VIROLOGICA SINICA, October 2007, 22 (5):366-373
CLC number: Q78 Document code: A Article ID: 1674-0769(2007)05-0366-08

Construction of shRNA of Fulminant Hepatitis Related Gene *mfgl2* and Investigation of Its Biological Effects *in vitro*[*]

Dong XI[#], Zhi-Mo WANG[#], Sui GAO, Chuan-Long ZHU, Jian-Wen GUO, Xiao-Ping LUO and Qin Ning[**]

(Laboratory of Infectious Immunology, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China)

Abstract: This study was designed to explore the RNA interference technique in inhibition of the expression of the mouse fibrinogen like protein 2 (*mfgl2*), which has been reported to be involved in the development a variety of diseases including fulminant viral hepatitis. A plasmid named p-*mfgl2*shRNA, complementary to the sequence of *mfgl2* was constructed, while another short hairpin RNA (shRNA) which was a mutated form of the *mfgl2*shRNA sequences was used as a control. A plasmid named pEGFP-*mfgl2* expressing the *mfgl2*-EGFP fusion protein was also constructed for the screening of the effect of p-*mfgl2*shRNA on *mfgl2* expression. By cotransfection of p-*mfgl2*shRNA and pEGFP-*mfgl2* or pcDNA3.1-*mfgl2* expression construct into CHO cells or HeLa cells, the inhibition of *mfgl2* expression by *mfgl2*shRNA was analyzed by direct observation through fluorescent microscopy, FACS, RT-PCR and immunohistochemistry staining. The experiments showed the significant inhibitory effect of p-*mfgl2*shRNA on *mfgl2* expression at 48h post-transfection in both CHO and Hela cell lines with the inhibitory efficiency as high as 80.1%. The study demonstrated that the construct of p-*mfgl2*shRNA successfully interfered with the *mfgl2* expression *in vitro*.

Key words: Fulminant hepatitis; *fgl2* prothrombinase; RNA interference; shRNA

Fibrinogen like protein 2 (*fgl2*) which encodes a serine protease is capable of directly cleaving prothrombin to thrombin to trigger the process of coagulation. In mice with fulminant viral hepatitis induced by murine hepatitis virus strain 3 (MHV-3) and patients with severe acute or chronic hepatitis B, it has been shown that the high expression of *fgl2* results in intravascular fibrin deposition within the liver, culminating in widespread hepatocyte necrosis, and is highly correlated with disease severity (3, 10, 12). *Fgl2* plays an important role in the development of MHV-3 induced fulminant hepatitis and severe

Received: 2007-04-05, Accepted: 2007-07-09
* Foundation items: National Natural Science Foundation of China (No.30571643, No.30672380) and National Key Basic Research Program of China (No. 2005CB2401, 2007CB512904)
# These authors contributed equally.
** Corresponding author. Tel: +86-27-83663181, Fax: +86-27-83662391, E-mail: qning@tjh.tjmu.edu.cn

acute on chronic hepatitis B in patients. The pharmacological blockage of *fgl2* may offer an important new therapeutic approach in hepatitis virus induced disease.

RNA interference has proven to be an extremely potent and versatile tool to specifically reduce expression of targeted genes. Research reports demonstrate the potential for use of small interfering RNAs (siRNAs) as therapeutic agents, especially in the areas of cancer and viral infection. siRNAs can be surprisingly effecient, for example, transfections done using subnanomolar concentrations of RNA sometimes achieve 90% reduction in mRNA levels (7). In this paper, a expression plasmid containing short hairpin RNA (shRNA) of mouse *fgl2* (*mfgl2*) was constructed and its interfering effect was investigated *in vitro*.

MATERIALS AND METHODS

Construction of p-mfgl2shRNA plasmid

Mouse U6 promoter (U6P) was amplified from genomic DNA extracted from mouse liver. The upstream primer (primer 1) was 5'-GTA**GGATCC**ATC CGACGCCGCCATCTCTA-3' and the downstream primer (primer 2) was 5'-GGCAGCC**AAGCTT**CACA AACAAGGCTTTTCTCCAA-3'. The boldface, underlined sequences correspond to the restriction enzyme sites for *Bam*H I and *Hin*d III, respectively. The amplified fragment was cloned to T-vector pMD-18 (Invitrogen Life Technologies, Carlsbad, USA) to construct pMD-18-U6. A 19 bp oligonucleotide (5'-GCAGTG GACAGTCTGAAGA-3', S1) in the exon1 of *mfgl2* and its inverted repeat (5'-TCTTCAGACTGTCCA CTGC-3', S2) formed the double strand of the constructed shRNA. The constructed shRNA was 52 bp and its sequence was 5'-GCAGTGGACAGTCTG AAGATTCAAGAGATCTTCAGAGTGTCCACTGC TTTTT-3' (S1+TTCAAGAGA+S2+TTTTT), which was produced by 3 rounds of PCR with following primers:

primer 3: 5'-CTTCAGACTGTCCACTGCAAACAA GGCTTTTCTCC-3',

primer 4: 5'-AGTCTGAAGATCTCTTGAATCTTCA GACTGTCCACTGC-3',

primer 5: 5'-AAGCTTAAAAAGCAGTGGACAGTC TGAAGATCTCTTGAAT-3' (Fig.1).

By using the transfect vector pMD-18, U6P and shRNA template were subcloned into pMSCVneo vector (Invitrogen Life Technologies) at the *Bam*H I and *Hin*d III restriction sites to construct the plasmid named p-*mfgl2*shRNA. By using pMSCVneo vector, a plasmid containing a fragment of the mutated shRNA acting as the experimental control was created (5'-GCAGTGGACACTCAGAGGATTCAAGAGAT CCTCTGAGTGTCCACTGCTTTTT-3'. Where the underlined nucleotides correspond to mutations with respect to the *mfgl2*shRNA) sequence.

Transfection

A 1.3 kb fragment of m*fgl2* was amplified (primer: 5 ´ -TCGAAGCTTGCCGCCACCATGGAGCTTCC TGGTG-3 ´ and 5 ´ -TCGGATCCGTGGCTTGAAA TTCTTGG-3 ´) from pcDNA3.1- *mfgl2* (constructed by our lab) and cloned into pEGFP-N2 (Clontech Company, USA) upstream of the GFP gene at the *Bam*H I and *Hin*d III restriction sites. CHO and Hela cell lines were individually cultured in 24-well or 6-well plates until 70%-80% confluence. The *mfgl2*shRNA plasmid (1.33μg for 6-well plates and 0.33μg for 24-well plates in each well) was mixed with pEGFP-*mfgl2* or pcDNA3.1-*mfgl2* (2.67μg for

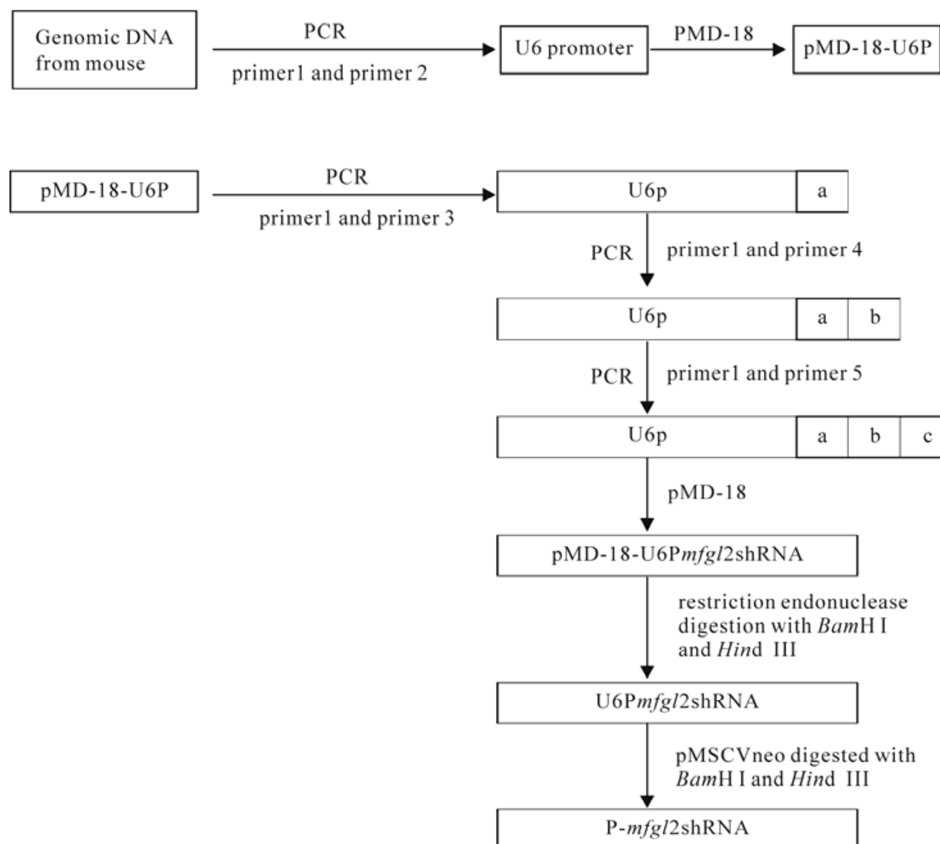

Fig.1. Flow chart of the construction of the p-*mfgl2*shRNA. The sequence of constructed *mfgl2*shRNA is 5'-GCAGTGGACAGT CTGAAGATTC –AAGAGATCTT-CAGAGTGTCCACTGCTTTTT-3'. a, b and c were one of the three parts of *mfgl2*shRNA from 5'- to 3', respectively.

6-well plates and 0.67μg for 24-well plates in each well) in serum-free F12-DMEM, and mixed with Lipofectamine (2 μg/μL, Invitrogen Life Technologies) according to the manufacture's protocol. The mutated shRNA plasmid was used as a control. After incubation at room temperature for 30 min, the mixture was added into CHO or Hela cells, and transfection was performed at 37℃ with 5% CO_2. Medium was replaced with fresh complete medium 5 h after transfection. At 48h post-transfection, cells were harvested for different assays. There were 4 groups which included the p-*mfgl2*-shRNA group, the mutated shRNA plasmid group, a no treatment group and a blank control.

Measuring the expression of EGFP

The expression of mfgl2-EGFP fusion protein was observed with a inverted fluorescent microscope. Positive rate of green fluorescent cells (α) was tested by FACS. 10 000 cells were counted per group. Inhibition efficacy=(α of no treatment group－α of p-mfgl2-shRNA group)/ α of no treatment group×100%.

RT-PCR

Total RNA was extracted from mfgl2shRNA and pcDNA3.1-mfgl2 plasmid transfected CHO cells or Hela cells using Trizol reagent (Invitrogen Life Technologies) according to the manufacture's standard protocol at 48h post transfection. 1μg RNA was used for reverse transcription according to the manufacture's standard protocol and 2 μL cDNA was amplified through PCR. The upstream primer was 5'-ACTGTGACATGGAGACCATG-3', and the down-

stream primer was 5'-TCCTTACTCTTGGTCAGAAG-3'. GAPDH was used as an internal reference.

Immunohistochemical staining

Cultured cell slices were blocked with 10% normal goat serum in PBS at room temperature for 2h. A polyclonal Ab to *mfgl2* prothrombinase was produced in rabbits by repeated injections with a 14-amino-acid hydrophilic peptide (CKLQADDHRDPGGN) from exon 1 of the *mfgl2* prothrombinase, which had been coupled to keyhole limpet hemocyanin. Ab was purified by affinity columns, and cultured cell slices were incubated with Ab (20μg/mL in PBS) at room temperature for 2h. Subsequently, slices were incubated with immunoperoxidase-conjugated goat IgG (6.7μg/mL) fraction to rabbit IgG Fc (Dako Cytomation) at room temperature for 1h, following which they were washed five times in PBS with 0.05% Tween20. Cultured cell slices were then air dried and photographed with a microscope.

Statistical analysis

Quantitative data were expressed as means ± SD. Statistical analysis was carried out by using a one-way analysis of variance, and a P value of less than 0.05 was considered statistically significant.

RESULTS

Construction of p-*mfgl2*shRNA plasmid

U6P was amplified from genomic DNA extracted from mouse liver. PCR products containing U6P and *mfgl2*shRNA are shown in Fig. 2A. The p-*mfgl2*-shRNA plasmid was successfully constructed as evidenced by the restriction enzyme mapping data (Fig.2B) and further confirmed by sequence analysis.

p-*mfgl2*shRNA inhibited the expression of mfgl2-EGFP fusion protein

The number of green fluorescent cells and their fluorescent intensity in cells treated with p-*mfgl2*-shRNA decreased significantly when viewed under an inverted fluorescent microscope at 24 h, 48 h and 72 h post-cotransfection, when compared with that in cells treated with either the irrelevant shRNA plasmid or no treatment (Fig.3A). This observation was further confirmed by FACS (Fig.3B). Inhibition efficacy of the p-*mfgl2*shRNA group was 80.1%. There was no significant difference between the pEGFP-*mfgl2* group and the mutated shRNA plasmid plus pEGFP-

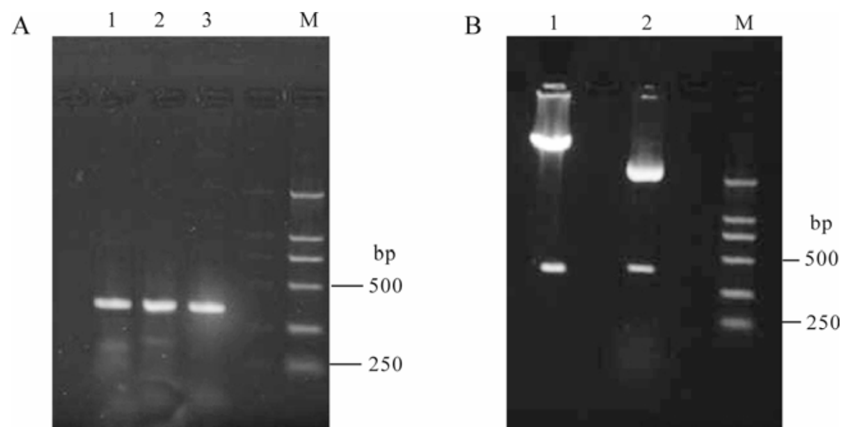

Fig. 2. Construction and identification of *mfgl2* shRNA plasmid. **A:** PCR products containing U6P plus entire *mfgl2* shRNA template (a+b+c, lane 1), U6P plus part of *mfgl2* shRNA template (a+b, lane 2) and U6P plus shorter part of *mfgl2* shRNA template (a, lane 3). **B:** Restriction endonuclease analysis of p-*mfgl2*shRNA (lane 1) and pMD18- U6P-*mfgl2*shRNA(lane 2) with *Bam*H I and *Hin*d III.

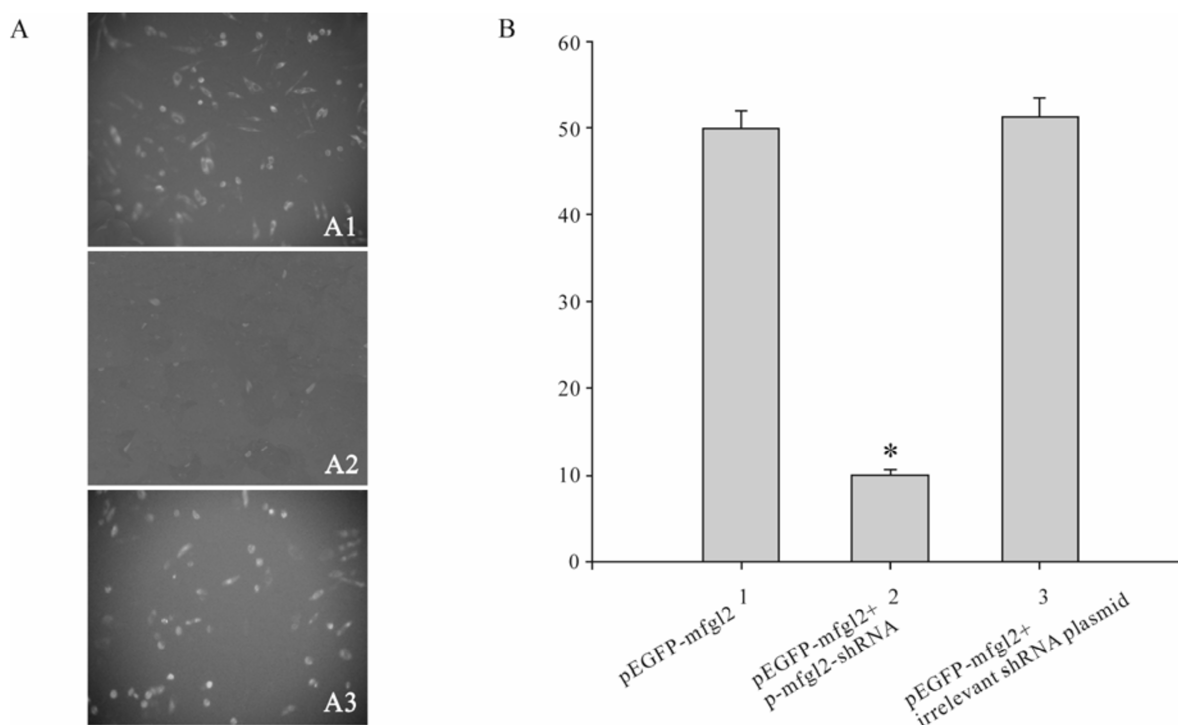

Fig.3. Effect of p-*mfgl2*shRNA on the expression of *mfgl2*-EGFP fusion protein. A: Under inverted fluorescent microscope, transfection of pEGFP-*mfgl2* (A1) and p-*mfgl2*shRNA plus pEGFP-*mfgl2* (A2) and mutated shRNA plasmid plus pEGFP-*mfgl2* (A3). B**:** by FACS, * $p<0.01$ compared with pEGFP-*mfgl2* group.

mfgl2 group.

p-mfgl2shRNA plasmid inhibited the expression of *mfgl2* significantly

p-*mfgl2*shRNA plasmid inhibited the expression of *mfgl2* not only at the mRNA level as shown by RT-PCR (Fig.4A) but also at the protein level as shown by immunohistochemistry staining (Fig.4B) in both CHO cell and Hela cells. There was no significant difference between the pcDNA3.1-*mfgl2* group and the mutated shRNA plasmid plus pcDNA 3.1-*mfgl2* group.

DISCUSSION

Worldwide about 400 million people have chronic infection of hepatitis B virus (HBV). With a 10%-20% HBV chronic carrier rate in China, safe and effective antiviral treatments are available but are still far from ideal. In the Far East, fulminant hepatic failure is mainly due to viral hepatitis. The mortality of fulminant viral hepatitis is over 80% in absence of immediate liver transplantation. Recently there has been significant interest in the use of gene therapy on fulminant viral hepatitis. Nakayama *et al*. used adenovirus encoding immunoglobin against CTLA-4 to suppress liver injury by inhibiting acquired immune responses in a mouse model of fulminant hepatitis induced by injection of Propionibacterium acnes and lipopolysaccharide (11). Arvelo *et al* reported that adenoviral mediated hepatic expression of A20, an anti-apoptotic protein, protected Balb/cJ mice from D-galactosamine/lipopolysaccharide acute toxic lethal hepatitis, which yielded an 85% survival rate compared with 15%-20% in control mice (2). Song *et al* used RNA interference targeting Fas to elevate the survival rate in mice with fulminant hepatitis and protect mice from fulminant hepatitis (15). Hecht *et al*.

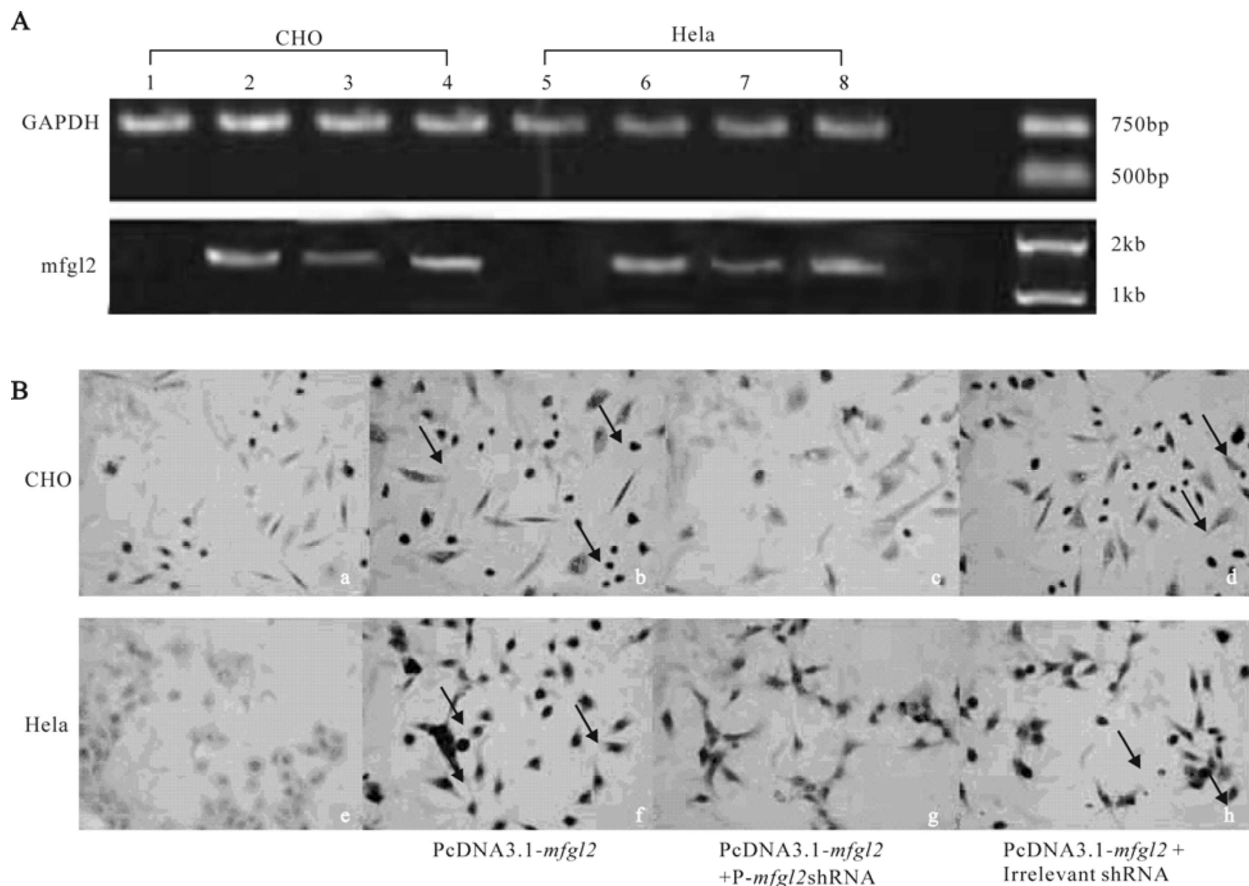

Fig.4 Effect of p-*mfgl2*shRNA on the expression of *mfgl2* in CHO and HeLa cells. **A**: RT-PCR results of the transfections of pcDNA3.1-*mfgl2* (lane 2 and lane 6), pcDNA3.1-*mfgl2* plus p-*mfgl2*shRNA (lane 3 and lane 7), and pcDNA3.1-*mfgl2* plus mutated shRNA plasmid (lane 4 and lane 8). The untransfected CHO (lane1) and HeLa (lane5) cells are shown as controls.**B:** Immunohistochemistry analysis of *mfgl2*.

reported that a designed human IL-6/sIL-6R fusion protein promoted liver regeneration and reversed severe hepatocellular injury (4).

Our previous reports both in an experimental animal model of fulminant viral hepatitis caused by MHV-3 and patients with acute on chronic hepatitis B, have shown a critical role for *fgl2* prothrombinase in the pathogenesis of fulminant viral hepatitis. *Fgl2* prothrombinase belongs to the fibrinogen family of proteins and encodes a serine protease, which is an immune coagulant with the ability of directly cleaving prothrombin to thrombin in the absence of factor VII or factor X (8), resulting in intravascular fibrin deposition within the liver and culminating in widespread hepatocyte necrosis (8, 9, 10). The importance of *mfgl2* prothrombinase in the pathogenesis of fulminant viral hepatitis is supported by the observation that a neutralizing antibody against *mfgl2* prevents both fibrin deposition and death from MHV-3 infection (9). Furthermore, recent studies have shown that inhibition of reticuloendothelial cell *mfgl2* expression through the use of gene-targeted *fgl2*-deficient mice results in the prevention of MHV-3 induced fibrin deposition, liver injury, and death (10). The human and murine genes for the *fgl2* have been localized to chromosome 7 and 5, respectively. The murine and human proteins share 78% overall identity, with greater conservation at the

C terminus. These studies argue that the *fgl2* prothrombinase is a logical target for therapeutic intervention in an attempt to ameliorate fulminant viral hepatitis.

We have recently successfully constructed a *mfgl2* antisense plasmid and therapeutic effects were achieved, which significantly ameliorated inflammatory infiltration, fibrin deposition and hepatocyte necrosis, prolonged the survival time period, and elevated the survival rate in Balb/cJ mice with MHV-3 induced fulminant hepatitis (17). Antisense technology has failed to gain widespread acceptance as a gene knockdown tool, largely due to the extensive experimental testing that often needs to be done to find effective target sites within the gene of interest, while it is now possible to obtain potent RNAi reagents without the extensive testing needed (1, 5, 6, 13, 14, 16). We successfully constructed a hairpin small interference RNA complementary to the sequence containing Ser89 of *mfgl2* and data showed its significant inhibitory effect on *mfgl2* expression *in vitro*. By 3 rounds of PCR a *mfgl2*shRNA fragment was obtained, which was convenient and avoided the difficulty of cloning a shorter DNA fragment. The recombinant vector of pEGFP-*mfgl2* provides a direct and simplified methodology for primary assessment of the effect of *mfgl2* siRNA on the *mfgl2* gene expression. FACS showed the inhibition efficacy was as high as 80.1%. p-*mfgl2*shRNA plasmid inhibited the expression of *mfgl2* significantly not only at the mRNA level but also at the protein level. This provides a foundation for further investigation for the application of these constructs *in vivo* and furthermore as a therapeutic strategy for a targeting intervention in the control of diseases to which the gene *fgl2* contributes.

Reference

1. **Amarzguioui M**, **Prydz H.** 2004. An algorithm for selection of functional siRNA sequences. **Biochem Biophys Res Commun,** 316: 1050-1058.
2. **Arvelo M B, Cooper J T, Longo C, *et al.*** 2002. A20 protects mice from D-galacto-samine/lipopolysaccharide acute toxic lethal hepatitis. **Hepatology**, 35: 535-543.
3. **Ding J W, Ning Q, Liu M F, *et al.*** 1997. Fulminant hepatic failure in murine hepatitis virus strain 3 infection: tissue-specific expression of a novel fgl2 prothrombinase. **J Virol,** 71: 9223-9230.
4. **Hecht N**, **Pappo O, Shouval D, *et al.*** 2001. Axelrod JH. Hyper-IL-6 gene therapy reverses fulminant hepatic failure. **Mol Ther,** 3: 683-687.
5. **Huesken D**, **Lange J, Mickanin C, *et al.*** 2005. Design of a genome-wide siRNA library using an artificial neural network. **Nat Biotechnol,** 23: 995-1001.
6. **Khvorova A**, **Reynolds A, Jayasena S D.** 2003. Functional siRNAs and miRNAs exhibit strand bias. **Cell**, 115: 209-216.
7. **Kim D H**, **Behlke M A, Rose S D, *et al.*** 2005. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. **Nat Biotechnol,** 23: 222-226.
8. **Levy G A**, **Liu M, Ding J, *et al.*** 2000. Molecular and functional analysis of the human prothrombinase gene (HFGL2) and its role in viral hepatitis. **Am J Pathol,** 156: 1217.
9. **Li C**, **Fung L S, Chung S**, ***et al.*** 1992. Monoclonal antiprothrombinase (3D4.3) prevents mortality from murine hepatitis virus (MHV-3) infection. **J Exp Med,** 176: 689-697.
10. **Marsden P A**, **Ning Q, Fung L S, *et al.*** 2003. The Fgl2/fibroleukin prothrombinase contributes to immunologically-mediated thrombosis in experimental and human viral hepatitis. **J Clin Inves**, 112: 58-66.
11. **Nakayama Y**, **Shimizu Y, Hirano K**, ***et al.*** 2005. Sugiyama T CTLA-4Ig suppresses liver injury by inhibiting acquired immune responses in a mouse model of fulminant hepatitis. **Hepatology,** 42: 915-924.
12. **Ning Q**, **Brown D, Parodo J, *et al.*** 1998. Ribavirin inhibits viral-induced macrophage production of TNF,

IL-1, the procoagulant fgl2 prothrombinase and preserves Th1 cytokine production but inhibits Th2 cytokine response. **J Immunol**, 160: 3487-3493.

13. **Reynolds A**, **Leake D, Boese Q,** ***et al.*** 2004. Rational siRNA design for RNA interference. **Nat Biotechnol,** 22: 326-330.
14. **Schwarz D S**, **Hutvagner G, Du T,** ***et al.*** 2003. Asymmetry in the assembly of the RNAi enzyme complex. **Cell**, 115: 199-208.
15. S**ong E**, **Lee SK, Wang J,** ***et al.*** 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. **Nat Med**, 8:347-351.
16. **Ui-Tei K, Naito Y, Takahashi F,** ***et al.*** 2004. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. **Nucleic Acids Res**, 32: 936-948.
17. **Zhu C L, Sun Y, Luo X P,** ***et al.*** 2006. Novel mfgl2 antisense plasmid inhibits mfgl2 expression and ameliorates MHV-3 induced fulminant hepatitis in Balb/cJ mice. **Human Gene Therapy**, 17: 589-600.