Short hairpin RNA silencing of TGF-βRII and FZD-7 synergistically suppresses proliferation and metastasis of hepatocellular carcinoma cells

CONG CHEN[1], YUYANG XUE[1], DEJUN ZHANG[2], WEI XU[1], HAO XU[1], HONG YAO[3], DONGSHENG PEI[3] and YUMING GU[1]

[1]Department of Interventional Radiology, Affiliated Hospital of Xuzhou Medical College, Xuzhou, Jiangsu 221002; [2]Department of Oncology, Huazhong University of Science and Technology, Wuhan, Hubei 430030; [3]Jiangsu Key Laboratory of Biological Cancer Therapy, Xuzhou Medical College, Xuzhou, Jiangsu 221002, P.R. China

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Abstract. Transforming growth factor-β (TGF-β) is a multi-functional regulator of cell growth, apoptosis, differentiation and migration. The Wnt/β-catenin signaling pathway has been implicated in a wide spectrum of diseases, including numerous cancers and degenerative disease. The aim of the present study was to investigate if simultaneous blocking of TGF-β and Wnt/β-catenin signaling pathways exerts synergistic anti-tumor effects on hepatocellular carcinoma (HCC) cells. Short hairpin (sh) RNA eukaryotic expression vectors, specific to TGF-β receptor II (RII) and Frizzled receptor (FZD)-7, were constructed by gene recombination. The expression vectors were transfected into human HCC HepG2 and Huh-7 cells using Lipofectamine 2000 to investigate the synergistic effects between TGF-β and Wnt/β-catenin signaling pathways on HCC cell proliferation, invasion and migration and the cell-cycle distribution. Western blot analysis was used to identify the expression of β-catenin, c-Myc and cyclin D1 in transfected cells to investigate the underlying mechanisms that cause TGF-β and Wnt/β-catenin signaling in HCC cells. shTGF-βRII-c and shFZD-7-2 were selected as the most efficient plasmids. A cell growth assay and colony-forming assay consistently demonstrated that the proliferative activity of the co-transfected group was significantly decreased compared to the single-transfected group. A wound healing invasion and migration assay demonstrated that co-transfection of shTGF-βRII-c and shFZD-7-2 decreased the invasion and migration abilities of the cells compared with either single-transfected group. In addition, the present study demonstrated that the observed reduction in cell proliferation was due to the cells arresting at the G1 phase of the cell cycle, and the downregulation of β-catenin, c-Myc and cyclin D1 impaired the proliferative and invasive abilities of the HCC cells. The present results demonstrate that simultaneous blocking of TGF-β and Wnt/β-catenin signaling by targeting TGF-βRII and FZD-7 may inhibit the proliferation and metastasis of HCC cells more effectively compared with blocking either the TGF-β or Wnt/β-catenin pathway.

Correspondence to: Professor Yuming Gu, Department of Interventional Radiology, Affiliated Hospital of Xuzhou Medical College, 99 Huaihai Road West, Xuzhou, Jiangsu 221002, P.R. China
E-mail: guyuming_2006@163.com

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Introduction

Human hepatocellular carcinoma (HCC) is one of the most widespread and severe types of malignancy in adults. An estimated 782,500 new liver cancer cases and 745,500 cancer-associated mortalities occurred worldwide during 2012 (1). Certain treatments, including liver transplantation, surgical resection, transcatheter arterial chemoembolization, stereotactic body radiation therapy and chemotherapy, are considered as the most effective therapies for the treatment of HCC; however, these treatments are only effective in certain patients (2). Therefore, novel potential therapeutic targets are urgently required for the management of HCC.

The Wnt/β-catenin signaling pathway has been demonstrated to be dysregulated in HCC (3) and gastric (4), breast (5) and colon cancers (6,7). It is one of the most significant pathways that regulates cell proliferation, differentiation, migration, apoptosis and survival. In normal epithelial cells, β-catenin interacts and binds to the cytoplasmic tail of E-cadherin and is sequestered to the membrane of the cell. In the cytoplasm of the cell, a small amount of free β-catenin forms complexes with glycogen synthase kinase 3β (GSK3β), adenomatous polyposis coli (APC) and Axin. This complex is phosphorylated by Casein kinase 1 and GSK3β and is subsequently degraded by the ubiquitination-proteasome pathway (8). When a Wnt ligand binds to a Frizzled receptor (FZD), GSK3β is released from the GSK3β/APC/Axin complex, leading to the activity of GSK3β becoming inhibited, and therefore liberating β-catenin. β-catenin accumulates in the cytoplasm of the cell and translocates to the nucleus where it regulates the transcription of target

genes through an interaction with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and Legless family docking proteins (9,10).

The transforming growth factor-β (TGF-β) signaling pathway has emerged as another key pathway in regulating tumor cell growth and differentiation and maintaining the tumor interstitium (11,12). In addition, TGF-β has been demonstrated to act as an oncogenic cytokine by inducing epithelial-mesenchymal transition (EMT), angiogenesis, and immune suppression (13). When TGF-β binds to a receptor complex composed of type I and type II serine/threonine kinase receptors, signals are propagated to the SMAD family of proteins, which activates the downstream signaling molecules (14,15).

The synergistic activities of the TGF-β and Wnt/β-catenin signaling pathways are pivotal during embryogenesis due to their interaction with downstream effectors Smad 3 and 4 and TCF/LEF (14,16). The effects of simultaneously targeting TGF-β and Wnt/β-catenin signaling pathways in HCC has, to the best of our knowledge, not been previously demonstrated. Consequently, the present study investigated the synergistic antitumorous effects caused by simultaneously blocking TGF-β and Wnt/β-catenin signaling pathways using short hairpin (sh)-TGF-βRII and shFZD-7 in human HCC HepG2 and Huh-7 cells.

Materials and methods

Cell culture. Human HCC HepG2 and Huh-7 cell lines were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and were cultured in Gibco Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). All cells were incubated at 37˚C in an atmosphere of 5% CO_2.

shRNA synthesis and transfection into pGPU6/GFP/Neo plasmids. shRNA sequences of TGF-βRII and FZD-7 were designed by siRNA Sequence-Selector software (Vector NTI® version 10; Thermo Fisher Scientific, Inc.). The oligonucleotide sequences were inserted into *Bam*HI and *Bbs*I sites of pGPU6/GFP/Neo plasmids (Shanghai GenePharma Co., Ltd., Shanghai, China). In total, 8 shRNAs were designed to target 4 regions in the mRNA of TGF-βRII and FZD-7. The 4 targeting sequences for TGF-βRII were as follows: 5'-GCC CATCCACTGAGACATATT-3', 5'-GGAGAAAGAATGACG AGAACA-3', 5'-GCTTTGCTGAGGTCTATAAGG-3' and 5'-GGAAGACAGAGAAGGACATCT-3'. The 4 targeting sequences for FZD-7 were as follows: 5'-GGTGGGTCATTC TGTCTCTCA-3', 5'-GCCGTCAAGACCATCACTATC-3', 5'-GTTCGTCTACCTCTTCATAGG-3' and 5'-GCACCATCA TGAAACACGACG-3'. Therefore, a total of 8 plasmids (sh-TGF-βRII-a; sh-TGF-βRII-b; sh-TGF-βR-c; sh-TGF-βRII-d; sh-FZD-7-1; sh-FZD-7-2; sh-FZD-7-3; sh-FZD-7-4) were obtained for transfection. Scrambled shRNA that did not cause specific degradation of any known cellular mRNA was used as a negative control (sh-NC; sense, 5'-CACCGTTCTCCGAACGTGTCACGTCAAGAGATT ACGTGACACGTTCGGAGAATTTTTTG-3' and antisense, 5'-GATCCAAAAAATTCTCCGAACGTGTCACGTAATC TCTTGACGTGACACGTTCGGAGAAC-3'). All the constructs were verified by sequence analysis.

shRNA transfection. The HCC cells were grown to 80% confluence prior to transfection. sh-TGF-βRII, sh-FZD-7 and sh-NC plasmids were transfected using Invitrogen Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The amounts of plasmid DNA in each transfection was equal (4 μg DNA and 10 μl Lipofectamine® 2000 in each group). The medium surrounding the cells was replaced 4-6 h post-transfection to alleviate toxicity.

Western blot analysis. The cells were homogenized in protein lysis buffer (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China), 48 h subsequent to transfection, and were centrifuged at 15,000 x g for 15 min. The supernatant was extracted to obtain the total cellular protein extracts. The concentration of the protein was determined using the bicinchoninic acid assay (BCA kit; Beyotime Institute of Biotechnology). The protein samples were denatured by mixing with 5X loading buffer (Beyotime Institute of Biotechnology). Following boiling for 5 min, the total cellular protein extracts were separated using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for β-catenin, 10% SDS-PAGE for TGF-βRII, FZD-7, c-Myc and β-actin and 12% SDS-PAGE for cyclin D1. The proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Uppsala, Sweden) using a wet or semi-dry transfer. The membranes were blocked with 5% skimmed milk and Tris-buffered saline with Tween 20 (TBST) for 2 h at room temperature, and rinsed three times with TBST for 30 min. The proteins were incubated with primary polyclonal rabbit anti-human antibodies against TGF-βRII, FZD-7, cyclin D1 and β-actin (dilution, 1:1,000; bioWORLD, Dublin, OH, USA; catalog no.'s., BS1696, BS2774, BS1741 and BS1002, respectively), and monoclonal mouse anti-human antibodies against β-catenin and c-Myc (dilution, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; catalog no.'s., sc-59737 and sc-40, respectively). The proteins were diluted with 0.5% skimmed milk in TBST overnight at 4˚C, followed by rinsing three times with TBST for 30 min. The proteins were incubated with the appropriate monoclonal goat anti-mouse (or anti-rabbit) immunofluorescence-conjugated secondary antibodies (dilution, 1:10,000; LI-COR Biotechnology, Lincoln, NE, USA). The bands of specific proteins on the nitrocellulose membranes were visualized with an Odyssey® CLx Infrared Imaging System (LI-COR Biotechnology).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. The expression of TGF-βRII and FZD-7 mRNA in the cells was assessed using RT-PCR to evaluate the efficiency of the shRNA transfection. Total RNA from the transfected cells was extracted using Trizol reagent (TianGen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. In total, 1 μg of RNA was reverse transcribed into cDNA using the Premium First Strand cDNA Synthesis kit (TianGen Biotech Co., Ltd.). PCR primers were ordered from Shanghai Genebase Gene-Tech Co., Ltd. (Shanghai, China). The primer sequences obtained were as follows: GADPH sense, 5'-AGA

AGGCTGGGGCTCATTTG-3' and antisense, 5'-AGGGGC CATCCACAGTCTTC-3', 258 bp; TGF-βRII sense, 5'-ATG CTGCTTCTCCAAAGTGC-3' and antisense, 5'-AGTGCT CGCTGAACTCCAT-3', 303 bp; FZD-7 sense, 5'-CTGTCG GGCTGCTACTTCAT-3' and antisense, 5'-GCCAGGATA GTGATGGTCTTG-3', 320 bp. The PCR reaction mixture contained 1 μg template cDNA, 10 μM of each primer, 12.5 μl 2×Master Mix (TianGen Biotech Co., Ltd.) and 25 μl ddH_2O. The PCR amplification cycle was as follows: Initial denaturation, 94˚C for 5 min; 30 cycles consisting of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 1 min; and storage, 72˚C for 5 min. An Eppendorf 5332 Mastercycler was used (Eppendorf, Hamburg, Germany). PCR products were separated by electrophoresis using 2% agarose gels.

Cell proliferation assay. The HepG2 and Huh-7 cells were incubated in 96-well ($5x10^3$ cells/well) plates (Corning Inc., Corning, NY, USA) in 100 μl medium. Subsequent to culturing for 24, 48, 72 and 96 h, the supernatant was removed and 100 μl serum-free DMEM and 10 μl cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) solution was added to each well, followed by incubation for 2 h at 37˚C. The absorbance at 450 nm was recorded using an ELX-800 Absorbance Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). All experiments were performed in quadruplicate and repeated ≥3 times.

Clonogenic assay. For clonogenic analysis, the cells were harvested at 48 h post-transfection and ~$3x10^3$ cells from each transfection group were seeded on 6-well culture plates. The cells were incubated for 12-14 days and fixed with 4% paraformaldehyde (Beijing Solarbio Sciences and Technology Co., Ltd., Beijing, China) and stained with crystal violet (Sigma-Aldrich). The cell colonies (≥50 cells) were counted using an Olympus microscope (IX71+DP721; Olympus Corporation, Tokyo, Japan) at x100 magnification.

Scratch wound healing assay. The cells were grown to confluence of 80-90% in 6-well culture plates to a density of $5x10^6$ cells/well. The confluent monolayer was disrupted with a 10-μl pipette tip and washed three times with phosphate-buffered saline (PBS) to remove cell debris. The wounded monolayer was photographed over the following 24 h using a fluorescent microscope (Ti-U; Nikon Corporation, Tokyo, Japan) at x100 magnification. The migration ability of the cells was determined by the ratio of the healing width at 24 h to the wound width at 0 h.

Cell invasion and migration assay. Transwell chambers (EMD Millipore Corporation, Billerica, MA, USA) containing an 8-μm pore polycarbonate membrane filter was coated with 60 μl/well Matrigel (for an invasion assay; BD Biosciences, San Jose, CA, USA) or without Matrigel (for a migration assay) and inserted in a 24-well culture plate. The lower chamber of the chamber was filled with 600 μl media supplemented with 10% FBS as a chemoattractant, and $1x10^5$ cells/well in 200 μl serum-free DMEM were seeded into the upper chamber. The chamber was incubated at 37˚C in a humidified chamber in an atmosphere of 5% CO_2 for 24 h (migration assay) or 48 h (invasion assay). Following incubation, the Transwell chambers were removed. The cells in the upper chamber that did not migrate were scraped away with a cotton swab. The cells that migrated through and adhered to the lower chamber were fixed in paraformaldehyde for 30 min, then washed twice with PBS and stained with crystal violet for 30 min. Images of the cells in the lower chamber were captured using a fluorescence microscope (Ti-U; Nikon Corporation) equipped with NIS-Elements F3.2. software (Nikon Corporation) at x200 magnification.

Cell-cycle analysis. Single-cell suspensions containing $1x10^6$ cells were collected at 48 h subsequent to transfection and fixed with 70% ethanol for 2 h at 4˚C. The cell cycle was monitored using propidium iodide (PI; Nanjing KeyGen Biotec, Nanjing, China) to stain the nuclei of the cells. The fluorescence of DNA-bound PI cells was measured with a BD FACScan flow cytometer (FACSCanto™ II; BD Biosciences), and the cell cycle distributions were analyzed with ModFit LT™ version 3.0 software (Verity Software House, Inc., Topsham, ME, USA).

Statistical analysis. All experiments were conducted ≥3 times independently. The experimental data were analyzed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA) and quantitative data were presented as the mean ± standard deviation. Multiple groups were compared using one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Downregulation of TGF-βRII and FZD-7 expression by shRNA in human HCC HepG2 and Huh-7 cells. A total of 8 shRNAs were designed to target 4 regions in the mRNA of TGF-βRII and FZD-7. Following transfection, the mRNA and protein levels of TGF-βRII and FZD-7 were examined by RT-PCR and western blot analysis to screen which shRNA specifically suppressed the expression of TGF-βRII and FZD-7. The mRNA and protein levels of TGF-βRII and FZD-7 were significantly reduced in cells transfected with sh-TGF-βRII and sh-FZD-7 plasmids compared with the control groups (Fig. 1). sh-TGF-βRII-c and sh-FZD-7-2 were demonstrated to be the most effective specific suppressors. Transfection with sh-NC did not affect TGF-βRII and FZD-7 expression at the mRNA or protein level in HCC cells. Therefore, HepG2 and Huh-7 cells transfected with sh-TGF-βRII-c and sh-FZD-7-2 plasmids were used for the following analysis.

Silencing of TGF-βRII and FZD-7 inhibited HCC cell proliferation. CCK-8 assays were used to determine if silencing TGF-βRII and FZD-7 affects the growth of HCC cells. As demonstrated by Fig. 2A, the proliferation of transfected cells was significantly suppressed at various time points (48, 72 and 96 h), particularly in the co-transfection group (sh-TGF-βRII and sh-FZD-7) compared with cells in the single-transfection group (sh-TGF-βRII or sh-FZD-7) at 96 h ($P<0.05$). However, no significant differences were observed between the blank and sh-NC groups ($P>0.1$). The proliferation ability of the HCC cells was also assessed using colony

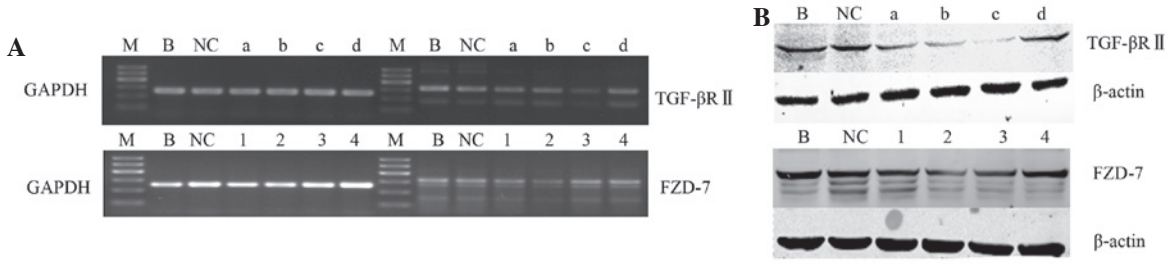

Figure 1. (A) The mRNA and (B) protein levels of TGF-βRII and FZD-7 in human hepatocellular carcinoma cells. GADPH and β-actin were used as the loading controls. Reverse transcription-polymerase chain reaction and western blot analysis results were obtained from 3 experiments. TGF-β, transforming growth factor-β; TGF-βRII, tranforming growth factor receptor II; FZD, frizzled receptor; NC, negative control; shRNA, short hairpin RNA; M, DNA marker; B, blank; NC, sh-NC; a, sh-TGF-βRII-a; b, sh-TGF-βRII-b; c, sh-TGF-βRII-c; d, sh-TGF-βRII-d; 1, sh-FZD-7-1; 2, sh-FZD-7-2; 3, sh-FZD-7-3; 4, sh-FZD-7-4.

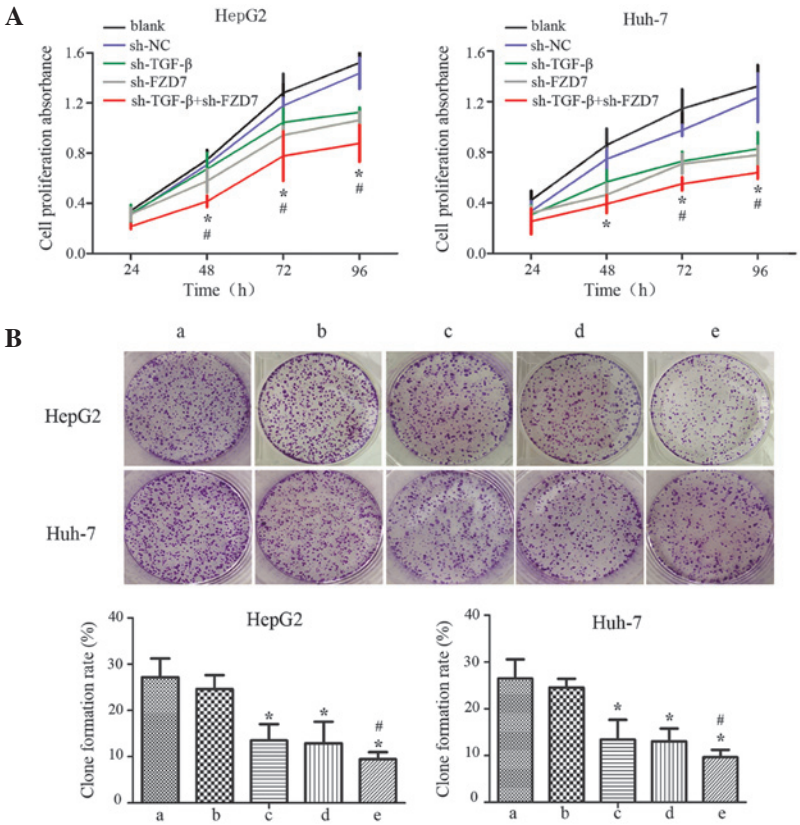

Figure 2. Anti-proliferation effects of sh-TGF-βRII and sh-FZD-7 on HCC cells . (A) A cell counting assay was performed to examine the anti-proliferative effects of the co-transfection of sh-TGF-βRII and sh-FZD-7 in HCC HepG2 and Huh-7 cells. *P<0.01 and #P<0.05. (B) The colony-forming abilities of the cells were downregulated in the co-transfection group compared with the single-transfection group in HepG2 and Huh-7 cells. Each bar represents three independent experiments exhibited as the mean ± standard deviation; error bars represent the standard deviation. *P<0.01 compared with a and b; #P<0.05 compared with c and d. TGF-βRII, transforming growth factor-β receptor II; FZD, frizzled receptor; shRNA, short hairpin RNA; HCC, human hepatocellular carcinoma; NC, negative control; a, blank; b, sh-NC; c, sh-TGF-βRII; d, sh-FZD-7; e, sh-TGF-βRII + sh-FZD-7.

formation assays; the results were consistent with those of the CCK-8 assays (Fig. 2B).

Cellular metastasis was impaired in HCC cells transfected with sh-TGF-βRII and sh-FZD-7. Cellular migration and invasion are two essential processes in cancer development. Therefore, the present study investigated the cellular metastasis of HCC to demonstrate the potential roles of TGF-β and Wnt/β-catenin signaling. The wound healing assay revealed that the wound was healed more slowly when cells were transfected with sh-TGF-βRII and sh-FZD-7 compared with the control cells (Fig. 3A). The cell migration assay demonstrated that co-transfection reduced the cell migration ability by 67 and 68% for HepG2 cells and by 23 and 29% for Huh-7 cells compared with the sh-TGF-βRII and sh-FZD-7 groups (Fig. 3B). Additionally, invasion assays were used to measure the invasion ability of the cells through filters coated with reconstituted basement membrane. The results demonstrated that simultaneous silencing of TGF-βRII and FZD-7 inhibited the invasive ability of the cell by 41 and 43% for HepG2 cells and by 41 and 38% for Huh-7 cells compared with the sh-TGF-βRII and sh-FZD-7 groups (Fig. 3C).

Cells arrest at the G1 phase, which contributes to the reduction in cell proliferation. To investigate the mechanisms

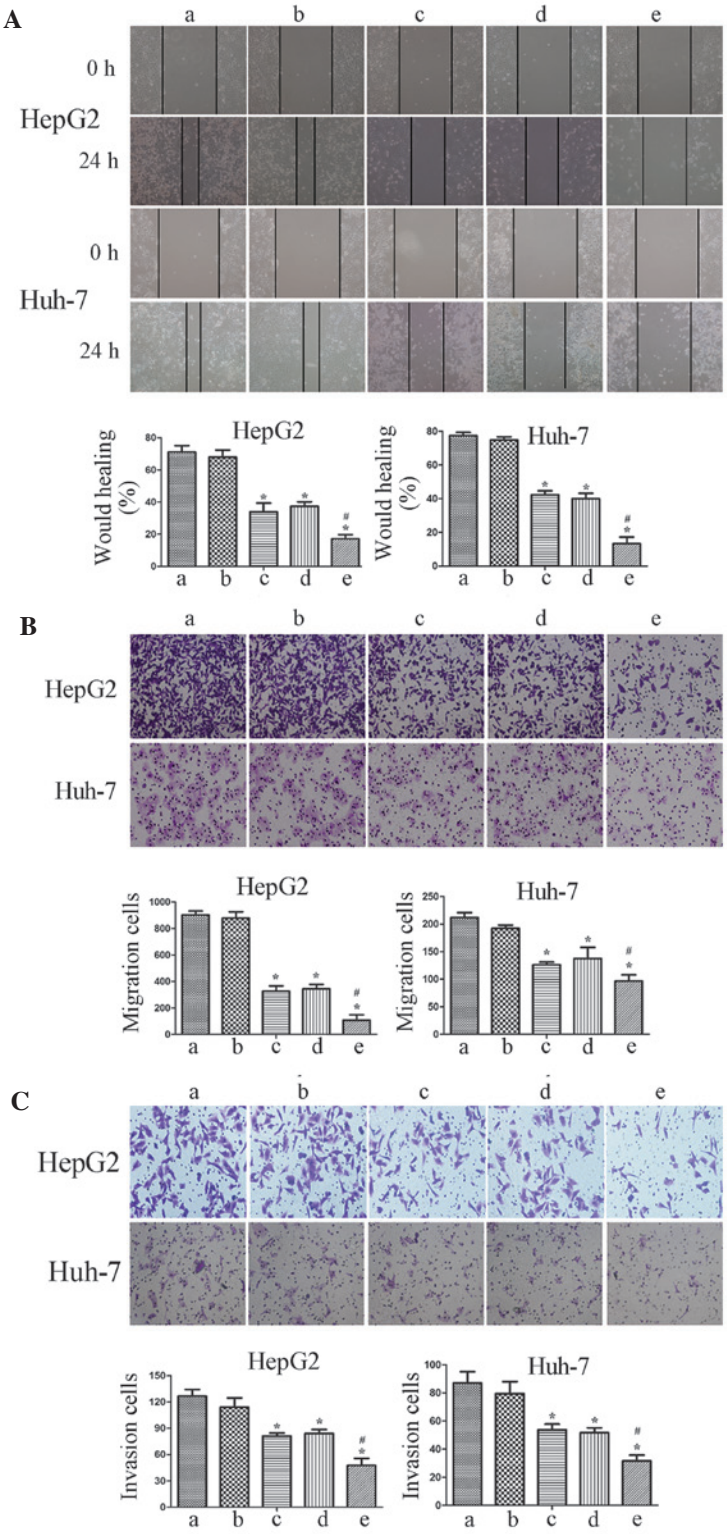

Figure 3. Transforming growth factor-β and Wnt/β-catenin signaling pathways regulate the migration and invasion of human hepatocellular carcinoma cells. (A) A wound healing assay was performed following TGF-βRII and FZD-7 knockdown in HepG2 and Huh-7 cells. (B) A cell migration assay was performed following TGF-βRII and FZD-7 knockdown in HepG2 and Huh-7 cells. (C) Matrigel cell invasion abilities were downregulated in the co-transfection group compared with the single-transfection group in HepG2 and Huh-7 cells. Images of the cells that migrated onto the lower chamber of a Transwell plate were captured under a light microscope, at x200 magnification. Each bar represents three independent experiments presented as the mean ± standard deviation; error bars represent the standard deviation. *P<0.01 compared with a and b; #P<0.05 compared with c and d. TGF-βRII, tranforming growth factor receptor II; FZD, frizzled receptor; NC, negative control; sh, short hairpin RNA; a, blank; b, sh-NC; c, sh-TGF-βRII; d, sh-FZD-7; e, sh-TGF-βRII + sh-FZD-7.

involved in the reduction of cell proliferation in TGF-βRII and FZD-7 knockdown cells, the present study performed flow cytometric analysis. As demonstrated in Fig. 4, TGF-βRII and FZD-7 knockdown cells led to a 18 and 13% increase in the percentage of cells in G0-G1 phase compared with the sh-TGF-βRII and sh-FZD-7 groups for Huh-7 cells. However,

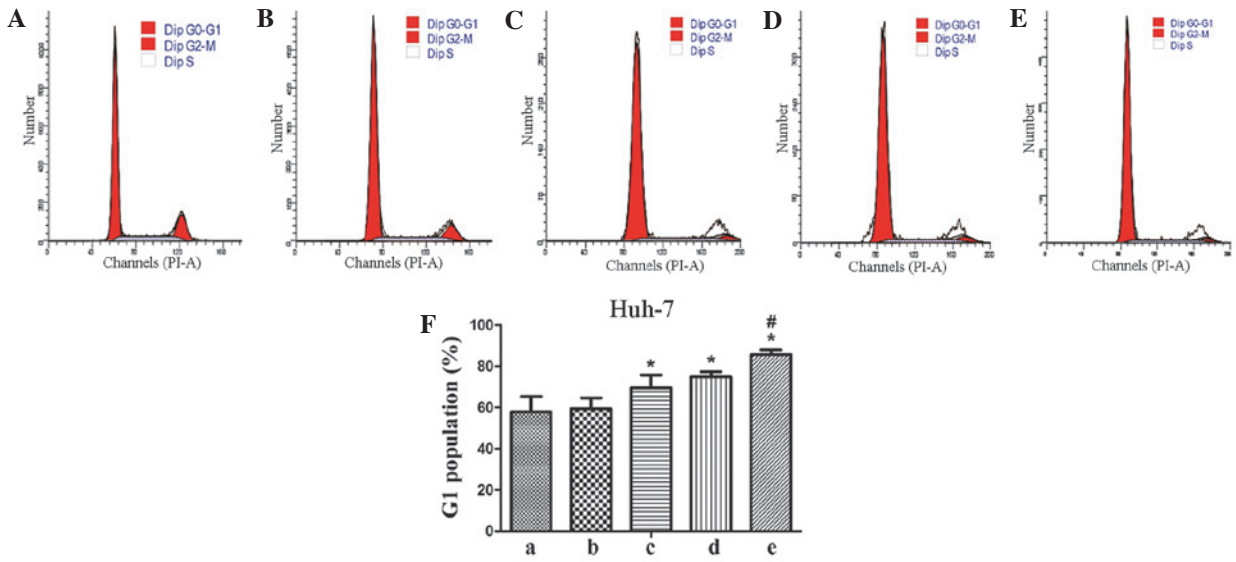

Figure 4. The percentage of G1 population cells was measured using flow cytometry following knockdown of TGF-βRII and FZD-7 in human hepatocellular carcinoma Huh-7 cells as follows: (A) Blank; (B) sh-NC; (C) sh-TGF-βRII; (D) sh-FZD-7; and (E) sh-TGF-βRII + sh-FZD-7. (F) Histogram of the results from flow cytometric analysis. Each bar represents three independent experiments presented as the mean ± standard deviation; error bars represent the standard deviation. *P<0.01 compared with A and B; #P<0.05 compared with C and D. TGF-βRII, tranforming growth factor receptor II; FZD, frizzled receptor; NC, negative control; shRNA, short hairpin RNA.

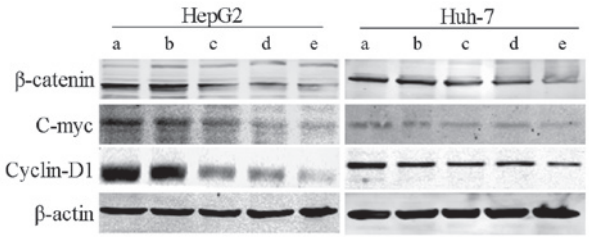

Figure 5. Knockdown of TGF-βRII and FZD-7 suppresses β-catenin, c-Myc and cyclin D1 expression in human hepatocellular carcinoma HepG2 and Huh-7 cells. β-actin was probed as a loading control. TGF-βRII, tranforming growth factor receptor II; FZD, frizzled receptor; NC, negative control; shRNA, short hairpin RNA; a, blank; b, sh-NC; c, sh-TGF-βRII; d, sh-FZD-7; e, sh-TGF-βRII + sh-FZD-7.

a statistical difference was not observed between control and knockdown groups in the percentage of HepG2 cells in G0-G1 phase (data not shown).

The expression of β-catenin, c-Myc and cyclin D1 are down-regulated in knockdown TGF-βRII and FZD-7 HCC cells. In order to investigate the possible underlying mechanisms of reduced proliferation and migration of HCC cells co-transfected with sh-TGF-βRII and sh-FZD-7, the protein expression levels of correlative genes, including β-catenin, c-Myc and cyclin D1, were analyzed. Western blot analysis demonstrated that the β-catenin, c-Myc, cyclin D1 protein levels were markedly reduced in the co-transfection group compared to the single-transfection groups (Fig. 5).

Discussion

TGF-β and Wnt/β-catenin signals composed of two groups of secreted proteins that act simultaneously to stimulate the development of hepatopathy and HCC (17). Accumulating evidence has revealed that TGF-β plays a dual role in cancer progression. At the early stages of tumorigenesis, particularly when the tumor is benign, TGF-β acts directly on the cell to suppress the tumor growth (11). However, as the tumor develops, genetic or biochemical alterations allow TGF-β to stimulate tumor progression in the cancer cell and surrounding non-malignant stromal cells. The stimulation of the migration and invasion abilities of cells by TGF-β may be of a greater clinical consequence compared to its tumor-suppressive role, since the majority of tumors retain functional TGF-β signaling pathways (18). In addition, β-catenin is an essential component of two cellular pathways; maintaining cell-to-cell adhesion and mediating the Wnt/β-catenin signal transduction pathway, which is pivotal in embryogenesis and malignant transformation of cells (19).

The Wnt receptor complex on the cell surface consists of a 7 transmembrane domain FZD protein and a single-pass transmembrane protein from the low density lipoprotein receptor-associated protein family. The activation of the Wnt receptor complex results in the stabilization of cytoplasmic β-catenin (8). Merle *et al* (20) reported that the overexpression of FZD-7 was detected in 90% of HCC cells, the majority of which were associated with chronic hepatitis B virus infection. In addition, a functional analysis demonstrated that the levels of FZD-7 mRNA was associated with enhanced cellular motility. By contrast, the TGF-β signaling pathway exerts its various effects through two transmembrane serine/threonine kinases, termed type I and type II receptors. The ligand-activated type II receptor associates, phosphorylates and activates the type I receptor, which in turn phosphorylates the members of the SMAD protein family (21). These findings indicate that FZD-7 and TGF-βRII are key gene targets for interfering with Wnt/β-catenin and TGF-β signaling pathways.

In the present study, pGPU6/GFP/Neo coding plasmids containing shRNA targeting TGF-βRII and FZD-7 were

constructed to investigate the effects of simultaneously blocking TGF-β and Wnt/β-catenin signaling pathways in HCC cells. The expression levels of TGF-βRII and FZD-7 were determined by RT-PCR and western blot analysis. The present study demonstrated that sh-TGF-βRII-c and sh-FZD-7-2 significantly downregulated the expression of TGF-βRII and FZD-7 in HCC HepG2 and Huh-7 cells. The present results demonstrated that simultaneously suppressing TGF-βRII and FZD-7 significantly inhibited the proliferation of HepG2 and Huh-7 cells. To additionally investigate the possible mechanisms of anti-proliferation efficacy, cell-cycle analysis was performed and a high proportion of cells at G1 phase arrest were observed following a blockade of TGF-βRII and FZD-7 in Huh-7 cells. The DNA content of the cells reflects the specific processes of cell growth and proliferation. In the cell cycle, cells go through various stages of DNA replication: G0 phase, cells are in a quiescent state; G1 phase, cells undergo pre-DNA synthesis; S phase, DNA is synthesized by the cells; G2 phase, DNA in the cells becomes tetraploid and cells reserve energy for mitosis (22). There are various checkpoints for the cells during the cell cycle. One checkpoint is the G1/S transition checkpoint, following which cells are no longer dependent on exogenous proliferative stimulation; therefore; the cells acquire the capacity to independently complete the cell cycle (22). Cyclin D1 forms a complex with cyclin-dependent kinases 4 and 6 and functions as a regulatory subunit of this complex. Cyclin D1 is required to facilitate the transition between G1 and S phases; therefore, it promotes the proliferation of cells and may contribute to tumorigenesis (23,24). In addition, cyclin D1 acts as a downstream effector molecule for Wnt/β-catenin signaling. In the present study, the decrease in cyclin D1 expression following RNA interference contributed to the cells arresting at the G1 phase, which may have prevented the proliferation of HepG2 and Huh-7 cells. As another direct target gene of the Wnt/β-catenin signaling pathway, c-Myc is also an oncogenic transcription factor, which participates in a broad spectrum of physiological and pathological processes, including cell proliferation, apoptosis, differentiation, senescence and angiogenesis (25). c-Myc regulates cells in the G1 phase to progress by positively regulating the activity of cyclin dependent kinases (26-28). Consequently, a suppression of c-Myc expression may reduce the proliferation and genesis of tumors. The present results revealed that the downregulation of cyclin D1 and c-Myc led to cells arresting at the G1 phase; therefore, contributing to the inhibition of the proliferative abilities of the cells, which is consistent with previous studies.

Cell invasion and migration are involved in a number of physiological processes. However, uncontrolled cell migration and invasion may lead to the development of metastasis, which is the cause of ~90% of human cancer-associated mortalities (29). Invasion and migration of cancer cells may be facilitated by TGF-β through various mechanisms, including regulating cell survival, angiogenesis, vascular integrity and interacting with the tumor microenvironment (21). An additional mechanism hypothesized to contribute to metastasis is reversible EMT, through which epithelial cells differentiate into mesenchymal cells (30). EMT usually occurs in tissue morphogenesis during cell development, wound repair and cancer progression in adult tissues (31). TGF-β has been demonstrated to induce a morphological alteration, which affects EMT. The transdifferentiation is accompanied by a reorganization of the actin cytoskeleton, downregulation of adhesion and cytoskeleton molecules and other extracellular matrix associations in the cells, which eventually leads to enhanced migratory and invasive properties of the cell (16). TGF-β may also contribute to the tyrosine phosphorylation of β-catenin, which disrupts the E-cadherin-β-catenin complex leading to the promotion of migration and invasive abilities of the cell (32). In the present study, silencing of TGF-β and Wnt/β-catenin signaling may lead to the E-cadherin-β-catenin complex stimulating the invasion and migration in HepG2 and Huh-7 cells.

Overall, the present results suggest that simultaneous targeting of TGF-β and Wnt/β-catenin signaling pathways exerts synergistic anti-tumor effects on HCC cells. Additional *in vitro*, *in vivo* and clinical studies are required to reveal more information concerning these pathways in the tumorigenesis of cells. The present findings may support the development of a novel treatment strategy that has greater efficacy in the management of HCC.

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