Regulatory mechanism of microRNA-128 in osteosarcoma tumorigenesis and evolution through targeting SASH1

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Abstract. Osteosarcoma, which commonly occurs in young individuals, is a type of malignant tumor of growing bones. MicroRNAs (miRNAs) have been found to be involved in various cancer-related processes. In the present study, it was reported that miRNA-128 (miR-128) was overexpressed in pathological tissues from patients with osteosarcoma. The present study investigated the possible regulatory mechanism of miR-128 on the progression of osteosarcoma and offered a foundation for clinical therapeutics in osteosarcoma. First, the expressions levels of miR-128 and its target gene, SAM and SH3 domain-containing 1 (SASH1), were measured in tissues from patients with osteosarcoma, and their correlation with osteosarcoma in terms of the pathological level were examined. The effects of miR-128 on osteosarcoma cell proliferation and apoptosis were examined, and its regulation of the expression levels of SASH1 and associated proteins was analyzed. Subsequently, the association between SASH1 and miR-128 was evaluated using a dual luciferase gene reporter assay. Finally, an *in vivo* xenograft tumor mouse model of osteosarcoma was established to confirm the *in vitro* results. The results demonstrated a higher expression of miR-128 in pathological tissues, compared with that in normal tissues. From examining the patient osteosarcoma tissues, marked correlations were found between the expression of miR-128 and that of SASH1, particularly with tumor size, invasion depth, lymph node metastasis, and tumor-node-metastasis stage. Compared with the negative control group and blank control group, the results showed that the inhibition of miR-128 led to a lower cell proliferation rate and higher apoptotic rate in MG-63 cells ($P<0.05$). Additionally, the expression of B-cell lymphoma 2 (Bcl-2) was downregulated in the miR-128-inhibited group, compared with that in the control group, whereas the expression levels of SASH1, Bcl-2-associated X protein and caspase-3 were upregulated in the group with miR-128 inhibition ($P<0.05$). SASH1 was confirmed as a direct target of miR-128 using a dual luciferase gene reporter assay. Finally, the downregulation of miR-128 was found to induce tumor suppressive effects on xenograft tumor models of osteosarcoma in mice *in vivo*. The results of the present study suggested that miR-128 may regulate the tumorigenesis and evolution of osteosarcoma through targeting SASH1.

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Introduction

Osteosarcoma is the most commonly occurring sarcoma in bones. It is derived from the progenitor cells in the osteoblast lineage with accumulated mutations to evade cell cycle checkpoints, resulting in excessive proliferation and defects in the ability to appropriately differentiate into mature bone-forming osteoblasts (1). The majority of osteosarcoma cases occur in young individuals, particularly children and adolescents (2). It is the second leading cause of cancer-associated mortality in children and young adults worldwide (3). As an aggressive sarcoma, patients with osteosarcoma are prone to relapse and the prognosis of osteosarcoma remains poor despite advances in therapeutic strategies, including surgery and chemotherapy (4). Statistics indicate that modern, multi-agent therapeutic strategies, for example dose-intensive chemotherapy in conjunction with surgery, can only achieve a 3-year event-free survival of 60-70% in particularly localized, non-metastatic disease (5). However, this aggressive sarcoma has a high metastatic potential, particularly to the lungs, with ~20% of patients presenting with lung metastases at initial diagnosis (6). It is reported that ~80% of patients with osteosarcoma eventually develop metastatic disease following treatment, which is the major cause of treatment failure and contributor to mortality rates (7). Fortunately, it has been noted that tumorigenesis, progression and the efficacy of osteosarcoma treatment are closely associated with gene expression (8). According to evidence, malignant transformation and tumorigenesis can result from the dysregulation of tumor suppressor genes (9), and previous studies have indicated that the dysregulation of tumor suppressor genes is a key contributor for the initiation and development of osteosarcoma (8,10). Therefore, tumor gene therapy, which is a form of tumor biotherapy, has promising potential in osteosarcoma treatment.

MicroRNAs (miRNAs) are single-stranded RNAs with a length of 20-23 nucleotides, which can control gene expression in a variety of cellular processes (11). The primary function of miRNAs is to act as negative regulators of gene expression at the post-transcriptional level (12). miRNAs typically reduce the translation and stability of mRNA, affect the output of numerous protein-coding genes and mediate processes in tumorigenesis, including cell cycle regulation, cell differentiation, invasion, apoptosis and inflammation (13). It has been found that >4,000 mature miRNAs in humans regulate ~30% of mammalian protein-coding genes (14). miRNA-128 (miR-128) is an important member of the miRNA family. It is a brain-enriched miRNA with tissue-specific and developmental-specific expression patterns, predominantly in neurons (14). However, in addition to tissues, the aberrant expression of miR-128 is detected in the blood of patients with types of malignant tumor, including leukemia, glioblastoma and prostate cancer (14,15). Studies have shown that miR-128 can regulate the proliferation, differentiation and apoptosis of various tumor cells through targeting several genes, indicating its importance in tumorigenesis and development (14,16). It has been reported that the expression of miR-128 is significantly increased in osteosarcoma tissues (17,18). However, the specific correlation between this ectopic overexpression of miR-128 and osteosarcoma, and the regulatory mechanism of the expression of miR-128 towards osteosarcoma cells remain to be fully elucidated.

In the present study, the regulatory mechanism of miR-128 in the progression of osteosarcoma cells through targeting SAM and SH3 domain-containing 1 (SASH1) was examined. Multifarious methods were applied to measure the expression of miR-128 and SASH1 in osteosarcoma cells, and to examine the effects of miR-128 on osteosarcoma cell proliferation, apoptosis, and the expression of SASH1 and certain associated proteins. The present study on the potential mechanism of miR-128 regulation through targeting SASH1 may offer a foundation for the clinical therapeutic treatment of osteosarcoma.

Materials and methods

Specimens. A total of 35 fresh osteosarcoma tissue and adjacent normal bone tissue specimens were collected from patients at the Second Hospital of Xiangya Medical College of Central South University (Changsha, China) from December 2015 to January 2017. The patients included 20 men and 15 women, 25 of which were aged <60 years and 10 were aged >60 years. All 35 cases were pathologically diagnosed as osteosarcoma postoperatively without any preoperative chemotherapy and radiotherapy. The present study was approved by the Second Hospital of Xiangya Medical College of Central South University (Changsha, China). Informed consent was obtained from each patient.

Cell culture. The MG-63 human osteoblastic cell line (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) was cultivated in RPMI-1640 medium supplemented with 20% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated at 37˚C in a 5% CO_2 humidified incubator. The cells were divided into three groups: i) miR-128-inhibited group (transfected with miR-128 inhibitor); ii) blank group (without miR-128 inhibitor); and iii) negative control (NC) group (to eliminate non-sequence specific effects). The logarithmic growth phase cells were trypsinized, counted and seeded in six-well plates at $1x10^6$ cells/well. The miR-128 inhibitors or NC vectors were then transfected into cells with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The transfection efficiency was measured using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The miR-128 mimics, inhibitor and NC oligonucleotides were purchased from Genepharma Co., Ltd. (Shanghai, China), and the sequences of the oligonucleotides were as follows: miR-128 mimics, 5'-UCACAGUGAACCGGUCUCUUU-3'; miR-128 inhibitor, 5'-AAAGAGACCGGUUCACUGUGA-3'; NC, 5'-UGUCCU CCUGGAAUUACACGU-3'.

Analysis of cell proliferation. At 72 h post-transfection, the cells were washed with PBS (pH 7.4), harvested by trypsinization, reseeded into a 96-well plate at a density of 10^5 cells/well and cultured in RPMI-1640 medium. The cells were then incubated for 6-48 h for the MTT cell proliferation assay. Briefly, 10 μl MTT reagent was added to the cells and incubated for 4 h at 37˚C until purple precipitate was visible. Subsequently, 100 μl detergent reagent was added and left at room temperature in the dark for 2 h. The absorbance in each well, including the blanks, was measured at 570 nm in a microtiter plate reader.

Analysis of cell apoptosis. The apoptosis of MG-63 human osteoblastic cells from the three groups was analyzed with an Annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (BD Biosciences, San Diego, CA, USA) using the flow cytometry method according to the manufacturer's protocol. Briefly, following transfection for 72 h, the cells were trypsinized and washed three times with pH 7.4 PBS buffer, followed by centrifugation at 1,000 x g for 5 min at room temperature and then dispersal in the buffer provided with the apoptosis kit. Subsequently, 5 μl each of Annexin V-FITC and propidium iodide (PI) solutions were added and mixed gently at room temperature. Following incubation on ice in the dark for 30 min, the cells were quantified by flow cytometry. Cells showing positive Annexin V-FITC and negative PI results were considered to be apoptotic. The basal apoptosis and necrosis were also determined in untreated cells.

Analysis of the expression of miR-128 via RT-qPCR analysis. Total RNA was extracted using an miRNeasy kit according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). To detect the expression of miR-128 in 35 paired tumor and adjacent tissues, as well as MG-63 cells, RT-qPCR analysis was performed using the TaqMan MicroRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, 2 μl RNA isolated from tissue samples or MG-63 cells was reverse transcribed into cDNA. The PCR reaction was subsequently performed with 2 μl cDNA, 10 μl TaqMan Universal Master Mix (2X), 1 μl primers and nuclease-free H_2O, with a total volume of 20 μl. The PCR conditions were as follows: Initial denaturing at 95˚C for 3 min, denaturing at 94˚C

for 30 sec, annealing at 56˚C for 30 sec, and extension at 72˚C for 30 sec. After 30 cycles, the final extension was performed at 72˚C for 10 min. The PCR products were detected using 1.5% agarose gel electrophoresis. U6 small nuclear RNA was used as an endogenous control. The sequences of the primers were as follow: U6 forward, CTCGCTTCGGCAGCACA and reverse, AACGCTTCACGAATTTGCGT; miR-128 forward, GGCTCACAGTGAACCGG and reverse, GTGCAGGGT CCGAGGT. The relative expression level of miR-128 was normalized to that of internal control U6 according to the $2^{-\Delta\Delta Cq}$ method (19).

Protein expression analysis by western blot analysis. The cells were lysed using M-PER protein extraction reagent supplemented with protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). The Bradford method was used to determine the concentration of proteins in the supernatant. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control. Protein (10 μg/lane) was separated by 10% SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked and incubated with rabbit anti-SASH1 (1:500; cat. no. A302-265A-1; Bethyl Laboratories, Inc., Montgomery, TX, USA), rabbit anti- B-cell lymphoma 2 (Bcl-2; 1:1,000; cat. no. ab59348; Abcam, Cambridge, MA, USA), rabbit anti-Bcl-2-associated X protein (Bax; 1:1,000; cat. no. ab182733; Abcam), rabbit anti-caspase-3 (1:1,000; cat. no. ab13847; Abcam) and rabbit anti-GAPDH (1:2,500; cat. no. ab9485; Abcam) at 4˚C overnight, and subsequently labeled with horseradish peroxidase-coupled secondary antibodies (1:5,000; cat. no. A0208; Beyotime Institute of Biotechnology, Shanghai, China) at room temperature for 2 h. Following washing with Tris-buffered saline/Tween-20 for 10 min three times, autoradiography was performed with chemiluminescence reagents. The relative protein expression levels of SASH1, Bax, Bcl-2 and caspase-3 were semi-quantified through the gray value ratio of each protein and internal control using ImageJ software (version 1.49; National Institutes of Health, Bethesda, MD, USA).

Dual luciferase reporter assay. The 293 cells (Type Culture Collection of the Chinese Academy of Sciences) were transfected with the wild-type SASH1 3'-UTR (SASH1-3'UTR) or mutant SASH1 3'-UTR (SASH1-MUT) were cloned into the p-MIR-reporter plasmid (Thermo Fisher Scientific, Inc.) together with either miR-128 mimics or NC using Lipofectamine RNAi Max (Thermo Fisher Scientific, Inc.). The cells were cultured at 37˚C for 48 h and the activities of the luciferases were measured using a dual-luciferase reporter kit (Beyotime Institute of Biotechnology).

In vivo xenograft tumor model. The MG-63 human osteoblastic cells were transfected with miR-128 inhibitor, and implanted into ten C57BL/6 female nude mice (4-week-old; weight range, 18.6-20.4 g). Mice were housed in specific pathogen-free conditions at 23±2˚C and 50±10% humidity, under a 12 h light/dark cycle with access to food and water *ad libitum*. The formation of tumors was examined weekly. The mice were sacrificed 4 weeks following implantation, and the tumor volumes and weights were measured.

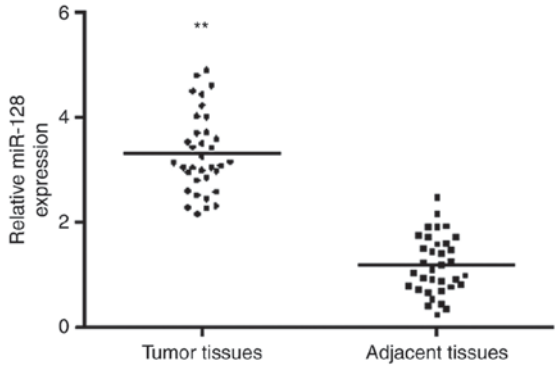

Figure 1. Comparison of the relative expression of miR-128 in osteosarcoma tissue and adjacent tissue. **P<0.01, vs. adjacent tissue. miR, microRNA.

Statistical analysis. SPSS 22.0 (IBM SPSS, Armonk, NY, USA) was used to analyze the data. Data are presented as the mean ± standard error of the mean. Statistical comparisons among groups were calculated using an independent t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-128 in osteosarcoma and normal tissues. In order to investigate the biological roles of miR-128 in the development of osteosarcoma, the expression of miR-128 in human osteosarcoma tumor tissues and human normal osteoblast tissues was compared. As shown in Fig. 1, the results indicated that the expression of miR-128 was increased significantly in tumor tissues, compared with that in normal tissues.

Expression of miR-128 and SASH1 in clinical pathology. To investigate the association between miR-128 and its target gene, SASH1, in osteosarcoma, and whether their expression levels are correlated with the progression of osteosarcoma, the present study evaluated the expression of miR-128 and SASH1 in 35 samples from patients with osteosarcoma (Table I). These patients included 20 men and 15 women, 25 of which were aged <60 years and 10 were aged >60 years. However, the results demonstrated that the expression of miR-128 in the osteosarcoma samples was independent of certain factors, including sex, age and histological grade (P=0.409, P=0.362 and P=0.172, respectively). The expression of SASH1 was also independent of these three factors (P=0.391, P=0.089 and P=0.192). By contrast, the expression of miR-128 and SASH1 showed significant correlation with tumor size (P=0.035 and P=0.044, respectively), invasion depth (P=0.034 and P=0.018, respectively), lymph node metastasis (P=0.026 and P=0.023, respectively) and TNM stage (P=0.015 and P=0.029, respectively). The expression value of miR-128 in the T3-T4 invasion depth group was 4.356±1.92, which was markedly higher than that in the T1-T2 invasion depth group, which had a value of 2.832±0.98. However, the expression value of SASH1 in the T3-T4 invasion depth group was 1.432±0.34, which was markedly lower than that in the T1-T2 invasion depth group, which had a value of 2.105±0.42. Similar trends were found in the different tumor size groups and different lymph node metastasis groups.

Table I. Expression of miR-128 and SASH1 in patient osteosarcoma tissues.

Factor	Cases (n)	miR-128 (mean)	P-value	SASH1 (mean)	P-value
Sex			0.409		0.391
Male	20	2.982±1.27		2.052±0.28	
Female	15	3.281±1.67		1.992±0.36	
Age (years)			0.362		0.089
<60	25	3.064±1.03		1.468±0.34	
≥60	10	2.763±1.32		1.336±0.37	
Tumor size (cm)			0.035		0.044
≥5	18	3.252±1.12		1.691±0.30	
<5	17	2.878±1.03		1.877±0.46	
Histological grade			0.172		0.192
Well/intermediately differentiated	15	3.481±1.16		1.646±0.33	
Poor differentiation	20	3.925±1.55		1.671±0.40	
Invasion depth			0.034		0.018
T1-T2	16	2.832±0.98		2.105±0.42	
T3-T4	19	4.356±1.92		1.432±0.34	
Lymph node metastasis			0.026		0.023
N0	27	2.696±1.55		1.993±0.38	
N1-N3	8	3.089±1.20		1.513±0.33	
Distant metastasis			0.467		0.356
N0	13	2.560±1.17		1.236±0.22	
M1	22	3.170±1.64		1.712±0.39	
TNM stage			0.015		0.029
I, II	21	4.695±2.04		2.052±0.35	
III, IV	14	2.647±1.11		1.443±0.29	

miR, microRNA; SASH1, TNM, tumor-node-metastasis.

Cell growth inhibition. The cells were transfected with miR-128 inhibitors, and the transfection efficiency was measured using RT-qPCR analysis (Fig. 2A). Subsequently, an MTT assay was used to monitor the effect of miR-128 on osteosarcoma cell growth (Fig. 2A and B). The results showed no significant difference in cell growth inhibition between the blank control and NC groups as time increased (P>0.05). As time increased, the miR-128-inhibited group showed significantly decreased cell viability, compared with those in the control groups (P<0.05), indicating that the absence of miR-128 may be able to inhibit the viability of osteosarcoma cells.

Cell apoptosis. To examine the effect of miR-128 on osteosarcoma cell apoptosis, an Annexin V-FITC apoptosis kit was used to analyze apoptotic cells (Fig. 3). The blank control and NC groups had apoptotic rates of 1.53% and 1.15%, respectively (Fig. 3A and B). The apoptotic rate of cells in the miR-128-inhibited group was 7.11%, which was higher, compared with the rate in the control groups (Fig. 3C), indicating that the absence of miR-128 may be able to promote osteosarcoma cell apoptosis.

Western blot analysis. To investigate the correlation between the miR-128 and SASH1 in the signaling pathway, the protein expression levels of SASH1 and associated proteins were measured using western blot analysis. The results showed no marked variation in the protein expression levels of SASH1, Bax, Bcl-2 or caspase-3 between the NC and blank groups (P>0.05; Fig. 4A-F). In the miR-128-inhibited group, the protein expression levels of SASH1, Bax and caspase-3 were significantly increased, whereas the expression level of Bcl-2 was significantly reduced, compared with the expression in the control groups (P<0.05).

SASH1 is a direct target of miR-128 in osteosarcoma. Using online bioinformatics tools TargetScan (20) and miRanda (21), SASH1 has been predicted as a target gene of miR-128, therefore, the present study further examined the association between SASH1 and miR-128 using a dual luciferase gene reporter assay. As shown in Fig. 5A and B, transfection in the miR-128 group showed significantly suppressed luciferase activity in the wild-type SASH1 3'UTR (SASH1-3'UTR) plasmid-transfected cells, whereas miR-128 had no effect on the mutant SASH1 3'UTR (SASH1-MUT) plasmid-transfected cells (P<0.01; Fig. 5B), suggesting that miR-128 directly targeted SASH1 *in vitro*.

Knockdown of miR-128 induces tumor suppressive effects on xenograft mice tumor models of osteosarcoma in vivo. Finally,

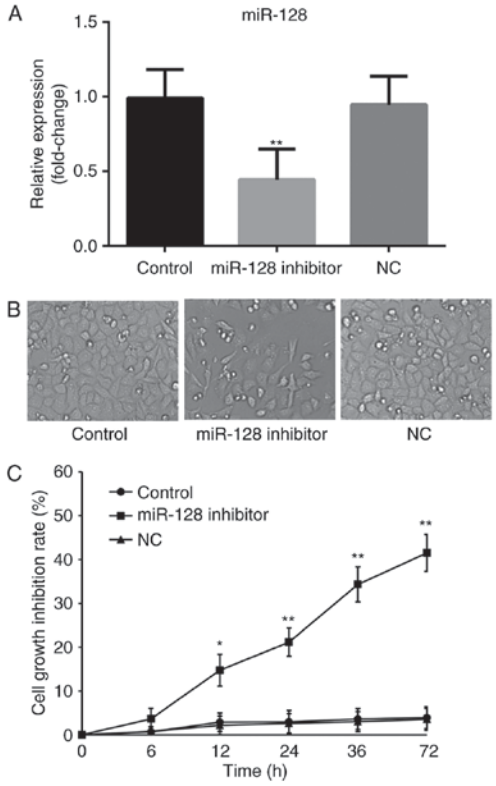

Figure 2. Effect of miR-128 on the proliferation of MG-63 cells. (A) Transfection efficiency of miR-128 inhibitor determined using reverse transcription-quantitative polymerase chain reaction analysis. (B) Morphological features of cells in different groups. (C) Proliferation rate of MG-63 cells in different groups. *P<0.05, vs. control group, **P<0.01, vs. control group. miR, microRNA; control, untransfected cells; miR-128 inhibitor, miR-128 inhibitor-transfected cells; NC, miR-128 negative control-transfected cells.

the present study examined the roles of miR-128 in xenograft mouse osteosarcoma models. As shown in Fig. 6A-C, transplantation of the mice with MG-63 human osteoblastic cells, which had been transfected with miR-128 inhibitor, led to significant decreases in tumor volume and weight (P<0.05).

Discussion

Osteosarcoma is the most commonly occurring sarcoma in young individuals, accounting for almost 60% of all bone sarcomas (21,22). This malignant bone tumor is an osteoid-producing solid tumor, which develops rapidly, particularly in large bones close to epiphyseal regions and has shown a high mortality rate in recent years (22). Despite studies reporting the importance of miR-128 in several types of malignant tumors, the potential mechanism and signaling pathway of miR-128 in regulating the development of osteosarcoma remain to been fully elucidated. Osteosarcoma is known for its high invasive ability and early metastasis, which contribute most to treatment failure and mortality rates (23). Therefore, it is crucial to investigate the molecular mechanism of miRNAs and signaling proteins in the development, invasion and metastasis of osteosarcoma in order to identify effective treatments. The present study demonstrated the overexpression of miR-128 in osteosarcoma tissue and suggested the regulatory effect of miR-128 in the pathogenesis of osteosarcoma through targeting SASH1. Studies have reported similar overexpression of miR-128 in other types of cancer(24). Zhu *et al* (25) found that miR-128 was expressed at high levels in the blood of 147 patients newly diagnosed with acute leukemia. It was reported that miR-128 was associated with to the prognosis of acute leukemia. According to another report, the expression level of miR-128 was increased in samples from patients with glioblastoma (26). Abnormal expression levels of miR-128 in other types of tumor tissue have also been reported (27,28).

In the present study, the effects of miR-128 on the progression of osteosarcoma at the cellular level were examined through the transfection of miR-128 inhibitor into MG-63 cells. The results showed that the absence of miR-128 markedly inhibited the growth and promoted the apoptosis of MG-63 cells. In addition, the knockdown of miR-128 induced tumor suppressive effects on xenograft tumor models of osteosarcoma in mice *in vivo*. Therefore, miR-128 may be able to promote osteosarcoma cell growth and prevent osteosarcoma cell apoptosis, indicating that miR-128 is important in tumorigenesis and development. SASH1 is a tumor suppressor candidate (29). According to previous studies, the decrease of SASH1 is linked to tumorigenesis, metastasis and poor prognosis in different types of cancer (10,29,30). Studies have demonstrated that SASH1 can inhibit cell proliferation (10,30). These studies suggested that the downregulation of SASH1 may be an important factor in tumorigenesis and evolution. Former studies have reported that functions, including the inhibition of cell growth, repression of invasive ability and promotion of apoptosis can suppress the metastasis of tumors (31). The data in the present study showed that the expression level of SASH1 was higher in the miR-128-inhibited group, compared with that in the negative control group and blank group, indicating that miR-128 may be able to downregulate the expression of SASH1 and prevent its inhibitory effect on tumor cell proliferation. The direct target association between SASH1 and miR-128 was confirmed using a dual luciferase gene reporter assay. Furthermore, the results of the present study revealed that the protein expression level of Bcl-2 was decreased in the miR-128-inhibited group, compared with that in the negative control group and blank group, whereas the expression levels of Bax and caspase-3 were markedly increased in the miR-128-inhibited group. This suggested that miR-128 may be able to upregulate the expression of Bcl-2 and downregulate the expression levels of Bax and caspase-3. Bcl-2 protein is well-known for its ability to repress a number of cell apoptotic processes (32), whereas Bax and caspase-3 are key factors in cell apoptotic processes (33,34). This further confirmed the hypothesis that miR-128 regulates the tumorigenesis and evolution of osteosarcoma through targeting SASH1 and correlative signal proteins. The clinical patient osteosarcoma samples also showed marked correlation between the expression of miR-128 and SASH1, particularly with tumor size, invasion depth, lymph node metastasis and TNM stage. With increased tumor size and more marked invasion and metastasis, there were higher expression levels of miR-128 and lower expression levels of SASH1 in the clinical osteosarcoma samples.

In conclusion, the present study investigated the role of miR-128 in the progression of osteosarcoma and the possible regulatory mechanism. The present study revealed that miR-128 downregulated the expression of SASH1 in osteosarcoma tumor cells, promoted osteosarcoma tumor cell

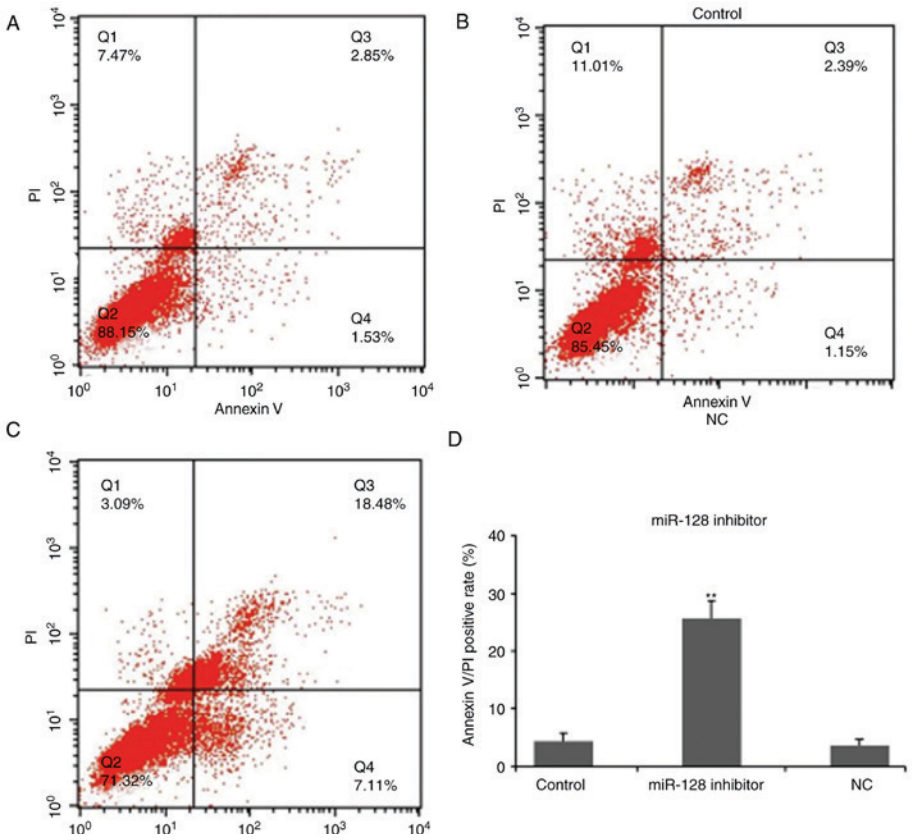

Figure 3. Effect of miR-128 on the apoptosis of MG-63 cells. (A) Blank control; (B) NC control; (C) miR-128 inhibitor; (D) Quantified results of the flow cytometry analysis. **P<0.01, vs. NC. miR, microRNA; control, untransfected cells; miR-128 inhibitor, miR-128 inhibitor-transfected cells; NC, miR-128 negative control-transfected cells.

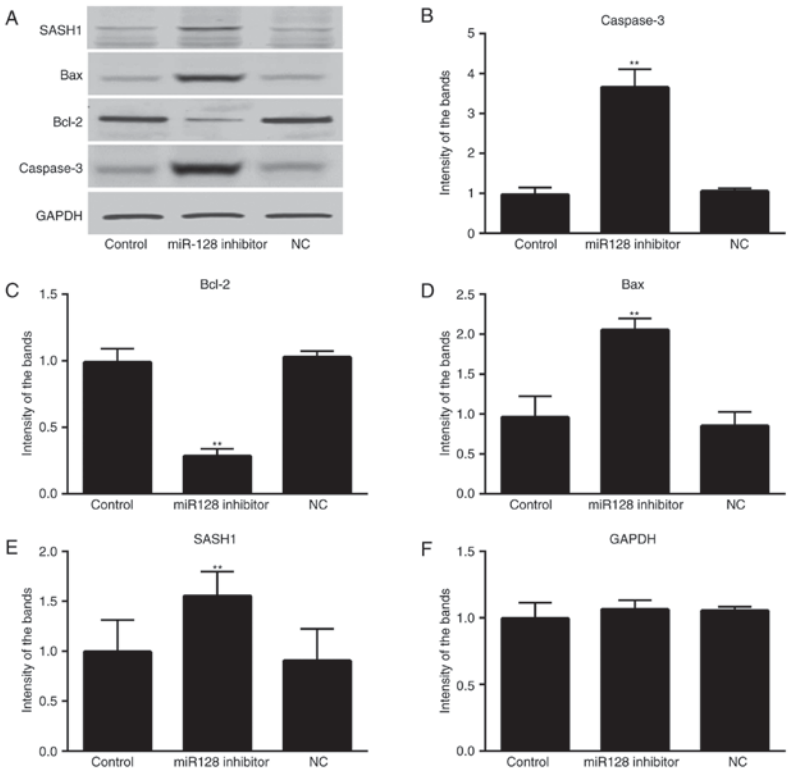

Figure 4. Effect of miR-128 on the expression of SASH1, Bcl-2, Bax and caspase-3 in MG-63 cells. (A) Protein expression of SASH1, Bcl-2, Bax, Caspase-3 and GAPDH in different groups; (B) Relative intensity of the Caspase-3 band; (C) Relative intensity of the Bcl-2 band; (D) Relative intensity of the BAX band; (E) Relative intensity of the SASH1 band; (F) Relative intensity of the GAPDH band; miR, microRNA; control, untransfected cells; miR-128 inhibitor, miR-128 inhibitor-transfected cells; NC, miR-128 negative control-transfected cells; SASH1, SAM-and SH3-domain containing 1; Bcl-2, B-cell lymphoma-2; Bax, Bcl2-associated X protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

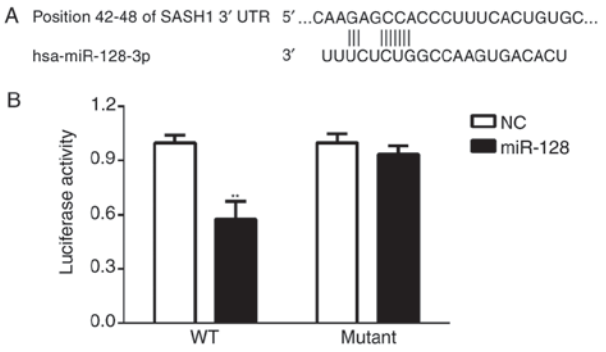

Figure 5. miR-128 directly targets SASH1 *in vitro*. (A) Sequence alignment of the paired site of the 3'-UTR of miR-128-3p and SASH1; (B) The activity of the luciferases in different groups. **P<0.01, vs. control group. miR, microRNA; WT, wild-type, miR, microRNA; miR-128, miR-128 mimics-transfected cells; NC, miR-128 mimics negative control-transfected cells; SASH1, SAM-and SH3-domain containing 1; UTR, untranslated region.

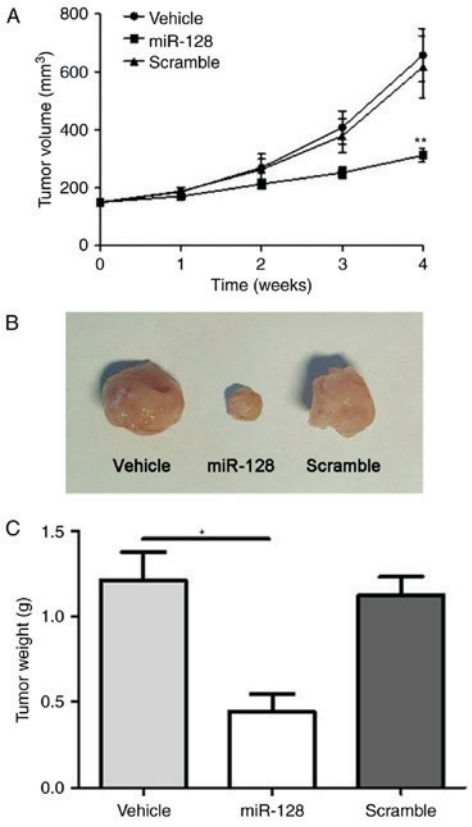

Figure 6. Knockdown of miR-128 induces tumor suppressive effects on xenograft tumor models of osteosarcoma in mice *in vivo*. (A) Tumor volume. (B) Representative images of tumors excised from mice in different groups. (C) Tumor size. *P<0.05, vs. control group; **P<0.01, vs. control group. miR, microRNA; Vehicle, mice transplanted with untreated MG-63 cells; miR-128, mice transplanted with MG-63 cells transfected with miR-128 inhibitor; Scramble, mice transplanted with MG-63 cells transfected with miR-128 inhibitor negative control.

proliferation and inhibited osteosarcoma tumor cell apoptosis. Therefore, gene therapy targeting miR-128 may offer a promising treatment method for osteosarcoma through inhibiting cell growth, repressing invasion and promoting apoptosis via the downregulation of miR-128 and upregulation of SASH1. Finally, the results of the *in vivo* animal experiment confirmed that the downregulation of miR-128 induced tumor suppressive effects on xenograft tumor mouse models of osteosarcoma. The findings of the present study may offer a foundation for future clinical therapeutics in osteosarcoma. However, further detailed investigations are required to thoroughly examine the regulatory mechanisms of miR-128 in osteosarcoma.

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Competing interests

The authors declare that they have no competing interests.

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