


## RESEARCH ARTICLE

# Silencing of lncRNA KLF3-AS1 represses cell growth in osteosarcoma via miR-338-3p/MEF2C axis

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**Abstract**

**Background:** Osteosarcoma (OS) is a highly recurrent malignancy occurring among adolescents. The goal of this research was to scrutinize the role and action mechanism of KLF3-AS1 in OS.

**Methods:** Western blotting and quantitative reverse transcription real-time PCR were conducted to ascertain the mRNA expressions of miR-338-3p, KLF3-AS1, and MEF2C in OS cell lines and tissue samples. Colony formation and CCK-8 experiments were done to evaluate the proliferative capacity of the cells. Western blotting was also executed to measure the relative expressions of the proteins Bcl-2 and Bax. RNA immunoprecipitation and dual luciferase reporter experiments were carried out to validate the target relationships among MEF2C, KLF3-AS1, and miR-338-3p. Mouse xenograft models were created to assess the influences of KLF3-AS1 on the growth of tumors in vivo.

**Results:** Elevated levels of KLF3-AS1 and MEF2C and reduced amounts of miR-338-3p were identified in OS. KLF3-AS1 targeted miR-338-3p, and miR-338-3p further targeted MEF2C. Silencing KLF3-AS1 induced apoptosis and attenuated proliferation in vitro and repressed the tumor growth in vivo. Inhibiting miR-338-3p inverted the cancer-suppressing effects of KLF3-AS1 silencing. Meanwhile, loss of MEF2C partially eliminated the effects brought about by miR-338-3p downregulation, namely the stimulation of cell growth and suppression of apoptosis.

**Conclusions:** Silencing of KLF3-AS1 could repress the growth of cells and induce apoptosis by regulating miR-338-3p/MEF2C in OS. This suggests that the regulatory axis KLF3-AS1/miR-338-3p/MEF2C is a prospective target for OS treatment.

**KEYWORDS**

apoptosis, KLF3-AS1, MEF2C, miR-338-3p, osteosarcoma, proliferation

## 1 | INTRODUCTION

Osteosarcoma (OS) is a severe bone malignancy mainly occurring among adolescents under twenty-five years old.<sup>1</sup> It is generally characterized by bone pain and dysfunction.<sup>2</sup> Although various options for OS treatment, such as aggressive surgery and chemoradiotherapy,

have improved the 5-year survival rate to approximately 60%, some OS patients still suffered from high rate of recurrence.<sup>3</sup> Therefore, comprehending the pathogenesis of OS is imperative to formulate efficacious therapeutic procedures.

Long non-coding RNA (lncRNA) are 200 nt in length and can regulate post-transcription.<sup>4</sup> LncRNAs have been reported to have

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a participation in the tumorigenesis of OS.<sup>5-7</sup> For instance, lncRNA TTN-AS1 is overexpressed in a tumor burden mouse model. It represses cancer cell apoptosis, eventually accelerating OS development.<sup>5</sup> On another case, the high expression of lncRNA SNHG4 can induce cell growth in OS<sup>6</sup> and is also closely associated with the poor prognosis of OS patients.<sup>7</sup> Notably, some recent researches demonstrated that lncRNA KLF3-AS1, located at chromosome 4p14, has been confirmed to serve an anti-tumor role in the development of esophageal squamous cell carcinoma<sup>8</sup> and gastric cancer.<sup>9</sup> Nonetheless, accurate knowledge on the function of KLF-AS1 in the occurrence and development of OS is relatively rare.

MicroRNAs (miRNAs), which are 16–25 nt in length, inversely regulate genes' expressions by degrading their target mRNAs.<sup>10</sup> Recently, the biological function of miRNAs in OS progression has drawn increasing attention.<sup>11-13</sup> For example, miR-1225-5p is a tumor-inhibiting factor in OS which impedes OS development.<sup>11</sup> Upregulating miR-505 can markedly reduce cell proliferation in OS.<sup>12</sup> At the same time, both miR-143-3p and miR-429 have been confirmed to be prognostic markers for OS patients.<sup>13</sup> Of note, miR-338-3p has been found to be a relevant tumorigenesis regulator in OS.<sup>14,15</sup> Jia et al. have reported that inhibiting miR-338-3p remarkably promotes the viability and increase the colony numbers of OC cells.<sup>14</sup> Additionally, it has been confirmed that miR-338-3p interacts with lncRNAs to act on OS progression. For instance, Zhang et al. have documented that lncRNA CASC15 modulates miR-338-3p to affect OS progression.<sup>15</sup> Until now, whether miR-338-3p can interact with KLF3-AS1 in OS progression remains unclear.

In the current report, we concentrated on KLF3-AS1's expression level and possible functions in OS cells and tumor xenograft models. In addition, this study also puts emphasis on the action mechanism of KLF3-AS1 in the cellular processes of OS cells. Our findings may contribute information and insights on possible clinical therapeutic biomarkers for OS.

## 2 | MATERIALS AND METHODS

### 2.1 | Reagents and animals

ScienCell provided the human normal osteoblast hFOB 1.19. OS cell lines HOS, Saos-2, and SW1353 were obtained from American Type Culture Collection (ATCC). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and the Lipofectamine 3000 were sourced from Invitrogen. KLF3-AS1-siRNA (si-lnc), MEF2C-siRNA (si-MEF2C) and its negative control (si-NC), small hairpin targeting KLF3-AS1 (sh-lnc) and its sh-NC, miR-338-3p mimic, mimic-NC, miR-338-3p inhibitor (inhibitor), and inhibitor-NC were all acquired from Sangon Biotech. The CCK-8 kit and dimethyl sulfoxide (DMSO) used for cell viability assays were procured from Aladdin. Crystal violet and RIP assay-related kit were from Sigma Aldrich. Primary antibodies (MEF2C, cat.no. ab211493; GAPDH, cat.no. ab8245; Bcl-2, cat.no. ab194583; and Bax, cat.no. ab32503), and HRP-conjugated

secondary antibody (cat.no. ab6789) were bought from Abcam. The radioimmunoprecipitation assay (RIPA) buffer, enhanced chemiluminescence (ECL) detection kit, and PARIS™ Kit were from Thermo Fisher Scientific. The Total RNA Extraction Kit was acquired from Promega; the First-Strand cDNA Synthesis Kit was from APEXBio Technology; and from Qiagen, we acquired a SYBR Green FAST Mastermix Kit (Dusseldorf, GER). Four- to five-week-old female BALB/c nude mice weighing 20–28 g, from Esebio, were used to establish tumor xenograft models.

### 2.2 | Collection of cancer samples

From May 2019 to June 2021, tumors and normal adjacent tissues were acquired from 37 patients suffering from OS. The inclusion criteria were as follows: (1) The patients diagnosed with OS through histological examinations; (2) the patients without any treatments prior their admission; (3) the patients without OS history. The exclusion criteria were as follows: (1) The patients diagnosed with concurrent malignant tumors, mental illness, heart dysfunction, or others; (2) the patients who were pregnant or lactating. All subjects, or their respective guardians, provided a written informed consent. The ethics committee of Huangshi Central Hospital, Affiliated Hospital of Hubei Polytechnic University, Edong Healthcare Group granted approval for this study.

### 2.3 | OS cell culture

HFOB 1.19 and 3 OS cell lines were grown in DMEM with an added 10% FBS and then kept in an environment with 5% CO<sub>2</sub> and a temperature of 37°C. After achieving 80%–90% confluence, cells at passages three to five were utilized for all the experiments.

### 2.4 | Cell transfection

The transfection had been conducted in strict compliance with the protocol. Si-KLF3-AS1, si-MEF2C, miR-338-3p mimic, miR-338-3p inhibitor, and their respective NCs were introduced individually into the OS cell lines for 48 h with the aid of a Lipofectamine 3000. Finally, the cells that had been successfully transfected were gathered for the subsequent experiments.

### 2.5 | Subcellular localization assay

A PARIS™ Kit was used on SW1353 and Saos-2 cells to separate their cytoplasmic and nuclear fragments and extract their RNA. The levels of the RNA expression were then assessed via qRT-PCR. The controls adopted for the cytoplasm and nucleus were GAPDH and U6, respectively.

## 2.6 | Western blotting

The Saos-2 and SW1353 cells were dissolved in a RIPA buffer to extract the total protein. Afterward, a total of 30 μg of protein that had been electrophoresed using a 10% SDS-PAGE gel was transferred onto polyvinylidene fluoride (PVDF) membrane. The membrane was blocked at room temperature with 5% bovine serum albumin. We then added the primary antibodies including Bax (dilution, 1:2000), Bcl-2 (dilution, 1:2000), cleaved caspase-3 (dilution, 1:2000), MEF2C (dilution, 1:2000), and GAPDH (dilution, 1:2000) on the membrane and incubated 24 h at 4°C. Thereupon, the horseradish peroxidase (HRP)-conjugated secondary antibody (dilution, 1:5000) was added onto the membrane, then it was incubated for another 1 h. GAPDH was adopted as the internal reference. Finally, an ECL detection Kit was utilized to better visualize the bands. They were then observed using Gel-Pro Analyzer 4.0 (Media Cybernetics, Silver Spring). All the antibodies were from Abcam.

## 2.7 | qRT-PCR

A Total RNA Extraction Kit was utilized for the extraction of the total RNA from the OS tumors and cells. The First-Strand cDNA Synthesis Kit was subsequently used to produce cDNA. PCR was accomplished under the Applied Biosystems (ABI) 7500 Real-Time PCR System with the aid of a SYBR Green FAST Mastermix. Table 1 lists all the primers used. The mRNA expressions of KLF3-AS1 and MEF2C were normalized to GAPDH while miR-338-3p was to U6. The gene expression was determined by applying the  $2^{-\Delta\Delta Ct}$  approach.

## 2.8 | Cell viability assay

Saos-2 and SW1353 cells were inoculated into culture plates with 96-wells (5000 cells/well) and then cultivated for 24, 48, and 72 h. Following the incubation at the specified durations, CCK-8 solution (10 μl) was pipetted into the wells before they were incubated for

two more hours at 37°C. Cell viability was then determined using a microplate reader with a 450nm filter.

## 2.9 | Cell colony assay

SW1353 and Saos-2 cells (1000 cells/well) were inoculated into 6-well culture plates and cultivated at 37°C. The culture medium was changed every 3 days. Around 14 days later, the cells were rinsed, fixed, stained, and maintained at 25°C. The number of cells was tallied with the aid of an Olympus light microscope.

## 2.10 | Flow cytometry

Annexin V-FITC Apoptosis Detection Kit (Beyotime) was used for flow cytometry.  $1 \times 10^6$  SW1353 and Saos-2 cells after transfection were collected and added 195 μl Annexin V-FITC binding buffer, 5 μl Annexin V-FITC, and 10 μl PI. After incubation cells for 20 min without light, the apoptosis rate was determined by flow cytometry (BD FACSCalibur).

## 2.11 | Dual luciferase reporter assay

The KLF3-AS1 and MEF2C sequences containing miR-338-3p binding sites were combined to pGL3 vectors to construct KLF3-AS1-wild type (WT), MEF2C-WT, KLF3-AS1-mutant (MUT), and MEF2C-MUT. The constructed vectors were individually transfected with either a miR-338-3p mimic or mimic-NC into the SW1353 and Saos-2 cells using Lipofectamine 3000. Afterward, the relative luciferase activities were estimated under a Dual-Glo Luciferase assay system (Promega).

## 2.12 | RNA immunoprecipitation (RIP) assay

SW1353 and Saos-2 were treated in a RIP lysis buffer, conjugated to magnetic beads, and then incubated with IgG or anti-Ago2 (1:3000). Subsequently, the proteins were digested using K buffer (150 μl). The enrichment of the RNA was determined via qRT-PCR.

## 2.13 | Mouse xenograft model

The nude BALB/c mice were separated into groups of 5 mice ad libitum: the sh-lnc and the sh-NC groups. Sh-NC and sh-lnc were each integrated first into lentivirus vectors prior their transfection into Saos-2 cells ( $1 \times 10^5$  cells/100 μl). Afterward, the transfected cells were administered subcutaneously into the nude mice. The tumor volumes were measured weekly. After 5 weeks, 50 mg/kg of pentobarbital sodium was used to anesthetize the mice before they were euthanized. The tumor xenografts were then collected and weighted.

TABLE 1 Primer sequences used for qRT-PCR analysis

Gene	Primer sequence (5'-3')
RNA KLF3-AS1	Forward: 5'-CTGTAGGCGCGCTCTTCTTT-3'
	Reverse: 5'-TCCGACCAAAGTTTGCCAAG-3'
miR-338-3p	Forward: 5'-TGCGGTCCAGCATCAGTGAT-3'
	Reverse: 5'-CCAGTGCAGGGTCCGAGGT-3'
MEF2C	Forward: 5'-GAACGTAACAGACAGGTGACAT-3'
	Reverse: 5'-CGGCTCGTTGTACTCCGTG-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACACA-3'
	Reverse: 5'-AACGCTTACGAATTTGCGT-3'
GAPDH	Forward: 5'-AGAAGGCTGGGGCTCATTTG-3'
	Reverse: 5'-AGGGGCCATCCACAGTCTTC-3'

## 2.14 | Statistical analysis

Data analysis was done in SPSS Statistics V22.0, and the values were indicated in the form of mean  $\pm$  SD. Student's t test was adopted to gauge the differences between two groups. Meanwhile, one-way ANOVA plus Tukey's post hoc test or two-way ANOVA with Sidak's multiple comparisons test were applied for multiple groups. MiR-338-3p's associations with KLF3-AS1 and MEF2C were ascertained using Pearson's correlation coefficient.  $p < 0.05$  was counted as statistically significant.

## 3 | RESULTS

### 3.1 | Silencing KLF3-AS1 induces apoptosis and represses the proliferation of OS cells in vitro and reduces in vivo tumor growth

The expression of KLF3-AS1 in OS tissues was determined first. As presented in Figure 1A, the expression levels of KLF3-AS1 were higher among the tumors than in normal tissues. As expected, unlike in hFOB 1.19 cells, we observed the overexpression of KLF3-AS1 among SW1353, HOS, and Saos-2 cells (Figure 1B). Saos-2 and SW1353 cells were chosen for the succeeding experiments. As presented in Figure 1C, KLF3-AS1 is predominantly distributed in the cytoplasmic region, with an approximate cytoplasm/nucleus ratio of 70%/30%, suggesting that KLF3-AS1 may exert post-transcriptional functions. Thereafter, si-lnc/NC were introduced into the OS cells to study the influences of KLF3-AS1 on their growth in vitro. As presented in Figure 1D, KLF3-AS1 levels were decreased after si-lnc transfection, indicating that the transfection experiments were successful. Afterward, we explored the consequences of silencing KLF3-AS1 on the proliferative capacity of OS cells. We uncovered that knocking-down KLF3-AS1 greatly repressed the cells' viability (Figure 1E). Meanwhile, the number of OS clones was less in those transfected with si-lnc than in those with si-NC (Figure 1F). Western blotting revealed that KLF3-AS1 silencing remarkably elevated the protein levels of Bax and cleaved caspase 3 but reduced Bcl-2 expression in OS cells (Figure 1G). The flow cytometry proved that KLF3-AS1 silencing increased apoptosis rate (Figure 1H). At last, the effect of KLF3-AS1 knockdown on solid tumor was investigated. As displayed in Figure 2, the injection of SW1353 cells stably transfected with sh-lnc remarkably reduced the tumor weight and tumor volume. This evidences that KLF3-AS1 knockdown repressed the growth of tumors in vivo. These outcomes suggest that KLF3-AS1 is a possible pathogenic lncRNA in OS and that its silencing can impede the progression of OS.

### 3.2 | Identifying miR-338-3p as a KLF3-AS1 target

As illustrated in Figure 3A, a latent miR-338-3p and KLF3-AS1 binding site was identified using LncBase Predicted v.2 ([http://carolina.imis.athena-innovation.gr/diana\\_tools/web/index.php?r=lncbase2/index-predicted](http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbase2/index-predicted)). Luciferase reporter assays revealed lower luciferase activities in the KLF3-AS1-WT/miR-338-3p mimic transfected ones than in those with KLF3-AS1-WT/mimic-NC. As for the KLF3-AS1-MUT/miR-338-3p mimic group, there had been no significant alterations in luciferase activities (Figure 3B). To further substantiate the binding, RIP assays were performed using Ago2 antibody, indicating the preferential enrichment of KLF3-AS1 on the Ago2-conjugated beads (Figure 3C). Lower levels of miR-338-3p have been observed among OS tumors (Figure 3D) and cell lines (Figure 3E) in comparison with the normal samples. Moreover, it was revealed that the miR-338-3p and KLF3-AS1 levels in the OS samples had a significantly negative association (Figure 3F). The above data show that KLF3-AS1 can target miR-338-3p, thus negatively modulating miR-338-3p expression.

sev2/index-predicted). Luciferase reporter assays revealed lower luciferase activities in the KLF3-AS1-WT/miR-338-3p mimic transfected ones than in those with KLF3-AS1-WT/mimic-NC. As for the KLF3-AS1-MUT/miR-338-3p mimic group, there had been no significant alterations in luciferase activities (Figure 3B). To further substantiate the binding, RIP assays were performed using Ago2 antibody, indicating the preferential enrichment of KLF3-AS1 on the Ago2-conjugated beads (Figure 3C). Lower levels of miR-338-3p have been observed among OS tumors (Figure 3D) and cell lines (Figure 3E) in comparison with the normal samples. Moreover, it was revealed that the miR-338-3p and KLF3-AS1 levels in the OS samples had a significantly negative association (Figure 3F). The above data show that KLF3-AS1 can target miR-338-3p, thus negatively modulating miR-338-3p expression.

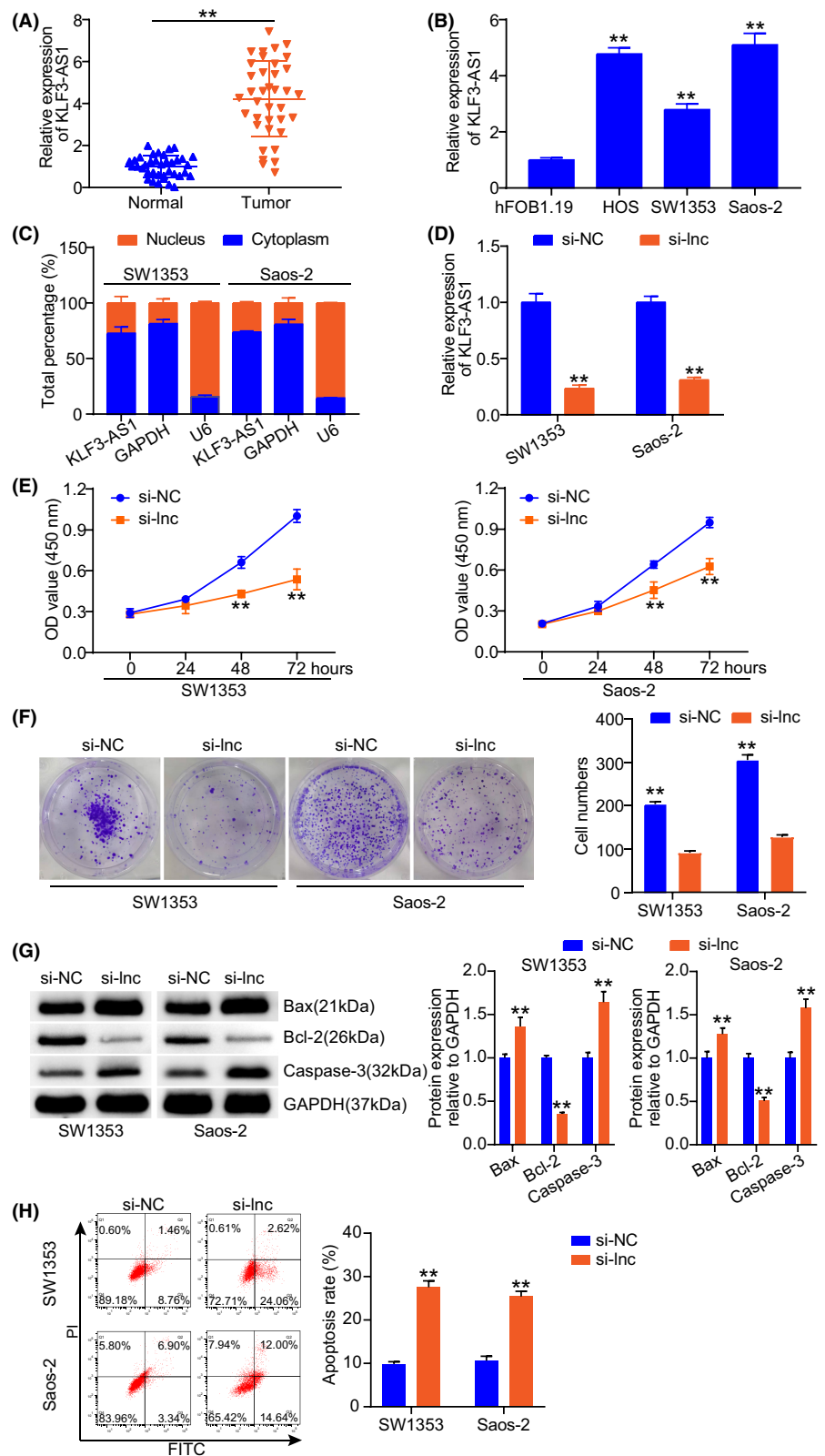
### 3.3 | MiR-338-3p inhibition attenuates the repressive effects of KLF3-AS1 silencing on OS cell growth

To further scrutinize the interaction of KLF3-AS1 with miR-338-3p during the progression of OS in vitro, the miR-338-3p inhibitor was introduced into the OS cells. MiR-338-3p expression was remarkably increased after silencing KLF3-AS1. Even so, this was reversible by miR-338-3p inhibitor transfection (Figure 4A). Through functional analyses, we uncovered that inhibiting miR-338-3p promoted cell viability and increased the number of cells (Figure 4B,C). Meanwhile, transfecting miR-338-3p inhibitor also reduced the protein levels of Bax and cleaved caspase 3 but elevated Bcl-2 protein levels (Figure 4D). The apoptosis rate was reduced after the cells with transfection of miR-338-3p inhibitor (Figure 4E). These findings imply that miR-338-3p inhibition could stimulate proliferation and repress apoptosis of OS cells. Interestingly, we further found that inhibiting miR-338-3p could invert the repressive effects of KLF3-AS1 silencing on proliferation and its stimulating effect on apoptosis in OS cells (Figures 4B-E). These findings further verify that KLF3-AS1 directly targets miR-338-3p to affect OS progression.

### 3.4 | MiR-338-3p targets MEF2C

The binding site of MEF2C and miR-338-3p was determined via starBase (<https://starbase.sysu.edu.cn/>) (Figure 5A) and further confirmed via luciferase reporter assay. The outcomes from the assay evidenced that miR-338-3p mimic was capable of reducing the luciferase activities of the MEF2C-WT vectors in both SW1353 and Saos-2 but had no impact on the luciferase activity of the MEF2C-MUT vector (Figure 5B). Expression analysis revealed the overexpression of MEF2C in OS tumors and cell lines in contrast to the controls (Figure 5C,D). Furthermore, miR-338-3p's expression in OS tumors was discovered to be inversely associated with that of MEF2C (Figure 5E). Our data support that miR-338-3p regulates MEF2C and that MEF2C is a downstream gene of miR-338-3p.

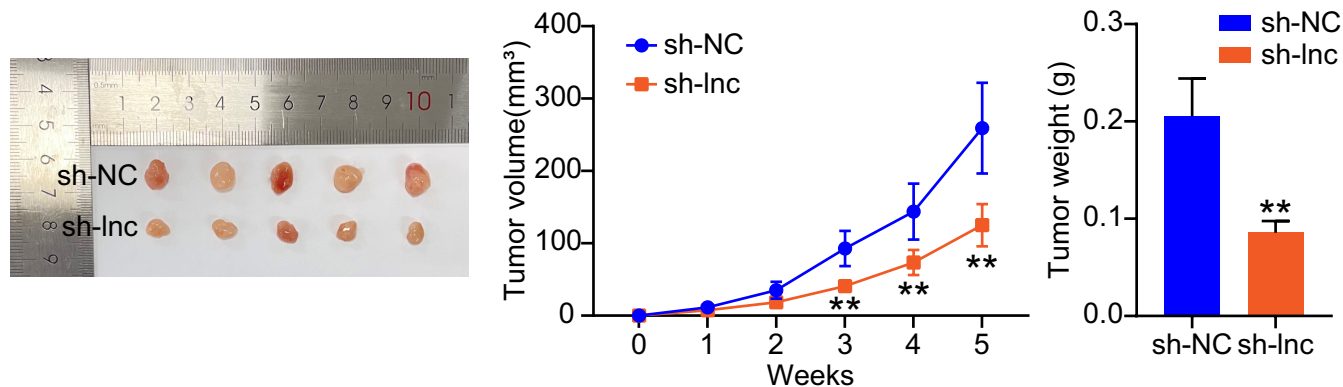
**FIGURE 1** Silencing KLF3-AS1 in OS cells induces apoptosis and represses proliferation in vitro and reduces in vivo tumor growth. (A) Relative KLF3-AS1 expression in tumors and normal tissues was ascertained via qRT-PCR.  $**p < 0.001$  vs. Normal. (B) Relative KLF3-AS1 expression in hFOB 1.19 cells and among the OS cell lines was determined via qRT-PCR.  $**p < 0.001$  vs. hFOB 1.19 cells. (C) Relative KLF3-AS1 levels within the nucleus and cytoplasm of Saos-2 and SW1353 cells was determined through the subcellular localization assay. (D) Relative KLF3-AS1 levels in SW1353 and Saos-2 after transfecting with si-KLF3-AS1 (si-INC) or si-NC were determined via qRT-PCR.  $**p < 0.001$  vs. si-NC. (E) The viability of SW1353 and Saos-2 cells after transfecting with si-NC or si-INC was estimated via CCK-8 experiment.  $**p < 0.001$  vs. si-NC. (F) The number of cell colonies of SW1353 and Saos-2 after transfecting with si-INC or si-NC was assessed by colony formation assay.  $**p < 0.001$  vs. si-NC. (G) Protein levels of Bcl-2, cleaved caspase 3, and Bax in SW1353 and Saos-2 cells after their transfection with si-INC or si-NC assessed via western blotting.  $**p < 0.001$  vs. si-NC. (H) Apoptosis rate in SW1353 and Saos-2 cells after their transfection with si-INC or si-NC was identified by flow cytometry.  $**p < 0.001$  vs. si-NC



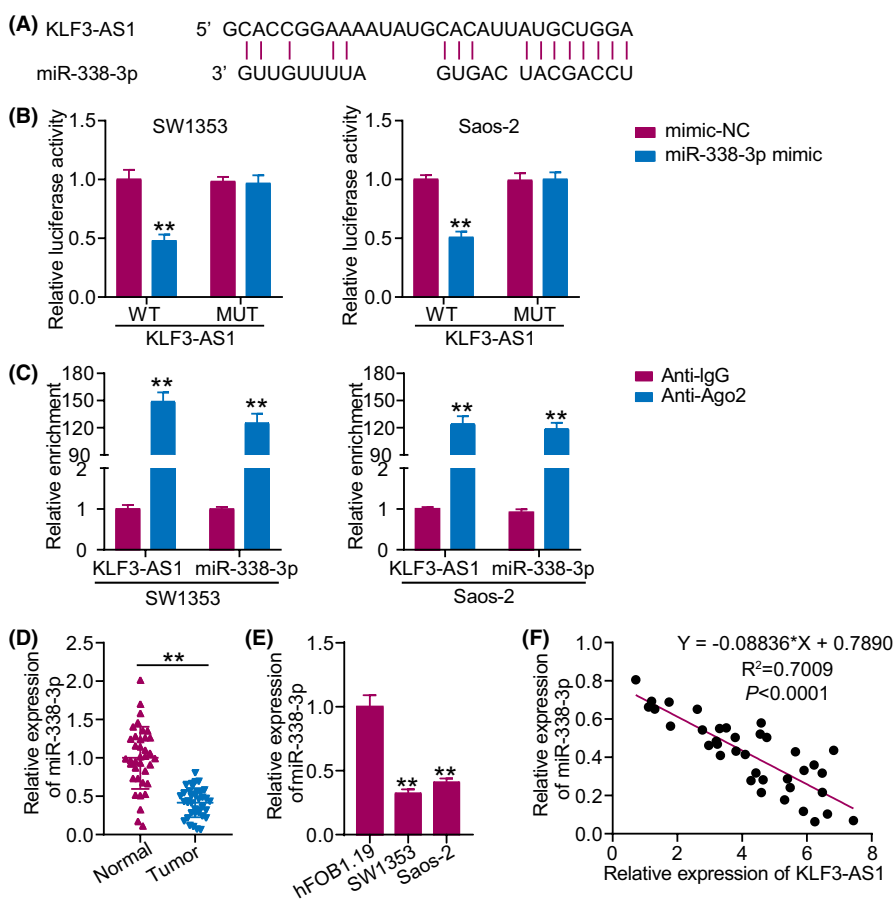
### 3.5 | MiR-338-3p downregulation suppresses OS cell growth in vitro via targeting MEF2C

Si-MEF2C was introduced into OS cells to look into the interaction of MEF2C with miR-338-3p. As displayed in Figure 6A, MEF2C was

significantly repressed after si-MEF2C transfection. Nonetheless, this outcome was reversible via miR-338-3p downregulation. Figure 6B,C shows that, in the si-MEF2C group, the number of colonies and viability of cells were reduced in contrast to that of the si-NC group. Western blotting showed that after the MEF2C

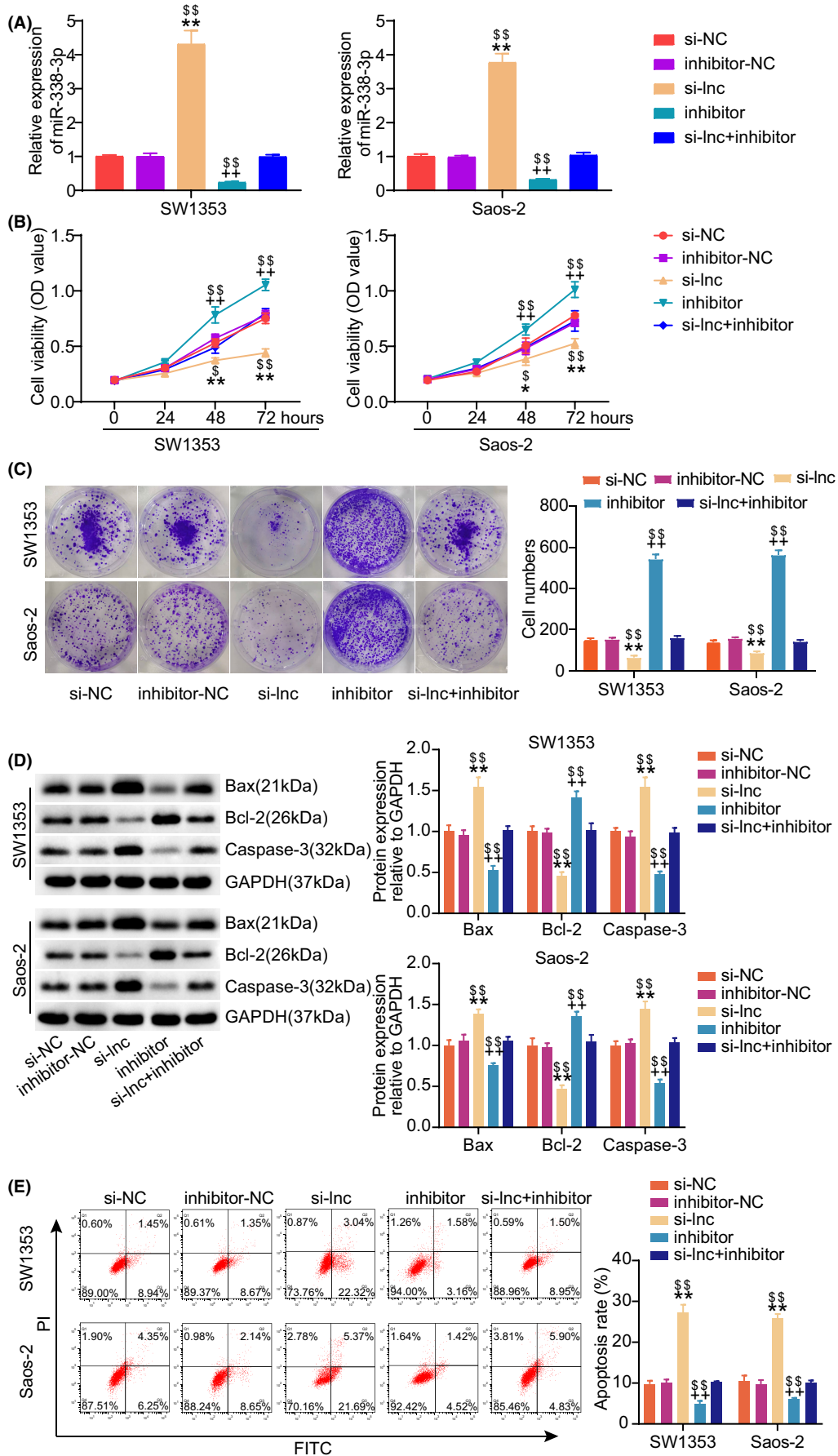


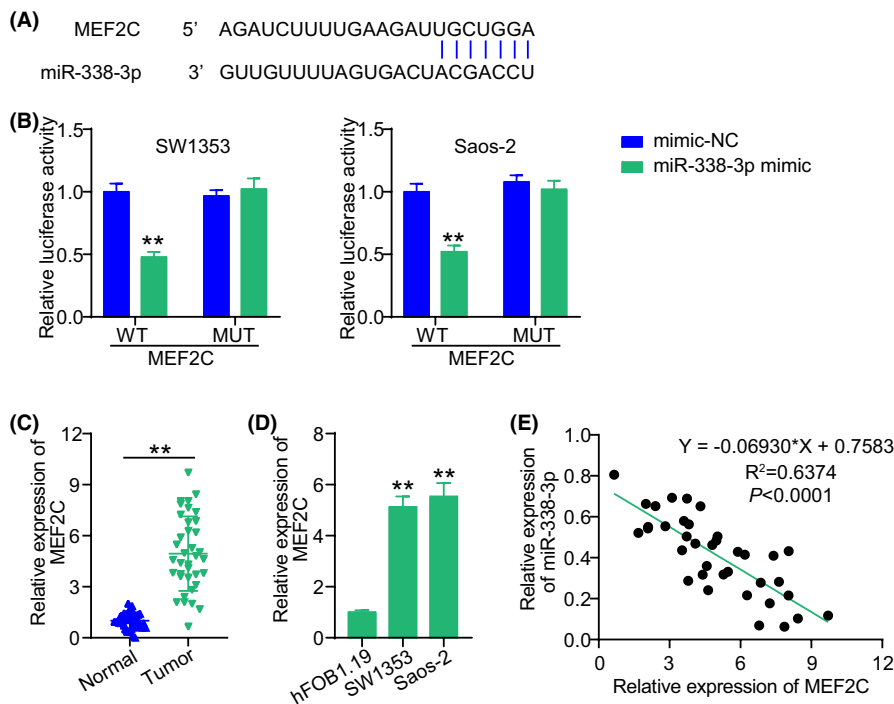
**FIGURE 2** Image, weight, and volume of the tumors from the mouse xenograft model after injecting with Saos-2 cells that were stably transfected with sh-KLF3-AS1 (sh-Lnc) or sh-NC.  $n = 5$  mice per group. \*\* $p < 0.001$  vs. sh-NC



**FIGURE 3** Identification miR-338-3p as a KLF3-AS1 target. (A) The KLF3-AS1 and miR-338-3p binding site predicted using LncBase Predicted v.2. (B) The luciferase activities among Saos-2 and SW1353 cells that had a transfection of pGL3-KLF3-AS1 WT or pGL3-KLF3-AS1 MUT plus either a mimic-NC or miR-338-3p mimic; were measured through a dual luciferase experiment. \*\* $p < 0.001$  vs. mimic-NC. (C) RIP method was used to validate miR-338-3p and KLF3-AS1's interaction. \*\* $p < 0.001$  vs. Anti-IgG. (D) Levels of miR-338-3p in normal tissues and tumors; quantified via qRT-PCR. \*\* $p < 0.001$  vs. Normal. (E) Relative miR-338-3p expression in hFOB 1.19 cells and among the OS cell lines as determined via qRT-PCR. \*\* $p < 0.001$  vs. hFOB 1.19 cells. (F) The correlation between KLF3-AS1 and miR-338-3p in OS tissues was determined via Pearson's correlation coefficient

**FIGURE 4** Inhibiting miR-338-3p attenuates the suppressive influence of KLF3-AS1 silencing on OS cell growth. (A) Relative miR-338-3p expression in SW1353 and Saos-2 cells after being transfected with si-Lnc, si-NC, miR-338-3p inhibitor (inhibitor), inhibitor-NC, or si-Lnc + inhibitor was determined via qRT-PCR. (B) Viability of Saos-2 and SW1353 cells after their transfection with si-Lnc, si-NC, inhibitor, inhibitor-NC, or si-Lnc + inhibitor was measured via CCK-8 experiment. (C) Number of colonies of SW1353 and Saos-2 cells transfected with si-Lnc, si-NC, inhibitor, inhibitor-NC, or si-Lnc + inhibitor were evaluated by means of a colony formation assay. (D) Bcl-2, cleaved caspase 3, and Bax protein levels in SW1353 and Saos-2 cells after their transfection with si-Lnc, si-NC, inhibitor, inhibitor-NC, or si-Lnc + inhibitor were determined by Western blotting. (E) Apoptosis rate in SW1353 and Saos-2 cells after their transfection with si-Lnc, si-NC, inhibitor, inhibitor-NC, or si-Lnc + inhibitor was identified by flow cytometry. \*\* $p < 0.05$  and \* $p < 0.001$  vs. si-NC; ++ $p < 0.001$  vs. inhibitor-NC; § $p < 0.05$  and §§ $p < 0.001$  vs. si-Lnc + inhibitor





**FIGURE 5** MiR-338-3p targets MEF2C. (A) The miR-338-3p and MEF2C binding site predicted using starBase. (B) The luciferase activity in SW1353 and Saos-2 cells that had a combined transfection of pGL3-MEF2C WT or pGL3-MEF2C MUT plus either a miR-338-3p mimic or a mimic-NC was measured via dual luciferase reporter experiment. \*\* $p < 0.001$  vs. mimic-NC. (C) Relative MEF2C levels in tumors and normal tissues as quantified via qRT-PCR. \*\* $p < 0.001$  vs. Normal. (D) Relative MEF2C levels in hFOB 1.19 cells and among the OS cell lines as determined via qRT-PCR. \*\* $p < 0.001$  vs. hFOB 1.19 cells. (E) Association of MEF2C and miR-338-3p expressions in OS tissues as indicated by Pearson's correlation coefficient

deletion in both SW1353 and Saos-2 cells, the protein levels of Bax and cleaved caspase 3 increased, and that of Bcl-2 decreased (Figure 6D). Flow cytometry identified that si-MEF2C led to the increase in apoptosis rate (Figure 6E). Furthermore, we learned that the deletion of MEF2C in OS cells partially eliminated miR-338-3p inhibitor's stimulating effect on proliferation and suppressive effect on apoptosis (Figure 6B–E). Our findings demonstrate that miR-338-3p's interaction with MEF2C can regulate tumorigenesis in OS.

## 4 | DISCUSSION

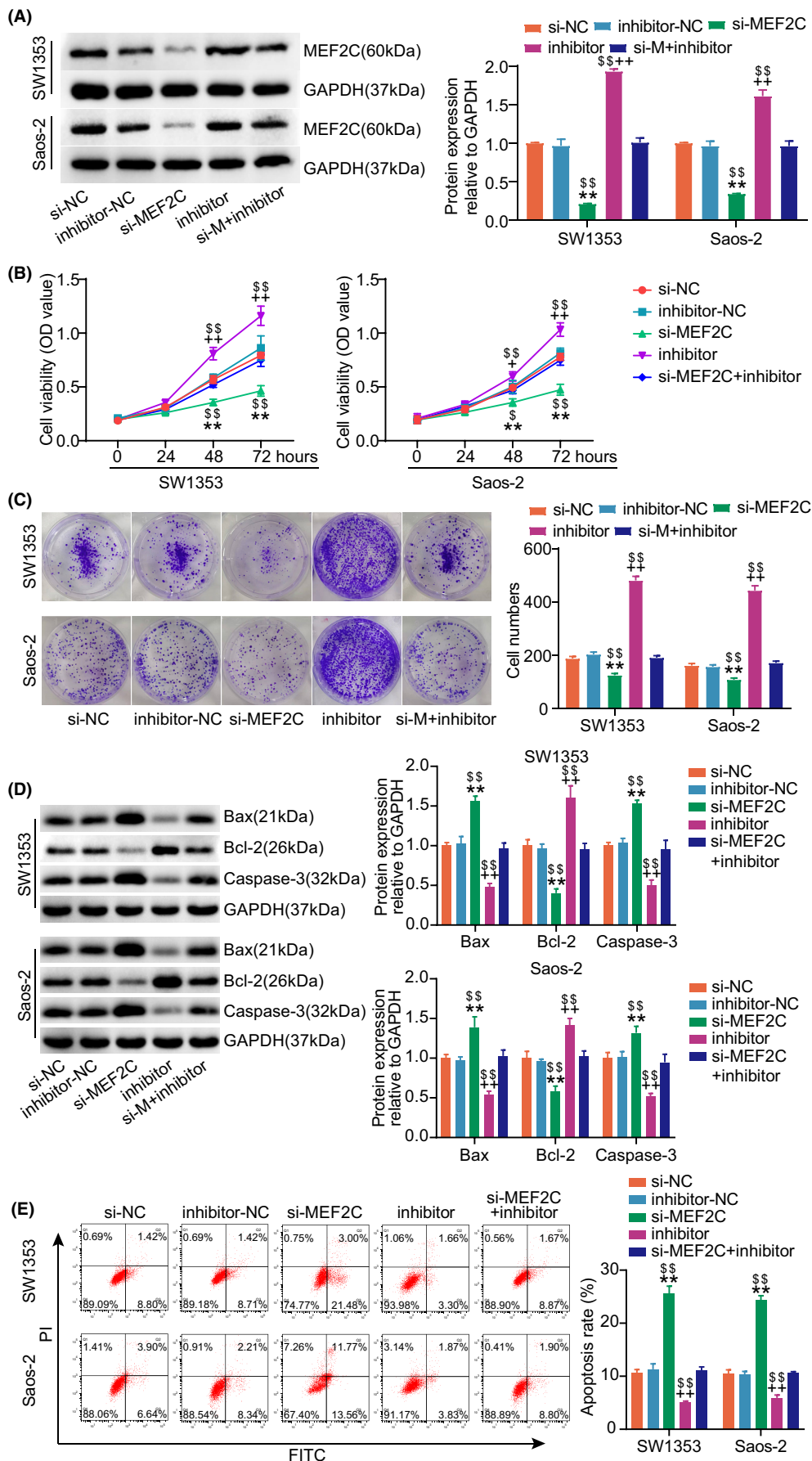
OS is one of the most prevalent bone malignancies among juveniles.<sup>16</sup> LncRNA KLF3-AS1 is abnormally expressed and serves as an inhibitor in numerous human cancers, including ESCC<sup>8</sup> and GC.<sup>9</sup> In the current study, we demonstrated that, contrary to previous results, KLF3-AS1 was overexpressed in OS cells and tumors. Therefore, we speculated that KLF3-AS1 may act as an oncogenic factor in OS. Additionally, the action mechanism of KLF3-AS1 was also uncovered, suggesting that silencing of KLF3-AS1 could repress OS cell growth via miR-338-3p/MEF2C.

Accumulating evidence have confirmed that the overexpressed lncRNAs can function as vital regulators in OS tumorigenesis.<sup>17,18</sup> For example, FLVCR1-AS1 repression can limit the proliferative and invasive capacities of OS cells.<sup>17</sup> PCAT6 interference decreases cell viability and metastasis in OS.<sup>18</sup> However, in both ESCC and GC, the metastasis and proliferation of cancer cells may significantly be suppressed by KLF3-AS1 overexpression.<sup>8,9</sup> Our results were opposite to these previous works as they indicated that knocking-down KLF3-AS1 did not only repress the in vitro cell proliferation, but it also limited in vivo tumor xenograft growth. Interestingly, previous studies have uncovered the inhibitory influence of KLF3-AS1 silencing on cell proliferation in osteoarthritis, a type of degenerative joint disease that manifest as chondrocytes apoptosis.<sup>19,20</sup> These data further imply that KLF3-AS1 silencing may attenuate the development of OS by controlling cancer cell proliferation.

Emerging studies have documented miR-338-3p's anti-tumor role in a variety cancer, such as cervical,<sup>21</sup> breast,<sup>22</sup> and colorectal<sup>23</sup> cancers. On those previous reports, miR-338-3p downregulation has been observed during cancer progression. Herein, a reduction in miR-338-3p was observed in OS cells and tissues. Similarly, numerous studies have discovered that miR-338-3p is downregulated

**FIGURE 6** Downregulating miR-338-3p inhibits the growth of OS cells in vitro via targeting MEF2C. (A) MEF2C protein levels in Saos-2 and SW1353 cells after transfecting with si-MEF2C, si-NC, inhibitor, inhibitor-NC, or si-MEF2C + inhibitor were determined via western blotting. (B) Viability of Saos-2 and SW1353 cells after transfecting with si-MEF2C, si-NC, inhibitor, inhibitor-NC, or si-MEF2C + inhibitor was measured via the CCK-8 experiment. (C) The cell colonies of SW1353 and Saos-2 cells transfected with si-MEF2C, si-NC, inhibitor, inhibitor-NC or si-MEF2C + inhibitor were evaluated via the colony formation assay. (D) Bax, cleaved caspase 3, and Bcl-2 protein levels in SW1353 and Saos-2 cells after their transfection with si-MEF2C, si-NC, inhibitor, inhibitor-NC, or si-MEF2C + inhibitor were determined via Western blotting. (E) Apoptosis rate in in SW1353 and Saos-2 cells after their transfection with si-MEF2C, si-NC, inhibitor, inhibitor-NC, or si-MEF2C + inhibitor was identified by flow cytometry. \*\* $p < 0.001$  vs. si-NC; + $p < 0.05$  and ++ $p < 0.001$  vs. inhibitor-NC; \$ $p < 0.05$  and \$\$ $p < 0.001$  vs. si-MEF2C + inhibitor





in OS tissues and that it inhibits the development of OS.<sup>14,24,25</sup> Our findings evinced that miR-338-3p may be a beneficial miRNA in OS tumorigenesis. Additionally, miR-338-3p has been confirmed to be regulated by lncRNAs, such as lncRNA CASC15<sup>15</sup> and lncRNA LINC00707,<sup>26</sup> to affect OS progression. Interestingly, miR-338-3p was also determined as KLF3-AS1's downstream target in the current study. Hence, we speculated that KLF3-AS1 may also serve as miR-338-3p's competing endogenous RNA to exert functions in OS tumorigenesis. As expected, we further found that miR-338-3p downregulation could invert KLF3-AS1 silencing's inhibitory effect on OS cell proliferation and stimulating effect on apoptosis. Therefore, we conclude that silencing of KLF3-AS1 represses OS cell growth via miR-338-3p regulation.

MEF2C, a member of MEF2 family, is generally characterized as a transcription factor associated with myogenic differentiation.<sup>27</sup> Recently, the oncogenic role of MEF2C in cancers has been drawing an increasing amount of attention. Agatheeswaran et al. found that MEF2C is overexpressed in myeloid leukemia.<sup>28</sup> Zhang et al. observed an increased MEF2C in liver cancer, whereas its knock-down inhibits cell growth and protects cancer cells against chemoresistance.<sup>29</sup> Ni et al. demonstrated that silencing of MEF2C not only represses endometrial carcinoma metastasis and proliferation of cells in vitro but also hampers tumor xenograft growth in vivo.<sup>30</sup> Herein, we also observed that MEF2C was overexpressed in OS cells and tumors, suggesting that MEF2C may also be an underlying oncoprotein in OS progression. Furthermore, MEF2C was verified to be miR-338-3p's direct target gene. Therefore, we further speculated MEF2C may evoke a miR-338-3p-mediated occurrence and development of OS. Unsurprisingly, loss of MEF2C partially diminished the stimulating effect of miR-338-3p downregulation on the proliferation of OS cells. Collectively, we believe that silencing of KLF3-AS1 limited the proliferation of cells in OS via the miR-338-3p/MEF2C axis.

Surely, there were limitations presented in this research. First, the clinical sample size for this investigation was small, so a relatively larger sample size should be considered in the future. Second, the interaction of KLF3-AS1 with the miR-338-3p/MEF2C axis was not demonstrated in animal models. Third, the detailed mechanisms of KLF3-AS1/miR-338-3p/MEF2C axis-related pathways associated with OS progression need further exploration.

## 5 | CONCLUSION

To sum up, KLF3-AS1 overexpression has been observed in OS cell lines and tumors. Loss of KLF3-AS1 attenuates OS progression by inhibiting cell growth through miR-338-3p/MEF2C axis. Our findings have uncovered new mechanism under the OS pathogenesis and contribute a prospective target for the treatment of OS.

## AUTHOR CONTRIBUTIONS

CFC and LL performed the experiments and data analysis. LL and CFC conceived and designed the study. LL and CFC acquired the

data. LL performed the analysis and interpretation of data. All authors read and approved this article.

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Not applicable.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## DATA AVAILABILITY STATEMENT

All data that have been generated or analyzed during this study are included in this article.

## ETHICAL APPROVAL

The present study was approved by the Ethics Committee of Huangshi Central Hospital, Affiliated Hospital of Hubei Polytechnic University, Edong Healthcare Group (Huangshi, China). The processing of clinical tissue samples had been in strict compliance with the ethical standards of the Declaration of Helsinki. All patients signed a written informed consent. This animal experiment had been executed with strict observance of the ARRIVE guidelines. This was authorized by the Ethics Committee of Huangshi Central Hospital, Affiliated Hospital of Hubei Polytechnic University, Edong Healthcare Group.

## CONSENT TO PARTICIPATE

All patients signed a written informed consent.

## CONSENT FOR PUBLICATION

Consent for publication was acquired from all participants.

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