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Down-regulation of MRPS23 inhibits LPS-induced proliferation and invasion *via* regulation of the NF-κB signaling pathway in osteosarcoma cells†

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Mitochondrial ribosomal protein S23 (MRPS23), encoded by a nuclear gene, is a participant in the translation of mitochondrial proteins. Recently, MRPS23 has been reported to be overexpressed in many types of cancers and have a close association with cancer progression. However, the specific roles of MRPS23 in osteosarcoma (OS) remain unknown. In this study, we investigated the expression pattern and biological functions of MRPS23 in OS cells. Our results demonstrated that MRPS23 was up-regulated in OS tissues and cell lines. Down-regulation of MRPS23 significantly inhibited OS cell proliferation and invasion induced by lipopolysaccharide (LPS) *in vitro*. Furthermore, the *in vivo* experiments showed that MRPS23 down-regulation markedly suppressed OS cell growth and metastasis induced by LPS. Mechanistically, down-regulation of MRPS23 inhibited the activity of NF-κB signaling pathway in OS cells. In conclusion, these findings indicated that MRPS23 may be a potential therapeutic target for OS treatment.

Introduction

Osteosarcoma (OS), a common type of bone sarcomas, frequently occurs in children, adolescents or young adults.[1,2] This malignant neoplasm arises from primitive transformed cells of mesenchymal origin, exhibits osteoblastic differentiation and produces malignant osteoid.[3–5] OS is characterized by aggressiveness, local recurrence and distant metastasis.[6] In the past, surgery was the main therapeutic measure for OS patients and the 5 year survival rate was only 20% due to the micrometastasis overlooked at diagnosis.[7] Currently, multimodal therapies have been applied for OS and the 5 year survival rate has been increased to 70% for patients with localized disease.[8,9] Unfortunately, OS patients with metastasis still suffer from a poor prognosis and their 5 year survival rate remains only 30%.[10,11] Therefore, exploring novel biomarkers and therapeutic approaches relevant to metastasis is urgently needed for a better clinical management of OS patients.

Mitochondria are bioenergetic, biosynthetic and signaling organelles.[12–14] They not only play a significant role in the adaptation to environmental changes but also function as the main energy and power centers in cells.[15,16] Increasing evidence has demonstrated that mitochondria take part in many other essential functions such as cell death, apoptosis and autophagy apart from energy metabolism.[17–19] In addition, mitochondria have been found serving as important mediators of tumorigenesis.[20,21] Thus, many researchers have focused on a group of unique proteins called mitochondrial ribosomal proteins (MRPs). These proteins are completely encoded by nuclear genes and mainly assist with the process of mitochondrial protein translation in mitochondria.[22] More importantly, many studies have reported other functions of MRPs in tumorigenesis.[23–25] For example, MRPS23, a member of the MRP family, has been demonstrated to be aberrantly expressed in some types of cancers and closely associated with development of these cancers.[26–28] However, the biological functions of MRPS23 in OS remain unclear.

In this study, we investigated the roles and mechanisms of MRPS23 in OS progression induced by lipopolysaccharide (LPS). The results showed that MRPS23 was up-regulated in OS tissues and cell lines. Furthermore, down-regulation of MRPS23 significantly inhibited LPS-induced OS cell proliferation and invasion *in vitro* and *in vivo* and these effects were mediated partly *via* the NF-κB pathway.

Results

Expression of MRPS23 is elevated in OS tissues and cell lines

The expression of MRPS23 in OS tissues was examined by immunohistochemistry, RT-PCR and western blot analysis. The

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results showed that MRPS23 staining was strong in OS tissues but rarely observed in corresponding normal bone tissues (Fig. 1A and B). In addition, MRPS23 mRNA and protein expression levels were markedly higher in OS tissues than in the adjacent normal bone tissues (Fig. 1C and D). Furthermore, we investigated the expression of MRPS23 in OS cell lines. As shown in Fig. 1E and F, MRPS23 was significantly up-regulated at both mRNA and protein levels in OS cell lines (SAOS2 and MG63) in comparison with the normal osteoblast cell line hFOB1.19.

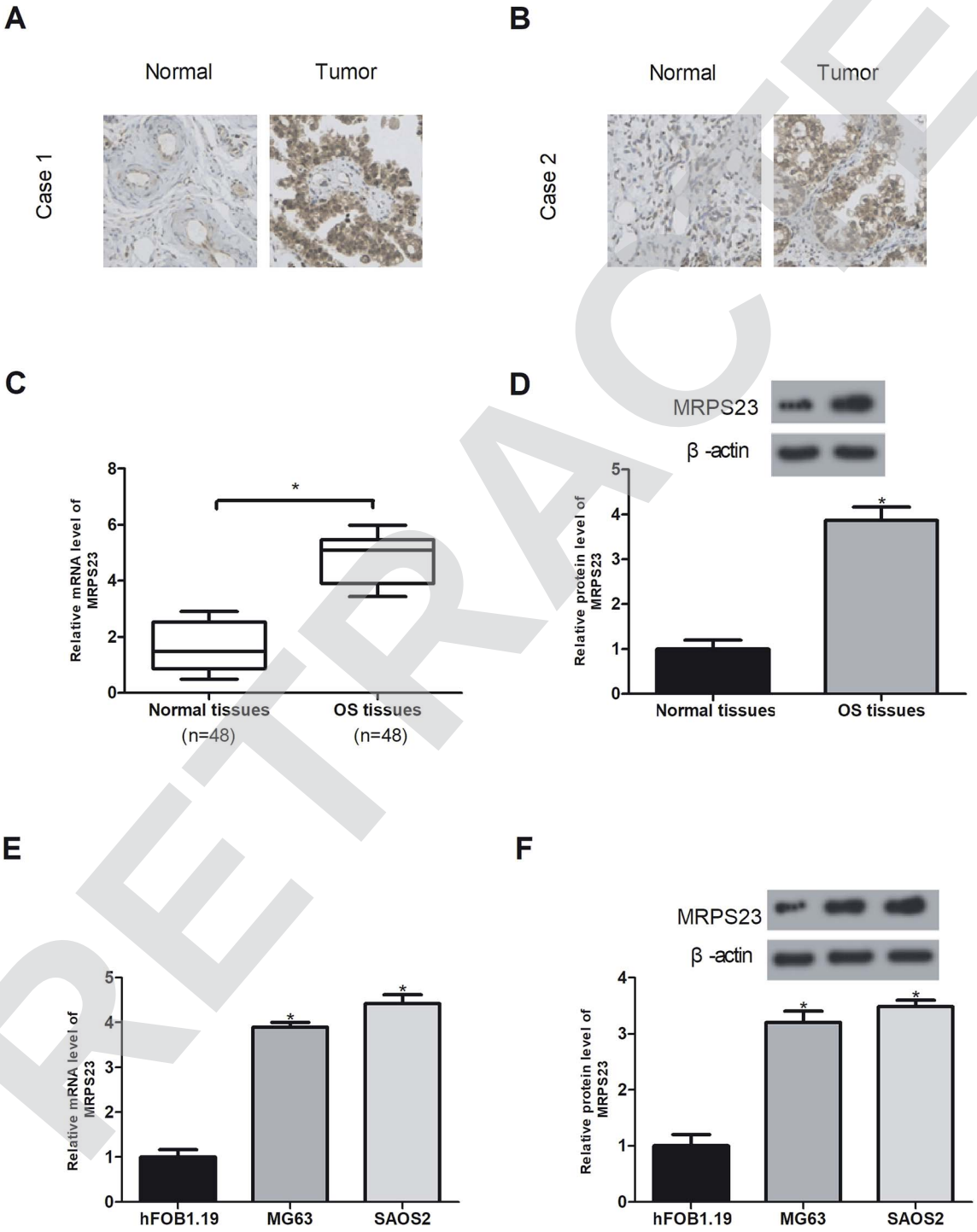

Fig. 1 Expression of MRPS23 is elevated in OS tissues and cell lines. (A and B) Representative immunostaining of MRPS23 expression in OS tissues and corresponding normal bone tissues. Magnification: 200×. (C and D) mRNA and protein expression of MRPS23 in OS tissues and matched normal bone tissues ($n = 48$). (E and F) mRNA and protein expression of MRPS23 in OS cell lines (MG63 and SAOS2) and the normal osteoblast cell line hFOB1.19. *$p < 0.05$.

Down-regulation of MRPS23 induces loss of mitochondrial membrane potential in OS cells and inhibits LPS-induced OS cell proliferation and invasion *in vitro*

To further investigate the role of MRPS23 in OS, we decreased the expression of MRPS23 in SAOS2 cells *via* shRNA transfection. RT-PCR and western blot assays were performed to verify the transfection efficiency (Fig. 2A and B). Considering the association of MRPS23 with the mitochondrial functions, we investigated the change of mitochondrial membrane potential in OS cells after sh-MRPS23 transfection. As shown in Fig. S1,† down-regulation of MRPS23 induced a significant decrease in mitochondrial membrane potential in SAOS2 cells. LPS is known as an inducer of cell proliferation and invasion in various cancers.[29,30] Therefore, we examined the effect of MRPS23 down-regulation on LPS-induced proliferation and invasion of OS cells. The MTT and transwell assays were performed to measure cell proliferation and invasion, respectively. As shown in Fig. 2C and D, LPS-treated SAOS2 cells showed enhanced proliferative and invasive abilities compared with corresponding control cells. However, these promoted effects were obviously prevented by MRPS23 down-regulation (Fig. 2C and D).

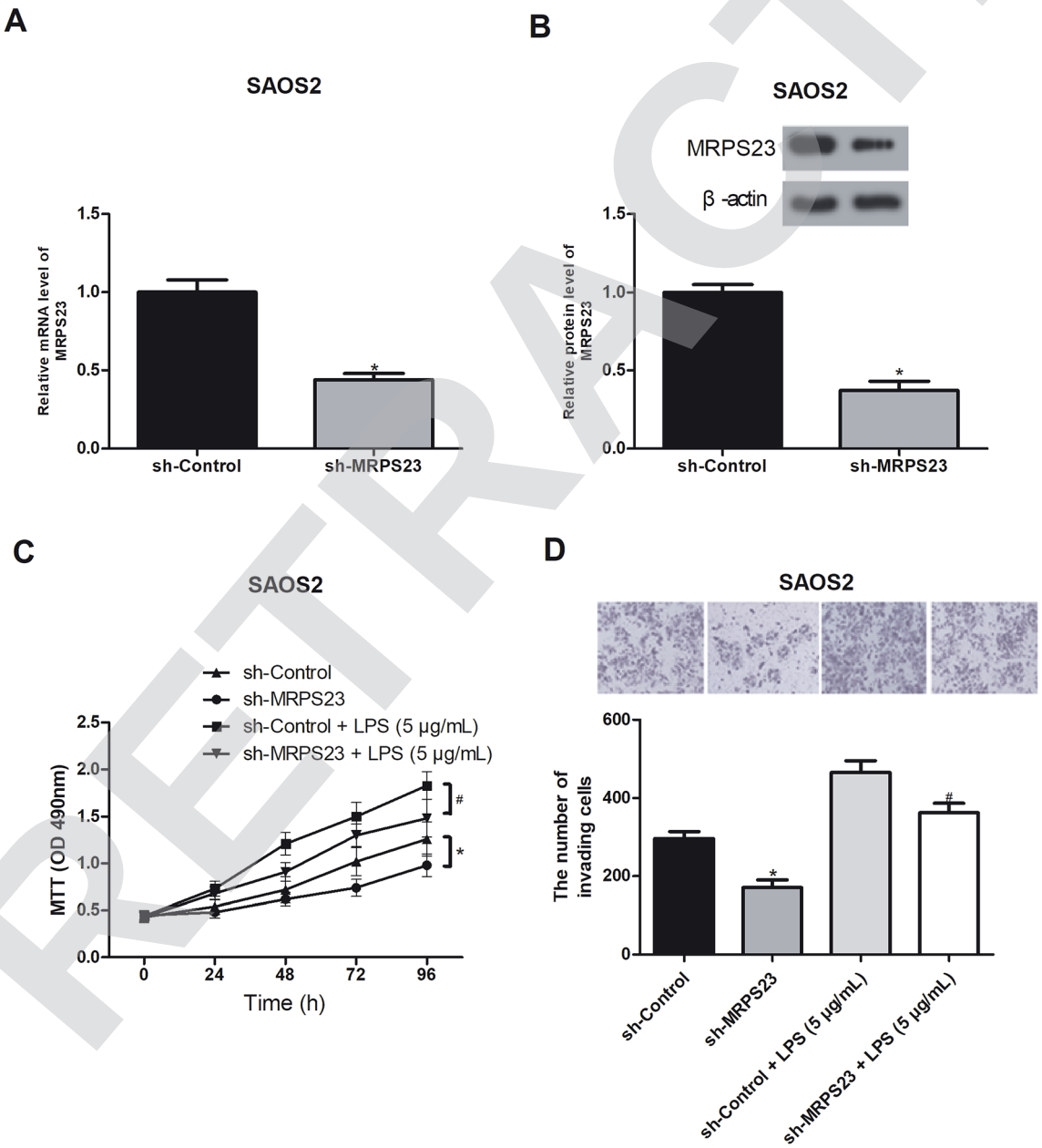

Fig. 2 Down-regulation of MRPS23 inhibits LPS-induced OS cell proliferation and invasion *in vitro*. (A and B) Down-regulation of MRPS23 was confirmed by RT-PCR and western blot assays after transfection of sh-MRPS23 or sh-Control in SAOS2 cells. (C and D) Cell proliferation and invasion were measured by the MTT and transwell assays, respectively. *$p < 0.05$ *vs.* shControl; #$p < 0.05$ *vs.* shControl + LPS.

Down-regulation of MRPS23 inhibits LPS-induced OS cell growth and metastasis *in vivo*

To determine whether MRPS23 down-regulation can suppress LPS-induced OS cell growth *in vivo*, SAOS2 cells transfected with sh-MRPS23 or sh-Control were treated with or without LPS and then injected into the left flank of mice. As shown in Fig. 3A, the growth rate of tumors was rapid in the SAOS2/LPS group but markedly slow in the SAOS2/sh-MRPS23 group in comparison with corresponding control groups. In addition, the average weight of tumors generated from LPS-treated SAOS2 cells was increased but greatly decreased in the SAOS2/sh-MRPS23 group in comparison with corresponding control groups (Fig. 3B). To further investigate the effect of MRPS23 down-regulation on OS cell metastasis *in vivo*, SAOS2 cells transfected with sh-MRPS23 or sh-Control were treated with or without LPS and then transplanted orthotopically into the bones of mice. As shown in Fig. 3C, more metastatic nodules were found in the SAOS2/LPS group while less metastatic nodules were detected in the SAOS2/sh-MRPS23 group, in comparison with corresponding control groups.

Down-regulation of MRPS23 abrogates LPS-induced activity of the NF-κB signaling pathway in OS cells

Previous studies have reported that LPS could promote cancer progression *via* regulating the NF-κB signaling pathway,[31–33] so we investigated whether MRPS23 down-regulation inhibited LPS-induced NF-κB pathway. As expected, LPS obviously increased the expression of NF-κB in SAOS2 cells while MRPS23 down-regulation significantly blocked this effect (Fig. 4A). To further confirm implication of the NF-κB pathway in the suppressive effect of MRPS23 down-regulation on LPS-induced OS cell proliferation and invasion, PDTC (NF-κB inhibitor) was used to treat OS cells. The results indicated that LPS-induced OS cell proliferation and invasion were inhibited by MRPS23 down-regulation and these effects were enhanced after PDTC treatment (Fig. 4B and C).

Discussion

Characterized by different degrees of mesenchymal differentiation, OS is a heterogeneous group of malignancies.[34] With a high rate of recurrence and metastasis, OS causes the death of a majority of OS patients.[35] Therefore, it is urgently required to explore more effective treatment alternatives.

Previous studies have demonstrated the abnormal expression and potential value of MRPS23 in several types of cancers.[26,27] The association of MRPS23 with cancer progression was first found in cervical cancer where overexpression of MRPS23 indicated high proliferative ability and oxygen consumption.[28] MRPS23 has also been reported to exert a similar effect on hepatocellular cancer where MRPS23 functions as a powerful driver of tumor proliferation.[36] In consistent with the previous findings, our study showed that MRPS23 was highly expressed in OS tissues and cell lines. Furthermore, down-regulation of MRPS23 inhibited OS cell proliferation and invasion. This *in vitro* result was further verified by our xenograft tumor assay which indicated that MRPS23 down-regulation suppressed OS cell growth and metastasis *in vivo*. All these observations suggested that MRPS23 might serve as a novel oncogene in cancer progression.

LPS is a strong inflammation stimulator and is associated with alteration of cytokine levels in tumor microenvironment.[37] The exposure to LPS may induce tumor proliferation and facilitate invasion.[29,30,32] For example, Wang *et al.* reported that hepatocellular cancer cell survival and proliferation were enhanced upon LPS stimulation.[38] Similarly, Yang *et al.* demonstrated that LPS could induce cell proliferation in colorectal cancer.[39] Consistently, Bedini *et al.* found that LPS exerted a promoting effect on glioblastoma cell proliferation.[40] Moreover, LPS is also an important player in mediation of the epithelial–mesenchymal transition, a key process with enhanced migration and invasion during cancer progression.[31,41] In this study, we found that LPS induced OS cell proliferation and invasion and this promoting effect was abrogated by MRPS23 down-regulation. We observed similar results in our *in vivo* experiments.

In regard to the mechanisms by which MRPS23 regulated LPS-induced OS progression, we focused on the NF-κB pathway. The NF-κB family consists of a group of transcription factors such as NF-κB1, NF-κB2, Rel A, Rel-B and Rel-C.[42,43] These members participate in various cellular processes and regulation.[44] A growing body of evidence has demonstrated that abnormal activation of the NF-κB pathway will cause aberrant expression of cancer-related genes, promote cell proliferation and accelerate tumor metastasis.[45,46] In this study, we examined the role of the NF-κB pathway in the inhibitory effect of MRPS23 down-regulation on LPS-induced OS progression. The results showed that LPS increased the expression of NF-κB in OS cells while MRPS23 down-regulation significantly reversed this effect. To further confirm involvement of the NF-κB pathway, OS cells were treated with PDTC (NF-κB inhibitor). The results indicated that LPS-induced OS cell proliferation and invasion were suppressed by MRPS23 down-regulation and these effects were potentiated after PDTC treatment. Based on these findings, we reasonably suggested that the NF-κB pathway could be responsible for part of the mechanisms by which MRPS23 mediated LPS-induced OS progression.

In conclusion, this study demonstrated that MRPS23 was highly expressed in OS tissues and cell lines and was involved in the regulation of LPS-induced OS cell proliferation and invasion *in vitro* and *in vivo*. Furthermore, MRPS23 exerted these effects partly *via* the NF-κB pathway. Taken together, our study provided evidence supporting MRPS23 as a potential therapeutic target for OS treatment.

Materials and methods

Ethics statement

This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985) and was approved by the Ethics

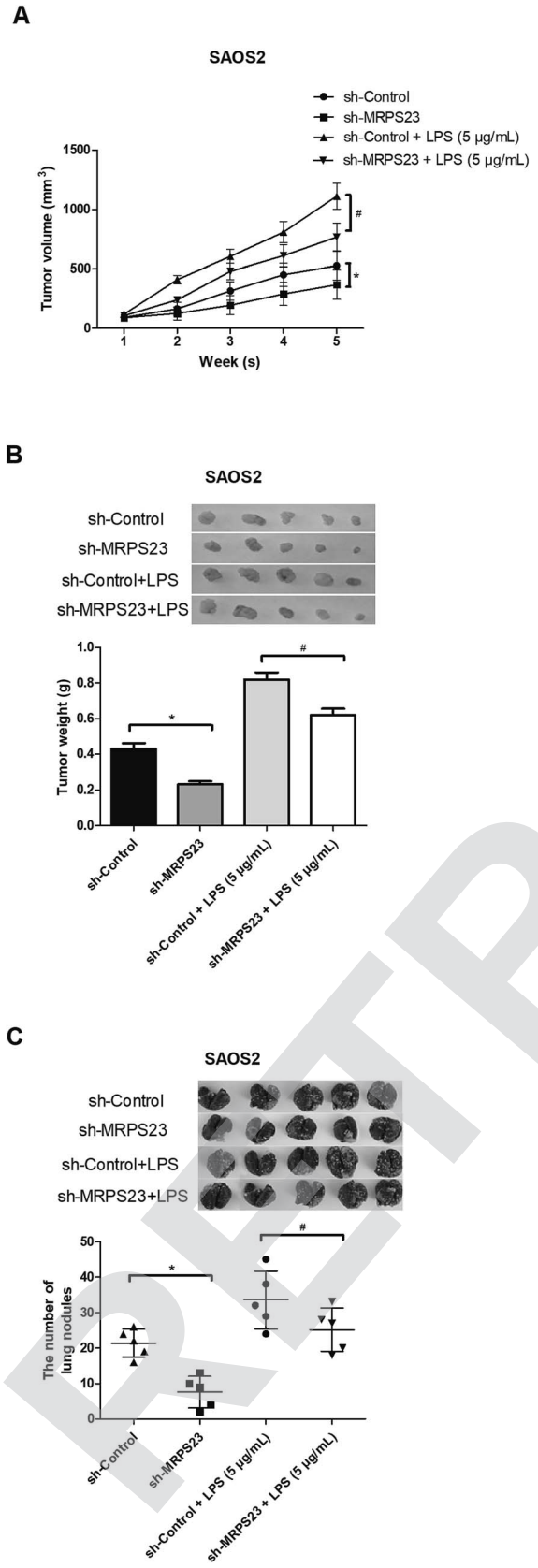

Fig. 3 Down-regulation of MRPS23 inhibits LPS-induced OS cell growth and metastasis *in vivo*. (A and B) Growth curves and average weight of tumors in different groups. (C) Quantification of metastatic lung nodules in different groups. *$p < 0.05$ *vs.* shControl; #$p < 0.05$ *vs.* shControl + LPS.

Committee of the First Affiliated Hospital of Henan University (Kaifeng, China).

Patients and tissue samples

A total of 48 OS patients from the First Affiliated Hospital of Henan University (Kaifeng, China) were enrolled in the study. The OS tissues and the adjacent normal bone tissues were collected during surgery and then frozen in liquid nitrogen and stored at −80 °C for future experiments. All tissue specimens were used with informed consent from each patient and with approval of the Ethics Committee of Henan University.

Cell lines and cell culture

Human OS cell lines (MG63 and SAOS2) and normal osteoblast cell line hFOB1.19 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin and then incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Immunohistochemistry (IHC)

Sliced tissues were washed with PBS containing 0.1% bovine serum albumin and then incubation with 3% hydrogen peroxide for 10 min. Subsequently, the slices were subjected to overnight incubation at 4 °C with primary anti-MRPS23 antibody (Cell Signaling Technology, Danvers, MA, USA), followed by incubation with HRP goat anti-mouse/rabbit IgG (Cell Signaling Technology) for 15 min at room temperature. Diaminobenzidine (DAB; Invitrogen) was used for color development. Representative images of tissues were captured using a light microscope (200×).

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

Total RNA was extracted from tissues or cells using TRIzol Reagent (Invitrogen) and then reversely transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The following primers were used: MRPS23, 5′-GGTTTGACGTATATGACGCCTT-3′ (forward) and 5′-CTCTAATCCGATCCTCGTGGTA-3′ (reverse); β-actin, 5′-CATGTACGTTGCTATCCAGGC-3′ (forward) and 5′-CTCCTTAATGTCACGCACGAT-3′ (reverse). The reaction conditions were as follows: 96 °C for 30 s, 40 cycles of 96 °C for 15 s, 62 °C for 1 min and 75 °C for 45 s. β-Actin was used as an internal control for normalization of the results. The relative mRNA expression was calculated by the $2^{-\Delta\Delta C_t}$ method.

Western blot analysis

Tissues or cells were lysed in lysis buffer. Cell lysates were collected after 5 min of centrifugation at 12 000*g*. The concentration of lysate proteins was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). An equal amount of protein was separated by 10% SDS-PAGE and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After

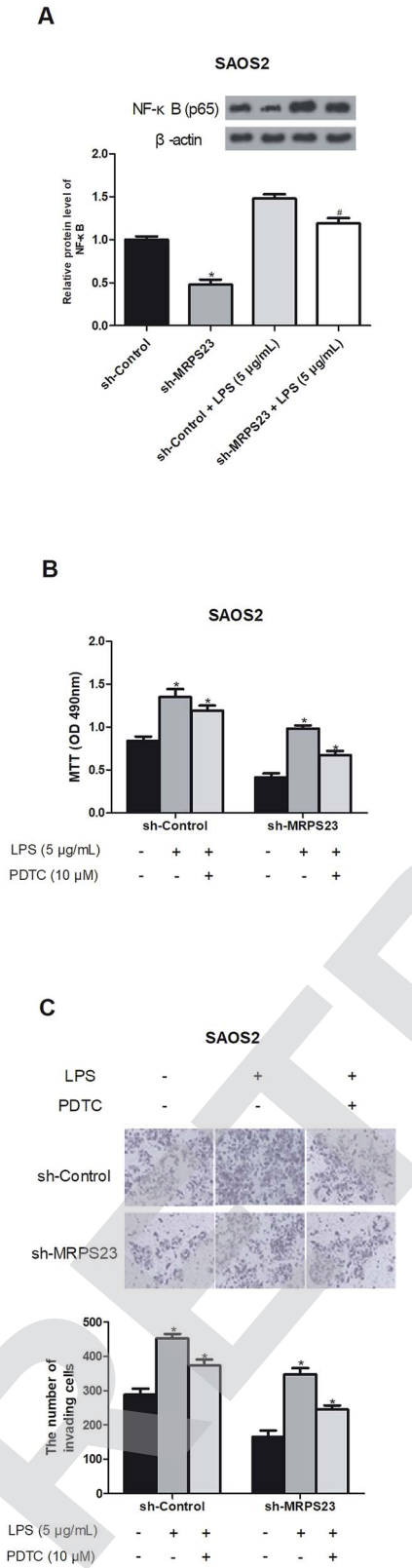

Fig. 4 Down-regulation of MRPS23 abrogates LPS-induced activity of the NF-κB signaling pathway in OS cells. (A) The protein expression of NF-κB was measured by western blot analysis in SAOS2 cells. (B and C) LPS-treated SAOS2 cells transfected with sh-MRPS23 or sh-Control were treated with or without PDTC (10 μM). Cell proliferation and invasion were determined using the MTT and transwell assays, respectively. *$p < 0.05$ *vs.* shControl; #$p < 0.05$ *vs.* shControl + LPS.

blocking in 5% non-fat milk, the membranes were incubated overnight at 4 °C with primary antibodies against MRPS23, NF-κB and β-actin. Subsequently, the membranes were washed three times with TBST and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. All antibodies were purchased from Invitrogen. Protein bands were detected using an enhanced chemiluminescence detection system (Cell Signaling Technology) and their intensity was analyzed using the Quantity One software (Bio-Rad, Hercules, CA, USA).

Cell transfection

The shRNA sequences targeting MRPS23 (GCAGAGTCTTGGAGAAACA) were purchased from GeneCopoeia (Rockville, MD, USA). Cells were plated in 6-well plates at a density of 1×10^5 cells per well. After reaching 80% confluence, cells were transfected with sh-MRPS23 or sh-Control using Lipofectamine 2000 reagent (Invitrogen). 48 h later, western blot assays were performed to confirm the transfection efficiency.

MTT assay

Cells were seeded in a 96-well plate at a density of 2×10^3 cells per well and cultured for different time. After treatment with LPS for 24 h, MTT (5 mg mL^{-1}; Sigma, St. Louis, MO, USA) was added to each well at different time points. After removal of culture medium, DMSO (Sigma) was added to each well. The absorbance was measured at 490 nm using a microplate reader.

Transwell assay

Cell invasion was detected using Matrigel-coated transwell chambers. Cells (1×10^5) were added to the upper chamber containing serum-free medium and the lower chamber was filled with culture medium containing 10% FBS. After incubation with LPS for 24 h, cells remaining on the upper surface of the filter were removed and cells invading to the lower surface of the filter were fixed and stained with 0.1% crystal violet. The number of invading cells from five random fields was counted under a microscope (200×).

Measurement of mitochondrial membrane potential

Changes in the mitochondrial membrane potential were measured using 3,3′-tetraethylbenzimidazolylcarbo-cyanine iodide (JC-1). In brief, cells were seeded in a 96-well plate and allowed to reach about 70% density. Subsequently, JC-1 was added and cells were further incubated for 30 min at 37 °C and then washed three times with PBS. Fluorescence was measured using a flow cytometry (excitation: 488 nm and emission: 525 nm).

In vivo xenograft tumor assay

Male BALB/c nude mice (4 to 6 weeks old) were obtained from Shanghai Laboratory Animal Center (Shanghai, China). All animal experiments were approved by the Animal Care and Use Committee of Henan University. For the tumor growth assay, mice were randomly divided into 4 groups ($n = 5$). 2×10^6

SAOS2 cells transfected with sh-MRPS23 or sh-Control with or without treatment of LPS (5 μg mL^{-1}) were resuspended in PBS and then subcutaneously injected into the left flank of mice. Tumors were measured every week. Tumor volume was calculated by the following formula: volume = 0.5 × width^2 × length. 35 days later, mice were sacrificed and tumors were harvested and weighed.

For the tumor metastasis assay, mice were randomly divided into 4 groups ($n = 5$). 2 × 10^6 SAOS2 cells transfected with sh-MRPS23 or sh-Control with or without treatment of LPS (5 μg mL^{-1}) were resuspended in PBS and then transplanted orthotopically into the bones of mice. 5 weeks later, mice were euthanized and their lungs were removed. The number of metastatic lung nodules was counted under a microscope.

Statistical analysis

All experiments were conducted three times and data were expressed as means ± standard deviation (SD). Statistical analysis was performed using SPSS 18.0 software. Student's *t*-test or one-way ANOVA was used for the comparison between different groups. $P < 0.05$ was considered statistically significant.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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