

# Overexpression of miR-100 inhibits growth of osteosarcoma through FGFR3

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**Abstract** Osteosarcoma (OS) is a prevalent, fast growing cancer. Identification of molecular regulation of OS growth may result in development of a novel therapy. Previous studies have highlighted a role of microRNAs (miRNAs) in the regulation of the carcinogenesis of OS, whereas the underlying mechanisms are not completely understood. Moreover, a role of miR-100 in the growth control of OS is not clear. Here we reported significantly higher levels of fibroblast growth factor receptor 3 (FGFR3) and significantly lower levels of miR-100 in the OS specimen, compared to those in the paired normal bone tissues. Bioinformatics analysis and luciferase reporter assay suggest that miR-100 binds to the 3'UTR of FGFR3 mRNA to prevent its translation. To prove it, we modified miR-100 levels in OS cells. We found that overexpression of miR-100 in OS cells decreased FGFR3 protein levels, whereas inhibition of miR-100 increased FGFR3 protein levels, without affecting FGFR3 transcripts. Moreover, overexpression of miR-100 suppressed the OS growth *in vitro* and *in vivo*, while inhibition of miR-100 significantly increased OS growth. Taken together, our data demonstrate that miR-100 may inhibit the growth of OS through FGFR3.

**Keywords** Osteosarcoma (OS) · Fibroblast growth factor receptor 3 (FGFR3) · miR-100

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## Introduction

Osteosarcoma (OS) is a prevalent primary bone malignancy and accounts for more than half of all malignant childhood bone tumors [1–6]. Although combined chemotherapy and surgery have been applied, the 5-year survival of patients remains pretty low [1–6]. Therefore, for controlling and treating osteosarcoma, new therapeutic targets are extremely needed. A number of human OS cell lines have been established, among which U2OS [7] cell line has been extensively characterized and widely used in research.

MicroRNAs (miRNAs) are a class of small, noncoding RNAs that regulate various biological processes [8–10]. Interestingly, bioinformatics approaches have predicted one third of all mammalian genes to be targeted and regulated by miRNAs [8–10]. Previous studies have shown that aberrant miRNA expression could impact normal biological processes, resulting in OS initiation and progression [11–15]. However, a role of miR-100 in the carcinogenesis of OS has not been studied.

The fibroblast growth factor receptors (FGFRs) have four different isoforms named 1–4. These FGFRs bind to more than 20 ligands from the members of the FGF family, and these signaling pathways have been reported to be involved in carcinogenesis of various cancers [16–18]. The FGFRs consist of a cellular ligand domain composed of three immunoglobulin-like domains, a single transmembrane helix domain, and an intracellular domain with tyrosine kinase activity [16–19]. The phosphorylation of FGFRs by FGFs initiates biological effects through activation of different signaling pathways including the phosphatidylinositol 3-kinase (PI3K) pathway, the extracellular-related kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, and the Jun N-terminal kinase (JNK) pathway [20–22]. FGFR3 is

overexpressed in some cancers to enhance tumor growth, whereas a specific function of FGFR3 in the tumor growth of OS is not completely clarified [23–25].

Here we reported significantly higher levels of FGFR3 and significantly lower levels of miR-100 in the OS specimen, compared to those in the paired normal bone tissues. Bioinformatics analysis and luciferase reporter assay suggest that miR-100 binds to the 3'UTR of FGFR3 mRNA to prevent its translation. To prove it, we modified miR-100 levels in OS cells. We found that overexpression of miR-100 in OS cells decreased FGFR3 protein levels, whereas inhibition of miR-100 increased FGFR3 protein levels, without affecting FGFR3 transcripts. Moreover, overexpression of miR-100 suppressed the OS growth *in vitro* and *in vivo*, while inhibition of miR-100 significantly increased OS growth. Taken together, our data demonstrate that miR-100 may inhibit the growth of OS through FGFR3.

## Materials and methods

### Patient tissue specimens

A total of 30 resected specimens from OS patients were collected for this study. OS specimen was compared with the paired normal bone tissue (NT) from the same patient. All specimens had been histologically and clinically diagnosed at the Department of Orthopedics, the First Affiliated Hospital of Liaoning Medical University from 2005 to 2014. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

### Culture of human OS cell line

U2OS is a widely used human OS line purchased from American Type Culture Collection (ATCC; Rockville, MD, USA), and has been described [7]. U2OS cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 20 % fetal bovine serum (Invitrogen).

### Animal manipulation

All animal procedures in the current study have been approved and conducted by the Institutional Review Boards of the First Affiliated Hospital of Liaoning Medical University. Female NOD/SCID mice were purchased from Charles River Laboratories (China), kept pathogen free, and used for study at 12 weeks of age. Luciferase-carrying U2OS cells (U2OS-LUC-2A-GFP) of  $10^6$  were injected subcutaneously to form a tumor in NOD/SCID mice. Adeno-associated viruses (AAV)

of  $10^9$  were injected in 100  $\mu$ l solution directly into the implanted tumor.

### Transfection of U2OS cells *in vitro*

U2OS cells were transfected with a miR-100 construct, or an antisense (as) of miR-100, or a scramble sequence (scr) as a control, and after which the transfected cells were purified based on green fluorescence protein (GFP) to generate U2OS-miR-100, U2OS-scr, or U2OS-as-miR-100 cells, respectively. The miR-100 sequence is 5'-AACCCGUAGA UCCGAACUUGUG-3', miR-100 antisense sequence 5'-CA-CAAGUUCGGAUCUACGGGUU-3', and control sequence 5'-UUUAGCGCCGAAAAGUAAUG-3'. Transfection was performed with Lipofectamine 2000 Reagent (Invitrogen), according to manufacturer's instructions.

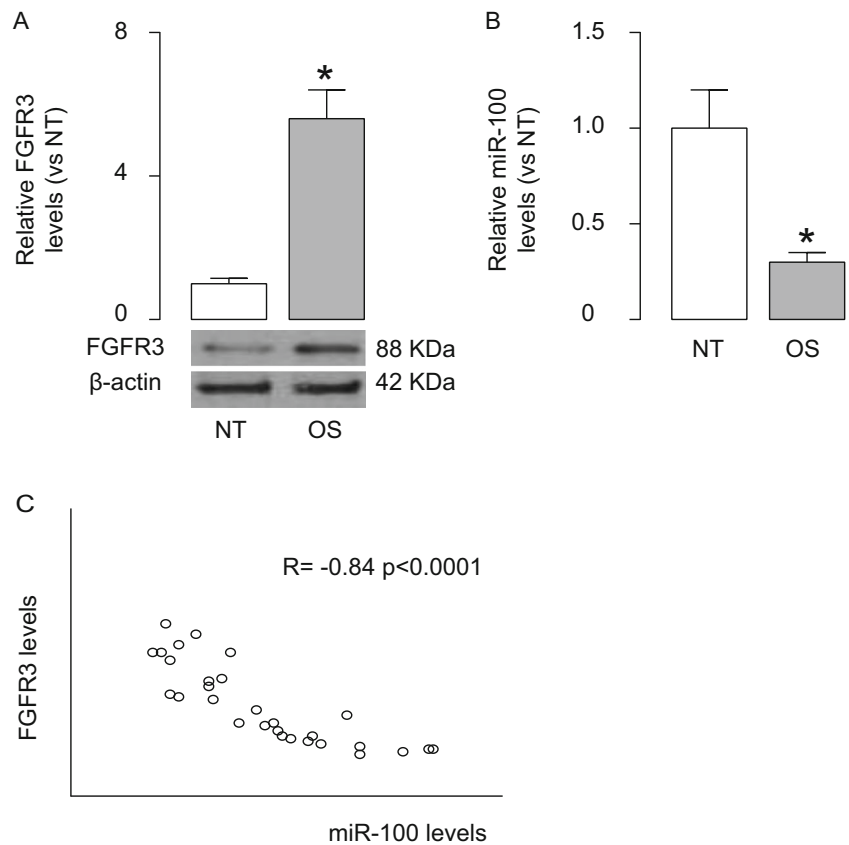
### Transduction of U2OS with AAV

U2OS cells were transduced with an AAV carrying luciferase and GFP construct (connected with a 2A sequence), to allow *in vivo* tracing in mice and purification of transduced cells by flow cytometry, respectively. Briefly, human embryonic kidney 293 cell line (HEK293) was used. We used a pAAV-CMV-LUC-GFP plasmid (Clontech, Mountain View, CA, USA), a packaging plasmid carrying the serotype 8 rep and cap genes, and a helper plasmid carrying the adenovirus helper functions (Applied Viromics, LLC, Fremont, CA, USA) in this study. AAV was prepared by triple transfection of the newly prepared plasmids, R2C8 (containing AAV2 Rep and AAV8 capsid genes) and pAd5 (containing adenovirus helper genes), into HEK293 cells by Lipofectamine 2000 Reagent (Invitrogen). The viruses were purified using CsCl density centrifugation and then titered by a quantitative densitometric dot-blot assay. Then, the U2OS cells were incubated with AAV at a MOI of 100 for 12 h.

### Luciferase reporter activity assay

Luciferase reporters were successfully constructed using molecular cloning technology. Target sequence was inserted into pGL3-Basic Vector (Promega, Madison, WI, USA) to obtain pGL3-FGFR3-3'UTR, which contains the miR-100 binding sequence (FGFR3-3'UTR sequence). U2OS-miR-100, U2OS-scr, or U2OS-antisense (as)-miR-100 cells were seeded in 24-well plates for 24 h, after which they were transfected with 1  $\mu$ g of luciferase reporter plasmids per well using PEI Transfection Reagent. Then, luciferase activities were measured using the dual-luciferase reporter gene assay kit (Promega), according to manufacturer's instructions.

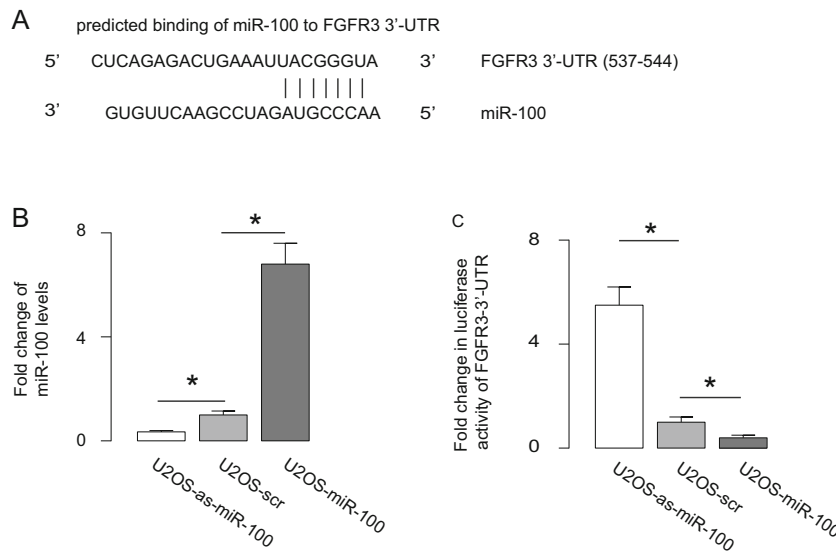
**Fig. 1** High levels of FGFR3 and low levels of miR-100 are inversely correlated in OS. We examined the FGFR3 levels and miR-100 levels in the resected OS specimens from 30 patients, and compared to those in the paired normal bone tissue (NT). **a–b** We detected significantly higher levels of FGFR3 by **a** Western blot and significantly lower levels of miR-100 by **b** RT-qPCR, in the OS samples. **c** A strong inverse correlation was detected between FGFR3 and miR-100 in OS specimen ( $R=-0.84, p<0.0001$ ).  $*p<0.05$



**Imaging of the implanted tumor by bioluminescence**

The tumor growth in the living animals was monitored and quantified by luminescence levels. Bioluminescence was measured

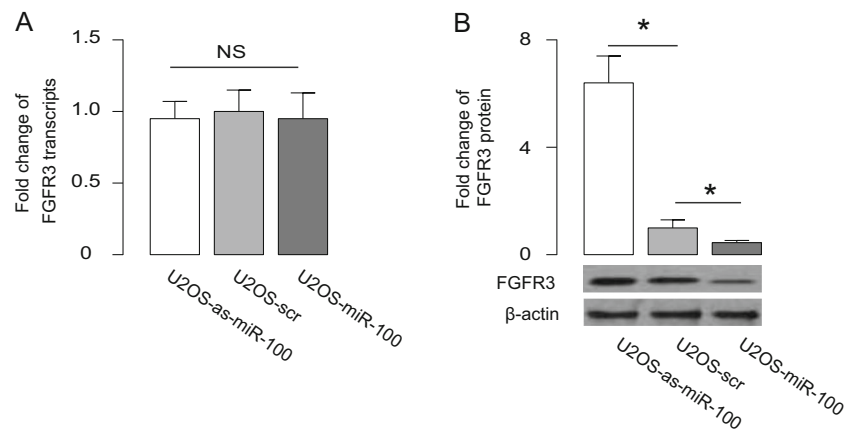
with the IVIS imaging system (Xenogen Corp., Alameda, CA, USA). All of the images were taken 10 min after intraperitoneal injection of luciferin (Sigma-Aldrich) of 150 mg/kg body weight, as a 60-s acquisition and 10 of binning. During image



**Fig. 2** MiR-100 targets 3'UTR of FGFR3 to inhibit its expression. **a** Bioinformatics analyses of FGFR3 target sequence show that the miR-100 binds to two sites at 3'UTR of FGFR3 mRNA. **b–c** We overexpressed or inhibited miR-100 expression in a human OS cell line, U2OS, to obtain U2OS-miR-100 and U2OS-as-miR-100 cells. The

cells transfected with a control plasmid carrying a scramble sequence (U2OS-scr). **b** RT-qPCR on miR-100. **c** U2OS-miR-100, U2OS-scr, and U2OS-as-miR-100 cells were transfected with 1  $\mu$ g of FGFR3-3' UTR luciferase reporter plasmid. The luciferase activities in these cells were then evaluated.  $*p<0.05$

**Fig. 3** MiR-100 decreases FGFR3 levels in OS cells. **a** RT-qPCR on FGFR3. **b** Western blot images and quantification for FGFR3. \* $p < 0.05$ . NS non-significant



acquisition, mice were sedated continuously via inhalation of 3 % isoflurane. Image analysis and bioluminescent quantification were performed using Living Image Software (Xenogen Corp).

### Quantitative PCR (RT-qPCR)

MiRNA and total RNA were extracted from cultured cells with miRNeasy mini kit or RNeasy kit (QIAGEN, Hilden, Germany), respectively, and used for cDNA synthesis. Quantitative PCR was performed in duplicates with QuantiTect SYBR Green PCR Kit (QIAGEN). All primers were purchased from QIAGEN. Values of genes were normalized against  $\beta$ -actin and then compared to controls.

### Western blot

The protein was extracted from the resected OS specimen or adjacent normal bone tissue (NT), or cultured cells. Primary antibodies were anti-FGFR3 and anti- $\beta$ -actin (Cell Signaling, San Jose, CA, USA).  $\beta$ -actin was used as a protein loading control. Secondary antibodies were HRP-conjugated anti-rabbit and were all purchased from Jackson Immuno Research Labs (West Grove, PA, USA). The protein levels were first normalized to  $\beta$ -actin and then normalized to control, quantified by NIH ImageJ software (Bethesda, MA, USA).

### Statistical analysis

All statistical analyses were carried out using the SPSS 17.0 statistical software package. All values are depicted as mean  $\pm$  standard deviation and are considered significant if  $p < 0.05$ . All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's exact test to compare two groups.

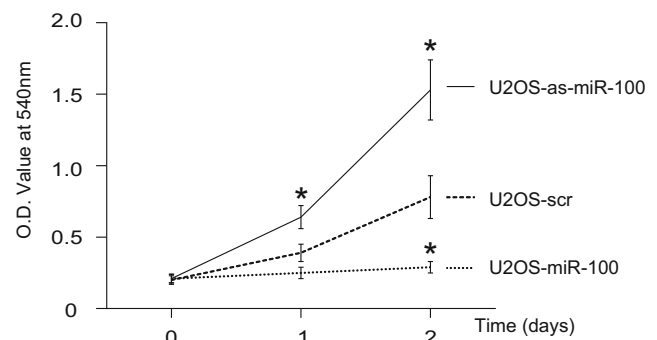
## Results

### High levels of FGFR3 and low levels of miR-100 are inversely correlated in OS

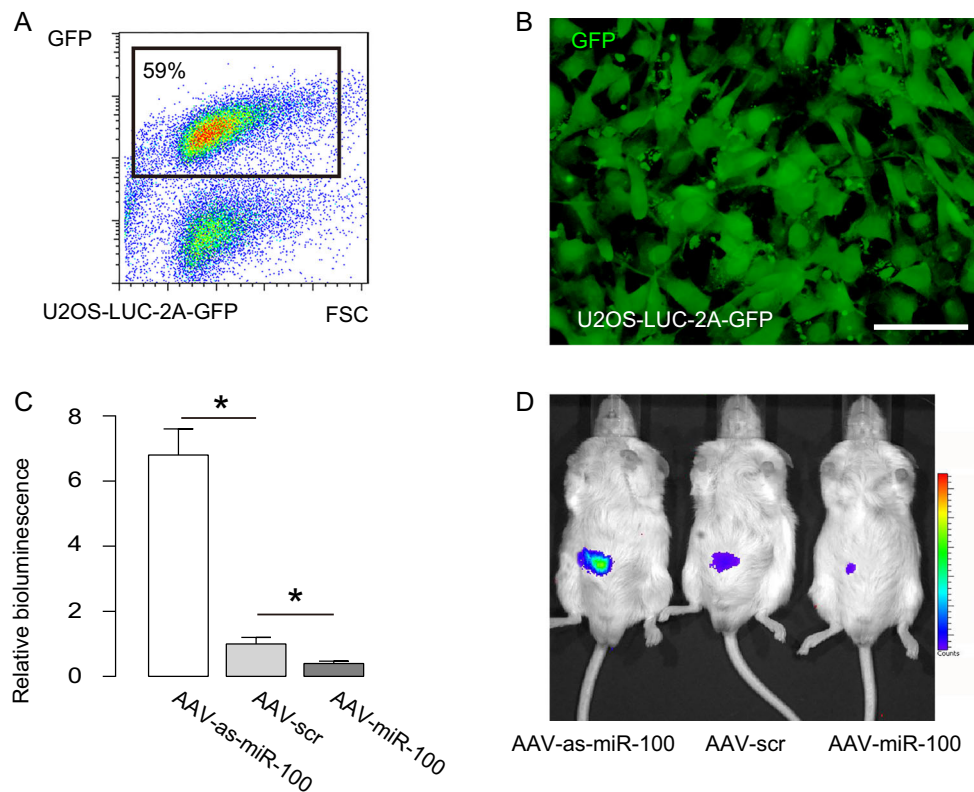
We examined the FGFR3 levels and miR-100 levels in the resected OS specimens from 30 patients, and compared to the paired normal bone tissue (NT). We detected significantly higher levels of FGFR3 by Western blot (Fig. 1a) and significantly lower levels of miR-100 by RT-qPCR (Fig. 1b), in the OS samples. Moreover, a strong inverse correlation was detected between FGFR3 and miR-100 in OS specimen (Fig. 1c,  $R = -0.84$ ;  $p < 0.0001$ ), suggesting a causal relationship.

### MiR-100 targets 3'UTR of FGFR3 to inhibit its expression

Based on our findings in the patients' samples, we performed bioinformatics analysis of FGFR3 target sequence, which shows that miR-100 binds to 3'UTR of FGFR3 mRNA at the 537th-544th base site (Fig. 2a). To prove the effect, we used a human OS cell line, U2OS, in our study. We transfected U2OS cells with either miR-100 or antisense for miR-100 (as-



**Fig. 4** MiR-100 inhibits OS growth in vitro. In an MTT assay, the increases in cell number of U2OS-as-miR-100 cells were significantly greater than those in the control U2OS-scr cells, while the luciferase activities in U2OS-miR-100 cells were significantly lower than those in the control U2OS-scr cells. \* $p < 0.05$



**Fig. 5** Suppression of miR-100 inhibits OS cell growth in vivo. **a** U2OS cells were transfected with a plasmid with luciferase and GFP construct (*U2OS-LUC-2A-GFP*), to allow in vivo tracing after purification of transduced cells by flow cytometry. **b** Purified U2OS-LUC-2A-GFP cells in culture. **c** We used 30 female NOD/SCID mice that were randomly separated into three groups of ten each. All the mice received subcutaneous injection of  $10^6$  U2OS-LUC-2A-GFP cells to form OS. **d**

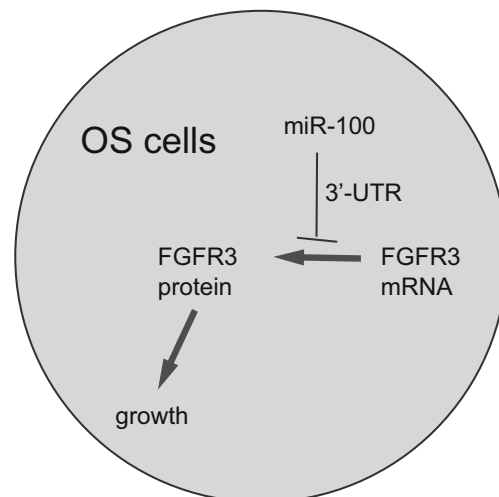
One month later, these mice received injection of either AAV-miR-100 or AAV-as-miR-100 or AAV-scr as a control. After 4 weeks, the tumor growth was monitored and quantified by luminescence levels. We detected significant lower tumor growth in AAV-as-miR-100-injected mice and significant higher tumor growth in AAV-miR-100-injected mice, compared to that in controls.  $*p < 0.05$

miR-100). U2OS cells were also transfected with a scramble sequence as a control (scr). Modulation of miR-100 levels in U2OS cells was confirmed by RT-qPCR (Fig. 2b). Then, U2OS-miR-100, U2OS-scr, and U2OS-as-miR-100 cells were transfected with 1  $\mu$ g of FGFR3-3'UTR luciferase reporter plasmid. We found that the luciferase activities in U2OS-as-miR-100 cells were significantly higher than those in the control, while the luciferase activities in U2OS-miR-100 cells were significantly lower than those in the control (Fig. 2c). These data suggest that miR-100 targets 3'UTR of FGFR3 to inhibit its translation.

**MiR-100 decreases FGFR3 protein but does not affect its transcription**

Since FGFR3 is well known to promote tumor growth, and specifically express in OS cells, we were thus prompted to evaluate whether FGFR3 may be regulated by miR-100 in OS cells. We found that although the FGFR3 transcripts did not change by miR-100 levels (Fig. 3a), the protein levels of FGFR3 in miR-100-overexpressing U2OS cells were significantly decreased, while the protein levels of FGFR3 in miR-

100-depleted U2OS cells were significantly increased (Fig. 3b). These data suggest that the translation of FGFR3 in OS cells may be regulated by miR-100.



**Fig. 6** Schematic of the model. MiR-100 inhibits growth of OS through FGFR3 suppression

### MiR-100 inhibited OS growth in vitro

Then, we examined whether the effects of miR-100 on FGFR3 may result in changes in cell growth in OS cells. In an MTT assay, the increases in cell number of U2OS-as-miR-100 cells were significantly higher than that of the control U2OS-scr cells, while the luciferase activities in U2OS-miR-100 cells were significantly lower than those in the control U2OS-scr cells (Fig. 4). These data suggest that miR-100 inhibits OS growth in vitro.

### Suppression of miR-100 inhibits OS cell growth in vivo

U2OS cells were transfected with a plasmid with luciferase and GFP construct (U2OS-LUC-2A-GFP), to allow in vivo tracing after purification of transduced cells by flow cytometry (Fig. 5a–b). We used 30 female NOD/SCID mice that were randomly separated into three groups of ten each. All the mice received subcutaneous injection of  $10^6$  U2OS-LUC-2A-GFP cells to form OS. One month later, these mice received injection of either AAV-miR-100 or AAV-as-miR-100 or AAV-scr as a control. After 4 weeks, the tumor growth was monitored and quantified by luminescence levels. We detected significant greater tumor growth in AAV-as-miR-100-injected mice and significant lower tumor growth in AAV-miR-100-injected mice, compared to controls (Fig. 5c–d), suggesting that inhibition of miR-100 suppresses OS cell growth in vivo. Our study thus demonstrates that miR-100 inhibits the growth of OS through FGFR3 suppression in OS cells (Fig. 6).

### Discussion

Previous studies have demonstrated an essential role of miRNAs in the growth of some cancers, whereas a role of miR-100 in the OS cell growth is unknown. In this study, we aimed to understand the molecular mechanisms that underlie the regulation of the tumor growth of OS by miR-100. Then, we used several human OS cell lines (including U2OS, HOS, etc.) to study the mechanism. We obtained similar results from these OS lines. Here we only showed our data using U2OS cells.

Of note, we detected strong inverse correlation between FGFR3 and miR-100 in OS specimen, suggesting presence of a regulatory relationship. Since FGFR3 plays a critical role in promoting cancer cell growth and has been shown to express in OS cells, we thus examined whether miR-100 may inhibit the expression of FGFR3. We either overexpressed or inhibited miR-100 expression in OS cells, which decreased or increased the protein levels of FGFR3, respectively, without affecting FGFR3 transcript levels. These data suggest that FGFR3 translational controls in OS cells may be regulated by miR-100, which is consistent with the function of

miRNAs. Moreover, using luciferase reporter assay, we identified a binding sites of miR-100 at 3'UTR on FGFR3 mRNA. Taken together, these findings strongly demonstrate a critical role of miR-100 in regulating FGFR3 translation, rather than in regulating the degradation of FGFR3 mRNA. FGFR3 is a protein that is modified by phosphorylation and sumoylation, which potentially affect its function. Here we did not study these protein modifications, since the total protein level is the first parameter to determine the activity of FGFR3. Future attempts may be taken to address protein modifications of FGFR3 to completely understand the relationship between FGFR3 and miR-100.

In addition, we found that the modification of miR-100 levels in OS cells significantly altered cell growth in vitro and in vivo. Our findings thus highlight miR-100/FGFR3 axis as a novel therapeutic target for inhibiting the growth of OS. In the future, experiments should be designed to determine the other possible targets of miR-100 to completely understand its role in the carcinogenesis of OS.

**Conflicts of interest** None

### References

1. Tsuchiya H, Tomita K, Mori Y, Asada N, Morinaga T, Kitano S, et al. Caffeine-assisted chemotherapy and minimized tumor excision for nonmetastatic osteosarcoma. *Anticancer Res.* 1998;18: 657–66.
2. Yang J, Zhang W. New molecular insights into osteosarcoma targeted therapy. *Curr Opin Oncol.* 2013;25:398–406.
3. Li G, Fu D, Liang W, Fan L, Chen K, Shan L, et al. *Cycl* silencing sensitizes osteosarcoma cells to trail-induced apoptosis. *Cell Physiol Biochem.* 2014;34:2070–80.
4. Liu Y, He J, Chen X, Li J, Shen M, Yu W, et al. The proapoptotic effect of formononetin in human osteosarcoma cells: involvement of inactivation of erk and akt pathways. *Cell Physiol Biochem.* 2014;34:637–45.
5. Wang Q, Cai J, Wang J, Xiong C, Zhao J. Mir-143 inhibits egfr-signaling-dependent osteosarcoma invasion. *Tumour Biol.* 2014;35:12743–8.
6. Xiao Q, Zhang X, Wu Y, Yang Y. Inhibition of macrophage polarization prohibits growth of human osteosarcoma. *Tumour Biol.* 2014;35:7611–6.
7. Ponten J, Saksela E. Two established in vitro cell lines from human mesenchymal tumours. *Int J Cancer.* 1967;2:434–47.
8. Sicard F, Gayral M, Lulka H, Buscail L, Cordelier P. Targeting mir-21 for the therapy of pancreatic cancer. *Mol Ther.* 2013;21:986–94.
9. Tavano F, di Mola FF, Piepoli A, Panza A, Copetti M, Burbaci FP, et al. Changes in mir-143 and mir-21 expression and clinicopathological correlations in pancreatic cancers. *Pancreas.* 2012;41:1280–4.
10. Ali S, Ahmad A, Banerjee S, Padhye S, Dominiak K, Schaffert JM, et al. Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of mir-200 and mir-21 expression by curcumin or its analogue cdf. *Cancer Res.* 2010;70:3606–17.
11. Luo XJ, Tang DG, Gao TL, Zhang YL, Wang M, Quan ZX, et al. MicroRNA-212 inhibits osteosarcoma cells proliferation and

- invasion by down-regulation of Sox4. *Cell Physiol Biochem.* 2014;34:2180–8.
12. Li F, Li S, Cheng T. Tgf-beta1 promotes osteosarcoma cell migration and invasion through the mir-143-versican pathway. *Cell Physiol Biochem.* 2014;34:2169–79.
  13. Xu H, Liu X, Zhao J. Down-regulation of mir-3928 promoted osteosarcoma growth. *Cell Physiol Biochem.* 2014;33:1547–56.
  14. Xu G, Wang J, Jia Y, Shen F, Han W, Kang Y. MiR-142-3p functions as a potential tumor suppressor in human osteosarcoma by targeting HMGA1. *Cell Physiol Biochem.* 2014;33:1329–39.
  15. Pan W, Wang H, Jianwei R, Ye Z. MicroRNA-27a promotes proliferation, migration and invasion by targeting MAP2k4 in human osteosarcoma cells. *Cell Physiol Biochem.* 2014;33:402–12.
  16. Friesel R, Maciag T. Fibroblast growth factor prototype release and fibroblast growth factor receptor signaling. *Thromb Haemost.* 1999;82:748–54.
  17. Jaye M, Schlessinger J, Dionne CA. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. *Biochim Biophys Acta.* 1992;1135:185–99.
  18. Wang X, Cao X. Regulation of metastasis of pediatric multiple myeloma by MMP13. *Tumour Biol.* 2014.
  19. Fujimoto J, Hori M, Ichigo S, Tamaya T. Expressions of the fibroblast growth factor family (FGF-1, -2 and -4) mRNA in endometrial cancers. *Tumour Biol.* 1996;17:226–33.
  20. Soundararajan P, Fawcett JP, Rafuse VF. Guidance of postural motoneurons requires MAPK/ERK signaling downstream of fibroblast growth factor receptor 1. *J Neurosci.* 2010;30:6595–606.
  21. Kuslak SL, Marker PC. Fibroblast growth factor receptor signaling through MEK-ERK is required for prostate bud induction. *Differentiation.* 2007;75:638–51.
  22. Williamson AJ, Dibling BC, Boyne JR, Selby P, Burchill SA. Basic fibroblast growth factor-induced cell death is effected through sustained activation of p38MAPK and up-regulation of the death receptor p75<sup>ntr</sup>. *J Biol Chem.* 2004;279:47912–28.
  23. Ren T, Qing Y, Dai N, Li M, Qian C, Yang Y, et al. Apurinic/aprimidinic endonuclease 1 induced upregulation of fibroblast growth factor 2 and its receptor 3 induces angiogenesis in human osteosarcoma cells. *Cancer Sci.* 2014;105:186–94.
  24. Mirabello L, Yu K, Berndt SI, Burdett L, Wang Z, Chowdhury S, et al. National osteosarcoma etiology study G: a comprehensive candidate gene approach identifies genetic variation associated with osteosarcoma. *BMC Cancer.* 2011;11:209.
  25. Jang JH. Identification and characterization of soluble isoform of fibroblast growth factor receptor 3 in human SaOS-2 osteosarcoma cells. *Biochem Biophys Res Commun.* 2002;292:378–82.