Pre-Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
50(1) 1–17

DOI: 10.1177/03000605211053717
journals.sagepub.com/home/imr
SAGE

SPOCD1 regulated by miR-133a-3p promotes hepatocellular carcinoma invasion and metastasis

Tianying Zheng, Xin Zhang, Yonggang Wang and Aijun Wang

Abstract

Objective: To investigate the tumorigenic role of spen paralogue and orthologue C-terminal domain-containing 1 (SPOCD1) in hepatocellular carcinoma (HCC) and identify the upstream regulatory mechanism.

Methods: We analyzed SPOCD1 and miR-133-3p expression in normal and HCC tissues from the Cancer Genome Atlas and UALCAN databases, and in normal hepatocytes and HCC cell lines by real-time quantitative polymerase chain reaction and western blot. We identified the miR-133a-3p-binding site on the SPOCD1 3′-untranslated region using TargetScan. Hierarchical regulation was confirmed by luciferase assay and miR-133a-3p overexpression/silencing. Cell proliferation, migration, invasion, and colony formation were assessed by MTT, scratch, transwell, and clonogenic assays, respectively.

Results: SPOCD1 was highly expressed in HCC tissues and cell lines, while miR-133a-3p expression was significantly downregulated. Kaplan–Meier analysis indicated that high SPOCD1 expression was significantly associated with poor survival. TargetScan and luciferase reporter assay revealed that SPOCD1 was the downstream target of miR-133a-3p. Overexpression of miR-133a-3p significantly inhibited the expression of SPOCD1, while miR-133a-3p knockdown significantly increased SPOCD1 expression.

Conclusion: SPOCD1, regulated by miR-133a-3p, promotes HCC cell proliferation, migration, invasion, and colony formation. This study provides the first evidence for the role of the miR-133a-3p/SPOCD1 axis in HCC tumorigenesis.

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Keywords
Spen paralogue and orthologue C-terminal domain-containing 1, miR-133a-3p, hepatocellular carcinoma, survival, tumorigenesis, gene expression

Date received: 5 May 2021; accepted: 28 September 2021

Introduction

According to global cancer statistics (GLOBOCAN), hepatocellular carcinoma (HCC) was the most common form of primary liver cancer (75%–85%), the sixth most commonly diagnosed cancer, and the third-leading cause of cancer-related deaths worldwide in 2020.[1] The highest incidences of HCC occur in East Asia, mainly in China, Korea, and sub-Saharan Africa. The diagnosis and staging of HCC are based on clinical, biological, and imaging features, and the main treatments include surgical resection, local ablation, and liver transplantation.[2,3] However, the lack of early specific manifestations and effective early diagnosis of HCC mean that most patients are initially diagnosed with advanced-stage disease.[4–6] Despite various treatment options, the SEER Cancer Statistics Review (1975–2016) reported that the overall prognosis of HCC patients in terms of survival remains dismal, with a 5-year survival rate of about 20%.[7] There is thus an urgent need to identify potential therapeutic targets for HCC and to clarify the underlying molecular mechanisms.

The spen paralogue and orthologue C-terminal domain-containing 1 protein (SPOCD1) belongs the transcription factor S-II (TFIIS) family. SPOCD1 was recently identified as an oncogene involved in tumor progression, which is upregulated in a variety of tumors, including ovarian cancer,[8] osteosarcoma,[9] and glioma.[10] Current studies showed that SPOCD1 promoted the progression of ovarian cancer and glioma via the phosphoinositide 3-kinase (PI3K)/Akt and pentraxin-related protein (PTX3) pathways, respectively. However, the upstream factors regulating SPOCD1, such as microRNAs (miRNAs), are still unclear. In addition, whether or not SPOCD1 acts as an oncogene in HCC to regulate the proliferation, migration, invasion, and colony formation of HCC cells remains unknown.

miRNAs are small non-coding endogenous RNAs with a length of approximately 22 nucleotides, which function to regulate gene transcription by binding to their target mRNA at the posttranscriptional level.[11] Abnormally expressed miRNAs may target the 5′- or 3′-untranslated regions (UTRs) of their target genes to regulate tumor proliferation, migration, and invasion. Dysregulation of miRNAs has thus been related to tumorigenesis and poor outcomes in patients with HCC.[12] Among these miRNAs, the tumorigenic role of miR-133a-3p has recently received widespread attention. In addition to alleviating oxidative stress,[13] miR-133a-3p expression has been related to tumor progression and it has been shown to be aberrantly expressed in various tumors, such as esophageal squamous cell carcinoma,[14] oral squamous cell carcinoma,[15] thyroid cancer,[16] gastric cancer,[17] and prostate cancer.[18] However, few studies have explored the role and possible regulatory mechanisms of miR-133a-3p in HCC. In addition, the hierarchical relationship between SPOCD1 and miR-133a-3p in HCC remains unclear.

Although various drugs, such as sorafenib, nivolumab, and lenvatinib, have been approved for the treatment of unresectable HCC, these treatments are not completely

satisfactory because of the rapid development of drug resistance and toxicity. There is thus an urgent need to identify more potential therapeutic targets for HCC treatment. In view of recent evidence indicating an oncogenic role for SPOCD1 in various cancers, we investigated its tumorigenic role in HCC and explored its upstream regulatory mechanism. This study aimed to evaluate the expression profiles of miR-133a-3p and SPOCD1 in HCC, and to explore the role of the miR-133a-3p/SPOCD1 axis in the proliferation, migration, invasion, and colony-forming ability of HCC cells. The results of this study shed light on the upstream regulation of SPOCD1 by miR-133a-3p, and indicate possible new therapeutic targets for HCC with SPOCD1 overexpression.

Materials and Methods

Analysis of microarray data and UALCAN dataset

HCC RNA-seq data were retrieved from The Cancer Genome Atlas (TCGA) database and analyzed using R (www.r-project.org). The screening conditions for differentially expressed mRNAs were $|\log \text{fold-change}| > 1.2$ and an adjusted P-value < 0.05. After removing unknown genes and missing information, SPOCD1 was identified as a potential differentially expressed gene in HCC. We used UALCAN (http://ualcan.path.uab.edu/cgi-bin/ualcan-res-mir.pl) to further confirm the impact and prognostic relevance of SPOCD1 in HCC patients. Transcripts per million values above and below the upper quartile were classified as high and low SPOCD1 expression, respectively. The TargetScan (http://www.targetscan.org/vert_72/) database was used to identify potential upstream regulatory miRNA candidates of SPOCD1. According to the site type and weighted contex++ score calculated by TargetScan, miR-133a-3p was identified as a potential upstream miRNA.

Cell culture and transfection

Normal human HL-7702 hepatocytes and HepG2, HUH-7, MHCC-97H, and PLC HCC cell lines were purchased from the BeNa Culture Collection (Beijing, China). All cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.), 100 U/mL streptomycin, and 100 U/mL penicillin at 37°C in an atmosphere of 95% air and 5% CO_2.

Cell transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. HepG2 cells grown to 70% confluency were transfected with scramble control (sh-NC), short hairpin RNA (shRNA) against miR-133a-3p (sh-miR-133a-3p), mimic control (mimic-NC), or miR-133a-3p mimics (miR-133a-3p). For luciferase reporter assay, HepG2 cells were co-transfected with pGL3 reporter constructs containing the wild-type 3′-UTR of SPCOD1 (SPOCD1-WT) or a 3′-UTR with a mutant miR-133a-3p-binding site (SPOCD1-Mut), and mimic-NC or miR-133a-3p. The above plasmids were purchased from Shanghai Genechem Company (Shanghai, China). The medium was replaced with fresh culture medium 6 hours after transfection, and the cells were cultured until the next experiment.

Real-time reverse transcription polymerase chain reaction (RT-qPCR)

Total RNAs were extracted from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Complementary DNA was created and subjected to

RT-qPCR using a PrimeScript RT reagent kit (Takara, Dalian, China) and SYBR Prime Script RT PCR kit (Takara), respectively. The primer sets used in this study were as follows: miR-133a-3p (forward primer: 5′-GCCTTTGGTCCCCTTCAAC-3′; reverse primer: 5′-TATGCTTGTTCTC GTCTCTGTGTC-3′), SPOCD1 (forward primer: 5′-CCCCATGGAGTGAAGCTTG T-3′, reverse primer: 5′-GCACCATGGG CCTTTTCTTC-3′), U6 (forward primer: 5′-CTCGCTTCGGCAGCACA-3′, reverse primer: 5′-AACGCTTCACGAATTTGCG T-3′), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer: 5′-GGAGCGAGATCCCTCCAAAAT-3′, reverse primer: 5′-GGCTGTTGTCATACT TCTCATGG-3′). Relative changes in expression levels of SPOCD1 and miR-133a-3p were calculated using the $2^{-\Delta\Delta CT}$ method. U6 and GAPDH were used as internal controls for miR-133a-3p and SPOCD1, respectively.

Western blot analysis

Western blot analysis was performed as described previously.[19] Briefly, cells used for western blot analysis were trypsinized and collected by centrifugation, and lysed in RIPA lysis buffer. Soluble proteins were collected and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes, and blocked with 5% skimmed milk for 1 hour. The membranes were then incubated with rabbit polyclonal antibody anti-SPOCD1 (Abcam, Cambridge, UK; #ab122188; 1:1000 dilution) or rabbit polyclonal antibody against GAPDH (Cell Signaling Technology, Cambridge, MA, USA; #2118; 1:4000 dilution) at 4°C for 2 hours. The immunoblots were washed twice with TBS containing 0.1% Tween 20 (TBST) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Abcam; #ab6721; 1:2000 dilution) at room temperature for 1 hour. After washing twice with TBST, the chemiluminescent signals on the immunoblots were visualized using a Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA; #170-5061) and imaged using a Tanon 5200 fully automatic chemiluminescence image analysis system (Tanon Science and Technology Co., Ltd., Shanghai, China).

Measurement of cell proliferation, migration, invasion, and colony-forming ability

Cell proliferation was assessed by MTT assay. Cells from each group were re-seeded at 5×10^3 cells per well in 96-well plates for the indicated times. MTT reagent (Sigma-Aldrich, St. Louis, MO, USA (10 μL) was then added into each well and further incubated at 37°C for 4 hours. After removing the supernatant and adding 200 μL of dimethyl sulfoxide, the absorbance was measured at 490 nm using a microplate reader (Bio-Rad Laboratories).

Cell migration ability was determined by wound-healing assay (scratch assay), as described previously.[20] Briefly, cells grown in DMEM containing 10% FBS were re-seeded in six-well plates for 24 hours to reach confluence. After aspirating the medium and adding fresh culture medium, a linear scratch was created on the cell monolayers using a sterile 200-μL pipette tip. The scratched area of each well was photographed using a Leica DMI3000B computer-assisted microscope (Leica, Buffalo Grove, IL, USA) and set as day 0. Images were captured in the same area at 24, 48, and 72 hours after scratching, and the open wound area was analyzed using Image-Pro Plus v6.0 image analysis

software (Media Cybernetics, Rockville, MD, USA).

To assess cell invasion, 1×10^5 HepG2 cells were resuspended in serum-free DMEM and seeded on the Matrigel-coated membrane of the upper transwell chamber (Corning Inc., Corning, NY, USA). The lower chamber was filled with DMEM medium with 10% FBS. After incubation for 12 hours, the cells in the upper chamber were wiped off and the invaded cells on the bottom of each well were stained with crystal violet at 25°C for 1 minute. Images of the stained cells were captured under a light microscope (Nikon Eclipse; Nikon, Tokyo, Japan; 100×), and the stained cells from five randomly selected fields were counted.

Colony-forming ability was assessed by clonogenic cell survival assay as described previously.[21] Briefly, cells were trypsinized to produce a single-cell suspension, seeded into six-well culture plates (100 cells per well), and incubated at 37°C to produce sufficiently large clones. The colonies were fixed and stained with 0.5% methylene blue in 50% ethanol for 12 hours. The number of colonies was counted manually and images of the colonies were captured to illustrate the colony size.

Dual luciferase reporter assay

The 3′-UTR activity of SPOCD1 was determined by dual luciferase reporter assay, as described previously.[22] In brief, HepG2 cells were transfected with mimic-NC or miR-133a-3p mimics for 24 hours, trypsinized, resuspended, and reseeded into 12-well culture plates at a density of 1×10^5 cells per well. After overnight culture, the cells were transfected with reporter constructs (SPOCD1-WT or SPOCD1-Mut) using Lipofectamine 3000 (Gibco Invitrogen) and incubated for a further 24 hours. The cells were harvested and lysed, and luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI, USA).

Statistical analysis

All statistical analyses were performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). Continuous data were presented as mean ± standard deviation. Differences between groups were analyzed by *t*-tests. All experiments in the study were repeated three times. A value of $P < 0.05$ was considered statistically significant.

Results

SPOCD1 was upregulated in HCC and was associated with poor survival

We investigated the relationship between SPOCD1 expression and HCC tumorigenesis by analyzing its expression levels in normal and HCC tissues using TCGA database. Expression of SPOCD1 was significantly upregulated in HCC tissues compared with normal liver tissues ($P < 0.001$; Figure 1a). The increased expression of SPOCD1 was further validated in normal hepatocytes (HL-7702 cells) and HCC cells (HepG2, HUH-7, MHCC-97H, and PLC cells). SPOCD1 mRNA levels were significantly increased in the four HCC cell lines by approximately two- to five-fold, compared with levels in HL-7702 hepatocytes ($P < 0.05$; Figure 1b). Western blot analysis also showed that SPOCD1 protein levels were significantly higher in HepG2, HUH-7, MHCC-97H, and PLC cells compared with HL-7702 hepatocytes (Figure 1c), indicating that SPOCD1 was upregulated in HCC tissues and cell lines. We also estimated the prognostic value of SPOCD1 in HCC patients. Survival analysis showed that higher SPOCD1 gene expression was significantly associated with poorer survival among patients with HCC ($P = 0.01$; Figure 1d).

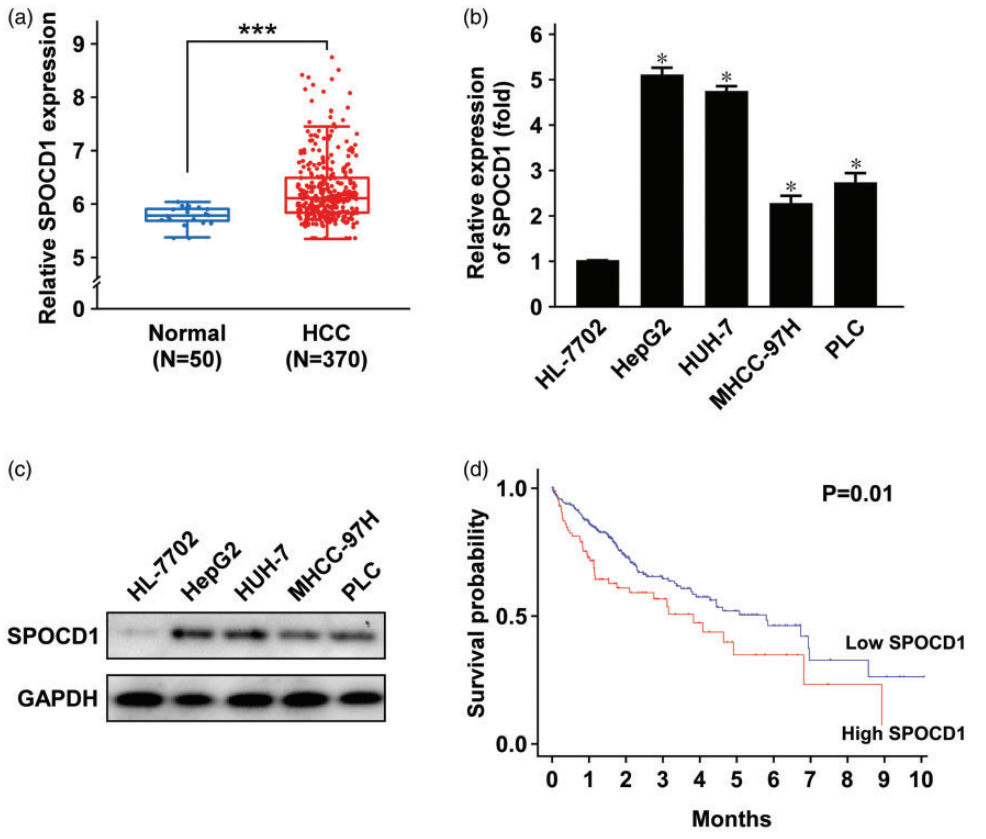

Figure 1. SPOCD1 is upregulated in hepatocellular carcinoma (HCC) and is associated with poor survival. (a) SPOCD1 expression levels were increased in HCC tissues (n = 370) compared with adjacent normal tissues (n = 50) based on RNA-seq data from The Cancer Genome Atlas. ***$P < 0.001$. (b) SPOCD1 expression levels in normal hepatocyte (HL-7702) and HCC cell lines (HepG2, HUH-7, MHCC-97H, and PLC) shown by real-time reverse transcription quantitative polymerase chain reaction. Transcript levels normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *$P < 0.05$. (c) SPOCD1 protein expression levels in normal hepatocyte (HL-7702) and HCC cell lines (HepG2, HUH-7, MHCC-97H, and PLC) shown by western blotting, with GAPDH as an internal control. (d) Survival probability based on SPOCD1 expression in 365 HCC patients. Higher SPOCD1 expression was associated with significantly poorer survival ($P = 0.01$). Transcript per million values above and below the upper quartile were classified as high and low SPOCD1 expression, respectively.

These results suggest that SPOCD1 may play a crucial role in HCC tumorigenesis.

Down-regulation of SPOCD1 inhibited cell proliferation, migration, invasion, and colony formation

We further explored the biological role of SPOCD1 in HCC tumorigenesis by examining its functional involvement in HCC cell proliferation, migration, invasion, and colony formation. SPOCD1 mRNA levels were significantly downregulated in HepG2 cells transfected with sh-SPOCD1 compared with cells transfected with sh-NC (data not shown). Western blot analysis indicated that SPOCD1 protein levels were significantly reduced at 48 hours after

transfection (upper right, Figure 2a). We also conducted MTT, scratch, transwell, and clonogenic assays to examine cell proliferation, migration, invasion, and colony-forming ability, respectively. MTT assay showed that cell proliferation was significantly reduced when SPOCD1 expression was inhibited ($P < 0.05$; Figure 2a), and scratch assay showed that transfection with sh-SPOCD1 significantly reduced cell migration compared with cells transfected with sh-NC ($P < 0.05$; Figure 2b). In addition, knockdown of SPCOD1 significantly reduced cell invasion by about 80% in transwell analysis ($P < 0.05$; Figure 2c). SPOCD1 knockdown also significantly reduced colony numbers by about 50% (Figure 2d) and reduced the colony size (Figure 2d, right panel), thus supporting an inhibitory effect of SPOCD1 on cell proliferation. In summary, the results of MTT, scratch, transwell, and clonogenic assays suggested that SPOCD1 played an oncogenic role in HCC and was functionally involved in cell proliferation, migration, invasion, and colony formation.

SPOCD1 was a downstream target of miR-133a-3p

To explore the molecular mechanism by which SPOCD1 promoted HCC cell proliferation, migration, invasion, and colony formation, we conducted TargetScan analysis to identify its upstream regulatory miRNAs. Among 1023 miRNAs, miR-133a-3p was identified as an miRNA that could bind to the SPOCD1 3′-UTR at positions 229 to 235 (Figure 3a). We examined the expression levels of miR-133a-3p in normal and HCC tissues, and showed that miR-133a-3p expression was significantly downregulated in HCC tissues ($P < 0.01$; Figure 3b). Downregulation of miR-133a-3p was further confirmed in HCC and normal hepatocyte cell lines, with significantly lower expression in HepG2, HUH-7, MHCC-97H, and PLC cells compared with HL-7702 hepatocytes ($P < 0.05$; Figure 3c). We determined if miR-133a-3p regulated the expression of SPOCD1 by transfecting HepG2 cells with miR-133a-3p mimics and inhibitors to overexpress and inhibit miR-133a-3p levels, respectively. Transfection with miR-133a-3p mimic significantly increased miR-133a-3p expression while transfection with miR-133a-3p inhibitor significantly reduced miR-133a-3p expression ($P < 0.05$; Figure 3d). Overexpression of miR-133a-3p significantly reduced SPOCD1 mRNA levels by about 55% ($P < 0.05$; Figure 3e). In addition, miR-133a-3p overexpression down-regulated SPOCD1 protein expression, as confirmed by western blot analysis, while inhibition of miR-133a-3p significantly upregulated SPOCD1 mRNA and protein levels ($P < 0.05$; Figure 3f). We further determined if miR-133a-3p regulated SPOCD1 expression by affecting the activity of its 3′-UTR. miR-133a-3p overexpression (miR-133a-3p mimic) significantly reduced the luciferase activity in HepG2 cells transfected with reporter constructs containing the SPOCD1 wild-type 3′-UTR compared with cells transfected with mimic control (mimic-NC) (SPOCD1-WT, $P < 0.05$; Figure 3g). However, miR-133a-3p mimic had no inhibitory effect on reporter constructs containing the mutant 3′-UTR (SPOCD1-Mut). These results indicate that miR-133a-3p acted as an upstream miRNA to negatively regulate the expression of SPOCD1 in HCC.

Down-regulation of miR-133a-3p increased HCC cell proliferation, migration, invasion, and colony formation

We determined if miR-133a-3p regulated the proliferation, migration, invasion, and colony-forming ability of HCC cells. Silencing of miR-133a-3p significantly increased cell proliferation by 260%

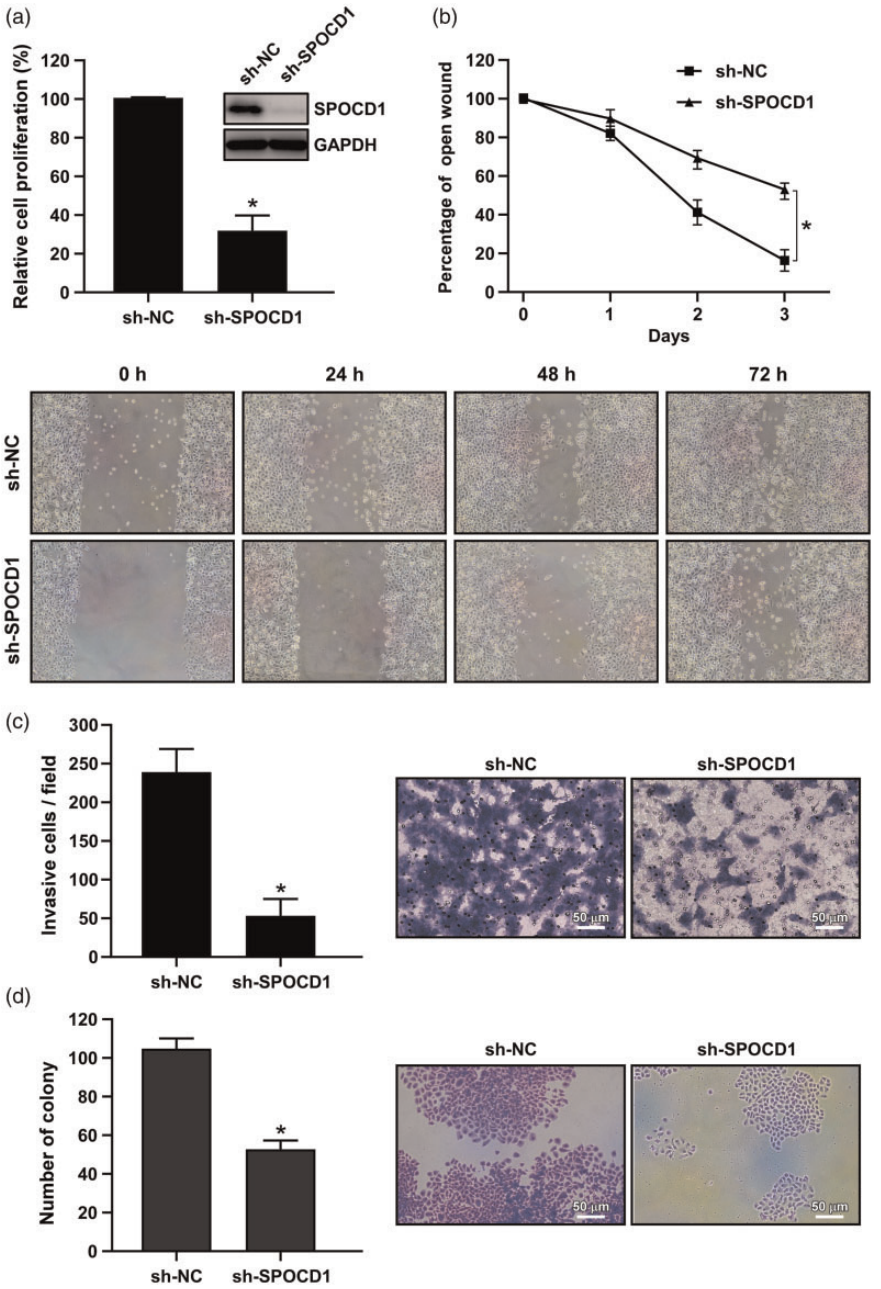

Figure 2. SPOCD1 down-regulation inhibited cell proliferation, migration, invasion, and colony formation. (a) HepG2 cells were transfected with shRNA negative control (sh-NC) or shRNA against SPOCD1 (sh-SPOCD1) for 48 hours, and the mRNA and protein expression levels of SPOCD1 were determined by real-time reverse transcription-quantitative polymerase chain reaction and western blot, respectively. The viability of HepG2 cells was significantly reduced after 72 hours of knockdown of SPOCD1 by sh-SPOCD1, as shown by MTT assay. (b) SPOCD1 promoted migration of HepG2 cells, as shown by scratch assay.

($P < 0.05$; Figure 4a). The wound closure rate on day 3 was significantly higher in cells transfected with sh-miR-133a-3p compared with cells transfected with sh-NC, according to scratch assay ($P < 0.05$; Figure 4b), suggesting that sh-miR-133a-3p silencing increased cell migration. The results of transwell and clonogenic assays showed that miR-133a-3p silencing significantly increased the numbers of invading cells ($P < 0.05$; Figure 4c) and colonies ($P < 0.05$; Figure 4d), while colony size was larger in cells transfected with sh-miR-133a-3p compared with sh-NC (Figure 4d, right panel). Overall, these results indicate that the miR-133a-3p/SPOCD1 axis promoted the proliferation, migration, invasion, and colony-formation of HCC cells.

SPOCD1 silencing rescued miR-133a-3p-mediated cell migration and invasion

We also performed rescue experiments to examine if miR-133a-3p regulated cell migration, invasion, and colony formation by regulating *SPOCD1*. miR-133a-3p knockdown significantly increased the expression of SPCOD1 ($P < 0.05$; Figure 5a), and this effect was significantly attenuated by further knockdown of SPOCD1 expression in sh-miR-133a-3p-transfected cells ($P < 0.05$). Subsequent MTT assay showed that cell proliferation increased by miR-133a-3p silencing was also significantly reduced after simultaneous knockdown of SPOCD1 expression ($P < 0.05$; Figure 5b). The rate of wound closure at 72 hours was significantly lower in cells transfected with sh-miR-133a-3p and sh-SPOCD1 compared with cells transfected with sh-miR-133a-3p (Figure 5c). In addition, simultaneous knockdown of SPOCD1 expression significantly reduced the increases in cell invasion ($P < 0.05$; Figure 5d) and colony formation ($P < 0.05$; Figure 5e) mediated by silencing miR-133a-3p. Collectively, these rescue experiments indicated that miR-133a-3p regulated cell migration, invasion, and colony formation by regulating the expression of SPOCD1.

Discussion

Liver cancer remains one of the most common causes of cancer-related death worldwide. Although the overall incidence and mortality of all cancer types has gradually declined, the incidence and mortality of liver cancer continue to rise.[23,24] A study using data from the Surveillance, Epidemiology and End Results (SEER) Program estimated that the incidence of HCC in the USA would exceed 56,000 cases in 2030.[25] Despite progress in the treatment of HCC, there is thus still an urgent need to identify novel potential therapeutic targets to improve the survival of patients with HCC. In the current study, we provided the first evidence showing that SPOCD1 was upregulated in HCC, and that its high expression was associated with a poor prognosis in patients with HCC. In contrast, miR-133a-3p expression

Figure 2. Continued
The wound closure rate at 72 hours was significantly lower in cells transfected with sh-SPOCD1 than in cells transfected with sh-NC. Error bars represent standard deviation of three independent experiments.
*$P < 0.05$. (c) SPOCD1 promoted HepG2 cell invasion, as shown by transwell assay. SPOCD1 knockdown significantly reduced the invasion ability of HepG2 cells. (d) SPOCD1 knockdown suppressed colony formation, as shown by clonogenic assay. Colonies formation was significantly reduced in HepG2 cells transfected with sh-SPOCD1 compared with sh-NC. Data presented as means of triplicate experiments.
*$P < 0.05$.

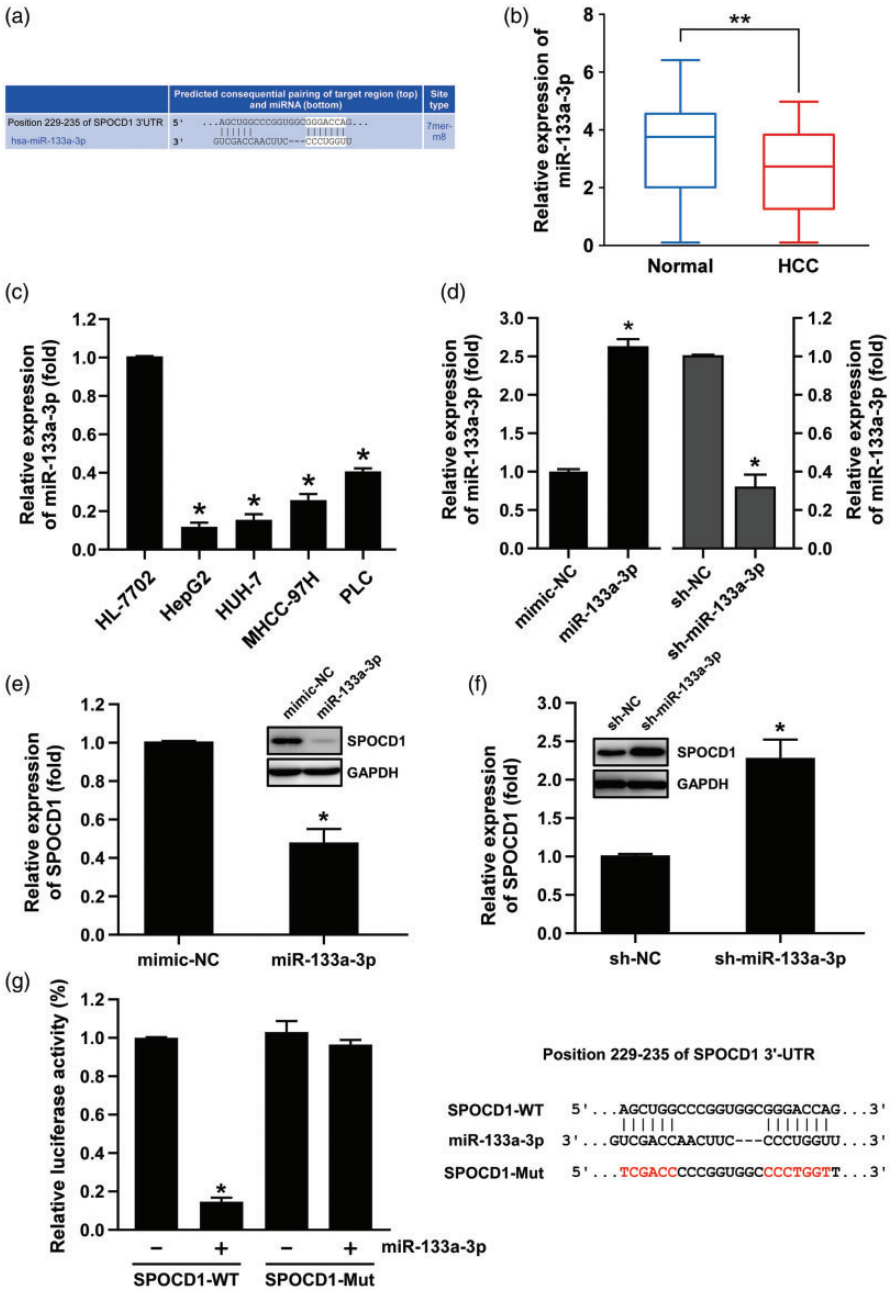

Figure 3. SPOCD1 is the downstream target of miR-133a-3p. (a) TargetScan prediction of the miR-133a-3p binding site on the 3′-untranslated region (UTR) of SPOCD1. (b) miR-133a-3p expression was significantly downregulated in hepatocellular carcinoma (HCC) tissues (n = 73) compared with non-tumor hepatic tissues (n = 70). **P < 0.01. (c) Expression levels of miR-133a-3p in HL-7702 hepatocytes and HCC cell lines (HepG2, HUH-7, MHCC-97H, and PLC cells) were determined by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). (d) miR-133a-3p expression levels were significantly increased

was negatively correlated with the expression of SPOCD1 and with HCC cell proliferation, migration, invasion, and colony formation. Bioinformatics analysis and cell experiments identified SPOCD1 as a downstream target of miR-133a-3p, and targeting SPOCD1 and miR-133a-3p dramatically reduced HCC cell proliferation, migration, invasion, and colony formation. These results thus revealed a new regulatory role for the miR-133a-3p/SPOCD1 axis in HCC tumorigenesis, in which miR-133a-3p binds to the 3′-UTR of SPOCD1 to inhibit its transcription and expression, subsequently weakening the tumorigenesis of HCC cells.

In this study, we identified SPOCD1 as an oncogene responsible for cell proliferation, migration, invasion, and colony formation in HCC. An oncogenic role of SPOCD1 has also been supported by other recent studies on glioma and ovarian cancer.[8,10] Liu et al.[10] showed that high SPOCD1 expression in glioma was associated with advanced tumor grade and poor prognosis, and that SPOCD1 positively regulated the expression of PTX3. However, expression levels of PTX3 vary among HCC cell lines,[26] and it is considered to have no diagnostic value in HCC patients because its expression is not related to the TNM classification of HCC.[27] The results of the current UALCAN analysis also found no relationship between PTX3 expression level and tumor grade or survival prognosis in HCC patients (data not shown). This suggests that SPOCD1 may promote HCC tumorigenesis through a PTX3-independent pathway. Liu et al.[8] recently showed that downregulation of SPOCD1 in ovarian cancer cells not only significantly reduced cell migration and invasion, but also inhibited the PI3K/Akt signaling pathway. Interestingly, the mammalian target of rapamycin/PI3K/Akt pathway has also been shown to be closely related to the development of hepatitis and fibrosis, HCC cell migration and invasion, and a poor prognosis in HCC patients.[28–30] In the current study, SPOCD1 was negatively regulated by miR-133a-3p, and miR-133a-3p was significantly downregulated in HCC tissues and cell lines. Although the correlation between miR-133a-3p and PI3K/Akt signaling in HCC is still clear, Tang et al.[18] demonstrated that downregulation of miR-133a-3p activated PI3K/Akt signaling to promote prostate cancer cell survival. Whether PI3K/Akt is the downstream signaling pathway of miR-133a-3p/SPOCD1 and participates in the proliferation, migration, invasion, and tumor formation of HCC cells thus warrants further investigation.

Recent studies suggested that abnormal expression of oncogenes is often caused by aberrant expression of miRNAs.[31] Several miRNAs have been identified as tumor suppressors in liver cancer, and their downregulation in tumors promotes cell proliferation, differentiation, apoptosis, migration, and invasion. Tumor suppressor

Figure 3. Continued
and decreased in HepG2 cells transfected with miR-133a-3p mimic and miR-133a-3p inhibitor, respectively, as shown by RT-qPCR. Transfection of (e) miR-133a-3p mimic and (f) miR-133a-3p inhibitors significantly reduced and increased SPOCD1 mRNA and protein expression levels in HepG2 cell, respectively. *$P < 0.05$. (g) Sequences of wild-type (SPOCD1-WT) and mutated (SPOCD1-Mut) SPOCD1 3′-UTR (right panel). Luciferase reporter assay was conducted in HepG2 cells co-transfected with reporter constructs containing SPOCD1-WT, SPOCD1-Mut, mimic-NC, or miR-133a-3p mimic. Data presented as means of triplicate experiments. *$P < 0.05$.

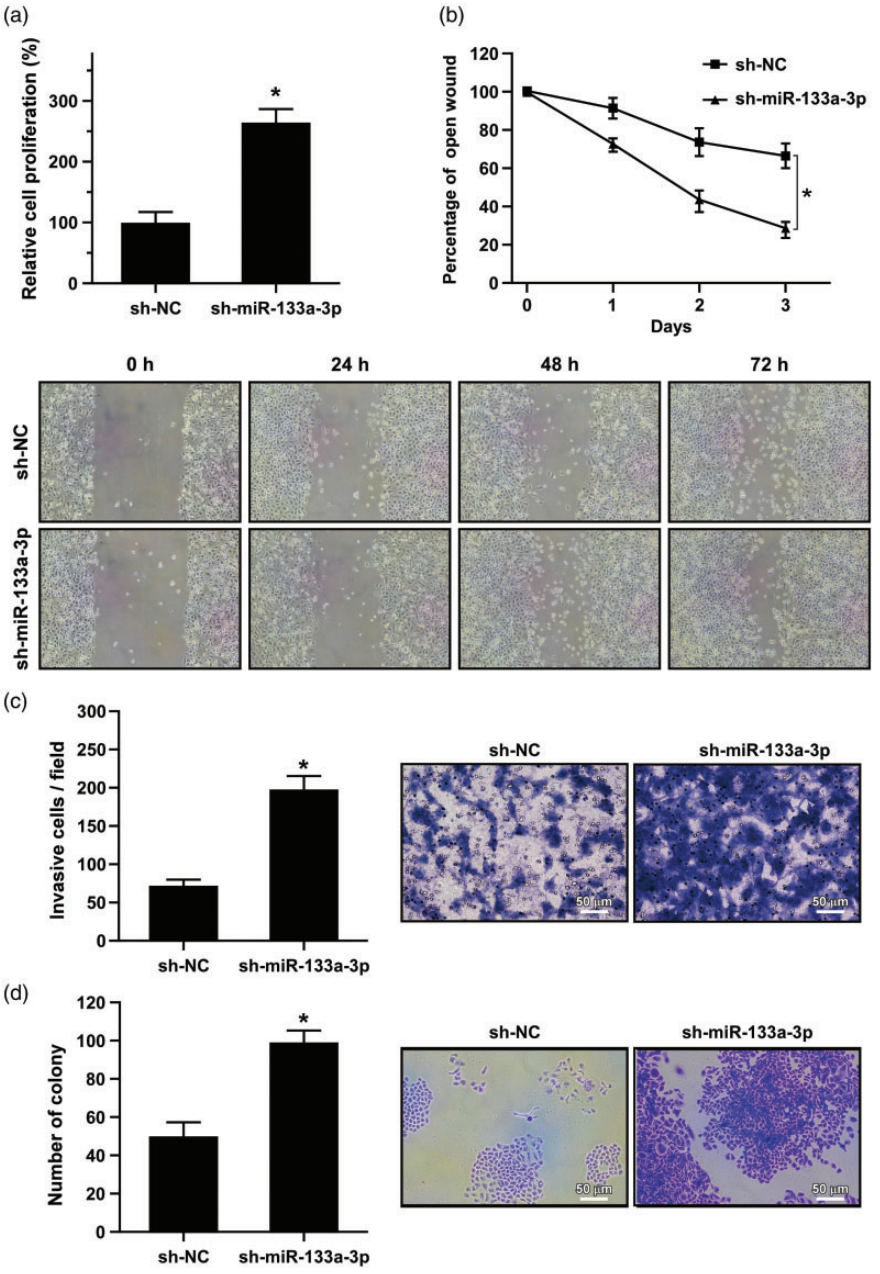

Figure 4. Silencing miR-133a-3p expression increased the proliferation, migration, invasion, and colony-forming ability of HepG2 cells. (a) HepG2 cells were transfected with shRNA negative control (sh-NC) or shRNA against miR-133a-3p (sh-133a-3p), and the cell viability was determined by MTT assay. (b) miR-133a-3p silencing increased the migration ability of HepG2 cells, as shown by scratch assay. The wound closure rate at 72 hours was significantly higher in cells transfected with sh-miR-133a-3p than in cells transfected with sh-NC. Error bars represent standard deviation of three independent experiments. *$P < 0.05$. (c) miR-133a-3p silencing promoted the invasion of HepG2 cells, as shown by transwell assay. (d) Silencing of miR-133a-3p increased the colony-forming ability of HepG2 cells, as shown by clonogenic assay. Data presented as means of triplicate experiments. *$P < 0.05$.

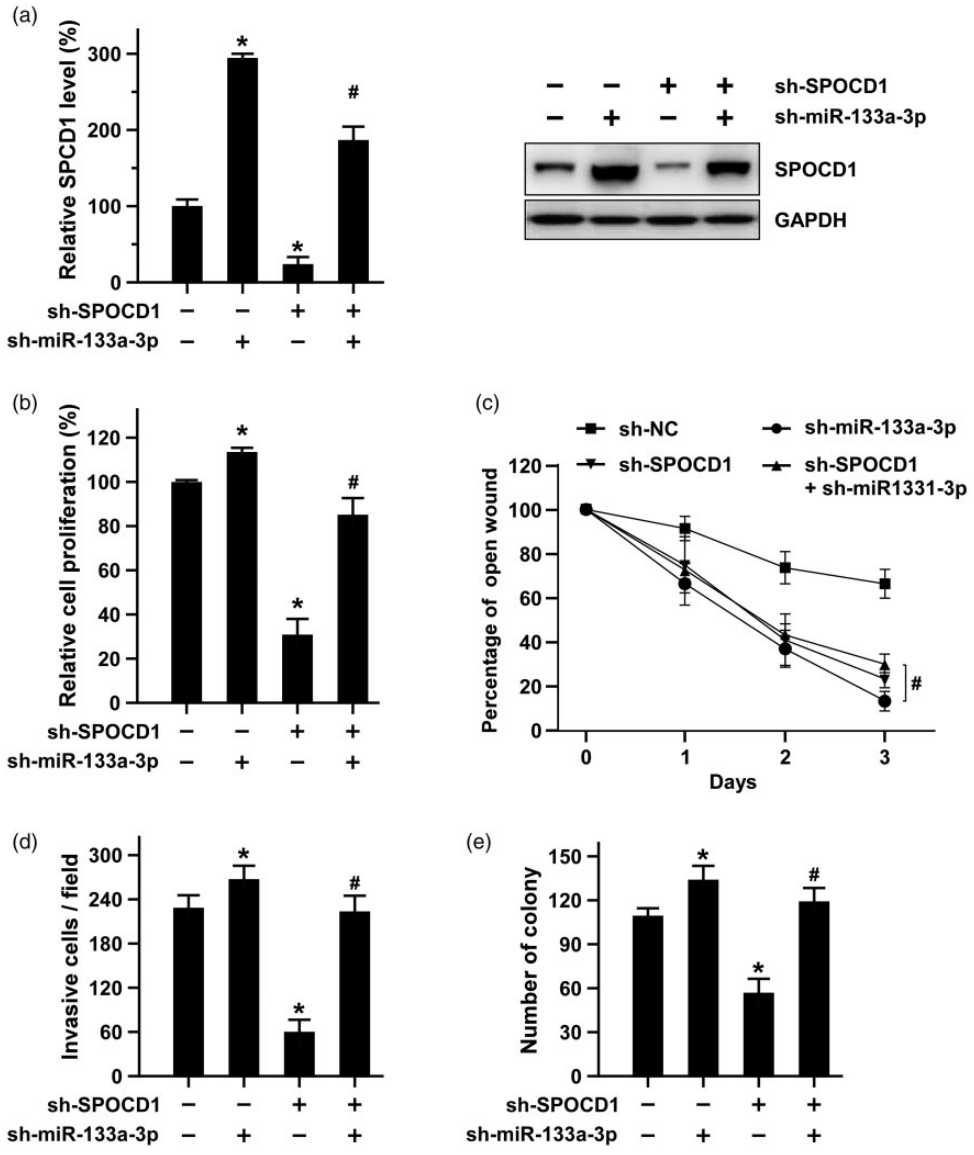

Figure 5. SPOCD1 knockdown reversed the regulatory effector of miR-133a-3p on cell proliferation, migration, invasion, and colony formation. (a) HepG2 cells were transfected with sh-SPOCD1 and/or sh-miR133a-3p, and SPOCD1 protein expression was examined by western blotting. The effects of SPOCD1 restoration on cell proliferation (b), migration (c), invasion (d), and colony formation (e) were determined by MTT, scratch, transwell, and clonogenic assays, respectively. *$P < 0.05$ compared with control group (sh-NC + mimic-NC); #$P < 0.05$ compared with sh-SPOCD1 group.

miRNAs in HCC include miR-29, miR-101, miR-122, miR-124, miR-145, miR-148, miR-193, miR-195, miR-199, miR-206, miR-370, miR-375, and miR-766.[32,33] We conducted a TargetScan analysis and cell experiments that further identified miR-133a-3p as a tumor suppressor in HCC, which could negatively regulate cell proliferation, migration, invasion, and colony formation by targeting SPOCD1. A single miRNA targets and regulates many downstream genes, and one miRNA can thus promote tumorigenesis by targeting different oncogenes or tumor suppressor genes in different tumors. Recent studies found that miR-133a-3p regulated different downstream oncogenes in different tumors. In oral squamous cell carcinoma, miR-133a-3p bound to the 3′-UTR and inhibited the expression of collagen type I alpha 1,[15] which is the main component of type 1 collagen and is responsible for tumor cell migration and invasion. In addition, miR-133a-3p positively regulated the expression of the transcription factors CREB1 and FOXP3 in retinoblastoma and gastric cancer cells, respectively, thereby affecting the cell cycle, proliferation, apoptosis, and autophagy.[17,34] Shi et al.[35] revealed the involvement of an miR-133a-3p/MAML1/DNMT3A axis in breast cancer cell proliferation, migration, and invasion. Hypermethylation of the miR-133a-3p promoter inhibited its expression and promoted the proliferation and metastasis of breast cancer cells by up-regulating the transcriptional coactivator MAML1 and subsequent DNMT3A expression. In a long non-coding RNA microarray study,[36] miR-133a-3p regulated osteosarcoma cell proliferation and apoptosis by parathyroid hormone type 1 receptor, and Han et al.[37] reported that miR-133a-3p regulated HCC cell behaviors by targeting the coronin-like actin-binding protein 1C gene (CORO1C), which plays a key role in regulating actin dynamics via effects on assembly by the actin-related protein 2/3 complex and disassembly by cofilin. Although SPOCD1 is functionally different from CORO1C, both genes are regulated by miR-133a-3p and are involved in cell migration and invasion. To the best of our knowledge, the regulatory relationship between CORO1C and SPOCD1 remains unknown. miR-133a-3p may therefore regulate cell proliferation, migration, invasion, and colony formation through two independent pathways (SPOCD1 and CORO1C), or SPOCD1 may directly or indirectly affect the expression of CORO1C, thereby regulating HCC migration and invasion (based on the transcriptional nature of SPOCD1). Further studies are needed to investigate a possible hierarchical relationship between SPOCD1 and CORO1C, and to evaluate the prognostic values of miR-133a-3p-regulated SPOCD1 and CORO1C in patients with HCC.

This study had some potential limitations. There was a lack of large clinical HCC samples to clarify the relationships between the expression of miR-133a-3p/SPOCD1 and HCC tumor grade, clinicopathologic parameters, and survival outcomes. A retrospective cohort study is therefore needed to analyze the expression levels of miR-133a-3p and SPOCD1 in a large number of HCC patients with different tumor grades, and to further examine the prognostic roles of miR-133a-3p and SPOCD1 in terms of overall survival of patients with HCC.

Conclusion

This study revealed a tumor suppressor role of the miR-133a-3p/SPOCD1 axis in HCC cell proliferation, migration, invasion, and colony formation. These results suggests that miR-133a-3p and SPOCD1 may thus provide novel therapeutic targets for the treatment of HCC. However, future population-based association studies are needed to examine the relationship between

targeting the miR-133a-3p/SPOCD1 axis, the expression levels of miR-133a-3p and SPOCD1, and survival outcomes.

Availability of data and materials

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

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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