Microsurgery of Hand and Foot, The Second People's Hospital of Dongying City, Dongying City, P.R. China

MiR-139 prompts the development of osteosarcomas mainly through targeting ROCK1

Shaojun Su, Xinguang Nie*

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**Corresponding author: Dr. Xinguang Nie, Microsurgery of Hand and Foot, The Second People's Hospital of Dongying City, #25 Changqing Road, Dawang Town, Dongying City, Shangdong Province, P.R. China niexingguang170118@163.com*

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Abnormal expression of miR-139 was found to be aberrantly expressed in various tumors. However, whether it is involved in osteosarcomas (OS) has never been explored. In the current study, we found that the level of ROCK1 was markedly increased in OS cancer tissues compared to that of noncancerous tissues. Meanwhile, the expression of miR-139 was markedly reduced in OS cancer tissues and cell lines. Enhanced miR-139 expression markedly suppressed colony-formation and cell invasion capacity of OS cancer cells. Dual luciferase reporter assay demonstrated that ROCK1 was a target gene of miR-139. Moreover, overexpression of ROCK1 also led to increased invasion capacity in OS cancer cells even when miR-139 was inhibited, suggesting the anti-invasion effects of miR-139 were mediated through ROCK1. In summary, our present findings indicate that miR-139 functions as a tumor suppressor in OS cancer cells mainly by targeting ROCK1.

1. Introduction

Osteosarcoma (OS) is a common primary bone malignancy which is characterized by the secretion of osteoid or immature bone among children and adolescents (Unni and Dahlin 1989). In the past years, the overall survival rate has been improved by the enhanced neoadjuvant chemotherapy and radical surgical *en bloc* resection technologies (Ciernik et al. 2011). Present studies have demonstrated that OS leads to a large proportion of death and disability in children and adolescents. Thus, it is important to explore the underlying mechanism in which OS is finely modulated.

MicroRNAs are small non-coding RNAs with approximately 22 nucleotides (Deng et al. 2016). In multiple biological processes, the involvement of miRNAs were widely reported through binding the 3'untranslated region (3'UTR) of target messenger RNAs (Fan et al. 2016; Fang et al. 2016; Huang et al. 2016). Multiple studies have indicated the aberrant expression of miRNAs in the progression of various tumors, including pancreatic cancer, lung cancer, and breast cancer. For instance, miR-25-3p was shown to target Sema4C in cervical cancer cells thereby regulating epithelial-mesenchymal transition (Song and Li 2017). And miR-638 was found to suppress OS cell proliferation mainly by inhibiting PIM1 in human OS (Wang et al. 2017). miR-139 was found to be aberrantly expressed in many tumors, including adult myeloid leukemia, breast cancer and bladder cancer (Krowiorz et al. 2016; Yonemori et al. 2016; Li et al. 2017). However, its specific expression pattern has never been explored in OS tissues.

Rho-associated protein kinase (ROCK) 1 is an important member of the rho-associated serine/threonine kinase family, that is involved in the malignant processes of cellular migration, invasion, and metastasis (Xu et al. 2015; Zhou et al. 2016). Enhanced expression of ROCK1 has been widely reported in various tumors, including glioma, osteosarcoma, prostate cancer, and gastric cancer (Hu et al. 2014). MicroRNAs were reported to be key regulator of ROCK1, including miR-135a, miR-145, and miR-148a (Li et al. 2013; Shin et al. 2014; Wan et al. 2014).

In the present study, we demonstrate that ROCK1 was a novel target gene of miR-139. Decreased miR-139 expression in OS cancer cells prompted the malignant processes of cancer cells mainly by targeting ROCK1.

2. Investigations and results

2.1. Reduced miR-139 level and enhanced ROCK1 expression in OS specimens

Firstly, we identified the expression of ROCK1 in OS tissues and noncancerous tissues. According to our data, the expression of ROCK1 was significantly increased in OS tissues (2.752±0.5875) compared with that of noncancerous tissues (1±0.328) (Fig. 1A). In addition, we explored the expression of miR-139 in OS tissues and noncancerous tissues. As shown in Fig. 1B, the expression of miR-139 was significantly decreased in OS tissues (0.438±0.0656), compared with noncancerous tissues (1±0.189).

2.2. ROCK1 as target gene of miR-139

Next, we further evaluated whether ROCK1 was the target gene of miR-139. Based on TargetScan (http://www.targetscan.org/vert_71/), a conserved binding site of miR-139 in the 3' untranslated region (3'UTR) of ROCK1 was identified (Fig. 2A). Then, the 3'UTR of ROCK1 was cloned into the dual luciferase reporter

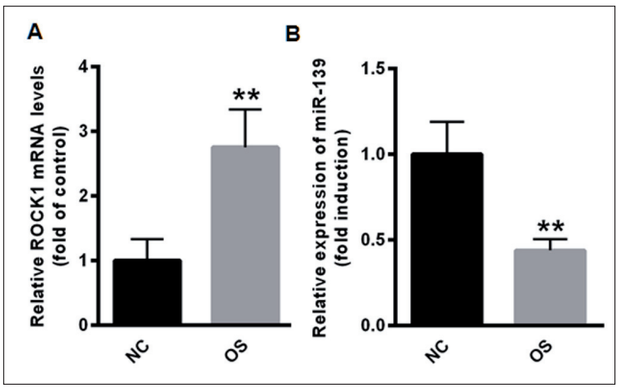

Fig. 1: Reduced miR-139 level and enhanced ROCK1 expression in OS specimens. (A) Real time PCR analysis of ROCK1. (B) The level of miR-139 in OS tissues and noncancerous tissues. *p<0.0.5, **p<0.01, vs. control.

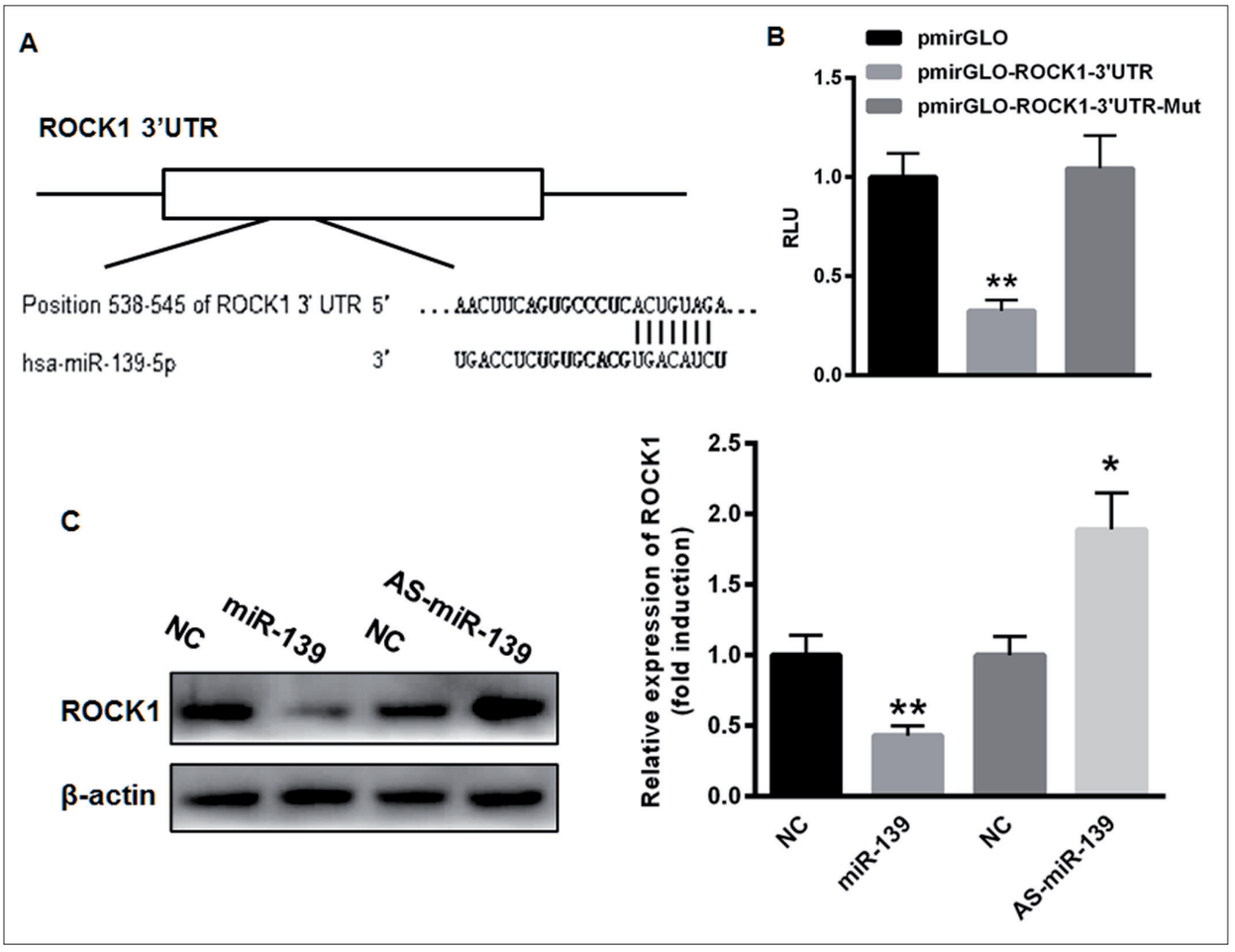

Fig. 2: ROCK1 was the target gene of miR-139. (A) Schematic analysis of miR-139 in the 3'UTR of ROCK1. (B) Dual luciferase reporter assay. (C) Western blot assay. *p<0.0.5, **p<0.01, vs. control.

vector, pmirGLO plasmid. Dual luciferase reporter assay showed that miR-139 could significantly suppressed the relative luciferase activity of ROCK1 (Fig. 2B). Meanwhile, the 3'UTR containing the binding site was mutated. As shown in Figure 2B, miR-139 could not suppress the relative luciferase activity of ROCK1-3'UTR-Mut. Western blot analysis demonstrated that overexpression of miR-139 could markedly inhibit the expression of ROCK1, while inhibition of miR-139 enhanced the protein level of ROCK1 (Fig. 2C). These data indicated that ROCK1 was the target gene of miR-139.

2.3. ROCK1 enhanced OS cancer cell migration and colony formation

Then, we explored the oncogenic role of ROCK1 in OS cancer cells. Overexpression of ROCK1 also increased the cell migration capacity compared with that of normal control (Fig. 3A). Moreover, transfection with Ad-ROCK1 significantly increased cell invasion capacity (Fig. 3B). We also explored the expression of ERK1 signaling. As shown in Fig. 3C, overexpression of ROCK1 markedly increased the phosphorylation level of ERK in OS cancer cells compared with that of Ad-NC. Furthermore, overexpression of ROCK1 markedly increased the phosphorylation level of p38 (Fig. 3C), suggesting an anti-apoptotic role of ROCK1 in OS cancer cells.

2.4. Reduced miR-139 exerts an oncogenic role mainly through ROCK1

To explore whether miR-139 exerted its role through ROCK1, a specific siRNA targeting ROCK1 was selected. As shown in Fig. 4A, silencing of ROCK1 markedly suppressed the expression of ROCK1, the activation of ERK1 and p38 even in OS cancer cells transfected with miR-139 inhibitor. Meanwhile, cell migration and colony formation capacity was also suppressed when ROCK1 was silenced (Fig. 4B and 4C).

3. Discussion

As the most common primary bone malignancy, osteosarcoma, leads to significant rate of disability and mortality (Orlandini et al. 2008; Nwankwo et al. 2013; Selmic et al. 2017). Recent studies have indicated the important role of miRNAs in the progression of various tumors (Hwang and Mendell 2007; Gandellini et al. 2015). Therefore, to identify the potential oncogenic miRNAs may shed light on the treatment of OS. In the current study, we first found that the level of miR-139 in OS tissues it was markedly reduced. For the first time, we demonstrated that ROCK1 is a novel target gene of miR-139. In OS cancer cells, miR-139 suppressed cancer cell migration and colony formation mainly through targeting ROCK1.

ROCK-1 is a serine/threonine kinase and belongs to the member of the Rho family of GTPase proteins. Studies have demonstrated that ROCK1 enhances the reorganization of the actin cytoskeleton and participates in multiple downstream signaling pathways, including cell migration, cell death, and survival (Narumiya et al. 2009; Matsubara and Bissell 2016). Recent studies have demonstrated that ROCK1 was significantly upregulated in bladder, lung, and prostate cancer (Liu 2011). For instance, the ROCK1 level was shown to be increased thereby enhancing cancer cell migration. Further studies have shown that ROCK1 is the target genes of multiple miRNAs, including miR-126, miR-335, miR-584, and

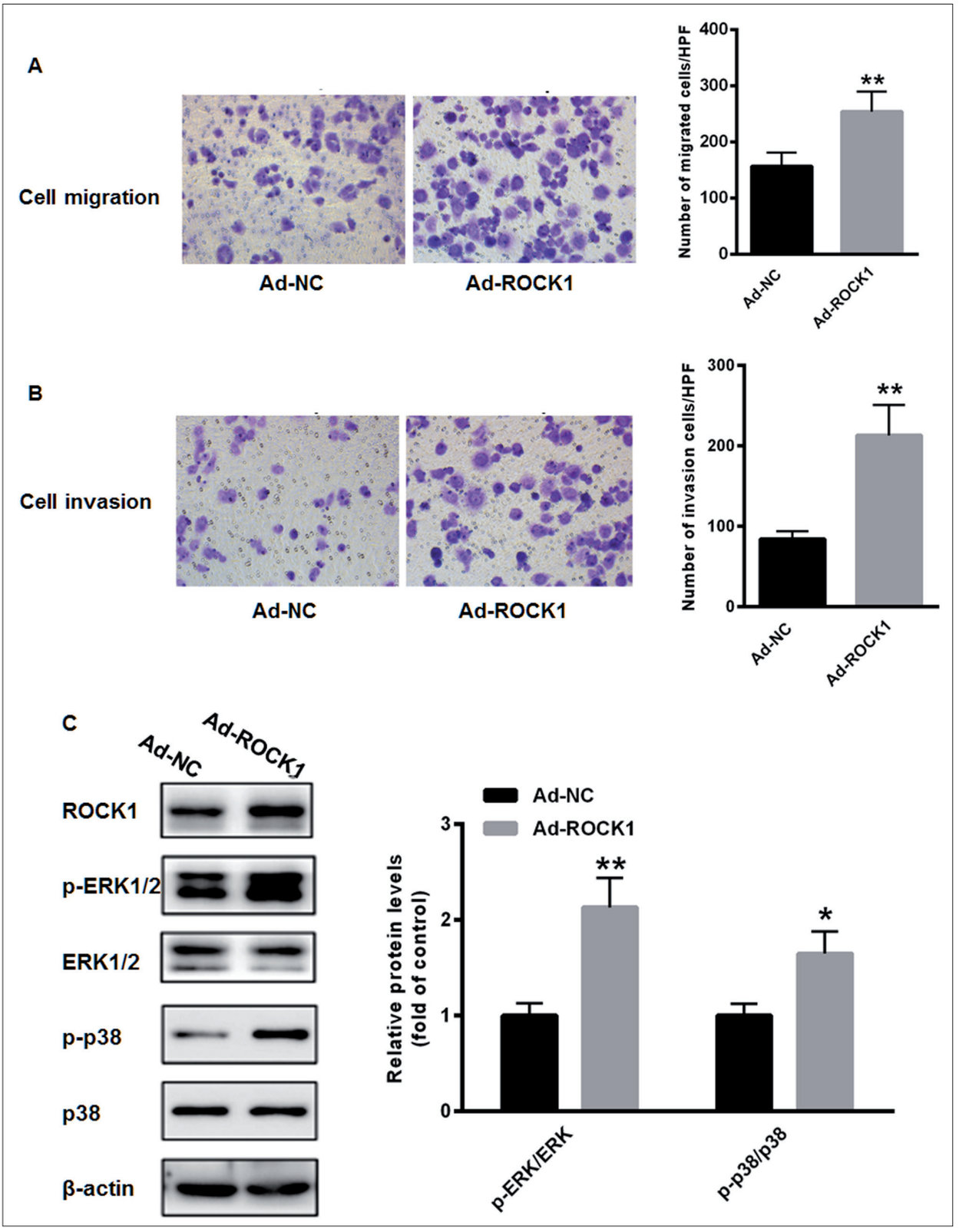

Fig. 3: ROCK1 enhanced OS cancer cell migration and colony formation. (A) Analysis of colony formation capacity. (B) Analysis of cell migration capacity. (C) Western blot analysis of ROCK1/ERK/p38 signaling after transfection of Ad-ROCK1 and Ad-NC into OS cancer cells. *p<0.0.5, **p<0.01, vs. control.

miR-186 (Lowery et al. 2008; Karius et al. 2012; Kong et al. 2012; Tufman et al. 2013). In these cancer cells, miRNAs were involved in cancer cell proliferation and/or invasion mainly by modulating the expression of ROCK1 in colon cancer, osteosarcoma and human renal cell carcinoma (Liang and He 2011; Karius et al. 2012; Kong et al. 2012). In the present study, we aimed to evaluate the possible role of miR-139 in the progression of OS. Thus, we first analyzed the expression of ROCK1 in OS tissues and cells. In line with previous studies, ROCK1 was found to be significantly upregulated in OS cancer cells, suggesting an oncogenic role in

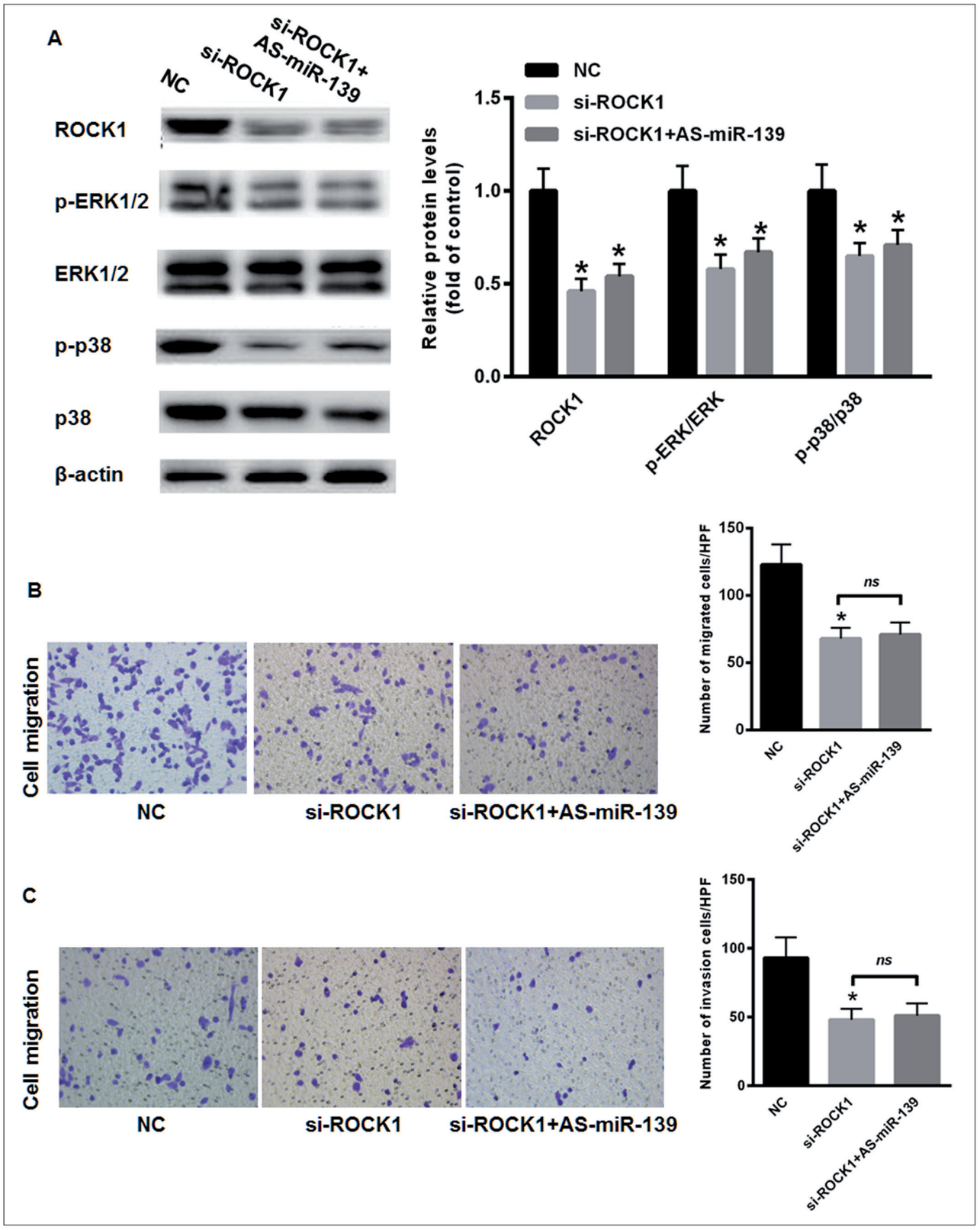

Fig. 4: Reduced miR-139 exerted an oncogenic role mainly through ROCK1. (A) Silencing of ROCK1 markedly suppressed the activation of ERK1 and p38 even in OS cancer cells transfected with miR-139 inhibitor even in OS cancer cells transfected with miR-139 inhibitor. Cell migration (B) and cell invasion capacity (C) was also suppressed when ROCK1 was silenced. *p<0.0.5, **p<0.01, vs. control.

OS cells. Then, dual luciferase reporter assay demonstrated that miR-139 suppressed the dual luciferase activity of ROCK1, indicating ROCK1 was a target gene of miR-139.

Furthermore, we evaluated the role of miR-139 in the progression of OS. We found that enhanced miR-139 expression markedly inhibited OS cancer cell proliferation and migration. In contrast, silencing of ROCK1 could partially reverse miR-139 inhibition-induced cancer cell invasion capacity. These data demonstrated that miR-139 functions as a tumor suppressor mainly by targeting ROCK1.

In conclusion, we first demonstrate that miR-139 acts as a tumor suppressor mainly by directly targeting ROCK1 in OS cancer cells. For the first time, we demonstrated that the expression of miR-139 was negatively correlated with ROCK-1 expression in primary OS tissues. These experimental data and finding in the current study may shed light on elucidating the biological functions of miR-139 in OS cancer cells. To further explore the molecular network involved in the ROCK1 and miR-139 regulation, a large quantity of clinical sample basis is necessary.

4. Experimental

4.1. Cell lines

Human OS cell line MG-63 (purchased from ATCC) was cultured in monolayer in RPMI 1640 (Life Technologies Invitrogen, Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C in a humid atmosphere with 5% CO_2.

4.2. Transient transfection

Cells were seeded at the density of 10^6 cells/well in the 6-well plates. Then, the siRNA targeting ROCK1 or a non-specific siRNA (NC) were pre-incubated with HiperFect transfection reagent (QIAGEN) at room temperature for 10 min. After that, the complex was added in to the culture medium for 48 h.

4.3. Western blotting

Total proteins were extracted from tissues or cells using RIPA buffer (Keygen, Nanjing, China). Protein 20 μg was separated using SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked with 5% fat-free milk at room temperature for 2 h. The primary antibodies used in the current study was ROCK1 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (1:1000 dilution; Santa Cruz Biotechnology) was used as a control. The signals were detected using a Super ECL Plus Kit (Keygen) and determined by quantitative analysis using UVP software (UVP, LLC, Upland, CA, United States). GAPDH was used as an internal control.

4.4. Real-time PCR

To analyze the expression of miR-139, TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) were applied. U6 snRNA was used to as an internal control for (Cheng et al. 2013). For the quantification of ROCK1, SYBR Green PCR mixture (Invitrogen) was used. And GAPDH was used as an internal control.

4.5. Apoptosis analysis

After treatment, the cells were washed with cold PBS for three times (5 min/time). To quantify cell apoptosis, an Annexin-V FITC–PI Apoptosis Kit (Invitrogen, Carlsbad, CA) was applied with strict accordance to the instructions. In brief, cells were washed with 1×PBS for three times and suspended at 2-3 × 10^6 cells/mL in 1 × Annexin-V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin-V FITC and propidium iodide buffer were added to the cells, which were then incubated for 15 min at room temperature in darkness. Cells without any treatment were used as internal control. After incubation, the cells were filtered by a filter screen and the cells were analyzed by FACS can (Becton–Dickinson, San Diego, CA) within 1 h of staining and 10,000 cells were evaluated in each sample.

4.6. Invasion and motility assays

Firstly, cells were seeded in the top chamber of each insert at 1.0×10^5 cells/well (BD Biosciences, San Jose, CA, USA) with 8.0-mm pores for motility assay. And for the invasion assays, 2.0×10^5 cells were cultured in a chamber (BD Biosciences) pre-coated with 0.2% Matrigel (Collaborative Research, Boston MA, USA) at 37 °C. As a chemoattractant, 10% fetal bovine serum was added to the culture medium in the lower chamber. After 24 hours, the cells remained in the upper compartment were removed by cotton swabs, and those invaded through the membrane were stained with a dye solution containing 20% methanol and 0.1% crystal violet. The cells were then imaged under a light microscope (Olympus) and ten individual fields were counted per insert. The results are presented as an average of three separate experiments.

4.7. Luciferase activity assay

HEK293T cells were seeded in 24-well plate at the density of 5×104 cells/well. After 24 h, wild-type or mutated ROCK1 3'-UTR luciferase reporter vector and miR-139 mimic or inhibitor were transfected into cells with Vigofect transfection reagent according to the manufacturer's instruction. After transfection for 48 h, the Dual-luciferase reporter assay system (Promega) was applied to determine the changes of relative luciferase units (RLU). Renilla activity was used as the internal control.

4.8. Statistical analysis

Data were presented as mean±SD from three independent experiments or five mice. Statistical analysis was carried out with Student's t test. $P < 0.05$ was considered as statistically significant difference.

Conflicts of interest: None declared

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