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SiO_2-induced release of sVEGFRs from pulmonary macrophages

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ABSTRACT

Background: The inhalation of silicon dioxide (SiO_2) particles causes silicosis, a stubborn pulmonary disease that is characterized by alveolar inflammation during the early stage. Soluble cytokine receptors (SCRs) play important roles in regulating inflammation by either attenuating or promoting cytokine signaling. However, the role of SCRs in silicosis remains unknown.

Methods and results: Luminex assays revealed increased soluble vascular endothelial growth factor receptor (sVEGFR) family levels in the plasma of silicosis patients. In an enzyme-linked immunosorbent assay (ELISA), cells from the differentiated human monocytic cell line U937 released sVEGFR family proteins after exposure to SiO_2 (50 μg/cm^2). Further Western blot experiments revealed that VEGFR expression was also elevated in U937 cells. In contrast, levels of sVEGFR family members did not change in the supernatants of human umbilical vein endothelial cells (HUVECs) after exposure to SiO_2 (50 μg/cm^2). Interestingly, VEGFR expression in HUVECs decreased after SiO_2 treatment. In a scratch assay, HUVECs exhibited cell migration ability, indicating the acquisition of mesenchymal properties.

Conclusion: Our findings highlight the important role of sVEGFRs in both inflammation and fibrosis induced by SiO_2, suggesting a possible mechanism for the fibrogenic effects observed in pulmonary diseases associated with fibrosis.

1. Introduction

The inhalation of silicon dioxide (SiO_2) particles causes silicosis, a stubborn pulmonary disease that is characterized by alveolar inflammation during the early stage and progressive pulmonary fibrosis during the late stage. The pathogenic mechanisms underlying silicosis remain unknown, and no effective treatment is available.

Considerable evidence suggests that alveolar macrophages (AMs), the first line of defense in the lung, initiate pulmonary dysfunction, which results from chronic inflammation (Keogh and Crystal, 1982; Ward and Hunninghake, 1998). AMs phagocytose silica and release cytokines and chemokines, which stimulate pulmonary fibroblasts (PFBs) to produce collagen, ultimately resulting in pulmonary fibrosis. In addition to cytokines and chemokines, soluble cytokine receptors (SCRs) play important roles in regulating inflammation, either by attenuating or promoting cytokine signaling (Levine, 2004, 2008). A key role of soluble cytokine receptors is preventing excessive inflammatory responses. For example, mutations in the 55-kDa extracellular domain of tumor necrosis factor receptor type 1 (TNFRSF1A, TNFR1) were identified in patients with TNF receptor-associated periodic syndrome (TRAPS). Moreover, a recombinant soluble human TNFR2-Ig fusion protein exerted therapeutic effects in these patients (Hull et al., 2002). SCRs also act as disease biomarkers. For example, elevated circulating levels of soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) are considered a biomarker of inflammation and predict all-cause mortality (Yuan et al., 2013). The mechanism underlying the generation of SCRs includes proteolytic cleavage of cell surface receptors (Blobel, 2005), alternative gene splicing, transcription and translation of cytokine-binding genes (Levine, 2004), and extracellular release of membrane-bound receptors within exosomes (Trajkovic et al., 2008).

In this study, we show that sVEGFRs family members are elevated in silicosis patients. Unlike macrophages, endothelial cells do not release sVEGFRs after SiO_2 exposure; instead, SiO_2 induces the decreased expression of VEGFRs in endothelial cells, which may undergo endothelial-mesenchymal transition (EndMT).

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2. Materials and methods

2.1. Animals

C57BL/6 mice, 6–8 weeks of age, were obtained from Dr. Tao Cheng at Nanjing Medical University Laboratories (Nanjing, China). All animals were male and housed (4 per cage) in a temperature-controlled room (25 °C, 50% relative humidity) on a 12-h light/dark cycle. All animal procedures were performed in strict accordance with ARRIVE guidelines, and animal protocols were approved by the Institutional Animal Care and Use Committee of Southeast University.

2.2. Establishment of a mouse model of silicosis

Animals were anesthetized with pentobarbital sodium via intraperitoneal injection, and their tracheae were surgically exposed. Prepared SiO_2 suspension (0.2 g/kg in 50 mg/ml saline) was instilled intratracheally. Control animals were given the same volume of sterile saline, as previously described (Liu et al., 2017). Plasma samples were collected after the administration of SiO_2 or saline for 7 days.

2.3. Reagents

SiO_2 was obtained from Sigma® (S5631), and 80% of the particles were less than 5 μm in diameter. The particles were selected via sedimentation according to Stokes' law, followed by acid hydrolysis and baking overnight (200 °C for 16 h). The silica samples used for the cell experiments were sterilized by autoclave and then suspended in sterile normal saline (NS) at a concentration of 5 mg/ml. Fetal bovine serum (FBS), normal goat serum and Dulbecco's modified Eagle's medium (DMEM; #1200-046) were purchased from Life Technologies™, and PenStrep (15140-122) was obtained from Fisher Scientific.

2.4. Luminex for cytokine detection

Cytokines were detected with the MILLIPLEX MAP Human Soluble Cytokine Receptor Panel (HSCR-32K-PMX14, Millipore®, USA), which included sCD30 (sTNFRSF8), sEGFR, sgp130, sIL-1RI (sCD121a), sIL-1RII (sCD121b), sIL-2Rα (sCD25), sIL-4R (sCD124), sIL-6R (sCD126), sRAGE, sTNFRI (sTNFRSF1A), sTNFRII (sTNFRSF1B), sVEGFR1 (sFlt-1), sVEGFR2 (sFlk-1) and sVEGFR3 (sFlt-4).

2.5. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell® and maintained in T75 flasks in 10% FBS in DMEM. HUVECs were stored in liquid nitrogen following passages 3-7 (P3-7). A vial of P3-7 HUVECs was thawed, plated, and passaged upon confluence for each experiment, and each experiment was performed using HUVECs between P10 and P15.

The human monocytic cell line U937 was purchased from ATCC and maintained in T75 flasks in 10% FBS in RPMI 1640 medium. Before experiments were performed, the cells were cultured at 8×10^5 cells/well in a 24-well plate. Then, 50 nM phorbol myristate acetate (PMA) was used to differentiate U937 cells for 24 h prior to the experiments.

2.6. Western blot analysis

Immunoblotting was performed as previously described (Carlson et al., 2004), with minor modifications. HUVECs were collected from culture dishes, washed with PBS and lysed using a mammalian cell lysis kit (MCL1-1KT, Sigma-Aldrich®) according to the manufacturer's instructions. Membranes were probed using primary antibodies, followed by alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (1:5000). Signals were detected using chemiluminescence (SuperSignal West Dura Chemiluminescent Substrate, Thermo Scientific). Western blots were repeated using cells from three different donors. A single representative immunoblot is shown in each figure. Densitometry analysis was performed using ImageJ software, and the results from all repeated experiments were combined into one plot.

2.7. Enzyme-linked immunosorbent assay (ELISA)

sVEGFR levels were measured by ELISA. ELISA kits were purchased from SenBeiJia® (Nanjing, China) and used following the manufacturer's protocols. Fifty-microliter aliquots of cell culture supernatant or plasma were added to each well of a 96-well plate for measurements. Each sample was tested in triplicate.

2.8. Lentiviral transduction of HUVECs with GFP

HUVECs were transduced using LV-GFP lentiviruses (Hanbio Inc., Shanghai, China) as previously described (Chao et al., 2014). Briefly, P3-4 HUVECs were cultured in 24-well plates at 1×10^4 cells/well in 10% FBS in DMEM for 48 h. The medium was then replaced with 1 ml of fresh medium and 8 μg/ml polybrene. Next, 100 μl of lentivirus solution (107 IU/ml) was added to each well, and the cells were incubated at 37 °C in 5% CO_2 for 24 h. After incubation, the treatment medium was replaced with fresh 10% FBS in DMEM, and the cells were cultured at 37 °C in 5% CO_2 until > 50% confluence was reached. Transduced cells were selected using puromycin. Specifically, the medium was replaced with 10 μg/ml puromycin and 10% FBS in DMEM, and the cells were cultured at 37 °C in 5% CO_2 for 24 h. The cells were then washed twice with fresh 10% FBS in DMEM. Pure, transduced HUVEC cultures were expanded and/or stored in liquid nitrogen as previously described (Carlson et al., 2004).

2.9. In vitro scratch assay

Cell migratory ability was tested using a 2D culture system with an *in vitro* scratch assay. Briefly, 1×10^5 HUVECs were seeded in 24-well tissue culture plates and cultured in growth medium for 24 h, at which time the HUVECs were approximately 70–80% confluent. Using a sterile 200-μl pipette tip maintained in a position perpendicular to the plate bottom, a straight line was carefully scratched in a single direction in the monolayer across the center of the well. A second straight line was scratched perpendicular to the first line to create a cross-shaped gap between cells within 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 software.

2.10. Statistics

The data are presented as the mean ± SEM. Unpaired numerical data were compared using unpaired *t*-tests (two groups) or an ANOVA (more than two groups), and statistical significance was set at $p < 0.05$.

3. Results

3.1. Changes in soluble cytokine receptor levels in silicosis patients

Soluble cytokine receptors play critical roles in different physiological and pathological settings. Thus, we first measured the levels of soluble cytokine receptors in plasma from healthy donors and silicosis patients (Table 1). As shown in Fig. 1, 5 of the 14 measured soluble cytokine receptors, specifically sIL-2Ra, sTNFRII, sVEGFR1, sVEGFR2 and sVEGFR3, showed significant increases in ten phase II silicosis patients compared with ten healthy donors. None of the analyzed soluble cytokine receptors exhibited a decrease. Interestingly, all three

Table 1
Basic information of the study subjects.

Group	Healthy	Silicosis
n	10	10
Age (years)	41.0 ± 7.7	42.1 ± 6.9
Duration of silicon exposure (years)	ND	15.7 ± 2.9
Smoking (%)	3(60.0)	4(80.0)
Duration of silicon exposure cessation (years)	ND	4.8 ± 1.7

sVEGFR family members demonstrated significant increases, indicating a potential role for this family in silicosis.

3.2. SiO_2 induces the release of sVEGFRs from differentiated U937 cells

As the first line of defense in the lung, AMs are the most important immune barrier against invading pathogens and environmental contaminants in pulmonary innate immunity and are also the primary sources of cytokines and chemokines (Kuo et al., 2015; Wang et al., 2016). We therefore examined the potential elevation of sVEGFR family release by AMs in response to silica exposure. Macrophages differentiated from U937 cells were used to study the effects of silica exposure on pulmonary cells. As shown in Fig. 2A and B, SiO_2 (50 μg/cm^2) induced rapid and sustained release of sVEGFR1 and sVEGFR2 from differentiated U937 cells. In contrast, sVEGFR3 showed a delayed but dramatic and sustained increase after 12 h of SiO_2 exposure.

3.3. SiO_2 induces VEGFR expression in differentiated U937 cells

Recent studies have shown that soluble cytokine receptors may be derived from the proteolytic cleavage of cell surface receptors or the release of exosome-like vesicles containing cytokine receptors (Levine, 2004, 2008). SiO_2 toxicity is initiated by acute exposure of AMs, but the potential increase in sVEGFR release from AMs remains to be characterized. We therefore measured VEGFR levels in differentiated U937 cells after SiO_2 exposure. As shown in Fig. 3A–D, SiO_2 (50 μg/cm^2) induced the expression of VEGFR1, VEGFR2 and VEGFR3 in differentiated U937 cells. The dosage of SiO_2 was selected based on our

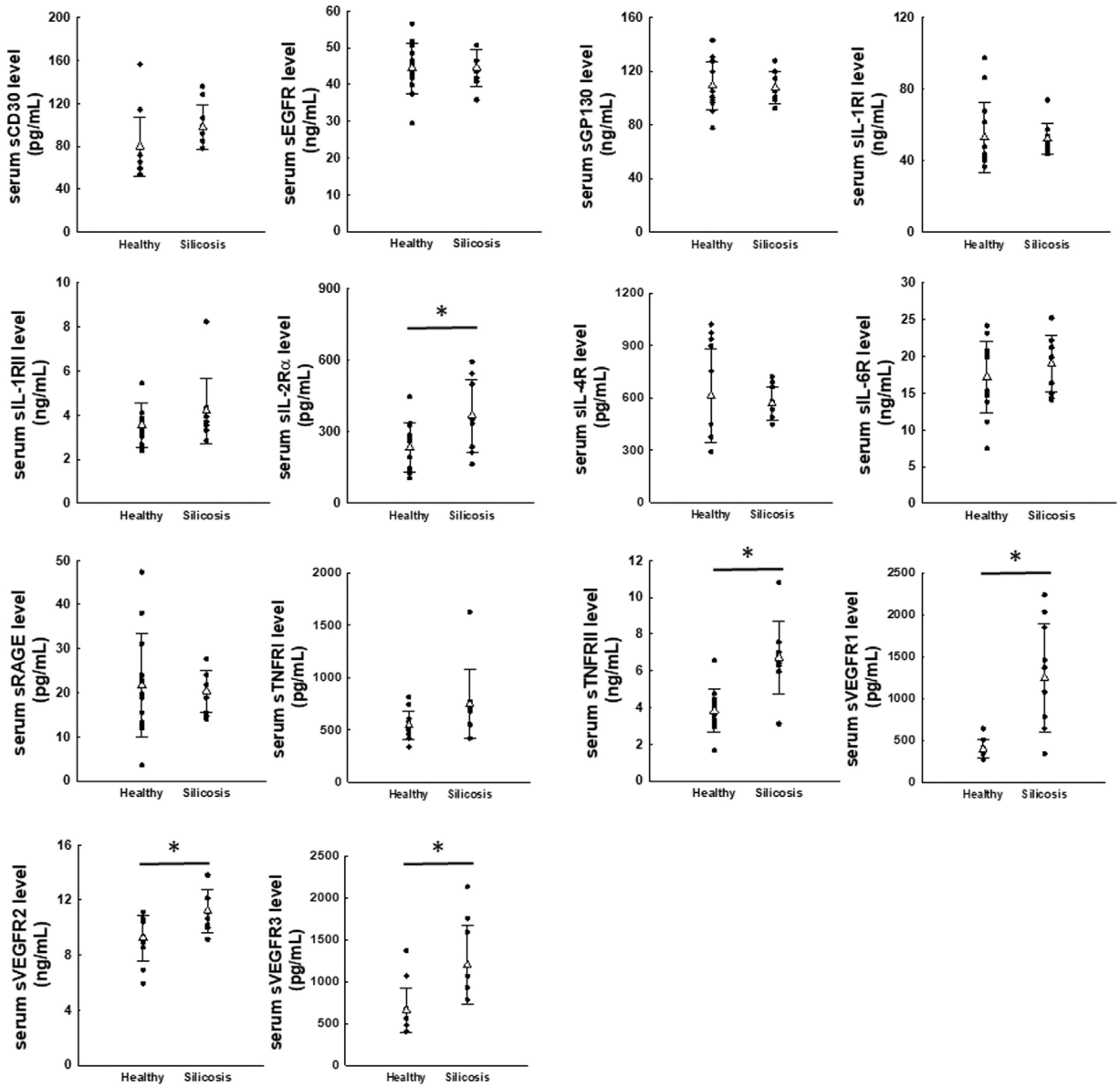

Fig. 1. SRC levels in healthy donors and silicosis patients.
Luminex assay showing the plasma levels of 14 SRCs in 10 patients with phase II silicosis compared with 10 healthy donors. The clinical characteristics of the donors are shown in Table 1. Data are presented as the mean ± SD, * $p < 0.05$ vs the healthy group.

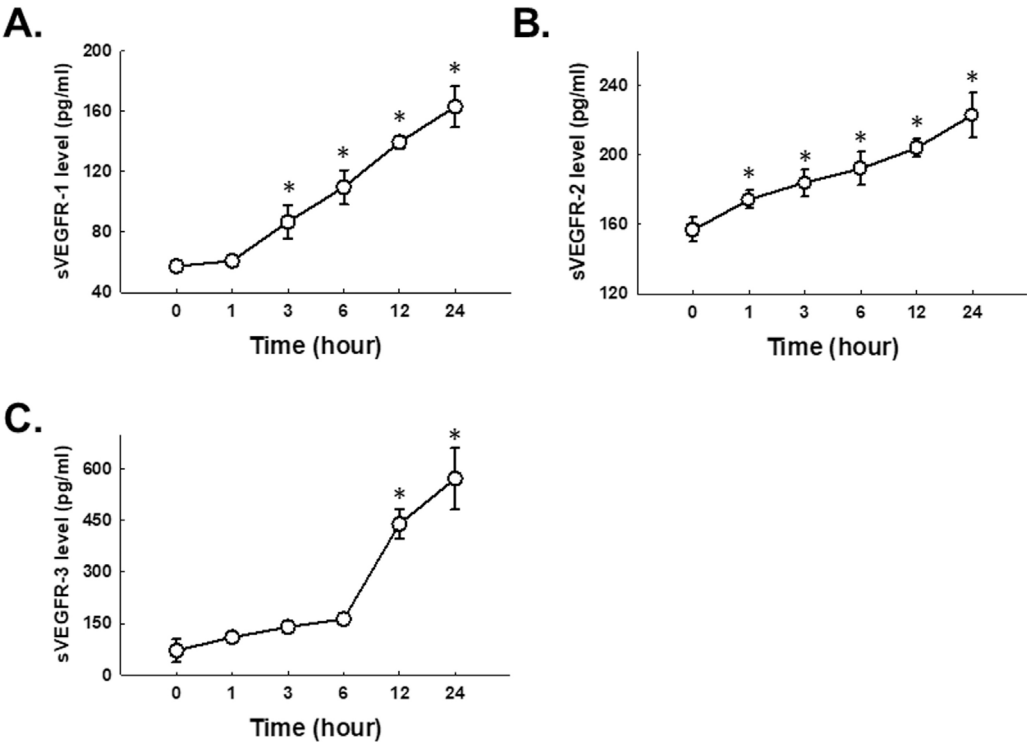

Fig. 2. SiO_2 induces the release of sVEGFRs from differentiated U937 cells.
ELISA assay showing that SiO_2 (50 μg/cm^2) induced increased levels of sVEGFR1 **(A)**, sVEGFR2 **(B)**, and sVEGFR3 **(C)** in a time-dependent manner in the supernatants of differentiated U937 cells. n = 5; * $p < 0.05$ vs the 0-h group.

previous study mimicking the *in vivo* status of pulmonary alveoli (Li et al., 2017; Wang et al., 2016; Zhang et al., 2016). Interestingly, although the time frame for sVEGFR1 and sVEGFR2 release (3 h after SiO_2 exposure) matched the expression of VEGFR1 and VEGFR2 in U937 cells, VEGFR3 expression increased 6 h after SiO_2 exposure, which was earlier than the release of sVEGFR3. This discrepancy suggests that different mechanisms are involved in the release of VEGFR family members.

3.4. SiO_2 induces the downregulation of VEGFR expression in HUVECs

sVEGFRs may be generated from endothelial cells via the cleavage of VEGFRs, the cell surface receptors that serve as markers of endothelial cells. We therefore measured sVEGFRs in the supernatants of HUVECs exposed to SiO_2 (50 μg/cm^2). Surprisingly, sVEGFR levels in HUVEC supernatants were very low and did not change after 24 h of SiO_2 treatment. We next measured the expression of VEGFRs in HUVECs after SiO_2 exposure. As shown in Fig. 4A–C, SiO_2 induced a rapid and sustained decrease in VEGFR1 and VEGFR2 in HUVECs. In contrast, VEGFR3 exhibited a slight, delayed decrease in HUVECs after 12 h of SiO_2 exposure.

3.5. SiO_2 induces increased cell migration in HUVECs

During pulmonary fibrosis, endothelial cells contribute to fibroblast accumulation via EndMT (Arciniegas et al., 2005; Hashimoto et al., 2010). Whether SiO_2-treated HUVECs undergo EndMT in the current setting remains to be determined. During EndMT, endothelial cells lose endothelial-specific markers, as shown in Fig. 4, and acquire mesenchymal cell functions such as increased cell migration (Piera-Velazquez et al., 2011). A scratch assay was performed to evaluate the effects of SiO_2 on cell migration by HUVECs. As shown in Fig. 5A and B, the cell migration ability of HUVECs was significantly increased after 12 h of SiO_2 exposure, indicating EndMT may occur.

3.6. sVEGFR levels increased in the plasma of mice with silicosis

To validate our sVEGFR findings in cell experiments, sVEGFR plasma levels in a mouse model of silicosis were detected in an ELISA assay. As shown in Fig. 6, mice treated with SiO_2 for 7 days exhibited high levels of all three forms of sVEGFR, indicating sVEGFRs are potential targets for treatment of silicosis.

4. Discussion

Silicosis is an occupational disease caused by silica inhalation and is characterized by progressive inflammation followed by pulmonary fibrotic reaction (Leung et al., 2012; Moore et al., 2003; Piguet et al., 1990; Rao et al., 2004). AMs are the primary producers of cytokines and chemokines, which are involved in the acquired immune response and provide the first line of defense in the lung by eliminating most foreign material from the distal airway (Fearon and Locksley, 1996; Sibille and Reynolds, 1990). However, the mechanisms of action of many cytokines and chemokines remain unclear. SCRs play important roles by targeting cytokines and modifying their biological activity in an antagonistic or agonistic fashion (Levine, 2004). The aim of the current study was to investigate whether SCRs are involved in silicosis.

Our study demonstrated that among 14 SCRs, the plasma levels of sIL-2Ra, sTNFRII, sVEGFR1, sVEGFR2 and sVEGFR3 were higher in silicosis patients than in healthy donors. Among these 5 elevated SCRs, all members of the sVEGFR family showed significant increases in patients. sVEGFR1 and sVEGFR3 demonstrated approximately 2-fold changes, whereas sVEGFR2 demonstrated an approximately 1.2-fold change. Similar changes in sVEGFRs were also observed in a mouse model of silicosis. VEGFR1 plays an important role in embryonic vascularization because VEGFR1 knockout mice die as a result of deficiencies in the structural organization of vessel walls (Fong et al., 1995). Moreover, VEGFR1 plays a critical role in inducing the paracrine release of tissue-specific growth factors (LeCouter et al., 2003). Interestingly, one of the primary characteristics of silicosis is the overproliferation of fibroblasts (Kuo et al., 2015; Liu et al., 2016, 2015; Wang et al., 2016; Ward and Hunninghake, 1998). To further

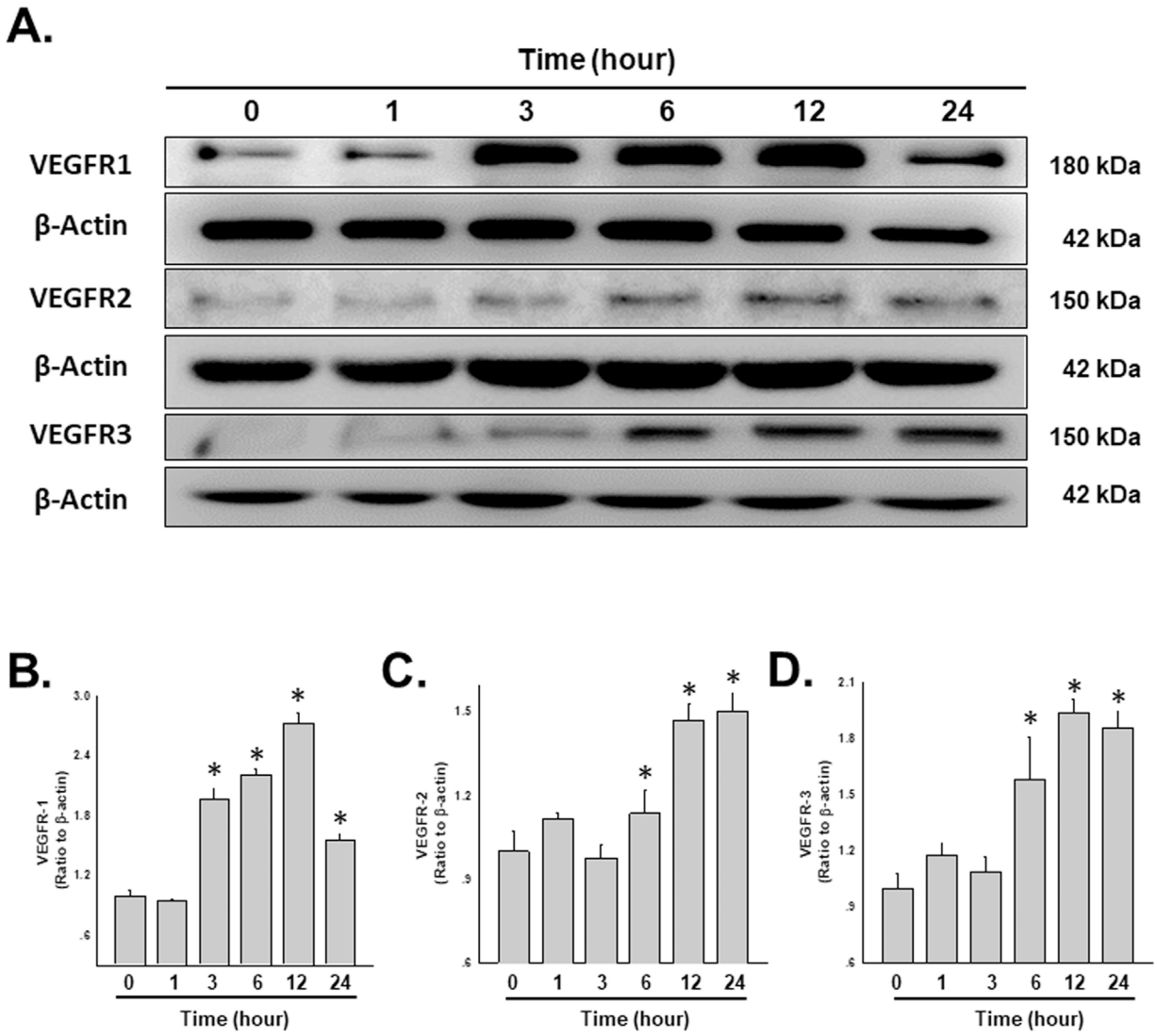

Fig. 3. SiO_2 induces the expression of VEGFRs in differentiated U937 cells.
A. Representative Western blot showing the effects of SiO_2 (50 μg/cm^2) on the expression of VEGFR1, VEGFR2 and VEGFR3 in differentiated U937 cells. Densitometric analyses from five separate experiments suggested that SiO_2 induced VEGFR1 (**B**), VEGFR2 (**C**) and VEGFR3 (**D**) expression in a time-dependent manner in differentiated U937 cells. * $p < 0.05$ vs the 0-h group.

understand the source of sVEGFRs, *in vitro* cell experiments were performed. Since AMs quickly respond to stimuli, short-term SiO_2 exposure was employed in the current study. Previous data from our lab have shown that conditioned medium from SiO_2-stimulated macrophages induces fibroblast proliferation (Wang et al., 2016); however, the underlying mechanism remains unclear. In the current study, the supernatants of SiO_2-stimulated macrophages showed increased levels of sVEGFR1, which may partially explain the fibroblast proliferation-promoting effects of AMs.

sVEGFR2 exhibited a slight increase in silicosis patients, whereas SiO_2 induced a rapid and sustained increase in sVEGFR2 in the supernatants of SiO_2-treated macrophages. VEGFR2 plays a pivotal role in cell proliferation, the production of NO and prostacyclin, angiogenesis, and vascular permeability (He et al., 1999; Terman et al., 1992). For example, VEGFR2 activation inhibits apoptosis in HUVECs (Gerber et al., 1998). VEGFR2 is a key receptor for the development of the vasculature (Tugues et al., 2011) and tumor angiogenesis (Jiang et al., 2012). Reduced levels of the circulating soluble receptor VEGFR2 have been observed in imatinib-resistant gastrointestinal stromal tumors (GIST), which results from the VEGF-mediated downregulation of VEGFR2 *in vitro* (Hansson et al., 2013). Thus, the implications of changes in sVEGFR2 expression are more complicated than those of sVEGFR1.

The function of sVEGFR3 in silicosis remains unclear, as it is normally present only on lymphatic and proliferating blood vascular endothelial cells. Some studies have shown that sVEGFR-3 is a predictive factor in GIST (Hansson et al., 2013) and breast cancer (Harris et al., 2016). Moreover, nonvascular VEGFR3 suppresses inflammatory angiogenesis in the cornea (Cursiefen et al., 2006). The increase in sVEGFR3 in the current setting may play a compensatory role in the anti-inflammatory effects induced by sVEGFR1 and sVEGFR2. Further experiments are needed to prove this hypothesis.

In the current study, VEGFRs were decreased in endothelial cells but not in the supernatants of cultured cells, indicating that endothelial cells may not be the source of sVEGFRs in plasma. The decrease in VEGFRs, a marker of endothelial cells, in HUVECs indicates the induction of EndMT by SiO_2. EndMT has been observed during fibrosis in organs such as the kidneys (Zeisberg et al., 2008), liver, and heart (Zeisberg et al., 2007) and in patients with diabetes (Widyantoro et al., 2010) and metastatic tumors (Zeisberg et al., 2007). During EndMT under physiological and pathological conditions, endothelial cells lose endothelial-specific markers, acquire a mesenchymal phenotype, and

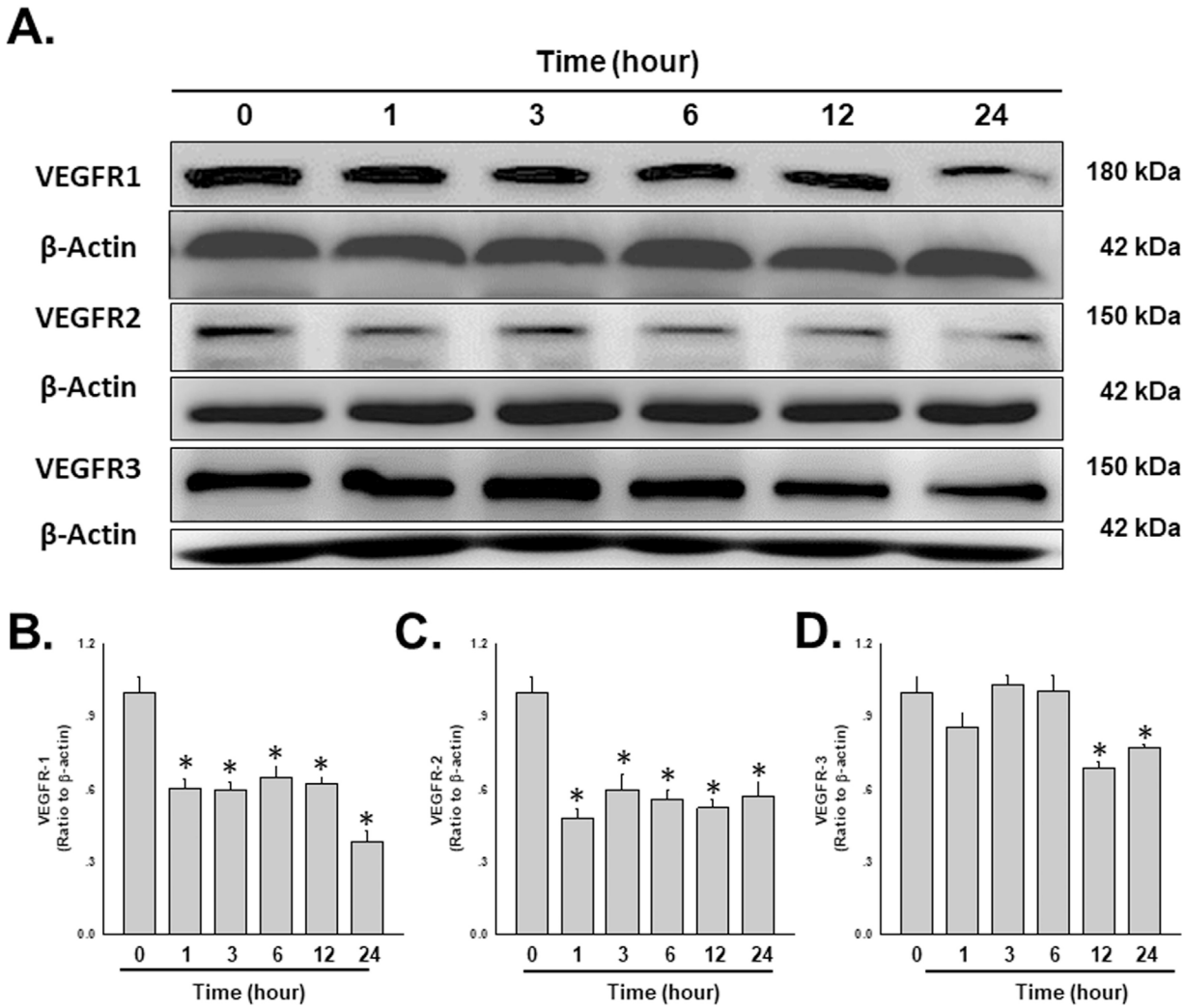

Fig. 4. SiO_2 inhibits the expression of VEGFRs in HUVECs.
A. Representative Western blot showing the effects of SiO_2 (50 μg/cm^2) on the expression of VEGFR1, VEGFR2 and VEGFR3 in HUVECs. Densitometric analyses from five separate experiments suggested that SiO_2 inhibited VEGFR1 (**B**), VEGFR2 (**C**) and VEGFR3 (**D**) expression in HUVECs. * $p < 0.05$ vs the 0-h group.

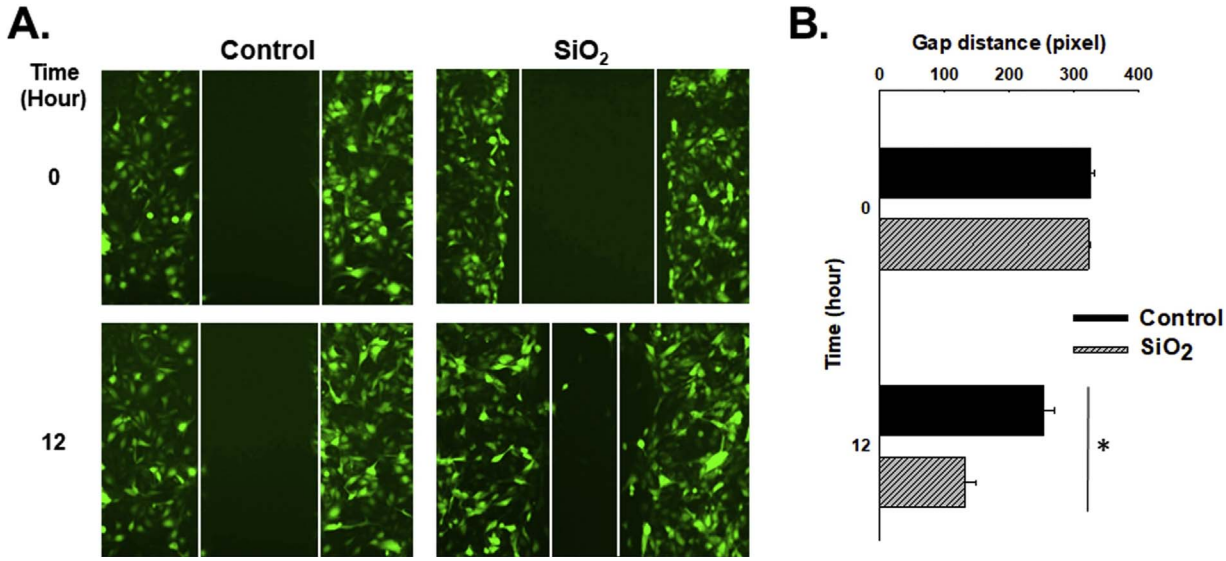

Fig. 5. SiO_2 induces cell migration in HUVECs.
A. Representative images showing the effects of SiO_2 on the migration of GFP-labeled HUVECs in scratch assays. Scale bar = 80 μm. **B.** Quantification of the scratch gap distances in six separate experiments. * $p < 0.05$ vs the corresponding time point in the control group.

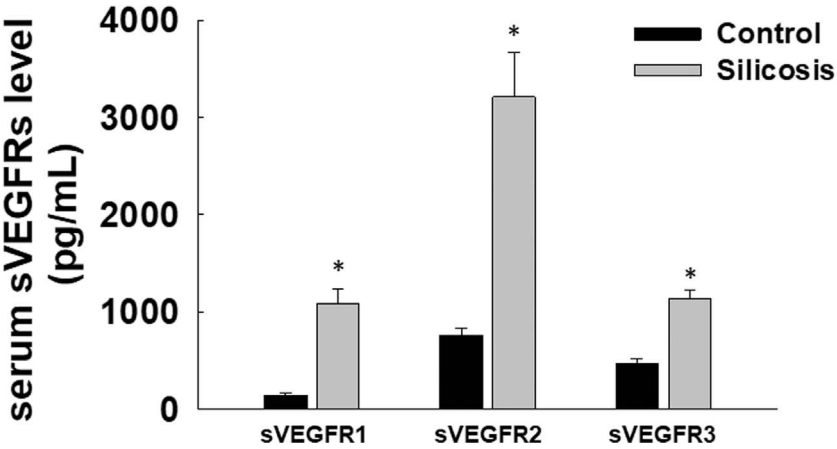

Fig. 6. sVEGFR levels increased in the plasma of mice exposed to SiO_2. ELISA assay showing that SiO_2 (0.2 g/kg in 50 mg/ml saline, one time) induced increased levels of sVEGFR1, sVEGFR2 and sVEGFR3 in mouse plasma after 7 days of treatment. n = 6; * $p < 0.05$ vs the control group.

begin to express mesenchymal cell products, such as type I collagen, type III collagen and α-smooth muscle actin (α-SMA) (Chao et al., 2016; Piera-Velazquez et al., 2011). EndMT of endothelial cells leads to a decrease in microvasculature and endothelial dysfunction, contributing to fibrosis (Zeisberg et al., 2007). Morphometric studies of idiopathic pulmonary fibrosis (IPF) have shown that capillary density is decreased in the fibrotic region (Ebina et al., 2004). In the current study, we observed a decrease in endothelial markers in HUVECs as well as an increase in migratory ability; these are both important properties of mesenchymal cells that indicate the occurrence of EndMT. A recent study from our laboratory suggested a role for MCPIP1 in EndMT induced by SiO_2. Further experiments are needed to investigate the role of sVEGFRs in EndMT induced by SiO_2.

In conclusion, SiO_2 induced the release of sVEGFR family proteins from pulmonary macrophages but not from endothelial cells. The decrease in VEGFRs in the endothelia indicates the occurrence of EndMT, which may contribute to the progression of fibrosis. Therefore, targeting VEGFR signaling represents a promising therapeutic strategy to prevent silicosis.

Ethics approval and consent to participate

All participants provided informed written consent prior to participating in the study. The use of human samples was in accordance with the approved guidelines of the Research and Development Committee of Nanjing Chest Hospital (2016-KL002-01), and all procedures were conducted in accordance with the Declaration of Helsinki.

Competing interests

The authors have no competing financial interests to declare.

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