

## Original Article

# MicroRNA-147 suppresses human hepatocellular carcinoma proliferation migration and chemosensitivity by inhibiting HOXC6

Cheng-Jun Sui\*, Feng Xu\*, Wei-Feng Shen, Bing-Hua Dai, Jiong-Jiong Lu, Min-Feng Zhang, Jia-Mei Yang

Department of Special Medical Care and Liver Transplantation, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China. \*Equal contributors.

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**Abstract:** Patients with hepatocellular carcinoma (HCC) experience poor prognosis and low survival rates. In this study, we explored the molecular mechanism of microRNA-147 (miR-147) in regulating human HCC. We firstly used quantitative RT-PCR (qRT-PCR) to compare the expression levels of miR-147 between 7 HCC and two normal liver cell lines, as well as 10 paired primary HCC tissues and their adjacent non-carcinoma tissues. We found miR-147 was down-regulated in both HCC cell lines and primary HCCs tissues. HCC cell lines HepG2 and HuH7 were transfected with lentiviral vector of miR-147 mimics. We found overexpressing miR-147 significantly inhibited HCC *in vitro* proliferation and migration, increased 5-FU chemosensitivity, and reduced *in vivo* tumorigenicity. Luciferase, qRT-PCR and western blot assays showed that HOXC6 was the downstream target of miR-147, and both gene and protein levels of HOXC6 were down-regulated by miR-147 in HCC cells. SiRNA mediated HOXC6 knockdown inhibited *in vitro* proliferation and migration, and increased 5-FU chemosensitivity in HCC. On the other hand, HOXC6 overexpression reversed the inhibitory effect of miR-147 on HCC *in vitro* proliferation. Therefore, our results suggest that miR-147 can modulates HCC development through the regulation on HOXC6.

**Keywords:** miR-147, hepatocellular carcinoma, HOXC6, migration, proliferation, chemosensitivity

## Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in male and the seventh most common cancer in female [1, 2]. Despite the tremendous efforts by physicians and researchers to develop early diagnostic methods or innovative treatment strategies to improve the clinical outcomes in patients with HCC, the majority of the HCC patients, especially those in the developing countries such as China, still experience very poor prognosis or low 5-year overall survival rate [3-5]. Thus, it is critical to elucidate the underlying molecular mechanisms of human HCC, search novel biomarker, and develop efficient treatment plans to suppress tumor growth, recurrence or metastasis in patients with HCC.

MicroRNA (miRNAs) are families of small strands, non-coding RNAs that transcriptionally attach to the 3'-untranslated regions (3'-UTR)

of targeted mRNAs to induce gene inhibition or protein degradation [6]. In both human and animals, miRNAs are found to play important roles in various aspects of organ development or pathology, such as embryogenesis, differentiation, apoptosis or cell death [7-10]. In various human cancers, miRNAs may act as either oncogenes or tumor-suppressor genes, depending on the differences in carcinoma forms or the downstream molecular pathways involved [11-14].

In many of the tumor-associated miRNAs, microRNA-147 (miR-147) could be either oncogene or tumor suppressive gene. MiR-147 is found to be up-regulated in human gastric cancer, suggesting a oncogenic role [15], but down-regulated in human breast cancer, indicating a tumor suppressor function [16]. In human HCC, miR-147 was found to be down-regulated in carcinoma tissues [17]. Interestingly, it was also reported that miR-147 was up-regulated in

recurrent HCC tissues [18]. Thus, definitive evidence is needed to explore the exact molecular mechanism of miR-147 in human HCC.

Families of homeobox (Hox) genes are transcription factors that play important roles in oncogenesis or blood cell differentiation [19, 20]. As Hox genes are mostly involved in liver development [21, 22], members of Hox genes, including HOXA7/A13 are also shown to regulate liver cancerogenesis [23]. One of the Hox genes, homeobox C6 (HOXC6), was shown to be differentially expressed in different stages of HCC tissues. However, the molecular mechanism of HOXC6 in HCC remains elusive.

In the present study, we investigated the possible molecular mechanisms of miR-147 in regulating HCC. We discovered that miR-147 was down-regulated in HCC cell lines and primary HCC tissues. Through functional assays of over-expressing miR-147 in HCC cell lines, we found miR-147 upregulation inhibited HCC proliferation and migration *in vitro* and tumorigenicity *in vivo*. Moreover, we used bioinformatics, western blotting, qPCR and functional assays to discover that the tumor suppressive effect of miR-147 was through the inverse regulation of HOXC6 in HCC.

### Materials and methods

#### *Human HCC cell lines and human samples*

In the present study, four HCC cell lines, HepG2, C3A, SNU-398, Hep3B and two normal hepatocytes cell lines THLE2 and THLE3 were purchased from ATCC (ATCC, USA). The other three HCC cell lines, HiH7, MHCC97L and MHCC97H were purchased from China Center for Type Culture Collection (CCTCC, WuHan, China). All cell types were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen, USA) in a humidified tissue culture chamber with 5% CO<sub>2</sub> at 37°C. Human HCC samples (T) and adjacent non-tumor (ANT) liver tissues were surgically retrieved from HCC patients registered in the Department of Special Medical Care and Liver Transplantation at Eastern Hepatobiliary Surgery Hospital of Second Military Medical University in Shanghai, China. Those clinical samples were immediately snap-frozen in liquid nitrogen after surgery and stored at -80°C until further experiment. All participating patients signed consent forms.

All experiments involving human subjects were reviewed and approved by the Clinical Research & Ethics Committee at the Eastern Hepatobiliary Surgery Hospital of Second Military Medical University.

#### *RNA extraction and qRT-PCR*

In both HCC cell lines and human samples, total RNAs were extracted by a Trizol reagent per manufacturer's instructions (Invitrogen, USA). Then, 5 µg total RNA was reverse-transcribed to cDNA by AMV reverse transcriptase (TaKaRa, Dalian, China) and a stem-loop RT primer (Applied Biosystems, USA). For has-miR-147 detection, qRT-PCR was conducted using a TaqMan MicroRNA Assays (Applied Biosystems, USA) in an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, USA) per manufacturer's instructions. U6 RNA was used as internal control. For HOXC6 detection, qRT-PCR was conducted using a SYBR Green Real-Time PCR Master Mix Kit (Applied Biosystems, USA) and GAPDH as internal control. Gene expression level was measured by a standard curve, and relative folder change was calculated using 2<sup>-ΔΔCt</sup> method and normalized to control.

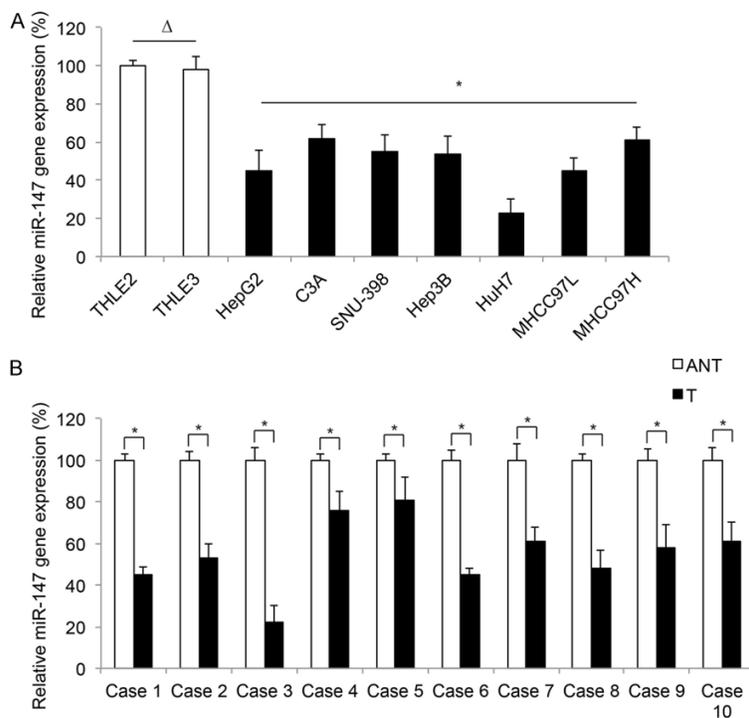
#### *MiR-147 overexpressing in HCC cells*

Lentiviral vector containing has-miR-147 mimic (miR-147-mimic) or a control lentiviral vector containing scramble miRNA (Control) were purchased from SunBio (SunBio Medical Biotechnology, China). To stably overexpress miR-147 in HCC cell lines, HepG2 and HuH7 cells were transfected with miR-147-mimic or Control lentivirus for 24 h at a multiplicity of infection (MOI) of 20 with 10 µg/ml polybrene. After replenished with fresh medium, cells were continuously grown *in vitro* and selected with 2 µg/ml puromycin to establish stable transfected cell lines. Transfection efficiency was then evaluated by qRT-PCR.

#### *Proliferation assay*

HCC proliferation was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Invitrogen, USA) per manufacturer's instructions. Briefly, HepG2 and HuH7 cells were plated into 96-well plates (5 × 10<sup>3</sup> cells/well) for 1, 2, 3, 4 and 5 days, respectively. Each day, cells were treated with 20 µl MTT (5 mg/ml, Sigma-Aldrich, USA) for 4

## miR-147 modulates HOXC6 in HCC



**Figure 1.** Expression of miR-147 in HCC cell lines and human tissues. A. Gene expression levels of miR-147 in seven HCC cell lines, HepG2, C3A, SNU-398, Hep3B, HuH7, MHCC97L and MHCC97H cells were measured by qRT-PCR and compared to the expression levels in two normal liver cell lines THLE-2 and THLE-3 cells (\*:  $P < 0.05$ ). B. Gene expression levels of miR-147 between paired HCC tissues (T) and their adjacent non-tumor tissues (ANT) in 10 HCC patients were compared by qRT-PCR (\*:  $P < 0.05$ ).

h, followed by 15 mins agitation of 100  $\mu$ L dimethyl sulfoxide solution (Sigma-Aldrich, USA) to dissolve crystalline formazan. The optical density (O.D.) at 490 nm was evaluated using a microplate reader (SpectraMax M5, Molecular Devices, USA) per manufacturer's instructions.

### Migration assay

HCC proliferation was evaluated by A QCM™ chemotaxis 96-well transwell assay (Chemicon, USA) per manufacturer's instructions. Briefly, HepG2 and HuH7 cells were placed into the Matrigel-coated upper chamber of transwell in 96-well plates ( $5 \times 10^3$  cells/well) in culture medium without serum. The bottom chambers were filled with 200  $\mu$ L culture medium, but with 5% FCS as chemoattractant. After 24 h, HCC cells in upper chambers were removed and those migrated into bottom chambers were fixed with Ethanol and treated with crystal violet. Fluorescent images were captured using an Olympus inverted microscope (IX-81, Olympus,

Japan) and the number of migrated cells in bottom chambers was quantified. The percentage of relative migration was evaluated by normalizing migrated cells under experimental conditions against the migrated cells under control conditions.

### Chemosensitivity assay

HepG2 and HuH7 cells were plated in 96-well plates and treated with 0, 1, 5, 10 and 20 mM 5-FU for 48 h, respectively. HCC cell viability was evaluated by a MTT assay. The O.D. was measured at 490 nm, and the relative viability was evaluated by normalizing the O.D. values under experimental conditions to the O.D. values under control conditions with 0 mM 5-FU.

### In vivo tumorigenicity assay

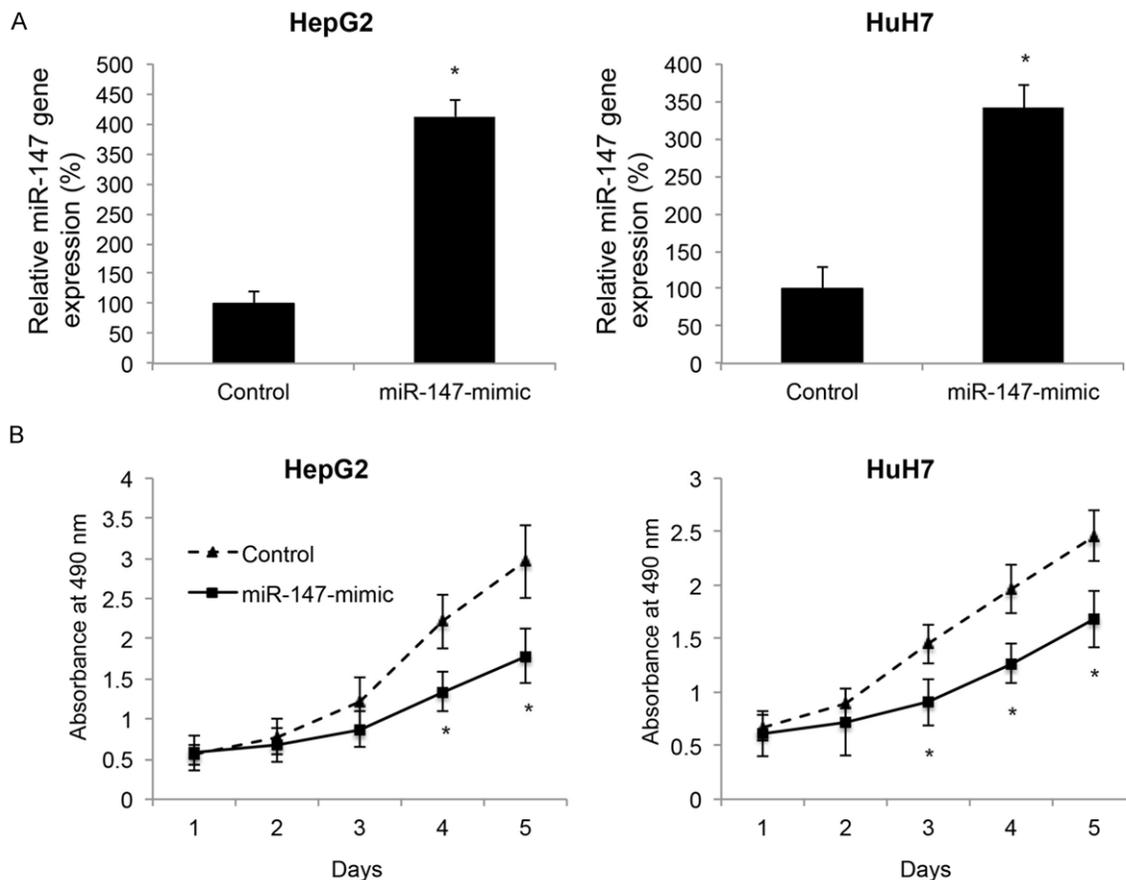
Healthy HepG2 cells (1 million in 100  $\mu$ L DMEM) with stably miR-147 overexpression or control transfections were

subcutaneously inoculated into the left flanks of female athymic mice (8-week old). The *in vivo* tumorigenicity was monitored 1, 2, 3, 4 and 5 weeks after inoculation, respectively. The volume of *in vivo* tumors was measured using the formula:  $L \times W \times W/2$ , where L is the length and W the width of tumor. Five weeks after inoculation, mice were sacrificed. Tumors were retrieved and prepared as formalin-fixed, paraffin-embedded sections, followed by Ki67 (Rabbit polyclonal, Sigma-Aldrich, USA) immunostaining.

### Dual-luciferase reporter assay

The 3'-untranslated region (UTR) of wild type human HOXC6 mRNA, including the binding sequence by has-miR-147, was cloned into the HindIII and SpeI restriction sites of luciferase reporter, pmiR-REPORT. In addition, the binding sequence of HOXC3 3'UTR was mutated using a Quik-Change™ Site-Directed Mutagenesis Kit (Stratagene, USA), and also cloned into pmiR-REPORT. In human HEK293T cells, co-transfec-

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**Figure 2.** Effect of overexpressing miR-147 on HCC proliferation. HepG2 and HuH7 cells were transfected with a lentivirus carrying hsa-miR-147 mimics (miR-147-mimic), or a control lentivirus (Control). A. The gene expression levels of miR-147 in HepG2 and HuH7 cells transfected with miR-147-mimic were measured by qRT-PCR, and normalized to the gene expression levels of miR-147 in control cells (\*:  $P < 0.05$ ). B. Proliferation of HepG2 and HuH7 cells was measured by a 5-day MTT assay (\*:  $P < 0.05$ ).

tion of luciferase reporters of Wild Type HOXC6 3'UTR, mutated HOXC6 3'UTR, or a control  $\beta$ -gal luciferase reporter, with lentivirus of miR-147-mimic was carried out using Lipofectamine 2000 (Sigma-Aldrich, USA) for 48 h. The relative luciferase activities were measured by a dual-luciferase reporter assay (Promega, USA) and normalized to the luciferase activity of  $\beta$ -gal control.

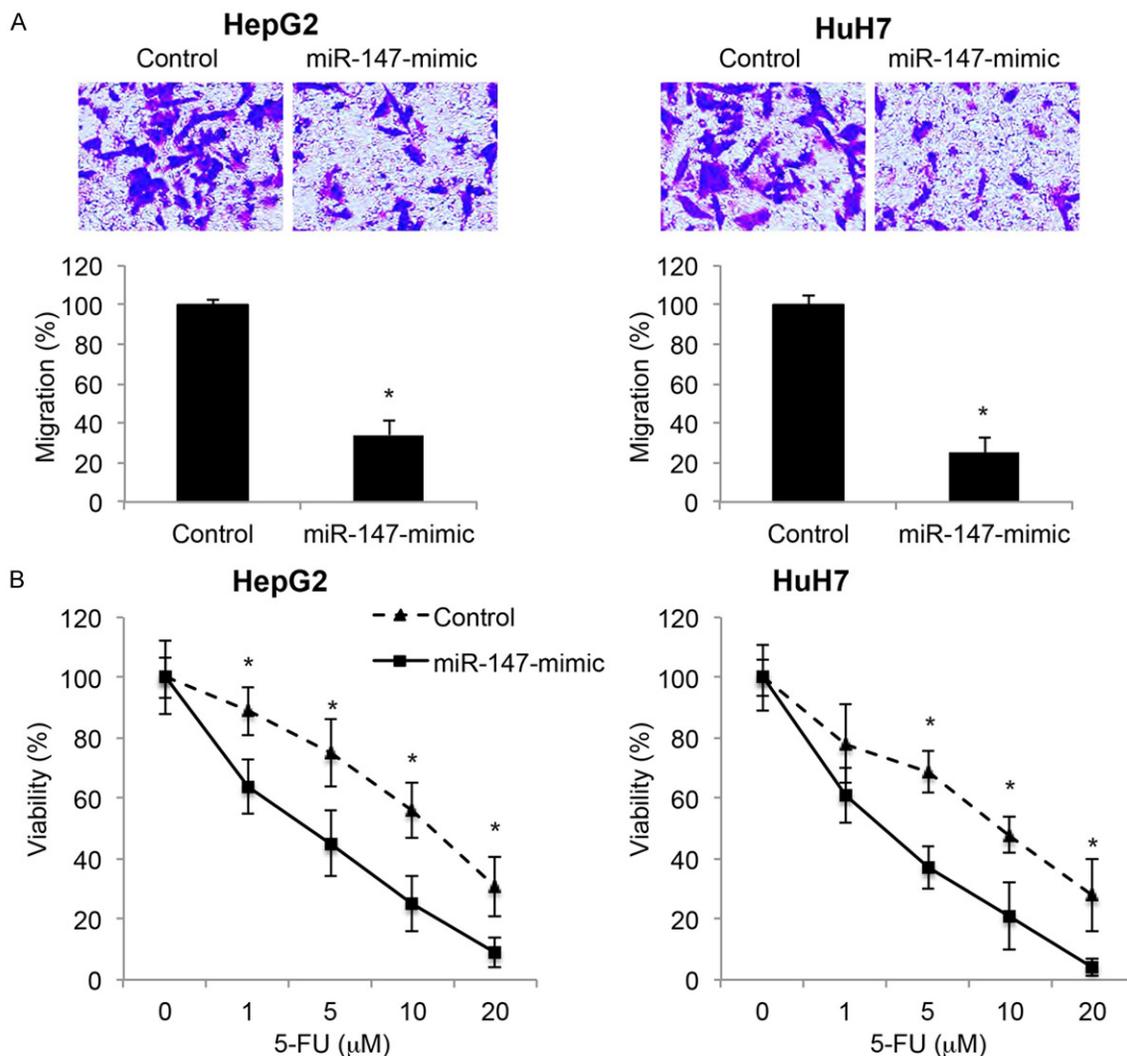
### Western blot assay

For protein extraction, HepG2 and Huh7 cells were harvested in an ice-cold lysis buffer with 10% glycerol and 1% NP-40 (Promega, USA). Then, 50  $\mu$ g protein of each sample was dissolved in 10% SDS-polyacrylamide gel electrophoresis and then electro-transferred onto PVDF membranes. After 1 h blocking with 1% BSA, the membrane were incubated with a rabbit polyclonal antibody against HOXC6 (Sigma-

Aldrich, USA) overnight at 4°C. An antibody against human Beta-actin was also used as control. The membranes were then washed with T-PBS (3  $\times$  10 mins) and incubated with horseradish peroxidase conjugated goat anti-rabbit antibody at 37°C for 1 h. The blots were visualized using an enhanced chemiluminescence film system (Amersham Pharmacia Biotechnology, USA) per manufacturer's instruction.

### HOXC6 down-regulation in HCC cells

Human HOXC6 specific siRNA (siHOXC6) and a scramble siRNA (Control) were purchased from RiboBio (RiboBio, Guangzhou, China). For siRNA transfection, HepG2 and HuH7 cells were maintained in 6-well plates (3  $\times$  10<sup>5</sup> cells/well) and infected with 50 nM siRNAs (siHOXC6 or Control) with Lipofectamine 2000 (Invitrogen, USA) for 24 h. After replenished with fresh



**Figure 3.** Effect of overexpressing miR-147 on HCC migration and 5-FU chemosensitivity. HepG2 and HuH7 cells were transfected with a lentivirus carrying hsa-miR-147 mimics (miR-147-mimic), or a control lentivirus (Control). A. In vitro migration was measured by a Transwell assay. Representative fluorescent images were shown for the migrated cells in bottom chamber (upper panel). The numbers of migrated cells were normalized to the control condition (lower panel, \*:  $P < 0.05$ ). B. HepG2 and HuH7 cells were treated with 5-FU at concentrations of 0, 1, 5, 10 and 20 M for 48 h, followed by a MTT assay to evaluate cell viability (\*:  $P < 0.05$ ).

medium, HCC cells were maintained for another 48 hours. Unhealthy cells were then removed and healthy ones were re-suspended in 96-well plates ( $5 \times 10^3$  cells/well). Transfection efficiency was evaluated by qRT-PCR.

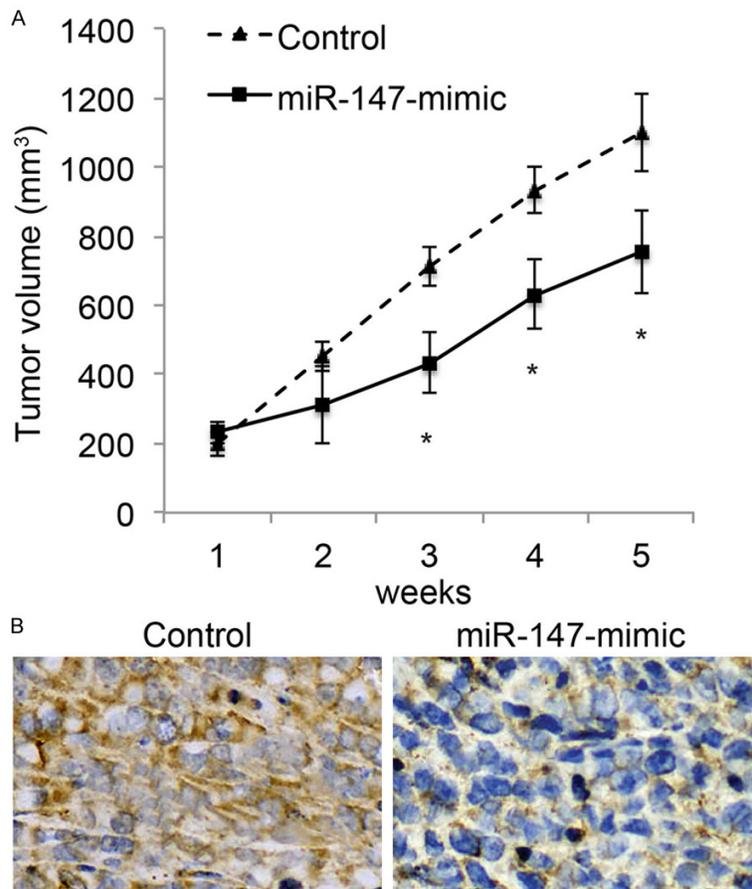
#### HOXC6 up-regulation in HCC cells

Gene of human HOXC6 was amplified from a human cDNA library and verified by sequencing. It was then cloned into the expression vector pcDNA3.1 (pcDNA3.1-HOXC6, Invitrogen, USA), and transfected into HepG2 and HuH7 cells by Lipofectamine 2000. Transfection of an empty pcDNA3.1 vector (Control) was also

performed. 24 h after HOXC6 overexpression in HCC cells, the culture was changed with fresh medium and maintained for another 48 h. The overexpression efficiency was evaluated by qRT-PCR.

#### Statistical analysis

All experimental data were presented as mean  $\pm$  standard errors. All experiments were at least repeated three times. Statistical difference was analyzed using a student's *t*-test (SPSS version 12.0), with a significance level of  $P < 0.05$ .



**Figure 4.** Effect of overexpressing miR-147 on in vivo HCC tumor growth. Lentivirus-transfected HepG2 cells ( $1 \times 10^6$ ) were subcutaneously injected into the left flank of 8-week-old female athymic mice. A. The tumor volumes were measured using the formula:  $L \times W \times W/2$ , where L is the length and W the width of tumor, 1, 2, 3, 4 and 5 weeks after inoculation, respectively (\*:  $P < 0.05$ ). B. Five weeks after inoculation, mice were sacrificed and Ki67 immunostaining was performed on paraffin-embedded tumor sections.

## Results

### *MiR-147 is down-regulated in HCC cell lines and human HCC tumors*

We used qRT-PCR to compare the gene expression levels of miR-147 in HCC cell lines and normal liver cells. We found that, as compared to the gene expression levels of miR-147 in two normal liver cell lines THLE-2 and THLE-3 cells, miR-147 gene expressions were significantly down-regulated in all 7 HCC cell lines examined in the study, including HepG2, C3A, SNU-398, Hep3B, HuH7, MHCC97L and MHCC97H cells (**Figure 1A**, \*:  $P < 0.05$ ). We then probed the gene expression levels of miR-147 in 10 paired clinical samples obtained from patients with HCC. The results of qRT-PCR demonstrated that miR-147 was also significantly down-regu-

lated in HCC (T) tissues, as compared to their adjacent non-tumor (ANT) liver tissues (**Figure 1B**, \*:  $P < 0.05$ ).

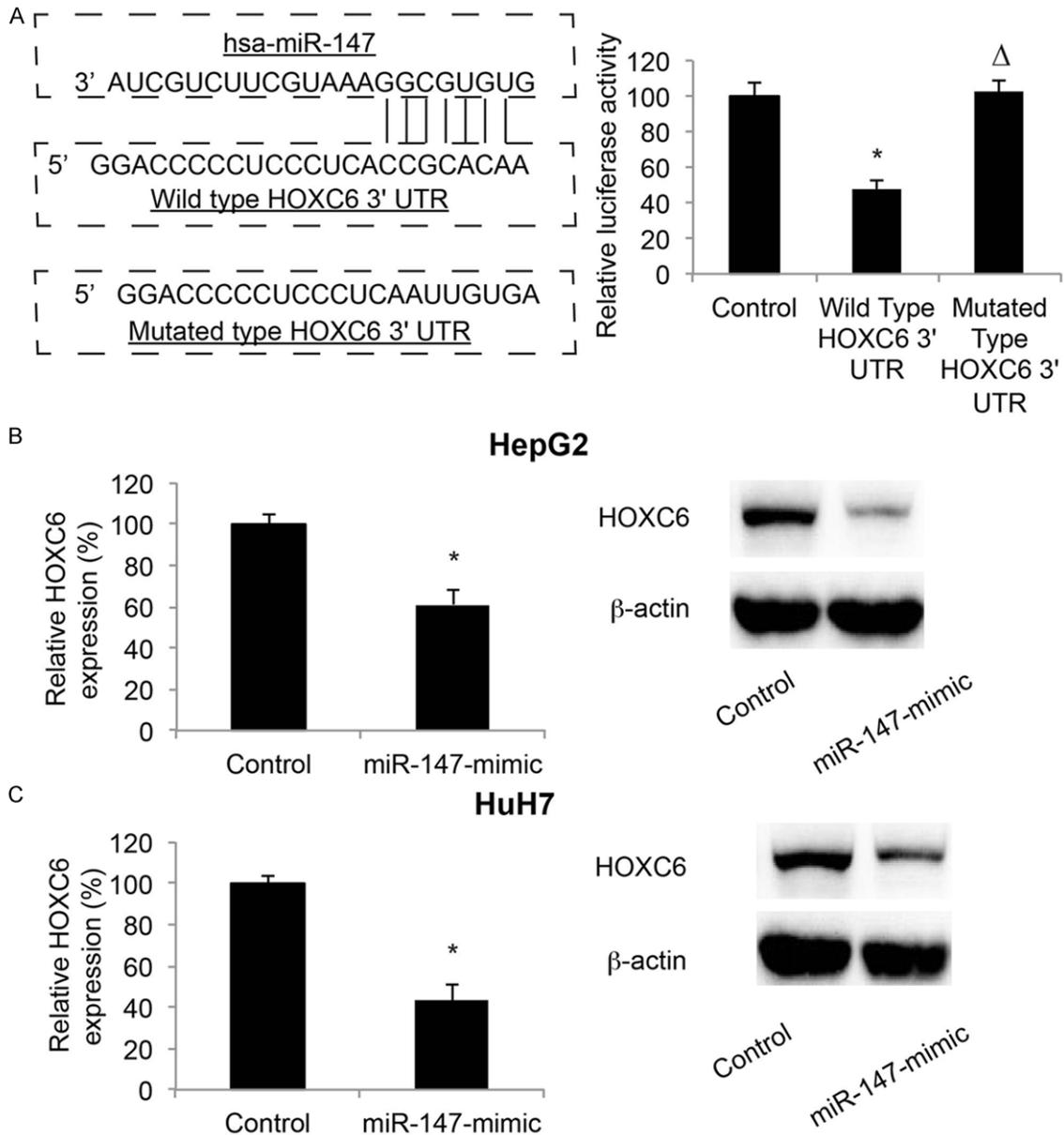
### *MiR-147 overexpression inhibits in vitro proliferation in HCC*

We then asked whether miR-147 played a functional role in modulating human HCC. In order to do so, we transfected two HCC cell lines, HepG2 and HuH7 cells with lentivirus carrying hsa-miR-147 mimics (miR-147-mimic) to ectopically over-express miR-147 in those HCC cells. For comparison, HepG2 and HuH7 cells were also transfected with a control lentivirus carrying scramble miRNAs (Control). The transfection efficiency of miR-147 overexpression was verified by qRT-PCR (**Figure 2A**, \*:  $P < 0.05$ ).

In addition, a 5-day MTT assay was used to examine the effect of miR-147 overexpression on HCC *in vitro* proliferation. We found that in both HepG2 and HuH7 cells, proliferation rates were significantly reduced by miR-147 overexpression (**Figure 2B**, \*:  $P < 0.05$ ).

### *MiR-147 overexpression inhibits in vitro migration and increased chemosensitivity in HCC*

We also examined whether overexpressing miR-147 would affect metastatic capability and chemosensitivity in HCC. In a transwell assay, we found that the migration capability was significantly reduced by miR-147 overexpression in both HepG2 and HuH7 cells (**Figure 3A**, \*:  $P < 0.05$ ). In addition, lentivirus-transfected HepG2 and HuH7 cells were treated with various concentrations of 5-FU (0~20 mM) for 48 hours, followed by a MTT assay to evaluate cell viability. We found that, at concentrations between 5 and 20 mM, 5-FU chemosensitivity was significantly increased by miR-147 overexpression in both HepG2 and HuH7 cells (**Figure 3B**, \*:  $P < 0.05$ ).

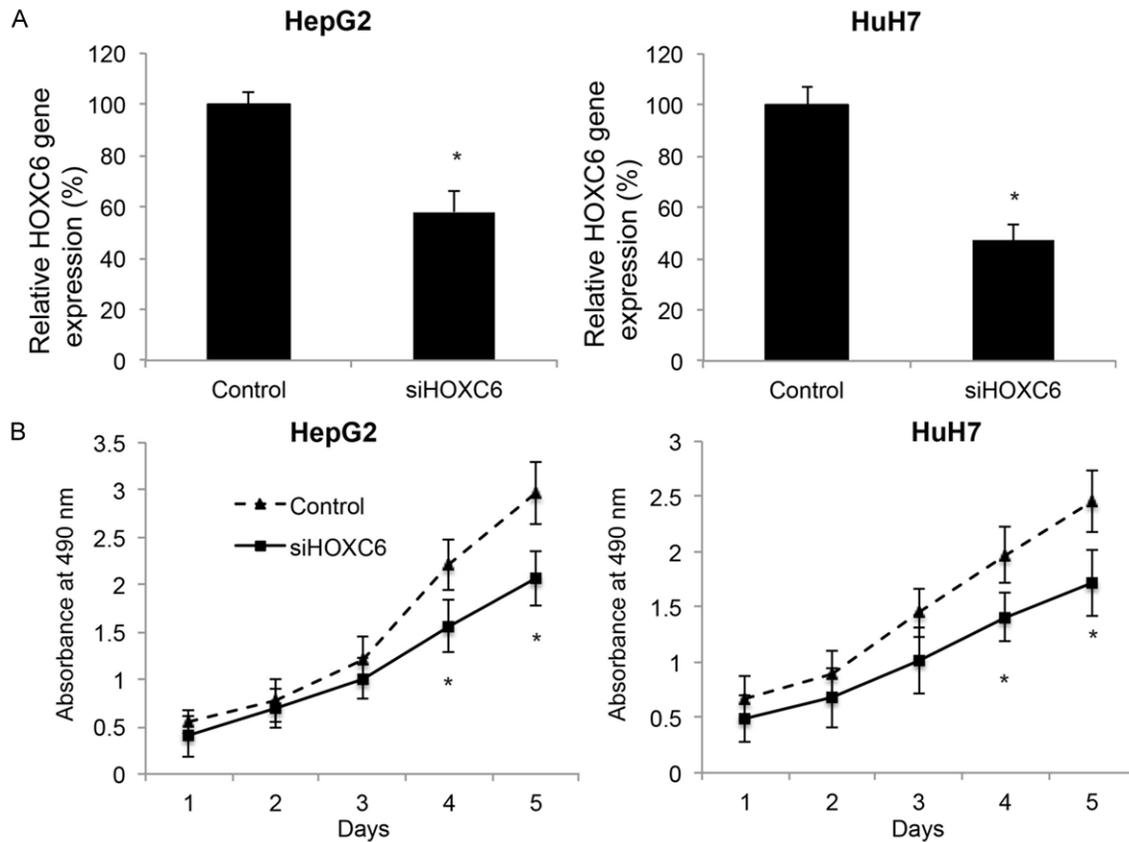


**Figure 5.** MiR-147 regulated HOXC6 in HCC cells. (A) Left: The predicted binding site of hsa-miR-147 on wild type HOXC6 3'-UTR was shown, along with the mutated HOXC6 3'-UTR. Right: In a luciferase assay, luciferase reporters of  $\beta$ -galactosidase control (Control), wild-type HOXC6 3'-UTR, or mutated HOXC6 3'-UTR were co-transfected with miR-147-mimic in HEK293 cells for 48 hours. The luciferase activities were measured and normalized to  $\beta$ -galactosidase of control (\*:  $P < 0.05$ ,  $\Delta$ :  $P > 0.05$ ). (B, C) In lentivirus transfected HepG2 (B) and HuH7 (C) cells, the gene expressions of HOXC6 were examined by qPCR (left), and protein expression of HOXC6 were examined by western blot (right).

*MiR-147 overexpression inhibits in vivo HCC tumorigenicity*

We also investigated whether overexpressing miR-147 may affect HCC tumorigenicity *in vivo*. One million healthy lentivirus-transfected HepG2 were subcutaneously inoculated into 2-month old female athymic mice. The *in vivo* growth of HCC tumor was monitored for 5

weeks. The weekly measurement on tumor volumes showed that miR-147 overexpression significantly inhibited the *in vivo* growth of HepG2 tumors between week 3 and week 5 after inoculation (Figure 4A, \*:  $P < 0.05$ ). Immunostaining of Ki-67 also demonstrated that *in vivo* tumor proliferation was significantly reduced by miR-147 overexpression (Figure 4B).



**Figure 6.** Effect of HOXC6 downregulation on HCC *in vitro* proliferation. HepG2 and HuH7 cells were transfected with a HOXC6 specific siRNA (siHOXC6), or a control siRNA (Control). A. The gene expression levels of HOXC6 in HepG2 and HuH7 cells transfected with siHOXC6 were measured by qRT-PCR, and normalized to the gene expression levels of HOXC6 in control cells (\*:  $P < 0.05$ ). B. Proliferation of HepG2 and HuH7 cells was measured by a 5-day MTT assay (\*:  $P < 0.05$ ).

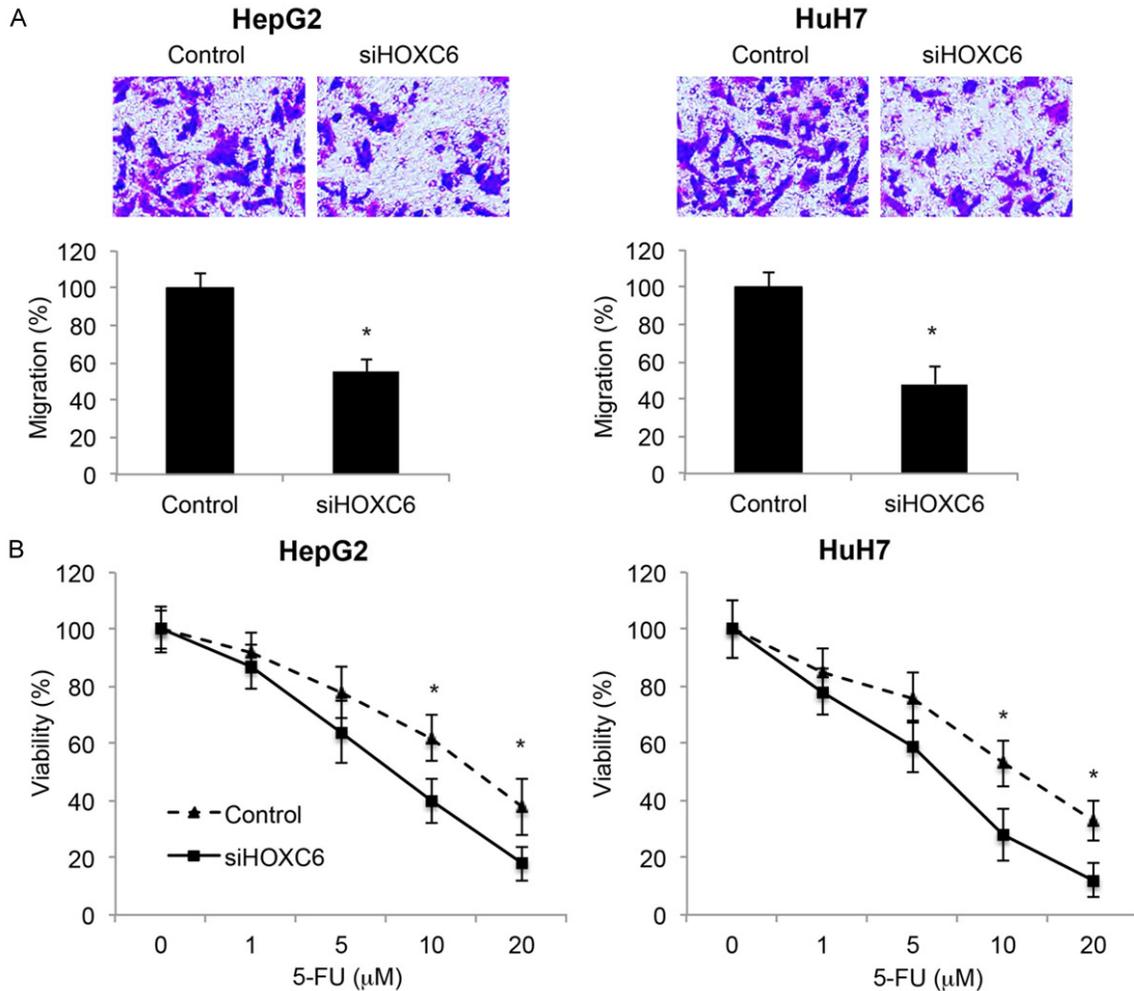
#### HOXC6 is regulated by miR-147 in HCC

We sought to identify the molecular pathway of miR-147 in HCC. Using some of the online bioinformatic tools, such as TargetScan, Pictar, miRANDA, we found that Homeobox (HOX)-containing gene 6 (HOXC6) may be a potential downstream target of miR-147 (Figure 5A, left panel). We then performed a dual-luciferase reporter assay to confirm that HOXC6 was directly bound by miR-147 (Figure 5A, right panel, \*:  $P < 0.05$ ). To further evaluate whether HOXC6 is directly regulated by miR-147 in HCC, we used qRT-PCR and western blot assay to re-examined the mRNA expression levels, as well as protein expression levels of HOXC6 in lentivirus-transfected HepG2 and HuH7 cells. The results showed that, both mRNA and protein levels of HOXC6 were significantly down-regulated by miR-147 overexpression in HepG2 and HuH7 cells (Figure 5B, 5C, \*:  $P < 0.05$ ).

#### HOXC6 downregulation regulates HCC proliferation migration and chemosensitivity

We then asked whether HOXC6 was playing a direct role in regulating human HCC. To do so, we performed siRNA mediated gene down-regulation of HOXC6 in HCC cells. In HepG2 and HuH7 cells, cells were transfected with HOXC6 specific siRNA (siHOXC6), or non-specific control siRNA (Control) for 24 hours. The results of qRT-PCR confirmed that siRNA mediated HOXC6 knockdown in these cells was efficient (Figure 6A, \*:  $P < 0.05$ ). Then, 48 h after siRNA transfection, a 5-day MTT assay was used on HepG2 and HuH7 cells to evaluate the effect of HOXC6 downregulation on HCC *in vitro* proliferation. It showed siRNA-mediated HOXC6 down-regulation was able to inhibit HCC cell proliferation (Figure 6A, \*:  $P < 0.05$ ).

We also examined the effects of HOXC6 down-regulation on HCC migration and 5-FU chemo-



**Figure 7.** Effect of HOXC6 downregulation on HCC migration and 5-FU chemosensitivity. HepG2 and HuH7 cells were transfected with a HOXC6 specific siRNA (siHOXC6), or a control siRNA (Control). A. In vitro migration was measured by a Transwell assay. Representative fluorescent images were shown for the migrated cells in bottom chamber (upper panel). The numbers of migrated cells were normalized to the control condition (lower panel, \*:  $P < 0.05$ ). B. HepG2 and HuH7 cells were treated with 5-FU at concentrations of 0, 1, 5, 10 and 20 M for 48 h, followed by a MTT assay to evaluate cell viability (\*:  $P < 0.05$ ).

sensitivity. The result of transwell assay showed that, in both HepG2 and HuH7 cells, HOXC6 downregulation inhibited the migration of HCC cells (**Figure 7A**, \*:  $P < 0.05$ ). Furthermore, the viability assay with 5-FU treatment showed that HOXC6 downregulation increased the 5-FU chemosensitivity in HepG2 and HuH7 cells (**Figure 7B**, \*:  $P < 0.05$ ).

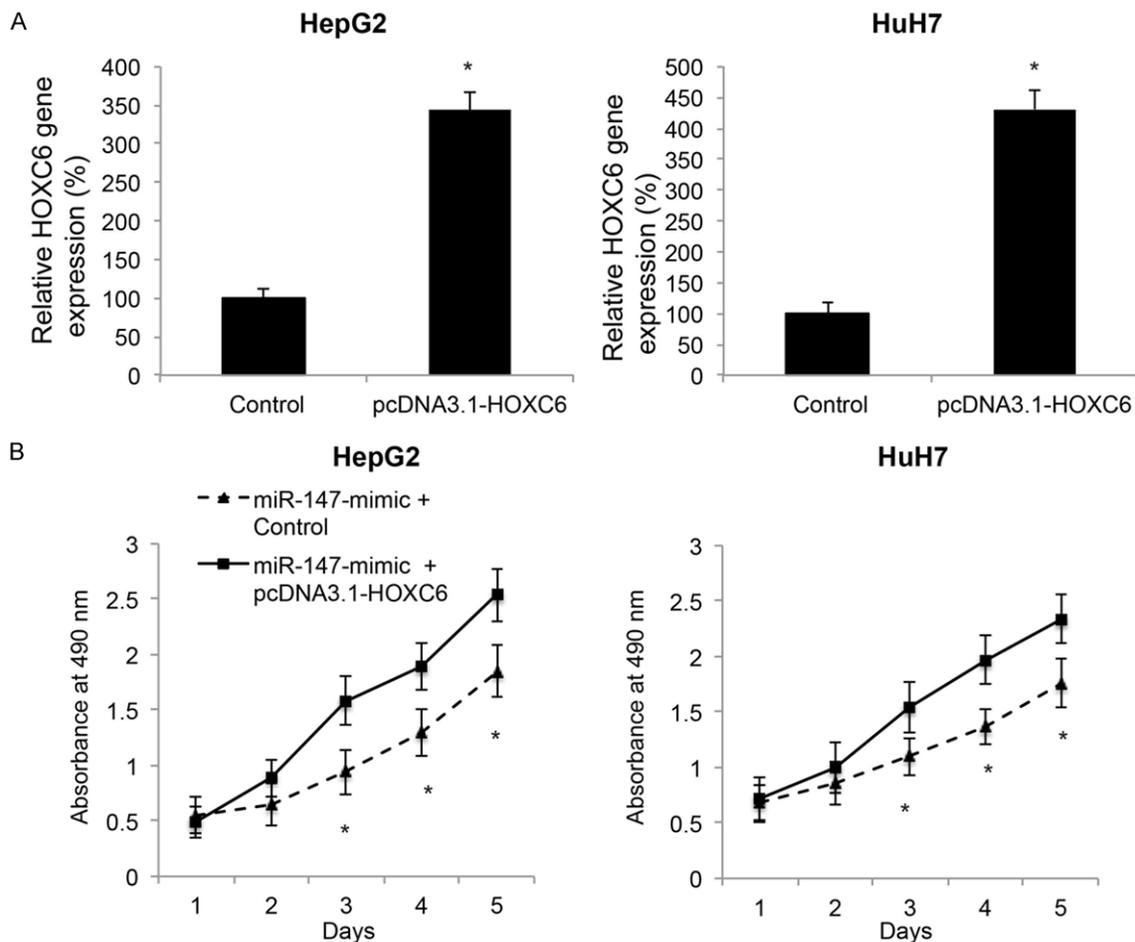
#### *HOXC6 upregulation reversed the regulation of miR-147 in HCC*

Finally, we asked whether HOXC6 overexpression might have opposite effect as miR-147 overexpression in HCC. To serve this purpose, we transfected the miR-147 overexpressed HepG2 and HuH7 cells with an overexpression

vector, pcDNA3.1-HOXC6. The transfection efficiency was confirmed by qRT-PCR, showing the gene expression levels of HOXC6 were both upregulated in HepG2 and HuH7 cells (**Figure 8A**, \*:  $P < 0.05$ ). Then, a 5-day MTT assay was applied on HCC cells to evaluate the effect of HOXC6 overexpression on miR-147 mediated HCC *in vitro* proliferation. It showed HOXC6 overexpression was able to reverse the inhibitory effect of miR-147 upregulation, thus promote cancer proliferation in both HepG2 and HuH7 cells (**Figure 8B**, \*:  $P < 0.05$ ).

#### **Discussions**

In human hepatocellular carcinoma, miRNAs play important roles in regulating carcinoma



**Figure 8.** Effect of overexpressing HOXC6 on HCC proliferation. The miR-147 upregulated HepG2 and HuH7 cells were transfected with a HOXC6 expression vector, pcDNA3.1-HOXC6, or a control vector (Control). A. The gene expression levels of HOXC6 in HepG2 and HuH7 cells were measured by qRT-PCR, and normalized to the gene expression levels of HOXC6 in control cells (\*:  $P < 0.05$ ). B. The effect of HOXC6 overexpression on proliferation of HepG2 and HuH7 cells was measured by a 5-day MTT assay (\*:  $P < 0.05$ ).

proliferation, metastasis and chemosensitivity. Our previous study showed that miR-218 played as a tumor suppressive factor in human HCC through the regulation on RET [24]. In the present study, we discovered another potential tumor suppressive miRNA, miR-147, to be down-regulated in both HCC cell lines and primary HCC tissues. In a genomic study, Varnholt and colleagues demonstrated that miR-147 was lowly expressed in hepatitis C virus-associated hepatocellular carcinoma [17], in line with our finding to suggest a tumor suppressive role of miR-147 in HCC. Intriguingly, in another study, Han and colleagues showed that miR-147 was upregulated in recurrent HCC after surgery, hinting an oncogenic role of miR-147 in resurgent liver tumor [18]. Therefore, it is important to further investigate the functional roles

of miR-147 to reconcile the seemingly conflict expression patterns of miR-147 in HCC, or at least, to explore the mechanisms of miR-147 in regulating common pathologic properties of HCC, such as cancer proliferation, metastasis or chemosensitivity.

To answer the question of whether miR-147 has a function role in HCC, we used lentiviral technology to stably over-express miR-147 in two HCC cell lines, HepG2 and HuH7 cells. After confirming the transfection efficiency with qRT-PCR, we used quantitative functional assays, including MTT, transwell and chemosensitivity assays *in vitro* and tumorigenicity assay *in vivo*, to evaluate the effect of miR-147 overexpression on HCC proliferation, migration and 5-FU chemosensitivity. The results, as presented

through **Figure 2** to **Figure 4**, all supported the notion that miR-147 inhibits the development of HCC. Together with our qRT-PCR results, and other study [17] showing miR-147 is down-regulated in HCC, all evidence point to a predominantly tumor suppressive role of miR-147 in HCC.

Also in the present study, result from luciferase assay showed that miR-147 directly binds HOXC6. SiRNA-mediated HOXC6 knockdown in HepG2 and Huh7 cells demonstrated that HOXC6 down-regulation also had inhibitory effects on HCC *in vitro* proliferation, migration and 5-FU chemoresistance, similar to the effect of miR-147 upregulation. In addition, HOXC6 upregulation was able to reverse the inhibitory effect of miR-147 overexpression on HCC growth. Therefore, it's very like that HOXC6 is the direct downstream target of miR-147 in HCC. Family of Hox genes are widely involved in liver development [21, 22]. In hepatocellular carcinoma cell lines, HOXA7/A13 is shown to be overexpressed and may be associated with eIF4E transcription factor [23]. However, little is known for the functional role of Hox genes in HCC. Thus, our study is the first one to report a functional role of HOXC6, most likely acting as an oncogene to directly regulation HCC growth.

In summary, we demonstrated in the present study that miR-147 was down-regulated in both HCC cell lines and primary HCC human tumors. Over-expression of miR-147 inhibited *in vitro* proliferation, migration, increased 5-FU chemosensitivity, and inhibited *in vivo* tumorigenicity in HCC. We also demonstrated that, HOXC6 was the downstream molecular target of miR-147 in HCC. The results of study may help to elucidate the regulatory mechanism of miRNA in HCC, and develop new bio-marker or therapeutic reagent for treating patients with HCC.

#### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Jia-Mei Yang, Department of Special Medical Care and Liver Transplantation, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, 800 Xiangyin Rd, Shanghai 200438, China. Tel: +86-021-81875552; E-mail: jiamei.yang@aol.com

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