

Downregulation of microRNA-143 promotes cell proliferation by regulating PKC ϵ in hepatocellular carcinoma cells

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Abstract. The abnormal expression of microRNAs (miRNAs) has been reported in hepatocellular carcinoma (HCC), however, the functional role of miR-143 in HCC remains to be fully elucidated. The present study aimed to investigate the effects of the downregulation of miR-143 on HCC cell proliferation and apoptosis, and elucidated the underlying mechanism. Hepg2 and Hep3B human hepatoma cell lines cells were transfected with miR-143 inhibitor. Following transfection, the cell viability and apoptosis were respectively determined using a 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide assay and flow cytometry, and the mRNA and protein levels of protein kinase C ϵ (PKC ϵ) were examined. The expression levels of PKC ϵ were downregulated by short hairpin (sh)RNA, and the effects of the downregulation of miR-143 on HCC cell proliferation were measured. The results showed that the miR-143 inhibitor significantly promoted cell proliferation and suppressed apoptosis in the Hepg2 and Hep3B cells. The miR-143 inhibitor significantly increased the protein levels of PKC ϵ in the Hepg2 and Hep3B cells; however, no significant differences were found in the mRNA levels of PKC ϵ . In addition, the downregulation of PKC ϵ markedly decreased the cell viability of the Hepg2 and Hep3B cells, and co-transfection with the miR-143 inhibitor and PKC ϵ shRNA significantly alleviated the miR-143 inhibitor-induced high cell proliferation. Taken together, these results suggested that miR-143 acts as a tumor suppressor gene in HCC. The downregulation of mi-143 promoted cell proliferation by regulating PKC ϵ in the HCC cells.

Introduction

Hepatocellular carcinoma (HCC) is one of the most life-threatening, aggressive and prevalent types of cancer worldwide, and is the third leading cause of cancer-associated mortality (1,2). It has been reported that the incidence of HCC continues to increase in several countries (1), particularly in East Asia where hepatitis B and C are endemic (3). Although there have been significant advances in the introduction of various screening programs for the prevention of this disease, the majority of patients with an advanced stage of disease show metastasis and have a poor prognosis (4). The median overall survival rate of patients at an advanced stage is <1 year (5). Therefore, an improved understanding of the molecular mechanism involved in the development and pathogenesis of HCC may provide novel therapeutic strategies.

MicroRNAs (miRNAs) are short, single-stranded, conserved, non-coding RNAs consisting of 18-25 nucleotides, which suppress the translation of target mRNAs or promote mRNA degradation by binding to the 3'-untranslated region of target transcripts (6,7). It has been demonstrated that miRNAs are involved in diverse biological functions, including cell proliferation, apoptosis and tumorigenesis (8). miRNAs function either as an oncogene or as a tumor suppressor in cancer, depending on the target genes (9). A variety of miRNAs have been reported to be responsible for the pathogenesis of HCC (10-12), including upregulated miR-21, miR-31 and miR-223, and downregulated miR-122, miR-145, miR-200c, miR-221 and miR-222 (13). The abnormal expression of miR-143 has been reported to be involved in several types of cancer, including colorectal cancer, non-small cell lung cancer and prostate cancer (14-16). A previous study found that miR-143 was also involved in HCC and was downregulated in HCC, compared with a control group (17). However, the exact functional role of miR-143 in HCC remains to be fully elucidated.

The present study aimed to examine the functional role of miR-143 in HCC. The effects of the downregulation of miR-143 on cell proliferation and apoptosis were investigated, and the underlying mechanisms were examined. The results of these investigations may provide novel insights into the pathogenesis of HCC and novel targets for HCC therapy.

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Materials and methods

Cell culture. Hepg2 and Hep3B human hepatoma cell lines were obtained from the Chinese Academy of Sciences Type Culture Collection (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium-F12 medium supplemented with 2.5% (v/v) fetal bovine serum (both from Thermo Fisher Scientific, Inc., Waltham, MA, USA), 50 mg/l gentamicin, 50 mg/l penicillin and 50 mg/l streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.). The cells were maintained in a humidified incubator containing 5% CO₂ and 95% air at 37°C.

Transient transfection. The miR-143 inhibitor and scrambled RNA were designed and produced by Guangzhou RiboBio Co., Ltd. (Guangzhou, China) according to the manufacturer's protocol. PKC ϵ short hairpin (sh)RNAs (shRNA1 and shRNA2) and the scramble control shRNA were designed and generated by GenePharma, Inc. (Shanghai, China). Cells at 70–80% confluence were used for transfection. Briefly, the cells were seeded in a 96-well plate (1 \times 10⁵ cells/well) prior to transfection. The miR-143 inhibitor (150 nM), PKC ϵ shRNAs (150 nM) or their scramble controls (150 nM) were transfected into the cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 48 h, the cells were harvested and cell lysates were prepared using radioimmunoprecipitation assay buffer (Sangon Biotech, Co., Ltd., Shanghai, China).

Cell viability. The cell viability of the Hepg2 and Hep3B cells was measured using a 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Briefly, the cells (1 \times 10⁵ cells/cm²) were seeded in 96-well plates and incubated for 12 h. Following transfection with miR-143 inhibitor, PKC ϵ shRNAs or their scramble controls, MTT solution (2.5 mg/ml; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was added to the plates and incubated for 4 h at 37°C. Dimethyl sulfoxide (Sigma-Aldrich; Merck Millipore) was added to each plate to dissolve the MTT formazan crystals. The absorbance was determined on an enzyme-linked immunosorbent microplate reader (Bio-Rad 2550; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 590 nm. Each experiment was performed in triplicate.

Cell apoptosis. The cell apoptosis was analyzed using an Annexin V-fluorescein-5-isothiocyanate (Annexin V-FITC) apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, cells (5 \times 10⁶) were seeded in 24-well plates and transfected with miR-143 inhibitor, PKC ϵ shRNAs or their scramble controls. The cells were then mixed with 10 μ l Annexin and incubated in the dark at room temperature for 20 min, followed by the addition of 5 μ l 10 mg/l propidium iodide. Finally, the cells were read using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The excitation wavelength and emission wavelength were set at 488 nm and 635 nm, respectively. The numerical values were analyzed using CellQuest software version 3.1 (BD Biosciences).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA

was extracted from the Hepg2 and Hep3B cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. miRNAs were isolated using an miRNeasy micro kit (Qiagen, Inc., Valencia, CA, USA). The RNA (8 ng) was reverse transcribed into complementary DNA (cDNA) using a TaqMan miRNA Reverse Transcription kit (Qiagen, Inc.). cDNAs were then subjected to qPCR using SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan) on a StepOne Plus system (Applied Biosystems; Thermo Fisher Scientific, Inc.). U6 and GAPDH were used as a loading control for miR-143 and PKC ϵ , respectively. PCR was performed as following: initial predenaturation for 1 min at 95°C, followed by 36 cycles of 95°C for 30 sec, 62°C for 30 sec and 72°C for 2 min, with a final extension at 72°C for 7 min. Primers for miR-143 and PKC ϵ were purchased from Qiagen, Inc. The specific primers were as following: miR-143 forward, 5'-AGTCAGTGAGATGAAGCACTG-3' and reverse, 5'-GT GCAGGGTCCGAGGT-3'; U6 forward, 5'-CTCGCT TCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATT TCGGT-3'; PKC ϵ forward, 5'-AGCCTCGTTCACGGTTCT-3' and reverse, 5'-TGTCCAGCCATCATCTCG-3'; GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'. The 2^{- $\Delta\Delta$ C_q} method (18) was used for quantitative analysis.

Western blot analysis. The expression levels of PKC ϵ were measured using western blot analysis. Briefly, protein was extracted from the Hepg2 and Hep3B cells following transfection with the miR-143 inhibitor, PKC ϵ shRNAs or their scramble controls. The protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc.). The protein samples (20 μ g/lane) were then separated by 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes (Takara Bio, Inc.). The membranes were probed with PKC ϵ antibody (1:1,000; cat no. ab124806) or GAPDH (1:2,000; cat no. ab9485) (both from Abcam, Cambridge, UK) at 4°C overnight, following which the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1,000; cat no. ab6721; Abcam) for 1 h at room temperature. The membranes were subsequently visualized using enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). The expression of PKC ϵ was normalized to that of GAPDH and was semi-quantified by densitometry using ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The data are presented as the mean \pm standard deviation. Statistical analysis was performed using the Statistics Package for Social Science (SPSS) software (version 19.0; IBM SPSS, Armonk, NY, USA). One way analysis of variance followed by a Tukey-Kramer post-hoc test, or a paired t-test was performed to confirm significance. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-143 is downregulated in Hepg2 and Hep3B cells following transfection. To investigate the functional role of miR-143

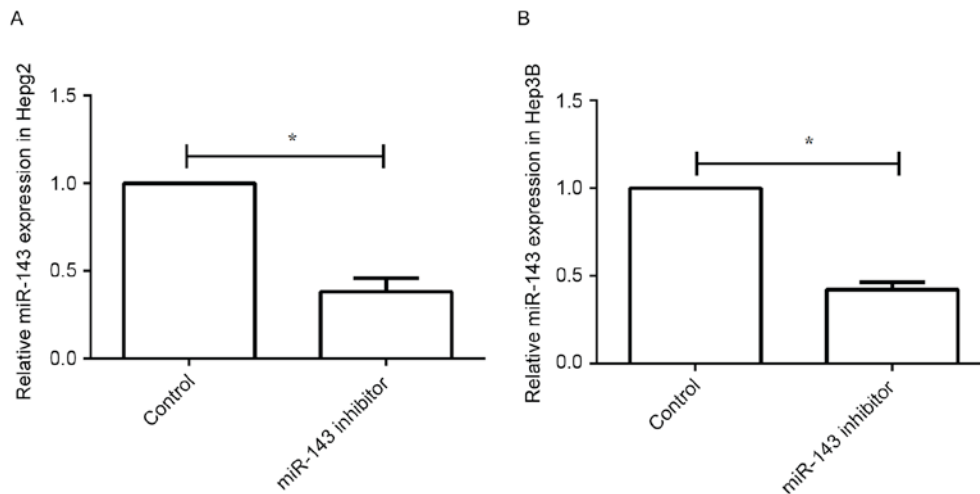


Figure 1. Relative expression levels of miR-143 following transfection with miR-143 inhibitor. (A) Relative expression levels of miR-143 following transfection in Hepg2 cells. (B) Relative expression levels of miR-143 following transfection in Hep3B cells. *P<0.05, compared with the control group. miR, microRNA.

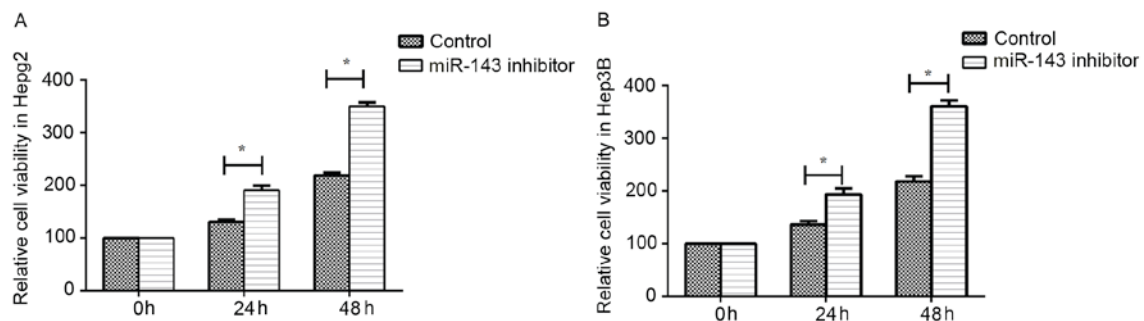


Figure 2. miR-143 inhibitor promotes the proliferation of Hepg2 and Hep3B cells. (A) Relative cell viability of Hepg2 cells following transfection with miR-143 inhibitor. (B) Relative cell viability of Hep3B cells following transfection with miR-143 inhibitor. *P<0.05, compared with the control group. miR, microRNA.

in HCC, the present study induced the downregulation of the expression of miR-143 in the Hepg2 and Hep3B human hepatoma cells. As shown in Fig. 1, the relative expression levels of miR-143 were significantly decreased in the Hepg2 and Hep3B cells following transfection with the miR-143 inhibitor, compared with those in the control group (P<0.05). These results indicated that the cells had been successfully transformed by the miR-143 inhibitor.

miR-143 inhibitor promotes cell proliferation of Hepg2 and Hep3B cells. The present study then determined the effect of the miR-143 inhibitor on cell proliferation in the Hepg2 and Hep3B cells. An MTT assay was then performed to confirm the cell viability at different time points (0, 24 and 48 h) following transfection with the miR-143 inhibitor. As indicated in Fig. 2A, no significant differences were found at 0 h post-transfection with the miR-143 inhibitor in the Hepg2 cells, however, the cell viability was significantly increased at 24 and 48 h post-transfection with the miR-143 inhibitor, compared with viability in the control group (P<0.05). Similar results were observed in the Hep3B cells (Fig. 2B). These results demonstrated that the miR-143 inhibitor promoted HCC cell proliferation.

miR-143 inhibitor suppresses the apoptosis of Hepg2 and Hep3B cells. The effects of the miR-143 inhibitor on apoptosis of the Hepg2 and Hep3B cells were analyzed using an Annexin V-FITC apoptosis detection kit. The percentages of apoptosis in the Hepg2 and Hep3B cells were recorded. As indicated in Fig. 3, it was found that the percentages of apoptosis in the Hepg2 and Hep3B cells were significantly reduced following transfection with the miR-143 inhibitor, compared with the control group (P<0.05), suggesting that the miR-143 inhibitor suppressed HCC apoptosis.

miR-143 inhibitor increases PKCε at the protein level, but not at the mRNA level in Hepg2 and Hep3B cells. To determine whether miR-143 was able to regulate the expression of PKCε in HCC, the present study determined the mRNA and protein levels of PKCε following transfection of Hepg2 and Hep3B cells with the miR-143 inhibitor. The results showed no significant change in the mRNA levels of PKCε following transfection with the miR-143 inhibitor. No significant differences in the mRNA levels of PKCε were found in either the Hepg2 or Hep3B cells (Fig. 4A and B). However, the protein levels of PKCε were markedly increased by transfection with the miR-143 inhibitor in the Hepg2 and Hep3B cells, compared

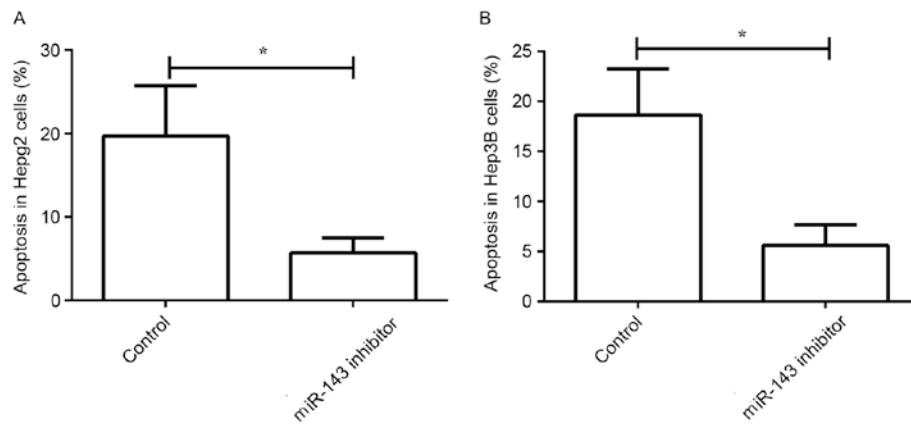


Figure 3. miR-143 inhibitor suppresses the apoptosis of Hepg2 and Hep3B cells. (A) Percentages of apoptosis of Hepg2 cells following transfection with miR-143 inhibitor. (B) Percentages of apoptosis of Hep3B cells following transfection with miR-143 inhibitor. *P<0.05, compared with the control group. miR, microRNA.

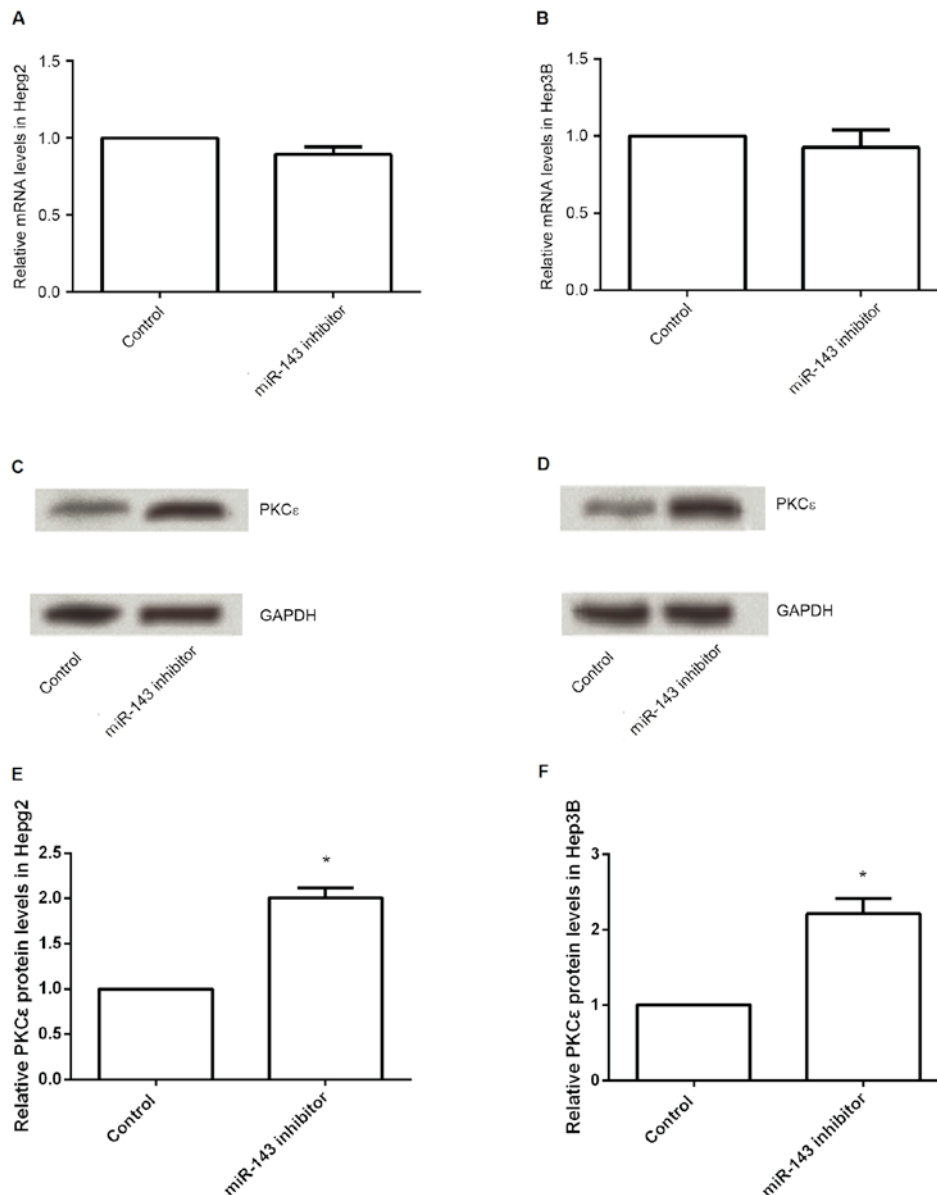


Figure 4. miR-143 inhibitor increases PKCε at the protein, but not the mRNA, level in in Hepg2 and Hep3B cells. Relative mRNA levels of PKCε in (A) Hepg2 and (B) Hep3B cells following transfection with miR-143 inhibitor. Representative images of western blots of the protein levels of PKCε in (C) Hepg2 and (D) Hep3B cells following transfection with miR-143 inhibitor. Relative protein levels of PKCε in (E) Hepg2 and (F) Hep3B cells following transfection with miR-143 inhibitor. *P<0.05, compared with the control group. PKC, protein kinase C; miR, microRNA.

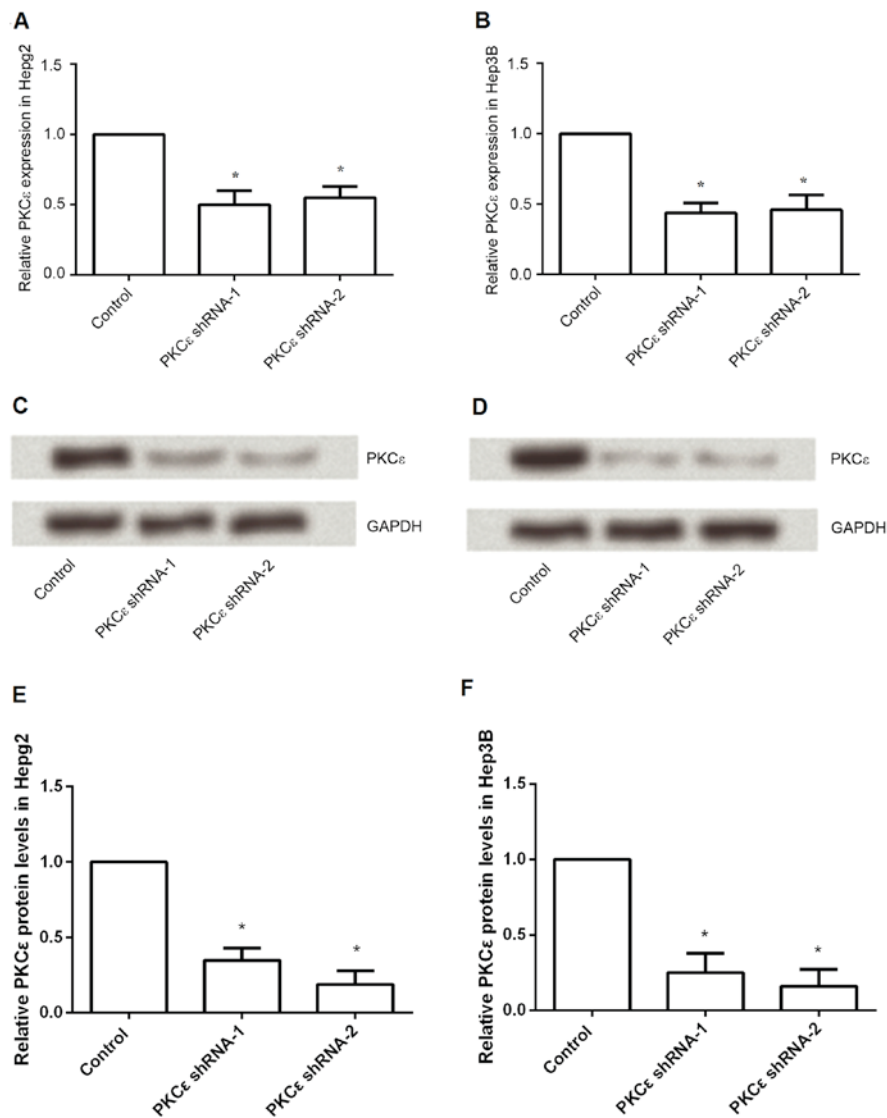


Figure 5. PKC ϵ is downregulated by shRNAs. Relative mRNA levels of PKC ϵ in (A) Hepg2 and (B) Hep3B cells following transfection with PKC ϵ shRNAs. Representative images of western blots of the protein levels of PKC ϵ in (C) Hepg2 and (D) Hep3B cells following transfection with PKC ϵ shRNAs. Relative protein levels of PKC ϵ in (E) Hepg2 and (F) Hep3B cells following transfection with PKC ϵ shRNAs. * $P < 0.05$, compared with the control group. PKC, protein kinase C; shRNA, short hairpin RNA.

with the control group ($P < 0.05$; Fig. 4C-F). The results indicated that the miR-143 inhibitor was able to alter the protein levels of PKC ϵ .

Downregulation of PKC ϵ eliminates the increased cell proliferation induced by miR-143 inhibitor in Hepg2 and Hep3B cells. To further examine whether the miR-143 inhibitor-induced promotion of cell proliferation was through regulating the expression of PKC ϵ , the present study investigated the effects of the downregulation of PKC ϵ on the proliferation of the Hepg2 and Hep3B cells. The mRNA and protein levels of PKC ϵ were assayed following transfection with PKC ϵ shRNAs (shRNA1 and shRNA2). As shown in Fig. 5, the results showed that the mRNA and protein levels of PKC ϵ were significantly downregulated by transfection with PKC ϵ shRNA1 and shRNA2, compared with levels in the control group ($P < 0.05$). As indicated in Fig. 6, the downregulation of PKC ϵ reduced the increase in cell proliferation induced by the

miR-143 inhibitor in the Hepg2 and Hep3B cells. These results suggested that the effects of the miR-143 inhibitor on HCC cell proliferation occurred through regulating the expression of PKC ϵ .

Discussion

The abnormal expression of miRNAs contributes to the progression and development of several types of tumor, including HCC (19,20). The biological roles of deregulated miRNA expression have received increased attention due to their unique features. In the present study, it was observed that the downregulation of miR-143 significantly promoted cell proliferation and reduced cell apoptosis of Hepg2 and Hep3B cells. In addition, the downregulation of miR-143 increased the protein levels of PKC ϵ , and the suppression of PKC ϵ reduced the miR-143 inhibitor-induced increase in cell proliferation.

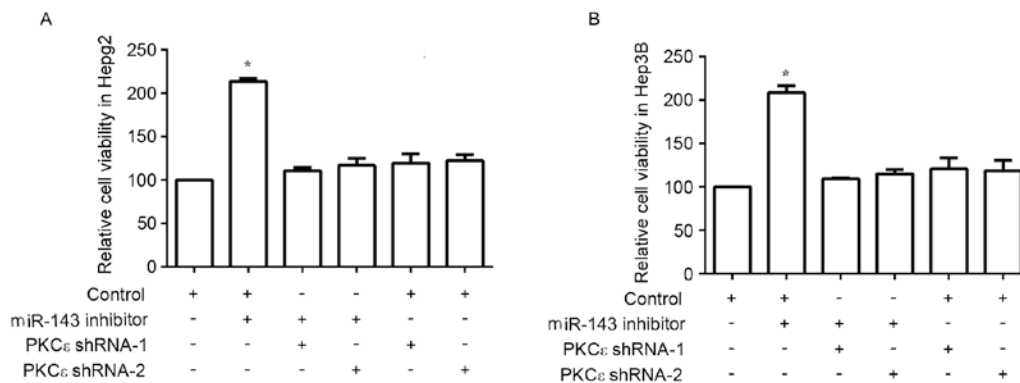


Figure 6. Downregulation of PKC ϵ eliminates the increased cell proliferation induced by miR-143 inhibitor in HepG2 and Hep3B cells. (A) Relative cell viability of HepG2 cells following transfection with miR-143 inhibitor and/or PKC ϵ shRNAs. (B) Relative cell viability of Hep3B cells following transfection with miR-143 inhibitor and/or PKC ϵ shRNAs. * $P < 0.05$, compared with the control group. PKC, protein kinase C; shRNA, short hairpin RNA; miR, microRNA.

There has been limited progress in understanding the mechanism and management of HCC. Currently, effective treatments for HCC are relatively unsystematic and unavailable, and HCC remains a major challenge for surgeons (21). Therefore, an improved understanding of the molecular mechanisms involved in the carcinogenesis and progression of HCC is essential. The identification and characterization of abnormal miRNA expression in the development and progress of HCC have received increased attention. Several deregulated miRNAs have been revealed in HCC, including miR-22, miR-29, miR-101 and miR-122, and are associated with cell proliferation, apoptosis, metastasis, tumorigenicity and the prognosis of HCC (22-25). Among the miRNAs, miR-143 has been investigated extensively and has been reported to be a tumor suppressor in several types of cancer, including bladder cancer, colon cancer and esophageal squamous cell carcinoma (26-28). However, the functional role of miR-143 in HCC remains to be fully elucidated.

In the present study, the functional role of miR-143 in HCC was investigated. The investigation focused on the effects of miR-143 on the proliferation and apoptosis of HepG2 and Hep3B cells. The expression of miR-143 was downregulated by transfection with miR-143 inhibitor, and the effects of the downregulation of miR-143 on cell proliferation and apoptosis were measured. The results revealed that miR-143 altered the proliferation and apoptosis of HepG2 and Hep3B cells. The downregulation of miR-143 significantly promoted cell proliferation 24 and 48 h following transfection, and significantly induced apoptosis. These results indicated that miR-143 may act as a tumor suppressor gene in HCC, which is consistent with previous reports (17,29). It has been demonstrated that deregulated cell proliferation is a key event in the progress and development of cancer, which is regulated by several factors (29). The inhibition of cell proliferation is a primary target in the prevention and treatment of cancer, one of the reasons being that the inhibition of cell proliferation is associated with increased apoptosis (30). The dysfunction of cell apoptosis is another important hallmark of cancer cells, which leads to uncontrolled cell growth, migration, invasion, and resistance to chemotherapy and radiotherapy (31).

The present study also examined the underlying mechanisms with respect to the effects of miR-143 on cell proliferation. It has been reported that the PKC serine-threonine kinases are

involved in different cellular processes and signal transduction pathways, which regulate cell proliferation, differentiation, apoptosis and migration (32-36). Among the members of the PKC family, PKC ϵ , a pro-proliferation and anti-apoptotic gene, has been identified as an oncogenic kinase and tumor biomarker (37,38). The overexpression of PKC ϵ has been reported to increase cell viability and cause the development of tumors *in vivo* (39). Several miRNAs have been identified in a range of diseases by regulating the expression of PKC ϵ . For example, Inoue *et al* suggested that decreased levels of miR-223 increased the levels of PKC ϵ and may be important in the pathogenesis of Gottron's papules (40). Körner *et al* identified PKC ϵ as a novel direct target of miR-31, with the downregulation of PKC ϵ resulting in enhanced apoptosis (41). A previous study revealed that miR-143 regulated cell apoptosis in lung cancer by targeting PKC ϵ (42). Therefore, the present study hypothesized that the effects of miR-143 on cell proliferation may also be through regulation of PKC ϵ . To confirm this hypothesis, the present study analyzed the mRNA and protein levels of PKC ϵ following transfection with miR-143 inhibitor. The resulting data showed that the protein levels of PKC ϵ , but not the mRNA levels, were significantly increased by the inhibitor. The expression of PKC ϵ was then downregulated by shRNAs to determine the effects of the downregulation of miR-143 on cell proliferation. The results showed that the downregulation of PKC ϵ reversed the increased cell proliferation induced by the miR-143 inhibitor, indicating that the downregulation of miR-143 promoted cell proliferation via the regulation of PKC ϵ .

In conclusion, the present study demonstrated that the suppression of miR-143 promoted cell proliferation and induced apoptosis, indicating that miR-143 functions as a tumor suppressor in HCC cells.

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