ORIGINAL RESEARCH

FGF5 promotes osteosarcoma cells proliferation via activating MAPK signaling pathway

Dunxin Han[1,]*
Mingming Wang[2,]*
Zhongkai Yu[3,]*
Long Yin[1]
Changli Liu[1]
Jianmin Wang[1]
Yongjun Liu[1]
Shengyang Jiang[1]
Zhongwu Ren[1]
Jun Yin[4]

[1]Department of Spine Surgery, 970 Hospital of the PLA Joint Logistic Support Force, Yantai, Shandong, People's Republic of China; [2]Department of Clinical Laboratory, Qingdao Women and Children's Hospital, Qingdao, People's Republic of China; [3]Department of Emergency, Liaocheng People's Hospital, Liaocheng, Shandong, People's Republic of China; [4]Department of Orthopedics, First People's Hospital of Yancheng, Yancheng, Jiangsu, People's Republic of China

*These authors contributed equally to this work

Correspondence: Zhongwu Ren
Department of Spine Surgery, 970th Hospital of People's Liberation Army, Yantai, Shandong 264002, People's Republic of China
Email renzhongwu1010@163.com

Jun Yin
Department of Orthopedics, First People's Hospital of Yancheng, Yancheng, Jiangsu 224006, People's Republic of China
Email younker197911@126.com

Objective: This study aimed to investigate the role of fibroblast growth factor-5 (FGF5) in osteosarcoma (OS) and explore the potential mechanisms.

Methods: OS gene expression data was downloaded from the Gene Expression Omnibus (GEO; GSE12865) and analyzed by R software. OS tissues and cell lines were collected. The expression level of FGF5 in tumor tissues and cell lines was detected using qRT-PCR. Knockout of FGF5 was performed using CRISPR/Cas9 system. The effects of FGF5 knockout on OS cell proliferation and tumor growth were determined through cell counting kit-8 assay and xenograft nude mice, respectively. Additionally, recombinant FGF5 (rFGF5) was added into OS cell and the effects of rFGF5 on the proliferation and apoptosis of OS cell lines were assayed. Furthermore, the protein expression levels of mitogen-activated protein kinase (MAPK) signaling pathway were detected through Western blot.

Results: FGF5 was significantly upregulated in OS tissues and cells, and closely associated with poor differentiation, larger tumor size, lymph node metastasis, and advanced TNM stage. FGF5 knockout could inhibit proliferation of OS cells and tumor growth in nude mouse model. Addition of exogenous rFGF5 promoted OS cell proliferation while inhibited OS cell apoptosis. The expression levels of MAPK signaling pathway proteins in FGF5 knockout group were significantly lower than that in control when there was no rFGF5. Additionally, their expression level in rFGF5 addition group was higher than that in without rFGF5 group.

Conclusion: We demonstrated for the first time that FGF5 was overexpressed in OS cell lines and clinical tissue samples and promotes OS cell proliferation by activating MAPK signaling pathway, which indicated that FGF5 was a potential therapeutic target for OS.

Keywords: fibroblast growth factor-5, osteosarcoma, cell proliferation, MAPK signaling pathway

Introduction

Osteosarcoma (OS) is the most universal bone malignancies in children and adolescents, which is characterized by direct formation of osteoid tissue or immature bone by tumor cells.[1,2] In spite of the advances in treatment strategies, including primary tumor resection, chemotherapy, or combination of surgery and chemotherapy, it is still far from satisfying the current medical requirements.[3,4] Currently, genetic abnormalities have been extensively recognized to play critical roles in OS tumorigenesis, and gene therapy has been gradually reported to be available in OS treatment.[5–7] However, the molecular mechanisms of OS are still elusive. Therefore, it is essential to elucidate the molecular mechanisms underlying the initiation and development of OS, which may contribute to identify reliable diagnostic and prognostic markers.

Fibroblast growth factor-5 (FGF5) is a member of the fibroblast growth factor (FGF) family.[8] The FGF family constitutes 18 polypeptides which transduce signals through receptor tyrosine kinases (RTK) named FGF receptors (FGFRs) 1 to 4. Several FGFs play important roles in embryonic development, tissue maintenance, and wound healing.[9] Additionally, FGFs are angiogenic and mitogenic, and overexpression or mutation of FGFs or FGFRs could contribute to malignancy.[10,11] FGF5 was first identified by a screening approach for transforming oncogenes.[12] Overexpression of FGF5 has been associated with prostate cancer, pancreatic cancer, breast cancer, renal cell carcinoma, and so on.[13,14] However, the roles of FGF5 and its signaling pathway in OS are still unknown. Specifically, FGF5 was reported to be frequently overexpressed in embryonic tissues but scarcely expressed in adult tissues.[15] Given that OS develops from embryonic cells and presents high incidence rate in adolescents, we speculated that it may be involved in the pathogenesis of OS.

Therefore, in this study, the expression level of FGF5 was detected in tumor tissues and cell lines. Further in vitro studies were performed to identify the functional role of FGF5 in proliferation and apoptosis of OS cell lines. Furthermore, the underlying molecular mechanism was also investigated.

Materials and methods
Tissue samples
A total of 15 pairs of tumor tissues and adjacent nontumor corresponding tissues obtained from 15 OS patients from 2014 and 2016 in the 107th Hospital of People's Liberation Army and First People's Hospital of Yancheng were used in this study. All the patients were pathologically confirmed and the tissue specimens were collected immediately after they were surgically resected, and then stored at −80°C. This study was approved by the ethics committee of 970th Hospital of People's Liberation Army and First People's Hospital of Yancheng and was conducted in accordance with the Declaration of Helsinki. All patients had provided their written informed consent before the study.

Cell culture
HEK293T, human normal osteoblast cells HOBC and NHOST, as well as OS cell lines U2OS, SAOS, MG63, and KHOS were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences

(Shanghai, China). HOBC and NHOST cells were cultured in DMEM/ham's F-12 (Invitrogen, Carlsbad, CA, USA) containing 10% FBS(Invitrogen) and 0.3 mg/ml G418. U2OS, MG63, and SAOS cells were maintained in DMEM medium (Invitrogen) containing 10% FBS (Invitrogen), 100 U/mL penicillin and 100 g/mL streptomycin (Life Technologies, Grand Island, NY, USA). KHOS cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS. All cells were incubated at 37°C in 5% CO_2 incubator.

CRISPR/Cas9-mediated FGF5 knockout
The gRNA targeting FGF5 gene was designed using E-CRISP database (5′-3′ GAAAATGGGTAGAGATATGC). pX330 vector in vitro transcription of Cas9 mRNA and gRNA was prepared as previously described.[16] Briefly, paired synthesized oligonucleotides were annealed and subcloned into the linearized pX330 vector. The constructed recombinant vector was used as the template. The production of gRNA by in vitro transcription was performed using the MEGAshortscript kit (Ambion, Austin, TX, USA) and then purified using the MEGAclearTM transcription clean-up kit (Ambion). Based on the Cas9 mRNA in vitro transcription vector as templates, Cas9 mRNA was produced and purified using the MEGAclearTM transcription clean-up kit (Ambion). To produce lentivirus, HEK 293T cells were transfected with pX330 vector. The lentivirus was then transfected into the MG63 and U20S cell lines. After 24 hours of transfection, puromycin was added for the selection of transfected cells. The puromycin selected cells were then expanded in regular culture medium. FGF5-knockout cell lines were determined by Western blot.

Mice
Female athymic nu/nu nude mice (AntiCancer Inc., San Diego, CA, USA), 4–6 weeks old, were purchased from Shanghai Institute of Zoology, China. They were maintained in pathogen-free conditions and were fed with autoclaved laboratory rodent diet. All animal experiments were conducted following the guidelines of Institutional Animal Care and Use Committee with ethical approval of 970th Hospital of People's Liberation Army and First People's Hospital of Yancheng.

Nude mouse orthotopic metastatic model
The mice were divided into four groups (N=10): MG63 transplatation (FGF5-knockout), MG63 transplatation

(FGF5-control), U2OS transplantation (FGF5-knockout), and U2OS transplantation (FGF5-control). All mice were anesthetized by subcutaneous injection of 0.02 mL ketamine mixture (15.2 mg/kg xylazine, 0.48 mg/kg acepromazine, and maleate 20 mg/kg ketamine). U2OS and MG63 cells (FGF5 knockout and control) (5×10^5) were respectively transplanted into the tibia of the nude mice as previously described.[17] The tumor size was measured once a week (six times in total). The mean tumor volume was calculated by the formula of width$^2\times$ length. Mice were euthanized when tumor volume became approximately 2.0 cm^3.

Expression profiling data collection and differential expression analysis

The expression profiling data (accession number: GSE12865) deposited in Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/) were extracted for analysis. The dataset included two replications of normal human osteoblasts and 12 osteosarcoma tissues. The differential expression analysis was performed using limma and pheatmap of R software[18] in R3.4.1. The *P*-values and logFC values were adjusted into false discovery rate (FDR) using multitest package (version 2.32.0).[19] FDR <0.05 and |logFC| >1 were chosen as cut-off criteria. The expression values of differentially expressed genes were extracted and performed bidirectional hierarchical clustering[20] based on Euclidean distance[21] using pheatmap package (version 1.0.8)[22] in R3.4.1. The results were displayed using heatmaps.

Cell counting kit-8 assay

Cell proliferation was determined using cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Japan). Briefly, the cells were seeded in 96-well plates with complete growth medium (with or without 10 ng/mL recombinant FGF5 (rFGF5) protein) at a concentration of 3,000 cells per well. After 1–5 days of incubation, the growth medium in each well was removed, and then filled with 100 ml of fresh medium containing 10 ml CCK-8 solutions. After incubated for 2 h at 37 °C, cell proliferation was determined by optical density measurement at 450 nm with a microplate reader.

Colony formation assay

After transfection, cells were seeded in six-well plates at a concentration of 500 cells per well, and cultured for approximately 10 days. Then cells were fixed with 4% paraformaldehyde. Following washing, the plates were air dried, and the colonies with more than 50 cells were counted using a microscope (IX83, Olympus, Japan).

Apoptosis assay

Cell apoptosis was determined by propidium iodide (PI) and fluorescein isothiocynate (FITC)-conjugated annexin V staining. Briefly, after treatment with the rFGF5 or isometric serum-free medium for 72 hours, cells were washed with PBSand then fixed in 70% ethanol. After washing, the fixed cells were stained in PI/FITC-annexin V with 50 μg/mL RNase A and then incubated at room temperature for 1 hour in the dark. Flow cytometry analysis was performed by using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA). The obtained data were analyzed using the Multicycle Cell Cycle Analysis software (Phoenix Flow System, San Diego, CA, USA).

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

Total RNA was extracted using TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, USA) and was reverse-transcribed into cDNA by Reverse Transcription kit (TaKaRa, Tokyo, Japan). The One Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, China) was to detect the expression level of FGF5 by real-time PCR analysis with GAPDH as internal reference. The relative expression levels were calculated by relative quantification ($2^{-\Delta\Delta Ct}$) method.

Western blot

Protein was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing protease inhibitors (Roche, Guangzhou, China) and then quantified with BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The Western blot system was constructed using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Primary antibodies (anti-FGF5, FGFR1, PARP, p-MEK2, p-ERK, p-Elk-1, and p-MNK1/2) were prepared in 5% blocking buffer at a dilution of 1:1000, and then incubated with the membrane at 4°C overnight. After washing, the membrane was incubated with secondary antibody marked by horseradish peroxidase for 1 hour at room temperature. The

polyvinylidene difluoride membrane carrying blots and antibodies was transferred into the Bio-Rad ChemiDoc™ XRS system, with coverage of 200 µL Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA). The signals were captured and the intensity of the bands was quantified using ImageJ software.

Statistical analysis

All experiments were repeated three times and the data of multiple experiments are presented as mean ± SD. Statistical analyses were performed using SPSS 16.0 and Graphpad 6.0. The *P*-values were calculated using a one-way ANOVA. A *P*-value of <0.05 was considered as statistically significant.

Results

FGF5 was upregulated in OS

After differential expression analysis of the dataset in GSE12865, FGF5 was found to be significantly upregulated between tumor and control groups, as shown in Figure 1A. To validate the analysis results above, the expression levels of FGF5 in tumor cells and tissues of all patients were determined using qRT-PCR and Western blot. As shown in Figure 1B, FGF5 was significantly upregulated in five OS cell lines compared with that in normal osteoblast cells HOBC and
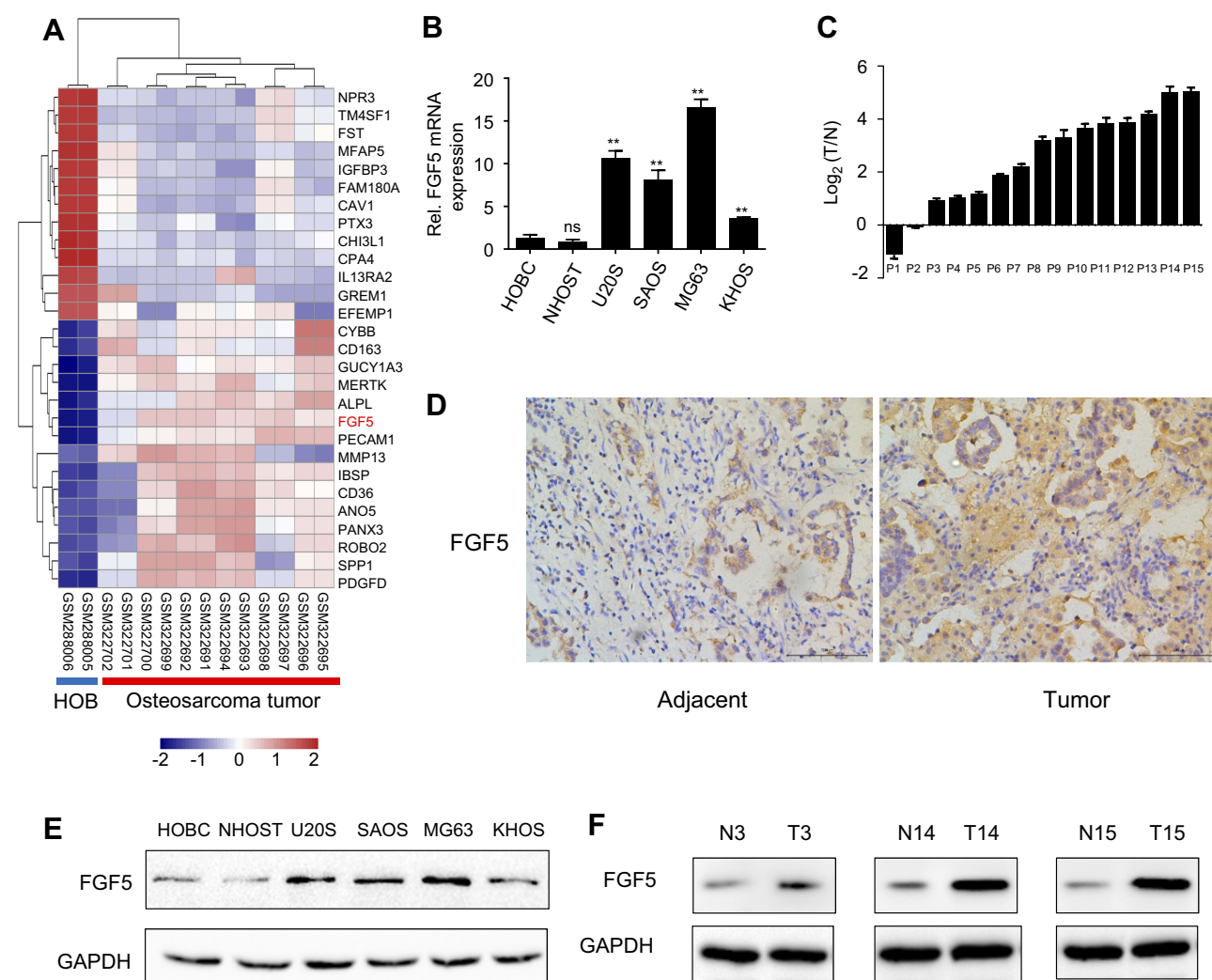

Figure I (**A**) Heatmap of differentially expressed genes between osteosarcoma tissues and tissues adjacent to cancer. (**B** and **C**) Expression levels of FGF5 in osteosarcoma cell lines and tissues detected by qRT-PCR, the results showed that FGF5 mRNA expression was significantly increased in osteosarcoma cell lines and tissues. (**D**) ICH staining for FGF5 in osteosarcoma and matched normal tissues. (**E** and **F**) Protein expression levels of FGF5 in osteosarcoma cell lines and tissues detected by Western blot, the results showed that FGF5 protein expression was upregulated in osteosarcoma cell lines and tissues. Error bars indicated mean ± SD (*P<0.01).
Abbreviations: HOB, human normal osteoblast; FGF5, fibroblast growth factor-5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 1 Correlation between FGF5 expression and clinical-pathological characteristic in 15 osteosarcoma patients

Characteristics	Case number	FGF5 expression		P-value
		Low	High	
Gender				
Male	9	4	6	0.706
Female	6	2	3	
Age				
≤25	12	6	6	0.185
25	3	0	3	
Tumor location				
Tibia/femur	10	4	6	0.706
Elsewhere	5	2	3	
Histology types				
Osteoblast type	8	5	3	
Chondroblast type	4	2	2	0.467
Other type	3	1	2	
X-ray types				
Osteogenesis type	5	3	2	
Osteolysis type	7	3	4	0.35
Mixed type	3	1	2	
Differentiated degree				
High	9	2	7	0.035
Low	6	5	1	
Tumor size				
8 cm	5	4	1	0.047
8 cm	10	2	8	
Lymph node metastasis				
Yes	11	2	9	0.011
No	4	4	0	
Tumor stage				
IIA	26	3	0	0.044
IIB/III	34	3	9	

NHOST ($P<0.01$). Additionally, FGF5 was found to be upregulated in OS tissues of 13 patients compared with adjacent nontumor tissues collected by us (Figure 1C). Moreover, the results of IHC staining and Western blot also showed that FGF5 was overexpressed in OS cells and tissues (Figure 1D–F). Importantly, high FGF5 expression was closely related to poor differentiation ($P=0.035$), larger tumor size ($P=0.047$),

lymph node metastasis ($P=0.011$), and advanced TNM stage ($P=0.044$) (Table 1).

Downregulation of FGF5 decreased OS cell proliferation and tumor growth

To explore the role of FGF5 in OS progression, we examined the effects of FGF5 suppression on OS cell proliferation, colony formation, and tumor growth. Because FGF5 had higher expression level in MG63 and U20S cell lines, the two cell lines were selected for this experiment. FGF5 in MG63 and U20S cell lines was knocked out by CRISPR/Cas9, which was confirmed by Western blot (Figure 2A). According to the OD450 value determined by CCK-8 assay, the OS cells proliferation in FGF5 suppression group was significantly inhibited compared with control group from the second day ($P<0.05$, Figure 2B). Similar results were found in colony formation assay. As shown in Figure 2C, colony formation experiment revealed that FGF5 knockout obviously inhibited colony formation. Additionally, by establishment of nude mouse orthotopic metastatic model, we found that mice with FGF5 knockout exhibited low FGF5 expression and the tumor value in mice significantly reduced in FGF5 knockout group from the second week ($P<0.05$, Figure 2D–E).

Exogenous rFGF5 promoted OS cell proliferation while inhibited OS cell apoptosis

To further investigate the effects of FGF5 on OS cell proliferation and apoptosis, we added 10 ng/mL exogenous rFGF5 into KHOS cell line. KHOS cell line was selected because FGF5 had a lower expression level in KHOS cell line than in the other three PS cell lines. The protein expression of FGF5 and its receptor FGFR1 was detected using Western blot. As shown in Figure 3A, FGF5 expression significantly increased after the addition of rFGF5, while the FGFR1 expression showed no obvious change. CCK-8 assay revealed that under 10% FBS, the proliferation promotion effect of rFGF5 addition on OS cells was not obvious. Whereas, under 1% FBS, addition of rFGF5 significantly increased cell proliferation from the third day ($P<0.05$, Figure 3B). Furthermore, flow cytometry analysis revealed that rFGF5 addition significantly inhibited apoptosis of OS cells under 1% FBS. The protein expression of cleaved PARP significantly decreased in rFGF5 group compared with control under 1% FBS (Figure 3C).
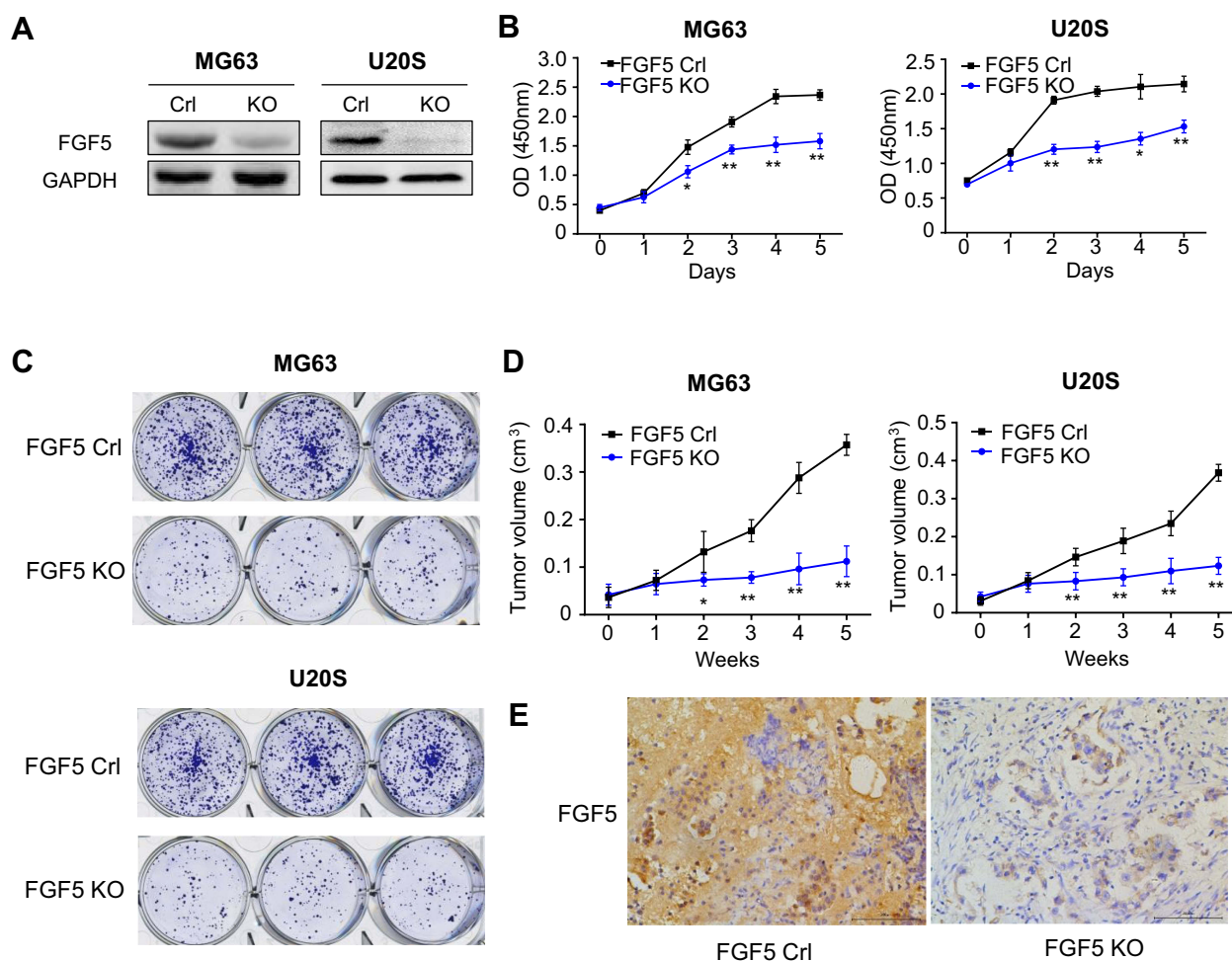
Figure 2 (**A**) The expression of FGF5 in two osteosarcoma cell lines after FGF5 knockout by Crispr/Cas9, the results showed that FGF5 was dramatically decreased in FGF5-KO MG63 an U20S cells. (**B** and **C**) Effects of FGF5 on cell proliferation and colony formation assessed by CCK-8 and colony formation assays. The results showed that knockout of FGF5 inhibited cell proliferation. (**D**) Effects of FGF5 on tumor growth determined by nude mouse orthotopic metastatic model. (**E**) ICH staining for FGF5 in nude mice. Error bars indicated mean ± SD (*P<0.05, **P<0.01).
Abbreviations: Crl, control; KO, knockout; FGF5, fibroblast growth factor-5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

FGF5 promoted OS cell proliferation via MAPK signaling pathway

Study has reported that mitogen-activated protein kinase (MAPK) signaling plays an important role in OS cell growth.[23] We speculated that FGF5 may function in OS cell proliferation through MAPK signaling pathway. Therefore, the expression level of FGF5, FGFR1 and MAPK signaling pathway associated proteins (p-MEK2, p-ERK, p-Elk-1, and p-MNK1/2) on OS cells without rFGF5 (FGF5 knockout and control) and with rFGF5 (FGF5 knockout and control) were detected through Western blot. As shown in Figure 4, the expression levels of FGF5, p-MEK2, p-ERK, p-Elk-1, and p-MNK1/2 in FGF5 knockout group were significantly lower than that in control when there was no rFGF5, however, the

expression of total MEK2, ERK, and MNK1 remained unchanged. Additionally, their expression level in rFGF5 addition group was higher than that in rFGF5 without group. Moreover, the Western blot results in vivo also confirmed this finding (Figure 5).

Discussion

In the present study, FGF5 was found to be significantly upregulated in OS tissues and cells, and FGF5 knockout could inhibit OS cell proliferation and tumor growth in nude mouse model. Subsequent experiments revealed that addition of exogenous rFGF5 promoted OS cell proliferation while inhibited OS cell apoptosis. Finally, we for the first time presented evidence that FGF5 exerted oncogenic activities in OS by activating MAPK signaling pathway.
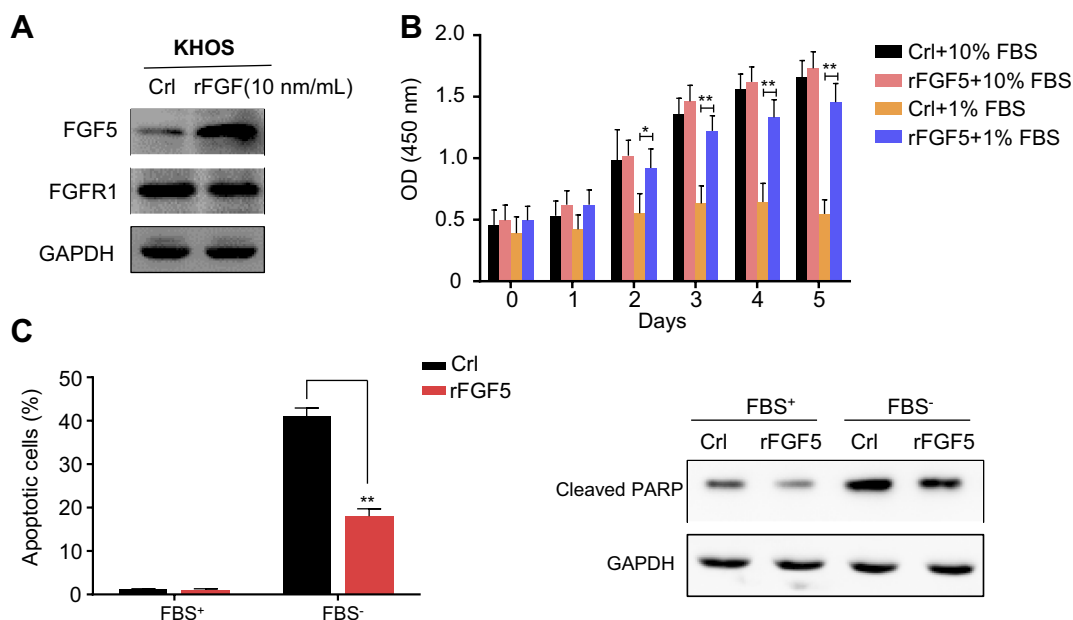
Figure 3 (**A**) The expression of FGF5 and FGFR1 in osteosarcoma cell after addition of exogenous rFGF5 into KHOS cell line. (**B**) The effect of rFGF5 on cell proliferation detected by CCK-8 assay. The results showed that exogenous rFGF5 enhanced cell proliferation. (**C**) The effect of rFGF5 on apoptosis detected by flow cytometry analysis and the protein expression of cleaved PARP. The results showed that exogenous rFGF5 inhibited cell apoptosis. Error bars indicated mean ±SD (*$P<0.05$, **$P<0.01$).
Abbreviations: Crl, control; FGF5, fibroblast growth factor-5; FGFR1, FGF receptor-1; rFGF5, recombinant FGF5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CCK-8, cell counting kit-8; PARP, poly (adenosine diphosphate) ribose polymerase.

FGFs are small polypeptide growth factors containing signal peptides to secrete to the extracellular environment to promote embryonic development, cell growth, morphogenesis, tumor growth and invasion.[9] In addition, FGF signaling has been implicated in a variety of pathological processes in angiogenesis.[24] FGF5 is originally identified as a transforming proto-oncogene, but the related data on oncogenic functions of FGF5 are relatively limited. Kornmann et al[25,26] have reported that overexpression of FGF5 is associated with the development of human pancreatic cancer. Hanada et al[27] have also reported that FGF5 is an overexpressed antigen in multiple human adenocarcinomas. In accordance with the findings above, our study also revealed a high expression of FGF5 in OS, further indicating the oncogenic potential of FGF5 in cancers.

It is well known that sustaining proliferative signaling as well as resisting cell death are the hallmarks of tumors.[28] To demonstrate the role of FGF5 in OS cell proliferation and apoptosis, we knocked out the FGF5 in OS cell lines through CRISPR/Cas9 system and added rFGF5 in OS cells to investigate the changes of OS cell proliferation and apoptosis. The results showed that silenced FGF5 significantly inhibited cell proliferation, while rFGF5 could promote OS cells proliferation but inhibit their apoptosis,

which was in line with a previous study of Allerstorfer et al.[15] They have suggested that siRNA-mediated FGF5 suppression significantly decreases glioblastoma cells proliferation, while rFGF5 significantly increases this parameter.

To further elucidate the molecular mechanisms underlying the oncogenic activities of FGF5, we detected the expressions of MAPK signaling pathways. MAPK is an insulin-mitogen activated protein kinase, which is related with cell survival, growth, and migration by regulating the signals from cell surface to nucleus through phosphorylation.[29,30] The MAPK pathway exerts a key role in integrating external signals from mitogens into signaling events to promote cell proliferation. Aberrant activation of MAPK signaling pathway has been demonstrated to be associated with tumor cell growth and proliferation, including in OS.[31,32] The present results showed that MAPK signaling pathway was activated in OS cell without FGF5 knockout, suggesting that FGF5 may promote OS cell proliferation via activation of MAPK signaling pathway.

Conclusion

We demonstrated for the first time that FGF5 was overexpressed in OS cell lines and clinical tissue samples.
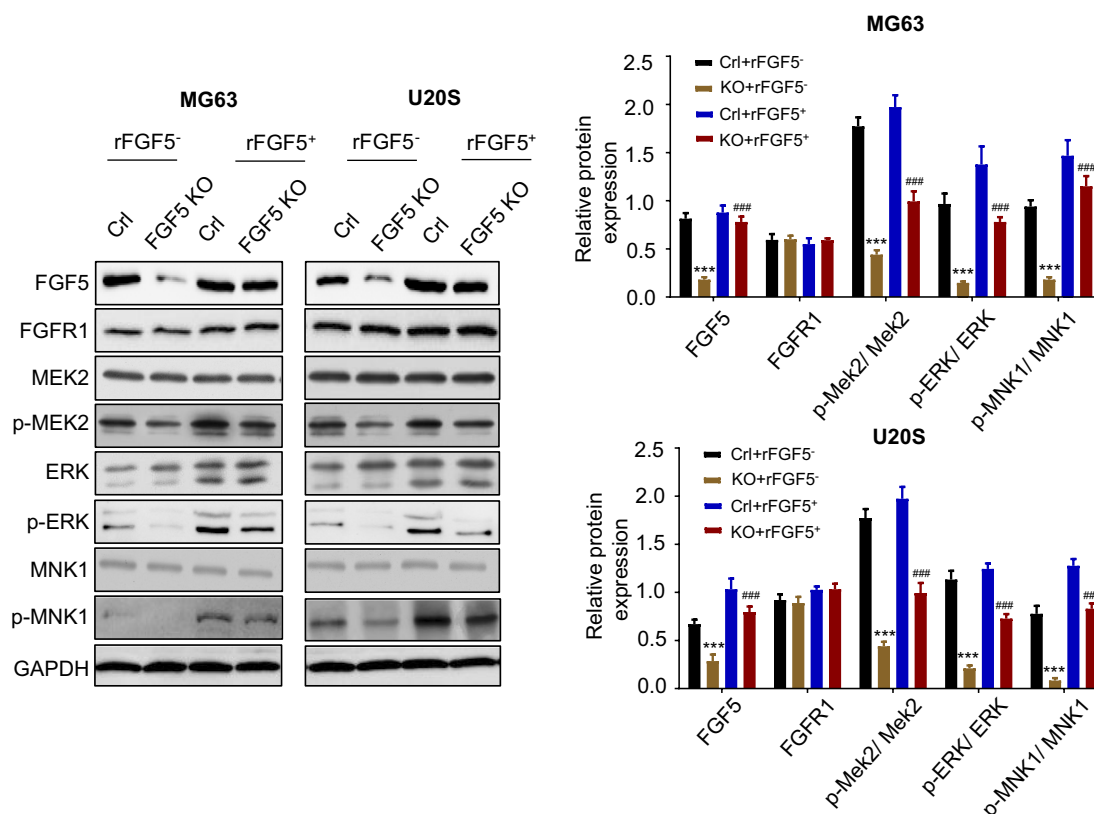
Figure 4 The expression level of FGF5, FGFR1 and MAPK signaling pathway associated proteins (p-MEK2, MEK2, p-ERK, ERK, p-MNK1, and MNK1) on control or FGF5 knockout OS cells without or with rFGF5 treatment and detected by Western blot. The results showed that FGF5 activiated the MAPK signaling. *** P <0.001 vs. Crl; ### P <0.001 vs rFGF5-

Abbreviations: Crl, control; KO, knockout; FGF5, fibroblast growth factor-5; FGFR1, FGF receptor-1; rFGF5, recombinant FGF5; OS, osteosarcoma; MAPK, mitogen-activated protein kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
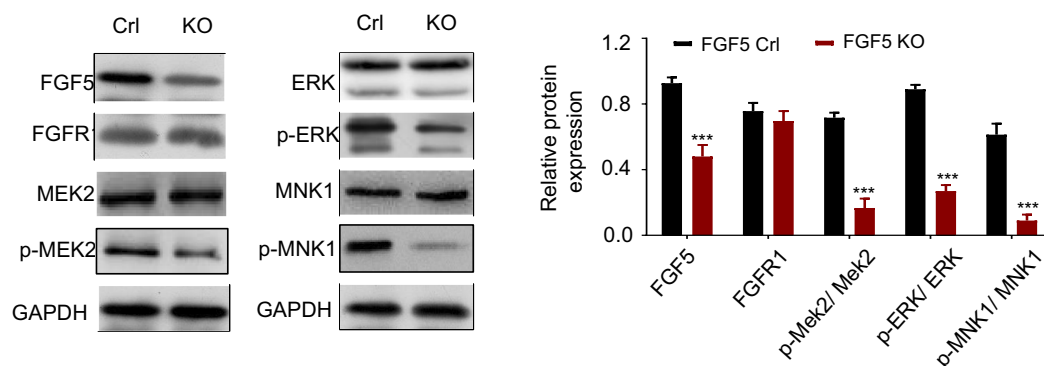

Figure 5 The expression levels of FGF5, FGFR1 and MAPK signaling pathway associated proteins (p-MEK2, MEK2, p-ERK, ERK, p-MNK1, and MNK1) in control and FGF5 knockout nude mice detected by Western blot. The results showed that FGF5 knockout dampen MAPK signaling in vivo. *** P <0.001

Abbreviations: Crl, control; KO, knockout; FGF5, fibroblast growth factor-5; FGFR1, FGF receptor-1; MAPK, mitogen-activated protein kinase; MAPK, mitogen-activated protein kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

High expression of FGF5 could promote OS cell proliferation. Importantly, we suggested that FGF5 may exert oncogenic function in OS cells by activating the MAPK signaling pathway. Therefore, FGF5 may serve as a potential therapeutic target for OS, and abrogation of FGF5 dependent signaling may have a role in OS therapy.

Disclosure

The authors report no conflicts of interest in this work.

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