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Circ-FOXM1 Promotes Cell Proliferation, Migration and EMT Process in Osteosarcoma Through FOXM1-Mediated Wnt Pathway Activation

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Abstract

Background: As the most common primary bone tumor in adolescents and children, osteosarcoma commonly occurs with high mortality rate and metastasis. Emerging evidence has illustrated that circular RNAs (circRNAs) are important regulatory RNAs that are involved in multiple biological activities of carcinomas. Circ-FOXM1 (hsa_circ_0025033) is a recently found circRNA and promotes the cellular activities of several cancers. However, the function and molecular mechanism of circ-FOXM1 in osteosarcoma have not been interrogated yet.

Methods: The qRT-PCR was utilized to test the expression of circ-FOXM1 in osteosarcoma cell lines. Loss-of-function assays including CCK-8, EdU, TUNEL, transwell and western blot assays were conducted to measure cell proliferation, cell migration, EMT process and cell apoptosis. Luciferase reporter assay and RIP assay were utilized to detect the interaction of circ-FOXM1 and RNAs.

Results: We discovered the high expression of circ-FOXM1 in osteosarcoma cells. Besides, it was indicated that circ-FOXM1 knockdown inhibited cell proliferation, cell migration and EMT process, as well as induced cell apoptosis of osteosarcoma cells. Furthermore, circ-FOXM1 was discovered to upregulate the expression level of forkhead box M1 (FOXM1) at post-transcriptional level. Moreover, it was proved that circ-FOXM1 sponged miR-320a and miR-320b so as to increase FOXM1 expression. Additionally, circ-FOXM1 could activate Wnt signaling pathway through upregulating FOXM1. In the end, rescue assays certified that FOXM1 overexpression could totally rescue the circ-FOXM1 silence-repressed cellular activities of osteosarcoma cells.

Conclusion: Circ-FOXM1 facilitated the progression of osteosarcoma cells via relieving FOXM1 from the inhibition by miR-320a and miR-320b.

Introduction

Osteosarcoma, the most common primary bone tumor, mainly happens in adolescents and children, with a high mortality rate [1,2]. In spite of the progresses in surgical removal, radiotherapy and neoadjuvant chemotherapy for patients suffering osteosarcoma, the prognosis still remains dismal as a result of the high rate of metastasis [3,4]. The 5-year survival rate of osteosarcoma patients possessing distant metastases is approaching 30% [5]. Nevertheless, deep understanding of the regulatory mechanisms in osteosarcoma is vitally important.

Mounting evidences have reported that dysregulated noncoding RNAs (ncRNAs) are related to the pathogenesis, initiation and even progression of osteosarcoma [6-8]. Circular RNAs (circRNAs), a new type of ncRNAs with a closed loop, are highly conserved due to their resistance to RNase R [9]. They can interact with RNA-binding proteins or act as sponges of miRNAs to facilitate their parental genes in terms of the development of tumors [10-12]. For examples, circRNA hsa_circ_0001564 modulates osteosarcoma cell proliferation and apoptosis via acting miRNA sponge [13]; circRNA_001569 targets miR-145 in the proliferation and invasion of colorectal cancer cells [14]; suppression of PABPN1

translation by circPABPN1 was relied on HuR [15]; and the circular RNA circβ-catenin promotes cell growth of liver cancer cells by activating the Wnt pathway [16].

Circ-FOXM1 (hsa_circ_0025033), located at chr12: 2966846-2983691, is a newly recognized cancer-related circRNA and its spliced length is 3410 nt. Circ-FOXM1 is a reported overexpressed gene in glioblastoma as screened by circRNA microarray [17]. Furthermore, circ-FOXM1 promotes cell progression by sponging miR-1304-5p that targets PPDPF and MACC1 in non-small cell lung cancer [18]. And the upregulated circ_0025033 facilitates cell proliferation and invasion through absorbing miR-1231 and miR-1304 in papillary thyroid cancer [19]. Circ-FOXM1 usually displays carcinogenic function in human cancers. However, the role of circ-FOXM1 in osteosarcoma is not fully investigated.

MicroRNAs (miRNAs) are 21-23 nucleotides long, single stranded, noncoding RNA molecules generated endogenously [20]. They can negatively regulate their downstream target messenger RNAs (mRNAs) and take part in the progression and development of human cancers. For example, miR-1231 is down-regulated in prostate cancer with prognostic and functional implications [21]. MiR-125 represses colorectal cancer proliferation and invasion via targeting TAZ [22]. MiR-223-3p inhibits osteosarcoma metastasis and progression through targeting CDH6 [23].

This research was aimed to investigate the biological function of circ-FOXM1 in osteosarcoma cells. Further, interaction of circ-FOXM1 and other RNAs such as miRNA and mRNA was also investigated deeply. Our research may offer a feasible therapeutic target for osteosarcoma patients, shedding light on the therapy of osteosarcoma.

Materials And Methods

Cell culture

Human normal osteoblastic cell line hFOB1.19 and OS cell lines including MG63, HOS, 143B and U2OS were acquired commercially from Chinese Academy of Sciences (Shanghai, China). All cell lines were allowed to grow in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) at 37℃ in 5% CO_2.

Cell transfection plasmids

143B and U2OS cells were seeded into 6-well plates. When cell density was reached 80-90%, cells were transfected with circ-FOXM1-specific short hairpin RNA (sh-circ-FOXM1#1/2/3) and negative control (sh-NC) in line with the user guide of Lipofectamine™3000 (Invitrogen). Overexpressing vector pcDNA3.1/FOXM1 and empty vector pcDNA3.1 serving as the control were purchased from (GenePharma, Shanghai, China). To overexpress or knockdown miR-320a and miR-320b, miR-320a and miR-320b mimics or inhibitor were designed by RiboBio (Guangzhou, China), along with their respective controls. After 48 h of transfection, cells were harvested for subsequent study.

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

Total RNA was extracted from OS tissues and cell lines using TRIzol reagent (Life Technologies Corporation). After measuring RNA concentration, 100 μg of RNA was used for reverse transcription. qRT-PCR analysis was carried out using SYBR Premix Ex Taq™ II (Takara, Japan) and the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The Ct value of samples was calculated by the $2^{-\Delta\Delta Ct}$ method. U6 small nuclear RNA was seen as the miRNA internal control and GAPDH was seen as the internal control for other genes. All reactions were performed in triplicate.

Cell proliferative assays

Cell counting kit-8 (CCK-8) assays were used to analyze OS cell proliferation. 143B and U2OS cells were put into 96-well plates and cultured with 10 μl/well CCK-8 (Beyotime, Jiangsu, China) solution for 48 h. The cellular absorbance was detected by microplate reader (Molecular Devices) at 450 nm. For EdU incorporation assay, cells were transfected and incubated with 100 μL of 50 μM EdU medium diluent (Ribobio, Guangzhou China) in culture plates for 3 h. After fixation, cells were treated with 100 μL of the 0.5% Troxin X-100 and 100 μL of 1 × Apollo® 488 fluorescent staining reaction liquid for 30 min at 37°C. Cell nuclei were dyed with DAPI. All proliferative assays were repeated for at least three times.

Cell apoptosis assay

Apoptotic cells were detected using the TUNEL assay (In Situ Cell Death Detection Kit, Roche). The transfected cell lines were reaped and rinsed in PBS for 5 min, then subjected to 0.1% TritonX-100 in 1% sodium citrate on ice for 2 min. Following labeling with the fluorescein-TUNEL reagent at 37°C for 1 h, DAPI was used to stain cell nucleus. The final images of three different experiments were visualized under an Olympus inverted microscope (Olympus, Japan).

Cell migration assay

After transfection, cells were trypsinized and adjusted to 2×10^5 cells/ml, followed by incubation in the upper chamber of separate polycarbonate membranes (8 μm pores) of a transwell (Costar, Lowell, MA). The lower chamber was supplied with 500 μl of culture medium with 10% FBS. A cotton swab scraped off the non-migrating cells in the upper chamber. The cells which filtered to the other side of the membrane were dyed with 2% crystal violet and counted under inverted microscope at × 200 magnification. Experiment was performed in triplicate.

Western blot assay

Cell lines were lysed in radio immunoprecipitation assay (RIPA) protein extraction reagent (Beyotime). Protein concentration was detected by BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL). Samples were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and blocked in TBST with 5% nonfat milk for 2 h. Protein was incubated with the primary antibodies (Abcam, Cambridge, USA) overnight at 4°C and washed thrice in

PBS, followed by incubation with secondary antibodies. An antibody against GAPDH served as an endogenous reference. The signals were detected using the ECL Plus Detection Kit (Pierce, Rockford, IL). Each procedure was performed for more than two times.

Immunofluorescence staining assay

After transfection, cells on coverslips were subjected to fixation with 4% paraformaldehyde, and then blocked with 5% BSA. After that, we cultivated cells with primary antibodies against β-catenin at 4°C for one night, following fluorescence-conjugated secondary antibodies at room temperature. DAPI was utilized to stain cell nuclear. Finally, we utilized the Olympus microscope to analysis. The experiment was repeated at least three times.

Luciferase reporter assay

FOXM1 promoter was amplified by PCR and cloned into pGL3-Basic vector (Promega Corporation, Madison, WI, USA). U2OS and 143B cells were seeded on 96-well plates (8000/well) and co-transfected with luciferase reporter plasmids containing FOXM1 promoter and sh-circ-FOXM1 or sh-NC. 48 h later, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA) in accordance with the user guide. For analyzing the interaction between miR-320a and miR-320b and circ-FOXM1 or FOXM1, U2OS and 143B cells were co-transfected with psiCHECK-circ-FOXM1-WT/MUT or psiCHECK-FOXM1-WT/MUT, and miR-320a and miR-320b mimics or miR-NC. Experimental data were obtained from three independent replicates.

RNA immunoprecipitation (RIP) assay

RIP assay was conducted using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) following the supplier's protocol. U2OS and 143B cells were rinsed in PBS and subjected to RIP buffer at 4℃ for 30 min, and incubated with protein A/G sepharose beads conjugated to antibodies against Ago2 (Millipore, Massachusetts, USA) or normal mouse immunoglobulin G (IgG; Millipore, California, USA). At length, immunoprecipitated RNA from cell lysates of three different experiments was extracted for qRT-PCR analysis.

Statistical analysis

All experimental results were expressed as the mean ± SD, and each experiment was repeated in triplicate. Statistical analyses and graphical depictions were conducted using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Student's t test or one-way analysis of variance was used to evaluate the differences between results. Statistical significance was set as $^{*}P < 0.05$, $^{**}P < 0.01$.

Results

Circ-FOXM1 was overexpressed in osteosarcoma cells

First of all, through UCSC (http://genome.ucsc.edu/) database, we found that circ-FOXM1 was located in chr12: 2966846-2983691 and FOXM1 was the host gene of circ-FOXM1 (Figure 1A). Following, according to the results from qRT-PCR, circ-FOXM1 was upregulated in osteosarcoma cell lines (MG-63, HOS, 143B and U2OS), compared with human osteoblast hFOB1.19 cells (Figure 1B). 143B and U2OS displayed the higher expression of circ-FOXM1, thus we selected these two cells for the later assays. Then we performed agarose gel electrophoresis and confirmed that circ-FOXM1 was a circRNA (Figure 1C). Subsequently, qRT-PCR detection found that, after RNase R treatment, FOXM1 expression was lowered whereas circ-FOXM1 expression varied not, further confirming that circ-FOXM1 had a closed loop structure (Figure 1D). Besides, after cells were treated with Actinomycin D, we discovered that the RNA level of circ-FOXM1 almost unchanged, while that of FOXM1 was significantly declined (Figure 1E). Collectively, circ-FOXM1 was expressed at high level in osteosarcoma.

Knockdown of circ-FOXM1 suppressed cell proliferation, migration and EMT process in osteosarcoma

To explore the function role of circ-FOXM1 in osteosarcoma, we transfected shRNAs specifically targeting circ-FOXM1 (sh-circ-FOXM1#1/2/3) into two osteosarcoma cells. The circ-FOXM1 expression was accordingly down-regulated in U2OS and 143B cells and sh-circ-FOXM1#1 and sh-circ-FOXM1#1 developed the better transfection efficiency (Figure 2A). CCK-8 assay demonstrated that cell viability was significantly inhibited by knockdown of circ-FOXM1 (Figure 2B). EdU assay showed that the ratio of EdU positive cells was declined in transfection groups, suggesting cell proliferation was obviously inhibited through transfection of circ-FOXM1 depletion (Figure 2C). In addition, TUNEL assay found that silencing circ-FOXM1 dramatically repressed the number of TUNEL positive cells, which indicated that cell apoptosis could be accelerated by circ-FOXM1 depletion (Figure 2D). In transwell assay, the number of migrated cells was distinctly reduced when circ-FOXM1 was silenced, which illustrated that cell migration could be restrained by knockdown of circ-FOXM1 (Figure 2E). It was also observed from western blot that the protein levels of MMP2 and MMP9 were relatively reduced by sh-circ-FOXM1#1. Meanwhile, E-cadherin (one epithelial marker) was increased and N-cadherin (one mesenchymal marker) was lessened after the down-regulation of circ-FOXM1, suggesting cell migration and EMT process could be repressed by silencing circ-FOXM1 (Figure 2F). Taken together, circ-FOXM1 suppression inhibited the proliferation, migration and EMT process of osteosarcoma cells.

Circ-FOXM1 could regulate the expression level of FOXM1 post-transcriptionally

Since FOXM1 was the host gene of circ-FOXM1, we assumed that circ-FOXM1 might influence the expression of FOXM1. Firstly, by browsing the GEPIA (http://gepia.cancer-pku.cn/) database, we discovered the overt upregulation of FOXM1 in sarcoma (SARC) tissues (Figure 3A). Through qRT-PCR analyses, we validated the overexpression of FOXM1 in osteosarcoma cells (Figure 3B). Moreover, we checked the direct impact of circ-FOXM1 on FOXM1 and found that FOXM1 mRNA expression and protein level were reduced in cells transfected with sh-circ-FOXM1 through RT-qPCR and western blot assay, suggesting the positive correlation of them (Figure 3C). Then, luciferase reporter assay was performed, and the results showed that sh-circ-FOXM1 transfection produced no change on the luciferase

activity of FOXM1 promoter (Figure 3D). And RIP assay confirmed that circ-FOXM1 and FOXM1 were both enriched in the mixture immunoprecipitated by anti-Ago2, which illustrated that they existed in RISC complex (Figure 3E). These data suggested that circ-FOXM1 might regulate FOXM1 expression through ceRNA network.

Circ-FOXM1 targeted miR-320a and miR-320b to modulate FOXM1

Competing endogenous RNA (ceRNA) model is constituted by lncRNAs or circRNAs with miRNAs for the upregulation of miRNAs-targeted mRNAs [24,25]. In order to explore the regulation mechanism of circ-FOXM1 on FOXM1, we initially sought for the candidate miRNAs targeting FOXM1 by the utilization of starBase (http://starbase.sysu.edu.cn/index.php) database. Through the prediction of PITA, miRanda, TargetScan and miRmap database in starBase, we selected four miRNAs binding to FOXM1 (miR-320a, miR-320b, miR-320c and miR-320d) (Figure 4A). For screening, we overexpressed the miRNAs (Figure S1A-B) and then luciferase reporter assay was implemented. The results indicated that the luciferase activity of FOXM1 was significantly restrained by both miR-320a upregulation and miR-320b upregulation, suggesting FOXM1 could bind with miR-320a and miR-320b (Figure 4B). Next, qRT-PCR and western blot assay indicated that the mRNA level and protein level of FOXM1 could be repressed by both miR-320a upregulation and miR-320b upregulation, which implied the negative correlation (Figure 4C-D). Further, we obtained the binding sites for FOXM1 and miR-320a or miR-320b from starBase (Figure 4E). Following, luciferase reporter assay indicated that the luciferase activity of FOXM1-WT was repressed by the overexpression of miR-320a or miR-320b, which further proved the binding between FOXM1 and miR-320a or miR-320b (Figure 4F). Then we detected the binding situation of circ-FOXM1 and miR-320a or miR-320b. Obviously, we obtained the binding sites of circ-FOXM1 and miR-320a or miR-320b from starBase and we also proved that the luciferase activity of circ-FOXM1-WT was inhibited via the upregulation of miR-320a or miR-320b, suggesting circ-FOXM1 could combine with miR-320a or miR-320b (Figure 4G-H). Additionally, RIP assays testified that circ-FOXM1, FOXM1, miR-320a and miR-320b co-existed in RNA inducing silencing complex (RISC) (Figure 4I). These results displayed that circ-FOXM1 sponged miR-320a and miR-320b to regulate FOXM1.

Circ-FOXM1 accelerated osteosarcoma progression through sponging miR-320a and miR-320b to upregulate FOXM1 expression and activate Wnt signaling pathway

FOXM1 has been reported to promote β-catenin into the nucleus, thus activating Wnt signaling pathway and playing a role in promoting cancer. Thus, we examined the regulatory role of circ-FOXM1/FOXM1 on Wnt pathway [26]. First of all, we overexpressed FOXM1 in U2OS cells by transfecting pcDNA3.1/FOXM1 and qRT-PCR analyzed that FOXM1expression was effectively upregulated by transfection (Figure 5A). Then through western blot assay, we detected the level of critical genes in Wnt pathway and discovered that the level of β-catenin and C-myc was significantly repressed by circ-FOXM1 knockdown and then it was totally reversed by overexpression of FOXM1. Similarly, the level of MMP2, MMP9, E-cadherin, and N-cadherin was also repressed by circ-FOXM1 inhibition and then totally reversed by FOXM1 upregulation (Figure 5B). Further, immunofluorescence assay proved that the part of β-catenin in nucleus was

decreased by circ-FOXM1 knockdown but then increased by FOXM1 upregulation (Figure 5C). These results indicated that circ-FOXM1 activated Wnt signaling pathway via upregulating FOXM1 expression. Then rescue functional assays were further conducted to verify the regulatory mechanism of circ-FOXM1 and FOXM1. Through CCK-8 and EdU assays, we proved that overexpression of FOXM1 could counteract the inhibitory effect of silencing circ-FOXM1 on cell proliferation (Figure 5D-E). Besides, TUNEL assay illustrated that the promoted cell apoptosis caused by the lack of circ-FOXM1 could be reversed by FOXM1 upregulation (Figure 5F). In the end, it was demonstrated from transwell assay that cell migration repressed by the silenced circ-FOXM1 could be recovered by overexpressing FOXM1 (Figure 5G).

In addition, we also conducted the rescue assay to detect the regulatory mechanism of circ-FOXM1, miR-320a and miR-320b. We silenced miR-320a or miR-320b by transfection miR-320a inhibitor or miR-320b inhibitor into cells. In CCK-8 and EdU assays, we discovered that the inhibited cell viability and proliferation caused by circ-FOXM1 depletion could be partially rescued by only miR-320a inhibitor but totally rescued by both miR-320a inhibitor and miR-320b inhibitor (Figure S2A-B). Then TUNEL assay indicated that circ-FOXM1 knockdown elevated cell apoptosis, while co-transfection of miR-320a inhibitor partially recovered this effect. However, co-transfection of miR-320a inhibitor and miR-320b inhibitor could fully recover this effect (Figure S2C). In the end, transwell and western blot assay further proved that cell EMT process and the activity of Wnt pathway repressed by circ-FOXM1 depletion could be partially rescued by only miR-320a inhibitor but totally rescued by both miR-320a inhibitor and miR-320b inhibitor (Figure S2D-E).

Taken together, as shown in Figure 6, this research proved that circ-FOXM1 was upregulated in osteosarcoma cells. The upregulated circ-FOXM1 accelerated osteosarcoma cell proliferation and migration through sponging miR-320a and miR-320b to upregulate FOXM1 expression and activate Wnt signaling pathway.

Discussion

Circular RNAs (circRNAs) are pivotal regulators for osteosarcoma patients. A novel circulating hsa_circ_0081001 serves as a potential biomarker for the diagnosis and prognosis of patients with osteosarcoma [27]. Down-regulated circ_HIPK3 suppresses cell proliferation, migration and invasion of osteosarcoma cells [28]. Circ-NT5C2 is an oncogene for the proliferation and metastasis in osteosarcoma by targeting miR-448 [29]. Circ_0001721 indicates poor prognosis in osteosarcoma and boosts cell progression through targeting miR-569 and miR-599 [30]. The involvement of circ-FOXM1 in osteosarcoma remains obscure.

Circ-FOXM1 (hsa_circ_0025033) has been identified in several kinds of cancers such as glioblastoma [17], non-small cell lung cancer [18] and papillary thyroid cancer [19], and exert the carcinogenic effects. In this research, we affirmed that circ-FOXM1 was heightened in osteosarcoma cells. Then we further tested the function role of circ-FOXM1 in osteosarcoma. In consistent with the conclusions from previous

researches, this study, for the first time, displayed the oncogenic role of circ-FOXM1 in the proliferation and cell motility of osteosarcoma cells.

Considering that forkhead box M1 (FOXM1), an overexpressed gene in sarcoma based on the data from GEPIA database, is the host gene of circ-FOXM1, we attempted to explore the possible relationship between FOXM1 and circ-FOXM1. Past works have indicated the tumor-promoting role of FOXM1 in several cancers. For instance, miR-26b-targeted DEPDC1 facilitates cell proliferation via upregulating FOXM1 in TNBC [31]. FOXM1 deubiquitinated by USP21 modulates cell cycle and paclitaxel sensitivity of basal-like breast cancer cells [32]. Repression of Wnt3a/FOXM1/β-Catenin pathway and stimulation of GSK3β and caspase are widely involved in the apoptotic impact of moracin D in breast cancer [33]. Consistently, we unveiled that FOXM1 was also expressed at high levels in osteosarcoma cells and FOXM1 was positively associated with circ-FOXM1. Moreover, circ-FOXM1 could regulate FOXM1 mRNA expression at post-transcriptional level but not at transcriptional level.

The participation of circRNAs in ceRNA network is universally known to be related to the post-transcriptional regulation of target mRNAs [34-36]. The ceRNA mechanism refers to that circRNA can act as a ceRNA to sponge miRNA, so as to regulate mRNA expression [24]. After browsing starBase database, we obtained four miRNAs targeting FOXM1. And only miR-320a and miR-320b exhibited the inhibitory effect on the luciferase activity of FOXM1 in U2OS and 143B cells. Additionally, miR-320a and miR-320b is recognized as a tumor suppressor in carcinomas, osteosarcoma included. For example, microRNA-320 family is reported to be down-regulated in colorectal adenoma and repress cell proliferation by targeting CDK6 [37]. MiR-320a can inhibit cell growth and chemosensitivity through regulating ADAM10 in gastric cancer [38]. Mir-320b suppresses pancreatic cancer cell proliferation through regulating FOXM1 [39]. Importantly, reports have proven that miR-320a and miR-320b function as a tumor inhibitor in osteosarcoma [40,41], which is consistence with our research. In short, mechanism assays showed that circ-FOXM1 acted as a miRNA sponge to modulate FOXM1 by targeting miR-320a and miR-320b.

Taken together, our research confirmed that circ-FOXM1 facilitated the biological processes of osteosarcoma cells via absorbing miR-320a and miR-320b to upregulate FOXM1 expression. All these findings displayed the modulation mechanism underlying circ-FOXM1 in osteosarcoma, offering a feasible therapeutic target for the treatment of osteosarcoma patients.

Abbreviations

Forkhead box M1 (FOXM1)

Circular RNAs (circRNAs)

Noncoding RNAs (ncRNAs)

Messenger RNAs (mRNAs)

MicroRNAs (miRNAs)

Competing endogenous RNA (ceRNA)

Fetal Bovine Serum (FBS)

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

Cell counting kit-8 (CCK-8)

5-Ethynyl-2'-deoxyuridine (EdU)

4',6-diamidino-2-phenylindole (DAPI)

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL)

RNA immunoprecipitation (RIP)

Radio immunoprecipitation assay (RIPA)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Wild-type (WT)

Mutant (Mut)

Standard deviation (SD)

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Authors confirmed that this work can be published. The content of this manuscript is original and it has not yet been accepted or published elsewhere.

Availability of data and material Not applicable.

Data Availability Statement Not applicable.

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Authors' contributions Hao Zhang conceived the projectct. Hao Zhang and Qiongqiong Zhou conducted functional assays. Hao Zhang made statistical analysis and edited the manuscript. Both of them revised the manuscript.

Competing interests Not applicable

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Figures

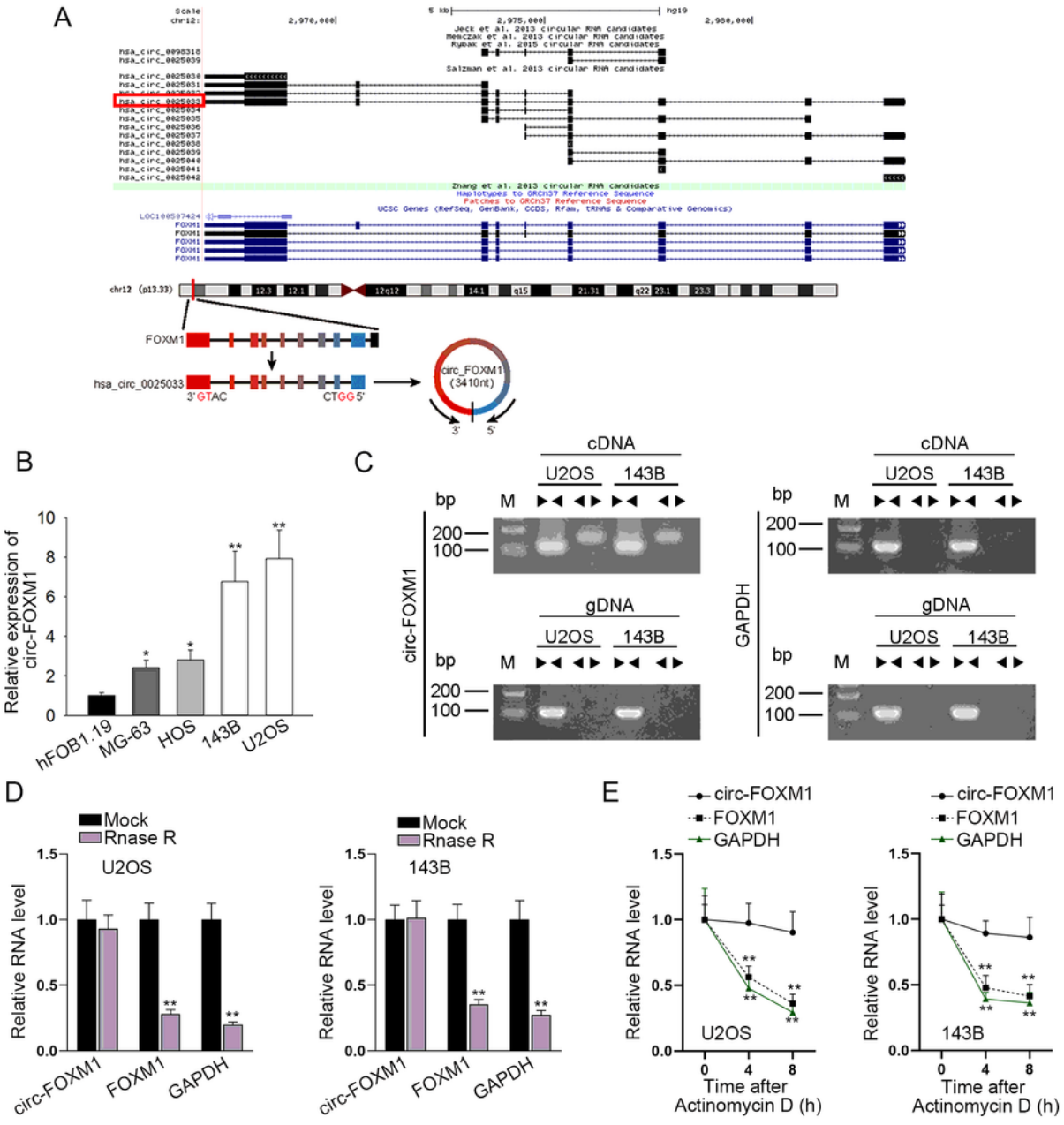

Figure 1

Circ-FOXM1 was overexpressed in osteosarcoma cells (A) The genomic loci of circ-FOXM1. (B) The overexpression of circ-FOXM1 in osteosarcoma cells (MG-63, HOS, 143B and U2OS) compared to normal osteoblast hFOB1.19 cells, as tested by qRT-PCR. (C-E) Agarose gel electrophoresis and qRT-PCR was used to examine circ-FOXM1 was a circRNA. *P < 0.05, **P < 0.01.

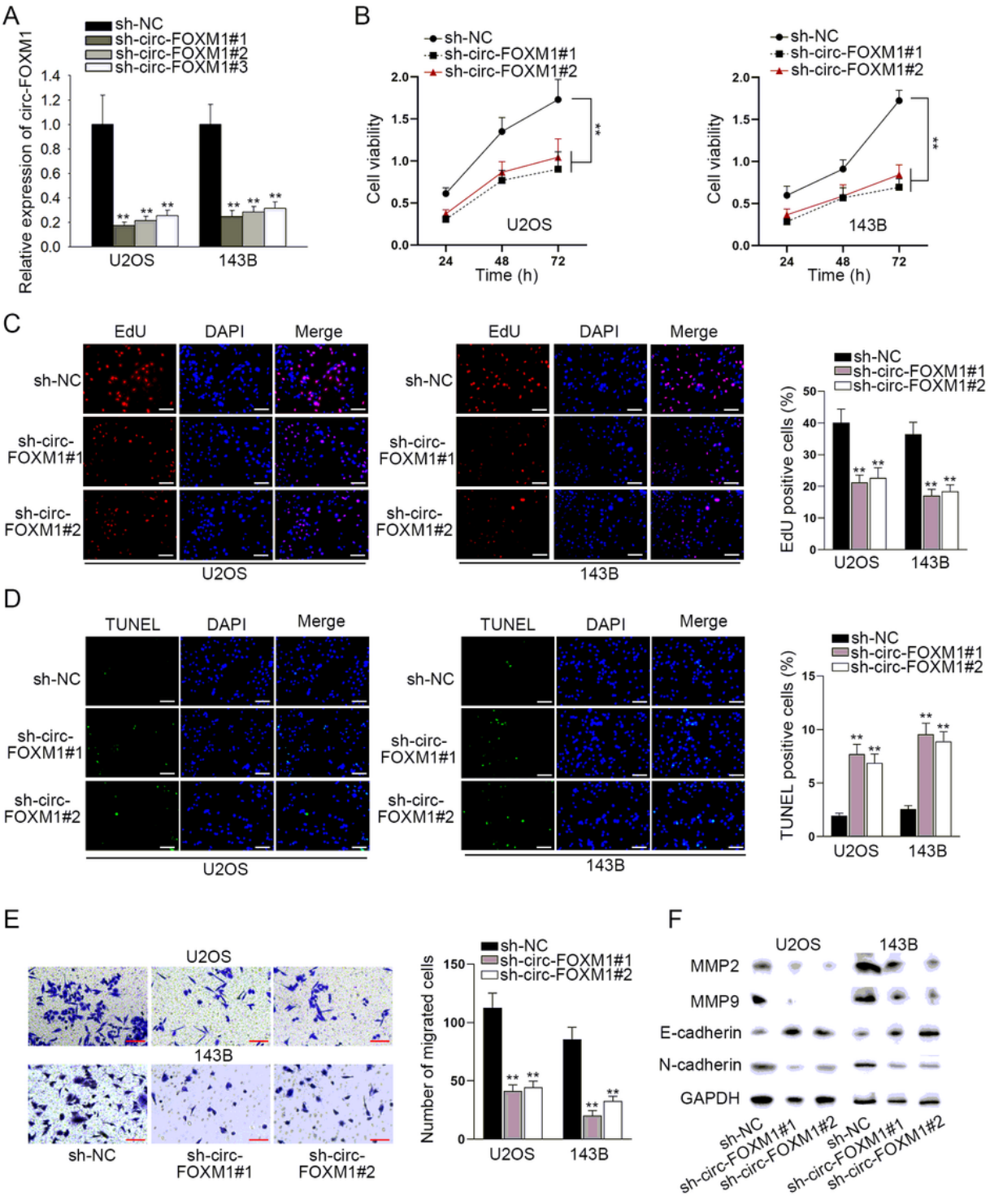

Figure 2

Knockdown of circ-FOXM1 suppressed cell proliferation, migration and EMT process in osteosarcoma (A) The inference efficacy of sh-circ-FOXM1 in two osteosarcoma cells was determined by qRT-PCR. (B-C) Cell proliferation was measured through CCK-8 and EdU (bar value = 120 μm) assays in sh-circ-FOXM1-transfected cells. (D) Cell apoptosis was estimated by the use of TUNEL (bar value = 120 μm) assay in sh-circ-FOXM1-transfected cells. (E) Transwell (bar value = 50 μm) assay was performed to evaluate cell migration in sh-circ-FOXM1-transfected cells. (F) The levels of EMT-associated proteins were respectively measured using western blot in sh-circ-FOXM1-transfected cells. **P < 0.01.

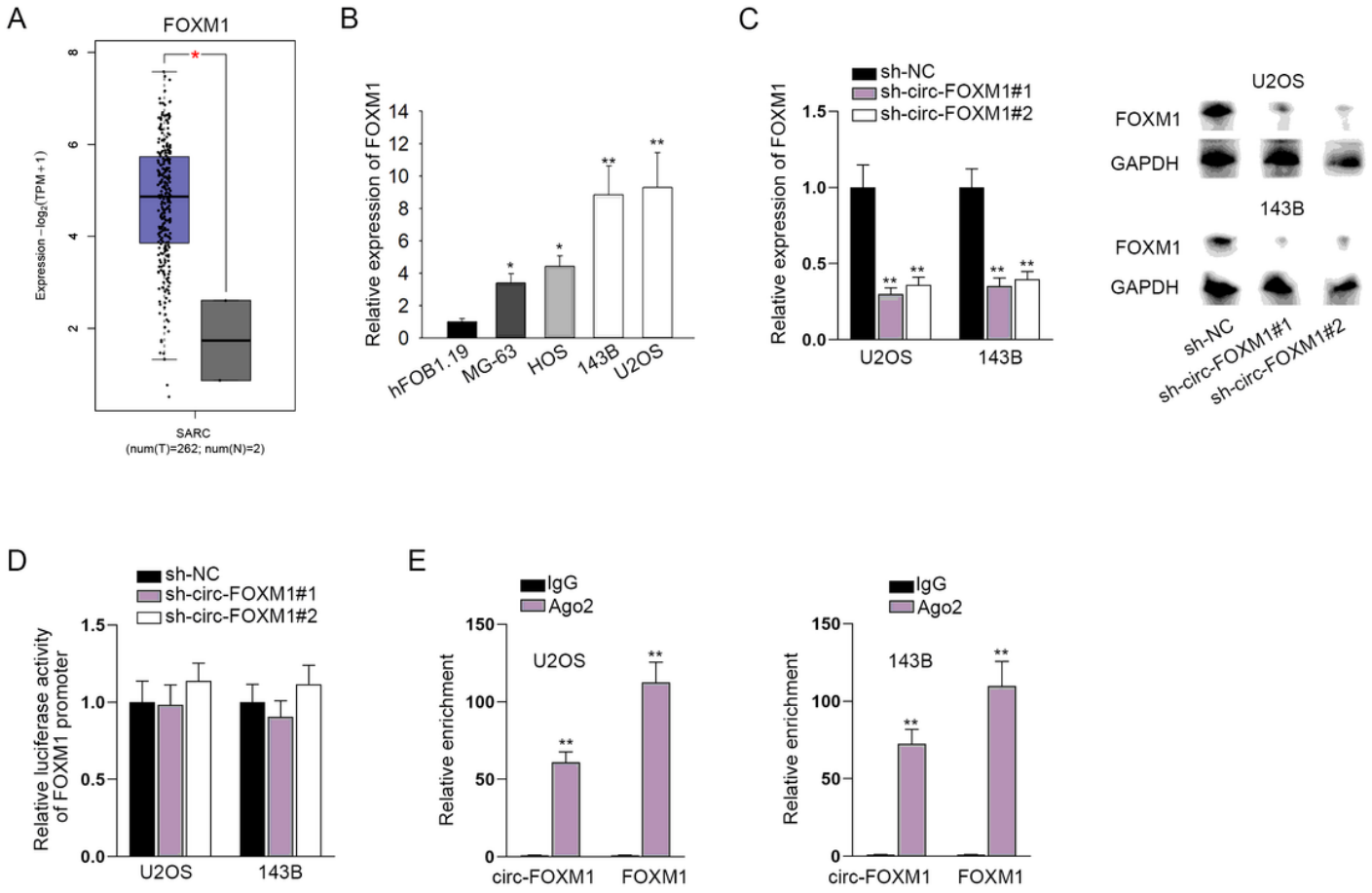

Figure 3

Circ-FOXM1 could regulate the expression level of FOXM1 post-transcriptionally (A) From GEPIA database, we found the upregulation of FOXM1 in sarcoma (SARC) tissues. (B) qRT-PCR examined the relatively high expression of FOXM1 in osteosarcoma cells, in comparison with normal hFOB1.19 cells. (C) The mRNA expression and protein level of FOXM1 were detected in cells transfected with sh-circ-FOXM1 through qRT-PCR and western blot assay. (D) Luciferase reporter assay detected the impact of

circ-FOXM1 knockdown on FOXM1 promoter. (E) RIP assay confirmed the co-existence of circ-FOXM1 and FOXM1 in RISC. *P < 0.05, **P < 0.01.

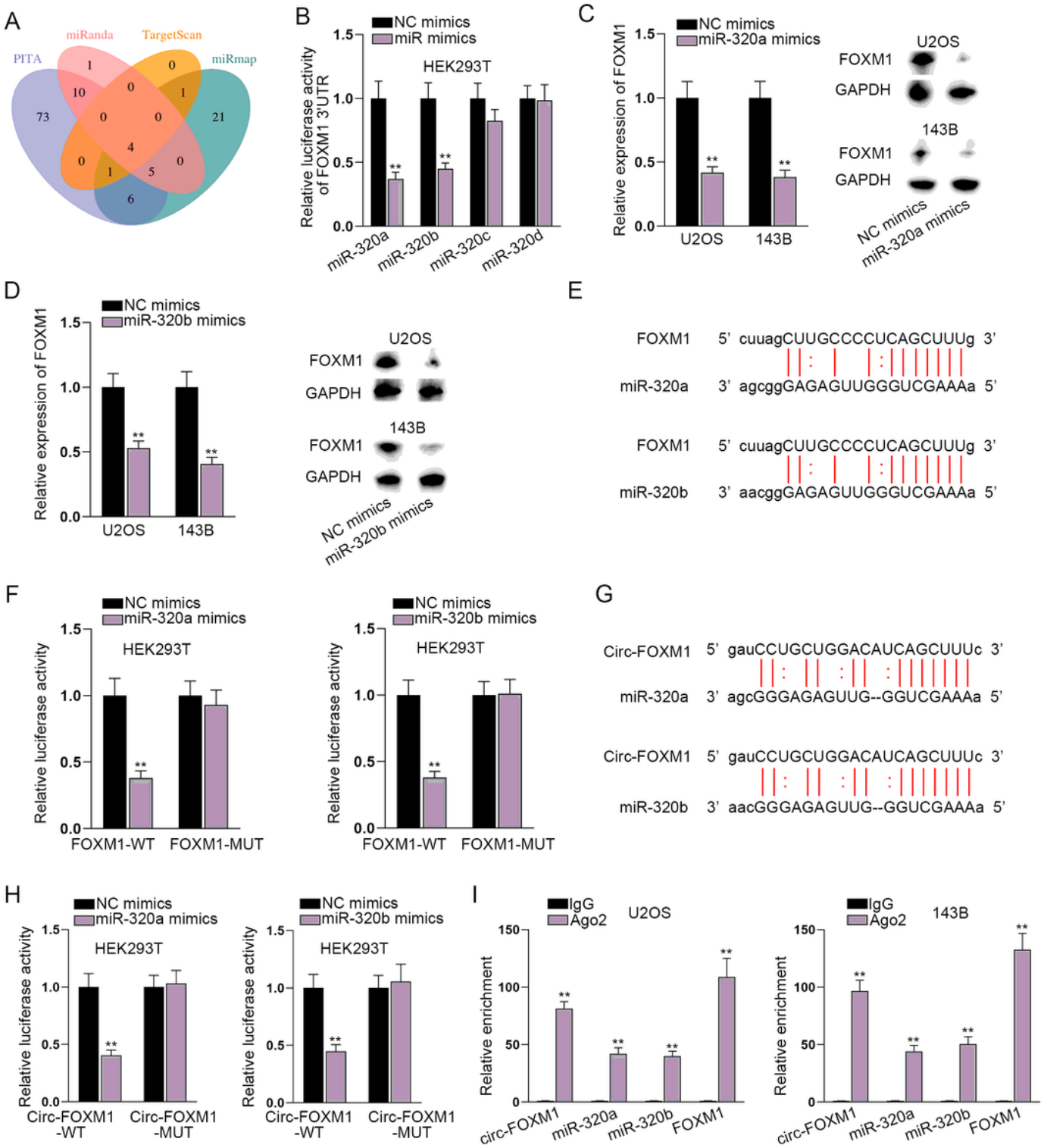

Figure 4

Circ-FOXM1 targeted miR-320a and miR-320b to modulate FOXM1 (A) The Venn diagram of the four miRNAs targeting FOXM1 from starBase websites (PITA, miRanda, TargetScan and miRmap database). (B) Luciferase reporter assay was utilized to screen out the miRNA that bind with FOXM1. (C-D) The

expression and protein level of FOXM1 were detected through qRT-PCR and western blot assay when miR-320a and miR-320b were overexpressed in cells. (E) The predicted binding sites between miR-320a or miR-320b and FOXM1. (F) Luciferase reporter assay was applied for proving the binding site of miR-320a or miR-320b and FOXM1. (G) The predicted binding sites between miR-320a or miR-320b and circ-FOXM1. (H) Luciferase reporter assay was applied for proving the binding site of miR-320a or miR-320b and circ-FOXM1. (I) The enrichment of miR-320a, miR-320b, circ-FOXM1 and FOXM1 was found in Ago2 group. **P < 0.01.

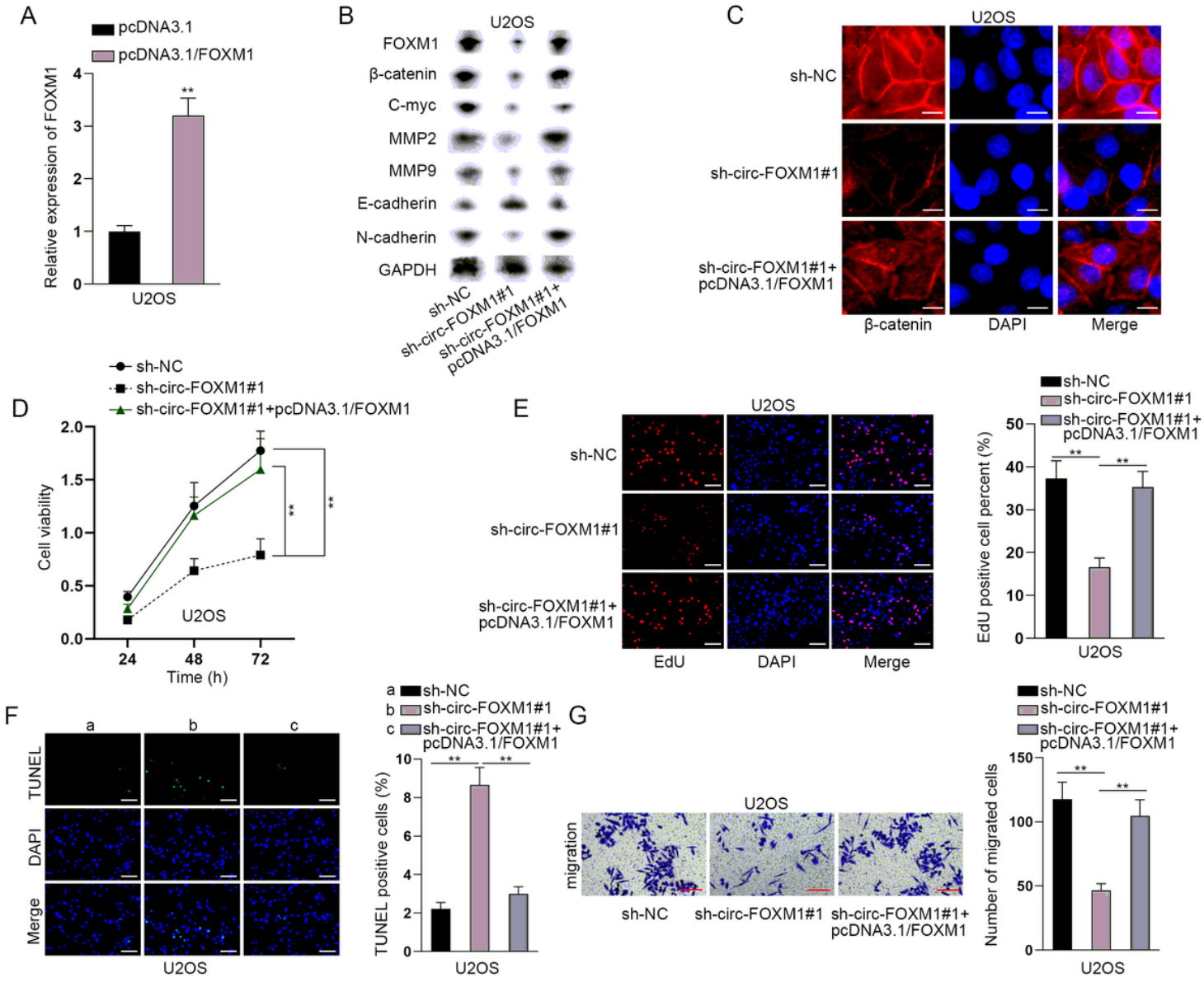

Figure 5

Circ-FOXM1 accelerated osteosarcoma progression through sponging miR-320a and miR-320b to upregulate FOXM1 expression and activate Wnt signaling pathway (A) qRT-PCR analysis of the overexpression efficacy of FOXM1 in U2OS cells. (B) Western blot assay detected the level of FOXM1, β-catenin, C-myc, MMP2, MMP9, E-cadherin and N-cadherin in different groups. (C) Immunofluorescence (bar value = 20 μm) assay detected the access to the nucleus of β-catenin in different groups. (D-E) CCK-8

and EdU (bar value = 120 μm) assays evaluated the proliferation in different groups. (F) TUNEL (bar value = 120 μm) assay measured the apoptosis of different groups. (G) Transwell (bar value = 50 μm) assay examined cell migration in different groups. **P < 0.01.

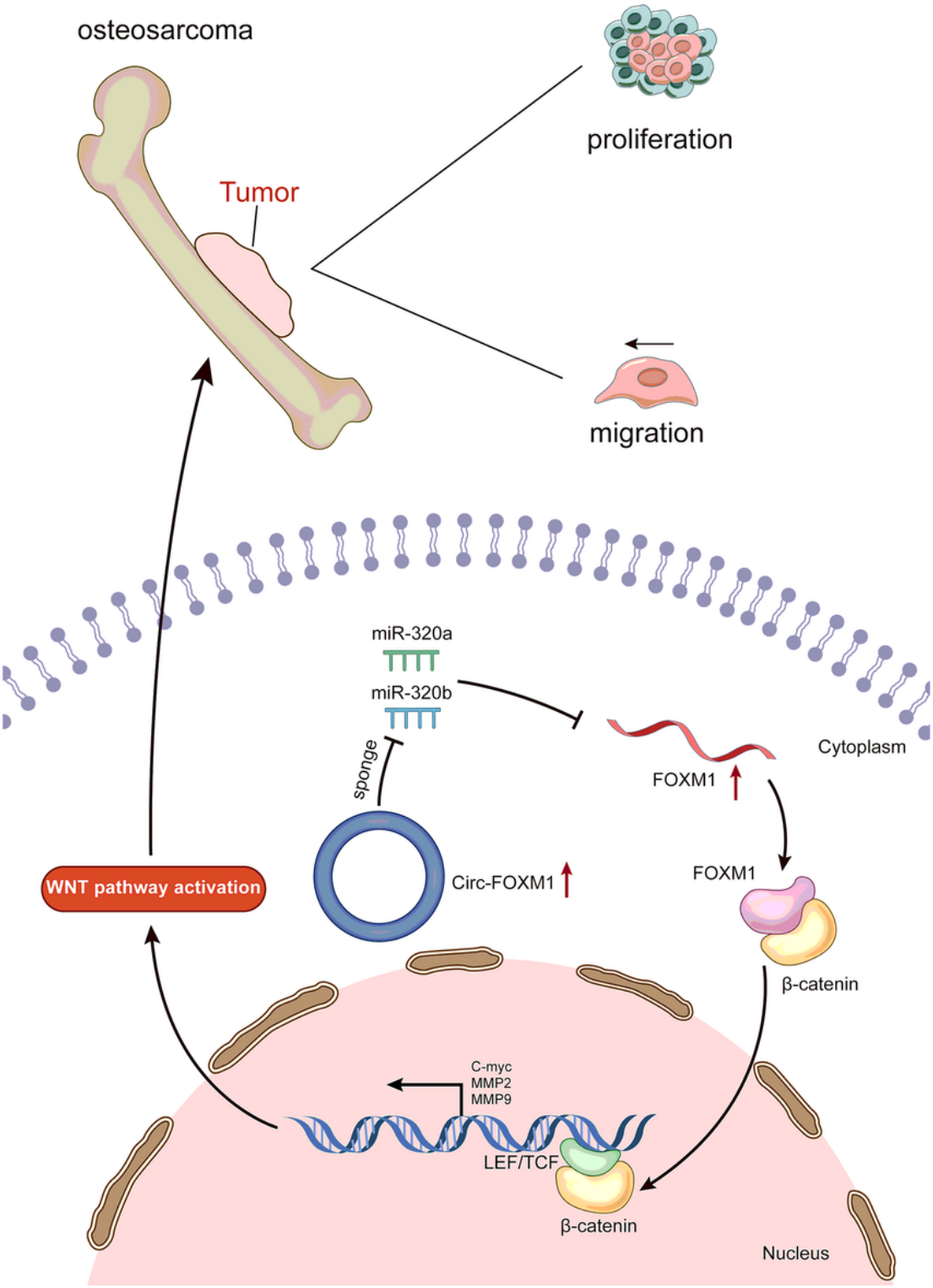

Figure 6

Graphical Abstract Circ-FOXM1 was upregulated in osteosarcoma cells. The upregulated circ-FOXM1 accelerated osteosarcoma cell proliferation and migration through sponging miR-320a and miR-320b to

upregulate FOXM1 expression and activate Wnt signaling pathway.

Supplementary Files

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