Received: 19 October 2022 | Revised: 27 November 2022 | Accepted: 23 December 2022

DOI: 10.1002/jcla.24828

RESEARCH ARTICLE

WILEY

Silencing circUSP48 suppresses osteosarcoma progression by regulating the miR-335/ smad nuclear interacting protein 1 pathway

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Abstract

Background: Circular RNAs (circRNAs) can have a critical function in the multi-processes of osteosarcoma (OS). Nevertheless, whether circUSP48 is involved in OS progression remains unclear.

Methods: In the current work, the expression of circUSP48, miR-335 and SNIP1 in OS cell lines and tissues were evaluated using qRT-PCR. Then, Sanger sequencing, RNase R treatment and FISH assay were performed for circUSP48 validation. Furthermore, the function and potential mechanisms of circUSP48 in OS were investigated by performing loss-of-function experiments.

Results: Silencing circUSP48 could suppress proliferation, invasion as well as migration of OS cells in vitro, also inhibiting the growth of tumor in vivo. Importantly, circUSP48 promoted OS malignancy by sponging miR-335 to upregulate SNIP1.

Conclusion: Overall, these findings suggested that circUSP48 acted as an oncogene in OS, which might become a new target for OS therapy.

KEYWORDS
circUSP48, miR-335, osteosarcoma, progression, SNIP1

1 | INTRODUCTION

With the potential for high metastasis and rapid progression, osteosarcoma (OS) is the most common bone cancer in adolescents and adults.[1] In addition, it has been pointed out that the 5-year survival rate for localized OS reaches approximately 70%, while the rate for the metastatic OS is only 15%–30%.[2] At present, almost 80% of OS tend to metastasize and spread to the lungs, and the prognosis of OS is not satisfactory.[3] Unfortunately, due to the complex karyotype and highly unstable genome of OS, treatment for OS remains a challenge for surgeons.[4] Thus, much remains to be developed in the treatment of OS.

Circular RNAs (circRNAs) have attracted the attention of researchers in recent years. They are formed during backsplicing with a covalently closed-loop structure.[5] According to increasing evidence, circRNAs can affect various biological processes and are also associated with tumor promotion and suppression.[6] For example, Circ-AASDH can promote the progression of lung adenocarcinoma through sponging miR-140-3p with the aim of upregulating E2F7 expression.[7] Moreover, based on recent studies, it can also be

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confirmed that various circRNAs are involved in the complex processes of OS.[8–11]

CircRNA can bind to microRNA to down-regulate the expression levels of downstream target genes.[11] CircRNA, microRNA, and mRNA can form an endogenous competitive network that can regulate the progression of tumors, including osteosarcoma. For example, circOMA1 could sponge miR-1270 to upregulate the expression of c-Myc, thus promoting the development of OS.[12] However, it remains unclear whether hsa_circ_0000028 (circUSP48) is involved in OS progression.

We hypothesized that circUSP48 could regulate OS progression through the endogenous competitive network. Then, the current work revealed that circUSP48 was strongly denoted in OS cell lines and tissues, and up-regulation of circUSP48 was related to OS progression. Furthermore, circUSP48 could sponge miR-335 to upregulate SNIP1 expression in OS. Therefore, our results demonstrated that circUSP48 could play the role of the new therapeutic target for patients with OS.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

Twenty-four paired OS tissues and adjacent normal tissues came from Renmin Hospital of Wuhan University. The tissues were frozen in liquid nitrogen and subsequently cryopreserved at −80°C. Furthermore, the approval of this study was obtained from the Ethics Committee of our hospital.

2.2 | Cell culture and transfection

OS cell lines (MG-63, 143B, U2OS, and Saos-2) and human osteoblasts (hFOB1.19) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with the addition of 1% penicillin/streptomycin. Negative control shRNA (sh-NC) and shRNA targeting circUSP48 (sh-circUSP48#1, sh-circUSP48#2) came from Servicebio. MiR-335 mimic, inhibitor, and corresponding negative control were obtained from GenePharma. Additionally, Lipofectamine® 2000 (Cell Signaling Technology, Inc.) was adopted for cell transfection.[7]

2.3 | Quantitative real-time PCR (qRT-PCR)

Total RNA extraction was performed from clinical specimens and cells using the RNeasy Plus Mini Kit (Life Technology). TaqMan Reverse Transcription Reagents were used to synthesize cDNA. The SYBR Green PCR Kit (Roche) was adopted to detect circRNA, miRNA, and mRNA expressions. In this study, GAPDH and U6 played the role of internal controls. Using $2^{-\Delta\Delta Ct}$ methods, we calculated the relative expression of the targeting factors.[8] The primers are listed in Supplementary Table S1.

2.4 | RNase R digestion

Total RNA was incubated with 3 U/mg of RNase R for 20 min. Subsequently, qRT-PCR was used to detect the expression of linear USP48 and circUSP48.[9]

2.5 | Sanger sequencing

The circUSP48 backspliced site was verified by divergent primers. Sanger sequencing was performed by Tsingke (Wuhan, China).[8]

2.6 | Cell proliferation and colony formation assay

To identify cell activity, cell counting kit 8 (CCK-8) was used. The cells were then seeded in 48-well plates and incubated overnight. The CCK-8 solution was then supplemented into each well. After that, a spectrophotometer was used to examine the OD value at 450 nm. Subsequently, the 5-ethynyl-2 ′-deoxyuridine (EdU) assay was carried out with the aim of evaluating cell proliferation based on a Click-iT EdU-488 kit (Servicebio, Wuhan, China). In terms of colony formation, cells were grown in a 6-well plate for 14 days to form colonies. Subsequently, we used 0.1% crystal violet for staining the cells.[11]

2.7 | Wound healing assay

The cells were seeded in 6-well plates and incubated overnight. A 200-μl pipette tip was used to vertically draw a straight line in the center of each well. Furthermore, the degree of wound healing was recorded at 0 and 48 h.[12]

2.8 | Transwell assays

The cells were seeded in the upper chamber with Matrigel, and the lower chamber was loaded with culture medium, including 20% FBS. The Transwell chamber was then placed in an incubator for 48 h. Finally, the invaded cells were stained with crystal violet and photographed.[12]

2.9 | Luciferase reporter assay

We designed fragments of circUSP48 3'-UTR that include mutant (Mut) and wild-type (Wt) miR-335 binding sequences. After that, luciferase reporter plasmids were synthesized using the psiCHECK vector (Invitrogen, USA). The SNIP1 plasmids were prepared using the same methods.[13]

2.10 | RNA immunoprecipitation assay (RIP)

This work performed the RIP assay with the use of the RNA-binding protein immunoprecipitation kit (Millipore, China). qRT-PCR was carried out to evaluate the expression of circUSP48 and miR-335.[14]

2.11 | Fluorescence in situ hybridization (FISH)

CircUSP48 probes (AAGGTTAGCTTAGGGAATCTTCA) and miR-335 probes (AAGACTCACTCCAACCGAACAA) were synthesized from Servicebio (Wuhan, China). According to the manufacturer's instructions, hybridization was carried out with miR-335 and circUSP48 probes overnight at 4°C. The images were obtained by a confocal microscope.[15]

2.12 | Western blot analysis

We extracted total protein with RIPA lysis buffer and quantified protein concentrations using the bicinchoninic acid assay. Proteins were resolved on an SDS-PAGE gel, and the gel was subsequently transferred to the PVDF membrane. The membrane was then nurtured with primary antibodies at 4 °C throughout the night (anti-SNIP1, 1:500, Servicebio, China; anti-GAPDH, 1:2000, Proteintech, China, secondary antibodies, 1:1000, Proteintech). In the end, the membrane was raised with second antibodies and photographed.[16]

2.13 | In vivo experiments

The animal experiments were carried out in accordance with the ARRIVE guidelines. BALB/c nude mice were subcutaneously injected with 143B cells transfected with sh-NC or sh-circUSP48, respectively. After injection, we monitored tumor volumes every 5 days. 4 weeks later, the tumors were weighed and stored for qRT-PCR and immunohistochemical assay.[17]

2.14 | Bioinformatics analysis

Differently expressed circRNAs of OS were screened using the Gene Expression Omnibus (GEO) database. Starbase, CircInteractome, and TargetScan predicted putative binding sites between circUSP48, miR-335, and SNIP1.

2.15 | Statistical analysis

SPSS 13.0 software was used to perform statistical analysis. The differences between the groups were checked according to Student's t-test for two groups and ANOVA for two or more groups. A p-Value <0.05 was shown to be of statistical significance.

3 | RESULTS

3.1 | Identification and characterization of circUSP48 in OS tissues and cells

We analyzed the GSE96964 microarray to detect circRNAs differently expressed in OS. The human osteoblast hFOB1.19 and seven OS cell lines (MG63, 143B, ZOS, ZOSM, U2OS, U2OS/MTX300, HOS) and were included in the GSE96964 dataset. CircUSP48 expression was strongly expressed in OS cell lines relative to human osteoblasts (Figure 1A). According to GSE96964 dataset, the top three upregulated circRNAs (hsa_circ_0092304, hsa_circ_0000028 and hsa_circ_0072932) were screened in OS cells than in human osteoblasts. Then, qRT-PCR results showed that hsa_circ_0000028 (circUSP48) was the most upregulated circRNA in OS cells compared to hFOB cell (Figure 1B). Thus, circUSP48 was selected for subsequent study. CircUSP48 comes from 13–15 exons of smad nuclear interacting protein 1 (SNIP1) on chromosome 1. Furthermore, Sanger sequencing identified the back-spliced region sequence of circUSP48 (Figure 1C). Subsequently, agarose gel electrophoresis revealed that divergent primers amplified circUSP48 in cDNA but not gDNA (Figure 1D). After treatment with RNase R, only USP48 mRNA was digested, not circUSP48 ($p = 0.032$, Figure 1E). qRT-PCR results showed that circUSP48 presented significant up-regulation in OS tissues and cell lines ($p = 0.0016$, Figure 1F; $p = 0.0021$, Figure 1G). The FISH assay revealed that circUSP48 was observed primarily in the cytoplasm (Figure 1H). Altogether, the results obtained suggested that circUSP48 could significantly affect OS progression.

3.2 | Silencing circUSP48 expression suppressed OS progression

Two shRNAs (sh-circUSP48#1, sh-circUSP48#2) targeting circUSP48 were transfected into OS cells to evaluate the cellular function of circUSP48. The expression of circUSP48 was downregulated after shRNA transfection in 143B and MG63 cells ($p = 0.026$, Figure 2A). While the expression of linear USP48 was unchanged in both cells ($p = 0.038$, Figure 2B). Since sh-circUSP48#1 exhibited better inhibition efficiency, it was selected to carry out future experiments. The CCK-8 assays revealed decreased cell viability in 143B and MG63 cells after the knockdown of circUSP48 ($p = 0.019$, Figure 2C). Similarly, Edu and colony formation assays proved that silencing circUSP48 hindered the proliferation ability of 143B and MG63 cells ($p = 0.037$, Figure 2D; $p = 0.026$, Figure 2E). Additionally, wound healing and Transwell assays were performed to assess the cell migration and invasion. The silencing of circUSP48 suppressed the migration and invasion ability of OS cells ($p = 0.033$, Figure 2F; $p = 0.025$, Figure 2G).

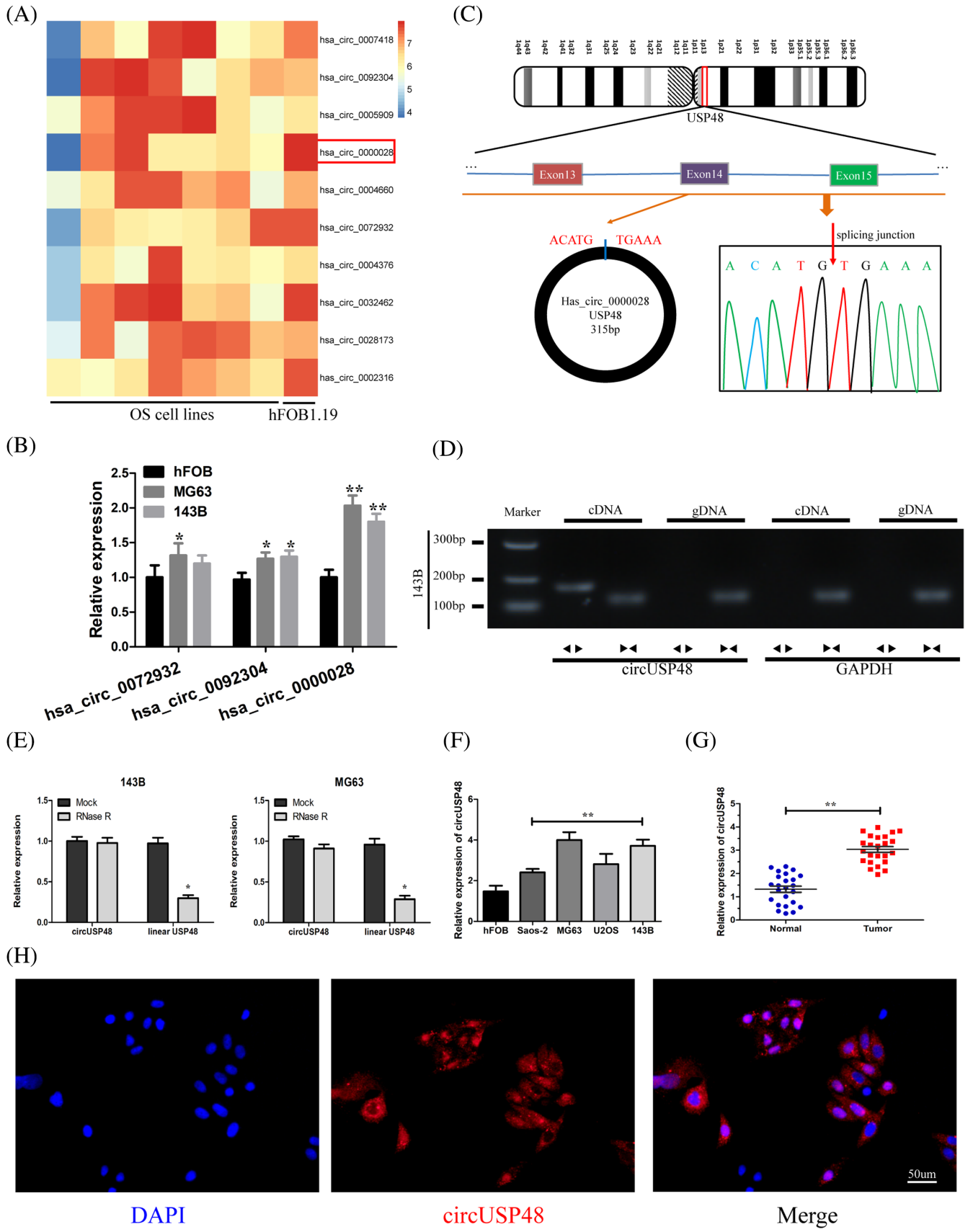

FIGURE 1 CircUSP48 Identification and characterization within OS tissues and cells. (A) Heatmap of differently expressed circRNAs in OS. (B) The expressions of top-three upregulated circRNAs. (C) Sanger sequencing identified the back-spliced region of circUSP48. (D) Gel electrophoresis confirmed the existence of circUSP48. (E) CircUSP48 levels were identified within OS cells after being subjected to RNase R treatment. (F) CircUSP48 levels within OS tissues. (G) CircUSP48 within OS cells. (H) FISH assay using the circUSP48 probe within 143B cell. $^{*}p < 0.05$, $^{**}p < 0.01$.

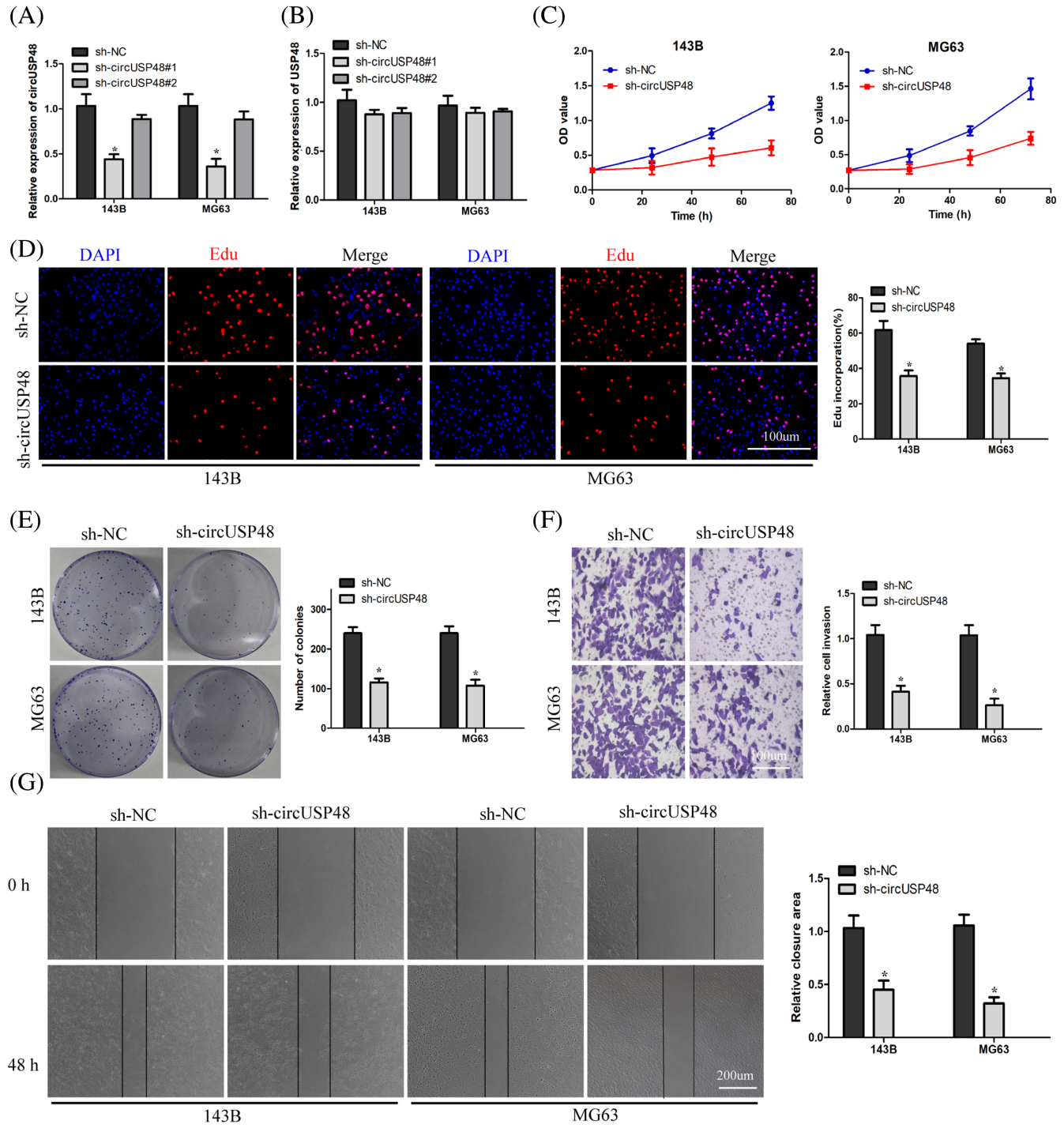

FIGURE 2 CircUSP48 knockdown suppressed OS development in vitro (A, B) QRT-PCR detected linear USP48 and circUSP48 levels in 143B and MG63 cells after being subjected to sh-NC or sh-circUSP48 transfection. (C) The evaluation of cell proliferation using CCK8. (D) Edu assays and (E) colony formation decided the proliferation capability. (F) Transwell assay determined cell invasion post-transfection. (G) The wound healing assay determined cell migration. $^{*}p<0.05$, $^{**}p<0.01$.

The results demonstrated that silencing circUSP48 suppressed OS progression in vitro.

3.3 | CircUSP48 sponged miR-335 in OS cells

We selected three miRNAs (miR-620, miR-335, and miR-140-3p) that potentially interacted with circUSP48 by searching the databases circBank and CircInteractome (Figure 3A). To explore the association between three miRNAs and circUSP48, qRT-PCR was adopted to detect the expression of the above miRNAs (Figure 3B). Since miR-335 was upregulated in 143B and MG63 cells, it was chosen for further experiments. Figure 3C presents the binding sites between circUSP48 and miR-335. We performed luciferase reporter assays to determine whether circUSP48 could bind to miR-335. The data proved that the luciferase activity

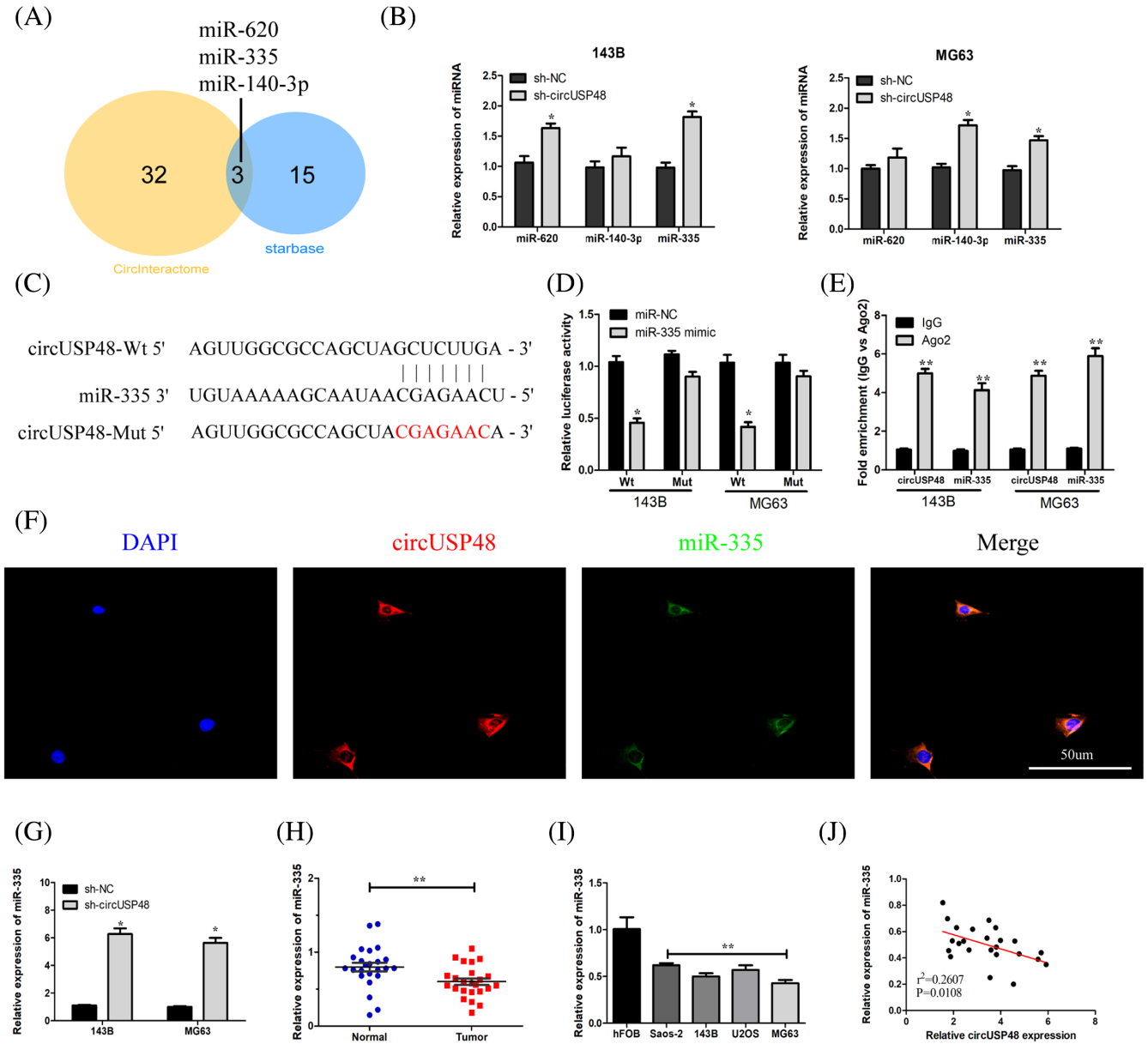

FIGURE 3 CircUSP48 serves as a sponge for miR-335 within OS cells (A) Intersection of miRNAs selected through CircInteractome and Starbase. (B) Three possible miRNAs levels within 143B and MG63 cells transfected with sh-NC or sh-circUSP48. (C) Binding sites in miR-335 for circUSP48. (D) After co-transfection, luciferase activity was calculated in 143B and MG63 cells. (E) The RIP assay confirmed the binding of circUSP48 to miR-335. (F) FISH experiment determined the location of circUSP48 and miR-335. (G) After the knockdown of circUSP48, miR-335 expression was examined in 143B and MG63 cells. (H) MiR-335 levels within OS tissue samples. (I) MiR-335 levels within OS cell lines. (J) Relationship between miR-335 and circUSP48. $^{*}p < 0.05$, $^{**}p < 0.01$.

in the circUSP48-Wt group was lowered by the miR-335 mimic (Figure 3D). RIP analysis revealed the enrichment of miR-335 and circUSP48 in Ago2-containing beads (Figure 3E). Furthermore, the FISH assay revealed that circUSP48 and miR-335 were primarily located in the cytoplasm (Figure 3F). The qRT-PCR results showed that silencing circUSP48 upregulated the expression of miR-335 in the OS cells ($p = 0.0261$, Figure 3G). Furthermore, miR-335 was negatively regulated in the OS tissues and cell lines ($p = 0.0037$, Figure 3H; $p = 0.00152$, Figure 3I), showing a negative correlation with circUSP48 ($p = 0.0108$, Figure 3J).

3.4 | CircUSP48 upregulated SNIP1 expression by sponging miR-335

Through bioinformatics analysis, we found four miR-335 overlapping targets, which are IRF2BP2, OLA1, SNIP1, and YWHAQ (Figure 4A). After being transfected with miR-335 mimic, SNIP1 expression was significantly reduced in 143B and MG63 cells (Figure 4B). SNIP1 had potential binding sites with miR-335 (Figure 4C). Luciferase reporter assays showed that miR-335 mimic decreased luciferase activity in the SNIP1-Wt group but not in the SNIP1-Mut group (Figure 4D).

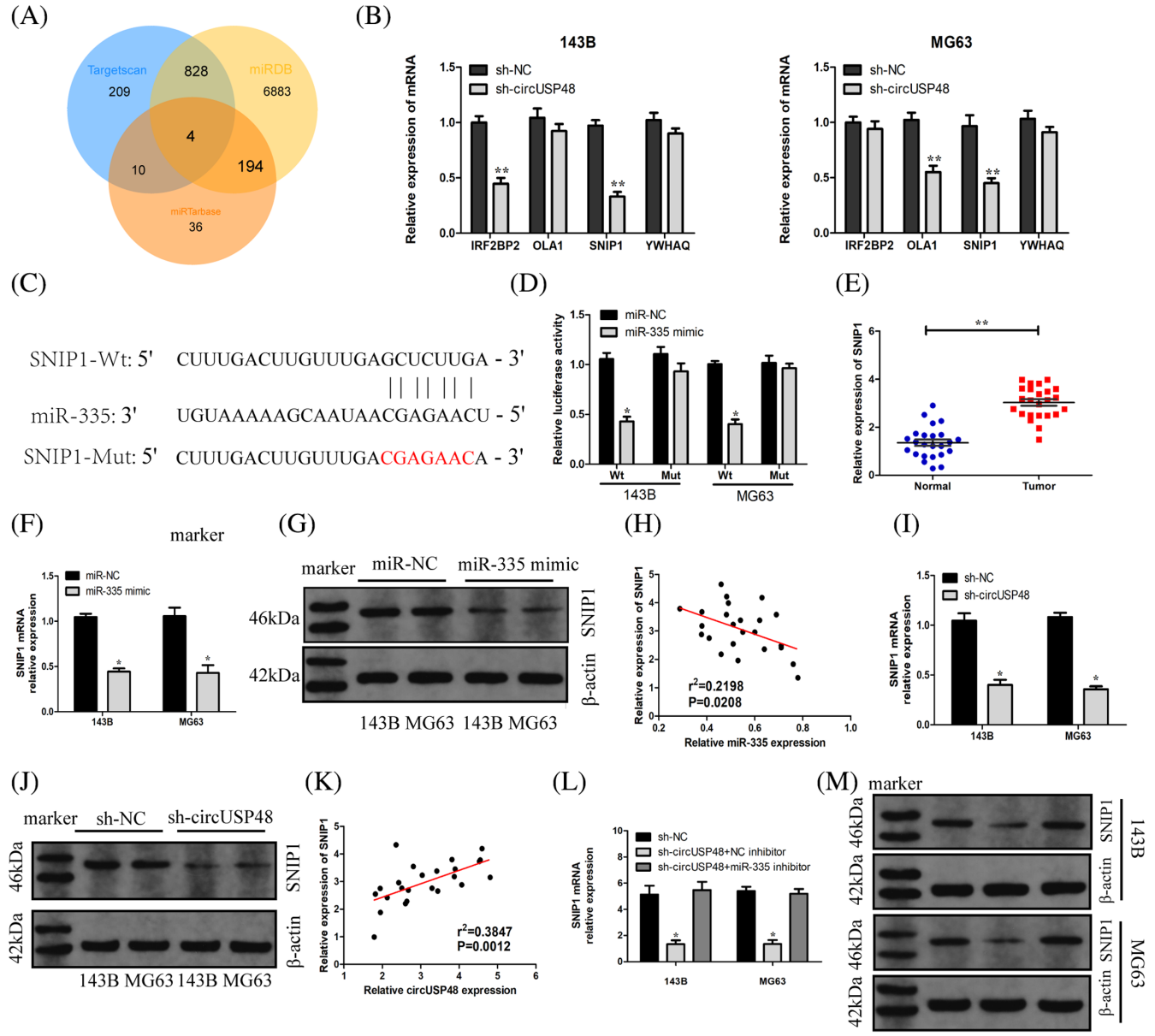

FIGURE 4 CircUSP48 upregulated SNIP1 expression through the sponge of miR-335. (A) Intersection among target genes of miR-335 determined through miRDB, Targetscan and miRTarBase. (B) After the transfection with miR-NC or miR-335 mimic, candidate target levels were detected within 143B and MG63 cells. (C) Binding sites in SNIP1 for miR-335. (D) Luciferase reporter assay conducted to verify binding sites in SNIP1 for miR-335. (E) SNIP1 expression within OS tissues. (F, G) The impact of miR-335 on SNIP1 protein and mRNA expression within 143B and MG63 cells. (H) Relationship of SNIP1 with miR-335. (I, J) The impact of circUSP48 on SNIP1 protein and mRNA expressions within OS cells. (K) Correlation of SNIP1 with circUSP48. (L, M) SNIP1 protein and mRNA expression within OS cells subjected to transfection using sh-circUSP48 and the NC inhibitor or miR-335 inhibitor. $^{*}p < 0.05$, $^{**}p < 0.01$.

The qRT-PCR findings revealed that the expression of SNIP1 was upregulated in OS tissues ($p = 0.0062$, Figure 4E). Additionally, miR-335 mimics reduced levels of SNIP1 mRNA and protein (Figure 4F, G). Importantly, SNIP1 expression showed a negative correlation with miR-335 ($p = 0.0208$, Figure 4H).

In order to investigate whether circUSP48 could control SNIP1, SNIP1 expression was detected after the depletion of circUSP48. The results showed that the suppression of circUSP48 caused a reduction in SNIP1 mRNA and protein levels ($p = 0.0382$, Figure 4I, J). Additionally, SNIP1 expression was negatively correlated with circUSP48 ($p = 0.0012$, Figure 4K). Importantly, rescue experiments showed that SNIP1 mRNA and protein levels improved significantly after cotransfected with the miR-335 inhibitor and sh-circUSP48 ($p = 0.0291$, Figure 4L, M). Together, these data demonstrated that circUSP48 generated the function of a sponge for miR-335 with the purpose of upregulating SNIP1.

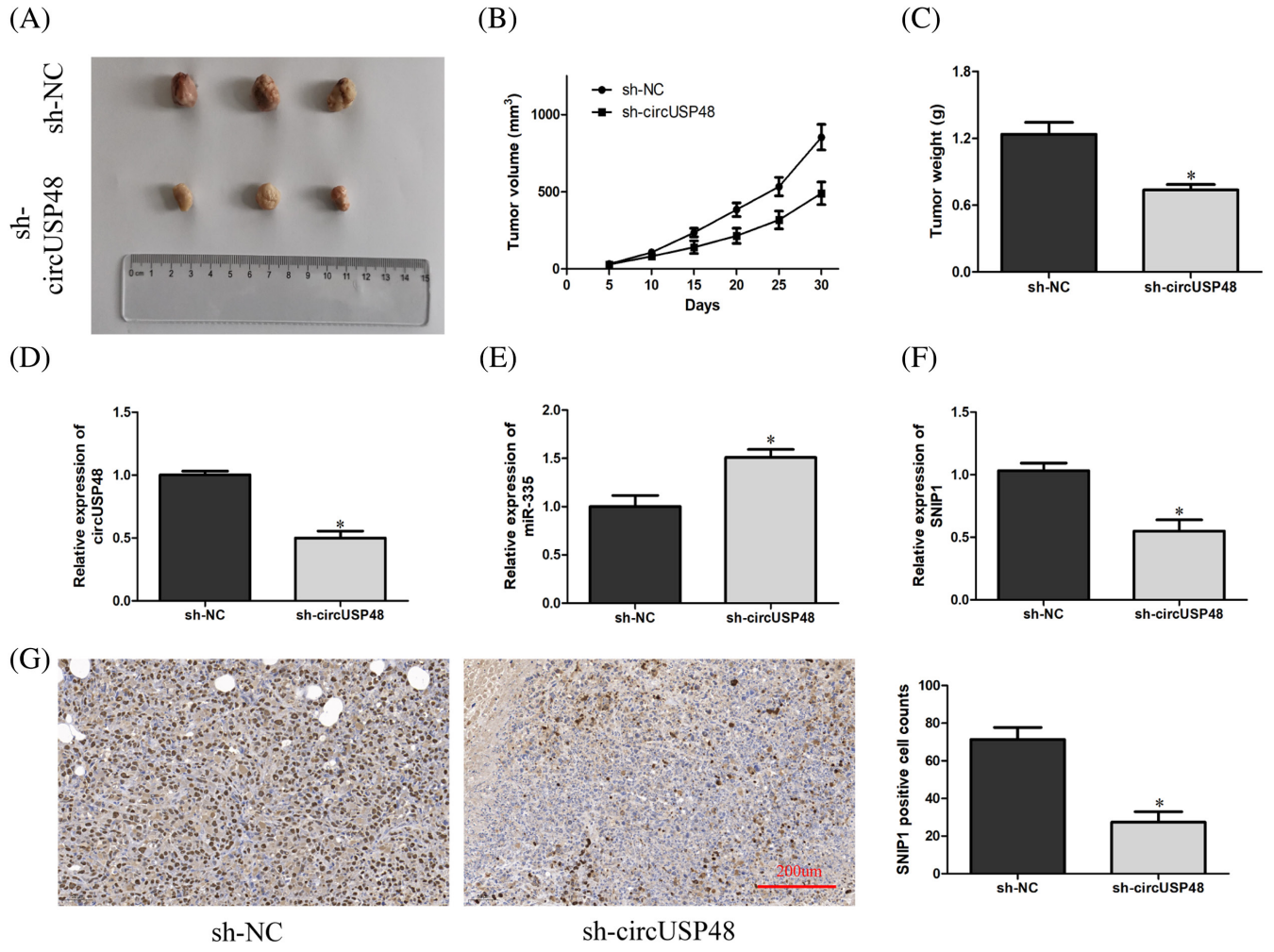

FIGURE 5 CircUSP48 knockdown suppressed tumor development in vivo (A) Representative images showing cancer masses of sh-circUSP48 and sh-NC groups. (B, C) Tumor volume and weight in two groups. (D–F) CircUSP48, miR-335 and SNIP1 levels of two groups. (G) IHC staining of SNIP1 in two groups. $^{*}p<0.05$, $^{**}p<0.01$.

3.5 | Silencing circUSP48 inhibited tumor growth in vivo

This study conducted a tumor xenograft experiment to assess the role of circUSP48 in the development of OS. Tumor growth was markedly reduced after circUSP48 knockdown (Figure 5A). Similarly, silencing of circUSP48 significantly reduced the average tumor volume and weight (Figure 5B, C). As expected, the expression of circUSP48 and SNIP1 was down-regulated in the sh-circUSP48 group, while the expression of miR-335 was the opposite (Figure 5D–F). Furthermore, the results of the IHC stain revealed that SNIP1 expression was markedly reduced in the sh-circUSP48 group compared to the sh-NC group (Figure 5G).

4 | DISCUSSION

CircRNAs have been shown to affect physiological and pathological processes.[13–15] Several studies have confirmed that dysregulated circRNAs can facilitate or suppress the biological characteristics of OS.[15,16] In addition, circRNAs are believed to be important therapeutic targets for OS. Therefore, new insights for the treatment of OS can be provided by fully understanding the role of circRNAs.[18] The current work identified and characterized a new circRNA named circUSP48 in OS tissues and cells. Our data demonstrated that silencing circUSP48 suppressed OS progression by sponging miR-335 and regulating SNIP1.

To date, more and more circRNAs have been discovered to be related to pathophysiological events in OS.[17,19,20] For example, circDOCK1 promotes the tumorigenesis of OS through the miR-339-3p/IGF1R axis.[21] However, it remains unclear whether circUSP48 exerts its functional role in OS. According to GSE96964 microarray, it was found that circUSP48 expression was strongly expressed in OS cell lines relative to human osteoblasts. Subsequently, through Sanger sequencing, we identified the back-spliced site of circUSP48 in OS. Additionally, agarose gel electrophoresis revealed that divergent primers amplified circUSP48 in cDNA but not gDNA. Furthermore, after RNase R treatment, only

linear USP48 mRNA was digested, not circUSP48. These results suggest that circUSP48 had a circular structure and was stable in OS. Subsequently, we found that circUSP48 was strongly denoted in OS tissues and cells. Additionally, the findings indicated that the knockdown of circUSP48 suppressed the migration and proliferation of OS cells. In summary, our research suggested that circUSP48 had an oncogenic function in OS.

Increasing evidence confirms that miRNAs could regulate the progression of different cancer types.[22,23] Circular RNAs have been shown to exert their function via sponging miRNAs.[24] Therefore, we assumed that circUSP48 could absorb miRNA to influence the development of OS. Through bioinformatic analysis, we show that miR-335 functions as a possible target of circUSP48. Subsequently, direct binding between miR-335 and circUSP48 was confirmed by adopting luciferase reporter and RIP assay. In addition, FISH assays revealed that circUSP48 and miR-335 were primarily located in the cytoplasm. Furthermore, Pearson's analysis showed that miR-335 expression had a negative correlation with circUSP48. Importantly, silencing circUSP48 upregulated miR-335 expression in OS cells. Collectively, these results suggested that circUSP48 exerted its function through the sponging of miR-335.

It has been proven that circRNAs can indirectly regulate target genes through binding to miRNAs.[24] SNIP1, a conserved nuclear protein, contains a forkhead structural domain. Researchers have found that SNIP1 was involved in a variety of biological processes, and increased SNIP promoted the progression of OS. Therefore, this work underwent rescue experiments to investigate the relationship between circUSP48, miR-335, and SNIP1. The inhibition of SNIP1 expression induced by sh-circUSP48 was counteracted by the miR-335 inhibitor, which confirmed that a circUSP48/miR-335/SNIP1 axis existed in OS.

5 | CONCLUSION

In conclusion, this research revealed that silencing circUSP48 suppressed OS progression through sponging miR-335 to down-regulate SNIP1. Therefore, the circUSP48/miR-335/SNIP1 axis could become a new target for OS therapy.

CONFLICT OF INTEREST

None.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

PATIENT CONSENT STATEMENT

All patients provided written informed consent.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

All authors approved permission to reproduce material from other sources.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Luo Y, Yang B, Yuan X, Zheng J. Silencing circUSP48 suppresses osteosarcoma progression by regulating the miR-335/ smad nuclear interacting protein 1 pathway. *J Clin Lab Anal*. 2023;37:e24828. doi:10.1002/jcla.24828