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MiR-124 suppresses cell proliferation in hepatocellular carcinoma by targeting PIK3CA

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ABSTRACT

MicroRNAs (miRNAs) have crucial roles in the development and progression of human cancers, including hepatocellular carcinoma (HCC). Recent studies have shown that microRNA-124 (miR-124) was downregulated in HCC; however, the underlying mechanisms by which miR-124 suppresses tumorigenesis in HCC are largely unknown. In this study, we report that phosphoinositide 3-kinase catalytic subunit alpha (PIK3CA) is a novel target of miR-124 in HepG2 cells. Overexpression of miR-124 resulted in decreased expression of PIK3CA at both mRNA and protein levels. We found that miR-124 overexpression markedly suppressed cell proliferation by inducing G1-phase cell-cycle arrest in vitro. Consistent with the restoring miR-124 expression, PIK3CA knockdown suppressed cell proliferation, whereas overexpression of PIK3CA abolished the suppressive effect of miR-124. Mechanistic studies showed that miR-124-mediated reduction of PIK3CA resulted in suppression of PI3K/Akt pathway. The expressions of Akt and mTOR, key components of the PI3K/Akt pathway, were all downregulated. Moreover, we found overexpressed miR-124 effectively repressed tumor growth in xenograft animal experiments. Taken together, our results demonstrate that miR-124 functions as a growth-suppressive miRNA and plays an important role in inhibiting the tumorigenesis through targeting PIK3CA.

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1. Introduction

MicroRNAs (miRNAs) are a class of endogenous, short (19–22 nucleotides), noncoding RNA molecules that function as critical gene regulators [1]. It is currently estimated that miRNAs could potentially regulate close to one third of the coding genes in human genome [2], indicating that miRNAs play substantial roles in physiological and pathological processes. Accumulating evidence has suggested that the deregulation of miRNAs is implicated in many human diseases, including cancers [3]. In human cancers, miRNAs are frequently located in genomic breakpoint regions and can function as tumor suppressor genes or oncogenes during tumor development and progression [4].

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer worldwide [5,6]. Despite the clinical implementation of numerous therapeutic strategies, HCC remains a major public health concern. The molecular pathogenesis of HCC is complicated and poorly understood. Recently, an increasing number of reports have showed that miRNAs play important roles in HCC progression, providing new avenues for HCC diagnostic and therapeutic application [7]. To date, multiple miRNAs have been shown to be dysregulated in HCC, such as miR-122, miR-7, miR-29, miR-221, and miR-151 [8–12], which contribute to the development and progression of HCC. Among them, miR-124 is found to be downregulated in HCC tissues [13,14]. MiR-124 has been previously shown to suppress cell proliferation in several cancers, including squamous cell carcinoma and gastric cancer [15,16]. These data suggest a potential tumour suppressive function of miR-124. However, the role of miR-124 in hepatocarcinogenesis and the molecular mechanisms by which miR-124 exerts its functions remain to be largely known.

In this study, we identified PIK3CA as a novel target of miR-124 in HepG2 cells. PIK3CA functions as an oncogene which plays important roles in many cancers, including HCC [17–19]. Furthermore, we show that overexpression of miR-124 in HepG2 cells suppressed cell proliferation and xenograft tumor growth through the repression of PIK3CA. Finally, we show that upregulation of miR-124 led to constitutive suppression of PI3K/Akt pathway. Our data suggest that miR-124 may be a new therapeutic target for HCC.

2. Materials and methods

2.1. Cell lines and cell culture

HepG2 and 293T cell lines were provided by Institute of Biochemistry and Cell Biology of Chinese Academy of Science (China) and originated from ATCC. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal

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bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate. Cells were incubated at 37 °C under a 5% CO_2 condition.

2.2. Vector constructs

The human pre-miR-124 sequence, the coding sequences of PIK3CA were amplified by PCR and cloned into pCDH-CMV-MCS-EF1-copGFP constructs (System Biosciences, California, USA) to generate pCDH-CMV-miR-124, and pCDH-CMV-PIK3CA. PIK3CA shRNA were cloned into pLVTHM vectors to generate pLVTHM-shPIK3CA. The sets of primers were: for pre-miR-124: forward 5′-CTAGTCTAGA GTCGCTGTTATCTCATTGTCTG-3′, reverse 5′-CGCGGATCCTCT GCTTC TGTCACAGAATC-3′; for PIK3CA: forward 5′-GACTAGTGAATCAGAAC AATGCCT CCACGACC, reverse 5′-CCCAAGCTTGACTTGACTTTTCTATT GACTCTTTT-3′.

The 3′UTR of PIK3CA was amplified by PCR with the following primers: 5′-GCTCTAGAGCTCTCAGCAGGCAAAGACCGA-3′ (forward) and 5′-TATAGGCCGGCCTATCATTTCTATATATTTTGGGGATTT-3′ (reverse). The 3′UTR was double-digested with *Xba*I/*Fse*I and inserted into the pGL3-control vector (Promega, USA). The mutant 3′UTR of PIK3CA, which carried the mutated sequence in the complementary site for the seed region of miR-124, was generated based on the pGL3-PIK3CA 3′UTR-WT plasmid by overlap-extension PCR.

2.3. Lentiviral vector production and infection

Virus particles were harvested 48 h after pCDH-CMV-miR-124 (or pCDH-CMV-PIK3CA) cotransfection with the packaging pRSV/pREV, pCMV/pVSVG and pMDLG/pRRE into 293T cells using Lipofectamine 2000 reagent (Invitrogen). HepG2 cells were infected with recombinant lentivirus-transducing units plus 8 mg/ml Polybrene (Sigma, St. Louis, Missouri, USA). The packaged lentiviruses were named LV-miR124, LV-PIK3CA, and LV-shPIK3CA. The empty lentiviral vector was used as a control.

2.4. RNA extraction and quantitative PCR

Total RNA containing miRNA and mRNA was extracted from cell lines using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. To quantitate PIK3CA, Akt, mTOR and β-actin mRNA expression, total RNA was reversely transcribed using First-Strand cDNA Synthesis kit (Invitrogen). Real-time PCR was performed using Quanti-Tect SYBR Green PCR mixture (Qiagen) on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The forward and reverse primers for PIK3CA were 5′-AAATGA AAGCTC ACTCTGGATTCC-3′ and 5′-TGTGCAATTCCTATGCAATC-3′. The forward and reverse primers for Akt were 5′-TTTGTCATGGAGTA CGCCAATG-3′ and 5′-CACAATCTCCGCACCGTAGAA-3′. The forward and reverse primers for mTOR were 5′-CGCTGTCATCCCTTTATCG-3′ and 5′-ATGCTCAAACACCTCCACC-3′. The forward and reverse primers for β-actin were 5′-GAGCTACGAGCTGCCTGACG-3′ and 5′-CCTAG AAGCATTTGCGGTGG-3′. The expression level of β-actin was used as an internal control. All samples were normalized to internal control and determined using the $2^{-\Delta\Delta Ct}$ analysis method.

To analyze miR-124 expression, specific stem-loop reverse transcription primers were used as the following: 5′-GGCACCTGAAATT AAGGC-3′ and 5′-TGATGGTGCCTACAGTGG-3′. RT primers of RNU6 were 5′-CTCGCTTCGGCAGCACA-3′ and 5′-AACGCTTCACGAATTTGC G T-3′. PCR was performed using ABI PRISM 7900 Sequence Detection System. All experiments were run in triplicate.

2.5. Luciferase reporter assay

HepG2-miR124 and control cells were transfected with pGL3-PIK3CA 3′UTR wild-type plasmid or mutant-type plasmid using lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's protocol. Renilla luciferase pRL-TK vector (Promega USA) was co-transfected as a transfection control. The luciferase activities were assayed 48 h later using the Dual-Luciferase Reporter Assay System (Promega, USA). Results were normalized to the Renilla luciferase.

2.6. Cell proliferation and cell cycle analysis

For analysis of cell proliferation, cells were seeded onto 24-well plates at 5×10^3 cells/well and the cell numbers were determined daily for one week. For analysis of cell cycle, cells were suspended in 1 ml solution containing 0.4 mM sodium citrate, 25 μg/ml propidium iodide (PI), and 50 μg/ml RNase. The stained cells were analyzed in a FACScan flow cytometer (BD Biosciences, USA) using the ModFit LT program (BD Biosciences, USA).

2.7. Western blot

Total cell protein extracts were separated by 10% SDS polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked for 1 h in PBST with 5% non-fat milk at 4 °C. Then, the blots were incubated with primary antibodies against PIK3CA, Akt, p-Akt (Ser473), mTOR, p-mTOR (Ser2448) and β-actin (Abcom, Cambridge, UK) followed by horseradish peroxidase-conjugated secondary antibody and detected by chemilu-minescence detection kit (Millipore, Billerico, Massochusatts, USA). The intensity of protein fragments was quantified using Image-Pro Plus software.

2.8. Tumor growth assay

Five-week-old female BALB/c nude mice were purchased from animal center of the Second Military Medical University in China. Animal handling was approved by Shanghai Medical Experimental Animal Care Commission. To evaluate hepatocellular carcinoma growth in vivo, HepG2-miR124 or control cells suspension of 5×10^6 cells in 100 μl was injected subcutaneously into the dorsal flank of nude mice. The tumor growth including length, width and weight was measured every 5 days for 25 days. The tumor volume (mm^3) was calculated according to the formula: volume (mm^3) = $1/2 \times \text{length} \times \text{width}^2$. After the mice were sacrificed, expression levels of miR-124, PIK3CA and key components of the PI3K/Akt pathway were tested by q-PCR and Western blot.

2.9. Statistical analysis

Statistical analysis was performed using a SPSS software package (SPSS Standard version 13.0, SPSS Inc). Data were expressed as mean ± standard deviation (SD) of at least three independent experiments. Data were considered to be statistically significant when $p < 0.05$ (*) and $p < 0.01$ (**).

3. Results

3.1. PIK3CA is a novel target of miR-124 in HepG2 cells

Using TargetScan, we identified PIK3CA as being a potential target of miR-124. The 3′-UTR of PIK3CA mRNA contains a complementary site for the seed region of miR-124 (Fig. 1A). To test whether PIK3CA could be directly targeted by miR-124, we cloned the PIK3CA 3′UTR (wt 3′UTR) or the mutant sequence (mt 3′UTR) into a luciferase reporter vector and performed luciferase reporter assays. The result showed that miR-124 overexpression decreased the PIK3CA 3′UTR luciferase reporter activity, and this effect was abolished when the 2 nucleotides in the miR-124 seed binding site

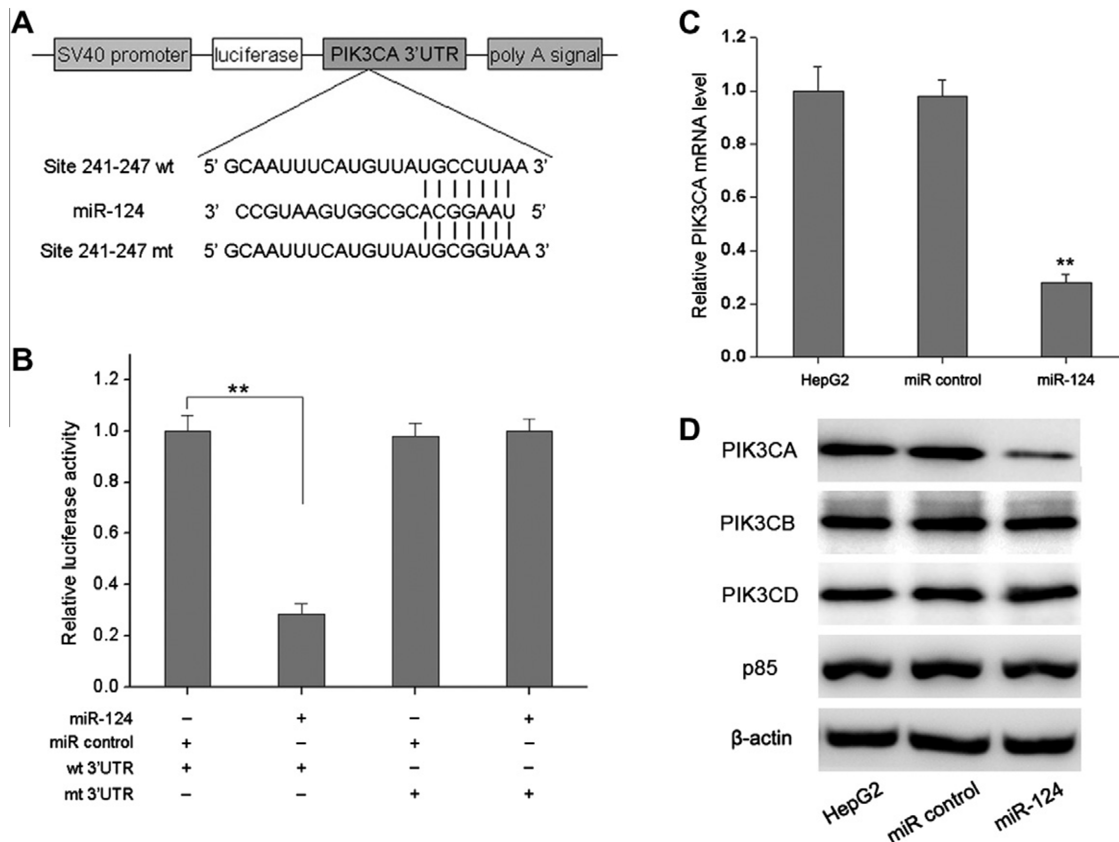

Fig. 1. PIK3CA is a direct target of miR-124 in HepG2 cells. (A) Diagram of PIK3CA 3′UTR-containing reporter constructs. (B) Dual luciferase reporter assays were performed. miR report construct, containing a wild-type or a mutated PIK3CA 3′-UTR, were transfected into HepG2 cells which were infected by miR-control-lentivirus or miR-124-lentivirus. Relative repression of firefly luciferase expression was standardized to a transfection control. (C) qRT–PCR analyze was performed to examine the effects of miR-124 on PIK3CA expression in HepG2 cells. HepG2 cells were infected with either lentivirus-miR-control or lentivirus-miR-124 for 72 h. PIK3CA expression was assessed by qRT–PCR. (D) Western blot was performed to examine the effects of miR-124 on the expressions of PIK3CA, PIK3CB, PIK3CD or their corresponding regulatory subunit, p85. All experiments were carried 3 times independently. $^{**}P < 0.01$ compared to HepG2-miR-control cells.

of the PIK3CA 3′UTR were mutated (Fig. 1B). We next determined whether overexpression of miR-124 leads to downregulation of endogenous PIK3CA expression in human HepG2 cells. qPCR assays showed that PIK3CA mRNA level was significantly reduced in miR-124-overexpressed cells compared to control (Fig. 1C). PIK3CA protein levels were also significantly suppressed in miR-124 group confirmed by Western blot (Fig. 1D). Taken together, these results indicated that PIK3CA was a direct target of miR-124 in HepG2 cells.

3.2. MiR-124 supresses cell proliferation by downregulating PIK3CA expression

Based on the observations above, we hypothesized that miR-124 might suppress cell proliferation by downregulating PIK3CA expression. Initially, we established a miR-124 over-expression model in HepG2 cells infected with miR-124 by Lentivirus pCDH-CMV system, which named as HepG2-miR-124, and cells infected with empty virus vector was used as a control. Increased expression of miR-124 in HepG2 cells was confirmed by qPCR (Fig. 2A). MiR-124 overexpression specifically suppressed PIK3CA protein expression, whereas the expression of the other two PI3K catalytic subunits (PIK3CB and PIK3CD) or their corresponding regulatory subunit, p85 were not affected (Fig. 1D). As shown in Fig. 2B, the results of MTT assay showed that overexpression of miR-124 significantly inhibited the growth of HepG2 cells when comparing to the corresponding control. MiR-124 overexpression increased the percentage of cells in G1 phase and decreased cells in S phase (Fig. 2C). These data indicate that miR-124 is able to suppress the growth of HepG2 cells by inducing G1-phase cell-cycle arrest.

Next, we performed gain-of-function and loss-of-function experiments to further verify that PIK3CA targeting is involved in miR-124-mediated growth inhibition in HepG2 cells. We found that shRNA knockdown of PIK3CA led to significant cell growth inhibition, which was similar to those induced by miR-124 (Fig. 2D). By contrast, transfection with PIK3CA open reading frame plasmid without 3′-UTR (cannot be targeted by miR-124) abolished miR-124-induced cell growth inhibition (Fig. 2E). Taken together, these results demonstrate that miR-124 supresses cell proliferation by downregulating PIK3CA expression.

3.3. MiR-124 regulates the PI3K/Akt pathway in HepG2 cells

Given that PIK3CA is a key member of PI3K family, we examined the expression of key components of the PI3K/Akt pathway in HepG2 cells with or without miR-124 overexpression. As depicted in Fig. 3A, the transcription of Akt and mTOR, which were major components of the PI3K/Akt pathway, was significantly down-regulated in HepG2-miR-124 cells; however, these deregulated expressions of miR-124 effectors could be restored by re-expression of PIK3CA (Fig. 3A). The total and phosphorylated protein levels of all these molecules described above showed the same results (Fig. 3B), indicating that miR-124 may be an important regulator of this signaling pathway.

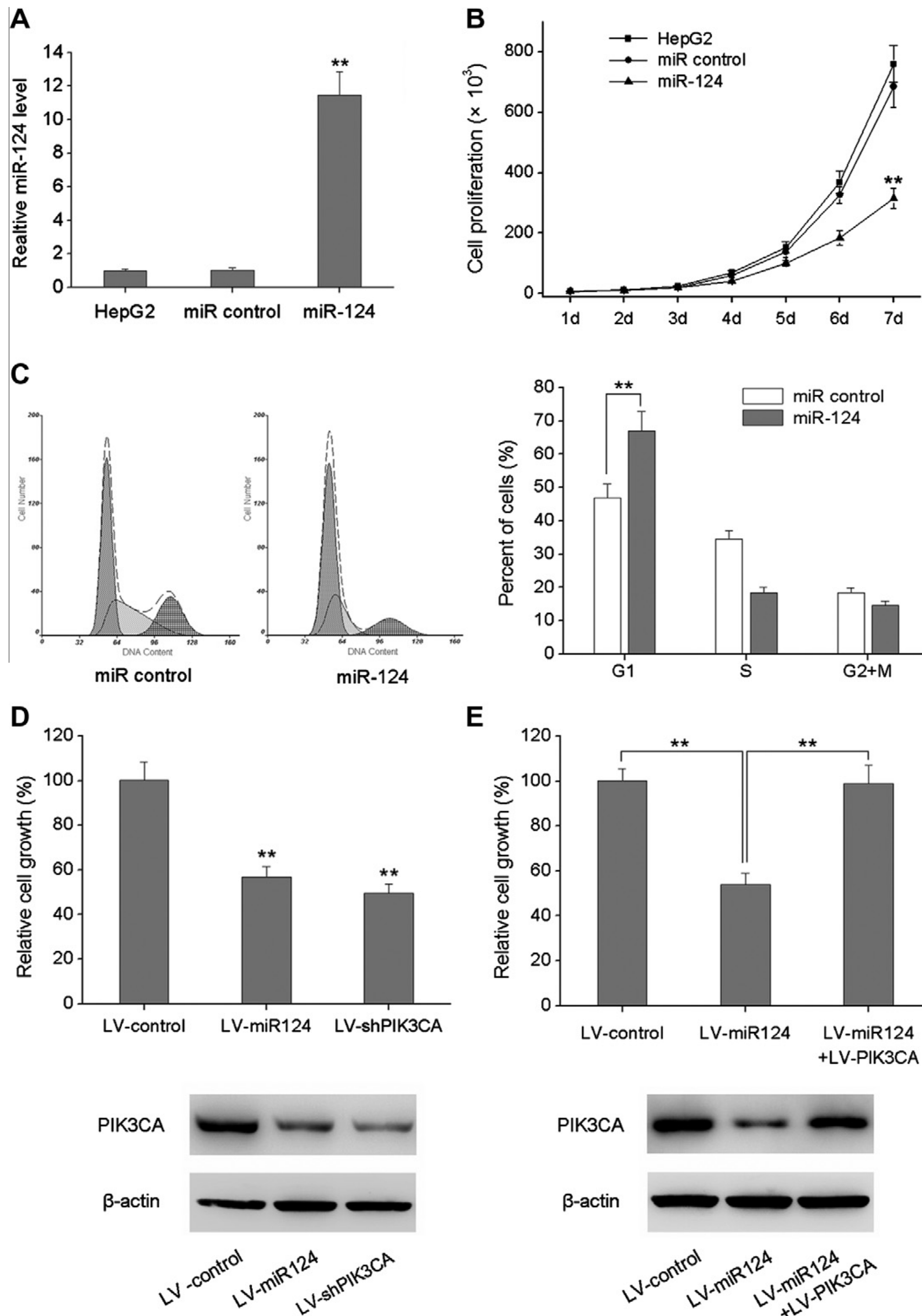

Fig. 2. Overexpression of miR-124 suppressed cell proliferation by downregulating PIK3CA expression. (A) HepG2 cells were infected with LV-miR-124 and control lentivirus respectively, and the stably transduced cells were analyzed for miR-124 levels by qRT-PCR. (B) Cell proliferation assay. (C) representative histograms for cell-cycle distribution of HepG2 cells transfected with miRNAs for 48 h. (D) HepG2 cells were infected with LV-shPIK3CA or LV-miR124. Cell growth rate and cell-cycle distribution were measured. (E) HepG2 cells were infected with LV-miR124 for 72 h, followed by infection with LV-PIK3CA. Cell growth rate was then performed. All experiments were carried 3 times independently. $^{**}P < 0.01$ compared to HepG2-miR-control cells.

3.4. MiR-124 overexpression inhibits the tumorigenesis in nude mice

To further study the function of miR-124 on inhibition of tumor growth in vivo, HepG2-miR-124 or control cells were injected subcutaneously into the dorsal flank of nude mice. As compared with control, the average tumor volume of HepG2-miR-124 group was markedly reduced (Fig. 4A). The average tumor weight was also significantly reduced in HepG2-miR-124 group (Fig. 4B). Morever, we measured the expression of miR-124, PIK3CA, Akt and mTOR in the harvested tumor tissues. Similar to our in vitro results, the mean level of miR-124 expression was significantly increased in the tumors derived from HepG2-miR-124 cells (Fig. 4C), and the

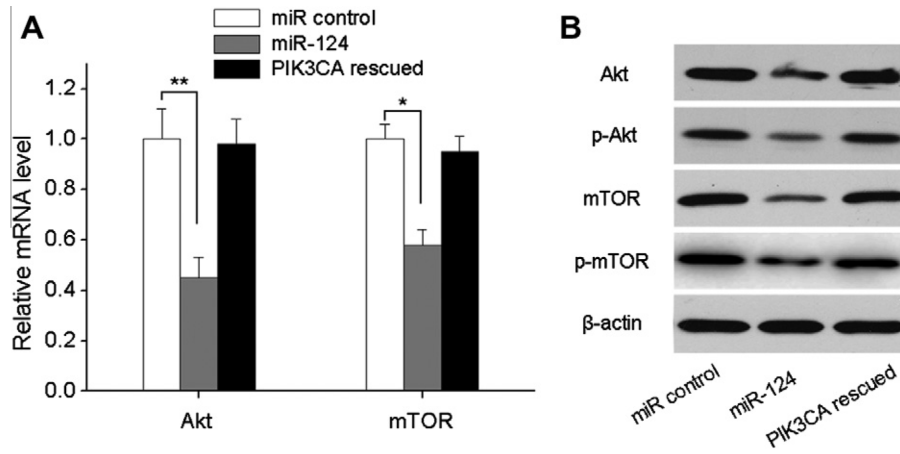

Fig. 3. miR-124 suppressed PIK3CA-mediated PI3K/Akt/mTOR signal pathway. (A) Expression profile changes of the Akt and mTOR genes at the mRNA level. The values were normalized to β-actin mRNA. (B) Expression profile changes of the Akt and mTOR genes at the protein level. miR-124-overexpression significantly inhibited the expression of Akt and mTOR protein; however, the inhibition was reversed by re-expression of PIK3CA. All experiments were carried 3 times independently. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to HepG2-miR-control cells.

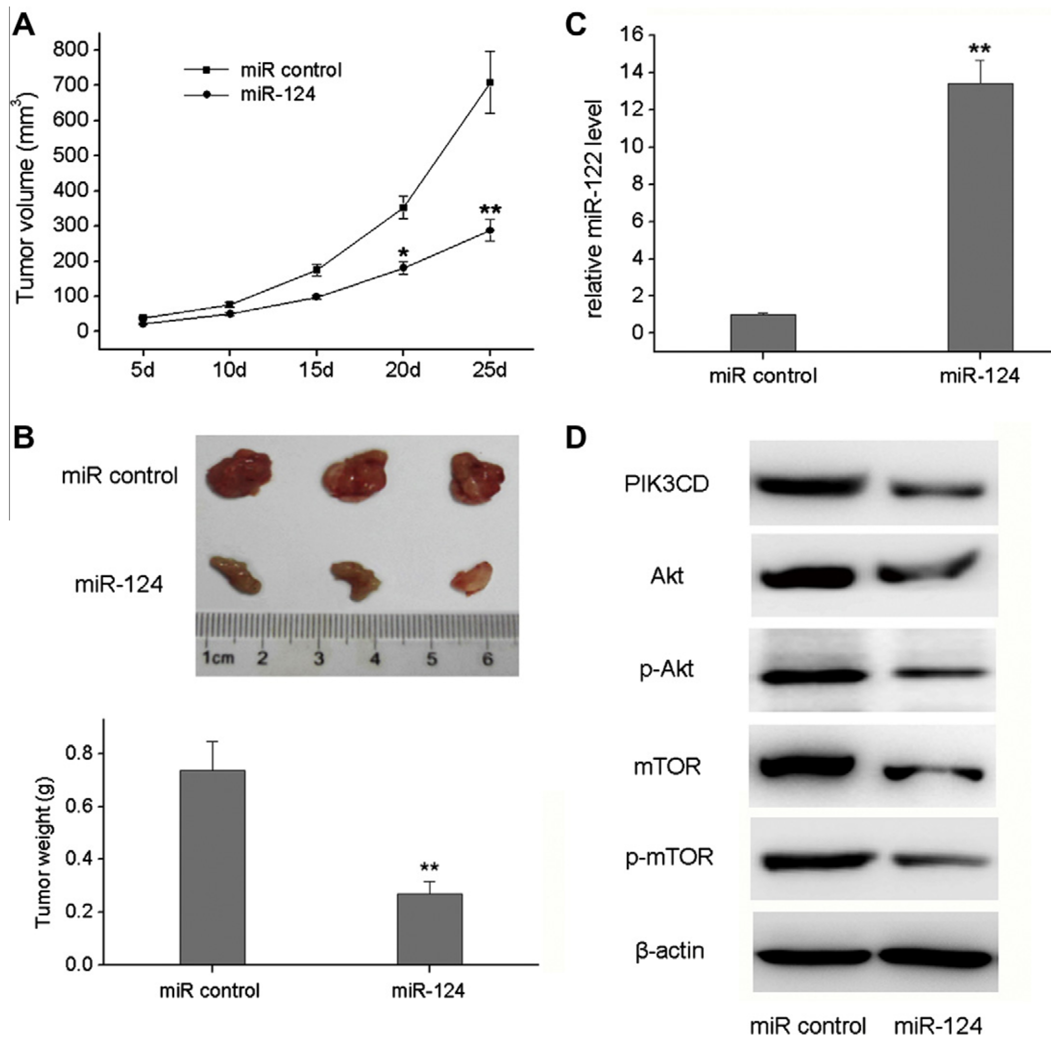

Fig. 4. miR-124 suppressed tumor growth in nude mice. HepG2-miR-124 or control cells (5×10^6) were inoculated subcutaneously into nude mice, and the mice were closed monitored for tumor growth. 25 days post inoculation, the mice were sacrificed and the tumors were recovered. (A) The volume of xenograft tumors. (B) Photography of xenograft tumor masses from nude mice, and the weight of xenograft tumors. (C) Expression of miR-124 in xenograft tumor tissues as determined by qRT-PCR. (D) Western blot analysis of the expression of PIK3CA and downstream components in miR-124-overexpressed and control xenograft tumor tissues. All experiments were carried three times independently. $^{**}P < 0.01$ compared to HepG2-miR-control cells.

expression levels of PIK3CA, Akt and mTOR were all decreased compared to the controls (Fig. 4D).

4. Discussion

Growing evidence has suggested that dysregulation of miRNAs contributes to tumorigenesis [20]. Changes in miRNA profiling are implicated in almost all aspects of cancer biology, including cell proliferation [21]. Thus, miRNAs are increasingly viewed as a potential diagnostic and therapeutic tool [22,23]. In this study, we focused on miR-124, which has been suggested to inhibit tumor growth in several human cancers, such as Glioblastoma multiforme [24] and cervical cancer [25]. Xie and colleagues recently found that the expression levels of miR-124 were frequently decreased in HCC cells and tissues, and low-level expression of miR-124 was significantly associated with a more aggressive and/or poor prognostic phenotype of patients with HCC [26]. However, the detailed mechanism(s) surrounding a role that miR-124 may play in HCC development needs to be further eluciated.

In this study, we provide the first evidence that miR-124 suppresses HCC progression by targeting a novel miR-124 target, PIK3-CA. Our results indicate that overexpression of miR-124 significantly suppressed HCC cell proliferation by inducing G1-phase cell-cycle arrest in vitro, and the tumorigenesis in vivo. We identified PIK3CA as a direct target of miR-124 in HepG2 cells, and this conclusion is supported by the following reasons: complementary sequence of miR-124 is identified in the 3′UTR of PIK3CA mRNA; overexpression of miR-124 led to a significant reduction in PIK3CA at both mRNA and protein level; miR-124 overexpression suppressed PIK3CA 3′UTR luciferase report activity and this effect was abolished by mutation of the miR-124 seed binding site. The functions of PIK3CA is further supported by the observations that PIK3CA knockdown induced cell growth inhibition similar to the phenotypes induced by miR-124 restoration, whereas PIK3CA overexpression could rescue the growth-suppressive effect of miR-124. These results indicate that miR-124 may function as a negative regulator or tumor suppressor for the cell growth partly mediated by repressing PIK3CA expression.

PIK3CA is known to be an oncogene component of phosphatidylinositol 3-kinase (PI3K) signaling pathway and is implicated in cell proliferation and carcinogenesis in many human cancers [27,28]. To further explore the molecular mechanisms of growth inhibition induced by miR-124, we examined the expression of key components of the PI3K/Akt pathway. The results suggested that the mRNA levels of Akt and mTOR were significantly suppressed. The total and phosphorylated protein levels of the molecules described above showed the same results, indicating that miR-124 may be an important regulator of this signaling pathway. More importantly, we found that these deregulated expressions of miR-124 effectors could be restored by overexpression of PIK3CA. All these results documented that miR-124 repressed PIK3CA expression, which, in turn, by regulating PI3K/Akt pathway, inhibited the growth and tumorgenicity of HepG2 cells.

In conclusion, the current study provides novel evidence that miR-124 suppresses HCC cell proliferation through repression of PIK3CA and downregulation of PI3K/Akt pathway. Our findings on miR-124 are encouraging and suggest that this miRNA could be a potential target for the treatment of HCC in future.

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