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MiR-298 suppresses the malignant progression of osteosarcoma by targeting JMJD6

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Abstract. – OBJECTIVE: To explore the role of microRNA-298 (miR-298) in affecting biological characteristics of osteosarcoma cells, and the possible molecular mechanism.

PATIENTS AND METHODS: Fifty clinical cases of osteosarcoma were collected for detecting differential expressions of miR-298 using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), and its level in osteosarcoma cell lines was determined as well. Proliferative and migratory changes in MG63 and U2OS cells overexpressing miR-298 were assessed by Cell Counting Kit-8 (CCK-8), transwell and wound healing assay. Candidate targets of miR-298 were predicted using online databases, and JMJD6, the most optimal target, was specifically analyzed by Dual-Luciferase reporter assay and rescue experiments.

RESULTS: MiR-298 was both expressed in osteosarcoma and healthy bone tissues, which was lowly expressed in the former tissues. It was downregulated in osteosarcoma cell lines as well. Low level of miR-298 predicted higher incidences of lymphatic metastasis, distant metastasis, and lower overall survival and progression-free survival in osteosarcoma. Overexpression of miR-298 weakened proliferative and migratory changes in MG63 and U2OS cells. JMJD6 was confirmed as the target gene binding miR-298, and negatively correlated to miR-298 level in osteosarcoma tissues. Overexpression of JMJD6 reversed the effect of overexpressed miR-298 on alleviating the malignant progression of osteosarcoma.

CONCLUSIONS: MiR-298 is less abundant in osteosarcoma samples, which is correlated to metastasis and prognosis of osteosarcoma. MiR-298, serving as a tumor suppressor, weakens proliferative and migratory abilities of osteosarcoma cells.

Key Words:
MiR-298, JMJD6, Osteosarcoma, Malignant progression.

Introduction

Osteosarcoma is a primary malignant bone tumor originating from mesenchymal tissues. It mainly affects adolescents in 13-25 years, with a higher incidence in men than women. Metaphysis of long bones, distal femur and proximal tibia are the common lesions involved by osteosarcoma, manifesting as bone-like tissues or tumor-like bones[1,2]. Hematogenous metastases of osteosarcoma are common, usually to the lung, and occasionally to the kidney, heart, brain and other important organs[3,4]. Osteosarcoma has a relatively poor prognosis because of high malignant level. The 5-year survival of osteosarcoma remains 60-70% even after active comprehensive treatment including surgery, neoadjuvant therapy, biological therapy, etc.[5,6]. Gene mutations are responsible for tumorigenesis, tumor progression, and tumor outcome. Gene variations and immune factors eventually decide the phenotypes of tumor cells[7,8]. At present, seeking for highly effective and specific targets for clinical treatment of osteosarcoma is of great significance[9,10].

MicroRNAs (miRNAs) have been emerged as future aspects in tumor treatment[11-13]. They are extensively distributed in eukaryotes with 22 nucleotides in transcripts[14,15]. Based on the principle of complementary base pairing, miRNAs are able to regulate expressions and functions of target genes by degrading or inhibiting translation of their mRNAs[16,17]. As a result, miRNAs are widely involved in cell behaviors and other life activities. MiR-298 is a tumor-associated miRNA, displaying regulatory effects on tumor cells *via* targeting CDK6, CDK9 and MYB[18-20].

The role of miR-298 in osteosarcoma is rarely reported. Through bioinformatic analyses using

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online databases, JMJD6 was predicted to be the most optimal target of miR-298. We designed a series of *in vitro* experiments to illustrate the molecular mechanisms of miR-298 and JMJD6 on regulating osteosarcoma progression. Our findings may provide evidence for targeted therapy of osteosarcoma.

Patients and Methods

Osteosarcoma Samples

Surgically resected osteosarcoma and adjacent healthy bone tissues were collected from 50 osteosarcoma patients, and stored at -80°C after labeling. Inclusion criteria: patients with no severe diseases in other organs, and none of patients had preoperative chemotherapy/radiotherapy or molecular targeted therapy. Exclusion criteria: patients with distant metastasis, those complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases, or those previously exposed to radioactive rays. Tumor node metastasis (TNM) staging of osteosarcoma was diagnosed based on the Union for International Cancer Control (UICC) criteria. This study was approved by the research Ethics Committee of Peking University Shenzhen Hospital and complied with the Helsinki Declaration. Informed consent was obtained from patients or their families.

Cell Lines and Reagents

Osteosarcoma cell lines (HOS, 143B, MG63, U2OS) and the osteoblast cell line (hFOB) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) at 37°C with 5% CO_2. Medium was replaced every 2-3 days. Cell passage was conducted at 90% confluence, and those in the logarithmic growth phase were collected for experiments.

Transfection

Transfection plasmids were provided by GenePharma (Shanghai, China). Cells were seeded in a 6-well plate and cultivated to 40-60% density and were subsequently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h cell transfection, cells were collected for verifying transfection efficacy and functional experiments.

Transwell Migration Assay

Cell suspension in serum-free medium was prepared at 5×10^5/mL. 200 μL of suspension and 700 μL of medium containing 20% FBS was respectively applied on the top and bottom of a transwell chamber (Corning, Corning, NY, USA). After cell culture for 48 h, migratory cells on the bottom were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 10 randomly selected fields per sample.

Wound Healing Assay

Cell suspension in serum-free medium was prepared at 5×10^5/mL, and implanted in 6-well plates. Until 90% of cell attachment, an artificial scratch was made using a sterilized pipette tip. Cells were washed in phosphate-buffered saline (PBS) for 2-3 times and cultured in the medium containing 1% FBS. 24 hours later, wound closure was captured for calculating the percentage of wound healing.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Osteosarcoma cells and tissues were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs, and purified by DNase I treatment. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan), followed by qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. MiR-298: forward: 5'-AGCAGAAGCAGGGAGGUUCUCCCA-3', reverse: 5'-CCGUGGUGAUACUGCGUUCC-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; JMJD6: forward: 5'-GGATCCATGTCCTACCCGCAGGGC-3', reverse: 5'-GAATTCCTATAGGTAGGGCTGGACGC-3'; GAPDH: forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3', reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) on ice for 15 min, and the mixture was centrifuged at 14000×g, 4°C for 15 min. The concentration of

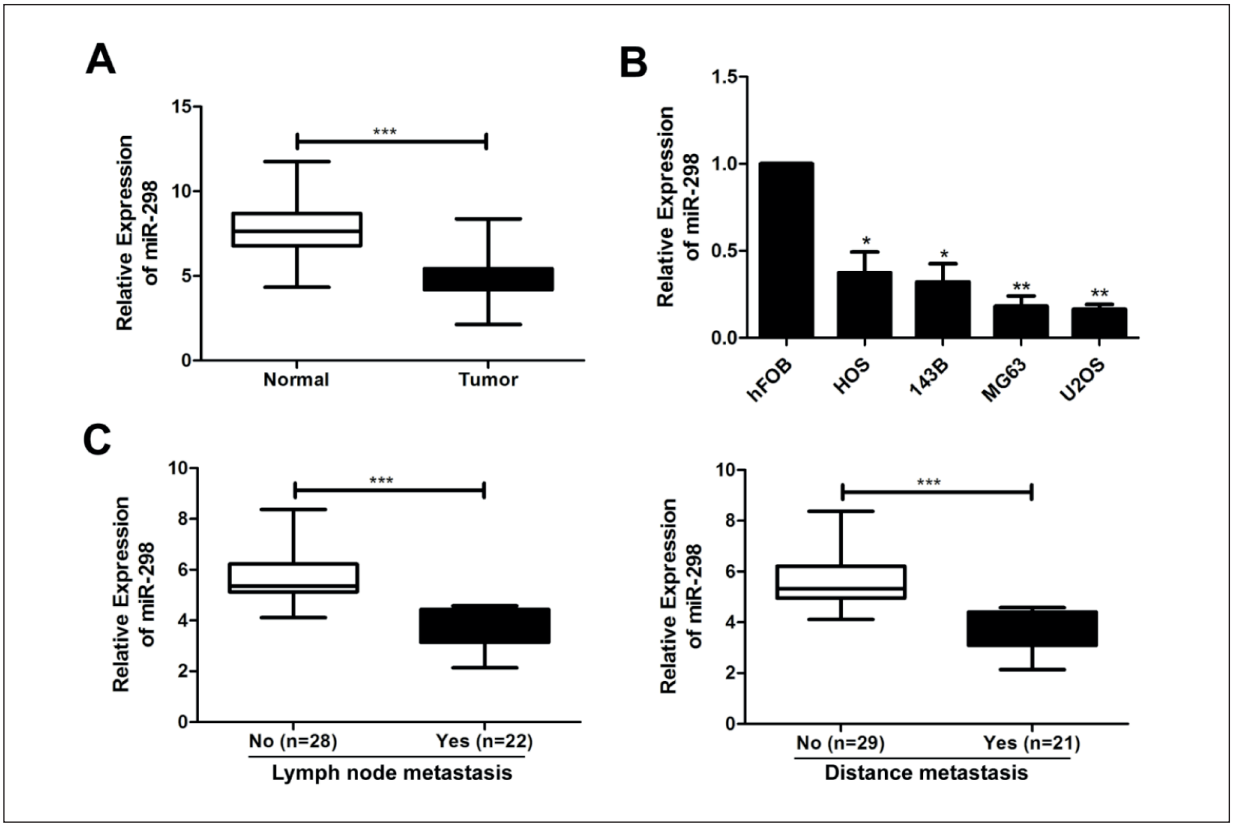

Figure 1. MiR-298 was lowly expressed in osteosarcoma tissues and cell lines. **A**, Differential level of miR-298 in osteosarcoma and healthy bone tissues. **B**, MiR-298 level in osteosarcoma cell lines. **C**, Differential level of miR-298 in osteosarcoma patients either with lymphatic metastasis/distant metastasis or not. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples with the adjusted same concentration were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses.

Dual-Luciferase Reporter Assay

pmirGLO-JMJD6-WT, pmirGLO-JMJD6-MUT and pmirGLO were co-transfected into HEK293T cells with NC mimic or miR-298 mimic, respectively. Luciferase activity (Promega, Madison, WI, USA) was measured after 48 h of co-transfection.

Statistical Analysis

GraphPad Prism 6 V6.01 (La Jolla, CA, USA) was used for statistical analyses. Differences between groups were compared by the *t*-test. Influences of miR-298 and JMJD6 on clinical data of osteosarcoma patients were analyzed Chi-square test. Kaplan-Meier survival curves were depicted, followed by log-rank test for comparing differences between curves. Data were expressed as mean ± standard deviation. $p<0.05$ was considered as statistically significant.

Results

MiR-298 Was Lowly Expressed in Osteosarcoma Tissues and Cell Lines

Firstly, we explored the expressions of miR-298 in osteosarcoma tissues and cell lines. Results demonstrated that miR-298 was both expressed in osteosarcoma and healthy bone tissues, which was lower in the former tissues (Figure 1A). Consistently, miR-298 was lowly expressed in osteosarcoma cell lines (Figure 1B). We subsequently analyzed the potential influence of miR-298 on pathology of osteosarcoma patients. A significant-

Table I. The clinicopathologic characteristics of the patients with osteosarcoma in high- miR-298 and low- miR-298 groups.

Index	Number of cases	High-miR-298 (n=26)	Low-miR-298 (n=24)	*p*-value
Age (years)				0.412
<15	22	10	12	
≥15	28	16	12	
Gender				0.426
Male	32	15	17	
Female	18	11	7	
Enneking stage				0.852
IA	4	2	2	
IIA	13	7	6	
IIB	25	13	12	
III	8	4	4	
Lymph node metastasis				0.011
No	28	19	9	
Yes	22	7	15	
Distance metastasis				0.025
No	29	19	10	
Yes	21	7	14	

ly higher rate of osteosarcoma patients expressing low level of miR-298 was detected in whom had lymphatic metastasis or distant metastasis (Table I). Meanwhile, lower level of miR-298 was examined in metastatic osteosarcoma cases in comparison to non-metastatic ones (Figure 1C). Kaplan-Meier survival curves were plotted for assessing the prognostic value of miR-298 in osteosarcoma.

MiR-298 Inhibited Proliferative and Migratory Abilities in Osteosarcoma

Prior to explore the biological function of miR-298 in osteosarcoma, we first successfully constructed miR-298 overexpression model in MG63 and U2OS cells by transfection of miR-298 mimic (Figure 2A). CCK-8 assay revealed a lower viability in osteosarcoma cells overexpressing miR-298 in comparison to controls (Figure 2B). In addition, both declined migratory cell number and percentage of wound closure by overexpression of miR-298 in osteosarcoma cells indicated the inhibited migratory ability (Figure 2C, 2D). All above findings suggested that miR-298 has an inhibitory effect on the proliferation and invasion of osteosarcoma.

Target Relationship Between MiR-298 and JMJD6

Using online databases, three candidates were searched as potential targets of miR-298. Among them, relative level of JMJD6 was the most affected one by overexpression of miR-298 in osteosarcoma cells (Figure 3A). Based on the binding sites in the 3'UTR of JMJD6 and miR-298, Dual-Luciferase reporter assay ascertained the binding relationship between them (Figure 3B). In 50 cases of osteosarcoma tissues, JMJD6 was highly expressed and negatively correlated to miR-298 level (Figure 3C). Identically, JMJD6 was upregulated in osteosarcoma cell lines (Figure 3D). Western blot analysis revealed that protein level of JMJD6 was markedly downregulated in MG63 and U2OS cells transfected with miR-298 mimic (Figure 3E). All above results demonstrated that JMJD6 was a target of miR-298.

Coregulation of MiR-298 and JMJD6 on Osteosarcoma

To further investigate the role of miR-298/JMJD6 in osteosarcoma, we used pcDNA-JMJD6 to transfect the osteosarcoma cells. We found that transfection of pcDNA-JMJD6 effectively upregulated protein level of JMJD6 in osteosarcoma cells overexpressing miR-298 (Figure 4A). Compared with those overexpressing miR-298, MG63 and U2OS cells co-overexpressing miR-298 and JMJD6 had higher viability and migratory cell number (Figure 4B, 4C). As a result, JMJD6 abolished the regulatory effect of miR-298 on osteosarcoma cell behaviors.

Discussion

Osteosarcoma is a malignant osteogenic tumor with a high degree of malignancy and poor progno-

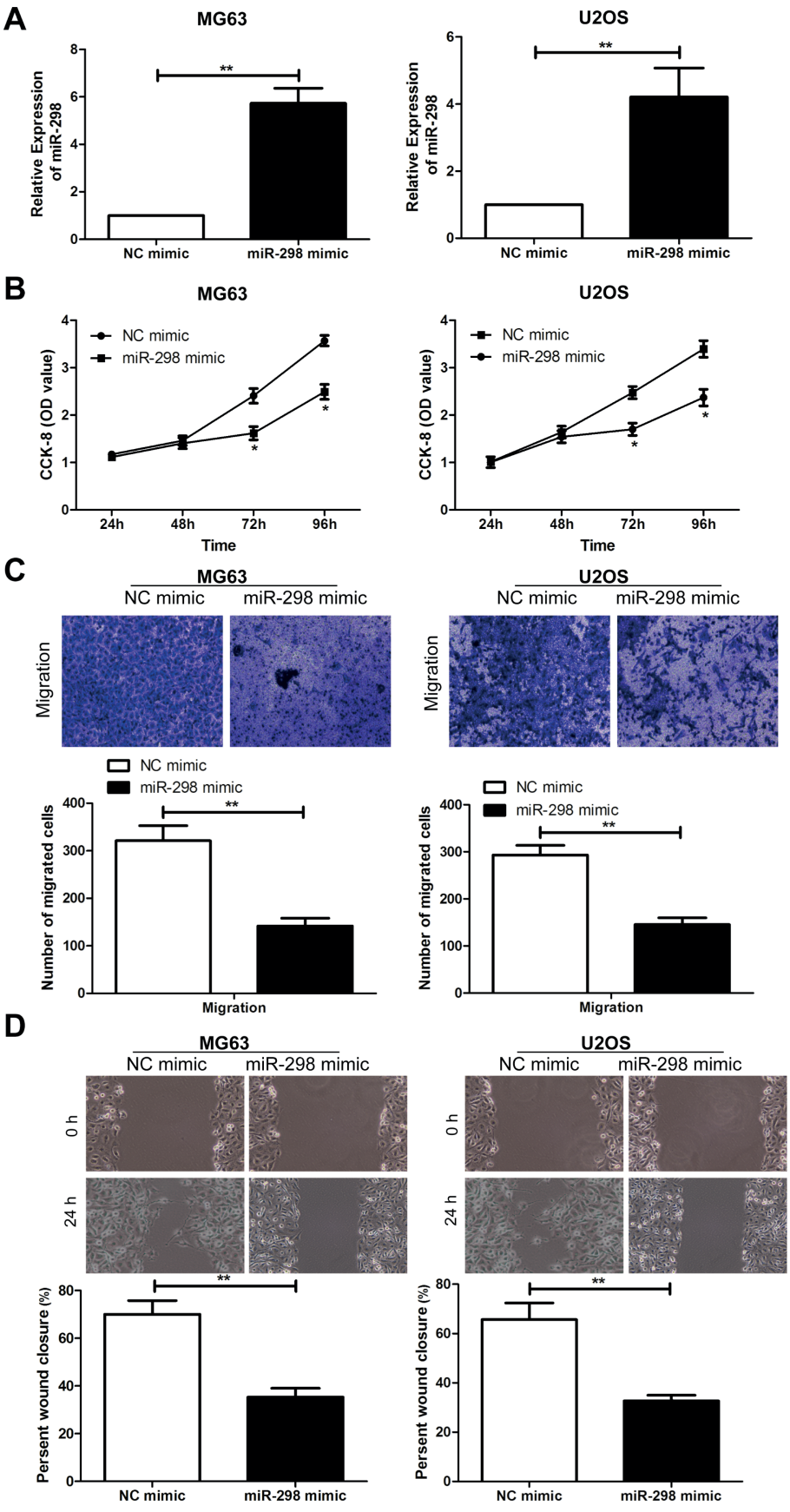

Figure 2. MiR-298 inhibited proliferative and migratory abilities in osteosarcoma. **A**, Transfection efficacy of miR-298 mimic in MG63 and U2OS cells. **B**, Viability in MG63 and U2OS cells overexpressing miR-298. **C**, Migration in MG63 and U2OS cells overexpressing miR-298. **D**, Wound closure in MG63 and U2OS cells overexpressing miR-298. *$p < 0.05$, **$p < 0.01$.

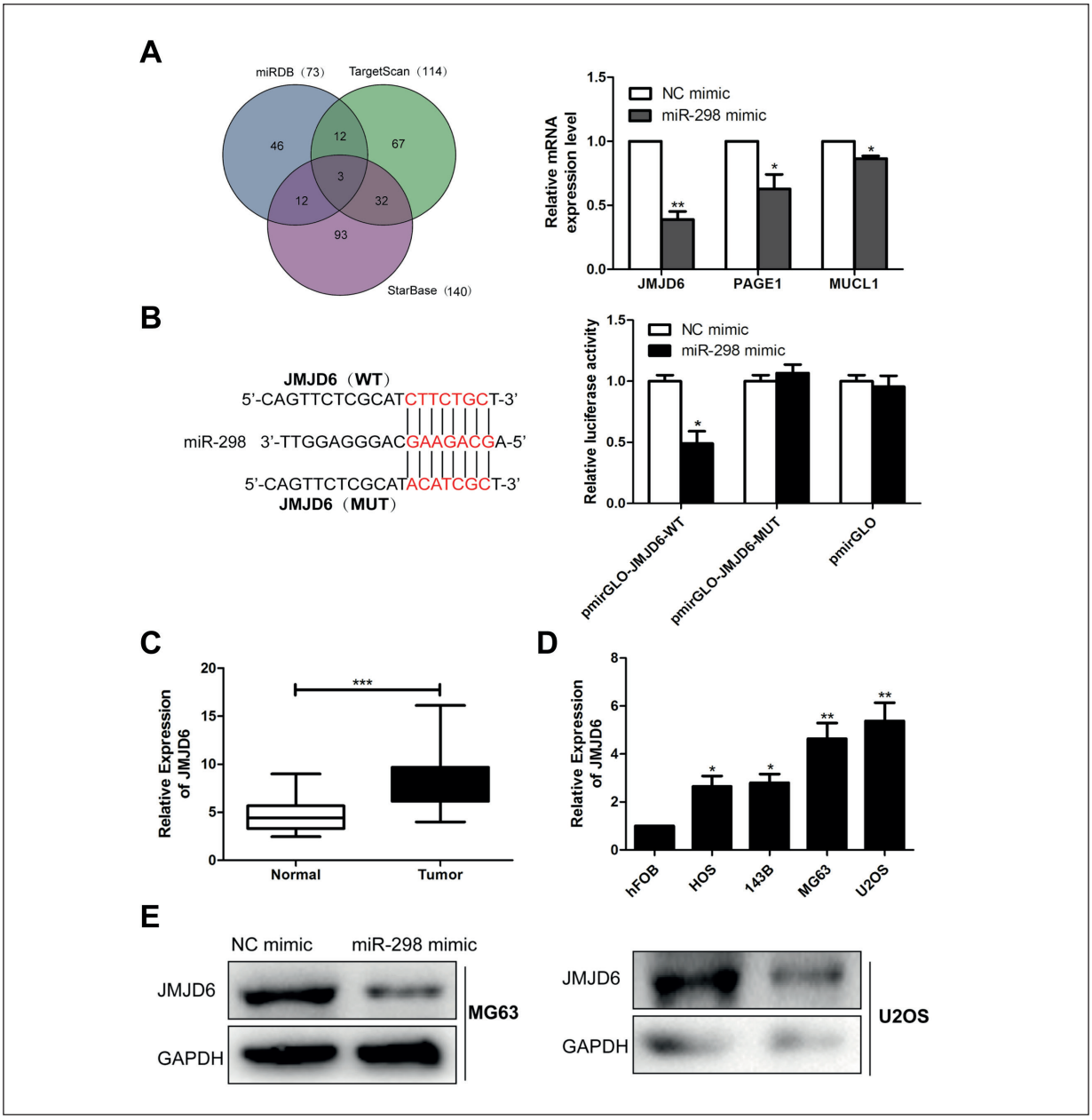

Figure 3. Target relationship between miR-298 and JMJD6. **A**, A Venn diagram depicted for candidate targets of miR-298 analyzed using miRDB, TargetScan and StarBase. Differential levels of JMJD6, PAGE1 and MUCL1 after overexpression of miR-298 were detected. **B**, A direct binding between miR-298 and JMJD6. **C**, Differential level of JMJD6 in osteosarcoma and healthy bone tissues. **D**, JMJD6 level in osteosarcoma cell lines. **E**, Protein level of JMJD6 in MG63 and U2OS cells overexpressing miR-298. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

sis. Most of patients would evolve metastasis or recurrence in the advanced stage of osteosarcoma[5,6]. Therefore, the management of osteosarcoma and its metastases, as well as improvement of prognosis and life quality of osteosarcoma patients are urgent tasks[8-10]. With the innovation of molecular biology techniques and the finish of human genome project, we have a deepening understanding of RNAs and their vital functions in biological activities[10,11]. Recently, miRNAs have been highlighted as their regulatory effects on tumor progression[10-13]. MiR-298 is a newly discovered miRNA involved in the maturation, differentiation and growth of cancer[18-20]. However, the association about miR-298 and osteosarcoma is unclear. In this study, the results showed that miR-298 was downregulated in

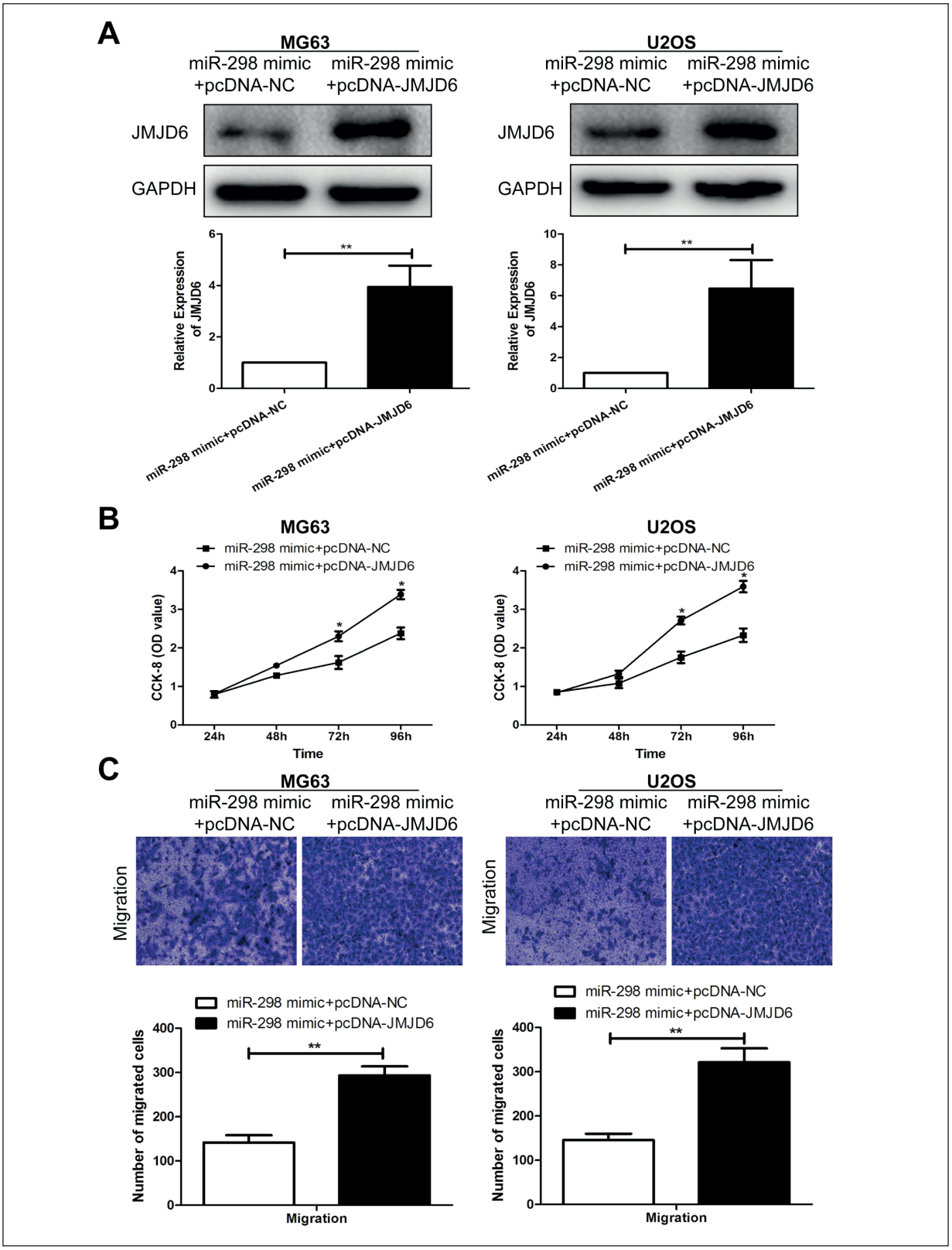

Figure 4. Coregulation of miR-298 and JMJD6 on osteosarcoma. **A**, Transfection efficacy of pcDNA-JMJD6 in MG63 and U2OS cells overexpressing miR-298. **B**, Viability in MG63 and U2OS cells overexpressing miR-298 or co-overexpressing miR-298 and JMJD6. **C**, Migration in MG63 and U2OS cells overexpressing miR-298 or co-overexpressing miR-298 and JMJD6 (magnification: 40×). *$p < 0.05$, **$p < 0.01$.

osteosarcoma tissues and cell lines. By analyzing clinical data of these patients, miR-298 was correlated to lymphatic metastasis, distant metastasis, overall survival and progression-free survival in osteosarcoma patients. Furthermore, overexpression of miR-298 decreased proliferative and migratory abilities in osteosarcoma cell lines. We thereafter suggested that miR-298 exerted an anti-cancer role in the development of osteosarcoma.

A single miRNA can simultaneously regulate multiple targets and pathways[16,17]. During the progression of osteosarcoma, miRNAs display either oncogenic role or anti-cancer role[21]. We have verified that JMJD6 was the target gene of miR-298 by bioinformatics analysis and luciferase reporter gene experiment. JMJD6 is one of the most popular demethylases recently studied, and it is also a member of the JmjC-domain-containing histone demethylases[22,23]. QRT-PCR data uncovered that JMJD6 was highly expressed in osteosarcoma tissues and cell lines. In addition, miR-298 was negatively correlated to JMJD6 in osteosarcoma tissues. As expected, protein level of JMJD6 in MG63 and U2OS cells overexpressing miR-298 was downregulated. Rescue experiments demonstrated the ability of JMJD6 to reverse the regulatory effects of miR-298 on osteosarcoma cell functions. To sum up, miR-298 was responsible for protecting the malignant progression of osteosarcoma by negatively regulating JMJD6, which confirmed the important effect of the miR-298/JMJD6 signal axis on the progression of osteosarcoma, and the regulatory network might be a new target for the diagnosis and treatment of osteosarcoma.

Conclusions

Summarily, miR-298 is less abundant in osteosarcoma samples, which is correlated to metastasis and prognosis of osteosarcoma. MiR-298, serving as a tumor suppressor, weakens proliferative and migratory abilities of osteosarcoma cells.

Conflict of Interests

The authors declare that they have no conflict of interest.

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