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Original Article

E2F3 promotes liver cancer progression under the regulation of circ-PRKAR1B

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Liver cancer is one of the most lethal malignant tumors in the world. The high recurrence and mortality rate make it urgent for scientists and clinicians to find new targets for better treatment of liver cancer. Here, we found that circ-PRKAR1B expression was increased in the paired intrahepatic metastasis sample through high-throughput sequencing. Further experiments also confirmed its high expression both in carcinoma and metastasis when compared to the paired para-carcinoma and the paired carcinoma, respectively. Mechanism study showed that circ-PRKAR1B could promote liver cancer progression through the miR-432-5p/E2F3 pathway, and microRNA-432-5p could directly target the 3′ untranslated region (UTR) of E2F3 mRNA to suppress its translation, thereby influencing liver cancer cell invasion and migration capacities. Clinical data obtained by using online databases based on The Cancer Genome Atlas (TCGA) samples and the clinicopathological data of liver cancer patients who underwent surgery in our hospital in the past 2 years also confirmed the significance of circ-PRKAR1B/miR-432-5p/E2F3 signaling in liver cancer progression. Animal experiments also indicated that targeting this newly identified signaling by overexpressing microRNA-432-5p could suppress the progression of liver cancer. Together, our study suggests that circ-PRKAR1B plays an important role in the regulation of liver cancer progression, and targeting this new circ-PRKAR1B/miR-432-5p/E2F3 signaling may help us find new treatment strategies to better suppress liver cancer progression.

INTRODUCTION

Primary liver cancer (PLC) is one of the main malignant tumors that seriously endanger human health. According to the histological classification of liver cancer, more than 90% of primary liver cancers are hepatocellular carcinoma (HCC). According to epidemiological survey data, PLC accounts for the fifth most commonly diagnosed and second most fatal cancer worldwide.[1] In China, the incidence and mortality of PLC are ranked 4th and 2nd, respectively.[2] The data from the American Cancer Society show that there will be 42,230 estimated new cases and 30,230 estimated new deaths of liver and intrahepatic bile duct cancer occurring in the United States in 2021.[1] The rate of metastasis and recurrence of HCC is very high among cancers.[3,4] In the past few decades, the therapeutic effect is still hard to satisfy clinicians and patients although progress has been made in the treatment of liver cancer. Therefore, finding new therapeutic targets to better treat liver cancer is the primary problem faced by liver surgeons and oncologists.

Circular RNA (circRNA) is a special type of non-coding RNA molecule. Unlike traditional linear RNA (linear RNA containing 5′ and 3′ ends), circRNA molecules are in a closed-loop structure, which is not affected by RNA exonuclease, has more stable expression, and is not easily degraded. circRNAs have been well documented to act as key molecules to regulate tumor initiation, progression, metastasis, and chemotherapy resistance. Sang et al.[5] reported that circRNA_0025202 could regulate the chemotherapy resistance of breast cancer through FOX signaling. Zeng et al.[6] showed that circHIPK3 could promote colorectal cancer growth and metastasis by sponging miR-7. Even though there are some papers published concerning the role of circRNAs in liver cancer,[7,8] to date, the detailed pathophysiological functions of circRNAs in the regulation of liver cancer remain to a large extent unknown.

As one type of non-coding RNAs, microRNAs (miRNAs) are short in length (20–24 nt) and have been demonstrated to affect the stability and translation of mRNAs through post-transcriptional regulation by binding to the 3′ untranslated region (UTR) mRNA of protein-coding genes. miRNAs have been reported to play important roles in the regulation of the progression of many cancers, including liver cancer.[9]

E2F transcription factor 3 (E2F3) encodes a member of a small family of transcription factors that function through binding of dimerization partner (DP) interaction partner proteins. The encoded protein recognizes a specific sequence motif in DNA and interacts directly with the retinoblastoma protein (pRB) to regulate

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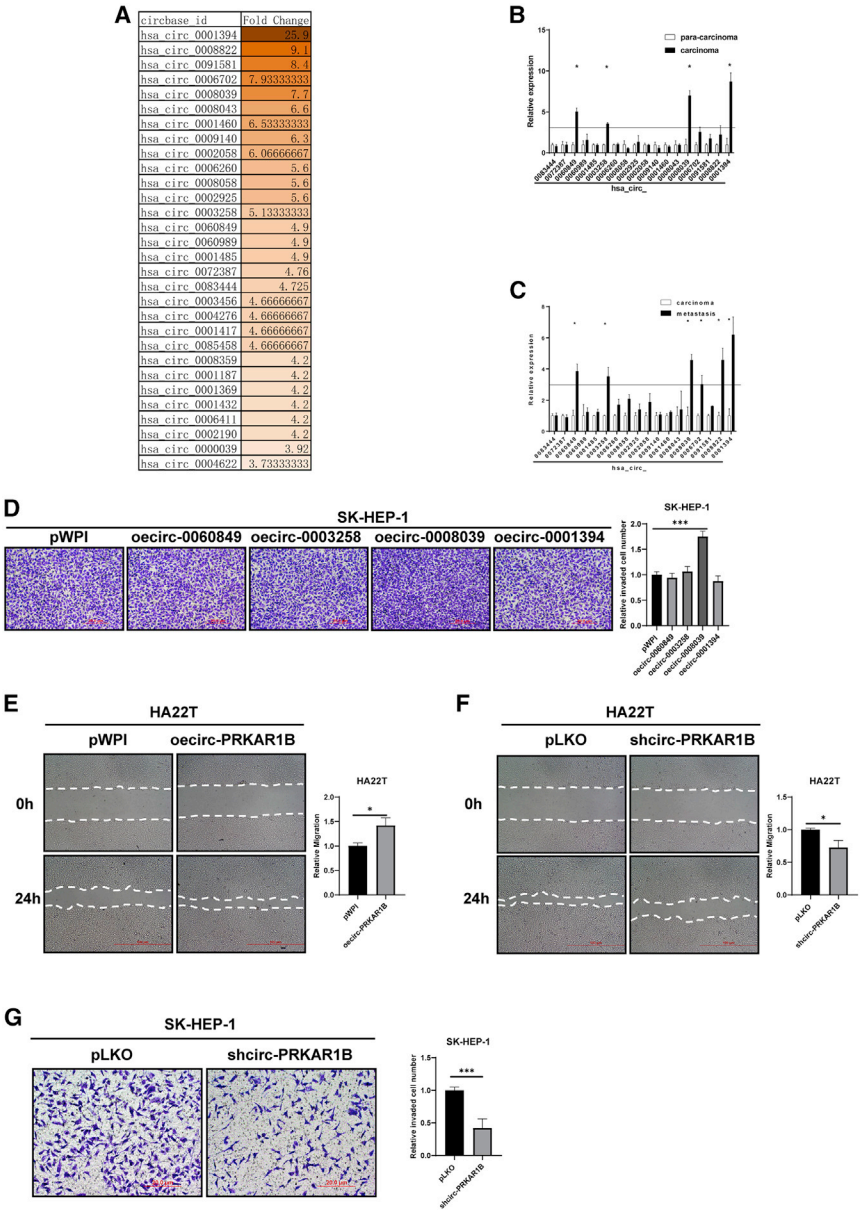


Figure 1. circ-PRKAR1B could promote liver cancer cell invasion and migration capacities

(A) The top 30 increased circRNAs conducted by high-throughput sequencing are shown, and the top 18 circRNAs were selected for further analysis. (B and C) qRT-PCR assay was used to check the top 18 circRNAs in the paired para-carcinoma and carcinoma (B) and the paired carcinoma and metastasis (C), respectively. (D) The invasion capacity was checked by Transwell invasion assay after overexpressing the selected 4 circRNAs in SK-HEP-1 cells. Right, statistical analysis of invading SK-HEP-1 cells. (E) The migration capacity was checked by wound-healing migration assay after overexpressing circ-PRKAR1B in HA22T cells. (F) The migration capacity was checked by wound-healing migration assay after knocking down circ-PRKAR1B in HA22T cells. (G) The invasion capacity was checked by Transwell invasion assay after knocking down circ-PRKAR1B in SK-HEP-1 cells. All quantifications are presented as mean ± SD and p values calculated by t test. **p < 0.01; ***p < 0.001; NS, no significance.

the expression of genes involved in the cell cycle. Altered copy number and activity of this gene have been observed in a number of human cancers. Feng et al.[10] reported that E2F3 could promote cancer growth and was overexpressed through copy number variation in human melanoma. Pei et al.[11] concluded that the E2F3/miR-125a/DKK3 regulatory axis promoted the development and progression of gastric cancer.

Here, we found that circ-PRKAR1B/miR-432-5p/E2F3 signaling was involved in the progression of liver cancer. Targeting this newly identified signaling may help us find new treatment strategies to better suppress liver cancer progression.

RESULTS

circ-PRKAR1B could promote liver cancer cell invasion and migration capacities

circRNAs have been well documented to act as key molecules to promote cancer progression. In our study, we first collected one HCC clinical tumor sample and the paired intrahepatic metastasis sample for high-throughput sequencing and selected the top 30 increased circRNAs in the metastasis sample (Figure 1A). To validate these in the clinic, we conducted qRT-PCR assay to check the expressions of the top 18 increased circRNAs in the paired para-carcinoma and carcinoma and the paired carcinoma and metastasis, respectively. The results showed circ-0060849, -0003258, -0008039, and -0001394 were increased both in carcinoma and in metastasis when compared to the paired para-carcinoma and the paired carcinoma, respectively (Figures 1B and 1C). Next, we overexpressed these four circRNAs (Figure S1A) to check their function in the invasion of liver cancer cells; the results indicated that circ-0008039 (circ-PRKAR1B) might be the candidate to promote liver cancer cell progression (Figure 1D), and the wound-healing migration also confirmed it (Figure 1E). In addition, we used 2 short hairpin RNA (shRNA) constructs with different sequences to knock down circ-PRKAR1B (shcirc-PRKAR1B 1#, shcirc-PRKAR1B 2#) in liver cancer cells, and the wound-healing migration assay and Transwell invasion assay also showed shcirc-PRKAR1B could decrease the invasion and migration abilities of liver cancer cells (Figures 1F and 1G; Figures S1B–S1D).

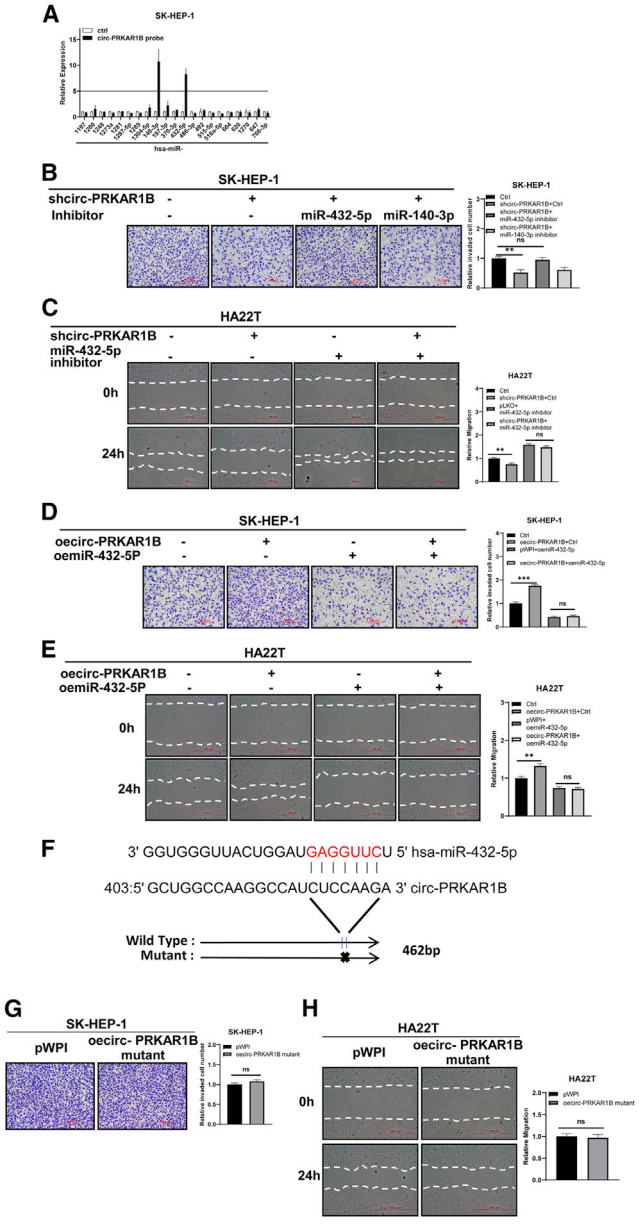


Figure 2. Mechanistic dissection showed that miR-432-5p was involved in circ-PRKAR1B-mediated cell invasion/migration of liver cancer cells

(A) RNA pull-down assay was used to choose the miRNAs that could potentially be targeted by circ-PRKAR1B. (B) The invasion capacity was checked by Transwell invasion assay after adding the miRNA inhibitor and shcirc-PRKAR1B in SK-HEP-1 cells. (C) The migration capacity was checked by wound-healing migration assay after shcirc-PRKAR1B/adding miR-432-5p inhibitor in HA22T cells. (D) The invasion capacity was checked by Transwell invasion assay after oecirc-PRKAR1B/oemiR-432-5p in SK-HEP-1 cells. (E) The migration capacity was checked by wound-healing migration assay after oecirc-PRKAR1B/oemiR-432-5p in HA22T cells. (F) The predicted binding nucleotides between miR-432-5p and circ-PRKAR1B. (G and H) Mutated form of circ-PRKAR1B failed to affect cell invasion (G) of SK-HEP-1 cells and cell migration (H) of HA22T cells. Left, representative images. Right, quantification of invading or migrating cells. All quantifications are presented as mean ± SD and p values calculated by t test. **p < 0.01; ***p < 0.001; NS, no significance.

Together, the results of Figure 1 and Figures S1A–S1D demonstrated that circ-PRKAR1B could promote liver cancer cell invasion and migration capacities.

Mechanistic dissection showed that miR-432-5p was involved in circ-PRKAR1B-mediated cell invasion/migration of liver cancer cells

It is widely shown that the most common mechanism of circRNAs in gene regulation is acting as a "sponge" to control miRNA activity. To investigate the underlying mechanism of circ-PRKAR1B-mediated cell invasion/migration of liver cancer cells, we first selected 21 miRNAs that were predicted by online databases that could potentially be sponged by circ-PRKAR1B, and the pull-down assay using circ-PRKAR1B biotin probe (5′-TTCCTTCCTCCTCTCGTTGT-3′) showed that miR-432-5p and miR-140-3p may be the candidates that could be sponged by circ-PRKAR1B (Figure 2A). Then we conducted reverse assay to verify the function of 2 miRNAs, and the data showed adding miR-432-5p inhibitor could partly reverse knocked down circ-PRKAR1B decreased cell invasion of SK-HEP-1 cells, but the miR-140-3p inhibitor could not (Figure 2B). The wound-healing migration assay of HA22T cells also confirmed it (Figure 2C). In addition, overexpressing miR-432-5p could also block circ-PRKAR1B-mediated cell invasion/migration of liver cancer cells (Figures 2D and 2E). At the same time, we mutated the nucleotides of circ-PRKAR1B that were binding to miR-432-5p (Figure 2F) and checked their function on liver cancer cell progression. The data demonstrated that invasion and migration abilities of liver cancer cells were not changed after inducing the mutated circ-PRKAR1B (Figures 2G and 2H), which indicated the important interaction between circ-PRKAR1B and miR-432-5p.

Together, the data from Figure 2 indicated that circ-PRKAR1B could promote liver cancer cell invasion and migration capacities by sponging miR-432-5p.

E2F3 may be the downstream gene of circ-PRKAR1B/miR-432-5p signaling-mediated cell invasion/migration of liver cancer cells

To explore the downstream genes that are involved in the circ-PRKAR1B/miR-432-5p signaling-mediated cell invasion/migration of liver cancer cells, we first selected some genes (MMP9,[12,13] E2F3,[14] CD44,[15] SOX9,[16] GSN,[17] FOXC2,[18] and SMAD4[19,20]) that were reported to play important roles in the progression of many cancers. The data of western blot assay showed E2F3 may be the candidate gene that could be regulated by circ-PRKAR1B, by overexpressing or knocking down circ-PRKAR1B in liver cancer cells (Figure 3A). Then we checked E2F3 protein expression by oecirc-PRKAR1B/oemiR-432-5p; the results showed oemiR-432-5p could block circ-PRKAR1B-mediated increased E2F3 expression (Figure 3B). Another reverse assay, adding miR-432-5p inhibitor could block shcirc-PRKAR1B-mediated decreased E2F3 expression, also confirmed E2F3 was under regulation of circ-PRKAR1B/miR-432-5p signaling (Figure 3C). In addition, the Transwell invasion assay and wound-healing migration assay by oecirc-PRKAR1B/shE2F3 or

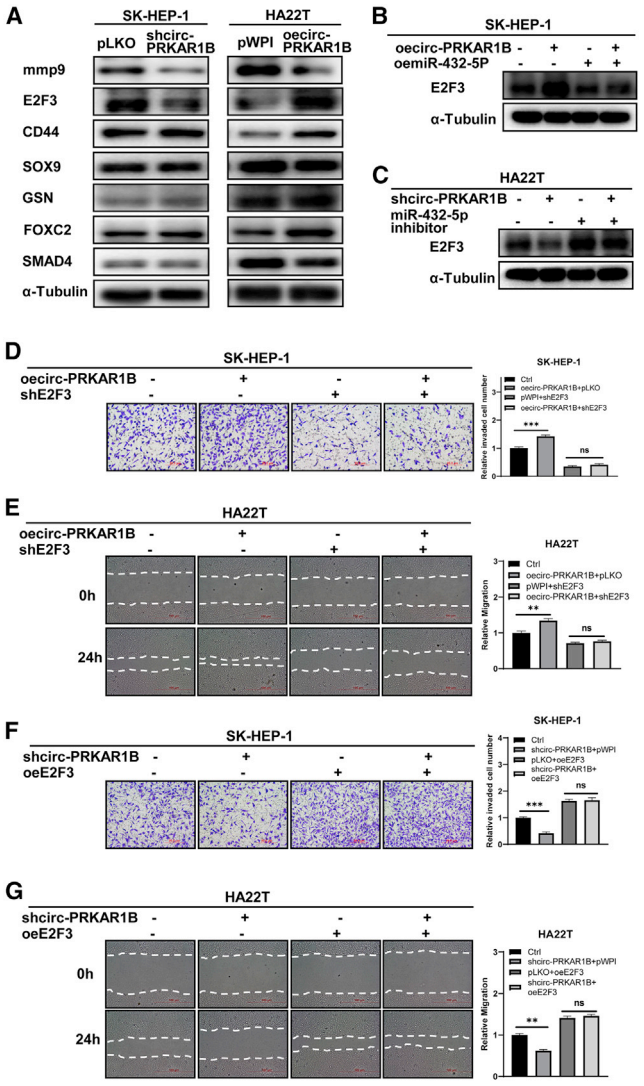


Figure 3. E2F3 may be the downstream gene of circ-PRKAR1B/miR-432-5p signaling-mediated cell invasion/migration of liver cancer cells

(A) Relative gene protein expressions were checked by western blot assay after sh/oecirc-PRKAR1B in liver cancer cells. (B) E2F3 protein expression was checked by western blot assay after oecirc-PRKAR1B/oemiR-432-5p in SK-HEP-1 cells. (C) E2F3 protein expression was checked by western blot assay after shcirc-PRKAR1B/adding miR-432-5p inhibitor in HA22T cells. (D) The invasion capacity was checked by Transwell invasion assay after oecirc-PRKAR1B/shE2F3 in SK-HEP-1 cells. (E) The migration capacity was checked by wound-healing migration assay after oecirc-PRKAR1B/shE2F3 in HA22T cells. (F) The invasion capacity was checked by Transwell invasion assay after shcirc-PRKAR1B/oeE2F3 in SK-HEP-1 cells. (G) The migration capacity was checked by wound-healing migration assay after shcirc-PRKAR1B/oeE2F3 in HA22T cells. All quantifications are presented as mean ± SD and p values calculated by t test. **p < 0.01; ***p < 0.001; NS, no significance.

shcirc-PRKAR1B/oeE2F3 also indicated E2F3 was a downstream gene of circ-PRKAR1B/miR-432-5p signaling-mediated cell invasion/migration of liver cancer cells (Figures 3D–3G).

Together, the data from Figure 3 indicated that E2F3 was one of the downstream genes of circ-PRKAR1B/miR-432-5p signaling-mediated cell invasion/migration of liver cancer cells.

Mechanism of how the circ-PRKAR1B/miR-432-5p axis can alter E2F3 expression: via directly binding to the 3′ UTR of E2F3-mRNA

To study the mechanism of how the circ-PRKAR1B/miR-432-5p axis can alter E2F3 expression, we first predicted the targeting sites where miR-432-5p located on the 3′ UTR of the mRNA of E2F3. We then constructed the wild-type and mutant 3′ UTR and conducted the reporter assay (Figure 4A). The data showed that adding miR-432-5p could decrease the luciferase activity in SK-HEP-1 cells transfected with wild-type E2F3 3′ UTR but not the mutant 3′ UTR (Figure 4B). At the same time, the addition of miR-432-5p inhibitor could increase the luciferase activity in HA22T cells transfected with wild-type E2F3 3′ UTR but not the mutant 3′ UTR (Figure 4C).

The results shown in Figure 4 indicated that the circ-PRKAR1B/miR-432-5p axis could directly bind to the 3′ UTR of E2F3-mRNA to alter E2F3 expression.

Clinical data also confirmed the importance of circ-PRKAR1B/miR-432-5p/E2F3 signaling in the regulation of liver cancer progression

To confirm the clinical significance of the circ-PRKAR1B/miR-432-5p/E2F3 signaling in the regulation of liver cancer progression, we analyzed the data through the GEPIA website (http://gepia.cancer-pku.cn/index.html); these data are based on The Cancer Genome Atlas (TCGA) samples, and the results showed that E2F3 expression was increased obviously in the liver hepatocellular carcinoma (LIHC) tumor group compared to normal liver tissues (Figure 5A). The immunohistochemistry (IHC) assay of clinical samples in our hospital also confirmed higher E2F3 expression in tumor tissues when compared with para-tumor tissues (Figure S5). At the same time, the data also indicated that the expression of E2F3 gradually increases as the tumor stage increases (Figure 5B). In addition, high expression of E2F3 often means low overall survival rate in LIHC (Figure 5C). We also analyzed the miR-432-5p expression in LIHC and normal liver tissues in the OncomiR database (http://www.oncomir.org/oncomir/index.html), and the results demonstrated that miR-432-5p expression was decreased in LIHC when compared to normal tissues (Figure 5D, top). The analysis of miRNA-Target co-expression in the ENCORI database (http://starbase.sysu.edu.cn/panCancer.php) also showed a negative correlation between miR-432-5p and E2F3P2 (Figure 5D, bottom). We also collected the clinicopathological data of liver cancer patients who underwent surgery in our hospital in the past 2 years (2019–2020), and the results of analysis showed that Barcelona Clinic Liver Cancer (BCLC) stage, Child-Pugh classification, tumor size, tumor number, T grade, tumor necrosis, vascular invasion, and histological grade had significant correlations with circ-PRKAR1B and/or miR-432-5p expression (Table 1).

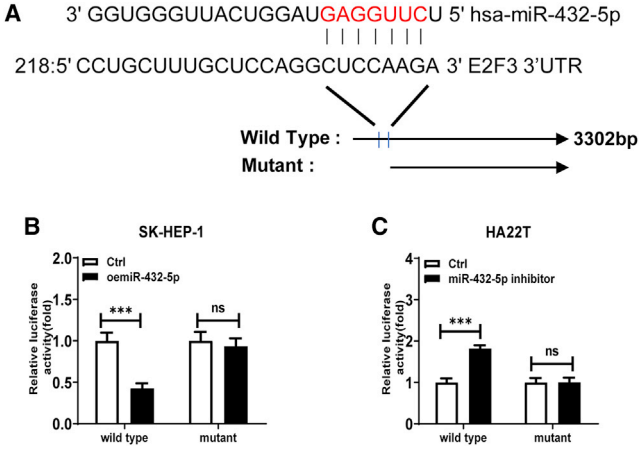


Figure 4. Mechanism of how the circ-PRKAR1B/miR-432-5p axis can alter E2F3 expression: via directly binding to the 3′ UTR of E2F3-mRNA

(A) Sequence alignment of the E2F3 3′ UTR with wild-type versus mutant potential miR-432-5p targeting sites. (B and C) Luciferase reporter assay was used to check the luciferase activity after transfection of wild-type or mutant E2F3 3′ UTR reporter in SK-HEP-1 and HA22T with overexpression of miR-149-5p or adding miR-432-5p inhibitor compared to the control cells. All quantifications are presented as mean ± SD and p values calculated by t test. ***$p < 0.001$; NS, no significance.

Together, the data from Figure 5 and Table 1 confirmed the importance of circ-PRKAR1B/miR-432-5p/E2F3 signaling in the regulation of liver cancer progression.

In vivo mouse model to demonstrate the role of circ-PRKAR1B/microRNA-432-5p/E2F3 signaling in the progression of liver cancer

To check the significance of circ-PRKAR1B/microRNA-432-5p/E2F3 signaling *in vivo*, we injected 1 × 106 SK-HEP-1-Luc cells into the left lobes of the liver *in situ* in the xenografted mouse model. The mice were divided into three groups: (1) pWPI + pLV, (2) oecirc-PRKAR1B + pLV, and (3) oecirc-PRKAR1B + oemiR-432-5p. Tumor growth and metastasis were checked by IVIS system once a week. After 8 weeks, we sacrificed the mouse and took the tumors and metastases for further studies. The results showed that the addition of circ-PRKAR1B could promote liver cancer metastasis, and adding miR-432-5p could block circ-PRKAR1B-mediated progression of liver cancer (Figure 6A). The data of metastatic foci in liver, mesentery, spleen, kidney, and stomach were also consistent with the IVIS signal (Figures 6B and 6C). IHC data confirmed that the addition of miR-432-5p could also reduce E2F3 expression mediated by circ-PRKAR1B (Figure 6D).

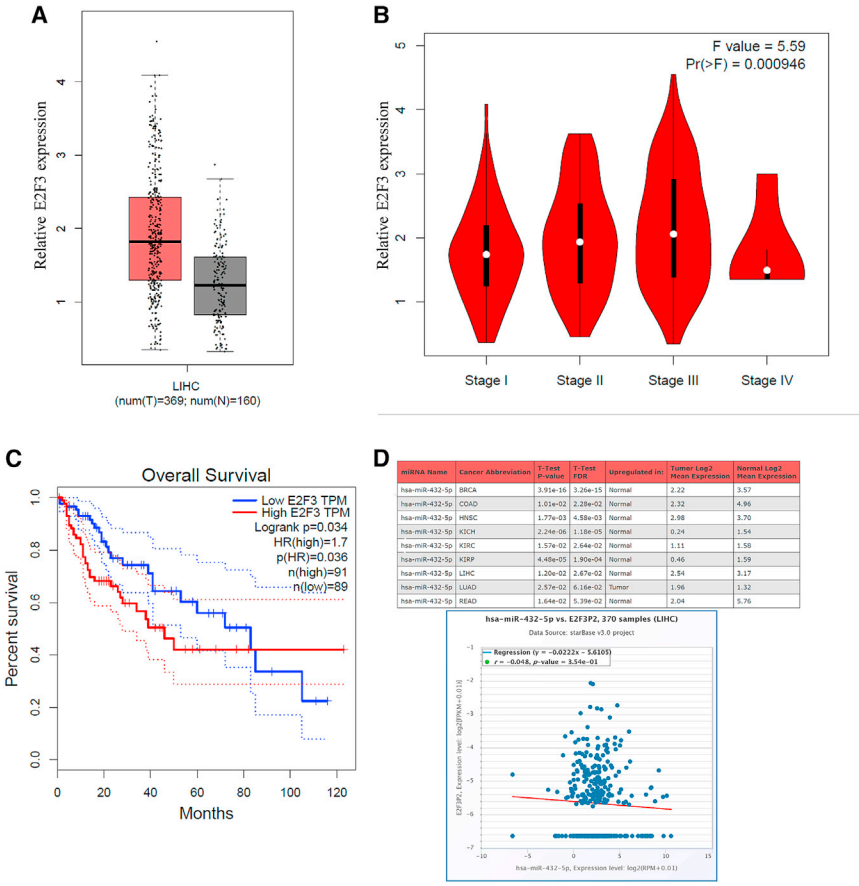
Together, the data from our *in vivo* mouse model in Figure 6 prove that circ-PRKAR1B may play an important role in promoting liver cancer metastasis via altering the miR-432-5p/E2F3 signaling axis.

DISCUSSION

PLC is a malignant tumor that affects people's life to a large extent, and its main type is HCC. In the past 30–40 years, the incidence of HCC in the United States has tripled, which makes it the fastest rising incidence of cancer in the US population.[21] The rate of metastasis and recurrence of HCC is very high, and the 5-year survival rate is usually less than 15%.[22] Finding new therapeutic targets to better treat liver cancer is still the primary problem faced by liver surgeons and oncologists. At present, scientists have made great contributions to improving the treatment of liver cancer, but it is still not enough; the task we face is daunting. Our study is another example to study the underlying mechanism of the progression of liver cancer, which may contribute to finding new therapeutic targets for better treatment of liver cancer.

circRNAs are a class of long, non-coding RNA molecules that shape a covalently closed continuous loop; they have no 5′–3′ polarity and contain no polyA tail, which has been proven to be widely present in many eukaryotic organisms.[23] circRNA is thought to exist in the cytoplasm or exosomes and has the features of tissue, disease, and timing specificity and high stability. In the past few years, many studies have demonstrated that circRNAs are connected to biological growth and development, stress response, and disease occurrence and development, but their biological function remains largely unknown. The biological functions of circRNA that are currently highly recognized are classified as follows:[24] (1) miRNA sponge. circRNA is rich in miRNA binding sites to act as a miRNA sponge, preventing miRNA from interacting with mRNA in the 3′ UTR, thereby indirectly regulating the expression of downstream target genes of miRNA. (2) Regulate protein binding. circRNA binds to mRNA-regulated binding protein (RBP) to change splicing patterns or mRNA stability. (3) Regulate gene transcription. circRNA interacts with RNA polymerase and regulates transcription, or EIcircRNA can interact with small ribonucleoproteins and then bind to RNA polymerase. (4) Coding function. Although circRNA is a non-coding RNA, some circRNAs can be translated by ribosomes and encode polypeptides to perform regulatory functions. Among the above four biological functions, sponge miRNA is the most common and is most widely documented by scientists. Today, the importance of circRNAs in the regulation of tumor initiation, progression, metastasis, and chemotherapy resistance has been gradually recognized. Chen et al.[25] reported that circRNA cRAPGEF5 inhibited the growth and metastasis of renal cell carcinoma via the miR-27a-3p/TXNIP pathway. Zhao et al.[26] concluded that circACAP2 promoted breast cancer proliferation and metastasis by targeting the miR-29a/b-3p-COL5A1 axis. Yu et al.[27] showed that circRNA-104718 acted as a competing endogenous RNA and promoted hepatocellular carcinoma progression through the microRNA-218-5p/TXNDC5 signaling pathway. Our study was another example showing the biological functions of circ-PRKAR1B by sponging miR-432-5p, influencing liver cancer cell invasion and migration. The reverse assay by overexpressing miR-432-5p or adding miR-432-5p inhibitor also confirmed the signaling. In our study, the data showed the reverse function was partial, which means sponging miR-432-5p is only one of the signals of circ-PRKAR1B-mediated liver cancer progression; the other mechanisms still need further study.

E2F3 belongs to the E2F family and has two distinct isoforms, E2F3a and E2F3b, functioning through binding of DP interaction partner



miRNA Name	Cancer Abbreviation	T-Test P-value	T-Test FDR	Upregulated in:	Tumor Log2 Mean Expression	Normal Log2 Mean Expression
hsa-miR-432-5p	BRCA	3.91e-16	3.26e-15	Normal	2.22	3.57
hsa-miR-432-5p	COAD	1.01e-02	2.28e-02	Normal	2.32	4.96
hsa-miR-432-5p	HNSC	1.77e-03	4.58e-03	Normal	2.98	3.70
hsa-miR-432-5p	KICH	2.24e-06	1.18e-05	Normal	0.24	1.54
hsa-miR-432-5p	KIRC	1.57e-02	2.64e-02	Normal	1.11	1.58
hsa-miR-432-5p	KIRP	4.48e-05	1.90e-04	Normal	0.46	1.59
hsa-miR-432-5p	LIHC	1.20e-02	2.67e-02	Normal	2.54	3.17
hsa-miR-432-5p	LUAD	2.57e-02	6.16e-02	Tumor	1.96	1.32
hsa-miR-432-5p	READ	1.64e-02	5.39e-02	Normal	2.04	5.76

Figure 5. Clinical data also confirmed the importance of circ-PRKAR1B/miR-432-5p/E2F3 signaling in the regulation of liver cancer progression

(A) E2F3 expression in LIHC tumor group compared to normal liver tissues in TCGA samples was analyzed by the GEPIA website (http://gepia.cancer-pku.cn/index.html). (B) GEPIA website was used to analyze the E2F3 expression in different LIHC tumor stages. (C) GEPIA website was used to analyze the survival rate connected to E2F3 in LIHC. (D) The miR-432-5p expression in LIHC and normal liver tissues was analyzed by the OncomiR database (http://www.oncomir.org/oncomir/index.html) (upper), and the miRNA-Target co-expression between miR-432-5p and E2F3P2 was analyzed by the ENCORI database (http://starbase.sysu.edu.cn/panCancer.php) (bottom).

proteins.[28] E2F3 has been reported as an oncogene in many cancers by many studies. Xue et al.[29] showed that circ-PRMT5 could accelerate proliferation and invasion of papillary thyroid cancer through regulation of the miR-30c/E2F3 axis. Huang et al.[30] reported that E2Fs could worked as promising diagnostic and prognostic markers in human hepatocellular carcinoma. Our study also proved that E2F3 was an oncogene under the regulation of circ-PRKAR1B/miR-432-5p signaling to promote liver cancer progression, which may work as a potential therapeutic target to develop new drugs for better treatment of liver cancer.

Conclusions

In conclusion, circ-PRKAR1B is increased in primary liver cancer tissues and paired intrahepatic metastasis samples. Elevated circ-PRKAR1B can promote liver cancer progression through miR-432-5p/E2F3 signaling, and targeting this newly exhibited signaling may help to find new therapies to better suppress the progression of liver cancer.

MATERIALS AND METHODS

Cell culture

SK-HEP-1 and HA22T liver cancer cells were cultured in DMEM with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin/streptomycin. All cells were cultured in a 5% (v/v) CO_2 humidified incubator at 37°C.

Transwell invasion assay

An 8-μm Transwell chamber was used to check the invasion capacity of liver cancer cells. First, the upper chamber was coated with 100 μL/well diluted Matrigel (1:20 dilution, BD Biosciences) and incubated in a 37°C humidified incubator for 2–4 h. Then, 5×10^4 cells/well of serum-free medium was added in the upper chamber and 750 μL media with 10% FBS/well was added into lower chambers. After incubation for 24 h, the upper chamber was washed, the invaded cells were fixed with methanol, and the cells were stained with 0.1% (w/v) crystal violet. Each experiment was repeated three times.

Wound-healing migration assay

Wound-healing migration assay was used to check the migration capacity of liver cancer cells. Cells were seeded in a 6-well plate, and a wound was created by using a sterile pipette tip when the cells were 90% or more confluent; at this point, photographs of the wound were taken under a microscope (marked as 0 h). 12 h later, photographs were taken again, and the distance of wound migration was measured for further analyses. Each experiment was repeated three times.

Western blot assay

RIPA lysis buffer was used to lyse liver cancer cells, and 30 μg of protein was run on SDS/PAGE gel. After electrophoresis, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was blocked for 1 h, rinsed with TBST three times, and then incubated in the corresponding primary antibody in a cold room at 4°C overnight. The membranes were rinsed with TBST three times again and incubated with secondary antibodies. Finally, images of the membrane were taken by the ECL

Table 1. Relations of circ-PRKAR1B and miR-432-5p expressions with clinicopathological characteristics of liver cancer

		circ-PRKAR1B			miR-432-5p		
Characteristics	Cases	Low	High	p	Low	High	p
All cases	138	69	69		69	69	
Age (years)							
<60	78	38 (48.7)	40 (51.3)	0.731	41 (52.6)	37 (47.4)	0.492
≥60	60	31 (51.7)	29 (48.3)		28 (46.7)	32 (53.3)	
Gender							
Male	105	54 (51.4)	51 (48.6)	0.549	50 (47.6)	55 (52.4)	0.318
Female	33	15 (45.5)	18 (54.5)		19 (57.6)	14 (42.4)	
HBsAg							
Negative	29	13 (44.8)	16 (55.2)	0.531	17 (58.6)	12 (41.4)	0.296
Positive	109	56 (51.4)	53 (48.6)		52 (47.7)	57 (52.3)	
BCLC stage							
A	81	47 (58.0)	34 (42.0)	**0.025**	30 (37.0)	51 (63.0)	**0.032**
B/C	57	22 (38.6)	35 (61.4)		39 (68.4)	18 (31.6)	
Child-Pugh classification							
A	93	45 (48.4)	48 (51.6)	0.586	39 (41.9)	54 (58.1)	**0.006**
B	45	24 (53.3)	21 (46.7)		30 (66.7)	15 (33.3)	
AFP(μg/L)							
<20	15	7 (46.7)	8 (53.3)	0.784	6 (40.0)	9 (60.0)	0.412
≥20	123	62 (50.4)	61 (49.6)		63 (51.2)	60 (48.8)	
Tumor size (cm)							
<5	68	47 (69.1)	21 (30.9)	**<0.001**	25 (36.8)	43 (63.2)	**0.002**
≥5	70	22 (31.4)	48 (68.6)		44 (62.9)	26 (37.1)	
Tumor number							
Solitary	77	30 (39.0)	47 (61.0)	**0.004**	45 (58.4)	32 (41.6)	**0.026**
Multiple	61	39 (63.9)	22 (36.1)		24 (39.3)	37 (60.7)	
Liver cirrhosis							
Absence	36	21 (58.3)	15 (41.7)	0.245	19 (52.8)	17 (47.2)	0.698
Presence	102	48 (47.1)	54 (52.9)		50 (49.0)	52 (51.0)	
T grade							
T1/T2	79	49 (62.0)	30 (38.0)	**0.001**	33 (41.8)	46 (58.2)	**0.025**
T3/T4	59	20 (33.9)	39 (66.1)		36 (61.0)	23 (39.0)	
N grade							
N0	132	68 (51.5)	64 (48.5)	0.095	65 (49.2)	67 (50.8)	0.404
N1–N3	6	1 (16.7)	5 (83.3)		4 (66.7)	2 (33.3)	
Tumor necrosis							
Absence	52	35 (67.3)	17 (32.7)	**0.002**	18 (34.6)	34 (65.4)	**0.005**
Presence	86	34 (39.5)	52 (60.5)		51 (59.3)	35 (40.7)	
Vascular invasion							
Absence	57	37 (64.9)	20 (35.1)	**0.003**	22 (38.6)	35 (61.4)	**0.025**
Presence	81	32 (39.5)	49 (60.5)		47 (58.0)	34 (42.0)	

(*Continued on next page*)

Table 1. *Continued*

Characteristics	Cases	circ-PRKAR1B		p	miR-432-5p		p
		Low	High		Low	High	
Histological grade							
G1/G2	95	53 (55.8)	42 (44.2)	**0.043**	39 (41.1)	56 (58.9)	**0.002**
G3/G4	43	16 (37.2)	27 (62.8)		30 (69.8)	13 (30.2)	

Data are presented as n (%). The data of gender, Hepatitis B virus surface antigen (HBsAg), BCLC stage, Child-Pugh classification, alpha-fetoprotein (AFP) expression, tumor size, tumor number, liver cirrhosis, T grade, N grade, tumor necrosis, vascular invasion, and histological grade based on different expression of circ-PRKAR1B or miR-432-5p are presented. The results of p value < 0.05 are marked in bold. Low, low expression; High, high expression.

system (Thermo Fisher Scientific). Information about the antibodies we used in the study is listed below: MMP-9 (E-11, Santa Cruz Biotechnology), E2F3 (Thermo Fisher Scientific), CD44 (EPR18668, Abcam), SOX9 (E-9, Santa Cruz Biotechnology), GSN (H-5, Santa Cruz Biotechnology), FOXC2 (G-7, Santa Cruz Biotechnology), SMAD4 (B-8, Santa Cruz Biotechnology), α-tubulin (TU-02, Santa Cruz Biotechnology).

qRT-PCR assay

The total RNA of liver cancer cells was extracted by Trizol reagent, and 2 μg of total RNA was taken for the reverse transcription. The Bio-Rad CFX96 system was used to conduct and calculate the expression of RNA (mRNA and miRNA). GAPDH (for mRNA) or U6 (for miRNA) was used to normalize the data, and relative expression was assessed by ΔΔCt values. All primers were purchased from Integrated DNA Technologies.

Plasmid construction

The pLKO.1-shcirc-PRKAR1B 1# 2#, pLKO.1-shE2F3, pWPI-circ-PRKAR1B, and pWPI-E2F3 plasmids; the psPAX2 packaging plasmid (10 μg); and the pMD2.G envelope plasmid (10 μg), were transfected into 293T cells using the standard calcium phosphate transfection method. 48 h later, lentivirus soups were collected and concentrated by density gradient for later use. Puromycin (5 μg/mL) was used to select the liver cancer cells infected with shRNA.

The plasmid sequences used were as follows:

shcirc-PRKAR1B 1#:

Forward: 5′-CCGG GACAACGAGAGGAGGAAGGAATTG-GATCCGTTCCTTCCTCCTCTCGTTGTCTTTTTG-3′

Reverse: 5′-AATTCAAAAAGACAACGAGAGGAGGAAGGAA CGGATCCAATTCCTTCCTCCTCTCGTTGTC-3′

shcirc-PRKAR1B 2#:

Forward: 5′-CCGGAACGAGAGGAGGAAGGAAGCATTGGA TCCGTGCTTCCTTCCTCCTCTCGTTTTTTTG-3′

Reverse: 5′-AATTCAAAAAAACGAGAGGAGGAAGGAAGCA CGGATCCAATGCTTCCTTCCTCCTCTCGTT-3′

oecirc-PRKAR1B:

To construct pWPI-circ-PRKAR1B (oecirc-PRKAR1B), the specific DNA fragments, which were shown to promote circularization, were first engineered in the pBSK vector as pBSK (circArm). The exons of the PRKAR1B gene were PCR amplified and then inserted into the pBSK (circArm) vector as the pBSK_circ-PRKAR1B.

The exon sequences inserted into the pBSK (circArm) vector are listed below:

Exons: oecirc-PRKAR1B contains three exons (exons 2, 3, and 4)

2 Forward: 5′-CTAATGACTTTTTTTTTATACTTCAGGAAG-GAAGCAGCCACCCTCG-3′

2 Reverse: 5′-CAAAATCTGCCTGTTTTCTTCCTTCTCCAG CTTCTCGAAGTG-3′

3 Forward: 5′-GAAGAAAACAGGCAGATTTTG-3′

3 Reverse: 5′-CTTCCTGACGTAGGACACGGC-3′

4 Forward: 5′-GCCGTGTCCTACGTCAGGAAGGTGATTCC-CAAGGACTACAAA-3′

4 Reverse: 5′-CTAATTCTTTTCCTTGCTTCTTACCTCCTCT CGTTGTCATCCAGG-3′

Then we subcloned the fragments including the exon of PRKAR1B and those two arms to the lentiviral pWPI vector for expressing circ-PRKAR1B.

circRNA pwpi Gibson:

Forward: 5′-GTGAGGAATTTCGACATTTAAATTTA ATAAA GTGCTGAGATTACAGGCG-3′

Reverse: 5′-ATTCCTGCAGCCCGTAGTTTTGCTGGGATTA-CAGGTGTGA-3′

oecirc-PRKAR1B mutant:

4 Forward: 5′-GCCGTGTCCTACGTCAGGAAGGTGATTCC-CAAGGACTACAAA-3′

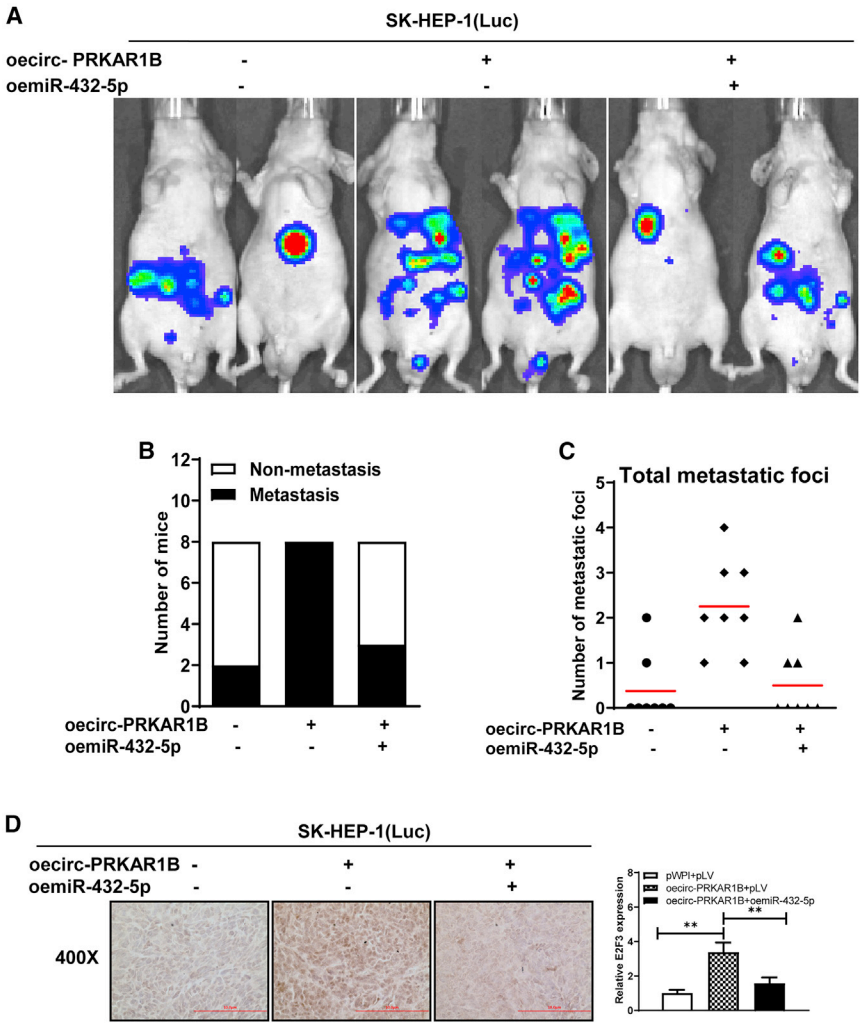


Figure 6. *In vivo* mouse model to demonstrate the role of circ-PRKAR1B/microRNA-432-5p/E2F3 signaling in the progression of liver cancer

(A) IVIS imaging was used to detect the various distal metastasis foci in mice with orthotopically xenografted tumor cells under the left lobes of liver capsule. (B) Quantification of the metastases in the mice. (C) Quantification of the total metastatic foci. (D) IHC assay was used to check E2F3 expression in mice tumor tissues in each group.

4 Reverse: 5′-CTAATTCTTTTCCTTGCTTCTTACATGGCC TTGGCCAGCGCAGTC-3′

In vivo studies

1 × 106 SK-HEP-1-Luc cells were injected into the left lobes of the liver *in situ* in the xenografted mouse model. The mice were divided into three groups: (1) pWPI + pLV, (2) oecirc-PRKAR1B + pLV, and (3) oecirc-PRKAR1B + oemiR-432-5p. Tumor growth and metastasis were checked by IVIS system once a week. After 8 weeks, we sacrificed the mouse and took tumors and any metastases for further studies. Animal experiments were approved by institutional animal care at Xiangya Hospital Central South University.

Clinicopathological data collection of liver cancer patients

We collected the clinicopathological data of liver cancer patients who underwent surgery in Xiangya Hospital Central South University in 2019 and 2020. 138 cases were collected, including 105 cases of males and 33 cases of females. The data of age, gender, HBsAg, BCLC stage, Child-Pugh classification, AFP (μg/L), tumor size (cm), tumor number, liver cirrhosis, T grade, N grade, tumor necrosis, vascular invasion, and histological grade were collected for further analysis. qRT-PCR assay was used to check circ-PRKAR1B and miR-432-5p expression of all patients. The data collection from patients was approved by the Ethics Committee of Xiangya Hospital of Central South University

Statistical analysis

SPSS 22.0 software was used to analyze the data. The data are presented as mean ± SE. Differences were analyzed with the one-way ANOVA test, and $p < 0.05$ was considered as statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.07.002.

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AUTHOR CONTRIBUTIONS

Y.X. ensured the integrity of the entire study. G.L. and X.O. performed the experiments and were major contributors in writing the manuscript. L.G., L.Y., and S.L. were in charge of data analysis. G.L., X.O., J.L., and Q.Z. were in charge of animal experiments. G.L., X.O., and Y.X. were responsible for revising the manuscript and checking all data. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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