



MiR-193b enhanced proliferation and migration and inhibits apoptosis through targeting RAB7A in osteosarcoma cell

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Abstract

Background Accumulating study indicated that microRNA (miRNA) played critical role in the osteosarcoma (OS). The role and mechanisms of miR-193b in OS cell lines were still unknown.

Objective We resolved the miR-193b expression in OS cell line and normal cell by RT-PCR assay. The effects of upregulated miR-193b on OS cell proliferation, migration, invasion and apoptosis were evaluated using CCK-8 assay, transwell assay, wound-healing assay and flow cytometric analysis *in vitro*, respectively. We investigated the effect of upregulated miR-193b on the mRNA level of cell cycle protein CCND1 and CCNE1 using RT-PCR assay and the protein level of epithelial to mesenchymal transition (EMT)-related protein E-cadherin, vimentin, and N-cadherin by western blotting assay in MG-63 and U2SO cells. Furthermore, luciferase reporter assays were employed to identify the candidate target gene RAB7A of miR-193b.

Results The expression of miR-193b was downregulated in OS cells. In MG-63 and U2SO cells, ectopic miR-193b expression inhibited cell proliferation, migration, invasion, and induced apoptosis. We found that miR-193b reduced the mRNA expression of CCND1 and CCNE1, and regulated the associated proteins of EMT including E-cadherin, vimentin and N-cadherin in MG-63 and U2SO cell lines. Moreover, the candidate target gene RAB7A was negatively regulated by miR-193b. In addition, upregulated RAB7A rescued the inhibitory effect of miR-193b mimics on the development of OS cell.

Conclusion In conclusion, this study suggested that miR-193b overexpression inhibited cell proliferation, migration, invasion, and induced cell apoptosis by down-regulating RAB7A in OS cell lines.

Keywords MiR-193b · Osteosarcoma · RAB7A · EMT

Introduction

Osteosarcoma (OS) is the most common and prevalent primary malignant tumor of bone in adolescents and young adults (Longhi et al. 2006). Osteosarcoma is likely aggressive, with early metastasis and rapid growth (Martin et al. 2012; Rosenberg 2013). Despite the improvement of surgery plus neoadjuvant chemotherapy, 30% in cases with metastatic disease and the 5-year survival for patients is

still poor, respectively (Schuetze 2017; Kleinerman 2016). Lung metastasis has a worse prognosis at the time of diagnosis (Nataraj et al. 2016; Bielack et al. 2016). Therefore, identification of studying the mechanisms that contribute to the development and progression of OS is important for advancing the OS treatment.

MicroRNAs (miRNAs) are a class of approximately 22–24 nucleotides in length, noncoding-small and endogenous RNA (Bartel 2004). MiRNAs play a key role in the regulation of transcriptional expression levels by binding to the 3'-untranslated region (3'-UTR) of target mRNAs (Makarova et al. 2016). The expression levels and roles of aberrant miRNAs are founded to be closely associated with the development of human cancer types (To et al. 2018; Fellenberg et al. 2019). MiRNAs have been reported to be differently expressed in OS and to function as tumor suppressors or oncogenes (Ram Kumar et al. 2016). The dysregulation of miR-193b has been found in several

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cancers (Khordadmehr et al. 2019; Zhang et al. 2017). The miR-193b is located on human chromosome 16p13.12, and has a different expression in colon cancer, prostate cancer, breast cancer, renal cell carcinoma, lung cancer and hepatocellular carcinoma (Fang et al. 2019; Chen et al. 2019a). MiR-193b enhance the chemosensitivity of OS cells by increasing FEN1-mediated autophagy (Dong et al. 2019). However, the expression levels and functional of miR-193b in OS cell lines is still unknown.

Ras-related protein Rab-7a (Rab7a) is located in late endosome, and effects transport to endocytic breakdown compartments (Bucci et al. 2000). Now, the studies found that the role of Rab7a in cancer (Heo et al. 2018; Guerra et al. 2019). Knockdown of RAB7A decreases cell proliferation and invasion in breast cancer cells (Xie et al. 2019). However, the relationship between the miR-193b-RAB7A axis involved in OS proliferation, migration, and apoptosis still needs further research.

The aim of the study to elucidate the expression and role of miR-193b in OS cells. Further, the biological functions of its proliferation, migration, and apoptosis in OS cell lines were researched.

Materials and methods

Cell culture

The normal human osteoblast hFOB1.19 cell line and the OS cell lines including U2SO, HOS, Saos-2, SW1353, and MG63 were got from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in the containing 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., USA) of Dulbecco's modified Eagle's medium (DMEM; Hyclone, UT, USA). Cells were cultured at 37 °C in an atmosphere containing 5% CO₂.

Cell transfection

MG-63 and U2SO cell were harvested at confluence of 75% to make cell transfections. Cell transfection was conducted using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. MiR-193b, miR-NC, RAB7A ectopic expression, NC was got from Sengong (Biotech, Shanghai, China). The NC was used as the negative control. miR-193b (5'-AAC UGGCCUCA AAGUCCCGC U-3') was synthesized from Sengong Biotech, Shanghai, China. The unspecific miRNA (5'-AC UGACACGUUCGGAGA AUU-3') was used as the negative control (ctrl miRNA).

Cell counting kit-8 (CCK-8) assay

MG-63 and U2SO cell were harvested at 24 h post-transfection. Cells were cultured in 96-well plates (3×10^4 cells) and cultured for 24, 48, 72 and 96 h. Cell proliferation was detected by the cell counting kit-8 (CCK-8) (Dojindo, Japan) by the manufacturer's instructions. Each well was added with 10 ul CCK-8 solution for 4 h. After cell culture was terminated by added with 10 ul DMSO, and OD values at 450 nm were detected.

Invasion assay

Transwell chambers were used to detect cell invasion. Briefly, cells were added on the upper surface of Matrigel-coated membrane inserts. After cultured 24 h, the across transwell membrane of cells were fixed with 4% paraformaldehyde. Then, the transwell membrane cell were stained with 0.5% crystal violet for 25 min. Final, invasive cells were counted and photographed by using optical microscope (Nikon, Tokyo, Japan).

Wound-healing assay

A wound-healing assay was measured to detect cell migration capability. The OS cells were added in six-well plates and were cultured to 75%. The cells were scratched using a sterile 200- μ L pipette tip. Then, the wound recovery was observed after 0 and 24 h.

Flow cytometric analysis

The cells were collected and assessed using Annexin V-APC / 7-AAD apoptosis Kit (Biolegend). The apoptosis rates were detected by flow cytometry (Beckman Coulter, FC500).

Dual-luciferase reporter assay

The target genes of miR-193b were assayed using TargetScan (http://www.targetscan.org/vert_72/). The RAB7A 3' untranslated region (UTR) was found at miR-193b binding sites. The wild-type (WT) and mutant RAB7A 3'UTR luciferase reporter gene plasmids were constructed. The MUT3'pGL3 vector or Luc-RAB7A-3'UTR WT were constructed by Shanghai Gene-Chem Co., Ltd. (Shanghai, China). The dual luciferase activity was detected using a Dual Luciferase Assay Kit (Promega Corporation, Madison, WI, USA).

Real-time quantitative polymerase chain reaction (PCR)

Total RNA was obtained from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and used for real-time PCR assay. RT-PCR was measured to detect the mRNA levels of CCND1 and CCNE1. The PCR reactions were conducted in a total volume of 20 μ l, including 10 μ l of 2XPower SYBR 1 Green PCR Master Mix (Applied Biosystems, Warrington, UK), 2 μ l of cDNA (5 ng/ μ l) and 1 μ l of primer mix (10 μ M each). PCR detection and amplification were assayed in a LightCycler 480 II (Roche Applied Science) as follows: an initial denaturation at 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Hairpin-itTM miR-193b qRT-PCR Primer Set (GenePharma) was used for the quantity of miR-193b. The mRNA levels of miR-193b were normalized to the endogenous levels of U6. The relative gene levels were accessed using the comparative Ct Method. $2^{-\Delta\Delta CT}$ method was measured to process data and three replicates were contained in each experiment (Table 1).

Western blotting

MG-63 and U2SO cells were cultured at 24-h post-transfection, and the cell total proteins were extracted using RIPA solution (Sangon). Then, electrophoresis was measured using a SDS-PAGE (12%) gel to separate proteins. Proteins were transferred to PVDF membranes. The membranes were soaked in 5% non-fat milk for 2-h room temperature. First antibody was performed using E-cadherin, N-cadherin, Vimentin, Bax, Bcl2, RAB7A, and GAPDH antibody (Cell Signaling Technology Inc.) primary antibodies for 4 h at 4 °C. The second blotting was measured using goat anti-rabbit IgG-HRP secondary antibody (1:1500, MBS435036, MyBioSource) for 2 h at room temperature. ECLTM Blocking Agent GE Healthcare (Sigma-Aldrich) was performed to incubate with membranes for 10 min to find signals and data were measured using Image J V1.34 software.

Table 1 The qRT-PCR primer sequences

Gene	Primer sequences (5'-3')
miR-193b	F: ACACTCCAGCTGGGAAGTGGC CCTCAAAGTC R: AGCCTCTGCGCACGTGTTC
U6	F: CTCGCTTCGGCAGCAC R: AACGCTTCACGAATTTGCGT
CCND1	F: TGGTGAACAAGCTC AAGTGGA R: AGGGCGGATTGGAA ATGAACT
CCNE1	F: TGACCTAAGGGACT CCCACAA R: TGATAATGTGGAGA GGGCAGC

Statistical analysis

The results were detected in at least three independent experiments. The quantitative data are presented as mean \pm standard deviation (SD). Student's *t* test was performed when only two groups using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA). For all comparisons, *P* value < 0.05 was used to indicate a statistically significant.

Results

miR-193b is downregulated in OS cell lines

To determine the expression levels of miR-193b in OS cell lines, we compared the expression levels of miR-193b in the hFOB 1.19 and the OS cell lines including U2SO, HOS, Saos-2, SW1353 and MG63 using qRT-PCR assay. As shown in Fig. 1a, the relative expression of miR-193b was significantly down-regulated in OS cell lines, including U2SO, HOS, Saos-2, SW1353 and MG63 compared with hFOB 1.19. The MG-63 and U2SO found lower expression than other three cell lines. MG63, U2SO were choose for the vitro experiments. We detected the transfection efficiency of miR-193b mimics using qRT-PCR. Transfection of miR-193b mimics to MG-63 and U2SO cell lines increased the levels of miR-193b compared with NC group (Fig. 1b).

Overexpression of miR-193b inhibited OS cell proliferation

To study the tumorigenic roles of miR-193b in OS cell, we transfected the miR-193b-mimics to MG-63, U2SO cell lines. CCK-8 assay results shown that upregulated miR-193b significantly decreased the cell proliferation of MG-63 cell lines (Fig. 2a). Moreover, upregulated miR-193b significantly decreased the cell proliferation of U2SO cell lines (Fig. 2b). Further, miR-193b also inhibited the mRNA expression levels of cell cycle regulatory proteins including CCND1 and CCNE1 in MG-63 and U2SO cell lines (Fig. 2c,d). Our results shown that miR-193b inhibited the proliferation of OS cells via down-regulating the levels of CCND1 and CCNE1.

Overexpression of miR-193b suppressed OS cell migration and invasion by regulating EMT-related protein

The wound-healing assay and Transwell assay were performed to detect the effects of miR-193b on the migration and invasion of OS cells in vitro. The wound-healing assay showed that overexpression of miR-193b significantly reduced the migration of MG-63 and U2SO cell

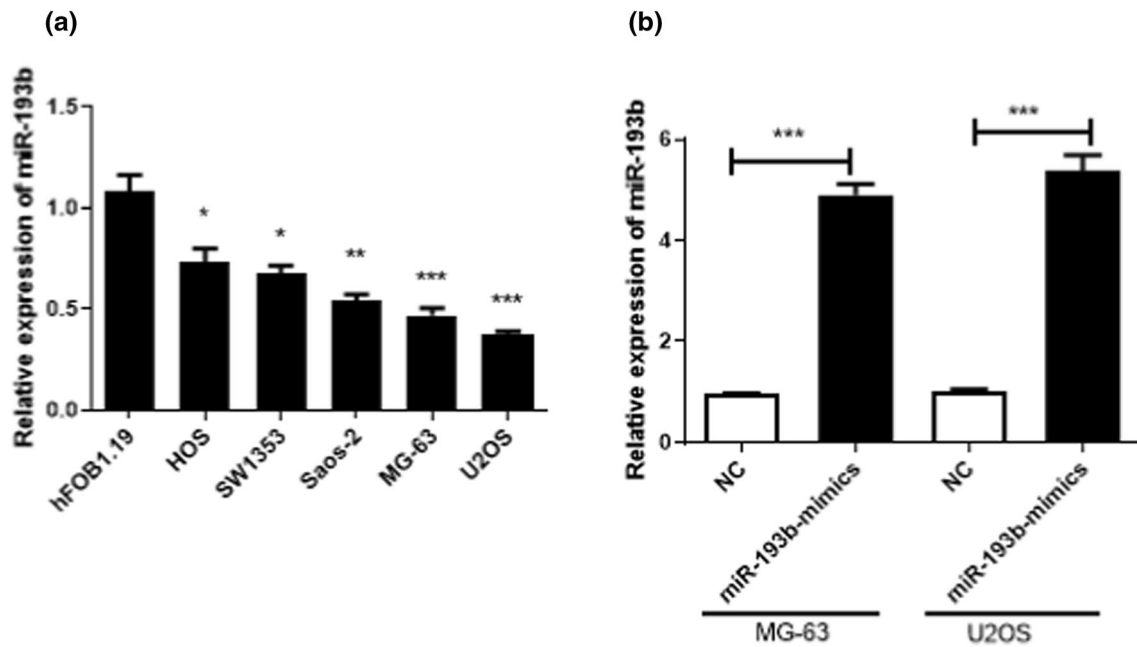


Fig. 1 MiR-193b is downregulated in OS cell lines. **a** RT-PCR assay was performed to measure the expression level of miR-193b in OS cell lines (MG-63, HOS, SW1353, Saos-2, U2OS) in comparison with hFOB1.19. ($n=3$). **b** miR-193b mimics were transfected

into MG-63 and U2OS cell lines, which was assessed by RT-PCR. ($n=3$). U6 was used as the standard reference. * $P<0.05$ vs control; ** $P<0.01$; *** $P<0.001$

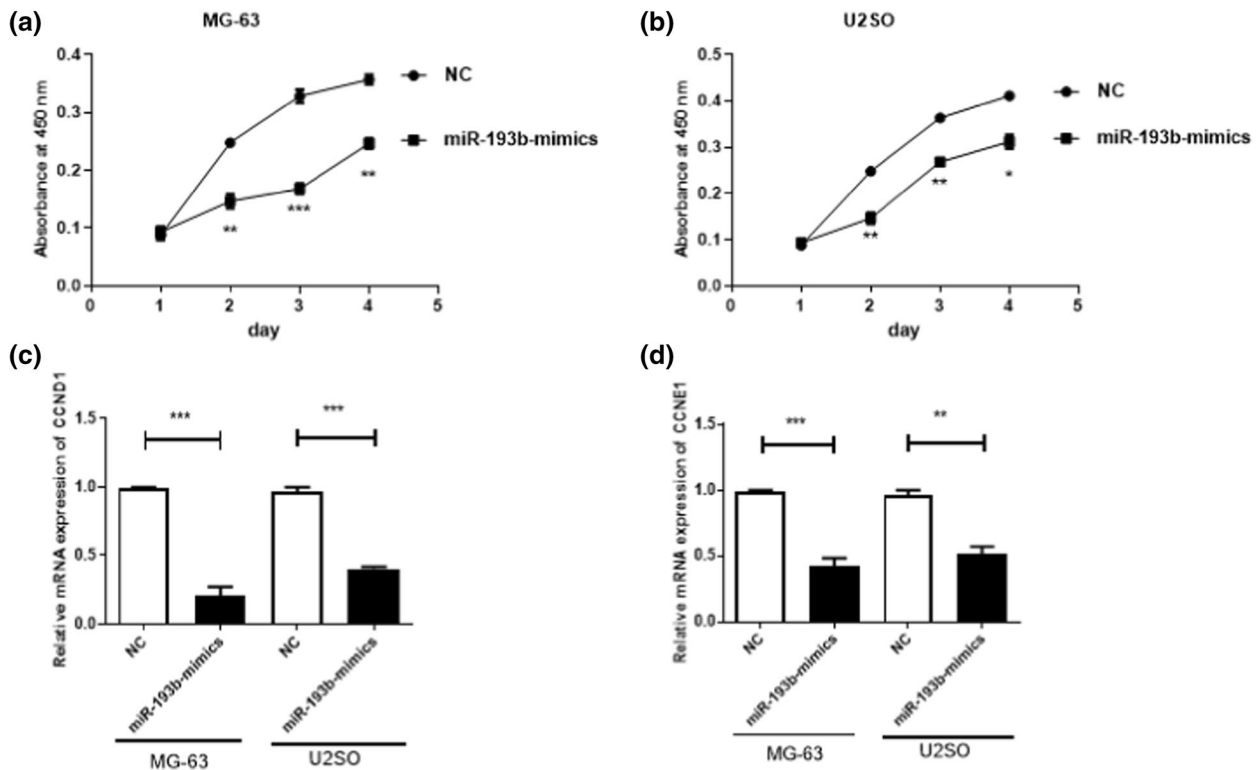


Fig. 2 Upregulated miR-193b reduced OS cell proliferation. **a, b** The CCK-8 assay was performed to measure the proliferation of miR-193b in MG-63 and U2OS cells. * $P<0.05$ vs the miR-NC group. **c, d** Overexpression of miR-193b inhibited the mRNA level

of CCND1 and CCNE1 in MG-63 and U2OS cell lines. All assays were performed at least three times. Data are presented as the mean values \pm SD. * $P<0.05$ vs control; ** $P<0.01$; *** $P<0.001$

lines (Fig. 3a). The Transwell assay supported the study that the upregulation of miR-193b remarkably decreased the invasive of MG-63 and U2SO cell lines (Fig. 3b). Further, we shown that overexpression of miR-193b significantly promoted the protein expression of E-cadherin, and decreased the expression of N-cadherin and vimentin (Fig. 3c,d). Our findings suggested that miR-193b decreased the invasion and migration of OS cells, and regulated the EMT related proteins.

Overexpression of miR-193b induced OS cell apoptosis

The cell apoptosis was detected using Annexin V/PI staining. As shown in Fig. 4a, the percentage of late-stage apoptotic cells was notably raised in the miR-193b overexpression group in MG-63 and U2SO cell lines. Further, we revealed that overexpression of miR-193b promoted the protein expression of Bax and decreased the protein expression of Bcl-2 using western blotting assay in MG-63 and U2SO cell lines (Fig. 4b). These

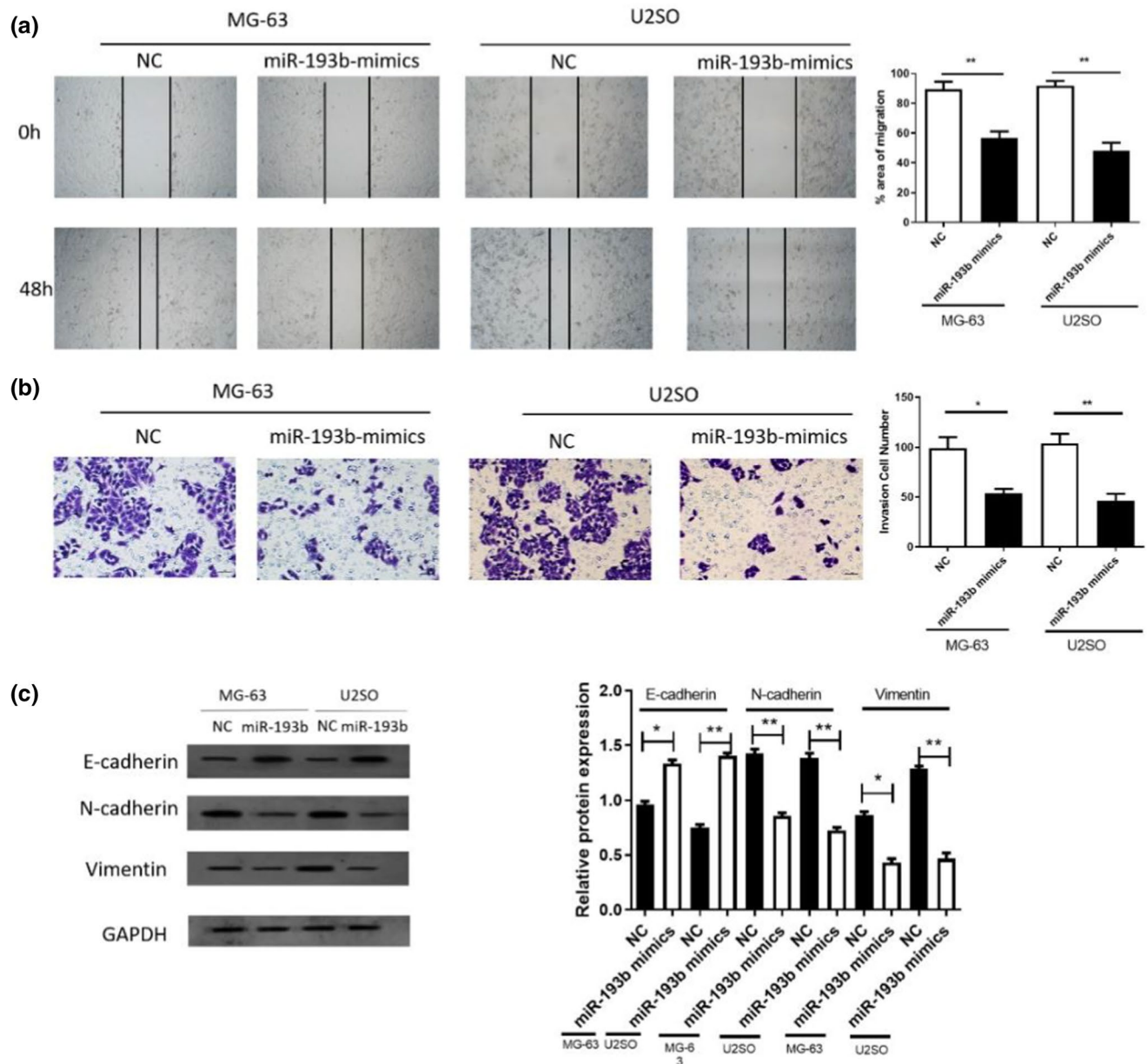


Fig. 3 Upregulated miR-193b decreased the migration and invasion of OS cell and EMT-related protein. **a**, **b** miR-193b markedly inhibited cell migration and invasion in MG63 and U2SO cells. **c** Western

blot assay that the protein of E-cadherin, N-cadherin and vimentin in MG-63 and U2SO cells. Data are presented as the mean values \pm SD. * $P < 0.05$ vs control; ** $P < 0.01$; *** $P < 0.001$

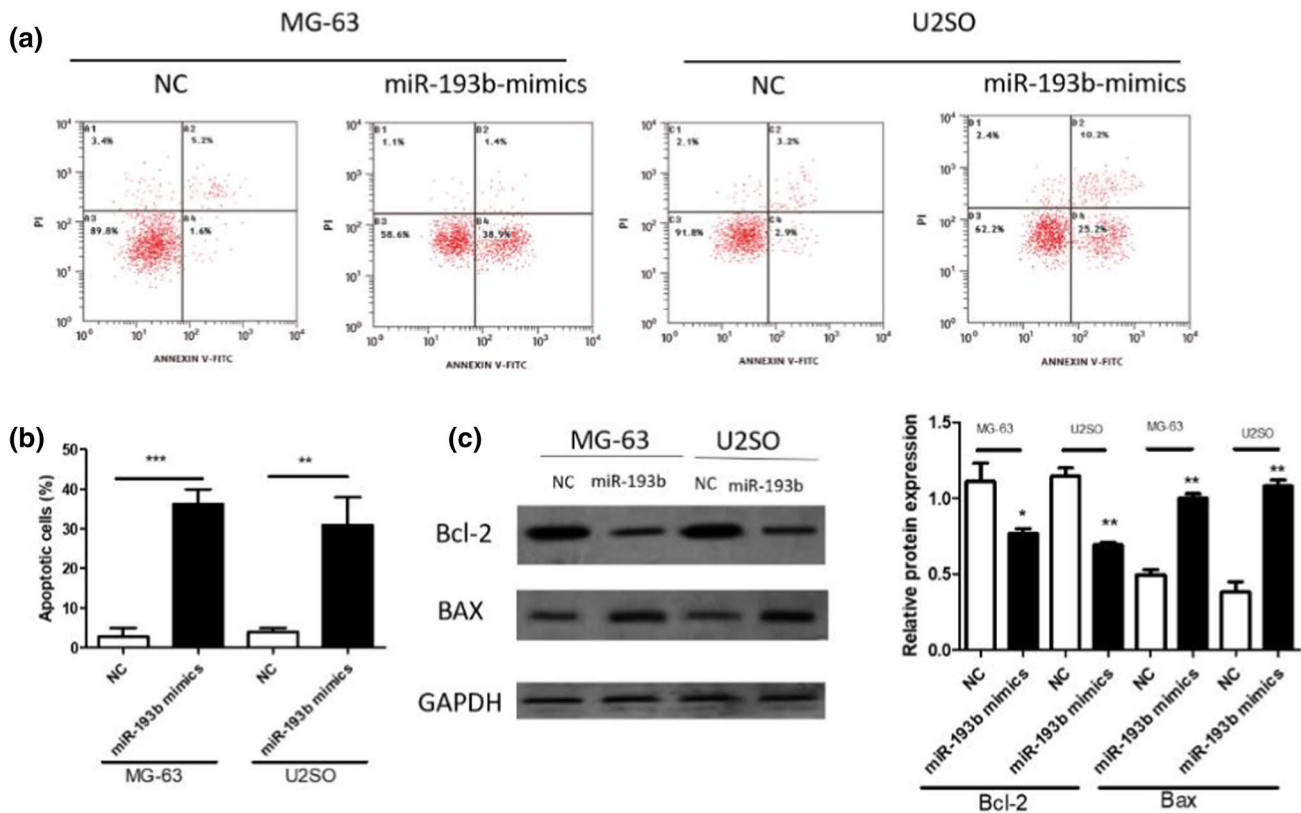


Fig. 4 Overexpression of miR-193b induced OS cell apoptosis. **a** Flow-cytometric analysis was measured to detect the apoptosis of miR-193b in U2SO and HOS cells. **b** Overexpression of miR-193b significantly upregulated the protein levels of Bax and downregulated

the protein levels of Bcl-2 using western blotting assay in MG-63 and U2SO cell lines. Data are presented as the mean values \pm SD. * $P < 0.05$ vs control; ** $P < 0.01$; *** $P < 0.001$

results indicated that miR-193b induced cell apoptosis in osteosarcoma cells, which may be related to the inhibition of Bcl-2 protein expression.

RAB7A is a target gene of miR-193b in OS cell lines

The potential target genes of miR-193b were found by TargetScan. RAB7A was one of the candidate targets (Fig. 5a). Dual-luciferase reporter assays showed that luciferase activity was reduced in the miR-193b mimics and pGL3-RAB7A-3'UTR-Wt group compared with NC group in MG-63 and U2SO cell lines (Fig. 5b). Western blotting assay and RT-PCR assay revealed that overexpression of miR-193b significantly inhibited the protein and mRNA level of RAB7A in MG-63 and U2SO cell lines (Fig. 5c,d). MiR-193b negatively regulated the RAB7A in OS cell line. These results suggested that miR-193b enhanced proliferation and migration and inhibits apoptosis through targeting RAB7A in osteosarcoma cell.

RAB7A participates in the regulation of miR-193b on the biological function of OS cells

Because RAB7A is a target gene of miR-193b in OS cells, we further explored whether RAB7A participates in the carcinogenic effects of miR-193b on OS cells. OS cells were co-transfected with miR-193b mimics or miR-NC and RAB7A or NC. As shown in Fig. 6a, the protein levels of RAB7A in miR-NC and miR-193b mimics + RAB7A group were higher than those in the miR-193b mimics + RAB7A group, indicating the successful transfection of overexpressed RAB7A. In terms of cell function experiments, the results revealed that overexpression of RAB7A promoted proliferation (Fig. 6b) and cell migration (Fig. 6c,d). Therefore, the results also demonstrated that overexpression of RAB7A partly reversed the suppressive effects on cell proliferation and migration induced by miR-193b mimics.

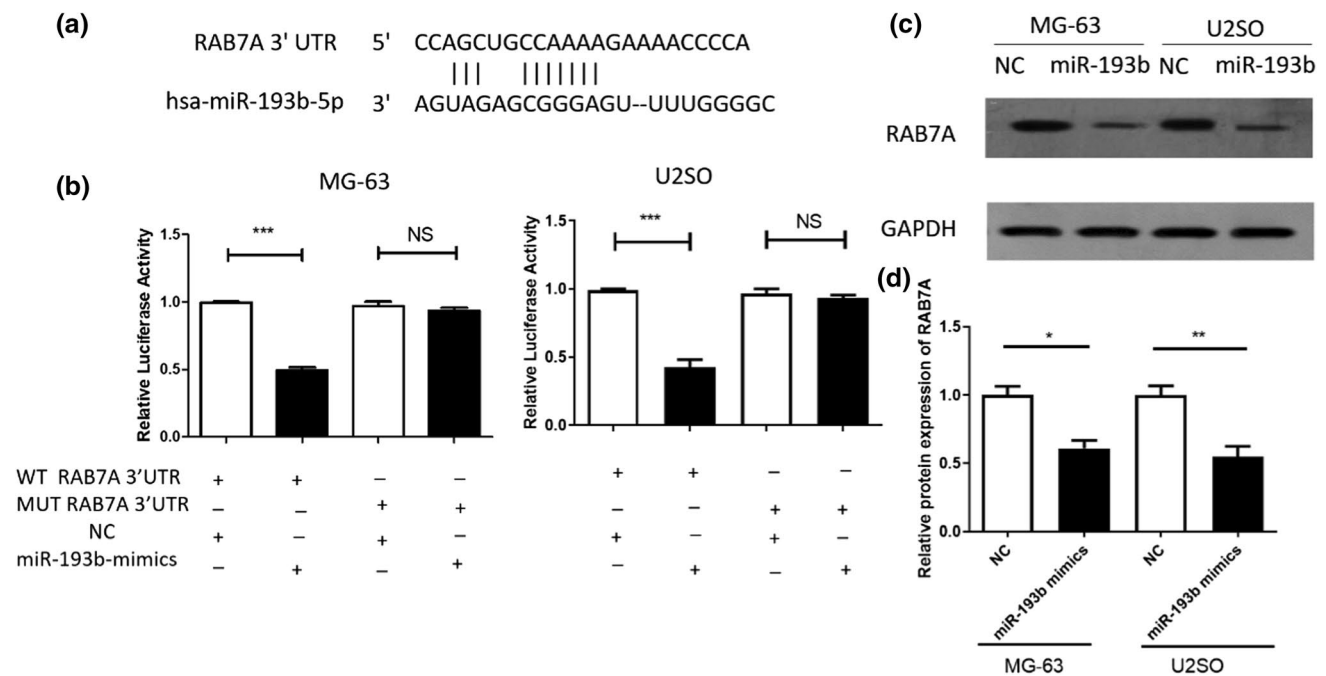


Fig. 5 RAB7A is a target gene of miR-193b in OS cell lines. **a** RAB7A was one of the candidate targets. **b** Dual-luciferase reporter assays was used to measure the luciferase activity. **c, d** Western blotting assay revealed that overexpression of miR-193b signifi-

cantly decreased the protein and mRNA level of RAB7A in MG-63 and U2SO cell lines. Data are presented as the mean values \pm SD. * $P < 0.05$ vs control; ** $P < 0.01$; *** $P < 0.001$

Discussion

An raising number of studies found that the aberrant miRNAs may be closely related to the progression and initiation of OS (Li et al. 2019; Chen et al. 2019b). The dysregulated of miRNAs contributed to the tumorigenic processes of OS, including cell migration, invasion, proliferation, and apoptosis of OS cell (Liu and Cui 2019). Hence, further research into the miRNAs is necessary to find candidate targets for the treatment of patients with OS. MiR-193b has been widely explored as a tumor suppressor in different cancers such as in colon cancer, prostate cancer, breast cancer, renal cell carcinoma, lung cancer, and hepatocellular carcinoma (Fang et al. 2019; Chen et al. 2019a; Choi et al. 2019; Hu et al. 2020). MiR-193b was downregulated in cancer (Mazzu et al. 2019). Therefore, miR-193b is a potential independent prognostic factor in glioma (Zhu et al. 2019). In the present study, we shown miR-193b as a novel biomarker for OS. The expression of miR-193b was notably down-regulated in OS cell lines.

MiR-193b performs tumor-suppressive functions in the cancer progression. Recently, the study has found that miR-193b is a typical multi-function miRNA that is mediated by its downstream genes. MiR-193b is associated with various pathological and physiological processes, including immunodeficiency, inflammation and the development and occurrence of tumors (Mullany et al. 2015). For

instance, upregulated miR-193b significantly reduced the progression of colon cancer cell cycle and its migration ability by the RAB22A-Ras signaling pathway (Fang et al. 2019). MiR-193b inhibited cell migration by targeting dimethylarginine dimethyl-laminohydrolase in breast cancer-1 (Hulin et al. 2017). Nevertheless, the roles of miR-193b are still unclear in OS cell lines. Herein, the functional experiments shown that overexpression of miR-193b reduced the proliferation, migration, and invasion of OS cell and induced cell apoptosis in MG-63 and U2SO cell. Further, miR-193b also reduced the mRNA of CCND1 and CCNE1 in MG-63 and U2SO cell lines. Our results indicated that miR-193 reduced the proliferation of OS cells via downregulated the expression of CCNA2 and CCNE1. Finally, the candidate target gene RAB7A was negatively regulated by miR-193b.

Epithelial to mesenchymal transition (EMT) is a major process to regulate cell invasion and migration in cancer (Dong et al. 2019; Cordani et al. 2019). EMT typical characteristic is the molecular changes of the epithelial cancer cells, which promote the acquisition of mesenchymal qualities and the epithelial feature loss. Additionally, EMT other characteristics contain inhibited E-cadherin and increased the levels of N-cadherin and vimentin (Wu et al. 2016). Our findings indicated that miR-193b decreased the migration and invasion of OS cells, and regulated the EMT related proteins. This study shown the profound involvement of

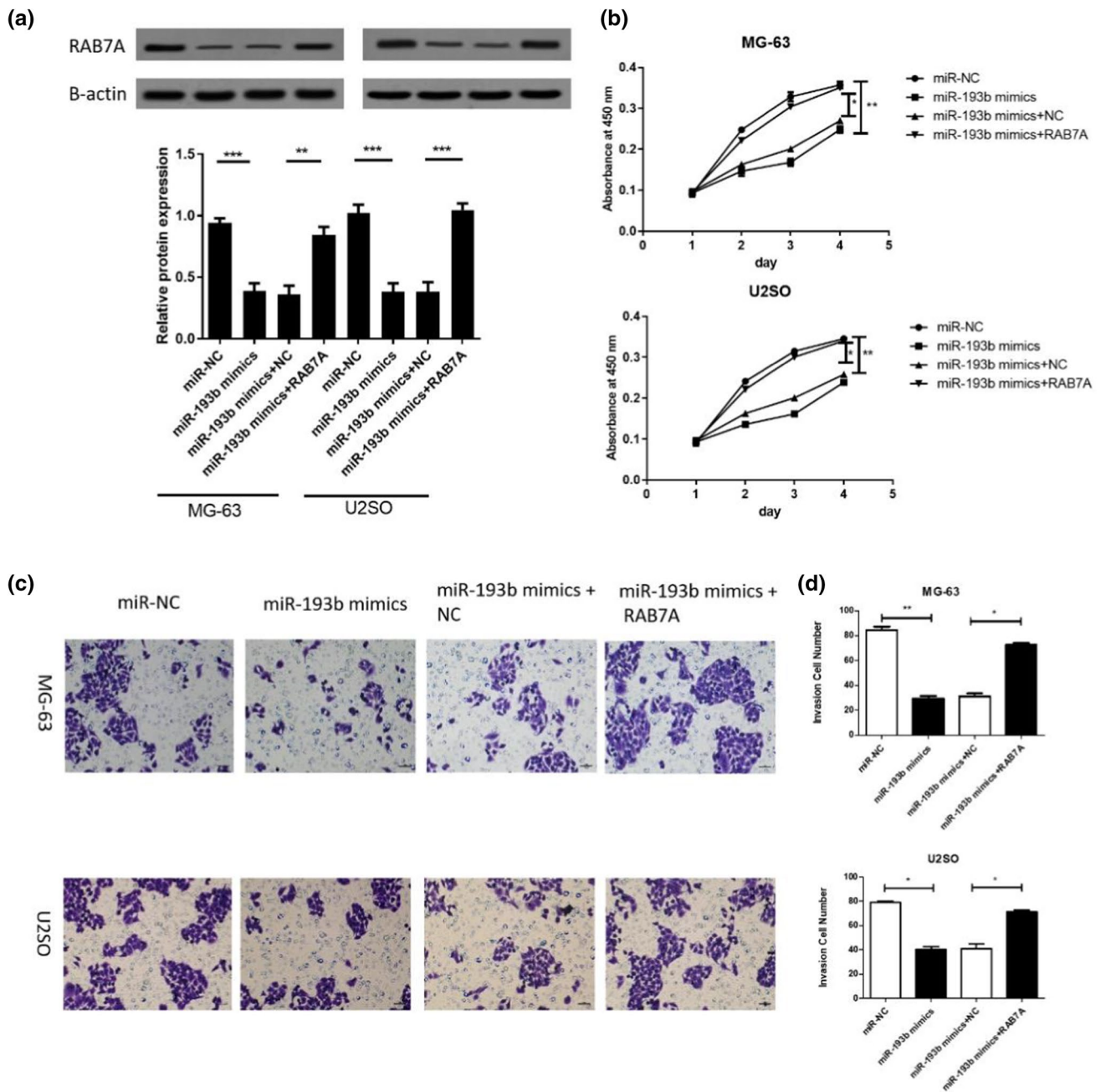


Fig. 6 Overexpression of RAB7A partly rescues the inhibitory effects on OS cells induced by miR-193b mimics. MG-63 and U2SO cells were transfected with miR-193b mimics or miR-NC and RAB7A and NC. **a** The protein level of RAB7A in MG-63 and U2SO cells was evaluated by qRT-PCR after 48 h of transfection. **b** The proliferation

of MG-63 and U2SO cells was identified by CCK-8 assay. **c, d** The migration of MG-63 and U2SO cells was analyzed by Transwell assay. Data from three independent experiments are expressed as the mean \pm SD. * $P < 0.05$ vs control; ** $P < 0.01$; *** $P < 0.001$

miR-193b in OS and suggests that miR-193b might be a potential target for treating patients with OS.

MiRNAs play a key part in the tumor progression and tumorigenesis by directly regulating the their target genes expression (Harries 2012). Accordingly, we further attempted to find the direct target gene of miR-193b in OS cells. Recently, some studies have found the role of Rab7a

in cancer (Heo et al. 2018; Guerra et al. 2019). Knockdown of RAB7A inhibits cell proliferation and migration in breast cancer cells (Xie et al. 2019). Bioinformatics analysis was performed to predict the target of miR-193b. The 3'-UTR of RAB7A was found to the binding site for of miR-193b. Then, the luciferase activity assay was used to detect the targeting of miR-193b to the 3'-UTR of RAB7A mRNA.

Furthermore, overexpression of miR-193b decreased the mRNA and protein expression of RAB7A in OS cell lines. These results provided meanfully evidence to designate RAB7A as a direct target gene of miR-193b in OS cells. Further, the results also demonstrated that overexpression of RAB7A partly reversed the suppressive effects on cell proliferation and migration induced by miR-193b mimics.

In conclusions, we found that miR-193b functions as tumor suppressor during the OS progression by directly targeting RAB7A. Hence, this study indicates function evidence fully supporting the hypothesis that miR-193b is a promising target for treatment OS.

Author contributions YYZ and HXY designed experiments; YYZ and JJD carried out experiments and wrote the manuscript; HXY and JJD analyzed experimental results.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval We confirmed that all methods in our study were performed in accordance with the relevant guidelines of CONSORT 2010.

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