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MiR-21 and miR-183 can simultaneously target SOCS6 and modulate growth and invasion of hepatocellular carcinoma (HCC) cells

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Abstract. – OBJECTIVE: Both miR-21 and miR-183 are upregulated in hepatocellular carcinoma (HCC) and are considered as oncomiR. However, their oncogenic roles are still not fully understood. This study aimed to explore the regulative role of miR-21 and miR-183 over suppressors of cytokine signaling 6 (SOCS6), a negative regulator of cytokine receptor signaling.

MATERIALS AND METHODS: qRT-PCR analysis was performed to assess miR-21 and miR-183 expression in tumor tissues obtained from HCC patients and in HCC cell lines HepG2 and Hep3B. Their regulation over SOCS6 is verified using dual luciferase assay and Western blot analysis. The function of miR-21/miR-183-SOCS6 axis in cell growth, invasion and apoptosis was studied.

RESULTS: MiR-21 and miR-183 expression in HCC tissues than in adjacent normal tissues. Knockdown of miR-21 and miR-183 in HepG2 and Hep3B cells could decrease cell viability, increase cell apoptosis and decrease cell invasion. Based on the dual luciferase assay and Western blot analysis, we confirmed that both miR-21 and miR-183 can simultaneously target SOCS6 and modulate its expression at protein level. Overexpression of SOCS6 without 3'UTR could significantly lower cell growth rate and invasion capability, but increase relative caspase 3/7 activity and the ratio of apoptotic cells. However, these effects could not be blocked by miR-21 or miR-183 mimics.

CONCLUSIONS: This study revealed a novel miR-21/miR-183-SOCS6 axis that might play an important role in modulating cell growth and invasion of HCC cells.

Key Words:
miR-21, miR-183, HCC, SOCS6.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of malignancy of liver cancer, which accounts for over 90% of all cases of liver cancer in China[1,2]. Currently, hepatectomy and liver transplantation are still the most effective therapeutic approaches[3]. The therapeutic effects of current targeting drugs are still limited[4]. Therefore, it is necessary to further explore the molecular mechanism of HCC for future targeting therapy.

The pathologic development HCC is associated with various molecular alterations. A series of miRNAs were aberrantly expressed in HCC cells or tissues compared with non-malignant hepatocytes or tissues. MiRNAs can be either tumor suppressors or oncogenes, depending on their downstream targets. In fact, the regulative network of miRNAs in HCC is quite complex and is far from clearly understanding[5,6]. For example, miR-141 can suppress the migration and invasion of HCC cells by targeting Tiam1[7], ZEB2 [8] and E2F3[9]. miR-24 can promote the proliferation and invasion of HCC cells by targeting SOX7[10]. miR-494 can initiate gene silencing of multiple invasion-suppressor miRNAs by direct targeting of TET1[11]. MiR-21 is a well identified tumor promoter of HCC[12-14]. High miR-21 expression usually predicts poor prognosis of HCC[13,14]. miR-183 is also significantly upregulated in HCC[15]. It can inhibit TGF-beta1-induced apoptosis through reducing PDCD4[16]. Besides, it is also a downstream effector of Wnt/beta-Catenin pathway which promotes HCC cell invasion[17]. However, due to the complex regulative network of miRNAs, the downstream regulation of miR-21 and miR-183 is still not fully understood.

The suppressors of cytokine signaling (SOCS) are a group of negative regulators of cytokine receptor signaling[18]. SOCS6 is a member of the family and is downregulated in several cancers, such as ovarian cancer, lung cancer, colorectal cancer, gas-

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tric cancer, stomach cancer, hepatocellular carcinoma, and pancreatic cancer. It is an important regulator of survival signaling since it can initiate ubiquitination of receptors or substrate proteins and, thereby, inducing apoptosis[18]. However, how it is dysregulated in HCC is still not clear.

In the current study, we demonstrated that miR-21 and miR-183 are overexpressed in HCC and can simultaneously target SOCS6, thereby, enhancing growth and invasion of HCC cells.

Materials and Methods

Human Specimen

This study was approved by the Ethic Committee of Zaozhuang Municipal Hospital. HCC tissues and the corresponding adjacent non-cancerous normal tissues were obtained from 10 HCC patients who received primary curative hepatectomy at Zaozhuang Municipal Hospital between 2013 and 2014. Written information consents were obtained from each patient. Before the resection, all of the patients had not received previous neoadjuvant therapy. The basic characteristics of the patients were summarized in Table I.

Cell Culture

Human hepatic immortalized cell line HL-7702 was obtained from Shanghai Cell Biology Institute, Chinese Academy of Sciences. Human hepatocellular carcinoma cell lines HepG2, Hep3B and PLC/PRF/5 (PLC) and HEK 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, VT, USA) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator.

Cell Transfection

HepG2 and Hep3B cells were transfected with 50 nM miR-21 or miR-183 mimics (RiboBio, China), 100 nM miR-21 or miR-183 inhibitors (anti-miR-21 or anti-miR-183) (RiboBio, China), co-transfected with 25 nM miR-21 and 25 nM miR-183 mimics, co-transfected with 50 nM anti-miR-21 and 50 nM anti-miR-183, or transfected with corresponding negative controls (RiboBio, China) using Lipofectamine 2000 (Invitrogen, Life Technologies, Grand Island, NY, USA). Hep3B cells were transfected with 50 nM si-SOCS6 (RiboBio, China) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies, Grand Island, NY, USA).

Human SOCS6 lentiviral vector (pLenti-SOCS6) without 3'-UTR were purchased from GENECHEM (China). The viral particles were co-transfected with the corresponding packaging mix into HEK 293T cells to produce pseudotyped lentivirus. HepG2 and Hep3B cells were treated with the viral supernatant with 5 μg/ml polybrene (Sigma-Aldrich). HepG2 and Hep3B cells with SOCS6 overexpression were further transfected with 50 nM miR-21 or miR-183 mimics (RiboBio, China) using Lipofectamine 2000 (Invitrogen, USA).

qRT-PCR Analysis of miR-21 and miR-183 Expression

Total RNAs in HCC tissues and cell samples were extracted using the mirVana PARIS Kit (Ambion, Foster City, CA, USA). Then, the cDNA was reversely transcribed using TaqMan MicroRNA Reverse Transcription Kit. To quantify the expression of miR-21 and miR-183, TaqMan MicroRNA Assay Kit (Applied Biosystems) was used according to manufacturers' instruction. U6 snRNA served as an internal control. qRT-PCR was conducted using Applied Biosystems 7300 Real Time PCR System. (Foster City, CA, USA).

Table I. The key characteristics of patients recruited

Parameters	Patients with HCC (n=10) No	%
Sex		
Male	7	70
Female	3	30
Age		
<60	6	60
≥60	4	40
Cirrhosis		
+	6	60
-	4	40
HBV		
+	8	80
-	2	20
Tumor size		
<5 cm	3	30
≥5 cm	7	70
Pathological tumor-node-metastasis stage		
Early (I and II)	7	70
Late (III and IV)	3	30
Child-Pugh		
A	8	80
B	2	20
C	0	0

Cell Viability Assay

Cells after transfection were then seeded in 96-well plates at 5×10^3 cells/well. Then cells were further incubated for 48h and the cell viability was determined using Cell Counting Kit-8 (CCK-8) (Dojindo, Tokyo, Japan) on the basis of manufacturer's instruction.

Cell Apoptosis Analysis

1×10^5 cells were seeded in 6-well plates and were used for transfection on the second day. 48 h after transfection, the relative caspase-3 and -7 activities were measured using caspase Glo-3/7 assay kit (Promega) and GloMax™ 20/20 Luminometer (Promega, Madison, WI, USA). Besides, the ratio of cells with active caspase 3 was also determined using Fluorescein Active Caspase 3 Staining Kit (Abcam, Cambridge, UK, ab65613) with a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA). The results were analyzed using ModFit software (BD Bioscience).

Transwell Analysis of Cell Invasion

1×10^5 cells were suspended in 200 μL serum free DMEM and plated into the upper chamber of the transwell insert chamber coated with Matrigel (BD Biosciences). To create chemoattractant environment, culture medium in the lower chamber was supplemented with 20% fetal bovine serum (FBS). The cells were further incubated at 37°C in a 5% CO_2 incubator for 24h. Then, the non-invaded cells on the top of the transwell were scraped off using a cotton swab. The invaded cells on the bottom surface were fixed with 4% polyoxymethylene and stained with 0.1% crystal violet for 20 min. The number of the cells were counted under a light microscope at 100× magnification.

Dual Luciferase Reporter Assay

By performing bioinformatic analysis in Targetscan 6.2, the putative miR-21/miR-183 binding sites of SOCS6 3'UTR were predicted. Three human SOCS6 wild-type-3'UTR sequences containing the miR-21 binding site (SOCS6 3'UTR position 1831-1853) and miR-183 binding sites (SOCS6 3'UTR position 79-100 and position 3655-3677) were synthesized and cloned into pGL-3 promoter vector. The reconstructed vectors were assigned as pGL3-SOCS6-WT1, pGL3-SOCS6-WT2 and pGL3-SOCS6-WT3 respectively. In parallel, another three reporter vectors containing the corresponding mutated 3'UTR sequence were also constructed and named as pGL3-SOCS6-MUT1, pGL3-SOCS6-MUT2 and pGL3-SOCS6-MUT3 respectively. These luciferase reporter vectors were co-transfected into HEK 293T cells with miR-183 or miR-21 mimics or negative control miRNA (50 nM) respectively. These luciferase reporter vectors were also co-transfected into Hep3B cells with anti-miR-21 or anti-miR-183 or negative controls (100 nM) respectively. 24h after transfection, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, USA). Firefly luciferase activity was normalized to that of Renilla luciferase.

Western Blot Analysis of SOCS6 Expression

48h after transfection, cells were lysed using a lysis buffer (Beyotime, China) and the protein concentration of the lysates were measured by BCA protein assay kit (Pierce, Rockford, IL, USA). The lysates were separated on 10% SDS-PAGE and then transferred onto a PVDF membrane. After blocking with 5% nonfat dry milk, the membranes were incubated with primary antibodies (anti-SOCS6, 1:1000, ab157168, Abcam; GAPDH, 1:2000, ab125247, Abcam) overnight at 4°C. Membranes were washed and incubated with corresponding HRP-labeled secondary antibodies (anti-rabbit IgG, 1:10,000, ab191866, Abcam; anti-mouse IgG, 1:10,000, ab6728, Abcam). The blot signals were visualized using ECL Western blotting substrate (Pierce) and the signal intensity of each band was quantified by ImageQuant 5.2 (GE Healthcare, Piscataway, NJ, USA).

Statistical Analysis

The result data was presented as mean ± SD based on at least three repeats. Group difference was assessed using Student's *t*-test. $p < 0.05$ was considered as statistically significant. Statistical significances are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

MiR-21 and miR-183 are Generally Significantly Upregulated in HCC

Based on HCC tissues and adjacent normal tissues obtained from 10 HCC patients, we observed that both miR-21 and miR-183 were significantly upregulated in 8 cases of HCC tis-

sues than in adjacent normal tissues, while two cases only had miR-21 or miR-183 significantly upregulated (Figure 1A and B). qRT-PCR analysis in three human hepatocellular carcinoma cell lines HepG2, Hep3B and PLC and one human hepatic immortalized cell line HL-7702 also showed that miR-21 and miR-183 expression were significantly higher in the cancer cell lines than in the normal cell line (Figure 1 C and D). These results suggest that MiR-21 and miR-183 are generally significantly upregulated in HCC.

Both miR-21 and miR-183 can Modulate Growth and Invasion of HCC Cells

To explore the role of miR-21 and miR-183 in HCC cells, HepG2 and Hep3B cells with high level expression of miR-21 and miR-183 were firstly transfected with anti-miR-21, anti-miR-183 or simultaneously transfected with miR-21 and miR-183 respectively. Knockdown of miR-21 or miR-183 could significantly reduce viability of HepG2 and Hep3B cells (Figure 2 A and B). In addition, knockdown of miR-21 or miR-183 significantly increased the relative caspase 3/7 activity (Figure 2 C and D). Simultaneous knockdown of miR-21 and miR-183 might have stronger effects than knockdown of miR-21 or miR-183 alone in inhibiting growth and promoting apoptosis (Figure 2 A, B, C and D). By the same time, we also measured the ratio of HepG2 and Hep3B with active caspase 3 after knockdown of miR-21 or miR-183. The results of flow cytometry assay showed that knockdown of either miR-21 or miR-183 could significantly increase the proportion of cells with the preapoptotic marker (Figure 2 E and F). Interestingly, knockdown of miR-21 and miR-183 simultaneously had stronger apoptosis inducing effect than either miR-21 or miR-183 alone (Figure 2 E and

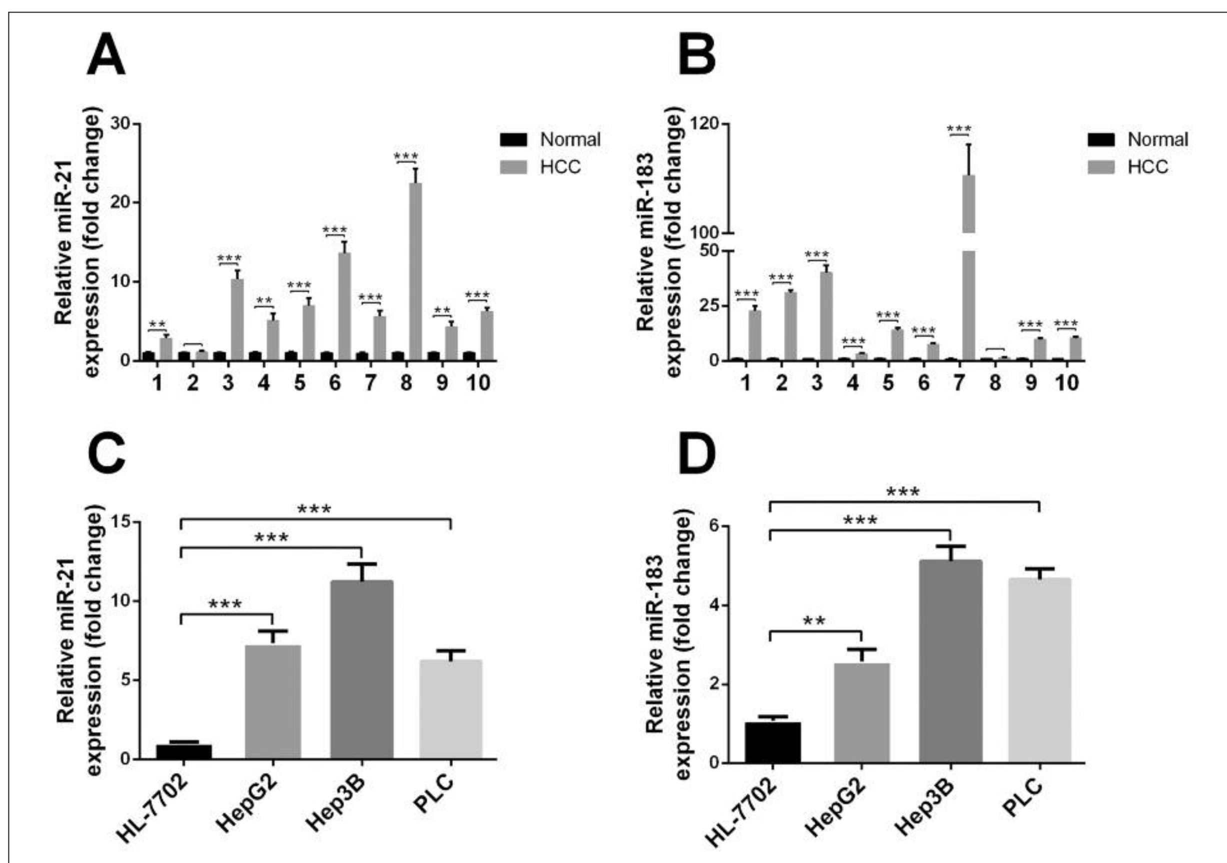

Figure 1. miR-21 and miR-183 expression are significantly increased in HCC tissue and cells. *(A* and *B)* qRT-PCR analysis of miR-21 *(A)* and miR-183 *(B)* expression in 10 cases HCC patients. Relative miRNAs expression were compared between HCC tissues and adjacent normal tissues. *(C* and *D)* qRT-PCR analysis of miR-21 *(C)* and miR-183 *(D)* expression in three human hepatocellular carcinoma cell lines HepG2, Hep3B and PLC and one human hepatic immortalized cell line HL-7702. Values are the average of triple determinations with the S.D. indicated by error bars. NSp ≥ 0.05, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

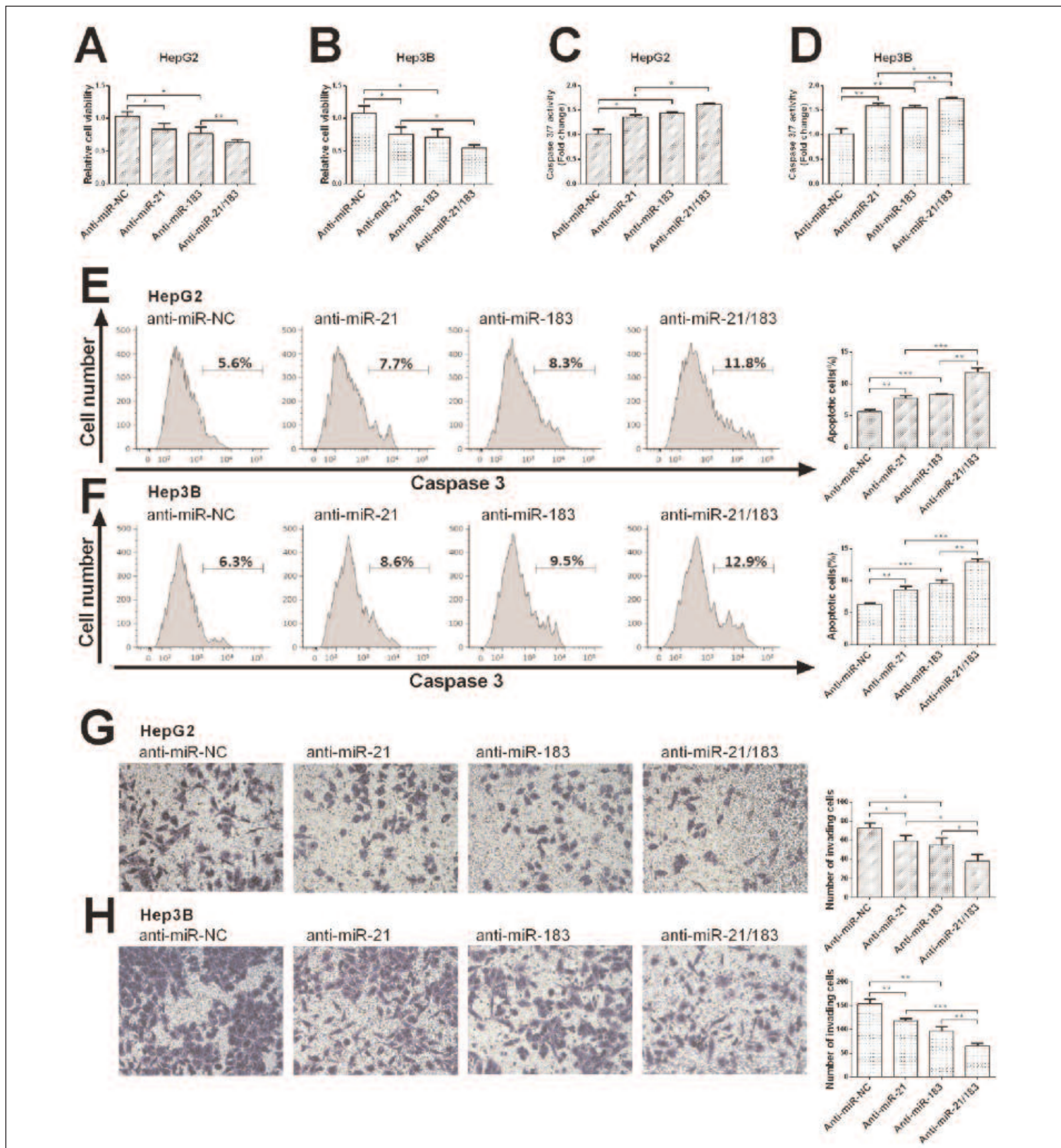

Figure 2. miR-21 and miR-183 can simultaneously modulate growth and invasion of HCC cells. *(A* and *B)* CCK-8 assay of cell viability of HepG2 *(A)* and Hep3B *(B)* cells 48h after transfected with 100 nM miR-21 inhibitors, 100 nM miR-183 inhibitors or co-transfected with 50 nM miR-21 and 50 nM miR-183 inhibitors. *C)* and *D)* Assessment of the activity of caspase 3/7 in HepG2 *(C)* and Hep3B *(D)* cells 48 h after transfected with 100 nM miR-21 inhibitors, 100 nM miR-183 inhibitors or co-transfected with 50 nM miR-21 and 50 nM miR-183 inhibitors. *(E* and *F)* Representative images and statistical analysis of flow-cytometry assay of the ratio of HepG2 *(E)* and Hep3B *(F)* cells with active caspase 3 after knockdown of miR-21, miR-183 or simultaneously knockdown of miR-21 and miR-183. *(G* and *H)* Representative images and statistical analysis of transwell assay of the invading HepG2 *(G)* and Hep3B *(H)* cells after knockdown of miR-21, miR-183 or simultaneously knockdown of miR-21 and miR-183. Values are the average of triple determinations with the S.D. indicated by error bars. NSp ≥ 0.05, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

F). By performing transwell assay, we observed that knockdown of either miR-21 or miR-183 could significantly decrease the invasion capability of the cancer cells. Similarly, knockdown of miR-21 and miR-183 simultaneously had stronger effect in inhibiting cell invasion than ei-

ther miR-21 or miR-183 alone (Figure 2 G and H). These results suggest that both miR-21 and miR-183 may have some synergic effects in modulating growth and invasion of HCC cells.

Both miR-21 and miR-183 can Directly Target SOCS6 and Modulate its Expression

Through prediction in bioinformatic database Targetscan 6.2, we observed that miR-21 has one, while miR-183 has two putative binding sites with SOCS6 (Figure 3A). To verify the binding sites, we constructed dual luciferase reporters with the wide type sequences (WT1, WT2 and WT3) and corresponding mutant sequences (MUT1, MUT2 and MUT3) (Figure 3A). In HEK 293T cells, miR-21 mimics could significantly inhibit luciferase activity of pGL3-SOCS6-WT2 (Figure 3 B) and miR-183 mimics could significantly inhibit luciferase activity of pGL3-SOCS6-WT1 and pGL3-SOCS6-WT3 (Figure 3 C). But these two miRNA mimics had no effect on the reporters with mutant sequences (Figure 3 B and C). In Hep3B cells, knockdown of endogenous miR-21 could significantly enhance luciferase activity of pGL3-SOCS6-WT2 (Figure 3 D), while knockdown of

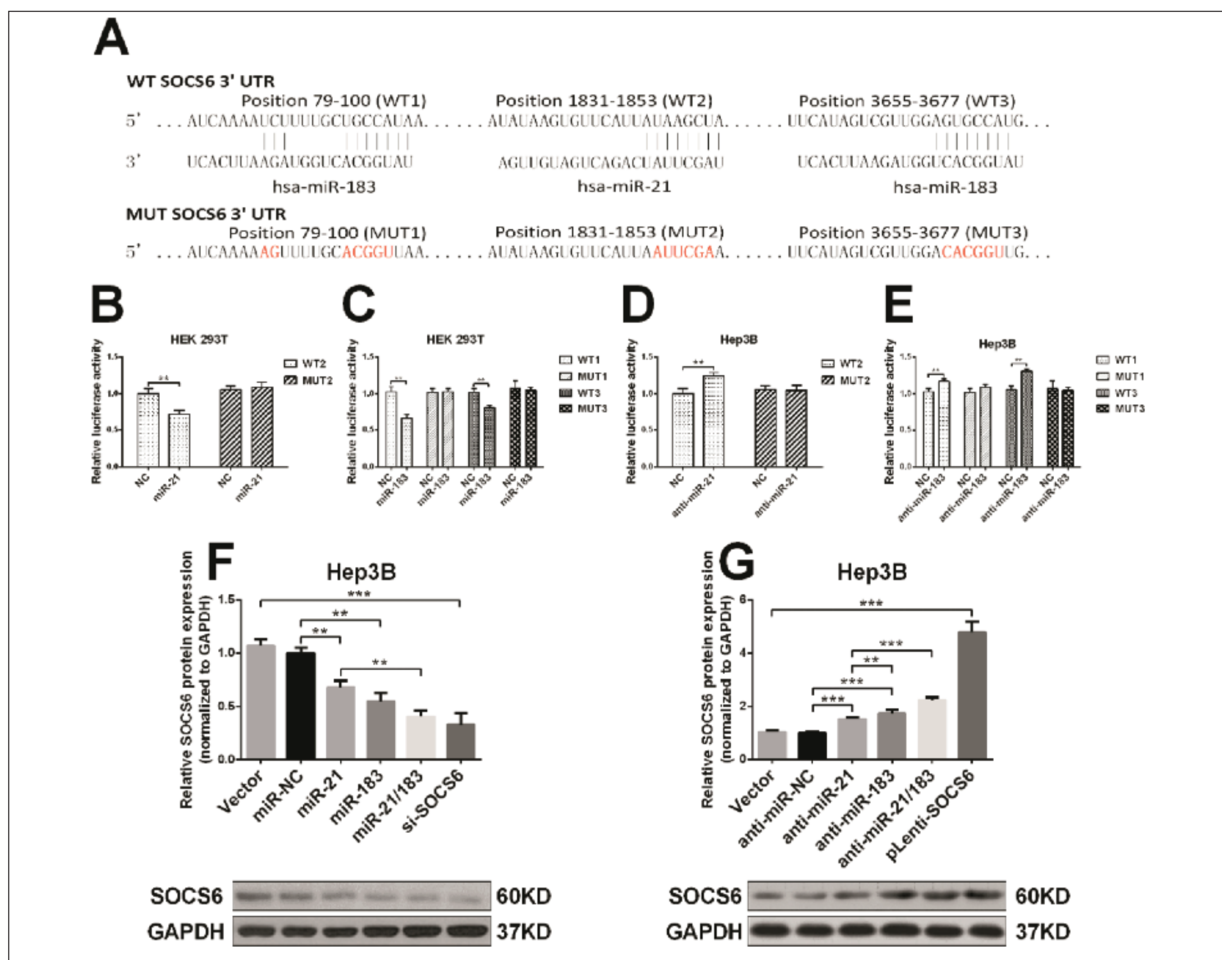

Figure 3. miR-21 and miR-183 can directly target SOCS6 and modulate its expression. ***(A)*** Putative binding sites between miR-21 and 3'UTR of SOCS6 (WT2) and between miR-183 and 3'UTR of SOCS6 (WT1 and WT3). The corresponding mutant sequences were also designed (MUT1, MUT2 and MUT3). ***(B** and **C)*** Dual luciferase assay of relative firefly luciferase activity in HEK 293T cells co-transfected with miR-21 mimics and pGL3-SOCS6-WT2 ***(B)*** or miR-183 mimics and pGL3-SOCS6-WT1 or pGL3-SOCS6-WT3 ***(C)***. (D and E) Dual luciferase assay of relative firefly luciferase activity in Hep3B cells co-transfected with anti-miR-21 and pGL3-SOCS6-WT2 ***(D)*** or anti-miR-183 and pGL3-SOCS6-WT1 or pGL3-SOCS6-WT3 ***(E)***. ***(F)*** Western blot analysis of SOCS6 expression in Hep3B cells with transfected with miR-21 mimics, miR-183 mimics, co-transfected with miR-21 mimics and miR-183 mimics or si-SOCS6. ***(G)*** Western blot analysis of SOCS6 expression in Hep3B cells with transfected with anti-miR-21, anti-miR-183, co-transfected with anti-miR-21 and anti-miR-183 mimics or pLenti-SOCS6. Values are the average of triple determinations with the S.D. indicated by error bars. NSp ≥ 0.05, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

endogenous miR-183 significantly promote luciferase activity of pGL3-SOCS6-WT1 and pGL3-SOCS6-WT3 (Figure 3 E). In Hep3B cells, overexpression of miR-21 and miR-183 could significantly reduce SOCS6 expression (Figure 3F). Overexpression of miR-21 and miR-183 simultaneously had stronger inhibiting effect than miR-21 alone (Figure 3F). Knockdown of endogenous miR-21 and miR-183 could significantly enhance SOCS6 expression (Figure 3G). Knockdown of miR-21 and miR-183 simultaneously had stronger effect in promoting SOCS6 expression than knockdown of miR-21 or miR-183 alone (Figure 3G). These results suggest that both miR-21 and miR-183 can directly target SOCS6 and modulate its expression.

miR-21 and miR-183 Modulates Cell Growth and Invasion Through SOCS6

Since miR-21 and miR-183 can modulate the expression of SOCS6, we further explored the role of miR-21/183-SOCS6 axis in growth and invasion of cancer cells. HepG2 and Hep3B cells transfected with pLenti-SOCS6 (without 3'UTR) had significantly lower cell growth rate (Figure 4 A and B). However, miR-21 and miR-183 mimics could not block these effects due to pLenti-SOCS6 transfection (Figure 4 A and B). Similarly, transfection with pLenti-SOCS6 significantly increased relative caspase 3/7 activity and increased the ratio of apoptotic cells (Figure 4 C, D, E and F). But miR-21 and miR-183 mimics could not rescue the apoptosis (Figure 4 C, D, E and F).

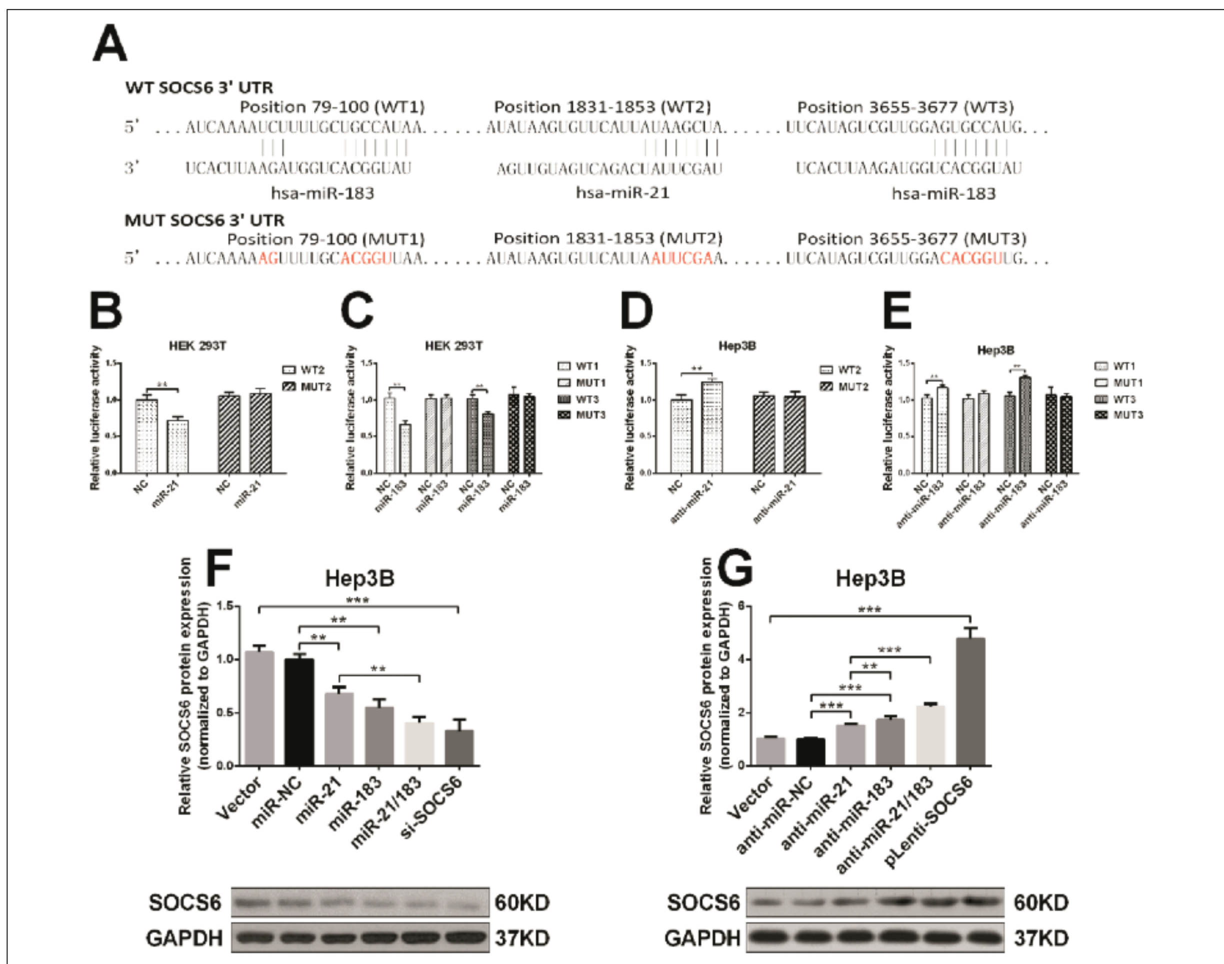

Figure 4. miR-21 and miR-183 modulates cell growth and invasion through SOCS6. HepG2 and Hep3B cells transfected with pLenti-SOCS6, pLenti-SOCS6 and 50 nM miR-21 or pLenti-SOCS6 and 50 nM miR-183 were used to analyze their cell viability, relative caspase 3/7 activity, active caspase 3 and invading capability. *(A* and *B)* CCK-8 assay of cell viability of HepG2 *(A)* and Hep3B *(B)* cells 48h after transfection. *(C* and *D)* Measurement of the activity of caspase 3/7 of HepG2 *(C)* and Hep3B *(D)* cells 48h after transfection. *(E* and *F)* Statistical analysis of HepG2 *(E)* and Hep3B *(F)* cells with active caspase 3 of 48h after transfection. *(G* and *H)* Statistical analysis of Transwell assay of the invading HepG2 *(G)* and Hep3B *(H)* cells. Values are the average of triple determinations with the S.D. indicated by error bars. NSp ≥ 0.05, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

In addition, transfection with pLenti-SOCS6 also reduced invasion capability of HepG2 and Hep3B cells, which could not be blocked by miR-21 or miR-183 mimics (Figure 4 G and H).

Discussion

Previous studies generally reported that miR-21 and miR-183 are upregulated in HCC tissues compared with healthy tissues[15,19-21]. In the current study, we also verified significantly higher miR-21 and miR-183 expression in HCC tissues than in adjacent healthy tissues. The oncogenic roles of miR-21 and miR-183 are widely reported in previous studies. High miR-21 or miR-183 expression is usually associated with poor prognosis of HCC[13,22-24]. In the current study, we also confirmed the important role of miR-21 and miR-183 in HCC cells. Knockdown of miR-21 and miR-183 in HepG2 and Hep3B cells could decrease cell viability, increase cell apoptosis and decrease cell invasion.

MiRNAs usually have multiple targets and, thus, can modulate multiple biological process[25-28]. During the past years, several studies have reported the molecular mechanism of miR-21 and miR-183 in HCC. High miR-21 expression is essential in the maintenance of tumorigenic phenotype of HCC cells *in vitro* and *in vivo*[29]. MiR-21 can promote migration and invasion of HCC cells through the miR-21-PDCD4-AP-1 feedback loop[12,20]. MiR-21 is also a downstream effector of high-mobility group Box 1 mediating matrix metalloproteinase activity during HCC development[30]. Knockdown of miR-21 can suppress hepatocellular carcinoma growth[29]. MiR-183 can inhibit TGF-beta1-induced apoptosis of HCC cells through inhibiting PDCD4 expression[16]. It is also a downstream effector of Wnt/beta-Catenin pathway which promotes HCC cell invasion[17].

Although the oncogenic roles of miR-21 and miR-183 in HCC were widely reported, whether they have other regulative roles should be further explored. SOCS6 is a member of the SOCS family and is generally downregulated in HCC[31]. It is an important regulator of survival signaling since it can initiate ubiquitination of receptors or substrate proteins and thereby inducing apoptosis[18]. Low SOCS6 expression correlates with aggressive tumor progression and poor prognosis of HCC[31]. Loss of SOCS6 expression enhances cells resistant to programmed cell death[32]. Overexpression of SOCS6 inhibited cell survival in oncogenic FLT3-dependent hematopoietic cells[33]. Therefore, it is generally considered as a tumor suppressor gene in multiple cancers. Recent studies showed that its expression is modulated by several miRNAs in different cancers. MiR-424-5p can downregulate SOCS6 expression in pancreatic cancer[34]. MiR-431 can downregulate SOCS6 expression in medulloblastoma and glioblastoma cells[35]. High miR-17-5p expression in gastric cancer inhibited SOCS6 expression[36]. However, whether other miRNAs can regulate its expression is not clear.

Through preliminary study and online prediction, we observed that both miR-21 and miR-183 have putative binding sites with SOCS6. Based on the dual luciferase assay and Western blot analysis, we confirmed that both miR-21 and miR-183 can target SOCS6 and modulate its expression at protein level. Considering the important role of SOCS6 in cancer cell apoptosis, we decided to further explore how the miR-21/miR-183-SOCS6 axis can affect cell viability and invasion. As expected, overexpression of SOCS6 without 3'UTR could significantly lower cell growth rate and invasion capability, but increase relative caspase 3/7 activity and the ratio of apoptotic cells. However, these effects could not be blocked by miR-21 or miR-183 mimics. These results directly suggest that miR-21/miR-183-SOCS6 axis might play an important role in modulating cell growth and invasion of HCC cells.

Conclusions

MiR-21 and miR-183 are overexpressed in HCC and can simultaneously target SOCS6, thereby enhancing growth and invasion of HCC cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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