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MicroRNA-384 downregulates SETD8 expression to suppress cell growth and metastasis in osteosarcoma cells

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Abstract. – OBJECTIVE: MiR-384 was reported to be downregulated and functioned as a tumor suppressor in several cancers. However, the expression and function of miR-384 in osteosarcoma (OS) have not been investigated. In the present study, we aimed to analyze the effect and mechanism of miR-384 in the progression of OS.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was used to determine the expression of miR-384 in OS tissues and cells. MTT assay, colony formation analysis, Transwell assays were performed to analyze the role of miR-384 in human OS cells. Western blotting was applied to analyze the expression of SETD8, and the luciferase reporter assay was used to assess the target gene of miR-384 in OS cells.

RESULTS: We found that miR-384 was significantly lowly expressed in OS tissues and OS cell lines compared with the adjacent noncancerous tissues and normal bone cell lines, respectively. Further functional analysis indicated that up-regulation of miR-384 significantly inhibited OS cells proliferation, migration, and invasion, but down-regulation of miR-384 had the opposite effects on OS cells in vitro. Moreover, SETD8 was identified as the potential target of miR-384 using dual luciferase assay, qRT-PCR and Western blot. Finally, we observed that upregulation of SETD8 reversed the effects of overexpressing of miR-384 on the proliferation, migration, and invasion of OS.

CONCLUSIONS: Our data provided the first evidence which supported the function of miR-384 as a tumor suppressor in OS by targeting SETD8.

Key Words:
miR-384, SETD8, Osteosarcoma, Proliferation, Migration, Invasion.

Introduction

Osteosarcoma (OS), the most common primary malignant bone tumor, predominantly affects children, adolescents, and young adults. 70-75% of patients are between 10 and 25 years old[1,2]. Despite the rapid development of diagnoses and therapies, the overall survival rate of osteosarcoma has not been substantially improved[3]. Because of the side effects and development of resistance, the common treatment, chemotherapy, is limited[4]. In addition, OS usually presents a high tendency to metastatic spread[5]. These problems result in high mortality. Therefore, it is vital to clarify the molecular mechanism of OS development for the treatment of this disease.

MicroRNAs (miRNAs) are a family of short non-coding RNAs that negatively regulate gene expression at the post-transcriptional level[6]. Recent studies[7,8] have revealed critical functions of miRNAs in essential biological processes, including proliferation, differentiation, and apoptosis. By regulating a variety of targets in cancer cells, miRNAs generally acted as oncogene or tumor suppressor[9,10]. For instance, miR-448 was reported to serve as a tumor suppressor in lung squamous cell carcinoma by targeting DCLK1[11]. Forced expression of miR-1271 suppressed endometrial cancer cells proliferation by targeting CDK1[12]. For OS, Xie et al[13] revealed miR-397 as a tumor suppressor by inhibiting the proliferation, migration, and invasion through targeting EIF4G2. Those results indicated the important potential of miRNAs as a therapeutic target for OS.

MiR-384, located in Xq21.1, has been reported to be down-regulated in several tumors, including colorectal cancer, non-small-cell lung cancer, and hepatocellular carcinoma[14-16]. Also, *in vitro* and *in vivo* assay suggested that miR-384 function as tumor suppressor in above tumors. However, the expression pattern and biological effect of miR-384 in the progression of OS have not been reported.

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Patients and Methods

Clinical Specimens

A total of 35 osteosarcoma samples and matched adjacent non-tumor samples were collected at Southern Medical University between January 2015 and January 2016. All the samples were collected before any treatment. The specimens were immediately frozen in liquid nitrogen and stored at -80°C until use. Written informed consent was obtained from all of the patients. Approval for this work was granted by the Ethics Committee of Southern Medical University.

Cell Culture and Transfection

Four OS cell lines (MG-63, HOS, SaOS-2, and U2OS) and human osteoblasts (hFOB1.19) cells were brought from Jennio Biotech Corp (Guangzhou, Guangdong, China). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 incubator.

MiR-384 mimics and miR-384 inhibitor and negative control were purchased from Ambion (Ambion Life Technologies, Austin, TX, UK). SETD8 overexpressed plasmids were obtained from Ribobio Co. (Guangzhou, Guangdong, China). Transfection of miRNA mimics or plasmid was conducted using 3 μL Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Transfection efficiencies were evaluated in every experiment by qRT-PCR.

RNA Extraction and qRT-PCR

Total RNA was extracted from clinical specimens and cells with TRIzol reagent according to the manufacturer's instructions. 1 μg total RNA was reverse transcribed into cDNA using the miScript reverse transcription kit according to the manufacturer's instructions. Quantitative reverse transcription-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan) and the ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The expression of miR-384 was normalized to U6 small nuclear RNA. The primers used are shown in Table I. The comparative $2^{-\Delta\Delta Ct}$ method was used for relative quantification and statistical analysis.

Cell Proliferation Assay

Cells were cultured in 96-well plates about 1.0×10^4 cells/mL. Then, the cells were incubated with 20 μl of 5 mg/ml methyl thiazolyl tetrazolium (MTT – Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37°C, and 150 μl of dimethyl sulfoxide (DMSO – Sigma-Aldrich, St. Louis, MO, USA) was added. Cell viability was assessed by the absorbance at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). All experiments were performed three times.

Colony Formation Assays

After translation, the cells were inoculated into six-well plates and maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium contained 10% fetal bovine serum (FBS) for two weeks. Then, colonies were fixed and stained with 0.05% crystal violet. Visible colonies were then manually counted. For each treatment group, wells were counted in triplicate.

Cell Migration and Invasion Assays

For migration assay, 1×10^5 cells in 100 μl serum-free DMEM were placed on the non-coated membrane in the upper chamber. For invasion assay, Matrigel (BD Biosciences, Beijing, China) was added. Media containing 20% FBS were added to the lower chamber. The cells attached to the lower surface of the membrane were fixed in 4% paraformaldehyde at room temperature for 30 min and stained with 4,6-diamidino-2-phenylindole (DAPI). Finally, cells were counted in five randomly assigned fields under a microscope (Olympus, Tokyo, Japan).

Table I. Sequence of the primers used in this study.

Genes	Sequences
MiR-384	Forward primer: 5'-TGTTAAATCAGGAATTTTAA-3' Reverse primer: 5'-TGTTACAGGCATTATGAA-3'
SETD8	Forward primer: CCATGGCTAGAGGCAGGAAG Reverse primer: TTCCGGCTAATGGTTTCCCC
U6	Forward primer: 5'-CGCTTCGGCAGCACATATAC-3' Reverse primer: 5'-TTCACGAATTTGCGTGTCAT-3'.

Luciferase Reporter Assay

Reporter constructs containing pGL3-SETD8 and pGL3-MUT SETD8 (with a mutated target seed sequence) were obtained from Bio-Asia (Haidian, Beijing, China). Cells (5 $\times 10^4$/well) were cultured in 24-well plates and, then, co-transfected with pGL3- SETD8-3'UTR and miR-384 mimic miR-384-MUT or control mimics using Lipofectamine 2000 Reagent. Forty-eight hours later, Luciferase activity was measured using the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA). Renilla-luciferase was used for normalization.

Western Blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the protein samples that were further transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Songjiang, Shanghai, China). Then, the membranes were exposed to primary and secondary antibodies at optimum dilution. Subsequently, the target bands were visualized with the Super Signals West Dura Extended Duration Substrate as instructed. The primary antibodies were to SETD8 (1:1000, ProteinTech, Donghu, Wuhan, China), GAPDH (1:5000; ProteinTech, Donghu, Wuhan, China), and anti-rabbit IgG-HRP were purchase from CST (Danvers, MA, USA). The experiments were carried out on three separate occasions.

Statistical Analysis

All the statistical analyses were performed using SPSS18.0 for Windows (SPSS Inc., Chicago, IL, USA). The Student *t*-test was used to estimate the significant differences between groups. $p < 0.05$ was considered as statistically significant.

Results

MiR-384 Was Commonly Downregulated in OS Tissues and Cell Lines

Expression of miR-384 was measured in OS samples and adjacent non-tumor tissues using Real-time PCR. As shown in Figure 1A, we observed that the expression of miR-384 was downregulated in the OS tissues compared with adjacent no-tumor tissues ($p < 0.01$). Moreover, we detected the levels of miR-384 in OS cell lines. As shown in Figure 1B, the data indicated that the expression of miR-384 was evidently decreased in human OS cell line (HOS, SaOS-2, MG-63, and U2OS) compared with osteoblast cell line. Taken together, our results suggested miR-384 as an anticancer miRNA.

MiR-384 Suppressed the Proliferation, Migration, and Invasion of OS Cells

Based on the results that miR-384 was down-regulated in OS, we next investigated the biological function of miR-384 in OS. As shown in Figure

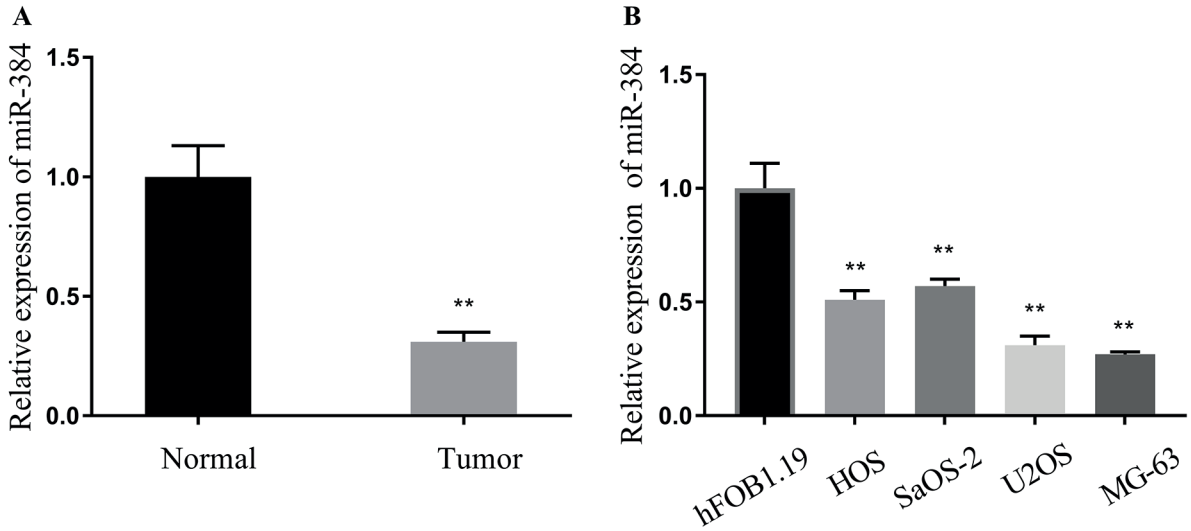

Figure 1. The expression levels of miR-384 in OS tissues and cell lines. ***(A)*** qRT-PCR was performed to measure relative miR-384 expression in NSCLC tissues and adjacent non-tumor tissues. ***(B)*** qRT-PCR of miR-384 expression in OS cell lines (MG-63, HOS, SaOS-2 and U2OS) and human osteoblasts (hFOB1.19). The relative expression of miR-384 was normalized to U6. *$p < 0.05$; **$p < 0.01$.

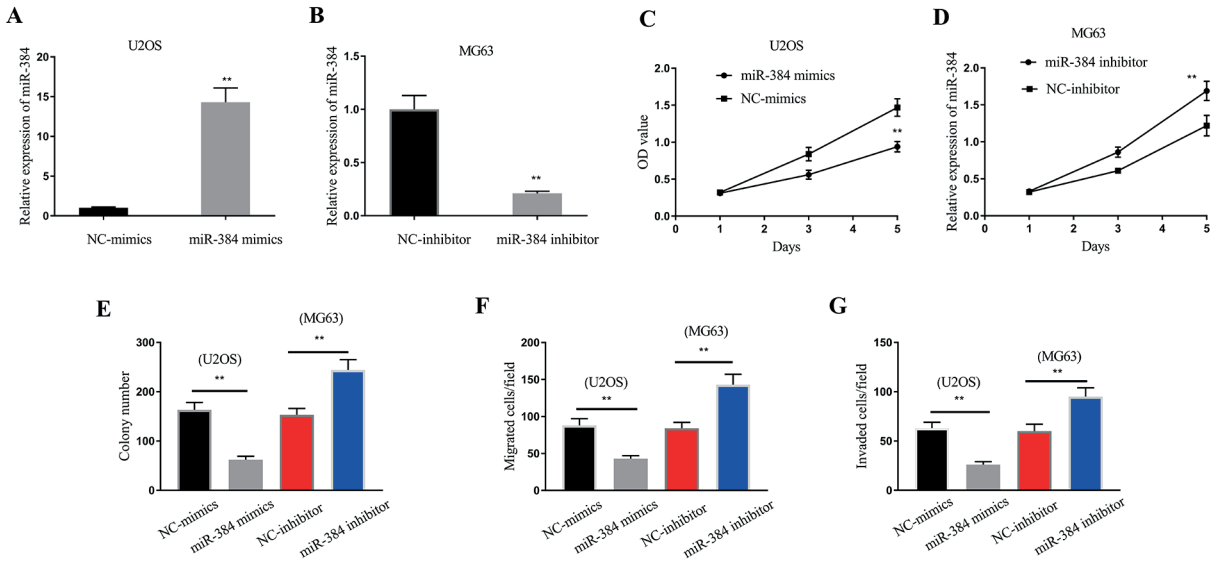

Figure 2. The effect of miR-384 on the proliferation, migration and invasion of OS cells. ***(A)*** The expression levels of miR-384 in U2OS cells transfected with miR-384 mimics. ***(B)*** The expression levels of miR-384 in MG63 cells transfected with miR-384 inhibitor. ***(C-D)*** The proliferation of U2OS and MG63 cells with aberrant expression of miR-384 was evaluated by MTT assay. ***(E)*** The long-term cell viability was evaluated using the colony formation assay. ***(F-G)*** Transwell assays were used to investigate the changes in the migratory and invasive abilities of U2OS and MG63 cells. $*p < 0.05$; $**p < 0.01$.

2A and 2B, miR-384 was overexpressed or repressed in MG-63 cells and U2OS cells by transfection with miR-384 mimic or miR-384 inhibitor. MTT and colony formation assay showed that U2OS cells with miR-384 mimics proliferated at a slower rate than control cells, and MG63 cells with miR-384 inhibitor proliferated at a faster rate than control cells (Figure 2C, 2D and 2E). Moreover, Transwell assays indicated that miR-384 overexpression significantly decreased the migration (Figure 2F; $p < 0.01$) and invasion (Figure 2G; $p < 0.01$) of U2OS cells. In contrast, down-regulation of miR-384 significantly increased the migration (Figure 2F; $p < 0.01$) and invasion (Figure 2G; $p < 0.01$) of MG63 cells.

SETD8 is a Direct Target of miR-384

To explore the mechanisms of miR-384 in OS cells, we used bioinformatics analysis to find the direct target of miR-384. We found that there was a putative miR-384 binding seed site in the 3'UTR of SETD8 (Figure 3A). Then, we performed luciferase reporter assay to confirm our hypothesis. The target sequence of the wild-type (WT) SETD8 3'-UTR or mutant (MUT) SETD89 3'-UTR was cloned into a luciferase reporter vector (Figure 3A). Then, we observed that up-regulation of miR-384 resulted in a significant downregulation in the luciferase activities of the pGL3-SETD8-3'UTR Wt (Figure 3B, $p < 0.05$), whereas pGL3-SETD8-3'UTR MUT blocked this effect (Figure 3B and 3C). Subsequently, we used RT-PCR to detect the levels of miR-384 and SETD8 mRNA in OS tissues. The results of Spearman's correlation test showed a significant inverse association between miR-384 and SETD8 mRNA (Figure 3D). Moreover, the protein expression of SETD8 was decreased by miR-384 mimic in U2OS and miR-384 inhibitor played an opposing role in SETD8 expression (Figure 3E). Together, these results suggest that SETD8 is a target of miR-384 in OS.

Overexpression of SETD8 Rescues the Effects of miR-384 in OS Cells

Having demonstrated that SETD8 is a direct target of miR-384, we further explore whether SETD8 re-expression could reverse the tumor-suppressor function of miR-384 in OS. MG63 cells were co-transfected with miR-384 mimics or NC-mimics and overexpressed SETD8 plasmid. Up-regulation of SETD8 was confirmed by qRT-PCR and Western blot (Figure 4A and 4B). The results of MTT and colony formation assay indicated that the forced SETD8 expression rescued the inhibition effect of miR-384 on cell proliferation (Figure 4C and 4D). Furthermore, transwell assay revealed that the inhibition effect of miR-

384 on cell migration and invasion was rescued by up-regulation of SETD8 (Figure 4E and 4F). These results suggested that the role of miR-384 as one tumor suppressor in OS was partly due to its regulation on SETD8.

Discussion

OS has become a serious burden for all countries of the world with, with an estimated worldwide yearly incidence rate of 4 million[17]. Several investigations have manifested the pivotal role of miRNAs in the development of OS, indicating a promising strategy against this disease[18]. In the present study, we found that miR-384 was significantly low expressed in OS tissues and cell lines. Overexpression of miR-384 could significantly inhibit OS cell proliferation, invasion, and migration, but inhibition of miR-384 exerted a contrary effect. Our data for the first time indicated that miR-384 may act as a tumor suppressor in OS.

According to previous researches, miR-384 was found to be abnormally expressed in several tumors. Growing studies reported the biological function and potential mechanism of miR-384 in tumor development. For instance, Song et al[19] reported that the expression levels of miR-384 was significantly down-regulated in renal cell carcinoma and its overexpression significantly inhibited the growth and invasion of RCC cells by targeting astrocyte elevated gene 1. Lai et al[20] revealed that miR-384 was significantly low expressed in hepatocellular carcinoma and exerted a suppressive effect on hepatocellular carcinoma cell proliferation through targeting IRS1. Wang et al[14] showed that low expression of miR-384 was closely correlated with poor prognosis of colorectal cancer patients. Functionally, forced miR-384 expression suppressed human colorectal cancer metastasis by targeting KRAS and CDC42. These results highlighted the potential role of miR-384 in tumor oncogenesis or progression. Then, we wondered whether miR-384 also exerted a similar effect in OS. Our *in vitro* experiment confirmed our hypothesis.

To explore the potential mechanism by which miR-384 suppressed proliferation and metastasis in OS, the identification of cancer-specific miRNAs and their target genes is essential. We performed bioinformatic analysis and found that SETD8 was a target of miR-384. SETD8 was an important oncogene in various tumors, including breast cancer, lung cancer, and OS[21-23]. Of note, a recent investigation by Zhang et al[24] showed that miR-127-3p targeted SETD8 to inhibit prolifera-

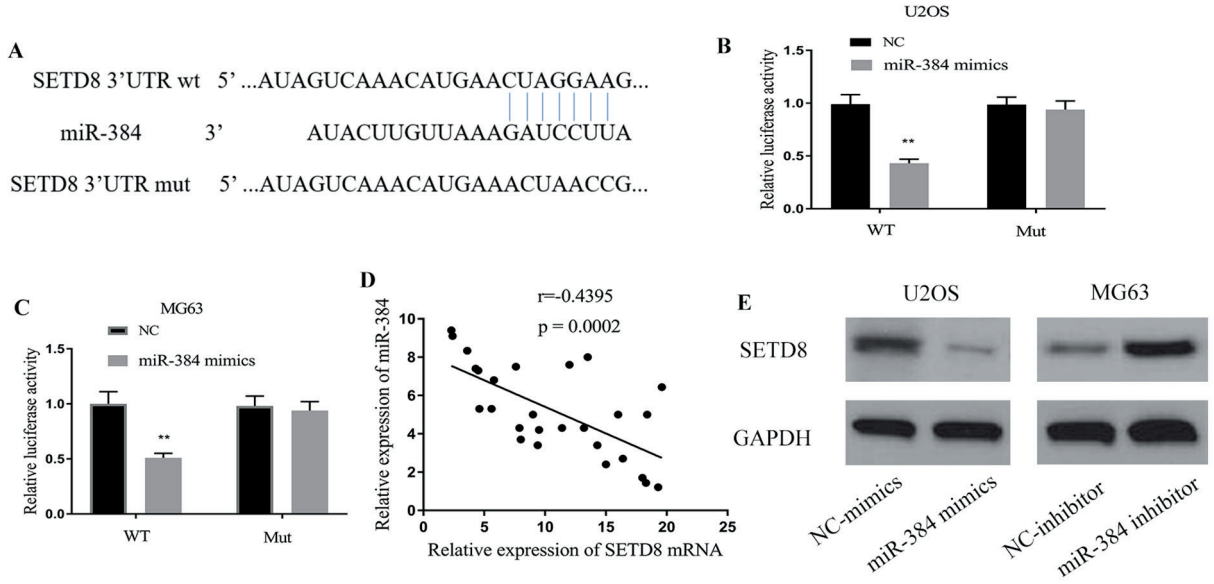

Figure 3. SETD8 is the direct target of miR-384. ***(A)*** Sequence alignment between miR-384 and the 3'UTR of human SETD8 mRNA and mutated nucleotides in the SETD8 3'-UTR (SETD8-3'UTR-MUT). ***(B-C)*** The analysis of the relative luciferase activities of SETD8-WT, SETD8-MUT in U2OS and MG63 cells. ***(D)*** An inverse correlation between miR-384 and SETD8 expression in the same set of OS was detected through Spearman's correlation analysis. ***(E)*** SETD8 protein expressions levels were detected after miR-384 mimics transfected in U2OS and miR-384 inhibitor transfected in MG63 by Western blot. *$p <$ 0.05; **$p < 0.01$.

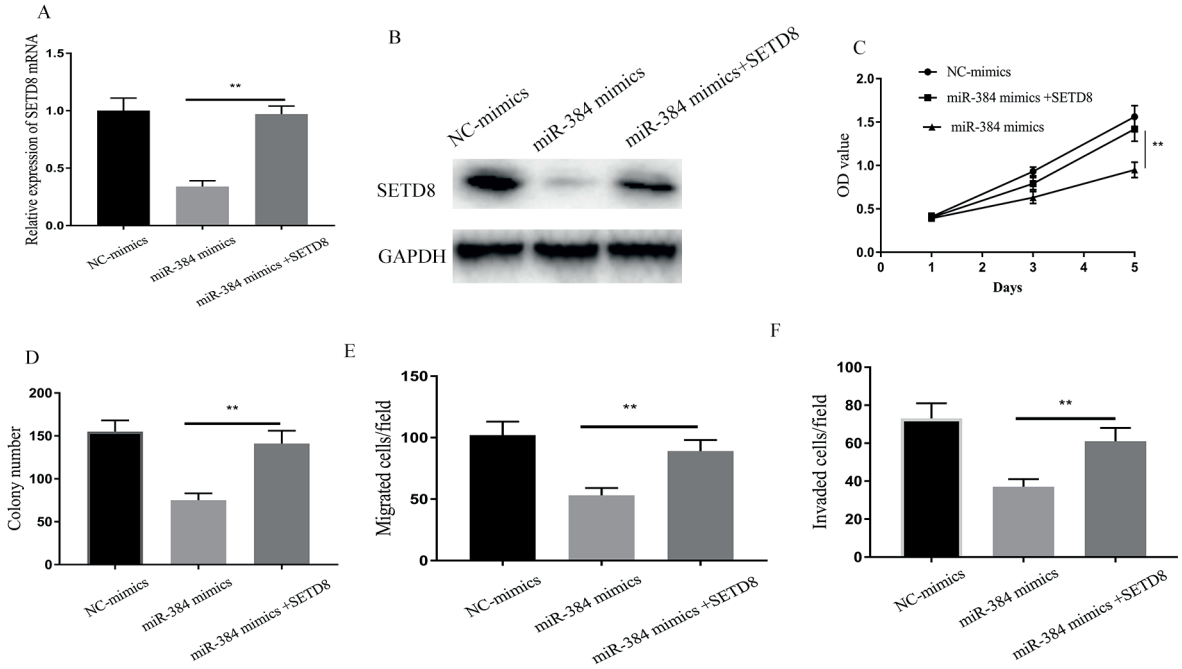

Figure 4. Overexpression of SETD8 rescues the effects of miR-384 in MG63 cells. ***(A-B)*** SETD8 mRNA and proteins in MG63 cells co-transfected with SETD8 overexpression plasmid and miR-384 mimic or NC-mimics by qRT-PCR and Western blot, respectively. ***(C)*** MTT assay was performed to detect the proliferation of MG63. ***(D)*** The long-term cell viability was evaluated using the colony formation assay. ***(E-F)*** Transwell assays were used to investigate the changes in the migratory and invasive abilities of MG63 cells transfected with miR-384 mimic with/without SETD8 overexpression plasmid. *$p < 0.05$; **$p < 0.01$.

tion and invasion in human osteosarcoma cells. Combining our previous results, we suggested that SETD8 may be a target of miR-384. Subsequently, luciferase reporter confirmed our suggestion. The data of PCR and Western blot also showed that overexpression of miR-384 could suppress the levels of SETD8 proteins and mRNA. Moreover, we performed *in vitro* experiment to validate whether overexpression of SETD8 could rescue the effects of miR-384 in OS cells. The results confirmed miR-384 as a tumor suppressor by targeting SETD8.

Conclusions

Our study firstly revealed that miR-384 inhibited the proliferation, migration, and invasion of OS cells *in vitro* by directly targeting SETD8, indicating that miR-384 can serve as a potential therapeutic target for OS.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Geller DS, Gorlick R. Osteosarcoma: a review of diagnosis, management, and treatment strategies. Clin Adv Hematol Oncol 2010; 8: 705-718.
2) Ottaviani G, Jaffe N. The epidemiology of osteosarcoma. Cancer Treat Res 2009; 152: 3-13.
3) Durnali A, Alkis N, Cangur S, Yukruk FA, Inal A, Tokluoglu S, Seker MM, Bal O, Akman T, Inanc M, Isikdogan A, Demirci A, Helvaci K, Oksuzoglu B. Prognostic factors for teenage and adult patients with high-grade osteosarcoma: an analysis of 240 patients. Med Oncol 2013; 30: 624.
4) Ferrari S, Serra M. An update on chemotherapy for osteosarcoma. Expert Opin Pharmacother 2015; 16: 2727-2736.
5) Osborne TS, Khanna C. A review of the association between osteosarcoma metastasis and protein translation. J Comp Pathol 2012; 146: 132-142.
6) Cullen BR. Viral and cellular messenger RNA targets of viral microRNAs. Nature 2009; 457: 421-425.
7) Lujambio A, Lowe SW. The microcosmos of cancer. Nature 2012; 482: 347-355.
8) Zhao B, Dong AS. MiR-874 inhibits cell growth and induces apoptosis by targeting STAT3 in human colorectal cancer cells. Eur Rev Med Pharmacol Sci 2016; 20: 269-277.

9) Zhang J, Yan YG, Wang C, Zhang SJ, Yu XH, Wang WJ. MicroRNAs in osteosarcoma. Clin Chim Acta 2015; 444: 9-17.

10) Cheng ZX, Song YX, Wang ZY, Wang Y, Dong Y. miR-144-3p serves as a tumor suppressor by targeting FZD7 and predicts the prognosis of human glioblastoma. Eur Rev Med Pharmacol Sci 2017; 21: 4079-4086.

11) Shan C, Fei F, Li F, Zhuang B, Zheng Y, Wan Y, Chen J. miR-448 is a novel prognostic factor of lung squamous cell carcinoma and regulates cells growth and metastasis by targeting DCLK1. Biomed Pharmacother 2017; 89: 1227-1234.

12) Li L, Qu YW, Li YP. Over-expression of miR-1271 inhibits endometrial cancer cells proliferation and induces cell apoptosis by targeting CDK1. Eur Rev Med Pharmacol Sci 2017; 21: 2816-2822.

13) Xie X, Li YS, Xiao WF, Deng ZH, He HB, Liu Q, Luo W. MicroRNA-379 inhibits the proliferation, migration and invasion of human osteosarcoma cells by targetting EIF4G2. Biosci Rep 2017; 37.

14) Wang YX, Chen YR, Liu SS, Ye YP, Jiao HL, Wang SY, Xiao ZY, Wei WT, Qiu JF, Liang L, Liao WT, Ding YQ. MiR-384 inhibits human colorectal cancer metastasis by targeting KRAS and CDC42. Oncotarget 2016; 7: 84826-84838.

15) Fan N, Zhang J, Cheng C, Zhang X, Feng J, Kong R. MicroRNA-384 represses the growth and invasion of non-small-cell lung cancer by targeting astrocyte elevated gene-1/Wnt signaling. Biomed Pharmacother 2017; 95: 1331-1337.

16) Chen Z, Yu C, Zhan L, Pan Y, Chen L, Sun C. LncRNA CRNDE promotes hepatic carcinoma cell proliferation, migration and invasion by suppressing miR-384. Am J Cancer Res 2016; 6: 2299-2309.

17) Clark JC, Dass CR, Choong PF. A review of clinical and molecular prognostic factors in osteosarcoma. J Cancer Res Clin Oncol 2008; 134: 281-297.

18) Sampson VB, Yoo S, Kumar A, Vetter NS, Kolb EA. MicroRNAs and potential targets in osteosarcoma: review. Front Pediatr 2015; 3: 69.

19) Song H, Rao Y, Zhang G, Kong X. MicroRNA-384 inhibits the growth and invasion of renal cell carcinoma cells by targeting astrocyte elevated gene 1. Oncol Res 2017 Aug 25. doi: 10.3727/096504017X15035025554553. [Epub ahead of print].

20) Lai YY, Shen F, Cai WS, Chen JW, Feng JH, Cao J, Xiao HQ, Zhu GH, Xu B. MiR-384 regulated IRS1 expression and suppressed cell proliferation of human hepatocellular carcinoma. Tumour Biol 2016; 37: 14165-14171.

21) Yu N, Huangyang P, Yang X, Han X, Yan R, Jia H, Shang Y, Sun L. microRNA-7 suppresses the invasive potential of breast cancer cells and sensitizes cells to DNA damages by targeting histone methyltransferase SET8. J Biol Chem 2013; 288: 19633-19642.

22) Chen T, Ren H, Thakur A, Yang T, Li Y, Zhang S, Wang T, Chen M. miR-382 inhibits tumor progression by targeting SETD8 in non-small cell lung cancer. Biomed Pharmacother 2017; 86: 248-253.

23) Abbas T, Shibata E, Park J, Jha S, Karnani N, Dutta A. CRL4(Cdt2) regulates cell proliferation and histone gene expression by targeting PR-Set7/Set8 for degradation. Mol Cell 2010; 40: 9-21.

24) Zhang J, Hou W, Chai M, Zhao H, Jia J, Sun X, Zhao B, Wang R. MicroRNA-127-3p inhibits proliferation and invasion by targeting SETD8 in human osteosarcoma cells. Biochem Biophys Res Commun 2016; 469: 1006-1011.