

TGF- β 1 Gene Silencing for Treating Liver Fibrosis

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Abstract: Small interfering RNA (siRNA) and short hairpin RNA (shRNA) targeting different regions of transforming growth factor β 1 (TGF- β 1) mRNA were designed and the silencing effect was determined after transfection into immortalized rat liver stellate cells (HSC-T6). There was not only significant decrease in TGF- β 1, tissue inhibitor of metalloproteinase 1 (TIMP-1), α -smooth muscle actin (α -SMA) and type I collagen after transfection with TGF- β 1 siRNAs, but also synergism in gene silencing when siRNAs targeting two different start sites were used as a pool for transfection. The two siRNA sequences which efficiently inhibited TGF- β 1 gene expression were converted to shRNAs via cloning into the pSilencer1.0. There was significant decrease in TGF- β 1 and TIMP-1 when HSC-T6 cells were transfected with pshRNA targeting the same regions of TGF- β 1 mRNA as siRNAs. Furthermore, TGF- β 1 gene silencing in HSC-T6 cells significantly decreased the levels of inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 beta (IL-1 β). In conclusion, both siRNA and shRNA showed sequence-specific and dose dependent TGF- β 1 gene silencing and have the potential to treat liver fibrosis.

Keywords: siRNA; shRNA; transforming growth factor β 1 (TGF- β 1); hepatic stellate cells; liver fibrosis

Introduction

Liver fibrosis is the excessive accumulation of extracellular matrix (ECM) proteins resulting from chronic liver damage. In general, it is an imbalance between the synthesis and degradation of ECM. In the presence of chronic liver injuries, hepatic stellate cells (HSCs) become activated and transform to proliferative myofibroblast-like cells, which account for the major source of ECM expression.^{1,2}

There is no standard treatment for liver fibrosis, and therefore effective antifibrotic medicines are needed urgently.^{1,3}

Among many inflammatory cytokines involved in liver fibrosis, TGF- β 1 appears to be the most important one (Figure 1).^{1,4} Cytokines of the TGF family affect a variety of cellular processes, including differentiation, proliferation, apoptosis and migration. Among them, TGF- β 1 is the most potent profibrogenic factor involved in initiation and maintenance of fibrogenesis in the liver.^{5,6} Stimulation of activated HSCs by TGF- β 1 is believed to be the key fibrogenic response in liver fibrosis because of following evidence: (i)

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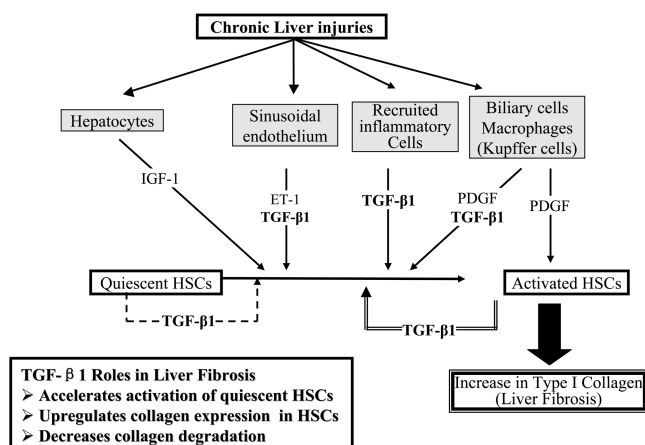


Figure 1. Role of TGF- β 1 in liver fibrosis. TGF- β 1 is the most potent single profibrogenic factor involved in initiation and maintenance of fibrogenesis in the liver. TGF- β 1 accelerates activation of quiescent hepatic stellate cells (HSCs), upregulates collagen expression, and decreases collagen degradation.

higher TGF- β 1 expression in activated HSC; (ii) potency of TGF- β 1 to upregulate ECM expression; (iii) higher expression of TGF- β receptors on HSC; (iv) TGF- β 1 increases the expression of TIMP-1.^{5–7} TGF- β 1 in the liver is secreted by hepatocytes, kupffer cells, stellate cells, endothelial cells and infiltrating mononuclear cells.⁵

Strategies aimed at disrupting TGF- β 1 expression or signaling pathways are extensively being investigated because blocking this cytokine may not only inhibit matrix production but also accelerate its degradation.⁸ Animal experiments using different strategies to block TGF- β 1 have demonstrated significant antifibrotic effect for liver fibrosis.^{6,9–14}

RNA interference (RNAi) is the phenomenon in which siRNA of 21–23 nt in length silences a target gene by binding to its complementary mRNA and triggering its degradation. Potent knockdown of the target gene with high sequence specificity makes siRNA a promising therapeutic strategy.² Compared to antisense oligonucleotides, neutralizing antibodies and soluble TGF- β receptor strategies, siRNA targeting TGF- β 1 has the potent knockdown of the target gene with high sequence specificity. siRNAs targeting other pathways have been proven effective in treating liver fibrosis^{15–18} and renal fibrosis.¹⁹ There are three ways to deliver siRNA: synthetic duplex, plasmid and viral vectors. While viral vectors give high transduction efficiency, their immune reactions limit their application in therapeutics. On the other hand, plasmid DNA complexes with cationic liposomes may not pass through the sinusoidal gaps, because these fenestrae get lost during liver fibrosis. In contrast, low molecular weight synthetic duplex siRNA is expected to pass through the sinusoidal gaps in fibrotic liver, and thus it may be an ideal candidate for treating liver fibrosis.

In this study, we designed and screened ten chemically synthesized siRNAs targeting different regions of TGF- β 1 mRNA and then converted the most potent siRNA sequences into shRNA via cloning into pSilencer1.0 vector. Both synthetic siRNAs and shRNA expression plasmids were tested in HSC-T6 cells for gene silencing and therapeutic efficacy.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), penicillin G (5000 U/mL), trypsin-EDTA, Trizol, DNase I,

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Table 1. Predesigned siRNAs for Rat TGF- β 1 Using BLOCK-iT RNAi Designer

start	sequence (DNA)
297	GCCAGATCCTGTCCAAACT
448	GGACTACTACGCCAAAGAA
499	CGCAATCTATGACAAAACC
640	GCAACACGTAGAACTCTAC
769	GAACCAAGGAGACGGAATA
888	GCACCATCCATGACATGAA
1033	GCAGCTGTACATTGACTTT
1036	GCTGTACATTGACTTTAGG
1167	CCCTCTACAACCAACACAA
1245	TCTACTACGTGGGTCGCAA

and Lipofectamine 2000 were purchased from Invitrogen Corporation (Carlsbad, CA). pSilencer1.0 was purchased from GenScript Corporation (Piscataway, NJ). Bovine serum albumin (BSA) (fraction V, purity >98%) was purchased from USB Corporation (Cleveland, OH). 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). TGF- β 1 enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems, Inc. (Minneapolis, MN). TNF- α and IL-1 β ELISA kits were purchased from eBioscience, Inc. (San Diego, CA).

siRNA Design and Synthesis. Ten synthetic siRNAs targeting TGF- β 1 mRNA (Accession no.: NM_021578) and one control siRNA were designed using BLOCK-iT RNAi Designer and purchased from Invitrogen Corporation (Carlsbad, CA), and their sequences are listed in Table 1. These siRNAs are of 19–21 nt with 2-thymidine deoxynucleotide overhangs at the 3'-end. All designed siRNA sequences were blasted against the rat genome database to eliminate cross-silence phenomenon with nontarget genes.

Design and Construction of shRNA Expression Plasmids. Following screening of different siRNA sequences, two effective and one control siRNA sequences were converted into shRNA (Table 2). pshRNA-769, pshRNA-1033 and control vectors were constructed using pSilencer 1.0 vector, which carries an shRNA expression cassette under the control of U6 promoter (GenScript Corporation, Piscataway, NJ). As shown in Table 2, these shRNAs contain two complementary oligonucleotides that were annealed to form a double-stranded DNA for ligation into pSilencer1.0 vector at corresponding sites under U6 promoter using T4 DNA ligase for 2 h at 25 °C. Following transformation in Top 10 supercompetent cells and amplification in broth media, plasmids were purified using QIAGEN Plasmid Mini Kit.

Transfection of siRNA and shRNA. HSC-T6 cells, kindly provided by Dr. Scott Friedman (Mount Sinai School of Medicine, New York), were seeded in 6-well plates at a density of 1.1×10^5 cells/well 12 h before transfection. siRNA duplexes were mixed with 4 μ L of Lipofectamine 2000 in 200 μ L of Opti-MEM I medium for 20 min at room temperature to allow complex formation. The transfection mixture was then added to each well with 1.3 mL of fetal

bovine serum (FBS) free DMEM medium at a concentration of 150 nM after washing cells with phosphate buffered saline (PBS). After 24 h of incubation, 0.5 mL of DMEM containing 10% FBS was added and incubated for another 24 h. For siRNA pool, the equal amounts of two siRNAs were mixed together and the final concentration of total siRNAs was 150 nM.

In the case of shRNA expression plasmids, HSC-T6 cells were transfected with shRNA expression plasmids pshRNA-769 and pshRNA-1033 after complex formation in 250 μ L of Opti-MEM I medium with pyridinium lipid (C16:1, amide linker, transisomer)/L- α -dioleoyl phosphatidylethanolamine (DOPE) (1:1 mol/mol) cationic liposomes as described by Zhu et al.²⁰ The transfection mixture was then added to each well with 2 mL of FBS free DMEM. At 3 h postincubation, 200 μ L of FBS was added and then incubated for an additional 42 h. The supernatant was collected for Western blot assay.

Real Time RT-PCR. Total RNA was isolated from cell pellets using Trizol reagent. Total RNA (1 μ g) was converted to cDNA using MultiScribe Reverse Transcriptase Reagent and random hexamers (Applied Biosystems, Inc., Branchburg, NJ). One hundred nanograms of the 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). We used the following primers. (i) TGF- β 1: forward, 5'-CATCCATGACATGAACCGACCCTT-3', and reverse, 5'-ACAGAAGT-TGGCATGGTAGCCCTT-3'. (ii) TIMP-1: forward, 5'-CCTCTGGCATCCTCTTGTGTCTAT-3', and reverse, 5'-CATTTCCCACAGCGTCGAATCCTT-3'. (iii) α 1(I)collagen forward, 5'-TGGTCCCAAAGTTCTCCTGGT-3', and reverse, 5'-TTAGGTCCAGGGAATCCCATCACA-3'. (iv) α -SMA: forward, 5'-ACAACGTGCCTATCTATGAGGGCT-3', and reverse, 5'-AGCGACATAGCACAGCTTCTCCTT-3'. We used 18S rRNA as an internal control: forward, 5'-GTCTGTGATGCCCTTAGATG-3', and reverse, 5'-AGCTTATGACCCGCACTTAC-3'. To confirm PCR specificity, the PCR products were subjected to a melting-curve analysis. Comparative threshold (C_t) method was used for calculating the relative amount of mRNA of treated sample compared to control samples.²¹ The illustrative real-time RT-PCR plot and calculation details were provided in Figure 2.

Western Blot Assay. Proteins in the cell culture medium were purified using Microcon YM-30 columns (Millipore) and then lysed using 2 \times 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. To immunodetect cellular proteins, cells were lysed directly with 1 \times Laemmli SDS sample buffer. The lysate was then boiled for 5 min and subjected to 6% or 10% SDS–polyacrylamide (SDS–PAGE) gel electrophoresis and subsequent transfer to Immobilon polyvi-

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Table 2. Sequences of shRNA against Different Target Regions of TGF-β1

Control shRNA	Antisense: 5'- ACTTCATAAGGCGCATGCTTCAAGAGAAGCATGCGCCTTATGAAGTTTTTTT -3' Sense: 5'-AATTAATAAA AACTTCATAAGGCGCATGCTTCTCTTGAAAGCATGCGCCTTATGAAGTGGCC -3
shRNA-769	Antisense: 5'- GAACCAAGGAGACGGAATTTCAAGAGATATTCGCTCTCCTTGGTTC TTTTTT-3' Sense: 5'-AATTAATAAA GAACCAAGGAGACGGAATATCTCTTGAAATATTCGCTCTCCTTGGTTCGGCC -3
shRNA-1033	Antisense: 5'- GCAGCTGTACATTGACTTTTCAAGAGAAAAGTCAATGTACAGCTGCTTTTTT -3 Sense: 5'-AATTAATAAA GCAGCTGTACATTGACTTTTCTCTTGAAAAGTCAATGTACAGCTGCGGCC -3

nylidene fluoride (PVDF) membrane (Millipore). After blocking with 5% nonfat dry milk in 1×PBST (PBS containing 0.05% Tween-20) for 1 h at room temperature, the membranes were incubated with rabbit antirat type I collagen (Calbiochem), TGF-β1 (Santa Cruz); β-actin (Santa Cruz) primary antibodies for 16 h at 4 °C. Membrane was then incubated with horseradish peroxidase-conjugated goat antirabbit secondary antibody (Santa Cruz) for 1 h at room temperature. Target proteins were detected by enhanced chemiluminescence (ECL) detection kit (GE Healthcare Life Sciences, Pittsburgh, PA).

ELISA of TGF-β1, TNF-α and IL-1β. HSC-T6 cells were transfected with shRNA expression vectors at a dose of 1 μg/well after complex formation with cationic liposomes prepared using pyridinium lipid (C16:1, amide linker, trans isomer) and DOPE at 1:1 molar ratio as described by Zhu et al.²⁰ Forty-eight hours after transfection, supernatant of the cultured cells was collected and the concentrations of TGF-β1, TNF-α and IL-1β were measured using ELISA kits according to the manufacturer's protocol.

Persistence of TGF-β1 Gene Silencing. HSC-T6 cells were seeded in 6-well plates and transfected with siRNA and shRNA when the cell confluence reached 40%. One microgram of siRNA duplexes or shRNA was mixed with 8 μL of pyridinium lipid (C16:1, amide linker, trans)/DOPE (1:1 mol/mol) cationic liposomes. The transfection mixture was then added to each plate with 2 mL of FBS free DMEM at a concentration of 150 nM for siRNA and 1 μg per well for shRNA; both conditions have been optimized for siRNA

and shRNA transfection in this case. After 3 h of incubation, 200 μL of FBS was added and incubated for another 24 h, 48 h and 72 h. At different time points, the cells were collected and total RNA was isolated for real time RT-PCR analysis.

Statistical Analysis. Data were expressed as the mean ± standard deviation (SD). Difference between any two groups was determined by ANOVA. *P* < 0.05 was considered statistically significant.

Results

Effect of siRNA Sequences and Doses on TGF-β1 Gene Silencing. To examine the ability of siRNA to silence gene expression, we selected 19bp synthetic siRNA duplexes targeting different regions of the rat TGF-β1 mRNA (Table 1). These siRNA duplexes were transfected into rat HSC-T6 cells after complex formation with Lipofectamine 2000. As shown in Figure 3, TGF-β1 gene silencing by siRNAs was sequence specific. Three siRNAs targeting TGF-β1 start sites of 769, 1033, and 1036 caused significant inhibition of TGF-β1 expression, while the control siRNA had no effect on TGF-β1 gene expression. Among all the siRNA sequences tested, siRNA-1036 showed the highest silencing effect up to 70%, while siRNA-1033 showed up to 55% of gene silencing. Therefore, we selected siRNA-1036 and siRNA-1033 to determine the effect of siRNA dose on TGF-β1 gene silencing. We also tested the pool of siRNAs targeting 769 and 1033, which showed higher TGF-β gene silencing effect than single siRNA. As shown in Figures 4A and 4B, there was significant increase in the TGF-β1 silencing effect as we increased the doses of siRNA-1033 and siRNA-1036 from 10 nM to 150 nM, with siRNA-1036 showing the highest TGF-β1 gene silencing effect at 150 nM.

We also determined the level of TGF-β1 secreted into the cell culture medium by Western blot analysis at day 2 post-transfection of HSC-T6 cells with siRNAs targeting TGF-β1 start sites of 769 and 1033 as well as the pool of these two siRNAs. As shown in Figure 5, the level of TGF-β1 protein secreted in the culture medium was significantly lower than that of nontransfected and control-siRNA transfected samples.

Effect of TGF-β1 Gene Silencing on TIMP-1 Expression. TGF-β1 is the key profibrogenic cytokine in response to chronic liver injuries. It not only enhances ECM synthesis but also inhibits ECM degradation by downregulating matrix-

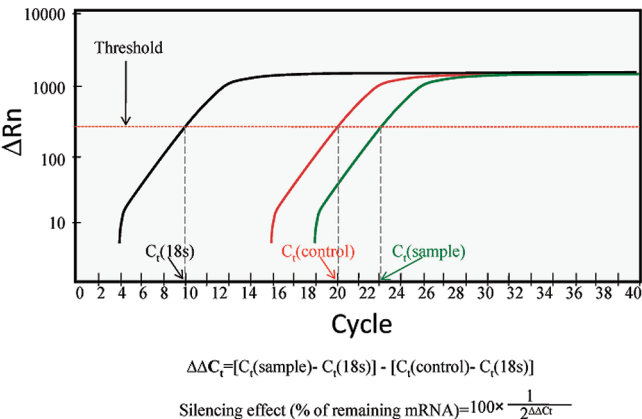


Figure 2. The real time PCR plot. Black curve is the intrinsic reference (18s). Red curve is the control group. Green curve is the group with treatment.

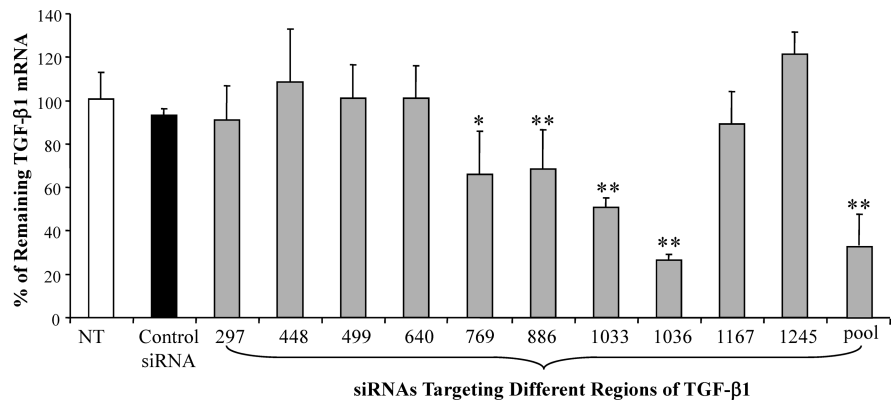


Figure 3. Effect of siRNA sequence on TGF-β1 gene silencing. Ten different siRNAs targeting different regions of TGF-β1 mRNA were transfected into HSC-T6 cells at a dose of 150 nM after complex formation with Lipofectamine 2000. TGF-β1 gene silencing was determined by real time RT-PCR at 48 h post-transfection. Results were represented as the mean ± SD (*n* = 3). **p* < 0.05, ***p* < 0.01.

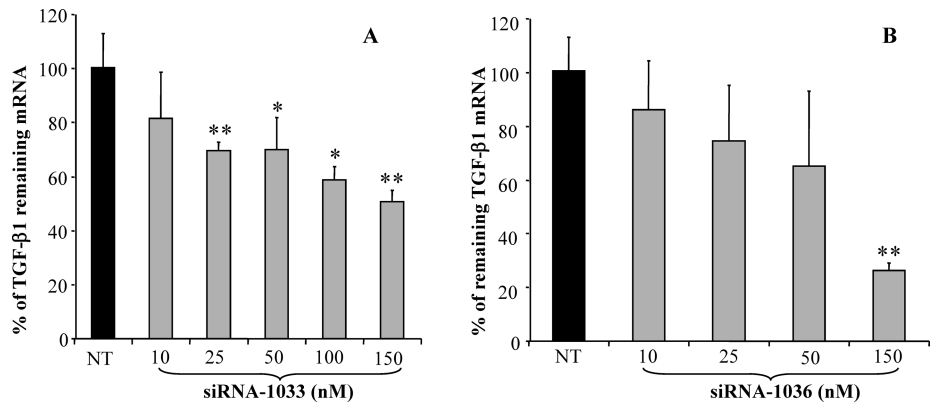


Figure 4. Effect of siRNA concentration on TGF-β1 silencing. Lipofectamine 2000/siRNA complexes were transfected into HSC-T6 cells at doses of 10, 25, 50, 100, and 150 nM. TGF-β1 gene silencing was determined by real time RT-PCR at 48 h post-transfection. Results were represented as the mean ± SD (*n* = 3). **p* < 0.05, ***p* < 0.01.

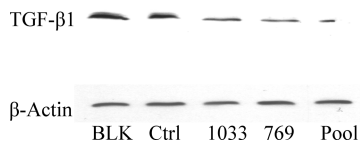


Figure 5. Western blot analysis for TGF-β1 gene silencing after transfection of HSC-T6 cells with Lipofectamine/siRNA complexes. From left to right, no siRNA, control siRNA, siRNA-1033, siRNA-769 and pool of siRNA-1033 and siRNA-769. From top to bottom, TGF-β1 and β-actin.

degrading enzymes and inducing TIMP-1.²² High level of TIMP-1 in the fibrotic accounts for the slow degradation of type I collagen. Therefore, we measured TIMP-1 mRNA expression of HSC-T6 cells after transfection with TGF-β1 siRNAs. As shown in Figure 6, both siRNA-1033 and siRNA-1036 inhibited TIMP-1 expression, while the control siRNA did not show any effect on TIMP-1 expression. These results suggested the similar inhibition profile of TIMP-1 and TGF-β1 after transfection of HSC-T6 cells with siRNA targeting TGF-β1.

Effect of siRNA on Type I Collagen and α-SMA Expression. Inhibition of TGF-β1 should enhance the degradation of ECM, in which type I collagen is the main

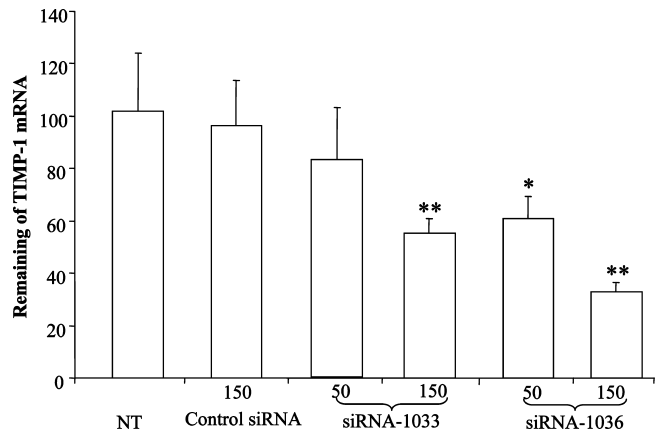


Figure 6. Effect of TGF-β1 gene silencing on TIMP-1 gene expression after transfection of HSC-T6 cells with Lipofectamine 2000/siRNA complexes. At 48 h post-transfection, cells were harvested, total RNA was extracted, and TIMP-1 gene expression was determined at mRNA level using real time RT-PCR. Results were represented as the mean ± SD (*n* = 3). **p* < 0.05, ***p* < 0.01.

component. Therefore, we designed collagen α1(I) mRNA specific primers to determine the effect of TGF-β1 siRNA

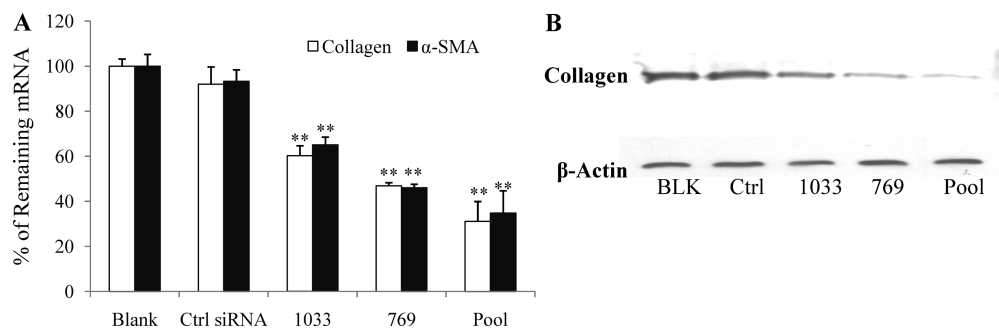


Figure 7. Effect of TGF- β 1 gene silencing on type α 1(I) collagen and α -SMA expression after transfection of HSC-T6 cells Lipofectamine 2000/siRNA-1033, 769 and pool complexes. A) At 48 h post transfection, cells were harvested, total RNA was extracted, and type α 1(I) collagen and α -SMA expression was determined at mRNA levels using real time RT-PCR. Results were represented as the mean \pm SD ($n = 3$). *: $P < 0.05$, **: $P < 0.01$ B) Western blot analysis for type α 1(I) collagen expression after TGF- β 1 gene silencing.

on collagen α 1(I) mRNA expression. At 150 nM, siRNA-1033, siRNA-769 and the pool of these two siRNAs showed significant inhibition of collagen α 1(I) mRNA (Figure 7A), while the control siRNA had little effect on collagen α 1(I) mRNA expression. We also determined the effect of TGF- β 1 gene silencing on type α 1(I) collagen protein expression by Western blot analysis of lysates of HSC-T6 cells after transfection with siRNAs targeting TGF- β 1 start sites of 769 and 1033 as well as the pool of these two siRNAs (Figure 7B). Consistent with the RT-PCR results, there was significant decrease in collagen of siRNA-769, siRNA-1033 and siRNA pool, while there was no decrease in collagen concentration for the control-siRNA treated sample.

Construction of shRNA Expression Vector. Following screening of different siRNA sequences for TGF- β 1 gene silencing, we selected two potent siRNA sequences, which contain unique restriction sites at the 5' and 3' ends for cloning and a TTTT stretch in the antisense to create the pol III terminal signal and TTCAAGAGA as a loop (Table 2). The constructed pshRNA-769 and pshRNA-1033 were confirmed by DNA sequencing using the forward primer 5'-TAAGTTGGGTAACGCCAGGG-3' and reverse primer 5'-GCTATGACCATGATTACGCC-3'.

Effect of shRNA on TGF- β 1 Silencing. TGF- β 1 gene expression was determined by real time RT-PCR and Western blot after transfection of HSC-T6 cells with pshRNA-769 and pshRNA-1033 complexed with pyridinium lipid (C16:1, amide linker, trans)/DOPE (1:1 mol/mol) cationic liposomes.²⁰ Both pshRNA-769 and pshRNA-1033 showed significantly higher TGF- β 1 gene silencing effect compared to control shRNA (Figure 8A). There was also similar decrease in the protein levels of TGF- β 1 and type I collagen (Figure 8B), which is in good agreement with the results of synthetic siRNAs.

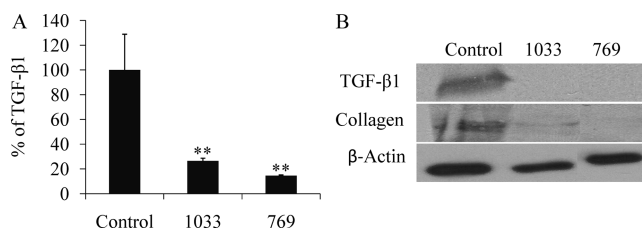


Figure 8. Effect of TGF- β 1 gene silencing on type α 1(I) collagen and TGF- β 1 expression after transfection of HSC-T6 cells with pshRNA-1033 or pshRNA-769 after complex formation with pyridinium lipid (C16:1, amide linker, trans)/DOPE (1:1 mol/mol) cationic liposomes.²⁰ At 42 h post-transfection, cells were harvested, total RNA was extracted, and TGF- β 1 expression was determined at mRNA levels using real time RT-PCR (A). In addition, protein levels of TGF- β 1 and type I collagen in the supernatant were determined using Western blot (B). Results were represented as the mean \pm SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$.

Effect of TGF- β 1 Gene Silencing on TNF- α and IL-1 β . Since liver inflammation promotes liver fibrosis, we wanted to determine whether TGF- β 1 gene silencing could decrease collagen gene expression by decreasing inflammation in the liver. Therefore, we measured the amount of TNF- α and IL-1 β secreted in the culture media upon transfection with pshRNA-1033 after complex formation with pyridinium lipid (C16:1, amide linker, trans)/DOPE (1:1 mol/mol) cationic liposomes²⁰ using ELISA kits. As can be seen in Figure 9A, the amount of TNF- α was decreased to $43.41 \pm 4.03\%$ and $51.85 \pm 4.75\%$, respectively, compared to the control group not treated with pshRNA-TGF- β 1. In the same trend, the level of IL-1 β also decreased to $49.12 \pm 5.78\%$ and $59.49 \pm 9.67\%$, respectively, compared to the control shRNA (Figure 9B).

Persistence of TGF- β 1 Gene Silencing. To compare siRNA and shRNA on TGF- β 1 gene silencing, we transfected HSC-T6 cells with siRNA-1033 and pshRNA-TGF- β 1-1033 after complex formation with our pyridinium lipid (C16:1, amide linker, trans)/DOPE (1:1 mol/mol) cationic

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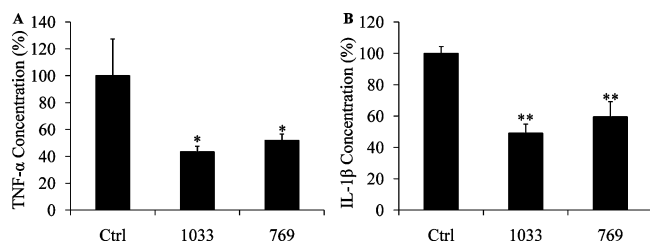


Figure 9. Effect of TGF- β 1 gene silencing on TNF- α and IL-1 β secretion. HSC-T6 cells were transfected with pshRNA-1033 and pshRNA-769 after complex formation with pyridinium lipid (C16:1, amide linker, transisomer)/DOPE (1:1 mol/mol) cationic liposomes. Following transfection, TNF- α and IL-1 β concentrations were measured by enzyme-linked immunosorbent assay (ELISA). Results were represented as the mean \pm SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$.

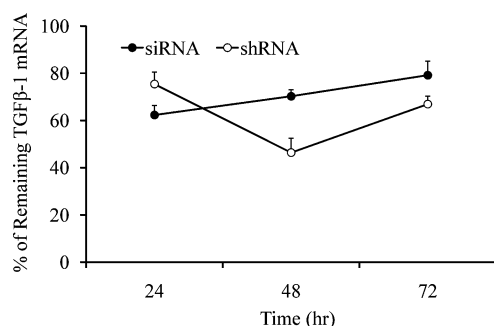


Figure 10. Persistence of siRNA and shRNA gene silencing. HSC-T6 cells were transfected with siRNA-1033 and shRNA-769 after complex formation with pyridinium lipid (C16:1, amide linker, trans)/DOPE (1:1 mol/mol) cationic liposomes as described above. After 3 h of transfection, 200 μ L of FBS was added and incubated for another 24 h, 48 h and 72 h. At different time points, the cells were collected and total RNA was isolated for real time RT-PCR analysis.

liposomes at 3.5/1 (+/-) ratio. As shown in Figure 10, the level of TGF- β 1 mRNA was significantly lower for the pSilencer-TGF- β 1 group, which was lowest at 48 h post-transfection compared to 24 h post-transfection for the siRNA-TGF- β 1 group. For siRNA treated group, the level of TGF- β 1 gene silencing significantly decreased with time, being the lowest at 72 h post-transfection. However, for the shRNA group, there was still significant silencing even after 72 h post-transfection.

Discussion

Liver fibrosis results from chronic injuries to the liver by chronic hepatitis, alcohol abuse, and toxic agents. Among all fibrogenic cytokines, TGF- β is the key mediator of liver fibrosis and thus has gained great attention.¹ TGF- β contains three isoforms including TGF- β 1, TGF- β 2 and TGF- β 3. Among them TGF- β 1 is the dominant stimulus to ECM production from HSCs and its expression increases during fibrogenesis.⁸ TGF- β 1 can be secreted by Kupffer cells, biliary cells and infiltrated inflammatory cells, while the

autocrine expression of TGF- β 1 by HSCs is the most important.⁴ Compared to quiescent HSCs, fully activated HSCs are relatively unresponsive to TGF- β 1, possibly due to the downregulation of TGF- β receptor. TGF- β 1 knockout mice have shown reduced collagen accumulation in response to liver injury compared to that of normal mice.²³ Disrupting TGF- β 1 synthesis or signaling pathways significantly decreased fibrosis in experimental animal model.^{6,9} However, due to its multiple actions, TGF- β represents a potentially important link between neoplasia and fibrosis in the liver. Mutation of TGF- β receptor or signaling intermediates has been found in various epithelial cancers.¹¹ Strategies aimed at disrupting TGF- β 1 expression or signaling pathways are being extensively investigated for treating liver fibrosis and various animal studies have demonstrated antifibrotic effect.^{6,9-14} Currently neutralizing antibodies, soluble TGF- β receptors, antisense oligonucleotide and siRNA were explored to block TGF- β 1 signaling pathway.

Selection of a potent siRNA sequence targeting a specific gene is a crucial step in developing its therapeutic applications. In this study, we used BLOCK-iT RNAi Designer from Invitrogen to design ten synthetic siRNAs (Table 1) targeting at different coding regions of TGF- β 1 mRNA. As shown in Figure 3, only 4 siRNAs showed significant silencing effect on TGF- β 1 gene expression, while other 6 siRNAs did not show any effect. All these 4 siRNAs are located in the coding regions of TGF- β 1 mRNA starting from 769 to 1036. Among these 4 siRNAs, siRNAs targeting 1033 and 1036 coding sites showed the highest TGF- β 1 gene silencing effect. These may be due to the different local secondary structures of the TGF- β 1 mRNA, and the 769–1036 region is more accessible for siRNAs.

Two selected siRNAs, siRNA-1033 and siRNA-1036, showed dose dependent inhibition of TGF- β 1 mRNA (Figure 4). The silencing effect of siRNA-1036 was similar to that of siRNA-1033 at low concentration, but more significant at high concentration (150 nM). At low concentrations, both siRNAs showed little inhibition, and therefore it is difficult to distinguish which one is more effective. Considering there is only a 3 base sequence shift between siRNA-1033 and siRNA-1036, the significant difference in silencing indicated that the silencing effect was very sequence specific.

Even though the dose increased to 3-fold, from 50 nM to 150 nM, the level of TGF- β 1 gene silencing did not increase that much. The gene silencing ability of siRNAs had no linear relationship with their dose. However, at the same total concentration, application of more than one siRNA targeting at different sites of mRNA produced enhanced TGF- β 1 gene silencing and significant decrease in type I collagen (Figures 3, 5 and 7), suggesting the use of more than one siRNA sequences as a pool may produce synergistic effect. These results are in good agreement with our recently published work where we demonstrated that a pool of siRNAs targeting

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different regions of HBsAg showed high gene silencing in HepG2.2.15 cells.²⁴

The *in vivo* stability of synthetic siRNA and the high cost for *in vivo* studies hamper its therapeutic application. Therefore, we decided to construct a plasmid based shRNA targeting the most potent region of the TGF- β 1 mRNA. The silencing effect of shRNAs targeted at 769 and 1033 showed relatively higher gene silencing, which lasted longer than synthetic siRNAs targeted at the same positions (Figures 3, 8 and 10). One reason might be the relatively higher stability of shRNA inside the cells than siRNA. Its consistent expression ability in the cells and ease to produce makes it a promising vector for therapeutic application. It is worth mentioning that HSC-T6 cells grow relatively fast and thus 72 h after transfection was the maximum time we could maintain healthy HSCs with 100% confluence. Beyond 72 h, the cells were too crowded and became unhealthy. Therefore, we could not determine gene silencing beyond 72 h post-transfection.

The development of chronic liver diseases is mediated by sustained hepatic inflammation. Many studies have shown various cytokines, which are crucial indicators of inflammation, such as TGF- β 1 and TNF- α , are important activators during the process of liver fibrosis.^{25–27} TNF- α has been reported closely related to liver fibrosis and cirrhosis.²⁷

Therefore, we determined the level of TNF- α secreted in the culture media after transfection of HSC-T6 cells with TGF- β 1 shRNA. TGF- β 1 gene silencing significantly decreased TNF- α concentration, suggesting TGF- β 1 gene silencing will decrease liver inflammation. IL-1 β is another important cytokine, which is known to promote local inflammatory response and consequently promotes chronic liver fibrosis.²⁸ Our results also showed that there is significant decrease in secreted IL-1 β upon TGF- β 1 gene silencing (Figure 9B). Both TNF- α and IL-1 β levels correlated with TGF- β 1 gene silencing levels.

In summary, we have successfully designed and validated TGF- β 1-specific siRNAs and then converted two potent siRNA sequences into shRNAs, which effectively silenced TGF- β 1 gene expression in HSC-T6 cells. TGF- β 1 gene silencing significantly reduced the production of type I collagen, TIMP-1, and inflammatory cytokines. Our results suggested that silencing of TGF- β 1 by siRNA and shRNA may be an efficient and more specific approach for therapy of liver fibrosis.

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