Cellular and Molecular Biology

Tian-Long et al., Cell Mol Biol 2018, 64:1

Research Article Open Access

High Expression of *NETO2* in Osteosarcoma Promotes Cell Proliferation and Migration

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3'/5'-UCCAC UACCA GUACU GUUA-3'; siENTO2 2: 5'-UCAAG CAUAU UCCUG CAAC- 3'/5'-GUUGC AGGAA UAUGC UUGA - 3'. Non-targeting siRNA controls (si-con) : 5'- UCAUA ACGUG GAUCG AUUC -3'/5'- GAAUC GAUCC ACGUU AUGA -3'. e transfection of si- *2* 1/2 and si-con were performed with Lipofectamine 2000 (ermo) according to the manufacturer's introductions. ese cells were then collected 24 h a er transfection to identify the e ciency of siRNA by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analyses.

qRT-PCR analysis

Total RNA from cultured cells was extracted using Trizol reagent (ermo) in accordance with the manufacturer's protocol. qRT-PCR was performed to synthesize and then amplify the cDNA using the PrimeScript™ RT reagent kit and SYBR Premix Ex Taq kit (TaKaRa Biotechnology, Shiga, Japan) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). e conditions of qPCR were as follows: 94°C for 10 min, 40 cycles of 94°C for 15 s, 60°C for 30 s. e primers are as follows: *2*: F: 5'-GGCGTGAAAAGCCCTCCATT -3', R: 5'- GCTCCCGAGAGCTCGAA -3'; GAPDH: F: 5'-GGAGCGAGATCCCTCCAA AAT -3', R: 5'-GGCTGTTGTCATACTTCTCATGG-3'. e relative expression levels of the mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Counting Kit-8

Cells treated with si- *2* and si-con was plated at a density of 3×10^3 cells/well in 96-well plates. At the di erent time points a er transfection, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well and the cells were incubated for 1 h at 37°C. e optical density (OD) was measured at 450 nm.

Scratch wound assay

Cells were cultured in 6-well plates in complete medium until they reached 90% con uence. e monolayer cell culture was scratched by a 200 μL pipette tip with a uniform wound. e stripped cells were washed away with serum-free culture medium, and the other cells were cultured in medium containing 10% FBS. Images were captured under an Olympus microscope (Olympus, Japan) at 0 and 24 h a er scratch. Migration rate was calculated as: (Migrated distance at measured time-initial distance)/Initial distance × 100%.

Western blot analysis

Total proteins from cultured cells were extracted using radio-immunoprecipitation (RIPA) assay lysis bu er (Beyotime Institute of Biotechnology, Shanhai, China). en, the protein concentration was quanti ed using bicinchoninic acid (BCA) method (Beyotime). Equal amounts of proteins (20 μg) were subjected to 10-12% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene di uoride (PVDF) membranes (Millipore, Billerica, MA, USA). e membranes were blocked with 5% non-fat milk, followed by incubation with corresponding diluted primary antibodies (anti-MEK, 1:1,000, ; anti-ERK, 1:1,000; anti-phosphorylated (p-)MEK and anti-p-ERK, 1:1000; anti- *2*, 1:1000, Cell Signaling Technology, Danvers, MA, USA; anti-GAPDH, 1:1000, Beyotime) at 4°C overnight. e membranes were incubated with horseradish peroxidase-labeled secondary antibody (Beyotime) at 37°C for 2 h. Proteins were visualized using electrochemiluminescence (ECL) reagents (Pierce Biotechnology, Inc., Rockford, IL, USA), and the scanned images were analyzed with Quantity One so ware (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Statistical analysis was done with using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (San Diego, CA, USA). All experiments were performed in triplicate. Data was expressed as mean and standard deviation (SD). Di erences between means were studied using a Student -test when two groups were compared. value<0.05 was considered to be statistically signi cant.

Results

Upregulated expression of *2* in OS tissues and cells

e analysis of the available clinical including OS tissues/normal and metastatic/ non-metastatic samples showed that a signi cant increase of *2* in OS tissues relative to normal tissue (Figures 1A-1C, P<0.01). Moreover, to validate our initial expression pro ling data, we performed qRT-PCR of OS cells and observed signi cant enhanced expression of *2* in OS cells compared with normal cells (Figure 1D, <0.01). ese data suggest a potential oncogenic role played by *2* in OS.

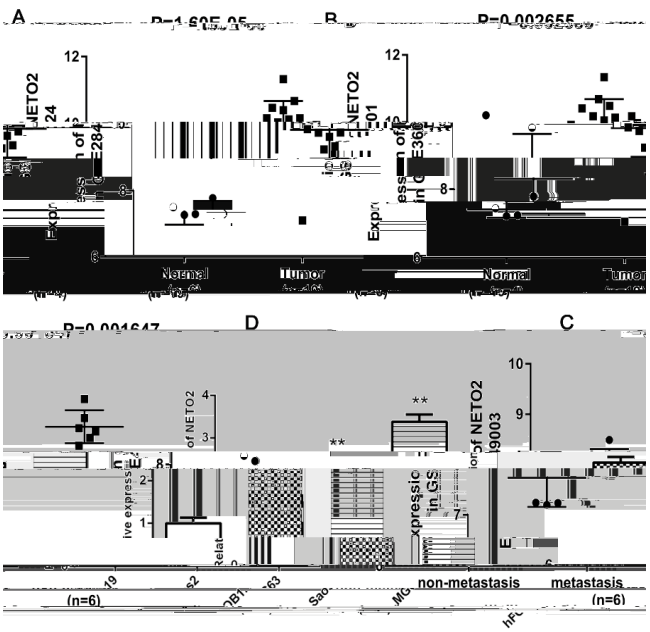
2 silencing impairs proliferation ability of OS cells

A er identifying and con rming di erential expression of *2* in OS tissues and cells, the e ect of *2* on OS cells proliferation was determined. Firstly, we successfully knocked down it by observing a decreased level of *2* both in mRNA and protein level a er transfection of si- *2* 1/2 (Figures 2A and 2B, <0.01). We chose one of si- *2* 1/2 which named as si- *2* to perform the subsequent experiments. Furthermore, OD values of OS cells measured by CCK-8 assay showed that reduced OD values was observed in si- *2* group compared with si-con group with a time-dependent manner (Figure 2C, <0.01). All results indicate that *2* silencing could inhibit OS cell proliferation.

2 silencing decreases migration ability

To further determine *2* in uencing OS cell migration capability, scratch wound assay was performed. e results showed that a lower migration distance was detected in si- *2* group on comparing si-con group (Figure 3). e result suggests a suppressive role of *2* silencing in OS cell migration property.

2 silencing inhibited cell proliferation and migration



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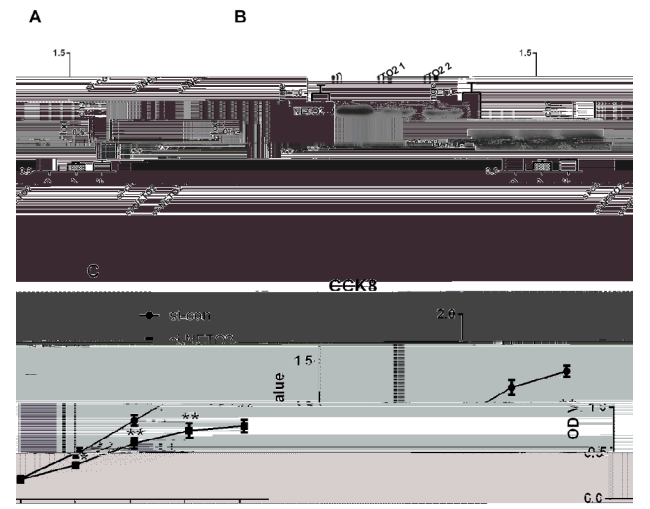

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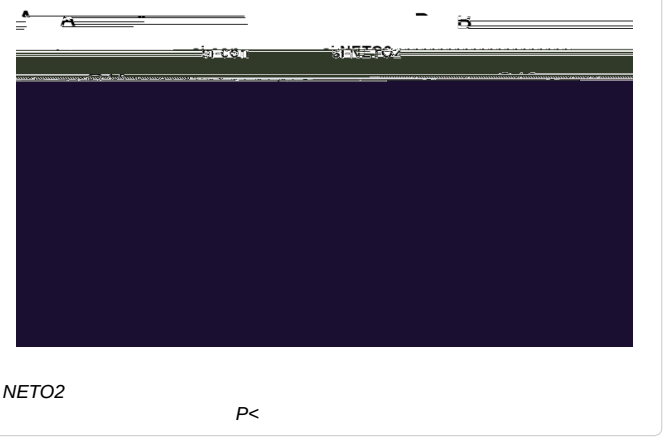
that *2* may function as an oncogene in OS, implying it will be a potential therapeutic target for the treatment of OS patients.

Cancer initiations and developments is a complex pathogenesis process, implicated various molecules and biological networks, and OS is no exception. It is beyond doubt that bioinformatics provides an



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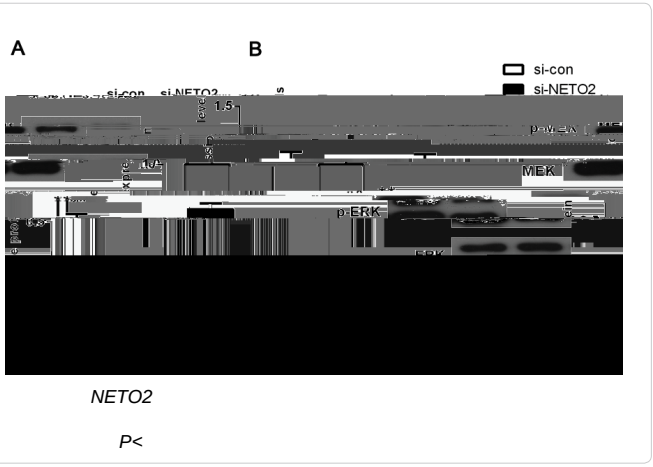

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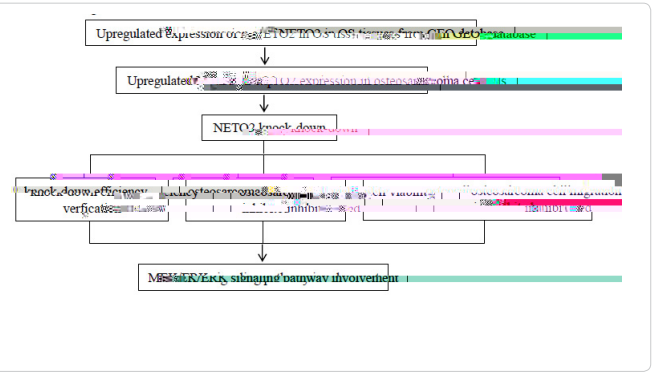

expedient tool for studying available molecules. We analyzed herein the mRNA expression patterns in OS tissues and normal samples available from GEO datasets. e data showed that an upregulated level of *2* was identi ed in OS tissues comparing with normal samples. To con rm it, we performed qRT-PCR to measure *2* mRNA expression level in OS cells. As expected, the result showed that *2* was signi cantly higher in OS cells than normal cells. Previous studies have suggested that *2* is overexpressed in a variety of cancers, but no reports about e ect of it on OS progression. ereby, this study o ers a good insight for comprehending the biological function of *2* on OS.

It is well-known that uncontrollable proliferation and aggressive migration are tightly correlated with knotty metastatic and recurrent

OS [16,17]. Hence, to address it, we assessed the e ect of *2* on OS proliferation and migration. OD values in OS cells showed that an obvious decreased OD value in si- *2* group was detected on comparing with si-con group. Additionally, the result in scratch wound assay suggested a delayed migration closure in si- *2* group in contrast with si-con group. In short, these data indicate that *2* could exert an inhibitory role in OS proliferation and migration.

Having well-documented that MEK/ERK signaling is crucial for cancer progression, to explore the mechanism underlying *2*-induced OS cell proliferation and migration, we examined its e ect on MEK/ERK signaling. In this work, hallmarks of this signaling including MEK/p-MEK and ERK/p-ERK were measured. e result of western blot analysis showed that p-MEK and p-ERK were signi cantly increased insi- *2* group on comparing si-con group while there were no obvious di erences of MEK and ERK in two groups. A previous study has suggested that inhibition in MEK/ERK signaling activity mediates repression in OS metastasis capability [18]. Another report has demonstrated that MEK1/2 and ERK1/2 phosphorylation are involved in Ewing sarcoma metastasis [19]. Altogether, our data in combination with previous studies suggests that MEK/ERK is participated in *2* facilitating OS progression. In spite of this, our current knowledge of the mechanisms responsible for these events and the regulatory components involved is still rudimentary at best and further deeper investigations is necessary [20-28].

Conclusion

Collectively, our present study demonstrated that *2*, which was markedly overexpressed in OS tissues and cells, played a signi cant role in OS proliferation and migration through the MEK/ERK pathway. A schematic ochart (Figure 5) was used to ravel out our ndings. erefore, these results shed some light on *2*

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