MicroRNA-340 promotes the tumor growth of human gastric cancer by inhibiting cyclin G2

GUOZHI YIN[1], HUAYOU ZHOU[2], YUMO XUE[1], BOWEN YAO[1] and WEI ZHAO[3]

[1]Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061;
[2]Department of General Surgery, Hanzhong Central Hospital, Hanzhong 723000; [3]Department of General Surgery,
The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, P.R. China

Abstract. Aberrant expression and function of microRNAs (miRNAs) play a critical role in the development and progression of various human cancers including gastric cancer. However, the clinical significance and underlying mechanisms of miR-340 remain largely unknown in gastric cancer. In the present study, we demonstrated that the expression of miR-340 was aberrantly elevated in both gastric cancer tissues and cells. Moreover clinical association analyses disclosed that the elevated level of miR-340 was significantly associated with unfavorable clinicopathological characteristics of the gastric cancer patients, such as poor differentiation, large tumor size and advanced tumor-node-metastasis (TNM) stage. Gastric cancer patients with high expression of miR-340 had prominently shorter overall survival and disease-free survival. Functionally, forced expression of miR-340 promoted cell viability, proliferation, colony formation and cell cycle progression in the SGC-7901 cells, while miR-340 silencing reduced cell viability, proliferation, colony formation and cell cycle progression in MGC-803 cells. Furthermore, *in vivo* experiments indicated that miR-340 knockdown suppressed the tumor growth of MGC-803 cells. Notably, alteration of miR-340 expression affected the luciferase activity of wild-type 3'-UTR of cyclin G2 (CCNG2) and regulated CCNG2 abundance in gastric cancer cells, indicating that CCNG2 is a direct target of miR-340. Moreover, CCNG2 knockdown eradicated the effects of miR-340 silencing on gastric cancer cells. In conclusion, our data suggest that miR-340 may potentially serve as a novel prognostic biomarker and therapeutic target for gastric cancer.

Correspondence to: Dr Wei Zhao, Department of General Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an 710061, P.R. China
E-mail: jdyfy_weiz@163.com

Introduction

Gastric cancer is the second leading cause of cancer-related deaths worldwide (1). Although remarkable progress has been made in the treatment of gastric cancer, the prognosis of gastric cancer patients remains poor (2). The main reasons for the unsatisfactory prognosis of gastric cancer patients include lack of symptoms for patients in the early stages and malignant growth and systemic metastasis for patients in the advanced stages (3). Therefore, it is of great importance to elucidate the molecular mechanisms involved in the development and progression of gastric cancer, which may contribute to the identification of novel biomarkers and therapeutic targets, thus promoting the early diagnosis and more effective treatment of gastric cancer.

MicroRNAs (miRNAs) are a group of endogenous short non-coding nucleotide RNAs which modulate the expression of downstream targets by binding to complementary sites within the 3'-untranslated region (3'-UTR) of target mRNAs (4). It has been well-recognized that miRNAs play crucial roles in various biological processes, including cell proliferation, apoptosis, and metastatic activity (5). Recently, various studies (6,7) have confirmed that miRNAs play critical roles in the development and progression of gastric cancer. The expression status, clinical significance and the biological function of miRNAs in gastric cancer remains largely unknown.

MicroRNA-340 (miR-340) has been found to play a critical role in human cancers, including breast (8,9), prostate (10,11), and esophageal cancer (12), hepatocellular carcinoma (13) and glioma (14). Functionally, miR-340 was found to inhibit the migration and invasion of breast cancer cells by inhibiting the Wnt pathway (8). In prostate cancer, miR-340 was found to inhibit cell proliferation and promote cell apoptosis by inhibiting high-mobility group nucleosome-binding domain 5 (11). Studies of glioma have shown that miR-340 inhibited the stem-like cell function by targeting tissue plasminogen activator (14). Notably, the expression of miR-340 has been found highly expressed in gastric cancer tissues (15,16). miR-340 can promote gastric cancer cell growth and reduce cell apoptosis effectively (17). However, the clinical significance and underlying mechanisms of miR-340 in human gastric cancer remain unknown.

In this study, miR-340 expression was found to be aberrantly increased in human gastric cancer tissues and cells. Elevated levels of miR-340 were found to be correlated with adverse clinicopathological features and poor prognosis of gastric cancer patients. Functionally, miR-340 promoted cell viability, proliferation, colony formation and cell cycle progression by targeting cyclin G2 (CCGN2).

Materials and methods

Clinical specimens and cell culture. Tumor tissues and the adjacent non-cancer tissues were obtained from 80 gastric cancer patients who received surgical resection at the Department of General Surgery, of the First Affiliated Hospital of Xi'an Jiaotong University. All clinical specimens were frozen and stored at -80°C. The patients did not receive any chemotherapy or radiotherapy before surgery. The demographic and clinicopathological features of the 80 patients are presented in Table I. All protocols involving clinical samples were approved by the Ethics Committee of Xi'an Jiaotong University according to the Declaration of Helsinki (as revised in Tokyo 2004).

Human gastric cancer cell lines (SGC-7901, MGC-803, MKN-45, and AGS) and a normal gastric epithelium cell line (GES-1) were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences. All cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen Life Technologies) at 37°C with 5% CO_2.

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated from the gastric cancer tissues and cells using TRIzol (Invitrogen Life Technologies) reagent. The TaqMan Human MiRNA Assay kit (Applied Biosystems, Foster City, CA, USA) and a SYBR® Premix Ex Taq™ II kit (Takara Bio, Shiga, Japan) were used for the PCR amplification. Primers for miR-340 (HmiRQP0434), CCGN2 (HQP021882), U6 (HmiRQP9001) and GAPDH (HQP006347) were purchased from Genecopoeia, Inc. (Guangzhou, China). U6 was used as an internal control for the relative expression level of miR-340, while GAPDH was used as the internal control for the relative expression of CCNG2.

Cell transfection. The plasmids and siRNA used in this study include miR-340 mimics (HmiR0090-MR04) and control vector (CmiR0001-MR03), miR-340 inhibitors (HmiR-AN0434-AM03) and negative control vector (CmiR-AN0001-AM03; all from Genecopoeia, Inc.) and CCNG2 siRNA (SR300634) and scrambled negative control siRNA (SR30004; both from OriGene, Technologies, Inc., Beijing, China). These vectors aforementioned were transfected into the gastric cancer cells with Lipofectamine 2000 (Invitrogen Life Technologies) based on the manufacturer's protocol.

Western blot analysis. RIPA buffer was used to extract the total protein from the gastric cancer cells, and 20-30 μg of isolated protein was separated by 10% SDS-PAGE and transferred onto 0.22-μm NC membranes (Sigma-Aldrich, St. Louis, MO,

Table I. The clinical significance of miR-340 expression in gastric cancer.

Clinicopathologic features	Total no. of patients (n=80)	miR-340		P-value
		Low n	High n	
Age (years)				
<65	44	24	20	0.369
≥65	36	16	20	
Gender				
Male	55	27	28	0.809
Female	25	13	12	
Histology				
Well, moderate	36	13	23	0.025[a]
Poor, signet	44	27	17	
Tumor Size (cm)				
<5	33	10	23	0.003[a]
≥5	47	30	17	
Depth of invasion				
T_1	15	7	8	0.775
T_2-T_4	65	33	32	
Lymph node metastasis				
Absent	29	12	17	0.245
Present	51	28	23	
Lymphatic invasion				
Absent	26	10	16	0.152
Present	54	30	24	
Venous infiltration				
Absent	56	25	31	0.143
Present	24	15	9	
TNM stage				
I, II	43	14	29	0.001[a]
III, IV	37	26	11	

[a]Statistically significant. T_1, mucosa (m), submucosal (sm); T_2-T_4, muscularis propria (mp), subserosa (ss), serosa exposed (se), serosa infiltrating (si); TNM, tumor-node-metastasis.

USA). After membrane transfer, the membranes were incubated with the GAPDH and CCNG2 antibodies (both from Cell Signaling Technology, Inc., Danvers, MA USA), overnight at 4°C. Then, the membranes were incubated with the secondary goat anti-mouse or anti-rabbit IgG antibody (ZSGB-BIO Co., Ltd., Beijing, China). GAPDH was used as control.

MTT, deoxyuridine (BrdU) incorporation and colony formation assays. For assessment of cell viability, gastric cancer cells ($4x10^3$) were seeded into 96-well plates and stained with sterile MTT for 4 h at 37°C, following which the culture medium
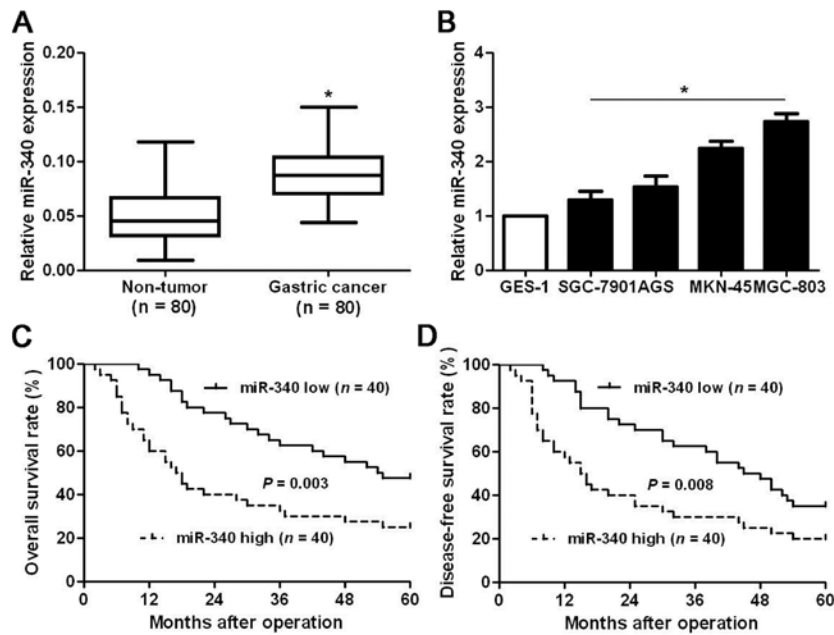
Figure 1. The expression status and prognostic value of miR-340 in gastric cancer. (A) Relative miR-340 expression levels in gastric cancer tissues and paired adjacent normal tissues were determined by qRT-PCR. (B) Expression levels of miR-340 in the gastric cancer cell lines was significantly increased compared to that in the GES-1 cells. *P<0.05. (C and D) Gastric cancer patients with high expression of miR-340 had poorer overall survival and disease-free survival.

was discarded and an extra 150 μl DMSO (both from Sigma-Aldrich) were then added into each well. The absorbance at 490 nm was examined 24, 48 and 72 h after transfection.

For assessment of proliferation, BrdU incorporation and colony formation assays were performed. Regarding the BrdU incorporation assay, cells after transfection were incubated with BrdU and then were stained with anti-BrdU antibody (Sigma-Aldrich) according to the manufacturer's instructions. Regarding the colony formation assay, gastric cancer cells were seeded on 6-well plates. Two weeks after cell seeding, the colonies were stained with 1% crystal violet and the number of colonies was counted.

Cell cycle assays. For the cell cycle analysis, 48 h after transfection, the gastric cancer cells were collected, washed with PBS, and fixed with 80% ethanol overnight at 4˚C. Then, the cells were incubated with RNaseA for 30 min at 37˚C, followed by incubation with propidium iodide (both from Sigma-Aldrich) for 20 min at room temperature. Next, the cells were subjected to flow cytometric analysis using a FACSCalibur (BD Biosciences, Bedford, MA, USA).

Tumor formation assay in a nude mouse model. Female BALB/c nude mice, 4-6 weeks old were used to establish the nude mouse xenograft model. MGC-803 cells transfected with miR-340 inhibitor or negative control vectors were suspended in 100 μl of PBS and were injected subcutaneously into the flank of each nude mouse. Tumor volumes were determined by measuring two of its dimensions with calipers every 3 days, and the equation for calculating the tumor volume was V = 0.5 x D x d² (V, volume; D, longitudinal diameter; d, latitudinal diameter). All nude mice were sacrificed at 3 weeks after the injection of MGC-803 cells. All *in vivo* protocols were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

Luciferase reporter assay. The wild-type 3'-UTR sequence of CCNG2 or the mutated sequence within the predicted target sites was synthesized and inserted into the pGL3 control vector (Promega Corp., Madison, WI, USA), to construct the wt CCNG2-3'-UTR or mt CCNG2-3'-UTR, respectively. Then, SGC-7901 cells were seeded into 24-well plates, and were cultured in OptimMEM reduced serum media (Invitogen Life Technologies), and were cotransfected with each luciferase reporter construct (the wild-type or mutant 3'-UTR of CCNG2) and miR-340 overexpression vector using FuGENE (Promega Corp). Forty-eight hours after transfection, SGC-7901 cells were harvested and luciferase activity was measured using the dual-luciferase reporter assay system (Promega Corp.). Results were obtained from three independent experiments performed in triplicate.

Statistical analysis. Data are presented as the mean ± SEM. GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. The differences between two or more groups were compared with a two-tailed Student's t-test or ANOVA. Pearson's correlation analysis was used to analyze the clinical correlation. To compare the difference of patient survival between two groups, the Kaplan-Meier method and the log-rank test were performed. Differences were considered statistically significant at P<0.05.

Results

miR-340 is aberrantly upregulated in gastric cancer tissues and cell lines. First, we measured the expression level of miR-340 in gastric cancer tissues and matched tumor-adjacent tissues with qRT-PCR. The results showed that miR-340 expression in gastric cancer tissues was significantly higher than that in the non-tumor tissues (P<0.05, Fig. 1A). As compared with GES-1 cells, miR-340 was upregulated in a

Table II. Multivariate Cox regression analysis of the 5-year overall and disease-free survival of 80 gastric cancer patients.

Variables	Overall survival			Disease-free survival		
	HR	95% CI	P-value	HR	95% CI	P-value
Histology	0.557	0.319-0.971	0.039[a]	0.686	0.390-1.205	0.190
Tumor Size (cm)	2.344	1.482-3.706	<0.001[a]	1.355	0.825-2.225	0.229
TNM stage	3.188	1.964-5.175	<0.001[a]	2.660	1.588-4.457	<0.001[a]
miR-340 expression	2.269	1.472-3.498	0.001[a]	1.604	1.011-2.543	0.045[a]

[a]Statistically significant. HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis.
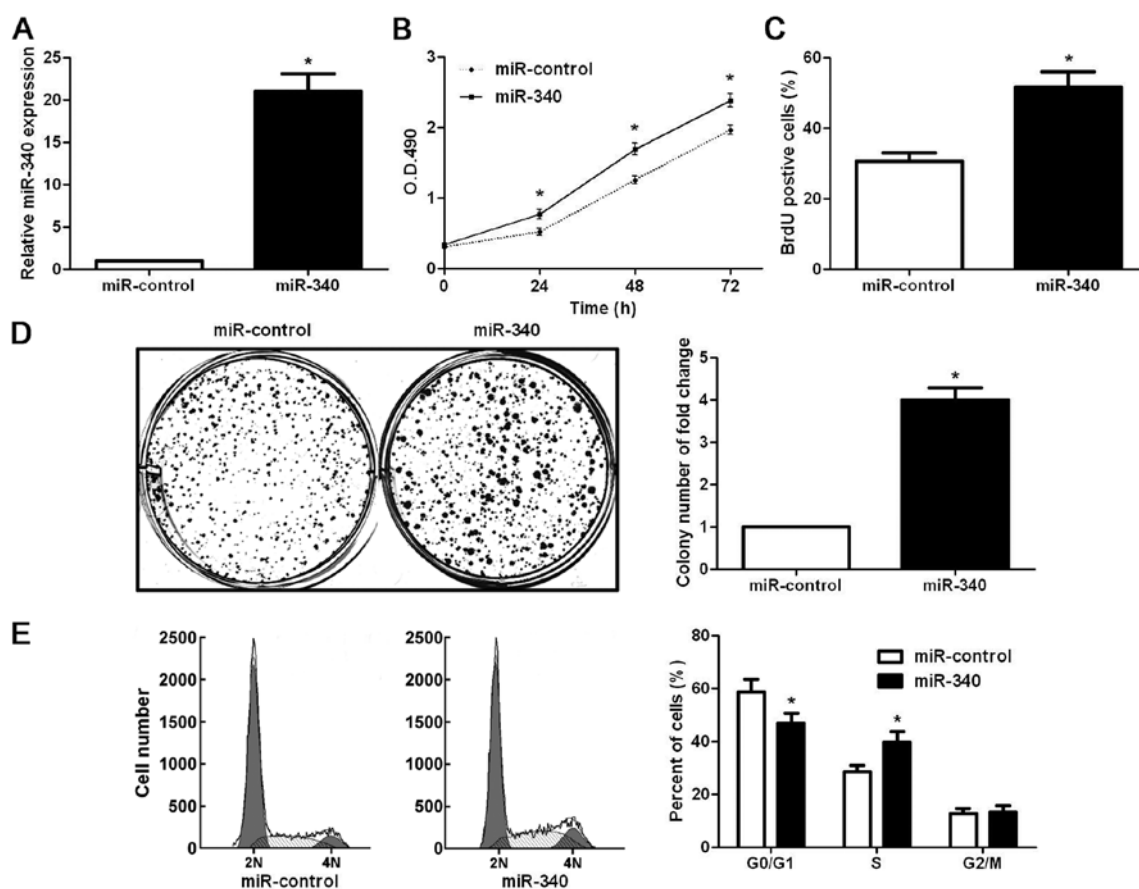

Figure 2. miR-340 overexpression increases cell viability, proliferation and cell cycle progression in the SGC-7901 cells. (A) Significant elevation of miR-340 in the SGC-7901 cells after transfection of miR-340 mimics. (B) Cell viability was significantly increased after transfection of miR-340 mimics as evaluated by an MTT assay. (C) Bromodeoxyuridine (BrdU) incorporation was significantly increased in the SGC-7901 cells after transfection of the miR-340 mimics. (D) Colony formation of the SGC-7901 cells was significantly increased after transfection of the miR-340 mimics. (E) Effects of miR-340 overexpression on the cell cycle progression of the SGC-7901 cells. Experiments were repeated at least three times with similar results. *P<0.05.

panel of gastric cancer cell lines (SGC-7901, AGS, MKN-45, and MGC-803) (P<0.05, Fig. 1B). The obvious elevation of miR-340 in gastric cancer tissues and cells suggest that miR-340 may play an oncogenic role in the development and progression of gastric cancer.

Elevated expression of miR-340 is correlated with adverse clinicopathological features and poor prognosis of the gastric cancer patients. The gastric cancer patients were divided into two groups (miR-340 high or low expression group) based on the median expression level of miR-340. Then we compared

the clinicopathological features and prognosis of gastric cancer patients between the two groups. As shown in Table I, high expression of miR-340 was associated with poor histological features (P=0.025), large tumor size (P=0.003), and advanced tumor-node-metastasis (TNM) stage (P=0.001). Furthermore, Kaplan-Meier analysis showed that increased miR-340 expression was significantly associated with shorter overall survival (P=0.003, Fig. 1C) and disease-free survival (P=0.008, Fig. 1D). Multi-variant Cox regression analysis demonstrated that miR-340 expression was an independent prognostic marker for predicting both overall survival
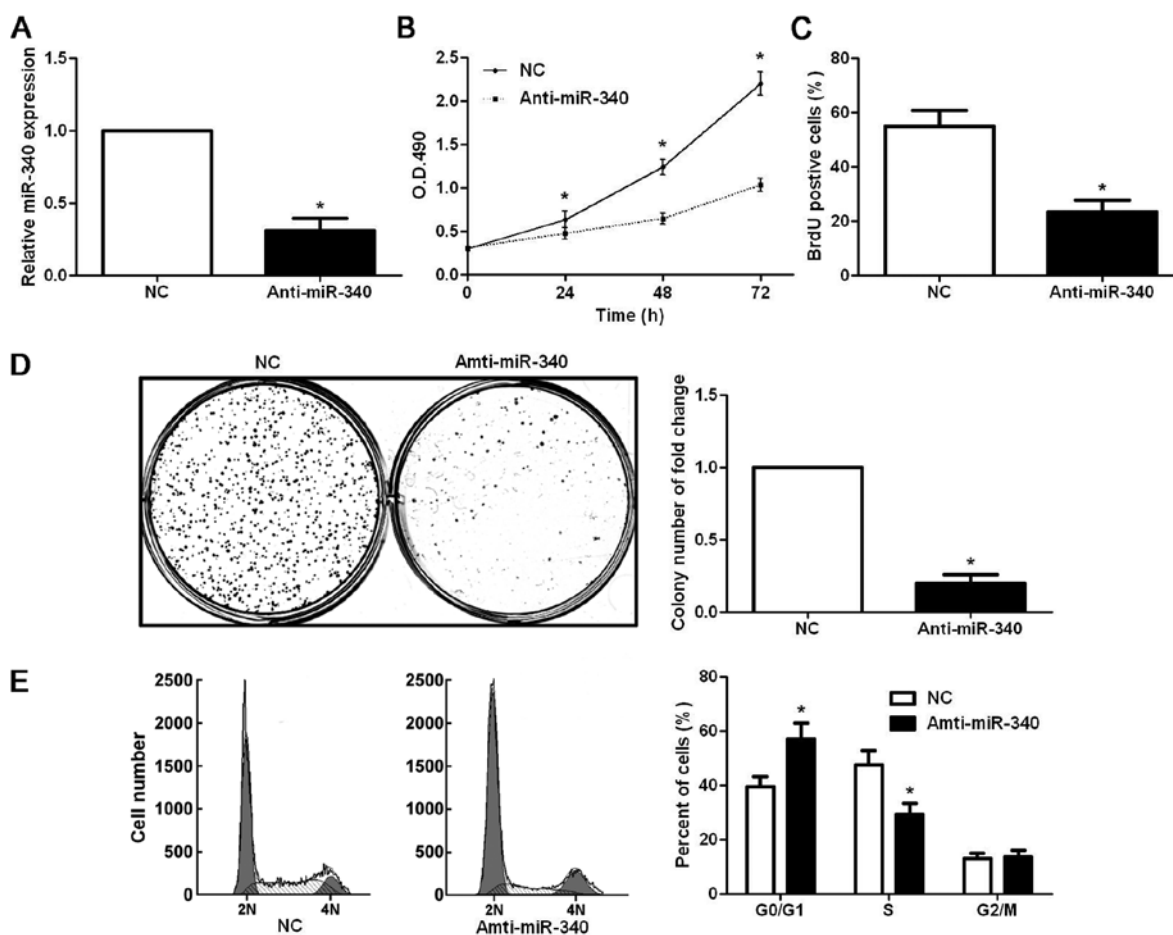
Figure 3. miR-340 silencing decreases cell viability, proliferation and cell cycle progression in the MGC-803 cells. (A) Significant decrease of miR-340 in the MGC-803 cells after transfection of miR-340 inhibitors. (B) Cell viability was significantly decreased after transfection of miR-340 inhibitors as evaluated by an MTT assay. (C) Bromodeoxyuridine (BrdU) incorporation was significantly decreased in the MGC-803 cells after transfection of miR-340 inhibitors. (D) Colony formation of the MGC-803 cells was significantly decreased after transfection of the miR-340 inhibitors. (E) Effects of miR-340 inhibition on the cell cycle progression of MGC-803 cells. Experiments were repeated at least three times with similar results. *$P<0.05$.

and disease-free survival in gastric cancer patients ($P=0.001$ and $P=0.045$, respectively, Table II). These data indicate that miR-340 can serve as a novel biomarker for the survival of gastric cancer patients.

miR-340 enhances the growth of gastric cancer cells by promoting cell viability, proliferation and cell cycle progression. To investigate the biological functions of miR-340 in gastric cancer cells, we transfected gastric cancer cell line SGC-7901 with miR-340 mimics. Transfection of miR-340 mimics significantly increased the miR-340 level in the SGC-7901 cells ($P<0.05$, Fig. 2A). MTT assays showed that ectopic expression of miR-340 significantly increased cell viability of the SGC-7901 cells ($P<0.05$, Fig. 2B). Furthermore, BrdU incorporation assay showed that the overexpression of miR-340 increased the proliferative ability of the SGC-7901 cells ($P<0.05$, Fig. 2C). Furthermore, miR-340 overexpression markedly increased the colony number of the SGC-7901 cells ($P<0.05$, Fig. 2D). Cell cycle analysis revealed that upregulation of miR-340 increased the percentage of cells in the S phase while it decreased the percentage of cells in the G0/G1 phase ($P<0.05$, Fig. 2E). In contrast, we downregulated the miR-340 level with corresponding inhibitors in the MGC-803 cells and examined the cell viability, proliferation,

colony formation and cell cycle. miR-340 inhibitor transfection significantly inhibited the expression level of miR-340 in the MGC-803 cells ($P<0.05$, Fig. 3A). Functionally, inhibition of miR-340 expression resulted in significantly decreased cell viability ($P<0.05$, Fig. 3B), proliferation ($P<0.05$, Fig. 3C), colony formation ($P<0.05$, Fig. 3D) and cell cycle progression ($P<0.05$, Fig. 3E). To further confirm these functional effects of miR-340 on gastric cancer cells, we performed tumor formation assay in a nude mouse model. Inhibition of the miR-340 expression level with its inhibitor significantly decreased the tumor growth of MGC-803 cells in the nude mice ($P<0.05$, Fig. 4). Collectively, these data indicate that miR-340 can promote the tumor growth of gastric cancer cells both *in vitro* and *in vivo*.

CCNG2 is a direct target of miR-340 in gastric cancer cells. To elucidate the underlying molecular mechanisms involved in the biological function of miR-340 in gastric cancer cells, we used TargetScanHuman 7.0 to search for the downstream targets of miR-340. The data in the TargetScanHuman 7.0 showed that 3'-UTR of CCNG2 contained the highly conserved putative binding sites for miR-340 (Fig. 5A), indicating that CCNG2 is a potential downstream target of miR-340. Then, we performed luciferase assays to elucidate whether miR-340
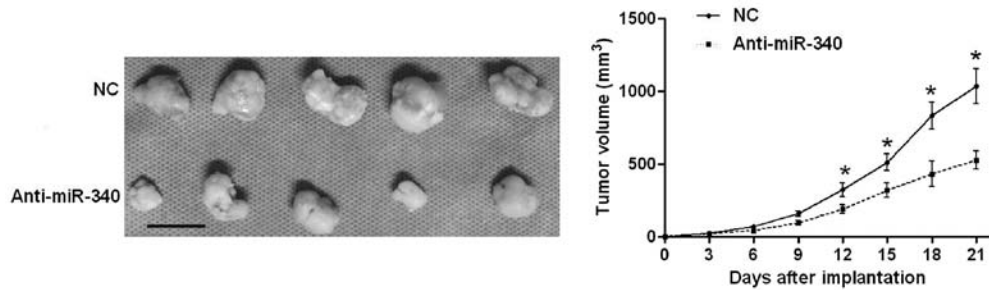
Figure 4. miR-340 knockdown inhibits the tumor growth in nude mice. MGC-803 cells that were transfected with the miR-340 inhibitor or negative control were subcutaneously implanted into nude mice. The tumor growth curve revealed that miR-340 knockdown significantly decreased the tumor growth *in vivo*. n=5, *P<0.05.
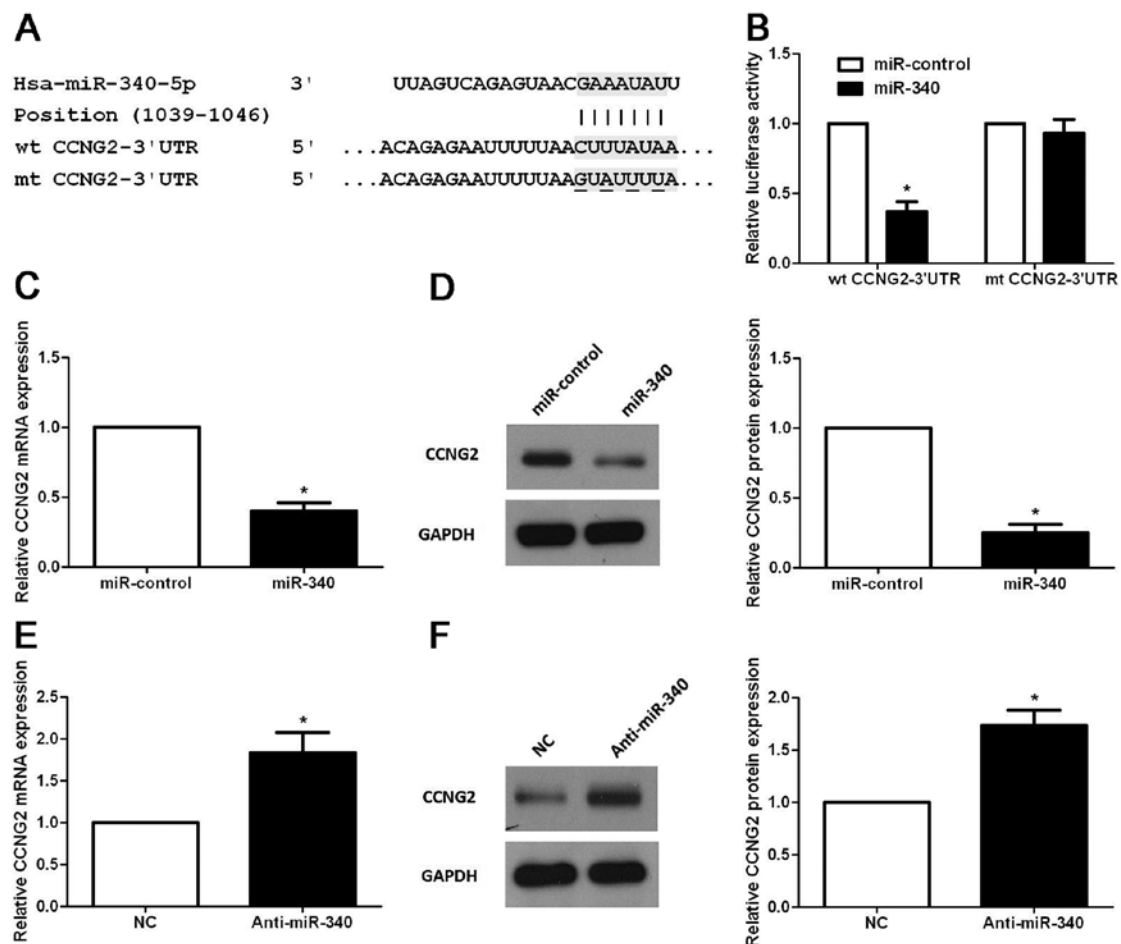

Figure 5. CCNG2 is a direct target of miR-340 in gastric cancer cells. (A) 3'-UTR of CCNG2 contains the highly conserved putative miR-340 binding sites. (B) miR-340 significantly suppressed the luciferase activity that carried the wild-type (wt) but not mutant (mt) 3'-UTR of CCNG2. (C) Overexpression of miR-340 reduced the expression of CCNG2 mRNA in SGC-7901 cells. (D) Overexpression of miR-340 reduced the expression of CCNG2 protein in the SGC-7901 cells. (E) Suppression of miR-340 increased the expression of CCNG2 mRNA in the MGC-803 cells. (F) Suppression of miR-340 increased the expression of the CCNG2 protein in the MGC-803 cells. n=3 repeats with similar results.*P<0.05.

could interact with the 3'-UTR region of CCNG2. The results of the luciferase assays showed that miR-340 overexpression significantly decreased the luciferase acitivy of the wt 3'-UTR of CCNG2 (P<0.05, Fig. 5B), while it had no effects on that of the mt 3'-UTR of CCNG2 in SGC-7901 cells. Next, we used qRT-PCR and western blot analysis to determine whether miR-340 could modulate the expression of CCNG2 in gastric cancer cells. Overexpression of miR-340 significantly reduced the expression level of CCNG2 mRNA in the SGC-7901 cells (P<0.05, Fig. 5C). The results from western blot analysis demonstrated that the protein level of CCNG2 in the SGC-7901 cells was also significantly decreased after miR-340 overexpression (P<0.05, Fig. 5D). Downregulation of miR-340 with its inhibitor significantly increased the mRNA level (P<0.05, Fig. 5E) and protein level (P<0.05, Fig. 5F) of CCNG2 in the MGC-803 cells. These data indicate that CCNG2 is a direct downstream target of miR-340 in gastric cancer cells.
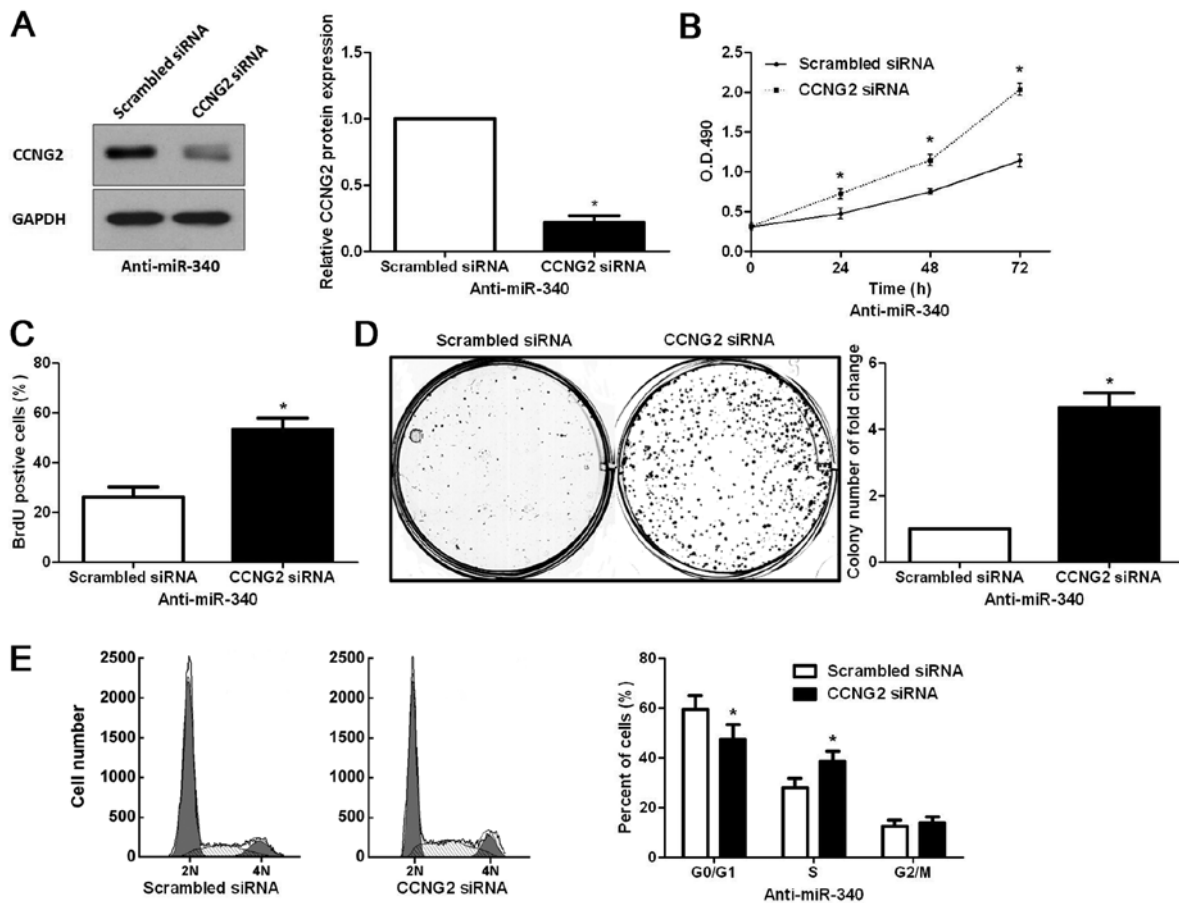
Figure 6. CCNG2 silencing abolishes the effects of miR-340 inhibitors on cell viability, proliferation and cell cycle progression. (A) CCNG2 siRNA significantly decreased the expression of CCNG2 in MGC-803 cells transfected with miR-340 inhibitor. CCNG2 knockdown abrogated the effects of miR-340 inhibitors on (B) cell viability, (C) proliferation, (D) colony formation, and (E) cell cycle progression. n=3 repeats with similar results. *P<0.05.

CCNG2 is critical for the functional effects of miR-340 in gastric cancer cells. As CCNG2 is a direct downstream target of miR-340, we further evaluated whether CCNG2 could mediate the biological function of miR-340 in gastric cancer cells. CCNG2 siRNA significantly reduced the protein level of CCNG2 in the MGC-803 cells transfected with miR-340 inhibitors (P<0.05, Fig. 6A). Knockdown of CCNG2 abrogated the effects of miR-340 knockdown on the MGC-803 cells with increased cell viability (P<0.05, Fig. 6B), proliferation (P<0.05, Fig. 6C), colony formation (P<0.05, Fig. 6D) and cell cycle progression (P<0.05, Fig. 6E). These data indicate that CCNG2 is not only a downstream target of miR-340, but also a functional mediator of miR-340 in gastric cancer cells.

Discussion

Accumulating studies demonstrate that aberrant expression and function of miRNAs play a critical role in the development of various types of human cancers (6,18), including gastric cancer (18). Examination of the expression status, biological function and underlying mechanisms of specific miRNAs in gastric cancer can contribute to the identification of novel biomarkers and therapeutic targets in human gastric cancer. Previous studies have shown that miR-340 plays an important role in human cancers (8-14). It was found to inhibit the metastatic behavior of breast cancer cells (14) while it decreased

the proliferation and promoted the cell apoptosis of prostate cancer cells (13,18). Moreover it was also found to modulate the stem-like cell function of glioma cells (10). However, the clinical significance and underlying mechanisms of miR-340 in gastric cancer remain unclear.

In the present study, we found that miR-340 expression was significantly increased in gastric cancer tissues compared with that in the paired non-tumor-adjacent tissues. Significant elevation of miR-340 was also observed in gastric cancer cells. Association analysis showed that elevated expression of miR-340 was significantly correlated with adverse clinicopathological features, including poor histological differentiation, large tumor size, and advanced TNM stage. Moreover, survival analysis showed that an elevated level of miR-340 was associated with poorer overall and disease-free survival of gastric cancer patients. These data indicate that miR-340 can serve as a novel prognostic indicator of the prognosis of gastric cancer patients.

Increased proliferative ability and cell cycle progression are important in the functional foundation for the development and progression of human cancers (19). The functional assays in this study showed that ectopic expression of miR-340 facilitated cell viability, proliferation, colony formation, and cell cycle progression in gastric cancer cells, while inhibition of miR-340 decreased these cellular processes in the gastric cancer cells. Therefore, these results demonstrated that

miR-340 contributes to the development and progression of gastric cancer by promoting cell viability, proliferation and cell cycle progression.

CCNG2, which is an important regulator of cell proliferation and cell cycle progression (20), has been found to be decreased in gastric cancer tissues (21,22). Moreover, CCGN2 was found to influence the cell viability and cell cycle progression of gastric cancer cells (22). In this study, we presented concrete data showing that CCGN2 is a novel downstream target of miR-340 in human gastric cancer cells. First, the 3'-UTR of CCNG2 contained the complementary sequence of miR-340. Second, overexpression of miR-340 significantly inhibited the luciferase activity of the wt 3'-UTR of CCNG2 while had no influence on that of the mt 3'-UTR of CCNG2. Third, overexpression of miR-340 significantly reduced the expression level of CCNG2, while inhibition of miR-340 increased the expression level of CCNG2 in the gastric cancer cells. Furthermore, this study showed that CCNG2 knockdown could reverse the effects of miR-340 inhibitors on cell viability, proliferation, colony formation and cell cycle progression. Therefore, CCNG2 is a direct functional target of miR-340 in gastric cancer cells.

In conclusion, we demonstrated that miR-340 is frequently overexpressed in gastric cancer tissues and cell lines. miR-340 plays an important role in the progression of gastric cancer by modulating the expression of CCNG2 in gastric cancer cells. Therefore, this study indicates that miR-340 can potentially serve as a promising prognostic factor and therapeutic target in gastric cancer.

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