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Original Research

Hsa_circ_0088212-mediated miR-520 h/APOA1 axis inhibits osteosarcoma progression

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ABSTRACT

Background: It has been known for decades that circRNAs are deregulated in cancer. Here, we characterized the role and underlying mechanism of circ_0088212 in osteosarcoma.

Methods: The expression levels of circ_0088212, miR-520 h, and APOA1 were determined by RT-qPCR. RNase R digestion was performed to verify the circular structure of circ_0088212. CCK8 and transwell invasion assays were conducted to examine the in vitro malignancy of osteosarcoma. Caspase-3 activity was also measured. An in vivo model of osteosarcoma was constructed to examine the in vivo effect of circ_0088212 on osteosarcoma. Luciferase reporter, RNA RIP, and RNA pull-down assays were performed to verify the interaction between miR-520 h and APOA1 or circ_0088212.

Results: Circ_0088212 and APOA1 were expressed at low levels in osteosarcoma tissues and cells, while miR-520 h was highly expressed. Overexpression of circ_0088212 was found to inhibit the in vitro and in vivo growth of osteosarcoma. Mechanistically, miR-520 h was the target of circ_0088212 and APOA1 was the target of miR-520 h. Circ_0088212 downregulated miR-520 h expression, while miR-520 h overexpression abolished the inhibitory effect of circ_0088212 on osteosarcoma cell proliferation and migration. Furthermore, miR-520 h overexpression led to reduced APOA1 expression, while APOA1 overexpression counteracted the oncogenic effect of miR-520 h in osteosarcoma cells.

Conclusion: Our findings demonstrated that circ_0088212 might exert a tumor-suppressive activity in osteosarcoma by sponging and sequestering miR-520 h away from APOA1. This suggests that the circ_0088212/miR-520 h/APOA1 axis may be a promising therapeutic target for osteosarcoma intervention.

Introduction

Osteosarcoma is a highly aggressive bone malignancy that is frequently diagnosed in children and adolescents [1]. Statistics suggest that approximately 3.5 million new cases are added every year worldwide [2]. Although great strides have been achieved in the diagnosis and treatment of osteosarcoma, its prognosis remains dismal [3]. Therefore, a better understanding of its underlying mechanism is important for developing effective novel treatment strategies and improving the clinical outcomes of affected patients.

Advances in transcriptomics have shown that non-coding transcripts are highly prevalent in the human transcriptome [4]. Novel circular RNAs (circRNAs), a type of single-stranded non-coding transcripts with covalently closed loops, are being discovered at an unprecedented pace. An increasing number of studies have demonstrated that circRNAs participate in the fine-tuning of gene expression and in various cellular processes [5]. Mechanistically, they act as miRNA sponges or decoys, or protein scaffolds, and influence gene expression dynamics to form complicated post-transcriptional regulatory networks. Recent studies have shown that circRNAs play a critical role in the pathophysiology of cancers, including osteosarcoma [6,7]. Many aberrantly expressed circRNAs could drive or suppress tumorigenesis and its progression via their gene-regulatory potential, resulting in the modulation of tumor cell characteristics [8]. For example, circ_001621 was discovered to boost the proliferative potential of osteosarcoma cells by acting as an miR-578 sponge [9]. High expression of circ_0000885 is related to advanced-stage osteosarcoma [10]. In the present study, we performed bioinformatics analysis and found that circ_0088212 was downregulated in osteosarcoma tissues; however, its role in osteosarcoma is yet to be discovered.

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APOA1, a gene located at 11q23.3, contains four exons. It encodes apolipoprotein A-I, which is the major protein component of high-density lipoprotein (HDL) in plasma. APOA1 has been found to be aberrantly expressed in different cancers [11]. For example, it is an independent negative risk factor for lung cancer progression [12]. Dufresne et al. detected an abundant expression of APOA1 in breast cancer [11]. Zhang et al. used the NCBI Gene Expression Omnibus (GEO) database and identified that APOA1 may be involved in liver metastasis in colorectal cancer [13]. However, its function in cancer has not been studied till date.

In the present study, we aimed to explore the differential expression of circRNAs in osteosarcoma via the circRNA microarray GSE96964. We characterized a novel downregulated circRNA circ_0088212, and established the existence of circ_0088212/miR-520 h/APOA1 axis. Our results indicate that circ_0088212 may function as a diagnostic and prognostic biomarker or therapeutic target for osteosarcoma.

Materials and methods

Clinical specimens

Osteosarcoma tissues and paracancerous normal tissues were obtained from patients undergoing surgical resection at the Renmin Hospital of Wuhan University. Ethical approval was obtained from the Ethics Committee of Renmin Hospital. Written informed consent was obtained from all patients. All tissue samples were validated by two independent pathologists and stored at −80 °C until analysis.

Cells and transfection

The normal osteoblast cell line hFOB1.19, and osteosarcoma cell lines (Saos-2, HOS, and SW1353) were purchased from the American Tissue Collection Center (ATCC). hFOB1.19 and SW1353 were cultivated in DMEM, Saos-2 in McCoy's 5A, and HOS in MEM. All media contained 10% FBS and 1% penicillin-streptomycin. The cells were maintained at 37 °C and under conditions of 5% CO2.

MiR-520 h mimic or mimic negative controls (NC), APOA1 overexpressing vector (pcDNA3.1-APOA1), and empty controls (OE-nc) were purchased from GenePharma (Shanghai GenePharma Co., Ltd, Shanghai, China). Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used to transfect 80% confluent SW1353 and HOS cells, which were then incubated for 48 h. Transfection efficiency was assessed by RT-qPCR.

For circ_0088212 overexpression, the amplified full sequence of circ_0088212 was fused into pLO5-ciR (Geneseed, Guangzhou, China) to generate pLO5-circ_0088212 vectors. The pLO5-circ_0088212 or pLO5-ciR vectors were delivered into 293T cells along with helper vectors (psPAX2 and pMD2.G). Next day, the medium containing the virus particles was subjected to sterile filtration and concentration through centrifugation. The viral titer of lentiviral particles was determined by qPCR through serial dilutions. The confluent SW1353 and HOS cells were infected with approximately 7×10^8 TU/mL virus particles for 48 h. The infected cells were maintained with 2 μg/ml of puromycin for 5 days. The transfection efficiency was validated using RT-qPCR.

RT-qPCR

Total RNA was isolated from osteosarcoma tissues and cells using TRIzol Reagent (Invitrogen, USA). First strand cDNA was synthesized from RNA using a First-strand cDNA Synthesis Kit (Merck, USA) and HyperScript III miRNA 1st Strand cDNA Synthesis Kit (Novabio, China). Quantitative PCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, USA), running on an ABI 7900HT Fast System (Applied Biosystems). Target gene expression was analyzed using the 2-ΔΔCt method, normalized to GAPDH or U6. The primers used in this study are listed in Table 1.

Table 1
PCR primers used in this study.

Targets	Primers	Sequences (5′ to 3′)
circ_0088212	Forward	CAGAAATATTTTGCCACTGTTGA
	Reverse	AGCGTGGGTCTTTGTTGTCT
miR-520h	Forward	TCGCGACAAAGTGCTTCCCT
	Reverse	GTGCAGGGTCCGAGGT
APOA1	Forward	CCCAGTTGTCAAGGAGCTTT
	Reverse	TGGATGTGCTCAAAGACAGC
GAPDH	Forward	CCACCCATGGCAAATTCCATGGGA
	Reverse	TCTAGACGGCAGGTCAGGTCCACC
U6	Forward	AAAGCAAATCATCGGACGACC
	Reverse	GTACAACACATTGTTTCCTCGGA

Western blots

Following centrifugation, the cell pellets were lysed in pre-cold RIPA buffer (Sigma-Aldrich, USA). The precipitate was discarded, and the soluble fraction was collected and assessed for protein concentration using a Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). Protein samples (15 μg) were loaded onto 10% SDS-PAGE before wet transfer onto PVDF membranes. Subsequently, the membranes were blocked using 5% bovine serum albumin (Sigma, USA) for 1 h at room temperature and incubated overnight with diluted primary antibodies at 4 °C. Following detection by HRP-conjugated mouse and rabbit secondary antibodies for 1 h at room temperature, the blots were developed using ECL western blotting detection system (Thermo Fisher Scientific, USA) and visualized using X-ray films.

Nuclear and cytoplasmic fractions

To determine the location of circ_0088212, a ReadiPrep nuclear/cytoplasmic fractionation kit (AAT Bioquest, USA) was used. Briefly, after rinsing with PBS, the cells were treated with 500 μL of cytosol extraction buffer and centrifuged for 20 s. Next, the supernatant was collected as a cytoplasmic extract, and the precipitates were suspended in 150 μL of 1X High Salt Buffer. After centrifugation, the supernatant was collected as a nuclear extract. The expression levels of circ_0088212 in the nuclear and cytoplasmic fractions were determined using RT-qPCR.

RNAse R digestion

RNase R (Thermo Fisher, USA) was added to 2 μg each of isolated total RNA samples from SW1353 and HOS cells and kept for 30 min at 37 °C to digest the linear RNA. Next, RT-qPCR was employed to examine circ_0088212 expression in treated and untreated SW1353 and HOS cells.

CCK8 assays

The SW1353 and HOS cells were seeded at a density of 1×10^5 cells per well into 96-well plates. After incubation for the indicated time, the cells were incubated with 10 μL of CCK-8 reagent (GLPBIO, USA) for an additional 2 h. Absorbance was measured using a microplate reader at 450 nm.

Transwell migration assay

Media containing 5×10^4 SW1353 and HOS cells were dropped onto ECM gel-coated Millicell cell culture inserts (Merck KGaA, USA). The inserts were positioned into the wells of a 12-well plate containing 1.5 mL medium with 10% FBS. After incubation for 48 h, the inserts were removed and cells that failed to invade through the pores were removed. The cells on the lower side of the insert membranes underwent fixation using 4% paraformaldehyde for 10 min followed by staining with 1%

crystal violet for 20 min. The number of invasive cells was counted under a microscope.

Luciferase reporter assay

The binding site of miR-520 h to circ_0088212 or 3'-UTR APOA1 (wild-type, WT) and the corresponding mutant binding site (MUT) were inserted into the psiCHECK2 vector to construct psiCHECK2-circ_0088212-WT, psiCHECK2-circ_0088212-Mut, psiCHECK2–3'-UTR APOA1-WT, and psiCHECK2–3'-UTR APOA1-MUT luciferase reporter vectors for the luciferase reporter assay. SW1353 and HOS cells (1×10^5) were transfected and incubated with miR-520 h mimic or mimic NC and luciferase reporter vectors for 48 h using Lipofectamine 3000. Next, luminescence signals were read using the Dual-Luciferase Reporter Assay System (GeneCopoeia, USA). Luciferase activity = firefly/Renilla luciferase.

RNA immunoprecipitation (RIP) assay

An RIP kit (Millipore Sigma, USA) was used to validate the interaction between circ_0088212 and miR-520 h, as described in the protocol. Briefly, 80% confluent SW1353 and HOS cells were collected and treated with 200 μL of harsh lysis buffer + protease inhibitor cocktail. Cytoplasmic lysates were isolated from debris pellets and incubated with the prepared beads along with IgG or anti-AGO2 antibody at 4 °C. The beads were then collected and washed thoroughly and RT-qPCR was employed to test circ_0088212 and circ_0088212 expression.

RNA pull-down assay

Eighty percent confluent SW1353 and HOS cells were lysed using RIPA buffer (Solarbio, USA). After centrifugation, the collected cell lysates were incubated with streptavidin magnetic beads attached to biotin-tagged miR-520 h or biotin-tagged NC for 5 h. The RNA bound to the streptavidin agarose beads was eluted and quantified by RT-qPCR.

Xenograft tumor formation

Six-week-old male nude mice, weighing approximately 25 g, were purchased from the Animal Experimental Center of Hubei Province (Wuhan, China). SW1353 cells (5×10^6) with pLO5-circ_0088212 vectors or pLO5-NC were administered anesthetically into the flanks of the mice. Tumor size, which was recorded weekly using a caliper, was calculated using the following equation: tumor volume = length × width × width/2. After 5 weeks, the mice were sacrificed, and the tumors were aseptically removed and weighed.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.0. Each measure is presented as mean ± standard deviation (SD). Comparisons between two groups were evaluated using Student's *t*-test or the Mann–Whitney U test. Comparisons among multiple groups were performed using one-way and two-way ANOVA. Pearson's correlation was used to determine the correlation between miR-520 h and circ_0088212 or APOA1. Statistical significance was set at $P < 0.05$.

Results

Identification of circ_0088212/miR-520 h/APOA1 axis in osteosarcoma

The key circRNA was first identified using the data from circRNA microarray dataset GSE96964, obtained from GEO DataSets. With $P < 0.05$ and logFC < -2, the top 5 downregulated circRNAs were screened (Supplementary Table 1). hsa_circ_0088212, with the lowest expression in osteosarcoma samples, was identified as the key gene. Based on circPrimer 1.2 analysis, its structure is shown in Fig. 1A. Next, 194 downregulated genes from the GSE16088 dataset were selected based on the criteria: $P < 0.05$ and logFC < -2. On uploading the 194 genes to STRING, six genes were confirmed to be related to cell adhesion (Fig. 1B). Among these, the expression of APOA1 was found to be the lowest in osteosarcoma (Supplementary Table 2), and its expression was widely distributed in the cell nucleus and cytosol (Fig. 1C). Therefore, APOA1 was identified as the gene of interest. To identify the target miRNA, CircInteractome was used to predict the miRNAs sponged by circ_0088212, and TargetScan was used to predict the miRNAs targeting APOA1. GSE65071 was used to screen the upregulated miRNAs based on the criteria: $P < 0.05$, and logFC > 2. Finally, three miRNAs (miR-330–5p, miR-520 h, and miR-671–5p) were found to be common among the three databases (Fig. 1D). As the effects of miR-330–5p and miR-671–5p on osteosarcoma have already been reported [14,15], miR-520 h was identified as the key miRNA to be explored.

Circ_0088212 is poorly expressed in osteosarcoma

We first examined the expression of circ_0088212 in osteosarcoma cells and clinical specimens and found that its expression was lower in the osteosarcoma cells and tissues compared with hFOB1.19 cells and normal tissues (Fig. 2A and 2B). In particular, SW1353 and HOS cells showed a strong downregulation of circ_0088212. To further characterize the biofunction of circ_0088212, we examined its subcellular localization in SW1353 and HOS cells. As shown in Fig. 2C, circ_0088212 was predominantly accumulated in the cytoplasm, verifying the competing endogenous RNAs (ceRNAs) activity of circ_0088212 in osteosarcoma tumorigenesis and progression. Additionally, circRNAs are generally known to be resistant to RNase A. As shown in Fig. 2D, RNAse A treatment substantially reduced linear RNA levels but showed no effect on that of circ_0088212, suggesting the circular characteristics of circ_0088212 in SW1353 and HOS cells.

Circ_0088212 elevation suppresses osteosarcoma cell growth and migration

To further explore the functional role of circ_0088212, we introduced circ_0088212-overexpressing vectors into SW1353 and HOS cells to increase the endogenous RNA level of circ_0088212 (Fig. 3A). The accumulation of circ_0088212 reduced the proliferation of SW1353 and HOS cells (Fig. 3B). Meanwhile, its overexpression increased caspase-3 activity, suggesting that circ_0088212 triggered the apoptosis of these cells (Fig. 3C). Additionally, the number of invaded cells in SW1353 and HOS cells overexpressing circ_0088212 were lower compared to that in other cells (Fig. 3D). These in vitro results indicate that circ_0088212 acts as a tumor suppressor in osteosarcoma cells. The anti-oncogenic properties of circ_0088212 were further examined in vivo using a xenograft model of SW1353 cells stably overexpressing circ_0088212. As shown in Fig. 3E, circ_0088212 overexpression resulted in a significant decrease in tumor volume and weight, suggesting that circ_0088212 suppresses tumorigenicity in vivo.

Circ_0088212 sponges miR-520 h

As shown in Fig. 4A, circ_0088212 contained sequences complementary to miR-520 h, based on the bioinformatics analysis. Next, luciferase activity in the SW1353 and HOS cells were assessed after cotransfection with miR-520 h mimic or mimic NC and luciferase-circ_0088212-WT or luciferase-circ_0088212-Mut. As shown in Fig. 4B, the miR-520 h mimic significantly increased the circ_0088212-WT-mediated luciferase activity, but had no effect on circ_0088212-WT-mediated activities. The RNA RIP assays validated the target binding between circ_0088212 and miR-520 h (Fig. 4C). Contrary to downregulation of circ_0088212, we found that miR-520 h was abundantly expressed in osteosarcoma tissues as well as in SW1353 and HOS cells

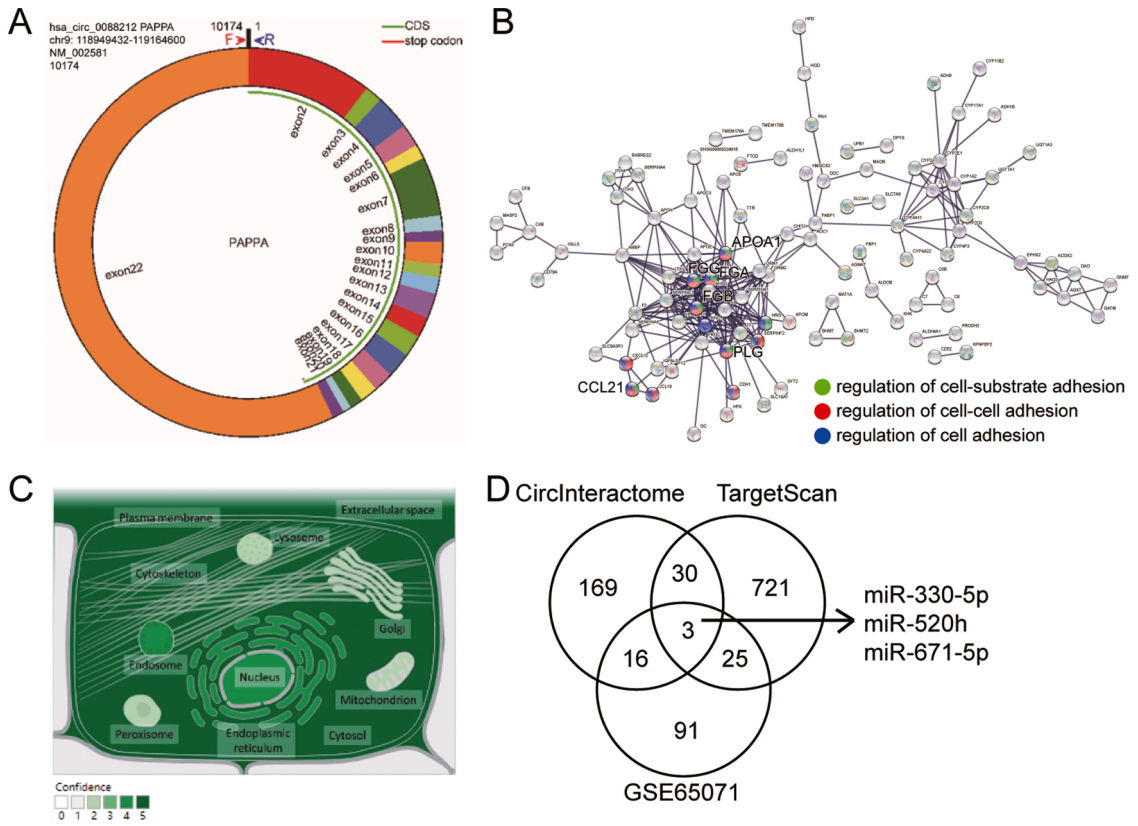

Fig. 1. The circ_0088212/miR-520 h/APOA1 axis might be crucial in osteosarcoma by bioinformatics analysis. A. The structure of circ_0088212 by circPrimer 1.2 analysis. B. The GO enrichment of downregulated genes from GSE16088 was analyzed by STRING. C. The distribution of APOA1 in cells. D. Three miRNAs were overlapped from CircInteractome, TargetScan, and GSE65071.

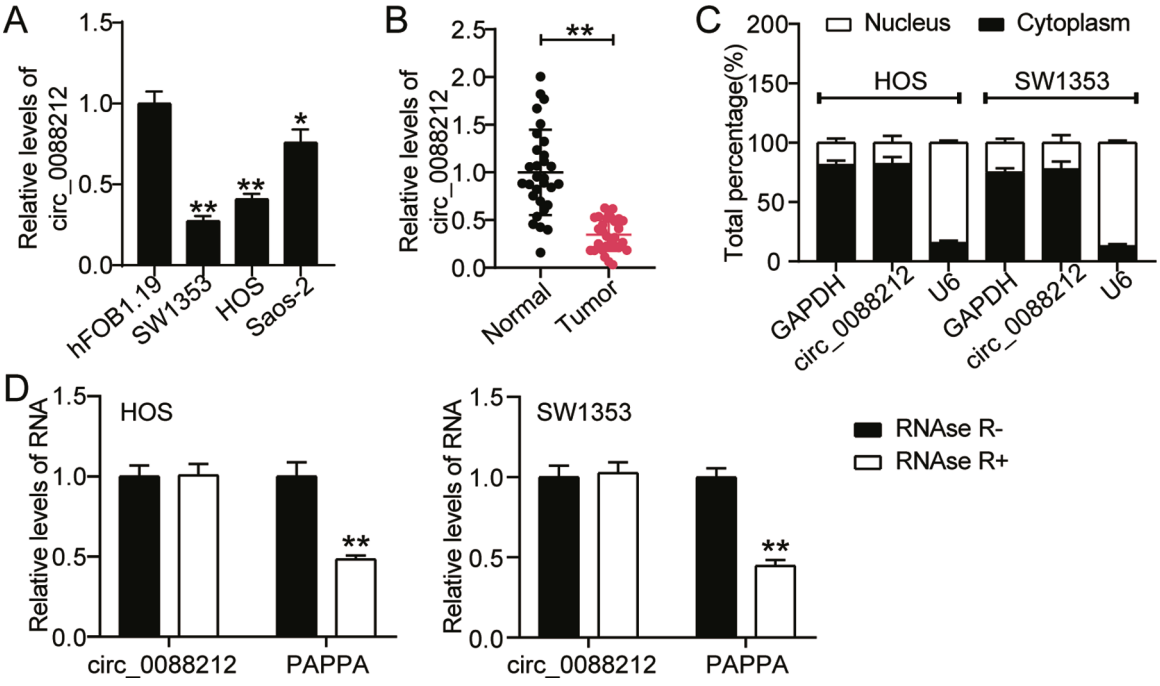

Fig. 2. Circ_0088212 is poorly expressed in osteosarcoma. A. The expression of circ_0088212 was examined in hFOB1.19, SW1353, HOS and Saos-2 cells by RT-qPCR. Vs.hFOB1.19,*$P<0.05$, **$P<0.001$; B. The expression of circ_0088212 was examined in osteosarcoma tissues and normal tissues by RT-qPCR. **$P<0.001$; C. Subcellular localization of the circ_0088212 examined by qRT-PCR after nuclear–cytoplasm fractionation. D. RNase digest R was used to treat circ_0088212 to determine its circularity in SW1353 and HOS cells, vs. RNAse R-, **$P<0.001$.

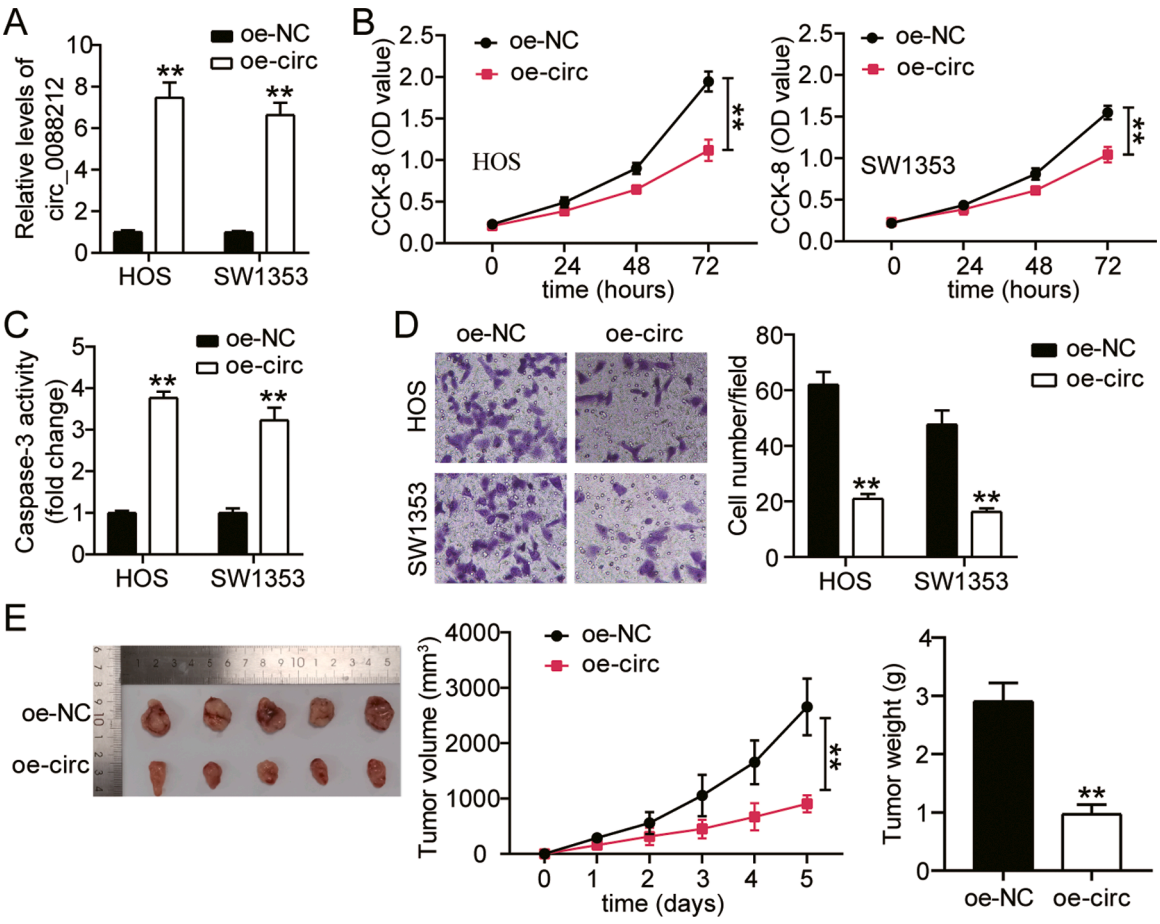

Fig. 3. circ_0088212 overexpression suppresses tumorigenicity in vitro and in vivo. A-D. Circ_0088212 overexpressing vectors (OE-circ) and empty vectors (oe-NC) were delivered into SW1353 and HOS cells for 48 h. The circ_0088212 was overexpressed in SW1353 and HOS cells by RT-qPCR (A), circ_0088212 overexpression impaired the cell-proliferation by CCK8 assays (B), circ_0088212 overexpression enhanced the casepase-3 activity (C), circ_0088212 overexpression suppressed the invasive capacities of SW1353 and HOS cells (D). SW1353 cells with stable empty vector or circ_0088212 overexpressing vectors were subcutaneously injected into nude mice. The effect of circ_0088212 overexpressing on tumor volume curve and tumor weight were analyzed. Vs. oe-NC, $^{**}P<0.001$.

(Fig. 4D and 4E), indicating a negative correlation between circ_0088212 and miR-520 h in osteosarcoma tissues (Fig. 4F). These findings suggest that miR-520 h and circ_0088212 form a ceRNA regulatory network and that circ_0088212 exerts a sequence-driven sponging effect on miR-520 h.

Circ_0088212 exerts its inhibitory effects on osteosarcoma by sequestering miR-520 h

Based on the above-mentioned findings, which suggested that circ_0088212 has a sponging effect on miR-520 h, we characterized the effect of this interaction on cellular behavior. Circ_0088212 overexpressing vectors and miR-520 h mimic were delivered into SW1353 and HOS cells. As shown in Fig. 5A, miR-520 h overexpression resulting from miR-520 h mimic transfection can be abrogated by circ_0088212 overexpressing vectors. Functional assays showed that miR-520 h overexpression boosted cell proliferation and diminished caspase-3 activity and that this phenomenon was nullified in SW1353 and HOS cells transfected with circ_0088212-overexpressing vectors and miR-520 h mimic (Fig. 5B and 5C). Fig. 5D also shows that circ_0088212 overexpression significantly abrogated the miR-520h-induced increase in invasiveness in SW1353 and HOS cells. Thus, circ_0088212 can impair miR-520 h oncogenicity through its ceRNA activity.

APOA1 is a direct target of miR-520 h

Given that APOA1 was predicted as a functional downstream mediator of the circ_0088212/miR-520 h ceRNA network, we performed a series of assays to validate the targeted relationship between miR-520 h and APOA1. As shown in Fig. 6A, miR-520 h shared seed sequences with the 3'-UTR APOA1. The luciferase reporter assay validated the direct binding of miR-520 h to the 3'-UTR of APOA1 (Fig. 6B). The RNA pull-down assay demonstrated the enrichment of APOA1 in SW1353 and HOS cells with bio-miR-520 h, but not in cells with bio-NC (Fig. 6C). The expression of APOA1 was found to be downregulated in the clinical osteosarcoma tissues (Fig. 6D). This phenomenon was also observed in the SW1353 and HOS cells (Fig. 6E). Interestingly, APOA1 expression levels were negatively correlated with miR-520 h expression in the clinical specimens (Fig. 6F).

MiR-520 h amplification downregulates APOA1 expression and increases osteosarcoma cell proliferation and migration

After recognizing the miR-520 h/APOA1 regulatory axis, we further examined whether their interaction affected the proliferation and migration of osteosarcoma cells. As shown in Fig. 7A, miR-520 h negatively reshaped APOA1 expression in SW1353 and HOS cells. Functionally, a low proliferative rate coupled with high caspase-3 activity was detected in APOA1-overexpressing SW1353 and HOS cells; however, this phenomenon was reversed on transfection with miR-520 h

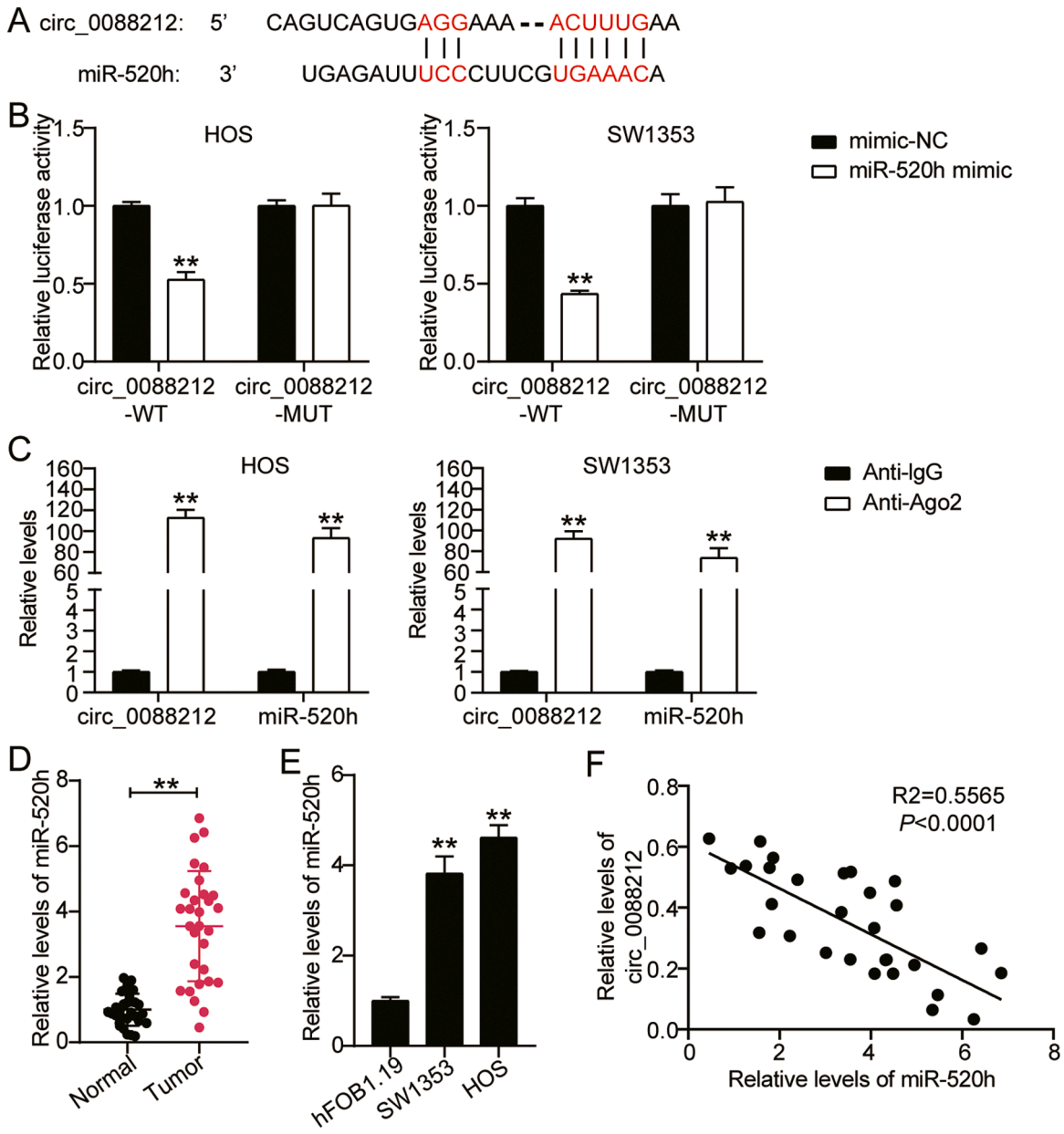

Fig. 4. circ_0088212 sponges miR-520 h. A. Graphical representation showing the predicted sites of miR-520 h for binding to circ_0088212. B. Luciferase activity of SW1353 and HOS transfected with luciferase-circ_0088212-WT and luciferase-circ_0088212-Mut. Vs. miR-NC,**P<0.001; C. The enrichment of miR-520 h and circ_0088212 in Ago2 and IgG detected by RIP assay. Vs. Anti-lgG,**P<0.001; D. qRT-PCR analysis of miR-520 h in osteosarcoma tissues. **P<0.001; E. qRT-PCR analysis of miR-520 h in hFOB1.19, SW1353 and HOS. Vs. hFOB1.19, **P<0.001; Pearson correlation analysis revealed the negative correlation between miR-520 h and circ_0088212.

(Fig. 7B and 7C). This regulatory axis was also adaptive to osteosarcoma cell invasion. MiR-520 h overexpression could offset the invasive defect resulting from ectopic APOA1 expression (Fig. 7D). These results show that the oncogenicity of miR-520 h is dependent on APOA1 downregulation.

Discussion

Accumulating evidence has implicated that circRNAs, either transcriptionally or post-transcriptionally, regulate coding genes in different malignancies, including osteosarcoma. In the present study, we found that circ_0088212 was downregulated in osteosarcoma tissues, and that its overexpression exerted inhibitory effects on tumor cell proliferation and migration, while also increasing cell apoptosis. The in vivo tumor suppressive effects of circ_0088212 in osteosarcoma were also observed in the SW1353-bearing mouse model. Furthermore, mechanical studies showed that circ_0088212 suppressed the proliferative and migratory phenotypes of osteosarcoma cells by competitively binding miR-520 h to unregulated APOA1 expression. Our findings suggest that the circ_0088212/miR-520 h/APOA1 axis might be a novel therapeutic target against osteosarcoma.

Furthermore, many circRNAs have been reported to be aberrantly expressed in osteosarcoma and typically contribute to activating oncogenes or inactivating tumor suppressors during osteosarcoma malignancy. For example, circ-XPR1, which is highly expressed in osteosarcoma tissues, is associated with an inferior prognosis in osteosarcoma patients [16]. Zhang et al. demonstrated that circ_0002052 activates the Wnt/β-catenin signaling pathway and acts as an oncogene in osteosarcoma [17]. In the present study, we first identified a novel circRNA circ_0088212, which is downregulated in osteosarcoma, using

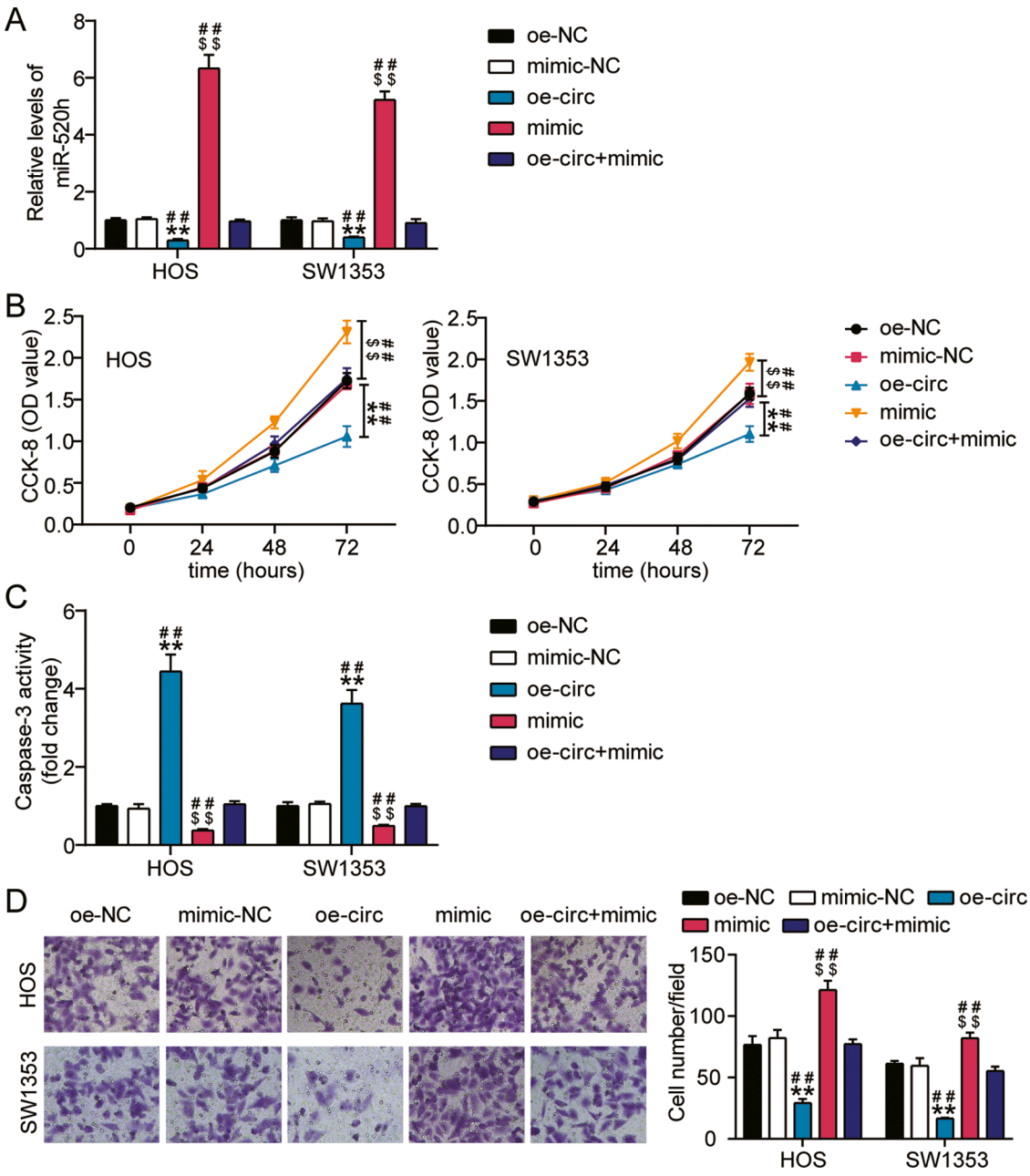

Fig. 5. Circ_0088212 exerts its tumor suppressive effect by sponging miR-520 h. SW1353 and HOS cells were transfected with circ_0088212-overexpressiong vectors (OE-circ), miR-520 h mimic, their corresponding NC, and OE-circ+OE-circ. A. RT-qPCR analyzing the expression of miR-520 h. B. CCK-8 assays of SW1353 and HOS cells. C. Caspase-3 activity in SW1353 and HOS cells assessed by a caspase-3 kit. D. Transwell assay of SW1353 and HOS cells. Vs. oe-NC, $^{**}P<0.001$; vs.mimic-NC, $^{\$}$ $^{\$}P<0.001$; vs. OE-circ+mimic, $^{\#\#}P<0.001$.

bioinformatics analysis. Compared with paracancerous tissues, we found that circ_0088212 was downregulated in osteosarcoma tissues and cells. In addition, our functional assays showed that circ_0088212 overexpression suppressed osteosarcoma cell proliferation and migration and increased caspase-3 activity. Furthermore, circ_0088212 overexpression inhibited tumor growth in the in vivo xenograft osteosarcoma model. Collectively, these results show that circ_0088212 inhibits osteosarcoma tumorigenesis both in vitro and in vivo.

To further explore the underlying mechanism of circ_0088212, we characterized circ_0088212 and found that it was primarily a cytoplasmic circRNA in osteosarcoma cells, which suggested that circ_0088212 might act as a miRNA sponge. According to our bioinformatics analysis, miR-520 h was predicted to be a target of circ_0088212. The interaction between circ_0088212 and miR-520 h was further validated by luciferase reporter and RIP assays. MiRNAs are well recognized as another subtype of noncoding RNAs. MiR-520 h has been found to be increased in breast cancer [18], renal cell carcinoma [19], and epithelial ovarian cancer and hence, it acts as an oncomir [20]. Conversely, miR-520 h is downregulated in colorectal [21] and pancreatic cancers [22] and suppresses the progression of these cancers. The variable expression and contrasting roles of miR-520 h in different cancers might suggest that its role is tissue- and cancer type-dependent. However, its expression status and function in osteosarcoma have not been reported till date. Our results demonstrated that miR-520 h is highly expressed in osteosarcoma cells and that its overexpression increases cell proliferation and migration and decreases caspase-3 activity, suggesting that miR-520 h acts as an oncomir during osteosarcoma progression. Regarding its interaction with circ_0088212, we found that

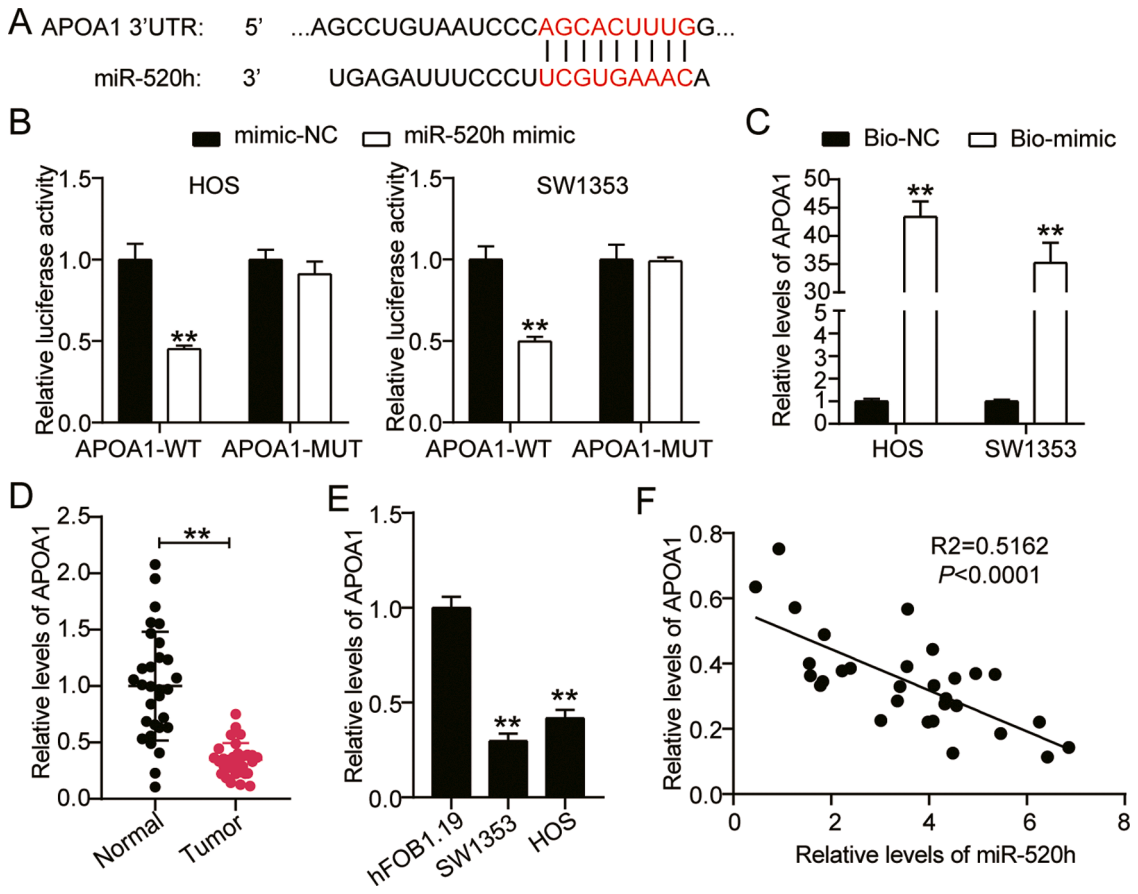

Fig. 6. APOA1 is a direct target of miR-520 h A. Putative binding sites of miR-520 h and APOA1. B. Luciferase activities in SW1353 and HOS cells co-transfected with miR-520 h and APOA1-WT or APOA1-MUT. Vs. miR-NC, $^{**}P<0.001$; C. RNA pull down assay explored the interaction between miR-520 h and its downstream APOA1 mRNAs. D. qRT-PCR for APOA1 mRNA in osteosarcoma tissues. Vs.Bio-NC, $^{**}P<0.001$; E. qRT-PCR for APOA1 mRNA in hFOB1.19, SW1353 and HOS cells. $^{**}P<0.001$; F. Pearson's correlation analysis revealed a negative correlation between APOA1 and miR-520 h.

the suppressive effect of circ_0088212 on the malignant behavior of osteosarcoma cells was abolished by miR-520 h overexpression, suggesting that circ_0088212 sequesters miR-520 h to exert its role. Our findings enrich the understanding of circRNA regulation in osteosarcoma progression.

As a participant of the ceRNA regulatory network, miRNAs exert their biofunction by targeting the 3'-UTR of mRNA and reshaping gene expression. In the present study, we verified APOA1 as a target of miR-520 h. APOA1 is a major protein component of high-density lipoprotein (HDL) in plasma and is responsible for de novo lipid biosynthesis. Perturbations of lipid or lipoprotein metabolism in the serum might impact tumorigenesis via inflammation and oxidative stress pathways [23]. Several studies have reported the deregulation of APOA1 in ovarian [24], breast [25], and lung cancers [12]. In addition, the modulation of APOA1 by ubiquitination is implicated in carcinogenesis in pancreatic cancer [26]. In a study on gastric cancer, low APOA1 expression was found to be associated with large tumors in the tumor-bearing gastric cancer mouse model [27]. All these results suggest an unfavorable role for APOA1 during malignancies. Zhang et al. used the NCBI GEO database and found that APOA1 might be a downstream effector of the miRNA-mediated regulatory network in colorectal cancer [13]. However, its role and regulatory mechanisms have not yet been reported. In this study, we confirmed the downregulation of APOA1 in osteosarcoma tissues and cells. Furthermore, APOA1 overexpression resulted in proliferative and migratory defects in osteosarcoma cells and increased apoptosis. Consistent with that of previous reports, our findings suggest that APOA1 inhibits tumor growth in osteosarcoma. As for the upstream regulatory mechanism of APOA1, we found that the tumor-suppressive effect of APOA1 can be abolished by miR-520 h overexpression. In the present study, we illustrated the tumor suppressive role of APOA1 and proposed a novel regulatory mechanism for miR-520 h via APOA1.

In addition, circRNAs or miRNAs harbor multiple binding sites and form various circRNA-miRNA-mRNA networks. Therefore, further studies are required to explore the underlying mechanism of circ-0,088,212 in osteosarcoma. Furthermore, the sample size was too small to support our analysis.

In summary, our results demonstrated that circ_0088212 is poorly expressed in osteosarcoma tissues and cells. Circ_0088212 serves as a tumor suppressor and markedly inhibits in vitro osteosarcoma cell proliferation and invasion and constrains in vivo tumorigenesis. Furthermore, it physically interacts with miR-520 h and exerts its tumor-suppressive function by downregulating APOA1 in osteosarcoma cells. The discovery of circ_0088212 enriches our understanding of the role of circRNAs in tumorigenesis and progression and may provide novel ideas for developing more efficient therapies for osteosarcoma.

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Ethics approval

The present study was approved by the Ethics Committee of Renmin Hospital of Wuhan University. The processing of clinical tissue samples is in strict compliance with the ethical standards of the Declaration of

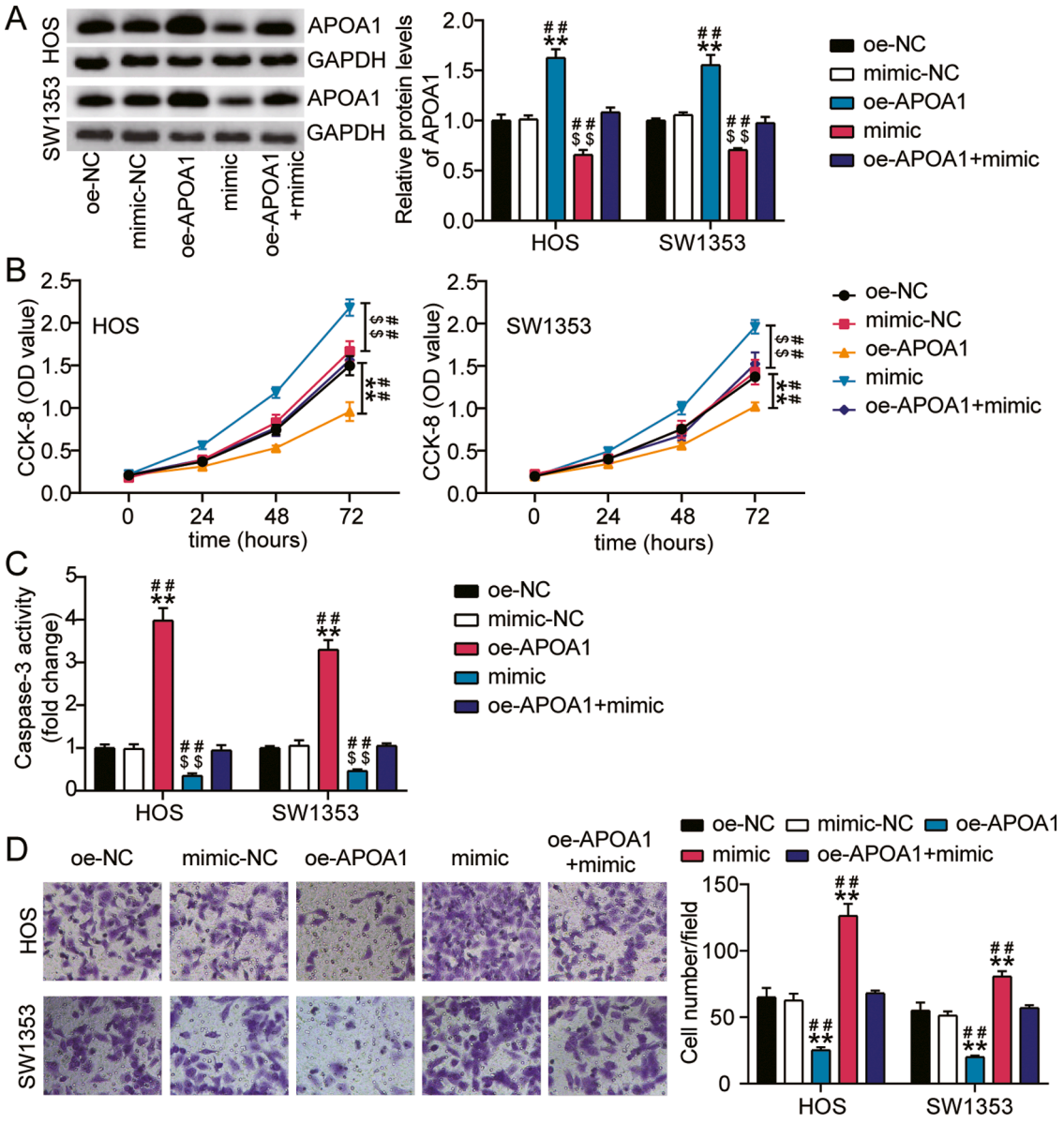

Fig. 7. miR-520 h overexpression reduced APOA1 expression and strengths osteosarcoma cell uncontrolling proliferation and migration. SW1353 and HOS cell were s co-transfected with miR-520 h mimics or miR-NC, APOA1-overexpressing vector (OE-APOA1) and empty vector (OE-NC), miR-520 h mimics +OE-APOA1. A. Western blot for APOA1 protein expression in SW1353 and HOS cells. B. CCK8 assays determining cell-proliferation in SW1353 and HOS cells. C. Caspase-3 activity in SW1353 and HOS cells was determined. D. Transwell invasion assay determining cell invasion. Vs. oe-NC, $^{**}P<0.001$; vs.mimic-NC, $^{\$\$}P<0.001$; vs. oe-APOA1+mimic, $^{\#\#}P<0.001$.

Helsinki. All patients signed written informed consent.

Consent to participate

All patients signed written informed consent.

Consent for publication

Consent for publication was obtained from the participants.

Availability of data and material

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

Code availability

Not available.

Authors' contributions

FL and XYZ performed the experiments and data analysis. FL and FW conceived and designed the study. FL and HP made the acquisition of data. HP did the analysis and interpretation of data. All authors read and approved the manuscript.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101219.

References

[1] L. Kager, G. Tamamyan, S. Bielack, Novel insights and therapeutic interventions for pediatric osteosarcoma, Future Oncol. 13 (4) (2017) 357–368.
[2] S. Simpson, M.D. Dunning, S. de Brot, L. Grau-Roma, N.P. Mongan, C.S. Rutland, Comparative review of human and canine osteosarcoma: morphology, epidemiology, prognosis, treatment and genetics, Acta Vet. Scand. 59 (1) (2017) 71.
[3] D.J. Harrison, D.S. Geller, J.D. Gill, V.O. Lewis, R. Gorlick, Current and future therapeutic approaches for osteosarcoma, Expert Rev Anticancer Ther 18 (1) (2018) 39–50.
[4] G. Romano, D. Veneziano, M. Acunzo, C.M. Croce, Small non-coding RNA and cancer, Carcinogenesis 38 (5) (2017) 485–491.
[5] L.S. Kristensen, T.B. Hansen, M.T. Venø, J. Kjems, Circular RNAs in cancer: opportunities and challenges in the field, Oncogene 37 (5) (2018) 555–565.
[6] Y. Wang, Y. Mo, Z. Gong, et al., Circular RNAs in human cancer, Mol. Cancer 16 (1) (2017) 25.
[7] J. Wang, L. Chen, Y. Zhang, et al., Association between serum vitamin B(6) concentration and risk of osteoporosis in the middle-aged and older people in China: a cross-sectional study, BMJ Open 9 (7) (2019), e028129.
[8] B. Wan, H. Hu, R. Wang, W. Liu, D. Chen, Therapeutic Potential of Circular RNAs in Osteosarcoma, Front Oncol 10 (2020) 370.
[9] X. Ji, L. Shan, P. Shen, M. He, Circular RNA circ_001621 promotes osteosarcoma cells proliferation and migration by sponging miR-578 and regulating VEGF expression, Cell Death Dis 11 (1) (2020) 18.
[10] Y. Chen, S. Zhang, C. Bai, Z. Guan, W. Chen, Circ_0000885 enhances osteosarcoma progression by increasing FGFR1 expression via sponging MiR-1294, Cancer Manag Res. 12 (2020) 6441–6452.
[11] J. Dufresne, P. Bowden, T. Thavarajah, et al., The plasma peptides of breast versus ovarian cancer, Clin. Proteomics 16 (2019) 43.
[12] S. Borgquist, T. Butt, P. Almgren, et al., Apolipoproteins, lipids and risk of cancer, Int. J. Cancer 138 (11) (2016) 2648–2656.
[13] T. Zhang, J. Guo, J. Gu, et al., Identifying the key genes and microRNAs in colorectal cancer liver metastasis by bioinformatics analysis and in vitro experiments, Oncol. Rep. 41 (1) (2019) 279–291.
[14] H. Wang, L. Liu, S. Fang, MicroRNA-330-5p inhibits osteosarcoma cell growth and invasion by targeting the proto-oncogene survivin, Mol. Med. Rep. 20 (3) (2019) 2236–2244.
[15] C. Xin, S. Lu, Y. Li, et al., miR-671-5p inhibits tumor proliferation by blocking cell cycle in osteosarcoma, DNA Cell Biol. 38 (9) (2019) 996–1004.
[16] X. Mao, S. Guo, L. Gao, G. Li, Circ-XPR1 promotes osteosarcoma proliferation through regulating the miR-214-5p/DDX5 axis, Hum. Cell 34 (1) (2021) 122–131.
[17] P.R. Zhang, J. Ren, J.S. Wan, R. Sun, Y. Li, Circular RNA hsa_circ_0002052 promotes osteosarcoma via modulating miR-382/STX6 axis, Hum. Cell 33 (3) (2020) 810–818.
[18] C.M. Su, M.Y. Wang, C.C. Hong, et al., miR-520h is crucial for DAPK2 regulation and breast cancer progression, Oncogene 35 (9) (2016) 1134–1142.
[19] W. Wang, Y. Yang, X. Chen, S. Shao, S. Hu, T. Zhang, MAGI1 mediates tumor metastasis through c-Myb/miR-520h/MAGI1 signaling pathway in renal cell carcinoma, Apoptosis 24 (11–12) (2019) 837–848.
[20] J. Zhang, W. Liu, F. Shen, et al., The activation of microRNA-520h-associated TGF-β1/c-Myb/Smad7 axis promotes epithelial ovarian cancer progression, Cell Death Dis. 9 (9) (2018) 884.
[21] T. Zhou, L. Wu, N. Ma, F. Tang, Z. Zong, S. Chen, LncRNA PART1 regulates colorectal cancer via targeting miR-150-5p/miR-520h/CTNNB1 and activating Wnt/β-catenin pathway, Int. J. Biochem. Cell Biol. 118 (2020), 105637.
[22] F. Wang, X. Xue, J. Wei, et al., hsa-miR-520h downregulates ABCG2 in pancreatic cancer cells to inhibit migration, invasion, and side populations, Br. J. Cancer 103 (4) (2010) 567–574.
[23] P.D. Chandler, Y. Song, J. Lin, et al., Lipid biomarkers and long-term risk of cancer in the Women's Health Study, Am. J. Clin. Nutr. 103 (6) (2016) 1397–1407.
[24] A.T. Marinho, H. Lu, S.A. Pereira, E. Monteiro, H. Gabra, C. Recchi, Anti-tumorigenic and platinum-sensitizing effects of apolipoprotein A1 and apolipoprotein A1 mimetic peptides in ovarian cancer, Front Pharmacol 9 (2018) 1524.
[25] J.X. Liu, Q. Yuan, Y.L. Min, et al., Apolipoprotein A1 and B as risk factors for development of intraocular metastasis in patients with breast cancer, Cancer Manag Res 11 (2019) 2881–2888.
[26] Y. Sun, D. Ren, C. Yang, et al., TRIM15 promotes the invasion and metastasis of pancreatic cancer cells by mediating APOA1 ubiquitination and degradation, Biochimica et biophysica acta Mol. Basis Dis. 1867 (11) (2021), 166213.
[27] P.K. Chong, H. Lee, J. Zhou, et al., Reduced plasma APOA1 level is associated with gastric tumor growth in MKN45 mouse xenograft model, J. Proteomics 73 (8) (2010) 1632–1640.