Increased Expression of Matrix Metalloproteinase in Clara Cell-Ablated Mice Inhaling Crystalline Silica

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We investigated the function of Clara cells *in vivo* during exposure to inhaled crystalline silica by examining pulmonary matrix metalloproteinase (MMP)-2 and MMP-9 mRNA levels in mice. The Clara cells of male FVB/n mice (8–12 weeks old) were ablated by intraperitoneal administration of naphthalene (300 mg/kg) in a corn oil vehicle. The mice were then exposed to crystalline silica (Min-U-Sil-5 silica, 97.1 ± 9.5 mg/m^2, 6 hr/day, 5 days/week) for up to 2 weeks. Transcriptional levels of mRNA extracted from the lungs were assessed by reverse transcription-polymerase chain reaction. Gene expression of both MMP-2 and MMP-9 was significantly more marked in the Clara cell-ablated group than in the group with Clara cells, indicating that Clara cells inhibit MMP expression. Our findings suggest that Clara cells inhibit pulmonary inflammation induced by crystalline silica via MMPs *in vivo*. *Key words*: Clara cell, crystalline silica, matrix metalloproteinase, naphthalene, reverse transcription-polymerase chain reaction. *Environ Health Perspect* 109:795–799 (2001). [Online 3 August 2001]
http://ehpnet1.niehs.nih.gov/docs/2001/109p795-799yatera/abstract.html

Clara cells are nonciliated, nonmucous secretory cells localized mostly in the bronchiolar surface epithelium, and they are one of the most multifunctional and heterogeneous cell types in the mammalian lung (*1*). Clara cells appear to play a role in pulmonary inflammation and fibrosis in the distal airways by serving as progenitors of bronchial epithelial cells (*2–5*). Clara cells are particularly likely to undergo apoptosis (*6,7*) when alveolar and bronchial epithelial cell apoptosis induces pulmonary fibrosis (*8*).

Clara cells are reported to have an inhibitory effect on pulmonary inflammation and fibrosis. For example, they secrete Clara cell secretory protein (CCSP) (*9*), which inhibits pulmonary inflammation (*10*); surfactant proteins, which prevent alveolar collapse (*11–15*); and protease inhibitors such as secretory leukocyte protease inhibitor (SLPI) (*16–18*) and elafin (*17,18*). Clara cells can also promote pulmonary inflammation and fibrosis. Of the different cell types identified in the lung, Clara cells appear to have the highest level of cytochrome P450 monooxygenases (*19*). Studies have also shown that the metabolic products of these monooxygenases can damage bronchial epithelial cells and that most bronchial epithelial cells produce various chemokines and proinflammatory cytokines during bronchial injury (*20–23*). Attention has therefore focused on the balance between the inhibitory and promotional effects of Clara cells in the process of pulmonary inflammation and fibrosis *in vivo*.

Plopper et al. (*24–26*) and many other researchers have created a mouse model in which Clara cells are selectively ablated with naphthalene, allowing the general role of Clara cells in pulmonary inflammation and fibrosis to be analyzed *in vivo*.

Various factors are involved in pulmonary inflammation and fibrosis. Like free radicals, matrix-degrading matrix metalloproteinase (MMP) enzymes directly cause airway and pulmonary injury and inflammation, but they also play an important role in repair (*27*). Increased expression of MMP-2 and MMP-9, enzymes that degrade type IV collagen and elastin, major structural components of the basement membrane, has been observed in the acute stages of pulmonary inflammation in animal studies (*28*). Expression of these MMPs is also increased in various types of inflammatory lung diseases in humans, such as bronchiolitis obliterans organizing pneumonia and idiopathic pulmonary fibrosis (*29*). These findings suggest that MMP-2 and MMP-9 are closely involved in inflammation and fibrosis.

In the present study, we investigated the role of Clara cells in inflammation and fibrosis *in vivo* by exposing Clara cell-ablated mice to crystalline silica, which is known to cause pulmonary fibrosis (*30–33*), and examined the gene expression of MMP-2 and MMP-9 in lung tissue using reverse transcription-polymerase chain reaction (RT-PCR).

Materials and Methods

Animals. Male FVB/n mice (8–12 weeks of age), as described previously (*3,34*), were used in the study. Age-matched mice were purchased from CLEA Japan, Inc. (Tokyo, Japan).

All of the animals were maintained according to the Guidelines for Animal Experimentation of the University of Occupational and Environmental Health, Japan, and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Washington, DC, USA).

Creation of Clara cell-ablated mice. Naphthalene (300 mg/kg body weight) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), dissolved in a corn oil vehicle, and injected intraperitoneally. Control mice were injected with vehicle only.

Inhalation of crystalline silica. Crystalline silica (Min-U-Sil 5 silica; U.S. Silica, Berkeley Springs, WV, USA) was used in the study. The mice were housed in an exposure chamber and exposed to crystalline silica for 6 hr/day, 5 days/week for up to 2 weeks. The mass concentration of the crystalline silica was 97.1 ± 9.5 mg/m^2 and was measured gravimetrically at 1-day intervals by the suction of air through a glass filter. Both the groups of wild-type mice and that of Clara cell-ablated mice were exposed to crystalline silica or fresh air. Each group, composed of five animals, was sacrificed after 1, 3, 7, and 14 days of exposure.

Preparation of RNA, cDNA synthesis, and polymerase chain reaction. RNA was extracted from the lung using guanidinium thiocyanate-phenol-chloroform. Total RNA (0.5 μg) was used to synthesize single-strand cDNA with Moloney murine leukemia virus-derived reverse transcriptase (Perkin Elmer, Norwalk, CT, USA). An equal amount of cDNA from each sample, standardized to give identical signals on the gel following amplification with β-actin primer, was amplified by specific primers for each gene (Table 1). The amplification was performed with a Thermocycler (Astech, Fukuoka, Japan) under the following conditions: denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 2

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We thank M. Tsunoda and M. Hirohashi for assistance with some of the experiments.

This work was supported in part by the Japanese Ministry of Education and Science.

Received 22 September 2000; accepted 15 February 2001.

min for *MMP-2*, *MMP-9*, and β-actin genes. β-actin was coamplified as an internal standard in a quantitative polymerase chain reaction (PCR) amplification of mRNA.

The fragment amplified by PCR was detected by electrophoresis on a 2% agarose gel with DNA markers and was visualized by ethidium bromide staining. The gels were photographed with Polaroid Type 665 positive/negative film (Polaroid Corp., Cambridge, MA, USA) under ultraviolet light at identical exposure and development times. The bands of the positive film were scanned, and the density of each PCR product was measured using National Institutes of Health (NIH) Image 1.61 software (written by Wane Rasband, National Institutes of Health, Bethesda, MD, USA). To quantify the transcriptional level of mRNA, the data were normalized to represent equivalent RNA loading based on the density of the β-actin product at the appropriate cycle of a given gene product.

Statistical analysis. Values are expressed as the mean ± standard error. We assessed the difference between values using the Mann-Whitney *U* test. *p*-Values < 0.05 were considered significant.

Histopathology and immunohistochemistry. The inflation-fixed lungs from the mice were washed in phosphate-buffered saline (PBS) three times and fixed in 10% buffered formalin. Paraffin-embedded specimens were sectioned at 5 μm and stained with hematoxylin and eosin for morphologic analysis by microscopy. For immunohistochemical staining for MMP-2 and MMP-9, the tissue sections were deparaffinized by washing in xylene four times for 10 min per wash, followed by dehydration through a series of 100% to 70% ethanol washes. The slides were placed in methanol containing 0.5% hydrogen peroxide to block endogenous peroxidase activity. Nonspecific binding was blocked by incubating the slides 0.1M-PBS for 1 hr at room temperature.

We incubated the lung sections overnight at 4°C with either affinity-purified goat polyclonal antibodies specific for MMP-2 and MMP-9 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; both at dilutions of 0.4 μg/mL) or rabbit anti-rat polyclonal antiserum specific for CCSP (courtesy of Gurmukh Singh, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; diluted 1:500–1:1000 before use).The sections were rinsed five times in 0.1 M PBS and incubated for 30 min at room temperature with biotinylated anti-goat secondary antibody made in rabbits (Vector Laboratories Inc., Burlingame, CA, USA) for MMP-2 and MMP-9, and anti-rabbit secondary antibody (DAKO JAPAN Co., Ltd., Kyoto, Japan) for CCSP, then incubated with avidin-biotin complex (DAKO JAPAN Co., Ltd.) for 30 min at room temperature. Sections were washed with PBS, rinsed briefly in 0.1 M acetate buffer (pH 6.0), incubated with diaminobenzidine for 3 min, and counterstained with hematoxylin. Incubations were carried out without the primary or secondary antibody as a labeling control.

Results

Immunohistochemistry for CCSP. Figure 1 shows that immunostaining patterns for CCSP 14 days after the intraperitoneal injection of naphthalene (Figure 1B) or corn oil vehicle only (Figure 1A) to the silica nonexposed group. The Clara cells were most ablated 3 days after the exposure (data not shown), and Figure 1B shows that > 70% of ablation continued 14 days after the exposure. Clara cells remained at the bifurcation of the bronchiole throughout the period.

MMP-2. The results for MMP-2 are shown in Figure 2. There was no difference in expression of MMP-2 between the unexposed groups of wild-type mice and that of Clara cell-ablated mice. MMP-2 expression was increased in both the Clara cell-ablated and nonablated groups exposed to crystalline silica, and comparison of these groups showed that the MMP-2 expression tended to be higher in the Clara cell-ablated group from the 3-day exposure and that the increase was statistically significant after 7 and 14 days of exposure.

MMP-9. The results for MMP-9 are shown in Figure 3. There was no difference in expression of MMP-9 between the unexposed groups of wild-type mice and that of Clara cell-ablated mice. In the two crystalline silica-exposed groups, the MMP-9 expression increased after 7 days of exposure and was statistically significantly higher in the Clara cell-ablated group than in the group of mice with Clara cells after 14 days of exposure.

Histopathology and immunohistochemistry. Histopathologic examination using hematoxylin and eosin staining revealed an increase in macrophage numbers after exposure in both the Clara cell-ablated and the nonablated groups. Of the two groups exposed, foamy macrophages were seen in the Clara cell-ablated group but not in the nonablated group (data not shown). No marked accumulation of crystalline silica was evident.

Table 1. Oligonucleotides of primers of target genes.

mRNA species	mRNA	PCR product	
		Cycle	bp
MMP-2	(sense) 5'-GAGATCTGCAAACAGGACAT (antisense) 5'-GGTTCTCCAGCTTCAGGTAA	26	476
MMP-9	(sense) 5'-CGACGAGTTGTGGTCGCTGG (antisense) 5'-GCACGCTGGAATGATCTGAG	33	624
β-actin	(sense) 5'-ATCATGTTTGAGACCTTCAACACC (antisense) 5'-TAGCTCTTCTCCAGGGAGG	22	357

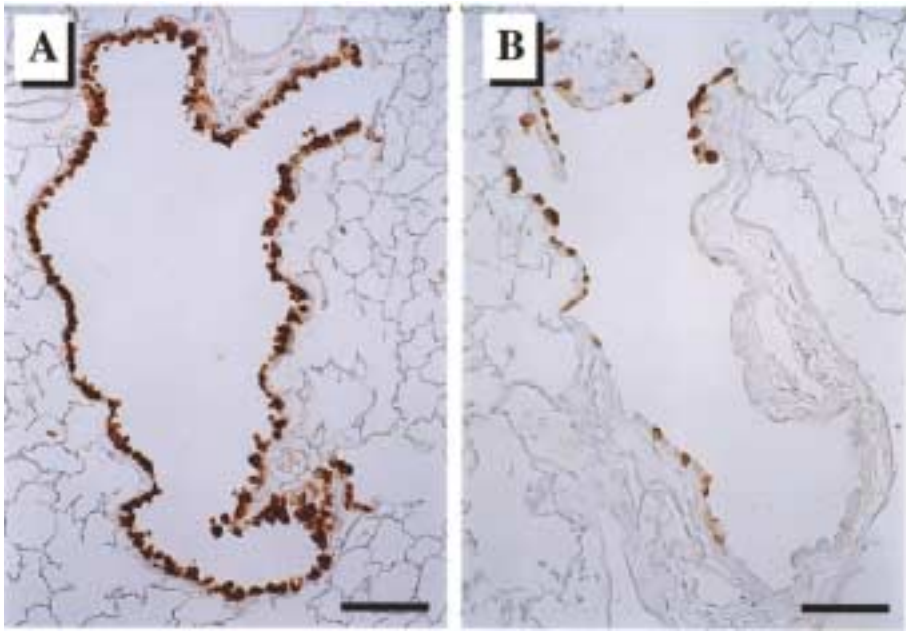

Figure 1. Immunohistochemistry for CCSP. (*A*) Mouse with Clara cells 14 days after intraperitoneal injection of corn oil vehicle only. (*B*) Clara cell-ablated mouse 14 days after intraperitoneal injection of naphthalene. Bar = 50 μm.

Immunostaining revealed the expression of MMP-2 and MMP-9 in alveolar macrophages, bronchial epithelial cells, alveolar epithelial cells, and vascular endothelial cells in both the Clara cell-ablated and the nonablated groups. The alveolar macrophages, bronchial epithelial cells, and vascular endothelial cells were particularly strongly stained (Figure 4). However, no newly strongly stained cell types were found after both crystalline silica exposure or Clara cell ablation.

Discussion

We ablated the Clara cells in mice using the cytoselective toxicity of the metabolic products of naphthalene mediated by cytochrome P450 monooxygenase (*3*,*24*,*25*,*34*). In a morphologic study of murine lungs using transmission electron microscopy, Plopper and colleagues (*24*–*26*) reported that intraperitoneally administered naphthalene is specifically toxic to Clara cells and does not cause changes in other epithelial cells, indicating that the toxicity of naphthalene to Clara cells is cytoselective.

Stripp et al. (*34*) reported that treatment with naphthalene at a concentration of 300 mg/kg results in the ablation of Clara cells in the bronchiolar region for at least 20 days in FVB/n mice (*34*), which seem to be more susceptible to naphthalene than do other mouse strains (*35*). Using the same method, we also confirmed that Clara cells are removed 1 day after naphthalene treatment and remain absent for at least 2 weeks (Figure 1B). The Clara cell-ablated mice were therefore exposed to crystalline silica for 2 weeks after naphthalene treatment.

In this study, the mice were exposed to a higher concentration of crystalline silica than in humans to investigate the role of Clara cells in the acute inflammatory response. We previously reported marked inflammatory cell accumulation and silica deposition in the lungs after intratracheal instillation of 2 mg crystalline silica in rats (*36*). The present histopathology findings using hematoxylin and eosin staining and phase-contrast microscopy of the lungs revealed an increase in inflammatory cell numbers in the alveoli, but showed no excessive deposition of crystalline silica in the lungs as was seen in our previous study. Although different animal species were used in the two studies, in the present study it was estimated that < 2 mg of crystalline silica was deposited in the lungs after inhalation, suggesting that this dose might not be excessive. In long-term inhalation studies conducted to date, we found that the maximum amount of dust deposited in the lungs is approximately 2 mg regardless of particle type (*37*).

In the current study, gene expression of MMP-2 and MMP-9 in the lungs tended to increase after crystalline silica exposure. We are the first to investigate MMPs induced by inhaled crystalline silica. Increased MMP-2 and MMP-9 expression was observed in

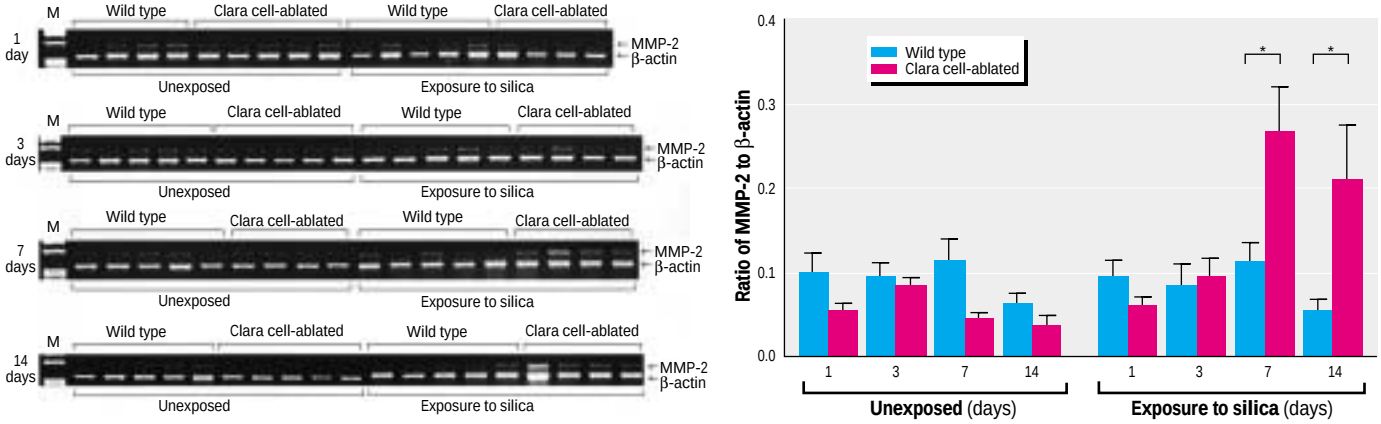

Figure 2. (*A*) Ethidium bromide staining of the PCR products separated on 2% agarose gel of MMP-2 mRNA in the lungs of crystalline silica-exposed and nonexposed mice. (*B*) Effect of crystalline silica exposure and the ablation of Clara cells on the expression of MMP-2 mRNA. Expression was significantly greater in the group of mice with Clara cells. The results are expressed as the ratio of MMP-2 to β-actin (mean ± SEM). M, DNA marker ϕX174/*Hae* III).
*Significantly different from the group of mice with Clara cells ($p < 0.05$).

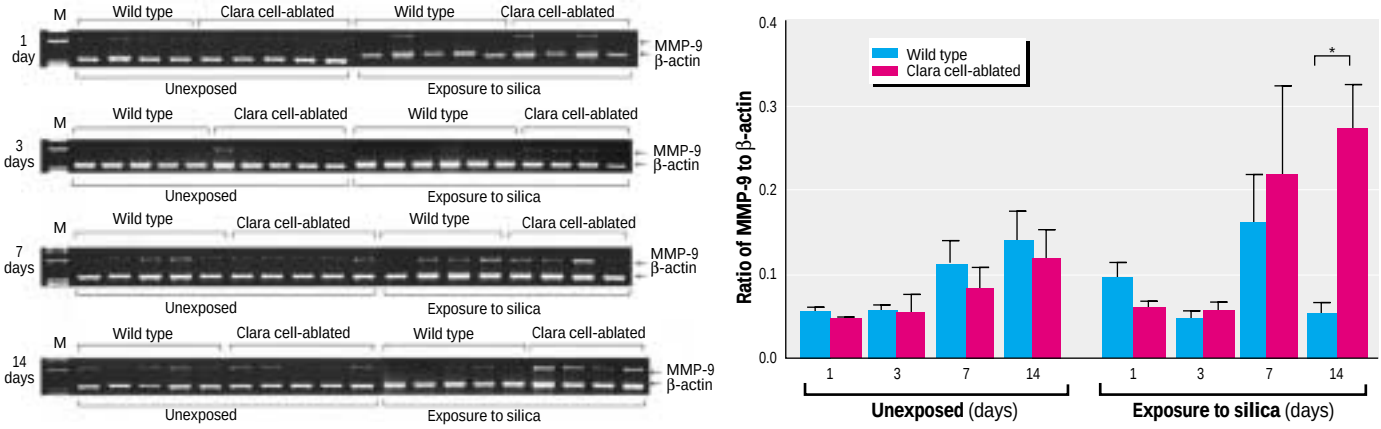

Figure 3. (*A*) Ethidium bromide staining of the PCR products separated on 2% agarose gel of MMP-9 mRNA in the lungs of crystalline silica-exposed and nonexposed mice. (*B*) Effect of crystalline silica exposure and the ablation of Clara cells on the expression of MMP-9 mRNA. Expression was significantly greater in the group of mice with Clara cells. The results are expressed as the ratio of MMP-9 to β-actin (mean ± SEM). M, DNA marker ϕX174/*Hae* III).
*Significantly different from the group of mice with Clara cells ($p < 0.05$).

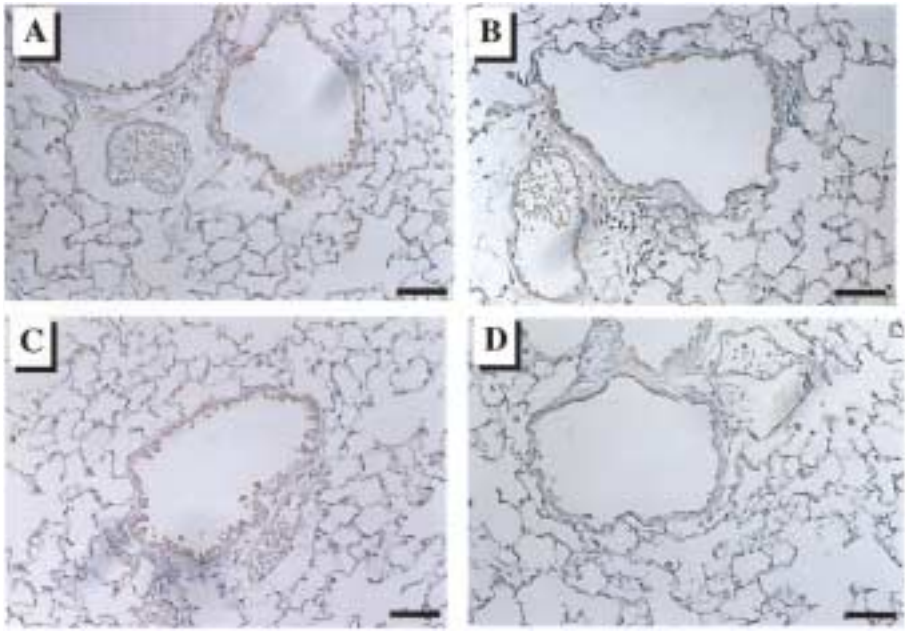

Figure 4. Immunohistochemistry for MMP-2 and MMP-9. (*A*) Immunohistochemistry for MMP-2 in the lung of a mouse with Clara cells exposed to crystalline silica for 14 days. (*B*) Immunohistochemistry for MMP-2 in the lung from a Clara cell-ablated mouse exposed to crystalline silica for 14 days. (*C*) Immunohistochemistry for MMP-9 in the lung from a mouse with Clara cells exposed to crystalline silica for 14 days. (*D*) Immunohistochemistry for MMP-9 in the lung from a Clara cell-ablated mouse exposed to crystalline silica for 14 days. Bar= 50 µm.

experimental silicosis induced by intratracheal instillation of crystalline silica in rats (*38*) and also in a previous study we conducted (*36*); both reflect the results of the present study.

Our current results show that Clara cells inhibit gene expression of MMP-2 and MMP-9 *in vivo* after crystalline silica exposure. In addition to producing factors that appear to stimulate MMPs expression, such as phospholipases (*22*,*39*), proinflammatory cytokines (*40*,*41*), and chemokines (*42*,*43*), bronchial epithelial cells, including Clara cells, also produce factors that inhibit MMPs expression, such as CCSP, SLPI (*16*–*18*), and elafin (*17*,*18*). However, our results confirm that Clara cells generally inhibit MMPs, although it is unclear which factors are responsible for this inhibition.

It is possible that CCSP produced specifically by Clara cells plays some part. Szabo et al. (*44*) reported a marked reduction in MMP-2 and MMP-9 expression in A549 cells in which CCSP was overexpressed. In the present study, immunohistologic examination showed that MMPs are produced mainly in alveolar macrophages, bronchial epithelial cells, and alveolar epithelial cells, and that CCSP secreted from Clara cells may inhibit the production of MMPs from these cells.

It is also possible that protease inhibitors produced by Clara cells are involved in the inhibition of MMPs. The two types of protease inhibitors produced in Clara cells, SLPI (*16*–*18*) and the elastase-specific inhibitor elafin (*17*,*18*), have an antiinflammatory effect. Studies have shown that SLPI and elafin inhibit MMP-2 and MMP-9 (*45*), suggesting that these protease inhibitors secreted by Clara cells may have inhibited the expression of MMP-2 and MMP-9 in our study.

In summary, we investigated gene expression of MMP-2 and MMP-9 in the lungs after inhalation of crystalline silica in mice in which the Clara cells had been ablated with naphthalene. Gene expression of MMP-2 and MMP-9 was significantly more marked in the Clara cell-ablated group than in the mice with Clara cells, indicating that Clara cells inhibited MMP-2 and MMP-9 expression. Our findings suggest that Clara cells inhibit pulmonary inflammation and fibrosis via MMPs *in vivo*.

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