ZC3H4 promotes pulmonary fibrosis via an ER stress-related positive feedback loop

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Abstract

Background

Pulmonary fibrosis is the sequela of many pulmonary diseases, such as pneumoconiosis and idiopathic pulmonary fibrosis. The principal characteristics of pulmonary fibrosis comprise myofibroblast proliferation, alveolar damage and deposition of extracellular matrix components, which causes abnormal lung structure remodeling and an irreversible decline in lung function; however, the detailed mechanisms remain unclear. The current study focused on the role of ZC3H4, a new member of the zinc finger protein family, in SiO₂-induced pulmonary fibrosis.

Methods

The expression of ZC3H4 and fibroblast activation markers (COL1A1, COL3A1 and ACTA1) was measured by western blotting and immunofluorescence staining after SiO₂ exposure (50 µg/cm²). The functional change in fibroblasts was studied with a scratch assay and a 3D migration assay. The CRISPR/Cas9 system was used to explore the regulatory mechanisms of ZC3H4 in pulmonary fibroblast cells.

Results

The expression levels of ZC3H4 and sigmar1 (a key regulator of ER stress) were increased in pulmonary fibroblast cells and were associated with fibroblast activation, as indicated by the increase in COL1A1, COL3A1 and ACTA1, as well as the migration ability. The SiO₂-enhanced fibroblast activation was attenuated by specific knockdown of ZC3H4 and inhibition of ER stress, demonstrating that ZC3H4 activated fibroblasts via the sigmar1/ER stress pathway. Interestingly, an ER stress blockade also inhibited ZC3H4 expression, indicating the positive feedback regulatory mechanism of ER stress on ZC3H4.

Conclusions

Our results demonstrate that ZC3H4 and sigmar1 might act as novel therapeutic targets for silicosis, providing a reference for further pulmonary fibrosis research.

Introduction

Silicosis is a chronic occupational disease caused by exposure to dust particles containing a high level of free silica-containing silicon dioxide (SiO₂), and these particles are often found in mining, quarry, metal foundries and other sandy industrial environments (1, 2). Silicosis is a severe problem in developing and

developed countries. Although preventive efforts have been implemented for many years, silicosis remains an incurable, fatal and disabling pulmonary disease characterized by pulmonary interstitial fibrosis and silicotic nodule formation (3). Silicosis progression is due to a lack of a hygienic measures and inadequate surveillance (4), and silicosis has a complex molecular and biological mechanism (5). Additionally, the prevalence and frequency of silicosis remain very high, and currently, no effective therapies are available (6).

Despite comprehensive studies examining the toxicity of crystalline silica over the last several years, the exact etiology and particular mechanism of silicosis remain unknown. The deposition of silica particles in the alveoli of the lungs is the beginning of the pathophysiological process of silicosis. Silica particles ingested by lung cells induce an inflammatory response that produces large amounts of collagen and stimulates fibroblast overproliferation (7). The principal characteristics of pulmonary fibrosis in silicosis include fibroblasts, myofibroblast proliferation, alveolar damage and deposition of extracellular matrix (ECM) components, which involve the abnormal remodeling of lung structure and an irreversible decline in lung function (8). Alveolar cells are the basic and essential immune barrier against a pathogenic organism and try to overcome invasion by this pathogenic organism and innate pulmonary immunity against environmental pollutants. When the free pathogenic bacteria and sand particles in the air reach the alveoli of the lung, they are triggered and removed by alveolar cells. Pulmonary fibroblasts pathologically indicate tissue fibrosis, fibroblast and myofibroblast proliferation with elevated expression levels of alpha-smooth muscle actin (α -SMA, ACTA1), collagen I (Col I, COL1A1) and collagen III (Col III, COL3A1) and deposition of ECM components (9, 10).

Mounting evidence has suggested that CCCH-type zinc finger proteins, such as ZC3H12A/MCPIP1, play an essential role in regulating cell proliferation and migration (11), modulating signaling pathways and targeting mRNA degradation (12–14). ZC3H4 is a newly found member of the CCCH-type zinc finger protein ZC3H12A (12), which has been shown to be involved in inflammation and fibrosis induced by SiO₂ via autophagy and endoplasmic reticulum (ER) stress (15, 16). The ER plays a significant role during the modification of posttranslational transmembrane and secretory proteins. Usually, proteins are modified in the ER before being transferred and folded. This process is energy-dependent and sensitive to ion variations, such as in the calcium levels, oxygen balance, and glucose supply. (17). Specific signaling pathways are activated due to ER stress, and this process is known as the unfolded protein response (UPR). Three essential proteins are involved in these signaling pathways, including inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like ER kinase (PERK) (18). ZC3H4-associated ER stress in pulmonary fibroblasts has received little consideration. In the current study, we focused on the role of ZC3H4 in pulmonary fibroblast activation via ER stress with the involvement of the MAP kinase pathway in silicosis, which will facilitate further discoveries in the progression of pulmonary fibrosis, and silicosis patients may provide a treatment strategy.

Materials And Methods

Reagents

Fetal bovine serum (FBS), 10x minimum essential medium (11430-030), Pen-Strep (15140-122) and Dulbecco's modified Eagle's medium (DMEM; 1200-046) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). GlutaMAX supplement (35050-061) and amphotericin B (BP2645) were purchased from Gibco (Thermo Fisher Scientific). Silicon dioxide (SiO₂) was obtained from MilliporeSigma (S5631; Billerica, MA, USA) and because it had more than 80% particle size diameter less than 5 µm, it was selected for use *via* sedimentation according to stocks' law, acid hydrolyzed and sterilized by baking overnight (200°C, 16 h) to inactivate infectious endotoxins (19). Phosphate-buffered saline (PBS) was used for the suspension of crystalline silica at a final concentration of 5 mg/ml, and for the cellular experiments, a dosage of 50 µg/cm² was applied. The crystalline silica dosage administered in this study was based on our previous experimental studies (19, 20).

Antibodies against ZC3H4 (20041-1-AP, rabbit), COL3A1 (22734-1-AP, rabbit) and ACTA1 (14395-1-AP, rabbit) were purchased from Proteintech, Inc. (Rosemont, IL, USA) antibodies against Sigmar1 were purchased from Invitrogen (Santa Cruz Biotechnology, Inc.); and antibodies against COL1A1 (BS1530, rabbit) and GAPDH (MB001, mouse) were purchased from BioWorld, Inc. Antibodies against BIP (3177, rabbit), ERN1 (3294, rabbit) and DDIT3 (2895, mouse) were purchased from CST, Inc., and an antibody against Actb (AP0060, rabbit) was obtained from BioWorld (Irving, TX, USA). Antibodies against p53 (SC6243, rabbit) were purchased from Santa Cruz Biotechnology, Inc.

Cell culture experiment

Mouse lung fibroblast (MLg 2908™) cells purchased from ATCC® and DMEM were cultured with 10% FBS, 100 µg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-GlutaMAX (Gibco) at 37°C in a humidified incubator and 5% CO₂ atmosphere (Thermo Heracell 150i CO₂ incubator, ThermoFisher Scientific, Inc., Germany). The cell concentration was adjusted according to the specific experimental requirements.

Western blot assay

The specific protein levels in pulmonary fibroblasts were determined by a western blot assay. After treatment, pulmonary fibroblasts cultured in 24-well plates and cold PBS were washed twice, and a cell lysis solution was used to lyse cells containing proteinase inhibitors (PI). The samples were freeze-thawed, and total cell proteins were harvested. The protein concentration was measured with the bicinchoninic acid assay (BCA) according to the manufacturer's instructions. Samples containing protein were separated via SDS-PAGE and transferred to PVDF membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline and Tween 20 for 1 hr at room temperature (25°C), 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 hr at room temperature. To identify the intensity of immune-reactive protein bands, chemiluminescence (ECL) was used. Each western blot was repeated independently >5 times. The expression level of GAPDH was used as a reference. The protein bands were quantified using ImageJ v1.48 software.

Cell counting kit 8 (CCK8)

Pulmonary fibroblasts were seeded into 96-well plates, and triplicate wells were used for a single sample (4×10^3 cells/well) and cultured overnight with exposure to silica at different time points (0, 1, 3, 6, 9, 12, 15, 21 and 24 hr). CCK8 reagents (10 μ l/well) were administered to the wells of the plate and incubated at 37°C for 2-4 hr. After incubation, the absorbance was measured at 450 nm by a Spectra Max Microplate Reader (Molecular Devices Instruments, Inc., Sunnyvale, California, USA).

Cell migration assays

Cell migration and motility assays were performed to observe pulmonary fibroblast migration after crystalline silicon dioxide (SiO₂) exposure as described previously (21). The cell gap was measured using ImageJ software.

CRISPR/Cas9 technology

Pulmonary fibroblasts were quickly transfected with CRISPR/Cas9 plasmids according to the manufacturer's protocol (Santa Cruz®) to delete/upregulate ZC3H4 and observe its downstream effects. The western blotting assay was used to determine the transfection efficiency. In brief, 24-well plates were used for cell seeding (2×10^5 cells /well), and the cells reached 40–80% confluency. The medium was changed to 200 μ l fresh antibiotic-free growth medium, and solutions A and B were added as follows. For solution A, transfection reagent (1 μ l) was poured into plasmid transfection medium (9 μ l), and for solution B, plasmid DNA (1 μ l) was poured into plasmid transfection medium (9 μ l). After 5 min, solution A was poured dropwise directly into solution B; the sample was then immediately vortexed and incubated at room temperature for >20 min. The mixed solution was added dropwise to 200 μ l of the medium in the 24-well plate, and the contents of the well were mixed by swirling the plate gently. The medium was added or replaced when necessary 12 hr after transfection. The pulmonary fibroblast cells were incubated for an additional 24–72 hr to conduct further experiments.

Immunocytochemistry staining

Immunofluorescence (IF) staining, a frequently used technique in clinical diagnosis and biological research, was performed to determine the fluorescence effect of ZC3H4 as previously described (22). Pulmonary fibroblasts were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. The fixed samples were permeabilized for 30 min at room temperature in PBS containing 0.3% Triton X-100 and then blocked with 10% normal goat serum (NGS; Life Technologies) in PBS containing 0.3% Triton X-100 at room temperature for 2 hr. The samples were blocked and incubated overnight at 4°C with pAb diluted in PBS containing 10% NGS and 0.3% Triton X-100. PBS was used to wash the samples three times, and the samples were incubated with donkey anti-rabbit (conjugated to Alexa Fluor 488) and donkey anti-mouse (conjugated to Alexa Fluor 576) secondary antibodies for 2 hr at room temperature. After PBS was used for three washes, the samples were fixed with mounting solution (ProLong Gold Antifade Reagent with DAPI; P36931, Life Technologies), and an EVOS FL fluorescence microscope was used to examine the prepared slides.

Real-time quantitative PCR

Total RNA was isolated from pulmonary fibroblasts and subjected to reverse transcription using a Prime Script RT Master Mix Kit (TaKaRa, RR036). Real-time quantitative PCR was performed by using the selected primers as follows: ZC3H4 (M) (forward primer (5'-3'): ATGCATGGTGACTTTCCGTGT; reverse primer (5'-3'): GCTTCTGCATCATCAGCCAAC), Sigmar1 (H) (forward primer (5'-3'): TATCGCAGTGCTGATCCA, reverse primer (5'-3'): TACTCCACCATCCACGTGTT) and GAPDH (M) (forward primer (5'-3'): ACCATCTTCCAGGAGCGAGAT; reverse primer (5'-3'): GGGCAGAGATGATGACCCTTT). The relative quantification was completed according to the manufacturer's instructions for TaKaRa SYBR Premix Taq.

Immunoprecipitation (IP)

The interactions between proteins were detected by IP assays as previously described (23). Briefly, equivalent amounts of the extracted proteins were incubated with anti-ZC3H4 antibodies overnight at 4°C, and then 20 µL of protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnology®, Inc.) was added for 90 min at 4°C. Then, the mixture was centrifuged (12000 × g, 3 min, 4°C), and the cell pellets were rinsed three times with RIPA lysis buffer. The cell pellets were later boiled in SDS loading buffer for 5 min. The mixture was then centrifuged (12000 × g, 3 min, 4°C). The collected supernatants were used in western blot assays to detect ZC3H4 and Sigma 1 receptor (Sigmar1).

Statistical analysis

The data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.). Unpaired numerical data were associated with an unpaired Student's t-test (2 groups) or ANOVA (2 groups). The level of significance was set at 0.05; values of $P < 0.05$ indicated statistical significance.

Results

SiO₂ increases pulmonary fibroblast activation

Pulmonary fibroblasts pathologically indicate tissue fibrosis, fibroblast and myofibroblast proliferation with elevated expression levels of ACTA, COL1A1, and COL3A1 and deposition of ECM components (9, 10). Fibroblast activation markers, such as COL1A1, COL3A1 and ACTA1, were measured by an immunoblotting assay (western blot), CCK8 cell counting kit and immunofluorescence staining after SiO₂ exposure (50 µg/cm²). Based on earlier experimental dosages, 50 µg/cm² was selected for all the related experiments (19, 24-27). Western blotting results confirmed that fibroblast activation markers such as COL1A1, COL3A1 and ACTA1 were elevated in the presence of SiO₂ after 3 hr (Fig. 1A-C). In addition, the cell viability of pulmonary fibroblasts increased after 12 h of addition of silica (50 µg/cm²) (Fig. 1D). Immunocytochemistry assays confirmed the upregulation of COL1A1, ACTA1 (Fig. 1E and F) and COL3A1 expression (Supplementary Fig. S1) induced by SiO₂ in pulmonary fibroblasts.

SiO₂ increases migration in pulmonary fibroblasts

Two-dimensional (2D) and 3-dimensional (3D) nested collagen matrix assays significantly represent the cell migration ability in *in vitro* culture (28-30). Compared with usual scratch assays, the nested collagen matrix (3D) assay is a reliable, quick, quantitative and easy method for determining fibroblast migration and motility in 3D models (6, 28, 31, 32). Here, we provide new insights into the novel roles that pulmonary fibroblasts exposed to SiO₂ were assessed via 2-dimensional and 3-dimensional nested collagen matrix gel assays, and their responses were examined in both models at the indicated time points. Although SiO₂ induced fibroblast migration and proliferation differently in 2-dimensional and 3-dimensional nested collagen matrix gel systems, the peak response of fibroblasts was observed at 24 hr. The scratch assay/wound healing assay (Fig. 2 A-B) demonstrated that SiO₂ significantly induced migration in MLg cells. Therefore, the schematic diagram of the 3D nested collagen matrix gel assay represents the migration of pulmonary fibroblasts after SiO₂ exposure (Fig. 2C). Pulmonary fibroblasts exhibited an increase in both migration distance (Fig. 2D-E) and number of migrated cells (Fig. 2D and F).

SiO₂ induces ZC3H4 in pulmonary fibroblasts

A previous study from our laboratory suggested that ZC3H4 is involved in the inflammatory stage of silicosis. To establish whether ZC3H4 is also involved in late fibrosis, MLg cells were exposed to SiO₂. Western blot assays confirmed that the ZC3H4 expression level was suddenly increased after SiO₂ exposure in pulmonary fibroblasts at different time points (0, 1, 3, 6, 12 and 24 hr) with a two-phase increase pattern (Fig. 3A-B). Immunocytochemistry confirmed the upregulation of ZC3H4 expression induced by SiO₂ in pulmonary fibroblasts (Fig. 3C).

To simplify the role of ZC3H4 in fibroblast activation induced by SiO₂, we transfected MLg cells with ZC3H4 NIC plasmids to specifically knock down ZC3H4. Western blot assays confirmed that the specific knockdown of ZC3H4 in pulmonary fibroblasts significantly inhibited the upregulation of ZC3H4 with or without SiO₂ (Fig. 3D-E). In the cell viability assay, ZC3H4 knockdown attenuated the increase in cell viability induced by SiO₂ (Fig. 3F), indicating that ZC3H4 mediated the proliferation induced by SiO₂ in fibroblasts. Moreover, ZC3H4 NIC plasmids abolished the effect of SiO₂ on the migration ability of MLg cells (Fig. 3G-H). Furthermore, the COL1A1 and ACTA1 expression levels induced by SiO₂ were assessed after the specific knockdown of ZC3H4 (Fig. 3I and J), in which all upregulated markers were inhibited, confirming the role of ZC3H4 in pulmonary fibroblast activation and migration.

SiO₂ induces ER stress in pulmonary fibroblasts

To depict the downstream molecular mechanism of ZC3H4 on fibroblast activation, ER stress was evaluated. The ER is a key factor for cellular activities (protein modification, folding, synthesis and transport) (33-35). Various pathological and physiological environmental factors could affect ER homeostasis, finally causing ER stress (36, 37). ER stress is responsible for pulmonary tract infections (38) and various types of lung disease (39, 40). UPRs are essential factors for maintaining homeostasis

and initiating the BIP and CHOP pathways (41). In a western blot assay, we measured the ER stress markers ERN1 (inositol-requiring enzyme 1, IRE1 α), BiP (binding immunoglobulin protein, BIP) and DDIT3 (C/EBP homologous protein, CHOP) in pulmonary fibroblasts after SiO₂ exposure. MLg cells treated with SiO₂ showed upregulation of ERN1, DDIT3 and BiP expression in a time-dependent manner with an ERN1 peak of 3 and 6 hrs, DDIT3 peak of 3 hrs and BiP peak of 12 hrs (Fig. 4 A-D). Immunocytochemistry assays were applied to confirm the colocalization of ZC3H4, BiP and DDIT3 (Fig. 4E-F). To further confirm the role of ZC3H4 in ER stress, ZC3H4 was specifically knocked down. As shown in Fig. 4G-H, ZC3H4 knockdown abolished the induction of ER stress induced by SiO₂. Collectively, these results indicated that ZC3H4 is responsible for fibroblast activation induced by SiO₂ via the ER stress pathway.

ZC3H4 induced ER stress via Sigmar1 in pulmonary fibroblasts

Having established the fundamental role of ZC3H4 in pulmonary fibroblast activation via ER stress in response to silica, the detailed molecular mechanism of activation of ER stress was further investigated. A previous study from our laboratory suggested that Sigmar1, a subclass of the sigma receptor family, mediated ER stress in silicosis (41). Sigmar1 is expressed in the ER with two steroid-binding domains and two transmembrane segments (42). The molecular action of Sigmar1 was previously discovered to be a ligand-regulated receptor chaperone via ER stress (43). In particular, whether ZC3H4 has a vital role in the activation of pulmonary fibroblasts via the sigmar1/ER stress pathway deserves investigation. To determine whether Sigmar1 is involved in silicosis, MLg were exposed to SiO₂ to assess the Sigmar1 level. Western blotting results confirmed that the expression level of Sigmar1 was increased in the presence of SiO₂ (Fig. 5A-B), which was confirmed by immunocytochemical staining (Fig. 5C). Further, specific inhibition of sigmar1 with the pharmacological agent BD1047 attenuated the upregulation of migration induced by SiO₂ (Supplementary Fig. S2A-B). Furthermore, the Sigmar1 expression level was assessed after the specific knockdown of ZC3H4, in which ZC3H4-NIC suppressed sigmar1 upregulation after SiO₂ exposure (Fig. 5D-E). Interestingly, the mRNA levels of ZC3H4 and Sigmar1 showed almost no change after SiO₂ exposure (Supplementary Fig. S3A-B). To further understand the regulatory effect of ZC3H4 on Sigmar1, Co-IP was conducted, in which there was a direct interaction between ZC3H4 and Sigmar1 (Fig. 5F). Taken together, these results verified that ZC3H4 and Sigmar1 are involved in the pulmonary fibroblast activation induced by SiO₂.

MAPK and PI3K pathways are involved in SiO₂-induced ZC3H4 upregulation

To further clarify the possible regulatory mechanism on ZC3H4, the involvement of the MAPK and P13K/Akt pathways was investigated since the MAPK and P13K/Akt pathways play an essential role in cell proliferation, migration and activation (44, 45). First, the short-term effect of SiO₂ on ZC3H4 was measured, in which a rapid and transient increase in ZC3H4 was observed. Then, the MAPK signaling pathway was assessed. As shown in Fig. 6C-D, phosphorylation of Mapk1, Mapk8 and Mapk14 was increased after SiO₂ exposure. Moreover, Akt phosphorylation showed a slight but significant increase after SiO₂ exposure (Fig. 6E-F). To assess the role of the MAPK or PI3K pathway on regulation of ZC3H4,

specific pharmacological inhibitors were applied. MLg cells were pretreated with a Mapk1 (SP600125) inhibitor, Mapk8 (SB203580) inhibitor, Mapk14 (U0126) inhibitor and P13K (LY294002) inhibitor separately for 1 hr, after which SiO₂ was applied. As shown in Fig. 6G-H, all inhibitors attenuated the increase in ZC3H4 and sigmar1 induced by SiO₂, confirming the role of the MAPK and PI3K pathways in the regulation of ZC3H4 and Sigmar1. Furthermore, fibroblast activation markers such as COL1A1 and ACTA1, as well as the ER stress markers DDIT3 and BiP, were inhibited by pretreatment with inhibitors (Supplementary Fig. 4A-E). These results demonstrated that the MAPK and PI3K/Akt pathways play a significant role in regulation of ZC3H4.

Induction of ZC3H4 by SiO₂ promotes a further increase in ZC3H4

Interestingly, SiO₂ induced a two-phase increase pattern in ZC3H4 (Fig. 3A-B), in which the early increase was due to the activation of MAPK and PI3K. The mechanism of the late increase in ZC3H4 deserves to be investigated since pulmonary fibrosis is a chronic pathological process. As ZC3H4-induced ER stress may increase the UPR in the ER lumen (46), whether ER stress affect the abnormal increase in ZC3H4 needs to be clarified. To our surprise, while salubrinal inhibited the increase in ER stress markers (Supplementary Fig. 5A-B), salubrinal reversed the SiO₂ effect in the upregulation of ZC3H4 in pulmonary fibroblasts (Fig. 7A-B). Furthermore, tunicamycin, a specific ER stress inducer, was applied to further explore the role of ER stress. As expected, tunicamycin induced the expression of ER stress markers in a dose-dependent manner, and ZC3H4 was also upregulated (Fig. 7. C-F). Furthermore, pulmonary fibroblasts were further exposed to tunicamycin, in which the ZC3H4 level was increased at a peak at 24 hrs, while there was no rapid increase within 6 hrs, which showed a different pattern compared with direct SiO₂ exposure (Fig. 7 G-H). Immunocytochemistry assays confirmed the upregulation of ZC3H4 expression after 50 µM tunicamycin in pulmonary fibroblasts (Fig. 7 I). These results demonstrated that ER stress was involved in the upregulation and activation of ZC3H4 in pulmonary fibroblasts in the late phase, indicating that the positive feedback loop (PFL) may be involved (Fig 8). Taken together, these results demonstrate that ZC3H4/ER stress plays a significant role in the activation of pulmonary fibroblasts.

Discussion

Pulmonary fibrosis is the sequela of many pulmonary diseases, such as idiopathic pulmonary fibrosis and pneumoconiosis. Pulmonary fibroblasts pathologically indicate tissue fibrosis and fibroblast and myofibroblast proliferation (9, 10). The current study principally focused on the role of ZC3H4 and sigmar1 on the proliferation, migration and activation of pulmonary fibroblasts exposed to SiO₂, which may provide a target for pulmonary fibrosis treatment, and the detailed molecular mechanism was also investigated.

Pulmonary fibroblasts clinically represent the proliferation of fibroblasts and myofibroblasts with the deposition of ECM components (9, 10). The zinc finger protein family has been shown to play a potential role in the inflammatory response and represents a downregulation of the inflammatory reaction, as well

as fibroblast activation, such as ZC3H12A/MCPIP1 (12, 13). ZC3H4 is similar to ZC3H12A, and both belong to the same family of CCCH-type zinc finger proteins (12). Interestingly, ZC3H12A may act as a molecular signal in the initiating pathway or as a functional factor to induce or inhibit inflammation, mainly depending on the time frame. The current study suggested a similar pattern of ZC3H4, in which ZC3H4 initiated ER stress as a signaling molecule in the early stage and promoted fibroblast migration and proliferation in the late stage, forming PFLs. PFLs are responsible for producing all-or-none responses by continuously converting graded inputs into discrete outputs (56, 57) and transforming fibroblasts to myofibroblasts (58). Mounting evidence has shown that fibrosis in pneumoconiosis and IPF is a chronic pathological process in which both signaling molecules and the TGF- β pathway are involved. CCCH-type zinc finger proteins participate in PFLs to initiate fibrosis and maintain the fibrosis process, indicating a suitable target for blocking or reserving this process. This two-phase increase pattern of ZC3H4 may guide new strategies for the treatment of fibrosis by an early blockade of ZC3H4 initiation.

Although the comprehensive mechanism of ZC3H4 in ER stress must be clarified, the present study recommends a novel role of ZC3H4 in ER stress. Previous studies have shown that ZC3H4 promotes epithelial-to-mesenchymal transition (EMT) via ER stress, which participates in the fibrosis induced by SiO₂ (16). Currently, the detailed mechanism by which ZC3H4 induces ER stress was investigated, in which ZC3H4 directly interacts with Sigmar1, which is expressed in the ER and a subclass of the sigma receptor family (42). The molecular action of Sigmar1 was previously discovered to be a ligand-regulated receptor chaperone via ER stress. Sigmar1 has been shown to be involved in fibroblast activation (41). Interestingly, the mRNA levels of both ZC3H4 and Sigmar1 did not show a significant change due to SiO₂ exposure, while these two proteins have been shown to be regulated by noncoding RNAs, such as circular RNAs (circRNAs) (15, 41). circRNAs are involved in cell proliferation and transformation, and their misregulation is related to diseases and can have phenotypes in animal models (47). However, given the low abundance of most circRNAs, whether the translation of certain circRNAs is crucial for their endogenous function remains unclear. Hence, the direct interaction between ZC3H4 and sigmar1 was investigated in the current study, but it did not rule out the role of circRNA in the regulation of these two proteins. Taken together, these results verified that ZC3H4 and Sigmar1 were involved in the pulmonary fibroblast activation induced by SiO₂.

Although the present study still focused on the activation of pulmonary fibroblasts, it delivered a new direction to treat silicosis. MAPK signaling pathways were responsible for the activation of transcription factors. Activation of MAPK was involved in the upstream signaling pathways for ER stress induction (48). In addition, MAPK pathways such as p38, JNK, and ERK were also stimulated by ER stress inducers and played a significant role in different cell signaling pathways and cell death (49–52). Inositol-requiring enzyme 1 (IRE1) is responsible for the activation and stimulation of MAPK (51). After stress, fibroblast cells inhibit migration due to abnormal signaling pathway responses, although ER stress is responsible for lung diseases (39, 40, 53). Due to the rapid and transient increase in ZC3H4 after SiO₂ exposure within 6 hr, MAPK initiated the expression of ZC3H4. Recently, Ets-like protein-1 (Elk-1) has been shown to

mediate ZC3H4 expression (54) in epithelial cells, while Elk-1 also induced ZC3H12A in macrophages (55), which indicates a similar upstream regulatory mechanism for the CCCH zinc finger protein family. Taken together, these results demonstrate that MAPK/ZC3H4/ER stress plays a significant role in the activation of pulmonary fibroblasts.

Conclusion

Our study revealed a link between ZC3H4 and pulmonary fibroblast activation via the sigmar1/ER stress pathway in silicosis; SiO₂ exposure enhanced the MAPK and P13K/Akt phosphorylation pathways with the involvement of ZC3H4. Interestingly, ZC3H4 has been shown to induce ER stress with the participation of a PFL, providing a novel therapeutic strategy for treating silicosis (Fig. 8).

Abbreviations

SiO₂: silicon dioxide; MCP-1: monocyte chemotactic protein-1; DMEM: Dulbecco's modified Eagle's medium; NS: normal saline; FBS: fetal bovine serum, CCK8: cell counting kit 8, PFBs: pulmonary fibroblasts, MAPK: mitogen-activated protein kinase, JNK: C-Jun N-terminal kinase, ERK: extracellular-regulated kinase, CHOP: C/EBP homologous protein, ERN1: inositol-requiring enzyme 1, DDIT3: DNA damage-inducible transcript 3, BiP: binding immunoglobulin protein, ERN1: ER to nucleus signaling 1, Mapk8: mitogen-activated protein kinase 8.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All of the relevant raw data and materials are freely available to any scientist upon request.

Competing interest

The authors have declared no financial or competing interests.

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Author contributions

Hammad Ghafoor performed the experimental work, interpreted the data, prepared the figures and wrote the manuscript. H.C., J.H., M.L., Z.W. and S.W. performed the experiments and interpreted the data. J.C. provided the funding and laboratory space and designed and monitored all the experiments. All the authors carefully read, discussed and approved the final manuscript.

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Figures

Figure 1

SiO₂ increases pulmonary fibroblast activation. A. Demonstrative western blotting assay showing that SiO₂ increased the expression level of the fibroblast inducers COL1A1 (COL I), COL3A1 (Col III) and ACTA1 (α-SMA) at different time points (hrs) in MLg cells. B. Densitometric analyses of COL1A1 (COL I) and COL3A1 (Col III) from five independent experiments at different time points (hrs) in MLg cells (n=5): *p<0.05 vs. the control group. C. Densitometric analyses of ACTA1 (α-SMA) from five independent experiments at different time points (hr) in MLg cells (n=5): *p<0.05 vs. the control group. D. CCK8 assay showing that SiO₂ increased fibroblast and myofibroblast activation in MLg cells (n=5); *p<0.05 vs. the

control group at the corresponding time point. E. Demonstrative immunocytochemistry assay representing the effect of SiO₂ that increased the expression level of COL1A1 (COL I) in MLg cells, scale bar=50 μm. F. Demonstrative immunocytochemistry assay representing the effect of SiO₂ that increased the expression level of fibroblast inducer ACTA1 (α-SMA) in MLg cells, scale bar= 50 μm.

Figure 2

SiO₂ increases migration in pulmonary fibroblasts. A. 2-dimensional assay represents migratory distance after SiO₂ exposure by using a scratch in pulmonary fibroblasts from 3 independent experiments. Scale bar = 100 μm. B. Quantification of gap distance from 5 independent experiments. *p<0.05 vs. the control group at the corresponding time point after SiO₂ exposure. C. The schematic diagram of the 3-dimensional nested collagen matrix gel assay represents the migration of pulmonary fibroblasts after

SiO₂ exposure. D. The demonstrative diagram of the 3D nested collagen matrix assay represents the induced migratory characteristic of pulmonary fibroblasts after SiO₂ exposure. Scale bar=100 μm. E. Quantification of the MLg cells was determined by using the migratory cell distance (pixel), *p<0.05 vs. the control group F. Quantification of the MLg cells was determined by using the migrated cells, *p<0.05 vs. the control group.

Figure 3

SiO₂ induces ZC3H4 in pulmonary fibroblasts. A. Demonstrative western blot assay showing the effect of SiO₂ on ZC3H4 protein with elevated expression levels at different time points (hrs) in MLg cells. B. Densitometric analyses of the ZC3H4 protein from five independent experiments in MLg cells at different time points, *p<0.05 vs. 0 h. C. Descriptive immunocytochemistry assay showing that SiO₂ increased the

expression of the ZC3H4 protein in MLg cells. Scale bar=50 μ m. D. Descriptive western blot assay representing the effects of SiO₂ after the transfection of pulmonary fibroblast cells with (ZC3H4-NIC) ZC3H4 double nickase plasmid to observe the downregulation of ZC3H4 proteins. E. Densitometric analyses of five independent experiments showing that SiO₂ induced the expression level of ZC3H4 with attenuated CRISPR/Cas9-mediated ZC3H4 silencing in MLg cells (n=5), *p< 0.05 vs. the Con-NIC of control group, #p< 0.05 vs. the Con-NIC of SiO₂ group. F. CCK8 assay showing the effects of SiO₂ after the transfection of pulmonary fibroblast cells with a ZC3H4 double nickase plasmid (ZC3H4-NIC) to observe the downregulation of ZC3H4, but SiO₂ induced increased fibroblast and myofibroblast cell viability in MLg cells, *p< 0.05 vs. the Con-NIC of control group, #p< 0.05 vs. the Con-NIC of SiO₂ group. G. Descriptive pictures demonstrating that GFP-labeled MLg cells induced cell migration in a 2D assay after SiO₂ exposure, but ZC3H4NIC silencing abolished pulmonary fibroblast migration. Scale bar =100 μ m. H. Quantification of the gap (scratch) width was detected in a 2D nested matrix assay from five independent experiments. *p< 0.05 vs. the Con-NIC of control group, #p< 0.05 vs. the ZC3H4-NIC of control group. at the conforming time points. The effect of CRISPR/Cas9-mediated ZC3H4NIC silencing abolished pulmonary fibroblast migration in a 2D nested matrix assay. *p< 0.05 vs. the Con-NIC of control group, #p< 0.05 vs. the Con-NIC of SiO₂ group. I. Demonstrative western blotting representing the effects of CRISPR/Cas9-mediated ZC3H4NIC on pulmonary fibroblasts revealed that SiO₂ increased the fibroblast activation inducers COL1A1 and ACTA1. J. Densitometric analyses of COL1A1 and ACTA1 expression levels from five independent experiments in MLg cells. *p< 0.05 vs. the Con-NIC of control group, #p< 0.05 vs. the Con-NIC of SiO₂ group.

Figure 4

SiO_2 induces ER stress in pulmonary fibroblasts. A. Demonstrative western blotting assay representing the effects of SiO_2 that increased the expression level of ER stress markers such as ERN1, DDIT3 and BiP in MLg cells. B. Densitometric analyses of ERN1 from five independent experiments indicated that SiO_2 induced the expression level of ERN1 in MLg cells in a time-dependent manner, $*p < 0.05$ vs. 0 h. C. Densitometric analyses of DDIT3 from five independent experiments indicated that SiO_2 induced DDIT3 expression in MLg cells in a time-dependent manner, $*p < 0.05$ vs. 0 h. D. Densitometric analyses of BiP from five independent experiments indicated that SiO_2 induced the expression level of BiP in MLg cells in a time-dependent manner, $*p < 0.05$ vs. 0 h. E. Demonstrative immunocytochemistry assay images demonstrating the effect of SiO_2 that increased the expression levels of ZC3H4 and BiP in MLg cells,

scale bar=50 μ m. F. Demonstrative immunocytochemistry assay images demonstrating the effect of SiO₂ that increased the expression levels of ZC3H4 and DDIT3 in MLg cells, scale bar =50 μ m. G. Demonstrative western blotting assay representing the effects of CRISPR/Cas9-mediated ZC3H4NIC on pulmonary fibroblast cells verified that SiO₂ increased ER stress, such as DDIT3 and BiP. H. Densitometric analyses of DDIT3 and BiP expression levels from five independent experiments in MLg cells. *p< 0.05 vs. the Con-NIC of control group, #p< 0.05 vs. the Con-NIC of SiO₂ group.

Figure 5

SiO₂ induces ZC3H4 and Sigmar1 expression in pulmonary fibroblasts. A. Demonstrative western blotting assay showing the effect of SiO₂ that increased the protein expression level of Sigmar1 in a time-dependent manner in MLg cells. B. Densitometric analyses represented the Sigmar1 protein from

five independent experiments in MLg cells in a time-dependent manner, * $p < 0.05$ vs. 0 h. C. Descriptive immunocytochemistry assay showing that SiO₂ increased the expression of Sigmar1 and ZC3H4 protein in MLg cells. D. Demonstrative western blotting assay representing ZC3H4NIC on pulmonary fibroblast cells that verified that SiO₂ increased the Sigmar1 protein. E. Densitometric analyses of Sigmar1 protein expression levels from five independent experiments in MLg cells. * $p < 0.05$ vs. the Con-NIC of control group, # $p < 0.05$ vs. the Con-NIC of SiO₂ group. F. To clarify that the interaction between ZC3H4 and Sigmar1 was assessed after SiO₂ exposure, we examined the immunoprecipitation of ZC3H4 in whole-cell lysates followed by immunoblotting for ZC3H4.

Figure 6

SiO₂ induces the MAPKinase (Mapk1, Mapk8 and Mapk14) and P13K/Akt phosphorylation pathways in pulmonary fibroblasts. A. Demonstrative immunoblotting assay demonstrating the effects of SiO₂ that increased the expression levels of ZC3H4 in MLg cells in a time-dependent manner. B. Densitometric analyses of ZC3H4 representing the effects of SiO₂ that increased the expression levels from five independent experiments in MLg cells in a time-dependent manner, *p<0.05 vs. 0 min. C. Demonstrative western blotting assay showing that Mapk1, Mapk8 and Mapk14 were elevated after SiO₂ exposure in MLg cells in a time (min)-dependent manner. D. Densitometric analyses of Mapk1, Mapk8, and Mapk14 representative the effects of SiO₂ that increased the expression levels from five independent experiments in MLg cells in a time-dependent manner, *p<0.05 vs. 0 min. E. Demonstrative western blotting assay showing that P13K/Akt was elevated after SiO₂ exposure in MLg cells in a time (min)-dependent manner. F. Densitometric analyses of P13K/Akt representative the effects of SiO₂ that increased the expression levels from five independent experiments in MLg cells in a time-dependent manner, *p<0.05 vs. 0 min. G. Demonstrative immunoblotting assay demonstrating the effects of SiO₂ that increased the expression levels of ZC3H4 and Sigmar1 were attenuated by pretreatment with MAPK (Mapk1, Mapk8, Mapk14) and P13K/Akt phosphorylation pathway inhibitors. H. Densitometric analyses representing the effects of SiO₂ that increased the expression levels of Sigmar1 and ZC3H4 (F) were attenuated by pretreatment with MAPK (Mapk1, Mapk8, Mapk14) and P13K/Akt phosphorylation pathway inhibitors, n=5, *p< 0.05 vs. the control group, #p< 0.05 vs. the SiO₂ group.

Figure 7

Induction of ZC3H4 by SiO₂ promotes a further increase in ZC3H4. A. Demonstrative western blotting assays showed that salubrinal was applied to MLg cells, indicating that ER stress was involved in the upregulation of ZC3H4. B. Densitometric analyses of ZC3H4, n=5, *p < 0.05 vs. the control group, #p < 0.05 vs. the SiO₂ group. C. Demonstrative western blotting assays showed that Tunicamycin was applied to MLg cells, indicating that ER stress was involved in the upregulation of ZC3H4. D. Densitometric analyses of five independent experiments indicated the effects of tunicamycin on ZC3H4 in MLg cells. *p < 0.05 vs. the control group. Densitometric analyses of five independent experiments indicated the effects of tunicamycin on ERN1 (E) and BiP (F) in MLg cells. *p < 0.05 vs. the control group. G. Demonstrative western blotting assays showed that tunicamycin induced the upregulation of ZC3H4 in time-dependent

manner. H. Densitometric analyses of five independent experiments indicated the effects of tunicamycin on ZC3H4 in MLg cells. *p< 0.05 vs. the 0 h group. I. Descriptive immunocytochemistry assay representing the effect of tunicamycin responsible for the elevated expression level of ZC3H4 in MLg cells.

Figure 8

Schematic diagram showing the mechanisms through which silica induced ZC3H4 and Sigmar1 expression in pulmonary fibroblast activation. ZC3H4 promotes pulmonary fibroblast activation with the involvement of MAPK (Mapk1, Mapk8 and Mapk14), and P13K/Akt phosphorylation pathways via sigmar1/ER stress in silicosis play a significant role in fibroblast proliferation, migration and differentiation. The interaction between ZC3H4 and Sigmar1 promotes the ER stress pathway with a PFL

that enhances the proliferation, migration and differentiation abilities with the increased synthesis of collagen, which may lead to fibrosis induced by SiO₂.

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