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# circHIPK2-mediated $\sigma$ -1R promotes endoplasmic reticulum stress in human pulmonary fibroblasts exposed to silica

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## Abstract

Silicosis is characterized by fibroblast accumulation and excessive deposition of extracellular matrix. Although the roles of SiO<sub>2</sub>-induced chemokines and cytokines released from alveolar macrophages have received significant attention, the direct effects of SiO<sub>2</sub> on protein production and functional changes in pulmonary fibroblasts have been less extensively studied. Sigma-1 receptor, which has been associated with cell proliferation and migration in the central nervous system, is expressed in the lung, but its role in silicosis remains unknown. To elucidate the role of sigma-1 receptor in fibrosis induced by silica, both the upstream molecular mechanisms and the functional effects on cell proliferation and migration were investigated. Both molecular biological assays and pharmacological techniques, combined with functional experiments, such as migration and proliferation, were applied in human pulmonary fibroblasts from adults to analyze the molecular and functional changes induced by SiO<sub>2</sub>. SiO<sub>2</sub> induced endoplasmic reticulum stress in association with enhanced expression of sigma-1 receptor. Endoplasmic reticulum stress promoted migration and proliferation of human pulmonary fibroblasts-adult exposed to SiO<sub>2</sub>, inducing the development of silicosis. Inhibition of sigma-1 receptor ameliorated endoplasmic reticulum stress and fibroblast functional changes induced by SiO<sub>2</sub>. circHIPK2 is involved in the regulation of sigma-1 receptor in human pulmonary fibroblasts-adult exposed to SiO<sub>2</sub>. Our study elucidated a link between SiO<sub>2</sub>-induced fibrosis and sigma-1 receptor signaling, thereby providing novel insight into the potential use of sigma-1 receptor/endoplasmic reticulum stress in the development of novel therapeutic strategies for silicosis treatment.

## Introduction

Silicosis is an occupational disease caused by long-term exposure to high levels of dust containing excessive free silica (crystalline silicon dioxide, SiO<sub>2</sub>) in mining and other dusty occupational environments<sup>1,2</sup>. Although preventive efforts have been made for many decades, silicosis remains a potentially fatal, incurable, and disabling

pulmonary disease characterized by silicotic nodule formation and pulmonary interstitial fibrosis<sup>3</sup>. Silicosis is especially prevalent in undeveloped countries because of poor surveillance<sup>4</sup>. The incidence and prevalence of silicosis are markedly increasing, and effective therapies are not currently available<sup>5</sup>. Despite the many studies conducted to investigate the toxicity of crystalline silica in the last several decades, to date, the exact mechanism of silicosis remains elusive.

Sigma-1 receptor ( $\sigma$ -1R) is a subtype of the sigma receptor family that is expressed in the endoplasmic reticulum (ER); it has two transmembrane segments and two steroid binding domains, forming a pocket that is the

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binding site for cholesterol, steroids, sphingolipids<sup>6</sup>, and a wide variety of natural or synthetic ligands from different classes, such as opioids, antipsychotics, psychostimulants, alkaloids, or antidepressants<sup>7</sup>.  $\sigma$ -1R has been associated with many diseases, including cocaine addiction<sup>8</sup>, stroke<sup>9</sup>, Alzheimer's disease<sup>10</sup>, amnesia<sup>11</sup>, retinal degeneration<sup>12</sup>, and cancer<sup>13–15</sup>. The molecular action of  $\sigma$ -1R was recently revealed to be a ligand-regulated receptor chaperone via ER stress (ERS)<sup>15</sup>. The ER is a specialized perinuclear organelle responsible for synthesis, folding, modification, and delivery of proteins to their target sites<sup>16–18</sup>. Various physiological and pathological conditions may affect ER homeostasis, ultimately causing ERS<sup>19,20</sup>. Although ERS is involved in different types of pulmonary disease<sup>21–23</sup>,  $\sigma$ -1R-associated ERS in pulmonary fibrosis has received little attention.

A recent study suggested that non-coding RNA is involved in  $\sigma$ -1R regulation<sup>24</sup>. Circular RNAs (circRNAs), as a new type of non-coding RNA, have been identified as competing endogenous RNAs that bind miRNAs by complementary base pairing<sup>25</sup>. Moreover, circRNAs are also reported to modulate the cell cycle in the formation of complexes with proteins<sup>26</sup>. Recent study suggest that circRNA-homeodomain-interacting protein kinase-2 (circHIPK2) may act as an endogenous miR-506-3p sponge, leading to an increase in  $\sigma$ -1R<sup>27</sup>, whereas its host gene-HIPK2 is involved in cell growth modulation, apoptosis, proliferation and tumor progression<sup>28–31</sup>. Therefore, these studies lend strong support to the hypothesis that circHIPK2, descendent from exon 2 of the HIPK2 gene, may be related in fibroblast activation.

In the current study,  $\sigma$ -1R-associated ERS was upregulated in human pulmonary fibroblasts exposed to SiO<sub>2</sub>, which subsequently induced fibroblast activation. Involvement of circHIPK2 in  $\sigma$ -1R regulation revealed novel functional circRNAs in SiO<sub>2</sub>-induced fibrosis and suggested that the circHIPK2/ $\sigma$ -1R/ERS pathway may be a potential therapeutic target for silicosis.

## Methods

### Reagents

SiO<sub>2</sub>, 80% of which had particle diameters of <5  $\mu$ m, was purchased from Sigma (S5631), selected via sedimentation according to Stokes' law, acid-hydrolyzed, and baked overnight (200 °C, 16 h)<sup>32</sup>. The silica samples for the cell experiments were suspended in phosphate-buffered saline (PBS) at a concentration of 5 mg/ml, and the volume of administration was 20  $\mu$ l/well in 24-well plates at a dosage of 50  $\mu$ g/cm<sup>2</sup>. Fetal bovine serum (FBS), normal goat serum and Dulbecco's modified Eagle's medium (DMEM; #1200-046) were purchased from Life Technologies. GlutaMax Supplement (35050-061) was obtained from Gibco. Lentiviral vectors carrying circHIPK2-siRNA were obtained from HANBIO. Control

siRNA (sc-37007) was purchased from Sigma-Aldrich. Antibodies against  $\sigma$ -1R were obtained from Invitrogen. Santa Cruz Biotechnology, Inc. The antibody against  $\alpha$ -SMA (SAB5500002) was purchased from Sigma, Inc. The antibody against COL1A2 (BS1530) and COL3A1 (BS1531) were purchased from BioWorld (St. Louis Park, MN, USA).

### Cell culture

Human pulmonary fibroblasts from adults (HPF-a) were purchased from ScienCell and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM L-GlutaMAX (Gibco) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

### Lentiviral transduction of HPF-a

P3-4 HPF-a was transfected with LV-GFP lentivirus (HANBIO Inc., Shanghai, China) as previously described<sup>32,33</sup>.

### Immunofluorescence staining

Immunofluorescence staining was performed as previously described<sup>34</sup>.

### Western blotting

Western blotting assays were used to determine the cellular protein levels as previously described<sup>32</sup>. Each western blot assay was repeated at least three times.

### Hoechst 33342 staining

HPF-a grown on poly-L-lysine-coated glass cover slips were staining using Hoechst 33342 (10  $\mu$ g/ml) after treated with BD1047 for 2 h and SiO<sub>2</sub> for 24 h. In brief, HPF-a were stained with Hoechst 33342 for 5 min at room temperature and then fixed in 4% PFA for 20 min. The population of apoptosis cells were observed under a fluorescence microscope. Five fields per well were randomly selected for apoptosis cell counting<sup>32</sup>.

### Bromodeoxyuridine (BrdU) labeling

Cells were prepared on poly-L-lysine-coated glass cover slips. 10  $\mu$ M BrdU (Yeasen, 40204ES60) in Dulbecco's phosphate-buffered saline was added 4 h before fixation. After fixing in 4% PFA at 4 °C overnight, cells were denatured in 2 N HCl at room temperature for 30 min and then rinsed in 0.1 M borate buffer (pH 8.0, 300  $\mu$ l/well) for 10 min. After incubation in blocking solution (PBS containing 10% normal goat serum and 0.3% TX-100) for 1 h, cells were then treated with mouse anti-BrdU antibody (1:200; SC-32323, Santa Cruz) overnight at 4 °C. After a wash stage with PBS, cells were incubated with donkey anti-mouse (conjugated to Alexa-Fluor 576) secondary antibodies for 2 h at room temperature. Washed three times in PBS, cells were mounted using mounting solution (Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI); P36931, Life Technologies). The

slides were imaged using a fluorescence microscope (Olympus IX70, Olympus America, Inc., Center Valley, PA, USA). Five fields per well were randomly selected for cell counting.

#### Cell migration assay

The cell migration capacity in a 2D culture system was evaluated using an *in vitro* scratch assay as previously described<sup>32</sup>.

#### Cell viability assessment using the MTT assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described<sup>32</sup>.

#### Real-time PCR

Total RNA was isolated from cells and subjected to reverse transcription using a PrimeScript RT Master Mix Kit (TaKaRa, RR036). Real-time PCR was performed using the following primers: human circHIPK2 (forward primer: 5'-CTGTGTGCTCCACCTACTTG-3'; reverse primer: 5'-TACCCAGTCATGTCCAGTTG-3'), human GAPDH (forward primer: 5'-ACCATCTTCCAGGAGCGAGAT-3'; and reverse primer: 5'-GGGCAGAGATGATGACCCTTT-3'). Relative quantification was performed using TaKaRa SYBR Premix Taq (TbI RNase H Plus) (TaKaRa, RR420).

#### Fluorescence *in situ* hybridization (FISH)

Cellular circHIPK2 expression was detected using FISH with a mixture of biotin-labeled DNA oligo probes that were specific for either endogenous or ectopically expressed circHIPK2. A biotin-labeled scrambled sequence was used as a negative control. In brief, cells were freshly fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature, followed by two washes with PBS, immersion in 70% ethanol overnight at 4 °C, permeabilization with 0.25% Triton X-100 for 15 min, and two 15-min washes with saline-sodium citrate (SSC) buffer. *In situ* hybridization was performed overnight at 37 °C with 10 pM biotin-labeled DNA oligo probes in hybridization buffer (HB), followed by serial washes with SSC buffer. The samples were incubated in blocking buffer (1% bovine serum albumin and 3% normal goat serum in PBS) for 1 h at room temperature and were then incubated with an anti-biotin HRP antibody (1:200) in blocking buffer overnight at 4 °C. The samples were washed with PBS twice, each for 2 min. DNA was stained with DAPI. Cell images were captured using a fluorescence microscope (Olympus BX53, Olympus America, Inc.).

#### Gel contraction assay

Gel contraction assay was performed as previously described<sup>5</sup>.

#### Statistical analyses

Statistical analysis was performed using Student's *t*-test or two-way analysis of variance (ANOVA) with SigmaPlot 11.0. The tests used are indicated in the figure legends. The ANOVA results were considered significant at a *p* < 0.05.

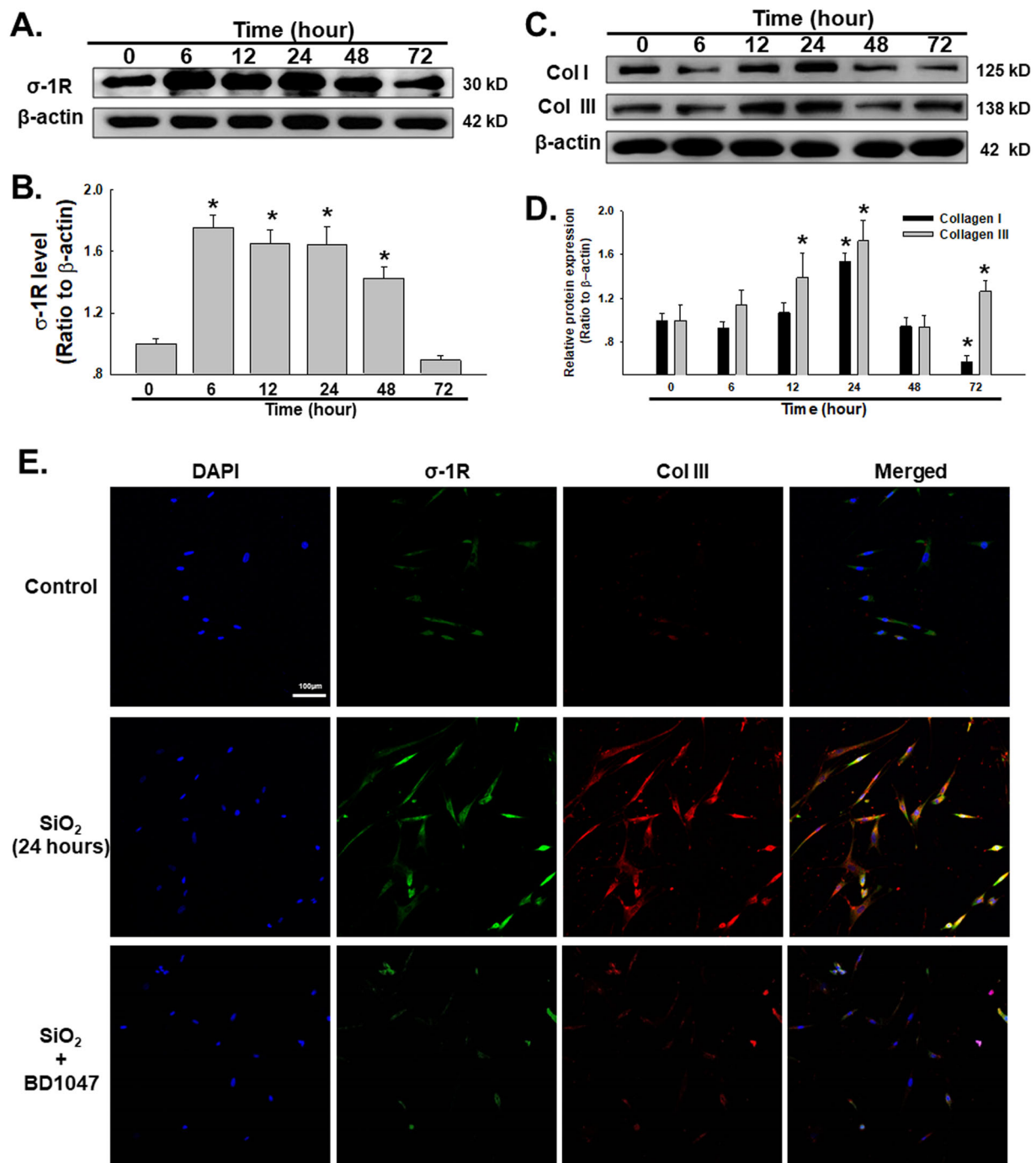
#### Results

##### Effects of SiO<sub>2</sub> on $\sigma$ -1R expression and collagen production in HPF-a

Previous reports have indicated that  $\sigma$ -1R is expressed in the lung; however, little is known about the effect of  $\sigma$ -1R on the progression of silicosis. Based on previous dosage experiments, 50  $\mu\text{g}/\text{cm}^2$  was selected for all the relating experiments<sup>35–39</sup>. To establish whether  $\sigma$ -1R is involved in silicosis, the HPF-a cell line was exposed to SiO<sub>2</sub> and assessed for  $\sigma$ -1R expression. Immunoblotting results demonstrated that  $\sigma$ -1R was upregulated in the presence of SiO<sub>2</sub> at 6, 12, 24 and 48 h (Fig. 1a and Fig. 1b). Moreover, collagen I and collagen III production levels were also significantly increased in the presence of SiO<sub>2</sub>, with a peak at 24 h (Fig. 1c and Fig. 1d). Immunocytochemistry assays confirmed the upregulation of  $\sigma$ -1R and collagen III induced by SiO<sub>2</sub> in HPF-a and the colocalization of  $\sigma$ -1R and collagen III, whereas collagen III induced by SiO<sub>2</sub> was attenuated by pretreatment of BD1047, a specific  $\sigma$ -1R inhibitor (Fig. 1e), indicating a role for  $\sigma$ -1R in fibroblast activation induced by SiO<sub>2</sub>.

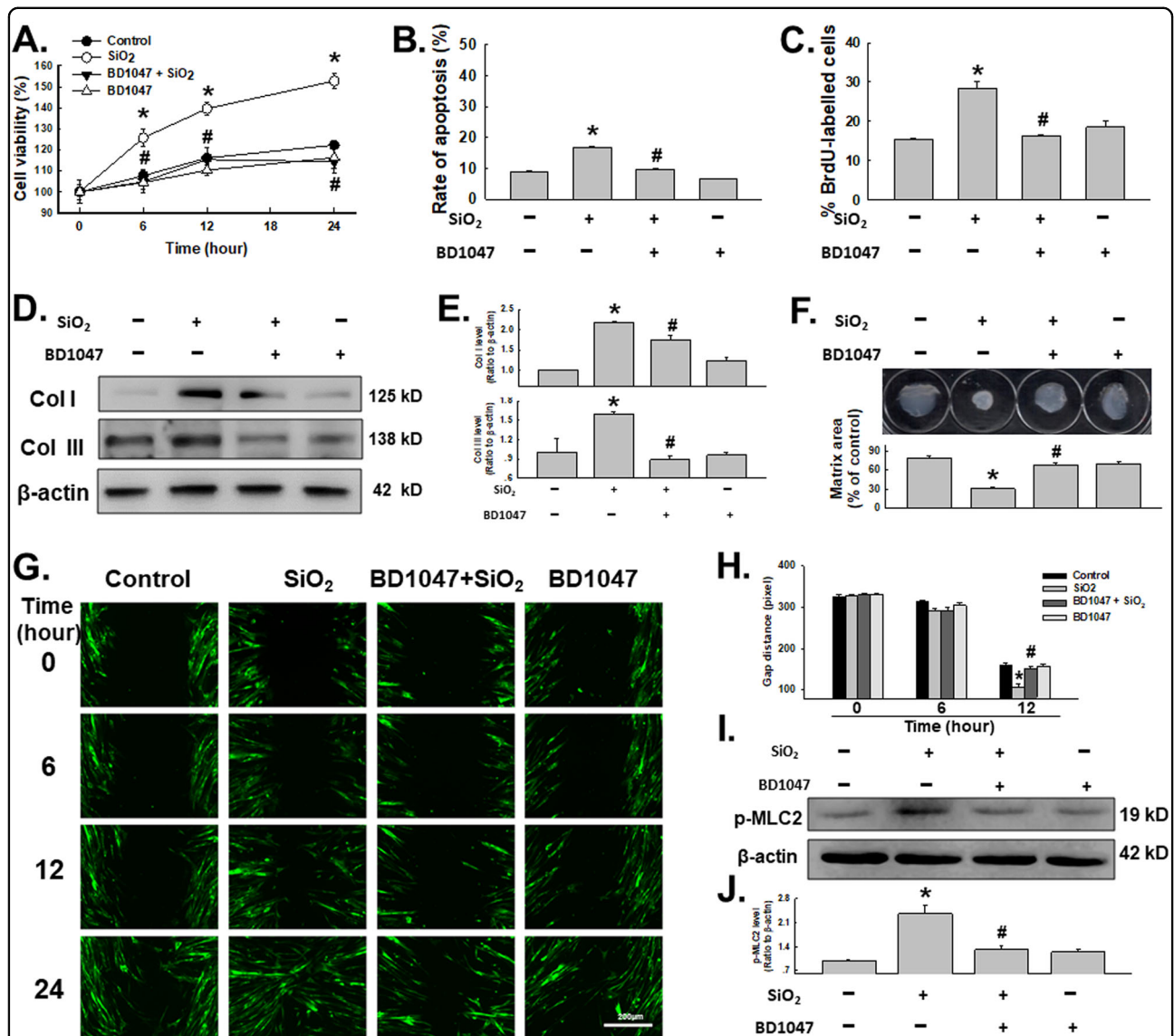
##### Involvement of $\sigma$ -1R in functional changes induced by SiO<sub>2</sub> in HPF-a

Pulmonary fibrosis in the late stage of silicosis is characterized by upregulation of fibroblast proliferation and migration<sup>40</sup>. Although  $\sigma$ -1R is involved in cell proliferation and migration in the central nervous system (CNS), the role of  $\sigma$ -1R in the peripheral system remains unclear. To establish the functional relevance of the changes in  $\sigma$ -1R expression, BD1047, a specific  $\sigma$ -1R inhibitor, was applied. As shown in Fig. 2a, SiO<sub>2</sub> induced a significant increase in HPF-a viability, which was attenuated by pretreatment with BD1047 (10  $\mu\text{M}$ ) for 2 h. To further understand the increase of cell viability induced by SiO<sub>2</sub>, both cell apoptosis and proliferation characteristic were analyzed. As shown in Fig. 2b and c, although SiO<sub>2</sub> induced slight increase in cell apoptosis in HPF-a (<20%), SiO<sub>2</sub> increase significant increase in cell proliferation, whereas both cell apoptosis and proliferation induced by SiO<sub>2</sub> were attenuated by pretreatment of BD1047. Meanwhile, pretreatment with BD1047 also inhibited collagen I and III induction by SiO<sub>2</sub> (Fig. 2d, e). Moreover, the matrix contraction assay was conducted, unveiling that pretreatment of BD1047 impaired gel contraction induced by SiO<sub>2</sub> exposure (Fig. 2f). Next, we explored the roles of  $\sigma$ -1R in SiO<sub>2</sub>-mediated cell migration. As shown



**Fig. 1** Effects of SiO<sub>2</sub> on  $\sigma$ -1R expression and collagen production in HPF-a. **a** Representative western blot showing SiO<sub>2</sub>-induced  $\sigma$ -1R upregulation in a time-dependent manner. **b** Densitometric analysis of  $\sigma$ -1R from five experiments; \*  $p < 0.05$  vs the control group. **c** SiO<sub>2</sub> upregulated collagen I and collagen III expression levels in a time-dependent manner. **d** Densitometric analysis of collagen I and collagen III from five experiments; \*  $p < 0.05$  vs the control group. **e** Representative immunocytochemical images showing that SiO<sub>2</sub> increased  $\sigma$ -1R and collagen III expression levels in HPF-a, which was attenuated by pretreatment of BD1047 (10  $\mu$ M)





**Fig. 2 Involvement of  $\sigma$ -1R in functional changes induced by SiO<sub>2</sub> in HPF-a.** **a** MTT assay showing that the SiO<sub>2</sub>-induced increase in cell viability was abolished by a 1-h pretreatment with BD1047 (10  $\mu$ M);  $n = 5$ ;  $*p < 0.05$  vs the control group at the corresponding time point. **b** Hoechst 33342 staining demonstrating that apoptosis induced by SiO<sub>2</sub> was attenuated by pretreatment of BD1047 (10  $\mu$ M) in HPF-a;  $n = 5$ ;  $*p < 0.05$  vs the control group,  $\#p < 0.05$  vs the SiO<sub>2</sub> group. **c** BrdU labeling assay demonstrating that proliferation induced by SiO<sub>2</sub> was attenuated by pretreatment of BD1047 (10  $\mu$ M) in HPF-a;  $n = 5$ ;  $*p < 0.05$  vs the control group,  $\#p < 0.05$  vs the SiO<sub>2</sub> group. **d** Representative western blot showing that SiO<sub>2</sub>-induced increased collagen I and III expression levels were attenuated by BD1047. **e** Densitometric analyses of collagen I and collagen III expression from five separate experiments;  $*p < 0.05$  vs the control group,  $\#p < 0.05$  vs the SiO<sub>2</sub> group. Representative immunocytochemical images showing that the  $\sigma$ -1R and collagen III co-localization induced by SiO<sub>2</sub> was attenuated by pretreatment with BD1047. **f** Representative images and data of gel contraction assay demonstrating that cell activation induced by SiO<sub>2</sub> was attenuated by pretreatment of BD1047 (10  $\mu$ M) in HPF-a;  $n = 5$ ;  $*p < 0.05$  vs the control group,  $\#p < 0.05$  vs the SiO<sub>2</sub> group. **g** Representative images showing the SiO<sub>2</sub>-induced increase in cell migration using a scratch assay was attenuated by pretreating HPF-a with BD1047. **h** Quantification of the scratch gap distances from six independent experiments;  $*p < 0.05$  vs. the corresponding time point in the control group;  $\#p < 0.05$  vs the corresponding time point in the SiO<sub>2</sub> group. **i** Representative western blot showing that SiO<sub>2</sub>-induced phosphorylation of MLC2 was attenuated by pretreatment with BD1047. **j** Densitometric analysis of the phosphorylation of MLC2 expression from five experiments;  $*p < 0.05$  vs the control group

in Figs. 2g, h, SiO<sub>2</sub> induced increased HPF-a migration, which was attenuated by pretreatment with BD1047. To further understand the mechanism of  $\sigma$ -1R in cell

migration, myosin light chain 2 (MLC2) levels were measured. Phosphorylation of MLC2 by Rho-associated coiled-coil-forming protein kinase (ROCK), demonstrated

to act as an effector downstream of Rho, can increase the contractility of actomyosin, which forms stress fibers and cell protrusions, resulting in cell migration<sup>41,42</sup>. As shown in Fig. 2I, j, MLC2 phosphorylation significantly increased in HPF-a exposed to SiO<sub>2</sub>, which was attenuated by pretreatment with BD1047. MLC2 phosphorylation has been shown to have important roles in promoting actin disassembly and cell detachment in non-muscle cells<sup>43,44</sup>. Excessive myosin activity may destabilize central stress fibers<sup>44,45</sup>, suggesting the role of the Rho-ROCK-MLC2 pathway in cell migration induced by  $\sigma$ -1R.

### SiO<sub>2</sub> induces ERS in HPF-a

$\sigma$ -1R is a ligand-regulated receptor chaperone triggered via ERS<sup>15</sup>. Whether upregulation of  $\sigma$ -1R also induces functional changes in HPF-a via ERS remains to be clarified. The protein kinase RNA-like ER kinase (PERK)/eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ )/C/EBP homologous protein (CHOP) pathway was tested first. SiO<sub>2</sub> administration induced increases in the expression levels of PERK, eIF2 $\alpha$ , CHOP (Fig. 3a–d). Binding immunoglobulin protein (BiP) is a master regulator of the ERS response; thus, we next investigated BiP expression and that of the ERS transcriptional enhancer associated activating transcription factor 6 $\alpha$  (ATF6 $\alpha$ ). HPF-a exposed to SiO<sub>2</sub> showed increased levels of BiP and elevated ATF6 $\alpha$  (Fig. 3e–g). Then, the involvement of the inositol-requiring protein-1 $\alpha$  (IRE1 $\alpha$ ) pathway in SiO<sub>2</sub>-induced ERS was investigated. As shown in Fig. 3h–i, HPF-a treated with SiO<sub>2</sub> showed upregulation of IRE1 $\alpha$  expression in a time-dependent manner. Taken together, these results demonstrate the activation of ERS in HPF-a cells exposed to SiO<sub>2</sub>.

### $\sigma$ -1R is involved in ERS induced by SiO<sub>2</sub>

To confirm the role of  $\sigma$ -1R in ERS upregulation induced by SiO<sub>2</sub>, BD1047 was applied. As shown in Fig. 4a, b, the exposure of SiO<sub>2</sub> induced not only upregulation but also phosphorylation of PERK, whereas pretreatment with BD1047 reversed the inductions by SiO<sub>2</sub>. Upregulation of nonphosphorylated and phosphorylated eIF1 $\alpha$  induced by SiO<sub>2</sub> was also attenuated by pretreatment with BD1047 (Fig. 4c–d). Moreover, the induction of CHOP, ATF6 $\alpha$  and IRE1 $\alpha$  by SiO<sub>2</sub> was attenuated by pretreatment with BD1047 (Fig. 4e–h), indicating an  $\sigma$ -1R/ERS pathway in fibroblast activation induced by SiO<sub>2</sub>.

### SiO<sub>2</sub> induced ERS-mediated fibroblast functional changes

After determining that the upregulated ERS associated with  $\sigma$ -1R was induced by SiO<sub>2</sub>, the roles of those changes in fibroblast migration and collagen production were investigated. To this end, salubrinal, a specific inhibitor of ERS, was applied. As shown in Fig. 5a–c, whereas

salubrinal did not affect  $\sigma$ -1R induction by SiO<sub>2</sub>, salubrinal attenuated collagen I and III upregulation induced by SiO<sub>2</sub>. In a 2D migration experiment, HPF-a migration was activated by the exposure to SiO<sub>2</sub>, which was then inhibited after blocking the function of ERS with salubrinal. (Fig. 5d–e)

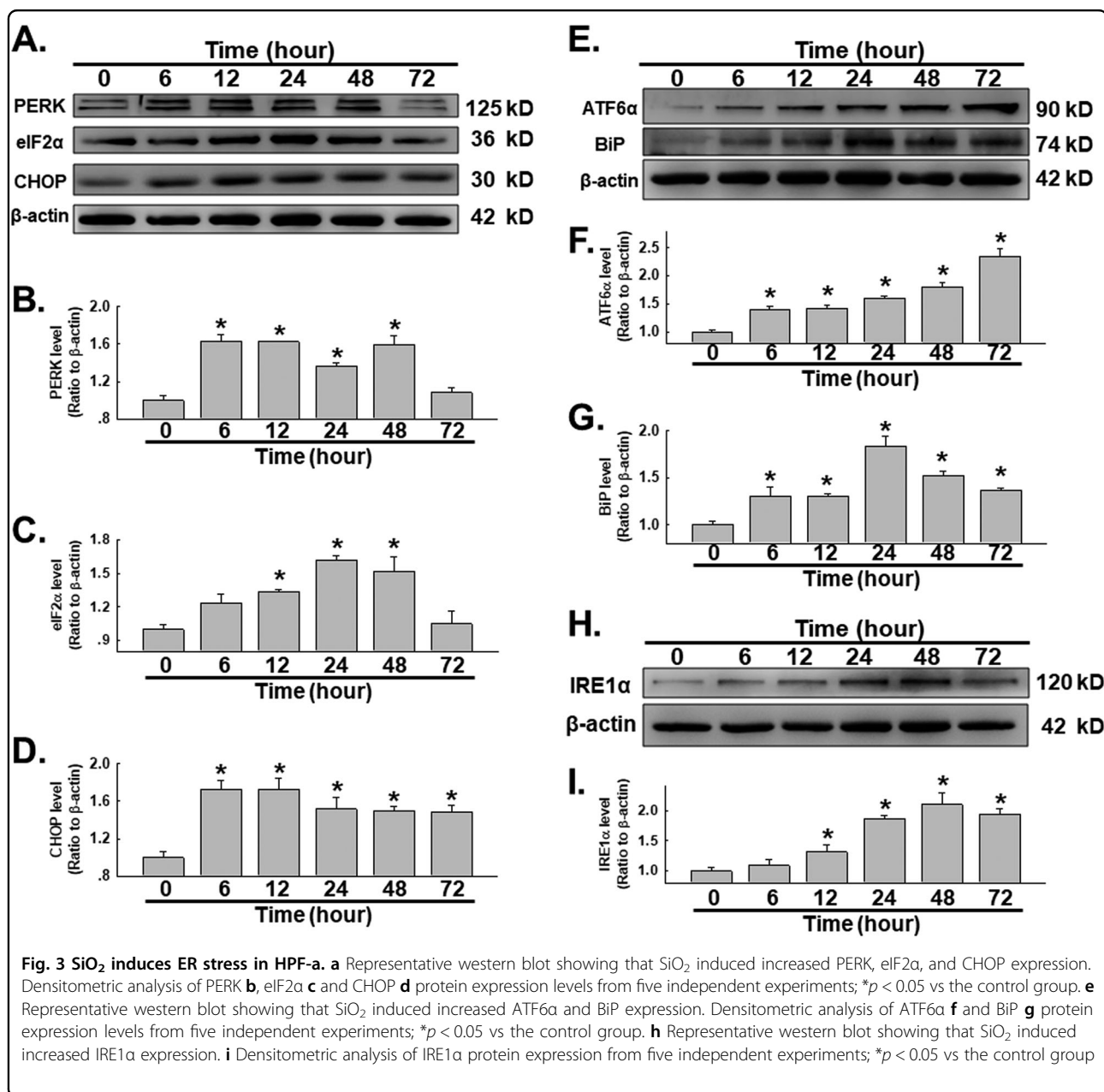
### circHIPK2 is involved in regulating $\sigma$ -1R after SiO<sub>2</sub> exposure in HPF-a

Non-coding RNA is an important post-transcriptional mechanism to regulate proteins, whereas  $\sigma$ -1R regulation associated with non-coding RNA was recently reported<sup>24</sup>. Bioinformatic analysis identified circHIPK2, which has two complementary residues with miR-506-3p; a conserved miR-506-3p binding site within the  $\sigma$ -1R 3'-untranslated region was defined as the putative target of miR-506-3p (Fig. 6a), indicating that circHIPK2 may regulate  $\sigma$ -1R expression by acting as a ceRNA. As shown in Fig. 6b, FISH assays revealed the expression of circHIPK2 in HPF-a, in which, expression of circHIPK2 was upregulated after SiO<sub>2</sub> exposure. Moreover, circHIPK2 and miR-506-3p levels were then detected by qRT-PCR (Fig. 6c–d). circHIPK2 showed a slight increase, whereas miR-506-3p remained stable with the time exposure of SiO<sub>2</sub>, indicating that ceRNA may be involved in  $\sigma$ -1R regulation. To further investigate the function of circHIPK2, siRNA was used to specifically silence the circRNAs (Fig. 6e). After silencing with circHIPK2-siRNA,  $\sigma$ -1R upregulation induced by SiO<sub>2</sub> was attenuated (Fig. 6f–g). Moreover, circHIPK2-siRNA also inhibited the upregulation of BiP (Fig. 6h–i), collagen I and III (Fig. 6j–k) induced by SiO<sub>2</sub>, indicating that circHIPK2 was upstream of functional changes in HPF-a (Fig. 7).

### Discussion

Mounting evidence has suggested that pulmonary fibrosis was mainly caused by abnormal activation of alveolar macrophages and pulmonary fibroblasts (PFBs)<sup>46</sup>. As the straightforward impacts of SiO<sub>2</sub> on PFBs tend to be relatively unnoticed, we focused on the effects of  $\sigma$ -1R expression in pulmonary fibroblasts on cell proliferation and migration after *in vitro* exposure to SiO<sub>2</sub>.

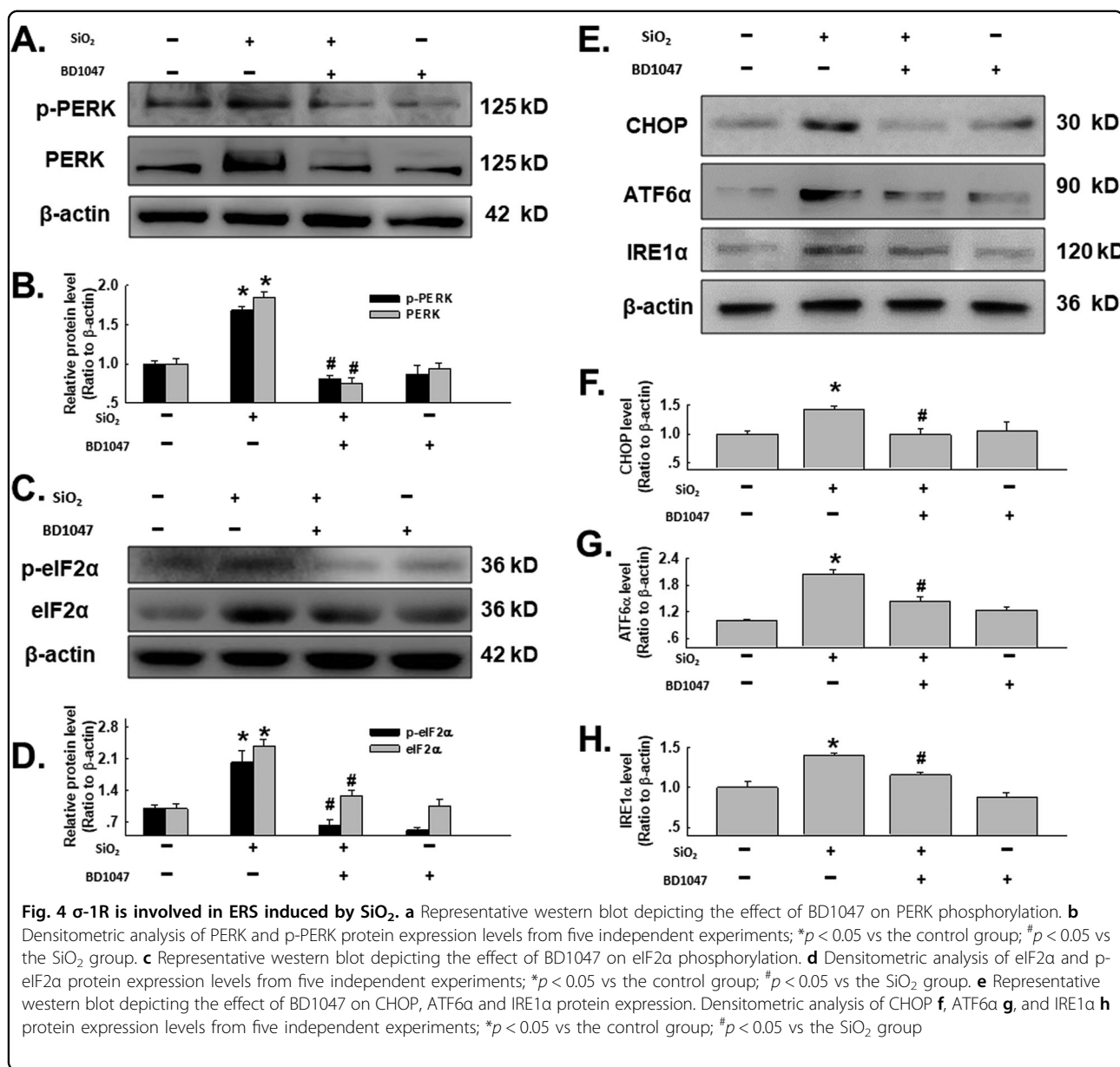
Several lines of evidence have demonstrated that  $\sigma$ -1R is a 25-kDa protein expressed in the endoplasmic reticulum that can facilitate cell proliferation and migration in the CNS. For instance,  $\sigma$ -1R receptor agonists exert potentiation of neurite outgrowth, myelination, long-term potentiation, and neuroprotection in *in vitro* studies and in animal models of neuropsychiatric diseases, such as ischemia and Alzheimer's disease<sup>47–53</sup>. In the current study, SiO<sub>2</sub>-induced  $\sigma$ -1R upregulation promoted cell proliferation and migration in PFBs, indicating the general role of  $\sigma$ -1R in determining cell fate. Moreover, effect of SiO<sub>2</sub> on Col I, Col III and  $\sigma$ -1R, as well as PERK and eIF $\alpha$ ,



was recovered after 24 h, which may due to the maximum effect reached. On the other hand, both ATF6 $\alpha$  and BiP showed a rapid and sustained increase after SiO<sub>2</sub> exposure because those two protein is upstream of other ERS marker, whereas further experiments need to confirm this different expression pattern of ERS marker.

The ER is an important organelle in the regulation of unfolded protein response (UPR), a protective mechanism accumulation of misfolded or unfolded proteins and autophagy, an intracellular degradation pathway for recycling and eliminating abnormal protein and damaged organelles<sup>16–18,54</sup>. These two mechanisms are correlative,

confirmed by studies shed light on the ability of ERS to stimulate or inhibit autophagy. On the other side, the UPR is distinguished by the action of three signaling proteins: PERK, ATF6, and IRE1 $\alpha$ <sup>17</sup>. Under physiological conditions, the luminal domains of PERK and ATF6 proteins are bound to the ER resident chaperone BiP, which keeps them inactivated<sup>55</sup>. It has been postulated that unfolded protein accumulation in the ER induces BiP release from these complexes to assist with the folding of accumulated proteins. Unlike these two associated with BiP, another UPR modulators IRE1 $\alpha$  induces signal transduction events ameliorating misfolded protein accumulation in

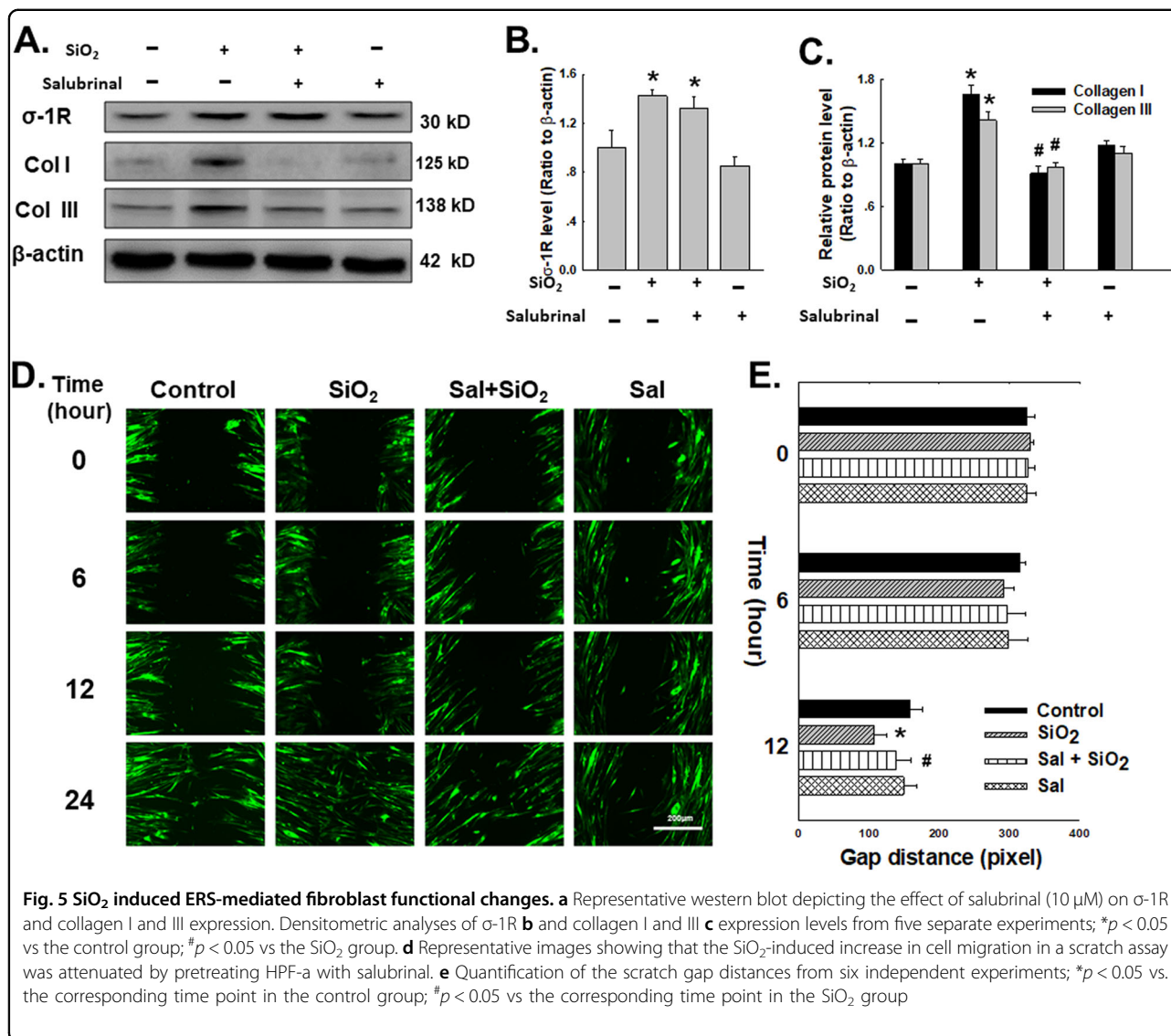


the ER by promoting the expression of ER chaperones, inhibiting protein entry into the ER via arresting mRNA translation and stimulating retrograde transportation of misfolded proteins from the ER into the cytosol for ubiquitination and destruction by a process known as ER-assisted degradation<sup>55,56</sup>. Therefore, in current study, SiO<sub>2</sub> exposure to fibroblast increase the accumulation of misfolded protein, thus activating UPR, which leads to the upregulation of ERS related protein.

$\sigma$ -1R is highly clustered at specialized ER subdomains that are physically associated with the mitochondrial outer membrane (MAM)<sup>15</sup>, a specialized ER membrane that regulates Ca<sup>2+</sup> signal transduction, bioenergetics, cell death, and lipid metabolism<sup>57,58</sup>. At the MAM,  $\sigma$ -1R acts

as a molecular chaperone and sustains the proper conformation of inositol triphosphate receptor type 3 to ensure proper Ca<sup>2+</sup> signaling from the ER into mitochondria to facilitate ATP production<sup>15,59,60</sup>. The  $\sigma$ -1R also chaperones an ERS sensor, inositol-requiring enzyme 1, to ensure the proper transmission of ERS into the nucleus to call for the enhanced production of anti-stress and antioxidant proteins<sup>61</sup>. Previous study has elucidated that  $\sigma$ -1R is highly assembled in MAM, forming a complex with BiP under physiological conditions. Once ERS is activated,  $\sigma$ -1R dissociates from BiP and becomes an active molecular chaperone. Mounting evidence has demonstrated that  $\sigma$ -1R is involved in ERS activation in different settings. All these studies indicated that the





**Fig. 5**  $\text{SiO}_2$  induced ERS-mediated fibroblast functional changes. **a** Representative western blot depicting the effect of salubrinal (10  $\mu\text{M}$ ) on  $\sigma$ -1R and collagen I and III expression. Densitometric analyses of  $\sigma$ -1R **b** and collagen I and III **c** expression levels from five separate experiments; \* $p < 0.05$  vs the control group; # $p < 0.05$  vs the  $\text{SiO}_2$  group. **d** Representative images showing that the  $\text{SiO}_2$ -induced increase in cell migration in a scratch assay was attenuated by pretreating HPF-a with salubrinal. **e** Quantification of the scratch gap distances from six independent experiments; \* $p < 0.05$  vs. the corresponding time point in the control group; # $p < 0.05$  vs the corresponding time point in the  $\text{SiO}_2$  group

mechanism of  $\sigma$ -1R was through ERS, whereas ERS was not thoroughly explored in silicosis. Here, we found that upregulation of  $\sigma$ -1R induced by  $\text{SiO}_2$  via ERS mediated a downstream cascade of fibroblast activation, indicating the therapeutic target of pulmonary fibrosis. Moreover, another of example of  $\sigma$ -1R involvement in disease development was unveiled, which is involved in cellular functional change and might be a general change in the disease pathology process.

Compared with studies on  $\sigma$ -1R function, research on its regulatory mechanism is far less. A recent study suggested a role for post-transcriptional mechanisms associated with non-coding RNAs in  $\sigma$ -1R regulation<sup>24</sup>. Newly found circRNAs form a class of novel non-coding RNAs that are relatively stable due to their cyclic structures. Therefore, circRNAs can resist excision enzymes, making them ideal disease marker candidates. circRNAs can act as

molecular sponges to combine and block the function of miRNAs, namely, via the ceRNA mechanism. Bioinformatic analysis suggests that circHIPK2 and miR-506-3p may be involved in  $\sigma$ -1R regulation via ceRNA. Study indicates that long non-coding RNA NEAT1 facilitates pancreatic cancer progression through negative modulation of miR-506-3p<sup>62</sup>. CircHIPK2 is derived from exon 2 of the HIPK2 gene, acting as a sponge for several microRNA. A recent study reported that circHIPK2 can directly bound to miR-124 and acted as an endogenous sponge for miR-124 to inhibit its activity, thus activate astrocyte via the regulation of autophagy and ERS through  $\sigma$ -1R<sup>27,62</sup>. In addition, HIPK2 is reported to be a crucial driver of kidney fibrosis by regulating pro-apoptosis, pro-fibrotic and pro-inflammatory pathways<sup>63</sup>, which may be a potential target for antifibrotic. These evidences suggest that circHIPK2 has a significant role in cell fate

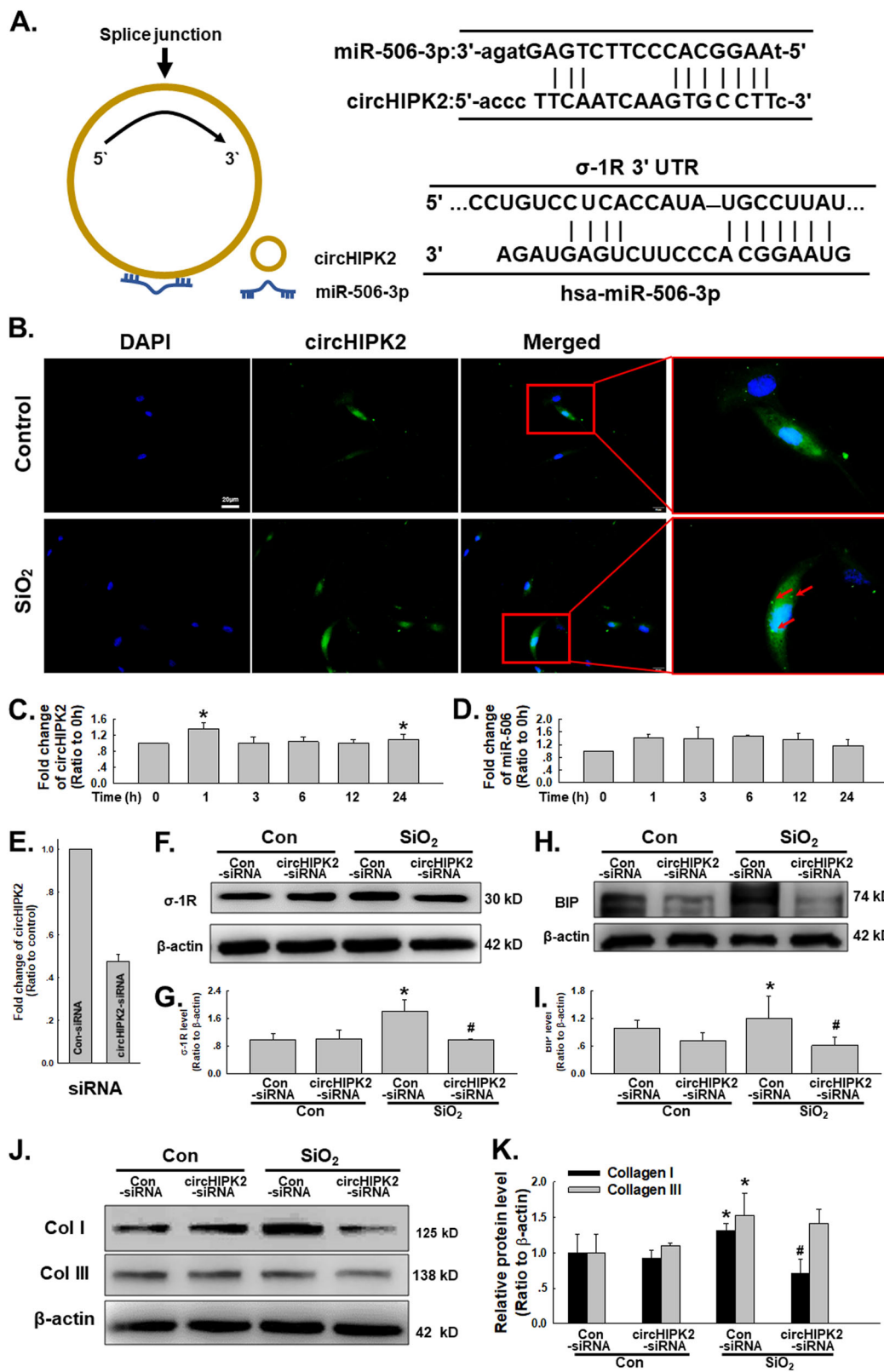
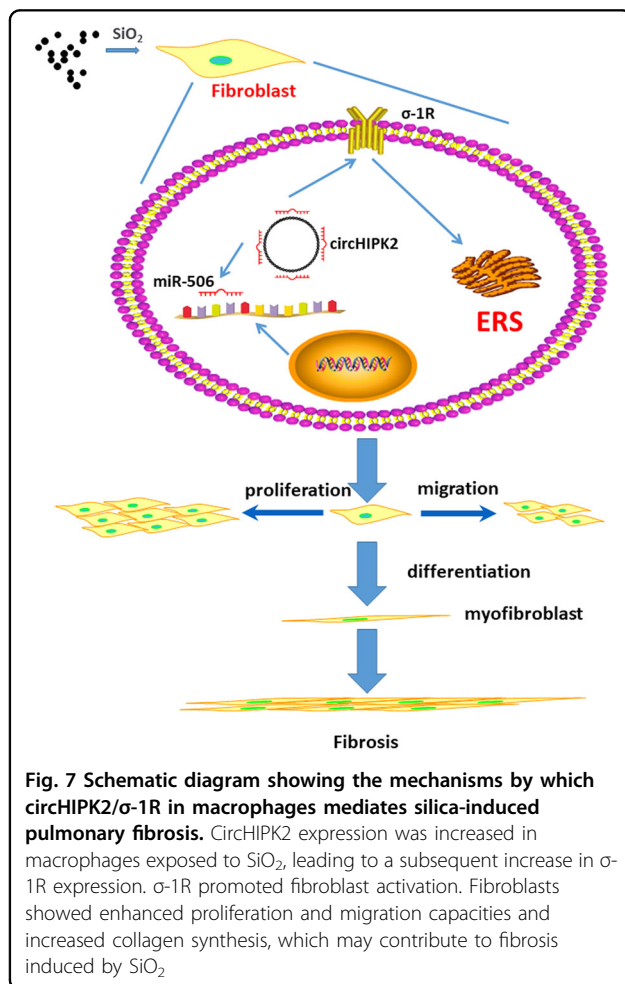


Fig. 6 (See legend on next page.)

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**Fig. 6 circHIPK2 is involved in regulating  $\sigma$ -1R after SiO<sub>2</sub> exposure in HPF-a.** **a** Bioinformatics analysis showing that circHIPK2 contains one site complementary to miR-506-3p and two miR-506-3p binding sites in  $\sigma$ -1R. **b** FISH assay showing circHIPK2 expression in HPF-a increased after SiO<sub>2</sub> exposure; circHIPK2 was labeled with fluorescein isothiocyanate. **c** qRT-PCR assay showing SiO<sub>2</sub> induced circHIPK2 upregulation from six independent experiments; \**p* < 0.05 vs the control group. **d** qRT-PCR assay showing SiO<sub>2</sub> had no effect on miR-506 expression in six independent experiments; \**p* < 0.05 vs the control group. **e** qRT-PCR assay showing siRNA knockdown of circHIPK2 inhibited circHIPK2 expression in HPF-a; *n* = 5. **f** Representative western blot depicting the effect of siRNA of circHIPK2 on  $\sigma$ -1R expression. **g** Densitometric analyses of  $\sigma$ -1R expression from five separate experiments; \**p* < 0.05 vs the control group; #*p* < 0.05 vs the SiO<sub>2</sub> group. **h** Representative western blot depicting the effect of siRNA of circHIPK2 on BiP expression. **i** Densitometric analyses of BiP expression from five separate experiments; \**p* < 0.05 vs the control group; #*p* < 0.05 vs the SiO<sub>2</sub> group. **j** Representative western blot depicting the effect of siRNA of circHIPK2 on collagen I and III expression. **k** Densitometric analyses of collagen I and III expression from five separate experiments; \**p* < 0.05 vs the control group; #*p* < 0.05 vs the SiO<sub>2</sub> group



determination. In current study found that circHIPK2 expression increased, whereas miR-506-3p remained stable, which matches the predicted ceRNA mechanism. Functional experiments and specific knockdown of circHIPK2 with siRNA confirmed the role of circHIPK2 in fibroblast activation induced by SiO<sub>2</sub>, suggesting that circHIPK2 may be a potential biomarker in diagnosing silicosis.

## Conclusions

Our study elucidated a link between SiO<sub>2</sub>-induced fibroblast activation and the circHIPK2/ $\sigma$ -1R pathway, thereby providing insight into the potential use of  $\sigma$ -1R to develop novel therapeutic strategies for treating silicosis.

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## Authors' contributions

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

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## Competing interests

The authors declare that they have no conflict of interest.

## Ethics approval and consent to participate

Not applicable.

## Availability of data and materials

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