Tumor Biology

Inhibition of miR-137 on cancer proliferation and metastasis is regulated by CDC42 in human hepatocellular carcinoma

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Short Title:	miR-137 regulates CDC42 in HCC
Article Type:	Original Article
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Title: **Inhibition of miR-137 on cancer proliferation and metastasis is regulated by CDC42 in human hepatocellular carcinoma**

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Running Title: miR-137 regulates CDC42 in HCC

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Abstract

Background We evaluated the mechanisms of CDC42 in association with microRNA-137 (miR-137) –induced inhibition in human hepatocellular carcinoma (HCC).

Methods 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, and correlation analysis on clinical tumor samples. In HCC cell line HuH7 and MHCC97L cells, miR-137 was upregulated to inhibit cell proliferation and metastasis *in vitro* and tumor growth *in vivo*. CDC42 was over-expressed in HuH7 and MHCC97L cells to evaluate its effect on miR-137 mediated anti-tumor effects. Furthermore, possible cross-talk between CDC42 and the other miR-137 target gene, AKT2, was evaluated by co-overexpressing CDC42 and AKT2 in HuH7 and MHCC97L cells and examining their effects on miR-137 mediated HCC regulation.

Results MiR-137 was confirmed to be down-regulated in HCC cell lines. Dual-luciferase assay showed CDC42 was directly targeted by miR-137, and western blot showed CDC42 was down-regulated by miR-137 upregulation in HuH7 and MHCC97L cells. In human tumors, expressions 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-overexpressing 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.

Conclusions Our work demonstrated that the CDC42 independently regulated the anti-tumor effects of miR-137 in human HCC.

Keywords: hepatocellular carcinoma, miR-137, CDC42

Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer in men an women over 50 years old. In the past decade, despite the great advances in HCC diagnosis and treatment, the incidence rates, as well as the mortality rates of world-wide HCC patients were suprisingly on the rise [1]. Especially in Asian or Chinese population, the numbers of HCC patients and HCC-related cancer death are almost twice the numbers of white 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 much accurate diagnosis and much advanced optimal treatment plans for the patients with HCC.

MicroRNA (miRNAs) are families of 18~22 of noncoding RNAs that bind to the 3'-untranslated regions (3'-UTR) of target mRNAs to negatively modulate gene and protein expressions by DNA or protein degradation in both animal and human [3]. In the past decades, mounting evidence shows that miRNAs play critical role in various stages of human cancers, including HCC, such as carcinogenesis, cancer proliferation and cancer metastasis [4-6]. In HCC, many of the cancer associated miRNAs act as either oncogenes, such as miR-494 / miR-93 / miR-184 [7-9], or tumor-suppressors, such as miR-31/ miR-29c / miR-148a [10-12]. Recently, a new study found that microRNA-137 (miR-137) was a tumor-suppressor miRNA in HCC, as miR-137 was down-regulated in HCC and its subsequent upregulation inhibited HCC proliferation, migration and in vivo xenograft [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 CDC42 (cell division cycle 42), 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]. Mouse model of CDC42 deficiency showed it induced development of HCC [21]. In human, CDC42 was found to be lowly expressed in clinical tumor samples [22]. While those evidence pointed a tumor-suppressing role of CDC42 in HCC, it was surprisingly shown that CDC42 could act as an oncogenic factor as knocking down CDC42 inhibited HCC migration *in vitro* [23]. Therefore, those seeming conflicting evidence suggest that complex signaling pathways may associated with CDC42 regulation in HCC.

In this study, we explored the possible molecular association between CDC42 and miR-137 in HCC regulation. We examined whether miR-137 was directly targeting CDC42 in HCC, and whether CDC42 exerted any regulatory effects on miR-137 induced inhibition on HCC proliferation and metastasis, the two key properties of human HCC. Furthermore, we examined whether there was cross-talk between CDC42 and other miR-137 target gene in HCC. The results of our work may help elucidate the molecular profile of miRNA regulation in human HCC.

Materials and methods

HCC cell lines

Human hepatocellular carcinoma cell lines, HuH7, BEL-7402, HEPG2, HEP3B cells and a normal human hepatocyte cell line (THLE-2) were obtained from Cell Bank

of Chinese Academy of Sciences (Shanghai, China). MHCC97L was kindly provided by Liver Cancer Institute of Zhongshan Hospital at Fudan University (Shanghai, China). All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, USA), 100 IU/mL penicillin G and 100 μg/mL streptomycin at 37 ^{o}C and 5% CO_2. Once confluent, cells were passaged with replenished medium every 3 or 4 days.

RNA isolation and quantitative real-time PCR (qRT-PCR)

To extract RNA, cell or tumor samples were homogenized in a TRIzol Reagent (Invitrogen, USA). Reverse-transcription was carried out by regular PCR using a RT-PreMix kit (SBS Genetech, China) per 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 snRBA, per manufacturer's protocol. To quantify mRNA expressions of CDC42 and AKT2, qRT-PCR was carried out using a SYBR Green PCR master mix (Applied Biosystems, USA) with internal control of 18s gene, per manufacturer's protocol. All primer sets were bought from SBS Genetech (SBS Genetech, China). Gene expression levels were quantified by $2^{-\Delta\Delta ct}$ methods against internal control genes and reported as relative fold-changes.

MicroRNA-137 upregulation

Lentiviruses carrying mature has-miR-137 mimic (miR-137), or its negative control miRNA (miR-C) were obtained from RiboBio (RiboBio, China). Lentiviral transfection in HuH7 and MHCC97L cells was carried out using a Lifofectamine 2000 reagent (Invitrogen, USA) per manufacturer's protocol for 24 h, followed by

replenishment with fresh medium.

Dual-luciferase reporter assay

The 3' UTR of human CDC42 including putative miR-137 binding site was amplified from a human liver cDNA library and then cloned into the SpeI / HindIII site of a pMIR-REPORT luciferase vector to generate wild type (WT) CDC42 luciferase reporter (CDC42-WT) (Ambion, USA). A mutant CDC42 3' UTR with modified miR-137 binding site was generated using a Site-Directed Mutagenesis Kit (SBS Genetech, China). It was also cloned into pMIR-REPORT vector to generate 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 renilla control.

Human tumor specimens

Thirteen human HCC specimens were collected through surgeries between June 2013 and April 2015 at the Department of Special Medical Care and Liver Transplantation, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University in Shanghai, China. Consent forms were signed by all patients. The clinical and laboratory protocols were approved by the Ethic Committees at the Eastern Hepatobiliary Surgery Hospital.

Western blot analysis

HuH7 and MHCC97L cells were colleted 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 in 10% SDS-PAGE gel, transferred to nitrocellulose membranes, and incubated with a

primary rabbit antibody against human CDC42 (1:200, Sigma Aldrich, USA) at 4 ^{0}C over night. On second day, after washing with Tris-buffered saline (3 X 10 mins), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Millipore, USA). The blot was visualized with enhanced chemiluminescence (Pierce, USA) per 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, USA) to make 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 a a Lifofectamine 2000 reagent (Invitrogen, USA) per manufacturer's protocol for 24 h, followed by replenishment with fresh medium.

In vitro proliferation assay

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

In vitro migration assay

Metastasis in HuH7 and MHCC97L cells was measured by a QCM chemotaxis

96-well migration assay (Chemicon, USA). On first day, HuH7 and MHCC97L were transfected with lentiviruses and/or overexpressing vectors. During the mean time, the upper chamber of the QCM Chemotaxis plate was coated with 0.1% gelatin (in PBS) over night. On second day, HuH7 and MHCC97L cells were resuspended and re-plated in upper chamber with RPMI-1640 medium. The lower chamber was filled with RPMI-1640 medium with the addition of 10% FBS as chemoattractant. After 24 h, HCC cells migrated into the lower chamber were fixed with 4% PFA and stained 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 lentiviruses and/or overexpressing vectors. After 24 h, cells were collected and resuspended. Approximate 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×width^2/2. At the end of 5-week transplantation, tumors were extracted and Ki-67 immunostaining (BD Bioscience) was carried out on paraffin sections.

Statistical analysis

In the present study, data were presented as mean ± standard deviation. Statistical analysis of two-tailed Student's *t* test (SPSS, version 11.0) was carried out to evaluate the difference between results. And the Differences were considered significant if $P < 0.05$. All experiments were repeated in triplicates.

Results

MicroRNA-137 is down regulated in HCC cell lines and targets CDC42

Previous study demonstrated that miR-137 was down-regulated in HCC cell lines [13]. In our study, we firstly verified this result by qRT-PCR. We found that the gene expression levels of miR-137 were lower in all seven probed HCC cell lines, than the expression level of miR-137 in THLE2, a normal human hepatocyte cell line (Fig. 1A, * $P < 0.05$). We then used lentiviral vector to ectopically upregulate miR-137 in two HCC cell lines, HuH7 and MHCC97L cells. The efficiency of lentivirus mediated miR-137 upregulation was confirmed by qRT-PCR (Fig. 1B, * $P < 0.05$).

In order to find the downstream molecular target of miR-137 in HCC, we explored several online miRNA target softwares, such as TargetScan (www.targetscan.org), microRNA (www.microRNA.org) and miTraget (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, CDC-42 was the downstream target of miR-137 (Fig. 1D, * $P < 0.05$).

MicroRNA-137 inversely correlates with CDC42 in both HCC tumor and cell lines

We then evaluated the correlation between mir-137 and CDC42 in both HCC tumors and cell lines. First, 13 clinically obtained HCC tumor samples were 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 mRNA expression level of CDC42 in human HCC tumors (Fig. 2A). We also examined the protein of CDC42 in HCC cell lines. We found that, while miR-137 was upregualted by

lentiviral transfection, CDC42 proteins were substantially down-regulated in HuH7 and MHCC97L cells (Fig. 2B). The down-regulation of CDC42 by miR-137 upregulation in HCC cells was further confirmed by qRT-PCR (Fig. 2C-D, miR-C vs. miR-137, * $P < 0.05$).

After CDC42 was down-regulated 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 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-D, miR-137 vs. miR-137+pcDNA3.1/+, $\Delta P > 0.05$), pcDNA3.1/CDC42 significantly upregualted CDC42 mRNA in HuH7 and MHCC97L cells (Fig. 2C-D, miR-137+pcDNA3.1/+ vs. miR-137+ pcDNA3.1/CDC42, * $P < 0.05$).

Inhibition of miR-137 on HCC proliferation was ameliorated by CDC42

Since we discovered that CDC42 was inversely regulated by miR-137 in HCC, we speculated CDC42 might play functional role in HCC. To test this hypothesis, we firstly upregulated miR-137 in HuH7 and MHCC97L cells with miR-137 lentivirus. Through a 5-day proliferation assay, we found that miR-137 upregulation significantly inhabited cancer growth in both HuH7 and MHCC97L cells (Fig. 3A, C, * $P < 0.05$), in line with previous report [13]. Secondly, 24 h after lentiviral transfection to upregulate miR-137, CDC42 was overexpressed in 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 (Fig. 3B, D, * $P < 0.05$). Thus, our results showed that overexpressing CDC42 ameliorated the inhibitory effect of miR-137 on HCC proliferation.

Inhibition of miR-137 on HCC metastasis was ameliorated by CDC42

We speculated CDC42 might play 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 lentivirus of miR-137 or miR-C, followed by a migration assay to assess the metastasis in 24 h. It showed that in both HCC cells, metastatic capability of cancer cells was inhibited by miR-137 upregulation. Immunostaining showed that significantly less migrated HCC cells were seen in the lower chambers (Fig. 4A, C, miR-C vs. miR-137). Quantitative measurement showed that miR-137 upregulation reduced the percentages of migrated cells to less than 40% in both HuH7 and MHCC97L cells,(Fig. 4B, 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 HuH7 and MHCC97L cells, followed by migration assay to evaluate the effect of CDC42 overexpression on HCC metastasis. Immunostaining showed that, after miR-137 upregulation, re-introduction of CDC42 promoted more HCC cells to migrate into lower chambers (Fig. 4A, 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 original levels (Fig. 4B, D, miR-137 + pcDNA3.1/+ vs. miR-137 + pcDNA3.1/+CDC42, * $P < 0.05$). It is also worth noting that, 2nd transfection of the empty vector had no effect on HCC metastasis (Fig. 4B, D, miR-137 vs. miR-137 + pcDNA3.1/+).

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

Inhibition of miR-137 on in vivo HCC tumor growth was ameliorated by CDC42

We then examined the effect of overexpressing CDC42 on miR-137 mediated inhibition on *in vivo* HCC tumor growth [13]. Firstly, we transfected HuH7 cells with lentivirus of miR-137 or miR-C. 24 h later, HuH7 cells were transplanted into 2-month old null mice. The *in vivo* growth of tumor was monitored for 5 weeks, followed by immunostaining of Ki67 at the end of *in vivo* assay. Both *in vivo* assay (Fig. 5A, * $P < 0.05$) 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 (Fig. 5C, * $P < 0.05$) and Ki67 immunostaining (Fig. 5D) showed that overexpressing CDC42 ameliorated the inhibitory effect of miR-137 on *in vivo* HCC tumor growth.

CDC42 and AKT2 were independently expressed in HCC

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 wondered whether CDC42 and AKT2 cross-talked in HCC. We firstly examined the effect of overexpressing CDC42 on mRNA expression level of AKT2 in HuH7 and MHCC97L cells. The results of qRT-PCR showed that CDC42 did not alter the expression level of AKT2 in HCC cells (Fig. 6A-B, $\Delta P > 0.05$). We then transfected 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 expression levels of AKT2 were significantly upregulated by pcDNA3.1/AKT2 (Fig. 6C-D, * $P < 0.05$), whereas expression levels of CDC42 were not changed (Fig. 6E-F, $\Delta P > 0.05$).

AKT2 contributed 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 regulating miR-137 induced HCC inhibition. Firstly, we studied the possible additive effect of AKT2 on HCC proliferation. We transfected HuH7 and MHCC97L cells with miR-137 lentivirus. 24 h after that, we co-transfected 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 (Fig. 7A-B, * $P < 0.05$). Secondly, we evaluated the additive effect of overexpressing AKT2 on HCC metastasis. 24 h after co-transfection of double overexpressing vectors, an *in vitro* migration assay was carried out. It showed that in both HCC cells, more cancer cells migrated into the lower chambers (Fig. 8A, C). Quantitative measurement also showed that AKT2 overexpression, in addition to CDC42 overexpression, further rescued HCC metastasis from miR-137 induced inhibition (Fig. 8B, D, * $P < 0.05$).

Discussions

It was recently reported that miR-137 was a new member of tumor-suppressing miRNAs in human HCC [13]. Liu and colleagues discovered that miR-137 was lowly expressed in HCC tumors and cell lines, and strongly associated with survivals in patients with HCC [13]. In addition, forced miR-137 over-expression was able to exert inhibitory effects on HCC proliferation and migration, both *in vitro* and *in vivo* [13]. In our work,

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 (THLE-2). Interestingly, one of the newly examined HCC cell line in our study, MHCC97L, was specifically derived from Chinese HCC patients with low metastasis capability [24]. Thus, our result showing miR-137 was down-regulated in MHCC97L cells, as in other HCC cell lines, suggests that low expression pattern of miR-137 may be universe regardless ethic background.

Also in our work, we used lentivirus to ectopically up-regulate miR-137 in HuH7 and MHCC97L cells. We found miR-137 upregulation inhibited HCC proliferation and metastasis in vitro and tumor growth in vivo (Fig. 3-5), further confirmed the anti-tumor effect of miR-137 in HCC as shown in previous study [13]. Moreover, we identified CDC42 was another downstream target gene of miR-137 in HCC. Dual-luciferase reporter assay and western blot assay showed CDC42 was directly regulated by miR-137 in HCC cells. Most importantly, while we used over-expression system to ectopically re-introduce CDC42 back into HCC cells after miR-137 upregulation, we were able to ameliorate or reverse the tumor-suppressing effects of miR-137 on HCC in vitro proliferation, migration and in vivo tumor growth. A previous report showed that CDC42 was lowly expressed in liver tumors than non-tumor liver tissues [22], suggesting CDC42 might act as an anti-tumor (or tumor suppressing) factor in HCC. Interestingly, another report showed CDC42 indeed acted as oncogene in HCC as CDC42 knockdown inhibited migration in QGY-7703 cells [23]. Though the results of our work supported the idea of CDC42 being an oncogene, caution shall be taken to draw such conclusion as

more complex signaling mechanisms may be associated with CDC42 to determine whether it's oncogene or tumor-suppressor in HCC.

In the final attempt of our study, we co-expressed CDC42 with AKT2, another known miR-137 target gene in HCC [13], in HuH7 and MHCC97L cells. We found that AKT2 contributed additively to CDC42 to rescue the inhibition of miR-137 on HCC proliferation and migration (Fig. 7 & 8), suggesting that CDC42 and AKT2 may exert their oncogenic effects independently, though 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 work discovered CDC42 is an independent target gene of miR-137 in regulating HCC. These results may help to identify possible bio-marker and elucidate the underlying molecular mechanisms of human HCC.

Conflict of Interest: None.

Figure Legends

Figure 1. Gene expression of miR-137 in HCC cell lines and its targeting on 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 miR-137 mimic lentivirus (miR-137) or a control miRNA lentivirus (miR-C). 24 h after transfection, qRT-PCR was used to evaluate the gene expression level of miR-137 in HuH7 and MHCC97L cells (*, $P < 0.05$). **(C)** Diagram was shown for the binding of hsa-miR-137

on 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). 24 h after transfection, relative luciferase activities were evaluated through a dual-luciferase reporter assay (*, $P < 0.05$).

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 upregualted by lentivirus in HuH7 and MHCC97L cells. The protein levels of CDC42 were evaluated by western blot. **(C-D)** 24 h after lentiviral transfection, HuH7 and MHCC97L cells were further transfected with pcDNA3.1/CDC42 to overexpress CDC42. The mRNA levels of CDC42 were evaluated by qRT-PCR (*, $P < 0.05$; Δ, $P > 0.05$).

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. 24 h after transfection, a MTT assay was carried out for 5 consecutive days to evaluate cell proliferation in vitro (*, $P < 0.05$). **(B)** 24 h after lentiviral transfection, 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-D)** The same experiments as (A) and (B) were also carried out in MHCC97L cells.

Figure 4. The effect of CDC42 on miR-137 mediated HCC metastasis (A-B) HuH7 cells were firstly transfected with lentiviruses of miR-137 or miR-C. 24 h after 1st transfection, 2nd transfection of pcDNA3.1/CDC42 or pcDNA3.1/+ was carried out. At the end of each transfection, migration assay was used to evaluate cancer metastasis *in vitro*. **(A)** The representative fluorescent images of the bottom chamber for each experimental condition were shown. **(B)** Quantification on migration capability was performed by calculating the relative percentages of migrated cells under each experimental condition and normalized them to control condition of miR-C transfection (*, $P < 0.05$; Δ, $P > 0.05$). **(C-D)** The same experiments as (A) and (B) were also carried out in MHCC97L cells.

Figure 5. The 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)** 5 weeks after inoculation, tumors were retrieved, sectioned and stained with Ki67. **(C)** HuH7 cells were firstly transfected with lentiviruses of miR-137 for 24 h, followed by 2nd transfection of pcDNA3.1/CDC42 or pcDNA3.1/+. 24 h after 2nd transfections cells were inoculated into the nude mice and tumor growth was monitored for 5 consecutive weeks (*, $P < 0.05$). **(D)** 5 weeks after inoculation, tumors were retrieved, sectioned and stained with Ki67.

Figure 6. CDC42 was independent of AKT2 in HCC (A-B) HuH7 and MHCC97L cells were transfected with either pcDNA3.1/CDC42 or pcDNA3.1/+. 24 h after transfection, mRNA expression levels of AKT2 were evaluated by qRT-PCR (Δ, $P > 0.05$). (**C-D)** HuH7 and MHCC97L cells were transfected with either pcDNA3.1/AKT2 or pcDNA3.1/+. 24 h after transfection, mRNA expression levels of AKT2 were evaluated by qRT-PCR (*, $P < 0.05$). (**E-F)** MRNA expression levels of CDC42 were also evaluated by qRT-PCR (Δ, $P > 0.05$).

Figure 7. AKT2 further rescued HCC proliferation than CDC42 (A) HuH7 cells and **(B)** MHCC97L cells were transfected with 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 on HCC proliferation (*, $P < 0.05$).

Figure 8. AKT2 further rescued HCC metastasis than CDC42 HuH7 cells and MHCC97L cells were transfected with miR-137 lentivirus for 24 h. They were 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/+. An *in vitro* migration assay was then carried out to evaluate cancer metastasis. In HuH7 cells **(A)** and MHCC97L cells **(C)**, the representative fluorescent

images of migrated cells in the bottom chambers were shown. Quantification on the percentages of migrated cells demonstrated that the metastasis capabilities were increased more than 150% with the additional AKT2 in both HuH7 cells **(B)** (*, $P < 0.05$) and MHCC97L cells **(D)** (*, $P < 0.05$).

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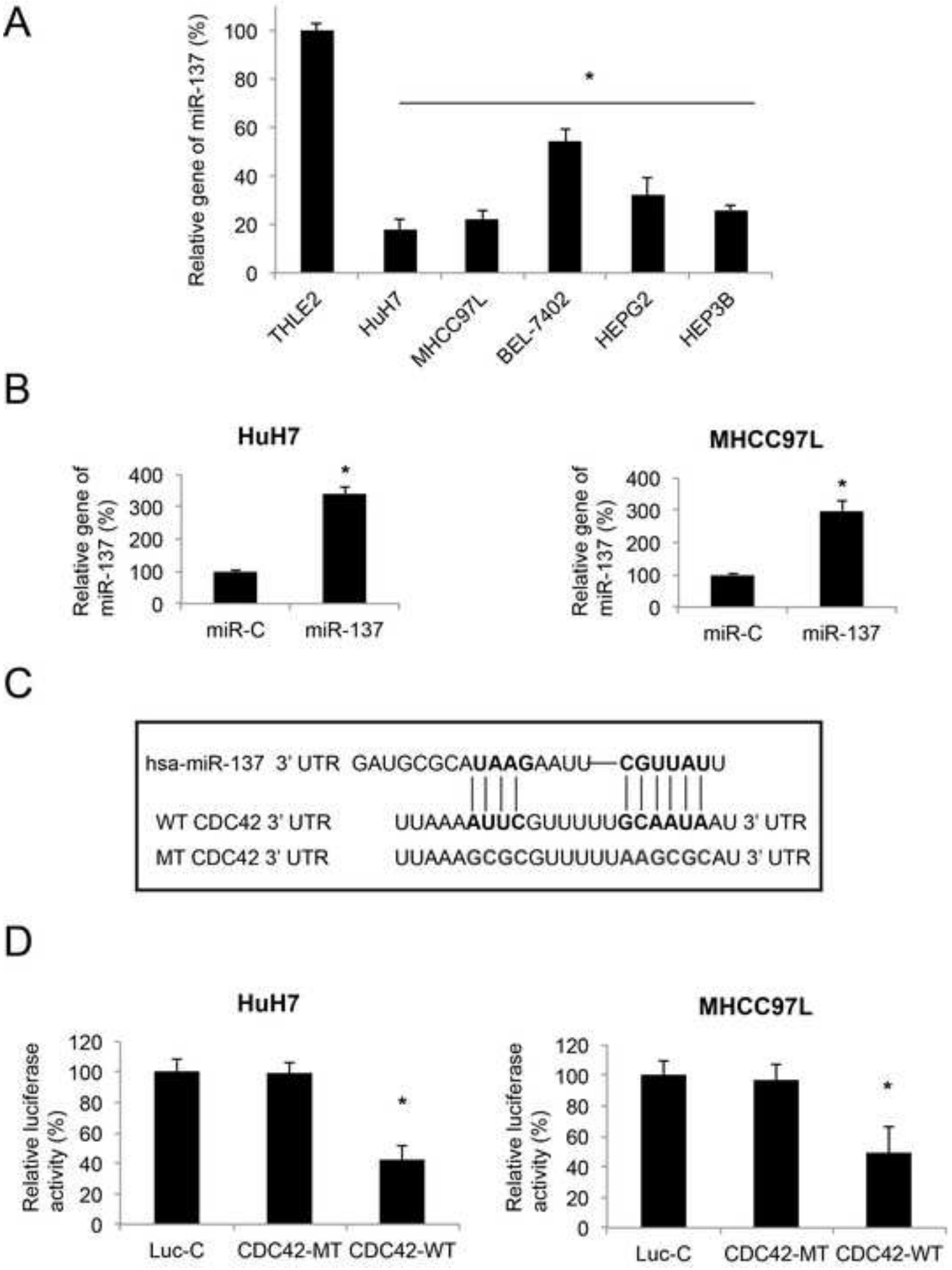

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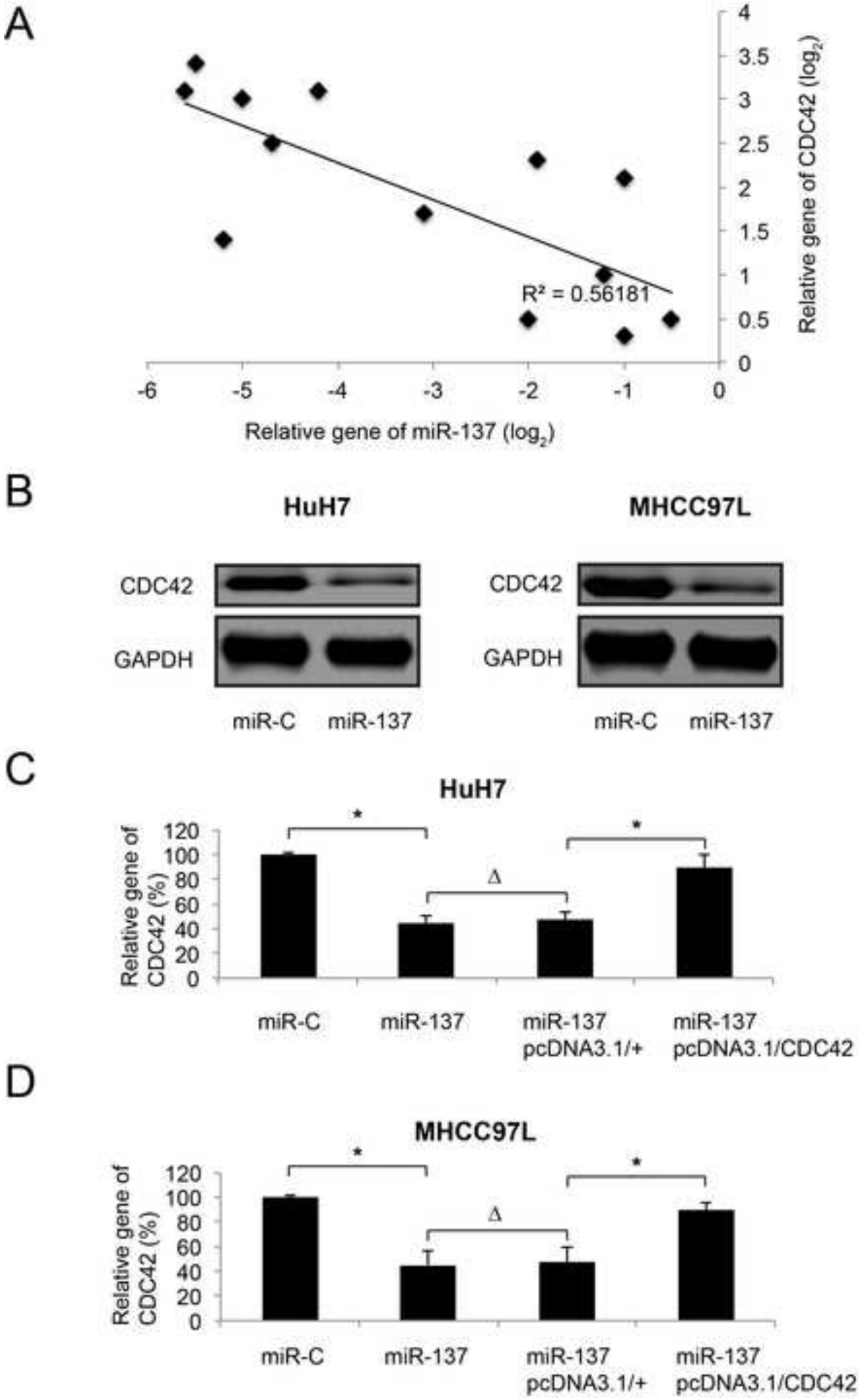

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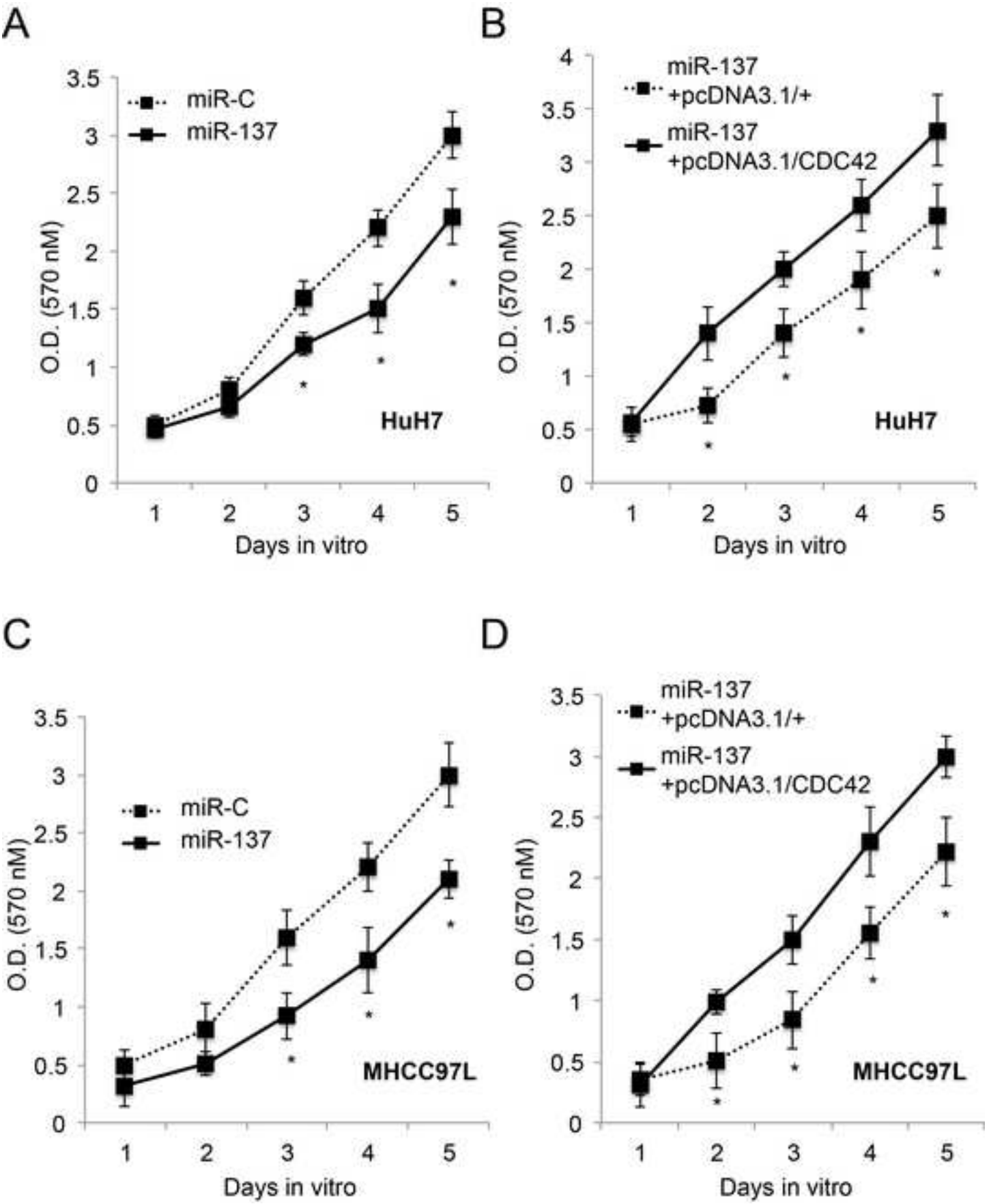

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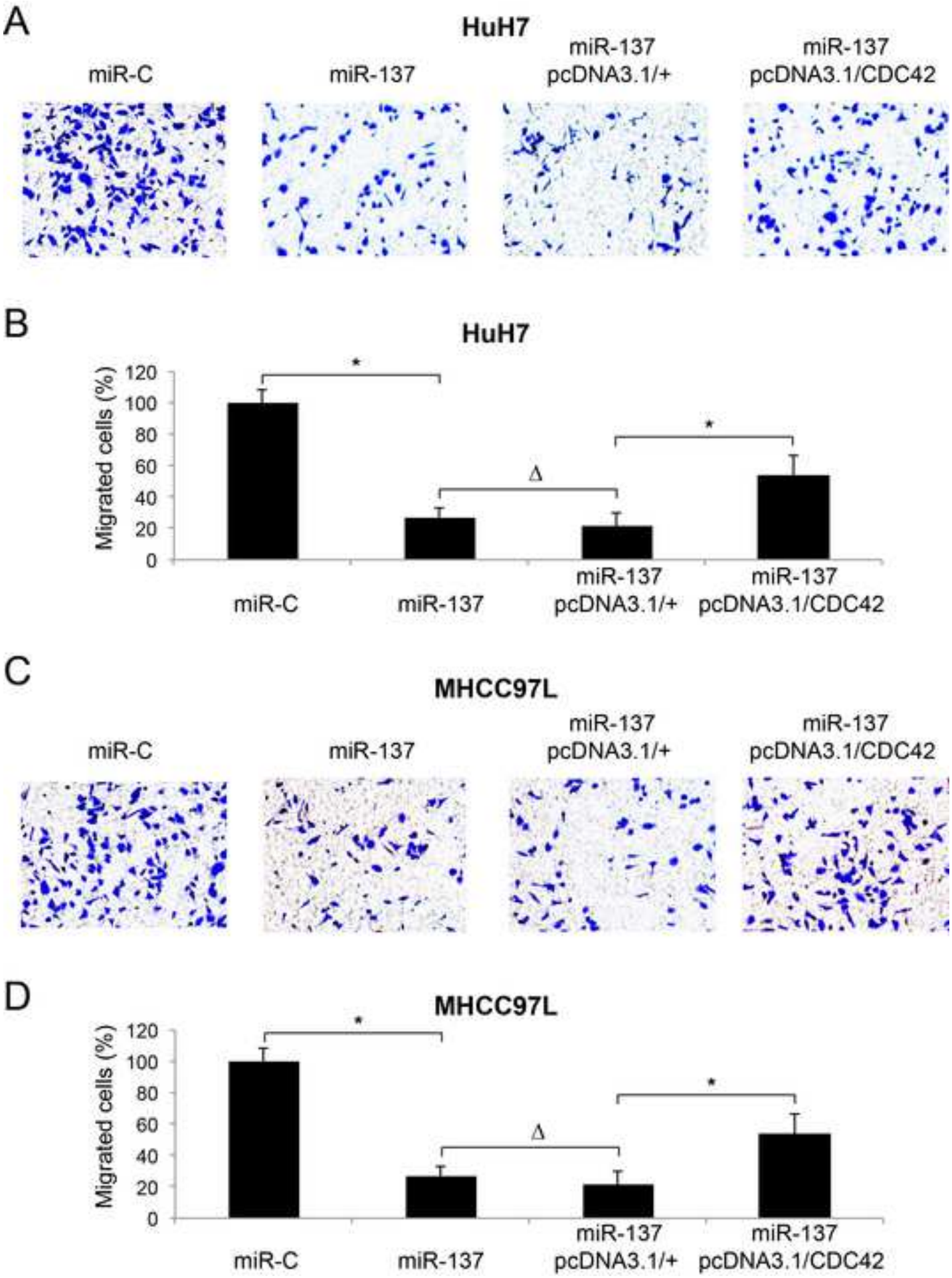

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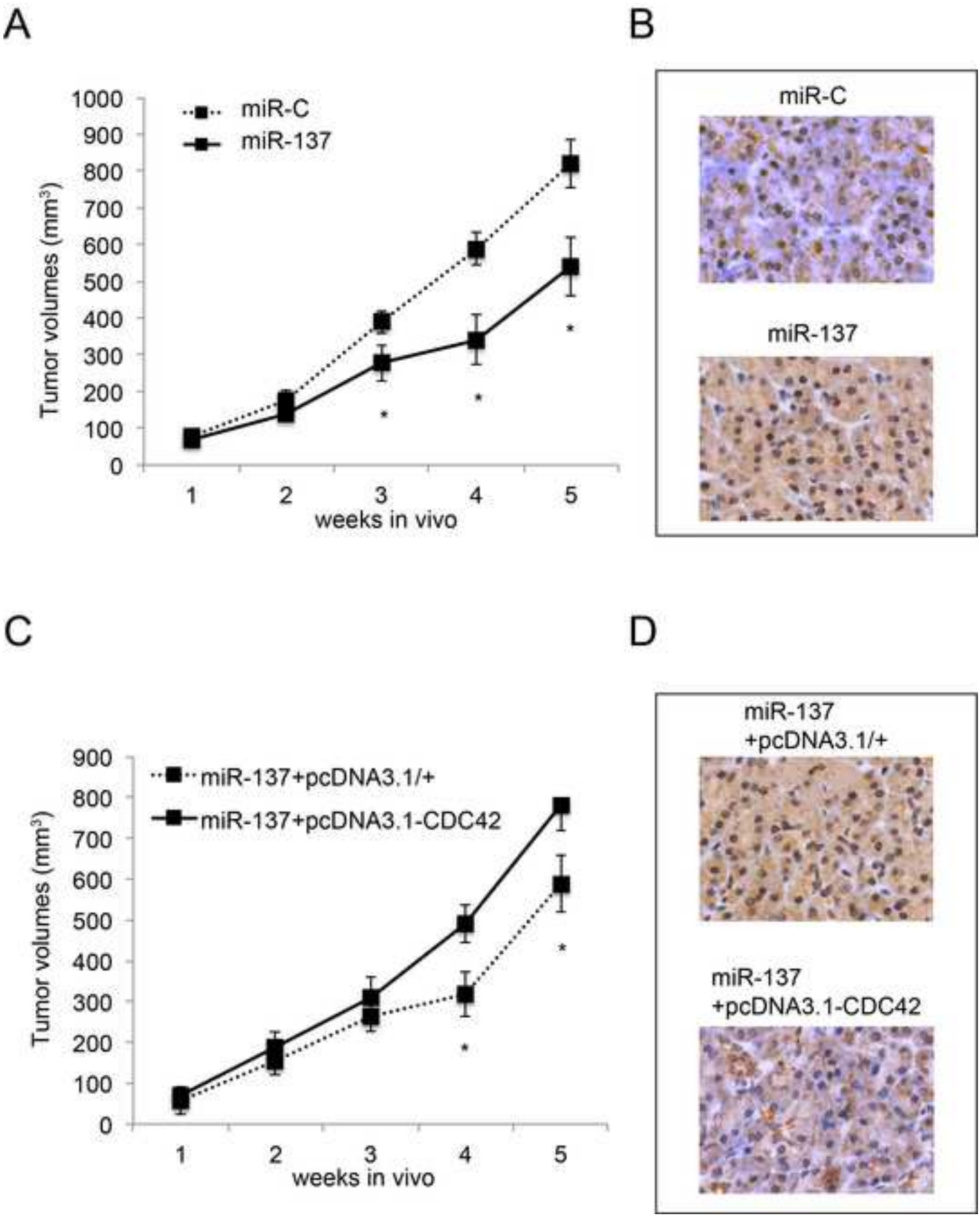

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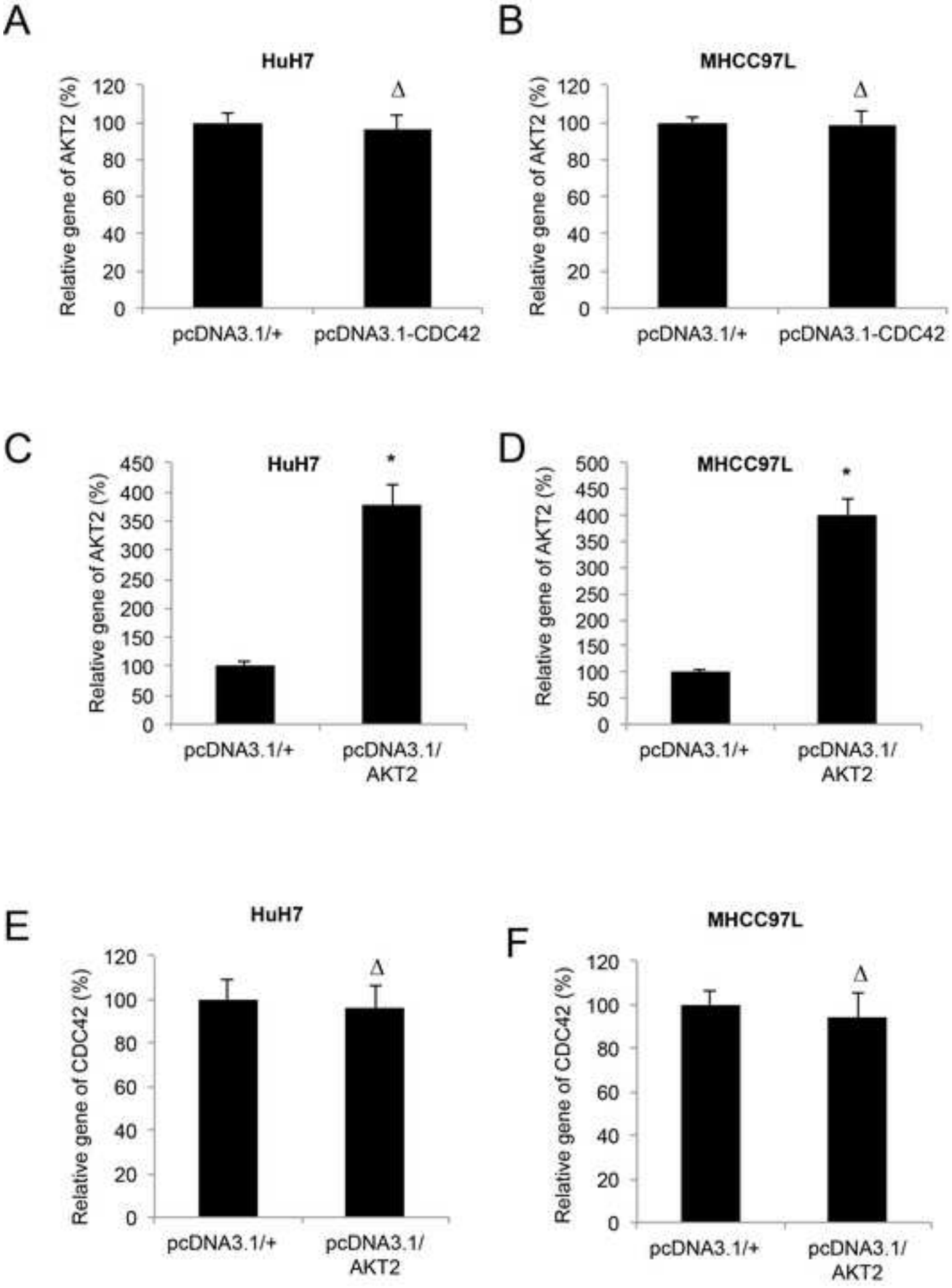

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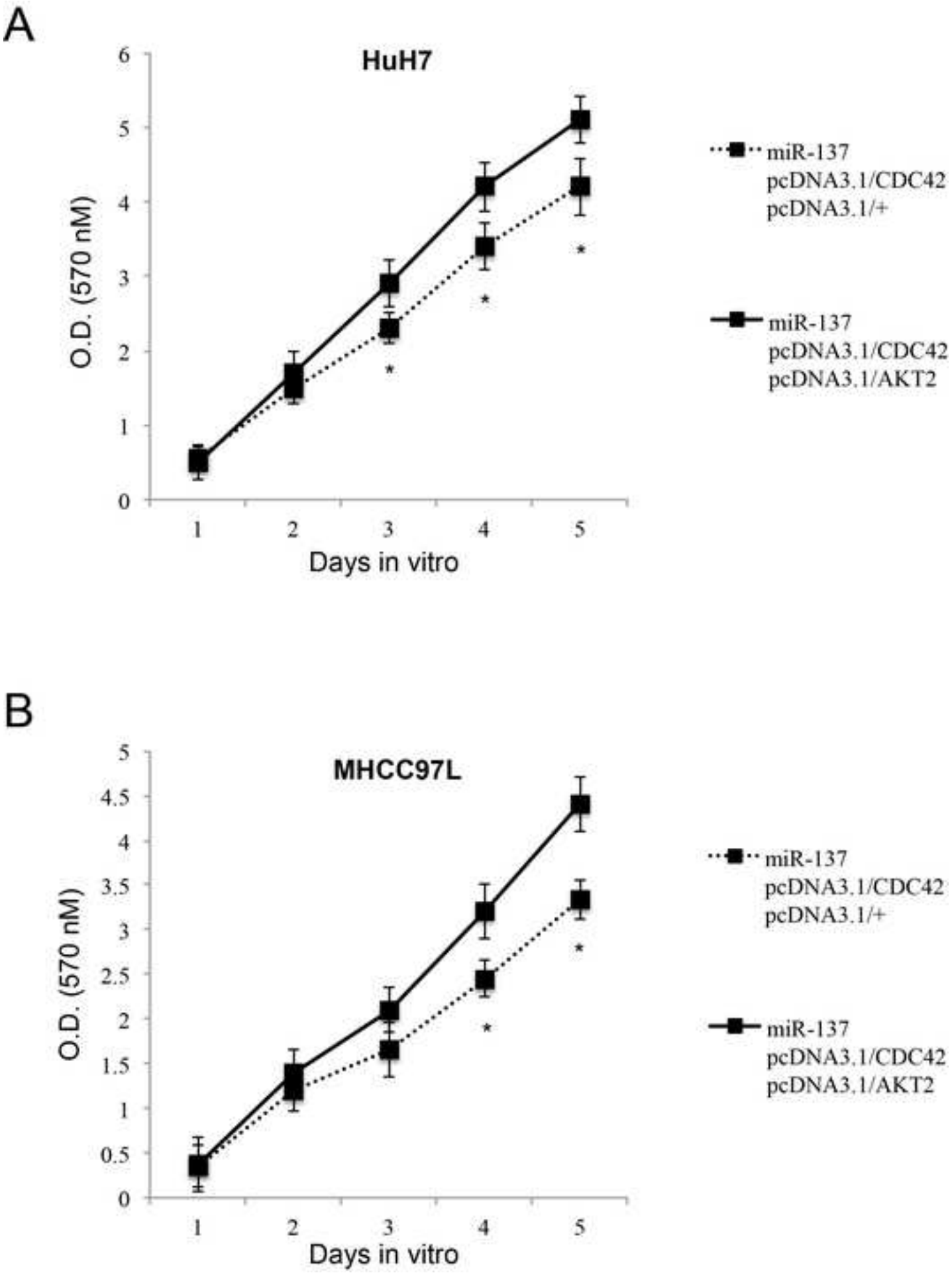

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