Inhibition of cell proliferation and metastasis of human hepatocellular carcinoma by miR-137 is regulated by CDC42

MING GAO[1], LIYING LIU[2], SHENGLEI LI[3], XUDONG ZHANG[1], ZHIWEI CHANG[1] and MINGZHI ZHANG[1]

[1]Department of Oncology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052;
[2]Department of Oncology, The First People's Hospital of Zhengzhou, Zhengzhou, Henan 450004;
[3]Department of Pathology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, P.R. China

Abstract. In the present study, we evaluated the mechanisms of CDC42 in association with the microRNA-137 (miR-137)-induced inhibition of human hepatocellular carcinoma (HCC). The gene expression levels of miR-137 were evaluated in HCC cell lines. Direct association of miR-137 with its downstream target, cell division control protein 42 (CDC42), was evaluated by dual-luciferase assay, western blot analysis and correlation analysis using clinical tumor samples. In the HCC HuH7 and MHCC97L cell lines, miR-137 was upregulated to inhibit cell proliferation and metastasis *in vitro* and tumor growth *in vivo*. CDC42 was overexpressed in the HuH7 and MHCC97L cells to evaluate its effect on the miR-137-mediated antitumor effects. Furthermore, possible crosstalk between CDC42 and another miR-137 target gene, AKT2, was evaluated by co-overexpressing CDC42 and AKT2 in the HuH7 and MHCC97L cells and examining their effects on miR-137-mediated HCC regulation. miR-137 was confirmed to be downregulated in the HCC cell lines. Dual-luciferase assay showed that CDC42 was directly targeted by miR-137, and western blotting showed that CDC42 was downregulated by miR-137 upregulation in the HuH7 and MHCC97L cells. In human tumors, the expression levels of CDC42 and miR-137 were inversely correlated. The inhibitory effects of miR-137 on HCC proliferation, metastasis and *in vivo* tumor growth were all ameliorated by CDC42 overexpression. Furthermore, co-overexpression of AKT2 in addition to CDC42 additively reduced the inhibition of miR-137 on HCC proliferation and metastasis, suggesting two independent pathways of CDC42 and AKT2 in miR-137-mediated HCC regulation. Our study demonstrated that CDC42 independently regulated the antitumor effects of miR-137 in human HCC.

Correspondence to: Dr Mingzhi Zhang, Department of Oncology, The First Affiliated Hospital of Zhengzhou University, 1 East Jianshe Road, Zhengzhou, Henan 450052, P.R. China
E-mail: mingzhiz@aol.com

Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer in men and women over 50 years of age. In the past decade, despite the great advances in HCC diagnosis and treatment, the incidence rates, as well as the mortality rates of HCC patients worldwide are increasing (1). Particularly in the Asian or Chinese population, the numbers of HCC patients and HCC-related cancer deaths are almost twice these numbers in Caucasian patients, due to common infection with *Helicobacter pylori* or hepatitis B virus (2). Thus, it is critical to elucidate the underlying mechanisms of HCC proliferation and metastasis in order to provide more accurate diagnosis and advanced optimal treatment strategies for HCC patients.

MicroRNAs (miRNAs) are families of 18-22 nucleotide non-coding RNAs that bind to the 3'-untranslated regions (3'-UTR) of target mRNAs to negatively modulate gene and protein expression by DNA or protein degradation in both animals and humans (3). In the past few decades, mounting evidence demonstrates that miRNAs play a critical role in various stages of carcinogenesis, cancer proliferation and cancer metastasis of human cancers, including HCC (4-6). In HCC, numerous cancer-associated miRNAs act as either oncogenes, such as miR-494, miR-93 and miR-184 (7-9), or tumor-suppressors, such as miR-31, miR-29c and miR-148a (10-12). A recent study found that miR-137 is a tumor-suppressor miRNA in HCC, as miR-137 was downregulated in HCC and its subsequent upregulation inhibited HCC proliferation and migration in *in vivo* xenografts (13). However, it is known that miR-137 may exert its cancer regulatory effects through multiple genes (14,15). Therefore, it is important to explore the full scope of the downstream genes associated with miR-137 to better understand its regulation in HCC.

One of the common target genes of miR-137 is cell division cycle 42 (CDC42), a GTPase of the Rho family (16-18). CDC42 itself is also highly associated with cancer regulation in many types of human carcinomas (19,20). A mouse model of CDC42 deficiency showed that it induced the development of HCC (21). In humans, CDC42 was found to be weakly expressed in clinical tumor samples (22). While this body of evidence points to a tumor-suppressing role of CDC42 in HCC, it is notable that CDC42 also acts as an oncogenic factor as knockdown of CDC42 inhibited HCC migration *in vitro* (23). Therefore, this

conflicting body of evidence suggests that complex signaling pathways may be associated with CDC42 regulation in HCC.

In the present study, we explored the possible molecular association between CDC42 and miR-137 in HCC regulation. We examined whether miR-137 directly targets CDC42 in HCC, and whether CDC42 exerts any regulatory effects on miR-137-induced inhibition of HCC proliferation and metastasis, the two key properties of human HCC. Furthermore, we examined whether there is crosstalk between CDC42 and other miR-137 target genes in HCC. The results of our study may help elucidate the molecular profile of miRNA regulation in human HCC.

Materials and methods

HCC cell lines. Human HCC cell lines, HuH7, BEL-7402, HEPG2 and HEP3B and a normal human hepatocyte cell line (THLE-2) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MHCC97L was kindly provided by the Liver Cancer Institute of Zhongshan Hospital at Fudan University (Shanghai, China). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Sigma-Aldrich, USA), 100 IU/ml penicillin G and 100 μg/ml streptomycin at 37˚C in 5% CO_2. Once confluency was achieved, the cells were passaged with replenished medium every 3 or 4 days.

RNA isolation and quantitative real-time PCR (qRT-PCR). To extract RNA, the cell or tumor samples were homogenized in TRIzol reagent (Invitrogen, USA). Reverse-transcription was carried out by regular PCR using a RT-PreMix kit (SBS Genetech, China) according to the manufacturer's protocol. To quantitatively measure the gene expression level of miR-137, miRNA qRT-PCR was carried out using a Hairpin-it™ miRNAs Real-Time PCR Quantitation kit (GenePharma, China) with internal control of U6 snRNA, according to the manufacturer's protocol. To quantify mRNA expression of CDC42 and AKT2, qRT-PCR was carried out using a SYBR Green PCR Master Mix (Applied Biosystems, USA) with internal control of the 18s gene, according to the manufacturer's protocol. All primer sets were purchased from SBS Genetech. Gene expression levels were quantified by $2^{-\Delta\Delta ct}$ methods against internal control genes and are reported as relative fold-changes.

MicroRNA-137 upregulation. Lentiviruses carrying the mature hsa-miR-137 mimic (miR-137) or its negative control miRNA (miR-C) were obtained from RiboBio (China). Lentiviral transfection of the HuH7 and MHCC97L cells was carried out using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol for 24 h, followed by replenishment with fresh medium.

Dual-luciferase reporter assay. The 3'-UTR of human CDC42 including the putative miR-137 binding site was amplified from a human liver cDNA library and then cloned into the *Spe*I/*Hin*dIII site of a pMIR-REPORT luciferase vector to generate the wild-type (WT) CDC42 luciferase reporter (CDC42-WT; Ambion, USA). A mutant CDC42 3'UTR with a modified miR-137 binding site was generated

using a Site-Directed Mutagenesis kit (SBS Genetech). It was also cloned into the pMIR-REPORT vector to generate the mutant CDC42 luciferase reporter (CDC42-MT). In both HuH7 and MHCC97L cells, miR-137 was co-transfected with CDC42-WT, CDC42-MT or a *Renilla* luciferase control vector (Luc-C) for 48 h. The luciferase activities were measured by a dual-luciferase reporter assay (Promega, USA), and normalized to the activity of the *Renilla* control.

Human tumor specimens. Thirteen human HCC specimens were collected by surgery between June 2013 and April 2015 at the Department of Oncology of The First Affiliated Hospital of Zhengzhou University, and the Department of Oncology of The First People's Hospital of Zhengzhou in Zhengzhou, China. Consent forms were signed by all patients. The clinical and laboratory protocols were approved by the Ethic Committees at the participating institutes.

Western blot analysis. HuH7 and MHCC97L cells were collected and lysed in a lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.5% NP-40 and protease inhibitor cocktail (Millipore, USA). The extracted cell proteins were dissolved on 10% SDS-PAGE gel, transferred to nitrocellulose membranes, and incubated with a primary rabbit antibody against human CDC42 (1:200; Sigma-Aldrich) at 4˚C overnight. On the second day, after washing with Tris-buffered saline (3 x 10 min), the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Millipore). Each blot was visualized with enhanced chemiluminescence (Pierce, USA) according to the manufacturer's protocol.

Overexpression assay. Whole DNA sequences of CDC42 and AKT2 were amplified from a human liver cDNA library and confirmed with sequencing. The DNA sequences were then cloned into a recombinant plasmid eukaryotic expression vector pcDNA3.1 (Invitrogen) to construct overexpressing vectors of CDC42 (pcDNA3.1/CDC42), and AKT2 (pcDNA3.1/AKT2). An empty vector (pcDNA3.1/+) was used as control. The transfection was carried out using Lipofectamine 2000 reagent according to the manufacturer's protocol for 24 h, followed by replenishment with fresh medium.

In vitro proliferation assay. HuH7 and MHCC97L cells were initially transfected with the lentiviruses and/or the overexpressing vectors for 24 h. After 24 h, the culture medium was replenished and the proliferation of HCC cells was characterized using a 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for 5 consecutive days. Briefly, after washing the cells with PBS, 1 ml of 0.5 mg/ml MTT was added into the culture for 4 h. MTT was aspirated and 300 μl isopropanol was immediately added. The optical density (OD) was measured at a wavelength of 570 nm.

In vitro migration assay. Metastasis in the HuH7 and MHCC97L cells was measured using a QCM chemotaxis 96-well migration assay (Chemicon, USA). On the first day, HuH7 and MHCC97L cells were transfected with the lentiviruses and/or the overexpressing vectors. During this time, the upper chamber of the QCM chemotaxis plate was coated
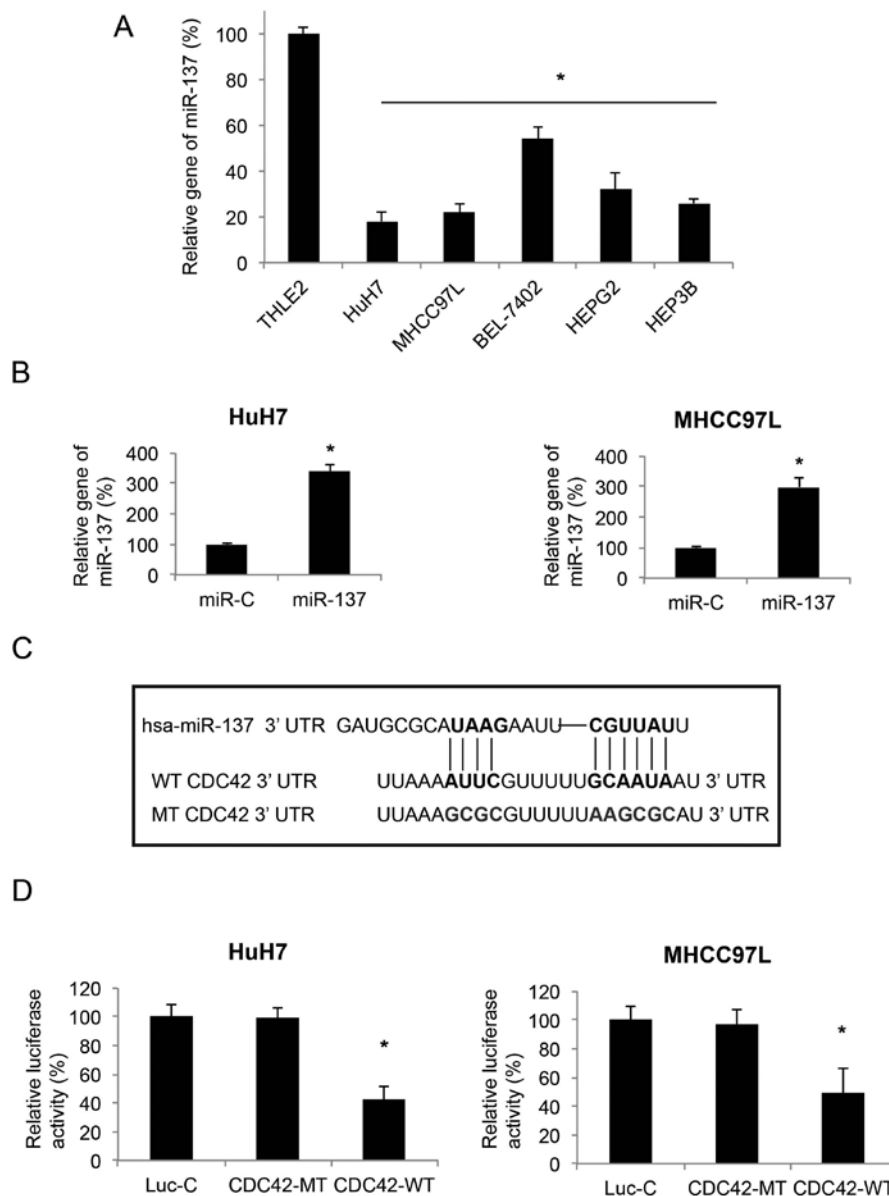
Figure 1. Gene expression of miR-137 in HCC cell lines and its targeting of CDC42. (A) Quantitative RT-PCR was used to compare the gene expression levels of miR-137 in 7 HCC cell lines to the expression level in a normal human hepatocyte cell line (THLE-2) (*P<0.05). (B) HuH7 and MHCC97L cells were transfected with an miR-137 mimic lentivirus (miR-137) or a control miRNA lentivirus (miR-C). Twenty-four hours after transfection, qRT-PCR was used to evaluate the gene expression level of miR-137 in the HuH7 and MHCC97L cells (*P<0.05). (C) A diagram illustrates the binding of hsa-miR-137 on the wild-type (WT) human CDC42 3'UTR. The binding site was genetically modified in a mutant (MT) CDC43 3'UTR. (D) HuH7 and MHCC97L cells were co-transfected with miR-137, and one of the three luciferase reporter vectors, including a luciferase reporter vector containing WT CDC42 3'UTR (CDC42-WT), a luciferase reporter vector containing MT CDC42 3'UTR (CDC42-MT) and a control *Renilla* luciferase reporter vector (Luc-C). Twenty-four hours after transfection, relative luciferase activities were evaluated through a dual-luciferase reporter assay (*P<0.05).

with 0.1% gelatin (in PBS) overnight. On the second day, HuH7 and MHCC97L cells were resuspended and re-plated in the upper chamber with RPMI-1640 medium. The lower chamber was filled with RPMI-1640 medium with the addition of 10% FBS as a chemoattractant. After 24 h, the HCC cells that migrated into the lower chamber were fixed with 4% PFA and stained with hematoxylin and eosin (H&E). The relative migration rates were measured by averaging the cell numbers in 5 random 0.1 x 0.1 mm regions and normalizing to the cell number under control conditions.

In vivo transplantation assay. HuH7 cells were transfected with the lentiviruses and/or the overexpressing vectors. After

24 h, the cells were collected and resuspended. Approximately 1 million cells were subcutaneously inoculated into the left flank of 2-month-old female nude mice. The *in vivo* tumor growth assay was carried out by measuring the *in vivo* tumor volume based on the equation: length x width^2/2. At the end of the 5-week transplantation, the tumors were extracted and Ki-67 immunostaining (BD Biosciences) was carried out on paraffin sections.

Statistical analysis. In the present study, data are presented as the mean ± standard deviation. Statistical analysis with the two-tailed Student's t-test (SPSS, version 11.0) was carried out to evaluate the difference between results. Differences
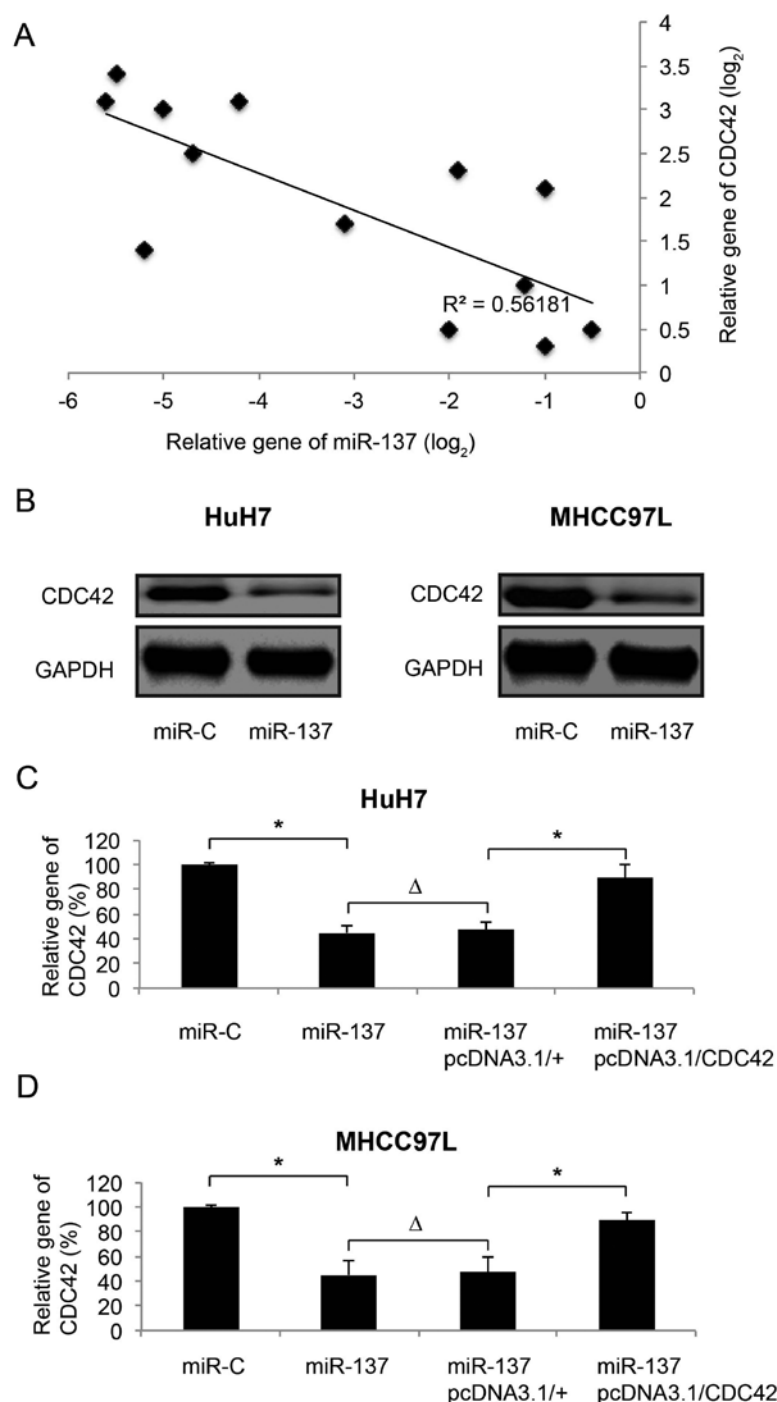
Figure 2. Inverse correlation of miR-137 and CDC42 in HCC. (A) Association between miR-137 and CDC42 gene expression levels was evaluated in 13 HCC tumor samples with a linear regression method. (B) miR-137 was ectopically upregulated by a lentivirus in the HuH7 and MHCC97L cells. The protein levels of CDC42 were evaluated by western blot analysis. (C and D) Twenty-four hours after lentiviral transfection, the HuH7 and MHCC97L cells were further transfected with pcDNA3.1/CDC42 to overexpress CDC42. The mRNA levels of CDC42 were evaluated by qRT-PCR ($^{*}P<0.05$; $^{\Delta}P>0.05$).

were considered significant at $P<0.05$. All experiments were repeated in triplicates.

Results

MicroRNA-137 is downregulated in HCC cell lines and targets CDC42. A previous study demonstrated that miR-137 is downregulated in HCC cell lines (13). In the present study, we firstly verified this result by qRT-PCR. We found that

the gene expression levels of miR-137 were lower in all 7 probed HCC cell lines, when compared with the expression level of miR-137 in THLE2, a normal human hepatocyte cell line ($P<0.05$, Fig. 1A). We then used a lentiviral vector to ectopically upregulate miR-137 in two HCC cell lines, HuH7 and MHCC97L. The efficiency of lentivirus-mediated miR-137 upregulation was confirmed by qRT-PCR ($P<0.05$, Fig. 1B).

In order to find the downstream molecular target of miR-137 in HCC, we explored several online miRNA target softwares,
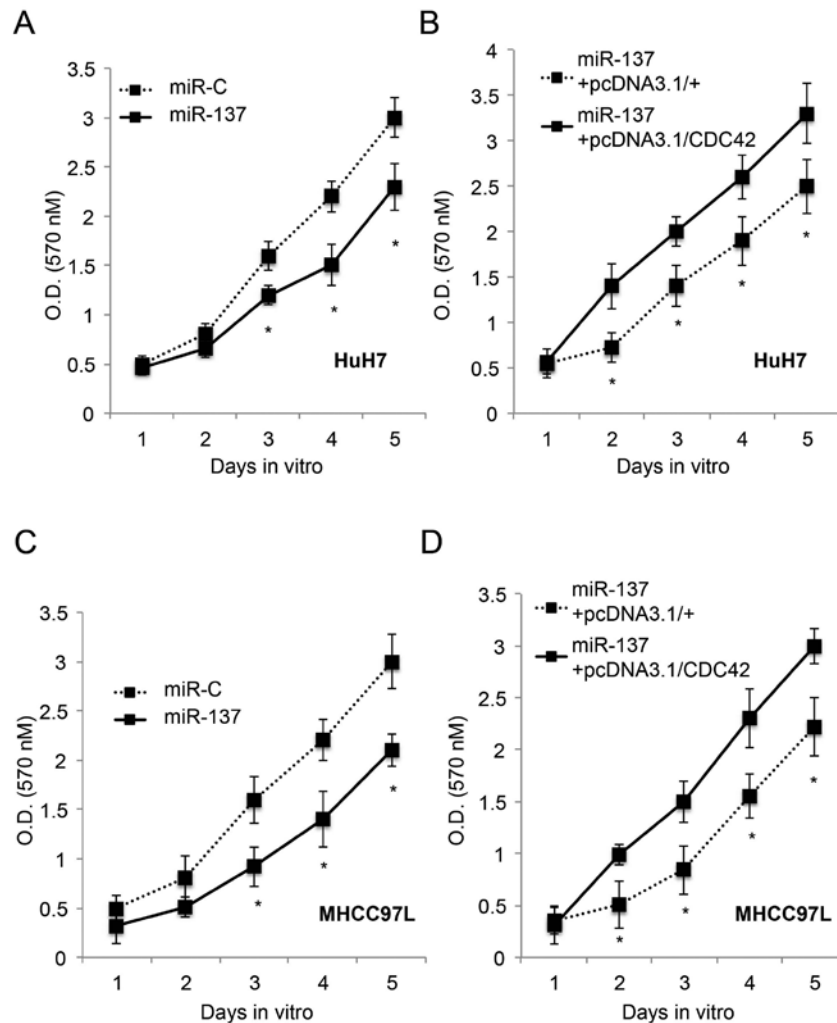
Figure 3. The effect of CDC42 on miR-137-mediated HCC proliferation. (A) HuH7 cells were transfected with lentiviruses of miR-137 or miR-C. Twenty-four hours after transfection, an MTT assay was carried out for 5 consecutive days to evaluate cell proliferation *in vitro* (*P<0.05). (B) Twenty-four hours after lentiviral transfection, the HuH7 cells were transfected with pcDNA3.1/CDC42 to ectopically overexpress CDC42 for another 24 h. Control HuH7 cells were transfected with an empty overexpression vector pcDNA3.1/+. MTT assay was then carried out for 5 days to evaluate cancer proliferation (*P<0.05). (C and D) The same experiments as (A) and (B) were also carried out in MHCC97L cells.

such as TargetScan (www.targetscan.org), microRNA (www.microRNA.org) and miTarget (www.cbit.snu.ac.kr/~miTarget) and found that CDC42 was a possible hit (Fig. 1C). We then carried out a dual-luciferase reporter assay and confirmed that in HuH7 and MHCC97L cells, CDC42 was the downstream target of miR-137 (P<0.05, Fig. 1D).

MicroRNA-137 is inversely correlated with CDC42 in both HCC tumors and cell lines. We then evaluated the correlation between miR-137 and CDC42 in both HCC tumors and cell lines. Firstly, 13 clinically obtained HCC tumor samples underwent gene expression analysis by qRT-PCR. We found that, through a linear regression method, the gene expression level of miR-137 was inversely correlated with the mRNA expression level of CDC42 in the human HCC tumors (Fig. 2A). We also examined the protein expression of CDC42 in HCC cell lines. We found that, while miR-137 was upregulated by lentiviral transfection, CDC42 protein levels were substantially downregulated in the HuH7 and MHCC97L cells (Fig. 2B). The downregulation of CDC42 by

miR-137 upregulation in HCC cells was further confirmed by qRT-PCR (Fig. 2C and D; miR-C vs. miR-137, P<0.05).

After CDC42 was downregulated by miR-137 upregulation, we re-introduced CDC42, through the transection of an overexpression vector pcDNA3.1/CDC42, back into HuH7 and MHCC97L cells. In the control experiment, the HCC cells were transfected with an empty vector pcDNA3.1/+. We found that, while pcDNA3.1+ had no effect on CDC42 gene expression (Fig. 2C and D; miR-137 vs. miR-137+pcDNA3.1/+, P>0.05), pcDNA3.1/CDC42 significantly upregulated CDC42 mRNA in the HuH7 and MHCC97L cells (Fig. 2C and D; miR-137+pcDNA3.1/+ vs. miR-137+ pcDNA3.1/CDC42, P<0.05).

Inhibition of HCC proliferation by miR-137 is ameliorated by CDC42. Since we discovered that CDC42 was inversely regulated by miR-137 in HCC, we speculated that CDC42 may play a functional role in HCC. To test this hypothesis, we firstly upregulated miR-137 in the HuH7 and MHCC97L cells with an miR-137 lentivirus. Through a 5-day proliferation assay, we found that miR-137 upregulation significantly
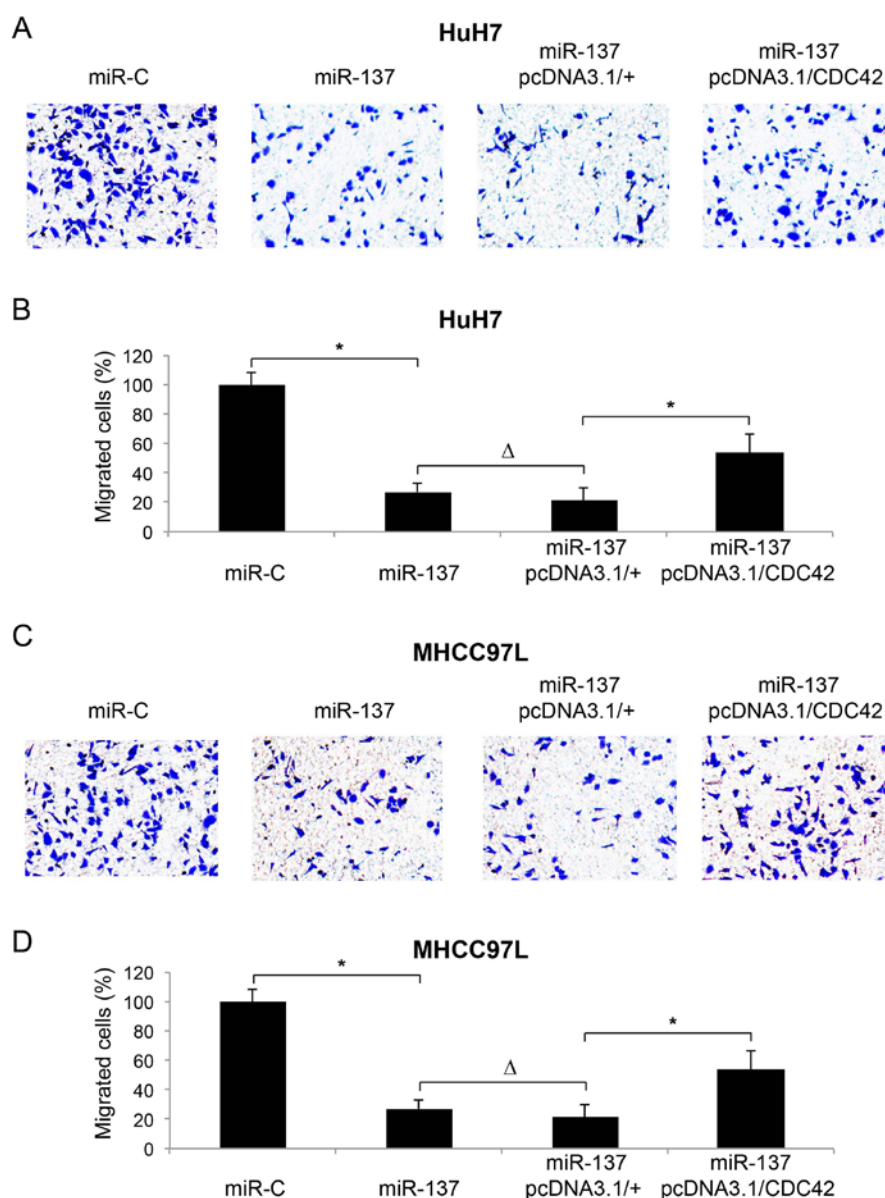
Figure 4. Effect of CDC42 on miR-137-mediated HCC metastasis. (A and B) HuH7 cells were firstly transfected with lentiviruses of miR-137 or miR-C. Twenty-four hours after the first transfection, a second transfection of pcDNA3.1/CDC42 or pcDNA3.1/+ was carried out. At the end of each transfection, a migration assay was used to evaluate cancer metastasis *in vitro*. (A) The representative fluorescent images of the bottom chamber for each experimental condition are shown. (B) Quantification of migration capability was performed by calculating the relative percentages of migrated cells under each experimental condition and normalizing them to the control condition of miR-C transfection ($^*P<0.05$; $^\Delta P>0.05$). (C and D) The same experiments as (A) and (B) were also carried out in MHCC97L cells.

inhabited cancer growth in both the HuH7 and MHCC97L cells (P<0.05, Fig. 3A and C), in line with a previous study (13). Secondly, 24 h after lentiviral transfection to upregulate miR-137, CDC42 was overexpressed in the HuH7 and MHCC97L cells for 24 h. Another 5-day *in vitro* proliferation assay showed that re-introduction of CDC42 restored the growth of HuH7 and MHCC97L cells (P<0.05, Fig. 3B and D). Thus, our results showed that overexpression of CDC42 ameliorated the inhibitory effect of miR-137 on HCC proliferation.

Inhibition of HCC metastasis by miR-137 is ameliorated by CDC42. We speculated that CDC42 may play a functional role in miR-137-mediated HCC metastasis. Firstly, we confirmed the inhibitory effect of miR-137 on HCC metastasis (13). We

transfected HuH7 and MHCC97L cells with a lentivirus of miR-137 or miR-C, followed by a migration assay to assess the metastasis in 24 h. The assay showed that in both HCC cell lines, the metastatic capability of the cancer cells was inhibited by miR-137 upregulation. Immunostaining showed that significantly less migrated HCC cells were noted in the lower chambers (Fig. 4A and C, miR-C vs. miR-137). Quantitative measurement showed that miR-137 upregulation reduced the percentages of migrated cells to <40% in both the HuH7 and MHCC97L cells (Fig. 4B and D; miR-C vs. miR-137, P<0.05).

Secondly, 24 h after miR-137 transfection, we carried out another transfection with either pcDNA3.1/CDC42 or pcDNA3.1/+ in the HuH7 and MHCC97L cells, followed by a migration assay to evaluate the effect of CDC42 over-
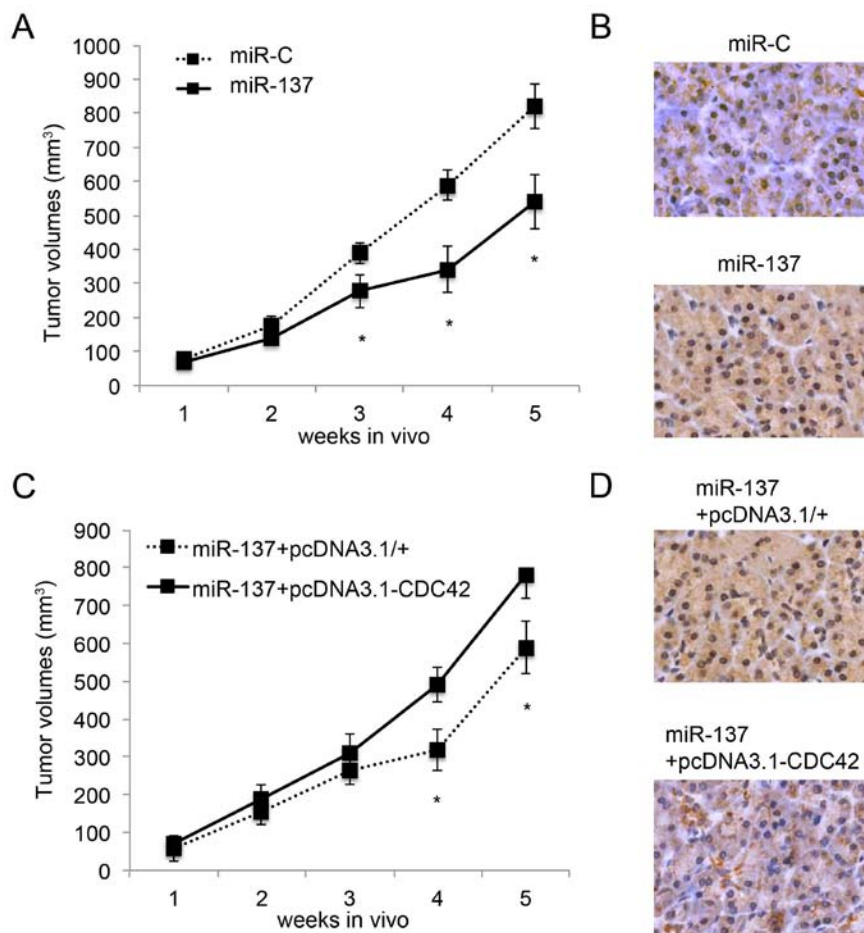
Figure 5. Effect of CDC42 on miR-137-mediated *in vivo* HCC tumor growth. (A) HuH7 cells were transfected with lentiviruses of miR-137 or miR-C, and then inoculated into the left flank of 2-month-old nude mice. *In vivo* tumor volume was monitored for 5 consecutive weeks (*P<0.05). (B) Five weeks after inoculation, the tumors were retrieved, sectioned and stained with Ki67. (C) HuH7 cells were firstly transfected with lentiviruses of miR-137 for 24 h, followed by a second transfection of pcDNA3.1/CDC42 or pcDNA3.1/+. Twenty-four hours after the second transfection, cells were inoculated into the nude mice and tumor growth was monitored for 5 consecutive weeks (*P<0.05). (D) Five weeks after inoculation, tumors were retrieved, sectioned and stained with Ki67.

expression on HCC metastasis. Immunostaining showed that, after miR-137 upregulation, re-introduction of CDC42 promoted more HCC cells to migrate into the lower chambers (Fig. 4A and C; miR-137 + pcDNA3.1/+ vs. miR-137 + pcDNA3.1/+CDC42). Furthermore, quantification demonstrated that CDC42 restored the percentages of migrated cells to ~60% of the original levels (Fig. 4B and D; miR-137 + pcDNA3.1/+ vs. miR-137 + pcDNA3.1/+CDC42, P<0.05). It is also worth noting that second transfection of the empty vector had no effect on HCC metastasis (Fig. 4B and D; miR-137 vs. miR-137 + pcDNA3.1/+).

Therefore, the results of our migration assay demonstrated that overexpression of CDC42 ameliorated the inhibitory effect of miR-137 on HCC metastasis.

Inhibition of in vivo HCC tumor growth by miR-137 is ameliorated by CDC42. We then examined the effect of the overexpression of CDC42 on miR-137-mediated inhibition of *in vivo* HCC tumor growth (13). Firstly, we transfected HuH7 cells with the lentivirus of miR-137 or miR-C. Twenty-four hours later, HuH7 cells were transplanted into 2-month-old null mice. The *in vivo* growth of tumors was monitored for 5 weeks, followed by immunostaining of Ki67 at the end of

the *in vivo* assay. Both the *in vivo* assay (P<0.05, Fig. 5A) and Ki67 immunostaining (Fig. 5B) confirmed that miR-137 inhibited HCC tumor growth. Secondly, 24 h after miR-137 transfection, HuH7 cells were further transfected with either pcDNA3.1/CDC42 or pcDNA3.1/+, followed by the transplantation assay. Both *in vivo* tumor growth assay (P<0.05, Fig. 5C) and Ki67 immunostaining (Fig. 5D) showed that overexpression of CDC42 ameliorated the inhibitory effect of miR-137 on *in vivo* HCC tumor growth.

CDC42 and AKT2 are independently expressed in HCC. A previous study demonstrated that miR-137 inhibited HCC through AKT2 (13). Since we demonstrated that CDC42 was also involved in miR-137-mediated HCC inhibition, we aimed to ascertain whether CDC42 and AKT2 undergoes crosstalk in HCC. We firstly examined the effect of the overexpression of CDC42 on the mRNA expression level of AKT2 in the HuH7 and MHCC97L cells. The results of qRT-PCR showed that CDC42 did not alter the expression level of AKT2 in the HCC cells (P>0.05, Fig. 6A and B). We then transfected the HuH7 and MHCC97L cells with another set of overexpression vectors of pcDNA3.1/AKT2 and its control pcDNA3.1/+. qRT-PCR showed that, in both HuH7 and MHCC97L cells, the
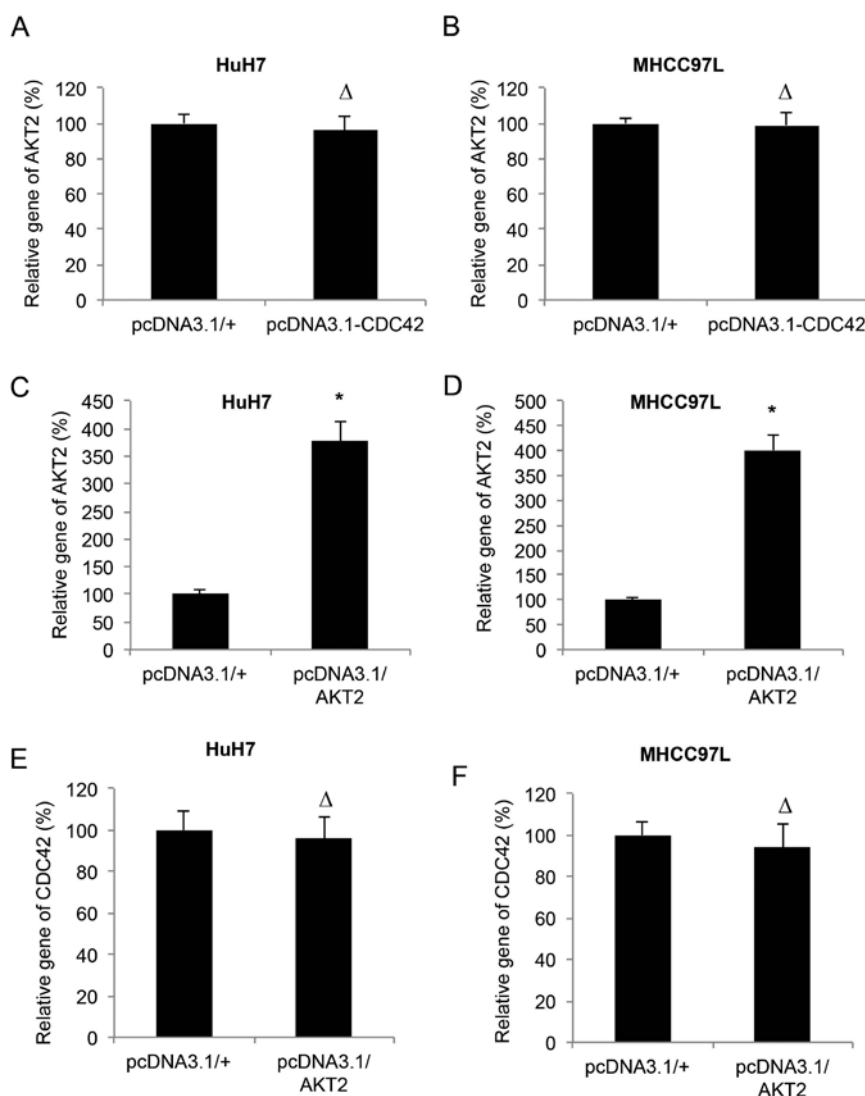
Figure 6. CDC42 is independent of AKT2 in HCC. (A and B) HuH7 and MHCC97L cells were transfected with either pcDNA3.1/CDC42 or pcDNA3.1/+. Twenty-four hours after transfection, mRNA expression levels of AKT2 were evaluated by qRT-PCR ($^\Delta$P>0.05). (C and D) HuH7 and MHCC97L cells were transfected with either pcDNA3.1/AKT2 or pcDNA3.1/+. Twenty-four hours after transfection, mRNA expression levels of AKT2 were evaluated by qRT-PCR (*P<0.05). (E and F) mRNA expression levels of CDC42 were also evaluated by qRT-PCR ($^\Delta$P>0.05).
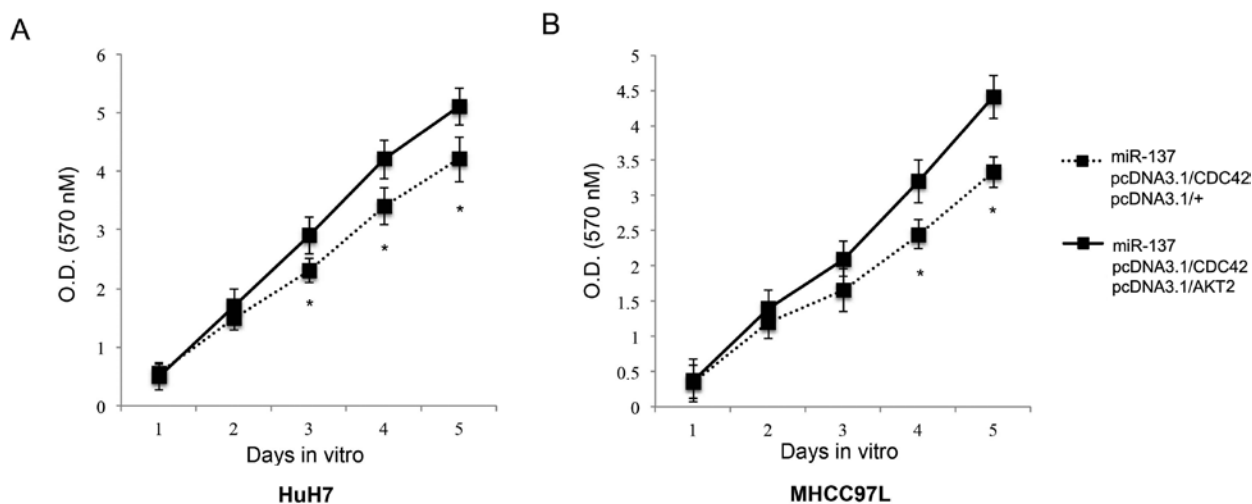

Figure 7. AKT2 further rescues HCC proliferation when combined with CDC42. (A) HuH7 and (B) MHCC97L cells were transfected with an miR-137 lentivirus for 24 h. They were then co-transfected with overexpressing vectors of pcDNA3.1/CDC42 and pcDNA3.1/AKT2 for another 24 h. The control cells were transfected with pcDNA3.1/CDC42 and pcDNA3.1/+. A 5-day *in vitro* proliferation assay was carried out to evaluate the combinational effects of CDC42 and AKT2 on miR-137-induced inhibition of HCC proliferation (*P<0.05).
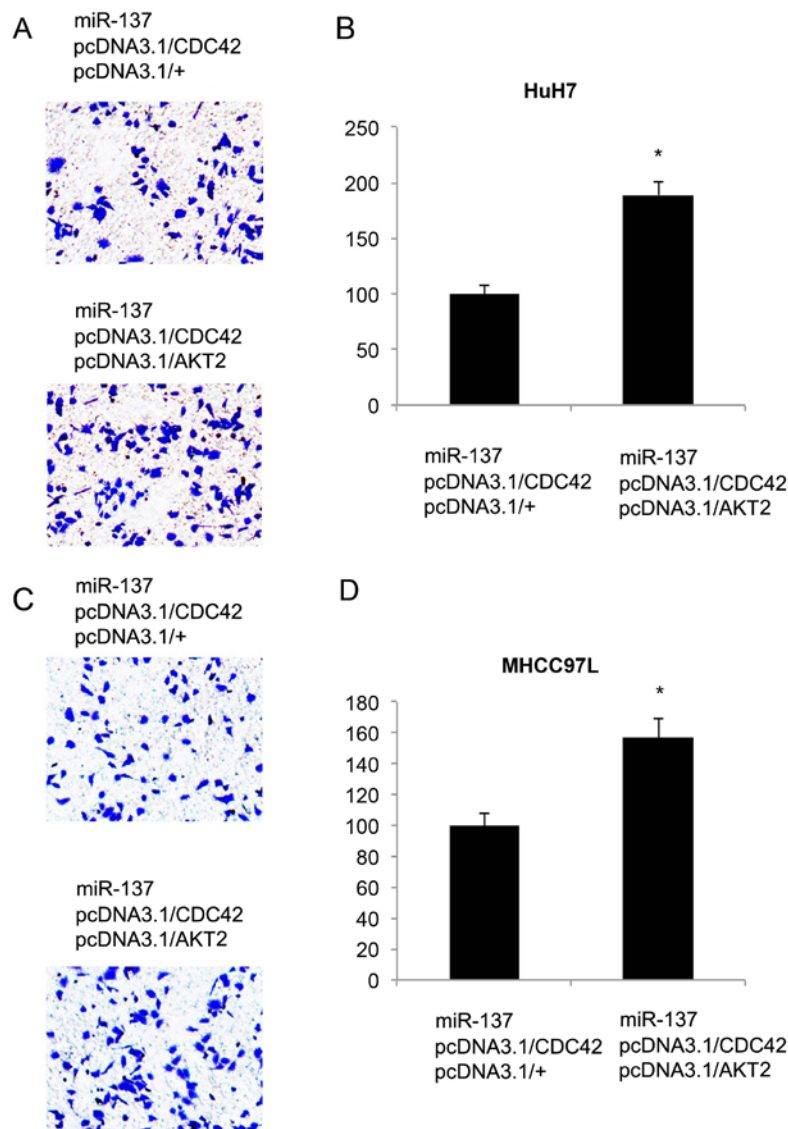
Figure 8. AKT2 further rescues HCC metastasis when combined with CDC42 in the HuH7 and MHCC97L cells transfected with an miR-137 lentivirus for 24 h. The cells were co-transfected with overexpression vectors pcDNA3.1/CDC42 and pcDNA3.1/AKT2 for another 24 h. The control cells were transfected with pcDNA3.1/CDC42 and pcDNA3.1/+. An *in vitro* migration assay was then carried out to evaluate cancer cell metastasis. In HuH7 (A) and MHCC97L cells (C), the representative fluorescent images of migrated cells in the bottom chambers are shown. Quantification of the percentages of migrated cells demonstrated that the metastasis capabilities were increased >150% with the addition of AKT2 in both HuH7 (B) (*P<0.05) and MHCC97L cells (D) (*P<0.05).

expression levels of AKT2 were significantly upregulated by pcDNA3.1/AKT2 (P<0.05, Fig. 6C and D), whereas expression levels of CDC42 were not changed (P>0.05, Fig. 6E and F).

AKT2 contributes additively to CDC42 in reversing the inhibitory effect of miR-137 on HCC proliferation and metastasis. We then investigated the correlation between AKT2 and CDC42 in the regulation of miR-137-induced HCC inhibition. Firstly, we studied the possible additive effect of AKT2 on HCC proliferation. We transfected HuH7 and MHCC97L cells with the miR-137 lentivirus. Twenty-four hours after that, we co-transfected the cells with pcDNA3.1/CDC42 and pcDNA3.1/AKT2. The control cells were co-transfected with pcDNA3.1/CDC42 and pcDNA3.1/+. The *in vitro* proliferation assay demonstrated that AKT2 overexpression, in addition to CDC42 overexpression, further rescued HCC proliferation from miR-137-induced inhibition (P<0.05, Fig. 7). Secondly,

we evaluated the additive effect of the overexpression of AKT2 on HCC metastasis. Twenty-four hours after co-transfection of the double-overexpressing vectors, an *in vitro* migration assay was carried out. It showed that in both HCC cell lines, more cancer cells migrated into the lower chambers (Fig. 8A and C). Quantitative measurement also showed that AKT2 overexpression, in addition to CDC42 overexpression, further rescued HCC metastasis from miR-137-induced inhibition (P<0.05, Fig. 8B and D).

Discussion

It was recently reported that miR-137 is a new member of the tumor-suppressing miRNAs found in human HCC (13). Liu *et al* found that miR-137 was lowly expressed in HCC tumors and cell lines, and was strongly associated with survival in patients with HCC (13). In addition, forced miR-137 overexpression

was able to exert inhibitory effects on HCC proliferation and migration, both *in vitro* and *in vivo* (13). In the present study, we firstly confirmed that the gene expression levels of miR-137 were low in 7 HCC lines, as compared to the expression level of miR-137 in a normal human hepatocyte cell line (THLE2). Importantly, one of the newly examined HCC cell lines in our study, MHCC97L, was specifically derived from Chinese HCC patients with low metastatic capability (24). Thus, our result showing that miR-137 was downregulated in MHCC97L cells, as in the other HCC cell lines, suggests that a low expression pattern of miR-137 may be universal regardless of ethic background.

Moreover, in our study, we used a lentivirus to ectopically upregulate miR-137 in the HuH7 and MHCC97L cells. We found that miR-137 upregulation inhibited HCC proliferation and metastasis *in vitro* and tumor growth *in vivo* (Figs. 3-5), further confirming the antitumor effect of miR-137 in HCC as shown in a previous study (13). Moreover, we identified CDC42 as another downstream target gene of miR-137 in HCC. Dual-luciferase reporter and western blot assays showed that CDC42 was directly regulated by miR-137 in HCC cells. Most importantly, while we used an overexpression system to ectopically re-introduce CDC42 back into HCC cells after miR-137 upregulation, we were able to ameliorate or reverse the tumor-suppressive effects of miR-137 on HCC *in vitro* proliferation, migration and *in vivo* tumor growth. A previous study showed that CDC42 was lowly expressed in liver tumors when compared to the expression in non-tumor liver tissues (22), suggesting that CDC42 may act as an antitumor (or tumor suppressing) factor in HCC. Interestingly, another study showed that CDC42 indeed acted as an oncogene in HCC as CDC42 knockdown inhibited the migration of QGY-7703 cells (23). Although the results of our study supported the idea of CDC42 as an oncogene, caution shall be taken to draw such a conclusion as more complex signaling mechanisms may be associated with CDC42 to determine whether it is an oncogene or a tumor-suppressor in HCC.

Finally, we co-expressed CDC42 with AKT2, another known miR-137 target gene in HCC (13), in the HuH7 and MHCC97L cells. We found that AKT2 contributed additively to CDC42 to rescue the inhibition of miR-137 on HCC proliferation and migration (Figs. 7 and 8), suggesting that CDC42 and AKT2 may exert their oncogenic effects independently, although both are regulated by miR-137. Future study may help to elucidate the differential signaling pathways associated with CDC42 or AKT2 in HCC regulation.

In summary, our study revealed that CDC42 is an independent target gene of miR-137 in regulating HCC. These results may help to identify possible biomarkers and elucidate the underlying molecular mechanisms of human HCC.

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