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Linc00460 promotes osteosarcoma progression via miR-1224-5p/FADS1 axis

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ABSTRACT

Aims: Previous studies have demonstrated that long non-coding RNAs (lncRNAs) were involved in tumorigenesis in various human neoplasms, including osteosarcoma (OS). However, the expression and specific role of lncRNA linc00460 in OS remain unknown.

Materials and methods: Bioinformatics analysis, Quantitative real-time polymerase chain reaction (qRT-PCR), CCK-8 assay, Colony formation assay, Wound healing assay, Transwell assay, Dual luciferase reporter assay, RNA immunoprecipitation and Western blot were utilized to analyze or detect survival, gene expression, cell proliferation, cell migration, cell invasion and interest protein levels, respectively.

Key findings: In this study, we found high linc00460 expression predicted poor prognosis of pan-cancer patients. Linc00460 was up-regulated in OS tissues and cells. High linc00460 expression was positively correlated with distant metastasis and poor overall survival of OS patients. Knockdown of linc00460 suppressed OS cells proliferation and metastasis in vitro. In addition, an inverse correlation between linc00460/miR-1224-5p and miR-1224-5p/FADS1 was observed in OS. Mechanistically, linc00460 functioned as a competitively endogenous RNA (ceRNA) to up-regulate FADS1 expression via sponging miR-1224-5p in OS, thereby promoting OS progression.

Significance: In conclusion, this study recognized linc00460 as a new oncogenic lncRNA in OS and suggests that the linc00460/miR-1224-5p/FADS1 axis might be a potential therapeutic target for OS.

1. Introduction

As the most common primary bone neoplasm, osteosarcoma (OS) ranks second in cancer-related deaths among children and adolescents [1,2]. Due to advances in multidisciplinary clinical therapy for OS in the past decades, patient outcomes have been improved [3]. However, the five-year overall survival of OS remains poor because of recurrence or metastasis [4]. The complexity of the initiation and development mechanisms of OS is considered as the major obstacle in improving patient survival [3,4]. Therefore, there is an urgent need to investigate the potential mechanism underlying tumorigenesis and to explore new molecular targets of OS.

Long non-coding RNAs (lncRNAs), > 200 nucleotides in length, are a class of molecules that have limited protein-coding potential. Increasing evidence has demonstrated that lncRNAs participated in the pathogenesis of many human malignancies [5–7]. Numerous studies reported that aberrantly expressed lncRNAs could serve as prognostic biomarkers and contributed to the development of many cancers, including OS [8–10]. Studies have shown that lncRNAs were implicated in the regulation of various cancer-related pathophysiological processes and behaviors, such as proliferation, metastasis, epithelial-mesenchymal transition (EMT), drug resistance and glycolysis [11–14]. Furthermore, lncRNAs could function as oncogenes or tumor suppressors by regulating the expression of key genes at the epigenetic level, transcriptional level, post-transcriptional level and post-translational level [15].

Long intergenic non-coding RNA 460 (linc00460), a newfound lncRNA located on chromosome 13q33.2, has recently been reported to work as an oncogene in several cancers. For example, linc00460 was up-regulated in tumor tissues and could promote cell metastasis by

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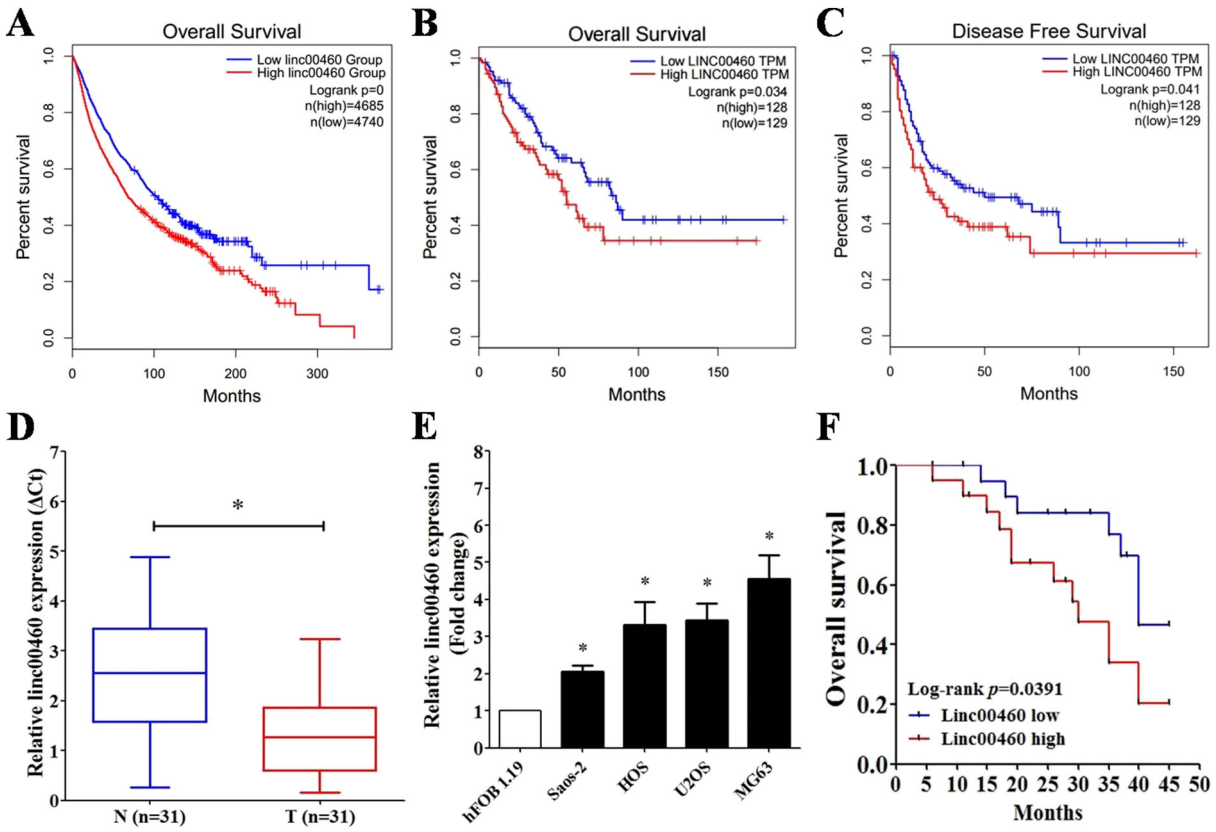

Fig. 1. Linc00460 is highly expressed in OS and predicts poor patient prognosis. (A) Result of TCGA data showed that high linc00460 expression predicted poor prognosis of pan-cancer patients. (B–C) Result of TCGA data showed that high 00460 expression predicted poor overall survival (B) and disease free survival (C) of sarcoma patients. (D) We used qRT-PCR to examine the relative expression of linc00460 in 31 paired tumor tissues and the matched normal tissues of OS patients. The result was expressed as ΔCt, $^{*}p < 0.05$ vs N. T: osteosarcoma tissues; N: Normal adjacent tissues. (E) The relative expression of linc00460 in one normal osteoblast cell line hFOB 1.19 and four OS cell lines Saos-2, HOS, U2OS and MG63 was determined by qRT-PCR. The result was expressed as fold change ($2^{-\Delta\Delta Ct}$ method), $^{*}p < 0.05$ vs hFOB 1.19. (F) Higher linc00460 expression correlated with shorter overall survival in OS patients.

Table 1
Correlation between linc00460 expression and clinical parameters in OS patients.

Clinical parameters	Total	Linc00460 expression		χ^2 test p value
		Low	High	
Gender				0.439
Male	18	8	10	
Female	13	7	6	
Age (year)				0.305
≥20	12	7	5	
< 20	19	8	11	
Tumor size (cm)				0.049
< 5	17	11	6	
≥5	14	4	10	
Enneking stage				0.602
I–II	21	10	11	
III	10	5	5	
Distant metastasis				0.020
Yes	13	3	10	
No	18	12	6	

affecting EMT in non-small cell lung cancer (NSCLC) [16]. Linc00460 knockdown inhibited cell proliferation and metastasis and increased apoptosis in epithelial ovarian cancer (EOC) [17]. Linc00460 also promoted NSCLC gefitinib resistance through the regulation of epidermal growth factor receptor (EGFR) [18]. However, the biological role and the underlying mechanism of linc00460 in OS remain unclear.

In this study, we found that linc00460 was highly expressed in OS when compared to the counterparts. Knockdown of linc00460 inhibited cell proliferation and metastasis of OS in vitro. We also revealed that linc00460 promoted OS development through the up-regulation of (Fatty acid desaturase 1) FADS1 via sponging miR-1224-5p. The results of this study suggest that linc00460 may be important for the progression of OS by targeting the miR-1224-5p/FADS1 axis.

2. Materials and methods

2.1. Clinical tissue specimens

A total of 31 cases of OS tumor tissues and their paired adjacent normal tissues were collected from patients who underwent surgical resection at the Zhengzhou Central Hospital Affiliated to Zhengzhou University between 2014 and 2018. No patients have been received chemotherapy or radiotherapy prior to surgery. All tissue specimens were immediately frozen in liquid nitrogen after resection and stored at −80 °C refrigerator for RNA extraction. All patients provided written informed consent and this study was approved by the Medical Ethics Committee of Zhengzhou Central Hospital Affiliated to Zhengzhou University.

2.2. OS cell lines and transfection

The osteoblastic cell line hFOB 1.19 and four human OS cell lines Saos-2, HOS, U2OS and MG63 were obtained from the Chinese Academy of Science (Shanghai, China). Cells were maintained in RPMI medium containing 10% fetal bovine serum (FBS) (Gibco, USA) at 37 °C in a cell incubator with 5% CO_2. Oligonucleotides or plasmids used for gene down-regulation (siRNA-linc00460, miR-1224-5p inhibitors) and

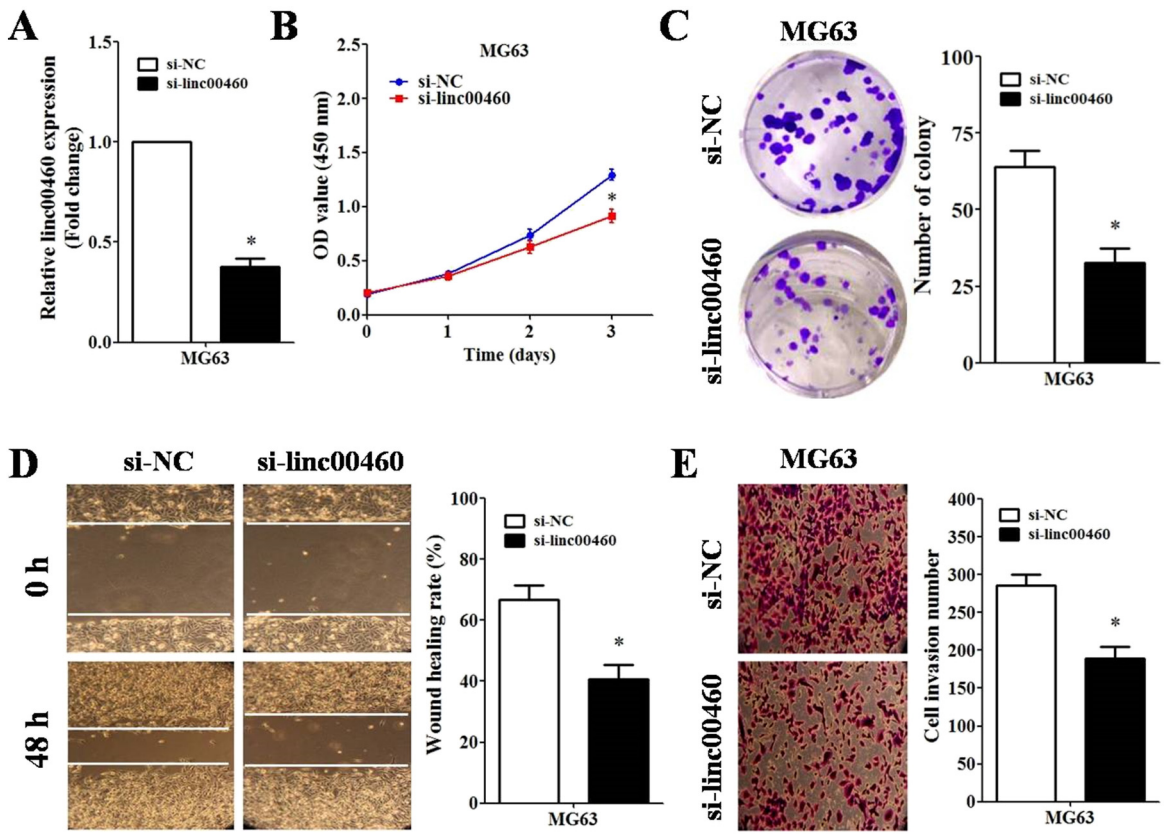

Fig. 2. Linc00460 promotes cell proliferation and metastatic potential of MG63 in vitro. (A) We used qRT-PCR to test knockdown effect of si-linc00460 in MG63 cells, $^*p < 0.05$ vs si-NC. (B–C) Effect of linc00460 knockdown on MG63 cells proliferation was examined by CCK-8 assay and colony formation assay, respectively, $^*p < 0.05$ vs si-NC. (D) Wound healing assay was performed to examine the effect of linc00460 knockdown on MG63 cells migration, $^*p < 0.05$ vs si-NC. (E) Transwell assay was used to detect the effect of linc00460 knockdown on MG63 cells invasion, $^*p < 0.05$ vs si-NC.

over-expression (miR-1224-5p mimics, pcDNA3.1-FADS1) as well as the corresponding negative control (siRNA-NC, miR-NC) were obtained from GenePharma. Cell transfection was performed using Lipofectamine 3000 (Invitrogen, USA) according to the procedures of user's manual.

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

Trizol regent (Invitrogen, USA) was used for total RNAs extraction from tissue specimens and cultured cells. After quality and integrity detection, total RNAs were synthesized into cDNA using the Reverse Transcriptase Kit (Thermo, USA). Then, the SYBR Green Master Mix Kit (DBI, Germany) and the ABI 7500 Fast Real-Time PCR System (Life, USA) were used for gene expression examination. The relative quantitative expression of interest genes were expressed as fold change ($2^{-\Delta\Delta Ct}$ method) or ΔCt. For subcellular distribution detection of linc00460, cytoplasmic and nuclear fractions were isolated using Nuclei Isolation Kit (Sigma, USA) before qRT-PCR. GAPDH and U6 were used for endogenous control as appropriate. Primer sequences are listed below.

Linc00460 Forward 5′-AGAAATCCTCCAGCCCTGTT-3′,
Reverse 5′-GGGTGACTCTTAGCCGAGAA-3′;
GAPDH Forward 5′-AGAAGGCTGGGGCTCATTTG-3′,
Reverse 5′-AGGGGCCATCCACAGTCTTC-3′;
MiR-1224-5p Forward 5′-GTCGTATCCAGTGCAGGGTCCGAGG.
TATTCGCACTGGATACGACCACTCCT-3′,
Reverse 5′-TCCGAGGTGGAGGGCTC-3′;
FADS1 Forward 5′-GTCTTCTTCCTGCTGTACCTG-3′,
Reverse 5′-GGTTCCACTTTGAGGTGCTGA-3′.
U6 Forward 5′-CTCGCTTCGGCAGCACA-3′,
Reverse 5′-AACGCTTCACGAATTTGCGT-3′.

2.4. Cell proliferation assay

Cell counting kit-8 (CCK-8) and colony formation assays were used to test cell proliferation. For CCK-8 assay, transfected cells were seeded in a 96-well plate, 10 μL of CCK-8 solution (Solarbio, China) was added to each well every day for three days, the absorbance at 450 nm was examined by a microplate reader. The detailed experimental procedures were following the manufacturer's instructions of CCK-8 kit. For colony formation assay, transfected cells seeded in a 24-well plate and cultured for one week. Then, cells were fixed with methanol and stained with crystal violet solution.

2.5. Cell migration and invasion assays

The Wound healing assay was used for detecting cell migratory ability. For this, transfected cells were seeded in a 6-well plate and cultured to about 70% confluence. Then, a 200 μL pipette tip was used to form artificial scratches for each well. Cell migration distance was monitored at 0 h and 48 h under an inverted microscope. The Transwell assay was used for examining cell invasive ability. Briefly, transfected cells were seeded onto the Matrigel-coated upper chambers with serum-free RPIM culture medium. A total of 500 μL RPIM medium containing 20% FBS was added to the lower chambers. After incubation for 48 h at 37 °C, the invasive cells were fixed with methanol and stained with crystal violet solution. The number of invasive cells was calculated under the inverted microscope.

Dual luciferase reporter assay.

Reporter plasmids (pmirGLO) of linc00460 (Wt-linc00460 and Mut-

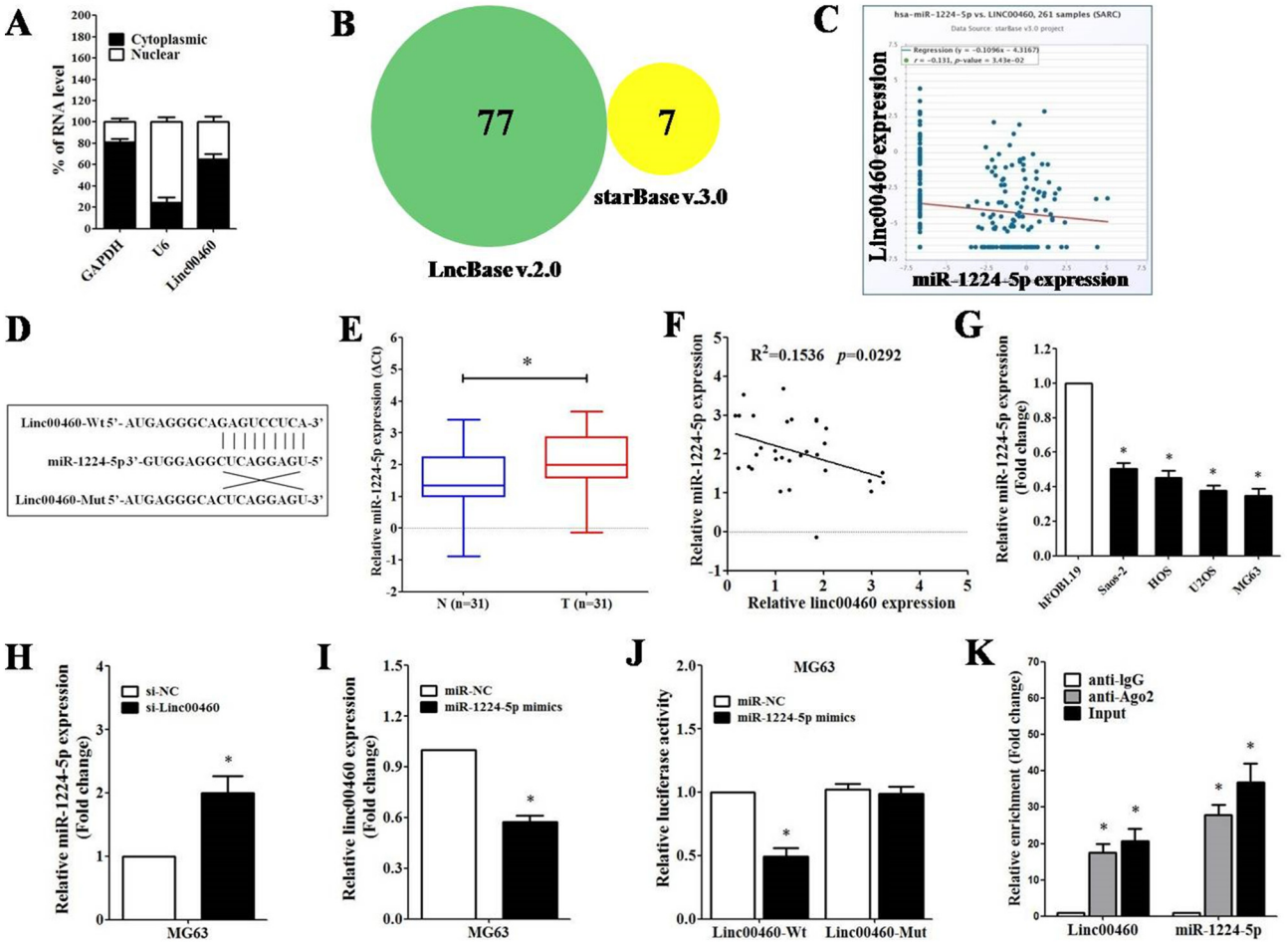

Fig. 3. Linc00460 directly interacts with miR-1224-5p in OS. (A) Sub-cellular distribution of linc00460 in MG63 cells was determined by qRT-PCR. (B) The candidate miRNAs targeting linc00460 was predicted by bioinformatic analysis. (C) The correlation between miR-1224-5p and linc00460 was analyzed from the TCGA data. (D) The putative binding site of miR-1224-5p in linc00460 is shown. (E) The expression level of miR-1224-5p in 31 paired OS tissues and normal tissues was detected using qRT-PCR, $*p < 0.05$ vs N. T: osteosarcoma tissues; N: Normal adjacent tissues. (F) Inverse correlation between miR-1224-5p and linc00460 expression in 31 cases of OS tissues. (G) The expression level of miR-1224-5p in OS cells was detected using qRT-PCR, $*p < 0.05$ vs hFOB 1.19. (H) The expression level of miR-1224-5p in MG63 cells after linc00460 knockdown was examined by qRT-PCR, $*p < 0.05$ vs si-NC. (I) Linc00460 expression in MG63 cells after miR-1224-5p overexpression was examined by qRT-PCR, $*p < 0.05$ vs miR-NC. (J) Dual luciferase reporter assay was used for detecting the targeted binding effect between linc00460 and miR-1224-5p in MG63 cells, $*p < 0.05$ vs miR-NC. (K) RIP was performed to examine the relative enrichment of linc00460 and miR-1224-5p in the co-precipitated RNAs, $*p < 0.05$ vs anti-IgG.

linc00460) and FADS1 (Wt-FADS1 and Mut-FADS1) were purchased from GenePharma (Shanghai, China). The Wt-linc00460/Wt-FADS1 or Mut-linc00460/Mut-FADS1 plasmids contain the wild type or mutant miR-1224-5p binding site, respectively. For this assay, the corresponding reporter plasmid was co-transfected with miR-1224-3p mimics or miR-NC into OS cells. 48 h after co-transfection, the dual luciferase reporter assay system (Promega, USA) was used for detecting the relative luciferase activity.

2.6. RNA immunoprecipitation

The RNA immunoprecipitation (RIP) assay was used to determine the binding effect between miR-1224-5p and its targets. The procedures were following user's manual of the Magna RIP Kit (Millipore, USA). In brief, OS cells were transfected miR-1224-5p or miR-NC. 48 h after transfection, the cell lysates were incubated with magnetic beads (conjugated with Ago2 antibody or rabbit IgG) in RIP buffer. Afterwards, the relative gene enrichment in the precipitated RNAs was detected by qRT-PCR and agarose gel electrophoresis.

2.7. Western blot

Total protein of each sample was extracted using RIPA buffer (containing 1% PMSF). After incubated for 10 min at 100 °C, equal amount of protein of each sample was subjected to polyacrylamide gel electrophoresis and PVDF membrane transfer. Then, the membrane was blocked in 5% skim milk, incubated with primary antibody (FADS1, Santa, USA; β-actin, Abcam, USA; Lamin B1, Abcam, USA), second antibody and ECL regent successively. Finally, the immunoreactive bands on the membrane were examined and analyzed by the scanning imaging system (Bio-Rad, USA).

2.8. Bioinformatics prediction and analysis

GEPIA (Website: http://gepia.cancer-pku.cn) was used to analyze the correlation between gene expression and patient survival in 262 cases of sarcoma from the TCGA data. Two independent online tools LncBase v2.0 (Website: http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted. Score threshold: > 0.7) and starBase v3.0 (Website: http://starbase.sysu.edu.cn. No score threshold is set) were used to predict miRNAs targeting

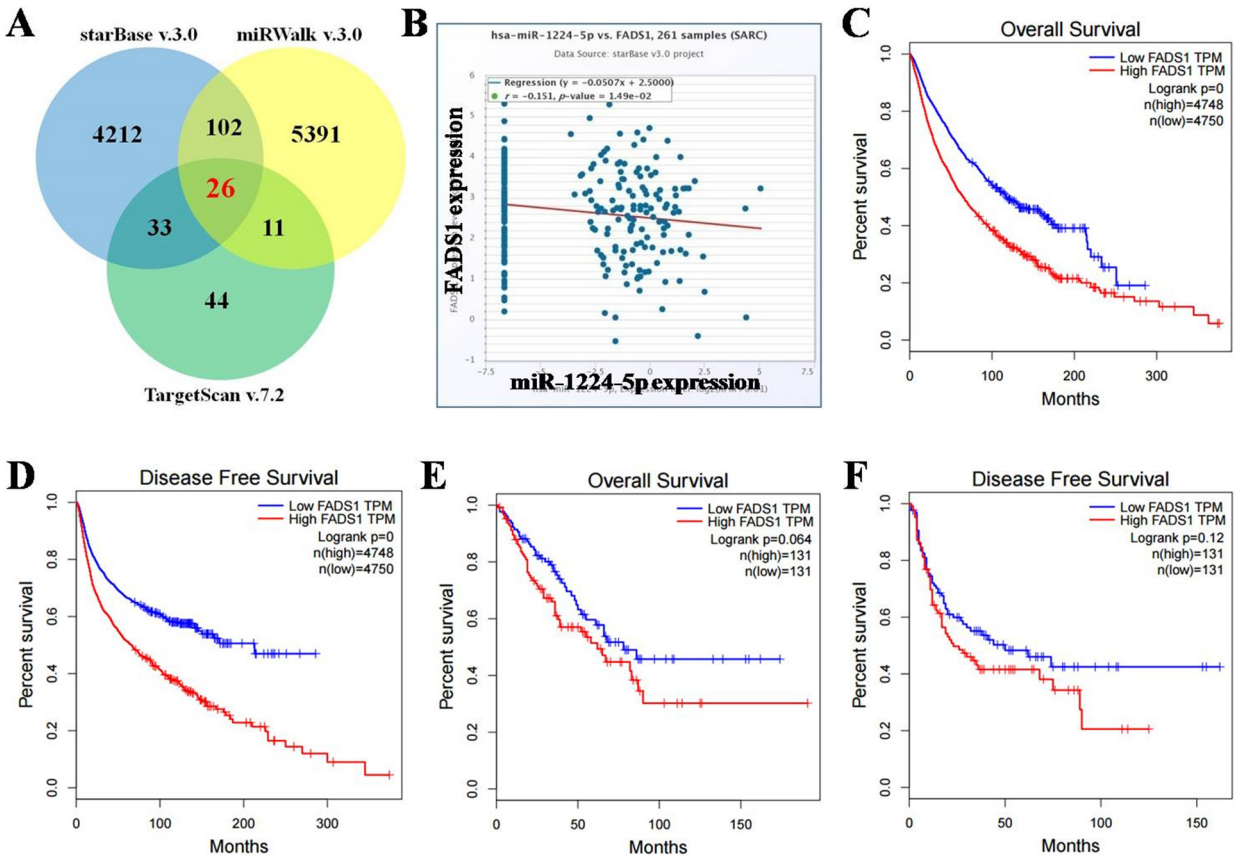

Fig. 4. High FADS1 expression predicts poor survival of cancer patients. (A) The potential targeted genes of miR-1224-5p were predicted by three online tools, respectively. (B) Correlation between of miR-1224-5p and FADS1 expression from the TCGA data was analyzed by starBase v3.0. (C-D) Result of TCGA data (GEPIA) showed that high FADS1 expression predicted poor overall survival (C) and disease free survival (D) of pan-cancer patients (31 types of cancers), high FADS1 expression group includes 4748 cases and low FADS1 expression group includes 4750 cases. (*E*-F) Result of TCGA data (GEPIA) showed that high FADS1 expression predicted poor overall survival (E) and disease free survival (F) of sarcoma patients; both high FADS1 expression group and low FADS1 expression group includes 131 cases.

linc00460. We used starBase v3.0 to analyze the correlation between each predicted miRNA and linc00460 in 261 cases of sarcoma tissues from the TCGA data, and to obtain the candidate miRNAs that were significantly negatively associated with linc00460 expression in sarcoma tissues.

To identify the potential genes that regulated by linc00460, three online tools starBase v3.0 (Website: http://starbase.sysu.edu.cn. No score threshold is set), TargetScan v7.2 (Website: http://www.targetscan.org/vert_72. No score threshold is set) and miRWalk v3.0 (Website: http://mirwalk.umm.uni-heidelberg.de. Score threshold is 1.0) were utilized to predict the possible target genes of miR-1224-5p. We compared the three prediction sets and obtained some genes simultaneously existed in the three sets of predictions. Next, starBase v3.0 was used to analyze the correlation between each of the candidate genes and miR-1224-5p in 261 cases of sarcoma tissues from the TCGA data, and to obtain genes that were significantly negatively correlated with miR-1224-5p expression in sarcoma tissues.

2.9. Statistical analysis

All experiments were performed in triplicate. All data in this study are expressed as mean ± SD. SPSS 19.0 and Graphpad prism 5.0 were used for statistical analysis. Correlation between gene expression and patients clinical parameters were analyzed by χ^2 test. Statistical differences between groups were analyzed by student's *t*-test or one-way ANOVA. Expression correlation between two different genes was determined by the Pearson's correlation coefficient. Kaplan-Meier and log-rank test was used to analyze the correlation between gene expression and patients overall survival. *P* value < 0.05 was considered to be statistically significant.

3. Results

3.1. Linc00460 is over-expressed in OS and correlates with poor patient prognosis

To observe the potential clinical value of linc00460 in various types of cancers, we first analyzed the relationship between linc00460 expression and the overall survival of pan-cancer patients (31 types of cancers) using an online tool called GEPIA [19] from TCGA data. Surprisingly, the result displayed that patients in the high linc00460 expression group (n = 4685) had poorer overall survival rate than patients in the low linc00460 expression group (n = 4740) (p < 0.0001, Fig. 1A). In addition, results from GEPIA (TCGA data) further showed that high linc00460 expression predicted poor overall survival (p = 0.034, Fig. 1B) and disease free survival (p = 0.041, Fig. 1C) of sarcoma patients (n_{high} = 128, n_{low} = 129). Then, we wonder whether linc00460 was aberrantly expressed in OS, qRT-PCR was used to determine the expression level of linc00460 in 31 paired OS tissue specimens (T) and their matched adjacent normal tissues (N). We found that linc00460 was significantly over-expressed in OS tissues when compared with normal tissues (p < 0.05, Fig. 1D). The expression level of linc00460 in OS cell lines was also examined using qRT-PCR. The result showed that linc00460 was up-regulated in four OS cell lines (especially in MG63 cell line) compared to the normal osteoblast cell line (p < 0.05, Fig. 1E). The clinical significance of linc00460

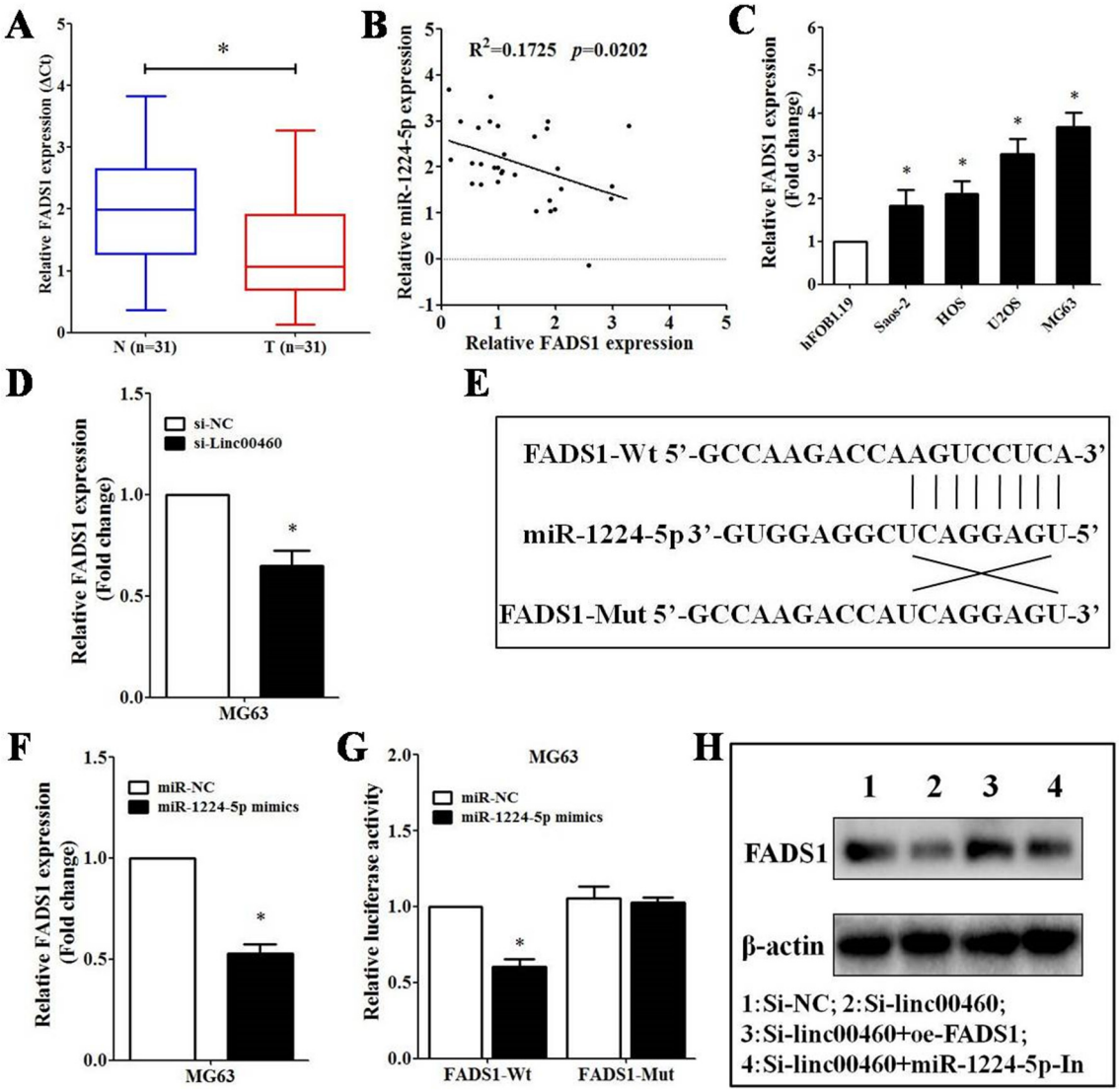

Fig. 5. Linc00460 sponges miR-1224-5p to enhance FADS1 expression in OS. (A) The expression of FADS1 in 31 paired OS tissues and normal tissues was detected using qRT-PCR, $*p < 0.05$ vs N. T: osteosarcoma tissues; N: Normal adjacent tissues. (B) Negative correlation between miR-1224-5p and FADS1 expression in 31 cases of OS tissues was observed. (C) The expression level of FADS1 in OS cells was determined by qRT-PCR, $*p < 0.05$ vs hFOB 1.19. (D, F) The expression of FADS1 in MG63 cells after linc00460 knockdown (D) or miR-1224-5p over-expression (F) was tested by qRT-PCR, $*p < 0.05$ vs si-NC or miR-NC. (E) The putative binding site between miR-1224-5p and FADS1 is shown. (G) Targeted binding effect between FADS1 and miR-1224-5p in MG63 cells was examined by dual luciferase reporter assay, $*p < 0.05$ vs miR-NC. (H) Western blot was used to determine the effect of linc00460/miR-1224-5p/FADS1 axis on FADS1 protein level in MG63 cells.

expression in OS was then investigated. To this end, we analyzed the correlation between linc00460 expression and clinicopathologic characteristics of OS patients. Based on the median value of linc00460 expression in 31 cases of OS tissues, we divided 31 cases of patients into two groups (low/high linc00460 expression group) ($n_{high} = 16$, $n_{low} = 15$). As shown in Table 1, the results indicated that high linc00460 expression was closely associated with large tumor size (≥ 5 cm) ($p = 0.049$) and distant metastasis ($p = 0.020$), but not related to gender ($p = 0.439$), age ($p = 0.305$) or tumor stage ($p = 0.602$). To better understand the relationship between linc00460 expression and patient prognosis in OS, we performed Kaplan-Meier survival analysis and log-rank test. We discovered that high linc00460 expression predicted poor overall survival of OS patients ($p = 0.0391$, Fig. 1F). These data indicated that linc00460 was up-regulated in OS and correlated with patient prognosis.

3.2. Down-regulation of linc00460 inhibits OS cell proliferation, invasion and migration

Considering that linc00460 is up-regulated in both OS tissues and cells and that high expression of linc00460 is closely associated with large tumor size and distant metastasis, we next used loss-of-function experiments to explore whether down-regulation of linc00460 affects OS cell proliferation and metastatic potential (invasion and migration) in vitro. An MG63 cell line with the highest linc00460 expression level was chosen for the following experiments. Specific siRNA was used to down-regulate linc00460 in MG63 cells, and the knockdown effect was confirmed by qRT-PCR ($p < 0.05$, Fig. 2A). Results of the CCK-8 and colony formation assays showed that down-regulation of linc00460 significantly inhibited cell proliferation ($p < 0.05$, Fig. 2B–C). In addition, we observed that linc00460 knockdown suppressed cell migration and invasion of MG63 using the Wound healing and Transwell assays ($p < 0.05$, Fig. 2D–E), respectively. These results indicated that down-regulation of linc00460 inhibited OS progression in vitro.

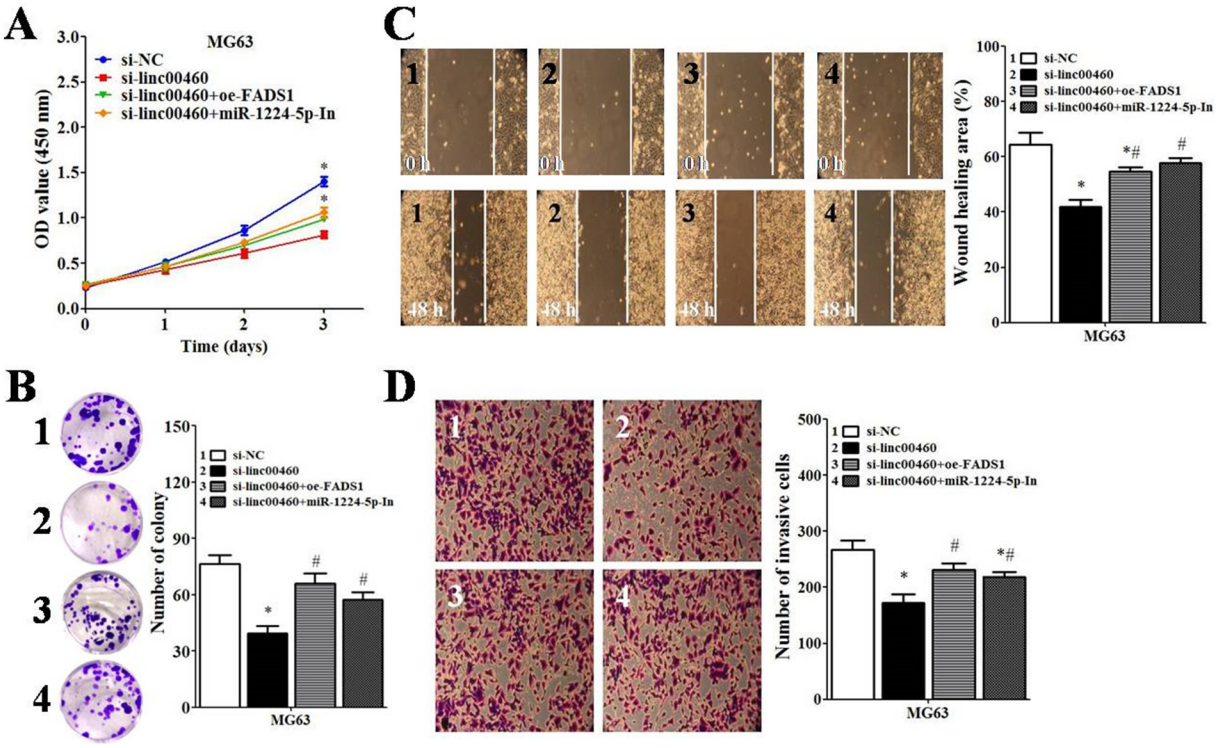

Fig. 6. Linc00460 promotes OS progression via miR-1224-5p/FADS1 axis. (A-B) CCK-8 (A) and colony formation assays (B) were performed to examine the effect of linc00460/miR-1224-5p/FADS1 axis on cell proliferation of MG63 in vitro, respectively, $*p < 0.05$ vs si-NC; $\#p < 0.05$ vs si-linc00460. (C-D) Wound healing assay (C) and Transwell assay (D) were used for evaluating cell metastatic ability of MG63 cells in each group, $*p < 0.05$ vs si-NC; $\#p < 0.05$ vs si-linc00460.

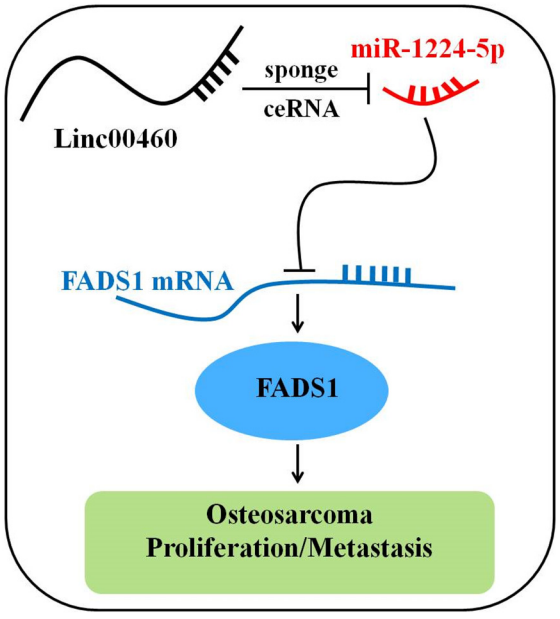

Fig. 7. The possible mechanism of linc00460 regulates OS progression.

3.3. Linc00460 works as a molecular sponge for miR-1224-5p in OS

To explore the possible regulatory mechanism of linc00460 in OS, the sub-cellular distribution of linc00460 in MG63 OS cells was determined. Results of Western blot indicate that the nuclear and cytoplasmic fractions are relatively clean (Supplementary Fig. 1A). We found that linc00460 was mainly localized in cytoplasm (Fig. 3A). As reported, cytoplasmic lncRNA probably exerted its function through ceRNA mechanism by acting as a molecular sponge of miRNAs. So we speculated that linc00460 might regulate OS progression in a ceRNA manner. We used two independent online tools LncBase v2.0 and starBase v3.0 to predict miRNAs targeting linc00460, and obtained a total of 84 candidate miRNAs (Fig. 3B). Next, we analyzed the correlation between each candidate miRNA and linc00460 in 261 cases of sarcoma tissues from the TCGA data using online software starBase v3.0 [20]. We found that only miR-1224-5p was significantly negatively associated with linc00460 expression in sarcoma tissues ($p = 0.0343$, Fig. 3C). Thus, miR-1224-5p was selected as the key candidate miRNA in subsequent studies. The predicted binding site of miR-1224-5p in linc00460 is shown in Fig. 3D. Then, we investigated the relationship between miR-1224-5p and linc00460 in both OS tissues and cell lines. The results of qRT-PCR showed that the expression of miR-1224-5p was obviously decreased in both OS tissues and four cell lines compared to the counterparts ($p < 0.05$, Fig. 3E, G), and that miR-1224-5p was significantly negatively correlated with linc00460 in 31 cases of OS tissues ($R^2 = 0.1536$, $p = 0.0292$, Fig. 3F). Down-regulation of linc00460 increased miR-1224-5p expression in MG63 cells ($p < 0.05$, Fig. 3H). Moreover, over-expression of miR-1224-5p decreased the expression level of linc00460 in MG63 cells ($p < 0.05$, Fig. 3I). The results of dual luciferase reporter assay and RIP assay confirmed that linc00460 could directly bind to miR-1224-5p ($p < 0.05$, Fig. 3J–K). The gel images of Fig. 3K are shown in Supplementary Fig. 1B. These above results suggested that linc00460 functioned as a molecular sponge for miR-1224-5p in OS.

3.4. Linc00460 functions as a ceRNA to up-regulate FADS1 expression via miR-1224-5p in OS

To verify that linc00460 acts as a ceRNA to regulate functional gene expression in OS through sponging miR-1224-5p, we used three online tools (starBase v3.0, TargetScan v7.2 and miRWalk v3.0) to first predict the possible target genes of miR-1224-5p. The above three software predicted 4373, 114 and 5530 genes to be candidate genes,

respectively, and a total of 26 genes were simultaneously identified in the three sets of predictions (Fig. 4A). Next, we analyzed the correlation between each of the 26 genes and miR-1224-5p in 261 cases of sarcoma tissues from the TCGA data using online software starBase v3.0. Only FADS1 was dramatically negatively correlated with miR-1224-5p expression in sarcoma tissues ($p = 0.0149$, Fig. 4B). So FADS1 was selected as the key candidate gene targeting miR-1224-5p in subsequent studies. To observe the potential clinical value of FADS1 in various types of cancers, we also analyzed the relationship between FADS1 expression and the overall survival of pan-cancer patients (31 types of cancers) using online tool GEPIA from TCGA data. The results displayed that patients in the high FADS1 expression group ($n = 4748$) had poorer overall survival ($p < 0.0001$, Fig. 4C) and disease free survival ($p < 0.0001$, Fig. 4D) than patients in the low FADS1 expression group ($n = 4750$). In addition, results from GEPIA (TCGA data) showed that high FADS1 expression also predicted poor overall survival ($p = 0.064$, Fig. 4E) and disease free survival ($p = 0.12$, Fig. 4F) of sarcoma patients ($n_{high} = 131$, $n_{low} = 131$).

Results of qRT-PCR indicated that FADS1 was up-regulated in both OS tissues and cell lines ($p < 0.05$, Fig. 5A, C), and that FADS1 and miR-1224-5p had an inverse expression correlation in 31 cases of OS tissues ($R^2 = 0.1725$, $p = 0.0202$, Fig. 5B). In addition, either linc00460 knockdown or miR-1224-5p over-expression could reduce the expression of FADS1 in MG63 cells ($p < 0.05$, Fig. 5D, F). The predicted binding site between miR-1224-5p and FADS1 is shown in Fig. 5E. We also discovered that linc00460 and FADS1 had the similar binding sites for miR-1224-5p. The direct binding effect between FADS1 and miR-1224-5p was confirmed by the dual luciferase reporter assay ($p < 0.05$, Fig. 5G). Furthermore, the effect of linc00460 knockdown decreasing FADS1 protein level in MG63 cells was partially reversed by over-expression of FADS1 or inhibition of miR-1224-5p ($p < 0.05$, Fig. 5H), respectively. These data suggested that linc00460 promoted FADS1 expression might through competitively binding to miR-1224-5p in OS cells.

3.5. Linc00460/miR-1224-5p/FADS1 axis regulates OS proliferation and metastatic potential in vitro

To demonstrate whether linc00460 promotes OS progression by targeting the miR-1224-5p/FADS1 axis, we performed rescue experiments using CCK-8 assay, colony formation assay, Wound healing assay and Transwell assay. Results showed that over-expression of FADS1 or inhibition of miR-1224-5p could partially reverse the effects of linc00460 knockdown on proliferation ($p < 0.05$, Fig. 6A-B) and metastatic potential ($p < 0.05$, Fig. 6C-D) in MG63 cells, respectively. These results suggested that linc00460 promoted OS progression might through up-regulating FADS1 via miR-1224-5p (Fig. 7).

4. Discussion

Accumulating studies have discovered that lncRNAs play important roles in the prognosis and (or) progression of many types of human tumors including OS. For example, lncRNA small nucleolar RNA host gene 4 (SNHG4) has been reported to be a potential indicator for overall survival and recurrence rates in OS patients [21]. LncRNA SNHG5 knockdown could inhibit OS cells growth and metastatic potential in vitro [22]. Although many lncRNAs in OS have been discovered in recent years, the functions and mechanisms of most lncRNAs in OS are still elusive. In this study, we revealed that a novel lncRNA linc00460 was significantly up-regulated in OS tissues and cell lines and that the mechanism underlying linc00460 promoted OS progression might be involved in the regulation of the miR-1224-5p/FADS1 axis.

Recently, it has been shown that linc00460 could function as a key factor to contribute to the development of various cancers. In lung cancer, linc00460 enhanced cell migration and EMT via interaction with its binding protein hnRNPK [23]. In esophageal squamous cell carcinoma, linc00460 might serve as potential predictor of patient survival when combined with two other lncRNAs [24]. In this study, high linc00460 expression predicted poor prognosis in pan-cancer patients, implying that linc00460 might be involved in cancer development. While high expression of linc00460 was found to be closely correlated with poor overall survival in OS patients, and that down-regulation of linc00460 significantly suppressed OS cell proliferation and metastatic potential. This suggests that linc00460 might work as an oncogene in OS.

As it is widely known, lncRNA located in the cytoplasm is likely to act as a ceRNA to sponge miRNAs, thereby regulating the function of the target gene downstream the miRNAs at the post-transcriptional level [25]. Therefore, the relative expression level of linc00460 in the cytoplasm and nucleus of MG63 cells was determined and found to be enriched in the cytoplasm. According to previous studies, linc00460 could promote tumor development in a ceRNA manner in meningioma, gastric cancer and papillary thyroid carcinoma [26–28]. To determine whether linc00460 worked as a ceRNA in OS via binding to miRNA, we performed bioinformatic analysis and screened out miR-1224-5p as a candidate miRNA targeting linc00460. Several previous studies indicated that miR-1224-5p was a tumor suppressor in both glioma and keloid [29,30]. Our results displayed that miR-1224-5p was down-regulated in OS and could directly bind to linc00460 in MG63 cells. These findings imply that miR-1224-5p may act as a tumor suppressor in OS and that linc00460 may exert its function via miR-1224-5p in OS.

In the ceRNA theory, miRNA regulates tumor development by suppressing its downstream target gene via direct interaction. Therefore, three independent online tools were used to predict the potential target genes of miR-1224-5p and obtain 26 candidate genes. After verification using bioinformatic analysis and dual luciferase reporter assay, FADS1 was found to be a possible target gene of miR-1224-5p in MG63 cells. As reported previously, FADS1 might be a prognostic biomarker in NSCLC [31]. However, to date, the expression and function of FADS1 in many human malignancies including OS are largely unexplored. It was observed that a high FADS1 expression could predict poor prognosis in pan-cancer patients, suggesting that FADS1 might play a certain role cancer progression. We found FADS1 was highly expressed in OS, which implies that FADS1 might be an oncogene in OS. Herein, it can be speculated that there might be a ceRNA axis among linc00460, miR-1224-5p and FADS1 in OS. The effects of linc00460 knockdown on FADS1 protein level, proliferation, migration and invasion were found to be reversed in part by over-expression of FADS1 or inhibition of miR-1224-5p in MG63 cells. These results imply that linc00460 promotes OS progression by targeting the miR-1224-5p/FADS1 axis. The specific regulatory role of the linc00460/miR-1224-5p/FADS1 axis in OS requires to be extensively explored in further studies.

In summary, linc00460 was shown to function as an oncogene that promoted OS cells proliferation, migration and invasion in this study. Additionally, linc00460 contributed to OS progression by up-regulating FADS1 via sponging miR-1224-5p in vitro. These findings suggest that linc00460 may be a novel prognostic and therapeutic target for OS.

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Authors' contributions

CYZ conceived and designed this study. HKL, PPX, XAZ and NWY performed the experiments and analyzed the data. HKL and CYZ wrote the manuscript. JYZ and JL participated in data collection of clinical parameters. All authors read and approved the manuscript for publication.

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Declaration of competing interest

No conflicts of interests.

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