Pharm Res (2011) 28:752–761
DOI 10.1007/s11095-011-0384-y

RESEARCH PAPER

GFAP Promoter-Driven RNA Interference on TGF-β1 to Treat Liver Fibrosis

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Received: 8 October 2010 / Accepted: 27 January 2011 / Published online: 23 February 2011

ABSTRACT

Purpose The objective was to determine the role of promoters and miRNA backbone in shRNA-based hepatic stellate cell (HSC)-specific transforming growth factor (TGF)-β1 gene silencing. This is expected to avoid the side effect of non-specific TGF-β1 gene silencing.

Methods Two most potent shRNAs targeting 769 and 1033 start sites of rat TGF-β1 mRNA were cloned into pSilencer 1.0 vector for enhanced TGF-β1 gene silencing. We then constructed HSC-specific pri-miRNA mimic and pri-miRNA cluster mimic expression plasmids in which shRNA expression was driven by a glial fibrillary acidic protein (GFAP) promoter to achieve HSC-specific TGF-β1 gene silencing to avoid nonspecific inhibition of TGF-β1 expression in other cells and organs.

Results These TGF-β1 pri-miRNA-producing plasmids showed the inhibition of proliferation and induced apoptosis of activated HSC-T6 cells. TGF-β1 pri-miRNA cluster mimic plasmids decreased TGF-β1 and collagen gene expression at both mRNA and protein levels.

Conclusions GFAP promoter driven TGF-β1 pri-miRNA producing plasmids have the potential to be used for site-specific gene therapeutics to treat liver fibrosis.

KEY WORDS GFAP promoter · liver fibrosis · pri-miRNA mimics · shRNA · TGF-β1

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INTRODUCTION

Fibrosis is a scarring response that occurs in almost all patients with chronic liver injury. If not controlled, liver fibrosis leads to cirrhosis, which is associated with nodule formation and organ contraction (1). Transforming growth factor beta 1 (TGF-β1) is a potent stimulus of hepatic stellate cells (HSCs), which produce excess collagen and other extracellular matrices (ECM) (2). The positive feedback production of TGF-β1 by activated HSCs makes liver injury worse. Therefore, TGF-β1 gene silencing produced by HSCs becomes crucial in treating liver fibrosis.

Strategies for disrupting TGF-β1 expression are extensively being investigated because inhibiting this cytokine may not only inhibit ECM production but also accelerate its degradation (3). The use of different strategies to inhibit TGF-β1 gene expression has shown antifibrotic effect for treating liver fibrosis in animal models (4–8). RNA interference (RNAi) is the phenomenon in which small interfering RNA (siRNA) of 21–23nt in length silences target gene expression by binding to its complementary mRNA and triggering mRNA degradation (9). Compared to antisense ODNs, antibodies and soluble TGF-β1 receptor, siRNA is likely to be quite potent in inhibiting TGF-β1 gene expression. We have also recently demonstrated siRNA sequence and dose-dependent TGF-β1 gene silencing (10).

The standard polymerase III (pol III) promoters, such as U6 and H1, have robust and constitutive activity across multiple cell types (11). However, these Pol III promoters do not provide the spatial or temporal control of target gene silencing that is desirable for treating liver fibrosis. TGF-β1 is a growth factor involved in many physiological activities. The promoter which can only drive TGF-β1

shRNA expression by specific cell types in the body should be utilized for cell-specific gene silencing.

In this study, to avoid the side effect of nonspecific TGF-β1 gene silencing, we constructed an HSC-specific expression plasmid. It is the first time to construct TGF-β1 pri-miRNA mimic plasmids driven by glial fibrillary acidic protein (GFAP)-promoter, a known biomarker for HSCs of fibrotic livers (12,13). Since GFAP promoter relies on RNA polymerase II (pol II) to achieve RNAi (14), this promoter-driven shRNA is unlikely to have inefficient nuclear export. Therefore, we inserted shRNA sequences targeting TGF-β1 into pri-miRNA backbone (Fig. 1). Even though we found that the silencing level of GFAP promoter-driven TGF-β1 pri-miRNA mimics was lower than that of U6 promoter-driven ones, the pri-miRNA cluster mimics of two shRNA inserts showed high silencing effect to compensate this phenomenon.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin G (5000 U/mL), trypsin-EDTA, Trizol, DNase I, and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). pSilencer1.0 was purchased from GenScript Corporation (Piscataway, NJ). All of the restriction enzymes were purchased from New England Biolabs (Ipswich, MA). SYBR Green-1 dye universal master mix and MultiScribe reverse transcriptase were purchased from Applied Biosystems, Inc. (Foster City, CA). All the primers for real-time PCR were purchased from Integrated DNA Technologies, Inc. TGF-β1 primary antibody, type α1(I) collagen primary antibody and rabbit anti-rat secondary antibody were purchased from Santa Cruz Biotech, Inc. (Santa Cruz, CA). TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience, Inc. (San Diego, CA).

Construction of shRNA Expression Plasmids

Based on our recently published results (10), we selected two potent siRNA sequences targeting 769 and 1033 start sites of TGF-β1 mRNA and converted them into shRNA sequences for cloning into pSilencer 1.0 vector, which carries a shRNA expression cassette under the control of a U6 promoter (Fig. 1). These shRNAs contain two complementary oligonucleotides, which were annealed to form a double-stranded DNA for ligation into pSilencer vector. pU6-shRNA-769, pU6-shRNA-1033, pU6-shRNA-769 +1033 and pU6-shRNA-scramble were constructed as mentioned in our previous study (10). For HSC-specific gene silencing, GFAP promoter was cloned by the Long Range PCR kit by using pGFA2 vector as a template, which is a gift from Dr. Michael Brenner of the Department of Neurology at the University of Alabama. Then, GFAP promoter fragment was inserted into pRNAT H1.1 to replace the H1 promoter in pRNAT H1.1 to make pGFA-RNAT H1.1 vector. pGFA-mi-shRNAs were constructed by inserting the pre-designed mi-shRNA sequence with miRNA 30 context to pGFA-RNAT H1.1. The sequences for all the predesigned mi-shRNA sequences are shown in Table 1.

Transfection

The day before transfection, HSC-T6 cells were seeded in a 6-well plate at a number of 1×10^6 cells per well. When the cell confluence reached 80 to 90%, cells were transfected with shRNA expression vectors at doses of 1 μg/well. The shRNA plasmids were mixed with Lipofectamine 2000 at a 1:3 w/v (μg/μl) ratio in 250 μl Dulbecco's Modified Eagle's Medium (DMEM) without serum for 20 min at room temperature to allow complex formation. The transfection mixture was then added to each plate with 2 ml fetal bovine serum (FBS) free DMEM. After 4 h of incubation, 200 μl FBS was added per well and incubated for additional 42 h.

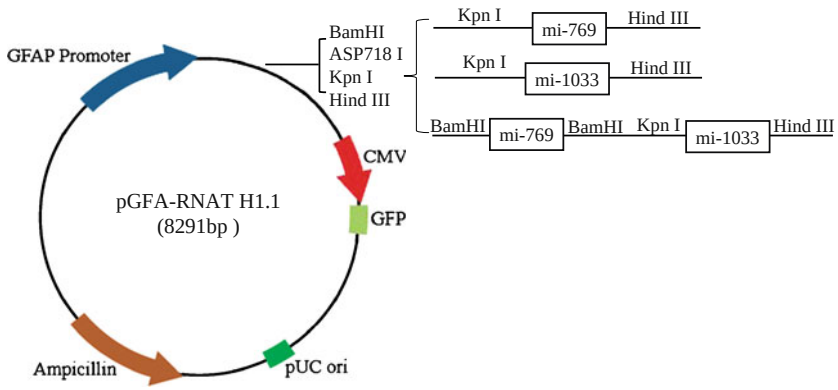

Fig. 1 Construction of a mi-shRNA expression vector, pGFA-RNAT H1.1, encoding single or multiple mi-shRNAs driven by a single GFAP promoter.

Real-Time PCR

Following transfection, total RNA was extracted from the cells by RNAeasy Mini Kit, and the RNA concentration was measured using a Nanodrop UV spectrophotometer (Thermo Scientific, Pittsburgh, PA). Then, 385 ng of the total RNA per sample was reverse transcribed into cDNA using MultiScribe Reverse Transcriptase Reagent and random hexamers. The obtained cDNA was amplified by real-time PCR using SYBR Green-1 dye universal master mix on ABI Prism 7700 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). The primers for TGF-β1 were used: forward, 5′-CATCCATGACATGAACCGACCCTT-3′, and reverse, 5′-ACAGAAGTTGGCATGGTAGCCCTT-3′. The primers for 18S as an internal control were used: forward, 5′-GTCTGTGATGCCCTTAGATG-3, and reverse, 5′-AGCTTATGACCCGCACTTAC-3.

Western Blot Assay

Transfected cells were lysed using 1× Laemmli sodium dodecyl sulfate (SDS) sample buffer containing 100 mM Tris (pH 6.8), 200 mM dithiothreitol (DTT), 4% SDS, 20% glycerol and 0.2% bromophenol blue. The lysed protein was then boiled at 100°C for 5 min and subjected to 4% or 10% sodium dodecyl sulfate -polyacrylamide (SDS-PAGE) gel electrophoresis and subsequent transfer by iBLot system. After blocking with 5% nonfat dried milk in 1× PBST containing 0.05% Tween-20 in PBS for 1 h at room temperature, the membranes were incubated with goat anti-rat type I collagen, rabbit anti-rat TGF-β1 and goat anti-rat β-actin primary antibodies for 16 h at 4°C. Membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. Target proteins were detected by enhanced chemiluminescence detection kit (ECL, GE Healthcare).

Trypan Blue Staining

Transfected HSC-T6 cells were suspended in DMEM medium at a concentration of 1×10^5/ml in a 1.5 ml tube. Five hundred microliters of cells and 0.1 ml 0.4% trypan blue staining buffer were mixed thoroughly. The staining samples were allowed to stand at room temperature for 5 min, and then 10 μl was applied to a hemocytometer for cell counting.

ELISA Assay of TNF-α

After transfection, the supernatant of the transfected cells was collected, and the concentration of TNF-α was measured by ELISA (eBioscience, San Diego, CA), according to the manufacturer's protocol.

Caspase Detection

Caspase-Glo 3/7 assay kit was used to determine the effect of TGF-β1 gene silencing on caspase 3/7 activity, as per the manufacturer's protocol (Promega, Madison, WI). Briefly, at 4 h post-transfection and 42 h of subsequent incubation in the presence of 10% FBS, 100 μl Caspase-Glo reagent was added to 100 μl of culture supernatants in 96-well plates and incubated at room temperature for 1 h. The contents were transferred into culture tubes, and luminescence was determined using a luminometer (Berthold, Germany).

HSC Wound Healing Assay

Wound healing assay of transfected HSC-T6 cells was carried out as described by Liu *et al.* (15), with minor modifications. Briefly, HSC-T6 cells were seeded in 6-well plates at a number of 1×10^6 per well and allowed to grow in DMEM growth medium containing 10% serum. The cells were then deprived of serum for 6 h when the cell's confluence reached 95%. Then, the cells were transfected with Lipofectamine 2000/pshRNA complexes at a dose of 1 μg plasmid per well. After transfection, the cells were still cultured in DMEM without serum. The cell monolayer was disrupted by a scratch to mimic wound. The cells were then cultured for additional 16 h, and the wound was observed under a microscope.

Cell Migration Assay

Migration assays were performed in Transwell membrane filter inserts in 24-well tissue culture plates (BD Labware, Bedford, MA) with the pore size in polycarbonate membranes of 8 μm. The lower surface of inserts membranes were pre-incubated with fibronectin diluted in 10 mmol/L $NaHCO_3$ 4°C overnight and then blocked with 0.1% heat-inactivated BSA at 37°C for 30 min. Cells transfected with pri-miRNA mimics were detached, washed once in PBS, and then resuspended in DMEM containing 0.1% BSA. A 300 μL cell suspension containing 8×10^4 cells was added to each insert. DMEM containing 1% FBS was added to the lower wells in the 24-well cell culture plate. Migration was allowed to proceed at 37°C for 4 h. Cells that did not migrate through the filters were removed by cotton swabs. After fixing and staining with Diff-Quick (Baxter Healthcare Corp., McGraw Park, IL), the cells were visualized under a fluorescent microscope.

Table 1 pri-miRNA Mimic Inserts Sequences

mi-shRNA-1033	GGATCCGTCGACTAGGGATAACAGGG TAATTGTTTGAATGAGGCTTCAGTAC TTTACAGAATCGTTGCCTGCACATCT TGGAAACAGCTGGGATTACTTCTTC aggTTAACCCAACAGAAGGCTCGAGAA GGTATATTGCTGTTGACAGTGAGCGC CGCAGCTGTACATTGACTTTGTGAA GCCACAGATGTAAAAGTCAATGTACA GCTGCTGCCTACTGCCTCGTCTAGA AAGGGGCTACTTTAGGAGCAATTATC TTGTTTACTAAAACTGAATACCTTGC TATCTCTTTGATACATTTTTTGGATCC
mi-shRNA-769	GGATCCGTCGACTAGGGATAACAGGG TAATTGTTTGAATGAGGCTTCAGTAC TTTACAGAATCGTTGCCTGCACATCT TGGAAACAGCTGGGATTACTTCTTC aggTTAACCCAACAGAAGGCTCGAGA AGGTATATTGCTGTTGACAGTGAGCG CCGAACCAAGGAGACGGAATAGTGA AGCCACAGATGTATATTCCGTCTCCT TGGTTCTGCCTACTGCCTCGTCTAG AAAGGGGCTACTTTAGGAGCAATTAT CTTGTTTACTAAAACTGAATACCTTG CTATCTCTTTGATACATTTTTTGGATCC
mi-shRNA-769+1033	GGATCCGTCGACTAGGGATAACAGGG TAATTGTTTGAATGAGGCTTCAGTAC TTTACAGAATCGTTGCAGCTGTACAT TGACTTTGTGAAGCCACAGATGTAA AAGTCAATGTACAGCTGCGCCTGCA CATCTTGGAAACAGCTGGGATTACTT CTTCAGGTTAACCCAACAGAAGGCT CGAGAAGGTATATTGCTGTTGACAGT GAGCGCCGAACCAAGGAGACGGAAT AGTGAAGCCACAGATGTATATTCCGT CTCCTTGGTTCTGCCTACTGCCTCG TCTAGAAAGGGGCTACTTTAGGAGC AATTATCTTGTTTACTAAAACTGAATA CCTTGCTATCTCTTTGATACATTTTTT GGATCC

RESULTS

Effect of shRNA Sequence on TGF-β1 Silencing

To examine the effect of shRNA sequence on TGF-β1 gene silencing, we transfected HSC-T6 cells with pU6-shRNA-scramble, pU6-shRNA-769, pU6-shRNA-1033 and pU6-shRNA-769+1033, respectively, after complex formation with Lipofectamine 2000. Real-time RT-PCR results (Fig. 2A) showed decrease in TGF-β1 gene expression in HSC-T6 cells to 53.60±1.33%, 48.05±7.51% and 24.09±0.25% by pU6-shRNA-769, pU6-shRNA-1033 and pU6-shRNA-769+1033, respectively, compared to pU6-shRNA-scramble. The plasmid encoding two shRNAs targeting two different regions such as 769 and 1033 start sites of TGF-β1 mRNA were more effective in TGF-β1 gene silencing, compared to the plasmids encoding single siRNA.

TGF-β1 gene silencing should enhance the degradation of type I collagen. We, therefore, determined the effect of TGF-β1 gene silencing on type I collagen protein expression by Western blot analysis. There was significant decrease in collagen concentration of pU6-shRNA-769, pU6-shRNA-1033 and pU6-shRNA-769+1033-treated groups, but little decrease in collagen concentration for the control shRNA-treated samples (Fig. 2B).

Effect of Promoters and pri-miRNA Mimics on TGF-β1 Gene Silencing

For HSC-specific gene silencing, we used pGFA-shRNA-1033 driven by GFAP promoter but failed to produce any TGF-β1 silencing effects (Fig. 3A). However, still directed by GFAP promoter, the pri-miRNA mimic, pGFA-mi-shRNA-1033, reduced the total amount of TGF-β1 mRNA to 44.05±1.60% of that in the HSC-T6 cell line administrated by pGFAP-RNAT H1.1 with scramble sequence as the control (Fig. 3A). pGFA-mi-shRNA-1033 driven by GFAP promoter had less silencing efficiency than pU6-shRNA-1033 driven by U6 promoter by comparing the third and fourth bars in Fig. 3A. The total amount of TGF-β1 mRNA in HSC-T6 treated by pU6-shRNA was only 29.28±1.59% of the control.

Transfection of HSC-T6 cells with GFAP promoter-driven shTGF-β1 expression plasmids decreased TGF-β1 mRNA level (Fig. 3B). Real-time RT-PCR results revealed TGF-β1 gene expression in HSC-T6 cells was decreased to 69.54±5.04%, 45.95±2.45% and 35.65±3.82% by pGFA-mi-shRNA-769, pGFA-mi-shRNA-1033 and pGFA-mi-shRNA-769+1033, respectively, compared to the control.

HSC-T6 Proliferation and Apoptosis

To determine the effect of GFAP-driven TGF-β1 pri-miRNA mimics and pri-miRNA cluster mimics on HSC-T6 proliferation and apoptosis, we did MTT assay. pGFA-mi-shRNA-769, pGFA-mi-shRNA-1033 and pGFA-mi-shRNA-769+1033 effectively prevented HSC-T6 cell proliferation (Fig. 4A). Similarly, Western blot analysis showed decrease in the phosphorylation of extracellular-signal-related kinase (p-ERK) due to TGF-β1 gene silencing, which further proved the inhibition of HSC-T6 proliferation after transfection of HSC-T6 cells with TGF-β1 pGFA-mi-shRNA plasmids (Fig. 4B). Western blot of ERK did not show any change in band thickness, suggesting an equal amount of proteins in each sample.

Trypan blue staining and caspase activity analysis demonstrated that TGF-β1 silencing also increased HSC-

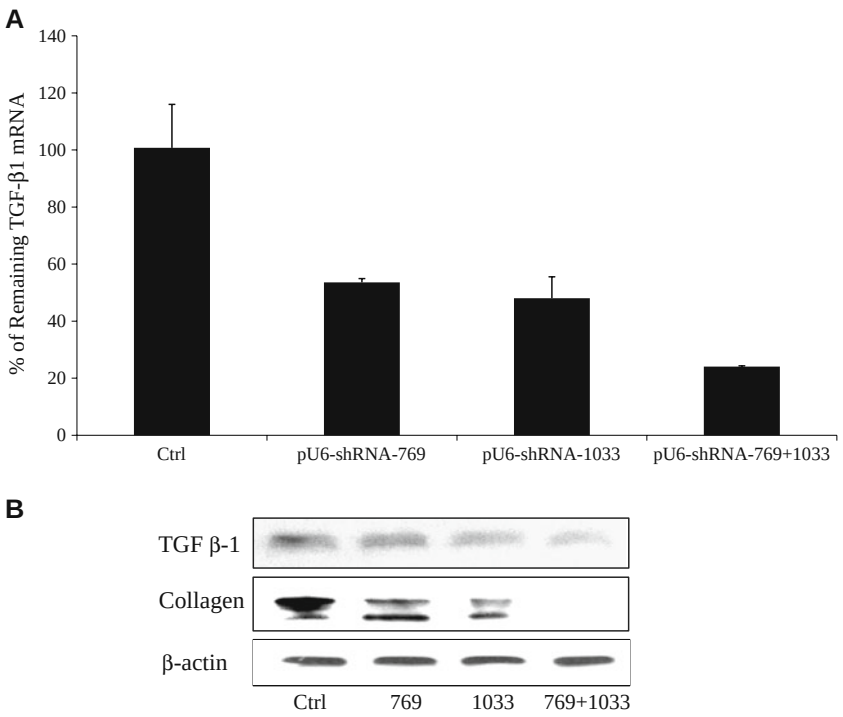

Fig. 2 Effect of shRNA sequences on TGF-β1 gene silencing and its effect on collagen gene expression. HSC-T6 cells were transfected by pU6-shRNA-scramble, pU6-shRNA-769, pU6-shRNA-1033 and pU6-shRNA-769+1033, respectively after complex formation with Lipofectamine 2000. (A) Real-time RT-PCR. Results are expressed as the mean ±S.D. ($n=4$). (B) Western blot analysis.

T6 apoptosis (Fig. 5A, B). Caspase 3/7 activity was significantly increased in HSC-T6 cells after transfection of HSC-T6 cells with pGFA-mi-shRNAs, especially for 769+1033 cluster (Fig. 5A), which correlated well with decrease in HSC-T6 cell viability (Fig. 5B).

pGFA-mi-shTGF-β1 Decreases TNF-α Amount in Cell Culture Medium

TNF-α concentration was decreased after transfection with pGFA-mi-shTGF-β1 plasmids. pGFA-mi-shRNA-769,

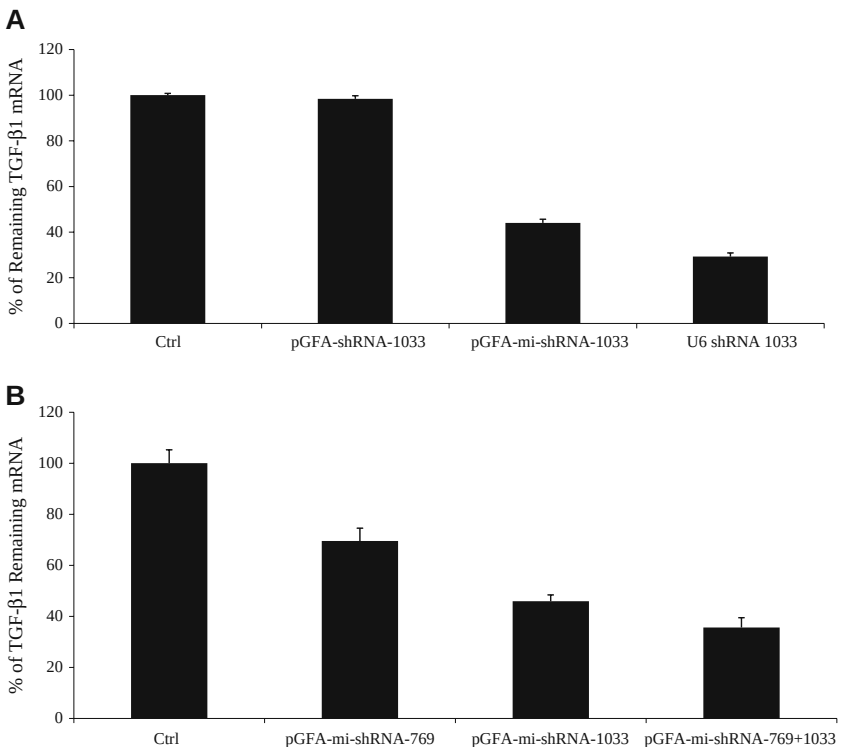

Fig. 3 Effects of promoters and pri-miRNA mimics on TGF-β1 silencing. After 42 h post transfection of pri-miRNA mimics and pri-miRNA cluster mimics, cells were harvested, total RNA was extracted, and TGF-β1 gene expression was determined at mRNA levels using real-time RT-PCR. Results were represented as the mean±SD ($n=3$). (**A**) pGFA-shRNA-1033 showed no TGF-β1 gene silencing, whereas pGFA-mi-shRNA-1033 reduced the total amount of TGF-β1 mRNA. pGFA-mi-shRNA-1033 had less silencing efficiency than pU6-shRNA-1033. (**B**) pGFA-mi-shRNA-769, pGFA-mi-shRNA-1033 and pGFA-mi-shRNA-769+1033 decreased TGF-β1 mRNA level in HSC-T6 cells. pGFA-mi-shRNA-769 +1033, producing pri-miRNA cluster mimics, had more silencing effects.

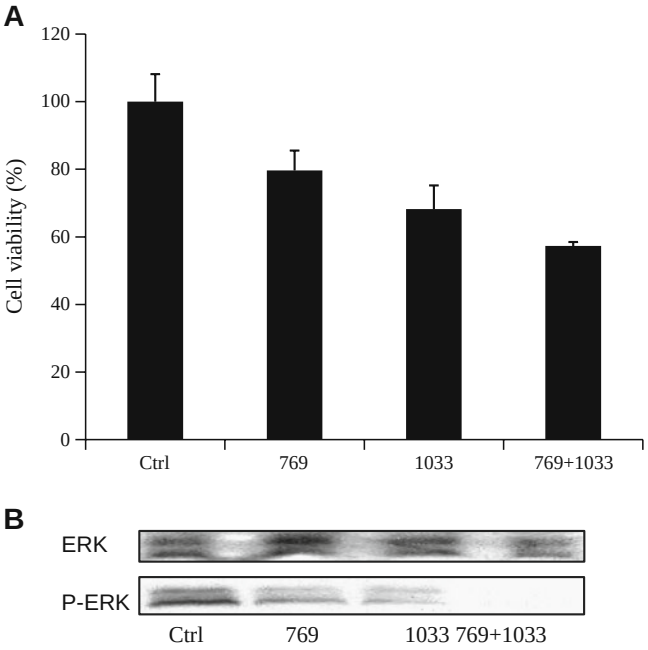

Fig. 4 Effects of GFAP-driven TGF-β1 pri-miRNA mimics and pri-miRNA cluster mimics on HSC-T6 proliferation. After 42 h post-transfection with TGF-β1 pri-miRNA mimics plasmids and pri-miRNA cluster mimics plasmids, (**A**) MTT assay showed the cell viability of the HSC-T6 cells transfected by pGFA-control, pGFA-mi-shRNA-769, pGFA-mi-shRNA-1033 and pGFA-mi-shRNA-769+1033. Results are expressed as the mean±S.D. ($n=3$). (**B**) Western blot analysis of p-ERK after transfection of HSC-T6 cells with pGFA-control, pGFA-mi-shRNA-769, pGFA-mi-shRNA-1033 and pGFA-mi-shRNA-769+1033. Western blot analysis of ERK was also performed for normalizing the amounts of proteins in each sample.

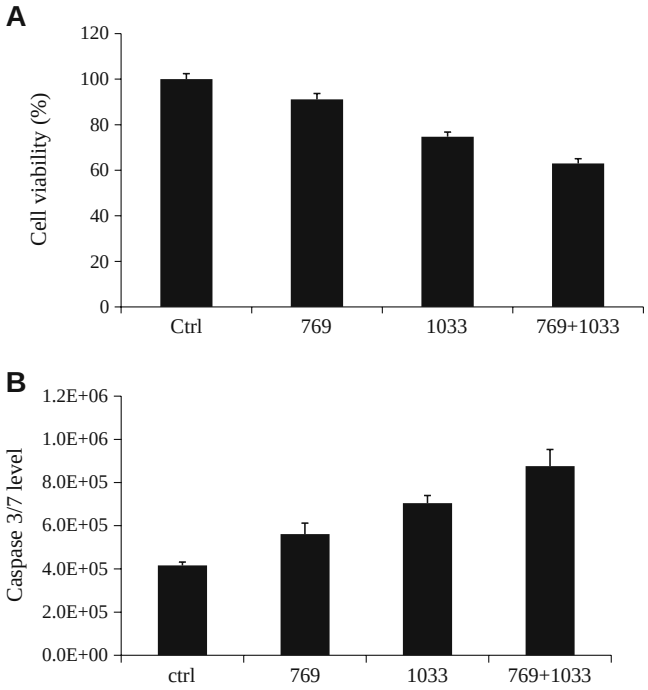

Fig. 5 Effects of GFAP-driven TGF-β1 pri-miRNA mimics and pri-miRNA cluster mimics on HSC-T6 apoptosis. After 42 h post-transfection with TGF-β1 pri-miRNA mimics plasmids and pri-miRNA cluster mimics plasmids, (**A**) caspase activity analysis demonstrated the change after transfection of HSC-T6 cells with pGFA-control, pGFA-mi-shRNA-769, pGFA-mi-shRNA-1033 and pGFA-mi-shRNA-769+1033, (**B**) trypan blue staining displayed the ratio of apoptotic HSC-T6 cells after transfection with pGFA-control, pGFA-mi-shRNA-769, pGFA-mi-shRNA-1033 and pGFA-mi-shRNA-769+1033. Results are expressed as the mean±S.D. ($n=4$).

pGFA-mi-shRNA-1033 and pGFA-mi-shRNA-769+1033 decreased TNF-α concentration in HSC-T6 cell culture medium to 281.27, 275.85 and 235.55 pg/ml from 483.50 pg/ml of the control group (Fig. 6).

pGFA-mi-shTGF-β1 Impedes HSC-T6 Migration

The wounded HSC-T6 monolayers transfected with GFAP promoter-driven scrambled shRNA expression vectors induced cell migration leading to wound closure at 16 h after wounding (Fig. 7Aa). However, transfection of pGFA-mi-shRNA-769 (Fig. 7Ab), pGFA-mi-shRNA-1033 (Fig. 7Ac) and pGFA-mi-shRNA-769+1033 (Fig. 7Ad) impeded wound closure and wound gaps remained.

In the directional cell migration toward fetal bovine sera, HSC-T6 transfected with pGFA-mi-shRNA-769+1033 showed the least migration ability (Fig. 7Bd) compared to the control group (Fig. 7Ba). Both groups transfected with pGFA-mi-shRNA-769 (Fig. 7Bb) and pGFA-mi-shRNA-1033 (Fig. 7Bc) also showed less migration.

Cell Specificity of GFAP Promoter-Driven shTGF-β1 Expression

After transfection with pGFA-mi-shRNA-1033 and U6-mi-siRNA-1033, the remaining TGF-β1 mRNA level in rat insulinoma INS-1E cell line was decreased to 57.77± 7.55% by U6 promoter-driven plasmids, compared to the

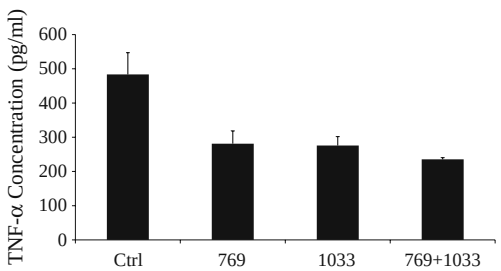

Fig. 6 Effects of GFAP-driven TGF-β1 pri-miRNA mimics and pri-miRNA cluster mimics on secretion of inflammatory cytokines by HSC-T6 cells. After 42 h post-transfection with TGF-β1 pri-miRNA mimics plasmids and pri-miRNA cluster mimics plasmids, concentration of TNF-α in cell culture medium was measured by ELISA. Results are expressed as the mean±S.D. ($n=4$).

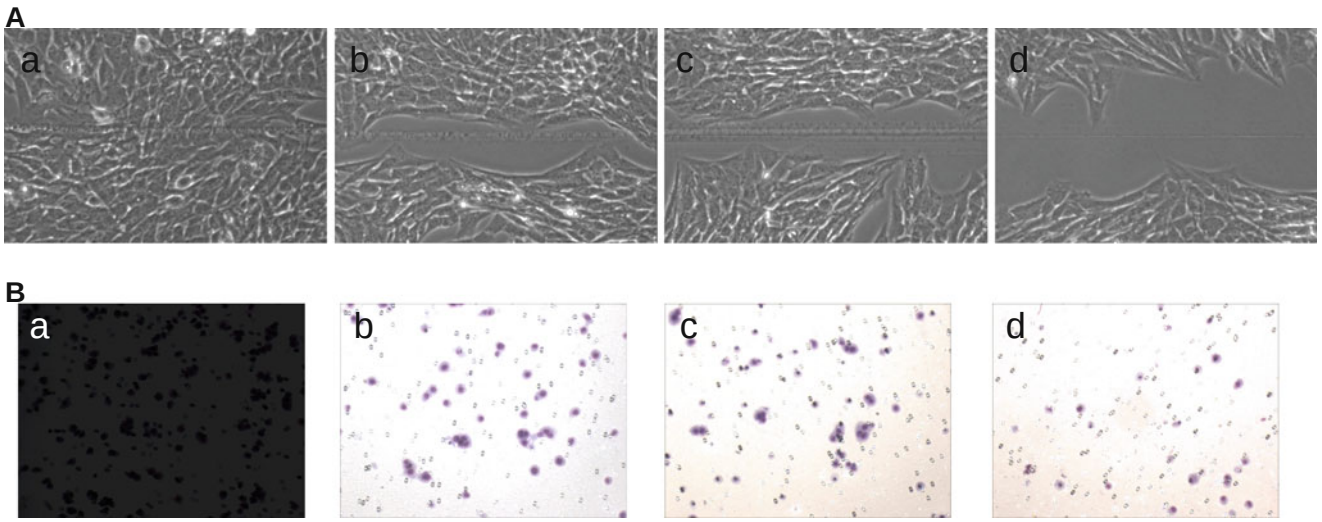

Fig. 7 Wound-healing and chamber migration assays. (**A**) HSCs were grown to confluence and were disrupted to generate a linear wound, and then transfected with a) pGFA-control, b) pGFA-mi-shRNA-769, c) pGFA-mi-shRNA-1033 and d) pGFA-mi-shRNA-769+1033 plasmids after complex formation with lipofectamine 2000 at a dose of 1 μg/well. After another 16 incubation in none serum medium, photographs were taken by microscope. (**B**) After transfection with a) pGFA-control, b) pGFA-mi-shRNA-769, c) pGFA-mi-shRNA-1033 and d) pGFA-mi-shRNA-769 +1033 plasmids, purple spots indicated the migrated HSC-T6 cells to the bottom of the chambers.

blank control (Fig. 8). However, GFAP promoter did not show this phenomenon.

DISCUSSION

Chronic liver injuries often activate HSCs to overproduce ECM proteins and several fibrogenic proteins. Among them, TGF-β1 is the key mediator of liver fibrosis. TGF-β1 knockout mice have shown reduced collagen accumulation in response to liver injury (16). Inhibition of TGF-β1 gene expression or its signaling has been shown to decrease fibrosis in experimental animal model (17,18). Therefore, in this study, we selected two potent siRNA sequences for converting into shRNA and then cloning into pSilencer 1.0 vector driven by a ubiquitous U6 promoter due to its relative ease of producing shRNA transcripts. Transfection of HSC-T6 cells with these plasmids showed decent down-regulation of TGF-β1 mRNA. Co-expression of two shRNAs targeting 769 and 1033 TGF-β1 mRNA start sites showed higher TGF-β1 gene silencing. This finding is in good agreement with our previous study (10), where we demonstrated that the mixture of two potent siRNA sequences, targeting 769 and 1033 start sites of TGF-β1 mRNA, showed higher TGF-β1 gene silencing compared to single siRNA application at the same total concentration.

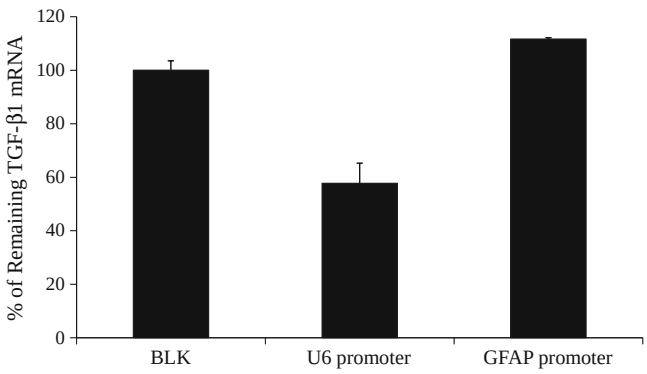

Fig. 8 Cell specificity of GFAP promoter-driven shRNA expression. After transfection with pGFA-mi-shRNA-1033 and U6-mi-siRNA-1033, the remaining TGF-β1 mRNA level in rat INS-1E cell line was decreased to 57.77±7.55% by U6 promoter driven plasmids, compared to the blank control.

Our purpose was to demonstrate whether the pool gene silencing effect similar to that achieved by the mixture of two siRNA pool is possible if we could co-express two shRNAs using a single plasmid. Just as the siRNA mixture was compared to single siRNA treatment, in this study, we also compared pU6-shRNA-769+1033 with single shRNA expression systems. We have previously demonstrated that co-expressing two shRNAs targeting different sites of hepatitis B virus surface antigen is more efficient and less toxic to the cells that mixing two different shRNA expression plasmids (19). We have also shown that co-expression of one cDNA and one shRNA or two different genes in a single plasmid is more effective and much safer than mixing two different plasmids, since the amount of bacterial backbone and cationic liposomes used is only half when we co-express two shRNAs in a single plasmid DNA (20,21).

TGF-β1 is overexpressed by HSCs, and U6 promoter drives high levels of shRNA expression by all cell types

which may elicit toxicity. Pri-miRNA mimics have the potential to mitigate this problem, as they can utilize low expression RNA pol II promoter. GFAP is an intermediate filament protein identified as a biomarker for both quiescent and activated HSCs (22). Furthermore, GFAP promoter has been utilized for HSC-specific gene expression (13,14) and is effective under the control of pol II, which can only recognize miRNA but not shRNA. Therefore, we selected miR-30 to be incorporated into the constructed plasmids. miR-30 has been investigated a lot in recent years and is involved in many areas in the body. miR-30 was upregulated in activated HSCs (23). Ji *et al.* demonstrated the expression of miRNA-30 was upregulated to more than 3-folds in activated HSCs than quiescent HSCs. This phenomenon facilitates TGF-β1 gene silencing in activated HSCs and reduces the unnecessary silencing in quiescent HSCs. However, some studies already demonstrated that the silencing effects of pri-miRNA mimics were decreased compared to matched shRNAs (24,25). It is good for the body because there may be some protective machinery from excessive intrinsic miRNA production to prevent toxicity in the cells, compared to artificial shRNA. In our study, the pri-miRNA mimics driven by GFAP promoter showed TGF-β1 gene silencing significantly less than the matched shRNA driven by U6 promoter (Fig. 3A). The choice between target expression and non-specific expression was affected by the balance between shRNAs and pri-miRNA mimics silencing effects. Fortunately, the pri-miRNA cluster mimics, which can target multiple sites in the same gene, in our case TGF-β1, controlled TGF-β1 level to a lower level relative to pri-miRNA mimics (Fig. 3B).

Besides the function of promoters, the secondary structure of pre-miRNA mimics plays an important role in efficient processing of miRNA-based silencing *in vitro* (26) and *in vivo* (27). The extended stem-loop structure in pri-miRNA mimics need to be bracketed by single-stranded RNA; otherwise, Drosha cannot recognize pri-miRNA transcriptions and process them correctly (28). From the secondary RNA structure software RNA structure version

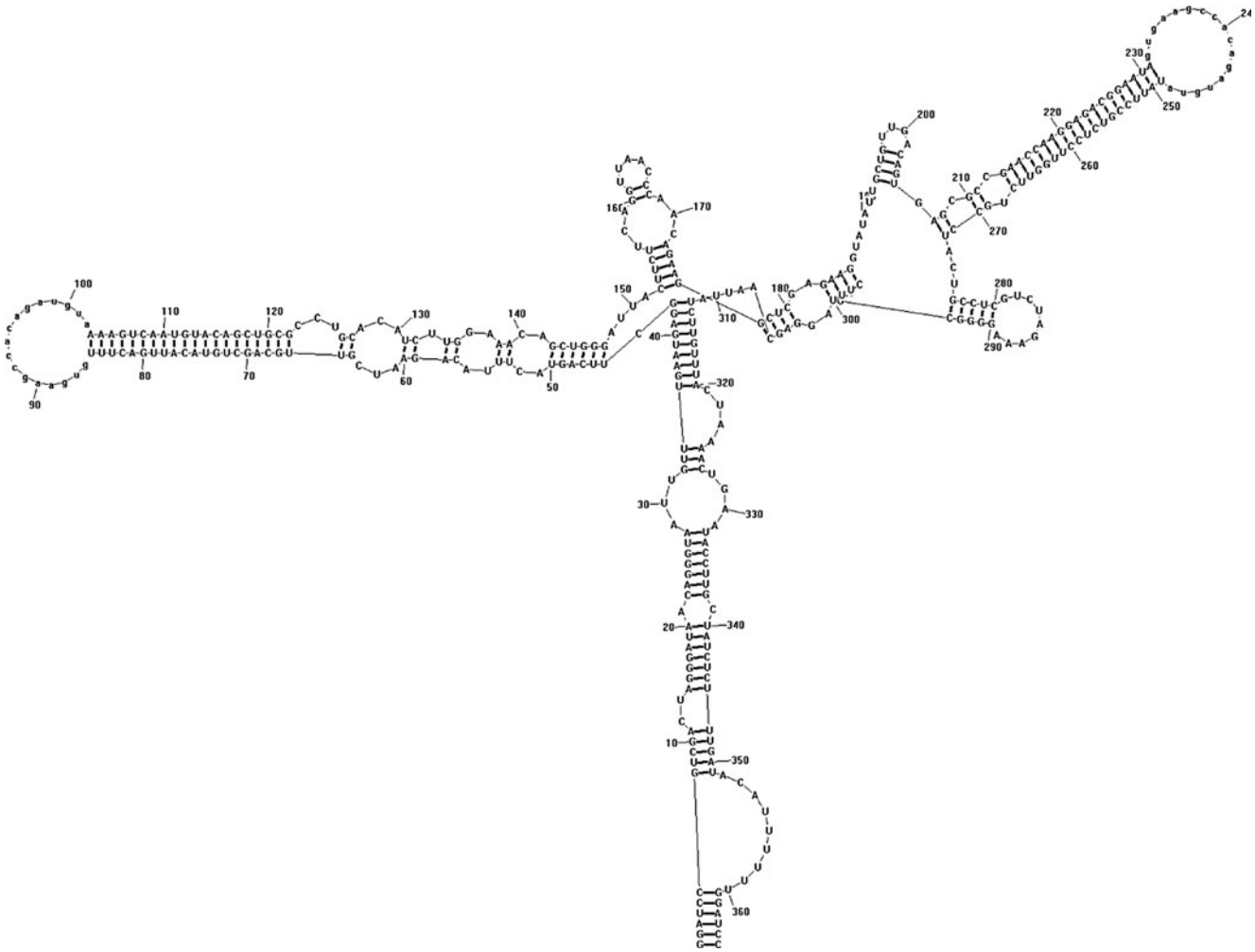

Fig. 9 The prediction of the secondary structure of the pri-miRNA cluster mimic transcribed from pGFA-mi-shRNA-769+1033 plasmid. The arrows points to the single strands flanking the extended stem-loops of pri-miRNA cluster mimics. The ovals indicate the shRNA inserts.

5.1, the pre-miRNA cluster mimic designed by our lab did have its extended stem-loop structure flanked by single-strand RNA (Fig. 9).

TGF-β1 is a protein that controls proliferation, apoptosis, and other functions in most cells. The TGF-β family is considered a negative cell proliferation controller and positive apoptosis inducer. TGF-β1 has been shown recently to increase the proliferation of variety of cells, for instance, airway smooth muscle cells (29), colon carcinoma cells (30, 31), and wounded epidermal cells (32). TGF-β1 is the most potent stimulus to HSCs in hepatic fibrosis. In quiescent HSCs, TGF-β1 inhibits the proliferation. However, once the quiescent HSCs are activated by the positive feedback activation mechanism, TGF-β1 loses its control to inhibit the proliferation of activated HSCs (33). Although the mechanisms of TGF-β1 to induce ECM accumulation have not been fully understood, TGF-β1 has been suggested to activate MAPK/ERK pathway (34), which is related closely to HSC proliferation. Although SMAD pathway has been reported to get activated after TGF-β1 application, it was also revealed that SMAD 2 pathway was not initiated by TGF-β1 (31). Our results suggest that TGF-β1 gene silencing inhibited the proliferation of HSC-T6 cells by MAPK/ERK pathway (Fig. 4B). The role of TGF-β1 on apoptosis was also changed after HSC activation. Saile *et al.* reported that DNA-NFκB binding ability was inhibited by TGF-β1 in activated HSCs (35), which meant TGF-β1 had anti-apoptotic effect on fibrotic HSCs. Thus, the inhibition of TGF-β1 allowed activated HSCs to undergo partial apoptosis (Fig. 4).

In conclusion, effective HSC-specific TGF-β1 gene silencing is possible using GFAP promoter, which is a pol II promoter to modulate miR30-shTGF-β1 expression in the pri-miRNA mimic plasmids. The pGFA-mi-shRNA 769+1033 showed higher efficiency in reducing HSC TGF-β1 mRNA level.

ACKNOWLEDGEMENTS

This study was supported by a grant from the National Institutes of Health (to RIM, grant number: EB003922).

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