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MicroRNA-200a Suppresses Cell Invasion and Migration by Directly Targeting GAB1 in Hepatocellular Carcinoma

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MicroRNA-200a (miR-200a) is frequently downregulated in most cancer types and plays an important role in carcinogenesis and cancer progression. In this study, we determined that miR-200a was downregulated in hepatocellular carcinoma (HCC) tissues and cell lines, consistent with the results of our previous study. Because a previous study suggested that downregulation of miR-200a is correlated with HCC metastasis, we aimed to elucidate the mechanism underlying the role of miR-200a in metastasis in HCC. Here we observed that overexpression of miR-200a resulted in suppression of HCC metastatic ability, including HCC cell migration, invasion, and metastasis, *in vitro* and *in vivo*. Furthermore, bioinformatics and luciferase reporter assays indicated that GAB1 is a direct target of miR-200a. Inhibition of GAB1 resulted in substantially decreased cell invasion and migration similar to that observed with overexpression of miR-200a in HCC cell lines, whereas restoration of GAB1 partially rescued the inhibitory effects of miR-200a. Taken together, these data provide novel information for comprehending the tumor-suppressive role of miR-200a in HCC pathogenesis through inhibition of GAB1 translation.

Key words: miR-200a; Hepatocellular carcinoma (HCC); Grb2-associated binding protein 1 (GAB1); Invasion and migration; Metastasis

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most prevalent and aggressive human malignancy and the third leading cause of cancer-related mortality worldwide¹. The poor prognosis of HCC is attributed to tumor invasiveness, frequent intrahepatic spread, and extrahepatic metastasis at initial diagnosis². Therefore, preventing the metastasis of cancer cells is a critical and effective therapeutic strategy for the successful management of HCC.

MicroRNAs (miRNAs) are a set of highly conserved noncoding small RNAs that regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of target mRNAs^{3,4}. Accumulating evidence suggests that miRNAs are involved in the development and progression of various human cancers^{3,5,6}. Some miRNAs have been identified to function as tumor suppressors or oncogenes and participate in the regulation of various fundamental biological processes in HCC, including tumorigenesis, angiogenesis, proliferation, apoptosis, invasion, and metastasis⁷⁻¹³.

Therefore, miRNAs may be potential therapeutic targets in HCC.

Emerging data have demonstrated that microRNA-200a (miR-200a) is frequently downregulated and may act as a tumor suppressor in many cancer types, including breast cancer, nasopharyngeal cancer, gastric cancer, colorectal cancer, and HCC¹⁴⁻¹⁸. Restoration of miR-200a expression has been proposed as a novel treatment strategy in HCC. We previously reported that miR-200a is downregulated in HCC tissues and cell lines and that reduced miR-200a expression is correlated with HCC metastasis and poor prognosis¹⁹. Experimental data suggest that miR-200a directly regulates several target mRNAs, including ZEB2¹⁵, β -catenin²⁰, CDK-6²¹, HDAC4¹⁸, IGF2²², EPHA2²³, TGF β 2²⁴, SIRT1¹⁴, and Ap-2 γ ²⁵. However, the potential mechanisms of miR-200a in affecting the malignant phenotype of HCC have not been completely elucidated. In the present study, we observed frequent downregulation of miR-200a in HCC tissues and cell lines, consistent with the results of our previous study¹⁹. *In vitro* and *in vivo*

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assays demonstrated that overexpression of miR-200a inhibits cell invasion and metastasis of HCC. Moreover, we determined that Grb2-associated binding protein 1 (GAB1), a novel tumor-suppressor gene, is a direct target of miR-200a. Silencing of GAB1 resulted in substantially decreased cell migration and invasion in HCC cell lines, whereas restoration of GAB1 reversed the inhibitory effects of miR-200a. Therefore, our results suggest a critical role for miR-200a in HCC pathogenesis and its possible application as a target for cancer therapy.

MATERIALS AND METHODS

Patients and Tissue Specimens

Forty-eight patients who were diagnosed with HCC at the Department of Hepatobiliary Surgery of Xijing Hospital of the Fourth Military Medical University between 2012 and 2015 were included in this study. The study protocol was approved by the Ethics Committee of Xijing Hospital. All participants provided informed written consent.

Cell Culture

The human HCC cell lines MHCC97-H, SMMC-7721, Huh7, and HepG2, the normal human hepatocyte cell line HL-7702, 293T cells (obtained from the Cell Bank of the Chinese Academy of Sciences) were cultivated in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin. In all experiments, cells were cultured at 37°C in a humidified 5% CO₂/95% air atmosphere.

Plasmids and Cell Transfection

Lentiviral plasmids encoding miR-200a (LV-miR-200a) and empty vector (LV-miR-NC) were designed and produced by Genechem (Shanghai, P.R. China). MHCC97-H and SMMC-7721 were plated in six-well plates and incubated overnight. The culture medium was replaced with transduction-enhancing solution containing 20 MOI lentivirus and 50 µg/ml polybrene. After 12 h, the medium was replaced with complete medium, and the cells were cultured for 72 h. The cells were transfected with miR-200a mimic, a mimic negative control, siRNA for GAB1 (si-GAB1), or siRNA-negative control (NC) (GenePharma, Shanghai, P.R. China) and pcDNA3.1-GAB1 plasmid lacking the 3'-UTR or pcDNA3.1-GAB1-NC (Genechem) using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions.

RNA Extraction and Real-Time PCR

Total RNA was isolated using RNAiso Plus reagent (TaKaRa, Dalian, P.R. China) according to the manufacturer's instructions. miR-200a expression levels were quantified using a miScript PCR system (Qiagen, Hilden,

Germany), a miScript II RT kit, miScript primer assays, and a miScript SYBR Green PCR kit. Small nuclear RNA U6 was employed for internal normalization.

For mRNA analysis, complementary DNA (cDNA) was generated with oligo-dT primers using the PrimeScript RT Reagent Kit (TaKaRa). The generated cDNA was amplified using SYBR Premix EX Taq II (TaKaRa) on a Bio-Rad IQTM5 detection system (Bio-Rad, Hercules, CA, USA). β-Actin was used as an internal control to normalize the mRNA expression levels of the target genes. All experiments were performed according to the manufacturer's instructions. All reactions were assessed in triplicate, and data were analyzed with the comparative Ct (2^{-ΔΔCt}) method.

Western Blot Analysis

The cells were washed three times with cold PBS and lysed in RIPA lysis buffer (Beyotime, Shanghai, P.R. China) containing fresh protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The protein concentration was determined using the Bio-Rad assay system (Bio-Rad). The total protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), and sequentially incubated with primary antibodies against GAB1 (1:1,000; Cell Signaling Technology) and β-actin (1:2,000; Cell Signaling Technology) overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; Abcam) at room temperature for 1 h. The blots were developed using enhanced chemiluminescence detection reagents (Pierce, Rockford, IL, USA) and scanned with a Molecular Imager System (Bio-Rad).

Migration and Invasion Assays

Cell migration ability was analyzed with non-Matrigel-coated Transwell cell culture chambers (8-µm pore size) (Millipore), and cell invasion ability was analyzed with Matrigel-coated Transwell cell culture chambers. A total of 3 × 10⁴ cells were added to the top chamber on the non-coated or Matrigel-coated membrane (24-well insert) and allowed to migrate toward the serum-containing medium in the lower chamber. The cells were incubated for 24 h, then fixed with 4% formaldehyde for 10 min and stained with 0.5% crystal violet for 30 min. The invaded and migrated cells were counted at 200× magnification from five different fields of each sample.

In Vivo Pulmonary Metastasis Assays

All experimental procedures involving mice were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the

Research Animal Care and Use Committee of the Fourth Military Medical University. For the in vivo pulmonary metastasis assay, 4- to 5-week-old female BALB/c nude mice ($n=8$ mice per group) were injected through the lateral tail vein with MHCC97-H cells (2×10^6) transfected with miR-NC or miR-200a. After 5 weeks, the mice were sacrificed, and the lungs were collected and fixed in 10% formalin. After paraffin embedding, the lung samples were sectioned and stained with hematoxylin and eosin (H&E).

Luciferase Reporter Assay

The luciferase reporter assay was performed in 293T cells. The 293T cells were seeded into a 24-well plate and cotransfected with luciferase reporter constructs encoding the wild-type 3'-UTR region of GAB1 (GAB1-WT-3'-UTR) or a mutated GAB1 3'-UTR region (GAB1-MUT-3'-UTR) (RIBOBIO, Guangzhou, P.R. China) and miR-200a mimic or mi-NC using Lipofectamine 2000 (Invitrogen). After 48-h of incubation, the cells were washed with PBS and lysed with Passive Lysis Buffer (Promega). Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer's protocol.

Statistical Analysis

Each experiment was repeated at least three times. All data are presented as the mean \pm SD. Student's *t*-test (two tailed) or the Student–Newman–Keuls test (SNK test, ANOVA) was employed to analyze the differences

using SPSS 13.0 software (Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

The Expression of miR-200a Is Downregulated in HCC Tissues and Cell Lines

We previously demonstrated that miR-200a is greatly downregulated in HCC cell lines and tissues and is associated with HCC metastasis¹⁹. To investigate the expression of miR-200a in HCC tissues and cells, the expression of miR-200a in tumor specimens and four HCC cell lines (MHCC97-H, SMMC-7721, Huh7, and HepG2) was detected by real-time PCR. Our results demonstrated that the expression of miR-200a was significantly decreased in HCC tissues and cell lines (Fig. 1A and B). These data suggest that miR-200a may function as a tumor-suppressive gene.

miR-200a Inhibits HCC Cell Migration and Invasion In Vitro

Because downregulation of miR-200a expression is correlated with HCC metastasis, we hypothesized that miR-200a might regulate this process. To test this hypothesis, we first transfected the lentiviral plasmid LV-miR-200a or a lentiviral control plasmid LV-miR-NC into the HCC cell lines MHCC97-H and SMMC-7721. As shown in Figure 2A, miR-200a was successfully overexpressed in the HCC cells transfected with LV-miR-200a. Then we investigated the role of miR-200a in HCC cell

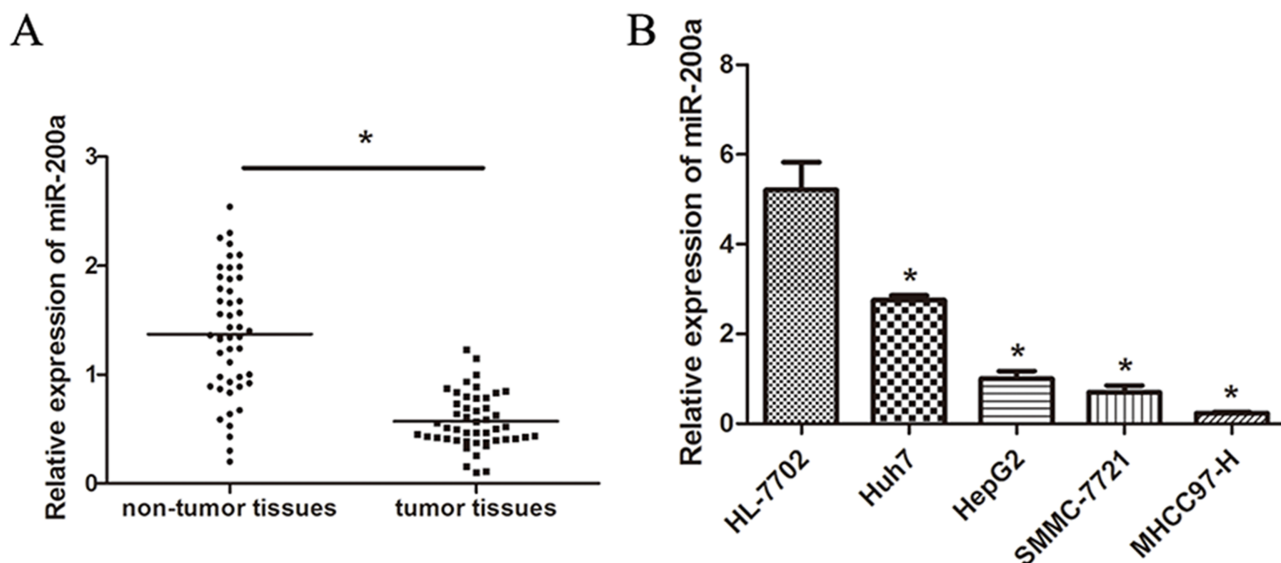


Figure 1. Expression of miR-200a in HCC tissues and cell lines. (A) Expression of miR-200a in HCC tissue and corresponding adjacent noncancerous tissues. (B) Relative expression of miR-200a in four HCC cell lines (Huh7, HepG2, SMMC-7721, and MHCC97-H) and a human normal liver cell line (HL-7702) as analyzed by RT-PCR. The data represent the mean \pm SD. * $p < 0.05$.

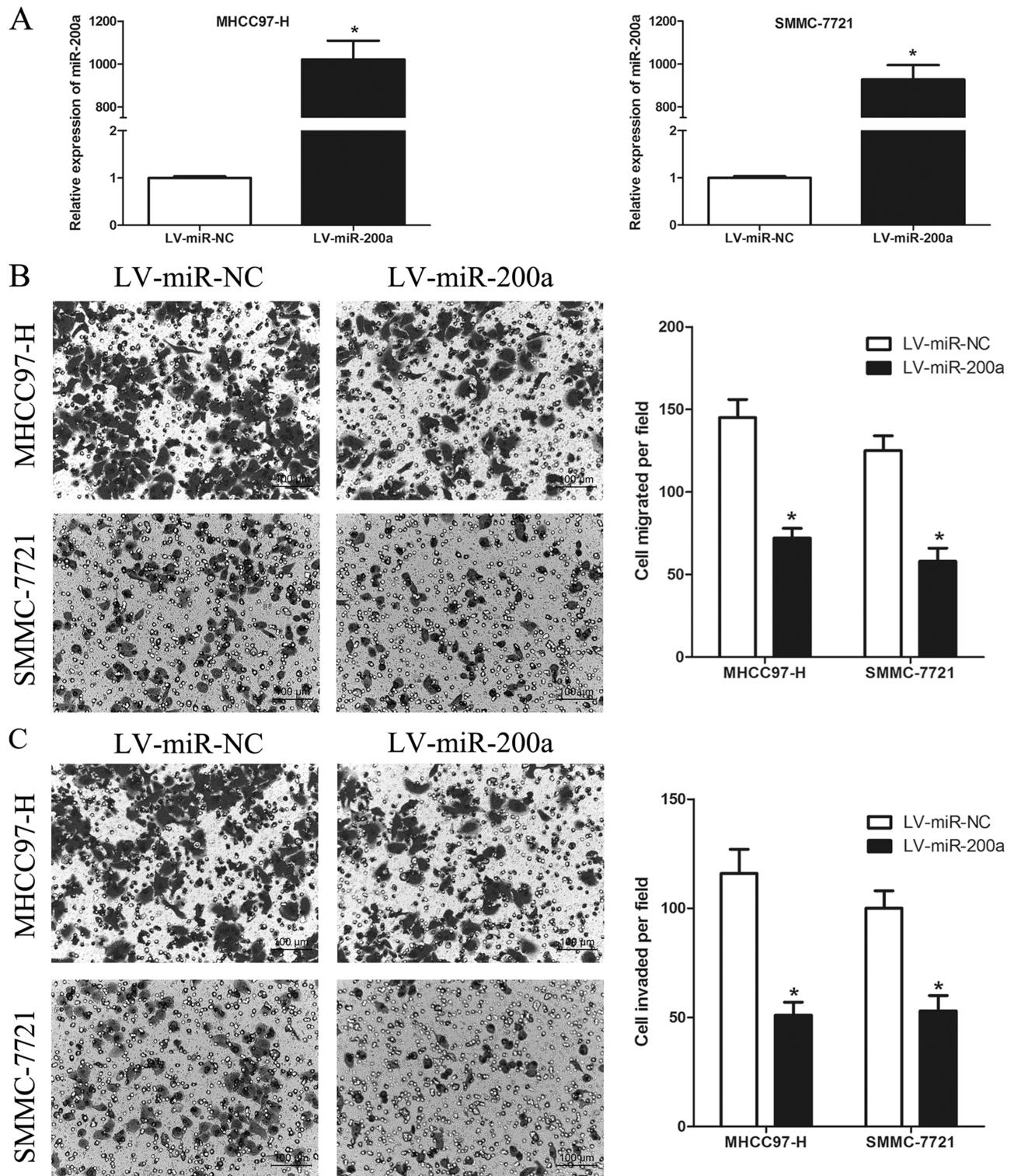


Figure 2. miR-200a inhibits HCC cell migration and invasion in vitro. (A) Expression of miR-200a following transfection in MHCC97-H and SMMC-7721 cells was confirmed by RT-PCR. (B and C) Overexpression of miR-200a significantly inhibited cell migration and invasion in MHCC97-H and SMMC-7721 cells. Migrated and invaded cells were counted in five randomly selected areas under a 200 \times microscope field. * $p < 0.05$ compared to the control.

migration and invasion using in vitro Transwell assays. The Transwell assays revealed that overexpression of miR-200a dramatically suppressed cell migration and invasion in MHCC97-H and SMMC-7721 cells ($p < 0.05$) (Fig. 2B and C). These data imply that miR-200a can inhibit the metastatic ability of HCC cells by suppressing their migration and invasion.

miR-200a Suppresses HCC Cells Metastasis In Vivo

To further demonstrate the association between miR-200a and HCC cell metastasis, in vivo pulmonary metastasis assays of HCC cells were performed in nude mice. MHCC97-H cells transfected with LV-miR-200a or LV-miR-NC were transplanted into nude mice via lateral tail vein injection. After 5 weeks, the mice were sacrificed, and the lungs were dissected and fixed for H&E staining. As shown in Figure 3A and B, overexpression of miR-200a decreased the incidence of lung metastasis and the number of metastatic lung nodules. Representative H&E staining of metastatic lung nodules is shown in Figure 3C. These data indicate that miR-200a may act as a metastatic suppressor of HCC in vivo.

GAB1 Is a Direct Downstream Target of miR-200a

To further explore the mechanism of action of miR-200a in HCC metastasis suppression, we used bioinformatics tools to predict the putative target genes of miR-200a. Among the candidates, GAB1 was selected for further experimental validation. A predicted complementary sequence of miR-200a was identified in the 3'-UTR of GAB1 mRNA (Fig. 4A). As shown in Figure 4B, miR-200a significantly decreased the luciferase activity of GAB1 containing a wild-type 3'-UTR but did not decrease the activity of GAB1 with a mutant 3'-UTR in 293T cells. RT-PCR and Western blotting further demonstrated that overexpression of miR-200a markedly suppressed the mRNA and protein levels of GAB1 in MHCC97-H and SMMC-7721 cells ($p < 0.05$) (Fig. 4C and D). Taken together, these results strongly suggest that GAB1 is a target of miR-200a in HCC.

Alterations of GAB1 Levels Influence the Effects of miR-200a on HCC Cells

To determine if downregulation of GAB1 accounts for the inhibition of cell migration and invasion by miR-200a, we transfected SMMC-7721 cells with GAB1 siRNA to knock down endogenous GAB1 expression. As shown in Figure 5A and B, GAB1 was significantly downregulated in SMMC-7721 cells, and knockdown of GAB1 markedly inhibited cell migration and invasion ($p < 0.05$) (Fig. 5C). These results are similar to those induced by miR-200a. To further confirm that GAB1 is a functional target of miR-200a, we constructed a plasmid with GAB1

lacking its 3'-UTR and introduced the construct into miR-200a-overexpressing SMMC-7721 cells. The inhibitory effects of miR-200a on HCC cell migration and invasion were partially antagonized by restoring GAB1 expression ($p < 0.05$) (Fig. 5D). These data suggest that GAB1 is a downstream functional mediator of miR-200a.

DISCUSSION

miR-200a suppresses different biological processes such as proliferation, invasion, metastasis, and tumorigenesis in HCC^{18,21,26,27}; however, the molecular mechanisms by which miR-200a inhibits HCC remain unclear. Therefore, in this study, we aimed to elucidate the biological functions and mechanism of miR-200a in HCC. Our results demonstrated that miR-200a is frequently downregulated in HCC tissues and cell lines, consistent with the results of our previous study. Furthermore, we elucidated the role of miR-200a deregulation in tumor invasion and metastasis using in vitro and in vivo assays. We observed that overexpression of miR-200a suppressed cell migration and invasion in vitro and restrained tumor metastasis in vivo. These results suggest that miR-200a is a novel tumor suppressor that plays an important role in the regulation of invasion and metastasis in HCC.

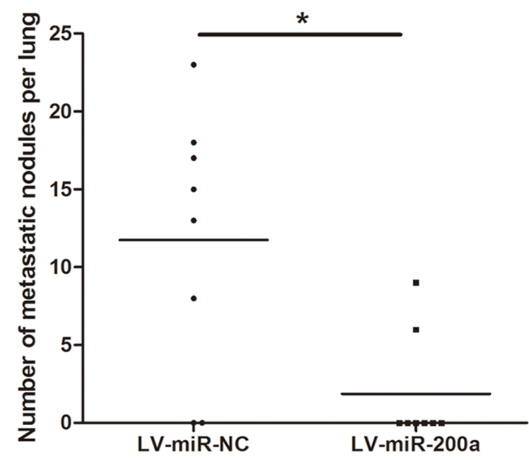
We also explored the exact molecular mechanism of miR-200a in suppressing invasion and metastasis in HCC. Luciferase reporter assays, RT-PCR, and Western blotting demonstrated that GAB1 is a direct target of miR-200a. More importantly, the effects of miR-200a modulation on cell migration and invasion were accompanied by alteration of GAB1 levels and activities. Overexpression of GAB1 abolished the effects induced by miR-200a. Thus, we confirmed that miR-200a plays a critical role in the inhibition of invasion and metastasis in HCC, partially by downregulating the expression of GAB1.

GAB1, which belongs to the Grb2-associated binder (Gab) family, has been reported to be involved in tumor occurrence, invasion, and metastasis, particularly in gastrointestinal tumors²⁸⁻³¹. GAB1 is most commonly and widely distributed in mammals and plays a crucial role in transmitting key signals that control cell growth, differentiation, and function from multiple receptors^{28,32,33}. Zhang et al.³⁴ demonstrated that GAB1 protein expression is significantly associated with aggressive tumor progression and poor prognosis in patients with HCC. Felic et al.³⁵ and Oka et al.³⁶ observed that Gab1 upregulates proto-oncogene expression by amplifying signaling pathways related to tumor biological behaviors. Downregulation of GAB1 inhibits cell proliferation, invasion, and migration in intrahepatic and hilar cholangiocarcinoma and colorectal cancer^{30,31,37}. Interestingly, we also observed that knockdown of GAB1 using siRNA can inhibit cell

A

Group	Lung metastasis
LV-miR-NC	6/8
LV-miR-200a	2/8

B



C

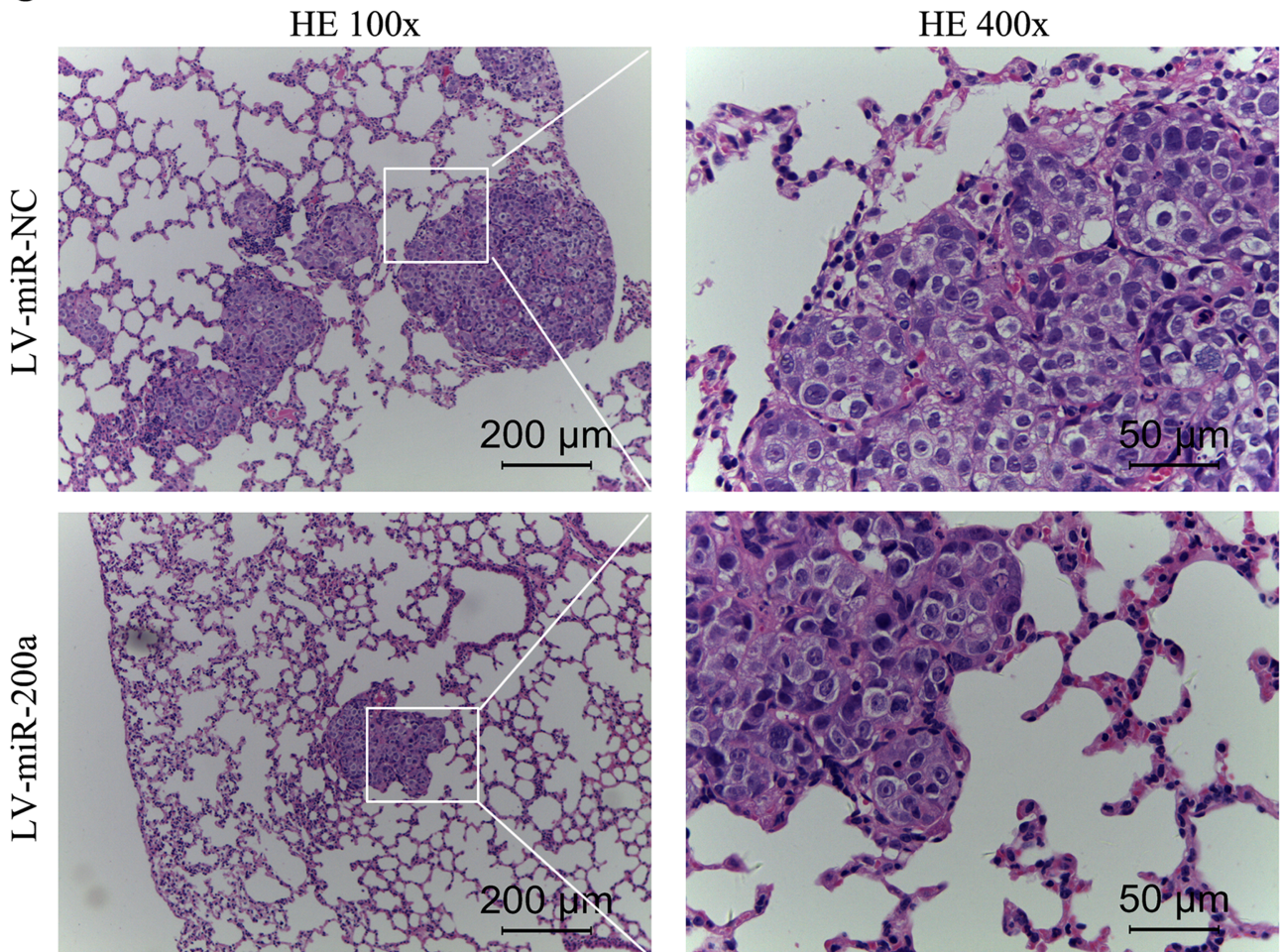


Figure 3. miR-200a suppresses tumor metastasis in vivo. (A) Incidence of lung metastasis in mice injected with MHCC97-H cells transfected with miR-NC or miR-200a cells through the lateral tail vein. (B) The number of metastatic nodules on the surface of the lungs in mice from the different groups. (C) Representative H&E staining of lung metastatic nodules. * $p < 0.05$ compared to the control.

invasion and migration in HCC. Recent studies have reported that GAB1 and FOXP1 expression are modulated by miR-150, resulting in proficient B-cell receptor signaling in chronic lymphocytic leukemia³⁸, and are also regulated by microRNA-409-3p, resulting in the suppression of colorectal cancer invasion and metastasis in colorectal cancer³⁷. Our study identified GAB1 as the target of miR-200a in HCC and revealed an important role for GAB1 dysregulation in HCC progression. Thus, the post-transcriptional regulation of GAB1 expression by miRNA

might be a novel mechanism by which GAB1 regulates cancer progression.

In summary, our results demonstrated that miR-200a is downregulated in HCC cell lines. Overexpression of miR-200a inhibits cell invasion and migration in vitro and metastasis of HCC in vivo partly by targeting GAB1. This study might provide new insight into the molecular mechanisms of HCC progression and metastasis and suggests that the miR-200a/GAB1 axis is a possible therapeutic strategy for the treatment of HCC.

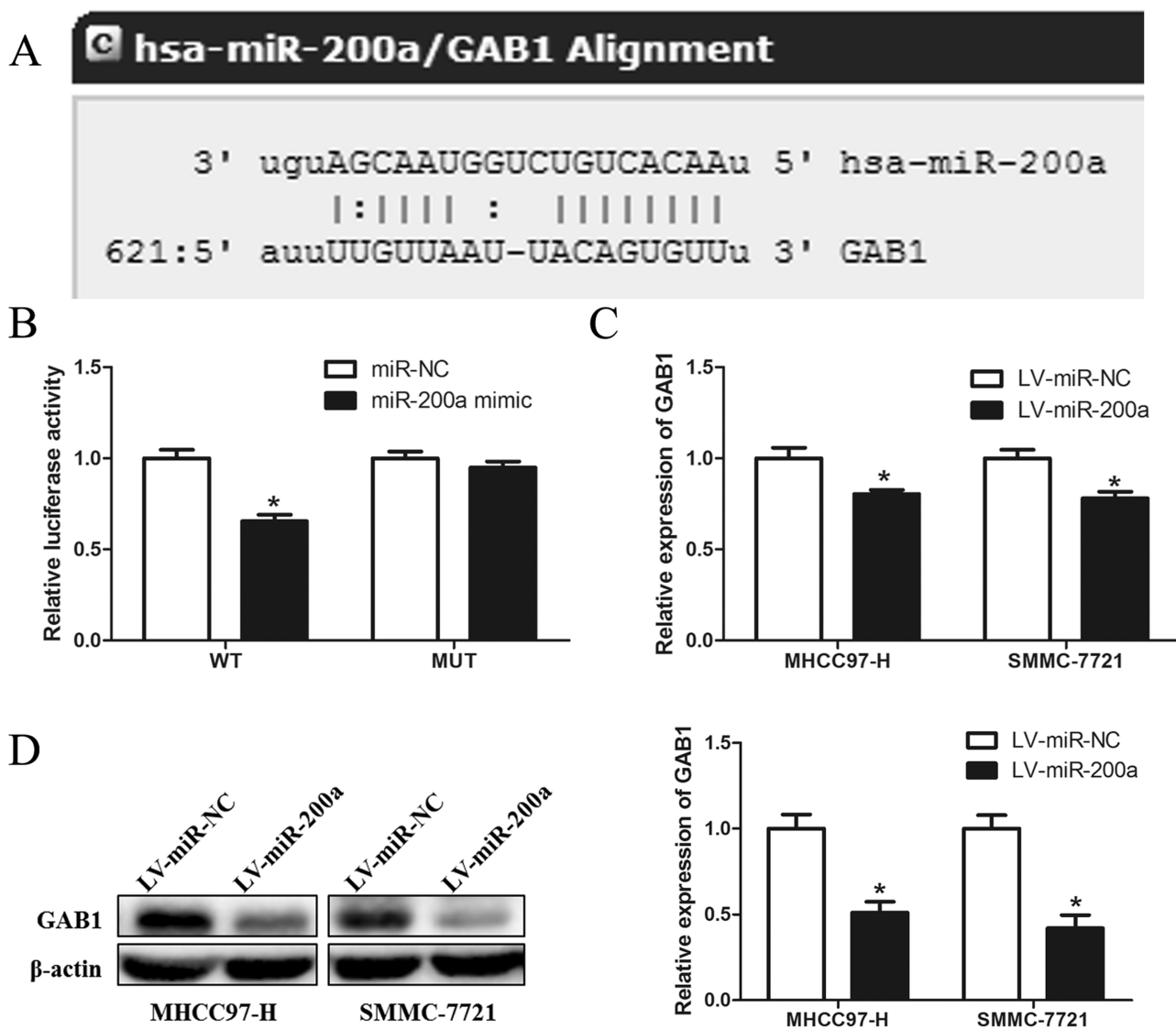


Figure 4. GAB1 is a direct downstream target of miR-200a. (A) GAB1 was bioinformatically predicted as a target of miR-200a using online software (<http://www.microrna.org/>). (B) A miR-200a mimic or negative control and a luciferase vector encoding the wild-type or mutant GAB1 3'-UTR region were introduced into 239T cells, and the relative luciferase activity was measured. (C) Quantitation of GAB1 mRNA levels in MHCC97-H and SMMC-7721 cells by RT-PCR after transfection with LV-miR-NC or LV-miR-miR-200a. (D) Detection of GAB1 protein in MHCC97-H and SMMC-7721 cells by Western blot analysis after transfection with LV-miR-NC or LV-miR-200a. * $p < 0.05$ compared to the control.

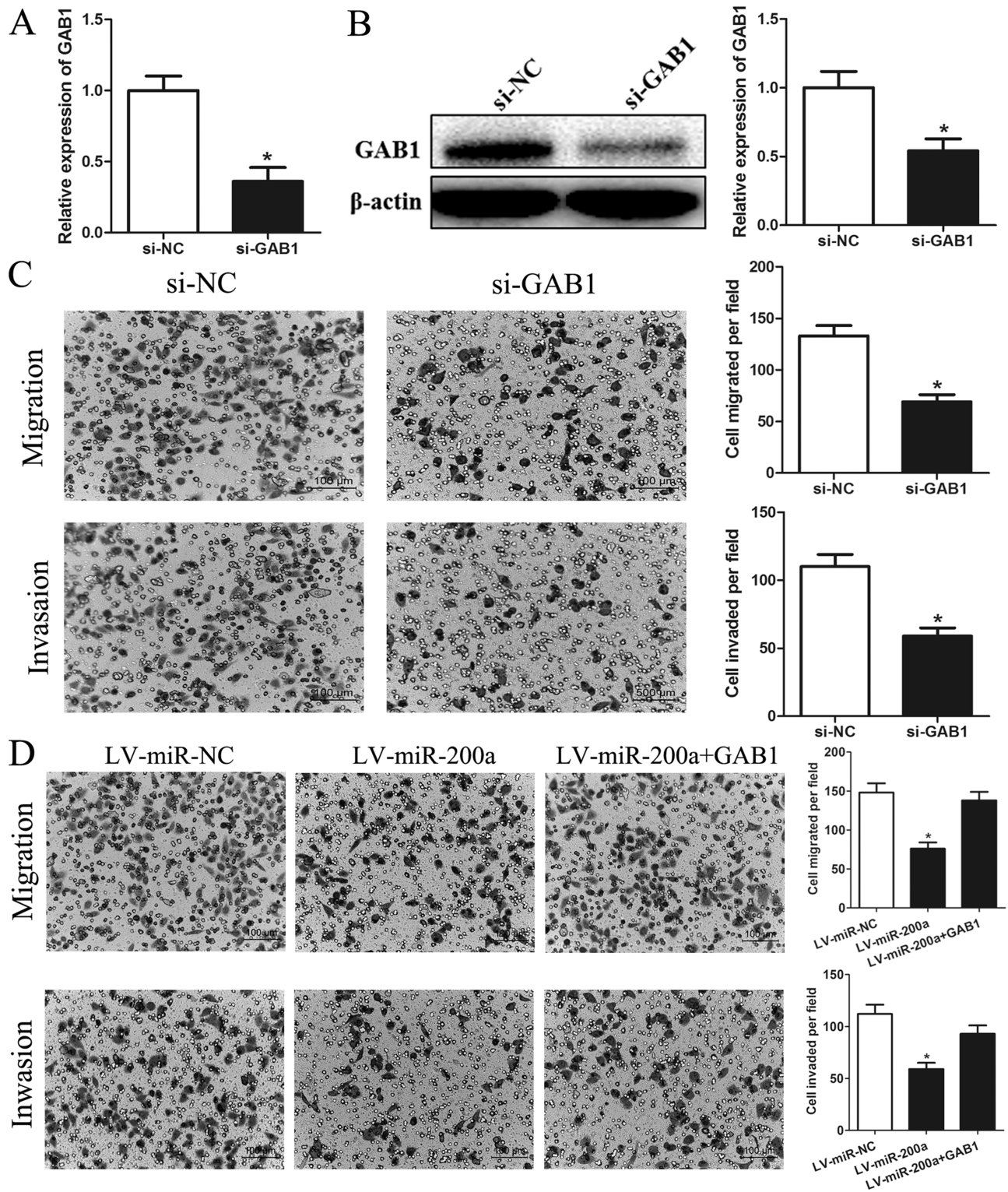


Figure 5. Alterations of GAB1 levels influence the effects of miR-200a on HCC cells. (A) Quantitation of GAB1 mRNA levels in SMMC-7721 cells by RT-PCR after transfection with siRNA targeting GAB1 (si-GAB1) or a negative control siRNA (si-NC). (B) Detection of GAB1 protein in SMMC-7721 cells by Western blot analysis after transfection with GAB1 siRNAs or si-NC. (C) GAB1 knockdown inhibited SMMC-7721 cell migration and invasion. (D) GAB1 reintroduction into SMMC-7721 cells partially rescued the miR-200a-mediated inhibition of cell migration and invasion. Migrated and invaded cells were counted in five randomly selected areas under a 200 \times microscope field. * $p < 0.05$ compared to the control.

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