MicroRNA-1 functions as a potential tumor suppressor in osteosarcoma by targeting Med1 and Med31

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Abstract. MicroRNA-1 (miR-1) has been shown to function as a critical gene regulator in multiple types of cancers. However, the role of miR-1 in osteosarcoma has not been totally clarified. In the present study, we investigated the effects of miR-1 on osteosarcoma and the underlying mechanism. We found that miR-1 was downregulated in osteosarcoma tissues and osteosarcoma cell lines. Restoration of miR-1 significantly suppressed osteosarcoma cell proliferation by inhibiting cell cycle progression. Mediator complex subunit 1 (Med1) and 31 (Med31) were validated as targets of miR-1 in osteosarcoma by luciferase reporter assay. Downregulation of Med1 and Med31 suppressed the proliferation of osteosarcoma cells, and overexpression of Med1 and Med31 abrogated the effects of miR-1 on cell proliferation. Furthermore, both miR-1 and knockdown of Med1 or Med31 reduced the expression of met proto-oncogene (MET) and blocked the downstream signaling of MET responding to hepatocyte growth factor (HGF). Taken together, the findings of this study suggest that Med1 and Med31 serve as potential gene therapeutic targets in osteosarcoma and miR-1 may prove to be a promising agent.

Introduction

Osteosarcoma is the most common primary malignant bone malignancy and usually occurs in children and adolescents, and is characterized by an aggressive clinical course (1). Although the combination treatment of chemotherapy and surgery has improved the prognosis of patients with primary bone osteosarcoma, the most effective drugs are still those developed decades ago, and there is the risk of local relapse after chemotherapy for a number of patients with osteosarcoma (1-3). For this reason, understanding the mechanisms of multiple genetic abnormalities synergistically contributing to osteosarcoma progression and investigating novel strategies for osteosarcoma treatment are necessary.

Recently, growing evidence suggests that miRNAs, an abundant class of small noncoding RNAs, play critical roles in the regulation of diverse biological processes (4). miRNAs post transcriptionally regulate gene expression through binding to the 3' untranslated regions (3'UTR) of target mRNAs and thus can function either as oncogenes or tumor-suppressor genes in tumorigenesis (5). Although dysregulation of miRNAs, including miR-34a (6), miR-376c (7) and miR-133a (8), has been associated with the initiation and progression of osteosarcoma, a clear understanding of the detailed roles and molecular mechanisms of miRNAs in osteosarcoma is lacking.

microRNA-1 (miR-1) is a highly conserved miRNA and plays critical role in skeletal and cardiac muscle development (9). Recent studies have shown that downregulation of miR-1 is a frequent event in various types of cancer including osteosarcoma and functions as a tumor-suppressor gene (10,11). In osteosarcoma, miR-1 has been shown to affect cell proliferation and the cell cycle (12). Several oncogenic genes have been validated to be the targets of miR-1, such as the met proto-oncogene (MET) (13,14), cyclin D2 (CCND2) (15) and prothymosin α (PTMA) (16). This implies that miR-1 is involved in tumorigenesis via a complex regulatory network, which, however, has not been totally elucidated in osteosarcoma.

In the present study, we confirmed two target genes of miR-1, Med1 and Med31, in osteosarcoma. Both Med1 and Med31 were overexpressed in osteosarcoma and involved in cell proliferation. Our data indicate that MET may mediate the downstream signaling and suggest the potential therapeutic application of miR-1 which functions by targeting multiple oncogenes in osteosarcoma.

Materials and methods

Patients and osteosarcoma tissues. Surgically resected paired osteosarcoma tumor tissues and adjacent normal bone and myeloid tissues were collected from 30 primary osteosarcoma patients who underwent surgical resection following informed consent between 2006 and 2009 at Shanghai 6th People's

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Hospital (Shanghai, China). Surgically removed tissues were snap-frozen in liquid nitrogen for further use. Both tumor and non-tumor samples were confirmed by pathological examinations. The experiments were approved by the Ethics Committee of Shanghai Jiaotong University (Shanghai, China).

Cell culture and transfection. Human osteoblasts hFOB and three human osteosarcoma cell lines, MG-63, U2OS and Saos-2, were purchased from the American Type Culture Collection. The cells were maintained in DMEM (MG-63 and U2OS) and RPMI-1640 medium (Saos-2) (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (Gibco) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37˚C.

For transfection, cells were plated in 6-well clusters or 96-well plates and incubated overnight, and then transfected with miRNA mimics or negative control (Ambion, Austin, TX, USA) at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

The expression of Med1 and Med31 was inhibited in MG-63 and U2OS cell lines using a lentiviral shRNA system (Lv-con, Lv-Med1 shRNA, Lv-Med31 shRNA) from Santa Cruz Biotechnology (Dallas, Texas, USA) with supporting siRNA transfection reagent (Santa Cruz). MG-63 and U2OS cells stably transfected with Med shRNA were selected in medium containing puromycin.

For overexpression of Med1 and Med31, the cDNA clones were purchased from OriGene Technologies (Rockville, MD, USA), and recombinant adenoviruses (Ad-Med1 and Ad-Med31) were constructed by Hanbio Co. Ltd. (Shanghai, China).

For the detection of MET signaling, hepatocyte growth factor (HGF; Sigma-Aldrich) was added to the medium as a MET ligand.

RNA extraction and real-time PCR. Total RNA, including miRNA, was extracted using the miRNeasy kit (Qiagen, Valencia, CA, USA). MicroRNA levels were quantified using the TaqMan MicroRNA assay kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. After reverse transcription, mRNA levels were analyzed using SYBR Green PCR master mix (Applied Biosystems) in the ABI PRISM 7900 Sequence Detection System (Applied Biosystems).

All samples were normalized to U6 small nuclear RNA or β-actin mRNA, and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

The primers used are listed as follows: 5'-CTAATGCTGG TCCCTTGGATAA-3' and 5'-AGGTCAGAAGGAGAGACA TAGT-3' for Med1; 5'-GTCGCTATGGAGACAGATGAT-3' and 5'-AGTAACCTCTTTGGGCAAGAA-3' for Med31; and 5'-CACTCTTCCAGCCTTCCTTC-3 and 5'-GTACAGGTCT TTGCGGATGT-3 for β-actin.

Cell proliferation assays. MG-63 and U2OS cells were seeded into 96-well plates ($5x10^3$ cells/well), transfected with the miRNA mimics or negative control and further incubated at 37˚C. At the indicated time periods, medium was replaced with fresh medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA). Cells were then incubated at 37˚C for 4 h and resolved by dimethyl sulfoxide (DMSO; Sigma-Aldrich). The absorbance was measured at 490 nm using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA). Each experiment was performed in triplicate and repeated three times.

Cell cycle analysis. After being serum-starved in 1% FBS-containing medium for 12 h, cells were transfected with or without the miRNA and incubated in complete medium for 48 h. For flow cytometric analysis, cells were washed twice with 1X phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol, resuspended in 1 ml solution containing 0.4 mM sodium citrate, 25 μg/ml propidium iodide (PI) and 50 μg/ml RNase. After being stained for 1 h, cells were analyzed in a FACScan flow cytometer (BD Biosciences) using ModFIT program (Verity Software House, Topsham, ME, USA).

3'UTR luciferase reporter assay. A fragment of 3'UTR of Med1 or Med31 containing the putative miR-1 binding site (420-427 or 1059-1069, respectively) was amplified by PCR using the following primers: wt-Med1 (forward), 5'-GAA AGGCATATCCAGACCCTATT-3' and wt-Med1 (reverse), 5'-GGAAGGCTGTCCTACACTAAAC-3'; wt-Med31 (forward), 5'-TCTCCTGGAACCTTACTGTCT-3' and wt-Med31 (reverse), 5'-GCAACTGATGATATTCCTGA AACC-3'. The PCR product was subcloned into a pMIR-REPORT vector (Ambion) to generate the pMIR-Report-Med1/Med31 wt plasmid. Site-directed mutagenesis of miR-1 binding sites was carried out using a QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA). All constructs were verified by DNA sequencing.

For reporter assays, U2OS cells were plated in 96-well clusters, then cotransfected with 0.3 μg wt or mutant reporter plasmids and 60 nM miR-1 precursors. At 48 h after transfection, luciferase activity was measured and normalized by the control vector containing *Renilla* luciferase.

Western blotting. Cultured or transfected cells were lysed using M-PER protein extraction reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with complete proteinase inhibitor mixture. Equal amounts of the extracts were loaded onto an SDS-PAGE, transferred to a PVDF membrane and then incubated with the following primary antibodies: mouse monoclonal Med1 and Med31 antibodies (both from Santa Cruz), rabbit monoclonal MET antibody (Abcam, Cambridge, MA, USA), mouse monoclonal β-actin antibody and rabbit monoclonal p-Erk1/2 (Thr202/Tyr204) and Erk1/2 antibodies (all from Cell Signaling Technology, Inc., Danvers, MA, USA). After incubation with HRP-conjugated secondary antibodies (Cell Signaling), protein bands were visualized using ECL substrates (Millipore, Billerica, MA, USA).

Statistical analysis. All data from three independent experiments are presented as the mean ± SD. Statistical comparisons between two groups were analyzed using the two-tailed Student's t-test. Variance analysis between multiple groups was performed using one-way ANOVA followed by a Student-Newman-Keuls test. The correlation between miR-1

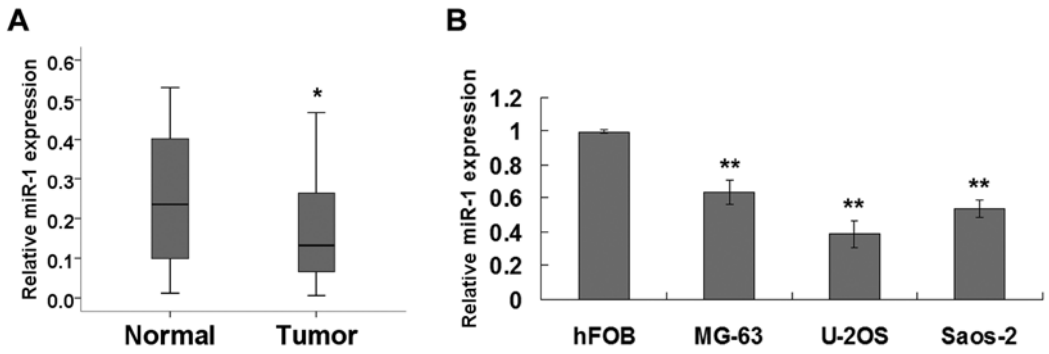

Figure 1. miR-1 is downregulated in osteosarcoma tissues and osteosarcoma cell lines. (A) Relative expression of miR-1 was examined in 30 paired clinical human osteosarcoma tissues and adjacent normal tissue using real-time PCR. (B) Relative expression of miR-1 was examined in an osteoblast cell line and three osteosarcoma cell lines. Expression of miR-1 was normalized to that of U6. $^{*}p<0.05$ compared with normal tissue, $^{**}p<0.01$ compared with the osteoblast cell line.

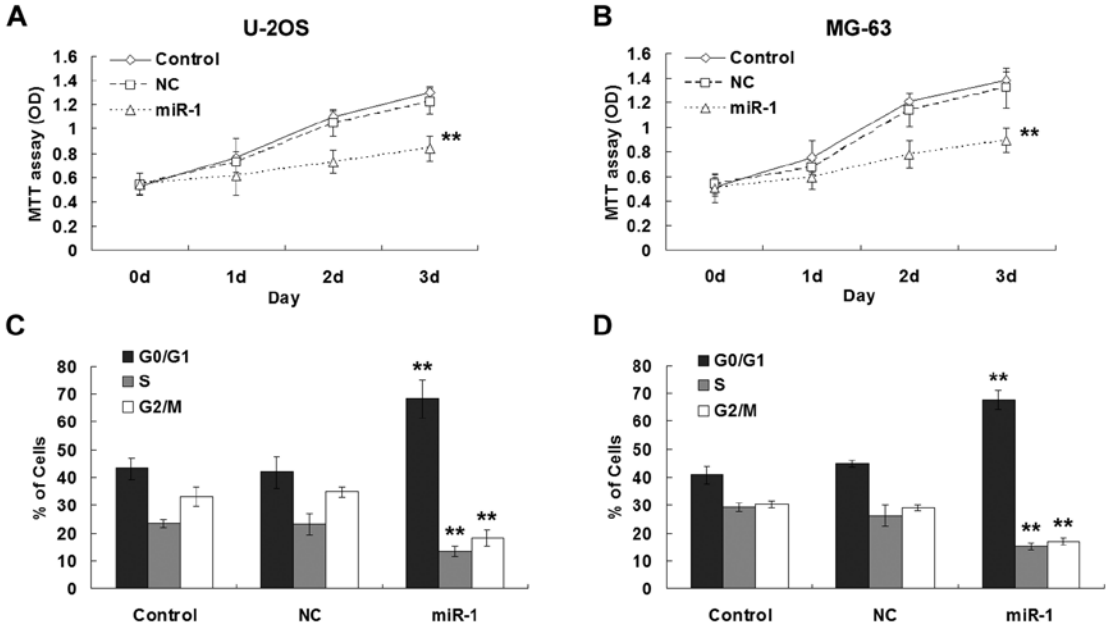

Figure 2. miR-1 inhibits osteosarcoma cell proliferation. U2OS and MG63 cells were transfected with or without negative control (NC) or miR-1 mimics. Cell proliferation of U2OS (A) and MG63 (B) cells was measured for the indicated time periods using MTT assay. Cell cycle progression of U2OS (C) or MG63 (D) cells was analyzed by flow cytometry. $^{**}p<0.01$ compared with the control.

and Med1 or Med31 expression was assessed by Pearson's correlation test. P-values of <0.05 were considered to indicate statistically significant results.

Results

miR-1 is downregulated in osteosarcoma. To determine the clinicopathologic significance of miR-1 in osteosarcoma development, we evaluated the expression level of miR-1 in 30 paired clinical human osteosarcoma tissues and adjacent normal tissue. As shown in Fig. 1A, miR-1 expression was significantly reduced in the tumor tissues as compared with that in the adjacent normal tissues. Furthermore, we extended our test to human osteosarcoma cell lines. Real-time PCR revealed that miR-1 expression was significantly decreased in the MG63, U2OS and Saos-2 osteosarcoma cells compared with the hFOB osteoblast cells (Fig. 1B).

Restoration of miR-1 inhibits cell proliferation. To investigate whether miR-1 functions as a tumor suppressor in osteosarcoma, we assessed the effect of miR-1 on osteosarcoma cell growth. In the U2OS and MG63 cells, transfection of miR-1 mimics significantly inhibited cell proliferation (Fig. 2A and B). Furthermore, analysis of DNA uptake by flow cytometry showed that miR-1 decreased the S and G2/M phase populations, with a concomitant increase in the proportion of cells in the G0/G1 phase in both osteosarcoma cell lines (Fig. 2C and D).

miR-1 directly targets Med1 and Med31. To further investigate the molecular mechanism, we aimed to identify the molecular targets of miR-1. Among the predicted targets of miR-1 from three programs, including miRDB, TargetScan and microrna.org, we were interested in Med1 and Med31. Both of them associate with the mediator complex (MED), which functions

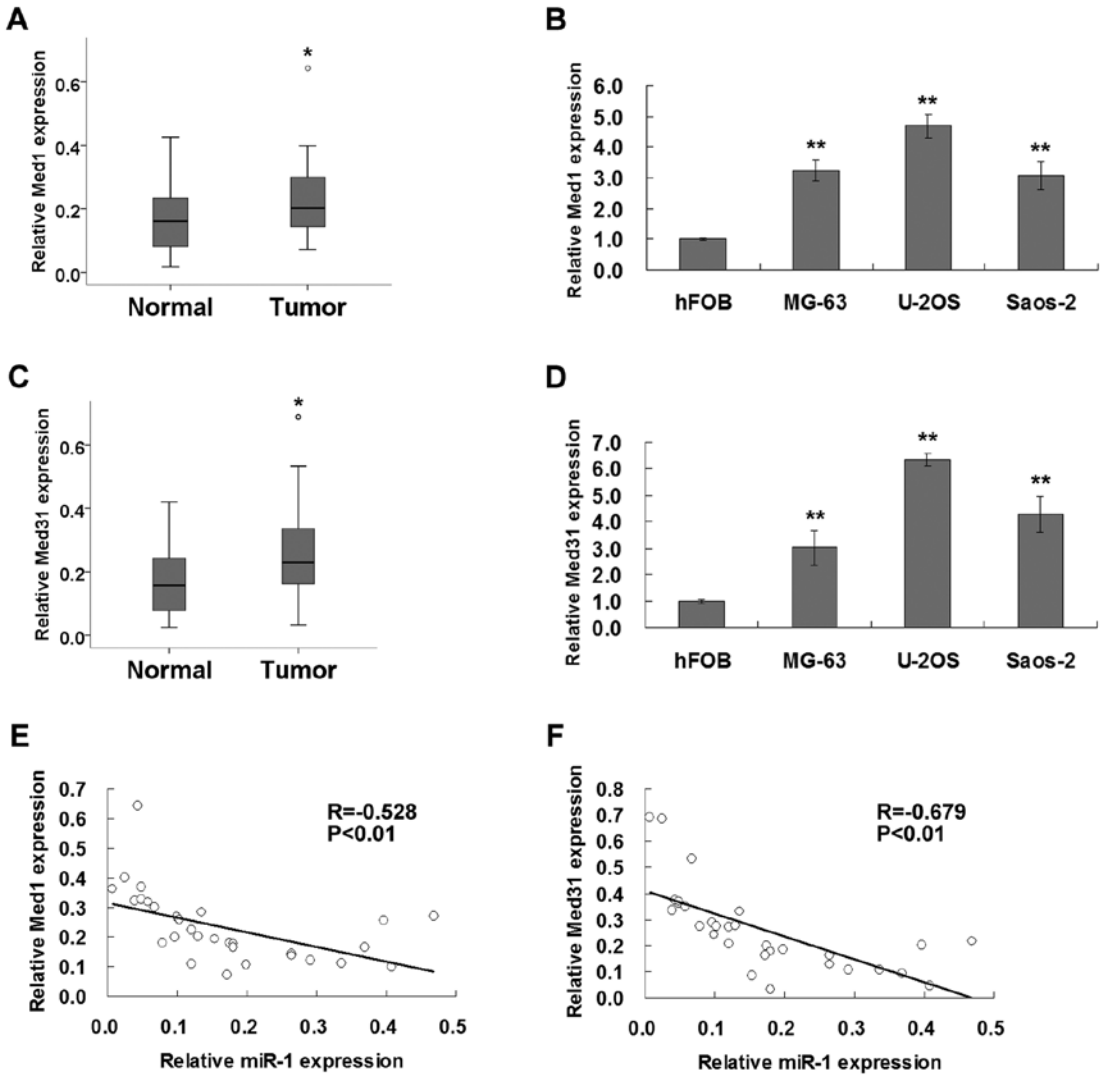

Figure 3. Med1 and Med31 are overexpressed in osteosarcoma tissues and osteosarcoma cell lines. Relative expression of Med1 (A) or Med 31 (C) was examined in 30 paired clinical human osteosarcoma tissues and adjacent normal tissues using real-time PCR. Relative expression of Med1 (B) or Med 31 (D) was examined in an osteoblast cell line and three osteosarcoma cell lines. Expression of Med1 or Med 31 was normalized to that of β-actin. Pearson's correlation analysis was used to determine the correlation between the expression levels of miR-1 and Med1 (E) or Med31 (F) in osteosarcoma tissues. R, regression coefficient; $^{*}p<0.05$ compared with normal tissue, $^{**}p<0.01$ compared with the osteoblast cell line.

as a component of the RNA polymerase II-mediated transcription machinery and plays an important role in transcription activation of eukaryotes (17).

We next tested the expression of Med1 and Med31 in clinical tissues and cell lines using real-time PCR. As shown in Fig. 3A-D, the expression levels of both Med1 and Med31 in the osteosarcoma tissues or osteosarcoma cell lines were significantly higher than the levels in normal tissue or osteoblast cells. Correlations between expression levels of miR-1 and Med1 or Med31 were further examined in primary human osteosarcoma tissues. Pearson's correlation analysis suggested that the expression levels of Med1 and Med31 were both significantly inversely correlated with miR-1 expression in the osteosarcoma tissues (Fig. 3E and F).

To verify whether miR-1 directly targets the 3'UTR of Med1 and Med31, a dual-luciferase reporter system was employed. miR-1 and luciferase reporter plasmids containing wild-type or mutated miR-1 binding sites in 3'UTR of Med1 or Med31 (Fig. 4A) were co-transfected into U2OS cells. Overexpression of miR-1 significantly suppressed the activity of the luciferase reporter containing wild-type Med1 or Med31 3'UTR, but not the activity of a reporter containing mutant Med1 or Med31 3'UTR (Fig. 4B and C). These data suggest that both Med1 and Med31 are directly targeted by miR-1. Moreover, enhanced miR-1 suppressed endogenous protein expression of Med1 and Med31 in the U2OS and MG63 cells (Fig. 4D and E).

Downregulation of Med1 and/or Med31 suppresses the proliferation of osteosarcoma cells. To elucidate the potential role of Med1 and Med31 in osteosarcoma development, we established stable Med1 and/or Med31-silenced osteosarcoma cell lines (Fig. 5A and B). MTT assay showed that separate or simultaneous silencing of Med1 and Med31 significantly inhibited proliferation of both U2OS and MG63 cells (Fig. 5C and D). Similar to induction of miR-1, reduced expression of Med1

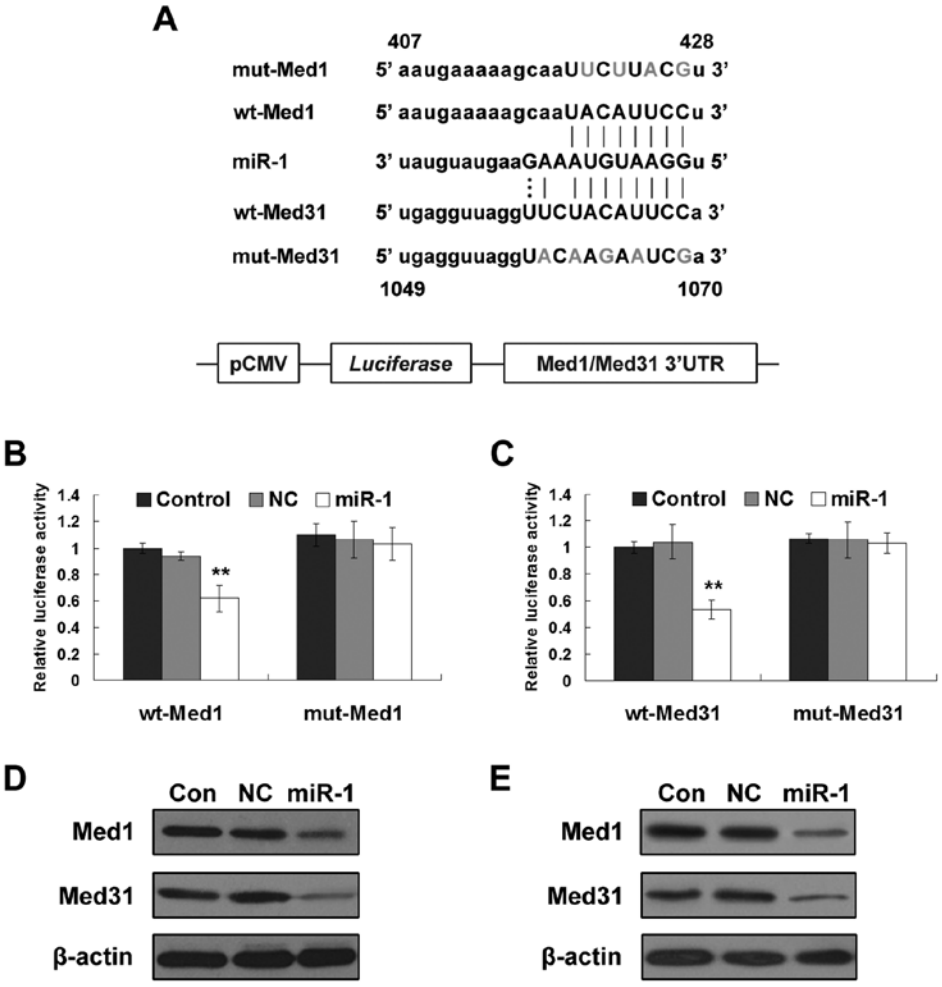

Figure 4. Med1 and Med31 are directly targeted by miR-1. (A) Predicted miR-1 binding sites within Med1 and Med31 3'UTR. The pMIR-Med1/Med31 vectors used in the luciferase assay are represented. The mutated miR-1 binding sites (mut-Med1 and mut Med31) are also shown. pMIR-reporter vectors and negative control (NC) or miR-1 mimics were cotransfected into U2OS cells. Relative luciferase activities of Med1 (B) or Med31 (C) wild-type (wt) and mutant (mut) 3'UTR regions were measured and normalized by *Renilla* luciferase activity. U2OS (E) and MG63 (F) cells were transfected with NC or miR-1 mimics for 48 h. Expression of Med1 and Med31 was detected by western blotting. $^{**}p<0.01$ compared with the control.

and/or Med31 blocked the cell cycle progression at the G0/G1 phase (Fig. 5E and F).

Overexpression of Med1 and/or Med31 abrogates the effects of miR-1. To further confirm that the tumor suppressive effect of miR-1 is mediated by supression of Med1 and Med31 in osteosarcoma cells, recombinant Ad-Med1 and Ad-Med31 were used to infect the U2OS and MG63 cells before miR-1 transfection. The decreased levels of Med1 and/or Med31 by miR-1 were significantly rescued via the infection of recombinant adenoviruses (Fig. 6A and B). Similarly, ectopic expression of Med1 and/or Med31 abrogated the growth suppressive effect induced by miR-1 in both U2OS and MG63 cells (Fig. 6C and D). Meanwhile, the restoration of expression of Med1 and Med31 significantly increased the proportion of cells in the S and G2/M phases (Fig. 6E and F). These data suggest that the growth suppressive effects of miR-1 were chiefly through inhibition of Med1 and Med31.

Med1 and Med31 modulate MET signaling. To further investigate the mechanism involved in the effect on cell proliferation by Med1 and Med31, we focused on MET signaling. Novello *et al* (12) showed that miR-1 could reduce the expression of MET in U2OS cells, implying that miR-1 modulates MET signaling in osteosarcoma. In contrast, Med1 deficiency resulted in the reduction of MET mRNA level and the block of the response to hepatocyte growth factor (HGF)/scatter factor (SF) in hepatocytes (18).

As shown in Fig. 7A, the downregulation of either Med1 or Med31 suppressed the expression of MET. Even in the presence of HGF, the expression of MET was still decreased by Med1/Med31 shRNA compared with the control (Fig. 7B). The phosphorylation of ERK1/2, a downstream target of MET, was also inhibited by Med1/Med31 shRNA (Fig. 7B). miR-1 exhibited effects similar to Med1/Med31 shRNA (Fig. 7A and B).

Discussion

In recent years, studies on the molecular mechanisms contributing to osteosarcoma carcinogenesis have revealed the critical role of miRNAs in the process. Several miRNAs have been found deregulated and related to osteosarcoma develop-

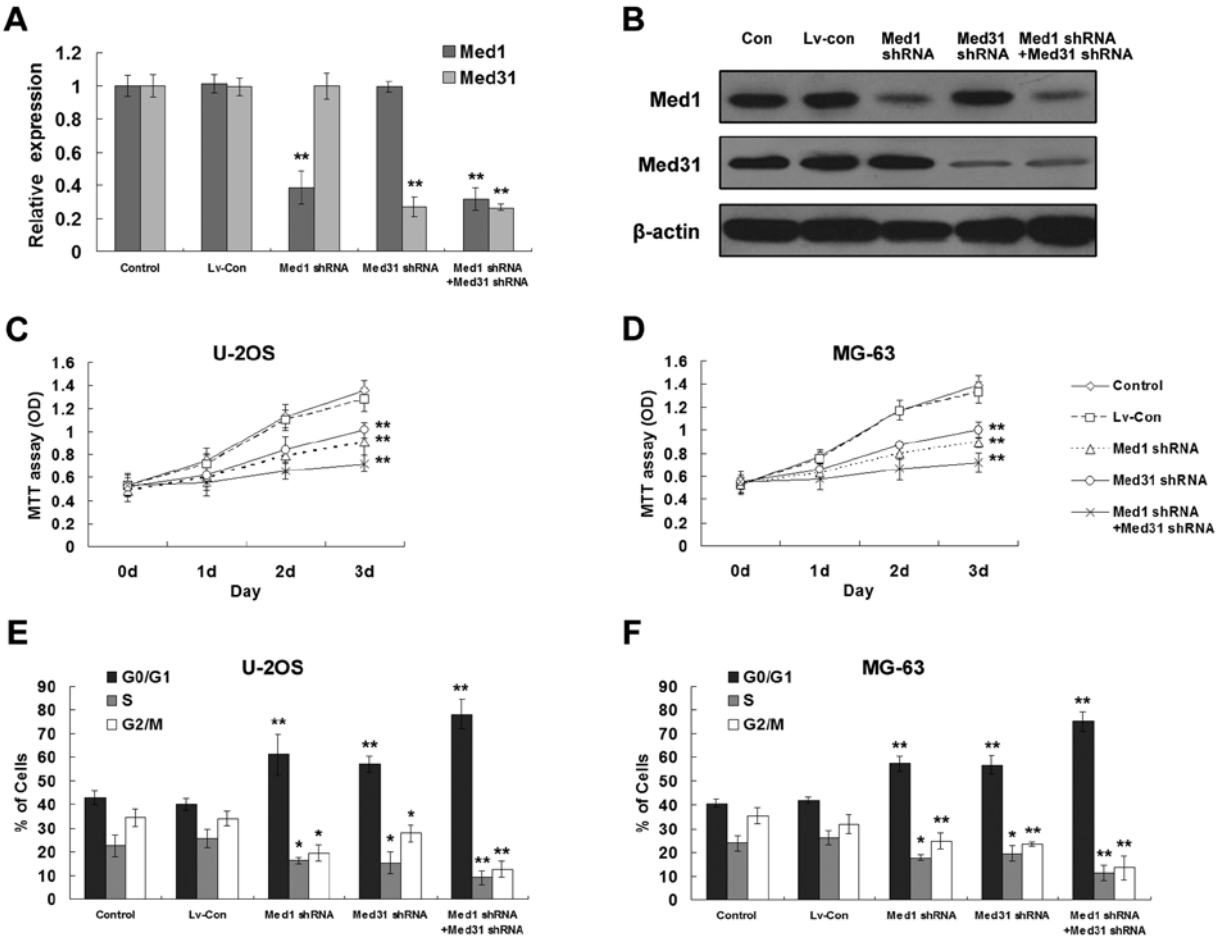

Figure 5. Downregulation of Med1 and/or Med31 suppresses the proliferation of osteosarcoma cells. U2OS cells were infected with Lv-con, Lv-Med1 shRNA, Lv-Med31 shRNA or Lv-Med1 shRNA plus Lv-Med31 shRNA. (A) Relative expression levels of Med1 and Med31 mRNA were detected by real-time PCR. (B) Expression levels of Med1 and Med31 were detected by western blotting. U2OS and MG63 cells were transfected with or without Lv-con, Med1 shRNA, Med31 shRNA or cotransfected with Med1 shRNA and Med31 shRNA. Cell proliferation of U2OS (C) and MG63 (D) cells was measured at the indicated time periods using MTT assay. Cell cycle progression of U2OS (E) or MG63 (F) cells was analyzed by flow cytometry. $^{*}p<0.05$, $^{**}p<0.01$ compared with the control.

ment (19). However, the detailed roles of these miRNAs in osteosarcoma progression are largely unknown. Here, we found that miR-1 was downregulated in osteosarcoma and targets Med1 and Med31. Both Med1 and Med31 were involved in the proliferation of osteosarcoma cells through influencing cell cycle progression. Furthermore, MET signaling may be an important downstream target of Med1 and Med31.

Previously studies have found that the expression levels of miR-1 are significantly reduced in lung cancer (13), colorectal cancer (20) and rhabdomyosarcoma (21). DNA methylation may partly explained miR-1 silencing (14). Our study showed that miR-1 was downregulated in osteosarcoma and restoration of miR-1 reduced cell proliferation, which was consistent with the findings of other researchers (11,12). Although several target genes of miR-1 have been validated in other cancers (13-16), no explicit target has been confirmed in osteosarcoma. In the present study, we found two new targets of miR-1, Med1 and Med31. Notably, both are subunits of MED.

MED is a multiprotein complex which plays a critical role in the regulation of eukaryotic mRNA synthesis through direct interactions with RNA Pol II and other transcriptional regulators, such as activators and transcription factors (17,22). To date, 30 distinct MED subunits (MEDs) have been found and different MED subunits may interact with the activation domain of different activators (17,23). Recent studies have suggested that a number of subunits, other than the complex itself, have a role in tumorigenesis. The deregulation of MEDs has been found in a variety of tumors including osteosarcoma (24-26). Med1 has equivocal functions in different tumors. It functions as a tumor-suppressor gene and inhibits invasion and metastasis in lung carcinomas (27) and melanoma cells (28). Conversely, deficiency of Med1 protects hepatocytes from chemical carcinogen-induced hepatocarcinogenesis (29), and loss of Med1 significantly decreased proliferation of prostate cancer cells (30). Med31 has not been found to be associated with tumorigenesis, but a recent study suggests that it is required for cell proliferation during mammalian development (31).

We found that both Med1 and Med31 were upregulated in osteosarcoma tissues or cell lines when compared with normal tissue or cells. Although Med1 and Med31 are located in different modules of MED (17), they showed similar effects in osteosarcoma progression. Downregulation of both Med1 and Med31 or either of them suppressed the proliferation of osteosarcoma cells, suggesting that they function as oncogenes in osteosarcoma. Both Med1 and Med31 were identified to be

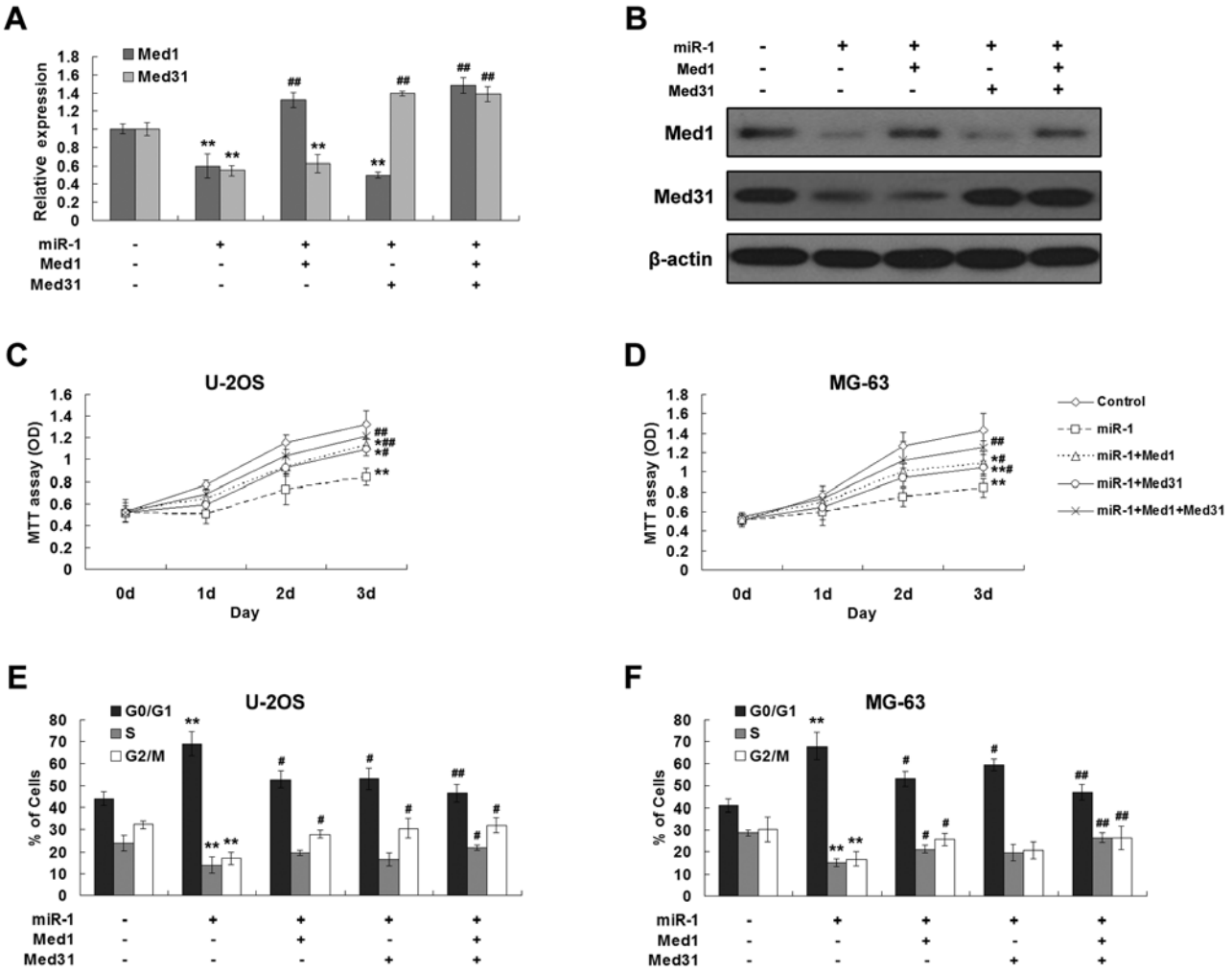

Figure 6. Overexpression of Med1 and/or Med31 restores the proliferation of osteosarcoma cells inhibited by miR-1. U2OS cells were infected with or without Ad-Med1 and/or Ad-Med31 for one day, and then were transfected with miR-1 for 48 h. (A) Relative expression levels of Med1 and Med31 mRNA were detected by real-time PCR. (B) Expression levels of Med1 and Med31 were detected by western blotting. U2OS and MG63 cells infected with or without Ad-Med1 and/or Ad-Med31 were transfected with miR-1. Cell proliferation of U2OS (C) and MG63 (D) cells was measured at the indicated time periods using MTT assay. Cell cycle progression of U2OS (E) and/or MG63 (F) cells was analyzed by flow cytometry. $^{**}p<0.01$ compared with the control, $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with miR-1.

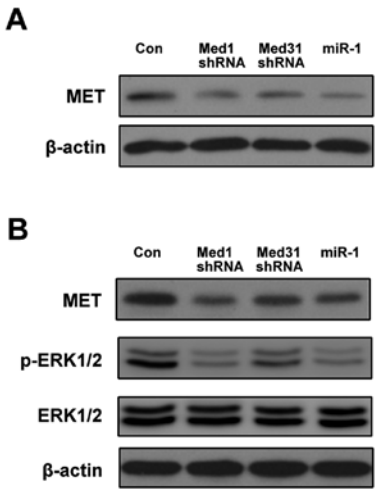

Figure 7. Downregulation of Med1 or Med31 suppresses MET signaling. U2OS cells were transfected with or without Med1 shRNA, Med31 shRNA or miR-1. (A) Protein expression of MET was detected by western blotting. (B) After stimulation with 30 ng/ml HGF for 48 h, protein expression levels of MET, ERK1/2 and p-ERK1/2 were evaluated by western blotting.

new direct targets of miR-1 in osteosarcoma, and overexpression abrogated the effects of miR-1, suggesting that miR-1 exerts its antitumor function via inhibition of Med1 and Med31 expression.

We further investigated the potential signaling pathway linking these Meds and cell proliferation and found that MET was a possible pathway. The Met gene encodes the tyrosine kinase receptor for HGF/SF. Met activation may induce proliferation, angiogenesis or stimulate motility to form micrometastases in tumor (32). Loss of Med1 resulted in the reduction of MET mRNA level in hepatocytes, suggesting Med1 could induce the expression of MET. Our data showed that the downregulation of either Med1 or Med31 suppressed the expression of MET and blocked the downstream signaling of MET responding to HGF. In combination with previous studies in which MET was identified as a target of miR-1 in other types of cancer (13,14), MET signaling may play a crucial role in osteosarcoma cell proliferation and may be regulated by miR-1 directly or through Med1 and Med31 indirectly.

In conclusion, taken together, our results demonstrate that miR-1 has great biological effect on the proliferation of osteosarcoma cells. This effect of miR-1 was mediated by the direct inhibition of Med1 and Med31. Both Med1 and Med31 were overexpressed in osteosarcoma and downregulation of Med1 and Med31 suppressed the proliferation of osteosarcoma cells. Furthermore, MET signaling may be involved in osteosarcoma cell proliferation regulated by miR-1. The present findings suggest that MEDs may serve as potential gene therapeutic targets in osteosarcoma and miR-1 may prove to be a promising agent.

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