MYCL promotes the progression of triple-negative breast cancer by activating the JAK/STAT3 pathway

HONGNAN JIANG[1], XIAOJUN LI[2], WEI WANG[1], YAOFENG HU[1] and DONGLIANG REN[1]

Departments of [1]Breast Surgery and [2]Radiology, The Second Hospital of Shanxi Medical University, Taiyuan, Shanxi 030001, P.R. China

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Abstract. The present study aimed to investigate the underlying regulatory mechanism of MYCL proto-oncogene (MYCL) in triple-negative breast cancer (TNBC) progression. *In vitro* experiments were performed to confirm the functional roles of MYCL in TNBC, and its effects on the JAK/STAT3 pathway through flow cytometric analysis, colony formation, wound healing and Transwell assays. In addition, the GSE45498 dataset demonstrated that MYCL was upregulated in TNBC and that it was significantly related to poor survival of patients with TNBC. Knockdown of MYCL induced the apoptosis, and suppressed the proliferation, migration and invasion of TNBC cells by inhibiting the JAK/STAT3 pathway. Notably, MYCL could activate the JAK/STAT3 pathway, whereas inhibition of the JAK/STAT3 pathway could eliminate the effect of MYCL on TNBC cells. Knockdown of MYCL also suppressed the growth of TNBC xenograft tumors. In conclusion, MYCL could promote TNBC progression by activating the JAK/STAT3 pathway.

Introduction

Breast cancer (BC) is one of the most common primary malignant tumors among women worldwide and is a serious threat to women's health (1). In recent decades, the incidence of BC has rapidly increased, with ≥1 million cases newly diagnosed each year (2). Triple-negative BC (TNBC) is the most aggressive subtype of BC, and is defined as a lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (3,4). At present, systemic chemotherapy is the main treatment for patients with TNBC (5). TNBC is more aggressive and prone to local recurrence and lymph node metastasis compared with other types of BC, leading to poor prognosis or clinical outcomes. In Chinese female patients, the 5-year survival rate of early-stage TNBC is 77%, which is much lower than that for other subsets of early-stage BC (6). Therefore, it is necessary to identify new alternatives for TNBC treatment.

MYC proto-oncogene (MYC) is a protein-coding transcription factor, and its family contains B-MYC, C-MYC, MYCL proto-oncogene (MYCL), N-MYC and S-MYC (7). In human primary small cell lung cancer (SCLC), MYCL was first reported to be amplified, which is usually accompanied by overexpression (8). Previous studies have shown that MYC is dysregulated in numerous types of cancer, including lung cancer, Burkitt's lymphoma, colon cancer and BC (8-11). In cancer, MYC has synergistic effects with other transcription factors and target genes to regulate numerous life processes, including cell growth, apoptosis, cell cycle and tumorigenesis (12,13). In addition, MYCL has been reported to accelerate proliferation and increase metastatic dissemination of SCLC (14,15). Previous studies have also revealed that MYC expression is dysregulated in 30-50% of cases of high-grade BC. Compared with in normal breast tissues, MYC expression in tumor tissues has been shown to be decreased, and among the major subclasses, MYC expression is relatively high in TNBC (16). Compared with C-MYC or N-MYC, which drive several types of human cancer (17-20), there are fewer studies on MYCL. Furthermore, to the best of our knowledge, no previous study has reported the genetic changes of the MYCL gene related to TNBC.

The JAK/STAT pathway is considered to be an evolutionarily conserved signaling pathway, which is regulated by a variety of interferons, cytokines, related molecules and growth factors (21). It is well known that the JAK/STAT pathway serves a key role in a variety of biological processes, including cell differentiation, apoptosis, proliferation, survival and immune response (22). It has previously been reported that the JAK/STAT3 autocrine activation loop is a vital driver of metastasis and progression of BC (23). However, it is unclear as to whether the JAK/STAT3 pathway can be regulated by MYCL in TNBC. The present study explored the role of MYCL in TNBC progression, and further explored the

Correspondence to: Dr Dongliang Ren, Department of Breast Surgery, The Second Hospital of Shanxi Medical University, 382 Wuyi Road, Taiyuan, Shanxi 030001, P.R. China
E-mail: dl2016543@163.com

Abbreviations: BC, breast cancer; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative BC

Key words: MYCL proto-oncogene, triple-negative breast cancer, proliferation, migration, JAK/STAT3 pathway

potential regulatory mechanism of MYCL in activating the JAK/STAT3 pathway.

Materials and methods

Cell lines and cell culture. The normal human breast epithelial cell line (MCF-10A), the 293 cell line and four TNBC cell lines (HCC1143, MDA-MB-231, BT-549 and MDA-MB-453) were acquired from American Type Culture Collection. Cells were incubated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin (all from Thermo Fisher Scientific, Inc.). The cells were fostered at 37˚C in an atmosphere containing 5% CO_2 and the medium was changed every 2-3 days.

Cell transfection. MYCL small interfering RNA (siRNAs) (si-MYCL#1, 5'-GACTACGACTCGTACCAGCACTAT T-3'; si-MYCL#2, 5'-CAGCACTATTTCTACGACTATGAC T-3'; and si-MYCL#3, 5'-CAAGCGACTCGGAGAATG AAGAAAT-3') and negative control (si-NC, 5'-TTCTCC GAACGTGTCACGT-3') were purchased from the Shanghai GenePharma Co., Ltd. The MYCL pcDNA3.1 overexpression vector (pc-MYCL) and negative control (pc-NC) were synthesized by Sangon Biotech Co., Ltd. The human TNBC cells ($1x10^5$) were seeded and cultured in 24-well plates for 1 day. After 72 h of cell culture, si-MYCL or si-NC (50 nM)was used to transfect MDA-MB-453 cells for 96 h at 37˚C using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) to construct a MYCL-silenced cell model. In addition, MDA-MB-231 cells were transfected with pc-MYCL or pc-NC (2 μg) using Lipofectamine® 2000 for 96 h at 37˚C to construct a MYCL-overexpressed cell model. Subsequent experiments were performed after 48 h of transfection. Furthermore, to confirm the effect of JAK on MYCL expression, a JAK inhibitor, 1 μM ruxolitinib (Ruxo; Shanghai YuanyeBio-Technology Co., Ltd.), was added into the medium to treat MDA-MB-231 cells transfected with pc-MYCL or pc-NC for 24 h at 37˚C.

Cell apoptosis analysis. The Annexin V-PE/7-AAD Apoptosis Detection Kit (Vazyme Biotech Co., Ltd.) was used to examine the apoptosis of MDA-MB-453 and MDA-MB-231 cells. Briefly, cells ($1x10^5$ cells/well in 6-well plates) were suspended in 100 μl binding buffer and incubated with 5 μl Annexin V-PE and 5 μl 7-AAD for 10 min in the dark at room temperature. Within 1 h of staining, flow cytometry (FACScan; BD Biosciences) was performed to evaluate the apoptotic rate of TNBC cells. The apoptotic rate was calculated as the percentage of early and late apoptotic cells.

Colony formation assay. After 48 h of transfection, MDA-MB-231 or MDA-MB-453 cells ($1x10^3$ cells/well) were transferred to 6-well plates and routinely cultured for 14 days to form obvious colonies. Subsequently, the colonies (>50 cells) were fixed in 4% formaldehyde at 37˚C for 20 min, followed by staining with 1% crystal violet (Sangon Biotech Co., Ltd.) for 15 min. Finally, images of the cell colonies were captured using a digital Sight camera (Nikon Corp.) and counted from five random fields under a light microscope (Olympus Corp.).

Wound healing assay. Briefly, after 48 h of transfection, MDA-MB-231 or MDA-MB-453 cells (50 μl; $5x10^5$ cells/ml) were inoculated into 6-cm culture plates with RPMI-1640 medium (Thermo Fisher Scientific, Inc.) containing 10% FBS. Once the cells reached 90% confluence, a single linear scratch was drawn in the middle of the cell monolayer with a 200-μl pipette tip. Subsequently, the cell debris was removed by flushing three times with PBS. The cells were then cultured in RPMI-1640 medium supplemented with 1% FBS for 48 h. Finally, images of the wounds were captured by a light microscope (Olympus Corp.). The relative migration rate was calculated as follows: Relative migration rate (%)=(wound distance at 0 h-wound distance at 48 h)/wound distance at 0 h x100.

Transwell assay. The Transwell assay was performed to evaluate cell invasion *in vitro*. The Transwell chambers (pore size, 8 μm) were coated with Matrigel (BD Biosciences) and incubated for 1 h at 37˚C. TNBC cells ($5x10^4$ cells/well in a 24-well plate) were suspended in RPMI-1640 serum-free medium on the upper surface of the chambers. Meanwhile, 500 μl RPMI-1640 medium with 10% FBS was added to the bottom chamber. After the cells were cultured at 37˚C (5% CO_2) for 1 day, cells on the upper side of the membrane were removed using a cotton swab, followed by washing with PBS three times. Subsequently, the cells on the opposite side of the membrane were fixed with 4% paraformaldehyde at room temperature for 20 min and stained with 0.2% crystal violet for 30 min at indoor temperature. The images were finally captured under a light microscope (Nikon Corp.). Five visual fields were randomly selected and the average number of stained cells was counted.

Reverse transcription-quantitative PCR (RT-qPCR). TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from TNBC cells. According to the manufacturer's protocol, total RNA (500 ng) was reverse transcribed to cDNA using the PrimeScript RT reagent Kit Perfect Real-Time kit (Takara Bio, Inc.). qPCR was performed to detect the mRNA expression levels of MYCL using SYBR Master Mixture (Takara Bio, Inc.) on a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: Pre-denaturation at 95˚C for 2 min; 45 cycles of denaturation at 95˚C for 5 sec, annealing at 55˚C for 15 sec and extension at 68˚C for 30 sec; and finally, a melting curve analysis was performed from 65 to 95˚C for 3 min. The final relative expression levels of MYCL were calculated using the $2^{-\Delta\Delta Cq}$ method (24) and normalized to GAPDH. The PCR primers of MYCL and GAPDH were as follows: MYCL, forward 5'-CCAAGCGACTCGGAGAATGA-3' and reverse 5'-TTGGGAGCAGCTTTCTGGAG-3'; GAPDH, forward 5'-CATGTTGCAACCGGGAAGGA-3' and reverse 5'-CGC CCAATACGACCAAATCAG-3'.

Western blotting. Total protein was extracted from cells and xenograft tumor tissues using RIPA lysis buffer with protease inhibitors (Roche Diagnostics) for 30 min on ice. The concentration of all proteins extracted from the supernatants of cell lysates was determined using a Protein Assay kit (BCA; Takara Bio, Inc.). Subsequently, 40 μg protein was separated by

SDS-PAGE on 12% gels and transferred to PVDF membranes (MilliporeSigma). The membranes were blocked with 5% non-fat dry milk dissolved in PBS -0.1% Tween for 1 h at room temperature, and primary antibodies against MYCL (cat. no. SAB1410807; 1:1,000), E-cadherin (cat. no. SAB5700789; 1:1,000), N-cadherin (cat. no. SAB5700641; 1:1,000), Vimentin (cat. no. SAB1305096; 1:1,000), phosphorylated (p)-JAK1 (cat. no. SAB4504446; 1:1,000), JAK1 (cat. no. SAB4300393; 1:1,000), p-JAK2 (cat. no. SAB4300124; 1:1,000), JAK2 (cat. no. SAB4501601; 1:1,000), p-STAT3 (cat. no. SAB5700362; 1:1,000), STAT3 (cat. no. SAB5700069; 1:1,000), C-MYC (cat. no. SAB5700727; 1:1,000), Cyclin D1 (cat. no. SAB4502602; 1:1,000), Bcl-2 (cat. no. SAB4500003; 1:1,000) and GAPDH (cat. no. ABS16; 1:1,000) (all from MilliporeSigma) overnight at 4˚C. After washing three times using TBS -0.1% Tween, the membranes were incubated with a HRP-conjugated secondary antibody (cat. no. A16096; 1:10,000; Thermo Fisher Scientific, Inc.) for 2 h at 25˚C. The protein expression intensity was determined using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.).

Bioinformatics analysis. The UALCAN database (http://ualcan.path.uab.edu) based on The Cancer Genome Atlas (TCGA) database was used to observe the mRNA expression of MYCL across various types of cancer and different breast cancer subtypes. The Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/index.html) was used to analyze MYCL expression in breast invasive carcinoma (BRCA). The Gene Expression Omnibus (GEO) TNBC GSE45498 dataset (25) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45498) was downloaded to analyze the MYCL mRNA expression in normal and TNBC tumor tissues using GraphPad Prism 7.0 (GraphPad Software, Inc.). The potential effect of MYCL on overall survival (OS) and disease-free survival (DFS) of patients with TNBC was analyzed using the Kaplan-Meier plotter (http://kmplot.com/analysis/). Gene Set Enrichment Analysis (GSEA) was conducted on the GSE45498 dataset using the R package clusterProfiler (26) to explore MYCL expression and predict the potential signaling pathway.

Lentiviral infection. MYCL short hairpin RNA (sh-MYCL) and a negative control (sh-NC) were packaged into the pGCSIL-GFP lentiviral vector, which was purchased from Shanghai GenePharma Co., Ltd. Lentivirus packaging was performed using the second-generation lentivirus packaging kit (Shanghai GeneChem Co., Ltd.) at 37˚C for 15 min. For lentivirus packaging, 10 μg lentiviral plasmid pGCSIL-GFP and two helper plasmids (5 μg pHelper 1 and 5 μg pHelper 2) were incubated with Lenti-Easy Packaging Mix (1 ml) at 37˚C for 15 min. Subsequently, the mixture was incubated for another 20 min in Lipofectamine 2000 and applied to 293T cells for transfection. The cells were transfected with lentiviruses at 37˚C for 6 h, the medium was changed and the viral particles were collected after 3 days. The transfected cells were filtered using a 0.45-μM mesh and were concentrated by ultracentrifugation at 70,000 x g at 4˚C for 2 h. The supernatant was collected for detecting viral titers. The MDA-MB-453 cell line was cultured to >80% confluence and was cultured with diluted lentiviruses at a multiplicity of infection of 10 and with polybrene (MilliporeSigma) for 24 h at 37˚C. Subsequently, fresh culture medium was used to replace the spent medium and GFP-labeled cells with a lentivirus transduction rate >80% at 72 h were screened out; stable expressing clones were selected using 4 μg/ml puromycin. The transduction efficacy of sh-MYCL was verified by western blotting.

Xenograft tumor assay. The experimental protocol of the present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals (27) and was approved by the Second Hospital of Shanxi Medical University (approval no. SXDW20210512; Taiyuan, China). Eight 6-week-old female BALB/c nude mice (weight, 20±2 g) were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd. All mice were housed in a specific pathogen-free animal facility with free access to water and food at 22±1˚C with 55±2% humidity and a 12-h light/dark cycle. The mice were randomly divided into two groups (n=4/group). MDA-MB-453 cells ($5x10^6$ cells) stably transduced with sh-MYCL or sh-NC were subcutaneously injected into the back of mice. The length and width of xenograft tumors were measured weekly. The volumes were calculated on the basis of the formula V (mm^3)=length x $width^2/2$. After 5 weeks, the mice were sacrificed via an intravenous injection of excess pentobarbital sodium (100 mg/kg), and the xenograft tumors were obtained, imaged and weighed. The protein expression levels of MYCL and JAK/STAT3 pathway-related proteins were detected using western blotting.

Statistical analysis. All *in vitro* experiments were carried out in triplicate. All data were analyzed using SPSS 20.0 (IBM Corp.) and are presented as the mean ± SD. The significant differences between two groups were determined by unpaired Student's t-test. The significant differences among multiple groups were determined by one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MYCL is upregulated in TNBC. The results of the pan-cancer analysis are shown in Fig. 1A, MYCL was revealed to be significantly upregulated in BRCA, cervical squamous cell carcinoma and uterine corpus endometrial carcinoma. Subsequently, the GEPIA was used to analyze MYCL expression in BRCA, and the results demonstrated that MYCL expression was significantly increased in tumor compared with in normal samples (Fig. 1B). To fully understand the expression of MYCL in patients with BC, MYCL expression based on major subclasses was analyzed using the UALCAN tool. As shown in Fig. 1C, the mRNA expression levels of MYCL were significantly upregulated in different subtypes of BC compared with in normal samples, and they were particularly higher in TNBC (Fig. 1C). Additionally, the Kaplan-Meier plotter indicated that compared with patients with low MYCL expression, patients with TNBC and high MYCL expression had poorer OS and DFS (Fig. 1D and E). Subsequently, MYCL expression was detected in several TNBC cell lines and in a normal human breast epithelial cell line by RT-qPCR and western blotting. Compared with in the MCF-10A normal breast epithelial cell line, the expression levels of MYCL in

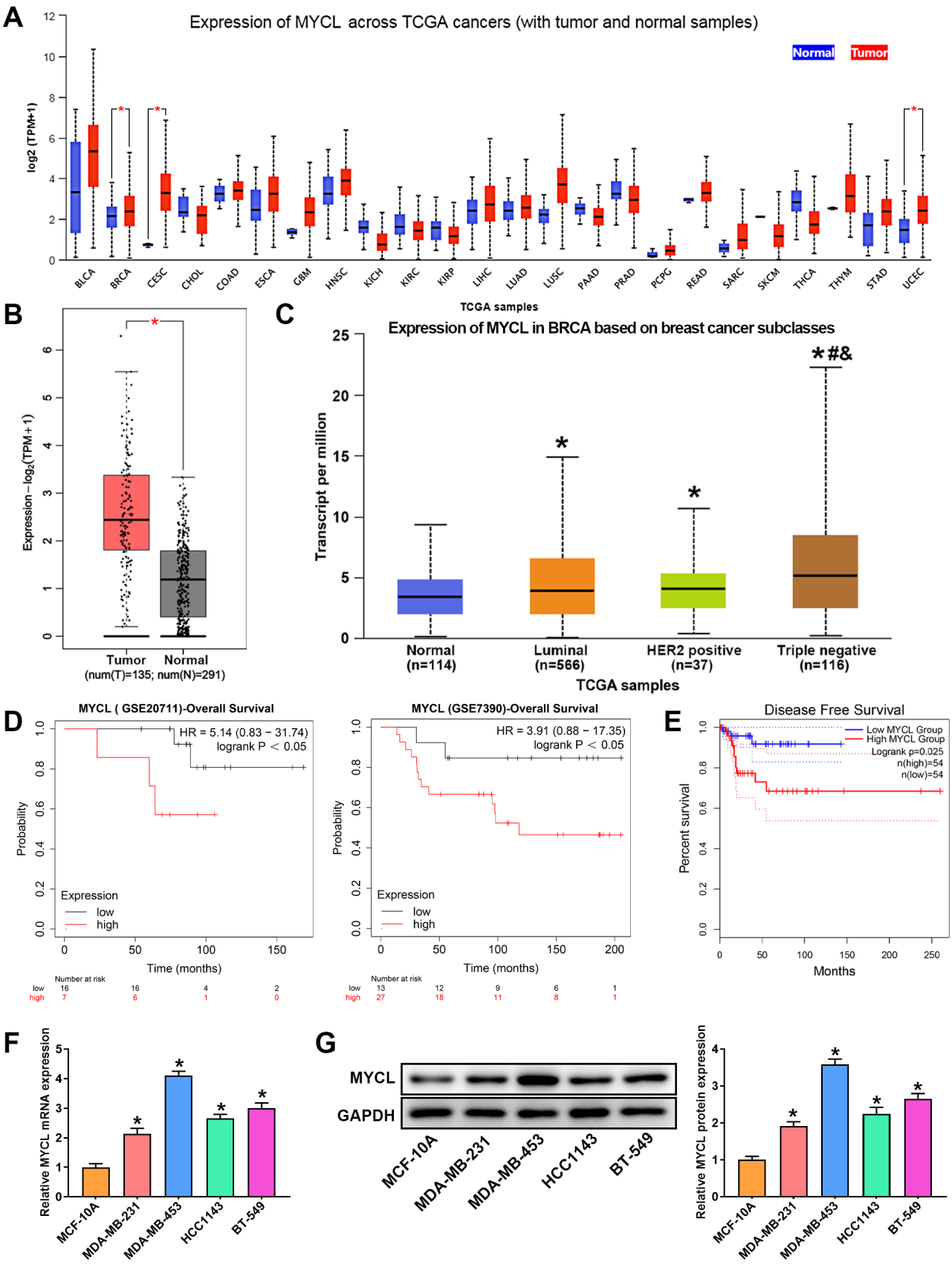

Figure 1. MYCL is highly expressed in TNBC. (A) TCGA dataset was used to analyze the expression of MYCL in cancer; MYCL was upregulated in BC. (B) TCGA analysis revealed MYCL was highly expressed in BC. (C) TCGA was used to analyze MYCL expression in diverse subtypes of BC; MYCL was highly expressed in TNBC. *P<0.05 vs. Normal; #P<0.05 vs. Luminal; &P<0.05 vs. HER2 positive. (D) High levels of MYCL expression were related to poor overall survival of patients with TNBC. (E) High levels of MYCL expression was related to poor disease-free survival of patients with TNBC. (F) mRNA expression levels of MYCL in TNBC cell lines was detected by reverse transcription-quantitative PCR. (G) Protein expression levels of MYCL in TNBC cell lines was detected by western blotting. *P<0.05 vs. MCF-10A. BC, breast cancer; HER2, human epidermal growth factor 2; TCGA, The Cancer Genome Atlas; TNBC, triple-negative BC; MYCL, MYCL proto-oncogene.

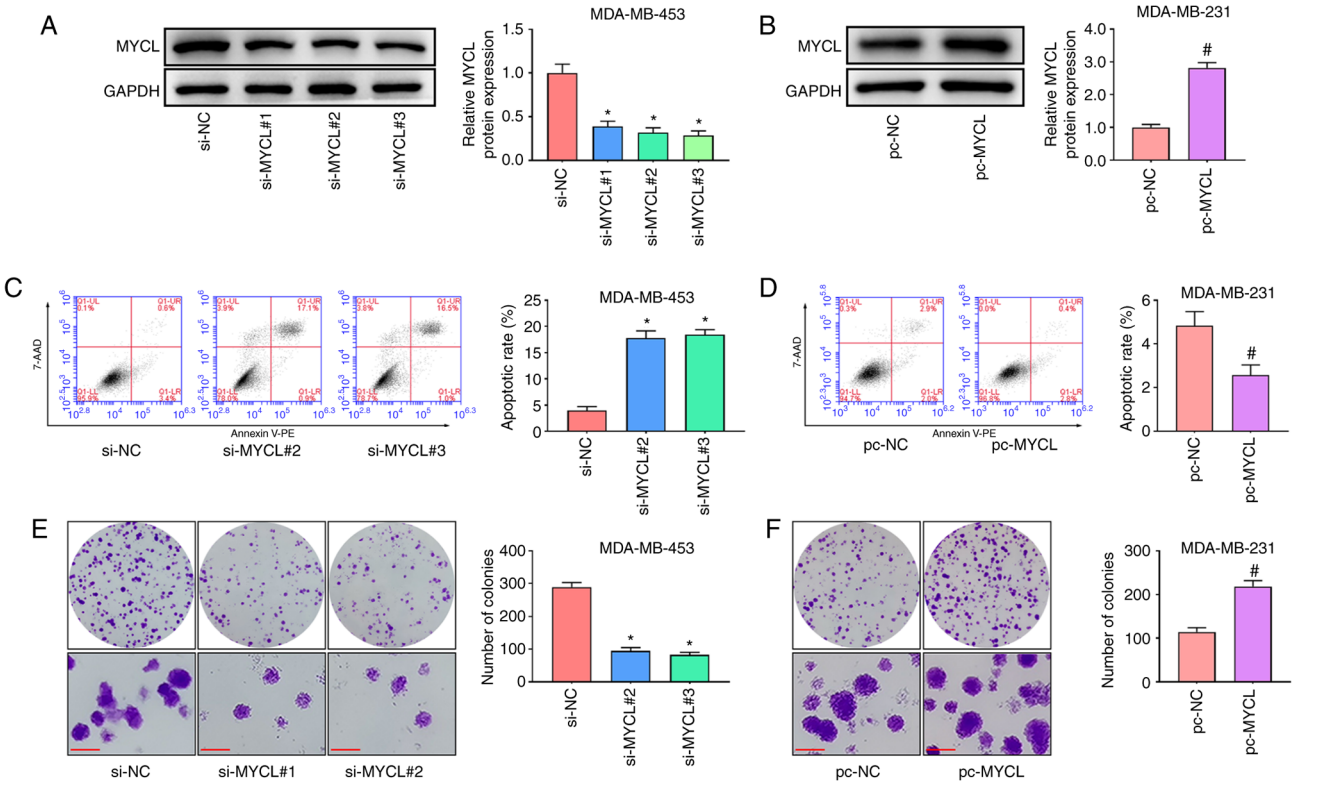

Figure 2. MYCL silencing promotes the apoptosis and inhibits the proliferation of triple-negative breast cancer cells. Protein expression levels of MYCL in (A) MDA-MB-453 cells transfected with MYCL siRNAs and (B) MDA-MB-231 cells transfected with pc-MYCL were detected by western blotting. Apoptosis of (C) MDA-MB-453 cells transfected with MYCL siRNAs and (D) MDA-MB-231 cells transfected with pc-MYCL was detected by flow cytometry. Colony formation was used to detect the effect of (E) MYCL silencing on MDA-MB-453 cell proliferation and (F) MYCL overexpression on MDA-MB-231 cell proliferation (scale bar, 500 μm). *P<0.05 vs. si-NC; #P<0.05 vs. pc-NC. NC, negative control; si/siRNA, small interfering RNA; MYCL, MYCL proto-oncogene.

TNBC cells were significantly upregulated, but not in a consistent manner. Among them, MYCL expression was the lowest in MDA-MB-231 cells and was the highest in MDA-MB-453 cells (Fig. 1F and G). These findings indicated that abnormal expression of MYCL may be related to TNBC progression.

MYCL silencing induces the apoptosis and suppresses the proliferation of TNBC cells. To further investigate the role of MYCL in TNBC progression, MDA-MB-453 and MDA-MB-231 cells were selected to perform *in vitro* analyses. Prior to functional experiments, the knockdown efficiencies of three siRNAs targeting the back-splicing sites of MYCL were verified in MDA-MB-453 cells and the efficiency of the MYCL overexpression vector in MDA-MB-231 cells was assessed using western blotting. MYCL expression was significantly reduced in all three si-MYCL groups, particularly in the si-MYCL#2 and si-MYCL#3 groups, compared with that in the si-NC group (Fig. 2A); therefore, these siRNAs were chosen for functional experiments. Furthermore, MYCL expression was significantly increased in the pc-MYCL group compared with that in the pc-NC group (Fig. 2B). Cell apoptosis and proliferation were subsequently detected using flow cytometry and colony formation assays, respectively. The results demonstrated that MYCL silencing induced the apoptosis and inhibited the proliferation of MDA-MB-453 cells compared with that in the cells transfected with si-NC (Fig. 2C and E). Conversely, MYCL overexpression suppressed the apoptosis and promoted the proliferation of MDA-MB-231 cells compared with that in the pc-NC group (Fig. 2D and F). These results indicated that MYCL silencing induced the apoptosis and suppressed the proliferation of TNBC cells.

MYCL silencing suppresses the migration and invasion of TNBC cells. To further explore whether MYCL knockdown or overexpression affected TNBC cell migration and invasion, further experiments were performed. The wound healing assay indicated that MYCL silencing significantly impaired the migratory ability of MDA-MB-453 cells in both si-MYCL#1 and si-MYCL#2 groups compared with that in the si-NC group (Fig. 3A). By contrast, MYCL overexpression had the opposite effect on MDA-MB-231 cell migration (Fig. 3B). Furthermore, the Transwell assay indicated that MYCL silencing significantly decreased the number of invasive MDA-MB-453 cells compared with that in the si-NC group (Fig. 3C), whereas the opposite effect was observed when MYCL was overexpressed in MDA-MB-231 cells (Fig. 3D). Furthermore, as expected, compared with those in the si-NC group, silencing of MYCL significantly promoted the expression levels of E-cadherin, and inhibited those of Vimentin and N-cadherin, in MDA-MB-453 cells (Fig. 3E). Conversely, MYCL overexpression reduced the expression levels of E-cadherin, and enhanced those of Vimentin and N-cadherin, in MDA-MB-231 cells compared with those in the pc-NC group (Fig. 3F). These findings provided strong evidence that the silencing of MYCL suppressed the tumorigenic potential of TNBC cells.

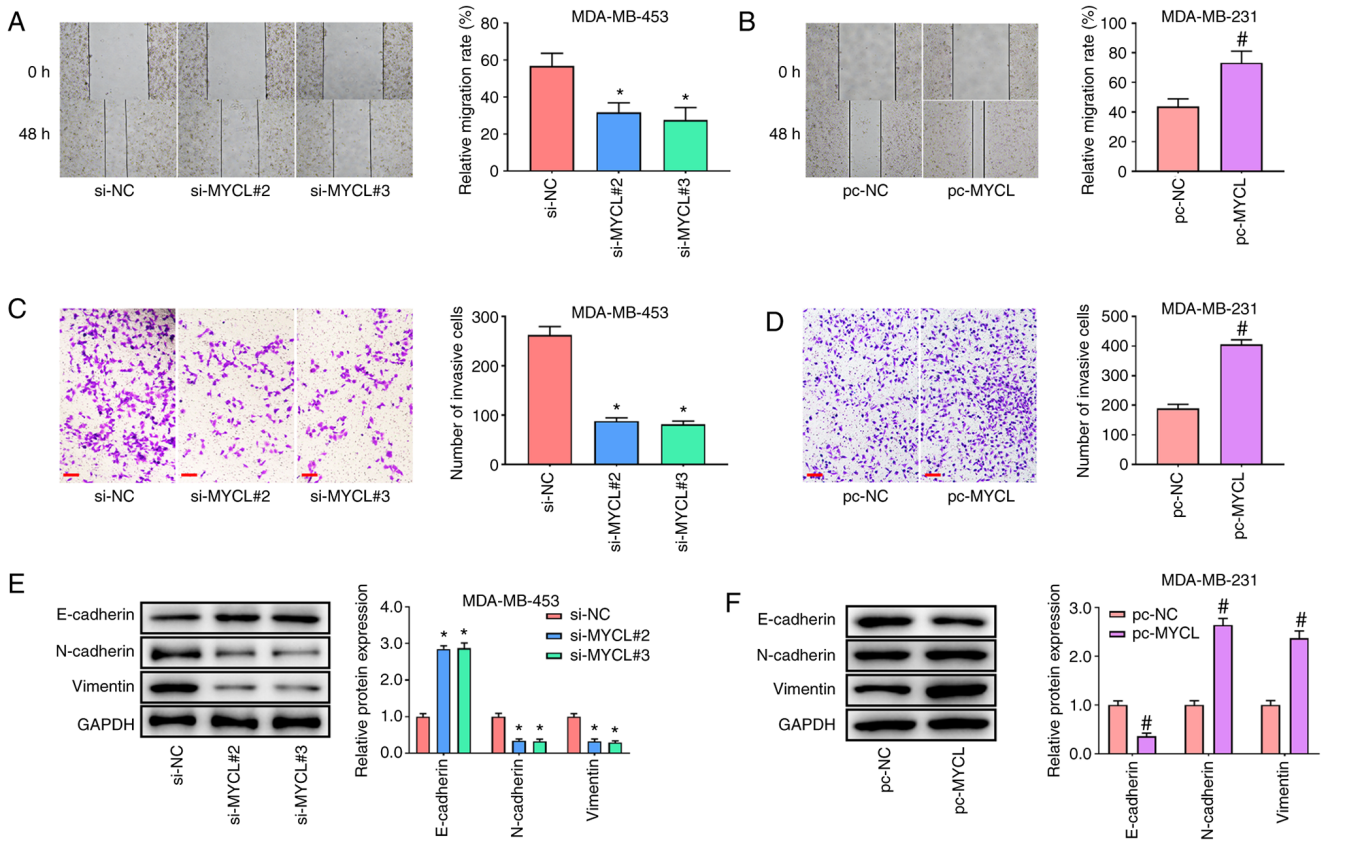

Figure 3. MYCL silencing inhibits triple-negative breast cancer cell migration and invasion. Migration of (A) MDA-MB-453 cells transfected with MYCL siRNAs and (B) MDA-MB-231 cells transfected with pc-MYCL was detected by wound healing assay (scale bar, 100 μm). Transwell assay detected the invasion of (C) MDA-MB-453 cells transfected with MYCL siRNAs and (D) MDA-MB-231 cells transfected with pc-MYCL (scale bar, 200 μm). Expression levels of E-cadherin, N-cadherin and Vimentin in (E) MDA-MB-453 cells transfected with MYCL siRNAs and (F) MDA-MB-231 cells transfected with pc-MYCL were detected by western blotting. *P<0.05 vs. si-NC; $^{\#}$P<0.05 vs. pc-NC. NC, negative control; si/siRNA, small interfering RNA; MYCL, MYCL proto-oncogene.

MYCL activates the JAK/STAT3 pathway. To investigate the underlying mechanisms of MYCL in TNBC, the GSE45498 TNBC dataset was used to predict the signaling pathway related to MYCL through GSEA. GSE45498 dataset analysis showed that MYCL mRNA expression was significantly upregulated in TNBC tissues compared with that in normal tissues (Fig. 4A). Furthermore, the GSEA showed that MYCL activated the JAK/STAT3 pathway (Fig. 4B). The proteins involved in the JAK/STAT3 pathway were subsequently evaluated by western blotting. The results indicated that silencing of MYCL significantly reduced the expression levels of p-JAK1/JAK1, p-JAK2/JAK2 and p-STAT3/STAT3 in MDA-MB-453 cells compared with in the si-NC group (Fig. 4C), whereas MYCL overexpression significantly increased the expression levels of these proteins compared with in the pc-NC group (Fig. 4D). Additionally, the protein expression levels of STAT3 downstream proteins, C-MYC, Cyclin D1 and Bcl-2, were also detected by western blotting. MYCL silencing significantly reduced the expression levels of C-MYC, Cyclin D1 and Bcl-2 in MDA-MB-453 cells compared with in the si-NC group (Fig. 4E), whereas MYCL overexpression significantly enhanced the expression levels of these proteins compared with in the pc-NC group (Fig. 4F). These findings demonstrated that MYCL could activate the JAK/STAT3 pathway in TBNC.

Inhibition of the JAK/STAT3 pathway eliminates the effect of MYCL on TNBC cells. To confirm the association between MYCL and the JAK/STAT3 pathway in TNBC development, Ruxo, a JAK inhibitor, was used to treat the transfected TNBC cells. Initially, western blotting revealed that compared with in the pc-MYCL group, the expression levels of p-JAK1/JAK1, p-JAK2/JAK2 and p-STAT3/STAT3 in the JAK/STAT3 pathway were significantly inhibited in the pc-MYCL + Ruxo group, indicating that inhibition of JAK could weaken the effect of MYCL overexpression on JAK/STAT3 signaling pathway in TNBC cells (Fig. 5A). Similar results were obtained in the detection of C-MYC, Cyclin D1 and Bcl-2 (Fig. 5B). Subsequently, experiments were performed to confirm whether MYCL affected the biological behaviors of TNBC cells through the JAK/STAT3 pathway. Flow cytometry revealed that MYCL overexpression significantly inhibited apoptosis, whereas Ruxo significantly enhanced the apoptosis of MDA-MB-231 cells compared with in the pc-NC group. Notably, the inhibitory effect of MYCL overexpression on apoptosis was significantly reversed by the JAK inhibitor Ruxo. The apoptosis of MDA-MB-231 cells was significantly increased in the pc-MYCL + Ruxo group compared with that in the pc-MYCL group (Fig. 5C and G). The colony formation assay revealed that MYCL overexpression enhanced cell proliferation, whereas Ruxo inhibited the proliferation of

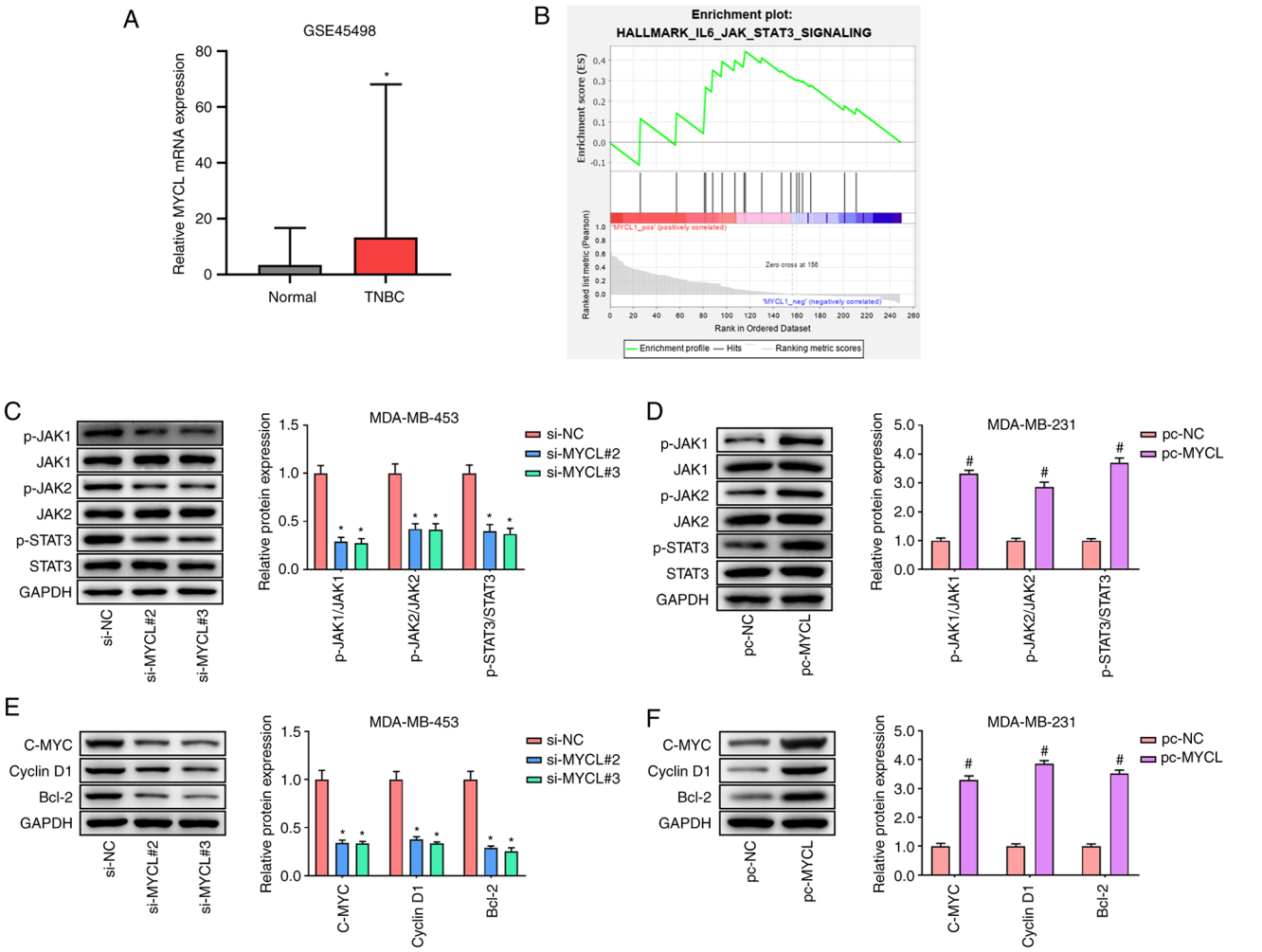

Figure 4. MYCL activates the JAK/STAT3 pathway. (A) Analysis of the Gene Expression Omnibus GSE45498 dataset showed that MYCL was highly expressed in TNBC. (B) Gene Set Enrichment Analysis of GSE45498 showed that MYCL activated the JAK/STAT3 pathway. In MDA-MB-453 cells transfected with MYCL siRNAs, the expression levels of (C) JAK/STAT3 pathway-related proteins and (E) STAT3 downstream proteins were assessed by western blotting. In MDA-MB-231 cells transfected with pc-MYCL, the expression of (D) JAK/STAT3 pathway-related proteins and (F) STAT3 downstream proteins were assessed by western blotting. *P<0.05 vs. si-NC; #P<0.05 vs. pc-NC. NC, negative control; si/siRNA, small interfering RNA; p, phosphorylated; TNBC, triple-negative breast cancer; MYCL, MYCL proto-oncogene.

MDA-MB-231 cells compared with in the pc-MYCL group. Furthermore, Ruxo reversed the effect of MYCL overexpression on the proliferative ability of MDA-MB-231 cells (Fig. 5D and H). Consistent with the aforementioned results, MYCL overexpression significantly promoted the migration and invasion of MDA-MB-231 cells compared with that in the pc-NC group, whereas Ruxo significantly suppressed the effect of MYCL overexpression on the migration and invasion of TBNC cells (Fig. 5E, F, I and J). Taken together, MYCL may affect the biological behaviors of TNBC cells by activating the JAK/STAT3 pathway.

MYCL silencing inhibits TNBC tumor growth in vivo. The expression levels of MYCL were higher in MDA-MB-453 cells than those in MDA-MB-231 cells; therefore, MDA-MB-453 cells were transfected with sh-MYCL to perform the xenograft tumor assay. The results revealed that tumor growth was markedly slower in mice injected with sh-MYCL cells compared with that in mice injected with sh-NC cells (Fig. 6B). In addition, the weight and size of the transplanted tumors formed by MDA-MB-453 cells transfected with sh-MYCL was smaller than that in the sh-NC group (Fig. 6A and C). Notably, western blotting indicated that MYCL expression was significantly reduced in xenograft tumors from the sh-MYCL group compared with that in the tumor tissues of the sh-NC group (Fig. 6D). Additionally, sh-MYCL significantly inhibited the JAK/STAT3 signaling pathway, as indicated by the significant decrease in the protein expression levels of p-JAK1/JAK1, p-JAK2/JAK2, p-STAT3/STAT3, C-MYC, Cyclin D1 and Bcl-2 in the tumor tissues of the sh-MYCL group compared with those in the sh-NC group (Fig. 6E and F). Briefly, MYCL silencing suppressed the growth of TNBC xenograft tumors.

Discussion

TNBC is one of the primary subsets of BC and is characterized by a lack of ER, PR and HER2 expression (28). TNBC is a type of high-grade invasive ductal carcinoma with highly aggressive biological behavior (29). In addition, TNBC is at a high risk of early recurrence, and is associated with bone,

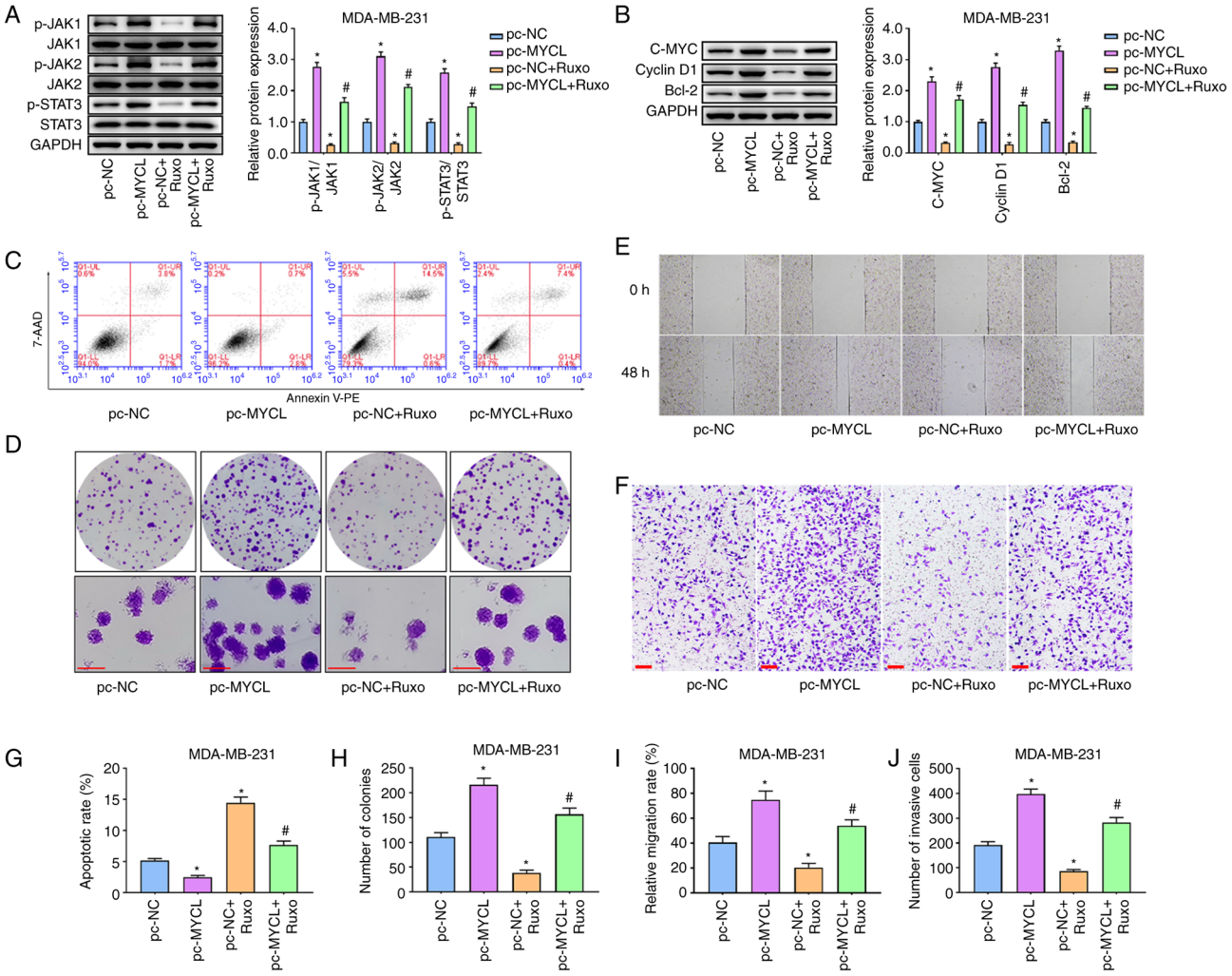

Figure 5. Inhibition of the JAK/STAT3 signaling pathway eliminates the effect of MYCL on triple-negative breast cancer cells. Expression levels of (A) JAK/STAT3 pathway-related proteins and (B) STAT3 downstream genes in MDA-MB-231 cells were assessed through western blotting. (C and G) MDA-MB-231 cell apoptosis was determined by flow cytometry. (D and H) MDA-MB-231 cell proliferation was estimated by colony formation assay (scale bar, 500 μm). (E and I) MDA-MB-231 cell migration was assessed by wound healing assay (scale bar, 100 μm). (F and J) Transwell assay was used to detect MDA-MB-231 cell invasion (scale bar=200 μm). *P<0.05 vs. pc-group; $^{\#}$P<0.05 vs. pc-MYCL. NC, negative control; p, phosphorylated; Ruxo, ruxolitinib; MYCL, MYCL proto-oncogene.

visceral and brain metastases, low DFS and OS rates, and poor prognosis (6,30). The evolution of TNBC is a multiple gene, step and stage pathological process, and an imbalance between cell growth and death caused by abnormal signal transduction pathways is an important mechanism underlying the occurrence and development of TNBC (31). Therefore, a better understanding of the molecular events associated with the malignancy may help to understand the pathological process of TNBC. The present study provided evidence to support the effect of MYCL expression and the JAK/STAT3 pathway on the biological behaviors of TNBC, including proliferation, apoptosis, migration and invasion.

A number of molecular markers have been demonstrated to regulate different types of cellular processes in TNBC (32). The MYC gene is a common intracellular proto-oncogene, which is highly expressed in a variety of tumors, including BC (16). Kato et al (15) reported that MYCL was highly expressed in SCLC cell lines. Furthermore, inhibition of MYC has been reported to decrease the proliferation of colon cancer cells (33). Notably, high expression of C-MYC protein is associated with a high risk to patients with BC, and C-MYC protein is considered a tumor marker of TNBC (34). In the present study, TCGA database analysis demonstrated that MYCL was significantly higher in BC tumor tissues, particularly in TNBC tumor tissues. The Kaplan-Meier Plotter analysis also revealed that higher expression of MYCL was associated with a higher risk of death. Furthermore, the present results showed that MYCL was markedly upregulated in all TNBC cell lines compared with in MCF-10A cells.

In recent years, increasing evidence has shown that MYC genes are extensively distributed in the human genome, and are involved in cell growth, apoptosis, proliferation and other cell activities (12,13). In TNBC progression, it has been reported that epithelial-to-mesenchymal transition (EMT) is responsible for the migration and invasion of tumor cells via the acquisition of invasive and mobile capabilities to facilitate metastasis, which is moderated by numerous related proteins, including E-cadherin, Vimentin and N-cadherin (35). Cadherins are transmembrane glycoproteins responsible for cell-cell adhesion and maintenance of normal tissue architecture (36).

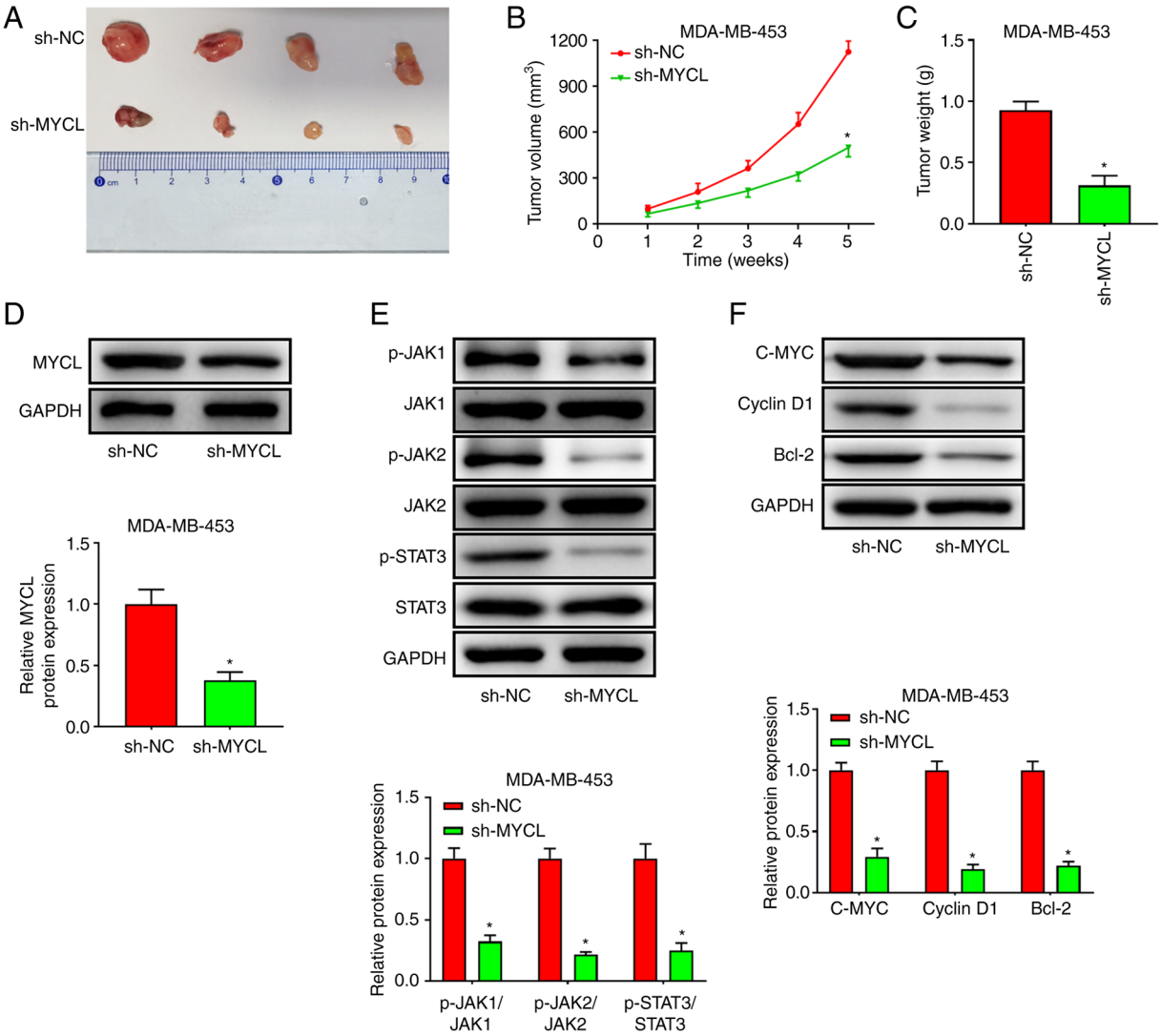

Figure 6. MYCL silencing inhibits triple-negative breast cancer tumor growth. (A) Tumors excised from sh-MYCL and sh-NC mice groups. (B) Changes in tumor volume. (C) Tumor weight. (D) Protein expression levels of MYCL in tumor tissue. Expression levels of (E) JAK/STAT3 pathway-related proteins and (F) STAT3 downstream proteins in tumor tissues were assessed by western blotting. *P<0.05 vs. sh-NC. NC, negative control; p, phosphorylated; sh, short hairpin; MYCL, MYCL proto-oncogene.

The role of cadherins in the process of cancer development has been studied widely and numerous studies have described E-cadherin as a tumor suppressor (37,38). In cancer, a number of tumors have been shown to exhibit N-cadherin upregulation at the onset of metastasis (39). Vimentin, a classic EMT biomarker, is upregulated during EMT in epithelial cells and induces a mesenchymal phenotype and motor behavior (40). In the present study, evidence indicated that silencing MYCL significantly inhibited the tumorigenesis, invasion and migration of TNBC cells. MYCL silencing significantly increased E-cadherin expression, and decreased the expression levels of N-cadherin and Vimentin in TNBC cells. Conversely, MYCL overexpression enhanced the migratory and invasive ability of TNBC cells, reduced E-cadherin expression, and increased the expression levels of N-cadherin and Vimentin. Furthermore, *in vivo* animal experiments reinforced the evidence that MYCL silencing suppressed the growth of TNBC tumors.

Among the most recently recognized carcinogenic signaling pathways, the JAK/STAT3 signaling pathway has an important role in the tumorigenesis of BC and other cancer types (23,41,42). It has been reported that persistent activation of the JAK/STAT3 pathway could promote tumor malignancy by facilitating tumor cell immune escape, proliferation, angiogenesis and survival (22,43). Activation of the JAK/STAT signaling pathway may also lead to accelerated proliferation of esophageal squamous cell carcinoma cells (44). Inhibition of the JAK/STAT3 pathway has been shown to suppress the *in vivo* growth of ovarian cancer cells (45). In the present study, GEO analysis revealed that MYCL activated the JAK/STAT3 pathway in TNBC. MYCL silencing suppressed the expression levels of p-JAK1/JAK1, p-JAK2/JAK2 and p-STAT3/STAT3 in TNBC cells. The downstream proteins of the JAK/STAT3 pathway, C-MYC, Cyclin D1 and Bcl-2, were also suppressed by MYCL silencing. The opposite results were obtained in MYCL-overexpressing cells. To further elucidate the association between MYCL and the JAK/STAT3 pathway in TNBC development, Ruxo, an effective inhibitor of the JAK/STAT3 pathway, was used to treat the transfected MDA-MB-231 cells. Inhibition of the JAK/STAT3 signaling pathway significantly suppressed the proliferation and migration of TNBC cells,

and partly abolished the effects of MYCL overexpression on TNBC.

In conclusion, MYCL could promote TNBC progression by activating the JAK/STAT3 pathway. Therefore, the present study indicated that MYCL could be regarded as a novel therapeutic target for the treatment of TNBC. However, there are some limitations in the present study. Firstly, in future studies, the sample of patients with TNBC should be collected to support the present findings; and secondly, the role of MYCL in TNBC and other subtypes of BC should be further investigated in future studies.

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Availability of data and material

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

Authors' contributions

Conception and design: HJ; Perform research: HJ and XL; Data analysis and interpretation: WW, YH and DR; Manuscript writing: All authors; Final approval of manuscript: All authors. All authors have read and approved the final manuscript, and met the authorship requirements stated earlier in this document, and each author believes that the manuscript represents honest work. HJ and DR confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The experimental protocol of the present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals and was approved by the Second Hospital of Shanxi Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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