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MicroRNA-340 suppresses osteosarcoma tumor growth and metastasis by directly targeting ROCK1

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ABSTRACT

MicroRNAs (miRNAs) play key roles in cancer development and progression. In the present study, we investigated the role of miR-340 in the progression and metastasis of osteosarcoma (OS). Our results showed that miR-340 was frequently downregulated in OS tumors and cell lines. Overexpression of miR-340 in OS cell lines significantly inhibited cell proliferation, migration, and invasion *in vitro*, and tumor growth and metastasis in a xenograft mouse model. ROCK1 was identified as a target of miR-340, and ectopic expression of miR-340 downregulated ROCK1 by direct binding to its 3′ untranslated region. siRNA-mediated silencing of ROCK1 phenocopied the effects of miR-340 overexpression, whereas restoration of ROCK1 in miR-340-overexpressing OS cells reversed the suppressive effects of miR-340. Together, these findings indicate that miR-340 acts as a tumor suppressor and its downregulation in tumor tissues may contribute to the progression and metastasis of OS through a mechanism involving ROCK1, suggesting miR-340 as a potential new diagnostic and therapeutic target for the treatment of OS.

1. Introduction

Osteosarcoma (OS) is a primary bone malignancy that mainly affects the rapidly growing bones of children and adolescents and is associated with high morbidity [1]. The development of multiple therapeutic strategies for OS including wide tumor excision, multi-agent chemotherapy and radiotherapy has significantly improved the prognosis of patients with this malignancy. However, 30% of those diagnosed with OS do not survive for more than 5 years and less than 50% live beyond 10 years. Approximately 15–20% of patients present with radiographically detectable metastases [2], and approximately 80% of patients develop metastatic disease after surgical resection [3]. Therefore, there is an urgent need to identify biomarkers and therapeutic targets for OS patients.

MicroRNAs (miRNAs) are a class of small (22-nucleotide) noncoding RNA molecules that control gene expression by binding to the 3′ untranslated region (UTR) of their target mRNAs, modulating mRNA stability and/or translation [4]. miRNAs are differentially expressed in various cells and tissues, underscoring their importance as biomarkers and therapeutic targets. Deregulation of miRNAs is associated with several diseases including cancer, and tumor-associated miRNAs can function as tumor suppressors or oncogenes depending on whether they target oncogenes or tumor suppressor genes [5,6]. To date, several miRNAs have been implicated in the development and progression of OS, such as miR-143, miR-199a-3p, miR-125b, miR-20a, and miR-34a [7–11]. In a recent array-based screen of miRNAs involved in the pathogenesis of OS, miR-340 was identified as one of 23 downregulated miRNAs in OS samples compared to control bone [12]. However, the specific roles and underlying mechanisms of miR-340 in OS have not been well established.

Rho-associated protein kinase 1 (ROCK1), which belongs to the AGC family of serine/threonine protein kinases, plays an important role in the regulation of the actin cytoskeleton through the phosphorylation of downstream substrates leading to actin filament stabilization and the modulation of actin-myosin contractility [13–15]. Increased expression of ROCK1 has been described in several human cancers and has been correlated with poor survival in breast cancer and in OS [16,17]. These findings indicate that ROCK1 may be a valuable therapeutic target.

In the present study, we showed that miR-340 was downregulated in OS cell lines and tissues. Ectopic expression of miR-340 inhibited cell proliferation and migration/invasion *in vitro* and tumor growth and metastasis *in vivo*. We identified ROCK1 as a direct target of miR-340 and showed that miR-340 functions as a tumor suppressor by downregulating ROCK1, providing a potential diagnostic and therapeutic target for the treatment of OS.

2. Materials and methods

2.1. Cell lines and patient samples

Human OS cell lines (HOS, SaOS2, MG63 and U2OS), and the human osteoblastic cell line (hFOB1.19) were purchased from the

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American Tissue Culture Collection. Human OS cells were cultured in RPMI 1640 (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). hFOB1.19 cells were cultured in Ham's F12/Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37 °C with 5% CO_2 in a humidified chamber. A total of 20 pairs of OS tissues and their matched adjacent normal bone were obtained from patients who underwent surgery at Renji Hospital, Shanghai Jiaotong University School of Medicine. The study was approved by the Ethics Review Committee of the Institutional Review Board of the hospital, and written informed consent was obtained from every patient.

2.2. Quantitative real-time PCR

miRNAs were isolated from cells or tissues using a miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RT and PCR primers for miR-340 and U6 were purchased from RiBoBio (Guangzhou, China). The levels of ROCK1 were examined using the forward primer, 5′-AGGAAGGCGGACATATTAGTCCCT-3′, and the reverse primer, 5′-AGACGATAGTTGGGTCCCGGC-3′. Reverse transcription PCR was done using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). qRT-PCR was performed using SYBR Premix Ex Taq II (TaKaRa) and the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). U6 or β-actin levels were used as internal controls, and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicate.

2.3. Lentiviral production and transduction

The pre-miR-340 sequence and ROCK1 coding sequence were purchased from Origene (Rockville, MD, USA). ROCK1 siRNA was designed and synthesized by RiBoBio (Guangzhou, China). The sequences were cloned into the pGCSIL-GFP lentiviral vector. The production, purification, and titration of lentivirus was performed as described by Xiong and colleagues [18]. SaOS2 and MG63 cells were infected with the recombinant lentivirus-transducing units at an MOI of 20 in the presence of 8 μg/mL polybrene (Sigma–Aldrich, St. Louis, MO, USA). Empty lentiviral vector was used as the negative control.

2.4. Cell proliferation assay

Cells were plated in 96-well plates at 1×10^3 per well in a final volume of 100 μL, and cultured for 1–4 and 5 days after transfection. Cell viability was determined using the WST-1 assay according to the manufacturer's protocol. Briefly, 10 μL WST-1 substrate was added to each well and incubated for 4 h at 37 °C and 5% CO_2. After incubating, the absorbance at 450 nm was measured by an enzyme-linked immunosorbent assay plate reader. Each experiment was performed in triplicate.

2.5. Cell migration and invasion assays

For transwell migration assays, 5×10^4 infected cells were plated into the top chamber of the insert (8 μm pore size; BD Bioscience, Bedford, MA, USA). For invasion assays, 1×10^5 cells were plated in the top chamber of the insert precoated with Matrigel (BD Bioscience). In both assays, cells were plated in medium without serum, and medium supplemented with 10% FBS was used as a chemoattractant in the lower chamber. The cells were incubated for 48 h and cells that migrated to the underside of the membrane were fixed with methanol and stained with Giemsa, imaged, and counted. Each experiment was performed in triplicate.

2.6. Western blotting

Cell lysates were prepared in RIPA buffer and total protein content was quantified by the BCA assay (Pierce). Lysates were separated by 10% SDS–PAGE and transferred to PVDF membranes. Blots were blocked in 5% non-fat dry milk for 2 h and incubated with primary antibodies against ROCK1 (1:500, Novus Biologicals, Littleton, CO, USA) and β-actin (1:2000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4 °C, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Protein was detected with Image Acquisition using ImageQuant™ LAS 4000 (GE Healthcare Life Sciences, MI, USA).

2.7. Plasmid construction and luciferase assay

A 293 bp fragment from the 3′ UTR of ROCK1 containing the miR-340 binding sites was cloned into the psiCHECK2 vector (Promega, Madison, WI, USA). The primers for ROCK1 3′ UTR were 5′-CGACTCGAGAAATGTAGAAGGTTGCACCAAC-3′ (forward) and 5′-AATGCGGCCGCTTA CATATCCATCAGTGCGGCT-3′ (reverse). Mutant (Mut) constructs were generated by mutating the seed region of the miR-340 binding site. miR-340 expressing or control cells were cultured in 24-well plates, and transfected with 100 ng luciferase reporter plasmid and 5 ng pRL-TK vector expressing the Renilla luciferase (Promega, Madison, WI, USA) using Lipofectamine 2000 reagent (Invitrogen). After 48 h, cells were harvested, lysed and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Renilla luciferase was used for normalization. All assays were performed in triplicate and repeated at least three times.

2.8. Animal experiments

For tumor growth assays, a total of 5×10^6 SaOS2 cells stably expressing miR-340 or negative control were injected subcutaneously into the left armpit of nude mice (5-week-old BALB/c-nu/nu, 6 per group). At 5 weeks after injection, the animals were euthanized and the tumors were weighed. For *in vivo* metastasis assays, 2×10^6 SaOS2 cells stably expressing miR-340 or negative control were injected into the caudal vein of nude mice (6 per group). After 6 weeks, the mice were euthanized, and lung metastatic colonization was monitored and quantified. All experimental procedures involving animals were approved by Shanghai Medical Experimental Animal Care Commission.

2.9. Statistical analysis

Data were expressed as the mean ± SD from at least three independent experiments. The Student's *t*-test was used to compare the differences between groups. All statistical analyses were performed using SPSS 15.0 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-340 is downregulated in OS cell lines and tissues

The expression of miR-340 was examined in four OS cell lines (HOS, SaOS2, MG63, U2OS) and the human osteoblastic cell line hFOB1.19. Quantitative reverse transcriptase PCR (qRT–PCR) analysis showed lower levels of miR-340 expression in all OS cells

compared to hFOB1.19 cells, with the lowest expression detected in MG63 and SaOS2 cells (Fig. 1A). These two cell lines were therefore used for subsequent experiments. The expression level of miR-340 was significantly downregulated in OS tissues versus corresponding normal tissues (Fig. 1B). Taken together, these results support the notion that miR-340 may act as a tumor suppressor in OS.

3.2. miR-340 suppresses OS cell proliferation, migration and invasion

To explore the biological significance of miR-340 in OS tumorigenesis, we established miR-340 stably expressing cell lines of SaOS2 and MG63 by lentivirus infection. Increased expression of miR-340 was confirmed by qRT-PCR (Fig. 2A). We found that overexpression of miR-340 significantly decreased the proliferation of SaOS2 and MG63 cells compared to their corresponding controls (Fig. 2B and C). Furthermore, transwell assays showed that miR-340 overexpression could suppress the migratory and invasive abilities of SaOS2 and MG63 OS cell lines (Fig. 2D and E). Taken together, these data demonstrate that miR-340 suppresses *in vitro* proliferation, migration and invasion of OS cells.

3.3. miR-340 suppresses tumor growth and metastasis of OS cells in nude mice

To further examine the effects of miR-340 on *in vivo* tumor growth, SaOS2 cells stably expressing miR-340 or NC were injected subcutaneously into the left armpit of nude mice and tumor size was measured after 5 weeks. As shown in Fig. 2F, miR-340-overexpressing tumors were smaller than control tumors. The average tumor weight was also significantly decreased in miR-340-overexpressing tumors compared to the controls. Furthermore, we investigated the effects of miR-340 on tumor metastasis *in vivo*. SaOS2 cells stably expressing miR-340 were transplanted into nude mice by intravenous tail injections to observe the metastatic nodules in the lungs. As shown in Fig. 2G, the average number of metastatic nodules on the surface of the lung was approximately 3-fold lower in the miR-340 transfection group than the control. Taken together, these findings confirm that miR-340 overexpression can suppress the tumorigenesis and metastasis of OS cells *in vivo*.

3.4. ROCK1 is a target gene of miR-340

To elucidate the underlying mechanisms by which miR-340 exerts its function, we explored miR-340 targets using the TargetScan bioinformatics algorithm. Our analysis revealed that ROCK1was a potential target of miR-340 based on putative target sequences at position 1317–1323 of the ROCK1 3′UTR (Fig. 3A). To confirm ROCK1 as a direct target of miR-340, we engineered luciferase reporter constructs containing the wild-type (WT) or mutant (Mut) 3′UTR of the ROCK1 gene. Luciferase reporter assays showed that miR-340 significantly decreased the luciferase activity of the ROCK1 3′UTR but not that of the mutant in SaOS2 and MG63 cells (Fig. 3B). qRT-PCR and Western blot analyses showed that overexpression of miR-340 significantly downregulated the expression of ROCK1 at the mRNA and protein levels in both OS cell lines (Fig. 3C). The association between ROCK1 and OS was further examined by analyzing the relative expression levels of ROCK1 in tissues from 20 OS patients, which showed that ROCK1 levels were more than two fold higher in OS tissues than in adjacent normal tissues (Fig. 3D). Then, we correlated ROCK1 with miR-340 expression in the same OS specimens. As shown in Fig. 3E, a significant inverse correlation was observed by Spearman's correlation analysis between the mRNA levels of miR-340 and ROCK1 ($R = -0.800$, $P < 0.001$; Fig. 3F). Taken together, these data strongly suggest that ROCK1 is a direct target of miR-340 in OS.

3.5. ROCK1 is involved in miR-340-induced suppression of OS cell proliferation, migration and invasion

The above results prompted us to examine whether the suppressive effect of miR-340 is mediated by repression of ROCK1 in OS cells. Therefore SaOS2 cells were infected with lentiviral constructs containing siRNA against ROCK1 or the negative control, and Western blot analysis confirmed that ROCK1 expression was significantly decreased (Fig. 4A). ROCK1 silencing significantly inhibited cell proliferation and suppressed migration and invasion (Fig. 4B and C), similar to those induced by miR-340. SaOS2 cells stably expressing miR-340 or NC were transfected with ROCK1 expressing plasmids without the 3′UTR region (Fig. 4D). As shown in Fig. 4E and F, overexpression of ROCK1 almost completely reversed the effect of miR-340 on the inhibition of proliferation, migration and invasion. Taken together, these findings indicated that ROCK1 is a functionally important target of miR-340 that is involved in the proliferation, migration and invasion of OS cells.

4. Discussion

In the present study, we investigated the roles of miR-340 in tumor growth and metastasis of OS. We found that miR-340 was frequently downregulated in OS cell lines and tissues. Further studies

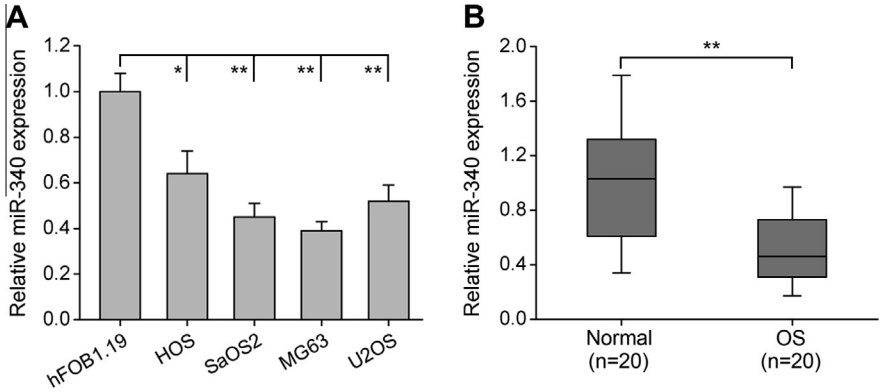

Fig. 1. miR-340 is downregulated in human OS cell lines and tissues. (A) The expression of miR-340 in the human osteoblastic cell line hFOB1.19 and four OS cell lines was measured by qRT-PCR. U6 was used as an internal control. (B) Expression of miR-340 in 20 paired primary osteosarcoma tissues (OS) and their corresponding normal tissues (Normal).*$P < 0.05$, **$P < 0.01$.

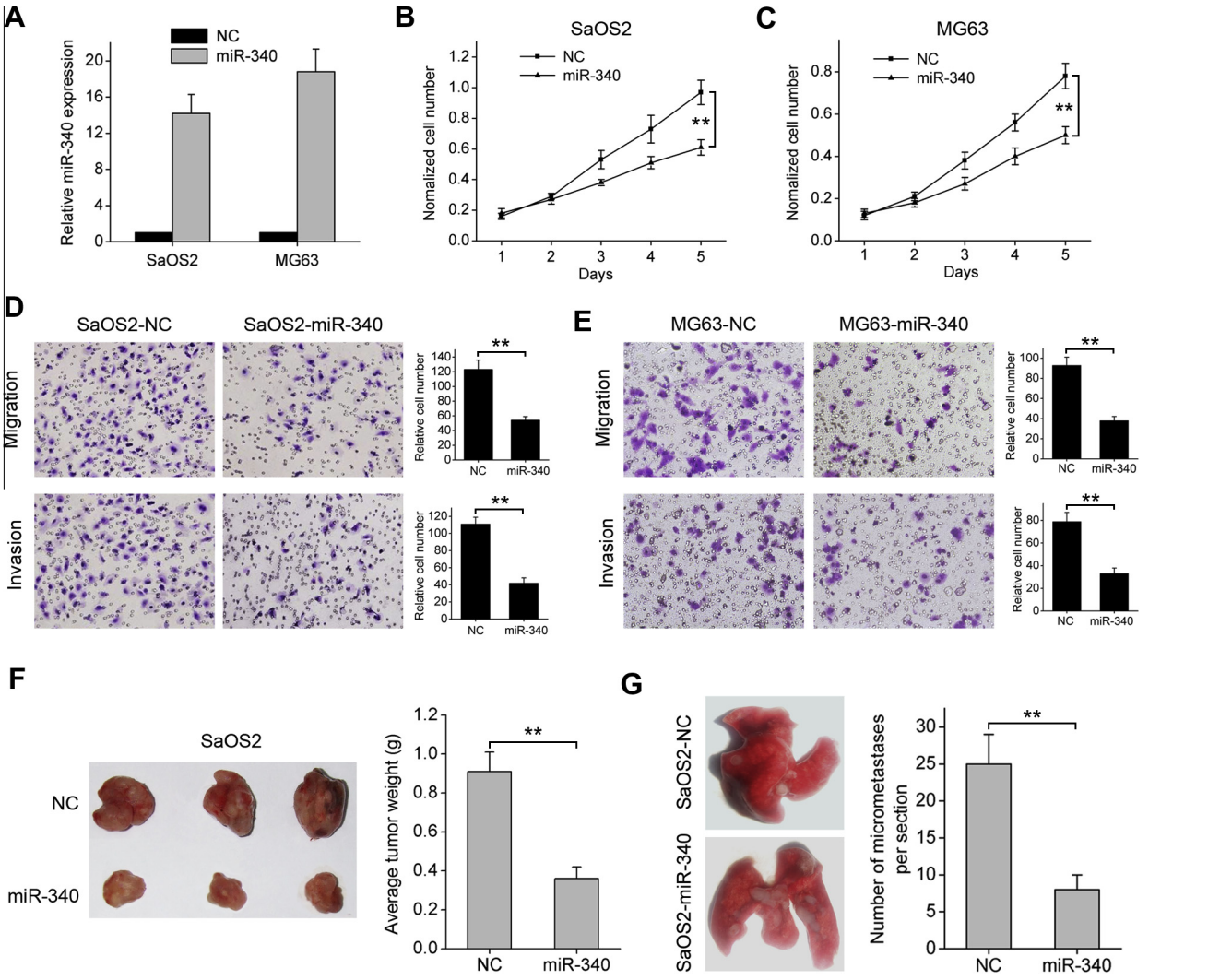

Fig. 2. miR-340 suppresses OS cell growth and metastasis. (A) Successful overexpression of miR-340 was confirmed by qRT-PCR after infection with miR-340-expressing or vector control lentivirus. (B, C) Cell proliferation was measured by WST-1 assay at different time points. (D, E) Transwell migration and invasion assays. Representative images are shown on the left, and the quantification of 10 randomly selected fields is shown on the right. (F) miR-340 attenuated tumor growth in a mouse xenograft model ($n = 6$). Representative images of xenograft tumors are shown on the left, and the average weight of tumors is plotted on the right. (G) Lung metastasis as revealed by the experimental metastasis animal model. Representative anatomical photos of lungs from mice are shown on the left. The mean number of visible tumor nodules in lungs from 6 mice is shown on the right. $^{**}P < 0.01$.

revealed that miR-340 overexpression suppressed *in vitro* cell proliferation, migration and invasion, and restrained *in vivo* tumor growth and metastasis. These results suggest that miR-340 acts as a tumor-suppressor whose downregulation may contribute to the progression and metastasis of OS.

Data on the involvement of miR-340 in cancer are limited and few potential targets of this miRNA have been identified. In a study designed to show that miRNAs target gene cohorts with similar functions, miR-340 was found to mimic the effects of TGF-β activation, inhibiting cell proliferation by modulating cell scattering and cell cycle arrest in lung cancer cells [19]. In neuroblastoma, miR-340 was associated with poor patient survival and its expression was regulated by methylation; the transcription factor SOX2 was identified as a direct target of miR-340 [20]. miR-340 was also shown to be involved in melanoma development by interacting with the 3′UTR of an isoform of microphtalmia-associated transcription factor often overexpressed in melanoma cells [21]. More recently, miR-340 was identified as a member of a miRNA combination with multiple target genes that acts synergistically to decrease the proliferation of human gastric cancer cell lines, and its downregulation in gastric cancer was associated with aberrant DNA methylation [22]. These studies together with our results confirm a tumor-suppressor role of miR340.

In the present study, we identified ROCK1 as a target of miR-340 and showed that miR-340 overexpression is correlated with ROCK1 downregulation leading to the inhibition of cell proliferation, migration and invasion. Our findings suggest that the tumor suppressor role of miR-340 is mediated by the regulation of ROCK1 expression. ROCK1 exists in a closed inactive conformation under quiescent conditions and it is activated by the direct binding of guanosine triphosphate-loaded Rho. It plays important roles in regulating cell polarity and migration by enhancing actomyosin contraction and focal adhesions, thus increasing cellular contraction, migration and chemotaxis [23]. Recent studies showed that ROCK1 is associated with cancer progression and ROCK1 expression is elevated in several cancers. For example, increased ROCK1 mRNA expression and decreased RhoE mRNA expression in hepatocellular carcinoma (HCC) were associated with cytoskeleton remodeling and the motility and invasiveness of HCC cells [24].

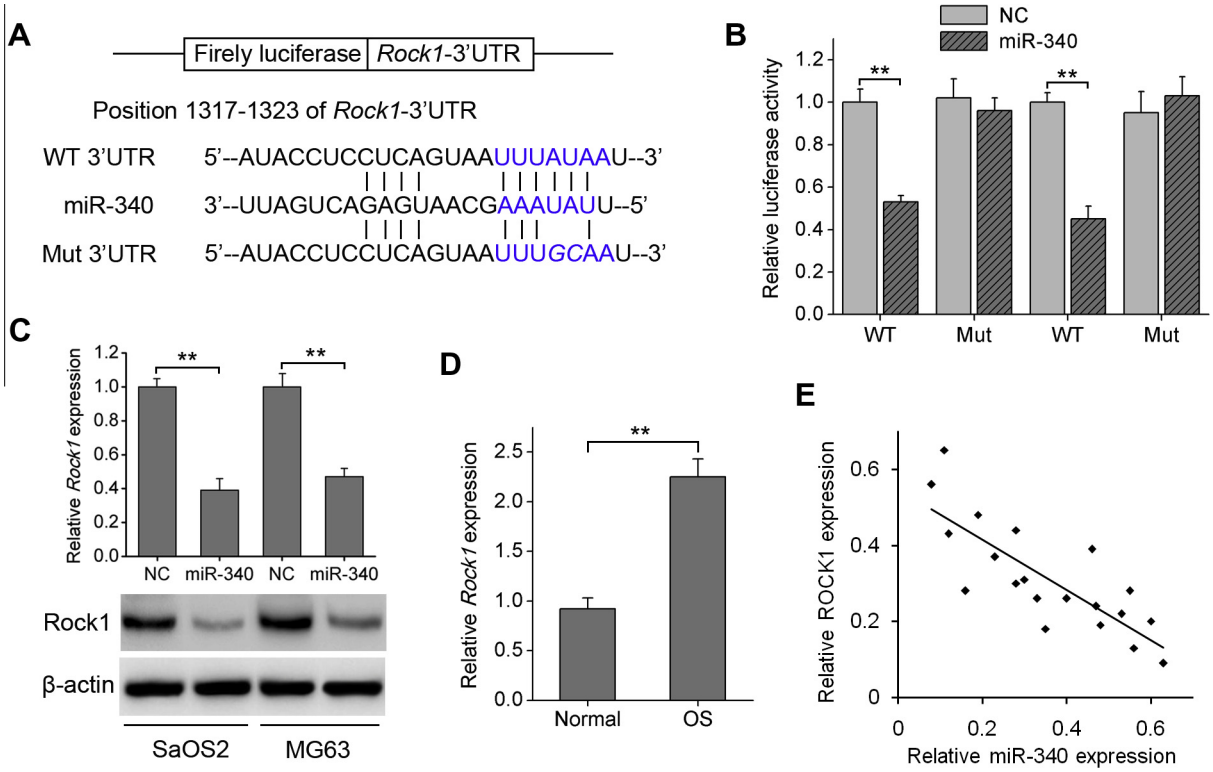

Fig. 3. miR-340 negatively regulates ROCK1 by binding to the ROCK1 3′UTR. (A) Diagram of the ROCK1 3′UTR-containing reporter construct. Schematic representation of the miR-340 sequences, putative miR-340 targeting site in the 3′UTR of ROCK1, and the generated mutant ROCK1 3′UTR. (B) A luciferase reporter assay showed the inhibitory effect of miR-340 on ROCK1–3′UTR luciferase activity in SaOS2 and MG63 cells. (C) ROCK1 mRNA levels were analyzed by qRT-PCR in SaOS2 and MG63 cells stably overexpressing miR-340. (D) ROCK1 protein levels were analyzed by Western blotting in SaOS2 and MG63 stably overexpressing miR-340. (E) Relative expression levels of ROCK1 in OS tissues and adjacent normal tissues. (F) Correlation of ROCK1 expression to miR-340 expression in 20 OS samples using simple linear regression analysis. $^{**}P < 0.01$.

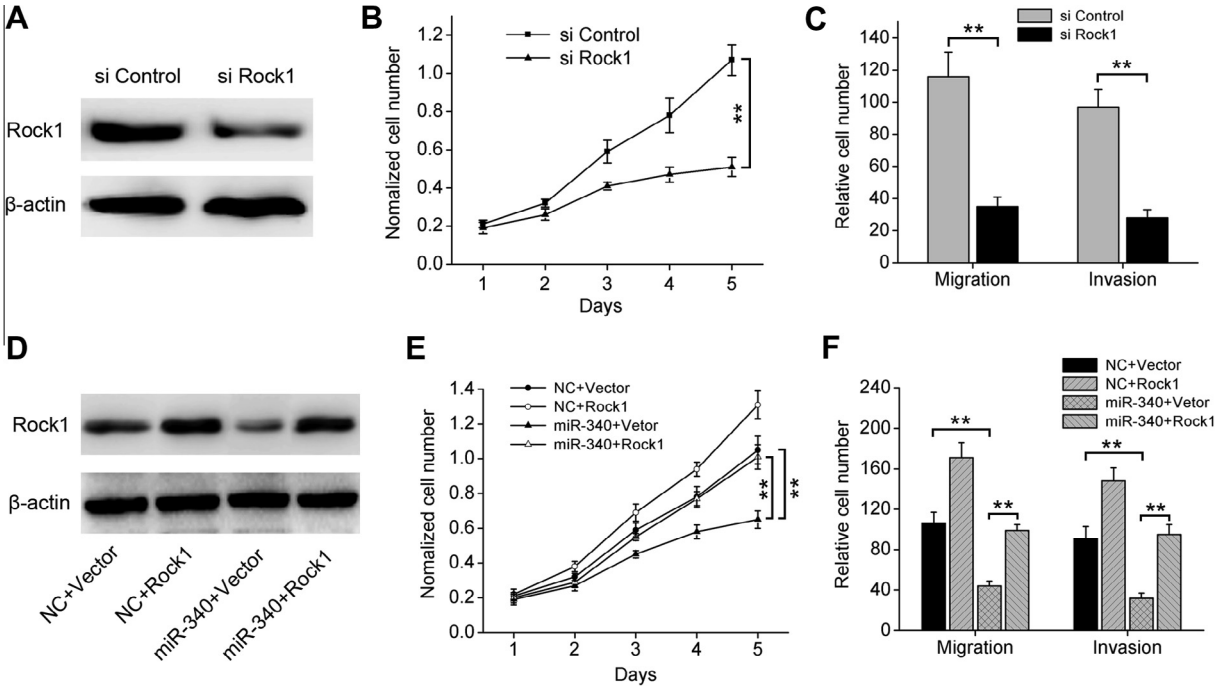

Fig. 4. Correlation between ROCK1 alterations and miR-340 in OS cells. (A) Knockdown of ROCK1 by siRNA was confirmed by Western blotting in SaOS2 cells. (B, C) Cell proliferation, migration and invasion assays were performed after infection with siROCK1 or siControl lentivirus. (D) SaOS2-NC or SaOS2-miR-340 cells were infected with ROCK1 expressing or vector control lentivirus, respectively. ROCK1 protein level was confirmed by Western blotting. (E) Cell proliferation, migration and invasion assays were performed. $^{**}P < 0.01$.

Furthermore, increased levels of ROCK1 have been associated with OS cell proliferation and poor outcomes of OS patients, highlighting the importance of targeting ROCK1 for the treatment of OS [17].

Although the exact mechanism underlying the miRNA-mediated regulation of ROCK1 is not clear, ROCK1 has been identified as a target of several miRNAs involved in carcinogenesis and tumor progression. In gastric cancer (GC), overexpression of miR-148a significantly inhibited GC invasion and metastasis by directly targeting the 3′UTR of ROCK1 [25]. The miRNA regulation of ROCK1 expression was also shown in bladder cancer, where miR-1280 acts as a tumor suppressor by directly targeting ROCK1 and inhibiting the migration/invasion of bladder cancer cells [26]. More recently, miR-148a was reported to suppress epithelial-to-mesenchymal transition by targeting ROCK1 in non-small cell lung cancer cells. These reports support the present findings that the growth and invasion of OS cells may be, in part, regulated by miR-340 modulation of ROCK1 expression.

In conclusion, the present study showed that miR-340 was downregulated in OS cell lines and tissues, and its ectopic expression inhibited cell proliferation, migration/invasion, tumor growth and metastasis. The tumor-suppressor function of miR-340 was mediated by the downregulation of its downstream target gene ROCK1. These results indicate that miR-340 deregulation may play important roles in tumor growth and metastasis and that miR-340 may be a potential therapeutic target for the treatment of OS.

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