

Original Article

MiR-346 promoted cell proliferation and cell cycle of human gastric cancer cells by suppressing sFRP4 expression

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Abstract: It has been well documented that aberrant expression of microRNAs is associated with carcinogenesis of Gastric cancer (GC). However, the underlying mechanisms were not fully elucidated. In the current study, our result suggested that miR-346 was identified to be significantly down-regulated in GC primary tumors and cell lines. The regulative role of miR-346 over GC cell growth was studied using MTT, Colony formation and flow cytometry, respectively. Our result showed that ectopic miR-346 expression led to a higher percentage of cells in S phase and a lower percentage of cells in G₁/G₀ phase and promoted GC cell proliferation. Furthermore, we found that miR-346 might exert its function by regulating SFRP4 expression. Taken together, our study has revealed miR-346 as a tumor suppressor in GC.

Keywords: MiR-346, gastric cancer, sFRP4, cell proliferation, cell cycle

Introduction

Gastric cancer (GC) is the second most common cause of cancer-related deaths all over the world [1-3]. Although advanced in treatment of GC, the overall outcome remains unsatisfactory [4, 5]. Therefore, it is an urgent need to find a novel molecular marker that predicting development or prognosis of cancer, and then improve the clinical management of cancer patient [6].

MicroRNAs (miRNAs) are a class of small, short non-coding RNAs (19-22 nucleotides) that play essential roles in multiple biological processes of cancer through their base pairing with the 3'-untranslated region (3'-UTR) of mRNA of their target genes [7-9]. More and more evidences showed that miRNAs can function as tumor oncogenes or suppressors in many types of cancers [10, 11]. Previous studies demonstrated that miR-346 functioned as an oncogene in cutaneous squamous cell carcinoma by regulating SRCIN1 expression [12]. However, the biological function of MiR-346 in GC remains largely unknown.

In the current study, our data demonstrated that the expression of miR-346 was higher in GC tissues and cancer cells. Ectopic expression of miR-346 promoted the GC cell proliferation, colony number and cell cycle through directly targeting Secreted frizzled-related protein 4 (sFRP4). Result of bioinformatics analyses showed that MiR-346 showed its function bounding to 3'-UTR of sFRP4 mRNA, which was confirmed by luciferase reporter assay. Result of Western blots showed that miR-346 might exert its function by regulating cyclin D1 and p27 via its influence on sFRP4. Together, our data suggested that overexpression of miR-346 may promote gastric cancer cell growth through the regulation of sFRP4 translation.

Materials and methods

Clinical specimens

Gastric carcinoma (GC) and normal gastric mucosal tissues (As Normal) were recruited from Department of Oncology, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of

Medicine (Shanghai, People's Republic of China). The study was approved by the ethics committee of Department of Oncology, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, People's Republic of China). All patients gave informed consent in written. A pathological diagnosis of gastric cancer was verified by at least two pathologists. Tissue samples were flash frozen and store at liquid nitrogen until used.

Cell culture

Human gastric cancer cell lines MGC-803, MKN-45, MKN-28, AGS and SGC-7901 were provided by the American Type Culture Collection (Manassas, VA, USA), and maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA), 100 units/ml of penicillin-streptomycin (Invitrogen, Carlsbad, CA), and human gastric epithelial cell (HGE) were purchased from Wuhan PriCells Biomedical Technology Co., Ltd (Wuhan, China) and maintained in PriCells Medium (Wuhan PriCells Biomedical Technology Co., Ltd, Wuhan, China). All cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Plasmids and transfection

The MiR-346 mimics, MiR-346 inhibitor and relative negative control were purchased from Shanghai GenePharma (Shanghai, China) and transfected into GC cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

RNA extraction and real-time quantitative GCR

Total RNA from clinical tissues and cells was extracted by using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The expression levels of MiR-346 were quantified using miRNA-specific TaqMan miRNA assay kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed using a SYBR-Green Master Mix (ABI, Foster, CA, USA) and a 7500 Fast Real-Time PCR system (Applied Bio-System, Foster City, CA) to detect the expression of sFRP4. PCR primer of sFRP4 (HQP016878) was synthesized by GeneCopoeia™. RNU6B (U6) or GAPDH were used as controls for normalization. The 2^{-ΔΔCt} method was used to quantify relative RNA

expression. Experiments were performed in triplicate.

MTT assays and colony formation

For analysis of cell proliferation, transfected MKN-45 cells (3×10³ cells/well) were seeded into 96-well plates, following incubation of cells for 1, 2, 3, 4, 5 and 6 days, 20 μl of 5 mg/ml MTT solution (Sigma-Aldrich) was added to each well were together incubated for 4 h, and the culture medium was removed and 150 μL DMSO (Sigma-Aldrich) was added, the absorbance in each well was measured with a microplate reader set at 490 nm.

For colony formation assay, transfected MKN-45 cells (1×10³ cells/well) were seeded onto six-well plate and incubated for 14 days in medium containing 10% FBS, and then cells were fixed with methanol, stained with 0.5% crystal violet for 10 min. Visible colonies were manually counted.

Cell cycle assays by flow cytometry

For analysis of cells cycle, transfected MKN-45 cells after 48 h post transfection were harvested, and then fixed in ice-cold 70% ethanol, incubated with RNase A at 37°C for 30 min, and then stained with propidium iodide (PI, Sigma, USA) at 4°C for 30 min in the dark. The distribution of cells was analyzed by cell flow cytometry.

Luciferase assays

The sFRP4 3'-UTR and the sFRP4 3'-UTR mutant were amplified and cloned into the downstream of pGL3/luciferase vector (Promega, China). Cells were co-transfected with miR-346 mimics, miR-346-in or the relative miR-NC control and sFRP4 3'-UTR or the mutant 3'-UTR, together with the controls. At 48 h after transfection, luciferase activity was detected using the dual-luciferase assay system (Promega, Madison, USA). Renilla luciferase was used for normalization.

Western blotting

Cells were harvested and lysed with RIPA lysis buffer on ice for 20 min, equal amounts of proteins (40 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), transferred onto PVDF membranes by

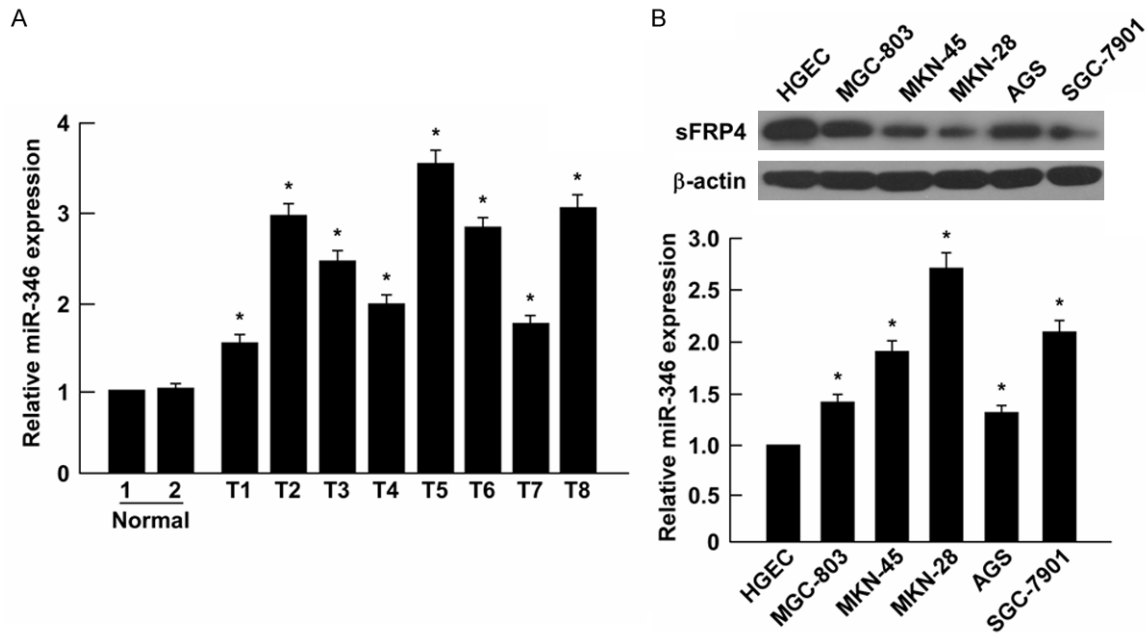


Figure 1. Upregulation of MiR-346 in human GC tissues and cell lines. A. Real-time PCR analysis of MiR-346 expression in human GC tissues and normal tissues. B. Relative expression of miR-346 in human gastric epithelial cell (HGEc) and GC cell lines, including MGC-803, MKN-45, MKN-28, AGS and SGC-7901. Experiments were repeated at least three times. Each bar represents the mean of three independent experiments. * $P < 0.05$.

electroblotting. After probed with 1:1000 diluted anti-sFRP4, anti-CyclinD1 and anti-P27 (Abcam, MA, USA) at 4°C overnight, the membrane was further probed with horseradish peroxidase (HRP)-conjugated corresponding second antibody for 2 h at room temperature. β -actin (Abcam, MA, USA) was used as the internal control. Immunocomplexes were visualized using the ECL detection reagent (Beyotime, China) according to the manufacturer's protocol.

Statistical analysis

All data are showed as mean $\pm$ standard deviation (SD) and performed using the SPSS 18.0 (SPSS, Chicago, IL), and all experiments were repeated at least three times independently. The Student's t-test was used to evaluate the statistical significance of differences between two groups of data in all pertinent experiments. $P < 0.05$ was considered significant.

Result

MiR-346 was up-regulated in human GC tissues and GC cell lines

Result of RT-PCR showed that levels of miR-346 were up-regulated in cancer tissues than

in normal tissues (**Figure 1A**). Similarly, higher levels of miR-346 were detected in Human gastric cancer cell lines MGC-803, MKN-45, MKN-28, AGS and SGC-7901 compared with human gastric epithelial cell (HGEc) (**Figure 1B**). Taken together, these results indicated that MiR-346 is upregulated in GC primary tumors and cell lines.

MiR-346 promoted GC cell proliferation and miR-346-in inhibited GC cell proliferation

To exam the biological function of miR-346 in GC, we used miR-346 or miR-346-in to gain or against miR-346 to achieve up-regulation or down-regulation of miR-346 in GC MKN-45 cells. Proliferation of the MKN-45 cells was measured by MTT assay and colony formation assay. Result of MTT assay showed that the MKN-45 cells transfected with the miR-346 exhibited significantly more proliferation compared with the negative control cells, while miR-346-in showed the opposite effect at various time points post-transduction (0, 1, 2, 3, 4, 5 and 6 d) (**Figures 2A and 3A**). Colony formation assays showed that miR-346 significantly increased the ability of colony formation in GC MKN-45 cells, while miR-346-in decreased its cell growth ability (**Figures 2B and 3B**). To inves-

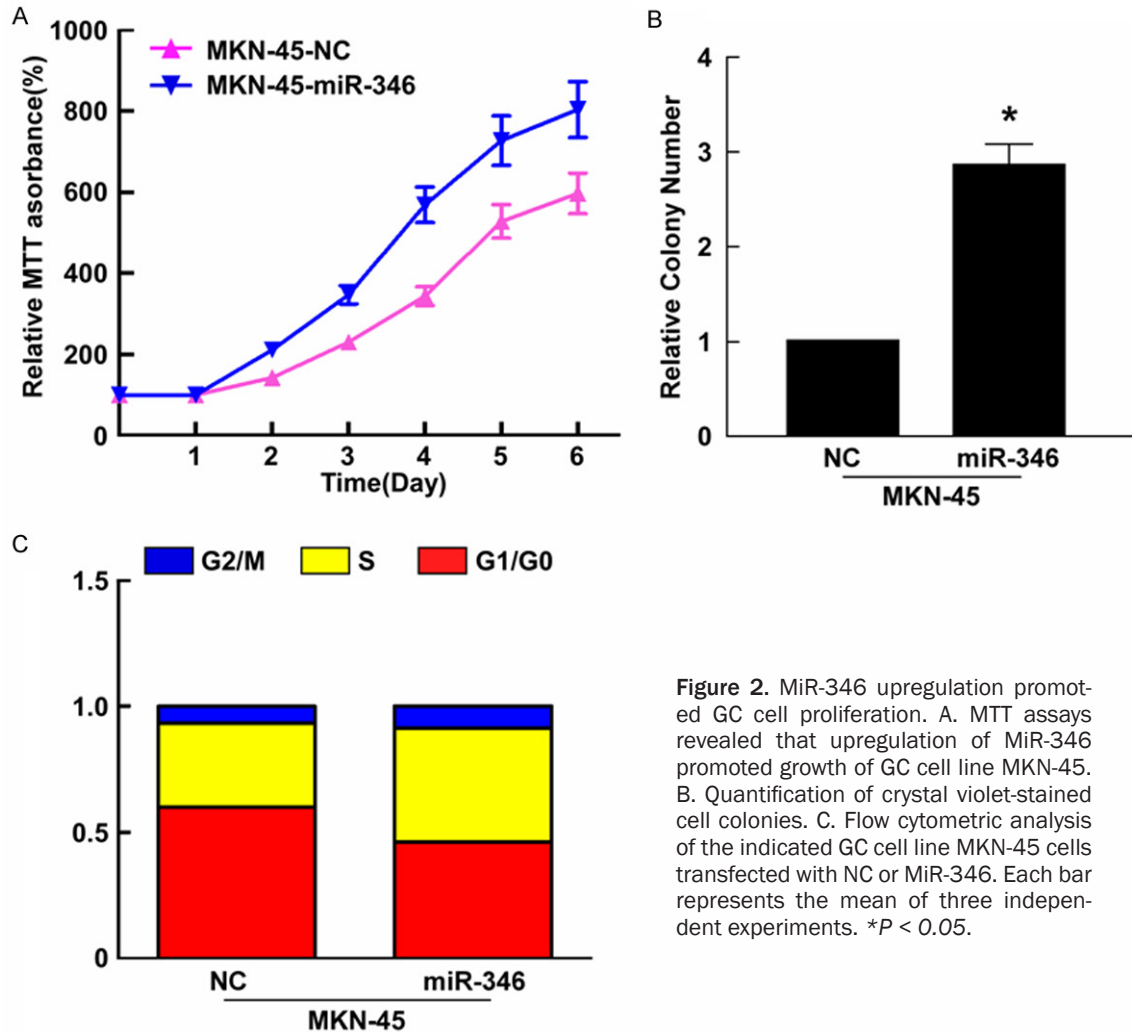


Figure 2. MiR-346 upregulation promoted GC cell proliferation. A. MTT assays revealed that upregulation of MiR-346 promoted growth of GC cell line MKN-45. B. Quantification of crystal violet-stained cell colonies. C. Flow cytometric analysis of the indicated GC cell line MKN-45 cells transfected with NC or MiR-346. Each bar represents the mean of three independent experiments. * $P < 0.05$.

tigate the mechanism mediating this pro-proliferative effect, cell cycle analysis was performed. Compared with the negative control cells The MKN-45 cells infected with the miR-346 increased the percentage of cells in the S phase while decreased the percentage of cells in the G_1/G_0 phase, and miR-346-in showed an increased in the number of cells in the G_1/G_0 phase and decreased in the number of cells in the S phase compared with the negative control cells (**Figures 2C** and **3C**).

MiR-346 directly targets sFRP4 by binding to its 3'-UTR and alter

Through prediction in online databases, we observed that miR-346 has a putative binding site in the 3'UTR of sFRP4 (**Figure 4A**). To confirm whether potential miR-346 binding sites in the 3'UTR of sFRP4, we used Luciferase assays.

The result showed that the suppression of the luciferase expression levels were enhanced upon expression of miR-346, but this effect was alleviated in cells with the mutant sFRP4 3'UTR luciferase reporter (**Figure 4B**). And then Western Blot analysis showed that expression of sFRP4 was markedly down-regulation in MKN-45 cells after transfection with miR-346, up-regulated in miR-346-in transfected MKN-45 cells (**Figure 4C**). In summary, our results suggested that miR-346 may target 3'-UTR of sFRP4 to inhibit its translation.

Growing reports showed that sFRP4 is closely correlated with Wnt/ β -catenin signaling activity. And then we examined the mRNA expression levels of downstream genes (Cyclin D1 and P27) in the Wnt/ β -catenin signaling pathway. As expected, result of Western blot showed that MKN-45 cells after transfection with miR-

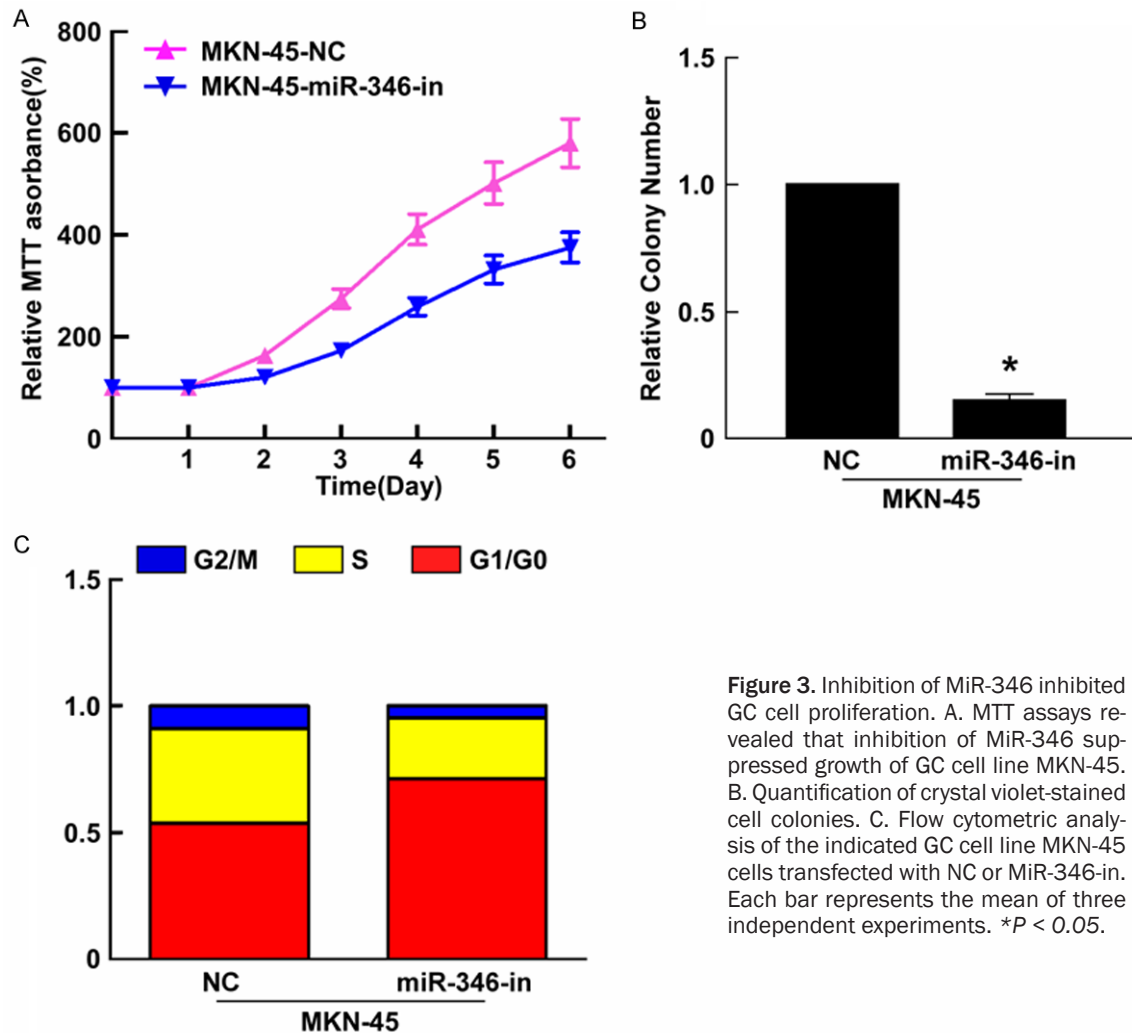


Figure 3. Inhibition of MiR-346 inhibited GC cell proliferation. A. MTT assays revealed that inhibition of MiR-346 suppressed growth of GC cell line MKN-45. B. Quantification of crystal violet-stained cell colonies. C. Flow cytometric analysis of the indicated GC cell line MKN-45 cells transfected with NC or MiR-346-in. Each bar represents the mean of three independent experiments. * $P < 0.05$.

346 enhanced the expression of Cyclin D1 and decreased p27 expression, while expression of Cyclin D1 was decreased and p27 expression was increased in miR-346-in transfected MKN-45 cells (Figure 4C).

Discussion

In our study, we focused on miR-346 which was increased in GC clinic tissues and five GC cell lines. Moreover, ectopic miR-346 expression led to a higher percentage of cells in S phase and a lower percentage of cells in G_1/G_0 phase, and then promoted GC cell proliferation and increased GC cell colony formation. Additionally, we identified sFRP4 as a direct and functional target of miR-346 in GC cells. In sum, these results indicated that miR-346 might function as an oncogene in GC.

It was reported that dysregulation of miRNAs contributed to the pathogenesis and progression of majority human malignancies [13]. Mounting evidence indicated that miRNAs were involved in GC carcinogenesis and patient prognosis. For example, miR-326 expression was decreased in gastric cancer, and is correlated with poor prognosis and decreased cell growth and metastasis by targeting FSCN1 in gastric cancer [8]. Finding by Zheng *et al.* indicated that miR-206 regulated c-Met Pathway and effectively inhibited GC Progression [14]. Wu *et al.* found that miR-449c level was reduced in GC, and miR-449c might target MET to promote GC cell growth [15]. However, there is still a need to clarify the significance and function of certain specific miRNA, which might play different roles in GC. In our study, our result showed that miR-346 was significantly up-regulated in

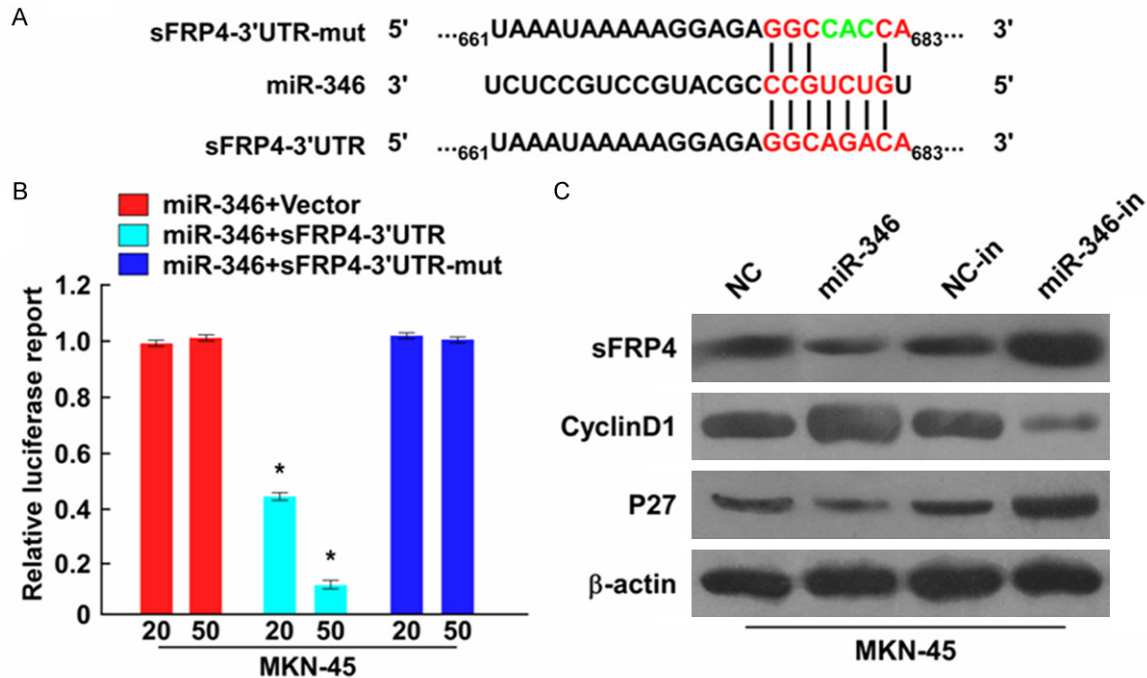


Figure 4. MiR-346 suppresses SFRP4 expression by directly targeting the SFRP4 3'-UTR and altered levels of proteins related to cell proliferation and cell cycle in GC cell line MKN-45. **A.** Predicted MiR-346 target sequence in the 3'-UTR of SFRP4 (SFRP4-3'-UTR) and positions of three mutated nucleotides (green) in the 3'-UTR of SFRP4 (SFRP4-3'-UTR mut). **B.** Luciferase reporter assay of the indicated MKN-45 cells transfected with the pGL3-SFRP4-3'-UTR reporter and MiR-346 or MiR-346 inhibitor oligonucleotides. **C.** Western blotting analysis of protein expression of SFRP4, cyclin D1 and p27 in indicated MKN-45 cells. β-actin served as the loading control. * $P < 0.05$.

GC clinical tissues and cancer cells. Ectopic miR-346 expression promoted GC cell proliferation and led to a higher percentage of cells in S phase and a lower percentage of cells in G_1/G_0 phase. Furthermore, the increased proliferation of the GC cells resulted from promoting cell cycle progression *in vitro*, miR-346 overexpression increased GC cell colony formation.

It is reported that canonical Wnt signaling pathway played a critical role in cancer cell cycle control [16, 17], and SFRP4 was a glycoprotein that acted as an antagonist of Wnt ligands, causing inhibition of the canonical Wnt signaling pathway, and then implicated in cell proliferation, which playing an important role in carcinogenesis [18, 19]. Finding by Carmon *et al.* showed that sFRP4 was significantly decreased in endometrial cancer cells and then suppressed cell growth [20]. In this study, we thus investigated whether miR-346, as a targeting miRNA for SFRP4 based on bioinformatics analyses, and might suppress SFRP4 expression. Moreover, result of luciferase reporter assay indicated that the 3'-UTRs of human SFRP4 contain a match of the seed sequence of miR-346, and binding of miR-346 to the seed region

of SFRP4 was important for blocking SFRP4 expression. Taken together, these findings strongly demonstrated a critical role of miR-346 in regulating SFRP4 translation.

In the current study, our data offered convincing evidence that miR-346 might function as an oncogenic miRNA in GC, partly by regulating SFRP4 expression. Hence, MiR-346 could be considered as a novel therapeutic target for GC patients.

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Disclosure of conflict of interest

None.

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