

The LINC00504/Mir-4739/KLK4 Axis Facilitates Cell Proliferation and Suppresses Cell Apoptosis Through Triggering The Wnt/B-Catenin Pathway In Triple-Negative Breast Cancer Cells

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Abstract

Background: Currently, long non-coding RNAs (lncRNAs) have been validated to exert critical influence on the malignant progression of triple-negative breast cancer (TNBC). LncRNA long intergenic non-protein coding RNA 504 (LINC00504) has been recently reported as a tumor facilitator in the cellular processes of several cancers. However, its function in TNBC remains unknown.

Methods: CCK-8 and colony formation assays were used to detect the cell viability and proliferation in TNBC. Flow cytometry analysis was utilized to measure the cycle and apoptosis of TNBC cells. The levels of key proteins associated with cell apoptosis or the β -catenin pathway were detected through western blot analysis. The activity of the Wnt/ β -catenin signaling pathway was measured by the TOP/FOP flash assay. ChIP assay was conducted to confirm the binding between LINC00504 and its transcription factor signal transducer and activator of transcription 3 (STAT3). RIP and luciferase reporter assays were used to detect and verify the interaction among LINC00504 and its downstream molecule.

Results: LncRNA LINC00504 was upregulated in TNBC, and silenced LINC00504 suppressed cell proliferation and triggers cell cycle arrest at G0/G1 stage and cell apoptosis in TNBC cells. STAT3 can transcriptionally activate LINC00504 and LINC00504 served as a molecular sponge of microRNA (miR-4379). Kallikrein related peptidase 4 (KLK4) was the target gene of miR-4379 and activates the Wnt/ β -catenin pathway. LINC00504 upregulated KLK4 via competitively binding with miR-4379 to activate the Wnt/ β -catenin pathway in TNBC. The suppression on TNBC cell proliferation and the promotion on TNBC cell cycle arrest and apoptosis under LINC00504 knockdown were rescued by miR-4379 depletion or KLK4 overexpression.

Conclusions: The LINC00504/miR-4379/KLK4 axis promotes cell growth and cell cycle progression as well as suppresses cell apoptosis through activating the Wnt/ β -catenin pathway.

1. Background

As a prevalent cancer among females globally, breast cancer seriously threatens the health of women [1]. Breast cancer is a dominant disease in gynecological oncology [2]. Triple-negative breast cancer (TNBC) is a particularly aggressive subtype of breast cancer characterized by the worst prognosis [3]. The definition of TNBC is adaptive to all tumors lacking the expression of ER, PR and HER2, which are all molecular targets for therapy [4]. Over the past few years, great progresses have been made in improving clinical therapeutics for breast cancer. However, now encouraging clinical activity is seen in the breast oncology community from molecularly targeted approaches to TNBC [5]. Thus, it is meaningful to figure out novel molecule targets for TNBC.

Long non-coding RNAs (lncRNAs) have over 200 nucleotides with limited or without coding ability. LncRNAs play pivotal regulatory roles in cell malignant behaviors including cell proliferation, migration, invasion and apoptosis [6, 7]. Recently, accumulating reports have revealed that lncRNAs act as tumor facilitators and suppressors, thereby affecting the development of tumors [8, 9]. For example, long non-

coding RNA MNX1-AS1 promotes TNBC development by enhancing phosphorylation of Stat3 [10]. PCAT6 promotes TNBC cell proliferation, migration, invasion, and EMT [11]. Upregulation of GATA3-AS1 contributes to TNBC cell proliferation and migration [12]. The role of lncRNA long intergenic non-protein coding RNA 504 (LINC00504) has been identified in several cancers. LINC00504 acts as a therapeutic biomarker for patients with non-small cell lung cancer [13]. LINC00504 promotes ovarian cancer cell development and stimulates aerobic glycolysis [14]. LINC00504 functions as a critical transcriptional regulator for c-Myc in cells of colon cancer [15]. Based on the Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia.cancer-pku.cn/>), we found that LINC00504 was highly expressed in breast cancer. Therefore, we aimed to figure out the function of LINC00504 in TNBC.

Many researches have indicated that lncRNAs act as competing endogenous RNAs (ceRNAs) by sponging microRNAs (miRNAs) and then modulate expression of target messenger RNAs (mRNAs) [16, 17]. Currently, ceRNA networks have been confirmed in progression of TNBC. For instance, lncRNA LRRC75A-AS1 acts as a ceRNA that promotes proliferation, invasion and EMT process of TNBC cells through the miR-380-3p/BAALC axis [18]. lncRNA HOST2 facilitates STAT3-mediated cell proliferation and migration as a ceRNA to decoy let-7b in TNBC [19]. lncRNA ARNILA serves as a ceRNA for miR-204 to upregulate Sox4, accelerating EMT, invasion and metastasis of TNBC [20]. Therefore, we planned to probe the functional meaning of LINC00504 and the putative ceRNA pattern mediated by LINC00504 in TNBC cells.

2. Methods

2.1 Clinical samples

Thirty-two pairs of TNBC tissues and adjacent non-tumor tissues were collected from TNBC patients. No patients had received chemotherapy or radiotherapy formerly at the First Hospital of Jilin University (Jilin, China). This research was approved by the Ethics Committee of the First Hospital of Jilin University (Jilin, China). All patients signed the informed consents in written form.

2.2 Cell culture

The TNBC cell lines (SKBR-3, MDA-MB-231 and BT-549) and the normal breast cell line (MCF-10A) were purchased from American Type Culture Collection (ATCC, USA) and were inoculated in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (ThermoFisher, Waltham, MA, USA) including 10% fetal bovine serum (FBS, ThermoFisher), 100 mg/mL of streptomycin (ThermoFisher) and 100 U/mL of penicillin (ThermoFisher) at 37°C in 5% CO₂.

2.3 Cell transfection

The sh-LINC00504#1/2 and negative control (sh-NC) were bought from OriGene Technologies (USA). The overexpression of kallikrein related peptidase 4 (KLK4) was provided by OriGene Technologies. MicroRNA 4739 (miR-4739) inhibitor, miR-4739 mimics, and their corresponding negative controls (NC-inhibitor and NC-mimics) were obtained from GenePharma (Shanghai, China). The lipofectamine 2000 (Invitrogen,

USA) was applied to achieve transfection. After incubated 4 h at 37 °C, the fresh RPMI 1640 medium with 10% FBS was applied to replace the medium. After being incubated 48 h at 37°C, the cells were collected for further analysis.

2.4 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The TRIzol (Life technologies, New York, NY, USA) kit was utilized to extract RNA, and the Prime Script RT reagent Kit (TaKaRa, Dalian, China) was used for reverse transcription. Next, the SYBR® Premix Ex Taq™ (TaKaRa) was utilized to conduct RT-qPCR analyses in the ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, CA, USA). Later the Genechem Co., Ltd. (Shanghai, China) synthesized the primer sequences for LINC00504, miR-4739 and KLK4. Glyceraldehyde phosphate dehydrogenase (GAPDH) functioned as the internal reference for LINC00504 and KLK4, and RNU6 (U6) acted as the internal reference for miR-4739. Relative expression of RNAs was calculated using $2^{-\Delta\Delta C_t}$ method.

2.5 Cell counting kit-8 (CCK-8) assay

Cells (with density of 3×10^3 cells/well) were incubated in a 96-well plate. CCK-8 solution (10 µL, Sigma-Aldrich, St. Louis, MO, USA) was added into each well and then the cells were cultured for 2 h. At 0, 24, 48, 72 h, cell viability was measured and qualified by the optical density value at 450 nm by a microplate reader (Molecular Devices, San Francisco, CA, USA).

2.6 Colony formation assay

SKBR-3 and MDA-MB-231 cells were seeded in a six-well plate, cultured with 10% FBS and preserved in RPMI 1640 medium for 10 days. Methanol was applied to fix colonies for 15 min at ambient temperature. After that, crystal violet dye was used to stain cells for 15 min. The Image J software was used to assess number of colonies (> 50 cells).

2.7 Cell cycle analysis

The cell cycle was measured in SKBR-3 and MDA-MB-231 cells using flow cytometry. The propidium iodide (PI) (Sigma-Aldrich) was applied to harvest and stain cells. After washing the cells by phosphate buffer saline (PBS) twice and centrifuging them, the cell suspension was fixed at chilled 70% ethanol and incubated 4°C overnight. Next, 100 µg/ml of RNase A was added, and the mixture was cultured at 37°C for 30 min. After adding PI solution, the mixture was incubated at 4°C for 30 min in the darkness. Finally, a FACSCalibur flow cytometry (BD Biosciences, Franklin Lake, NJ, USA) and the ModifitLT software were used to analyze samples.

2.8 Cell apoptosis

Cells (5×10^4 cells/well) were grown in six-well plate and cultured overnight. After treatment, cold PBS was re-suspended in binding buffer (BD Biosciences) and were applied to wash the harvested cells twice. Cells were dyed using 5 µl of Annexin V- Fluorescein Isothiocyanate (FITC) and 5 µl of PI, lightly mixed

and reacted in conditions devoid of light for 10–15 min at room temperature. Eventually, a FACSCalibur flow cytometer (BD Biosciences) was used to analyze the cell apoptosis.

2.9 Western blot

Ice-cold PBS was used to collect and wash the cells twice. Proteins were extracted with RNeasy lysis buffer. After being centrifuged and heated, the electrophoresis in 12% sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to fractionate lysates. Protein lysates were transferred to polyvinylidene difluoride (PVDF) membranes and closed by 5% skim milk for 2 h at ambient temperature. Later, these membranes were cultured with primary antibodies including: anti-Bax (ab182733, 1:1000, Abcam, Cambridge, UK), anti-Bcl-2 (ab182858, 1:2000, Abcam), anti- β -catenin (ab32572, 1:5000, Abcam), anti-c-Myc (ab32072, 1:1000, Abcam). The mixture was then incubated overnight at 4°C with continuous agitation. Secondary antibodies were used to culture the membranes for 2 h at 37°C. The Tris Buffered Saline Tween (TBST) was used to wash the membranes for three times. The ImageJ software was used to detect densitometry analysis of the band intensity.

2.10 Caspase-3 activity detection

The relative caspase-3 activity was measured by the Caspase-3 Colorimetric Assay Kit (Keygen, Jiangsu, China) in line with the manufacturer's protocols.

2.11 Chromatin immunoprecipitation (ChIP) assay

The crosslinked chromatin was sheared and immunoprecipitated with anti-STAT3 antibody (ab68153, Abcam) or anti-IgG antibody (ab6789, Abcam) as control, along with the magnetic beads. At last, precipitates were purified for RT-qPCR.

2.12 Luciferase reporter assay

The sequence of LINC00504 promoter was subcloned into the luciferase reporter pGL3-vector (Promega, Madison, WI, USA). LINC00504-WT/Mut pmirGLO vectors or KLK4 3'untranslated region (UTR)-WT/Mut pmirGLO vectors (GenePharma, Shanghai, China) were constructed and transfected into SKBR-3 and MDA-MB-231 cells with miR-4739 mimics, NC-mimics, miR-4739 inhibitor or NC-inhibitor. 48 h after transfection, the luciferase activity was determined by the Dual-Luciferase Reporter Assay System (Promega, USA).

2.13 RNA immunoprecipitation (RIP) assay

A Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore, Danvers, MA, USA) was utilized to perform RIP assay. Next, precooled PBS was utilized to wash cells, and the supernatant was taken out.

After the cells were lysed and centrifuged, the supernatant was discarded. The RIPA buffer was used to resuspend the magnetic beads-antibody complex incubated at 4 °C. Next, the magnetic bead-protein complex was collected via the sample placed on the magnetic pedestal. The antibodies were anti-Ago2 (ab 186733, Abcam), and anti-IgG (ab6715, Abcam) served as the negative control (NC). The endogenous miR-4739 that combined with LINC00504/KLK4 was precipitated. Subsequently, cell lysis in the RIPA buffer was incubated with magnetic bead conjugated with human Ago2 antibody (Millipore, USA). The purified RNA concentration was determined using RT-qPCR analysis.

2.14 TOP/FOP flash assay

The TOP or FOP plasmids were co-transfected with expression regulation plasmids into TNBC cells. After 48 h of incubation, a Dual Luciferase Reporter Assay Kit (Promega) was used to measure the activity of the Wnt/ β -catenin signaling pathway.

2.15 Subcellular fraction assay

The nuclear and cytoplasmic LINC00504 were isolated by the PARIS™ kit (Thermo Fisher Scientific). LINC00504 from isolated nuclear and cytoplasmic fractions was reversely transcribed and applied for PCR analysis after purification. U6 was used as the internal reference for the nucleus, and GAPDH as the internal reference for the cytoplasm.

2.16 Statistical Analysis

SPSS 21.0 (SPSS, USA) software was utilized to conduct the data analysis. We used mean \pm standard deviation (SD) to exhibit the obtained data. The Student's t-test was applied for comparisons of two groups, and one-way/two-way analysis of variance was utilized for comparisons among different groups, and then a Tukey's post hoc test was conducted. Each experiment was repeated at least three times. $p < 0.05$ was considered as significantly statistical.

3. Results

3.1 LncRNA LINC00504 is upregulated in TNBC tissues and cells

LncRNA LINC00504 has been indicated as an oncogene to promote progression of many cancers. Here, we explored the expression level of LINC00504 in TNBC. The overexpression of LINC00504 in breast cancer tissues was revealed from the database of GEPIA (Fig. 1A). The results from RT-qPCR showed that LINC00504 was highly expressed in TNBC cell lines, in comparison to control cell line (Fig. 1B). Furthermore, we verified that LINC00504 was overexpressed in TNBC tissues in contrast with adjacent

noncancerous tissues (Fig. 1C). After that, it was confirmed that LINC00504 expression was higher in advanced-stage tissues than in early-stage tissues (Fig. 1D). All these data revealed that LINC00504 was upregulated in TNBC cell lines and tissues.

3.2 Downregulation of LINC00504 suppresses cell proliferation and triggers cell cycle arrest and cell apoptosis in TNBC cells

Next, shRNA-LINC00504 plasmids were transfected into TNBC cells, and the knockdown efficiency of LINC00504 was verified through RT-qPCR analysis (Fig. 2A). Cell viability of SKBR-3 and MDA-MB-231 cells after silencing LINC00504 was accessed via CCK-8 assay. It was suggested that LINC00504 knockdown inhibited cell viability (Fig. 2B). In addition, the effects of sh-LINC00504#1/2 on cell proliferation was assessed. As revealed in Fig. 2C, inhibition of LINC00504 reduced the number of colonies in colony formation assay. Additionally, we studied the influence of LINC00504 silencing on cell cycle through flow cytometry. The LINC00504 knockdown elevated the percentage of SKBR-3 and MDA-MB-231 cells in the G0/G1 phase and declined that in the S phase, suggesting that LINC00504 knockdown can trigger cell cycle arrest at G0/G1 phase (Fig. 2D). Moreover, we detected cell apoptosis in TNBC cells using flow cytometry. After silencing LINC00504, cell apoptotic rate was increased (Fig. 2E). Additionally, we detected the activity of caspase-3, and found that the activity of caspase-3 was significantly increased by silencing of LINC00504 (Fig. 2F). We conducted the western blot to determine the expression of apoptosis-related proteins (Bax and Bcl-2) in SKBR-3 and MDA-MB-231 cells. Results depicted that Bax level was elevated and Bcl-2 expression was repressed after silencing LINC00504 (Fig. 2G). These findings showed that LINC00504 downregulation repressed cell growth and induced cell apoptosis and cell cycle arrest in the G0/G1 phase in TNBC cells.

3.3 LINC00504 is transcriptionally activated by STAT3

After that, we sought to examine the upstream transcription factor for LINC00504. As was predicted from UCSC, STAT3 is putative to serve as the transcription factor for LINC00504. We effectively overexpressed expression of STAT3 via transfection of pcDNA3.1-STAT3 and validated that up-regulation of STAT3 significantly increased expression of LINC00504 (Fig. 3A). Spearman's correlation analysis from the GEPIA database suggested that there was a positive relationship between LINC00504 expression and STAT3 expression in TNBC tissues (Fig. 3B). Next, through RT-qPCR, the expression of STAT3 was revealed to be upregulated in TNBC tissues than adjacent normal tissues (Fig. 3C). In addition, STAT3 was confirmed to be highly expressed in TNBC cell lines than control cell line (Fig. 3D). Then, the DNA motif of STAT3 was predicted from JASPAR website (<http://jaspar.genereg.net/matrix/MA0079.1/>) (Fig. 3E). ChIP assay showed that anti-STAT3 significantly precipitated LINC00504 promoter in TNBC

cells (Fig. 3F). As was shown in Fig. 3G, overexpression of STAT3 increased the luciferase activity of pGL3 vectors containing LINC00504 promoter in TNBC cells. To conclude, STAT3 served as the transcription factor for LINC00504.

3.4 LINC00504 serves as a molecular sponge of miR-4739

Subsequently, we aimed to explore the potential mechanism underlying LINC00504 on TNBC cell malignant behaviors. It was demonstrated that lncRNAs act as ceRNAs to sponge miRNAs at the post-transcriptional level, thereby regulating cancer tumorigenesis [16, 17]. Thus, we detected the subcellular location of LINC00504 through subcellular fraction assay. As was revealed in Fig. 4A, LINC00504 was mainly located in the cytoplasm of SKBR-3 and MDA-MB-231 cells. Then, we predicted the potential miRNAs binding with LINC00504 by usage of DIANA tool (<http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index>). Among 5 candidate miRNAs, only miR-4739 was under-expressed in TNBC cells (Fig. 4B). Then through RT-qPCR analysis, miR-4739 was revealed to be downregulated in TNBC tissues than adjacent normal tissues (Fig. 4C). Next, we upregulated miR-4739 expression through transfection of miR-4739 mimics and inhibited the expression of miR-4739 through transfection of miR-4739 inhibitor (Fig. 4D-E). The binding site between LINC00504 and miR-4739 was predicted from DIANA tool. and mutated the binding site of LINC00504 for miR-479 for the next assay (Fig. 4F). The luciferase activity of wild-type LINC00504 reporters was decreased by miR-6754-5p mimics and increased by miR-4739 inhibitor, while the luciferase activity of LINC00504-Mut reporters showed no significant change (Fig. 4G). Moreover, relative LINC00504 enrichment precipitated by anti-IgG and anti-Ago2 was measured using the RIP assay. The finding depicted that miR-4739 mimics elevated the relative enrichment of LINC00504 precipitated by anti-Ago2, indicating that LINC00504 could interact with miR-4739 (Fig. 4H). Thus, LINC00504 is a molecular sponge for miR-4739.

3.5 KLK4 is the downstream target of miR-4739 and activates the Wnt/ β -catenin pathway

To detect the potential target of miR-4739, we predicted five mRNAs which might interact with miR-4739 using TargetScan (http://www.targetscan.org/vert_72/) (Fig. 5A). After overexpressing miR-4739 using miR-4739 mimics, KLK4 expression exhibited the most significant downregulation, while expression of other four candidate mRNAs depicted no significant changes (Fig. 5B). The upregulation of KLK4 expression in TNBC cells and tissues was validated by RT-qPCR (Fig. 5C-D). Furthermore, upregulation of miR-4739 repressed the KLK4 expression and upregulation of LINC00504 elevated KLK4 expression; miR-4739 inhibition increased KLK4 expression and silencing of LINC00504 reduced KLK4 expression (Fig. 5E). Results of RIP assay revealed that LINC00504, miR-4739 and KLK4 were enriched in anti-Ago2 group in comparison with the control group, revealing that miR-4739 could interact with LINC00504 and KLK4 (Fig. 5F). Subsequently, the binding sequence between miR-4739 and 3'UTR of KLK4 were

identified, and the binding site was mutated for the luciferase reporter assay (Fig. 5G). Luciferase activity of the KLK4 3'UTR-WT reporter was suppressed by the miR-4739 overexpression using miR-4739 mimics and elevated by the miR-4739 downregulation using miR-4739 inhibitor. However, no significant change in luciferase activity of KLK4 3'UTR-Mut was detected (Fig. 5H). Moreover, the SKBR-3 and MDA-MB-231 cells were transfected by sh-LINC00504#1/2. Transfection of sh-LINC00504#1/2 significantly reduced KLK4 expression (Fig. 5I). KLK4 has been documented to activate the Wnt/ β -catenin pathway [21]. We aimed to investigate whether LINC00505 could activate the Wnt/ β -catenin pathway via regulating KLK4. It was revealed from TOP/FOP flash assay that silencing of LINC00504 reduced the luciferase activity of TOP vector, while such effect was reversed by pcDNA3.1-KLK4, indicating that LINC00504 activated the Wnt/ β -catenin pathway by upregulation of KLK4 (Fig. 5J). Importantly, overexpression of KLK4 reversed the suppressive effects of sh-LINC00504#1/2 on protein levels of β -catenin and c-Myc, suggesting that LINC00504 activated the Wnt/ β -catenin pathway through upregulating KLK4 (Fig. 5K). In summary, these results showed that LINC00504 could directly interact with miR-4739 to upregulate KLK4 and trigger the Wnt/ β -catenin pathway.

3.6 The LINC00504/miR-4739/KLK4 axis promotes cell growth and cell cycle progression as well as suppresses cell apoptosis in TNBC

Finally, we decided to explore whether LINC00504 exerts its biological function in TNBC cells via upregulation of KLK4. CCK-8 and colony formation assays confirmed that KLK4 upregulation increased cell viability and proliferation, which were previously decreased by sh-LINC00504#1; knockdown of miR-4739 also counteracted the suppressive effects of sh-LINC00504#1 on cell viability and proliferation (Fig. 6A-B). Then, the influence of silencing LINC00504 on cell cycle was reversed by overexpression of KLK4 or downregulation of miR-4739 in MDA-MB-231 cells (Fig. 6C). Subsequently, we detected cell apoptosis rate through flow cytometry analysis and apoptosis-related protein levels through western blot analysis in MDA-MB-231 cells. It was demonstrated that the increased cell apoptosis caused by sh-LINC00504#1 was further decreased by KLK4 overexpression or miR-4739 downregulation (Fig. 6D-E). Finally, KLK4 overexpression or miR-4739 downregulation counteracted the suppressive effect of sh-LINC00504#1 on caspase-3 activity (Fig. 6F). These results confirmed that the LINC00504 could promote cell viability and cell cycle progression and suppress cell apoptosis through the positive modulation on KLK4 at a miR-4739-dependent manner.

4. Discussion

This study provides the material significance for LINC00504 in TNBC cells. The results revealed that LINC00504 was upregulated in TNBC tissues and cell lines. LINC00504 knockdown suppressed TNBC cell proliferation, leading to cell cycle arrest in the G0/G1 phase, and accelerated cell apoptosis in TNBC cells. Our study subsequently indicated that STAT3 was the transcription factor for LINC00504. STAT3 serves as the transcription factor for lncRNA SNHG17 [22]. Moreover, STAT3 also plays an important role in TNBC. LEPRb-induced STAT3 activation is critical for the induction and maintenance of TNBC cancer stem cells [23].

The ceRNA pattern is a typical regulatory mechanism in progression of multiple cancers, including TNBC [19, 24]. Thus, we further investigated the underlying ceRNA network of LINC00504 in TNBC. Our findings showed that LINC00504 acted as a molecular sponge of miR-4739, whose expression is negatively regulated by VPS9D1-AS1 in prostate cancer cells [25]. Moreover, other two investigations have documented the functions of miR-4739, which is associated with unilineage hematopoietic stem cell differentiation, the β -catenin signaling pathway and gastric cancer, as well as in the pathogenesis of negative anaplastic large cell lymphoma [26, 27]. Our study revealed that KLK4 served as the direct target of miR-4739. LINC00504 competitively interacted with miR-4739 to upregulate KLK4. Additionally, KLK4 protein expression could play the role as a selective predictive tissue-based biomarker on breast cancer patient's response to adjuvant systemic cancer therapy [28].

The Wnt/ β -catenin pathway can be activated in plentiful cellular processes, including cell proliferation, differentiation, migration and polarity, survival and self-renewal in stem cells. As a complicated intracellular cell signaling cascade, the Wnt/ β -catenin pathway is confirmed as a highly conserved and tightly regulated signaling that controls carcinogenesis in various cancers [29, 30]. It was declared that the Wnt/ β -catenin pathway participated in TNBC progression. HePTP facilitates migration and invasion ability of TNBC cells via triggering the Wnt/ β -catenin signaling pathway [31]. LGR6 promotes tumor proliferation and metastasis through the Wnt/ β -catenin signaling pathway in TNBC [32]. Abundant studies have depicted that KLK4 can activate the Wnt/ β -catenin signaling. Silencing of KLK4 inhibits the growth of oral squamous cell carcinoma cells through the Wnt/ β -catenin signaling pathway [33]. Overexpression of LINC01314 down-regulated KLK4 to suppress the Wnt/ β -catenin signaling pathway in gastric cancer [21]. Our study implied that KLK4 activated the Wnt/ β -catenin pathway in TNBC cells and that LINC00504 triggered the Wnt/ β -catenin pathway through upregulation of KLK4. After rescue assays, we demonstrated that KLK4 could counteract the influences of LINC00504 downregulation on cell proliferation, cell cycle progression, cell apoptosis, revealing that LINC00504 promoted TNBC cell malignant behaviors via overexpression of KLK4 to activate the Wnt/ β -catenin pathway.

In summary, we elucidated that the LINC00504/miR-4739/KLK4 axis promotes TNBC cell proliferation ability, cell cycle and suppresses cell apoptosis through the Wnt/ β -catenin pathway, which may shed a new insight into TNBC treatment.

Abbreviations

lncRNAs
long non-coding RNAs
TNBC
triple-negative breast cancer
ceRNAs
competing endogenous RNAs
miRNAs
microRNAs
GAPDH
glyceraldehyde phosphate dehydrogenase
RT-qPCR
Reverse transcription quantitative polymerase chain reaction
CCK-8
cell counting kit-8
PI
propidium iodide
RIPA
RNA immunoprecipitation assay
SDS-PAGE
sodium dodecyl sulfate, polyacrylamide gel electrophoresis
ChIP
Chromatin immunoprecipitation
RIP
RNA immunoprecipitation
NC
negative control
SD
standard deviation

Declarations

Ethics approval and consent to participate

This research was approved by the Ethics Committee of the First Hospital of Jilin University (Jilin, China).

Consent to publish

Not applicable.

Availability of data and materials

The datasets applied during the present study is available from the corresponding author on reasonable request.

Competing interests

The authors declare that no conflict of interest exists.

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Authors' contributions

Study concepts and design: D.W. and H.J.;

Data acquisition and analysis: Z.Z. and S.L.;

Experimental studies and statistical analysis: D.W. and H.J.;

Manuscript preparation and editing: D.W. and H.J.;

Manuscript review: D.W., H.J., Z.Z. and S.L.

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Figures

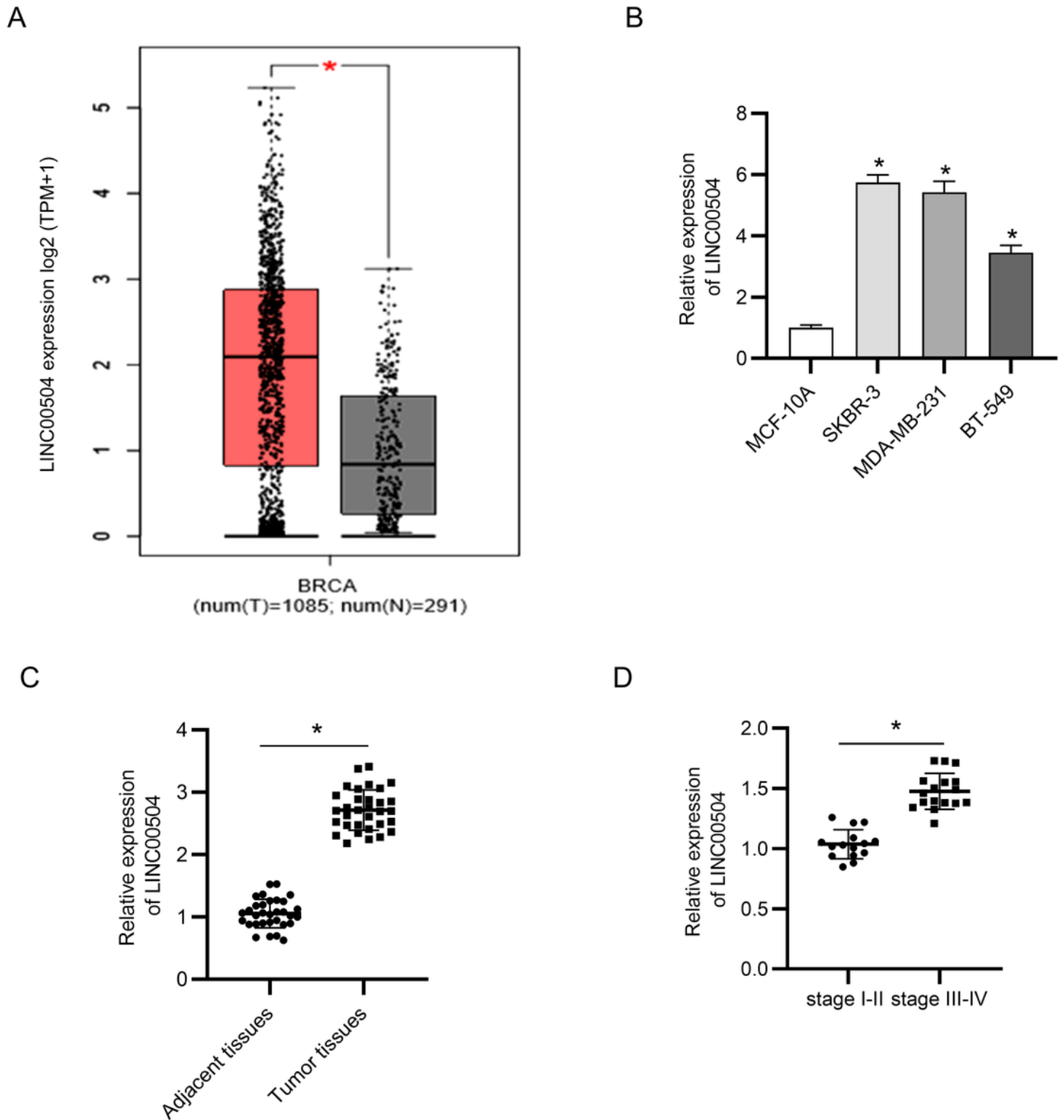


Figure 1

LncRNA LINC00504 is upregulated in TNBC tissues and cells. (A) The GEPIA database showed the expression of LINC00504 in breast cancer (BRAC). (B) Relative expression of LINC00504 in TNBC cell lines (SKBR-3, MDA-MB-231 and BT-549) and normal breast cell line (MCF-10A). (C) Relative LINC00504 expression in TNBC tissues and adjacent non-tumor breast tissues. n=32. (D) Relative expression level of

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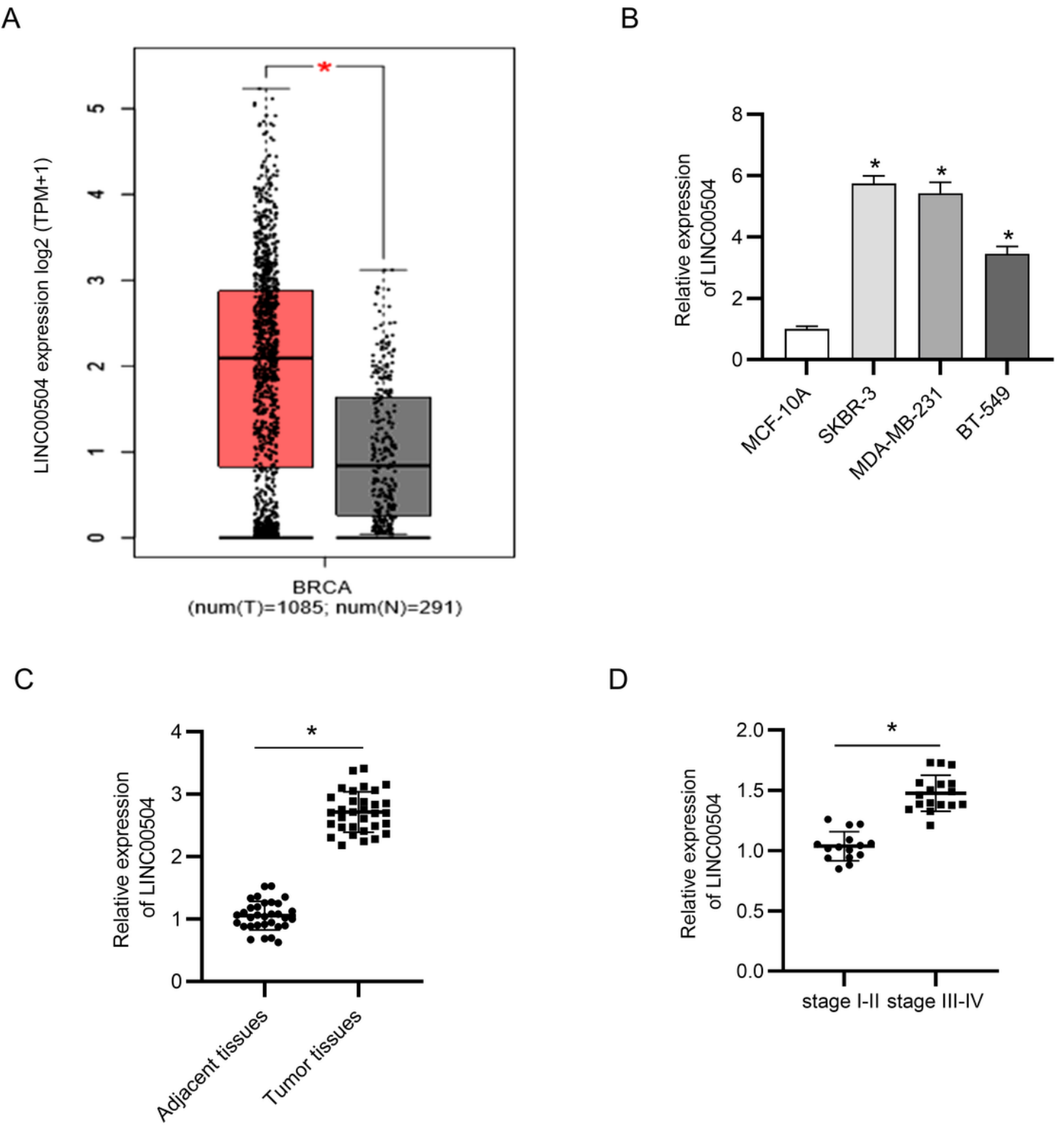


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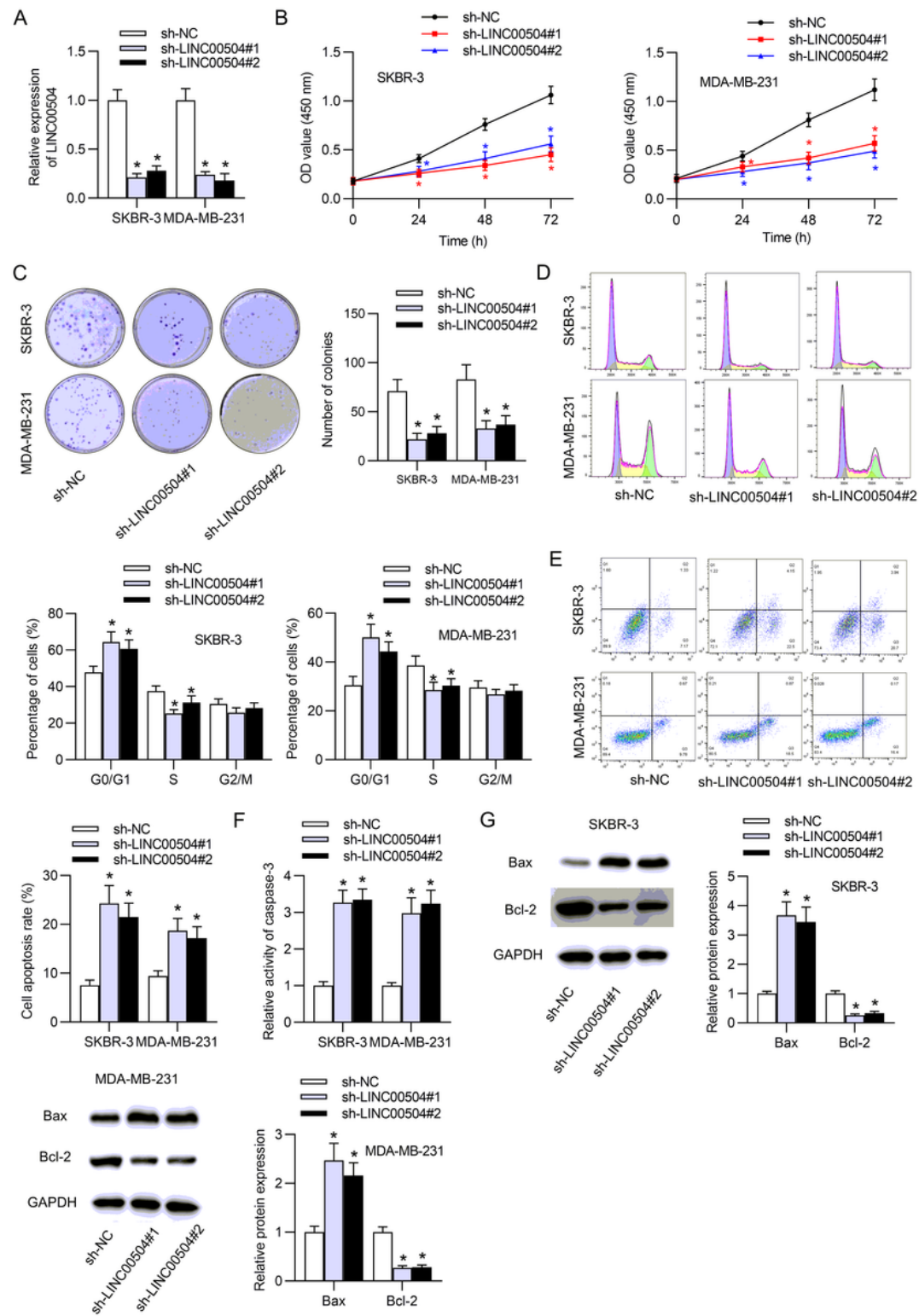


Figure 2

Downregulation of LINC00504 suppresses TNBC cell proliferation and triggers TNBC cell cycle arrest and cell apoptosis. (A) RT-qPCR determined relative expression of LINC00504 in MDA-MB-231 cells and SKBR-

3 cells after silencing LINC00504. (B) CCK-8 assays measured cell viability of SKBR-3 and MDA-MB-231 cells after LINC00504 knockdown. (C) Colony formation assays measured the colony numbers of SKBR-3 and MDA-MB-231 cells. (D) Flow cytometry detected the effect of silencing LINC00504 on cell cycle progression. We revealed percentage of cell population at different phases of cell cycle. (E) Flow cytometry measured cell apoptosis rate. (F) Caspase-3 Colorimetric Assay Kit determined the activity of caspase-3. (G) Levels of Bax and Bcl-2 was examined by western blot. Full-length blots are presented in Supplementary material. The ImageJ software was used to detect densitometry analysis of the band intensity. * $p < 0.05$.

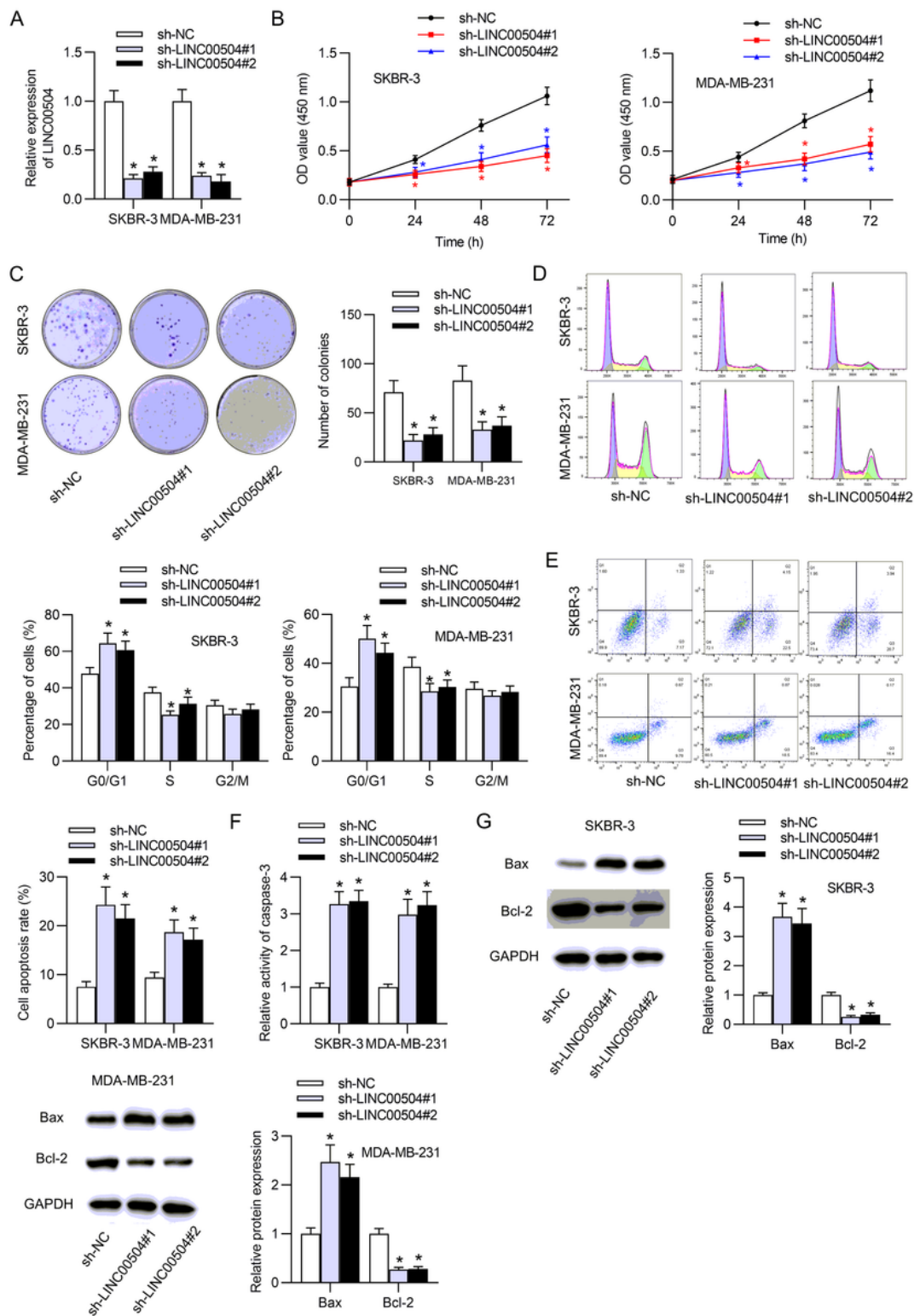


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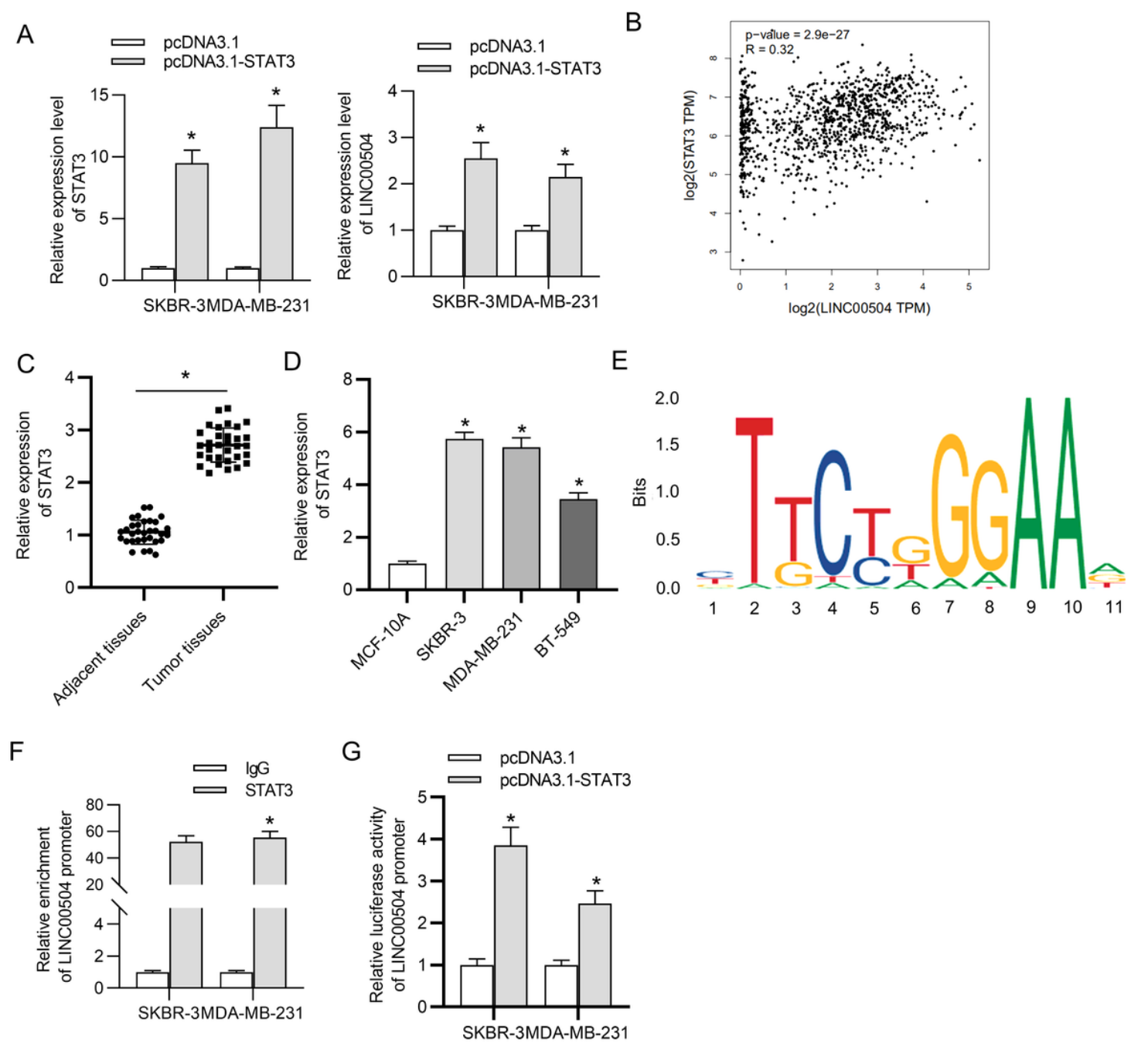


Figure 3

LINC00504 is transcriptionally activated by STAT3. (A) The left panel: overexpression efficiency of STAT3 was verified by RT-qPCR analysis; the right panel: the effects of STAT3 overexpression on LINC00504 expression. (B) The GEPIA database showed the correlation between LINC00504 and STAT3 in breast cancer. (C) RT-qPCR analysis was used to detect the expression of STAT3 in TNBC tissues and adjacent

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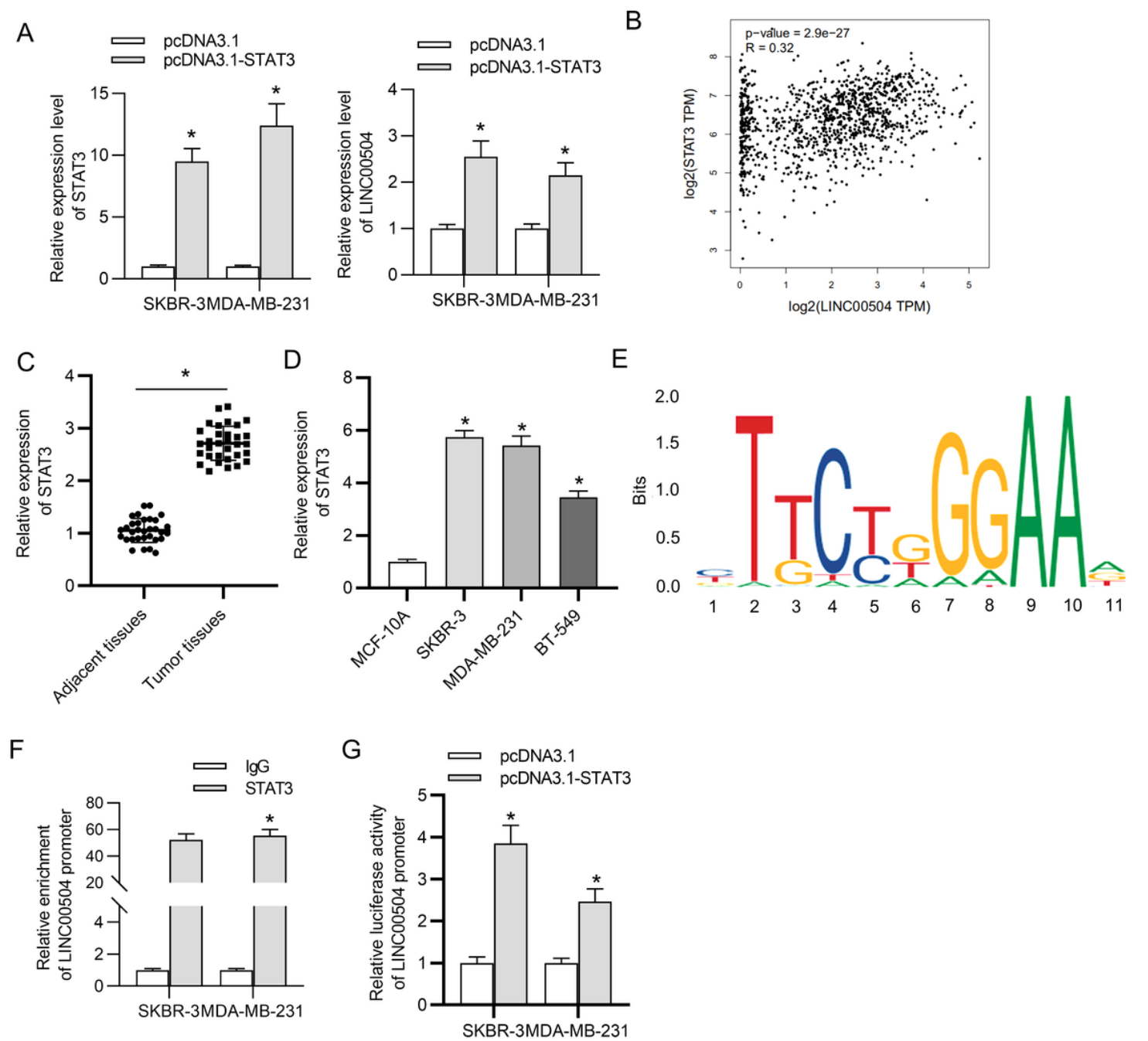


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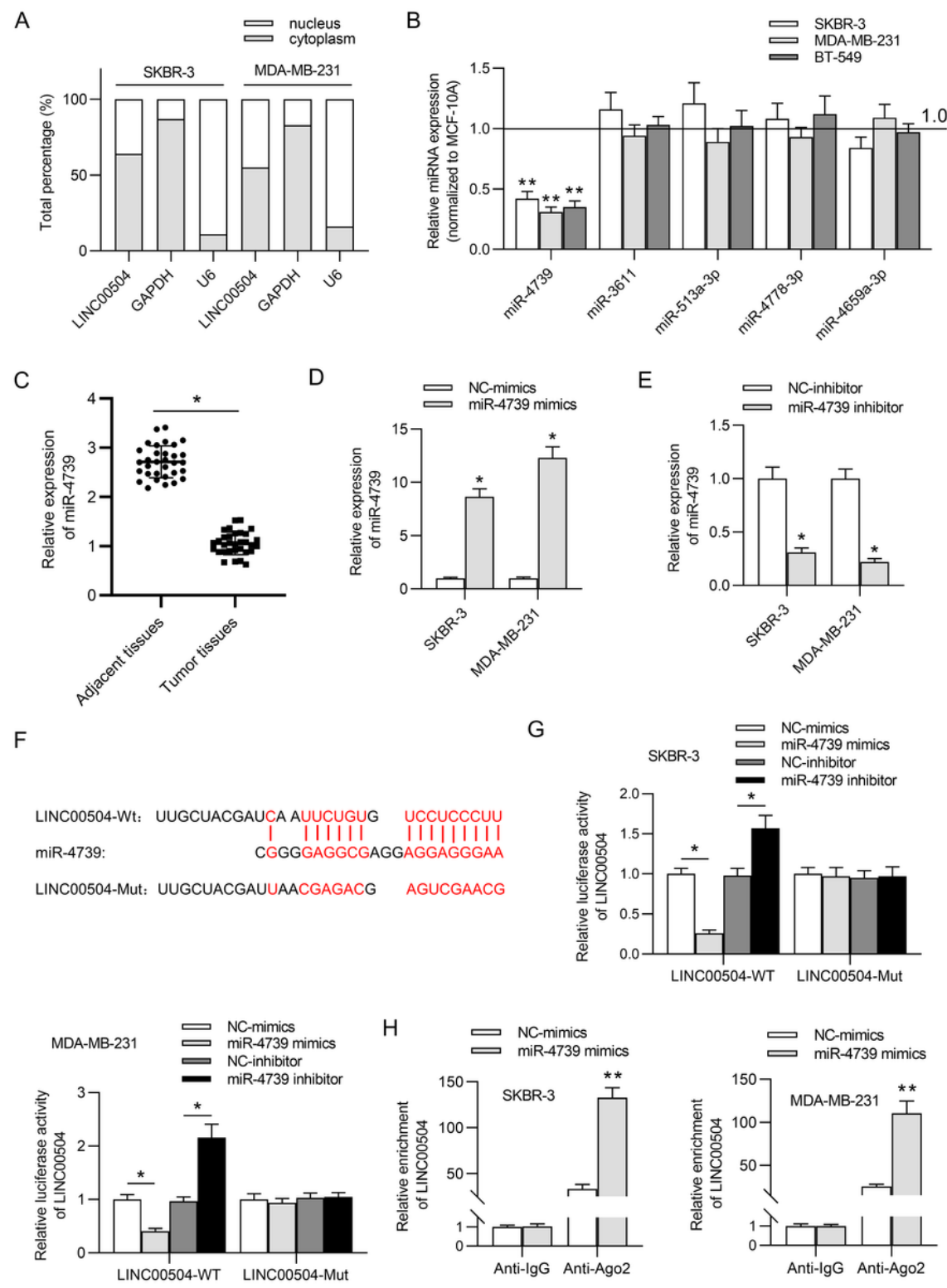


Figure 4

LINC00504 serves as a molecular sponge of miR-4739. (A) Subcellular fraction assay disclosed the location and distribution of LINC00504 in the cytoplasm and nucleus in TNBC cells. (B) RT-qPCR detected the relative expression of 5 potential miRNAs in TNBC cells. (C) The miR-4739 expression in TNBC tissues and adjacent normal tissues was detected. (D) RT-qPCR measured the relative expression of miR-4739 in NC-mimics, miR-4739 mimics group. (E) RT-qPCR determined the relative expression of miR-4739 in NC-inhibitor or miR-4739 inhibitor group. (F) The binding site between miR-4739 and LINC00504 was predicted through starBase v2.0. (G) Luciferase report assay revealed luciferase activity of LINC00504 by miR-4739 mimics or miR-4739 inhibitor. (H) RIP assay revealed relative enrichment of LINC00504 precipitated by anti-IgG and anti-Ago2 in NC-mimics and miR-4739 mimics group. * $p \leq 0.05$, ** $p < 0.01$.

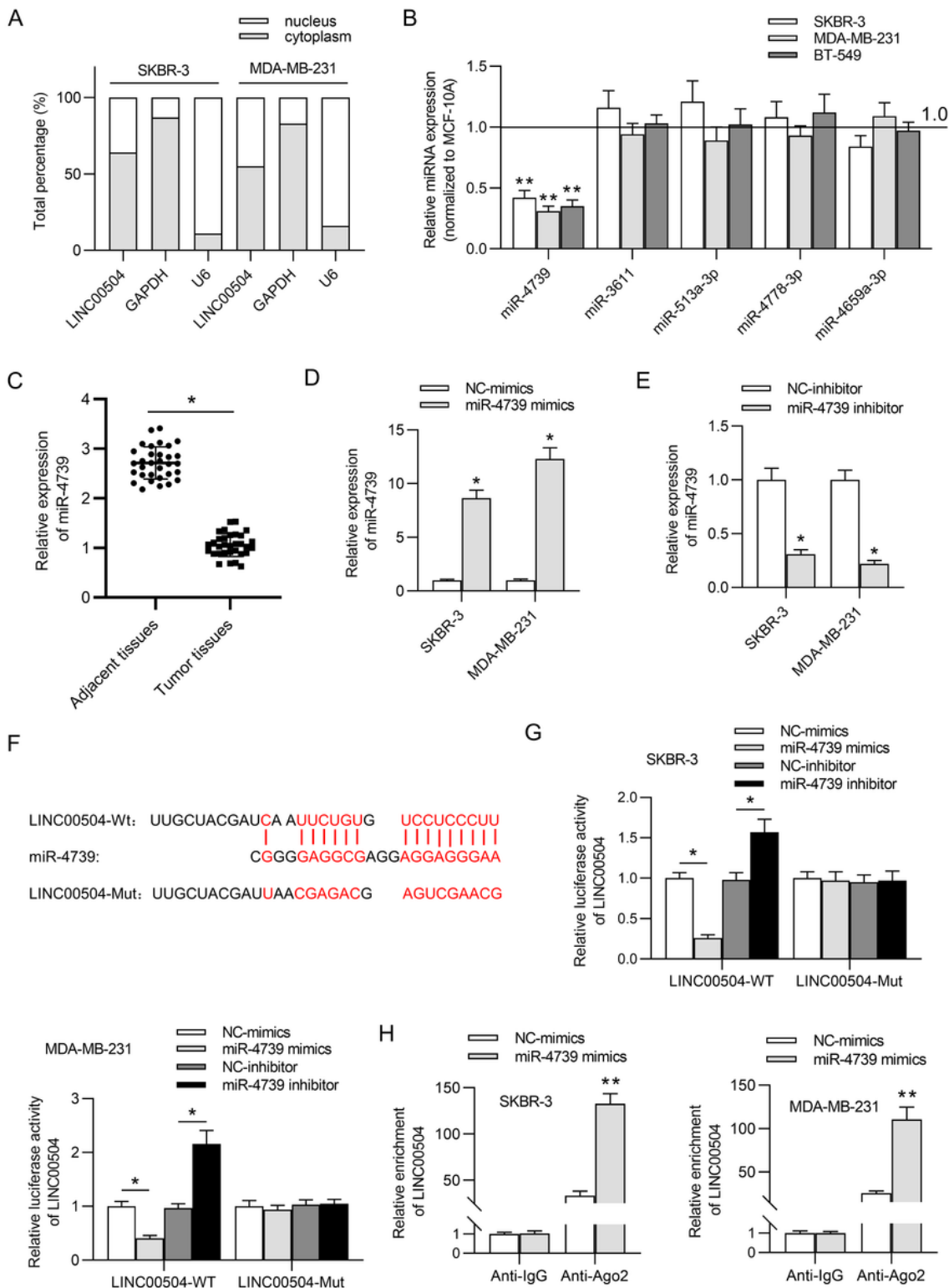


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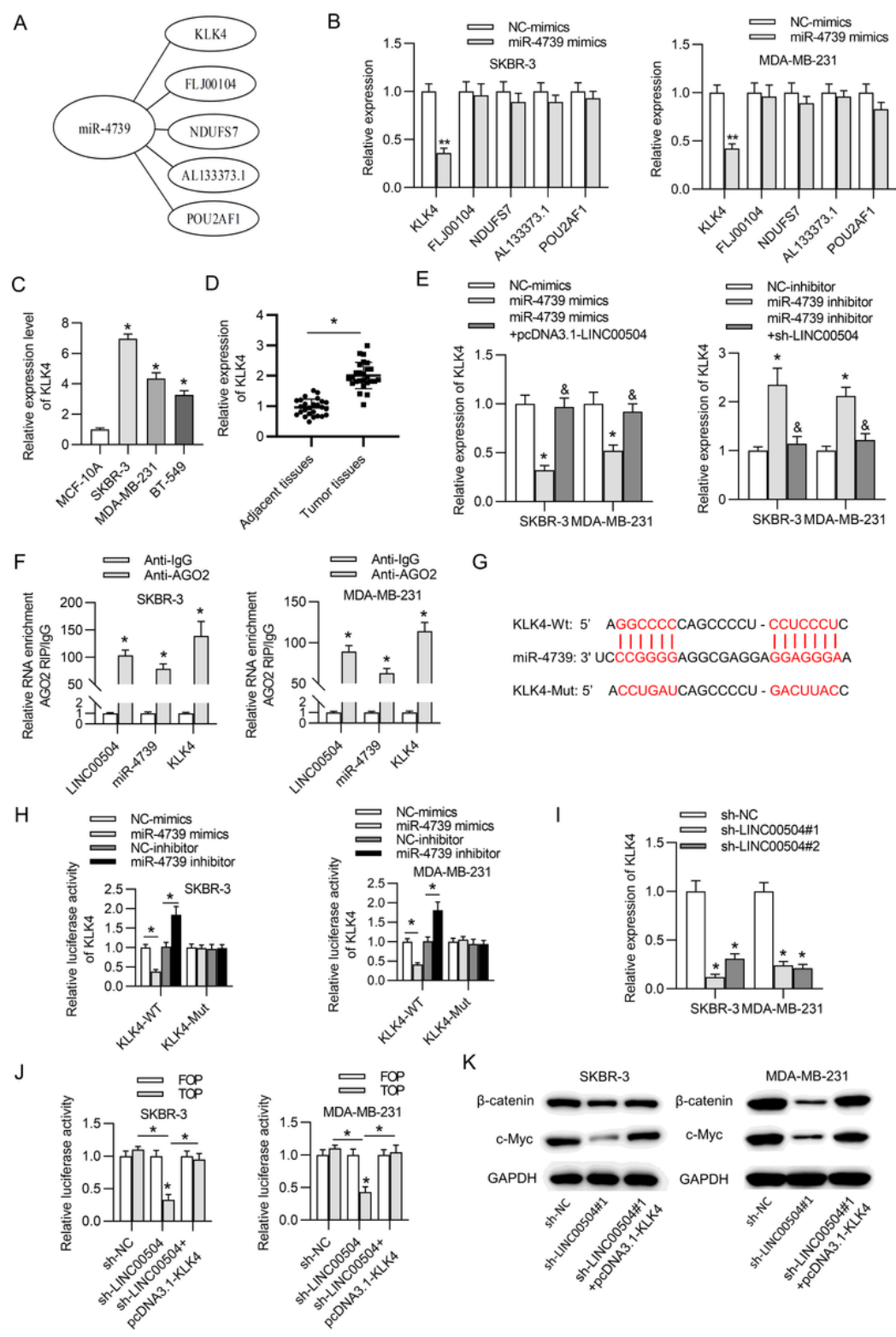


Figure 5

KLK4 is the downstream target of miR-4739. (A) TargetScan website revealed 5 potential target genes of miR-4739. (B) RT-qPCR measured the expressions of five candidate mRNAs after upregulating miR-4739. (C-D) RT-qPCR determined KLK4 expression in TNBC cells and tissues. (E) Relative expression levels of KLK4 in SKBR-3 and MDA-MB-231 cells after transfection with miR-4739 mimics, miR-4739 mimics+pcDNA3.1-LINC00504, miR-4739 inhibitor, miR-4739 inhibitor+pcDNA3.1-LINC00504 were measured by RT-qPCR. (F) RIP assay revealed the relative enrichment of LINC00504, miR-4739 and KLK4. (G) The binding site between miR-4739 and 3'UTR of KLK4 was predicted by TargetScan. (H) Luciferase report assay validated the direct interaction among LINC00504, miR-4739 and KLK4. (I) Knockdown efficiency of KLK4 was verified through RT-qPCR. (J) TOP/FOP flash assay was used to verify the activity of the Wnt/ β -catenin pathway. (K) Western blot showed the rescue effects of KLK4 on the inactivated Wnt/ β -catenin pathway caused by silenced LINC00504. Full-length blots are presented in Supplementary material. The ImageJ software was used to detect densitometry analysis of the band intensity. * $p < 0.05$, ** $p < 0.01$.

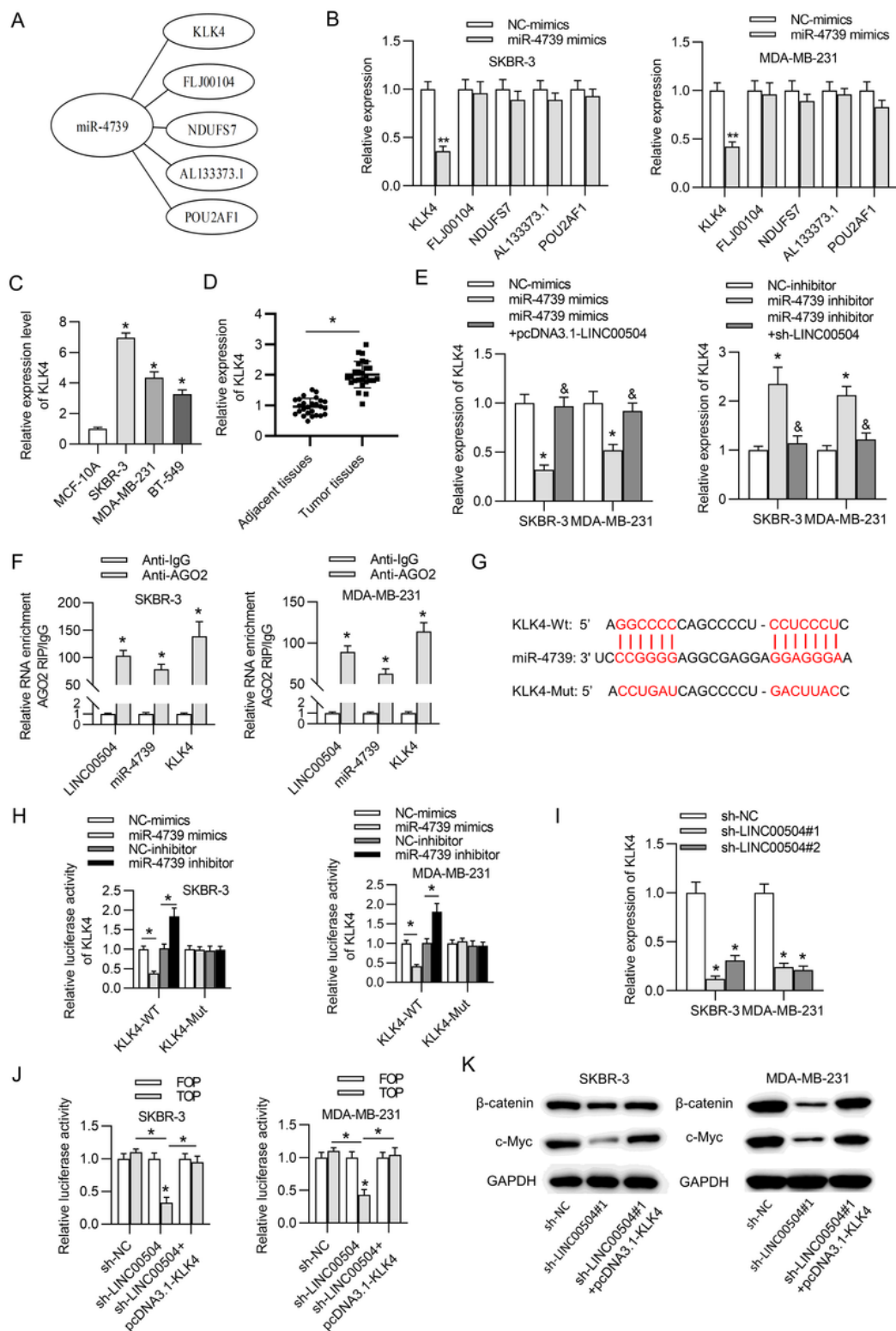


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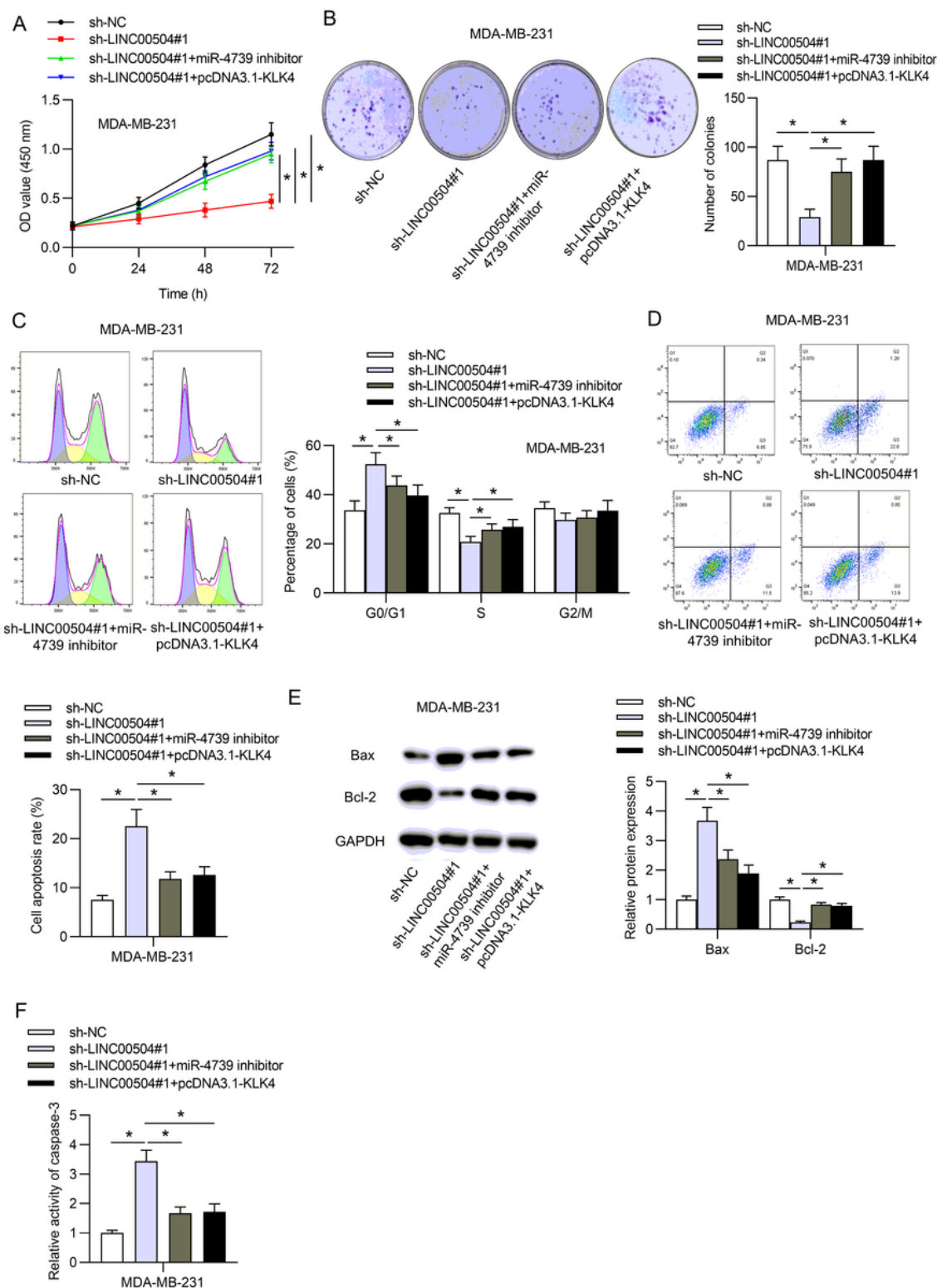


Figure 6

The LINC00504/miR-4739/KLK4 axis promotes cell proliferation, cell cycle progression, and suppresses cell apoptosis by activating the Wnt/ β -catenin pathway in TNBC. (A) Cell viability of MDA-MB-231 cells was detected by CCK-8 assays in 4 groups: sh-NC, sh-LINC00504#1, sh-LINC00504#1+miR-4739 inhibitor, sh-LINC00504#1+ pcDNA3.1-KLK4. (B) Colony formation assay was applied to evaluate the colony numbers. (C) Flow cytometry detected the cell cycle progression. (D) Flow cytometry analysis showed the

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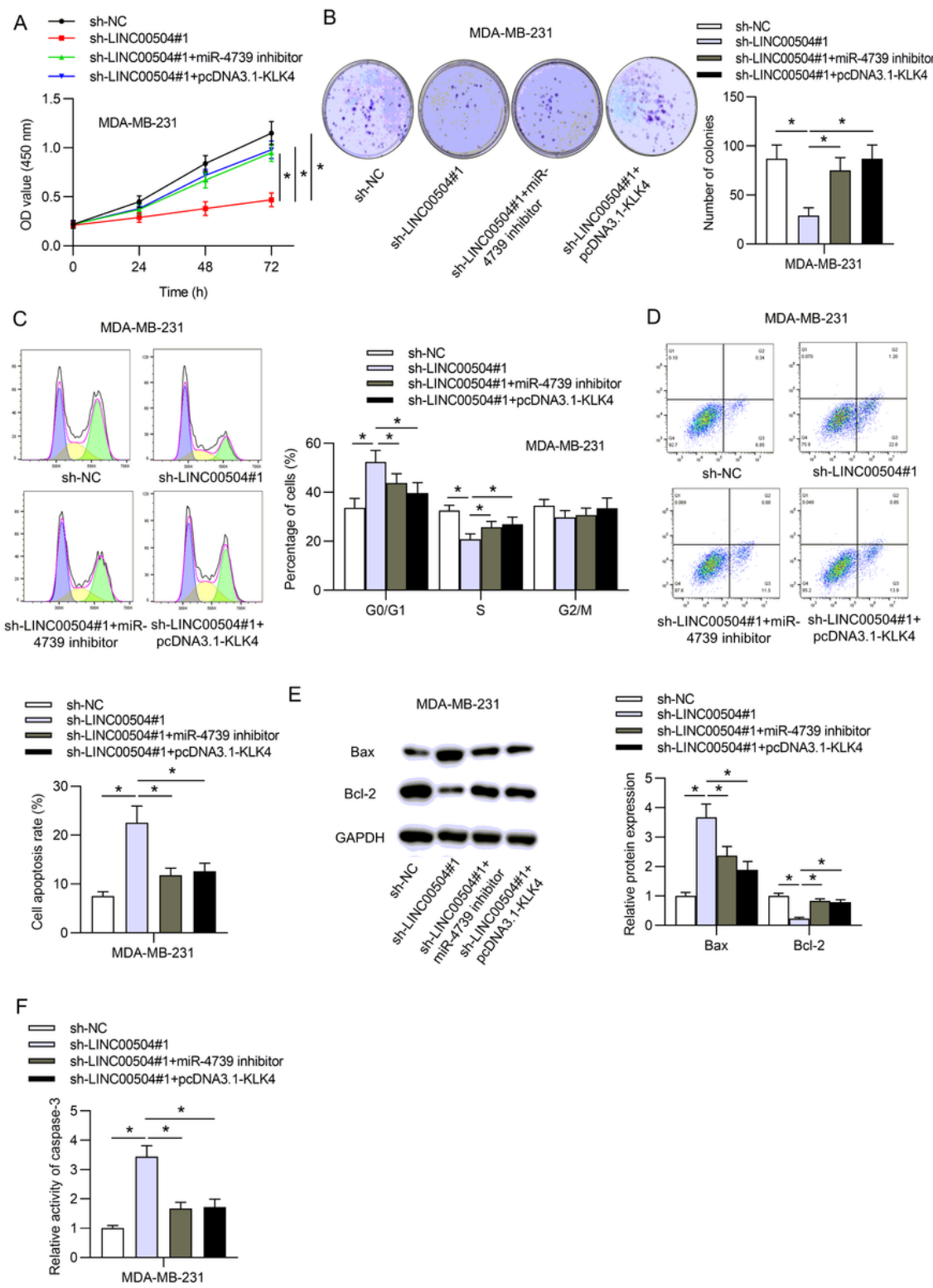


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