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Original Article

MicroRNA-34a promotes cell cycle arrest and apoptosis and suppresses cell adhesion by targeting DUSP1 in osteosarcoma

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Abstract: MicroRNAs are often deregulated in most cancer types and have important functions in carcinogenesis and cancer progression. Here, we studied the function of microRNA-34 (miR-34a) in osteosarcoma MG63 and U-2OS cells by expressed with pre-miR-34a, anti-miR-34a and corresponding negative controls, respectively. Cells proliferation, cell cycle and apoptosis was measured by MTT and flow cytometry assay. The effect of miR-34a on DUSP1 expression was evaluated by luciferase assays, real-time PCR and western blot assay. The data showed that miR-34a reduced the proliferation of MG63 cells through prompting cell cycle arrest at G0/G1 phase, cell apoptosis, and suppressed cell adhesion ability. Whereas anti-miR-34a increases U-2OS cell proliferation by preventing cell apoptosis, and promotes cell adhesion. Finally, we identified Dual-specificity phosphatase 1 (DUSP1) as the target gene of miR-34a in osteosarcoma cells and confirmed that DUSP1 enhanced the proliferation through inhibiting cell cycle arrest at G0/G1 phase and apoptosis, and inhibits the decreased cell adhesion induced by miR-34a. However, inhibition of DUSP1 resulted in substantially decreased proliferation and adhesion, and cell cycle arrest in G0/G1 phase and cell apoptosis similar to that observed with miR-34a in U-2OS cells. Our findings find out an important function of miR-34a as a novel tumor-suppressor in osteosarcoma pathogenesis through inhibition of DUSP1.

Keywords: miR-34a, osteosarcoma, DUSP1, G0-G1 phase, adhesion

Introduction

Osteosarcoma is the most prevalent primary malignant bone cancer which happens generally in young people affecting rapidly growing bones [1] and with an annual estimated incidence of about 4 million worldwide [2]. During the last decade, surgery combined with chemotherapy for osteosarcoma treatment by inhibiting cell growth or invasion, has been investigated [3]. Still, despite aggressive treatment, the progress of metastasis and chemotherapy resistance stays main problems responsible for the failure of treatment. The overall survival rate of osteosarcomas is only 50% to 80% [4] and local relapse happens in about 10% of patients [5] and 70% of patients with recurrence die despite second-line treatment [6]. Therefore, there is a marked requirement to develop effective therapeutic treatments that inhibit metastasis, the major reason of death in osteosarcoma.

Finding out the underlying molecular mechanisms that drive the progression and metastasis of osteosarcoma would help the advance of better therapeutic strategies to improve the patient prognosis and management, and it could also help to identify novel molecular prognostic factors and therapeutic targets. Reports have explored the genes related to the metastasis of osteosarcoma, and miRNAs have grown into a novel exploration hotspot [7, 8]. miRNAs are small non-coding RNAs, constituting about 22 nucleotides, which classically participating in post-transcriptional regulation of gene [9]. Growing evidences have demonstrated that miRNAs have vital functions in biological and

pathological processes, for example cell proliferation, apoptosis, stress response, and development [10, 11]. Moreover, frequent dysregulations of miRNAs have been detected in many types of cancer, including ovarian [12], liver [13], bladder [14], and colorectal cancer [15]. Especially, miR-34a is a recognized tumor inhibitor that controls cell proliferation, invasion, and other courses in various kinds of tumor [16, 17]. Yet, the functions of miR-34a in OS have not been fully clarified. Thus, it is important to find out the functions and underlying mechanisms of miR-34a in OS.

miR-34a has many target genes that have numerous functions in shaping the cellular function in OS, such as p53, Eag1, Wnt and Notch [18, 19]. Mitogen-activated protein kinases (MAPKs) constitute an extremely conserved clan of kinases that transmit signals from outside to the intracellular molecules has been implicated in pathogenesis of OS [20, 21]. The largest groups of enzymes that mediate the dephosphorylation of MAPK are the protein tyrosine phosphatase superfamily, termed the dual-specificity protein phosphatases (DUSPs). DUSP1 was firstly replicated as a gene induced by growth factor involved in the G0/G1 phase transition and apoptosis [22, 23]. miR-101 has been found target DUSP1 to affect the secretion of TGF-β in sorafenib [24]. miR-940 inhibition retains MKP1 expression and attenuates JNK-mediated apoptosis in lung cancer cells [25]. However, the regulation of DUSP1 by miR-34a in OS remains unknown.

The current study intends to study the roles of miR-34a and DUSP1 in proliferation, apoptosis and adhesion of OS cells and the involved molecular mechanisms. This may help develop potential diagnostic and therapeutic targets for OS.

Materials and methods

Cell culture and transfection

Two human OS cells (U-2OS and MG63) were purchased from the American Type Culture Collection (Rockville, USA). Cells were cultured in DMEM (Life technology) complemented with 10% fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin (Life Technologies) in 5% CO_2 at 37°C in a humidified incubator. MG63 cells were transfected with miR-34a or negative control (NC) (Ambion, TX) using HiPerFect transfection reagent (Qiagen, CA) according to the manufacturer's instructions. For inhibition assay, U-2OS cells were transfected with anti-miR-34aor control (NC) (Ambion, TX). The coding sequence of DUSP1 was amplified from MG63 cells, and ligated into the vector pcDNA3. This DUSP1 expression vector or blank pcDNA3 vector was introduced into MG63 cells using TransIT-LT1 transfection reagent (Mirus, WI). U-2OS cells were also transfected with DUSP1 small interfering RNAs (siRNAs) or negative control siRNA (siControl) (Invitrogen) to knockdown of DUSP1.

Quantitative real-time PCR (qRT-PCR)

MiRNAs were isolated from osteosarcoma cell lines using a miRNeasy Nini Kit (Aiagen, Valencia, CA, USA). Total RNA was extracted by the TRLzol (Invitrogen) according to the manufacturer's protocols. Synthesis of complementary DNA (cDNA) was completed according to the manufacturer's instruction of Superscript III Kit (Invitrogen™, Carlsbad, CA). qRT-PCR was done on the iCycleriQ Real-time detection System (Bio-Rad). The comparative CT was calculated to analyze the expression levels of miR-34a and DUSP1 in osteosarcoma cells. DUSP1 forward primer sequence, 5'-TGGAGGAAGGGTGTTTGTCC-3' and reverse primer, 5'-CAAGGCAGATGGTGGCTGA-3'; GAPDH forward primer, 5'-ACAACTTTGGTATCGTGGAAGG-3' and reverse primer, 5'-GCCATCACGCCACAGTTTC-3'. Experiments were done in triplicate, and data analysis was made by iCycleriQ Optical System software (version 3.0). 5S and GAPDH were analyzed as a reference gene for microRNA and mRNA.

Immunoblot analyses

Cells were lysed in ice-cold RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) added with protease inhibitors. 50 μg protein were loaded into 10% SDS-PAGE and were shifted onto a PDVF membrane (Millipore, USA). Membranes were incubated with a rabbit polyclonal antibody to Bax, Bcl-2, Cyclin D1, Cyclin E, E-cadherin, β-catenin or GAPDH at 4°C overnight, and then cultured with horseradish peroxidase-conjugated anti-rabbit antibody (Jackson ImmunoReseach) for 60 min at room temperature before the specific protein bands were visualized using the chemiluminescent

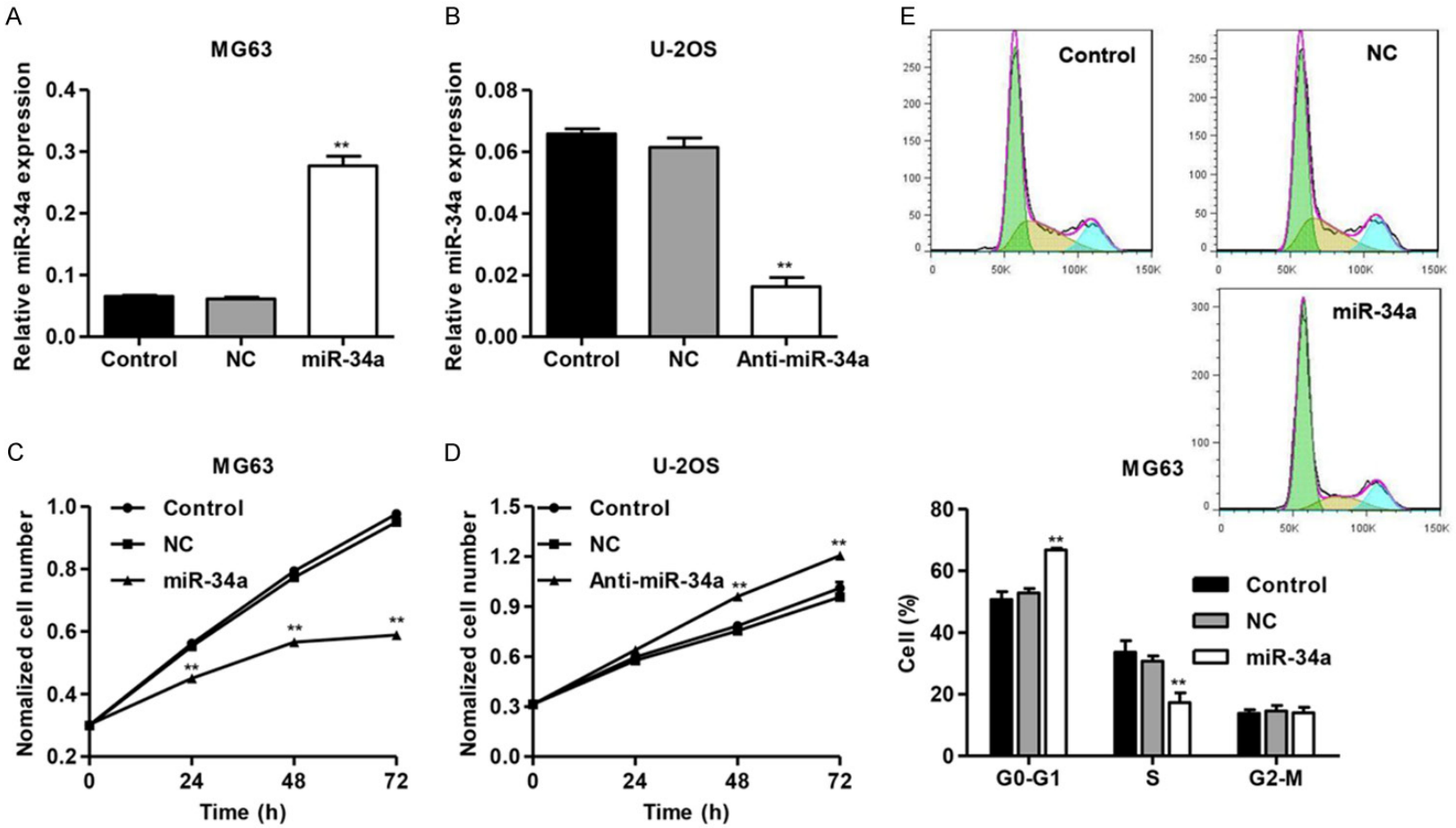

Figure 1. miR-34a suppresses osteosarcoma cell proliferation and induces G0-G1 phase arrest. A. High expression of miR-34a in MG63 cells was established after transfection with miR-34a. B. Successful knockdown of miR-34a in U-2OS cells was confirmed by QRT-PCR after transfection with miR-34a inhibitor or negative control (NC). C, D. Cell proliferation of MG63 and U-2OS cells was measured by MTT at indicated time points. E. Cell cycle of MG63 cells was analysed by flow cytometry assay. **P<0.01 compared with NC.

substrate (Pierce, USA) and spotted by Image Quant LAS 4010 Imaging System (GE Healthcare, Piscataway, NJ).

MTT

Cells proliferation ability was analyzed by MTT. Briefly, cells were plated in 96-well plate with 2,000 cells/well. Every 24 h break, 20 μl of 5 mg/ml MTT (Sigma) was added to each well with a final concentration of 10 μmol/L and the plates were further incubated at 37°C in the dark for 2 hours. Then the medium was aspired, and added 100 μl of DMSO (dimethyl sulfoxide) to melt the formazan. The optical density (OD) at 595 nm was assessed in a microtiter plate reader. Blank wells without cells were designated as controls.

Cell cycle analysis

1×10^4 cells/well were seeded in 6-well plate and allowed to grow to 60%-80% confluence. Then cells were fixed in 70% chilled ethanol before cultured in 10 mmol/L RNase at 37°C for 10 min. The cells were stained with 10 μmol/L propidium iodide (Sigma, USA) for 20 min. The fluorescence intensities were assessed by FACS using a FL-2A filter (BD FACS Calibur, USA). Data were studied with Cyflogic software (CyFlo Ltd, Turku, Finland).

Apoptosis assay

Following double staining with 10 μmol/L annexin V-fluorescein isothiocyanate (Santa Cruz, USA) and 5 μmol/L of PI, the cells were evaluated using flow cytometry (FACScan®; BD Biosciences). Cell death determinations included early apoptotic cells, which is annexin V positive/PI negative and late apoptotic cells, which is annexin V positive/PI positive with Cyflogic software (CyFlo Ltd, Turku, Finland).

Cell adhesion assay

The 12-well plate was pre-coated with fibronectin (5 μg/ml) at room temperature for 2 h. Cell were then seeded into the coated wells with a density of 1×10^5 cells/well and stay set for 1 h at 37°C. Non-adherent cells were detached by phosphate-buffered saline. Remaining cells were fixed in paraformaldehyde and then stained by 0.2% crystal violet.

Dual-luciferase reporter assay

The 3'UTR of DUSP1 covering the miR-34a binding site was amplified (Forward primer sequences: 5'-GCGCTCTAGAAAGGCCACGGGAGGTGAGG-3' and Reverse primer sequences: 5'-GCGCTCTAGACCATTGTATAAAAAAGTCAT-3') and cloned into the pGL3 luciferase vector (Promega). Mutant constructs were made via changing the seed sequence of the miR-34a binding site. MiR-34a expressing or control cells were transfected with constructed reporter vectors and pRL-TK vector (Promega) by the lipofectamine 3000 (Invitrogen, Carlsbad, CA). Reporter tests were done 48 h post-transfection by Dualluciferase assay system (Promega).

Statistical analysis

Data are expressed as the mean ± SD. All of the statistical analyses were made using GraphPad Prism v5.0 (GraphPad Software, Inc.). Differences between each groups were evaluated with Student's t test. *P* value less than 0.05 were considered to be statistically significant.

Results

Overexpression of miR-34a prevents OS cell proliferation and prompts cell cycle arrest

To explore the functions of miR-34a in osteosarcoma cells, MG63 cells were transfected with miR-34a or NC for overexpression and U-2OS cells were transfected with anti-miR-34a or negative control RNA (NC) for inhibition of miR-34a function. As revealed in **Figure 1A** and **1B**, the level of miR-34a was significantly augmented by 3.54-fold in MG63 cells and decreased by 73.5% in U-2OS cells compared with corresponding NC groups. Then, the MTT assay was done to observe the outcome of miR-34a on the proliferation ability of human osteosarcoma cells 0, 24, 48 and 72 h after the transfection of miR-34a mimic or anti-miR-34a and its corresponding NC. As a result, the cell proliferation ability of MG63 cells was significantly poorer in miR-34a group than the NC, while that of U-2OS cells was significantly higher in anti-miR-34a group than the NC (**Figure 1C** and **1D**).

Because miR-34a mimic evidently suppressed proliferation of osteosarcoma cells, we rea-

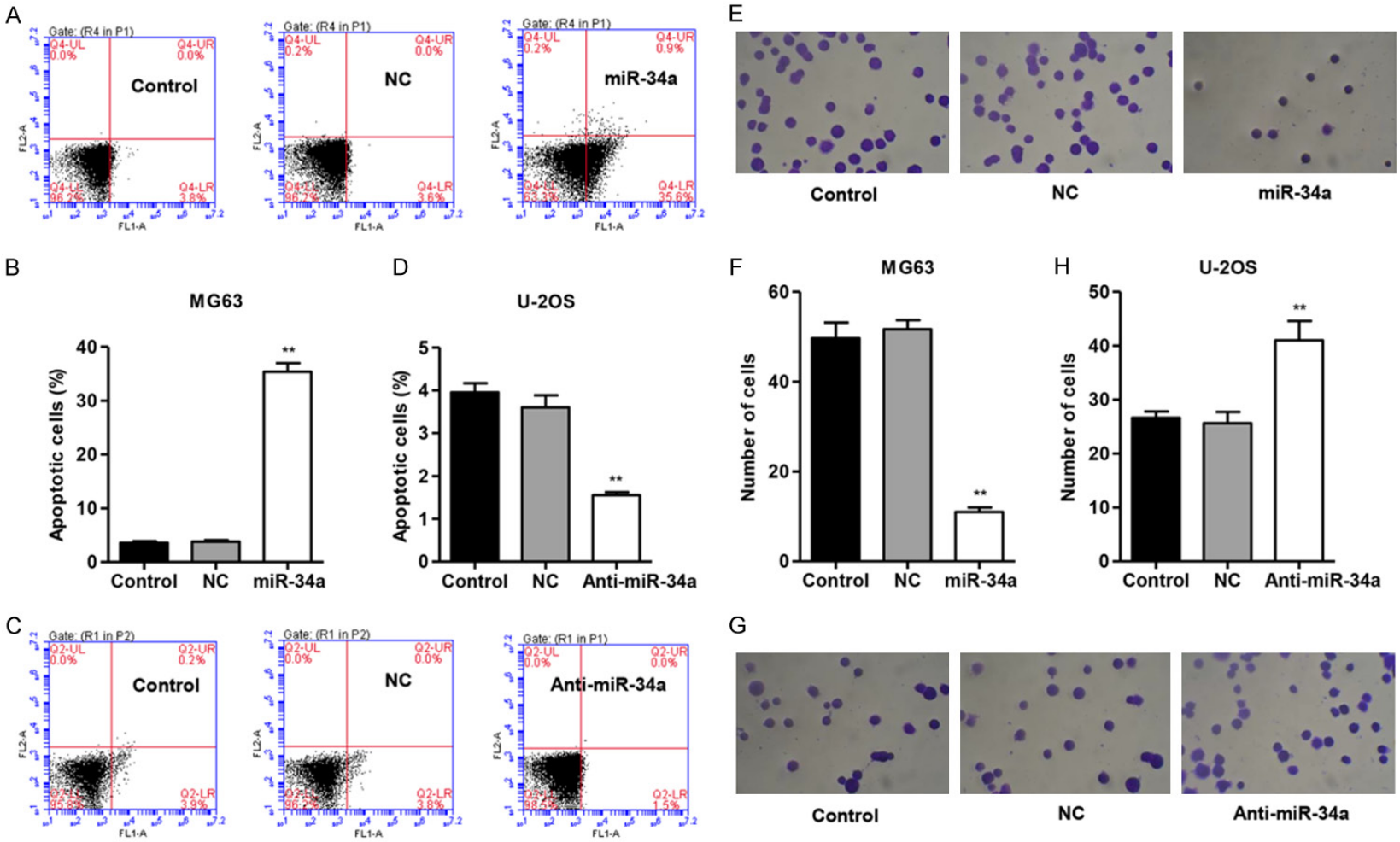

Figure 2. miR-34a induces osteosarcoma cell apoptosis andinhibits osteosarcoma cell adhesion. After MG63 cells transfected with miR-34a oligoribonucleotides (A, B) and U-2OS cells transfected with anti-miR-34a (C, D), the cell apoptosis was measured by flow cytometry. After MG63 cells transfected with miR-34a oligoribonucleotides (E, F) and U-2OS cells transfected with anti-miR-34a (G, H), cell adhesion was measured. Magnification, ×200. **P<0.01 compared with NC.

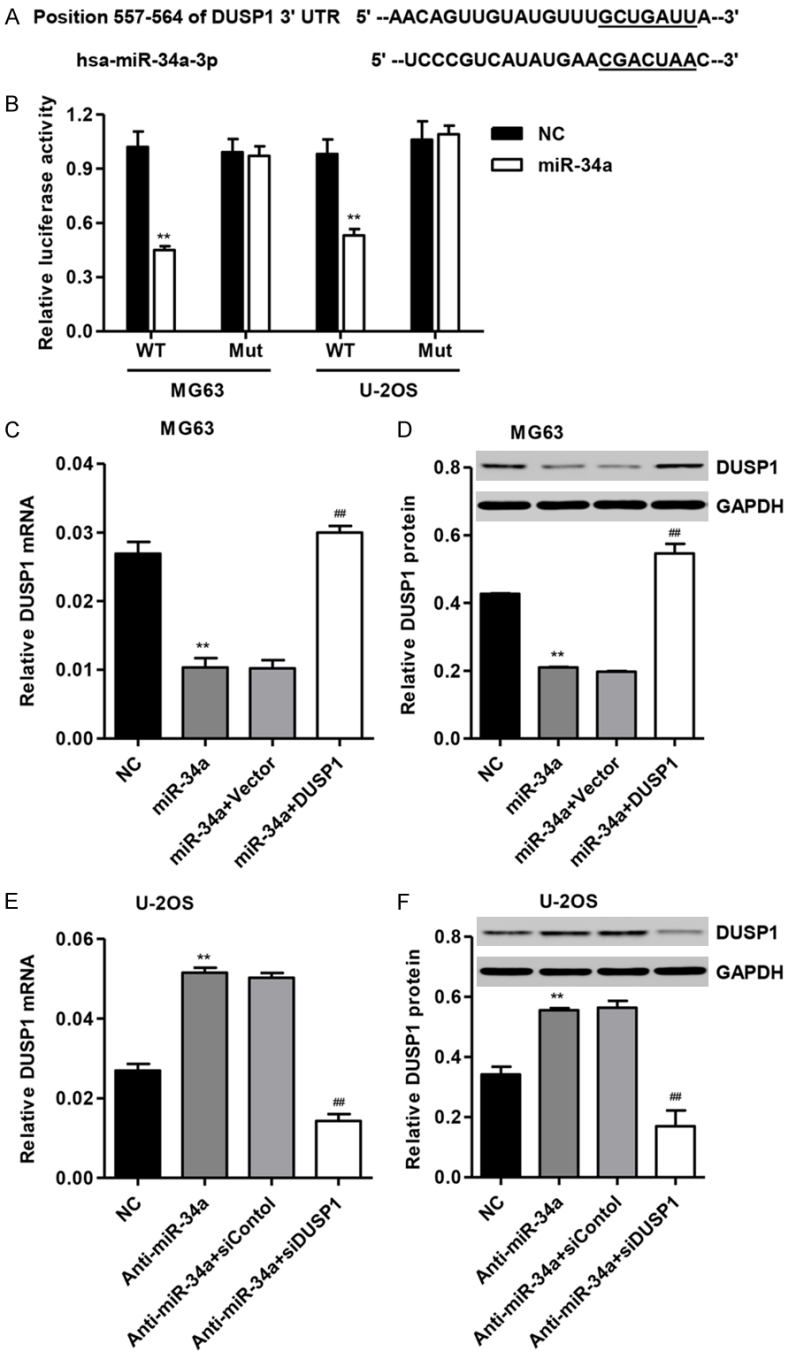

Figure 3. miR-34a negatively regulates DUSP1 by binding to the DUSP1 3'UTR. (A) Schematic diagram of potential miR-34a-target site in DUSP1 3'UTR. (B) A luciferase reporter assay showed the inhibitory effect of miR-34a on DUSP1-3'UTR in MG63 and U-2OS cells. After miR-34a-mediated MG63 cells transfected with pcDNA3-DUSP1 or blank pcDNA3 expression vector and anti-miR-34a-mediated U-2OS cells transfected with DUSP1-siRNA (siDUSP1) or negative control siRNA (siControl), the DUSP1 mRNA and protein levels were analyzed by QRT-PCR and Western blotting in MG63 (C, D) and U-2OS cells (E, F). **P<0.01 compared with NC. ##P<0.01 compared with miR-34a+Vector or Anti-miR-34a+siControl.

soned that miR-34a might arrest the cell cycle of osteosarcoma cells. The results of flow cytometry exhibited that the high expression of miR-34a significantly augmented the cells in the G0/G1 and reduced the cells in the S phase in MG63 cells compared to NC (**Figure 1E**). However, there was no significant change of anti-miR-34a on cell cycle arrest of U-2OS cells (data not shown).

Overexpression of miR-34a prompts osteosarcoma cell apoptosis and prevents cell adhesion

The Annexin-VFITC/PI staining method was used to spot the apoptosis of OS cells. The data revealed that the percentage of cell apoptosis was increased by 9.30-fold following transfection with the miR-34a in MG63 cells (**Figure 2A** and **2B**) and was decreased by 56.9% after transfected the anti-miR-34a in U-2OS cells (**Figure 2C** and **2D**). Since migration is a key characteristic of malignant tumor, we next assessed the properties of miR-34a on the cell adhesion. The data demonstrated that adhesive ability of MG63 was significantly suppressed by 78.4% in miR-124 mimic group (**Figure 2E** and **2F**) and that of U2OS cells was significantly elevated-by 60.1% in anti-miR-34a group compared with its corresponding NC groups (**Figure 2G** and **2H**).

DUSP1 is a direct target gene of miR-34a in OS cells

To delineate the molecular mechanism that miR-34a repressed osteosarcoma cell growth

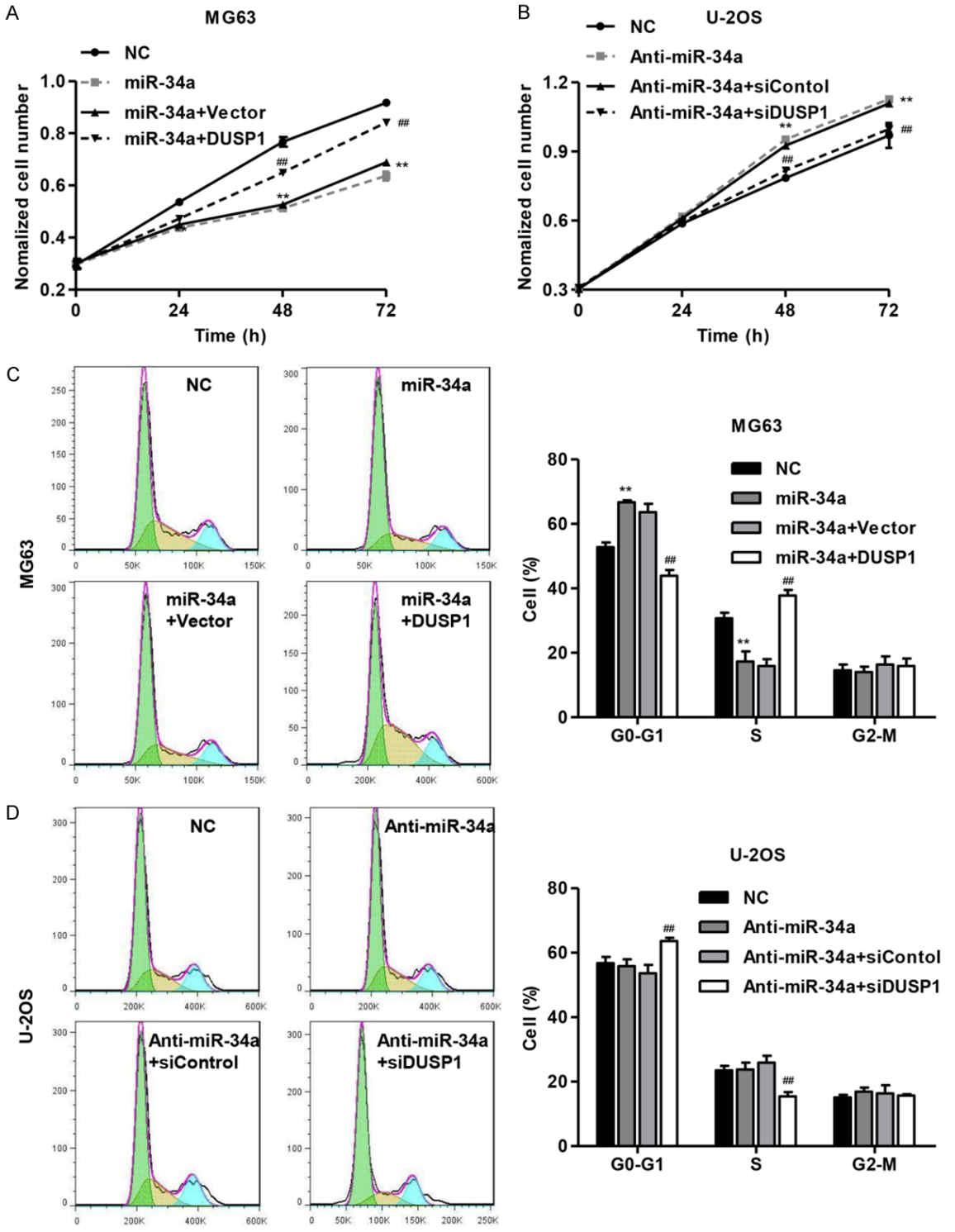

Figure 4. Overexpression of DUSP1 reverses the effects of miR-34a on proliferation and cell cycle. After miR-34a-mediated MG63 cells transfected with pcDNA3-DUSP1 or blank pcDNA3 expression vector and anti-miR-34a-mediated U-2OS cells transfected with DUSP1-siRNA (siDUSP1) or negative control siRNA (siControl), the proliferation and cell cycle was measured by MTT assay (A, B) and flow cytometry (C, D), respectively. **P<0.01 compared with NC. ##P<0.01 compared with miR-34a+Vector or Anti-miR-34a+siControl.

and adhesion, miR-34a target genes were searched using the TargetScan (**Figure 3A**). Next, we further demonstrated whether DUSP1 was a direct target gene of miR-34a via lucifer-

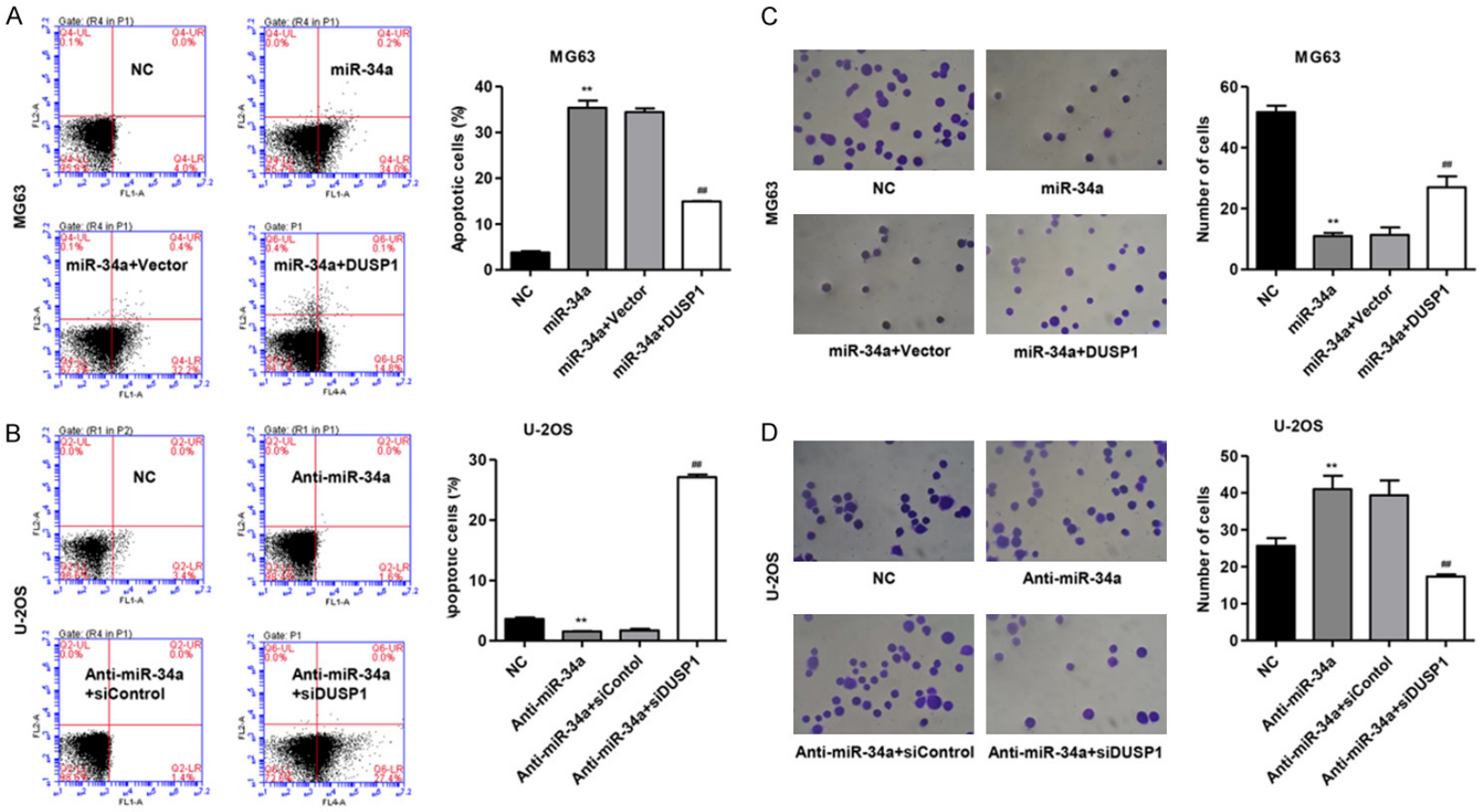

Figure 5. Overexpression of DUSP1 reverses the effects of miR-34a on cell apoptosis and cell adhesion. After miR-34a-mediated MG63 cells transfected with pcDNA3-DUSP1 or blank pcDNA3 expression vector and anti-miR-34a-mediated U-2OS cells transfected with DUSP1-siRNA (siDUSP1) or negative control siRNA (siControl), the cell apoptosis and cell motility was measured by flow cytometry (A, B) and adhesion assay (C, D), respectively. **P<0.01 compared with NC. ##P<0.01 compared with miR-34a+Vector or Anti-miR-34a+siControl.

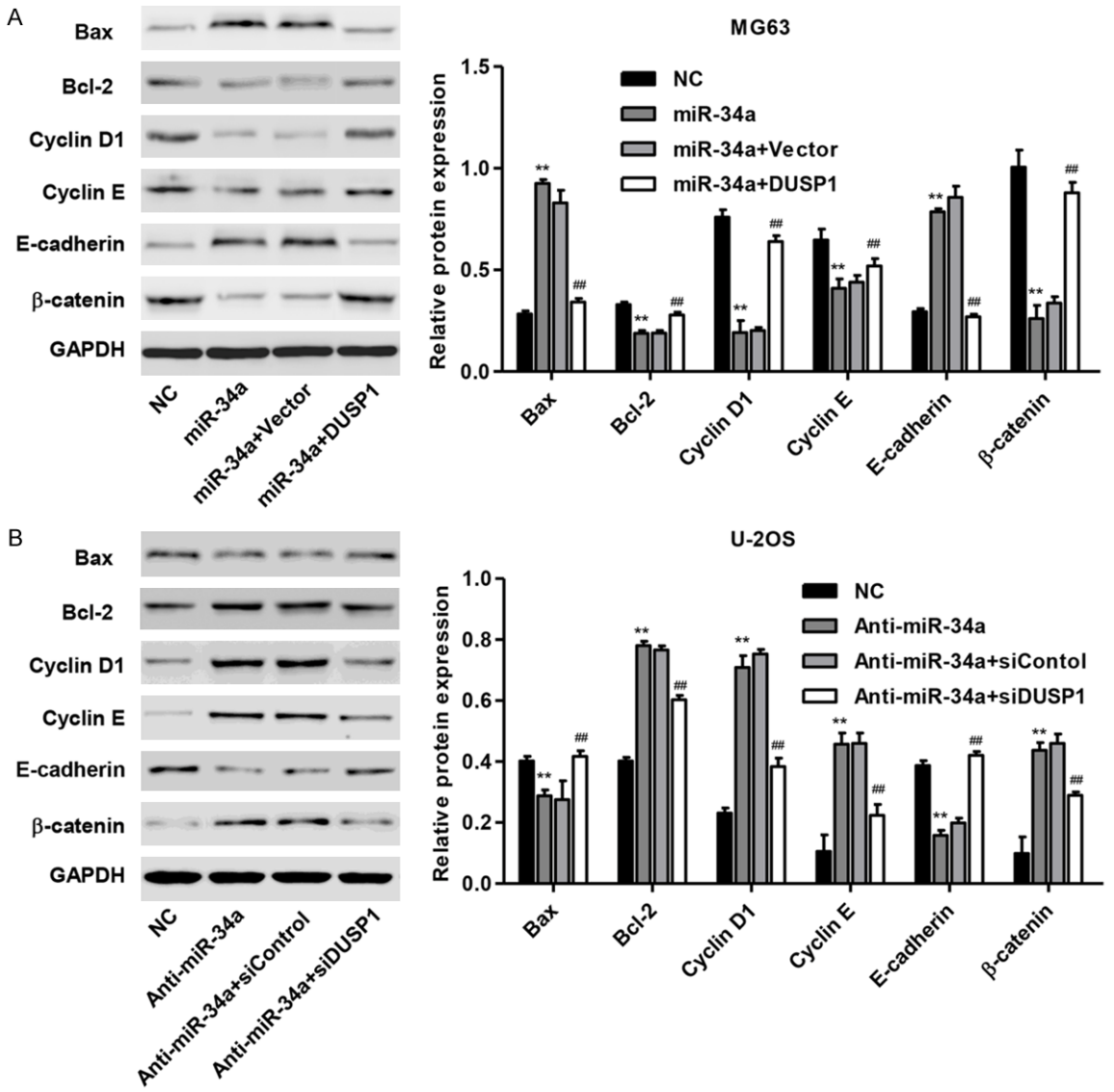

Figure 6. Overexpression of DUSP1 reverses the effects of miR-34a on the protein expressions. After miR-34a-mediated MG63 cells transfected with pcDNA3-DUSP1 or blank pcDNA3 expression vector (A) and anti-miR-34a-mediated U-2OS cells transfected with DUSP1-siRNA (siDUSP1) or negative control siRNA (siControl) (B), the protein expression of Bax, Bcl-2, Cyclin D1, Cyclin E, E-cadherin and β-catenin was measured by Western blotting. **P<0.01 compared with NC. ##P<0.01 compared with miR-34a+Vector or Anti-miR-34a+siControl.

ase reporter assay. The 3'UTR of DUSP1 was inserted into a luciferase reporter vector with or without the mutated miR-34a binding site in the 3'UTR of DUSP1. The data displayed that highly expression of miR-34a significantly repressed the luciferase activity of pGL3-DUSP1 3'UTR WT but not the Mut, demonstrating that miR-34a can bind to the 3'UTR of DUSP1 directly (**Figure 3B**).

To investigate whether miR-34a inhibited the abilities of proliferation and adhesion of OS cells by downregulation of DUSP1, we cotransfected MG63 with miR-34a mimic and pcDNA3-DUSP1 and U2OS cells with anti-miR-34a and siDUSP1. QRT-PCR and Western blotting assay observed that the level of DUSP1 on mRNA and protein levels in MG63 cells transfected with miR-34a and pcDNA3-DUSP1 was increased by 1.94- and 1.76-fold significantly (**Figure 3C** and **3D**), and that in U-2OS cells introduced with anti-miR-34a and siDUSP1 was significantly reduced by 71.6% and 69.9% (**Figure 3E** and **3F**).

Up-regulation of DUSP1 reverses cell proliferation and cell cycle induced by miR-34a overexpression in osteosarcoma cells

As presented at **Figure 4A** and **4B**, overexpression of DUSP1 significantly improved the cell proliferation of MG63 cells transfected with miR-34a, and siRNA silencing of DUSP1 significantly decreased the cell proliferation of U-2OS cells transfected with anti-miR-34a and siDUSP1. Flow cytometry assay showed that high expression of DUSP1 significantly decreased the cells in the G0/G1 phase and enlarged the cells in the S phase of MG63 cells transfected with miR-34a, and knockdown of DUSP1 significantly augmented the cells in the G0/G1 phase and increased the cells in the S phase of U-2OS cells expressing anti-miR-34a and siDUSP1 (**Figure 4C** and **4D**). Our data clearly showed that miR-34a repressed proliferation and prompted cell cycle arrest in osteosarcoma cells by down-regulating DUSP1 expression, and that decreased DUSP1 expression was essential for the inhibition of miR-34a on proliferation and cell cycle in osteosarcoma cells.

Up-regulation of DUSP1 reverses cell apoptosis and cell adhesion induced by miR-34a overexpression in osteosarcoma cells

Annexin-VFITC/PI staining showed that high expression of DUSP1 reduced the apoptosis of MG63 cells transfected with miR-34a mimics significantly, and knockdown of DUSP1 significantly augmented the cell apoptosis of U-2OS cells expressing anti-miR-34a and siDUSP1 (**Figure 5A** and **5B**). The cell adhesion assay showed that high expression of DUSP1 amplified cell adhesion of MG63 cells transfected with miR-34a significantly, and knockdown of DUSP1 decreased cell adhesion of U-2OS cells transfected with anti-miR-34a and siDUSP1 significantly (**Figure 5C** and **5D**). Our data clearly exhibited that miR-34a prompted cell apoptosis and inhibits cell adhesion in osteosarcoma cells by down-regulating DUSP1 expression, and that decreased DUSP1 expression was essential for the promotion of miR-34a on apoptosis and decreasing cell adhesion in osteosarcoma cells.

Up-regulation of DUSP1 reverses related protein expression induced by miR-34a overexpression in osteosarcoma cells

The proliferation ability, cell cycle and cell adhesion were reduced in osteosarcoma cells transfected with miR-34a. Thus, Bax, Bcl-2, Cyclin D1, Cyclin E, E-cadherin and β-catenin were determined by a Western blot. Our data exhibited that the protein levels of Bax and E-cadherin displayed dramatic up-regulation in miR-34a group than those in NC (**Figure 6A**). However, the protein levels of Bcl-2, Cyclin D1, Cyclin E, and β-catenin were significantly downregulated when overexpression of miR-34a in MG63. Interestingly, overexpression of DUSP1 reversed the effect of miR-34a on these protein expressions in MG63 cells. In addition, anti-miR-34a reduced the levels of Bax and E-cadherin, but improved Bcl-2, Cyclin D1, Cyclin E, and β-catenin significantly, which were reversed by siDUSP1 transfection in U-2OS cells (**Figure 6B**).

Discussion

The improvements and early diagnosis in OS cure has certainly improved the survival of OS patients in the past decade [26]. Targeted therapies designed against molecules participating in the pathogenesis of cancer were reported as effective ways in cancer recently. Our study displayed that miR-34a expression inhibited the proliferation and adhesion but induced the cell cycle arrest and apoptosis of the MG63 cells. Inhibition of miR-34a increased proliferation and adhesion but suppressed the cell apoptosis of the U-2OS cells. Moreover, we found that miR-34a played as a new tumor-suppressor via directing DUSP1.

Growing evidences have presented that miRNAs are often deregulated in a many cancers [11, 13]. We chose miR-34a not only because findings verified that the level of miR-34a was reduced in OS tissues as compared to adjacent normal tissues [18], but the induction of cell cycle arrest and apoptosis by miR-34a was also mediated by p53 [27]. Reports have indicated that miR-34a might function as a new tumor suppressor in OS. Unrestrained cell proliferation and hostile metastasis are vital stages for cancer progression. Thus, in this study we explored the functions of miR-34a on OS cell proliferation and adhesion. We demonstrated that overexpression of miR-34a decreased the cell proliferation and adhesion of MG63 cells significantly, and knockdown of miR-34a markedly increased the proliferation and adhesion of U-2OS cells. Furthermore, high expression of miR-34a induced cell cycle arrest at G0/G1

phase and apoptosis of MG63 cells significantly, and knockdown of miR-34a markedly decreased cell apoptosis of U-2OS cells. These outcomes show that miR-34a plays an vital portion in the progress of OS, which in line with the previous studies [16, 18, 19].

Recognizing the miR-34a-targeting genes provide molecular base for exploration of regulatory mechanisms of miR-34a. Through TargetScan 5.1, DUSP1, which has multipotent roles in cancer progression, was found as a target gene of miR-34a. Recently, it has been shown that high DUSP1 expression is related to decreased sensitivity to cisplatin-induced cell death [28] and the reduction of DUSP1 gene expression resulted in reduced cell proliferation, migration and invasion abilities in OS cells [21]. Consistently, our study showed that high expression of DUSP1 significantly inhibited miR-34-induced the decreased cell proliferation and adhesion and the stimulation of cell cycle arrest and cell apoptosis of MG63 cells, but inhibition of DUSP1 markedly inhibited anti-miR-34-induced the increased cell proliferation and adhesion and reduction of cell apoptosis of U-2OS cells.

Since DUSP1 has been associated a several cell processes in cancer, we decided to deepen our research about the role of DUSP1 in the OS pathogenesis. After DUSP1 overexpressing and silencing in OS cell lines, we observed that DUSP1 is elaborate in cell cycle-, apoptosis- and adhesion-related proteins in OS. Overexpression of miR-34a increased the levels of Bax and E-cadherin, but decreased the expression of Bcl-2, Cyclin E, Cyclin D1 and β-catenin in MG-63 cells, which was reversed by DUSP1 overexpression. Additionally, inhibition of miR-34a decreased the levels of Bax and E-cadherin, but increased the level of Bcl-2, Cyclin E, Cyclin D1 and β-catenin in U-2OS cells, which was reversed by DUSP1 gene silencing. We have previously demonstrated high expression of the Cyclin E and Cyclin D1 in c-Fos transgenic osteosarcomas affected cell cycle progression and differentiation [29]. β-catenin pathway activator could increase cell proliferation and metastasis of OS cells [30], while inhibition of E-cadherin promotes osteosarcoma cell proliferation and metastasis [31], suggesting a negative role between β-catenin and E-cadherin in cell proliferation and invasion of OS cells.

In conclusion, our esults verified that miR-34a has crucial functionson the proliferation, apoptosis and adhesion of OS cells. High expression of miR-34a repressed DUSP1, suggestive of that miR-34a functions as an novel and important tumor suppressor might via targeting DUSP1 in osteosarcoma.

Disclosure of conflict of interest

None.

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