Circ_001422 aggravates osteosarcoma progression through targeting miR-497-5p/E2F3 axis

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Case Report

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Abstract

Circ_001422 has been confirmed to be involved in regulating osteosarcoma progression, but its specific mechanism has not been clearly studied. This work aimed to analyze circ_001422's role in osteosarcoma (OS) cell biological behaviors and the possible molecular mechanisms. This work carried out RT-qPCR for detecting circ_001422, E2F3 and miR-497-5p levels, whereas CCK-8 together with Transwell assays for measuring cell growth, migration as well as invasion abilities. Relation of miR-497-5p with E2F3, as well as circ_001422 with miR-497-5p was analyzed through dual-luciferase reporter gene assay. Protein level was identified by westernblot. According to our results, circ_001422 expression within osteosarcoma tissue significantly increased compared with corresponding healthy samples. Inhibition of circ_001422 significantly decreased OS cell growth, invasion and migration. From mechanism research, miR-497-5p was proved as circ_001422's target, and E2F3 was miR-497-5p's target. Besides, miR-497-5p down-regulation or E2F3 overexpression abolished circ_001422 inhibition-mediated inhibition on OS cell proliferation, invasion and migration. Collectively, this study has first suggested circ_001422's role in enhancing OS proliferation, migration as well as invasion via miR-497-5p/E2F3 axis. Our results will offer new ideas and new anti-OS targets.

Introduction

Osteosarcoma (OS) has been the commonly seen bone cancer, with a high incidence and poor prognosis among pediatric and adolescent patients[1–2]. Osteosarcoma shows high aggressiveness and susceptibility to early metastases. For metastatic or advanced OS patients, their 5-year survival remains as low as 20%[3]. Recently, although neoadjuvant therapy and adjuvant chemotherapy have been widely used in clinical practice, they still cannot totally suppress cancer proliferation, so that OS still has a high mortality[4]. Consequently, discovering novel therapeutic methods and targets is urgently needed. Finding ideal biomarkers and targets for treating OS is important for improving the diagnosis and treatment of osteosarcoma.

Circular RNAs (circRNAs), the novel non-coding RNAs (ncRNAs), have been widely distributed within eukaryote cytoplasm[5]. Different from linear RNA molecules, circRNAs, as cyclic closed molecules, have a high degree of stability[6]. CircRNAs have multiple activities, like transcription and splicing modulation, RNA transport and protein binding[7, 8]. Numerous circRNAs are found to be abnormally expressed within different tumors, while their abnormal expression is closely related to tumor progression, acting as tumor suppressors or oncogenic genes within tumors[9–11]. To take an example, circ_0008259 can suppress OS occurrence and development via the miR-21-5p/PDCD4 axis[12]. Circ-LRP6 expression increases in OS cells as well as tissues, whereas its down-regulation can inhibit the OS cell growth, migration and invasion by miR-141-3p/HDAC4/HMGB1 axis[13]. Circ_001422, the newly reported circRNA, has been reported to promote OS tumorigenesis[14]. However, the mechanism of the involvement of circ_001422 in OS is not completely clear at present.

MicroRNAs (miRNAs) are able to modulate target levels through incomplete complementation with target gene 3'UTR, thereby regulating malignant biological behavior of cancer cells[15]. MiR-497-5p, belonging to miR-15 family, shows low expression within diverse tumor tissues like gastric cancer (GC), OS, hepatocellular carcinoma (HCC) as well as non-small-cell lung cancer (NSCLC), which is considered as the vital tumor suppressor[16–19]. However, the current functional research on miR-497-5p within OS remains lacking. In recent years, studies found that circular RNAs may be miRNAs' "molecular sponges" and inhibit their function[12–13]. For instance, as revealed by Ma and colleagues, circ_UBAP2 sponged miR-637 as the ceRNA to regulate HMGB2 expression to facilitate OS progression[20]. Zhang et al. showed that circ_0002137 modulated OS development via miR-433-3p/IGF1R axis[21]. As reported by Liu and coworkers, circ_0081001 upregulated BACH1 expression through miR-494-3p, thus promoting OS cell invasion and growth [22]. Here, miR-497-5p was reported as circ_001422's potential target. However, it remains unclear about circ_001422/miR-497-5p axis' effect on OS development.

E2F transcription factor 3 (E2F3), which belongs to the E2F transcription factor (TF) family, regulates the progression of the cell cycle and has been confirmed with high levels within diverse cancers and can accelerate cancer cell growth[23–25].

E2F3, which belongs to the E2F TF family, participates in cancer genesis and progression, and its expression is increased within diverse cancers like bladder cancer (BLCA), laryngeal cancer, osteosarcoma and breast cancer (BRCA)[26–29]. In addition, various miRNAs like miR-22, miR-194-5p or miR-195-5p, can regulate cancer genesis and progression via E2F3, suggesting that E2F3 may be the possible anticancer therapeutic target[30, 26, 27]. TargetScan online prediction identified E2F3 as miR-497-5p's potential target. However, it remains unclear about miR-497-5p/E2F3's effect on OS.

The present work examined circ_001422's role in OS cell biological behavior and investigated how circ_001422/miR-497-5p axis affected OS for the first time. Our results will offer the possible targets to diagnose and treat OS clinically.

Materials And Methods

Clinical specimens

This work obtained cancer tissues and paracancerous counterparts in 40 cases with osteosarcoma from October 2019 to July 2020 in the department of Orthopedics, The First Affiliated Cancer Hospital of Zhengzhou University. Each specimen was confirmed with the pathological diagnosis of osteosarcoma and was harvested under patient informed consent. The present work gained approval from Ethics Committee of The First Affiliated Cancer Hospital of Zhengzhou University.

Cell culture and transfection

This work obtained human osteoblast (OB) hFOB1.19 cells and different OS cells (HOS, U-2OS, Saos-2, MG63) in Cell Bank of Shanghai Chinese Academy of Sciences. After collection, this experiment cultivated cells within RPMI-1640 medium that contained 1% penicillin-streptomycin (P-S) as well as 10% fetal bovine serum (FBS), and later incubated under 5% CO_2, saturated humidity, and 37 °C conditions. Thereafter, this work inoculated HOS and Saos-2 cells ($1.5x10^5$/well) into the 6-well plates, and transfected them using miR-497-5p siRNA or inhibitor at 24 h later following the $lipofectamine^{3000}$ (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 24 h, transfection efficiency was detected.

qRT-PCR

This study utilized TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) to isolate total tissue and cellular RNAs. Later, RNA was prepared to cDNA through reverse transcription (Takara Bio). Subsequently, Bio-Rad system was utilized for qPCR using the TaqMan Universal Master Mix II kit. Reaction conditions were as follows: 10-min pre-denaturation under 95℃ , 30-s denaturation under 95℃, 30-s annealing under 60℃, as well as 30-s extension under 74℃ for altogether 40 cycles. Data were analyzed by $2^{-\triangle\triangle ct}$ approach, with GAPDH being the endogenous control for determining target gene mRNA level. The primer sequences are shown in Table 1.

Cell counting kit-8 (CCK-8) assay

This work carried out CCK-8 assay in line with CCK-8 kit instructions. The HOS and SaOS-2 transfected cells (1.5×10^3/well) were added to a 96-well plate, followed by 24-, 48- and 72-h incubation under 37°C, separately, and every well was poured 5 mg/mL CCK-8 solution (20 μL) to incubate under 37°C for a 4-h period. Later, absorbance (OD) values were determined at 450 nm using the microplate reader.

Transwell migration and invasion assays

This work transfected Trypsin in HOS and SaOS-2 cells from diverse groups for 24 h, then serum-free DMEM was added for cell resuspension. For invasion experiment: bottom Transwell chamber was added with DMEM (600 μL) that contained 10% FBS, whereas the top chamber (coated with Matrigel) was added with 200 μL (about 2×10^4 cells) cell suspension to routinely culture for a 24-h period. Afterwards, migrating cells were stained by 0.1% crystal violet for a 20-min period, followed by phosphate buffered saline (PBS) washing as well as crystal violet staining. Later, this work randomly chose 5 fields of view (FOVs) with the microscope to determine invading cell quantity. For Migration experiment: Transwell chamber does not need to lay Matrigel to simulate an artificial basement membrane and the subsequent experimental procedures were identical to those in cell migration experiments.

Digestion with RNAse R

This assay focused on testing RNA stability. First of all, we extracted total RNAs from HOS and Saos-2 cells, followed by the addition of RNase R (Thermo Fisher, USA) and mixing for a 20-min period under

37°C to remove linear RNA. Then, this work carried out RT-qPCR for determining circ_001422 and NSD2 levels.

Nuclear and cytoplasmic fractions

This work utilized ReadiPrep Nuclear/Cytoplasm Isolation Kit (AAT Bioquest, USA) for determining circ_001422 localization. Briefly, cytosol extraction buffer was used to process cells, followed by centrifugation for 20 seconds to collect the supernatant as a cytoplasmic extract. Thereafter, resuspend the pellet in 1X high salt buffer (150 µL). The samples were then centrifuged to collect the supernatant as a nuclear extract. Subsequently, we conducted RT-qPCR for analyzing cytoplasmic/nuclear fraction of circ_001422.

Pull-down assay

Briefly, circ_001422 was labeled with biotin, followed by overnight incubation using streptavidin beads under 4°C. Thereafter, the mixture was then centrifuged washed with wash buffer I. The bead-biotin complex was assed to the lysate and incubate under ambient temperature for a 1-h period. The sample was then rinsed by Wash Buffer II, bead-bound RNA was captured and RT-qPCR assay was then determined.

Dual-luciferase reporter gene assay

This work first constructed the 3'UTR mutant (MUT) and wild-type (WT) luciferase expression vectors E2F3-MUT (circ_001422-MUT) and E2F3-WT (circ_001422-WT). Following dual-luciferase assay kit protocols, these vectors were co-transfected within HOS and SaOS-2 cells using miR-NC together with miR-497-5p mimics with Lipofectamine^{TM} 2000, respectively. Dual-luciferase reporting kit was employed for determining luciferase activities after 48 h.

Western-blot (WB) assay

After 48 h, we collected HOS and SaOS-2 cells, followed by addition of 100 µL of RIPA solution (Thermo Fisher Scientific, Inc.) into 6-well plate for lysis. Then, this work utilized the BCA method (Pierce; Thermo Fisher Scientific, Inc.) for protein quantification. Protein aliquots were later subject to SDS-PAGE for separation and transferred on PVDF membrane, and blocking by 5% defatted milk powder for a 2-h period under ambient temperature. Thereafter, membrane was then subjected to overnight incubation using primary antibodies under 4 °C, washing by TBST, and incubated using diluted secondary antibody for another 2-h period on a shaker. Following TBST washing, ECL chemiluminescence was added into the membrane for exposure. Image J software was employed for analyzing band gray level.

Statistical analysis

Results were represented by mean±SD (X±SD) and data measurements were completed by SPSS21.0. This work then conducted Student's t-test for comparing 2 groups, whereas one-way ANOVA for

comparing diverse groups. Correlation of circ_001422 with miR-497-5p, as well as miR-497-5p with E2F3 within osteosarcoma samples was analyzed through Pearson method. *P*<0.05 stood for statistical significance.

Results

Circ_001422 shows high expression within OS tissues as well as cells

RT-qPCR detected circ_001422 levels within 40 osteosarcoma as well as paracancerous samples, which markedly increased within osteosarcoma tissues compared with paracancerous counterparts (Figure 1A). Relationship of circ_001422 level with clinicopathological parameters of osteosarcoma was analyzed. circ_001422 level was not related to patient age, gender, or tumor sizes. Compared with low-expression group, patients in circ_001422 high expression group had higher clinical stage and higher metastasis rate (Table 2). Compared with hFOB1.19 osteoblasts, circ_001422 expression was significantly increased within OS cells. Particularly, HOS and SaOS-2 cells exhibited higher circ_001422 level, and they were selected for the following functional experiments (Figure 1B). Next, we analyzed subcellular localization of circ_001422's subcellular localization within osteosarcoma cells, and circ_001422 showed major cytoplasmic enrichment, validating the role of circ_001422 as the competing endogenous RNA (ceRNA) during OS development (Figure 1C-D). Furthermore, RNAse R exposure significantly reduced linear NSD2 mRNA expression, while circ_001422 levels remained unchanged, indicating the circulation of circ_001422 within OS cells (Figure 1E-F).

Silencing circ_001422 suppressed OS cell growth and invasion

For exploring circ_001422's effect on OS cells, this work transfected si-circ_001422 in HOS and SaOS-2 cells, followed by qRT-PCR for detecting transfection efficiency. As a result, following transfection with circ_001422 siRNA, circ_001422 expression was significantly reduced, indicating that the transfection was effective and might be applied in later analyses (Figure 2A). Since si-circ_001422$^{1\#}$ has the higher knockout efficiency, it was selected for the next experiment. Then, CCK-8 along with Transwell assay was carried out for analyzing how silencing circ_001422 affected osteosarcoma cells. According to Figure 2B-E, after transfection of siRNA to inhibit circ_001422 expression, cell growth, invasion and migration significantly decreased. According to the obtained results, circ_001422 is associated with the growth, invasion and migration of OS cells, and has the cancer-promoting effect.

MiR-497-5p serves as circ_001422's target

circRNAs often play a role of miRNA sponges for regulating the expression of miRNAs, thereby participating in cellular functions [13,14]. The StarBase database estimated miR-497-5p containing circ_001422 binding sites (Figure 2A). For verifying relation of circ_001422 with miR-497-5p, this work performed luciferase reporter gene assay. As a result, miR-497-5p mimic significantly reduced normal group (WT) luciferase activity by luciferase assay, but not the mutant group (MUT) (Figure 2B). RNA pull-down analysis also confirmed that circ_001422 interacted with miR-497-5p (Figure 2C). miR-497-5p

expression in OS cells and tissues was analyzed. miR-497-5p expressed decreased within osteosarcoma tissues whereas negative relation to circ_001422 level (Figure 2D-E). Apart from that, miR-497-5p level decreased in OS cells (Figure 2F). More importantly, based on RT-qPCR detection results, si-circ_001422 group had markedly increased miR-497-5p level relative to the si-circ_001422 NC group (Figure 2G). Taken together, circ_001422 can target and regulate miR-497-5p expression.

Circ_001422 enhances growth, invasion as well as migration of OS cells through sponging miR-497-5p

Next, for exploring how circ_001422/miR-497-5p axis affected OS, we co-transfected HOS and SaOS-2 cells with si-circ_001422 and miR-497-5p inhibitor. We conducted qRT-PCR for analyzing miR-497-5p inhibitor transfection efficiency, consequently, miR-497-5p level increased within circ_001422-knockdown (Figure 4A). By conducting various functional assays, circ_001422 silencing alone remarkably inhibited OS cell proliferation, migration along with invasion, while miR-497-5p down-regulation partially abolished inhibitory role of low expression of circ_001422 in invasion and migration of OS cells (Figure 4B-D). In line with the above findings, circ_001422 exerts a tumor-promoting effect in osteosarcoma cells through modulating miR-497-5p.

E2F3 serves as miR-497-5p's target

For exploring miR-497-5p's mechanism in OS occurrence and development, the TargetScan bioinformatics database was utilized for predicting miR-497-5p's possible target. According to predicted results, there were binding sites in E2F3 3'UTR for miR-497-5p (Figure 5A). To test this prediction, this work conducted the luciferase reporter assay. As a result, cells co-transfected with E2F3-WT miR-497-5p had evidently reduced luciferase activity relative to miR-NC group (Figure 5B). To verify miR-497-5p's effect on the possible target molecule E2F3, as well as on the expression of E2F3, this work carried out WB and RT-qPCR assays. As a result, miR-497-5p mimic markedly decreased E2F3 mRNA and protein levels (Figure 5C-D). Expression of E2F3 was also detected in OS. E2F3 showed high expression within OS cells and tissues (Figure 5E-F). More importantly, E2F3 level showed negative relation to miR-497-5p level, whereas positive relation to circ_001422 level within OS tissues (Figure 5G-H). Taken together, circ_001422 specifically combined with miR-497-5p to modulate its level.

Circ_001422 promotes osteosarcoma cell proliferation, invasion and migration via miR-497-5p/E2F3 axis

Based on these findings, miR-497-5p served as circ_001422's target, whereas E2F3 served as miR-497-5p's target. Therefore, we were curious whether circ_001422 enhanced OS development through modulating miR-497-5p/E2F3 axis. First, OS cells were transfected with pc-E2F3 overexpression or pcDNA3.1 empty vector to test the transfection efficiency. As a result, pc-E2F3 group had remarkably increased E2F3 level, indicating successful transfection (Figure 6A). si-circ_001422, pc-E2F3 and miR-497-5p inhibitor in various combinations were co-transfected into HOS and SaOS-2 cells, followed by CCK-8 as well as Transwell assay. According to our results, circ_001422 silencing alone markedly suppressed growth, invasion and migration of OS cells, while the inhibitory effect was partly abolished

via suppressing miR-497-5p or up-regulating E2F3 (Figure 6B-D). Collectively, circ_001422 promotes OS development via miR-497-5p/E2F3 axis.

Discussion

circRNAs are increasingly found to be related to tumor occurrence and development, becoming potential new targets for tumor therapy[6–7]. Many articles indicate that circRNAs also act as important players in tumorigenesis and development in osteosarcoma[8–10]. For example, circ-LRP6 showed high expression within osteosarcoma, which enhanced OS proliferation, migration and invasion[13]. Additionally, circ_0000658 level decreased within osteosarcoma, while circ_0000658 down-regulation suppressed OS malignant biological behavior as a tumor suppressor gene[31]. As found by a previous study, circ_001422 was confirmed to promote OS metastasis and development by miR-195-5p/FGF2/PI3K/Akt axis[14]. This experiment also confirmed that circ_001422 was abnormally increased within OS, while circ_001422 up-regulation within tissues was tightly associated with high patient clinical stage as well as metastatic events. These results suggest that circ_001422 is likely to be involved in the metastasis of osteosarcoma, which is consistent with presious findings, suggesting that circ_001422 has a tumor-promoting effect in tumors.

CircRNAs can exert their biological functions indirectly through sponge miRNAs[12–13]. Our bioinformatics results predicted miR-497-5p to be circ_001422's target, while qRT-PCR method confirmed that circ_001422 could negatively regulate miR-497-5p level. More importantly, simultaneously inhibiting miR-497-5p expression abolished circ_001422 knockdown's effect on growth, invasion together with migration of OS cells. Knockdowning circ_001422 expression could suppressed growth, invasion together with migration of OS cells through up-regulating miR-497-5p.

Studies have found that miRNAs regulate tumor progression by participating in tumor cell growth, invasion, apoptosis, and migration[32–34]. For exploring miR-497-5p's effect on osteosarcoma. We conducted dual-luciferase reporter assay and targetScan bioinformatics software, which verified E2F3 as miR-497-5p's target. E2F3 has been widely suggested to participate in tumor cell occurrence and development[23, 25]. For example, E2F3 showed high expression within bladder cancer, and it showed positive relation to cancer malignancy[30]. E2F3 level within prostate cancer was related to patient survival[35]. E2F3 also exhibited abnormally high expression within endometrial cancer tissues, which was related to pathological grade and tumor stage[36]. Currently in this work, E2F3 expression markedly increased within osteosarcoma tissues, which exhibited negative relation to miR-497-5p level whereas positive relation to circ_001422 level within OS tissues.

As an important participant in the ceRNA network, circRNAs can adsorb miRNAs through sponges, thereby affecting the latter downstream target gene levels, resulting in changes in cell phenotypes[12–14]. Here, we have demonstrated miR-497-5p as circ_001422's target, while E2F3 as miR-497-5p's target. We were curious that whether circ_001422/miR-497-5p/E2F3 axis was related to OS development. As

revealed by diverse experiments, circ_001422 silencing alone remarkably inhibited growth, invasion together with migration in OS cells, while the inhibitory effect was partly abolished through inhibiting miR-497-5p or up-regulating E2F3. These results suggest that circ_001422's oncogenic effect was partly determined via miR-497-5p/E2F3 axis (Fig. 7).

Collectively, this work confirmed the promoting activity of circ_001422 for OS cell migration and invasion in vitro, and preliminarily determined its mechanism of promoting metastasis. More importantly, we demonstrated for the first time that circ_001422 negatively regulates miR-497-5p and in turn regulates E2F3 to play an oncogenic role in OS. However, this study still lacks animal experiments for confirming circ_001422's mechanism of action in vivo, and more experiments are needed. Nevertheless, the present work offers certain research basis for anti-metastasis and clinical research on osteosarcoma.

Declarations

Conflicts of interest

None.

Acknowledgement

None.

Author's Contribution

Xinyu Li designed the experiments, performed the periments and drafted the manuscript. Xin Zhao undertook the statistical analysis. Jin Li and Xiaozhan Zhang helped revised the manuscript. All authors read and approved the final manuscript.

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References

1. Anderson ME. Update on Survival in Osteosarcoma. Orthop Clin North Am. 2016;47(1):283-92.
2. Chen C, Xie L, Ren T, Huang Y, Xu J, Guo W. Immunotherapy for osteosarcoma: Fundamental mechanism, rationale, and recent breakthroughs. Cancer Lett. 2021;500:1-10.
3. Harrison DJ, Geller DS, Gill JD, Lewis VO, Gorlick R. Current and future therapeutic approaches for osteosarcoma. Expert Rev Anticancer Ther. 2018;18(1):39-50.
4. Kristensen LS, Andersen MS, Stagsted LVW, Ebbesen KK, Hansen TB, Kjems J. The biogenesis, biology and characterization of circular RNAs. Nat Rev Genet. 2019;20(11):675-691.

5. Li Z, Li X, Xu D, Chen X, Li S, Zhang L, Chan MTV, Wu WKK. An update on the roles of circular RNAs in osteosarcoma. Cell Prolif. 2021;54(1):e12936.
6. Li J, Sun D, Pu W, Wang J, Peng Y. Circular RNAs in Cancer: Biogenesis, Function, and Clinical Significance. Trends Cancer. 2020;6(4):319-336.
7. Zhang Z, Yang T, Xiao J. Circular RNAs: Promising Biomarkers for Human Diseases. EBioMedicine. 2018;34:267-274.
8. Lei M, Zheng G, Ning Q, Zheng J, Dong D. Translation and functional roles of circular RNAs in human cancer. Mol Cancer. 2020;19(1):30.
9. Chen HH, Zhang TN, Wu QJ, Huang XM, Zhao YH. Circular RNAs in Lung Cancer: Recent Advances and Future Perspectives. Front Oncol. 2021;11:664290.
10. Solé C, Lawrie CH. Circular RNAs and cancer: Opportunities and challenges. Adv Clin Chem. 2020;99:87-146.
11. He J, Xie Q, Xu H, Li J, Li Y. Circular RNAs and cancer. Cancer Lett. 2017;396:138-144.
12. Guan K, Liu S, Duan K, Zhang X, Liu H, Xu B, Wang X, Jin X. Hsa_circ_0008259 modulates miR-21-5p and PDCD4 expression to restrain osteosarcoma progression. Aging (Albany NY). 2021;13(23):25484-25495.
13. Yu Y, Dong G, Li Z, Zheng Y, Shi Z, Wang G. circ-LRP6 contributes to osteosarcoma progression by regulating the miR-141-3p/HDAC4/HMGB1 axis. Int J Oncol. 2022;60(4):38.
14. Yang B, Li L, Tong G, Zeng Z, Tan J, Su Z, Liu Z, Lin J, Gao W, Chen J, Zeng S, Wu G, Li L, Zhu S, Liu Q, Lin L. Circular RNA circ_001422 promotes the progression and metastasis of osteosarcoma via the miR-195-5p/FGF2/PI3K/Akt axis. J Exp Clin Cancer Res. 2021;40(1):235.
15. Lu TX, Rothenberg ME. MicroRNA. J Allergy Clin Immunol. 2018;141(4):1202-1207.
16. Feng L, Cheng K, Zang R, Wang Q, Wang J. miR-497-5p inhibits gastric cancer cell proliferation and growth through targeting PDK3. Biosci Rep. 2019;39(9):BSR20190654.
17. Xu GS, Li ZW, Huang ZP, Brunicardi FC, Jia F, Song C, Zou HJ, Sun RF. MiR-497-5p inhibits cell proliferation and metastasis in hepatocellular carcinoma by targeting insulin-like growth factor 1. Mol Genet Genomic Med. 2019;7(10):e00860.
18. Ma W, Gao Y, Zhang J, Yao X, Jia L, Xu Q. Long noncoding RNA LINC01410 promotes tumorigenesis of osteosarcoma cells via miR-497-5p/HMGA2 axis. J Biochem Mol Toxicol. 2021;35(12):e22921.
19. Li G, Wang K, Wang J, Qin S, Sun X, Ren H. miR-497-5p inhibits tumor cell growth and invasion by targeting SOX5 in non-small-cell lung cancer. J Cell Biochem. 2019;120(6):10587-10595.
20. Ma W, Zhao X, Gao Y, Yao X, Zhang J, Xu Q. Circular RNA circ_UBAP2 facilitates the progression of osteosarcoma by regulating microRNA miR-637/high-mobility group box (HMGB) 2 axis. Bioengineered. 2022;13(2):4411-4427.
21. Zhang M, Yu GY, Liu G, Liu WD. Circular RNA circ_0002137 regulated the progression of osteosarcoma through regulating miR-433-3p/ IGF1R axis. J Cell Mol Med. 2022;26(6):1806-1816.

22. Liu S, Duan K, Zhang X, Cao X, Wang X, Meng F, Liu H, Xu B, Wang X. Circ_0081001 down-regulates miR-494-3p to enhance BACH1 expression and promotes osteosarcoma progression. Aging (Albany NY). 2021;13(13):17274-17284.
23. Liu G, Ouyang X, Gong L, Yao L, Liu S, Li J, Zhang Q, Xiao Y. E2F3 promotes liver cancer progression under the regulation of circ-PRKAR1B. Mol Ther Nucleic Acids. 2021;26:104-113.
24. Wang T, Du M, Zhang W, Bai H, Yin L, Chen W, He X, Chen Q. MicroRNA-432 Suppresses Invasion and Migration via E2F3 in Nasopharyngeal Carcinoma. Onco Targets Ther. 2019;12:11271-11280.
25. Al Ahmed HA, Nada O. E2F3 transcription factor: A promising biomarker in lung cancer. Cancer Biomark. 2017;19(1):21-26.
26. Wang Y, Sun G, Wang C, Guo W, Tang Q, Wang M. MiR-194-5p inhibits cell migration and invasion in bladder cancer by targeting E2F3. J BUON. 2018;23(5):1492-1499.
27. Zhou M, Wang Y, Zhang C, Qi M, Yao M, Sun L, Xu X. MicroRNA-195-5p suppresses the proliferation, migration, invasion and epithelial-mesenchymal transition of laryngeal cancer cells in vitro by targeting E2F3. Exp Ther Med. 2021;22(4):1078.
28. Ma C, Han J, Dong D, Wang N. MicroRNA-152 Suppresses Human Osteosarcoma Cell Proliferation and Invasion by Targeting E2F Transcription Factor 3. Oncol Res. 2018;26(5):765-773.
29. Tan PY, Wen LJ, Li HN, Chai SW. MiR-548c-3p inhibits the proliferation, migration and invasion of human breast cancer cell by targeting E2F3. Cytotechnology. 2020;72(5):751-761.
30. Guo J, Zhang J, Yang T, Zhang W, Liu M. MiR-22 suppresses the growth and metastasis of bladder cancer cells by targeting E2F3. Int J Clin Exp Pathol. 2020;13(3):587-596.
31. Jiang X, Chen D. Circular RNA hsa_circ_0000658 inhibits osteosarcoma cell proliferation and migration via the miR-1227/IRF2 axis. J Cell Mol Med. 2021;25(1):510-520.
32. Guan H, Liu J, Lv P, Zhou L, Zhang J, Cao W. MicroRNA-590 inhibits migration, invasion and epithelial-to-mesenchymal transition of esophageal squamous cell carcinoma by targeting low-density lipoprotein receptor-related protein 6. Oncol Rep. 2020;44(4):1385-1392.
33. Guan H, Shang G, Cui Y, Liu J, Sun X, Cao W, Wang Y, Li Y. Long noncoding RNA APTR contributes to osteosarcoma progression through repression of miR-132-3p and upregulation of yes-associated protein 1. J Cell Physiol. 2019;234(6):8998-9007.
34. Zhao X, Xu Y, Sun X, Ma Y, Zhang Y, Wang Y, Guan H, Jia Z, Li Y, Wang Y. miR-17-5p promotes proliferation and epithelial-mesenchymal transition in human osteosarcoma cells by targeting SRC kinase signaling inhibitor 1. J Cell Biochem. 2019;120(4):5495-5504.
35. Olsson AY, Feber A, Edwards S, Te Poele R, Giddings I, Merson S, Cooper CS. Role of E2F3 expression in modulating cellular proliferation rate in human bladder and prostate cancer cells. Oncogene. 2007;26(7):1028-37.
36. Zhang Y, Wang Z, Ma J, Huo J, Li Y, Wang Y, Chen H, Shan L, Ma X. Bioinformatics Identification of the Expression and Clinical Significance of E2F Family in Endometrial Cancer. Front Genet. 2020;11:557188.

Tables

Table 1

The primer sequences

Gene	Position	Sequence（5 → 3）
circ_001422	Forward	CAAGCACAGTCTTCGGAAGT
	Reverse	TTCTGGTGCCTGCTTCATCT
GAPDH	Forward	CAAGGTCATCCATGACAACTTTG
	Reverse	GTCCACCACCCTGTTGCTGTAG
miR-497-5p	Forward	TGCGCCAGCAGCACACTGTGG
	Reverse	CAGTGCGTGTCGTGGAGT
U6	Forward	TCCGATCGTGAAGCGTTC
	Reverse	GTGCAGGGTCCGAGGT
NSD2	Forward	AGACAGATGGCAAAGGGTGG
	Reverse	GGGTGATGTCGTTCTCGTGT
E2F3	Forward	TGACCCAAT GGTAGGCACAT
	Reverse	CATCTAGGACCACACCGACA

Table 2

Correlation between circ_001422 expression and clinically-related pathological parameters in patients with osteosarcoma

Clinical parameters	Cases	circ_001422 expression		P value
		Low (n=20)	High (n=20)	
Gender				0.500
Male	19	10	9	
Female	21	10	11	
Age (years)				0.366
<18	12	5	7	
≥18	28	15	13	
Tumor size (cm)				0.376
<5	22	12	10	
≥5	18	8	10	
TNM stage				0.005*
I-II	19	14	5	
III	21	6	15	
Distant metastasis				0.005*
No	17	13	4	
Yes	23	7	16	

Figures

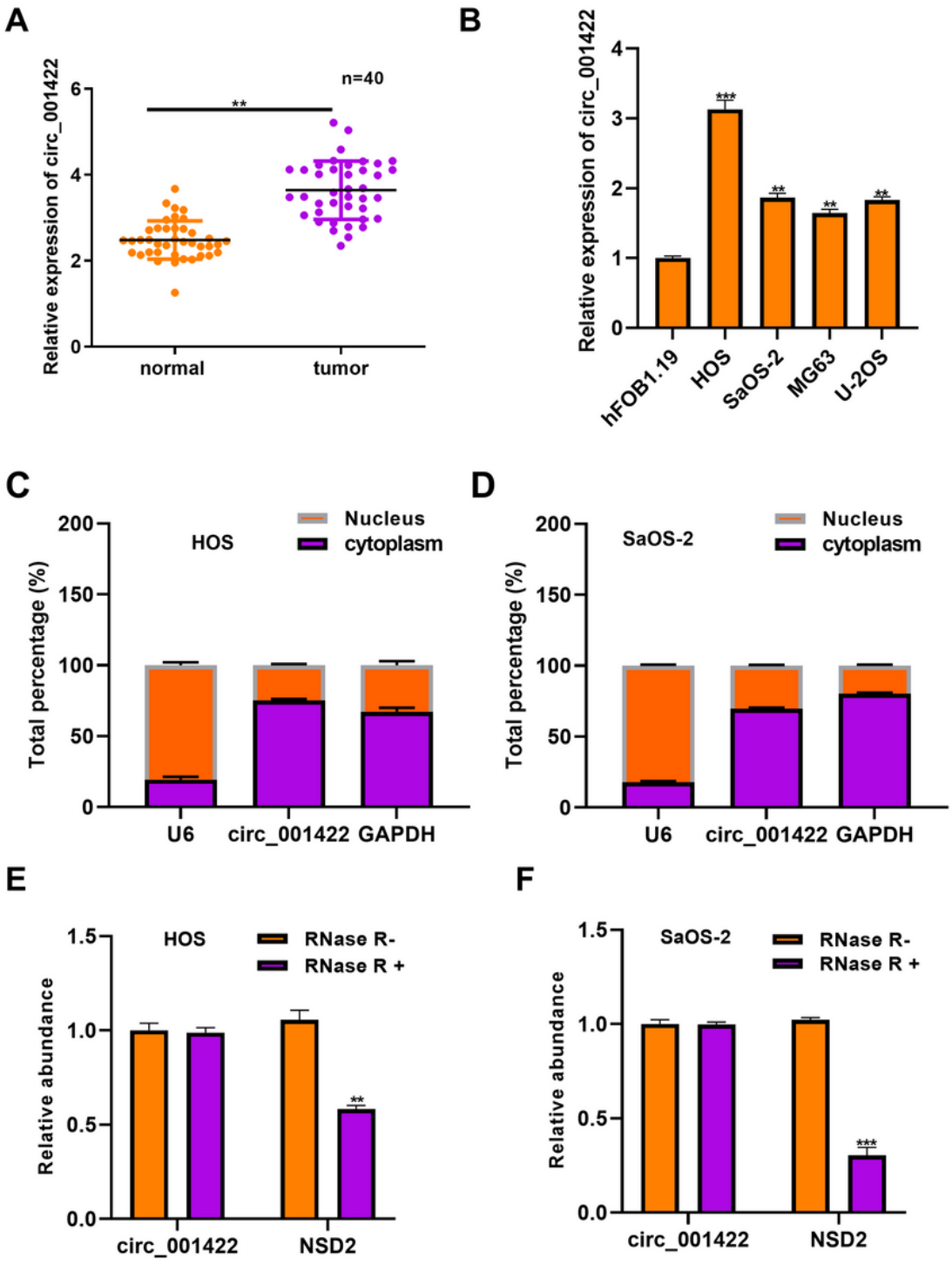

Figure 1

Circ_001422 shows high expression within osteosarcoma tissues and cells

(A) circ_001422 levels within osteosarcoma as well as adjacent tissues detected through qRT-PCR. (B) circ_001422 expression in osteosarcoma cells measured through qRT-PCR. (C) Circ_001422 localization detected through qRT-PCR in HOS cells. (D) Circ_001422 localization detected through qRT-PCR in SaOS-2

cells. (E) Detection of stability of circ_001422 in HOS cells. (F) Detection of stability of circ_001422 in SaOS-2 cells. **P<0.01, ***P<0.001.

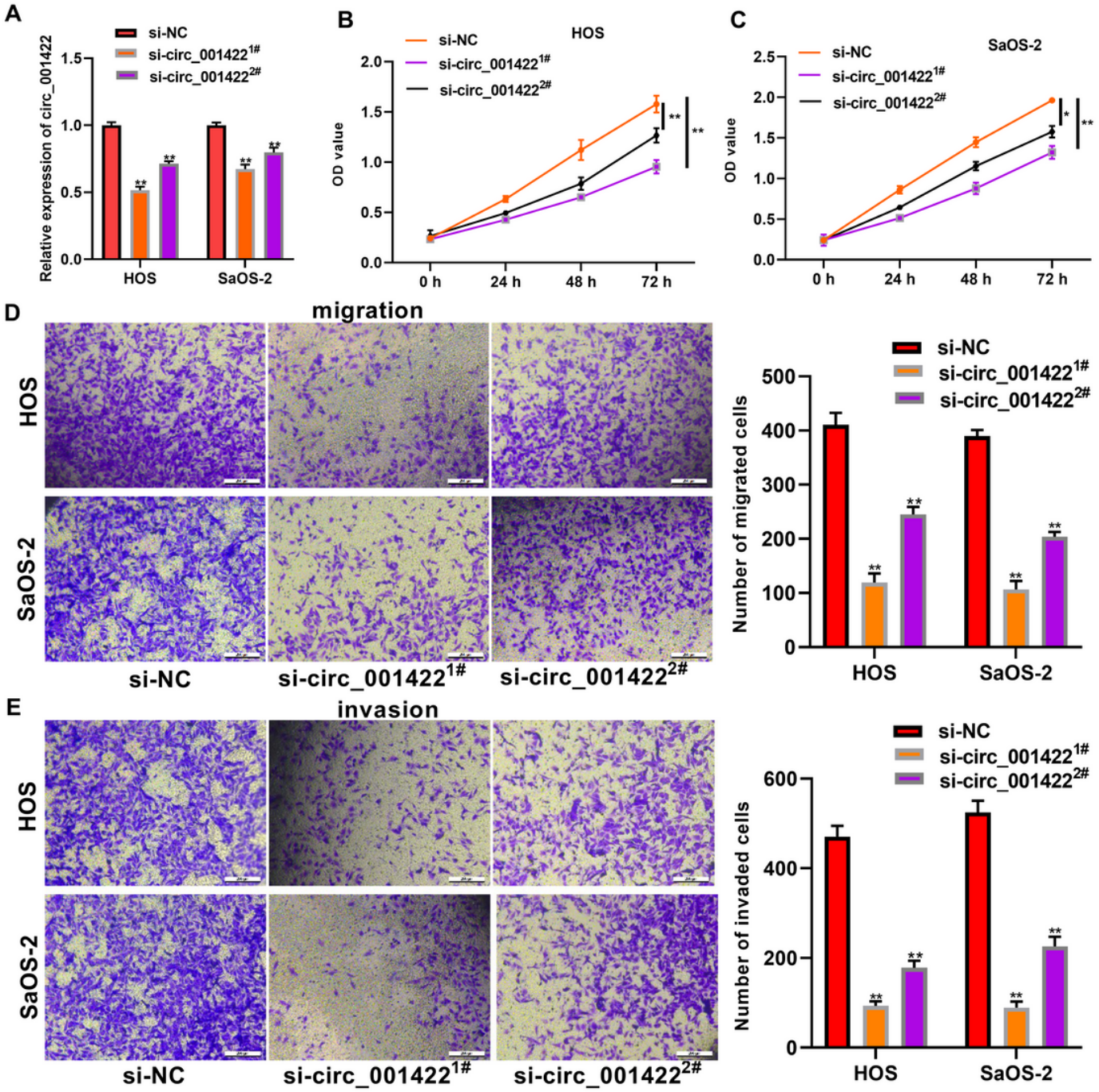

Figure 2

Silencing circ_001422 suppresses proliferation and migration of osteosarcoma cells

(A) Detection of knockout efficiency in OS cells measured by qRT-PCR. (B) Silencing circ_001422 inhibited the proliferation in HOS cells. (B) Circ_LDLR silencing inhibited cell proliferation in SaOS-2 cells. (D) Circ_LDLR silencing inhibited cell migrated abilities of OS cells. (E) Circ_LDLR silencing inhibited cell invaded abilities of OS cells. *P<0.05, **P<0.01.

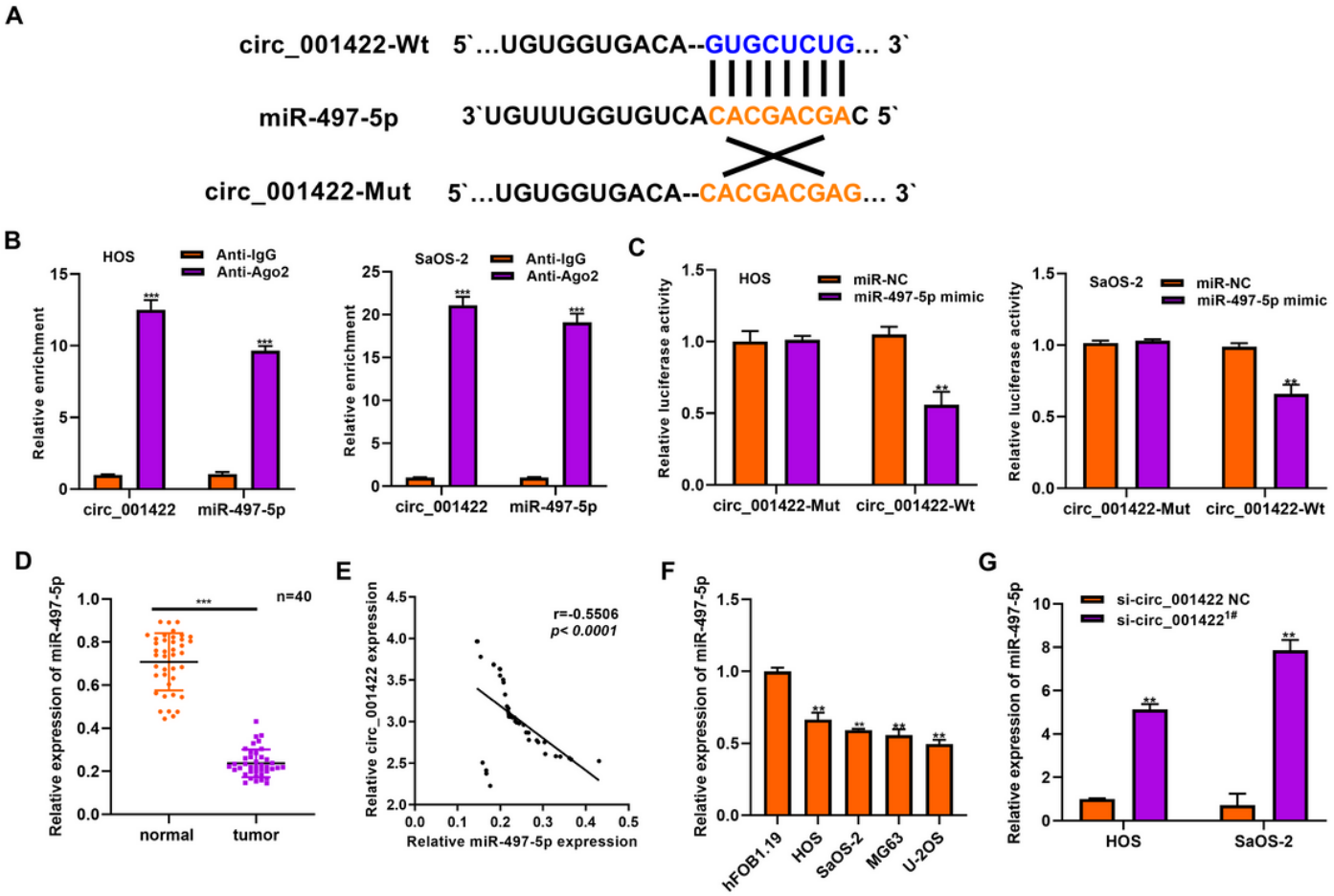

Figure 3

MiR-497-5p serves as circ_001422's target

(A) MiR-497-5p serves as circ_00142's target by StarBase database prediction. (B) circ_00142 and miR-497-5p levels enriched via IgG and Ago2 in RNA pull-down assay. (C) Targeting relation of circ_00142 with miR-497-5p through luciferase assay. (D) miR-497-5p expression in osteosarcoma and adjacent tissues measured through qRT-PCR. (E) Negative relation between circ_00142 level and miR-497-5p expression in OS tissues. (F) miR-497-5p level within osteosarcoma cells measured through qRT-PCR. (G) miR-497-5p level increased in si-circ_00142-transfectd cells. **P<0.01, ***P<0.001.

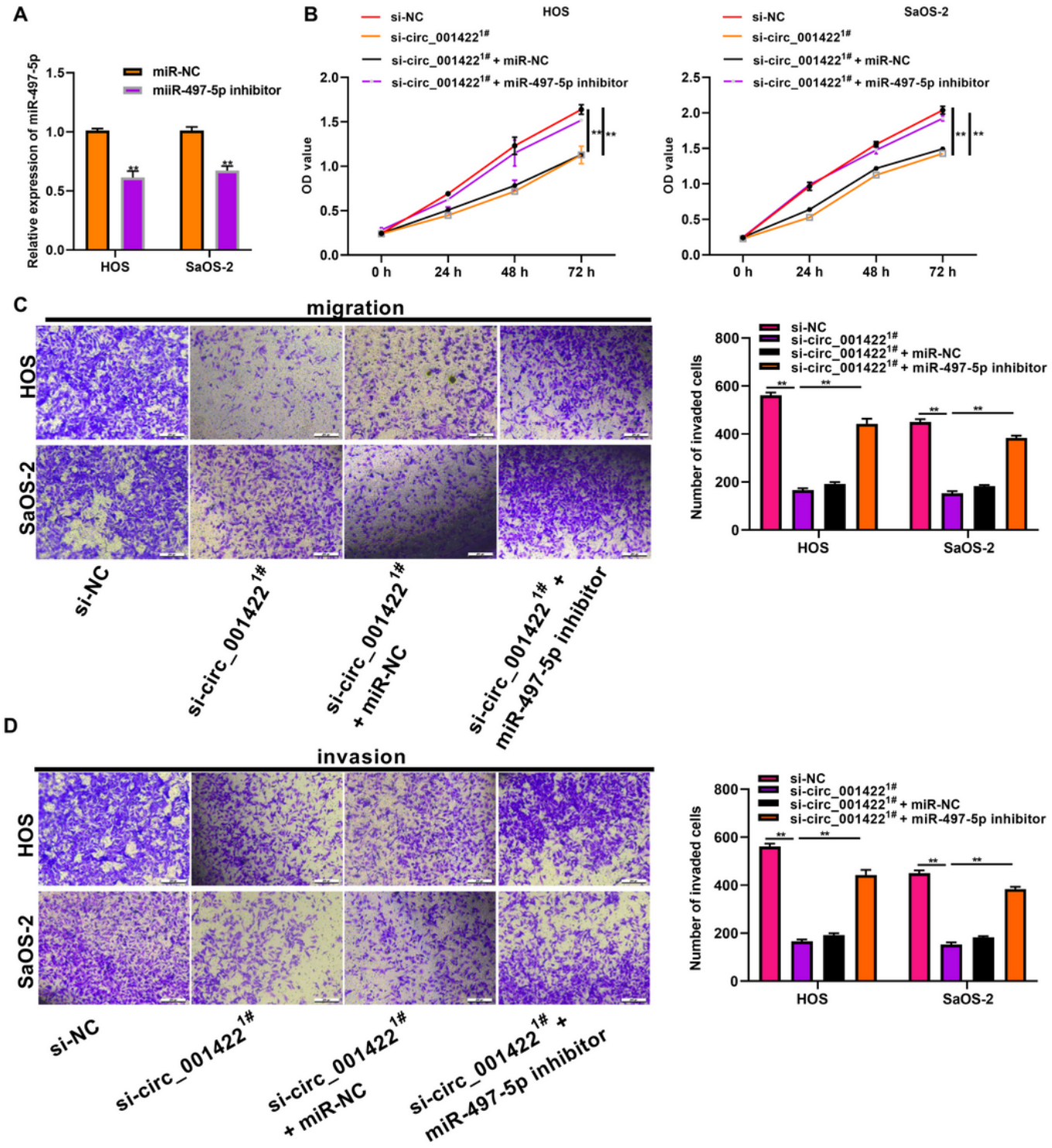

Figure 4

Circ_001422 enhances OS cell growth, invasion and migration via sponge of miR-497-5p

(A) miR-497-5p level declined in miR-497-5p inhibitor-transfected cells. (B) OS cell growth analyzed through CCK-8 assay. (C) OS cell migration analyzed through Transwell assay. (D)) OS cell invasion explored by adopting Transwell assay. $^{**}P < 0.01$.

Figure 5

E2F3 is miR-497-5p's target

(A) E2F3 is E2F3's target by Bioinformatics analysis prediction. (B) Luciferase assay for testing targeting association between E2F3 and miR-497-5p. (C) E2F3 mRNA level was decreased in miR-497-5p mimics-transfected cells. (D) E2F3 protein level decreased in miR-497-5p mimics-transfected cells. (E) E2F3 levels within osteosarcoma and non-carcinoma samples measured via qRT-PCR. (F) miR-497-5p levels within osteosarcoma cells measured through qRT-PCR. (G) Negative association of E2F3 expression with miR-497-5p expression within OS tissues. (H) E2F3 level showed positive relation to circ_00142 level within OS tissues. $^{**}P<0.01$, $^{***}P<0.001$.

Figure 6

Circ_001422 promotes osteosarcoma cell growth, invasion and migration through modulating miR-497-5p/E2F3 axis

(A) E2F3 expression was increased after cells transfected with pc-E2F3. (B) The detection of OS cell proliferation through CCK-8 assay. (C) OS cell migration measured through Transwell assay. (D)) OS cell invasion measured through Transwell assay. $^{**}P<0.01$, $^{***}P<0.001$.

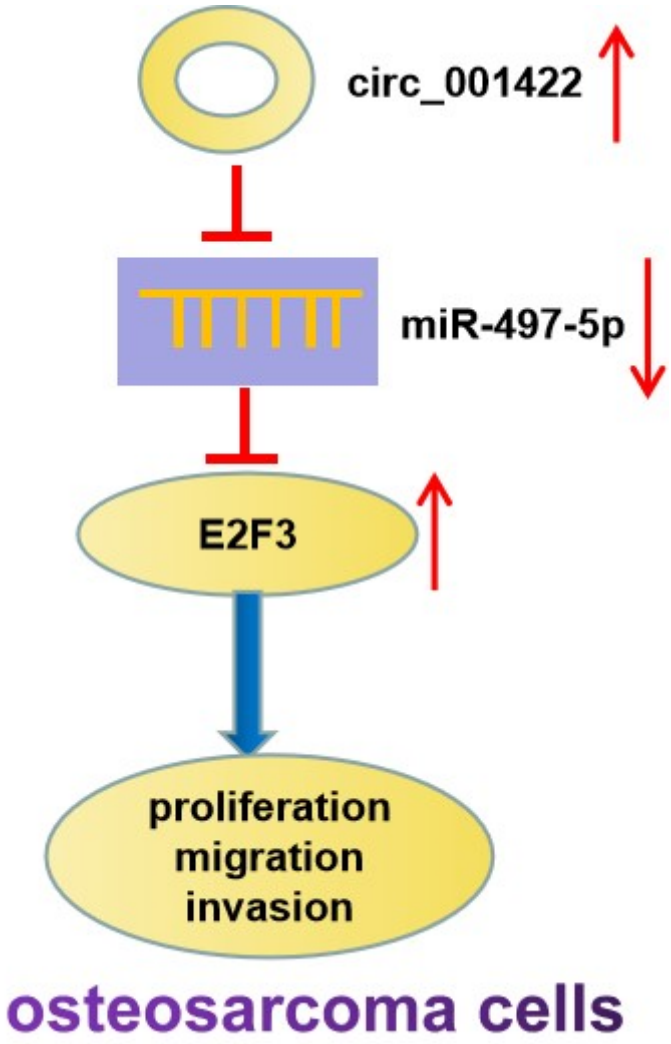

Figure 7

Schematic diagram of circ_001422/miR-497-5p/E2F3 axis regulation in OS