Original Article
microRNA-363 plays a tumor suppressive role in osteosarcoma by directly targeting MAP2K4

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Abstract: Abnormal expression of microRNAs plays important functions in osteosarcoma. The aim of this study was to investigate expression, functions and molecular mechanisms of microRNA-363 in osteosarcoma. Quantitative Real-time PCR was used to detect the expression level of microRNA-363 in osteosarcoma tissue samples and cell lines. After transfection, CCK8 assay, cell migration and invasion assay, western blot and Dual-Luciferase report assay were performed in human osteosarcoma cells. According to the results, we found that microRNA-363 was down-regulated in osteosarcoma tissues and cell lines. In addition, low expression level of microRNA-363 was associated with tumor size, clinical stage and distant metastasis. Moreover, microRNA-363 targeted MAP2K4 to inhibit osteosarcoma cell growth, migration and invasion. In conclusion, microRNA-363 played a tumor suppressive role in osteosarcoma by directly targeting MAP2K4. These findings indicated that microRNA-363 may have therapeutic value in treating osteosarcoma.

Keywords: microRNA-363, osteosarcoma, MAP2K4, growth, metastasis

Introduction

Osteosarcoma (OS), the most common primary bone tumor, mainly occurs in children and young adults characterized by malignant osteoid production and osteoblastic differentiation [1, 2]. It predominantly arises from metaphysis regions of the long bones with active bone growth and repair, such as knee joint, lower femur, and upper tibia [3]. In United States, it is estimated that there would be approximately 900 newly diagnosed OS patients per year [4]. Increasing evidences have demonstrated that OS should be considered as a type of differentiation disease caused by genetic and epigenetic changes [5]. These changes prevent normal osteoblast differentiation from mesenchymal stem cell progenitor [6]. Currently, the standard therapies for OS patients are aggressive surgical resection combined with anti-OS drugs chemotherapy, and sometimes radiotherapy [7]. Thanks to progresses have been developed in treatments for OS, the 5-year survival rate of patients with localized disease has improved to 60%-70% [8-10]. However, for patients with metastatic or recurrent diseases, the 5-year survival rate is less than 20% [11]. Hence, it is important to investigate the fundamental molecular mechanisms that underlie the OS progression of rapidly growth and metastasis to identify novel therapies for patients with OS.

A large number of studies have demonstrated that abnormal expression of microRNAs (miRNAs) is associated with many human cancers, also including OS [12-14]. miRNAs are a family of endogenous, conserved, non-coding and short RNA molecules approximately 22-24 nucleotides in length, which regulate gene expression at post-transcription level [15]. miRNAs regulate more than 30% of all human genes by binding to their target genes in a base-pairing manner and resulting in mRNA degradation or disrupting mRNA from being translated [16]. More importantly, emerging studies indicated that miRNAs play important roles in various critical biological processes, such as development, differentiation, proliferation, apoptosis, angiogenesis, metastasis and metabolism, and

consequently their alterations have been verified to play a role in cancer initial and progression [17]. miRNAs can act as either oncogenes or tumor suppressors, depending on their expression and target mRNAs [18]. miRNAs, up-regulated in tumors, function as oncogenes which enhance tumor progression through down-regulation of tumor suppressor genes. In this regard, miRNAs, down-regulated in tumors, are considered as tumor suppressors which block tumor development via blockade of oncogenes [19-21]. Therefore, further exploration of the roles of miRNAs will provide insight into the mechanisms of OS progression and identify therapeutic targets.

In this study, we studied the expression, functions and molecular mechanism of miR-363 in OS. We found that miR-363 was down-regulated in OS tissues and cell lines. In addition, low expression level of microRNA-363 was associated with tumor size, clinical stage and distant metastasis. Ectopic expression of miR-363 significantly inhibited OS cell proliferation, migration and invasion. It functioned as a tumor suppressor in OS. Moreover, mitogen-activated protein kinase 4 (MAP2K4) was demonstrated as a direct target of miR-363 in vitro. Taken together, these results suggested that miR-363 may play important roles in regulating OS rapidly growth and metastasis. It could be investigated as a targeted therapy to block rapidly growth and metastasis of OS.

Material and methods

Clinical specimens

For analysis expression level of miR-363, a total of seventy-four specimens of OS and matched non-cancerous bone tissue samples were obtained from OS patients who undergo surgical resection at Yidu Central Hospital of Weifang from 2006 to 2012. All patients had not received any therapy treatments before surgery. OS tissues and non-cancerous bone tissues were immediately snap-frozen at liquid nitrogen and transferred to -80°C refrigerator until use. In addition, the clinicopathological information was also collected from all OS cases involved in this study. The present study was approved by Yidu Central Hospital of Weifang's Medical Ethics Committee. Written informed consent was obtained from all OS patients.

Cell culture and miRNA mimics, siRNAs transfection

OS cell lines MG63, HOS, U2OS, Saos2, human normal osteoblastic cell line hFOB 1.19 and HEK293T were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin (Gibco, Grand Island, NY) and 100 U/ml streptomycin (Gibco, Grand Island, NY). All these cell lines were maintained in conditions of 95% air and 5% CO_2 at 37°C.

To enforce miR-363 expression, miR-363 mimics and NC were used. Meanwhile, miR-363 inhibitor and NC inhibitor were adopted to decrease miR-363 expression. miR-363 mimics, NC, miR-363 inhibitor and NC inhibitor were purchased from GenePharma (Shanghai, China). MAP2K4 siRNA and NC siRNA (GenePharma, Shanghai, China) were transfected into OS cells for reducing MAP2K4 expression. Cell transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Before transfection, cell culture medium was replaced with complete medium without antibiotics.

RNA isolation and Quantitative Real-time PCR (qRT-PCR)

Total RNA was isolated from OS tissues, noncancerous bone tissues and cell lines by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following to the manufacturer's instructions. PrimeScript RT reagent Kit (TaKaRa, Japan) was used to perform reverse transcription. For miR-363 expression detection, qRT-PCR was performed using SYBR Premix Ex Taq II (TaKaRa, Japan). SYBR Green PCR Master Mix (Applied Biosystems) was used to detect MAP2K4 mRNA expression. U6 and GADPH were used for normalization.

Cell counting kit-8 (CCK-8) assay

CCK8 assay (Dojindo, Kumamoto, Japan) was adopted to evaluate cell growth. After transfection, 3000 cells in 100 ul complete medium were seeded into 96-well plate. After incubation 24, 48, 72 and 96 h, 10 ul CCK8 assay solution was added into each well. Then, after
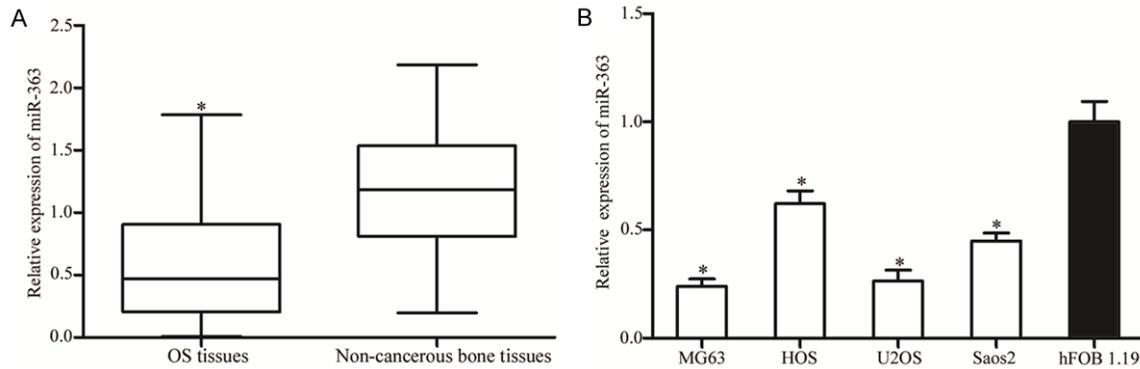
Figure 1. miR-363 was down-regulated in OS tissues and cell lines. A. The expression level of miR-363 was decreased in OS tissue samples compared with matched non-cancerous bone tissue samples. B. miR-363 was also down-regulated in OS cell lines compared with hFOB 1.19. *P<0.05 compared with their matched non-cancerous bone tissue samples and hFOB 1.19, respectively.

Table 1. Correlation of miR-363 expression level with clinicopathological features in osteosarcoma patients

Variables	Case number	miR-363 expression		P value
		Low (n = 42)	High (n = 32)	
Gender				0.371
Male	41	24	17	
Female	33	18	15	
Age				0.225
<50 years	41	27	14	
≥50 years	33	15	18	
Anatomical location				0.810
Tibia/femur	37	26	21	
Elsewhere	27	16	11	
Tumor size (cm)				0.004
<8 cm	43	18	25	
≥8 cm	31	24	7	
Clinical stage				0.019
I-II	32	13	19	
III	42	29	13	
Distant metastasis				0.006
Present	25	20	5	
Absent	49	22	27	

incubation another 2 h, optical density (OD) at 450 nm was measured with Enzyme immuno-assay analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Each sample was measured in triplicate.

Cell migration and invasion assay in vitro

Cell migration and invasion assay were carried out in transwell chambers (Costar, Cambridge, MA) with 8 mm pore filter inserts. For cell migra-

tion assay, 1×105 transfected cells in 300 µL DMEM medium without antibiotics were added into the upper chamber. Meanwhile, 500 ul DMEM medium supplemented with 20% FBS was added into the lower chamber as a chemoattractant. After incubation 24 h, the non-migrated cells were carefully removed with cotton swabs. Filtered cells were fixed, stained with crystal 0.5% violet (Beyotime Institute of Biotechnology, Haimen, China), and washed with phosphate-buffered saline (PBS; Gibco, Grand Island, NY). For cell invasion assay, the procedure was the same as the cell migration assay except that trasnwell chamber was coated with Matrigel (BD Biosciences, San Jose, CA). The cells were counted under a inverted microscope in five random fields per transwell chamber. All experiments were repeated at least three times.

Protein extraction and western blot

Cells were lysed with radioimmunoprecipitation buffer (RIPA) Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 3% proteinase inhibitors (Sigma, St. Louis, MO, USA). Total protein concentration was detected with BCA protein assay kit (Pierce, Rockford, IL, USA).

For western blot, equal amount of proteins was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk in Tris-buffered
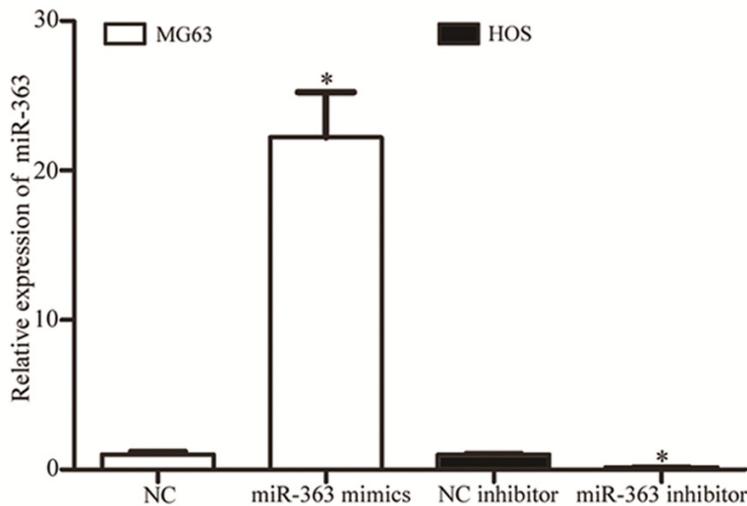
Figure 2. miR-363 was up-regulated in miR-363 mimics-transfected MG63 cells, while miR-363 inhibitor resulted in down-regulation of miR-363 in HOS cells. *$P<0.05$ compared with cells trasnfected with NC.

saline (TBS) at room temperature, the membrane was incubated with primary antibodies for overnight at 4°C, followed by incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, CA, USA). The protein signals were visualized with ECL (Pierce, Rockford, IL, USA). In this study, MAP2K4 (1:1000 dilution; sc-376838) and β-actin (1:1000 dilution; sc-271344) primary antibodies were purchased from Santa Cruz Biotechnology. The protein expression levels were normalized to β-actin.

Dual-Luciferase report assay

To explore whether MAP2K4 was a direct target of miR-363, Dual-Luciferase Reporter Assay System (Promega, Madison, WI, Germany) was performed. PGL3-MAP2K4-3'UTR Wt and PGL3-MAP2K4-3'UTR Mut was also purchased from GenePharma (Shanghai, China). HEK293T cells were transfected with miR-363 mimics or NC, and co-transfection with PGL3-MAP2K4-3'UTR Wt or PGL3-MAP2K4-3'UTR Mut by using Lipofectamine 2000, following to the manufacturer's instructions. After transfection 48 h, firefly activity and renilla luciferase activity in each well were both detected. The firefly luciferase activity was normalized to the Renilla luciferase activity.

Statistical analysis

The data were presented as mean ± S.D., and compared using SPSS 13.0 statistical software

(SPSS Inc, Chicago, IL, USA). A difference was considered statistically significant when $P<0.05$.

Results

miR-363 was down-regulated in human OS tissue samples and cell lines

To evaluate the expression level of miR-363, qRT-PCR was performed. As shown in **Figure 1A**, miR-363 was significantly down-regulated in human OS tissues compared with matched non-cancerous bone tissue samples ($P<0.05$). Meanwhile, miR-363 expression in OS cell lines and human normal osteoblastic cell line hFOB 1.19 was also detected. As shown in **Figure 1B**, expression level of miR-363 was decreased in all four OS cell lines compared with hFOB 1.19 ($P<0.05$). These results indicated that miR-363 may play a potential tumor-suppressive role in OS.

Correlation between miR-363 expression level and clinicopathologic features in OS patients

To further explore the possible roles of miR-363 in OS carcinogenesis and progression, we performed an investigation into the correlation between miR-363 expression level and clinicopathological features in OS patients. As shown in **Table 1**, low expression level of miR-363 was associated with tumor size (P = 0.004), clinical stage (P = 0.019) and distant metastasis (P = 0.006). In contrast, there is no significant association with gender, age and anatomical location (P>0.05). Taken together, these findings suggested that miR-363 expression was associated with OS carcinogenesis and development.

miR-363 expression in OS cells after transfection

To investigate the roles of miR-363, we performed both gain and loss of function strategies in OS cell lines. miR-363 expression in MG63 was the lowest in these four OS cell lines, while its expression in HOS was the highest. Therefore, MG63 was transfected with
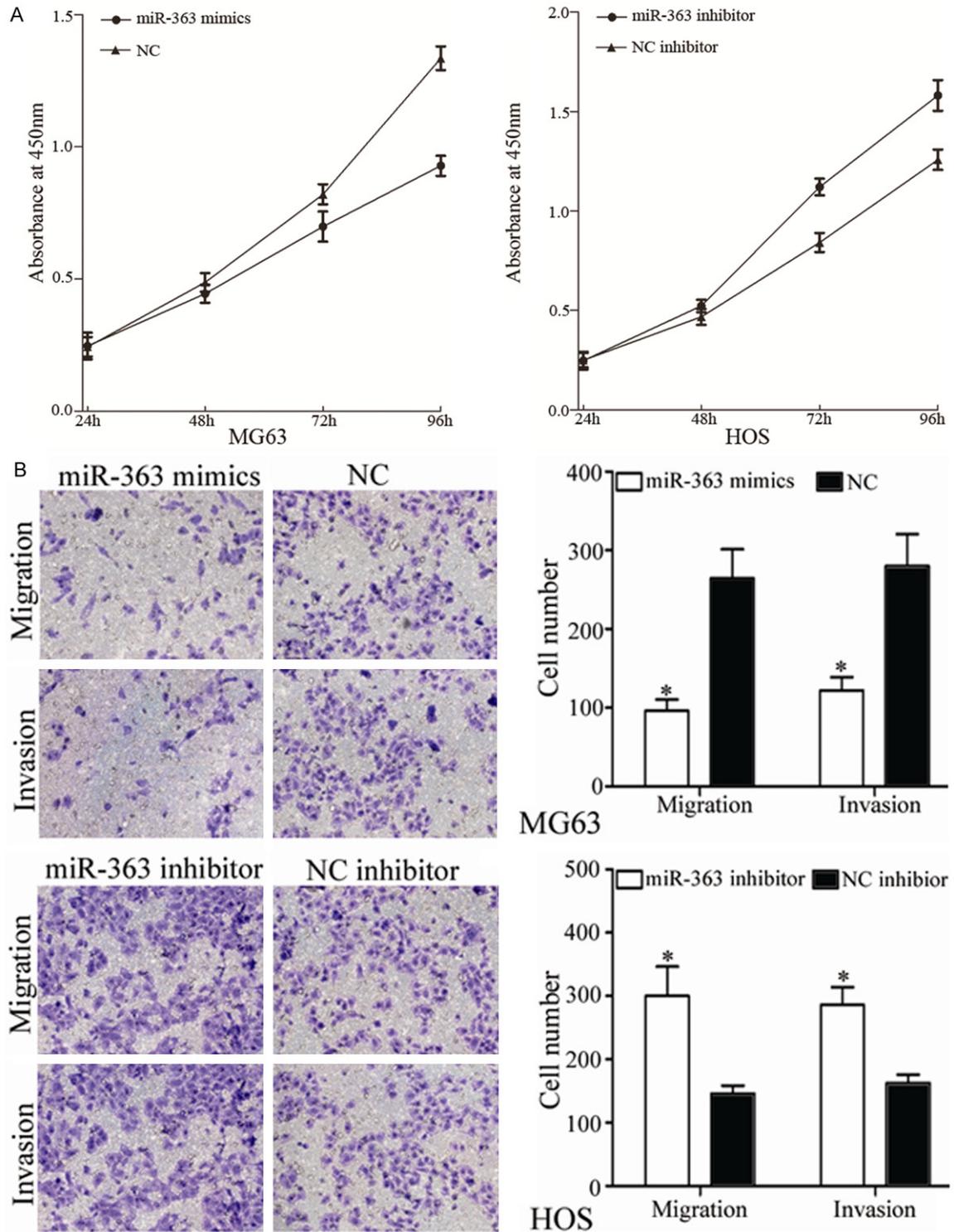
Figure 3. miR-363 inhibited OS cell proliferation, migration and invasion. A. Over-expression of miR-363 by miR-363 mimic inhibited MG63 cell proliferation, whereas down-regulation of miR-363 by miR-363 inhibitor enhanced HOS cell growth. B. miR-363 mimics inhibited MG63 cell migration and invasion. By contrast, miR-363 inhibitor promoted HOS cell migration and invasion. $^{*}P<0.05$ compared with the NC groups.

miR-363 mimics and HOS was transfected with miR-363 inhibitor. After transfection, qRT-PCR was performed to assess cell transfection efficiency. The results showed that miR-363 mim-
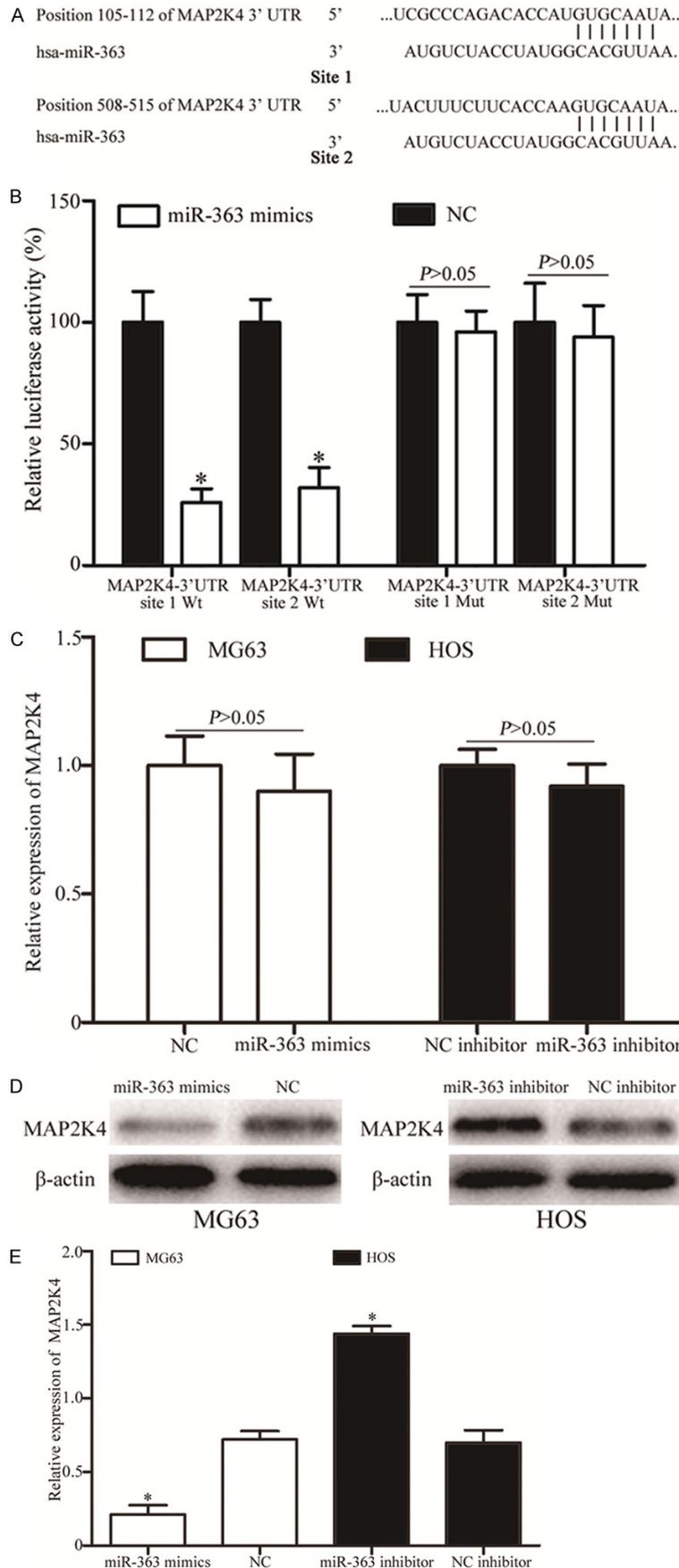
Figure 4. MAP2K4 was identified as a direct target of miR-363 in vitro. A. The miR-363 binding site1 and site2 in the 3'-UTR of MAP2K4. B. Dual-Luciferase report assay revealed that miR-363 significantly inhibited the PGL3-MAP2K4-3'UTR Wt (site1 and site2) luciferase activity, but not the PGL3-MAP2K4-3'UTR Mut (site1 and site2) luciferase activity in HEK293T cells. C. qRT-PCR showed that MAP2K4 expression at mRNA level was not altered after transfection with miR-363 mimics or miR-363 inhibitor. D, E. MAP2K4 protein expression was down-regulated in miR-363 mimics-transfected MG63 cells, whereas MAP2K4 protein was up-regulated in miR-363 inhibitor-transfected HOS cells. *$P<0.05$ compared with the NC groups.

ics caused the up-regulation of miR-363 expression in MG63 cells, while the miR-363 inhibitor resulted in down-regulation of miR-363 expression in HOS cells (shown in **Figure 2**, $P<0.05$).

miR-363 inhibited OS cell proliferation, migration and invasion

To assess the effects of miR-363 in cell growth, CCK8 assays were adopted. As shown in **Figure 3A**, up-regulation of miR-363 by miR-363 mimic inhibited MG63 cell proliferation, while down-regulation of miR-363 by miR-363 inhibitor enhanced HOS cell growth ($P<0.05$).

Migration and invasion assays were performed to explore the functions of miR-363 in OS cell motility. As shown in **Figure 3B**, miR-363 mimics inhibited MG63 cell migration and invasion. By contrast, miR-363 inhibitor promoted HOS cell migration and invasion ($P<0.05$). These results suggested that miR-363 play-
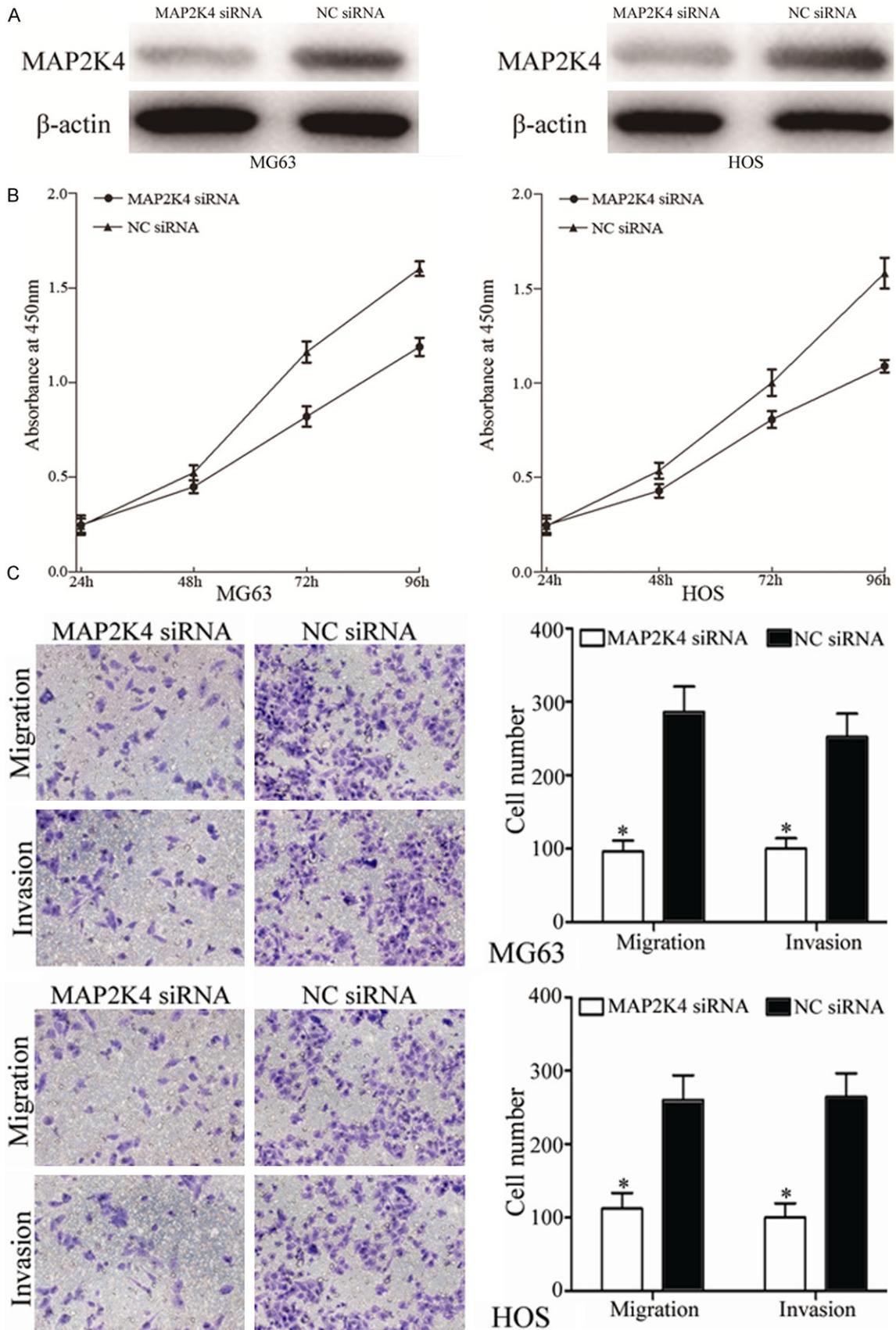
Figure 5. Effects of MAP2K4 in MG63 and HOS cells. A. After transfection with MAP2K4 siRNA, MAP2K4 was significantly down-regulated in MG63 and HOS cells. B. MAP2K4 siRNA inhibited MG63 and HOS cell growth. C. MAP2K4 siRNA decreased MG63 and HOS cell migration and invasion. $^*P<0.05$ compared with the NC groups.

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MAP2K4 was identified as a direct target gene of miR-363 in vitro

To explore the molecular mechanism of miR-363 in OS, TargetScan and miRanda were used. Bioinformatics analysis predicated that MAP2K4 was a target of miR-363. Two potential binding sites for miR-363 were demonstrated which were located at 105-112 bp and 508-515 bp downstream from the 5' end of the MAP2K4 3'-UTR (shown in **Figure 4A**).

Dual-Luciferase report assays were performed to explore whether miR-363 directly targeted to the 3'UTR of MAP2K4. As shown in **Figure 4B**, miR-363 significantly inhibited PGL3-MAP2K4-3'UTR site1 Wt and PGL3-MAP2K4-3'UTR site2 Wt luciferase activity, but not the PGL3-MAP2K4-3'UTR site1 Mut and PGL3-MAP2K4-3'UTR site2 Mut luciferase activity in HEK293T cells (*P*<0.05).

Next, we performed qRT-PCR and western blot of assess the regulatory functions of miR-363 on MAP2K4. qRT-PCR demonstrated that MAP2K4 expression at mRNA level was not altered during these treatments (shown in **Figure 4C**, *P*>0.05). However, western blot showed that MAP2K4 expression at protein level was significantly down-regulated in miR-363 mimics-transfected MG63 cells, whereas MAP2K4 protein was up-regulated in miR-363 inhibitor-transfected HOS cells (shown in **Figure 4D** and **4E**, *P*<0.05). The results suggested that miR-363 negatively regulated MAP2K4 expression at the post-transcriptional level. Taken together, MAP2K4 was a direct target gene of miR-363.

MAP2K4 was involved in miR-363-induced functions in OS cells

MAP2K4 was found up-regulated in OS and over-expression of MAP2K4 was obviously correlated with poor response to treatment, tumor progression and worse overall survival [22]. To further explore whether the suppressive roles of miR-363 on OS cell growth and metastasis

was exerted via MAP2K4, CCK8 assay, migration and invasion assay were performed in MG63 and HOS cells after transfection with MAP2K4 siRNA.

Firstly, after transfection, western blot was adopted to assess transfection efficiency. The results showed that MAP2K4 was both significantly down-regulated in MG63 and HOS cells after transfection with MAP2K4 siRNA compared with cells transfected with NC siRNA (shown in **Figure 5A**; *P*<0.05).

Then, the CCK8 assay revealed that knockdown of MAP2K4 inhibited MG63 and HOS cell growth (shown in **Figure 5B**; *P*<0.05). Furthermore, cell migration and invasion assays demonstrated that silencing of MAP2K4 decreased MG63 and HOS cell migration and invasion abilities (shown in **Figure 5C**; *P*<0.05). These findings suggested that the functions of MAP2K4 siRNA in cell growth and metastasis were similar to those functions exerted by miR-363 in OS cells, rendering MAP2K4 as a functional target of miR-363 in OS.

Discussion

OS are aggressive primary tumor of the bone [23]. With the development of therapy treatments in OS, the 5-year survival rate was increased to approximately 60-70% [1]. However, like to most malignant tumor, recurrence and metastasis usually caused poor prognosis in OS. OS is apt to metastasis and often spreads to pulmonary, bones and brain, even after surgery and chemotherapy [24]. OS patients with metastasis are not suitable for surgery and result in 5-year survival rate of less than 30% [25]. Therefore, it is very emergent to explore the molecular mechanisms governing rapidly growth and metastasis of OS and investigate new targeted therapy for OS growth and metastasis.

Increasing evidences demonstrated that miRNAs involved into cancer growth and metastasis, also including OS [12, 13, 26]. Recent reports suggested that the expression of miR-363 was down-regulated in some cancers,

such as hepatocellular carcinoma [27], colorectal cancer [28], neuroblastoma [29], head and neck squamous cell carcinoma [30] and breast cancer [31]. However, in prostate cancer [32], gastric cancer [33] and uterine leiomyoma [34], the expression of miR-363 was reported to be up-regulated in tumor tissues. These conflicting studies suggested that the expression levels of miR-363 in cancers have tissue specificity. In this study, we found that miR-363 was down-regulated in OS tissues and cell lines. In addition, low expression level of miR-363 was correlated with tumor size, clinical stage and distant metastasis. These findings indicated that miR-363 may act as a tumor suppressor in OS.

miR-363 was proved to act as a tumor suppressor in the malignant phenotype of cancers. For example, in hepatocellular carcinoma, miR-363 targeted S1PR1 to inhibit cell growth. In addition, miR-363 was found to be down-regulated in hepatocellular carcinoma patients with cisplatin-based chemotherapy and cisplatin-resistant hepatocellular carcinoma cell lines. Enforced miR-363 expression blocked cisplatin resistance in hepatocellular carcinoma cells, whereas down-regulation of miR-363 enhanced cell viability during cisplatin treatment [35]. In colorectal cancer, miR-363 suppressed tumourigenesis by directly targeting GATA6 [28]. Qiao et al. reported that endogenous expression of miR-363 decreased clone formation, anchorage-independent growth, and invasion in vitro via blockade of GRP-R [29]. Sun and his colleague found that the expression level of miR-363 was decreased in metastatic head and neck squamous cell carcinoma tissues and highly invasive cell lines. Ectopic expression of miR-363 suppressed head and neck squamous cell carcinoma migration and invasion through down-regulation of PDPN [30].

miR-363 was also demonstrated as an oncogene in cancers. In gastric cancer, miR-363 enhanced cell growth, viability, progression, epithelial-mesenchymal transition and tumorsphere formation by down-regulation of tumor suppressor MBP-1 [33]. In prostate cancer, miR-363 targeted c-myc to promote cell proliferation, transformation property and epithelial-mesenchymal transition [32]. These studies suggested that functions of miR-363 in cancers also have tissue specificity. They also indi-cated that miR-363 may function as a potential therapeutic gene for the treatment of these cancers.

In our study, we found that miR-363 significantly inhibited OS cell growth and motility. In addition, MAP2K4 was identified as a direct target of miR-363 in vitro. Firstly, bioinformatics analysis predicted that MAP2K4 contained miR-363 seed match at position 105-112 and 508-515 of the MAP2K4 3'-UTR. Secondly, Dual-Luciferase report assay demonstrated that miR-363 directly targeted to MAP2K4 3'-UTR. Thirdly, Real-time PCR and western blot revealed that miR-363 negatively regulated MAP2K4 expression at the post-transcriptional level. Finally, knockdown of MAP2K4 also decreased OS cell proliferation, migration and invasion. These results indicated that miR-363 targeted MAP2K4 to decrease OS cell growth, migration and invasion.

MAP2K4, a member of the MAPK signaling pathway, is located on chromosome 17 and encodes a protein of 399 amino acids [36]. It is a dual-specificity protein kinase that phosphorylates serine, threonine and tyrosine residues [37]. MAP2K4 plays functions through typically activates p38 MAPK and c-Jun N-terminal kinase (JNK) [38]. In OS, MAP2K4 was found up-regulated and over-expression of MAP2K4 was obviously correlated with poor response to treatment, tumor progression and worse overall survival, which indicated that MAP2K4 could play important functions in OS carcinogenesis and progression [22]. Therefore, MAP2K4 was served as a potential therapeutic gene for the treatment of OS. In this study, for the first time, we demonstrated that miR-363 targeted MAP2K4 to inhibit OS cell growth and metastasis. Taken together, miR-363/MAP2K4 based targeted therapy could be a novel treatment for OS.

In conclusion, this was the first study to show that miR-363 was down-regulated in OS and significantly associated with tumor size, clinical stage and distant metastasis. We also observed that miR-363 inhibited cell proliferation, migration and invasion. In addition, for the first time, MAP2K4 was identified as a direct target of miR-363. These findings indicated that miR-363 could be a novel target for OS therapy in the future.

Disclosure of conflict of interest

None.

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