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Journal of Cellular Biochemistry 112:3140–3150 (2011)

Journal of Cellular Biochemistry

shRNA-Mediated Silencing of Gli2 Gene Inhibits Proliferation and Sensitizes Human Hepatocellular Carcinoma Cells Towards TRAIL-Induced Apoptosis

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ABSTRACT

Aberrant activation of the Hedgehog (Hh) signaling pathway has been reported in various cancer types including hepatocellular carcinoma (HCC). As a key effector of this signaling, Gli2 plays a crucial role in carcinogenesis, including the activation of genes encoding apoptosis inhibitors and cell-cycle regulators. In this study, we examined the role of Gli2 proliferation and survival of HCC cells. First, the expression levels of Hh pathway components were detected in a subset of HCC cell lines. To establish the role of Gli2 in maintaining the tumorigenic properties of HCC cells, we developed small hairpin RNA (shRNA) targeting Gli2 and transfected it into SMMC-7721 cell, which was selected with high level of Hh signaling expression. Next, effects of Gli2 gene silencing, on cell proliferation and on the expression of cell cycle-related proteins were evaluated, then, whether down-regulation of Gli2 renders HCC cell susceptible to TRAIL was examined in vitro. Knockdown of Gli2 inhibited cell proliferation and induced G1 phase arrest of cell cycle in SMMC-7721 cell through down-regulation of cyclin D1, cyclinE2, and up-regulation of p21-WAF1. Also, Gli2 gene siliencing sensitized SMMC-7721 cell to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by reducing the expression of the long and short isoform of c-FLIP and Bcl-2, and then augmented the activation of initiator caspases-8/-9 and effector caspases-3, which induces PARP cleavage. In conclusion, our data suggest that Gli2 plays a predominant role in the proliferation and apoptosis resistance of HCC cells, and that knockdown of Gli2 may be a novel anticancer strategy for the treatment of HCC. J. Cell. Biochem. 112: 3140–3150, 2011.

KEY WORDS: Gli2; shRNA; HCC; TRAIL; APOPTOSIS

The role of the Hedgehog (Hh) signaling pathway has been well documented in tissue patterning, cell differentiation and proliferation, and tumorigenesis [Ingham and McMahon, 2001; Rubin and de Sauvage, 2006]. The signaling pathway is initiated by the binding of ligands (Sonic, Indian, and Desert hedgehog) to the transmembrane receptor patched 1(Ptch1). Ptch1 constitutively inhibits a second transmembrane protein Smoothened (Smo). After Sonic hedgehog (Shh) binds to Ptch1, inhibition of Smo is lost, which leads to the activation of a group of transcription factors, including Gli1, Gli2, and Gli3 [Lum and Beachy, 2004]. Gli3 acts primarily as a transcriptional repressor [Ruiz et al., 2002], whereas Gli2 is reported to be the primary activator of Hh pathway and Gli1 is a transcriptional target and downstream of Gli2, which also acts as a transcriptional activator [Thayer et al., 2003], and its up-regulation in response to the activation of the Hh signaling pathway depends on Gli2 protein stabilization.

Gli2 seems to be the major nuclear effector of Hedgehog signaling [Bai et al., 2002; Lipinski et al., 2006] in the nucleus. Gli2 binds to and activates transcription of target genes Gli2 is involved in oncogenesis through the up-regulation of genes encoding apoptosis

Abbreviations: HCC, hepatocellular carcinoma; TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand; Gli, glioma-associated oncogene; Hh, Hedgehog; Shh, Sonic hedgehog; ASO, antisense oligonucleotides; c-FLIP, cellular FLICE-inhibitory protein; shRNA, short hairpin RNA; RT-PCR, reverse transcription-polymerase chain reaction; GFP, green fluorescent protein; FADD, fas-associated protein with death domain; PARP, poly ADP-ribose polymerase; DFF45, DNA fragment factor 45.

Dawei Zhang and Jianping Liu contributed equally to this work, and should be regarded as joint first authors.

Grant sponsor: Science and Technology Planning Project of Guangdong Province, China; Grant numbers: 2005B30301018, 2008B080703054.

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Received 20 January 2011; Accepted 14 June 2011 • DOI 10.1002/jcb.23240 • © 2011 Wiley Periodicals, Inc.
Published online 21 June 2011 in Wiley Online Library (wileyonlinelibrary.com).

inhibitors (e.g., Bcl-2, cFLIP) [Regl et al., 2004; Kump et al., 2008], cell-cycle regulators (e.g., cyclin D1 and c-Myc) [Grachtchouk et al., 2003], and inducers of angiogenesis (e.g., vascular endothelial growth factor) [Eichberger et al., 2006] and the role in carcinogenesis has been shown in several cancer [Berman et al., 2003; Thayer et al., 2003; Katoh and Katoh, 2005; Mukherjee et al., 2006; Thiyagarajan et al., 2007; Velcheti and Govindan, 2007; Ji et al., 2008]. Gli2 protein has been reported to be highly expressed in hepatocellular carcinoma (HCC) cell lines and human HCC tissues [Patil et al., 2006; Sicklick et al., 2006; Kim et al., 2007; Cheng et al., 2009]. Gli2 down-regulation with antisense oligonucleotides (ASO) can inhibit proliferation of various HCC cell lines through down-regulation of c-Myc and Bcl-2 and up-regulation of p27, apart from this, Gli2 down-regulation could overcome the unresponsiveness of some HCC lines to Shh antibody or Smo inhibitor-cyclopamine because of ligand-independent activation mechanisms or frequent epigenetic changes at Smo or its downstream components [Siegelin et al., 2009]. Therefore, because of the critical role of Gli2 in tumor cell survival, proliferation, and angiogenesis, blocking Gli2 transcription factor would be an ideal strategy to combat HCC.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, induces apoptosis in a variety of cancer cells with little or no effect on normal cells [Daniels et al., 2005]. However, recent reports have demonstrated that many tumor cells including HCC cells acquire resistance to the apoptotic effects of TRAIL and this resistance appears to be closely related to deregulated expression of anti-apoptotic molecules [Yamanaka et al., 2000; Koehler et al., 2009]. As target genes of Gli2, c-FLIP (cellular FLICE-inhibitory protein) and Bcl-2 plays a pivotal role in modulating intrinsic and extrinsic apoptotic pathways in HCC cells. The sensitivity of HCC cells to TRAIL can be increased by combining TRAIL with some compounds, which can reduce expression level of c-FLIP or Bcl-2 [Carlisi et al., 2009; Jin et al., 2009]. Siegelin et al. [2009] have reported that KAAD-cyclopamine facilitated TRAIL-mediated apoptosis by up-regulation of DR5 and down-regulation of c-FLIP and Bcl-2 in malignant glioma cells [Mukherjee et al., 2006]. Previous studies demonstrated that Gli2 down-regulation is effective in inducing apoptosis in HCC cells. But little is known about the sensitization to TRAIL induced by Gli2 down-regulation in HCC cells; we have performed a research on this subject.

In this study, the expression of Shh pathway components in human HCC cell lines was analyzed. We then constructed plasmid, which could encode shRNA against Gli2 and transfected it into SMMC-7721 cells, to demonstrate that Gli2 down-regulation overcame resistance to TRAIL via down-regulation of c-FLIP and Bcl-2, consequently leading to induction of caspase-8- or caspase-9-dependent apoptosis pathway in SMMC-7721 cells.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

The human HCC cell lines HepG2 and SMMC-7721 were purchased from Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences (Shanghai, China), PLC/PRF/5 was generously provided by Dr. Junyao Xu (Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, China). These cells were cultured in DMEM (Hyclone), and supplemented with 10% fetal bovine serum (Hyclone) at 37°C in a humidifed 5%CO_2/95% air atmosphere. Adult liver cells were established from the normal liver tissues of HCC patients who underwent curative surgery at Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, China and were cultured in RPMI 1640 supplemented with penicillin and streptomycin. Recombinant human TRAIL (amino acids 114–281) was purchased from PeproTech, Inc. (Rocky Hill), reconstituted at 20 μg/ml in sterile PBS and stored at −20°C.

CONSTRUCTION OF THE Gli2-SPECIFIC SHORT HAIRPIN RNA (SHRNA) EXPRESSION PLASMID

The pGPU6/GFP/Neo siRNA plasmid vector was provided from GenePharma Co., Ltd (Shanghai, China). Four DNA temple oligonucleotides corresponding to Gli2 gene (GenBank NM_005270.3) and a negative control oligonucleotide having no homology with human beings or mice were designed and synthesized as Table I. BLAST search against EST libraries was performed to confirm that no other human gene was targeted. All these sequences were inserted between Bbs I and BamH I enzyme sites of the pGPU6/GFP/Neo vector, respectively. The recombinant plasmids were named pGPU6-sh1, pGPU6-sh2, pGPU6-sh3, pGPU6-sh4 pGPU6-NC. The pGPU6/GFP/Neo was used as vector control and named as pGPU6-VC. The recombinant vectors were confirmed by the digestion analysis of restriction endonuclease, and all the constructed plasmids were

TABLE I. Oligonucleotides Sequence Used in the Construction of shRNA Expression Vectors

Plasmid name sequences of oligonucleotides (S, sense; A, antisense)
pGPU6-sh1 S: 5′-CACCGCAAACACATGACCACCATGCTTCAAGAGAGCATGGTGGTCATGTGTTTGCTTTTTTG-3′
A: 5′-GATCCAAAAAA***GCAAACACATGACCACCATGC***TCTCTTGAAGCATGGTGGTCATGTGTTTGC -3′
pGPU6-sh2 S: 5′-CACCGCAACAAAGCCTTCTCCAACGTTCAAGAGACGTTGGAGAAGGCTTTGTTGCTTTTTTG-3′
A: 5′-GATCCAAAAAA***GCAACAAAGCCTTCTCCAACG***TCTCTTGAACGTTGGAGAAGGCTTTGTTGC-3′
pGPU6-sh3 S: 5′-CACCGGAAGATCTGGACAGGGATGATTCAAGAGATCATCCCTGTCCAGATCTTCCTTTTTTG-3′
A: 5′-GATCCAAAAAA***GGAAGATCTGGACAGGGATGA***TCTCTTGAATCATCCCTGTCCAGATCTTCC-3′
pGPU6-sh4 S: 5′-CACCGCCCTGTCATCTCTGACATCTTTCAAGAGAAGATGTCAGAGATGACAGGGCTTTTTTG-3′
A: 5′-GATCCAAAAAA***GCCCTGTCATCTCTGACATCT***TCTCTTGAAAGATGTCAGAGATGACAGGGC-3′
pGPU6-NC S: 5′-CACC***GTTCTCCGAACGTGTCACGT***CAAGAGATTACGTGACACGTTCGGAGAATTTTTTG-3′
A: 5′-GATCCAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGACGTGACACGTTCGGAGAAC-3′

Target sequences are marked in bold and italic type.

confirmed by DNA sequencing. Each vector contains the neomycin resistance gene to enable G418 selection in mammalian cells.

STABLE TRANSFECTION OF PLASMIDS AND SELECTION

The SMMC-7721 cells were seeded in six-well plates at a density of 2.0×10^4cells/well and cultured overnight to about 80% confluence prior to transfection. Then, cells were transfected with pGPU6-VC, pGPU6-NC, pGPU6-sh1, pGPU6-sh2, pGPU6-sh3, or pGPU6-sh4 using Lipofectamine2000 (Invitrogen) following the manufacturer's specifications and the ratio of the plasmids and the transfection reagent was 1 μg: 1.5 μl. SMMC-7721 cells stably expressing shRNA were selected with medium containing 800 μg/ml G418 (Sigma) 48 h after transfection, After 2 weeks, individual clones were trypsinized and maintained with 400 μg/ml G418. Names of the stable transfected cells were SMMC-7721-VC, SMMC-7721-NC, SMMC-7721-sh1, SMMC-7721-sh2, SMMC-7721-sh3, and SMMC-7721-sh4, they were verified by RT-PCR and Western blot analysis.

RNA EXTRACTION AND REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from Adult liver, PLC/PRF/5, HepG2, and SMMC-7721 cells (untransfected or stably transfected) using the TRIzol reagent (Invitrogen), the concentration and purity of the total RNA were detected with ultraviolet spectrophotometer. RT-PCR reaction was performed using a Primescript™ RT-PCR kit (TaKaRa, Japan) according to the manufacturer's guidelines. Briefly, 1 μg of total RNA was used in a 20 μl reverse transcription assay. Subsequently, 2.5 μl of these cDNA was used in a 25 μl PCR reaction. The reactions were carried out in a DNA Engine Peltier Thermal Cycler (Bio-Rad) with the following conditions: denaturation at 94°C for 30 s, annealing at 53–58°C for 30 s, and extension at 72°C for 40 s for 25–32 cycles, followed by a final extension at 72°C for 7 min. After amplification, 4 μl of PCR product was run on a 2% agarose gel and visualized by ethidium bromide staining. The specific primers sequences, annealing temperature, and cycles are shown in Table II, the house-keeping gene GAPDH from each sample was used as an internal control. The inhibition ratio of Gli2 mRNA expression was calculated with the following formula: inhibition ratio = [1-(SMMC-7721-sh Gli2 density/SMMC-7721-sh GAPDH density)/(SMMC-7721 Gli2 density/SMMC-7721 GAPDH density)] × 100%.

WESTERN BLOT ANALYSIS

Adult liver, PLC/PRF/5, HepG2, and untransfected or stably transfected SMMC-7721 cells treated with 100 ng/ml TRAIL were harvested, washed twice with cold PBS, and lysed with RIPA lysis buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, 100 μg/ml phenylmethylsulfonylfluoride (PMSF), and 1%Triton X-100) on ice for 30 min and cell lysates were centrifuged at 12,000 rpm/min for 30 min at 4°C. The amount of protein in the supernatant was determined using bicinchoninic acid (BCA) protein assay reagent (Pierce). Equal amounts proteins samples (30 μg) were separated on 10% SDS–PAGE gels, and electroblotted onto polyvinylidene fluoride (PVDF) membrane (Millipore). After blocking with 5% nonfat milk in TBST buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for 2 h at room temperature, the membranes were probed with anti-Gli2, anti-cFLIP, anti-β-actin (Santa Cruz, CA), anti-Bcl-2, anti-Bax (Cell Signaling Technology) primary antibodies at 4°C overnight, followed by incubation with secondary antibodies conjugated to horseradish peroxidase (Boster Biological Technology, Wuhan, China) for 1 h at room temperature. Protein bands were visualized by chemiluminescence using Super-ECL kit (Applygen Tech, Beijing, China), followed by exposure to Kodak X-ray film. To measure the expression of each gene, the relative intensity was calculated by comparing it with the intensity of β-actin, using densitometry (Quantity One 4.6.2, Bio-Rad). The cells that had higher inhibitory rate of Gli2 expression were harvested and used for further assays.

MTT ASSAY

The MTT assay was performed to assess the effect of Gli2 on cell proliferation and chemosensitivity to TRAIL. SMMC-7721 and three stably transfected cells (SMMC-7721-VC, SMMC-7721-NC, and SMMC-7721-sh1) were plated in 96-well plates at a density of 5.0×10^3cells/well for proliferation assay. Then for 7 days, every 24 h a batch of cells were stained with 20 μl sterile MTT dye (5 mg/ml; Sigma) at 37°C for 4 h, then culture medium was removed and 150 μl of DMSO was added and thoroughly mixed in for 10 min. Spectrometric absorbance (A) at 490 nm was measured by using a

TABLE II. The Primers Sequence, Annealing Temperature, Product Size, and Cycle of Each Target Gene for RT-PCR

Gene	Primer	Sequence (5'–3')	T_m (°C)	Product size (bp)	Cycle
Shh	Forward	GATGTCTGCTGCTAGTCCTCG	53	289	30
	Reversed	CACCTCTGAGTCATCAGCCTG			
Ptch1	Forward	CAGAGAAGGCTTGTGGCCAC	56	374	30
	Reversed	GCTCAATGACTTCCACCTTCG			
Smo	Forward	GTTCTCCATCAAGAGCAACCAC	54	258	32
	Reversed	CGATTCTTGATCTCACAGTCAGG			
Gli1	Forward	TTCCTACCAGAGTCCCAAGT	57	185	29
	Reversed	CCCTATGTGAAGCCCTATTT			
Gli2	Forward	TGGCCGCTTCAGATGACAGATGTTG	58	200	30
	Reversed	CGTTAGCCGAATGTCAGCCGTGAAG			
c-FLIP	Forward	ATGTCTGCTGAAGTCATCC	55	512	28
	Reversed	ATCCTCACCAATCTCCTGCC			
Bcl-2	Forward	GACTTCGCCGAGATGTCCAG	60	218	28
	Reversed	GTGCAGGTGCCGGTTCAGG			
GAPDH	Forward	ATCTTCCAGGAGCGAGATCCC	58	450	25
	Reversed	CGTTCGGCTCAGGGATGACCT			

microplate reader. The cell proliferation curve was plotted using the absorbance at each time point. The inhibition rate was calculated as [1–A value (transfected cells)/A value (untransfected cells)] ×100%. To assess chemosensitivity to TRAIL, the aforementioned four kinds of cells were seeded in 96-well plates and were cultured for 24 h. Then, the cells were administered with different concentrations of TRAIL at 0, 50, 100, 200, 300, and 400 ng/ml for another 24 h. At the end of the incubation, the cells were treated with MTT as described earlier. The cell survival value index was calculated as [A490 (TRAIL+)/A490(TRAIL−)] ×100%. Each group contained five wells and the experiments were performed in triplicate.

COLONY-FORMATION ASSAY

Approximately 4.0×10^2 untransfected SMMC-7721, stably transfected SMMC-7721-VC, SMMC-7721-NC, and SMMC-7721-sh1 cells were plated in 10-cm culture dishes, respectively. After 15 days, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted.

FLOW CYTOMETRY ANALYSIS OF CELL CYCLE AND APOPTOSIS

SMMC-7721, VC, NC, and sh1 cells were collected during the logarithmic growth phase and then single cell suspensions were prepared and incubated with 70% alcohol overnight at 4°C. The cells were resuspended in phosphate-buffered saline (PBS) containing 20 μg/ml propidium iodide (PI) and 10 μg/ml RNase A for 30 min at room temperature, DNA quantities in different cell cycles (G0/G1, S, and G2/M phases) were analyzed by flow cytometry (BD Biosciences). A total of 5.0×10^5 SMMC-7721, VC, NC, and sh1 cells were seeded in the six wells and cultured for 24 h. Then, the cells were administered with TRAIL (100 ng/ml) for another 24 h and collected, the cells were washed twice with ice cold PBS and resuspended in binding buffer. Annexin V-fluorescein isothiocyanate (FITC; 0.5 μg/ml) and propidium iodide (0.6 μg/ml) were then added to this cell suspension. After 15 min incubation in the dark at room temperature, stained cells were immediately analyzed by FACSCalibur (BD Biosciences). All of the samples were assayed in triplicate.

DETERMINATION OF CASPASE ACTIVITY

The enzymatic activity of the caspase-8 and caspase-3 was measured using a colorimetric assay kit (Keygen Biotech, Nanjing, China) following the manufacturer's protocol. In brief, a total of 5.0×10^5 untransfected and stably transfected SMMC-7721 cells were seeded in the six wells and cultured for 24 h. Then, the cells were administered with TRAIL (100 ng/ml) for another 24 h and harvested, resuspended in 50 μl of lysis buffer and incubated on ice for 30 min. The lysed cells were centrifuged at 12,000 rpm for 30 min, and equal amounts of protein (100 μg per 50 μl) were incubated with 50 μl of 2 × reaction buffer and 5 μl of peptide substrate, DEVD-pNA for caspase-3, IETD-pNA for caspase-8, respectively, at 37°C for 4 h in the dark. The caspase activities were quantified spectrophotometrically at a wavelength of 405 nm.

STATISTICAL ANALYSIS

Data are expressed as mean ± SD and all statistical analyses were performed by using SPSS16.0. Comparisons among all groups were performed with the one-way analysis of variance (ANOVA) test or unpaired Student's *t*-test. Differences were considered significant at $P < 0.05$. The results shown in each of the figures in this article are representative of at least three independent experiments.

RESULTS

DIFFERENTIAL EXPRESSIONS OF SHH PATHWAY GENE IN HCC CELL LINES

In order to analyze whether Shh signaling is present in HCC, we examined expression level of the pathway components in HCC cell lines PLC/PRF/5, HepG2, SMMC-7721, compared with normal adult liver. RT-PCR analyses showed that all of the components of the pathway were expressed in HCC cell lines to different extents, Ptch1, Smo, Gli1, and Gli2 levels were much higher in HCC cell lines, especially in SMMC-7721 relative to adult liver cell (Fig. 1A). Gli2, a transcription factor, considered to be a downstream target of Shh pathway, was significantly overexpressed in SMMC-7721 but almost undetectable in adult liver cell (Fig. 1B). However, the elevated expression of Gli1 was only observed in PLC/PRF/5 and SMMC-7721. To further confirm these findings, we performed western blot for Gli2, the protein was highly expressed in HCC cell lines particularly in SMMC-7721, but not in adult liver cell cells. Therefore, we used SMMC-7721 for the subsequent experiments.

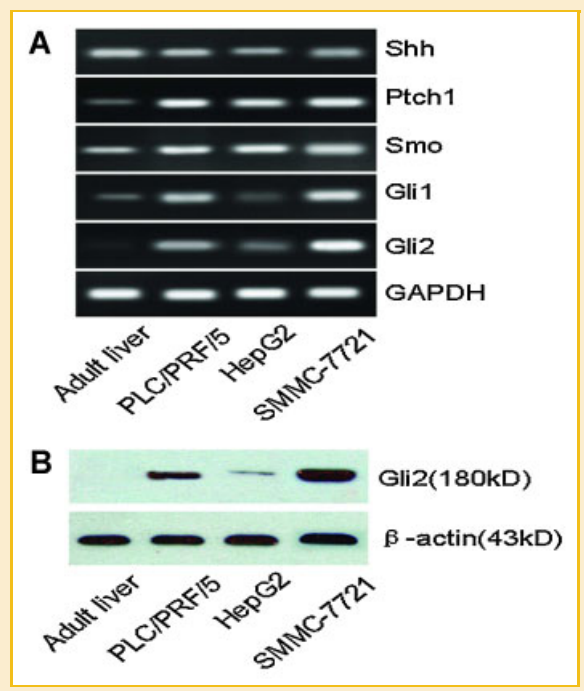

Fig. 1. Expressions of Shh pathway component genes in HCC cell lines and adult liver. A: RT-PCR was performed to detect the expression of Shh, Ptch1, Smo, Gli1, and Gli2. GAPDH level is for normalization purposes. All three HCC cell lines expressed these gene mRNAs and Gli2 is almost undetectable in adult liver. B: Gli2 protein expression was detected by Western blot in HCC cell lines and adult liver. Gli2 protein is present in all the three human HCC cell lines, but not in adult liver cells. Highest expression level of Gli2 was found in SMMC-7721 cells.

Gli2 RNAI CAUSES EFFECTIVE AND SPECIFIC DOWN-REGULATION OF Gli2 EXPRESSION

SMMC-7721 cells were transfected with plasmid pGPU6-VC, pGPU6-NC, pGPU6-sh1, pGPU6-sh2, pGPU6-sh3, and pGPU6-sh4, respectively. Twenty-four hour after transfection, green fluorescent protein (GFP) within the vector pGPU6 was expressed within the cytoplasm of cancer cells, as shown in Figure 2A. Several stably transfected cell colonies were obtained by G418 selection for 2 weeks. Subsequently, RT–PCR and Western blot assays were performed to testify the silencing effect of shRNA-targeting Gli2 gene. As shown in Figure 2B, the expression levels of Gli2 mRNA in SMMC-7721-sh1, SMMC-7721-sh2, and SMMC-7721-sh3 are decreased compared with untransfected SMMC-7721 cells, and the inhibitory rates were 73% for SMMC-7721-sh1, 50% for SMMC-7721-sh2 and 55% for SMMC-7721-sh3. In addition, Western blot analysis (Fig. 2C) showed a reduced expression of Gli2 protein in the same SMMC-7721-sh1, SMMC-7721-sh2, and SMMC-7721-sh3 cells, and the expression inhibitory rates were 68% for SMMC-7721-sh1, 44% for SMMC-7721-sh2 and 51% for SMMC-7721-sh3, respectively. There was no obviously difference in the Gli2 mRNA and protein expression levels among SMMC-7721-sh4, SMMC-7721-VC, SMMC-7721-NC, and SMMC-7721 ($P > 0.05$). The results suggested that pGPU6-sh system could effectively knockdown the endogenous Gli2 expression in SMMC-7721 cells, especially pGPU6-sh1, so we chose SMMC-7721-sh1 cells (stably expressing pGPU6-sh1) for further assays.

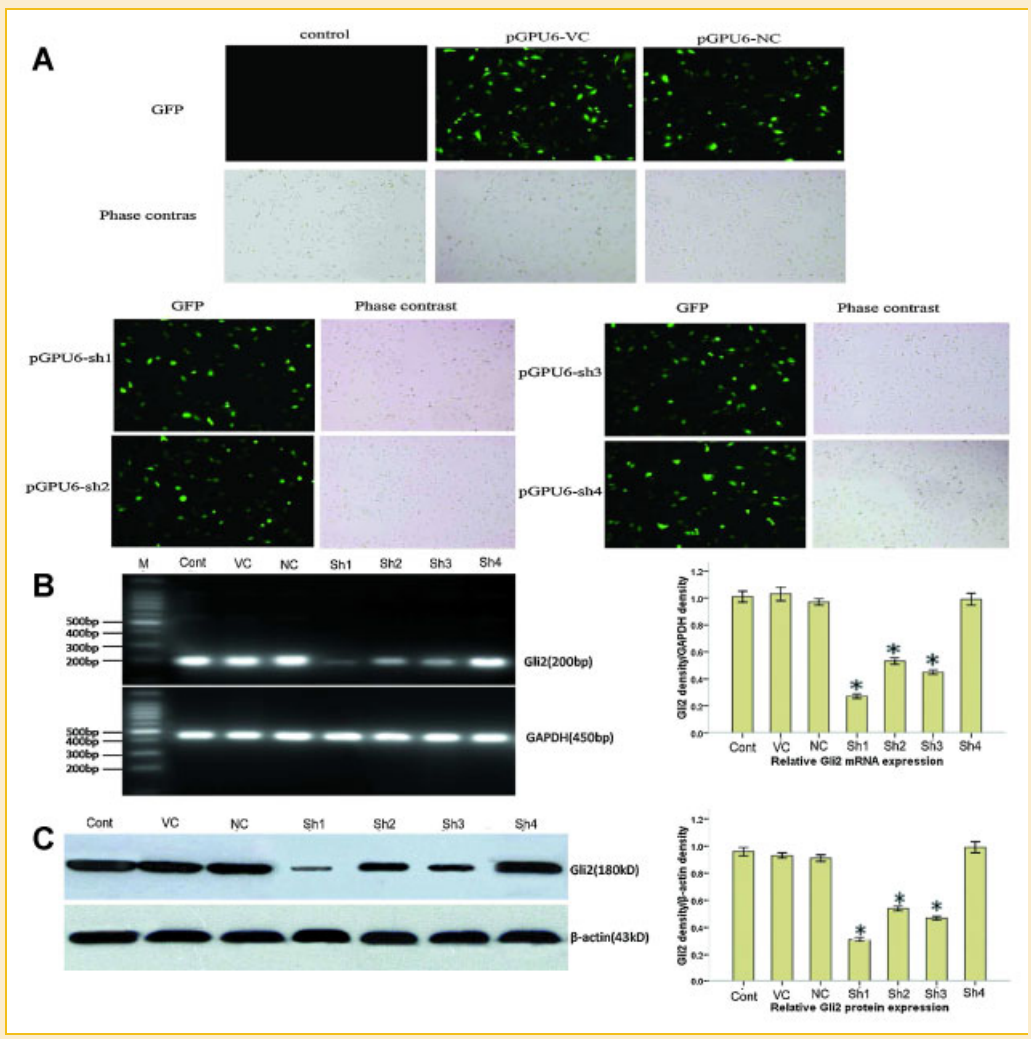

Fig. 2. Gli2-specific shRNAs suppresse expression of Gli2 mRNA and protein in SMMC-7721 cells. A: The green fluorescent protein (GFP) expression of SMMC-7721 cells after shRNA plasmids transfection. The SMMC-7721 cells were transfected with pGPU6-VC, pGPU6-NC, pGPU6-sh1, pGPU6-sh2, pGPU6-sh3, and pGPU6-sh4 using Lipofectamine2000. The transfection efficacy was monitored by GFP reporter connected to pGPU6 vectors. GFP emitted green light (509nm) and the phase contrast images of the same areas were taken after transfection 48 h under a microscope with the blue light (470 nm) excitation. B: The level of Gli2 mRNA was assessed by semi-quantitative RT-PCR. Total RNA was extracted from SMMC-7721(Control), SMMC-7721-VC (VC), SMMC-7721-NC(NC), SMMC-7721-sh1(sh1), SMMC-7721-sh2(sh2), SMMC-7721-sh3(sh3), and SMMC-7721-sh4(sh4). GAPDH was used as normalization control. C: The level of Gli2 protein expression was assessed by Western blotting. β-actin was used as loading control. Densitometric analysis was performed using Quantity one 4.6.2 software. $^{*}P < 0.05$ compared with control. These experiments were performed in triplicate. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

EFFECTS OF Gli2 SILENCING ON CELL PROLIFERATION AND CELL CYCLE DISTRIBUTION IN SMMC-7721 CELLS

MTT assays were performed to evaluate the effect of specific pGPU6-sh1 on SMMC-7721 cell proliferation. The cell growth curve is shown in Figure 3A, compared with SMMC-7721, the growth rate of SMMC-7721-sh1 was inhibited significantly in a time-dependent manner, and the highest inhibitory rate was $41 \pm 1.1\%$ ($P < 0.01$) on day 4. However, there was no obvious difference among SMMC-7721-VC, SMMC-7721-NC, and SMMC-7721 ($P > 0.05$). Next, we explored whether Gli2-shRNA affected colony formation of SMMC-7721 cells, as shown in Figure 3B. The number of colonies of SMMC-7721-sh1 cells were much less than that of SMMC-7721-VC, SMMC-7721-NC, and SMMC-7721 ($P < 0.01$). All these results showed that shRNA-mediated Gli2 down-regulation resulted in marked inhibition of HCC cell proliferation in vitro. The mechanisms of inhibition of cell proliferation were then examined by investigating the cell cycle distribution. As shown in Figure 3C, compared with SMMC-7721, SMMC-7721-VC, or SMMC-7721-NC group, the percentage of SMMC-7721-sh1 cells in the G0-G1 phase were significantly increased ($P < 0.05$), while the percentage in the S phase were significantly decreased ($P < 0.05$). In contrast, SMMC-7721 group showed no significant differences compared with SMMC-7721-VC,

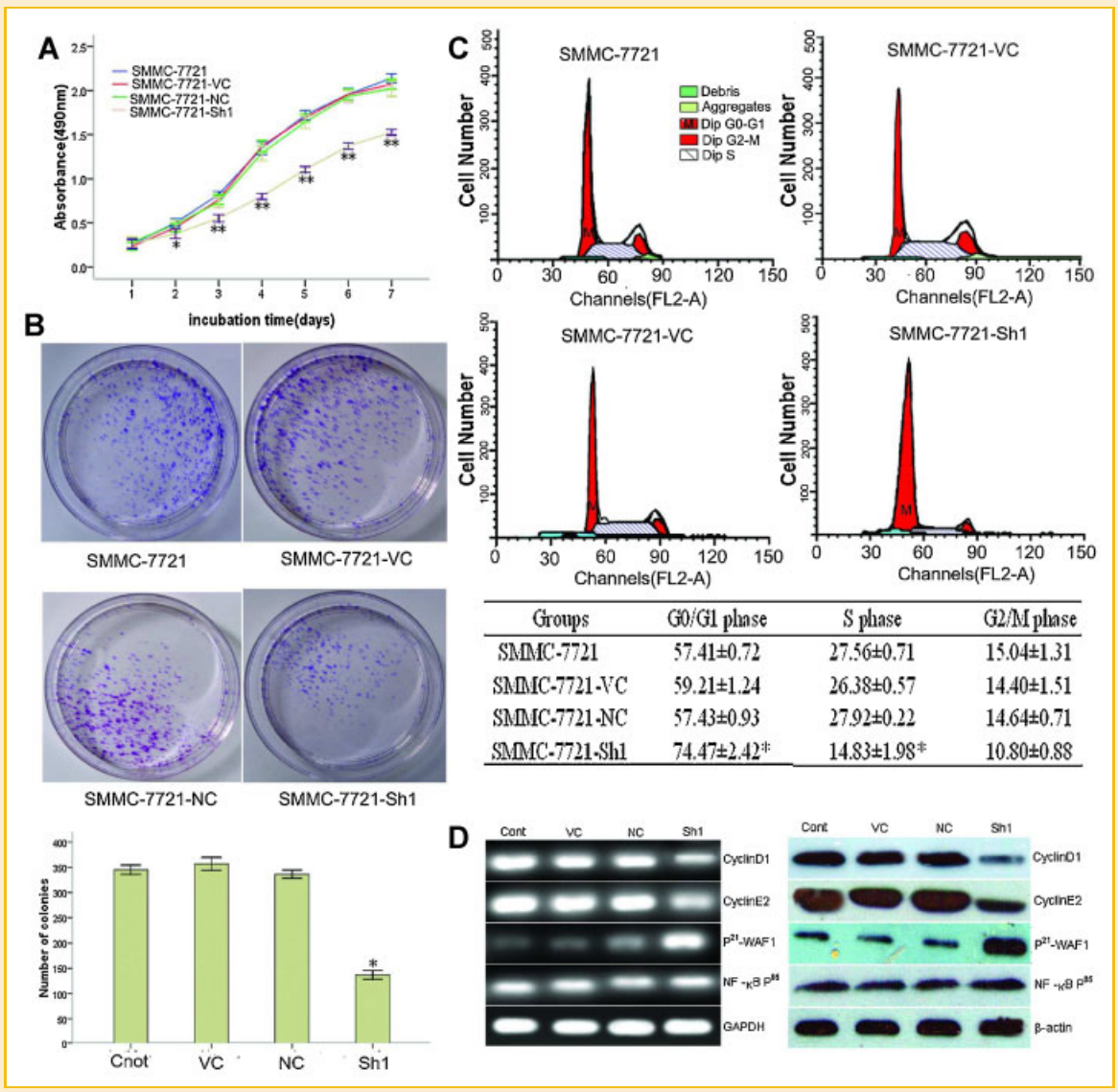

Fig. 3. Effects of down-regulation of Gli2 on cell proliferation and cell cycle. A: Cell viability by MTT. Sh1, VC, NC, and Control cells seeded in 96-well plates were cultured for 7 days; their numbers were determined by absorbance at 490 nm every day. B: Colony-formation assay. Sh1, VC, NC, and Control cells were plated in 10-cm culture dishes, respectively. After 15 days, cells were fixed with methanol and stained with 0.1% crystal violet, visible colonies were manually counted. C: The changes of cell cycle were detected by flow cytometry., Sh1, VC, NC, and Control cells were collected and subsequently analyzed for their DNA content by flow cytometry. D: Effects of Gli2 silencing on expression of cyclin D1, cyclin E2, p21-WAF1, NF-κB p65 were analyzed by RT-PCR and Western blot. GAPDH andβ-actin was used as internal control. These experiments were performed in triplicate, $^{*}P < 0.05$ compared with control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

and SMMC-7721-NC groups ($P > 0.05$). Figure 3D showed the modulation of genes correlated with cell cycle by Gli2 silencing, compared with SMMC-7721, SMMC-7721-VC, and SMMC-7721-NC groups. Levels of cyclin D1 and cyclin E2 decreased in SMMC-7721-sh1 cells, whereas levels of p21-WAF1 were up-regulated in both mRNA and protein levels. We next evaluated the effect Gli2 silencing on activation of the NF-κB pathway, however, Gli2 silencing did not affected the mRNA and protein expression of NF-κB p65. These results indicated that shRNA-mediated down-regulation of Gli2 induced cell cycle arrest in the G0-G1 phase by regulating expression of several cell cycle genes, which correlated with G1-S phase progress.

INHIBITION OF Gli2 SENSITIZES SMMC-7721 CELLS TO TRAIL-MEDIATED APOPTOSIS

The activation of shh signaling pathway has been reported to be associated with chemoresistance in some cancers. We tested if Gli2 silencing could increase the cytotoxic effects of TRAIL in HCC SMMC-7721 cells. MTT assay was performed after the SMMC-7721, SMMC-7721-VC, SMMC-7721-NC, and SMMC-7721-sh1 cells were treated with various concentrations of TRAIL for 24 h. We found that the survival rate in SMMC-7721-sh1 group was significantly lower than the other three groups, and the 50% inhibitory concentration (IC_{50}) values of TRAIL in SMMC-7721-sh1, SMMC-7721, SMMC-7721-VC, and SMMC-7721-NC cells were 159.61 ± 8.13 ng/ml,

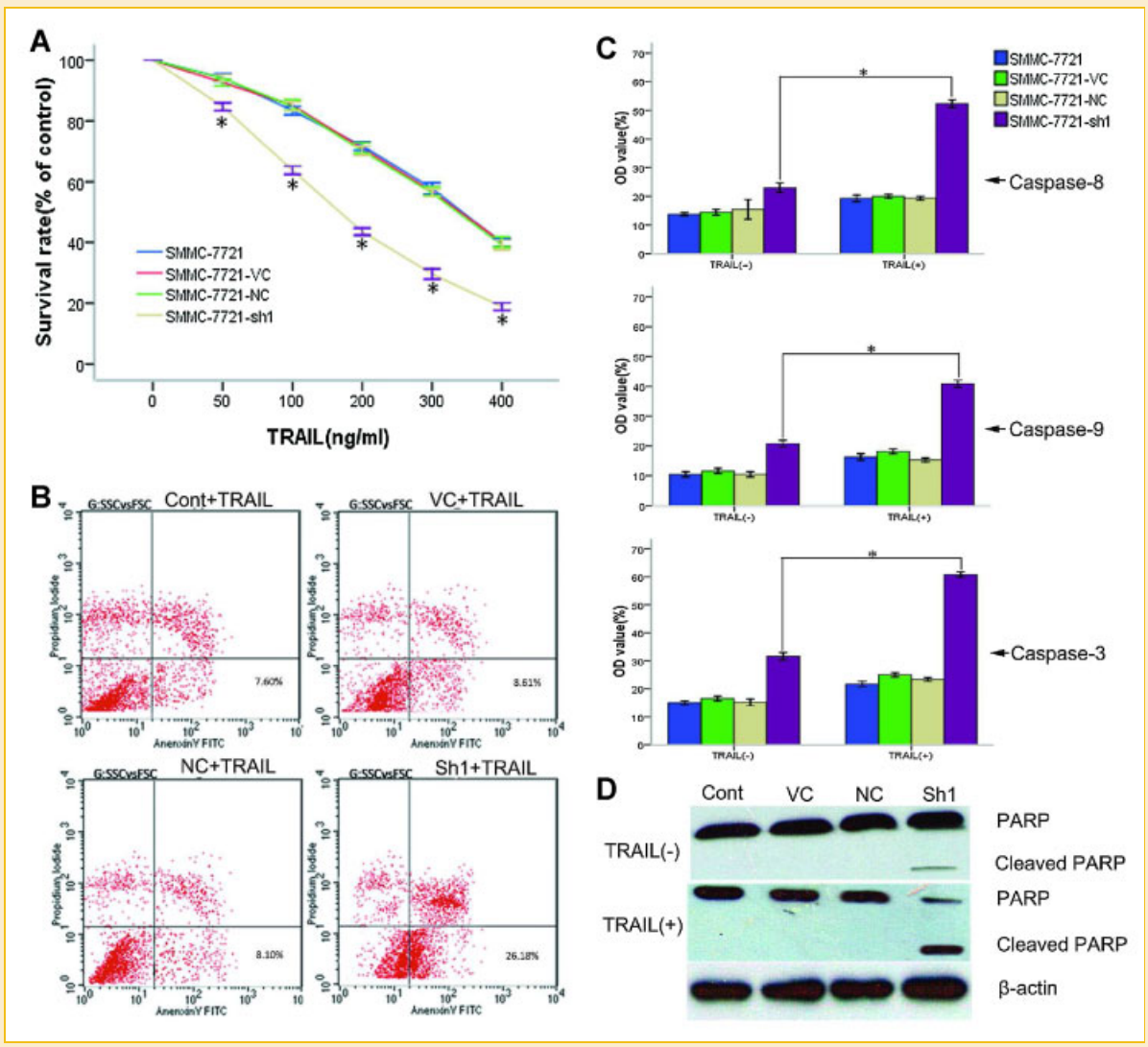

Fig. 4. Effects of Gli2 silencing on the sensitivity of SMMC-7721 cells to TRAIL-induced apoptosis. A: Sh1, were treated with vehicle (TRAIL-) or increasing concentrations of TRAIL for 24 h, cell survival rate was calculated as: Survival rate = [A490 (TRAIL +)/A490(TRAIL-)] × 100%, the IC_{50} value of TRAIL in Sh1 cells was significantly lower than VC, NC, and Control cells**. B: TRAIL-induced apoptosis in Sh1, VC, NC, and Control cells. Apoptotic levels were determined 24 h after TRAIL (100 ng/ml) treatment. C: Sh1, VC, NC, and Control cells were treated with TRAIL (100 ng/ml) for 24 h. Enzymatic activities of -3, -8, and -9 were measured by Colorimetric Assay Kit, caspases levels in the absence of TRAIL were used as control. Data are expressed as the mean ± S.D. of three independent experiments. *$P < 0.05$ compared with control. D: Cleaved PARP expression after TRAIL (100ng/ml) treatment for 24 h in Sh1, VC, NC, and Control cells. Protein was extracted for detection of PARP and cleaved PARP by Western blotting, andβ-actin was used as an internal control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

334.96 ± 10.62 ng/ml, 338.59 ± 9.08 ng/ml, 338.54 ± 10.28 ng/ml ($P < 0.05$), respectively (Fig. 4A). These results indicated that Gli2 knockdown made the cells more sensitive to TRAIL. To determine the effects of Gli2 silencing on TRAIL-mediated apoptosis, SMMC-7721 cells were treated with TRAIL (100 ng/ml) for 24 h. Flow cytometric analysis indicated that the apoptotic rate of SMMC-7721-sh1 cells combined with TRAIL treatment markedly increased to 26.18 ± 1.8% ($P < 0.05$), while there were no obvious differences in apoptotic rates among the SMMC-7721, SMMC-7721-VC, SMMC-7721-NC ($P > 0.05$), which was 7.60 ± 0.8%, 8.61 ± 1.0%, 8.10 ± 1.1%, respectively (Fig. 4B). Caspases are known to act as important mediators of apoptosis through the cleavage of various cellular substrates, so we investigated the activity of caspase-3, -8, and -9 in the Gli2-down-regulated SMMC-7721 cells. As shown in Figure 4C, SMMC-7721-sh1 cells combined with TRAIL showed a significant increase in the activity of caspase-3, -8, and -9, compared with other control cells. Furthermore, SMMC-7721-sh1 cells treated with TRAIL expressed higher levels of cleaved poly ADP-ribose polymerase (PARP) products compared with control, VC, and NC cells treated with TRAIL (Fig. 4D).

KNOCKDOWN OF Gli2 COULD DOWN-REGULATE THE EXPRESSION LEVELS OF c-FLIP AND Bcl-2

To further elucidate the mechanism underlying the enhancement of TRAIL-induced apoptosis by the silencing of Gli2, we analyzed the expression levels of c-FLIP and Bcl-2, which had been reported to inhibit activation of caspase-8, caspase-9, respectively, and confer resistance to TRAIL in some malignancies. As showed in Figure 5A, the levels of mRNA expression of c-FLIP and Bcl-2 in SMMC-7721-sh1 cells were down-regulated compared with those in corresponding control cells. Consistent with the reduction of mRNA, Western blotting showed significantly decreased expression of c-FLIP$_L$, c-FLIP$_S$, and Bcl-2 protein in SMMC-7721-sh1 cells exposed to TRAIL, but there were no obvious change in the level of Bax expression (Fig. 5B). Therefore, it was concluded that the enhancement of chemosensitivity by knockdown of Gli2 in HCC cells might be associated with down-regulation of c-FLIP and Bcl-2 gene which was important in mediating caspases-induced apoptosis.

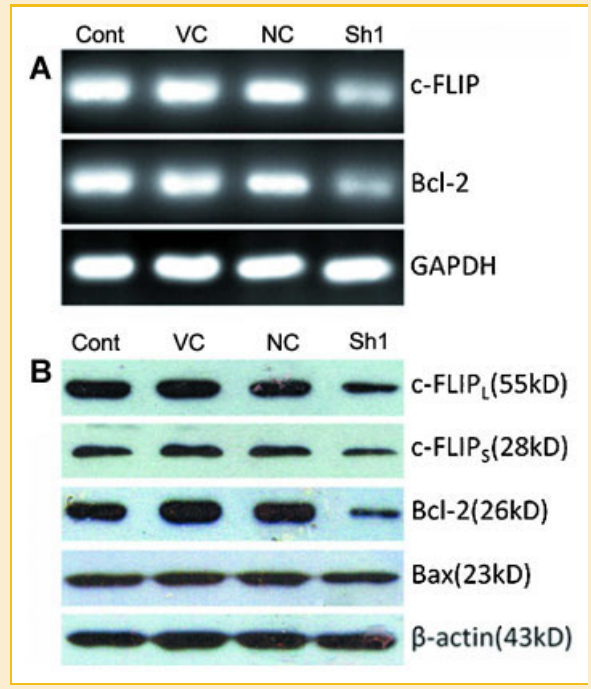

Fig. 5. Effects of Gli2 silencing on expression of anti-apoptotic genes. A: RT-PCR analysis of c-FLIP and Bcl-2 mRNA. After Sh1, VC, NC, and Control cells were treated with TRAIL (100 ng/ml) for 24 h, cells were harvested and total RNA was extracted for detection of c-FLIP and Bcl-2. GAPDH was used as an internal control. B: Western blot analysis of c-FLIP$_L$, c-FLIP$_S$, Bcl-2, and Bax. After Sh1, VC, NC, and Control cells were treated with TRAIL (100 ng/ml) for 24 h, cells were harvested and protein was extracted for detection of c-FLIP$_L$, c-FLIP$_S$, Bcl-2, and Bax. β-actin was used as an internal control. Triplicate experiments showed consistent results. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

DISCUSSION

Previous evidence has demonstrated that constitutive activation of the Hh pathway occurs in various types of malignant tumors such as prostate cancer, pancreatic carcinomas, breast cancer, basal cell carcinoma, and gastrointestinal cancer. Recent studies revealed that the Shh pathway is abnormally activated in human HCC. In this study, we found that HCC cell lines express all the components of the Shh pathway, albeit to different extents. These dates are in line with previous observations that the Shh signaling pathway is constitutively activated in HCC cells.

Since Shh signaling pathway contributes to the induction and maintenance of HCC, it may be a novel therapeutic target in HCC. Several approaches are now being attempted to block the Shh pathway of HCC, including Shh antibody [Thiyagarajan et al., 2007], cyclopamine or KAAD-cyclopamine-antagonists of Smo [Huang et al., 2006; Patil et al., 2006], Gli-1 and Gli2 RNA interference [Kim et al., 2007; Chen et al., 2008]. Treatment with cyclopamine inhibited proliferation, induced apoptosis and repressed c-Myc and cyclinD expression in a subset of HCC cell lines [Patil et al., 2006; Kim et al., 2007]. However, many HCC cell types do not respond to cyclopamine because of the presence of mutations at Smo or its downstream components. As final transcription factors of Shh pathway, specifically down-regulating the expression of Gli may be a preferred approach for blocking the pathway. Previous studies concentrated on the role of Gli1 oncogene in tumorigenicity, Chen et al. [2008] showed that Gli-1 siRNA decreased cell viabilities in Huh7 cells via inducing apoptosis. But, Gli1 was not sufficient to inhibit the proliferation of HCC cells and had little effect on the expression of downstream target genes, whereas Gli2 regulates Gli1 transcription and plays a critical role in the malignant phenotype of several carcinomas, including basal cell carcinoma, medulloblastomas, breast cancer, prostate cancer and HCC [Fulda et al., 2002; Okano et al., 2006; Sicklick et al., 2006; Jin et al., 2009]. Knockdown of Gli2 decreased both Gli1 and other target gene levels and could overcome the unresponsiveness of some HCC cell lines to Shh antibody or the Smo inhibitor-cyclopamine [Siegelin et al., 2009].

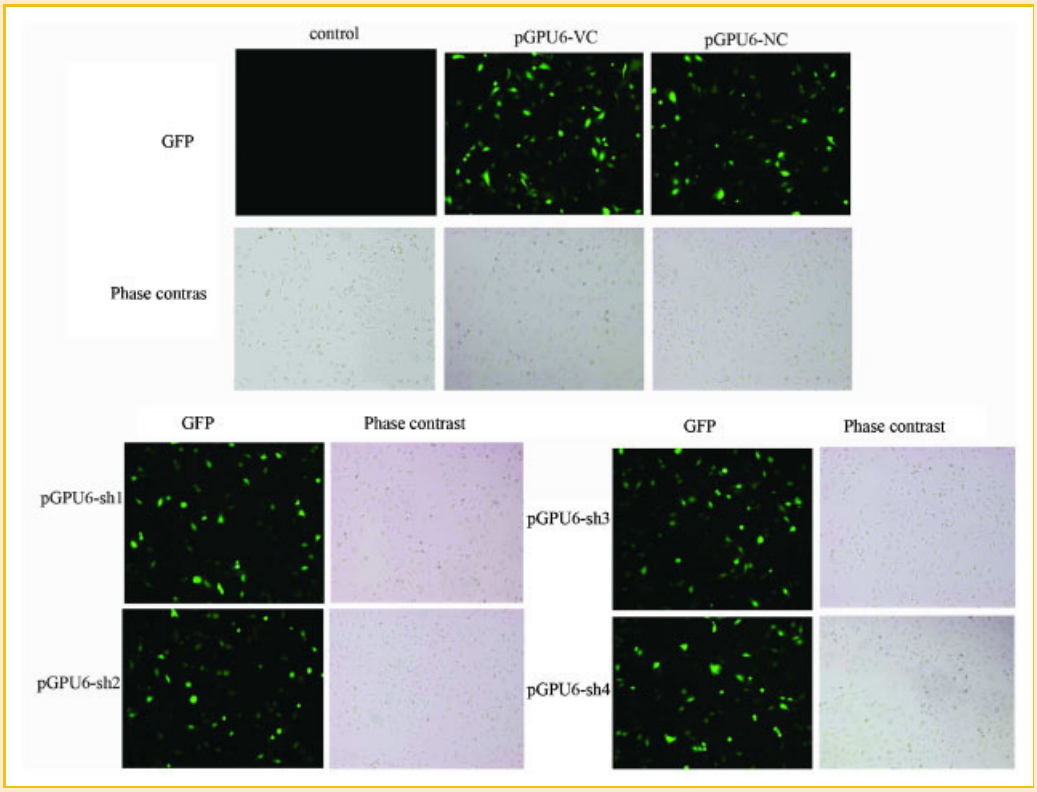

Fig. 6. Higher magnification of Figure 2A. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

To establish the role of Gli2 in maintaining the tumorigenic properties of HCC cells, we constructed four Gli2-specific shRNA expression plasmid (pGPU6-sh1, pGPU6-sh2, pGPU6-sh3, and pGPU6-sh4), and successfully transfected them into SMMC-7721 cells. We measured their inhibitory effects on Gli2 mRNA and protein expression and found that pGPU6-sh1 has a higher inhibitory rate. The present study demonstrated that Gli2 silencing inhibited the proliferation and induced cell-cycle arrest at G1 phase in HCC SMMC-7721 cells. Moreover, we showed that this cell-cycle arrest was accompanied by down-regulation of cyclin D1 and cyclin E2, and up-regulation of p21-WAF1, a known cell cycle inhibitors. Previous studies also showed that Shh inhibition results in cells spending more time in G1 and G2 phase [Agathocleous et al., 2007]. Interestingly, in this study, down-regulation of Gli2 induced cell cycle arrest in the G1 phase, but not G2 phase, and induces changes of several gene associated with cell cycle control, such as cyclin D1, cyclin E2, and p21-WAF1, which have been reported as downstream target genes of Gli2 [Fan and Khavari, 1999]. Shh signaling has also been shown to act upstream of the WNT, TGF-β, and BMI pathways [Taipale and Beachy, 2001; Kopper and Hajdu, 2004; Leung et al., 2004], however, we have not found effects of Gli2 silencing on activation of the NF-κB pathway.

Direct targeting of Gli2 by antisense oligonucleotide has already been shown to inhibit HCC cell proliferation. However, we did not observe enhanced TRAIL-induced apoptosis upon depletion of Gli2. TRAIL can selectively induce apoptosis in human cancer cells and is therefore currently under clinical trials as a cancer therapeutic agent [Ashkenazi et al., 2008; Herbst et al., 2010; Soria et al., 2010]. Previous studies have shown that although HCC cell lines are resistant to TRAIL, combinations of TRAIL with several chemotherapeutic agents have successfully enhanced TRAIL-mediated cancer cell death [Yamanaka et al., 2000; Lacour et al., 2001]. In this study, we found that the depletion of Gli2 resulted in an increased sensitivity to TRAIL and enhance TRAIL-induced apoptosis in SMMC-7721 cells.

Resistance to apoptosis is a major obstacle to TRAIL treatment. Because c-FLIP is known to regulate TRAIL-mediated apoptosis, we analyzed the expression of c-FLIP. We found a significant down-regulation of both mRNA and protein levels of c-FLIP followed by Gli2 silencing in SMMC-7721 cells. Previous studies have demonstrated that c-FLIP is an intracellular inhibitor of caspase-8 that has homology to caspase-8 but has no proteolytic activity. When c-FLIP binds to Fas-associated protein with death domain (FADD), it was thought to antagonize the recruitment and activation of procaspase-8, hereafter, prevent cleavage of procaspase-3 and engagement of extrinsic apoptotic pathway [Krueger et al., 2001; Sakai et al., 2003]. Du et al. [2009] showed that c-FLIP was over expressed in HCC and c-FLIP gene silencing enhanced doxorubicin-induced apoptosis. Activation of the intrinsic apoptotic pathway is another hallmark of TRAIL-induced cell death and is regulated by the proteins of the Bcl-2 family. Several studies showed over-expression of Bcl-2 conferred protection against TRAIL in HCC, Bcl-2 over-expression has been shown to block cleavage of caspases-9, -7, and -3 into active subunits and cleavage of the caspase substrates DFF45 or PARP [Fulda et al., 2002; Han et al., 2008]. In this current study, we also found Gli2

knockdown remarkably decreased expression of Bcl-2 and increased TRAIL-induced apoptosis, but, the expression of Bax protein was invariant. In line with our data, Regl et al. [2004] showed dysregulation of Shh signaling protected tumor cells from apoptosis and expression of the anti-apoptotic factor Bcl-2 is predominantly activated by Gli2 compared with Gli1, which was consistent with Kim report that Bcl-2 level decreased following Gli2 down-regulation and increased significantly only when Gli2 was overexpressed but not when Gli1 was overexpressed [Kim et al., 2007].

Caspases are essential during the process of apoptosis in response to various stimuli; its activation is regulated by various cellular proteins, including c-FLIP and Bcl-2, as mentioned earlier. Active caspases cleave PARP, a protein that acts as a marker of apoptosis. In the present study, Gli2 silencing combined with TRAIL down-regulate c-FLIP and Bcl-2 levels, followed by elevation of caspase-8, -9, -3 enzymatic activity and PARP cleavage. The results, in the light of the fact that c-FLIP and Bcl-2 are specific inhibitor of caspase-8 and caspase-9 activation, respectively, and block caspase-3-mediated apoptosis pathway. Nevertheless, the accurate apoptotic pathway of Gli2 shRNA sensitizing TRAIL-induced apoptosis in SMMC-772 cells through down-regulation of c-FLIP and Bcl-2 needs to be further elucidated in the future research.

In conclusion, we demonstrated for the first time that the knockdown of endogenous Gli2 expression contributes to sensitizing HCC cell line SMMC-7721 to TRAIL-induced apoptosis. The synergistic action is associated with decreased levels of Bcl-2, c-FLIP, and activation of caspase-dependent pathways. The effects of the strategies need further investigation in other HCC cell lines and in vivo.

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