

Circular RNA 0001666 inhibits colorectal cancer cell proliferation, invasion and stemness by inactivating the Wnt/ β -catenin signaling pathway and targeting microRNA-1229

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Abstract. A previous bioinformatics study suggested that circular RNA 0001666 (circ_0001666) and its target microRNA (miR)-1229 were associated with colorectal cancer (CRC) pathogenesis. However, the role of this interaction in the regulation of CRC cell malignancy remains unclear. Thus, the aim of the present study was to examine the interaction between circ_0001666 and miR-1229, and its effects on CRC cell malignancy. circ_0001666 overexpression or knockdown plasmids were transfected into the HT-29 and HCT-116 cell lines. In addition, in rescue experiments, circ_0001666 or miR-1229 overexpression plasmids were transfected into the HT-29 cell line, either alone or in combination. Following transfection, cell proliferation, apoptosis, invasion and the number of CD133⁺ cells were analyzed. The protein expression level of proteins in the Wnt/ β -catenin pathway was also examined. In both HT-29 and HCT-116 cell lines, circ_0001666 overexpression increased apoptosis, whilst inhibiting cell proliferation and invasion, and reducing the frequency of CD133⁺ cells. By contrast, circ_0001666 knockdown reduced apoptosis, but increased cell proliferation and the number of CD133⁺ cells. However, cell invasion remained unaffected. In addition, circ_0001666 expression levels negatively regulated those of miR-1229, whereas miR-1229 expression did not

affect circ_0001666, in both the HT-29 and HCT-116 cell lines. Furthermore, a luciferase reporter assay confirmed that miR-1229 directly bound to circ_0001666. In the HT-29 cell line, miR-1229 overexpression activated the Wnt/ β -catenin pathway, and promoted cell proliferation, invasion and stemness, while suppressing cell apoptosis. In addition, miR-1229 overexpression reversed the effects of circ_0001666 overexpression. In conclusion, circ_0001666 suppresses CRC cell proliferation, invasion and stemness by inhibiting the Wnt/ β -catenin signaling pathway by targeting miR-1229, and may represent a potential target for CRC treatment.

Introduction

Colorectal carcinoma (CRC) is the fourth leading cause of cancer-related mortality worldwide, accounting for ~900,000 deaths annually in 2018 (1). The pathogenesis of CRC starts as low-grade dysplasia and often progresses slowly to form a polyp in the colorectal lumen, which eventually develops into a tumor with considerable size and symptoms (including pain, cramping and bleeding) (2). Therefore, most patients with CRC are at an advanced or metastatic stage when diagnosed, at which point tumor cells have already invaded into regional lymph nodes and spread into distal organs (such as the liver, lung and pancreas), which accounts for the major comorbidity in CRC (3,4). To overcome the aforementioned problems and develop therapeutic interventions, understanding the mechanism of CRC carcinogenesis (such as cell viability, invasion and stemness) is necessary.

Circular (circ)RNA is a type of non-coding RNA molecule, with a circular structure, which participates in gene regulation under both physiological and pathological conditions (5,6). Previous studies have demonstrated that circRNA molecules [such as circular homeodomain interacting protein kinase (circ-HIPK) 3, circular SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 5 (circ-SMARCA5), circ_0026416 and circ_103809] were associated with CRC pathogenesis (7-10). circ_0001666, a recently identified circRNA derived from the circulation of FAM120B gene, regulates multiple microRNA (miRNA/miR) targets and

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Abbreviations: CRC, colorectal carcinoma; circRNAs, circular RNAs; miRNAs, microRNAs; NC, negative control; CD133⁺, CD133 positive; RT-qPCR, reverse transcription-quantitative PCR; WT, wild-type; MT, mutant type; ceRNA, competitive endogenous RNA

Key words: colorectal cancer, circ_0001666, miR-1229, Wnt/ β -catenin pathway, cell malignant behaviors

downstream genes, such as ETS variant transcription factor 4, to promote papillary thyroid carcinoma cell proliferation and migration (11). Furthermore, a previous bioinformatics study demonstrated that, in CRC, circ_0001666 targeted miR-1229 as part of a circRNA/miRNA network (12). In addition, miR-1229 has been reported to regulate the Wnt/ β -catenin signaling pathway to promote the malignancy of several cancer cell types. The Wnt/ β -catenin signaling pathway is a key signaling pathway associated with oncogenesis, including in breast cancer, liver cancer and CRC (10,13-15). Based on these aforementioned studies, it may be hypothesized that circ_0001666 regulates the Wnt/ β -catenin signaling pathway via miR-1229 in CRC. However, to the best of our knowledge, this hypothesis has yet to be investigated.

The aim of the present study was to examine the interaction between circ_0001666, miR-1229 and the Wnt/ β -catenin signaling pathway, as well as the potential effect of this network on cell viability, invasion and stemness in CRC.

Materials and methods

Cell culture and reagents. The human CRC cell lines (HT-29, HCT-116, SW480 and SW-620) and the human intestinal epithelial cell line (HIEC) (https://web.expasy.org/cellosaurus/CVCL_6C21) were purchased from American Type Culture Collection. All the cell lines used in the current study were authenticated using short tandem repeat profiling. The cells were cultured in McCoy's 5a medium (HyClone; Cytiva), containing 10% FBS (HyClone; Cytiva). The circ_0001666 and negative control (NC) overexpression plasmids were constructed using a pCD5-ciR backbone, and were purchased from Guangzhou Genesee Biotech Co., Ltd. The circ_0001666, miR-1229 and NC knockdown plasmids were constructed using a pGPH1 backbone purchased from Shanghai GenePharma Co., Ltd. In addition, the NC and miR-1229 overexpression plasmids were constructed using a pGPU6 backbone purchased from Shanghai GenePharma Co., Ltd. TRIzol[®], Lipofectamine[®] 3000 Transfection reagent and PVDF Membranes were purchased from Thermo Fisher Scientific, Inc. RNase R was purchased from Epicentre[®] Biotechnologies (Illumina, Inc.). The reverse transcription (RT) kit and Fast quantitative (q)PCR Mix were purchased from Takara Biotechnology Co., Ltd. The Cell Counting Kit (CCK)-8, One Step TUNEL Apoptosis Assay kit, RIPA lysis buffer, BCA Protein assay kit, BeyoECL kit, 293T cells, pGL6 plasmid and Luciferase Reporter Gene Assay kit were purchased from Beyotime Institute of Biotechnology. The 293T cell line was cultured in DMEM containing 10% FBS (both from HyClone; Cytiva). The Matrigel[®] Basement Membrane Matrix and Transwell chambers were purchased from BD Biosciences. The antibodies were purchased from Abcam.

circ_0001666 plasmid transfection. The 0.8 μ g pCD5-ciR-NC (empty vector) (16), 0.8 μ g pCD5-ciR-circ (the forward cloning primer, 5'-CGGAATTCAGTCTATACTCTTGTCTTGTGA-3' and reverse, 5'-CGGGATCCAGCAACATGCGTACCAC-3'; expected product length, 13,733 bp), 0.8 μ g pGPH1-NC (5'-CACCGAGTGAAACAGTGCAGCTGCGAACAGCTGCACTGTTTAC-3') and 0.8 μ g pGPH1-circ

(5'-CACCGATGACCATTCAGATCCTTTTCGAAAAAGGATCTGGAATGGTCATC-3') plasmids were mixed with Lipofectamine[®] 3000 and transfected into the HT-29 and HCT-116 cell lines for 6 h at 37°C. Untransfected HT-29 and HCT-116 cell lines were also used as a control. At 24 h post-transfection, circ_0001666 and miR-1229 expression, cell proliferation, apoptosis and invasion, as well as the number of CD133⁺ cells were evaluated.

miR-1229 plasmid transfection. The 0.8 μ g pGPU6-NC (5'-AACACCGAACGAGACAGGATT-3'), 0.8 μ g pGPU6-miR (5'-CTCTCACCCTGCTCCCTCCACAG-3'), 0.8 μ g pGPH1-NC (5'-AAGAACAACACAAAAGAACAG-3') and 0.8 μ g pGPH1-miR (5'-CTGTGGGAGGGCAGTGGTGAGAG-3') plasmids were transfected into the HT-29 and HCT-116 cell lines using Lipofectamine[®] 3000 for 6 h at 37°C, according to the manufacturer's instructions. Untransfected HT-29 and HCT-116 cell lines were also used as a control. At 24 h post-transfection, circ_0001666 and miR-1229 expression was evaluated.

Rescue experiments. For the rescue experiments, the following co-transfections were performed in the HT-29 cell line: i) 0.8 μ g pCD5-ciR-NC + 0.8 μ g pGPU6-NC; ii) 0.8 μ g pCD5-ciR-circ plasmid + 0.8 μ g pGPU6-NC; iii) 0.8 μ g pCD5-ciR-NC + 0.8 μ g pGPU6-miR; and iv) 0.8 μ g pCD5-ciR-circ + 0.8 μ g pGPU6-miR. To perform these transfections, Lipofectamine[®] 3000 was used according to the manufacturer's protocol for 6 h at 37°C. At 24 h post-transfection, circ_0001666 and miR-1229 expression, cell proliferation, apoptosis and invasion, as well as the number of CD133⁺ cells were evaluated.

RT-qPCR. The cells were collected 48 h following transfection for RT-qPCR. Total RNA was isolated from the cells using TRIzol. Subsequently, for the determination of circ_0001666 expression levels, total RNA was digested using RNase R. The RT reagent kit was then used to reverse transcribe RNA to cDNA with thermal cycling conditions of 37°C for 15 min and 85°C for 5 sec. qPCR was performed using the TB Green Fast qPCR Mix (Takara Biotechnology Co., Ltd.) with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions for qPCR were as follows: 95°C for 30 sec for 1 cycle; followed by 95°C for 5 sec and 61°C for 15 sec for 40 cycles. The 2^{- $\Delta\Delta$ C_q} method was used to calculate the relative expression levels of the targets (17). The following primer sequences were used: i) circ_0001666 forward, 5'-TCTACTCACTCTTACTGGAGGACTG-3' and reverse, 5'-CTTGCTTCGGTGGTGCTCTG-3'; expected product length, 252 bp; ii) GAPDH forward, 5'-GAGTCCACTGGCGTCTTCAC-3' and reverse, 5'-ATCTTGAGGCTGTTGTCATACTTCT-3'; expected product length, 149 bp; iii) miR-1229 forward, 5'-ACACTCCAGCTGGGCTCTCACCCTGCCT-3' and reverse, 5'-TGTCGTGGAGTCTGGCAATTC-3'; expected product length, 64 bp; and iv) U6 forward, 5'-GCTCGCTTCGGCAGCACATA-3' and reverse, 5'-AATATGGAACGCTTCACGAATTTGC-3'; expected product length, 102 bp. The detailed procedure of designing circ_0001666 primers were in accordance with a previous study (18). In addition, the forward and reverse primers for miR-1229 were design according to previous studies (19,20).

Western blot analysis. The cells were collected 48 h following transfection for western blot analysis and was performed using RIPA lysis buffer, BCA protein assay kit and BeyoECL kit, as described in a previous study (21). The following antibodies were used: i) Anti-GSK3 β (rabbit; monoclonal; 1:10,000; cat. no. ab32391); ii) anti-phosphorylated (p)-GSK3 β (rabbit; monoclonal; 1:15,000; cat. no. ab131097); iii) anti- β -catenin (rabbit; monoclonal; 1:5,000; cat. no. ab32572); iv) anti-GAPDH (rabbit; polyclonal; 1:3,000; cat. no. ab9485); v) anti-C-X-C motif chemokine receptor 5 (CXCR5; rabbit; monoclonal; 1:1,000; cat. no. ab254415); and vi) HRP-conjugated goat anti-rabbit IgG H&L (1:10,000; cat. no. ab181662). ImageJ (version 1.8.0; National Institutes of Health) was used for semi-quantification and analysis of the western blot bands.

Cell proliferation assay. CCK-8 assay solution was added and incubated with the cells 0, 24, 48 and 72 h post-transfection. After a 2-h incubation, the optical density was measured using a microplate reader.

Apoptosis detection. Apoptosis was detected using a One Step TUNEL Apoptosis assay kit 48 h following transfection. Briefly, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton X-100 for 5 min at room temperature, incubated with TUNEL solution at 37°C for 60 min, then counterstained with 0.5 μ g/ml DAPI for 5 min at room temperature. The cells were then observed under an inverted fluorescence microscope and the TUNEL-positive cells were counted. The stained cells were counted in five fields of view.

Matrigel assay. A total of 5×10^4 cells were harvested 48 h following transfection and counted. The cells in FBS-free McCoy's 5a medium were then seeded in a Transwell chamber (cat. no. 353097, Corning, Inc.) pre-coated with Matrigel Basement Membrane Matrix (37°C for 60 min) and incubated for 24 h at 37°C. The lower chamber was filled with 10% FBS-containing McCoy's 5a medium. After being fixed with 4% paraformaldehyde for 30 min at room temperature, the cells on the bottom surface of the chamber were stained with crystal violet for 30 min at room temperature and counted under a light microscope. The stained cells were counted in five fields of view.

Flow cytometry. The cells were harvested and stained in the dark for 60 min on ice with Alexa Fluor[®] 488-conjugated rabbit monoclonal antibody against CD133 (cat. no. ab271092; 1:2,500) or Alexa Fluor[®] 488-conjugated rabbit IgG isotype control (cat. no. ab172730; 1:500) 48 h following transfection. The numbers of CD133⁺ cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and Flowjo 7.6 (BD Biosciences). A general viable cell gate was used to exclude apoptotic and dying cells, and to distinguish cells based on size and granularity (22).

Luciferase reporter gene assay. The binding site of circ_001666 and miR-1229 was predicted by RNAhybrid (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid>). The wild-type (WT) or mutant type (MT) circ_0001666 DNA fragments were cloned into the pGL6 plasmid. The plasmid constructs were co-transfected with pGPU6-NC/pGPU6-miR into the 293T cell line

(American Type Culture Collection) using Lipofectamine[®] 3000, according to the manufacturer's instructions. The cells were then lysed at 48 h after transfection and luciferase activity was detected using a Luciferase Reporter Gene Assay kit. The luciferase activity of the circ_0001666 WT/pGPU6 group was set as 1. The luciferase activity was normalized to *Renilla* luciferase activity. The 293T cells were cultured in 10% FBS-containing DMEM (HyClone; Cytiva) under 5% CO₂ at 37°C.

Fluorescence in situ hybridization (FISH). FISH was used to detect the location of circ_0001666 expression in the CRC cell lines and to visualize circ_0001666 and miR-1229 binding. Briefly, the CRC cell lines were fixed at room temperature using 4% paraformaldehyde solution for 10 min at room temperature. Subsequently, the cells were permeabilized by 0.5% Tween X-100 at 4°C for 5 min and hybridized with 50 pM Cy3-labeled circ_0001666 probes (5'-AGATGACCATTCCAGATCCTT-3') (Sangon Biotech Co., Ltd.) overnight at 37°C. After incubation, the cells were washed by 0.1% Tween 20 containing 4X saline-sodium citrate (SSC) for 5 min at 42°C, 2X SSC for 5 min at 42°C and 1X SSC for 5 min at 42°C, successively. DAPI was added for nuclear staining at room temperature for 10 min and the images were visualized under a fluorescence microscope. All procedures were conducted according to the Ribo[™] Fluorescent In Situ Hybridization kit instructions (Guangzhou RiboBio Co., Ltd.).

Statistical analysis. All the data are presented as the mean \pm SD and were analyzed using GraphPad Prism v7.01 (GraphPad Software, Inc.). To compare the differences between multiple groups one way ANOVA followed by Tukey's post hoc test was used. All experiments were conducted in triplicate. P<0.05 was considered to indicate a statistically significant difference.

Results

circ_0001666 expression following transfection. circ_0001666 mRNA expression level was reduced, while miR-1229 mRNA expression level was increased in the CRC cell lines compared with that in the HIEC cell line (Fig. S1). The HT-29 and HCT-116 CRC cell lines were transfected with pCD5-ciR-circ, pCD5-ciR-NC, pGPH1-circ or pGPH1-NC plasmids. In both the cell lines, the relative mRNA expression levels of circ_0001666 were upregulated in the pCD5-ciR-circ group compared with that in the pCD5-ciR-NC group (both P<0.001). In addition, relative mRNA expression levels of circ_0001666 were downregulated in the pGPH1-circ group compared with that in the pGPH1-NC group (both P<0.01) (Fig. 1). In addition, FISH was performed to determine the cellular localization of circ_0001666. The results demonstrated that circ_0001666 was expressed intracellularly in the CRC cell lines (Fig. S2).

circ_0001666 suppresses CRC cell proliferation, apoptosis and invasion. In the HT-29 cell line, circ_0001666 overexpression reduced cell proliferation at 48 (P<0.05) and 72 h (P<0.01), as well as the number of invading cells (P<0.01). In addition, it also promoted apoptosis, as evidenced by the number of TUNEL-positive cells (P<0.001; Fig. 2A-E). However,

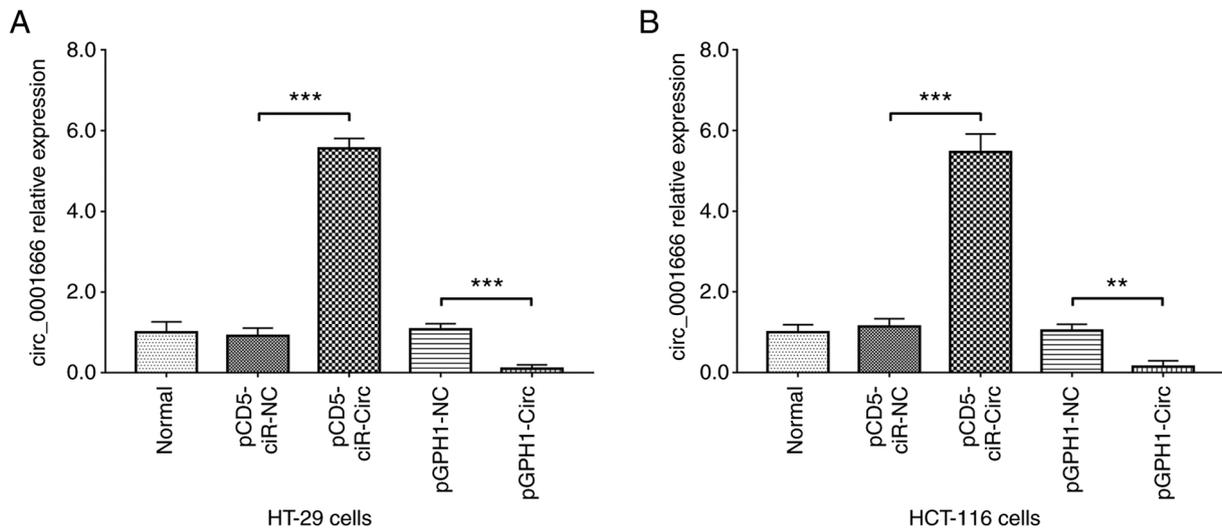


Figure 1. circ_0001666 expression following circ_0001666 overexpression or knockdown. Relative mRNA expression levels of circ_0001666 following transfection with circ_0001666 overexpression or knockdown plasmids in (A) HT-29 and (B) HCT-116 cell lines. ** $P < 0.01$, *** $P < 0.001$. circ, circular RNA; NC, negative control. pCD5-ciR-Circ represented circ_0001666 overexpression plasmids, pCD5-ciR-NC represented overexpression NC plasmids. pGPH1-Circ represented circ_0001666 knockdown plasmids, pGPH1-NC represented knockdown NC plasmids.

circ_0001666 knockdown had the opposite effect on proliferation and apoptosis; however, it did not affect cell invasion. In addition, the same results were observed in the HCT-116 cell line following circ_0001666 overexpression and knockdown (Fig. 2F-J). These data suggested that circ_0001666 inhibited cell proliferation and invasion, whilst promoting apoptosis, in the CRC cell lines.

circ_0001666 inhibits cancer stemness. In the HT-29 cell line, the numbers of CD133⁺ cells were reduced following circ_0001666 overexpression ($P < 0.01$), but was increased following knockdown of expression ($P < 0.05$) (Fig. 3A and B). Furthermore, the same results were observed in the HCT-116 cell line (Fig. 3C and D). The forward-side scatter cryptogram of CD133⁺ cell is displayed in Fig. S3. As CD133 is a biomarker for cancer stem cells, these findings indicated that circ_0001666 inhibited cancer stemness in the CRC cell lines (23).

circ_0001666 directly binds to miR-1229. In both the HT-29 and HCT-16 cell lines, circ_0001666 overexpression downregulated miR-1229 mRNA expression level, while knockdown of circ_0001666 expression upregulated miR-1229 mRNA expression level (both $P < 0.01$) (Fig. 4A and C). In addition, miR-1229 mRNA expression level was increased in the HT-29 and HCT-16 cell lines following transfection with miR-1229 overexpression plasmid, while miR-1229 expression level was decreased in the HT-29 and HCT-16 cell lines following transfection with miR-1229 knockdown plasmid, indicating a successful transfection (all $P < 0.01$) (Fig. S4A and B). However, neither miR-1229 overexpression nor knockdown affected circ_0001666 expression (both $P > 0.05$) (Fig. 4B and D). Furthermore, the results from a luciferase reporter assay and FISH suggested that miR-1229 could bind to circ_0001666 (Figs. 4E and F, and S5). These data suggested that circ_0001666 negatively regulated miR-1229 expression level; however, miR-1229 expression

did not affect the expression level of circ_0001666 in the CRC cell lines.

Analysis of circ_0001666, miR-1229, CXCR5 and the Wnt/ β -catenin pathway in rescue experiments. To determine whether circ_0001666 regulated CRC cell function by targeting miR-1229, rescue experiments were performed. The transfection efficiencies of each plasmid in these experiments are shown in Fig. 5A and B. Western blot analysis indicated that, in the HT-29 cell line, miR-1229 overexpression reduced p-GSK-3 β protein expression levels and increased those of β -catenin. In addition, it also attenuated the effect of circ_0001666 overexpression on the Wnt/ β -catenin pathway (Fig. 5C and D). These data suggested that circ_0001666 regulated the Wnt/ β -catenin pathway by targeting miR-1229 in CRC cells.

To investigate the effect of circ_0001666 and miR-1229 on CXCR5, the mRNA and protein expression level of CXCR5 in rescue experiments was determined. In the HT-29 cell line, circ_0001666 suppressed CXCR5 mRNA and protein expression level, while miR-1229 increased CXCR5 mRNA and protein expression level. In addition, miR-1229 attenuated the effect of circ_0001666 overexpression on regulating CXCR5 mRNA and protein expression level (Fig. S6). This data suggested that circ_0001666 regulated the expression level of miR-1229, which was mediated by CXCR5 in the CRC cell lines.

Cell proliferation, apoptosis, invasion and stemness in rescue experiments. In the HT-29 cell line, miR-1229 overexpression promoted cell proliferation at 48 ($P < 0.05$) and 72 h ($P < 0.01$), the numbers of invading cells ($P < 0.01$), and the number of CD133⁺ cells ($P < 0.01$). By contrast, miR-1229 overexpression inhibited apoptosis, as evidenced by the number of TUNEL-positive cells ($P < 0.01$; Fig. 6). In addition, miR-1229 overexpression abolished the effect of circ_0001666 overexpression on CRC cell proliferation,

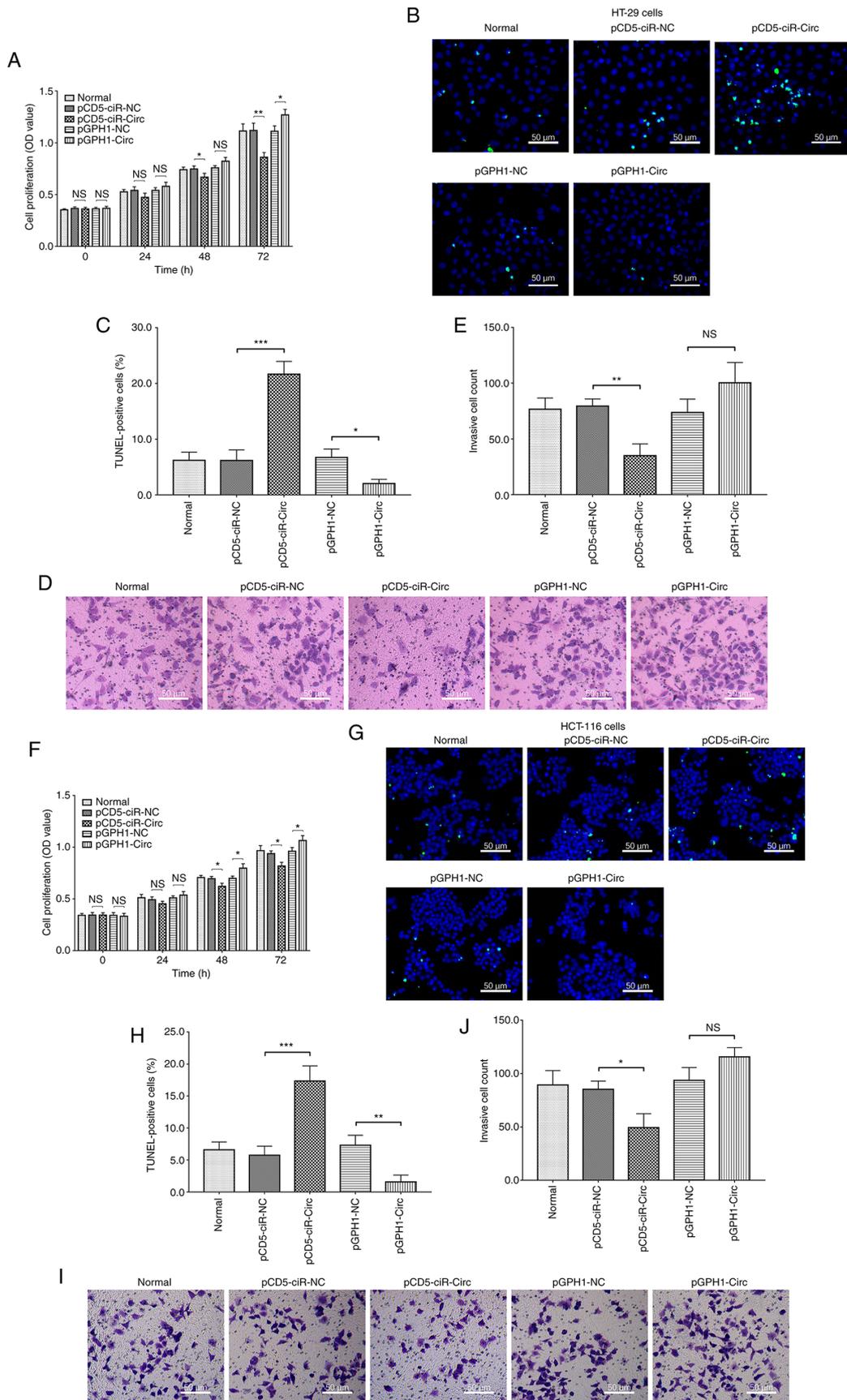


Figure 2. Cell proliferation, apoptosis and invasion following circ_0001666 overexpression or knockdown. (A) Proliferation in the HT-29 cells following transfection. (B and C) Cell apoptosis rate and (D and E) the number of invasive cells in the HT-29 cell line following transfection. (F) Proliferation in the HCT-116 cell line following transfection. (G and H) Apoptosis rate and (I and J) the number of invasive cells in the HCT-116 cell line following transfection. *P<0.05, **P<0.01, ***P<0.001. NS, not significant; circ, circular RNA; NC, negative control. pCD5-ciR-Circ represented circ_0001666 overexpression plasmids, pCD5-ciR-NC represented overexpression NC plasmids. pGPH1-Circ represented circ_0001666 knockdown plasmids, pGPH1-NC represented knockdown NC plasmids.

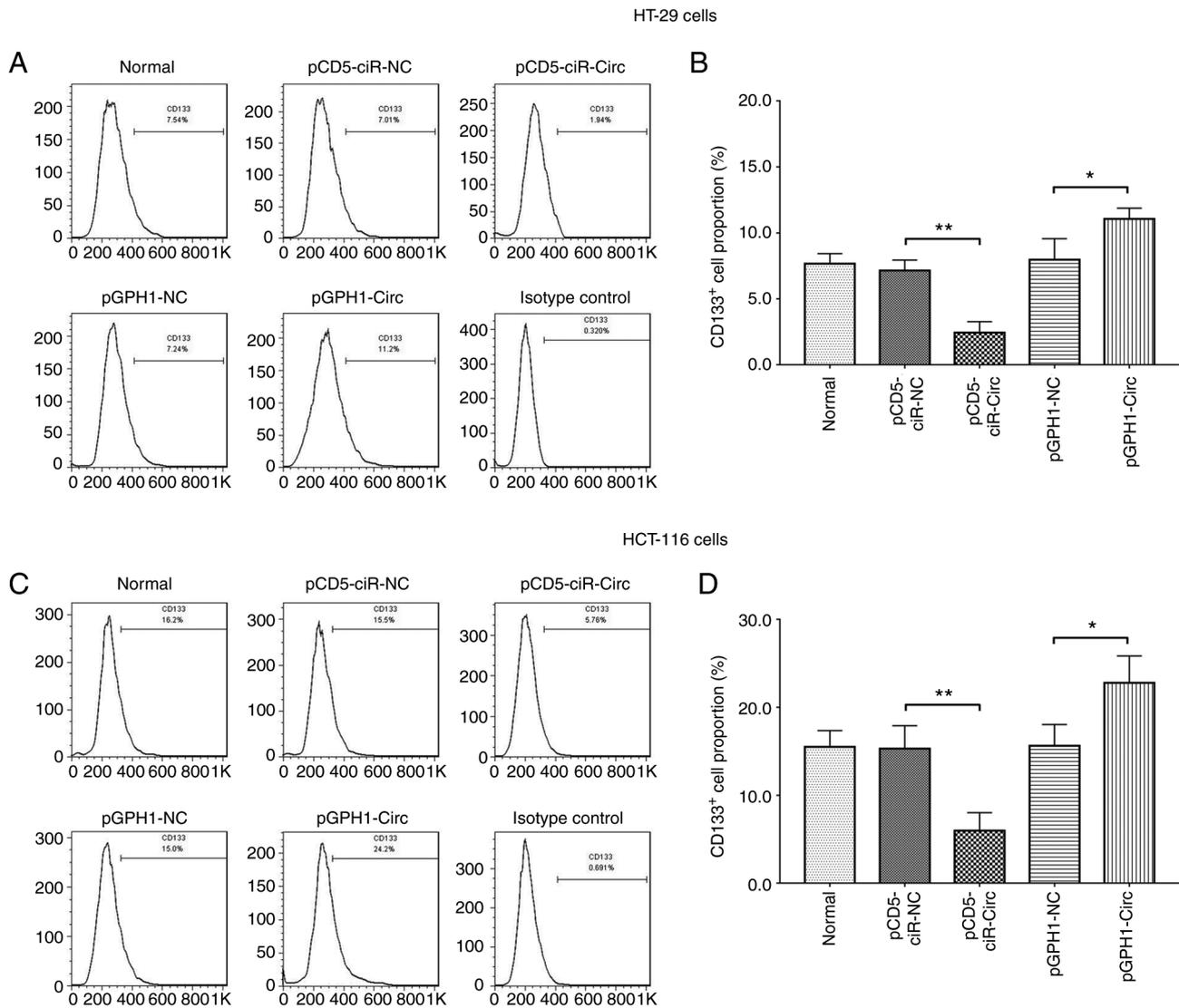


Figure 3. Number of CD133+ cells following circ_0001666 overexpression or knockdown. Number of CD133+ cells in (A and B) the HT-29 and (C and D) HCT-116 cell lines following transfection. * $P < 0.05$, ** $P < 0.01$. circ, circular RNA; NC, negative control. pCD5-ciR-Circ represented circ_0001666 overexpression plasmids, pCD5-ciR-NC represented overexpression NC plasmids. pGPH1-Circ represented circ_0001666 knockdown plasmids, pGPH1-NC represented knockdown NC plasmids.

apoptosis, invasion and stemness (Fig. 6). These data suggested that circ_0001666 regulated CRC cell malignancy by targeting miR-1229.

Discussion

CRC places a huge medical burden across the world. The majority of patients with CRC are diagnosed at an advanced stage and miss the therapeutic window (2,3). Even after aggressive management, multiple recurrence and metastasis are often reported in patients with CRC resulting in an unfavourable survival profile (4). To further improve the CRC prognosis and individualize the management options are still a critical issue for clinicians.

Understanding the role of circRNA tumorigenicity for therapeutic intervention has attracted increased attention from researchers. Previous studies have demonstrated that several circRNA molecules were associated with CRC tumorigenesis by modulating cell proliferation and migration. For example,

circ-HIPK3 suppressed miR-7 mRNA expression level to promote proliferation and migration in CRC cells (9). In addition, circ_0026416 regulated the mRNA expression level of miR-346 and nuclear factor IB to promote proliferation and migration in CRC cells (8). By contrast, other circRNA molecules may serve as tumor suppressors in CRC. For example, circ-SMARCA5 was found to suppresses the mRNA expression level of miR-39-3p, which resulted in reduced CRC cell proliferation and migration (7). Another circRNA, circ_103809, served as a sponge for miR-532-3p, leading to inhibition of cell proliferation and migration in CRC cells (10). Altogether, this indicates that several circRNA molecules have been associated with CRC tumorigenesis and may serve as potential therapeutic targets.

As a newly identified circRNA, few studies have described the role of circ_0001666 in carcinogenesis, to the best of our knowledge. An *in vitro* study suggested that circ_0001666 regulated the expression levels of several miRNA targets (including miR-330-5p, miR-193a-5p and miR-326) to promote papillary

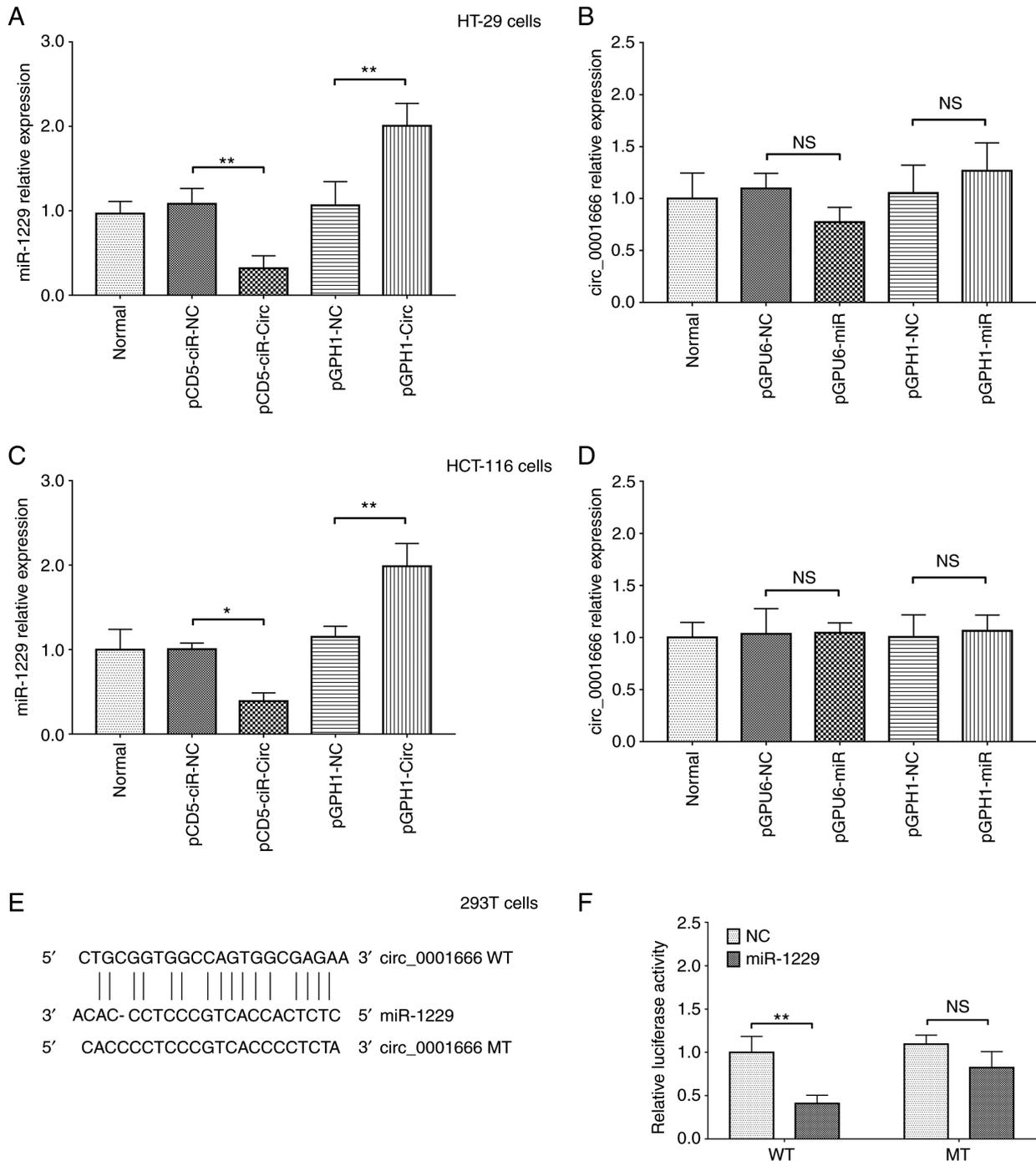


Figure 4. Regulatory mechanism between circ_0001666 and miR-1229. miR-1229 mRNA expression levels in the (A) HT-29 and (C) HCT-116 cell lines following circ_0001666 overexpression and knockdown. circ_0001666 mRNA expression level in the (B) HT-29 and (D) HCT-116 cell lines following miR-1229 overexpression and knockdown. (E) Binding sequences between circ_0001666 and miR-1229. (F) Effect of miR-1229 on the luciferase activity of circ_0001666 WT and circ_0001666 MT in the 293T cell line. *P<0.05, **P<0.01. NS, not significant; WT, wild-type; MT, mutant; circ, circular RNA; NC, negative control; miR, microRNA. pCD5-ciR-Circ represented circ_0001666 overexpression plasmids, pCD5-ciR-NC represented overexpression NC plasmids. pGPH1-Circ represented circ_0001666 knockdown plasmids, pGPH1-miR represented miR-1229 knockdown plasmids, pGPH1-NC represented knockdown NC plasmids. pGPU6-miR represented miR-1229 overexpression plasmids, pGPU6-NC represented overexpression NC plasmids.

thyroid carcinoma cell proliferation and migration (11). In addition, another study identified an immune-related risk signature of CRC, in which circ_0001666 was a critical component of a circRNA/miRNA/hub gene network (24). Furthermore, according to a bioinformatics study, circ_0001666 and miR-1229 may be involved in CRC pathogenesis (12). Despite these bioinformatics studies and the finding that circ_0001666 is part of an important competing endogenous RNA (ceRNA) network

in CRC, the molecular mechanism underlying the role of circ_0001666 in CRC carcinogenesis has not been determined.

The results from the present *in vitro* study suggested that circ_0001666 inhibited cell proliferation, invasion and stemness in CRC. A possible reason to explain this result is that circ_0001666 overexpression reduces β -catenin protein expression, which, in turn, would increase the protein expression levels of E-cadherin, a key protein in intercellular

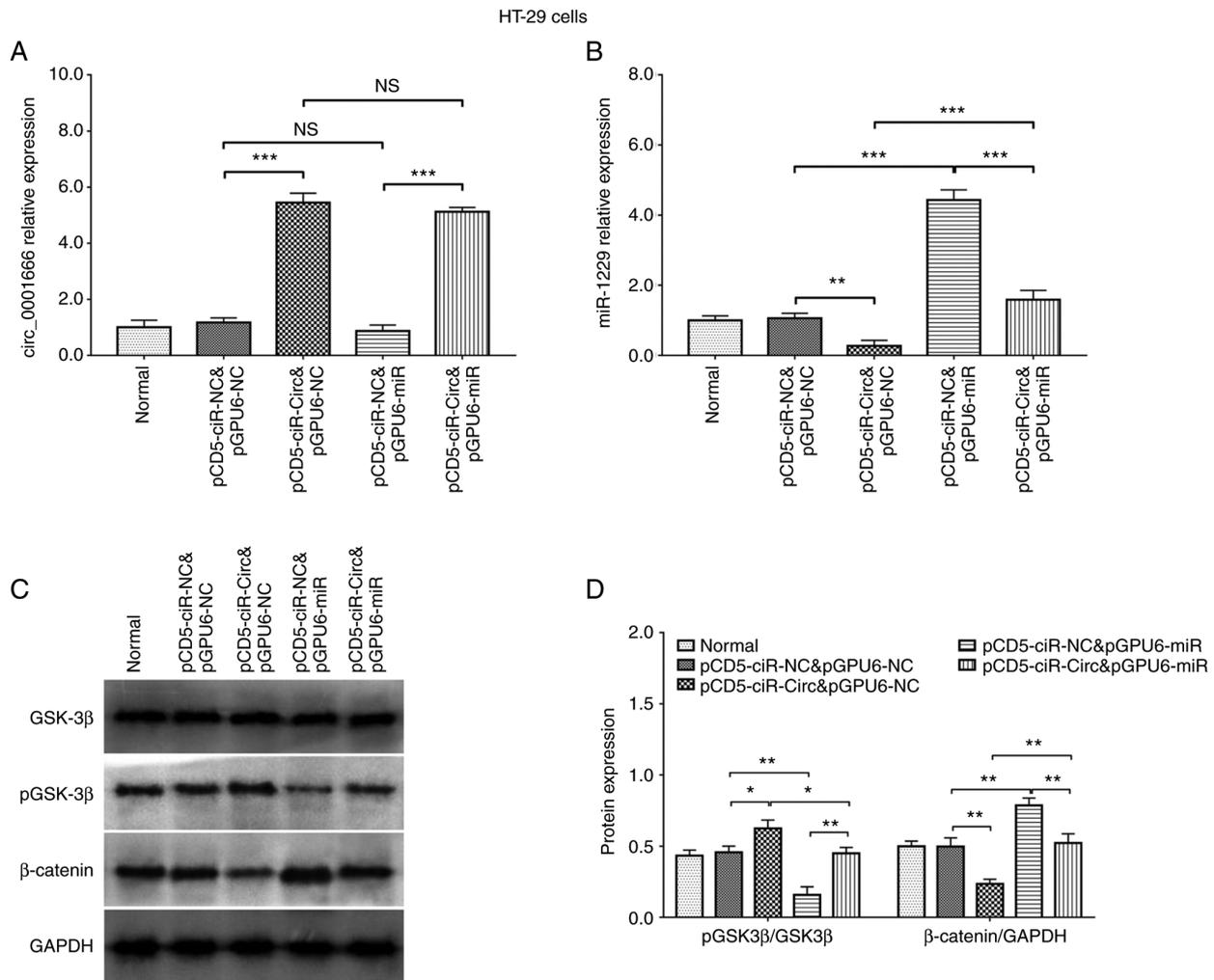


Figure 5. Analysis of circ_0001666 and miR-1229 expression, and the Wnt/ β -catenin pathway in rescue experiments. (A) circ_0001666 and (B) miR-1229 mRNA expression level and (C and D) protein expression level of proteins in the Wnt/ β -catenin signaling in the HT-29 cells line in rescue experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NS, not significant; p, phosphorylated; miR, microRNA; circ, circular RNA; NC, negative control. pCD5-ciR-Circ represented circ_0001666 overexpression plasmids, pCD5-ciR-NC represented overexpression NC plasmids. pGPU6-miR represented miR-1229 overexpression plasmids, pGPU6-NC represented overexpression NC plasmids.

junctions (25). Therefore, increased E-cadherin protein expression would lead to tightening of intercellular junctions, and eventually prevent local invasion and metastasis of CRC cells (25). It was also hypothesized that overexpression of circ_0001666 decreases cancer stemness, as evidenced by the reduced numbers of CD133⁺ cells in the present study; further experiments are required to validate this. Therefore, circ_0001666 overexpression would lead to reduced tumor cell formation and reduced tumor cell numbers.

A major role of circRNA molecules is to serve as ceRNA, which inhibit target miRNA expression and downstream gene regulation (26). miRNA is a type of small non-coding RNA involved in multiple cellular processes, including cell proliferation, differentiation and apoptosis (27). Accumulating evidence suggests that miRNA molecules are also involved in tumorigenesis, including CRC pathogenesis. A previous study demonstrated that miR-1229 was a target of circ_0001666, based on a circRNA/miRNA network analysis of CRC (12). In addition, miR-1229 promoted breast cancer cell proliferation and tumor growth by activating the Wnt/ β -catenin signaling pathway (13). Furthermore, an *in vivo* study into

CRC suggested that miR-1229 regulated the protein expression levels of HIPK2 and promoted angiogenesis, highlighting its oncogenic role in CRC (21). In the present study, miR-1229 promoted CRC cell proliferation, invasion and stemness.

Wnt/ β -catenin is a signaling pathway with a critical role in cancer biology (10). Wnt binds to the surface receptor Frizzled and its coreceptor low-density lipoprotein receptor-related protein group 5/6, causing β -catenin to dissociate from its degradation complex, leading to transactivation of target genes (14). In addition to its function in normal physiological conditions, Wnt/ β -catenin also plays a critical role in CRC pathogenesis by promoting cell proliferation, modulating epithelial-to-mesenchymal transition, disrupting intercellular junctions and regulating angiogenesis (14,15). A previous study suggested that miR-1229 regulated the Wnt/ β -catenin signaling pathway to induce breast cancer cell malignancy (13). In the present study, circ_0001666 regulated the Wnt/ β -catenin signaling pathway by targeting miR-1229 in the CRC cell lines. This finding could be explained as follows: miR-1229 overexpression upregulated p-GSK-3 β protein expression levels and p-GSK-3 β targeted β -catenin to regulate its activity (10). In

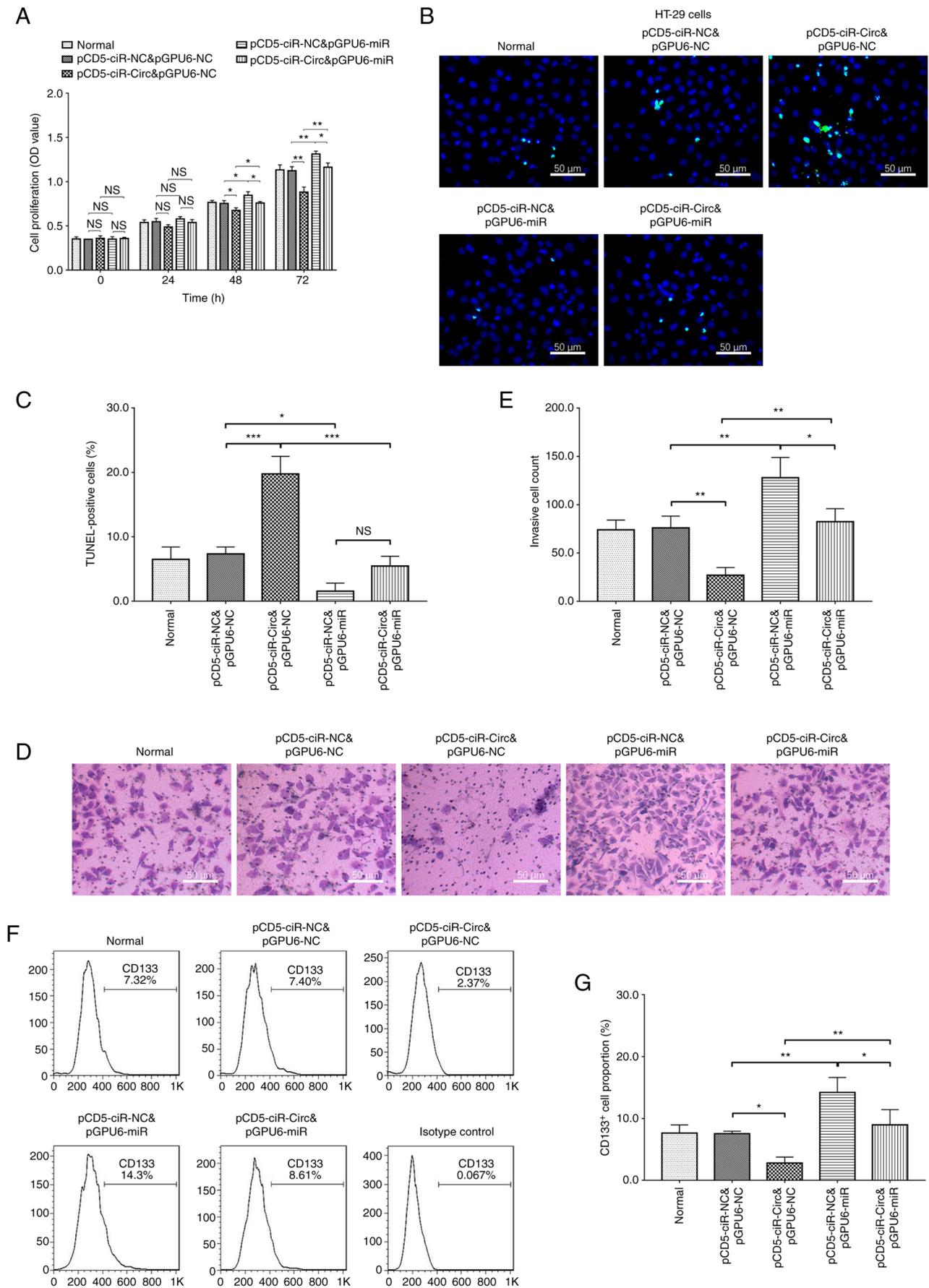


Figure 6. Cell proliferation, apoptosis, invasion and stemness in rescue experiments. (A) Cell proliferation, (B and C) apoptosis and (D and E) invasion in rescue experiments in the HT-29 cell line. (F and G) Number of CD133⁺ cells in rescue experiments in the HT-29 cell line. *P<0.05, **P<0.01, ***P<0.001. NS, not significant; miR, microRNA; circ, circular RNA; NC, negative control. pCD5-ciR-Circ represented circ_0001666 overexpression plasmids, pCD5-ciR-NC represented overexpression NC plasmids. pGPU6-miR represented miR-1229 overexpression plasmids, pGPU6-NC represented overexpression NC plasmids.

addition, circ_0001666 overexpression resulted in the regulation of β -catenin protein expression. miR-1229 overexpression promoted cell proliferation, invasion and stemness to a greater extent than circ_0001666 overexpression in the CRC cell lines. These data suggested that the circ_0001666/miR-1229/ β -catenin signaling pathway may represent a potential area for metastatic CRC therapeutic intervention.

There are several limitations in the current study. Firstly, circ_0001666 and miR-1229 mRNA expression levels were not evaluated in human CRC samples. Secondly, RNA immunoprecipitation experiment was not performed to determine the binding between circ_0001666 and miR-1229 in the CRC cell lines.

In conclusion, circ_0001666 suppressed CRC cell proliferation, invasion and stemness by targeting miR-1229 and the Wnt/ β -catenin signaling pathway, which may represent potential targets for CRC treatment.

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Availability of data and materials

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

Authors' contributions

ZY conceived and designed the study. FB and CZ performed the experiments and data analysis. YO and KX contributed to acquisition of data and interpretation of data. ZY and FB confirm the authenticity of the raw data. ZH was involved in data interpretation, and wrote the manuscript. All authors reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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