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Knockdown of microRNA-181a inhibits osteosarcoma cells growth and invasion through triggering NLRP3-dependent pyroptosis

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Abstract. – OBJECTIVE: This study aimed to investigate the physiological function and molecular mechanism of microRNA-181a (miRNA-181a) in the carcinogenesis of osteosarcoma.

MATERIALS AND METHODS: The relative expression of miRNA-181a in tissues and cultured cells was detected by quantitative real time-polymerase chain reaction (qRT-PCR). MiR-181a inhibitor and miR-181a mimics were used to manipulate its level in cells. Cell proliferation and invasion were measured using Cell Counting Kit-8 (CCK-8) assay and transwell assay, respectively. The protein levels of the target genes were detected by Western blotting and immunohistochemistry. Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) assay was employed to detect cell apoptosis. Moreover, a xenograft tumor bearing mice model was used to evaluate the effect of miR-181a *in vivo*.

RESULTS: We found that miRNA-181a was aberrantly elevated in osteosarcoma tissues and cells. Moreover, the overexpression of miRNA-181a could facilitate cell proliferation and migration. By contrast, miRNA-181a knockdown reverses these effects. Additionally, downregulation of miRNA-181a could activate NLR-like receptor protein 3 (NLRP3)-dependent pyroptosis, as evidenced by the increased levels of pyroptosis-related genes, NLRP3, caspase-1, interleukin-18, and interleukin-1β (IL-1β). Moreover, miRNA-181a inhibitor transfected cells compared with the control. Further mechanistic studies identified that miRNA-181a knockdown suppressed cell proliferation and invasion by activating NLRP3-dependent pyroptosis. Silencing NLRP3 could effectively reverse the effects mediated by miRNA-181a inhibitor. Consistently, *in vitro* results also demonstrated that knockdown of miRNA-181a notably suppressed tumor growth *via* activating pyroptosis.

CONCLUSIONS: These results provide that miRNA-181a might serve as potential therapeutic target for osteosarcoma patients.

Key Words
MiRNA-181a, Osteosarcoma, NLRP3, Pyroptosis.

Introduction

Osteosarcoma (OS) is an aggressive primary bone malignancy usually deriving from the metaphysis of the long bones. Osteosarcoma commonly occurs among adolescents and children[1]. The treatment of osteosarcoma including surgery resection, adjuvant chemotherapy, and radiotherapy has been rapidly progressed over the past two decades. The survival rate of 5-year for patients with metastatic osteosarcoma has been improved significantly. Unfortunately, most patients at later or more advanced stages are associated with high mortality and poor prognosis[4]. Therefore, screening novel biomarkers and therapeutic targets is very imperative for osteosarcoma prevention and treatment.

Pyroptosis is a novel proposed inflammatory form of programmed cell death in recent years[5]. Although pyroptosis shares biochemical and morphological characteristics with necrosis and apoptosis, pyroptosis remains a unique process triggered by various stimuli. The main features of pyroptosis include cell swelling and lysis, concurrent with the release of proinflammatory cytokines, such as interleukin-1β (IL-1β) and interleukin-18 (IL18)[6]. Caspase-1, the key effector protease of the inflammasome, is activated during pyroptosis and maturates the pro-IL-1β and pro-IL-18 into active forms IL-1β and IL-18. Then, both IL-1β and IL-18 are released to extracellular space leading to recruitment of inflammatory cells and aggravation of inflammatory response[7]. Accumulating evidence has revealed that pro-inflammatory microenvironment is favorable for tumor initiation and progression, and several pro-inflammatory cytokines, includes IL-1β and IL-18, were aberrantly increased in various human malignant tumors[8]. Therefore, several studies have been set out to explore whether pyroptosis could serve as a reliable therapeutic target for cancer treatment.

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MicroRNAs are a class of endogenous single-stranded non-coding RNA with a length of approximately 21-25 nucleotides. MicroRNAs are ubiquitously expressed in plants and animals[9,10]. It has been well-established that miRNAs exert function in physiological and pathological processes by identifying and binding to the complementary sequences located at 3'-untranslated regions (UTRs) in their target mRNAs. The binding between microRNAs and target mRNA leads to inhibition of translation or degradation of mRNA[11]. In the last decade, the functional roles of miRNAs have been widely studied in human diseases, especially in cancer. It has been proved that miRNAs were involved in multiple biological processes of tumor cells including proliferation, apoptosis, and metastasis by directly targeting tumor suppressor genes or oncogenes. Among numerous microRNAs, the pro-tumourigenic function of miRNA-181a has been identified in various human malignancies. Of note, increasing evidence also revealed that miRNA-181a could promote osteosarcoma progression through multiple distinct mechanisms. For instance, some authors[12-15] have reported that miRNA-181a functions as an oncogenic miRNA in osteosarcoma *via* directly downregulating CYLD, RASSF1A, and PTEN. In addition, triptolide and lncRNA CASC2 have also been found to alleviate osteosarcoma progression by downregulating miRNA-181a[16,17]. Collectively, [illegible] literature has inferred the important role of miR-181a in the tumorigenesis and progression of osteosarcoma. However, very little is known about association between miR-181a and inflammatory response in osteosarcoma.

The objective of this research was to elucidate the role of miR-181a in osteosarcoma and address whether miR-181a participates in the regulation of the NLRP3 inflammatory pathway. Our results revealed that miR-181a could enhance the proliferation and invasiveness of osteosarcoma cells by blocking the activation of NLRP3-dependent pyroptosis. These findings illuminated that miR-181a serves as a therapeutic target in osteosarcoma progression.

Materials and Methods

Clinical Specimens

A total of 20 paired osteosarcoma and non-cancerous tissues were collected from May 2017 to June 2018. All collected specimens were directly frozen in liquid nitrogen and stored at -80°C before analysis was performed. All clinical investigations in the current study have obtained consent from all patients and were approved by the Ethics Committee of Wuxi Traditional Chinese Medicine Hospital (Wuxi, Jiangsu, China).

Cell Lines and Culture

Three human osteoblasts cell lines (U2OS, Saos-2, and MG-63), and one human osteoblasts hFOB (human fetal osteoblastic) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in the Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) and 1% streptomycin and penicillin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C under 5% CO_2.

Transfection

To explore the functional role of miRNA-181a in osteosarcoma cells, we manipulated miRNA-181a level in osteogenic-2 (Saos-2) and human bone osteosarcoma epithelial cells (U2OS cells) by transfecting miRNA-181a mimics, Negative control (NC), and miRNA-181a inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). The NOD-like receptor protein 3 (NLRP3) shRNA (Cell Signaling Technology, Danvers, MA, USA) was used to repress the expression of NLRP3. Briefly, the cells in the logarithmic growth phase were cultured in 6-well plates at concentration of 1×10^6 cell/well for 24 h. Following 80-90% confluence, Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to facilitate the penetration of miRNA-181a across cell membrane according to the manufacturer's protocols. Transfection efficiency of miRNA-181a inhibitor was determined by examining the miRNA-181a level using quantitative real-time PCR (qRP-PCR; Thermo Fisher Scientific, Waltham, MA, USA).

Assessment of Cell Viability

Change of cell viability in each group was determined by using Cell Counting Kit-8 (CCK-8) assay (Abcam, Cambridge, MA, USA) following miRNA-181a mimic and miRNA-181a inhibitor transfection. Briefly, transfected Saos-2 and U2OS cells were plated in a 96-well plate (5×10^3 cells/well) for 48 h. Then, culture media were discarded and incubated with 10 μL CCK-8 working solution for 1.5 h at 37°C. A microplate reader was employed to detect absorbance value at 450

nm. Cell viability was calculated according to the formula: Cell viability (%) = Optical Density (OD) value of experimental group - OD value of blank group/OD value of control group (OD value) – OD value of blank group.

Western Blotting Analysis

Western blotting analysis was conducted following the protocols described in Han et al[18]. The primary antibodies used in the current study were as follows: Ki-67 (1:2000, #ab16667) and cleaved Interleukin (IL-18) (1:1500, ab71495) were purchased from Abcam (Cambridge, MA, USA); Proliferating cell nuclear antigen (PCNA) (1:1000, #13110), Matrix metalloproteinase-2 (MMP-2) (1:1000, #87809), MMP-9 (1:1000, #13667), Metalloproteinase inhibitor 3 (TIMP-3) (1:1000, #5673), NLRP3 (1:1000, #91413), cleaved caspase-1 (1:1000, #238979), cleaved IL-1β (1:1000, #2021) were obtained from Cell Signaling Technology (Danvers, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 1:3000; (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as internal reference. Anti-mouse horseradish peroxidase (HRP)-conjugated antibody (1:[illegible] #20001) and anti-rabbit-Horseradish-per[illegible] (anti-rabbit-HRP) (1:3000, #20002) were ob[illegible]d from Absin Bioscience Inc. (Zhangjiang, Sh[illegible] hai, China) and were used as secondary antibod[illegible]

Cell Invasion Assays

The effect of miRNA[illegible] on c[illegible]nvasive ability was evaluated by [illegible]swell[illegible] swell assay was cond[illegible]d [illegible] to the [illegible]-cedure used by Ha[illegible] al[19]. B[illegible] the cells in logarithmic growth phase were plated at the upper chamber of transwell (8-l m pore size; BD Biosciences, San Jose, CA, USA) at a density of 1 × 10^5 cells/well. Lower chamber with filled with [illegible] 10% FBS medium. Following incubati[illegible] for 24 h, non-invasive cells at the [illegible]r side o[illegible] membrane were removed using [illegible]on swabs, while invasive cells adhered to lo[illegible]mbrane were observed and counted [illegible]der a [illegible]cope (magnification: 200×) in 5 [illegible]omly chos[illegible]d following 4% formald[illegible]de and [illegible]hemato[illegible] staining.

Quantitative [illegible] Tim[illegible] (qRT-PCR)

The tota[illegible] Saos-2 and [illegible]S cells was isolated [illegible]using [illegible]Izol reagent (Invitrogen, Carlsbad, CA, USA). [illegible]primers were designed and [illegible]ized by Sha[illegible] GenePharma Comp[illegible] (Shanghai, China). [illegible]he prime sequences [illegible] in current st[illegible]dy were listed in Table I. En[illegible]nous refere[illegible] for miRNA-181a and pyro-p[illegible] related g[illegible] (NLRP3, caspase-1, IL-1β, and [illegible]2) w[illegible]mall RNA U6 and GAPDH, respect[illegible]T-PCR results were calculated by [illegible]sing the $2^{-\Delta\Delta Ct}$ method.

[illegible]ac[illegible] Dehydrogenase (LDH) Release Assays

MiRNA-181a inhibitor-induced cytotoxicity in Saos-2, and U2OS cells were evaluated by determining the release of Lactate Dehydrogenase (LDH) using commercial kit (#88954, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction.

Table I. The pri[illegible] seque[illegible] quantitative real-time RT-PCR.

Genes	Sequences
miR[illegible]forward	5′-CUCUAGAGGGAAGCGCUUUCUG-3′
m[illegible]a reve[illegible]	5′-GA AAGCGCUUCCCUCUAGAGUU-3′
NL[illegible]	5′-ATTACCCGCCCGAGAAAGG-3′
NLRP[illegible]	5′-TCGCAGCAAAGA TCCACACAG-3′
[illegible]ase-1 [illegible]	5′-GGTCTTGTGACTTGG AGGACAT-3′
C[illegible]se-1 reve[illegible]	5′-TTTCAGTGGTTGGCATCTGTAG-3′
I[illegible]forward	5′-TGACCTGTTCTTTGAGGCTGAC-3′
[illegible]	5′-GATGCTGCTGTGAGATTT GAAG-3′
L-18 forward	5′-ACAACCGCAGTAATACGGAGCA-3′
-18 reverse	5′-TGTGCTCTGCTTGAGAGG TGCT-3′
[illegible]ward	5′-CTCGCTTCGGCAGCACA-3′
U[illegible]reverse	5′-AACGCTTCACGAATTTGCGT-3′
GAPDH forward	5′-TGAACGGGAAGCTCACTGG-3′
GAPDH reverse	5′-TCCACCACCCTGTTGCTGTA-3′

Animal Studies

All animal experiments were performed in accordance with the Laboratory Animal Care Guidelines of the Animal Ethics Committee of Experimental Animal Center of Nanjing University (Nanjing, Jiangsu, China). Briefly, Saos-2 transfected with or without miRNA-181a inhibitor were injected into the back of 6-8-week-old nude mice (n=8). Tumor volumes in each group were measured and recorded every three days. 27 days later, nude mice were executed following anesthesia and tumor weight was measured. Cell apoptosis in tumor tissues was determined by using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL assay; Abcam, Cambridge, MA, USA). The expression levels of Ki-67, PCNA, NLRP3, cleaved caspase-1, IL-1β, and IL-18 in tumor tissues were detected by immunohistochemistry (IHC) assays (Thermo Fisher Scientific, Waltham, MS, USA).

Statistical Analysis

All data were analyzed by using SPSS 20.0 statistical software (IBM, Armonk, NY, USA) and GraphPad Prism 7.0 (San Diego, CA, USA) and expressed as mean ± standard errors. The comparisons between groups were analyzed by using Student's *t*-test or One-way ANOVA. $p<0.05$ was considered statistically significant.

Results

MiRNA-181a Was Aberrantly Increased in Osteosarcoma Tissues and Cell Lines

We first examined the miRNA-181a levels in osteosarcoma samples and adjacent non-cancerous tissues using qRT-PCR. As presented in Figure 1A, the expression level of miRNA-181a expression was abnormally elevated in osteosarcoma tissues in comparison with corresponding non-cancerous tissues ($p<0.05$). To further confirm the oncogenic role of miRNA-181a in osteosarcoma, we also detected miRNA-181a in MG63, SAOS-2 and U20S cells and human normal osteoblast hFOB.19. As expected, miRNA-181a was distinctively up-regulated in osteosarcoma cell lines compared with normal osteoblast hFOB.19 ($p<0.05$, Figure 1B). Furthermore, SAOS-2 and U20S cells show higher levels of miRNA-181a. Therefore, SAOS-2 and U20S cells were chosen as the model cell lines *in vitro* experiments.

MiRNA-181a Enhances the Proliferation and Invasion of Osteosarcoma Cells

To investigate the effect of miRNA-181a on cell viability and aggressiveness of osteosarcoma cells, we transfected SAOS-2 and U20S with miRNA-181a mimic or miRNA-181a inhibitor. As illustrated in Figure 1C and Figure 1D, miRNA-181a level in SAOS-2 and U20S cells was significantly increased after transfection with miRNA-181a mimic while decreased in miRNA-181a inhibitor-transfected cells ($p<0.05$). Up-regulation miRNA-181a markedly increased the cell ability and invasive activities of SAOS-2 and U20S cells ($p<0.01$, Figure 2A and Figure 2B). Meanwhile, cell proliferation and invasion were effectively suppressed by miRNA-181a knockdown ($p<0.01$, Figure 2C and Figure 2D). Additionally, Western blotting analysis also confirmed that proliferating cell nuclear antigen (PCNA) and Ki-67 as hallmarks for cells proliferating activity, were tremendously elevated after miRNA-181a up-regulation in SAOS-2 and U20S cells (Figure 2E). On the contrary, knockdown of miRNA-181a could markedly reduce the level of PCNA and Ki-67 compared with control. Consistently, overexpression of miRNA-181a in osteosarcoma cells could significantly increase the expression of matrix metalloproteinases-2 (MMP-2), matrix metalloproteinases-9 (MMP-9), while decreased the level of tissue inhibitor of metalloproteinasas-3 (TIMP-3; $p<0.05$, Figure 2D). Nevertheless, miRNA-181a inhibitor exerted an opposite effect on the levels of MMP-2, MMP-9, and TIMP3 ($p<0.05$, Figure 2D). These findings suggested the oncogenic role of miRNA-181a in osteosarcoma.

MiRNA-181a Knockdown Induces NLRP3-Dependent Pyroptosis in Osteosarcoma Cells

Accumulating evidence[20] reported that miRNAs modulate carcinogenesis and cancer progression by regulating pyroptotic process. Thus, we also validated whether pyroptosis was involved in the pro-tumorigenic functions of miRNA-181a. We first examined the effect of miRNA-181a on the release of lactate dehydrogenase (LDH), as a key marker of pyroptosis. MiRNA-181a inhibitor significantly elevated the release of LDH compared with control (Figure 3A). Moreover, the levels of pyroptosis-related genes (NLRP3, caspase-1, IL-1β, and IL-18) were determined by qRT-PCR and Western blotting. Figure 3B illustrated that miRNA-181a inhibitor notably elevated the expression of NLRP3, caspase-1, IL-1β, and IL-18 at mRNA level. In addi-

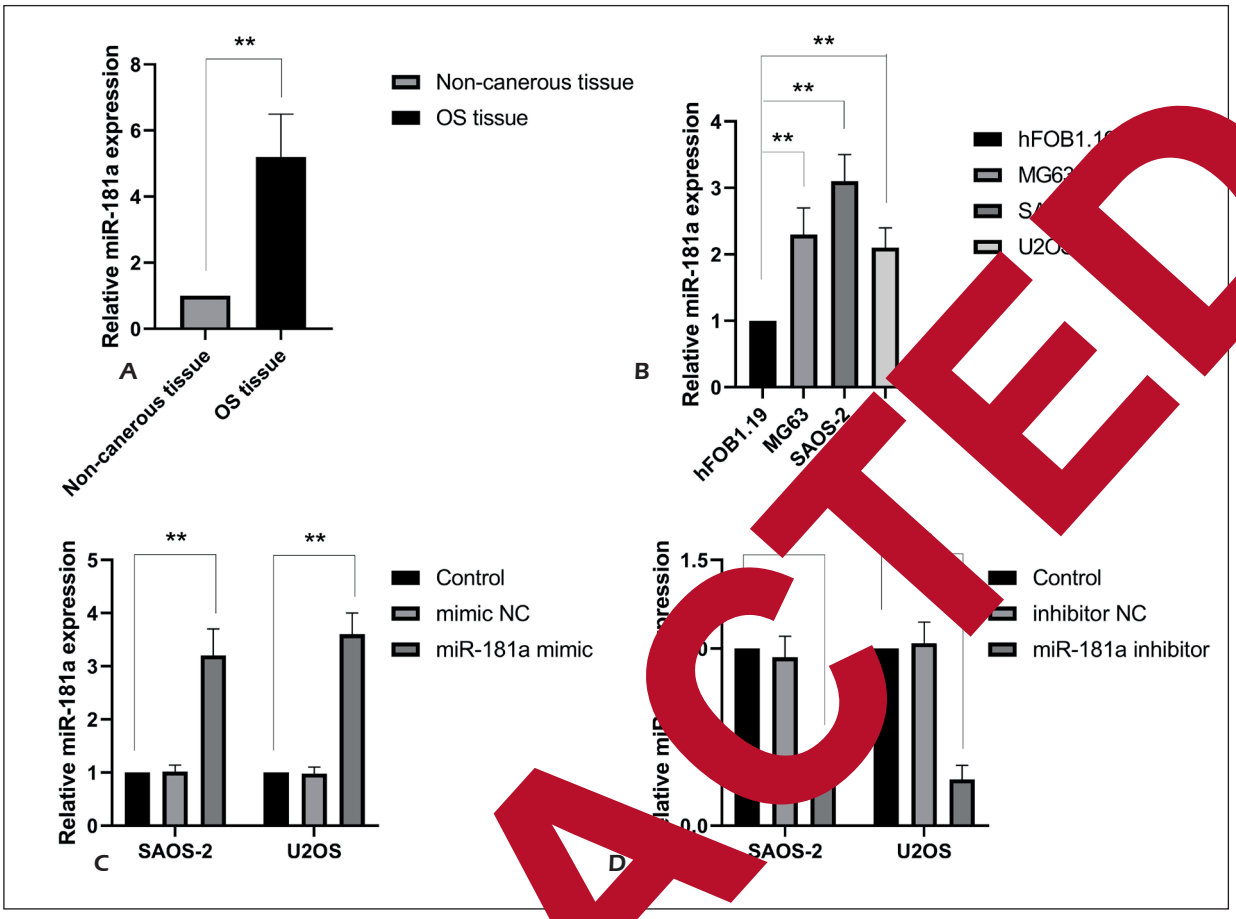
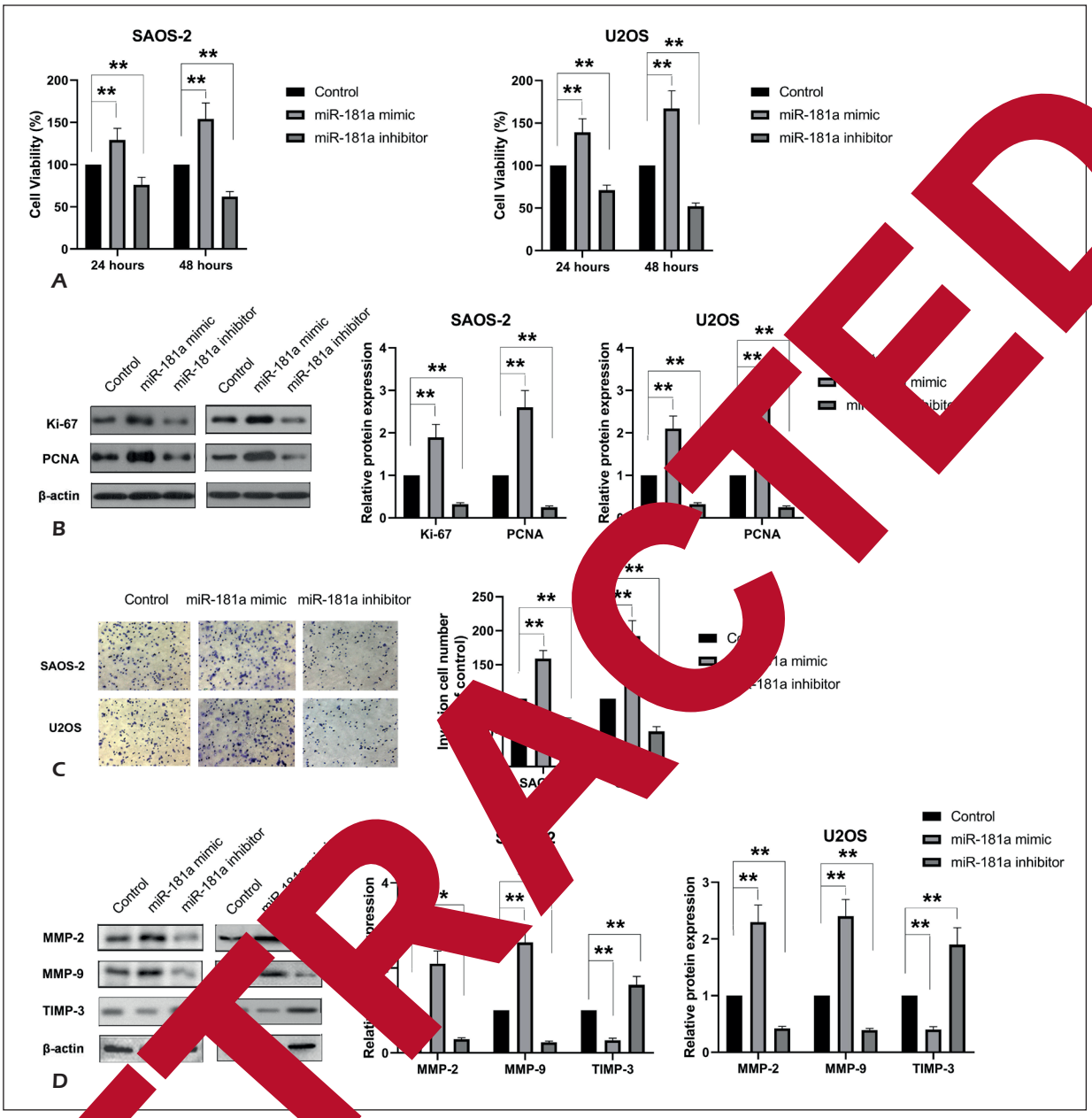


Figure 1. MiRNA-181a was aberrantly regulated in t[illegible]eosarcoma tissues and cell lines. **A**, Expression level of miRNA-181a in osteosarcoma tissues w[illegible]mpared w[illegible]t in the correspondingly non-cancerous adjacent tissues. **B**, Levels of miRNA-181a in human [illegible]sarcom[illegible]ll lines (M[illegible] SAOS-2, and U20S) were higher than that in human normal osteoblast hFOB.19. $^{**}p<0.01$ [illegible]ared with [illegible]B.19. **C**, Re[illegible]tive expression of miRNA-181a was increased in miRNA-181a mimic-transfected cells, $^{**}p<0$[illegible]pare[illegible] Relative expression of miRNA-181a in SAOS-2, and U20S were increased in miRNA-181a[illegible]ic [illegible]ecreased [illegible]miRNA-181a inhibitor group. $^{**}p<0.01$.

tion, miRNA-18[illegible] markedly [illegible]eased the expression of [illegible]LRP3, [illegible]d caspase-1, mature IL-1β, and [illegible]8 at protein [illegible](Figure 3C). Given the key [illegible] of NLRP3 in py[illegible]sis, SAOS-2 and U20S [illegible]s were [illegible]nsfected with NLRP3 shRNA. As [illegible]ted i[illegible]gure 4A, NLRP3 expression was drama[illegible]duced i[illegible]LRP3 shRNA-transfected [illegible]ells c[illegible]red w[illegible]ontrol. Our results showed [illegible]e viab[illegible]nvasion of SAOS-2 and U20S cell[illegible]ere ma[illegible]y decreased by miRNA-181a kn[illegible], which were reversed by NLRP3 shRNA [illegible] and 4C). Furthermore, as shown in [illegible]gure 4D, NLRP3 siRNA markedly diminished [illegible]miRNA-181a inhibitor-induced downregulation o[illegible]7, PCNA, MMP-2, and MMP-9 protein levels, as well as the up-regulation of TIMP-3 ($p<0.01$). Overall, these results indicated that miR-181 knockdown suppresses cell growth and invasiveness by activating NLRP3 inflammasome and pyroptosis in osteosarcoma cells.

Downregulation of MiRNA-181a Suppresses Tumor Growth and Induces Pyroptosis In Vivo

We verified *in vitro* observations in a xenograft murine model. As shown in Figure 5A and 5B, silencing miR-181a effectively reduced tumor growth. We showed that the tumor volume and tumor weight in miR-181a inhibitor group were profoundly smaller than that in control group. Consistently, the proportion of TUNEL-positive cells was dramatically elevated in miR-181-a inhibitor group compared with control (Figure 5C). Meanwhile, IHC results demonstrated that the abundance of PCNA and Ki-67 was also markedly decreased by miR-181a knockdown. Furthermore, we also val-



Figur MiRNA 181a enhances c l proliferation and invasion of osteosarcoma cells. SAOS-2 and U20S cells were transfect n miR 181a mimic or miRNA-181a inhibitor. **A**, Change of cell viability was assessed by CCK-8 assay. **B-D**, Rela tei els of K 7, PCNA, MMP-2, MMP-9, and TIMP3 in SAOS-2 and U20S cells as analyzed by Western blotting as chos internal control. **C**, Number of invasive cells was quantified by transwell assay (magnification:). **

ida the effe miR-181a knockdown in the ex levels of NLRP3 inflammasome-relat- ot onsistent with *in vitro* observations, e levels of NLRP3, caspase-1, IL-18, and IL-1β umor tissues were significantly increased in m 1a inhibitor group in comparison with control group (Figure 5D). Collectively, these results suggested that miR-181a could regulate pyroptosis, thus modulating osteosarcoma progression.

Discussion

As an oncogene, miR-181a could facilitate osteosarcoma progression through inhibition of NLRP3-dependent pyroptosis. MiRNA-181a inhibitor and NLRP3 knockdown effectively alleviate cell viability and invasiveness of osteosarcoma cells. In summary, miRNA-181a promotes the progression of osteosarcoma, at least partially, by blocking pyroptosis.

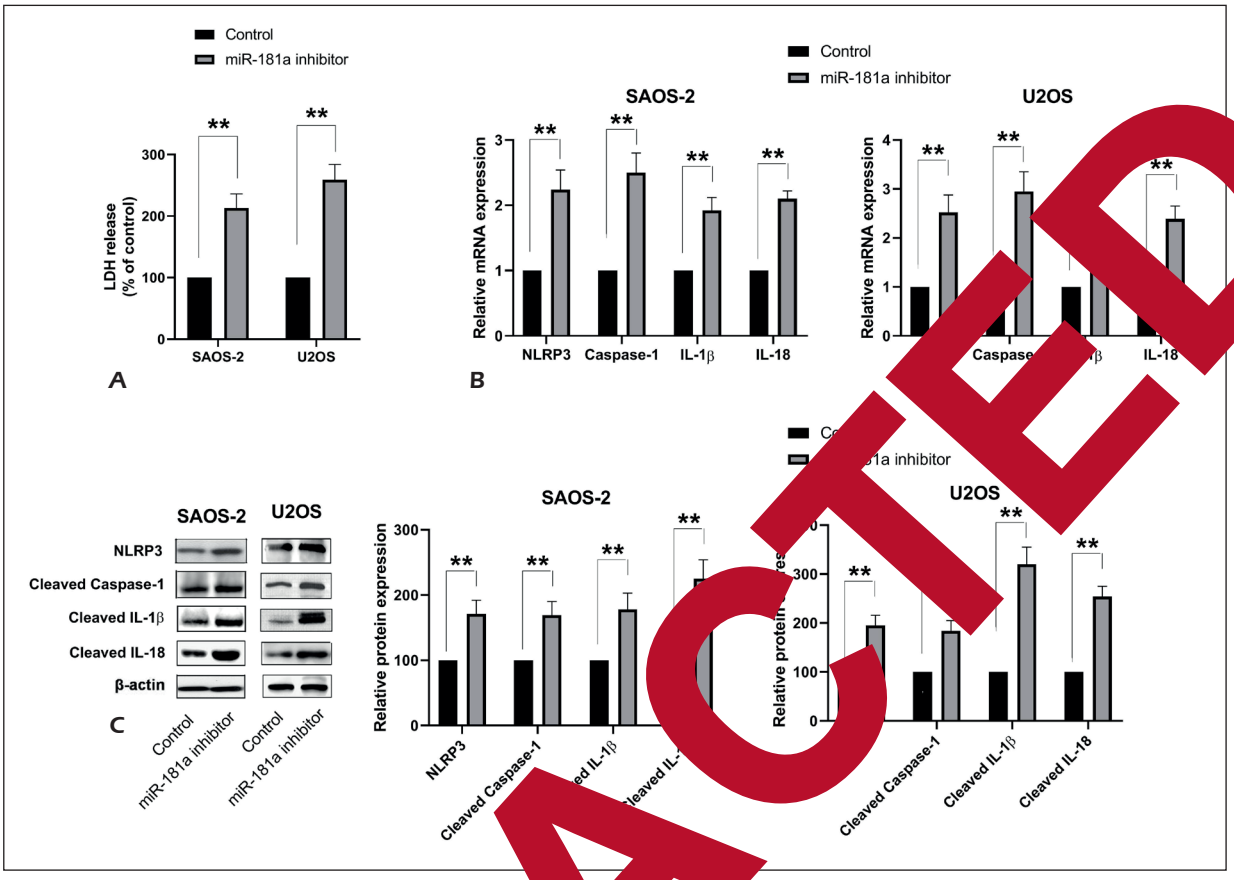


Figure 3. Silencing miRNA-181a promotes pyroptosis [illegible]arcoma cells. **A**, Down-regulation of miRNA-181a increases the release of LDH in SAOS-2 and U20[illegible]ls. **B**, Both [illegible]A and (**C**) protein levels of NLRP3, cleaved caspase-1, IL-1β, and IL-18 were elevated in miRNA-1[illegible]ed SAOS[illegible] U20S cells. β-actin was chosen as internal control, $^{**}p<0.01$.

Recently, the funct[illegible]al [illegible]iRNA[illegible]a in human diseases, especially [illegible]cancer, have been widely exp[illegible]. Existing e[illegible]e reveals that miRNA-18[illegible]y plays a cr[illegible]l role in facilitating tu[illegible]r prog[illegible]n and metastasis, but also exert[illegible]nti-tumorige[illegible]roperties. For instance, [illegible] *in vitro* and *in v*[illegible]xperiments have revea[illegible] that mi[illegible]A-181a is frequently elevated an[illegible]ngly [illegible]elated with the aggressive cancer pr[illegible]n multi[illegible]myeloma[21]. On the con[illegible]y, ac[illegible]latin[illegible]dies also have confirmed t[illegible]press[illegible] miRNA-181a in human malig[illegible]y. For i[illegible]nce, a recent study[22] conducted in [illegible]blastoma has reported that co-delivery [illegible]RNA[illegible] with switchable lipid nanoparticles [illegible]uld effectively potentiate the anti-tumor po[illegible]y of Melphalan, a common chemotherapeutic[illegible]nt in clinical setting. Additionally, it has been reported that[23] miRNA-181a was down-regulated in cervical cancer and lncRNA LUCAT1 promoted tumorigenesis and progression in cervical cancer by modulating miRNA-181a. Here, our results demonstrated that miRNA-181a was aberrantly increased in osteosarcoma. *In vitro* and *in vivo* experiments suggested that blockade of miRNA-181a could effectively abolish its tumor-promoting ability, supporting the role of miRNA-181a as an oncogene in osteosarcoma. Given these combined results, it is reasonable to believe the role of miRNA-181a in human malignancy is tissue specific.

Pyroptosis is a programmed cell death process which depends on the activation of NLRP3/caspase-1 pathway[24]. Recently, the multifaceted roles of pyroptosis in cancer have been extensively reported. It has been reported that pyroptotic cell death is one of the mechanisms used by chemotherapeutic agents to eradicate cancerous cells[25,26]. Therefore, activating pyroptotic cell death is increasingly considered an important therapeutic target for the intervention and treatment of cancer[27]. Ding et al[28] reported

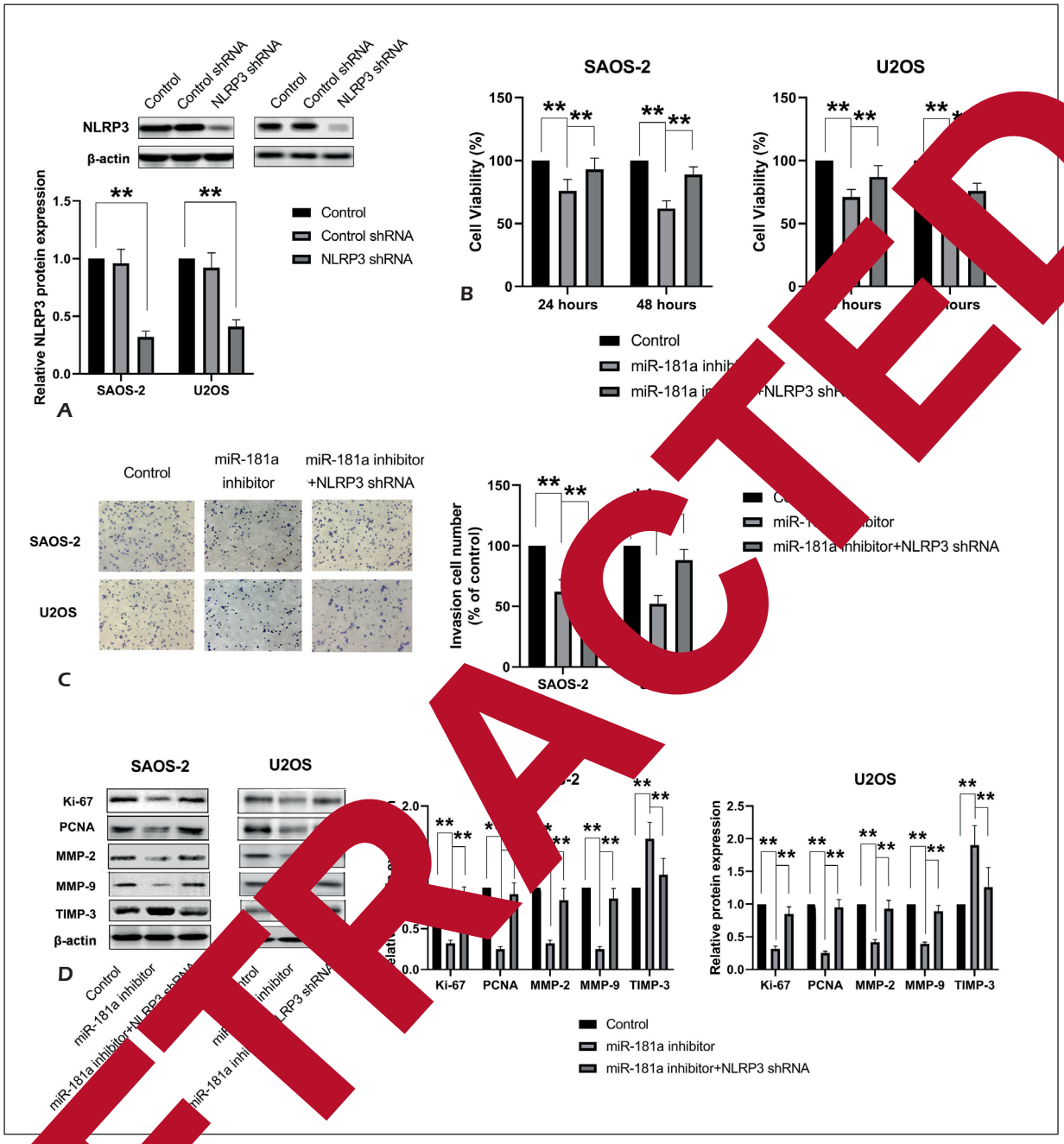


Figure Knockdown attenuates the inhibitory of miRNA-181a inhibitor on osteosarcoma cell viability and invasion. **A**, Relative expression level of NLRP3 was significantly reduced after transfected SAOS-2 and U20S cells with NLRP3 shRNA. **B**, Transfection with NLRP3 shRNA reverses the decrease of cell viability-induced by miRNA-181a inhibitor. **C**, Number of invasive cells in control group, miRNA-181a inhibitor group, and miRNA-181a inhibitor+NLRP3 shRNA group was quantified by transwell assay (magnification: 200 ×). **D**, Western blotting analysis was carried out to detect the expression of Ki-67, PCNA, MMP-2, MMP-9 and TIMP3 in SAOS-2 and U20S cells in the three groups. β-actin was chosen as internal control, $^{**}p<0.01$.

Dioscin exerts tumor-suppressing ability in osteosarcoma by triggering GSDME-dependent pyroptotic cell death and cell apoptosis. Of note, several studies also pointed out that miRNAs were involved in various tumors and other diseases by regulating pyroptosis[29]. In this way, microRNA-30c-5p protects endothelial cells from NLRP3 inflammasome-dependent pyroptosis *via* targeting FOX3[30]. MiRNA-214 could also suppress glioma cells growth and invasiveness by

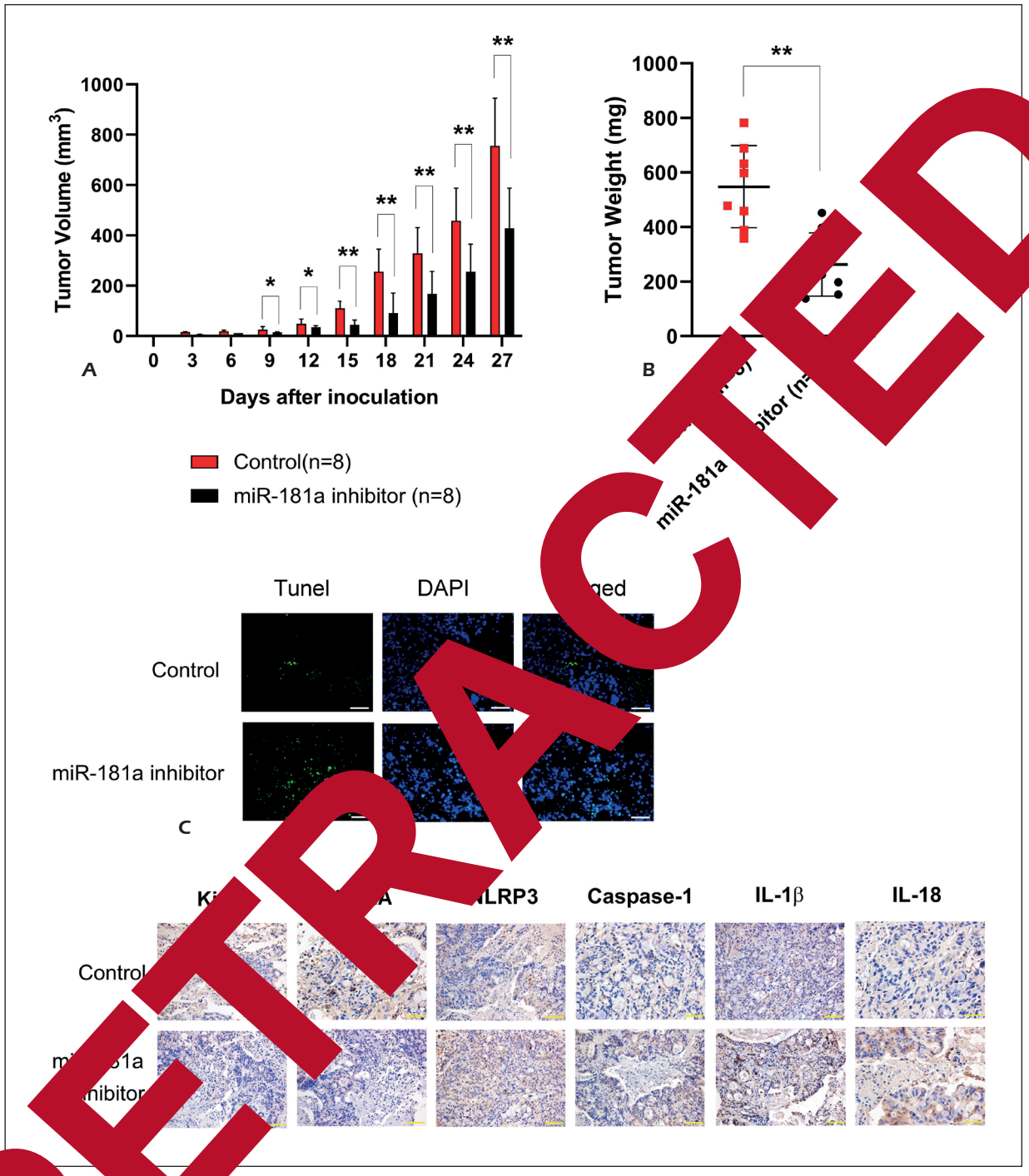


Fig. 5. Down-regulation of miRNA-181a suppresses tumor growth in vivo. **A**, Tumor volume and (**B**) weight decreases in miRNA-181a inhibitor group (n=8) compared with control group (n=8). **C**, TUNEL-positive cells were increased in miRNA-181a inhibitor compared with control (magnification: 200 ×). **D**, Abundance of Ki-67, PCNA, NLRP3, cleaved caspase-1, IL-1β and IL-18 in tumor tissues were detected by IHC (magnification: 200 ×). $^{**}p<0.01$, $^{*}p<0.05$.

down-regulating caspase-1, which subsequently inhibits pyroptosis[20]. Similarly, our current study reveals that down-regulation miRNA-181a suppresses cell growth and invasion by inducing pyroptotic cell death. Blockade of pyroptosis process with NLRP3 shRNA dramatically reversed the inhibitory of miRNA-181a inhibitor on cell proliferation and invasiveness. Although

we revealed that down-regulation of miRNA-181a could suppress osteosarcoma progression via inducing pyroptotic cell death, the limitations of the current study should be pointed out and addressed in the future. So, how miRNA-181a regulates pyroptosis process in osteosarcoma also remains to be explored. It has been well-established that most miRNAs play their roles in the development and progression of cancer by directly targeting downstream targets. Hence, the potential targets that contribute to the role of miRNA-181a still need to be investigated.

Conclusions

In summary, we demonstrated that miRNA-181a serves as an oncogenic miRNA in osteosarcoma and knockdown miRNA-181a could effectively suppress growth and invasiveness of osteosarcoma cells by inducing pyroptotic cell death.

Conflict of Interests

The authors declare that they have no conflict of interests.

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