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miR-200bc/429 Inhibits Osteosarcoma Cell Proliferation and Invasion by Targeting PMP22

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Background: MicroRNAs (miRNAs) are small non-coding RNAs which play a crucial role in diverse biological processes and could contribute to cancer development and progression. MiR-200bc/429 have been found to be aberrantly expressed in osteosarcoma (OS). However, the features of miR-200bc/429 in the tumorigenesis and progress of OS remain poorly understood.

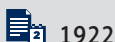
Material/Methods: The miR-200bc/429 expression was firstly identified in human OS clinical samples and cell lines by quantitative real-time PCR (qRT-PCR). After transfection with miR-200bc/429 mimics or negative control in U2OS or MG63 cells, cell proliferation was measured by CCK-8 assay. Following that, wound-healing assay and Transwell invasion assay were performed to evaluate cell migration and invasion, respectively. Finally, luciferase reporter assay and Western blot analysis were performed to determine if peripheral myelin protein-22 (PMP22) is a direct target of miR-200bc/429.

Results: Results revealed that miR-200bc/429 were significantly depressed in human OS tissues and cell lines by qRT-PCR. Then, restoration of miR-200bc/429 significantly inhibited cell proliferation ($P < 0.05$) and invasion ($P < 0.05$) *in vitro*. Luciferase reporter assay and Western blot analysis revealed that miR-200bc/429 could directly target PMP22 3' untranslated region (UTR) and inhibit its expression in U2OS and MG63 cells.

Conclusions: These findings suggest that miR-200bc/429 inhibit OS cells proliferation and invasion by targeting PMP22, and function as a tumor suppressor and may be a patent molecular marker as well as a potential target for OS therapy.

MeSH Keywords: **Cell Proliferation • MicroRNAs • Osteosarcoma**

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Background

Osteosarcoma (OS), a fatal malignant neoplasm predominantly affecting children and adolescents, is characterized by high local aggressiveness and poor therapeutic outcome [1]. Due to the introduction of neoadjuvant chemotherapy using cisplatin, doxorubicin, ifosfamide, and methotrexate, 5-year survival rate among OS patients has fallen to 60–75%. However, the 5-year survival rate among children and adolescents has reached a plateau since the mid-1980s [2,3]. Besides no substantial improvement in survival rate has been achieved in the past 20 years. Hence, there is an increasing sense of urgency to elucidate the underlying molecular mechanisms of OS.

MicroRNAs (miRNAs) are small non-coding RNAs (19–23 nucleotides) that post-transcriptionally regulate gene expression in diverse biological processes and have been found to play a crucial role in tumor initiation and progression through modulation of tumor growth, progression, metastasis, and drug resistance [4]. Increasing evidence revealed that a host of miRNAs are aberrantly expressed in OS patients [5–8]. These deregulated miRNAs might be either proto-oncogenes or anti-oncogenes, depending on their target mRNAs. Therefore, identification of novel miRNAs related with OS development should contribute to a better understanding of genetic mechanisms and new clinical methods for OS therapy in the future.

Previous studies have shown that miR-200 is a family of tumor-suppressor miRNAs which are significantly involved in inhibition of epithelial-to-mesenchymal transition (EMT), repression of cancer stem cells (CSCs) self-renewal and differentiation, modulation of cell division and apoptosis, and reversal of chemoresistance in various human cancers [9]. Recently, miR-200 has also been reported to be frequently downregulated in OS cells [10,11]. Thus, miR-200 might be a potential target for cancer therapy. However, to date, the biological function of miR-200bc/429 in OS remains largely unknown.

In this study, the miR-200bc/429 expression was first identified to be significantly downregulated in human OS clinical samples and cell lines by qRT-PCR. We found that overexpression of miR-200bc/429 in OS cell lines U2-OS and MG63 significantly inhibited cell proliferation and invasion through decreasing the expression of PMP22.

Material and Methods

Clinical tissue samples

Fresh OS tissue clinical samples were collected from routine therapeutic operations at our department. The research protocol was permitted by the Research Ethics Committee of

Tianjin Third Central Hospital. All patients gave written informed consent.

Cell culture

Human normal osteoblast hFOB1.19 cells and OS U2OS and MG63 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in the Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 100 U penicillin/ml and 100 µg streptomycin/ml at 37°C in a humidified atmosphere with 5% CO₂.

Total RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cell lines and frozen tissues using the Trizol reagent (Invitrogen, USA) and reversely transcribed to cDNA with M-MLV (Promega, USA) following standard protocols. EzOmics SYBR qPCR, miRNA qRT-PCR kit, and miR-200bc/429 primer, which were purchased from Biomics, were analyzed in a qRT-PCR detection system (ABI, USA). The miR-200bc/429 relative expression levels of each group were calculated using the 2^{-ΔΔct} method and normalized using RNU6B as endogenous reference genes.

Transfection with miR-200bc/429 mimics

MiRNA mimics for miR-200bc/429, as well as the negative control, were purchased from Biomics Biotechnology, Inc. (Nanjing, China). Transfections were performed with Lipofectamine™ 2000 Reagent (Invitrogen, CA) following the standard protocol.

Cell proliferation assay

We seeded 4×10³ U2OS or MG63 cells in 96-well plates. Overnight, the cells were treated with miR-200bc/429 mimics or the negative control. After 12, 24, 48, and 72 h incubation, 10 µl of CCK-8 was added into each well, followed by 4 h incubation. Absorbance value at 450 nm was then measured.

Wound-healing assay

After 48 h transfection, U2OS or MG63 cells monolayers were wounded with a P-200 pipette tip, and wounded monolayers were gently washed 3 times with phosphate buffer solution (PBS), and then serum-free medium was added for further incubation. Images were captured at 0 and 48 h, and the distances between 2 edges were scaled for 3 positions each time at over 3 time points.

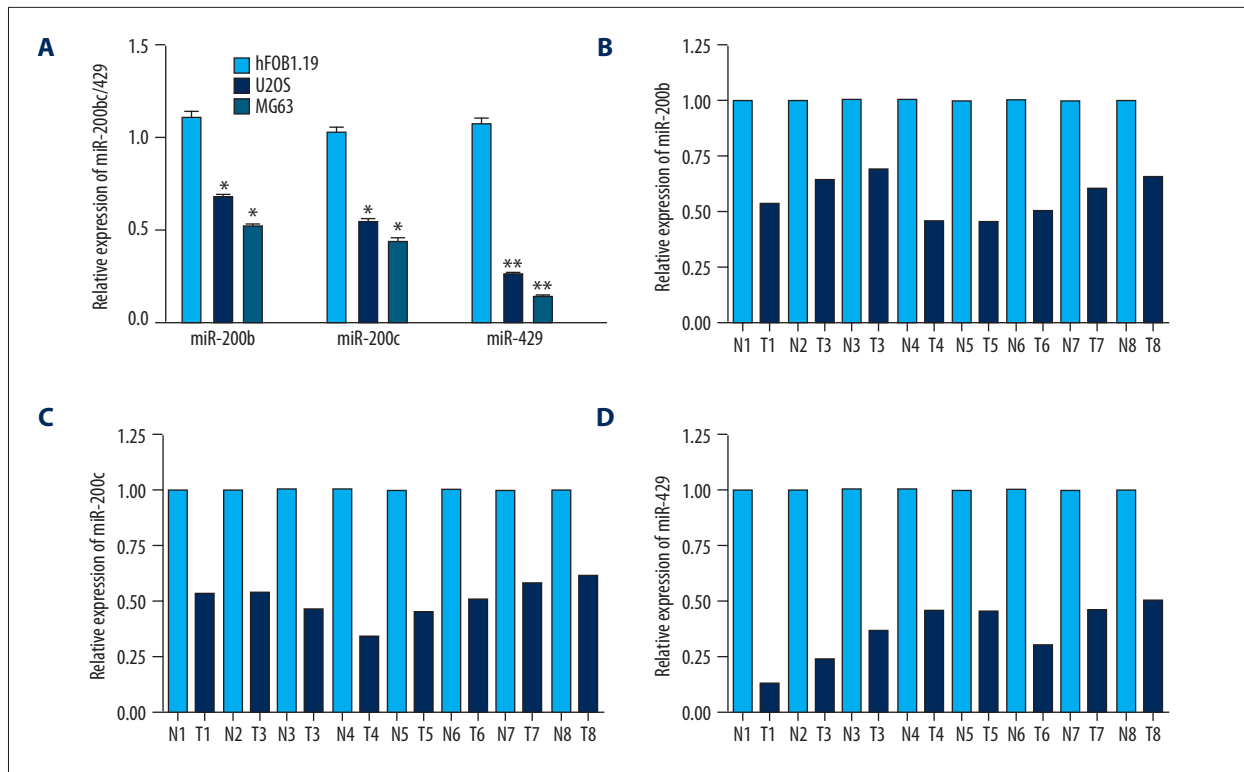


Figure 1. Quantitative qRT-PCR analysis of miR-200bc/429 expression in OS cell lines and tissues. (A) Relative expression of miR-200bc/429 in different OS cell lines and normal osteoblasts. (B) Relative expression of miR-200b in OS tissues compared with corresponding normal tissues. (C) Relative expression of miR-200c in OS tissues compared with corresponding normal tissues. (D) Relative expression of miR-429 in OS tissues compared with corresponding normal tissues. Data are expressed as means \pm SD of 3 independent experiments. * $P < 0.05$ compared with the normal osteoblastic cell line hFOB1.19 or normal tissues.

Transwell invasion assay

To assay the invasive capacity of tumor cells, Transwell migration assays were carried out by using 24-well MILLI cell Hanging Cell Culture inserts 8 mm PET (MILLIPORE) coated with Matrigel matrix gel (BD Biosciences) according to the manufacturer's protocol. After transfection for 48 h, cells suspended in serum-free medium were added into the upper chamber. After 24 h incubation, cells on the underside of the membrane were fixed with 90% alcohol and stained with 0.1% crystal violet solution. The values for invasion were obtained by counting 6 fields per membrane.

Luciferase reporter assay

The predicted binding site of PMP22 mRNA 3' UTR for miR-200bc/429 was introduced in pMiR-report Fluc vectors (Ambion). Both sense and antisense oligonucleotide templates were synthesized and cloned into pMiR-Report at HindIII and SpeI sites. All recombinant plasmids were verified by DNA sequencing. For luciferase reporter assays, the constructed luciferase reporter plasmid, β -galactosidase (β -gal) and miR-200bc/429 mimics or

the negative control were co-transfected into U2OS and MG63 cells using Lipofectamine 2000. After 48 h transfection, luciferase activity was measured using a GloMax 96 luminometer (Promega) according to the manufacturer's protocols. The luciferase activity was normalized to β -gal.

Western blot analysis

Cells were harvested 48 h after transfection with miRNA mimics and lysed in buffer RIPA. We separated 30 μ g protein of each sample by denaturing 12% SDS-PAGE and then transferred it to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA and then incubated with primary antibodies against PMP22 (Abcam, Cambridge, MA, 1:1000) or β -actin (Abcam, Cambridge, MA, 1:5000) overnight at 4°C followed by incubation with horseradish peroxidase-labeled secondary antibodies for 2 h at room temperature. β -actin was used as a control.

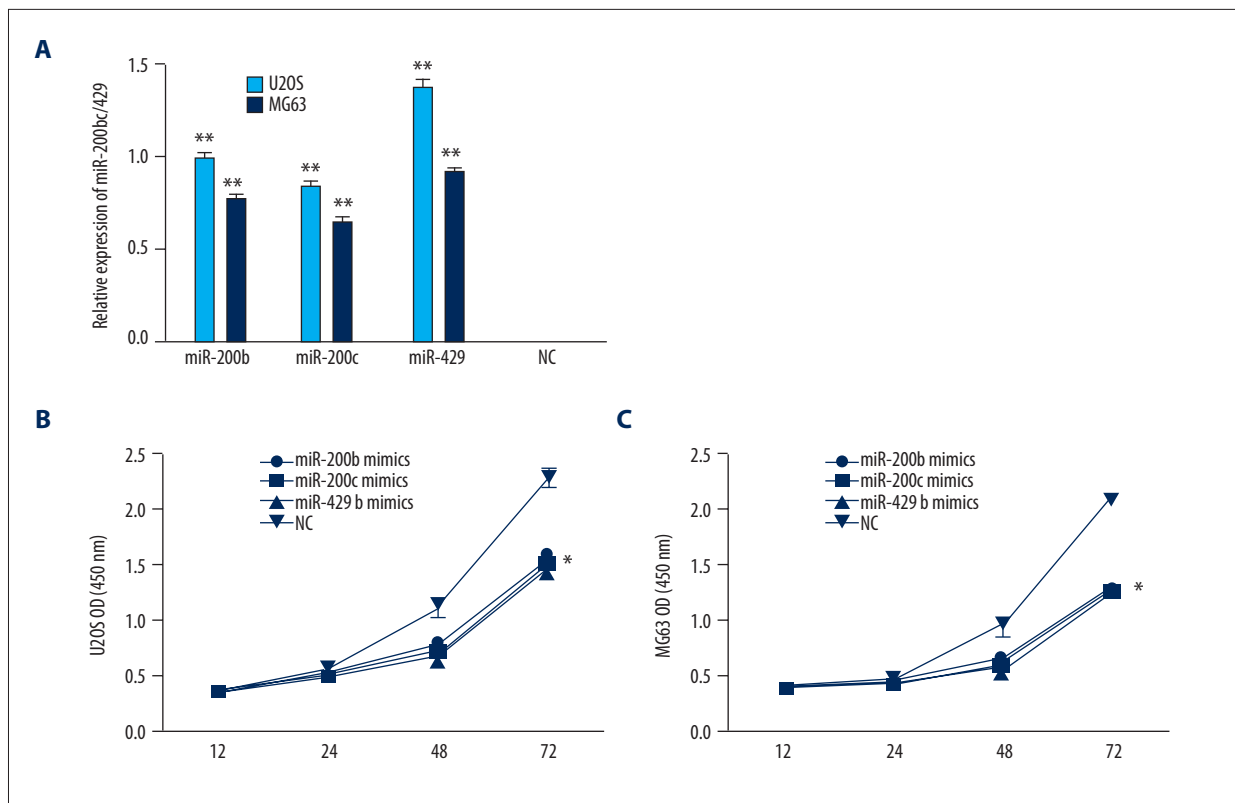


Figure 2. Restoration of miR-200bc/429 inhibits cell proliferation *in vitro*. (A) The efficiency of transfection was confirmed by qRT-PCR. (B) The cell proliferative potential (CCK-8) was determined in U2OS. (C) The cell proliferative potential (CCK-8) was determined in MG63. * $P < 0.05$, ** $P < 0.01$ between 2 groups.

Statistical analysis

The experimental results are shown as means \pm SD. For comparisons, Student's t test was performed between 2 groups. P value < 0.05 was considered significant.

Results

miR-200bc/429 is downregulated in OS cell lines and tissues

We first assessed the expression of miR-200bc/429 in OS cell lines and tissues by qRT-PCR. As shown in Figure 1A, in U2OS cell line, miR-200bc/429 expression levels were significantly downregulated 1.64 \pm 0.04, 1.92 \pm 0.21, and 4.15 \pm 0.28-fold compared with hFOB1.19, respectively; in MG63 cell line, miR-200bc/429 expression levels were significantly downregulated 2.15 \pm 0.13, 2.34 \pm 0.18, and 7.75 \pm 0.78-fold compared with hFOB1.19, respectively. Consistently, the expression of miR-200bc/429 was further confirmed in 8 pairs of human OS clinical samples (Figure 1B–1D). These data suggest the key roles of miR-200bc/429 in the tumorigenesis and tumor progression of OS.

Restoration of miR-200bc/429 inhibit cell proliferation.

To investigate the clinicopathologic significance of miR-200bc/429 in OS development, we first assessed the effect of miR-200bc/429 on OS cell growth in U2OS and MG63 cells by CCK-8 *in vitro*. As shown in Figure 2A, the efficiency of transfection was confirmed by qRT-PCR. Transfection of miR-200bc/429 mimics can significantly inhibit cell proliferation (Figure 2B, 2C). These results suggest that miR-200bc/429 may function as a tumor suppressor, at least by suppressing cell proliferation.

Restoration of miR-200bc/429 inhibit cell invasion and migration.

To further evaluate the function of miR-200bc/429 on the migratory capability, we employed wound-healing and Transwell chamber assay. After transfection with miR-200bc/429 mimics, we observed a dramatic inhibition of migration in U2OS and MG63 cells (Figure 3A, 3B). In the Transwell assay, the result showed that the number of invasive cells transfected with miR-200bc/429 was significantly lower than that transfected with negative miRNA (Figure 3C). These results suggest that the upregulation of miR-200bc/429 inhibits the invasion and migration of OS cells.

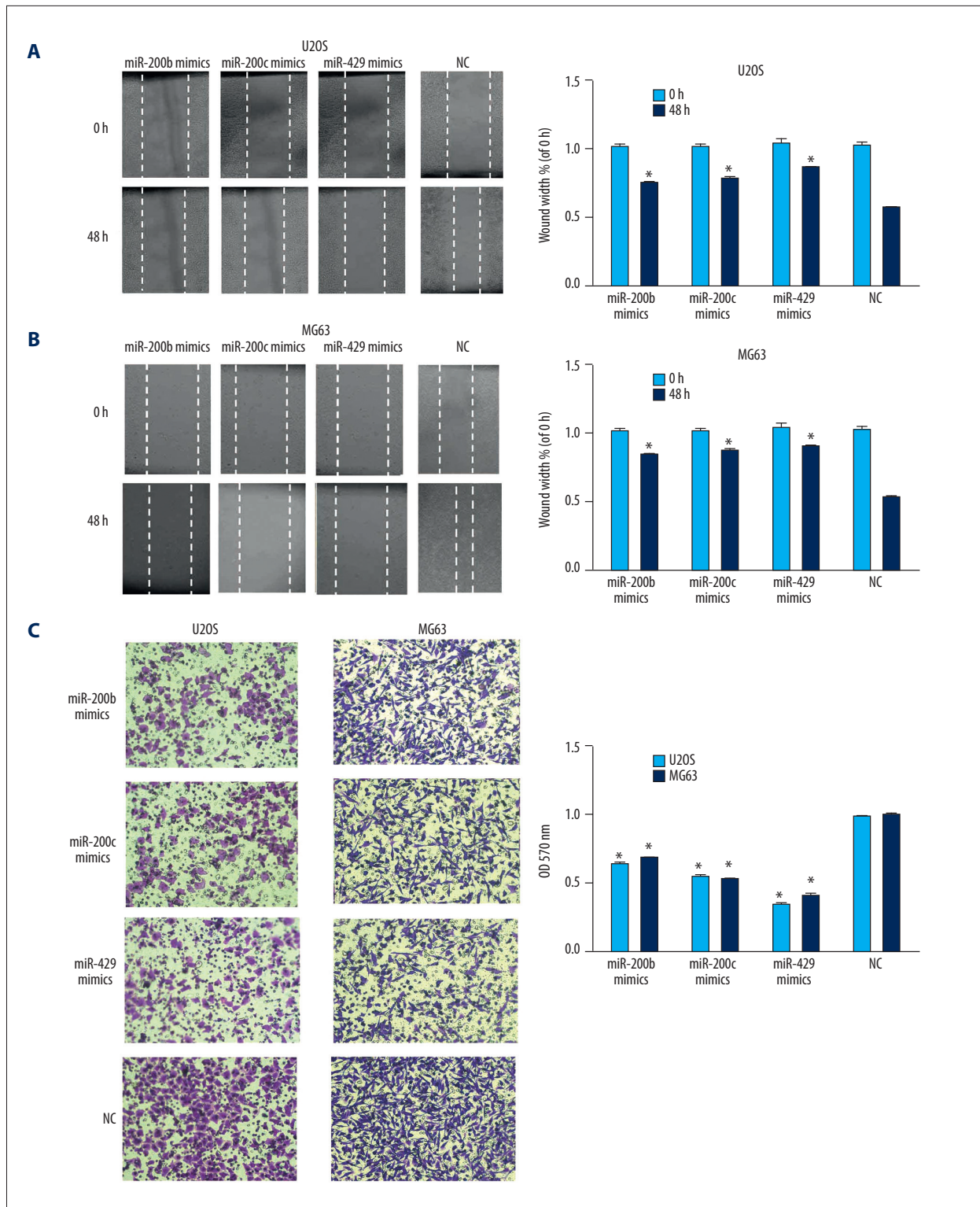


Figure 3. Restoration of miR-200bc/429 inhibits cell invasion and migration *in vitro*. **(A)** Wound-healing assay for U2OS cells transfected with miR-200bc/429 mimics or the negative control. **(B)** Wound-healing assay for MG63 cells transfected with miR-200bc/429 mimics or the negative control. **(C)** Representative micrographs (left) and quantification (right) of the Transwell invasion assay in the U2OS and MG63 cells. Magnification $\times 200$. Data are expressed as means \pm SD of 3 independent experiments. * $P < 0.05$ compared with the negative control group.

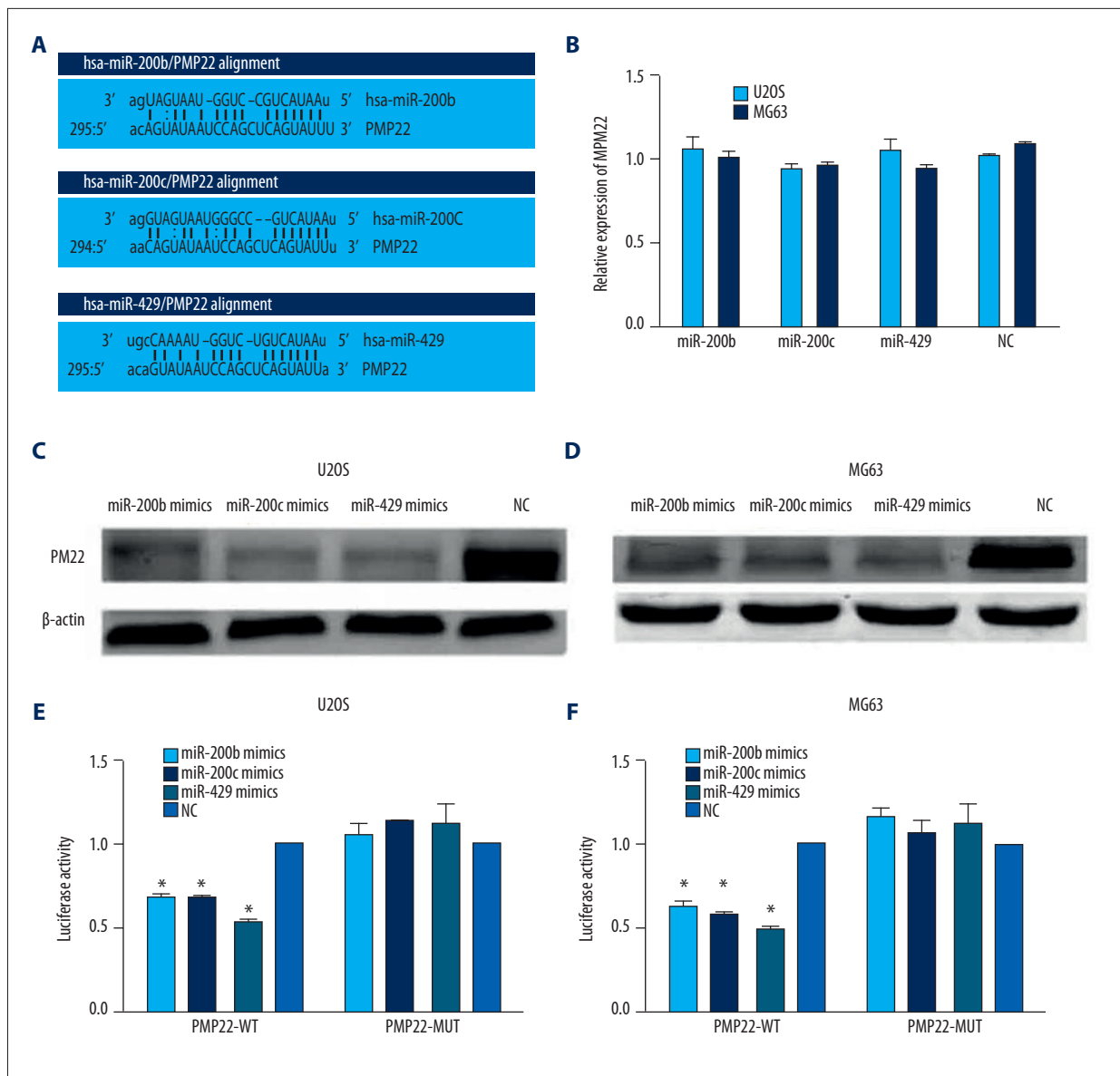


Figure 4. MiR-200bc/429 directly target the PMP22 in OS cells. (A) Predicted binding sequences of miR-200bc/429 in the 3'UTR of PMP22. (B) The gene expression of PMP22 was detected by qRT-PCR in the U2OS and MG63 cells. (C) The protein expression of PMP22 was determined by Western blot in U2OS cells. (D) The protein expression of PMP22 was determined by Western blot in MG63 cells. (E) Luciferase reporter assays in U2OS cells. (F) Luciferase reporter assays in MG63 cells. Data are expressed as means ±SD of 3 independent experiments. * P<0.05 compared with the negative control group.

miR-200bc/429 directly target the PMP22 in OS cells.

To clarify the mechanism of miR-200bc/429, through use of bioinformatics software (*microRNA.org*), we found that the gene encoding PMP22 contains a potential miR-200bc/429 binding site (Figure 4A). To explore whether miR-200bc/429 can directly target PMP22, we constructed a luciferase reporter vector containing the putative PMP22 3'UTR target site downstream of the luciferase gene. When treated with miR-200bc/429, the relative luciferase activity of the reporter containing wild-type

PMP22 3'-UTR were obviously suppressed, while the luciferase activity of the reporter containing mutant 3'-UTR was unaltered (Figure 4E, 4F). Meanwhile, we observed a dramatic decrease of PMP22 protein level in transfected miR-200bc/429 mimics cells compared with the negative control cells (Figure 4C, 4D). However, there was no correlation between miR-200bc/429 and the PMP22 expression in mRNA level (Figure 4B). These results suggest that PMP22 is the direct target gene of the miR-200bc/429.

Discussion

The miRNA-200 family, consisting 5 members (miR-200a, miR-200b, miR-200c, miR-429, and miR-141), has received much recent cancer research attention. Based on the seed sequences and potential similar target gene profiles, the miR-200 family can be divided into 2 clusters: the miR-200bc/429 cluster containing AAUACUG, and the miR-200a/141 cluster containing AACACUG. A different biological function can be caused by the different seed sequence [12]. Increasing evidence now supports that miR-200 plays a significant role in epithelial-to-mesenchymal transition (EMT), an important process in tumor progression and embryonic development. Recent studies have established that the miR-200 family inhibits EMT and determines the epithelial phenotype of cancer cells by direct targeting of E-cadherin repressors ZEB1 and ZEB2, suggesting that the miR-200 family members may be a metastasis-suppressor [13–16]. Moreover, Zhu et al. reported that miR-200bc/429 could regulate multidrug resistance in both vincristine-resistant gastric cancer cells and cisplatin-resistant lung cancer cells, partially by modulation of apoptosis via targeting B-cell lymphoma 2 gene (BCL-2) and X-linked inhibitor of apoptosis protein gene (XIAP) [17]. However, the expression and functional implications of the miR-200 family in OS remain poorly understood.

PMP22, a member of the growth arrest-specific gene-3 (*gas-3*) family, contributes to peripheral myelination in the peripheral nervous system [18]. It was reported that PMP22 is mainly expressed by Schwann cells (SCs) and frequently dysregulated expression in various cancers, involved in multiple critical biological processes, including proliferation, invasion, and apoptosis, and is proposed as a diagnostic and prognostic marker [19–22]. In OS, previous studies showed frequent amplification and overexpression of PMP22 in high-grade OS may be involved in the cell growth, cell cycle and tumorigenesis [23]. Recently, Liu et al. found that PMP22 participates in the proliferation, invasion, migration, and colony formation of OS cells, possibly via the MAPK signal transduction pathway [24]. Taken

together, these studies support a potential oncogenic function for PMP22 in OS. PMP22 regulate proliferation, invasion, migration, and apoptosis, possibly through the modulation of Rho small GTPase [25].

In the present study, we found that miR-200bc/429 were downexpressed in OS tissues and multiple cell lines by qRT-PCR. Reservation of miR-200bc/429 significantly inhibited the proliferation in U2OS and MG63 OS cells by CCK-8 assay. Furthermore, the results of wound-healing and Transwell chamber assay showed that overexpression of miR200bc/429 suppressed OS cell migration. In addition, luciferase reporter assay and Western blot analysis further revealed that miR-200bc/429 could directly target the 3'UTR of PMP22 and inhibit its expression in U2OS and MG63 cells.

Conclusions

Our present study demonstrates an inverse correlation between the miR-200bc/429 and PMP22 in human OS cells for the first time. In addition, the effects of miR-200bc/429 on cell proliferation and invasion suggest that it suppresses tumorigenesis in OS. We also provide evidence that miR-200bc/429 can inhibit the proliferation and invasion of OS cells by targeting PMP22. These results suggest that miR-200bc/429 have great potential to become a novel biomarker for tumorigenesis and progression and a target for new drug development against OS.

Conflicts of interest

The authors have declared no conflict of interest.

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