circ-ANKS1B facilitates osteosarcoma cell proliferation and invasiveness via upregulating Ki-67 expression by sponging miR-149-5p

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Abstract

Background: Mounting evidence has shown that Circular RNAs (circRNAs) are associated with initiation and progression of human cancers. However, the expression and function of circRNAs in the development of osteosarcoma (OS) remain unclear.

Methods: In this study, the expression profiles of circRNA circ-ANKS1B in OS were identified through qRT-PCR and in situ hybridization (ISH). The relationships between expression of circ-ANKS1B and clinicopathological features of OS patients was analyzed. Cell proliferation potential, migration and invasion ability of OS cells were evaluated through CCK8, colony formation, transwell and wound healing assays *in vitro*. Xenograft nude mouse experiment was performed to investigate tumor formation ability *in vivo*. The downstream regulated microRNA of circ-ANKS1B was proved via qRT-PCR and dual-luciferase reporter.

Results: We found expression of circ-ANKS1B was markedly overexpressed in OS cell lines and tumor tissues, and high expression of circ-ANKS1B was correlated with advanced TNM stage and poor prognosis of OS patients. The results of functional experiments showed that depletion of circ-ANKS1B could inhibit proliferation and invasion ability of OS cells *in vitro*, and tumor formation ability *in vivo*. Further mechanistic studies revealed that circ-ANKS1B could sponge endogenous miR-149-5p and partially reversed the suppressive effect of miR-149-5p in OS cells. Furthermore, we demonstrated that circ-ANKS1B regulated Ki-67 expression by sponging miR-149-5p. **Conclusions:** In summary, our data showed that circ-ANKS1B accelerated cell growth and invasion in OS by sponging miR-149-5p and regulating Ki-67.

Background

Osteosarcoma (OS) is one of the most common primary skeletal highly aggressive tumors in children and adolescents[1]. OS frequently originates from transformed cells characterized by immature bone formation or malignant osteoid producing[2]. Although surgical resection combined with neoadjuvant chemotherapy had greatly improved the prognosis of OS, patients with recurrence and metastasis remain suffer a dismal prognosis and the most clinical challenging burdens [3]. It is, hence, urgently needed to identify the molecular mechanisms underpinning the malignant transformation of this deadly disease.

Circular RNAs (circRNAs) are proved to be a special type of non-coding RNAs [4, 5]. Although increasing number of novel circRNAs have been identified, but their biological functions remain largely unknown. Promising evidences have proven that circRNA participates in various physiological and pathological processes[6]. Additionally, several circRNAs have been reported to be closely associated with tumor progression [7, 8]. circRNAs could indirectly regulate gene expression and cancer progression through the competitive endogenous RNAs (ceRNAs) manner [7]. For instance, circRNA_000284 promotes cervical cancer cell aggressive behaviors by sponging miR-506[9]. In colorectal cancer, circ-CBL.11 suppresses

cancer growth through sponging miR-6678-5p[10]. However, the interaction mechanisms of circRNAs and miRNA are waiting for be further explored in OS.

Previous studies also confirmed that multiple circRNAs contribute to the OS progression. Previously, Zhou et al. reported that circRNA-0008717 promoted OS cell proliferation, migration and invasion ability via specific sponging with miR-203[11]. Zhang *et al*. proved that circRNA UBAP2 could promote OS progression via inhibiting miR-143 through sponge adsorption[12]. Huang *et al.* showed that circRNA-0002052 inhibited OS cell proliferation, migration and invasion through affecting activation of miR-1205/APC2 axis[13]. Moreover, it has been suggested that circNASP and circRNA-100876 were involved in the progression of OS [14, 15]. Therefore, it is essential to reveal the functional role of circRNAs in OS.

In this study, we observed that expression of circ-ANKS1B was elevated in OS tissues and cell lines. Overexpression of circ-ANKS1B expression was correlated with the dismal prognosis of OS patients. Additionally, suppression of circ-ANKS1B could inhibit proliferation, migration and invasion ability *in vitro*, tumor formation ability *in vivo*. Moreover, circ-ANKS1B could suppress the Ki-67 level of OS cells via sponging with miR-149. Our findings revealed the crucial role of the circ-ANKS1B/miR-149/Ki-67 axis in OS progression and provide a potential novel therapeutic target for treatment of OS.

Materials And Methods

Human specimens

The human study was approved by the Institutional Ethical Board of the First Affiliated Hospital of Zhengzhou University, Zhengzhou Central Affiliated Hospital to Zhengzhou University, the Affiliated Cancer Hospital of Zhengzhou University. All adult patients or parents/legally authorized representatives of participants under age 18 signed the written informed consents before enrolled into the study. All of recruited patients were diagnosed between 2008-2013 were totally enrolled. Surgical removed samples included adjacent benign tissue and primary tumor were fixed in 10% formalin then embedded with paraffin for immunohistochemistry analyses.

Construction of tissue microarray (TMA)

We combined all OS samples from the above mentioned three hospital to create a relative large-scale cohort with more OS samples[16, 17]. The osteosarcoma TMA used in present study was constructed with 120 osteosarcoma specimens and 65 normal specimens.

Cell lines and cell culture

MG63, U2OS, and 143B were purchased from the American Type Culture Collection (Rockville, USA). Normal osteoblast cells HOBC and HFOB was purchased from Shanghai Institute for Biological Science (Shanghai, China). All cell lines were maintained at 37°C in a humidified incubator (Thermo Fisher Scientific, USA) containing 5% CO2.

Immunohistochemical (IHC) and in situ hybridization (ISH)

IHC was performed as previous description[18]. ISH was performed through digoxygenin (DIG)-labeled circ-ANKS1B ISH probes as described previously[19]. circ-ANKS1B expression in OS tumor tissues and adjacent non-tumor tissues was detected by fluorescence. The intensities and proportion of circ-ANKS1B dyeing was calculated as following method: 1-3 scores were the low expression, while 3-5 scores were represented high expression.

Western blotting

Western blotting was performed as previous description [20]. And the primary antibodies were as following: Ki-67 (Proteintech, 27309-1-AP), GAPDH (Proteintech, 10494-1-AP).

Quantitative real- time PCR

Total RNA was obtained via the method of Trizol (Life Technologies, USA) extraction. And the target cDNA was synthesized through the PrimeScript RT reagent Kit (Promega, USA). Quantitative real-time PCR reactions were performed using SYBR Green kit (ABI, USA). The relative gene expression was normalized to control using $2^{-\Delta\Delta}$Ct method. The primers used in this study were listed in Supplemental Table 1.

Cell Transfection

MG63 or U2OS cells (1×10^6 cells/well) were seeded into a 6-well plate. 24 h later, cells were transiently transfected with the miR-149 mimics or negative control (NC), shRNA targeting circ-ANKS1B or negative control through lipofectamine 3000 (Invitrogen, USA). The RNAi sequences used in this study were listed in Supplemental Table 2.

CCK-8, Cell invasion and Colony formation assay

CCK-8, cell migration and invasion assays were performed according to previously described method[21].

Dual luciferase reporter gene assay

PmirGLO, pmirGLO-circ-ANKS1B or pmirGLO-circ-ANKS1B-mut vectors, pmirGLO-Ki-67 or pmirGLO-Ki-67-mut vectors were cotransfected with miR-149 by Lipofectamine™ 2000 Transfection Reagent (Invitrogen); 24 hours later, the luciferase intensity was measured and normalized to the renilla luciferase intensity using the Dual-Luciferase® Reporter Assay System (Promega).

Xenograft model

Male nude mice were ready for subcutaneously injection. U2OS cells stably transfected with sh-circ-ANKS1B or ctrl (5×10^6 cells) were inoculated subcutaneously into the flanks of the nude mice. Tumor size and volumes were measured every week. 4 weeks later, all the mice were sacrificed and the tumor

weights were measured. All animal experiments were approved by the Animal Care Committee of the First Affiliated Hospital of Zhengzhou University. All experimental procedures involving animals were strictly followed in accordance with the Guide for the Care and Use of Laboratory Animals as described previously[20].

Statistical analysis

All of the statistical analyses were performed using GraphPad Prism software. Two-tailed Student's t-tests were used to compare mean values of two groups. Two-way ANOVA was conducted to compare mean values of more than two groups. Pearson $\chi 2$ tests were adopted to analyze the association of circ-ANKS1B expression with clinicopathological variables. For the survival analysis, Kaplan-Meier plots and log-rank tests were used. Two-tailed Fisher's exact test was performed as appropriate. p value < 0.05 was considered as statistically significant difference.

Results

circ-ANKS1B is overexpressed in OS tissues and cell lines.

Based on human reference genome GRCh37/hg19, circ_0007294 is located at chr12:100166699-100175875 and is assumed to derive from the gene ANKS1B. Therefore, we termed hsa_circ_0007294 as "circ-ANKS1B". To detect the expression of circ-ANKS1B in OS, qRT-PCR was conducted in 30 OS tissues and paired adjacent non-tumor tissues. We observed that circ-ANKS1B was significantly overexpressed in OS tissues compared to matched nonneoplastic counterparts (**Fig. 1A, 1B**). Similarly, circ-ANKS1B level was also higher in OS cell lines, including MG63, U2OS and 143B cells than that in normal human osteoblast cell lines (HFOB and HOBC) (**Fig. 1C**). Subsequently, we verified circ-ANKS1B expression in OS TMA using the ISH assay. Based on the result of ISH staining, we divided circ-ANKS1B expression levels into five stratifications (**Fig. 1D**), then we found that circ-ANKS1B expression was overexpressed in OS tissues comparing with adjacent non-tumor tissues (**Fig. 1E**). Taken together, we proved that circ-ANKS1B expression is upregulated during OS tumorigenesis.

High expression of circ-ANKS1B predicts poor prognosis of OS patients

Then, we investigated the correlation between circ-ANKS1B expression and clinicopathological characteristics (**Table 1**). We found that circ-ANKS1B expression is significantly related to later TNM stage and large tumor size (**Fig. 2A, 2B**). Meanwhile, circ-ANKS1B expression was higher in metastatic tumors than in primary osteosarcoma tissues (**Fig. 2C**). Additionally, there was a remarkable tendency for increased circ-ANKS1B expression in recurrent OS tissues (**Fig. 2D**). Moreover, we found that OS patients who with overexpression of circ-ANKS1B always lead to poor overall survival and disease-free survival (**Fig. 2E, 2F**). Furthermore, circ-ANKS1B expression was found to be independent prognostic factors by multivariate analysis (**Table 2**). These findings indicated that circ-ANKS1B might contribute to OS progression and spired us to investigate the biological mechanism of circ-ANKS1B.

Knockdown of circ-ANKS1B suppresses OS proliferation and invasion *in vitro*

To analyze the potential function of circ-ANKS1B, we performed functional experiments. Because circ-ANKS1B level was the highest in U2OS and 143B cells, we knocked down the expression of circ-ANKS1B in these two cell lines using specific shRNAs targeting circ-ANKS1B (**Fig. 3A, 3B**). Notably, circ-ANKS1B silencing did not affect the expression of its linear mRNA ANKS1B (**Fig. 3C**). Subsequently, to evaluate the function of circRNA-000284 on cell proliferation, CCK-8 assay was conducted and the results showed that suppression of circ-ANKS1B could dramatically inhibit the cell proliferation in contrast to control cells (**Fig. 3D**). And we also observed that knockdown of circ-ANKS1B could suppress the colony formation and DNA synthesis rate compared with that in the negative control (**Fig. 3E, 3F**). Additionally, results of migration and invasion assays suggested that the invasive and migratory capacity was significantly inhibited by transfection of sh-circ-ANKS1B (**Fig. 3G, 3H**). In a word, these findings indicated that circ-ANKS1B acted as a important role during progression of OS cells.

Depletion of circ-ANKS1B suppresses OS growth *in vivo*

To further determine whether circ-ANKS1B could affect OS tumorigenesis *in vivo*, we conducted stable circ-ANKS1B-depleted U2OS cells and injected them subcutaneously into the flanks of nude mice (**Fig. 4A**). The circ-ANKS1B-depleted group (sh-circ-ANKS1B) mice developed weaker luciferase signal compared with the control group (NC) (**Fig. 4B**). At five weeks after inoculation, tumor volume and weight were noticeably decreased in the circ-ANKS1B silencing group compared with the NC group (**Fig. 4C and 4D**). Moreover, Ki-67 IHC staining intensive from the xenograft tumors also declined in the circ-ANKS1B-depleted group (**Fig. 4E**), suggesting that suppression of circ-ANKS1B could inhibit progression of OS *in vivo*.

circ-ANKS1B functions as a sponge for miR-149-5p

Given that it has been widely identified that circRNAs exerted biological function mainly through sponging to specific miRNAs, then by using Circinteractome tool (https://circinteractome.nia.nih.gov/), we identified that miR-149-5p, a known tumor suppressor in OS, contains complementary sequence to circ-ANKS1B (**Fig. 5A**). In addition, Pearson correlation analysis suggested that circ-ANKS1B was negatively correlated with miR-149-5p expression (**Fig. 5B**). Additionally, we observed that miR-149-5p was evidently low-expressed in OS tissues and cell lines (**Fig. 5C, 5D**). Moreover, as shown in **Fig. 5E and 5F**, overexpression circ-ANKS1B significantly inhibited miR-149-5p expression, while circ-ANKS1B-depleted dramatically increased miR-149-5p expression level. More importantly, luciferase reporter assay was performed, and results confirmed miR-149-5p could directly interact with circ-ANKS1B **(Fig. 5G)**. Taken together, these findings suggest that circ-ANKS1B directly bind to miR-149-5p and inhibit its activity.

circ-ANKS1B affects proliferation and invasion abilities of OS cells via miR-149-5p

We further examined whether circ-ANKS1B functions as an oncogene by sponging with miR-149-5p, U2OS or 143B cells were transfected with miR-149-5p mimics or mock control, with/without circ-ANKS1B overexpression vector. Cell proliferation and colony formation assays indicated that overexpression of miR-149-5p could inhibit cell proliferation, while overexpression of circ-ANKS1B partially reversed the suppressive effect of miR-149p-5p in U2OS or 143B cells (**Fig. 6A and 6B**). Consistently, transwell assay showed that circ-ANKS1B reversed the inhibition effect of miR-149-5p on cell invasion ability (**Fig. 6C**). Based on these results, circ-ANKS1B affected the proliferation and invasion abilities of OS cells, at least partly by miR-149-5p.

circ-ANKS1B-mediated miR-149 negatively regulates Ki-67 in OS

Recently studies revealed that circRNA could exert biological function by involving in circRNA-miRNA-mRNA crosstalk. Based on this, we performed TargetScan to identify the potential gene targeting by miR-149-5p. We found the 3'UTR of Ki-67, a well-known tumor proliferation marker, bears one potential miR-149-5p binding sites (**Fig. 7A**). In addition, we found expression of Ki-67 was negative correlated with miR-149-5p in OS samples (**Fig. 7B**). Luciferase reporter assay was performed to prove the effect of miR-149-5p on Ki-67 mRNA level. And the results showed that miR-149-5p could obviously inhibit luciferase activity of Ki-67-3'UTR wild-type compared with negative control (**Fig. 7C**). Furthermore, we proved that miR-149-5p could significantly regulate the mRNA and protein levels of Ki-67 in OS cells (**Fig. 7D and 7E**). Co-transfected with circ-ANKS1B and miR-149 mimic could reverse the function of circ-ANKS1B on Ki-67 expression (**Fig. 7E**). Consistently, Ki-67 was positively correlated with expression of circ-ANKS1B in OS tissues (**Fig. 7F**).

Functional experiments showed that Ki-67 overexpression reversed the inhibition effect of circ-ANKS1B on cell proliferation (**Fig. 8A, 8B**), as well as invasion ability (**Fig. 8C**). These finding indicated that circ-ANKS1B sponges miR-149-5p, which subsequently allows for Ki-67 translation. circ-ANKS1B promotes the proliferation of OS partly via the circ-ANKS1B/miR-149/Ki-67 axis.

Discussion

Comparing with miRNA and lncRNA, circRNA displayed greater advantages as a biomarker for tumor diagnosis due to highly conserved and stable feature[7]. Increasing evidence have revealed that abnormal expression of circRNAs could significantly affect the progression of tumors through specific sponging with miRNA[8]. Recent studies proved that circRNA_0008035 was markedly overexpressed in gastric cancer tissue, and suppression of circRNA_00080350 could substantially affect the proliferative rate of cells by regulating miR-375/YBX1[22]. Other studies also proved that knockdown of circ_0002024 in bladder cancer could significantly suppresses cell proliferation and invasion by sponging miR-197-3p[23]. circRNA_0103809 promoted progression of hepatocellular carcinoma through regulating activation of miR-490-5p/SOX2 signaling pathway[24]. All these findings demonstrated that circRNAs acted as important role during initiation and progression of tumors.

In this study, we uncovered that circRNA circ-ANKS1B was up-regulated in the OS tissues and cell lines for the first time. We found that high level of circ-ANKS1B was significantly associated with advanced TNM stage, highly metastasis and recurrence incidence. Additionally, high expression of circ-ANKS1B always lead to poor prognosis of OS patients. Functional assays revealed that circ-ANKS1B silencing significantly decreased cell proliferation, migration and invasion abilities *in vitro*, and tumor formation ability *in vivo*. These findings indicated that circ-ANKS1B may act as a biomarker or therapeutic target for OS.

Recent studies have proved that circRNAs affected progression of tumor through specific sponging with miRNAs[6]. In present study, we demonstrated that miR-149-5p was target sponging by circ-ANKS1B through using bioinformatics analysis and luciferase reporter assay. Many studies have proved that miR-149 could act as a tumor suppressor role during progression of solid tumor [25, 26]. Moreover, miR-149 was proved markedly downregulated in human OS tissues and could significantly inhibit proliferation and migration of OS cells[27]. To explore the deep molecular mechanism between circ-ANKS1B and miR-149-5p, functional experiments were conducted to investigate the interaction between them during progression of OS. Firstly, we found that expression of circ-ANKS1B and miR-149 was negative correlation both in human OS tissues and cell lines. Secondly, we also confirmed that circ-ANKS1B inhibited expression of miR-149-5p in OS cells. More importantly, the inhibitory effect of proliferation and invasion caused by miR-149-5p could be recovered by circ-ANKS1B overexpression, demonstrating that circ-ANKS1B exerted oncogene role in OS via negatively targeting miR-149-5p.

It is widely acknowledged that miRNAs could regulate gene expression post-transcriptionally[28]. Hence, we further investigated the downstream mechanism of miR-149-5p in OS cells. Through bioinformatics analysis and functional experiments, we identified Ki-67 as a potential target of miR-149-5p. Moreover, we also confirmed that circ-ANKS1B promoted Ki-67 expression via inhibiting miR-149-5p in OS cells. Ki-67 is commonly used for evaluating proliferate ability of cells because its abnormal expression is associated with cell growth[29]. High level of Ki67 had been proved to reflect the high cellular proliferation rate in most aggressive tumors, including OS[30]. To determine the effect of circ-ANKS1B in miR-149-5p /Ki-67 signaling axis, we preformed rescued experiments. We found both mRNA and protein levels of Ki-67 were decreased in OS cells under the condition of miR-149-5p mimics, while such phenomenon was restored by adding the circ-ANKS1B. Additionally, we testified the decreased Ki-67 expression in tumors from mice treated with circ-ANKS1B-delepted OS cells. All these results suggested that circ-ANKS1B positively regulated Ki-67 by interacting with miR-149-5p. Nevertheless, considering the specific configuration and ambiguous function, the more detailed molecular mechanism of circ-ANKS1B involved in the tumorigenesis of OS is still inconclusive and needs to be further studied.

Conclusion

In summary, the expression of circ-ANKS1B was upregulated in OS cell lines and tissues. Furthermore, its expression was associated with poor prognosis of OS patients. circ-ANKS1B silencing significantly suppressed OS cell proliferation and invasion *in vitro* and *in vivo*. Mechanistically, circ-ANKS1B could

promote the expression of Ki-67 by acting as a sponge of miR-149-5p. These results demonstrated that the circ-ANKS1B/miR-149-5p/Ki-67 axis may play critical regulatory roles in the pathogenesis of OS and may serve as a novel therapy target in OS.

Declarations

Authors' contributions

H.Y, Y.J.L and L.M.W conceived and designed the study. H.Y, C.L, Y.L.L, L.Z and Y.Q.W performed experiments; Y.Z, C.L and M.Z collected the clinical samples; All authors reviewed and approved the manuscript.

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Disclosure of Potential Conflict of Interest

The authors report no conflicts of interest in this work.

Availability of data and materials

All the data and material could be traced from the paper we have published before

Consent for publication

All the listed authors have participated actively in the study, and have seen and approved the submitted manuscript.

Ethics approval and consent to participate

The study was approved by the human ethic committee of The First Affiliated Hospital of Zhengzhou University. All patients provided written informed consent and the project was in accordance with the Helsinki Declaration of 1975.

Abbreviations

Hepatocellular Carcinoma (HCC); overall survival (OS)；Tissue microarrays (TMA); Quantitative real-time PCR (qRT-PCR); Cell counting kit-8 (CCK-8); 3'UTR (3' untranslated region); The gene set enrichment analysis (GSEA)；standard deviation (SD);

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Tables

Table 1. Correlation of clinic-pathological features with circ-ANKS1B expression in osteosarcoma TMA corhort

Clinicopathological Features Variables		circRNA-0007294 expression		*P-value*
		Low expression (n=57)	High expression (n=63)	
Age (years)	≤18	32 (56.1\%)	35 (55.6\%)	1.000
	>18	25 (43.9\%)	28 (44.4\%)	
Gender	Female	36 (63.2\%)	35 (55.6\%)	0.509
	Male	21 (36.8\%)	28 (44.4\%)	
Primary tumor site	Tibia/femur	26 (45.6\%)	29 (46.0\%)	1.000
	Elsewhere	31 (54.4\%)	34 (54.0\%)	
Histologic subtypes	chondroblastic	14 (24.6\%)	11 (16.4\%)	0.620
	Fibroblastic	21 (36.9\%)	24 (38.2\%)	
	Osteoblastic	4 (10.8\%)	8 (9.1\%)	
	Mixed	18 (27.7\%)	20 (36.4\%)	
TNM stage	Stage I/II	49 (86.0\%)	41 (65.1\%)	**0.015**
	Stage III	8 (14.0\%)	22 (34.9\%)	
Distant metastasis	Absent	45 (78.9\%)	21 (33.3\%)	**0.000**
	Present	12 (21.1\%)	42 (66.7\%)	
Recurrence	No	39 (68.4\%)	22 (34.9\%)	**0.000**
	Yes	18 (31.6\%)	41 (65.1\%)	
Tumor size	≤7.5cm	49 (86.0\%)	24 (38.1\%)	**0.000**
	>7.5cm	8 (14.0\%)	39 (61.9\%)	
Chemotherapy response	Good	21 (36.8\%)	26 (41.2\%)	0.645
	Poor	20 (35.1\%)	19 (30.1\%)	
	NA	16 (28.1%)	18 (28.5%)	

[a]Bold values indicate statistical significance, $P < 0.05$

Table 2. Correlation of clinic-pathological features with circ-ANKS1B expression in OS cohort

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Univariate and multivariate analysis of overall survival in OS patients (n=120)						
Age (>18 vs ≤18)	1.167	0.906-1.463	0.188			
Gender (Male *vs* Female)	1.017	0.882-1.022	0.256			
Primary tumor site (Tibia/femur *vs* Elsewhere)	0.905	0.721-1.081	0.376			
TNM stage (III/IV *vs* I/II)	3.584	2.473-4.786	**0.001**	3.097	2.469-3.902	**0.003**
Distant metastasis (Present *vs* Absent)	3.081	2.989-4.326	**0.005**	2.601	2.022-3.258	**0.034**
Recurrence (Present *vs* Absent)	3.050	2.303-3.377	**0.011**	2.598	1.978-3.341	**0.026**
Tumor size (>7.5cm *vs* ≤7.5cm)	1.604	1.279-2.017	0.067			
Chemotherapy response (Poor *vs* Good)	2.484	1.988-2.807	**0.022**	1.685	1.359-1.984	0.156
circ-ANKS1B expression (High *vs* Low)	2.590	1.989-3.067	**0.008**	2.559	2.022-3.258	**0.027**
Univariate and multivariate analysis of disease-free survival in OS patients (n=120)						
Age (>18 vs ≤18)	1.047	0.783-1.247	0.315			
Gender (Male *vs* Female)	0.970	0.878-1.155	0.375			
Primary tumor site (Tibia/femur *vs* Elsewhere)	0.913	0.75-1.144	0.283			
TNM stage (III/IV *vs* I/II)	3.256	2.479-3.651	**0.023**	2.987	2.278-3.755	**0.005**
Distant metastasis (Present *vs* Absent)	2.732	2.008-3.196	**0.019**	3.019	2.330-3.606	**0.031**
Recurrence (Present *vs* Absent)	2.219	2.058-2.717	**0.016**	2.135	1.955-3.347	**0.027**
Tumor size (>7.5cm *vs* ≤7.5cm)	1.580	1.385-1.895	0.089			
Chemotherapy response (Poor *vs* Good)	2.127	1.904-2.555	**0.040**	1.795	1.438-2.214	0.195
circ-ANKS1B expression (High *vs* Low)	2.365	2.030-3.206	**0.026**	2.426	2.124-2.703	**0.019**

Figures

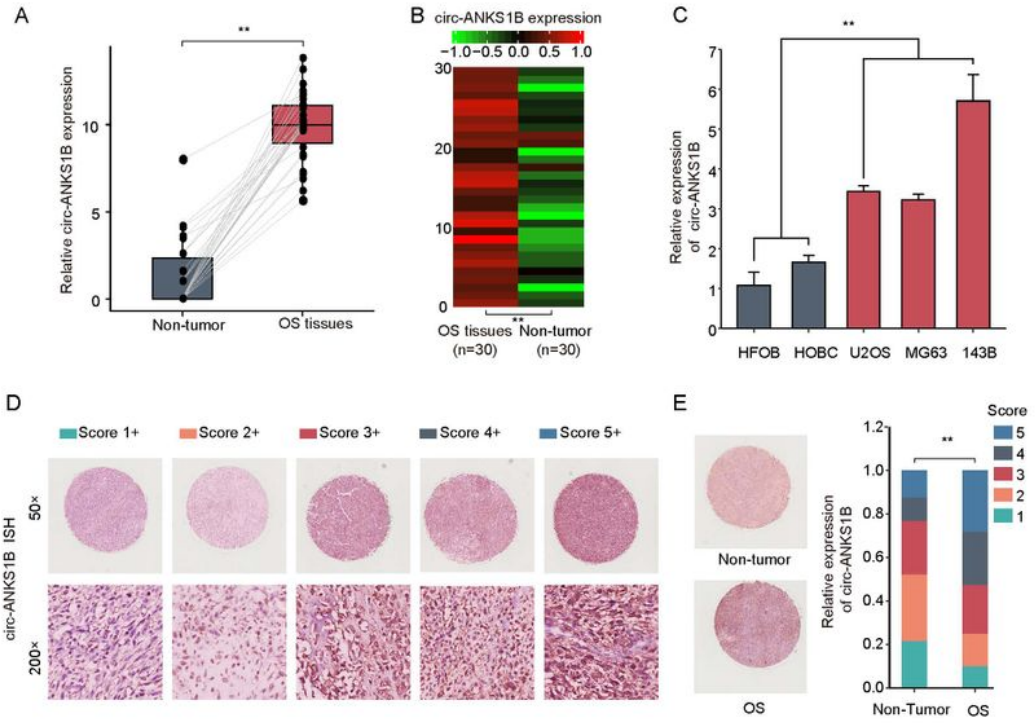

Figure 1

circ-ANKS1B expression is upregulated in OS tissues and cell lines (A-B) Relative expression of circ-ANKS1B in human OS tissues (n=30) compared with corresponding adjacent normal tissues (n=30). circ-ANKS1B expression was examined by qPCR and normalized to GAPDH expression. (C) circ-ANKS1B expression levels of OS cell lines (U2OS, MG63 and 143B), compared with that in human osteoblast cells (HFOB and HOBC). (D) Representative circ-ANKS1B staining patterns via ISH assays. (E) circ-ANKS1B

expression was significantly higher in OS tissues (n = 120) compared with that in non-tumor tissues (n = 65). *p < 0.05, **p < 0.01.

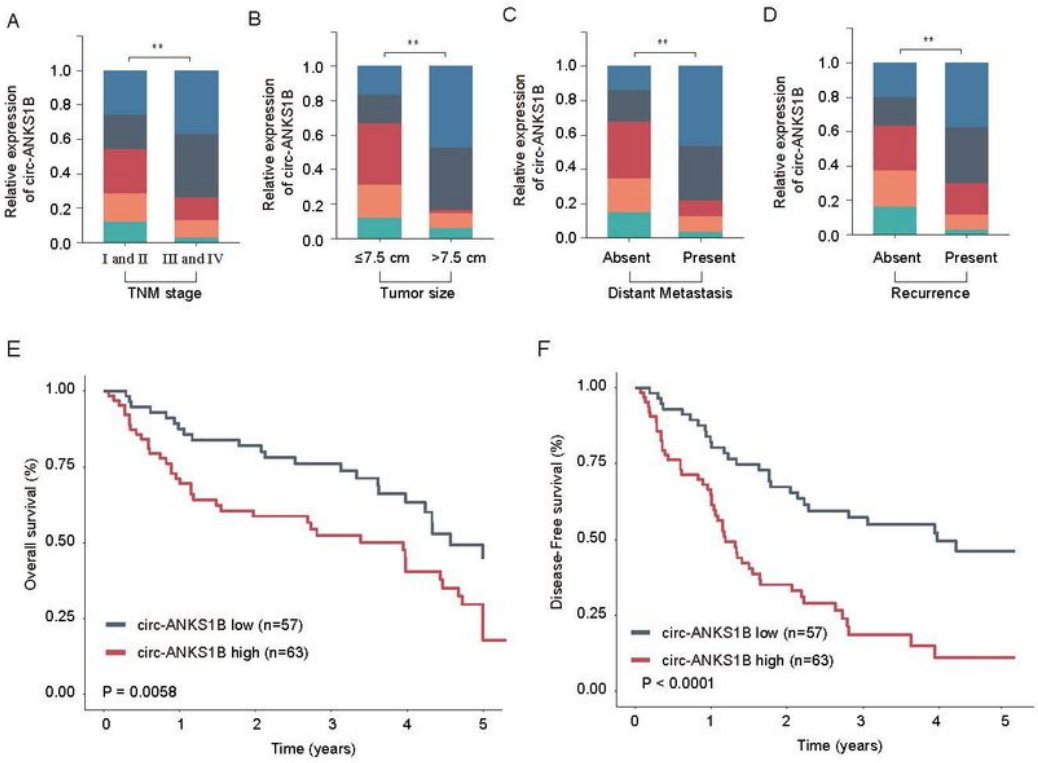

Figure 2

circ-ANKS1B expression predicts OS patients' survival. The correlation of circ-ANKS1B expression level with TNM stage (A), tumor size (B), distant metastasis (C) and recurrence status (D). Kaplan-Meier

analysis revealed that high expression of circ-ANKS1B was related to poorer overall survival (E) and disease-free survival (F) of OS patients. *p < 0.05, **p < 0.01.

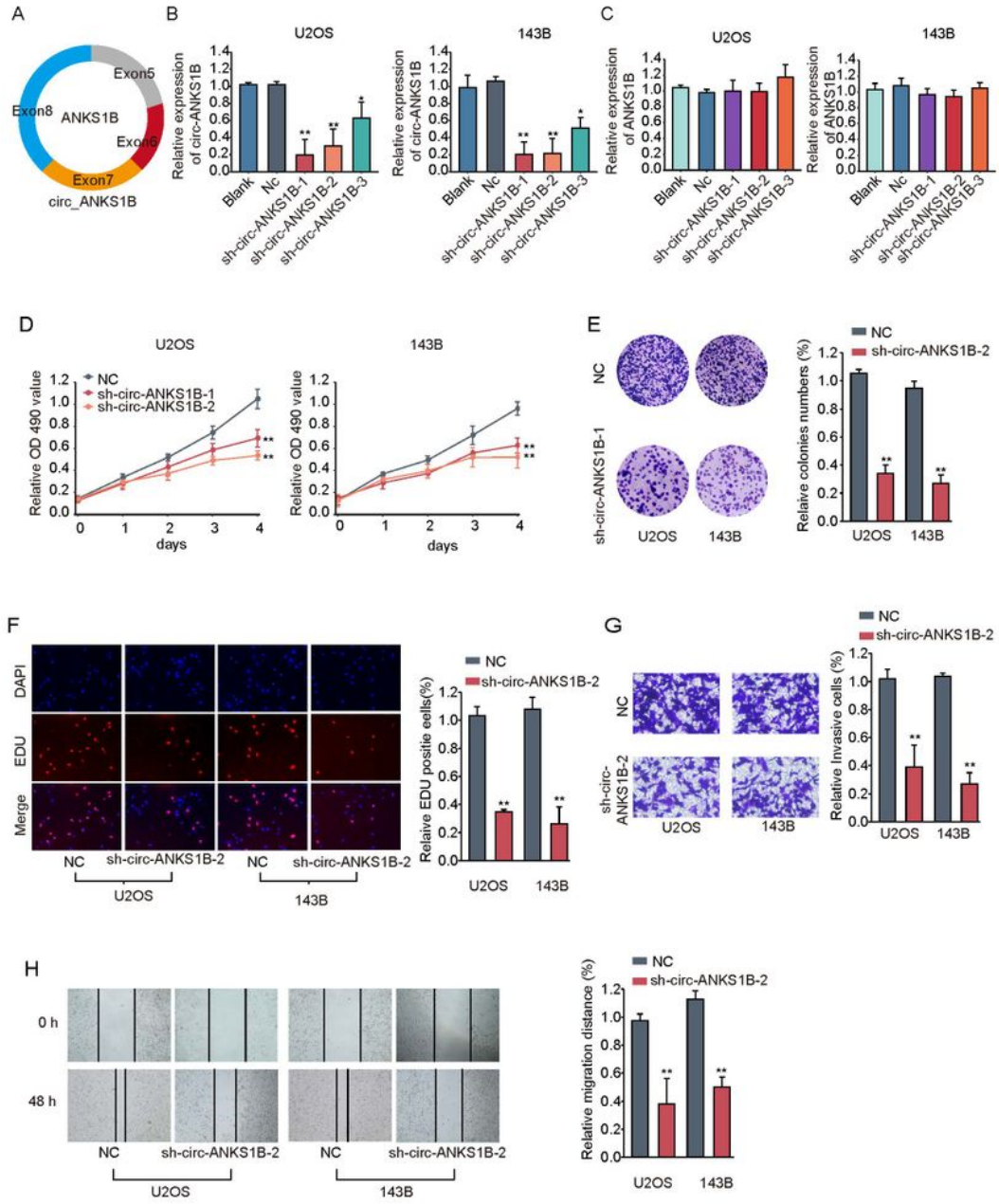

Figure 3

Silencing of circ-ANKS1B inhibits OS cell proliferation, migration and invasion in vitro. (A-C) Expression levels of circ-ANKS1B and its linear mRNA ANKS1B were detected by qRT-PCR in U2OS and 143B cells transfected with shRNAs targeting circ-ANKS1B (sh-circ-ANKS1B-1/2/3) or negative control

oligonucleotide (NC). (D) CCK-8 assay showed that the proliferation of U2OS and 143B cells was inhibited with circ-ANKS1B silencing. (E) Colony formation assays suggested that circ-ANKS1B knockdown inhibited the colony-forming ability of U2OS and 143B cells. (F) Edu assay showed that the DNA synthetics of U2OS and 143B cells was inhibited with circ-ANKS1B silencing. (G, H) Transwell and wound healing assays showed that circ-ANKS1B knockdown significantly inhibited the migration and invasion of U2OS and 143B cells. Data are listed as means ± s.d. of at least three independent experiments. *p < 0.05, **p < 0.01.

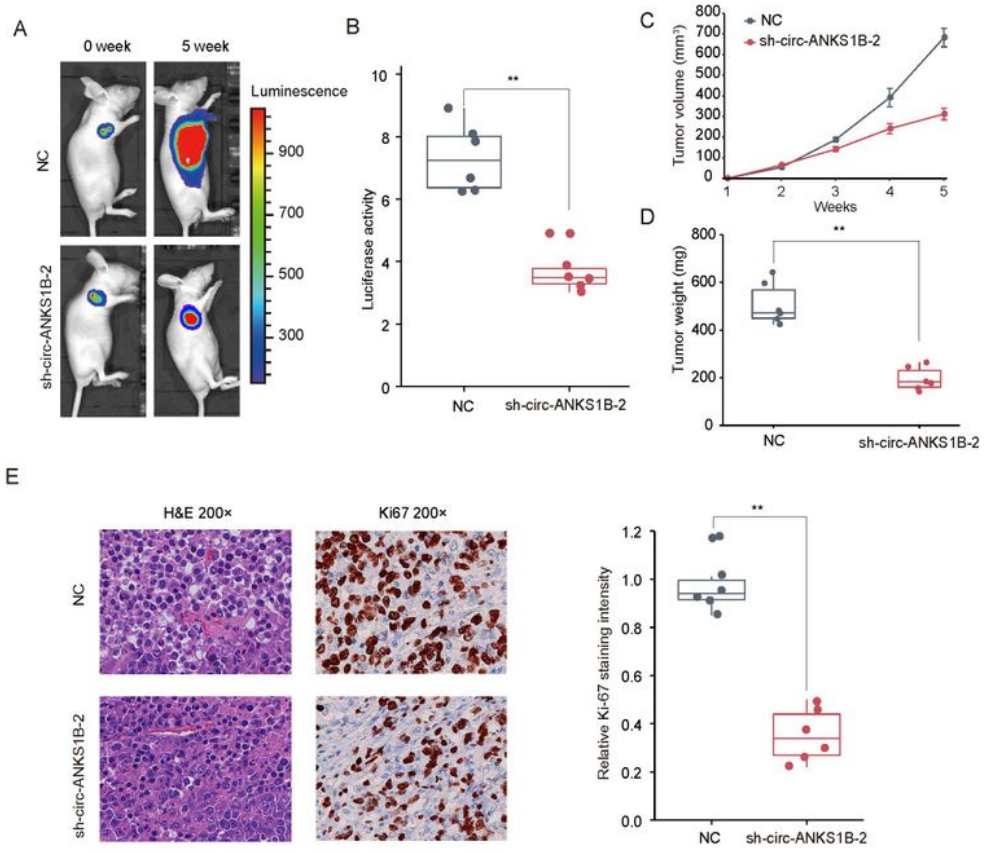

Figure 4

Knockdown of circ-ANKS1B suppresses OS tumorigenesis in vivo. U2OS cells stably transfected with sh-circ-ANKS1B (sh-circ-ANKS1B-2) or negative control (NC) were implanted subcutaneously into nude mice and tumor growth was monitored. (A) Representative living image photos of nude mice. (B) The relative photon flux of tumors from NC or sh-circ-ANKS1B-2 group was analyzed. n = 6 per group. (C, D) The relative tumor volume and tumor weight of tumors from NC or sh-circ-ANKS1B group was analyzed. n = 6 per group. (E) Representative IHC staining and the expression levels of Ki-67 in tumor tissues from NC or sh-circ-ANKS1B-2 group. *p < 0.05, **p < 0.01.

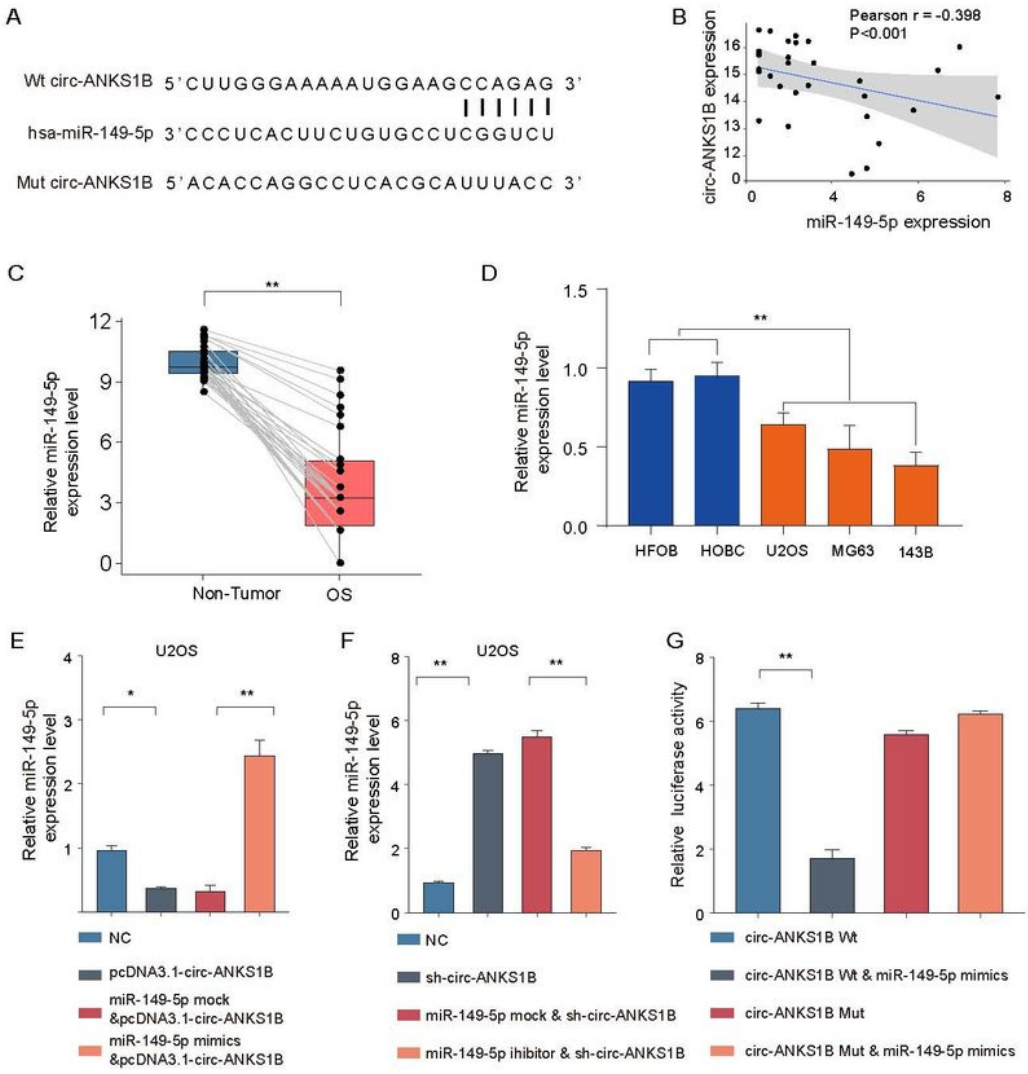

Figure 5

The interaction between circ-ANKS1B and miR-149-5p. (A) The putative binding sites of miR-149-5p on circ-ANKS1B are predicted. (B) The correlation between miR-149-5p and circ-ANKS1B expression in OS tissues. (C) RT-qPCR analysis of miR-149-5p expression levels in OS tissues (n=30) compared with corresponding adjacent normal tissues (n=30). (D) miR-149-5p expression levels of OS cell lines (U2OS, MG63 and 143B), compared with that in human osteoblast cells (HFOB and HOBC). RT-qPCR analysis of

miR-149-5p expression levels in U2OS and 143B cells after circ-ANKS1B overexpression (E) or silencing (F). (G) Dual-luciferase reporter assay showed the luciferase activity of the combination between miR-149-5p and circ-ANKS1B. *p < 0.05, **p < 0.01.

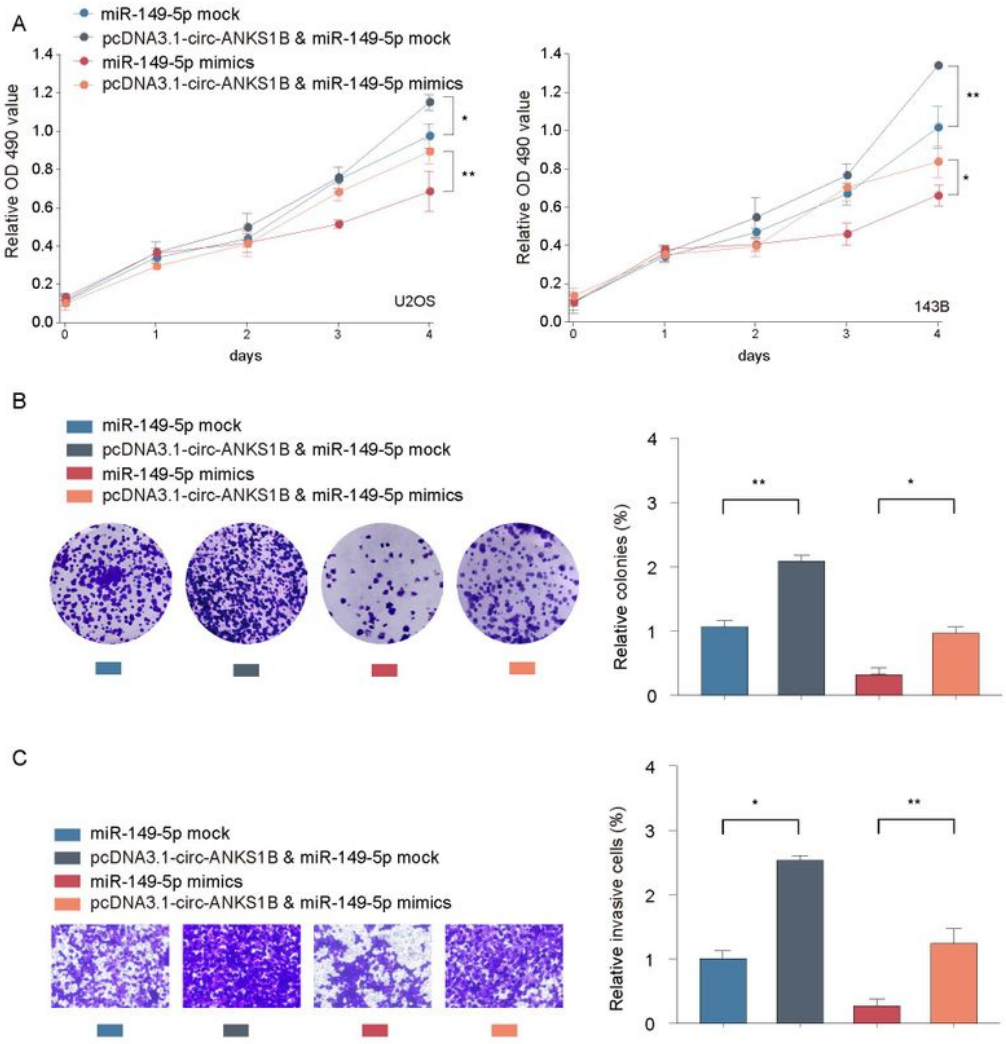

Figure 6

Overexpression of circ-ANKS1B partially reverses the suppressive effect of miR-149-5p in OS cells. U2OS and 143B cells were transfected with miR-149-5p mock or miR-149-5p mimics, with or without circ-

ANKS1B overexpression vector. (A) Cell proliferation of U2OS and 143B was analyzed at indicated time points by CCK-8 kit. Colony formation (B) and cell invasion ability (C) of U2OS was analyzed by colony formation assay and transwell assay, respectively. *p < 0.05, **p < 0.01.

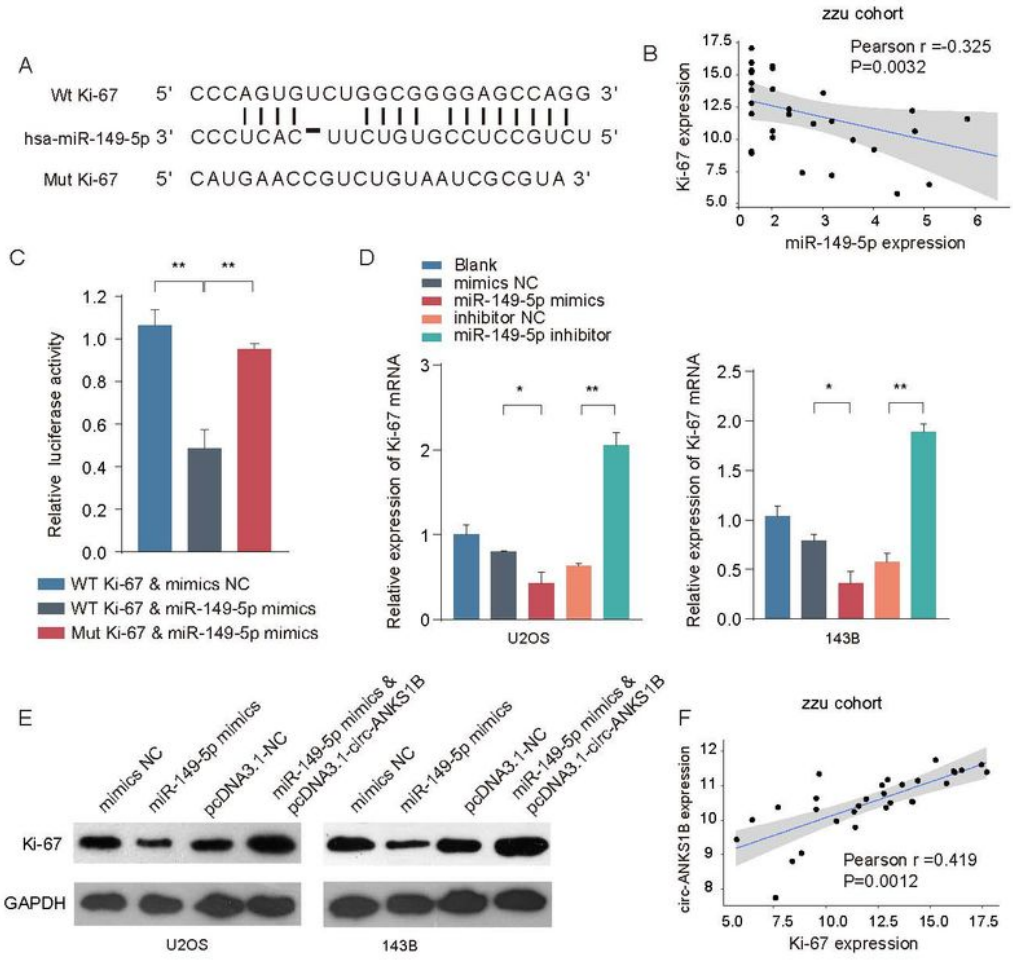

Figure 7

circ-ANKS1B inhibits Ki-67 expression by regulating miR-149-5p (A) The putative binding sites of miR-149-5p on Ki-67 are predicted. (B) Correlation of the Ki-67 mRNA expression level between miR-149-5p

expression. (C) Dual-luciferase reporter assay showed the luciferase activity of the combination between miR-149-5p and Ki-67. The mRNA expression level (D) and protein level (E) of Ki-67 expression was significantly decreased by miR-149-5p mimics, but this effect could be restored by circ-ANKS1B overexpression. (F) Correlation of the Ki-67 mRNA expression level and circ-ANKS1B expression. *p < 0.05, **p < 0.01.

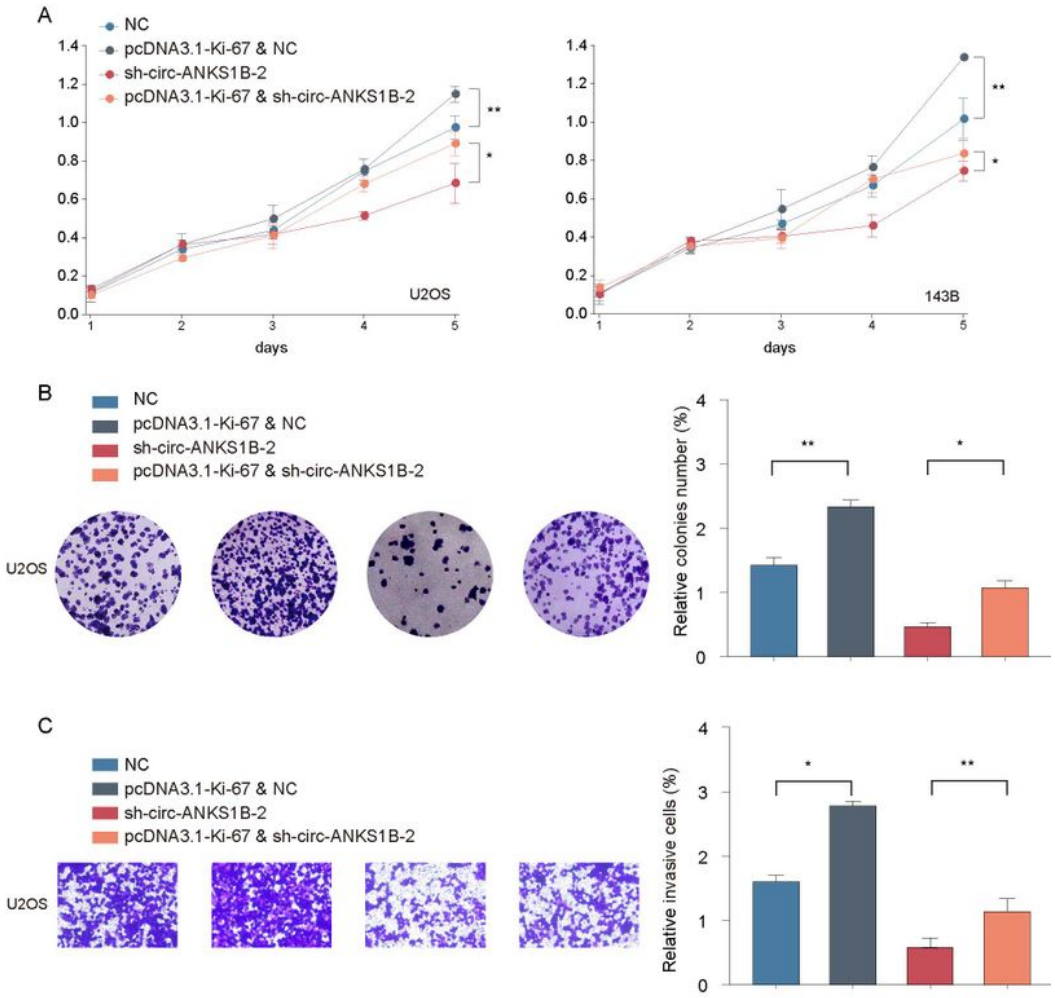

Figure 8

circ-ANKS1B promote OS cell proliferation and invasion through upregulating Ki-67. U2OS and 143B cells were transfected with sh-circ-ANKS1B-2 or NC, with or without pcDNA3.1 overexpressed Ki-67. (A) Cell proliferation of U2OS and 143B was analyzed at indicated time points by CCK-8 kit. (B) Colony formation and cell invasion ability (C) of U2OS was analyzed by colony formation assay and transwell assay, respectively. *p < 0.05, **p < 0.01.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryTable12.docx