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# ZC3H4 mediates silica-induced EndoMT via ER stress and autophagy

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ARTICLE INFO	A B S T R A C T
Edited by Dr. Alan Hargreaves Keywords: ZC3H4 Silicosis EndoMT ER stress Autophagy	<i>Background:</i> Inflammatory reactions induced by alveolar macrophages and excessive fibroblast activation lead to pulmonary fibrosis in silicosis. The endothelial-mesenchymal transition (EndoMT) is a key source of myofibroblasts. ZC3H4 is a member of the CCCH zinc finger protein family that participates in macrophage activation and epithelial mesenchymal transition (EMT). However, whether ZC3H4 is involved in EndoMT in silicosis has not yet been elucidated. Therefore, we conducted further studies into the role of ZC3H4 in silica-induced EndoMT in pulmonary rescale.
	<i>Methods</i> : Western blotting and immunofluorescence staining were used to detect the regulatory influences of SiO <sub>2</sub> on pulmonary fibrosis and EndoMT. ZC3H4 was specifically downregulated using CRISPR/Cas9 to explore whether ZC3H4 regulated EndoMT during silicosis. C57BL/6 J mice were administered with SiO <sub>2</sub> via the trachea to establish a silicosis animal model. <i>Results:</i> 1) SiO <sub>2</sub> exposure increased ZC3H4 expression in pulmonary vessels. 2) ZC3H4 was involved in EndoMT induced by silica. 3) ZC3H4 mediated EndoMT via endoplasmic reticulum stress (ER stress) and autophagy. <i>Conclusions</i> : ZC3H4 greatly affects the progression of SiO <sub>2</sub> -induced EndoMT via ER stress and autophagy, which provides the possibility that ZC3H4 may become a novel target in pulmonary fibrosis treatment.

# 1. Introduction

Silicosis, one of the most common occupational diseases, is caused by exposure to high concentrations of free silicon dioxide (SiO<sub>2</sub>) particles and is characterized by pulmonary fibrosis and silicon nodule formation (M, 2019). The pathogenic processes of silicosis include abnormal activation of macrophages, excessive secretion of inflammatory cytokines or fibrogenic factors (Mossman and Churg, 1998; Fujimura, 2000), severe destruction of pulmonary alveolar epithelial cells and vascular endothelial cells, occurrence of the epithelial-mesenchymal transition (EMT) or endothelial-mesenchymal transition (EndoMT), and resultant excessive myofibroblast proliferation as well as excessive extracellular matrix (ECM) deposition (Jiang et al., 2019a; Yao et al., 2018; Fang et al., 2018). EndoMT is a newly discovered cellular program that was recently considered to be a vital source of tissue myofibroblasts in addition to EMT (Piera-Velazquez et al., 2011); notably, EndoMT has been implicated in the pathogenic changes of fibrosis in response to endothelial injury. Multiple studies have shown that EndoMT plays a much more important role than EMT in lung fibrosis progression because EndoMT is the basis for the development of EMT (Fang et al., 2018; Chao et al., 2016). However, compared to EMT, which has been studied extensively, EndoMT has not been deeply studied in pulmonary fibrosis induced by silica.

Several studies have shown that multiple molecular regulators are involved in the steps of EndoMT development. Among these, monocyte chemotactic protein 1 (MCP-1)-induced protein-1 (MCPIP1, encoded by the *zc3h12a* gene) has attracted much attention in recent years due to its crucial impact on inflammation and EndoMT stages in pulmonary fibrosis (Liu et al., 2016; Wang et al., 2016). MCPIP1 is a well-studied member of the CCCH zinc finger protein family, which destabilizes several inflammatory factors via its intrinsic RNase activity. Zinc finger CCCH-type containing 4 protein (ZC3H4) is another member of the

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Abbreviations: EndoMT, endothelial-mesenchymal transition; EMT, epithelial-mesenchymal transition; SiO<sub>2</sub>, silicon dioxide; HUVEC, Human umbilical vein endothelial cells; VE-cad, VE-cadherin; Col1, type I collagen;  $\alpha$ -SMA, alpha-smooth muscle actin; NS, normal saline; ER stress, Endoplasmic reticulum stress.

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Fig. 1. EndoMT occured in silica-induced pulmonary fibrosis mice (A) Representative micro-CT images of lungs isolated from the mice with a single dose of 5 mg silica for 56 days and the vehicle mice (NS treatment for 56 days). The yellow arrowhead indicates granular high-density shadows and reticular fibrous shadows. The images are representative of several mice from each group (n = 6). (B) Representative Sirius red staining images illustrate that silica increased collagen deposition. The images are representative of several mice from each group (n = 6). Scale bar =  $20 \ \mu$ m. (C) Hydroxyproline assay demonstrating silica treatment (5 mg each, 56 days) was associated with increased the pulmonary hydroxyproline content (n = 6); The data are showed as a mean ± SEM; \*p < 0.05 vs. the vehicle group. (D) Representative immunohistofluorescence images show that SiO<sub>2</sub> downregulated the expression of endothelial marker VE-cad (green fluorescence) and upregulated the expression of mesenchymal marker  $\alpha$ -SMA (red fluorescence) in the pulmonary vessels (red rectangle) of mice with silicosis. The images are representative of several mice from each group (n = 6). Fluorescence intensity analysis of the immunofluorescence images was expressed as a red/green fluorescence ratio (mean ± SEM). \*p < 0.05 vs. the vehicle group.



Fig. 2. SiO<sub>2</sub>-induced EndoMT in endothelial cells.

(A) CCK8 showing that SiO<sub>2</sub> increases HUVEC proliferation in a dose-dependent manner. The data are showed as a mean  $\pm$  SEM; \*p < 0.05 vs. the vehicle group; n = 5. (B) Representative Western blots showing SiO<sub>2</sub> increased the expression of CoI I and  $\alpha$ -SMA and decreased the expression of VE-cad in HUVECs in a dose-dependent manner. (C) Densitometric analyses of five independent experiments illustrate that SiO<sub>2</sub> increased CoI I / $\alpha$ -SMA expression and decreased VE-cad expression in HUVECs in a dose-dependent manner (n = 5); The data are showed as a mean  $\pm$  SEM; \*p < 0.05 vs. the vehicle group. (D) CCK8 showing that SiO<sub>2</sub> increases HUVEC proliferation in a time-dependent manner. The data are showed as a mean  $\pm$  SEM; \*p < 0.05 vs. the 0 -h group; n = 5. (E) Representative Western blots showing the effects of SiO<sub>2</sub> on the expression of VE-cad, CoI I, and  $\alpha$ -SMA in HPAECs in a time-dependent manner. (F) Densitometric analyses of five independent experiments illustrate that SiO<sub>2</sub> increased the expression of CoI I and  $\alpha$ -SMA and decreased the expression of VE-cad in HPAECs in a time-dependent manner (n = 5); The data are showed as a mean  $\pm$  SEM; \*p < 0.05 vs. the 0 -h group is a time-dependent manner (n = 5); The data are showed as a mean  $\pm$  SEM; \*p < 0.05 vs. the 0 -h group.

CCCH zinc finger protein family, and it is similar to MCPIP1/ZC3H12A (Jiang et al., 2019a). ZC3H4 was first discovered in 2006, and the functions of this protein have only just begun to be uncovered. Based on our earlier studies of silica-induced pulmonary fibrosis, we discovered a link between the ZC3H4 protein and inflammation in macrophages. Additionally, ZC3H4 affects EMT of alveolar epithelial cells via ER stress (Jiang et al., 2019a; Yang et al., 2018a). These findings are the basis of the hypothesis that ZC3H4 participates in various cellular processes. However, whether ZC3H4 regulates EndoMT and how to regulate

EndoMT in endothelial cells during silicosis is completely unknown. Our study explores the regulatory mechanisms of ZC3H4 in EndoMT, which is conducive to further study of the pathogenesis of silica-induced pulmonary fibrosis.

### 2. Materials and methods

#### 2.1. Reagents

Eighty percent of the SiO<sub>2</sub> particles were between 2 and 5 µm in diameter, and they were purchased from Sigma® (S5631). The silicon dioxide particles were sterilized overnight as previously described (200 °C for 16 h) (Zhou et al., 2018), and they were then diluted in sterilized phosphate buffer solution (PBS) or normal saline (NS) at a concentration of 5 mg/mL or 50 mg/mL, respectively. The SiO<sub>2</sub> dosage used in cellular or animal experiments was the same as our experimental results (Figs. 1 and 2) and what was used in our previous studies (Yang et al., 2018b). Antibodies against ZC3H4 (20041-1-AP) and  $\alpha$ -SMA (14395-1-AP) were purchased from Protein Tech Company. Antibodies against COLI (BS1530, rabbit) and GAPDH (MB001, mouse) were purchased from BioWorld, Inc. An antibody against VE-cad (sc-9989, mouse) was purchased from Santa Cruz Biotechnology, Inc.

# 2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) and human pulmonary artery endothelial cells (HPAECs) were obtained from ATCC®, and they were cultured in DMEM containing 10 % fetal bovine serum (FBS) at 5% CO<sub>2</sub> and 37 °C. The cells used in the experiments were between passages 5 and 15.

# 2.3. Cell model establishment

Cells were cultured on 24-well plates and then treated with silica. The silica samples were resuspended in PBS at a concentration of 5 mg/mL, and the volume applied to each well of a 24-well plate was 20  $\mu$ l, corresponding to a silica dosage of 50  $\mu$ g/cm<sup>2</sup> for the cell experiments (Yang et al., 2018a).

#### 2.4. Animal models establishment

C57BL/6 J mice were purchased from the Model Animal Research Center of Nanjing Medical University. C57BL/6 J mice were 5-8 weeks old and were administered abdominal pentobarbital sodium for anaesthesia. After basic anaesthesia, the mice were administered with a SiO<sub>2</sub> suspension (5 mg particles in 100  $\mu$ L of NS) via the trachea a single time, and the control group was treated with 100 µL of sterilized NS (Zhou et al., 2018). The dose was based on calculations of human lifetime exposure to respirable crystalline silica and an equivalent exposure for mice, and represent approximately 60-120 % of a human lifetime exposure at the recommended of National Institute for Occupational Safety and Health (NIOSH) exposure limit (Mayeux et al., 2018). In this study, a single dose of 5 mg silica was used, which might be considered high when compared to calculated human exposures. In the absence of a standardized dose and method of administration, we selected, after comparing different routes and doses from previous studies, the single dose of 5 mg silica by intratracheal instillation. Silica treatment for 56 days was exhibited abnormal lung fibrosis through chest radiographic images (Fig. 1A), which were similar to the imaging features of chronic human silicosis. Therefore, a prepared SiO<sub>2</sub> suspension (0.2 g/kg in 50 mg/mL saline) was instilled intratracheally, which corresponds to a silica dosage of about 5 mg per mouse. Pulmonary tissues were obtained after SiO<sub>2</sub> suspension treatment for 56 days.

# 2.5. Western blot analysis

Western blot analysis was measured as previously reported (Cao et al., 2017). Briefly, the HUVECs and HPAECs were collected in RIPA lysis buffer (P0013B, Beyotime) after being washed several times with cold PBS buffer. Nuclear and cytoplasmic proteins were extracted using an extraction kit (P0027, Beyotime). The concentration of each sample

was detected using a BCA kit (P0011, Beyotime). Equal concentrations of each sample were separated by 8 %-12 % SDS-PAGE and were transferred to PVDF membranes. PVDF membranes were incubated in 5% nonfat dry milk for 1 h under ambient conditions. After that, the membranes were incubated with the primary antibodies against GAPDH, α-SMA, VE-cad, ZC3H4, COLI, BIP, CHOP, BECN, ATG5 and LC3B (1:800 dilution) at 4 °C for a whole night. The next day, PVDF membranes were washed four times with tris-buffered saline + tween (TBST) and then incubated for 1 h at room temperature with goat anti-mouse and anti-rabbit IgG secondary antibodies (1:5000 dilution) conjugated with horseradish peroxidase (Thermo Fisher Scientific) in TBST. After PVDF membranes were washed four times in TBST. Protein bands were visualized using a chemiluminescent reagent (SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific) and a Kodak IS4000 MM Pro Imaging System (Carestream Health, Rochester, NY). Each Western blot was representative of at least five independent experiments. The protein bands were evaluated using ImageJ 1.48v software.

# 2.6. Computed tomographic analysis (CT analysis) for animals

CT images were detected under an Hiscan VM Pro CT machine, and the parameters were setup as follows: 20-90 kV;  $200 \text{ }\mu\text{A}$ ;  $18 \text{ }\mu\text{m}$  image resolution; and  $75 \text{ }\mu\text{m}$  pixel size. The quantification of CT images were performed using Image J software

# 2.7. Cell counting Kit-8 (CCK8) assay

Briefly, the HUVECs were incubated in 96-well plates at an initial density of

 $1\times 10^4$  cells/well and cultured for 24 h in a 5 % CO<sub>2</sub> incubator at 37 °C. After treatment, cells were applied with CCK8 (10  $\mu$ L/well), and then the plates were cultured for 4 h in the incubator. The absorption values were detected at 450 nm. Each experiment was conducted at least five times.

#### 2.8. Immunofluorescence staining

HUVECs cover-slips or the tissue slides were fixed with 4% paraformaldehyde throughout the night, after that the cells were permeabilized for half an hour with 0.3 % Triton X-100 in PBS. Then the cells or the tissue slides were blocked for 2 h at room temperature (RT) with 0.3 % Triton X-100 containing 10 % normal goat serum (NGS) (blocking buffer). HUVECs or the tissue slides were incubated with primary antibodies in blocking buffer overnight at 4 °C and then washed with PBS for three times. Next, HUVECs or the tissue slides were incubated secondary antibodies for 2 h at RT (AlexaFluor 488/576-conjugated anti-mouse/rabbit IgG secondary antibody;1:250; Invitrogen, Carlsbad, USA). After three washes in PBS, the samples were mounted with Prolong® Gold antifade reagent with DAPI (P36931, Life Technologies) and observed through the fluorescence microscope (Zeiss, Carl Zeiss, Göttingen, Germany). Green (488 nm) and red (576 nm) fluorescence intensity values were analyzed by Image J software. Red/green fluorescence ratios were calculated from the green and red intensity values in at least five images for each group.

#### 2.9. Sirius red staining

Sirius red staining (G1470, Solarbio Science & Technolog Company) was used for detecting for collagen. According to the manufacturer's recommended protocol, tissue sections were fixed with 10 % formal-dehyde solution at 4 °C and then dewaxed and hydrated. After that, dyed the slides with weigert hematoxylin for about 10–20 min, and then washed the slides with running water for about 5–10 min. After washing, the slides were dyed with sirius red staining solution for 1 h at room temperature and then washed again with running water. At last,



Fig. 3. SiO<sub>2</sub> induced ZC3H4 expression.

(A) Representative immunofluorescence images showing VE-cad and ZC3H4 expression in lung tissues from NS-treated mice and SiO<sub>2</sub>-treated mice. ZC3H4 expression was increased, while VE-cad expression was decreased in silicosis mice. The images are representative of several mice from each group (n = 6). Scale bar = 20  $\mu$ m. (B) Fluorescence intensity analysis of the immunofluorescence images was expressed as red/green fluorescence ratio (mean  $\pm$  SEM). \*p < 0.05 vs. the vehicle group. (C) Representative Western blots show the effects of SiO<sub>2</sub> on the expression of ZC3H4 in HUVECs. (D) Densitometric analyses of five independent experiments illustrate that SiO<sub>2</sub> increased ZC3H4 expression in HUVECs (n = 5); The data are showed as a mean  $\pm$  SEM; \*p < 0.05 vs. the 0 -h group. (E) Representative immunocytofluorescence staining images show VE-cad and ZC3H4 expression in HUVECs (n = 5). Scale bar = 20  $\mu$ m.

the slides were fixed with neutral balata and observed through the light microscope.

# 2.10. Hydroxyproline assay

The whole mouse lung samples were weighed and homogenized in distilled water ( $dH_2O$ ). After which, lung samples were hydrolyze in 1 ml of 12 N HCl at 110°C for 18 h. The protocol of analyzing hydroxy-proline content was based on BioVision hydroxyproline assay kit

(Milpitas, CA) manufacturer's instructions (Chung et al., 2014). The hydroxyproline content was expressed as micrograms of the total lung tissue.

2.11. Cell transfection via CRISPR-associated protein 9 (Cas9) technology

A CRISPR/Cas9 double nickase plasmids (sc-437281, sc-411693-NIC), CRISPR activation plasmids (sc-437275, sc-411693-ACT),



(caption on next page)

# Fig. 4. ZC3H4 was involved in SiO<sub>2</sub>-induced EndoMT.

(A) Representative Western blots show that the CRISPR/Cas9 system successfully downregulated ZC3H4 expression in HUVECs. (B) Densitometric analyses of five separate experiments suggest that transfection with ZC3H4 double nickase plasmid (ZC3H4 NIC) downregulated ZC3H4 expression in HUVECs (n = 5); The data are showed as a mean  $\pm$  SEM; \*p < 0.05 vs. the control group. (C) Representative Western blots show that downregulating ZC3H4 inhibited the SiO<sub>2</sub>-induced increase in Col I and  $\alpha$ -SMA expression and reversed the SiO<sub>2</sub>-induced decrease in VE-cad expression. (D) Densitometric analyses of five independent experiments show that CRISPR/Cas9-mediated ZC3H4 silencing attenuated the SiO<sub>2</sub>-induced increase in Col I and  $\alpha$ -SMA expression and ameliorated the SiO<sub>2</sub>-induced decrease in VE-cad expression (n = 5); The data are showed as a mean  $\pm$  SEM; \*p < 0.05 vs. the control group; <sup>#</sup>p < 0.05 vs. the SiO<sub>2</sub> group. (E) Representative immunocytofluorescence staining images show that SiO<sub>2</sub> decreased the expression of VE-cad and increased the expression of  $\alpha$ -SMA in HUVECs. ZC3H4 silencing inhibited the SiO<sub>2</sub>-induced decrease in VE-cad expression. Scale bar = 20 µm.

transfection medium (sc-36868) and transfection reagent (sc-29528) were obtained from Santa Cruz Biotechnology. The transfection volume listed here was for a single well of the 24-well cell-culture plate. In brief, 1  $\mu$ L of plasmids were mixed with 9  $\mu$ L of transfection medium; at the same time, 1  $\mu$ L of transfection reagent were mixed with 9  $\mu$ L of transfection medium. Both the mixtures stood for 5 min, after that, mix the transfection solution and stood for an additional 30 min. The mixed solution was then co-incubated with HUVECs in an incubator containing 5% CO<sub>2</sub> at 37 °C for about 12 h–24 h, and then the medium were replaceed by DMEM containing 10 % fetal bovine serum (FBS) to conduct further experiments.

# 2.12. Statistics

The data analyses were performed using SigmaPlot 11.0 software. The data are presented as the mean  $\pm$  standard error of the mean (mean  $\pm$  SEM). Unpaired *t*-test (2 groups) or analysis of variance (ANOVA; > 2 groups) were used to calculate the significant differences. *P*<0.05 was used to analyze the significant differences.

#### 3. Results

#### 3.1. EndoMT occurred in mice with silica-induced pulmonary fibrosis

C57BL/6 J mice were administered a single dose of 5 mg silica via intratracheal injection to establish the silicosis model as described in a previous study (Zhou et al., 2018). To increase similarity to clinical practice, noninvasive imaging evaluation-computed tomography (CT) was used to observe progressive anatomical pulmonary changes. We found that 56 days after SiO<sub>2</sub> administration, the chest radiographic images from the silicosis model animals exhibited abnormal lung fibrosis, including increased granular high-density shadows or reticular fibrous shadows (arrowhead), obliteration of the costophrenic angle and serious lung collapse (Fig. 1A), which were similar to the imaging features of chronic human silicosis. Based on this finding, 56 days after SiO<sub>2</sub> exposure was chosen for the rest of the in vivo assays. Pulmonary fibrosis is characterized by the excessive deposition of ECM, including collagens, fibronectins, laminins, proteoglycan and others in the lung. Collagens are the most important components of the ECM (Eijgenraam et al., 2019). Sirius red staining, one of the best understood histochemical techniques to highlight collagens, showed that compared to the vehicle group (NS group), the experimental group exhibited more serious alveolar damage, and large amounts of red-stained collagen were deposited in the pulmonary interstitium, as well as around the blood vessels and bronchi (Fig. 1B). To quantify the extent of fibrosis, the pulmonary hydroxyproline level, an index to evaluate systemic fibrosis, was chosen to determine the fibrosis degree in the silicosis models, and pulmonary hydroxyproline was found to be significantly elevated compared to control levels on day 56 (Fig. 1C). Then we examined the effects of SiO<sub>2</sub> on EndoMT in pulmonary vessels. Immunofluorescence staining (Fig. 1D) showed that expression of the endothelial marker VE-cadherin (VE-cad) (green fluorescence) in the pulmonary vasculature (red rectangle) was decreased and discontinuous, but alpha-smooth muscle actin ( $\alpha$ -SMA) expression (red fluorescence) was upregulated in the pulmonary vasculature of mice after SiO<sub>2</sub> treatment for 56 days, suggesting that EndoMT occurred in areas of pulmonary fibrosis stimulated by silica. Fig. 1E displays the fluorescence ratios of the immunofluorescence staining and shows that the red fluorescence intensity was increased in SiO<sub>2</sub>-treated lung tissues, but the green fluorescence intensity was decreased (statistical data not shown). The red/green fluorescence ratio in this group was significantly higher than that of the vehicle group (NS group). These results confirmed that EndoMT was stimulated in mice with pulmonary fibrosis after exposure to a silica suspension for 56 days.

#### 3.2. SiO<sub>2</sub>-induced EndoMT in endothelial cells

Because reactive pulmonary endothelial cells always undergo increased cell proliferation (Li et al., 2018; Vadivel et al., 2014), we first explored the effect of SiO<sub>2</sub> on HUVEC proliferation. HUVECs were treated with different concentrations of SiO<sub>2</sub> for 24 h and analyzed by the CCK-8 assay, which was used to determine the effect of SiO<sub>2</sub> on cell proliferation. As shown in Fig. 2A, the CCK-8 assay indicated that SiO<sub>2</sub> increased HUVEC proliferation in a dose-dependent manner. SiO<sub>2</sub> at a concentration of 50 µg/cm<sup>2</sup> significantly increased HUVEC proliferation, while 100 µg/cm<sup>2</sup> SiO<sub>2</sub> exhibited an inhibitory effect on cell proliferation compared with that of the vehicle group. Moreover, SiO<sub>2</sub> induced  $\alpha$ -SMA and type I collagen (Col1) expression while downregulated VE-cad expression in a dose-dependent manner, with a peak response at 50 µg/cm<sup>2</sup> (Fig. 2B-C). These proteins are important, physiologically physiological relevant indicators of EndoMT. Based on this dose experiment, we chose 50 µg/cm<sup>2</sup> for the rest of the in vitro assays.

To select the appropriate time to estimate functional changes, we incubated HUVECs for different times with SiO<sub>2</sub> (50 µg/cm<sup>2</sup>). A previous study from our lab showed that SiO<sub>2</sub> induced the expression of EndoMT markers in HUVECs in a time-dependent manner, with a peak response at 24 h. Moreover, the CCK-8 assay (Fig. 2D) showed that SiO<sub>2</sub> increased the HUVEC proliferation, with the peak response observed at 24 h. Therefore, the 24 h time point after SiO<sub>2</sub> exposure was selected to maximize the likelihood of detecting the effects of ZC3H4 because this time point corresponded to marked EndoMT occurrence and increased cell proliferation in HUVECs after SiO<sub>2</sub> exposure.

Then, we verified that silica also induced EndoMT in HPAECs, a representative cell line of the pulmonary endothelium (Fig. 2E-F), which was characterized by decreased expression of the endothelial marker VE-cad and increased expression of mesenchymal proteins. These results confirmed that silica stimulated EndoMT in vitro.

## 3.3. SiO<sub>2</sub>-induced ZC3H4 expression

Under EndoMT conditions, we examined ZC3H4 protein expression and observed the colocalization of ZC3H4 and VE-cad in lung tissues. As shown in Fig. 3A, ZC3H4 expression (red fluorescence) exhibited an increasing trend, while VE-cad expression (green fluorescence) decreased in the pulmonary vasculature (red rectangle) of SiO<sub>2</sub>-induced mice. Fluorescence analysis also showed that the red/green fluorescence ratio was significantly higher in the SiO<sub>2</sub>-treated group than in the vehicle group (Fig. 3B). Next, we extended the in vivo examinations to cellular experiments. We found that SiO<sub>2</sub> promoted ZC3H4 expression in HUVECs, as shown by Western blotting (Fig. 3C-D). This finding was confirmed by immunofluorescence staining of ZC3H4 in HUVECs (Fig. 3E). These results confirmed that silica stimulated ZC3H4



Fig. 5. ZC3H4 mediated EndoMT through ER stress.

(A) Representative immunofluorescence staining images show that SiO<sub>2</sub> upregulated the expression of the ER stress marker CHOP and downregulated the expression of VE-cad in the pulmonary vessels (red rectangle) of mice with silicosis. The images are representative of several mice from each group (n = 6). Scale bar = 20  $\mu$ m. (B) Fluorescence intensity analysis of the immunohistofluorescence images was expressed as red/green fluorescence ratio (mean  $\pm$  SEM). \*p < 0.05 vs. the vehicle group. (C) A representative Western blot shows that downregulating ZC3H4 inhibited the SiO<sub>2</sub>-induced increase in BIP and CHOP expression of HUVECs. (D) Densitometric analyses of five independent experiments show that CRISPR/Cas9-mediated ZC3H4 silencing attenuated the SiO<sub>2</sub>-induced increase in BIP and CHOP expression; The data are showed as a mean  $\pm$  SEM; \*p < 0.05 vs. the control group; #p < 0.05 vs. the SiO<sub>2</sub> group. (D) Representative Western blots show the effects of the ER stress inhibitor salubrinal on SiO<sub>2</sub>-induced EndoMT in HUVECs. (E) Densitometric analyses of five independent experiments indicate that salubrinal reversed SiO<sub>2</sub>-induced EndoMT; The data are showed as a mean  $\pm$  SEM; \*p < 0.05 vs. the control group; #p < 0.05 vs. the SiO<sub>2</sub> group.

expression.

#### 3.4. ZC3H4 was involved in SiO<sub>2</sub>-induced EndoMT

The previous results revealed that SiO2 promoted EndoMT and

ZC3H4 expression in pulmonary vessels. Whether there was a link between these two phenomena was unclear. To explore the regulatory effects of ZC3H4 on EndoMT, the CRISPR/Cas9 system was used to knock down ZC3H4 (Fig. 4A-B). Western blotting showed that downregulating ZC3H4 reversed the decrease in VE-cad and the upregulation



Fig. 6. ZC3H4 mediated EndoMT through autophagy.

(A) Representative immunohistofluorescence staining images show that SiO<sub>2</sub> upregulated the expression of the autophagy marker BECN and downregulated the expression of VE-cad in the pulmonary vessels (red rectangle) of mice with silicosis. The images are representative of several mice from each group (n = 6). Scale bar = 20  $\mu$ m. (B) Fluorescence intensity analysis of the immunohistofluorescence images was expressed as a red/green fluorescence ratio (mean ± SEM). \*p < 0.05 vs. the vehicle group. (C) A representative Western blot shows that downregulating ZC3H4 inhibited the SiO<sub>2</sub>-induced increase in ATG5, LC3B and BECN expression of HUVECs. (D) Densitometric analyses of five independent experiments show that CRISPR/Cas9-mediated ZC3H4 silencing attenuated the SiO<sub>2</sub>-induced increase in ATG5, LC3B and BECN expression; The data are showed as a mean ± SEM; \*p < 0.05 vs. the control group; #p < 0.05 vs. the SiO<sub>2</sub> group. (E) Representative Western blots show the effects of the autophagy inhibitor 3-MA on SiO<sub>2</sub>-induced EndoMT in HUVECs. (F) Densitometric analyses of five independent experiments indicate that 3-MA reversed SiO<sub>2</sub>-induced EndoMT (n = 5); The data are showed as a mean ± SEM; \*p < 0.05 vs. the control group; #p < 0.05 vs. the SiO<sub>2</sub> group.

of Col I and  $\alpha$ -SMA induced by SiO<sub>2</sub> treatment for 24 h (Fig. 4C-D). Taken together, these results demonstrate that the activation of EndoMT is regulated by ZC3H4 in silicosis. This finding was confirmed by immunofluorescence staining (Fig. 4E).

# 3.5. ZC3H4 mediated EndoMT through ER stress

Endoplasmic reticulum stress (ER stress) has been shown to participate in the occurrence of EndoMT in many disease models (Bouvier et al., 2009; Gunther et al., 2012). Some studies showed that highly oxidized protein products modulated EndoMT in renal glomerular endothelial cells via ER stress (Liang et al., 2016); other studies showed that ER stress influenced EMT through the Src pathway in HUVECs (Ying et al., 2016). Based on these studies, we hypothesized that ER stress was also involved in pulmonary vascular EndoMT induced by SiO<sub>2</sub>. To test this hypothesis, we first measured ER stress-related markerexpression in the pulmonary tissues of mice with silicosis. The C/EBP homologous protein (CHOP) pathway, which is one of three pathways involved in ER stress, was examined in lung tissues. As shown in Fig. 5A and 5B, SiO<sub>2</sub> upregulated CHOP protein expression (red fluorescence) in damaged pulmonary vessels (red rectangle), while downregulating VE-cad protein expression (green fluorescence), and the results are expressed as an increase in the red/green fluorescence ratio. To further understand the regulatory effects of ZC3H4 in SiO<sub>2</sub>-induced ER stress, we knocked down ZC3H4 expression specifically in HUVECs with ZC3H4 NIC plasmids. This treatment significantly decreased ZC3H4 levels in HUVECs and reversed the SiO<sub>2</sub>-induced upregulation of CHOP and binding immunoglobulin protein (BIP), which are primary regulators of the ER stress response (Fig. 5C-D). These results suggested that the activation of ER stress in SiO<sub>2</sub>-induced HUVECs was regulated by ZC3H4. Then, the ER stress inhibitor salubrinal was used to assess the molecular mechanism underlying the induction of EndoMT by SiO<sub>2</sub> treatment for 24 h. As shown in Fig. 4D-E, pretreatment with salubrinal for 1 hattenuated the SiO2-induced upregulation of Col1 and α-SMA and downregulation of VE-cad, suggesting that ER stress was involved in EndoMT. These results indicated that ZC3H4 stimulated EndoMT through ER stress.

# 3.6. ZC3H4 mediated EndoMT through autophagy

Autophagy, which appears to be an evolutionarily conserved process, was proposed as an explanation for the restoration of homeostasis by the elimination of misfolded proteins and toxic metabolic products (Gui et al., 2019). The activation of autophagy also appears to be a molecular modulator of EndoMT (Singh et al., 2015). Initially, we sought to determine the expression of autophagy-related markers in lung tissue samples from mice with silicosis. As shown in Fig. 6A, the level of the autophagy-related marker BECN (red fluorescence) was upregulated, while VE-cad (green fluorescence) was downregulated in damaged pulmonary vessels (red rectangle) following treatment with SiO<sub>2</sub>. The results were confirmed by fluorescence ratio analysis (Fig. 6B). Then, ZC3H4 NIC plasmids were transfected into endothelial cells to examine the effects of ZC3H4 on autophagy. As shown in Fig. 6C-D, silencing ZC3H4 protein expression specifically in HUVECs markedly reversed the upregulation of ATG5, LC3B and BECN (markers of autophagy) induced by SiO<sub>2</sub>, suggesting that ZC3H4 regulated autophagy in endothelial cells as well. To assess the effect of autophagy on EndoMT induced by silica treatment for 24 h, SiO<sub>2</sub> was administered after pretreatment with the specific autophagy inhibitor 3-methyladenine (3-MA) for 1 h. As shown in Fig. 5D-E, pretreatment with 3-MA markedly inhibited the effect of SiO2 on EndoMT. Therefore, these results showed that ZC3H4 mediated EndoMT through autophagy. Collectively, these data showed that ZC3H4 participated in regulating SiO2-induced EndoMT not only through ER stress but also autophagy.

### 4. Discussion

When the lungs are exposed to air containing free silica particles for a long time, the pathological process of pulmonary fibrosis begins with pulmonary endothelial cell impairment, which is followed by limited self-recovery of endothelial cells (Almale et al., 2019; Chen et al., 2018), inflammatory cytokine secretion, gradual fibroblast proliferation, and myofibroblast activation (Yao et al., 2019). In past studies, histopathological examination was used to detect the different stages of lung fibrosis in dead animals, which was not similar to clinical practice. To provide more clinically relevant data, imaging detection technology was used to track fibrosis progression in living animals in this study. As a result, we found that the time it takes to successfully establish a

pulmonary fibrosis mouse model (according to the radiographic findings) was 56 days after  $SiO_2$  administration, which was different from the time point (28 days) used in our past studies (Wang et al., 2016; Zhou et al., 2018).

EndoMT, described as a phenotypic conversion that gives rise to matrix-producing fibroblasts and myofibroblasts, participates in the generation of new tissue types during embryonic development and plays a vital role in the inflammatory response and wound healing in damaged tissues (Platel et al., 2019; Tsai et al., 2019). EndoMT is also influential in fibrogenesis in many tissues, such as the kidney, heart, lung and liver (Gui et al., 2019; Platel et al., 2019; Lu et al., 2019; Sallustio et al., 2019). Our results showed that during silica-induced lung fibrosis, the expression level of the classic endothelial marker VE-cad was decreased in the silica model group compared with the vehicle group in vivo and in vitro, indicating the loss of endothelial characteristics. However, the expression levels of COL1 and  $\alpha$ -SMA were increased, showing upregulated fibroblast activity (Jiang et al., 2019b). These abnormalities in protein expression indicate that EndoMT occurred in pulmonary fibrosis induced by silica.

EndoMT is controlled by complex regulatory loops and signaling pathways, and ZC3H4 is regarded as a critical protein that positively regulates EMT in lung fibrosis. Blockade of ZC3H4 expression was shown to attenuate EMT in pulmonary fibrosis (Jiang et al., 2019a), suggesting that ZC3H4 may be an effective target for pulmonary fibrosis treatment. However, there is no information in the literature concerning whether ZC3H4 can regulate or how it regulates EndoMT in pulmonary fibrosis. In this study, we measured the protein expression of ZC3H4 and found that it increased significantly after SiO<sub>2</sub> treatment, and EndoMT also increased, indicating that ZC3H4 is involved in SiO<sub>2</sub>-induced EndoMT.

The molecular mechanisms by which ZC3H4 regulates EndoMT remain unclear. It has been indicated that ER stress might be a molecular regulator that contributes to cell transformation and proliferation. For example, ER stress exerts potential EMT effects on many kinds of epithelial cells (Ulianich et al., 2016; Delbrel et al., 2019). Our previous study showed that ER stress promoted EMT in alveolar epithelial cells, which was followed by cellular morphological changes from an initial "cobblestone-like" to a spindle-shaped "fibroblast-like" morphology (Jiang et al., 2019a). Moreover, ER stress has been shown to account for EndoMT in cardiac and kidney fibrosis (Bouvier et al., 2009; Ying et al., 2016). To explore whether ER stress participates in EndoMT of pulmonary vessels, in this study, we conducted in vitro and in vivo experiments and determined that ZC3H4 plays a key role in regulating EndoMT through ER stress.

Autophagy is recognized as an adaptive response that plays a critical role in diseases, even cancer, because autophagy possesses the powerful ability to regulate protein and organelle degradation (Cinque et al., 2015; Garcia-Prat et al., 2016). Recent research has revealed that autophagy plays a crucial role in tissue fibrosis and the generation of myofibroblasts through EMT (Skarkova et al., 2019; Hill et al., 2019). However our previous study showed that autophagy was not activated by SiO<sub>2</sub> during the EMT process (Jiang et al., 2019a). In the present study, HUVECs treated with SiO<sub>2</sub> exhibited activated autophagy and EndoMT, which suggests that although the pathological process of EndoMT is similar to that of EMT, there are still differences in the precise mechanisms linking autophagy to SiO<sub>2</sub>-induced mesenchymal transition.

Taken together, these data suggest that ZC3H4 participates in EndoMT by regulating ER stress and autophagy. In addition, researchers have found that ER stress is closely related to autophagy, and excessive accumulation of unfolded proteins in the endoplasmic reticulum can promote autophagy (Zhang et al., 2019). Therefore, further research will be required to elucidate the interactions between these two molecular mechanisms in pulmonary fibrosis-associated EndoMT.

As shown in our research, while silica-induced EndoMT appears to be



Fig. 7. Schematic diagram showing the molecular mechanisms through which ZC3H4 regulated SiO<sub>2</sub>-induced EndoMT. ZC3H4 expression was upregulated in endothelial cells exposed to silica. Moreover, ZC3H4 reinforced EndoMT through its association with ER stress and autophagy in silicosis.

partial, it seems likely that; 1) ZC3H4 is only one component of a regulatory network, which may result in the transition from an endothelial to mesenchymal phenotype. Similar to EMT, EndoMT is induced by many important signaling pathways. Recent studies suggest that the E3 ubiquitin ligase HECTD1 and CCCH zinc-finger-containing protein-MCPIP1 mediate pulmonary EndoMT in the context of silicosis (Fang et al., 2018; Chao et al., 2016). Moreover, noncoding RNAs are involved in EndoMT in lung fibrosis, although the detailed mechanisms remain unclear (Bai et al., 2018; Huang et al., 2018). 2) The EndoMT observed under some experimental conditions is transient and possibly reversible, rather than indicative of a stable transition. The reversal of mesenchymal phenotypes can be into epithelial phenotypes achieved using stimuli that scavenge reactive oxygen species (ROS) (Wang et al., 2010). Compared to EMT, which has been widely studied, EndoMT in fibrotic diseases has received little attention. Stable or transient cellular phenotypes have not been clearly described in fibrotic disease-associated EndoMT. Although the reversible transition of EndoMT in pulmonary fibrosis deserves further investigation, several studies of liver fibrosis associated with EndoMT have indicated that ROS might play a key role in EndoMT, as restoring ROS levels allowed EndoMT to proceed, while EndoMT was blocked by either pharmacological inhibition of ROS with a small-molecule compound or depletion of NAPDH oxidase 4 (NOX4) expression (Li et al., 2019).

## 5. Conclusions

In summary, our findings provide the first evidence that ZC3H4 reinforces EndoMT through its association with ER stress and autophagy in the context of silicosis (Fig. 7). These novel findings provide insight into the use of ZC3H4 as a potential marker of pulmonary fibrosis.

## Ethical approval and consent to participate

The Animal Ethics Board of the Nanjing Medical University approved all animal experimental procedures.

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#### **Consent for publication**

None.

## Availability of supporting data

The obtained results of the current study are available on reasonable request.

#### Contributions

R.J. designed the experiments, performed the experiments, prepared the figures, and wrote the manuscript. Q.G. and T.Y. performed the experiments and interpreted the data. R.J. and J.C. provided laboratory space and designed the experiments, interpreted the data, wrote the manuscript and directed the project. All authors read, discussed and approved the final manuscript.

## **Transparency document**

The Transparency document associated with this article can be found in the online version.

### **Declaration of Competing Interest**

The authors declare no conflict of interest.

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