Life Sciences 235 (2019) 116799

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Knockdown of TRIM66 inhibits cell proliferation, migration and invasion in colorectal cancer through JAK2/STAT3 pathway

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ARTICLE INFO

Keywords:
Colorectal cancer (CRC)
Tripartite motif (TRIM) family
TRIM66
JAK2/STAT3 signaling pathway

ABSTRACT

Colorectal cancer (CRC) is one of the most common malignancies in the world. Emerging evidence has shown that dysregulation of tripartite motif (TRIM) family proteins is strongly correlated with the tumorigenesis of CRC. Here, we evaluated the biological roles of TRIM66, a member of TRIM family, in the progression of CRC. The results demonstrated that TRIM66 was markedly up-regulated in both CRC tissues and cell lines. To further investigate the functions of TRIM66 in CRC, CRC cells were infected with lentivirus expressing anti-TRIM66 shRNA (sh-TRIM66) or control lentivirus (sh-con). We found that knockdown of TRIM66 significantly inhibited cell proliferation, migration, invasion of CRC cells. TRIM66 knockdown also suppressed epithelial-mesenchymal transition (EMT), as proved by the increased E-cadherin expression and decreased expressions of N-cadherin and vimentin. Furthermore, TRIM66 knockdown markedly inhibited tumor growth in a mouse xenograft model. Knockdown of TRIM66 reduced the activation of JAK2/STAT3 signaling pathway in CRC cells. Treatment with AG490, an inhibitor of JAK2/STAT3 signaling pathway, enhanced the inhibitory effects of TRIM66 knockdown on cell proliferation, migration and invasion. These findings suggested that knockdown of TRIM66 exhibited anti-tumor activity through inhibiting the JAK2/STAT3 signaling pathway in CRC cells.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies and its incidence rates are 30% to 40% higher in men than in women [1]. Most CRC cases are due to old age as well as lifestyle factors such as unhealthy diet, obesity, and smoking. There is strong evidence that the prognosis of CRC is better with an earlier diagnosis [2,3]. Treatments used for CRC include some combination of surgery, radio-/chemo-therapies and targeted therapy [4]. Although great advances in therapeutic approaches have been achieved in recent decades, the 5-year survival rate of patients with late stage CRC is very poor because of recurrence and metastasis [2,3]. Thus, it is important to improve diagnostic markers and elucidate the molecular mechanisms of CRC progression.

Tripartite motif (TRIM) family proteins are also referred to as RING B-box coiled-coil (RBCC) proteins, Most of TRIM proteins have E3 ubiquitin ligase activities, thereby exhibiting various functions in cellular processes such as development, apoptosis, autophagy, protein quality control, and intracellular signaling [5–7]. Accumulating evidence suggest that TRIM proteins have unique and important roles in several diseases classified as developmental disorders, immunological disease, or cancers [6,8]. Recent pathological studies have shown that dysregulation of some TRIM proteins is strongly correlated with the malignancy and prognosis of CRC [9–13]. The cancer-related TRIM proteins act as either oncogenic factors or onco-suppressors.

TRIM66 is a member of TRIM protein family that has been shown to play a role in tumor development and progression of several cancers [14,15]. However, the expression and biological function of TRIM66 in CRC remain unclear. Therefore, we evaluated the expression of TRIM66 in CRC tissues and cell lines. CRC cells with TRIM66 knockdown were used to investigate the biological behaviors including proliferation, migration/invasion and EMT process. Finally, the mechanism of TRIM66-regulates CRC cell proliferation and migration was explored. The results showed that TRIM66 was markedly up-regulated in CRC and might function as an oncogenic factor.

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https://doi.org/10.1016/j.lfs.2019.116799
Received 2 June 2019; Received in revised form 17 August 2019; Accepted 27 August 2019
Available online 28 August 2019

2. Materials and methods

2.1. Patients and CRC tissue collection

A total of 17 CRC tissues and paired adjacent normal tissues were collected from patients who were diagnosed as stage II-IV CRC and subjected to tumor resection at the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). The clinical specimens were stored at −80 °C until use. The present study has been approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University. Written informed consent had been obtained from each participant.

2.2. Cell culture

Human normal colorectal cell lines NCM460 and human CRC cell lines including HCT116, HT29, CaCo2 and SW620 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 or Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/G-streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) in an incubator with 5% CO_2 at 37 °C. For the AG490 treatment group, cells with different infections were incubated with 10 nM AG490 (Sigma) for 48 h.

2.3. Small hairpin RNA (shRNA)

Lentivirus expressing an anti-TRIM66 shRNA (sh-TRIM66) and control lentivirus (sh-con) were packaged by Genechem (Shanghai, China). The infection process was performed according to the protocol. Briefly, lentiviral stocks diluted with culture medium were added to the cells and incubated for 24 h at 37 °C. After that, the medium was replaced with fresh medium and incubated for another 72 h. Finally, the cells were harvested for the selection of stable transfection.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). And cDNA was synthesized using the obtained total RNA with PrimeScript RT reagent Kit (TaKaRa, Shiga, Japan). The qRT-PCR amplification was performed using SYBR Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Waltham, MA, USA) on ABI 7500 fast RT-PCR System (Applied Biosystems, Foster City, CA, USA) with the following primers: TRIM66 forward, 5'-GCCC TCTG TGCT ACTT ACTC-3′, and reverse 5'-GCTG GTTG TGGG TTAC TCTC-3′; GAPDH forward, 5'-CCAT CAAT GACC CCTT CATT G-3′, and reverse 5'-CATG GGTG GAAT CATA TTGG AAC-3′. GAPDH were used as internal control.

2.5. Western blot

Cells were lysed with RIPA lysis buffer with protease inhibitor cocktail (Sigma). After determination of protein concentration using BCA protein assay (Thermo Fisher), equal amounts of protein were subjected to SDS-PAGE gels and transferred to PVDF membranes (Thermo Fisher). After blocking with 5% nonfat milk at 37 °C for 1 h, the membranes were immunolotted with primary antibodies against E-cadherin, N-cadherin, vimentin (Abcam, Cambridge, MA, USA), JAK2, p-JAK2, STAT3, p-STAT3, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C for 10 h. Then the membranes were incubated with secondary antibodies (Santa Cruz) at 37 °C for 1 h, and the protein bands were detected with an enhanced chemiluminescence (ECL) kit (Bio-Rad Laboratories, Hercules, CA, USA). The intensity of signals was determined using Image J (National Institutes of Health, NIH, Bethesda, MD, USA).

2.6. Cell proliferation assay

Cell proliferation was assessed using CCK-8 assay kit (Dojindo, Kumamoto, Japan). Cells (1×10^4 cells/well) were seeded into 96-well plates and incubated for 48 h. After attachment, cells were subjected to different transfection. Subsequently, 10 μl CCK-8 solution was added to the cells at different time points and incubated for another 2 h. The absorbance was measured spectrophotometrically at 450 nm using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

2.7. Migration and invasion assays

Cell migration and invasion ability were tested by transwell assay using 8.0 μm 24-well Boyden chamber. Cells (1×10^4 cells/well) in serum-free medium were added into the upper chamber, while culture medium containing 10% FBS was added to the lower chamber. After 24 h incubation, the non-migrative cells were removed. The migrative cells were fixed with 4% formaldehyde and stained with 0.1% crystal violet. Five random selected visual fields (× 200) were selected and the cell numbers were counted under the microscope. For invasion assays, the membranes were pre-coated with Matrigel.

2.8. Tumor growth in a mouse xenograft model

Fifteen female BALB/c (nu/nu) mice (Shanghai Slac Laboratory Animals Ltd., Shanghai, China) were randomly separated into 2 groups ($n = 5$): a) negative control group, mice were injected subcutaneously with sh-con infected cells; b) TRIM66 knockdown group, mice were injected subcutaneously with sh-TRIM66 infected cells. After different injection into the right flank of the mice, the tumor size was measured with calipers every week since tumors were palpable. After 4 weeks, the mice were sacrificed, and the tumors were removed and weighted. The animal experiments were approved by Animal Research Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University and carried out in accordance with the guidelines.

2.9. Statistical analysis

Results are presented as the mean ± SD from at least three independent experiments. Statistical analyses were performed using SPSS 17.0 (IBM, Armonk, NY, USA). Differences between two groups were compared using two-tailed Student's *t*-test. Additionally, multiple group comparisons were analyzed with one-way ANOVA. $P < 0.05$ was considered as statistical significance.

3. Results

3.1. TRIM66 was highly expressed in human CRC tissues and cell lines

First, we examined the expression levels of TRIM66 in clinical samples using qRT-PCR and western blot. The results showed that TRIM66 was highly expressed in human CRC tissues when compared with paired adjacent normal tissues (Fig. 1A and B). Furthermore, the TRIM66 expressions in normal colorectal cell lines NCM460 and four human CRC cell lines including HCT116, HT29, CaCo2 and SW620 cells. Compared with the NCM460 cells, TRIM66 expressions were markedly up-regulated in CRC cell lines, especially in CaCo2 and SW620 cells (Fig. 1C and D).

3.2. Knockdown of TRIM66 inhibited the proliferation of CRC cells

To further investigate the role of TRIM66 in CRC, TRIM66 was knocked down in CaCo2 and SW620 cells through transfection with sh-TRIM66, respectively. Then the mRNA and protein levels were analyzed using RT-PCR along with western blot analysis, respectively. As shown

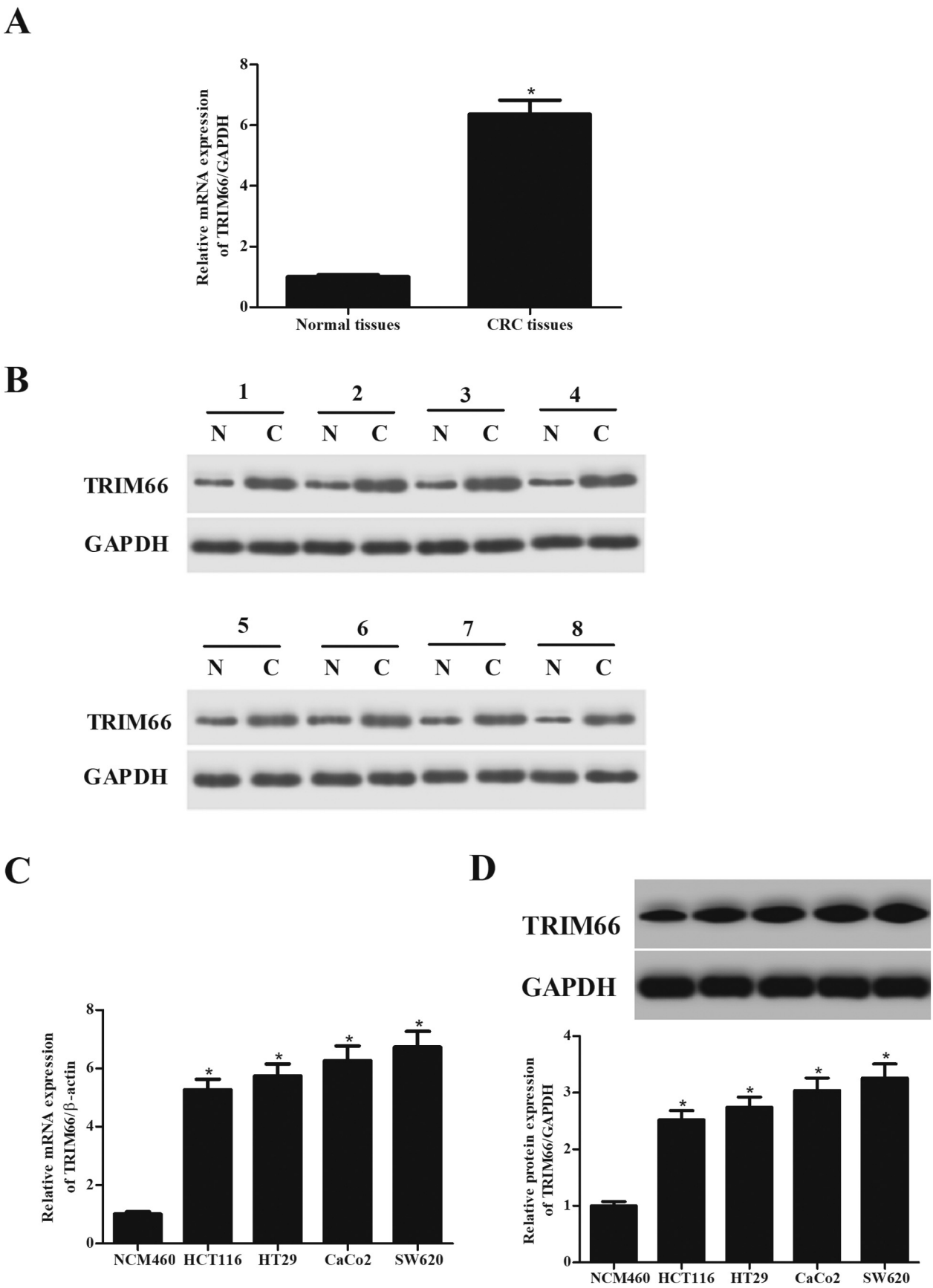

Fig. 1. TRIM66 expressions were upregulated in human CRC tissues and cell lines. (A and B) TRIM66 expressions in CRC tissues and paired adjacent normal tissues. N represents "Normal tissues"; C represents "CRC tissues". (C and D) TRIM66 expressions in normal colorectal cell lines and four human CRC cell lines. *$p < 0.05$.

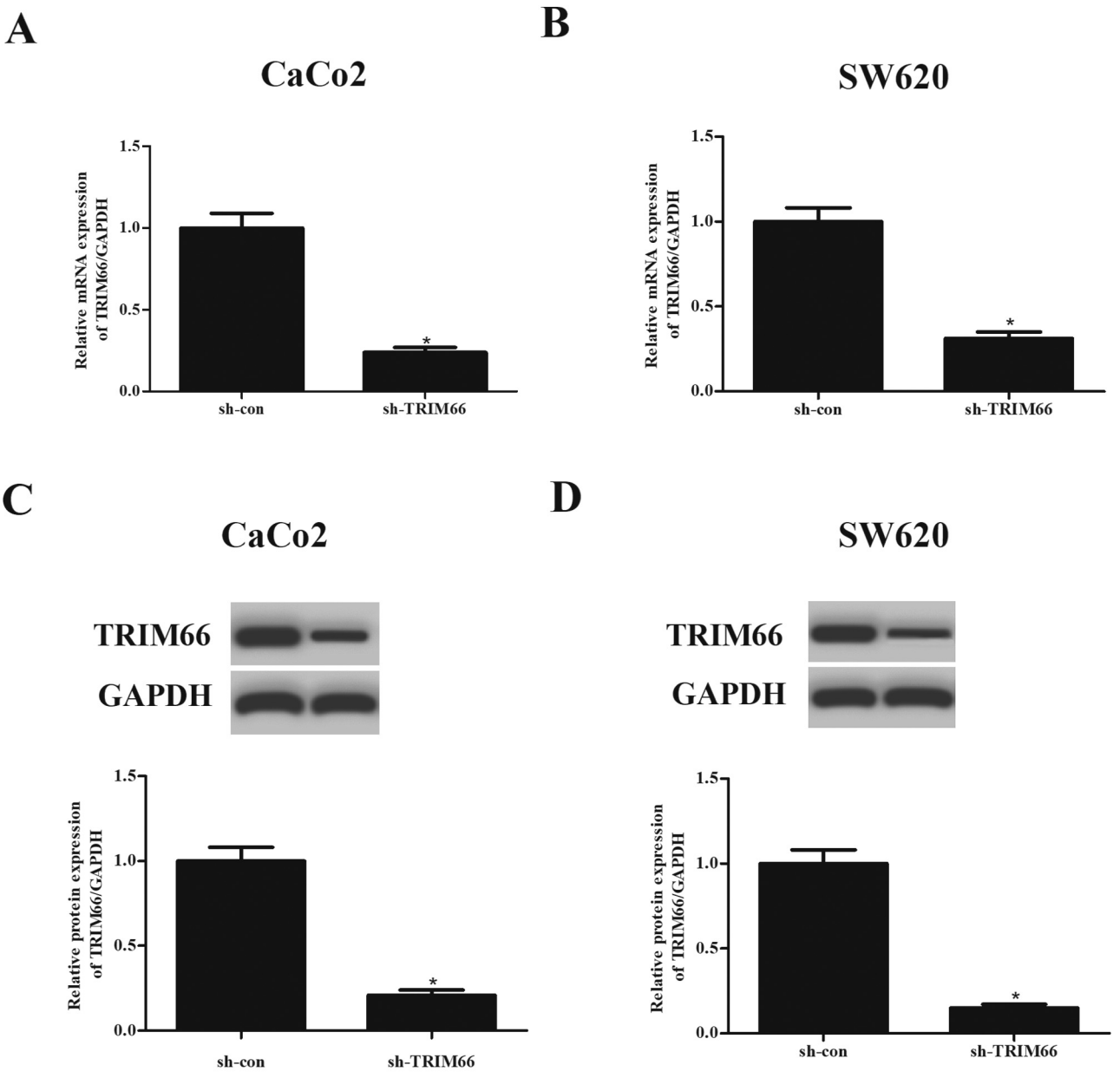

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Fig. 2. CRC cells proliferation was inhibited by knockdown of TRIM66. CaCo2 and SW620 cells were transfected with lentivirus expressing an anti-TRIM66 shRNA (sh-TRIM66) and control lentivirus (sh-con). The mRNA and protein levels of TRIM66 were analyzed using RT-PCR (A and B) and western blot analysis (C and D). Cell proliferation of CaCo2 (E) and SW620 cells (F) was measured using CCK-8 assay. *$p < 0.05$.

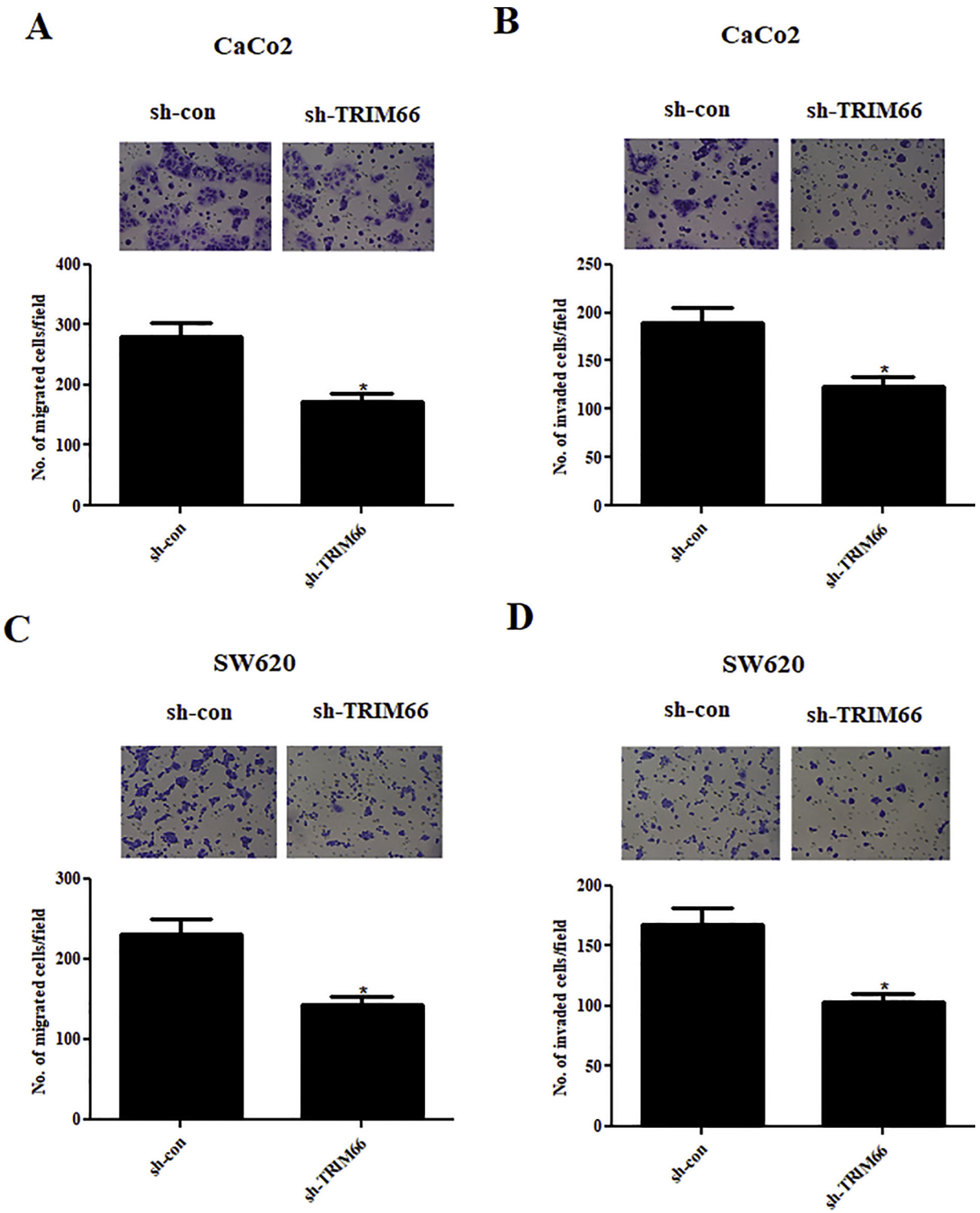

Fig. 3. CRC cells migration and invasion were suppressed by knockdown of TRIM66. Cell migration and invasion of CaCo2 and SW620 cells were evaluated using transwell assay. (A and C) Cell migration of CaCo2 and SW620 cells. (B and D) Cell invasion of CaCo2 and SW620 cells. *$p < 0.05$.

in Fig. 2A and C, TRIM66 was markedly decreased in mRNA and protein levels after transfection with sh-TRIM66 in CaCo2 cells. Similar results were observed in SW620 cells (Fig. 2B and D). TRIM66 protein expression was significantly decreased in CaCo2 and SW620 cells transfected with sh-TRIM66 by 78 and 84%, respectively (Fig. 2C and D), indicating the transfection was successful. Furthermore, we found that transfection of sh-TRIM66 resulted in considerable decrease in cell proliferation of CaCo2 and SW620 cells (Fig. 2E and F).

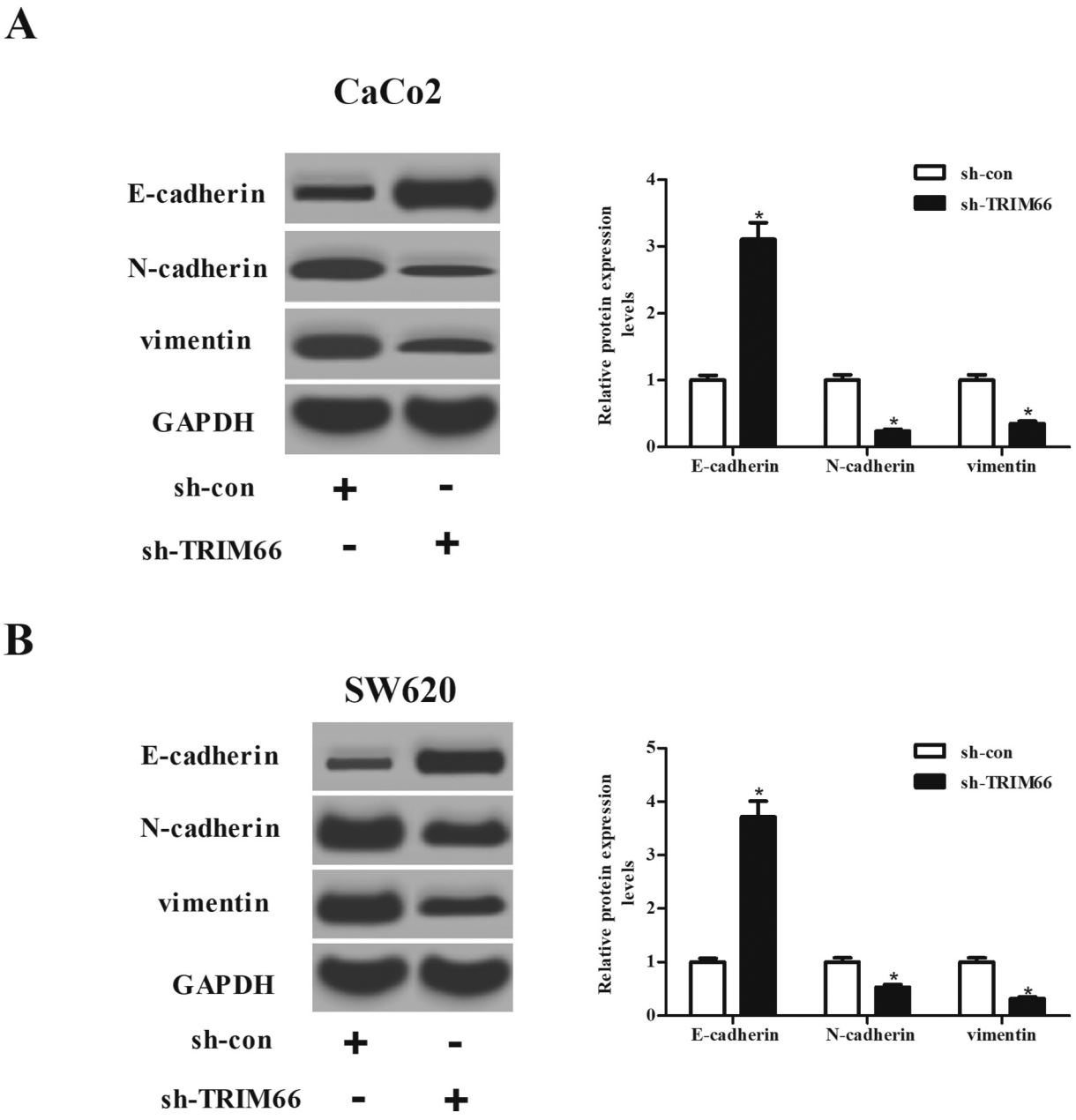

Fig. 4. EMT process was blocked by knockdown of TRIM66 in CRC cells. (A and B) The expressions of the EMT-related markers including E-cadherin, N-cadherin, and vimentin were detected using western blot analysis in CaCo2 and SW620 cells. *$p < 0.05$.

3.3. Knockdown of TRIM66 inhibits the migration and invasion of CRC cells

Next, we evaluated the effects of TRIM66 knockdown on cell migration and invasion using transwell assay. As indicated in Fig. 3A and C, cell migration of CaCo2 and SW620 cells was significantly inhibited after transfection with sh-TRIM66. Moreover, TRIM66 knockdown also greatly suppressed cell invasion of CaCo2 and SW620 cells (Fig. 3B and D).

3.4. Knockdown of TRIM66 inhibits the epithelial-mesenchymal transition (EMT) process in CRC cells

Then we investigated whether TRIM66 knockdown could affect EMT process by detecting the expressions of the related markers including E-cadherin, N-cadherin, and vimentin. Western blot analysis showed that the E-cadherin expression was increased, while N-cadherin and vimentin expressions were decreased in the sh-TRIM66 infected CaCo2 and SW620 cells (Fig. 4A and B).

3.5. Knockdown of TRIM66 attenuated tumor growth in a xenograft model in vivo

Subsequently, the anti-tumor effect of TRIM66 knockdown was confirmed *in vivo*. As illustrated in Fig. 5A, tumor growth was markedly decreased in the TRIM66 knockdown group as compared to the negative control group. And the final tumor weight showed significant decrease in TRIM66 knockdown group when compared with negative control group (Fig. 5B).

3.6. Knockdown of TRIM66 repressed the activation of JAK2/STAT3 pathway in CRC cells

It has been demonstrated that JAK2/STAT3 signaling pathway plays

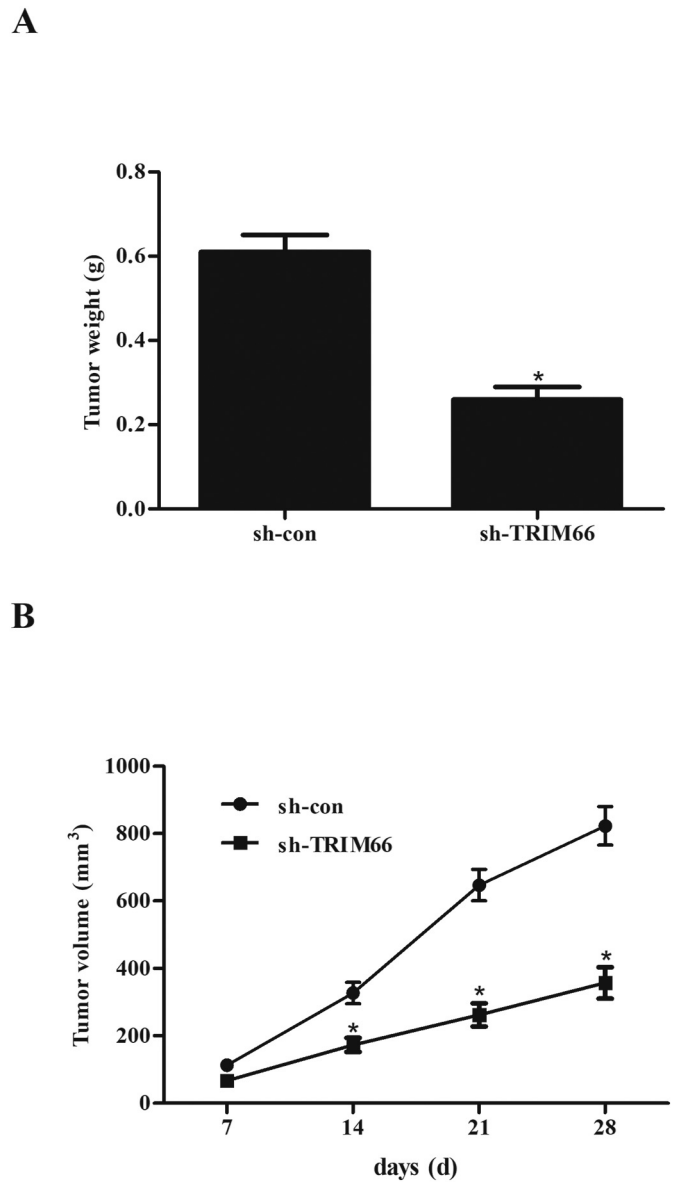

Fig. 5. Knockdown of TRIM66 suppressed tumorigenesis *in vivo*. Mice in negative control group and TRIM66 knockdown group were injected subcutaneously with sh-con or sh-TRIM66 infected cells, respectively. Mice in control group did not receive any treatment. (A) Tumors were removed and weighted after 4 weeks. (B) Tumor size was measured every week since tumors were palpable. *$p < 0.05$.

an important role in the tumorigenesis of CRC [16,17]. Therefore, we assessed the effect of TRIM66 knockdown on the activation of JAK2/STAT3 signaling pathway. The results in Fig. 6A indicated that knockdown of TRIM66 significantly inhibited the expressions of p-JAK2 and p-STAT3 in SW620 cells. Treatment with AG490, an inhibitor of JAK2/STAT3 signaling pathway enhanced the inhibitory effects of cell proliferation, migration and invasion in SW620 cells (Fig. 6B–D).

4. Discussion

Previous studies have demonstrated that many TRIM family proteins are aberrantly expressed in multiple diseases, especially in cancers [8,18,19]. Copious evidence has highlighted the strong associations between TRIM family proteins and CRC. TRIM25 has been found to be up-regulated in CRC tissues and cell lines and acts as an oncogene in CRC. CRC cells overexpressing TRIM25 exhibits higher proliferative and migrative abilities [20]. Zhang et al. [21] reported that TRIM27 is upregulated in CRC tissues, and the elevated expression levels of TRIM27 are significantly associated with tumor invasion, metastasis and prognosis. TRIM27 overexpression in CRC cells promotes cell proliferation and EMT process, indicating that TRIM27 is an oncogenic protein in the progression of CRC. Additionally, TRIM58 expression has been observed to be significantly decreased in human CRC tissues and inversely correlated with CRC progression [11]. Overall survival is significantly reduced in CRC patients with low TRIM58 expression. Further investigations prove that ectopic overexpression of TRIM58 inhibits CRC cell invasion and EMT. These findings suggest that TRIM58 functions as a tumor-suppressor *via* inhibiting cell invasion of CRC cells [11].

TRIM66 has been found to be highly expressed in non-small cell lung cancer (NSCLC) tissues when compared with normal paracancerous tissues [14]. The increased TRIM66 expressions are closely associated with lymph node metastasis and TNM stage in NSCLC patients. Besides, high TRIM66 expression is related to short survival time and poor prognosis of NSCLC patients. Besides, TRIM66 was reported to be up-regulated in NSCLC cell lines [22]. Knockdown of TRIM66 suppresses cell proliferation, invasion, migration, clonogenic ability and EMT progress of A549 cells. These findings suggest that TRIM66 serves as a molecular marker for predicting the prognosis of NSCLC. In addition, TRIM66 expression level in osteosarcoma tissues is much higher than that in normal tissues. High TRIM66 expression is correlated with high rate of local recurrence and lung metastasis, and short survival time [15]. Knockdown of TRIM66 in osteosarcoma cell lines significantly induces G1-phase arrest and cell apoptosis, while inhibits cell proliferation, migration, invasion. *In vivo* studies demonstrate that TRIM66 knockdown suppresses tumorigenicity in nude mice [15]. The data indicate that TRIM66 may be a prognostic factor and potential therapeutic target in osteosarcoma. However, the expression and biological roles of TRIM66 in CRC remains unknown. Our results showed that TRIM66 was markedly up-regulated in CRC tissues and cell lines. TRIM66 might function as an oncogenic factor, which is evidenced by the inhibitory effects of TRIM66 knockdown on cell proliferation, migration, invasion, EMT and tumor growth.

JAK/STAT signaling pathway is recognized as an evolutionarily conserved signaling pathway that can be activated by diverse cytokines, interferons, growth factors, and related molecules [23]. JAK/STAT signaling pathway has been found to be involved in multiple processes such as cell division, differentiation, cell death, immunity, and tumor formation [24,25]. Among the several members of JAK and STAT proteins, abnormalities in the JAK2/STAT3 pathway have been observed to be involved in the oncogenesis of several cancers. JAK2/STAT3 pathway plays important roles in tumor cell proliferation, survival, tumorigenesis, angiogenesis and tumor resistance to cancer therapy [16,26,27]. Several other studies have reported that the JAK2/STAT3 signaling pathway is overactive in CRC and implicated in CRC cell growth, survival, invasion, and migration. For instance, inhibition of JAK2/STAT3 signaling induces CRC cell apoptosis *via* mitochondrial pathway and suppresses CRC xenograft tumor growth [17]. Inhibition of JAK2/STAT3 signaling induces cell apoptosis and cell cycle arrest, while reduces tumor cell invasion in CRC cells [16]. Additionally, hispidulin suppresses cell growth and metastasis through regulation of JAK2/STAT3 signaling in CRC cells [28]. These studies imply that JAK2/STAT3 signaling pathway might serve as a therapeutic target for the treatment of CRC. In the present study, we demonstrated that knockdown of TRIM66 suppressed the activation of JAK2/STAT3 pathway in CRC cells. Inhibition of JAK2/STAT3 signaling pathway by AG490 enhanced the anti-tumor effect of TRIM66 knockdown, suggesting that TRIM66 exerted its roles *via* regulating JAK2/STAT3 signaling pathway.

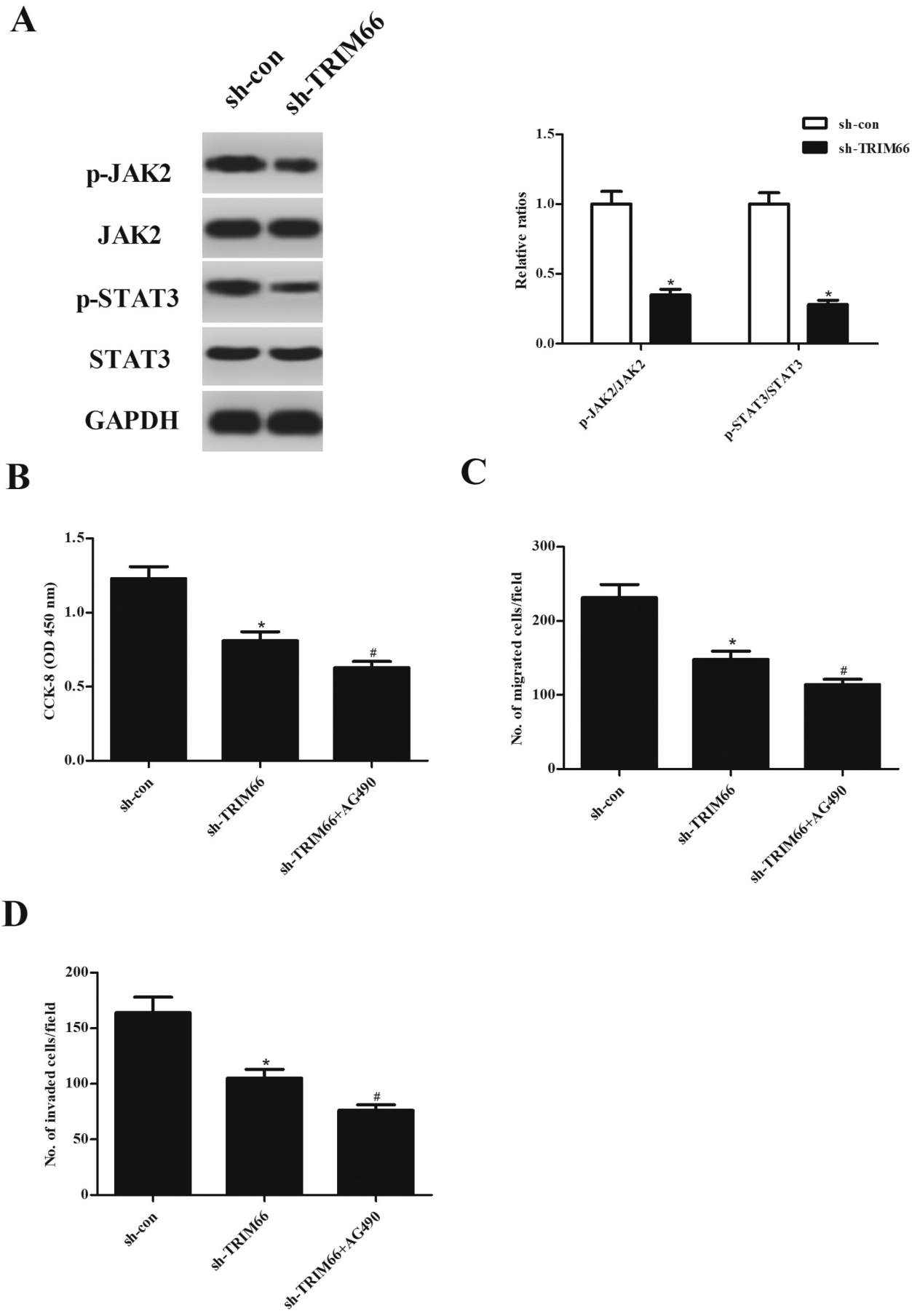

Fig. 6. AG490 enhanced the anti-tumor effect of TRIM66 knockdown in SW620 cells. (A) Effect of TRIM66 knockdown on the activation of JAK2/STAT3 signaling pathway. (B-D) Effect of AG490 on cell proliferation, migration and invasion in SW620 cells. *$p < 0.05$ sh-con; #$p < 0.05$ sh-TRIM66.

5. Conclusion

In conclusion, our study demonstrated that TRIM66 expression was increased in CRC tissues and cell lines. Mechanistic studies proved that knockdown of TRIM66 inhibited cell proliferation, migration, invasion, EMT of CRC cell lines. *In vivo* study revealed that TRIM66 knockdown suppressed tumor growth in nude mice. The anti-tumor effect of TRIM66 knockdown might be attributed to the inhibition of JAK2/STAT3 signaling pathway.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgement

This study was supported by the Foundation for the Central Universities in China (No: xjj2018113) and the National Natural Science Foundation of China (No: 81803015).

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