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Original Article

MicroRNA-181b promotes osteosarcoma cell proliferation, invasion and migration in vitro via targeting RASSF8

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Abstract: microRNAs (miRNA) are a class of small, non-coding RNA that involved in different cancer-related processes. Previous studies have been indicated miR-181b as a tumor onco-miR during multi-cancer tumorigenesis. However, the role of miR-181b in osteosarcoma is still unclear. In this study, we demonstrate miR-181b was up-regulated in osteosarcoma tissues compared to adjacent non-tumor tissue. Functional study suggests miR-181b promotes the osteosarcoma cell proliferation, invasion and migration. We also identified that RASSF8, a functional tumor suppressor was a direct target of miR-181b by the results of luciferase reportor assay, western blot assay and real-time PCR. These findings provide profound evidence that miR-181b plays a key role in promoting osteosarcoma cell proliferation, invasion and migration and also can regulate RASSF8 expression in osteosarcoma cells. These findings strongly suggest that exogenous miR-181b may have therapeutic value in treating osteosarcoma.

Keywords: miR-181b, RASSF8, osteosarcoma, MG-63, U2OS

Introduction

Osteosarcoma (OS) is one of the most common type of bone cancer that leading cause of cancer death among adolescents and young adults [1, 2]. With the current surgical techniques and chemotherapeutic treatments, the 5-year survival rate with localized OS is only about 60-80%, while it is only 15-30% with metastatic OS [3-5]. Among the genetic factors that present in OS, mutations and copy number variations are causative resultant for OS oncogenic transformation progress [6]. Therefore, it's vital to illustrate the cellular events that initiate and propagate osteosarcoma genesis [6].

MicroRNAs (miRNAs) are short non-coding RNAs with 18-22 nucleotides that post-transcriptional modify gene expression through direct interaction with the 3'untranslated region (3'UTRs) of specific target mRNAs in eukaryotic cells [7]. Expression of a single miRNA can silence numbers of target genes, granting these molecules extensive control over many cellular functions, such as cell differentiation, proliferation and metastasis [8-10]. Deregulation of miRNAs can act as tumor suppressors or oncogenes contributing to these phenotypes [11-13]. Several upregulated miRNAs signature known as oncomiRs have been found in the OS, such as miR-190 [14], miR-10b [15], miR-7 [16], miR-214 [17], and miR-210 [18]. Kevin B. Jones [19] surveyed a well-characterized group of OS tissues using array-based technologies and qRT-PCR, which confirmed that miR-181b was significantly upregulated. However, little is known about the role of miR-181b in the pathogenesis of OS.

RASSF8 is a member of the Ras-association domain family (RASSF), several of which are believed to be tumor suppressor genes [20, 21]. RASSF proteins have been found to play key roles in biological processes, such as cell death, cell cycle control, microtubule stability, promoter methylation, vesicle trafficking, and response to hypoxia [22, 23]. RASSF8 is ubiquitously expressed in all major tissues, and

serves as a suppressor gene [24]. In lung cancer, downregulated RASSF8 can increase cell migration and growth [24].

In the current study, we found that miR-181b was upregulated in OS tissues and cell lines. Overexpression of miR-181b promoted cell viability, growth, invasion and migration *in vitro*. In addition, RASSF8 was identified as a direct target of miR-181b and RASSF8 was found downregulated in OS tissues and cells. Taken together, our findings collectively suggest that miR-181b serves as an onco-miRNA via direct targeting and suppressing RASSF8 expression and may thus be a promising therapeutic target for OS.

Materials and methods

Human OS tissues and cell culture

18 paired fresh surgically resected osteosarcoma tumor tissues and adjacent normal bone tissues, which were diagnosed by an independent pathologist, were collected from the Qilu Hospital of Shandong University, between December 2014 and Decemeber 2015. All study procedures were approved by the Institutional Review Board of the Qilu Hospital of Shandong University. Informed consent was given by all participants. The human OS cell lines (Saos-2, MG-63 and U2OS) and normal osteoblastic cell lines NHOst or hFOB1.19 (Shanghai Cell Bank, Chinese Academy of Sciences) were cultured in Dulbecco's modiedEagle's medium (DMEM) with 10% fetal bovine serum (FBS) and incubated at 37°C in 5% CO_2.

RNA oligonucleotides, plasmids and transfection

The 2'-O-me-miR-181b mimic (miR-181b mimics) and negative control (miR control) were chemically synthesized and purified by high-performance liquid chromatography by GenePharma (Shanghai, China) and used for transfection at a final concentration of 20 nM with Lipofectamine 2000 (Invitrogen).

Luciferase reporter gene assay

The 3'-UTR of *RASSF8* in the pMirGLO reporter vector was purchased from Origene Technologies. The miR-181b binding site was mutated using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). WT and mutant constructs were transfected into MG-63 and U2OS cells concurrently with miR-181b mimic or miR control. At 48 hours after transfection, cells were assayed for relative luciferase activity using the luciferase assay kits (Promega, Madison, WI, USA). The data depicted represent three independent experiments performed on different days.

Cell proliferation assays

Cell viability was tested with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to the manufacturer's instruction (Sigma). Cells were transfected and seeded at 1000 cells per well in a 96-well plate in quadruplicate. The absorbance at 570 nm was measured using an IQuant Universal Microplate Spectrophotometer (BioTek, Winooski, VT).

For colony formation assay, transfected cells were placed at 300 cells per well in a 6-well plate in quadruplicate and maintained for two weeks, the culture medium was replaced every 3 days. Colonies were then fixed with methanol and stained with 0.1% crystal violet (Sigma) in PBS for 15 minutes, then the stained colonies were counted under a microscope.

Wound-healing assay

Then transfected cells were seeded and cultured to 95% confluence in six-well plates, then the cell layers were scratched with a 20 ml tip to form wound gaps, washed twice with PBS and cultured. The wound healing was photographed at different time points, and each wound was analyzed by measuring the distance migrated by the cells in three different areas.

Cell invasion and migration assay

Cell invasion assay was performed using Transwell chambers with or without BD Matrigel (BD Biosciences) following the manufacturer's protocol. Cells were suspended in serum-free DMEM culture medium at a concentration of 1×10^4 cells/ml and then added to the upper chamber with a Matrigel-coated or Matrigel-free filter. The lower chambers were filled with 10% FBS as a chemoattractant, and incubated in the chambers for 48 h. At the end of the experiments, the cells on the upper surface of the membranes were removed using a cotton swab, and the cells on the lower surface were fixed and stained with 0.1% crystal violet. Five visual fields of each insert were randomly chosen and counted under a microscope and imaged to count the migrated cells.

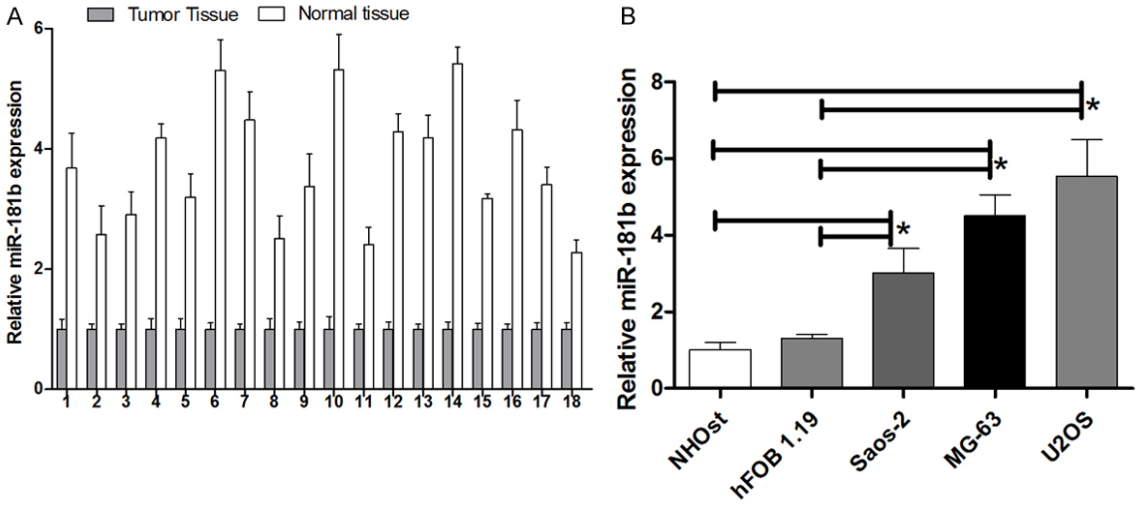

Figure 1. Relative expression of miR-181b in OS tissues and different OS cell lines. A. A column plot of miR-181b expression in 18 pairs of OS and healthy bones (Normal); B. Endogenous levels of miR-181b in OS cell lines were measured by qRT-PCR and normalized to snoRNA U6. Error bars are SD. Experiments were conducted in triplicates.

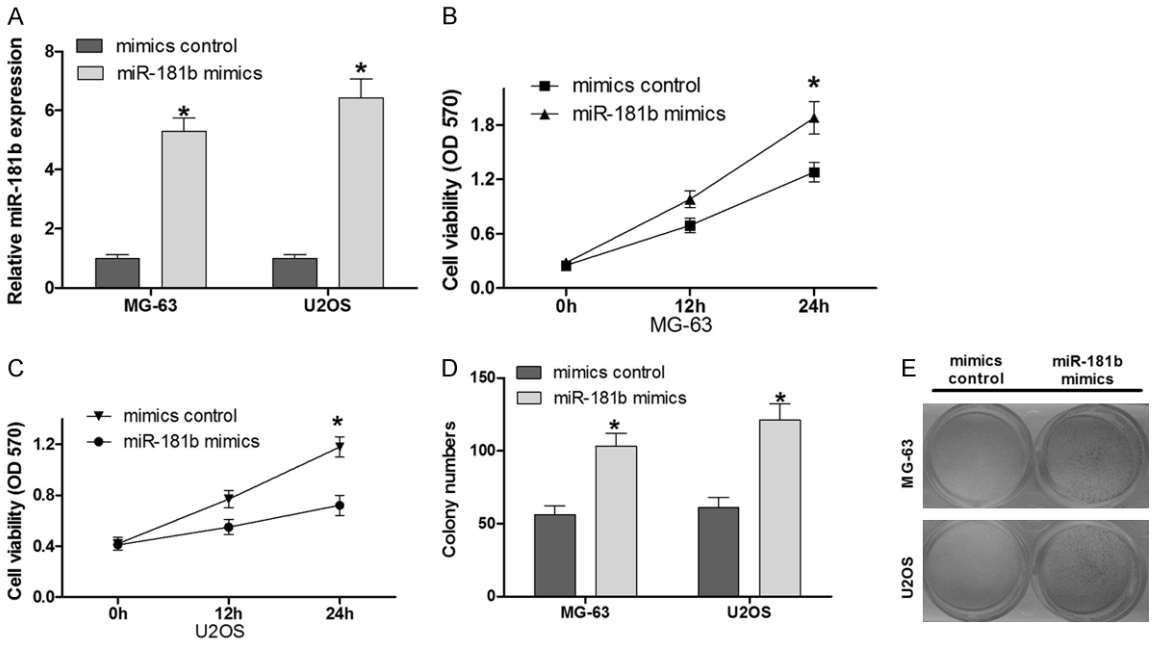

Figure 2. miR-181b promotes the OS cells viability and growth. A. miR-181 mimics were transfected in MG-63 and U2OS cells and assessed by qRT-PCR; B and C. MTT assay was performed with MG-63 and U2OS cells transfected with mimics or miR-181b mimics, OD570 was measured at the indicated time; D and E. Colony formation assay was performed in cells transfected with mimics control or miR-181b mimics.

Western blot analysis

Western blotting was performed according to the standard methods as described [25] using anti-RASSF8 antibody (ab12610, Abcam, Cambridge, MA, USA) and the anti-GAPDH antibody (Sigma-Aldrich) was used as a loading control. The experiments were repeated three times.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from tissues and cells using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Total RNA was extracted from tissues and cells using a commercially available kit (mirVana; QIAGENE, Shanghai, China) according to the manufactur-

er's protocol. Reverse transcriptions were performed by using miScript Reverse Transcription Kit (218061, QIAGEN) and QuantiTect SYBR Green RT-PCR Kit (204243, QIAGEN) was used for real-time qPCR analysis with the ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The relative expression level of miR-181b and RASSF8 were normalized to that of internal control U6 or GAPDH using the comparative delta CT ($2^{-\Delta\Delta Ct}$) method. Each sample was analyzed in triplicate and the mean expression level was calculated.

Statistical analysis

Statistical analyses were performed by one-way ANOVA. $P \leq 0.05$ was considered as statistically significant differences (GraphPad Prism 5; GraphPad software, Inc., San Diego, CA, USA).

Results

miR-181b was overexpressed in OS tissues and cell lines

To understand the role of miR-181b in OS, miR-181b expression was examined by qRT-PCR in 18 paired OS tissues and the corresponding normal bone tissues. Here, we found that miR-181b was upregulated in OS relative to healthy bone tissues (**Figure 1A**). Moreover, we examined the expression of miR-181b in different human OS cell lines using qRT-PCR. Upregulated expression of miR-181b was found in all OS cells with a prominently highest level in U2OS cells compared to normal osteoblastic cell lines (NHOst and hFOB1.19) (**Figure 1B**). These results suggest that miR-181b may serves as an onco-miRNA in OS development.

Overexpression of miR-181b promoted OS cell proliferation

Since our findings have demonstrated significant upregulation of miR-181b in human OS samples and cells, we examined the potential tumor promoting role of miR-181b in OS cells. We investigated the functional significance of miR-181b on cell viability and cell growth in MG-63 and U2OS cells using miR control or miR-181b mimics. Our data showed a significant increase of miR-181b levels (~5.3-folds in MG-63 cells and ~6.4-folds in U2OS cells) in miR-181b-transfected cells compared to that of miR control cells (**Figure 2A**). Then we compared the cell viability and growth changes in miR-181b-transfected cells. Our results showed that the ectopic expression of miR-181b increased cell viability in a time-dependent manner (**Figure 2B** and **2C**). Furthermore, treatment with miR-181b mimics prominent induced OS cell growth as demonstrated by colony formation assay (**Figure 2D** and **2E**), indicating that miR-181b can promote OS cells proliferation.

miR-181b overexpression promoted OS cell invasion and migration

To further detect whether miR-181b is associated with the metastasis ability of OS cells, we analyzed the effect of miR-181b expression on the migratory and invasive behavior of MG-63 and U2OS cells. The ectopic expression of miR-181b promoted invasion (**Figure 3A** and **3B**) and migration (**Figure 3C**) capacity of OS cells using transwell assays with or without matrigel. Moreover, would healing assay also confirmed that the overexpression of miR-181b induced by miR-181b mimics transfection remained smaller wound breadth in MG-63 and U2OS cells (**Figure 3D**). These results showed that miR-181b expression contributes to the regulation of OS cells metastasis in vitro.

RASSF8 is a target of miR-181b and downregulated in OS tissues and cell lines

Bioinformatics research was performed to find potential targets of miR-181b using Targetscan. As shown in **Figure 4A**, RASSF8 was identified as a potential target of miR-181b. In order to address the molecular mechanism underlying the tumor promoting functions of miR-181b, we cloned 3'UTR sequences (wild type or mutated) binding site of miR-181b into the pmiRGLO luciferase vector, respectively, and co-transfected with the miR-181b or miR control into OS cells. Data from the luciferase assay showed that overexpression of miR-181b remarkably suppressed the luciferase activity of the reporter gene with the wild-type construct but not the mutant in MG-63 and U2OS cells (**Figure 4B** and **4C**). In addition, overexpression of miR-181b resulted in a reduction of RASSF8 mRNA and protein expression in OS cells (**Figure 4D** and **4E**).

We further investigated the functional role of RASSF8 with qRT-PCR on OS tissue and cells. The RASSF8 mRNA is obviously downregulated in 18 Osteosarcoma tissues compared with

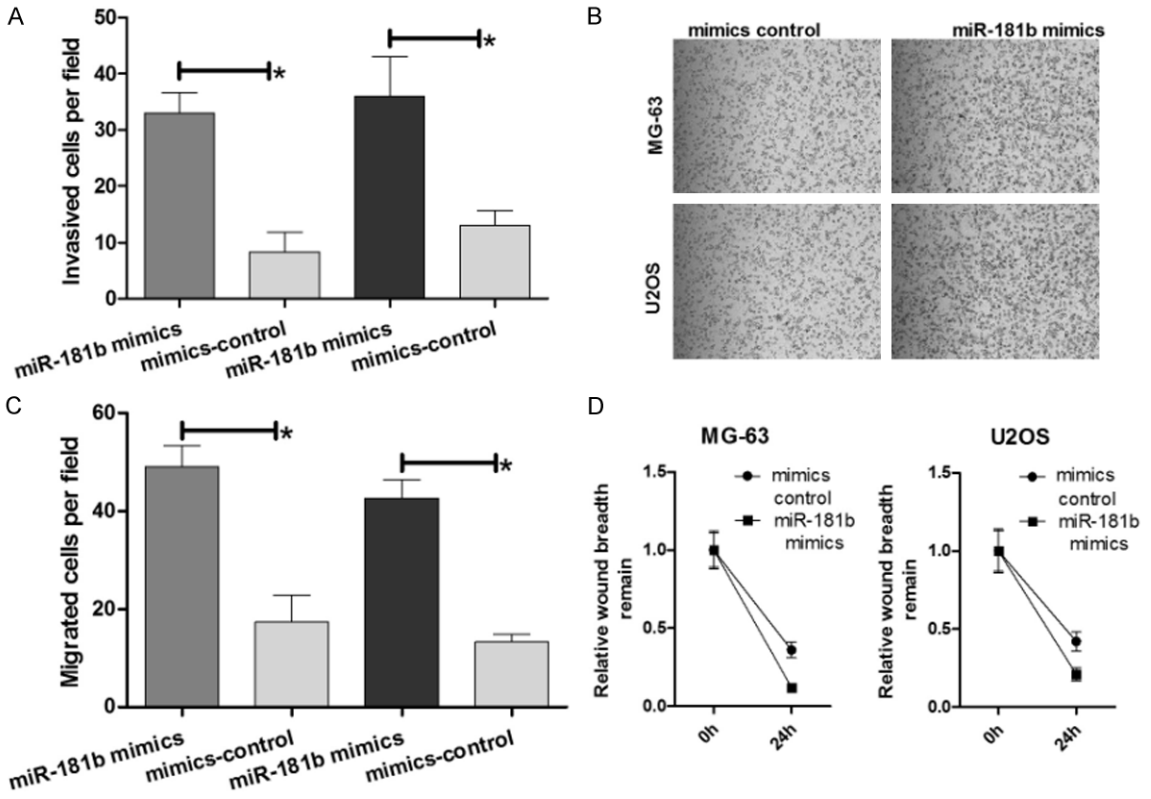

Figure 3. miR-181b overexpression promoted OS cell invasion and migration. miR-181b mimics or control were transfected in MG-63 and U2OS cells and then transwell assay were performed with matrigel (A and B) or without matrigel (C); (D) Overexpression of miR-181b increased cell migration ability with wound healing assay.

Normal bone tissues as presented in **Figure 4F**. In addition, we found that the RASSF8 mRNA was also obviously downregulated in OS cells MG-63 and U2OS (**Figure 4G**).

Discussion

miR-181b belongs to the miR-181 family and plays important and different regulatory roles in the cellular functions in different cancer cell lines [26-28]. The varying effects of miR-181b in different cell types mainly depend on the cancer types and their target genes and co-regulatory functions. MiR-181b facilitates HCC and breast cancer cells progression via suppressing TIMP3 expression [29, 30], miR-181b can sensitize lung and gastric cancer cells to chemotherapy by targeting BCL-2 [31, 32]. Previous study showed that miR-181b stimulated HeLa cell growth and inhibited cell death via targeting adenylyl cyclase 9 (AC9) [33, 34]. miR-181b upregulation is associated with the progression of leukoplakia to oral carcinoma [35], as well as poor prognosis and therapeutic outcome in colon cancer [36]. However, miR-181b was downregulated in human glioma cells [37] and astrocytic tumors [38], which suggested that miR-181b may have a tumor-type specific role.

In this study, we investigated the biological role of miR-181b in the progression of OS. We found significant upregulation of miR-181b in OS tissues and cells compared with matched normal bone tissues. Additionally, functional experiments demonstrated that miR-181b promoted cell viability, growth, invasion and migration. We demonstrated RASSF8 as a potential target of miR-181b. Fluorescent reporter assays showed that miR-181b directly bonded to the RASSF8 3'UTR. Furthermore, qRT-PCR and Western blot analyses confirmed that miR-181b negatively regulated RASSF8 by reducing RASSF8 mRNA and protein levels. In addition, we clarified that RASSF8 was downrgulated in OS tissues and cell lines. Our work may be the first time to reveal the functional role of miR-181b targeting RASSF8 in OS.

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 377-383 of RASSF8 3' UTR	5' . . . CAUGUAAUCAAUGUGUGAAUGUU. . .	7mer-m8	-0.11	79	-0.11	5.744	0.77
hsa-miR-181b-5p	3' UGGGUGGCUGUCGUUACUUACAA						

RASSF8-3'UTR mut: 5'...CAUGUAAUCAAUGUGUGA **CACA**U...3'

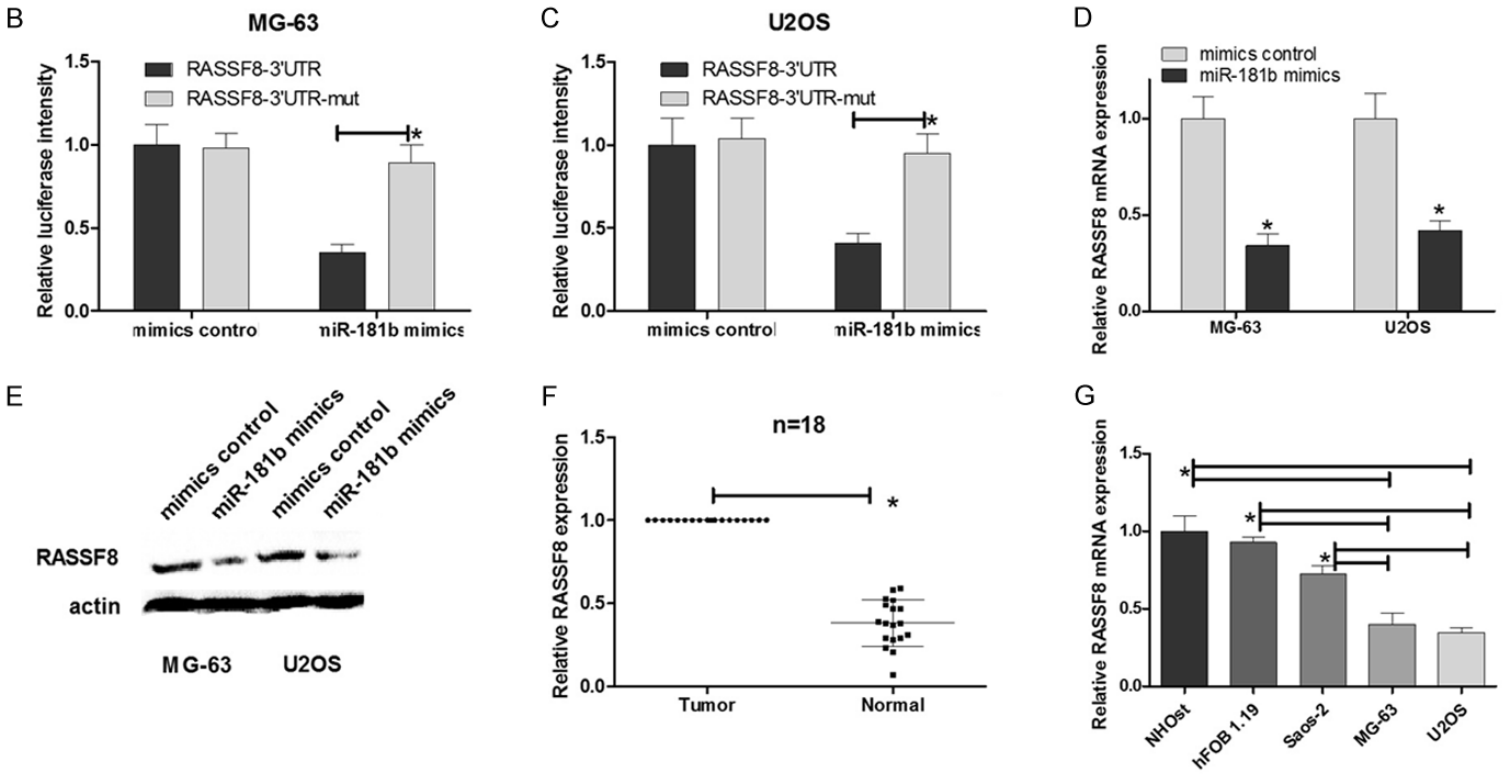

Figure 4. *RASSF8* is a target of miR-181b and downregulated in OS tissues and cells. A. Computational analysis showed that miR-181b potentially targeted the RASSF8 gene. The mutate base pairs in the predicted seed region targeted by miR-181b in the RASSF8 3'UTR was also showed. B and C. Luciferase assay of the effect of miR-181b on expression of reporter luciferase gene, which contains a RASSF8 3'-UTR fragment with wild type (RASSF8 3'-UTR) or mutant (RASSF8 3'-UTR mut) miR-181b binding site. Transfections of MG-63 cells were conducted in triplicates, 48 hours after transfections cells were lysed and luciferase activities were measured. Relative luciferase activity normalized to renilla luciferase activity is shown. D and E. Effect of miR-181b overexpression in MG-63 and U2OS cells on endogenous RASSF8 mRNA and protein expression as assessed by qRT-PCR and Western blot. F. A column plot of RASSF8 expression in 18 pairs of OS and healthy bones (Normal). G. Endogenous levels of RASSF8 in OS cell lines were measured by qRT-PCR and normalized to GAPDH. Error bars are SD. Experiments were conducted in triplicates.

Previous study has demonstrated that RASSF8 downregulation can promote ESCC metastasis by enhancing cell motility and invasiveness and by increasing lymphangiogenesis via the NF-κB/VEGF-C axis [39]. RASSF8 depletion can reduced the cellular levels of IkB-a and increased NF-κB/p65 levels [40]. Both *in vivo* and *in vitro* studies conducted by Wang et al [41] showed that overexpression of RASSF8 inhibited melanoma cells' growth, migration and invasion as a result of downregulating P65 which lead to G1-S arrest and apoptosis induction through increasing p53 and p21 expression. In addition, following RASSF8 depletion can increase its accumulation in the cytoplasm and then delocalized to the nucleus to activate the canonical Wnt signaling pathway [42, 43]. Since, we have confirmed that miR-181b can promoted OS proliferation, invasion and migration through down regulating RASSF8. The detailed molecular mechanisms of RASSF8 and whether miR-181b involved in these pathways in OS should be investigated in the future.

In summary, the present study suggested miR-181b can promoted OS cells proliferation, migration and invasion through direct targeting and suppressing RASSF8. These findings provide us a basis to further investigate the underlying mechanisms of OS and may facilitate the development of novel therapeutic strategies for OS clinical application.

Disclosure of conflict of interest

None.

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