Silica-induced initiation of circular ZC3H4 RNA/ZC3H4 pathway promotes the pulmonary macrophage activation

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ABSTRACT: Phagocytosis of silicon dioxide (SiO2) into lung cells causes an inflammatory cascade that results in fibroblast proliferation and migration, followed by fibrosis. Circular RNAs (circRNAs) are a subclass of noncoding RNAs that are present within mammalian cells; however, researchers have not determined whether circRNAs are involved in the pathophysiologic process of silicosis. To elucidate the role of these RNAs in SiO2-induced inflammation in pulmonary macrophages, we investigated the upstream molecular mechanisms and functional effects of circRNAs on cell apoptosis, proliferation, and migration. Primary cultures of alveolar macrophages from healthy donors and from patients and the RAW264.7 macrophage cell line were used to explore the functions of circZC3H4 RNA in macrophage activation. The experimental results indicated the following: 1) SiO2 concomitantly increased circZC3H4 RNA expression and increased ZC3H4 protein levels; 2) circular ZC3H4 (circZC3H4) RNA and ZC3H4 protein participated in SiO2-induced macrophage activation; and 3) SiO2-activated macrophages promoted fibroblast proliferation and migration via the circZC3H4 RNA/ZC3H4 pathway. The up-regulation of the ZC3H4 protein was confirmed in tissue samples from patients with silicosis. Our study elucidates a link between SiO2-induced macrophage activation and the circZC3H4 RNA/ZC3H4 pathway, thereby providing novel insight into the potential use of ZC3H4 to develop novel therapeutic strategies for silicosis.—Yang, X., Wang, J., Zhou, Z., Jiang, R., Huang, J., Chen, L., Cao, Z., Chu, H., Han, B., Cheng, Y., Chao, J. Silica-induced initiation of circular ZC3H4 RNA/ZC3H4 pathway promotes the pulmonary macrophage activation. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: miR-212 · migration · silicosis · inflammation · circRNA

Silicosis, a chronic, fibrotic pulmonary disease caused by the inhalation of silica, is one of the most serious occupational diseases worldwide, and it occurs in many industries. Silicosis is a fatal lung condition that is characterized by chronic and late pulmonary fibrosis, and no effective diagnostic tools are available for its early detection (1, 2). Even when the patient is removed from the environment containing silica dust, lung function damage and disease progression continue. For individuals suffering from end-stage silicosis, the only effective treatment is lung transplantation (3, 4). In addition, some patients diagnosed with silicosis may develop lung cancer at a late stage of the disease process (5). The pathophysiology of silicosis begins with the deposition of silica particles in the alveoli of the lung. Ingestion of silica particles by macrophages initiates an inflammatory response that stimulates fibroblasts to overproliferate and produce large amounts of collagen. Silica particles then become encased by collagen, leading to fibrosis and the nodular lesions characteristic of the disease. The steps in the development of silicosis, including acute and chronic inflammation and fibrosis, have different molecular and cellular requirements, suggesting that silica-induced inflammation and fibrosis are important components (6). In response to external stimuli, macrophages are activated by cytokines secreted by lymphocytes of the adaptive immune
system. Those stimuli not only lead to the differentiation of macrophages into classically (M1) activated macrophages that possess enhanced antimicrobial, inflammatory, and antigen-presentation properties but also promote an alternatively activated macrophage phenotype (M2) that is characterized by anti-inflammatory actions (7–9).

Circular RNAs (circRNAs), a large class of highly abundant RNAs, have recently come into focus as a newly appreciated class of noncoding RNAs that possess important capabilities as gene regulators in mammals (10). Because of their closed loop structure, circRNAs are not translated into proteins; another consequence of their lack of free ends is that they are not degraded by exonucleases (11, 12). Most circRNAs are generated by exon RNA backsplicing, in which an upstream 3′ splicing site is joined to a downstream 5′ site, and circRNAs are primarily localized in the cytoplasm (13). Although significant progress has been made in exploring their biogenesis and regulation, because of the wide range of species and quantities of circRNAs found in cells, there is currently little understanding of their function. EliciRNA refers to a class of circRNAs that consist of exons and retained introns; these circRNAs are found in the nucleus and seem to act as expression regulators for their parental genes (14). However, most circRNAs are found in the cytoplasm, in which, intronic lariat species of circRNAs can interact directly with proteins (15). Fusion circRNAs arise from translocated chromosomal regions and are involved in proliferation and tumorigenesis (16). Among the numerous effects of circRNAs, 1 crucial function is their activity as microRNA (miRNA) sponges, where they can bind to miRNAs and act as natural miRNA sponges to inhibit the miRNAs’ regulatory actions (17, 18). CircRNAs have received considerable attention in the fields of growth and development, life processes, the cell cycle, and disease (19–23), providing a comprehensive landscape of circRNAs that will facilitate further discoveries in the progression of pulmonary fibrosis.

Monocyte chemotactic protein 1 (MCP-1)–induced protein-1 (MCPIP1) is a recently identified CCCH zinc finger protein that is induced in human monocyte-derived macrophages upon stimulation by MCP-1. MCPIP1 regulates immune responses by targeting mRNA degradation and by modulating signaling pathways. Immune responses are also regulated by the effects of cytokine production, immune cell activation, and immune homeostasis. Many studies have shown that MCPIP1 has a vital role in the down-regulation of the inflammatory response (24, 25). In our previous studies of the activation of alveolar macrophages (AMOs) by MCPIP1 in silicosis, we found that MCPIP1 regulates AMO activation through its effects on cytokine production and signaling pathways that affect fibroblast migration (26–28). MCPIP1 is also known as ZC3H12A. ZC3H4 is a newly identified protein that is similar to ZC3H12A, and both belong to the family of CCCH-type zinc finger proteins. There are very few correlative reports about ZC3H4. In particular, whether ZC3H4 has a role in the activation of AMOs and in the development of silicosis is completely unknown. Our study reveals a novel regulatory mechanism of circZC3H4 RNA/ZC3H4 in silicosis, providing a comprehensive landscape of circZC3H4 RNA/ZC3H4 that will facilitate further discoveries in the progression of pulmonary fibrosis.

**MATERIALS AND METHODS**

**Animals**

C57BL/6N mice (male, 6–8 wk old) were purchased from the Comparative Medicine Center, Yangzhou University (Yangzhou, China). All animal procedures were performed in strict accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and the animal protocols were approved by the Institutional Animal Care and Use Committee of Southeast University.

**Reagents**

Fetal bovine serum (FBS), normal goat serum (DMEM; 1200-046), and 10× minimum essential medium (11430-030), and Pen-Strep (15140-122) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Amphotericin B (BP2645) and GlutMax supplement (35050-061) were Gibco brand (Thermo Fisher Scientific). Silicon dioxide (SiO2) was purchased from MilliporeSigma (S5631; Billerica, MA, USA); it was prepared for use via sedimentation according to Stokes’ law, acid hydrolyzed, and baked overnight (200°C, 16 h) to inactivate contaminating endotoxins. The silica samples were suspended in PBS at a concentration of 5 mg/ml, and the volume applied was 20 μl/well in a 24-well plate, which corresponds to a silica dosage of 50 μg/cm² for the cell experiments. Rabbit pAb to ZC3H4 (20041-1-AP) and NOS2 (18985-1-AP) were obtained from the Proteintech Group (Rosemont, IL, USA), and ARG1 (9819S) and SOCS-3 (2923S) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; SC32233) were from Santa Cruz Biotechnology (Dallas, TX, USA), and β-actin (AP0060) was obtained from Bioworld (Irving, TX, USA). F4/80 (ab100790) were purchased from Abcam (Cambridge, MA, USA). The short interfering RNA (siRNA) transfection reagent (SC29528) and the DUX2 clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) knockout plasmid (SC-417443) were purchased from Santa Cruz Biotechnology.

**Cell culture**

The RAW264.7 and L929 (ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in T25 flasks in DMEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO₂ atmosphere. To conduct experiments, we seeded cells in 24-well plates at a concentration of 1 × 10⁵ cells/ml for 24 h before further treatment. The cell concentration was adjusted according to the requirements of specific experiments.

**Western blotting**

The expression levels of specific proteins in RAW264.7 macrophages were determined by Western blotting in conjunction
with our experiments. After treatment, RAW264.7 cells cultured in 24-well plates were washed twice with cold PBS and lysed with cell lysis solution containing proteinase inhibitors. The samples were subjected to freeze-thawing, and total cell proteins were harvested. The protein concentration was quantified with the bicinchoninic acid assay, according to the manufacturer’s instructions. Protein samples were separated via SDS-PAGE gel electrophoresis and transferred to PVDF membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline and Tween 20 for 2 h at room temperature, the membranes were incubated with primary antibodies at 4°C overnight and then with secondary antibodies in 5% nonfat dry milk in Tris-buffered saline and Tween 20 for 1 h at room temperature. Chemiluminescence was used to detect the immunoreactive protein bands. Each Western blot was repeated independently ≥3 times. The expression level of GAPDH was used as a reference.

Immunofluorescence staining

After experimental treatment of cells seeded in 24-well plates on coverslips, the cells were washed twice with cold PBS to remove the remaining medium and fixed in 4% paraformaldehyde at 4°C overnight. The following day, the coverslips were blocked with 10% normal goat serum in 0.3% Triton X-100 for 2 h at room temperature and incubated with primary antibodies (ZC3H4 or F4/80) at 4°C overnight. The next day, the cells were incubated with the appropriate fluorescent secondary antibodies (Alexa Fluor, Thermo Fisher Scientific), and the nuclei were stained with DAPI. A fluorescence microscope (Olympus IX70, Olympus America, Inc., Center Valley, PA, USA) was used to capture images of the cells.

siRNA-mediated knockdown

We knocked down circRNAs with siRNA. The RNA interference protocol for a single well of a 24-well plate was as follows. Briefly, 9 μl of serum-free DMEM was combined with 1 μl of transfection reagent, and 1 μl of siRNA stock was added to 9 μl of serum-free DMEM; both solutions were then incubated at room temperature and incubated with primary antibodies (ZC3H4 or F4/80) at 4°C overnight. The next day, the cells were incubated with the appropriate fluorescent secondary antibodies (Alexa Fluor, Thermo Fisher Scientific), and the nuclei were stained with DAPI. A fluorescence microscope (Olympus IX70, Olympus America, Inc., Center Valley, PA, USA) was used to capture images of the cells.

Target DNA deletion by CRISPR/Cas9 technology

The DUX2 CRISPR/Cas9 knockout plasmid was purchased from Santa Cruz Biotechnology. The protocol described here is for a single well of a 24-well cell-culture plate; the cell and reagent amounts were adjusted according to the demands of the experiments. RAW264.7 cells were seeded in 24-well plates at a density of 2 × 10⁶ cells/well in antibiotic-free standard growth medium 24 h before transfection. When the cells reached 40–80% confluency, the medium was replaced with 200 μl of fresh antibiotic-free growth medium, and solutions A and B were prepared as follows: For solution A, 1 μl of transfection reagent was added to 9 μl of plasmid transfection medium; for solution B, 1 μl of plasmid DNA was added to 9 μl of plasmid transfection medium. After 5 min, solution B was dropped dropwise directly to solution A; the sample was then immediately vortexed and incubated at room temperature for ≥20 min. The mixed solution was added dropwise to the 200 μl of medium in the 24-well plate, and the contents of the well were mixed by swirling the plate gently. Medium was added or replaced when necessary 12 h after transfection. The cells were incubated for an additional 24–72 h to conduct further experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were seeded in a 96-well plate at a density of 6 × 10⁴ cells/well and cultured in an incubator containing 5% CO₂ at 37°C for 24 h. Different seeding densities were employed in the initial experiments. After treatment, 20 μl of a 5 mg/ml MTT solution was applied to the treated cells in each well, and the plates were incubated in a 5% CO₂ incubator for 1–4 h at 37°C. Finally, the medium was removed from each well, and 200 μl of DMSO was added. To fully dissolve the formazan crystals, the plate was agitated on a shaker for at least 10 min. The absorbance of each well was then measured with a microplate reader (BioTek Instruments, Winooski, VT, USA) at a reference wavelength of 570 nm. Each experiment was repeated ≥3 times.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) was performed to determine the relative expression of circRNA-0001544, miR-212, and ZC3H4 mRNAs. Total RNA was isolated from RAW264.7 macrophages using Trizol reagent (Thermo Fisher Scientific). After the extraction of total RNA, the concentration of RNA was detected by NanoDrop-One (Thermo Fisher Scientific). The various samples were normalized to contain 400 ng RNA and reverse-transcribed into cDNA; the products of reverse transcription were used as templates for real-time qRT-PCR in specialized, 48-well plates for PCR. The intersection points of the amplification curve and the threshold lines were used to calculate the cycle threshold values of the samples. The relative quantitative expression of RNA was normalized to that of the recognized marker gene GAPDH.

Nested-matrix model and cell migration assay

A 3-dimensional (3D) migration model was used, as described previously, with some modifications (26). For the nested, attached matrix, a standard fibroblast-populated, 3D collagen matrix (FPCM) was incubated in the attached state for 48 h in DMEM containing 5% FBS. The FPCM was then removed from the culture well and placed in 60 μl of fresh acellular collagen matrix solution centered inside a 12-mm-diameter, scored area on the bottom of a new culture well. Next, 140 μl of acellular collagen matrix solution was used to cover the newly transferred FPCM. The matrix system was allowed to polymerize for 1 h at 37°C in an atmosphere of 5% CO₂; then, 1 ml of DMEM containing 10% FBS was added to the well. In these experiments, we used conditioned medium from RAW264.7 macrophages that had undergone 1 of 4 treatments. Fresh medium was mixed with the prepared conditioned medium at a ratio of 1:1. Cell migration from the nested FPCM to the acellular matrix was observed by fluorescence microscopy at 24, 48, and 72 h and compared with migration in samples observed at 0 h. Digital images of the interface of the nested FPCM with the acellular matrix were captured with an EVOS FL cell-imaging microscope (Thermo Fisher Scientific). Pulmonary fibroblast (PFB) migration from the nested FPCM was quantified by counting the number of cells that had clearly migrated from the nested matrix to the cell-free matrix; the
maximum migration distance was quantified by identifying the cell that had migrated the greatest distance from the nested matrix to the cell-free matrix and measuring that distance. The number of cells per field that had migrated from the nested matrix and the maximum migration distance per field were averaged.

**FISH**

We seeded RAW264.7 cells on coverslips at $1 \times 10^4$ cells/well in a 24-well plate. After experimental treatment, the cells were washed twice with cold PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.25% Triton X-100 for 15 min and prehybridized in hybridization solution for 1 h at 37°C. The cells were then incubated with the labeled probe in hybridization solution at 37°C overnight. The following day, the coverslips were washed and incubated in blocking buffer for 1 h at room temperature. The samples were then incubated with primary antibodies (antidigoxigenin-horseradish peroxidase or FITC) at 4°C overnight. The next day, the cells were incubated with the appropriate fluorescent secondary antibodies (cyanine 5) and mounted with DAPI. A fluorescence microscope was used to capture the cell images.

**RNA pulldown**

Probes for miR-212 were biotinylated and transfected into cells in culture dishes. After 72 h of transfection, the cells were collected and lysed on ice. Dynabeads (Thermo Fisher Scientific) were incubated with RNase-free bovine serum albumin and yeast RNA pulldown before use to reduce nonspecific binding. The samples were incubated with beads for 3 h at 4°C and centrifuged at 3000 rpm for 5 min to separate the beads from the samples. The beads were then washed with buffer solution. The sample RNAs were extracted and analyzed by qRT-PCR, as described previously.

**Human bronchoalveolar lavage fluid**

Human bronchoalveolar lavage fluid (BALF) was obtained from Nanjing Chest Hospital (Nanjing, China). Primary AMOs derived from human BALF were used to perform the experiments in our study in accordance with the approved guidelines of the Research and Development Committee of Nanjing Chest Hospital. After the BALF was filtered through multilayered gauze, it was centrifuged in a 50-ml centrifuge tube at 4°C for 10 min at 1800 rpm. After discarding the supernatant, the cells were resuspended in serum-free medium, counted, seeded in a 24-well plate at $5 \times 10^4$ cells/well, and incubated for 2 h at 37°C in an atmosphere of 5% CO₂. The serum-free medium was then removed, and each well was washed twice with cold PBS to remove nonadherent cells and cell debris. The remaining cells, 95% of which were macrophages, were cultured in DMEM containing FBS and used in further experiments.

**circBase database**

The interaction between circZC3H4 RNA and miR-212 was predicted based on information in the circBase database (Max Delbruck Center for Molecular Medicine, Berlin, Germany).

**Statistical analysis**

The data analyses were conducted using Sigma Plot 11.0 software (Systat Software, San Jose, CA, USA). Unpaired numerical data were compared with an unpaired Student’s $t$ test (2 groups) or ANOVA (>2 groups). The significance level was set at 0.05; values of $P < 0.05$ were regarded as indicating statistical significance.

**RESULTS**

**SiO₂ induces AMO activation associated with ZC3H4 protein**

Recent evidence suggests that AMO polarization and activation have a crucial role in early inflammatory responses associated with fibrosis induced by SiO₂ (29–31). In this study, we examined the expression of F4/80, a macrophage marker, in an in vivo animal model and observed that the expression of F4/80 was significantly increased after 28 d of SiO₂ treatment compared with that in a normal saline (NS) (Fig. 1A). Concurrently, in vitro, we examined whether SiO₂ induced AMO activation in RAW264.7 cells. Macrophages comprise a heterogeneous population of cells that are essential for the initiation and resolution of pathogen- or damage-induced inflammation and are classified according to their functional polarization. M1 macrophages produce proinflammatory cytokines, whereas M2 macrophages secrete anti-inflammatory cytokines and promote tissue repair and remodeling as well as tumor progression. After SiO₂ treatment, we found that the levels of NOS2 (a marker of M1 macrophages) and ARG and SOCS3 (markers of M2 macrophages) in RAW264.7 cells were significantly increased in a time-dependent manner (Fig. 1B–D). These results showed that SiO₂ induced AMO activation not only in vivo but also in vitro.

Under conditions of AMO activation, we examined the ZC3H4 protein and discovered that ZC3H4 protein levels in RAW264.7 cells increased in a time-dependent manner within 48 h. ZC3H4 levels reached a peak at 6 h after the SiO₂ treatment (Fig. 1E, F). This finding was confirmed by immunostaining of the ZC3H4 protein in RAW264.7 cells (Fig. 1G).

**ZC3H4 is involved in SiO₂-induced AMO activation**

Although previous studies have shown that ZC3H4 protein levels increase with AMO activation, whether or not there exists a link between these 2 phenomena remains unknown. To study the effect of ZC3H4 protein on AMO activation after SiO₂ treatment, the CRISPR/Cas9 system was used to knock down ZC3H4 specifically (Fig. 2A, B). We measured the expression of AMO activation markers under these conditions and found that the increase in NOS2 and SOCS3 that is normally induced by SiO₂ treatment decreased to some extent (Fig. 2C, D). Moreover, previous studies have shown that SiO₂ causes AMO viability to decrease in a dose- and time-dependent manner. ZC3H4 protein knockdown rescued the decreased cell viability of AMOs induced by SiO₂ (Fig. 2E). These results indicated that the ZC3H4 protein was involved in AMO...
Figure 1. SiO\textsubscript{2} induces macrophage activation associated with ZC3H4 protein. A) Immunohistochemistry of the macrophage marker F4/80 and ZC3H4 protein in the lung tissues of mice exposed to NS or SiO\textsubscript{2}. Colocalization of F4/80 and ZC3H4 protein is shown. The images are representative of several individuals from each group (n = 5). Scale bar, 100 \mu m. B) Representative Western blots showing the effects of SiO\textsubscript{2} (50 \mu g/cm\textsuperscript{2}) on the expression of the M1 marker NOS2, the M2a marker ARG1, and the M2c marker SOCS3 in RAW264.7 cells. Densitometric analyses of Western blots from 5 separate experiments suggested that SiO\textsubscript{2} induced NOS2 (C) and ARG1 and SOCS3 (D) were expressed in a time-dependent manner. *P < 0.05 vs. the corresponding protein expression at 0 h. E) Representative Western blots showing the effects of SiO\textsubscript{2} (50 \mu g/cm\textsuperscript{2}) on the ZC3H4 protein in RAW264.7 cells. F) Densitometric analyses of Western blots from 5 separate experiments suggested that SiO\textsubscript{2} induced ZC3H4 expression in a time-dependent manner. *P < 0.05 vs. 0 h. G) Representative images of immunocytochemical staining showing that SiO\textsubscript{2} (50 \mu g/cm\textsuperscript{2}) increased the ZC3H4 protein in RAW264.7 cells 24 h after the initiation of SiO\textsubscript{2} treatment. Scale bars, 10 \mu m.
activation and that it influenced cell viability during AMO activation after SiO2 treatment.

To assess the functional relevance of the changes in the ZC3H4 protein in AMOs to SiO2-induced fibrosis, conditioned medium from AMOs was collected and used in a 3D cell migration assay involving fibroblasts. Considerable evidence suggests that PFB migration is a critical aspect of pulmonary fibrosis. The use of 3D

Figure 2. ZC3H4 protein is involved in SiO2-induced functional changes in cells. A) Representative Western blot showing that transfection with Notch intracellular domain (NIC) down-regulated the ZC3H4 protein in RAW264.7 cells. B) Densitometric analyses of Western blots from 5 separate experiments suggested that transfection with ZC3H4 NIC down-regulated the ZC3H4 protein in RAW264.7 cells (n = 5). *P < 0.05 vs. the control (Con) group. #P < 0.05 vs. the Con-NIC of the SiO2 group. C) Representative Western blots showing the effects of ZC3H4 NIC transfection on SiO2-induced NOS2 and SOCS3 expression in RAW264.7 cells. D) Densitometric analyses of Western blots from 5 separate experiments suggested that SiO2-induced NOS2 and SOCS3 expression was attenuated by ZC3H4 NIC transfection. *P < 0.05 vs. the corresponding Con-NIC group at 0 h, #P < 0.05 vs. the corresponding Con-NIC group. E) MTT assay showing the effect of ZC3H4 NIC transfection on the viability of RAW264.7 cells (n = 5). *P < 0.05 vs. the Con group, #P < 0.05 vs. the Con-NIC of SiO2 group. F) Representative images of the nested collagen matrix show the effect of conditioned medium from RAW264.7 cells on the migration of GFP-labeled L929 cells. Scale bar, 200 μm. Quantification of the number of cells that migrated from the nested gel (G) and the maximum migrated distance (H) from 6 independent experiments. *P < 0.05 vs. the Con group, #P < 0.05 vs. the Con-NIC of SiO2 group.
culture systems has facilitated the analysis of fibroblast physiology under conditions that more closely resemble the in vivo environment. Fibroblasts cultured in a 3D system were exposed to conditioned medium from AMOs. As shown in Fig. 2F–H, conditioned medium from SiO₂-treated AMOs significantly increased PFB cell migration, both as measured by the maximum migration distance and as measured by the number of migrating cells, whereas conditioned medium from the ZC3H4 protein knockdown group suppressed the increase in cell migration induced by SiO₂ treatment. These results suggested that SiO₂-induced activation related to the ZC3H4 protein could facilitate the migration of fibroblasts.

Expression profile of circZC3H4 in AMOs after SiO₂ treatment

The results described above showed that the ZC3H4 protein was closely associated with AMO activation and the migration induced by that activation. However, the mechanism underlying the regulation of those activities by the ZC3H4 protein remained unclear. To further elucidate the possible regulatory mechanism, our group conducted high-throughput screening of fibroblast samples from experimental and control groups. Compared with control mice, mice subjected to SiO₂ treatment for 28 d differentially expressed circRNAs (Fig. 3A). Among those RNAs, circRNA-0001544, which is transcribed from the Zc3h4 gene, caught our attention because the expression of that circRNA was increased in comparison to the control (Fig. 3B, C). Taking into account the special structure of circRNAs, we designed a specific primer for circZC3H4 RNA and examined its expression in AMOs in vitro (Fig. 3D). As shown in Fig. 3E, circZC3H4 RNA expression was up-regulated within 24 h after the model was established; its peak expression occurred at 3 and 6 h of treatment. We confirmed the expression of circZC3H4 RNA in SiO₂-treated AMOs by in situ hybridization (Fig. 3F).

Effect of circZC3H4 on ZC3H4 expression in AMOs

Having shown that the expression of circZC3H4 RNA and ZC3H4 protein increased in AMOs after SiO₂ treatment, we were interested in the potential involvement of that circZC3H4 RNA in the regulation of the expression of ZC3H4 protein and in AMO activation induced by SiO₂. To identify the connection between circZC3H4 RNA and ZC3H4 protein in AMOs after SiO₂ treatment, siRNA for circZC3H4 RNA was used to specifically knock down circZC3H4 RNA. We examined the expression of ZC3H4 protein and found that SiO₂ significantly up-regulated ZC3H4 protein expression, whereas knockdown of circZC3H4 RNA significantly attenuated the effect of SiO₂ (Fig. 4A, B). That phenomenon was confirmed by immunofluorescence staining (Fig. 4C). To determine the functional effects of circZC3H4 RNA on ZC3H4-related AMO activation, we measured the levels of NOS2 and SOCS3 and discovered that their expression decreased when circZC3H4 RNA was knocked down during SiO₂ treatment (Fig. 4D, E).

circZC3H4 RNA mediates macrophage activation by targeting miR-212

Although the results above show that circZC3H4 RNA can regulate both the expression of the ZC3H4 protein and AMO activation, the specific mechanism underlying that effect remains unclear. We, therefore, explored the potential mechanism by which circZC3H4 RNA regulates ZC3H4. Bioinformatics analysis indicated the presence of complementary base pairs between circZC3H4 RNA and miR-212, indicating the existence of potential binding sites between those RNAs, as shown in Fig. 5A. RNA in situ hybridization assays were conducted to verify the direct binding of circZC3H4 RNA to miR-212. Figure 5B shows the colocalization of labeled circZC3H4 RNA and miR-212. Using an RNA pulldown assay, we found that biotin-labeled circZC3H4 RNA could pull down miR-212 (Fig. 5C). Base-pair mutations of circZC3H4 RNA abrogated the ability to pull down miR-212, suggesting that circZC3H4 RNA and miR-212 bind directly to each other via base-pairing interactions.

circZC3H4 RNA/miR-212 regulates the expression of ZC3H4 protein

Based on the ability of circZC3H4 RNA to regulate ZC3H4 protein and on the demonstrated interaction between circZC3H4 RNA and miR-212, we speculated that circZC3H4 RNA may regulate ZC3H4 protein via miR-212. Further bioinformatics analysis revealed the existence of complementary base pairs in miR-212 and Zc3h4 mRNA (Fig. 6A). We examined the expression of miR-212 as well as that of Zc3h4 mRNA in AMOs after SiO₂ exposure and found that the expression of miR-212 showed an increasing trend and that Zc3h4 mRNA levels increased after 6 h of SiO₂ treatment (Fig. 6B, C). To confirm that miR-212 affects the expression of ZC3H4 protein, we employed an inhibitor (anti-miR-212) and a mimic (mimic–miR-212) of miR-212. The expression of ZC3H4 protein significantly increased after treatment with anti–miR-212 (Fig. 6D, E), whereas mimic–miR-212 suppressed ZC3H4 protein expression (Fig. 6F, G). These results demonstrated the effect of miR-212 on ZC3H4 protein and showed that miR-212 negatively regulated the expression of the ZC3H4 protein. To further demonstrate that the effect of miR-212 on circZC3H4 RNA regulated ZC3H4 protein, circZC3H4 RNA and miR-212 inhibitors were cotransfected to the cell line (Fig. 6H, I). We found that the effect of anti–miR-212 on ZC3H4 protein was abolished by circZC3H4-siRNA, indicating that circZC3H4 RNA regulated ZC3H4 protein via miR-212.
Figure 3. Differential expression of circRNAs in mouse lung tissues. A) Hierarchical clustering analysis of circRNAs that were differentially expressed in lung tissues of NS- and SiO2-treated mice. Each group contained 3 individuals (greater than a 2-fold difference in expression). $P < 0.05$. The expression levels are presented in different colors and indicate expression levels above and below the median expression level across all samples. B) Volcano plots were constructed using fold change values and $P$ values. The vertical lines correspond to 2.0-fold up- and down-regulation between the NS- and SiO2-treated groups, and the horizontal line represents the $P$ value. The red points in the plot represent the circRNAs that showed statistically significant differential expression. C) The scatter plot is a method used to visually assess the variations in circRNAs expression between NS- and SiO2-treated samples. The values corresponding to the $x$ and $y$ axes in the scatter plot are the normalized signals of the samples (log$_2$ scaled). The green lines indicate fold changes. The circRNAs above the top green line and below the bottom green line show a difference $>2.0$-fold in expression in the NS- and SiO2-treated groups. D) Divergent primers amplified circRNAs from cDNAs but not genomic DNA (gDNA). E) As shown in the qRT-PCR analysis, circZC3H4 was expressed in RAW264.7 cells exposed to SiO2 ($n = 5$). *$P < 0.05$ vs. circZC3H4 expression at 0 h. F) FISH assay showing circZC3H4 RNA expression in RAW264.7 cells exposed to SiO2; circZC3H4 RNA was labeled with FITC. Scale bar, 20 μm.
ZC3H4 protein levels increased in a human macrophage cell line and in AMOs from patients with silicosis

Previous data from our laboratory suggested that macrophage activation and apoptotic marker expression were increased in macrophages from the BALF of patients with silicosis (27, 32). We, therefore, extended our cell culture experiments to include examination of a human macrophage cell line and cells from patients with silicosis to validate our findings. As shown in Fig. 7A, B, ZC3H4 protein levels were significantly increased in human macrophage U937 cells after SiO2 treatment. Moreover, ZC3H4 protein levels were significantly increased in AMOs from the BALF of patients with silicosis (Fig. 7E). These results indicate that macrophages from patients with silicosis express increased levels of ZC3H4.
protein and that those cells undergo apoptosis and activation, thus promoting the development of silicosis (Fig. 8).

**DISCUSSION**

In the present study, we focused our attention on the newly identified CCCH-type protein ZC3H4. The official designation of ZC3H4 is zinc finger CCCH-type containing 4. In a previous study, our group demonstrated that another CCCH-type protein, MCPIP1, has a crucial role in AMO activation after exposure to SiO2 (28), and that activation is influential for the migration of downstream effector cells, that is, pulmonary fibroblasts. It has been reported that MCPIP1 is very widely expressed in many tissues and organs, especially in spleen, lung, thymus, and intestine; all of which contain macrophages in high abundance. MCPIP1 negatively regulates macrophage activation and has important effects in immunity and inflammatory diseases (33, 34). Here, we present a study of ZC3H4, in which we explored whether ZC3H4 has an effect similar to that of MCPIP1 on AMO activation and the activation-induced migration of pulmonary fibroblasts.

Zinc finger proteins comprise a superfamily of proteins that possess a typical zinc finger domain and are involved in many aspects of biology. Zinc finger proteins can be divided into different classes according to the types of fingers they possess. Most zinc finger proteins are of the CCHH or CCCC type. The CCCH-type zinc finger proteins are less common than other types in mammalian genomes and represent ~0.8% of all zinc finger proteins (35, 36). CCCH zinc finger proteins contain a motif consisting of 3 cysteines and 1 histidine residue. Among the numerous CCCH-type proteins, the tristetraprolin (TTP) family has been well studied. TTP can bind to adenylate-uridylate-rich elements within the 3'-UTRs of mRNAs, thereby affecting mRNA degradation. TTP knockout mice display a marked increase in the secretion of TNF and show an inflammatory phenotype (37). ZAP, another CCCH-type zinc finger protein, binds to viral RNAs and causes specific loss of some mRNAs (38). Roquin, another CCCH-zinc finger protein, functions as an ubiquitin ligase and is required for the repression of autoimmunity (39). We measured the expression of ZC3H4 protein and found that it increased significantly after SiO2 treatment, along with AMO activation. To further explore the functional effect of ZC3H4 protein on PFB, we collected conditioned medium and studied its effect on the 3D migration of PFB. The active constituents
Figure 6. circZC3H4 RNA acts as a sponge for miR-212. A) Bioinformatics analysis showing the 3 potential binding sites of miR-212 to Zc3h4 mRNA. B) As shown in the qRT-PCR analysis, SiO2 had no significant effect on the expression of miR-212 in 6 independent experiments. C) qRT-PCR analysis showing the effect of SiO2 on Zc3h4 mRNA expression. *P < 0.05 vs. the 0-h
of the collected, conditioned medium from the AMOs include cytokines and fibrosis factors. Our previous studies (27) have shown that macrophage-derived MCPIP1, another CCCH-type zinc finger protein, mediates silica-induced pulmonary fibrosis via autophagy and apoptosis, which includes some momentous signaling pathways. Here, our intent was to explore whether there existed other pathways. Our results suggest that ZC3H4 protein affects the migration of PFBs and thereby influences the development of pulmonary fibrosis via AMO activation.

The mechanism by which the ZC3H4 protein is regulated remains unknown. To explore its regulatory mechanism, we conducted high-throughput screening of samples from experimental animal models and controls, which showed differential expression of a number of circRNAs. Among those RNAs, expression of circZC3H4 RNA increased vs. the control. Because of the characteristics of circRNAs, that newly identified, noncoding RNA species has attracted a great deal of interest in recent years (12, 40, 41). However, many questions exist concerning the function of circRNAs. Some reports have demonstrated the enormous potential effects of circRNAs in diseases, even cancer, because circRNAs possess a powerful ability to regulate protein expression, a significant component of which is due to their miRNA sponge function. Recent research has revealed that complex interactions between miRNAs and regulatory proteins occur in eukaryotic cells. The 3'-UTR–located Alu elements may have a role in mobile regulatory modules by providing binding sites for miRNA regulation, and their abundance and ability to

group. D) Representative Western blot showing the effects of anti–miR-212 transduction on SiO2-induced ZC3H4 expression in RAW264.7 cells. E) Densitometric analyses of 5 separate experiments suggested that transduction of cells with anti–miR-212 significantly enhanced the ZC3H4 protein. *P < 0.05 vs. the control (Con) group, **P < 0.05 vs. the Con-anti of SiO2 group. F) Representative Western blot showing the effects of mimic–miR-212 transduction on SiO2-induced ZC3H4 protein in RAW264.7 cells. G) Densitometric analyses of 5 separate experiments suggested that transduction of cells with mimic–miR-212 significantly inhibited the SiO2-inhibited ZC3H4 protein. *P < 0.05 vs. the Con group, **P < 0.05 vs. the Con-mimic of SiO2 group. H) Representative Western blot showing the effects of cotransfection of anti–miR-212 and circZC3H4-siRNA on ZC3H4 protein in RAW264.7 cells. I) Densitometric analyses of 5 separate experiments suggested that circZC3H4-siRNA abolished the effect of anti–miR-212 on ZC3H4 protein. *P < 0.05 vs. the Con group.

![Figure 7.](image)

**Figure 7.** ZC3H4 protein is increased in a human macrophage cell line and AMOs from patients with silicosis. A) Representative Western blot showing ZC3H4 protein in the human macrophage cell line U937 after exposure to SiO2. B) Densitometric analyses of 5 separate experiments suggested that the ZC3H4 protein in U937 cells was elevated after SiO2 treatment. *P < 0.05 vs. the 0-h group. C) Representative Western blot showing ZC3H4 protein in AMOs from healthy donors and patients with silicosis. D) Densitometric analyses of macrophage samples from 5 healthy donors and 5 patients with silicosis suggested that the ZC3H4 protein was elevated in macrophages from patients with silicosis. *P < 0.05 vs. the healthy group. E) Immunohistochemistry of the macrophage marker F4/80 and the ZC3H4 protein in the lung tissues of patients with silicosis. Colocalization between F4/80 and ZC3H4 protein is shown. Scale bar, 100 μm.
present specific sets of miRNA target sites may be an important role (42). Although the number of verified, human miRNAs is still expanding, only a few human miRNAs have been functionally annotated. In this study, we explored the effect of circZC3H4 RNA on ZC3H4 protein. Our results showed that this circRNA affected the expression of the ZC3H4 protein and AMO activation related to ZC3H4 after SiO2 treatment. We also examined the expression of circZC3H4 RNA and zc3h4 mRNA and found both were up-regulated in a time-dependent manner after SiO2 stimulation. Because circZC3H4 RNA is derived from the parent gene Zc3h4, which encodes ZC3H4 protein, the expression of this circRNA and the corresponding mRNA may compete with each other (43), leading to changes in their expression. Indeed, research has shown that circRNA biogenesis competes with pre-mRNA splicing. Other research has demonstrated the interaction between circRNAs and miRNAs (12, 44–46). Bioinformatics analysis showed the presence of complementary base pairs in circZC3H4 RNA and miR-212, indicating that these RNAs possess potential binding sites that are the same as the complementary base pairs present in miR-212 and Zc3h4 mRNA. This information leads us to explore the regulation of the ZC3H4 protein by circZC3H4 RNA. Our data suggest that miR-212 significantly regulates expression of the ZC3H4 protein, and that regulation can be abolished by circZC3H4 RNA knockdown.

Taken together, the results of our study demonstrate that circZC3H4 RNA and miR-212 have competing endogenous effects. Through its binding sites for miR-212, circZC3H4 RNA is able to regulate miR-212 activity, thereby relieving the inhibition of the ZC3H4 protein by miR-212 and affecting AMO activation and its downstream effects on PFB migration after SiO2 exposure. We consider it likely that the circZC3H4 RNA and ZC3H4 pathway in SiO2-activated macrophages that we observed in cell culture, as well as the promotion of fibroblast proliferation and migration by the activated macrophages, also occur in actual pulmonary fibrosis but recognize that, at present, this is in only a speculative, albeit plausible, extrapolation in a clinical setting.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grant 81773796) and the National Key R&D Program of China (Grant 2017YFA0104303), the Natural Science Foundation of Jiangsu Province (Grant BK20141347), and the Postgraduate Research and Practice Innovation Program of Jiangsu Province (Grant KYCX17-0165). The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

X. Yang, J. Wang, and Z. Zhou performed the experiments, interpreted the data, prepared the figures, and wrote the manuscript; R. Jiang, J. Huang, L. Chen, Z. Cao, H. Chu, B. Han, and Y. Cheng performed the experiments and interpreted the data; J. Chao provided laboratory space and funding, designed the experiments, interpreted the data, wrote the manuscript, and directed the project; and all authors read, discussed, and approved the final manuscript.

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Received for publication October 10, 2017. Accepted for publication January 8, 2018.