

# MicroRNA-744-5p Suppresses Tumorigenesis and Metastasis of Osteosarcoma Through the MAPK/ERK Signaling Pathway by Targeting TGFB1

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Primary research

**Keywords:** osteosarcoma, miR-744-5p, TGFB1, MAPK/ERK signaling pathway

**Posted Date:** June 1st, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-545532/v1>

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# Abstract

**Background:** Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents, and accumulating evidence has revealed that microRNAs (miRNAs) exert a crucial part in the progression of OS.

**Methods:** GSE65071 from the GEO database was analyzed and miR-744-5p was found to be the lowest expressed miRNA. Real-time quantitative PCR (qRT-PCR), Western blotting (WB), colony formation assay, 5-Ethynyl-2-Deoxyuridine (EdU) incorporation assay and Transwell migration and invasion assay were performed to examine the effects of miR-744-5p in vitro, Luciferase-reporter assay was performed to detect the interactions between miR-744-5p and its specific target gene. Subcutaneous tumor-forming animal models and tail vein injection lung metastatic models were conducted in animal experiments to detect the effects of miR-744-5p in vivo.

**Results:** miR-744-5p expression was down-regulated in OS cells and tissues. Higher expression of miR-744-5p was related with better clinical prognosis and lower malignancy degree of OS, including cell proliferation, migration and invasion in vitro and vivo. Transforming growth factor- $\beta$ 1 (TGFB1) was negatively regulated by miR-744-5p and could reverse the effects of miR-744-5p on OS proliferation, migration and invasion. The MAPK/ERK signaling pathway was involved in the miR-744-5p/TGFB1 axis.

**Conclusions:** In general, this study suggests that miR-744-5p is a negative regulator of TGFB1, and suppresses OS progression and metastasis via MAPK/ERK signaling pathway.

## 1. Background

Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents, originating from mesenchymal cells[1, 2]. With a poor prognosis, the mortality could be over 90% before polychemotherapy was introduced to clinical practice[3]. Significant progress has been made in treatment of OS in the past 30 years, effective therapies like neoadjuvant chemotherapy combined with surgical resection has been introduced into clinical treatment, and the prognosis and quality of life has visibly improved compared to decades before[4–6]. Nevertheless, the 5-year survival rate of OS patients is still less than 50%[7]. Lung metastasis is the main problem for OS therapy, and the 5-year survival rate is < 30% with metastatic OS[8]. Therefore, more potent therapeutic strategies and approaches for OS are urgently needed.

Epithelial-to-mesenchymal transition (EMT) is a complex process through which epithelial cells obtain features of mesenchymal cells and lose original polarity. This reversible phenotypic change was supposed to be a stimulative element of tumor migration and invasion[9]. Accumulating evidence have revealed that EMT is closely related to tumor occurrence and development[10, 11]. Therefore, suppressing the progression of EMT may be a potentially crucial approach to OS treatment.

MicroRNAs (miRNAs) are a family of endogenous small non-coding RNAs, regulating the expression of target gene by combining with the 3'-untranslated regions (3'-UTRs)[12]. Accumulating evidence suggests that miRNAs play a crucial role in the occurrence and development of various tumors by regulating multiple signaling pathways[13, 14]. Moreover, partial miRNAs participate in the course of EMT and act on the regulation of tumors[15, 16]. miR-744-5p has been reported to be up-regulated in several tumors and was closely related with the clinical characteristics[17–20]. However, the functions of miR-744-5p in OS has not been reported and the mechanism of action of miR-744-5p still needs further study.

Mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway is reported to be related with different stages of tumor growth through diversified mechanisms[21, 22]. The MAPK pathway plays a crucial part in partial miRNAs regulation of various tumors[23–25]. Suppressing the MAPK/ERK pathway is supposed to be connected with inhibiting the viability and migration, and promoting the senescence and apoptosis of tumor cells.

Transforming growth factor- $\beta$  (TGF- $\beta$ ), the prototype of the TGF- $\beta$  family, with a bifunctional of regulating cell proliferation, has been reported to have a promoting regulation effect on EMT, and sufficient evidence has demonstrated that there's conspicuous increased level of TGF- $\beta$  in tumor cells[26–28]. Moreover, it has also been reported that MAPK/ERK was associated with TGFB1[29, 30]. Nevertheless, the specific effects of TGFB1 in OS have not been clearly illuminated yet, and the detailed function of TGFB1/MAPK/ERK axis remains investigation.

In this study, we found that miR-744-5p was remarkably down-regulated in osteosarcoma, and it suppresses the proliferation, migration and invasion of osteosarcoma cells by negatively regulating TGFB1 and MAPK/ERK signaling pathways. These findings may provide a new therapeutic strategy for osteosarcoma.

## 2. Materials And Methods

### Tissue samples

This study was approved by the ethics committee of the Second Affiliated Hospital of Southern Medical University. All human osteosarcoma and para-carcinoma samples were obtained from a total of 25 patients undergoing biopsies before receiving chemotherapy and radiotherapy at the department of joint and orthopedics. Tissue samples obtained from biopsy were collected and instantly frozen in liquid nitrogen. The pathological and personal clinical information is listed in Table 1.

### Obtainment and analysis of original data

GES65071 from the GEO database was downloaded. The R package affy was used in background correction and normalization processing. The R package limma was used to detect the difference of the miRNA expression level between normal samples and OS samples. The filter criterion: Log [fold change (FC)] > 1 and adj. P value < 0.05.

## Cells and cell culture

All human OS cells, including Saos-2, U-2 OS, MG-63, MNNG, 143B and normal osteoblast cell line hFOB 1.19 were obtained from the American Type Culture Collection (ATCC, Manassas, US). OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, US) replenished with 10% fetal bovine serum (FBS) (Gibco, NY) and 1% penicillin/streptomycin (PS, Gibco, CA). The hFOB 1.19 cells were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) (Life Technologies, NY) with 0.3mg/ml of G418, 10% FBS and 1% PS. All cells were incubated with 5% CO<sub>2</sub> at 37°C.

## Establishment of transfected cells

Plasmids overexpressed miR-744-5p and TGFB1 were used in the vitro experiments. The cells were cultured in 6-well plates. After washing with DMEM, the complex liquid of transfection was added into the plates and incubated for 24 h. Then the cells were cultured with DMEM containing 10% FBS for 48 h. G418 selective media was used to screen out the transfected cells. The lentiviral transfection was conducted in vivo experiments. The cells were cultured in 24-well plates for 24 h. The medium with 2 µg/ml polybrene was used to replace the original medium, and lentivirus transfected with miR-744-5p or TGFB1 were added into the wells. After incubating for 24 h, the cells were cultured with DMEM for another 72 h. The transfection efficiency was examined via qRT-PCR .

## Quantitative real-time PCR (qRT-PCR)

Total RNAs from cells and tissue samples in Trizol (Invitrogen, US) were extracted from the frozen pulverized samples according to the manufacturer's protocol. 500 ng of total RNA was reverse transcribed into cDNA. The cDNA was diluted five times with enzyme-free water. One-step qRT-PCR was performed in a 10 µL reaction system. The purity and integrity of the total RNAs were examined through the absorbance at 260nm and 280 nm with. The primers of TGFB1, U6 and GAPDH were purchased from TsingKe (Beijing, China) and the primers of miR-744-5p were designed personally based on the purchased primers. Reverse transcription (RT) was performed with SuperScript™ Preamplification System for First Strand cDNA Synthesis according to the protocol of manufacture, and qPCR was performed with LightCycler® Real Time PCR. The expression of U6 or GAPDH served as endogenous control. The sequences of the primers are as follows: TGFB1 forward: 5'-GGCCAGATCCTGTCCAAGC-3'; TGFB1 reverse: 5'-GTGGGTTTCCACCATTAGCAC-3'; GAPDH forward: 5'-GGAGCGAGATCCCTCCAAAAT-3'; GAPDH reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'; U6 forward: 5'-CTCGCTTCGGCAGCAC-3'; U6 reverse: 5'-AACGCTTCACGAATTTGCGT-3'; miR-744-5p forward: 5'-AATGCGGGGCTAGGGCTA-3'; miR-744-5p reverse: 5'-GTGCAGGGTCCGAGGT-3'.

## Western Blotting (WB)

Proteins of the cells were extracted, and the concentration of proteins was assessed with the BCA protein assay kit (Beyotime, China). Then the proteins were electrophoresed through 10% SDS-PAGE for 4 h at 40 volts and then transferred to the PVDF membranes. The proteins were incubated with specific primary

antibodies at 4°C overnight. After washing with TBST, the proteins were incubated with the secondary antibodies at indoor temperature for 2 h. Rat anti-TGFB1 (1:1000, Abcam), GAPDH (1: 10,000, Proteintech), N-cadherin (1:1000, Abcam), E-cadherin (1:1000, Abcam), Vimentin (1:1000, Abcam), p-P38 (1:1000, Abcam), t-P38 (1:1000, Abcam), antibodies were used to detect the proteins. Reacting bands were acquired with ECL reagent and the quantity analysis was performed with ImageJ normalized to GAPDH.

#### Colony formation assay

About 800 OS cells were seeded into the six-well plate and cultured with DMEM and 10% FBS at 37°C for 1 week. When the colonies turned invisible they were washed with PBS and fixed with 4% paraformaldehyde, then satined with 0.1% crystal violet. The images were captured with a scanner and the counts were calculated manually.

#### 5-Ethynyl-2-Deoxyuridine (EdU) Incorporation assay

The EdU incorporation assays were performed in accordance with the manufacturer's protocol.  $1 \times 10^4$  cells/well of OS cells were seeded into 96-well plate and cultured with 100  $\mu$ L of 50  $\mu$ M EdU medium for 2 h. Then the cells were fixed with 4% paraformaldehyde and destained with 2mg/mL glycine. Next, Apollo staining was performed with 1X Apollo dyeing reaction fluid. 1X Hoechst 33342 reaction mixture was used in DNA staining. At least 50 cells per well were selected randomly. The intensity was measured from five random fields and the photos were taken with fluorescent microscope (Carl Zeiss, Germany).

#### Transwell Migration and invasion assay

Transwell migration assay was performed to detect the cell migratory ability. Total of  $4.0 \times 10^4$  cells were seeded in the upper chamber with 200  $\mu$ L of DMEM, while the lower chamber was immersed with 600  $\mu$ L of DMEM with 10% FBS. After incubating for 24 h, the lower chamber was moved away and cells were fixed with 4% paraformaldehyde for 30 min. Then stained the cells with 0.1% crystal violet for 20 min, unmigratory cells on the upper chamber were wiped with swab. After removing the crystal violet, five randomized fields were observed and photographed with a microscope. As for the Transwell invasion assay, Matrigel (BD 5mg/ml) was diluted to 1mg/ml with serum-free medium. 100  $\mu$ L of resulting Matrigel was put on the upper chamber and incubated at 37°C for 1 h. The following steps were the same with the Transwell migration assay.

#### Luciferase reporter assay

Possible miR-744-5p-binding sites were obtained from the miRDB database. Wild-type TGFB1 (WT-TGFB1-3'-UTR) and mutant TGFB1 (MUT-TGFB1-3'-UTR) were synthesized by GenePharma (Shanghai, China). Cells overexpressing miR-744-5p was transfected with WT-TGFB1-3'-UTR and the negative controls were transfected with MUT-TGFB1-3'-UTR. 48 h later after transfection, the luciferase activity was determined with the Dual-Luciferase Assay System (Promega, WI, US) and normalized using Renilla luciferase.

## Immunohistochemistry (IHC)

The slides were immersed in the miscible liquids of potassium dichromate and concentrated sulfuric acid, then flushed for 1 h. Polylysine was smeared on the surface. Tissues were conducted through paraffin embedding. Tissue sections were dewaxed with xylene and ethanol. Then the sections were immersed in 0.01 mol/L sodium citrate buffer for 10 min and 3% hydrogen peroxide for 30 min at indoor temperature. Next, the sections were put into phosphate-buffered saline (PBS) for 5 min, and sealed with 5% bovine serum for 0.5 h at 37°C. The tissue sections were incubated with primary antibodies overnight at 4°C. After washing three times with PBS, the sections were incubated with secondary antibodies for 0.5 h at 37°C and then incubated with SABC for another 0.5 h. Color developing agents were added after wiping up the sections and then hematoxylin staining was performed. Finally, the sections were dehydrated with ethanol and xylene, and sealed with resin. The main antibodies were Ki-67, E-cadherin, N-cadherin and Vimentin (Abcam, UK). Photos were captured with an orthophoto microscope.

## Hematoxylin-eosin (HE) staining

The tissues were immersed in stationary liquid containing 10% methanol. After dehydrating with ethanol and xylene, the samples were embedded in the paraffin. The sections were dewaxed with xylene and ethanol before staining. The sections were successively immersed in Hematoxylin, hydrochloric acid, ammonium hydroxide and flushed with distilled water for 1 h. Next, the sections were dehydrated in the ethanol and dipped in eosin staining solution. Finally the sections were immersed in ethanol and xylene, and sealed with gums.

## Animal experiments

Nude mice in the study were purchased from Animal Core Facility of Southern Medical University and were randomly divided into 5 groups, 5 in each group. OS cells with fluorescent protein RFP were inoculated into subcutaneous tissue of the nude mice. Pulmonary metastasis models were conducted through tail vein injection. The volume and size of tumors were recorded every 3 days and the tumors were separated and imaged on day 28th. The mice were sacrificed at the end of experiments.

## Statistical analysis

All experiments were repeated at least 3 times and data were demonstrated as means  $\pm$  standard deviation. Independent Students' t-test and One-way ANOVA were used to compare the difference between two groups in clinical characteristics. Paired t-test was used to evaluate the differences in the miRNA expression between TGFB1 and miR-744-5p in tissue samples. Pearson's chi-squared test was performed to detect the relation between miR-744-5p and TGFB1. Log-rank test was conducted to evaluate the prognosis and overall survival of OS patients. Statistical analyses were performed with SPSS, v. 23.0.  $p < 0.05$  was considered statistically significant. Data are presented as the means  $\pm$  SD. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

## 3. Results

### 3.1 miR-744-5p was down-regulated in OS cells and tissues.

In order to investigate how miRNAs express in OS cells and tissue samples, GSE65071 from the GEO database was analyzed with R package. The volcano plot demonstrated the differences of miRNAs expression between OS and normal tissues (Fig. 1a). According to the miRNA expression levels, the up- and down-regulated miRNAs were displayed with a cluster heat map (Fig. 1b). Totally, 89 miRNAs were down-regulated in OS tissues compared to normal tissues (fold change > 1, FDR < 0.05), and the top ten down-regulated miRNAs were shown in **Fig. 1c**. We found that miR-744-5p was the most down-regulated miRNA among these miRNAs. Based on above results, we attempted to figure out the specific function of miR-744-5p in OS. qRT-PCR was performed to assess miR-744-5p expression in 25 paired OS and adjacent normal tissues, and results figured that miR-744-5p was significantly down-regulated in OS tissues (Fig. 1d). Furthermore, low expression of miR-744-5p was found in OS cells including 143B, MNNG, U-2 OS, MG-63 and Saos-2 compared with in hFOB 1.19 cell lines (Fig. 1e).

### 3.2 miR-744-5p was closely related to better clinical characteristics of OS patients.

As shown in (Fig. 2a-d), expression level was significantly associated with OS clinical characteristics, higher expression of miR-744-5p was found in patients with earlier pathological stage, smaller tumor size, localized growth and higher long-time survival rate. Detailed clinical data was demonstrated in Table 1.

### 3.3 Up-regulation of miR-744-5p inhibited OS cell proliferation, invasion and migration *in vitro*.

MG-63 and 143B cell lines were used *in vitro* for further experiments in this study. qRT-PCR was performed to evaluate the transfection efficiency of miR-744-5p mimics (Fig. 3a). Colony formation and EdU assays were conducted to detect the effect of miR-744-5p on cell proliferation. Figure 3b-e demonstrated that over-expressed miR-744-5p significantly inhibited the proliferation of the OS cells. Transwell migration assays were performed to assess the influence of miR-744-5p on the migratory ability of the OS cells *in vitro* and results showed that over-expressed miR-744-5p significantly decreased migration of OS cells. Similarly, the invasive ability of the OS cells were detected with Transwell invasion assays, and up-regulating miR-744-5p suppressed the invasive ability of both 143B and MG-63 cells (Fig. 3f-i). Furthermore, WB analysis demonstrated that miR-744-5p decreased the expression of tumorigenesis-related proteins, N-cadherin, vimentin, and increased the level of E-cadherin in both MG-63 and 143B OS cells (Fig. 3j).

### **3.4 miR-744-5p suppressed xenograft tumor growth and metastasis *in vivo*.**

In order to investigate the effect of miR-744-5p *in vivo*, OS cells transfected with Lv-miR-NC, Lv-miR-744-5p were implanted subcutaneously in nude mice. Figure 4a showed the lung tissues obtained from the mice sacrificed in week 4. It was shown that tumors volume was smaller and the average weight was lighter in the Lv-miR-744-5p group compared with the NC group (Fig. 4b, **c**). IHC was performed to assess the expression of proliferation and invasion-related factors including Ki-67, E-cadherin, N-cadherin and vimentin. The results demonstrated that Ki-67, N-cadherin and vimentin were obviously down-regulated in the Lv-miR-744-5p group versus controls, indicating that miR-744-5p suppressed OS cell proliferation and invasion of OS cells *in vivo*. On the contrary, E-cadherin was observed with higher expression compared with Lv-miR-NC group (Fig. 4d). Less OS cells were observed in the over-expressed miR-744-5p group through HE-staining (Fig. 4e).

### **3.5 TGFB1 was up-regulated in OS tissues and was a target of miR-744-5p.**

The main function of miRNAs was inhibiting the translation or promoting the degradation of the target genes. Thus we attempted to figure out the downstream mechanism of miR-744-5p in the occurrence and development of OS, miRDB database was checked and 111 genes in total were found to be a potential target of miR-744-5p. KEGG pathway enrichment analysis demonstrated that MAPK signaling pathway was the most relative center of the mentioned genes (Fig. 5a). Among all candidate genes in MAPK axis, we found TGFB1 was down-regulated with the overexpression of miR-744-5p in both 143B and MG-63 OS cells (Fig. 5b, **c**). Luciferase-reporter assay was performed to detect the specific relationship between TGFB1 and miR-744-5p, results showed that miR-744-5p directly targeted TGFB1, and over-expressed miR-744-5p significantly suppressed the luciferase activity of OS cells (Fig. 5d, **e**). WB verified that miR-744-5p negatively regulated the expression of TGFB1, and MAPK-related proteins were down-regulated in the miR-744-5p mimics group (Fig. 5f). qRT-PCR was performed to investigate the expression of TGFB1 in OS cell lines and tissue samples, higher expression of TGFB1 was found in various OS cells, especially in MG-63 and 143B, and TGFB1 was significantly higher expressed in OS tissues compared with adjacent tissues (Fig. 5g, **h**). Furthermore, TGFB1 was negatively related with miR-744-5p with an  $r$  of -0.7326 in OS tissues (Fig. 5i).

### **3.6 TGFB1 was connected with poor clinical characteristics of OS patients.**

qRT-PCR were conducted to detect the relevance between TGFB1 and clinical characteristics. Figure 6a-c demonstrated that higher expression level of TGFB1 was found in more patients with advanced, larger

and metastatic tumor. Although there was no statistical difference in overall survival, patients with higher expression level of TGFB1 tend to have a poorer prognosis (Fig. 6d).

### **3.7 miR-744-5p suppressed OS proliferation, migration and invasion by regulating MAPK signaling pathway through TGFB1 *in vitro*.**

In order to verify that miR-744-5p regulates proliferation, migration and invasion of OS cells through via TGFB1, a series of rescue experiments were TGFB1 was over-expressed artificially, and qRT-PCR showed that TGFB1 was successfully transfected into the OS cells (Fig. 7a). Colony formation and EdU assays were performed, it was found that over-expressed miR-744-5p significantly suppressed the proliferation of OS cells, and the effects were restored with the up-regulation of TGFB1 (Fig. 7b-e). Transwell migration and invasion assays were performed, and results revealed that TGFB1 reversed the protective effects of miR-744-5p on OS migration and invasion (Fig. 7f-i). WB were performed to detect the downstream mechanism of miR-744-5p/TGFB1 axis regulating OS cells. Figure 7j, k showed that over-expressed miR-744-5p inhibited the expression of N-cadherin, vimentin, TGFB1, and t-P38, and promoted the expression of E-cadherin, indicated that EMT and MAPK pathways played essential roles in the process of miR-744-5p/TGFB1 axis regulating the OS promotion.

### **3.8 miR-744-5p suppressed OS growth and metastasis by regulating MAPK signaling pathway through TGFB1 *in vivo*.**

Rescue assays were performed in animals to verify how miR-744-5p and TGFB1 worked *in vivo*. OS cells transfected with Lv-miR-NC, Lv-miR-744-5p and Lv-miR-744-5p with TGFB1 were respectively implanted subcutaneously in nude mice. Figure 8a-c demonstrated that over-expression of miR-744-5p suppressed the growth of tumor, and there was significant difference in weight and volume, however, the introduction of TGFB1 inhibited the effects of miR-744-5p, made tumors larger and heavier remarkably. Moreover, IHC showed that higher expression of TGFB1 increased the expression level of ki-67, N-cadherin and vimentin which were down-regulated by miR-744-5p (Fig. 8d). Figure 8e demonstrated that Lv-miR-744-5p inhibited the invasion of OS cells, while inversely over-expression of TGFB1 promoted the metastasis conspicuously.

## **4. Discussion**

OS is the most frequent primary malignant tumor of bones which basically occurs in the adolescents[31]. However, though accumulating researches have been conducted on, poor prognosis of OS has not been solved well. Therefore, it's necessary to develop novel potential targets on OS therapies.

miRNAs have been reported to be directly related to the regulation of gene expression, and substantial evidence have revealed that abnormal miRNAs expression appears in numerous tumors[32–34]. Numerous studies have shown that various miRNAs have different functions in the process of OS developing[35–39]. Shaoxuan He[40] demonstrated that miR-217 inhibits the proliferation, migration and invasion of OS cells via directly targeting SIRT1. On the contrary, miR-652 is reported to promote tumorigenesis and metastasis through targeting RORA[41]. miR-744-5p has been reported to play a negatively regulatory role in some cancers like ovarian cancer and non-small cell lung cancer, however, few studies investigate the effects and mechanism of miR-744-5p in OS[17–19]. In accordance with the data from GEO database and results of qRT-PCR, we found that miR-744-5p was down-regulated in the OS cells and tissue samples, which was consistent with previous studies.

EMT participates in the migration and invasion of tumor cells, and promotes cancer progression and metastasis. During EMT process, epithelial cancer cells acquire characteristic of mesenchymal cells and lose the cell polarity and adhesion between cells. These features accelerate the migration and invasion of OS cells, promote tumor metastasis and increase drug resistance in OS therapies[42–44]. Furthermore, reduction of E-cadherin and induction of N-cadherin and vimentin levels are reported in the conversion from epithelial cell to mesenchymal cell[45, 46]. In this study, a series of experiments were undertaken to investigate the function and molecular mechanism of miR-744-5p in OS. We found that miR-744-5p was lowly expressed in OS tissues and played a negative regulatory role in tumor developing. Results indicated that miR-744-5p downregulated the cell proliferation, migration and invasion of OS, and could be a novel target in the treatment of OS. Moreover, via Luciferase-reporter assay, we proved that there was a binding sequence between miR-744-5p and TGFB1, and TGFB1 was found to be suppressed by miR-744-5p in the study.

TGFB1, a regulatory cytokine participating in multiple signaling pathways, has been reported to play dual roles in cell growth by regulating cell autophagy[47]. TGFB1 acts as a tumor suppressor in the early stage of tumor or normal tissues, and promotes tumorigenesis and metastasis in advanced tumors[26, 28, 48]. Recent studies have demonstrated that TGFB1 played a vital part in the progression in various tumors including ovarian cancer, colorectal cancer, cervical cancer, gastric cancer[26, 28, 49–51]. It was also found that TGFB1 induced EMT in the tumorigenesis and metastasis in cancer[52, 53]. In this study, we found that TGFB1 was significantly upregulated in tumor tissues and was highly correlated to the development of OS, indicating that TGFB1 could play a stimulative role in tumorigenesis and metastasis in OS. Moreover, the relationship between clinical characteristics and TGFB1 was verified in the study, results demonstrated that a high expression level could result in a poor prognosis of OS patients, which was quite similar with other related studies[54, 55].

Through bioinformatic analysis we have found that MAPK signal pathway was remarkably correlated with miR-744-5p. To detect the specific mechanism of this axis in OS, we performed various experiments. WB demonstrated that over-expression of miR-744-5p significantly suppressed the EMT-related and MAPK/ERK-related proteins in both 143B and MG-63 OS cells. Moreover, TGFB1 was then over expressed in the miR-744-5p overexpressed group, the results revealed a trend back to the results of the control

group. Similar outcomes were found in the rescue assays in vivo. ERK belongs to the MAPK family, and MAPK/ERK signaling pathway is supposed to be a central section that regulates proliferation, differentiation and apoptosis in cells[56, 57]. Accumulating evidence have shown that there's a close relationship between the MAPK/ERK pathway and tumor growth and metastasis, and enhanced ERK expression has been perceived in various tumors[58–60].

This study still remains shortcomings. We constructed the OS mice models through subcutaneous injection due to the limitation of experiment conditions, and orthotopic models were considered to be used to examine the results in the further study. Furthermore, function deficiency assay was expected to be performed to verify the effects of miR-744-5p and TGFB1. Besides, liquid biopsy has been noticed as a convenient and efficacious checkup method in recent years, and miRNAs have been detected in multiple body liquids[61–64]. miRNAs could become a potential therapeutic direction in the treatment of cancers and the expression profile analysis of miRNAs in body liquids could be conducted to figure out whether miR-744-5p could be utilized in clinical treatment of OS patients. Except the insufficiency, this study revealed the relationship between miR-744-5p and OS for the first time. OS patients with a high expression level of miR-744-5p was found to have better clinical characteristics and prognosis, indicating that miR-744-5p could be a latent target in prediction and assessment during treatment. Meanwhile, we detected the downstream mechanism of miR-744-5p and found that TGFB1 was a target gene of the miRNA and MAPK/ERK signaling pathway was involved in the process.

## 5. Conclusions

In conclusion, we found miR-744-5p was negatively related to the progression and metastasis of osteosarcoma via downregulating TGFB1 through MAPK/ERK signaling pathway. We demonstrated that miR-744-5p suppressed the proliferation, migration and invasion of OS cells through MAPK/ERK signaling pathway by directly targeting TGFB1. Thus miR-744-5p/TGFB1 signal pathway could be a potential therapeutic target for OS and may provide further insight into the molecular mechanism of OS.

## Declarations

### Ethics approval and consent to participate

The Ethics Committee of Zhujiang Hospital of Southern Medical University approved the experiments involving patients tissues in this study.

The Ethics Committee of Southern Medical University approved the animal experiments in this study.

### Consent for publication

Authors involved in this paper all signed written consent for publishing in your journal.

### Availability of data and materials

The datasets generated and analysed during the current study are available in the [GEO DataSets] repository, [ <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65071> ]

## Competing interests

The authors declare that they have no competing interests.

## Fundings

This study was funded by Guangdong Foundation of Science and Technology: 2017ZC0113

## Author's contributions

Conceptualization, Lj.L. and Hf.L.; methodology, Lj.L. and Hf.L.; software, Hf.L. and L.L.; validation, Lj.L., Gl.C. and L.L.; formal analysis, Lj.L., Gl.C., L.L. and Bs.Y.; investigation, Lj.L., Gl.C. Hf.L. and L.L.; resources, Lj.L., Hf.L., L.L. Jy.T., Xp.W., Gf.W. and C.X.; data curation, Lj.L., Gl.C., Hf.L. and L.L.; writing—original draft preparation, Lj.L. and Hf.L.; writing—review and editing, Gl.C., L.L., Jy.T., S.Z., Gf.W., Lt.L., Zw.L., Hq.S. and Yc.L.; visualization, Lj.L., Hf.L.; supervision, Lj.L. and Gl.C.; project administration, Lj.L.; funding acquisition, Lj.L.. All authors read and approved the final manuscript.

## Acknowledgements

Not applicable

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## Tables

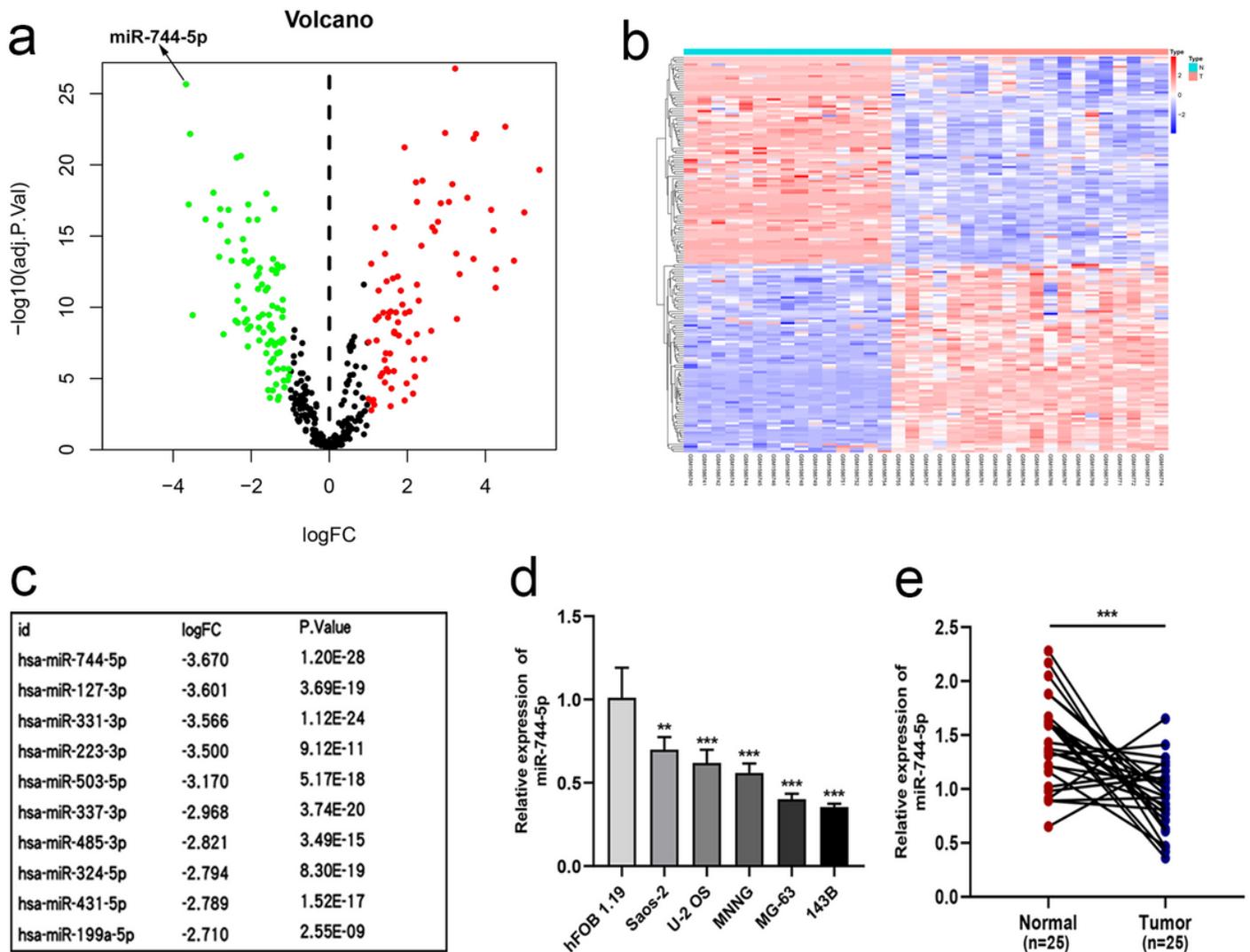
Table 1

Expression of miR-744-5p and TGFB1 according to patients' clinical characteristics

characteristics	miR-744-5p expression				TGFB1 expression		
	total	Tumor tissue	para-carcinoma tissue	P value	Tumor tissue	para-carcinoma tissue	P value
Age (y)				0.291			0.377
< 18	14	0.93 ± 0.38	1.34 ± 0.40	0.024*	2.15 ± 0.86	1.24 ± 0.61	0.001***
≥ 18	11	0.91 ± 0.26	1.46 ± 0.47	0.017*	2.42 ± 0.65	1.42 ± 0.74	0.003**
Gender				0.391			0.374
Female	13	0.98 ± 0.37	1.30 ± 0.41	0.103	2.14 ± 0.77	1.40 ± 0.68	0.002**
Male	12	0.86 ± 0.27	1.56 ± 0.41	0.002**	2.41 ± 0.79	1.23 ± 0.66	0.001***
Location				0.572			0.531
Arm/hand	11	0.96 ± 0.32	1.72 ± 1.05	0.029*	2.38 ± 0.77	1.35 ± 0.66	0.004**
Leg/foot	14	0.89 ± 0.34	1.41 ± 0.44	0.015*	2.18 ± 0.80	1.30 ± 0.69	0.001***
TNM stage				0.135			0.014*
I	6	1.04 ± 0.25	1.26 ± 0.38	0.401	1.80 ± 0.60	1.21 ± 0.50	0.054
II	11	1.03 ± 0.30	1.40 ± 0.41	0.075	2.09 ± 0.67	1.27 ± 0.74	0.004**
III/IV	8	0.67 ± 0.29	1.59 ± 0.47	0.005**	2.88 ± 0.71	1.46 ± 0.72	0.003**
Tumor size				0.017*			0.014*
< 5cm	11	1.09 ± 0.29	1.31 ± 0.41	0.272	1.86 ± 0.46	1.25 ± 0.53	0.001***
≥ 5cm	14	0.89 ± 0.24	1.43 ± 0.43	0.006*	2.44 ± 0.84	1.37 ± 0.84	0.004**
Lung metastasis				0.005**			0.005**
Yes	8	0.67 ± 0.29	1.59 ± 0.47	0.005**	2.88 ± 0.71	1.46 ± 0.72	0.003**

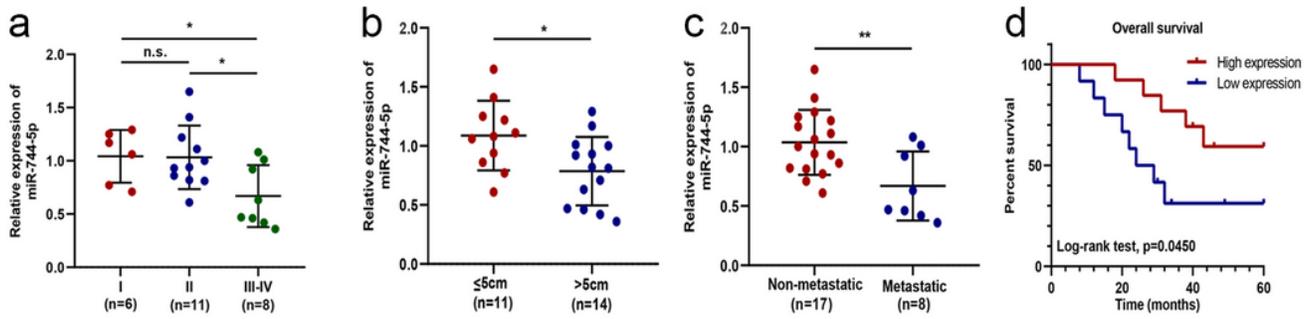
		miR-744-5p expression		TGFB1 expression			
No	17	1.04 ± 0.27	1.35 ± 0.39	0.043*	1.99 ± 0.64	1.25 ± 0.65	0.000***

## Figures



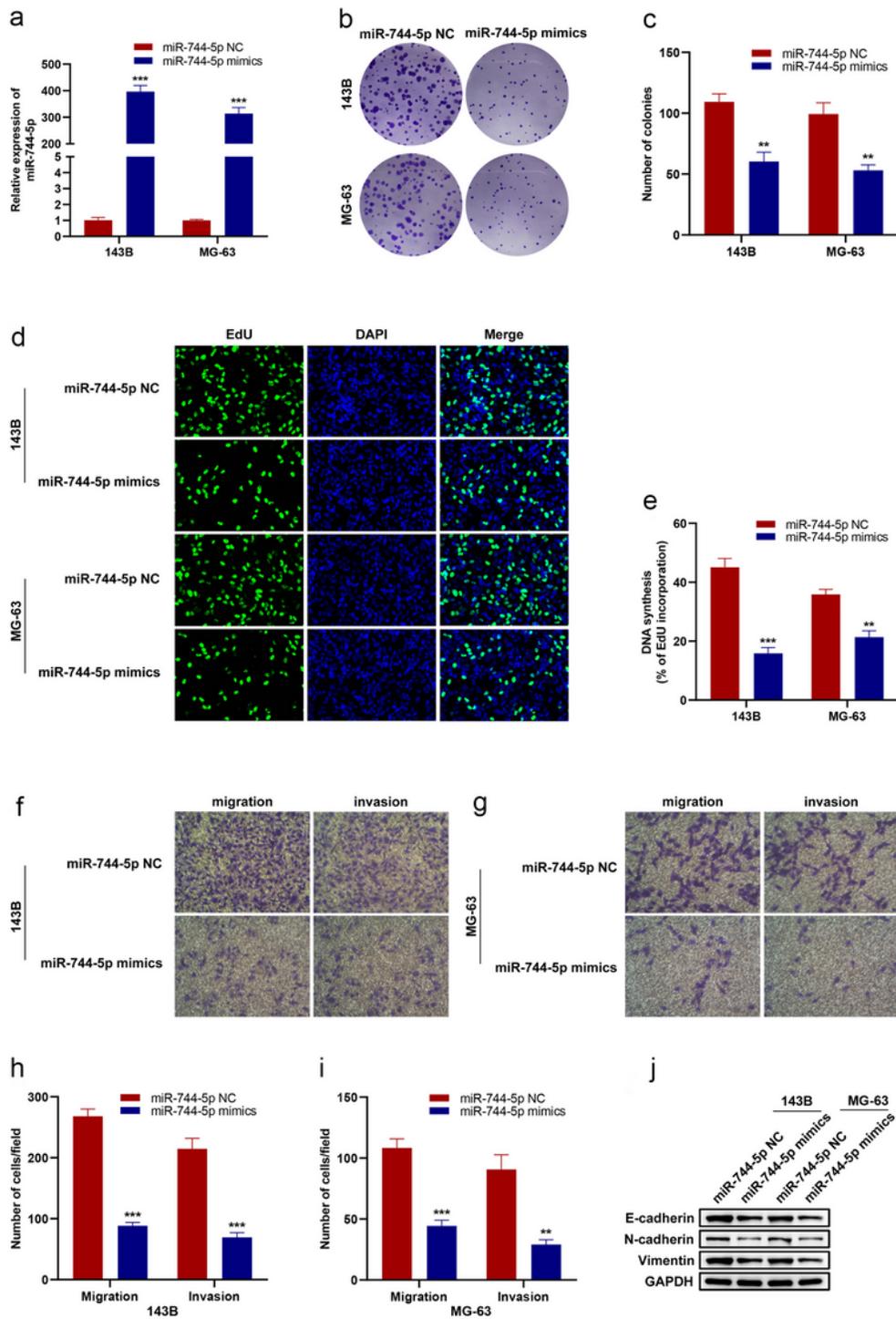
**Figure 1**

miR-744-5p is down-regulated in osteosarcoma cell lines and clinical tissues. (a) Volcano plot demonstrated the expressive diversity of miRNAs between OS and normal tissues from GSE65071. (b) The cluster heat map showed the up-regulated and down-regulated miRNAs in GSE65071. (c) The top ten down-regulated miRNAs are listed. (d) The relative expression of miR-744-5p was remarkably suppressed in OS cell lines. (e) Expression of miR-744-5p was significantly down-regulated in OS clinical tissues than para-carcinoma tissues.



**Figure 2**

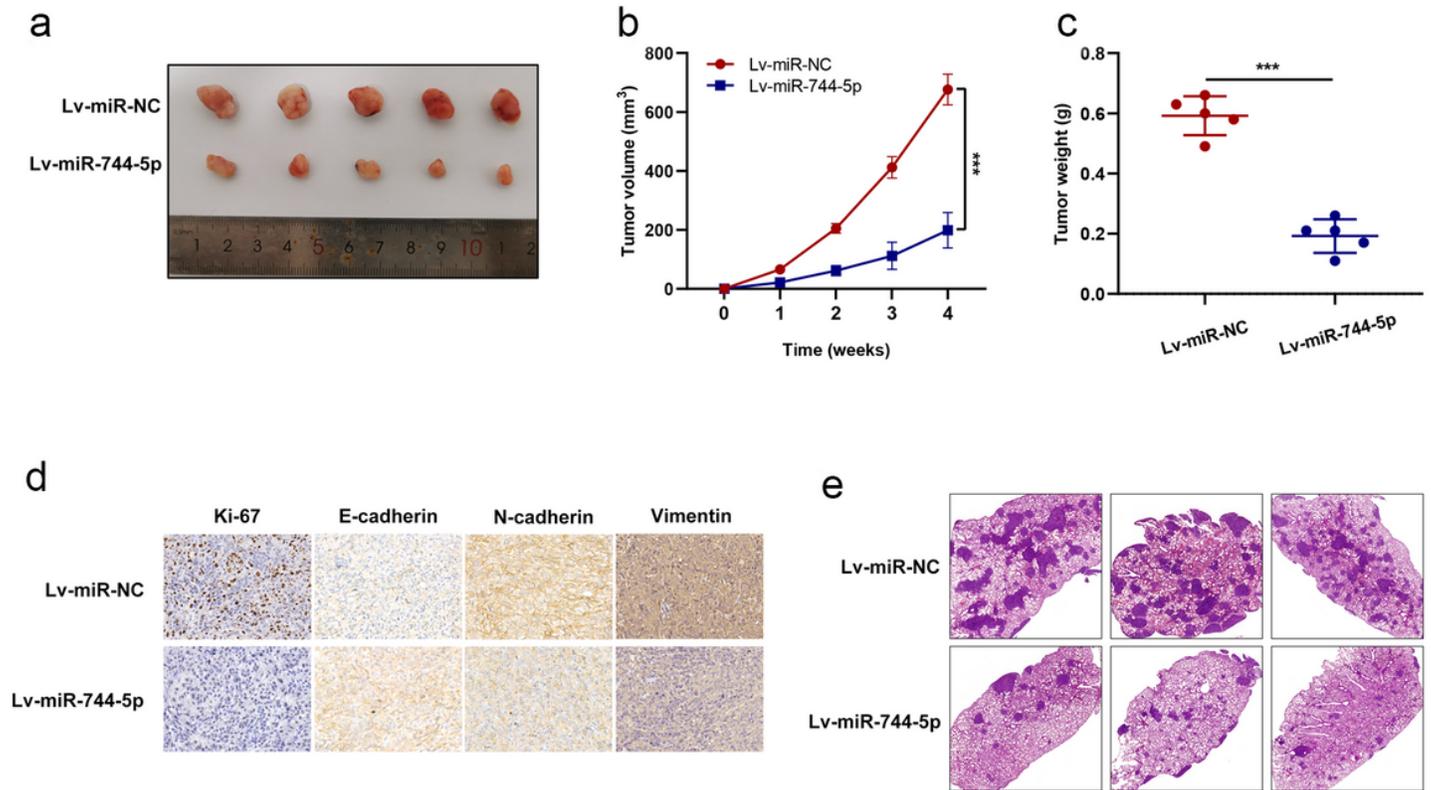
miR-744-5p is closely related to clinical features of OS patients. (a) Lower expression of miR-744-5p was found in the middle and advanced stage of OS compared to the early stage. (b) Lower expression of miR-744-5p was related to larger tumors. (c) Lower expression of miR-744-5p was found in more patients with metastasis. (d) Log-Rank test demonstrated that patients with higher miR-744-5p expression had a better prognosis.



**Figure 3**

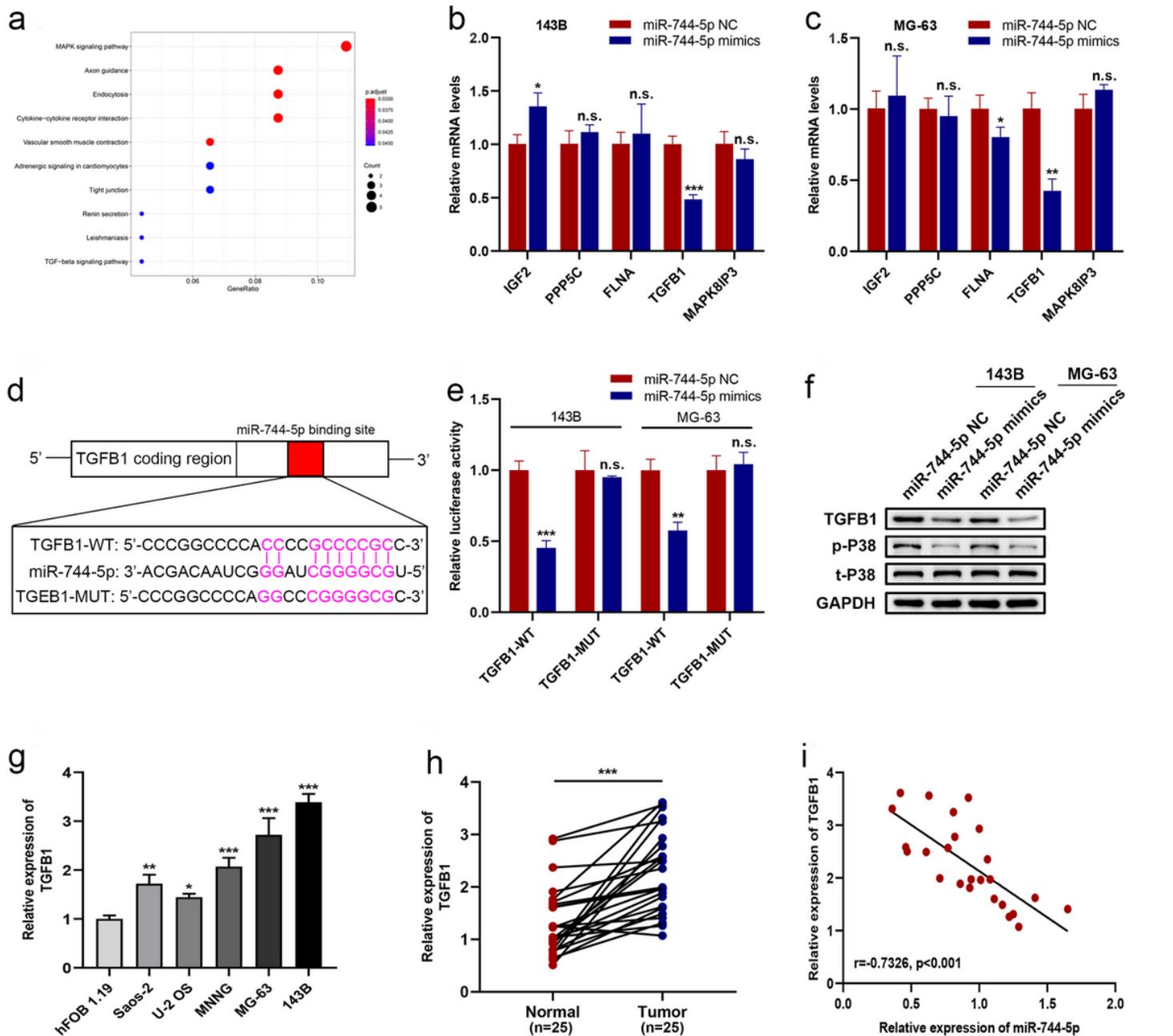
Over-expression of miR-744-5p suppressed OS cells EMT, migration and invasion in vitro. (a) miR-744-5p mimics were successfully transfected into 143B and MG-63 cell lines. (b-e) Colony formation and EdU assays demonstrated that over-expression of miR-744-5p suppressed the proliferation of OS cells. (f-i) Transwell migration and invasion assays showed that over-expressed miR-744-5p remarkably inhibited

the migratory and invasive ability of OS cells. (j) WB indicated that over-expression of miR-744-5p suppressed the expression level of metastasis-related proteins in OS cells.



**Figure 4**

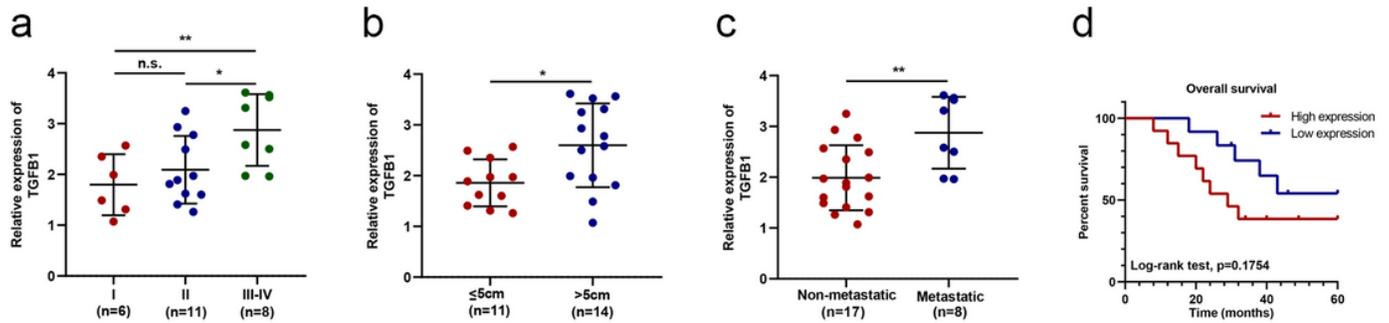
miR-744-5p suppressed xenograft tumor growth and pulmonary metastasis in vivo. (a-c) miR-744-5p mimics suppressed the tumor growth in nude mice, and the volume and weight were smaller and lighter compared to the miR-744-5p-NC group. (d) Higher expression of E-cadherin and lower expression of Ki-67, N-cadherin and vimentin were found in the Lv-miR-744-5p group according to IHC. (e) The HE staining demonstrated that less OS cells were found in lungs of nude mice of Lv-miR-744-5p group, indicating that miR-744-5p suppressed pulmonary metastasis.



**Figure 5**

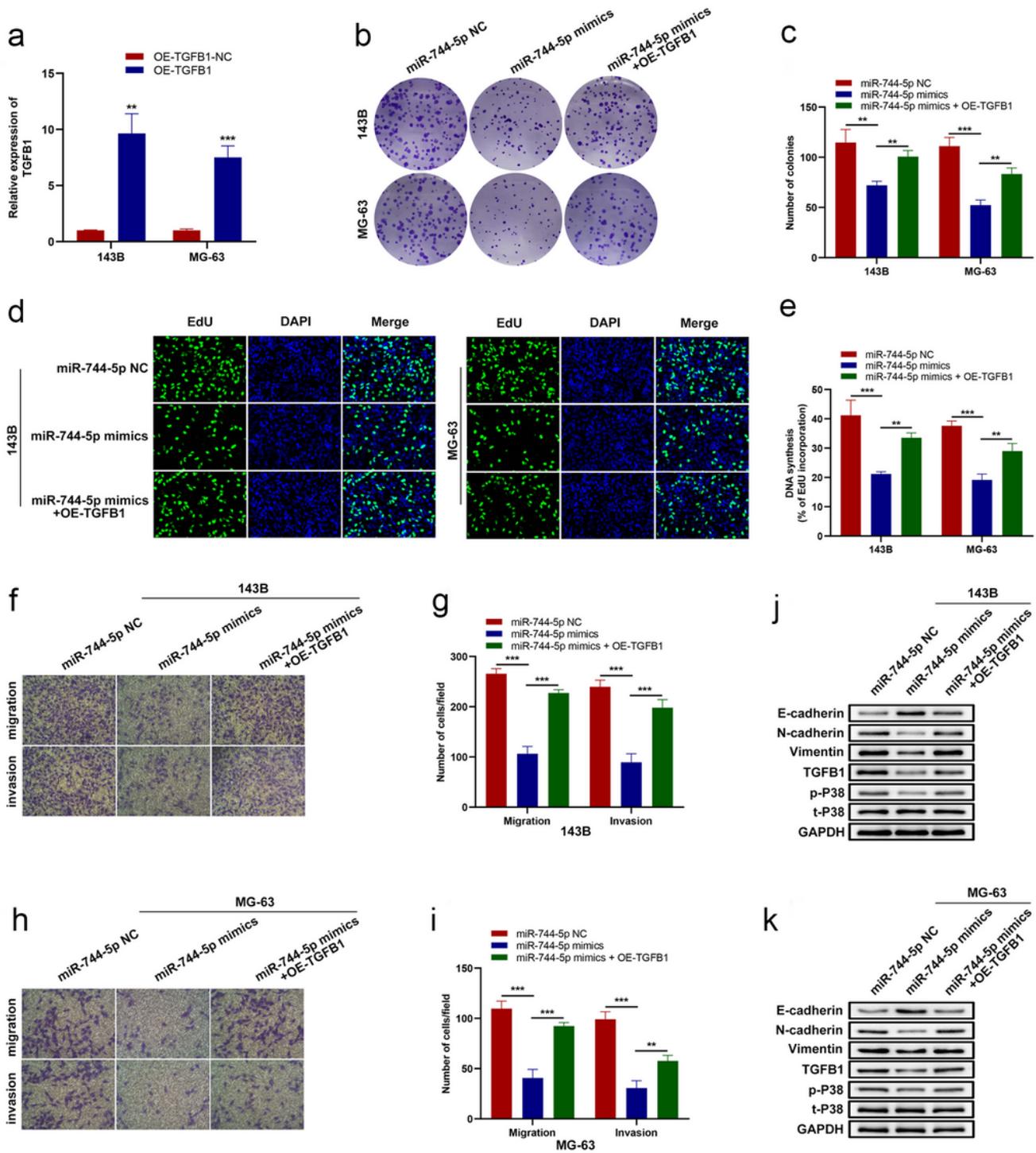
TGFβ1 expression was up-regulated in OS cell lines and tissues and was a target of miR-744-5p. (a) miRDB database demonstrated a total of 111 genes targeted to miR-744-5p, and MAPK signaling pathway was the most relative one according to KEGG pathway enrich analysis. (b, c) Among all candidate genes in MAPK axis, TGFβ1 was significantly downregulated by miR-744-5p both in 143B and MG-63 cells at the same time. (d, e) The WT-TGFβ1-3'-UTR and MUT-TGFβ1-3'-UTR were synthesized. Over-expressed miR-744-5p significantly suppressed the luciferase activity of WT-TGFβ1-3'-UTR but no effect on MUT-TGFβ1-3'-UTR in 143B and MG-63 cells. (f) WB showed that miR-744-5p down regulated the expression level of TGFβ1 and p-P38. (g) Higher expression of TGFβ1 was found in OS cell lines,

especially in 143B and MG-63 cells. (h) Expression of TGFB1 was significantly up-regulated in OS clinical tissues than para-carcinoma tissues. (i) TGFB1 expression level was negatively related to miR-744-5p in OS tissues.



**Figure 6**

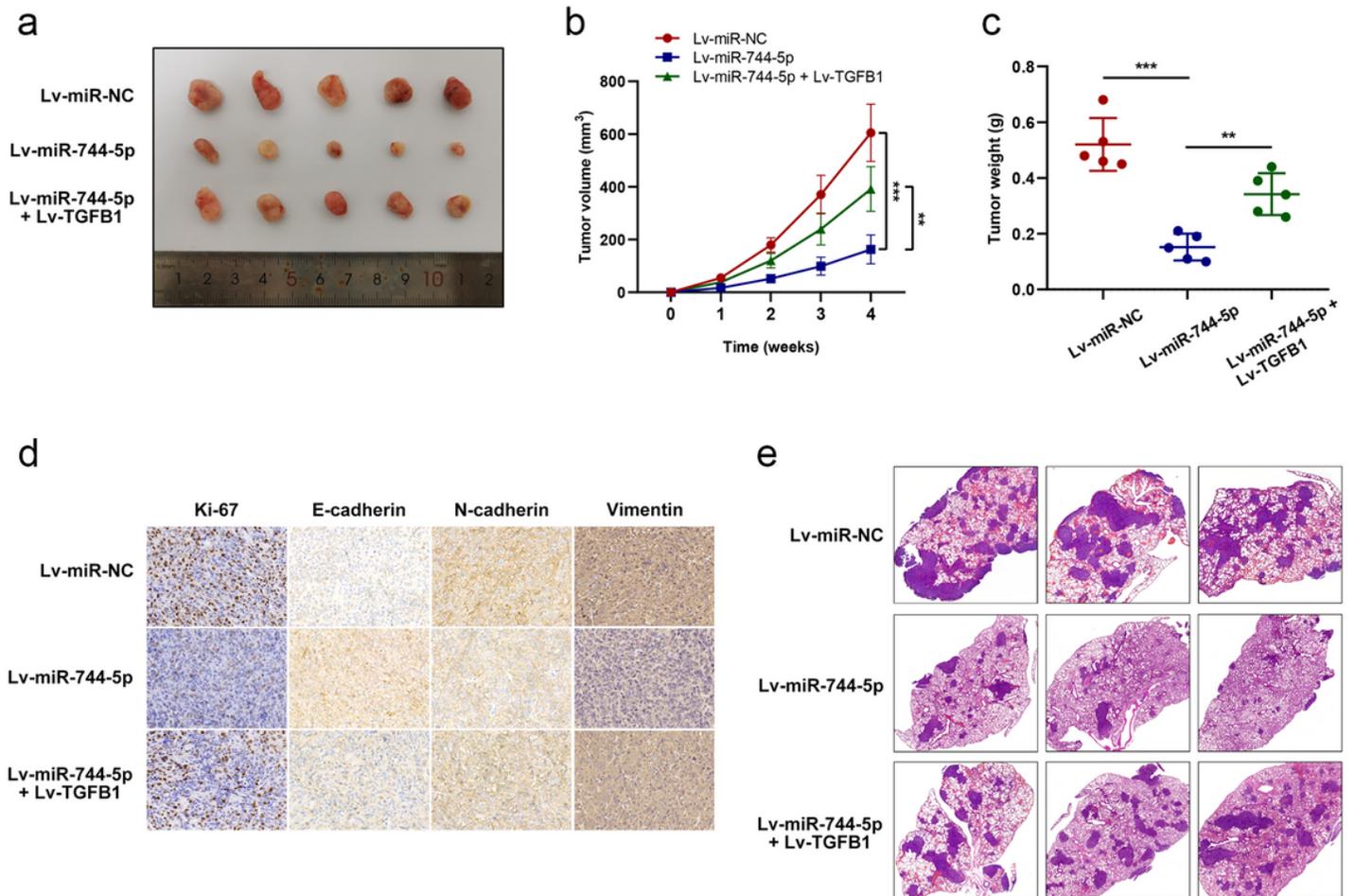
TGFB1 is related to clinical features of OS patients. (a) Higher expression of TGFB1 was found in the middle and advanced stage of OS compared to the early stage. (b) Higher expression of TGFB1 was related to larger tumors. (c) Higher expression of TGFB1 was found in more patients with metastasis. (d) Although there was no statistical difference in overall survival between two groups, patients with lower expression level of TGFB1 tend to have a better prognosis.



**Figure 7**

miR-744-5p down-regulated MAPK signaling pathway through inhibiting TGFB1 expression. (a) TGFB1 was successfully transfected into 143B and MG-63 cell lines. (b-e) Colony formation and EdU assays demonstrated that miR-744-5p suppressed the proliferation of OS cells, and over-expression of TGFB1 could reverse the effect. (f-i) Transwell migration and invasion assays indicated that over-expressed miR-744-5p significantly suppressed the migratory and invasive ability of OS cells, and over-expressed TGFB1

could abolish the influence. (j, k) Western blotting assays showed that miR-744-5p down-regulated metastasis related, MAPK-related and TGFB1 proteins in OS cells, while TGFB1 own contrary functions.



**Figure 8**

miR-744-5p suppressed tumor growth and pulmonary metastasis through TGFB1 in vivo. (a-c) miR-744-5p inhibited metastasis and growth of xenograft tumor and TGFB1 reversed the effects of miR-744-5p. (d) IHC showed that TGFB1 up-regulated the expression level of Ki-67, N-cadherin and vimentin which were suppressed in miR-744-5p mimics group. (e) TGFB1 promoted the decreased pulmonary metastasis caused by miR-744-5p.