Oncology Research, Vol. 26, pp. 1183–1189
Printed in the USA.

0965-0407/18 $90.00 + .00
DOI: https://doi.org/10.3727/096504018X15151495109394
E-ISSN 1555-3906
www.cognizantcommunication.com

MicroRNA-338-3p Suppresses Proliferation of Human Liver Cancer Cells by Targeting SphK2

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Recent studies have revealed abnormal expression of miRNAs in various tumors. Although microRNA-338-3p (miR-338-3p) plays an important role in many types of tumors, its influence on liver cancer (LC) is unknown. In this study, we found that expression of miR-338-3p was decreased in LC cells and tissues. Colony formation and cell proliferation were suppressed by enhanced expression of miR-338-3p in LC cells. Moreover, miR-338-3p targeted sphingosine kinase 2 (SphK2). Silencing of SphK2 had an identical influence as overexpression of miR-338-3p in LC cells. Overexpression of SphK2 without the 3′-untranslated region remarkably enhanced the growth suppression triggered by miR-338-3p in LC cells. These findings indicate that miR-338-3p influences the development of LC by targeting SphK2, suggesting that miR-338-3p can be targeted as an innovative therapeutic strategy for LC.

Key words: Liver cancer (LC); Proliferation; MicroRNA-338-3p; Sphingosine kinase 2 (SphK2)

INTRODUCTION

As one of the most prevalent cancers and second greatest contributor to cancer-specific mortality worldwide, liver cancer (LC) has an incidence rate of more than 0.7 million people every year. The mortality rate of LC is 0.75 million people per year, with half of this number attributable to China[1,2]. Although therapies such as surgery or chemotherapy are employed, patients with LC have a high rate of recurrence because of invasion and metastasis[3]. Thus, it is necessary to identify new targets to develop innovative treatments for LC.

MicroRNAs (miRNAs) are noncoding single-stranded RNAs consisting of 22–25 nucleotides. They bind to the target 3′-untranslated region (3′-UTR) and lead to its decomposition[4,5]. miRNAs are prevalent in various reactions, such as apoptosis, metabolism, and differentiation[6–8]. Additionally, they have been shown to play an essential role in tumorigenesis by modulating oncogenes and the expression of tumor suppressors, or by serving as oncogenes or tumor suppressors[9,10]. In aggressive LC, hypernomic expression of some miRNAs including miR-200a, miR-125b, and miR-214 has been detected, whereas downregulation of other miRs has been observed, such as that of miR-155, miR-183, and miR-550a[11–13]. A recent study also demonstrated that miRNA signatures are a subgroup of potential prognostic biomarkers in LC patients[14].

MicroRNA-338-3p (miR-338-3p) is located in the seventh intron of the AATK gene, whose expression is modulated by miR-338-3p in murine neurons[15]. miR-338-3p was first discovered in neurodegenerative diseases triggered by prions, as its expression decreases in murine brains that have undergone infection by mouse-adapted scrape[16]. In the generation and development of cancer, downregulation of miR-338-3p is prevalent in various tumor types[17–19]. However, the influence of miR-338-3p in LC is undetermined.

Thus, in this study, the possible role of miR-338-3p in LC was evaluated. miR-338-3p expression was detected in both tissues and cells of LC. The influence of miR-338-3p was also evaluated in vitro. Moreover, the essential mechanisms of the function of miR-338-3p in LC cells were explored, providing insight into LC.

MATERIALS AND METHODS

Cell Culture

All cell lines used in our research were derived from the Cell Bank of the Chinese Academy of Sciences

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(Shanghai, P.R. China). Normal hepatocellular cells (HL-7702) and LC cells (HepG2, SMMC-7721, and BEL7402) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS; HyClone, Logan, UT, USA) and antibiotics, reaching a terminal concentration of 10%. Cells were incubated under conditions of 5% CO_2 and 37°C.

Tissue Samples

Fresh tissues obtained from 39 patients who underwent surgical excision at The 4th Hospital of Hebei Medical University from January 2015 to September 2016 were evaluated in this study. The tissues consisted of 39 LC specimens and 21 surrounding normal mucosa specimens. Our research was approved by the Ethics Review Committees of The 4th Hospital of Hebei Medical University. Written informed consent was obtained from all participants.

Cell Proliferation and Colony Formation Assay

A water-soluble tetrazolium salt assay was conducted to detect cell proliferation using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, the cells were seeded into 96-well plates and incubated for 4 days at 5% CO_2 and 37°C. At the beginning of the assay, the medium was supplemented with 10 µl of CCK-8, and the cells were incubated for 2 h at 37°C. Absorbance at 450 nm was measured to evaluate cell counts using a plate reader. For the colony generation assay, cells were seeded into six-well plates and incubated for 9 days. Methanol/acetone (1:1) was utilized to fix the colonies, and crystal violet was used for staining.

RNA Isolation and qRT-PCR

Total RNA was obtained from specimens and cells using the Qiagen RNeasy Kit (Hilden, Germany). Electrophoretic and spectrophotometric methods were used to evaluate the quantity and quality of RNA. Quantitative reverse transcription(qRT)-polymerase chain reaction (PCR) was applied to estimate the expression of miR-338-3p according to the TaqMan protocol (Applied Biosystems, Foster City, CA, USA). U6 snRNA (RNU6B; Applied Biosystems) was used to normalize miR-338-3p expression using the $2^{-\Delta\Delta Ct}$ method.

Construction of 3′-UTR Luciferase Plasmid and Reporter Assays

PCR was conducted to amplify the sphingosine kinase 2 (SphK2) 3′-UTR, which was subsequently added to the pmiRGLO blank vector (Promega, Madison, WI, USA) at the *Xba*I and *Xho*I sites located on the 3′ side of the luciferase gene. The following primers were used: forward, 5′-AUGGGACCAGACGUGAUGCUGGA-3′; reverse, 5′-GUUGUUUUAGUGACUACGACCU-3′. Sequencing was conducted to verify the 3′-UTR of SphK2, which was named pmiR-GLO-WT. The QuikChange Site-Directed Mutagenesis Kit (Promega) was applied for miR-338-3p in the 3′-UTR of SphK2 (pmiR-GLO-mut), during which pmiR-GLO-WT was used as the template. For the luciferase reporter assay, 96-well plates were used to culture SMMC-7721 and HepG2 cells. The two cell lines were cotransfected with either mutant or wild-type reporter plasmid (100 nM) as well as miRNA (100 nM) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). A dual-luciferase assay system (Promega) was applied to assess luciferase activity 48 h after cotransfection.

Plasmid Construction and Cell Transfection

Coding sequences of SphK2 with the 3′-UTR absent were cloned into the pcDNA3.1 vector (Invitrogen) to produce the pcDNA3.1-SphK2 expression vector. Cells

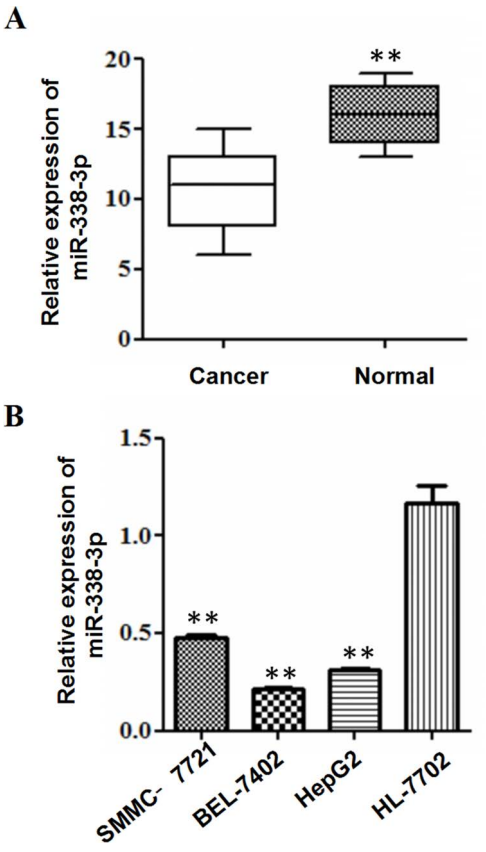

Figure 1. Decreased microRNA-338-3p (miR-338-3p) expression in liver cancer (LC) tissues and cells. (A) The expression of miR-338-3p in LC ($n=39$) and surrounding normal tissues ($n=21$) was evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (**$p<0.001$, unpaired t-test tissues study). (B) miR-338-3p expression in LC cells (HepG2, SMMC-7721, and BEL-7402) was evaluated by qRT-PCR and compared to a normal liver mucosa cell line (HL-7702). Data are the mean ± standard error. **$p<0.001$.

were cultured at 37°C and 5% CO_2. Lipofectamine 2000 was used for transfection after confluence reached 70%.

Western Blotting

The RIPA buffer consisted of 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 1% Triton X-100, 1% SDS, and 1% sodium deoxycholate. RIPA buffer, protease inhibitor cocktail without EDTA (Kodak, Rochester, NY, USA), phosphatase inhibitors (5 mM sodium orthovanadate), and 1 mM phenylmethylsulfonyl fluoride were added for cell lysis. SDS-PAGE was conducted to resolve protein lysates, which were subsequently transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Amersham, UK) and detected with antibodies. ECL measurement agents (Kodak) were used for observation. Primary antibodies for β-actin and SphK2 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

The mean ± standard deviation (SD) was used to describe the data. A Student's *t*-test or simple one-way analysis of variance was applied to compare groups. SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was applied for analyses. Values were regarded as significant when the two-tailed value was $p < 0.05$.

RESULTS

Downregulated Expression of miR-338-3p in Human LC Cells and Tissues

To investigate the influence of miR-338-3p on tumorigenesis of LC, we detected its expression in 39 LC specimens and 21 surrounding normal mucosa specimens. Using qRT-PCR, we found that miR-338-3p expression was noticeably decreased in LC specimens compared to surrounding normal specimens (Fig. 1A). Furthermore, miR-338-3p expression remarkably decreased in LC cells (SMMC-7721, BEL-7402, and HepG2) compared to normal cells (HL-7702) (Fig. 1B), indicating that downregulation of miR-338 in LC cells is associated with the tumorigenesis and progression of LC.

miR-338-3p Suppresses LC Cell Proliferation In Vitro

To evaluate the efficiency of miR-338-3p and its inhibitors, we performed qRT-PCR to examine miR-338-3p expression in HepG2 cells transfected with miR-338-3p or

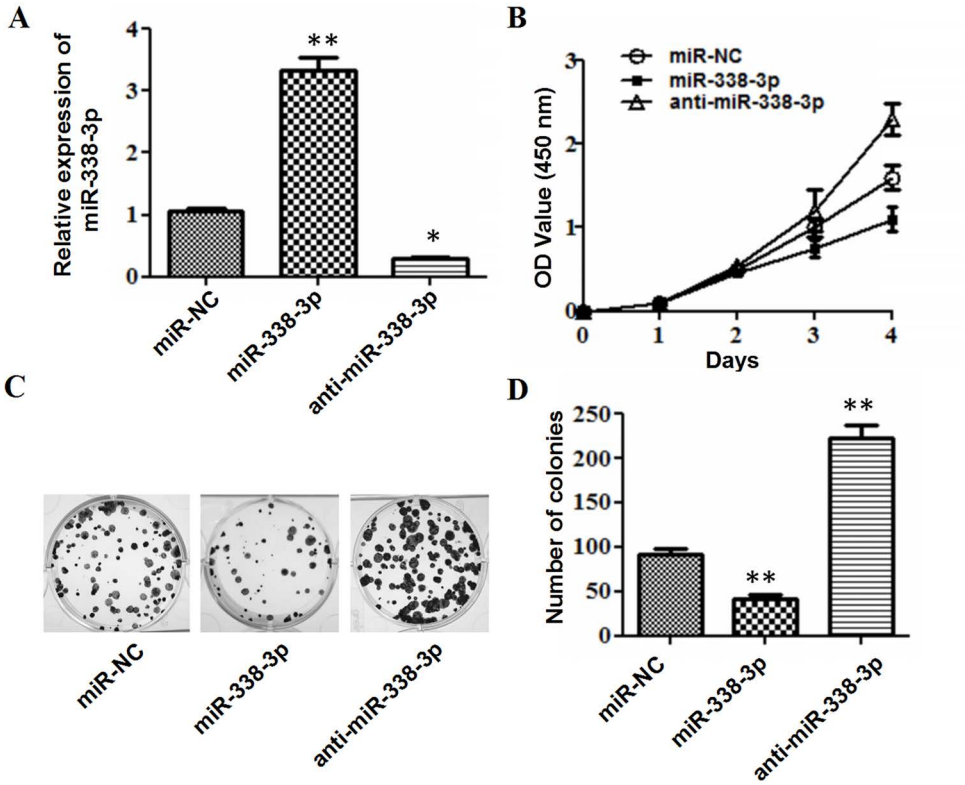

Figure 2. miR-338-3p suppresses proliferation of LC cells in vitro. (A) miR-338-3p expression was detected by RT-PCR in HepG2 cells transfected with miR-338-3p or its inhibitors. (B) Analysis of cell proliferation assay in HepG2 cells upon miR-338-3p or its inhibitors. (C) Colonies formed in HepG2 cells transfected with miR-338-3pa or its inhibitors. (D) Quantification of the relative colony formation in HepG2 cells transfected with miR-338-3p or its inhibitors. Data are the mean ± standard error. NC, negative control. *$p < 0.01$; **$p < 0.001$.

its inhibitors or their relative negative controls. Figure 2A shows that the concentration of miR-338-3p was elevated following transfection with mimics. Moreover, it decreased significantly following transfection with inhibitors. The influence of miR-338-3p on cell growth was also evaluated. As shown in Figure 2B, miR-338-3p expression in HepG2 cells markedly suppressed cell growth at the fourth day compared to in HepG2/miR-NC cells. In contrast, anti-miR-338-3p expression in HepG2 cells increased cell growth compared to that in the blank control. Colony formation analysis also showed that miR-338-3p expression decreased the colony number, while anti-miR-338-3p expression enhanced the colony number compared to their counterpart controls (Fig. 2C and D). Taken together, our results demonstrate that miR-338-3p expression inhibited LC cell proliferation.

miR-338-3p Directly Targets SphK2

Bioinformatic analysis suggested that the SphK2 3′-UTR contains a predicted binding site for miR-338-3p based on TargetScan facilitation (Fig. 3A). Modulation of SphK2 by miR-338-3p was verified by luciferase reporter assays and Western blotting, which suggested downregulation of SphK2 in SMMC-7721 and HepG2 cells subsequent to transfection with miR-338-3p (Fig. 3B). The dual-luciferase reporter system with vectors consisting of mutant or wild-type SphK2 3′-UTR was employed to determine whether SphK2 is a target of miR-338-3p. Luciferase activity from the wild-type reporter was obviously inhibited by miR-338-3p cotransfection. However, it failed to suppress that of the mutant reporter in HepG2 cells (Fig. 3C). Additionally, miR-338-3p cotransfection remarkably suppressed the activity of luciferase from the wild-type reporter in SMMC-7721 cells (Fig. 3D). Thus, miR-338-3p directly targets SphK2 by negatively modulating SphK2 expression once bound to the 3′-UTR.

Inhibitory Effects of miR-338-3p on LC Cells Are Modulated Through Downregulation of SphK2

To determine if the inhibitory influence of miR-338-3p on LC cells is based on downregulation of SphK2, SphK2-siRNA was used to suppress the expression of SphK2 (Fig. 4A). The CCK-8 assay showed that SphK2-siRNA inhibited the proliferation of HepG2 cells compared to negative controls, which was similar to cells transfected with miR-338-3p (Fig. 4B). The colony formation assay indicated that SphK2-siRNA reduced the number of colonies of HepG2 cells compared to negative controls,

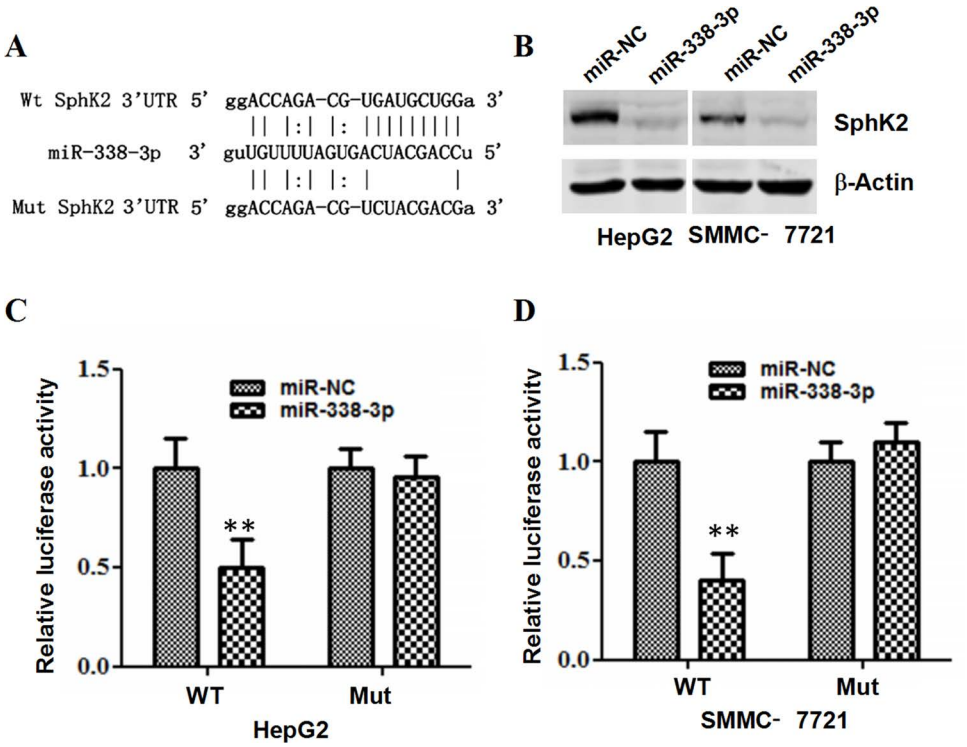

Figure 3. miR-338-3p aims directly at sphingosine kinase 2 (SphK2). (A) The mutant and wild-type SphK2 3′-untranslated regions (3′-UTRs) were not comparable. (B) Expression of SphK2 was measured by Western blotting. (C) Luciferase activity was detected in HepG2 cells transfected with reporter vectors consisting of wild-type 3′-UTR (or mutant 3′-UTR) of SphK2. (D) Luciferase activity was measured in SMMC-7721 cells transfected with reporter vectors consisting of wild-type 3′-UTR (or mutant 3′-UTR) of SphK2. Data are the mean ± standard error. $^{**}p < 0.001$.

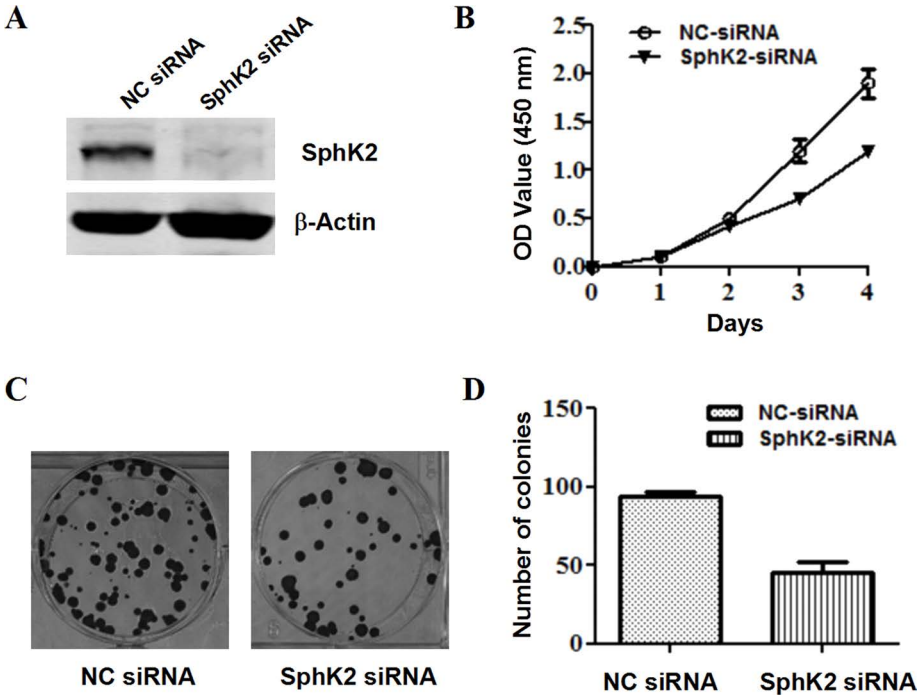

Figure 4. Suppressive effects of miR-338-3p on LC cells are modulated by downregulating SphK2. (A) SphK2 expression was measured by Western blotting in HepG2 cells transfected with NC siRNA or SphK2-siRNA. (B) Analysis of cell proliferation assay in HepG2 cells transfected with NC siRNA or SphK2-siRNA. (C) Colonies formed in HepG2 cells transfected with NC siRNA or SphK2-siRNA. (D) Quantification of the relative colony generation in HepG2 cells transfected with NC siRNA or SphK2-siRNA. Data are the mean ± standard error.

which was similar to cells transfected with miR-338-3p (Fig. 4C and D). We also overexpressed SphK2 lacking the 3′-UTR in HepG2 cell lines cotransfected with miR-338-3p. Western blotting showed that SphK2 expression was decreased in HepG2 cells subsequent to transfection with miR-338-3p and was overexpressed in cells cotransfected with pcDNA3.1-SphK2 (without the 3′-UTR) and miR-338-3p (Fig. 5A). The results of the CCK-8 assay and colony formation assay demonstrated that the proliferation-suppression effects of miR-338-3p on HepG2 cells were partly restored by pcDNA3.1-SphK2 lacking the 3′-UTR (Fig. 5B–D), suggesting that suppression of miR-338-3p in LC cells was modulated by downregulation of SphK2.

DISCUSSION

To efficiently treat cancer, it is necessary to understand the mechanisms of tumorigenesis. Previous studies showed that malfunctions in miRNA expression are closely associated with cancer and often lead to cancer development. Thus, it is important to determine the influence of malfunctioning miRNAs in cancer. Downregulation of miR-338-3p has been reported in LC[20], gastric malignancy[21], and colorectal malignancy[22]. However, upregulated miR-338-3p was observed in pancreatic neoplasms[23]. In contrast, miR-338-3p expression in LC is poorly understood. In our study, PCR was conducted to evaluate changes in miR-338-3p expression. We verified that miR-338-3p expression was noticeably downregulated in LC tissues compared to the surrounding normal tissues. Our results indicate that miR-338-3p influences LC development by targeting SphK2, suggesting that miR-338-3p can serve as an innovative therapeutic strategy for LC.

Identifying the role of miR-338-3p is necessary for revealing the mechanisms of LC, and thus we conducted bioinformatics analysis to predict its target gene. Taking gene overlap determined by PicTarget, miRBase targets, and TargetScan into consideration, SphK2 was predicted as the target. Luciferase reporter, Western blotting, and qRT-PCR assays verified that miR-338-3p targets SphK2 by interacting with the binding site at the 3′-UTR. Sphingolipids comprise a large family[24] and participate in multiple reactions such as apoptosis and invasion[25]. Sphingosine kinases are important in the reaction of sphingoid-base phosphates and have two different isoforms: SphK1 and SphK2[26,27]. SphK1 is oncogenic and plays an important role in tumorigenesis[28]. However, the mechanism of SphK2 in LC remains unclear. Thus, we investigated the influence of miR-338-3p on SphK2 and LC proliferation. We found that the hypernomic expression of miR-338-3p suppressed the levels of

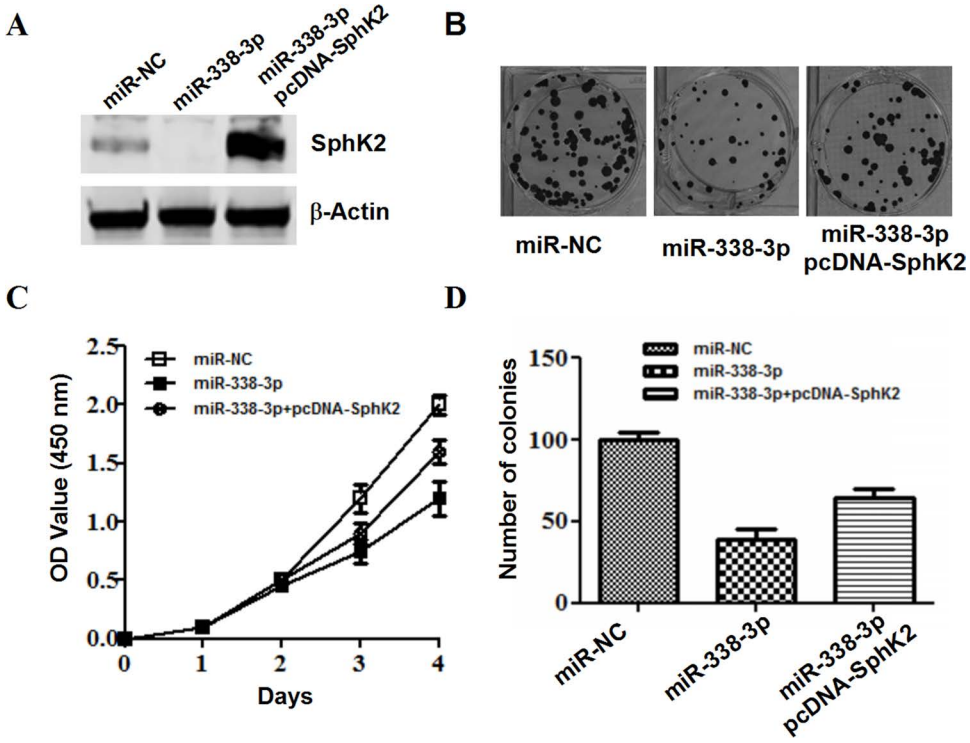

Figure 5. Overexpression of SphK2 lacking the 3′-UTR rebuilds miR-338-3p's influence on proliferation of LC cells. (A) SphK2 expression was measured by Western blotting in HepG2 cells transfected with the indicated plasmid. (B) Analysis of cell proliferation assay in HepG2 cells transfected with the indicated plasmid. (C) Colonies formed in HepG2 cells transfected with the indicated plasmid. (D) Quantification of relative colony generation in HepG2 cells transfected with the indicated plasmid. Data are the mean± standard error.

SphK2, while expression of anti-miR-338-3p increased the expression level of SphK2. Knockdown of SphK2 clearly mimicked the suppressive role of miR-338-3p in LC proliferation. Overexpression of SphK2 lacking the 3′-UTR also significantly restored miR-338-3p-triggered growth suppression in LC cells. These results strongly indicate that SphK2 is a promising target for anticancer treatment because of its oncogene function.

In summary, we demonstrated the function of miR-338-3p as a growth-suppressive miRNA in human LC, at least partially through downregulation of SphK2, providing innovative perspectives into the mechanisms of LC progression.

ACKNOWLEDGMENTS: This work was supported by the Key Project of Medical Science Research in Hebei Province (20150335). The authors declare no conflicts of interest.

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