Roles of microRNA-146a and microRNA-181b in regulating the secretion of tumor necrosis factor-α and interleukin-1β in silicon dioxide-induced NR8383 rat macrophages

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Abstract. Despite increasing evidence to suggest that microRNA (miR)-146a and miR-181b are involved in the regulation of immune responses and tumor progression, their roles in silicosis remain to be fully elucidated. Therefore, the present study examined the roles of miR-146a and miR-181b in inflammatory responses, and their effect on the expression of the tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) inflammatory chemokines in silicon dioxide (SiO_2)-induced NR8383 rat macrophages. Alterations in the expression levels of miR-146a and miR-181b in rats with silicosis have been previously investigated using miRNA arrays. In the present study, the expression levels of miR-146a and miR-181b were assessed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The NR8383 cells were transfected with miRNA-146a and miR-181b mimics or inhibitors, and the cells and culture supernatants were collected following SiO_2 treatment for 12 h. The expression levels of TNF-α and IL-1β were detected using western blotting, RT-qPCR and ELISA. Analysis of variance and Student's two-tailed t-test were used to perform statistical analyses. The expression level of miR-146a was significantly increased, while the expression level of miR-181b was significantly decreased in the fibrotic lungs of the rats with silicosis, compared with the levels in the normal rats. It was observed that, following treatment of the NR8383 cells with SiO_2 for 12 h, the levels of TNF-α were significantly increased following miR-181b knockdown and the levels of IL-1β were significantly increased following miR-146a knockdown, compared with the inhibitor-treated controls ($P<0.05$). By contrast, miR-181b mimic transfection led to a significant reduction in the levels of TNF-α ($P<0.05$), and miR-146a mimics were responsible for the decrease in IL-1β ($P<0.05$). The results of the present study provide evidence supporting the roles of miR-146a and miR-181b in the pathogenesis of silicosis, and suggest that they may be candidate therapeutic target in this disease.

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Introduction

Silicosis is a form of lung disease, which is caused by the inhalation and deposition of occupational dust containing silicon dioxide (SiO_2) (1). Silicosis is considered to be one of the most severe occupational diseases, with no effective treatment (2-7). Therefore, elucidation of the mechanisms underlying the development and progression of silicosis is critical in order to develop effective therapeutic methods. At present, the mechanisms of silicosis remain to be fully elucidated. Previous studies have indicated that pulmonary alveolar macrophages are pivotal in the pathological process of silicosis (8,9). Resident and recruited pulmonary macrophages have been observed to be in intimate contact with silica at the beginning of deposition and throughout the process, with the particles remaining in the lung (9). These macrophages appear to be stimulated to secrete mediator substances, which alter the function and behavior of other cells. Tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) are two mediators, of which the levels are commonly increased in airway inflammation, and are involved in the initiation and progression of silicosis (2,8,10,11).

MicroRNAs (miRNAs) are small regulatory RNAs, which control gene expression by translational suppression and destabilization of target mRNAs (12). Numerous miRNAs have

been implicated in various biological processes, including miR-125b and let-7, which are associated with cell proliferation control, miR-15a and miR-16-1 that contribute to heart disease, lung disease and oncogenesis, and miR-155 and miR17 that function as tumor suppressors or as oncogenes (13,14). There is increasing evidence that miRNAs are key regulators of genes in inflammatory responses and fibrosis of the lung (15-18). Aberrant expression of miR-146a and miR-181b have been reported to be involved in the formation of lung cancer (19,20). However, studies investigating their roles in silicosis have been limited (21,22).

In our previous study, a large-scale screen for miRNAs potentially involved in experimental silicosis in rats was performed, and it was revealed that miR-146a is upregulated and miR-181b is downregulated in response to stimulation with SiO_2 (21). However, the specific roles of these two miRNAs and the underlying mechanism of action in silicosis remain to be elucidated. Therefore, the aim of the present study was to determine the roles of miR-146a and miR-181b in inflammatory responses, and determine their effect on the expression of the TNF-α and IL-1β inflammatory chemokines in SiO_2-induced NR8383 rat macrophages.

Materials and methods

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for miRNA. All experimental procedures in the present study were approved by the Experimental Animal Ethics Committee of Sichuan University (Chengdu, China). RT-qPCR for miRNA was performed using an EpiScript™ Reverse Transcriptase kit (EpiCentre, Palmerston North, New Zealand), as reported previously (21). A total of 20 male Sprague Dawley rats (age, 5-7 weeks; weight, 160-200 g; Dashuo Center of Experimental Animals, Chengdu, China), were used for the experiments of the present study. The animals were housed in standard conditions at 22±1˚C with relative humidity of 59% and a 12 h light/dark cycle, and were provided with *ad libitum* access to food and water. The rats were randomly divided into two experimental groups: A control group (n=10) and a silicosis group (n=10). The rats were then sacrificed through loss of blood under 10% chloral hydrate anesthesia. Following sacrifice on day 40, the lungs were harvested for total RNA isolation and histological analysis. Briefly, 5 μm sections of lung tissue were obtained from the inferior lobe of the left lungs and fixed with 10% formalin (Kelon, Beijing, China), embedded in paraffin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and stained with hematoxylin and eosin (Beijing Solarbio Science & Technology Co., Ltd.) and Masson (Beijing Solarbio Science & Technology Co., Ltd.) dyes for histological examination of the collagen fibers. The tissue samples were cryopulverized using Biopulverizer™ (Biospec Products, Inc., Bartlesville, OK, USA) and homogenized using a Mini-Bead-Beater-16 (BioSpec, Shanghai Yuan Sheng Co., Ltd., Shanghai, China). Total RNA was isolated from the tissue samples using TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA) and further purified using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (600 ng), 10 μM stem-loop RT primer (Table I), 10X RT buffer, 2.5 mM each of dATP, dGTP, dCTP and dTTP (HyTest Ltd., Turku, Finland), 10 U/μl reverse transcriptase and 40 U/μl RNase inhibitor (EpiCentre) were subjected to RT reactions. PCR Master Mix (Qiagen) and 0.4 μM of each primer (Table I) were used for RT-qPCR. The reactions were conducted with the following thermocycling conditions: 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec, and 60˚C for 60 sec. Each sample was normalized to endogenous U6 RNA content. RT-qPCR was performed using a CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each experiment was performed in triplicate. The Data were analyzed using Microsoft Excel. The relative expression levels of miR-146a were evaluated by the $2^{-\Delta\Delta CT}$ method.

Cell culture, transfection and SiO_2 treatment. NR8383 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology and were cultured in Ham's F12K medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 15% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA). The NR8383 cells were seeded in triplicate into 6-well plates at a density of $1x10^5$ cells/well, and were grown to 30-50% confluence at 37˚C in an atmosphere containing 5% CO_2. Turbofect transfection reagent from Thermo Fisher Scientific (Pittsburgh, PA, USA) was used for transfection, according to the manufacturer's instructions. The miRNA mimic control, miR-146a mimic, miR-181b mimic, miRNA inhibitor control, miR-146a inhibitor and miR-181b inhibitor (Guangzhou RiboBio Co. Ltd., Guangzhou, China) were transfected at a final concentration of 25 nM. Following 24 h of transfection, >90% of the cells exhibited green fluorescence under an inverted fluorescence microscope (Nikon ECLIPSE Ti-U; Nikon, Tokyo, Japan).. The cells were then treated with 20 μg/cm^2 SiO_2 (cat. no. S5631; Sigma-Aldrich). The silica content of the quartz dust was >99%, particle size of the dust was 0.5-10 μm, and 80% of the dust particles were 1-5 μm. The cells and culture supernatants were collected following SiO_2 treatment for 12 h at 37˚C in an atmosphere containing 5% CO_2. RT-qPCR, western blotting and ELISA were then performed to detect the expression levels of TNF-α and IL-1β.

RT-qPCR for mRNA. The NR8383 cells were suspended at ~$1x10^7$ cells/well in TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and the total RNA was isolated, according to the manufacturer's instructions. Total RNA (1 μg) was subjected to reverse transcription using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio, Inc., Tokyo, Japan). SYBR® Premix Ex Taq™ II (Takara Bio, Inc.) and the following specific primers were used for RT-qPCR: TNF-α, forward 5'-CATGGATCTCAAAGACAACCAA-3' and reverse 5'-CTCCTGGTATGAAATGGCAAAT-3'; IL-1β, forward 5'-CTTCAAATCTCACAGCAGAATC-3' and reverse 5'-GCTGTCTAATGGGAACATCACA-3'; β-actin, forward 5'-GGAGATTACTGCCCTGGCTCCTA-3' and reverse 5'-GACTCATCGTACTCCTGCTTGCTG-3'. The PCR reactions were run using the following thermal cycling parameters: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 15 sec and 72˚C for 45 sec. Each experiment was performed in triplicate.

Western blot analysis. Total protein (30 μg) was extracted using a total protein extraction kit (Nanjing KeyGEN Biotech

Table I. Oligonucleotide primers used in the present study.

Primer	Primer sequence	
	Reverse transcription	Quantitative polymerase chain reaction
U6	5'-CGCTTCACGAATTTGCGTGTCAT-3'	F: 5'-GCTTCGGCAGCACATATACTAAAAT-3' R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'
Rno-miR-146a	5'-GTCTTATCCAGTGCGTGTCGTGGAGTCG GCAATTGCACTGGATACGACAACCA-3'	GSP: 5'-CAGTGCGTGTCGTGGAGT-3' R: 5'-GGGTGAGAACTGAATTCCA-3'
Rno-miR-181b	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCG GCAATTGCACTGGATACGACAGCCTAT-3'	GSP: 5'-GGGTTCAAGTAATCCAGG-3' R: 5'-TGCGTGTCGTGGAGTC'-3'

miR, microRNA; F, forward; R, reverse; GSP, gene-specific primer.

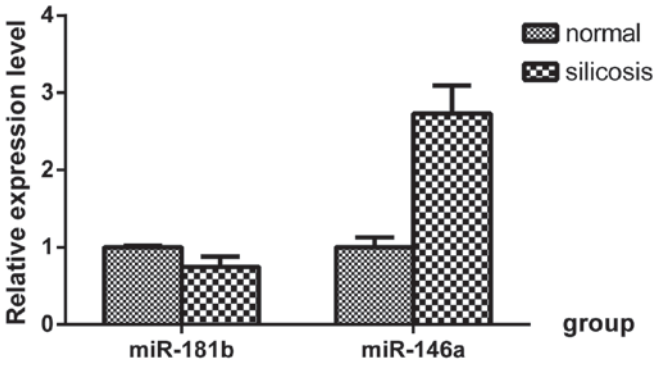

Figure 1. Expression levels of miR-146a and miR-181b in SiO_2-treated lungs. miR, microRNA.

Co., Ltd., Nanjing, China) according to the manufacturer's instructions, and loaded onto 15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) using a semi-dry blotting system (Invitrogen Life Technologies). The membranes were blocked with 5% skim milk (w/v) containing 0.2% Tween-20 (Sigma-Aldrich) for 2 h at room temperature, and then were incubated overnight with the following primary antibodies in blocking buffer at 4˚C: Polyclonal goat anti-rat TNF-α (1:300; cat. no. sc-1349; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and polyclonal goat anti-rat IL-1β (1:500; cat. no. sc-1251; Santa Cruz Biotechnology, Inc.). These were used according to the manufacturer's instructions. The membranes were washed three times for 6 min with 1X Tris-buffered saline with Tween-20 were then incubated with polyclonal rabbit anti-goat secondary antibody (1:2,000; cat no. ZB-2306; ZSGB-BIO, Beijing, China) for 1 h at 37˚C in an atmosphere containing 5% CO_2. Following final washes, the signals were detected using enhanced chemiluminescence reagents (Beyotime Institute of Biotechnology, Shanghai, China). The intensity of each signal spot was transformed into digital data, with auto-background subtraction during spot density analysis, using the Image-Pro Plus software, version 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA).

ELISA. The levels of TNF-α and IL-1β in the supernatants were determined using TNF-α and IL-1β ELISA kits (NeoBioscience Technology Co., Ltd., Beijing, China), according to the manufacturer's instructions. Briefly, the samples were diluted and added to the wells (100 μl) prior to being covered with a closure plate membrane, and incubated for 90 min at 37˚C. The closure plate membrane was removed and the liquid was carefully discarded, prior to five washes in phosphate-buffered saline containing 1% bovine serum albumin and 0.05% Tween 20, each for 30 sec. Horseradish peroxidase-conjugated anti-rat TNF-α or IL-1β polyclonal antibody (from the TNF-α and IL-1β ELISA kits; NeoBioscience Technology Co., Ltd.; 100 μl) was added to each well, except the blank well, and incubated for 60 min at 37˚C. The closure plate membrane was removed, the liquid discarded, and the plates were dried prior to further washing with washing buffer, repeated five times for 30 sec. A total of 100 μl Chromogen Solution B (NeoBioscience Technology Co., Ltd.) was added to each well, and incubated in the dark for 15 min at 37˚C. A total of 100 μl stopping solution (NeoBioscience Technology Co., Ltd.) was then added to each well to stop the reaction. Absorbance was measured at 450 nm using a Thermo Scientific Multiskan GO (Thermo Fisher Scientific, Pittsburgh, PA, USA) as compared with the blank well 15 min following the end of the reaction.

Statistical analysis. R software from the The Comprehensive R Archive Network (http://cran.r-project.org/) was used to perform all statistical analyses. A one-way analysis of variance and two-tailed Student's t-test were used to analyze the data.

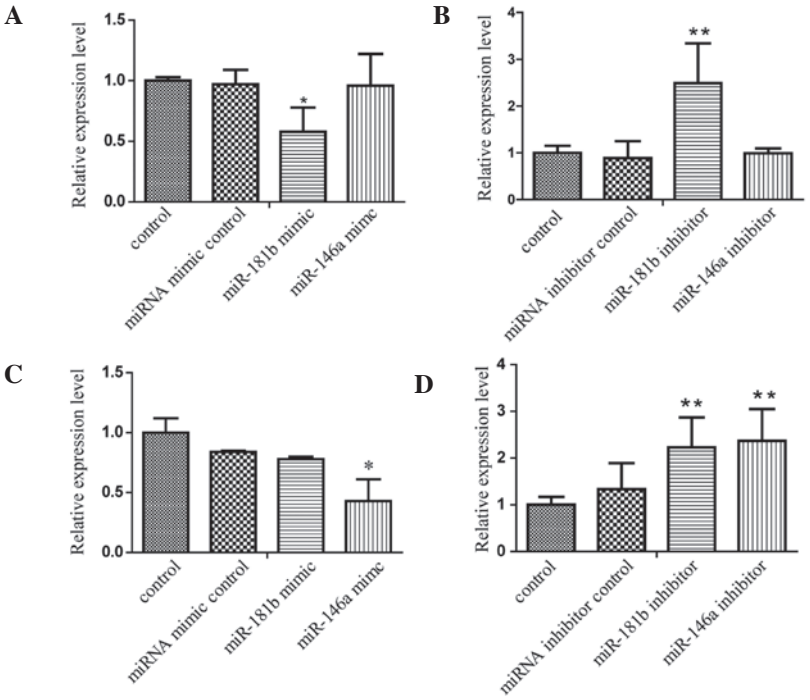

Figure 2. mRNA expression levels of TNF-α and IL-1β. (A) Effects of overexpression of miR-146a or miR-181b on the expression levels of TNF-α. (B) Effects of knockdown of miR-146a or miR-181b on the expression of TNF-α. (C) Effects of overexpression of miR-146a or miR-181b on the expression of IL-1β. (D) Effects of knockdown of miR-146a or miR-181b on the expression of IL-1β. The error bars represent the mean ± standard deviation. *P<0.05, vs. miRNA mimic control group; **P<0.05, vs. miRNA inhibitor control group. TNF-α; tumor necrosis factor-α; IL-1β, interleukin; miR, microRNA.

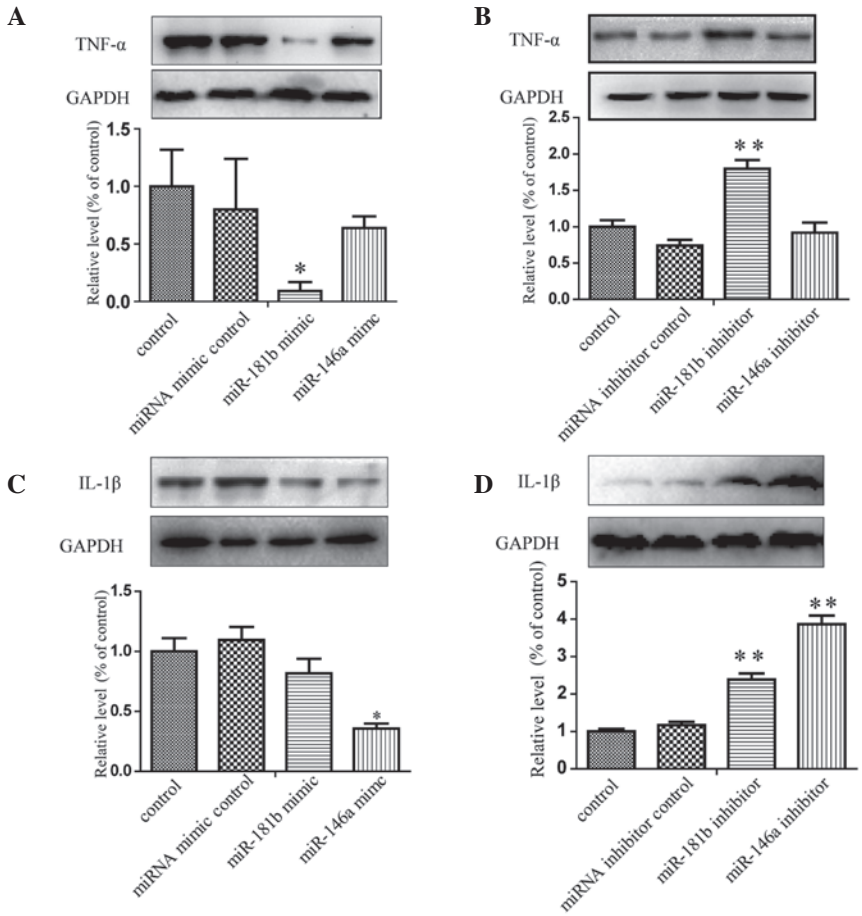

Figure 3. Protein expression levels of TNF-α and IL-1β. (A) Effects of overexpression of miR-146a or miR-181b on the expression of TNF-α. (B) Effects of knockdown of miR-146a or miR-181b on the expression of TNF-α. (C) Effects of overexpression of miR-146a or miR-181b on the expression of IL-1β. (D) Effects of knockdown of miR-146a or miR-181b on the expression of IL-1β. The error bars represent the mean ± standard deviation. *P<0.05, vs. miRNA mimic control group; **P<0.05, vs. miRNA inhibitor control group. TNF-α; tumor necrosis factor-α; IL-1β, interleukin; miR, microRNA.

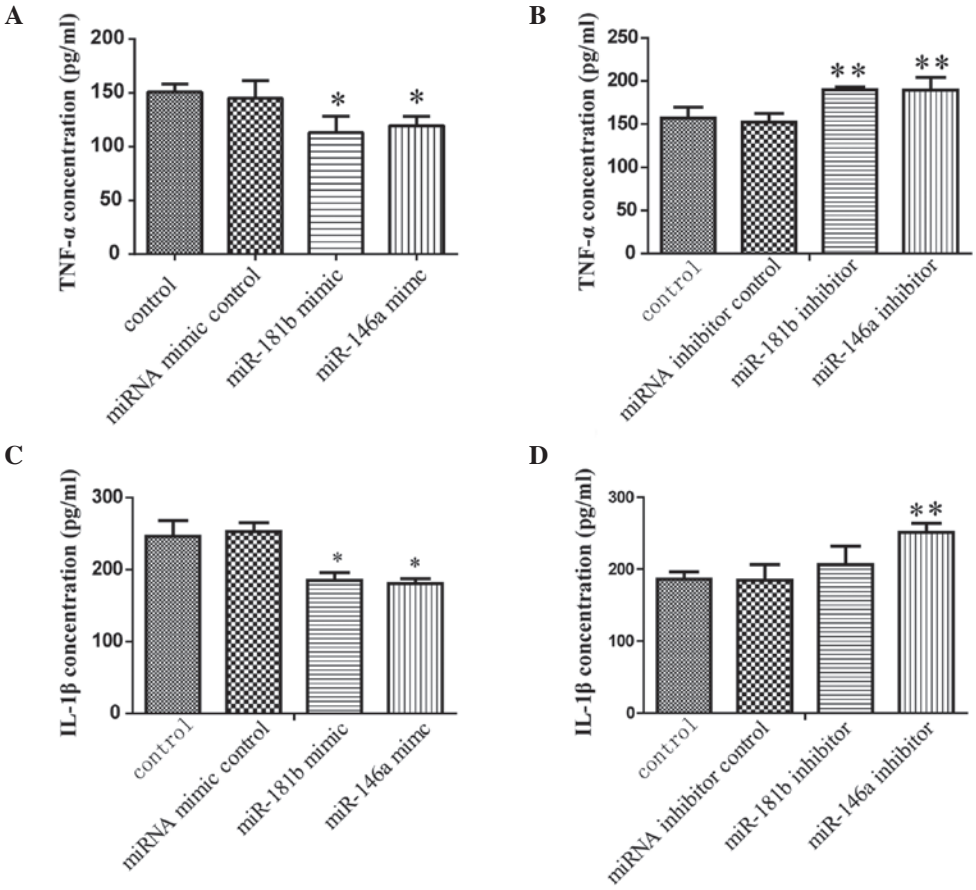

Figure 4. Expression levels of TNF-α and IL-1β, determined using ELISA. (A) Effects of overexpression of miR-146a or miR-181b on the expression of TNF-α. (B) Effects of knockdown of miR-146a or miR-181b on the expression of TNF-α. (C) Effects of overexpression of miR-146a or miR-181b on the expression of IL-1β. (D) Effects of knockdown of miR-146a or miR-181b on the expression of IL-1β. The error bars represent the mean ± standard deviation. *P<0.05, vs. miRNA mimic control group; **P<0.05, vs. miRNA inhibitor control group. TNF-α; tumor necrosis factor-α; IL-1β, interleukin; miR, microRNA.

P<0.05 (two-tailed) was considered to indicate a statistically significant difference.

Results

Expression levels of miR-146a increase and miR-181b decrease in SiO_2-treated lungs. Typical fibrosis was observed in the SiO_2-treated lungs, which involved infiltration of macrophages in alveolar spaces and increased collagen deposition (21). miRNA array analysis of the total RNA samples was performed on the day 40-SiO_2-treated lungs, and these were compared with the respective controls, as described in our previous study (21). Of 1,890 miRNAs examined, 39 miRNAs were either up- or downregulated (P<0.05; fold changes >2) in the SiO_2-treated lungs (21). In addition, the results of the RT-qPCR revealed that the expression of miR-146a was significantly increased and the expression of miR-181b was significantly decreased in the SiO_2-treated lungs (Fig. 1).

mRNA expression levels of TNF-α and IL-1β, detected using RT-qPCR, are altered by miRNA transfection. As shown in Fig. 2A, when the NR8383 cells were transfected with the miR-181b mimic for 24 h following 12 h of SiO_2 treatment, the expression of TNF-α was significantly decreased, compared with the miRNA mimic control group (P<0.05). Simultaneously, miR-181b inhibitors increased the mRNA expression of TNF-α significantly (P<0.05; Fig. 2B), whereas the miR-146a mimic and inhibitors had no significant effect on the mRNA expression levels of TNF-α (Fig. 2A and B). As shown in Fig. 2C, 12 h following SiO_2 treatment of the NR8383 cells transfected with either the miR-146a mimics, miR-181b mimics or miRNA mimic control, the miR-146a mimics significantly attenuated the expression of IL-1β (P<0.05). The expression levels of IL-1β in response to 12 h of SiO_2 treatment, subsequent to transfection with the miR-146a or miR-181b inhibitors were significantly increased, compared with those in the inhibitor control group (P<0.05; Fig. 2D).

Protein expression levels of TNF-α and IL-1β, detected using western blot analysis, are significantly altered by miRNA transfection. The results of the western blot analysis demonstrated that overexpression of miR-181b following SiO_2 treatment for 12 h significantly reduced the protein levels of TNF-α, compared with the miRNA mimic control group (P<0.05; Fig. 3A). Subsequent to transfection with miR-181b inhibitors, followed by SiO_2 treatment for 12 h, the protein levels of TNF-α were significantly upregulated (P<0.05; Fig. 3B). However, the miR-146a mimics or inhibitors had no significant effect on the protein expression levels of TNF-α. The NR8383 cells transfected with the miR-146a mimic exhibited lower expression levels of IL-1β in response to the subsequent SiO_2 stimulation (P<0.05; Fig. 3C) and IL-1β was significantly

upregulated in the NR8383 cells, which were transfected with the miR-146a or miR-181b inhibitors (P<0.05; Fig. 3D).

Expression levels of TNF-α and IL-1β, detected using ELISA, are significantly altered by miRNA transfection. When the NR8383 cells were transfected with the miR-181b mimic, the expression of TNF-α was significantly decreased in the culture supernatants, compared with the miR-181b mimic control group (P<0.05; Fig. 4A). Simultaneously, miR-181b inhibitors increased the protein levels of TNF-α significantly (P<0.05), while the miR-146a mimic and inhibitors had no effect on the protein expression of TNF-α (Fig. 4A and B). As shown in Fig. 4C, when the NR8383 cells were transfected with miR-146a or miR-181b mimics for 24 h, followed by SiO_2 treatment for 12 h, the expression levels of IL-1β were significantly decreased, compared with the mimic control group (P<0.05). The expression of IL-1β in response to SiO_2 treatment, following transfection with miR-146a or miR-181b inhibitors, was significantly increased, compared with the inhibitor control group (P<0.05; Fig. 4D).

Discussion

It has been increasingly accepted that aberrant expression levels of certain miRNAs are significantly involved in the underlying pathophysiology of lung diseases (15,17,18). However, investigations focusing on the association between alterations in miRNA expression and the formation of silicosis are limited. In order to identify miRNAs potentially involved in SiO_2-induced lung fibrosis, our previous study characterized the miRNA profile of SiO_2-treated lungs using an miRCURY™ LNA array (version 16.0; Exiqon A/S, Vedbaek, Denmark) (21). In the present study, RT-qPCR revealed that the expression of miR-146a was significantly increased, while the expression of miR-181b was significantly reduced in fibrotic lungs. In order to elucidate the underlying mechanisms, the NR8383 cells were transfected with miRNA-146a and miR-181b mimics or inhibitors, and the cells and culture supernatants were collected following SiO_2 treatment for 12 h. The results demonstrated that the mRNA and protein levels of TNF-α were significantly reduced by miR-181b mimic, and the opposite effect was observed following knockdown of miR-181b. Simultaneously, the expression mRNA and protein expression levels of IL-1β were significantly decreased by the miR-146a mimic and increased by the miR-146a inhibitor. Taken together, the results of the present study provided support for the roles of miR-146a and miR-181b in the pathogenesis of silicosis, and suggested that they may be candidate therapeutic targets in this disease.

Alterations in the expression of miR-146a are important events in the pathogenesis of several human diseases, including autoimmune disorders, viral infections, cancer, muscle disorders and myelodysplastic syndrome (23-28). In the present study, transfection of NR8383 cells with the miR-146a mimic resulted in reduced IL-1β release, wheraes transfection with the miR-146a inhibitor increased IL-1β release in response to SiO_2 stimulation. This suggested that miR-146a may directly or indirectly suppress the expression of IL-1β. Liu *et al* (29) reported that miRNA-146a was upregulated in human bronchial epithelial cells in response to stimulation by transforming growth factor β1 and cytomix, a mixture of IL-1β, interferon γ and TNF-α. Zhong *et al* (30) provided evidence that miR-146a exerts negative feedback in neutrophil elastase-stimulated MUC5AC production from human bronchial epithelial cells. Sato *et al* (31) demonstrated that reduced levels of miR-146a increase the release of the inflammatory mediator prostaglandin E2, indicating a crucial role for this miRNA in the abnormal inflammatory response in chronic obstructive pulmonary disease. A number of previous reports have suggested that miR-146a transcription is regulated by nuclear factor κB (30,32,33). The results of the present study suggested that miR-146a may act through post-translational inhibition of target genes and regulate innate responses of macrophages to SiO_2 stimulation.

miR-181b belongs to the miR-181 family and has an important regulatory role in cell cycle and differentiation (34-36). miR-181b is also involved in different cancer cell lines (37,38). In the present study, the mRNA and protein levels of TNF-α were significantly reduced by the mimic, and the opposite effect was observed following knockdown of miR-181b with the inhibitor. This indicated that the miR-181b mimic enhanced the phagocytic ability of the NR8383 cells towards SiO_2. Despite the prominent biological importance of miR-181b, its response to SiO_2 stimulation remains to be fully elucidated. The effects of miR-181b in NR8383 cells predominantly depend on the target genes and their co-regulatory functions. For example, miR-181b has been reported to sensitize pancreatic ductal adenocarcinoma, gastric and lung cancer cells to chemotherapy by targeting B cell lymphoma-2 (39-41). Yang *et al* (20) demonstrated that miR-181b is markedly downregulated in clinical non-small cell lung cancer tissues, compared with non-tumorous lung tissues. The precise molecular mechanisms for the altered expression of miR-181b in silicosis, and how this affects TNF-α require investigation.

The present study investigated the roles of miR-146a and miR-181b in regulating TNF-α and IL-1β secretion in SiO_2-induced NR8383 rat macrophages. miR-146a and miR-181b were characterized in SiO_2-treated lungs, which indicated the importance of miR-146a and miR-181b in silicosis, and assisted in elucidating the mechanisms underlying the development and progression of silicosis. A limitation of the present study was that the investigation was *in vitro*, thus it remains to be elucidated whether manipulation of the expression of miR-146a and miR-181b *in vivo* has a significant effect on the development of pulmonary fibrosis. Therefore, examination of the effect of miR-146a and miR-181b *in vivo* is a priority for future investigations. In addition, the basis for cell-type-specific expression of individual miR-146 and miR-181 family members and, in certain cases, their ability to target specific genes, requires further investigation. The present study, to the best of our knowledge, is the first to indicate that alterations in the expression of miR-146a and miR-181b may be correlated with silicosis, suggesting that miR-146a and miR-181b may be involved in silicosis and act as potential therapeutic targets for silicosis. Further investigation of the molecular mechanisms by which miR-146a and miR-181b contribute to the initiation and progression of silicosis are required.

In conclusion, the present study investigated the association between alterations in the expression of miR-146a and

miR-181b, and silicosis. The results provide support for the role for miR-146a and miR-181b in the pathogenesis of silicosis, and suggested the possibility for their use as therapeutic targets in this disease. The specific mechanism underlying this interaction requires further investigation.

Acknowledgements

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