Knockdown of IncRNA MEG3 inhibits viability, migration and invasion and promotes apoptosis by sponging miR-127 in osteosarcoma cell†

Running title: Role of MEG3 by sponging miR-127 in osteosarcoma

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Abstract:
Osteosarcoma (OS) is one of the most common bone malignancies and occurs almost exclusively in children and adolescents. This study aimed to explore the role of lncRNA maternally expressed gene 3 (MEG3) in OS cells growth and metastasis, and to uncover the possible underlying mechanism. In this study, the expressions of MEG3 in five OS cell lines (MG63, OS-732, SaOS, G292, and 143B) and in a human osteoblast cell line hFOB1.19 were measured by qRT-PCR analysis. The expressions of MEG3, miR-127 and ZEB1 in OS-732 cells were overexpressed or suppressed by transfection. Cell viability, migration, invasion and apoptosis were then assessed. The results showed that MEG3 was highly expressed in OS cell lines when compared to hFOB1.19 cell. MEG3 silence significantly suppressed OS-732 cells growth and metastasis, as evidenced by the decreases in cell viability, migration, invasion, and increase in apoptotic cell rate. MEG3 acted as an endogenous sponge by binding to miR-127. More interestingly, MEG3 silence could not suppress OS-732 cells growth and metastasis when miR-127 was knocked down. ZEB1 was a target gene of miR-127, and miR-127 overexpression-induced impairments in cell growth and metastasis were attenuated when ZEB1 was overexpressed. Moreover, miR-127 suppression activated JNK and Wnt signaling pathways, while these activations were recovered by ZEB1 silence. To conclude, our findings suggest that lncRNA MEG3 promoted OS cells growth and metastasis in vitro through sponging miR-127. This study provides the evidence that MEG3 may be a potential therapeutic target for OS. This article is protected by copyright. All rights reserved

Keywords: Osteosarcoma; MEG3; miR-127; ZEB1
Introduction

Osteosarcoma (OS) is one of the most common bone malignancies, accounts for about 20% to 35% of all primary bone malignancies, and it occurs almost exclusively in children and adolescents [Jemal et al., 2011; Ottaviani and Jaffe, 2009]. OS is highly aggressive and rapidly metastasizes, the cancer cells can spread to distant sites including liver, bone, brain, and other organs [Wen et al., 2017]. Despite advances in the available treatment modalities including surgery, chemotherapy or combination of both, the survival rate of patients with OS still remains low [Mirabello et al., 2009; Ta et al., 2009], and the molecular mechanisms of this disease are poorly understood [Jin et al., 2017]. Thus, understanding the mechanisms of OS, and finding novel drug targets is of great significance which may help to treat this devastating cancer.

Long non-coding RNAs (lncRNAs) are one kind of endogenous RNA comprises a sequence larger than 200 nucleotides but with no significant or functional open reading frame(s) [Yang et al., 2017]. Various studies have explored the role of lncRNAs in different biological processes like gene expression, cell proliferation, and differentiation [Bartel, 2004; Cerk et al., 2016; Malek et al., 2014; Nicoloso et al., 2009; Palazzo and Lee, 2015; Silva et al., 2015; Zhang et al., 2016a]. LncRNAs are also involved in different types of cancers. For example, lncRNA HOTAIR was upregulated in breast cancer and promoted metastasis [Yan et al., 2015; Zhuang et al., 2015]. Similarly, Zhang and his colleagues demonstrated that increased expression of SPRY4-IT1 in clear cell renal cell carcinoma led to poor prognosis, and vice versa [Zhang et al., 2014]. Aberrant expression of lncRNA maternally expressed gene 3 (MEG3) in several cancers especially in gastric, colorectal, ovarian, cervical cancers, and OS have been reported recently [Cao et al., 2016; Sheng et al., 2014; Tian et al., 2015; Zhang et al., 2016b; Zhou et al., 2015]. However, the precise molecular mechanism(s) behind the altered expression of MEG3 in OS and its function are still unclear.

Similar with lncRNAs, miRNAs are also involved in various physiological and pathological processes including cancer [Bartel, 2004]. Recent studies proposed an interesting viewpoint that lncRNA plays a role in preventing mRNA from degradation by miRNA, as this lncRNA works like sponge in having miRNA exhausted, leaving mRNA alone without being decay [Cai et al., 2017; He et al., 2016]. By doing so, the lncRNA forms specific structures of circular nucleic acid strand, or called circular endogenous RNA (ceRNA). To date, MEG3 has been described to be a
ceRNA that regulate miR-421 [Zhang et al., 2017b], miR-21 [Zhang et al., 2016b], miR-141 [Zhou et al., 2015], and miR-140-5p [Li et al., 2017]. Thus, we speculated that MEG3 may serve as a ceRNA which connecting some other miRNAs in OS.

In this study, we aimed to explore the role of MEG3 in OS growth and metastasis, and to uncover the possible underlying mechanism by analyzing the cross-regulation between MEG3 and miR-127, since the tumor suppressive effect of miR-127 on OS has been revealed previously [Zhang et al., 2016a].

Materials and methods

Cell culture and treatment

Human OS cell lines MG63, 143B G292 and human osteoblast cell line hFOB1.19 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). OS-732 cell line was obtained from Orthopaedics Graduate School in Beijing Jishuitan Hospital. SaOS cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). G292 and SaOS cells were cultured in McCoy’s medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated bovine serum (FBS, Gibco). All other cells were cultured in DMEM (Gibco, Grand Island, NY) containing 10% heat-inactivated FBS. The cells were plated in tissue culture dishes at 37°C in a humidified 5% CO\textsubscript{2} incubator and cultured for 2–4 days until confluence or sub-confluence was reached. Subcultures were prepared using 0.05% trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO) and seeded in 6- or 96-well tissue culture plates.

qRT-PCR

Total RNA was extracted from cells using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer’s instructions. For detection the expression levels of MEG3 and ZEB1 in cells, PrimeScript RT reagent Kit (Promega, Madison, WI, USA) and the One Step SYBR® PrimeScript®PLUS RT PCR Kit (TaKaRa Biotechnology, Dalian, China) were used. The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) were used for testing the expression levels of miR-127 in cells. The internal control genes were GAPDH for MEG3 and ZEB1, and
U6 snRNA for miR-127. Relative expressions of MEG3, miR-127 and ZEB1 were calculated by relative quantification \(2^{-\Delta\Delta Ct}\) method [Livak and Schmittgen, 2001].

Transfection and generation of stably transfected cell lines
Short-hairpin RNA (shRNA) directed against human lncRNA MEG3 was ligated into the pGPU6/GFP/Neo plasmid (GenePharma, Shanghai, China) and was referred as to sh-MEG3. The sequences for MEG3 shRNAs were as follows: shRNA#1, 5’-UUA GGU AAG AGG GAC AGC AGC UGG CUG G-3’; shRNA#2, 5’-GGA AUG AGC AUG CUA CUG AAU-3’. For the analysis of the ZEB1 functions, the full-length ZEB1 sequences and shRNA directed against ZEB1 were constructed in pEX-2 and pGPU6/GFP/Neo plasmids (GenePharma), respectively, and they were referred as to pEX-ZEB1 and sh-ZEB1. The sequences for ZEB1 shRNA are: 5’-GAA CUU GUC UUG CGC AAA ATT-3’. The plasmid carrying a non-targeting sequence was used as a negative control (NC) of sh-MEG3 and sh-ZEB1 that was referred as to sh-NC. miR-127 mimic, inhibitor and their respective NC were synthesized (Life Technologies Corporation, MD, USA) and transfected into cells to alter miR-127 expression in cell. The sequences of miR-127 mimic and inhibitor are: 5’-CUG AAG CUC AGA GGG CUC UGA U-3’ and 5’-AUC AGA GCC CUC UGA GCU UCA G-3’, respectively. The lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) was used for the cell transfection according to the manufacturer’s instructions. The stably transfected cells were selected by the culture medium containing 0.5 mg/ml G418 (Sigma-Aldrich). After approximately 4 weeks, G418-resistant cell clones were established.

Cell viability assay
Cell viability was measured by using a Typan Blue Staining Cell Viability Assay Kit (Beyotime, Shanghai, China). In brief, \(1 \times 10^5\) OS-732 cells were seeded in duplicate in 60-mm dishes. After transfection, \(5 \times 10^3\) cells were collected and stained with 1 ml Trypan Blue Solution at room temperature for 5 min. The stained cells were counted under a hemocytometer, and the ratio of living/total cells was calculated.
Apoptosis assay
Cell apoptosis analysis was performed using Annexin V-FITC Apoptosis Detection Kit (Beyotime). Briefly, 1 × 10^5 transfected cells were collected and washed in phosphate buffered saline (PBS) twice and fixed in 70 % ethanol. Fixed cells were then washed twice in PBS and stained with 5 μl Annexin V-FITC and 10 μl PI in the presence of 50 μg/ml RNase A (Sigma-Aldrich), and then incubated for 1 h at room temperature in the dark. Flow cytometry analysis was done by using a FACS can (Beckman Coulter, Fullerton, CA, USA). The data were analyzed by using FlowJo software (Treestar, San Carlos, CA).

Migration and invasion assay
Cell migration was determined by using a modified two-chamber migration assay with a pore size of 8 mm. For migration assay, cells suspended in 200 ml of serum-free medium were seeded on the upper compartment of 24-well Transwell culture chamber, and 600 ml of complete medium was added to the lower compartment. After incubation at 37°C, cells were fixed with methanol. Non-traversed cells were removed from the upper surface of the filter carefully with a cotton swab. Traversed cells on the lower side of the filter were stained with crystal violet and counted.

The invasion behavior of was determined using 24-well Millicell Hanging Cell Culture inserts with 8 mm PET membranes (Millipore, Bedford, Massachusetts, USA). Briefly, after the cells were treated for indicated condition, 5 × 10^4 cells in 200 μl serum-free DMEM medium were plated onto the upper chamber, while complete medium containing 10% FBS was added to the lower chamber. After processing the invasion chambers for 48 hours (37 °C, 5% CO2) in accordance with the manufacturer’s protocol, the non-invading cells were removed with a cotton swab; the invading cells were fixed in 100% methanol and then stained with crystal violet solution and counted microscopically. The data are presented as the average number of cells attached to the bottom surface from five randomly chosen fields.

Reporter vectors constructs and luciferase reporter assay
The fragment from MEG3 and ZEB1 containing the predicted miR-127 binding site was amplified by PCR and then cloned into a pmirGlo Dual-luciferase miRNA Target Expression
Vector (Promega, Madison, WI, USA) to form the reporter vector MEG3-wild-type (MEG3-Wt) and ZEB1-wt, respectively. To mutate the putative binding site of miR-127 in the MEG3 and ZEB1, the sequence of putative binding site was replaced and was named as MEG3-mutated-type (MEG3-mt), and ZEB1-mt. Then the vectors and miR-127 mimics were co-transfected into cells, and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) were used for testing the luciferase activity.

**Western blot**

The protein used for western blotting was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Basel, Switzerland). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer’s instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4°C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 hour at room temperature. After rinsing, the Polyvinylidene Difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

**Statistical analysis**

All experiments were repeated three times. The results of multiple experiments are presented as mean ± standard deviation (SD). Statistical analyses were performed using Graphpad 6.0 statistical software (GraphPad Software Inc., San Diego, CA). P-values were calculated using one-way analysis of variance (ANOVA). A P-value of < 0.05 was considered to indicate a statistically significant result.
Results

Expression of MEG3 in different OS cell lines
Initially, we measured the expression of lncRNA MEG3 in five OS cell lines (MG63, OS-732, SaOS, G292, and 143B) and a human osteoblast cell line (hFOB1.19) to reveal whether MEG3 was abnormal expressed in OS cell lines. qRT-PCR analytical results showed that, the expression levels of MEG3 in these five OS cell lines were much higher than the level in hFOB1.19 cells ($P < 0.05$, and $P < 0.01$, Figure 1). This finding suggested that MEG3 was highly expressed in OS cell lines and hit us that MEG3 might be involved in the tumorigenesis and development of OS.

Suppression of MEG3 inhibited cell viability, migration, invasion, and promoted apoptosis in OS-732 cells
To explore the functional effects of MEG3 on OS-732 cells, the expressions of MEG3 in OS-732 cells were altered by transfection with two different sequences of MEG3 shRNA (sh-MEG3#1 and sh-MEG3#2). After transfection, the expression of MEG3 in cells was measured by qRT-PCR, and we found that both sh-MEG3#1 and sh-MEG3#2 significantly reduced MEG3 expression when compared to the shNC group ($P < 0.01$ or $P < 0.001$, Figure 2A). The expression of MEG3 was much lower in sh-MEG#1 group of cells than in sh-MEG#2 group of cells, and thus sh-MEG3#1 transfected cells were used as a test group for the following experiments. Next, we detected the impacts of MEG3 silence on OS-732 cells viability, migration, invasion and apoptosis. Data given in Figure 2B-2G showed that sh-MEG3#1 significantly decreased cell viability ($P < 0.05$), migration ($P < 0.05$), invasion ($P < 0.05$), and increased apoptotic cell rate ($P < 0.001$); besides, metastasis-related proteins S100A4, MMP-2 and MMP-2, and anti-apoptotic protein Bcl-2 were downregulated, while pro-apoptotic protein Bax and cleaved capsase-3 and -9 were upregulated by sh-MEG3#1. These data indicated the anti-growth and anti-metastatic roles of MEG3 silence on OS-732 cells.

MEG3 acted as a ceRNA for miR-127
An increasing literature has evidenced that MEG3 can act as a ceRNA via sponging several miRNAs [Zhang et al., 2017b][Li et al., 2017; Zhang et al., 2016b; Zhou et al., 2015]. Herein, we
detected the cross-regulation between MEG3 and miR-127, since in a recent literature the tumor suppressive functions of miR-127 on OS has been reported [Zhang et al., 2016a]. We found that the expression level of miR-127 was significantly overexpressed after MEG3 was silenced ($P < 0.01$, Figure 3A). To verify whether miR-127 was able to bind to the sequences of MEG3 in BON1 cells, MEG3-wt and MEG3-mt containing the wild-type and mutant binding sequences of miR-127 was generated, respectively (Figure 3B). Dual-luciferase reporter assay revealed that the luciferase activity was significantly reduced by co-transfection with MEG3-wt and miR-127 mimic ($P < 0.05$, Figure 3C). However, no significant difference change of luciferase activity was observed after co-transfection with MEG3-mt and mimic NC ($P > 0.05$). Thus, we inferred that MEG3 may serve as an endogenous sponge of miR-127.

**Role of miR-127 in cell viability, migration, invasion, and apoptosis of OS-732 cell**

To detect whether miR-127 was implicated in MEG3-modulated cell growth and metastasis of OS-732 cells, sh-MEG3#1 and miR-127 inhibitor were co-transfected into OS-732 cells. We found that sh-MEG3#1-induced deceases in cell viability, migration and invasion, and sh-MEG3#1-induced increase in apoptotic cell rate were all attenuated by addition of miR-127 inhibitor ($P < 0.05$, or $P < 0.01$, Figure 4A-4C and 4E). Meanwhile, sh-MEG3#1-induced downregulations of S100A4, MMP-2, MMP-9, Bcl-2, and sh-MEG3#1-induced upregulations of Bax, cleaved capsase-3, and -9 were all remarkably abolished by addition of miR-127 inhibitor (Figure 4D and 4F). These data indicated that MEG3 silence suppressed OS cells growth and metastasis possibly via negative regulation of miR-127.

**ZEB1 was a target of miR-127**

A recent study has reported that ZEB1 was frequently upregulated in OS tissues, and it acted as an oncogene in OS cells [Li et al., 2016a]. Herein, we detected whether ZEB1 was involved in miR-127 modulated OS-732 cells growth and metastasis. qRT-PCR and western blotting analyses results showed that, the expressions of ZEB1 were downregulated in cells which were transfected with miR-127 mimic ($P < 0.05$), and while were upregulated in miR-127 inhibitor transfected cells ($P < 0.01$, Figure 5A-5B). Furthermore, ZEB1-wt and ZEB1-mt containing the wild-type and mutant binding sequences of miR-127 within the 3’UTR of ZEB1 was generated,
respectively (Figure 5C), and the luciferase activity was significantly decreased by co-transfection with ZEB1-wt and miR-127 mimic ($P < 0.05$, Figure 5D). No significant change of luciferase activity was observed after co-transfection with ZEB1-mt and mimic NC ($P > 0.05$). These data suggested that ZEB1 was a target of miR-127, and it was negatively regulated by miR-127 in OS-732 cells.

**Role of ZEB1 in cell viability, migration, invasion, and apoptosis of OS-732 cells**

As ZEB1 was a target of miR-127 in OS cells, we speculated that ZEB1 might be involved in miR-127-mediated inhibition of OS cells growth and metastasis. To test this hypothesis, miR-127 mimic and a ZEB1 expressing vector (pEX-ZEB1) were co-transfected into OS-732 cells. qRT-PCR and western blot analytical results showed that both the mRNA and protein levels of ZEB1 were reduced by miR-127 mimic ($P < 0.05$), and the levels were upregulated after the addition of pEX-ZEB1 ($P < 0.001$, Figure 6A-6B), indicating the highly efficiency of transfection. Data in Figure 6C-6F showed that, miR-127 overexpression-induced decreases in cell viability, migration, invasion, and miR-127 overexpression-induced increase in apoptotic cell rate were all attenuated when ZEB1 was overexpressed ($P < 0.05$, or $P < 0.01$). Moreover, western blot analytical results showed that miR-127 overexpression-induced downregulation of Bcl-2, upregulation of Bax, and activations of caspase-3 and caspase-9 were abolished when ZEB1 was overexpressed (Figure 6G). Based on these data, we inferred miR-127 overexpression suppressed OS cells growth and metastasis possibly via targeting ZEB1.

**Role of miR-127 in JNK and Wnt signaling pathways**

To further explore the underling mechanism(s) via which miR-127 and ZEB1 mediated OS cells growth and metastasis, we focused on JNK and Wnt pathways. We found that overexpression of miR-127 led to decreased expressions of p-JNK, p-c-Jun, Wnt3a, Wnt5a, and β-Catenin, while miR-127 suppression affected these proteins resulted in completely opposite impacts (Figure 7A-7B), suggesting the blockage effect of miR-127 on JNK and Wnt pathways. Moreover, miR-127 suppression-induced upregulations of p-JNK, p-c-Jun, Wnt3a, Wnt5a, and β-Catenin were all recovered when ZEB1 was overexpressed, indicating miR-127 blocked JNK and Wnt signaling pathways via negative regulation of ZEB1.

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**Discussion**

Although several studies have described the role of lncRNA MEG3 in different types of cancers, only few studies have explored the role MEG3 in OS [Cao et al., 2016; Guo et al., 2013; Sheng et al., 2014; Sun et al., 2016; Zhang et al., 2016b; Zhou et al., 2015]. In this study, we explored the functional effects of MEG3 on OS and detected the cross-regulation between MEG3 and miR-127, which may provide a better understanding of MEG3 in OS.

MEG3, a newly recognized lncRNA, is widely expressed in normal tissues while it was lost in an expanding list of primary human tumors [Sheng et al., 2014; Sun et al., 2014; Yin et al., 2015; Zhang et al., 2017a]. In a recent literature, MEG3 also been reported to low express in OS tissues when compared with adjacent non-tumor tissues [Tian et al., 2015]. In contrast to this previous study, we demonstrated that MEG3 was highly expressed in five kinds of OS cell lines (MG63, OS-732, SaOS, G292, and 143B) when compared to osteoblast cell line hFOB1.19. This discrepancy may be due to that we measured the expression of MEG3 in OS cell lines, while Tian et al. measured it in OS tissues. Anyway, our study together with the study of Tian et al. hit us that abnormal expression of MEG3 may be involved in the occurrence and development of OS.

MEG3 has been verified in several tumors to function as tumor suppressors. For instance, upregulation of MEG3 inhibited breast cancer cells proliferation and invasion capacities [Zhang et al., 2017b]. MEG3 was able to inhibit cell growth and induced apoptosis in esophageal squamous cell carcinoma EC109 cells [Huang et al., 2017]. However, to our knowledge, there is only one literature reported the functional impacts of MEG3 on OS cells, and in which MEG3 was indicated as a tumor suppressor [Sun et al., 2016]. Our data were inconsistence with the study of Sun et al., indicated that MEG3 exerted tumor-promoting activity in OS, as MEG3 silencing reduced OS-732 cells viability, migration, invasion, and induced apoptosis. This discrepancy may be due to, the cell line used in this study was OS-732 while Sun et al. used were MG63 and HOS cells. The phenomenon of this discrepancy called for intense efforts in the search of MEG3 on other immortalized OS cell lines and primary OS cells, which may explain MEG3 exerted either tumor suppressive or promoting role in OS.

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miR-127 has been reported to function as a tumor suppressor in a variety of human cancers, including prostate cancer and breast cancer [Saito et al., 2006; Wang et al., 2014]. miR-127 also been demonstrated to be low expressed in OS tissues and cell lines, and that overexpression of miR-127 significantly inhibited OS cells proliferation, migration and invasion [Zhang et al., 2016a]. Consistence with these previous, our data also suggested that miR-127 exhibited anti-tumor activities, as OS cells viability, migration, and invasion were significantly increased, and apoptosis was induced by miR-127 downregulation. Moreover, dual-luciferase reporter assay revealed that MEG3 could act as an endogenous sponge by directly binding to miR-127 and suppressed miR-127 expression. Actually, previous studies have demonstrated the cross-regulation between MEG3 and miR-144 in gastric cancer [Zhou et al., 2015], and between MEG3 and miR-26a in tongue squamous cell cancer [Jia et al., 2014]. However, our results for the first time indicated MEG3 can crosstalk with miR-127, and MEG3 silence suppressed OS cells growth and metastases via sponging miR-127.

ZEB1, also known as TCF8 or delta EF1, is a transcriptional repressor of E-cadherin and polarity factor genes [Burk et al., 2008]. Expression of ZEB1 promotes metastasis of tumor cells in a mouse xenograft model, indicating a role of ZEB1 in invasion and metastasis of human tumors [Spaderna et al., 2008]. Recently, ZEB1 was found to be significantly higher in the OS tissues when compared to their matched adjacent normal tissues, and inhibition of ZEB1 expression suppressed the proliferation, migration, and invasion of OS cells [Li et al., 2016a]. In this study, we found that miR-127 could target ZEB1. Overexpression of ZEB1 reversed miR-127 overexpression-induced decreases of cell viability, migration, invasion, and reversed miR-127 overexpression-induced apoptosis in OS-732 cells. Based on these findings we speculated that miR-127 suppressed OS cells growth and metastases possibly via negative regulation of ZEB1. Evidence showed that JNK is a master protein kinase that plays an important role in osteoblast proliferation, differentiation and apoptosis, and it is well-established that JNK pathway is involved in pathogenesis and therapeutics of OS [Li et al., 2016b]. In addition to JNK, Wnt is also known as an important regulator in osteoblastogenesis, and it is believed that antagonizing the Wnt pathway might yield inhibition of OS cells as osteoblastogenesis is impaired [Angulo et al., 2017]. Numerous scientific evidences have implicated miRNAs as regulators of the Wnt-signaling pathway in the context of many cancers, including hepatocellular carcinoma, breast...
cancer, and meningiomas [Drago-Ferrante et al., 2017; Saydam et al., 2009; Wang et al., 2012]. Besides, the network involving both miR-127 and JNK signaling pathway have been implicated in regulating tumorigeneses in hepatocellular carcinoma [Yang et al., 2013]. In this study, we demonstrated that miR-127 suppression upregulated the expressions of p-JNK, p-c-Jun, Wnt3a, Wnt5a, and β-Catenin, and while these upregulations induced by miR-127 suppression were abolished by ZEB1 silence. These data suggested that miR-127 blocked JNK and Wnt pathways via negatively regulation of ZEB1, and the tumor suppressive role of miR-127 was via suppression of JNK and Wnt pathways.

This study revealed one of the possible mechanisms involving the tumor-promoting functions of MEG3 on OS cells (Figure 8). In OS-732 cell line, MEG3 acted as a ceRNA for miR-127, and ZEB1 was a target gene of miR-127. MEG3 exerted tumor-promoting functions at least in part via sponging miR-127, leaving ZEB1 alone without being decay, and thus activated JNK and Wnt signaling pathways.

In conclusion, our findings suggest that lncRNA MEG3 might be contribute to the development of OS. MEG3 silence suppressed OS cells viability, migration, invasion, and induced apoptosis in vitro through inhibiting miR-127 pathway. This study provides the evidence that MEG3 may be a potential therapeutic target for OS.
References


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Figure legends

Figure 1 LncRNA MEG3 was highly expressed in OS cell lines. The expression of MEG3 in five OS cell lines (MG63, OS-732, SaOS, G292, and 143B) and in a human osteoblast cell line hFOB1.19 was measured by qRT-PCR analysis. Data represented mean ± SD. *P < 0.05, ***P < 0.001 vs. hFOB1.19 cells.

Figure 2 Suppression of MEG3 inhibited cell viability, migration, invasion, and promoted apoptosis in OS-732 cells. (A) OS-732 cells were transfected with two different sequences of MEG3 shRNA (sh-MEG1#1 and sh-MEG1#2), the expression of MEG3 in the transfected cells were then measured by qRT-PCR. OS-732 cells were transfected with sh-MEG1#1, and then (B) cell viability, (C) migration, (D) invasion, (E) the expressions of metastasis-related proteins (F) apoptosis, and (G) the expressions of apoptosis-related proteins were respectively assessed. Data represented mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs. shNC group.

Figure 3 MEG3 acted as a ceRNA for miR-127. (A) OS-732 cells were transfected with MEG3 targeted shRNA (sh-MEG1#1), the expression of miR-127 in cells was then measured by qRT-PCR. (B) The sequence of miR-127 and the binding site in the sequence of MEG3. (C) Dual-luciferase reporter assay was performed to detect whether miR-127 could bind to MEG3. Data represented mean ± SD. *P < 0.05 vs. mimic NC; **P < 0.01 vs. shNC.

Figure 4 Suppression of MEG3 inhibited OS-732 cells growth and metastasis via sponging miR-127. OS-732 cells were co-transfected with MEG3 targeted shRNA (sh-MEG1#1) and miR-127 inhibitor. Thereafter, (A) cell viability, (B) migration, (C) invasion, (D), the expression of metastasis-related proteins (E) apoptosis, and (F) the expressions of apoptosis-associated proteins were respectively assessed. Data represented mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5 ZEB1 was a target of miR-127. The (A) mRNA and (B) protein levels of ZEB1 in OS-732 cells, which were transfected with miR-127 mimic or miR-127 inhibitor, were detected by
qRT-PCR and western blot analyses. (C) The sequence of miR-127 and the binding site in the 3’UTR of ZEB1. (D) Dual-luciferase reporter assay was performed to detect whether ZEB1 was a target of miR-127. Data represented mean ± SD.* P < 0.05, ** P < 0.01.

**Figure 6** miR-127 overexpression inhibited OS-732 cells growth and metastasis via targeting ZEB1. OS-732 cells were co-transfected with miR-127 mimic and ZEB1 expressing vector (pEX-ZEB1). Thereafter, the (A) mRNA, and (B) protein levels of ZEB1 in cells were detected by qRT-PCR and western blot analyses. (C) Cell viability, (D) migration, (E) invasion, (F) apoptosis, and (G) the expression of apoptosis-associated proteins were respectively assessed. Data represented mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001.

**Figure 7** miR-127 blocked JNK and Wnt signaling pathways via negative regulation of ZEB1. OS-732 cells were co-transfected with miR-127 inhibitor and ZEB1 targeted shRNA (sh-ZEB1). The expression of main factors in (A) JNK, and (B) Wnt signaling pathways were tested by western blot analysis.

**Figure 8** Schematic diagram of the regulatory relations between MEG3 and miR-127, and between miR-127 and ZEB1 revealed in this study.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

A. Relative expression of ZEB1/control

B. ZEB1

C. Cell viability (% of control)

D. Relative migration (%)

E. Relative invasion (%)

F. Apoptotic cells (%)

G. Western blot analysis of Bcl-2, Bax, pro-Caspase-3, cleaved-Caspase-3, pro-Caspase-9, cleaved-Caspase-9, and GAPDH.
Figure 7
Figure 8