

TROP2 promotes cell proliferation and migration in osteosarcoma through PI3K/AKT signaling

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Abstract. Human trophoblast cell surface antigen 2 (TROP2) has been noted to serve an important role in the proliferation and migration of various types of human cancers. However, the potential role and the molecular mechanisms of TROP2 in osteosarcoma (OS) remain largely unclear. In the present study, high expression of TROP2 in human OS tissues and cell lines was observed. Overexpression of TROP2 promoted the proliferation and migration of OS cell lines, while TROP2 knockdown markedly decreased cell growth and migration. Furthermore, it was revealed that TROP2 overexpression significantly activated the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway. Collectively, these results suggested that TROP2 may promote OS cell proliferation and migration via PI3K/AKT signaling and may serve as a novel treatment target for OS.

Introduction

Osteosarcoma (OS) is the most common malignant tumor of the skeletal system, with high rates of local invasion and early metastasis. OS mostly occurs among children and adolescents, with a reported incidence rate of 4-5 per million (1). The overall survival rate of patients with OS is reported to be 60-80% (2-4). However, despite the advances in multiagent chemotherapy and surgical technique, the survival rate of patients with locally advanced or metastatic tumors at diagnosis is still very low (<20%) (5-9). Thus, there is urgent need to reveal detailed signal pathways involved in OS pathogenesis and the molecular mechanism of its metastasis, which may provide novel therapeutic targets for the clinical management of OS.

Human trophoblast cell surface antigen 2 (TROP2) is a 36-kDa single-pass transmembrane protein encoded by the tumor-associated calcium signal transducer 2 (Tacstd2) gene, with low to no expression in normal tissues (10-13). Accumulating studies have demonstrated TROP2 to be a candidate tumor prognostic marker, which was highly expressed by various tumors, such as pancreatic cancer (14,15), gastric cancer (11,16), lung cancer (17,18), ovarian cancer (19), and colorectal cancers (20). TROP2 overexpression correlates with increased tumor recurrence, invasiveness, and poor clinical outcome (21-23). Although TROP2 has mostly been reported to be highly expressed in epithelial cancers, previous studies have also found TROP2 expression in stem cells in various tissue types, such as human and mouse prostate (10,24). In addition, TROP2 is also expressed in bone tissues and regulates the proliferation and differentiation of bone marrow stromal cells (25-27). However, so far the potential role and the molecular mechanisms of TROP2 in OS remain largely unclear.

The present study aimed to investigate the role of TROP2 in OS. We evaluated the expression of TROP2 in human OS tissues and cell lines. Furthermore, the specific effects of TROP2 on OS cells proliferation, cell migration, together with the possible mechanism involved in this process were also explored for the first time. The results of the present study suggest that TROP2 may be a potential prognostic biomarker and a potential therapeutic target for OS.

Materials and methods

Clinical tissue samples. This study was approved by the Medical Ethics Committee of The First Affiliated Hospital of Xinjiang Medical University (Urumqi, China). Ten OS specimens and paired adjacent normal bone tissues were collected during surgery from OS patients without prior chemotherapy or radiotherapy (Table I). Written informed consent was obtained from each patient. Tissue samples were immediately stored in liquid nitrogen for further analysis.

Immunohistochemistry (IHC). TROP2 expression was detected by IHC in paraffin-embedded specimens, using the standard immunoperoxidase staining procedure. The 5 μ m thick slides were incubated overnight with anti-TROP2 antibody (1:50; Cell Signaling Technology, Inc., Danvers, MA, USA). Sections were then stained with Diaminobenzidine (DAB) and

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hematoxylin after the application of a secondary antibody for 30 min at room temperature. The degree of immunostaining was reviewed and scored independently by two observers as previously described (6,28).

Cell culture. The normal osteoblast cells hFOB1.19 and OS cell lines U2OS, MG63, and MNNG/HOS were purchased commercially from Academia Sinica Cell Bank (Shanghai, China). hFOB1.19 cells were cultured in DMEM/F-12 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to American Type Culture Collection (ATCC; Manassas, VA, USA) protocols. MNNG/HOS and MG63 cells were cultured in Eagle's minimum essential medium (Thermo Fisher Scientific, Inc.) containing 10% FBS (15140-122; Gibco; Thermo Fisher Scientific, Inc.). The U2OS cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Thermo Fisher Scientific, Inc.) medium containing 10% FBS (15140-122; Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂.

Lentiviral and retroviral infection. A lentiviral short hairpin RNA (shRNA) construct targeting TROP2 was obtained from Jikai Corporation (Shanghai, China), with a sequence as follows: 5'-GCGGCAGAACACGTCTCAGAACTCGAGTTC TGAGACGTGTTCTGCCGC-3'. The oligonucleotides were phosphorylated, annealed, and cloned into the pLKO.1 vector according to the manufacturer's instructions. Lentiviral over-expression particles were prepared by GenePharma (Shanghai, China). Lentiviral and retroviral infection of MG63, U2OS and MNNG/HOS cells were performed according to the manufacturer's protocols. The expression of TROP2 was determined by western blot analyses and quantitative polymerase chain reaction (qPCR).

Cell proliferation. To evaluate the effects of TROP2 on OS cells, MG63, U2OS and MNNG/HOS cells were seeded into 96-well plates on days 1, 2, 3, and 4 post-infection (3,000 cells/well). After 24 h, the cell proliferation rate of MG63 and MNNG/HOS cells was detected with the CCK-8 Assay kit. Experiment was performed with three replicates.

Wound healing assay. For wound healing assay, MG63 and MNNG/HOS cells were seeded into six-well plates and cultured to 100% confluence. The cell layer was carefully scratched to create a wound using a sterile 1,000 μ l pipette tip. Then, the cells were washed twice with PBS and treated with complete medium without FBS. The evaluation of wound healing was done under a light microscope at 24 h after scratching. The percentage of wound closure was calculated using Image J software (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA).

Transwell assay. Transwell assay was performed to determine the effect of TROP2 on the migration of OS cells. Cells were suspended in serum-free medium and added to the upper chamber (Corning Costar, Rochester, NY, USA) and 10% FBS-medium was added in the lower chamber. After incubation for 24 h, the non-migratory cells were removed, while the migratory cells below the membrane were fixed and stained with crystal violet. The number of migration cells were

counted under a light microscope (magnification, x200) using five randomly chosen visual fields.

RNA isolation and reverse transcription (RT)-qPCR. Total cellular RNA was extracted from cell lines using Trizol reagent (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA by using the reverse transcriptional kit (Takara Bio, Inc.). The expression level of TROP2 was quantified by RT-qPCR using SYBR Green PCR PrimeScript RT-PCR Kit (Takara Bio, Inc.) on the ABI Step One Plus System. The following primers were used: TROP2, forward: 5'-CCTCATCGCCGTCATCGT-3' and reverse: 5'-CGGTTTCCTTTCTCAACTCCC'; GAPDH, the internal control, were forward: 5'-GACTCATGACCACAG TCCATGC-3' and reverse: 5'-AGAGGCAGGGATGATGTT CTG-3'. The 2^{- $\Delta\Delta C_q$} method was used to calculate relative gene expression.

Western blot analysis. Total protein of tissue samples and cells was extracted by using radioimmunoprecipitation assay buffer with a protease inhibitor (Beyotime Institute of Biotechnology, Haimen, China). After the protein concentration was measured by the BCA Protein Assay Kit (Beyotime Institute of Biotechnology), equivalent amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). After blocking in 5% nonfat milk for 1 h, the polyvinylidene difluoride membranes were incubated overnight at 4°C with antibodies specific to TROP2 (1:1,000; Cell Signaling Technology, Inc.), β -actin (1:2,000; Cell Signaling Technology, Inc.), p-phosphoinositide 3-kinase (p-PI3K; 1:1,000; Cell Signaling Technology, Inc.), PI3K (1:1,000; Cell Signaling Technology, Inc.), p-protein kinase B (p-AKT; 1:1,000; Cell Signaling Technology, Inc.), and AKT (1:1,000; Cell Signaling Technology, Inc.). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1,000; Beyotime Institute of Biotechnology) was applied for 2 h at room temperature. An enhanced chemiluminescent detection reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to detect immunoreactive bands, and all protein expression was quantified using Bio-Rad XRS chemiluminescence detection system (Bio-Rad Laboratories, Inc.).

Statistical analysis. All experiments were repeated at least three times. Results were presented as mean \pm standard deviation and analyzed using SPSS 23.0 software (IBM Corp., Armonk, NY, USA). Statistical differences between the means of the various groups were analyzed using one-way and two-way analysis of variance (for cell proliferation rate and TROP2 mRNA levels at all time points) followed by the post hoc Bonferroni's test. Differences between two groups were evaluated by using the Student's t-test when appropriate. For all analyses, $P < 0.05$ was considered to indicate a statistically significant difference.

Results

TROP2 was significantly up-regulated in OS cell lines and clinical specimens. RT-qPCR and western blotting analysis were employed to detect the expression of TROP2 in OS cell

Table I. Clinical profiles of the 10 patients with osteosarcoma.

Clinical variable	No. of patients (n=10)
Sex	
Female	5
Male	5
Age, years	
Median	27.7
Range	9-64
Tumor location	
Femur/tibia	8
Humerus	2
Tumor size, cm	
≤5	4
>5	6
Distant metastasis	
Yes	0
No	10

lines. The results show that the expression of TROP2 was markedly up-regulated in three different OS cell lines (U2OS, MG63, and MNNG/HOS) compared to normal osteoblast cells hFOB1.19 ($P<0.05$; Fig. 1). To determine the expression of TROP2 in OS specimens and paired adjacent normal bone tissues, IHC was performed. Significantly higher TROP2 levels were identified in OS tissues than in adjacent normal bone tissues ($P<0.05$; Fig. 2).

TROP2 promotes cell proliferation. The effects of TROP2 on the proliferation rate of OS cells were identified by CCK-8 assay, which revealed the overexpression of TROP2 significantly promoted the proliferation of MG63, U2OS and MNNG/HOS cells in a time-dependent manner. When TROP2 was knocked-down, the proliferation rate decreased significantly (Fig. 3, S1 file). In addition, to check the efficiency of TROP2 overexpression and knockdown, TROP2 mRNA levels at all time points after infection were detected and shown in S2 file.

TROP2 promotes cell migration. To explore the biological effects of TROP2 on OS cell migration, a wound migration assay was performed. Relevant photographs were taken at 0 and 24 h after wound induction. Western blotting analysis showed successful upregulation and downregulation of TROP2 levels in OS cell lines (Fig. 4A and B). The wound-healing abilities of TROP2-overexpressing OS cells were significantly higher than those of the control group. In contrast, TROP2 depletion markedly decreased the wound-closure capacity (Fig. 4C and D). To further explore the function of TROP2 in OS, a Transwell migration assay was performed. As shown in Fig. 4E, migration was markedly increased in cells overexpressing TROP2. In contrast, TROP2 downregulation inhibited the migration of OS cells (Fig. 4E and F).

TROP2 knockdown inhibits the progression of OS by regulating the PI3K/AKT pathway. To explore the possible molecular mechanism underlying the effects of TROP2 on OS cells, we

detected the activation of PI3K/AKT signaling pathway, which is a key regulator of OS development. The result showed that TROP2 overexpression significantly upregulated the levels of p-PI3K and p-AKT. In addition, p-PI3K and p-AKT levels were decreased in TROP2-knockdown cells (Fig. 5A).

Blocking the PI3K/AKT signaling pathway rescues the increased cell proliferation and migration induced by TROP2 overexpression. To verify the role of the PI3K/AKT signaling pathway, LY294002, a specific PI3K/AKT inhibitor, was used to inhibit p-AKT expression. The concentration of LY294002 used was $10\text{ }\mu\text{M}$, based on previous studies (29-31). LY294002 significantly inhibited p-AKT expression (Fig. 5B). The migration ability of OS cells exposed to LY294002 were markedly decreased. The increased migration induced by TROP2 overexpression were also reduced by LY294002 exposure (Fig. 5C).

Discussion

Although outstanding advances in multiagent chemotherapy and surgical technique have been achieved in recent decades, the prognosis for OS patients with locally advanced or metastatic remains poor (2-4). Thus, there is an urgent need to research the molecular mechanisms and deregulated genes responsible for OS carcinogenesis. In this study, we found that TROP2 was significantly upregulated in human OS tissues. Overexpression of TROP2 increased the proliferation and migration of OS cells, while TROP2 knockdown significantly decreased cell growth and migration. Moreover, upregulation of TROP2 activated PI3K/AKT pathway. The increased migration observed after TROP2 overexpression were rescued by inhibition of PI3K/AKT. These novel findings indicate that TROP2 promotes OS cell proliferation and migration through PI3K/AKT signaling.

Previous studies have linked TROP2 to increased tumor growth and demonstrated TROP2 to be a candidate tumor prognostic marker in various tumors. TROP2 is found to be overexpressed in the majority of human epithelial cancers, including pancreatic cancer, gastric cancer, lung cancer, ovarian cancer, and colorectal cancers (10,14,32). Chen *et al* (33) investigated the expression of TROP2 and epithelial-mesenchymal transition (EMT) indicator proteins in 93 patients with gallbladder cancer (GBC) and demonstrated that increased expression of TROP2 in GBC is associated significantly with aggressive progression and poor prognosis. In another study based on clinical samples, Guan *et al* (34) found that overexpression of TROP2 could be an independent predictor for poor clinical outcome in nasopharyngeal carcinoma. In cervical cancer cell line CaSki cells, Liu *et al* (35) found that TROP2 knockdown significantly inhibited the proliferation and colony formation of CaSki cells, and demonstrated that TROP2 might become a novel target for cervical cancer treatment. Similarly, in our study, IHC revealed higher TROP2 expression in OS samples, suggesting that high TROP2 expression may be associated with a poor prognosis. Overexpression of TROP2 increased the proliferation and migration of two different OS cell lines, indicating that high TROP2 levels may promote the generation, development, and metastasis of OS.

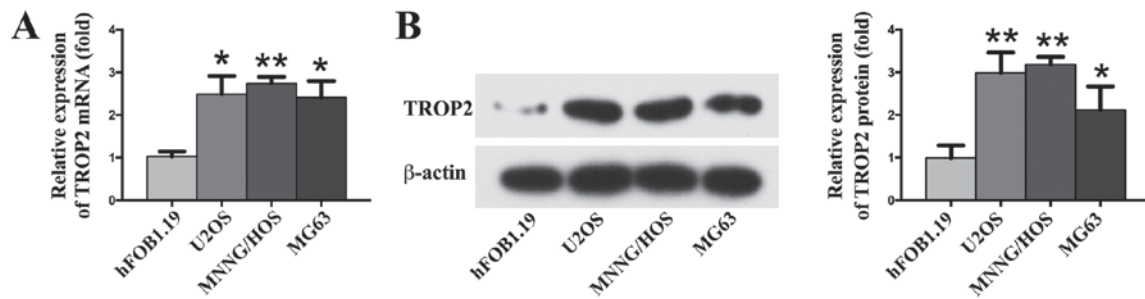


Figure 1. TROP2 is upregulated in OS cell lines. (A) Reverse transcription-quantitative polymerase chain reaction analysis revealed the expression levels of TROP2 in three OS cell lines (U2OS, MG63 and MNNG/HOS) and the normal osteoblast cells hFOB1.19. (B) Western blot analysis was performed to determine the expression of TROP2 in the OS cell lines (U2OS, MG63, and MNNG/HOS) and the normal osteoblast cells hFOB1.19. These results indicated that the expression of TROP2 was upregulated in OS cell lines. * $P < 0.05$ and ** $P < 0.01$ vs. hFOB1.19. TROP2, trophoblast cell surface antigen 2; OS, osteosarcoma.

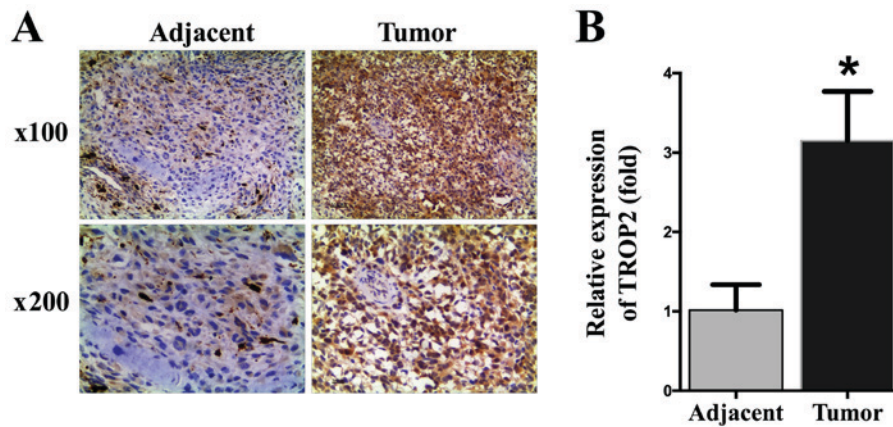


Figure 2. TROP2 is upregulated in clinical OS specimens. (A) Representative immunohistochemical staining image of OS tissues and paired adjacent normal bone tissues. Magnification, x100 and x200 as indicated. (B) Relative quantitative comparison of TROP2 expression in OS tissues and paired adjacent normal bone tissues. * $P < 0.05$ vs. adjacent normal bone tissues. TROP2, trophoblast cell surface antigen 2; OS, osteosarcoma.

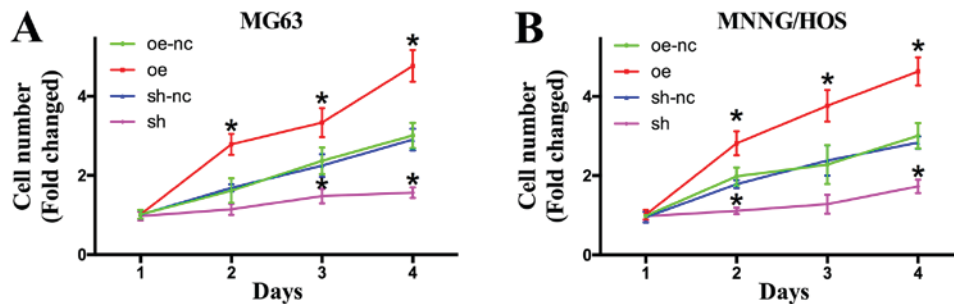


Figure 3. Effects of TROP2 overexpression and knockdown on OS cell proliferation. (A) Cell proliferation was assessed in MG63 cell lines by CCK-8 assay on days 1, 2, 3 and 4 following lentiviral infection. (B) Cell proliferation was assessed in MNNG/HOS cell lines by CCK8 assay on days 1, 2, 3 and 4 following lentiviral infection. * $P < 0.05$ vs. oe-nc. TROP2, trophoblast cell surface antigen 2; OS, osteosarcoma; Oe, overexpression of TROP2; sh, short hairpin RNA knockdown of TROP2; nc, negative control; oe-nc, the negative control group of TROP2 overexpression; sh-nc, the negative control group of TROP2 knockdown using nc short hairpin RNA; CCK-8, Cell Counting Kit-8.

The PI3K/AKT pathway plays a crucial role in tumorigenesis. Previous studies have shown that alteration of the PI3K/AKT pathway is strongly implicated in OS pathogenesis. Moreover, studies have suggested that inhibition of the PI3K/AKT pathway disrupts functions essential for OS progression (36,37). Graziano *et al* (38) found that Wilms' tumor gene 1 (WT1) silencing inhibits OS cell proliferation by down-regulating PI3K/AKT pathway. Huang and Jin (39) reported that the activation of the PI3K/AKT signal pathway is essential

for the effects of zinc finger transcription factor ZIC2 on OS cells, and the effects of ZIC2 on the OS cells were reversed by a PI3K/AKT inhibitor. Interestingly, a previous study indicated that TROP2 promotes PI3K/AKT activation (40). Considering the critical role of PI3K/AKT in cell proliferation and migration, we assessed the levels of PI3K/AKT signaling pathway in OS cells. Being consistent with previous studies (26,40,41), TROP2 increased the expression of total p-PI3K and p-AKT via PI3K/AKT pathway. Furthermore, the increased migration

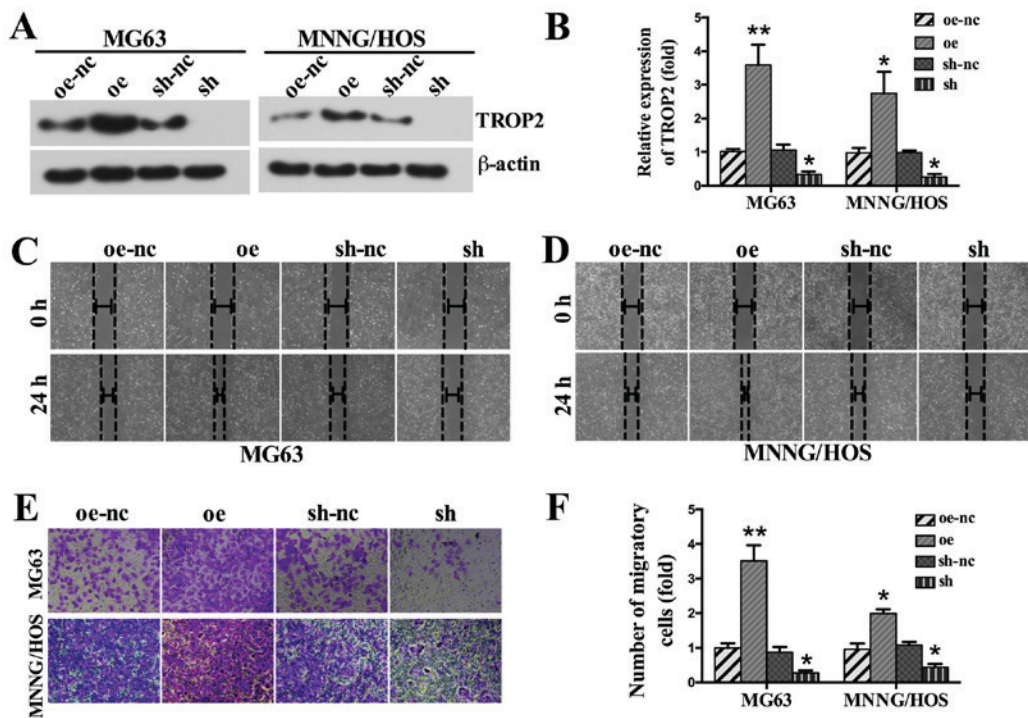


Figure 4. Effects of TROP2 knockdown and overexpression on the migration of OS cells. (A) Western blot analysis was performed to (B) confirm the upregulation of TROP2 protein expression by lentiviral infection, and the downregulation of TROP2 protein expression by shRNA in MG63 and MNNG/HOS cell lines. A wound-healing assay was performed to assess migration in the (C) MG63 and (D) MNNG/HOS cell lines (magnification, $\times 100$). (E) Transwell assays were performed to assess migration in the MG63 and MNNG/HOS cell lines (magnification, $\times 200$). (F) Relative quantitative comparison of migratory cells. Images are representative of three independent experiments. All data are expressed as means \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. oe-nc. TROP2, trophoblast cell surface antigen 2; OS, osteosarcoma; Oe, overexpression of TROP2; sh, short hairpin RNA knockdown of TROP2; nc, negative control; oe-nc, the negative control group of TROP2 overexpression; sh-nc, the negative control group of TROP2 knockdown using nc short hairpin RNA.

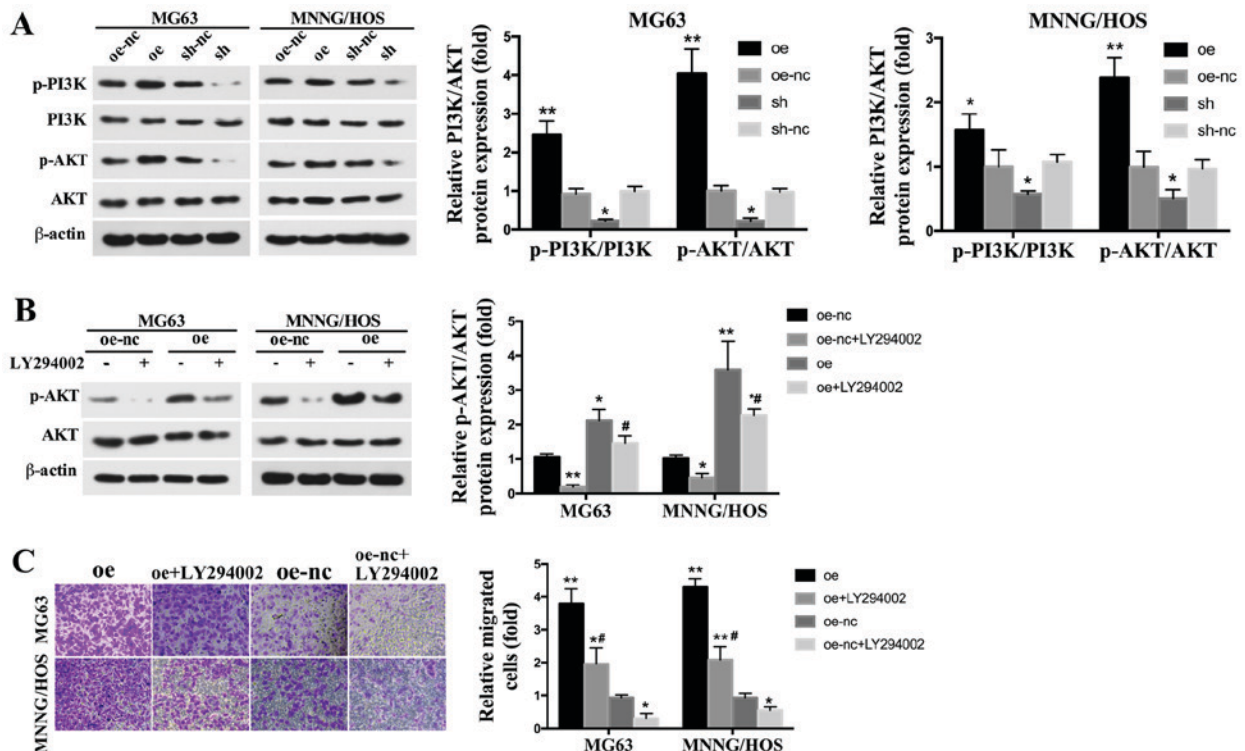


Figure 5. Effects of TROP2 knockdown and overexpression on the PI3K/AKT signaling pathway. (A) The protein levels of p-PI3K, PI3K, p-AKT and AKT were assessed by western blot analyses. (B) The effect of TROP2 overexpression on p-AKT expression level following the application of LY294002. (C) The effect of TROP2 overexpression on MG63 and MNNG/HOS cell migration following the application of LY294002 (magnification, $\times 200$). * $P < 0.05$ and ** $P < 0.01$ vs. oe-nc; # $P < 0.05$ vs. oe group. TROP2, trophoblast cell surface antigen 2; OS, osteosarcoma; Oe, overexpression of TROP2; sh, short hairpin RNA knockdown of TROP2; nc, negative control; oe-nc, the negative control group of TROP2 overexpression; sh-nc, the negative control group of TROP2 knockdown using nc short hairpin RNA.

and proliferation induced by TROP2 overexpression was rescued by a PI3K/AKT inhibitor. Thus, we inferred that up-regulating TROP2 causes aberrant activation of PI3K/AKT in OS cells.

To the best of our knowledge, this is the first study of the effect of TROP2 on OS cells. However, some limitations of the present study should be noted. First, this was an *in vitro* study without *in vivo* evaluation, which may decrease the robustness of the results. Thus, further *in vivo* application of TROP2-related treatment, such as molecular targeted drug for TROP2 should be used to verify these findings. Second, as most of the findings in this study were obtained from MG63 and MNNG/HOS cells, more OS cell lines should be studied in the future. Third, due to the small patient population size, we have limited ability to reveal the correlation between TROP2 expression and the prognosis of OS. In addition, the carcinogenesis of OS is complicated, which means TROP2 may also target other signaling pathways, further studies are therefore required. Nevertheless, our study provides useful insight into the effect of TROP2 on cell proliferation and migration in OS cell lines. Taken together, our data suggest that TROP2 promotes human OS cell proliferation and migration via activation of PI3K/AKT pathway.

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Availability of data and materials

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

Authors' contributions

ZT conceived and designed the present study. QG, AN and XG performed the experiments and were also the predominant contributors to the writing of the manuscript. KT and CL analyzed and interpreted the data. XF made contributions to the interpretation of data and critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of The First Affiliated Hospital of Xinjiang Medical University (Xinjiang, China). Written informed consent was obtained from each patient.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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