

The siRNA silencing of DcR3 expression induces Fas ligand-mediated apoptosis in HepG2 cells

TUANJIE ZHAO, YINGCHEN XU, SHULIN REN, CHAOJIE LIANG, XIAONA ZHOU and JIXIANG WU

Department of General Surgery, Beijing Tongren Hospital, Capital Medical University, Beijing 100730, P.R. China

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Abstract. Dysfunctional Fas ligand (FasL) may inhibit the apoptosis of tumor cells. FasL contains two receptors, Fas and Decoy Receptor 3 (DcR3). DcR3 competitively binds to FasL over Fas, resulting in the inhibition of FasL-mediated apoptosis. Therefore, it was suggested that the downregulation of DcR3 expression enhances FasL-mediated apoptosis. In the current study, the expression of DcR3 was silenced in liver cancer HepG2 cells in order to study the effect of FasL on HepG2 cell activity and invasiveness. DcR3 siRNA knock-down HepG2 cells (KD), DcR3 blank plasmid control HepG2 cells and wild-type HepG2 cells (WT) were treated with FasL (10 ng/ml). Flow cytometry was used to detect changes in the cell cycle and apoptosis. MTS, clonogenic, wound healing and Transwell assays were performed to examine changes in cell activity, proliferation, migration and invasiveness. Reverse transcription polymerase chain reaction and western blot analysis were performed to measure the expression of DcR3, matrix metalloproteinase 9 (MMP9), vascular endothelial growth factor (VEGF)-C and VEGF-D. The results demonstrated that, compared with WT cells, the proportion of KD cells in the G2/M phase decreased following treatment with FasL. KD cells were more sensitive to FasL-induced apoptosis. Following treatment with FasL, the activity and proliferation, migration and invasion of KD cells were reduced, and the expression of MMP9, VEGF-C and VEGF-D decreased. Furthermore, it was demonstrated that DcR3 is involved in the proliferation and invasion of HepG2 cells, and this mechanism may be associated with the regulatory effect of the expression of MMP9, VEGF-C and VEGF-D; however, the exact mechanism of action remains unclear. Following DcR3 silencing, FasL-mediated apoptosis increased in HepG2 cells. Therefore, DcR3 combined with FasL may be a potential target for the treatment of liver cancer.

Introduction

Liver cancer is a growing problem in less developed countries and, according to the GLOBOCAN 2012 (1), it is the second most common cause of cancer-associated mortality worldwide. The disease burden of liver cancer is increasing markedly across the world (2). If liver cancer is diagnosed at an early stage, patients are able to undergo effective treatment via liver resection, transplantation or ablation, and the 5-year survival rate of such patients is >50% (3). However, the prognosis of patients with advanced liver cancer is very poor and the 5-year survival rate of such patients is <5% (4).

Hepatoblastoma is a highly malignant liver cancer that occurs most commonly in children (5). The 5-year overall survival rate of patients with stage IV hepatoblastoma is ~40% (6). The standard method of treating patients with hepatoblastoma is surgery combined with high dose, non-targeted chemotherapy; however, such chemotherapy induces severe side effects in patients, including ototoxicity and cardiotoxicity (7,8). Therefore, novel therapeutic strategies to treat patients with hepatoblastoma are required.

Due to advances in molecular biology, a number of tumor-associated genes have been identified, including CTNNB1 and β -Catenin in hepatoblastoma (9). Current studies focusing on gene therapy aim to develop molecular biological methods to inhibit the *in vivo* expression of such genes in order to treat patients with cancer. Studies investigating methods of treating cancer via gene therapy tend to focus on genes that induce the apoptosis of cancer cells (10-12).

The abnormal activation of apoptosis pathways may induce the onset and progression of numerous diseases, including different types of cancer. The Fas/Fas ligand (FasL) system is one such important apoptotic pathway (12). Ogasawara *et al* (13) demonstrated that the liver is more sensitive to Fas-mediated apoptosis compared with other organs. FasL, a type II membrane protein, belongs to the tumor necrosis factor (TNF) superfamily (14). Fas, which is a member of the TNF receptor (TNFR) family, is widely expressed in normal cells, including liver, kidney and heart cells, and binds to FasL (15). Fas is involved in transferring the apoptotic signal, resulting in cell apoptosis (16) and the Fas/FasL system is a key physiological regulator of programmed cell death (17). It has been demonstrated that resistance to apoptosis due to loss of Fas function may serve an important role in the pathogenesis of several malignancies (18).

Correspondence to: Professor Jixiang Wu, Department of General Surgery, Beijing Tongren Hospital, Capital Medical University, 1 Dongjiaominxiang Street, Beijing 100730, P.R. China
E-mail: wjx95@hotmail.com

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FasL contains two receptors, Fas and decoy receptor 3 (DcR3). DcR3, initially identified by Pitti *et al* (19), does not contain a death domain and so cannot transfer apoptotic signals (20). DcR3 competitively binds to FasL over Fas, thus inhibiting FasL-induced apoptosis (21) and may contribute to tumor growth in this manner (22). Furthermore, DcR3 binds to FasL and inhibits FasL-induced apoptosis. DcR3 is highly expressed in many malignant tumors, including liver cancer (23-25). It has been demonstrated that the expression of DcR3 mRNA is 60.4% in liver cancer tissues; however, it is not expressed in the adjacent normal tissues. Furthermore, DcR3 expression is associated with tumor size, clinical stage, tumor invasion and metastasis (23). Yu *et al* (26) demonstrated that decreasing DcR3 expression in SW480 colon cancer cells inhibited cell growth and metastatic ability, induced apoptosis and altered the cell cycle profile of these cells. However the exact molecular mechanism underlying the oncogenic property of DcR3 in liver cancer remains unclear.

Our previous study by the current study transfected human liver cancer HepG2 cells with lentivirus-based short hairpin RNA vector targeting DcR3 stably and indicated that loss of DcR3 impaired the growth and invasive ability of HepG2 (27). The present study also used the HepG2 cell line, which was established in 1979 and mistakenly reported as a hepatocellular carcinoma cell line (28). However, in 2009, it was demonstrated that HepG2 cells originated from hepatoblastoma, not hepatocellular carcinoma (29). The results of the current study are relevant to hepatoblastoma and liver cancer in general, however, they may not be useful regarding hepatocellular carcinoma.

Therefore, in the current study, HepG2 cells that had undergone DcR3 knockdown by small interfering (si)RNA knockdown were treated with FasL to assess the effect of FasL on HepG2 cell activity and invasive capabilities.

Materials and methods

Reagents. Reagents and kits included: Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), a prime Script RT reagent kit and SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan), Transwell inserts (Axygen; Corning Inc., Corning, NY, USA), Matrigel (BD Biosciences, San Jose, CA, USA), human recombinant FasL (R&D Systems, Inc., Minneapolis, MN, USA), rabbit anti-human GAPDH polyclonal antibody (cat. no. 5714; Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit anti-human matrix metalloproteinase 9 (MMP9) polyclonal antibody (cat. no. ab38898), rabbit anti-human vascular endothelial growth factor C (VEGF-C) monoclonal antibody (cat. no. ab191274), rabbit anti-human VEGF-D polyclonal antibody (cat. no. ab155288) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (cat. no. ab205718) (all Abcam, Cambridge, UK).

Cell culture. Hepatoblastoma HepG2 cells were purchased from the Cell Bank at Peking Union Medical College Cancer Hospital (Beijing, China). Cells were cultured at 37°C, in 5% CO₂ and saturated humidity in DMEM with 10% FBS, 100 U/ml ampicillin, and 100 µg/ml streptomycin.

Cell transfection. In our previous study (27), HepG2 cells were successfully transfected with target lentivirus Lv-DcR3-EGFP-shRNA [the sequence with a stem-loop structure was CGCTGGTTTCTGCTTGGAGCA-CTC GAG-TGCTCCAAGCAGAAACCAGCG (Beijing TransGen Biotech, Co., Ltd., Beijing, China)] and blank lentivirus Lv-NC-EGFP-shRNA (Beijing TransGen Biotech, Co., Ltd.) using the Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The ratio of plasmid DNA (µg)/Lipofectamine 2000 reagent (µl) was 1:3.5, and DcR3 siRNA knockdown in HepG2 cells (KD) was achieved and DcR3 blank plasmid negative control HepG2 cells were utilized (negative control, NC). The interval between transfection and subsequent experimentation was 4 weeks. Wild-type HepG2 cells (WT) were used as a normal control.

Detection of apoptosis. HepG2 WT cells and KD cells were seeded into 6-well plates at 5x10⁵ cells/well. Following 24 h incubation, KD cells were incubated with dimethyl sulfoxide (DMSO) containing FasL (10 ng/ml; KD + Fas L). KD and WT cells incubated with DMSO alone acted as controls. Following 48 h incubation, cells were digested with trypsin and centrifuged at 1,000 x g for 5 min at room temperature. Cells were then washed with PBS and centrifuged at 1,000 x g for 5 min at room temperature. Cells were resuspended in PBS, 10x10⁴ cells were transferred into a centrifuge tube and centrifuged at 1,000 x g for 5 min at room temperature. Apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC) cell apoptosis assay kit (Beyotime Institute of Biotechnology, Haimen, China). Cells were resuspended in 195 µl Annexin V-FITC; then 5 µl Annexin V-FITC was added and mixed well. Following 10 min incubation at 20-25°C, cells were centrifuged at 1,000 x g for 5 min and the supernatant was discarded. Cells were then resuspended in 190 µl Annexin V-FITC and 10 µl propidium iodide (PI) was added and mixed well. PI staining was performed at room temperature for 20 min in the dark. A flow cytometer was used to assess the results; cells stained with Annexin V-FITC exhibited green fluorescence and those stained with PI exhibited red fluorescence. Flow cytometry data were analyzed using CellQuest Pro 5.10 (BD Biosciences, Franklin Lakes, NJ, USA). The cells were properly gated and a dual-parameter dot plot of FL1-H (x-axis; Annexin V fluorescence) vs. FL2-H (y-axis; PI fluorescence) was demonstrated to be logarithmic in fluorescence intensity.

Cell cycle analysis. HepG2 WT cells and KD cells were seeded into 6-well plates at 5x10⁵ cells/well. Following 24 h incubation, KD + Fas L cells were incubated with DMSO containing Fas (10 ng/ml). KD and WT cells incubated with DMSO alone acted as controls. Following 48 h, cells were washed with PBS and digested with trypsin. Cells were then transferred to a centrifuge tube and centrifuged at 1,000 x g for 5 min at room temperature; the supernatant was then discarded. The cells were resuspended with 0.9% NaCl and then the supernatant was discarded. Subsequently, cells were fixed with 1 ml 75% ethanol overnight at -20°C. Following fixation, cells were centrifuged at 1,000 x g at 4°C for 5 min and the supernatant was discarded. The cells were washed twice with NaCl 0.9% with 1% bovine serum albumin (Sigma-Aldrich;

Merck KGaA, Darmstadt, Germany) added. Following the addition of 50 μ l RNase (Sigma-Aldrich; Merck KGaA) and 50 μ l PI for 20 min, cells were left in the dark at 4°C. Cells were mixed gently on ice in the dark and then flow cytometry was performed. Flow cytometry data were analysed using Cell Quest Pro 5.10 (BD Biosciences). The DNA content (x-axis, PI fluorescence) vs. counts (y-axis) was plotted as a histogram.

MTS cell proliferation assay. Cells in the WT, NC, and KD groups were seeded into 96-well plates at 2,000 cells/well. Following 24 h incubation, each group of cells (WT, NC and KD) were incubated either with DMEM containing FasL (10 ng/ml) or DMEM alone. Cells were incubated at 37°C in 5% CO₂ for 8 days. Subsequently, 20 μ l CellTiter 96® AQueous One Solution Reagent (Promega Corporation, Madison, WI, USA) was added to each well and incubated at 37°C for 3 h in 5% CO₂. Optical density was measured at 490 nm everyday using Plate reader spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Clonogenic assay. For each experimental group (WT, NC and KD cells), 800 cells/well were seeded into a 6-well plate. Following 24 h incubation, each group of cells (WT, NC and KD) were incubated either with DMEM containing FasL (10 ng/ml) or DMEM alone. When clones became visible, the supernatant was discarded and clones were washed with PBS for 3 times. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Following three washes in PBS, cells were stained with 1% crystal violet solution at 37°C for 10-30 min. Plates were then air-dried and the numbers of clones were calculated. The colony formation rate (%) was calculated using the following formula: (number of clones/number of inoculated cells) x100.

Wound healing assay. WT, NC and KD cells were seeded at 1x10⁵/ml into 6-well plates and cultured until cells grew to filled the plates. A 200 μ l pipet tip was used to evenly scratch across the well (at intervals of 0.5-1.0 cm) 5 times, with the tip kept vertical. Removed cells were washed with PBS (3 times) and each group of cells (WT, NC and KD) was incubated either with DMEM containing FasL (10 ng/ml) or DMEM alone. Plates were incubated at 37°C with 5% CO₂ for 48 h, and samples were photographed at 0, 24 and 48 h.

Cell invasion assay. The apical side of the membrane of the Transwell inserts was coated with 50 mg/l Matrigel at a dilution of 1:8 and was then air-dried at 4°C. A total of 3x10³ cells from each group in 200 μ l serum-free medium was added to the upper chamber of the Transwell assay. DMEM containing FasL (10 ng/ml) and 1% FBS (Gibco; Thermo Fisher Scientific, Inc.) was added to the chamber below the insert, DMEM without FasL was used as the control. Following incubation at 37°C for 24 h, the inserts were taken out and washed twice with PBS. A cotton swab was used to remove the membrane matrix and the cells on the apical side of the insert. Cells on the basal side of the insert were fixed using formaldehyde for 30 min at room temperature and subsequently stained with crystal violet for 20 min at room temperature. Cells were washed with water 3-5 times. The mean cell count of five randomly selected microscopic fields using a light microscope was used for each

sample. For each group, 3 repeats were carried out. The relative invasion rate (%) was calculated using the following formula: (The number of invasive cells in the treated sample/the number of invasive cells in the control) x100%.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). WT, NC and KD cells were inoculated in 6-well plates at 5x10⁵ cells/well. Following 24 h incubation, each group of cells (WT, NC and KD) were incubated either with DMEM containing FasL (10 ng/ml) or DMEM alone. After 48 h, total RNA was extracted from each group using TRIzol and reverse transcribed into cDNA using the Prime Script RT reagent kit following the manufacturers' protocol. qPCR was performed using the SYBR Premix Ex Taq (Takara Bio, Inc.). The primer sequences used were as follows: DcR3, forward, 5'-GGTACCAGGAGCTGAGGAGTGT'-3' and reverse, 5'-CCTTGGTGTCTGGACCCCA-3'; MMP 9, forward, 5'-TGTACCGCTATGGTTACACTCG-3' and reverse, 5'-GGCAGG GACAGTTGCTTCT-3'; VEGF-C, forward, 5'-GGCTGG CAACATAACAGAGAA-3' and reverse, 5'-CCCCACATC TATACACACCTCC-3'; VEGF-D, forward, 5'-TCCCATCGG TCCACTAGGTTT-3' and reverse, 5'-AGGGCTGCACTG AGTTCTTTG-3'; GAPDH (the internal control), forward, 5'-GTCGGAGTCAACGGATTGG-3' and reverse, 5'-AAA AGCAGCCCTGGTGACC-3'.

The qPCR conditions were: Initial denaturation at 94°C 2 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 60 sec; and final extension at 72°C for 10 min. Relative mRNA expression was calculated using the 2^{- $\Delta\Delta C_q$} method (30).

Western blot analysis. Each group of cells was washed in PBS at 4°C and then radioimmunoprecipitation assay lysis buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) containing phenylmethylsulfonyl fluoride was added for 5 min. Lysates were centrifuged at 12,000 x g for 5 min at 4°C. The protein concentration in the supernatant was quantified using a BCA kit. Protein samples (30 μ g) were separated using 12% SDS-PAGE and then transferred to nitrocellulose membranes. Following blocking with 5% skimmed milk for 1 h at room temperature, rabbit anti-human MMP9 polyclonal antibody (1:1,000), rabbit anti-human VEGF-C polyclonal antibody (1:1,000), rabbit anti-human VEGF-D monoclonal antibody (1:1,000), rabbit anti-human GAPDH antibody (1:5,000) were added and membranes were incubated at 4°C overnight. Following washing with TBST, horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:5,000) was added and incubated at room temperature for 1 h. Enhanced chemiluminescent agent (EMD Millipore, Billerica, MA, USA) was added following washing with TBST. A Bio-Rad gel imaging system (BioradGelDoc XR; Bio-Rad Laboratories, Inc.) was used to capture the images, and GAPDH was used as the internal control for the analysis of DcR3, MMP9, VEGF-C, and VEGF-D.

Statistical analysis. Values are expressed as the mean \pm standard deviation. All statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The statistical significance among groups was determined by two-tailed

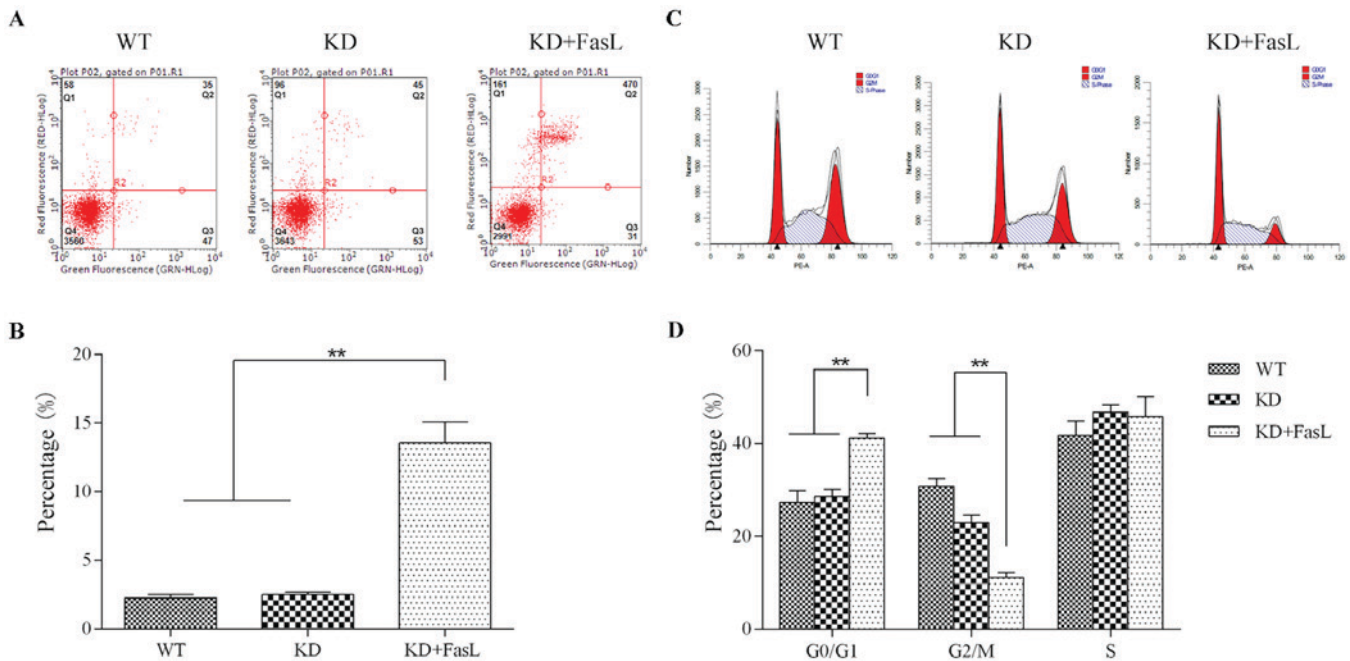


Figure 1. Effects of FasL on the apoptosis and cell cycle of various groups of cells. (A) Flow cytometry presenting the apoptosis of cells in each group; (B) the proportion of apoptotic cells in each group; (C) cell cycle analysis; (D) quantification of cell cycle analysis. ** $P < 0.01$. FasL, Fas ligand; WT, wild-type HepG2 cells; KD, DcR3 knockdown HepG2 cells; NC, negative control HepG2 cells; DcR3, Fas and decoy receptor 3.

analysis of variance followed by Dunnett's multiple comparisons test. $P < 0.05$ was considered to indicate a significant difference. All experiments were performed in triplicate and repeated at least three times.

Results

Following DcR3 knockdown, HepG2 cells became more sensitive to FasL-induced apoptosis. Flow cytometric analysis indicated that, following treatment with FasL, the proportion of KD cells exhibiting signs of early apoptosis was $12.29 \pm 1.12\%$, which was significantly higher than that of the KD ($1.12 \pm 0.10\%$) and WT ($0.99 \pm 0.10\%$) groups ($P < 0.01$; Fig. 1A and B).

Cell cycle analysis by flow cytometry indicated that the proportion of cells in the KD + FasL group in the G₀/G₁ phase ($41.12 \pm 1.02\%$) was significantly greater than that of the KD ($28.59 \pm 1.51\%$) and WT ($27.34 \pm 2.52\%$) groups ($P < 0.01$; Fig. 1C and D). Furthermore, the proportion of cells in the G₂/M phase in the KD + FasL group ($11.11 \pm 1.10\%$) was significantly lower than that of the KD ($22.98 \pm 1.71\%$) and the WT ($30.84 \pm 1.61\%$) groups ($P < 0.01$).

Following DcR3 knockdown, FasL decreases the proliferation, migration and invasion of HepG2 cells. The results of the MTS assay demonstrated that, compared with the WT and NC groups, the proliferation rates of cells in the KD, WT + FasL, NC + FasL and WT + FasL groups from day 3 onwards were significantly decreased compared with the WT group ($P < 0.05$ or $P < 0.01$; Fig. 2A). The proliferation rate of cells in the KD + FasL group was significantly lower. These results indicate that FasL decreases cell proliferation and that this impact is enhanced following DcR3 knockdown.

The results of the wound healing assay indicated that FasL reduced cell migration, with the most significant decrease identified in the KD + FasL group. Cell migration was significantly decreased in the KD + FasL group compared with all other groups at 48 h ($P < 0.01$; Fig. 2B and C).

The results of the clonogenic assay demonstrated that compared with the WT group, the number of clones in the KD, WT + FasL, NC + FasL and KD + FasL groups all decreased (Fig. 3A and B). The greatest decrease was found in the KD + FasL group; the number of clones was significantly decreased in this group compared with all other groups, ($P < 0.01$). These results indicate following DcR3 silencing, treatment with FasL further decreases HepG2 cell proliferation.

Furthermore, the results of the Transwell cell migration assay demonstrated that the number of cells in the KD + FasL group passing through the Matrigel membrane was significantly decreased compared with all other groups ($P < 0.01$; Fig. 3C and D). These results suggest that FasL decreases the invasiveness of HepG2 cells.

Following DcR3 knockdown, the expression of VEGF-C, VEGF-D and MMP9 decreases following treatment of HepG2 cells with FasL. The results of RT-qPCR demonstrated that, compared with the other groups, the expression of VEGF-C, VEGF-D and MMP 9 mRNA in the KD + FasL group was significantly decreased ($P < 0.01$; Fig. 4). The same trend was observed regarding the expression of VEGF-C, VEGF-D and MMP9 protein following western blotting.

Discussion

The Fas/FasL system is an apoptotic pathway that serves an important role in maintaining immune homeostasis (31) and tumorigenesis (32). Under normal circumstances, Fas is highly

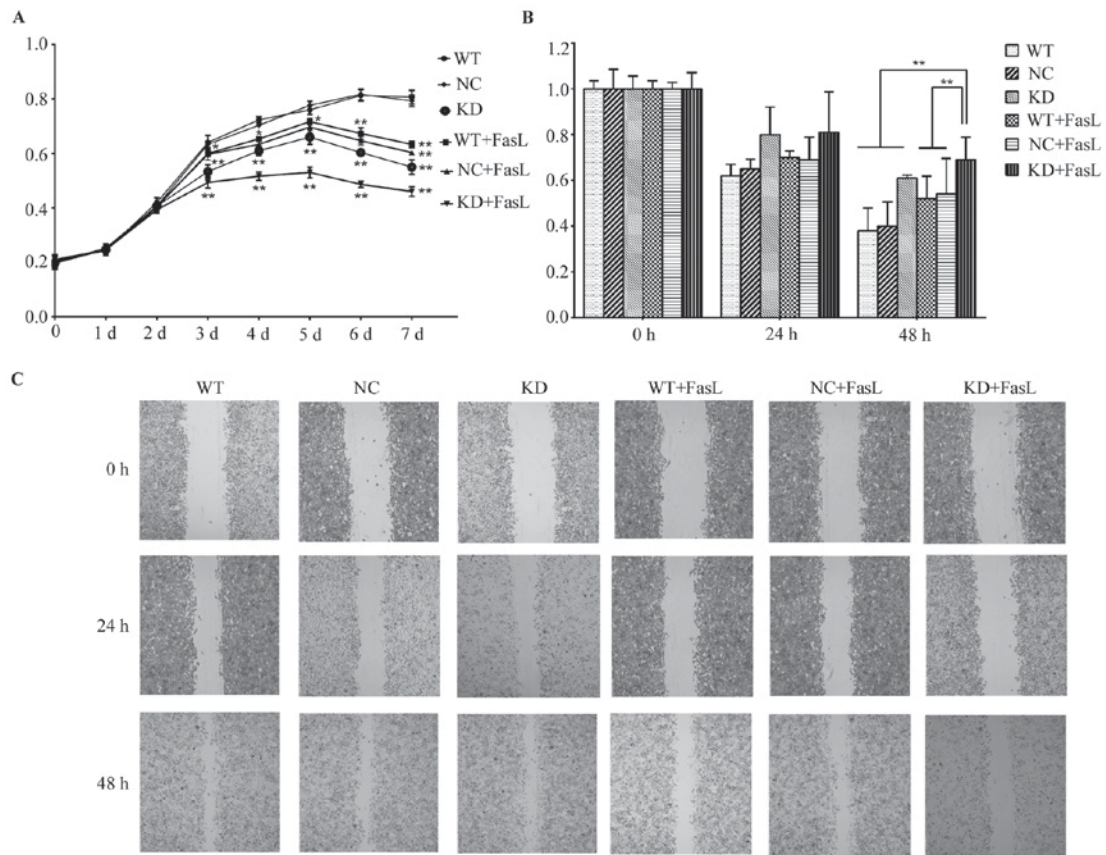


Figure 2. Effects of FasL on the activity and migration of HepG2 cells. (A) The results of the MTS assay. From Day 3, the OD values of the KD, WT + FasL, NC + FasL and KD + FasL groups were lower than the WT group, respectively. The OD value in the KD + FasL group was the lowest. (B and C) The results of the wound healing assay indicated that following the addition of FasL, cell migration was reduced in all treatment groups; the decrease in the KD cells was the most significant. * $P < 0.05$, ** $P < 0.01$. OD, optical density; FasL, Fas ligand; KD, DcR3 knockdown HepG2 cells; WT, wild-type HepG2 cells; NC, negative control HepG2 cells; DcR3, Fas and decoy receptor 3.

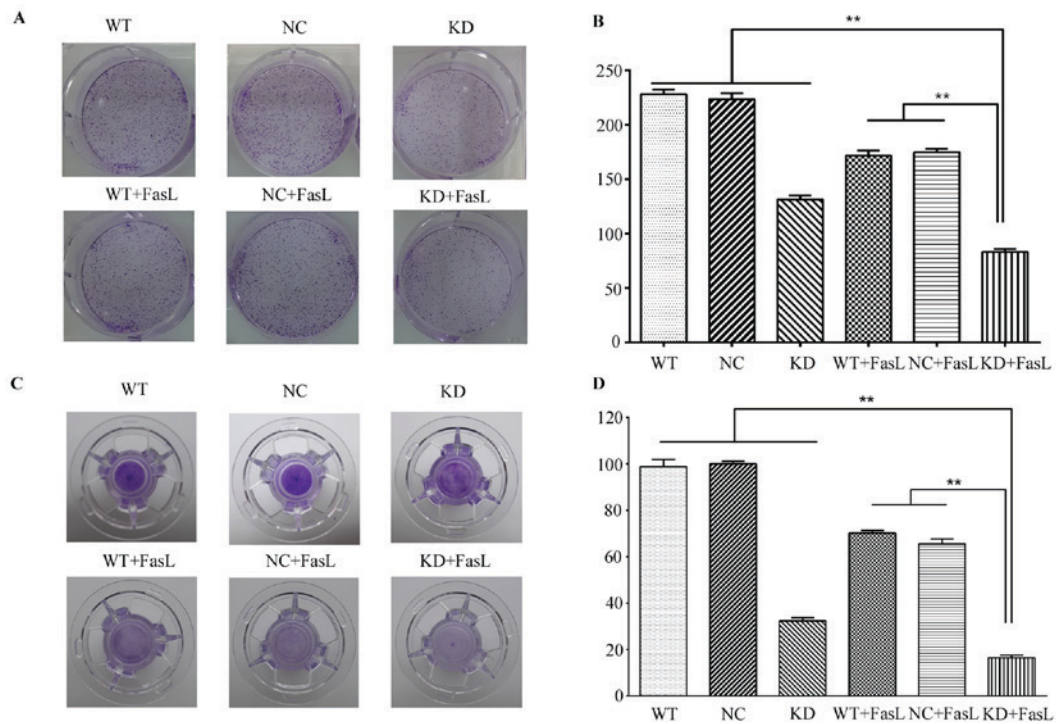


Figure 3. Effects of FasL on the proliferation and invasiveness of HepG2 cells. (A and B) Results of the clonogenic assay demonstrated that following treatment with FasL, the clonogenic ability of the KD group was decreased compared with other groups. ** $P < 0.01$. (C and D) The results of the Transwell cell migration assay indicated that the invasive ability of the KD cells decreased significantly following addition of FasL. ** $P < 0.01$. FasL, Fas ligand; WT, wild-type HepG2 cells; KD, DcR3 knockdown HepG2 cells; NC, negative control HepG2 cells; DcR3, Fas and decoy receptor 3.

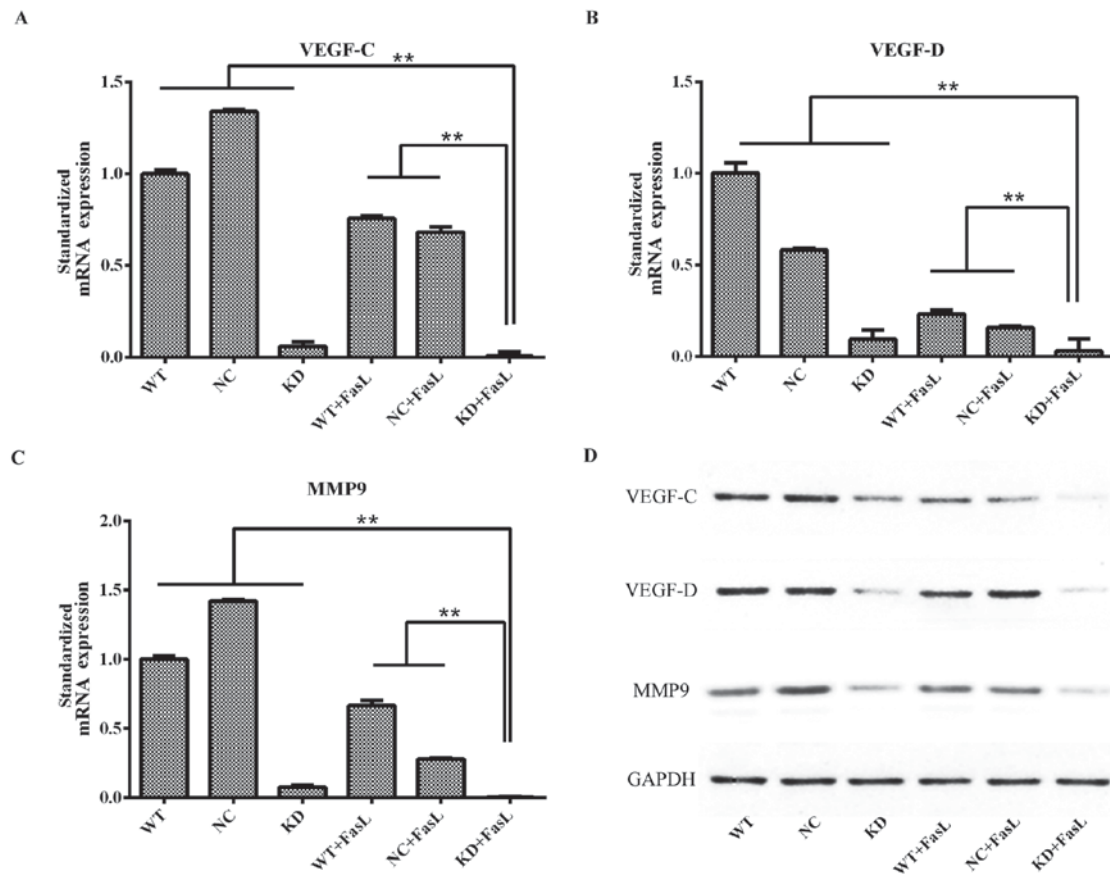


Figure 4. Changes in the expression of (A) VEGF-C, (B) VEGF-D and (C) MMP9 mRNA in HepG2 cells following exposure to FasL. ** $P < 0.01$. (D) Results of western blotting presenting the expression of VEGF-C, VEGF-D and MMP9 protein in each of the groups. MMP9, matrix metalloproteinase 9, VEGF-C, vascular endothelial growth factor C, VEGF-D, vascular endothelial growth factor D; FasL, Fas ligand; WT, wild-type HepG2 cells; KD, DcR3 knockdown HepG2 cells; NC, negative control HepG2 cells; DcR3, Fas and decoy receptor 3.

expressed in various cells and the development of tumors is often associated with a lack of Fas expression or function, and the increased expression of FasL in tumor cells (33). It has been established that Fas/FasL not only induces apoptosis, but also triggers multiple signaling pathways to inhibit apoptosis; furthermore, its abnormal expression is closely associated with tumorigenesis (34). It may stimulate tumor cell proliferation and promote tumor cell migration and invasion via the nuclear factor- κ B, mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways (35). FasL contains two receptors, Fas and DcR3. DcR3 competitively binds to FasL over Fas, thereby inhibiting FasL-induced apoptosis.

The DcR3 gene was identified in 1998 by Pitti *et al* (19) and is closely associated with tumorigenesis. In the majority of tissues, the expression of DcR3 is negligible; however, it is highly expressed in many different types of cancer, including esophageal (19), gastric (19), colon (19), rectal (19), pancreatic (23), liver (23-25), lung (19,23), kidney (36), breast (23), ovarian (37) and skin cancer (38).

Connolly *et al* (21) intravenously injected FasL to Fas⁺ mice and demonstrated that the mice succumbed to acute fatal fulminant hepatic necrosis in ≤ 2 h; however, this did not occur in mice pre-treated with DcR3. It was therefore hypothesized that DcR3 reduces Fas-mediated liver cell necrosis. Hayashi *et al* (39) demonstrated that downregulating DcR3 expression using siRNA may increase Fas-mediated apoptosis

and using TNF- α to increase the expression of DcR3 could inhibit Fas-mediated apoptosis in rheumatoid arthritis synovial fibroblasts. Li *et al* (40) indicated that DcR3 expression in the human gastric cancer BGC823 and breast cancer MCF-7 cell lines were 35.3 and 21.6%, whereas the apoptosis induced by FasL was 15.6 and 58.2%, respectively. Therefore, it was suggested that FasL-mediated apoptosis was directly associated with levels of DcR3 expression (40). The results of the current study demonstrated that FasL-induced apoptosis was increased when DcR3 in HepG2 cells was silenced, which is in accordance with the aforementioned experimental results. Furthermore, FasL significantly decreased the proliferation and invasiveness of KD cells. These results indicate that FasL may impair the proliferation and invasiveness of HepG2 cells more effectively when DcR3 expression is decreased.

Tumor invasion and metastasis is a complex multi-step process. When leaving the primary tumor and moving in and out of blood vessels, cancer cells must initially break through the extracellular matrix and basement membrane barrier to infiltrate the adjacent fibrous connective tissue before moving to more distant sites (41). The degradation of the extracellular matrix and the basement membrane is generally conducted by MMPs, hyaluronidase, elastase, cathepsin and large numbers of macrophages (42). MMPs are proteolytic enzymes that are closely associated with tumor invasion and metastasis (43).

They are directly involved in tissue damage, induce VEGF and stimulate endothelial cells to synthesize proteases that lead to endothelial cell proliferation and migration (44). MMPs are considered to be important inducers and regulators of angiogenesis (45). MMP9 is a type IV collagenase that acts as a protease in tumor invasion and metastasis primarily by degrading collagen type III and IV in the extracellular matrix and the basement membrane of blood vessel walls (46). MMP9 is also involved in the formation of blood vessels in tumors (47). The increased expression of MMP9 is associated with tumor malignancy and prognosis (48,49) and it has been demonstrated that disease-free and 5-year survival rates are significantly decreased in patients with gliomas that are MMP9 positive compared with those that are MMP9 negative. In the present study, the results of RT-qPCR and western blotting demonstrated that the expression of MMP9 in DcR3-knockdown HepG2 cells treated with FasL was significantly lower than in normal and FasL-treated normal HepG2 cells, indicating that FasL may reduce the expression of MMP9 and inhibit tumor angiogenesis invasion and metastasis in HepG2 cells following DcR3 silencing.

There have been few studies investigating tumor lymphangiogenesis; attention has focused on angiogenesis, due to its importance during the tumorigenesis of solid tumors (50-52). In fact, due to their structure and function, lymphatic vessels are not only involved in maintaining tumor growth, but are also the major cause of metastasis in different types of cancer and are key factors determining cancer stage and patient prognosis (53). Lymph node metastasis is commonly used as a guide for treatment selection (54). The liver is an organ containing abundant blood vessels and liver cancer tissues exhibit a large amount of lymphangiogenesis that is closely associated with liver cancer cell migration and invasion (55).

Tumor lymphangiogenesis is extremely complex. Tumor cells, stromal cells, macrophages and activated platelets all can secrete a variety of lymphatic growth factors that induce the formation of new tumor lymphatic vessels and promote tumor lymphatic metastasis (56). The dimeric glycoproteins VEGF-C and VEGF-D, are the most well-studied lymphatic endothelial growth factors. VEGF-C was the first lymphatic endothelial growth factor identified to serve a role in promoting lymphangiogenesis (57). VEGF-D was identified in 1998 and was determined to have a similar structure, function and common specific receptor to VEGF-C (58). It has been demonstrated that VEGF receptor 3 (VEGFR3) has the highest affinity in binding to VEGF-C/D (59). VEGFR3 is primarily expressed in embryonic and adult lymphatic endothelial cells and is an important signal that mediates lymphangiogenesis. Following the binding of VEGFR-3 to VEGF-C/D, lymphatic endothelial cell proliferation and migration is stimulated, tumor lymphangiogenesis is promoted, tumor cells move into lymphatic vessels and cancer metastasis occurs via the lymphatic system (60-62). It has been demonstrated that VEGF-C exhibits the strongest promoting effect on lymphangiogenesis (63). It is highly expressed in lung, breast, stomach and colon cancer tumors and is closely associated with tumor lymphoid metastasis (64). Tumor cells are able to secrete large amount of growth factors to promote angiogenesis, providing essential nutrients to the tumor tissue, promoting tumor lymphangiogenesis and mediating tumor invasion. It has been reported that tumor angiogenesis is associated with VEGF-A/VEGFR-2 and that lymphangiogenesis is

primarily regulated by VEGF-C, VEGF-D and VEGFR-3 (65). Under normal physiological conditions, VEGF-C and VEGF-D are able to mediate endothelial cell mitosis, cell migration and apoptosis (66). However, when cells are in abnormal states of proliferation and activation (i.e., tumorigenesis), tumor cells stimulate the surrounding tissues and induce lymphangiogenesis via autocrine and paracrine signaling. Growth of the newly formed lymphatic vessels is promoted, as is tumor lymphatic metastasis (67). The results of the current study demonstrated that the expression of VEGF-C and VEGF-D in DcR3-silenced cells following treatment with FasL was significantly decreased compared with normal and FasL-treated normal cells, indicating that FasL reduces the expression of VEGF-C and VEGF-D following the downregulation of DcR3 *in vitro*, thus impairing the ability of lymphangiogenesis and lymph node metastasis of HepG2 cells.

In conclusion, the results of the current study indicate that FasL increases the apoptosis of HepG2 cells and significantly decreases the proliferation and invasion of HepG2 cells following DcR3 silencing. This may be due to the downregulation of MMP9, VEGF-C, VEGF-D expression; however, its exact mechanism of action remains unclear and further studies are required. The results of the present study suggest that DcR3 silencing combined with FasL treatment may be a novel method of treating patients with liver cancer, particularly those with hepatoblastoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TZ designed the study, performed experiments, analyzed the data and wrote the paper. YX, SR and XZ performed experiments. CL analyzed data. JW initiated, organized and designed the study, and wrote the paper

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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