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Original Article

# MiRNA-218 inhibits cell proliferation, migration and invasion by targeting Runt-related transcription factor 2 (Runx2) in human osteosarcoma cells

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ABSTRACT

*Purpose:* The deregulation of miRNA-218 has been found in a number of cancers. Using miRNA-218 as a target for Runt-related transcription factor 2 (Runx2), we sought to understand the role of miRNA-218 in osteosarcoma (OS).

*Methods:* The expression of miRNA-218 was detected in the OS tumor tissues and OS cells. The Runx2 expression level was evaluated in Saos-2, 143B, U2OS, and MG-63. miRNA-218 overexpressed U2OS cells were achieved by transfection with miRNA-218 mimics. The role of miRNA-218 in inhibiting OS tumorigenesis was explored by CCK8, colony formation, cell wound scratch and Transwell assay. TargetScan and dual-luciferase reporter assay identified the interaction between miRNA-218 and Runx2. The inhibitive effect of miRNA-218 on OS through targeting Runx2 was also evaluated.

*Results:* MiRNA-218 levels were remarkably down-regulated in OS tumor tissues and cell lines. The overexpression of miRNA-218 suppressed U2OS cell development and metastasis. The target interaction between miRNA-218 and Runx2 was validated, and their expression showed a negative correlation in U2OS cells. The suppressed U2OS cell development and metastasis were remarkably reversed by Runx2 overexpression.

*Conclusion:* MiRNA-218 showed an inhibitive effect on the development and metastasis of osteosarcoma cell proliferation by targeting Runx2. Our findings may provide novel clues for OS treatment.



## 1. Introduction

Osteosarcoma (OS) is a malignant bone tumor most frequently diagnosed in adolescents and young adults [1]. Patients with OS often complain of pain and fracture-related symptoms. OS is responsible for 20% of all primary bone cancers in patients. Each year, the incidence of OS varies by population, ranging from 4.6 per million whites to 6.8 per million blacks, and there is a gender disparity [2]. Surgery and chemotherapy are the primary treatments for OS. Despite advancements in management, current therapies have not been shown to affect recurrent or metastatic OS significantly. The 5-year survival rate for metastatic OS patients is reported to be low, ranging between 20% and 30% [3,4]. OS has been a global health concern, and a novel treatment is urgently needed.

Numerous studies have concentrated on the discovery of novel biomarkers for prognosis prediction and the development of targeted therapies. A recent study demonstrated that osteopontin, which is expressed in bone tissues, is involved in OS growth and metastasis and has been proposed as a therapeutic target for OS [5]. Inactivation of the tumor suppressor genes p53, pRB, and RecQL helicase has been reported in OS, and these genes have been proposed as potential therapeutic targets [6,7]. Runt-related transcription factor 2 (Runx2), a member of the RUNX transcription factor family, is also known as CBFa-1. It is a heterodimeric complex

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that can bind DNA as a subunit and is involved in osteoblast differentiation and skeletal morphogenesis [8]. Runx2 is required for early osteoblast differentiation and serves as the master gene for bone formation [9]. Runx2 was identified as a critical molecular marker in the etiology of osteosarcoma by Nathan et al. [10]; Lucero et al. demonstrated that Runx2 promoted human osteosarcoma cell proliferation [11]; and Del et al. demonstrated that WWOX inhibited osteosarcoma metastasis by decreasing Runx2 expression [12]. Runx2 overexpression in the osteoblast lineage of transgenic mice inhibits osteoblast maturation, increases bone resorption, and results in osteopenia with multiple fractures [11]. These studies established that osteoblasts can circumvent Runx2-mediated strict growth regulation and form tumor-like osteoblasts.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that play a critical role in gene expression regulation. By post-transcriptionally regulating target genes, miRNA deregulation is associated with the progression and development of OS [13,14]. The miRNA-218 expression has been found to be abnormal in a variety of tumor types, including colon cancer [15], glioblastoma [16], and head and neck squamous tumor [17]. miR-218 was reported to modulate osteoclast differentiation and inflammation response through MMP-9 in periodontitis [18]. A growing body of evidence indicates that miRNA-218 has a suppressive effect on cancer proliferation and invasion by targeting Runt-related transcription factor 2 (Runx2) [19,20]. However, the interaction between miRNA-218 and Runx2 in OS remains unknown.

As a result, we concentrated on elucidating the role of miRNA-218 in the growth and metastasis of OS cells, as well as the miRNA-218-Runx2 target interaction. We sought to elucidate the tumor-suppressive role of miRNA-218 in OS by regulating Runx2, hoping that our findings would provide new insights into how to improve OS treatment.

## 2. Methods and materials

### 2.1. Tissue sample collection

Between February 2012 and January 2019, 82 OS patients (41 males and 41 females) undergoing surgery at Huai'an Second People's Hospital had their paired tumor and adjacent normal tissues harvested after providing informed consent. Patients between the ages of 15 and 24 years were included, but those who had received radiotherapy or chemotherapy prior to surgery were excluded. Table 1 summarizes the basic characteristics of the patients included.

### 2.2. Cell culture

The human OS cell lines (Saos-2, 143B, U2OS, and MG-63) and human osteoblast cell line hFOB1 were used. hFOB1 cells were grown in D-MEM/F-12 medium (GIBCO, USA), Saos-2 cells in McCoy's 5a medium (GIBCO, USA), 143B cells in Eagle's minimum essential medium (Corning, USA), and U2OS and MG-63 cells in RPMI 1640 medium (GIBCO, USA). All cell lines were cultured in the same manner as described in the Cell Bank of the Chinese Academy of Sciences (https://www.cellbank.org.cn/index.php).

### 2.3. RT (reverse transcription)-PCR analysis

Total RNA was extracted from tissue and cell samples using the TRIzol method (Invitrogen, USA). TaqMan miRNA reverse transcription kit (Thermo Fisher Scientific, USA) was used to reverse transcribe the specific cDNA for miRNAs according to the manufacturer's instructions. MiRNA-218 expression was measured in comparison to U6 using PCR. The expression of Runx2 relative to GAPDH was achieved with the primers of Forward: 5′-TGACCAGTCTTACCCCTCCT-3′, Reverse: 5′-CTGAAGCACCTGAAATGCG-3'. There were four replications for each assay.

### 2.4. Transfection

The miRNA-218 mimics (5′ UUGUGCUUGAUCUAACCAUGU 3′), siRNA (5′ ACAUGGUUAGAUCAAGCACAA 3′), and the control sequence (5′ GUGGAUAUUGUUGCCAUCA 3') inserted plasmids were obtained from Shanghai GenePharma Co., China. The miRNA-218-overexpression and miRNA-Ctrl U2OS cells were achieved by lipofection transfection (Invitrogen, USA) after transfection with 40 nM miRNA-218 mimics or its control. The adenovirus expressing Runx2 vector and controls were constructed and transfected as per the previous description [21]. After transfection for 24 h, the expressions of miRNA-218 and Runx2 were detected by RT-qPCR analysis.

### 2.5. Colony formation and CCK-8 assay

After transfection, cells were collected and transferred to the fresh culture media for further analysis. When the cells grew to 50% confluence, cells (600 cells/well) were plated in a 6-well plate and cultured for two weeks. Following incubated with 0.1% crystal violet for 10 min, the photos of colony formation were taken and analyzed.

For Cell Counting Kit-8 assay, cells were plated on the 96 well plate and cultured. Cells were incubated with CCK-8 solution (100 μL/well, Beyotime, China) for 12, 24, 48, 72 h, and the optical density (OD) value of cultures was determined at 480 nm under a microplate spectrophotometer (Thermo, Waltham, MA, USA).

### 2.6. Cell migration and invasion assay

For wound-healing, cells were seeded in the 6-well plate and cultured for 8–24 h. When cells grew to 90% confluence, each well with cultured cells was scratched by a 200 μl pipette tip followed by 48 h culture. The photos of cultured cells were captured at 0, and 48 h in the same position and the area of wound-healing was analyzed.

Transwell assay was applied to evaluate the changes in cell metastasis ability. Cells were seeded in the Matrigel-coated Transwell or normal Transwell plate (Costar, USA) of the upper chamber at $5 \times 10^4$ cells/well. The bottom of the Transwell plate was added with 400 μL DMEM with 10% PBS. Following 24 h incubation, cells on the upper surface of the Transwell filter membrane were wiped off, and the membrane and lower chamber cultures were stained with 0.1% crystal violet. After dried, the staining cells were observed at three random fields under a microscope.

### 2.7. Prediction of the target interaction between miRNA-218 and Runx2

The target interaction between miRNA-218 and Runx2 was predicted with the application of the TargetScan online tool and determined by the dual-luciferase reporter assay (Abcam, Cambridge, UK), as the manufacturer described. Briefly, the wide-type or mutant 3′-UTR sequences of Runx2 were cloned to the luciferase reporter vector. The recombinant plasmid, miRNA-218 mimics, and miRNA-218-ctrl vector was co-transfected to U2OS cells. Then, the transfected cells were maintained for 48 h, and the luciferase signals were evaluated by fluorescence microplate.

**Table 1**
The basic information of osteosarcoma patients included in our study.

| Characteristics | | n | miRNA-218 | | P |
|---|---|---|---|---|---|
| | | | Low | High | |
| Age (years) | | | | | NS |
| | <20 | 29 | 13 | 16 | |
| | ≥20 | 53 | 28 | 25 | |
| Gender | | | | | NS |
| | Male | 41 | 20 | 21 | |
| | female | 41 | 18 | 23 | |
| Tumor size (cm) | | | | | NS |
| | <8 | 36 | 20 | 16 | |
| | ≥8 | 46 | 24 | 22 | |
| Tumor stage | | | | | 0.033 |
| | I | 23 | 18 | 5 | |
| | II/III | 59 | 31 | 28 | |
| Metastasis | | | | | 0.016 |
| | Absent | 57 | 27 | 30 | |
| | Present | 25 | 19 | 6 | |

NS: no significant difference.

### 2.8. Western blotting

The indicated cells were lysed using RIPA lysis reagent (Sigma, USA). The concentration of the supernatant was quantified by BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Then the protein was separated by 10% sodium dodecyl polyacrylamide gels and transferred onto PVDF membrane (Millipore, USA). After blocking with 5% nonfat milk for 1h, the membrane was incubated with primary antibody against Runx2 (Abcam, ab236639, 1:1000) and β-actin (Abcam, ab5694, 1:1000) at 4 °C overnight, followed by incubated with HRP-linked secondary antibody (Abcam, ab7090, 1:1000) for 1h. The protein bands were quantified by ImageJ software and β-actin functioned as the internal control.

### 2.9. Statistical analysis

The data were displayed as mean ± standard deviation (SD) and analyzed by SPSS software. The multi-group comparison was analyzed by the one-way ANOVA method followed by Tukey's analysis. Student's t-test was used to analyze the difference between the two groups. Differences with a $p$-value < *0.05* were considered as statistically significant.

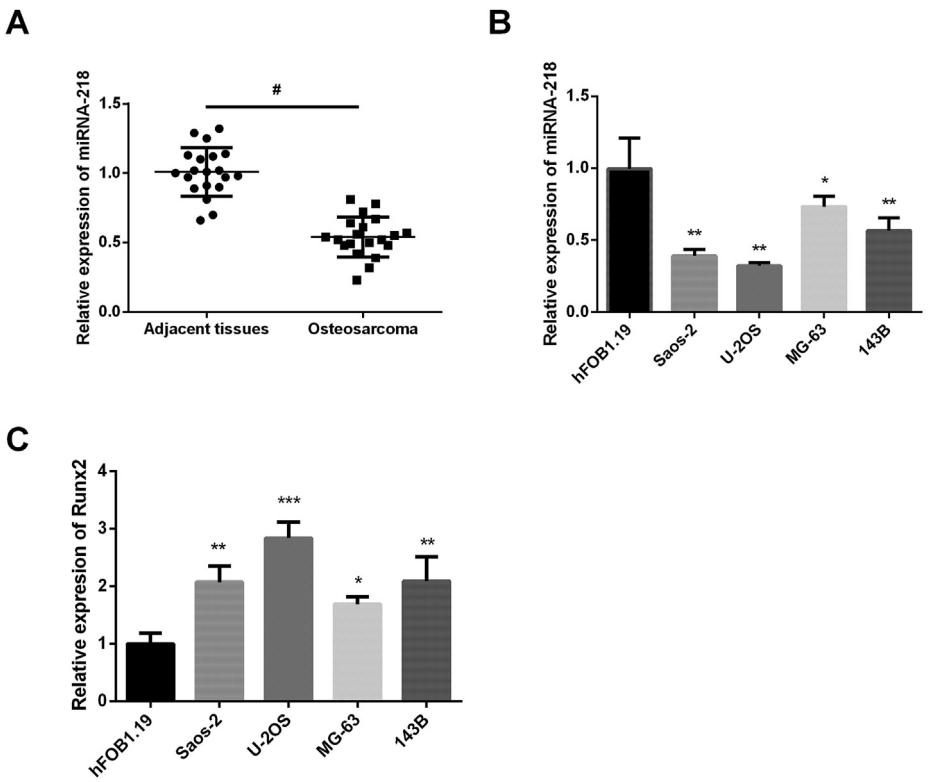


**Fig. 1. The expression of miRNA-218 and Runx2 in osteosarcoma tissues and cell lines.** (**A**) The expression of miRNA-218 was detected in 20 paired tumor tissues and normal tissues. The expression of miRNA-218 was significantly declined in osteosarcoma tissues, compared with controls. The expression of miRNA-218 (**B**) and Runx2 (**C**) in hFOB1.19, Saos-2, U-2OS, MG-63 and 143B cell lines. MiRNA-218 expression was significantly lower in osteosarcoma cell lines. #$P$ < 0.01, compared with normal tissues. *$P$ < 0.05, **$P$ < 0.01 and ***$P$ < 0.001, compared with hFOB1.19 cells.

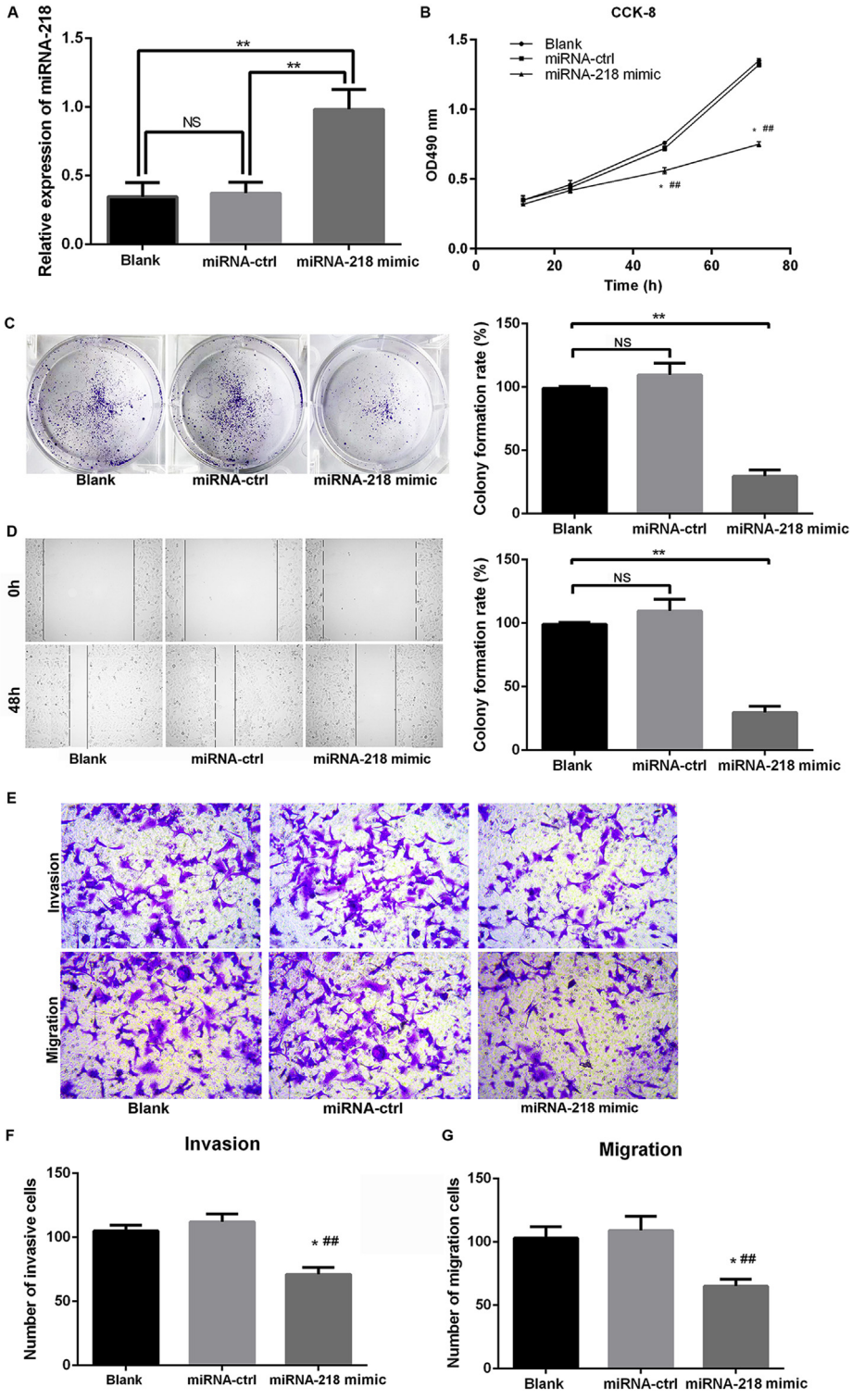


**Fig. 2. The inhibitive effect of miRNA-218 overexpression on the proliferation, colony formation, migration and invasion of U-2OS cells.** (**A**) The miRNA-218 overexpression cells were achieved by transfection with miRNA mimics. The positive transfection was detected by qRT-PCR analysis. The expression of miRNA-218 was significantly elevated in cells transfected with miRNA-218 mimics. (**B**) The effect of miRNA-218 overexpression on cell proliferation was detected by CCK-8 analysis. miRNA-218 overexpression significantly inhibited cell proliferation, compared with controls. (**C**) The colony formation of miRNA-218 overexpression cells was analyzed. The number of colonies was the lowest in cells transfected with miRNA mimics. (**D**) The changes of migration ability after miRNA-218 overexpression were observed by cell scratch assay. miRNA-218 overexpression obviously suppressed the cell wound-healing ability. The effect of the overexpression of miRNA-218 on cell invasion (**E and F**) and migration (**E and G**) was measured by Transwell assay. miRNA-218 overexpression remarkably inhibited cell invasion and migration. *$P < 0.01$ compared with miRNA-ctrl group; ##$P < 0.01$ compared with blank group; **$P < 0.01$ compared with indicated group; NS: no significant difference.

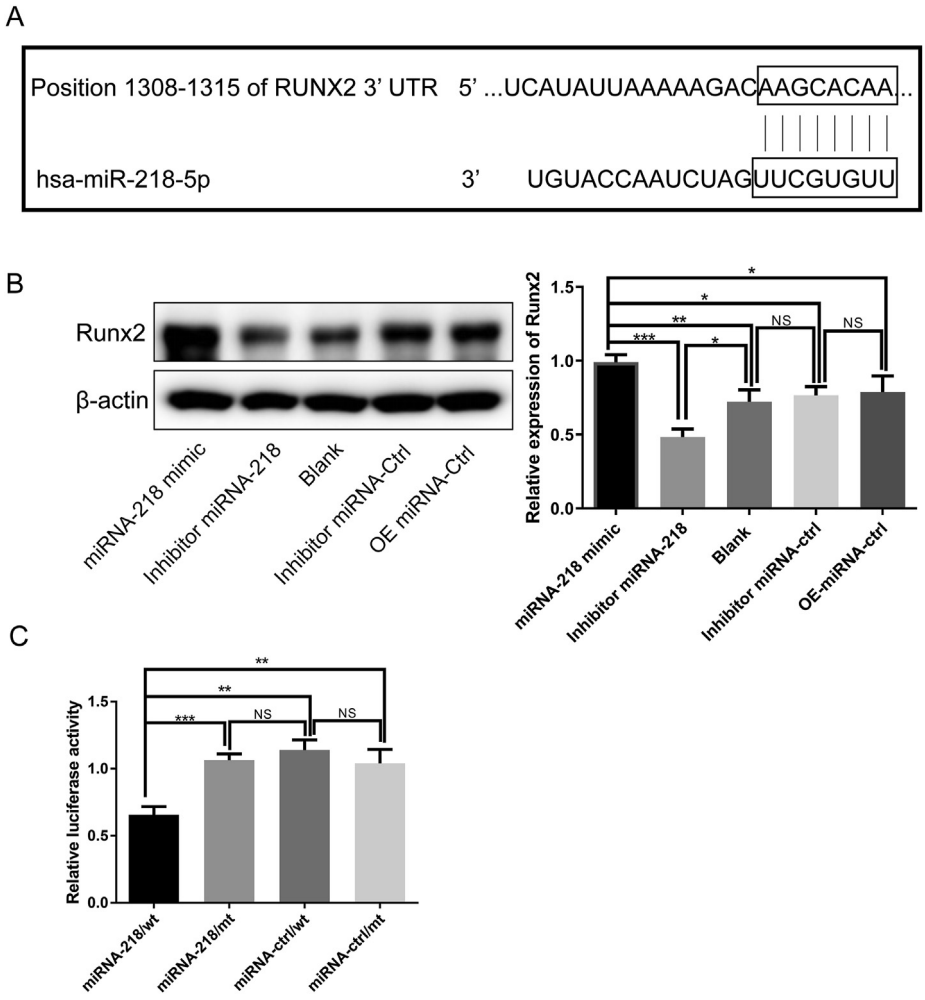


**Fig. 3. The target interaction between miRNA-218 and Runx2.** (**A**) The gene target Runx2 of miRNA-218 was predicted by TargetScan. Runx2 was a direct target for miRNA-218. (**B**) The interaction between Runx2 and miRNA-218 was determined by Western blot analysis. Runx2 expression in U2OS cells were detected by transfection with miRNA-218 mimics and miRNA-218 inhibitors, separately. The expression of Runx2 was significantly declined in miRNA-218 mimic group and obviously accumulated in miRNA-218 inhibitor group. (**C**) The target association between miRNA-218 and Runx2 was determined by a luciferase assay. The relative luciferase signaling was lowest in wide-type 3′UTR of Runx2 group co-transfected with miRNA-218 mimics. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and NS, no significant difference.

## 3. Results

### 3.1. MiRNA-218 was poorly expressed, and Runx2 was highly expressed in OS tissues and cell lines

The expressions of miRNA-218 and Runx2 were investigated using RT-qPCR. Compared with normal controls, miRNA-218 expression was significantly down-regulated in OS tumor tissues ($P < 0.01$, Fig. 1A). The human OS cell lines (Saos-2, 143B, U2OS, and MG-63) and human osteoblast cell line hFOB1 were used to detect the expressions of miRNA-218 and Runx2. When MG-63, 143B, Saos-2, and U2OS cells were compared to FOB1.19, MiRNA-218 expression was reduced in all four OS cell lines tested, with U2OS cells showing the greatest reduction ($P < 0.01$) (Fig. 1B). In terms of Runx2 expression, all four OS cell lines were significantly higher than FOB1.19 cells ($P < 0.05$). The accumulation of Runx2 expression was found to be highest in U2OS ($P < 0.01$) (Fig. 1C). As a result, U2OS cells were used for further investigation.

### 3.2. The role of miRNA-218 in the development and metastasis of U2OS cells

The miRNA-218 overexpression U2OS cells were generated by transfecting miRNA-218 mimics vector via Lipo3000 reagent (Invitrogen, USA) to assess the role of miRNA-218 in the development and metastasis of OS cells. The positive transfection was determined by RT-qPCR analysis. After transfection for 24 h, the expression of miRNA-218 in the U2OS cell line was remarkably increased in the miRNA-218 mimic group than the control groups ($P < 0.01$). MiRNA-218 expression in miRNA-218-ctrl group was comparable to the blank control group ($P > 0.05$) (Fig. 2A). MiRNA-218 overexpression obviously inhibited the cell proliferation at 48 h and 72 h of culture (Fig. 2B). The colony formation assay showed that miRNA-218 overexpressed U2OS cells displayed decreased colony formation ability compared to controls (Fig. 2C). To evaluate the antagonistic effects of miRNA-218 on metastasis of tumor cells, cell wound scratch and Transwell assays were conducted. As shown in Fig. 2D, the miRNA-218 overexpression obviously inhibited the

cell migration ability of U2OS cells, compared with miRNA-ctrl and blank control groups ($P < 0.01$). Meanwhile, the Transwell assay revealed that invaded cells were strikingly declined than that in the miRNA-218-ctrl and blank control groups ($P < 0.01$) (Fig. 2E and F). Similarly, miRNA-218 overexpression dramatically declined the number of migration cells compared with controls ($P < 0.01$) (Fig. 2E and G).

### 3.3. MiRNA-218 targets Runx2 interaction in OS development

MiRNA-218 was predicted to have a target binding site in the Runx2 3′UTR region between 1.3k and 1.4k using the TargetScan online tool (Fig. 3A). Western blot analysis was used to determine the target interaction between miRNA-218 and Runx2 following transfection with miRNA-218 mimics or a miRNA inhibitor. The results indicated that Runx2 protein levels were significantly increased in miRNA-218 mimic transfected U2OS cells, but significantly decreased following miRNA-218 silencing by siRNA (Fig. 3B). The luciferase assay also revealed a negative relationship between miRNA-218 and Runx2 expression. The luciferase activity of the wide-type 3′UTR of Runx2 was significantly reduced in U2OS cells co-transfected with miRNA-218 mimics, compared to the mutant 3′UTR of Runx2. There were no significant changes in luciferase activity in miRNA-218 mimics + mutant 3′-UTR, miRNA-Ctrl + wide-type 3′-UTR, and miRNA-Ctrl + mutant 3′-UTR sequence of Runx2 groups ($P > 0.05$, Fig. 3C).

### 3.4. MiRNA-218 attenuates U2OS cell proliferation and metastasis by targeting Runx2

To evaluate whether miRNA-218 functions via Runx2, U2OS cells were randomly divided into four groups, including miRNA-218 mimic + vector group, miRNA-218 mimic + Runx2 overexpression group, miRNA-218 ctrl + vector group, and miRNA-218 ctrl + Runx2 overexpression group. As expected, the protein expression of Runx2 was significantly lower in the miRNA-218 mimic + vector group, while it was reversed in the miRNA-218 mimic + Runx2 overexpression group (Fig. 4A). As determined by the CCK8 assay, the cell viability in miRNA-218 mimic transfected U2OS cells was the lowest among groups, while the miRNA-218-ctrl + OE-Runx2 group was the highest. No significant difference was detected in the cell viability of miRNA-218-ctrl + vector and miRNA-218 mimics + OE-Runx2 group (Fig. 4B). Similar results were obtained in the colony formation assay. The number of clones was lowest in miRNA-218 mimics transfected cells but significantly increased in the miRNA-218 mimics + OE-Runx2 group. Most clones were found in the miRNA-218-ctrl + OE-Runx2 group. The ability of U2OS cells to form colonies in the miRNA-218 mimics + OE-Runx2 group was comparable to that of the miRNA-218-ctrl group (Fig. 4C).

The cell wound scratch assay, and Transwell analysis were used to investigate the effect of miRNA-218 on the metastasis ability of U2OS cells by targeting Runx2. The reconstituted U2OS cells with miRNA-218 mimics showed markedly elevated migration ability than those treated with miRNA-218 mimics + OE-Runx2. The migration ability of cells in miRNA-218-ctrl + OE-Runx2 group was highest among groups (Fig. 4D). In the Transwell assay, the number of migrating and invasive cells were significantly higher in miRNA-218-ctrl + OE-Runx2 group, compared with miRNA-218 mimics + OE-Runx2 and miRNA-218-ctrl + vector group ($P < 0.01$); while there was no obvious difference between miRNA-218 mimics + OE-Runx2 and miRNA-218-ctrl + vector groups ($P > 0.05$) (Fig. 4E, F and G).

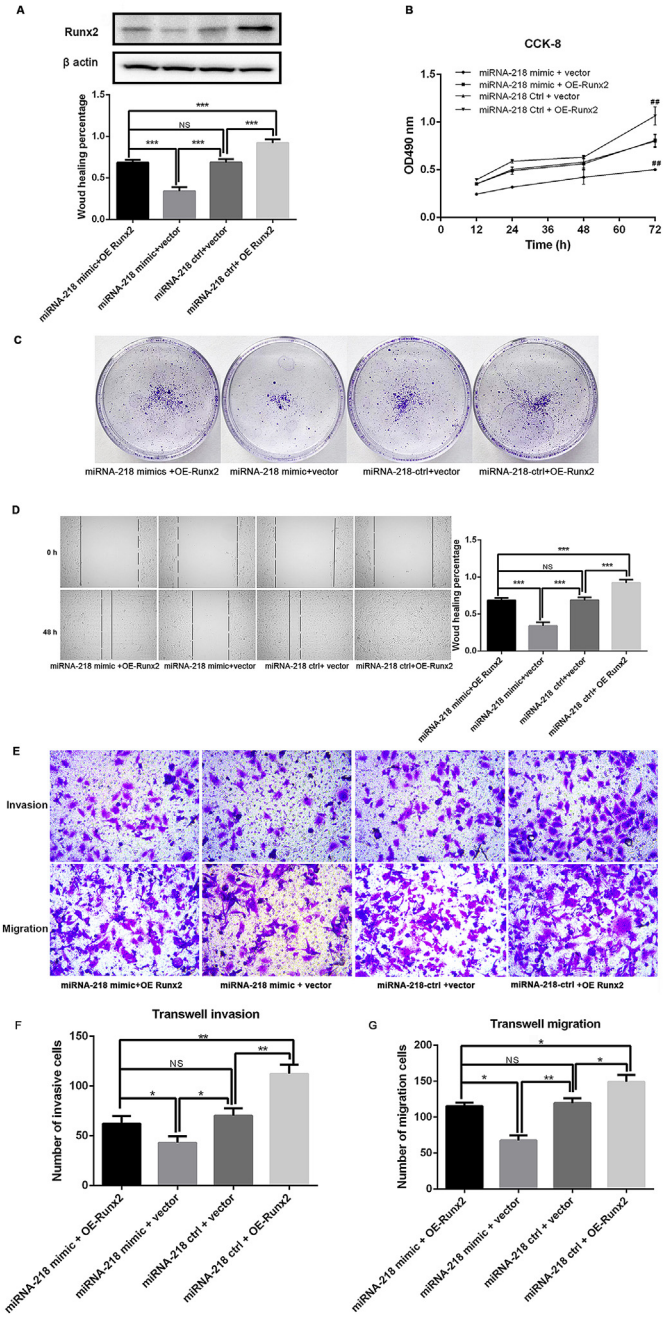


**Fig. 4. The inhibitive effect of miRNA-218 on cell proliferation, invasion and migration by targeting Runx2.** U2OS cells were assigned to four groups, including miRNA-218 mimics + vector, miRNA-218 mimics + OE-Runx2, miRNA-218-ctrl + OE-Runx2, and miRNA-218-ctrl + vector. (**A**) Runx2 expression after miRNA-218 mimics and Runx2 transfection intervention was detected by Western blot. Runx2 expression was significantly decreased in miRNA-218 mimics + OE-Runx2, which was rescued by Runx2 vector transfection. (**B**) Cell proliferation signaling after Runx2 overexpression was captured at 12, 24, 48, 72 h by CCK8 assay. U2OS cells proliferation was significantly elevated after transfected with miRNA-218 mimics and declined after transfection with Runx2 vector. (**C**) The cell proliferation ability was also measured by colony formation assay. Results revealed that the number of cell colonies were significantly increased in cells transfected with miRNA-218 mimics and relieved by Runx2 vector transfection. (**D**) Cell scratch assay indicated that the scratch area significantly declined in miRNA-218 overexpression group and reversed after Runx2 vector transfection. Transwell assay revealed that the cell invasion (**E and F**) and migration (**E and G**) in miRNA-218 overexpression cells was obviously declined after Runx2 vector transfection. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ## indicates $P < 0.01$ compared with miRNA-218 mimics + OE-Runx2 or miRNA-ctrl + vector; NS, no significant difference.

## 4. Discussion

OS is a common type of bone cancer that originates in bone-forming mesenchymal cells. It is distinguished by rapid bone growth and a proclivity for metastasis [22]. Despite advances in therapeutic management, high-grade OS has a poor prognosis. OS treatment remains a challenge. Numerous studies have established that miRNAs play critical roles in the progression and development of OS [23,24]. MiRNA-199a-3p, a tumor suppressor gene, has been implicated in the growth and metastasis of OS cells [25]. MicroRNA-154-5p is not abundant in OS tissues, but its overexpression significantly inhibits OS tumorigenesis [26]. MiRNA-542-5p facilitated OS tumorigenesis by increasing cell proliferation and was proposed as a prognostic biomarker [27]. All of the preceding evidence points to miRNAs playing a critical role in the development and progression of OS.

MiRNA-218, a tumor suppressor, is found to be significantly down-regulated in several cancers, including pancreatic cancer [28], cervical carcinoma [29], and thyroid cancer [30]. Numerous previous studies have established that miRNA-218 regulates target genes and thus plays a role in tumorigenesis [31–33]. MiRNA-218 expression was found to be significantly decreased in the serum of laryngeal cancer patients and was reported to be negatively correlated with the prognosis of laryngeal cancer patients [34]. MiRNA-218 increased the chemosensitivity and apoptosis in cervical cancer cells by suppressing surviving expression [35]. In addition, miRNA-218 inhibits the proliferation and metastasis of OS cells by mediating TIAM1, MMP2, MMP9, and E2F2 expression and function [36,37]. However, the mechanism by which miRNA-218 contributes to OS is not entirely understood. In our study, miRNA-218 expression was significantly down-regulated in OS tumor tissues and cell lines, which was consistent with previous findings [37]. Furthermore, increasing miRNA-218 expression significantly reduced cell development and metastasis as measured by CCK8, colony formation, cell wound scratch, and Transwell assays, indicating that miRNA-218 plays a tumor-suppressive role in OS.

Runx2 is the first transcription factor detected in preosteoblasts and plays an essential role in different stages of osteogenesis through canonical Wnt signaling pathways [38]. The abnormal expression of Runx2 has been found in various tumor cells, which has been linked to tumor cell migration and oncogenesis [39]. Runx2 was recently identified as a direct target of miRNA-218, and its overexpression abolished the suppressive activity of miRNA-218 in ovarian cancer cell growth and metastasis [19]. The role of miRNA-218 by interaction with Runx2 has not been elucidated in OS genesis. Therefore, we investigated the role of miRNA-218 and Runx2 in OS oncogenesis in this study. In the present study, using the publicly available TargetScan online tool, we discovered that Runx2 is the direct target of miRNA-218. Furthermore, Runx2 was found to be significantly overexpressed in U2OS cells, while miRNA-218 expression was found to be the weakest in U2OS cells. The oncogenesis-related Runx2 gene was suppressed considerably by miRNA-218 overexpression, which was accompanied by clearly inhibited U2OS cell proliferation, migration, and invasion.

## 5. Conclusion

Overexpression of miRNA-218 inhibited the development and metastasis of OS, in part by regulating Runx2. Our study shed new light on miRNA-218's role in inhibiting OS tumorigenesis, and we believe miRNA-218 can be a new prognostic marker and therapeutic target in OS.

## Ethical approval

The study was carried out in accordance with the Helsinki Declaration and was approved by the Ethics Committee of Huaian Second People's Hospital (approval No. JSHA2012-077-T).

## Consent to participate

The written informed consent was obtained from all involved subjects.

## Availability of data and material

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

## Consent to publish statement

N/A.

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## Author contributions

Qiang Guo investigated, data analysis and wrote the draft-manuscript; Junan Ma participated in data analysis; Jing Wu designed and modified the manuscript. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Declaration of competing interest

The authors declare no conflict of interest.

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