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Original Article

MiR-374a promotes the proliferation of human osteosarcoma by downregulating FOXO1 expression

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Abstract: MiRNAs play crucial roles in development of cancer. However, the underlying mechanisms of miRNAs in osteosarcoma (OS) are poorly understood. In the present study, we reported that the expression of miR-374a was markedly upregulated in OS tissues and OS cells compared with the matched adjacent normal tissues and human osteoclast h-FOB cell lines. Overexpression of miR-374a promoted the proliferation and anchorage-independent growth of OS cells, whereas inhibition of miR-374a showed opposite effect. Furthermore, we identified that FOXO1 is the functional target of miR-374a. MiR-374a-induced proliferation was correlated with FOXO1, upregulating of the cell cycle regulator cyclin D1 and downregulating of cyclin-dependent kinase inhibitors p27. In functional assays, FOXO1 downregulation is required for miR-374a-induced OS cell proliferation. In sum, our data provide compelling evidence that a novel mechanism of FOXO1 suppression mediated by miR-374a in OS.

Keywords: miR-374a, osteosarcoma, FOXO1, cell proliferation

Introduction

Osteosarcoma is the most common primary malignant bone tumors diagnosed childhood and young adult [1]. In the last several decades, significant advances have been made in understanding the mechanism of OS carcinogenesis [2, 3]. However, the detailed mechanism of OS carcinogenesis remains largely unknown. Therefore, elucidating the potential mechanism that mediate the initiation and progression of OS is urgent and of great interest [4].

Accumulating evidences have demonstrated that microRNAs (miRNAs), are highly conserved, small noncoding RNAs, which have important functions in a variety of cellular processes, such as cell proliferation, apoptosis, invasion and differentiation [5-7]. Previous studies have found that miR-374a is upregulated in several kinds of tumors, suggest that miR-374a may play pivotal roles in cancer tumorigenesis [8-11]. In the present study, we investigated the biological effects and the potential mechanisms of miR-374a in OS. Furthermore, ectopic overexpression of miR-374a promoted the cell proliferation of OS *in vitro*. Bioinformatics analysis further revealed forkhead box protein O1 (FOXO1), as a putative target of miR-374a. Further investigations revealed that miR-374a directly targeted the 3'-UTR of FOXO1 to suppress the expression of this gene, which in turn promoted the proliferation of OS.

Materials and methods

Clinical specimens

Eight human Osteosarcoma (OS) tissues and the matched adjacent normal tissues (ANT) were obtained from OS patients at Affiliated Hospital of Jining Medical University (Shandong, People's Republic of China). The study was approved by the ethics committee of Affiliated Hospital of Jining Medical University (Shandong, People's Republic of China). Written informed consent was obtained from all patients. Tissue samples were collected at surgery, immediately frozen in liquid nitrogen and stored until total RNAs or proteins were extracted.

Cell culture

Human osteosarcoma cell lines (SAO-2, SOSP-9607, MG-63, and U2-OS) and human osteo-

blast h-FOB cell lines were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA), 100 units/ml of penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cell lines were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

Plasmids, small interfering RNA and transfection

For ectopic expression of FOXO1, FOXO1 ORFs with 3'-UTR was amplified using PCR and subcloned into pEGFP-N1 by using the following primers, sense 5'-GGCGAGCTCGTGAGCAGGTTACACTTAAAAGTAC-3', antisense 5'-GGCTCTAGAGCACAT AACCTGCACACATTG-3' (Invitrogen, USA). MiR-374a mimic, miR-374a inhibitor and negative control were purchased from GeneCopoeia (Guangzhou, China) and transfected into OS cells using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions.

For FOXO1 depletion, FOXO1-siRNA [siRNA sequence used is listed as: GCUCAAAUGCUAGUACUAUTT (sense), AUAGUACUAGCAUUUGAGCTA (antisense)] and Negative Control siRNA were purchased from Qiagen. Transfection of siRNAs were performed using lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's protocol.

RNA extraction and real-time quantitative PCR

Total RNA including microRNAs was extracted from culture cells and patient samples using mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions, and then cDNA was synthesized from 5 ng of total RNA using the Taqman® miRNA reverse transcription kit (Applied Biosystems). The expression levels of miR-374a were quantified using the miRNA-specific TaqMan® MiRNA Assay Kit (Applied Biosystems). The relative miR-374a expression levels after normalization to U6 small nuclear RNA were calculated using $2^{-[(Ct\ of\ miR\text{-}374a)\ -\ (Ct\ of\ U6)]}$.

Real-time PCR was performed using the Applied Biosystems 7500 Sequence Detection system using the following primers for cyclin D1 (forward primer: 5'-AACTACCTGGACGCTTCCT-3' and reverse primer: 5'-CCACTTGAGCTTGTTCACCA-3') and p27 (forward primer: 5'-TGCAACCGACGATTCTTCTACTCAA-3', p27 reverse primer: 5'-CAAGCAGTGATGTATCTGATAAACAAGGA-3'). Expression data were normalized to the geometric mean of GAPDH (forward primer 5'-GACTCATGACCACAGTCCATGC-3'; reverseprimer 3'-AGAGGCAGGGATGATGTTCTG-5') to control the variability in expression levels and calculated as $2^{-[(Ct\ of\ CyclinD1\ and\ p27)\ -\ (Ct\ of\ GAPDH)]}$.

MTT assays and colony formation

For MTT assay, SOSP-9607 cells were seeded in 96-well plates in medium containing 10% FBS at approximately 3,000 cells/well. During the last 4 h of each day of culture, cells were treated with MTT (50 mg per well, Sigma, USA). The generated formazan was dissolved in DMSO, and the absorbance was recorded.

For colony formation assay, SOSP-9607 cells were plated into three 60 mm dishes (1×10^3 cells per well) and incubated for 10 days in medium containing 10% FBS. The colonies were stained with 1.0% crystal violet for 30 s after fixation with 10% formaldehyde for 5 min. The number of colonies, defined as > 50 cells/colony were counted.

Anchorage-independent growth assay

Cells were trypsinized, and 1000 cells were resuspended in 2 ml complete medium plus 0.3% agar (Sigma). The agar-cell mixture was plated on top of a bottom layer consisting of 1% agar in complete medium. Cells were incubated for 14 days at 37°C until colony formation and colonies were stained with 0.5% Crystal Violet for counting under microscope and cell colonies were photographed at an original magnification of 100 ×. Only cell colonies containing more than 50 cells were counted.

Luciferase assays

Cells were seeded in triplicate in 24-well plates (5×10^4/well) and cultured for 24 hours. The pGL3-luciferase reporter gene plasmids pGL3-FOXO1-3'-UTR, or the control-luciferase plasmid were cotransfected into the cells with the control pRL-TK Renilla plasmid (Promega) using Lipofectamine 2000 Reagent (Invitrogen). Dual-luciferase activity assays were assayed 48 hours after transfection.

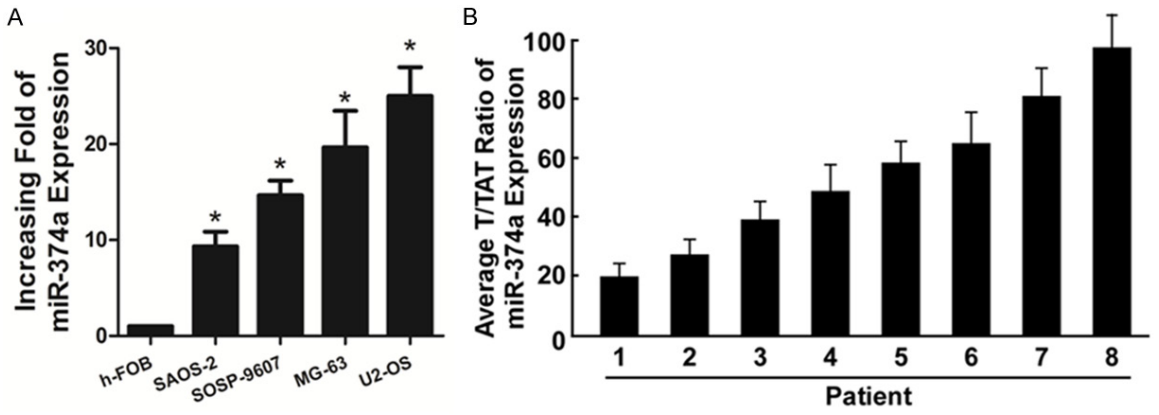

Figure 1. Expression of miR-374a in human osteosarcoma (OS) cell lines and tissues. A. Real-time PCR analysis of miR-374a expression in human osteoblast h-FOB cell lines and OS cell lines, including SAOS-2, SOSP-9607, MG-63 and U2-OS. B. Relative miR-374a expression levels in 8 paired primary OS tissues (T) and the tumor adjacent normal tissues (TAT) from the same patient were detected by PCR analysis. Each bar represents the mean of three independent experiments. *$P < 0.05$.

Western blotting

Protein lysates were prepared, the equivalent aliquots of proteins were electrophoresed on a 10% SDS/polyacrylamide gel and transferred to nitrocellulose membranes. The membrane was incubated overnight with anti-FOXO1, anti-cyclin D1 and anti-p27 (1:1000; Cell Signaling Technology). To control sample loading, the blotting membranes were stripped and re-probed with an anti-a-tubulin antibody (Sigma-Aldrich). And signals were visualized by enhanced chemiluminescence (ECL, Thermo).

Statistical analysis

Statistical analysis was performed using the SPSS 16.0 (SPSS Inc, Chicago, IL, USA). Statistical analyses were done by analysis of variance (ANOVA) or Student's t test. Statistical significance was defined as a value of $P < 0.05$.

Result

MiR-374a expression was upregulated in OS tissues and OS cell lines

Real-time PCR analysis revealed that the expression levels of miR-374a in all 4 tested OS cell lines (SAO-2, SOSP-9607, MG-63, and U2-OS) was markedly upregulated miR-374a levels than those in the human osteoblast h-FOB cell lines (**Figure 1A**), and miR-374a expression was markedly upregulated in the OS tissues than in the tumor adjacent normal tissues (TAT) (**Figure 1B**), indicating that miR-374a played a crucial role in OS. Together, these results suggest that miR-374a is significantly increased in OS and may serve as a prognostic marker for patients with OS.

MiR-374a promoted OS cell proliferation cell cycle progression

Because miR-374a was significantly upregulated in OS tissues and OS cell lines, we investigated the function of miR-374a in OS cells. SOSP-9607 cells were transfected with miR-374a mimics, miR-374a inhibitor or the respective controls, the result of PCR revealed that both of them showed great transfection efficiency (**Figures 2A** and **3A**).

Using MTT assay and colony formation assays showed that cell growth rate was significantly increased in miR-374a-transduced SOSP-9607 cells, compared to negative control (NC)-transfected cells (**Figure 2B** and **2C**). Strikingly, we found that enforced expression of miR-374a in SOSP-9607 cells drastically enhanced their anchorage-independent growth ability (**Figure 2D**). In contrast, the cell growth rates and colony numbers of SOSP-9607 cells transfected with miR-374a-in were significantly decreased than those transfected with NC (**Figure 3B** and **3C**). In addition, the anchorage-independent growth ability of SOSP-9607 cells was significantly decreased in response to miR-374a-in (**Figure 3D**). These results showed that miR-374a promoted OS cell tumorigenicity *in vitro.*

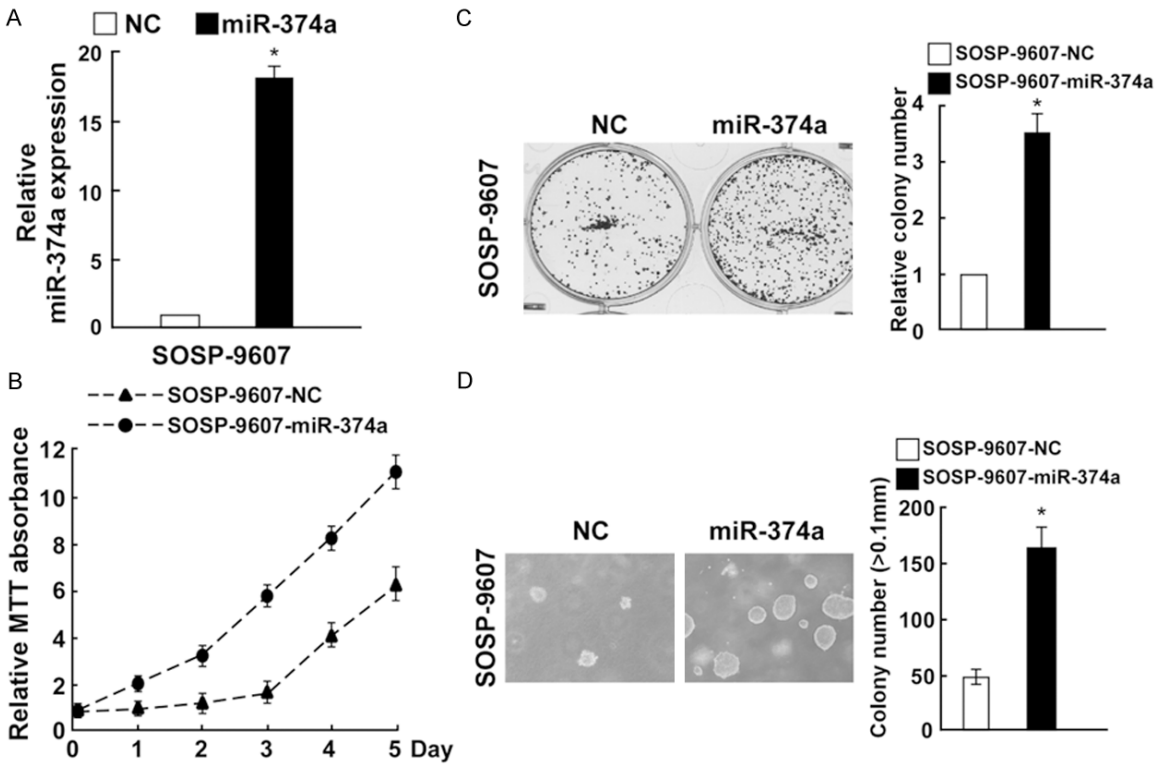

Figure 2. miR-374a upregulation promoted OS cell proliferation. A. Validation of miR-374a expression levels after transfection by PCR analysis. B. MTT assays revealed that inhibition of miR-374a promoted growth of SOSP-9607 OS cell line. C. Representative quantification of crystal violet-stained cell colonies. D. Upregulation of miR-374a promoted the anchorage-independent growth of SOSP-9607 cells. Representative micrographs (left) and quantification of colonies that were > 0.1 mm (right). Each bar represents the mean of three independent experiments. *$P < 0.05$.

MiR-374a directly targets FOXO1 by binding to its 3'-UTR in OS

It is generally accepted that miRNAs regulate the expression of mRNAs by targeting the 3'UTR of relative mRNAs. Our analysis using publicly available algorithm TargetScan demonstrated that miR-374a may directly target the 3'UTR of FOXO1 (**Figure 4A**).

To determine whether miR-374a affects FOXO1 expression, expression of FOXO1 were detected in the SOSP-9607 cells, which were transfected with miR-374a mimics, miR-374a-in or the respective controls. Western blotting analysis showed that miR-374a mimics markedly suppressed FOXO1 protein levels in SOSP-9607 cells (**Figure 4B**), while miR-374a-in clearly promoted FOXO1 protein expression. To verify the effect of miR-374a on the inhibition of FOXO1 expression, we examined whether FOXO1 is regulated by miR-374a through direct binding to its 3'UTR. FOXO1 3'-UTR vector were cotransfected in SOSP-9607 cells with miR-374a mimic, miR-374a-in or miR-374a-mut, followed by measurement of luciferase activity. Significant repression of luciferase activities were observed in was observed in SOSP-9607 cells transfected with miR-374a mimic, whereas the positive effect of miR-374a-in increased wild-type FOXO1 luciferase activity. Meanwhile, overexpressing miR-374a-mut had no effect on the luciferase activity of FOXO1 3'-UTR (**Figure 4C**). These results, taken together, demonstrated that FOXO1 is a bona fide target of miR-374a.

MiR-374a altered levels of proteins related to proliferation and cell cycle in OS cells

A previous study revealed that FOXO1 can regulate a series of genes relevant to the cell proliferation and cell cycle, including the CDK inhibitors p27and the CDK regulator cyclin D1 [12]. To investigate the mechanism underlying cell proliferation and cell cycle, we tested the effect

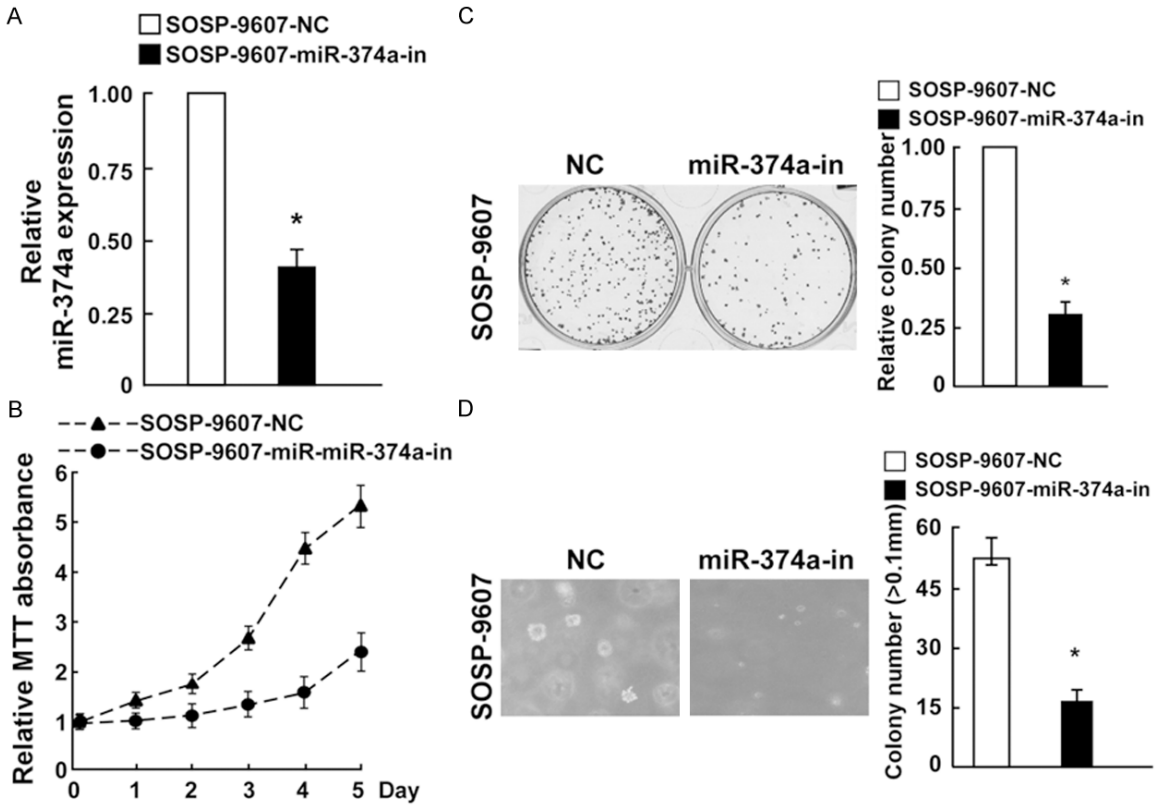

Figure 3. Inhibition of miR-374a inhibited OS cell proliferation. A. Validation of miR-374a expression levels after transfection by PCR analysis. B. MTT assays revealed that upregulation of miR-374a inhibited growth of SOSP-9607 OS cell line. C. Representative quantification of crystal violet-stained cell colonies. D. Inhibition of miR-374a inhibited the anchorage-independent growth of SOSP-9607 cells. Representative micrographs (left) and quantification of colonies that were > 0.1 mm (right). Each bar represents the mean of three independent experiments. **P < 0.05*.

of miR-374a on critical cell-proliferation and cell cycle related regulators p27and cyclin D1. Using real-time PCR and Western blotting analysis, we observed that p27 mRNA and protein were downregulated and cyclin D1 mRNA and protein were upregulated in miR-374a-transfected SOSP-9607 cells, compared to NC-transfected cells (**Figure 4D** and **4E**). Thus providing further evidence that indicated that miR-374a modulated the expression of p27 and cyclin D1 by regulating FOXO1.

FOXO1 downregulation is required for miR-374a-induced proliferation of OS cells

To further investigate the role of FOXO1 in OS cell proliferation, we first examined the effects of FOXO1 downregulation on cell proliferation of OS cells transfected with miR-374a-in. As predicted, Western blot analysis verified that FOXO1-siRNA effectively decreased the expression of FOXO1 in miR-374a-in-transfected SOSP-9607 cells (**Figure 5A**). Colony formation and anchorage-independent growth assays revealed that FOXO1-silenced in miR-374a-in-transfected SOSP-9607 cells have an additive effect on cells proliferation (**Figure 5B**). Taken together, these results demonstrated that direct FOXO1 downregulation is required for miR-374a-induced OS cell proliferation.

Discussion

In this present study, we demonstrated that miR-374a was upregulated in OS tissues and OS cell lines, compared to the tumor adjacent normal tissues and human osteoblast h-FOB cell lines. Upregulation of miR-374a promoted the cell proliferation and anchorage-independent growth of OS cells. Conversely, inhibition of miR-374a reduced this effect, probably through downregulating FOXO1 expression post-translationally by targeting its mRNA 3'-UTR. The negative regulation of FOXO1 by

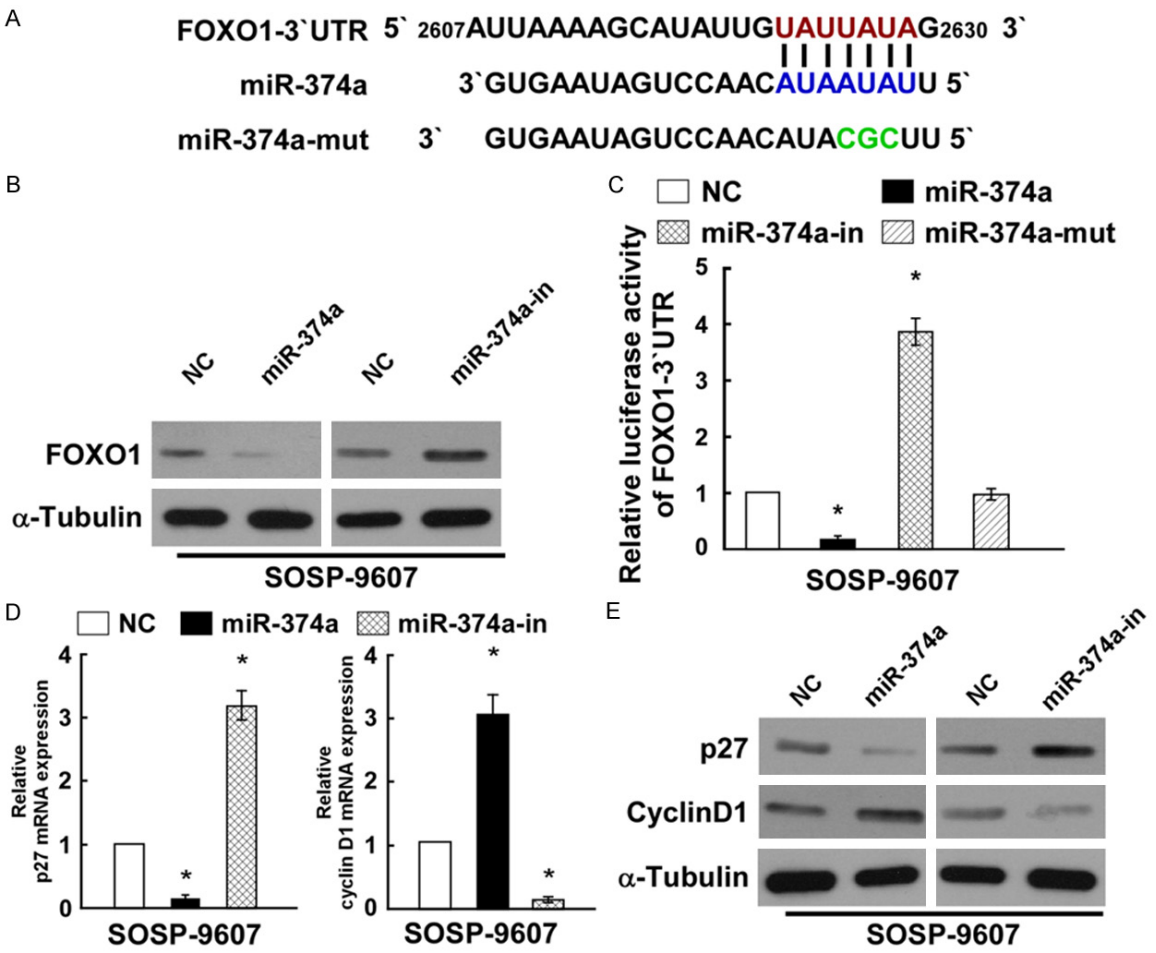

Figure 4. miR-374a suppresses FOXO1 expression by directly targeting the FOXO1 3'-UTR. A. Predicted miR-374a target sequence in the 3'-UTR of FOXO1 (FOXO1-3'-UTR) and positions of three mutated nucleotides (red) in the 3'-UTR of miR-374a (miR-374a-mut). B. Western blotting analysis of FOXO1 expression in cells transfected with miR-374a or the miR-374a inhibitor. α-Tubulin served as the loading control. C. Luciferase reporter assay of the indicated cells transfected with the pGL3-FOXO1-3'-UTR reporter and miR-374a or miR-374a-in or miR-374a with oligonucleotides. D. Real-time PCR analysis of expression of cyclin D1 and p27 in indicated SOSP-9607cells. E. Western blotting analysis of expression of cyclin D1 and p27 protein in SOSP-9607 cells. α-Tubulin served as the loading control. *$P < 0.05$.

miR-374a leads to upregulation of CyclinD1 and downregulation of p27. Collectively, these findings suggest that miR-374a may promote the initiation and progression of OS.

It has been demonstrated that microRNAs negatively regulate their target mRNAs in a sequence-specific manner [6, 13], which play crucial roles in development of human cancers, including osteosarcoma [14-16]. The effects of miR-374a have been mostly described in motile kinds of cancer cells [8, 9, 11]. Growing evidence suggests that FOXO1 expression is regulated by several microRNAs [17-19]. However, there was no report about FOXO1 regulated by miR-374a in OS to promote cell proliferation. It is the first time to uncover the new relationship of regulation of FOXO1 and miR-374a in OS. In the present study, we found that miR-374a expression was significantly overexpressed in human OS tissues and OS cells (SAOS-2, SOSP-9607, MG-63 and U2-OS), compared to the matched adjacent normal tissues and human osteoclast h-FOB cell lines, and miR-374a overexpression in SOSP-9607 cells promotes OS cell proliferation *in vitro*, suggesting that miR-374a plays an important role in malignant processes in OS.

By using bioinformatic analysis, we identified FOXO1 as the potential target gene of miR-374a. The luciferase activity assay demonstrat-

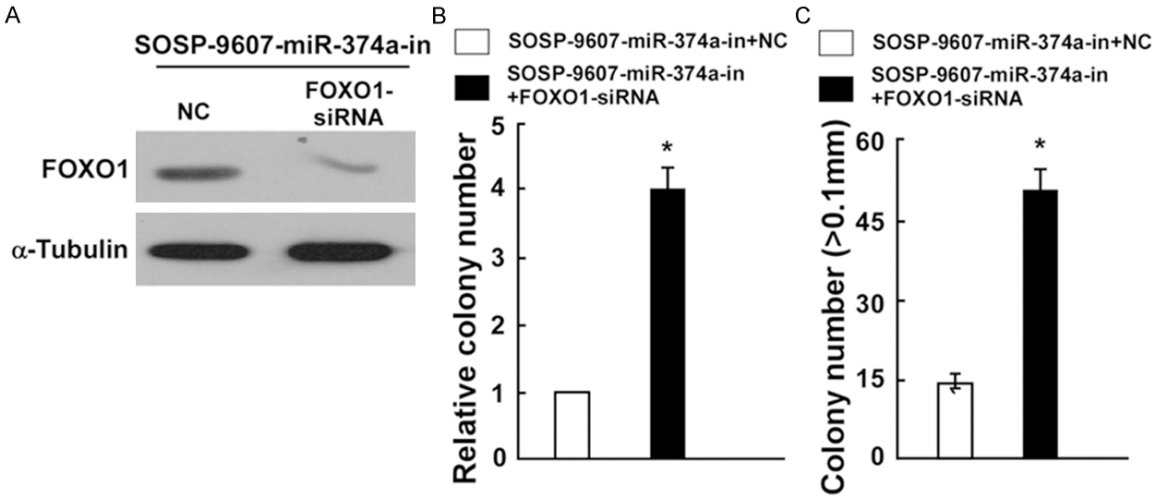

Figure 5. FOXO1 upregulation is required for miR-374a-in-induced proliferation of OS cells. A. Western blot analysis verified that silencing FOXO1 effectively decreased the expression of FOXO1 in miR-374a-in-transfected SOSP-9607 cells. B. miR-374a-in-transfected SOSP-9607 cells after transfection with FOXO1-siRNAs promoted cell colonies formation. C. miR-374a-in-transfected SOSP-9607 cells after transfection with FOXO1-siRNAs promoted the anchorage-independent growth. Representative quantification of colonies that were > 0.1 mm. Each bar represents the mean of three independent experiments. **P < 0.05*.

ed that the downregulation of FOXO1 was mediated by miR-374a through the FOXO1-3'-UTR. Our study identified FOXO1 as a genuine target of miR-374a, suggesting a crucial functional role of FOXO1 in OS tumorigenesis. FOXO1 is a key effector regulates many biological processes, such as cell cycle regulation, cell differentiation and tumorigenesis [19, 20]. FOXO 1 expression leads to G_1/S arrest, resulting from downregulation of cyclin D1 and increased expression of cyclin dependent kinase inhibitors p27 [12, 21]. In our study, expression of FOXO1 proteins was significantly downregulated in miR-374a-overexpressing SOSP-9607 cells, while its inhibition of miR-374a increases FOXO1 expression. The downstream genes (cyclin D1, p27) regulated by FOXO1 are crucial in the regulation of cell proliferation, cycle, differentiation, apoptosis and other cellular functions [17, 22-24]. Our studies here showed that the molecular mechanism by which miR-374a promotes OS cancer cell proliferation by suppressing FOXO1, upregulation of cyclin D1 and downregulation of p27 in cells overexpressing miR-374a. Furthermore, FOXO1-silenced in miR-374a-in-transfected SOSP-9607 cells have positive effect to promote cell proliferation, suggesting that direct FOXO1 downregulation is required for miR-374a-induced OS cell proliferation.

In conclusion, the current study revealed that miR-374a can increase the proliferation of prostate cancer cell lines by targeting expression of FOXO1, suggesting miR-374a plays an essential role in the regulation of OS cell proliferation and may function as an onco-miRNA. These findings collectively implicate miR-374a might serve as a promising prognostic and therapeutic target for future OS therapy.

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Disclosure of conflict of interest

None.

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