Self-assembled Micelle Interfering RNA for Effective and Safe Targeting of Dysregulated Genes in Pulmonary Fibrosis

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RNA interference (RNAi) has been used as a method to regulate the expression of specific target gene in vitro and in vivo. However, the effectiveness of delivery and the nonspecific immune stimulatory function of siRNA are the limiting factors for therapeutic application of siRNAs. To overcome these limitations, we developed self-assembled micelle inhibitory RNA (SAMiRNA) nanoparticles made of individually bi-conjugated siRNAs with hydrophilic polymer and lipid on their ends and characterized their stability, immune stimulatory function and in vivo silencing efficacy. SAMiRNAs form very stable nanoparticles with no significant degradation in the size distribution and polydispersity index over 1 year. Overnight incubation of SAMiRNAs (3µM) on murine PBMCs did not cause any significant elaboration of innate immune cytokines such as TNF-α, IL-12 or IL-6, while unmodified siRNAs or liposome or liposome complex significantly stimulated the expression of these cytokines. Lastly, in vivo silencing efficacy of SAMiRNAs was evaluated by targeting amphiregulin (AR) and connective tissue growth factor (CTGF) in bleomycin or TGF-β transgenic (Tg) animal models of pulmonary fibrosis. Only two or three times of intratracheal (i.t.) or intravenous (i.v.) delivery of AR or CTGF SAMiRNAs significantly reduced the bleomycin- or TGF-β-stimulated collagen accumulation in the lung and substantially restored the lung function of TGF-β Tg mice. These studies demonstrated that SAMiRNA nanoparticles as a less-toxic, stable siRNA silencing platform for efficient in vivo targeting of genes implicated in the pathogenesis of pulmonary fibrosis.
as a powerful tool to target specific mRNA in a sequence-specific manner *in vitro* and *in vivo* experimental systems (1). Due to its high selectivity, the RNAi silencing approach was also suggested as a promising platform for therapeutic application on the diseases with aberrant transcriptional expression of specific gene (2,3). However, there are still several issues that significantly restrict the effectiveness and safety in therapeutic application. Naked nucleic acid molecules including synthetic small interfering RNA (siRNA) are easily degraded by ubiquitous nucleases either in the circulation or inside cells and they are unable to enter the cells though passive diffusion mechanisms due to large molecular weight and polycationic nature of chemical structure (4). In addition, non-specific innate immune stimulatory function of siRNA could be a serious problem especially for repetitive and high dose therapeutic application (5,6). In the last decade, various approaches have been developed to overcome these issues. They include chemical modification of siRNA itself to resist nuclease degradation or the use of liposome or lipid conjugation for efficient cellular uptake and effective silencing (7-10). Although several modified or naked siRNAs are currently being tested for clinical use (10), no specific siRNA platform is currently approved for therapeutic application yet. Thus, there is a critical need to develop effective and safe methods of delivery for better therapeutic targeting of genes *in vivo*.

Pulmonary fibrosis is a fatal progressive lung disease characterized by epithelial damage, fibroproliferative matrix deposition and parenchymal remodeling (11-13). TGF-β is believed to play a central role in this dysregulation. TGF-β expression is exaggerated fashion in patients with pulmonary fibrosis where, in contrast to controls, a sizable percentage of this cytokine is biologically active (14-16). The important role that TGF-β may play in this disorder can be seen in studies that demonstrate that TGF-β is a critical mediator of pulmonary fibrosis after bleomycin lung injury (17,18), adenoviral transfer or transgenic overexpression of TGF-β caused a progressive fibrotic pulmonary response *in vivo* (19-21). Recent studies from our laboratory and others have further identified that TGF-β regulated genes, such as amphiregulin (AR) or connective tissue growth factor (CTGF), mediate the effector function of TGF-β in the pathogenesis of pulmonary fibrosis (22-24). In these studies, targeted silencing of AR or CTGF expression with either genetic ablation or chemical inhibition significantly reduced collagen accumulation in animal models of pulmonary fibrosis, suggesting that these molecules are reasonable therapeutic target for the intervention of pulmonary fibrosis.

For effective and safe *in vivo* delivery of siRNAs, here we developed a modified siRNA nanoparticles consisted of individually bi-conjugated siRNAs with hydrophilic polymer and hydrophobic synthetic lipid on each ends of individual siRNA. In solution, the modified siRNAs spontaneously form stable and less toxic self-assembled micelle interfering RNA (SAMiRNA) nanoparticles. Our studies demonstrated that *in vivo* delivery of AR or CTGF SAMiRNAs via either intratracheal (i.t) or intravenous (i.v.) injections effectively silenced the expression of target genes as well as collagen accumulation in the lungs in animal models of pulmonary fibrosis. These studies highlighted that a potential use of SAMiRNA nanoparticles as an effective and safe delivery platform to target critical gene(s) implicated in the pathogenesis of pulmonary fibrosis or other diseases with dysregulate gene expression.

**Experimental procedures**

*Mice used in these experiments* - C57BL/6 mice were purchased from the
Jackson Laboratory (Bar Harbor, ME) and were housed in animal facilities at the Korean Institute of Toxicology (KIT) and Brown University until they were used. TGF-β Tg mice were maintained and characterized according to the procedures previously described (21,22). All murine procedures were approved by the Institutional Animal Care and Use Committees at KIT and Brown University.

**SAMiRNA synthesis and physicochemical characterization** - The detailed procedure and materials used for SAMiRNA nanoparticle synthesis are described in Supplemental Information. To prepare homogenous nanoparticles, synthesized SAMiRNAs were dissolved in 1.5 ml Dulbecco's Phosphate Buffered Saline (DPBS) at a concentration of 50 μg/ml, followed by lyophilization at -75 °C and 5mTorr for 48 hours. The lyophilized SAMiRNAs were resuspended with DPBS just before use. The size distribution and polydispersity index (PDI) of SAMiRNAs were measured by zeta-potential measurement using Zetasizer Nano-ZS (MALVERN, UK). A one-time measurement consists of 15 repetitive size measurements, and this measurement was repeated six times.

**Ex vivo organ imaging analysis** - To track SAMiRNA delivery to lung and other organs ex vivo imaging analysis was performed. In brief, Male C57/BL6 mice stimulated by bleomycin or TGF-β transgene expression were used for ex-vivo imaging of Cy5.5 labeled SAMiRNA in organ. Labeled SAMiRNAs were delivered at a dose of 5 mg/kg i.v. or i.t.; 12, 24 or 48 hours post treatment, mice were sacrificed and organ of interest, liver, lung and spleen were collected for imaging analysis. After the organs were gently washed in PBS, fluorescence images were acquired under the IVIS200 system (PerkinElmer, USA) at Cy5.5 wavelengths (Emission, 695-770). The ROI data were analyzed with the IVIS software (Living Image Software).

**Construction of siRNA encapsulated liposomes** - siRNA encapsulated liposomes, called as stable nucleic acid particles (SNALP), were prepared according to previously reported method (25). A DODMA (1,2-Dioleyloxy-3-(dimethylamino)propane), DSPC (1,2-dioctadecanoyl-sn-glycerol-3-phosphocholine), Cholesterol (2,15-dimethyl-14-(1,5-dimethylhexyl)tetracyclo-heptadec-7-en-5-ol), PEG2000PE (1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and were dissolved in ethanol at a molar ratio of 40:10:47:3, respectively. The lipid mixture was added into β-gal siRNA solution (366 μg in citrate buffer, pH 4) while mixing to the final ethanol and lipid concentrations of 30% (vol/vol) and 6.1 mg/ml, respectively. The resulted emulsion was extruded through a stacked 100 nm poresize filter (Millipore) at room temperature using Avanti mini-Extruder for total of 20 passes through membrane followed by dialyses against pH 4.0 50mM citrate and pH7.4 phosphate buffered saline (PBS) at room temperature. The encapsulation efficacy of siRNA with liposome was assessed using HPLC chromatogram and RiboGreen assay (Molecular Probes/Invitrogen, Eugene, OR) for siRNA quantitation.

**Isolation of mouse peripheral blood mononuclear cells (PBMCs) Isolation** - Mouse whole blood obtained from ICR strain (8 week, Female) was diluted with PBS to 3ml at the ratio of 1:1 (2ml blood diluted with 2ml of 1X PBS) and loaded on 3ml of Percoll hypaque in a 15ml falcon tube. After centrifugation at 1,400rpm for 30 min at 20°C, buffy coat containing PBMCs over the Percoll layer was separated by 5ml pipette and washed with 20ml 1X PBS by spinning at 1,400rpm, 20°C for 15min. Then isolated PBMCs were resuspended by RPMI medium and viable cells were counted. After
centrifugation at 1,400rpm, 4°C for 10min, supernatant was discarded and cell pellet was resuspended by RPMI medium supplemented with 10% FBS for cell seeding.

**Bleomycin challenge** - Sex-matched, 8-wk-old wild-type C57BL/6 mice were exposed to a single bleomycin HCl (1mg/kg, Nippon Kayaku, Tokyo, Japan) dissolved in sterilized 0.9% saline via intratracheal administration (26). For accurate instillation of the bleomycin into the lung, an automatic video instillator was used as described previously (27).

**siRNA transfection to mouse PBMC cells** - Mouse PBMCs (0.5x10^6 cells/250µl) were seeded on each well of 48-well plate. According to manufacture’s protocol for siRNA transfection, 12.5µl of OptiMEM containing 5µM of siRNA was added to 12.5µl of OptiMEM containing 2µl of Lipofectamine 2000 (Invitrogen, USA) after 5min incubation at RT. After gentle mixing, the siRNA-Lipofectamine mixture was incubated at RT for 10min, and treated to the mouse PBMCs. The specific siRNAs targeted AR, CTGF, and β-gal were selected and synthesized at Bioneer Inc (Daejeon, Korea) and used for the evaluation. After siRNA transfection, PBMCs were incubated for 24hr and harvested culture media was stored at -80°C until being assayed.

**Treatment of lyophilized SAMiRNA or encapsulated liposome to mouse PBMCs** - Mouse PBMCs (0.5x10^6 cell / 250µl) were seeded on each well of 48-well plate. Lyophilized SAMiRNAs of AR, CTGF, Control (siCon), and β-gal were prepared 10 µM solution with PBS and were directly applied to the mouse PBMCs to 1 µM or 5 µM concentration. The 1 µM siRNA (β-gal) encapsulated liposome was used for this evaluation. After SAMiRNAs or siRNA encapsulated liposome treatment, PBMCs were incubated for 24hr and harvested culture media was stored at 80°C until being assayed.

**Luminex multiplex screening assay** - After 24hr incubation of mouse PBMC, 200µl media was harvested and stored at -80°C for luminex multiplex assay. Multiplex analysis was performed by customer service (Woongbee Meditech Biotechnology Inc., Seoul, Korea) and released cytokines (TNF-α, MCP-1, IFN-γ, IL-12 (p70), IL-6) in the supernatant were measured.

**In vitro SAMiRNA silencing of AR and CTGF** - The mouse fibroblast cell line (NIH3T3, ATCC) were cultured in a 12-well plate in the recommended culture medium (1ml of RPMI-1640 medium with 10% FBS) for 18 hours, then the medium was replaced with of Opti-MEM medium. After 30 min incubation, the medium was replaced with Opti-MEM containing SAMiRNA (100-400nm) and cultured at 37°C in 5% CO2 incubator.

**In vivo AR and CTGF silencing in animal models of pulmonary fibrosis** - TGF-β transgenic or bleomycin-challenged mice were used to evaluate in vivo silencing efficacy of SAMiRNA in the development and progression of pulmonary fibrosis. 6-8 weeks-old TGF-β Tg and control littermates were randomized to receive AR- or CTGF-specific or control SAMiRNAs via intratracheal (i.t.) or intravenous (i.v.) injections (1-5mg/kg per mouse) on 7, 9 and 11 days after transgene induction, then the fibrotic changes in the lungs were evaluated. Similarly, SAMiRNAs were delivered to the mice on day 7 and 9 with and without bleomycin-challenged mice via i.t or i.v. injections.

**Flow cytometric analysis on the SAMiRNA targeted cells** - SAMiRNA targeted cells in fibrotic lung were assessed using flow cytometric analysis on the enzyme-digested lung tissue were performed using cell-specific markers (FITC–labeled Clara Cell 10 Kd (CC10) for airway epithelial cells, Surfactant Protein C (SPC) for alveolar epithelial cells, CD140a (PDGFRα) for fibroblasts and mesenchymal cells, CD68 for
macrophages, and CD3 for T cells) and Cy5.5-labeled SAMiRNA. TGF-β Tg mice were used for this evaluation. In brief, the TGF-Tg mice were incubated for 2 weeks for induction of pulmonary fibrosis. Then 3 mg/kg Cy5.5 conjugated SAMiRNA was introduced to the lung via i.t. injection. Lungs from these mice were digested and cells were isolated as described (28). Isolated cells were incubated for 2 hrs at 4°C with cell-specific antibodies for airway epithelial cell (CC10, Santa Cruz), alveolar type II cell (SPC, Santa Cruz), macrophage (CD68 and F4/80, ebioscience), fibroblast (CD140, ebioscience) and pan T cell (CD3, BD Pharmigen). At least 10,000 cells per sample were analyzed using FACS Aria II flow cytometer (BD Biosciences).

Clinical chemistry and hematologic evaluation on the blood - To evaluate systemic toxicity of SAMiRNA, we screened the levels of various biochemical indicators of liver and kidney toxicity that include the levels of AST, ALT, ALP kidney function (BUN, Creatine, total(T)-protein, Albumin, T-cholesterol, and T-bilirubin) and hematologic parameters such as total leukocyte or platelet counts, hemoglobin, or hematocrit 1 day after administration of SAMiRNA with i.v. or i.t. delivery.

mRNA analysis - Total cellular RNA was obtained using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. mRNA was measured using real-time RT-PCR as described previously (29,30). The primer sequences for extracellular matrix genes were obtained from PrimerBank (pga.mgh.harvard.edu/primerbank/) or the same as previously used (22,29,31).

Histologic analysis - Mouse lungs were removed en bloc, inflated to 25 cm pressure with PBS containing 0.5% low melting point agarose gel, fixed, embedded in paraffin, sectioned, and stained. Hematoxylin and eosin, and Mallory's trichrome stains were performed in the Research Histology Laboratory of the Department of Pathology at the Yale University School of Medicine. BAL and lung inflammation was assessed as described previously (29).

Quantification of lung collagen - Animals were anesthetized, median sternotomy was performed, and right heart perfusion completed with calcium and magnesium-free PBS. The heart and lungs were then removed. The right lung was frozen in liquid nitrogen and stored at −80°C until used. Collagen content was determined by quantifying total soluble collagen using the Sircol Collagen Assay kit (Biocolor, Accurate Chemical & Scientific Co., Westbury, NY) according to the manufacturer's instructions.

Lung function measurements - Measurements of lung function were obtained by forced maneuvers using the flexiVent®-FX2 (SIREQ Sientific Respiratory Equipment Inc., Montreal, QC, Canada). Animals were anesthetized with Xylazine (10 mg/kg) and ketamine (100 mg/kg), tracheotomized, and mechanically ventilated. Respiratory mechanics were determined by application of predefined pressure/volume perturbations to the airways. Dynamic readouts, including total lung capacity, resistance, and compliance were obtained by the linear single-compartment model using multiple linear regression. Measurements of respiratory system input impedance; tissue damping and tissue elastance were obtained using multiple low-frequency forced oscillations. All measurements were carried out until three acceptable readings (coefficient of determination >0.95) were recorded for each animal, and the average calculated. After lung function test, the mice lungs were harvested for further histologic evaluation and also for RNA and protein expression analysis.

Statistical evaluation - Values are expressed as mean ± S.E.M. As appropriate, groups were compared by two-tailed Student t test. A p value of ≤ 0.05 was considered to be significant.
Results

Physico-chemical properties of SAMiRNA nanoparticles - For effective and safe in vivo delivery of siRNAs, we developed nanoparticles consisting of siRNAs conjugated with hydrophilic polymer polyethylene glycol (PEG) and hydrophobic synthetic lipid on 3' and 5’ ends of sense (passenger) strand of each siRNAs (see Supplemental Information for details of conjugation process). The conjugated siRNA molecules spontaneously form a globular micelle structure with a hydrophobic lipid in center and a hydrophilic polymer outside coat (Fig. 1A), so it is designated as SAMiRNA (self-assembled micelle interfering RNA). The uniform globular structure of SAMiRNA nanoparticles were well visualized by cryo-transmission electron microscopy (TEM) (Fig. 1B). For in vivo application of SAMiRNA in animal models of pulmonary fibrosis, we generated SAMiRNAs of amphiregulin (AR) and CTGF, the genes induced by TGF-β stimulation and critical mediators of pulmonary fibrosis, were specifically targeted (22,23,32). The size of AR and CTGF SAMiRNAs nanoparticles are relatively uniform and similar to each other (about 100nm ±20 and 0.2 ± 0.01 for average size and polydispersity index (PDI), respectively) (Fig. 1, C and D). Importantly, the size and the PDI of these nanoparticles were not significantly changed over 1 year of observation under room temperature in solution (22°C, 55±5% humidity) (Fig. 1E), indicating long term stability of SAMiRNA nanoparticles.

In vitro silencing efficacy and non-specific immune stimulatory function of SAMiRNA nanoparticles - In vitro silencing efficacy of AR and CTGF SAMiRNAs was evaluated and compared to unmodified siRNAs. The silencing efficacy evaluated by qRT-PCR was approximately 50% and 75% for unmodified AR and CTGF siRNAs, respectively. When the same cells were treated with SAMiRNAs, comparable level of silencing efficacy to unmodified siRNA was achieved for both AR and CTGF silencing (Fig. 2, A and B).

To evaluate nonspecific immune stimulatory activity of SAMiRNAs, PBMCs were isolated and incubated with 1µM or 5µM of unmodified siRNAs, SAMiRNAs or liposome encapsulated siRNAs for 24hrs. Next, the levels of innate immune cytokines (IFN-γ, IL-12(p70), IL-6, TNF-α, MCP-1) elaboration in culture supernatant was assessed using multiplex magnetic luminex assay. Incubation of murine PBMCs with unmodified AR, CTGF or β-gal siRNAs showed significant increases in the levels of TNF-α, IL-12 or IL-6 in culture supernatant (Fig. 2C) at both concentrations. In this evaluation, β-gal siRNAs encapsulated with liposome, a stable nucleic acid lipid particles (SNALPs) served as a control for SAMiRNA. Interestingly, β-gal SNALP caused the most significant increase of all the cytokines measured. Interestingly, lipofectamine (Lipofectamine RNAiMAX, Life Technologies, Grand Island, NY) itself showed low levels of cytokines, but exhibited a similar pattern of nonspecific cytokine stimulation. However, no detectable cytokines were noted in the supernatants of murine PBMCs incubated with AR, CTGF or β-Gal SAMiRNAs at both concentrations. These studies suggested that SAMiRNA nanoparticles have much less non-specific immune stimulatory activity compared to unmodified siRNAs or SNALP liposome complex.

Blood chemistry and hematologic evaluation after SAMiRNAs treatment – No significant changes suggesting liver or kidney toxicity were noted in biochemical or hematologic evaluations in the serum of mice after suggesting abnormale after administration of SAMiRNAs via i.t. or i.v. (Supplemental Fig. 1). These studies suggest
that the SAMiRNAs doses tested in current studies were safely delivered without significant systemic toxicity.

**Biodistribution and pharmacokinetics of SAMiRNA nanoparticles in animal models of pulmonary fibrosis** - To confirm the delivery of SAMiRNA nanoparticles to the lung of animals with and without fibrosis, *ex vivo* fluorescence images were taken 12, 24 and 48 hrs after intratracheal (i.t.) or intravenous (i.v.) injections of labeled SAMiRNAs with Cy5.5. With i.t. injection, strong fluorescence was detected in the lungs of bleomycin-challenged or TGF-β Tg mice as well as WT animals (Fig. 3A). The fluorescence in the lungs was gradually decreased, but a strong level of fluorescence was noted until 48 hrs after injection (Fig. 3A). Similar patterns were observed for fluorescence-labeled SAMiRNA accumulation in the lungs of bleomycin-challenged or TGF-β Tg mice was observed with i.v. injection (Fig. 3B). Interestingly, fluorescence levels in the lungs of bleomycin-challenged or TGF-β Tg mice with i.v. injection remained strong while only a weak signal were noted in wild type animals. These findings suggest enhanced retention rate of these SAMiRNAs at the sites of injury or fibrosis in the lungs (Fig. 3B). With i.v. injection, we observed SAMiRNA nanoparticles were accumulated in the liver as well as in the lung as noted previously (33). It is also interesting to note the fluorescence levels are more consistently maintained in the lungs of the mice with bleomycin challenge or TGF-β overexpression up to 48 hrs with i.v. delivery than i.t. injection (Fig. 3B). SAMiRNA targeted cells were further assessed by flow cytometric analysis using various cell-specific markers and fluorescence labeled SAMiRNAs (Fig 3C). These studies identified that about 25-30% of total lung cells were Cy5.5-positive cells. However, the percentage of Cy5.5 positive cells were variable depending on the cell types: about 35%, 20%, 75%, 70%, 63% for CC10, SPC cells, CD140a, CD68 and CD3 positive cells, respectively. It is of interesting to note that highest targeting cells with SAMiRNA in fibrotic lung were CD140a positive cells including fibroblasts, the major cells responsible for the development of pulmonary fibrosis.

We further evaluated the pharmacokinetics of SAMiRNA after i.t. or i.v. injections of AR SAMiRNA on bleomycin-challenged or TGF-β Tg mice. As expected, AUC (area-under-the curve), Cmax (maximal concentration), and Tmax (time to reach Cmax) were higher with i.v. delivery than i.t. injection, but time to reach half maximal concentration (T1/2) was similar to each other (5.1 hrs and 4.3 hrs for i.t. and i.v., respectively), suggesting that the degradation and excretion of SAMiRNA nanoparticles in the circulation are similarly regulated regardless of routes of SAMiRNA delivery (Fig. 4A). Although serum levels of siRNA are less than bleomycin-challenged mice, a similar pattern of pharmacokinetics was observed with AR SAMiRNA in the circulation of TGF-β Tg mice (Fig. 4B).

**In vivo silencing efficacy of AR and CTGF SAMiRNA in a bleomycin model of pulmonary fibrosis** - In vivo efficacy of SAMiRNA was first evaluated in a bleomycin animal model of pulmonary fibrosis. To establish the time points of SAMiRNA treatment, wild type mice were exposed to vehicle (PBS) or bleomycin and kinetic changes in the expression of AR and CTGF and other genes associated with pulmonary fibrosis were determined (Fig. 5). Bleomycin treatment significantly induced the expression of AR and CTGF on days 7 and 9. The AR and CTGF expression corresponded well with the level of collagen contents in the lung and the expression of extracellular matrix-associated genes such as collagen 3α1, fibronectin and elastin compared to the lungs from the mice challenged with vehicle (PBS) (Fig. 5). Based on these kinetic studies, eight
weeks old male C57BL/6 mice were treated with AR or CTGF SAMiRNAs (3mg/kg/each mouse) twice via i.t. or i.v. injections on days 7 and 9 after bleomycin challenge (Fig. 6A). The mice sacrificed on day 14 after bleomycin challenge showed increased expression of AR, CTGF, collagen $\alpha_1$, fibronectin and inflammatory responses, and collagen accumulation in the lung (Fig. 6, B-D). However, i.t. administration of AR or CTGF SAMiRNAs on these mice significantly reduced the bleomycin-stimulated expression of AR and CTGF as well as the expression of collagen and fibronectin and inflammation and collagen accumulation in the lung compared to the mice treated with control SAMiRNAs (Fig 6, B-D). Delivery of AR or CTGF SAMiRNAs via i.v. injection (3mg/kg/mouse) also showed effective silencing of AR and CTGF and significant reduction of collagen accumulation in the lung compared to the mice received control SAMiRNA nanoparticles (Figure 7 and data not shown).

**In vivo Silencing efficacy of AR and CTGF SAMiRNAs in TGF-$\beta$-Tg model of pulmonary fibrosis** - Although bleomycin has been commonly used to induce injury and fibrosis for various studies of fibrosis in the lung and other organs, it has a significant limitation when representing actual disease phenotype of human pulmonary fibrosis. To compensate this deficiency, further studies were undertaken to evaluate in vivo silencing efficacy of SAMiRNA using TGF-$\beta$-Tg mice in which active TGF-$\beta$ is overexpressing specifically in the lung (21). It is well documented that AR and CTGF are induced by TGF-$\beta$ stimulation and mediate the essential TGF-$\beta$ effector function in the pathogenesis pulmonary fibrosis (22,32). The 6-8 weeks old, WT and TGF-$\beta$ Tg male mice were used for this evaluation. Since apparent fibrotic tissue responses in the lung of TGF-$\beta$ transgenic mice starts about a week after TGF-$\beta$ transgene induction with administration of doxycycline drinking water (21), AR and CTGF SAMiRNAs and control SAMiRNAs were delivered to WT and TGF-$\beta$ Tg mice on day 7, 9, and 11 days after transgene induction via i.v. injection (5mg/kg) and the mice were sacrificed and evaluated on day 14 (Fig. 8A). The mRNA expression of AR or CTGF in the lungs from TGF-$\beta$ Tg was significantly reduced by treatment of AR or CTGF SAMiRNA, respectively (Fig. 8B). In addition, TGF-$\beta$-stimulated mRNA expression of collagen or fibronectin gene was also significantly down regulated by treatment of AR, CTGF SAMiRNA alone or combination (Fig. 8B). As expected, lung collagen levels were significantly induced in the TGF-$\beta$ Tg mice treated with control SAMiRNA compared to WT animals (Fig. 8, C and D). The AR or CTGF SAMiRNA treatment significantly reduced the levels of TGF-$\beta$-stimulated accumulation of collagen in the lung measured by Sircol collagen assay or Mallory trichrome staining (Fig. 8, C and D). Interestingly, we noted that the mice treated with AR and CTGF SAMiRNAs in combination (2.5mg/kg each, i.v.) showed more consistent silencing effects in collagen accumulation in the lung compared to the mice treated with AR or CTGF SAMiRNA alone. These findings suggest that there is a potential additive or synergistic interaction between AR and CTGF in TGF-$\beta$-stimulated pulmonary fibrosis (Fig. 8, C and D). These studies demonstrated that in vivo SAMiRNA silencing of AR and CTGF effectively inhibits TGF-$\beta$-stimulated pulmonary fibrosis. It is also important to note that the effects of AR- or CTGF SAMiRNAs in fibrotic tissue response of TGF-$\beta$ Tg mice were not from the changes in the levels of transgene expression, since no changes of transgene expression in the lungs from TGF-$\beta$ Tg mice were detected with and without SAMiRNAs treatment (data not shown).

**Effect of AR and CTGF SAMiRNAs silencing on lung function of TGF-$\beta$ Tg mice** -
In human pulmonary fibrosis, a lung function test provides informative and important clinical indices frequently used for the evaluation of disease development and progression. Thus, we evaluated the effect of SAMiRNA silencing of AR and CTGF alone or in combination on the lung function of TGF-β Tg mice. TGF-β Tg mice showed typical obstructive phenotypes characterized by increased respiratory system resistance and elastance, central airway resistance and decreased levels of compliance (Fig. 9). All of these parameters were significantly improved after SAMiRNA silencing of AR or CTGF alone or in combination compared to control SAMiRNA treated mice (Fig. 9). It is also interesting to note that SAMiRNA silencing of AR and CTGF in combination showed more consistent restoration of lung function in central airway resistance and respiratory system elastance than silencing of AR or CTGF alone (Fig. 9, B and C).

Discussion

After discovery of short synthetic double strand RNAs with silencing ability of specific mRNA in mammal cells, small interfering RNAs (siRNAs) have been extensively used to target specific gene expression (1,2). However, in vivo therapeutic application of siRNA is still under development, mainly because issues limiting optimal delivery of the siRNAs to target tissue or cells in vivo have not been sufficiently addressed yet. Unmodified naked siRNAs are rapidly degraded by the nuclease in the circulation and efficiently removed through renal clearance (34,35). In addition, activation of non-specific innate immune stimulation of short RNA sequences could be a significant problem in repetitive and high dose therapeutic application of siRNAs (5,6). Thus, to achieve optimal in vivo siRNA delivery and specific silencing, it is imperative to develop modified siRNA or strategies that allows protecting siRNAs from nuclease degradation and facilitate cellular uptake for efficient gene silencing without significant immunogenicity.

A number of delivery carriers including liposomes, lipids, polymers, peptides and viral vectors have been suggested to improve in vivo delivery and cellular uptake (10,36). In particular, advances in the development of siRNA conjugation with lipid, polymers, and peptides significantly improved efficiency and cell-specific delivery with less cytotoxicity, and as a result, several modified siRNAs are at the stage of clinical development (7,10). To build upon these efforts, we developed a novel siRNA nanoparticle platform with conjugation of polymer and lipid on both ends of the passenger (sense) strand of siRNA. In this modification, all the single siRNAs are individually modified, so in a solution state, nanoparticles spontaneously generated as self-assembled micelle with a hydrophobic lipid core inside and a hydrophilic polyethylene glycol (PEG) coat outside. The use of current bi-conjugated modification of a single siRNA using polymer and synthetic lipid provide several advantages over conventional modification of siRNAs. First, the modification generates stable nanoparticles with uniform size for effective in vivo delivery of siNRAs. It has been suggested that the optimal particle size for in vivo delivery is more than 20nm but less than 100nm, that enables the particle to escape renal clearance but remains small enough to penetrate through vasculature to reach specific target cells (37). Due to the nature of vasculature at the sites of injury or fibrosis with active inflammation and because neovascularization is leaky and permeable compared to normal healthy tissues, the nanoparticles with appropriate size will be readily accumulated at the pathologic sites by enhanced permeation and retention (EPR) effect (38). We also see significant injury/leak responses in the either in the lungs from...
bleomycin-challenged or TGF-\(\beta\) Tg mice as we observed increased total BAL proteins in these mice compared to control mice (data not shown). Accordingly, when we injected SAMiRNA nanoparticles in animal models of fibrosis via i.v. injections, increased retention of fluorescence-labeled SAMiRNAs in the compromised lungs from the mice with TGF-\(\beta\) overexpression compared to uncompromised lungs from wild type mice (Fig. 3). The pharmacokinetic studies also provide supporting evidence that the clearance rate in the circulation is much slower with SAMiRNA modification than with unmodified siRNAs, since \(T_{1/2}\) of SAMiRNAs was more than 4 hrs compared to that of unmodified siRNAs (15min-1hr) reported in the previous studies (33). It is also interesting to note that the SAMiRNAs are effectively delivered to the mesenchymal cells that include fibroblasts and myofibroblasts, the major cells responsible for pulmonary fibrosis as well as other inflammatory and structural cells. In addition, the stability of SAMiRNA nanoparticles was excellent since no significant degradation was noted both in the size distribution or polydispersion index in a solution over 12 months. When viewed in combination, current studies demonstrated that SAMiRNA nanoparticles with uniform size distribution, longstanding physical stability, delayed clearance and enhanced retention rate confer strong advantages in intact and effective in vivo delivery of siRNAs at sites of injury and fibrosis.

Another advantageous point in the use of SAMiRNA nanoparticles for in vivo application is a very low, or lack of nonspecific immune stimulatory function. We did not note significant cytokine expression associated with innate immune response at concentrations that unmodified siRNA or liposome or lipofectamine itself induced significant levels of cytokine stimulation. We also did not note significant changes in the BAL inflammation or in the expression of these innate cytokines in the BAL or in the lung with i.t. or i.v. injection of SAMiRNA nanoparticles, further supporting a lack of, or low immunogenicity of SAMiRNA nanoparticles (Supplemental Fig.2). This decreased immunogenicity might simply be the result of a shielding effect of the PEGylated coat of SAMiRNAs that prevented direct exposure of double strand (ds) siRNAs to immune cells or cell surface receptors such as Toll-like receptors (TLRs). Of various TLRs, TLR3 binds with dsRNA and TLRs 7/8 binds to single-stranded RNA, but all three receptors can recognize the synthetic siRNAs (5). Since these receptors are primarily localized in the endosomal compartment, naked siRNAs or siRNAs simply complexed with cationic lipids or polymers readily bind to these receptors during cellular uptake and internalization process. The SAMiRNAs nanoparticles made of individually bi-conjugated siRNA with polymer and lipid on their ends can evade this innate immune surveillance system since free siRNA is not released in acidic endosomal compartment. It is also important to note that lipid conjugation greatly affect the cellular cytotoxicity as well as the levels of cellular uptake. In general, positively charged lipids improve entrapment of negatively charged siRNA and cellular uptake and aid endosome escape (39,40). However, the nature of lipids also greatly affect the efficacy of cellular uptake as well as the levels of cytotoxicity (10,41). Thus, it is reasonable to speculate that the siRNA conjugation of simple synthetic lipid instead of employing strong cationic lipid (42) in the preparation of SAMiRNA nanoparticles might lead to less cytotoxic immunogenicity, at least in part. Further detailed mechanistic studies will be necessary to understand the pathways leading to immune responses to siRNAs.

Lastly, we confirmed that in vivo delivery of SAMiRNA nanoparticles efficiently silenced the expression of target
gene expression as well as significantly improve the pathologic changes associated with dysregulated gene expression. In this evaluation process, we employed two target genes, amphiregulin (AR) and CTGF, since they are well known to play a critical role in the pathogenesis of pulmonary fibrosis (22,24). Pulmonary fibrosis is a devastating lung disease with high mortality rate and few therapeutic option. More than 75% of the patients die within 5 years of diagnosis. Currently only 2 drugs are approved as therapeutic drugs, but they only delay the progress of the disease with improved lung function (43-45). Thus, pulmonary fibrosis is a lung disease with high unmet medical need; the development of interventional tools is critical to prevent or reverse the fibrosis. It has been shown that TGF-β plays a central role in pulmonary fibrosis as well as fibrotic tissue response in other organs (46,47). However, to pleiotrophic and essential roles of TGF-β in cellular development and immune regulation, targeting downstream mediators of TGF-β such as AR and CTGF, rather than TGF-β itself could be an alternative and effective way to block pulmonary fibrosis. In this study, based on the kinetic changes in the expression of AR and CTGF, we treated bleomycin challenged mice with AR or CTGF SAMiRNAs, at two times, on day 7 and 9 during a 14 day evaluation period. With less or similar doses of conventional siRNA treatment (1 - 5mg/kg/mouse), AR or CTGF SAMiRNAs significantly reduced in vivo expression of AR and CTGF, collagen accumulation in the lung and the expression of genes directly associated with pulmonary fibrosis, compared to the mice with control SAMiRNA. Similarly to bleomycin-challenged mice, we also achieved a significant silencing effect of AR and CTGF expression as well as collagen accumulation in the lung with 3 injections of AR or CTGF SAMiRNAs on TGF-β Tg mice. This is an impressive in vivo effect compared to previous studies that used naked AR siRNA silencing (22), since current studies showed treatment effect rather than preventive measure with less than one third of the dosing schedule. In addition, silencing of these target genes significantly restored the lung function of TGF-β Tg animals. Either AR or CTGF SAMiRNAs treatment improved the obstrucive phenotype of TGF-β Tg mice especially in central airway resistance and compliance. It is also of interest to note that more prominent and consistent inhibitory effects on collagen accumulation in the lung and lung function restoration were seen in the mice treated with AR and CTGF SAMiRNAs in combination than AR or CTGF alone. Although the benefits of combination is limited depending on the animal models of fibrosis and route of delivery, these findings suggest potential synergistic or additive interactions between AR and CTGF in the pathogenesis of pulmonary fibrosis. Interestingly, recent studies reported that CTGF is a ligand for epidermal growth factor receptor (EGFR) and mediates TGF-β-induced EGFR activation (48). If this is the case, both AR and CTGF could be required for optimal stimulation of EGFR signaling pathway that is essential for TGF-β-stimulated pulmonary fibrosis as we have shown previously (22). Thus, further mechanistic studies to define the interaction of these two mediators are warranted to develop the most effective antifibrotic strategy for the intervention of pulmonary fibrosis.

In this study, we demonstrated that a newly developed SAMiRNA nanoparticles consisting of individually bi-conjugated siRNAs with a polymer and a synthetic lipid provide effective in vivo delivery of siRNAs with stable, decreased immunogenicity compared to naked siRNA or liposome-based delivery. We also demonstrated a significant in vivo efficacy of SAMiRNA nanoparticles targeting AR and CTGF in the intervention of
collagen accumulation and lung function restoration in animal models of pulmonary fibrosis. These findings suggest SAMiRNA nanoparticles as an effective siRNA delivery tool for the intervention of pulmonary fibrosis we well as other diseases with dysregulated gene expression.

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Author Contributions – P.O.Y., H.O.P., K.L. conceived the idea for the project and acquired and analyzed data. H.N.K., Y.K., S.J.B., J.H.P., T.K., W.S.K., J.L., S.Y. conducted SAMiRNA synthesis, pharmacokinetic evaluation and chemiochemical characterization. JSY conducted immunogenicity assay. J.W.P., C.M.L., H.R.K., W.K.C. acquired and analyzed the data of TGF-β transgenic mice. Q.L., performed lung function test and data analysis. S.H.K., K.L., conducted in vivo challenge in bleomycin model of lung fibrosis. J.A.E. evaluate the data. CGL conceived idea for the project and analyzed data and wrote manuscript with P.O.Y.

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**FIGURE LEGENDS**

**Figure 1.** Physico-chemical properties of the SAMiRNA. A, schematic diagram of SAMiRNA nanoparticles. B, a representative Cryo-TEM image of SAMiRNA nanoparticles (scale bar = 100 nm). C-D, size distribution and polydispersity index (PDI) of amphiregulin (AR) and CTGF SAMiRNAs measured by Nano-Zetasizer (ZS). E, Long term stability of AR and CTGF SAMiRNAs measured by nanoparticle size and PDI. d, diameter. Panel B is a representative of a minimum of 3 independent experiments. The values in panels C and E represent mean ± SEM of evaluations with a minimum of 4 independent experiments. Panel D is a representative plot with 6 replicates.

**Figure 2.** Silencing efficacy and nonspecific immune stimulatory effects of naked siRNAs and SAMiRNAs. A and B, NIH3T3 cells were treated with amphiregulin (AR) and CTGF naked siRNAs and SAMiRNAs then the expression of AR and CTGF mRNA was measured by real-time RT-PCR and compared to scrambled siRNAs or SAMiRNAs (Sc siRNA or Sc SAMiRNA), respectively. C, the expression of nonspecific innate immune cytokine was evaluated using mouse PBMCs stimulated by naked siRNAs or SAMiRNAs of AR (siAR), CTGF (siCTGF), β-galactosidase (si-β-gal) and their control siRNA (si-Cont) or SAMiRNA (SAMiRNA-Cont). 5x10^5
mouse PBMCs were seeded onto 48-well plate and treated with PBS, 5 µM naked siRNAs, 1 or 5 µM lyophilized SAMiRNAs, 1 µM of encapsulated liposome called stable nucleic acid lipid particles (SNALP), Lipofectamine RNAiMax only, and Concanavalin A (ConA; at 20µg/ml). After 24hr incubation, cell culture supernatant were harvested and the levels of representative innate immune cytokines (TNF-α, MCP-1, IFN-γ, IL-12(p70), IL-6) were measured by luminex multiplex screening assay. The values in the panels represent mean ± SEM of a minimum of 3 separate evaluations with duplicates.

Figure 3. In vivo biodistribution of SAMiRNA in bleomycin and TGF-β Tg animal models of fibrosis. A, Time kinetic ex vivo fluorescence images after intratracheal instillation of Cy5.5-labeled SAMiRNA in bleomycin (BLM)-induced fibrosis and TGF-β Tg mice (left). Quantitative kinetic evaluation of fluorescence intensity by measuring Region of Interest (ROI) values (right panel). B, time kinetic ex vivo fluorescence images after intravenous (i.v.) injection of Cy5.5-labeled SAMiRNA nanoparticles in WT, BLM-challenged and TGF-β Tg mice (left panel). Quantitative kinetic evaluation of fluorescence intensity by measuring ROI (region of interest) values (right panel). C. Representative flow cytometric analysis on the SAMiRNA targeted cells in the lung. 24 hrs after i.t. injection of 3mg/kg APC-Cy5.5-labeled SAMiRNAs to TGF-β Tg mice, lung cells were isolated and subjected to flow cytometric evaluation using FITC-labeled cell specific markers: Clara cell 10Kd (CC10) for airway epithelial cell), Surfactant Protein C(SPC for alveolar epithelial cell), CD140a (mesenchymal cells including fibroblast and myofibroblast), CD68 (macrophages), CD3 (T cell). At least 10,000 cells per sample were analyzed. Percentages of Cy5.5 positive (+) SAMiRNA targeted cells in each group of cells defined by cell surface marker were separately represented (n=4 each, right panel). Ex vivo fluorescence images in panels A and B are representative of a minimum of 4 mice each group. ROI quantitation in panels A and B represent mean ± SEM of evaluations with a minimum of 4 mice.

Figure 4. Pharmacokinetic evaluation of SAMiRNA. Serum levels of amphiregulin (AR) siRNA were evaluated by qRT-PCR at indicated time points after i.t. or i.v delivery of AR SAMiRNAs (SAMiRNA-AR) (5mg/kg). A, pharmacokinetic evaluation of SAMiRNA-AR in bleomycin-challenged mice. B, pharmacokinetics of SAMiRNA-AR in TGF-β Tg mice. Each panel is a representative plot of a minimum of 3 independent evaluations.

Figure 5. Time kinetic changes in the expression of CTGF, AR and fibrosis-related genes and collagen deposition in the lungs of bleomycin-induced fibrosis. A-E, time kinetics in the expression of connective tissue growth factor (CTGF), amphiregulin (AR), collagen 3A1, fibronectin, and elastin in the lungs of mice exposed to intratracheal bleomycin. F, kinetic changes of soluble collagen protein deposition in lungs measured by the Sircol assay. The values in each panel are represent means ± SEM of minimum of 4 mice treated with bleomycin. *p<0.05, **p<0.01, ***p<0.001.

Figure 6. In vivo silencing of AR and CTGF expression in bleomycin model of pulmonary fibrosis. 8 weeks old WT mice were challenged with bleomycin (BLM) and AR and CTGF SAMiRNAs (3mg/kg) were delivered via intratracheal (i.t.) injection on days 7 and 9 after bleomycin challenge and the mice were sacrificed and evaluated on day 14. A, schematic illustration of the protocol used in this evaluation. B, the silencing effects on the expression of AR, CTGF, and genes associated with fibrosis evaluated by qRT-PCR. C, H &E stains of lungs from mice treated with vehicle (PBS), bleomycin (BLM), bleomycin and control (BLM+SAM-Con), AR (BLM-SAM-AR), and CTGF (BLM-SAM-CTGF) SAMiRNAs. D, Representative Mallory-
trichrome staining of the lungs from these mice. Values in panel B are mean ± SEM of a minimum of 5 mice each group. Panel C is a representative of a minimum of 5 mice each group. ##p<0.01, ###p<0.001 compared to vehicle (PBS) control; **p<0.01, ***p<0.001 compared to bleomycin-challenged mice treated with control SAMiRNA (BLM+SAM-Cont). All scale bars are 400 µm.

Figure 7. *In vivo* silencing of bleomycin model of pulmonary fibrosis AR and CTGF by SAMiRNAs *via* i.v delivery. 8 weeks old WT mice were challenged with bleomycin (BLM) and treated with control AR and CTGF SAMiRNAs (3mg/kg) were delivered via i.v. injection on days 7 and 9 after bleomycin challenge then the mice were sacrificed and evaluated on day 14. A, collagen content is the lungs from these mice evaluated by Sircol collagen assay. B, Mallory-trichrome staining of lungs treated with vehicle (PBS), bleomycin (BLM), bleomycin and control (BLM+SAM-Cont), AR (BLM+SAM-AR), CTGF (BLM+SAM-CTGF) SAMiRNAs, and CTGF+AR combination (BLM+SAM-Combo). Values in panel A are mean±SEM. ##p<0.01 compared to PBS control, *p<0.05 compared to BLM-SAM-Cont. All scale bars are 400 µm.

Figure 8. *In vivo* silencing of AR and CTGF SAMiRNAs significantly suppress TGF-β-stimulated collagen accumulation in the lung and restore the lung function TGF-β Tg mice. 8 weeks old WT and TGF-β Tg mice were treated with control, AR and CTGF and SAMiRNAs alone or together via i.v. injection (5mg/kg, each) on day 7, 9 and 11 after transgene induction with doxycycline (Dox). A, schematic illustration of the protocol used in this evaluation. B, Total collagen quantitation in the lung by Sricol assay. C, The silencing effects on the expression of AR, CTGF, and genes associated with fibrosis evaluated by qRT-PCR. D, Representative Mallory-Trichrome staining of the lung from WT and TGF-β Tg mice challenged with control (SAM-Cont), AR (SAM-AR), CTGF (SAM-CTGF), AR+CTGF (SAM-Combo) SAMiRNAs. Values in panels B and C represent the mean ± SEM of a minimum of 5 mice each group. Panel D is a representative of a minimum of 5 mice each group. ##p<0.01 compared to WT mice; *p<0.05, **p<0.01 compared to TGF-β Tg mice treated with control SAMiRNA (SAM-Cont). Scale bar in panel C, 500 µm.

Figure 9. *In vivo* silencing of AR and CTGF SAMiRNAs restores the lung function of TGF-β Tg mice. 8 weeks old WT and TGF-β Tg mice were treated with control, AR and CTGF and SAMiRNAs alone or together via i.v. injection (5mg/kg, each) on day 7, 9 and 11 after transgene induction with doxycycline (Dox). Lung functions were measured by Flexivent system. A, Respiratory system resistance (Rrs). B, Respiratory system elastance (Ers). C, Central airway resistance (Rn). D, Respiratory system compliance (Crs). Values represent the mean ± SEM of a minimum of 5 mice each group. #p<0.05, ##p<0.01; *p<0.05, **p<0.01 compared to TGF-β Tg mice treated with control SAMiRNA (SAM-Cont). Scale bar in panel C, 500 µm.
Figure 1

A

Hydrophilic polymer/
Lipid bi-conjugated siRNA
Lipid
siRNA
Hydrophilic polymer

B

C

D

E

SAMiRNA-AR Stability
SAMiRNA-CTGF Stability

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Figure 3

A

|              | WT mice | BLM-induced mice | TGF-β tg mice |
|--------------|---------|------------------|---------------|
| Non-treated  | Liver   | Lung             | Spleen        |
|              | Liver   | Lung             | Spleen        |
| 12hr         | Liver   | Lung             | Spleen        |
| 24hr         | Liver   | Lung             | Spleen        |
| 48hr         | Liver   | Lung             | Spleen        |

B

|              | WT mice | BLM-induced mice | TGF-βtg mice |
|--------------|---------|------------------|---------------|
| Non-treated  | Liver   | Lung             | Spleen        |
|              | Liver   | Lung             | Spleen        |
| 12hr         | Liver   | Lung             | Spleen        |
| 24hr         | Liver   | Lung             | Spleen        |
| 48hr         | Liver   | Lung             | Spleen        |

C

- **Total Cells**
- **CC10**
- **SPC**

- **CD 140a**
- **CD 68**
- **CD 3**

Bar graphs showing the percentage of Cy5.5+ cells.
Figure 4

A

![Graph showing siRNA concentration (ng/ml) vs. hours after administration for i.t. and i.v. administrations.](image)

| SAMiRNA-AR | Administration route | I.T. | I.V. |
|------------|----------------------|------|------|
| Dose (mg/kg) | 3                   | 5    |      |
| AUC_{0-24} (hr*ng/ml) | 3164.283 | 6076.164 | 
| Cmax (ng/ml) | 980.688          | 6341.65 | 
| Tmax (hr) | 5.134            | 4.318 | 
| T1/2 (hr) | 4.331            | 4.475 | 

B

![Graph showing siRNA concentration (ng/ml) vs. hours after administration for i.t. and i.v. administrations.](image)

| SAMiRNA-CTGF | Administration route | I.T. | I.V. |
|--------------|----------------------|------|------|
| Dose (mg/kg) | 3                   | 5    |      |
| AUC_{0-24} (hr*ng/ml) | 97.266         | 1029.77 | 
| Cmax (ng/ml) | 19.171           | 1029.828 | 
| Tmax (hr) | 1                  | 1812.203 | 
| T1/2 (hr) | 4.331            | 4.475 | 

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Figure 5

A. CTGF

B. AR

C. Collagen 3A1

D. Fibronectin

E. Elastin

F. Collagen Contents

Day

Soluble Collagen (ug/g)

Relative mRNA expression (%)

Relative mRNA expression (%)

Relative mRNA expression (%)

Relative mRNA expression (%)

Control mice
BLM-induced mice
Figure 6

A

BLM

0 7 9 14 (days)

SAMiRNA, i.t. Sacrifice

B

AR  CTGF  Collagen 3A1  Fibronecin

mRNA expression levels (%)

PBS  BLM+PBS  BLM+SAM-Cont  BLM+SAM-AR  BLM+SAM-CTGF

C

PBS  BLM+PBS  BLM+SAM-Cont  BLM+SAM-AR  BLM+SAM-CTGF

D

PBS  BLM+PBS  BLM+SAM-Cont  BLM+SAM-AR  BLM+SAM-CTGF
Figure 7

A

Soluble Collagen ug/g

PBS  BLM+SAM-Cont  BLM+SAM-CTGF  BLM+SAM-AR  BLM+SAM-Combo

B

PBS  BLM+PBS  BLM+SAM-Cont

BLM+SAM-CTGF  BLM+SAM-AR  BLM+SAM-Combo
Figure 8

A

Dox water

0 7 9 11 13

WT TGF-β Tg

SAMiRNAs, i.v. Sacrifice

B

AR

WT SAM-Cont SAM-AR TGF-β TG

CTGF

WT SAM-Cont SAM-CTGF TGF-β TG

Col1a1

WT SAM-Cont SAM-AR SAM-CTGF SAM-Combo TGF-β TG

Fibronectin

WT SAM-Cont SAM-AR SAM-CTGF SAM-Combo TGF-β TG

C

Soluble Collagen (ng/mg)

WT SAM-Cont SAM-AR SAM-CTGF SAM-Combo TGF-β TG

D

WT SAM-Cont SAM-AR SAM-CTGF SAM-Combo TGF-β TG
Figure 9

A

B

C

D

WT SAM-Cont SAM-AR SAM-CTGF SAM-Combo

TGF-β TG

Rrs (cm H20.s/mL)

60.00

50.00

40.00

30.00

20.00

10.00

0.00

WT SAM-Cont SAM-AR SAM-CTGF SAM-Combo

TGF-β TG

Ers (cm H20/mL)

60.00

50.00

40.00

30.00

20.00

10.00

0.00

WT SAM-Cont SAM-AR SAM-CTGF SAM-Combo

TGF-β TG

Rn (cm H20.s/mL)

60.00

50.00

40.00

30.00

20.00

10.00

0.00

WT SAM-Cont SAM-AR SAM-CTGF SAM-Combo

TGF-β TG

Crs (cm H20/mL)

60.00

50.00

40.00

30.00

20.00

10.00

0.00

WT SAM-Cont SAM-AR SAM-CTGF SAM-Combo

TGF-β TG

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Self-assembled Micelle Interfering RNA for Effective and Safe Targeting of Dysregulated Genes in Pulmonary Fibrosis

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