

ORIGINAL ARTICLE

MEX3C promotes osteosarcoma malignant progression through negatively regulating FGF14

Yile Cao¹, Fan Jiang², Sheng Zhang², Lei Yang³, Yu Sun⁴

¹Department of Clinical Medicine, School of Medicine, Yangzhou University, Yangzhou 225000, China. ²Department of Orthopedics, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China. ³Department of Orthopedic Surgery, Nanjing First Hospital, Nanjing Medical University, Nanjing 210006, China. ⁴Clinical Medical College, Yangzhou University, Northern Jiangsu People's Hospital, Yangzhou 225001, China.

Summary

Purpose: Previous studies have shown that MEX3C is an oncogene; however, its role in osteosarcoma (OS) development has not been reported. We aimed at investigating whether MEX3C could be engaged in the malignant progression of OS through regulating FGF14.

Methods: SNHG1 was knocked down using small-interfering RNAs in gastric cancer cell line MGC-803 and then the changes in the expression levels of SNHG1 and Notch1 in each group of cells were evaluated via quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Results: MEX3C had increased expression both in OS tissue samples and in OS cell lines. High expression of MEX3C was predictive of high incidence of nodal involvement or distant metastasis. Silencing MEX3C remarkably attenuated the migration ability of OS cells, while, conversely, overexpres-

sion enhanced that. Bioinformatics analysis and luciferase assay confirmed that MEX3C bind to FGF14 directly, and the expression of FGF14 was significantly reduced in OS tumor tissue specimens, and was negatively correlated with MEX3C. Overexpression of FGF14 was able to reverse the promoting effect of MEX3C on the crawling ability and invasiveness of OS cells.

Conclusions: MEX3C was remarkably increased in OS tissues and was remarkably correlated with the incidence of metastasis of OS patients. In addition, MEX3C accelerated the malignant progression of OS through negatively modulating FGF14.

Key words: lncRNA SNHG1, Notch1, gastric cancer cells, proliferation, apoptosis

Introduction

Osteosarcoma (OS), a common primary bone malignant tumor, accounts for about 15% of all bone tumors, second only to multiple myeloma [1,2]. With a high grade of malignancy, OS tends to occur in adolescents. At present, the main treatment for this tumor is surgery combined with adjuvant chemotherapy, however, the 5-year survival of non-metastatic osteosarcoma is only 60% to 70% in recent years [3,4]. Nowadays, due to lack of effective methods for OS diagnosis, the vast

majority of micrometastases are difficult to be detected in the early stage, which leads the majority of OS patients already in the middle or late stage at the time of definite diagnosis and the long-term survival of patients with metastasis or recurrence is less than 20% [5,6]. Therefore, it is of great significance to search for more effective diagnostic indicators, prognostic markers and new therapeutic targets to improve the survival rate of patients with OS [7,8].

Corresponding author: Yu Sun, PhD. Department of Orthopedics, Orthopedic Institute, Clinical Medical College of Yangzhou University, Northern Jiangsu People's Hospital, 98 Nantong West Rd, Yangzhou, Jiangsu 225001, P.R. China.
E-mail: docsunyu@126.com
Received: 11/01/2020; Accepted: 19/02/2020

MEX3C protein, a member of the protein family containing the KH domain contains two KH domains [9,10]. It is essential, as reported in the literature, for the normal development and maintenance of germ cell totipotency in early embryos of nematode worms. In addition, homologous proteins of nematode MEX3C were also found in humans and mice in the following few years [11,12]. In addition to a C-terminal containing a RING domain with E3 ubiquitin ligase activity, mammalian MEX3C proteins contain two RNA-bound KH domains, like nematode MEX3C proteins, and can be engaged in many post-transcriptional regulations [13,14] and thus it has been shown to be associated with many diseases, including hypertension, energy metabolism, immune response, and cancer. However, the specific role and mechanism of MEX3C in OS still remains elusive [11,14,15]. In this study, we examined the MEX3C expression in OS tissues and cell lines, and further explored the possible impact of the mutual regulation between MEX3C and FGF14 on OS progression, so as to provide clues for the etiology and new treatment modalities exploration of this disease.

Methods

Patients and OS samples

52 pairs of tumor tissues specimens and normal ones were obtained from OS patients admitted to Orthopedics Clinic in The First Affiliated Hospital of Nanjing Medical University and Nanjing First Hospital. All the cases were diagnosed by clinical, pathological and imag-

ing, and were followed up. All subjects signed informed consent forms. Our study was approved by the hospital ethics committee and complied with the Helsinki Declaration clinical practice guidelines.

Cell lines and reagents

OS cell lines HOS, U2OS, SOSP-9607, MG63, 143B, SaOS-2 and normal osteoblast hFOB cell line were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA). The cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) at 37°C with 5% CO₂.

Transfection

Lentiviral vectors containing MEX3C overexpression and knockdown sequences (MEX3C or anti-MEX3C), and plasmids containing FGF14 overexpression and knockdown sequence (pcDNA-FGF14 or si-FGF14) were all provided by GenePharma (Shanghai, China). Transfection was conducted when the cell density reached 30-50%.

Transwell assay

The OS cells transfected for 48 h were centrifuged and resuspended in RPMI 1640 medium without FBS, and the density was adjusted to 5×10⁵ cells / mL. Transwell chamber was used to determine the cell migration ability.

Wound healing assay

The cell monolayer membrane was scratched with a 200 ul pipette tip after cells were fully adhered. Six-well plates were observed under a microscope (magnification 40x) and images were photographed.

Table 1. Association of MEX3C expression with clinicopathologic characteristics of osteosarcoma

Characteristics	Number of cases	MEX3C expression		p value
		Low (n=27)	High (n=25)	
Age (years)				0.416
<15	24	11	13	
≥15	28	16	12	
Gender				0.447
Male	32	15	17	
Female	20	12	8	
Enneking stage				0.928
IA	9	6	3	
IIA	14	8	6	
IIB	20	8	12	
III	9	5	4	
Lymph node metastasis				0.027
No	31	20	11	
Yes	21	7	14	
Distant metastasis				0.013
No	30	20	10	
Yes	22	7	15	

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from OS cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). qRT-PCR reactions were conducted using SYBR® Premix Ex Taq™ (TaKaRa, Tokyo, Japan). Primers used in the qPCR reaction: MEX3C: forward: 5'-CTCCGGAGAAA-GAGCGTCAA-3', reverse: 5'-GTGCTTTAATTTTACAACC-CTGGC-3'; FGF14: forward: 5'-CTACTTGCAAATGCACC-CCG-3', reverse: 5'-AGGGGTAAAAAGTTCTGATGGGT-3'; β -actin: forward: 5'-CCTGGCACCCAGCACAAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'.

Western blot

The transfected cells were lysed using RIPA lysis buffer, shaken on ice for 30 min, and centrifuged at 14400×g for 15 min at 4°C. The total protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The extracted protein was separated with a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures.

Luciferase assay

pcDNA-NC and pcDNA-FGF14 vectors, wild-type MEX3C and mutant MEX3C 3'-UTR plasmids were constructed and introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight h later, luciferase activity was normalized to the

control using luciferase reporter assay system (Promega, Madison, WI, USA).

Statistics

Statistical analysis was performed using GraphPad Prism 6 V6.01 software (La Jolla, CA, USA). Student's t-test and one-way analysis of variance (ANOVA) were used to analyze the statistical difference between two groups and multiple groups. The data are presented as mean±SD (standard deviation). $P < 0.05$ was considered statistically significant.

Results

MEX3C was highly expressed in OS tissues and cell lines

Figure 1A and 1B show that MEX3C was highly expressed both in tumor tissue samples collected from OS patients and in OS cell lines as compared to normal controls. Based on the expression of MEX3C, we divided the 52 cases of OS patients into high and low expression group and then analyzed the relationship between MEX3C expression and clinicopathological parameters in patients with OS via χ^2 test. Table 1 indicates that high MEX3C expression was positively correlated with the incidence of distant or lymph node metastasis of OS patients (Figure 1C), regardless of age, sex, or Enneking stage.

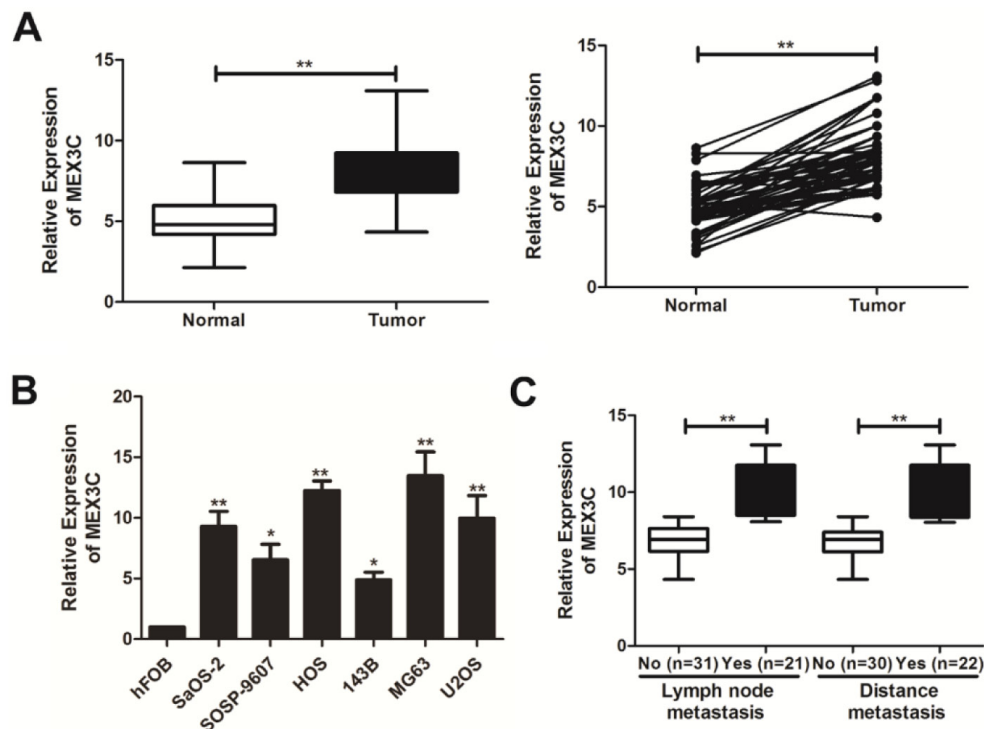


Figure 1. MEX3C is highly expressed in osteosarcoma (OS) tissues and cell lines. **A:** qRT-PCR was used to detect differences in the expression of MEX3C in OS tumor tissues and adjacent tissues. **B:** qRT-PCR was used to detect the expression level of MEX3C in OS cell lines. **C:** qRT-PCR was used to detect the expression of MEX3C in OS tumor tissues with or without lymph node metastasis. Data are average±SD. * $p < 0.05$, ** $p < 0.01$.

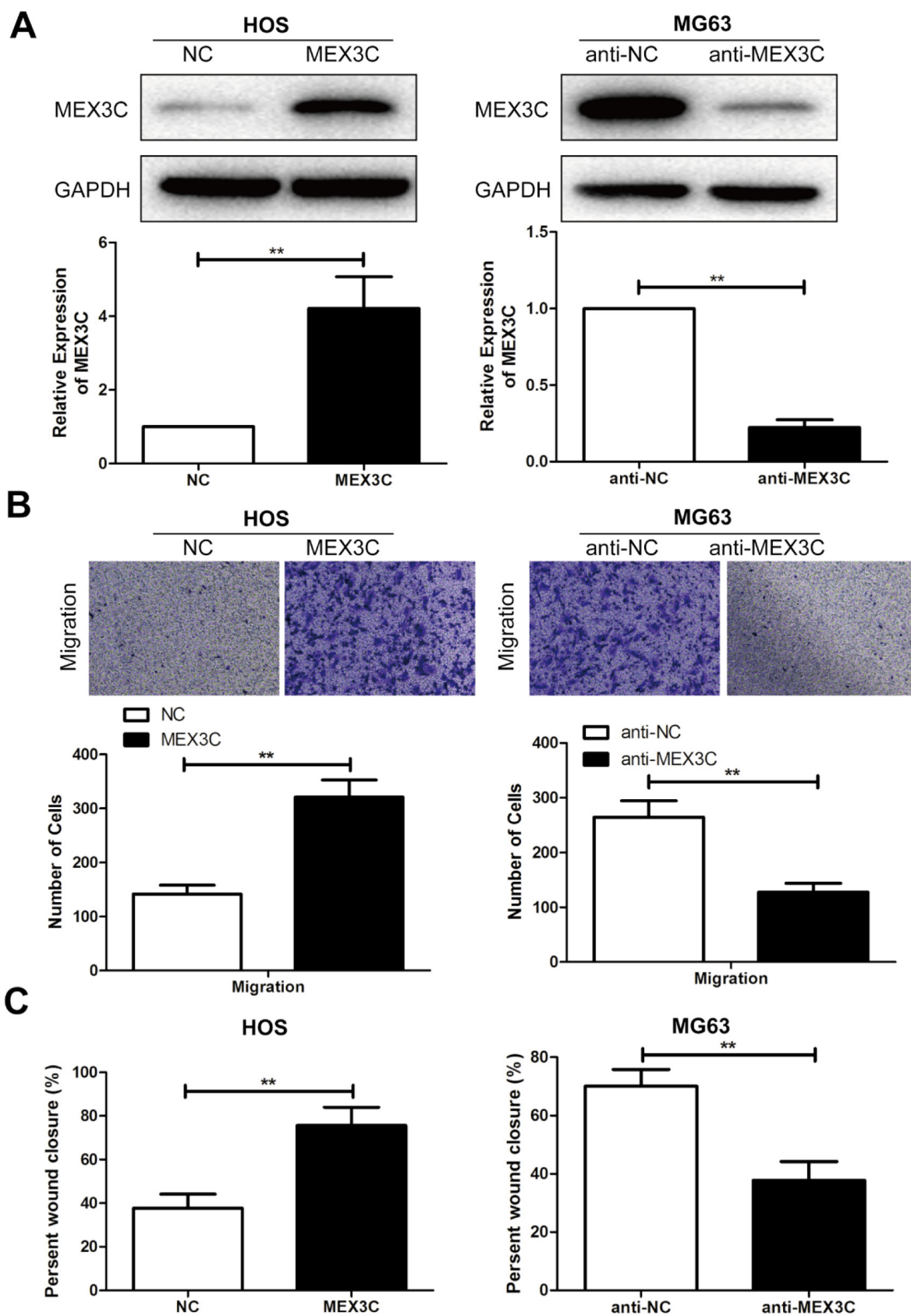


Figure 2. MEX3C can promote the metastatic ability of osteosarcoma (OS) cells. **A:** Western blot and qRT-PCR verified the transfection efficiency of MEX3C overexpression or knockdown vector in OS cell lines HOS and MG63, respectively. **B:** Transwell migration assay was used to detect the migration ability of OS cells after transfection of MEX3C overexpression or vector knockdown in OS cell lines HOS and MG63 (Magnification: 40 ×). **C:** The cell wound healing test was used to detect the crawling ability of OS cells (magnification:40×) after transfection with MEX3C overexpression or vector knockdown in the OS cell lines HOS and MG63, respectively. Data are average ± SD, **p<0.01.

MEX3C promoted cell migration and invasion in OS cell lines

We then constructed MEX3C overexpression and knockdown cell models in OS cell lines HOS and MG63, respectively, and verified the transfection efficiency at both mRNA and protein levels (Figure 2A). Transwell assay showed that knockdown of MEX3C markedly reduced the number of membrane-penetrating OS cells in transwell compartment compared with the anti-NC group, suggesting an inhibited migration ability. On the contrary, overexpression of MEX3C resulted in an opposite result (Figure 2B). Meanwhile, cell wound healing assay also revealed that MEX3C was able to enhance the crawling ability of OS cells (Figure 2C).

MEX3C modulated FGF14 OS in cell lines

To further explore the pathways by which MEX3C promotes the malignant progression of this tumor, we used bioinformatics analysis to search for a downstream gene of MEX3C and then predict a possible relationship between MEX3C and FGF14. Luciferase assay confirmed that overexpression of FGF14 could attenuate the luciferase activity of wild-type MEX3C group, while did not alter mutant MEX3C group, suggesting that MEX3C is capable of binding to FGF14 at specific binding sites (Figure 3A). Subsequently, we found that FGF14 expression was markedly reduced in the tumor tissue specimens of OS patients in comparison to the adjacent ones (Figure 3B), demonstrating a negative correlation between MEX3C and FGF14 in OS tumor samples (Figure 3C). In addition, FGF14 was also found to be less expressed in OS cell lines compared with hFOB *in vitro* (Figure 3D), and the expression of FGF14 in OS cells was increased after knockdown of MEX3C, while the opposite results were observed after overexpression of MEX3C (Figure 3E).

MEX3C interacted with FGF14 and regulated its expressions in OS cell lines

Co-transfection of MEX3C and FGF14 overexpressed/knocked down vectors in OS cell lines HOS and MG63 was performed, respectively, to evaluate the interaction between MEX3C and FGF14 in OS cells. In HOS cells, simultaneous overexpression of the above two genes reduced the elevated MEX3C expression in single MEX3C overexpression group, while the opposite results were observed in MG63 cells (Figure 4A). Meanwhile, in transwell experiments, the number of OS cells penetrating the membrane in the transwell compartment after co-transfection of MEX3C and FGF14 overexpressed

vectors was remarkably reduced in comparison to the single upregulation of MEX3C, suggesting inhibited migration ability. On the contrary, simultaneous knockdown of these two genes resulted in an opposite observation (Figure 4B). Consistently, cell wound healing assay revealed same tendency in crawling ability of OS cells to their migration capacity (Figure 4C).

Discussion

OS, the most common primary bone malignant tumor among adolescents is currently one of the most difficult diseases to overcome in the Orthopedic field [1-3]. Recent clinical data abroad have shown that the incidence of OS occupies the first place in primary malignant bone tumors [2,3]. Due to the extremely high grade of malignancy and early metastasis and also the lack of effective treatment, the average survival time of OS patients is less than 2 years, and the prognosis is extremely poor [4-6]. In recent years, different hypotheses appeared about the pathogenesis of OS, but the specific one still remains unclear [5,8]. Thus, it will be of great theoretical and clinical value to look for effective biomarkers and therapeutic targets for diagnosis and treatment of OS [7,8].

MEX3C protein is a member of the RNA binding protein family containing KH domain [9-11]. The total length of MEX3C protein contains 443 amino acid residues, with two KH domains in series at its N-terminal [10,12]. Currently, the specific mechanism of MEX3C in OS process has not been reported at home and abroad, and the relationship between MEX3C and OS cell functions has been rarely explored [15]. The present research showed that the mRNA level of MEX3C was significantly up-regulated in OS tissues and positively correlated with the incidence of metastasis of OS patients, suggesting that MEX3C may act as an oncogene in OS. As we know, the strong invasion and metastasis abilities of OS cells are the main reason for its poor prognosis. In this study, we performed overexpression and knockdown of MEX3C in OS cell lines, respectively, and examined the changes in the cell functions and we verified that MEX3C is able to enhance the invasiveness and migratory capacity of OS cells; however, the specific molecular mechanism remains elusive.

Although many studies have demonstrated that MEX3C can be engaged in the regulation of translation of many mRNAs, it is not clear whether MEX3C binds its RNA targets either directly or indirectly [9,13]. Through Bioinformatics analysis we screened out FGF14, one of the target genes of MEX3C and then confirmed their binding relation-

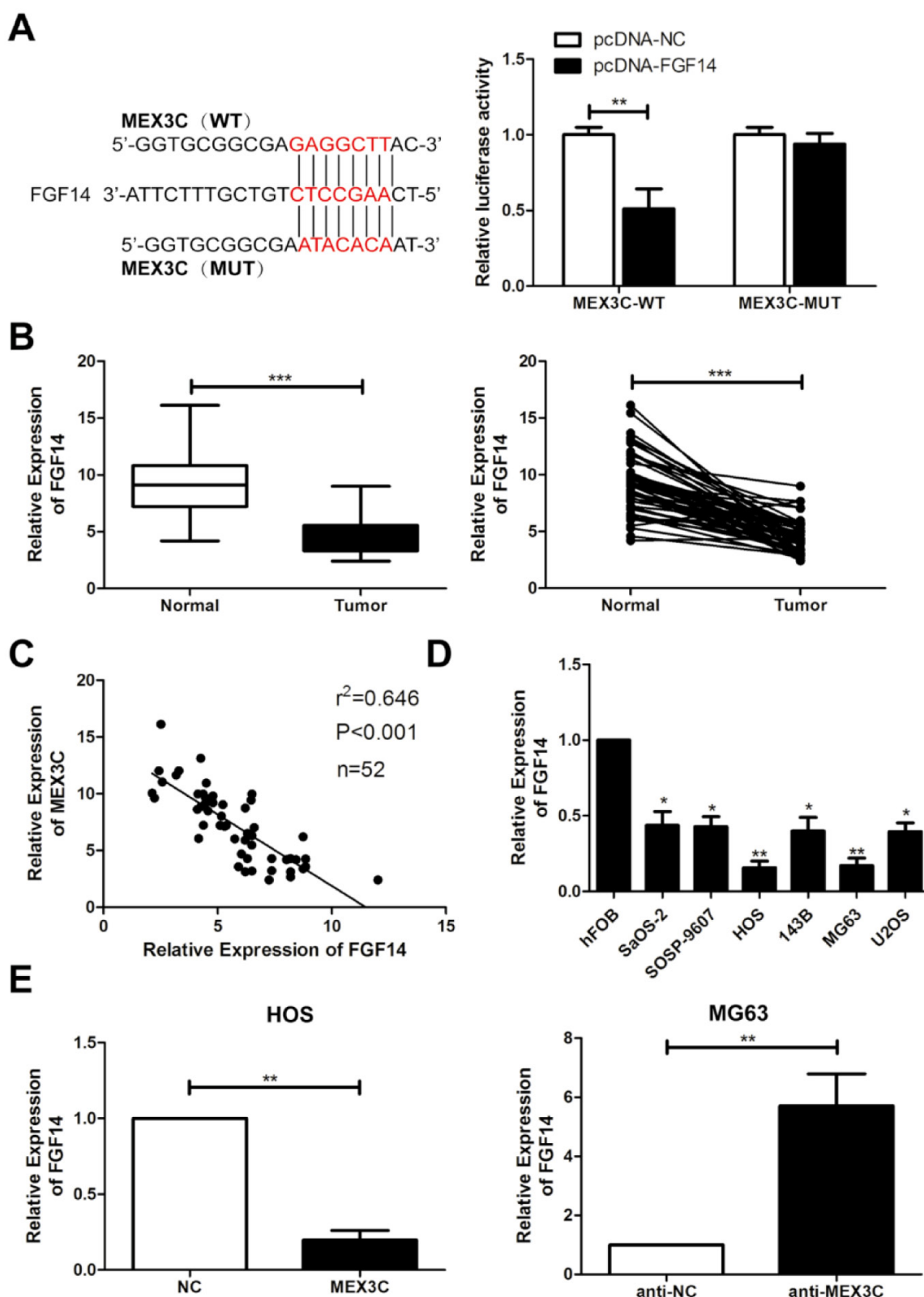


Figure 3. MEX3C can bind to FGF14. **A:** The luciferase reporter gene experiment suggested the binding between MEX3C and FGF14. **B:** qRT-PCR was used to detect the expression of FGF14 in OS tumor tissues and adjacent tissues. The results showed that FGF14 expression was markedly reduced in the tumor tissue specimens of OS patients compared with the adjacent ones. **C:** qRT-PCR showed that the expression levels of MEX3C and FGF14 in OS tissues were significantly negatively correlated. **D:** qRT-PCR was used to detect the expression level of FGF14 in OS cell lines. The results showed that FGF14 was found less expressed in OS cell lines compared with hFOB in vitro. **E:** qRT-PCR examined the expression of FGF14 in the OS cell lines HOS and MG63 after transfection of MEX3C overexpression or knockdown vectors. The results showed that the expression of FGF14 in OS cells was increased after knockdown of MEX3C, while the opposite results were observed after overexpression of MEX3C. Data are average±SD, *p<0.05, **p<0.01, ***p<0.001.

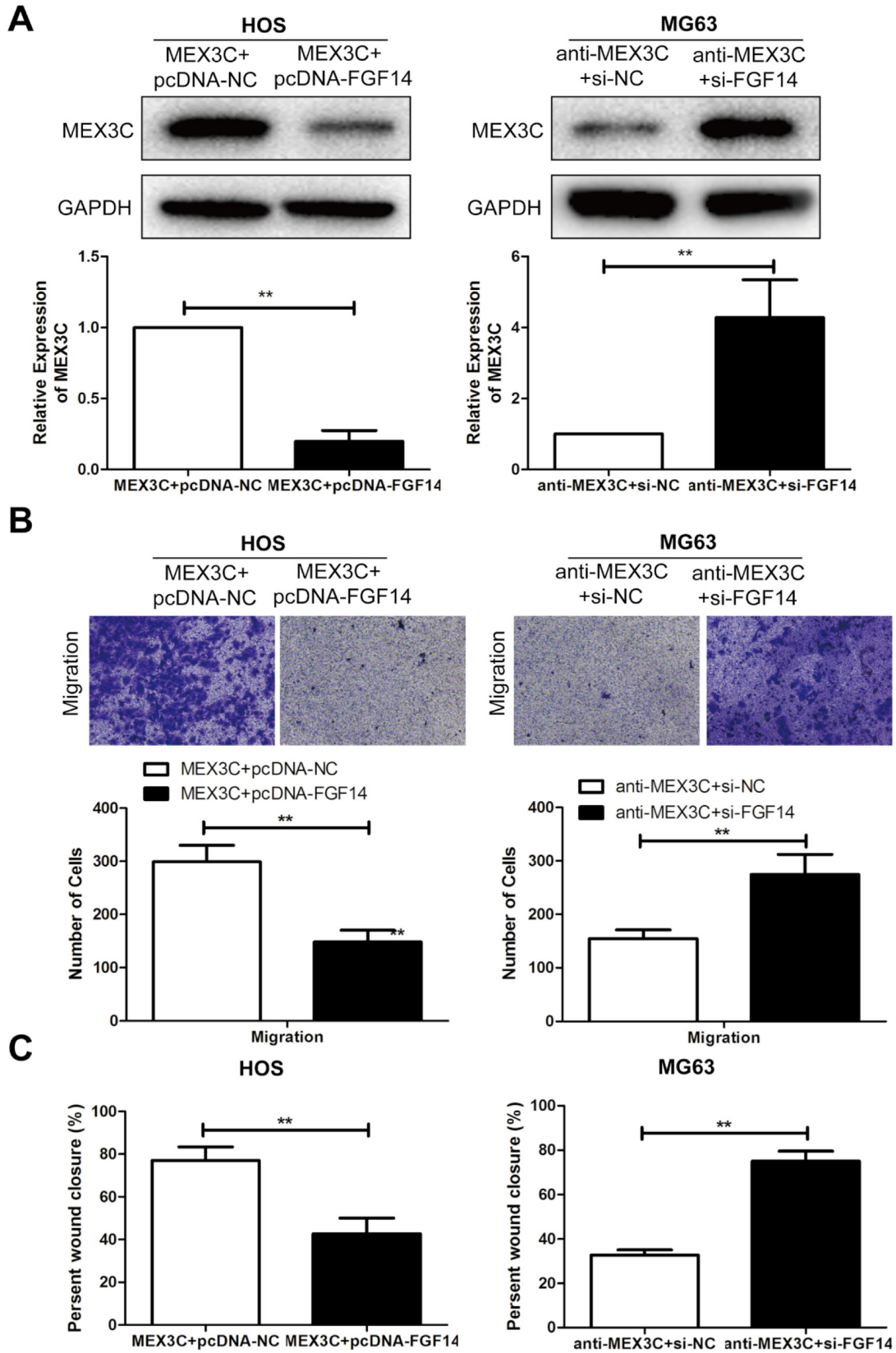


Figure 4. MEX3C regulated the metastatic ability of osteosarcoma (OS) cells through modulating the expression of FGF14. **A:** Western blot and qRT-PCR verified the transfection efficiency of MEX3C and FGF14 overexpression or knock-down vectors in the OS cell lines HOS and MG63, respectively. **B:** Transwell migration assay was used to detect the migration ability of OS cells after overexpression of MEX3C and FGF14 or knockdown of these two proteins in the OS cell lines HOS and MG63 (Magnification: 40×). **C:** The cell wound healing assay was used to detect the crawling ability of OS cells (Magnification:40×) after co-transfection of MEX3C and FGF14 overexpression or knockdown vector in the OS cell lines HOS and MG63, respectively. Data are average±SD. **p<0.01.

ship by luciferase assay. In addition, we found that the mRNA expression of FGF14 was markedly reduced in OS tumor tissues and cell lines, which was negatively correlated with MEX3C. Meanwhile, it was also confirmed by cell reverse experiments that FGF14 reversed the influence of MEX3C on the invasion and metastasis of OS cells. With the deepening of the research, further understanding of the interaction between MEX3C and FGF14 and their roles in the progression of OS will be more conducive to the diagnosis, treatment and prognosis assessment of this tumor.

Conclusions

In summary, MEX3C, with increased expres-

sion in OS tissues, was remarkably correlated with the poor prognosis and high metastasis rate of OS patients. In addition, MEX3C may accelerate the malignant progression of OS through negative regulation of FGF14.

Funding

This study was supported by the National Natural Science Fund from the National Natural Science Foundation of China (Grant No. 81802149).

Conflict of interests

The authors declare no conflict of interests.

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