

Downregulation of the expression of HDGF attenuates malignant biological behaviors of hilar cholangiocarcinoma cells

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Abstract. Hepatoma-derived growth factor (HDGF) has been reported to be a potential predictive and prognostic marker for several types of cancer and important in malignant biological behaviors. However, its role in human hilar cholangiocarcinoma remains to be elucidated. Our previous study demonstrated that high expression levels of HDGF in hilar cholangiocarcinoma tissues correlates with tumor progression and patient outcome. The present study aimed to elucidate the detailed functions of the HDGF protein. This was performed by downregulating the protein expression of HDGF in the FRH0201 hilar cholangiocarcinoma cell line by RNA interference (RNAi) *in vitro*, and revealed that downregulation of the HDGF protein significantly inhibited the malignant biological behavior of the FRH0201 cells. In addition, further investigation revealed that downregulation of the protein expression of HDGF significantly decreased the secretion of vascular endothelial growth factor, which may be the mechanism partially responsible for the inhibition of malignant biological behaviors. These findings demonstrated that HDGF is important in promoting malignant biological behaviors, including proliferation, migration and invasion of hilar cholangiocarcinoma FRH0201 cells. Inhibition of the expression of HDGF downregulated the malignant biological behaviors, suggesting that downregulation of the protein expression of HDGF by RNAi may be a novel therapeutic approach to inhibit the progression of hilar cholangiocarcinoma.

Introduction

Hilar cholangiocarcinoma is a life threatening malignancy, which is difficult to diagnose and is associated with high mortality rates (1). Surgery, either in the form of liver resection or liver transplantation, is the only effective curative therapy for hilar cholangiocarcinoma (2,3) and only 50-70% of the patients who undergo surgery are candidates for curative resection (4), however, the recurrence rates remain high even following curative resection (5). Adjuvant therapy, including chemotherapy and radiation therapy, has not been confirmed to reduce the risk of recurrence (6). Therefore, identifying novel biomarkers, which are involved in tumor development and progression may assist in improving therapeutic strategies and patient outcomes.

Hepatoma-derived growth factor (HDGF), as a member of the heparin-binding growth factors, was originally purified from cultured media with the HuH-7 human hepatoma cell line (7,8). Previous studies have demonstrated that HDGF is upregulated in various types of malignancy and is predictive of a poor survival outcome, and that HDGF possesses aggressive biological behaviors *in vitro*, including proliferation, migration, invasiveness and angiogenesis (9-12). However, the correlation between the malignant behaviors and HDGF in the hilar cholangiocarcinoma cell line remains to be fully elucidated.

The present study was designed to clarify the malignant behaviors of the FRH0201 human hilar cholangiocarcinoma cell line by using small interfering (si)RNA *in vitro*, and aimed to provide novel experimental evidence for the targeted therapy of human hilar cholangiocarcinoma.

Materials and methods

Cell lines and cell culture. The human FRH0201 hilar cholangiocarcinoma cell line was provided by Professor Xiaopeng Wu (Qilu Hospital of Shandong University, Jinan, China). The cells were incubated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA), at 37°C with 5% CO₂.

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siRNA transfection. The sequence of the siRNA targeting HDGF (5'-AACCGGCAGAAGGAGUACAAA-3') used was adopted from a previous study (10). The HDGF siRNA and negative control siRNA were chemically synthesized by GenePharma Co., Ltd. (Shanghai, China). The corresponding cells were divided as follows: Control, negative-siRNA and HDGF-siRNA group. *In vitro* transfections were performed using Lipofectamine RNAiMax (Invitrogen Life Technologies), according to the manufacturer's instructions. All siRNAs were dissolved in sterilized and RNase-free water at a final concentration of 20 mM. Briefly, the FRH0201 cells were seeded in a six-well plate at a density of 5×10^5 cells/well at 37°C with 5% CO₂ and were incubated until they reached 80% confluence. The cells were incubated in the siRNA-Lipofectamine complex-containing medium for 6 h, following which the medium was replaced with DMEM containing 10% FBS at 37°C with 5% CO₂. The cells were incubated for 48 h and were then harvested for analysis of the mRNA and protein expression levels of HDGF.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was extracted from the FRH0201 cells in 96-well plates (seeded at a density of 2×10^3 /200 μ l) using TRIzol reagent (Takara Bio, Inc., Tokyo, Japan). The quantity of total RNA was 2 μ g as per the instructions. First strand cDNA was synthesized from the mRNA using a PrimescriptTM RT reagent kit (Takara, Bio., Inc.), and the RT-qPCR was performed using a SYBR Green PCR kit (Takara, Bio., Inc.) on a Light Cycler system 2.0 (Roche Diagnostics, Mannheim, Germany). The sequence of the primers (GenePharma Co., Ltd., Shanghai, China) used were as follows: HDGF, sense 5'-CAGCCAACAAATACCAAGTCT-3' and antisense 5'-GTTCTCGATCTCCCACAGC-3', and GAPDH, sense 5'-GGTGGTCTCCTCTGACTTCAACA-3' and antisense 5'-GTTGCTGTAGCCAAATTCGTTGT-3'. The PCR conditions were as follows: Initial denaturation at 95°C for 10 sec, followed by 45 cycles at 95°C for 5 sec, 60°C for 30 sec and 72°C for 10 sec. The comparative threshold cycle (Ct) method ($2^{-\Delta\Delta C_t}$) was used to analyze the relative changes in gene expression and the levels were normalized against GAPDH (13). The experiment was repeated twice with triplicate measurements in each experiment.

Western blot analysis. All the grouped cells were harvested and rinsed twice with phosphate-buffered saline (PBS). The total cellular protein was extracted using a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology, Shanghai, China). Following extraction, the protein concentration was measured using an Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology), and an equal quantity of protein (30 μ g) from each group was subjected to 10% SDS-PAGE (Beyotime Institute of Biotechnology). The proteins were subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Following blocking with Tris-buffered saline with Tween-20 (pH 7.6; Beyotime Institute of Biotechnology), containing 5% non-fat milk for 2 h at room temperature, the PVDF membranes were incubated with rabbit anti-human HDGF polyclonal antibody (1:100; Proteintech Group, Inc., Chicago, USA) and rabbit anti-human β -actin monoclonal antibody

(1:1,000; Santa Cruz Biochemistry, Inc., Santa Cruz, CA, USA) overnight at 4°C. The membranes were subsequently incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:1,000; Beyotime Institute of Biotechnology) at room temperature. The immunoreactive bands were visualized using a Chemiluminescent ECL Detection system (EMD Millipore), according to the manufacturer's instructions. The intensity of each band was quantified using ImageJ software version 1.43 (National Institutes of Health, Bethesda, MD, USA). The experiment was repeated twice with triplicate measurements in each experiment.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Prior to siRNA transfection, 10^4 cells were seeded into a 96-well plate (200 μ l/well), with three wells for each group. At 48 h post-transfection, MTT (5 mg/ml; 20 μ l/well; Beyotime Institute of Biotechnology) was added and the cells were cultured at 37°C for 4 h. Following discarding of the supernatant, the cells were mixed with dimethyl sulfoxide (150 μ l/well; Beyotime Institute of Biotechnology) for 10 min. The absorbance of each well was measured at 570 nm (A570) using an ultraviolet spectrophotometer, UV9100 (LabTech, Inc., Beijing, China) and the cell proliferation rate was calculated.

Wound healing migration assay. Briefly, the cells in each group were seeded into a 12-well plate, at equal densities, in complete medium and were incubated at 37°C with 5% CO₂ until the cells had grown to 80% confluence. Scratching wounds of an identical width were created in the monolayer using a sterile pipette tip. The wells were rinsed with PBS three times to remove floating cells and debris, and the remaining cells were cultured in serum-free DMEM, with the culture plates incubated at 37°C and in 5% CO₂. Following incubation, wound healing was measured and images were captured with a light microscope Olympus IX81 (Olympus, Tokyo, Japan) at 0, 8 and 16 h. The experiment was repeated twice with triplicate measurements in each experiment.

Transwell invasion assay and migration assay. The invasive capability of tumor cells was determined using Matrigel-coated Transwell invasion chambers (8 μ m pore size; BD Biosciences, Bedford, MA, USA). At 48 h post-siRNA treatment, the cells in the groups were collected and 10^5 cells from each group were added to upper Transwell chambers in 100 μ l serum-free medium. The lower chamber was filled with 500 μ l DMEM, containing 10% FBS. Following incubation at 37°C for 24 h, the cells that had invaded through the membrane were fixed with methanol for 10 min, stained with Trypan blue (Beyotime Institute of Biotechnology) for 10 min and counted under a light microscope Olympus IX81 (Olympus). The migration assay were performed in a similar manner, using a Transwell chamber without Matrigel, and incubation conditions were 37°C for 16 h. Triplicate measurements were performed in each experiment.

Enzyme-linked immunosorbent assay (ELISA). The cells from all the groups were seeded into six-well plates (5×10^5 cells/well) and cultured in serum-free DMEM for 24 h at 37°C with 5% CO₂. The supernatant was collected, centrifuged at 1,000 \times g for 15 min at 4°C, filtered through a 0.22 μ m filter (EMD Millipore) and stored at -80°C until use. Additionally, the

supernatant levels of vascular endothelial growth factor (VEGF) were detected using a VEGF ELISA kit (Pierce Biotechnology, Rockford, IL, USA), to recognize VEGF165 and VEGF121. The ELISA was performed, according to the manufacturer's instructions. The concentrations of VEGF in the supernatant was measured in duplicate.

Statistical analysis. All results are presented as the mean \pm standard deviation. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). The differences between groups were assessed using Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HDGF siRNA effectively suppresses the mRNA and protein expression levels of HDGF. siRNA targeting HDGF was used to decrease the mRNA expression levels of HDGF in the FRH0201 cells. RT-qPCR revealed that the mRNA expression of HDGF was markedly decreased to 25.3% by the HDGF siRNA at 48 h post-transfection, compared with the control group ($P < 0.01$; Fig. 1A). Similarly, western blot analysis demonstrated that the protein expression of HDGF was inhibited to 34.9% of that observed in the control group ($P < 0.01$; Fig. 1B and C). This confirmed that HDGF siRNA effectively inhibited the expression of its target gene, HDGF, at the transcriptional and translational levels.

Downregulation of the expression of HDGF decreases the proliferative ability of FRH0201 cells. The results of the MTT assay revealed that the proliferative ability of the FRH0201 cells treated with HDGF siRNA was 46.0% of that observed in the control cells 48 h post-transfection ($P < 0.01$; Fig. 1D).

Downregulation of the expression of HDGF inhibits the migratory ability of FRH0201 cells. In the present study, a cell wound healing migration assay and a Transwell migration assay were used to detect the migratory ability of tumor cells *in vitro*. In the cell wound healing migration assay, no statistical difference was observed between the groups, although the migratory ability was decreased 8 h post-transfection with HDGF siRNA. By contrast, the migratory ability of the HDGF siRNA group was significantly decreased at 16 h post-transfection, and was 41.7% of that observed in the control cells ($P < 0.05$; Fig. 2). In addition, the Transwell migration assay confirmed in, three-dimensional culture medium, that the migration of the cells transfected with HDGF siRNA was inhibited and the number of migrating cells was 26.5% of that observed in the control cells ($P < 0.01$; Fig. 3).

Downregulation of the expression levels of HDGF inhibits the invasive ability of FRH0201 cells. The Transwell invasion assay revealed that the invasive ability of the cells transfected with HDGF siRNA was inhibited. The number of invading cells was 22.7% of that observed in the control cells ($P < 0.01$; Fig. 3).

Downregulation of the protein expression of HDGF inhibits the secretion of VEGF in FRH0201 cells. The levels of VEGF, which were detected in the supernatants of the different

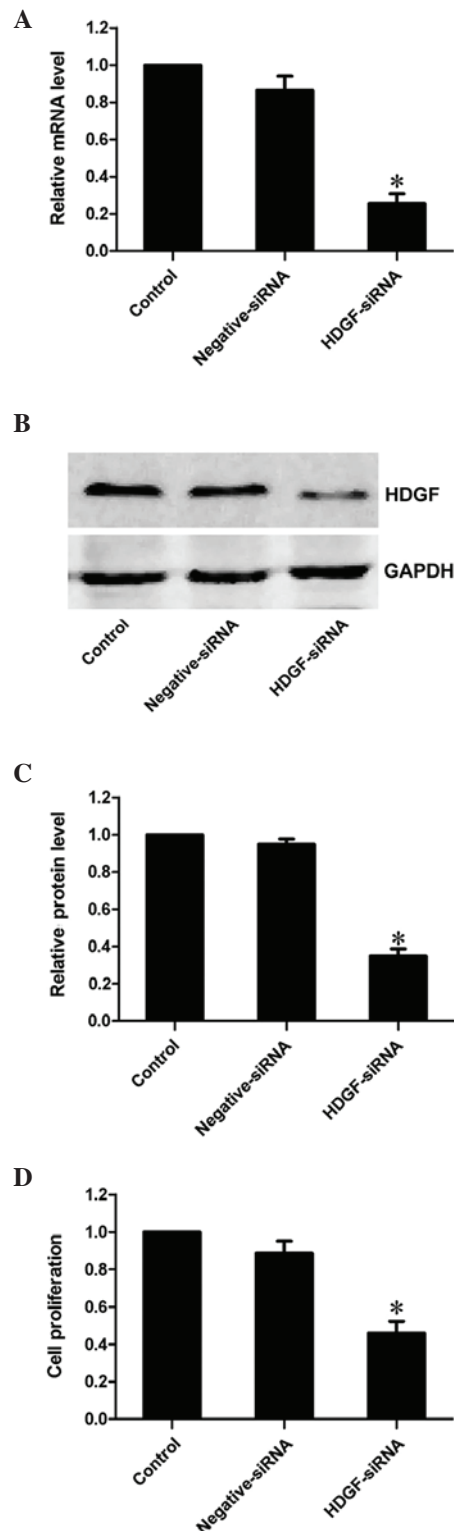


Figure 1. Following siRNA transfection into hilar cholangiocarcinoma cells, the changes in the mRNA and protein expression levels of HDGF were recorded. (A) Results of the reverse transcription-quantitative polymerase chain reaction analysis revealed that the mRNA expressions levels of HDGF were markedly decreased to 25.3% by transfection with HDGF siRNA at 48 h post-transfection, compared with the control group ($P < 0.01$). (B and C) Results of the western blot analysis revealed that the protein expression of HDGF was inhibited to 34.9% of that observed in the control group ($P < 0.01$). (D) A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay revealed that the proliferative ability of the FRH0201 cells transfected with HDGF siRNA was only 46.0% of that observed in the control cells 48 h after transfection ($P < 0.01$). Data are presented as the mean \pm standard deviation. HDGF, hepatoma-derived growth factor; siRNA, small interfering RNA.

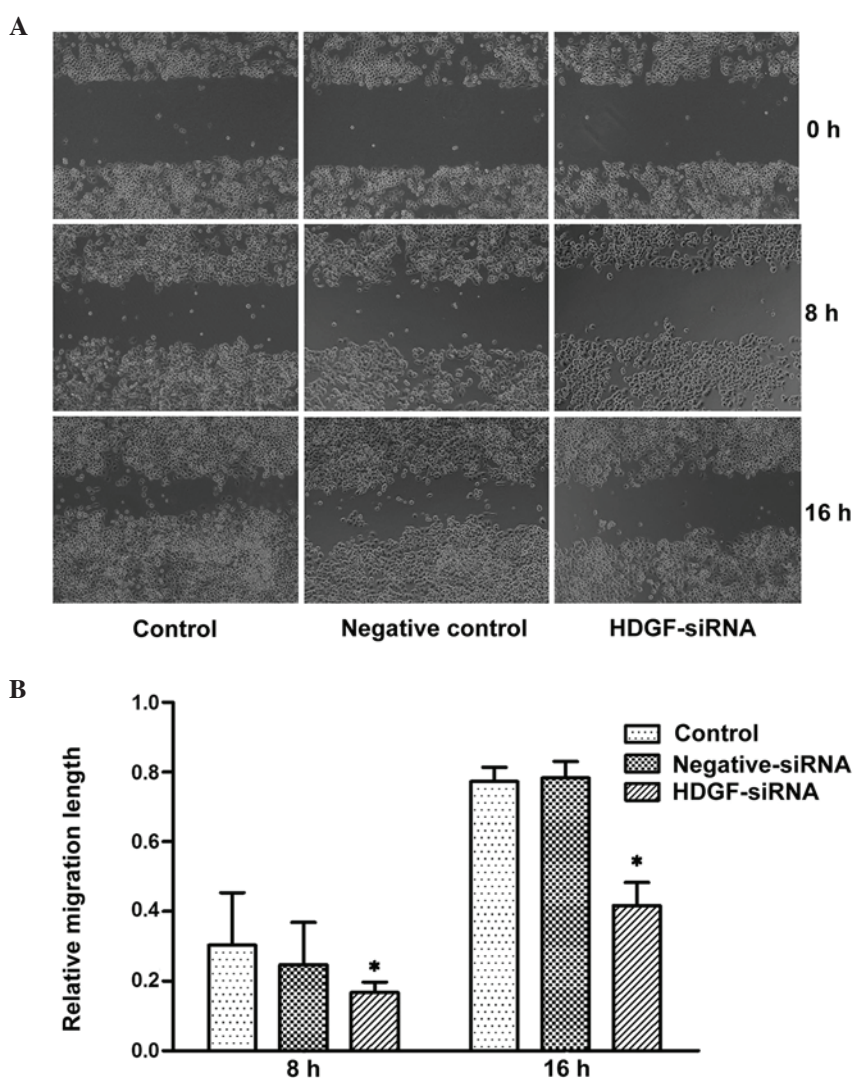


Figure 2. A wound healing migration assay was performed to determine the migratory ability of the cells prior to and following transfection with HDGF siRNA. (A) Representative images of the wound healing assay (magnification, x200). (B) No significant differences were observed between the migration of the cell groups at 8 h post-HDGF siRNA transfection. However, the migration ability of the HDGF siRNA group decreased significantly at 16 h post-transfection and was 41.7% of that observed in the control cells (* $P < 0.05$). Data are presented as the mean \pm standard deviation. HDGF, hepatoma-derived growth factor; siRNA, small interfering RNA.

groups revealed that the secretion of VEGF was significantly inhibited by HDGF siRNA, the level of which was 66.51% of that observed in the control supernatant ($P < 0.001$; Fig. 4).

Discussion

The potential capacities of malignant tumors, including sustaining proliferative signaling, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis, make its therapeutic strategies more complicated and difficult (14). Therefore, identifying and screening potential molecular targets or tumor biomarkers involved in one or more malignant processes is beneficial for tumor diagnosis and therapy, and for improving patient survival rates and prognosis.

As a nuclear-targeted mitogen, HDGF has mitogenic activity for various cells following translocation into the nucleus, including hepatic, gastric, lung cancer cells, fibroblasts, endothelial cells, smooth muscle cells and neuronal cells (7,15-20). The downregulation of HDGF suppresses

cancer cell growth and invasion, induces apoptosis, and may function as a tumor survival factor (21,22). Previously, a systematic proteomic investigation of human metastatic hepatocellular carcinoma (HCC) cell lines demonstrated that the HDGF protein was one of the metastasis-associated proteins, and that knockdown of HDGF induced cell apoptosis in metastatic HCC cells (23). Therefore, HDGF may act as an oncogene, being important in tumor pathogenesis and progression, and may be a key target for future therapy. However, the roles of HDGF protein in the malignant biological behaviors of the human hilar cholangiocarcinoma cell line, including proliferation, migration, invasion and angiogenesis remain to be elucidated.

Tumorigenesis and progression is the result of cell proliferation and apoptosis in a condition of disequilibrium (24). Several studies have indicated that, following nuclear translocation, HDGF promotes the proliferation of numerous tumor cells, whereas HDGF interference, knockdown or neutralizing antibodies significantly increase the rate of apoptosis

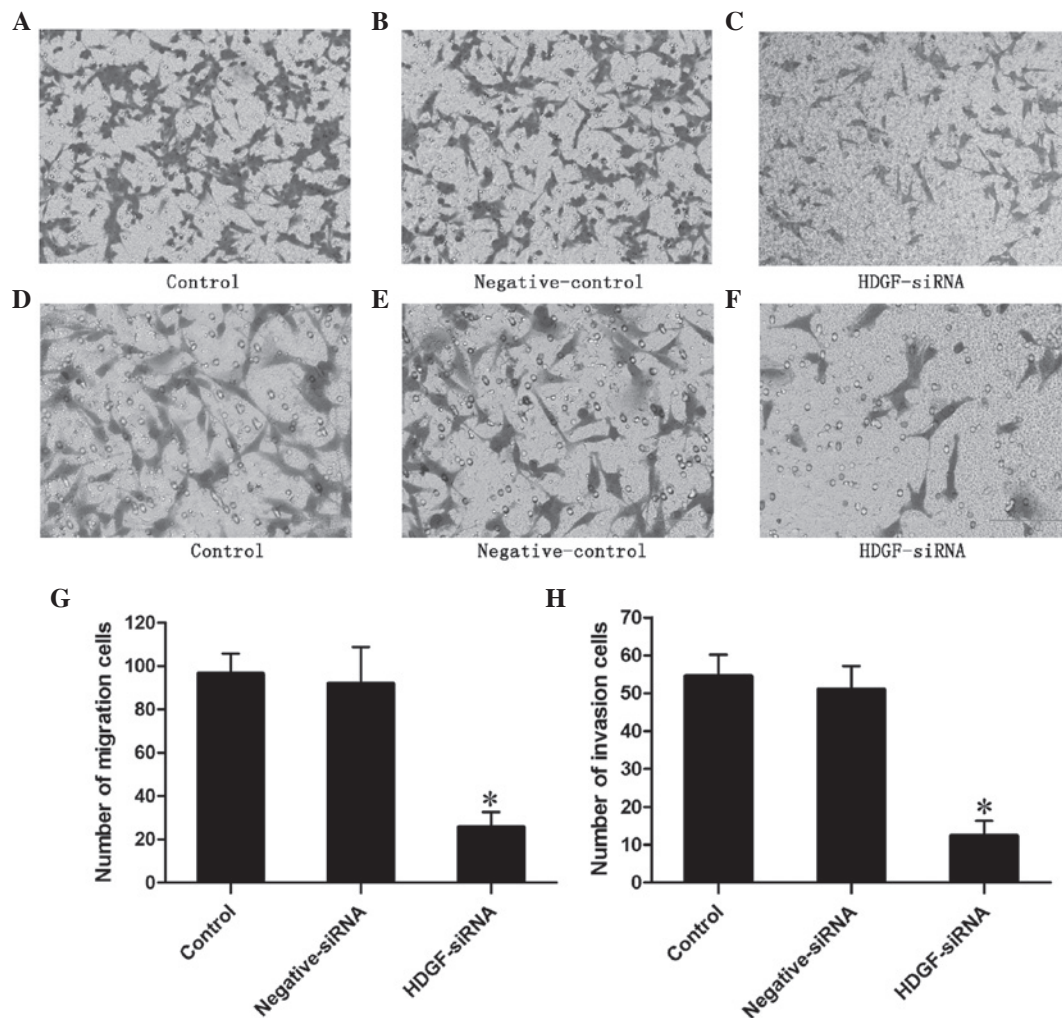


Figure 3. Downregulation of the expression of HDGF inhibits the migration and invasive abilities of the FRH0201 cells. (A-C) Representative images of the Transwell migration assay. (A) Control FRH0201 cells; (B) negative-siRNA FRH0201 cells; (C) HDGF-siRNA FRH0201 cells. (D-F) Representative images of the Transwell invasion assay (D) Control FRH0201 cells; (E) negative-siRNA FRH0201 cells; (F) HDGF-siRNA FRH0201 cells. (G) Transwell migration assay in three-dimensional culture medium demonstrated that the migration of the cells transfected with HDGF siRNA was inhibited, being 26.5% of that observed in the control cells ($P < 0.01$). (H) Transwell invasion assay revealed that the invasive ability of the cells transfected with HDGF siRNA was inhibited, being 22.7% of that observed in the control cells ($P < 0.01$). Data are presented as the mean \pm standard deviation. HDGF, hepatoma-derived growth factor; siRNA, small interfering RNA.

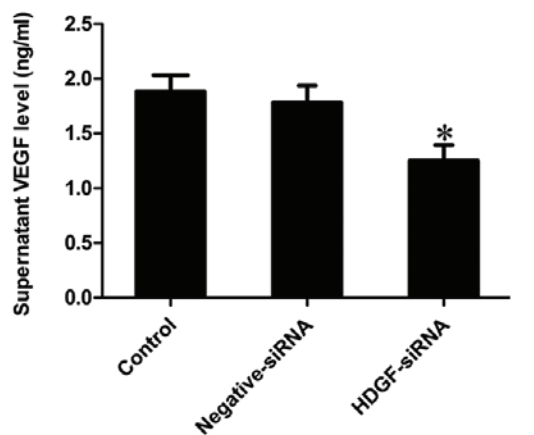


Figure 4. Levels of VEGF in the supernatants revealed that the secretion of VEGF was significantly inhibited by HDGF siRNA, with levels being 66.51% of those observed in the control supernatant ($P < 0.001$). Data are presented as the mean \pm standard deviation. HDGF, hepatoma-derived growth factor; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.

and inhibit proliferation (25-27). The results of the present study demonstrated that, following downregulation of the HDGF protein by HDGF siRNA, the proliferative capacity of the FRH0201 cells was significantly decreased.

Tumor cell migration, invasion and metastasis is a complicated process involving multiple steps and factors (28). Although primary tumors can be effectively controlled or cured with surgery, radiotherapy, chemotherapy and other local therapies, these methods fail to manipulate disease progression for disseminated tumors, which is an important cause of malignancy-associated mortality (29). Therefore, inhibiting the process of tumor cell migration and invasion is an important therapeutic strategy. Previous studies have demonstrated that knockdown of the expression of HDGF inhibits tumor cell migration and invasion in hepatic, lung and prostate carcinoma (12,21,30). Our previous study demonstrated that tumor tissues with high expression levels of HDGF exhibit increased invasive abilities, compared with tissues expressing a low level HDGF. Similarly, patients with

increased expression of HDGF have been observed to have a significantly poorer outcome, compared with those exhibiting low expression levels of HDGF (31). In the present study it was confirmed that inhibition of the protein expression of HDGF significantly decreased the migration and invasion of FRH0201 cells.

It is generally accepted that tumor growth, invasion and metastasis require angiogenesis, and that vascularization is closely associated with tumor invasion and patient prognosis (32). HDGF, a member of the heparin-binding growth factor family, exhibits a wide range of biological functions and can promote tumor neovascular formation and development as a potential endothelial mitogen (19,33). Previous studies have demonstrated that high expression levels of HDGF activate the extracellular signal-regulated kinase pathway and upregulate the secretion of VEGF (34-36). In addition, another study revealed that HDGF is a potent endothelial mitogen *in vivo* and regulates endothelial cell migration by mechanisms distinct from VEGF (11). Okuda *et al* (37) reported that HDGF-induced tumor formation and angiogenesis *in vivo* involves the direct angiogenic activity and induction of VEGF secretion. In the present study downregulation of the expression of HDGF decreased the secretion of VEGF, which demonstrated that HDGF may induce angiogenesis partially by the action of VEGF.

In conclusion, the present study demonstrated that HDGF was important in promoting the malignant biological behaviors (proliferation, migration and invasion) of the FRH0201 hilar cholangiocarcinoma cell line, and inhibition of the expression of HDGF downregulated the malignant biological behaviors. These results provide novel insights and indicate the potential clinical use of HDGF as an effective therapeutic target for hilar cholangiocarcinoma by inhibiting proliferation, migration and invasion of cancer cells. Further investigation of the mechanism underlying the action of HDGF in hilar cholangiocarcinoma is required.

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