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RESEARCH ARTICLE

Circular RNA hsa_circ_0002052 promotes osteosarcoma via modulating miR-382/STX6 axis

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Abstract

Circular RNAs (circRNAs) exert pivotal effects on regulating the progression of osteosarcoma (OS). It was found through microarray analysis that *circ-0002052* is abnormally expressed in OS, but the role of *circ-0002052* in OS remains obscure. The results of this research manifested that relative to that in non-tumor controls, *circ-0002052* level was raised in OS tissues. Up-regulated *circ-0002052* was associated with advanced stage, tumor size, and metastasis. Additionally, *circ-0002052* elevation indicated a low survival rate in OS patients and silencing of *circ-0002052* suppressed proliferation, migration, and invasion of OS cells. It was proved that *circ-0002052* sponged miR-382 and stimulated *STX6* expression, thus activating Wnt/β-catenin. The function of *circ-0002052* reduction in OS cells was effectively reversed by miR-382 suppression. To sum up, it can be concluded that *circ-0002052*, functioning as a sponge for miR-382, enhances the activation of Wnt/β-catenin mediated by *STX6* to stimulate the progression of OS, and *circ-0002052* may be an underlying treatment target and a biomarker for prognosis of osteosarcoma.

Keywords *Circ-0002052* · Osteosarcoma · miR-382 · Migration · Proliferation

Introduction

OS is an invasive cancer of the bone and usually occurs among teenagers [1]. In adolescent malignant tumors, the incidence rate of OS ranks only second to lymphoma [2]. OS has a high degree of malignancy, early hematogenous metastasis, high incidence of lung metastasis, rapid development of the disease, and very high mortality rate [3–5]. OS cases have a rather low 5-year survival rate [6]. Up to now, there are no efficacious regimens for radically curing OS. Particularly, the growth mechanism of OS still needs to explore. Today, there is an urgent need to investigate novel targets for OS therapy and improve patients' outcomes.

CircRNAs are a type of ncRNAs that are extensively expressed in numerous cell species [7] and their 3′ and 5′ ends join to one another forming a circle-like structure [8]. Increasing studies have manifested that circRNAs exert crucial effects in varying processes related to pathology, including those in cancer [9–11]. For example, Ma et al. found that *circ-000284* facilitates cells to proliferate and invade in cervical cancer [12]. Li et al. discovered that *circDDX17* acts as a tumor suppressor in colorectal cancer [13]. Li et al. found that circRNA *BCRC4* overexpression modulates miR-101/*EZH2* signaling and cell apoptosis in bladder cancer [14]. In recent years, research has denoted that circRNAs are intimately associated with the pathogenesis of osteosarcoma [15, 16]. So, the microarray GSE96964 was reviewed and it was found that *circ-0002052* is abnormally expressed in OS.

The role of circRNA in regulation of miRNAs has always been of concern [17]. MiRNAs, with a length of about 22–25 nt, are a kind of non-coding RNA. It is able to recognize the target gene mRNA by base complementary matching, and directly degrade or inhibit translation, so as to exert post-transcriptional regulation of genes [18, 19]. Studies have shown that hsa-miR-19-3p and hsa-miR106b-3p may be reliable markers for predicting and treating OS [20]. Besides, Xu et al. found that miR-382-suppressing tumors

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and that miR-382 overexpression is a new strategy to inhibit tumor metastasis and prevent CSC-induced relapse in OS [21].

In conclusion, this study clarified the effect of *circ-0002052* on the proliferation, invasion, and migration of OS cells, and verified that *circ-0002052* exerts its biological function by binding to miR-382 to regulate *STX6* expression. In addition, it may provide us with a new perspective for the in-depth study of the diagnosis of OS.

Materials and methods

Human samples

OS and non-cancer tissues were harvested from the OS patients of the First People's Hospital of Qujing and kept in liquid nitrogen following operation. All cases received no chemoradiotherapy prior to operation. They singed the informed content. This research received the approval from the Ethic Committee of the First People's Hospital of Qujing.

Culture and transfection of cells

The American Type Culture Collection (USA) supplied OS cell lines, non-cancer cell line hFOB 1.19 and 293T cell line, which were then raised in DMEM with 10% FBS provided by Gibco (USA), streptomycin (100 mg/mL), and penicillin (100 U/mL). The *circ-0002052* overexpressing plasmids, shRNAs targeting *circ-0002052*, and negative control were purchased from RiboBio (Guangzhou, China). miR-382 mimics and inhibitors were obtained from GeneCopoeia (Guangzhou, China). Transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

Quantitative real-time PCR (qRT-PCR)

Based on the TRIzol total RNA manual, centrifugation was performed at 4 °C, and isopropanol precipitates in the upper aqueous phase were harvested, rinsed, and dried at room temperature. Subsequently, 20–30 mL DEPC-treated water was added, RNA concentration was calculated, and RNAs were preserved in a refrigerator at − 80 °C. With reference to the Takara OneStep PrimeScript® miRNA cDNA Synthesis Kit instructions, RT was conducted, followed by PCR detection using SYBR Green I fluorescence method. *STX6* primer sequences: F: 5′-CACCAACGAGCTGAGAAATAACC-3′, R: 5′-CCCTGACAACTTGCCGAGT-3′. *U6* primer sequences: F: 5′-CTCGCTTCGGCAGCACA-3′, R: 5′-AACGCTTCACGAATTTGCGT-3′. *GAPDH* primer sequences: F: 5′-GTCAACGGATTTGGTCTGTATT-3′, R: 5′-AGTCTTCTGGGTGGCAGTGAT-3′. $2^{-\Delta\Delta Ct}$ was utilized to calculate the relative concentration of the samples to be examined. Each experiment was repeated thrice for averaging.

Examination of proliferation

To implement CCK-8 assay, 2×10^3 cells were raised for specified time following inoculation into a ninety-six-well plate, followed by incubation with CCK-8 solution acquired from Dojindo Laboratories (Japan) for 2 h. After that, a microplate reader supplied by TECAN (Austria) was employed to measure absorbance at 450 nm. EdU experiment was implemented to test cell proliferation. Each assay was implemented thrice one by one.

Wound healing assay

Following 24 hours of transfection, a micropipette tip was used to make a straight scratch in each well center. In each well, cells migrated to the scratch, which was observed to analyze cell migration. 24 hours later, the speed of wound closure was measured and normalized to the length at 0 h. Each assay was conducted in triplicate.

Transwell invasion assay

Transwell chambers (Corning, NY, USA) coated with Matrigel (BD Biosciences) were applied to test cell invasion. The upper Transwell chamber was inoculated with the transfected cells (2.0×10^4 per well) in medium without serum, whereas the lower Transwell chamber was added with DMEM (10% FBS) as a chemoattractant. Following 48 h of incubation at 37 °C, the cells not subjected to invasion on the upper chamber were rubbed away using cotton swabs, but those undergoing invasion on the lower chamber underwent fixation in 4% paraformaldehyde for 20 min, followed by 15 min of 1% crystal violet (Sigma) staining. Cells undergoing invasion were photographed and calculated in five fields of view randomly selected in each filter using an optical microscope (Olympus).

Luciferase reporter assays

Circ-0002052 WT, *circ-0002052* MUT, *STX6* WT or *STX6* MUT sequence were put into pmiR-reporter attained from Promega (USA). Subsequently, OS cells were treated with miR-382 mimics or miR-NC, at 48 h after which, the dual-luciferase reporter assay kit supplied by Promega (USA) was utilized to test the relative activity of luciferases.

Fractionation in the nucleus and cytoplasm

Nuclear and cytoplasmic fractions were picked with the use of PARIS Kit obtained from Life Technologies (USA) by reference to the regimen of the manufacturer. Then qRT-PCR was carried out. U6 was considered to be the nuclear control, whereas GAPDH to be the cytoplasmic control.

Statistical analysis

GraphPad Prism 6.0 from GraphPad Inc. (USA) was utilized for data processing. Experimental results were represented as mean ± standard deviation (SD). ANOVA or Student's *t* test was carried out for the assessment of differences. Pearson's correlation coefficient was used to test associations among *circ-0002052*, miR-382, and *STX6*. Besides, the log-rank test and Kaplan–Meier method were adopted for the assessment of survival rates. $p < 0.05$ denoted a statistically significant difference.

Results

Circ-0002052 elevation is related to poor prognosis of OS

Through analyzing the microarray GSE96964 from platform GPL19978, *circ-0002052* was found to be highly expressed in OS cells (Fig. 1a). To ascertain the possible functions of *circ-0002052* in OS, qRT-PCR was implemented to test

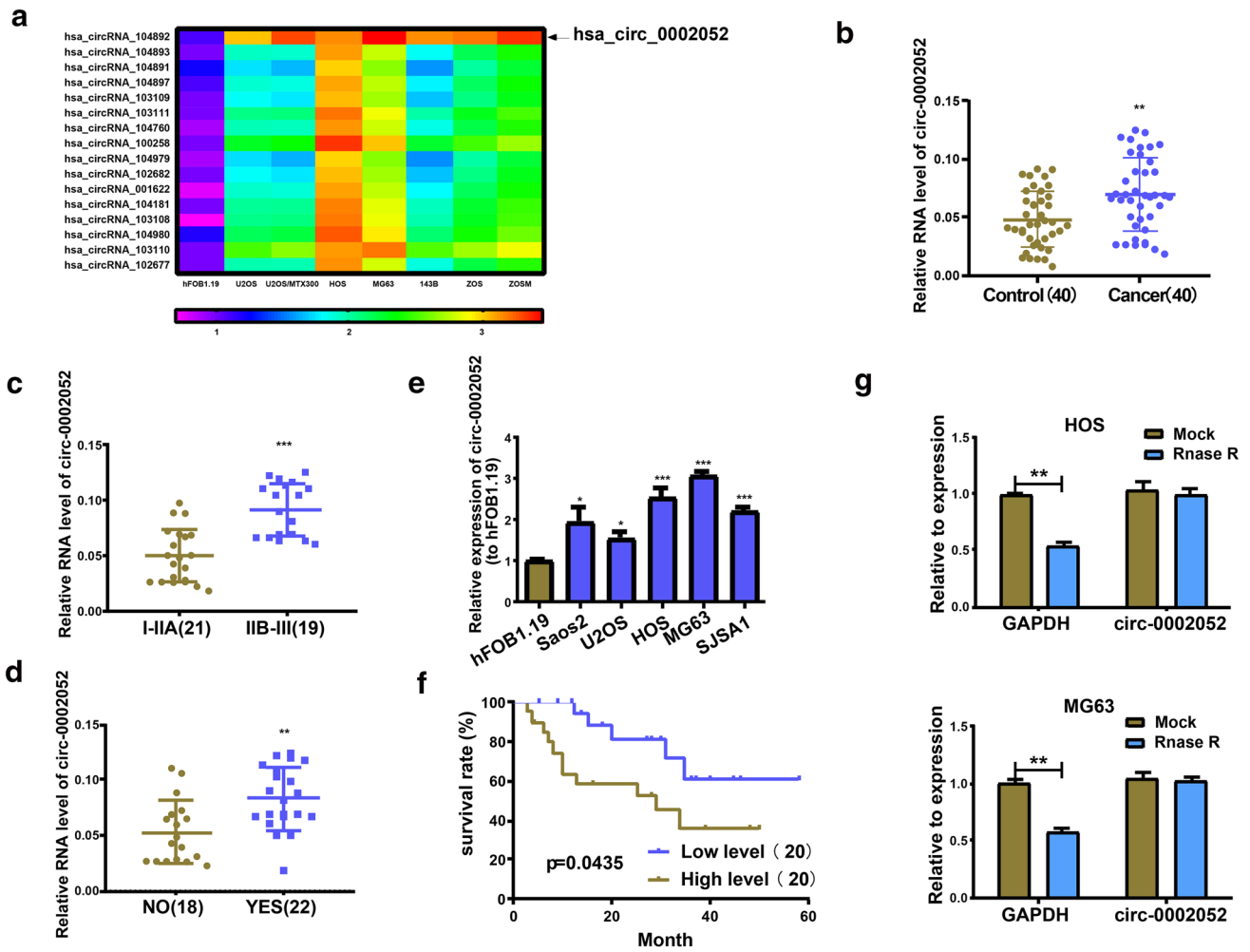

Fig. 1 *Circ-0002052* elevation is related to poor prognosis of OS. **a** Analysis of microarray GSE96964 from platform GPL19978 containing even human OS cell lines (U2OS, U2OS/MTX300, HOS, MG63, 143B, ZOS, ZOSM) and the human osteoblast hFOB1.19. CircRNAs with varying expressions are identified on the basis of the criteria of fold change > 2 and $p < 0.05$. **b** *Circ-0002052* expression is tested in forty OS tissues and forty non-cancer controls. **c** Assessment of *circ-0002052* expression in OS tissues at denoted stages. **d** Assessment of *circ-0002052* expression in OS tissues undergoing distant metastasis or not. **e** Assessment of *circ-0002052* expression in OS cells. **f** Kaplan–Meier curve is utilized to determine overall survival rate. **g** *Circ-0002052*'s resistance to RNase R digestion in OS cells. Each value represents mean ± SD in three independent experiments. $*p < 0.05$, $**p < 0.01$, $**p < 0.001$

its levels in OS tissues and matching non-tumor controls collected. *Circ-0002052* expression was increased in OS tissues (Fig. 1b). Obviously, the levels of *circ-0002052* in OS tissues at the late stage (Fig. 1c) and those subjected to metastasis (Fig. 1d) were higher. Additionally, the association between clinical characteristics and *circ-0002052* was assessed. It was affirmed that *circ-0002052* expression was positively correlated with late tumor stage as well as tumor size and metastasis in a positive manner, and *circ-0002052* level was not overtly associated with sex or age (Table 1). Identically, *circ-0002052* expression went up in OS cells (Fig. 1e). The assessment of the overall survival rate unraveled that the higher *circ-0002052* expression was accompanied with a lower survival rate (Fig. 1f), suggesting that *circ-0002052* is a possible marker for the prognosis of OS cases. As *circ-0002052* is resistant to RNase R digestion, it was verified to possess the characteristics of circRNAs (Fig. 1g).

Circ-0002052 sponges miR-382 in OS

To date, whether *circ-0002052* could be a miRNA sponge remains unknown. We performed nuclear/cytoplasmic fractionation assay and found that *circ-0002052* was mainly located in the cytoplasm of OS cells (Fig. 2a). Then, we designed shRNAs targeting *circ-0002052* and confirmed their efficiency (Fig. 2b). Bioinformatic analysis using circinteractome (https://circinteractome.nia.nih.gov/) denoted that miR-382 might be a target. Moreover, *circ-0002052* reduction in OS cells led to a remarkable rise in miR-382 level (Fig. 2c), and *circ-0002052* expression had an inverse relationship to miR-382 in OS tissues (Fig. 2d), implying the regulatory correlation between them. Since sh-*circ-0002052*#2 showed the greatest knockdown efficiency, sh-*circ-0002052*#2 (named as sh-*circ-0002052*) was used for subsequent experiments. Then *circ-0002052*-WT or -MUT reporter was utilized for luciferase reporter assay, so as to ascertain the interaction between the two. MiR-382 mimics in HOS and MG63 cells repressed the activity of *circ-0002052*-WT reporter (Fig. 2e, f). What's more, reduction assay manifested that miR-382 reduced *circ-0002052* in OS cell lysates in a direct manner (Fig. 2g). Hence, *circ-0002052* sponges miR-382 in OS in a direct way.

Table 1 Association of *circ-0002052* expression with clinicopathological features of osteosarcoma

Feathers	Number	High	Low	*p* value
All cases	40	20	20	
Age (years)				0.7475
< 18	24	13	11	
≥ 18	16	7	9	
Gender				0.7512
Male	18	10	8	
Female	22	10	12	
Tumor size (cm)				**0.0187**
< 5	14	3	11	
≥ 5	26	17	9	
Distant metastasis				**0.0248**
Absent	18	5	13	
Present	22	15	7	
Anatomic location				0.7524
Tibia/femur	20	9	11	
Elsewhere	20	11	9	
Clinical stage				**0.0104**
I ~ IIA	21	6	15	
IIB ~ III	19	14	5	

Total data from 40 tumor tissues of osteosarcoma patients were analyzed. The expression of *circ-0002052* was assayed by qRT-PCR, the median expression level was used as the cutoff. Data were analyzed by Chi-squared test and Fisher's exact test

p value in bold indicates statistically significant

Circ-0002052-miR-382 regulatory loop plays a vital role in cellular functions

Afterwards, the potential roles of *circ-0002052* in OS were investigated. CCK-8 and EdU incorporation assays were carried out and indicated that *circ-0002052* knockdown suppressed the proliferation of HOS and MG63 cells (Fig. 3a, b). To explore whether *circ-0002052* regulates metastasis and invasion, wound healing assay and Transwell assay was performed. As shown, decreased expression of *circ-0002052* led to attenuated migration and invasion of HOS and MG63 cells (Fig. 3c, d). More importantly, transfection with miR-382 inhibitors reversed the effects of *circ-0002052* knockdown on proliferation, migration, and invasion (Fig. 3a–d).

We next examined whether miR-382 affected cell proliferation, migration, and invasion in OS cells. Compared with negative controls, overexpression of miR-382 obviously inhibited capability of cell proliferation, migration, and invasion, and *circ-0002052* up-regulation could partially reverse miR-382 suppression effect (Fig. 4a–d). Thus, *circ-0002052* promotes OS progression by sponging miR-382.

STX6 up-regulation by *circ-0002052*-caused miR-382 suppression induces activation of Wnt/β-catenin signaling

To further explore the molecular mechanism of *circ-0002052*/miR-382 axis, we further investigated downstream signaling. Through bioinformatic analysis (TargetScan7) and literature review, we selected *STX6* as a potential target of miR-382. To validate it, luciferase reporter assay was conducted. MiR-382 mimics markedly reduced the activity of WT-*STX6*-reporter (Fig. 5a, b). Furthermore, *STX6* expression had an inverse association

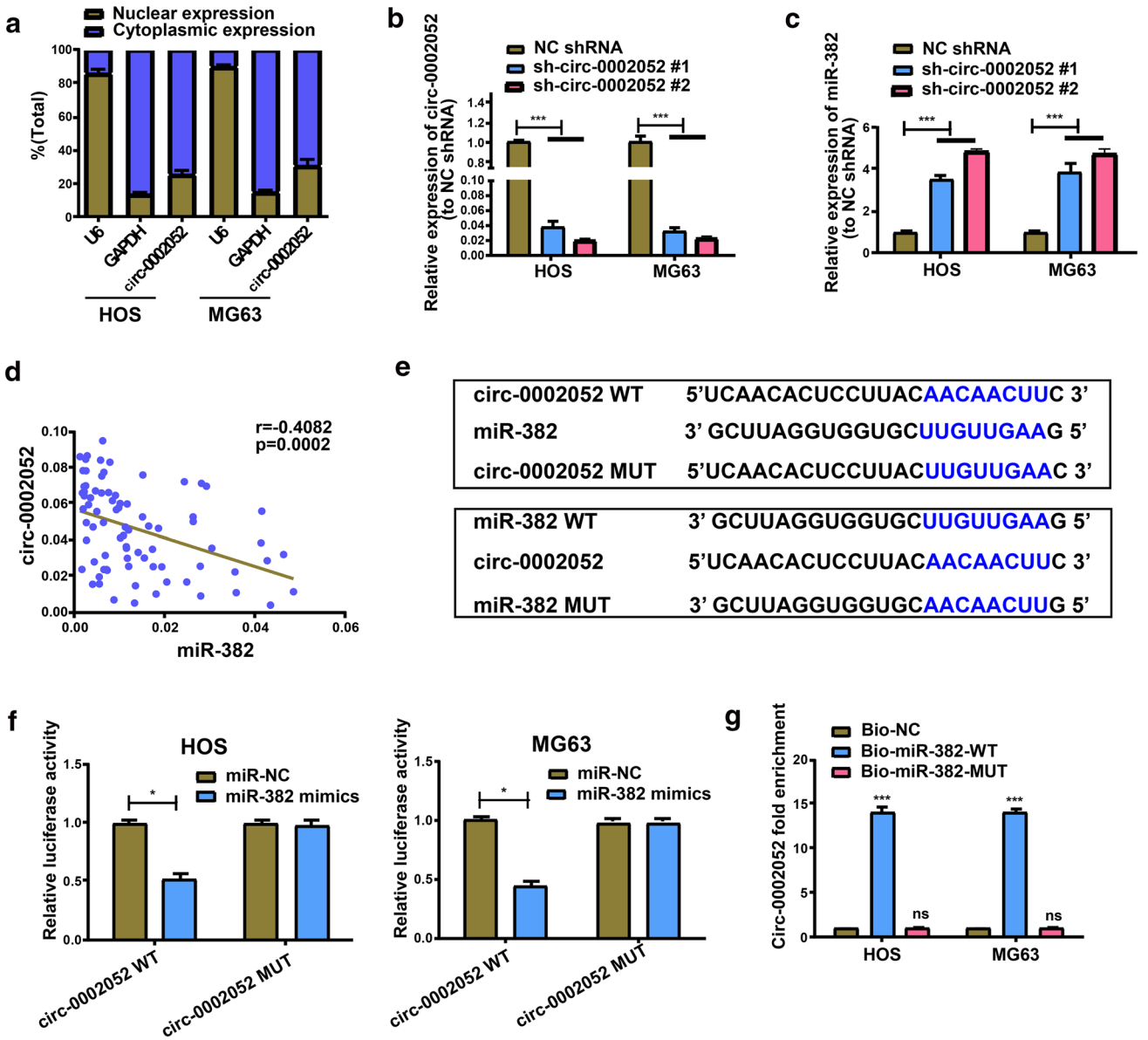

Fig. 2 *Circ-0002052* sponges miR-382 in OS. **a** Fractionation assays are implemented in the nucleus and cytoplasm to detect *circ-0002052* expression. **b** *Circ-0002052* expression is lowered with the use of two shRNAs specific to it. **c** *Circ-0002052* reduction raises miR-382 expression. **d** Inverse relationship between *circ-0002052* and miR-382 in OS tissues ($r = -0.4082$). **e**, **f** *Circ-0002052-WT* activity is weakened by miR-382 displayed in luciferase reporter assay. **g** Biotin-miR-382-WT mutually acts with *circ-0002052* by reduction assay. Each value represents mean ± SD in three independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$; *ns* no significance

with miR-382, but was positively related to *circ-0002052* in OS tissues (Fig. 5c). Moreover, miR-382 inhibited *STX6* expression in OS cells (Fig. 5d). Also, *circ-0002052* reduction caused the lowered *STX6* expression, but treatment with miR-382 inhibitor abolished this effect (Fig. 5d). Hence, *circ-0002052* facilitates *STX6* expression through impeding miR-382. Numerous studies have shown that Wnt/β-catenin is closely related to OS [22–24]. Hence, we sought to explore whether *circ-0002052* eventually regulates activation of Wnt/β-catenin signaling and promotes OS progression. The effects of *circ-0002052* on expressions of the target genes (*MYC*, *SOX4*, and *CCND1*) of Wnt/β-catenin signaling were tested. It was discovered that *circ-0002052* reduction lessened their expressions in a manner dependent on *STX6* (Fig. 5e). To sum up, *circ-0002052* mediates miR-382/*STX6* axis in OS to modulate the activation of Wnt/β-catenin signaling.

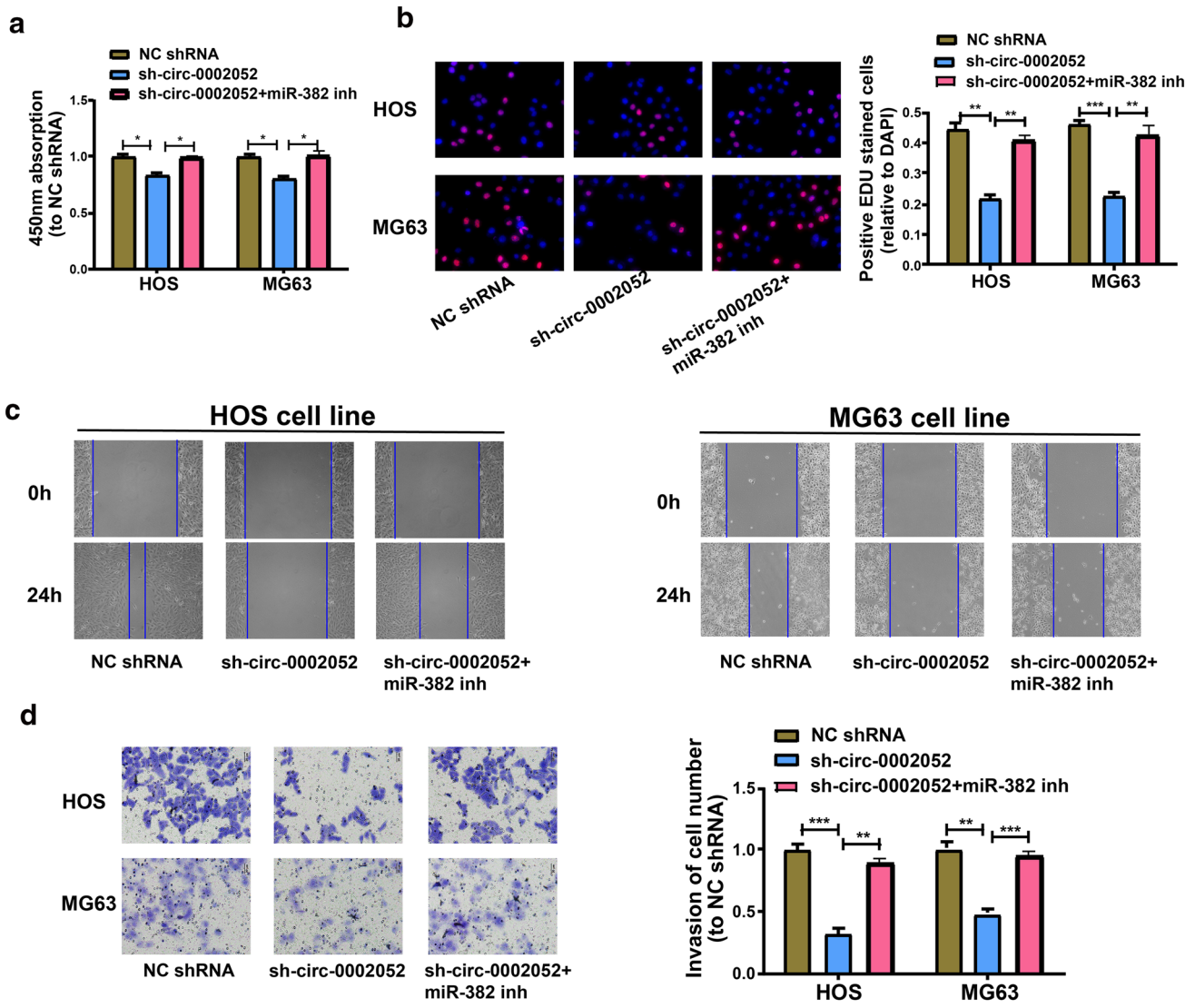

Fig. 3 *Circ-0002052* promotes OS progression through suppressing miR-382. **a** CCK-8 and **b** EdU assays are conducted to test cell proliferation. **c** Wound healing assay is implemented to assess HOS and MG63 cell migration. **d** Invasion of HOS and MG63 cells is evaluated via Transwell assay. HOS and MG63 cells are treated with NC shRNA, sh-*circ-0002052* or co-treated with sh-*circ-0002052* and miR-382 inhibitor. Each value represents mean ± SD in three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Discussion

Elucidating the molecular mechanism of OS development is still urgently required. It was shown in this research that *circ-0002052* rose in OS tissues and had associations with late tumor stage as well as tumor size and metastasis. Apart from that, high *circ-0002052* expression represented a low survival rate. Reduction of *circ-0002052*-suppressed OS cells to migrate, proliferate, and invade. Nevertheless, the result of invasion assay only due to proliferation and migration, or also with matrix degradation by MMPs needs further study.

The mutual action between circRNA and miRNA has been extensively researched [25, 26]. Through regulating the activity of miRNAs, circRNA indirectly promotes expression of specific mRNAs [27]. For instance, *circRNA_010567* boosts myocardial fibrosis by repressing miR-141 through targeting TGF-β1 [28]. However, whether *circ-0002052* has target miRNAs remains to be figured out. In the present research, *circ-0002052* was discovered to be present in the OS cell cytoplasm, implying that *circ-0002052* may sponge miRNA. After analysis, *circ-0002052* was predicted to sponge miR-382. The mutual action between the two was subsequently confirmed by reduction assay and luciferase reporter assay. Apart from that, *circ-0002052* impeded miR-382 expression in OS. MiR-382 acts as a tumor suppressor in many cancers [29, 30], including OS [31]. Thus, *circ-0002052* sped up the growth of OS via repressing miR-382.

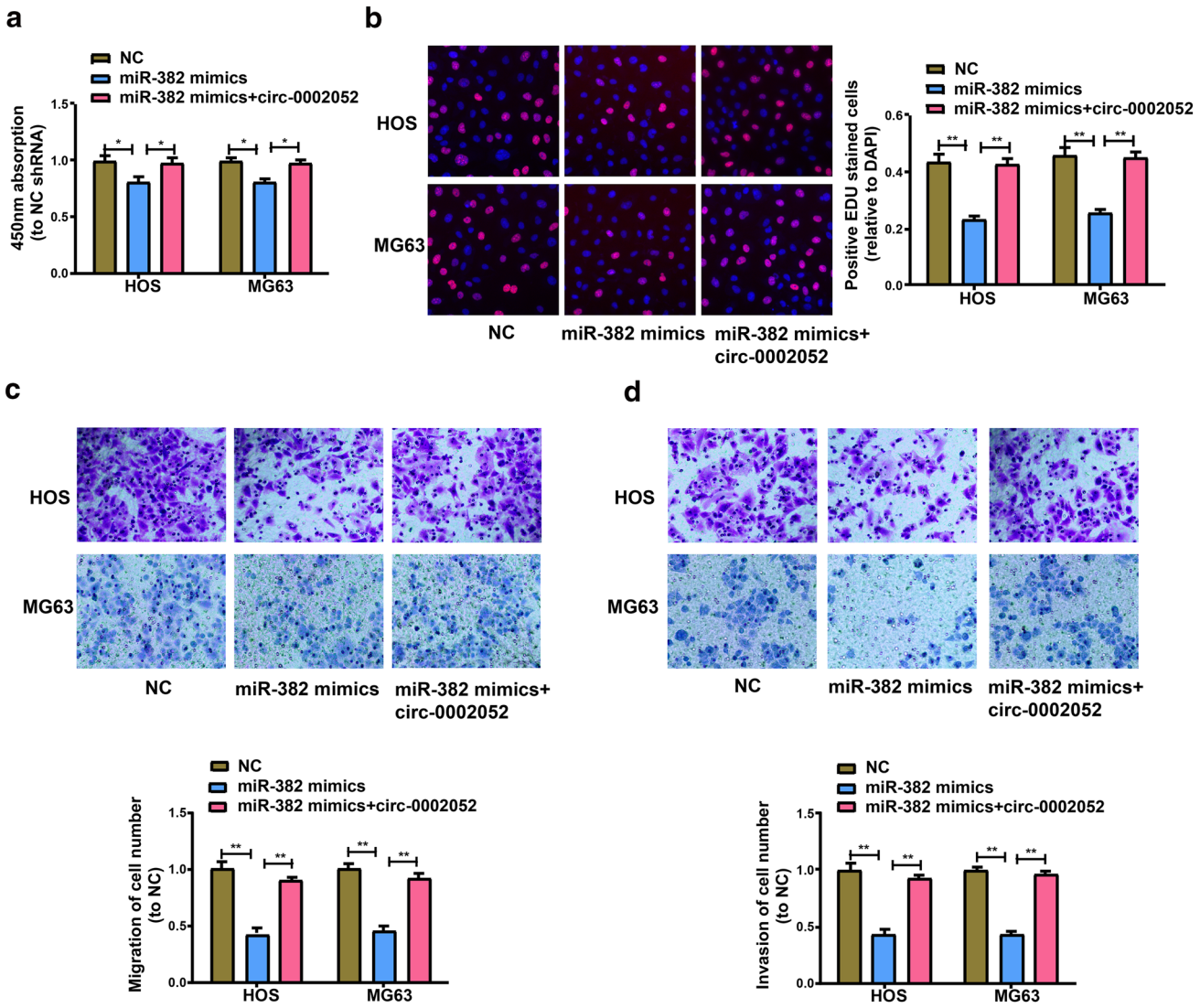

Fig. 4 *Circ-0002052*-miR-382 regulatory loop plays a vital role in cellular functions. **a** CCK-8 and **b** EdU assays are conducted to test cell proliferation. **c** Wound healing assay is implemented to assess HOS and MG63 cell migration. **d** Invasion of HOS and MG63 cells is evaluated via Transwell assay. HOS and MG63 cells are treated with negative controls, miR-382 mimics or co-treated with *circ-0002052* overexpressing plasmids and miR-382 mimics. Each value represents mean ± SD in three independent experiments. *$p < 0.05$, **$p < 0.01$

Wnt/β-catenin signaling is a widely accepted pathway in OS, so its correlation with *circ-0002052* was examined. According to the findings, *circ-0002052* contributed to Wnt/β-catenin signaling activation through elevating *STX6* in OS cells. Nevertheless, whether PI3K/AKT, MEK/ERK, JNK, NF-kB, or Notch signaling pathways are involved in *circ-0002052*/miR-382/*STX6* axis needs further study.

To conclude, our work demonstrated *circ-0002052* as a novel oncogene in OS. Moreover, we elucidated that *circ-0002052* activates Wnt/β-catenin signaling through miR-382/*STX6* axis to promote OS progression.

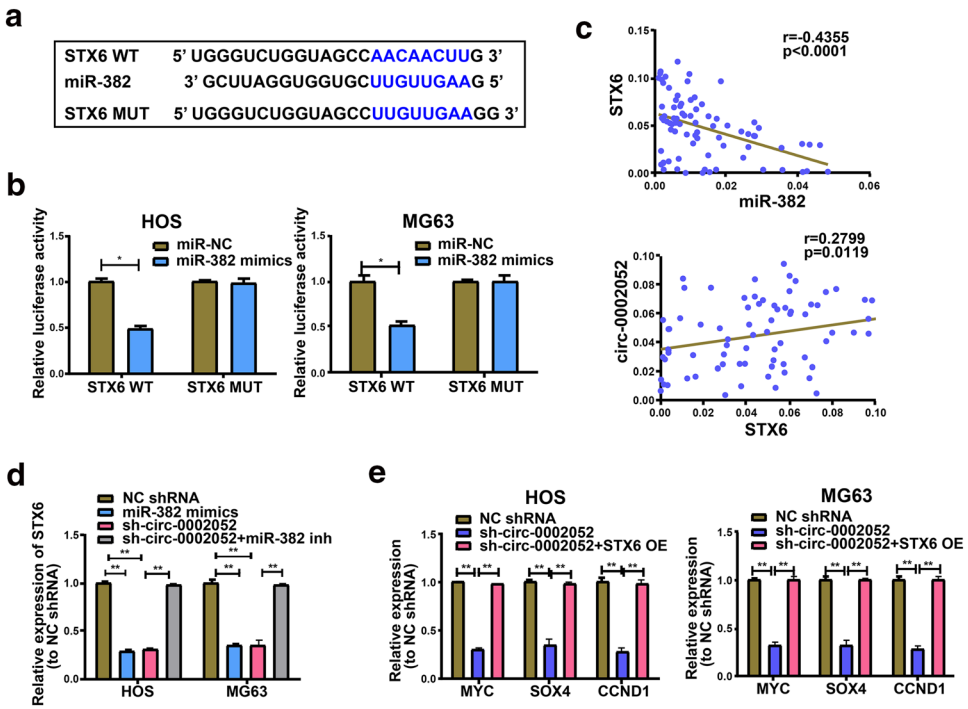

Fig. 5 *STX6* up-regulation by *circ-0002052*-caused miR-382 suppression induces activation of Wnt/β-catenin signaling. **a**, **b** MiR-382 targets *STX6* in a direct way demonstrated by luciferase reporter assay. **c** *STX6* in OS is negatively correlated with miR-382 ($r=-0.4355$), but positively related to *circ-0002052* ($r=0.2799$). **d** Expression of *STX6* was measured by qRT-PCR in OS cells transfected with described plasmids. **e** Expression of target genes (MYC, SOX4, and CCND1) of Wnt/β-catenin signaling was tested by qRT-PCR transfected with described plasmids. Each value represents mean ± SD in three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$

Author contributions PZ made substantial contributions to conception and design. JR, RS, and YL made acquisition of data. PZ and JR performed the experiments. JW and RS wrote the draft manuscript. All authors contributed to the reviewing of the manuscript, and approved the final manuscript for submission.

Availability of data and materials The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

Ethical standards The study was performed in accordance with the Declaration of Helsinki and obtained the approval from the Ethics Committee of the First People's Hospital of Qujing.

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