

Vitamin D3 mediates miR-15a-5p inhibition of liver cancer cell proliferation via targeting E2F3

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Abstract. Vitamin D3 has been demonstrated to suppress the development and progression of liver cancer, but the mechanism is unclear. The effects of vitamin D3 and microRNA (miR)-15a-5p on liver cancer cells were investigated in the present study using MTT and colony formation assays, flow cytometry, western blotting and reverse transcription-quantitative PCR. A dual-luciferase reporter assay was performed to determine whether E2F transcription factor 3 (E2F3) was a target of miR-15a-5p. The effects of silencing the E2F3 gene expression in liver cancer cells were investigated using a small interfering RNA. Vitamin D3 suppressed liver cancer cell proliferation, induced apoptosis and increased miR-15a-5p expression. Treatment with the miR-15a-5p mimics significantly suppressed liver cancer cell proliferation compared with that of the controls. Bioinformatics analysis and a dual-luciferase reporter assay demonstrated that E2F3 was a target of miR-15a-5p and that silencing E2F3 inhibited liver cancer cell proliferation. Therefore, Vitamin D3 suppressed cell proliferation by miR-15a-5p-mediated silencing of E2F3 gene expression. These findings suggested a role for vitamin D3 and E2F3 targeting as potential novel liver cancer therapies.

Introduction

Liver cancer is a common malignancy worldwide. Liver cancer is not sensitive to chemotherapy and radiotherapy. Liver resection, local cytoreductive therapies and liver transplantation are considered curative for patients with early hepatocellular carcinoma (1). The majority of patients with advanced disease at diagnosis do not receive curative treatment (2). The carcinogenesis of liver cancer appears to be multifactorial, involving

multiple genetic changes that may provide clues for identifying novel therapeutic targets. Vitamin D3 may be involved in certain types of cancer; previous studies have demonstrated that a sufficient serum concentration of 25-hydroxyvitamin D, a metabolite of vitamin D3, may decrease the risk of bladder cancer (3-6). Vitamin D3 is the biologically active form of vitamin D, and its ability to regulate cell proliferation, apoptosis and angiogenesis may influence cancer risk, development, and progression (7-9). Nuclear vitamin D receptor is a ligand-dependent nuclear transcription factor that binds vitamin D3 to form a hormone-receptor complex that either initiates or inhibits the transcription of a target gene by binding to the promoter (10).

The anticancer effects of vitamin D may be mediated by changes in micro (mi)RNA expression (11). miRNAs may function as either tumor suppressors or oncogenes (12). Multiple deregulated miRNAs have been identified in liver cancer, including miRNA (miR)-363-3p, miR-187-3p, miR-99a and miR-143 (13-16). miR-15a, located on human chromosome 13q14, is abnormally expressed in numerous types of tumors (17), and has been demonstrated to suppress hepatocellular carcinoma (HCC) cell proliferation and invasion through the regulation of target genes, including BDNF and cMyb (18,19). The present study investigated the antiproliferative effects of vitamin D3 and its molecular mechanism in liver cancer cells.

Materials and methods

Cell culture. HepG2 and Hep3B human hepatoma cells, and 293 human embryonic kidney cells were obtained from the Key Laboratory of Environment and Genes Related to Diseases at Xi'an Jiaotong University. The cells were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂ in a humidified atmosphere. HepG2 cells were authenticated by short tandem repeat analysis. miR-15a-5p mimic, mimic control, si-E2F3 and si-control were synthesized by Shanghai GenePharma Co., Ltd. miR-15a-5p-inhibitor and inhibitor-ctrl were synthesized by Sangon Biotech Co., Ltd. Cells were transfected with miR-15a-5p mimic (50 nM), mimic-ctrl (50 nM), miR-15a-5p inhibitor (1 µg/ml), inhibitor-control (1 µg/ml), si-E2F3 or

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si-control (50 nM) by using JetPrime transfection reagent (Polyplus-transfection SA) according to the manufacturer's instructions. The sequences are presented in Table SI.

MTT assay. Hep3B and HepG2 cells were incubated in 96-well plates at the density of 3,000 cells/well for 24 h prior to treatment with 0, 300, 600 or 1,200 nM vitamin D3 at 37°C for 24, 48 and 72 h or prior to transfection with a miR-15a-5p mimic, mimic control (mimic-ctrl), miR-15a-5p inhibitor, inhibitor control, small interfering (si)-RNA targeting E2F transcription factor 3 (si-E2F3) or si-control for an additional 24, 48 and 72 h. MTT (Sigma-Aldrich; Merck KGaA) was added to each well and incubated for 4 h at 37°C. After discarding the supernatant, 150 μ l/well of DMSO was added, and the absorbance was read at 492 nm using a microplate reader.

Colony formation assay. Hep3B and HepG2 cells were incubated at the density of 2,000 cells/well for 24 h and transfected with a miR-15a-5p mimic, mimic-ctrl, miR-15a-5p inhibitor, inhibitor-control, si-E2F3 or si-control, and cultured for 2 weeks. The colonies were stained with 0.1% crystal violet for 30 min, rinsed with phosphate buffered saline (PBS) and images were acquired using Quantity One software (version 4.3.1; Bio-Rad Laboratories, Inc.). The supernatant was discarded, crystal violet staining was solubilized using DMSO, and the absorbance was read at 492 nm on a microplate reader.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from Hep3B and HepG2 cells following treatment with vitamin D3 or transfection by using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Prime-Script RT reagent kit (Takara Bio, Inc.) was used to reverse transcribe RNA according to the manufacturers' instructions. The RT-qPCR assays were conducted with SYBR[®] Green Ex Taq II (Takara Bio, Inc.) using a FTC-3000TM System (Funglyn Biotech Inc.). The reactions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and at 60°C for 30 sec. miRNA expression was normalized against a U6 endogenous control. E2F3 expression was normalized to β -actin. The $2^{-\Delta\Delta Cq}$ (20) method was used to determine the relative expression of miR-15a-5p. The primer sequences are presented in the Table SI.

Plasmid construction. Overexpression of miR-15a-5p in Hep3B and HepG2 cells was induced by the miRNA mimics synthesized by GenePharma (Shanghai GenePharma Co.). The mimic and control sequences are presented in Table SI. The wild-type (WT) and mutant (MUT) 3'-untranslated regions (UTRs) of the human *E2F3* mRNA were synthesized from oligonucleotides and cloned into the *SacI* and *XhoI* sites of a pmirGLO Vector (Promega Corporation).

Apoptosis assay. Hep3B and HepG2 cells cultured in 12-well plates at the density of 2×10^5 cells/well for 24 h, then treated with 1,200 nM of vitamin D3 at 37°C for 48 h, harvested and washed with PBS. Following staining with an Annexin V-FITC Apoptosis Detection Kit (7Sea Biotech) according to the manufacturer's instructions, apoptosis was evaluated by flow cytometry (ACEA Bioscience, Inc.) by calculating early and late apoptosis and analyzed by NovoExpress (ACEA Bioscience, Inc.).

Dual-luciferase reporter assay. miR-15a-5p target genes were identified using TargetScan (<http://www.targetscan.org>). The dual-luciferase reporter assay was conducted in 293 cells seeded in 96-well plates at the density of 5×10^4 cells/well. After a 24-h incubation, miR-15a-5p were co-transfected with WT E2F3 3'-UTR or MUT E2F3 3'-UTR or pmirGLO Vector (Promega Corporation) using JetPrime Polyplus transfection reagent (Polyplus-transfection SA) according to the manufacturer's instructions. After a 48-h transfection, Luciferase activity was examined using the Dual-Glo Luciferase Assay system and results were normalized to Renilla luciferase activity (Promega Corporation).

Western blotting. Total protein was extracted from Hep3B and HepG2 cells using RIPA lysis buffer (Xi'an Wolsen Biotechnology Co., Ltd). Protein concentration was determined using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Proteins (30 μ g) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocking with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h, the membranes were incubated overnight at 4°C with primary antibodies against Bcl-2 (cat. no. 12789-1-AP; 1:500; ProteinTech Group, Inc.), Bax (cat. no. 50599-2-Ig; 1:500; ProteinTech Group, Inc.) and β -actin (cat. no. sc-47778; 1:1,000; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Subsequently, the membranes were washed with TBST and incubated with secondary goat anti-rabbit or goat anti-mouse antibody (cat. nos. 111-035-144; 115-035-003; 1:1,000; Jackson ImmunoResearch Laboratories, Inc.) for 2 h at room temperature. The protein expression in each sample was normalized to that of β -actin.

Statistical analysis. Assays were performed in triplicate and the results are reported as the mean \pm standard error of the mean (SEM). Student's t-test or one-way ANOVA followed by a Tukey's post hoc test was performed to analyze the differences between the groups. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with vitamin D3 inhibits liver cancer cell proliferation. The MTT assay results demonstrated that vitamin D3 significantly inhibited the proliferation of Hep3B and HepG2 cells compared with the controls (Fig. 1A). The Annexin-V assay results revealed that 1,200 nM vitamin D3 significantly increased apoptosis in HepG2, but not in Hep3B cells compared with their respective controls (Fig. 1B). The antiproliferative mechanism of vitamin D3 was investigated by RT-qPCR, which demonstrated that miR-15a-5p expression was significantly increased in Hep3B and HepG2 cells treated with 1,200 nM vitamin D3 and that the expression levels of miR-203a-3p and miR-214-3p did not change compared with the controls (Fig. 1C).

miR-15a-5p mimics inhibit liver cancer cell proliferation. The function of miR-15a-5p was studied by gain-of-function experiments following transfection of Hep3B and HepG2

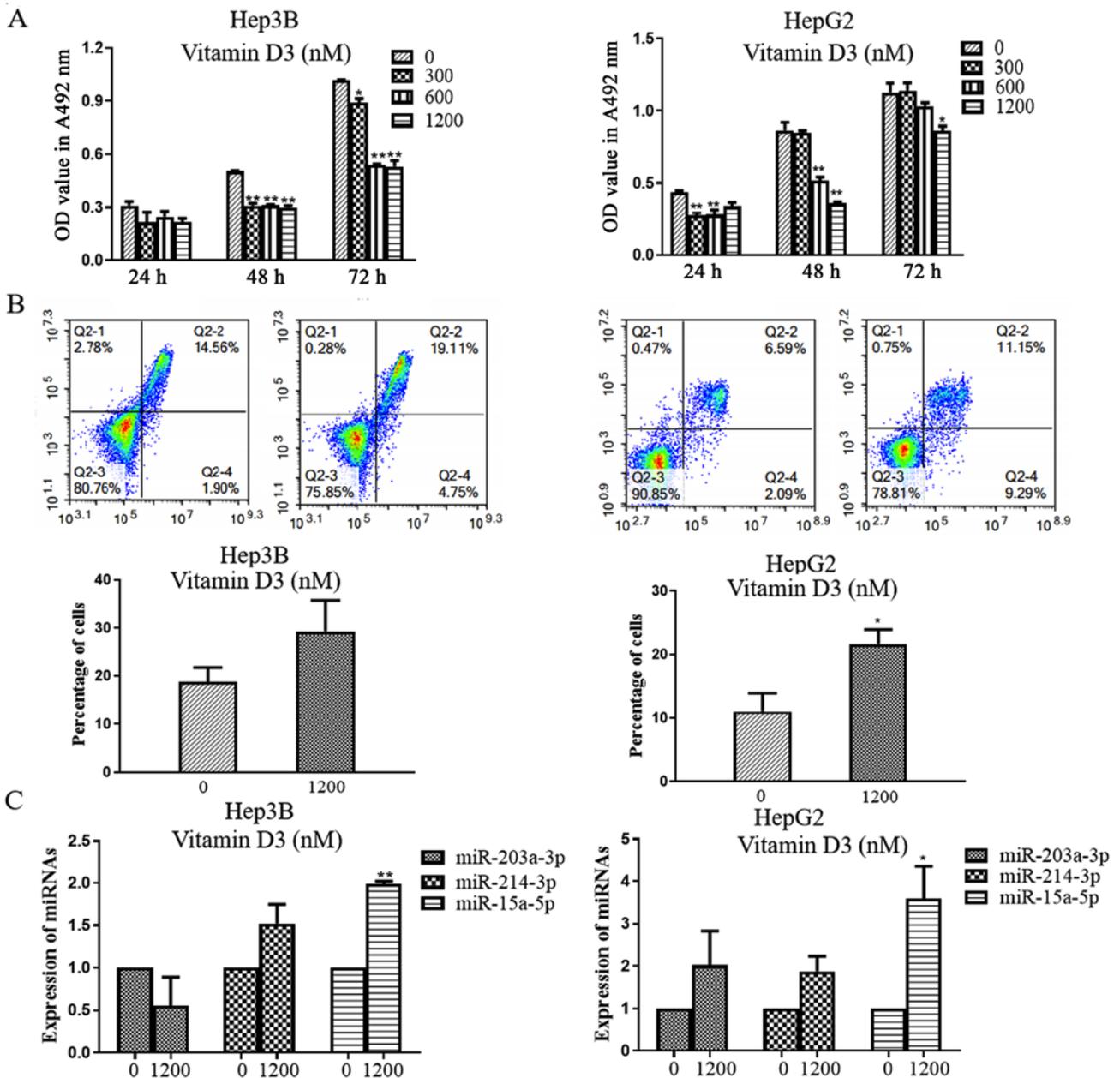


Figure 1. Vitamin D3 inhibits liver cancer cell proliferation. (A) Hep3B and HepG2 cells were treated with vitamin D3 for 24, 48 or 72 h prior to the MTT assay. (B) Annexin V assay of the effects 1,200 nM vitamin D3 on the apoptosis of Hep3B and HepG2 cells determined by flow cytometry. (C) miR-15a-5p, miR-203a-3p and miR-214-3p expression assayed by reverse transcription-quantitative PCR. *P<0.05 and **P<0.01 vs. mimics-ctrl. miR, microRNA.

cells with the miR-15a-5p mimic. The expression of miR-15a-5p in Hep3B and HepG2 cells transfected with the miR-15a-5p mimic was significantly higher compared with that in cells transfected with the mimic-ctrl (Fig. 2A). The MTT and colony formation assays revealed significant proliferation inhibition following transfection with the miR-15a-5p mimic compared with mimic-ctrl. No significant differences were observed between the miR-15a-5p mimic and mimic-ctrl in Hep3B cells at 72 h (Fig. 2B and C). Bcl-2 was downregulated and Bax was upregulated in the miR-15a-5p mimic-transfected Hep3B and HepG2 cells compared with the mimic-ctrl-transfected cells (Fig. 2D). These findings demonstrated that miR-15a-5p was downregulated and may serve a role in suppressing cell proliferation and inducing apoptosis in liver cancer cells.

Inhibition of miR-15a-5p contributes to carcinogenesis in liver cancer cells. miR-15a-5p expression was decreased in HepG2 cells transfected with an miR-15a-5p inhibitor compared with that in the inhibitor control group (Fig. 3A). The results from MTT assay revealed that vitamin D3 inhibited Hep3B and HepG2 cell proliferation, and that the inhibitor could partly rescue the inhibiting effect of vitamin D3, however, the difference was not statistically significant (Fig. S1). The proliferation and colony formation of Hep3B and HepG2 cells transfected with the miR-15a-5p inhibitor were increased compared with the inhibitor control-transfected cells (Fig. 3B and C). Bcl-2 protein expression was increased in Hep3B and HepG2 cells transfected with the miR-15a-5p inhibitor, compared with cells transfected with the inhibitor control (Fig. 3D).

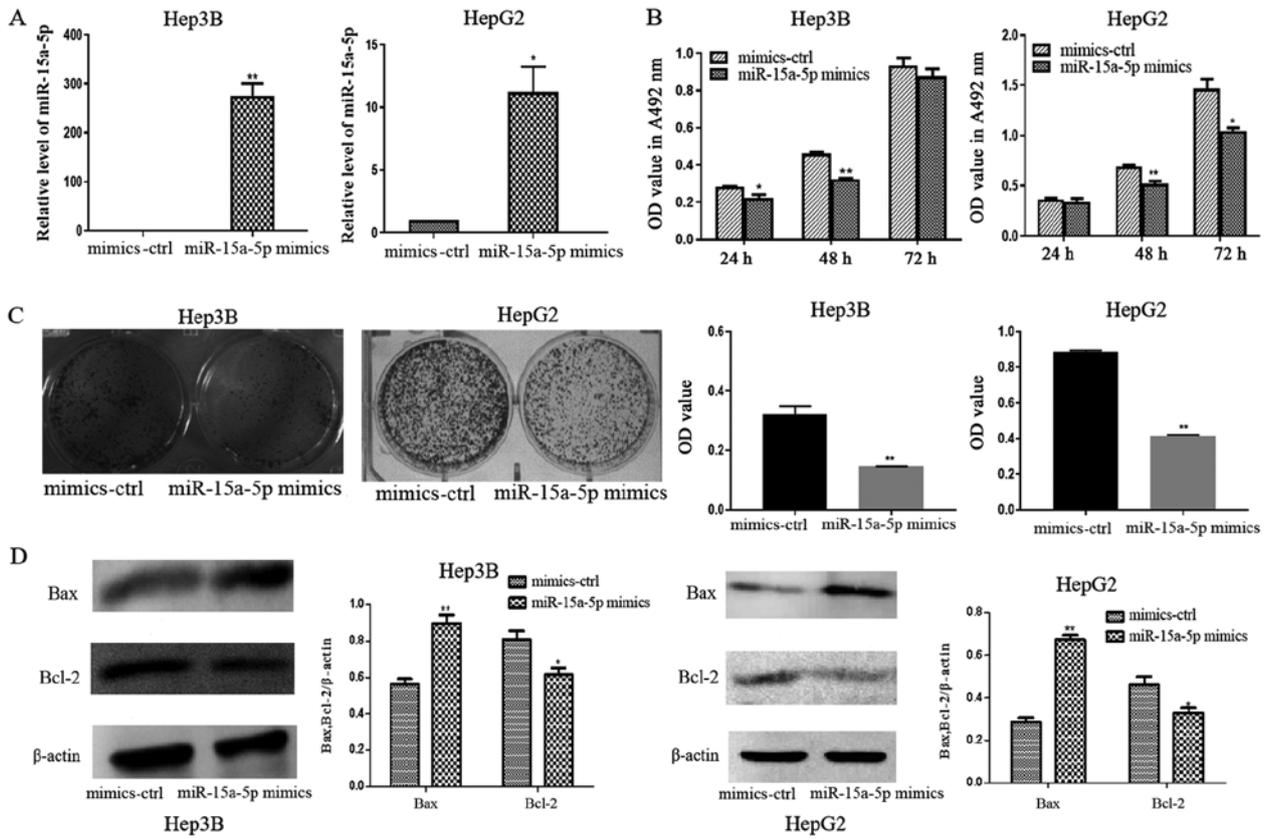


Figure 2. miR-15a-5p inhibits proliferation of liver cancer cells. (A) miR-15a-5p expression in cells transfected with miR-15a-5p mimics and mimic controls. (B) Proliferation of Hep3B and HepG2 cells 24, 48 and 72 h after transfection with the miR-15a-5p mimics or mimics-ctrl. (C) Colony formation of Hep3B and HepG2 cells after miR-15a-5p mimic or control transfection. (D) Western blotting of Bcl-2, Bax and β-actin expression. *P<0.05 and **P<0.01 vs. mimics-ctrl. miR, microRNA; mimics-ctrl, mimic control; OD, optical density.

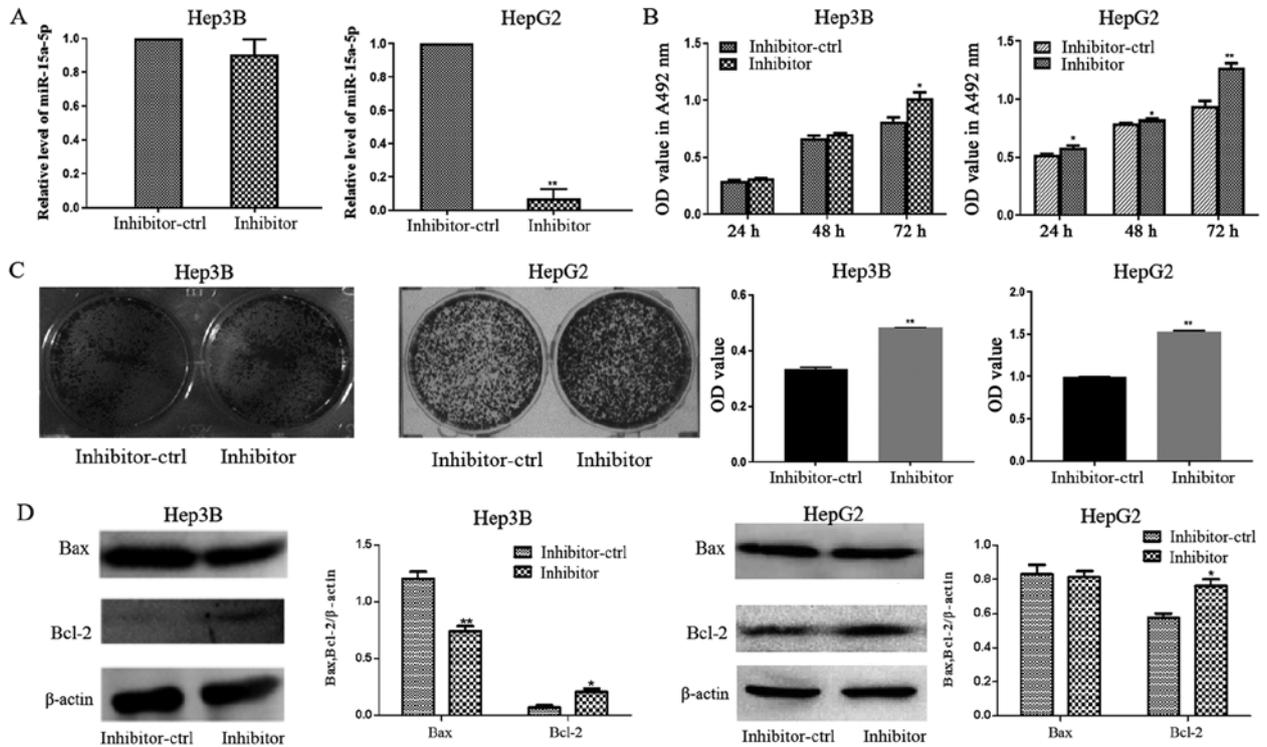


Figure 3. miR-15a-5p inhibition promotes liver cancer cell proliferation. (A) Reverse transcription-quantitative PCR of miR-15a-5p expression in Hep3B and HepG2 cells transfected with miR-15a-5p inhibitor compared with inhibitor-ctrl. (B) MTT assay of cell proliferation 24, 48 and 72 h after transfection with miR-15a-5p inhibitor or inhibitor-ctrl. (C) Colony formation after transfection with miR-15a-5p inhibitor or inhibitor-ctrl. (D) Western blotting of Bcl-2, Bax and β-actin expression. *P<0.05 and **P<0.01 vs. inhibitor-ctrl. Inhibitor-ctrl, inhibitor-control.

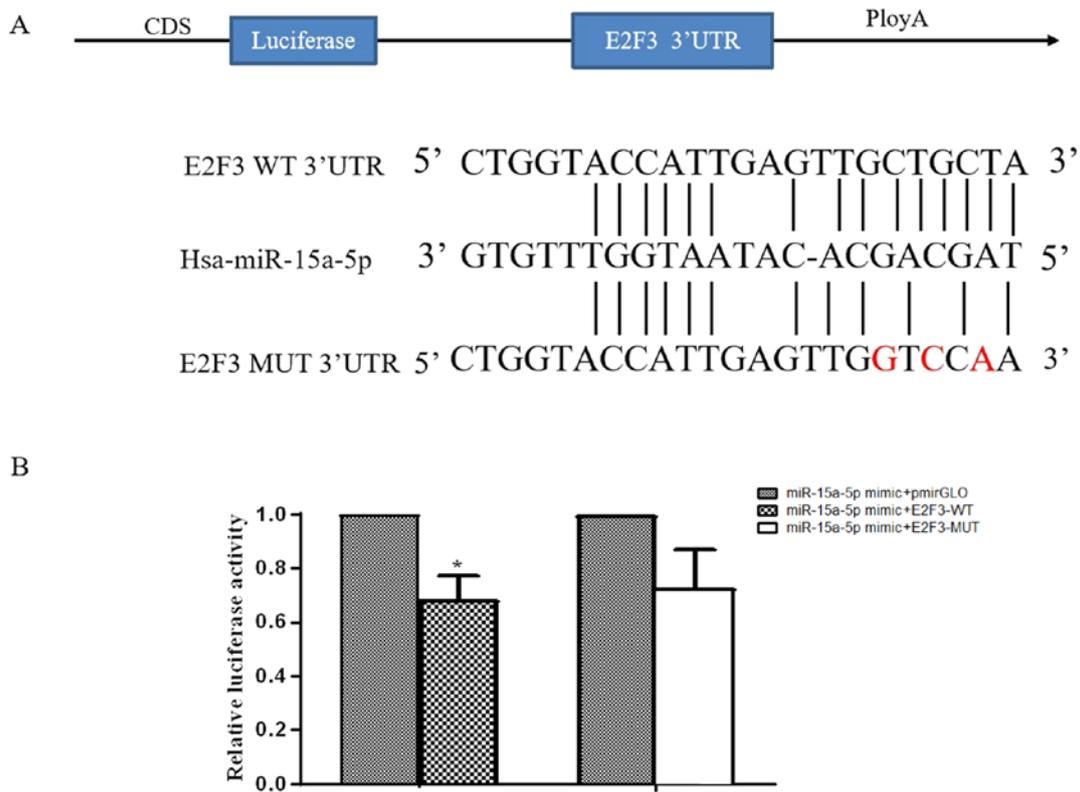


Figure 4. E2F3 is the target of miR-15a-5p. (A) The binding sites of miR-15a-5p in the 3'-UTR of human E2F3. (B) miR-15a-5p mimic was co-transfected with E2F3-WT, E2F3-MUT or the pmirGLO vector in 293 cells. Luciferase activity was significantly reduced in cell transfected with the E2F3-WT vector compared with the controls. *P<0.05 vs. pmirGLO. UTR, untranslated region; WT, wild-type; MUT, mutant; E2F3, E2F transcription factor 3.

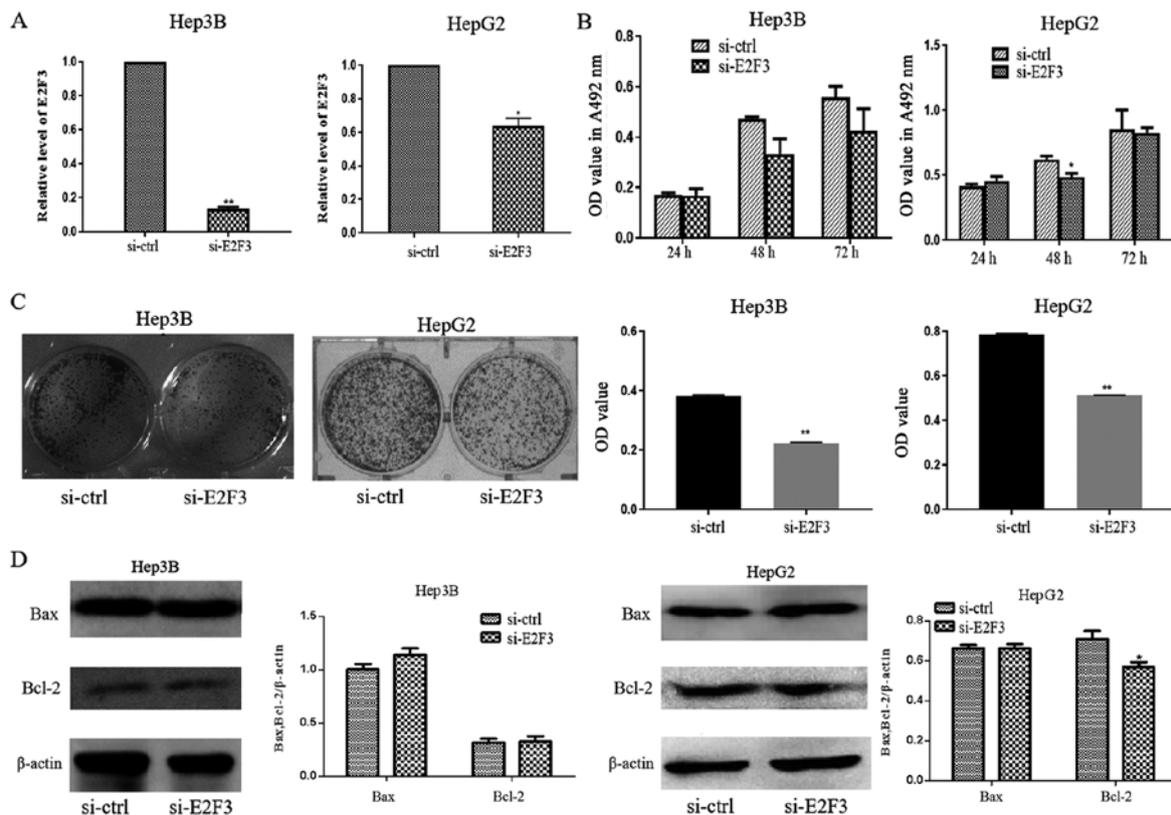


Figure 5. Silencing E2F3 regulates liver cell proliferation. (A) E2F3 expression was assayed in Hep3B and HepG2 cells transfected with si-E2F3 or the si-ctrl by reverse transcription-quantitative PCR. (B) MTT assay of Hep3B cells at 24, 48 and 72 h after transfection with si-E2F3 or si-ctrl. (C) Colony formation of Hep3B and HepG2 cells transfected with si-E2F3 or si-ctrl. (D) Western blotting of Hep3B and HepG2 cells transfected with si-E2F3 or si-ctrl. *P<0.05 and **P<0.01 vs. si-ctrl. si, small interfering; si-ctrl, small interfering-control; E2F3, E2F transcription factor 3.

E2F3 is a target of miR-15a-5p. The 3'-UTR of E2F3 contained a potential miR-15a-5p binding site (Fig. 4A). E2F3 expression has been demonstrated to be upregulated in various types of cancer (21,22); thus, E2F3 was selected as a candidate miR-15a-5p target gene. A luciferase reporter assay was performed in 293 cells co-transfected with miR-15a-5p mimics and E2F3-WT or -MUT vectors, or the pmirGLO vector. Luciferase activity was significantly reduced in cells co-transfected with the miR-15a-5p mimics and E2F3-WT vector compared with co-transfected with the miR-15a-5p mimics and pmirGLO vector (Fig. 4B), which suggested that miR-15a-5p directly targeted E2F3.

Silencing E2F3 suppresses liver cancer cell proliferation. E2F3 mRNA expression was significantly decreased in si-E2F3-transfected cells compared with si-control (ctrl)-transfected cells (Fig. 5A). Additionally, MTT assay results demonstrated that cell proliferation was decreased at 48 h in si-E2F3-transfected HepG2 cells compared with si-ctrl transfected HepG2 cells (Fig. 5B). Hep3B and HepG2 cells transfected with si-E2F3 formed fewer colonies compared with those of si-ctrl-transfected cells (Fig. 5C). Bcl-2 was downregulated in si-E2F3-transfected HepG2 cells (Fig. 5D). These results indicated that miR-15a-5p may function as a tumor suppressor by directly targeting E2F3 transcription and that vitamin D3 may suppress the proliferation of liver cancer cells by regulating the miR-15a-5p/E2F3 axis.

Discussion

Vitamin D is a fat-soluble steroid hormone; physiological events associated with the activation of vitamin D signaling may influence the prevention and treatment of various types of cancer (23). Mediation of the anticancer activity of vitamin D by changes in miRNA expression has been demonstrated both *in vitro* and *in vivo* (24,25). In the present study, vitamin D3, the active form of vitamin D, suppressed the proliferation and induced apoptosis in liver cancer cells, which was consistent with its activity as a tumor suppressor against the development of liver cancer. Deregulation of miRNAs, including miR-15a-5p, has been reported in various types of cancer (18-27). In the present study, MTT assays in Hep3B and HepG2 cells demonstrated significant proliferation inhibition following transfection with the miR-15a-5p mimic compared with that in the controls. The results suggested that miR-15a-5p functioned as a tumor suppressor gene and that vitamin D3 may exert its anticancer effects by increasing miR-15a-5p expression in liver cancer cells.

miRNAs interact with 3'-UTRs to inhibit the expression of the target mRNA (28). In the present study, Bioinformatics analysis revealed that the 3'-UTR of E2F3 contained a potential miRNA-15a-5p binding site. The E2F family of transcription factors regulates the cell cycle, proliferation and apoptosis (29,30). E2F3 has been reported to be involved in various types of human cancer, including nasopharyngeal carcinoma (31), hepatocellular carcinoma (32) and bladder cancer (33). It was demonstrated that E2F3 is upregulated in HCC compared with normal controls, and that overexpression of E2F3 could be associated with a poor prognosis in HCC (34). The 3'-UTR of E2F3 mRNA includes a number

of miRNA seed sequences; in the present study, the dual luciferase reporter assay revealed that E2F3 was a target gene of miR-15a-5p. siRNA silenced E2F3 expression, and E2F3 silencing suppressed the proliferation, induced apoptosis and decreased colony formation in liver cancer cells.

In conclusion, the results of the present study provided novel evidence for the inhibition of Hep3B and HepG2 cell proliferation by vitamin D3 and the molecular mechanisms involved. These results provide a new therapeutic rationale and target for the early diagnosis and treatment of liver cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JW conceived the study. YL and SC performed the experiments. QL and RZ analyzed the data. YL wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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