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Small Interfering RNAs (siRNAs) Targeting TGF- β_1 mRNA Suppress Asbestos-Induced Expression of TGF- β_1 and CTGF in Fibroblasts

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Abstract

Interstitial lung disease (ILD) afflicts millions of people worldwide. ILD can be caused by a number of agents, including inhaled asbestos, and may ultimately result in respiratory failure and death. Currently, there are no effective treatments for ILD. Transforming growth factor- β_1 (TGF- β_1) is thought to play an important role in the development of pulmonary fibrosis, and asbestos has been shown to induce TGF- β_1 expression in a murine model of ILD. To better define the role of TGF- β_1 in ILD, we developed several small interfering RNAs (siRNAs) that target TGF- β_1 mRNA for degradation. To assess the efficacy of each siRNA in reducing asbestos-induced TGF- β_1 expression, Swiss 3T3 fibroblasts were transfected with TGF- β_1 siRNAs and then treated with chrysotile asbestos for 48 h. Two independent siRNAs targeting TGF- β_1 mRNA knocked-down asbestos-induced expression of TGF- β_1 mRNA by 72–89% and protein by 70–84%. Interestingly, siRNA knockdown of TGF- β_1 also reduced asbestos-induced expression of connective tissue growth factor (CTGF). CTGF can be upregulated by TGF- β_1 and appears to play an important role in the development of pulmonary fibrosis. These results suggest that siRNAs could be effective in preventing or possibly arresting the progression of pulmonary fibrosis. Studies are underway in vivo to test this postulate.

Keywords

interstitial lung disease; RNA interference; small interfering RNA; transforming growth factor- β_1 ; connective tissue growth factor; asbestos

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Introduction

Interstitial lung disease (ILD) afflicts millions of individuals worldwide.¹ This chronic disease can be caused by environmental agents such as asbestos² or silica³ and antineoplastic drugs such as bleomycin.⁴ Most often the cause remains unknown, or idiopathic.^{1,5–7} The pathogenesis of ILD involves failure of alveolar reepithelialization, persistence of fibroblasts/myofibroblasts, consequent deposition of extracellular matrix, and distortion of lung architecture, which ultimately results in respiratory failure.^{5,6} Currently, there are no effective treatments for ILD.⁷

Transforming growth factor- β_1 (TGF- β_1) has been shown to play an important role in the process of ILD. TGF- β_1 is a multifunctional protein that regulates cell proliferation,⁸ differentiation,⁹ apoptosis,¹⁰ cell cycle,¹¹ embryogenesis,¹² development,^{13,14} wound healing,^{15,16} tissue repair,¹⁷ angiogenesis,^{18,19} and tumor development.²⁰ TGF- β_1 has been implicated as one of the key cytokines in the induction of fibrosis in many organs, including the lung. In the lung, it stimulates differentiation of fibroblasts to myofibroblasts, induces the synthesis of matrix proteins, and inhibits collagen degradation. TGF- β_1 -deficient mice suffer from multifocal inflammatory disease and die from organ failure after birth.²¹

Introducing a TGF- β_1 expression vector in a rat lung induced significant proliferation of fibroblasts and deposition of collagen fibrils.²² Moreover, overexpression of TGF- β_1 induces fibroproliferative pulmonary disease in mice that are naturally resistant to fibrosis²³ and in tumor necrosis factor- α (TNF- α) receptor knockout mice that also are resistant to fibrogenesis.²⁴ TGF- β_1 is also upregulated in the foci of activated fibroblasts and alveolar epithelial cells from patients with idiopathic pulmonary fibrosis.^{25,26} Together this evidence suggests that TGF- β_1 plays an important role in the pathogenesis of pulmonary fibrosis.

Connective tissue growth factor (CTGF) is a member of the CTGF/cysteine-rich 61/nephroblastoma overexpressed (CCN) family.²⁷ CTGF is thought to be a key determinant of progressive fibrosis. Many studies have correlated increased expression of CTGF with fibroproliferative disorders, and downregulation of CTGF expression seems to offer protection from fibrosis.²⁸ TGF- β_1 induces CTGF expression through different signaling pathways and a specific TGF- β_1 responsive element in the CTGF promoter.²⁹ CTGF is considered to be a downstream mediator of TGF- β_1 signaling in the fibrosis process.²⁹

RNA interference (RNAi) technology can suppress specific gene expression and thus could have great potential to cure diseases that currently have no promising treatment.^{30–32} Several RNAi-technology—based therapies have already been evaluated in clinical trials for the treatment of age-related macular degeneration (AMD) and respiratory syncytial virus (RSV) infection.³³ We are exploring the use of RNAi-technology to inhibit TGF- β_1 -induced fibrosis as a potential treatment for ILD.

Materials and Methods

siRNAs and Reagents

siRNAs targeting TGF- β_1 mRNA were designed using SMARTpool technologies (Dharmacon, Lafayette, CO) and synthesized by Dharmacon. The negative control siRNAs were purchased from Ambion (Austin, TX). Fetal bovine serum (FBS), bovine calf serum (BCS), trypsin/EDTA, and penicillin/streptomycin were purchased from Invitrogen (Invitrogen Corp., Carlsbad, CA). All other reagents were purchased from Sigma Chemicals (Sigma-Aldrich, St. Louis, MO).

Cell Culture

Swiss 3T3 fibroblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultivated in Dulbecco's modified Eagle's medium (Sigma-Aldrich) with nonessential amino acids, L-glutamine, 10% bovine calf serum (Invitrogen), 1% penicillin, and 1% streptomycin.

Treatment of Cells with Chrysotile Asbestos

Chrysotile asbestos was provided by the National Institution of Environmental Health Sciences [(NIEHS), Research Triangle Park, NC]. Aliquots of 10 mg asbestos were autoclaved and diluted in sterile PBS to a final concentration of 1 mg/mL. The asbestos was sheared by sequential passage through 18, 20, 22, 23, and 25 gauge needles for 20 times each, followed by sonication for 2 h prior to its administration to the cells to increase the fiber numbers and reduce their size. Doses were expressed as micrograms of asbestos/centimeters squared of dish surface to standardize the treatments among experiments as previously reported in a number of laboratories.^{34,35}

Transfection

Transfections were performed using Amaxa nucleofector with solution R (Amaxa, Koeln, Germany). Briefly, 1×10^6 Swiss 3T3 fibroblasts were resuspended in 100 μ L of solution R and mixed with 2 μ g pmaxGFP plasmid (from Amaxa) or 1 μ g siRNA. Cells were transfected using program U-30 and were immediately transferred into wells containing 37°C prewarmed culture medium in six-well plates. After transfection, cells were cultured for 5 h and washed with PBS twice. Cells were further cultured in medium containing 10% bovine calf serum and 1% penicillin/streptomycin. Twenty-four hours after transfection, cells were observed by fluorescent microscopy to determine the transfection efficiency.

Analysis of mRNA

TGF- β_1 mRNA was measured by real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Contaminating DNA was eliminated using TurboDNase (Ambion). Reverse transcription of 500 ng total RNA was performed in a total volume of 20 μ L using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). One microliter of cDNA was amplified by PCR in 20 μ L reactions containing specific primers and iQ SYBR Green Supermix (Bio-Rad). PCR was performed for 35 cycles consisting of 95°C for 15 s and 60°C for 45 s using iCycle iQ Real Time Detection System (Bio-Rad). The primers used for PCR were purchased from Integrated DNA Technologies, Inc. (Coralville, IA), and the sequences are shown in Table 1. All samples were run in triplicate. Negative controls, such as cDNA reactions without reverse transcriptase or RNA, and PCR mixtures lacking cDNA were included in each PCR to detect possible contaminants. Following amplification, specificity of the reaction was confirmed by melt curve analysis. Relative quantitation was determined using the comparative C_T method with data normalized to 36B4 riboprotein mRNA and calibrated to the average ΔC_T of untreated controls. Data are expressed as percentage of control that was set to 100%.

Analysis of TGF- β_1 Protein

Supernatants from each sample were collected, centrifuged at 1.6×10^4 g for 1 min to remove any cells and then frozen at -80°C . Total TGF- β_1 protein was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D System, Minneapolis, MN). Data were normalized to total protein concentration as determined by DC Protein Assay (Bio-Rad).

Statistical Analysis

Data are presented as the means $\pm$ standard error of mean (SEM), where $n = 3$ except where indicated. The statistical significance was determined by using the unpaired Student's *t* test to assess the difference between two groups. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test were performed when multiple groups were compared to a single control. *P* values of < 0.05 were considered significant.

Results

Four siRNAs targeting murine TGF- β_1 mRNA were designed using Dharmacon SMARTpool technologies and further tested in our laboratory. The sense and antisense sequence of each siRNA is shown in Table 2. A nontargeting, negative control siRNA (N) was purchased from Ambion. The sequence of this negative control siRNA is proprietary.

We first tested the ability of individual siRNAs to knock down basal levels of TGF- β_1 expression in Swiss 3T3 fibroblasts. siRNAs targeting TGF- β_1 mRNA were transfected into Swiss 3T3 fibroblasts by nucleofection. After transfection, cells were cultured overnight, washed with PBS two times, and further cultured with medium containing 10% bovine calf serum and 1% penicillin/streptomycin. Seventy-two hours later, total RNA and conditioned medium were collected for analysis of TGF- β_1 expression by quantitative real-time RT-PCR and ELISA, respectively. Each of the four siRNAs reduced the levels of TGF- β_1 mRNA by at least 50%. However, S1 and S2 were the most efficient, knocking down expression by 80 and 74%, respectively (Fig. 1A). Consistent with these results, TGF- β_1 protein levels in the cell culture supernatant from siRNA-transfected cells were also reduced from ~ 600 pg/mL to < 300 pg/mL (Fig. 1A). To assess the specificity of siRNA-mediated knockdown, we analyzed expression of two housekeeping genes, β -actin and GAPDH. None of the siRNAs showed statistically significant knockdown of either of these genes, although S2 knocked down β -actin mRNA by $\sim 40\%$ and GAPDH mRNA by 30% (Fig. 1B).

We next asked whether S1 and S2, the two most efficient siRNAs, could knock down asbestos-induced TGF- β_1 mRNA and protein in Swiss 3T3 fibroblasts *in vitro*. To determine the optimal dose of asbestos and time of treatment, 3T3 fibroblasts were treated with various concentrations of chrysotile asbestos fibers. At the specified time points after asbestos treatment, TGF- β_1 mRNA was measured by quantitative real-time RT-PCR. As shown in Figure 2A, treatment of Swiss 3T3 fibroblasts with as little as $5 \mu\text{g}/\text{cm}^2$ asbestos resulted in a $\sim 1000\%$ increase in TGF- β_1 mRNA expression at 24 h. Increasing the dose of asbestos to $16.6 \mu\text{g}/\text{cm}^2$ resulted in a 3000% increase by 48 h. Because Swiss 3T3 fibroblasts demonstrated TGF- β_1 mRNA induction in response to asbestos treatment, we further measured the levels of TGF- β_1 protein in supernatants from the asbestos-treated Swiss 3T3 fibroblasts. As shown in Figure 2B, there was a time- and dose-dependent increase in the level of TGF- β_1 protein that correlated with the mRNA data above. We chose a dose of $10 \mu\text{g}/\text{cm}^2$ for 48 h for our siRNA experiments because this dose induced near-maximal levels of TGF- β_1 protein and did not cause significant cell death.

We transfected Swiss 3T3 fibroblasts with S1 and S2 by nucleofection. After transfection, cells were cultured for 5 h, washed with PBS twice, and further cultured with medium containing 1% bovine calf serum and 1% penicillin/streptomycin. Twenty-four hours later, transfected cells were treated with $10 \mu\text{g}/\text{cm}^2$ chrysotile asbestos. After 48 h of treatment with chrysotile asbestos, total RNA and supernatants were collected for analysis by quantitative real-time RT-PCR and ELISA, respectively. S1 knocked down asbestos-induced expression of TGF- β_1 mRNA by 89% and TGF- β_1 protein by 84%, whereas S2 knocked down their expression by 72 and 70%, respectively (Fig. 3A). The nontargeting siRNA, did not knock down asbestos-induced TGF- β_1 mRNA levels; however, protein levels were

reduced by 41%. The siRNA-mediated knockdown of TGF- β_1 appeared to be specific, because neither S1 nor S2 had any significant effect on β -actin or GAPDH mRNA levels (Fig. 3B).

CTGF is considered to be a downstream mediator of TGF- β_1 signaling in the fibrogenic process and is upregulated in Swiss 3T3 fibroblasts exposed to asbestos (Fig. 4). Therefore, we measured CTGF mRNA levels in asbestos-treated cells that had been transfected with S1, our most efficient siRNA. As shown in Figure 5, TGF- β_1 mRNA was knocked down to ~ 10% of controls and CTGF mRNA was reduced to ~ 15% of controls. These results suggest that siRNA-mediated knockdown of TGF- β_1 abrogates asbestos-induced expression of CTGF mRNA as well.

Discussion

The design and selection of effective siRNAs are the keys to successful RNAi experiments. Not all siRNAs directed against a given target silence with equivalent efficiencies. There are several guidelines and online siRNA design tools available to help researchers develop siRNAs; however, the designed siRNAs still need to be tested in order to ensure they are efficient and have no significant off-target effects. The siRNAs designed to target TGF- β_1 mRNA significantly knocked down both basal and asbestos-induced expression of TGF- β_1 mRNA and protein (Figs. 1A and 3A). In particular, TGF- β_1 siRNA1 (S1) performed the best and it knocked down asbestos-induced TGF- β_1 mRNA and TGF- β_1 protein by 89 and 84%, respectively (Fig. 3A). These TGF- β_1 siRNAs are currently being tested in animal models of pulmonary fibrosis to see if they can prevent/cure fibrogenic disease.

RNAi gene silencing was initially thought to function very specifically; however, off-target effects^{36–39} and potential interferon responses⁴⁰ have been reported. In our experiments, two different siRNAs targeting separate sequence regions of the TGF- β_1 mRNA significantly reduced asbestos-induced expression of TGF- β_1 mRNA and protein. These siRNAs did not significantly affect two unrelated mRNAs (Figs. 1B and 3B); however, in the absence of comprehensive whole genome array profiling, it is impossible to rule out all possible off-target effects. As a control for nonspecific effects due to introduction of siRNAs into cells, we used a commercially available nontargeting siRNA (N). The sequence of this siRNA is proprietary but reported to have minimal sequence similarity to known mouse genes and thus should not affect gene expression. In our experiments, this nontargeting siRNA had no effect on TGF- β_1 mRNA level (Fig. 3A). However, it resulted in reduced levels of TGF- β_1 protein (Fig. 3A). One explanation for this observation is that this nontargeting siRNA shares partial homology with TGF- β_1 mRNA and acts like microRNAs (miRNAs) inhibiting translation.⁴¹

Increased expression of TGF- β_1 in human pulmonary fibrotic disease has been demonstrated by Khalil and Greenberg.⁴² Similarly, TGF- β_1 gene expression and protein production are increased in a variety of animal models of pulmonary fibrosis, including scarring consequent to asbestos exposure.⁴³ Analysis of TGF- β_1 mRNA expression in fibroproliferative lesions collected by laser capture microdissection from lungs of mice exposed to chrysotile asbestos showed an approximate 15-fold increase.⁴⁴ We previously showed by in situ hybridization that the cell types expressing TGF- β_1 mRNA in the lung after exposure to asbestos include bronchiolar-alveolar epithelial cells, macrophages, and fibroblasts.^{43,45} In preliminary experiments, fibroblast (Swiss 3T3), lung epithelial (C10), and macrophage (RAW264.7) cell lines each showed increased expression of TGF- β_1 after exposure to asbestos, with Swiss 3T3 having the greatest response (data not shown). A key feature of ILD is the “fibroblastic focus,” composed of fibroblasts and fibroblast-like cells.⁴⁶ Fibroblasts are believed to be the main cellular source of extracellular matrix deposition that typifies

fibrosis, and there is ample evidence that fibroblasts produce a number of factors, including TGF- β_1 .⁴⁷ Therefore, we chose to use Swiss 3T3 fibroblasts to test the hypothesis that siRNAs could effectively knock down expression of TGF- β_1 in response to asbestos. Although the use of a cell line as a surrogate for what may be happening in primary cells and in vivo can be criticized, we have extensive experience with the Swiss 3T3 fibroblast cell line and have shown that levels of cytokine expression and the signaling cascades controlling TGF- β_1 gene expression are identical in primary lung cells and in the Swiss 3T3 fibroblast cell line.⁴⁸ Furthermore, Swiss 3T3 fibroblasts and human lung fibroblasts (HFL-1) show similar patterns of TGF- β_1 expression in response to TNF- α ,⁴⁹ a key mediator of TGF- β_1 expression.^{48,50,51} Thus, it is reasonable to expect the knockdown of RNA expression demonstrated here to be representative of what would occur in primary cell cultures and in the human lung.

TGF- β_1 is produced in a latent form that must be activated to be biologically active. We have previously shown that reactive oxygen species generated by the iron in chrysotile asbestos can activate latent TGF- β_1 protein in vitro.⁵² Consistent with these results, low levels of active TGF- β_1 were detected in supernatants from asbestos-treated cells by ELISA, whereas no active TGF- β_1 could be detected in supernatants from cells transfected with S1 or S2 siRNAs prior to asbestos exposure (data not shown). CTGF is considered to be a downstream mediator of TGF- β_1 signaling in the fibrogenic process and has been demonstrated in models of fibrogenesis⁵³ and in human lungs.⁵⁴ Exposure of fibroblasts to asbestos also resulted in significantly higher levels of CTGF mRNA compared to untreated controls (Fig. 4). Therefore, we measured CTGF mRNA levels in cells transfected with S1, the siRNA that showed the most significant knockdown of TGF- β_1 mRNA and protein. CTGF was significantly knocked down by S1 with 84% efficiency (Fig. 5). These results suggest that knocking down TGF- β_1 may prevent a fibrogenic cascade induced by asbestos.

TGF- β_1 plays an important role in the development of pulmonary fibrosis and reducing TGF- β_1 may prevent the fibrosis; however, TGF- β_1 also plays important roles in diverse biological functions. TGF- β_1 null mutation in mice can cause infertility in both male⁵⁵ and female⁵⁶ genders, excessive inflammation, and early death.⁵⁷ These studies imply that knocking down TGF- β_1 as a therapy for fibrotic diseases should be carried out in a targeted fashion because systemic effects could ensue. Regulating the degree of TGF- β_1 knockdown so that normal biological functions are undisturbed is a potentially important issue that could limit its use.

As a therapeutic approach, RNAi could have many advantages over other pharmaceuticals. siRNAs are easy to produce compared to recombinant proteins and monoclonal antibodies. Furthermore, siRNAs are potentially more potent than antisense oligonucleotides. The drug discovery and development phases of RNAi-based therapy are much shorter than traditional small molecule drugs.⁵⁸ However, RNAi-based therapy still has barriers to overcome, such as efficient systemic delivery in humans and avoiding off-target effects. Once these barriers are overcome, RNAi-based therapy could benefit millions of individuals.

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FIGURE 1.

Knockdown of TGF- β_1 mRNA and protein by siRNAs. **(A)** Swiss 3T3 fibroblasts were transfected with 1 μ g of siRNA targeting four different sites within the TGF- β_1 mRNA or mock transfected (control). Seventy-two hours later, total RNA was collected for analysis of TGF- β_1 mRNA expression by real-time qRT-PCR (open bars, left y-axis) and supernatants were collected for analysis of TGF- β_1 expression by ELISA (solid bars, right y-axis). **(B)** Specificity of siRNAs was assessed by measuring β -actin (open bar) or GAPDH mRNAs (closed bar) by real-time qRT-PCR. Data are expressed as the mean $\pm$ SEM of triplicate plates for each treatment group. * $P < 0.05$ versus control. S1: TGF- β_1 siRNA1, S2: TGF- β_1 siRNA2, S3: TGF- β_1 siRNA3, and S4: TGF- β_1 siRNA4.

FIGURE 2.

Asbestos induces TGF- β_1 expression in fibroblasts. Confluent monolayers of Swiss 3T3 fibroblasts were rendered quiescent in medium containing 1% serum for 48 h and then treated with 0, 5, 10, or 16.6 $\mu\text{g}/\text{cm}^2$ of chrysotile asbestos. After 6, 12, 24, or 48 h, (A) total RNA was collected for analysis of TGF- β_1 mRNA expression by real-time qRT-PCR and (B) supernatants were collected for analysis of TGF- β_1 expression by ELISA. Data are expressed as the mean $\pm$ SEM of triplicate plates for each treatment group at each time point. * $P < 0.05$ versus control.

FIGURE 3.

siRNAs targeting TGF- β_1 mRNA inhibit asbestos-induced expression of TGF- β_1 . Swiss 3T3 fibroblasts were transfected with 1 μ g of siRNAs targeting TGF- β_1 mRNA (S1 and S2), a nontargeting siRNA (N), or mock transfected (control). Twenty-four hours later, transfected cells were treated with 10 μ g/cm² chrysotile asbestos. After 48 h of treatment with chrysotile asbestos, total RNA and supernatants were collected. **(A)** TGF- β_1 mRNA levels were measured by real-time qRT-PCR (open bars, left y-axis) and TGF- β_1 protein levels (solid bars, right y-axis) were measured by ELISA. **(B)** Specificity of siRNAs was assessed by measuring β -actin (open bars) or GAPDH mRNAs (solid bars) by real-time qRT-PCR. Data are expressed as the mean $\pm$ SEM of triplicate plates for each treatment group. * $P < 0.05$ versus control.

FIGURE 4.

Asbestos induces CTGF mRNA expression in fibroblasts. Confluent monolayers of Swiss 3T3 fibroblasts were rendered quiescent in medium containing 1% serum for 24 h and then treated 10 $\mu\text{g}/\text{cm}^2$ of chrysotile asbestos. After 48 h, CTGF mRNA was measured by real-time qRT-PCR. Data are expressed as the mean $\pm$ SEM of triplicate plates for each treatment group. * $P < 0.05$ versus control.

Figure 5. siRNA-mediated knock down of TGF-β₁ inhibits asbestos-induced CTGF mRNA expression. Swiss 3T3 fibroblasts were transfected with 1 μg of siRNA1 (S1), a non-targeting siRNA (N) or mock transfected (control). Twenty-four hours later, transfected cells were treated with 10 μg/cm² chrysotile asbestos. After 48 h of treatment with chrysotile asbestos, total RNA and supernatants were collected. CTGF mRNA levels were measured by real-time qRT-PCR. Data are expressed as the mean ± SEM of triplicate plates for each treatment group. *P<0.05 versus control.

TABLE 1

PCR Primer Sequences

Gene	Primer sequences (5'–3') Forward and Reverse	Product (bp)	Refs.
Murine TGF- β_1	GGATACCAACTATTGCTTCAGCTCC AGGCTCCAAATATAGGGGCAGGGTC	146	Sullivan et al. ⁴⁸
Murine CTGF	GCAGCGGTGAGTCCTTCC AATGTGTCTTCCAGTCGGTAGG	232	Howell et al. ⁵⁹
Human/murine 36B4	CGACCTGGAAGTCCAACACTAC ATCTGCTGCATCTGCTTG	109	Simpson et al. ⁶⁰
Murine GAPDH	TTCACCACCATGGAGAAGGC GGCATGGACTGTGGTCATGA	236	Overbergh et al. ⁶¹
Murine β -Actin	AGAGGGAAATCGTGCCTGAC CAATAGTGATGACCTGGCCGT	148	Overbergh et al. ⁶¹

TABLE 2Sequences of the siRNAs Targeting Mouse TGF- β_1 Gene (NM_011577)

Name	Position in Gene	Sequence
S1	1165–1183	GAAGCGGACUACUAUGCUAUU (sense, 5'–3') UUCUUCGCCUGAUGAUACGAU-P (antisense, 3'–5')
S2	1091–1109	AGGCGGUGCUCGCUUUGUAUU (sense, 5'–3') UUUCCGCCACGAGCGAAACAU-P (antisense, 3'–5')
S3	1217–1235	ACAACGCCAUCUAUGAGAAUU (sense, 5'–3') UUTGUUGCGGUAGAUAGUCUU-P (antisense, 3'–5')
S4	990–1008	GAAACGGAAGCGCAUCGAAUU (sense, 5'–3') UUCUUUGCCUUCGCGUAGCUU-P (antisense, 3'–5')

The sense sequence is 19 bases of targeted sequence with two-nucleotide, 3'-UU, overhang added. The antisense sequence is 19 bases with two-nucleotide, 3'-UU, overhang added and is phosphorylated (P) at the 5' end. S1: TGF- β_1 siRNA1, S2: TGF- β_1 siRNA2, S3: TGF- β_1 siRNA3, and S4: TGF- β_1 siRNA4.