Int J Clin Exp Med 2016;9(11):21075-21083
www.ijcem.com /ISSN:1940-5901/IJCEM0036508

Original Article

MicroRNA-211 inhibits tumor growth and invasion through directly targeting RUNX2 in osteosarcoma

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Received July 23, 2016; Accepted September 18, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Osteosarcoma is the most common primary neoplasm of the bone and mainly occurs in adolescents and young adults. Despite great progress in current treatments, approximately one-third of patients with OS will not survive for more than five years, and fewer than 50% will live more than ten years. Aberrantly expressed microRNAs contribute to the carcinogenesis and progression of human cancers, including osteosarcoma. Increasing studies suggested that microRNA-211 (miR-211) might play important roles in the development of various cancer types; however, the expression patterns, roles and its underlying mechanism of miR-211 in osteosarcoma remain largely unexplored. In this study, we demonstrated that miR-211 was significantly downregulated in osteosarcoma tissues and cell lines compared with their adjacent normal tissues and human normal osteoblastic cell line, respectively. Functional studies showed that resumption of miR-211 inhibited cell proliferation, migration and invasion in osteosarcoma. Runt-related transcription factor 2 (Runx2) was validated as a direct target gene of miR-211. In addition, RUNX2 mRNA expression was upregulated in osteosarcoma tissues and inversely correlated with miR-211 expression. Furthermore, upregulation of RUNX2 could partially rescue the antitumor effects of miR-211 in OS cells. Taken together, these findings indicated the essential roles of miR-211 in suppressing osteosarcoma progression, suggesting miR-211 as a potential therapeutic target for the treatment of osteosarcoma.

Keywords: microRNA-211, osteosarcoma, runt-related transcription factor 2, proliferation, migration, invasion

Introduction

Osteosarcoma (OS) is the most common primary neoplasm of the bone which mainly occurs in adolescents and young adults [1]. The higher prevalence of OS in patients between 12-19 years old suggested that there may be a closely correlation between this disease and bone development [2]. Currently, the standard treatments for patients with OS are surgical management, chemotherapy and radiotherapy [3]. Despite great progress in current treatments, approximately one-third of patients with OS will not survive for more than five years, and fewer than 50% will live more than ten years [4, 5]. OS patients with metastases have poor prognosis with a long-term survival rate of 10-30% [6]. Therefore, elucidation the molecular mechanisms of OS tumorigenesis and development would definitely play essential roles in improving the survival rate of patients with this disease.

microRNAs (miRNAs) are a new class of endogenous, non-coding and short RNAs of 18-22 nucleotides in length [7]. Production of miRNAs require a set of proteins collectively referred to as the miRNA machinery [8]. Longer primary miRNA transcripts are processed into mature miRNAs through two steps of cleavage mediated through the endonucleases Drosha and Dicer [9]. The mature miRNAs are then incorporated into an RNA-induced silencing complex (RISC), interaction with the 3'-untranslated regions (3'UTRs) of their direct target genes in base pairing manner and induced translational inhibition or mRNA degradation [10, 11]. A single miRNA could negatively regulate a great deal of target genes and therefore act as important regulators in diverse biological processes of cancer, including developmental biology, cellular differentiation programs, and oncogenesis [12]. Increasing studies reported that the miRNAs is dysregulated in various kinds of human

cancer, such as OS [13], gastric cancer [14], lung cancer [15], bladder cancer [16] and so on. In cancer, miRNAs might serve as tumor suppressors and/or oncogenes according to the roles of their target genes. Downregulated miRNAs may normally function as tumor suppressor genes in human cancer through negatively regulation of oncogenes. Likewise, upregulation of miRNAs may act as oncogenes by down-regulate tumor suppressor genes. Therefore, miRNAs may have potential as diagnostic, prognostic, and therapeutic targets in anticancer treatments.

In this study, the objective was to determine the expression, roles of miR-211 in OS and its underlying mechanism. To this end, RT-qPCR was performed to measure miR-211 expression in OS tissues and cell lines. The roles of miR-211 in OS were evaluated by using MTT assay, migration and invasion assays. Furthermore, the direct target genes of miR-211 in OS were also analyzed. Finally, our results showed that miR-211 was downregulated in OS and acted as a tumor suppressor through down regulated a novel target, RUNX2.

Material and methods

Tumor specimens

Osteosarcoma tissues (n=21) and their adjacent normal tissues (n=21) were obtained from OS patients who underwent surgery at Department of Orthopaedic Surgery, the Second Affiliated Hospital of Zhejiang University School of Medicine. None of these OS patients were treated with radiotherapy and/or chemotherapy before surgery. All these tissue specimens were stored at -80°C until use. This study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine. All patients were given written informed consent.

Cell lines and culture condition

Human OS cell lines (MG-63, SAOS-2, SOSP-9607, HOS, U2OS) and a human normal osteoblastic cell line (hFOB 1.19) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Gibco, Grand Island, NY) at 37°C in 5% CO_2 atmosphere.

Oligonucleotides transfection

The miR-211 mimics and negative control miRNA mimics (NC) were purchased from RiboBio (Guangzhou, China). RUNX2 overexpressed plasmid (pcDNA3.1-RUNX2) and negative control blank plasmid (pCDNA3.1) were synthesized by Chinese Academy of Sciences (Changchun, China). For transfection, cells were seeded in 6-well plates at a density of 30%-40% confluence. After incubation overnight, transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

miR-211 and RUNX2 mRNA expression was measured using RT-qPCR. Briefly, total RNA from homogenized tissues and cell lines were harvested using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. All RNA samples were examined as to their concentration and purity. Total RNA purity and concentration was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized by using Moloney Murine Leukemia Virus Reverse Transcription system (Promega Corporation, Madison, WI, USA). qPCR was then conducted using a SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd., Dalian, China). RT-qPCR was performed on an Applied Biosystems® 7900 HT Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The U6 and GADPH were used as an endogenous control for miR-211 and RUNX2 mRNA expression, respectively. Data was calculated using the $2^{-\Delta\Delta Ct}$ method.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Transfected cells were harvested, counted and re-seeded in 96-well plates at a density of 3×10^3 cells each well. Cells were then incubated at 37°C in 5% CO_2 atmosphere for 24, 48, 72 and 96 h. At the indicated time periods, MTT assay (Sigma-Aldrich, St. Louis, MO, USA) was performed. Briefly, culture medium was

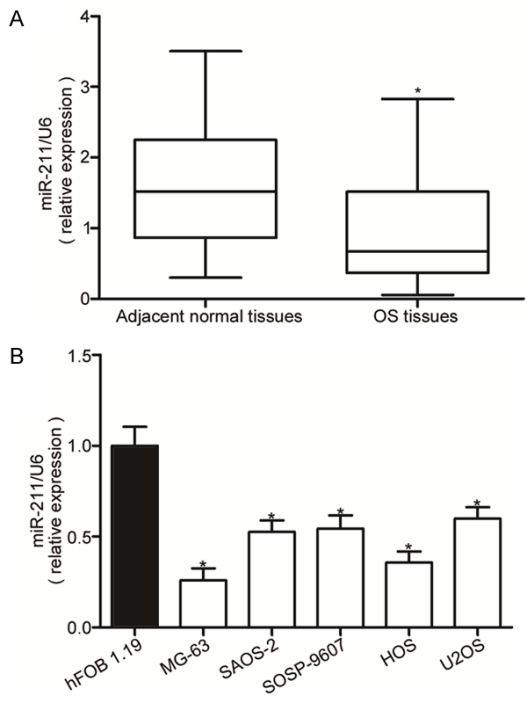

Figure 1. Expression of miR-211 in OS tissues and cell lines. A. RT-qPCR analysis of the miR-211 expression in OS tissues and their adjacent normal tissues. B. RT-qPCR analysis of the miR-211 expression in a human normal osteoblastic cell line hFOB 1.19 and OS cell lines (MG-63, SAOS-2, SOSP-9607, HOS, U2OS). *P<0.05 compared with respective control.

replaced with 0.5 mg/ml MTT solution and incubated at 37°C for 4 h. The medium containing MTT solution was removed and 150 μl dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added into each well to resolve MTT formazan. The optical density (OD) at 490 nm was determined with an automatic multi-well spectrophotometer (Bio-Rad, Richmond, CA, USA).

Migration and invasion assay

Transwell chambers (8 μm; Costar, Corning Incorporated, Corning, NY, USA) was used to investigate the effects of miR-211 on the migration and invasion capacities of OS cells. For migration assay, transfected cells were harvested, counted and resuspended in FBS-free culture medium. 5×10^4 cells were added in the upper chamber and 500 μl culture medium with 20% FBS was added into the lower chamber. The transwell chambers were then incubated at 37°C in 5% CO_2 atmosphere for 48 h. The cells remained on the upper surface of the transwell chambers were carefully removed with cotton swabs; while the migrated cells were fixed with 4% paraformaldehyde for 10 min, stained with 0.5% crystal violet for 30 min and then washed with PBS (Gibco, Grand Island, NY). Cells were then counted under an inverted microscope (×200; Olympus Corporation, Tokyo, Japan). The procedure of invasion assay was similar with migration assay, except that transwell chambers were pre-coated with Matrigel (BD Bioscience, San Jose, CA, USA).

Bioinformatic predication.

TargetScan (http://www.targetscan.org/index.html) was used to predicate the potential target genes of miR-211.

Luciferase reporter assay

HEK293T was purchased from ATCC and used for luciferase reporter assay. Luciferase reporter plasmids, including pGL3-RUNX2-3'UTR wild-type (Wt) and pGL3- RUNX2-3'UTR mutant-type (Mut), were synthesized and confirmed by GenePharma (Shanghai, China). For luciferase reporter assay, HEK293T cells were seeded in 24-well plates at a density of 30%-40% confluence and transfected with pGL3-RUNX2-3'UTR Wt or pGL3-RUNX2-3'UTR Mut, together with miR-211 mimics or NC using Lipofectamine 2000. The transfected cells were then incubated at 37°C in 5% CO_2 atmosphere for 48 h. Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) was used to determine firefly and renilla luciferase activities. Renilla luciferase activities was employed as an internal control of transfection efficiency.

Western blot analysis

Total protein was isolated from transfected cells by using RIPA buffer with protease inhibitors and phosphate inhibitors (Roche, Indianapolis, IN, USA). Protein was quantified by using a BCA quantification kit (Beyotime Institute of Biotechnology, Jiangsu, Haimen, China). Equal amounts of protein was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) and then blocked in Tris-buffered saline-Tween 20 (TBST) containing 5% milk at room temperature for 2 h. The membranes were then probed with primary antibodies at 4°C for overnight. The primary antibodies

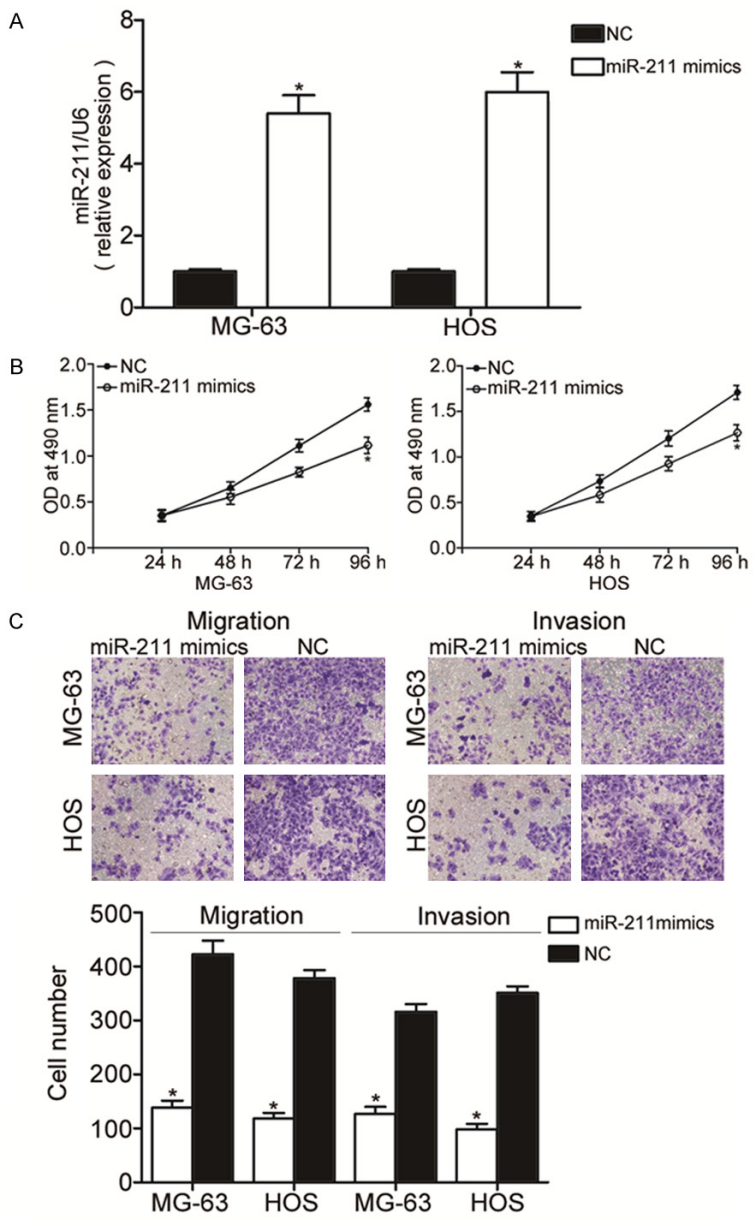

Figure 2. miR-211 inhibited proliferation, migration and invasion in MG-63 and HOS cells. A. MG-63 and HOS cells that were transfected with miR-211 mimics or NC, were subjected to RT-qPCR for miR-211 expression. B. MTT assay showed that upregulation of miR-211suppressed the proliferation of MG-63 and HOS cells. C. MiR-211 inhibited capacities of cell migration and invasion in MG-63 and HOS cells compared with NC group. *P<0.05 compared with respective control.

used in this study includes mouse anti-human monoclonal RUNX2 antibody (1:1000 dilution; sc-390715; Santa Cruz Biotechnology, CA, USA) and mouse anti-human monoclonal β-actin (1:1000 dilution; sc-47778; Santa Cruz Biotechnology, CA, USA). After washing with TBST, the membranes were incubated with goat anti-mouse horse-radish peroxidase (HRP)-conjugated secondary antibody (1:1000 dilution; sc-2005; Santa Cruz Biotechnology, CA, USA). The signals were visualized with SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Inc., Rockford, IL, USA) and analyzed using Quantity One software (version 4.62; Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were presented as mean ± S.D. The difference between groups was compared with student's t-test by using SPSS 13.0 statistical software (SPSS Inc, Chicago, IL, USA). Differences were considered significant at P<0.05.

Results

miR-211 was downregulated in OS tissues and cell lines

To gain insight into the biological roles of miR-211 in human OS, we examined miR-211 expression in OS tissues and their adjacent normal tissues. As show in **Figure 1A**, miR-211 expression was lower in OS tissues than adjacent normal tissues (P<0.05). In this study, we also measured the miR-211 expression in OS cell lines (MG-63, SAOS-2, SOSP-9607, HOS, U2OS) and a human normal osteoblastic cell line (hFOB 1.19). The results showed that levels of miR-211 were reduced in OS cell lines compared with that in hFOB 1.19 (**Figure 1B**, P<0.05).

miR-211 inhibited cell proliferation, migration and invasion of OS

The downregulation of miR-211 prompted us to investigate whether miR-211 acted as a tumor suppressor in OS carcinogenesis and progression. Therefore, miR-211 mimics or NC was injected in MG-63 and HOS cells which

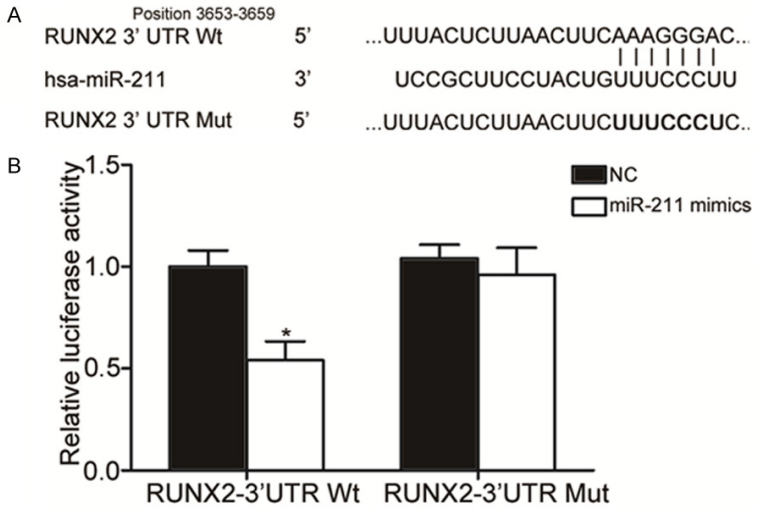

Figure 3. RUNX2 was a direct target of miR-211. A. Predicted wild type and mutant-type sequence in the 3'UTR of RUNX2. B. HEK293T cells were injected with pGL3-RUNX2-3'UTR Wt or pGL3-RUNX2-3'UTR Mut, and together with miR-211 mimics or NC. Luciferase activities were determined after transfection 48 h. *P<0.05 compared with respective control.

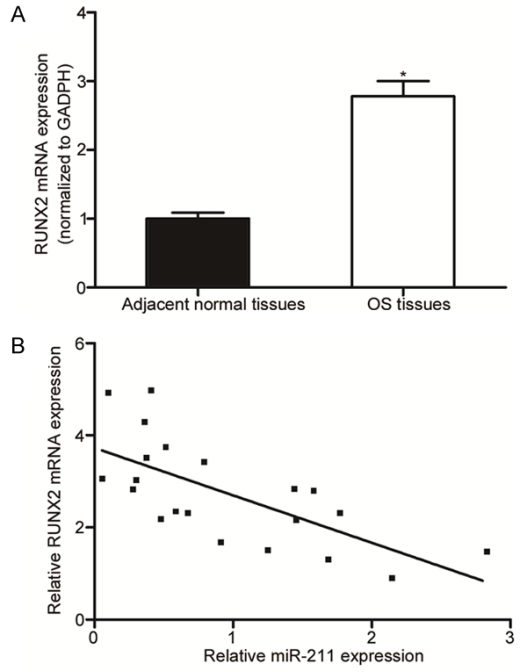

Figure 4. RUNX2 mRNA was upregulated in OS tissues and was inversely correlated with miR-211 expression. A. RT-qPCR analysis of the RUNX2 mRNA expression in OS tissues and their adjacent normal tissues. B. Negative correlation was found between miR-211 and RUNX2 mRNA expression in OS tissues. *P<0.05 compared with respective control.

expressed relatively lower miR-211 expression in five examined OS cell lines. RT-qPCR was performed to evaluate its transfection efficiency and revealed that miR-211 was obviously increased in MG-63 and HOS transfected with miR-211 mimics (**Figure 2A**, P<0.05).

The effect of miR-211 overexpression on OS cells was assessed by using MTT assay. As shown in **Figure 2B**, upregulation of miR-211 significantly decreased MG-63 and HOS cells proliferation (P<0.05). Furthermore, the roles of miR-211 on OS cells migration and invasion were investigated using migration and invasion assay. As shown in **Figure 2C**, the number of the migrated (P<0.05) and invaded (P<0.05) cells transfected with miR-211 mimics were significantly fewer than cells transfected with NC. These data demonstrated that miR-211 functioned as a tumor suppressor in OS through inhibiting cell growth and metastasis.

RUNX2 was a direct target of miR-211

To identify target genes of miR-211, bioinformatics analysis was performed to predict potential targets of miR-211 and identified RUNX2 as a putative miR-211 target gene (**Figure 3A**). Luciferase reporter assays were adopted to investigate whether the 3'UTR of RUNX2 could be directly targeted by miR-211. HEK293T cells were transfected with luciferase reporter plasmids along with miR-211 mimics or NC. As shown in **Figure 3B**, miR-211 overexpression decreased luciferase activities of pGL3- RUNX2-3'UTR Wt (P<0.05); however, mutation of the putative target site in the RUNX2 3'UTR abolished this suppression by miR-211. These results indicated that RUNX2 was a direct target of miR-211.

RUNX2 was inversely correlated with miR-211 expression in OS tissues

To explore the association between miR-211 and RUNX2 expression, we firstly measured RUNX2 mRNA expression in OS tissues and their adjacent normal tissues. The results

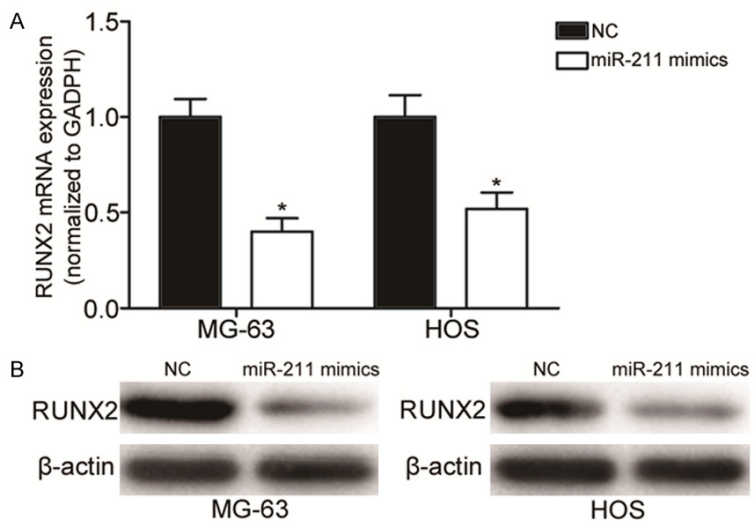

Figure 5. miR-211 down regulated RUNX2 expression in MG-63 and HOS cells. A. RT-qPCR analysis of the RUNX2 mRNA expression in MG-63 and HOS cells transfected with miR-211 mimics or NC. B. Western blot analysis was performed to determine RUNX2 protein expression in MG-63 and HOS cells transfected with miR-211 mimics or NC. *P<0.05 compared with respective control.

showed that RUNX2 mRNA was significantly upregulated in OS tissues compared with that in their adjacent normal tissues (**Figure 4A**, P<0.05). We also investigated the association between miR-211 expression and RUNX2 mRNA expression in clinical OS tissues. Spearman's correlation analysis revealed that RUNX2 mRNA expression was inversely correlated with miR-211 expression in OS tissues (**Figure 4B**; r=-0.6838, P=0.0006).

miR-211 negatively regulated RUNX2 expression in OS cells

To evaluate the regulation effects of miR-211 on RUNX2 expression, RT-qPCR and western blot was performed. The results of RT-qPCR showed that upregulation of miR-211 reduced RUNX2 mRNA expression in MG-63 and HOS cells (**Figure 5A**, P<0.05). In addition, western blot indicated that RUNX2 protein (**Figure 5B**, P<0.05) expression was decreased by miR-211 overexpression in MG-63 and HOS cells. These data suggested that miR-211 may negatively regulate RUNX2 expression at both mRNA and protein level in OS.

The antitumor effects of miR-211 in OS were mediated through the repression of RUNX2

Based on the findings above, we hypothesized that miR-211 suppressed MG-63 and HOS cells proliferation, migration and invasion through downregulation of RUNX2. Hence, MG-63 and HOS cells were transfected with pcDNA3.1-RUNX2 or pcDNA3.1. After transfection, RUNX2 was significantly upregulated in MG-63 and HOS cells (**Figure 6A**, P<0.05). Subsequently, gain-of-function experiments were performed. As shown in **Figure 6B** and **6C**, upregulation of RUNX2 could partially rescued the antitumor effects of miR-211 in MG-63and HOS cells. Taken together, these findings suggested that RUNX2 was a functional downstream target for miR-211 in OS.

Discussion

Up to now, the exact mechanisms underlying OS tumorigenesis and tumor development is complicated and has not been clarified yet [17]. Accumulated studies have demonstrated that miRNAs are abnormally expressed in OS and may represent as a novel therapeutic targets for the treatments of this disease [18-20]. Our data showed that expression level of miR-211 was significantly lower in OS tissues and OS cell lines than in their adjacent normal tissues and human normal osteoblastic cell line, respectively. Additionally, functional experiments showed that miR-211 overexpression suppressed cell proliferation, migration and invasion in OS. Furthermore, RUNX2 was demonstrated to be the direct target gene of miR-211. RUNX2 mRNA was downregulated in OS tissues and was inversely correlated with miR-211 expression. Ectopic of miR-211 expression down regulated RUNX2 at both mRNA and protein levels in OS cells. Upregulation of RUNX2 could partially rescued the suppressive effects of miR-211 overexpression on OS cells proliferation, migration and invasion. Our work may be the first time to reveal the expression and roles of miR-211 in OS and its underlying mechanism.

Many studies have reported that miR-211 was closely implicated with various cancers. For example, Mazar found that miR-211 was reduced or absent in nonpigmented melanoma

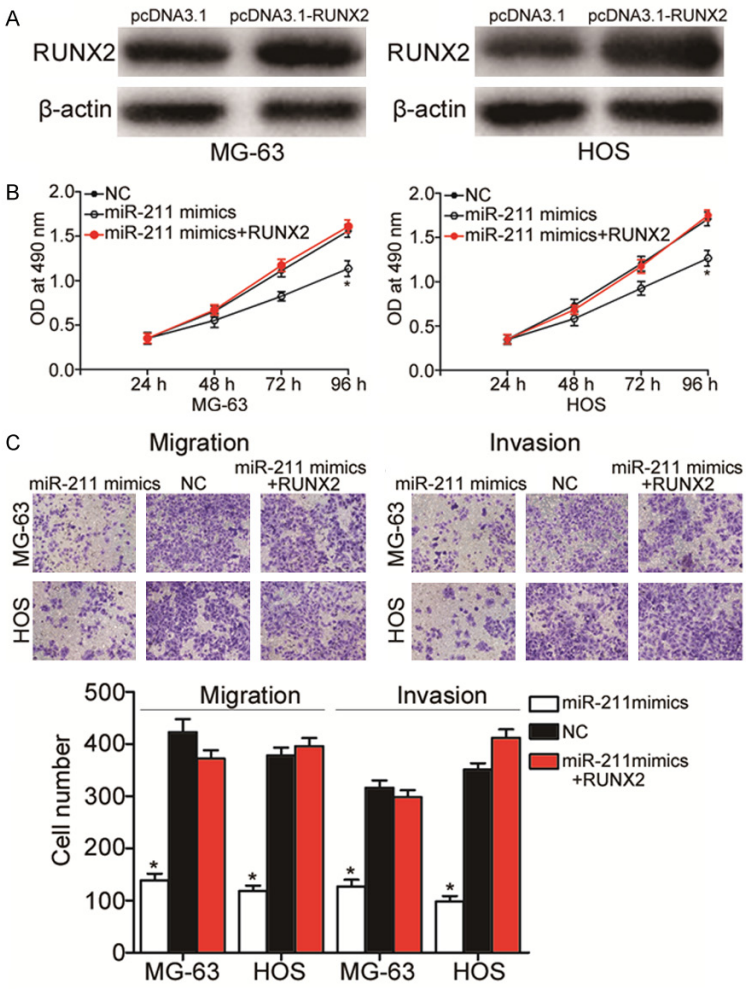

Figure 6. RUNX2 overexpression rescued the antitumor effects of miR-211 on proliferation, migration and invasion in MG-63 and HOS cells. (A). MG-63 and HOS cells that were transfected with pcDNA3.1-RUNX2 or pcDNA3.1, were subjected to western blot analysis for RUNX2 protein expression. Up-regulation of RUNX2 could partially rescued the suppressive effects of miR-211 on MG-63 and HOS cells proliferation (B) migration and invasion (C). *P<0.05 compared with respective control.

cells compared with normal human melanocytes [21]. In colorectal cancer, miR-211 was upregulated in tumor tissues. Kaplan-Meier showed that patients with high miR-211 expression had a significantly worse median survival than those with a low expression. The univariate and multivariate analysis indicated that miR-211 expression was an independent prognostic factor for patients with colorectal cancer [22]. Jiang and his colleagues found that levels of miR-211 were reduced in hepatocellular carcinoma tissues compared with adjacent normal liver tissues [23]. Chang et al. revealed that miR-211 expression was higher in oral carcinoma tissues. Its expression was obviously correlated with advanced nodal metastasis, vascular invasion, and poor prognosis of oral carcinoma [24]. These studies suggested that abnormal expression of miR-211 might be a prognostic marker of cancer patients.

miR-211 has been demonstrated to play important roles in multiple types of human cancer. For example, in melanoma, reintroduction of miR-211 decreased cell growth and invasion through down-regulation of PDK4 [21]. Song et al. reported that miR-211 overexpression inhibited cell growth, cell cycle, migration, and invasion of triple-negative breast cancer via directly targeting CDC25B [25]. Cai et al. demonstrated that resumption of miR-211 improved cell proliferation, tumor growth, and cell migration of colorectal cancer in vitro and in vivo via blockade of CHD5 [26]. Study by Wang et al. revealed that miR-211 re-expression reduced gastric cancer cells proliferation and invasion through negatively regulation of SOX4 [27]. In hepatocellular carcinoma, enforced miR-211 expression decreased cell growth in vitro and in vivo, and invasion by targeting SATB2 [23]. Xia *et al.* revealed that miR-211 overexpression arrested epithelial ovarian cancer cells in the G0/G1-phase, suppressed proliferation and increased apoptosis through regulation of Cyclin D1 and CDK6 [28]. In oral carcinoma, upregulation of miR-211 enhanced cell proliferation, migration, anchorage-independent colony formation and tumorigenicity of only SAS high-grade oral carcinoma cells [24]. These findings suggested that miR-211 may have therapeutic value in treating these human cancers.

Identification of cancer-specific miRNAs and their target genes is pivotal for understanding their roles in tumorigenesis and tumor develop-

ment [29, 30]. In present study, RUNX2 was identified as a novel direct target of miR-211. The RUNX2 gene is approximately 220 kb on chromosome 6 near the border between cytobands 6p21.1 [31]. RUNX2, a member of the RUNX family, is an osteoblast-specific transcriptional factor and highly expressed OS tissues and cell lines [30, 32]. Won *et al*. demonstrated that high RUNX2 expression was significantly correlated with the clinical stage, metastasis and prognosis in OS [32]. Previous study demonstrated that regulating RUNX2 expression could vary the local internal environment of osteocytes, affect osteosarcoma maturity, control apoptosis and matrix metalloproteinase activity and thereby affect OS formation [33]. It also found that Runx2 underexpression decreased motility of OS cells [34]. Heng et al. reported that RUNX2 underexpression suppressed the invasion of OS cells by downregulation of VEGF, MMP-2 and MMP-9 [35]. Taken together, our study demonstrated that the tumor suppressive abilities of miR-211 to inhibit OS cells growth and metastasis were achieved by regulation of RUNX2 expression. miR-211/RUNX2 based targeted therapy may be an effective treatment for patients with OS.

In conclusion, this study demonstrated that miR-211 acted as a tumor suppressor in the regulation of OS cells proliferation, migration and invasion, to a certain extent, via targeting RUNX2. Accordingly, miR-211 may serve as potential therapeutic target for OS.

Acknowledgements

This work was supported by Zhejiang Medicines Health Science and Technology Program General Studies Program (2016: Item number: 2016134432) and Zhejiang Provincial Natural Science Foundation of Youth Project Application (2016; Item Number: LQ16H060001).

Disclosure of conflict of interest

None.

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