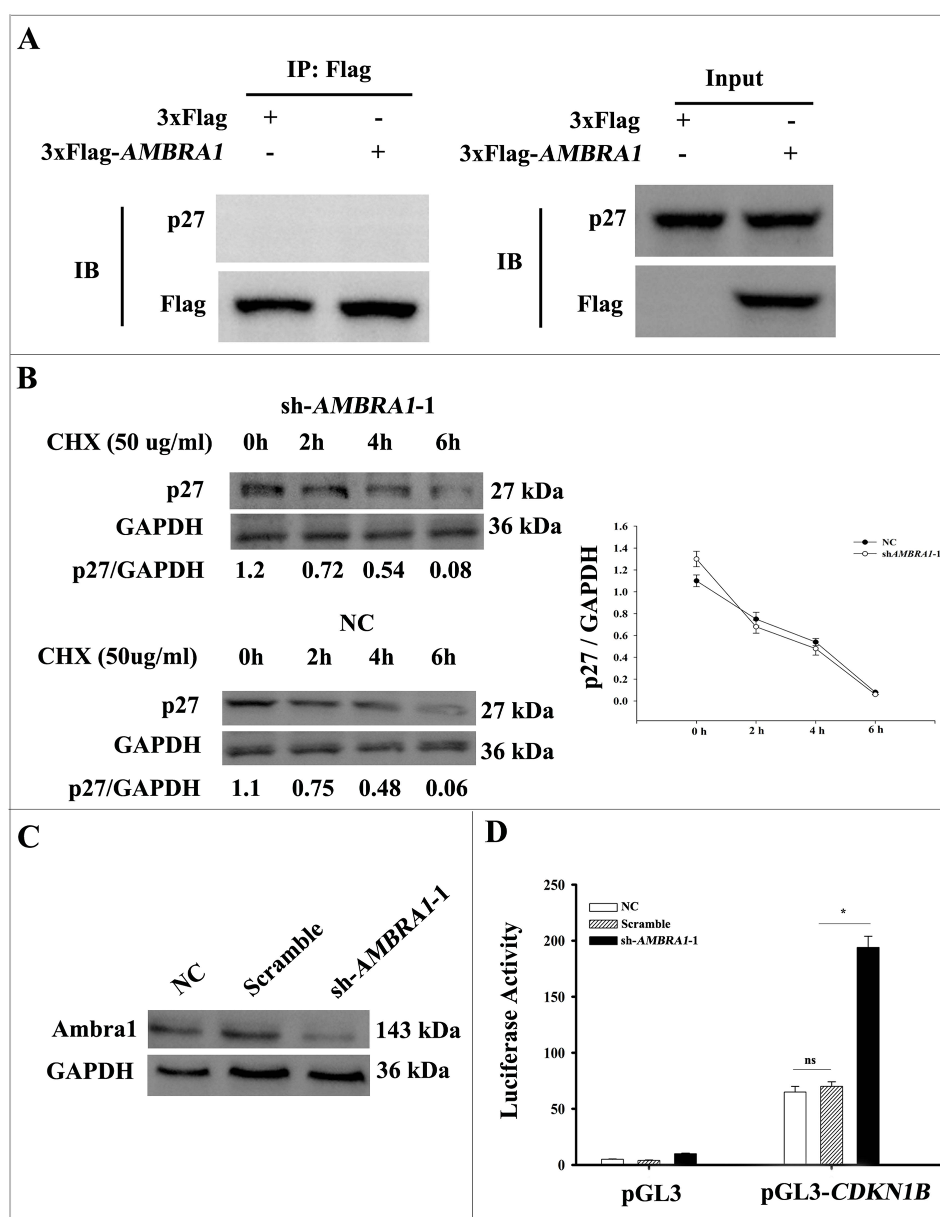

Figure 3 Knockdown of Ambra1 promotes the transcription of p2**7** (**A**) MDA-MB-231 cells were transfected with 3 × Flag or 3 × Flag-*AMBRA1* for 48 h, and co-IP was performed with anti-Flag or anti-p27 antibodies. (**B**) MDA-MB-231 cells were treated with 50 μg/mL CHX with or without sh*AMBRA1*-1 for 0, 2, 4 or 6 h, and then p27 was detected by Western blotting. (**C**) 293T cells were transfected with scramble or sh*AMBRA1*-1 for 48 h, after which the protein of Ambra1 was tested by Western blotting. (**D**) pGL3-*CDKN1B* or pGL3-basic vector was co-transfected with scramble or sh*AMBRA1*-1 into 293T cells for 48 h, and *CDKN1B* promoter activity was detected. The results (means ± SDs) were derived from three independent experiments (*$p < 0.05$, ns $p > 0.05$).

Ambra1 Regulates the Phosphorylation and Subcellular Localization of FoxO1

The transcriptional activity of FoxO1 depends mainly on its phosphorylation status and subcellular localization.[32,36–38] In general, only FoxO1 located in the nucleus has transcriptional functions. When FoxO1 is phosphorylated, it is expelled from the nucleus into the cytoplasm and degraded, resulting in the inhibition of target gene transcription.[39] In our previous study, we reported that Ambra1 could modulate the phosphorylation and intracellular localization of FoxO1, thereby influencing the transcription of Bim by FoxO1.[34] However, which residue of FoxO1 is regulated by Ambra1 for its phosphorylation has not been determined.

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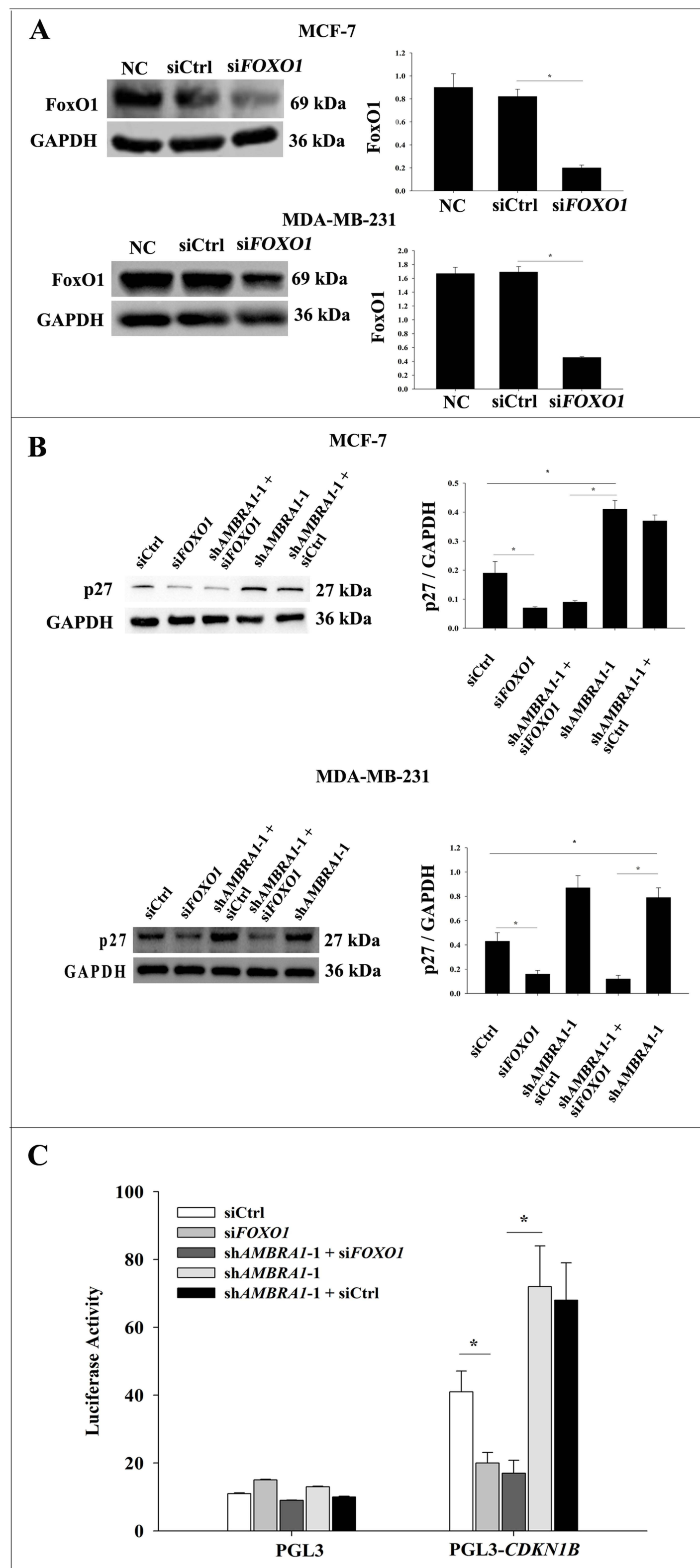

Figure 4 FoxO1 mediates the regulation of p27 transcription by Ambra1 (**A**) MCF-7 and MDA-MB-231 cells were transfected with siCtrl or si*FOXO1* for 48 h, after which the expression of p27 was tested by Western blotting. (**B**) MCF-7 and MDA-MB-231 cells were transfected with siCtrl, si*FOXO1*, sh*AMBRA1*-1, sh*AMBRA1*-1 + siCtrl or sh*AMBRA1*-1 + si*FOXO1* for 48 h, after which the expression of p27 was tested by Western blotting. (**C**) pGL3-*CDKN1B* or pGL3-basic vectors were co-transfected with siCtrl, si*FOXO1*, sh*AMBRA1*-1, sh*AMBRA1*-1 + siCtrl or sh*AMBRA1*-1 + si*FOXO1* into 293T cells for 48 h, and *CDKN1B* promoter activity was detected. The results (means ± SDs) were derived from three independent experiments (*$p < 0.05$).

To determine the residue sites at which Ambra1 phosphorylates FoxO1, we first transfected MCF-7 and MDA-MB-231 cells with scramble or sh*AMBRA1*-1 for 48 hours. Subsequently, Western blotting was used to detect the expression of FoxO1, FoxO1 phosphorylated at serine 256 ($pFoxO1^{s256}$), FoxO1 phosphorylated at threonine 24 ($pFoxO1^{T24}$), and FoxO1 phosphorylated at serine 319 ($pFoxO1^{s319}$). The results revealed that the expression of $pFoxO1^{s256}$ was significantly decreased, whereas there were no significant changes in the expression of FoxO1, $pFoxO1^{T24}$ or $pFoxO1^{s319}$ (Figure 5A, * $p < 0.05$, ns $p > 0.05$). Therefore, Ambra1 mainly regulates the phosphorylation status of the serine 256 residue of FoxO1.

To verify the effect of Ambra1 on the phosphorylation and distribution of FoxO1 in the nucleus, MCF-7 and MDA-MB-231 cells were treated with sh*AMBRA1*-1 or scramble for 48 h, and then Western blotting was used to detect the nuclear FoxO1 ($FoxO1_n$) and $pFoxO1^{s256}$ ($pFoxO1_n^{s256}$). The results showed that sh*AMBRA1*-1 reduced the expression of $pFoxO1_n^{s256}$, while increasing $FoxO1_n$ (Figure 5B, * $p < 0.05$).

Therefore, downregulation of Ambra1 has no effect on the expression of FoxO1, but inhibits its phosphorylation, resulting in more FoxO1 accumulating in the nucleus to exert transcriptional activity.

Ambra1 Regulates the Transcription of p27 via Akt-Mediated Phosphorylation of FoxO1

Akt plays a crucial role in the regulation of the phosphorylation of FoxO1.[37,39] To confirm the role of Akt in regulating FoxO1 phosphorylation by Ambra1, we overexpressed Ambra1 in MCF-7 and MDA-MB-231 cells using LV-*AMBRA1* (Figure 6A, * $p < 0.05$). As expected, the overexpression of Ambra1 led to an increase in the expression of both Akt and pAkt (Figure 6B, * $p < 0.05$, ns $p > 0.05$).

We subsequently explored whether Ambra1 also regulates the phosphorylation of FoxO1 through Akt to control the transcription of p27. 1 nM GSK-690693 was used to inhibit the phosphorylation of Akt. Figure 6B showed that GSK had no significant effect on the expression of Akt, but could significantly inhibit the expression of pAkt; in addition, the increase in pAkt induced by the LV-*AMBRA1* was also significantly inhibited by GSK (Figure 6B, * $p < 0.05$, ns $p > 0.05$). After MCF-7 and MDA-MB-231 cells were transfected with Empty, LV-*AMBRA1*, or LV-*AMBRA1* + GSK for 48 h, the expression of total FoxO1 and $pFoxO1^{s256}$, as well as the expression of $FoxO1_n$ and $pFoxO1_n^{s256}$ in nucleus, was detected by Western blotting. The results revealed that the overexpression of Ambra1 had no significant effect on total FoxO1, but increased the expression of $pFoxO1^{s256}$, which was reversed by GSK (Figure 6C, * $p < 0.05$). However, in the nucleus, LV-*AMBRA1* increased the level of $pFoxO1_n^{s256}$ while decreasing that of $FoxO1_n$, and the addition of GSK reversed the situation (Figure 6D, * $p < 0.05$). Meanwhile, the decrease in p27 caused by Ambra1 overexpression was also reversed by GSK (Figure 6E, * $p < 0.05$).

The above results fully demonstrate that Ambra1 regulates the phosphorylation of FoxO1 through Akt, thereby modulating the distribution of FoxO1 in the nucleus and the transcription of p27.

Ambra1 Regulates Breast Cancer Cell Proliferation Through p27 in vivo

To confirm whether Ambra1 regulates the proliferation of BC cells through p27 in vivo, we inoculated BALB / c nude mice with MDA-MB-231 cells that had previously been transfected with scramble, sh*AMBRA1*-1 or sh*AMBRA1*-1 + sh*CDKN1B* for 48 h. On the 31st day after cell inoculation, the tumors were removed and their volume and weight were measured. The tumors were subsequently embedded in paraffin in preparation for HE staining. The results revealed that both the volume and weight of the tumors in the scramble group were significantly greater than those in the sh*AMBRA1*-1 + sh*CDKN1B* group, whereas no tumors formed in the sh*AMBRA1*-1 group (Figure 7A–D, * $p < 0.05$). HE staining revealed many tumor cells in the tumors of both the scramble group and the sh*AMBRA1*-1 + sh*CDKN1B* group, indicating that the xenograft tumor model in nude mice was successfully established (Figure 7E). Specifically, in vivo, the silencing of Ambra1 significantly inhibited the proliferation of BC cells, whereas the inhibition of p27 expression partially reversed the inhibitory effects of Ambra1 silencing on the proliferation of BC cells. Therefore, p27 plays an important role in the regulation of BC cell proliferation by Ambra1.

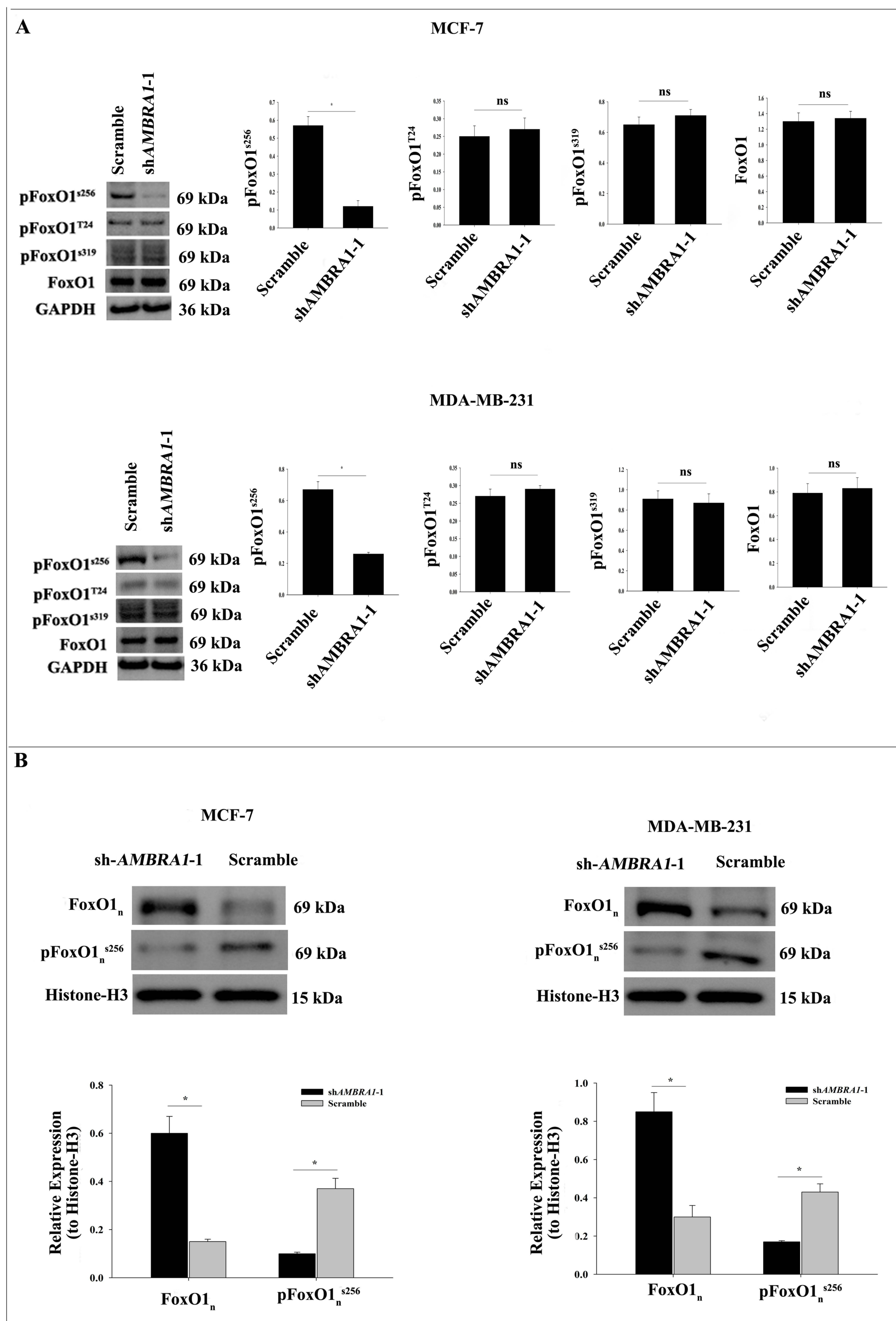

Figure 5 Ambra1 regulates the phosphorylation and subcellular localization of FoxO1 MCF-7 and MDA-MB-231 cells were transfected with scramble or sh*AMBRA1*-1 for 48 h, (**A**) the expression of FoxO1, pFoxO1^{s256}, pFoxO1^{T24} and pFoxO1^{s136} was tested by Western blotting; (**B**) the expression of FoxO1$_n$ and pFoxO1$_n$^{s256} was tested by Western blotting. The results (means ± SDs) were derived from three independent experiments (*p < 0.05, ns p > 0.05).

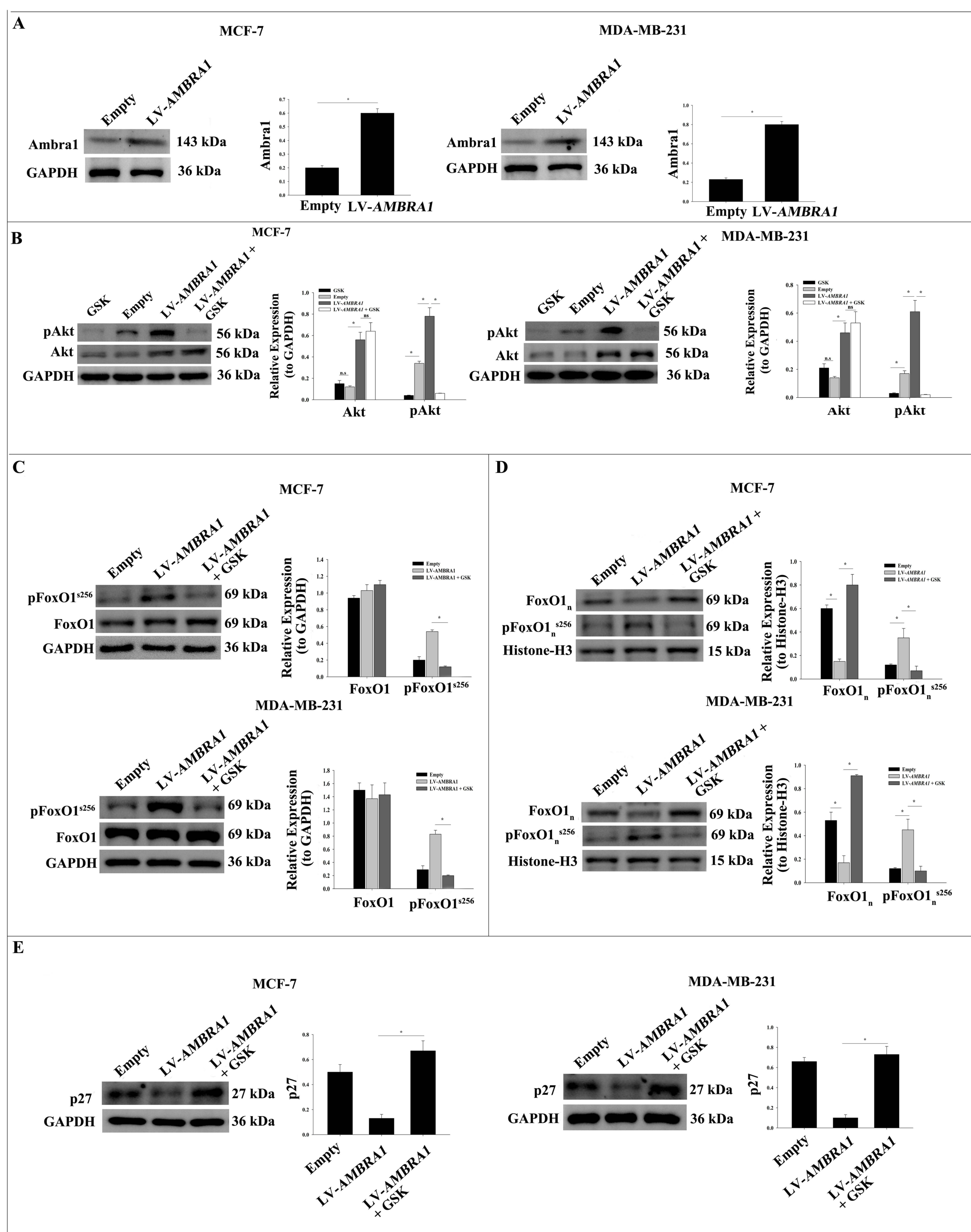

Figure 6 Ambra1 regulates the transcription of p27 via Akt-mediated phosphorylation of FoxO1 (**A**) MCF-7 and MDA-MB-231 cells were transfected with Empty or LV-*AMBRA1* for 48 h, the expression of Ambra1 was tested by Western blotting. (**B**) MCF-7 and MDA-MB-231 cells were treated with or without 1 nm GSK combined with Empty or LV-*AMBRA1* for 48 h, then the expression of Akt and pAkt was tested by Western blotting; (**C**) the expression of FoxO1 and pFoxO1^{s256} was tested by Western blotting; (**D**) the expression of FoxO1$_n$ and pFoxO1$_n^{s256}$ was tested by Western blotting; (**E**) the expression of p27 was tested by Western blotting. The results (means ± SDs) were derived from three independent experiments (*p < 0.05, ns p > 0.05).

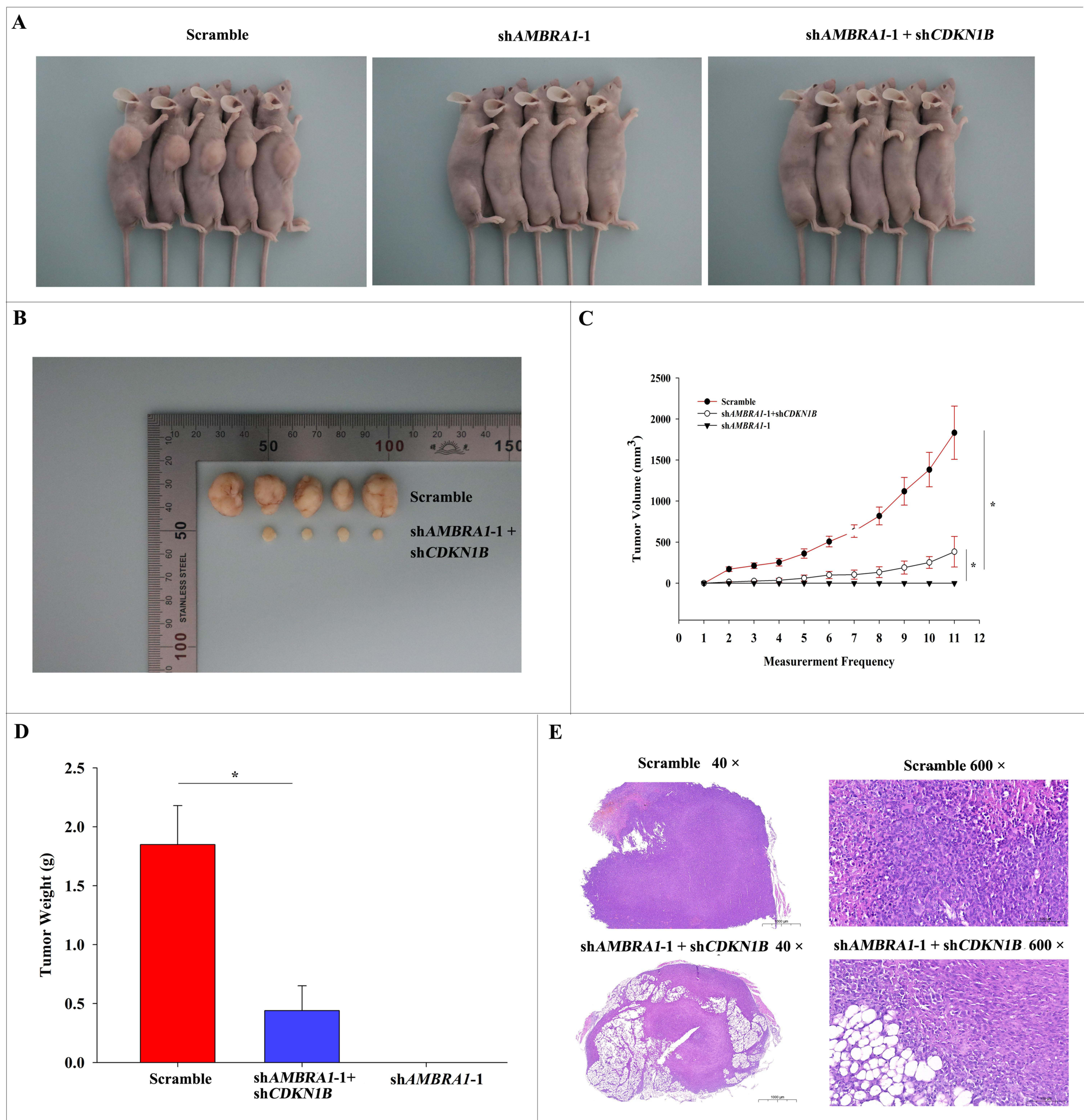

Figure 7 Ambra1 regulates breast cancer cell proliferation through p27 in vivo (**A**) After transfection with scramble, sh*AMBRA1*-1 or sh*AMBRA1*-1 + sh*CDKN1B* for 48 h, MDA-MB-231 cells were inoculated into BALB/ c nude mice. (**B**) After the cells were inoculated for the indicated times, the tumors in the nude mice were removed, and (**C**) the volume and (**D**) weight of the tumors were measured. (**E**) Xenograft tumors were detected by HE staining (*$p < 0.05$).

Discussion

In this study, we demonstrated that Ambra1 is a critical factor in the development of BC and that a decrease in its expression can inhibit the proliferation of BC cells. Moreover, p27, a key downstream target of Ambra1, plays an important role in this process. Additionally, we clarified that Ambra1 regulates the expression of p27 at the transcriptional level through the Akt-FoxO1 signaling pathway.

Ambra1 is a key component of the autophagy core complex.[6] As a scaffold protein, it is involved in regulating multiple cellular signaling pathways.[7–19] Recently, it has been discovered that Ambra1 is also involved in the

regulation of cell proliferation. Cianfanelli et al reported that in MEFs, the absence of Ambra1 can increase the phosphorylation and stability of c-Myc, which is an important protein that promotes cell proliferation.[22] Therefore, Ambra1 can regulate cell proliferation through the c-Myc pathway. As mentioned earlier, Ambra1 can regulate the cell cycle by controlling the turnover of D-type cyclins.[13–17] In uveal melanoma cells and mantle cell lymphoma (MCL) cells, high expression of Ambra1 promoted the ubiquitination and degradation of cyclin D1, arresting the cell cycle at the G1/S phase and thereby inhibiting cell proliferation.[40,41] Additionally, in a mouse melanoma model carrying $Braf^{V600E/+}$, $Pten^{-/-}$ mutations, the depletion of Ambra1 led to the accumulation of cyclin D1, which in turn promoted tumor proliferation, invasion, and metastasis.[42,43] Therefore, Ambra1 can regulate cancer cell proliferation in a cyclin D1-dependent manner. All the above results suggest that Ambra1 is a factor that inhibits cell proliferation. In contrast, Ye et al reported that Ambra1 deficiency significantly inhibited the expression of cyclin D1 and the proliferation of gastric adenocarcinoma cells.[23] In this study, we explored the regulatory effects of Ambra1 on the proliferation of BC cells. The results showed that downregulating the expression of Ambra1 significantly inhibited cell proliferation. Moreover, the downregulation of Ambra1 significantly inhibited the growth of xenograft tumors in nude mice. These results are consistent with those of Ye et al.[23] Thus, Ambra1 seems to play a dual role in promoting and inhibiting cell proliferation. The dual effect of Ambra1 on cell proliferation is most likely related to the type of cancer. In addition, other factors may also have an impact, such as the tumor microenvironment and the activation of different signaling pathways.[9,10,19]

p27 can effectively arrest cells in the G1 phase and inhibit cell proliferation.[24–29] Knocking down Ambra1 led to arrest of the cell cycle in BC cells in the G1 phase. Therefore, p27 may play an important role in this process. To prove that p27 is involved in the regulation of BC cell proliferation by Ambra1, we first verified that Ambra1 has a regulatory effect on the expression of p27. To further confirm the correlation between Ambra1 and p27, we detected the expression of these two proteins in the samples of 50 patients with breast cancer. The results revealed that the expression of p27 was relatively low in patients with high expression of Ambra1, and *vice versa*. This further illustrates the negative correlation between these two proteins. As expected, our in vitro and in vivo experiments demonstrated that the slowdown in the proliferation of BC cells induced by the downregulation of Ambra1 was reversed by the silencing of p27. Therefore, p27 plays an indispensable role in the regulation of BC cell proliferation by Ambra1. It should be noted that inhibition of p27 expression could only partially reverse the inhibitory effect of Ambra1 silencing on breast cancer cell proliferation in nude mouse xenograft tumor models. This might be due to the complex internal environment, where the inhibition of the p27 pathway may be compensated by other mechanisms such as cyclin Ds and autophagy. This result also suggests that the regulatory mechanism of Ambra1 on breast cancer cell proliferation is relatively complex, and p27 pathway is only one of them. The relevant mechanisms still need further exploration.

p27 is a downstream target gene of FoxO1.[30–33] In MCF-7 and MDA-MB-231 cells, Ambra1 regulated the expression of p27 at the transcriptional level. These findings suggest that FoxO1 may play an important role in the regulation of p27 expression by Ambra1. This was confirmed by the fact that the increase in p27 expression caused by the downregulation of Ambra1 was inhibited by the silencing of FoxO1. Therefore, FoxO1 is essential for the regulation of p27 expression by Ambra1. We previously demonstrated that Ambra1 can promote the phosphorylation of FoxO1 through Akt.[31] In this study, we confirmed that Ambra1 mainly regulates the phosphorylation of FoxO1 at serine 256 via Akt, thereby altering the nuclear distribution and transcriptional activity of FoxO1. Moreover, the decrease in p27 expression caused by high Ambra1 expression was reversed by the Akt inhibitor GSK. Thus, Ambra1 also regulates the expression of p27 through the Akt-FoxO1 pathway.

Conclusions

Ambra1 plays a pivotal role in regulating BC cell proliferation through the Akt-FoxO1-p27 signaling axis. Therefore, Ambra1 is an important factor in the development of breast cancer and also a potential therapeutic target.

Data Sharing Statement

The raw data in this study may be obtained from the corresponding author upon reasonable request.

Ethics Approval and Informed Consent

This study was performed in line with the principles of the Declaration of Helsinki and GB/T 42011-2022 Laboratory Animals-General Principles of Welfare. Approval was granted by the Ethics Committee of the Second Affiliated Hospital of Guangxi Medical University (February 19th, 2021/KY-0007 and KY-0008).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report there are no competing interests to declare.

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