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Original Article

Tumor-suppressive miR-99a inhibits cell proliferation via targeting of TNFAIP8 in osteosarcoma cells

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Abstract: Osteosarcoma (OS) has been described as the most common primary malignant bone tumor in adolescents and young adults worldwide. MicroRNAs (miRNAs) have demonstrated playing critical role on the cellular biology and development of cancer. However, the essential mechanisms of miRNAs underlying osteosarcoma oncogenesis and progression have not fully understood. In this study, we found that the expression of miR-99a was repressed in OS tissues and cells using qRT-PCR assays. We demonstrated that overexpression of miR-99a inhibits OS cell viability and growth with MTT, colony formation and in vivo mice experiment. In addition, FACS and Annexin V assays identified that miR-99a can induce OS cell cycle progression and cell apoptosis. Furthermore, we demonstrated that TNFAIP8 is a direct target of miR-99a and is upregulated in OS samples and cells. Knockdown of TNFAIP8 significantly attenuated OS cell viability and growth through inhibiting cell cycle and inducing cell apoptosis in vitro and in vivo. These findings establish that miR-99a plays a significant tumor-suppressing role in OS and proposes it as a potential diagnostic and therapeutic target in managing OS metastases.

Keywords: miR-99a, TNFAIP8, osteosarcoma, MG-63, U2OS

Introduction

Osteosarcoma (OS) is one of the most common type of bone cancer that occurs predominantly in children and young adults worldwide [1]. With the current surgical techniques and chemotherapeutic treatments, the 5-year survival rate with localized OS is about 60-80%, while the rate is only 15-30% when the OS has already spread to lung or other organs [2-4]. Therefore, there is an urgent need for a comprehensive understanding of underlying mechanism of OS biology to optimize the diagnosis, therapy and prognosis prediction of this disease.

MicroRNAs (miRNAs), a type of non-coding small RNAswith 18-22 nucleotides in length, exert their functions through directinteraction with the 3'untranslated region (3'UTRs) of specific target mRNAs to regulate their expression at the post-transcriptional levels [5]. A large number of studies have proved that deregulation of miRNAs act as tumor suppressors or oncogenes contributing to cancer development [6-8]. Several reports have suggested that the deregulation of miRNA can regulate tumor development, proliferation, invasion and metastasis [9, 10]. It has been described that in head and neck squamous carcinoma, decreased expression of miR-99a has been found, and restoring the expression of miR-99a inhibited the cell proliferation and enhanced apoptosis [11]. Furthermore, it has been demonstrated that miR-99a was also down regulated in numerous cancers, including cervical carcinoma, pancreatic ductal adenocarcinoma, pancreatic cancer and small cell carcinoma of cervix [12, 13]. However, little is known about the role of miR-99a in the pathogenesis of OS.

TNFAIP8 (also called SCC-S2/GG2-1/NDED) is originally identified from an expression profile by comparing a primary human head and neck squamous cell carcinoma cell line with its matched metastatic cell line [14] '¼ D/7. Recently, experimental evidences have supported the notion that TNFAIP8 serves as an oncogene in human cancersand is associated with enhanced DNA synthesis, cell survival and inhibition of apoptosis through inhibiting caspase 8 and caspase 3 [15-19]. In addition, its expression can be upregulated by TNF-α and

NF-κB in various cells [15]. However, the role of TNFAIP8 and its upstream regulation in Osteosarcoma remains to be unraveled.

In the current study, we found that miR-99a was down regulated in OS tissues and cell lines. Restoration of miR-99a expression reduced cell viability and growth *in vitro* and suppressed tumorigenicity *in vivo*. In addition, TNFAIP8 was identified as a direct target of miR-99a; down-regulation of TNFAIP8 suppressed the viability and growth in vitro and in vivo. Furthermore, TNFAIP8 was found up-regulated in OS tissues and cells. Taken together, our findings collectively suggest that miR-99a acts as a tumor suppressor via regulation of TNFAIP8 expression and may thus be a promising therapeutic target for OS.

Materials and methods

Human OS tissues and cell culture

23 paired fresh surgically resected osteosarcoma tumor tissues and adjacent normal bone tissues, which were diagnosed by an independent pathologist, were collected from the Departments of Orthopedics, The Fourth Harbin hospital between November 2013 and November 2015. Specimens were freshly frozen in liquid nitrogen immediately after collection and stored at -80°C until use. Both tumor and non-tumor samples were confirmed by pathological examination. All samples were obtained with informed consent andthis research was approved by the Research Ethics Committee of The Fourth Harbin hospital. The human OS cell lines (KHOS, 143b, LM7, U2OS and MG-63) and normal osteoblastic cell lines NHOst (Shanghai Cell Bank, Chinese Academy of Sciences) were cultured in Dulbecco's modied Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and incubated at 37°C in 5% CO_2.

RNA oligonucleotides, plasmids and transfection

The FAM modified 2'-OMe-oligonucleotides were chemically synthesized and purified by high-performance liquid chromatography by GenePharma Co. Ltd. (Shanghai, China). The 2'-O-me-miR-99a mimic was composed of RNA duplexes with the following sequence: miR-99 amimics: (forward) 5'-AACCCGUAGAUCCGAUCUUGUG-3', (reverse) 5'CAAGAUCGGAUCUACGGGUUUU-3'; negative control (NC): (forward) 5'-UUCUCCGAACGUGUCACGUTT-3', (reverse) 5'-ACGUGACACGUUCGGAGAATT-3'. siRNA (Cat. No. HSS119329) for TNFAIP8 and control siRNA (Cat. No. 12935-300) were bought from Thermo Fisher Scientific and used at a final concentration of 20 nM. Lipofectamine 2000 (Invitrogen) was used for the transfection. Cells were grown to 70-80% confluence, transfection was performed using the Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. At 4 h after infection, the medium was replaced with fresh medium containing 10% fetal bovine serum.

Luciferase reporter gene assay

The 3'-UTR of *TNFAIP8* in the pMirGLO reporter vector was purchased from Origene Technologies Inc. The miR-99a 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-99a mimic or miR control. At 48 hours after transfection, cells were assayed for relative luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Transfections were conducted in triplicate.

Cell proliferation assays

Cell viability was tested with MTT kit (Sigma) according to the manufacturer's instruction. Cells were seeded at 800 cells per well in a 96-well plate in quadruplicate. Then the cell viability was measured at the indicated time by incubating cells with 0.5 mg/ml MTT reagent in PBS (Sigma-Aldrich) for 2 hours, subjected to lysis with 100% DMSO. Plates were read at 590 nm using the Tecan SpectraFluor Microplate Reader and Magellan 6 software (Tecan Group Ltd.) at 0, 6, 12, 24, 48 hours after cell plating. For colony formation assay, transfected cells were placedat 200 cells per well in a 6-well plate in quadruplicate and maintained in proper media containing 10% FBS for two weeks, during which the 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. Stained colonies werecounted under a microscope.

Cell cycle analysis

Transfected MG-63 or U2OS cells were collected and fixed in 75% ethanol at -20°C for 16 h,

then the cells were washed twice in cold phosphate buffered saline (PBS) and stained with propidium iodide (PI) (Invitrogen). Then the cells were examined with a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The percentage of the cells in G0-G1, S, and G2-M phase were counted and compared. The experiments were repeated in triplicate.

Apoptosis analysis

The percentage of apoptotic cells was determined by Annexin V-FITC and PI staining (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. The apoptotic morphology was determined by 4,6-diami dino-2-phenylindole (DAPI) staining. Control or miRNA transfected cells were stained with DAPI (1 μg/μl; Sigma-Aldrich) to visualize apoptotic cells with fragmented or condensed nuclei. At least five visual fields were observed under a fluorescence microscope for each sample (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

Tumor-bearing nude mouse model in vivo

5-week-old BALB/c mice were maintained under specific pathogen-free conditions and manipulated according to protocols approved by the Harbin Experimental Animal Care Commission. MG-63 or U2OS cells transfected with miR-99a or TNFAIP8 siRNA, or their controls were harvested at a concentration of 2×10^7 cells/ml. The suspending cells (0.1 ml) were subcutaneously injected into either side of the posterior flank of the nude mouse. Tumor volumes (length × width^2 × 0.5) were examined every 7 days when the implantations were starting to grow bigger. 35 days after injection, the mice were killed and tumor weights were measured. All experiments were performed under the guidelines for animal experiments as stipulated by The Fourth Harbin hospital of Medical Science.

Western blot analysis

Western blotting was performed according to the standard methods as previously described [20] using anti-TNFAIP8 antibody (ab64988, 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 usinga commercially available kit (mirVana; QIAGENE, Shanghai, China) according to the manufacturer's protocol. Then 2 μg of totalRNA was reversely transcribed by M-MLV reverse transcriptase (Invitrogen). A stem-loop RT primer was used for the reverse transcription as follows, miR-99a-RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAGA; miR-99a-forward: GCTGGAGAACCCGTAGATCCGAT; miR-99a-reverse: GTGCAGGGTCCGAGGT; U6-RT AAAATATGGAACGCTTCACGAATTTG; U6-forward: CTCGCTTCGGCAGCACATATACT; U6-reverse: ACGCTTCACG-AATTTGCGTGTC. Quantitative RT-PCR was performed in anABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The relative expression level of miR-99a and TNFAIP8 were normalized to that of internal control U6 or GAPDH using the comparative delta CT (2-ΔΔCt) 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-99a expression in OS tissues and cells

To understand the role of miR-99a in OS, miR-99a expression was examined by qRT-PCR in 23 OS tissues and the corresponding normal bone tissues.Here, we found that miR-99a is downregulated in OS relative to healthy bones (**Figure 1A**). Moreover, we examined the expression of miR-99a in different human OS cell lines using qRT-PCR. We found down regulated expression of miR-99a in all OS cells with a prominently low level of expression in MG-63 cells compared with normal osteoblastic cell lines NHOst (**Figure 1B**, $P<0.05$). These results suggest that miR-99a plays an anti-cancer role in OS development.

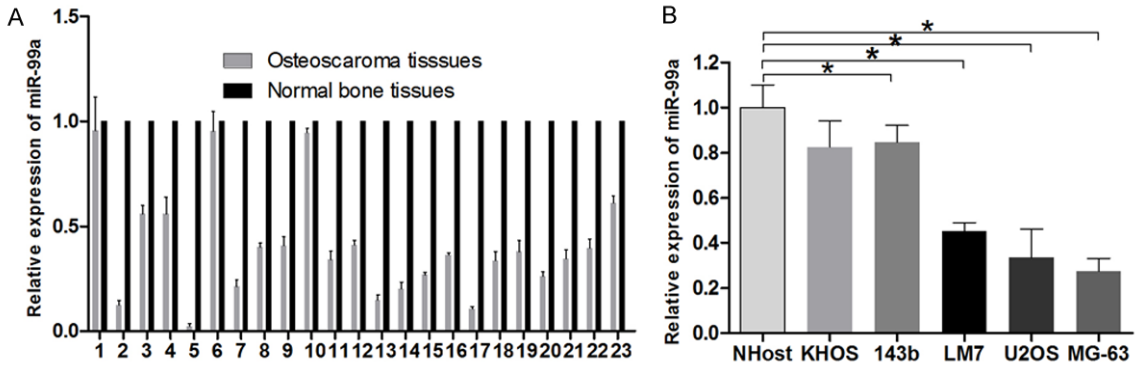

Figure 1. Relative expression of miR-99a in OS tissues and different OS cell lines. A. A column plot of miR-99a expression in 23 pairs of OS and healthy bones (Normal); B. Endogenous levels of miR-99a in OS cell lines were measured by qRT-PCR and normalized to snoRNA U6. Error bars are SD. Experiments were conducted in triplicates.

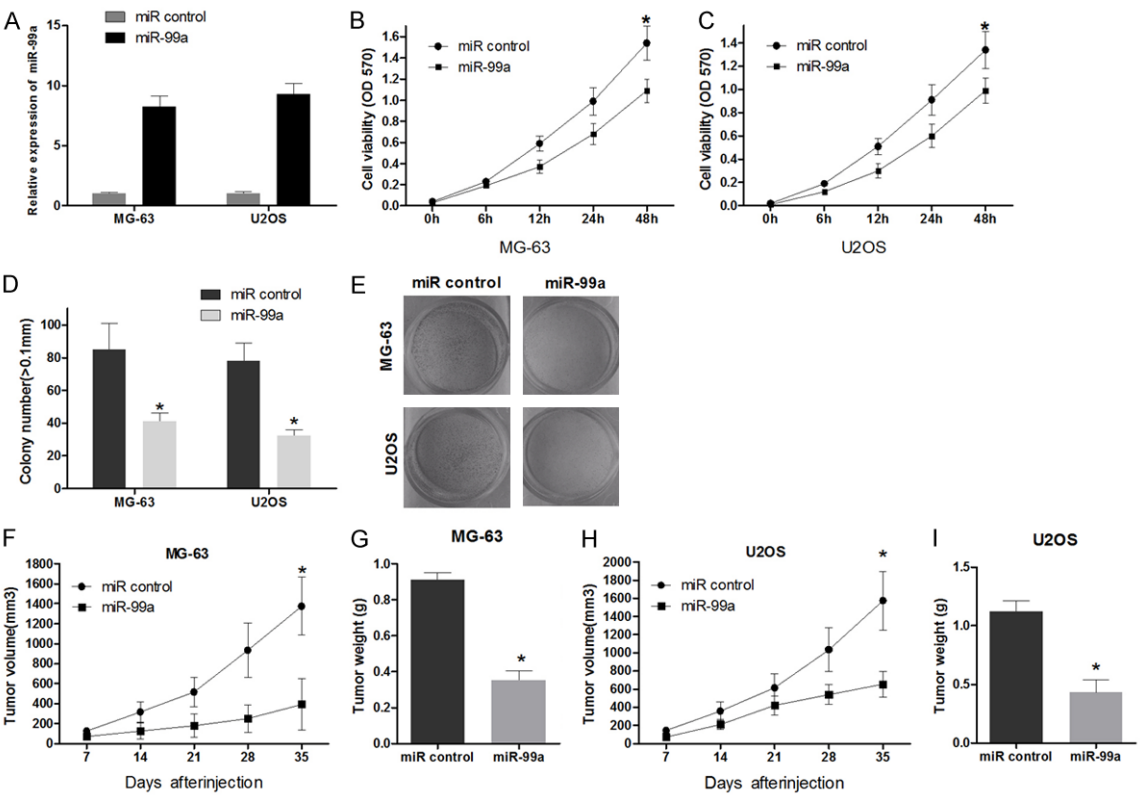

Figure 2. miR-99a suppresses the in vitro and in vivo OSviability and growth. A. miR-99a overexpression in MG-63 and U2OS cells are assessed by qRT-PCR; B and C. MTT assay with MG-63 and U2OS cells, OD570 was measured at the indicated time, Ectopic expression of miR-99a significantly inhibited U2OS cell proliferation in 12, 24 and 48 h compared with cells expressing scramble mimics.; D and E. Colony formation assay; F-I. NOD/SCID mice were injectedwith miR-99a or miR control transfected cells. Six mice were assigned to each group. Tumor volume was measured at the indicated time. The mice were sacrificed at 35 days and then the tumors weight was measured.

Restoration of miR-99a expression inhibited OS proliferation in vitro and in vivo

Since our findings have demonstrated significant downregulation of miR-99a in human OS samples and cells, we examined the potential tumor suppressing role of miR-99a in OS cells. We investigated the functional significance of miR-99a on cell viability and cell growth in MG-63 and U2OS cells using miR control or miR-99a. Our data showed a significant increase of miR-99a levels (~7-folds in MG-63

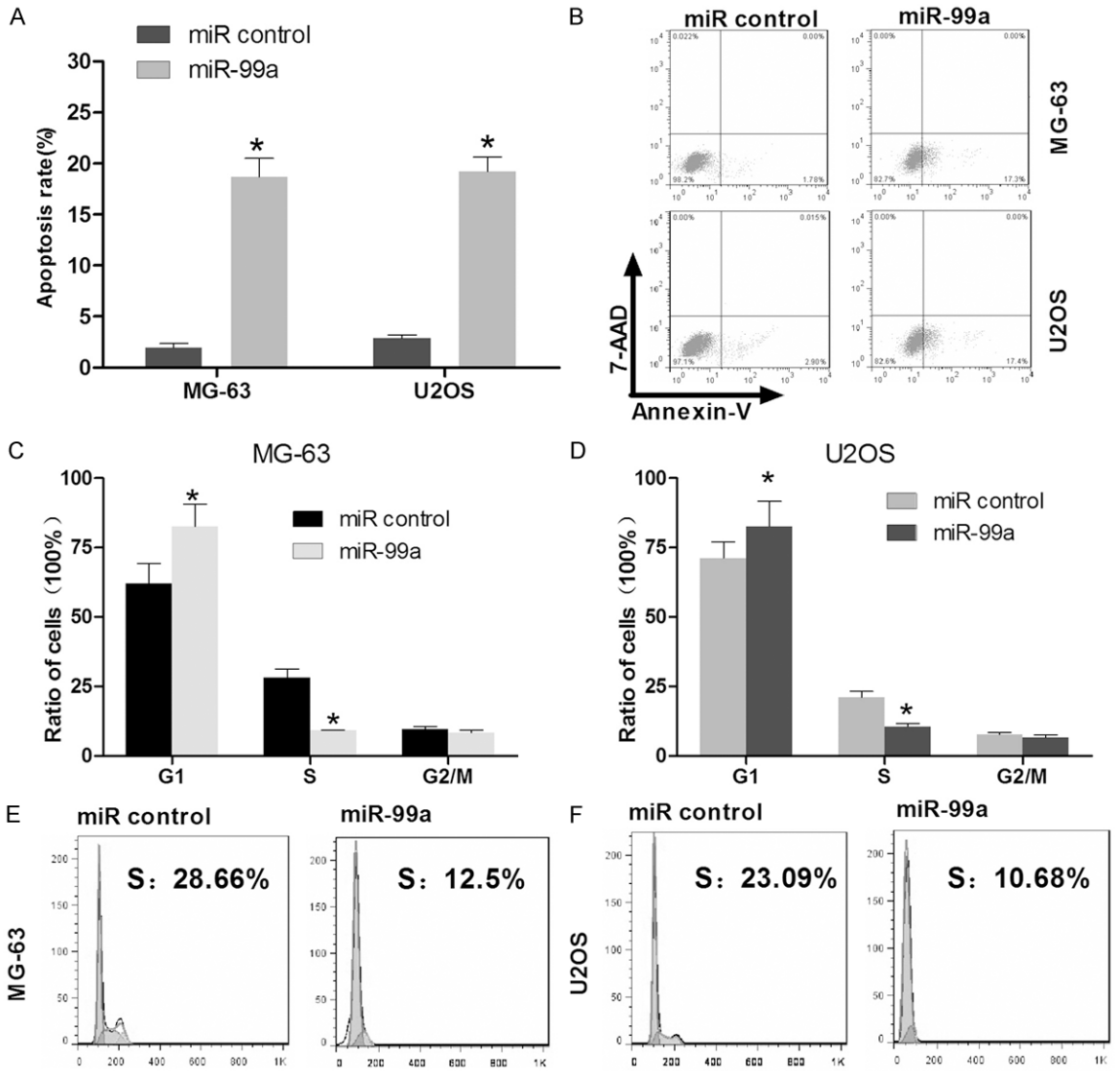

Figure 3. Role of miR-99a in cell cycle and apoptosis of OS cells. A and B. Overexpression of miR-99a in the MG-63 and U2OS OS cell line induced apoptosis. Cells were stained with the Annexin V-FITC apoptosis detection kit. C-F. Cell cycle distribution in MG-63 and U2OS cells following transfection with miR-99a or miR control. miR-99a caused cell cycle arrest at the G0/G1 phase. Data are presented as the means ± SD and are representative of three independent experiments. *P<0.05.

cells and ~9.2-folds in U2OS cells) in miR-99a-transfected cells compared to that of miR control cells (**Figure 2A**). Then we compared the cell viability and growth changes after miR-99a overexpression in OS cells. Our results showed that the ectopic expression of miR-99a suppressed cell viability in a time-dependent manner (**Figure 2B** and **2C**, *P*<0.05). Furthermore, treatment with miR-99a resulted in a significant growth inhibition as demonstrated by colony formation assay (**Figure 2D** and **2E**, *P*<0.05). We next investigated the efficacy of miR-99a against tumor growth *in vivo*. The introduction of miR-99a into MG-63 cells resulted in decreased growth of subcutaneous xenografted tumors in nude mice (**Figure 2F** and **2G**, *P*<0.05). Similarly, U2OS cells transfected with miR-99a showed statistically smaller tumor volumesand weight in mice than untreated and NC miRNA-transfected cells (**Figure 2H** and **2I**, *P*<0.05), indicating that miR-99a also inhibits the growth of OS cells *in vivo*.

Restoration of miR-99a expression induced OS cell apoptosis and inhibited G1/S transition

Subsequently, we investigated apoptosis and cell cycle progression in MG-63 and U2OS cells

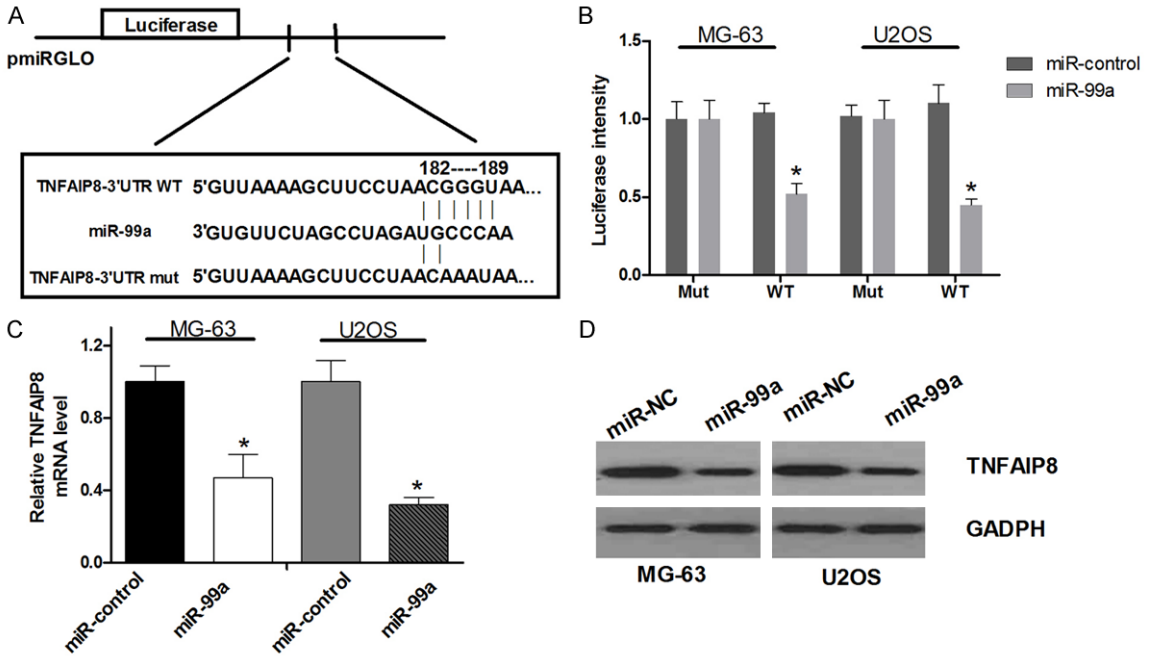

Figure 4. TNFAIP8is a target of miR-99a. A. Computational analysis showed that miR-99a potentially targeted the TNFAIP8 gene. The mutate base pairs in the predicted seed region targeted by miR-99a in the TNFAIP8 3'UTR was also showed. B. Luciferase assay of the effect of miR-99a on expression of *reporter luciferase* gene, which contains a *TNFAIP8* 3'-UTR fragment with wild type (TNFAIP8 3'-UTR) or mutant (TNFAIP8 3'-UTR mut) miR-99a 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. C. Effect of *miR-99a* overexpression in MG-63 and U2OS cells on endogenous *TNFAIP8mRNA* expression as assessed by qRT-PCR. D. Relative protein expression of TNFAIP8 in different OS cell lines as assessed by Western blot. *Indicates *P* value < 0.05 as estimated by the Student's *t*-test.

using miR-99a or miR control. Our results showed that the ectopic expression of miR-99a resulted in the increased induction of apoptotic cells by 19-fold as demonstrated by Annexin v-FITC Apoptosis Detection kit (**Figure 3A** and **3B**, $P<0.05$). Furthermore, miR-99a induced elevation of the percentage of G1-phase cells and reduction of S-phase cells (by 16.16% in MG-63 cells and 12.41% in U2OS cells), respectively (**Figure 3C-F**, $P<0.05$). Taken together, these results indicated that miR-99a efficientlyinduced cell apoptosis and inhibited cell cycle progression *in vitro*, thus manifesting the tumor suppressive role of miR-99a in OS cells.

TNFAIP8 is a target of miR-99a

Bioinformatics research was performed to find potential targets of miR-99a using Targetscan and miRanda. As shown in **Figure 4A**, TNFAIP8 was identified as a potential target gene of miR-99a, with the predicted binding site at the base from positions 182 to 189. In order to address the molecular mechanism underlying the tumor suppressing functions of miR-99a, we studied the effect of overexpressing of miR-99a on expression of the putative target TNFAIP8. To validate whether the 3'-UTR of TNFAIP8 is a functional target of miR-99a, a dual-luciferase reporter system was employed. We cloned 3'UTR sequences containing wild type or mutated binding site of miR-99a into the pmiRGLO luciferase vector, respectively, and co-transfected with the miR-99a or miR control into OS cells. Data from the luciferase assay showed that overexpression of miR-99a remarkably suppressed the luciferase activity of the reporter gene with the wild-type construct but not with the mutant TNFAIP8 3'UTR construct in OS cells (**Figure 4B**, $P<0.05$). In addition, overexpression of miR-99a resulted in a reduction of TNFAIP8 mRNA and protein expression in OS cells (**Figure4 C** and **4D**, $P<0.05$).

TNFAIP8 acts as a tumor suppressor in OS cells

In order to directly address the question whether TNFAIP8 functions as a tumor suppressor in

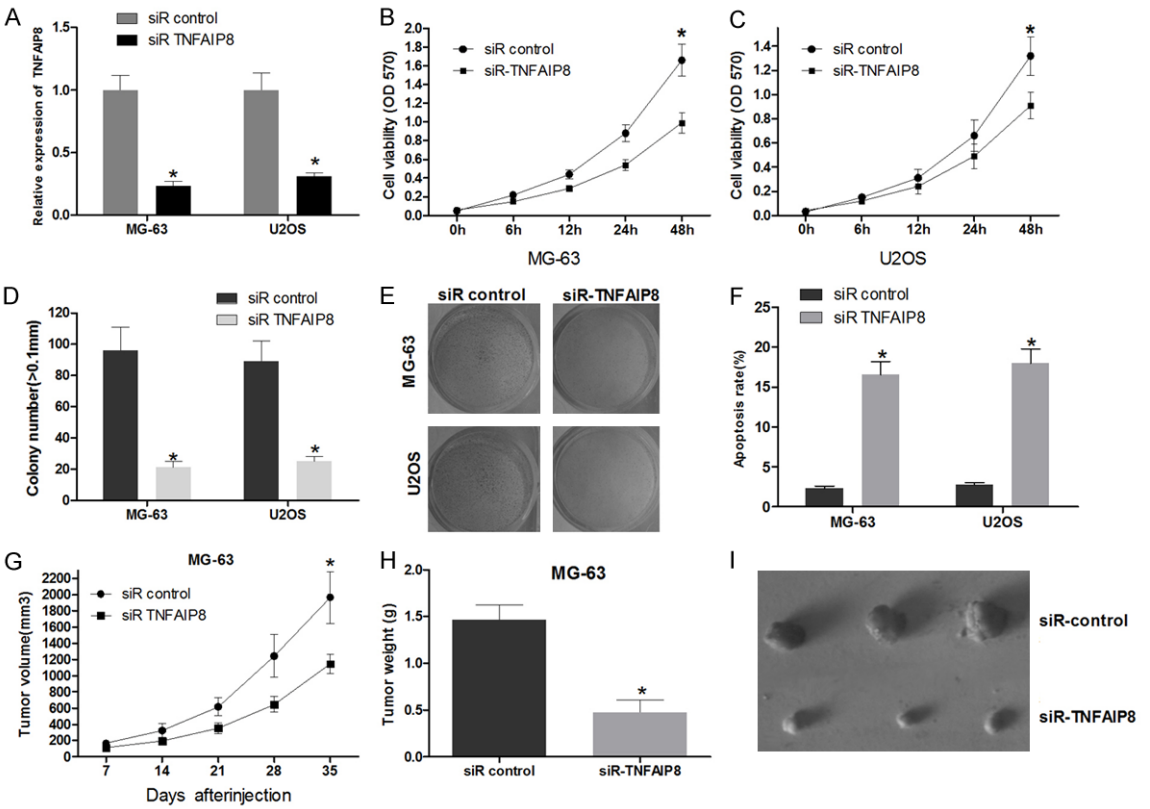

Figure 5. Effect of TNFAIP8 overexpression in OS cells. A. TNFAIP8 downregulation by siRNA in MG-63 and U2OS cells are assessed by qRT-PCR; B and C. MTT assay with MG-63 and U2OS cells, OD570 was measured at the indicated time; D and E. Colony formation assay; (F) Knockdown of TNFAIP8 in the MG-63 and U2OS OS cell line induced apoptosis. Cells were stained with the Annexin V-FITC apoptosis detection kit; (G-I) NOD/SCID mice were injected with miR-99a or miR control transfected cells. Six mice were assigned to each group. Tumor volume was measured at the indicated time. The mice were sacrificed at 35 days and then the tumors weight was measured.

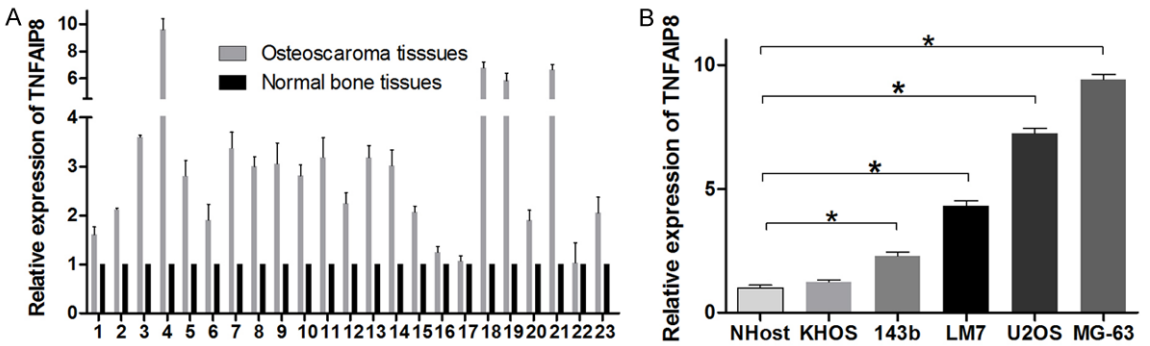

Figure 6. Relative expression of TNFAIP8 in OS, healthy bones and different OS cell lines. A. A column plot of TNFAIP8 expression in 23 pairs of OS and healthy bones (Normal); B. Endogenous levels of TNFAIP8 in OS cell lines were measured by qRT-PCR and normalized to GAPDH. Error bars are SD. Experiments were conducted in triplicates.

MG-63 and U2OS, a TNFAIP8 siRNA was transfected in the two cells which showed obviously suppression of the expression of TNFAIP8 (**Figure 5A**, $P<0.05$). Knockdown of *TNFAIP8* led to a significant decrease in the cell viability (**Figure 5B** and **5C**, $P<0.05$). Then we observed significant decrease in the number of colonies and promoted apoptosis upon *TNFAIP8* knocking down (**Figure 5D-F**, $P<0.05$). In addition, the introduction of TNFAIP8 siRNA into MG-63 cells resulted in decreased tumor volume and weight of subcutaneous xenografted tumors in nude

mice (**Figure 5G-I**, $P<0.05$), which was consistent with the results of miR-99a overexpression. These results indicated that downregulation of TNFAIP8 inhibits the growth of OS cells *in vivo*.

TNFAIP8 is upregulated in OS tissues and cells

We further investigated the functional role of TNFAIP8 with qRT-PCR on OS tissue and cells. The TNFAIP8 mRNA is upregulated in 23 Osteosarcoma tissues compared with Normal bone tissues as presented in **Figure 6A**. In addition, we found that the TNFAIP8 mRNA upregulated in OS cells compared with NHOst cells (**Figure 6B**, $P<0.05$). This finding is consistent with the results that *TNFAIP8* acts as a tumor suppressor in OS.

Discussion

In this study, we investigated the biological role of miR-99a in the progression of OS. We found significant down-regulation of miR-99a in OS tissues and cells compared with matched normal bone tissues. Additionally, functional experiments demonstrated that miR-99a suppressed cell growth *in vitro* and inhibited tumor growth *in vivo*. Our data regarding the cell cycle showed that miR-99a inhibited the proliferation of OS cells via induction of the cell cycle arrest at the G1/S phase and reduction of cell apoptosis. Our work may be the first time to reveal the role of miR-99a in OS.

In line of these evidences, we identified TNFAIP8 as a direct functional target of miR-99a using a prediction program. Computational analysis revealed binding sites for the miR-99a seed sequence at 3'UTR of TNFAIP8. Furthermore, restoration of miR-99a expression led to the decrease in luciferase activity of wild-type TNFAIP8 3'UTR whereas the site-directed mutation abrogated miR-99a regulation. In addition, results from the qRT-PCR and protein expression analysis indicated that the ectopic expression of miR-99a suppressed mRNA and protein levels of TNFAIP8 simultaneously. Taken together, these results suggest that miR-99a regulated the expression of TNFAIP8 by directly targeting 3'UTR of TNFAIP8 in OS cells.

We next examined the functions of TNFAIP8 in the regulation of OS cell lines. In this study, we clarified that the high expression of TNFAIP8 was expressed in OS tissues and cells. In our in vitro TNFAIP8 suppression analysis, significant apoptosis induction and reduced proliferation were observed in the TNFAIP8 siRNA group compared with those in the control groups. TNFAIP8 was first identified as one of the more highly expressed genes in metastatic head and neck tumors than in primary tumor cells [14]. Recently, TNFAIP8 has been demonstrated to be associated with proliferation, apoptosis, invasion, and metastasis in several cancers [21]. It has been demonstrated that TNFAIP8 act a suppressor of TNF-a mediated cell apoptosisthrough inhibiting caspase-3 and caspase-8 activity [22, 23]. Moreover, mouse lung metastasis model of breast cancer cellsrevealed that suppressing of TNFAIP8 expression attenuated lungmetastasis via inhibiting MMP1 and MMP2 in tumor cellsand VEGFR2 in endothelial cells [24]. Recently, TNFAIP8 hasbeen identified to be associated with Gαi-coupled dopamine-D2 short receptor-mediated cell death reduction and cell transformationenhancement by directly interacting with Gαi [23]. Since we have identified that miR-99a can target TNFAIP8, we may speculate that miR-99a may regulate the above pathways, which deserve our further investigation.

In summary, the present study suggested, for the first time, the correlation of miR-99a and TNFAIP8 in OS cells. Our results provided evidence that the expression levels of miR-99a in OS tissues and cells were significantly reduced, while the TNFAIP8 expression levels were upregulated. And that miR-99a plays an important role in OS cell proliferation and tumorigenesis by targeting TNFAIP8 both *in vitro* and *in vivo*. These findings would be beneficial for determining the underlying mechanisms of OS and may facilitate the development of novel therapeutic strategies for clinical application.

Disclosure of conflict of interest

None.

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