Anti-fibrotic effects and the mechanism of action of miR-29c in silicosis

LEI HUANG[1,2], YANG ZHANG[3,4], JIE YANG[1], JUAN LI[5], JU WU[6], FAXUAN WANG[7], YAJIA LAN[1] and QIN ZHANG[1]

Departments of [1]Environmental Health and Occupational Medicine and [2]Occupational Hazard Assessment, West China School of Public Health and West China Fourth Hospital; [3]Department of Periodical Press and National Clinical Research Center for Geriatrics; [4]Chinese Evidence-Based Medicine Center, West China Hospital, Sichuan University, Chengdu, Sichuan 610041; Departments of [5]Occupational Medicine and [6]Respiratory and Critical Care Medicine, The Second People's Hospital of Panzhihua, Panzhihua, Sichuan 611300; [7]Department of Occupational and Environmental Medicine, School of Public Health, Ningxia Medical University, Yinchuan, Ningxia 750004, P.R. China

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Abstract. Despite increasing evidence suggesting a role for the miR-29 family in the suppression of fibrosis, its role in silicosis remains unknown. The present study aimed to examine the anti-fibrotic effects and specific mechanism of action of microRNA (miR)-29c in pulmonary silicosis using animal and cell models. miR-29c expression levels were examined in the lungs of silicotic rats via reveres transcription-quantitative (RT-q)PCR. A Transwell system employing co-cultures of pulmonary fibroblasts and macrophages was used to establish an *in vitro* cell model of silicosis, and lentivirus was used to overexpress or knockdown miR-29c in cultured cells. Changes in collagen type I α I (COL1α1), COL3α1, α-smooth muscle actin (α-SMA) and TGF-β1 expression levels were determined via RT-qPCR and western blotting. Data analysis was performed using R software. miR-29c expression was significantly downregulated in the lungs of silicotic rats and in the pulmonary fibroblasts of the *in vitro* model of silicosis. Furthermore, COL1α1, COL3α1, α-SMA and TGF-β1 expression levels were significantly increased in cultured fibroblasts following 12 or 18 h exposure to SiO_2. Lentiviral-mediated knockdown of miR-29c resulted in increased the expression levels of COL1α1, COL3α1, α-SMA and TGF-β1, while lentiviral-mediated miR-29c overexpression significantly suppressed the expression levels of these fibrosis-related genes. Taken together, these results demonstrated that miR-29c was significantly associated with silica-induced pulmonary fibrosis and the expression levels of COL1α1, COL3α1, TGF-β1 and α-SMA are under the regulation of miR-29c to different extents. This study therefore identified possible candidate molecular targets for preventing or delaying the occurrence and progression of silicosis.

Introduction

Silicosis is an occupational disease caused by the inhalation of free silica dust, and is characterized by diffuse fibrosis in the lung tissue (1). In 2017, 23,395 patients were diagnosed with silicosis worldwide (2). The exact mechanisms contributing to the pathogenesis of pulmonary silicosis remain unclear, limiting the scope for the development of targeted therapies for this disease. Nonetheless, it is generally considered that alveolar macrophages and lung fibroblasts are the main target and effector cells contributing to the fibrosis that underlies silicosis (3,4). Upon phagocytosing large quantities of silica dust, alveolar macrophages secrete various bioactive substances and demonstrate altered plasma membrane characteristics (1). Moreover, the secretion of bioactive substances can stimulate fibroblasts to release substantial amounts of collagen and fibronectin, leading to an irreversible structural change in the lung tissue (5). While the critical factors involved in the pathogenesis of silicosis have yet to be fully elucidated, accumulating evidence indicates that TGF-β (6), collagen type I α I (COL1α1) (6), COL3α1 (7) and α-smooth muscle actin (α-SMA) (8) serve important roles in the process of pulmonary fibrosis.

The microRNA (miRNA/miR)-29 family of miRNAs comprises of miR-29a, miR-29b and miR-29c (9,10). The abnormal expression of miR-29 family members has been implicated in the fibrosis of various tissues, including those

Correspondence to: Professor Qin Zhang, Department of Environmental Health and Occupational Medicine, West China School of Public Health and West China Fourth Hospital, Sichuan University, 16, Section 3, South Renmin Road, Chengdu, Sichuan 610041, P.R. China
E-mail: zhang_q@scu.edu.cn

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of the lung, liver, kidney and heart (11-15), and there is increasing evidence to suggest that decreased levels of miR-29 are associated with numerous respiratory disorders, including lung cancer and idiopathic pulmonary fibrosis (16-18). The expression of miR-29 family miRNAs was significantly down-regulated in the lung tissue of animals in a bleomycin-induced mouse model of pulmonary fibrosis, while knockdown of the miR-29 family in IMR-90 cells resulted in the derepression of fibrosis-related genes (19). In addition, miR-29 has been shown to be negatively regulated by TGF-β/Smad signaling, while miR-29 overexpression has, in turn, been revealed to negatively regulate TGF-β expression and Smad3 signaling (20). Lian *et al* (21) reported that miR-29b could inhibit extracellular matrix (ECM) synthesis in lung fibroblasts stimulated with supernatants from silica-treated lung macrophages. Another study has shown that silica can dynamically regulate miR-29b expression and thereby influence the promotion of mesenchymal-epithelial transition in RLE-6TN cells (22).

Although numerous studies have confirmed that miR-29 is involved in pulmonary fibrosis, little is known regarding the association between miR-29 and silicosis. Silicosis is a specific disease resulting from accumulated exposure to silica dust, and is distinct from other pulmonary fibrotic diseases (1). Our previous study revealed that miR-29c expression was significantly decreased in the lungs of silicotic rats, as determined by miRNA microarray analysis (23). The present study aimed to examine the role and mechanism of action of miR-29c in the pathogenesis of silicosis and the associated fibrotic process.

Materials and methods

Animal model for the study of pulmonary silicosis. A total of 20 Sprague-Dawley male rats (Dashuo Center of Experimental Animals, Chengdu, China; age, 5-7 weeks; weight, 160-200 g) were randomly assigned evenly into two groups (silicosis and control group). The animals had free access to food and water, and were housed at an environmental temperature of 22±1˚C, under 12-h light/dark cycles with a relative humidity of 59%. The silicosis group was anesthetized with etherization and received a single intratracheal installation of 1 ml silica suspension (equal to 50 mg silica dust). While the control group was treated in the same way, it was administered an injection of 1 ml sterilized saline instead. After the rats were anesthetized with 5% chloral hydrate (400 mg/kg; Tianjin Kemiou Chemical Reagent Co., Ltd.), they were euthanized by exsanguination from the heart after 40 days. Our previous study already established the experimental silicosis rats via disposable intratracheal instillation of silica dust suspension (23). Pathological examination and analysis of hydroxyproline levels in lung tissues confirmed the successful establishment of silicosis (23). The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, and all experiments approved by and complied with the regulations of the Experimental Animal Ethics Committee of Sichuan University (approval no. K2015028).

In vitro model for the analysis of silicosis. A schematic diagram describing the *in vitro* cell model for the study of silicosis is presented in Fig. 1. Pulmonary fibroblasts were isolated from the lung tissue of a healthy adult male Sprague-Dawley rat (age, 3 months; weight, 425 g) and cultured in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). Rat NR8383 pulmonary macrophages (Shanghai Institute of Biochemistry and Cell Biology) were cultured in Ham's F12K cell culture medium (Sigma-Aldrich; Merck KGaA) supplemented with 15% FBS. Cell cultures were maintained at 37˚C in a humidified incubator containing an atmosphere of 5% CO_2.

Passage 4-8 pulmonary fibroblasts in the logarithmic phase of growth were seeded at a density of $6x10^5$ cells/well in 6-well plates in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and incubated for 12 h at 37˚C to achieve cultures that were 80-90% confluent. Subsequently, Transwell chambers (EMD Millipore; pore size, 0.4 μm) were inserted into the 6-well culture plates, such that the Transwell membranes were immersed in the culture medium and positioned immediately above the underlying fibroblast monolayer. NR8383 cells ($6x10^5$ cells/chamber) in Ham's F12K medium supplemented with 15% FBS (Gibco; Thermo Fisher Scientific, Inc.) were then seeded into the upper compartment of the Transwell chamber. The plate was incubated for 24 h at 37˚C under normal cell culture conditions. Half of the Transwells were then treated with silica suspension at a final concentration of 40 $\mu g/cm^2$ (experimental group), and the other half with an equal volume of serum-free Ham's F12K medium (control group; Table S1 and Data S1). The 6-well plates containing the co-cultures were returned to the incubator at 37˚C and the cells were then processed for downstream assays at 6, 12 and 18 h timepoints. Cells were observed using a confocal microscope (magnification, x100; Nikon Corporation).

miR-29c overexpression and inhibition studies. Lentivirus for the overexpression and inhibition of miR-29c was purchased from Shanghai GeneChem Co., Ltd. The lentiviral cassettes encoded an enhanced green fluorescence protein (eGFP) reporter for the evaluation of cell transduction efficiency. Fibroblasts were infected with virus at a multiplicity of infection of 20-50 in the presence of Enhanced Infection Solution (ENi.S; Shanghai GeneChem Co., Ltd.), to achieve an optimal transduction efficiency of ~80% after 4 days. Prior to viral transduction, fibroblasts were seeded into 6-well plates at a density of $6x10^5$ cells/well and then cultured at 37˚C for 24 h. Cell cultures were divided into five groups (overexpression, overexpression control, inhibitor, inhibitor control and blank control) and prepared in triplicate. The culture medium was then replaced with ENi.S reagent before the addition of lentivirus. The cells of the overexpression group were transduced with LV-rno-miR-29c lentivirus that encodes full-length miR-29c, while the cells of the overexpression control group were transduced with CON063 lentivirus encoding only the eGFP reporter. The cells of the inhibition group were transduced with LV-rno-miR-29c-inhibition lentivirus that encodes small interfering RNA (5'-CCGGTAACCGATTTCAAATGGTGC TATTTTTG-3') directed against miRNA-29c, while the cells of the inhibition control group were transduced with CON157 lentivirus encoding non-sense miRNA. The blank control group contained cells that were not infected with virus. An inverted fluorescence microscope (magnification, x100; Nikon

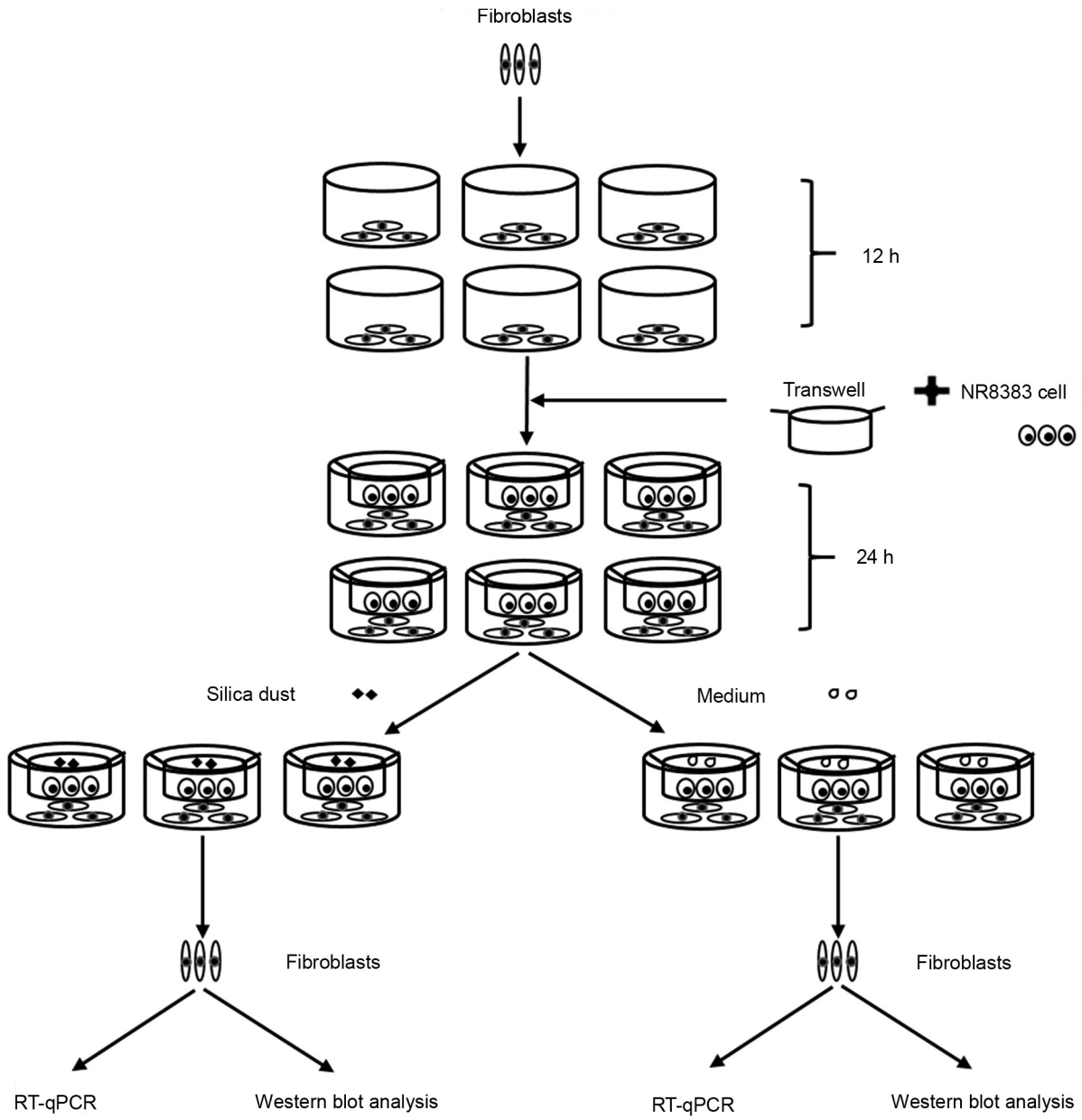

Figure 1. Schematic diagram of the silicosis cell model *in vitro*. Fibroblasts in the logarithmic phase of growth were seeded into 6-well plates and incubated for 12 h to 80-90% confluence. Subsequently, Transwell chambers were inserted into the 6-well culture plates. NR8383 cells were seeded into the upper compartment of the Transwell chamber. The plate was incubated for 24 h under normal cell culture conditions. Subsequently, half of the Transwell chambers were treated with 40 μg/cm^2 silica suspension and the other half were treated with an equal volume of serum-free Ham's F12K medium. Following incubation, fibroblasts were processed via RT-qPCR and western blotting at 6, 12 and 18 h time points. RT-qPCR, reverse transcription-quantitative PCR.

ECLIPSE Ti-U; Nikon Corporation) was used to examine eGFP expression in the cell cultures, which provided a readout for the efficiency of viral transduction. Virally transduced fibroblasts were co-cultured with NR8383 macrophages and treated with 40 μg/cm^2 silica suspension as aforementioned, and then harvested for downstream assays at the 12 h time-point. Reverse transcription-quantitative PCR (RT-qPCR) was performed to confirm the overexpression and suppression of miR-29c in virally transduced cells.

RT-qPCR analysis. Total RNA was isolated from lung tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. The quality and concentration of total RNA isolated from each group were evaluated by 1.2% gel electrophoresis and analysis using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Inc.). miR-29c expression was determined via RT-qPCR using the All-in-One miRNA qRT-PCR Detection kit (cat. no. AOMD-Q020; GeneCopoeia, Inc.). For RT, the reactions contained 150 ng purified total RNA, 0.3 μl stem-loop RT primer (Table I), 2 μl 10x RT buffer, 2 μl dNTP, 0.2 μl reverse transcriptase, 0.3 μl RNase Inhibitor and RNase-free H_2O, to a total volume of 20 μl. Thermal cycling was performed as follows: 30 min at 16˚C, 40 min at 42˚C, 5 min at 85˚C, and a final temperature hold at -20˚C. RT-qPCR reactions contained 5 μl 2x PCR master mix, 0.5 μl each primer (Table I), 2 μl cDNA and RNase-free H_2O, to a total volume of 10 μl. Thermocycling parameters were as follows: 95˚C

Table I. Oligonucleotides for miR-29c mRNA detection.

Primer name	Reverse transcription primer	Reverse transcription-quantitative PCR primer
U6	5'CGCTTCACGAATTTGCGTGTCAT3'	F: 5'GCTTCGGCAGCACATATACTAAAAT3' R: 5'CGCTTCACGAATTTGCGTGTCAT3'
rno-miR-29c-3p	5'GTCGTATCCAGTGCGTGTCGTGGAGTC GGCAATTGCACTGGATACGACTAACCG3'	F: 5'GGGTAGCACCATTTGAAA3' R: 5'TGCGTGTCGTGGAGTC3'

miR, microRNA; F, forward; R, reverse.

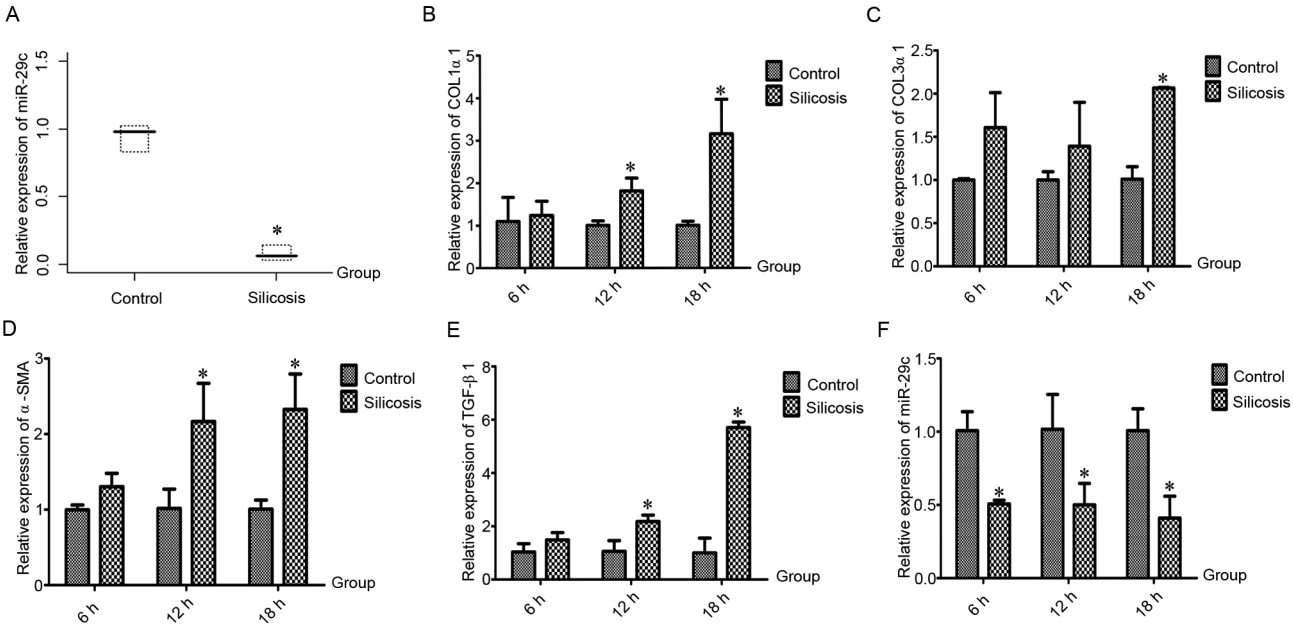

Figure 2. mRNA expression levels of miR-29c, COL1α1, COL3α1, α-SMA and TGF-β1. (A) Altered miR-29c expression level of experimental silicosis rats. Reverse transcription-quantitative PCR results of the expression levels of (B) COL1α1, (C) COL3α1 and (D) α-SMA and (E) TGF-β1 in the *in vitro* silicosis cell model. (F) Relative expression level of miR-29c following silica suspension exposure at different time-points. *P<0.05 vs. corresponding control group. miR-29c, microRNA-29c; COL1α1, Collagen type I α 1; COL3α1, Collagen type III α 1; α-SMA, α-smooth muscle actin.

for 5 min; followed by 40 cycles of 95˚C for 10 sec, 60˚C for 60 sec and 95˚C for 15 sec. Endogenous U6 RNA expression was used for the normalization of each sample.

Total RNA from fibroblasts and culture supernatants was isolated using TRIzol reagent. RT was performed using the PrimeScript RT reagent kit (Takara Bio, Inc.) and qPCR was performed using the All-in-One miRNA qRT-PCR Detection kit (GeneCopoeia, Inc.). Endogenous β-actin mRNA was used for the normalization of each sample. Primers for the specific detection of ACTB, COL1α1, COL3α1, TGF-β1 and α-SMA were purchased from GeneCopoeia, Inc. (cat. nos. RQP051050, RQP054226, RQP051466, RQP050181 and RQP050919). The $2^{-\Delta\Delta Cq}$ method was used to evaluate the expression level of target genes (24).

Western blotting. Total protein was extracted from fibroblasts and culture supernatants using a total protein extraction kit (Nanjing KeyGEN Biotech Co., Ltd.) and the protein concentration was determined using a BCA assay (Nanjing KeyGen Biotech Co., Ltd.) in accordance with the manufacturer's instructions. Protein extracts (40 μg) were loaded onto 10% polyacrylamide gels, electrophoretically separated and transferred onto PVDF membranes (EMD Millipore). PVDF membranes were then blocked with 5% skimmed milk for 2 h at room temperature. The membranes were then washed three times in TBS/20% Tween 20 (TBST) before incubation with primary antibody at 4˚C for 12 h. Membranes were probed with antibodies against COL1α1 (Purified rabbit polyclonal antibody; 139 kDa; cat. no. NB600-408; Novus Biologicals, LLC), COL3α1 (Purified rabbit polyclonal antibody; 138 kDa; cat. no. NB600-594; Novus Biologicals, LLC), TGF-β1 (Purified rabbit polyclonal antibody; 25 kDa; cat. no. sc-146; Santa Cruz Biotechnology, Inc.), α-SMA (Purified rabbit polyclonal antibody; 42 kDa; cat. no. abs107717; Absin Bioscience, Inc.) and β-actin (Purified mouse polyclonal antibody; cat. no. sc-4778; Santa Cruz Biotechnology, Inc.). Membranes were then washed in TBST and incubated with HRP-conjugated goat anti-rabbit IgG (cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) or goat anti-mouse IgG (cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) secondary antibodies for 1 h at room temperature. The PVDF membranes were then incubated with ECL reagents (Beyotime Institute of Biotechnology) at room

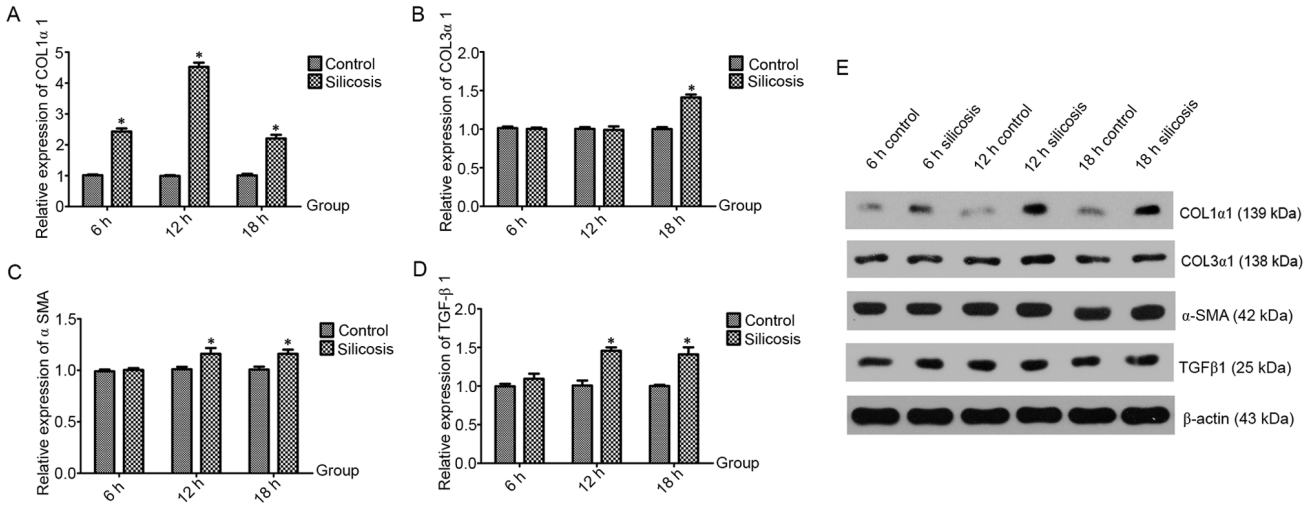

Figure 3. Western blotting results of COL1α1, COL3α1, α-SMA and TGF-β1 expression levels in the *in vitro* silicosis cell model. Protein expression levels of (A) COL1α1, (B) COL3α1, (C) α-SMA and (D) TGF-β1 in the *in vitro* silicosis cell model. (E) Western blotting results of COL1α1, COL3α1, α-SMA and TGF-β1 expression levels. *P<0.05 vs. corresponding control group. miR-29c, microRNA-29c; COL1α1, Collagen type I α 1; COL3α1, Collagen type III α 1; α-SMA, α-smooth muscle actin.

temperature in the dark for 2-3 min. Detection of immunoreactivity and subsequent densitometry analysis of protein bands was performed using the Bio-Rad Gel Imaging system (Bio-Rad Laboratories, Inc.) and ImagePro Plus software (version 6.0; Media Cybernetics, Inc.). Endogenous β-actin expression was used for sample normalization during the densiometric analysis.

Statistical analysis. Each experiment was performed in triplicate. R software (www.r-project.org) was used to perform data analysis. The Student's t-test and one-way ANOVA were used to evaluate the significance of differences in expression between various groups. When the results of ANOVA were statistically significant, Student-Newman-Keuls was used for further analysis. P<0.05 was considered to indicate a statistically significant difference (two-tailed).

Results

miR-29c expression is downregulated in the silicotic rat lung. RT-qPCR analysis revealed that the expression of miR-29c was significantly downregulated in the lung tissues of silicotic rats, when compared with control animals (P<0.05; Fig. 2A).

COL1α1, COL3α1, α-SMA, TGF-β1 and miR-29c expression levels are altered in an in vitro cell model of silicosis. As presented in Fig. 2B-E, treatment of cultured cells with 40 $\mu g/cm^2$ SiO_2 for 12 and 18 h resulted in a significant increase in the mRNA expression levels of COL1α1, α-SMA and TGF-β1, when compared with the controls (P<0.05). A significant increase in the expression of COL3α1 mRNA in these cells was only observed after 18 h treatment with SiO_2 (P<0.05). No significant change in the expression of any of these genes was observed at the 6 h time point (P>0.05). However, the treatment of cultured cells with SiO_2 resulted in a significant reduction in the expression of miR-29c at all time points analyzed, when compared with the untreated controls (P<0.05; Fig. 2F).

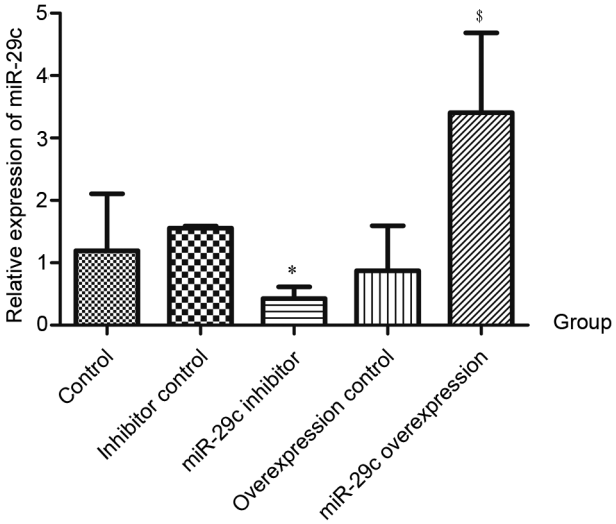

Figure 4. Relative expression level of miR-29c when transfected with corresponding viruses. *P<0.05 vs. inhibitor group; $P<0.05 vs. overexpression control group. miR-29c, microRNA-29c.

COL1α1, COL3α1, α-SMA and TGF-β1 protein expression levels are significantly upregulated in an in vitro cell model of silicosis. Western blot analysis results demonstrated that the treatment of cell cultures with SiO_2 for 12 h resulted in a significant increase in COL1α1, α-SMA and TGF-β1 protein expression levels, when compared with the untreated controls (P<0.05). Indeed, a significant increase in COL1α1 protein expression was observed as early as 6 h after SiO_2 treatment (P<0.05). Following induction in response to SiO_2 treatment, COL1α1, COL3α1, α-SMA and TGF-β1 protein expression levels remained elevated relative to controls for the duration of the experimental period (P<0.05; Fig. 3).

miR-29c regulates COL1α1, COL3α1, α-SMA and TGF-β1 expression in rat lung fibroblasts in an in vitro cell model of silicosis. Lentiviral-mediated gene delivery was used to

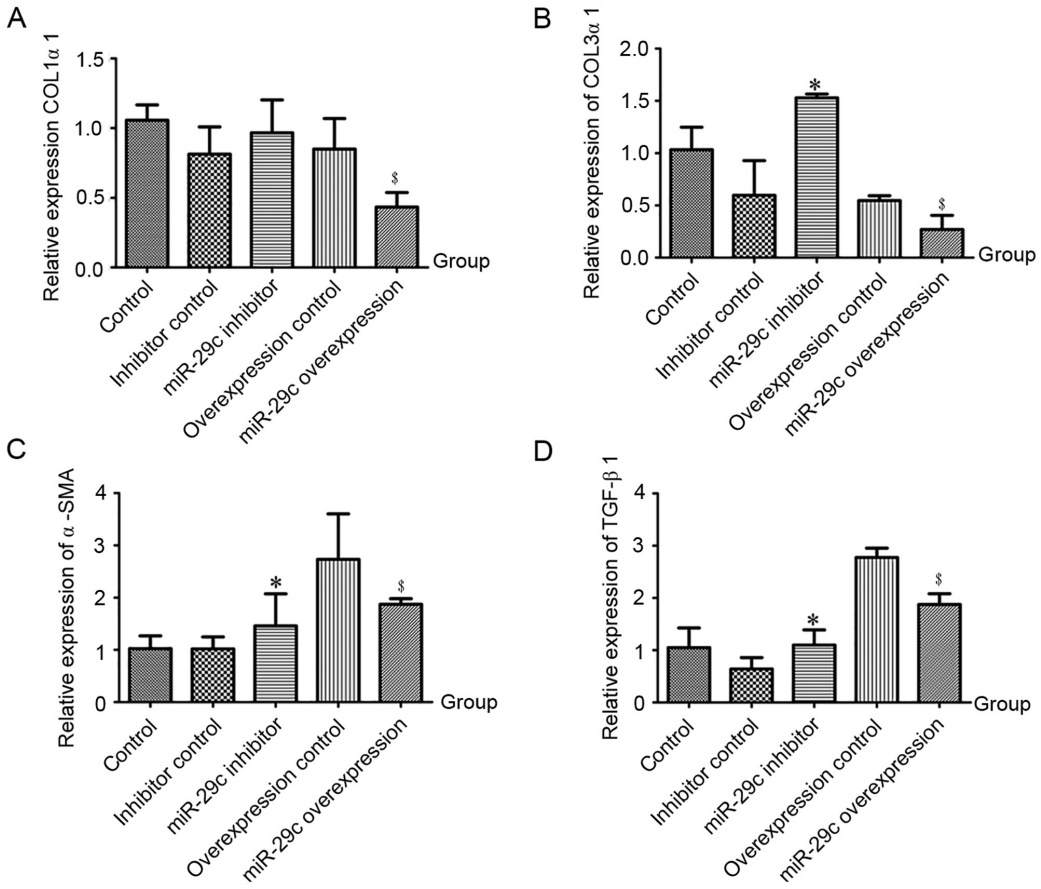

Figure 5. mRNA expression levels of COL1α1, COL3α1, α-SMA and TGF-β1 in miR-29c overexpression and inhibition cell model. mRNA expression levels of (A) COL1α1, (B) COL3α1, (C) α-SMA and (D) TGF-β1. *P<0.05 vs. corresponding inhibitor control group; $P<0.05 vs. corresponding overexpression control group. miR-29c, microRNA-29c; COL1α1, Collagen type I α 1; COL3α1, Collagen type III α 1; α-SMA, α-smooth muscle actin.

either enhance or suppress miR-29c expression in cultured rat lung fibroblasts, which was performed to determine the role of this miRNA in the regulation of key genes associated with the fibrotic process. Following viral transduction and subsequent SiO_2 treatment for 12 h, miR-29c expression levels were analyzed in the cells of the various treatment groups. As expected, no significant differences in miR-29c expression were observed among the cells of the blank control, overexpression control and inhibitor control groups (P>0.05). However, miR-29c expression was significantly decreased in the cells of the miR-29c inhibitor group (LV-rno-miR-29c-inhibition) when compared with those of the inhibitor control (CON157) group (P<0.05). Conversely, miR-29c expression was significantly higher in the cells of the miR-29c overexpression group (LV-rno-miR-29c), when compared with the cells of the overexpression control (CON063) group (P<0.05; Fig. 4).

RT-qPCR analysis revealed that lentiviral-mediated overexpression or inhibition of miR-29c resulted in differential effects on the mRNA expression levels of COL1α1, COL3α1, α-SMA and TGF-β1 in the rat lung fibroblasts of the *in vitro* silicosis cell model (Fig. 5). The fibroblasts of the miR-29c inhibitor group (LV-rno-miR-29c-inhibition) demonstrated a significant upregulation in the expression of COL3α1, α-SMA and TGF-β1, when compared with those of the inhibitor control (CON157) group (P<0.05). Conversely, all four fibrosis-related genes were significantly downregulated in the cells of the miR-29c overexpression (LV-rno-miR-29c) group, when compared with those of the control (CON063) group (P<0.05). In addition to the RT-qPCR analysis, western blotting identified that COL1α1, COL3α1 and α-SMA protein expression levels were significantly higher in the cells of the miR-29c inhibitor group, when compared with those of the inhibitor control group (P<0.05), while the expression of COL3α1 was suppressed in the cells of the miR-29c overexpression group, when compared with those of the overexpression control group (P<0.05; Fig. 6).

Discussion

The pathogenesis of silica dust-induced pulmonary fibrosis involves a complicated network of biological processes that include inflammation, immunological changes, cell cytotoxicity and tissue repair (5), as well as a variety of cell types and bioactive substances (1). Our previous miRNA microarray analysis revealed that miR-29c expression was decreased in lung tissue in an experimental rat model of pulmonary silicosis (23), thus suggesting a potential association between miR-29c and silicosis. To test this hypothesis, and to further examine the mechanistic relationship between miR-29c and key proteins involved in the fibrotic process, the present study developed an *in vitro* cell model of silicosis to complement the *in vivo* model. The current RT-qPCR analysis also identified miR-29c downregulation in the lungs of silicotic rats, thereby validating our previous findings. Subsequently,

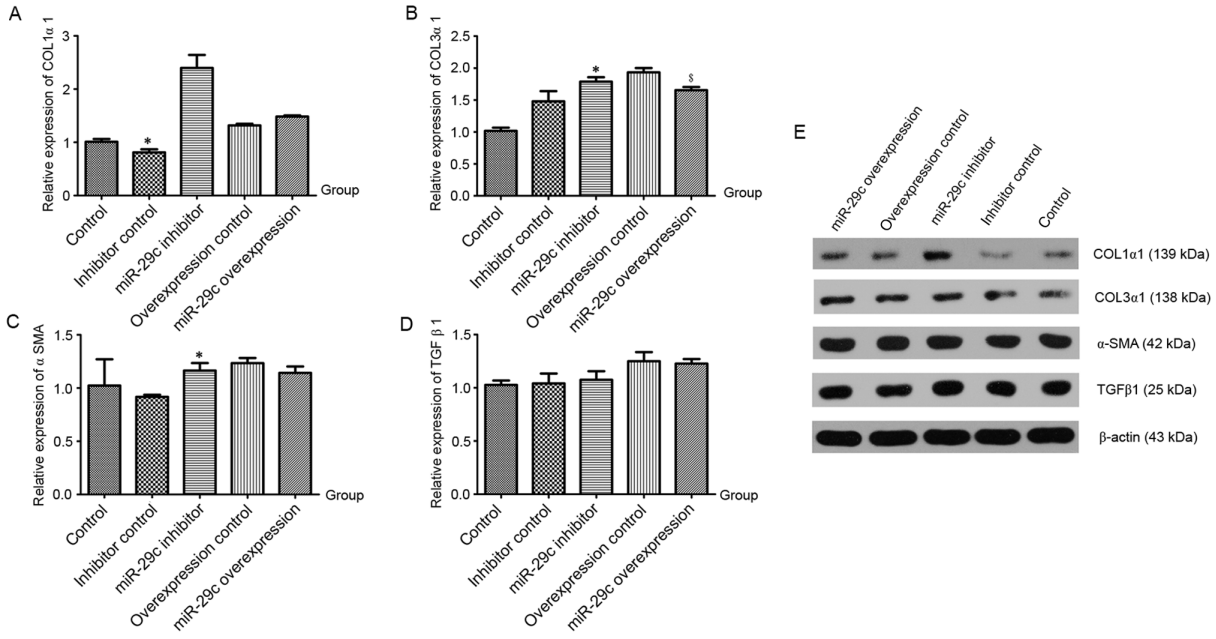

Figure 6. Protein expression levels of COL1α1, COL3α1, α-SMA and TGF-β1 in miR-29c overexpression and inhibition cell model. Protein expression levels of (A) COL1α1, (B) COL3α1, (C) α-SMA and (D) TGF-β1. (E) Western blotting results of COL1α1, COL3α1, α-SMA and TGF-β1. *P<0.05 vs. corresponding inhibitor control group; $P<0.05 vs. corresponding overexpression control group. miR-29c, microRNA-29c; COL1α1, Collagen type I α 1; COL3α1, Collagen type III α 1; α-SMA, α-smooth muscle actin.

primary rat lung fibroblasts were co-cultured with NR8383 rat macrophages in a Transwell system to establish an *in vitro* model of silicosis that permitted a more detailed analysis of miR-29c function. The fibroblasts in these *in vitro* co-cultures were then transduced with lentivirus to experimentally alter miR-29c expression, which was used to determine the potential role of this miRNA in the fibrotic process. RT-qPCR and western blotting analysis revealed that miR-29c regulated the expression of the four fibrosis-related proteins, COL1α1, COL3α1, α-SMA and TGF-β1. These experiments therefore identified a potential mechanism via which miR-29c can contribute to the pathogenesis of silicosis. Furthermore, the relevance of miR-29c in this disease was confirmed at both the whole-organism and cellular level. The molecular functions of miR-29c in silicosis are summarized in Fig. 7.

Previous studies have reported a relationship between miR-29 and fibrotic diseases (25,26). In the present study, it was found that miR-29c was downregulated in the lungs of silicotic rats, a finding that is consistent with those of Xiao *et al* (20), who used a bleomycin-induced model of pulmonary fibrosis. The present study used an *in vitro* Transwell culture system that enabled the treatment of microphages with silica dust and their simultaneous co-culture with fibroblasts. This system allowed interaction between fibroblasts and microphages through the Transwell filter membrane, and was adopted to simulate the internal environment of lung tissue. Using this assay, the mRNA and protein levels of COL1α1, COL3α1, TGF-β1 and α-SMA were examined, and it was identified that these were all increased in response to SiO_2 treatment. The present findings are consistent with those of Li *et al* (27), who have also observed increases in the expression of COL1 and TGF-β using this *in vitro* cell model.

The TGF-β family of cytokines, which are predominantly expressed by macrophages, hyperplastic alveolar epithelial

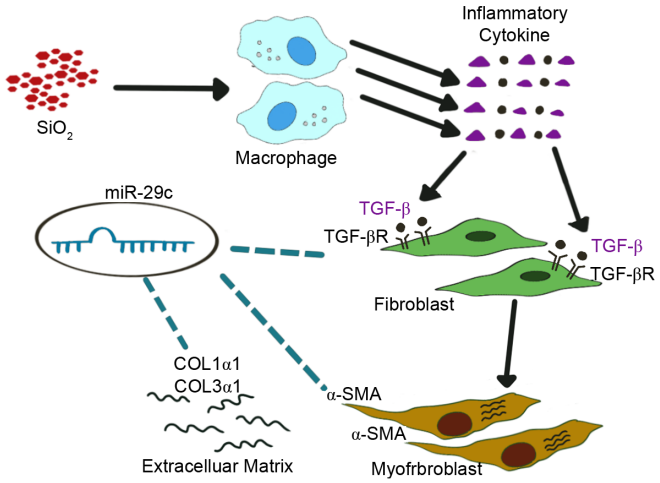

Figure 7. Molecular functions of miR-29c in silicosis. Under the stimulation of SiO_2, lung macrophages secreted some inflammatory factors, including TGF-β1. TGF-β1 interacted with the TGF-β1 receptor of fibroblasts to stimulate transformation into myofibroblasts and secretion of extracellular matrix, including COL1α1 and COL3α1. miR-29c regulated the expression of the four fibrosis-related proteins, COL1α1, COL3α1, α-SMA and TGF-β1. miR-29c, microRNA-29c; COL1α1, Collagen type I α 1; COL3α1, Collagen type III α 1; α-SMA, α-smooth muscle actin.

cells and bronchial epithelial and mesenchymal cells, are capable of activating fibroblasts to promote the deposition of collagen, which ultimately leads to fibrosis (28,29). TGF-β has been reported to suppress miR-29 expression in several cell types, including HK2 cells (30), human embryonic lung fibroblasts (19), mouse and human hepatic stellate cells (13), human trabecular meshwork cells (31), renal fibroblasts and renal tubular epithelial cells (12,32,33). Smad7 serves as an inhibitory Smad to suppress Smad2 and Smad3

activation (34). Smad7 expression is induced by TGF-β1 via a Smad3-dependent mechanism (34). Overexpression of Smad7 has been shown to block renal fibrosis by restoring the expression of miR-29b in a mouse model of obstructive nephropathy. In contrast, inhibition of Smad7 was found to promote renal fibrosis by enhancing the loss of miR-29b (35). The current research has indicated that TGF-β1 mRNA was upregulated in response to miR-29c inhibition, and downregulated following miR-29c overexpression. The present study concluded that miR-29c can inhibit the fibrotic process that underlies silicosis via its impact on TGF-β signaling. It should be noted that there was no statistically significant change in TGF-β protein expression; however, this observation was probably a consequence of the delay between gene upregulation and subsequent protein expression.

The fibrosis-related protein α-SMA is a biomarker of activated myofibroblasts (36). Myofibroblasts differentiate from fibroblasts in response to particular extracellular stimuli and demonstrate enhanced secretory properties, when compared with their undifferentiated counterparts (37). The present study demonstrated that α-SMA expression was significantly upregulated in the fibroblasts of the miR-29c inhibition group and was decreased in the fibroblasts of the miR-29c overexpression group. These results suggest that miR-29c overexpression inhibits silicosis-associated myofibroblast differentiation via the inhibition of the TGF-β signaling pathway, resulting in the suppression of α-SMA expression.

Changes in the content of COL1 and COL3 proteins within the ECM are a major indicator of pulmonary fibrosis (7). Moreover, a genome-wide analysis of endogenous miR-29 target genes in nasopharyngeal carcinomas revealed that miR-29 inhibits the expression of 15 collagen genes, including those that encode the interstitial collagens COL1α1, COL1α2 and COL3α1 (38). Previous studies have confirmed that miR-29 targets COL1α1 (39,40) and COL3α1 (41,42) and other ECM-related genes. The current results indicated that COL3α1 expression was significantly upregulated in the cells of the miR-29c inhibition group and downregulated in the cells of the miR-29c overexpression group. It is therefore possible that overexpression of miR-29c can inhibit the expression of ECM-related proteins implicated in silicosis.

There are some limitations associated with the present study. Unfortunately, due to the budget and time constraints, the present study was unable to monitor dynamic changes in miR-29c expression in silicotic rats. Therefore, only miR-29c expression at day 40, which was the final day of the experimental period, was determined. Furthermore, the current study only examined the expression of miR-29c in the animal and cell experiments, and not other members of miR-29 family. While the present study demonstrated that COL1α1, COL3α1, TGF-β1 and α-SMA expression levels were differentially regulated by miR-29c, direct interactions could not be determined as dual luciferase reporter assays were not employed. In future studies, dual luciferase reporter assays and the association between miR-29a/b expression and silicosis will be evaluated.

In conclusion, the present study demonstrated that miR-29c expression was significantly decreased in *in vivo* and *in vitro* models of silicosis. It was identified that COL1α1, COL3α1, TGF-β1 and α-SMA, which are key fibrosis-related proteins, were regulated by miR-29c expression. miR-29c expression not only inhibited the expression of a variety of ECM proteins, but it also suppressed the TGF-β signaling pathway, which is closely associated with fibrosis. The results of the present study therefore provide new insights into the mechanisms underlying pulmonary fibrosis associated with silicosis, and highlight potential gene targets for the clinical treatment of fibrotic diseases.

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Availability of data and materials

The datasets analyzed of this study are available from the corresponding author on reasonable request.

Authors' contributions

Conceived and designed the experiments: LH, YZ, YL and QZ. Conducted the experiments: LH, YZ, JL, JW, JY and FW. Drafted the work: LH, YZ and JY. Involved in revising the manuscript critically for important intellectual content: JL, JW, YL, FW and QZ. All the authors have read and approved the submission of the manuscript for publication.

Ethics approval and consent to participate

The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, and all experiments approved by and complied with the regulations of the Experimental Animal Ethics Committee of Sichuan University (approval no. K2015028).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Leung CC, Yu IT and Chen W: Silicosis. Lancet 379: 2008-2018, 2012.
2. Shi P, Xing X, Xi S, Jing H, Yuan J, Fu Z and Zhao H: Trends in global, regional and national incidence of pneumoconiosis caused by different aetiologies: An analysis from the global burden of disease study 2017. Occup Environ Med 77: 407-414, 2020.
3. Arcangeli G, Cupelli V and Giuliano G: Effects of silica on human lung fibroblast in culture. Sci Total Environ 270: 135-139, 2001.

4. Lehnert BE, Valdez YE, Lehnert NM, Park MS and Englen MD: Stimulation of rat and murine alveolar macrophage proliferation by lung fibroblasts. Am J Respir Cell Mol Biol 11: 375-385, 1994.
5. Greenberg MI, Waksman J and Curtis J: Silicosis: A review. Dis Mon 53: 394-416, 2007.
6. Shimbori C, Bellaye PS, Xia J, Gauldie J, Ask K, Ramos C, Becerril C, Pardo A, Selman M and Kolb M: Fibroblast growth factor-1 attenuates TGF-β1-induced lung fibrosis. J Pathol 240: 197-210, 2016.
7. Van Hoozen BE, Grimmer KL, Marelich GP, Armstrong LC and Last JA: Early phase collagen synthesis in lungs of rats exposed to bleomycin. Toxicology 147: 1-13, 2000.
8. Gregory AD, Kliment CR, Metz HE, Kim KH, Kargl J, Agostini BA, Crum LT, Oczypok EA, Oury TA and Houghton AM: Neutrophil elastase promotes myofibroblast differentiation in lung fibrosis. J Leukoc Biol 98: 143-152, 2015.
9. Noetel A, Kwiecinski M, Elfimova N, Huang J and Odenthal M: microRNA are central players in anti- and profibrotic gene regulation during liver fibrosis. Front Physiol 3: 49, 2012.
10. Dostie J, Mourelatos Z, Yang M, Sharma A and Dreyfuss G: Numerous microRNPs in neuronal cells containing novel microRNAs. RNA 9: 180-186, 2003.
11. Cushing L, Kuang P and Lü J: The role of miR-29 in pulmonary fibrosis. Biochem Cell Biol 93: 109-118, 2015.
12. Qin W, Chung ACK, Huang XR, Meng XM, Hui DS, Yu CM, Sung JJ and Lan HY: TGF-β/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. J Am Soc Nephrol 22: 1462-1474, 2011.
13. Roderburg C, Urban GW, Bettermann K, Vucur M, Zimmermann H, Schmidt S, Janssen J, Koppe C, Knolle P, Castoldi M, *et al*: Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. Hepatology 53: 209-218, 2011.
14. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA and Olson EN: Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci USA 105: 13027-13032, 2008.
15. Yang T, Liang Y, Lin Q, Liu J, Luo F, Li X, Zhou H, Zhuang S and Zhang H: miR-29 mediates TGFβ1-induced extracellular matrix synthesis through activation of PI3K-AKT pathway in human lung fibroblasts. J Cell Biochem 114: 1336-1342, 2013.
16. Kwon JJ, Factora TD, Dey S and Kota J: A systematic review of miR-29 in cancer. Mol Ther Oncolytics 12: 173-194, 2018.
17. Mestdagh P, Vandesompele J, Brusselle G and Vermaelen K: Non-coding RNAs and respiratory disease. Thorax 70: 388-390, 2015.
18. Tang Y, He R, An J, Deng P, Huang L and Yang W: The effect of H19-miR-29b interaction on bleomycin-induced mouse model of idiopathic pulmonary fibrosis. Biochem Biophys Res Commun 479: 417-423, 2016.
19. Cushing L, Kuang PP, Qian J, Shao F, Wu J, Little F, Thannickal VJ, Cardoso WV and Lü J: miR-29 is a major regulator of genes associated with pulmonary fibrosis. Am J Respir Cell Mol Biol 45: 287-294, 2011.
20. Xiao J, Meng XM, Huang XR, Chung AC, Feng YL, Hui DS, Yu CM, Sung JJ and Lan HY: miR-29 inhibits bleomycin-induced pulmonary fibrosis in mice. Mol Ther 20: 1251-1260, 2012.
21. Lian X, Chen X, Sun J, An G, Li X, Wang Y, Niu P, Zhu Z and Tian L: MicroRNA-29b inhibits supernatants from silica-treated macrophages from inducing extracellular matrix synthesis in lung fibroblasts. Toxicol Res (Camb) 6: 878-888, 2017.
22. Sun J, Li Q, Lian X, Zhu Z, Chen X, Pei W, Li S, Abbas A, Wang Y and Tian L: MicroRNA-29b mediates lung mesenchymal-epithelial transition and prevents lung fibrosis in the silicosis model. Mol Ther Nucleic Acids 14: 20-31, 2019.
23. Faxuan W, Qin Z, Dinglun Z, Tao Z, Xiaohui R, Liqiang Z and Yajia L: Altered microRNAs expression profiling in experimental silicosis rats. J Toxicol Sci 37: 1207-1215, 2012.
24. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
25. Roncarati R, Viviani Anselmi C, Losi MA, Papa L, Cavarretta E, Da Costa Martins P, Contaldi C, Saccani Jotti G, Franzone A, Galastri L, *et al*: Circulating miR-29a, among other up-regulated microRNAs, is the only biomarker for both hypertrophy and fibrosis in patients with hypertrophic cardiomyopathy. J Am Coll Cardiol 63: 920-927, 2014.
26. Knabel MK, Ramachandran K, Karhadkar S, Hwang HW, Creamer TJ, Chivukula RR, Sheikh F, Clark KR, Torbenson M, Montgomery RA, *et al*: Systemic delivery of scAAV8-encoded MiR-29a ameliorates hepatic fibrosis in carbon tetrachloride-treated mice. PLoS One 10: e0124411, 2015.
27. Li J, Yao W, Zhang L, Bao L, Chen H, Wang D, Yue Z, Li Y, Zhang M and Hao C: Genome-wide DNA methylation analysis in lung fibroblasts co-cultured with silica-exposed alveolar macrophages. Respir Res 18: 91, 2017.
28. Massagué J, Blain SW and Lo RS: TGF beta signaling in growth control, cancer, and heritable disorders. Cell 103: 295-309, 2000.
29. Attisano L and Wrana JL: Signal transduction by the TGF-beta superfamily. Science 296: 1646-1647, 2002.
30. Du B, Ma LM, Huang MB, Zhou H, Huang HL, Shao P, Chen YQ and Qu LH: High glucose down-regulates miR-29a to increase collagen IV production in HK-2 cells. FEBS Lett 584: 811-816, 2010.
31. Luna C, Li G, Qiu J, Epstein DL and Gonzalez P: Role of miR-29b on the regulation of the extracellular matrix in human trabecular meshwork cells under chronic oxidative stress. Mol Vis 15: 2488-2497, 2009.
32. Wang B, Komers R, Carew R, Winbanks CE, Xu B, Herman-Edelstein M, Koh P, Thomas M, Jandeleit-Dahm K, Gregorevic P, *et al*: Suppression of microRNA-29 expression by TGF-β1 promotes collagen expression and renal fibrosis. J Am Soc Nephrol 23: 252-265, 2012.
33. Jiang L, Zhou Y, Xiong M, Fang L, Wen P, Cao H, Yang J, Dai C and He W: Sp1 mediates microRNA-29c-regulated type I collagen production in renal tubular epithelial cells. Exp Cell Res 319: 2254-2265, 2013.
34. Lan HY and Chung ACK: Transforming growth factor-β and Smads. Contrib Nephrol 170: 75-82, 2011.
35. Chung AC, Dong Y, Yang W, Zhong X, Li R, Lan HY: Smad7 suppresses renal fibrosis via altering expression of TGF-β/Smad3-regulated microRNAs. Mol Ther 21: 388-398, 2013.
36. Zhang HY and Phan SH: Inhibition of myofibroblast apoptosis by transforming growth factor beta(1). Am J Respir Cell Mol Biol 21: 658-665, 1999.
37. Hu B and Phan SH: Myofibroblasts. Curr Opin Rheumatol 25: 71-77, 2013.
38. Sengupta S, den Boon JA, Chen IH, Newton MA, Stanhope SA, Cheng YJ, Chen CJ, Hildesheim A, Sugden B and Ahlquist P: MicroRNA 29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins. Proc Natl Acad Sci USA 105: 5874-5878, 2008.
39. Wang M, Yang ZK, Liu H, Li RQ, Liu Y and Zhong WJ: Genipin inhibits the scleral expression of miR-29 and MMP2 and promotes COL1A1 expression in myopic eyes of guinea pigs. Graefes Arch Clin Exp Ophthalmol 258: 1031-1038, 2020.
40. Han Z, Zhang T, He Y, Li G, Li G, Jin X: Inhibition of prostaglandin E2 protects abdominal aortic aneurysm from expansion through regulating miR-29b-mediated fibrotic ECM expression. Exp Ther Med 16: 155-160, 2018.
41. Chen Y, Mohammed A, Oubaidin M, Evans CA, Zhou X, Luan X, Diekwisch TG and Atsawasuwan P: Cyclic stretch and compression forces alter microRNA-29 expression of human periodontal ligament cells. Gene 566: 13-17, 2015.
42. Maurer B, Stanczyk J, Juengel A, Akhmetshina A, Trenkmann M, Brock M, Kowal-Bielecka O, Gay RE, Michel BA, Distler JH, *et al*: MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. Arthritis Rheum 62: 1733-1743, 2010.