

## EXPERIMENTAL STUDY

# MEG3 long non-coding RNA prevents cell growth and metastasis of osteosarcoma

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**OBJECTIVE:** This study aimed to investigate the role of long non-coding RNA MEG3 (lncRNA MEG3) in osteosarcoma (OS) and further explore the underlying molecular mechanism.

**MATERIALS AND METHODS:** The expression profiles of MEG3 in OS cell lines and normal osteoblast cell line were detected by qRT-PCR. MEG3 was over-expressed in OS cell line by using LV-MEG3. MTT and colony-formation assays were applied for cell proliferation analysis. Cell migration assay was applied to investigate the cell migration ability. In addition, the expression levels of cell growth and metastasis related factors (Notch1, Hes1, TGF- $\beta$ , N-cadherin and E-cadherin) were determined to illustrate the mechanisms.

**RESULTS:** We found that compared with normal osteoblast hFOB1.19 cell line, MEG3 was significantly down-regulated in MG63 and U2OS cell lines, particularly in MG-63 cells. MEG3 was significantly up-regulated in MG63 cells by LV-MEG3. Cell proliferation and migration ability were obviously repressed by MEG3 over-expression. In addition, MEG3 over-expression markedly inhibited Notch1, Hes1, TGF- $\beta$  and N-cadherin expression, and the expression level of E-cadherin was improved.

**CONCLUSIONS:** These results indicated that MEG3 could prevent cell growth and metastasis of OS by repressing Notch and TGF- $\beta$  signaling pathway, thus providing a potential therapeutic target for OS treatment (Tab. 1, Fig. 4, Ref. 30). Text in PDF [www.elis.sk](http://www.elis.sk).

**KEY WORDS:** lncRNA MEG3, osteosarcoma, cell growth, metastasis.

**Introduction**

Osteosarcoma (OS), the most common primary malignant bone tumor in children and adolescents, is frequently derived from the long bones and characterized by immature bone or osteoid tissue production by malignant cells (1–3). OS has a very high degree of malignancy and a poor prognosis. Even with surgery combined with chemotherapy regimens, the main method for OS treatment, the patient's annual survival rate is only 55–68 % (2, 4). The main reason for the poor prognosis of OS is the early occurrence of metastases. Patients with OS suffer from soft tissue swelling and severe pain which seriously affect the quality of patient's life. Therefore, OS treatment has become an urgent problem for researchers to solve. Treatment at gene level has always been a hot spot in OS research, but its specific mechanism still needs further study, and finding effective therapeutic targets is the key to gene therapy.

Long non-coding RNAs (lncRNAs), a class of transcribed RNA of the genome without protein-coding potential, more than 200 nucleotides in length, play an important role in regulating gene

expression by different mechanisms (5–7). Recent studies have demonstrated the expression profiles and functions of lncRNAs in various cancers (8). For example, lncRNA HOTAIR has been found to be increased in breast tumors and can promote metastasis via the regulation of Polycomb Repressive Complex 2 (9). High SPRY4-IT1 expression has been observed in renal cancer and down-regulation of SPRY4-IT1 reduces renal cancer cell proliferation, invasion and migration (10). EWSAT1 and SNHG12 were all found to be up-regulated in OS and act as tumor promoter (11, 12).

MEG3, a well studied lncRNA, is encoded by the MEG3 transcript of the DLK1/MEG3 locus on human chromosome, or Meg3 on mouse chromosome (13). A number of studies have demonstrated that lncRNA MEG3, a tumor suppressor gene, was down-regulated in various cancers and was associated with poor prognosis (14–18). A previous study indicated that the expression of lncRNA MEG3 was clearly lower in osteosarcoma tissues compared with adjacent non-tumor tissues (19). However, the exact role of lncRNA MEG3 in OS and the underlying molecular mechanism remains unclear. Thus, in the present study, we aim to investigate the role of lncRNA MEG3 in OS and further explore the underlying molecular mechanism.

**Materials and methods***Cell culture*

The human osteosarcoma (HOS) cell lines (MG63 and U2OS), and normal osteoblast hFOB1.19 cell lines were purchased from

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American Type Culture Collection (ATCC). MG63 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, America) containing 10 % fetal bovine serum (FBS) (Gibco, America), 100 U penicillin and 100 mg/mL streptomycin (Invitrogen). The U2OS cells were cultured in RPMI 1640 medium. The hFOB1.19 cells were cultured in F12/DMEM (v/v: 1:1, Hyclone) medium supplemented with 2.5 mM/L glutamine (without phenol red), 10 % FBS and 0.3 mg/ml G418 (Sigma, St. Louis, MO, USA).

#### Plasmids and cell transfection

To over-express MEG3 in MG63 cells, lentivirus expressing whole MEG3 (LV-MEG3) and an empty vector (LV-GFP) were obtained from Genscript (Nanjing, China). The LV-MEG3 or LV-GFP was transfected into MG63 cells by using Lippo2000 (Invitrogen) according to the manufacturer's instructions. After incubation for 48h, cells were collected for following experiments. To verify the stable over-expression of MEG3 in MG63 cells, real time PCR analysis was applied. Each experiment was repeated three times.

#### MTT assay

In the present study, cell viability was measured by performing MTT assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma). In short, cells were seeded into 96-well plates ( $3 \times 10^3$  cells per well), after incubation for certain times, 20  $\mu$ l MTT was added to each well and then incubated for another 4h at 37 °C. Cell viability was assessed by detecting the absorbance at 490 nm using a microplate reader. All tests were performed in quadruplicate.

#### Colony-formation assay

For colony formation assay, the transfected cells (200 per well) were plated in a 6-well plate. The cells were cultured for 14 days under the standard culture conditions, and cell culture medium was replaced every 2–3 days. After fixing with methanol, stained by 0.5 % crystal violet, formed colonies were photographed by Nikon camera (Nikon, Japan). All tests were performed at least three times.

**Tab. 1. Primer sequence for PCR.**

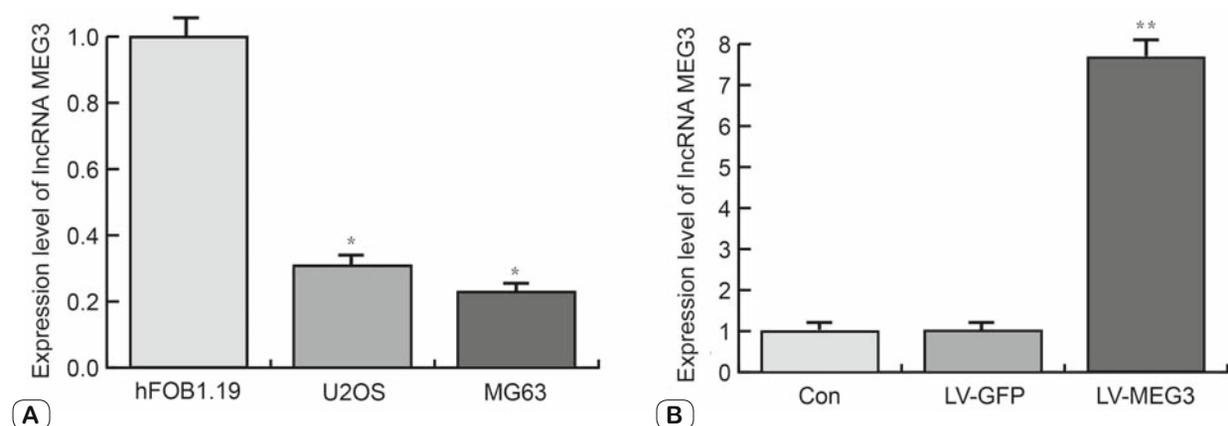
Sequence (5'-3')
TGF- $\beta$ -Forward: 5'GCTGTGAAGCCTTGAGAGTAATGG3'
TGF- $\beta$ -Reverse: 5'TTCCTGTTGACTGAGTTGCGATAA3'
N-cadheren-Forward: 5'GACAATGCCCTCAAGTGTT3'
N-cadheren-Reverse: 5'CCATTAAGCCGAGTGATGGT3'
E-cadheren-Forward: 5'CAATGCCGCCATCGCTTAC3'
E-cadheren-Reverse: 5'ATGACTCCTGTGTTCTCTGTTAATG3'
Notch1-Forward: 5'AGT GCG ACT GTG ACC CTG G3'
Notch1-Reverse: 5'GAC ATG GGT TGG ACG CAC AC3'
Hes1-Forward: 5'AGA TAG CTC GCG GCA TTC C3'
Hes1-Reverse: 5'ACC TCG TTC ATG CAC TCG C3'
MEG3-Forward: 5'CTGCCCATCTACACCTCACG 3'
MEG3-Reverse: 5'CTCTCCGCCGTCTGCGCTAGGGGCT3'
GAPDH-Forward: 5'AGCCACATCGCTCAGACAC3'
GAPDH-Reverse: 5'GCCCAATACGACCAAATCC3'

#### Cell migration assay

To investigate the cell migration ability in different groups, cell migration assay was applied. Shortly, 48 h after cell transfection, cells were trypsinized and then re-seeded ( $1.0 \times 10^5$  cells per well) into serum-free DMEM in the upper chamber of a 24-well transwell (Corning, America). And DMEM supplemented with 20 % FBS was added into the lower chambers. After incubation for 24 h, cells in the upper chamber were removed, and the cells in the lower chambers were stained by crystal violet. Tests were performed in triplicate.

#### Quantitative real time PCR

Total RNA from cells was isolated using the TRIzol reagent (Invitrogen, America) following the manufacturer's instructions. Reverse transcription assay was carried out by using the cDNA Synthesis SuperMix (Trangene, Beijing, China) according to the manufacturer's instructions. Real-time PCR was applied with ABI 7500 Real-time PCR instrument using the SYBR Green Master Mixture (Roche, America) reagent. GAPDH served as an internal control. The  $2^{-\Delta\Delta Ct}$  method was performed for the calculation



**Fig. 1. Expression level of MEG3 in OS cells.** The expression level of MEG3 in OS cells and the normal osteoblast cell was detected by qRT-PCR. A: expression level of MEG3 in OS cells (MG63 and U2OS) and the normal osteoblast cell (hFOB1.19); B: expression level of MEG3 in MG63 cells in different groups. \*  $p < 0.05$  vs hFOB1.19; \*\*  $p < 0.01$  vs Con; ##  $p < 0.01$  vs LV-GFP.

of the relative expression of the genes. Primers used for PCR are listed in Table 1.

#### Western blotting

Cells were collected and lysed in RIPA buffer containing protease inhibitor cocktail. Cell lysates were centrifuged, and the supernatant was harvested. Protein samples were resolved by subjecting to 10% SDS-PAGE and then transferred to a nitrocellulose membrane. After blocked with 5 % fat-free milk for 1 h at room temperature, the membranes were incubated with the primary antibodies (TGF- $\beta$ /N-cadheren/ E-cadheren/Notch1/Hes1, 1:1000, CST, USA;  $\beta$ -actin, 1:2000, CST, USA) at 4°C overnight. Then, the membranes were incubated with a secondary antibody at room temperature for 2 h. The ECL detection system (Thermo Scientific, Waltham, MA, USA) was applied for the visualization of the bands.

#### Statistical analysis

Data were present as means  $\pm$  SD. SPSS 16.0 software was carried out to calculate differences among groups by using one-way analysis of variance (ANOVA) or Student's test. A value of  $p < 0.05$  was considered significant.

## Results

#### MEG3 is down-regulated in OS cell line

The expression level of lncRNA MEG3 in human osteosarcoma cell line MG-63, U2OS and osteoblast cell line hFOB1.19 was detected by qRT-PCR, and the results are shown in Figure 1A. Significant down-regulation of MEG3 was observed in OS cell lines compared with the normal osteoblast hFOB1.19 cell line. As MEG3 is less expressed in OS MG63 cells than in U2OS cells, MG63 cell line was selected for following experiments.

To investigate the role of MEG3 in OS cells, MEG3 was over-expressed in MG63 cells by transfection with LV-MEG3. As shown in Figure 1B, MEG3 was effectively up-regulated in MG63 cells by LV-MEG3.

#### MEG3 over-expression inhibits MG63 cell proliferation

48 h after cell transfection, MG63 cell proliferation was determined by performing MTT and colony-formation assays. We

found that cell viability and cell clonality were obviously lower in LV-MEG3 MG63 cells than in LV-GFP and control MG63 cells (Fig. 2). The data indicated that MEG3 over-expression could prevent MG63 cell proliferation.

#### MEG3 over-expression inhibits MG63 cell migration

Cell migration ability of MG63 cells after cell transfection was detected by performing cell migration assay using a 24-well transwell. As shown in Figure 3, compared with the controls, the cell migration ability was markedly decreased in MG63 cells transfected with LV-MEG3. This data indicated that over-expression of MEG3 could inhibit MG63 cell migration.

#### MEG3 over-expression affects the expression of tumor related factors

To further explore the underlying molecular mechanisms of the functional role of MEG3 in OS cells, the expression levels of tumor related factors (TGF- $\beta$ , N-cadheren, E-cadheren, Notch1 and Hes1) were determined by qRT-PCR and western blotting, respectively. As shown in Figure 4, both the mRNA and protein expression levels of TGF- $\beta$ , N-cadheren, Notch1 and Hes1 were decreased when MEG3 was over-expressed. And E-cadheren was significantly up-regulated when MEG3 was over-expressed in MG63 cells.

## Discussion

LncRNAs are involved in almost all cellular events, including cell proliferation, differentiation, apoptosis, etc. Accumulating evidence indicated that LncRNAs play critical roles in the progression of tumor. LncRNA MEG3, which is considered as a tumor suppressor gene, is well studied in a variety of caners (20). MEG3 was down-regulated in cervical cancer, and is a potential marker for diagnosis and prognosis of patients with cervical cancer (21). Ectopic expression of MEG3 in nasopharyngeal carcinoma cells could inhibit cell proliferation, colony formation and induce cell cycle arrest (22). Guo et al found that MEG3 inhibits the proliferation of endometrial carcinoma cells via preventing Notch signaling pathway (23). Luo et al reported that MEG3 acts as a cancer inhibitor by inhibiting cell proliferation and inducing

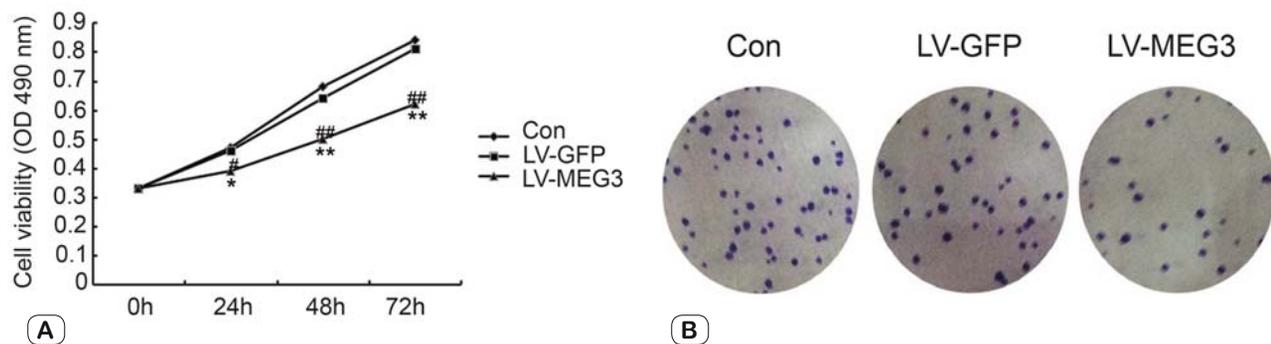


Fig. 2. Effect of MEG3 on MG63 cell growth. 48 h after cell transfection, MG63 cell viability and colony formation ability were determined by MTT (A) and Cell colony formation assay (B) respectively. \*, \*\*  $p < 0.05$ ,  $0.01$  vs Con. #, ##,  $p < 0.05$ ,  $0.01$  vs LV-GFP.

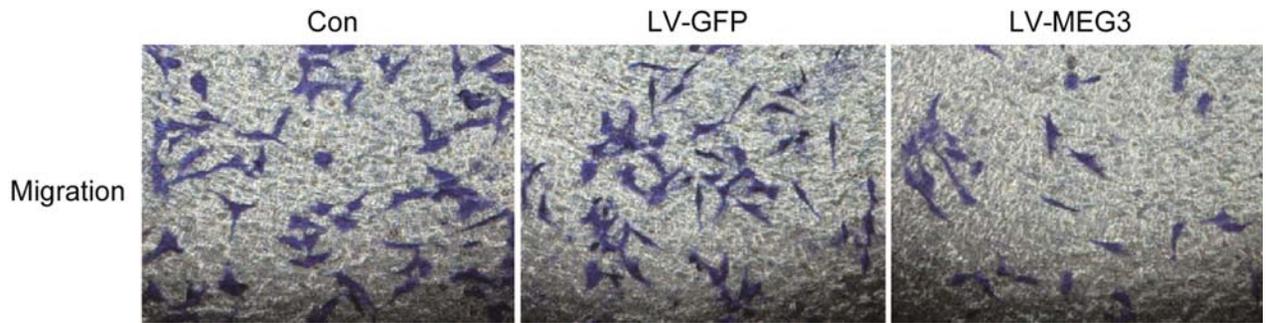


Fig. 3. Effect of MEG3 on MG63 cell migration. 48 h after cell transfection, MG63 cell migration ability was analyzed by cellular migration transwell assay.

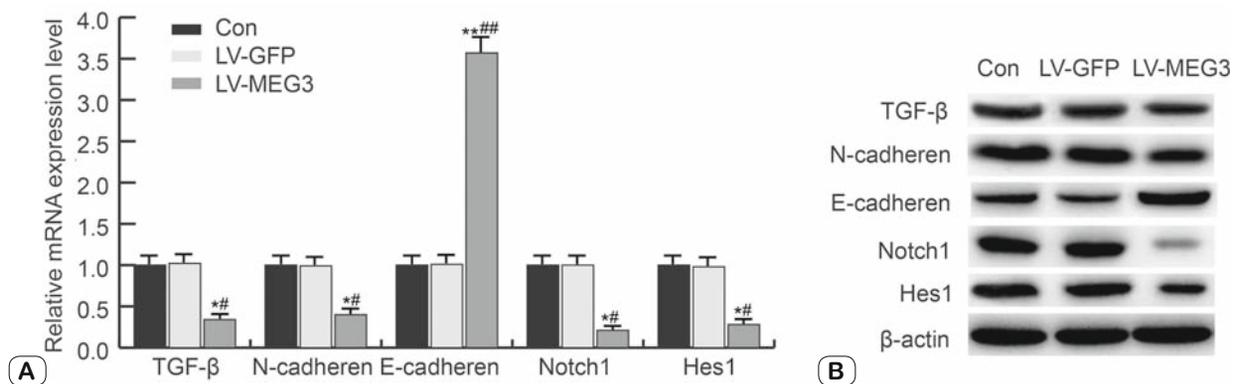


Fig. 4. Effect of MEG3 on tumor related factors expression. 48 h after cell transfection, the expression levels of TGF-β, N-cadheren, E-cadheren, Notch1 and Hes1 were measured by qRT-PCR (A) and western blot analysis (B) respectively. \*, \*\*  $p < 0.05$ , 0.01 vs Con. #, ##  $p < 0.05$ , 0.01 vs LV-GFP.

apoptosis in prostate cancer (24). Zhu et al suggested that MEG3 functions as a tumor suppressor in hepatoma cells through interacting with p53 protein (18). In addition to these studies, the role of MEG3 has been investigated in gallbladder cancer, NSCLC, neuroendocrine tumor, gastric cancer, thyroid carcinoma and so on (25, 14–17). However, to the best of our knowledge, the potential roles of MEG3 in OS remain largely unknown.

As lower expression of MEG3 has been verified in osteosarcoma tissues compared with adjacent non-tumor tissues in a previous study, in the present study, we first detected the expression level of MEG3 in human osteosarcoma cell lines (MG-63 and U2OS) and the normal osteoblast cell line (hFOB1.19). We found MEG3 is low expressed in the osteosarcoma cell line MG-63 and U2OS, especially in MG-63 cell line. We next investigated the effects of MEG3 on MG63 cell growth and migration by performing MTT, colony-formation assay and cellular migration transwell assay via MEG3 over-expression. The results of our study indicated that MEG3 over-expression significantly repressed the growth and migration ability of MG63 cells. Thus, we confirmed the tumor suppressor role of MEG3 in OS process which is consistent with previous research.

Notch signaling pathway has been demonstrated to be involved in the development of various cancers, and is a potential therapeutic target for cancer treatment (26, 27). TGF-β signaling

pathway, one of the most important pathways in tumor cell EMT (an important factor of tumor cell metastasis), plays an important role in controlling tumor cell metastasis (28, 29). Previous studies have proved that MEG3 is related to Notch and TGF-β signaling pathways (23, 30). To further explore the mechanism of the effect of MEG3 on OS cell behavior, Notch and TGF-β signaling pathways were analyzed in our study. And we found that MEG3 over-expression inhibited the expression of Notch1, Hes1, TGF-β and N-cadheren, and enhanced E-cadheren expression.

Taken together, the data of the present study indicated that MEG3 prevents cell growth and metastasis of OS by inhibiting Notch and TGF-β signaling pathway. MEG3 may be used as a novel therapeutic target for OS treatment.

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