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Upregulated miRNA-1236-3p in osteosarcoma inhibits cell proliferation and induces apoptosis via targeting KLF8

Y. SUN[1], L. CAO[2], J.-T. LIN[1], Y. YUAN[3], Z.-L. CAO[4], J.-D. JIA[5]

[1]Trauma Department of Orthopedics, Yantaishan Hospital, Yantai, China.
[2]Department of Orthopedics, Jinan Zhangqiu District Hospital of TCM, Jinan, China.
[3]Department of Orthopedics, Binzhou Medical University, Binzhou, China.
[4]Department of Orthopedics, Yantaishan Hospital, Yantai, China.
[5]Department of Pathophysiology, Binzhou Medical University, Binzhou, China.

Abstract. – OBJECTIVE: To elucidate the regulatory effect of miRNA-1236-3p on the cellular behaviors of osteosarcoma (OS) cells, and to provide novel hallmarks and therapeutic targets for the diagnosis, treatment, and prognosis of OS.

PATIENTS AND METHODS: Relative level of miRNA-1236-3p in OS tissues and cell lines was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Regulatory effects of miRNA-1236-3p on the proliferative ability of HOS and U-2OS cells were evaluated by cell counting kit-8 (CCK-8) assay. Through flow cytometry, the potential influences of miRNA-1236-3p on cell cycle progression and apoptosis of OS cells were examined. Subsequently, the dual-luciferase reporter gene assay was conducted to explore the binding of KLF8 (Krüppel-like factor 8) to miRNA-1236-3p. Regulatory effects of KLF8 on cellular behaviors of OC cells were also evaluated.

RESULTS: MiRNA-1236-3p was downregulated in OS tissues relative to controls. Meanwhile, miRNA-1236-3p was lowly expressed in OS with worse TNM stage or distant metastasis. The overexpression of miRNA-1236-3p in HOS and U-2OS cells suppressed the proliferative ability, arrested the cell cycle in the G0/G1 phase and induced apoptosis. Conversely, miRNA-1236-3p knockdown obtained the opposite trends. KLF8 was verified to bind to miRNA-1236-3p, and its expression was negatively regulated by miRNA-1236-3p in OS cells. A series of functional experiments displayed the oncogenic role of KLF8 in OS.

CONCLUSIONS: MiRNA-1236-3p is downregulated in OS tissues and cell lines. The overexpression of miRNA-1236-3p suppresses the proliferative ability and induces apoptosis of OS cells via downregulating KLF8.

Key Words:
Osteosarcoma, MiRNA-1236-3p, Proliferation, Apoptosis, KLF8.

Introduction

Osteosarcoma (OS) is the most common primary bone malignancy, which mainly affects areas with rapid bone turnover and growth[1]. OS especially affects adolescent long bone metaphysis. The pathogenesis of OS is considered to be related to various factors, such as chemical substances, internal and external irradiation, chronic inflammatory irritation, and viral infection[2]. Currently, the preferred treatment regimen for OS is preoperative chemotherapy-surgery-postoperative chemotherapy, which markedly decreases the disability rate and prolongs the overall survival of OS patients. Nevertheless, some patients experience postoperative recurrence and secondary malignancies, leading to a poor prognosis[3]. Novel strategies and therapeutic targets are urgent to be searched for OS treatment[4].

MiRNAs are small, non-coding RNAs containing multiple nucleotides[5]. Although miRNAs are unable to encode proteins, they exert a vital regulation on gene expressions. MiRNAs are capable of inhibiting the translation of target genes through the incomplete base-paring[6]. Due to the special complementary binding, multiple targets may have a common miRNA, and meanwhile, several miRNAs could regulate a common target miRNA. The precise and complex miRNA regulatory network is of great significance in different stages of cellular behaviors[7]. Some studies[8,9] have identified the biological functions of miRNAs in OS. However, the specific mechanism of miRNAs in regulating the etiology of OS remains unclear.

MiRNA-1236-3p is reported[10-14] to be downregulated in many types of tumors, which is closely related to migratory and invasive abilities of OS

Corresponding Author: Yan Sun, MM; e-mail: khqrel@163.com

cells. Nevertheless, the effect of miRNA-1236-3p on mediating the progression of OS is rarely reported. This study determined the expression pattern of miRNA-1236-3p in OS and further explored its target gene KLF8 (Krüppel-like factor 8). Regulatory effects of miRNA-1236-3p and KLF8 on cellular behaviors of OS cells were evaluated.

Patients and Methods

Sample Collection

OS tissues and adjacent normal tissues were surgically resected from 30 OS patients who underwent surgery in Yantaishan Hospital from February 2016 to December 2017. None of the patients received preoperative treatment and they denied family history. Samples were labeled and immediately preserved in liquid nitrogen. Patients were volunteered to participate in this study, which was approved by the Ethics Committee of Yantaishan Hospital.

Cell Culture

Human-derived osteoblast cell line hFOB and OS cell lines (HOS, U-2OS, MG-63, and Saos-2) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 2.5 mmol/L L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 20% fetal bovine serum (FBS) (Hyclone, South Logan, UT, USA) in a 5% CO_2 and 37°C incubator.

Cell Transfection

Cells in good viability were seeded in the 6-well plate applied with 1.5 ml of serum-free medium and 500 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) containing transfection vectors per well. Fresh medium was replaced 4-6 h later. MiRNA-1236-3p mimics, inhibitor and negative control were constructed by GenePharma (Shanghai, China); pcDNA-KLF8, KLF8 siRNA-1, and KLF8 siRNA-2 were constructed by GeneChem (Shanghai, China). Transfection efficacy was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RNA Extraction and qRT-PCR

Tissues or cells were lysed in TRIzol (Invitrogen, Carlsbad, CA, USA). The extracted RNA was quantified using NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA) and subjected to reverse transcription for obtaining complementary deoxyribose nucleic acid (cDNA) using the PrimeScript TM RT reagent kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was conducted using SYBR® Green Master Mix and examined on ABI7900. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were served as internal references. The relative level of the target gene was calculated using $2^{-\Delta\Delta CT}$. Primer sequences were displayed in Table I.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in the 96-well plate with 5×10^5 cells/mL per well. Absorbance (OD) at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

Cell Cycle Determination

Cell density was adjusted to 5×10^4 cells/mL and fixed in pre-cold 75% ethanol overnight. Before cell cycle determination, cells were washed with

Table I. The sequences related to the study.

Gene	Primer sequence
miR-1236-3p F:	GGGCCTCTTCCCCTTGTCT
miR-1236-3p R:	TATGGTTGTTCTGCTCTCTGTCTC
KLF8 F:	GCTCACCGCAGAATCCATACA
KLF8 R:	GTGCACCGAAAAGGCTTGAT
GAPDH F:	AGCCACATCGCTCAGACAC
GAPDH R:	GCCCAATACGACCAAATCC
U6 F:	CTCGCTTCGGCAGCAGCACATATA
U6 R:	AAATATGGAACGCTTCACGA

phosphate-buffered saline (PBS) twice, incubated with 100 μL of RNaseA in a 37°C water bath in the dark for 30 min, and then, incubated with 400 μL of Propidium Iodide (PI) at 4°C in the dark for 30 min. Flow cytometry was used for determining the absorbance at 488 nm.

Apoptosis Determination

Cell density was adjusted to 5×10^5 cells/mL and fixed in pre-cold 70% ethanol for 30 min. Subsequently, cells were incubated in 5 mL of Annexin V-FITC (fluorescein isothiocyanate) and 1 mL of PI (50 mg/mL) and subjected to apoptosis determination. Apoptotic rate of cells was finally calculated.

Dual-Luciferase Reporter Gene Assay

Wild-type (WT) and mutant-type (MUT) KLF8 luciferase vectors were constructed based on the predicted binding sequences between miRNA-1236-3p and KLF8 on TargetScan. Luciferase activity was examined using a standard protocol.

Western Blot

Total protein from cells was extracted using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and quantified by bicinchoninic acid (BCA) method (Abcam, Cambridge, MA, USA). 50 μg protein sample was loaded for electrophoresis. After transferring on a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland), it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by electrochemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Measurement data were analyzed by the *t*-test, and categorical data were analyzed using the chi-square test. $p<0.05$ was considered statistically significant. GraphPad Prism 8 (La Jolla, CA, USA) was used in this study for depicting figures.

Results

MiRNA-1236-3p Was Downregulated in OS

The expression level of miRNA-1236-3p in OS tissues and adjacent normal tissues was determined by qRT-PCR. The data showed a lower level of miRNA-1236-3p in OS tissues relative to controls (Figure 1A). Clinical data of enrolled OS patients were collected for correlation analysis. It is observed that miRNA-1236-3p expression remained lower in OS tissues with worse TNM stage or distant metastasis (Table II). Identically, miRNA-1236-3p was lowly expressed in OS cell lines relative to the hFOB cell line (Figure 1B). Here, HOS and U-2OS cell lines were chosen for subsequent experiments.

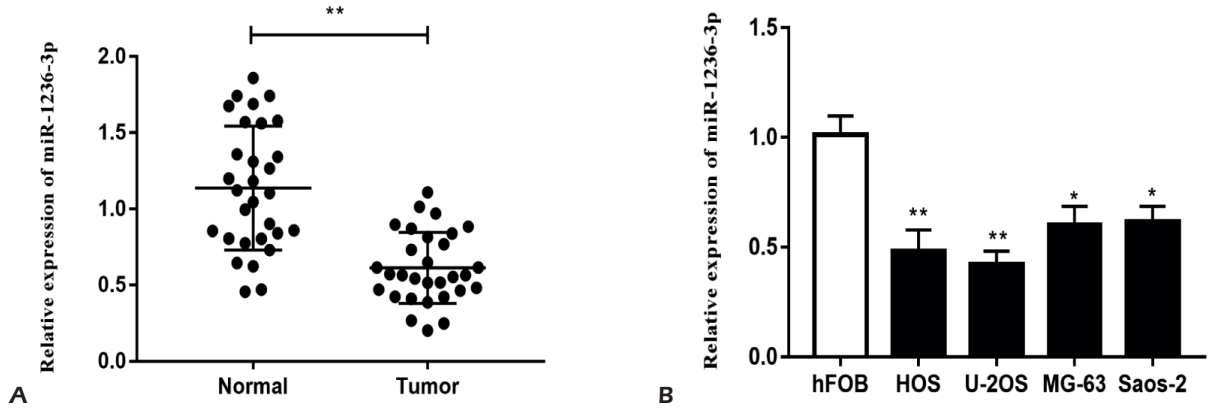

Figure 1. MiR-1236-3p was downregulated in OS. ***A,*** Expression level of miR-1236-3p was lower in OS tissues relative to adjacent normal tissues determined by qRT-PCR. ***B,*** Expression level of miR-1236-3p was lower in OS cell lines relative to hFOB cell line determined by qRT-PCR. *$p<0.05$, **$p<0.01$.

MiRNA-1236-3p Suppressed Proliferative Ability and Induced Apoptosis of OS Cells

MiRNA-1236-3p was lowly expressed in OS tissues and cell lines, suggesting its crucial role in the progression of OS. We first constructed miRNA-1236-3p mimics and inhibitor, and tested their transfection efficacy in OS cells (Figure 2A, 2B). CCK-8 assay revealed the increased viability in OS cells overexpressing miRNA-1236-3p, whereas those with miRNA-1236-3p knockdown presented decreased viability (Figure 2C). After transfection of miRNA-1236-3p mimics, OS cells were mainly arrested in G0/G1 phase; and miRNA-1236-3p knockdown accelerated cell cycle progression into S phase (Figure 2D). Moreover, the apoptotic rate markedly elevated in OS cells overexpressing miRNA-1236-3p (Figure 2E).

MiRNA-1236-3p Targeted KLF8

The binding sequences between miRNA-1236-3p and KLF8 were predicted through online bioinformatics website (Figure 3A). OS cells were co-transfected with KLF8 WT/KLF8 MUT and miRNA-1236-3p mimics/NC, respectively. As the data revealed, luciferase activity markedly decreased in cells co-transfected with KLF8/WT and miRNA-1236-3p mimics relative to those co-transfected with KLF8 WT and NC, suggesting the binding between KLF8 and miRNA-1236-3p (Figure 3B). By detecting KLF8 level in OS tissues, it was upregulated in OS tissues than those of controls (Figure 3C). KLF8 level was identically upregulated in OS cell lines (Figure 3D). Moreover, transfection of miRNA-1236-3p mimics inhibited KLF8 level, and conversely, miRNA-1236-3p knockdown upregulated KLF8 at both mRNA and protein levels (Figure 3E-3H). It is demonstrated that KLF8 expression was negatively regulated by miRNA-1236-3p in OS cells.

KLF8 Overexpression Accelerated Proliferative Ability and Suppressed Apoptosis of OS Cells

Transfection efficacy of pcDNA-KLF8 and KLF8 siRNAs (KLF8 siRNA-1, KLF8 siRNA-2) in OS cells was verified by qRT-PCR (Figure 4A, 4B). Overexpression of KLF8 markedly increased the viability, and knockdown of KLF8 achieved the opposite trend (Figure 4C). As flow cytometry revealed, OS cells were arrested in the G0/G1 phase by transfection of pcDNA-KLF8, demonstrating the arrested cell cycle progression (Figure 4D). Meanwhile, KLF8 overexpression reduced the apoptotic rate in HOS and U-2OS cells (Figure 4E). Converse

Table II. Correlation of miR-1236-3p expression with clinicopathological feature of osteosarcoma.

		Expression level		
Clinical parameters	**Cases (n)**	**miR-1236-3p high**	**miR-1236-3p low**	***p*-value**
Ages (years)				
<20 years	19	11	8	0.078
≥20 years	11	4	7	
Gender				
Male	16	10	6	0.23
Female	14	7	7	
Tumor size (cm)				
<5	13	8	5	0.015
≥5	17	7	10	
Anatomical location				
Tibia/femur	20	10	10	0.236
Elsewhere	10	4	6	
TNM stage				
I+II	13	9	4	<0.001
III+IV	17	5	12	
Distant metastasis				
Yes	18	6	12	<0.001
No	12	8	4	

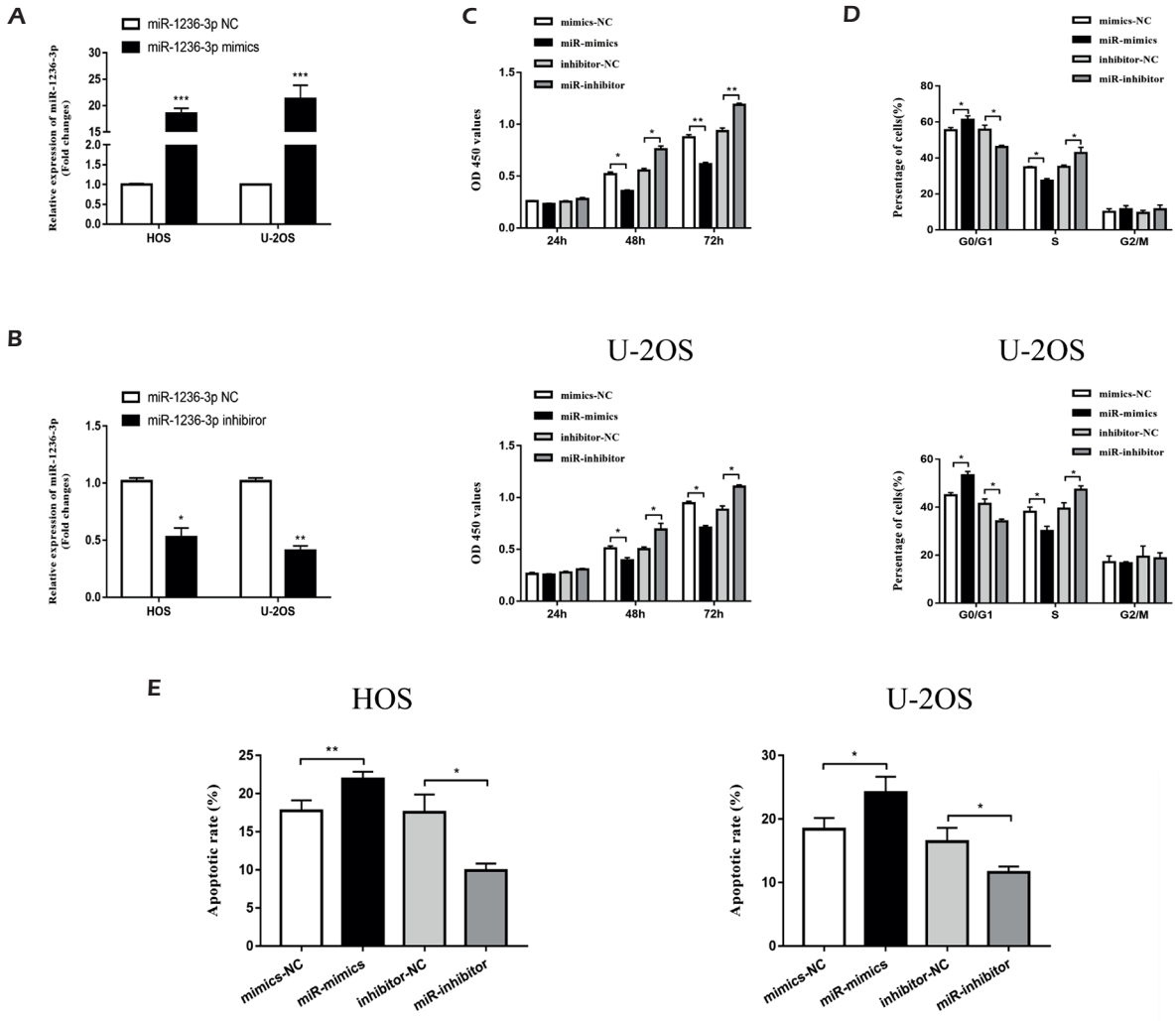

Figure 2. MiR-1236-3p suppressed proliferative ability and induced apoptosis of OS cells. ***A, B,*** Transfection efficacy of miR-1236-3p mimics and inhibitor in HOS and U-2OS cells determined by qRT-PCR. ***C,*** CCK-8 assay showed viability in HOS and U-2OS cells transfected with mimics-NC, miR-1236-3p mimics, inhibitor-NC or miR-1236-3p inhibitor, respectively. ***D,*** Flow cytometry showed cell cycle distribution in HOS and U-2OS cells transfected with mimics-NC, miR-1236-3p mimics, inhibitor-NC or miR-1236-3p inhibitor, respectively. ***E,*** Flow cytometry showed apoptotic rate in HOS and U-2OS cells transfected with mimics-NC, miR-1236-3p mimics, inhibitor-NC or miR-1236-3p inhibitor, respectively. *p<0.05, **p<0.01, ***p<0.001.

to the biological function of miRNA-1236-3p in OS, KLF8 accelerated proliferative ability and suppressed apoptosis of OS cells.

Discussion

MiRNA is a key regulator in tumor progression. There are several miRNAs that have been identified to influence multiple aspects of OS cells, serving as oncogenes or tumor-progression tumors[15,16]. These miRNAs exert diagnostic, therapeutic, and prognostic potentials for tumors. For instance, downregulated miR-30c is correlated with high incidence, high invasive rate, and poor prognosis of OS[17]. Overexpression of miR-30c could suppress the invasive ability of OS. MiR-199b-5p promotes malignant progression of osteosarcoma by regulating HER2[18]. MiR-423-5p inhibits proliferative and migratory abilities of OS cells by directly targeting STMN1[19]. MiRNA-17 accelerates the proliferative rate and inhibits apoptosis of OS cells through mediating SASH1[20].

Our research group previously screened differentially expressed miRNAs in OS, and the

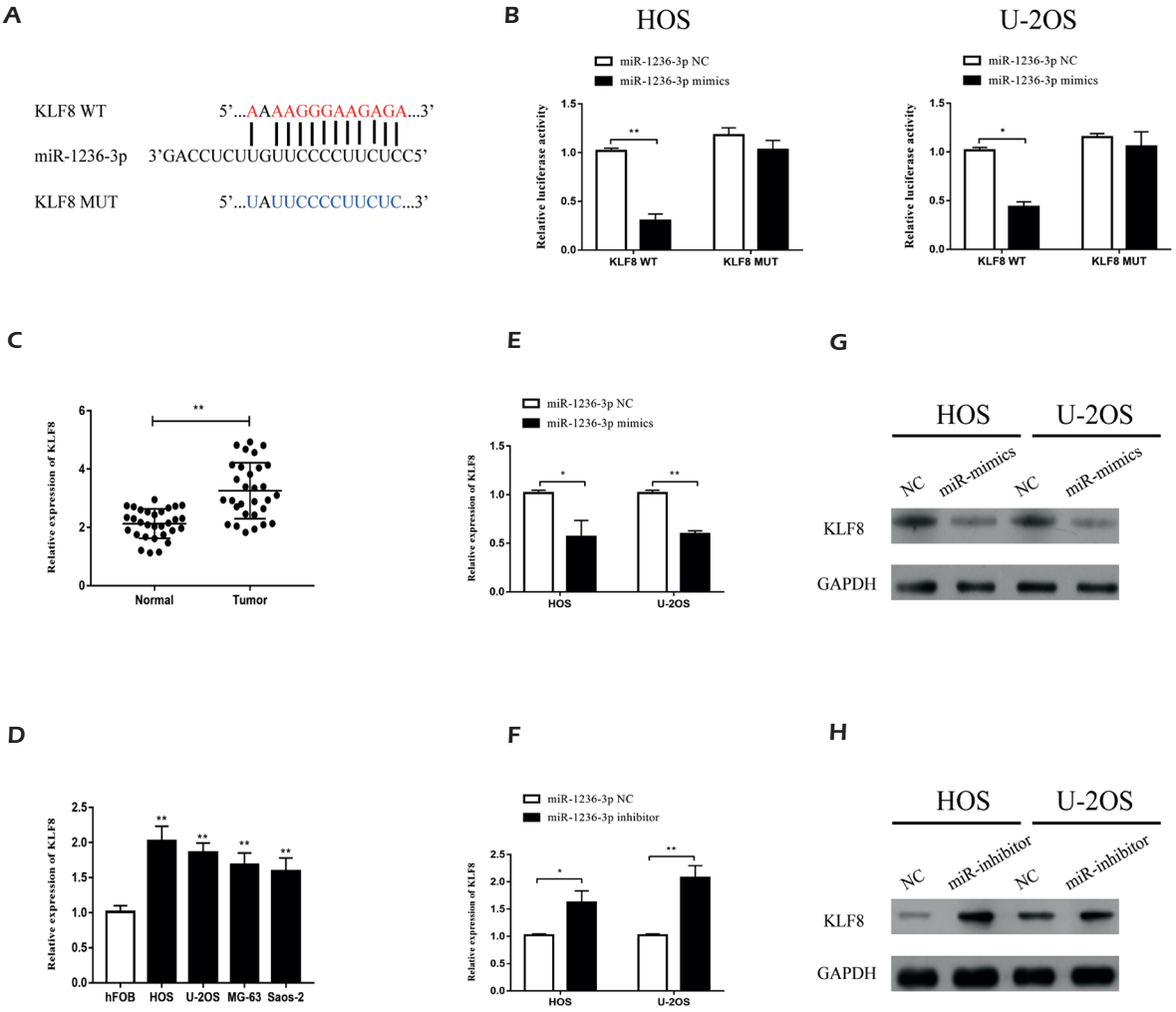

Figure 3. MiR-1236-3p targeted KLF8. ***A,*** The binding sequences between miR-1236-3p and KLF8 predicted through online bioinformatics website. ***B,*** OS cells were co-transfected with KLF8 WT/KLF8 MUT and miR-1236-3p mimics/NC, respectively. Luciferase activity markedly decreased in cells co-transfected with KLF8/WT and miR-1236-3p mimics relative to those co-transfected with KLF8 WT and NC. ***C,*** Expression level of KLF8 was higher in OS tissues relative to adjacent normal tissues determined by qRT-PCR. ***D,*** Expression level of KLF8 was higher in OS cell lines relative to hFOB cell line determined by qRT-PCR. ***E,*** Relative level of KLF8 in HOS and U-2OS cells transfected with NC or miR-1236-3p mimics determined by qRT-PCR. ***F,*** Relative level of KLF8 in HOS and U-2OS cells transfected with NC or miR-1236-3p inhibitor determined by qRT-PCR. ***G,*** Protein level of KLF8 in HOS and U-2OS cells transfected with NC or miR-1236-3p mimics determined by Western blot. ***H,*** Protein level of KLF8 in HOS and U-2OS cells transfected with NC or miR-1236-3p inhibitor determined by Western blot. **p*<0.05, ***p*<0.01.

downregulated miRNA-1236-3p has attracted our attention. So far, the role of miRNA-1236-3p in OS has not been reported yet. In high-grade serous ovarian cancer, miRNA-1236-3p inhibits tumor cells to migrate and invade by targeting ZEB1. MiRNA-1236-3p suppresses invasive and metastatic rates of gastric cancer cells *via* targeting MTA2. Through binding to KLF8, miRNA-1236-3p inhibits A549 cells to migrate and invade. Based on the relevant reports, we speculated that miRNA-1236-3p may serve as a tumor-suppressor gene in OS. Firstly, miRNA-1236-3p was found to be downregulated in OS tissues and cell lines. Cellular experiments illustrated that overexpression of miRNA-1236-3p in HOS and U-2OS cells suppressed proliferative ability, arrested cell cycle in G0/G1 phase, and induced apoptosis. It is suggested that miRNA-

1236-3p exerted a tumor-inhibiting effect on OS.

We next explored the biological function of miRNA-1236-3p in mediating the progression of OC. Through dual-luciferase reporter gene assay, it is found that miRNA-1236-3p could directly bind to the 3'UTR of KLF8 mRNA. Furthermore, both mRNA and protein levels of KLF8 were negatively regulated by miRNA-1236-3p.

KLF8 (Krüppel-like factor 8) is a member of the first subgroup of the KLF transcription factor family. Members in the KLF family have three highly conserved zinc finger protein structures at the N-terminus, where specifically binds to promoter regions enriched with GC[21]. KLF transcription factors exert a vital regulatory effect on physical development and pathological progressions. KLF8 is reported to be upregulated in multiple types of tumors[22]. KLF8

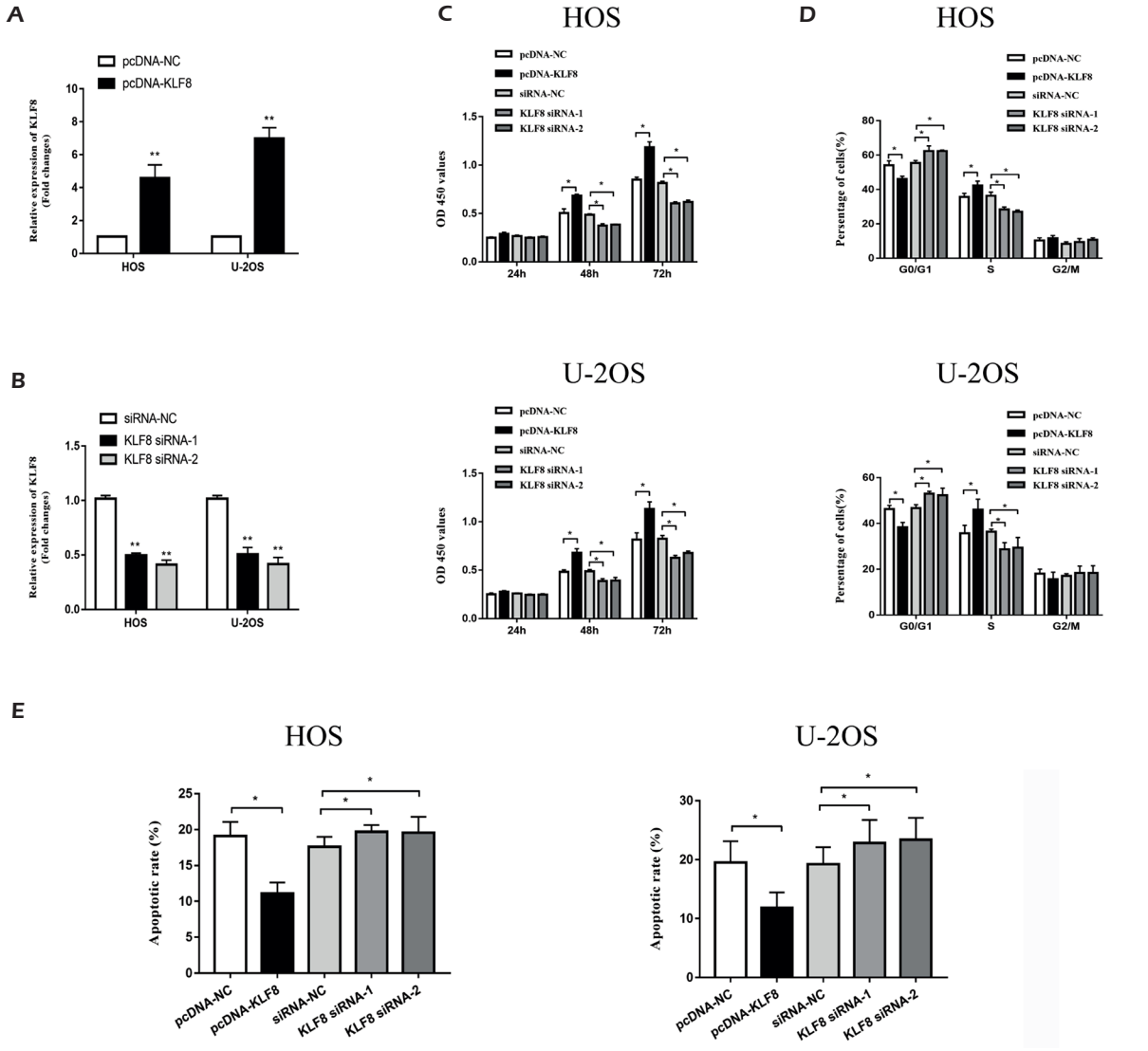

Figure 4. KLF8 overexpression accelerated proliferative ability and suppressed apoptosis of OS cells. ***A,*** Transfection efficacy of pcDNA-NC or pcDNA-KLF8 in HOS and U-2OS cells determined by qRT-PCR. ***B,*** Transfection efficacy of siRNA-NC, KLF8 siRNA-1, or KLF8 siRNA-2 in HOS and U-2OS cells determined by qRT-PCR. ***C,*** CCK-8 assay showed viability in HOS and U-2OS cells transfected with pcDNA-NC, pcDNA-KLF8, siRNA-NC, KLF8 siRNA-1, or KLF8 siRNA-2, respectively. ***D,*** Flow cytometry showed cell cycle distribution in HOS and U-2OS cells transfected with pcDNA-NC, pcDNA-KLF8, siRNA-NC, KLF8 siRNA-1, or KLF8 siRNA-2, respectively. ***E,*** Flow cytometry showed apoptotic rate in HOS and U-2OS cells transfected with pcDNA-NC, pcDNA-KLF8, siRNA-NC, KLF8 siRNA-1, or KLF8 siRNA-2, respectively. *$p<0.05$, **$p<0.01$.

is capable of mediating cell cycle progression, oncogenic transformation, and EMT[23,24]. The specific function of KLF8 in tumor progression has been elucidated, and its role in OS, however, remains unclear. In this work, KLF8 was highly expressed in OS, which was able to accelerate the proliferative rate and inhibit apoptosis of OS cells.

Conclusions

We found that MiRNA-1236-3p is downregulated in OS. Overexpression of miRNA-1236-3p suppresses proliferative ability and induces apoptosis of OS cells *via* downregulating KLF8. MiRNA-1236-3p may be utilized as a novel therapeutic target for OS.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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