Supplementary methods

Western Blot

In brief, cells or lung homogenates were lysed in Laemmli lysis buffer and the lysates were incubated for 5 minutes at 95°C. Afterwards, protein samples were separated by 10% SDS gel electrophoresis and transferred to a PVDF membrane (Millipore, Billerica, MA). Membranes were blocked for 1 hour in 4% milk in TBST and incubated overnight with monoclonal antibodies against α-smooth muscle actin (a-SMA), β-actin, GAPDH, collagen (all Santa Cruz, CA), phospho-ERK1/2 or total ERK1/2 (both Cell Signaling, Leiden) at 4°C. All secondary antibodies were horseradish peroxidase (HRP)-conjugated from DakoCytomation (Glostrup, Denmark) and diluted according to the manufacturer's instructions. Blots were imaged using Lumilight plus ECL substrate from Roche (Almere, The Netherlands) on an ImageQuant LAS 4000 biomolecular imager from GE Healthcare (Buckinghamshire, U.K). For quantification, densitometry was performed with ImageJ (NIH, Maryland, U.S) using the histogram function in a selected area of mean gray value for each band. Values for the protein of interest were corrected for those of β-actin or GAPDH.

Wound scratch assays

Cells were seeded in six-well plates in DMEM supplemented with 10% FCS. After the cells formed a confluent monolayer, a scratch was created in the center of the monolayer by a sterile p200 pipette tip. Next, medium was removed and cells were washed with serum-free medium to remove floating debris. The cells were subsequently incubated for 18 hours with serum-free medium (negative control), serum-free medium supplemented with 100 μM PAR-1-AP, or serum-free medium containing 100 μM PAR-1-AP and 10 μM P1pal-12. When indicated, cells were pre-incubated with 10 μM P1pal-12 for 30 minutes before scratching. The ability of cells to close the wound was assessed by comparing the 0- and 18-hour phase-contrast micrographs of 6 marked points along the wounded area. The percentage of non-recovered wound area was calculated by dividing the non-recovered area after 18 hours by the initial area at 0 hour as previously described.

Hydroxyproline Assay

Right lungs were homogenized in saline (100 mg lung in 900 μl saline) and stored at -20°C. Next, hydroxyproline content was analyzed using the hydroxyproline assay kit (Sigma, Netherlands) as per the manufacturer's instructions. In detail, 40 μl homogenate was added to

60 µl water after which 100 µl 12N HCL was added, Samples were hydrolyzed at 120°C for 3 hours after which 20 µL of the hydrolyzed samples were transferred to a 96 well plate. Subsequently, the plate (also containing a hydroxyproline standard curve) was incubated at 60°C till all fluid was evaporated (approximately 2 hours) after which the chloramine T/Oxidation buffer mixture was added. After 5 minute incubation on a shaker at room temperature, 100 µL of the Diluted DMAB Reagent was added and the samples were incubated for 90 minutes at 60 °C. Finally, the absorbance was measured at 560 nm and hydroproline content was calculated according to the standard curve. Right lung collagen content was calculated by multiplying the hydroxyproline values with a factor 7.4 (because collagen contains on average 13.5% hydroxyproline [1]). Finally, right lung collagen levels were multiplied by a factor 2 to obtain total lung collagen content [2].

Histological Analysis

Briefly, the excised lung was fixed in formalin, embedded in paraffin and 4-µm-thick slides were subsequently deparaffinized, rehydrated and washed in deionized water. Slides were stained with hematoxylin and eosin (H&E) and Masson's trichrome according to routine procedures. In H&E staining, severity of fibrosis was assessed according to the Ashcroft scoring system using a 200× magnification. Two independent observers were blinded to the treatment group and an average of 10 fields of each lung section were selected and scored. The average Ashcroft score was calculated by averaging the individual field scores.

Immunohistochemistry

Four-µm sections were first deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol. Smooth muscle actin (α-SMA) and collagen staining were performed with an anti-α-smooth muscle actin antibody (1:1000, 24 hour at 4°C, Santa Cruz, CA) or anti-collagen-I antibodies (1:800, overnight at 4°C, GeneTex, CA). A horseradish peroxidase-conjugated polymer detection system (ImmunoLogic, Duiven, the Netherlands) was applied for visualization, using an appropriate secondary antibody and diaminobenzidine (DAB) staining. Slides were photographed with a microscope equipped with a digital camera (Leica CTR500).

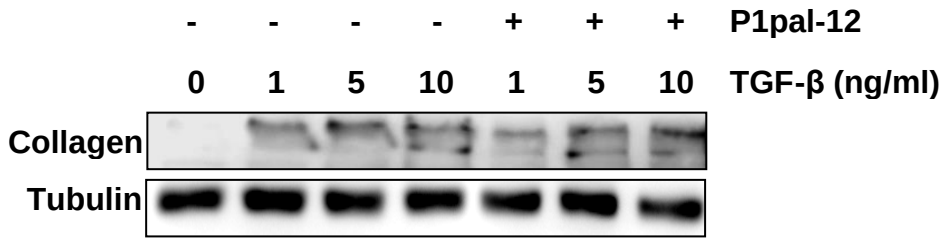
qPCR

Total RNA was isolated from lung homogenates with TriPure (Roche, Almere, Netherlands) following the manufacturer's recommendations. q-PCR was performed with SYBR Green

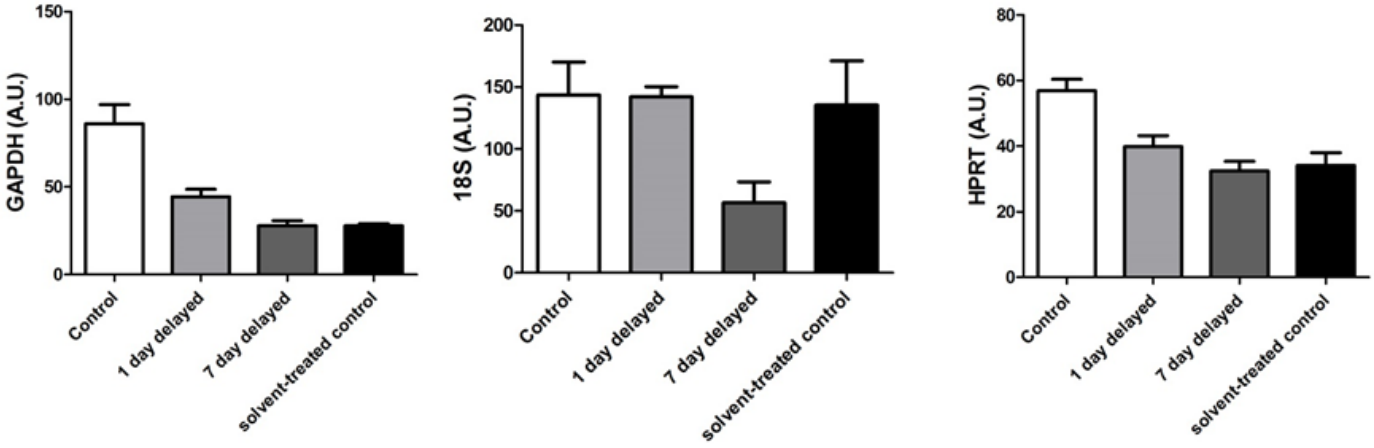
PCR master Kit (Roche) using the following primers: α-SMA: forward 5'-TCCCTGGAGAAGAGCTACGAACT-3' and reverse 5'-GATGCCCGCTGACTCCAT-3'; collagen 1A1: forward 5'-ACCTAAGGGTACCGCTGGA-3' and reverse 5'-TCCAGCTTCTCCATCTTTGC-3'; Fibronectin forward 5'-CCATGTAGGAGAACAGTGGCA-3' and reverse 5'-GAAGCACTCAATGGGGCA-3'. The results were calculated as Efficiency^{-Cp}.

Reference

1. Neuman RE, Logan MA. The determination of hydroxyproline. *J Biol Chem* 1950;**184**:299–306.
2. Kliment CR, Englert JM, Crum LP et al. A novel method for accurate collagen and biochemical assessment of pulmonary tissue utilizing one animal. *Int J Clin Exp Pathol* 2011;**4**:349-55.



Supplementary figure 1: TGFbeta induced collagen production is independent from P1pal-12 treatment. Western blot analysis of collagen expression by NIH3T3 cells stimulated with TGFbeta in the presence and absence of P1pal-12. Tubulin was used as loading control.



Supplementary figure 2: Expression of GAPDH, HPRT and 18S rRNA mRNA levels in lung homogenates obtained 14 days after bleomycin instillation in untreated mice and mice treated with 2.5 mg/kg P1pal-12 from day 1 or day 7 post-bleomycin instillation as assessed by real-time RT-PCR.