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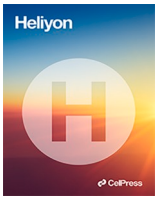

Research article

CircGNAO1 strengthens its host gene GNAO1 expression for suppression of hepatocarcinogenesis

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is one of the most prevalent primary liver carcinoma. Guanine nucleotide-binding protein, α-activating activity polypeptide O (GNAO1) was reported to be under-expressed in HCC tissues. This study aimed to investigate the GNAO1-derived circular RNA (circRNA) and its molecular mechanisms in HCC.

Methods: Real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot were applied to examine RNA and protein levels. Functional experiments were performed to study HCC cell proliferation, cell cycle and cellular senescence. The interactions among circGNAO1, GNAO1 and DNA methyltransferase 1 (DNMT1) were examined by mechanism assays. The methylation level was analyzed by bisulfite sequencing PCR (BSP).

Results: CircGNAO1 is down-regulated and positively associated with GNAO1 in HCC tissues. Overexpression of circGNAO1 inhibits cell proliferation, induces cell cycle arrest and facilitates cell senescence in HCC cells. CircGNAO1 facilitates the progression of HCC via modulating GNAO1. Mechanistically, circGNAO1 enhances the transcription of GNAO1 by sequestering DNMT1, thereby up-regulating GNAO1 expression in HCC cells.

Conclusions: CircGNAO1 up-regulates its host gene GNAO1 expression for suppression of hepatocarcinogenesis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent primary liver cancers and the third leading reason of cancer-related mortality across the globe [1]. Risk factors for HCC encompass chronic hepatitis B, hepatitis C [2,3], alcohol, primary biliary cirrhosis [4], and autoimmune hepatitis [5,6]. Dietary toxins, like aflatoxins and aristolochic acid, have also been reported to increase the risk of HCC occurrence [7]. Liver transplantation and surgical resection remains the first-line treatment options for HCC patients [8]; sorafenib, lenvatinib and regorafenib are regarded to be the standard treatments for advanced HCC, improving the overall survival (OS) of HCC patients [9]. Despite the fact that great progresses have been made in therapeutic methods, the survival rate of HCC patients still

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remains low owing to the high recurrence rate and heterogeneity [10]. Hence, it is desperately urgent to explore the specific molecular mechanism underlying occurrence and development of HCC.

Circular RNAs (circRNAs) are a type of endogenous non-coding RNAs characterized with closed loop structures [11]. The regulatory roles of circRNAs have been investigated in hepatocarcinogenesis and the development of HCC [12]. It is noteworthy that dysregulated circRNA-miRNA-mRNA axis plays important roles in HCC and has been widely discussed [13]. For example, circRNA-5692 hampers the development of HCC by acting as a sponge for miR-328-5p to up-regulate DAB2IP expression level [14]; circRNA-104718 modulates miR-218-5p and TXNDC5 via competing endogenous RNA (ceRNA) mode, thus facilitating HCC progression [15]; hsa_circ_0091570 as a ceRNA sequesters miR-1307 to hinder HCC progression [16]. However, the roles of most circRNAs in HCC have not been thoroughly studied.

Guanine nucleotide-binding protein, α-activating activity polypeptide O (GNAO1) belongs to the subunit family of Gα proteins, which are molecular switchers controlling signal transduction, and the dysregulation of Gα proteins can promote tumorigenesis [17]. GNAO1 was found to be under-expressed in HCC tissues [18], and its down-regulation was reported to be due to promoter methylation mediated by DNA methyltransferase 1 (DNMT1) [19]. Nevertheless, the potential upstream circRNA of GNAO1 in HCC remained unknown. As reported previously, a circRNA can affect the progression of diseases by regulating the expression of its host gene [20–22]. Based on the above research status, our study aimed to explore the effect of GNAO1-derived circGNAO1 on the cellular progression in HCC, and the underlying mechanism concerning its host gene GNAO1 in HCC. With this study, we intended to gain a better understanding of molecular mechanism underlying HCC progression for the treatment improvement.

2. Materials and methods

2.1. Bioinformatics analysis

circBase (http://circbase.org/) was used to analyze the circRNAs derived from GNAO1, and identified five candidates: hsa_circ_0105616, hsa_circ_0039422, hsa_circ_0105617, hsa_circ_0105618, and hsa_circ_0105619.

2.2. Specimen collection

50 HCC tissue samples and 50 pericarcinoma liver tissue (PCLT) samples were collected from HCC patients who undergone surgical resection in the First Affiliated Hospital of Xi'an Jiaotong University. Prior to surgery, no patients had received radio- or chemotherapy. The signed informed consents were obtained from the patients. The protocols of our study were approved by the First Affiliated Hospital of Xi'an Jiaotong University. Subsequent to the collection, the samples were frozen by liquid nitrogen, followed by being stored at −80 °C for long-term storage.

2.3. Cell line culture

HCC cell lines (LM3, Huh7, Hep3B, and MHCC97H) and immortalized normal liver cell lines (THLE-3) were all commercially attained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were placed in RPMI-1640 medium (A4192301, Gibco, Grand Island, NY, USA) with 10 % fetal bovine serum (FBS, #12483020, Gibco) and 1 % penicillin-streptomycin (#15070063, Gibco). All cells were maintained in an incubator at 37 °C containing 5 % CO_2.

2.4. Plasmids construction

Two interference sequences of GNAO1 and the control sequence were inserted into pLKO.1 vector to construct sh-GNAO1#1/2 and sh-NC, with sh-NC as the negative control (NC). We obtained the overexpression vectors of circGNAO1 and DNMT1 by inserting the full sequences of circGNAO1 and DNMT1 into vectors, with empty vector utilized as the NC. Abovementioned plasmids were transfected with application of Lipofectamine Reagent.

2.5. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from THLE-3, LM3, Huh7, Hep3B, and MHCC97H cells, HCC tissues, and PCLT using TRIzol (#15596018, Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. Total RNA was then converted into complementary DNA (cDNA) via reverse transcription using TaqMan® MicroRNA Reverse Transcription Kit (#4366596, Applied Biosystems™, Foster City, CA, USA). Afterwards, qPCR was implemented on StepOnePlus™ Real-time PCR systems (#4376600, Applied Biosystems™) using SYBR™ Green PCR Master Mix (#4364346, Applied Biosystems™). The $2^{-\Delta\Delta Ct}$ approach was employed to calculate the relative expression levels. GAPDH served as the internal reference for RT-qPCR. The assays were conducted with bio-repeats and each repeats were implemented in triplicate.

2.6. Western blot

Total protein was subjected to extraction from LM3 and MHCC97H cells in RIPA buffer, followed by separation utilizing SDS-PAGE. Then the extracted protein was transferred onto PVDF membranes. After the membranes were blocked by non-fatted milk at room

temperature, the primary antibodies encompassing Anti-GNAO1 (1/1000; #3975, Cell signaling Technology, Boston, MA, USA), Anti-GAPDH (1/1000; ab9485, Abcam, Cambridge, MA, USA), and Anti-DNMT1 (1/1000; sc-271729, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were adopted to incubate with the membranes overnight at 4 °C. Next, HRP-conjugated secondary antibodies (1/2000) were incubated with the membranes. BCA Protein Assay kit (Beyotime, Shanghai, China) was employed for quantification of protein concentration. The blots were visualized by ELC detection kit (Pierce Biotechnology, Rockford, IL, USA). GAPDH acted as the internal reference. The assays were carried out with bio-repeats and each repeats were conducted in triplicate.

2.7. Fluorescence in situ hybridization (FISH)

CircGNAO1-FISH probe was acquired from RiboBio (Guangzhou, China). LM3 and MHCC97H cells were subjected to 15 min of fixation in 4 % PFA, followed by 15 min of permeabilization in 0.5 % TritonX-100 at 4 °C. Afterwards, digoxigenin (DIG) was labeled with circGNAO1-FISH probe or control probe, followed by 4 h of incubation with cells at 55 °C. The signal was detected using Hoechst-conjugated Anti-DIG antibodies. Laser confocal microscope was used for image obtaining. DAPI (D9542, Sigma-Aldrich, St. Louis, MO, USA) was adopted to counterstain nucleus. The assays were performed with bio-repeats and each repeats were implemented in triplicate.

2.8. Subcellular fractionation analysis

Subcellular fractionation analysis was conducted to analyze the localization of circGNAO1 in HCC cells. Nuclear and cytoplasmic RNAs of LM3 and MHCC97H cells were separated and purified using PARIS kit (AM1921, Invitrogen) based on the manufacturer's instructions. ACTB and U6 acted as the cytoplasmic and nuclear controls separately. The assays were implemented with bio-repeats and each repeats were implemented in triplicate.

2.9. Cell counting kit-8 (CCK-8) assay

CCK-8 reagent (ck-04, DOJINDO, Kumamoto, Kyushu, Japan) was conducted to evaluate cell viability. Transfected LM3 and MHCC97H cells were placed into 96-well culture plates. Next, all the transfected cells underwent 24, 48 or 72 h of incubation, and then CCK-8 reagent was added in each well. The cells were incubated for another 4 h after the addition. The absorbance at 450 nm was evaluated by Absorbance Microplate Reader (ELx808™, BioTek, Winoski, VT, USA). The assays were carried out with bio-repeats and each repeats were implemented in triplicate.

2.10. 5-Ethynyl-2′-deoxyuridine (EdU) assay

EdU assay was carried out by using BeyoClick™ EdU-488 Cell Proliferation Kit with Alexa Fluor 488 (C0071S, Beyotime) following the manufacturer's instructions. After the transfection, cells were subjected to incubation with EdU reagent, fixation by para-formaldehyde and permeabilization by Triton X-100. Besides, the nucleus of cells was stained with DAPI. Lastly, the proliferation of LM3 and MHCC97H cells was determined with a fluorescence microscope (FL400, Leica, Weztlar, Germany). The assays were carried out with bio-repeats and each repeats were implemented in triplicate.

2.11. Flow cytometry analysis

Transfected LM3 and MHCC97H cells were subjected to the treatment of trypsin, followed by the washing in pre-cooled PBS. Subsequently, cells were treated with PI/RNase Staining Buffer (#550825, BD Biosciences, Franklin Lake, NJ, USA). Finally, cell apoptosis was analyzed by Flow Cytometer (FACSCanto II, BD Biosciences). The assays were carried out with bio-repeats and each repeats were implemented in triplicate.

2.12. Senescence assay

Senescence β-Galactosidase Staining Kit (#9860, Cell Signaling Technology) was utilized in SA-β-gal staining assay to analyze HCC cell senescence under the manufacturer's instruction. The assays were performed with bio-repeats and each repeats were carried out in triplicate.

2.13. Luciferase reporter assay

To construct the reporter vector, GNAO1 promoter was inserted into the pGL3 vector (E1751, Promega, Madison, WI, USA). After that, the reporter vector was co-transfected with overexpression vector of circGNAO1 or empty vector into LM3 and MHCC97H cells. 36 h later, the luciferase reporter activity was determined on Dual Luciferase Report Assay System (E1910, Promega), with Renilla luciferase being the internal reference. The assays were carried out with bio-repeats and each repeats were implemented in triplicate.

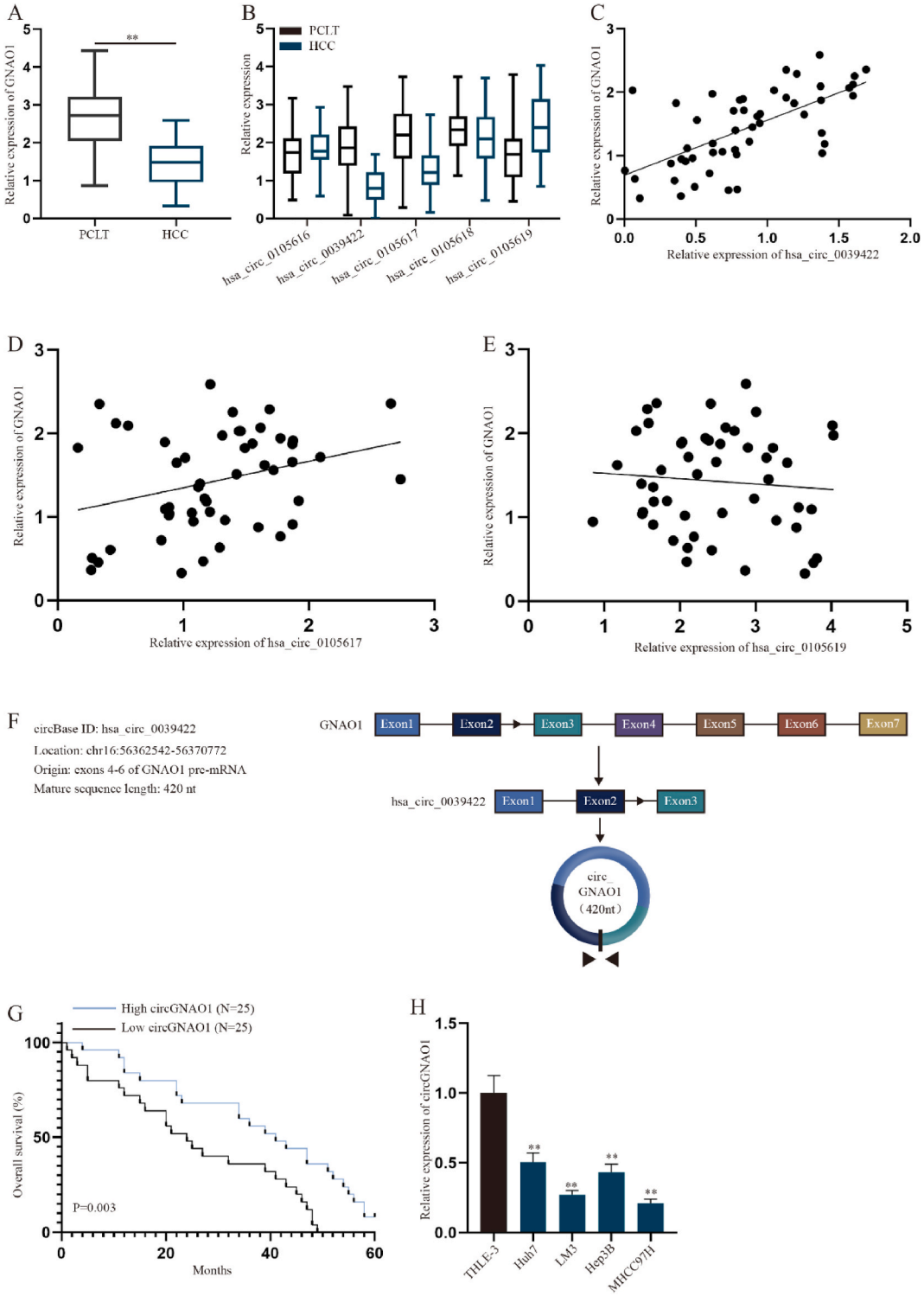

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Fig. 1. CircGNAO1 is down-regulated and positively correlated with GNAO1 in HCC tissues. (A) GNAO1 expression level was detected in HCC tissues and PCLT by RT-qPCR analysis. GNAO1 expression was significantly downregulated in HCC tissues. (B) RT-qPCR examined the expression of hsa_circ_0105616, hsa_circ_0039422, hsa_circ_0105617, hsa_circ_0105618 and hsa_circ_0105619 in HCC tissues and PCLT. hsa_circ_0039422, hsa_circ_0105617, and hsa_circ_0105619 showed significant differences in expression, while the rest showed no significant differences. (C–E) Pearson correlation analyses evaluated the correlation between GNAO1 and hsa_circ_0039422, GNAO1 and hsa_circ_0105617, GNAO1 and hsa_circ_0105619 in HCC tissues. hsa_circ_0039422 was the most closely associated with GNAO1 compared with the other two circRNAs. (F) The information about hsa_circ_0039422 (circGNAO1) was shown. (G) The OS of HCC patients with high and low circGNAO1 expression was displayed by Kaplan-Meier curve. P = 0.003. High expression of circGNAO1 was associated with high OS, while low expression of circGNAO1 was associated with low OS. (H) CircGNAO1 expression level in HCC cell lines (Huh7, LM3, Hep3B and MHCC97H) and normal THLE-3 cells was measured by RT-qPCR analysis. circGNAO1 expression was significantly downregulated in HCC cells compared with THLE-3 cells. **P < 0.01.

2.14. RNA pulldown assay

CircGNAO1 probe and control probe were attained from RiboBio. Structure buffer was added to biotin-labeled circGNAO1 probe to form secondary structure. After that, the probe was heated and ice-bathed for denaturation. M − 280 streptavidin beads (#6013, Takara, Dalian, China) were subjected to 2 h of incubation with circGNAO1 probe at 4 °C. The cell lysate was prepared for 3 groups (Marker, Control probe, and circGNAO1 probe), followed by being incubated with beads-probe complex at 4 °C for one night. Subsequent to the incubation, the proteins were eluted from the beads and separated by SDS-PAGE. The gel was silver stained, followed by being subjected to mass spectrometry. The assays were performed with bio-repeats and each repeats were implemented in triplicate.

2.15. RNA-binding protein immunoprecipitation (RIP) assay

EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (17–701, Sigma-Aldrich) was utilized in RIP assays for the verification of the binding between circGNAO1 and DNMT1, circGNAO1 and Ago2. Immunoglobulin G (IgG) was utilized as the NC. Firstly, the transfected cells were lysed. After that, 50 μg Protein A/G Agrose magnetic beads were incubated with 5 μg Anti-IgG (CS200621, Millipore, Billerica, MA, USA) and Anti-DNMT1 or Anti-Ago2 (ab186733, Abcam) at 4 °C for one night., Next, the cell lysate was co-incubated with antibody-magnetic beads complex at 4 °C for another whole night. After washing and purification, circGNAO1 was extracted from the complex and quantified by RT-qPCR analysis. The assays were conducted with bio-repeats and each repeats were implemented in triplicate.

2.16. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted with application of ChIP Kit (#17–295, Sigma-Aldrich). LM3 and MHCC97H cell lysates were sonicated to be fragments and immunoprecipitated using Anti-DNMT1, Anti-RNA pol II (ab5408, Abcam) and Anti-IgG antibodies. The enrichment of promoter was detected by RT-qPCR analysis. PCR Cleanup kit (AP-PCR-50, Axygen, Silicon Valley, CA, USA) was used for DNA purification. The assays were carried out with bio-repeats and each repeats were implemented in triplicate.

2.17. Bisulfite sequencing PCR (BSP)

EZ DNA Methylation-Gold Kit (D5005, ZYMO, Orange County, CA, USA) was employed to perform BSP as instructed by the protocol. Firstly, genomic DNA was isolated from LM3 and MHCC97H cells, followed by bisulfite treatment. GNAO1 promoter was amplified using PCR methods, with bisulfite-treated DNA as template. PCR products were purified by PCR Cleanup kit, followed by being cloned into the pMD19-T vector (#6013, TaKaRa). The clones were randomly selected and sequenced. The assays were carried out with bio-repeats and each repeats were implemented in triplicate.

2.18. Statistical analysis

All the experiments in this study contained three biological repeats and each repeats were implemented with technical triplicate. Data in this research were displayed as mean ± standard deviation (SD), with SPSS software as the analysis tool for data. One-way/two-way analysis of variance (ANOVA) and student's *t*-test were adopted to analyze difference between groups. Pearson correlation was used to analyze the correlation between two variables in HCC tissues. Kaplan-Meier curve was used to exhibit the OS of HCC patients and log-rank test was applied for comparisons in this curve. P value under 0.05 was regarded as the threshold for statistically significance.

3. Results

CircGNAO1 is down-regulated and positively correlated with GNAO1 in HCC.

To identify the GNAO1-derived circRNA, we searched circBase (http://circbase.org/) and found 5 candidates, hsa_circ_0105616, hsa_circ_0039422, hsa_circ_0105617, hsa_circ_0105618, and hsa_circ_0105619. We applied RT-qPCR analysis to detect the expression level of GNAO1 in HCC tissues and PCLT. GNAO1 was significantly down-regulated in HCC compared to that in PCLT (Fig. 1A). Also, we measured the expression levels of the five candidate circRNAs in HCC tissues and PCLT. It turned out that, hsa_circ_0039422 and

hsa_circ_0105617 expression levels of were down-regulated while that of hsa_circ_0105619 was up-regulated in HCC tissues, compared to PCLT. As for the rest, no marked differences could be found between HCC tissues and PCLT (Fig. 1B). Pearson correlation analysis verified that hsa_circ_0039422 had the closest correlation with GNAO1 (Fig. 1C–E). Therefore, we conjectured that hsa_circ_0039422 was the most relevant upstream circRNA of GNAO1 in HCC. The relevant information of hsa_circ_0039422 (written as circGNAO1) was shown in Fig. 1F. Then, we investigated the association of circGNAO1 with HCC. Based on Kaplan-Meier curve, OS of HCC patients with up-regulated circGNAO1 expression were higher than those with down-regulated circGNAO1 expression (Fig. 1G), suggesting that high expression of circGNAO1 indicates a good prognosis. Subsequently, we conducted RT-qPCR to examine the expression level of circGNAO1 in Huh7, Hep3B, LM3, MHCC97H and THLE-3 cell lines. As shown in Fig. 1H, circGNAO1 expressed at low levels in HCC cell lines compared to that in THLE-3. To sum up, circGNAO1 is low-expressed in HCC tissues and cell lines and positively correlated with its host gene GNAO1.

CircGNAO1 overexpression inhibits HCC cell proliferation, triggers cell cycle arrest and promotes cellular senescence.

Next, we investigated the biological functions of circGNAO1 in HCC cells. The efficiency of overexpression vector of circGNAO1 in LM3 and MHCC97H cells was examined at first (Fig. 2A). CCK-8 assay was performed to determine the influence of circGNAO1 on HCC

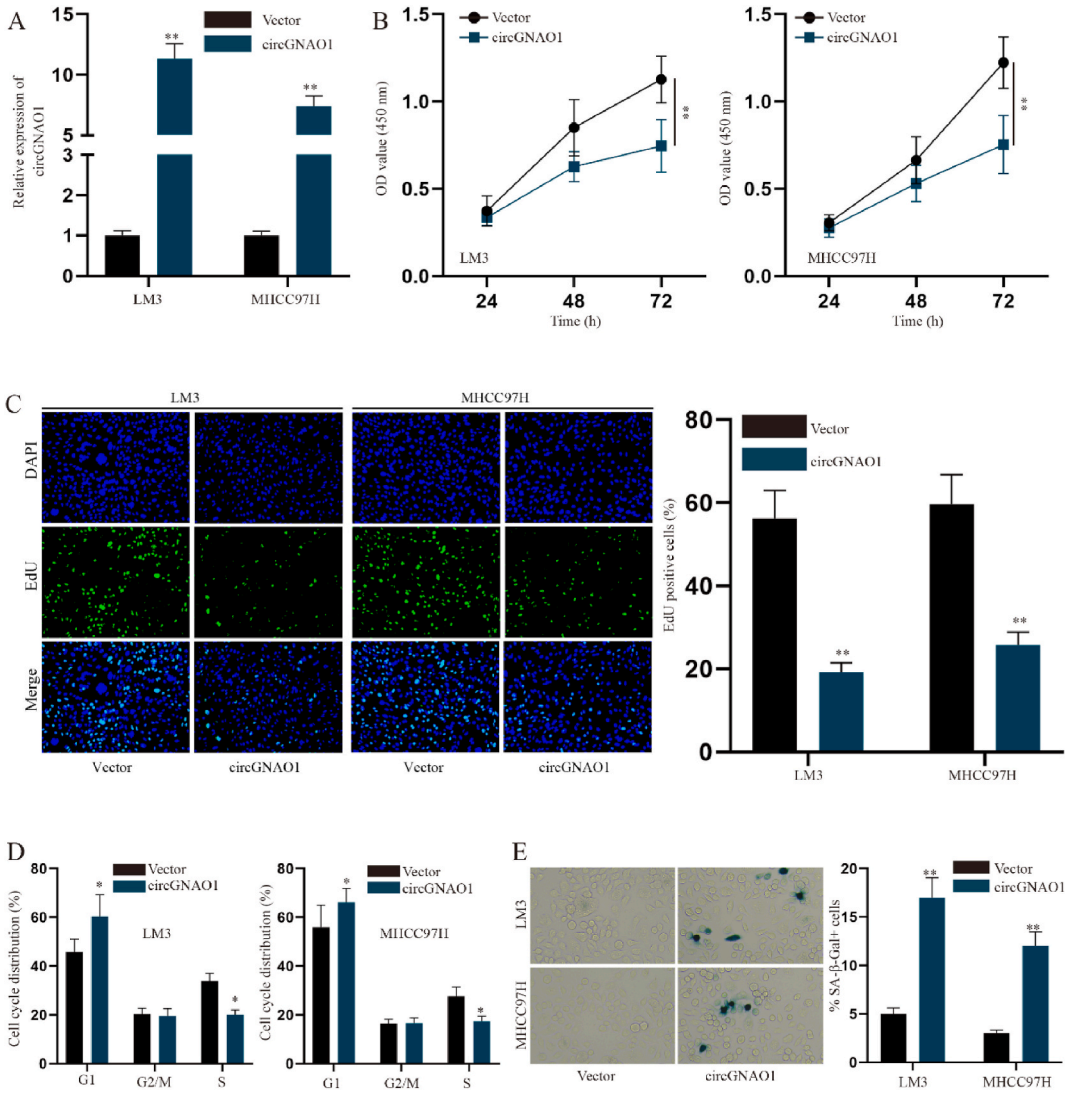

Fig. 2. CircGNAO1 overexpression impairs cell proliferation, induces cell cycle arrest and promotes cell senescence in HCC cells. (A) The overexpression efficiency of overexpression vector of circGNAO1 was detected in HCC cells via RT-qPCR analysis. (B) CCK-8 assays explored the effect of circGNAO1 enhancement on HCC cell viability. Overexpression of circGNAO1 decreased the viability of HCC cells. (C) EdU assays analyzed the influence of circGNAO1 overexpression on HCC cell proliferation. Overexpression of circGNAO1 decreased the proliferation of HCC cells. (D) Flow cytometry analysis uncovered the influence of circGNAO1 overexpression on cell cycle. The cell cycle was hampered at G1 phase in HCC cells by circGNAO1 overexpression. (E) SA-β-gal staining assay explored the influence of circGNAO1 overexpression on HCC cell senescence. Overexpression of circGNAO1 increased the proportion of SA-β-Gal + cells. **P < 0.01.

cell viability. As exhibited in Fig. 2B, overexpressed circGNAO1 weakened the viability of LM3 and MHCC97H cells. EdU assay was then conducted to assess the effect of circGNAO1 on the proliferation of HCC cells. It was disclosed that, overexpressed circGNAO1 repressed HCC cell proliferation (Fig. 2C). In addition, flow cytometry analysis was applied to evaluate the effect of circGNAO1 on cell cycle. As displayed in Fig. 2D, the cell cycle was hampered at G1 phase in HCC cells by circGNAO1 overexpression. Furthermore, SA-β-gal staining assay was carried out to determine the influence of circGNAO1 on cellular senescence (Fig. 2E). The results uncovered that the proportion of SA-β-Gal + cells was increased by up-regulated circGNAO1, which indicates that circGNAO1 promotes cellular senescence. Collectively, circGNAO1 hampers cell growth, induces cell cycle arrest and propels cellular senescence in HCC.

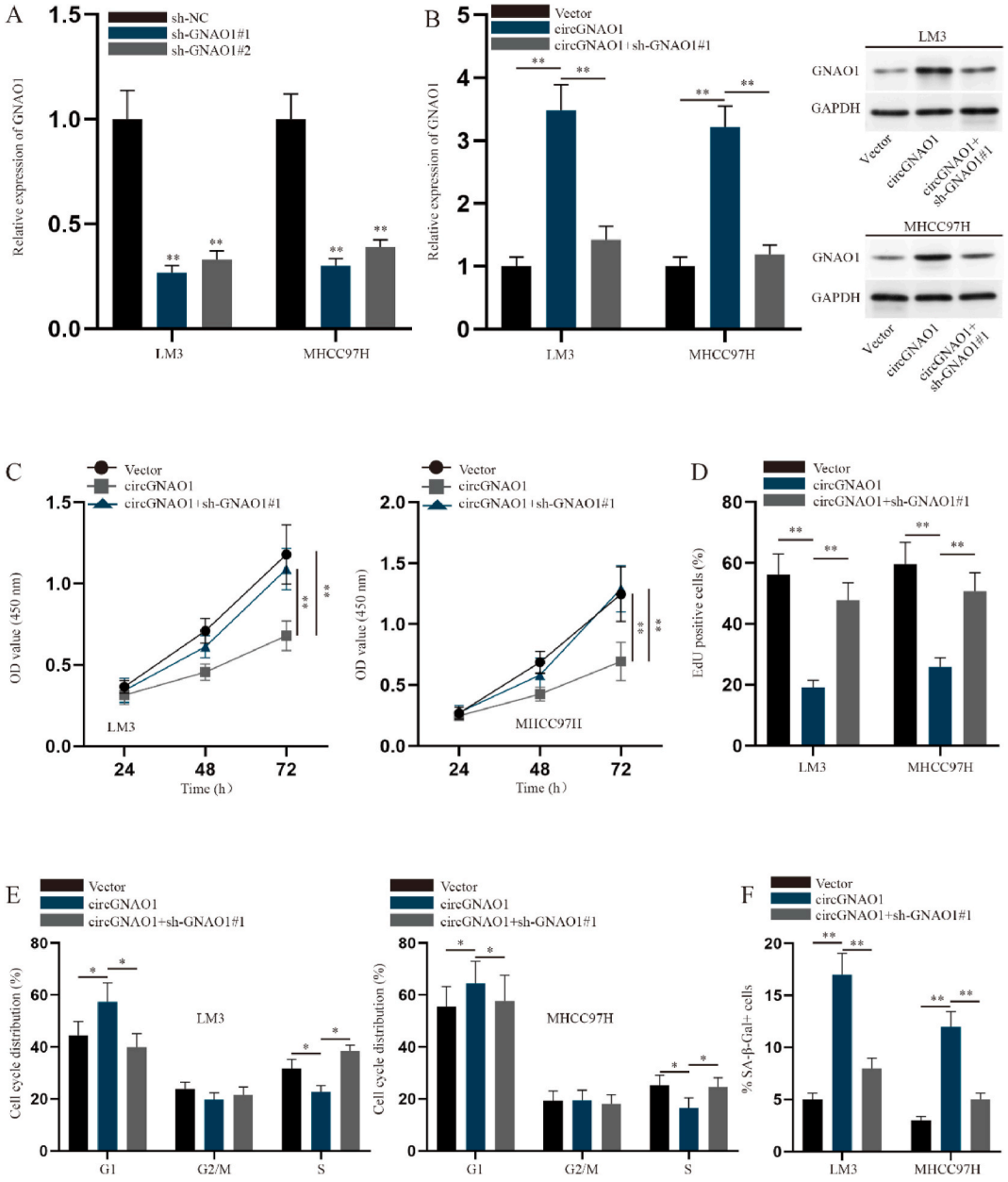

Fig. 3. CircGNAO1 suppresses HCC progression by GNAO1. (A) The knockdown efficiency of sh-GNAO1#1/2 was verified by RT-qPCR analysis. (B) The level of GNAO1 was measured under different conditions (Vector, circGNAO1 overexpression vector, and circGNAO1 overexpression vector + sh-GNAO1#1) via RT-qPCR and Western blot analyses. Knocking down GNAO1 reversed the upregulation of GNAO1 mRNA and protein levels induced by overexpression of circGNAO1. (C–F) CCK-8, EdU, flow cytometry and SA-β-gal staining assays disclosed the changes in cell function under different conditions. Knocking down GNAO1 reversed the effects of overexpression of circGNAO1 on cell function. *P < 0.05, **P < 0.01.

3.1. CircGNAO1 suppresses HCC progression via GNAO1

Further, we performed rescue assays to validate whether circGNAO1 affects the progression of HCC cells through modulating GNAO1. Firstly, we verified the efficiency of sh-GNAO1#1/2 in LM3 and MHCC97H cells (Fig. 3A), and sh-GNAO1#1 was chosen for ensuing experiments. Through RT-qPCR and Western blot analyses, GNAO1 mRNA and protein levels were elevated by circGNAO1 overexpression, and then fully reversed by GNAO1 knockdown (Fig. 3B). After that, CCK-8, EdU, flow cytometry and SA-β-gal staining assays were conducted to evaluate the functional change of HCC cells. As shown in Fig. 3C–F, GNAO1 knockdown completely counteracted the suppressive effect of circGNAO1 overexpression on cell growth and cell cycle and the promoting effect on cellular senescence in HCC. Taken together, circGNAO1 plays a suppressive role in HCC cells via up-regulating GNAO1 expression.

3.2. CircGNAO1 positively regulates the expression of its host gene GNAO1 at the transcriptional level

Next, we explored how circGNAO1 affected the expression of GNAO1 in HCC cells. We firstly detected GNAO1 expression in HCC

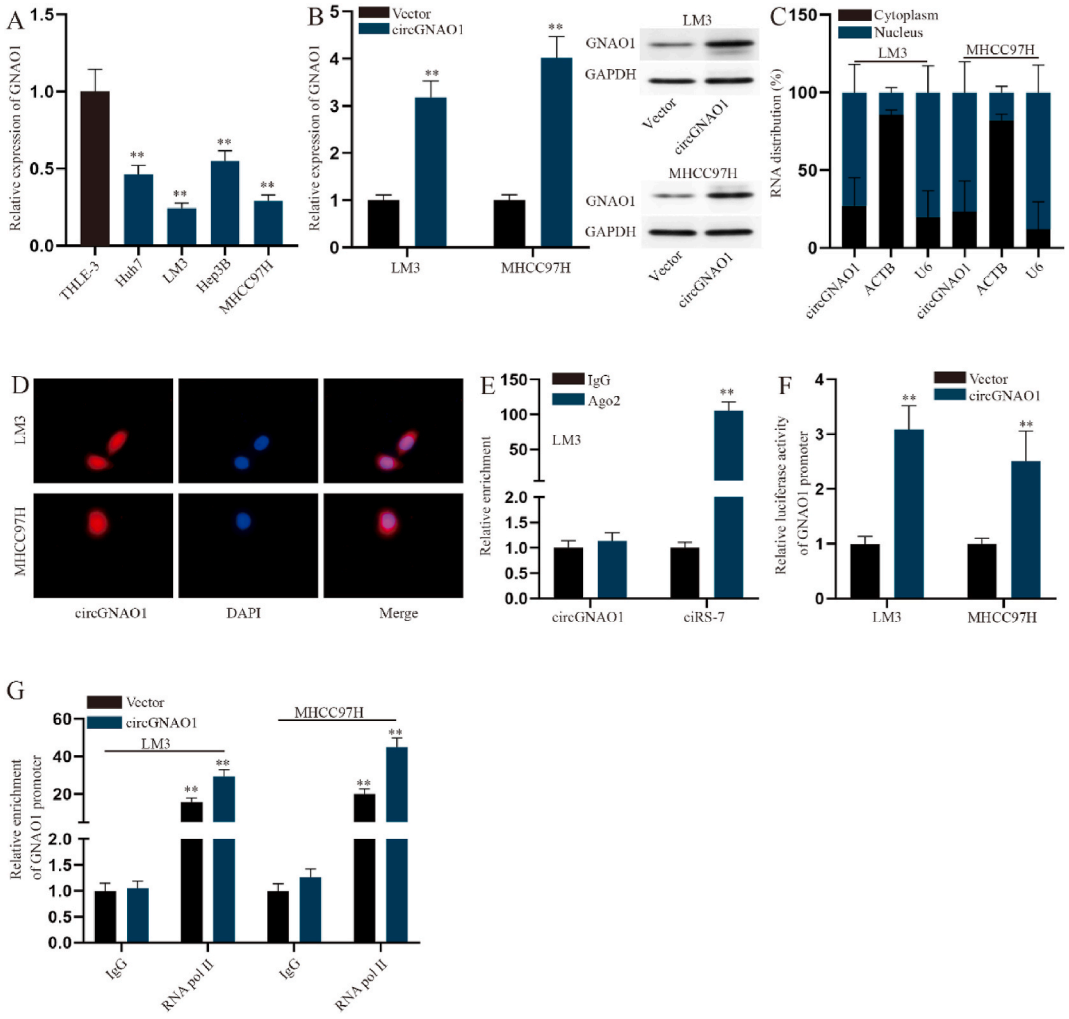

Fig. 4. CircGNAO1 positively regulates its host gene GNAO1 expression at the transcription level in HCC cells. (A) RT-qPCR analysis detected GNAO1 expression level in Huh7, LM3, Hep3B, MHCC97H and THLE-3 cells. GNAO1 expression was significantly downregulated in HCC cells compared with THLE-3 cells. (B) RT-qPCR and Western blot analyses explored the effect of circGNAO1 enhancement on the mRNA and protein level of GNAO1. Overexpression of circGNAO1 upregulated the mRNA and protein expression of GNAO1. (C–D) The localization of circGNAO1 in HCC cells was unveiled by FISH and subcellular fractionation assays. circGNAO1 was mainly localized in the nucleus. (E) RIP assays were used to detect the enrichment of circGNAO1 and ciRS-7 in Ago2 group. No significant increase in circGNAO1 enrichment was observed in the Ago2 group compared to the IgG group, while the positive control ciRS-7 showed a significant abundance in the Ago2 group compared to the IgG group. (F) The effect of circGNAO1 overexpression on GNAO1 transcription was explored by luciferase reporter assays. Overexpression of circGNAO1 enhanced the luciferase activity of the GNAO1 promoter. (G) The effect of circGNAO1 up-regulation on GNAO1 transcription was detected by ChIP assays. circGNAO1 overexpression significantly enhanced the abundance of GNAO1 promoter precipitated by RNA pol II. **P < 0.01.

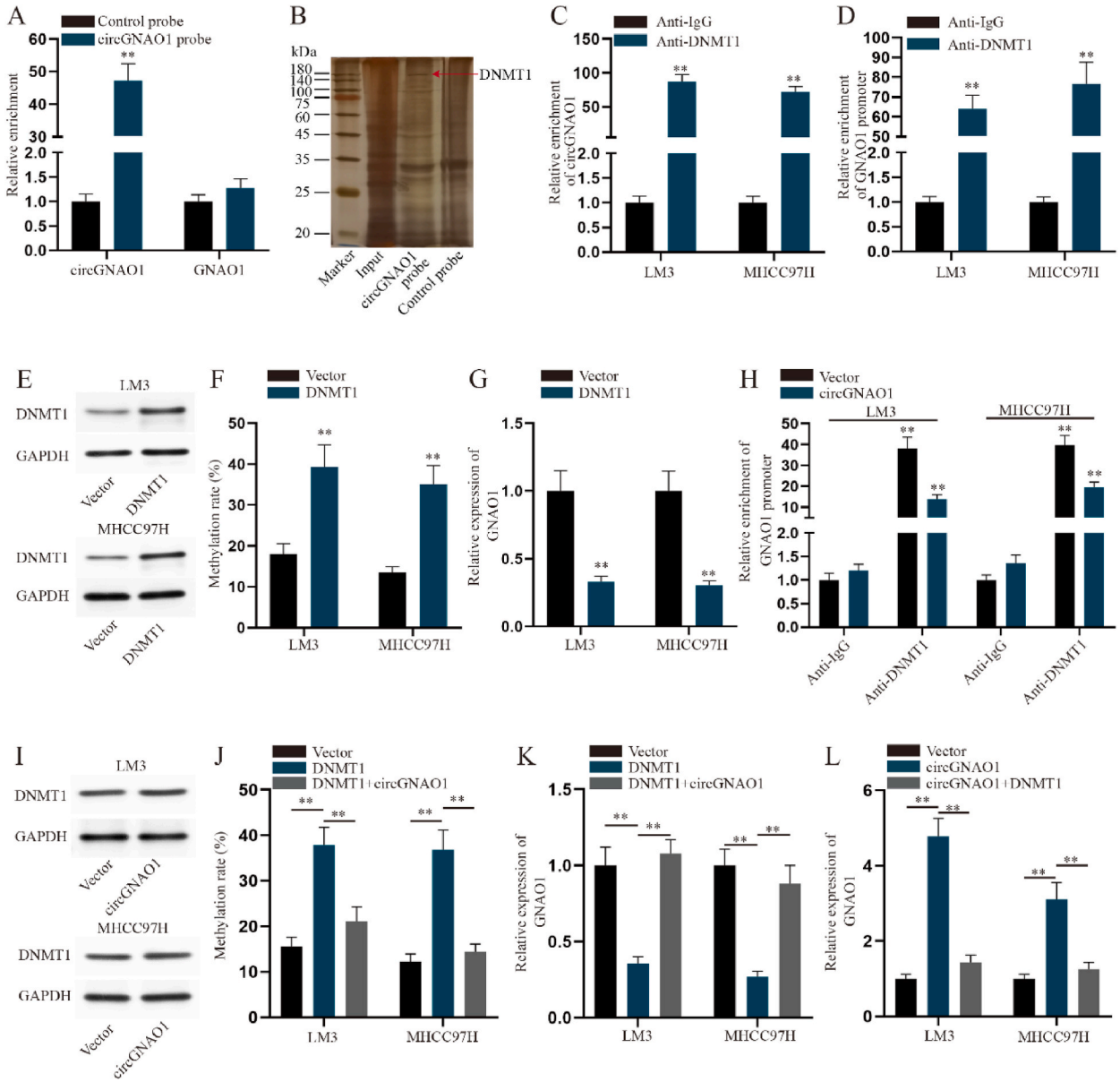

Fig. 5. CircGNAO1 impedes the inhibition of DNMT1 on GNAO1 transcription. (A) RNA pulldown assays investigated the interaction between circGNAO1 probe and circGNAO1/GNAO1. circGNAO1 probe specifically pulled down circGNAO1. (B) RNA pulldown assays explored the interacting proteins of circGNAO1. DNMT1 was determined as the RBP of circGNAO1 through silver staining and mass spectrometry (183 kDa). (C) The combination between circGNAO1 and DNMT1 was analyzed by RIP assays. circGNAO1 was dramatically enriched in Anti-DNMT1 groups compared to control. (D) ChIP assays certified the binding between DNMT1 and GNAO1 promoter. (E) The overexpression efficiency of DNMT1 overexpression vector was proved by Western blot analysis. (F) BSP analysis detected the influence of DNMT1 enhancement on the methylation of GNAO1 promoter. DNMT1 overexpression promoted the methylation of GNAO1 promoter. (G) RT-qPCR analyzed the impact of DNMT1 overexpression on the expression of GNAO1. Overexpression of DNMT1 promoted the expression of GNAO1. (H) ChIP assays explored the influence of circGNAO1 upregulation on the binding between DNMT1 and GNAO1 promoter. Overexpression of circGNAO1 reduced the enrichment of the GNAO1 promoter in the Anti-DNMT1 group. (I) Western blot analysis detected the effect of circGNAO1 overexpression on DNMT1 level. Overexpression of circGNAO1 had no significant effect on the protein level of DNMT1. (J) BSP analysis was performed to assess the impact of different conditions (Vector, DNMT1 overexpression vector, DNMT1 overexpression vector + circGNAO1 overexpression vector) on the methylation of the GNAO1 promoter. Overexpression of circGNAO1 reversed the promoting effect of overexpressed DNMT1 on the methylation of the GNAO1 promoter. (K) RT-qPCR analysis was performed to assess the impact of different conditions (Vector, DNMT1 overexpression vector, DNMT1 overexpression vector + circGNAO1 overexpression vector) on the expression of GNAO1. Overexpression of circGNAO1 reversed the inhibitory effect of overexpressed DNMT1 on the expression of GNAO1. (L) GNAO1 expression was disclosed by RT-qPCR analysis under different conditions (Vector, circGNAO1 overexpression vector, circGNAO1 overexpression vector + DNMT1 overexpression vector). Overexpression of DNMT1 reversed the promoting effect of circGNAO1 overexpression on the expression of GNAO1. **P < 0.01.

and normal cell lines (Fig. 4A). The results unveiled that GNAO1 was lowly expressed in HCC cell lines relative to that in the normal control. RT-qPCR and Western blot analyses were then implemented to examine the influence of circGNAO1 on GNAO1. We found that circGNAO1 overexpression increased both GNAO1 mRNA and protein levels (Fig. 4B). As exhibited by the subcellular fractionation and FISH assays, circGNAO1 was located both in the cytoplasm and nucleus of HCC cells, but mainly in HCC cell nucleus (Fig. 4C and D). CircRNA was reported to work as a miRNA sponge and affect target gene expression [23]. Therefore, we tried to figure out whether circGNAO1 influences GNAO1 expression in this way using Ago2 RIP assay. It was revealed that no remarkable up-regulation of circGNAO1 enrichment could be found in Ago2 group relative to IgG group, while the positive control ciRS-7 was significantly abundant in Ago2 group relative to IgG group (Fig. 4E). The results in Fig. 4E excluded the possibility that circGNAO1 could act as a miRNA sponge to regulate GNAO1. Since nuclear circRNA mainly affects the transcription of target genes [24,25], we conducted the luciferase reporter assays to unclose the relation between circGNAO1 and GNAO1 promoter (Fig. 4F). It was unearthed that circGNAO1 overexpression enhanced the luciferase activity of GNAO1 promoter, verifying the binding between circGNAO1 and GNAO1 promoter. Besides, ChIP assays uncovered that circGNAO1 overexpression significantly enhanced the abundance of GNAO1 promoter precipitated by RNA pol II (Fig. 4G). The results in Fig. 4F and G indicated that circGNAO1 propels GNAO1 transcription. Overall, circGNAO1 positively regulates the expression of its host gene GNAO1 at the transcription level.

3.3. CircGNAO1 sequesters DNMT1 to enhance the transcription of GNAO1 and up-regulate GNAO1 expression

Next, we investigated the specific molecular mechanism of circGNAO1 in regulating GNAO1 expression in HCC. After designing circGNAO1 probe and the control probe, we implemented RNA pulldown assay and certified that circGNAO1 probe could specifically pull down circGNAO1 while GNAO1 was enriched in neither circGNAO1 probe nor the control probe (Fig. 5A). This proved the specificity of circGNAO1 probe. Afterwards, RNA pulldown assay followed by silver staining was carried out to disclose the interacting protein of circGNAO1. In accordance with the results of mass spectrometry, DNMT1 was specifically pulled down by circGNAO1 probe (Fig. 5B), indicating that DNMT1 is the potential interacting protein of circGNAO1 in HCC. RIP assay verified the combination between circGNAO1 and DNMT1, as circGNAO1 was dramatically enriched in Anti-DNMT1 groups compared to control (Fig. 5C). Given that circRNAs can combine with RNA-binding proteins (RBPs) to modulate the expression of target genes [26], in this study, DNMT1 might also serve as the RBP of circGNAO1 to influence the transcription of GNAO1. Moreover, previous study has reported that DNMT1-induced hypermethylation of GNAO1 promoter triggers the inhibition of GNAO1 transcription, leading to the down-regulation of GNAO1 level in HCC [19]. Thereof, we speculated that circGNAO1 might bind to DNMT1 to impede the binding between DNMT1 and GNAO1 promoter, resulting in the hypomethylation of GNAO1 promoter to promote the transcription of GNAO1. To verify this hypothesis, we firstly performed ChIP assays to elucidate the binding between DNMT1 and GNAO1 promoter. As shown in Fig. 5D, GNAO1 promoter was highly abundant in Anti-DNMT1 groups, which verified that DNMT1 binds to GNAO1 promoter. The efficiency of overexpression vector of DNMT1 was examined by Western blot (Fig. 5E). Then, BSP was conducted to detect the effect of DNMT1 on the promoter methylation of GNAO1. As shown in Fig. 5F, DNMT1 promoted the promoter methylation of GNAO1. According to RT-qPCR analysis, up-regulated DNMT1 diminished the mRNA levels of GNAO1 (Fig. 5G). The results in Fig. 5D–G indicated that DNMT1 binds to GNAO1 promoter and induces the promoter hypermethylation of GNAO1 to down-regulate GNAO1 expression.

Furthermore, we investigated the effect of circGNAO1 on the binding between DNMT1 and GNAO1 promoter. ChIP assays showed that the abundance of GNAO1 promoter in Anti-DNMT1 group was largely reduced by overexpressed circGNAO1 (Fig. 5H), indicating that circGNAO1 inhibits the binding between DNMT1 and GNAO1 promoter. As displayed in Western blot analysis, circGNAO1 had no marked effect on DNMT1 expression (Fig. 5I), suggesting that circGNAO1 directly inhibits the binding between DNMT1 and GNAO1 promoter. Furthermore, BSP analysis results revealed that overexpression of DNMT1 promoted the methylation of the GNAO1 promoter, but this promoting effect was reversed by the overexpression of circGNAO1 (Fig. 5J). RT-qPCR analysis indicated that DNMT1 overexpression inhibited GNAO1 expression, but circGNAO1 overexpression reversed this inhibition (Fig. 5K). Also, based on RT-qPCR analysis, DNMT1 overexpression could rescue the promoting effect of up-regulated circGNAO1 on GNAO1 expression (Fig. 5L). Taken together, circGNAO1 sequesters DNMT1 to promote the transcription of GNAO1 and up-regulate GNAO1 expression.

4. Discussion

Former study unclosed that GNAO1 is down-regulated in HCC tissues, and it hampers proliferation and facilitates senescence of HCC cells [18]. In the present study, we found that GNAO1 is low-expressed in HCC tissues and cell lines, which is consistent with the former study. Via bioinformatics and relevant experiments, we identified the target circRNA of GNAO1 as hsa_circ_0039422 (written as circGNAO1). The positive correlation between circGNAO1 and its host gene GNAO1 was also validated. As GNAO1 has been reported to modulate HCC cell proliferation and senescence, we wondered whether GNAO1-derived circGNAO1 could also exert the same functions in HCC cells. Moreover, emerging evidence supports that circRNAs play regulatory roles in cancer biology, including HCC [27]. For example, cSMARCA5 inhibits cell growth and cell migration in HCC [28]; circRHOT1 hampers HCC development and progression [29]; circ_0001955 promotes HCC tumorigenesis [30]. In this study, we carried out the gain-of-function assays to determine the functions of circGNAO1 in HCC cells. By performing CCK-8, EdU, flow cytometry and SA-β-gal staining assays, we proved that circGNAO1 suppresses cell proliferation, induces cell cycle arrest and facilitates cellular senescence.

Next, we further investigated the molecular mechanism of circGNAO1 underlying HCC regulation. In recent years, increasing attention has been focused on the circRNA-related ceRNA network (i.e. circRNA-miRNA-mRNA axis) and the interaction between circRNA and its host gene [31,32]. Given that, we turned to concentrate on the interaction between circGNAO1 and GNAO1 in HCC. We found that circGNAO1 facilitates the progression of HCC via modulating GNAO1 through rescue experiments. However, despite the

existence in both the nucleus and cytoplasm of HCC cells, we conducted Ago2 RIP assay and found that circGNAO1 cannot work as miRNA sponge to regulate GNAO1 expression level in ceRNA network.

Herein, DNMT1 was validated as the RBP of circGNAO1 in HCC through RNA pulldown and RIP assays. Through RIP, BSP and RT-qPCR, we verified that DNMT1 binds to GNAO1 promoter and induces GNAO1 promoter hypermethylation, causing the down-regulation of GNAO1 in HCC. The relationship between DNMT1 and GNAO1 proved in our report is in line with previous study [19]. Previous studies have reported that circRNAs can act as RBP sponges to modulate target gene expression [33]. In this study, we found that circGNAO1 directly inhibits the binding between DNMT1 and GNAO1 promoter and even can completely reverse the promoting effect of DNMT1 overexpression on GNAO1 promoter methylation. In the meantime, the promoting effect of up-regulated circGNAO1 on GNAO1 expression can be thoroughly countervailed by DNMT1 overexpression. The above results proved that circGNAO1 sequesters its RBP DNMT1 to facilitate the transcription and expression of GNAO1 in HCC.

Nevertheless, the current study is subject to some limitations. First of all, the downstream target of GNAO1 still requires further investigations. Moreover, owing to shortage of materials, we couldn't conduct animal assays to explore the influence of circGNAO1 on xenografts. In the future study, we will carry out relevant assays to explore the functions of circGNAO1 *in vivo*, and the downstream mechanisms of GNAO1 in HCC.

In conclusion, circGNAO1 strengthens its host gene GNAO1 expression for suppression of hepatocarcinogenesis. Our study firstly probed into whether GNAO1-derived circRNA existed in the upstream of GNAO1 and played a role in regulating HCC progression via interacting with GNAO1. Furthermore, for the first time, we verified that circGNAO1 propelled the transcription of GNAO1 by impeding the DNMT1-induced methylation of GNAO1 promoter. The findings in this study might offer new perspectives into HCC treatment.

CRediT authorship contribution statement

Hongwei He: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Qing Zhang:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis. **Qiyun Gu:** Writing – review & editing, Writing – original draft, Software, Resources, Investigation, Data curation. **Hui Yang:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Conceptualization. **Caibin Yue:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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