RESEARCH ARTICLE

Knockdown of GCF2/LRRFIP1 by RNAi Causes Cell Growth Inhibition and Increased Apoptosis in Human Hepatoma HepG2 Cells

Jing-Ping Li[1,2], Nai-Xia Cao[1], Ri-Ting Jiang[1], Shao-Jian He[1], Tian-Ming Huang[1], Bo Wu[1], De-Feng Chen[1], Ping Ma[1,3], Li Chen[1], Su-Fang Zhou[1], Xiao-Xun Xie[1], Guo-Rong Luo[1]*

Abstract

Background: GC-binding factor 2 (GCF2) is a transcriptional regulator that represses transcriptional activity of the epidermal growth factor receptor (EGFR) by binding to a specific GC-rich sequence in the EGFR gene promoter. In addition to this function, GCF2 has also been identified as a tumor-associated antigen and regarded as a potentially valuable serum biomarker for early human hepatocellular carcinoma (HCC) diagnosis. GCF2 is high expressed in most HCC tissues and cell lines including HepG2. This study focused on the influence of GCF2 on cell proliferation and apoptosis in HepG2 cells. Materials and Methods: GCF2 expression at both mRNA and protein levels in HepG2 cells was detected with reverse transcription (RT) PCR and Western blotting, respectively. RNA interference (RNAi) technology was used to knock down GCF2 mRNA and protein expression. Afterwards, cell viability was analyzed with a Cell Counting Kit-8 (CCK-8), and cell apoptosis and caspase 3 activity by flow cytometry and with a Caspase 3 Activity Kit, respectively. Results: Specific down-regulation of GCF2 expression caused cell growth inhibition, and increased apoptosis and caspase 3 activity in HepG2 cells. Conclusions: These primary results suggest that GCF2 may influence cell proliferation and apoptosis in HepG2 cells, and also provides a molecular basis for further investigation into the possible mechanism at proliferation and apoptosis in HCC.

Keywords: GC-binding factor 2 - hepatocellular carcinoma - tumor associated-antigen - proliferation - apoptosis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors of the digestive system (Venook et al., 2010). Most typical cases of HCC develop from a viral hepatitis infection (such as hepatitis B, C or D) or cirrhosis (Cho et al., 2011). Although many previous reports have suggested cellular changes and etiological agents can cause HCC, the molecule pathogenesis of HCC is still unclear.

GC-binding factor 2 (GCF2) / leucine-rich repeat (in FLII) interacting protein 1 (LRRFIP1) gene is located on chromosome 2 q37.3 and encodes a protein with a deduced molecular weight of 160 kDa (Reed et al., 1998). GCF2 mRNA was found to widely express as a 4.2 kb mRNA in most human tissues and cell lines. However, it is noteworthy that GCF2 mRNA was strongly expressed in a variety of cancer cell lines (Rikiyama et al., 2003). Many previous reports pertaining to the function of GCF2 have revealed GCF2 is a transcriptional repressor that negatively regulates the promoter activities of the epidermal growth factor receptor (EGFR) (Rikiyama et al., 2003), platelet-derived growth factor A-chain (PDGF-A) (Khachigian et al., 1999), Insulin-like grow factors Π (IGF2) (Eden et al., 2001), tumor necrosis factor alpha (TNF-α) (Suriano et al., 2005) and the glutamine transporter EAAT2 (Mallolas et al., 2006). In addition to its function of transcription repression, GCF2 has recently been identified as a regulator of toll-like receptor (TLR) pathway signaling (Dai et al., 2009). Furthermore, GCF2 was found as the key mediator for the Wnt pathway, the knockdown of GCF2 repressed remarkably cancer cell adhesion, migration and invasion (Ohtsuka et al., 2011; Ariake et al., 2012). Hence, these reports above suggest the functions of GCF2 may be multifarious and closely related to biological behaviors of tumors. Using the technology of serological identification of antigens by recombinant expression cloning (SEREX). We previously identified GCF2 as a HCC associated antigen, which was a valuable serum biomarker to improve early HCC diagnosis (Stenner-Liewen et al., 2000). Recently, an observation in our lab showed one of the alternative splicing regions of

*[1]Department of Histology and Embryology, School of Preclinical Medicine, Guangxi Medical University, Nanning, [2]Department of Histology and Embryology, School of Preclinical Medicine, Guilin Medical University, Guilin, [3]Clinical Skill Learning Center of Zunyi Medical University at Zhuhai, Zhuhai, China *For correspondence: gwongluo@yahoo.com*

GCF2, namely the fifth alternative splicing region (D5), was only found to express in HCC cell lines and HCC tissues, which suggested that D5 of GCF2 was correlated to the development of HCC (unpublished data). Besides, our other observation showed GCF2 was high-expressed in many HCC cell lines such as HepG2, BEL-7404, QGY-7703 and SMMC-7721, especially in HepG2 cells (Jiang et al., 2012). However, the possible role of GCF2 on pathogenesis of HCC is still unclear. Because abnormal cellular proliferation and apoptosis play an important role in pathogenesis of HCC (Tannapfel et al., 1999), we wonder if GCF2 influences cell proliferation and apoptosis in HCC.

On basis of high-expression of GCF2 in HepG2 cells, we chose HepG2 cell line as the subject investigated in the present study. We first designed four siRNAs targeting different sites of human GCF2 mRNA to select the most effective small interference RNA (siRNA) that greatly decreased GCF2 gene expression. Afterwards, the most effective siRNA (namely siRNA-1) was transiently transfected to HepG2 cells to knock down GCF2 protein expression. Finally, we detected cell viability, apoptosis and caspase 3 activity after down-regulation of GCF2 in HepG2 cells. Our data show that specific down-regulation of GCF2 causes cellular proliferation inhibition and apoptosis increase in HepG2 cells. These results provide a molecular basis for further investigation into the possible mechanism at cell proliferation and apoptosis in HCC.

Materials and Methods

Cell lines and culture

Human primary hepatocellular cancer cell lines HepG2 and normal liver cell line HL-7702 were purchased from Shanghai Institute of Cell Biology of Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal boveine serum (Gibco) and 1×10^5 U/L penicillin G and streptomycin at 37°C in a 5% CO_2 incubator.

Selection of the most effective siRNA

In order to select the most effective siRNA to knock down GCF2 expression, four siRNAs targeting different sites of human GCF2 mRNA (GenBank accession no. NM-001137551) were designed and synthesized by GenePharma Co. (Shanghai, China), and a control siRNA that could not target GCF2 mRNA was synthesized as a negative control (N.C. for short). Additionally, normal HepG2 cells served as an untreated control (Untreated for short). All the siRNAs mentioned above were detailed in Table 1.

HepG2 cells (1×10^5) were seeded on 24-well plates and incubated for 24hours before transfection. The next day, cells were grown to 50-60% confluence and initially transfected with 0.8 μg of siRNA by using lipofactamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

The most effective siRNA was selected from the four siRNAs by comparing GCF2 mRNA expression level after transfection in HepG2 cells. On basis of the data in our preliminary experiment (unpublished data), we selected 48 hours after transfection as the optimal time point for assessing the interference effect of these siRNAs in the present study.

Therefore, at 48 hours after transfection, total RNA in HepG2 cells was extracted using a RNAiso Plus kit (TaKaRa Bio, Japan) according to the manufacturer's instructions. RT-PCR was performed using a PrimeScript™ One Step RT-PCR Kit (TaKaRa) as described by the manufacturer. The specific primers for tested genes were as follows: 5'-GAC TTC CGA CAC CCT CAA T-3' (forward) and 5'-GGG GAC CTC TAC CAT ACA TT-3' (reverse). GAPDH was served as an internal control for normalization and the primers for PCR were as follows: 5'-CAA GGT CAT CCA TGA CAA CTT TG-3' (forward) and 5'- GTC CAC CAC CCT GTT GCT GTA G-3' (reverse). Thermal cycle conditions of PCR amplification were as follows: 94 for 3 min, followed by 35 cycles of 94°C 30 s, 55°C 30 s, 72°C 30 s, and then a final extension at 72°C for 10 min. The PCR product of GCF2 and GAPDH
were 592 bp and 496 bp, respectively.

Detection of GCF2 protein expression after transfection

In order to confirm the interference effect of the most effective siRNA on GCF2 protein expression, GCF2 protein in HepG2 cells transfected with the siRNA was measured by western blot and the procedure was performed as before (Zhou et al., 2006). Meanwhile, as control subjects, GCF2 protein expression in untreated control (Untreated) and negative control (N.C.) were also measured to help to assess interference effect of the siRNA.

Cell viability assay

Cells (1×10^3) were seeded on 96-well plates. Cell viability was assessed with a Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) at 24 hours, 48 hours, 72 hours and 96 hours after transfection. The absorbance was measured at 450 nm (OD450) with an ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA). Experiment was done in triplicate and average OD450 was used to calculate the growth inhibition rate (IR) by the formula: IR= (1- average OD450 of treated group/average OD450 of normal control) ×100%.

Detection of apoptotic incidence by flow cytometry

Cell apoptosis at 48 hour after transfection was performed using a Alexa Fluor® 488 Annexin V/

Table 1. siRNA Sequence Used in this Study

Name	siRNA Sequence
siRNA-1	Sense : 5'-GGAAAUCAAGGACUCUCUAGCAGA A-3'
	Anti-sense: 5'-UUCUGCUAGAGAGUCCUUGAUUUCC-3'
siRNA-2	Sense: 5'- GCUAGACAAUGAAAAGACATT-3'
	Anti-sense: 5'- UGUCUUUUCAUUGUCUAGCTT-3'
siRNA-3	Sense: 5'- CAGUAUACUGCAAUUUCAGUUUGCU-3'
	Anti-sense:5'-AGCAAACUGAAAUUGCAGUAUACUG-3'
siRNA-4	Sense: 5'- CCGACACCCUCAAUAAUGUTT-3'
	Anti-sense: 5'- ACAUUAUUGAGGGUGUCGGTT-3'
N.C.	Sense: 5'-UUCUCCGAACGUGUCACGUTT-3'
	Anti-sense: 5'-ACGUGACACGUUCGGAGAATT-3'

*Negative control is called N.C. For short

Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer's instructions. Analysis was repeated three times and results shown were averages from three analysis.

Caspase 3 activity assay

Caspase 3 activity was tested by Caspase 3 Activity Kit (Beyotime) in the light of the manufacture's instructions. The kit is based on the ability that caspase 3 catalyzes acetyl-Asp-Glu-Val-Asp p-nitroanilide to the yellow formazan product, p-nitroaniline. According to the manufacturer's instruction, HepG2 cells were lysed and washed with cold PBS. The mixture containing 80μL of reaction buffer, 10μL of cell lysates and 10μL of caspase 3 substrate (2 mM acetyl-Asp-Glu-Val-Asp p-nitroanilide) was prepared in 96-well microtiter plate and then incubated at 37°C for 2 hours. Caspase 3 activity was measured in the samples by microplate spectrophotometer at an absorbance of 405 nm (OD405). Caspase 3 activity was expressed as the OD405 produced by the yellow formazan product (p-nitroaniline) in catalytic reaction. Analysis was done three times and average OD405 was used to assess caspase 3 activity.

Statistical analysis

The data of experiment were expressed as means±standard deviation (SD). Statistical analyses were performed using SPSS Version 17. 0 for Windows. A value of $p<0.05$ was considered statistically significant.

Results

siRNA-1 was the most effective siRNA

The RT-PCR analysis results showed the relative level of GCF2 mRNA in HepG2 cells transfected with siRNA-1 was lowest in all HepG2 cells transfected with different siRNAs or controls at 48 hours after transfection (Figure 1A). Hence, the siRNA-1 was selected as the most effective siRNA for the continued experiment.

siRNA-1 down-regulates GCF2 protein expression

To confirm the interference effect of siRNA-1 on protein expression of GCF2, GCF2 protein expression was examined in HepG2 cells by western blot. The results revealed that GCF2 protein expression was decreased at 48 hours after transfection with siRNA-1 (Figure 1B). The protein expression inhibition rate of GCF2 was about 47.17 %. The result further confirmed that siRNA-1 was an effective interference sequence which can be used for the continued experiment.

Down-regulation of GCF2 inhibits cell proliferation

The results from Cell Counting Kit-8 assay showed that cell viability was reduced at 24 hours, 48 hours, 72 hours and 96 hours after transfection with siRNA-1 (Figure 2), and cell proliferation inhibition rates were 25.08 %, 43.65 %. 18.76 % and 16.55 %, respectively. Furthermore, the cell viability at 48 hours after transfection with siRNA-1 was reduced significantly as compared with negative control (N.C.) and untreated control (Untreated). Thus, these data imply that GCF2 may influence cellular proliferation in HepG2 cells.

Knockdown of GCF2 increases cell apoptosis

Analysis of apoptotic cells revealed an obvious increase of the percentage of apoptotic cells in HepG2 cells after knockdown of GCF2 expression. In comparison with negative control (N.C.) or untreated control (Untreated), the average of the percentage of apoptotic cells was more higher in HepG2 cells after transfection with siRNA-1

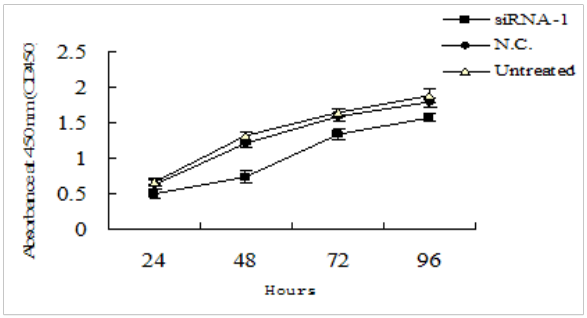

Figure 2. Cell Viability was Measured in the Samples by ELISA Reader at an Absorbance of 450 nm (OD405). The vertical axis represents OD450 and axis horizontal represents the time after transfection. Experiment was done in triplicate and average OD450 was used to calculate the growth inhibition rate (IR) by the formula: IR= (1- average OD450 of treated group/average OD450 of normal control) ×100%. The figure shows a significantly lower level of the OD450 at 48h after transfection with siRNA-1 compared with N.C. or Untreated control

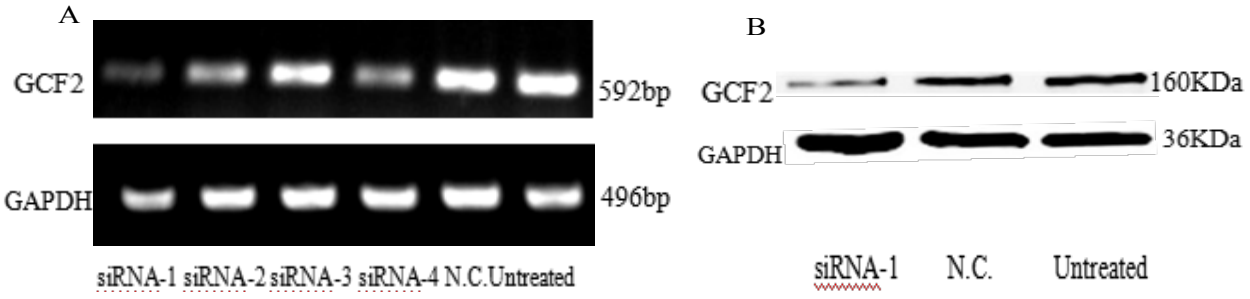

Figure 1. GCF2 Expression in HepG2 Cells Transfected with siRNAs for 48 hours. (A) PCR products for GCF2 mRNA were electrophoresed through a 1.0 % agarose gel. Lane 1 to 5, HepG2 cells transfected with siRNA-1, siRNA-2, siRNA-3, siRNA-4 and negative control sequence (N.C.), respectively. Lane 6, normal HepG2 cells (Untreated control). (B) Western blot analysis for GCF2 protein expression in HepG2 cells. Lane 1, HepG2 cells transfected with siRNA-1, Lane 2, HepG2 cells transfected with N.C; Lane 3, Untreated control. GAPDH in each lane served as an internal control for normalization. These results above confirmed that siRNA-1 was an effective interference sequence

(Figure 3, Table 2). Evidently, the results suggest that GCF2 may influence cellular apoptosis in HepG2 cells.

Reduced GCF2 expression improves caspase 3 activity

Since caspase activation is one of the characteristic symbols of cell apoptotic process and caspase 3 severs as an executioner in the execution-phase of cell apoptosis (Frejlich et al., 2013; Donato et al., 2014), we detected caspase 3 activity in HepG2 cells after transfection. The result from the enzyme activity analysis showed an increase of caspase 3 activity in HepG2 cells transfected with siRNA-1 than that of negative control (N.C.) or untreated control (Untreated) (Figure 4). Furthermore, the caspase 3 activity at 48 hours after transfection with siRNA-1 was increased significantly as compared with negative control (N.C.) and untreated control (Untreated). These data suggest that GCF2 may influence cell apoptosis and some cell death signals may be activated along with down-regulation of GCF2.

Discussion

The unbalance between proliferation and apoptosis plays a role in the pathogenesis of cancer (Tannapfel et al., 1999; Wong 2011). GCF2 is a transcriptional repressor that decreased the promoter activity of EGFR, PDGF-A and other several genes by binding to GC-rich sequences in their promoters (Reed et al., 1998; Khachigian et al., 1999; Rikiyama et al., 2003). In addition to its function of transcription repression, GCF2 can promote adhesion, migration, metastasis, and invasion via RhoA activation in cancer cells (Ohtsuka et al., 2011; Ariake et al., 2012). These studies suggest the functions of GCF2 may be multifarious and closely related to cancer. Using the SEREX approach, we previously constructed cDNA expression library derived from HCC patients in Guangxi population and identified three cDNA clones (Stenner-Liewen et al., 2000; Zhou et al., 2006). We found one of the cDNAs, namely HOM-HCC 2.5.1, shared part sequences with GCF2 cDNA and coded some amino acid sequences of GCF2 protein. Surprisingly, frequency analysis of antibody responses further revealed that GCF2 antibody appeared at a high percentage in allogenic serum of HCC patients detected, but normal individuals were rarely antibody-positive reaction, hence GCF2 was identified as a HCC associated antigen and linked to HCC (Stenner-Liewen et al., 2000). In common with other solid tumors, a major feature of HCC is a combination of unrestrained cell proliferation and impaired apoptosis (Li et al., 2014). Since GCF2 is transcriptional regulator, we wonder if GCF2 influences these two kinds of biological behaviours in HCC cell lines HepG2.

Efficient gene silencing is the cornerstone of the whole experiment. RNAi (RNA interference) is a naturally occurring mechanism that causes gene silencing by the introduction of double-stranded RNAs (dsRNAs) (Peng

Table 2. Cell Apoptosis Analysis by Flow Cytometry (%)

Groups	First time	Second time	Third time	Mean±SD
siRNA-1	15.7±1.64	12.5±1.37	18.2±1.79	15.4±1.60*
N.C.	8.5±1.35	7.9±1.06	9.0±1.25	8.37±1.22
Untreated	8.1±1.07	7.2±0.95	8.2±1.19	7.83±1.07

**p<0.05;Data are expressed as mean±SD for three experiments. A significantly higher of apoptotic cells percentage after transfection was present compared with N.C or Untreated control

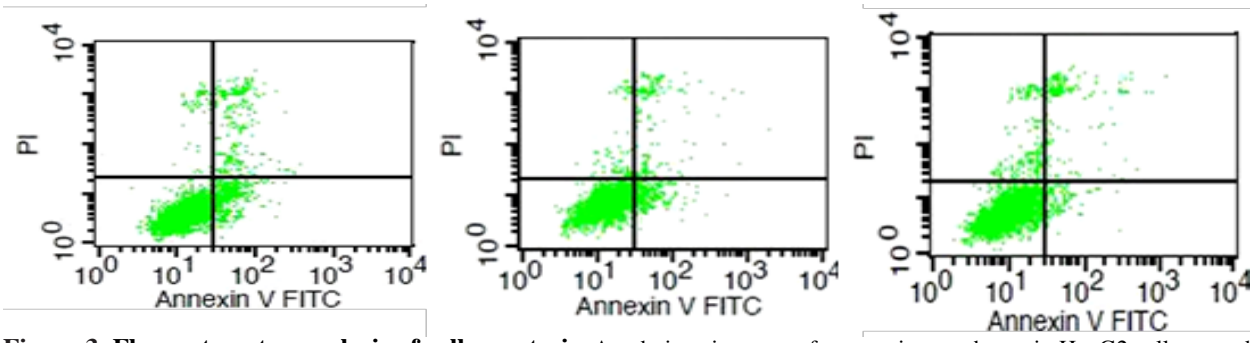

Figure 3. Flow cytometry analysis of cell apoptosis. An obvious increase of apoptosis was shown in HepG2 cells treated with siRNA-1 as compared with N.C or Untreated control. Analysis was repeated 3 times and the average was calculated

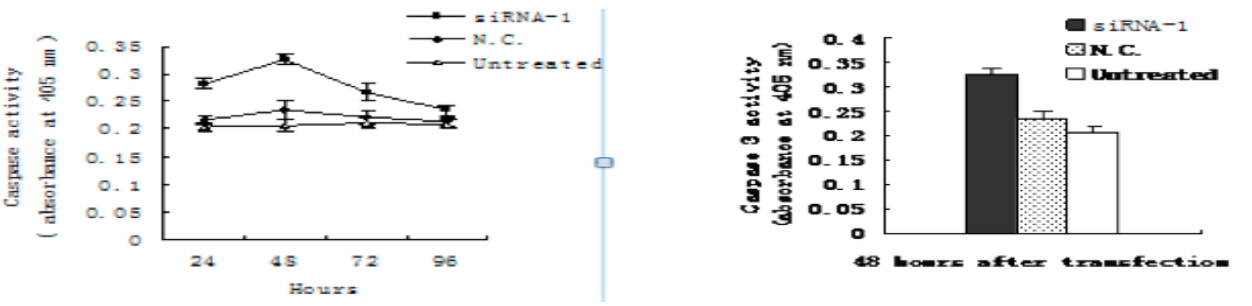

Figure 4. Caspase 3 Activity was Measured in the Samples by Microplate Spectrophotometer at an Absorbance of 405 nm (OD405). Caspase 3 activity was expressed as the OD405 produced by the yellow formazan product (p-nitroaniline) in catalytic reaction. The vertical axis shows the absorbance at 405 nm (OD405), and the axis horizontal shows the time after transfection. A significantly increase of absorbance at 48 hours after transfection with siRNA-1 was present compared with N.C or Untreated control

et al., 2013; Giraud et al., 2014). One of critical steps for efficient gene silencing by RNAi is the design and selection of siRNAs that maximally knock down the expression of target genes. In the present study, we first designed four siRNAs targeting different sites of human GCF2 mRNA, and then selected the most effective siRNA on the basis of the relative expression level of GCF2 mRNA after transfection. The results have revealed the siRNA-1 is the most effective siRNA which effectively inhibits GCF2 expression in HepG2 cells. Thus, selection of siRNA-1 gives a basis for the continued observation of GCF2's role on proliferation and apoptosis in HepG2 cells.

It is generally known that uncontrolled cell proliferation is one of characteristics of cancer. In the present study, we have observed that down-regulation of GCF2 can inhibit cell growth in HepG2 cells. The result suggests some programs involving with cell proliferation may be launched after knockdown of GCF2 in HepG2 cells, although the exact mechanisms are not understood. Therefore, GCF2 may play important role in the process of cell proliferation in HCC. This idea is supported by some observations. First, GCF2 is over-expressed in a variety of HCC cell lines, including HepG2 (Reed et al., 1998; Rikiyama et al., 2003). Second, GCF2 can negatively regulate some growth factors expression, such as PDGF-A, vascular endothelial cell growth factor (VEGF) and IGF2 (Khachigian et al., 1999; Eden et al., 2001). Third, GCF2 partakes in Wnt signaling that regulates cell proliferation by controlling proteolysis of a key signaling protein (Liu et al., 2014) .

Apoptosis plays an important role in the balance between the production of new cells and cell death in a tissue. However, too little apoptosis occurs in cancer so that malignant cells will not die (Wong, 2011). The activation of caspase activation is one of the characteristic symbols in apoptotic process, and caspase 3 severs as an executioner of apoptosis. The apoptosis analysis in this study has revealed that an obvious increase both on cell apoptosis percentage and caspase 3 activity after knockdown of GCF2, suggesting some apoptotic signal paths may be opened again in HepG2 cells. Cell proliferation and apoptosis are often related to each other (Wang et al., 2013). The result is also consistent with the preceding result that down-regulation of GCF2 inhibits cell growth in HepG2 cells in this study. Thus, GCF2 may play important roles in the process of cell apoptosis in HCC. This idea is also supported by several observations. First, GCF2 is a transcriptional repressor that repressed TNF-a expression, whereas TNF-a mediates some cell death signal paths (Suriano et al., 2005; Schlatter et al., 2011). Second, GCF2 is over-expressed in many HCC cell lines such as HepG2 and SMMC-7721, but too little apoptosis occurs in these cancer cells (Jiang et al., 2012). Third, GCF2 interacts with the tumor suppressor spleen tyrosine kinase and negatively regulates nuclear factor kappa B activation induced by TNF (Zhou and Geahlen, 2009). Lastly, using technology of chromatin immunoprecipitation and hybridization of high-density promoter microarray (ChIP-chip), we have recently identified many candidate target genes which specifically bind GCF2 in HepG2 cells, and one of the candidate target genes, such as fibroblast growth factor receptor2 (FGFR2), vascular endothelial growth factor-A (VEGFA), inhibitor of growth protein 2 (ING2) and tumor necrosis factor receptor-associated factor 3 (TRAF3) are closely related to cell proliferation and apoptosis (data not published), although the exact mechanism are not understood.

In conclusion, our findings suggest that down-regulation of GCF2 causes cell growth inhibition and apoptosis increase in HepG2 cells. These primary results indicate that GCF2 may influence the process of proliferation and apoptosis in HepG2 cells. Future studies will be performed to identify the exact mechanisms which are responsible for cell proliferation and apoptosis in signaling pathways.

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