Additional file 1

Additional methods

Cell cultures

Cells were isolated from mouse lungs as described previously [1]. In the respective experiments, prominin-1\(^+\) cells were isolated either from the healthy or inflamed lungs (7 days after BLM instillation) by magnetic cell sorting using anti-prominin-PE antibody (eBioscience) and anti-PE magnetic beads (Miltenyi Biotec). For expansion of prominin-1\(^+\) progenitors, isolated cells were resuspended in the Culture Expansion Medium (CEM) containing Iscove’s Modified Dulbecco’s Medium, 2% foetal calf serum, 100 mM, b-mercaptoethanol, 100 U penicillin, 100 mg/mL streptomycin, 2mM L-glutamine, 25 mM, N-2-hydroxyethylpiperazine-N9-ethane sulfonic acid (all Invitrogen), and plated at 5x10\(^6\) cells into 6-cm diameter tissue culture dishes. To generate single prominin-1\(^+\) cell-derived clones, prominin-1\(^+\) cells from lung explants of C57Bl/6-EGFP mouse were sorted with magnetic beads as described above and 1 to 5 sorted cells were plated on non-transgenic lung-derived feeder layer in the CEM. For type II lung alveolar epithelial differentiation, cells were cultured in the modified Small Airway Growth Medium (SAGM; Cambrex) as described previously [1]. Macrophage differentiation was induced with 10 ng/mL macrophage-colony stimulating factor (M-CSF, PeproTech) in the CEM. 10 ng/mL TGF-\(\beta\) (PeproTech) was added to the CEM to stimulate myofibroblast differentiation. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO\(_2\). The culture medium was changed two to three times a week.
**Reverse Transcription and Real-Time Polymerase Chain Reaction**

RNA isolation and cDNA synthesis were performed as described [1]. cDNA was amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and oligonucleotides complementary to transcripts of the analyzed genes using the 7500 Fast Real-Time PCR System (Applied Biosystems). The following oligonucleotides were used in this study: αSMA (Acta2): 5’- cgctgctcaggagcctctgaga-3’, 5’- cgaagccgctcctacaga-3’; collagen I (Coll1a1): 5’- gatgacgtgcaatgcaatgaa-3’, 5’- ccctgactcactatatctgta-3’; fibronectin (Fn1): 5’- ttcaaggcttcacaccc-3’, 5’- cagatggcaaagaaggacaggg-3’; gapdh (Gapdh): 5’- ctgacacacatcttccctgc-3’, 5’- ggcattggactgtggcatgag-3’; SP-C (Sftpc): 5’- ttgacttccagggctc-3’, 5’- gtttctacgcccttgga-3’. Transcript levels of Gapdh were used as endogenous reference, and relative gene expression was analyzed using the 2^-ΔΔCt methods.

**Histology, immunocytochemistry and phagocytosis assay**

Formalin-fixed, paraffin-embedded lung sections were stained with hematoxylin and eosin for histological analysis and with Masson’s trichrome staining for detection of collagen fibers. The degree of fibrosis, analyzed as collagen I depositions, was calculated as percentage of the fibrotic area in relation to the total lung area. Calculations represent the average of 5 independent sections for each studied lung tissue. Immunofluorescence analysis was performed on frozen tissue sections and cells cultured on gelatin-coated cover slips as described previously [1]. For prominin-1 detection, frozen sections and cultured cells were stained with the appropriate primary antibody
prior to fixation with 4% paraformaldehyde. The following primary, secondary antibodies and dilutions were used in this study: rat-anti-prominin-1 1:200, anti-prominin-1-PE 1:200 (eBioscience), anti-F4/80-PE 1:400, anti-Cxcr4-FITC 1:400 (both from BD Bioscience), rabbit anti–surfactant protein-C 1:400 (SP-C, Abcam), mouse anti-β-tubulin IV 1:400 (Sigma), rabbit anti-collagen I 1:400 (Sigma), rabbit anti-fibronectin 1:400 (Milipore), mouse anti-smooth muscle actin 1:1000 (Sigma), donkey anti-rat AlexaFluor488 1:400, chicken anti-rabbit Alexa Fluor 488 1:400, chicken anti-rabbit Alexa Fluor 546 1:600, and goat anti-mouse Alexa Fluor 546 1:600 (all from Molecular Probes). Phagocytosis activity assay was performed using the Texas Red-conjugated *E. coli* BioParticles (Invitrogen) according to manufacture’s recommendations.

**Western-blot**

Prominin-1\(^+\) cells were challenged with TGF-β (PeproTech) for 1, 6 and 24 hours. Control cells were cultured in the absence of TGF-β. Cell lysates were blotted and incubated with rabbit anti-Phospho-Smad2 (1:500) and rabbit anti-Smad2/3 (1:1000; both Cell Signaling Technology), and β-actin (1:20000, Sigma).

**Flow cytometry**

Cells were filtered through 70-µm nylon mesh filter, stained for 30 minutes on ice with the appropriate antibodies, and analyzed on a CyAN ADP (Dako-Cytomation) using FlowJo 8.7.3 software (TreeStar). The following mouse specific antibodies and dilutions were used: anti–prominin-1-PE 1:200, biotin anti–c-kit 1:200, biotin anti-CD45 1:400 (all eBioscience), anti-F4/80-PE 1:400 and streptavidin-APC 1:600 (both BD Bioscience).
References

Additional Figure legends

**Figure S1. Prominin-1\(^+\) progenitors protect from induction, but do not affect progression of bleomycin-induced experimental pulmonary fibrosis**

Administration of prominin-1\(^+\) cells 2 hours after bleomycin treatment protected the mice from fibrosis and collagen I deposition in contrast to later cell administration (24h, 3d, 14d). The degree of fibrosis, analysed as collagen I depositions, was calculated as percentage of the fibrotic area (collagen I-positive area) in relation to the total lung area. Lung tissue sections were stained with Masson’s trichrome and analyzed at day 21 after injections of prominin-1\(^+\) cells 2 or 24 hours, and 3 or 14 days after bleomycin (BLM) instillation. Control healthy mice (PBS) or bleomycin-challenged mice (BLM) were injected only with saline (PBS). Calculations represent the average of 5 independent sections for each studied lung tissue. Differences were considered as statistically significant for p<0.05 (*), p<0.01 (**), p<0.001 (***)

**Figure S2. The prominin-1 expression correlates with the disease state of the bleomycin-treated mice.**

A, Immunofluorescent of healthy lung section (d0) showed the distinct cellular distribution of prominin-1- and \(\alpha\)SMA-expressing cells. B, Upon bleomycin (BLM) treatment the mice develop pulmonary inflammation (d7) characterized by the massive cell infiltration within the lung tissue. At that stage prominin-1-expressing, but \(\alpha\)SMA-negative cells represent an abundant fraction. C-D, Thorough the chronic stage prominin-1 expression was down-regulated whereas \(\alpha\)SMA\(^+\) fibroblasts become the
major cellular component of the lung tissue. A-D, DAPI visualized cell nuclei. Bars = 20\(\mu\)m.

**Figure S3. Bone marrow-derived prominin-1\(^+\) cells accumulated within the inflamed lung.**

C57Bl/6 mice were lethally irradiated and reconstituted with bone marrow of syngeneic C57Bl/6-EGFP animals. 6 weeks after bone marrow reconstitution, chimeric mice received bleomycin to induce pulmonary fibrosis. In the chimeric mice 7 days after bleomycin (BLM) instillation, EGFP-positive cells accumulated in the inflamed lung tissue, and around 30-40\% expressed prominin-1 (A). At day 21 after the bleomycin treatment the microphotograph of the lung from the chimeric mice showed no co-localization of EGFP and \(\beta\)-tubulin IV (B). DAPI visualized cell nuclei. Bars = 20\(\mu\)m.

**Figure S4. Chimeric mice develop BLM-induced pulmonary fibrosis.**

Hematoxylin and eosin (H&E)–stained histopathological sections and Masson’s Trichrome staining for collagen I deposition from lungs of healthy (d0) C57Bl/6 mice (A) and of animals at day 7 (B) and 21 (C) after bleomycin (BLM) exposure. Original magnifications x100 (for H&E and Masson’s trichrome).

**Figure S5. TGF-\(\beta\) mediates fibroblast differentiation of lung inflammatory prominin-1\(^+\) cells \textit{in vitro}.**

Western blotting analysis of Smad2 phosphorylation (P-Smad2) in lung inflammatory prominin-1\(^+\) cells cultivated \textit{in vitro} in the presence of TGF-\(\beta\) for 0, 1, 6 and 24 hours.
Samples from two independent experiments are shown for each time point. In total n= 4.
Figure S1

Collagen I (% of positive area)

- PBS
- BLM
- Prom1+ 2h
- Prom1+ 24h
- Prom1+ 3d
- Prom1+ 14d

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Figure S2

Prominin-1/αSMA

A  BLM, d7

B  BLM, d14

C  BLM, d21

D

Figure S2
Figure S4
Figure S5