MicroRNA-148a Acts as a Tumor Suppressor in Osteosarcoma via Targeting Rho-Associated Coiled-Coil Kinase

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MicroRNAs (miRs) have been demonstrated to be involved in the development and progression of osteosarcoma (OS), but the molecular mechanism still remains to be fully investigated. The present study investigated the function of miR-148a in OS, as well as its underlying mechanism. Our data showed that miR-148a was significantly downregulated in OS tissues compared to their matched adjacent normal tissues, and also in OS cell lines compared to normal human osteoblast cells. Low expression of miR-148a was significantly associated with tumor progression and a poor prognosis for OS patients. Rho-associated coiled-coil kinase 1 (ROCK1) was then identified as a target of miR-148a in Saos-2 and U2OS cells, and the expression of ROCK1 was significantly increased in OS tissues and cell lines. Moreover, the protein expression of ROCK1 was markedly reduced in miR-148a-overexpressing Saos-2 and U2OS cells, but significantly increased in miR-148a-downregulated Saos-2 and U2OS cells. Further investigation indicated that miR-148a had a suppressive effect on the proliferative, migratory, and invasive capacities of Saos-2 and U2OS cells. Moreover, overexpression of ROCK1 attenuated the inhibitory effects of miR-148a upregulation on the malignant phenotypes of Saos-2 and U2OS cells. In addition, overexpression of miR-148a significantly inhibited the tumor growth of U2OS cells in nude mice. Taken together, these data demonstrate that miR-148a acts as a tumor suppressor in OS, at least partly, via targeting ROCK1. Therefore, the miR-148a/ROCK1 axis may become a potential therapeutic target for OS.

Key words: Osteosarcoma; MicroRNAs (miRs); Rho-associated coiled-coil kinase 1 (ROCK1);
Tumor suppressor

INTRODUCTION

Osteosarcoma (OS) is the most common cancer that is primarily present around regions with active bone growth and repair[1,2]. Recent studies have suggested that some noncoding RNAs and proteins act as tumor suppressors or oncogenes in OS, deregulations of which are tightly associated with the malignant progression of OS[1,3]. Therefore, it is necessary to explore the underlying molecular mechanisms for identifying targets or candidates for the treatment of patients with OS.

MicroRNAs (miRs), a class of noncoding RNAs that are 18–25 nucleotides in length, have been demonstrated to suppress the protein expression of their targets via directly binding to the 3′-untranslated region (3′-UTR)

of their target mRNAs[4,5]. Moreover, it has been well established that miRs act as key regulators in tumorigenesis via regulating the expression of oncogenes or tumor suppressors[6–8]. For instance, Jin et al. reported that miR-218 inhibited OS cell migration and invasion by targeting TIAM1, MMP2, and MMP9[9]. Duan et al. found that miR-199a-3p was frequently downregulated in OS and played a suppressive role in OS cell proliferation and migration[10].

Recently, miR-148a has been demonstrated to play a suppressive role in a variety of human cancers. For instance, it suppresses the proliferation and migration of pancreatic cancer cells by targeting ErbB3[11]. Yu et al. reported that miR-148a acted as a tumor suppressor in

human gastric carcinogenesis by targeting CCK-BR via inactivating STAT3 and Akt[12]. Xu et al. found that miR-148a could suppress metastasis and serve as a prognostic indicator in triple-negative breast cancer[13].

A previous study has shown that miR-148a is upregulated in the peripheral blood of OS patients compared to healthy controls, and high circulating miR-148a levels are significantly associated with tumor size and distant metastasis, as well as shorter overall survival and disease-specific survival after 5 years, suggesting that the circulating miR-148a level may be used as an indicator of progressive phenotype as well as a novel diagnostic biomarker for OS patients[14]. On the contrary, Chang et al. reported that overexpression of miR-148a significantly inhibited the proliferation, migration, invasion, and stemness of cancer stem cells derived from primary OS cells[15]. In addition, Bhattacharya et al. showed that the reduced expression of miR-148a-3p contributed to the suppression of OS cell death[16]. Therefore, miR-148a may act as a tumor suppressor in OS. However, the underlying mechanism of miR-148a in the regulation of OS progression remains largely unknown.

The aim of this study was to investigate the expression pattern and clinical significance of miR-148a in OS. We also investigated the regulatory mechanism of miR-148a in the malignant phenotypes of OS cells.

MATERIALS AND METHODS

Clinical Specimens

Our study was approved by the ethics committee of Xiangya Hospital, Central South University, Changsha, P.R. China. A total of 76 cases of OS tissues and their matched adjacent normal tissues were collected from our hospital from January 2010 to March 2014. Written informed consent was obtained from each patient. The clinical characteristics of OS patients are summarized in Table 1. Tissue samples were stored in liquid nitrogen until use.

Cell Cultures

Human OS cell lines including Saos-2, 143B, and U2OS, and a human osteoblast cell line, hFOB1.19, were obtained from the Cell Bank of Central South University (Changsha, P.R. China). Cells were cultured in

Table 1. Association Between miR-148a Expression and Clinicopathological Characteristics in Osteosarcoma

Variables	Cases ($n=76$)	Low miR-148a ($n=39$)	High miR-148a ($n=37$)	p Value
Age				1.00
<20	32	16	16	
≥20	44	23	21	
Sex				0.484
Male	45	25	20	
Female	31	14	17	
Tumor size				0.018*
<5 cm	30	10	20	
≥5 cm	46	29	17	
Location				0.808
Femur or tibia	51	27	24	
Others	25	12	13	
Lung metastasis				0.015*
No	51	21	30	
Yes	25	18	7	
TMN stage				0.013*
I/IIA	24	7	17	
IIB/III	52	32	20	
Serum lactate dehydrogenase				0.816
Normal	31	15	16	
Elevated	45	24	21	
Serum alkaline phosphatase				0.353
Normal	32	14	18	
Elevated	44	25	19	

*The difference is statistically significant.

Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher, Carlsbad, CA, USA) with 10% fetal calf serum added (Thermo Fisher) at 37°C in a humidified atmosphere with 5% CO_2.

Real-Time RT-PCR Assay

Total RNA was extracted using TRIzol reagent (Thermo Fisher) according to the manufacturer's instructions. For analysis of mRNA expression, RNA was reverse transcribed into cDNA using 1 μg of total RNA with M-MLV Reverse Transcriptase (Promega, Beijing, P.R. China) according to the manufacturer's instructions. GAPDH was used as the internal control. The specific primers were as follows: rho-associated coiled-coil kinase 1 (ROCK1), 5′-AAGTGAGGTTAGGGCGAAATG-3′ (forward) and 5′-AAGGTAGTTGATTGCCAACGAA-3′ (reverse); GAPDH, 5′-CTGGGCTACACTGAGCACC-3′ (forward) and 5′-AAGTGGTCGTTGAGGGCAATG-3′ (reverse). For miR analysis, real-time PCR was performed using PrimeScript® miRNA RT-PCR Kit (Takara, Dalian, P.R. China) according to the manufacturer's instructions. U6 was used as the internal reference. The reaction conditions were 95°C for 5 min, and 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 30 s. The relative expression was analyzed by the $2^{-\Delta\Delta Ct}$ method.

Transfection

Cells were cultured to 70% confluence and resuspended in serum-free medium. Lipofectamine 2000 (Thermo Fisher) was used to perform transfection according to the manufacturer's instructions. In brief, serum-free medium was used to dilute the miR-148a mimic (Genechem, Shanghai, P.R. China), miR-148a inhibitor (Genechem), pcDNA3.1-ROCK1 plasmid (Yearthbio, Changsha, P.R. China), and Lipofectamine 2000, respectively. The diluted Lipofectamine 2000 was then added into the diluted miR or plasmid, respectively. After incubation for 20 min at room temperature, they were added into the cell suspension. After incubation at 37°C 5% CO_2 for 6 h, the medium was replaced by DMEM with 10% fetal calf serum. After transfection for 48 h, the following assays were performed.

Western Blot

Western blotting assay was performed to detect the protein expression level. In brief, tissues and cells were solubilized in cold RIPA lysis buffer. Proteins were separated with 10% SDS-PAGE and transferred onto PVDF membrane (Thermo Fisher), which was blocked by 5% skim milk for 1 h, and then incubated overnight at 4°C with rabbit anti-human ROCK1 antibody (1:100; Abcam, Cambridge, MA, USA) and rabbit anti-human GAPDH

antibody (1:200; Abcam), and then with goat anti-rabbit secondary antibodies (1:10,000; Abcam) for 40 min. An ECL kit (Thermo Fisher) was used to visualize the protein bands.

Bioinformatical Prediction and Luciferase Assays

Bioinformatical analysis was used to predict the potential target genes of miR-148a using TargetScan (http://www.targetscan.org/). ROCK1 was predicted to be a potential target gene of miR-148a. The wild type (WT) and mutant type (MT) of ROCK1 3′-UTR were amplified by PCR and cloned in pMIR-REPORT miRNA (Thermo Fisher). Saos-2 and U2OS cells were transfected with WT-ROCK1-3′UTR or MT-ROCK1-3′UTR reporter plasmid plus miR-148a mimics or miR-NC using Lipofectamine 2000 according to the manufacturer's instructions. After transfection for 48 h, the firefly luciferase activity and *Renilla* luciferase activity were determined using the Firefly & Renilla Luciferase Assay Kit (Thermo Fisher) according to the manufacturer's protocols. The firefly luciferase activity was normalized to that of the *Renilla* luciferase.

Cell Proliferation Assay

Saos-2 and U2OS cells in each group were suspended in 100 μl of fresh serum-free DMEM containing 0.5 g/L MTT (Sigma-Aldrich, St. Louis, MO, USA) and seeded into 96-well plates. After incubation at 37°C for 4 h, the MTT medium was removed by aspiration, and 50 μl of dimethyl sulfoxide (Sigma) was added, and then incubated at 37°C for 10 min. The optical density (OD) at 570 nm was measured using the ELx800 Absorbance Microplate Reader (Biotek, Winooski, VT, USA).

Wound Healing Assay

Cells were cultured to full confluence, and wounds of approximately 1-mm width were created with a plastic scriber. OS cells in each group were washed with PBS and incubated in serum-free DMEM. After wounding for 24 h, cells were incubated in DMEM with 10% FBS. After being cultured for 48 h, cells were photographed using a microscope.

Cell Invasion Assay

Cell invasion assay was performed using the Transwell chamber with a Matrigel-coated filter (BD Biosciences, Franklin Lakes, NJ, USA). A total of 200 μl of the Saos-2 and U2OS cell suspension (1×10^4 cells) in serum-free DMEM was added to the upper chamber, and 500 μl of DMEM containing 10% fetal calf serum was added to the lower chamber. After incubation for 24 h, cells on the upside of the filter were removed using cotton swabs. The invasive cells on the lower side were fixed, stained

with 0.1% crystal violet (Sigma), and counted using a light microscope.

Tumor Growth In Vivo

BALB/C-nu/nu nude mice (male, 10 weeks) were maintained under specific pathogen-free conditions at The Animal Center of Central South University. U2OS cells were stably transfected with blank pLP/VSVG vector or pLP/VSVG-miR-98 plasmid, respectively. These transfected cells (1×10^7) were then injected subcutaneously into the dorsal flank of mice. After tumor implantation for 60 days, the animals were sacrificed, and the tumor tissue in each mouse was photographed and weighed. The tumor volume was then calculated using the formula V (mm^3)$=0.5 \times a \times b2$ (a is the maximum length to diameter, and b is the maximum transverse diameter).

Statistical Analysis

All data were expressed as mean±standard deviation (SD). Difference was analyzed using one-way analysis of variance (ANOVA) with SNK-q test. SPSS17.0 software was used to perform statistical analysis. A value of $p < 0.05$ was considered statistically significant.

RESULTS

miR-148a Is Downregulated in OS, Associated With Disease Progression

To reveal the role of miR-148a in OS, we first performed RT-PCR to examine the expression levels of miR-148a in 76 cases of OS tissues and paired adjacent nontumor bone tissues. miR-148a was significantly downregulated in OS tissues compared to matched adjacent normal tissues (Fig. 1A). We further examined the miR-148a levels in OS cell lines including Saos-2, 143B, and U2OS, as well as in the human osteoblast cell line hFOB1.19. We found that the expression of miR-148a was also reduced in OS cell lines compared to hFOB1.19 cells (Fig. 1B). Therefore, downregulation of miR-148a may play a role in the development and progression of OS.

We then studied the clinical significance of miR-148a expression in OS. Using the mean value of miR-148a expression as a cutoff, the OS patients were divided into a high miR-148a expression group and a low miR-148a expression group. Low expression of miR-148a was significantly associated with tumor size, lung metastasis, and TMN stage, but showed no association with age,
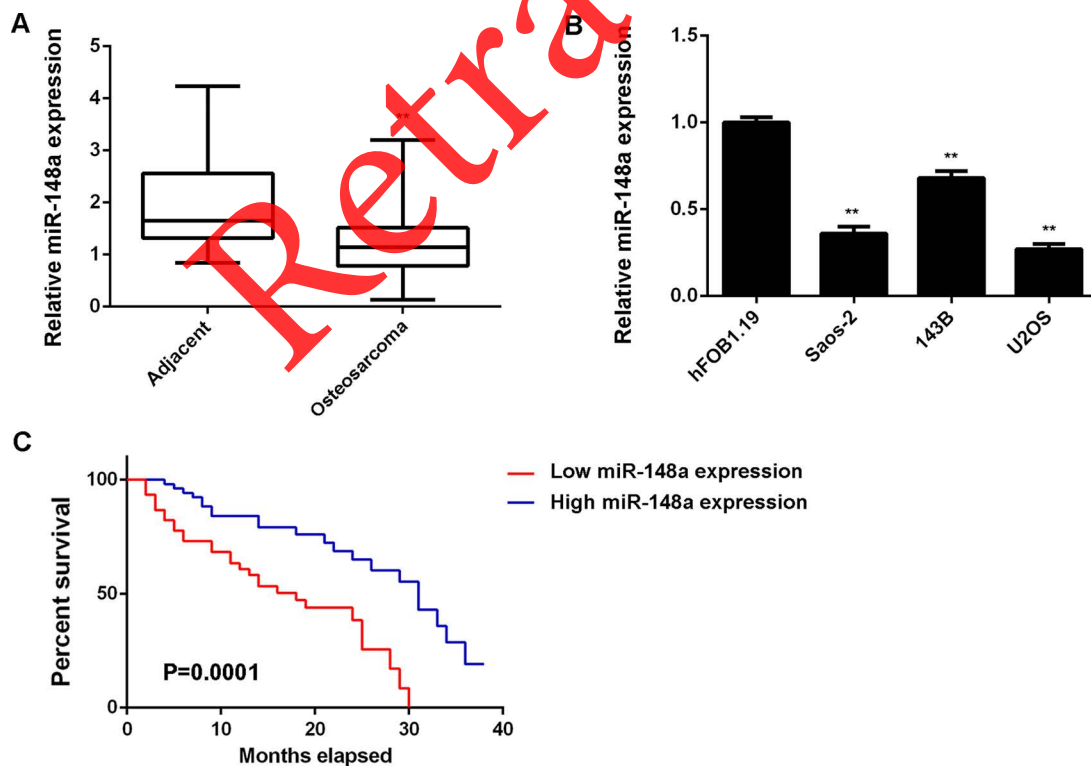

Figure 1. RT-PCR was used to detect the expression of miR-148a in (A) osteosarcoma (OS) tissues and paired adjacent nontumor bone tissues, and (B) human OS cell lines (Saos-2, 143B, and U2OS) and the human osteoblast cell line hFOB1.19. **p<0.01 versus hFOB1.19. (C) OS patients with low miR-148a expression showed shorter survival time, when compared with those having high miR-148a expression.

gender, location, serum lactate dehydrogenase, or serum alkaline phosphatase (Table 1). These findings suggest that reduced miR-148a expression may contribute to the malignant progression of OS. Further investigation showed that OS patients with a low miR-148a expression showed a shorter survival time when compared with those having a high miR-148a expression, suggesting that miR-148a may become a predictor for the prognosis of OS patients (Fig. 1C).

miR-148a Directly Targets ROCK1 and Inhibits its Protein Expression in OS Cells

We further focused on the potential targets of miR-148a, and ROCK1 was predicted to be a direct target gene of miR-148a (Fig. 2A). To verify whether miR-148a directly binds to the 3′-UTR of ROCK1, we generated WT-ROCK1 and MT-ROCK1 vectors that contain the WT and MT seed sequences of miR-148a within the 3′-UTR of ROCK1 mRNA, respectively (Fig. 2B). We conducted

a luciferase reporter assay, and our data showed that the luciferase activity was only decreased in Saos-2 and U2OS cells cotransfected with the WT-ROCK1 vector and the miR-148a mimic, but showed no difference in Saos-2 and U2OS cells cotransfected with the MT-ROCK1 vector and the miR-148a mimic, when compared to the control group (Fig. 2C and D), indicating that ROCK1 is a target gene of miR-148a in Saos-2 and U2OS cells.

ROCK1 Is Significantly Upregulated in OS

We then performed RT-PCR to examine the mRNA expression of ROCK1 in OS tissues and paired adjacent nontumor bone tissues. ROCK1 was frequently upregulated in OS tissues compared to matched adjacent normal tissues (Fig. 2A). In addition, we found that the protein levels of ROCK1 were upregulated in OS cell lines compared with those in the human osteoblast cell line hFOB1.19 (Fig. 3B). These data indicate that ROCK1 is upregulated in OS.
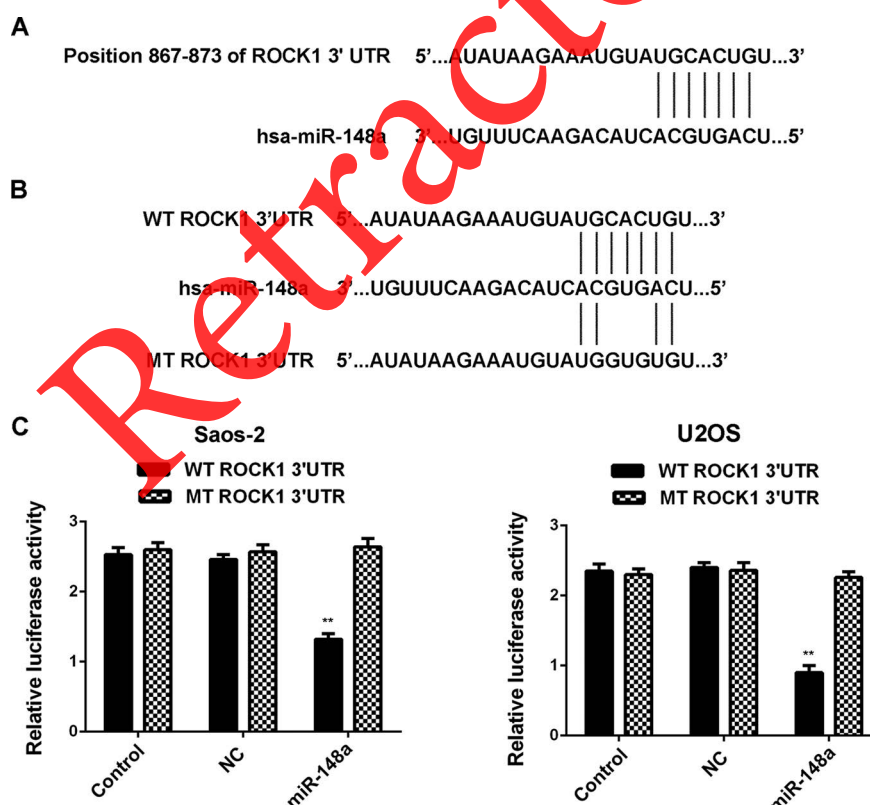

Figure 2. (A) Rho-associated coiled-coil kinase 1 (ROCK1) was predicted to be a direct target gene of miR-148a. (B) The wild or mutant seed sequences of miR-148a within the 3′-untranslated region (3′-UTR) of ROCK1 mRNA were indicated. (C) Luciferase activity was only reduced in Saos-2 and U2OS cells cotransfected with the miR-148a mimic and the vector containing wild type (WT) of ROCK1 3′-UTR, but unchanged in cells cotransfected with the miR-148a mimic and the vector containing mutant type (MT) of ROCK1 3′-UTR, when compared to the control group. Control: Saos-2 and U2OS cells transfected with WT-ROCK1 or MT-ROCK1, respectively. NC (negative control): Saos-2 and U2OS cells cotransfected with WT-ROCK1 or MT-ROCK1 and scramble miR mimic, respectively. **$p < 0.01$ versus Control.
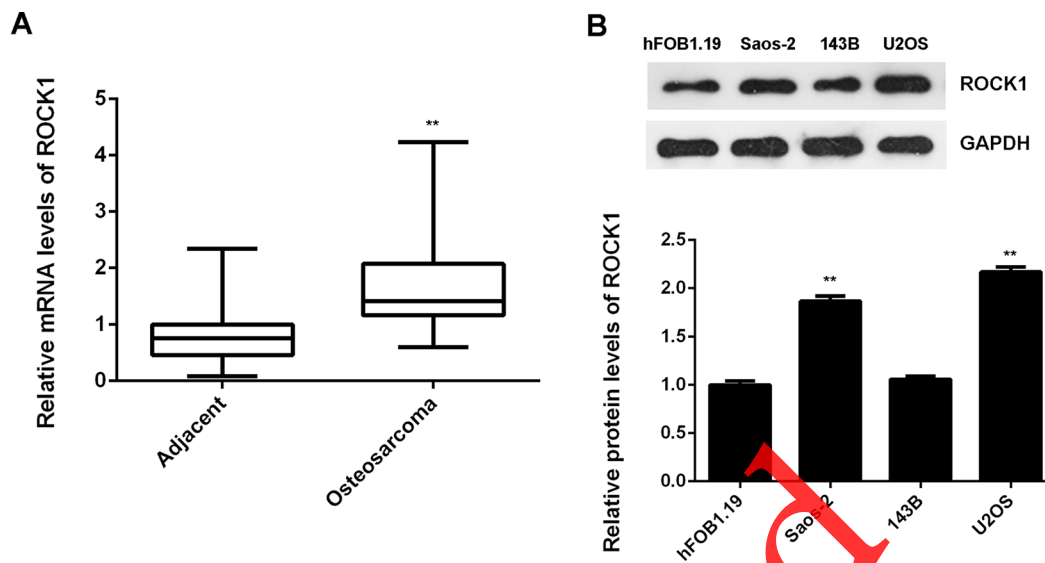
A

B

Figure 3. RT-PCR was used to detect the mRNA expression of ROCK1 in (A) OS tissues and paired adjacent nontumor bone tissues, and (B) human OS cell lines (Saos-2, 143B, and U2OS) and the human osteoblast cell line hFOB1.19. **$p < 0.01$ versus hFOB1.19.

miR-148a Negatively Regulates the Protein Expression of ROCK1 in OS Cells

As miRs generally inhibit the protein translation of their target genes, we further investigated the effects of miR-148a on the protein expression of ROCK1 in Saos-2 and U2OS cells. Saos-2 and U2OS cells were transfected with miR-NC, miR-148a mimic, NC inhibitor, or miR-148a inhibitor, respectively. RT-PCR data showed that transfection with the miR-148a mimic enhanced miR-148a expression when compared with the miR-NC group, while transfection with the miR-148a inhibitor resulted in a significant decrease in miR-148a expression when compared with the NC inhibitor group in Saos-2 and U2OS cells (Fig. 4A and B). We then performed Western blot assay to determine the protein levels of ROCK1 and found that overexpression of miR-148a significantly inhibited the protein expression of ROCK1, while knockdown of miR-148a significantly enhanced the protein expression of ROCK1 in Saos-2 and U2OS cells (Fig. 4C–F). Therefore, miR-148a negatively regulates the protein expression of ROCK1 in OS cells.

miR-148a Inhibits the Proliferation, Migration, and Invasion of OS Cells

Afterward, we examined the effects of miR-148a overexpression or downregulation on the malignant phenotypes of Saos-2 and U2OS cells. MTT assay data showed that overexpression of miR-148a markedly inhibited the proliferation of Saos-2 and U2OS cells, while inhibition of miR-148a expression significantly enhanced the proliferation of Saos-2 and U2OS cells, suggesting that miR-148a negatively mediates the proliferation of OS cells

(Fig. 5A–D). Moreover, wound healing and Transwell assay data indicated that overexpression of miR-148a significantly inhibited the migration and invasion of Saos-2 and U2OS cells, suggesting that miR-148a may have a suppressive effect on OS metastasis (Fig. 6A–D).

Overexpression of ROCK1 Attenuates the Inhibitory Effects of miR-148a on the Malignant Phenotypes of OS Cells

We further explored the roles of miR-148a and ROCK1 in the regulation of OS cell proliferation and invasion. Saos-2 and U2OS cells were cotransfected with miR-148a mimic and blank pcDNA3.1 vector, or with miR-148a mimic and ROCK1 plasmid, respectively. After transfection, we first detected the protein levels of ROCK1 in each group. The protein levels of ROCK1 were higher in Saos-2 and U2OS cells cotransfected with the miR-148a mimic and ROCK1 plasmid when compared with those in cells transfected with the miR-148a mimic and blank vector (Fig. 7A and B). We then performed an MTT assay and found that overexpression of miR-148a suppressed the proliferation of Saos-2 and U2OS cells, which was significantly reversed by overexpression of ROCK1 (Fig. 7C and D), suggesting that miR-148a inhibits cell proliferation via targeting ROCK1 in OS cells. We performed wound healing and Transwell assays to examine cell migration and invasion. Our data indicated that the migration and invasion of Saos-2 and U2OS cells were significantly higher in the miR-148a+ROCK1 group when compared with the miR-148a+blank group (Fig. 8). These findings suggest that miR-148a inhibits the malignant phenotypes of OS cells via targeting ROCK1.
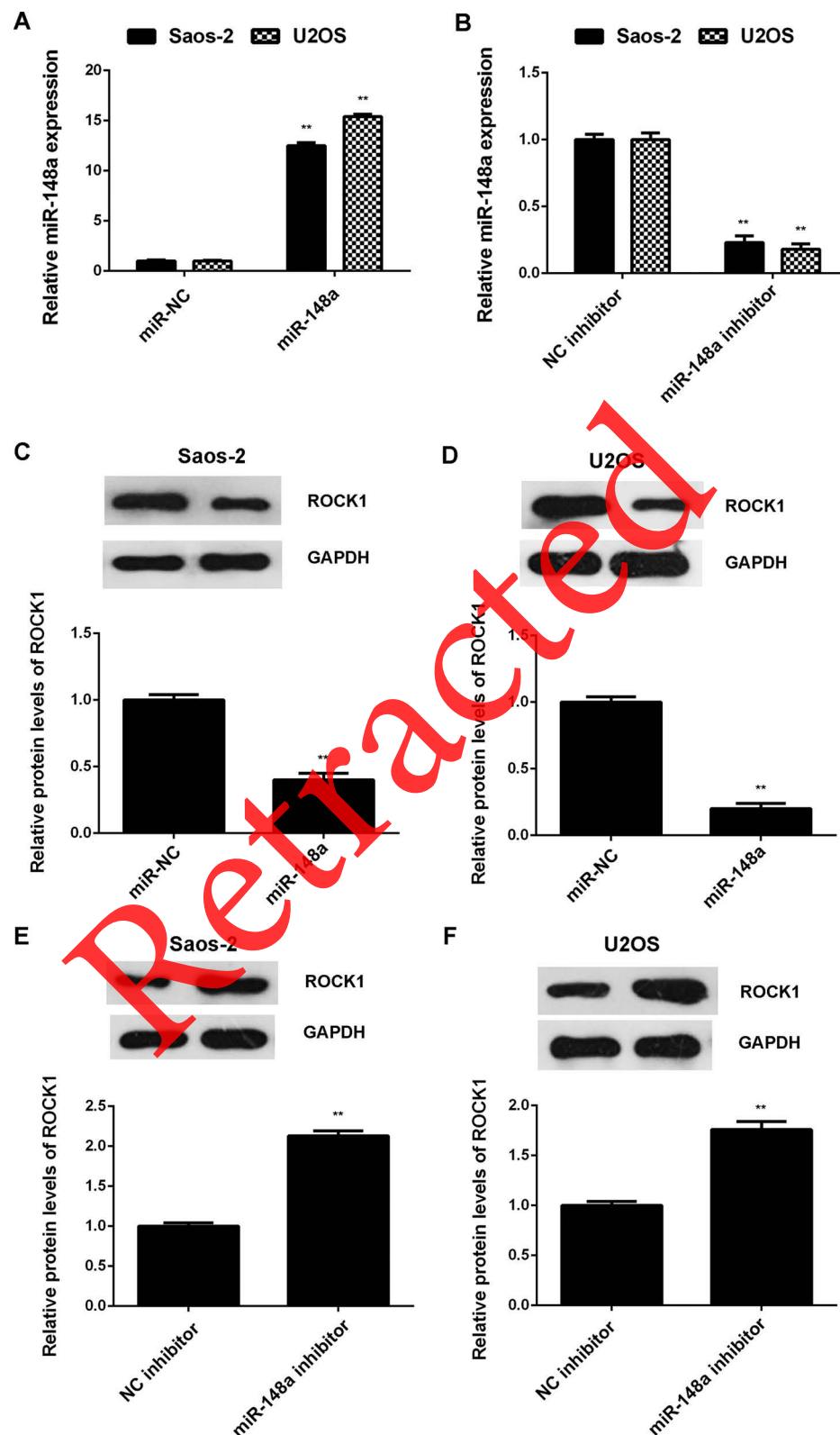
Figure 4. Saos-2 and U2OS cells were transfected with miR-NC, miR-148a mimic, NC inhibitor, or miR-148a inhibitor, respectively. (A, B) RT-PCR was performed to determine the relative expression of miR-148a. (C–F) Western blot assay was performed to examine the protein levels of ROCK1. **$p < 0.01$ versus miR-NC or NC inhibitor, respectively.
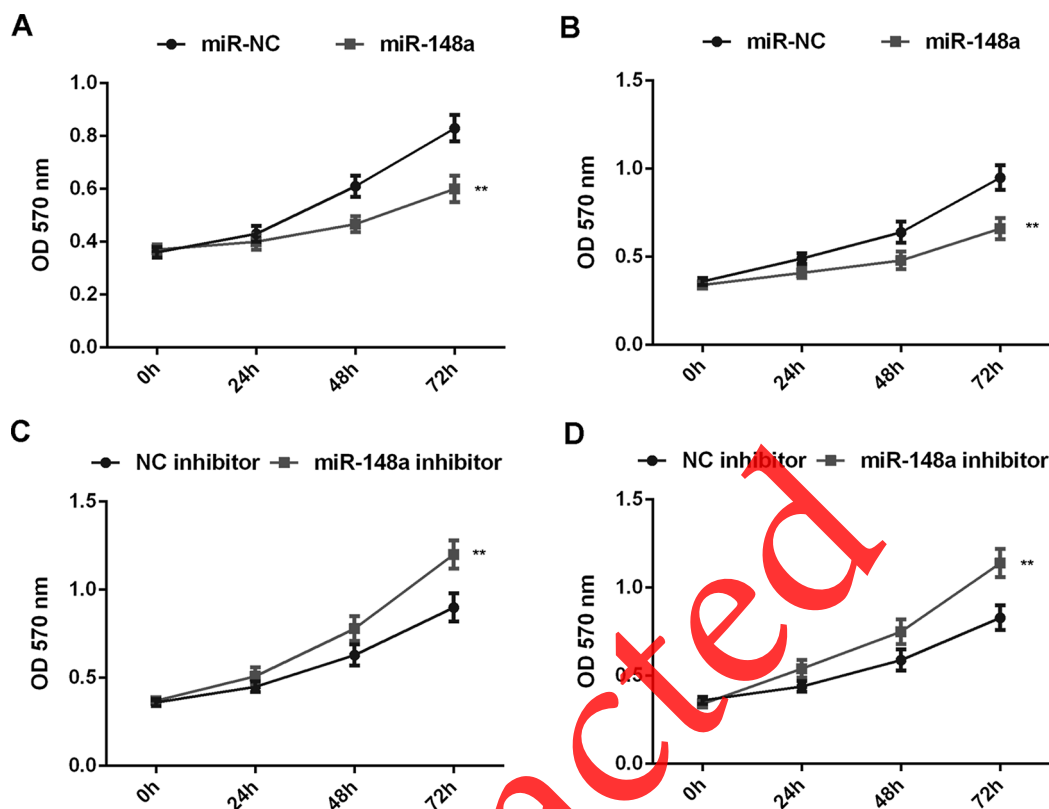
Figure 5. Saos-2 and U2OS cells were transfected with miR-NC, miR-148a mimic, NC inhibitor, or miR-148a inhibitor, respectively. (A–D) MTT assay was performed to examine the cell proliferation. **$p<0.01$ versus miR-NC or NC inhibitor, respectively.

Overexpression of ROCK1 Reduces the Tumor Growth of U2OS Cells in Nude Mice

To further study the potential effect of miR-148a overexpression on OS growth in vivo, the xenograft model of nude mice was generated. U2OS cells were injected subcutaneously into the dorsal flank of nude mice. Sixty days after tumor implantation, the animals were sacrificed. The tumors were significantly smaller in the miR-148a group compared with those in the miR-NC group (Fig. 9A). Moreover, overexpression of miR-148a decreased tumor volume and weight when compared with the miR-NC group (Fig. 9B and C). Therefore, overexpression of miR-148a inhibits tumor growth of U2OS cells in vivo.

DISCUSSION

miRs have been demonstrated to act as key regulators in human cancers, including OS. However, the underlying mechanism of miR-148a in the regulation of the malignant phenotypes of OS cells still needs to be fully uncovered. Here we reported that miR-148a was significantly downregulated in OS tissues and OS cell lines and that low miR-148a expression is significantly associated with OS progression. ROCK1 was then identified as a target of miR-148a in Saos-2 and U2OS cells, and the

expression of ROCK1 was significantly increased in OS tissues and cell lines. Moreover, the protein expression of ROCK1 was negatively regulated by miR-148a in Saos-2 and U2OS cells. Further investigation showed that miR-148a had a suppressive effect on the proliferation, migration, and invasion of Saos-2 and U2OS cells. Furthermore, overexpression of ROCK1 attenuated the inhibitory effects of miR-148a upregulation on the malignant phenotypes of Saos-2 and U2OS cells. In addition, miR-148a overexpression significantly reduced the tumor growth of U2OS cells in nude mice.

Previous studies showed some seemingly contradictory findings on the role of miR-148a in OS. Hu et al. studied the differential expression profiles of miRs between OS MG-63 cells and osteoblast cells using an miR microarray and found a total of 268 miRs that were significantly deregulated. They further showed that miR-148a was among the upregulated miRs in MG-63 cells compared with osteoblast cells[17]. Moreover, Ma et al. showed that miR-148a was upregulated in the peripheral blood of OS patients compared to healthy controls, which was associated with OS progression and poor prognosis[14]. The above two studies suggest that miR-148a may play an oncogenic role in OS. However, Chang et al. reported
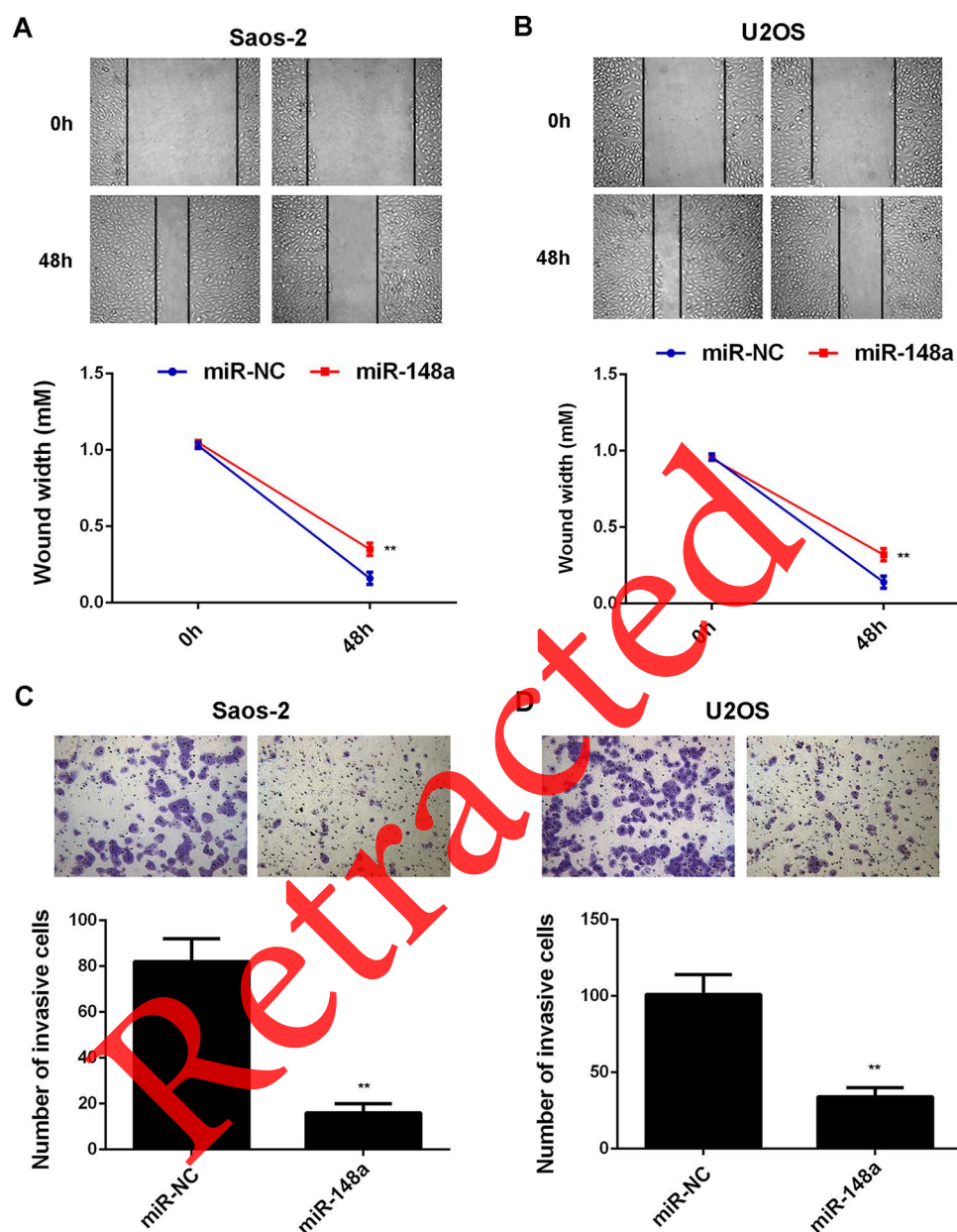
Figure 6. Saos-2 and U2OS cells were transfected with miR-NC and miR-148a mimic, respectively. (A, B) Wound assay and (C, D) Transwell assay were performed to examine the cell migration and invasion. $**p < 0.01$ versus miR-NC or NC inhibitor, respectively.

that overexpression of miR-148a significantly inhibited the proliferation, migration, invasion, and stemness of cancer stem cells derived from the OS MG-63 cell line, and then showed that miR-148a was significantly downregulated in OS tissues compared with nontumor tissues[15]. Similarly, Bhattacharya et al. showed that overexpression of miR-148a could inhibit OS cell proliferation and induce cell apoptosis[16]. Therefore, these findings suggest that miR-148a may act as a tumor suppressor in OS. However, the exact mechanism of miR-148a in OS is

unclear. In the present study, we, for the first time, showed that miR-148a was downregulated in OS, and the reduced miR-148a levels were associated with tumor size, lung metastasis, and TNM stage in OS. These findings further suggest that miR-148a downregulation may contribute to OS progression. Moreover, in vitro and in vivo studies showed that miR-148a has a suppressive effect on the proliferation, migration, invasion, and tumor growth of OS cells. Therefore, miR-148a indeed acts as a tumor suppressor in OS.
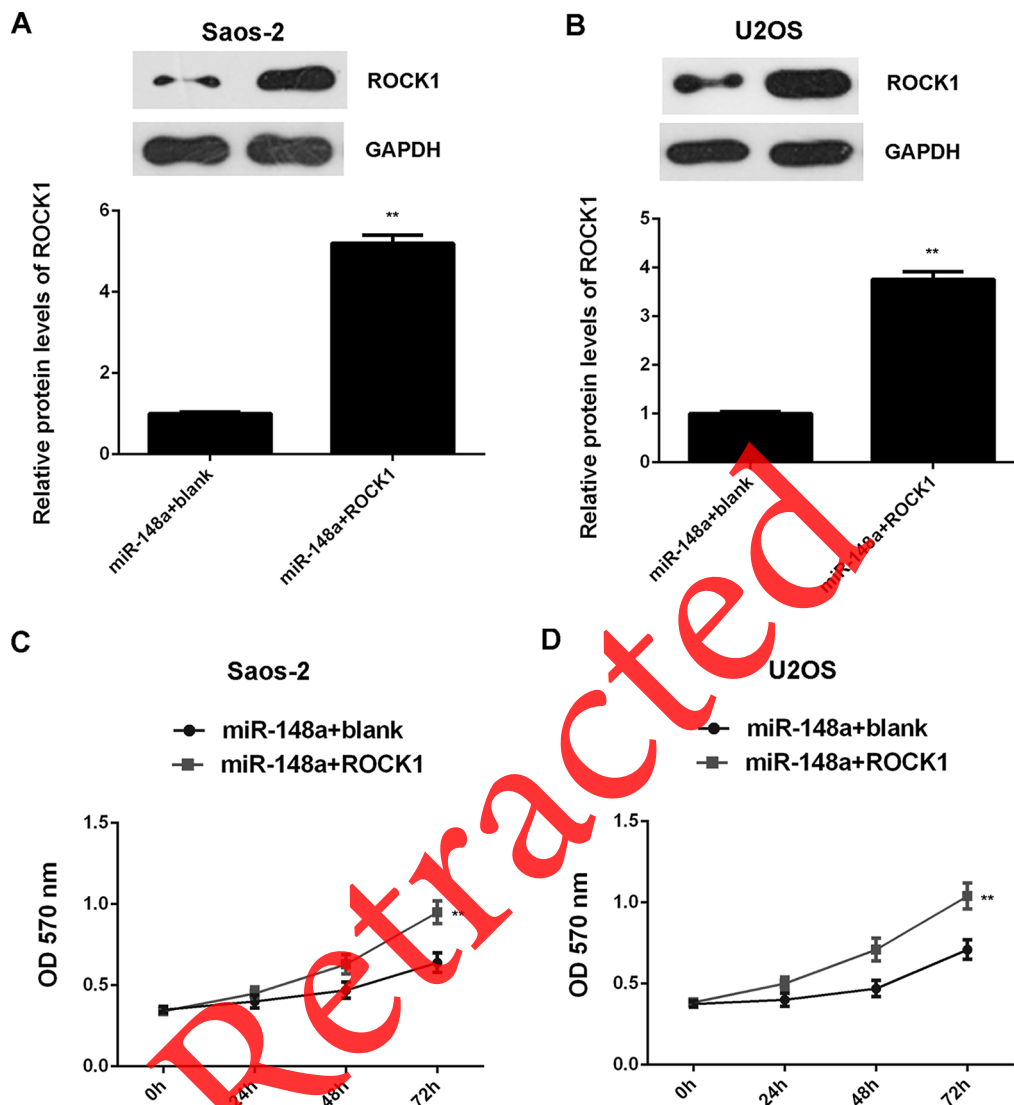
Figure 7. Saos-2 and U2OS cells were cotransfected with miR-148a mimic and blank pcDNA3.1 vector, or with miR-148a mimic and pcDNA3.1-ROCK1 expression plasmid, respectively. (A, B) Western blot assay was performed to examine the protein expression of ROCK1. (C, D) MTT assay was performed to examine cell proliferation. $**p < 0.01$ versus miR-148a + blank.

As miRs generally function through regulating the protein expression of their target genes, we then studied the potential targets of miR-148a in OS. Bioinformatics analysis and dual luciferase reporter gene assay data showed that ROCK1 was a target gene of miR-148a in OS cells. ROCK1, a serine/threonine kinase in the Rho family of GTPase proteins, is involved in the reorganization of the actin cytoskeleton and thus participates in mediating cell motility[18–20]. Moreover, ROCK1 acts as an important oncogene in a variety of human cancers, such as lung cancer, bladder cancer, prostate cancer, and so forth[21–23]. Recently, ROCK1 has been implicated in OS. Liu et al. showed that the expression of ROCK1 was frequently increased in OS tissues, which was associated with poor outcomes in OS patients[24]. In our study, we

also reported that ROCK1 was upregulated in OS tissues and cell lines when compared to paired adjacent nontumor tissues and normal osteoblast cells, consistent with the previous findings[24]. Moreover, Liu et al. showed that knockdown of ROCK1 decreased cell proliferation, viability, and induced apoptosis in OS cells, which further suggest that ROCK1 acts as an oncogene in OS[24]. In this study, as we found that ROCK1 was negatively regulated by miR-148a in OS cells, we speculated that it might also be involved in the miR-148a-mediated OS cell proliferation, migration, and invasion. To clarify this speculation, miR-148a-overexpressing OS cells were transfected with ROCK1 expression plasmid to upregulate its expression. We showed that ROCK1 upregulation significantly attenuated the suppressive effect of
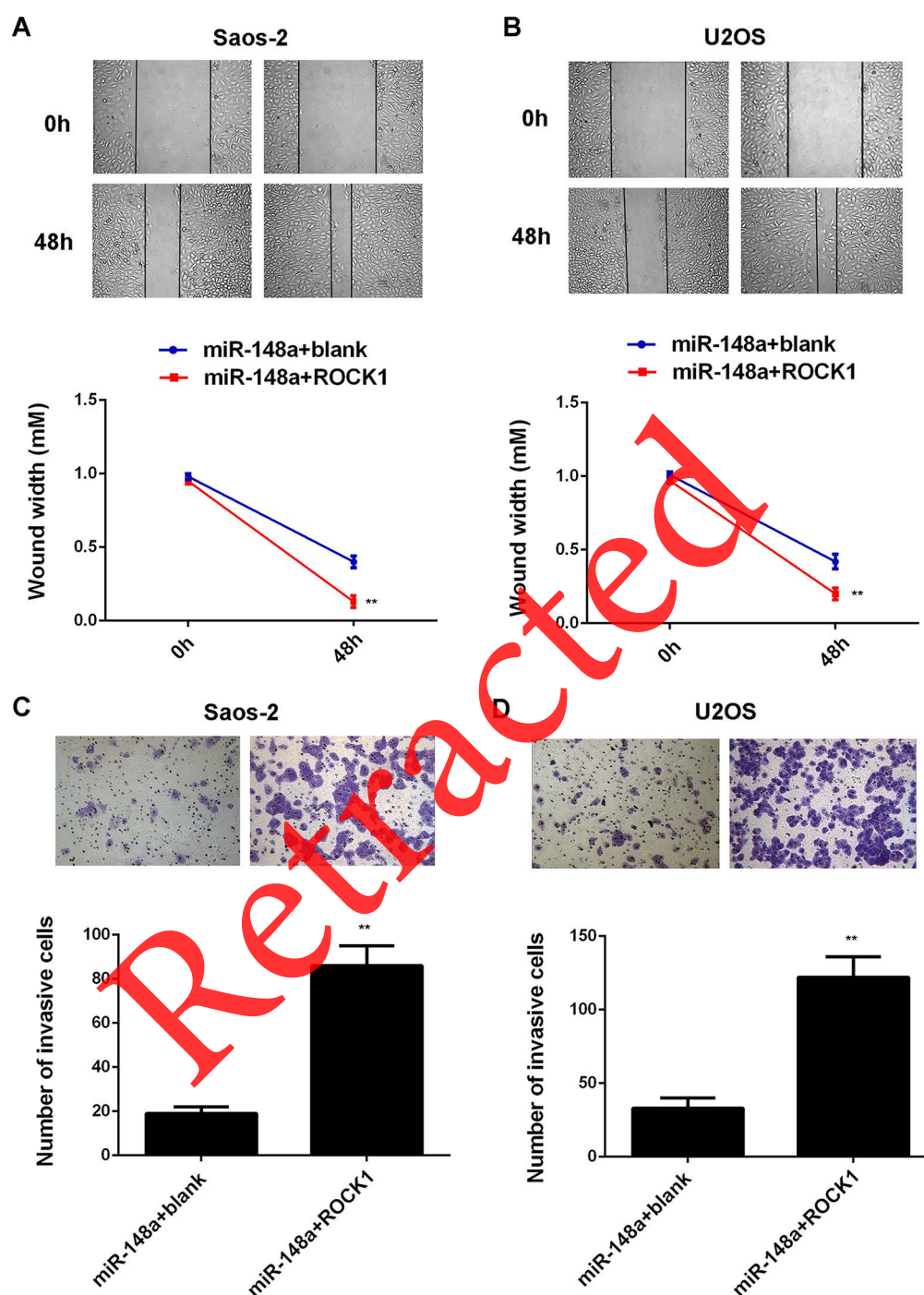
Figure 8. Saos-2 and U2OS cells were cotransfected with miR-148a mimic and blank pcDNA3.1 vector, or with miR-148a mimic and pcDNA3.1-ROCK1 expression plasmid, respectively. (A, B) Wound healing assay and (C, D) Transwell assay were performed to examine cell migration and invasion, respectively. **$p < 0.01$ versus miR-148a + blank.

miR-148a overexpression on the proliferation, migration, and invasion of Saos-2 and U2OS cells, which confirms that miR-148a plays a suppressive role in the regulation of OS malignant phenotypes, at least partly, via inhibition of ROCK1.

In addition, the targeting relationship between miR-148a and ROCK1 has been reported in non-small cell lung cancer (NSCLC) and gastric cancer[25,26]. Li et al. reported that miR-148a suppressed the epithelial-to-mesenchymal transition of NSCLC cells by targeting ROCK1[25]. Zheng
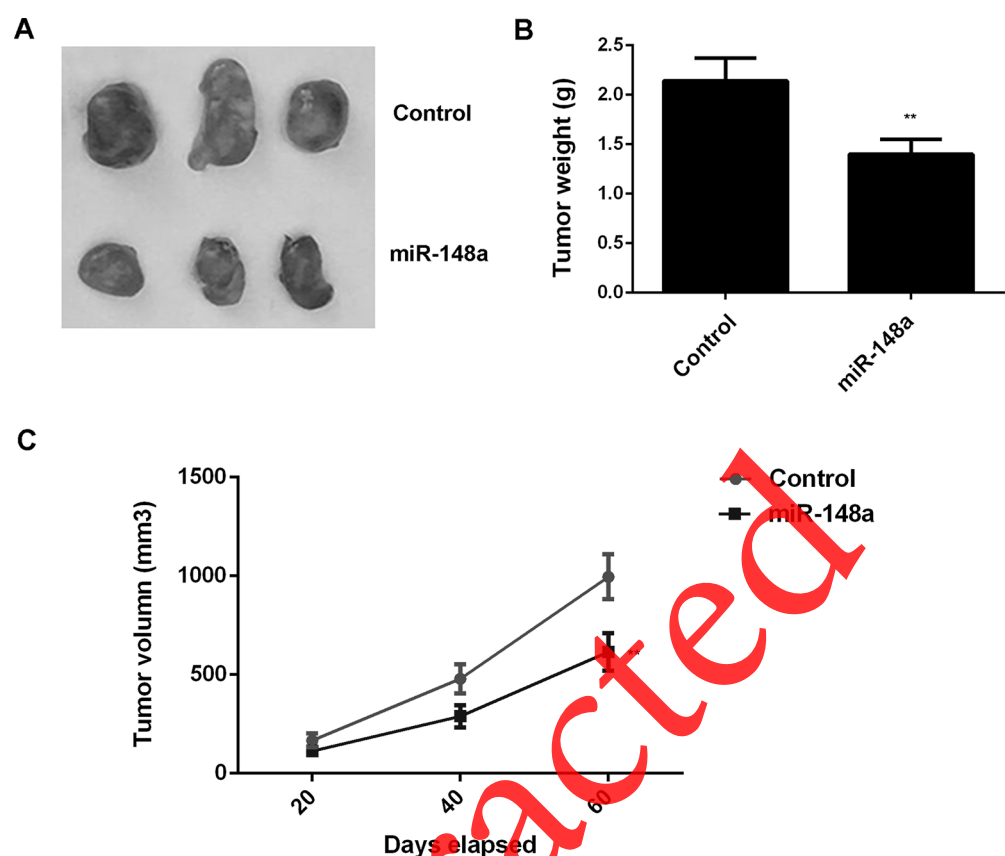
Figure 9. U2OS cells were stably transfected with pLP/VSVG-miR-148a lentiviral plasmid or with the blank pLP/VSVG vector as the control group. Nude mice were then subcutaneously injected with the above cells. (A) After implantation for 60 days, mice were sacrificed, and the xenograft was obtained. (B) Tumor weight and (C) tumor volume were calculated. $**p < 0.01$ versus Control.

et al. found that miR-148a inhibited the invasion and metastasis of gastric cancer cells by inhibition of ROCK1[26]. Therefore, our study expands the understanding of miR-148a/ROCK1 signaling in human cancers.

To our knowledge, this study demonstrates, for the first time, that miR-148a acts as a tumor suppressor in OS via directly targeting ROCK1, thus suggesting that miR-148a may become a potential candidate for the treatment of OS.

REFERENCES

1. Thompson LD. Osteosarcoma. Ear Nose Throat J. 2013; 92:288–90.

2. Wu X, Zhong D, Gao Q, Zhai W, Ding Z, Wu J. MicroRNA-34a inhibits human osteosarcoma proliferation by down-regulating ether a go-go 1 expression. Int J Med Sci. 2013; 10:676–82.

3. Murray E, Hernychova L, Scigelova M, Ho J, Nekulova M, O'Neill JR, Nenutil R, Vesely K, Dundas SR, Dhaliwal C, Henderson H, Hayward RL, Salter DM, Vojtěšek B, Hupp TR. Quantitative proteomic profiling of pleomorphic human sarcoma identifies CLIC1 as a dominant pro-oncogenic receptor expressed in diverse sarcoma types. J Proteome Res. 2014;13:2543–59.

4. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human microRNA targets. PLoS Biol. 2004; 2:e363.

5. Liu X, Fortin K, Mourelatos Z. MicroRNAs: Biogenesis and molecular functions. Brain Pathol. 2008;18:113–21.

6. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature 2005; 435:834–8.

7. Lujambio A, Calin GA, Villanueva A, Ropero S, Sanchez-Cespedes M, Blanco D, Montuenga LM, Rossi S, Nicoloso MS, Faller WJ, Gallagher WM, Eccles SA, Croce CM, Esteller M. A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci USA 2008; 105:13556–61.

8. Nana-Sinkam SP, Croce CM. Clinical applications for microRNAs in cancer. Clin Pharmacol Ther. 2013;93: 98–104.

9. Jin J, Cai L, Liu ZM, Zhou XS. miRNA-218 inhibits osteosarcoma cell migration and invasion by down-regulating of TIAM1, MMP2 and MMP9. Asian Pac J Cancer Prev. 2013;14:3681–4.

10. Duan Z, Choy E, Harmon D, Liu X, Susa M, Mankin H, Hornicek F. MicroRNA-199a-3p is downregulated in

human osteosarcoma and regulates cell proliferation and migration. Mol Cancer Ther. 2011;10:1337–45.

11. Feng H, Wang Y, Su J, Liang H, Zhang CY, Chen X, Yao W. MicroRNA-148 suppresses the proliferation and migration of pancreatic cancer cells by downregulating ErbB3. Pancreas 2016;45:1263–71.

12. Yu B, Lv X, Su L, Li J, Yu Y, Gu Q, Yan M, Zhu Z, Liu B. MiR-148a functions as a tumor suppressor by targeting CCK-BR via inactivating STAT3 and Akt in human gastric cancer. PLoS One 2016;11:e0158961.

13. Xu X, Zhang Y, Jasper J, Lykken E, Alexander PB, Markowitz GJ, McDonnell DP, Li QJ, Wang XF. MiR-148a functions to suppress metastasis and serves as a prognostic indicator in triple-negative breast cancer. Oncotarget 2016; 7:20381–94.

14. Ma W, Zhang X, Chai J, Chen P, Ren P, Gong M. Circulating miR-148a is a significant diagnostic and prognostic biomarker for patients with osteosarcoma. Tumour Biol. 2014; 35:12467–72.

15. Chang Y, Zhao Y, Gu W, Cao Y, Wang S, Pang J, Shi Y. Bufalin inhibits the differentiation and proliferation of cancer stem cells derived from primary osteosarcoma cells through mir-148a. Cell Physiol Biochem. 2015;36:1186–96.

16. Bhattacharya S, Chalk AM, Ng AJ, Martin TJ, Zannettino AC, Purton LE, Lu J, Baker EK, Walkley CR. Increased miR-155-5p and reduced miR-148a-3p contribute to the suppression of osteosarcoma cell death. Oncogene 2016;35:5282–94.

17. Hu H, Zhang Y, Cai XH, Huang JF, Cai L. Changes in microRNA expression in the MG-63 osteosarcoma cell line compared with osteoblasts. Oncol Lett. 2012;4:1037–42.

18. Tang AT, Campbell WB, Nithipatikom K. ROCK1 feedback regulation of the upstream small GTPase RhoA. Cell Signal. 2012;24:1375–80.

19. Surma M, Wei L, Shi J. Rho kinase as a therapeutic target in cardiovascular disease. Future Cardiol. 2011;7:657–71.

20. An L, Liu Y, Wu A, Guan Y. microRNA-124 inhibits migration and invasion by down-regulating ROCK1 in glioma. PLoS One 2013;8:e69478.

21. Rentala S, Chintala R, Guda M, Chintala M, Komarraju AL, Mangamoori LN. Atorvastatin inhibited Rho-associated kinase 1 (ROCK1) and focal adhesion kinase (FAK) mediated adhesion and differentiation of CD133+CD44+ prostate cancer stem cells. Biochem Biophys Res Commun. 2013;441:586–92.

22. Vigil D, Kim TY, Plachco A, Garton AJ, Castaldo L, Pachter JA, Dong H, Chen X, Tokar B, Campbell SL, Der CJ. ROCK1 and ROCK2 are required for non-small cell lung cancer anchorage-independent growth and invasion. Cancer Res. 2012;72:5338–47.

23. Majid S, Dar AA, Saini S, Shahryari V, Arora S, Zaman MS, Chang I, Yamamura S, Chiyomaru T, Fukuhara S, Tanaka Y, Deng G, Tabatabai ZL, Dahiya R. MicroRNA-1280 inhibits invasion and metastasis by targeting ROCK1 in bladder cancer. PLoS One 2012;7:e46743.

24. Liu X, Choy E, Hornicek FJ, Yang S, Yang C, Harmon D, Mankin H, Duan Z. ROCK1 as a potential therapeutic target in osteosarcoma. J Orthop Res. 2011;29:1259–66.

25. Li J, Song Y, Wang Y, Luo J, Yu W. MicroRNA-148a suppresses epithelial-to-mesenchymal transition by targeting ROCK1 in non-small cell lung cancer cells. Mol Cell Biochem. 2013;380:277–82.

26. Zheng B, Liang L, Wang C, Huang S, Cao X, Zha R, Liu L, Jia D, Tian Q, Wu J, Ye Y, Wang Q, Long Z, Zhou Y, Du C, He X, Shi Y. MicroRNA-148a suppresses tumor cell invasion and metastasis by downregulating ROCK1 in gastric cancer. Clin Cancer Res. 2011;17:7574–83.