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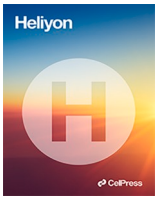

Research article

The role of m6A modified circ0049271 induced by MNNG in precancerous lesions of gastric cancer

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ABSTRACT

Gastric cancer (GC) is a malignant cancer with the highest global rates of morbidity and death. Dietary factors have a close relationship with the occurrence of GC. Circular RNAs (circRNAs) and N6-methyladenine (m6A) are important factors in the onset and progression of GC and other malignancies. However, little is known about the role of circRNA m6A modifications in the occurrence and development of GC. Initially, a transformed malignant cell model generated by the chemical carcinogen N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was established in this investigation. Furthermore, following exposure to MNNG, circ0049271 is substantially expressed in gastric epithelial cells (GES-1). Subsequent research revealed that the knockdown of circ0049271 prevented the epithelial-mesenchymal transition (EMT) as well as the migration, invasion, and proliferation of gastric epithelial cells induced by long-term exposure to MNNG. The opposite effects were observed when circ0049271 was overexpressed. Mechanistically, circ0049271 activates the TGFβ/SMAD signaling pathway and has m6A modifications mediated by WTAP. Our findings indicate that circ0049271 promotes the occurrence of GC by regulating the TGFβ/SMAD pathway, and WTAP may mediate the methylation of circ0049271 m6A. This study provides new insights into the regulation of circRNA-mediated m6A modifications and the discovery of early GC induced by dietary factors such as nitrite.

1. Introduction

Gastric cancer (GC) is a malignant tumor with high morbidity and mortality rates worldwide. New cases of GC and GC-related deaths rank fifth and third in the common malignancies, respectively [1]. Despite the progress in various treatment methods such

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as surgery, chemotherapy, and biological therapy in recent years, early detection of GC remains challenging. The prognosis for advanced GC is poor, with a low survival rate [2].

Gastric mucosal carcinogenesis typically involves a prolonged precancerous lesion stage (PLGC), which is a crucial step in the progression of GC [3]. Environmental factors (diet and exogenous chemicals), gastric carcinogens, and Helicobacter pylori infection can trigger the occurrence and development of GC [4]. In daily life, consuming too many pickled foods can lead to the accumulation of excessive nitroso compounds in the body. Due to an unbalanced diet, this can cause a variety of lesions and possibly even GC. N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), an activated N-nitroso compound, is commonly used to simulate gastric mucosal carcinogenesis induced by the ingestion of nitroso compounds in vivo.

Circular RNAs (circRNAs) are a class of closed circular RNAs without 5′ and 3′ ends. They are less susceptible to endogenous RNases because of their closed structure [5]. CircRNAs may be useful as therapeutic targets and molecular markers for both physiological and pathological processes, according to data published in recent years [6,7].

Transforming growth factor-β (TGF-β) plays an important role in cancer progression, including three subtypes of TGFβ1, TGFβ2, and TGFβ3. Two type I and two type II serine/threonine kinase receptors make up the heterotetramer complex formed by the 12 serine/threonine kinase receptors. The TGF-β subfamily signals through a receptor complex composed of TGFBR1 and TGFBR2, phosphorylates SMAD2 and SMAD3, and then phosphorylates SMAD2/3 proteins to form a complex with SMAD4. This complex is transported to the nucleus and regulates the expression of target genes [8].

Cancer molecular genetics research is now centered on epigenetic mechanisms including methylation and histone modification due to the rapid advancement of molecular medicine. The N6 -methyladenosine (m6A) modification is the most abundant and prevalent co-transcriptional modification in eukaryotes [9]. The regulatory function of m6A is mainly accomplished by three homologous factors known as "writer," "eraser," and "reader." M6A "writers" are proteins responsible for the formation of methyltransferase complexes, including METTL3, METTL14, METTL16, WTAP, and others. Some demethylases (erasers), such as FTO and ALKBH5, maintain the balance of m6A levels in the transcriptome. Furthermore, a diverse class of RNA-binding proteins (readers), including IGF2BP1/2/3, are necessary for the unique biological functions carried out by m6A-modified RNAs [10].

M6A modification is widely present in circRNAs [11,12]. A previous study showed that circ0008399 binds to WTAP and regulates the expression of target RNA through m6A modification, therefore reducing the sensitivity of bladder cancer cells to cisplatin [13]. CircITGB6 enhances the stability of the EMT-promoting gene PDPN mRNA by directly interacting with IGF2BP3 [14]. In addition to its classical function of regulating the stability of circRNAs, a recent study reported that m6A methylation regulates the expression of its downstream molecules by affecting the subcellular localization of circRNAs. This discovery offers a novel approach to the identification and management of colorectal liver metastasis and is highly significant in the malignant progression of colorectal cancer [15]. However, the significance of circRNA methylation in diet-induced cancers remains unclear.

In this study, we investigated the effects of long-term low-dose MNNG exposure on the malignant cytological properties of normal gastric mucosal cells and the role of m6A-modified circ0049271 in promoting the occurrence of GC. We discovered that circ0049271 is a highly expressed molecule in malignant transformed cells (TGES-1) that triggers the TGFβ/SMAD pathway by bioinformatics analysis and experimental validation. GC is promoted by WTAP-modified circ0049271 in an IGF2BP3-dependent way. This study provides a novel therapeutic target for the treatment of GC.

2. Materials & methods

2.1. Cell and cell culture

Human gastric epithelial cells (GES-1) were purchased from Shanghai Enzyme Linked Biotechnology Co., LTD. (Shanghai, China). GES-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Meilunbio，China) with 10 % fetal bovine serum (FBS) (Gibco, USA). All cells were cultured and treated at 37 °C and 5 % CO_2 conditions. The procedure for inducing TGES-1 cells was as follows: MNNG (Macklin, China) concentrations of 0.5 and 1 μmol/L (μM) were were chosen based on the cell survival rate for subsequent experiments. GES-1 and TGES-1 cells were grown in DMEM containing 10 % FBS after being treated with 1 μM MNNG.GES-1 cells and TGES-1 cells treated with or without 1 μM MNNG were cultured in DMEM with 10 % FBS. In the experimental group, MNNG was diluted to 1 μM with fresh DMEM culture solution the next day and treated continuously for 30 generations.

2.2. QRT-PCR assay

Total RNA was extracted from the cells using TRIzol reagent (Vazyme, China). Isolated RNA was used for reverse transcription with HiScript QRT SuperMix for qPCR, following the manufacturer's instructions. (Vazyme). Quantitative RT-PCR was conducted using SYBR Green PCR Master Mix (Vazyme) on an Applied Biosystems QuantStudio 3 system (Thermofisher, USA). Results were calculated using the $2^{-\Delta\Delta Ct}$. GAPDH was used as the internal control.

2.3. Cell counting kit-8 (CCK8) assay

Cells in the logarithmic growth phase were seeded in 96-well plates (1×10^3 cells/well)，with three replicate wells in each group. The cells were incubated for 24, 48, 72, and 96 h. Phosphate-buffered saline (PBS) was added to the surrounding pores during the determination in order to stop the medium from volatilizing too much. 10 % CCK-8(Vazyme, China) medium was added to each well and incubated in the incubator for 3 h in the dark. Finally, the absorbance value (OD) of each sample at 450 nm was determined using

an enzyme labeling instrument, and the determination was repeated three times.

2.4. Cell cloning formation assay

Cells in the logarithmic phase of growth were trypsinized, and 1×10^3 cells were seeded in 3.5 cm dishes and incubated at 37 °C for 14 days. The cell colonies were fixed in 4 % paraformaldehyde and stained with 0.5 % crystal violet. Cell colonies were counted and analyzed after being washed with PBS to remove the floating color.

2.5. Transwell assay

Following digestion, the cells were resuspended in serum-free DMEM and planted at a density of 3×10^4 cells/well in the upper chamber of a 24-well transwell plate. Matrigel (BD Bioscience, USA)-coated chambers were used for invasion assays, while Matrigel-uncoated chambers were utilized for migration assays. 600 μL of complete medium was added to the lower chamber. After 24 h, the cells in the upper chamber were wiped with a cotton swab, and the cells in the lower chamber were fixed with a 4 % paraformaldehyde solution for 20 min. Subsequently, they were stained with 1 % crystal violet for 10 min, and the number of invading or migrating cells was counted under a 10 × 10 microscope.

2.6. Western blot

Western blotting was performed to estimate the protein levels of N-cadherin (CST, 1:500 dilution), E-cadherin (CST, 1:500 dilution), Vimentin (CST, 1:500 dilution), PCNA (CST, 1:500 dilution), Nanog (CST, 1:500 dilution), OCT4 (CST, 1:500 dilution), Lin28 (CST, 1:500 dilution), SOX2 (CST, 1:500 dilution), GAPDH (CST, 1:2000 dilution), CD81 (Abcam, 1:500 dilution), CD9 (Abcam, 1:500 dilution), HSP70 (Abcam, 1:500 dilution), Calnexin (Abcam, 1:500 dilution), TGFβ1 (Abcam, 1:500 dilution), SMAD2 (Abcam, 1:500 dilution), PSMAD2 (Abcam, 1:500 dilution), SMAD3 (Abcam, 1:500 dilution), PSMAD3 (Abcam, 1:500 dilution), and SMAD2/3 (Abcam, 1:500 dilution). The cells were collected, and the total proteins were extracted from the cells using RIPA lysate. Total protein was measured by a NanoDrop 1000 spectrophotometer (Thermofisher, USA), followed by separation through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the samples were transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5 % skimmed milk. The membranes were then incubated with primary antibodies overnight at 4 °C. After washing the samples with Tris Buffered Saline with Tween (TBST), they were incubated with secondary antibodies. The results were visualized using an enhanced chemiluminescence (ECL) (Vazyme, China) substrate.

2.7. Animal experiment

Six-week-old BALB/c nude mice were purchased from the Animal Research Center of Jiangsu University. Mice were housed in polypropylene cages at 20–22 °C and 40–60 % humidity with 12-h light and dark cycles at the Animal Center of Jiangsu University. Six male nude mice were randomly divided into two groups, and each mouse was subcutaneously injected with 5×10^7 cells into the upper back. Except for the different types of inoculated cells, the feeding conditions of the two groups of nude mice are completely identical. Approximately 4 weeks after injection, the mice were sacrificed by cervical dislocation, and tumors were dissected. We further observed the tumor occurrence in two groups of mice inoculated with different cells. All mouse experiments were approved by the Animal Care and Use Committee of Jiangsu University (UJS-IACUC-AP-2021030816), and efforts were made to minimize suffering and distress. Due to immune deficiency, malignant transformed cells and GC are more likely to form tumors in nude mice, so we chose the nude mouse model.

2.8. Bioinformatics analysis

Microarray data from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) obtained using the GEO2R online differentiation analysis. Fold change ≥ 2 and P value < 0.05 were set as the threshold for significantly differential expression. Download differential circRNAs for intersection analysis and draw a heat map. Using the SRAMP database (http://www.cuilab.cn/sramp/) to predict the circ0049271 m6A modification site. Through the CircInteractome website (https://circinteractome.nia.nih.gov/) prediction with circ0049271 proteins.

2.9. RNase R treatment

TGES-1 cells were seeded in 6-well culture plates and incubated overnight. After extracting total cellular RNA, 2 μg of RNA was incubated in a metal bath at 37 °C for 15 min with or without RNase R (3 U) (Lucigen, USA). The experiment was then stopped and kept at −20 °C for 30 min, after which the supernatant was removed by centrifugation. After reverse transcription immediately following RNase R treatment, the expression of linear GAPDH and circ0049271 was detected by qRT-PCR.

2.10. Actinomycin D treatment

TGES-1 cells were seeded in 6-well plates. After treating cells with 5 μg/mL of actinomycin D (MCE, USA) for 4, 8, 12, 18, and 22 h,

RNA was extracted at each time point, and the expression was measured by qRT-PCR.

2.11. Nuclear-cytoplasmic fractionation

Nuclear and cytoplasmic RNA from TGES-1 cells were isolated using the Paris Kit (Gaithersburg, MD, USA) following the manufacturer's instructions. U6 and actin were used as positive controls for nuclear and cytoplasmic components, respectively, for the qRT-PCR assay.

2.12. Plasmid transfection

To inhibit and overexpress circ0049271, specific small interfering RNAs (siRNAs) by GenePharma (Shanghai, China) and overexpression plasmids by Fenghui (Hunan, China) were synthesized and established. Non-targeting siRNA (si-NC) and empty plasmids were used as the NC. The 6-well plate was inoculated with logarithmic cell culture to reach 50–70 % confluence. siRNA and plasmid were transfected into cells in serum-free medium with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. 6 h after transfection, the cells were transferred to the complete culture medium and cultured for an additional 30h.

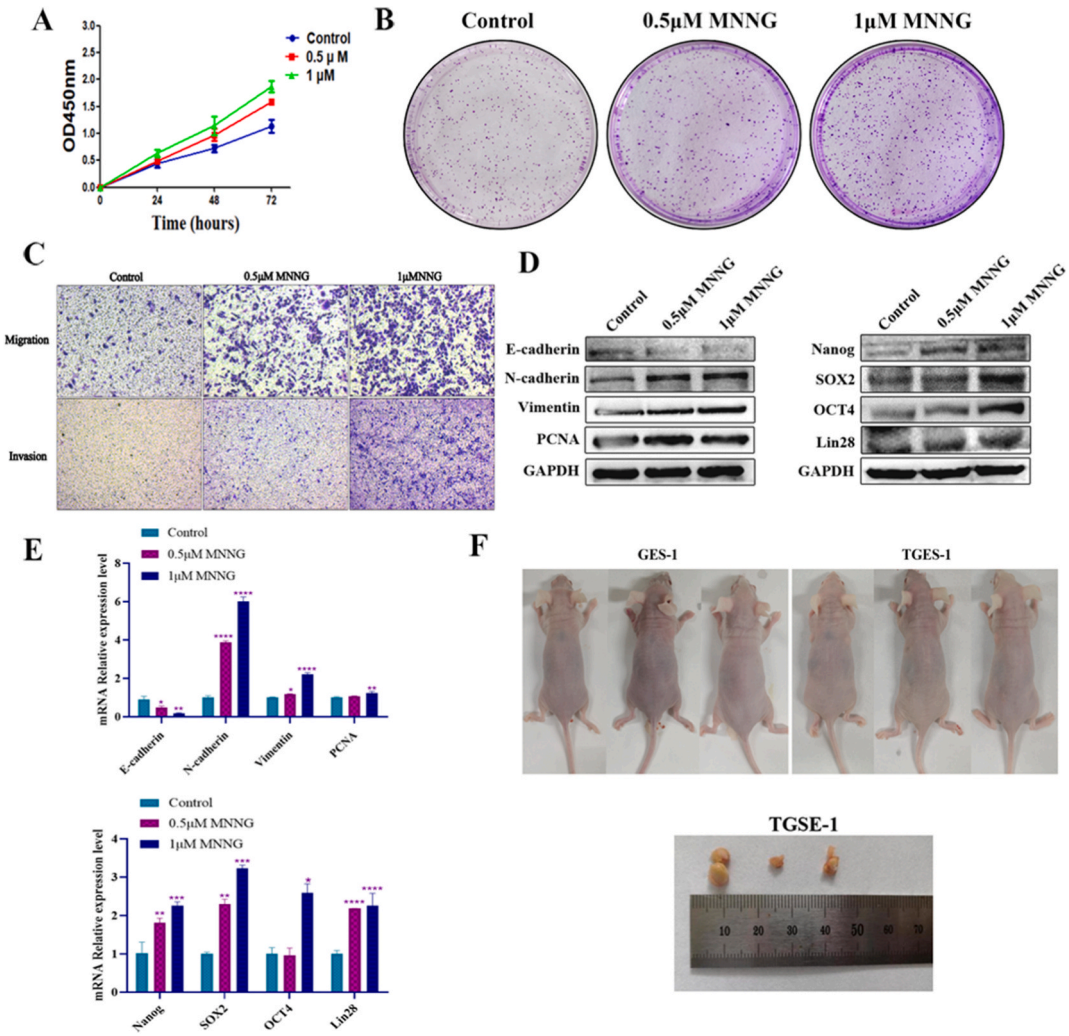

Fig. 1. MNNG induces malignant transformation of GES-1 cells in vitro and tumorigenesis in nude mice in vivo. (A) The proliferation of GES-1 cells treated with MNNG for 30 passages by CCK8 assay. (B) The cloning ability of GES-1 cells treated with MNNG. (C) The migration and invasion ability of GES-1 cells after continuous treatment for 30 generations. (D) The protein levels of EMT and stemness were detected by Western blot. (E) QRT-PCR was used to detect the levels of EMT and stemness mRNA expression. (F) Nude mice were subcutaneously injected with 5×10^7 TGES-1 cells, and the tumor size was observed 4 weeks later. The results represent mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 , ****P < 0.0001.

2.13. Statistical analysis

All data were analyzed using GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA) and expressed as mean ± SD. According to the actual situation, the difference between the two groups was analyzed by an independent sample T-test, and the difference between the multiple groups was analyzed by a one-way ANOVA. Differences were considered statistically significant at $p < 0.05$. **** indicates a p-value of less than 0.0001, *** indicates a p-value of less than 0.001, ** indicates a p-value of less than 0.01, and * indicates a p-value of less than 0.05.

3. Results

3.1. MNNG induces malignant transformation of GES-1 cells in vitro and tumorigenesis in nude mice in vivo

GES-1 cells were exposed to 0.5 μM or 1 μM MNNG for 30 generations. Next, we used cell growth, flat cloning, and transwell experiments to evaluate the proliferation, migration, and invasion abilities of cells exposed to MNNG. The cell growth curve demonstrated that GES-1 cells treated with 1 μM MNNG had enhanced proliferation (Fig. 1A), and the colony formation, migration, and invasion abilities were also significantly increased (Fig. 1B and C) . Furthermore, Western blotting showed that the expression of mesenchymal markers (N-cadherin and Vimentin) increased, while the expression of the epithelial marker E-cadherin decreased, and the stemness markers (Nanog, SOX2, Lin28, and OCT4) were upregulated (Fig. 1D). The qRT-PCR results were consistent with those of western blotting (Fig. 1E). From the perspective of cell morphology, the shape of GES-1 cells treated with 1 μM for an extended period also changed, transitioning from a spindle shape to a diamond shape. (Fig. 1F). Based on the above results, 1 μM MNNG treatment of

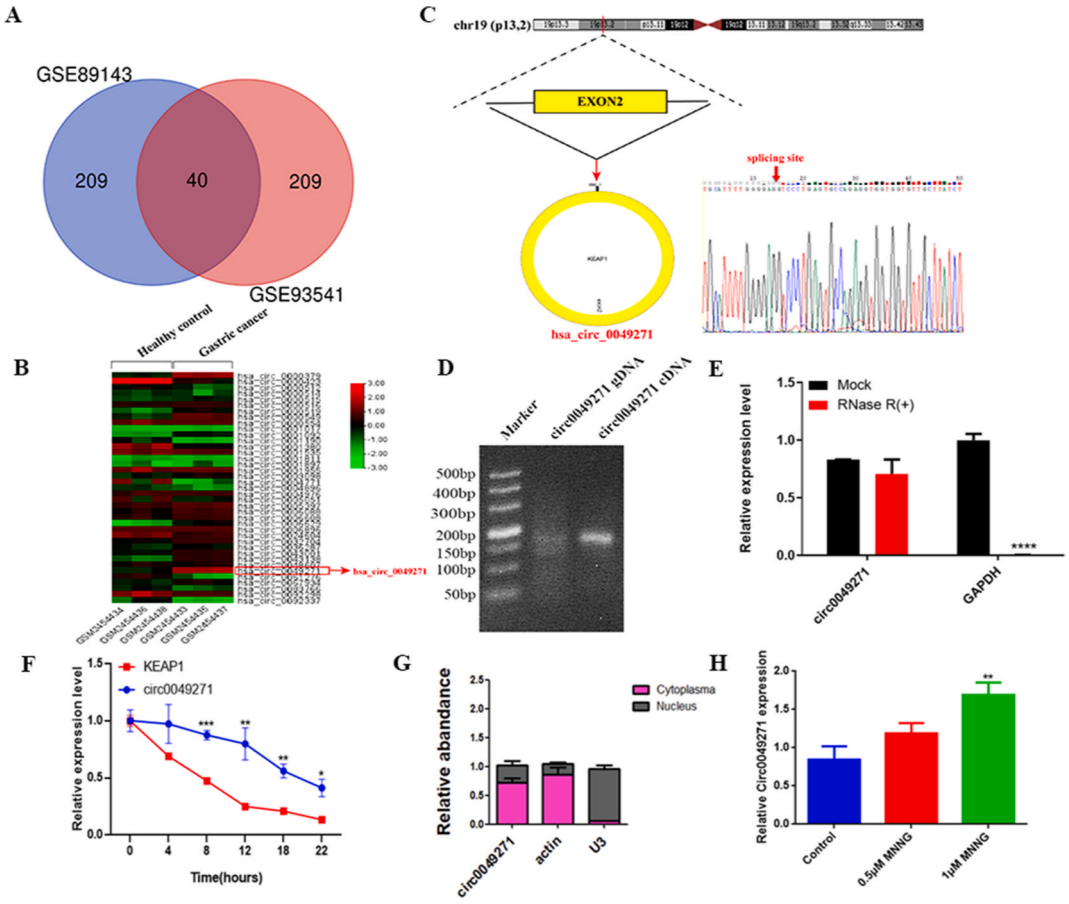

Fig. 2. Identification of circ0049271 as a candidate gene for malignant transformation of GES-1 cells. (A) Coincidence of differentially expressed circRNAs in GEO public database. (B) The heat map of differentially expressed circRNAs. (C) circ0049271 cyclization mechanism and sanger sequencing. (D) Agarose gel electrophoresis verification of circ0049271 gDNA (E) The expression levels of circ0049271 before and after RNase R treatment by QRT-PCR. (F) The expression levels circ0049271 and its mother gene KEAP1 after actinomycin D treatment of TGES-1. (G) Expression of circ0049271, actin and U3 in nuclear and cytoplasmic fractions of TGES-1 cells. (H) circRNADb website was used to predict the ORF and IRES of circ0049271. (I) NCBI CDD site be used to predict circ0049271 translation polypeptide. (J) The expression of circ0049271 in GES-1 cells induced by 0.5 μM and 1 μM MNNG by QRT-PCR. The results represent mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 , ****P < 0.0001.

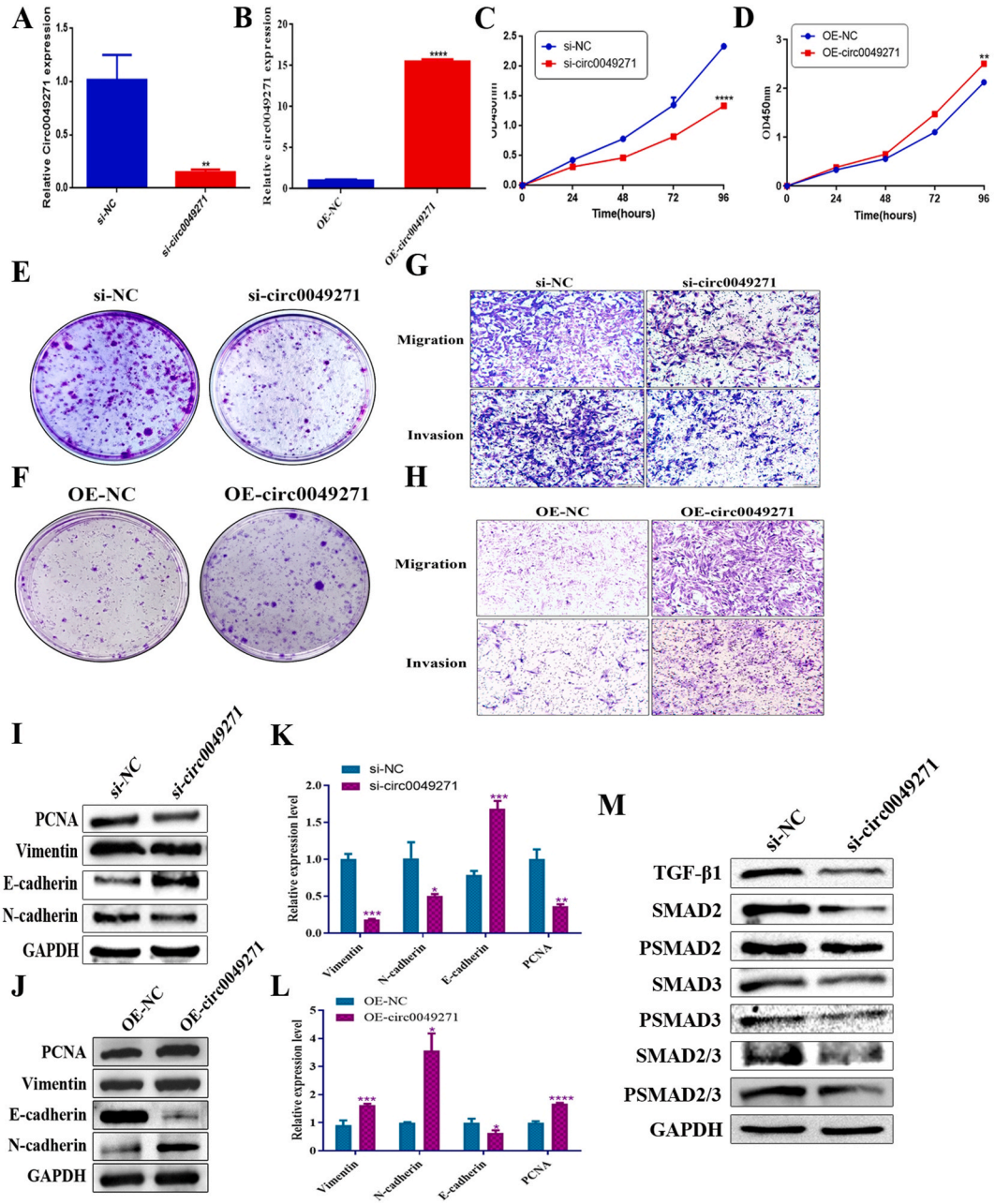

Fig. 3. Circ0049271 accelerates proliferation, migration, invasion, EMT, and stemness of TGES-1 cells in vitro. (A) QRT-PCR was used to detect the level of circ0049271 after TGES-1 transfection with si-circ0049271. (B) QRT-PCR was used to detect the level of circ0049271 after TGES-1 transfection with overexpression plasmid. (C) The proliferation of TGES-1 cells after knocking down circ0049271 by CCK-8 assay. (D) The proliferation of TGES-1 cells after overexpression of circ0049271 by CCK-8 assay. (E) The cloning ability of TGES-1 cells after knocking down circ0049271. (F) The cloning ability of TGES-1 cells after overexpression of circ0049271. (G) The migration and invasion ability of TGES-1 cells after knocking down circ0049271. (H) The migration and invasion ability of TGES-1 cells after overexpression of circ0049271. (I)The expression levels of EMT and PCNA proteins of TGES-1 cells after knocking down circ0049271 by Western blot. (J) The expression levels of EMT and PCNA proteins of TGES-1 cells after overexpression of circ0049271 by Western blot. (K) The expression levels of EMT and PCNA mRNA of TGES-1 cells after knocking down circ0049271 by qRT-PCR. (L) The expression levels of EMT and PCNA mRNA of TGES-1 cells after overexpression of circ0049271 by qRT-PCR. (M) The expression levels of TGFβ、PSMAD2 and PSMAD3 after knocking down circ0049271 by Western blot. (N) The morphology of TGES-1 cells after knockdown circ0049271. The results represent mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 , ****P < 0.0001.

GES-1 cells (TGES-1 cells) was used as the subsequent malignant transformation cell model. The TGES-1 cells demonstrated the ability to induce subcutaneous tumor growth in nude mice (Fig. 1G). Tumorigenicity is one of the gold indicators for successfully establishing malignant transformation cells. We found that under the same cell number and treatment conditions, GES-1 cells do not have tumorigenicity, while TGES-1 cells induced by MNNG have tumorigenicity in nude mice. These results indicate that long-term chronic MNNG exposure successfully induced the malignant transformation of GES-1 cells, promoting their proliferation, migration, invasion, EMT, and stemness. It also demonstrated that the malignant transformed cell model we constructed can be used to study the mechanism of MNNG-promoted gastric carcinogenesis.

3.2. *Identification of circ0049271 as a candidate gene for malignant transformation of GES-1 cells*

Numerous studies have demonstrated that circRNAs play an important role in the occurrence and progression of GC. To identify differentially expressed circRNAs, bioinformatics analysis was performed using the GEO database (GEO, https://www.ncbi.nlm.nih.gov/geo/)(Fig. 2A and B). Hsa_circ_0049271, which is highly expressed in both GC tissues and serum, was selected as the target molecule for this study. Circ0049271 is a circular RNA looped from exon 2 of human chromosome 19. Sanger sequencing results were consistent with the expected amplified fragment (Fig. 2C). The circ0049271 qRT-PCR result was likewise shown by agarose gel electrophoresis to be a single band with the anticipated size. Crucially, it did not exhibit any contamination from genomic DNA (gDNA), indicating that the primer was successful in amplifying the product from complementary DNA (cDNA) rather than gDNA (Fig. 2D). In addition, circ0049271 was more resistant to RNase R and more stable than the linear RNA GAPDH (Fig. 2E). The half-life

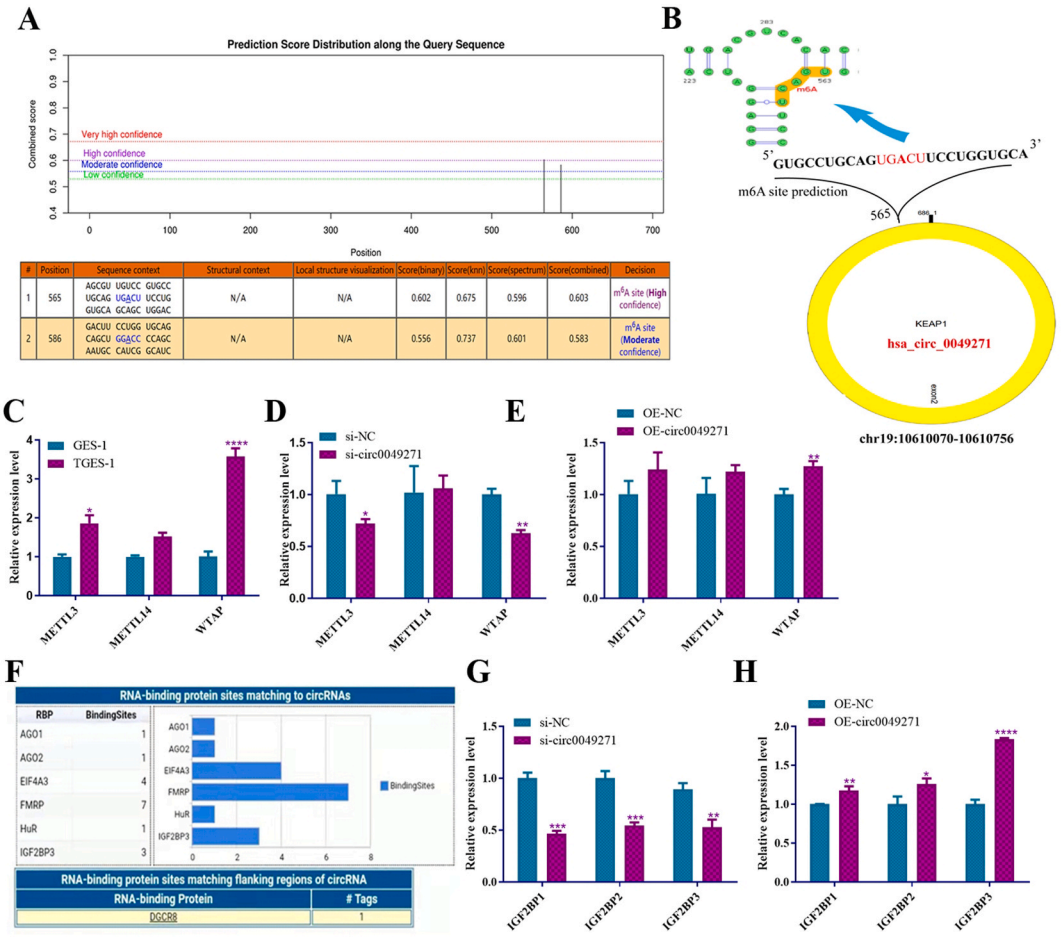

Fig. 4. WTAP mediated the modification of circ0049271 m6A and circ0049271 promoted the level of IGF2BP3. (A) SRAMP website was used to predict the level of circ0049271 m6A modification. (B) SRAMP website predicted the m6A motif structure of circ0049271. (C) The expression levels of METTL3, METTL14, and WTAP in TGES-1 cells by qRT-PCR. (D) The expression levels of METTL3, METTL14, and WTAP after knocking down circ0049271 in TGES-1 cells by qRT-PCR. (E) The expression levels of METTL3, METTL14, and WTAP after overexpression of circ0049271 in TGES-1 cells by qRT-PCR. (F) CircInteractome database showed that IGF2BP3(m6A reader protein) had a high binding ability with circ0049271. (G) The mRNA expression levels of IGF2BP1, IGF2BP2, and IGF2BP3 after knocking down of circ0049271 in TGES-1 cells by qRT-PCR. (H) The expression levels of IGF2BP1, IGF2BP2, and IGF2BP3 after overexpression of circ0049271 in TGES-1 cells by qRT-PCR.

of KEAP1 mRNA was found to be approximately 8 h, while that of circ0049271 was approximately 18 h After TGES-1 cells were treated with actinomycin D to suppress gene transcription, (Fig. 2F). These results of two experiments above indicate that circ0049271 is a stable circular RNA. Circ0049271 was primarily located in the cytoplasm of TGES-1 cells, according to the results of qRT-PCR (Fig. 2G). The circRNADb website predicted that circ0049271 had an ORF and an IRES sequence element (Fig. 2H). Furthermore, the NCBI CDD website also predicted that the ORF coding micropeptide of circ0049271 belonged to the BTB protein family (Fig. 2I). Thus, the possibility of its translation into micropeptides exists. Total RNA was extracted from GES-1 cells and 0.5 μM and 1 μM MNNG-induced GES-1 cells. QRT-PCR showed that circ0049271 was significantly highly expressed in GES-1 cells treated with 1 μM for an extended period (Fig. 2J). These results indicate that circ0049271 is a circular RNA that is highly expressed during gastric carcinogenesis.

3.3. Circ0049271 accelerates proliferation, migration, invasion, and EMT of TGES-1 cells by the TGFβ/SMAD pathway in vitro

To investigate the role of circ0049271 in the occurrence of GC, we designed and synthesized siRNA fragments, a circ0049271 overexpression plasmid, and transfected TGES-1 cells. After circ0049271 siRNA or overexpression plasmids were transfected, the expression level of circ0049271 was measured by QRT-PCR. The results showed that the si-circ0049271 fragment reduced the expression level of circ0049271 in TGES-1 to less than 50 % (Fig. 3A). After transfection with the overexpression plasmid, the expression level of circ0049271 increased by approximately 15-fold (Fig. 3B). CCK8, plate cloning, migration and invasion assays were conducted on TGES-1 cells transfected with the si-circ0049271 fragment. The results showed that after the knockdown of circ0049271, cell proliferation slowed (Fig. 3C), the number of plate colony-forming cell colonies was significantly reduced (Fig. 3E), and the migration and invasion abilities were also significantly reduced (Fig. 3G). In addition, Western blotting showed decreased expression of EMT markers (N-cadherin and Vimentin) and increased expression of E-cadherin (Fig. 3I). The qRT-PCR results were consistent with those of western blotting (Fig. 3K). Therefore, the knockdown of circ0049271 inhibited the proliferation, migration, and invasion of TGES-1 cells. Overexpression of circ0049271 resulted in opposite effects (Fig. 3D–F, H, J and L). Knockdown of circ0049271

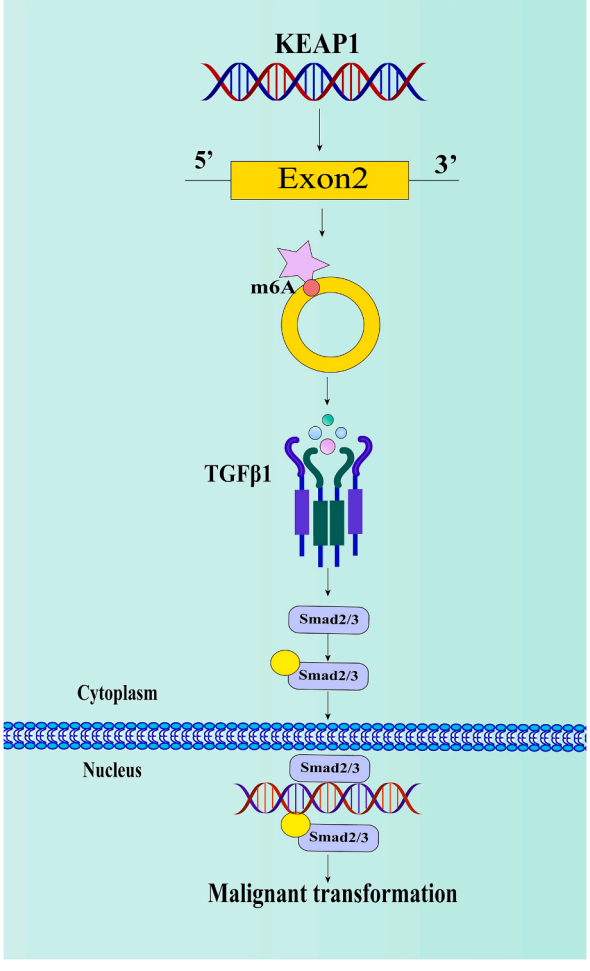

Fig. 5. Schematic diagram of MNNG induced m6A modified circ0049271 promotes precancerous lesions in gastric cancer.

suppressed the expression of TGFβ1, PSMAD2, and PSMAD3, as determined by western blotting (Fig. 3M).

3.4. WTAP mediated the modification of circ0049271 m6A and circ0049271 promoted the level of IGF2BP3

To investigate the presence of m6A modifications in circ0049271, we utilized the SRAMP online platform (http://www.cuilab.cn/sramp/) for prediction. The obtained results demonstrated the existence of a m6A methylation site within circ0049271 (Fig. 4A). An m6A motif structure was also predicted (Fig. 4B). Based on this prediction, three m6A writer proteins (METTL3, METTL14, and WTAP) were further detected in GES-1 and TGES-1 cells. It was found that compared with METTL3 and METTL14, the expression level of WTAP in TGES-1 cells was higher than that in GES-1 cells (Fig. 4C) . After the circ0049271 knockdown, the expression of WTAP and METTL3 decreased, but the reduction in WTAP was more pronounced (Fig. 4D). WTAP increased after the overexpression of circ0049271 (Fig. 4E). The above experimental results suggest that WTAP might be the writer protein for circ0049271 m6A methylation. Additionally, the CircInteractome website (https://circinteractome.nia.nih.gov/) predicted circ0049271 to interact with IGF2BP3 (m6A reader protein) (Fig. 4F). Next, we performed validation, and qRT-PCR results revealed that the expression of IGF2BP1, IGF2BP2, and IGF2BP3 decreased after the knockdown of circ0049271 in TGES-1 cells (Fig. 4G). After circ0049271 overexpression, the expression of IGF2BP1, IGF2BP2, and IGF2BP3 increased, the increase in IGF2BP3 was more significant than increases in IGF2BP1 and IGF2BP2 (Fig. 4H). These result also corroborates the predicted results to a certain extent. We hypothesized that WTAP-mediated m6A modification promotes the occurrence of GC through circ0049271 binding to IGF2BP3. Naturally, a series of experimental verifications are needed (see Fig. 5).

4. Discussion

In China, dietary factors significantly influence the incidence of gastrointestinal cancer. The early diagnosis rate of GC is less than 20 %, and most patients are diagnosed at an advanced stage, with an overall survival rate of less than 50 % [16]. Currently, the carcinogenic chemical MNNG is frequently used to mimic the ingestion of nitrate in the stomach and its conversion to nitric acid and other carcinogens, thereby simulating normal gastric mucosal lesions [17,18]. Our previous studies revealed that MNNG induces autophagy, EMT, and cell proliferation in the gastric tissues of Wistar rats [19]. In this study, we used MNNG to induce gastric mucosal GES-1 cells to establish a cell model. By detecting the proliferation, migration, and invasion of GES-1 cells, we found that cell proliferation, migration, invasion, and EMT processes increased after MNNG treatment. In addition, we found that MNNG-treated cells possessed tumorigenic abilities in vivo (Fig. 1). Previous studies, as well as this study, have demonstrated that MNNG promotes the occurrence of GC, but the specific molecular mechanism remains unclear.

Several circRNAs are inappropriately expressed in GC tissues or cell lines, and these aberrant expressed circRNAs have been associated with the development, metastasis, recurrence, and treatment resistance of GC [20–23]. In this study, the highly expressed circRNAs in the tissues and plasma of GC patients were analyzed using GEO databases (GSE89143 and GSE93541). Circ0049271 was also highly expressed in the malignant transformed cells (Fig. 2). Gao et al. analyzed the expression profile of circRNA host genes in lung adenocarcinoma (LUAD) samples from the GEO and the TCGA. Their results showed that mRNA regulated by circ0049271 can serve as diagnostic and therapeutic biomarkers for LUAD [24]. Li et al. reported that circ0049271 has a diagnostic value in non-small cell lung cancer (NSCLC) and may play key roles in NSCLC pathogenesis through signaling pathways and cell cycles [25]. However, the molecular mechanism and function of circ0049271 in the occurrence and development of tumors such as gastric cancer have not been reported yet. Consequently, circ0049271 was chosen as the research subject for this experiment. In order to clarify the specific mechanism through which circ0049271 promotes carcinogenesis, we knocked down and overexpressed circ0049271 to observe its effect on the occurrence of gastric cancer. These results showed that circ0049271 could promote the proliferation, migration, invasion, and EMT of malignantly transformed cells. Members of the transforming TGF-β family play important roles in tumors initiation and progression [26]. TGF-β1 binds to membrane receptors to form complexes that initiate intracellular signaling and activate downstream SMAD2 and SMAD3 proteins [27]. CircCCDC66 promotes GC growth and metastasis by activating c-Myc and TGF-β signaling pathways [28]. Song et al. reported that HOXA10 can activate TGFβ/SMAD signaling and promote the interaction between SMAD2/3 and METTL3 in the nucleus, thereby enhancing the invasion and metastasis of GC cells [29]. The TGF-β signaling pathway is a promising target for cancer therapy, and its significance is still being discovered. Our experiments confirmed that the expression of TGFβ1 and SMAD was downregulated after the circ0049271 knockdown. Circ0049271 regulates TGFβ1/SMAD signaling to promote MNNG-induced gastric carcinogenesis (Fig. 3).

M6A is the most common RNA modification in eukaryotes [30]. M6A has a significant impact on the occurrence and progression of tumors by influencing RNA splicing, translation, stability, and the epigenetics of non-coding RNA [31,32]. It was found that circRNAs containing m6A residues can be translated without the cap's assistance [33]. circMAP3K4 modified by m6A encodes 455 novel peptides that prevent liver cancer cell apoptosis, a process enhanced by IGF2BP1 [34]. METTL14-mediated m6A modification of circORC5 inhibits GC progression by regulating the miR-30c-2-3p/AKT1S1 axis [35]. Wilms' tumor 1-associating protein (WTAP) is a widely expressed nuclear protein associated with WT, most of which depends on m6A modification of methyltransferase activity [36]. Recent reports have shown that WTAP-mediated m6A modification of UNC-51-like kinase 1 (ULK1) enhances the stability of its mRNA in an IGF2BP3-dependent manner and promotes the progression of epithelial ovarian cancer [37]. These findings open up new avenues for the investigation of circRNAs modified by m6A. Nevertheless, little is understood about how m6A modification affects circRNAs in GC. In this study, we predicted that circ0049271 has highly credible m6A modification sites that can interact with WTAP, a key m6A writing protein. We detected the expression levels of three m6A writer proteins (METTL3, METTL14, and WTAP) in malignantly transformed cells. We then either knockdown or overexpressed circ0049271 in these cells, respectively. Among them, WTAP was

significantly positively correlated with the malignancy of cells and the expression level of circ0049271. IGF2BP3 belongs to a conserved family of RNA-binding oncoembryonic proteins [38]. CircARID1A can bind to IGF2BP3 in GC and promote cancer proliferation by forming a circARID1A-IGF2BP3-SLC7A5 RNA-protein ternary complex [39]. Circ0049271 binding protein was predicted online through the website, and then qRT-PCR assay was used to detect the expression level of IGF2BP3 in cells with knockdown or overexpressed circ0049271. The results indicated that IGF2BP3 is a probable binding protein for circ0049271. Of course, this requires further validation through the RNA binding protein immunoprecipitation (RIP) assay. These findings elucidate a new mechanism of circ0049271 in the development of GC for the first time. It also suggests that circ0049271 can bind to the key writer of m6A, revealing a new role for circ0049271 in the regulation of mRNA the m6A modification. WTAP mediates m6A modification of circ0049271 to promote the malignant transformation of GES-1 cells by regulating IGF2BP3(Fig. 4). However, the mechanism by which circ0049271 regulates IGF2BP3 remains unclear. Our recent findings suggest new therapeutic strategies and targets for MNNG-induced GC.

4.1. Limitations

This study suggests that circ0049271 may promote gastric cancer occurrence by regulating the TGFβ/SMAD pathway, providing new insights into the role of circRNA-mediated m6A modifications in the occurrence of GC induced by dietary factors such as nitrite. However, more investigation is needed to determine the clinical significance of these findings and their applicability to patients. This study has only been a partial part of our research, which has laid the foundation and provided research directions for future studies. In the coming years, we will strengthen the research on clinical relevance and clinical diagnostic and therapeutic targets.

5. Conclusions

We revealed the role of a novel circRNA, circ0049271, in the occurrence of MNNG-induced GC and elucidated the possible mechanism. We found that circ0049271 promotes the occurrence of MNNG-induced GC by regulating the TGFβ/SMAD pathway. In addition, WTAP mediates the m6A modification of circ0049271 by regulating IGF2BP3. These findings provide new insights into the mechanisms and therapeutic strategies of MNNG-induced gastric carcinogenesis.

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Data availability statement

All data generated or analyzed in this study are included in this published article. After the publication of the manuscript, the relevant data can be stored in the publicly available repository.

Ethics

All mouse experiments were approved by the Animal Care and Use Committee of Jiangsu University, and efforts were made to minimize animal suffering and distress (2021030816).

Patient consent for publication

Not applicable.

CRediT authorship contribution statement

Yue Zhang: Writing – original draft, Methodology, Data curation. **Zhiqiang Chen:** Formal analysis, Data curation, Conceptualization. **Jiajia Song:** Methodology, Formal analysis. **Hui Qian:** Methodology, Investigation, Conceptualization. **Yue Wang:** Writing – review & editing, Funding acquisition, Data curation, Conceptualization. **Zhaofeng Liang:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35654.

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