Supplemental Materials

Materials and methods

Primary mouse lung fibroblast culture. Fresh lung tissues from eight-week-old C57BL/6J mice were promptly excised after euthanization, washed with PBS, and cut to ~1 mm^3-sized tissue pieces. These tissue pieces were then suspended in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Grand Island, NY, USA) containing 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific) and 1x Antibiotic-Antimycotic (Thermo Fisher Scientific), and cultured on culture plates at 37°C in a humidified 5% CO_2 incubator. The culture medium was changed every three days. When the fibroblasts reached 80% confluence on the plate, cells were passaged at 1:5 dilution. The fibroblasts from passages five to eight were used for experiments.

Detailed immunohistochemistry and immunofluorescence. Formalin-fixed, paraffin-embedded lung tissue sections (left lung lobe, 5 μm) were deparaffinized, antigen-unmasked, and used to perform immunohistochemistry assays with ImmPRESS Polymer Detection system (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's protocol, as previously described (Dong, et al., 2016). Peroxidase activities were detected with the peroxidase substrate ImmPACT NovaRED (Vector Laboratories), which generates positive staining with red color. To stain nuclei, the slides were counterstained with Hematoxylin QS (Vector Laboratories), which visualizes nuclei with blue-violet color. The primary antibodies used for Immunohistochemistry included anti-TIMP1 (Santa Cruz, Dallas, TX, USA), anti-Collagen I (Abcam, Cambridge, MA, USA), anti-FN1 (Abcam), anti-α-SMA (Sigma-Aldrich), anti-Ki-67 (Cell Signaling, Danvers, MA, USA), and anti-PCNA (GeneTex, Irvine, CA, USA). Images were photographed using Olympus Provis AX-70 system (Olympus, Center Valley, PA, USA). Positive staining was measured using ImageJ program (National Institutes of Health, Bethesda, MD, USA), and relative intensity was presented as mean ± SD (*n*=4).

Cryostat sections from frozen lung tissues (left lung lobe, 7 μm) were fixed with 4% paraformaldehyde at room temperature for 10 min and used for immunofluorescence staining. After being blocked for 1 hr at room temperature, slides were immunostained with primary antibodies at 4°C overnight, incubated with Alexa Fluor 405-, Alexa Fluor 488-, or Alexa Fluor 594-conjugated secondary antibodies (Thermo Fisher Scientific, Rockford, IL, USA) for 1 hr at room temperature in dark, and mounted with ProLong Diamond Antifade Mountant with or without DAPI (Thermo Fisher Scientific). When mouse primary antibody was applied, the blocking reagent and antibody diluent from the M.O.M. Immunodetection Kit (Vector Laboratories) were used to eliminate background staining. The primary antibodies used for immunofluorescence were anti-TIMP1 (R&D Systems, Minneapolis, MN, USA), anti-Mac2 (Cedarlane, Burlington, NC, USA), anti-Hsp47 (EMD Millipore, Billerica, MA, USA), anti-E-cadherin (Abcam), anti-Collagen I (Abcam), anti-FN1 (Abcam), anti-Vimentin (Santa Cruz), anti-α-

SMA (Abcam or Sigma-Aldrich), anti-PDGFR-β (Abcam), anti-Ki-67 (Cell Signaling), anti-PCNA (GeneTex), anti-CD63 (R&D Systems), anti-integrin β1 (Santa Cruz), and anti-p-ERK1/2 (Thr202/Tyr204) (Cell Signaling). Images were taken with a Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy, Jena, Germany). Quantification of positive staining was performed using ImageJ program to derive relative intensity as mean ± SD (*n*=4).

***In vitro* cell proliferation and inhibition.** Primary fibroblasts were derived from adult C57BL/6J mouse lungs. The cells were pre-cultured in serum-free DMEM for 24 h and were then treated with DM or 5 μg/ml MWCNTs, with or without TIMP1 neutralizing antibodies (R&D Systems, 1 μg/ml), in DMEM containing 0.5% FBS for 24 h. Immunofluorescence of Ki-67 was performed to detect cell proliferation with DAPI for nuclear staining. Quantification of positive staining was expressed as the percentages of Ki-67+ and Ki-67- cells, derived from the count of around 150 cells per treatment.

PCR array, quantitative RT-PCR (qRT-PCR), and microarray analyses. Total RNA was extracted from lung tissue samples. Pooled RNA samples (*n*=6 per group) were reverse-transcribed, and the Mouse Fibrosis RT² Profiler PCR Array (QIAGEN, Valencia, CA) was applied. qRT-PCR was performed using RT² SYBR Green ROX qPCR Mastermix (Qiagen) as described previously (Dong, et al., 2015). Mouse glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as an internal control for normalization. Primer sequences are available upon request. Fold changes are presented as the mean ± SD (*n*=4). Microarray gene expression analysis (*n*=4) was performed using the MouseWG-6 v2.0 Expression BeadChip (Illumina, San Diego, CA) through a service at the Yale Center for Genome Analysis (Yale University School of Medicine, New Haven, CT).

Enzyme-linked immunosorbent assay (ELISA). BAL fluid was obtained following the method described previously (Dong, et al., 2016). Serum samples were obtained using the BD Microtainer tube with serum separator (Becton, Dickinson and Company, Franklin Lakes, NJ). Protein levels of TIMP1 were determined using Mouse TIMP-1 DuoSet ELISA kit (R&D Systems). Samples from five or six animals per group were measured to derive the mean ± SD.

Immunoblotting and quantification. Lung tissue samples were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific). Twenty micrograms of whole protein extract were resolved on a 4-15% or 8-16% Criterion Tris-HCl Gel (Bio-Rad, Hercules, CA) and transferred to a PVDF membrane (EMD Millipore, Billerica, MA). GAPDH was examined as a loading control. The primary antibodies used for immunoblotting were anti-FN1 (Abcam), anti-FSP1 (EMD Millipore), anti-PCNA (GeneTex), and anti-GAPDH (Fitzgerald, Acton, MA). Band intensity was measured using the ImageJ program. Relative band intensity was presented by the fold change between an experimental sample and the DM-treated WT control at each time point (*n*=3).

References

Dong J, Porter DW, Batteli LA, Wolfarth MG, Richardson DL and Ma Q. 2015. Pathologic and molecular profiling of rapid-onset fibrosis and inflammation induced by multi-walled carbon nanotubes. Archives of toxicology 89:621-633.

Dong J, Yu X, Porter DW, Battelli LA, Kashon ML and Ma Q. 2016. Common and distinct mechanisms of induced pulmonary fibrosis by particulate and soluble chemical fibrogenic agents. Archives of toxicology 90:385-402.

Figure legends

Figure S1. Detection of TIMP1 in lung cells. C57BL/6J mice were exposed to DM or MWCNTs (40 μg) for 7 days. Induction and localization of TIMP1 in macrophages, fibroblasts, and epithelial cells in the alveolar region were analyzed by double immunofluorescence staining of TIMP1 (green) and a cell type-specific marker (red) on lung sections. Scale bar: 20 μm.

Figure S2. MWCNTs induce TIMP1 expression in cultured lung fibroblasts. Lung fibroblasts were isolated from C57BL/6J mice and cultured *in vitro*. Cells were treated with DM or 5 μg/ml MWCNTs for 24 h. TIMP1 expression was determined by immunofluorescence (green). Scale bar: 20 μm.

Figure S3. Enlarged images of Masson's Trichrome staining presented in Figure 2A. The time course of the response is shown, with reduced formation of fibrotic foci in Timp1 KO lungs compared with WT. Scale bar: 100 μm.

Figure S4. Reduced collagen deposition in Timp1 KO lungs. WT and Timp1 KO mice received DM or MWCNTs (40 μg). Deposition of total collagen or Collagen I and III on 7 day post-exposure was detected by Masson's Trichrome staining (upper panel, collagen stained blue) or Picro-Sirius Red staining (lower panel, Collagen I and III stained red). The micrograph was taken to demonstrate reduced collagen deposition within dense fibrotic foci in Timp1 KO lungs compared with WT (scale bar: 20 μm). Marked deposition of collagen fibers is indicated in the enlarged rectangle insert.

Figure S5. The induction of fibrosis markers by MWCNTs. WT and Timp1 KO mice were exposed to DM or 40 μg MWCNTs. Collagen I (upper panel) and FN1 (lower panel) levels were determined by immunohistochemistry (red indicates positive staining, blue represents nuclear counterstaining) on lung sections from mice on day 7 post-exposure. Scale bar: 20 μm.

Figure S6. Band intensity of FN1 relative to GAPDH in immunoblotting. WT and Timp1 KO mice received DM or MWCNTs (40 μg) for 1, 3, 7 and 14 days. Whole protein lysates from lung tissues were analyzed (*n*=3).

Figure S7. Band intensity of FSP1 relative to GAPDH in immunoblotting. WT and Timp1 KO mice received DM or MWCNTs (40 μg) for 1, 3, 7 and 14 days. Whole protein lysates from lung tissues were analyzed (*n*=3).

Figure S8. The time course of α-SMA expression. WT and Timp1 KO mice received DM or MWCNTs (40 μg). α-SMA level was detected by immunohistochemistry (red indicates positive staining, blue represents nuclear counterstaining) on days 1, 3, 7 and 14 post-exposure. Scale bar: 20 μm.

Figure S9. Enlarged images of anti-Ki-67 and anti-PCNA immunohistochemistry presented in Figure 4A. Red indicates positive staining, and blue indicates nuclear counterstaining. Scale bar: 50 μm.

Figure S10. Band intensity of PCNA relative to GAPDH in immunoblotting. WT mice received DM or MWCNTs (40 μg) for 1, 3, 7 and 14 days. Whole protein lysates from lung tissues were analyzed (*n*=3).

Figure S11. Band intensity of PCNA relative to GAPDH in immunoblotting. WT and Timp1 KO mice received DM or MWCNTs (40 μg) for 1, 3, 7 and 14 days. Whole protein lysates from lung tissues were analyzed (*n*=3).

Figure S12. TIMP1 mediates MWCNT-induced proliferation of cultured lung fibroblasts. Lung fibroblasts were isolated from C57BL/6J mice, cultured *in vitro*, and treated with DM or 5 μg/ml MWCNTs, with or without TIMP1 neutralizing antibodies (1 μg/ml), for 24 h. Immunofluorescence of Ki-67 was performed to detect cell proliferation. (A) Staining of Ki-67 (red) and nucleus (DAPI, blue). Scale bar: 20 μm. (B) Percentages of Ki-67+ and Ki-67- cells, derived from the count of around 150 cells per treatment.

Figure S13. Quantification of immunofluorescence staining presented in Figures 6A, 6C and 6D. The numbers of triple-positive cells are shown as the mean ± SD (*n*=4).

Figure S14. MWCNTs stimulate the co-localization of TIMP1, CD63, and integrin β1 in cultured lung fibroblasts. Lung fibroblasts were isolated from C57BL/6J mice and cultured *in vitro*. Cells were treated with DM or 5 μg/ml MWCNTs for 24 h. (A) The co-localization of TIMP1, CD63, and integrin β1 was examined by triple immunofluorescence of TIMP1 (green), CD63 (red), and integrin β1 (blue). (B) Corresponding IgG controls showed negative staining. Scale bar: 20 μm.