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Long non-coding RNA ZFAS1 regulates NOB1 expression through interacting with miR-646 and promotes tumorigenesis in osteosarcoma

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Abstract. – OBJECTIVE: Zinc finger antisense 1 (ZFAS1), a newly identified lncRNA, is aberrantly regulated in various cancers including osteosarcoma (OS). However, the underlying molecular mechanisms of ZFAS1 in OS remain to be elucidated.

MATERIALS AND METHODS: We used transfection, luciferase report assay, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), colony formation assay, transwell migration, invasion assays, and Western blot to determine the potential mechanisms.

RESULTS: Our study showed that ZFAS1 was up-regulated in OS cells and promoted the colony formation, migration, and invasion of OS cells via activating the MAPK signaling pathway. Furthermore, the experimental results indicated that miR-646 was a target of ZFAS1 and there was a negative relationship between ZFAS1 and miR-646 expression. Additionally, we found that ZFAS1 in OS cells up-regulated the expression of NOB1 through sponging miR-646, finally facilitating the growth of the OS cells.

CONCLUSIONS: These results demonstrated that ZFAS1/miR-646/NOB1 axis might play an important role in the development of OS, and ZFAS1 and miR-646 can be considered as potential biomarkers for the diagnosis and treatment of OS.

Key Words:
ZFAS1, MiR-646, NOB1, Osteosarcoma, LncRNA, CeRNA.

Introduction

Osteosarcoma (OS) is the most common primary bone malignant tumor in children and adolescents[1]. It is classically described as an aggressive osteoid-producing tumor of mesenchymal origin[2]. Currently, the surgery combined with adjuvant and neoadjuvant chemotherapy is regarded as the standard treatment procedure for OS[3]. Despite significant advances in the 1970s and 1980s, the outcomes and treatments have likewise improved very little in several decades[3,4]. The 5-year overall survival remains about 70% for patients with a localized OS, while patients with recurrent or metastatic disease poorly fare with overall survival rates of approximately 20%[4]. Thus, multiple efforts are needed to improve therapeutic efficacy. Based on the uncertain etiology of OS, a better understanding of the molecular pathology of OS will facilitate the development of diagnosis and therapy for OS patients[2]. Recent studies on the mechanisms of carcinogenesis have gradually focused on long non-coding RNAs (lncRNAs)[5]. LncRNAs constitute non-protein coding RNA molecules with more than 200 nucleotides in size[6]. LncRNAs play an important role in various biological processes such as gene expression, cell cycle regulation, and chromatin remodeling[7]. LncRNAs are also associated with the development of many diseases, especially cancers[7]. With regard to their role in cancer, lncRNAs facilitate tumor initiation and development through various mechanisms ranging from epigenetic regulation of key cancer genes and enhancer-associated activity to post-transcriptional processing of mRNAs[5,8]. Besides, they are frequently and aberrantly expressed in many cancers such as OS[9], prostate[10], kidney[11], and breast cancer[12]. Thus, lncRNAs can be considered as new biomarkers for cancer diagnosis, prognosis, and therapy. Zinc finger antisense 1 (ZFAS1), a newly identified lncRNA, is aberrantly regulated in myocardial infarction[13] and several cancers (e.g., ovarian cancer, breast cancer, and gastric cancer)[14-16]. For instance, suppression of ZFAS1 expression seems to promote the pro-

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liferation, migration, and invasion of breast cancer cells, which indicated that ZFAS1 might be a tumor suppressor in breast cancer[15]. However, ZFAS1 overexpression in ovarian cancer could promote proliferation rate, migration activity, and development of chemoresistance in ovarian cancer[14]. Therefore, ZFAS1 can be classified as either tumor suppressor or oncogene. Although ZFAS1 has been reported to be upregulated in OS[17,18], there is a little known about the potential molecular mechanisms of ZFAS1 in correlation with OS tumorigenesis and progression. In this study, we explored the expression level, biological function, and underlying mechanism of ZFAS1 in the development of OS.

Materials and Methods

Cell Culture

OS cell lines (U2OS, Saos-2, and MG-63) and normal human osteoblastic cell line (NHOst) were obtained from Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were incubated in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in 5% CO_2.

Transient Transfection

Small interfering RNA si-ZASF1, si-NOB1, and corresponding negative control siRNA were obtained from Shanghai GenePharma CO., Ltd. (Shanghai, China). MiR-646 mimics, miR-646 inhibitor, and corresponding control were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, Guangdong, China). To overexpress ZFAS1 and NOB1, they were amplified by PCR and cloned into pCDNA3.1 (+) vector (Invitrogen, Carlsbad, CA, USA) to generate pCDNA3.1-ZFAS1 plasmids and pCDNA3.1-NOB1 plasmids. Cells were seeded in six-well plates at a concentration of 1.0×10^5 per well. After incubation for 24 h, cells were separately transfected with si-ZASF1, pCDNA-ZFAS1, miR-646 mimics, miR-646 inhibitor, si-NOB1, and pCDNA-NOB1 plasmids using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 h of incubation, the transfected cells were collected for the following experiments.

Luciferase Report Assays

The full-length ZFAS1 and 3'UTR of NOB1 gene, containing the putative miR-646 binding sites, were amplified by PCR and was inserted into the psiCHECK2 vector (Promega, Madison, WI, USA). The coding sequences of ZFAS1 were generated by PCR and cloned into pCDNA3.1 (+) vector (Invitrogen, Carlsbad, CA, USA) to generate pCDNA3.1-ZFAS1 plasmids. Cells were co-transfected with the plasmids using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After transfection for 48 h, cells were harvested. Then, the luciferase activities were detected using a dual-luciferase reporter assay system (Promega, Madison, WI, USA). The relative luciferase activity was normalized to Renilla luciferase activity. All the experiments were performed in triplicate.

Quantitative Real Time-PCR (qRT-PCR)

Total RNAs from cells were exacted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNAs were converted into cDNA using the Reverse Transcriptase Kit (Takara, Dalian, Liaoning, China). qRT-PCR analyses were performed using the SYBR Green Mixture (TaKaRa, Dalian, Liaoning, China) in the ABI StepOne-Plus System. The samples were amplified as the following protocol: 95°C for 5 min, 40 cycles of 95°C for 30 s, 60°C for 40 s, and 72°C for 1 min. β-actin was used as an internal control. The relative expression was calculated and normalized using the $2^{-\Delta\Delta Ct}$ method. Each sample was evaluated for three independent experiments. Primers sequences are as follows: ZFAS1 forward, 5'-AAG CCA CGT GCA GAC ATC TA-3', reverse, 5'-CTA CTT CCA ACA CCC GCA TT-3'; miR-646: forward, 5'-ACA CTC CAG CTG GGA AGC AGC TGC CTC-3', reverse, 5'-CTC AAC TGT GCT GCA TTA GTT AGC TCA GA-3'; NOB1 forward, 5'-ATC TGC CCT ACA AGC CTA AAC-3', reverse, 5'-TCC TCC TCC TCC TCC TCA C-3'; β-actin forward, 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3', reverse, 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'.

Colony Formation Assay

Cells were seeded in 6-well plates and incubated in complete medium for two weeks. During this period, the fresh medium was replaced every three or four days. After two weeks, the cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet. The assays were performed in triplicate. Visible colonies were imaged and counted.

Transwell Migration and Invasion Assays

Cell migration and invasion assays were performed using 24-well plates (8 μm pores, BD Biosciences, Lake Franklin, NJ, USA). A total of 1.0×10^5 cells suspended in 100 μL serum-free RPMI-1640 medium were cultured in the upper chamber of a transwell or Matrigel-coated transwell insert. The lower chamber was filled with RPMI-1640 medium containing 10% FBS. After 24 h of incubation, cells in the upper chamber were removed by cotton swabs, and cells that had traversed the membrane were fixed with paraformaldehyde, stained with 0.1% crystal violet, washed with PBS, and then counted under an Olympus BX51 microscope (Olympus Corp., Shinjuku, Tokyo, Japan) at 200 × magnification in five random fields in each well. The assays were performed in triplicate.

Western Blot

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholic acid, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail; Santa Cruz Biotechnology, Santa Cruz, CA, USA) on ice for 30 min. The protein concentration of cell lysates was measured by the BCA method. The quantified protein was equally separated on SDS-PAGE and transferred into a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% non-fat milk for 1 h and incubated overnight at 4°C with primary antibodies (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA) against Raf1, MEK, and ERK. After washed with TBST buffer, the membranes were incubated with secondary antibodies for 1 h at 25°C. Then, the proteins of the membranes were detected by the ECL system and quantified by Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Actin was used as a loading control for the normalization of the protein quantity. Each experiment was performed in triplicate.

Statistical Analysis

Data were expressed as mean ± standard (SD). Statistical analyses were performed by SPSS Statistics software (Version 22.0. IBM SPSS Statistics for Windows, Armonk, NY, USA). The differences between groups were evaluated by the Student's *t*-test or one-way ANOVA, followed by a Bonferroni post-hoc test. A *p*-value of less than 0.05 was considered statistically significant.

Results

The Role of ZFAS1 in OS Cells

To explore the role of ZFAS1 in OS, we initially analyzed the expression levels of ZFAS1 in OS cell lines (U2OS, Saos-2, and MG-63) by qRT-PCR. Compared with the normal osteoplastic cell line (Nhost), the expression of ZFAS1 was significantly upregulated in OS cell lines, especially in the U2OS cell line (Figure 1A). Thus, we selected the U2OS cell line for the following experiments. Besides, qRT-PCR analysis results showed that ZFAS1 expression was downregulated and upregulated by ZFAS1 siRNA and overexpression plasmid transfection, respectively (Figure 1B and 1C). Next, we determined the effect of ZFAS1 on the proliferation, migration, and invasion of OS cells using colony formation assay and transwell assays. Colony formation assay indicated that the number of colony formation was considerably decreased in OS cells with ZFAS1 knockdown, while the cell numbers were increased in OS cells with ZFAS1 overexpression (Figure 1D). Transwell assays revealed that the knockdown of ZFAS1 significantly inhibited the migration and invasion of OS cells, whereas the upregulation of ZFAS1 promoted OS cells migration and invasion abilities (Figure 1E and 1F). To further investigate the potential mechanism of ZFAS1 on the activity of OS cells, MAPK signaling pathway was measured by Western blot. Our results showed that ZFAS1 overexpression phosphorylated and activated Raf1, MEK, and ERK expression, but the knockdown of ZFAS1 remarkably decreased the phosphorylation expression of Raf1, MEK, and ERK (Figure 1G). Therefore, ZFAS1 overexpression induced the activation of the MAPK signaling pathway, which might influence the colony, migration, and invasion of OS cells.

MiR-646 Is a Target of ZFAS1

We used DIANA TOOLS LncBase to predict potential ZFAS1 targets and obtained multiple miRNAs. We selected the number one miR-646 for further analysis. We used TargetScan to identify the potential sites of ZFAS1 bound to miR-646 (Figure 2A). We inserted ZFSA1 with miR-646, predicted binding sites into luciferase reporter vectors and co-transfected with miR-646 mimics or miR-control into OS cells. As shown in Figure 2B, a significant decrease in luciferase activity was observed during miR-646 transfection. Besides, qRT-PCR demonstrated that miR-646 expression was significantly increased

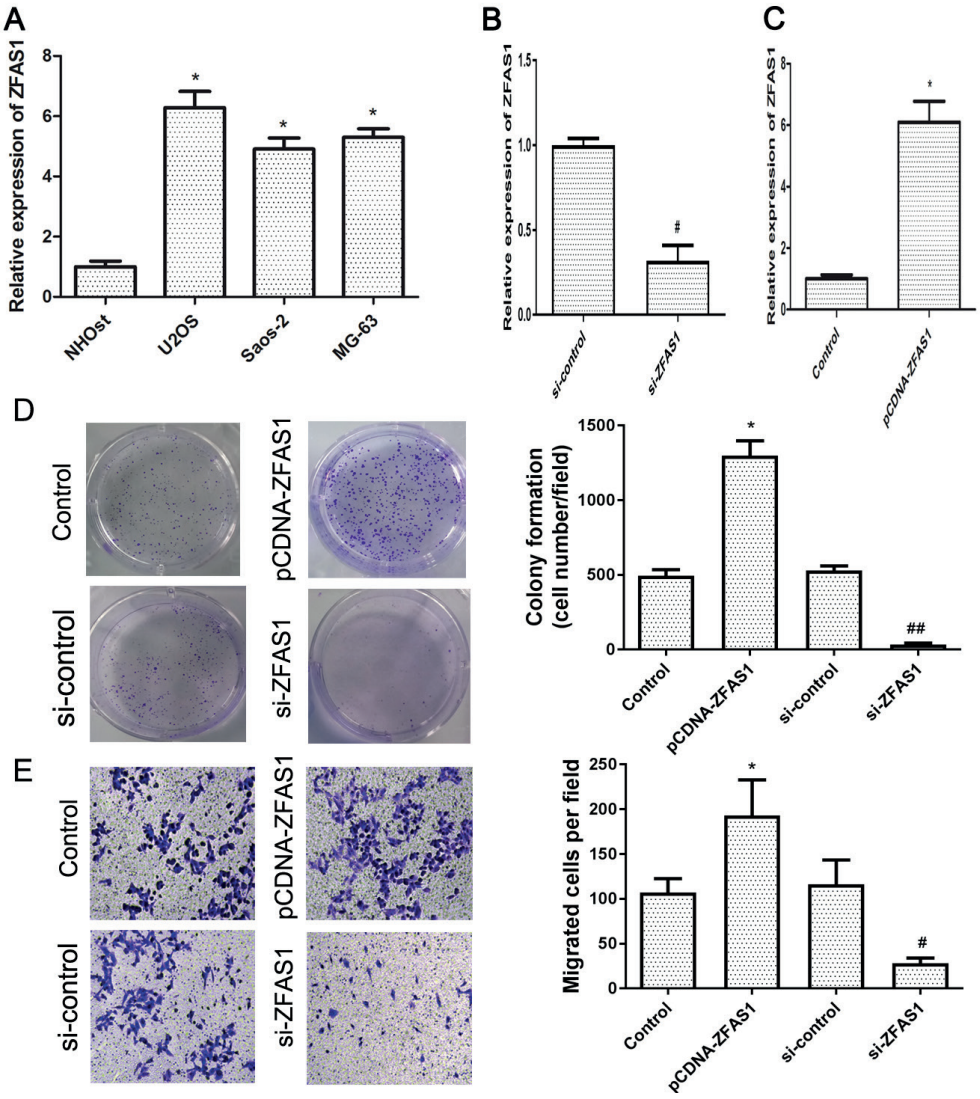

Figure 1. The role of ZFAS1 in OS cells. ***A,*** Relative expression levels of ZFAS1 in the three OS cell lines (U2OS, Saos-2 and MG-63) and the normal osteoplastic cell line (Nhost) were detected by qRT-PCR. ***B,*** Relative expression levels of ZFAS1 were determined by qRT-PCR in U2OS cells transfected with ZFAS1 siRNA (si-ZFAS1) or control siRNA (si-control). ***C,*** Relative expression levels of ZFAS1 were measured by qRT-PCR in U2OS cells transfected with ZFAS1 overexpression plasmids (pCDNA-ZFAS1) or empty vector (control). After U2OS cells were transfected with si-ZFAS1 or pCDNA-ZFAS1, we evaluated the effect of ZFAS1 on the cell proliferation, migration, and invasion using colony formation assay ***D,*** transwell migration assay (magnification 200x) ***E,*** and transwell invasion assay (magnification 200x).

Figure continued

in OS cells with ZFAS1 knockdown (Figure 2C). Therefore, the results showed that miR-646 was a target of ZFAS1.

The Role of MiR-646 in OS Cells

To investigate the effect of miR-646 on OS cells, we used a colony formation assay and transwell assays to determine the proliferation, migration, and invasion of OS cells. qRT-PCR showed that the expression of miR-646 was increased or decreased by miR-646 mimics or miR-646 inhibitor transfection, respectively (Figure 3A and 3B). Colony formation assay indicated that miR-646 significantly inhibited the colony forming efficiency of OS cells (Figure 3C). Transwell assays showed that miR-646 effectively inhibited the migration and invasion of OS cells, while miR-646 inhibitor promoted the migration and invasion of OS cells (Figure 3D and 3E).

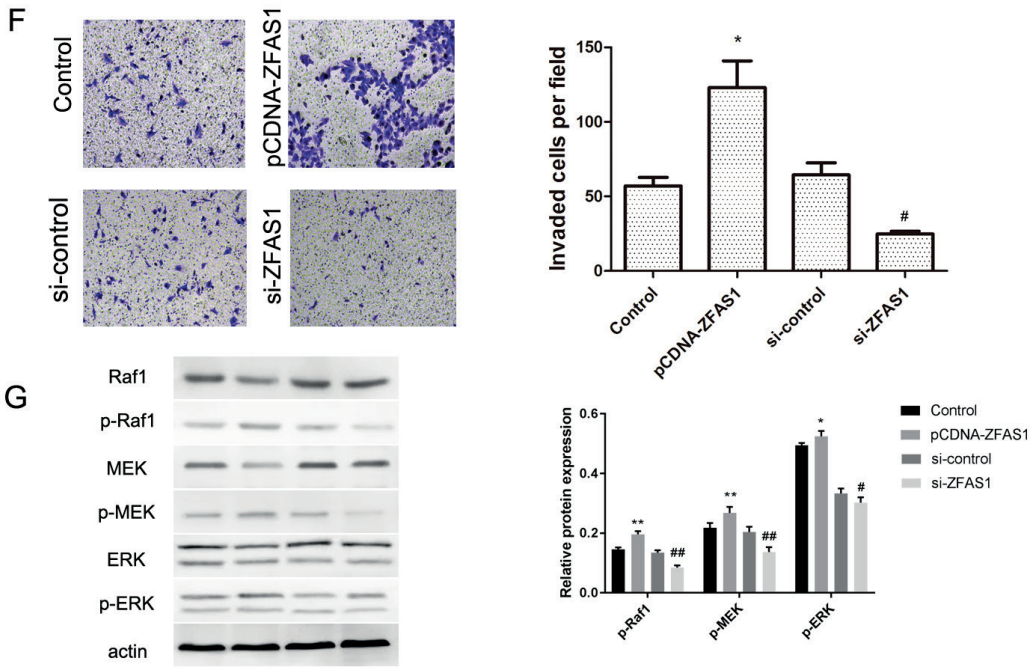

Figure 1. *Continued.* ***F,*** respectively. (magnification 200x) ***G,*** The role of ZFAS1 on the MAPK signaling pathway in U2OS cells transfected with si-ZFAS1 or pCDNA-ZFAS1. **p*<0.05 and ***p*<0.01 *vs.* control group; $^{\#}p$<0.05 and $^{\#\#}p$<0.01 *vs.* si-control group.

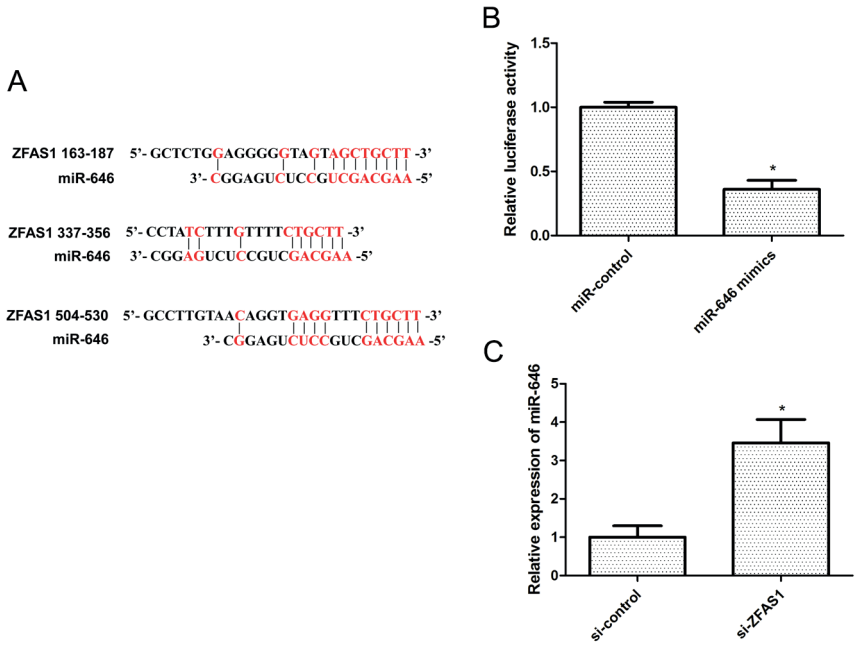

Figure 2. miR-646 is a target of ZFAS1. ***A,*** The putative binding sites of miR-646 and ZFAS1. ***B,*** The luciferase activity of ZFAS1 in U2OS cells following transfection with the miR-646 mimics or mimics control (miR-control) was measured using luciferase report assays. ***C,*** The relative expression levels of miR-646 in OS cells with ZFAS1 knocked down were analyzed by qRT-PCR. **p*<0.05 *vs.* control group

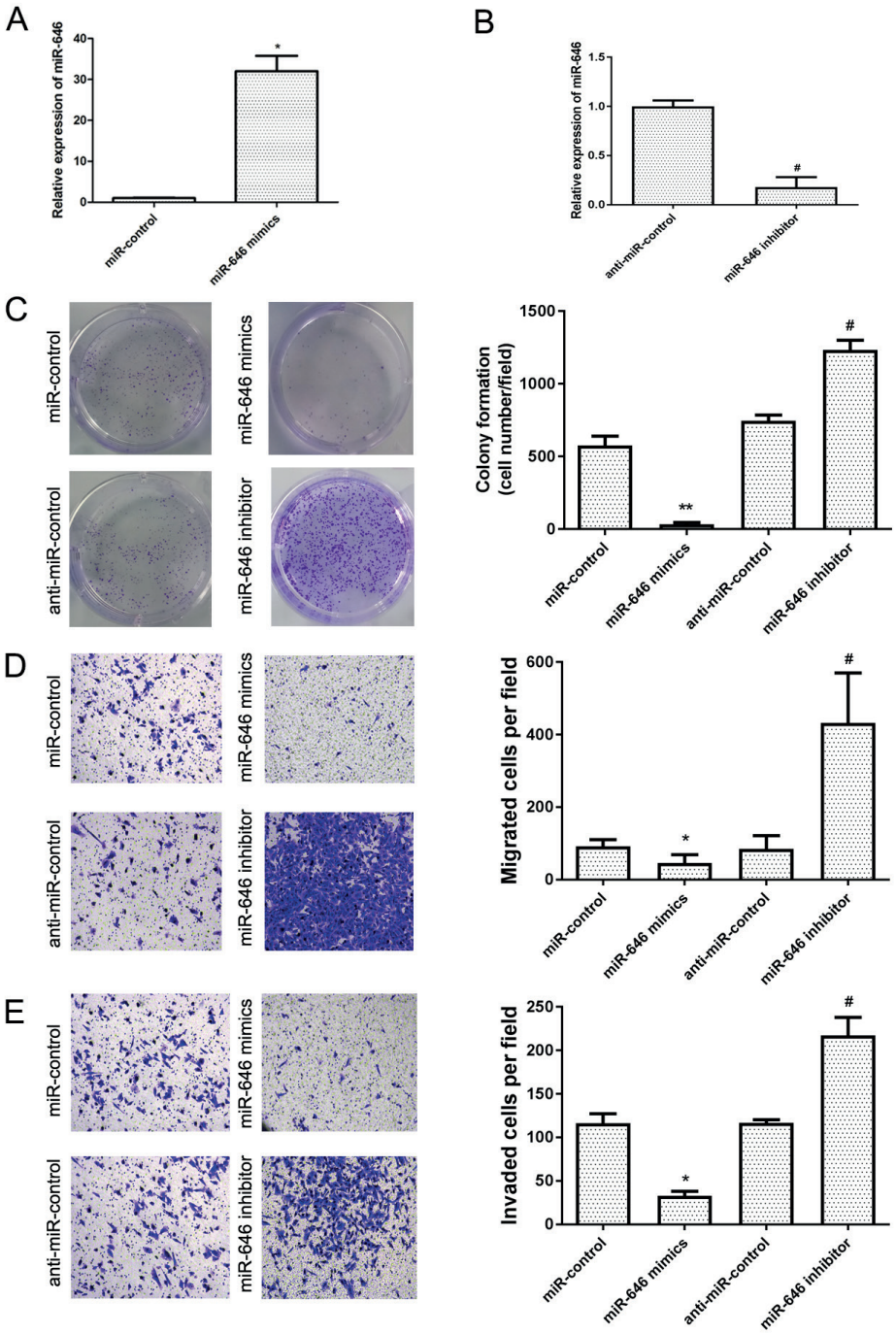

Figure 3. The role of miR-646 in OS cells. ***A,*** Relative expression levels of miR-646 were determined by qRT-PCR in U2OS cells transfected with miR-646 mimics or corresponding mimics control (miR-control). ***B,*** Relative expression levels of miR-646 were determined by qRT-PCR in U2OS cells transfected with miR-646 inhibitor or corresponding inhibitor control (anti-miR-control). After U2OS cells were transfected with miR-646 mimics or miR-646 inhibitor, we determined the effect of miR-646 on the cell proliferation, migration and invasion using colony formation assay ***(C),*** transwell migration assay (magnification 200x) ***(D),*** and transwell invasion assay (magnification 200x) ***(E),*** respectively. *p<0.05 and **p<0.01 *vs.* miR-control group; $^{\#}p$<0.01 *vs.* anti-miR-control group (magnification 200x).

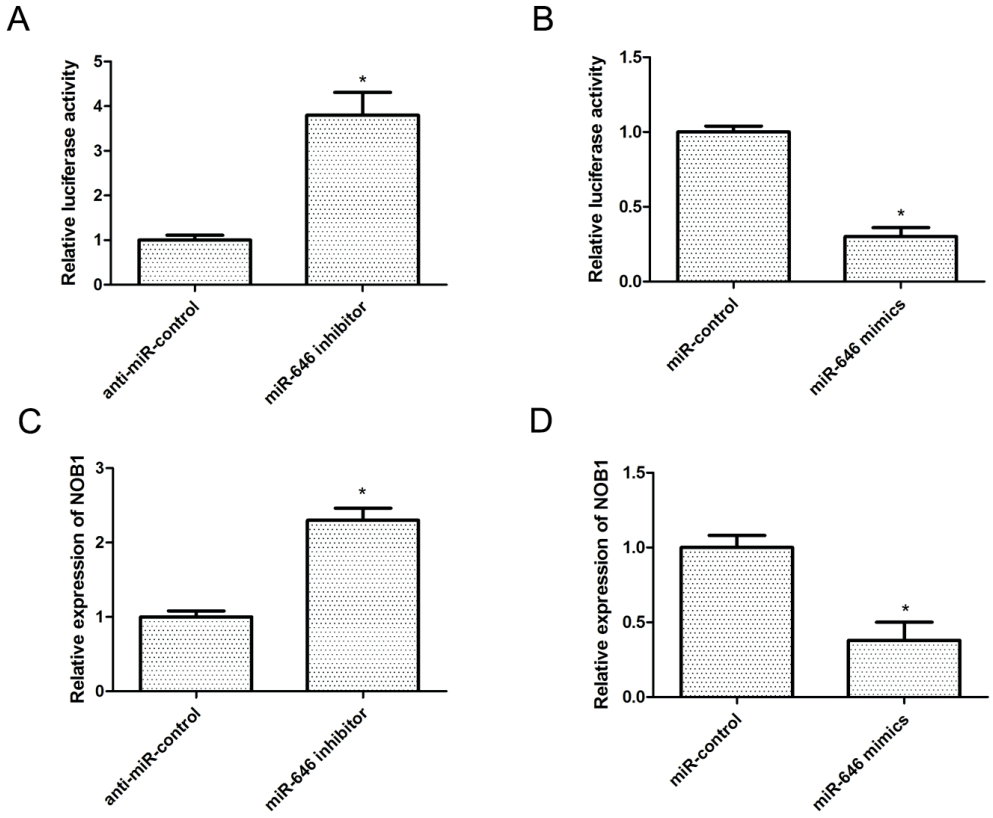

Figure 4. NOB1 is a target for miR-646. ***A,*** The luciferase activity of NOB1 in U2OS cells after transfection with the miR-646 inhibitor or corresponding inhibitor control (anti-miR-control) was measured using luciferase report assays. ***B,*** The luciferase activity of NOB1 in U2OS cells after transfection with the miR-646 mimics or mimics control (miR-control) was measured using luciferase report assays. ***C,*** Relative expression levels of NOB1 were determined by qRT-PCR in U2OS cells transfected with miR-646 inhibitor or anti-miR-control. ***D,*** Relative expression levels of NOB1 were determined by qRT-PCR in U2OS cells transfected with miR-646 mimics or miR-control. *$p<0.05$ *vs.* control group

NOB1 Is a Target for MiR-646

It is reported that NOB1 is a target of miR-646 in OS cells[19]. To verify this finding, we constructed luciferase reporter plasmid with the NOB1 3'-UTR region. The luciferase reporter plasmid was cotransfected into OS cells along with miR-646 or miR-646 inhibitor, and the luciferase activity was measured. The results showed that miR-646 considerably suppressed the luciferase activity compared with the control group, while miR-646 inhibitor elevated the luciferase activity (Figure 4A and 4B). We next examined the expression of NOB1 in OS cells transfected with miR-646 or miR-646 inhibitor. We observed that the mRNA level of NOB1 was markedly reduced after miR-646 mimic transfection, but significantly enhanced after inhibition of miR-646 compared with the control group (Figure 4C and 4D). Collectively, the results suggested that NOB1 was a direct target for miR-646 in OS cells.

ZFAS1 Induces NOB1 Up-Regulation by Inhibiting MiR-646 Expression

As mentioned above, ZFAS1 could target miR-646, and NOB1 was a direct target for miR-646 in OS cells. Thus, we speculated that ZFAS1 might induce NOB1 up-regulation by inhibiting miR-646 expression. To test this speculation, we first evaluated the expression of NOB1 protein in OS cells transfected with ZFAS1 siRNA or overexpression plasmid. The results showed that ZFAS1 overexpression elevated the protein expression of NOB1, but the knockdown of ZFAS1 reduced the expression of NOB1 (Figure 5A). Then, we knocked down NOB1 in OS cells with ZFAS1 overexpression and found that the colony, migration, and invasion ability of OS cells were remarkably decreased (Figure 5B). Meanwhile, we transfected NOB1 overexpression plasmid into OS cells with ZFAS1 knockdown and observed that the colony, migration, and in-

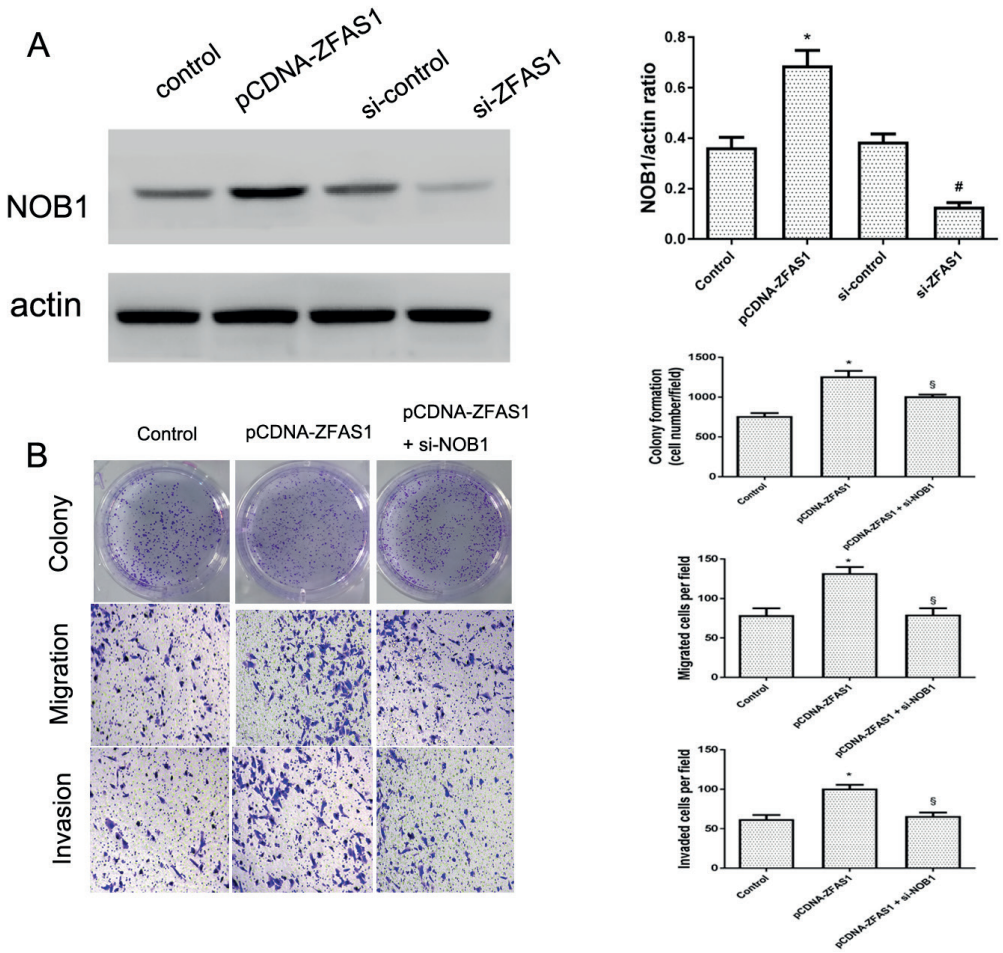

Figure 5. ZFAS1 induces NOB1 up-regulation by inhibiting miR-646 expression. ***A,*** The expression of NOB1 protein was measured by Western blot in OS cells transfected with ZFAS1 siRNA (si-ZFAS1), corresponding control siRNA (si-control), ZFAS1 overexpression plasmid (pCDNA-ZFAS1), or empty vector (control). ***B,*** After the U2OS cells were transfected with ZFAS1 overexpression plasmids (pCDNA-ZFAS1), empty vector (control), pCDNA-ZFAS1 + NOB1 siRNA (si-NOB1), the cell colony, migration and invasion ability were determined using colony formation assay, transwell migration assay, and transwell invasion assay, respectively (magnification 200x).

Figure continued

vasion ability of OS cells were partially rescued (Figure 5C). Therefore, ZFAS1 could up-regulate the expression of NOB1.

Subsequently, we used OS cells with ZFAS1 overexpression to co-transfect with miR-646 mimic and determined the NOB1 expression by qRT-PCR. Although ZFAS1 overexpression markedly increased the expression of NOB1 compared with the control group, this situation was reversed by miR-646 overexpression in OS cells after transfection with miR-646 mimic (Figure 5D). Meanwhile, OS cells with ZFAS1 knockdown were co-transfected with miR-646 inhibitors, and NOB1 expression was also detected by qRT-PCR. As shown in Figure 5E, miR-646 inhibitors in OS cells with ZFAS1 knockdown partially rescued the down-regulation of NOB1 induced by ZFAS1 knockdown. As mentioned above, ZFAS1 induced NOB1 up-regulation, which might be induced by inhibiting miR-646 expression.

Discussion

In the present study, our results indicated that ZFAS1 was up-regulated in OS cells, and ZFAS1 overexpression promoted the colony, migration, and invasion ability of OS cells via activating MAPK signaling pathway. Additionally, the experimental results showed that miR-646 was a target of ZFAS1 and inhibited the OS cell colony, migration, and invasion ability. Meanwhi-

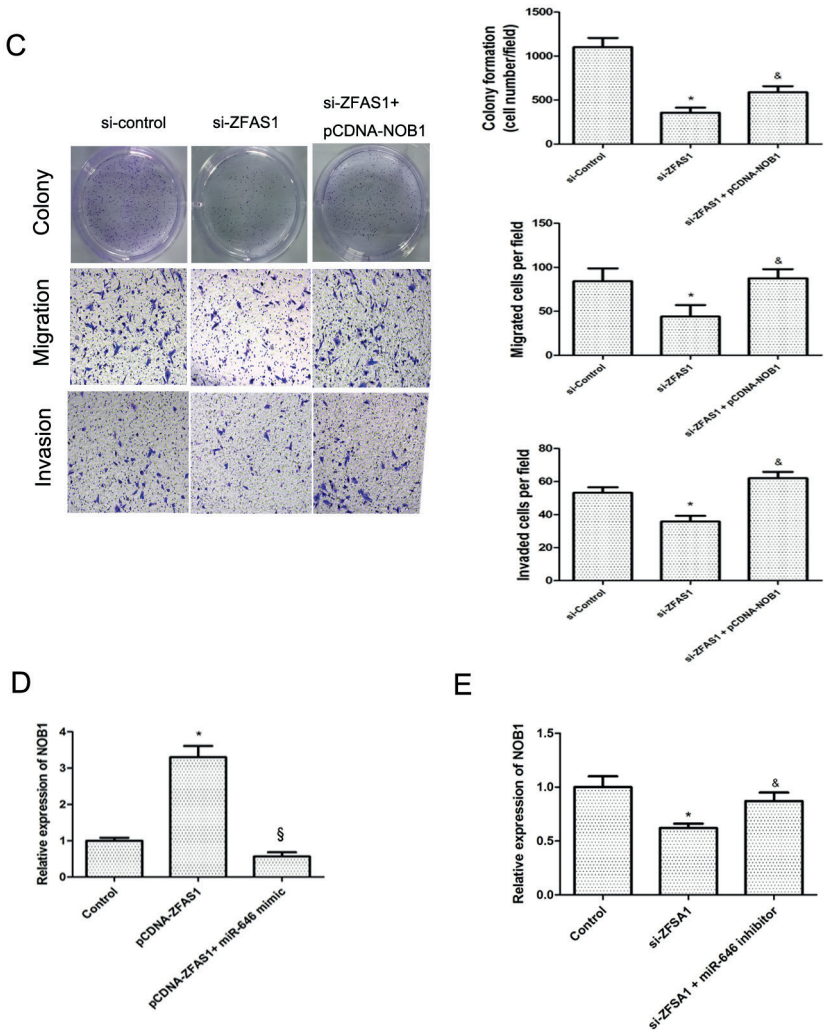

Figure 5. *Continued.* ***C,*** After the U2OS cells were transfected with ZFAS1 siRNA (si-ZFAS1), corresponding control siRNA (si-control), si-ZFAS1 + NOB1 overexpression plasmid (pCDNA-NOB1), the cell colony, migration, and invasion ability were measured using colony formation assay, transwell migration assay, and transwell invasion assay, respectively (magnification 200x). ***D,*** The relative expression levels of NOB1 were determined by qRT-PCR in U2OS cells after transfection with ZFAS1 overexpression plasmids (pCDNA-ZFAS1), empty vector (control), pCDNA-ZFAS1 + miR-646 mimic. ***E,*** The relative expression levels of NOB1 were determined by qRT-PCR in U2OS cells after transfection with ZFAS1 siRNA (si-ZFAS1), corresponding control siRNA (si-control), si-ZFAS1 + miR-646 inhibitor. *$p<0.05$ *vs.* control group; §$p<0.05$ *vs.* pCDNA-ZFAS1 group; &$p<0.05$ *vs.* si-ZFAS1 group.

le, we verified that NOB1 was a direct target for miR-646 in OS cells. Furthermore, we found that ZFAS1 induced NOB1 up-regulation by inhibiting miR-646 expression.

ZFAS1 has been reported to be an oncogene in hepatocellular carcinoma[20], colorectal cancer[21], and nasopharyngeal carcinoma[22], but to be a tumor suppressor gene in breast cancer[15]. Although several researches suggest that ZFAS1 is upregulated in OS[18], few studies about the potential molecular mechanism of ZFAS1 in OS have been reported. Hence, we tried to do the relevant research in this study. In agreement with previous investigations, our study demonstrated that ZFAS1 was up-regulated in OS cells. To further explore this mechanism, the biological experiments showed that ZFAS1 overexpression markedly promoted the colony, migration, and invasion ability of OS cells via activating MAPK signaling pathway. Thus, ZFAS1 can be considered as an oncogene in the OS.

Accumulating evidence[23,24] suggests that lncRNAs may function as competing endogenous RNAs (ceRNAs) to sponge miRNAs, which can regulate the depression of miRNA targets and impose an additional level of post-transcriptional regulation. Thus, we hypothesized that ZFAS1 might act as a ceRNA in OS. To verify our hypothesis, we used DIANA TOOLS LncBase to predict potential ZFAS1 targets, and we obtained the ranking first miR-646. Luciferase report assays showed that miR-646 might be a target of ZFAS1. It is reported[19,25,26] that miR-646 can be classified as a tumor suppressor in various cancers. Consistent with the previous researches, our study revealed that miR-646 was down-regulated in OS cells and miR-646 knockdown promoted the colony, migration, and invasion ability of OS cells.

MiR-646 acts as a tumor suppressor through targeting NOB1 expression[19,27]. In agreement with previous studies, our work showed that NOB1 was a direct target for miR-646 in OS cells, and miR-646 decreased the expression of NOB1. According to these results, we speculated that ZFAS1 might regulate NOB1 expression through acting as ceRNA of miR-646. The results indicated that ZFAS1 overexpression enhanced the expression of NOB1, whereas ZFAS1 induced NOB1 upregulation which was reversed by miR-646 overexpression. Therefore, ZFAS1 could induce NOB1 up-regulation by inhibiting miR-646 expression, and ZFAS1/miR-646/NOB1 axis facilitated the colony, migration, and invasion ability of OS cells.

Conclusions

Our study indicated that ZFAS1 in OS cells up-regulated the expression of NOB1 by acting as ceRNA of miR-646. Moreover, the results demonstrated that ZFAS1/miR-646/NOB1 axis might play an important role in the development of OS, and ZFAS1 and miR-646 can be regarded as potential biomarkers for the diagnosis and treatment of OS.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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Ethics approval and consent to participate

Not applicable.

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