Triptolide inhibits migration and viability, and promotes apoptosis by targeting the PI3K/Akt signaling pathway via upregulation of miR-125a-5p in SW1353 human chondrosarcoma cells

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Abstract. Malignant chondrosarcoma is a rare type of bone cancer, for which an effective comprehensive treatment strategy is lacking. Triptolide (TPL) is an active chemical component originating from the Chinese herb *Tripterygium wilfordii* Hook F, which exerts inhibitory effects on various cancer cells. However, to the best of our knowledge, little is currently known about the effect of TPL on chondrosarcoma. In the present study, SW1353 human chondrosarcoma cells were used as *in vitro* cell model. Cell Counting Kit-8, Annexin V/PI staining, wound healing assay, Transwell invasion assay and the detection of proinflammatory cytokines were performed to determine the effects of TPL on SW1353 chondrosarcoma cell viability, apoptosis, migration, invasion and inflammation. In addition, the protein expression levels of phosphorylated (p)-PI3K, PI3K, p-AKT and AKT were detected to determine whether TPL exerted its antitumor effects via the PI3K/Akt signaling pathway. Furthermore, the microRNA (miR)-125a-5p-inhibitor was introduced into cells to determine whether TPL exerted its effect on SW1353 cells via miR-125a-5p. TPL inhibited SW1353 chondrosarcoma cell viability, migration, invasion and proinflammatory cytokine expression in a dose-dependent manner. The most obvious effect observed in the current study was in the 50 nM TPL group. In addition, TPL inhibited PI3K/Akt signaling pathway activation and upregulated miR-125a-5p expression in a dose-dependent manner. By contrast, miR-125-5p inhibition accelerated the viability, migration, invasion and proinflammatory cytokine expression of SW1353 cells compared with the control. Notably, miR-125a-5p inhibition reversed the effects of TPL on SW1353 cells. In conclusion, TPL exerted an antitumor effect on SW1353 human chondrosarcoma cells and miR-125a-5p served an essential role in inhibiting the tumorigenic phenotype of chondrosarcoma cells. TPL exerted its inhibitory effects on chondrosarcoma cells via upregulating miR-125a-5p and inhibiting the PI3K/Akt signaling pathway.

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Introduction

Malignant chondrosarcoma is a rare type of bone cancer that affects cartilaginous tissues; it accounts for 20-25% of bone sarcomas in Norway and 20-30% of cases in the United States, making it the second most common type of sarcoma in the United States (1,2). The main treatment for chondrosarcoma is surgery (3), and tumor size and location determine the treatment. The affected bone and the surrounding tissue may need to be removed, and this may be followed by radiation therapy, chemotherapy or a combination of both (4). However, when the cancer has spread to other parts of the body, surgery may not be an option (4); in these cases, chemotherapy or radiation may be used to control the cancer. Although chondrosarcoma is generally considered a slow-growing cancer, it can be difficult to treat. When the tumor is located in a difficult-to-operate area, such as intracranial chondrosarcoma and chondrosarcoma of the axial skeleton (including pelvis and spine), or has already spread to multiple organs, surgery is not always possible or surgery alone cannot completely remove the lesion Notably, the 10-year survival rate of patients with high-grade chondrosarcoma is <30% (5); therefore, it is essential to develop alternative options to alleviate the prognosis of chondrosarcoma.

Triptolide (TPL) is an active chemical component that originates from the Chinese herb *Tripterygium wilfordii* Hook F (6). *Tripterygium wilfordii* Hook F has a long history in clinical practice for treating inflammatory diseases, such as rheumatoid arthritis, and autoimmune disorders. TPL has also attracted the interest of researchers due to its potential therapeutic effects in cancer treatment. Notably, TPL has been reported to slow the proliferation of various types of cancer cells, such as prostate, breast, lung, colon and ovarian cancer (7-12). Studies have also shown that TPL promotes apoptosis of cancer cells (13). In addition, TPL has been reported to suppress angiogenesis, or the formation of new blood vessels, which is vital for cancer cell proliferation and metastasis (14). In an unpublished clinical trial focused on pancreatic cancer, Gene Ontology analysis of assay for transposase-accessible chromatin with sequencing data from paired biopsies showed that TPL was able to downregulate the

expression of genes negatively regulating the cell cycle and to upregulate genes controlling cell cycle checkpoints (15), thus indicating its potential in cancer treatment. Furthermore an animal study revealed that TPL may be effective in the treatment of different types of malignant tumor (16), such as lung cancer and prostate cancer (17-20), due to its effects on inhibiting cancer cell growth and migration, and enhancing sensitivity to chemotherapy However, the effect of TPL on chondrosarcoma is not fully understood; therefore, the present study aimed to determine the potential of TPL on treating chondrosarcoma cells.

The PI3K/Akt signaling pathway is known to participate in the development of chondrosarcoma; this pathway is overactivated in chondrosarcoma cells, leading to increased cell survival and proliferation (21,22). The increased expression of these oncogenic proteins, including PI3K and Akt, leads to enhanced cell proliferation and survival, which are hallmarks of chondrosarcoma (23-25). In addition, it has been reported that mutations in the PI3K/Akt signaling pathway are associated with an increased risk of developing chondrosarcoma (26); thus, the PI3K/Akt signaling pathway is considered to play a vital role in its pathogenesis. Overactivation of this pathway due to mutations in PI3K or Akt genes leads to increased expression of several oncogenic proteins, including mTOR, VEGF and cyclin D1, as well as increased tumor vascularization and an increased risk of developing chondrosarcoma (27,28). Therefore, developing a strategy that inhibits PI3K/Akt may be effective in treating chondrosarcoma.

In the present study, the inhibitory effects of TPL on SW1353 human chondrosarcoma cell viability, apoptosis, migration, invasion and inflammation were assessed, and the underlying molecular mechanism associated with the miR-125a-5p/PI3K/Akt axis was investigated.

Materials and methods

miR-125a-5p-inhibitor transfection. miR-125a-5p-inhibitor (cat. no. miR20000443-1-5) and miR-control (Table I) were purchased from Guangzhou RiboBio Co., Ltd. and were transfected using a transfection kit (cat. no. C10511-05; Guangzhou RiboBio Co., Ltd.) into SW1353 human chondrosarcoma cells (cat. no. CL-0447; Wuhan Pricella Biotechnology Co., Ltd.). The cells were cultured in DMEM (cat. no. CGM101.05; CellMax) supplemented with 10% FBS (cat. no. SA211.02; CellMax) at 37˚C in an atmosphere containing 5% CO_2. Briefly, $1x10^5$ cells/well were seeded into 24-well plates. After 24 h, 100 nM miR-125a-5p-inhibitor or miR-control was added at 37˚C for 24 h. A total of 24 h post-transfection, the expression levels of miR-125a-5p in both groups were detected by reverse transcription-quantitative PCR (RT-qPCR) and further experiments were conducted 48 h after transfection.

Cell Counting Kit (CCK)-8 assay. The CCK-8 cell viability assay (cat. no. C0038; Beyotime Institute of Biotechnology) was conducted according to the manufacturer's instructions. Briefly, SW1353 cells were seeded into a 96-well plate at a density of $5x10^3$ cells/well. Subsequently, the cells were incubated with 0, 5, 10, 20, 50, 100, 200 and 500 nM TPL (cat. no. HY-32735; MedChemExpress) for 24 h. After 24 h, cells were treated with 10% CCK-8-DMEM at 37˚C for 2 h.

Table I. miR-125a-5p inhibitor and miR-control sequences.

Name	Sequence, 5'-3'
miR-125a-5p-inhibitor	UCACAGGUUAAAGGGUCUCAGGGA
miR-control	CAGUACUUUUGUGUAGUACAA

miR, microRNA.

Subsequently, measurement was conducted at OD 450 nm using a microplate reader. For miR-125a-5p inhibition experiments, cells transfected with miR-control and miR-125a-5p-inhibitor were cultured with or without 50 nM TPL at 37˚C for 24 h. The CCK-8 analysis of cells transfected with miR-control and miR-125a-5p-inhibitor was performed as aforementioned.

Apoptosis assay. SW1353 human chondrosarcoma cells were seeded into a 6-well plate at a density of $1x10^6$ cells/well. Subsequently, 10, 20 and 50 nM TPL was added at 37˚C for 24 h. After treatment, the cells were collected with trypsin, and Annexin V-FITC and PI staining (cat. no. C1062L; Beyotime Institute of Biotechnology) was conducted at room temperature for 10 min according to the manufacturer's instructions. The FITC and PI channels were selected and 10,000 events were counted by flow cytometry (Accuri™ C6 PLUS; BD Biosciences) followed by analysis using CSampler software (version 1.0.264.21; BD Biosciences).

Wound healing migration assay. SW1353 chondrosarcoma cells were seeded in a 6-well plate. When the confluence of the seeded SW1353 chondrosarcoma cells reached 100, 10% FBS-containing DMEM was removed from the SW1353 cells and they were starved in serum-free DMEM for 12 h at 37˚C. Subsequently, a 200-μl pipette tip was used to generate a wound in the middle of the cell monolayer. The cells were then washed three times with PBS, and were treated with DMEM containing 0, 10, 20 and 50 nM TPL for 24 h at 37˚C. For miR-125a-5p inhibition experiments, SW1353 cells transfected with miR-control and miR-125a-5p-inhibitor were incubated in the presence or absence of 50 nM TPL for 24 h at 37˚C. Subsequently, images of the cells were captured at 0 and 48 h (magnification, x40) using a light microscope. ImageJ software (version 1.53; National Institutes of Health) was used to measure the migration area and the migration rate (%) was calculated as follows: (Area at 0 h-Area at 48 h)/Area at 0 h x100.

Transwell invasion assay. For the invasion assay, a 24-well Transwell system (pore size, 8 μm) was used. Before cells were seeded, the chambers were precoated with Matrigel at 37˚C for 12 h. Trypsin was used to collect the SW1353 chondrosarcoma cells, and the cells were resuspended in serum-free DMEM and seeded into the upper chamber at an initial density of $1x10^5$ cells/well. DMEM containing 10% FBS was added into the lower chamber. The cells were treated with 0, 10, 20 and 50 nM TPL for 24 h at 37˚C. For miR-125a-5p inhibition experiments, SW1353 cells transfected with

Table II. Primer sequences.

Target name	Forward, 5'-3'	Reverse, 5'-3'
IL-1β	CCAAACCTCTTCGAGGCACA	AGCCATCATTTCACTGGCGA
IL-6	CGCCTTCGGTCCAGTTGC	TCTGAGGTGCCCATGCTACA
TNF-α	CTGGGCAGGTCTACTTTGGG	CTGGAGGCCCCAGTTTGAAT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
miR-125a-p	GGCGTCCCTGAGACCCTTTAA	GTGCAGGGTCCGAGGT
U6	GAAAGAAGACGCCGAGAAAGG	GGGAGATGTGGATCTATGTCGT

miR, microRNA.

miR-control and miR-125a-5p-inhibitor were incubated in the presence or absence of 50 nM TPL for 24 h at 37˚C. After 24 h of treatment, the cells were fixed in paraformaldehyde for 20 min and were washed with PBS. Hoechst 33342 staining solution was used to stain the cells that invaded from the upper chamber at room temperature for 10 min. After staining, $\lambda_{ex/em}$=346/460 nm was measured using a microplate reader and images were captured using an inverted fluorescence microscope (magnification, x40).

RT-qPCR. SW1353 chondrosarcoma cells were treated with 0, 10, 20 and 50 nM TPL for 24 h at 37˚C with or without lipopolysaccharide (LPS; cat. no. HY-D1056; MedChemExpress) stimulation. For miR-125a-5p inhibition experiments, SW1353 cells transfected with miR-control and miR-125a-5p-inhibitor were incubated in the absence or presence of 50 nM TPL for 24 h at 37˚C. After treatment, total RNA was extracted from SW1353 human chondrosarcoma cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). For miRNA RT-qPCR detection, the TaqMan miRNA kit (cat. no. 4427975; Applied Biosystems; Thermo Fisher Scientific, Inc.) was applied for RT and qPCR, according to the manufacturer's protocol. For mRNA RT-qPCR detection, the PrimeScript™ RT reagent kit (cat. no. RR037Q; Takara Biotechnology Co., Ltd.) was used to reverse transcribe mRNA, according to the manufacturer's protocol. qPCR detection of mRNA was conducted using the SYBR Green kit (cat. no. D7262; Beyotime Institute of Biotechnology) under the following conditions: 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec, 58˚C for 15 sec and 72˚C for 1 min on a PCR machine. For miRNA detection, the following conditions were performed: 95˚C for 3 min, followed by 40 cycles at 95˚C for 15 sec, 58˚C for 30 sec and 72˚C for 30 sec. The mRNA and miRNA expression levels were normalized to GAPDH and U6, respectively, and $2^{-\Delta\Delta Cq}$ was used to quantify expression levels (29). The primer sequences are shown in Table II.

Western blotting. The SW1353 cells were incubated with 0, 10, 20 and 50 nM TPL for 24 h at 37˚C. For miR-125a-5p-inhibitor experiments, SW1353 cells transfected with miR-control and miR-125a-5p-inhibitor were incubated in the presence or absence of 50 nM TPL for 24 h at 37˚C. After treatment, total protein was isolated from SW1353 human chondrosarcoma cells using RIPA reagent (cat. no. P0013B; Beyotime Institute of Biotechnology). Subsequently, the loading buffer was added to the protein samples and boiled for 5 min and protein samples were quantified by BCA kit (cat. no. P0010S; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Protein samples (20 μg) were separated by SDS-PAGE on 10% gels and transferred to PVDF membranes, and then blocked with 5% nonfat milk for 30 min at room temperature. The membranes were incubated with PI3K (1:1,000; cat. no. ab302958; Abcam), AKT (1:1,000; cat. no. ab300473; Abcam), phosphorylated (p)-AKT (1:1,000; cat. no. ab192623; Abcam) and p-PI3K (1:1,000; cat. no. ab278545; Abcam) and GAPDH (1:5,000; cat. no. 10494-1-AP; Proteintech Group, Ltd.) primary antibodies overnight at 4˚C, and were then incubated with HRP-conjugated Goat Anti-Rabbit IgG(H+L) secondary antibodies (1:20,000; cat. no. SA00001-2; Proteintech Group, Ltd.) for 1 h at 37˚C. Subsequently, the blots were visualized with an ECL reagent (cat. no. SQ202; Epizyme, Inc.) and the membranes were exposed using the Bio-Rad imaging system (Bio-Rad Laboratories, Inc.). Image Lab software (version 6.0.1; Bio-Rad Laboratories, Inc.) was used for semi-quantification.

Statistical analysis. GraphPad Prism 8.0 software (Dotmatics) was used for statistical analysis. Data are presented as the mean ± standard deviation of three biological replicates. One-way ANOVA was used to determine the significance of the differences between groups, followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TPL suppresses SW1353 chondrosarcoma cell viability. The CCK-8 cell viability assay was conducted to determine the cytotoxic effects of TPL on SW1353 cells. The results demonstrated that 10, 20, 50, 100, 200 and 500 nM TPL reduced the viability of SW1353 cells in a dose-dependent manner (Fig. 1A). As shown in Fig. 1B, the IC_{50} value of TPL in SW1353 cells was 100.5 nM. Since concentrations >100 nM were larger than the IC_{50} value, TPL concentrations at 10, 20 and 50 nM were selected for further experiments.

TPL promotes SW1353 chondrosarcoma cell apoptosis. Annexin V/PI staining was conducted to determine whether TPL has pro-apoptotic effects on SW1353 chondrosarcoma

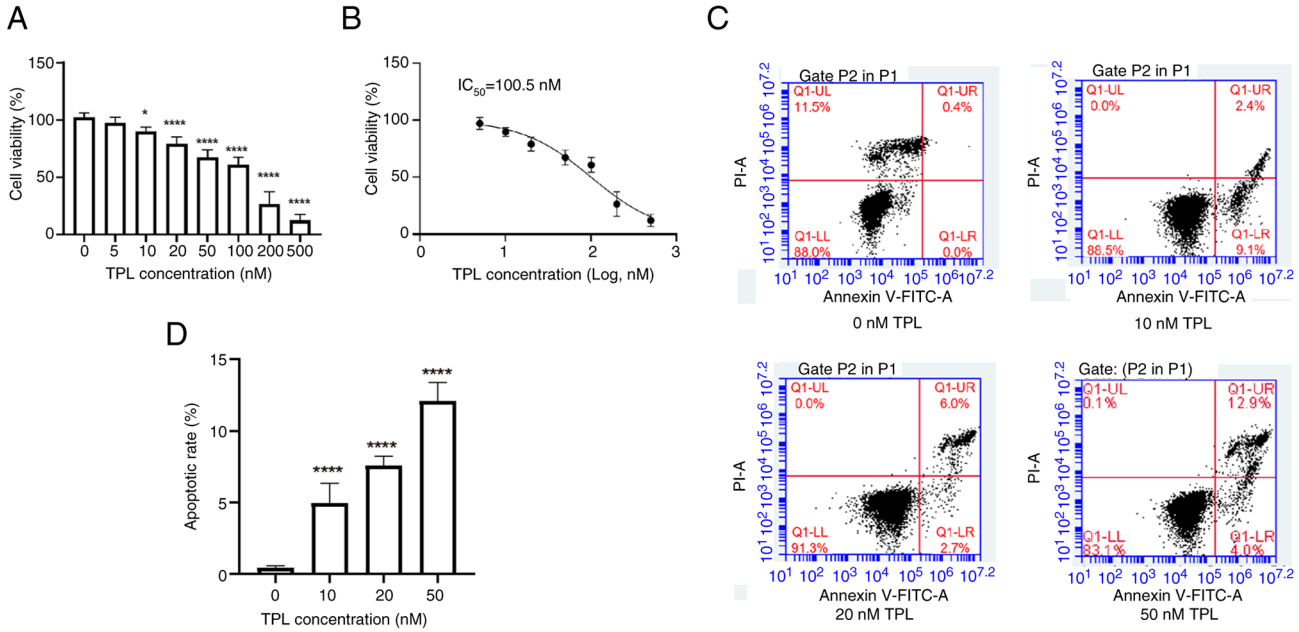

Figure 1. Effect of TPL on SW1353 human chondrosarcoma cell viability and apoptosis. (A) Cell Counting Kit-8 analysis of various concentrations of TPL on SW1353 cell viability. (B) IC_{50} value of TPL in SW1353 cells. (C) Annexin V/PI staining revealed the change in apoptotic rate after TPL treatment of SW1353 cells. (D) Statistical results of (C) Statistical analysis was performed using one-way ANOVA; *P<0.05, ****P<0.0001 vs. control. TPL, triptolide.

cells. As shown in Fig. 1C and D, the apoptotic rate was increased by TPL in a dose-dependent manner. This outcome indicated that TPL enhanced the apoptosis of SW1353 human chondrosarcoma cells.

TPL inhibits SW1353 chondrosarcoma cell migration. A wound healing migration assay was performed to assess whether TPL inhibited the migration of SW1353 chondrosarcoma cells. As shown in Fig. 2A, 10 nM TPL slightly inhibited wound closure, 20 nM TPL moderately inhibited wound closure and 50 nM TPL markedly inhibited wound closure. As shown in Fig. 2B, the migration rate of the control, 10, 20 and 50 nM TPL groups were 82.67±1.70, 72.33±2.50, 57.67±3.09 and 33.67±5.79%, respectively. These results demonstrated that TPL inhibited wound healing *in vitro* in a dose-dependent manner.

TPL inhibits SW1353 chondrosarcoma cell invasion. The Transwell invasion assay was conducted to investigate whether TPL reduced the invasion of SW1353 cells. The Transwell chamber coated with Matrigel gelatinous protein mixture was used, and the cells that successfully invaded through the Matrigel to the lower Transwell chamber were stained with Hoechst 33342 and fluorescence intensity was recorded. As shown in Fig. 2C and D, 10, 20 and 50 nM TPL markedly inhibited SW1353 human chondrosarcoma cells invasion in a dose-dependent manner compared with that in the control group; notably, 20 and 50 nM TPL resulted in a significant decrease in invasion.

Effect of TPL on proinflammatory cytokines. To determine whether TPL had any effect on SW1353 human chondrosarcoma cell inflammation, the mRNA expression levels of proinflammatory cytokines, IL-1β, IL-6 and TNF-α, were detected. As shown in Fig. 3A, IL-1β expression was upregulated by LPS stimulation, which was used to induce abundant pro-inflammatory cytokine levels in SW1353 cells, whereas 20 and 50 nM TPL significantly downregulated the LPS-induced upregulation of IL-1β in a dose-dependent manner and 50 nM exhibited the best effect. Similar trends were identified regarding TNF-α and IL-6 expression (Fig. 3B and C). These results indicated that TPL downregulated LPS-induced activation of proinflammatory cytokines in SW1353 human chondrosarcoma cells.

TPL downregulates the PI3K/Akt pathway. To determine whether TPL exerts its antitumor effects via inhibiting the PI3K/Akt signaling pathway, the protein expression levels of p-PI3K, PI3K, p-PI3K/PI3K, p-Akt, Akt and p-Akt/Akt were detected. As shown in Fig. 4, 10, 20 and 50 nM TPL downregulated the expression levels of p-PI3K and p-AKT in a dose-dependent manner, whereas there was no difference in PI3K and Akt expression compared with the 0 nM control; among the concentrations 10, 20 and 50 nM TPL significantly decreased the p-PI3K/PI3K ratio compared with 0 nM, whereas 20 and 50 nM TPL significantly decreased the p-AKT/Akt ratio compared with 0 nM. These results demonstrated that TPL inhibited PI3K/Akt activation in SW1353 human chondrosarcoma cells and this may be the mechanism underlying the inhibitory effects of TPL on SW1353 chondrosarcoma cells.

TPL promotes miR-125a-5p expression. To verify whether TPL regulates the PI3K/AKT signaling pathway directly or indirectly, the expression levels of miR-125a-5p were detected, due to its pivotal role in tumor progression (30). As shown in Fig. 5A, miR-125a-5p was upregulated by 10, 20 and 50 nM TPL treatment in a dose-dependent manner. These results indicated that miR-125a-5p might mediate the antitumor

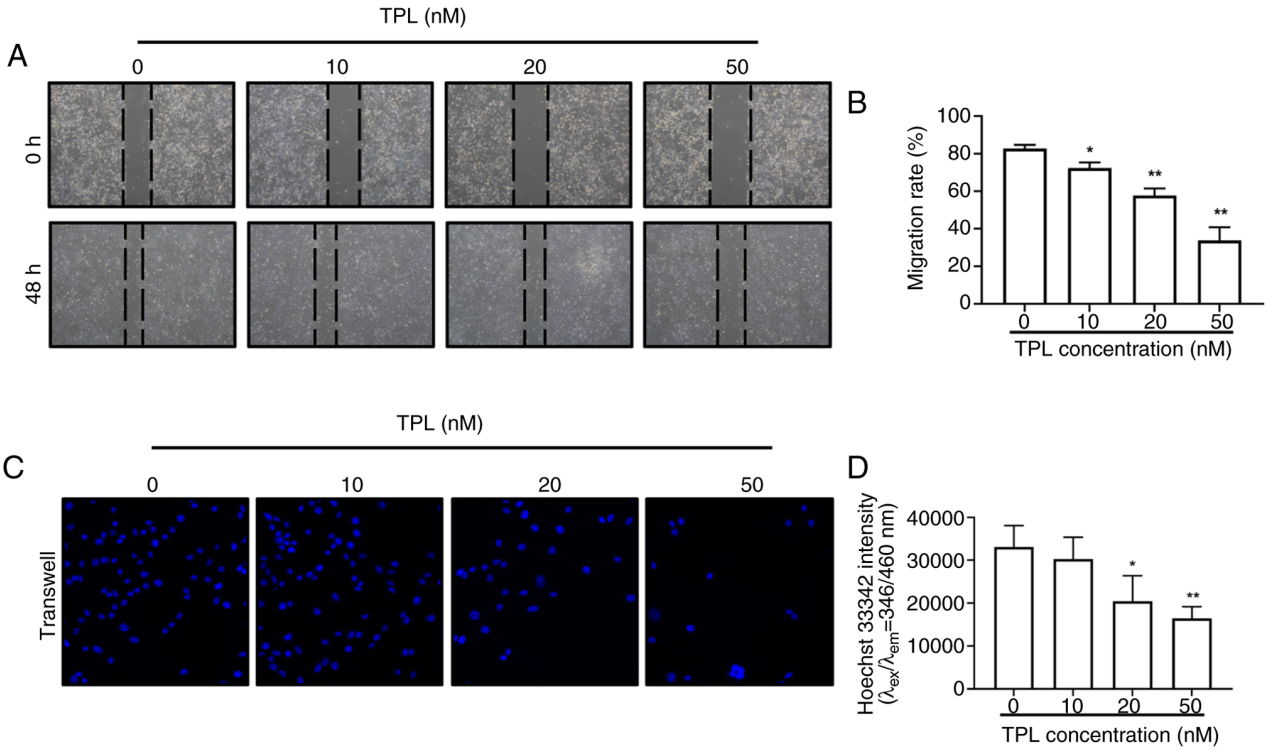

Figure 2. Effect of TPL on SW1353 human chondrosarcoma cell migration and invasion. (A Wound healing assay revealed that TPL inhibited SW1353 cell migration and (B) TPL exerted its anti-migratory effect in a dose-dependent manner. (C) Transwell invasion assay revealed that TPL inhibited SW1353 cell invasion and (D) TPL exerted its anti-invasive effect in a dose-dependent manner. Statistical analysis was performed using one-way ANOVA; *P<0.05, **P<0.01 vs. control. TPL, triptolide.

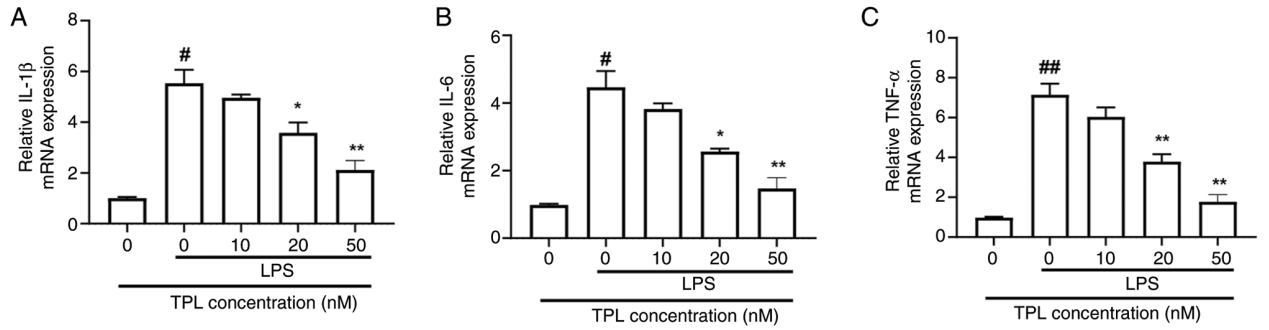

Figure 3. Effect of TPL on LPS-induced inflammatory cytokine expression in SW1353 human chondrosarcoma cells. TPL reduced LPS-induced (A) IL-1β, (B) IL-6 and (C) TNF-α mRNA expression in a dose-dependent manner in SW1353 chondrosarcoma cells. Statistical analysis was performed using one-way ANOVA; *P<0.05, **P<0.01 vs. LPS treated group; $^{\#}$P<0.05, $^{\#\#}$P<0.05 L vs. control group. LPS, lipopolysaccharide; TPL, triptolide.

effects of TPL on SW1353 human chondrosarcoma cells and TPL might exert its regulatory effect on PI3K/Akt via upregulating miR-125a-5p. Furthermore, SW1353 chondrosarcoma cells were transfected with a miR-125a-5p-inhibitor, and miR-control was used as a control, to verify the effect of TPL on miR-125a-5p; transfection success is shown in Fig. 5B. TPL significantly promoted miR-125a-5p expression compared with that in the miR-control group, whereas transfection with a miR-125a-5p-inhibitor blocked this trend (Fig. 5B). Thus, the present results suggested that the miR-125a-5p-inhibitor successfully downregulated the expression levels of miR-125a-5p and reversed the TPL-induced upregulation of miR-125a-5p in SW1353 chondrosarcoma cells.

Inhibiting miR-125a-5p reverses TPL-induced inhibition of SW1353 chondrosarcoma cell viability. To verify whether TPL exerted its inhibitory effect on SW1353 chondrosarcoma cell viability via miR-125a-5p, the CCK-8 cell viability assay was conducted. As shown in Fig. 6A, cell viability was significantly increased by miR-125a-5p inhibition compared with that in the miR-control group. By contrast, 50 nM TPL reduced cell viability, whereas miR-125a-5p inhibition blocked this trend. These results indicated that 50 nM TPL exerted its inhibitory effect on SW1353 chondrosarcoma cell viability by promoting miR-125a-5p expression, and inhibiting miR-125a-5p reversed the trend.

Inhibiting miR-125a-5p reverses TPL-induced SW1353 chondrosarcoma cell apoptosis. To determine the underlying

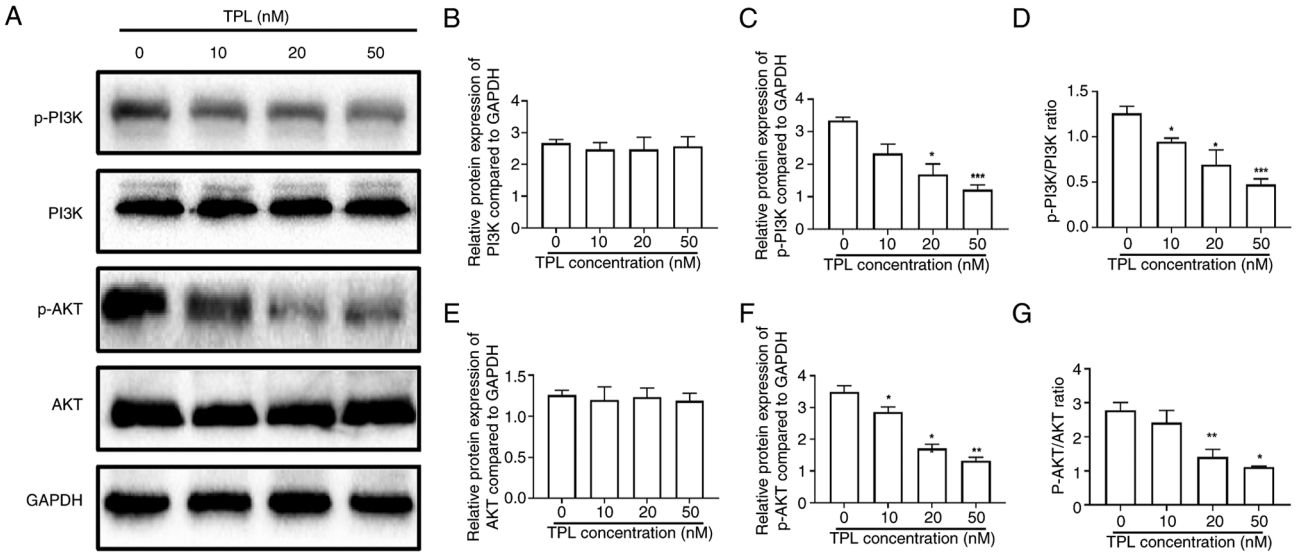

Figure 4. Effect of TPL on PI3K/Akt signaling pathway activation. (A) Representative western blot images of p-PI3K, PI3K, p-AKT and AKT. TPL reduced p-PI3K and p-AKT activation, whereas PI3K and AKT expression was not significantly affected. Statistical analysis results of (B) PI3K, (C) p-PI3K, (D) p-PI3K/PI3K ratio, (E) AKT, (F) AKT and (G) p-AKT/AKT ratio. Statistical analysis was performed using one-way ANOVA; *P<0.05, **P<0.01, ***P<0.001 vs. control. p-, phosphorylated; TPL, triptolide.

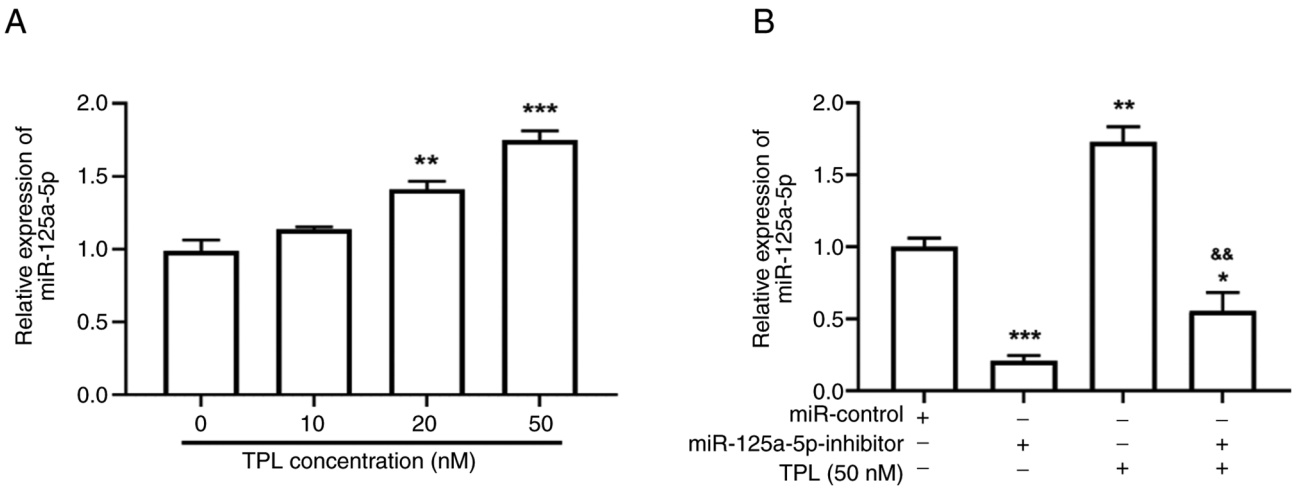

Figure 5. Effect of TPL on miR-125a-5p expression and transfection of SW1353 cells with the miR-125p-5a-inhibor. (A) TPL induced upregulation of miR-125a-5p expression in a dose-dependent manner. (B) miR-125a-5p-inhibitor downregulated miR-125a-5p expression and reversed TPL-induced upregulation of miR-125a-5p. Statistical analysis was performed using one-way ANOVA; *P<0.05, **P<0.01 ***P<0.001 vs. control; $^{\&\&}$P<0.01 vs. TPL group. miR, microRNA; TPL, triptolide.

mechanism of TPL in inducing the apoptosis of SW1353 chondrosarcoma cells, Annexin V/PI apoptosis analysis was conducted. As shown in Fig. 6B, the apoptotic rate of miR-125a-5p-inhibitor-transfected SW1353 cells was significantly reduced, whereas 50 nM TPL significantly promoted apoptosis compared with that in the miR-control group. Notably, 50 nM TPL-treated miR-125a-5p-inhibitor-transfected SW1353 cells showed no significant differences in apoptosis compared with the miR-125a-5p-inhibitor group. These data indicated that TPL exerted its pro-apoptotic effect on SW1353 chondrosarcoma cells via miR-125a-5p and inhibiting miR-125a-5p may block the effect of TPL.

Inhibiting miR-125a-5p diminishes the effects of TPL on cell migration. A wound healing migration assay was conducted to verify whether TPL inhibited SW1353 human chondrosarcoma cell migration via miR-125a-5p. As shown in Fig. 7A and B, the miR-125a-5p-inhibitor promoted chondrosarcoma cell migration compared with the miR-control. By contrast, 50 nM TPL-treated miR-control-transfected chondrosarcoma cells exhibited reduced cell migration compared with that in the miR-control group. However, 50 nM TPL-treated miR-125a-5p-inhibitor-transfected cells showed no difference in cell migration compared with the miR-125-a-5p inhibitor group. These data demonstrated that miR-125a-5p inhibition may block the TPL-induced inhibitory effect on the migration of SW1353 chondrosarcoma cells.

Inhibiting miR-125a-5p diminishes the effects of TPL on cell invasion. The Transwell invasion assay was conducted

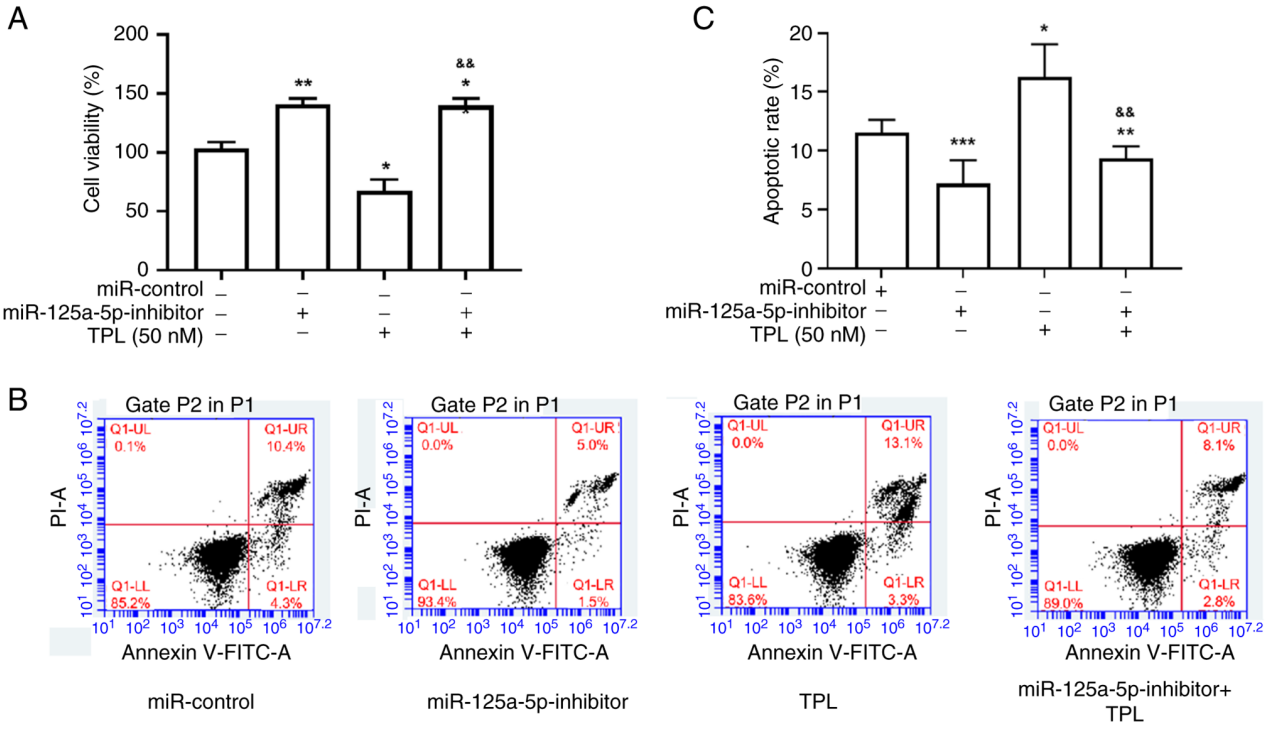

Figure 6. Effect of the miR-125a-5p-inhitor on SW1353 cell viability. (A) TPL-induced inhibition of SW1353 cell viability was reversed by the miR-125a-5p-inhibitor. (B) Flow cytometry revealed TPL-induced apoptosis of SW1353 cells was reversed by the miR-125a-5p-inhibitor and (C) statistical results revealed the pro-apoptotic effect of TPL. statistical analysis was performed using one-way ANOVA; *P<0.05, **P<0.01, ***P<0.001 vs. control; $^{\&\&}$P<0.01 vs. TPL group. miR, microRNA; TPL, triptolide.

to verify whether inhibiting miR-125a-5p diminished the inhibitory effects of TPL on chondrosarcoma cell invasion. As shown in Fig. 7C and D, 50 nM TPL significantly inhibited cell invasion through Matrigel, whereas miR-125a-5p-inhibitor-transfected cells exhibited enhanced invasion compared with that in the miR-control group. Cells treated with 50 nM TPL and transfected with the miR-125a-5p-inhibitor showed no differences in invasion compared with non-TPL-treated cells. These data verified that the inhibitory effect of TPL on SW1353 human chondrosarcoma cell invasion was blocked by the miR-125a-5p-inhibitor.

Inhibiting miR-125a-5p diminishes TPL-induced anti-inflammatory effects. To determine the whether TPL exerted its anti-inflammatory effects through regulating miR-125a-5p, the mRNA expression levels of proinflammatory cytokines were detected. As shown in Fig. 8A, after LPS treatment, IL-1β was upregulated in miR-125a-5p-inhibitor-transfected cells compared with that in the miR-control group, whereas 50 nM TPL significantly downregulated IL-1β expression. By contrast, 50 nM TPL-treated miR-125a-5p-inhibitor-transfected SW1353 cells exhibited no differences in IL-1β expression compared with in non-TPL-treated cells. Similar results were revealed regarding IL-6 and TNF-α expression (Fig. 8B and C). These data revealed that TPL exerted its inhibitory effect on proinflammatory cytokines via regulating miR-125a-5p, and blocking miR-125a-5p reversed the effects of TPL.

TPL regulates the PI3K/Akt signaling pathway via miR-125a-5p. To determine whether TPL regulated the PI3K/Akt signaling pathway via miR-125a-5p, the protein expression levels of p-PI3K, PI3K, p-AKT and AKT were detected. Notably, 50 nM TPL inhibited the expression levels of p-PI3K, p-PI3K/PI3K ratio, p-AKT and p-AKT/AKT ratio compared with that in the miR-control group (Fig. 9). By contrast, p-PI3K and p-Akt expression was upregulated in miR-125a-5p-inhibitor-transfected SW1353 cells and the addition of 50 nM TPL did not reverse this trend. These data suggested that miR-125a-5p inhibition blocked the TPL-induced regulation of the PI3K/Akt pathway, thus miR-125a-5p is essential for the antitumor effects of TPL on chondrosarcoma.

Discussion

In terms of primary bone malignancies, chondrosarcoma is the second most common type after osteosarcoma (31). Chondrosarcoma, regardless of its origin, responds best to locoregional therapy, such as surgical excision (32). Notably, surgical intervention is the most effective treatment option, whereas chemotherapy and radiotherapy are less effective (33). As a result of the absence of an effective adjuvant therapy, chondrosarcoma does not have a good prognosis (34). Therefore, novel chondrosarcoma-sensitive treatments with low toxicity and high efficacy are required. Research has focused on using natural products to treat cancer and Chinese herbal medicine has been used in cancer adjuvant treatment (35). Among the natural herb products, TPL has been considered as a potential supplementary option to standard cancer treatments due to its anticancer effects on various types of cancer (36).

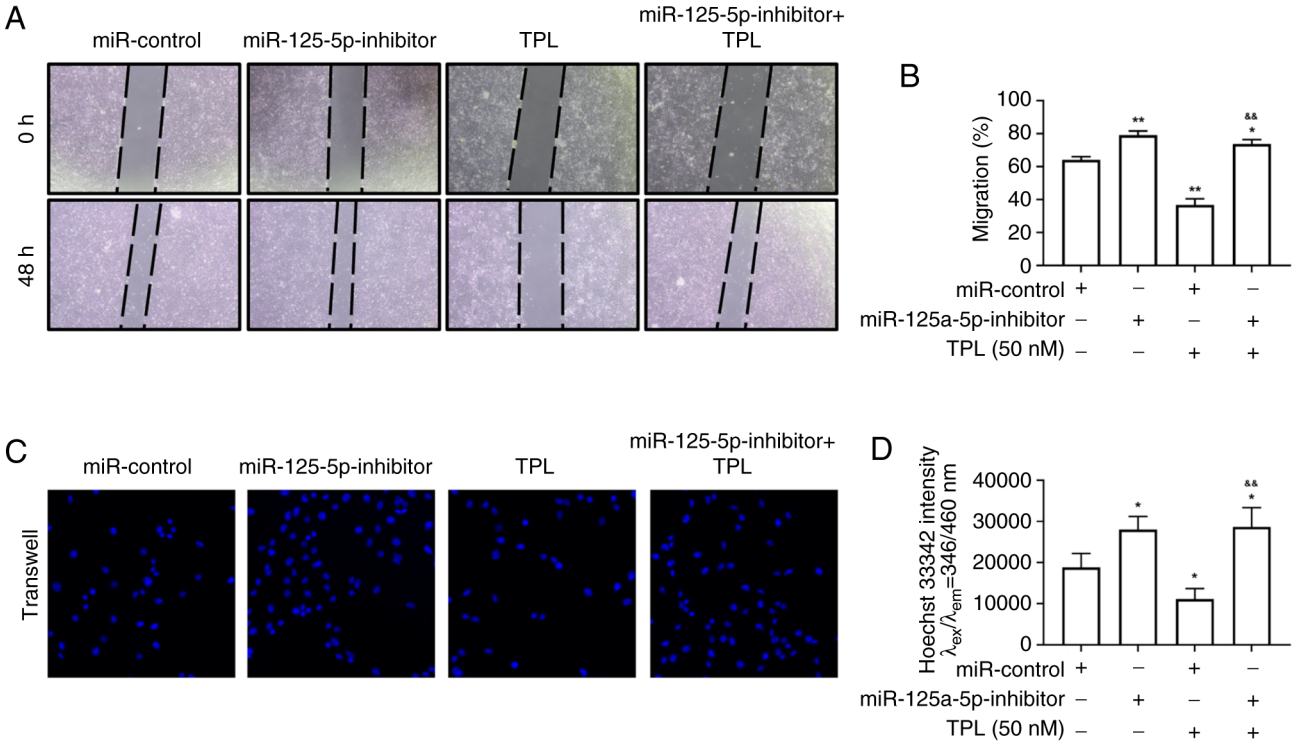

Figure 7. Effect of the miR-125a-5p-inhibitor on SW1353 cell migration and invasion. (A) Wound healing images revealed that the miR-125a-5p-inhibitor reversed TPL-induced inhibition of SW1353 cell migration and (B) significant differences were found. (C) Transwell invasion assay revealed that the miR-125a-5p-inhibitor reversed TPL-induced inhibition of SW1353 cell invasion and (D) significant differences were found. Statistical analysis was performed using one-way ANOVA; *P<0.05, **P<0.01 vs. miR-control; $^{\&\&}$P<0.01 vs. TPL group. miR, microRNA; TPL, triptolide.

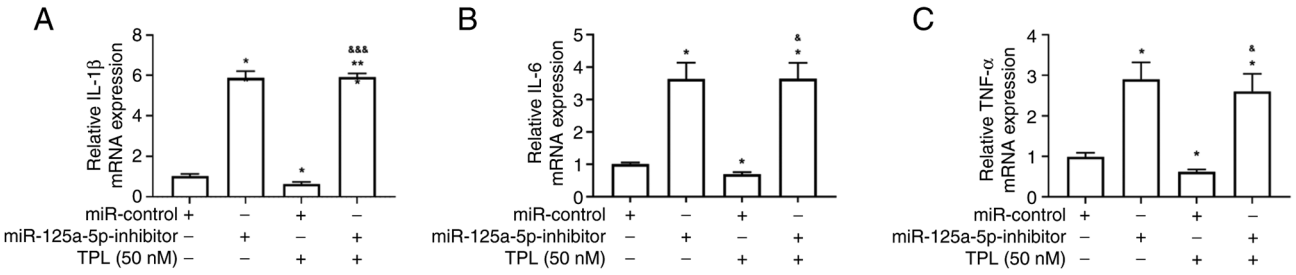

Figure 8. Effect of the miR-125a-5p-inhibitor on proinflammatory cytokine expression in SW1353 cells. The miR-125a-5p-inhibitor reversed TPL-induced inhibition of (A) IL-1β, (B) IL-6 and (C) TNF-α expression in LPS-treated SW1353 cells. Statistical analysis was performed using one-way ANOVA; *P<0.05, **P<0.01 vs. miR-control; $^{\&}$P<0.05, $^{\&\&\&}$P<0.001 vs. TPL group. miR, microRNA; TPL, triptolide.

TPL has been reported to exert antitumor effects in different animal models of cancer, including melanoma (37), pancreatic cancer (38), ovarian cancer (39), breast cancer (40) and cholangiocarcinoma (41). In addition, TPL has been shown to exert its antitumor effects via multiple pathways, including AKT/mTOR (41), NRF2 (42) and PI3K/AKT/NFATc1 (42). Therefore, TPL may have a complex role in cancer therapy. Previous studies have focused on the potential combination of TPL with nanoparticles in treating cancer, including breast cancer, colon cancer and melanoma (37,43-45). However, only a few studies have identified the potential of using TPL to treat sarcoma (46,47). Considering the reported positive effect of TPL in treating cancer, it is reasonable to hypothesize the further use of TPL in treating sarcoma, particularly chondrosarcoma. However, whether TPL has antitumor effects on chondrosarcoma cells remains unclear.

The results of the present study suggested that TPL reduced human chondrosarcoma cell viability in a dose-dependent manner. The IC_{50} value of TPL on SW1353 cells was reported to be 100.5 nM. Moreover, TPL decreased SW1353 human chondrosarcoma cell invasion and migration, as determined using wound healing and Transwell assays, which demonstrated that TPL may inhibit chondrosarcoma cell metastasis. Furthermore, TPL reduced LPS-induced upregulation of proinflammatory cytokines, including IL-1β, IL-6 and TNF-α. Notably, the progression of multiple types of cancer is related to inflammation (48,49). These results indicated that TPL exerted antitumor effects on cancer cell viability, migration, invasion and inflammation.

As a means of elucidating how TPL inhibited SW1353 human chondrosarcoma cell progression, the expression levels of PI3K/Akt signaling pathway-related proteins were

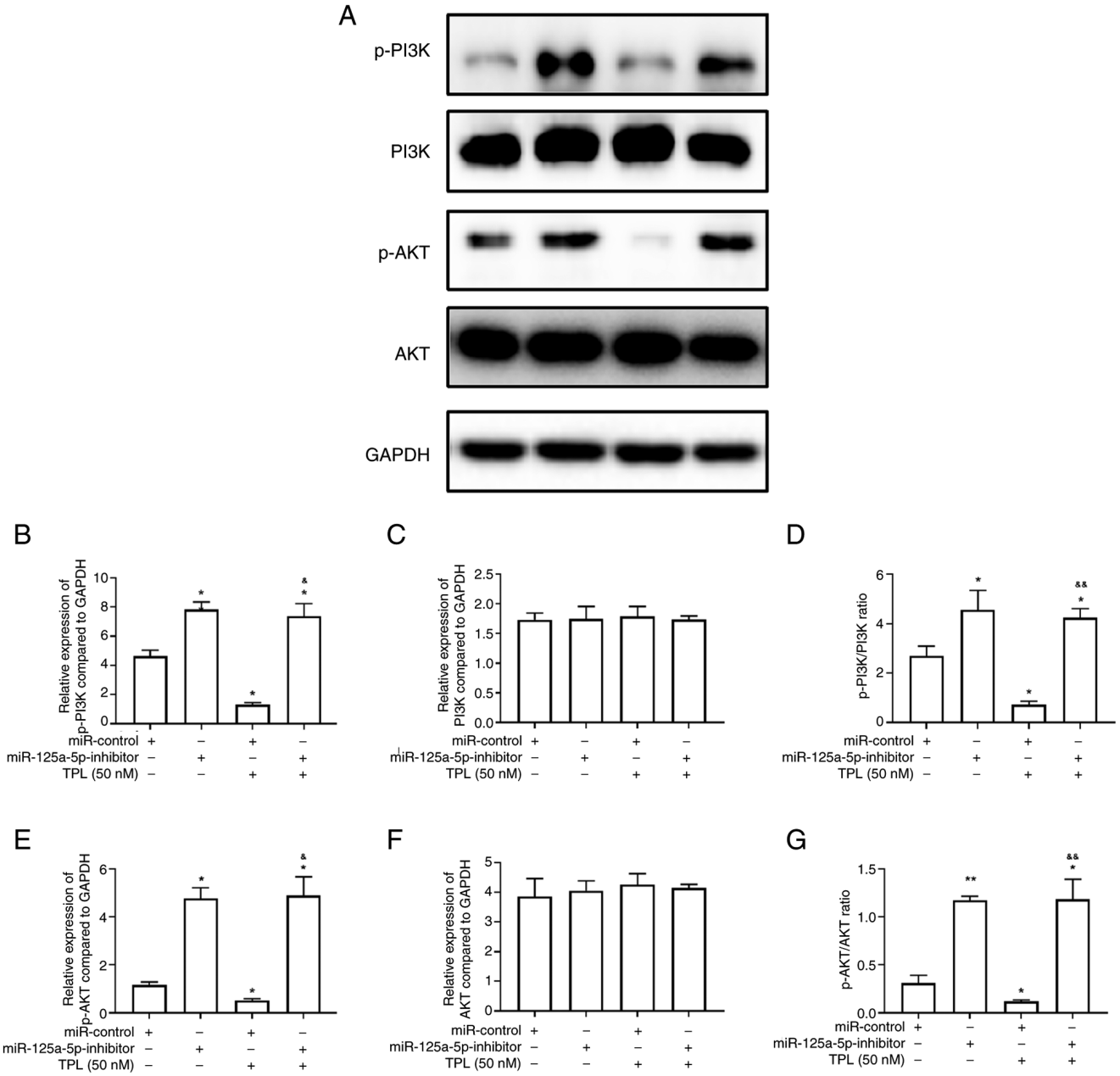

Figure 9. Effect of the miR-125a-5p-inhibitor on PI3K/Akt pathway activation in SW1353 cells. (A) Representative western blot images of p-PI3K, PI3K, p-AKT and AKT. The miR-125a-5p-inhibitor upregulated p-PI3K and p-AKT activation, and reversed the TPL-induced inhibitory effects on p-PI3K and p-AKT. Statistical results of (B) p-PI3K, (C) PI3K, (D) p-PI3K/PI3K ratio, (E) p-AKT, (F) AKT and (G) p-AKT/AKT ratio. Statistical analysis was performed using one-way ANOVA; *P<0.05, **P<0.01 vs. miR-control; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. TPL + miR-control group. miR, microRNA; p-, phosphorylated; TPL, triptolide.

measured. The activation of PI3K/Akt is essential for the oncogenesis, development and metastasis of chondrosarcoma (33,50). By contrast, inhibition of the PI3K/Akt pathway is associated with the suppression of cancer cell metastasis (51,52). In addition, PI3K/Akt upregulation can lead to the activation of inflammatory factors, including IL-1β, IL-6 and TNF-α (53,54). The present study demonstrated that TPL decreased the activation of p-PI3K and p-Akt, whereas there were no significant differences in the expression of total PI3K and Akt. The PI3K/Akt pathway is directly associated with cellular quiescence, proliferation and metastasis (55). Notably, PI3K/Akt inhibition has been reported to reduce the progression of chondrosarcoma (56). The present findings supported that TPL significantly inhibited PI3K/Akt signaling pathway activation. Thus, the present data demonstrated that TPL could reduce SW1353 chondrosarcoma cell viability, migration, invasion and LPS-induced inflammatory cytokine levels by inhibiting PI3K/Akt activation.

Chondrosarcoma is associated with various genetic and epigenetic changes, such as alterations in miRNAs (57). miR-125a-5p belongs to the miR-125 family and is known to suppress tumor growth (58,59). A previous study revealed the role of miR-125a-5p in inhibiting cancer, suggesting that it plays a vital role in the development and progression of tumors, including hepatocellular carcinoma, cervical cancer and breast cancer (60). There is evidence that miR-125a-5p regulates the expression of several genes involved in angiogenesis, cell cycle progression and apoptosis. For example,

miR-125a-5p has been reported to inhibit the expression levels of the cell cycle-related genes CDK6 and Bcl-2 (61). Moreover, miR-125a-5p has been shown to downregulate the expression levels of the pro-angiogenic gene VEGFA (62). According to these findings, miR-125a-5p may serve an important role in regulating cell cycle progression, angiogenesis and apoptosis, which are all important processes in the development and progression of chondrosarcoma. The current study demonstrated that TPL inhibited SW1353 chondrosarcoma cell progression via upregulating miR-125a-5p, since inhibition of miR-125a-5p reversed the therapeutic effects of TPL on chondrosarcoma. It was hypothesized that TPL may modulate miR-125a-5p expression through mechanisms such as transcription factor regulation, epigenetic modification, and antioxidant and anti-inflammatory effects. Future studies will focus on elucidating the specific pathways by which TPL influences miR-125a-5p, providing a clearer understanding of its role in miRNA-mediated regulation and potential therapeutic applications.

The present study has certain limitations. First, this study did not include *in vivo* experiments. Second, there may be more downstream targets of miR-125a-5p; however, only the PI3K/Akt signaling pathway was investigated. Further studies should aim to determine the effects of TPL *in vivo* and the underlying mechanism of miR-125a-5p in chondrosarcoma.

In conclusion, TPL inhibited SW1353 chondrosarcoma cell viability, migration, invasion and inflammation via upregulating miR-125a-5p-mediated inhibition of the PI3K/Akt signaling pathway. By contrast, inhibiting miR-125a-5p diminished the therapeutic potential of TPL.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CL, performed manuscript writing and interpreted the data. ZZ conducted the *in vitro* experiments LY provided the transfection technique guidance and interpreted the data. CL and ZB analyzed the data. CL and ZZ confirm the authenticity of all the raw data. ZB conceived and designed, and provided essential support for the current study. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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