Transcription factor KLF16 activates MAGT1 to regulate the tumorigenesis and progression of breast cancer

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Abstract. Breast cancer is the most frequent cause of cancer-related mortality among women worldwide. The present study aimed to explore the role of magnesium transporter protein 1 (MAGT1) in breast cancer and to illustrate the potential underlying molecular mechanisms. Bioinformatic analysis was performed to explore the association between MAGT1 expression and patients with breast cancer. MTT, colony formation, wound healing and Transwell assays were performed to examine the proliferative, migratory and invasive abilities of MCF-7 cells. Western blot analysis was conducted to determine the corresponding protein expression. Chromatin immunoprecipitation and luciferase reporter assays were carried out to reveal the interaction between MAGT1 and the Kruppel-like factor 16 (KLF16). In addition, an experimental animal model was established by the subcutaneous injection of MCF-7 cells into BALB/c nude mice, and tumor weight and size were measured. The results revealed that MAGT1 expression was upregulated in breast cancer. MAGT1 knockdown significantly suppressed the MCF-7 cell proliferative, migratory and invasive abilities, and downregulated the protein expression of Ki67, proliferating cell nuclear antigen, MMP2 and MMP9. MAGT1 knockdown also markedly suppressed tumor growth *in vivo*. Moreover, KLF6 could bind to the MAGT1 promoter and positively regulate MAGT1 expression. The inhibitory effects of KLF6 knockdown on cell proliferation, migration and invasion *in vitro*, and tumor growth *in vivo* were partly abolished by MAGT1 overexpression. On the whole, the findings of the present study suggest that MAGT1 knockdown exerts notable inhibitory effects on the progression of breast cancer, providing a potential therapeutic target for the treatment of breast cancer.

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Introduction

Breast cancer is one of the most common malignant tumors and the most frequent cause of cancer-related mortality among females worldwide, severely endangering the life and health of women (1,2). Even though notable improvements have been made in recent years, 30% of patients with breast cancer are suffer from relapse and metastasis, and patients with metastasis have a 5-year survival rate of only 26%, thus leading to a great burden for families and society (3,4). Breast cancer still remains a major health concern and represents a top biomedical research priority. Epidemiological research has revealed various pathogenic factors that can result in the development of breast cancer, including estrogen, alcohol consumption, obesity and progestin use, as well as genetic mutations (5,6). Recently, advances in genetic testing for individuals who are at a high risk of developing cancer and in targeted gene therapy for breast cancer are rapidly emerging, contributing to a new era in cancer treatment (7). Thus, seeking for novel potential target genes for providing effective therapeutic strategies for breast cancer is of utmost importance.

Magnesium transporter protein 1 (MAGT1), an evolutionally conserved Mg^{2+}-specific ion transport facilitator, possesses five predicted transmembrane regions with a putative signaling sequence and a number of COOH-terminal phosphorylation consensus sites (8,9). It has been demonstrated that MAGT1 is a critical regulator of intracellular free Mg^{2+} levels, and plays a crucial role in coordinating natural killer (NK) and $CD8^+$ T-cell activation (10). Notably, recent studies have indicated

that MAGT1 is overexpressed in hepatocellular carcinoma, glioma and colorectal cancer, demonstrating that it is also associated with the overall survival time and chemotherapeutic efficacy; thus, it may be a novel therapeutic target for cancer treatment (9,11,12). However, the role of MAGT1 has not yet been reported in breast cancer, at least to the best of our knowledge.

The Kruppel-like factors (KLFs), a type of transcription factors, are characterized by three zinc finger DNA binding domains near their C terminus (13). KLFs can bind to GC-rich DNA elements where they can regulate transcription promoter-dependently (14). As a critical member of the KLF family, KLF16 has been reported to coordinate various biological processes including cell growth, death and metabolism, and to participate in multiple diseases (15-17). KLF16 has been reported to affect tumorigenesis and the development of malignant tumors, such as prostate and gastric cancer (14,18). Notably, recent evidence has indicated that KLF16 has critical oncogenic functions in breast cancer, as KLF6 is involved in the proliferation, migration and invasion of breast cancer cells (19).

The present study first investigated the role of MAGT1 in breast cancer. Subsequently, the potential mechanisms of action of MAGT1 and KLF16, particularly their associated roles in the regulation of breast cancer progression were explored. The findings presented herein provide a novel potential biomarker for predicting the development and progression of breast cancer.

Materials and methods

Bioinformatics analysis. The expression of MAGT1 and KLF16 in breast cancer and normal breast samples was retrieved from the Encyclopedia of RNA Interactomes (ENCORI; http://starbase.sysu.edu.cn/index.php), including 1,104 tumor and 113 normal samples. The Human Protein Atlas (HPA; https://www.proteinatlas.org/) was also applied for the analysis of MAGT1 and KLF16 expression by immunohistochemical assay. The binding between MAGT1 promoter and KLF6 was predicted using the JASPAR database (https://jaspar.genereg.net/).

Cells, cell culture and transfection. The MCF-10A (cat no. CRL-10317), MCF-7 (cat no. HTB-22), MDA-MB-231 (cat no. HTB-26) and SK-BR-3 (cat no. HTB-30) cell lines were obtained from the American Type Culture Collection (ATCC). The SUM190PT (cat no. YS1334C) cell line was obtained from Shanghai Yaji Biological Technology Co., Ltd. All cells were cultured in DMEM supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), and maintained at 37˚C in a humidified incubator containing 5% CO_2.

For transfection, short hairpin RNAs (shRNA; pGPU6) targeting KLF6 (shRNA-KLF6-1/2) and MAGT1 (shRNA-MAGT1-1/2) and an empty pGPU6 plasmid which was used as the negative control (shRNA-NC) were obtained from Shanghai GenePharma Co., Ltd. In addition, the pcDNA3.1(+) KLF16 overexpression vector (Oe-KLF16) and empty vector NC (Oe-NC) were supplied by Shanghai GenePharma Co., Ltd. The full-length sequence of MAGT1 was amplified and cloned into the pcDNA3.1 plasmid (Shanghai GenePharma Co., Ltd.) to generate pcDNA-MAGT1, and the pcDNA3.1 empty plasmid was used as its negative control (pcDNA-NC). When the cells reached 70-80% confluency, they were transfected with the corresponding aforementioned plasmids (50 nM) using Lipofectamine 3000® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions at 37˚C for 48 h. Following 48 h of transfection, subsequent experiments were conducted.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the cells using the RNeasy Mini kit (Qiagen, Inc.) and then reverse transcribed into complementary DNA (cDNA) using the Strand cDNA Synthesis kit (Takara Bio, Inc.) using the following thermocycling conditions: 70˚C for 5 min, 42˚C for 1 h and 70˚C for 15 min. qPCR was subsequently carried out using a PrimeScript RT-PCR kit (Qiagen, Inc.) in accordance with the manufacturer's instructions. The thermocycling conditions were as follows: Initial denaturation at 94˚C for 5 min; followed by 22 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec. The mRNA levels were analyzed using the $2^{-\Delta\Delta Cq}$ method (20) and normalized to the reference gene, GAPDH. The primer sequences used were as follows: MAGT1 forward, 5'-CTCAGCCTCTGCCCAAAGAA-3' and reverse, 5'-CAC AAGGCGACGGAACTTGT-3'; KLF16 forward, 5'-CAAGTC CTCGCACCTAAAGTC-3' and reverse, 5'-AGCGGGCGA ACTTCTTGTC-3'; GAPDH forward, 5'-CCATGGGGAAGG TGAAGGTC-3' and reverse, 5'-AGTGATGGCATGGACTGT GG-3'.

Western blot analysis. The MCF-7 cells and mouse tissues were collected and lysed with RIPA buffer on ice for 30 min. After determining the protein concentration using a BCA kit (Beyotime Institute of Biotechnology), the same amount of protein (30 μg/lane) was separated on a 12% SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membranes (MilliporeSigma). The membranes were blocked with 5% (w/v) skimmed milk in TBST for 1 h at room temperature, followed by probing with primary antibodies against MAGT1 (dilution 1:600; cat no. 27994-1-AP, ProteinTech Group, Inc.), Ki67 (dilution 1:1,000; cat no. ab16667, Abcam), proliferating cell nuclear antigen (PCNA; dilution 1:1,000; cat no. ab18197, Abcam), MMP2 (dilution 1:1,000; cat no. ab92536, Abcam), MMP9 (dilution 1:1,000; cat no. ab283575, Abcam), KLF16 (dilution 1:1,000; cat no. orb39548, Biorbyt, Ltd.) and GAPDH (dilution 1:2,500; cat no. ab9485, Abcam) at 4˚C overnight, followed by incubation with goat anti-rabbit horseradish peroxidase-labeled secondary antibody (dilution 1:2,000; cat no. ab6721, Abcam) at room temperature for 2 h. The bands were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia). ImageJ software (version 1.8.0; National Institutes of Health) was used for densitometry.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability was assessed using MTT assay. The transfected cells were seeded in 96-well plates at 37˚C for incubation of 24, 48 and 72 h, respectively. MTT solution (5 mg/ml; MilliporeSigma) was added to each well to incubate

the cells at 37˚C for a further 4 h. Dimethyl sulfoxide (DMSO) was used to dissolve the formazan. The absorbance at 570 nm was measured using a multi-well scanning spectrophotometer (Bio-Rad Model 2550 EIA Reader).

Cell colony formation assay. The MCF-7 cells were plated in six-well plates in DMEM containing 10% FBS at 37˚C in a humidified incubator containing 5% CO_2. The medium was refreshed every 3 days. After 2 weeks, the cells were washed with PBS, fixed with 4% formaldehyde (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature and stained with crystal violet (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature for observation.

Wound healing assay. The MCF-7 cells were plated in six-well plates in DMEM containing 10% FBS at 37˚C in a humidified incubator containing 5% CO_2. After 24 h, a straight line was drawn using a 200 μl pipette tip. The cells were then washed with PBS three times and incubated in serum-free medium for 48 h. The healing of the scratches was observed under a light microscope (Olympus Corporation) at 0 and 24 h. The gap distance was quantitatively evaluated using ImageJ software (1.8.0 172 version; National Institutes of Health). Migration (%)=[(0 h average scratch distance-24 h average scratch distance)/0 h average scratch distance] x100.

Transwell assay. A total of $1x10^5$ MCF-7 cells was suspended in 200 μl DMEM without FBS and inoculated into the upper chamber of 24-well Transwell inserts (Corning Falcon; Corning, Inc.) pre-coated with Matrigel (BD Biosciences). Subsequently, 500 μl DMEM containing 10% FBS were added to the lower chamber. The Transwell was incubated at 37˚C in a humidified incubator containing 5% CO_2. After 24 h, the non-invaded cells were wiped out using a cotton swab, and the cells that had invaded to the bottom of the insert was fixed with 4% formaldehyde for 10 min and stained with crystal violet for 10 min at room temperature. The stained cells were observed under a light microscope (Olympus Corporation).

Dual luciferase reporter gene assay. KLF16 binding motif and MAGT1 promoter (full length, FL) or serial truncations (E1 Del and E2 Del) were cloned into the pGL3-basic vector (E1761; Promega Corporation). A total of $1x10^5$ MCF-7 cells were seeded in 24-well plates. On the following day, the cells were transfected with the pGL3-based reporter constructs (2 μg) and pRL-SV40 vector (2 μg) using Lipofectamine 3000® (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, the Firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega Corporation), and normalized to *Renilla* activity.

Chromatin immunoprecipitation (ChIP) assay. The MCF-7 cells were cross-linked with 1% formaldehyde at room temperature for 10 min and quenched in 125 mM glycine for 5 min. The cell lysates were then sonicated into fragments. Subsequently, 100 μl lysates containing chromatin fragments were immunoprecipitated with antibody against KLF16 (5 μg; cat. no. sc-377519; Santa Cruz Biotechnology, Inc.) or IgG (5 μg; cat. no. 2729; Cell Signaling Technology, Inc.) at 4˚C overnight. The immune complexes were recovered by the addition of A/G-agarose beads (Santa Cruz Biotechnology, Inc.). The precipitated DNA was purified using the ChIP DNA Clean & Concentrator kits (Zymo Research) and then analyzed using RT-qPCR as described above.

In vivo experiments. A total of 36 male BALB/c nude mice weighing 18-22 g were obtained from HFK Bioscience Co, Ltd. and housed in a standard environment with a controlled temperature (20±2˚C), humidity (50±5%), a 12/12-h light/dark cycle and free access to water and food. The mice were allowed to acclimatize to their environment for 1 week prior to the experiments. All animal experiment procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals of The Second Clinical Medical College of North Sichuan Medical College and were approved by the Ethics Committee of The Second Clinical Medical College of North Sichuan Medical College (Nanchong, China; approval no. NSMC-2021-94). Mice (6 mice per group) were randomly divided into three groups in the first section as follows: The control group, shRNA-NC group and shRNA-MAGT1 group. In subsequent experiments, mice (6 mice per group) were randomly divided into three groups as follows: The control group, sh-KLF16 group and sh-KLF16s+pcDNA-MAGT1 group. Approximately $1x10^7$ transfected MCF-7 cells were subcutaneously injected into the right axillary of the mice. After the tumor was formed, the body weight and tumor size of the mice were recorded every 3 days, and the tumor size was calculated using the following equation: 1/2 x length x $width^2$. At the end of the 21st day, the 36 mice were euthanized by an intraperitoneal injection of 120 mg/kg sodium pentobarbital, and the tumors were removed after the heartbeat cessation and respiratory arrest of the nude mice were confirmed. The mice were monitored every day, and the humane endpoints were the following: A marked reduction in food or water intake, labored breathing, inability to stand and no response to external stimuli. No abnormal signs that signified the humane endpoints of the experiment were observed in any of the mice during the experiment. The tumors were frozen at -80˚C for further analysis.

Statistical analysis. The mean ± standard deviation (SD) for each independent assay was used to present the experimental data. The association of survival with MAGT1 expression was estimated using the Kaplan-Meier analysis with the log-rank test. An unpaired student's t-test or one-way ANOVA assay followed by Tukey's post hoc test were applied for comparisons between two groups or among more than two groups, respectively. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

MAGT1 is upregulated in tumor samples of breast cancer. Firstly, bioinformatics analysis was conducted to examine the expression level of MAGT1 in breast cancer. As shown in Fig. 1A, the analysis of the ENCORI database (http://starbase.sysu.edu.cn/index.php) revealed a relatively high expression of MAGT1 in breast cancer samples in comparison to normal samples ($P<0.01$). The immunohistochemical data obtained from the HPA database (https://www.proteinatlas.org/) further verified the upregulated expression level of MAGT1 in breast

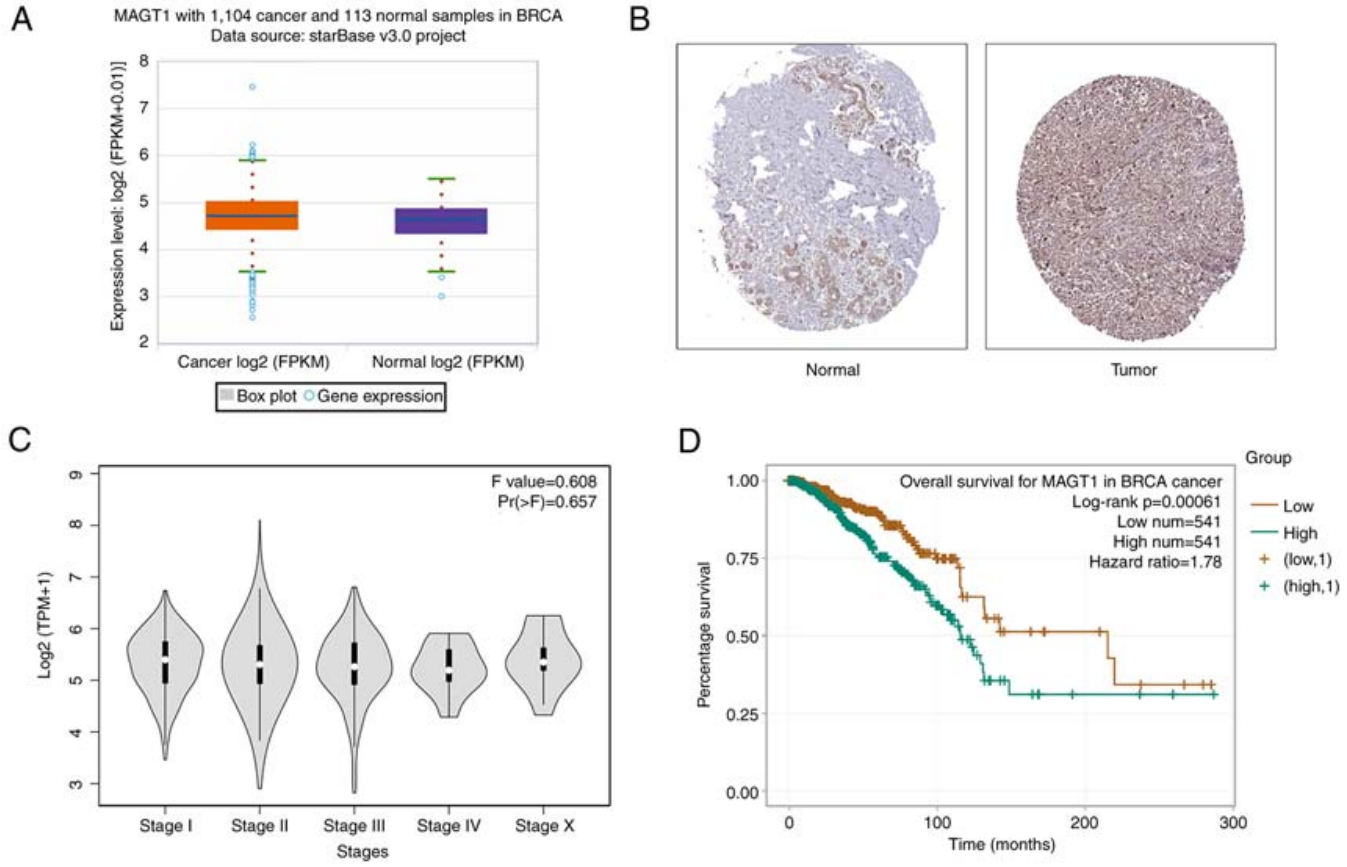

Figure 1. MAGT1 is upregulated in tumor samples of breast cancer. (A) The ENCORI database (http://starbase.sysu.edu.cn/index.php) was used to analyze the expression level of MAGT1 in cancer samples and normal samples in breast cancer. (B) The immunohistochemical data on MAGT1 expression in cancer samples and normal samples in breast cancer were obtained from the Human Protein Atlas database (https://www.proteinatlas.org/). The association between MAGT1 expression and (C) tumor stage, and (D) survival time, was analyzed using the ENCORI database. MAGT1, magnesium transporter protein 1; ENCORI, Encyclopedia of RNA Interactomes.

tumor tissues (Fig. 1B). Further analysis revealed that there was no obvious association between MAGT1 expression and the tumor stage of breast cancer (Fig. 1C). The overall survival assay revealed that the high expression of MAGT1 was significantly associated with a poor survival time (Fig. 1D). These data suggested that MAGT1 expression was upregulated in breast cancer, and the upregulated expression of MAGT1 predicted a poor outcome of patients.

MAGT1 knockdown suppresses the proliferation, migration and invasion of MCF-7 cells. To explore the specific role of MAGT1 in breast cancer, the MCF-7 cells were selected for use in further experiments, as these cells exhibited a significantly high expression of MAGT1 in comparison to the MCF-10A cells, and exhibited the highest expression level of MAGT1 among several breast cancer cell lines (MCF-7, MDA-MB-231, SK-BR-3 and SUM190PT cells) (Fig. 2A). Due to the high expression level of MAGT1 in breast cancer, the MCF-7 cells were transfected with shRNA-MAGT1-1 or shRNA-MAGT1-2 to achieve MAGT1 knockdown. As shown in Fig. 2B and C, transfection with both shRNA-MAGT1-1 and shRNA-MAGT1-2 resulted in the significantly decreased mRNA and protein expression of MAGT1; however, as shRNA-MAGT1-2 decreased MAGT1 to a greater degree, it was thus applied for use in subsequent experiments. A series of cellular biological experiments were then carried out to evaluate the effects of MAGT1 on the biological behaviors of breast cancer cells. MTT and colony formation assays revealed that MAGT1 knockdown markedly restricted cell viability and the formation of cell colonies, suggesting that the cell proliferative ability was suppressed upon MAGT1 knockdown in MCF-7 cells. This was also verified by the downregulated protein expression of Ki67 and PCNA in the shRNA-MAGT1 group, compared to the shRNA-NC group (Fig. 2D-F). In addition, the decreased wound healing and invasive abilities of the cells in the shRNA-MAGT1 group, accompanied by the decreased protein expression of MMP2 and MMP9, demonstrated that MAGT1 knockdown also restricted the cell migratory and invasive abilities of the MCF-7 cells (Fig. 2G-J).

MAGT1 knockdown inhibits tumor growth in vivo. To further validate the aforementioned findings *in vitro*, and *in vivo* experiment was conducted. Male BALB/c nude mice were subcutaneously injected with MCF-7 cells transfected with shRNA-NC or shRNA-MAGT1. As shown in Fig. 3A-C, the tumor size and tumor weight were markedly decreased when MAGT1 was knocked down. In particular, the mouse body weight and tumor size were monitored every 3 days before the mice were sacrificed. No evident differences in body weight were observed; however, the tumor volume was significantly decreased upon MAGT1 knockdown (Fig. 3D and E). Furthermore, the protein expression level of Ki67, PCNA,

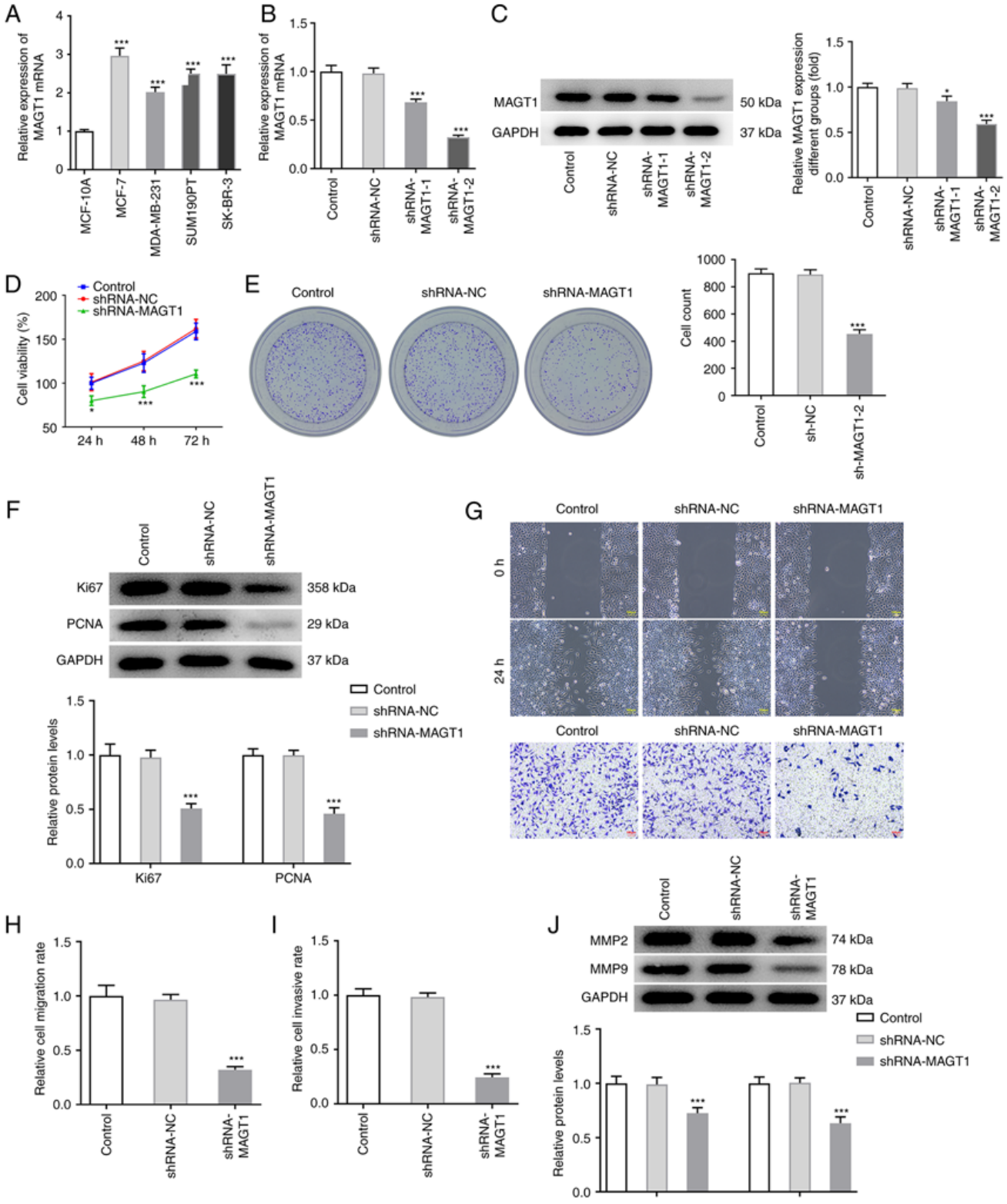

Figure 2. MAGT1 knockdown suppresses the proliferation, migration and invasion of MCF-7 cells. (A) The expression level of MAGT1 in multiple breast cancer cell lines (MCF-7, MDA-MB-231, SK-BR-3 and SUM190PT cells) and MCF-10A cells was determined using RT-qPCR. ***P<0.001 vs. MCF-10A cells. MCF-7 cells were transfected with shRNA-MAGT1-1 or shRNA-MAGT1-2, or shRNA-NC, and the expression level of MAGT1 was measured using (B) RT-qPCR and (C) western blot analysis. MCF-7 cells were transfected with shRNA-NC and shRNA-MAGT1, respectively. (D) Cell viability was then evaluated using MTT assay. (E) Cell colony formation assay was conducted. (F) Protein expression of Ki67 and PCNA was detected using western blot analysis. (G) Wound healing and Transwell assays were performed to examine cell migration and invasion, respectively. (H) Quantification of cell migration rate. (I) Quantification of cell invasion rate. (J) Protein expression of MMP2 and MMP9 was determined using western blot analysis. All experiments were performed in triplicate. *P<0.05 and ***P<0.001 vs. shRNA-NC. MAGT1, magnesium transporter protein 1; RT-qPCR, reverse transcription-quantitative PCR.

MMP2 and MMP9 exhibited a notable decrease in the tumor tissues of mice injected with cells in which was MAGT1 knocked down (Fig. 3F). This finding was consistent with the *in vitro* findings.

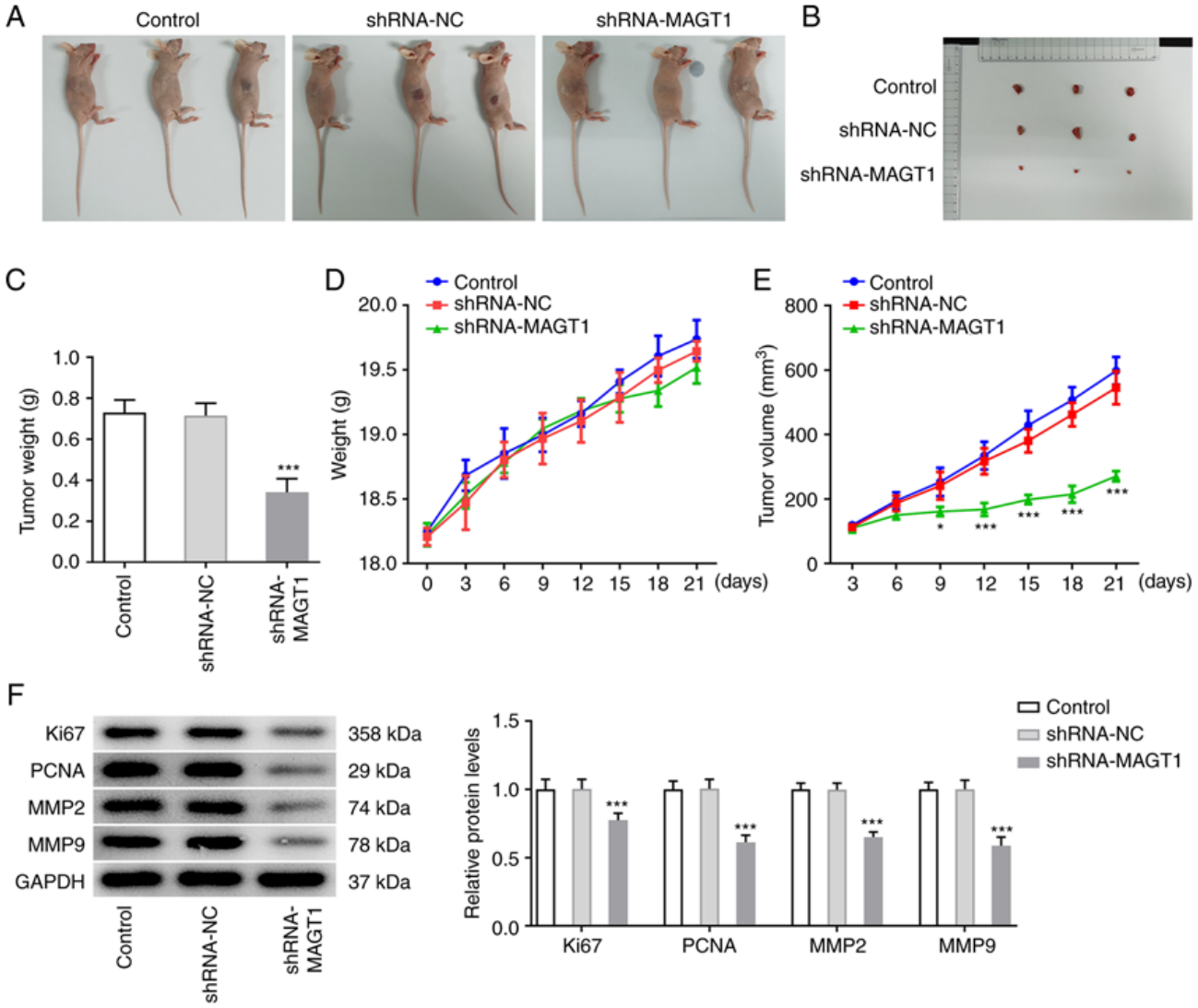

Figure 3. MAGT1 knockdown inhibits tumor growth *in vivo*. (A) Male BALB/c nude mice were subcutaneously injected with MCF-7 cells transfected with shRNA-NC or shRNA-MAGT1. After sacrifice, the mice with tumors were imaged. (B) The tumors were removed and imaged. (C) Tumor weight was recorded. Before sacrifice, the (D) body weight and (E) tumor volume was recorded every 3 days. (F) The protein expression of Ki67, PCNA, MMP2 and MMP9 in tumor tissues of mice was examined using western blot analysis. All experiments were performed in triplicate. *P<0.05 and ***P<0.001 vs. shRNA-NC. MAGT1, magnesium transporter protein 1; PCNA, proliferation cell nuclear antigen.

KLF16 is upregulated in tumor tissues of breast cancer and regulates MAGT1 through transcriptional activation. Subsequently, to identify the role of KLF16 in breast cancer, its expression in breast cancer was examined using bioinformatics analysis. As exhibited in Fig. 4A and B, the expression of KLF16 in tumor samples was higher than that in normal samples, according to the ENCORI and HPA databases. Even though KLF16 expression was not associated with tumor stage (Fig. 4C), these results also indicated a potential involvement of KLF16 in breast cancer. Of note, it was predicted using the JASPAR database that there were two potential KLF16 response elements (E1 and E2) binding to the MAGT1 promoter (Fig. 5A). To further ensure the association between KLF16 and MAGT1, the MCF-7 cells were first transfected with sh-KLF16-1/2 and sh-NC, respectively. The results revealed that the expression level of KLF16 was markedly decreased by transfection with sh-KLF16-1/2 (Fig. 5B and C). Due to a higher transfection efficacy, sh-KLF16-1 was selected for use in further experiments. KLF16 knockdown was then found to exert an inhibitory effect on the expression level of MAGT1 (Fig. 5D). Subsequently, luciferase reporter assay demonstrated that KLF16 knockdown reduced the transcriptional activity of MAGT1 (Fig. 5E). To determine which responsive elements were mainly responsible for the regulatory effects of KLF16 on MAGT1, the MCF-7 cells were co-transfected with serial truncations (E1 Del and E2 Del) of the MAGT1 promoter and Oe-NC/Oe-KLF16. As shown in in Fig. 5F, the transcriptional activity of MAGT1 was strictly limited in the E1 Del group upon KLF16 overexpression, indicating that E1 in the MAGT1 promoter was the main response element for this binding association between KLF16 and the MAGT1 promoter. This result was then verified by ChIP assay, as KLF16 was enriched at the ZNF217 promoter within the E2 region (Fig. 5G).

The inhibitory effects of KLF16 on the cell proliferation, migration and invasion, and tumor growth are diminished by MAGT1 overexpression. As was expected, KLF16 was also highly expressed in breast cancer cell lines, particularly

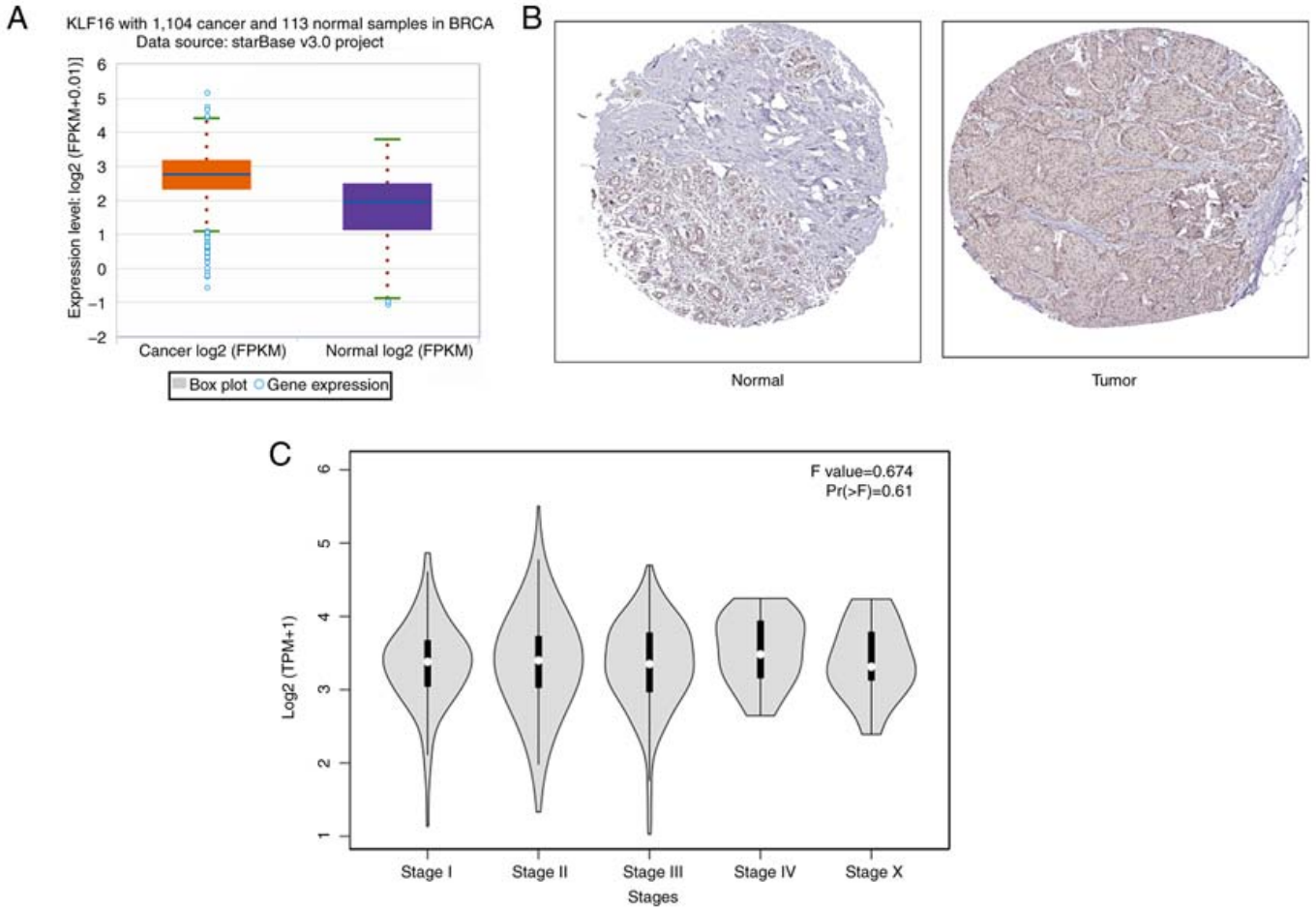

Figure 4. KLF16 is upregulated in breast tumor tissues. (A) The ENCORI database (http://starbase.sysu.edu.cn/index.php) was used to analyze the expression level of KLF16 in cancer samples and normal samples in breast cancer. (B) The immunohistochemical data on KLF16 expression in cancer samples and normal samples in breast cancer were obtained from the Human Protein Atlas database (https://www.proteinatlas.org/). (C) The association between KLF16 expression and tumor stage was analyzed using the ENCORI database. KLF16, Kruppel-like factor 16; ENCORI, Encyclopedia of RNA Interactomes.

in the MCF-7 cells (Fig. 6A). Subsequently, through gain- and loss-of-function experiments, the effects of KLF16 and MAGT1 on breast cancer progression were investigated. Firstly, transfection with pcDNA-MAGT1 successfully overexpressed MAGT1 expression at the mRNA and protein level in MCF-7 cells (Fig. 6B and C). The MCF-7 cells were then transfected with shRNA-NC or shRNA-KLF16 alone, or co-transfected with shRNA-KLF16 and pcDNA-NC/pcDNA-MAGT1. The mRNA and protein expression level of MAGT1 was decreased by KLF16 knockdown, followed by a restoration by a simultaneous transfection with pcDNA-MAGT1 (Fig. 6D and E).

In addition, a series of cellular biological behaviors were assessed using MTT, colony formation, wound healing and Transwell assays, as aforementioned. The results revealed that KLF16 knockdown markedly reduced cell viability and cell colonies, and also decreased the protein expression of Ki67 and PCNA, suggesting that KLF16 knockdown suppressed MCF-7 cell proliferation (Fig. 6F-H). In addition, the suppressive effects of KLF16 knockdown on cell migration and invasion were also evidenced by the hindered wound healing and decreased number of invasive cells, accompanied by the downregulated protein expression of MMP2 and MMP9 (Fig. 6I-L). Nevertheless, simultaneous transfection with pcDNA-MAGT1 and sh-KLF16 partly abolished these suppressive effects of KLF16 knockdown on cell proliferation, migration and invasion compared with transfection with sh-KLF16 alone (Fig. 6F-L). Eventually, these *in vitro* findings were also verified *in vivo*. Male BALB/c nude mice were subcutaneously injected with MCF-7 cells transfected with shKLF16 or co-transfected with sh-KLF16 and pcDNA-MAGT1. As illustrated in Fig. 7A-C, the reduced tumor size and tumor weight induced by KLF16 knockdown were partly reversed by MAGT1 overexpression. The mouse body weight continued to increase with time prolonging before, without notable differences among the groups (Fig. 7D). The speed of tumor growth during this period was hindered by KLF16 knockdown, which was partly abolished by MAGT1 overexpression (Fig. 7E). Furthermore, the reduced protein expression of Ki67, PCNA, MMP2 and MMP9 by KLF16 knockdown was markedly elevated by MAGT1 overexpression (Fig. 7F).

Discussion

Breast cancer has continued to be the leading cause of cancer-related mortality among females worldwide for years (21). Despite the fact that the current therapeutic management of breast cancer can control primary tumor growth, the high invasiveness of breast cancer cells predisposes the tumor

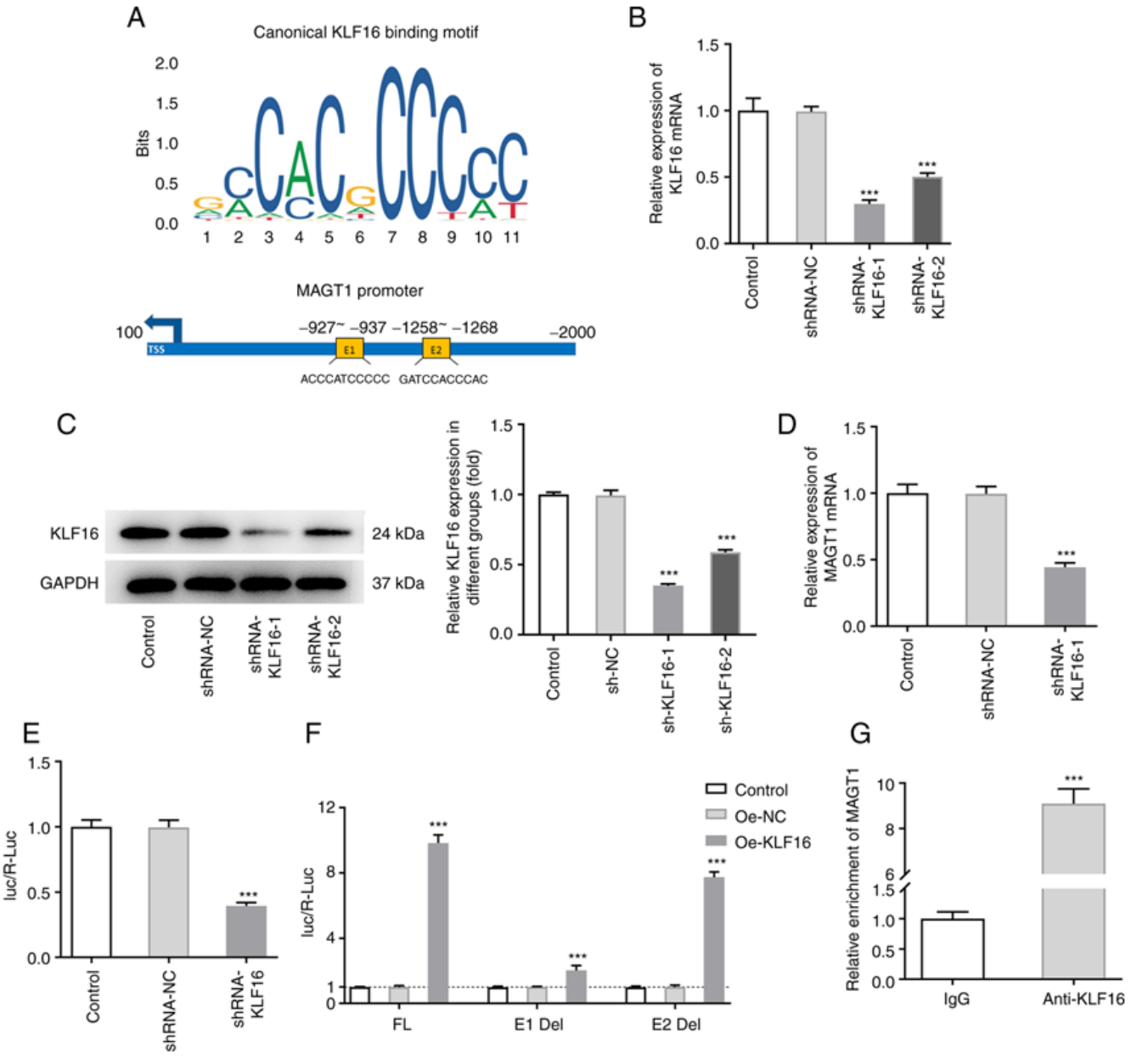

Figure 5. KLF16 regulates MAGT1 through transcriptional activation. (A) The JASPAR database (https://jaspar.genereg.net/) predicted that there were two potential KLF16 responsive elements (E1 and E2) binding to MAGT1 promoter. MCF-7 cells were first transfected with sh-KLF16-1/2 and sh-NC, respectively, and the expression level of KLF16 was then detected using (B) RT-qPCR and (C) western blot analysis. (D) MCF-7 cells were transfected with sh-KLF16 and sh-NC, and the expression level of MAGT1 was detected using RT-qPCR. (E) MAGT1 promoter transcriptional activity upon KLF16 silencing was evaluated by luciferase reporter assay. ***P<0.001 vs. sh-NC. (F) The relative luciferase activity of MAGT1 promoter deletion mutants (E1 Del and E2 Del) upon KLF16 overexpression was detected using luciferase reporter assay. All experiments were performed in triplicate. ***P<0.001 vs. Oe-NC. (G) Immunoprecipitated chromatin fragments were analyzed using RT-qPCR. ***P<0.001 vs. IgG. KLF16, Kruppel-like factor 16; MAGT1, magnesium transporter protein 1; RT-qPCR, reverse transcription-quantitative PCR.

to metastasis, resulting in relapses and deterioration (22). Thus, it is necessary to expand the knowledge of the pathogenesis of breast cancer, and to identify strategies with which to prevent or attenuate the metastasis of breast cancer.

The mechanisms of invasion and metastasis of breast cancer cells are complex and involve the abnormal expression of various genes (23). It has been reported that gremlin-1 (GREM1) expression is significantly higher in breast carcinoma tissues than that in corresponding normal tissues. GREM1 contributes to the proliferation, migration and invasion of breast cancer cells (24). Additionally, Aldo-keto reductase family 1, member B10 has also been found to be overexpressed in breast cancer tissues, which was then demonstrated to promote breast cancer cell migration and invasion (25). Thus, targeting an effective gene functioning on cell proliferation, migration and invasion is an alternative option to develop therapies for breast cancer. MAGT1 is a chromosome X-linked gene encoding a highly selective Mg^{2+} transporter, and its critical role in temporally coordinating NK and $CD8^+$ T-cell activation has been widely recognized (10,26). Recently, the importance of MAGT1 in tumor progression has been focused on by scholars and illustrated in multiple studies. For instance, Li *et al* (27) disclosed that MAGT1 functioned as a crucial targeted gene for miR-628-5p, which was responsible

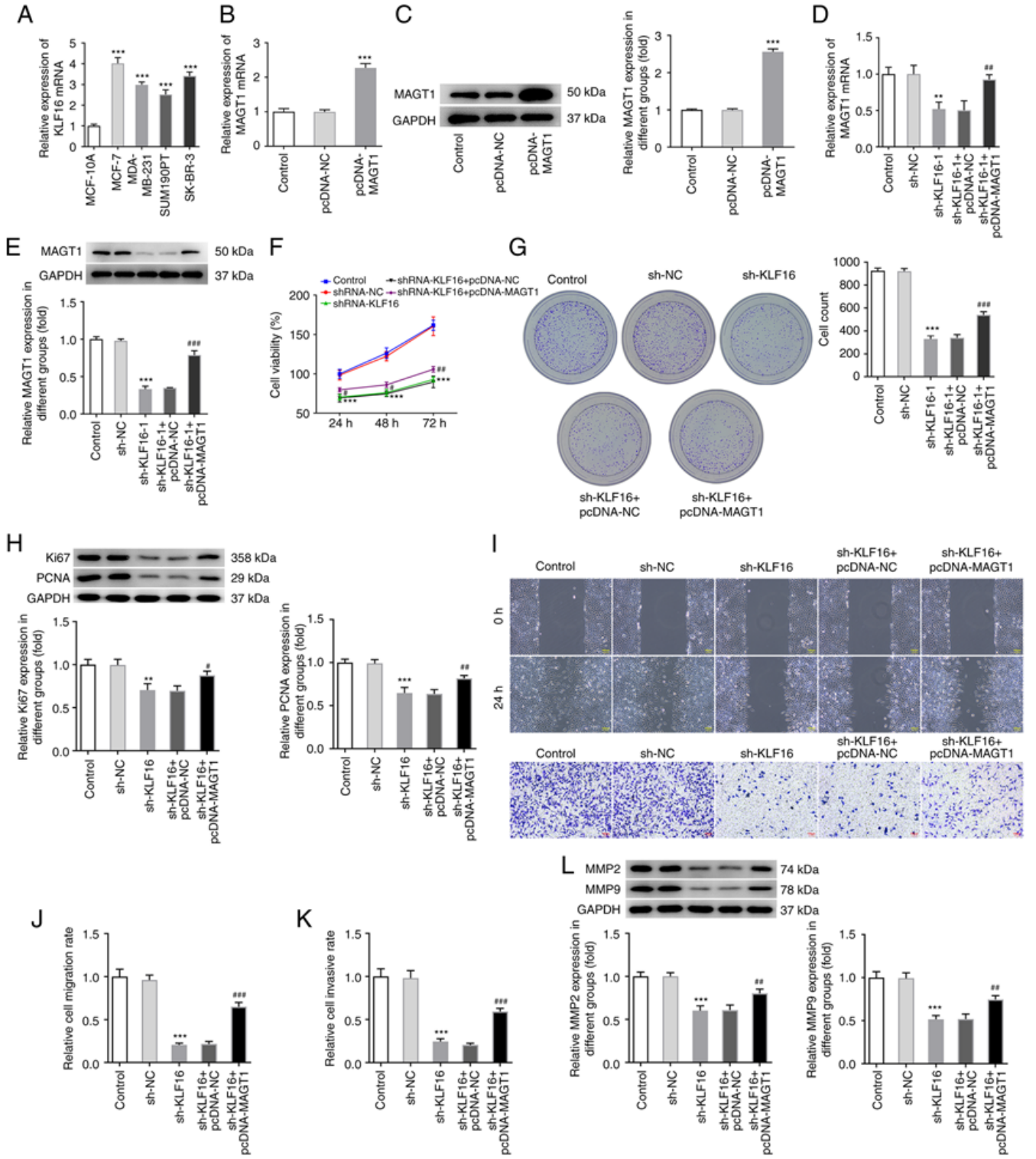

Figure 6. The inhibitory effects of KLF16 on the cell proliferation, migration and invasion are diminished by MAGT1 overexpression. (A) The expression level of KLF16 in multiple breast cancer cell lines (MCF-7, MDA-MB-231, SK-BR-3, and SUM190PT cells) and MCF-10A cells was determined using RT-qPCR. ***P<0.001 vs. MCF-10A cells. MCF-7 cells were transfected with pcDNA-NC or pcDNA-MAGT1, and the expression level of MAGT1 was measured using (B) RT-qPCR and (C) western blot analysis. MCF-7 cells were transfected with shRNA-NC or shRNA-KLF16 alone, or co-transfected with shRNA-KLF16 and pcDNA-NC/pcDNA-MAGT1. The expression level of MAGT1 was measured using (D) RT-qPCR and (E) western blot analysis. (F) Cell viability was then evaluated using MTT assay. (G) Cell colony formation assay was conducted. (H) Protein expression of Ki67 and PCNA was detected using western blot analysis. (I) Wound healing and Transwell assays were performed to examine cell migration and invasion, respectively. (J) Quantification of cell migration rate. (K) Quantification of cell invasion rate. (L) Protein expression of MMP2 and MMP9 was determined using western blot analysis. All experiments were performed in triplicate. **P<0.01 and ***P<0.001 vs. shRNA-NC; #P<0.05, ##P<0.01 and ###P<0.001 vs. sh-KLF16 + pcDNA-NC. Kruppel-like factor 16; MAGT1, magnesium transporter protein 1; RT-qPCR, reverse transcription-quantitative PCR; PCNA, proliferation cell nuclear antigen.

for sevoflurane-mediated glioma progression. Bi *et al* (28) demonstrated that MAGT1 was indispensable for cervical cancer cell proliferation and cell cycle progression by modulating the ERK/p38 MAPK signaling pathway. In addition, the

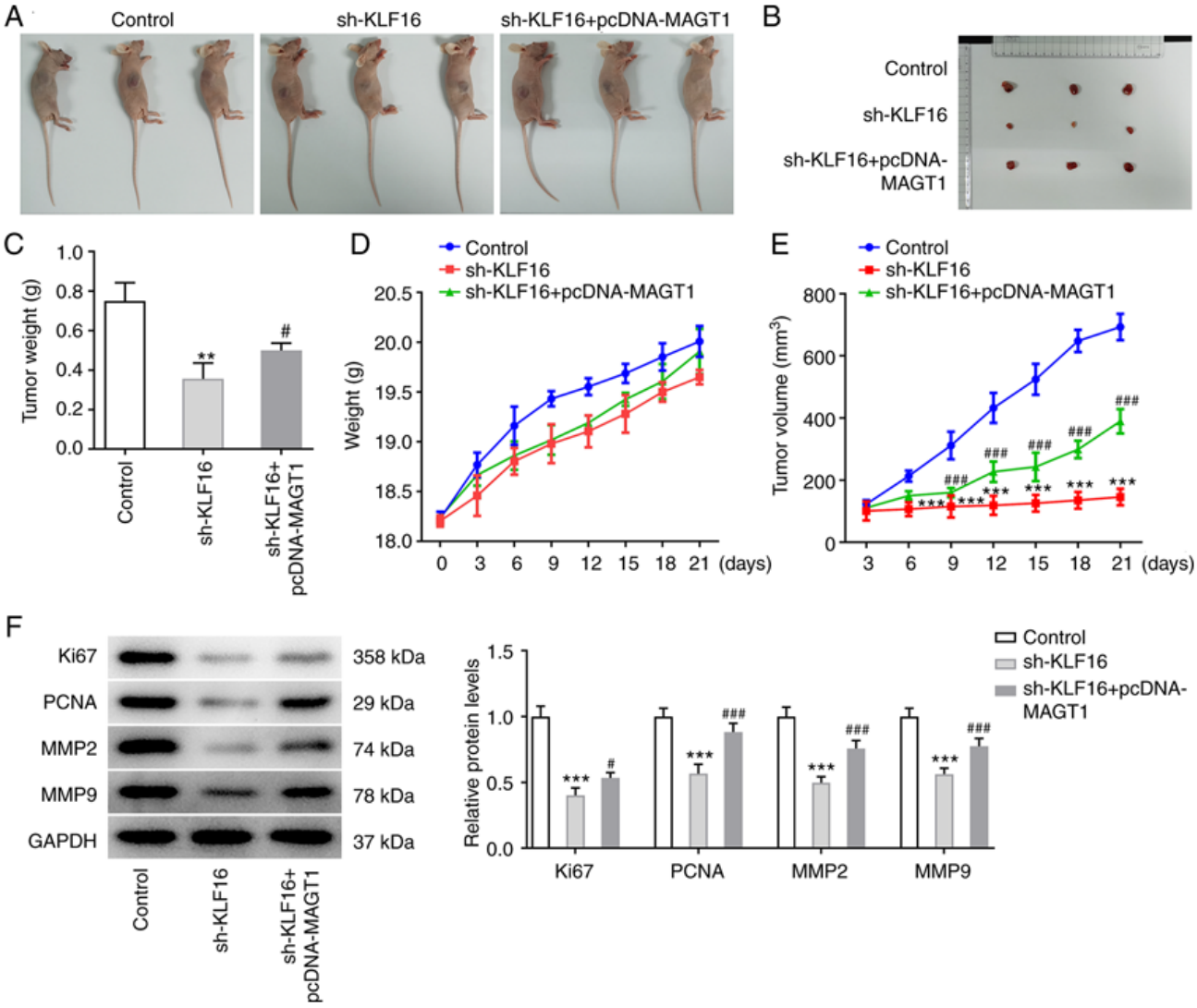

Figure 7. The inhibitory effect of KLF16 on tumor growth is diminished by MAGT1 overexpression. (A) Male BALB/c nude mice were subcutaneously injected with MCF-7 cells transfected with sh-KLF16 or co-transfected with sh-KLF16 and pcDNA-MAGT1. After sacrifice, the mice with tumors were imaged. (B) The tumors were removed and imaged. (C) The tumor weight was recorded. Before sacrifice, the (D) body weight and (E) tumor volume was recorded every 3 days. (F) The protein expression of Ki67, PCNA, MMP2 and MMP9 in tumor tissues of mice was examined using western blot analysis. All experiments were performed in triplicate. **P<0.01 and ***P<0.001 vs. shRNA-NC; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. sh-KLF16 + pcDNA-NC. Kruppel-like factor 16; MAGT1, magnesium transporter protein 1; PCNA, proliferation cell nuclear antigen.

overexpression of MAGT1 has been linked to tumor metastasis and anticancer drug resistance in colorectal cancer (12). Nevertheless, the role of MAGT1 in breast cancer has not been addressed to date, at least to the best of our knowledge. The present study was the first time to demonstrate that MAGT1 was abnormally upregulated in tissue samples of breast cancer patients and breast cancer cell lines. The oncogenic activity of MAGT1, as evidenced by the restricted cell proliferative, migratory and invasive abilities of MCF-7 cells upon MAGT1 knockdown was first demonstrated in breast cancer. Moreover, MAGT1 knockdown attenuated tumor growth *in vivo*, further verifying the oncogenic role of MAGT1. These novel findings manifest that targeting MAGT1 may be a promising strategy for the treatment of breast cancer.

Transcription factors drive cell fate transitions by determining global transcriptional, epigenetic and topological alterations (29). Indeed, transcription factors were not originally considered ideal targets for drug development; however, the advanced understanding of these transcription factors, in terms of the their structure, interaction with proteins and the dynamic mode of binding to DNA, provide immense potential for novel therapeutic strategies targeted against transcription factors (30). At present, numerous transcription factors have been reported to be associated with multiple tumor biomarkers, such as NF-κB, p53, forkhead box O and others (31-33). The KLF family (KLF1-KLF17), a type of zinc finger-containing transcription factor, has been found to play a crucial role in tumorigenesis and development by modulating cancer-promoting or cancer-suppressive genes via binding to the GC-rich DNA sequence in primer regions of these genes (34,35). A previous study indicated that KLF16 could transcriptionally repress the expression of mitochondrial transcription factor A, which plays an oncogene role in cancer, by interacting with its promoter, thereby suppressing human glioma cell proliferation and tumorigenicity (36). Consistently, a potential binding association between transcription factor

KLF16 and the MAGT1 promoter was also found through the JASPAR database (https://jaspar.genereg.net/) in the present study, and this connection was subsequently verified by luciferase reporter and ChIP assays. In addition, the dysregulation of KLF16 in breast cancer positively influenced MAGT1 expression. Further *in vitro* and *in vivo* experiments not only revealed the oncogenic role of KLF16 in breast cancer due to the suppressive effects on cell proliferation, migration and invasion, and tumor growth upon KLF16 knockdown, but also revealed a rescue of its antitumor activity by simultaneous transfection with pcDNA-MAGT1, suggesting that the anticancer effects of KLF16 knockdown may be dependent on its inhibitory effect on MAGT1 expression.

In conclusion, the present study demonstrated that MAGT1 and KLF16 were upregulated in tumor tissues of breast cancer patients, as demonstrated from bioinformatics data and in breast cancer cell lines. The knockdown of MAGT1 or KLF16 hindered the development of breast cancer via restricting cell proliferation, migration and invasion, and tumor growth. Mechanistically, KLF16 could directly bind to the MAGT1 promoter and transcriptionally activated MAGT1 expression, thus regulating the oncogenic role of MAGT1 and influencing the progression of breast cancer. The present study provides a novel target for the treatment of breast cancer and discloses its potential regulatory mechanisms.

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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

XiaofenZ designed the study. LL, XiZ, YL, BX, SP and HJ conducted the experiments and collected the data. LL, XiZ, YL and BX analyzed and interpreted the data. LL and XiZ wrote the manuscript. XiaofenZ revised the manuscript. All authors have read and approved the final manuscript. XiaofenZ, LL and XiZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All animal experiment procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals of The Second Clinical Medical College of North Sichuan Medical College and were approved by the Ethics Committee of The Second Clinical Medical College of North Sichuan Medical College (Nanchong, China; approval no. NSMC-2021-94).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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