Exosome-derived microRNA-22 ameliorates pulmonary fibrosis by regulating fibroblast-to-myofibroblast differentiation both in vitro and in vivo

Running title: miR-22 in myofibroblast differentiation

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Abstract

Background

Although aberrant proliferation and activation of lung fibroblasts are implicated in the initiation and progression of idiopathic pulmonary fibrosis (IPF), the underlying mechanisms are not well characterized. Numerous microRNAs (miRNAs) have been implicated in this process; however, miRNAs derived from exosomes and their relevance to fibroblast-to-myofibroblast differentiation have not been fully elucidated. In this study, we aimed to identify exosome-derived miRNAs relevant to fibrosis development.

Methods

We profiled exosome-derived miRNAs expression in sera of C57BL/6 mice exhibiting bleomycin-induced pulmonary fibrosis by miRNA array analysis. After validating a selected miRNA by quantitative reverse-transcription polymerase chain reaction, its effect on fibroblast-to-myofibroblast differentiation was investigated using human lung fibroblasts. Furthermore, we determined the role of the selected miRNA in an in-vivo pulmonary fibrosis model.

Results

MiRNA array analysis revealed that miR-22 expression was increased by up to 2 fold on day 7 after bleomycin treatment compared with that in vehicle-treated mice. In vitro, miR-22 transfection suppressed TGF-β1-induced α-SMA expression. This was mediated via the
inhibition of the ERK1/2 pathway. Baseline α-SMA expression was increased upon miR-22 inhibitor transfection. Furthermore, miR-22 negatively regulated connective tissue growth factor expression in the presence of TGF-β1. In vivo, administration of a miR-22 mimic on day 10 after bleomycin challenge ameliorated pulmonary fibrosis lesions accompanied by decreased α-SMA expression in the model mice.

Conclusions

Exosomal miR-22 modulates fibroblast-to-myofibroblast differentiation. The present study warrants further investigations to shed light on miR-22 as a novel therapeutic target for patients with IPF.

Key words

Idiopathic pulmonary fibrosis, exosome, microRNA, myofibroblast differentiation
Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating lung disorder with poor prognosis\(^1\). The pathogenesis of IPF is not fully understood; however, the formation of fibroblastic foci and excessive deposition of extracellular matrix proteins are regarded as factors that directly cause IPF\(^2\). Although disease progression is variable and unpredictable, the median survival time from diagnosis is estimated to be 2–4 years\(^3,4\). As available therapeutics are limited\(^5,6\), the development of novel therapeutic strategies is urgently needed.

MicroRNAs (miRNAs) are small, non-coding, single-stranded RNA molecules that mediate mRNA cleavage, translational repression, and mRNA destabilization\(^7\). Emerging evidence indicates the involvement of miRNAs in the development of organ fibrosis\(^8\). In patients with IPF, data that suggest significant changes in the expression of miRNAs, including \(miR-21\)^9,10, \(Let-7d\)^11, and \(miR-200\)^12, have been accumulating. Recently, Liu et al.\(^13\) showed that decreased expression of \(miR-30a\) might be associated with IPF progression. Moreover, Ren et al.\(^14\) indicated that \(miR-541\) might play a key role in the formation of lung fibrosis. MiRNAs are encapsulated in exosomes, which are 40–100-nm vesicles that are released from several cell types participating in intercellular communication\(^15\).

Although multiple classes of cells are involved in the pathogenesis of IPF, lung fibroblast-to-myofibroblast differentiation is a key event in the initiation and progression of the
Among numerous growth factors, the transforming growth factor-β1 (TGF-β1) signaling pathway is the main cascade implicated in myofibroblast differentiation. After binding to its receptor, TGF-β1 induces numerous events, including extracellular matrix production and α-smooth muscle actin (α-SMA) expression, which are signaled in a Smad-dependent or -independent manner. Kang recently summarized several miRNAs involved in the TGF-β signaling-mediated lung fibrosis, and miR-101, miR-9-5p, miR-1343, and miR-27b have been found to interfere with fibroblast-to-myofibroblast differentiation. Similarly, miR-27a has been proven to repress myofibroblast differentiation.

In this study, we aimed to identify exosome-derived miRNAs that are relevant to fibroblast-to-myofibroblast differentiation. To achieve this, we utilized a well-characterized bleomycin (BLM)-induced murine pulmonary fibrosis model, and exosome-derived miRNAs were subjected to a miRNA array analysis to characterize time-dependent changes in miRNA expression within exosomes in the serum.

Materials and Methods

Animals and BLM treatment

In the present study, we used 12-week-old male SPF C57BL/6 mice (Charles River Laboratories, Japan, Yokohama, Japan). Osmotic pumps (ALZET, model 2001; DURECT Corporation,
Cupertino, CA, USA) containing 200 μL of saline with or without BLM (100 mg/kg body weight; Nippon Kayaku Co., Tokyo, Japan) were implanted subcutaneously through a small incision in the back according to the manufacturer’s instructions on day 0 (Fig. 1A) \(^{24, 25}\). Incision wounds were sealed with a surgical suture. BLM was continuously infused via the pumps for 7 days, according to the manufacturer’s instructions. The experimental protocols were approved (approval number, 29-056) by the Animal Care and Use Committee of the Nippon Medical School, Tokyo, Japan.

**Exosome extraction and miRNA array analysis**

Exosomes were extracted using ExoQuick™ (System Biosciences, LLC, Palo Alto, CA, USA) from mouse sera on day 0 before BLM-challenge, and on days 7, 14, 21, and 28 after BLM challenge (Fig. 1A). Briefly, ExoQuick™ Exosome Precipitation Solution was added to the serum, which was then refrigerated according to the manufacturer’s protocol. The ExoQuick/biofluid mixture was centrifuged at 1500 × g at 4 °C for 30 min. After centrifugation, the exosomes appeared as a beige or white pellet and were resuspended in sterile water. Exosome presence and purity were confirmed by western blot analysis (see below) of the lysates using anti-CD63, CD81, and TSG101 antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). MiRNAs within exosomes were extracted using the mirVana™ miRNA Isolation Kit
(Life Technologies, Carlsbad, CA, USA) and were analyzed using the 3D-GENE® miRNA oligo chip (Toray Industries Inc., Tokyo, Japan), according to the manufacturer’s protocol.

**Quantitative reverse-transcription (qRT)-PCR analysis of miRNA expression**

The isolated miRNAs were converted to complementary DNA. The expression of miR-22 and snoRNA423 (a constitutively expressed “housekeeping” gene used as a control) was quantified by qRT-PCR using the TaqMan Gene Expression Assay Kit (Applied Biosystems Japan, Ltd., Tokyo, Japan), the THUNDERBIRD Probe qPCR Mix (Toyobo, Osaka, Japan), and the 7500 Fast Real-Time PCR System (Applied Biosystems). MiR-22 expression was normalized to that of snoRNA423.

**Cell culture**

Human fetal lung fibroblast (HFL-1) cells (RCB0521, Riken BRC Cell Bank, Ibaraki, Japan) were maintained in 75-cm² Nunc EasyFlask cell-culture flasks (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal calf serum, 100 µg/mL penicillin, and 250 µg/mL streptomycin sulfate (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at 37 °C, with 5% CO₂, in a humidified atmosphere. Subconfluent cells (passage 14–18) were treated with miR-22 mimic,
miR-22 inhibitor (see below), and/or TGF-β1 (5 ng/mL; R&D Systems Inc., Minneapolis, MN, USA).

**MiRNA mimic or inhibitor transfection**

MiR-22 mimic, miR-22 inhibitor, or a negative control oligonucleotide consisting of a random sequence of bases (Ambion, Foster City, CA, USA) was transiently transfected into HFL-1 cells using Lipofectamine® iMAX (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions.

**Western blot analysis**

Cells from subconfluent cultures were washed twice with PBS, scraped into PBS, and pelleted via centrifugation. Whole-cell lysates were prepared in Radio-Immunoprecipitation Assay (RIPA) buffer (FUJIFILM Wako Pure Chemical Corporation) for immunoblotting. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc.), according to the manufacturer’s instructions, using bovine serum albumin as the standard. Samples were resolved via sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% skim milk in
Tris-buffered saline (0.15 M NaCl and 0.05 M Tris-HCl [pH 8.0], and 0.05% [v/v] Tween 20) and incubated with primary antibodies at the manufacturer’s recommended dilutions. Primary antibodies against extracellular-signal-regulated protein kinase (ERK)1/2, phospho (p-)ERK1/2, (p-)Smad2, (p-)Smad3, and TGFβRI were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). A mouse monoclonal anti-α-SMA antibody was purchased from Dako (Glostrup, Denmark). A mouse monoclonal anti-β-actin antibody (clone AC-74) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and they were used as the loading and transfer controls. After incubation with either anti-mouse IgG2b HRP-conjugated secondary antibody (SouthernBiotech, Birmingham, AL, USA) or anti-rabbit IgG HRP-conjugated secondary antibody (SouthernBiotech), protein bands were detected using the ImmunoStar® LD system (FUJIFILM Wako Pure Chemical Corporation), according to the manufacturer’s instructions, and were photographed using an Amersham Imager 600 (GE Healthcare Life Sciences, Marlborough, MA, USA). The bound antibodies were then removed by incubating the membranes in Restore Western Blot Stripping Solution (FUJIFILM Wako Pure Chemical Corporation), according to the manufacturer’s protocol, and then reprobed. Protein band intensity was quantified via densitometry using ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA).
**Immunocytochemistry**

Changes in α-SMA protein expression were visualized by immunofluorescence as previously described\(^2^6\). Briefly, HFL-1 cells were seeded in a 12-well plate (Thermo Fisher Scientific Inc.), and after 48 h of transfection with miR-22 mimic (50 nM), the cells were treated with TGF-β1 (5 ng/mL) for another 72 h. The cells were then washed with PBS and fixed in 4% paraformaldehyde at room temperature for 30 min. Subsequently, the cells were blocked with 3% goat serum at room temperature for 30 min, and incubated for 10 min with PBS containing 0.1% TritonX™-100 (Sigma-Aldrich Japan, Tokyo, Japan) before stained with anti-human α-SMA antibody (Dako) at 4 °C overnight. Thereafter, the cells were incubated with secondary biotin-conjugated anti-rabbit IgG antibody at room temperature for 1 h and stained with Alexa Fluor® 488-conjugated streptavidin (Life Technologies corporation, CA, USA) at room temperature for 1 h. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Finally, the sections were observed using a fluorescence microscope BZ-X800 (Keyence, Osaka, Japan).

**Small interfering RNA (siRNA) transfection**

SiRNA targeting connective tissue growth factor (CTGF) and non-targeting control, which were
synthesized by Thermo Fisher Scientific, and were transfected into cells at a final concentration of 50 nM with Lipofectamine® RNAiMAX (Thermo Fisher Scientific) 48 h after cell seeding, according to the manufacturer’s instructions. Then, cells were incubated at 37°C for 48 hours.

**RNA extraction from lung fibroblasts and qRT-PCR**

Total RNA was extracted from cultured HFL-1 cells, using ISOGEN reagents with spin columns (Nippon Gene, Tokyo, Japan), and converted to complementary DNA. CTGF and GAPDH mRNA expression was measured by qRT-PCR using the TaqMan Gene Expression Assay (Applied Biosystems Japan, Ltd.) and THUNDERBIRD Probe qPCR Mix on an Applied Biosystems 7500 Fast Real-Time PCR System. The relative amounts of CTGF mRNA were normalized to GAPDH mRNA expression levels.

**Administration of a miR-22 mimic to mice exhibiting BLM-induced pulmonary fibrosis**

Either a miR-22 mimic or scrambled RNA (1 nmol/mouse) (GeneDesign Inc., Osaka, Japan) was administered intravenously via the tail vein on day 10 after BLM-challenge.

Mice were sacrificed on day 28, and the lungs were removed and subjected to histological and biochemical analyses.
Histological examination of pulmonary tissues

Lung samples were fixed in 10% formalin buffer (FUJIFILM Wako Pure Chemical Corporation) and embedded in paraffin. Paraffin sections were cut at 3 µm thickness, stained with hematoxylin and eosin (H&E) and Masson’s trichrome stain to assess gross morphology and collagen deposition, respectively, and examined by microscopy. Lung fibrosis was measured by quantitative histology using Ashcroft’s method\textsuperscript{27}.

Evaluation of lung fibrosis with collagen measurement

Total lung collagen was determined using a Sircol Collagen Assay kit (Biocolor Ltd., Carrickfergus, Norther Ireland, UK) as per the manufacturer’s instructions. Briefly, lungs were harvested on day 28 and homogenized in 0.5 M acetic acid (50 volumes to wet lung weight) containing approximately 1 mg pepsin/10 mg tissue residue. Each sample was incubated for 24 h at room temperature with stirring. After centrifugation, 100 µL of each supernatant was assayed. One millilitre of Sircol dye reagent, which binds to collagen, was added to each sample and mixed for 30 min. After centrifugation, the pellets were suspended in 1 mL of the alkali reagent included in the kit and read at 540 nm using a spectrophotometer. Collagen standard solutions were used to construct a standard curve. Collagens contain approximately 14% hydroxyproline by weight, and the collagen values obtained with this method correlated well
with the hydroxyproline content reported in the manufacturer’s data.

**Immunohistochemistry for α-SMA**

Sections of paraffin-embedded lung lobes were deparaffinized and rehydrated. Antigen retrieval was achieved by boiling at 105 °C for 7 min in 10 mM citrate buffer (pH 6.0), followed by gradual cooling to room temperature. Then, the sections were treated with 3% hydrogen peroxide in methanol for 20 min and blocked with 10% normal goat serum (NICHIREI BIOSCIENCES, INC., Tokyo, Japan) at room temperature for 20 min. Sections were incubated with an anti-α-SMA antibody (Abcam, Cambridge, MA, USA) for 1 h at room temperature. For α-SMA staining, tissue sections were incubated with a secondary anti-rabbit antibody, Histofine Simple Stain Mouse MAX-PO(R) (NICHIREI BIOSCIENCES, INC.), for 30 min at room temperature. Then, an ImmPACT DAB Peroxidase Substrate Kit (VECTOR, Burlingame, CA, USA) was used to visualize α-SMA expression and counter stained with hematoxylin.

**Statistical analysis**

Comparisons between multiple groups were made using a one-way analysis of variance, and Tukey–Kramer post-hoc correction to adjust for multiple comparisons. An unpaired two-tailed Student’s *t*-test was used for all single comparisons. The data were analyzed using JMP 9.
software, version 9.0.3 (SAS Institute Inc., Cary, NC, USA), and expressed as mean ± standard deviation. Results with $P$ value < 0.05 were considered statistically significant.

**Results**

**Tentative upregulation of exosomal miR-22 in sera from mice with BLM-induced pulmonary fibrosis**

As an initial assessment, we screened for exosome-derived miRNAs implicated in lung fibrosis by observing time-dependent changes in exosomal miRNAs during the course of BLM-induced murine pulmonary fibrosis. To this end, we first isolated exosomes from sera of mice with BLM-induced pulmonary fibrosis (Fig. 1A). Exosomes were characterized at the protein level by the presence of CD63, CD81, and TSG101 (Fig. 1B). Then, we extracted miRNAs from the exosomes and hybridized them to a miRNA array containing 1900 miRNAs. As shown in Fig. 1C, we successfully identified ten 2-digit miRNAs that exhibited the highest degree of up- or downregulation. In this panel, miR-22 was the third most strongly upregulated miRNA after BLM challenge; however, its effect on myofibroblast differentiation in the lungs has not been determined. Therefore, we focused on this miRNA in subsequent analyses. Validation analysis of miR-22 expression by qRT-PCR revealed that its expression in exosomes derived from BLM-treated mouse sera was upregulated by up to 2-fold on day 7 when
compared with that of control mice, and declined thereafter (Fig. 1D).

**MiR-22 mimic suppresses TGF-β1-induced α-SMA expression in human lung fibroblasts in vitro**

Fibroblast-to-myofibroblast differentiation is induced upon TGF-β1 treatment, and is characterized by α-SMA expression\(^{18,19}\). The inhibitory effect of miR-22 on myofibroblast differentiation has been shown using cardiac fibroblasts isolated from rats\(^{28}\); however, the effect of miR-22 has not been determined in human lung fibroblasts. Therefore, we transfected HFL-1 cells with miR-22 mimic before the addition of TGF-β1 and then evaluated α-SMA expression by western blot analysis. As shown in Fig. 2A, TGF-β1-induced α-SMA expression was significantly attenuated by the miR-22 mimic. To further confirm this result, we performed immunofluorescence staining of α-SMA. As shown in Fig. 2B, TGF-β1-induced cytosolic α-SMA expression (green) was ameliorated by miR-22 transfection. Next, we evaluated whether miR-22 inhibitor would affect α-SMA expression. As shown in Fig. 2C, miR-22 inhibitor slightly enhanced α-SMA expression under normal physiological condition.

**MiR-22 modulates the ERK signaling pathway in human lung fibroblasts**

Hong *et al.*\(^{28}\) demonstrated that miR-22 affects TGF-β1 signaling by interfering with
TGFβRI expression. Therefore, we probed whether miR-22 inhibits TGFβRI expression in human lung fibroblasts. However, the results showed that miR-22 had no effect on TGFβRI expression (data not shown). Fierro-Fernandez et al.\textsuperscript{21} reported that miR-9-5p inhibits TGF-β1 signaling by regulating Smad2 phosphorylation; thus, we next assessed whether miR-22 inhibits Smad signaling. Again, the results indicated this was not the case (data not shown). As Midgley \textit{et al.}\textsuperscript{29} reported that TGF-β1-induced myofibroblast differentiation is mediated via ERK1/2, we examined whether this pathway is a target of miR-22. As shown in Fig. 3, western blot analysis revealed that miR-22 decreased TGF-β1-induced ERK1/2 phosphorylation at the indicated time points.

\textbf{MiR-22 mimic modulates myofibroblast differentiation via regulation of CTGF}

CTGF is known to upregulate α-SMA expression in human lung fibroblasts\textsuperscript{30}. In support of this finding, we observed that siCTGF attenuated both baseline and TGF-β1-induced α-SMA expression (Fig. 4A). To assess whether miR-22 affects CTGF expression, we transfected HFL-1 cells with miR-22 mimic and investigated changes in TGF-β1-induced \textit{CTGF} mRNA expression. As shown in Fig. 4B, the miR-22 mimic inhibited \textit{CTGF} mRNA expression in the presence of TGF-β1.
Effect of miR-22 mimic on BLM-induced murine pulmonary fibrosis

The above results prompted us to hypothesize that inhibition of the fibroblast differentiation by replenishing miR-22 would prevent the development of pulmonary fibrosis. To test this, we intravenously administered miR-22 mimic to mice with BLM-induced pulmonary fibrosis via the tail vein on day 10 after BLM challenge. On day 28, the mice were sacrificed, and the lungs were removed and subjected to immunohistochemical and biochemical analyses (Fig. 5A). Lung histological data revealed focal fibroplasias with destruction of the alveolar wall in the group receiving BLM (Fig. 5B and C). Injection of the miR-22 mimic on day 10 ameliorated the lesions (Fig. 5D and E). To quantify the antifibrotic effects of miR-22 in the lungs of BLM-treated mice, we determined the extent of lung fibrosis by quantitative histology according to Ashcroft’s method on day 28 post treatment. Because BLM administration via osmotic pumps causes lung fibrosis predominantly in the subpleural regions, subpleural fibrosis between the groups was compared using a numerical scale. Two blinded observers [NK and MI] quantified fibrosis in each section. Fibrosis scores were significantly lower in mice that had received the miR-22 mimic on day 10 (Fig. 5F, *P < 0.05). As shown in Fig. 5G, collagen content in the lungs was attenuated when the miR-22 mimic was infused to BLM-treated mice. We further examined whether α-SMA expression in the lungs of BLM-treated mice was altered by miR-22 mimic administration on day 10 post BLM challenge using immunohistochemical
staining. Increased immunoreactivity for α-SMA was observed in fibrotic regions in BLM-treated mice (Fig. 5H), whereas this increase was attenuated upon miR-22 injection, along with amelioration of fibrosis (Fig. 5I).

Discussion

In the present study, we observed a robust increase in the expression of exosomal miR-22 in sera of mice with BLM-induced pulmonary fibrosis on day 7 post BLM challenge. In vitro, transfection of a miR-22 mimic into human lung fibroblasts significantly inhibited TGF-β1-induced α-SMA expression. Conversely, TGF-β1-induced α-SMA protein expression was increased upon transfection of a miR-22 inhibitor. The effect of the miR-22 mimic was proven to be mediated via TGF-β1-induced p-ERK1/2 inhibition. Furthermore, we demonstrated that miR-22 inhibited CTGF expression, which could lead to the suppression of CTGF-induced α-SMA expression. In vivo, injection of the miR-22 mimic into mice with BLM-induced pulmonary fibrosis ameliorated the lesions, accompanied by a decrease in α-SMA expression in the lungs. These results delineated a novel regulatory mechanism involved in fibroblast-to-myofibroblast differentiation in response to miRNA.

Interestingly, although we demonstrated that miR-22 expression was upregulated in exosomes derived from mouse sera after BLM challenge, several studies have reported
downregulation of miRNAs, including miR-101\textsuperscript{20} and miR-27b\textsuperscript{23}, after BLM administration. Differences in study design could account for the inconsistency; we detected upregulation of miR-22 expression in serum-derived exosomes, whereas miR-101 and miR-27b were found to be downregulated in the lungs of BLM-treated mice. The precise origin of the exosomes detected in this study has yet to be defined; however, they are likely to have been secreted in some tissue other than the lungs and to have been delivered to the lungs, whose components are decreased as a result of BLM administration. Furthermore, the approach used to establish the BLM-induced pulmonary fibrosis model likely affected the results; we used osmotic pumps to induce pulmonary fibrosis because lung fibrosis is observed in the subpleural regions and the distribution of lesions is similar to that observed in human IPF, whereas Huang \textit{et al.}\textsuperscript{20} and Zeng \textit{et al.}\textsuperscript{23} adopted intranasal administration of BLM in their studies on miR-101 and miR-27b, respectively. Investigating miR-22 expression in the lungs using the same model could help understand the roles of this miRNA and exosomes.

Exosomes are secreted by most cell types via exocytosis, containing and transferring various biomolecules, such as DNA, RNA, proteins, and lipids\textsuperscript{33, 34}. Studies on the relevance of exosomes in the pathogenesis of IPF have yielded contradictory results. Tan \textit{et al.}\textsuperscript{35} reported that exosomes derived from human amnion epithelial cells ameliorated experimental lung fibrosis, whereas Martin-Medina \textit{et al.}\textsuperscript{36} showed that WNT-5A-carrying exosomes derived from
bronchoalveolar lavage fluid from experimental fibrotic lungs and patients with IPF accelerated
disease progression. Thus, it would be of interest to further explore the effects of miR-22 -carrying exosomes on BLM-induced pulmonary fibrosis.

In this study, we observed that miR-22 interferes with the ERK1/2 signaling pathway, resulting in the amelioration of TGF-β1-induced α-SMA expression. However, it is important to note that Hong et al.\textsuperscript{28} demonstrated that in rat cardiac fibroblasts, miR-22 inhibits myofibroblast differentiation by modulating TGFβRI expression. The present study was designed to use fibroblasts from human lungs, while Hong et al. observed the effect of miR-22 in cardiac fibroblasts from rats. Fibroblasts are heterogeneous, and the different sources of fibroblasts used in their and our studies might, at least in part, account for the discrepant results.

In addition, the current study suggested that CTGF mRNA expression was attenuated by miR-22. CTGF plays an essential role in tissue repair and pulmonary fibrogenesis. Yang et al.\textsuperscript{30} reported that CTGF stimulated lung fibroblast differentiation \textit{in vitro} and \textit{in vivo}. In a previous report, Yang et al.\textsuperscript{37} reported that miR-18a regulated CTGF expression via the TGF-β1 signaling pathway in lung fibroblasts. Further, miR-19a, 19b, and 26b reportedly are involved in CTGF expression and pulmonary fibroblast differentiation\textsuperscript{38}. However, the association between miR-22 and CTGF expression had not been previously discussed. Therefore, this study has revealed a novel CTGF regulatory mechanism.
According to the current miRNA array results, miR-16 was the most strongly upregulated after BLM challenge. This miRNA targets the 3’ untranslated region of the mTORC2 component-encoding gene Ribcor39. Notably, we previously observed that miR-16 inhibited BLM-induced murine pulmonary fibrosis by targeting mTORC2-secreted protein acidic and rich in cysteine (SPARC) axis40. The second most strongly induced miRNA, miR-15a, was among the 161 miRNAs that were previously reported to be differentially expressed in the lungs of BLM-treated and control mice8. This study was the first to assess the role of miR-22 in the context of pulmonary fibrosis. Thereby, we focused on the function of miR-22 throughout the study, demonstrating that this miRNA regulated myofibroblast differentiation.

In conclusion, we demonstrated that exosomal miR-22 modulates fibroblast-to-myofibroblast differentiation. Further studies are warranted to investigate miR-22 as a potential novel therapeutic target for IPF.

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Figure legends

Fig. 1. Identification of important exosome-derived microRNAs (miRNAs) that might be involved in bleomycin (BLM)-induced murine pulmonary fibrosis. (A) Outline of the experimental design used for the selection of miRNA candidates using a comprehensive miRNA array-based approach. Osmotic pumps containing 200 μL of saline, with or without BLM (100 mg/kg mouse body weight), were implanted subcutaneously through a small incision in the back according to the manufacturer’s instructions. BLM was infused continuously from days 0 to 6. Exosomes were extracted using ExoQuick™ from sera of mice collected on day 0 before BLM challenge, and on days 7, 14, 21, and 28 post BLM challenge. MiRNAs extracted from the exosomes were then hybridized to the Toray 3D-Gene® miRNA array. n = 2–3 mice per time point. (B) Lysates prepared from the extracted exosomes were subjected to western blot analysis to confirm the presence of exosomes using anti-CD63, CD81, and TSG101 antibodies. (C) Heat maps displaying differential expression of miRNAs at the indicated time points after BLM challenge. The 10 most strongly up- (red) and downregulated (blue) 2-digit miRNAs were selected for further analyses. (D) Validation of exosome-derived miR-22 expression. miRNAs were extracted from exosomes and converted to cDNA. Relative expression of miR-22 and snoRNA432 was quantified by quantitative reverse-transcription PCR. The relative expression of miR-22 was normalized against that of snoRNA432, and the fold-change values relative to
that of a saline-treated group are presented. Results from two independent experiments assayed in duplicate are presented. The relative expression of exosome-derived miR-22 in BLM-treated mice was upregulated by up to 2-fold on day 7 as compared to that in the control mice.

**Fig. 2.** Effect of miR-22 mimic on TGF-β1-induced α-smooth muscle actin (α-SMA) expression in human lung fibroblasts. (A) Monolayer-cultured human fetal lung fibroblasts (HFL-1 cells) were serum-starved for 24 h before transfection of a miR-22 mimic (50 nM). The cells were cultured for 48 h with a miR-22 mimic before the addition of TGF-β1 (5 ng/mL), and further cultured for 72 h. Whole-cell lysates were prepared and subjected to western blot analysis using an antibody against α-SMA. A representative blot from three independent experiments is presented (left). α-SMA signal intensity was normalized to that of β-actin and expressed relative to that in cells treated with scrambled miRNA (right). ImageJ software was used for signal quantification. The miR-22 mimic significantly reversed TGF-β1-induced α-SMA expression. *P < 0.05 by Student’s t-test. (B) HFL-1 cells were grown in a 12-well plate, and transfected with miR-22 mimic (50 nM) for 48 h before incubation with TGF-β1 (5 ng/mL) for another 72 h. HFL-1 cells were stained with an anti-α-SMA antibody. Nuclei were counterstained with DAPI. Original magnification, 200×. (C) HFL-1 cells were cultured with a miR-22 inhibitor (50 nM) for 72 h. Whole-cell lysates were prepared and subjected to western blot analysis using an
antibody against α-SMA. A representative blot from two independent experiments is presented (left). α-SMA signal intensity was normalized to that of GAPDH and expressed relative to that in cells treated with scrambled miRNA (right). ImageJ software was used for signal quantification. The miR-22 inhibitor slightly stimulated α-SMA expression under normal physiological condition.

**Fig. 3.** MiR-22 mimic modulates TGF-β1-induced extracellular-signal-regulated kinase (ERK)1/2 signaling. Monolayer-cultured human fetal lung fibroblasts (HFL-1 cells) were serum-starved for 24 h before transfection of a miR-22 mimic (50 nM). The cells were cultured for 48 h with the miR-22 mimic before the addition of TGF-β1 (5 ng/mL). Cells were harvested at 0.5, 1.0, and 4.0 h after the addition of TGF-β1, and whole-cell lysates were subjected to western blot analysis using antibodies against ERK and phospho (p)-ERK. A representative blot from two independent experiments is presented in the upper panel. Relative intensity analysis of each band was conducted using ImageJ software. Fold-changes in p-ERK/ERK expression are presented in the lower panel. The miR-22 mimic decreased TGF-β1-induced p-ERK at each time point.

**Fig. 4.** Connective tissue growth factor (CTGF) is regulated by miR-22 in response to TGF-β1.
(A) Monolayer-cultured human fetal lung fibroblasts (HFL-1 cells) were serum-starved for 24 h before transfection of small interfering (si)CTGF (50 nM). The cells were cultured for 48 h with siCTGF before the addition of TGF-β1 (5 ng/mL) and were then cultured for another 72 h. Whole-cell lysates were prepared and subjected to western blot analysis using an antibody against α-SMA. A representative blot from three independent experiments is presented (left). α-SMA signal intensity was normalized to that of GAPDH and expressed relative to that in cells treated with scrambled siRNA (right). ImageJ software was used for signal quantification. siCTGF attenuated α-SMA expression under both normal physiological (lane 4) and TGF-β1-stimulated (lane 3) conditions. (B) HFL-1 cells cultured in monolayers were serum-starved for 24 h before being treated with the miR-22 mimic (50 nM) for 48 h. Then, the cells were treated with TGF-β1 (5 ng/mL) for another 48 h. Total cellular RNA was harvested to analyze CTGF mRNA expression by qRT-PCR. The miR-22 mimic inhibited CTGF mRNA expression in the presence of TGF-β1.

Fig. 5. MiR-22 mimic suppresses bleomycin (BLM)-induced murine pulmonary fibrosis. (A) Outline of the experimental design used for the administration of miR-22 mimic. Animals were subcutaneously implanted with osmotic pumps that contained BLM (100 mg/kg body weight) or saline vehicle. BLM was infused continuously from day 0 to 6. The miR-22 mimic (1
nmol/mouse) was injected intravenously via the tail vein on day 10 after initiating BLM treatment. On day 28 post BLM injection, the mice were sacrificed, and their lungs were collected for analyses. (B–E) Increased fibrosis and collagen deposition observed in the lungs of BLM-treated mice were attenuated by injection of the miR-22 mimic on day 10 post BLM treatment. Representative photomicrographs of H&E and Masson’s trichrome staining of the left lungs from BLM-treated mice, with or without miR-22 mimic injection. Magnification, 40×. (F) The extent of lung fibrosis was measured by quantitative histology according to Ashcroft’s method on day 28 to determine the antifibrotic effects of the miR-22 mimic in the lungs of BLM-treated mice. Because BLM administration with osmotic pumps causes lung fibrosis predominantly in the subpleural regions, subpleural fibrosis was compared between the groups using a numerical scale. Administration of the miR-22 mimic on day 10 significantly attenuated the increase in the subpleural fibrosis score induced by BLM administration. *P < 0.05 by Student’s t-test. n = 4 mice for BLM group, n = 6 mice for BLM + miR-22 mimic group. (G) The amount of collagen was quantified to assess the antifibrotic effects of the miR-22 mimic in the lungs of BLM-treated mice. Infusion of the miR-22 mimic decreased the collagen content in the lungs of BLM-treated mice. n = 4 mice for each group. (H–I) Immunohistochemical staining for α-SMA (brown) in lungs of mice with BLM-induced pulmonary fibrosis, without or with miR-22 mimic injection (H and I, respectively).
α-SMA-positive cells were markedly increased by BLM treatment (H), and this increase was attenuated by miR-22 (I).
Fig. 1

A. Implantation of osmotic pump

B. Day

| CD63  | CD81  | TSG101 |
|-------|-------|--------|
| 0     | 7     | 14     | 28     |

C. Day 0, Day 7, Day 14, Day 21, Day 28

mumu-miR-16-5p
mumu-miR-15a-5p
mumu-miR-22-3p
mumu-miR-15b-5p
mumu-miR-21a-5p
mumu-miR-25-3p
mumu-miR-23a-5p
mumu-miR-24-1-5p
mumu-miR-9-3p
mumu-miR-23a-3p
mumu-miR-23a-5p
mumu-miR-15a-3p
mumu-miR-29c-5p
mumu-miR-29b-1-5p
mumu-miR-28a-3p
mumu-miR-23b-5p
mumu-miR-28a-1-3p
mumu-miR-34a-3p
mumu-miR-34c-5
mumu-miR-21a-3p

D. mR37 (fold induction)

Day 0  Day 7  Day 14  Day 28
Fig. 2

A

\[ \text{Relative intensity} \]

\[ \alpha\text{-SMA} / \beta\text{-actin} \]

TGF-\( \beta \)1

miR-22 mimic

- - + +

B

Control

TGF-\( \beta \)1

TGF-\( \beta \)1 + miR-22 mimic

C

\[ \alpha\text{-SMA} / \text{GAPDH} \]

miR-22 inhibitor

- +
Fig. 3

![Image of Western Blot and Bar Graph]

| TGF-β1 | miR-22 mimic | 0.5 h | 1.0 h | 4.0 h |
|--------|--------------|-------|-------|-------|
| -      | -            | -     | -     | -     |
| +      | +            | -     | -     | -     |
| -      | -            | -     | -     | -     |
| +      | +            | -     | -     | -     |

**Western Blot Images:**
- p-ERK 1/2
- ERK 1/2
- β-actin

**Bar Graph:**
- Relative intensity (p-ERK/ERK)
- Bars for each condition with error bars.
Fig. 4

A

\[ \text{α-SMA} \]
\[ \text{GAPDH} \]

| TGF-β1 | siCTGF |
|-------|--------|
| -     | -      | +   | +   |

\[ \text{α-SMA/GAPDH} \]

Relative intensity

B

\[ C_{\text{TGF-β1}} / \text{GAPDH} (\text{fold induction}) \]

| NC | TGF-β1 | miR-22 mimic | TGF-β1 + miR-22 mimic |
|----|--------|--------------|----------------------|
| 1  | 2      | 1            | 1                    |

* *
Fig. 5

A study design showing the timeline and treatment groups:

- **Day 0**: Implantation of osmotic pump
- **Day 10**: Bleomycin from day 0 to 5
- **Day 28**: Sacrifice

**Study groups**
- **Group 1**: Saline
- **Group 2**: Bleomycin
- **Group 3**: Bleomycin + miR-22 mimic

**F**
Numerical score:

|         | BLM | BLM + miR-22 mimic |
|---------|-----|-------------------|
| Score   | 3   | 2                 |

**G**
Collagen content (mg/g lung weight):

|         | BLM | BLM + miR-22 mimic |
|---------|-----|-------------------|
| Content | 200 | 100               |

**B** and **C**
Images showing H&E and Masson's trichrome staining.

**D** and **E**
Additional images showing BLM and BLM + miR-22 mimic staining.

**H** and **I**
Further images showing BLM and BLM + miR-22 mimic.