miR-4295 promotes cell proliferation, migration and invasion of osteosarcoma through targeting interferon regulatory factor 1

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Received March 30, 2019; Accepted July 23, 2020

DOI: 10.3892/ol.2020.12123

Abstract. Osteosarcoma (OS) is the most common form of primary malignant bone tumor. Despite encouraging progress in the treatment of OS, the survival rate for patients with OS has remained unchanged over the past 40 years. It has been established that miRNA plays a crucial regulatory role in the progression and development of OS. To explore the potential association of miRNAs with OS, bioinformatics techniques were used to screen for differentially expressed miRNA genes in OS in the Gene Expression Omnibus database. In the GSE70367 database, it was revealed that miR-4295 expression was abnormally elevated in the expression of OS cells. To characterize the potential function of miR-4295 in OS, the expression levels of miR-4295 in 30 samples of OS and adjacent normal tissues was examined. The results revealed that the expression of miR-4295 was significantly increased in OS tissues compared with the paired normal tissues. Moreover, the expression levels of miR-4295 in OS cell lines (MG-63 and Saos-2) were significantly higher compared with those in the normal human mesenchymal stem cells. In addition, miR-4295 was associated with OS cell proliferation, migration and invasion. Furthermore, it was demonstrated that the expression of interferon regulatory factor (IRF)1, a tumor suppressor, was regulated by miR-4295 directly in OS cells. Taken together, the present results revealed that miR-4295 may act as a tumor activator by targeting IRF1 during the progression of OS. Investigating miR-4295 may provide novel insight into the mechanisms of OS metastasis, and inhibition and targeting miR-4295 may be a novel therapeutic strategy for the treatment of OS.

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Key words: miR-4295, osteosarcoma, interferon regulatory factor 1, proliferation, migration, invasion

Introduction

Osteosarcoma (OS) is the most frequent form of primary malignant bone tumor and it mainly affects children, adolescents or young adults (1). The peak incidence of the disease is in patients aged 15-25 years old, and is more commonly observed in males compared with females (1). OS exhibits a high degree of malignancy and tends to metastasize early, with a clinical metastasis rate of ~20%, with lung metastases being the most common (2-4). The course of OS progresses rapidly, which is life threatening and has a high mortality rate (5). The prognosis for OS distant metastasis cases remains poor (5). The mechanisms underlying the pathogenesis of OS remains unclear. Studies have reported that a variety of oncogenes and tumor suppressor genes have significant contributions in the development of OS, but the specific molecular mechanisms remain unclear (6-9). Therefore, a better understanding of the mechanisms underpinning the development of OS may provide an important theoretical basis for the clinical treatment of OS.

MicroRNAs (miRNAs/miRs), small non-coding RNAs of 20-25 nucleotides in length, were first discovered by Lee *et al* in 1993 in *Caenorhabditis elegans*. Gene expression can be regulated by miRNAs after transcription, regulating protein abundance by promoting mRNA degradation or translational inhibition, thereby acting as an oncogene or tumor suppressor (10,11). Studies have revealed that miRNAs play an important role in various biological processes, such as cell differentiation, proliferation, apoptosis, migration, metabolism and defense (12-14). In OS, a variety of abnormally expressed miRNAs had been identified, which are involved in biological processes, including invasion and metastasis, and affect the malignant biological behavior of OS cells by regulating different target genes, such as AKT, TRAF3 and p57 (15,16). However, it is still unclear which specific miRNAs specifically regulate the development and progression of OS.

Recently, several studies have shown that miR-4295 may act as an oncogene and a potential biomarker for cancer diagnosis and treatment (17,18). It was reported that miR-4295 can inhibit the G_0/G_1 arrest and apoptosis of glioma cells and promote cell proliferation and cell activity in glioma cells (19). High expression of miR-4295 may contribute to proliferation and invasion of pancreatic ductal adenocarcinoma (20). Taken together, it was hypothesized that miR-4295 may serve an important role in the development of OS.

Interferon regulatory factor 1 (IRF1) is a member of the interferon family (IRFs) and widely expressed in various tissues (21). IRFs are a family of transcription factors that regulate interferon expression, antivirus and antitumor activity. The *IRF1* gene has been studied in multiple species (22) and IRF1 selectively modulates different sets of genes, such as p53, p21 and TRAIL, and contributed to the cellular immune response (23). Several malignant blood system diseases, such as acute leukemia and myelodysplastic syndrome, and a variety of cancer types, were accompanied by abnormal expression of the *IRF1* gene (22,24).

The present study aimed to explore the potential function of miR-4295 in OS and examine the influence of the miR-4295 target genes.

Materials and methods

Tissue specimens and lentivirus All tissue specimens obtained after surgery were immediately frozen using liquid nitrogen and then stored at -80˚C until use. No patients received any treatment, including chemotherapy or radiation therapy, before surgery. A total of 15 tumor tissues and adjacent normal tissues (>3 cm away from the tumor tissues) were collected from 10 men and 5 women (age range, 10-65 years; median age, 32 years). Amputation was performed in 9 cases whereas local resection of mass was performed in 6 cases. This study was based on the latest revised principles outlined in the Helsinki Declaration. The acquisition and analysis of the OS cancer specimens was approved by the Ethics Committee of Xiangyang No. 1 People's Hospital and the patients provided written informed consent. Lentivirus used in the present study was provided by Shanghai GenePharma, Co., Ltd. LV-vector lentivirus was the negative control. LV-IRF1 corresponds to the IRF1 expression group.

Cell culture. Human OS cell lines (MG-63 and Saos-2) and normal human mesenchymal stem cells (hMSC) were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) and incubated at 37˚C with 5% CO_2.

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from OS cells using TRIzol® (Thermo Fisher Scientifi, Inc.) and was reverse transcribed into cDNA using the 5xPrimeScript RT Master mix (Takara Bio, Inc.) according to the manufacturer's protocols. qPCR was performed using the 2xT5 Fast qPCR mix (SYBR Green) (TsingKe Biological Technology) with a LightCycler® 96 Real-Time qPCR Detection system (Roche Diagnostics). RT-qPCR reactions were performed as follows: 40 cycles, denaturation, 94˚C for 15 sec; annealing, extension at 62˚C for 30 sec. GAPDH or U6 was used as a reference gene. The relative mRNA and miRNA expressions were calculated using the $2^{-\Delta\Delta Cq}$ method (25). The primers used were as follows: GAPDH, Forward: 5'-ATCACCATCTTCCAGGAGGGA-3' and reverse: 5'-CCTTCTCCATGGTGGTGAAGAC-3'; U6, forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'; IRF1, forward: 5'-CTGTGCGAGTGTACCGGATG-3' and reverse: 5'-ATCCCCACATGACTTCCTCTT-3'; miR-4295 RT: 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAAGGAA-3'; PCR, forward: 5'-GGGCAGUGCAAUGUU-3' and reverse: 5'-CAGTGCGTGTCGTGGAGT-3'. All results were derived from three independent amplifications.

Cell transfection. Cells ($3x10^5$) were seeded in six-well dishes, and transfected transiently with 200 pmol miR-4295 mimic/inhibitor/mimic non-targeting, scrambled/inhibitor non-targeting, scrambled (NC; Shanghai GenePharma, Co., Ltd.) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. During transfection, DMEM without FBS was used. Cells were harvested after 48 h post transfection for RT-qPCR and the dual luciferase assay.

Cell proliferation assay. Cell proliferation was determined using a Cell Counting Kit (CCK)-8 assay (Dojindo Molecular Technologies, Inc.) in accordance with the manufacturer's protocol. To conduct this experiment, $5x10^3$ cells were seeded in 96-well dishes and cultured in 100 μl DMEM medium at 37˚C. After 12 h, 10 μl CCK-8 solution was added to each well and incubated for 1 h at 37˚C. Absorbance was then measured with a multifunctional microplate reader at 450 nm. The assays were independently repeated ≥3 times.

Cell migration and invasion assay. The migration and invasion ability of MG-63 and Saos-2 cells were evaluated using a Transwell chamber (Corning, Inc.). In total, $1x10^4$ cells were seeded in the upper chamber without FBS. Then, 600 μl DMEM containing 50 ml/l FBS was added to the lower chamber and incubated for 24 h at 37˚C. The chamber was fixed with 4% paraformaldehyde at room temperature for 20 min, washed with PBS three times, and stained with 0.1% crystal violet (Beyotime Institute of Biotechnology) for 15 min at room temperature. The unmigrated cells were wiped off gently by using cotton swabs. The migration ability of the cells was determined by counting manually the number of transmembrane cells. At least three fields of view were randomly observed in each group using light microscopy (magnification, x100).

The invasion ability of the cells was assayed in a Transwell chamber precoated with Matrigel (Corning Inc.). In total, 50 μl of Matrigel collagen was diluted (Corning, Inc.) with 400 μl of FBS-free DMEM medium (ice operation). Then, 60 μl of the dilution was added to the upper chamber of the Transwell chamber and incubated for 4 h at 37˚C. The subsequent steps are the same as the migration assay. The invasive ability of the cells was determined by manually counting the number of transmembrane cells. At least three fields of view were randomly observed in each group by using Leica DMi8 microscope (Leica Microsystems, Inc.; magnification, x100).

Wound healing assay. The cells were plated in a 6-well plate one day in advance. A 20 μl pipette tip was used to create a scratch wound across the cells on the plate, and then the plate was washed twice with PBS to remove any floating cells. Low serum medium containing 2% FBS was replaced after scraping. Images of the cells migrated across the wounds were

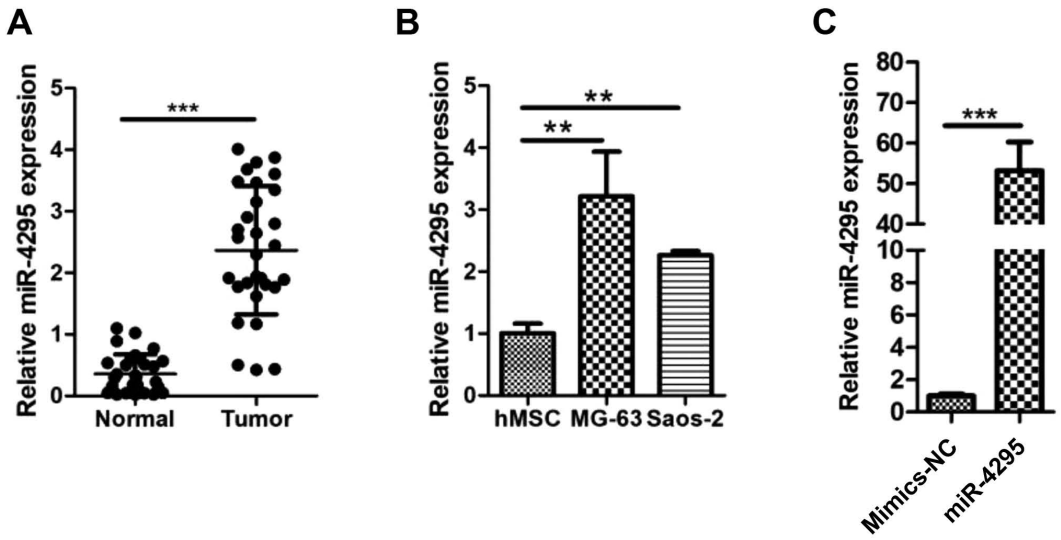

Figure 1. Expression levels of miR-4295 mRNA are significantly increased in OS tissues and cells. (A) Expression levels of miR-4295 in 30 samples of OS and adjacent normal tissues analyzed using reverse transcription PCR. Statistical analysis was evaluated using a paired Student's t-test. (B) Expression levels of miR-4295 in MG-63 and Saos-2 cell lines. Statistical analysis was evaluated using one-way analysis of variance and Tukey's post hoc test. (C) Expression levels of miR-4295 in the MG-63 cell line after transfection of miR-4295 mimics. Statistical analysis was evaluated using two-sided independent Student's t-test. **P<0.01, ***P<0.001. miR, microRNA; OS, osteosarcoma.

captured immediately and again at 24-h after scrapping using light microscopy (magnification, x100).

Dual luciferase assay. A dual luciferase assay was used to detect the direct binding of miR-4295 to the target gene *IRF1*. The binding sites of the IRF1 3' untranslated region (UTR) and miR-4295 were identified using the RNAhybrid database (http://alk.ibms.sinica.edu.tw/cgi-bin/RNAhybrid/RNAhybrid.cgi). Segmented 3'UTRs of the *IRF1* gene were amplified. After sequencing and blast validation, these fragments were inserted into the PMIRGLO (Addgene, Inc.) vector to construct recombinant plasmids. The firefly luciferase gene contained in the vector was used as a reference for normalization. A total of $5x10^4$ MG-63/Saos-2 cells per well were seeded into 12-well dishes and transfected with IRF1 luciferase reporter plasmids (100 ng) using Lipofectamine 2000. For overexpression and inhibition of miR-4295 experiments, 200 pmol mimics/inhibitor or miRNA NC were co-transfected along with plasmids, and luciferase activity was measured 48-h later. To measure the luciferase activity, cells were lysed in 1x lysis buffer provided with the Dual-Luciferase® Reporter Assay system (Promega Corporation) and luminescence was measured by adding luciferase assay reagent as per the manufacturer's protocol. Luciferase activity was measured using the Dual-Luciferase® Reporter Assay system (Promega Corporation). The assays were independently repeated ≥ three times.

Statistical analysis. Statistical analysis was performed using SPSS software version 13.0 (SPSS, Inc.). All experiments were performed in triplicate. The data shown are the mean ± SD. Multiple group comparisons were analyzed using one-way analysis of variance and Tukey's post hoc test, with virus infection treatment as the betweenvsubjects factor. Paired Student's t-test was used to analyze the significance of mRNA levels in OS and adjacent normal tissues. Unpaired Student's t-test was used to analyze the significance of cell proliferation assay and the Transwell migration/invasion assay. A paired t-test was used to analyze the relative miR-4295 expression levels between paired normal and tumor tissues. Correlation analysis was evaluated using Pearson's correlation. All comparisons were two-tailed. P<0.05 was considered to indicate a statistically significant difference.

Results

Levels of miR-4295 are significantly increased in OS tissues and cells. To investigate the potential function of miR-4295 in OS, the expression levels of miR-4295 in 30 samples of OS and adjacent normal tissues were detected using RT-qPCR. As shown in the Fig. 1A, miR-4295 expression was significantly increased in OS tissues compared with the paired normal tissues (P<0.01). Moreover, the expression levels of miR-4295 in OS cell lines (MG-63 and Saos-2) were significantly higher compared with those in the normal hMSC cells (Fig. 1B). The data indicated that miR-4295 expression may be associated with OS progression. As shown in the Fig. 1C, miR-4295 expression was significantly increased after transfection with miR-4295 mimics compared with the NC.

miR-4295 promotes OS cell proliferation. To explore the effects of miR-4295 on OS cells *in vitro*, miR-4295 mimics/mimics-NC, miR-4295 inhibitor/inhibitor-NC were transfected into MG-63 and Saos-2 cells. Upregulated miR-4295 expression significantly increased the proliferation ability of OS cells. In contrast, miR-4295-knockdown decreased MG-63 and Saos-2 cells proliferation ability (Fig. 2A and B, respectively). These data showed that miR-4295 promotes OS cell proliferation.

miR-4295 promotes the migration and invasion of OS cells. A Transwell and wound-healing assay were carried out to

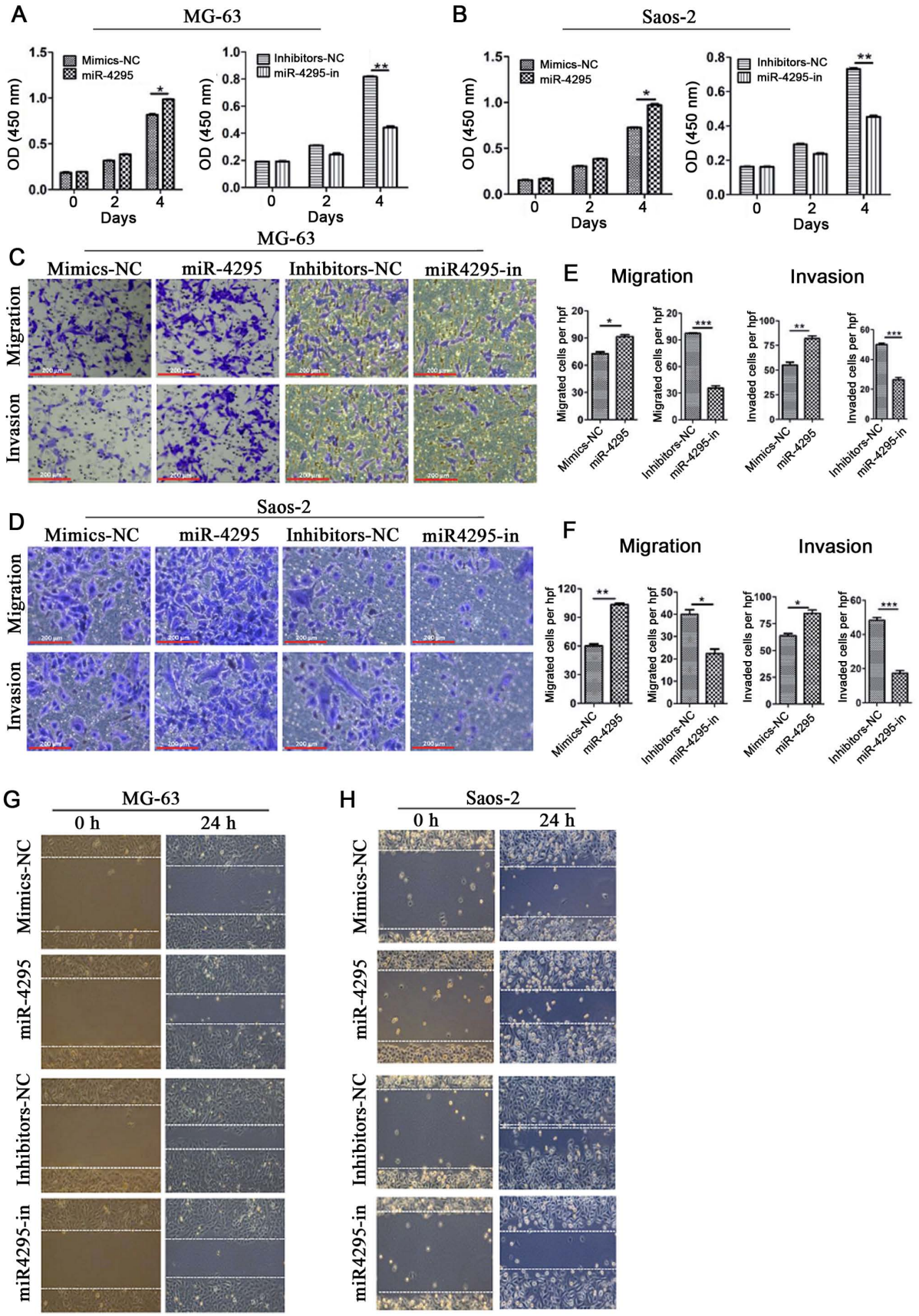

Figure 2. Effects of miR-4295 on the proliferation, migration and invasion of OS cells. (A and B) Cell Counting Kit-8 assay of MG-63 and Saos-2 cells after transfection with miR-4295 mimics/mimics-NC and miR-4295 inhibitor/inhibitor-NC. Statistical analysis was evaluated using two-sided independent Student's t-tests. (C and D) Migration and invasion of MG-63 and Saos-2 cells after transfection with miR-4295 mimics/mimics-NC and miR-4295 inhibitor/inhibitor-NC. (E and F) Three fields of the indicated cells were evaluated. Means + SD are shown from three independent experiments performed in triplicate Statistical analysis was performed using two-sided independent Student's t-tests. (G and H) The wound-healing assay of MG-63 and Saos-2 cells after transfected with miR-4295 mimics/mimics-NC, miR-4295 inhibitor/inhibitor-NC, respectively. Scale bar, 200 μm. *P<0.05, **P<0.01, ***P<0.001. miR, microRNA; OS, osteosarcoma; NC, negative control.

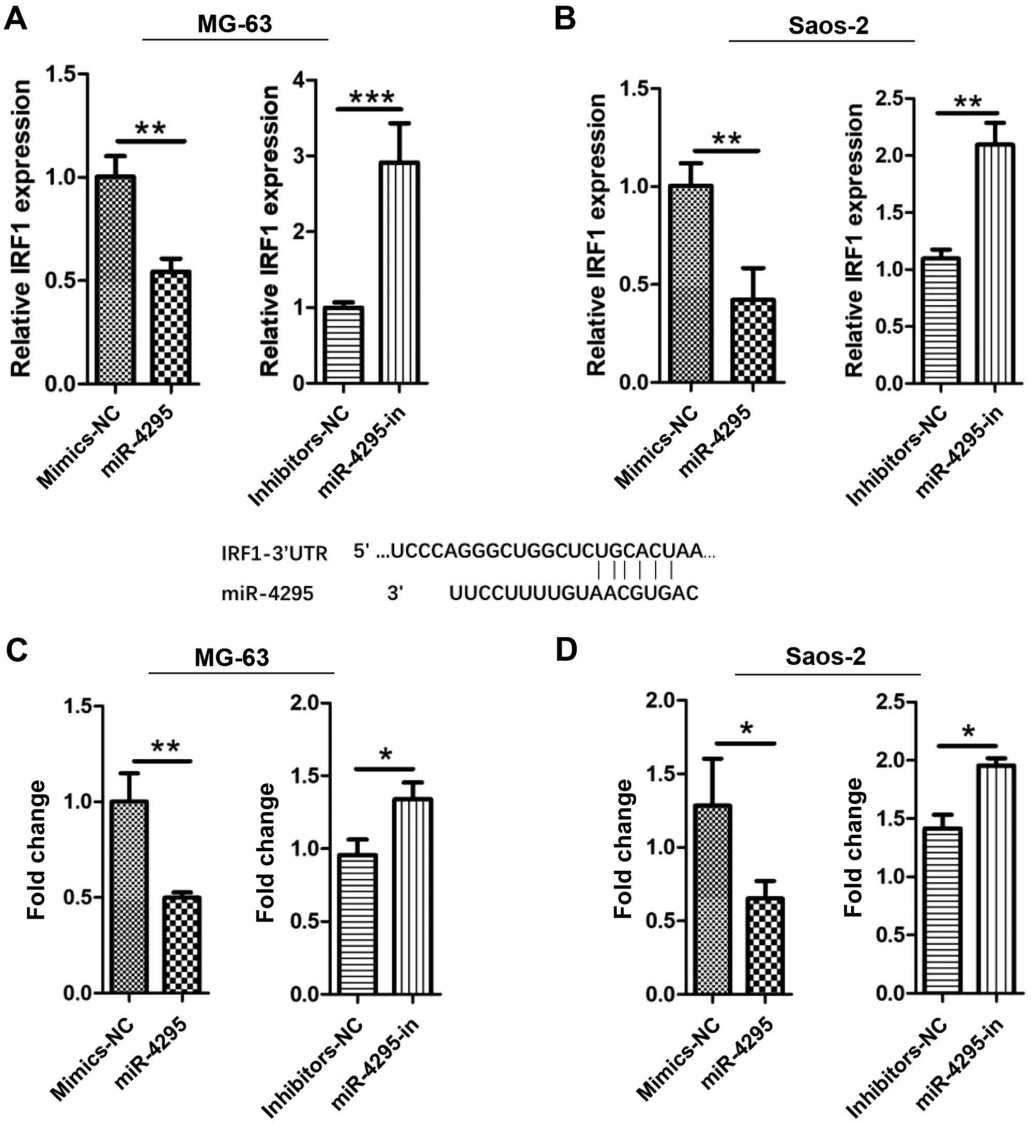

Figure 3. miR-4295 targets the *IRF1* gene directly. (A and B) Expression levels of IRF1 after upregulation or knockdown of miR-4295 in MG-63 and Saos-2 cells. (C and D) Luciferase activity of the PMIRGLO-IRF1 reporter in MG-63 and Saos-2 cells was identified after co-transfection of the IRF1 3'UTR plasmid with miR-4295 mimics/mimics-NC or miR-4295 inhibitor/inhibitor-NC. Statistical analysis was evaluated using independent Student's t test. *P<0.05, **P<0.01, ***P<0.001. IRF1, interferon regulatory factor 1; miR, microRNA; NC, negative control; UTR, untranslated region. Scale bar, 200 μm.

determine the influence of miR-4295 on the cell migration and invasion *in vitro*. It was demonstrated that MG-63 and Saos-2 cells transfected with miR-4295 mimics had increased migration and invasion compared with cells transfected with miR-NC (Fig. 2C-F), while cells treated with miR-4295 inhibitor exhibited a significant decrease in cell migration and invasion compared with cells treated with inhibitor-NC (Fig. 2C-F). Taken together, these results showed that overexpression of miR-4295 facilitated the migration and invasion of OS cells.

miR-4295 influences OS cell proliferation, migration and invasion by targeting the IRF1 gene. To further elucidate the underlying mechanisms of miR-4295-influenced cell proliferation, migration and invasion, the downstream signaling pathways of miR-4295 were analyzed. The Target Scan Human7.1 (http://www.targetscan.org/vert_71/) database was applied to predict target genes of miR-4295. *IRF1*, one of the top 20 candidate target genes, as our previous study was associated with IRF1 (data are not shown). The mRNA expression levels of *IRF1* were examined after overexpression or knockdown of miR-4295 in OS cells. The mRNA expression levels of *IRF1* were significantly suppressed when miR-4295 expression was upregulated, while *IRF1* expression increased when miR-4295 was knocked down in OS cells (Fig. 3A and B). To determine whether miR-4295 directly targeted the *IRF1* gene, the *IRF1* 3'UTR was predicted through using Target Scan Human7.1 online tool and cloned into PMIRGLO vector. Dual luciferase reporter assays were performed to assess the interaction between miR-4295 and IRF1. As shown in Fig. 3C and D, there was a significant downward trend of the luciferase activity in the co-transfection of the *IRF1* 3'UTR plasmid with miR-4295 mimic compared with the mimics-NC. Furthermore, the luciferase activity of PMIRGLO-IRF1 reporter in OS cells was found to be decreased by compared with the miR-4295 inhibitor-NC. These data suggested that miR-4295 could

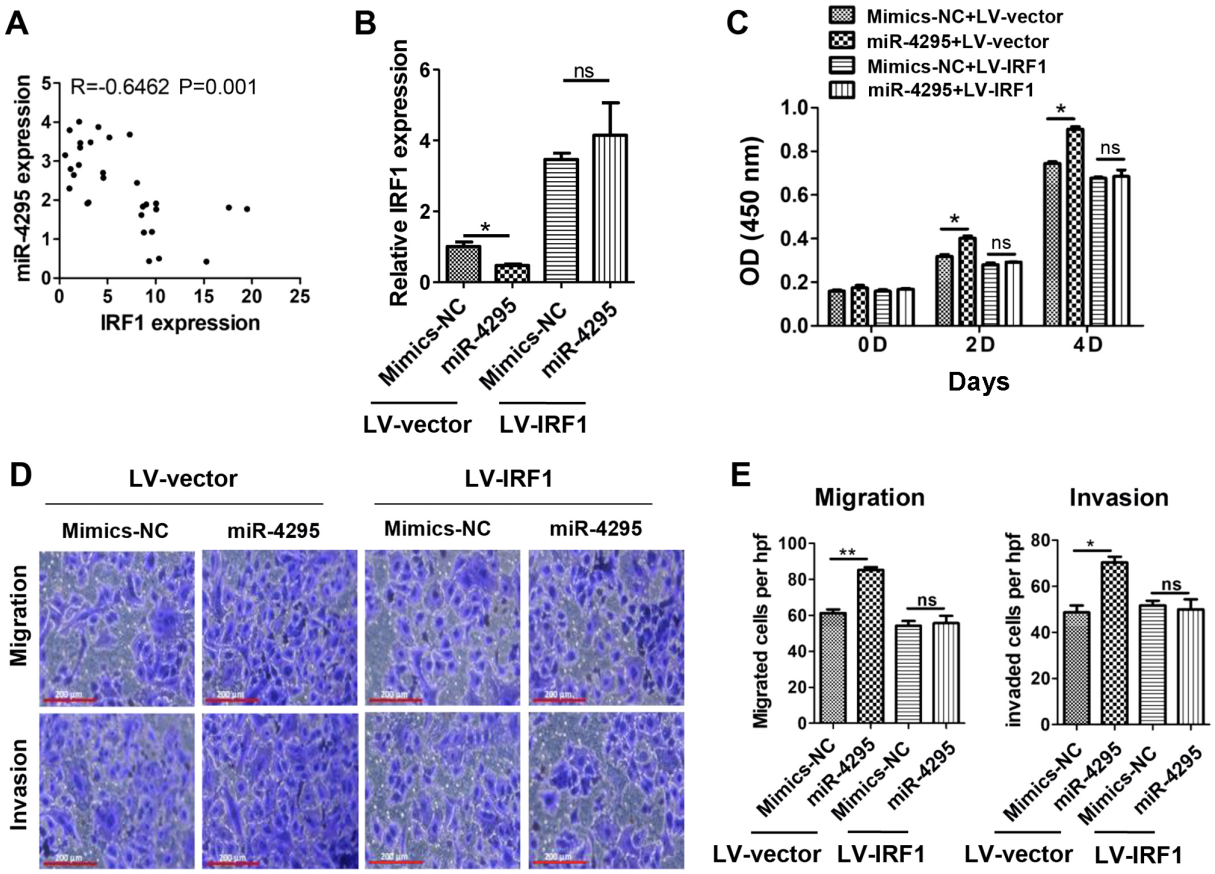

Figure 4. miR-4295 promotes Saos-2 cell migration and invasion by targeting the *IRF1* gene. (A) Correlation of expression levels of miR-4295 and *IRF1* in 30 samples of OS tissues analyzed using reverse transcription PCR. Statistical analysis was evaluated with the Pearson's correlation test. (B) Expression levels of IRF1 after transfection with LV-IRF1 and LV-NC lentivirus in Saos-2 cells transfected with mimics-NC or mir-4295 mimics. Statistical analysis was analyzed using one-way analysis of variance and Tukey's post hoc test. (C) Cell Counting Kit-8 assay of Saos-2 cells after transfection with LV-IRF1 and LV-NC lentivirus. Statistical analysis was analyzed using one-way analysis of variance and Tukey's post hoc test. (D) Migration and invasion of Saos-2 cells after transfection with LV-IRF1 and LV-NC lentivirus. (E) Three fields of the indicated cells were evaluated. Means + SD are shown from three independent experiments performed in duplicates. Statistical analysis was evaluated using one-way analysis of variance and Tukey's post hoc test. *P<0.05, **P<0.01. miR, microRNA; IRF1, interferon regulatory factor 1; OS, osteosarcoma; NC, negative control; ns, not significant. Scale bar, 200 μm.

suppress the translation of IRF1 by targeting its 3'-UTR. Then, the mRNA expression levels of *IRF1* in 30 OS tissues were detected, and the results indicated that the expression levels of miR-4295 were correlated with *IRF1* (Fig. 4A).

To verify whether IRF1 could inhibit the miR-4295-mediated promotion of OS cell proliferation, migration and invasion, the LV-vector and LV-IRF1 lentivirus was used to transfect Saos-2 cells that were subsequently used in CCK-8 and Transwell assays (Fig. 4C-E). Cell proliferation, migration and invasion in miR-4295+LV-IRF1 group were similar to those in the mimics-NC+LV-IRF1 group. The results indicated that the miR-4295-mediated promotion of Saos-2 cell proliferation, migration and invasion was inhibited by transfection with LV-IRF1 lentivirus. These results suggested that miR-4295 promotes OS cell proliferation, migration and invasion by targeting *IRF1* gene.

Discussion

OS, the most common form of primary bone tumor, primarily affects children and adolescents (5). In the past few years, the prognosis of patients with OS had progressed very little, especially for patients with metastatic disease (26-28). Despite encouraging progression in therapeutic management, the survival rates still remained unchanged over the past 40 years (28,29). In recent years, with the development of genomics and the deepening of miRNA research, our understanding of the mechanisms underlying occurrence, progression and metastasis of OS have improved, especially in in terms of occurrence and progression (30,31). It has been established that miRNAs play a key regulatory function in the progression of OS (31).

To explore the association of miRNAs with OS, bioinformatics techniques were used to screen for differentially expressed miRNAs in OS in the GEO database. In the GSE70367 database, it was demonstrated that miR-4295 expression was abnormally elevated in OS cell lines. Recent studies have found that miR-4925 might exert some function as a cancer-related miRNA in several types of cancer (20,31-33). It was reported that miR-4295 could inhibit glioma cell arrest at the G_0/G_1 phase, cause apoptosis and promote cell proliferation (19). Also, inhibition of miR-4295 suppressed cell proliferation and invasion through antagonizing the Wnt/β-catenin signaling pathway in pancreatic ductal adenocarcinoma (20). In bladder cancer, overexpression of miR-4295 could promote cell proliferation, colony formation and migration, and downregulation of miR-4295

induced cell cycle arrest and apoptosis (34). To characterize the potential role of miR-4295 in OS, the expression levels of miR-4295 were examined in 30 samples of OS and adjacent normal tissues in the present study. The results revealed that the expression of miR-4295 was significantly increased in OS tissues compared with the paired normal tissues. Moreover, the expression levels of miR-4295 in MG-63 and Saos-2 cell lines were significantly higher compared with in the normal hMSC cells. In addition, the functional assays showed that upregulation of miR-4295 could promote the proliferation, migration and invasion of OS cells. Taken together, these findings indicated that miR-4295 might act as a novel tumor activator in OS.

To elucidate the underlying molecular mechanisms of miR-4295, the downstream signaling pathways of miR-4295 were analyzed. Using the Target Scan Human7.1 database, the target genes of miR-4295 were predicted. The 20 top candidate target genes of miR-4295 were as follows: Lysine demethylase 2A (KDM2A), SLAIN motif family member 1 (SLAIN1), MDM4 regulator of P53 (MDM4), kruppel like factor 7 (KLF7), poly(A) specific ribonuclease Subunit PAN3 (PAN3), ectonucleotide pyrophosphatase/phosphodiesterase family member 5 (NPP5), F-Box protein 28 (FBXO28), MYB proto-oncogene like 1 (MYBL1), activin A receptor type 1 (ACVR1), cytoplasmic polyadenylation element binding protein 1 (CPEB1), carbohydrate sulfotransferase 1 (CHST1), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (PIK3CB), RNA 3'-terminal phosphate cyclase (RTCA), syntabulin (SYBU), interferon regulatory factor 1 (IRF1), ribosomal protein S6 kinase A5 (RPS6KA5), MAF BZIP transcription factor (MAF), dynein light chain LC8-type 2 (DYNLL2) and SET binding factor 2 (SBF2; data not shown). Our previous study showed that siRNA-mediated IRF1-knockdown could promote the proliferation and migration of Saos-2 cells (unpublished data). IRF1, a member of IRF family, could serve as a transcriptional regulator and tumor suppressor playing a crucial role in tumor cell growth and immune responses (35). Various studies have demonstrated that IRF1 can act as a tumor suppressor contributing several types of cancer (36). A recent study demonstrated that overexpression of IRF1 suppresses cell proliferation, migration and invasion, and blocked cell cycle progression of cholangiocarcinoma cells (36). As a tumor suppressor, upregulation of IRF-1 suppresses the transformed phenotype of ovarian cancer cells (37). IRF1 exerts its antiproliferative effect through repressing the transcription of a novel proliferation-related downstream target, the *Ki-67* gene, in a dose-dependent manner in renal carcinoma cells (38). Notably, upregulation of IRF-1 had been found to result in a 15-fold downregulation of survivin protein levels in breast carcinoma cells (39). The present study observed that the expression of *IRF1* was suppressed by overexpression of miR-4295 and increased by knockdown of miR-4295 in OS cells. Meanwhile, the data demonstrated that the 3'-UTR region of *IRF1* had a conserved miR-4295 binding site. The 3'-UTRs of the *IRF1* gene were inserted into the pMIRGLO vector. The results of the luciferase assay confirmed that *IRF1* was a target gene of miR-4295 in OS cells. Meanwhile, the mutant 3'UTRs of the *IRF1* gene were constructed but these were not successfully inserted into pMIRGLO vector. Experiments using such vectors could validate the present conclusion. Taken together, the results revealed that miR-4295 may act as a tumor activator by targeting *IRF1* during the progression of OS.

In conclusion, miR-4295 promoted proliferation, migration and invasion of OS cells by targeting the *IRF1* gene. The resolved functions of miR-4295 may provide novel insight into the mechanisms of OS metastasis, and inhibition of miR-4295 may have value as a therapeutic strategy for the treatment of OS.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

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

Authors' contributions

ZZ provided the samples. JC and BH performed the experiments. JC, JD and KY analyzed the data. JC wrote the manuscript. JC and ZZ designed and supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Xiangyang No. 1 People's Hospital (Hubei, China). Written informed consent was obtained from each patient prior to participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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