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Original Article

The effect of Smad2- and Smad3-targeting RNA interference on extracellular matrix synthesis in rat fibroblasts of peritoneal adhesion tissues

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Abstract: Fibroblasts migrating to peritoneum injuries play an important role in the development of postoperative peritoneal adhesions due to the excessive synthesis and deposition of extracellular matrix (ECM). This effect is mainly induced by the transforming growth factor-β (TGF-β). Studies indicate that elevated TGF-β1 levels and TGF-β1/Smad signaling are both implicated in the formation of peritoneal adhesions. To confirm the effect of TGF-β1/Smad signaling interference in regulating excessive ECM synthesis, a total of four different R-Smad-targeting small interference RNA (siRNA) duplexes (Smad2-500, Smad2-956, Smad3-378, Smad3-1385) were tested in this study using a TGF-β1-stimulated adhesion tissue fibroblasts (ATFs) cell model. The *in vitro* assessments show that all proposed siRNAs are capable of significantly downregulating the mRNA and protein levels of Smad2 and Smad3 in ATFs. They also inhibit the phosphorylation of both Smads, which confirms their effect in blocking the TGF-β1/Smad signaling pathway. Moreover, the siRNA duplexes can appreciably lower the elevated levels of fibronectin and collagen 3 alpha 1 (COL3A1) in TGF-β1-stimulated ATFs, and the Smad3-378 siRNA can actually restore both molecules (fibronectin and COL3A1) to normal levels. Therefore, the Smad3-378 siRNA is suitable for both adhesion prevention and wound healing. Overall, our results indicate that postoperative adhesion prophylaxis may be achieved by temporarily blocking TGF-β1/Smad signaling transduction.

Keywords: TGF-β/Smads, peritoneal adhesions, fibroblast, small interference RNA (siRNA)

Introduction

Following abdominal laparotomy, many patients (55%-100%) suffer from peritoneal adhesions that may undermine the efficiency of the surgical treatment, leading to long-term complications such as intestinal obstruction, infertility, and chronic pelvic pain [1-3]. Surgical operations and locally applied physical barriers are commonly used to prevent the formation of such adhesions. However, these techniques cannot completely protect against peritoneal adhesions due to the multifactorial nature of adhesion pathogenesis [4]. Ongoing research efforts have led to a greater understanding of the adhesion formation process, which in turn has enabled the development of targeted treatments based on the use of pharmacological agents such as recombinant tissue plasminogen (tPA) and neutralizing antibodies for plasminogen activator inhibitor 1 (PAI-1), transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and vascular endothelial growth factor (VEGF). These treatments aim at correcting the aberrant fibrinolytic activity that occurs during the development of adhesions, and they have demonstrated moderate success in different experimental models [3]. In general, three to five days of treatment are required to completely heal peritoneal surfaces. However, the half-lives of the treatment

agents mentioned above are short (a few minutes), which restricts their applicability [5, 6]. Drugs such as steroids, non-selective and selective cyclooxygenase inhibitors, heparin, and neurokinin-1 receptors have also been investigated as anti-adhesion treatments [7], along with the anti-IL-6 receptor antibody and the inhibitors of hypoxia-inducible factor and thrombin-activated fibrinolysis [8-10]. Despite the numerous proposed treatments, a resounding clinical practice for the prevention of adhesion formation is yet to be established.

Studies available in the literature show that the formation of adhesions in the peritoneum involves the migration, proliferation, and physiological function alteration of fibroblasts [11, 12]. These processes are regulated by TGF-β1, a protein that directly transforms fibroblasts to myofibroblasts and modulates extracellular matrix (ECM) synthesis [13-15]. The available studies suggest that peritoneal adhesion pathogenesis is controlled by TGF-β1/Smad signaling. For example, Uyama et al. demonstrated that following adhesion formation, phosphorylated Smad2 is formed in the serosa [9]. Deng et al. suggested that peritoneal injury induced up-regulation of P-glycoprotein (P-gp) expression via the TGF-β1/Smad signaling pathway [16]. *In vivo* analyses conducted by Guo et al. showed that Smad2/3 activation in the injured peritoneum may be blocked by the Smad7 transgene, which prevents adhesion formation [17].

In this study, we investigated the role of TGF-β1/Smad signaling in modulating fibroblasts for peritoneal adhesion prophylaxis. For this purpose, we designed four different small interfering RNA (siRNA) duplexes that target Smad regulating receptors (R-Smad). Generally speaking, the RNA interference technology mediated by small double-stranded RNA molecules is widely applied in the development of experimental therapeutic for various diseases [18]. Previously, it had been shown that the siRNAs targeting hypoxia inducible factor 1 (HIF-1) and plasminogen activator inhibitor 1 (PAI-1) minimize adhesion formation in rats [19]. Therefore, siRNAs targeting R-Smad may be used to temporarily block TGF-β1/Smad signaling transduction and prevent peritoneal adhesions. Herein, we analyzed the effects of the designed siRNAs on R-Smad expression and ECM synthesis in ATFs.

Methods

Rat peritoneal adhesion model

Sprague-Dawley (SD) rats (8-10 weeks, 250-300 g) were purchased from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China). The animals were cared for and used according to the institutional and national guidelines. All rats were fasted for 12 h and then anesthetized by groin injection of 4% pentobarbital solution in water (1 mL/kg dosage). Subsequently, each rat was placed on its back on an operating table and covered with an appropriate incision sterile gauze after hair removal. The abdominal skin was sterilized with 1% antiseptic povidone-iodine solution, and a vertical midline incision was performed (3 cm long). The cecum was stretched to the sterile gauze and kept moist. Its surface was clamped with hemostatic forceps, and then the left and right sides of the incision were scraped by eye scissors until serosal petechiae. After replacing the cecum inside the abdomen, the incision was closed with continuous 3-0 polypropylene sutures. Two weeks after surgery, the animals were sacrificed and their abdominal cavities were opened under septic conditions. Adhesion tissues were collected for primary fibroblast culture.

Primary adhesion tissue fibroblast culture and treatment

The ATFs were cultured as per the method detailed in Deng LL et al. [16]. Briefly, the adhesion tissue was rinsed with phosphate buffer solution (PBS) three times then cut into small pieces (size of 1-2 mm^3) in a sterile culture disk. The pieces were transferred to a T-25 flask for primary explant culture at 37°7 in a humidified atmosphere of 5% CO_2. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS), 100 U/mL streptomycin, and 100 U/mL penicillin. Outgrowth of fibroblasts generally took 1 weeks. When 70%-80% confluence was reached, the cells were sub-cultured by trypsinization. The ATFs were randomly divided into seven groups, namely the blank, TGF-β1, negative, TGF-β1 + Smad2-500 siRNA, TGF-β1 + Smad2-956 siRNA, TGF-β1 + Smad3-378 siRNA, and TGF-β1 + Smad3-1385 siRNA groups. Recombinant human TGF-β1 (Peprotech, Rocky Hill, NJ, USA) was added to all groups,

Table 1. The siRNA oligo used in this study

siRNA name	Forward (5'-3')	Reverse (5'-3')
Smad2-500	GGCUGCCACAUGUUAUAUATT	UAUAUAACAUGUGGCAGCCTT
Smad2-956	GGUGUUCAAUCGCAUAUUATT	UAAUAUGCGAUUGAACACCTT
Smad3-378	GUGAACACCAAGUGCAUUATT	UAAUGCACUUGGUGUUCACTT
Smad3-1385	GAUCGAGCUACACCUGAAUTT	AUUCAGGUGUAGCUCGAUCTT
Negative	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Table 2. Quantitative real-time polymerase chain reaction primers used in this study

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	GATGAGATTGGCATGGCTTT	CACCTTCACCGTTCCAGTTT
fibronectin	AGGAGAACCAGGAGAGCACA	TCGGTCACTTCCACAAACTG
COL3A1	CGGGCAAGAATGGAGCAAAG	ACCAGGGAAACCCATGACAC
Smad2	TCACAGCCATCATGAGCTCAAGG	TCACAGCCATCATGAGCTCAAGG
Smad3	CTCCTGGCTACCTGAGTGA	GCTGTGAAGCGTGGAATGTC

except the blank group, at a final concentration of 10 ng/mL for 24 h. The TGF-β1 group was considered as positive control, and it was administered with TGF-β1 only, whereas the negative group was also transfected with scrambled siRNA. As for the blank group, it was not treated with any siRNA or TGF-β1. Cells were identified as fibroblasts by immunofluorescence with a monoclonal antibody against vimentin. The ATFs used in all experiments were taken from the 3rd, 4th, or 5th passage.

siRNA preparation and gene-silencing assays

The siRNA duplexes were designed and synthesized by GenePharma Corporation (Shanghai, China), as shown in **Table 1**. The transfection was conducted using INTERFERin® (Polyplus, France), as per the published protocol. The ATFs were seeded in a 6-well plate, at the concentration of 5 × 10^5 cells per well. For 24 h before transfection, TGF-β1 was added to all groups, except the blank group, at a final concentration of 10 ng/mL. Afterward, 10 μL of INTERFERin® was mixed with 990 μL Opti-MEM (Gibco, Waltham, MA, USA) and the mixture was incubated for 10 min at room temperature. Subsequently, 200 μL of the siRNA duplex (100 nM) were dispersed in 800 μL Opti-MEM for 5 min at room temperature, and then the solution was added to the INTERFERin®-Opti-MEM mixture. The final solution was incubated for 20 min at room temperature, and during the incubation period, the medium was replaced, and the cells were washed three times with PBS. Then, the transfection mixture was incubated with the cells for 6 h at 37°C in a humidified atmosphere of 5% CO_2. After transfection, the cell culture was replaced with serum-containing DMEM, and the cells were grown for 48 h before incubation for total RNA, or total protein extraction.

Cell viability assay

The viability of ATFs was assessed using cell counting kit-8 (CCK-8) (DOJINDO, Kumamoto, Japan), according to the manufacturer's protocol. The cells were seeded in 96-well plates at the density of 5.0 × 10^3 cells per well and allowed to attach and grow overnight. The medium was then replaced with serum-free DMEM. After 8 h, TGF-β1 was subsequently added at the final concentration of 10 ng/mL, and the cells were transfected with different concentrations (0, 50, 100, 200, or 400 nM) of negative siRNA, Smad2-500 siRNA, Smad2-956 siRNA, Smad3-378 siRNA, or Smad3-1385 siRNA for 24 h. Then, the mixtures were incubated with 100 μL 10% WST-8 reagent, and 3 h later, the number of newly formed formazan dye was quantified by microplate reader at 450 nm. The measured absorbance values were used to calculate cell viability according to the following formula: cell viability (%) = OD value of siRNA group/OD value of blank group × 100%. The experiment was repeated three times.

Quantitative real-time PCR (qPCR)

The total RNA in ATFs was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), and its purity was assessed based on the A_{260}/A_{280} ratio. Subsequently, 1.0 μg of the extracted RNA was reverse transcribed using the PrimeScript™ RT reagent kit (Takara, Dalian, China), according to the manufacturer's instructions. The obtained cDNA was analyzed by the CFX96 PCR instrument (Bio-Rad, Hercules, CA, USA) using the SYBR Premix Ex Taq™ kit (Takara) with primer pairs specific to the target genes, as shown in **Table 2**. The primer PCR conditions were set to 30 s hot

start at 95°5, followed by 40 cycles of 5 s at 95°5 and 30 s at 60°0. The qPCR data were processed and exported using the ΔΔCT method [20].

Protein extraction and determination

The protein content in ATFs was extracted using a cell lysis buffer (Beyotime, Shanghai, China) supplemented with protease inhibitors and phosphatase inhibitors (Roche, San Francisco, CA). The extraction solutions were kept on ice for 30 min and then centrifuged at 12,000 rpm and 4°n for 15 min. Samples were taken from each extract to determine the protein contents, and the remaining solutions were divided into 10 μL aliquots and stored at -70°7 until further use. The protein concentrations were measured by paper spot protein assay against a bovine serum albumin (BSA) standard curve.

WES™ automatic western blot analysis

Automatic western blot analyses were performed on a ProteinSimple™ Wes instrument using reagents purchased from ProteinSimple (San Jose, CA, USA), and all samples were prepared according to the manufacturer's recommendations. First, the protein concentration in each sample was adjusted to 4 μg per 2.5 μL using the buffer solution. Then, the Master Mix was added at the ratio of 4 to 1. The mixture was blended with Biotinylated Ladder in a boiling water bath for 15 min before being transferred to sample plates. The final samples were centrifuged at 3000 rpm for 5 min, and then they were separated by Simple Western using the following settings: 200 s to fill the capillaries with the separation matrix, 16.7 s to stack the matrix, and 10 s to load the protein samples. The separation voltage was set to 375 V, and the process was carried out for 25 min. Afterward, the capillaries were washed three times with the washing buffer (150 s each time), and then they were blocked with antibody diluent for 5 min to prevent non-specific binding by the primary antibody. The capillaries were incubated with the primary antibody for 30 min, washed, then incubated with HRP conjugated secondary antibodies (1:500, A0208/A0216, Beyotime) and chemiluminescent substrate (ProteinSimple) for another 30 min.

The primary antibodies detecting Smad2 (1:1000, 3103, Cell Signaling Technology, Boston, MA, USA), Smad3 (1:1000, 9523, Cell Signaling Technology), phospho-Smad3 (P-Smad3, 1:200, Ser423/425, C25A9, Cell Signaling Technology), phospho-Smad2 (P-Smad2, 1:200, Ser456/467, 3108, Cell Signaling Technology), collagen III (1:500, ab7778, Abcam, Cambridge, UK), and fibronectin (1:500, ab24-13, Abcam) were applied, and chemiluminescence was determined using a ProteinSimple™ Wes instrument. Semi-quantitative densitometric analysis was carried out using the ImageJ software.

Statistical analysis

All experiments were repeated three times and the data were processed by SPSS 22.0 (IBM, Armonk, NY, USA). The obtained numerical values are expressed as mean ± standard deviation (SD). Statistical analyses were performed according to Student's t test or one-way analysis of variance (ANOVA) method, and *P*-values less than 0.05 were considered statistically significant.

Results

Identification of cultured adhesive tissue fibroblasts (ATFs)

As shown in **Figure 1A** and **1B**, the cultured ATFs were thin, long, spindle-shaped, and locally aligned in parallel clusters. Based on immunofluorescence analysis, these ATFs presented positive monoclonal antibody staining for vimentin (**Figure 1C**), the marker of fibroblasts.

Effect of siRNA transfection concentration on cell viability

The CCK-8 assay was used to determine the viability of ATF cells under varying transfection concentrations of R-Smad siRNA. The results illustrated in **Figure 2** show that at 50 and 100 nM, the designed siRNAs exhibited no obvious cytotoxicity. However, at 200 and 400 nM, the viability of ATFs appreciably decreased. Therefore, the final transfection concentration of 10 nM used herein is largely safe.

R-Smad-targeting siRNAs block TGF-β1/Smad signaling transduction

To determine whether TGF-β1/Smad signaling transduction is blocked by the R-Smad siRNA duplexes, the gene and protein expressions of Smad2 and Smad3 in ATFs transfected with

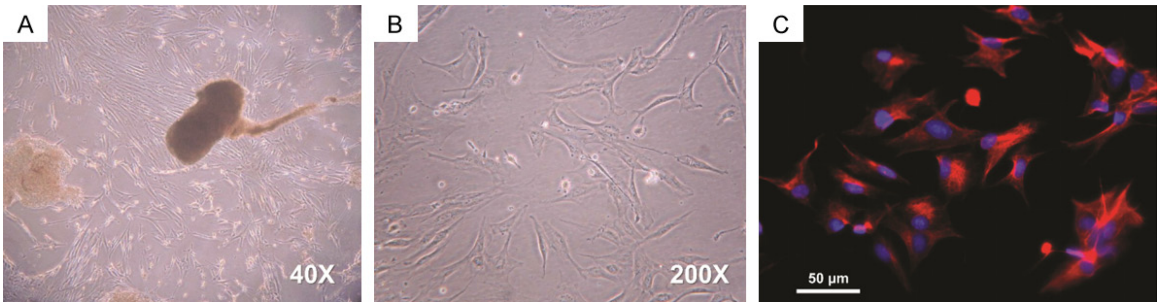

Figure 1. Identification of cultured adhesive tissue fibroblasts (ATF). Microscopic images at (A) 40 × and (B) 200 × magnification. (C) Immunofluorescence detection of SABC-CY3-labeled vimentin (red fluorescence) in cultured ATFs.

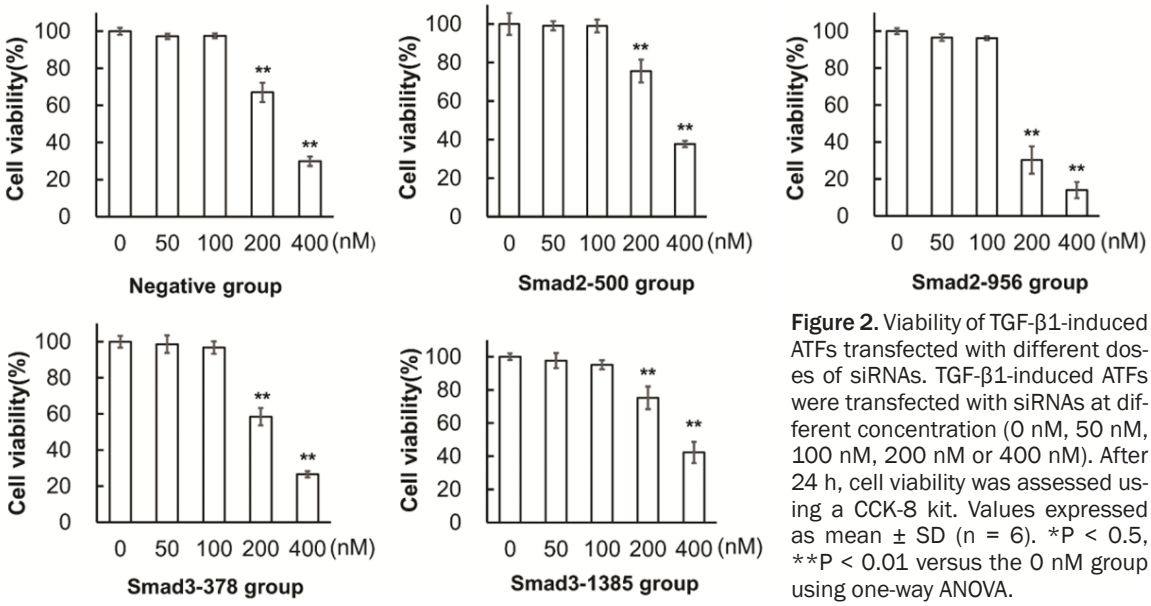

Figure 2. Viability of TGF-β1-induced ATFs transfected with different doses of siRNAs. TGF-β1-induced ATFs were transfected with siRNAs at different concentration (0 nM, 50 nM, 100 nM, 200 nM or 400 nM). After 24 h, cell viability was assessed using a CCK-8 kit. Values expressed as mean ± SD (n = 6). *P < 0.5, **P < 0.01 versus the 0 nM group using one-way ANOVA.

different siRNAs were analyzed. As shown in **Figure 3A** and **3B**, the relative mRNA expressions of Smad2 and Smad3 in the TGF-β1 group were significantly greater (more than 1.5 times) than those observed in the blank group (P < 0.01). Meanwhile, all four R-Smad siRNA groups presented downregulated expressions of Smad2 and Smad3, compared with the TGF-β1 group (P < 0.01). Western blot analyses showed that the corresponding protein expressions exhibited a similar trend. Similarly, the protein expressions of Smad2 and Smad3 in the four R-Smad siRNA groups were obviously lower than those detected in the TGF-β group (**Figure 3C** and **3D**, P < 0.01).

The phosphorylation of R-Smads is a critical step in the activation of TGF-β1/Smad signaling. Therefore, the effects of different R-Smad siRNAs on Smad2 and Smad3 phosphorylation (P-Smad2 and P-Smad3 levels) were also analyzed. The results illustrated in **Figure 3E** and **3F** show that compared with the blank group, the P-Smad2 and P-Smad3 levels in the TGF-β1 group increased by approximately 40% (P < 0.01) and approximately 30% (P < 0.05), respectively. In contrast, the addition of different R-Smad siRNAs significantly reduced these levels (**Figure 3E** and **3F**, P < 0.01), which indicates that all R-Smad-targeting siRNA duplexes designed herein can block TGF-β signaling transduction.

R-Smad-targeting siRNAs reduce ECM synthesis in ATFs

Knowing that TGF-β1 can significantly enhance the synthesis of ECM molecules such as fibronectin and collagen, the effects of the proposed R-Smad siRNAs on the ECM levels in TGF-β1-stimulated ATFs were also assessed. As shown in **Figure 4**, the increased mRNA and protein expressions of fibronectin and

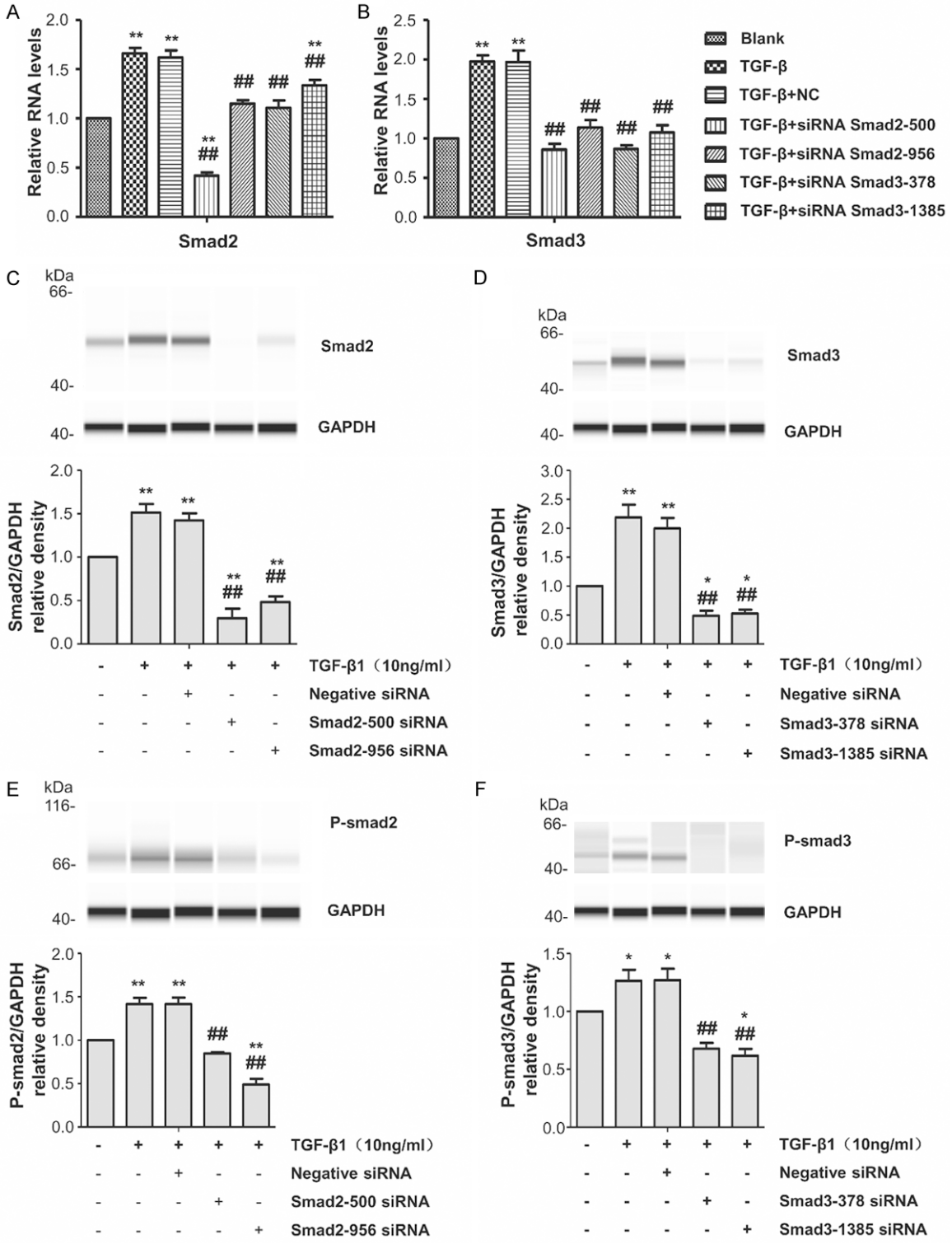

Figure 3. Effect of R-Smad-targeting siRNAs on TGF-β1/Smad signaling transduction in ATFs. TGF-β1-induced ATFs were transfected with different R-Smad siRNAs and negative controls. At 24 h post-transfection, mRNA and protein expression of R-Smad were detected using qRT-PCR and Western blot. qRT-PCR analysis of the (A) Smad2 and (B) Smad3 mRNA levels. Western blot analysis of the (C) Smad2, (D) Smad3, (E) P-Smad2, and (F) P-Smad3 protein expressions. (C-F) Representative western blot (upper) and densitometric analysis (lower). Each data point was normalized against its corresponding GAPDH level and then against the value in the blank group. Values expressed as mean ± SD (n = 3). *P < 0.5, **P < 0.01 versus the blank group (one-way ANOVA). #P < 0.5, ##P < 0.01 versus the TGF-β1 group (one-way ANOVA).

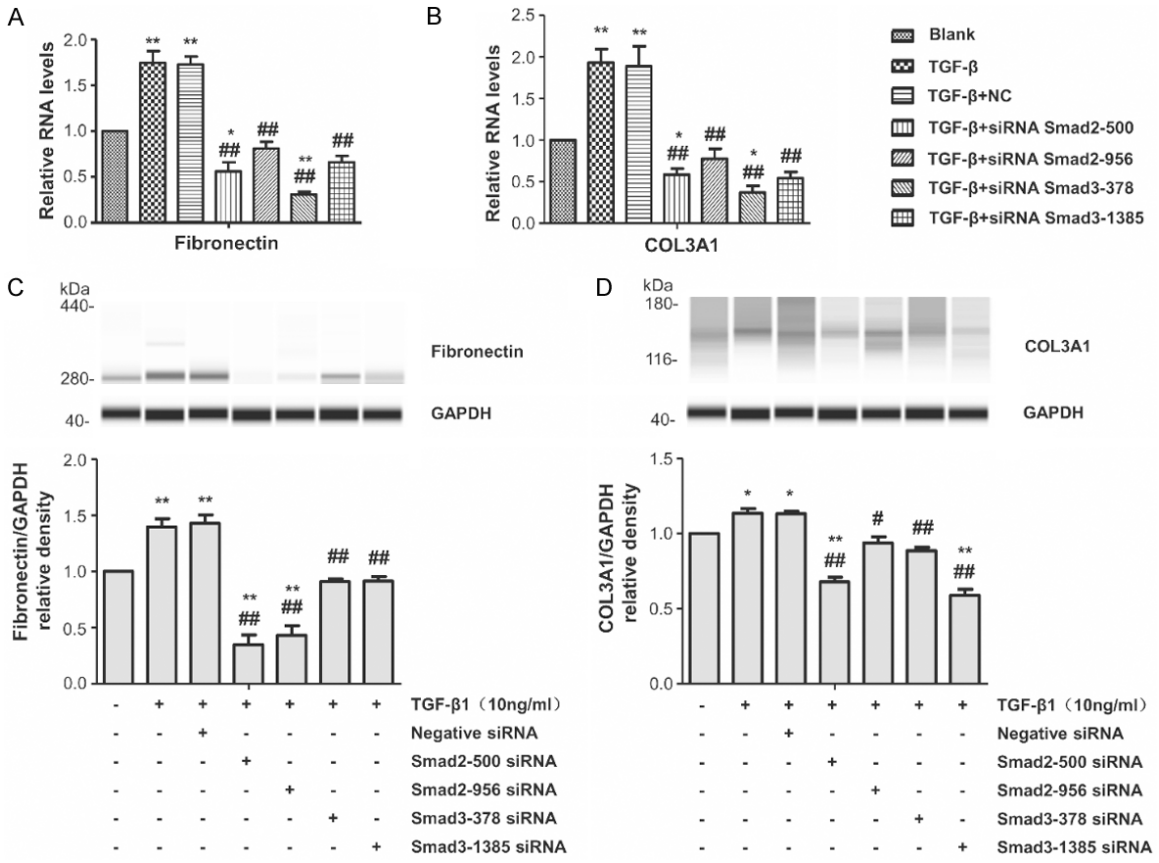

Figure 4. Effect of R-Smad-targeting siRNAs on fibronectin and COL3A1 expressions in TGF-β1-stimulated ATFs. TGF-β1-induced ATFs were transfected with different R-Smad siRNAs and negative controls. At 24 h post-transfection, mRNA and protein expression of fibronectin and COL3A1 were detected using qRT-PCR and Western blot. qRT-PCR analysis of (A) fibronectin and (B) COL3A1 mRNA levels. Western blot analysis of (C) fibronectin and (D) COL3A1 protein expressions. (C, D) Representative western blot (upper) and densitometric analysis (lower). Each data point was normalized against its corresponding GAPDH or β-actin level and then against the value in the blank group. Values expressed as mean ± SD (n = 3). *P < 0.5, **P < 0.01 versus the blank group (one-way ANOVA). #P < 0.5, ##P < 0.01 versus the TGF-β1 group (one-way ANOVA).

COL3A1 in TGF-β1-induced cells (P < 0.05 and P < 0.01 versus the blank group, respectively) were remarkably downregulated by Smad2 and Smad3 siRNAs (P < 0.05 and P < 0.01 for fibronectin and COL3A1 levels, respectively). As for the negative control siRNA, it had practically no effect on ECM synthesis. Notably, the amount of fibronectin in TGF-β1-stimulated ATFs transfected with Smad3-378 and Smad3-1385 was similar to that detected in the blank group (**Figure 4C**, P > 0.05). The COL3A1 levels in Smad2-956- and Smad3-378-transfected ATFs were also similar to those in the blank group (**Figure 4D**, P > 0.05).

Discussion

As previously reported, fibroblasts play an important role in the formation of peritoneal postoperative tissue adhesions. When injured peritoneal tissues do not heal properly, fibroblasts signal for the deposition of extracellular matrix via cell growth factors and cytokines. Eventually, adhesion fibroblasts develop into myofibroblasts, and both phenotypes secrete massive amounts of extracellular matrix molecules that create a weak fibrous bridge between tissues. When the bridge is vascularized and ECM deposition occurs, tissue adhesions are said to be formed [1]. The excessive secretion of TGF-β1 by macrophages following operative injury also plays an important role in adhesion formation by activating the Smad2/Smad3 signaling pathway. TGF-β1 protein molecules bind to TGF-β receptors II (TβRII) on cell membranes, which signals for the phosphorylation of TGF-β receptors I (TβRI). In turn, the phosphorylated TβRI activates R-Smads

(Smad2 or Smad3), also by phosphorylation. The receptor-activated R-Smads then bind to co-Smad (Smad4), resulting in the formation of complexes that translocate across the nuclear membrane. In the nucleus, the Smad complexes, in association with co-activators and co-repressors, as well as other transcription factors, modulate the expression of the target gene, thereby stimulating the synthesis of collagens and fibronectin. These ECM molecules promote the formation of permanent adhesions at the sites of early fibrinous adhesions [21]. Based on this mechanism, we believe that postoperative adhesions may be effectively prevented by modulating the excessive secretion and deposition of ECM species via the temporary blockage of TGF-β signaling transduction.

In this study, we designed four different siRNA duplexes (Smad2-500, Smad2-956, Smad3-378, Smad3-1385) that target R-Smads in ATFs, and we assessed their efficiency in blocking TGF-β1/Smad signaling and regulating excessive ECM synthesis in TGF-β1-stimulated ATFs. The obtained results demonstrated that all investigated siRNAs can significantly downregulate the mRNA and protein levels of Smad2 and Smad3, as well as reduce the phosphorylation of both Smads. This confirms that all four siRNAs proposed herein are capable of inhibiting the ATF TGF-β1/Smad signaling pathway. Further analyses showed that TGF-β1-stimulated ATFs treated with any one of the siRNAs exhibit significantly diminished fibronectin and collagen 3 alpha 1 (COL3A1) levels, which validates our hypothesis.

Although collagen and fibronectin contribute to the formation of undesirable adhesions, both species are essential for wound healing. Collagen, a molecule produced by fibroblasts, is involved in all three phases of the wound healing cascade. It stimulates cellular migration and contributes to new tissue development [22]. As for fibronectin, it promotes the migration of neutrophils, monocytes, fibroblasts, and endothelial cells to the wound region, and it is also involved in tissue organization and remodeling [23]. Therefore, reduced levels of collagen and fibronectin will delay injury repair, thereby increasing the risk of infection and inflammation. Interestingly, the downregulated levels of fibronectin in TGF-β1-stimulated ATFs treated with Smad3-378 or Smad3-1385 siRNA duplexes are similar to that detected in the blank. Similarly, Smad2-956 and Smad3-378 siRNAs do not reduce COL3A1 below the level observed in the blank group. This indicates that among the investigated siRNA duplexes, Smad3-378 is highly promising for the prevention of adhesion formation without affecting the efficiency of wound healing, as it can recover the increased amounts of fibronectin and COL3A1 to normal levels.

Postoperative peritoneal adhesion is an attractive target for gene therapy due to several inherent biological features, such as the localization of the disease at the site of peritoneal trauma and its rapid development over a short period of time (first few days following surgical operation). These characteristics lend themselves perfectly to gene therapy, as the gene vector can be applied locally following completion of the operation. Moreover, the short duration of gene expression coincides with the period of altered molecular aberrations occurring after surgery (e.g., depressed tPA, elevated PAI-1, TGF-β1, and HIF-1α) [3]. Recently, several gene therapy studies have been employed to reduce adhesions, and they were moderately successful. Most of these studies tackled the molecular imbalance between tPA and PAI-1, which plays a central role in adhesion formation. According to Nair S et al. [24], Ad5-Sigma-luc and Ad5-MSLN-CRAd-luc exhibit high reporter gene expressions in human adhesion fibroblasts, and so, they can be used to deliver therapeutic genes such as tPA to postoperative adhesion sites. Alternatively, adhesions may be prevented by downregulating PAI-1. This may be achieved using an adenovirus vector encoding human tPA [25] or a cationic polymer containing siRNAs that target PAI-1 and HIF-1α (the gene inducer of PAI-1) [19]. Adhesions may also be reduced using the adenovirus encoding genes of hepatocyte growth factor (HGF) or of HGF's downstream signaling molecule sphingosine kinase 1 (SK-1). Both genes have simultaneous effects on the proliferation and migration of mesothelial cells [26, 27]. The role of the TGF-β1/Smad pathway in regulating adhesion formation has also been examined, and the available study shows that Smad7 transgene partially inhibited the production of fibrogenic

factors and attenuated peritoneal adhesion in rats [17].

Currently, various clinical trials for locally delivered siRNA drugs are widely reported. These trials are concerned with the treatment of numerous diseases including ocular diseases, lung infections/disorders, hypertrophic scars and keloids, and cancer [28]. In this study, we proposed R-Smad-targeting siRNAs for the prevention of adhesion formation via the alleviation of excessive ECM synthesis in TGF-β-induced ATFs. Based on the obtained results, it may be concluded that the temporary blockage of TGF-β1/Smad signaling is a promising strategy for the prophylaxis of postoperative adhesions. *In vivo* transfection in rat adhesion models will be analyzed in future studies in order to further examine the adhesion prevention efficiency of the proposed R-Smad siRNAs.

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Disclosure of conflict of interest

None.

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