MCPIP1 mediates silica-induced cell migration in human pulmonary fibroblasts

Running Title: MCPIP1 induced pulmonary fibroblast migration

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**ABSTRACT**

**Background:** Silicosis is a systemic disease caused by inhaling silicon dioxide (SiO₂). Phagocytosis of SiO₂ in the lungs initiates an inflammatory cascade that results in fibroblast proliferation and migration followed by fibrosis. According to previous data from our lab, monocyte chemotactic protein-1 (MCP-1) plays a critical role in fibroblast proliferation and migration in conventional 2-dimensional (2D) monolayer cultures. The current study aimed to explore the downstream cascade of MCP-1 in both 2D and 3-dimensional (3D) cell culture models of silicosis.

**Methods and results:** Experiments using primary cultured adult human pulmonary fibroblasts (HPF-a) demonstrated the following: 1) SiO₂ treatment induces expression of MCP-1-induced protein (MCPIP1) in a time- and dose-dependent manner in both 2D and 3D cultures; 2) the MAPK and PI3K/Akt pathways are involved in SiO₂-induced MCPIP1 expression; and 3) MCPIP1 induction mediates the SiO₂-induced increase in cell migration in both 2D and 3D cultures.

**Conclusion:** The effect of MCP-1 in silicosis occurs mainly through MCPIP1, which, in turn, mediates the observed SiO₂-induced increase in pulmonary fibroblast migration. However, the time frame for MCPIP1 induction differed between 2D and 3D cultures, indicating that compared with conventional 2D cell culture systems, 3D culture may be useful for analyses of fibroblast physiology under conditions that more closely resemble *in vivo* environments. Our study determined the link between fibroblast-derived MCPIP1 and SiO₂-induced cell migration, and this finding provides novel evidence of the potential of MCPIP1 in the development of novel therapeutic strategies for silicosis.

**Key words:** MCPIP1; pulmonary fibroblast; migration; silicosis; 3-dimensional cultures
**Background**

Silicosis, which is caused by the inhalation of silica, is one of the most serious occupational diseases worldwide. The pathogenic characteristics of silicosis include chronic inflammation and late pulmonary fibrosis. Furthermore, lung function impairment increases with disease progression, even when the patient is no longer exposed to silica (26).

Increasing evidence suggests that pulmonary fibroblast migration is a critical aspect of pulmonary fibrosis (7, 21, 26, 29, 35, 43, 44). Although fibroblast function in silicosis has been studied extensively in 2-dimensional (2D) cell culture models (19), discrepancies between the behaviors of cells in culture and *in vivo* have motivated an increasing number of research groups to utilize 3-dimensional (3D) models, which better represent the microenvironment of living tissue (8).

The extracellular matrix (ECM) plays important roles during migration processes, including aiding in the establishment of the cellular environment and providing mechanical support and a framework for cells and tissues (12, 18, 23, 40). Cell migration/motility in a 3D matrix is a complicated process that involves cell-matrix adhesion, cell-matrix interaction, and global/local matrix remodeling (38). Studies of fibroblasts cultured in collagen matrices have been used to model fibrosis *in vitro*, with the fibroblasts developing a myofibroblast-like appearance and exerting isometric tension on the matrix similar to that found *in vivo* (9, 10, 19, 23). Significant differences in effects on cell physiology have been observed between 2D and 3D *in vitro* culture systems (14, 34, 37, 38). Compared with conventional 2D cell culture systems, the fibroblast-populated collagen matrix (FPCM) culture system has facilitated the analysis of fibroblast physiology under conditions that more closely resemble the *in vivo* environment (14, 25, 34, 37, 38). Nonetheless, the mechanisms by which SiO2 affects the function of fibroblasts under conditions of a 3D-collagen matrix system remain unknown. Previous data from our laboratory demonstrated that SiO2 induces functional changes in fibroblast physiology, such as the induction of cellular apoptosis and increases in matrix contraction and cellular migration (29). However, details...
regarding how SiO₂ regulates fibroblast migration in a 3D-collagen matrix have not been described to date.

Although a previous study demonstrated that chemokine/cytokine monocyte chemotactic protein-1 (MCP-1) is released by pulmonary fibroblasts treated with SiO₂ (29), details of the downstream mechanistic role of MCP-1, specifically in 3D culture systems, have not been determined. MCP-1-induced protein 1 (MCPIP1 or ZC3H12A) was recently identified in human peripheral blood monocytes treated with MCP-1 (49), and studies suggest that MCPIP1 is MCP-1-inducible and plays a fundamental role in immune regulation (27). For instance, a study by Liang, J. et al. indicated that MCPIP1 is a negative regulator of macrophage activation (28), and further investigations indicated that MCPIP1 has a significant anti-inflammatory function by inhibiting the generation of a set of major proinflammatory cytokines (27, 30). With the emerging interest in the molecular mechanisms that control fibroblasts in silicosis, a growing number of new molecules have been identified in recent years (22, 36). MCPIP1 is expressed by immune cells, in which it targets proteins involved in regulating inflammation and consequently affects the magnitude of the immune response (33). The function of MCPIP1 in fibroblast migration and subsequent fibrosis, however, remains poorly defined. Given its well-established role in human umbilical vein endothelial cell (HUVEC) migration (50), MCPIP1 is a promising candidate regulator of fibroblast migration.

The present study aimed to explore the mechanisms by which SiO₂ mediates MCPIP1 induction in human fibroblasts cultured in both 2D and 3D models and to reveal the potential role of MCPIP1 in fibroblast migration following SiO₂ exposure. Understanding how MCPIP1 expression is regulated and determining its functional relevance in the process of silicosis may provide us with a better understanding of fibrosis and offer insights into potential therapeutic targets for silicosis.

Materials and Methods

Reagents
SiO$_2$ was obtained from Sigma® (S5631, 1-5 µm). Fetal bovine serum (FBS), normal goat serum, Dulbecco’s modified Eagle’s medium (DMEM, #1200-046), and 10X-MEM (11430-030) were acquired from Life Technologies™. Amphotericin B (BP2645) and GlutaMax™ Supplement (35050-061) were obtained from Gibco®, and Pen Strep (15140-122) was purchased from Fisher Scientific. PureCol® type I bovine collagen (3 mg/mL) was obtained from Advanced Biomatrix. Antibodies against p53 (SC6243, rabbit), MCPIP1 (SC136750, goat) and β-actin (SC8432, mouse) were obtained from Santa Cruz Biotechnology®, Inc. The short interfering RNA (siRNA) transfection reagent (SC29528) and MCPIP1 siRNA (SC78944) were purchased from Thermo Scientific.

**Cell culture**

Adult human pulmonary fibroblasts (HPF-a) were purchased from ScienCell and maintained in T75 flasks in DMEM containing 10% FBS. The cells were stored in liquid nitrogen at passages 3-7 (P3-7). For each experiment, a vial of P3-7 HPF-a was thawed, plated, and passaged upon confluence; fibroblasts were used between P10 and P15.

**Fibroblast-populated collagen matrix (FPCM)**

A collagen matrix model was utilized as previously described (29). The final matrix parameters were as follows: volume = 0.2 mL; diameter = 12 mm; collagen concentration = 1.5 mg/mL; and cell concentration = 1.0 × 10$^6$ cells/mL. Matrices were established in 24-well plates (BD#353047) and incubated in the attached state with 5% FBS in DMEM (supplemented with 50 μg/mL ascorbic acid) for approximately 48 h prior to the initiation of an experiment.

**MTT assay**

Cell viability was measured via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (29). Briefly, cells were collected and seeded into 96-well plates; different seeding densities were optimized at the beginning of the experiments. The cells were then exposed to SiO$_2$. After incubation for 24 h, 20 μL of MTT tetrazolium salt dissolved in Hank’s balanced salt solution was added to each well at a final concentration of 5 μg/mL, and the plates were
incubated in a CO₂ incubator for 1-4 h. Finally, the medium was aspirated from each well, and 200 μL of dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance of each well was measured using a microtiter plate spectrophotometer at reference wavelengths of 570 nm and 630 nm. Each of the experiments was repeated at least three times.

Establishment of green fluorescence- and red fluorescence-labeled primary HPF-a

HPF-a were transduced with LV-RFP or LV-GFP lentivirus (Hanbio Inc., Shanghai, CN) as previously described (6). Briefly, P3-4 primary HPF-a were cultured for 48 h in 10% FBS in DMEM in a 24-well plate (1×10⁴ cells/well). The medium was replaced with 1 mL of fresh medium and 8 μg/mL polybrene; 100 μL of lentivirus solution (10⁷ IU/mL) was added to each well, followed by incubation at 37°C in 5% CO₂ for 24 h. The treatment medium was then replaced with fresh DMEM containing 10% FBS, and the cells were cultured at 37°C in 5% CO₂ until >50% confluence was achieved. The transduced cells were selected using blasticidin, as follows. The medium was replaced with DMEM containing 10 μg/mL puromycin and 10% FBS, and the cells were cultured at 37°C in 5% CO₂ for 24 h. The cells were then washed twice with fresh DMEM containing 10% FBS. Purified transduced cultures of HPF-a were expanded and/or stored in liquid nitrogen as previously described (3). Previous data from our lab have shown no significant effect of GFP or RFP expression on fibroblast morphology, contraction, migration, or population growth (6).

In vitro scratch assay

Cell migration ability in a 2D culture system was evaluated using an in vitro scratch assay. Briefly, 1×10⁵ HPF-a were seeded in 24-well tissue culture plates and cultured in growth medium for 24 h, a time when the HPF-a were approximately 70-80% confluent. Using a sterile 200-μL pipette tip, a straight line was carefully scratched in the monolayer across the center of the well in a single direction while keeping the tip perpendicular to the bottom of the plate. Similarly, a second straight line was scratched perpendicular to the first line to create a cross-shaped cellular gap in each well. Each well was washed twice with 1 mL of
fresh growth medium to remove any detached cells. Digital images of the cell gap were captured at different time points, and the gap width was quantitatively evaluated using ImageJ (http://rsb.info.nih.gov/ij/) software.

**Nested matrix model and cell migration assay**

A nested collagen matrix model was utilized as previously described (15, 29), with certain modifications. For the nested attached matrix, a standard FPCM was incubated in the attached state for 72 h in DMEM containing 10% FBS. The FPCM was then removed from the culture well and placed in a 60-µL aliquot of fresh acellular collagen matrix solution (NeoMatrix solution) in the center of a 12-mm-diameter score on the bottom of a new well. The newly transferred FPCM was covered with a 140-µL aliquot of NeoMatrix solution, which was allowed to polymerize for 1 h at 37˚C in 5% CO₂; 2 mL of DMEM containing 10% FBS was then added to the well.

Cell migration out of the nested FPCM and into the acellular NeoMatrix was quantified via fluorescence microscopy at 24 h after nesting. Digital images (constant dimensions of 1000 × 800 µm) were captured using an EVOS® FL Cell Imaging microscope (Life Technologies, Grand Island, NY) for 3-5 randomly selected microscopic fields at the interface of the nested FPCM and the acellular NeoMatrix. Migration of HPF-a out of the nested FPCM was quantified by counting the number of cells that had clearly migrated from the nested matrix into the cell-free matrix. The maximum migration distance was quantified by identifying the cells that had migrated the greatest distance into the cell-free matrix. The number of cells per field that had migrated out of the nested matrix and the maximum migration distance per field were averaged from the digital micrographs.

**MCPIP1 RNA interference using siRNA**

MCPIP1 RNA interference was performed on FPCMs as previously described (4, 29), with some modifications. The protocol for a single collagen matrix is as follows. Briefly, 17.5 µL of serum-free DMEM was separately combined with 3 µL of transfection reagent or 3 µL of siRNA stock, followed by incubation at room temperature (RT) for 15 min. The transfection reagent and siRNA solutions were
then mixed together and incubated at RT for another 15 min. HPF-a were prepared at a concentration of 5.0x10^6 cells/mL in serum-free DMEM according to the FPCM protocol. The siRNA-vehicle solution was mixed with 40 µL of the cell suspension and incubated at RT for 15 min, and the mixture was added during the FPCM setup. The transfected matrices were cultured for 24 h in serum-free DMEM, which was then replaced with 5% FBS in DMEM (supplemented with 50 µg/mL ascorbic acid) for 48 h prior to conducting further experiments. The knock-down efficiency of siRNAs was determined after 2 days of transfection by Western blot analysis.

**Gel contraction assay**

FPCM contraction was determined using the floating matrix contraction assay as previously described (1), with minor modifications. Briefly, matrices were polymerized, covered with DMEM containing 5% FBS, released from the culture well using a sterile spatula, and incubated at 37°C. The matrices were fixed overnight at 4°C at different time points after release using 4% paraformaldehyde in phosphate-buffered saline (PBS), and images were obtained using a flatbed scanner connected to a desktop. The matrix area was measured using ImageJ software, and the data are presented as the ratio of the released matrix area to the attached matrix area.

**Immunoblotting**

Immunoblotting was performed as previously described (3, 29), with minor modifications. FPCMs were collected from culture dishes and washed with PBS, and HFFs were lysed using a Mammalian Cell Lysis kit (MCL1-1KT, Sigma-Aldrich®) according to the manufacturer’s instructions. Western blots were probed with antibodies recognizing p53 (1:1,000) and β-actin (1:10,000) and alkaline phosphatase-conjugated goat anti-mouse or rabbit IgG secondary antibodies (1:5,000). Signals were detected by chemiluminescence (SuperSignal West Dura Chemiluminescent Substrate, Thermo Scientific). Each Western blot was repeated for three different donors, and a single representative immunoblot for all related blots is shown in each figure. Densitometry was performed using ImageJ (http://rsb.info.nih.gov/ij), and the results for all repeated experiments
were combined into one plot.

**Immunocytochemistry**

HPF-a were fixed with 4% paraformaldehyde in PBS at 4˚C overnight. The fixed samples were permeabilized for 30 min at room temperature (RT) 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 RT for 2 h. The blocked samples were incubated overnight at 4°C with primary antibodies diluted in PBS containing 10% NGS and 0.3% Triton X-100. The samples were then washed three times with PBS and incubated with donkey anti-rabbit (conjugated to Alexa-Fluor® 488) and donkey anti-mouse (conjugated to Alexa-Fluor® 576) secondary antibodies for 2 h at RT. After three washes with PBS, the samples were mounted with mounting solution (Prolong® Gold antifade reagent with DAPI; P36931, Life Technologies), and the slides were examined using an EVOS FL fluorescence microscope.

**Statistics**

Data are expressed as the mean ± SEM. Unpaired numerical data were compared using an unpaired t-test (two groups) or analysis of variance (ANOVA; more than two groups), with statistical significance set at p < 0.05.

**Results**

**SiO2 induces MCPIP1 expression in a dose-dependent manner in HPF-a**

Previous data from our lab suggested that SiO2 induces a rapid increase in MCPIP1 expression in a time-dependent manner in HPF-a grown in 2D culture (29). However, it is unclear whether those effects were dependent on the dosage of SiO2. In the present study, we first examined the effect of SiO2 dosage on the expression of MCPIP1 in 2D and 3D models. As shown in Figure 1, the maximal effect of a 3-h SiO2 treatment on MCPIP1 expression resulted from a dose of 50 µg/cm² in the 2D model (Figure 1A-B) and from doses of 50 and 100 µg/cm² in the 3D model (Figure 1D-E), consistent with the results from other studies regarding the differences in cell physiology between 2D and 3D models (14, 34, 37, 38). SiO2 also induced α-SMA expression in a dose-dependent manner in
both 2D and 3D models, with a peak response at 50 and 100 µg/cm² (Figure 1A, 1C, 1D, 1E). Interestingly, α-SMA expression decreased in the 2D model at 200 µg/cm² (Figure 1A and 1C), whereas α-SMA expression in the 3D model at the same dosage remained elevated (Figure 1D and 1E). In addition, 200 µg/cm² SiO₂ exhibited a toxic effect in both models, as cell viability was significantly decreased compared with the other groups (Figure 1G), indicating an unspecified effect of a high SiO₂ dosage. Based on this dosage experiment and on results from previous studies (2, 11, 17), we chose 50 µg/cm² for all of the subsequent experiments.

**SiO₂ induces MCPIP1 expression in a time-dependent manner in HPF-a**

Because the results of the dose-response experiments indicated a difference in responses to SiO₂ by HPF-a in 2D and 3D models, we investigated the timing of the responses in both models. Previous data suggest a rapid induction of MCPIP1 expression in SiO₂-treated HPF-a. Due to the multifunctionality of MCPIP1 in process of cell migration, we extended our time-course experiments to 24 h. As shown in Figure 2A-B, SiO₂ induced MCPIP1 and α-SMA expression in a time-dependent manner, with a peak at 1 h in the 2D model that returned to the control level after 24 h; this finding was confirmed by immunostaining (Figure 2C). Although SiO₂ induced MCPIP1 and α-SMA expression in a time-dependent manner in the 3D model, the peak responses of MCPIP1 and α-SMA differed: MCPIP1 expression peaked at 3 and 6 h and then returned to control levels, whereas α-SMA exhibited a rapid and sustained increase in the 3D model (Figure 2D-E). Because α-SMA is a marker of fibroblast activation, these results suggest that compared with conventional 2D cell culture systems, the fibroblasts exhibited increased motility in the ECM, indicating that the cells in the 3D model had physiological characteristics that more closely resembled those observed in the in vivo environment.

**SiO₂ induces MAPK (ERK, JNK, p38) and PI3K/Akt phosphorylation**

Because the MAPK kinase and PI3K/Akt pathways play critical roles in migration signaling (45, 46), we next examined the involvement of MAPK and
PI3K/Akt in an *in vitro* silicosis model. First, we examined the effect of SiO$_2$ on the activation of MAPKs in the 2D culture system. As shown in Figure 3A-H, SiO$_2$ exposure resulted in rapid ERK, JNK, p38 and Akt phosphorylation. The specificity of these signaling pathways was assessed using a pharmacological approach. Pretreatment of cells with a JNK (SP600125), p38 (SB203580), or PI3K (LY294002) inhibitor for 1 h decreased the SiO$_2$-induced increase in MCPIP1 and α-SMA expression after 3 h of SiO$_2$ treatment (Figure 3I-J). Interestingly, the MEK inhibitor (U0126) only inhibited the SiO$_2$-mediated induction of α-SMA, not of MCPIP1. We then further examined the MAP and PI3K/Akt pathways in the 3D culture system. As shown in Figure 4C-D and 4G-H, p38 and Akt phosphorylation showed increases similar to those in the 2D culture system. However, Erk phosphorylation showed a delayed increase (Figure 4A-B), whereas JNK phosphorylation increased at an earlier time point in the 3D culture system compared with the 2D culture system (Figure 4E-F). Interestingly, all inhibitors decreased the SiO$_2$-induced increase in MCPIP1 and α-SMA expression after 3 h of SiO$_2$ treatment (Figure 4I-J). Nevertheless, SiO$_2$ induced MAPK and PI3K/Akt pathway activation in both culture systems, which could be blocked by pretreatment with specific pharmacological inhibitor of MAPKs and PI3K/Akt.

**MCPIP1 is involved in SiO$_2$-induced fibroblast migration in 2D and 3D culture systems**

Because increasing evidence suggests that pulmonary fibroblast migration is a critical component of pulmonary fibrosis, we explored the role of MCPIP1 in SiO$_2$-mediated cell migration. The results of the scratch assay shown in Figure 5A-B demonstrate that SiO$_2$ induced a significant increase in the migration of HPF-a, which was inhibited by MCPIP1 RNAi (Figure 5C-D).

After determining that SiO$_2$ exposure induces the migration of HPF-a in a scratch assay, we sought to validate these findings by monitoring migration in the 3D cell culture system. The experimental protocols are illustrated in Figure 6A. Briefly, the FPCM was established and stabilized for 48 h before SiO$_2$ treatment. To rule out the effect of SiO$_2$ accessibility to cells, it was added to the cultured
cells either 12 h before (pretreatment, Pre) or after (post-treatment, Post) embedding the FPCM in a fresh acellular collagen matrix. Both pre- and post-treatment of HPF-a with SiO$_2$ significantly increased the maximum migrated distance and the number of migrated cells (Figure 6B-D), with no difference found between the pre- and post-treatment groups. These results were similar to those of the 2D scratch assay.

To further understand the role of MCPIP1 in SiO$_2$-mediated migration, RNAi experiments were conducted in a nested matrix cell migration model, which facilitates the analysis of fibroblast physiology under conditions that more closely resemble the \textit{in vivo} environment compared with conventional 2D cell culture. As shown in Figure 6 E-G, SiO$_2$ induced a significant increase in cell migration in the control RNAi group, similar to the results observed in the scratch assay. Interestingly, although MCPIP1 RNAi abolished the increase in the number of migrated cells (Figure 6E-F), it only partially limited the SiO$_2$-induced maximum migrated distance (Figure 6E and 6G). Meanwhile, knocking down MCPIP1 did not affect gel contraction (Figure 7).

**Discussion**

Silica exposure causes lung inflammation and fibrosis, which is histologically characterized by areas of inflammation, matrix deposition, and fibroblastic foci (31, 36). In addition to the role of alveolar macrophages (AMOs) in SiO$_2$-induced toxicity (16, 20, 26, 42), recent studies have focused on the direct effect of SiO$_2$ on dendritic cells such as fibroblasts, which play an important role in the pathogenesis of fibrosis. However, most studies on fibroblast function have been conducted in conventional 2D cell culture models (19), and there is abundant evidence suggesting significant discrepancies in cell behavior between 2D and 3D culture systems (5, 8). In the current study, we focused on the effects of MCPIP1 expression in pulmonary fibroblasts on cell migration after \textit{in vitro} SiO$_2$ exposure, especially with regard to the comparison of cell function between 2D and 3D systems.
Fibroblast migration/motility, which has an important role in silicosis (21, 26, 29, 43, 44), is a complicated process that involves cell-matrix adhesion, cell-matrix interaction, and global/local matrix remodeling (38). Compared with classic scratch assays, the nested collagen matrix model is an easy, rapid, reliable, and quantitative method for measuring fibroblast migration/motility in 3D models (15, 29, 41, 48). Fibroblasts within the embedded matrix of the nested model have a morphology that is similar to that of normal fibroblasts in tissue (13, 24). Although progress has been made in 2D cell culture systems for studying fibroblast migration (39), the mechanisms underlying cell migration in 3D cell culture systems remain less clear. Here, we provide new insights into the novel roles played by MCPIP1 in regulating fibroblast migration in experimental models of silicosis.

SiO$_2$ was found to induce MCPIP1 expression by activating the MAPK and PI3K/Akt pathways in both 2D and 3D culture systems. However, the timing of pathway activation was different in each system, suggesting that fibroblasts respond differently to SiO$_2$ between 2D and 3D systems. The properties of fibroblasts in 3D matrix assays result from a combination of cell-matrix adhesion, cell-matrix interaction, and global/local matrix remodeling, which may represent the situation in vivo. Moreover, abundant evidence suggests that SiO$_2$-induced fibrosis is a delayed response (26), which is consistent with findings in vitro, whereby a different response of cell migration to SiO$_2$ was observed between a scratch assay (3 h from start to migration) and a nested model (12 h from start to migration; data not shown).

Our study indicates that MCPIP1, a well-known anti-inflammatory molecule, is involved in fibroblast migration, as SiO$_2$ exposure resulted in increased fibroblast migration with a concomitant increase in MCPIP1 expression. Our findings are in agreement with a previous report demonstrating a role for MCPIP1 in angiogenesis with a scratch assay using HUVECs (32). This consistency could be considered to be a general effect on MCPIP1 during cell migration in different cell types and 2D/3D cell culture systems. Moreover, multiple lines of evidence have indicated that SiO$_2$ promotes fibroblast cell viability (29), though others
have reported that MCPIP1 overexpression induces apoptosis in various cell lines (49). Thus, we could not rule out the possibility that SiO₂-mediated fibroblast migration was due to an increase in the number of fibroblasts after SiO₂ exposure. The role of MCPIP1 in pulmonary fibroblast viability should be further investigated.

In addition to fibroblast migration, other silicosis-mediated processes such as collagen matrix contraction are also important functions of fibroblasts in the ECM. Fibroblast-mediated collagen matrix contraction is an *in vitro* model of tissue remodeling that is widely used to investigate the repair functions of fibroblasts in a variety of tissues, including the lungs and skin. Our laboratory previously showed that SiO₂ could increase matrix contraction, which was abolished by pretreatment with the MCP-1-specific receptor-CCR2 blocker RS102895 (29). Further results from studies of rat pulmonary fibroblasts indicated that MCPIP1 mediates SiO₂-induced matrix contraction (unpublished data). Although it remains unclear whether MCPIP1-mediated matrix contraction is a general phenomenon, our findings rule out the possibility that MCPIP1 is involved in collagen matrix contraction because knocking down MCPIP1 did not affect gel contraction (Figure 7) induced by SiO₂. Thus, MCPIP1 is specifically involved in the regulation of fibroblast migration rather than in a widespread cellular response to SiO₂.

In the present study, SiO₂ exposure resulted in JNK, p38, Erk, MAPK and PI3K/Akt phosphorylation, findings that were similar to a previous report on the effect of FPCM release on fibroblasts in a 3D collagen matrix (25). Interestingly, although the JNK, p38 MAPK, and PI3K/Akt pathways were found to be involved in MCPIP1 expression, blockade of the Erk pathway did not affect the expression of this protein in 2D culture system (Figure 3I-J). This discrepancy could be due to a compensatory response to PI3K pathway inhibition that resulted in increased MCPIP1 expression, a hypothesis that is supported by previous reports describing a novel compensatory feedback mechanism for the PI3K and MEK pathways in cancer cells (47).

In summary, our findings demonstrate a molecular pathway involving the
SiO$_2$-mediated induction of MCPIP1 in migrating fibroblasts. MAPK and PI3K/Akt pathway activation resulted in increased MCPIP1 expression, which, in turn, induced cell migration. Our findings also suggest that a 3D culture system may be a reliable model for investigating the physiological functions of fibroblasts. Moreover, these findings provide new potential targets for treating SiO$_2$-induced fibrosis.
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Additional Information

The authors declare no competing financial interests.

Author contributions

JC designed the experiments, analyzed the data, wrote the paper, and supervised the project. HL designed and performed the experiments, analyzed the data, and performed the statistical analyses. XD, YC, SF, YZ, XW and WZ performed the experiments and analyzed the data. HY and HL designed the experiments, analyzed the data, and supervised the project. All authors critiqued and approved the manuscript.

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Figure legends:

**Figure 1. SiO\textsubscript{2} mediates increases in MCPIP1 and α-SMA expression in HPF-a in a dose-dependent manner.**

**A.** Representative Western blot showing the induction of MCPIP1 and α-SMA expression by SiO\textsubscript{2} in HPF-a in a 2D culture system. Densitometric analyses showing the SiO\textsubscript{2}-mediated induction of MCPIP1 (B) and α-SMA (C) protein expression in a dose-dependent manner. **D.** Representative Western blot showing the induction of MCPIP1 and α-SMA expression by SiO\textsubscript{2} in HPF-a in a 3D culture system. Densitometric analyses showing the SiO\textsubscript{2}-mediated induction of MCPIP1 (E) and α-SMA (F) protein expression in a dose-dependent manner. *p<0.05 vs. the corresponding control group; #p<0.05 vs. the corresponding 25 µg/cm\textsuperscript{2} group; $p<0.05$ vs. the corresponding 50 µg/cm\textsuperscript{2} group; &p<0.05 vs. the corresponding 100 µg/cm\textsuperscript{2} group; n=5. **G.** MTT assay showing that SiO\textsubscript{2} increased the viability of HPF-a in a dose-dependent manner. *p<0.05 vs. the corresponding control group. *p<0.05 vs. the corresponding control group; n=5.

**Figure 2. SiO\textsubscript{2} mediates increases in MCPIP1 and α-SMA expression in HPF-a in a time-dependent manner.**

**A.** Representative Western blot showing the induction of MCPIP1 and α-SMA expression by SiO\textsubscript{2} in HPF-a in a 2D culture system. **B.** Densitometric analyses showing the SiO\textsubscript{2}-mediated induction of MCPIP1 and α-SMA protein expression in a time-dependent manner. *p<0.05 vs. MCPIP1 in the control group; #p<0.05 vs. α-SMA in the control group; n=5. **C.** Representative immunocytochemical images showing the induction of MCPIP1 expression by SiO\textsubscript{2} in HPF-a. Scale bar=10 µm. **D.** Representative Western blot showing the induction of MCPIP1 and α-SMA expression by SiO\textsubscript{2} in HPF-a in a 3D culture system. **E.** Densitometric analyses showing the SiO\textsubscript{2}-mediated induction of MCPIP1 and α-SMA protein expression in a time-dependent manner. *p<0.05 vs. MCPIP1 in the control group; #p<0.05 vs. α-SMA in the control group; n=5.
**Figure 3. SiO₂ induces ERK1/2, JNK, p38 MAPK and PI3K/Akt pathway activation in a 2D culture system.**

Representative Western blots showing the induction of ERK (A), p38 (C), JNK (E) and Akt (G) phosphorylation by SiO₂ in HPF-a cultured in a 2D system. Densitometric analyses of p-ERK (B), p-p38 (D), p-JNK (F) and p-Akt (H) expression from five separate experiments. *p<0.05 vs. the corresponding 0 min group. I. Representative Western blot showing that SiO₂-induced MCPIP1 and α-SMA expression was attenuated by pretreatment of HPF-a with a MAPK or PI3K/Akt pathway inhibitor. Densitometric analyses of MCPIP1 (J) and α-SMA (K) expression from five separate experiments. *p<0.05 vs. the control group; #p<0.05 vs. the SiO₂ group.

**Figure 4. SiO₂ induces ERK1/2, JNK, p38 MAPK and PI3K/Akt pathway activation in a 3D culture system.**

Representative Western blots showing the induction of ERK (A), p38 (C), JNK (E) and Akt (G) phosphorylation by SiO₂ in HPF-a cultured in a 2D system. Densitometric analyses of p-ERK (B), p-p38 (D), p-JNK (F) and p-Akt (H) expression from five separate experiments. *p<0.05 vs. the corresponding 0 min group. I. Representative Western blot showing that SiO₂-induced MCPIP1 and α-SMA expression was attenuated by pretreatment of HPF-a with a MAPK or PI3K/Akt pathway inhibitor. Densitometric analyses of MCPIP1 (J) and α-SMA (K) expression from five separate experiments. *p<0.05 vs. the control group; #p<0.05 vs. the SiO₂ group.

**Figure 5. MCPIP1 mediates SiO₂-induced HPF-a migration in 2D cultures.**

A. Representative images of SiO₂-induced migration of GFP-labeled HPF-a cultured as a monolayer. Scale bar=80 μm. B. Quantification of the scratch gap distance from six separate experiments. *p<0.05 vs. the control group at the corresponding time points. C. Representative images showing that SiO₂-induced cell migration was abolished by MCPIP1 RNAi. Scale bar=80 μm. D. Quantification of the scratch gap distance from six separate experiments. Small
panel showed efficiency of siRNA of MCPIP1. *p<0.05 vs. the control siRNA-treated group at the corresponding time points.

**Figure 6. SiO₂ induces HPF-a migration in a nested matrix model.**

A. Schematic of the nested matrix model and the experimental protocol. B. Representative images of SiO₂-induced migration of GFP-labeled HPF-a cultured in a 3D matrix model. Scale bar=80 μm. Quantification of the maximum migrated distance (C) and the number of cells that migrated from the nested gel (D) from six independent experiments. *p<0.05 vs. the corresponding control group. E. Representative images of SiO₂-induced cell migration in a nested gel matrix; this migration was abolished by MCPIP1 RNAi. Scale bar=80 μm. Quantification of the number of migrated cells from the nested gel matrix (F) and the maximum migrated distance (G) from six separate experiments. Small panel showed efficiency of siRNA of MCPIP1. *p<0.05 vs. the corresponding control RNAi group; # p<0.05 vs. the SiO₂- and control siRNA-treated group.

**Figure 7. MCPIP1 is not involved in SiO₂ induced gel contraction.**

A. Representative images of the collagen gel size, showing that SiO₂ resulted in increased gel contraction (indicating fibroblast activation), a result that was not affected by MCPIP1 RNAi. B. Quantification of the gel size at different time points after SiO₂ exposure. *p<0.05 vs. the control group at the corresponding time point.