MCPIP1 mediates silica-induced cell migration in human pulmonary fibroblasts

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Submitted 10 August 2015; accepted in final form 20 November 2015

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 two-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 three-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 matrixes 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 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.

MCPIP1: pulmonary fibroblast; migration; silicosis; 3-dimensional cultures

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Although a previous study demonstrated that chemokine/cytokine monocyte chemotactic protein-1 (MCP-1) is released...
by pulmonary fibroblasts treated with SiO$_2$ (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 et al. (28) indicated that MCPIP1 is a negative regulator of macrophage activation, 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 SiO$_2$ 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 SiO$_2$ 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, no. 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. 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 −80°C. MTT/2-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (29).

**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 no. 353047) and incubated in the attached state with 5% FBS in DMEM (supplemented with 50 μg/ml ascorbic acid) for ~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 Hanks’ balanced salt solution was added to each well at a final concentration of 5 μg/ml, and the plates were incubated in a CO$_2$ 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 by using a microtiter plate spectrophotometer at reference wavelengths of 570 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 lentiviral vector-red fluorescent protein (LV-RFP) or lentiviral vector-green fluorescent protein (LV-GFP) lentivirus (Hanbio, Shanghai, China) 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 (1×10$^5$ cells/well). The medium was replaced with 1 ml of fresh medium and 8 μg/ml polybrene; 100 μl of lentivirus solution (10$^7$ IU/ml) was added to each well, followed by incubation at 37°C in 5% CO$_2$ 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% CO$_2$ until >50% confluence was achieved. The transduced cells were selected by using blastcidin, 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% CO$_2$ 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 laboratory 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, 1×10$^5$ 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 ~70–80% confluent. Using a sterile 200-μl pipette tip, we carefully scratched a straight line 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 with 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% CO$_2$; 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 1,000 × 800 μm) were captured by using an EVOS FL Cell Imaging microscope (Life Technologies, Grand Island, NY) for three to five 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

L122 MCPIPI INDUCED PULMONARY FIBROBLAST MIGRATION
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 5.0 × 10^5 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 knockdown efficiency of siRNAs was determined after 2 days of transfection by Western blot analysis.

**GeL contraction assay.** FPCM contraction was determined by 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 with 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 via a flatbed scanner connected to a desktop. The matrix area was measured with 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 HPF-a were lysed by 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 with 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. Cutaneous wounds 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 and incubated at RT for 15 min. HPF-a were prepared at a concentration of 5.0 × 10^5 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 knockdown efficiency of siRNAs was determined after 2 days of transfection by Western blot analysis.

**Statistics.** Data are expressed as means ± SE. Unpaired numerical data were compared by unpaired t-test (two groups) or analysis of variance (ANOVA; more than two groups), with statistical significance set at P < 0.05.
2D culture system. However, Erk phosphorylation showed a delayed increase (Fig. 4, A and B), whereas JNK phosphorylation increased at an earlier time point in the 3D culture system compared with the 2D culture system (Fig. 4, E and F).

Interestingly, all inhibitors decreased the SiO$_2$-induced increase in MCPIP1 and α-SMA expression after 3 h of SiO$_2$ treatment (Fig. 4, I and 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 Fig. 5, A and B, demonstrate that SiO$_2$ induced a significant increase in the migration of HPF-a, which was inhibited by MCPIP1 RNAi (Fig. 5, C and 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 Fig. 6A. Briefly, the FPCM was established and stabilized for 48 h before SiO2 treatment. To rule out the effect of SiO2 accessibility to cells, it was added to the cultured cells either 12 h before (pretreatment, Pre) or after (posttreatment, Post) embedding the FPCM in a fresh acellular collagen matrix. Both pre- and posttreatment of HPF-a with SiO2 significantly increased the maximum migrated distance and the number of migrated cells (Fig. 6, B–D), with no difference found between the pre- and posttreatment groups. These results were similar to those of the 2D scratch assay.

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 in SiO2-induced toxicity (16, 20, 26, 42), recent studies have focused on the direct effect of SiO2 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 present study, we focused on the effects of MCPIP1 expression in pulmonary fibroblasts on cell migration after in vitro SiO2 exposure, especially with
Fig. 3. SiO₂ induces ERK1/2, JNK, p38 MAPK, and phosphatidylinositol-3-kinase (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 5 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 5 separate experiments. *P < 0.05 vs. the control group; #P < 0.05 vs. the SiO₂ group.
Fig. 4. SiO$_2$ 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 SiO$_2$ 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 5 separate experiments. *P < 0.05 vs. the corresponding 0 min group. I: representative Western blot showing that SiO$_2$-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 5 separate experiments. *P < 0.05 vs. the control group; #P < 0.05 vs. the SiO$_2$ group.
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.

SiO₂ 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 SiO₂ 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 SiO₂-induced fibrosis is a delayed response (26), which is consistent with findings in vitro, whereby a different response of cell migration to SiO₂ 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, since SiO₂ 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
Fig. 6. SiO$_2$ induces HPF-a migration in a nested matrix model. A: schematic of the nested matrix model and the experimental protocol. Pre, pretreatment; Post, posttreatment. B: representative images of SiO$_2$-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 6 independent experiments. *P < 0.05 vs. the corresponding control group. E: representative images of SiO$_2$-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 6 separate experiments. Representative blots showing efficiency of siRNA of MCPIP1. *P < 0.05 vs. the corresponding control RNAi group; #P < 0.05 vs. the SiO$_2$- and control siRNA-treated groups.


evidence have indicated that SiO\textsubscript{2} promotes fibroblast cell viability (29), although others have reported that MCPIP1 overexpression induces apoptosis in various cell lines (49). Thus we could not rule out the possibility that SiO\textsubscript{2}-mediated fibroblast migration was due to an increase in the number of fibroblasts after SiO\textsubscript{2} 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 SiO\textsubscript{2} 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 SiO\textsubscript{2}-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 (Fig. 7) induced by SiO\textsubscript{2}. Thus MCPIP1 is specifically involved in the regulation of fibroblast migration rather than in a widespread cellular response to SiO\textsubscript{2}.

In the present study, SiO\textsubscript{2} 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 (Fig. 3, I and 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\textsubscript{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\textsubscript{2}-induced fibrosis.

ACKNOWLEDGMENTS

This study is the result of work that was partially supported by the resources and facilities of the core laboratory at the Medical School of Southeast University.
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