miR-142 acts as a tumor suppressor in osteosarcoma cell lines by targeting Rac1

ZHONGHUI ZHENG, MULIANG DING, JIANGDONG NI, DEYE SONG, JUN HUANG and JUNJIE WANG

Department of Orthopaedics, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, P.R. China

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Abstract. Although the 5-year survival rate of osteosarcoma (OS) has risen to ~60-70%, a substantial portion of patients still respond poorly to chemotherapy and have a high risk of relapse or metastasis even after curative resection. In this study, we found that the expression of miR-142 was significantly reduced in OS tissues and OS cell lines, while Ras-related C3 botulinum toxin substrate 1 (Rac1) expression was increased in the OS tissues and OS cell lines compared with expression in the controls. We then demonstrated that miR-142 regulated Rac1 expression at the transcriptional and translational levels by directly targeting its 3'-untranslated region (3'UTR). In addition, by loss- and gain-of function experiments, we investigated the role of miR-142 in OS cell lines and found that miR-142 acted as a tumor suppressor in the OS cell lines and inhibited cell proliferation and cell invasion and arrested cell cycle in the S phase. Furthermore, miR-142 inhibited osteosarcoma cell invasion by inducing E-cadherin expression and reducing expression of matrix metalloproteinase 2 (MMP2) and MMP9. Thus, overexpression of miR-142 and/or knockdown of Rac1 would be a novel target for OS therapy in the future.

Introduction

Osteosarcoma (OS), the most common malignant tumor of the bone, occurs mainly in adolescents and young adults with a morbidity of ~5 cases per million (1). It originates from common mesenchymal stem cell (MSC) progenitors that undergo disruption to normal osteoblast differentiation (2). Although the 5-year survival rate has risen to ~60-70%, a substantial percentage of patients still respond poorly to chemotherapy and have a high risk of relapse or metastasis even after curative resection (3).

microRNAs (miRNAs), a class of short (~22 nt) endogenous non-coding RNAs, post-transcriptionally regulate the expression of target genes involved in many types of cancers including osteosarcoma (4) and act as oncogenes or tumor-suppressor genes. A single miRNA can silence a large number of genes, allowing these molecules extensive control of many cellular functions (5). Emerging evidence of individual miRNAs affecting developmental biology, cellular differentiation programs and oncogenesis continues to grow (6).

In osteosarcoma, expression levels of miRNAs as tumor suppressors, such as miR-34 and miR-142, were recently demonstrated to be downregulated. It was suggested that miR-142 may play an important role in maintaining the self-renewal capacity of bronchioalveolar stem cells (7). It was also found that esophageal squamous cell carcinoma patients with high expression of miR-142 had poorer survival rates than those with low expression of miR-142, suggesting that miR-142 may act as a tumor suppressor (8). miR-142 was found to be downregulated in osteosarcoma cell lines (9), however, the role of miR-142 in osteosarcoma remains unknown.

Ras-related C3 botulinum toxin substrate 1 (Rac1) is a member of the Ras homologue (Rho) family that plays a vital role in multiple cell functions, including cell migration, invasion and cell cycle arrest (10). Rac1 has been found to be upregulated in many cancers, including glioma (11), breast cancer (12) and skin tumors (13). Rac1 as a novel target of miR-142 has been demonstrated in hepatocellular carcinoma cells (14). However, the underlying mechanism of miR-142 and Rac1 in osteosarcoma remains to be elucidated.

In the present study, we found that the expression of miR-142 was significantly reduced in OS tissues and OS cell lines, while Rac1 expression was increased in OS tissues and OS cell lines compared with expression levels in the controls. We then demonstrated that miR-142 regulated Rac1 expression at the transcriptional and translational levels by directly targeting its 3'-untranslated region (3'UTR). In addition, by loss- and gain-of-function experiments, we investigated the role of miR-142 in OS cell lines, and found that miR-142 acted as a tumor suppressor in the OS cell lines, inhibiting cell proliferation and cell invasion and arresting the cell cycle in the S phase. Furthermore, miR-142 inhibited osteosarcoma cell invasion by inducing E-cadherin expression and reducing expression of matrix metalloproteinase 2 (MMP2) and MMP9. Thus, overexpression of miR-142 and/or knockdown of Rac1 may be a novel target for OS therapy.

Correspondence to: Dr Muliang Ding, Department of Orthopaedics, The Second Xiangya Hospital, Central South University, No. 139 Middle Renmin Road, Changsha, Hunan 410011, P.R. China

E-mail: dmldoc@gmail.com

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Materials and methods

Cell culture and cancer tissue collection. hFOB1.19, Saos-2, MG63, U2OS and HOS cells were obtained from the American Type Culture Collection (ATCC). All the cells were cultivated in RPMI-1640 (Gibco) basic medium supplemented with 10% FBS. All of the cells were cultured in conditions of 95% air and 5% carbon dioxide (CO₂) at 37°C. A total of 6 OS tissue and 3 adjacent tissue samples were obtained from The Second Xiangya Hospital of Central South University according to the legislation and the Ethics Board of The Second Xiangya Hospital. Informed consent was acquired from all subjects or from their caregivers. All samples were collected and classified according to the World Health Organization (WHO) criteria. All of the samples were stored at -80°C until used.

Cell treatment. To investigate the role of miR-142 in OS cells, ectopic expression of miR-142 was achieved by transfecting Lv-pre-miR-142 or Lv-anti-miR-142 using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Rac1 siRNA oligonucleotides were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Rac1 siRNA and antisense, 5'-AAGGGACAGGACCAAGAACGA-3'; used: Rac1 sense, 5'-GAGAAACTGAAGGAGAAGAAG-3'. The group design consisted of a blank control group (con), an empty vector group (vector) and transfection groups (si-Rac1).

Quantitative polymerase chain reaction (qPCR) analysis

Real-time PCR for miRNA. Total RNA was extracted from the indicated cells using TRIZol reagent (Invitrogen) following the manufacturer's instructions. The specific primers for miRNA-142 and U6 were purchased from GeneCopoeia. The relative expression of miR-142 was measured using the miScript SYBR®-Green PCR kit (Qiagen). Expression of U6 was used as an endogenous control. Data were processed using the 2-ΔΔCT method.

Real-time PCR for mRNA. Total RNA (0.5 µg) was reverse transcribed using the RevertAid First Strand cDNA synthesis kit (Fermentas). The following PCR amplification was carried out in a Thermal Cycler Dice Real-Time System using SYBR-Green qPCR Mix (Toyobo). For each sample, the relative mRNA level was normalized to β-actin. The following primer pairs were used: Rac1 sense, 5'-GAGAAACTGAAGGAGAAGAAG-3' and antisense, 5'-AAGGGACAGGACCAAGAACGA-3'; β-actin sense, 5'-AGGGGCGGACTCGTACATACT3' and antisense, 5'-GCGGCCACACCATGTACCT3'.

Western blotting. Total protein was extracted from the indicated cells using RIPA buffer (Auragene, Changsha, China). Protein concentration was determined with the BCA protein assay kit (Beyotime, Hangzhou, China). Fifty micrograms of proteins mixed in loading buffer was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Corning Inc., Corning, NY, USA). After incubation with the appropriate primary antibody overnight at 4°C, the membrane was washed and the antigen-antibody complex was incubated using a horseradish peroxidase-conjugated secondary anti-body. The signal was visualized by the Chemi-Lumi One system (Nacalai Tesque, Kyoto, Japan). Data were analyzed by densitometry using Image-Pro Plus software 6.0 and normalized to β-actin expression. Antibodies were obtained from the following sources: mouse monoclonal anti-Rac1 antibody from Cell Signaling Technology, rabbit polyclonal anti-E-cadherin, anti-MMP2 and anti-MMP9 antibodies from Immunoway (Newark, DE, USA); mouse monoclonal anti-β-actin antibody from Boster (Wuhan, China); and horseradish peroxidase-conjugated secondary anti-rabbit/mouse IgG from Cell Signaling Technology.

Dual luciferase reporter assay. A wild-type 3'UTR of Rac1 (wt-Rac1) or a mutant 3'UTR of Rac1 (mut-Rac1) was constructed into the dual luciferase reporter vector. For luciferase assay, 10⁵ cells were seeded in 6-well plates for 12 h. Then, the cells were co-transfected with wt-Rac1 or mut-Rac1 dual luciferase reporter vector and miR-142 mimic (pre-miR-142), or miR-142 inhibitor (anti-miR-142), respectively. Following a 5-h incubation with transfection reagent, the medium was refreshed with fresh complete medium. After transfection for 48 h, the luciferase activities in each group were measured by using the dual luciferase reporter gene assay kit and detected on an LD400 luminometer (both from Promega). Renilla luciferase activity was normalized to firefly luciferase activity.

CCK-8 cell proliferation assay. CCK-8 was used to evaluate cell proliferation. Cells (5x10³) were seeded in each 96-well plate for 24 h, treated with the indicated drugs, and further incubated for 0, 24, 48 and 72 h, respectively. One hour before the end of the incubation, 10 µl CCK-8 reagents was added to each well. Optical density (OD) 570 nm value in each well was determined by an enzyme immunooassay analyzer.

Transwell assay. The cells treated with the indicated drugs for 72 h were starved in serum-free medium for 24 h and were then resuspended in serum-free medium. The cells were added to the upper chamber, while the lower chamber was filled with base medium containing 10% FBS. After incubation for 24 h, the cells that attached to the bottom were fixed and stained with crystal violet for 20 min. The redundant crystal violet was washed by 0.1 M PBS, and dried in air. The OD of the crystal violet dissolved by 10% acetic acid at 570 nm was detected by an enzyme immunooassay analyzer.

Flow cytometric analysis of the cell cycle. Cells were treated with the indicated drugs for 72 h. After trypsinization and washing with ice-cold PBS, the cell suspensions were stained using BD Cyclest™ Plus (BD Biosciences) according to the manufacturer's instructions, and then the cell cycle was analyzed by flow cytometry (Beckman Coulter, Fullerton, CA, USA). The experiments were performed in triplicate.

Statistical analysis. A t-test or one-way ANOVA were used to analyze the statistical data by GraphPad Prism 5 software, depending on the experimental conditions. All data are presented as mean ± SD. Compared with the respective controls, P-values of <0.05 were considered to indicate statistically significant differences.
Results

miR-142 is downregulated in OS tissues and OS cell lines, while Rac1 is upregulated in OS tissues and OS cell lines. The average expression level of miR-142 was significantly downregulated (P<0.001) in the OS tissue samples from the 6 OS patients compared with the 3 normal controls, as indicated by qPCR (Fig. 1A). Similar results were observed in the OS cell lines, particularly in the Saos-2 and MG63 cells (Fig. 1B). In addition, we also detected the expression of Rac1 in the OS tissue samples and OS cell lines. As shown in Fig. 1C and D, we found that Rac1 was upregulated in the OS tissues and OS cell lines, which was inversely correlated with the expression of miR-142.

miR-142 regulates Rac1 expression at the transcriptional and translational levels by directly targeting its 3’UTR. Considering the inverse correlation between miR-142 and Rac1, we then aimed to ascertain whether the 3’UTR of Rac1 contains a direct target site for miR-142. A dual luciferase reporter assay was performed using a vector encoding the wild-type (Wt) and mutant (Mut) 3’UTR of Rac1 mRNA. We found that the luminescence activity was significantly suppressed in the miR-142 transfectants compared to the negative control transfectant. Moreover, miR-142-mediated repression of luciferase activity was abolished by the mutant type 3’UTR of Rac1 (Fig. 2A and B). Furthermore, Rac1 mRNA and protein expression levels were markedly downregulated by pre-miR-142 transfection, compared with the control in the Saos-2 and MG63 cell lines (Fig. 2C-F). These results determined that miR-142 directly targets Rac1 and regulates its expression at the transcriptional and translational levels.

Effects of miR-142 on cell proliferation and the cell cycle in the OS cell lines. CCK-8 was used to detect the Saos-2 and MG63 cell proliferation affected by miR-142. It was found that overexpression of miR-142 induced inhibition of proliferation,
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A miR-142 regulates Rac1 expression at the transcriptional and translational levels by directly targeting its 3’UTR. (A and B) miR-142 mimic (pre-miR-142) or the miR-142 inhibitor (anti-miR-142) was co-transfected with wt-3’UTR of Rac1 (wt-Rac1) or mutant-3’UTR of Rac1 (mut-Rac1) into the Saos-2 or MG63 cells, respectively. Compared to the negative control, the luciferase activity of wt-Rac1 was significantly decreased by pre-miR-142, which was abrogated by mut-Rac1. The luciferase activity had no significant changes in the Saos-2 or MG63 cells treated with anti-miR-142. (C) After pre-miR-142 or anti-miR-142 transfection, qPCR was used to detect the expression of miR-142 in the Saos-2 and MG63 cells. (D) After pre-miR-142 or anti-miR-142 transfection, qPCR was used to detect the mRNA expression of Rac1 in the Saos-2 and MG63 cells. (E and F) After pre-miR-142 or anti-miR-142 transfection, western blotting was used to detect the expression of Rac1 protein in the Saos-2 cells and MG63 cells. Data are presented as means ± SD. *P<0.05 and **P<0.01 vs. the control. Rac1, Ras-related C3 botulinum toxin substrate 1.

while downregulation of miR-142 promoted cell proliferation in the Saos-2 and MG63 cell lines (Fig. 3A and B). Flow cytometric analysis was used to analyze cell cycle alterations after pre-miR-142 or anti-miR-142 treatment. Upregulation of
miR-142 induced cell cycle arrest at the S phase and decreased the percentage of cells in the S phase in the Saos-2 and MG63 cell lines. Treatment of Saos-2 and MG63 cells with anti-miR-142 significantly increased the percentage of cells in the S phase compared with the percentage in the control group (Fig. 3C and D).

Overexpression of miR-142 suppresses cell invasion and regulates invasion-related genes. A Transwell assay was used to measure the invasive ability of the Saos-2 and MG63 cells following pre-miR-142 or anti-miR-142 transfection. The results showed that overexpression of miR-142 significantly decreased the invasive ability, while downregulation of miR-142 induced the invasive ability of the Saos-2 and MG63 cells (Fig. 4A and B). In addition, to explore the potential downstream molecular pathway underlying miR-142 targeting to Rac1, we tested the expression of invasion-related genes including E-cadherin, MMP2 and MMP9 by western blotting in the Saos-2 and MG63 cells after pre-miR-142 or anti-miR-142 transfection. We observed a significant decrease in expression of MMP2 and MMP9 proteins and a significant increase in expression of E-cadherin protein in the cells treated with pre-miR-142. Inversely, downregulation of miR-142 induced the expression of MMP2 and MMP9 proteins and significantly decreased the expression of E-cadherin in the Saos-2 and MG63 cells compared with that of control (Fig. 4C and D).
Knockdown of Rac1 suppresses cell proliferation and invasion, and induces cell cycle arrest at the S phases. To investigate the role of Rac1 in OS cells, a loss-of-function experiment was performed. As shown in Fig. 5A and B, siRac1 treatment significantly decreased the expression of Rac1 in the Saos-2 and MG63 cells, indicating that the efficiency was satisfied for further analysis. CCK-8 was used to detect the Saos-2 and MG63 cell proliferation affected by Rac1. It was found...
Figure 5. Knockdown of Rac1 regulates cell proliferation, invasion and cell cycle. (A and B) After si-Rac1 transfection, western blotting was used to detect the expression of Rac1 in Saos-2 cells and MG63 cells. (C and D) After si-Rac1 transfection, CCK-8 assay was used to measured cell proliferation of Saos-2 and MG63 cells. (E and F) After si-Rac1 transfection, flow cytometry was used to analyze cell cycle alterations in the Saos-2 and MG63 cells. (G and H) After si-Rac1 transfection, a Transwell assay was used to measure the cell invasive ability of the Saos-2 and MG63 cells. Data are presented as means ± SD. *P<0.05, **P<0.01 and ***P<0.001 vs. the control. Rac1, Ras-related C3 botulinum toxin substrate 1.
that knockdown of Rac1 promoted cell proliferation in the Saos-2 and MG63 cell lines (Fig. 5C and D). Flow cytometric analysis was used to analyze cell cycle alterations following knockdown of Rac1. Knockdown of Rac1 induced cell cycle arrest at the S phase and markedly decreased the percentage of cells in the S phase in the Saos-2 and MG63 cell lines compared with the control group (Fig. 5E and F). Transwell assay was used to measure the invasive ability of the Saos-2 and MG63 cells after knockdown of Rac1. The results showed that knockdown of Rac1 significantly decreased the invasive ability (Fig. 5G and H).

Discussion

miRNAs have been implicated in cancer growth and metastasis (15). Moreover, miRNAs function as tumor suppressors or oncogenes by targeting oncopgenes or suppressor genes (16). In this study, we found that miR-142 expression was downregulated in the osteosarcoma tissues and cell lines compared with expression in the paired normal bone tissues and osteoblastic hFOB1.19 cell line, whereas upregulated Rac1 expression was observed in the osteosarcoma tissues and cell lines compared with expression in the paired normal bone tissues and osteoblastic cell lines. In addition, we identified Rac1 as a direct target of miR-142, which regulates Rac1 expression at the transcriptional and translational levels. Furthermore, we also found that overexpression of miR-142 suppressed osteosarcoma cell proliferation and invasion and arrested the cell cycle in the S phase in the osteosarcoma Saos-2 and MG-63 cells. Moreover, our findings showed that the ability of miR-142 to inhibit osteosarcoma cell invasion involved E-cadherin, MMP2 and MMP9 expression. Similarly, knockdown of Rac1 by si-Rac1 transfection suppressed the ability of cell growth and invasion, and induced cell cycle arrest in the S phase. Taken together, our findings suggest that miR-142 plays a fundamental role in tumorigenesis and cancer cell invasion by targeting Rac1.

miR-142 has been found to be downregulated in several types of cancers, including hepatic cancer (17), squamous cell lung cancer (18) and human acute lymphoblastic leukemia (19), and acts as a tumor suppressor. It was reported that lower miR-142 expression in hepatocellular carcinoma was significantly associated with poor survival (17). Rac1 expression is frequently upregulated in several types of cancers, including hepatic cancer (14) and breast cancer (20). In line with previous studies, in the present study, we also found that miR-142 was downregulated, while Rac1 was upregulated in 6 osteosarcoma tissue samples compared with the paired normal bone tissues. We also found similar results in the osteosarcoma Saos-2 and MG63 cells.

To further verify the role of miR-142 in the development of osteosarcoma, loss- and gain-of-function experiments were performed. Upregulation of miR-142 significantly inhibited cell proliferation and invasion and arrested the cell cycle in the S phase in the osteosarcoma cell lines, indicating that upregulation of miR-142 may inhibit tumor progression in osteosarcoma carcinogenesis. Meanwhile, downregulation of miR-142 significantly induced cell proliferation and invasion and increased the percentage of cells in the S phase in the osteosarcoma cell lines, indicating that downregulation of miR-142 may promote tumor progression in osteosarcoma carcinogenesis. These results suggest that miR-142 acts as a tumor-suppressor whose downregulation may contribute to the progression and metastasis of osteosarcoma.

Rac1 as a target of miR-142 has been demonstrated in diffuse large B-cell lymphoma cells (21) and hepatocellular carcinoma cell lines (14). Consistent with previous studies, we also identified Rac1 as a direct target of miR-142 in the osteosarcoma Saos-2 and MG63 cells. miR-142 overexpression suppressed Rac1 3'UTR luciferase report activity, which was abolished by mutation of the Rac1 3'UTR binding site. Overexpression of miR-142 induced a significant decrease in Rac1 at both the mRNA and protein levels. These results indicate that miR-142 may function as a tumor suppressor, at least partly, mediated by suppressing Rac1 expression in osteosarcoma cells.

Rac1 activation is correlated with metastatic progression in many types of cancers such as medulloblastoma (22) and breast cancer (20). Increasing evidence reveals that Rac1-dependent cell signaling activation can promote cell adhesion, invasion and metastasis in a variety of cancers, including osteosarcoma (23), suggesting that Rac1 plays an important role in cancer invasion (24). In the present study, we confirmed that Rac1 is upregulated in osteosarcoma. Furthermore, our data showed that knockdown of Rac1 by si-Rac1 suppressed cell proliferation and invasion, and induced cell cycle arrest at the S phase. In addition, we found that the ability of miR-142 to act as a tumor suppressor not only suppressed Rac1 expression, but also induced E-cadherin expression and decreased MMP2 and MMP9 expression. E-cadherin is a member of the type I classical cadherin family. Studies, including those in osteosarcoma, have demonstrated that E-cadherin plays a tumor-suppressor role (25). E-cadherin is frequently downregulated during carcinoma metastasis (26). Loss of E-cadherin facilitates the initial invasive behavior of cancer (27). MMPs promote cell invasion and migration in several types of cancers, such as osteosarcoma, prostate, lung, colon and pancreas cancer (28). Compared with normal tissues, a higher expression of MMPs is often observed in malignant tumor tissues (29). Taken together, our study demonstrated that the ability of miR-142 to inhibit osteosarcoma cell invasion was achieved by inducing E-cadherin expression and reducing expression of MMP2 and MMP9.

In conclusion, the present study provides novel evidence that miR-142 functions as a tumor-suppressor miRNA in osteosarcoma via targeting the 3'UTR of Rac1. Furthermore, miR-142 inhibits osteosarcoma cell invasion by inducing E-cadherin expression and reducing expression of MMP2 and MMP9. Our findings revealed that upregulation of miR-142 could be a potential target for the treatment of osteosarcoma in the future.

References


