Distinct niches within the extracellular matrix dictate fibroblast function in (cell-free) 3D lung tissue cultures

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Running head: The instructive ECM of 3D-LTCs

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Abstract

Instructive cues from the extracellular matrix (ECM) and their functional interplay with cells play pivotal roles for development, tissue repair, and disease. However, the precise nature of this interplay remains elusive. We used an innovative 3D cell culture ECM model by decellularizing 300 µm thick *ex vivo* lung tissue scaffolds (d3D-LTCs) derived from diseased and healthy mouse lungs, which widely mimics the native (patho)physiological *in vivo* ECM microenvironment. We successfully repopulated all d3D-LTCs with primary human and murine fibroblasts, and moreover demonstrated that the cells populated also the innermost core regions of the d3D-LTCs in a real 3D fashion. The engrafted fibroblasts revealed a striking functional plasticity depending on their localization in distinct ECM niches of the d3D-LTCs, affecting the cells' tissue engraftment, cellular migration rates, cell morphologies, and protein expression and phosphorylation levels. Surprisingly, we also observed fibroblasts, which were homing to the lung scaffold's interstitium, as well as fibroblasts which were invading fibrotic areas. To date, the functional nature and even the existence of 3D cell-matrix adhesions *in vivo* as well as in 3D culture models is still unclear and controversial. Here, we show that attachment of fibroblasts to the d3D-LTCs evidently occurred via focal adhesions, thus advocating for a relevant functional role *in vivo*. Furthermore, we found that protein levels of talin, paxillin and zyxin, phosphorylation levels of paxillinY118, as well as the migration-relevant small GTPases RhoA, Rac and CDC42 were significantly reduced compared to their attachment to 2D plastic dishes. In summary, our results strikingly indicate that inherent physical or compositional characteristics of the ECM act as instructive cues altering the functional behavior of engrafted cells. Thus, d3D-LTCs might aid to obtain more realistic data *in vitro*, with a high relevance for drug discovery and mechanistic studies alike.

Introduction

Cells in tissues and organs are closely embedded in an extracellular matrix (ECM), which consists of basement membranes and fibers as its main structural components. The ECM is a crosslinked and highly insoluble matrix that in mammals consists of a "core matrisome" of ~300 proteins (collagens, proteoglycans, and glycoproteins) and "ECM-affiliated" proteins (43). For long the ECM has been thought of being a mere structural passive support for cells, but the ECM contains cues which tightly instruct cell behaviors such as adhesion, determination, differentiation, proliferation, survival, polarity, and migration (35). Cellular behavior is not only influenced by the ECM's composition, but also by its biomechanical properties.

Standard cell culture techniques based on plastic and glass surfaces are currently still highly used to investigate fundamental cellular processes, albeit these models by no means can reflect the complexity of native tissues regarding molecular composition, topology, structure and biomechanics. Culturing cells in two dimensional (2D) artificial environments leads to abnormal cellular behaviors like flattened cell morphologies, aberrant polarization, changed migration patterns, different responses to pharmaceutical reagents or variations in cellular differentiation (2, 10). Therefore, a multitude of three dimensional (3D) cell culture systems based on natural, synthetic or hybrid materials exists, which all try to bridge the gap between conventional plastic dishes and the *in vivo* situation (10). Nevertheless, these 3D cell culture systems are mostly tunable in their biomechanical properties, although fail in recapitulating the molecular composition, topology and structure of native ECM.

In bioengineering and regeneration medicine, the decellularization of tissue and whole organs by detergent perfusion and their recellularization with various cell types, has recently become an ambitious approach in creating functional bioartificial organs ready for transplantation (44, 45, 50). In order to study living tissue *ex vivo*, especially for physiological and pharmacotoxicological studies, various

strategies have emerged to develop an exciting 3D cell culture technology by culturing viable and functional tissue slices from different species and organs under regular cell culture conditions (19, 25, 31, 40, 47, 54, 55). These precision-cut tissue slices (PCTS) or 3D lung tissue cultures (3D-LTCs) can be obtained from healthy and diseased human tissue, as well as from organs of different animal disease models, such as the bleomycin or elastase mouse models, which mimic fibrosis or emphysema in the lung, respectively.

In all tissues of the human body, fibroblasts are well-known to be the main producers and maintainers of the ECM. Apart from that, fibroblasts play major roles in wound healing, inflammation, angiogenesis, as well as in a pathological context in tissue fibrosis and cancer progression (reviewed in (37)). During tissue injury and repair, resident fibroblasts are activated by various chemical signals, whereas TGFβ1 is the most prominent one, leading to increased cell migration and invasion, proliferation, ECM production and transdifferentiation to highly contractile myofibroblasts (7, 12, 14, 20, 49). Hallmarks of myofibroblasts are the neo-expression of a-SMA in cytoskeletal stress fibers and the formation of supermature focal adhesion contacts (FAs), which are integrin-based cell-ECM adhesions (32).

Here we used decellularized *ex vivo* 3D-LTCs (d3D-LTCs) as a novel 3D cell culture tool to study the functional behavior of engrafted human and murine lung fibroblasts. We discovered an extensive functional plasticity of fibroblasts with key changes in engraftment, protein expression, phosphorylation patterns, cellular morphology and migratory behavior, all of which were apparently dependent on the fibroblasts' localization to distinct niches (alveolar, fibrotic, emphysematous, airway/vessel, mesothelium) within the d3D-LTCs, as well as when compared to conventional 2D plastic dishes. Our results indicate that inherent physical or compositional characteristics of the ECM act as instructive cues altering the functional behavior of the engrafted fibroblasts. Importantly, native tissue matrices (3D-LTCs) might become a valuable tool to substitute traditional cell culturing techniques, such as 2D plastic

dishes. Thus, *in vitro* assays with cells engrafted in d3D-LTCs will produce more realistic data by mimicking an *in vivo* scenario, which all together will be of highest relevance for mechanistic studies, functional analyses, and pharmacological testing alike. Moreover, understanding the underlying mechanisms of the interaction of real tissue's ECM and cells, especially fibroblasts which keep up the tissue's ECM structure and architecture, will help to get better insights and experimental control over recellularization processes. Such knowledge is urgently needed for fabricating bioengineered tissue that can replace diseased tissues and whole organs (6).

Material and Methods

Cells and cell culture

Mouse lung fibroblasts MLg (Mlg 2908) were purchased from ATCC (CCL-206) and cultivated in DMEM/HAM's F12 (catalog# E15-813, PAA) medium containing 10% FBS. MLg fibroblasts were not used at passage numbers higher than 15. Primary mouse fibroblasts were isolated by outgrowth from mouse lung tissue. Whole lungs were minced with a scalpel in 10 cm cell culture dishes filled with 5 ml prewarmed DMEM/HAM's F12 medium containing 20% FBS into small pieces. The minced lung tissue together with the medium was transferred into a 50 ml Falcon tube and digested by 5 mg of Collagenase I (Biochrom) at 37°C for 1 hour. The suspension was filtered through nylon filters with a pore size of 70 µm (BD Falcon) and the filtrate was centrifuged at 400 g at 4°C for 5 minutes. The cell pellets were resuspended in prewarmed DMEM/HAM's F12 medium containing 20% FBS and the cells plated on 10-cm cell culture dishes. Primary human fibroblasts were isolated by outgrowth from human lung tissue derived from lung explants or tumor-free areas of lung resections as previously described (6). Primary cells were used at passages < 6. All cells were cultivated and passaged at standard conditions (5 % CO2 and 37°C).

Animals

Pathogen-free female C57Bl/6-N mice (C57BL/6NCrl, Charles River, Germany) between the ages of 8-12 weeks were used. Mice were housed with water and food ad libitum. All experiments were performed in accordance with the guidelines of the Ethics Committee of the Helmholtz-Center Munich and approved by the Regierungspräsidium Oberbayern, Germany (projectnr: 55.2-1-54-2532-88-12).

132

133 **Animal disease models**

134 For the induction of lung fibrosis mice were subjected to intratracheal bleomycin instillation. Bleomycin
135 sulfate (Almirall, Barcelona, Spain) was dissolved in sterile phosphate-buffered saline and applied using
136 the Micro-Sprayer Aerosolizer, Model IA-1C (Penn-Century,Wyndmoor, PA, US), as a single dose of 0.08
137 mg in 50 µl solution per animal (3 U/kg body weight). Emphysema was induced by oropharyngeal (100
138 U/kg body weight in 80 µl PBS) application of porcine pancreatic elastase (Sigma, Taufkirchen,
139 Germany), which was dissolved in sterile PBS (Gibco, Carlsbad, CA, USA). Control mice were treated
140 with 50 µl and 80 µl PBS, respectively. Mice were sacrificed at day 14 after instillation for the generation
141 of 3D-LTCs.

142

143 **Human tissue**

144 The experiments with human tissue were approved by the Ethics Committee of the Ludwig-Maximillian
145 University Munich, Germany (projectnr. 455-12). All samples were provided by the Asklepios Biobank for
146 Lung Diseases, Gauting, Germany (projectnr. 333-10). Written informed consent was obtained from all
147 subjects. Tumor or tumor-free tissue from patients that underwent lung tumor resection was used.

148

149 **Generation of murine 3D *ex vivo* lung tissue cultures (3D-LTCs)**

150 For the murine 3D-LTCs healthy, fibrotic and emphysematous mice were anaesthetized with a mixture of
151 ketamine (bela-pharma, Germany) and xylazinhydrochloride (cp-pharma, Germany). After intubation
152 and dissection of the diaphragm, lungs were flushed via the heart with sterile sodium chloride and a

broncho-alveolar lavage (BAL) was taken (2x 500 µl sterile PBS). Using a syringe pump, lungs got infiltrated with warm low melting agarose (2 wt-%, Sigma, Germany, kept at 40°C) in sterile cultivation medium (DMEM/F12, Gibco, Germany, supplemented with penicillin/streptomycin and amphotericin B, both Sigma). The trachea was closed with a thread to keep the agarose inside the lung. Afterwards, the lung was excised, transferred into a tube with cultivation medium and cooled on ice for 10 min, to allow gelling of the agarose. The lobes were separated and cut with a vibratome (Hyrax V55, Zeiss, Germany) to a thickness of 300 µm. The 3D-LTCS were cultivated for up to seven days in sterile conditions.

Decellularization of 3D-LTCs

The 3D-LTCs (300 µm thick) were washed three times for 5 minutes in sterile deionized water, followed by incubation in 50 ml in deionized water (Falcon tubes) for 16 hours at 4°C on a tube roller. Followed by a washing step in deionized water, the 3D-LTCs were incubated in a 50 ml 0.1% SDS solution for 4 hours at room temperature. Followed by two washing steps in deionized water for 10 minutes each, the 3D-LTCs were incubated in 1 M NaCl for 16 hours at 4°C on a tube roller. The 3D-LTCs were washed twice in deionized water for 10 minutes each and were then incubated in 7.5 ml PBS together with 5 mM $MgCl_2$ and 30µg/ml DNAse for 3 hours at 37°C. Finally, the 3D-LTCs were washed for three times in deionized water for 10 minutes each, and stored in 24-well plates (TPP Techno Plastic Products, Switzerland) containing PBS which was supplemented with penicillin/streptomycin (Sigma). Quality control of the decellularization process was accomplished by histological stainings, immunofluorescence stainings, protein content measurement (BCA), Western blotting and qPCR.

Recellularization of d3D-LTCs with fibroblasts and categorization of ECM niches

The d3D-LTCs were recellularized in a suspension of 3x 10^6/ml MLg, primary mouse or human fibroblasts. d3D-LTCs and cells were put together in either 15 ml Falcon tubes or 2 ml Eppendorf tubes in DMEM/HAM's F12 (catalog# E15-813, PAA) medium containing 10% FBS. To allow gas exchange, the tubes were sealed with parafilm. The tubes were rotating at 10-15 rpm on a tube roller at standard cell culture conditions (5 % CO2 and 37°C). After 16 hours of incubation the d3D-LTCs were separated from the cells and incubated in 24-well plates DMEM/HAM's F12 medium containing 10% FBS at standard cell culture conditions for two to nine days. Experiments with fibroblasts cultured on 2D plastic dishes were performed in the same serum and environmental conditions as fibroblasts that were cultured on d3D-LTCs.

Within d3D-LTCs we defined five ECM niches, which we used as a spatially restricted ECM region in the functional assays (engraftment, cell proliferation, morphology, and migration): alveolar, fibrotic, emphysematous regions, and airway/vessel, and the mesothelium. Alveolar regions in 3D-LTCs had to be free from large/small airways/bronchioles as well as large vessels, and were chosen according to their characteristic alveolar structure with diameters of 30-40 µm. Fibrotic regions were best chosen first in the brightfield mode of the microscope. Here, the regular alveolar structural network was lost and substituted by a dense, tightly packed ECM network, which became easily visible in the brightfield microscope. Emphysematous regions in 3D-LTCs had to be free from large/small airways/bronchioles as well as large vessels and were chosen according to their characteristic enlarged air spaces (<80 µm). For airway/vessels we chose only those that were cut longitudinally. The mesothelium usually was found in the very periphery of the 3D-LTCs, with a characteristic fiber orientation perpendicular to the subpleural alveolar regions. Depending on the cutting process, the mesothelium appeared either as a thin (10 µm) or as a broad (100 µm) sheet (see also figure 2b or figure 5a). Alveolar regions, airway/vessels and the mesothelium were assessed in the same 3D-LTCs derived from control mouse lungs (PBS).

198

199 **Histochemistry of 3D-LTCs and microscopy**

200 Native and decellularized 3D-LTCs were fixed in 4% paraformaldehyde (pH 7.0) and embedded in
201 paraffin. The paraffin sections (2 µm) were deparaffinized and rehydrated. These sections were stained
202 with Masson Trichrome (HT15-1KT, Sigma) according to the manufacturer's instruction, or with Mayer`s
203 Hämalaun (T865, Roth) and Eosin Y solution (0,5%) (X883, Roth). Imaging of the sections was
204 accomplished at an upright AxioImager.M2 (Zeiss) using an EC PLN-NEOF 10x/0.3 M27 objective and an
205 AxioCam MRc (Zeiss). The microscope system was driven by AxioVision 4.8 (Zeiss) software.

206

207 **Immunohistochemistry of 3D-LTCs , confocal fluorescence microscopy and 3D cell shape analysis**

208 3D-LTCs were washed twice in PBS (138 mM NaCl, 26 mM KCl, 84 mM Na_2HPO_4, 14 mM KH_2PO_4, pH
209 7.4), fixed in 4% paraformaldehyde (PFA) in PBS for 30 minutes and permeabilized in 4% PFA/0.3% Triton
210 X-100 in PBS for 5 minutes. Alternatively, 3D-LTCs were fixed in ice-cold methanol for 2 min. and washed
211 in PBS. Primary antibodies were diluted in 1% bovine serum albumin (BSA, Sigma) in PBS, incubated for
212 16 hours at 4°C and subsequently washed three times with PBS for 5 minutes each. Secondary
213 antibodies were diluted in 1% bovine serum albumin (BSA, Sigma) in PBS, incubated for 4 hours at room
214 temperature and subsequently washed three times with PBS for 5 minutes each. Cell nuclei were
215 stained with DAPI (40,6-diamidino-2-phenylindole, Sigma-Aldrich, 1:2,000). For imaging the 3D-LTCs
216 were placed into a glass bottomed 35 mm CellView cell culture dish (Greiner BioOne), adding a drop of
217 PBS on top of the 3D-LTCs, putting a wet tissue in a circular fashion to the periphery of the dish, and
218 tightly sealing the lid with parafilm to prevent dehydration. Images of 3D-LTCs were acquired as z-stacks
219 using an inverted stand with an LSM 710 (Zeiss) module operated in multitrack mode using the following

objectives: Plan-Apochromat 20×/0.8 M27 and Plan-Apochromat 63×/1.4 M27. The automated microscopy system was driven by ZEN2009 (Zeiss) software. Assessment of 3D cell morphologies and 3D cell shape analyses were performed as previously described (7). In short, fibroblasts were either transfected with EGFP-N2 vector, or stained with Phalloidin (A12381, Invitrogen) after fixation with paraformaldehyde (4% in PBS). High-resolution confocal z-stacks were acquired with an LSM 710 using an LD C-Apochromat 40x/1.1 NA water objective lens (Carl Zeiss). The confocal fluorescent z-stacks were volume rendered with Imaris 9.0 software (Bitplane) and its statistical analysis tool (Measurement Pro) was used for the readout of 3D cell shape, cell surface area, and cell volume. 3D cell shape analysis in Imaris software calculates prolate and oblate parameters, whereas high values for prolate and low values for oblate indicate an elongated spheroid. Low values for prolate and high values for oblate indicate a rather flat and disk-shaped spheroid.

Antibodies and dyes for immunohistochemistry

For fluorescence immunolabeling the following primary (1) and secondary (2) antibodies (monoclonal = mc; polyclonal = pc) were used: 1) αSMA (A5228, Sigma, 1:5000, mouse, mc), Collagen 1 (600-401-103-0.1, Rockland, 1:100, rabbit, pc), Collagen 4 (ab6586, Abcam, 1:100, rabbit, pc), Fibronectin (sc-9068, Santa Cruz, 1:100, rabbit, pc), Ki67 (RBK027, Zytomed, 1:100, rabbit, mc), Laminin5 (ab14509, Abcam, 1:100, rabbit, pc), Talin (T3287, Sigma, 1:100, mouse, mc); and 2) donkey anti-rabbit IgG Alexa Fluor-488 (Invitrogen, 1:500) and donkey anti-rat IgG Alexa Fluor-568 (Invitrogen, 1:500). Cell nuclei were stained with Dapi (D9564-10MG, Sigma, 1:2000) and Actin stress fibers with Phalloidin (A12381, Invitrogen, 1:300).

242

243 **Transient transfections, cDNA constructs and recellularization of 3D-LTCs with transfected fibroblasts**

244 Cells were transiently transfected in DMEM/HAM's F12 medium containing 10% FBS using
245 Lipofectamine^{TM} 2000 (Invitrogen) in a 6-well format according to the manufacturer's manual.
246 Experiments were carried out 16–24 h after transfection. The αActinin1-EGFP cDNA construct (pGB9)
247 encoding fusion protein was generated by subcloning the human αActinin1 cDNA from a pGEX-4T-1 via
248 EcoRI sites into a pEGFP-N2 (Clontech) vector. The original αActinin1-pGEX-4T-1 plasmid was a generous
249 gift of Dr. Kristina Djinovic-Carugo (Max F.Perutz Laboratories, Vienna). The transfected cells were
250 trypsinized, resuspended in fresh cell culture medium and incubated in a rolling fashion together with
251 native 3D-LTCS in a 2 ml Eppendorf tube without the plastic lid but sealed with parafilm for 16 hours.
252 Next the 3D-LTCS were transferred to 24-well plates and either stained for ECM proteins or directly used
253 for microscopy.

254

255 **Confocal 4D live cell imaging**

256 Confocal time-lapse microscopy was implemented on an LSM710 system (Carl Zeiss) containing an
257 inverted AxioObserver.Z1 stand equipped with phase-contrast and epi-illumination optics and operated
258 by ZEN2009 software (Carl Zeiss). 3D-LTCs were kept in DMEM/HAM's F12 medium containing 10% FBS
259 and 15 mM HEPES during the whole period of observation. The nuclear staining of cells or tissue was
260 accomplished by incubation with HOECHST (bisBenzimide H 33342 trihydrochloride, Sigma, 1:500) for 10
261 min. A tissue imaging chamber, as previously described (8), containing the 3D-LTCS or cells was placed
262 into a PM S1 incubator chamber or an incubator XLmulti (PeCon/Carl Zeiss) and kept at 37°C and 5%
263 CO_2. Time-lapse images in various intervals were acquired by using the following objective lenses: EC
264 Plan-Neofluar DICI 10x/ 0.3 NA (Carl Zeiss), LD C-Apochromat 40x/1.1 NA water objective lens (Carl

Zeiss) and LCI PLN-NEOF DICIII 63x/ 1.30 NA water objective lens (Carl Zeiss). Z-stacks were taken according to the thickness of the 3D-LTCS and were ranging between 150 µm and 300 µm. The confocal 4D data sets were either maximum intensity projected in the ZEN2009 software (Carl Zeiss) or imported into Imaris 7.6.5 or 8.0.0 software (Bitplane). Within the Imaris software the confocal 4D data sets were either volume or surface rendered and exported either as time-lapse movies or figures. Migration of the HOECHST stained, reseeded fibroblasts was assessed with Imaris (Bitplane) software using the spots-algorithm and the automatic tracking function therein, whereas per each single time-lapse we analyzed 60-400 tracks, depending on how many cells were found in the region of interest. A biological n=3 (using lung slices from different mice and condition/niche and 4-9 technical replicates) was analyzed.

Protein isolation from 3D-LTCs, SDS-PAGE and Western Blotting

Native, decellularized and recellularized 3D-LTCs were washed three times in PBS. Five lung slices of each condition were pooled in an Eppendorf tube and lysed in 500 µl ice-cold RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate) containing 1× Roche complete mini protease inhibitor cocktail. After an incubation of 2 hours rotating at 4°C, the lung slices were removed from the lysates and the protein content was measured. Samples were mixed with 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 1% bromphenol blue, and 10% glycerol, and proteins were separated using standard SDS-10% PAGE. For immunoblotting, proteins were transferred to PVDF (Millipore (Billerica, MA, (USA)), 0.45µm or 0.2µm) membranes, which were blocked with 5% milk in TBST (0.1% Tween 20/TBS) and incubated with primary, followed by HRP-conjugated secondary antibodies over night at 4°C and at room temperature for 1 hour, respectively. The following primary antibodies were used for immunoblotting: αSMA (A5228, Sigma, 1:1000, mouse, mc), β-Actin (A3854, Sigma,1:40000, mouse, HRP-conjugate, mc), ß-Tubulin (2146, Cell Signaling, rabbit, pc), Caveolin1 (13267, Cell Signaling, 1:1000,

rabbit, mc), E-Cadherin (610181, BD, 1:2500, mouse, mc), GAPDH (3683, Cell Signaling, 1:1000, rabbit, HRP-conjugate, mc), LaminA/C (2032, Cell Signaling, 1:1000, rabbit, pc) and Vimentin (sc-7557-R, Santa Cruz, 1:1000, rabbit, pc).

mRNA Isolation and qRT-PCR

Native, decellularized and recellularized 3D-LTCs were washed three times in PBS. Five lung slices of each condition were pooled in pre-cooled cryotubes together with a grinding pool and frozen in liquid nitrogen. The frozen 3D-LTCs were homogenized with a micro-dismembrator (Sartorius, Göttingen, Germany). RNA extraction from mouse tissue was performed using the Roti Quick Kit (Carl Roth, Karlsruhe, Germany) followed by RNA isolation using the PeqGold RNA kit (Peqlab) according to the manufacturer's instruction. The concentration of the isolated RNA was assessed spectrophotometrically at a wavelength of 260 nm (NanoDrop 1000). cDNA was synthesized with the GeneAMP PCR kit (Applied Biosystems (Foster City, CA, USA)) utilizing random hexamers using 1 µg of isolated RNA for one reaction. Denaturation was performed in an Eppendorf Mastercycler with the following settings: lid=45°C, 70°C for 10 minutes and 4°C for 5 minutes. Reverse transcription was performed in an Eppendorf Mastercycler with the following settings: lid=105°C, 20°C for 10 minutes, 42°C for 60 minutes and 99°C for 5 minutes. qRT-PCR reactions were performed in triplicates with SYBR Green I Master in a LightCycler® 480II (Roche (Risch, Switzerland)) with standard conditions: 95°C for 5 min followed by 45 cycles of 95°C for 5 s (denaturation), 59°C for 5 s (annealing) and 72°C for 20 s (elongation). Target genes were normalized to GAPDH expression. Mouse primer sequences used for qPCR:

Gene	Orientation	Sequence 5' ➔ 3'
Actin	fwd	GGCACCACACCTTCTACAATG
Actin	rev	GGGGTGTTGAAGGTCTCAAAC
αSMA	fwd	GCTGGTGATGATGCTCCCA
αSMA	rev	GCCCATTCCAACCATTACTCC

Cav1	fwd	CGACGACGTCAAGATTGACTT
Cav1	rev	TGCACGGTACAACCGCCCAG
Desmin	fwd	TCAACCTTCCTATCCAGACCT
Desmin	rev	GCTGACAACCTCTCCATCC
E-Cadherin	fwd	CCATCCTCGGAATCCTTGG
E-Cadherin	rev	TTTGACCACCGTTCTCCTCC
Elastin	fwd	GCATCGGTGGCTTAGGAG
Elastin	rev	AACCGAGCTCCTGTTCCT
Fibronectin	fwd	GTGTAGCACAACTTCCAATTACGAA
Fibronectin	rev	GGAATTTCCGCCTCGAGTCT
LaminA	fwd	CCACCGTGGTTCCCACT
LaminA	rev	CACCGGCAGCCTTGTCAG
Rock1	fwd	GTCGACTGGGGACAGTTTTG
Rock1	rev	AGGCAGGAAAATCCAAATCA
SM22	fwd	CCTCCAGCTCCTCGTCATAC
SM22	rev	CCGAAGCTACTCTCCTTCCA
Tln1	fwd	GGAGTTTGGAGATGCCATTG
Tln1	rev	CAGTCCTTGCTGTCCAGCTT
Vimentin	fwd	AGACGGTTGAGACCAGAGATGG
Vimentin	rev	TGTTGCACCAAGTGTGTGCAAT
Vinculin	fwd	AAAGCCATTCCTGACCTCAC
Vinculin	rev	TCTGATCCTCAGTGGTCTGAA

Statistics

Statistical analyses and tests were performed using GraphPad PRISM4 software (GraphPad software). Data are presented as mean and standard deviation (SD) or standard error of the mean (SEM), as indicated in the text. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Results

Effective decellularization of murine three-dimensional ex vivo tissue cultures

The generation of 300 µm 3D-LTCs from normal and diseased (fibrotic, emphysematous) murine lung tissue was performed as described earlier in one of our prior studies (54). In order to extract all cellular and nuclear material from the native 3D-LTCs we applied a sequential incubation in various solutions including sterile deionized water (16 hours), 0.1% SDS solution (4 hours), 1M NaCl (16 hours) and DNAse (3 hours) with several washing steps in between all before mentioned steps. As any remaining cellular components might have had a possible negative impact on experiments with repopulated cells, we performed control experiments demonstrating the effective removal of cellular components. Therefore, we initially performed Hematoxylin/Eosin (HE) and Masson Trichrome (MT, data not shown) histological stainings of paraffin-embedded d3D-LTCs. These experiments clearly disclosed the complete extraction of all cells and nuclei from the normal and diseased (fibrotic, emphysematous) 3D-LTCs after the decellularization procedure (figure 1a). Next, we PFA-fixed the 300 µm thick native and decellularized 3D-LTCs derived from normal and diseased lungs, and immunofluorescently labelled them for the ECM protein collagen1. Additionally, nuclei were stained with DAPI and cytoskeletal actin stress-fibers with Phalloidin. By applying 3D-confocal immunofluorescence microscopy, native 3D-LTCs exhibited a distinct nuclear and cytoskeletal actin staining, which was clearly missing after the decellularization process (figure 1b). However, for the ECM protein collagen1, a strong staining signal was found in both, native and decellularized 3D-LTCs. This unambiguously demonstrated that all cellular components were successfully extracted from the 3D-LTCs, whereas components of the ECM, here collagen1, remained complete. To further investigate the integrity of ECM molecules subsequent to decellularization, we immunofluorescently labelled in addition to the interstitial ECM component collagen1, the basal membrane components collagen4 and laminin5 (figure 1d). When we compared native and decellularized 3D-LTCs, no evident differences in the staining pattern or signal intensities for collagen1, collagen 4 and laminin5 were detectable. Finally, to further verify the complete removal of cellular components on a molecular level, we quantified the overall amount of soluble proteins by protein content measurements (data not shown), as well as the amount of cell-specific proteins (E-cadherin, lamin A/C, vimentin, β-tubulin, β-actin, αSMA, GAPDH, caveolin1) by Western blotting (figure 1c). The overall amount of soluble proteins was largely reduced in the d3D-LTCs (on average 90.1% (+/- 1.1% SD)) (data not shown). Moreover, cell-specific proteins in the d3D-LTCs were undetectable by Western blotting, though the same proteins extracted from native 3D-LTCs noticeably gave a signal at their

corresponding molecular weight (figure 1c). Finally, qPCR analysis revealed that RNA transcripts of diverse cellular targets (rock1, talin1, αSMA, SM22, fibronectin, desmin, vimentin, E-cadherin, β–actin, caveolin1, vinculin, elastin, laminC) were absent in d3D-LTCs (data not shown). Additionally, we performed high-resolution confocal imaging of the entire height (300 µm) of d3D-LTCs, which were immunofluorescently stained for Collagen 1. Software-based surface rendering and orthoview of volume rendered z-stacks, impressively revealed various layers of entire alveoli and alveolar ducts, demonstrating an intact lung ECM structure after decellularization (figure 1e and figure 1f, supplementary movie1). All these data confirmed the effective clearance of cells and cellular components from normal, fibrotic and emphysematous 3D-LTCs after decellularization. Moreover, the 3D architecture of the 3D-LTCs as well as the integrity of specific ECM proteins remained intact after decellularization.

Engraftment of fibroblasts to the ECM of d3D-LTCs and differential protein expression compared to 2D plastic dishes

Next, we wanted to investigate whether fibroblasts successfully engraft the d3D-LTCs. Here, we applied 4D confocal live-cell imaging of lung fibroblasts, which were stained with the non-toxic cell tracker dye CMTPX. Live-cell imaging was started immediately after the d3D-LTCs were incubated for four hours together with CMTPX-stained lung fibroblasts. At the beginning of the live-cell imaging (0 hours) the fibroblasts were already attached to the ECM of the d3D-LTCs and evidently started to spread and migrate within the next four hours (figure 2a and supplementary movie2). Thus spread fibroblasts adopted an elongated cellular shape in alveolar regions (yellow arrow in the magnified view of a region of interest taken from movie2 and depicted in figure 2a). Intriguingly, high-resolution confocal imaging of the entire height (300 µm) of d3D-LTCs, which were immunofluorescently stained for Collagen 1 and fibroblasts stained for Phalloidin, clearly demonstrated that first, fibroblasts in alveolar regions adopted a sheet-like 3D morphology, and secondly, that reseeded fibroblasts also populated the inner regions of 3D-LTCs after 120 hours (figure 2a and supplementary movie3). Similarly, primary mouse lung fibroblasts and also patient derived human lung fibroblasts (pHFibs) successfully engrafted the d3D-LTCs (data not shown). After culturing the lung fibroblasts for 5 days, we fixed and stained the recellularized d3D-LTCs with a nuclear-specific dye, and concomitantly imaged the scaffold's ECM either by its autofluorescence or by immunostaining of the ECM proteins emilin-2 and fibronectin. Surprisingly, we found an uneven distribution of the engrafted fibroblasts, with a significantly higher amount of

fibroblasts found in emphysematous (4.9 fold/3.0 fold; p=0.0061/p=0.015), airway/vessel (6.0 fold/3.6 fold; p=0.0013/p=0.0032), and mesothelial regions (6.6 fold/3.9 fold; p=0.0002/p=0.0007) compared to alveolar or fibrotic areas, respectively (figure 2b). In emphysematous, airway/vessel and mesothelial regions the fibroblasts appeared as a confluent cell layer, whereas in the alveolar or fibrotic regions fibroblasts were found to be rather sparsely engrafted (figure 2b). We hypothesized that structural or compositional alterations in the investigated ECM niches (alveolar, fibrotic, emphysematous, airway/vessel, mesothelium) might affect the proliferative capacity of the engrafted fibroblasts. Therefore, we applied a Ki67 immunofluorescence staining (figure 2c). Quantification of the fibroblasts' proliferative activity based on Ki67 staining revealed an increase between alveolar/fibrotic and the airway/vessel (5-10%), emphysematous (5-12%) and mesothelial regions (15-20 %) within the 3D-LTCs, though neither of these changes was statistical significant (figure 2c). However, when compared to fibroblasts cultured on conventional 2D-PDs, the proliferative capacity of fibroblasts that engrafted the 3D-LTCs was significantly lower ($p<0.0001$) (figure 2c). Thus, these data demonstrate that the engraftment of fibroblasts into d3D-LTCs might primarily depend on the initial attachment of the fibroblasts to various niches, probably due to diverse physicochemical, mechanical and topological properties of the ECM, but cannot completely rule out an additional contribution of differential proliferative activities of the fibroblasts.

It has been shown before, that culturing cells in unique ECM environments causes variations in gene-expression and cellular behavior (3, 16, 17, 29). Therefore, we were interested whether the engraftment of pHFibs in d3D-LTCs would change the protein expression of specific cellular markers when compared to the same fibroblasts cultured in conventional 2D-PDs. Hence, we chose markers which reportedly play a role in mechanotransduction (yes-associated protein 1 (YAP), tropomyosin) (11, 34), (myo)fibroblast transdifferentiation (αSMA, calponin h1, desmin) (33), cellular contraction (calponin h1, caldesmon) (48), and well-known secreted ECM proteins (fibronectin, collagen1) (37). By immunoblotting and subsequent densitometric analyses we found that the protein expression of YAP (0.15 fold, p=0.003), tropomyosin (0.13 fold, p=0.0004) and fibronectin (0.7 fold, p=0.02) were significantly downregulated in pHFibs that engrafted the d3D-LTCs (figure 2d). Yet, for all other markers under investigation, that is αSMA, calponin h1 , desmin, caldesmon, and collagen1, we could not detect major alterations in their protein expression (figure 2d). Thus, we concluded that culturing fibroblasts on d3D-LTCs distinctly changes the expression of specific proteins compared to fibroblasts cultured on conventional 2D-PDs.

412

413 ***Anchorage of fibroblasts to 3D-LTCs occurs by focal adhesion contacts (FAs) and differential protein***
414 ***expression of distinct FAC proteins***

415 We recently found that mouse lung fibroblasts, which are externally added to native (non-
416 decellularized) 3D-LTCs would integrate into the *ex vivo* tissue and adhere to the ECM by forming focal
417 adhesions (FAs), which are integrin-based cell-matrix contacts (8). Thus, we were curious whether
418 repopulated fibroblasts would adhere to d3D-LTCs by forming FAs, which are sometimes heavily
419 discussed as being mere artefacts of culturing cells in 2D-PDs (56). Indeed, by ectopically expressing
420 EGFP-αActinin1, which is a well-known marker for FAs and cross-linker of actin filaments in stress-fibers,
421 in MLg lung fibroblasts, and by applying 4D-confocal microscopy, we plainly could point out streak-like
422 structures reminiscent of FAs, both in their shapes and dynamics (yellow arrows in figure 3a and
423 supplementary movie4). In order to corroborate these findings, we performed 3D-confocal microscopy
424 on methanol-fixed 3D-LTCs, which were repopulated with pHFibs for 3 days and immunostained for the
425 FA component talin (5) and the ECM protein fibronectin. Figure 3b distinctly displays talin-positive
426 streak-like structures reminiscent of FAs (yellow arrows), which are partially co-localizing with
427 fibronectin fibers. The white rectangle in the merged image indicates a magnified interior cell region
428 which is presented at the bottom of the panel. Moreover, we also performed immunofluorescence
429 stainings using antibodies to talin and fibronectin on native (non-decellularized) 3D-LTCs. Interestingly,
430 3D-confocal fluorescent microscopy revealed that likewise in native (non-decellularized) 3D-LTCs streak-
431 like structures (yellow arrows in figure 3c) can be found which are reminiscent of 2D cell-matrix adhesive
432 components (FAs, fibrillar adhesions) and partially co-localized with fibronectin fibers (blue arrows in
433 figure 3c). Likewise, as 3D-LTCs engrafted pHFibs differentially expressed specific proteins compared to
434 those cultured on 2D-PDs (figure 2d), we were eager to know whether components of FAs were similarly
435 deregulated. Indeed, by immunoblotting and subsequent densitometric analyses, we observed that the
436 expression of the FA markers talin (0.28 fold, p=0.006), paxillin (0.22 fold, p=0.006) and zyxin (0.34 fold,
437 p=0.013) was significantly reduced in pHFibs cultured on 3D-LTCs (figure 3d). Additionally,
438 phosphorylation levels of paxillinY118 (0.08 fold down-regulation, p=0.0012) were significantly
439 deregulated, whereas phosphorylation levels of zyxinS142/143 were not (figure 3d). Surprisingly,
440 protein levels of tensin (3.4 fold, p=0.032) were significantly elevated in pHFibs engrafted in 3D-LTCs,
441 while levels of αActinin1 (0.9 fold, p=0.251) were found to be mainly unchanged (figure 3d). Taken
442 together, fibroblasts adhered to the surface of decellularized 3D-LTCs by means of FAs, whose
443 composition might be altered compared to 2D-FAs of fibroblasts cultured in conventional 2D-PDs.

444

445 ***Morphological plasticity of fibroblasts is a function of their surrounding microenvironment***

446 Next, we wanted to investigate whether distinct niches within the ECM of 3D-LTCs (alveolar, fibrotic,
447 emphysematous, airway/vessel, mesothelium) would induce alterations in the morphology of
448 fibroblasts. By repopulating d3D-LTCs with MLg fibroblasts, which ectopically expressed EGFP in their
449 cytosol, subsequent 3D confocal live-cell imaging and software-based isosurface rendering, we were
450 able to assess the fibroblasts' morphology. We observed that fibroblasts adopted their morphology
451 according to their surrounding microenvironment, as in alveolar regions we found fibroblasts, which
452 engrafted the d3D-LTCs by displaying two distinct morphologies: flat and dome-like fibroblasts with
453 moderately sized cell bodies lining alveoli, and elongated, spindle-shaped ones (figure 4a and
454 supplementary movie5). Interestingly, when we utilized 4D confocal live-cell imaging (16 hours) of a
455 single EGFP-expressing lung fibroblast, which was lining an alveolus, we observed that although this
456 fibroblast produced cellular protrusions, it largely kept up its dome-like morphology over time and
457 stayed completely stationary (figure 4b and supplementary movie6). An in-depth classification of
458 numerous fibroblastic morphologies in distinct niches within the ECM scaffold of d3D-LTCs is illustrated
459 in figure 4c, showing software-based surface rendered confocal z-stacks. Interestingly, fibroblasts that
460 engrafted in the mesothelium were flat with a small cell body (figure 4c). Likewise, in regions of
461 airways/vessels fibroblasts took on small cell bodies with mostly elongated, bipolar and bulbous shapes
462 (figure 4c). Contrarily, in emphysematous regions fibroblasts were flat and multipolar, with mostly huge
463 cell bodies, though noticeably missing the dome-like phenotype found in alveolar areas (figure 4c). In
464 fibrotic regions fibroblasts adopted long, elongated, bipolar and spindle-shaped cell bodies (figure 4c).
465 Software-based quantitation of 3D cell shapes (figure 4d) confirmed our previous observations:
466 fibroblasts that engrafted either fibrotic or airways/vessels were quantified as elongated spheroids
467 (prolate > oblate), fibroblasts in alveolar and mesothelial niches were quantified as flattened disk-
468 shaped spheroids (prolate < oblate), and fibroblasts on 2D-PD and in emphysematous niches were
469 quantified as flat ellipsoids (prolate = oblate) (figure 4d). Quantitation of cell volumes and cell surface
470 areas indicated that the largest cells are found on 2D-PD and in emphysematous niches, whereas the
471 smallest cells were those that engrafted to the mesothelium and airways/vessels (figure 4e). In
472 conclusion, fibroblasts reseeded in d3D-LTCs took on a large diversity of different cell shapes and cell
473 sizes which were highly dependent on their surrounding microenvironment in the d3D-LTCs.

474

3D-migration of lung fibroblasts engrafted on decellularized 3D-LTCs differs between distinct ECM niches and 2D plastic dishes

Beforehand, we observed an altered re-population behavior and a remarkable morphological plasticity of fibroblasts, both of which apparently were a function of their surrounding microenvironment in the 3D-LTCs. Therefore, we wanted to determine whether different niches within the 3D-LTCs might additionally have any impact on cell migration. For that we had MLg lung fibroblasts repopulating decellularized 3D-LTCs, which were derived from lungs from mice treated with either PBS (control), bleomycin (fibrotic), or elastase (emphysematous). After 3 days of incubation the nuclei of lung fibroblasts were stained with HOECHST and migration of fibroblasts was assessed by 4D confocal live-cell imaging for 24 hours by putting the emphasis on distinct niches (2D-PDs, alveolar, fibrotic, emphysematous, airway/vessel, mesothelium) for imaging and analyses thereof (figure 5a and supplementary movie7). By plotting the measured migration speed against the straightness of the migration tracks, we evidently could discern distinct migration patterns of the repopulated fibroblasts (figure 5a). In alveolar and fibrotic regions, fibroblasts mostly exhibited a preference towards non-migration (red rectangles in figure 5a). In contrast, fibroblasts that engrafted in emphysematous areas displayed a more uniform distribution of migration speeds and straightness of tracks, which was very similar to the pattern found in fibroblasts migrating on a 2D-PD (figure 5a). Strikingly, in regions of the airway/vessel and the mesothelium, the high amount of parallel tracks indicated that most fibroblasts migrated here in a parallel or antiparallel fashion (figure 5a). The diversity in migration patterns was also reflected by the migration speeds of the repopulated fibroblasts (figure 5b). The migration speed of fibroblasts on 3D-LTCs was overall significantly slower (5.71 fold) when compared to fibroblasts migrating on the surface of 2D-PDs (0.07 µm/min vs 0.4 µm/min, $p < 0.0001$). Moreover, by looking at various ECM niches in the 3D-LTCs, fibroblasts engrafted in the airway/vessel (1.0 µm/min) or the mesothelium (0.08 µm/min) displayed a significantly (* $p < 0.05$; ** $p = 0.0024$; *** $p = 0.001$) higher migration rate than those found in the alveolar (0.06 µm/min) or fibrotic (0.04 µm/min) regions (figure 5b). However, migration speeds of fibroblasts on emphysematous 3D-LTCs were found to be only slightly increased compared to alveolar and fibrotic areas, as well as slightly decreased in contrast to airway/vessel and mesothelial regions (figure 5b).

As we observed that migration of fibroblasts largely differed on decellularized 3D-LTCs compared to 2D-PDs, we intended to investigate whether regulators of migration were differentially expressed. By immunoblotting and subsequent densitometric analyses, we detected that the protein expression of

RhoA (0.20 fold, p=0.0045), Rac1,2,3 (0.25 fold, p<0.0001), CDC42 (0.34 fold, p=0.043), and Src-kinase (0.48 fold, p=0.0004) was significantly diminished in pHFibs cultured on 3D-LTCs (figure 5c). However, for ROCK1 we could not detect substantial changes in protein expression levels.

In our analyses of fibroblastic migration on 3D-LTCs we identified a strong bias towards non-migrating cells in alveolar and fibrotic niches. By subsequent confocal 3D microscopy we identified fibroblasts in healthy alveolar as well as in fibrotic areas which were completely surrounded by collagen1, indicating that these fibroblasts did not sit on the surface of the ECM scaffold but are either located in the interstitium or had invaded the fibrotic ECM, respectively (figure 5d and figure 5e). A complete embedding of fibroblasts in either the interstitium or fibrotic regions might explain the decline of migration rates of fibroblasts in these areas.

Discussion

Qualities of the ECM, such as its composition, topology, biomechanical properties, post-translational modifications of proteins, or its reservoir function for growth factors and soluble mediators, produce instructive cues which are interpreted and processed by cells. Subsequently, these signals phenotypically transform the cells by affecting their fundamental biological processes including gene-expression, proliferation, adhesion and migration, survival and apoptosis, and differentiation (6). First, we showed here the effective decellularization of 3D-LTCs derived from healthy and diseased mouse lung tissue. Second, by using patient derived primary human fibroblasts as well as mouse fibroblasts we demonstrated the successful engraftment and proliferation of the fibroblasts in the ECM of the 3D-LTCs. Third, we observed that fibroblasts adhered to decellularized 3D-LTCs by means of FAs. Fourth, we discerned considerable differences in respect of protein expression, cellular morphology and migratory behavior when comparing fibroblasts cultured in conventional 2D-PDs to cells cultured on decellularized 3D-LTCs and distinct ECM niches therein.

Functional studies which directly address how changes in natively derived ECM instruct alterations in cellular phenotypes are still very rare. For instance, Parker and colleagues reported that primary human lung fibroblasts derived from healthy lungs and were cultured on decellularized fibrotic ECM, resulted in changes of gene expression patterns as well as on alterations on the translational level. Both observed effects were corresponding to the pathological expression levels found in idiopathic pulmonary fibrosis (IPF) (46). Similarly, normal primary human fibroblasts, which were cultured on decellularized fibrotic ECM derived from IPF lungs, transdifferentiated into αSMA positive myofibroblasts independently of TGFβ1 (4). In aging, decellularized lung ECM derived from old mice negatively affected the gene expression of the basal lamina components laminin-α3 and -α4 in reseeded primary human bronchial epithelial as well as in primary human lung fibroblasts (27). However, bone-marrow derived mesenchymal stem cells (MSCs) largely retained their mesenchymal phenotype when they were used for the recellularization of decellularized whole mouse lungs, although the same MSCs showed alterations in spreading and proliferation when they encountered lung compartments which were rich in type I collagen, type IV collagen and laminin (18). Here, we applied decellularized 3D-LTCs to examine how different cues of the ECM, stemming from different niches (2D-PDs, alveolar, fibrotic, emphysematous, airway/vessel, mesothelium), would affect the cellular phenotype of primary and immortalized fibroblasts. The successful engraftment of transformed dermal fibroblasts into decellularized rodent lung scaffold slices was shown before, whereas the authors of this study revealed

559 that the initial adhesion of the fibroblasts was reliant on β1-integrin and FAK by an ERK-dependent
560 pathway (52). By using 4D confocal live-cell imaging of fluorescently labeled immortalized mouse
561 fibroblasts (MLg) we could, for the very first time, directly observe the early engraftment of fibroblasts
562 into decellularized 3D-LTCs. After four hours of incubating the fibroblast with the decellularized 3D-LTCs,
563 the cells were already attached to the ECM of the 3D-LTCs and started to spread, in alveolar areas
564 obtaining an elongated morphology, and clearly showed activities of migration within the next four
565 hours. Interestingly, blade-cutting of the 3D-LTCs sometimes produced around large airways a
566 displacement of the ECM, which usually underlies the epithelial cell layer from the surrounding alveolar
567 area, leading to a dilated structure filled with a loose network of ECM. In these dilated areas fibroblasts
568 were able to attach but during the whole observation period of the time-lapse were neither spreading
569 nor started to migrate. These fibroblasts remained their spherical cellular morphology that they usually
570 adopt after trypsinization and as well as being in suspension in cell culture medium. At the magnification
571 (10x objective) used for 4D confocal live-cell imaging, we could not make out any delicate structures but
572 we speculate that the behavior of the fibroblasts in this specialized microenvironment might be close to
573 fibroblasts cultured in free-floating collagen matrices, in which the cells' morphologies were described
574 to be roundish and include the formation of a mixture of ruffles and filipodia at the cell margins (30).
575 After culturing primary human fibroblasts for five days on 3D-LTCs, we witnessed that engraftment of
576 the cells differed significantly within distinct ECM niches, exhibiting the highest cell numbers in
577 emphysematous, airway/vessel, and mesothelial regions, and low amount of cells in alveolar and fibrotic
578 areas. Such a finding would advocate for an impact on the fibroblasts' proliferation rate due to diverse
579 ECM properties in the various niches within the 3D-LTCs. Low tissue elasticity was reported to inhibit the
580 cell-cycle in fibroblasts and other cells as a widely conserved mechanism (38), and fibroblasts were
581 shown to tightly couple cell tension and proliferation in a Rho-Rock-myosin dependent manner below a
582 tissue stiffness (measured as Young's elastic modulus in pascals (Pa)) of 6 kPa (42). In the normal human
583 lung the physiological stiffness range is 0.2 - 2 kPa, whereas in fibrotic lungs derived from IPF patients
584 the tissue stiffness can reach values of up to 35 kPa (4). However in the mouse, stiffness mapping by
585 atomic force microscopy in non-decellularized tissue slices revealed a median stiffness of 0.5 kPa in
586 normal and 3 kPa in fibrotic lung (41). In line with these published work, we observed that the
587 fibroblasts displayed the highest proliferation rate when cultured on 2D-PDs, which usually have a
588 stiffness in the GPa range (9). Surprisingly, by using Ki67 immunofluorescence stainings, we could not
589 detect any statistically relevant effects on proliferation in mechanically stiff fibrotic regions, albeit we
590 detected a trend of higher cell numbers and higher amount of Ki67 positive cells in fibrotic compared to

591 normal alveolar regions, and the highest amount of Ki67 positive fibroblasts was detected in the
592 mesothelium. Thus, proliferation might be affected, but in our case were at least in a statistical sense
593 not assessable by Ki67 immunofluorescence stainings or, the initial adhesion of cells to different ECM
594 niches within the decellularized 3D-LTCs might lead to the identified increase in cell numbers in
595 emphysematous, airway/vessel, and mesothelial regions.

596 Correspondingly, by repopulating decellularized 3D-LTCS with fibroblasts, we found differences in
597 migration patterns as well as significant alterations in migration speeds in various ECM niches of the 3D-
598 LTCs. Remarkably, the lowest migration speeds were measured for fibroblasts that engrafted fibrotic
599 and the alveolar areas, and the fastest migrating fibroblasts were found in airway/vessels and the
600 mesothelium. However, when cultured on 2D-PD, the fibroblasts displayed the highest migration speed.
601 The migration of cells can be influenced by the physical properties (confinement, rigidity, adhesion, and
602 topology) of their complex 3D tissue environment and the cells' intrinsic biochemical state (protein
603 expression and signaling activity) (reviewed in (13)). Ultrastructurally, different regions of 3D-LTCs
604 (alveolar, fibrotic, emphysematous, airway/vessel, and mesothelium) were found to be considerable
605 different in their collagen and elastin fiber organization as well as in their topology (4, 24, 53). These
606 differences might partially account for the altered migration patterns that we observed in ECM niches.
607 Especially in the airway/vessel and mesothelium, the reseeded fibroblasts were migrating on the ECM's
608 surface in a highly directional, either parallel or antiparallel, fashion, thus following the course of the
609 airway/vessel and mesothelium. A special net orientation of fibers in the ECM could account for the
610 identified directional migration, although, as ultrastructurally demonstrated by scanning electron
611 microscopy, for instance the collagen network in the mesothelium consists of interwoven and
612 crisscrossed collagen fibers with no apparent net fiber orientation (53). Quite intriguingly, it was shown
613 for fibroblasts in *in vitro* micropattern-assay that migration speeds were highest for fibroblasts migrating
614 along a 1D fibrillar line (21). Thus, a similar topography might exist on the surface of the airway/vessel
615 and mesothelium niche, otherwise it remains obscure which properties of the ECM would contribute to
616 the directional migration of the fibroblasts in these ECM niches. Particularly in the fibrotic and alveolar
617 compartment, we observed a strong bias towards stationary fibroblasts, which was unexpected for the
618 fibrotic niche, as the increased stiffness due to fibrosis should have increased the overall migration
619 speed. However, by confocal 3D fluorescent microscopy we identified fibroblasts, which were
620 completely surrounded or embedded in the collagen matrix in either the alveolar or fibrotic niche,
621 respectively. In conclusion, we speculated that fibroblasts are rendered quiescent in the alveolar niche,
622 and slowly invading cells in the fibrotic niche. Thus, both observed states would contribute to an overall

reduced net migration speed in the alveolar as well as in the fibrotic niche. Interestingly, Southern et. al. reported that normal decellularized lung ECM promotes the motility of fibroblasts, while fibrotic ECM immobilizes fibroblasts mediated by non-muscle myosin II activities (51). This is in line with our data, as we also observed that fibroblasts that engrafted in the fibrotic niche display lower migration rates compared to normal alveolar regions (0.042 µm/min vs 0.062 µm/min ($p = 0.06$), respectively). Anyway, the underlying mechanisms of this observation might differ from each other, as Southern et. al. used 10 µm thick lung tissue sections, which is essential the thickness of a cell nucleus, whereas we used 300 µm thick 3D-LTCs. Apparently, in our 300 µm thick 3D-LTCs also repopulated also the inner parts of the thick tissue sections (compare figure 2a, figure 4a, figure 5d and supplementary movie3), whereas on 10 µm thick tissue sections fibroblasts assumingly only interact with the very surface of the slices but would not integrate into the tissue in a true 3D fashion. We also observed that after 5 days of recellularization the engraftment of fibroblasts in fibrotic and alveolar regions was much lower, compared to emphysematous regions, airway/vessel and the mesothelium, which further might reflect that fibroblasts in fibrotic and alveolar regions have to invade the ECM to reach the inner parts of the 300 µm thick lung tissue section, a process which would include proteolytic activity and might slow down the migration of fibroblasts.

Prominent cell-matrix adhesions are usually found as focal adhesions (FAs) in cells which are cultured on rigid plastic surfaces in conventional 2D-PDs. Apart from adhesive functions, FAs were shown to be involved in signaling, mechanosensing and cell migration, although their functional nature and even existence *in vivo* as well as in 3D culture models are still under heavy debate (22, 26, 39, 56). However, we demonstrated in our earlier work that fibroblasts cultured in a 3D collagen invasion model as well as in native and non-decellularized 3D-LTCs clearly formed adhesive structures reminiscent of FAs (7, 8). In the current study, we corroborated these results by 4D confocal fluorescence live-cell imaging as well as immunofluorescence 3D confocal microscopy, as fibroblasts which were repopulating the decellularized 3D-LTCs undoubtedly formed FAs. These findings go in line with data published by Sun et al. who demonstrated that the initial adhesion of fibroblasts during their engraftment in decellularized 3D-LTCs depends on the FAs component integrin-β1 (52). Above all, by immunofluorescence stainings against the FA component talin, we also found elongated talin-stained structures, which co-localized with fibronectin fibers in native, non-decellularized 3D-LTCs. This, together with our previously published results, strongly advocate for a physiological role of FAs in complex tissue environments, though at this point we cannot rule out that for instance serum in the culturing medium might cause an artificial effect

654 on the formation of FAs, as even the addition of serum was shown to influence cell contractility,
655 adhesion and assembly of FAs in fibroblasts (23).

656 Fibroblasts which are cultured in various ECM environments display a high-degree of morphological
657 plasticity (16). Usually, fibroblasts are conventionally defined, amongst other hallmarks, by their spindle-
658 shaped morphology, especially in tissue. Here, we demonstrated that fibroblasts adopted a variety of
659 morphological phenotypes, ranging from flat with either small or huge cell bodies to elongated and
660 spindle-shaped ones, depending on their engraftment in different niches of 3D-LTCs (alveolar, fibrotic,
661 emphysematous, airway/vessel, and mesothelium). Quite strikingly, fibroblasts, which engrafted in a
662 kind of 2D fashion on the surface of the mesothelium or airway/vessel, had a morphological phenotype
663 (flat cell bodies) similar to those fibroblasts which were growing on 2D-PDs. These data also correlated
664 well with migration speeds, which were highest for fibroblasts that engrafted ECM niches of
665 airway/vessel and the mesothelium, though the measured migration speeds were still significantly lower
666 compared to fibroblasts cultured on 2D-PDs. In contrast, in fibrotic and partially in alveolar regions, we
667 found fibroblasts with elongated, spindle-shaped and bipolar cell shapes, reminiscent of morphologies
668 of fibroblasts completely embedded in a collagen matrix (7, 36). Apart from elongated cell
669 morphologies, we found fibroblasts in the alveolar niche lining the inner part of the alveoli, developing
670 huge dome-like cell bodies with a morphological phenotype that was remarkably quite stable over time.
671 Conclusively, the engraftment of fibroblasts in native tissue matrices of 3D-LTCs can trigger a huge
672 plasticity in cellular morphology as a function of the ECM's compositional and/or physical properties.

673 By studying the transcriptome and translatome of primary fibroblasts that both were cultured on
674 decellularized ECM of either fibrotic or healthy lung, Parker and colleagues demonstrated that the
675 fibrotic ECM triggered the expression of fibrotic related proteins, the regulation of which was
676 preferentially directed on a translational level (46). We compared the expression of selected proteins of
677 primary human fibroblasts that were either cultured on 2D-PDs or 3D-LTCs. We observed a striking
678 differential protein expression for markers which play a role in mechanotransduction (YAP,
679 Tropomyosin), secreted ECM proteins (Fibronectin), components of FAs (Talin, Paxillin, Zyxin),
680 phosphorylated proteins (pPaxillin), and regulators of cellular migration (RhoA, Rac, CDC42, Src). Our
681 findings are also in line with reports that the cultivation of fibroblasts on 2D substrates compared to
682 either murine tissue-derived 3D matrices or cell-derived 3D matrices led to changes in the composition
683 of FAs and the phosphorylation status of proteins such as focal adhesion kinase (FAK) and paxillin (16). It
684 might be intriguing to figure out a method to spatially separate different ECM niches (alveolar, fibrotic,

emphysematous, airway/vessel, and mesothelium) in the 3D-LTCs, in order to further investigate in detail variations in the transcriptional/translational programs of fibroblasts as a function of the ECM. In light of that and to further increase the distinction of the niches, we applied laser dissection, though unfortunately this method was not convincingly working in our hands to cut the 300 µm thick 3D-LTCs.

Our findings demonstrated that culturing fibroblasts on native decellularized tissue matrices led to functional changes in cellular behavior, specifically in their engraftment, morphology, migration, protein expression, and phosphorylation patterns, as a consequence of the ECM's instructive properties. Furthermore, such functional changes might also have huge consequences for entire signaling pathways (28). Additionally, we also demonstrated, to the best of our knowledge for the very first time for d3D-LTCs, that reseeded fibroblasts attached to native (8) as well as to decellularized lung ECM via focal adhesions, which apparently are rich in the focal adhesion protein talin. It will be interesting to see in future studies, whether the functional properties of the 3D focal adhesions (size, numbers, dynamics, composition) in fibroblasts engrafted to various ECM niches are substantially different a) within the niches themselves, b) compared to focal adhesions/fibrillar adhesions found in 2D plastic dishes, and c) compared to 3D matrix adhesions found in collagen gel/cell-derived matrix embedded fibroblasts. Finally, we described that 300 µm thick d3D-LTCs have an intact architecture with various layers of alveoli, and that reseeded fibroblasts also repopulated the innermost regions of d3D-LTCs, and besides evidently homed to the interstitium.

Still, sodium dodecyl sulfate (SDS), the detergent used in the decellularization process, is known to ultrastructurally altering ECM molecules, removing glycosaminoglycans and growth factors (15), which all altogether could have some negative influences on cell behavior, although here we could not observe any obvious cytotoxic effects on reseeded fibroblasts in our experiments.

Taken together, it might be of high value and uttermost importance, especially for the pharmacological testing of compounds *in vitro*, to substitute traditional techniques of culturing cells for native tissue matrices, such as decellularized 3D-LTCs. For example in translational studies on interstitial lung diseases (ILD) and idiopathic pulmonary fibrosis (IPF)), primary human fibroblasts cultured on 2D plastic dishes are easily transdifferentiated into αSMA-expressing fibroblasts by treatment with TGFβ1 within 16 hours. However, in native non-ILD/IPF lung slices TGFβ1 treatment alone is not sufficient to induce a pathological αSMA expression. On the contrary, it takes a special pro-fibrotic cocktail, containing several pro-fibrotic mediators, to induce αSMA-expression starting after 48 hours (1).

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Author Contributions

GB, MK and OE conceived and initiated the research. GB, AS and SV performed all experiments. JB, GP and ML performed surgical work. GB, AS, SV, MK and OE analyzed the data. GB, AS, MK, SV and OE interpreted results of the experiments. GB and AS prepared the figures. GB drafted and wrote the manuscript. GB, AS, MK, JB and OE edited and revised the manuscript. All authors read the manuscript and discussed the interpretation of the results.

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884

885 # Figure legends

886

887 **Figure 1. Murine three-dimensional *ex vivo* tissue cultures (3D-LTCs) are effectively decellularized. (a)**
888 Hematoxylin/Eosin (HE) staining demonstrating the effective decellularization (dec) of 3D-LTCs derived
889 from PBS, Elastase and Bleomycin treated mice, compared to a native (nat) control 3D-LTC. Scale bar =
890 100 µm. **(b)** Immunofluorescence stainings depicting the efficient removal of cells from 3D-LTCs derived
891 from PBS (normal), Bleomycin (fibrotic) and Elastase (emphysematous) treated mice. In all images cell
892 nuclei are stained for DAPI (blue), extracellular Collagen 1 (green) and intracellular Actin stress-fibers
893 (red). Note that after the decellularization (dec), in comparison to the non-decellularized, native (nat)
894 3D-LTC, no fluorescent signals are visible for cell nuclei and Actin stress-fibers, though the staining for
895 the ECM-protein Collagen 1 remains intact. The white encircled area in the fibrotic panel indicates an
896 ECM-dense fibrotic region. Scale bar = 100 µm. **(c)** Western blot analysis exhibiting that there are no cell
897 specific proteins left after decellularization (dec) of 3D-LTCs derived from PBS (normal), Bleomycin
898 (fibrotic) and Elastase (emphysematous) treated mice, compared to native (nat) control 3D-LTCs. **(d)**
899 Confocal immunofluorescence microscopy of decellularized PBS 3D-LTCs showing the integrity of ECM
900 proteins after detergent treatment. Native (nat) and decellularized (dec) 3D-LTCs derived from PBS
901 (normal) treated mice were immunofluorescently labelled with antibodies against the interstitial ECM-
902 protein Collagen 1 (Col1). Note that, by comparing native (nat) and decellularized 3D-LTCs (dec), no
903 differences in the staining pattern or signal intensities for Col1 are visible. Very similar results were
904 obtained when antibodies for components of the basal lamina like Collagen 4 (Col4) and Laminin 5
905 (Lam5) were used. Confocal z-stacks are shown as maximum intensity projections. Scale bar = 50 µm. **(e)**
906 3D surface rendered confocal z-stack of a normal (=healthy) d3D-LTC immunofluorescently labeled for
907 Collagen 1 (grey). The graphical reconstruction shows various layers of entire alveoli and alveolar ducts,
908 demonstrating an intact lung ECM structure after decellularization. Scale bar = 50 µm. **(f)** Orthoview of a
909 confocal z-stack of a normal d3D-LTC immunolabeled for Collagen 1 (grey). The orthoview shows various
910 layers of entire alveoli and alveolar ducts, demonstrating an intact lung ECM structure after
911 decellularization. Scale bar = 50 µm.

912

913 **Figure 2. Engraftment of fibroblasts to the ECM of d3D-LTCs and differential protein expression**. **(a)** 4D
914 confocal live-cell imaging of the engraftment of MLg lung fibroblasts which where stained with the non-

toxic cell-tracker dye CMTPX (red). Live-cell imaging started 4 hours after stopping the rolling incubation of the d3D-LTCs in the cell suspension of fluorescently (CMTPX) labeled fibroblasts. The reseeded fibroblasts effectively attached to, spread and started to migrate on the decellularized 3D-LTCs. In the middle panel, 15 magnified frames (0 h 0 min, until 12 h 24 min) of a time-lapse movie (Supplementary Movie 2) indicate a spreading and migrating fibroblast (yellow arrow). Confocal z-stacks are shown as maximum intensity projections. Scale bars = 100 µm and 30 µm. The rightmost picture in the panel displays a 3D surface rendered confocal z-stack of a d3D-LTC that was repopulated with mouse lung fibroblasts (MLgs, stained for Phalloidin and depicted in red) and immunolabeled for Collagen 1 (yellow). The graphical reconstruction clearly demonstrates that fibroblasts occupy also niches deep within the d3D-LTCs. Scale bar = 70 µm. **(b)** The amount of engrafted primary human lung fibroblasts noticeably differed between distinct ECM niches (Alv = alveolar, Fibr = fibrotic, Emph = emphysematous; A/V = airway/vessel; MT = mesothelium) of decellularized 3D-LTCs. Alveolar and fibrotic regions exhibited low levels of cellular engraftment, whereas airway/vessels, the mesothelium and emphysematous regions where heavily populated after long-term cultivation (5 days). Cell nuclei were stained for DAPI (here depicted in white) and the ECM-proteins fibronectin (FN) and emilin-2 (E2) were immunofluorescently labelled (green). AF = autofluorescence signal of lung tissue. Confocal z-stacks are shown as maximum intensity projections. Scale bar = 100 µm. Statistics: one-way Anova with Bonferroni's multiple comparison test. **(c)** Ki67 staining (red) of MLg fibroblasts demonstrated that the cells proliferated significantly lower when cultured for 5 days on the 3D-LTCs compared to conventional 2D-PDs. Here, no significant differences were measured between distinct ECM niches (Alv = alveolar, Fibr = fibrotic, Emph = emphysematous; A/V = airway/vessel; MT = mesothelium) of decellularized 3D-LTCs. Cell nuclei were stained for DAPI (blue) and the ECM-protein fibronectin was immunofluorescently labelled (green). Scale bars = 100 µm. Statistics: one-way Anova with Bonferroni's multiple comparison test. **(d)** Immunoblot (two representative blots of an n=4 are shown) and its associated densitometric analysis, which demonstrate that the cultivation of pHFibs on 3D-LTCs significantly changes the expression of specific proteins when compared to the cultivation of the same cells in conventional 2D-PDs. The relative protein expression was normalized for each protein to its corresponding value measured for the 2D plastic dish condition (2D). YAP = yes-associated protein 1; TM = tropomyosin; Cph1 = calponin h1; αSMA = α-smooth muscle actin; Des = desmin; Cald = caldesmon; FN = fibronectin; Col1 = collagen 1. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Statistics: two-tailed paired t-tests. n=4 (4 different fibroblast cell lines derived from four patients).

946

947 **Figure 3. Anchorage of fibroblasts to 3D-LTCs by focal adhesion contacts (FAs) and differential protein**
948 **expression of distinct FA proteins. (a)** 4D confocal live-cell imaging of an MLg lung fibroblast. The
949 fibroblast ectopically expressed EGFP-αActinin1 and was found migrating on a decellularized 3D-LTC.
950 The yellow arrows point out streak-like structures reminiscent of focal-adhesion contacts (FAs) usually
951 found in fibroblasts and other cells cultured in conventional 2D-PDs. Four different frames (0 min, 19
952 min, 39 min, and 59 min) of a time-lapse movie are shown. The confocal z-stack is shown as a maximum
953 intensity projection. Scale bar = 10 µm. **(b)** Confocal 3D immunofluorescent microscopy of fixed primary
954 human fibroblasts, which repopulated a d3D-LTC for 3 days. The fibroblasts were double-
955 immunolabeled using antibodies to talin (green), a common FA marker, and fibronectin (red), a protein
956 of the ECM. Cell nuclei were stained for DAPI (blue). The yellow arrows identify streak-like structures
957 reminiscent of FAs, which are partially co-localizing with fibronectin fibers. The white rectangle in the
958 merged image indicates a magnified interior cell region which is displayed at the bottom of the panel.
959 Again, yellow arrows identify streak-like structures which are highly reminiscent of FAs. The confocal z-
960 stack is shown as a maximum intensity projection. Scale bars = 10 µm. **(c)** Confocal 3D fluorescent
961 microscopy of native 3D-LTCs. The 3D-LTCs were double-immunolabeled using antibodies to talin
962 (green) and fibronectin (red). Cell nuclei were stained for DAPI (blue). Note that also in native 3D-LTCs
963 streak-like structures (yellow arrows) can be found that are reminiscent of 2D adhesive components
964 (FAs, fibrillar adhesions) in cells cultured in 2D-PDs. Blue arrows indicate fibronectin fibers. The merged
965 image displays co-localizing talin-positive structures and fibronectin fibers. The confocal z-stack is
966 shown as a maximum intensity projection. Scale bar = 10 µm. **(d)** Immunoblot (two representative blots
967 of an n=4 are shown) and its associated densitometric analysis, which demonstrate that the cultivation
968 of primary human lung fibroblasts on 3D-LTCs significantly changes the expression of specific FA proteins
969 and also their phosphorylation status when compared to the cultivation of the same cells in
970 conventional 2D-PDs. The relative protein expression was normalized for each protein to its
971 corresponding value measured for the 2D plastic dish condition (2D). αAct = αActinin1; Tal = talin; Tns =
972 tensin; pPax = pPaxillinY118; Pax = paxillin; pZyx = pZyxinS142/143; Zyx = zyxin. * $p<0.05$; ** $p<0.01$; ***
973 $p<0.001$. Statistics: two-tailed paired t-tests. n=4 (4 different fibroblast cell lines derived from four
974 patients).

975

Figure 4. Morphological plasticity of fibroblasts as a function of their surrounding microenvironment. **(a)** 3D confocal live-cell imaging of MLg lung fibroblasts which ectopically express EGFP. Note that the fibroblasts adopt their morphology according to their surrounding microenvironment. Two fibroblasts (each denoted by one yellow arrow), which engrafted into an alveolar region, with distinct morphologies (flat and dome-like; elongated and spindle-shaped) are shown as isosurface rendered structures (red). Nuclei are rendered in blue and the autofluorescence of the decellularized 3D-LTCs' ECM is depicted in green. Scale bar = 10 µm. **(b)** 4D confocal live-cell imaging of an MLg lung fibroblast, which ectopically expresses EGFP, demonstrates that this cell does not migrate, though creates protrusions and largely keeps up its dome-like morphology over time, thus lining the inner side of an alveolus. Four different frames (0 h, 4 h, 8 h, and 16 h) of a time-lapse movie are shown. Scale bar = 10 µm. **(c)** The morphology of engrafted MLg lung fibroblasts dramatically differed between distinct ECM niches (2D = 2D plastic dish, Alv = alveolar, Fibr = fibrotic, Emph = emphysematous; A/V = airway/vessel; MT = mesothelium) of the decellularized 3D-LTCs. The fibroblast ectopically expressed EGFP and z-stacks were taken by 3D confocal live-cell imaging. The z-stacks were isosurface rendered (red) and views from two different angles (xy and xz) of the rendered fibroblasts are shown. Scale bar = 10 µm. **(d)** Quantitation and statistical evaluation of 3D cell shapes of mouse lung fibroblasts (MLg), which repopulated various ECM niches (2D plastic dish, alveolar, fibrotic, emphysematous, airway/vessel, mesothelium) in d3D-LTCs. Data shown represent mean values (± s.d.) from randomly chosen cells (n = 24-29). **(e)** Quantitation and statistical evaluation of cell volume (µm3) and cell surface area (µm2) of mouse lung fibroblasts (MLg), which repopulated various ECM niches (2D plastic dish, alveolar, fibrotic, emphysematous, airway/vessel, mesothelium) in the d3D-LTCs. Data shown represent mean values (± s.d.) from randomly chosen cells (n = 24-29). One-way Anova test $p < 0.0001$. Data shown as log10 values.

Figure 5. 3D-migration of lung fibroblasts in decellularized 3D-LTCs differs between distinct ECM niches and 2D plastic dishes. (a) 4D confocal live-cell imaging of HOECHST stained MLg lung fibroblasts that engrafted on decellularized 3D-LTCs for 72 hours. Different ECM niches (2D = 2D plastic dish, Alv = alveolar, Fibr = fibrotic, Emph = emphysematous; A/V = airway/vessel; MT = mesothelium) were chosen for imaging. Fibrotic areas, airway/vessel and part of the mesothelium are indicated by an encircled, red-dashed line. Note that plotting the measured migration speed of the fibroblasts against the straightness of the tracks exhibits different modes of migration. This indicates that migration of fibroblasts differs between distinct niches of the ECM. Every single line represents the track path of

every cell measured, indicating also the track length. Red rectangles indicate slowly migrating or stationary cells. The red arrows indicate an overall directed migration of the fibroblasts. Scale bar = 100 µm. **(b)** The automatic quantification of the migration speed of fibroblasts in various ECM niches (2D = 2D plastic dish, Alv = alveolar, Fibr = fibrotic, Emph = emphysematous; A/V = airway/vessel; MT = mesothelium) of 3D-LTCs shows an overall highly significant reduction in cellular migration on the 3D-LTCs in comparison to fibroblasts cultured on 2D plastic dishes. Fibroblasts engrafted in the airway/vessel or the mesothelium display a significantly higher migration speed than those found in the alveolar or fibrotic regions. Statistics: one-way Anova with Bonferroni's multiple comparison test; two-tailed paired t-tests. n=3 (biological replicates) **(c)** Immunoblot (two representative blots of an n=4 are shown) and its associated densitometric analysis, which demonstrate that the cultivation of primary human lung fibroblasts on 3D-LTCs significantly changes the expression of proteins, which are known as regulators of cellular migration, when compared to the cultivation of the same cells in conventional 2D-PDs. The relative protein expression was normalized for each protein to its corresponding value measured for the 2D plastic dish condition (2D). RhoA = RhoA; Rac = Rac1,2,3; CDC42 = CDC42; R1 = ROCK1. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Statistics: two-tailed paired t-tests. n=4 (4 different fibroblast cell lines derived from four patients). **(d)** Orthoview of a confocal z-stack of primary human fibroblasts that engrafted in alveolar regions. Nuclei were stained for DAPI (blue/white). Fibroblasts were stained with the cell-tracker dye CMTPX (red) and Collagen 1 (green/red). The yellow arrows indicate a fibroblast which is completely surrounded by Collagen 1, indicating that this fibroblast homed to the interstitium. Scale bar = 10 µm. Picture on the right: 3D surface rendered confocal z-stack of a d3D-LTC that was repopulated with mouse lung fibroblasts (MLgs, stained for Phalloidin and depicted in red) and immunolabeled for Collagen 1 (grey). The yellow arrow in the graphical reconstruction depicts one fibroblast that sits between alveolar structures (Av) in the interstitial space. Scale bars = 40 µm. **(e)** Orthoview of a confocal z-stack of primary human fibroblasts that engrafted in fibrotic regions. Nuclei were stained for DAPI (blue/white). Fibroblasts were stained with the cell-tracker dye CMTPX (red) and Collagen 1 (green/red). Note that in the dense fibrotic tissue fibroblasts might be completely surrounded by Collagen 1 (here in red), indicating that these cells (here in white) have invaded the ECM. Scale bar = 100 µm. The blue arrows in the orthoview to the right indicate an EGFP-expressing MLg lung fibroblast (green) with a spindle-shaped morphology completely surrounded by fibrotic ECM (red). Scale bar = 10 µm.

Supplementary information – movie legends

Movie 1. 360° animated graphical reconstruction of a d3D-LTC.

3D surface rendered confocal z-stack of a normal (=healthy) d3D-LTC immunofluorescently labeled for Collagen 1 (grey). The movie displays a 360° horizontal animation of the graphical reconstruction demonstrating various layers of entire alveoli and alveolar ducts. All in all, this clearly shows intact lung ECM architecture after decellularization. Scale bar = 50 µm.

Movie 2. Engraftment of fibroblasts to the ECM of d3D-LTCs

4D confocal live-cell imaging of the engraftment of MLg lung fibroblasts which where stained with the non-toxic cell-tracker dye CMTPX (red). Live-cell imaging started 4 hours after stopping the rolling incubation of the d3D-LTCs in the cell suspension of fluorescently (CMTPX) labeled fibroblasts. The reseeded fibroblasts effectively attached to, spread and started to migrate on the decellularized 3D-LTCs. Confocal z-stacks are shown as maximum intensity projections. Scale bar = 100 µm.

Movie 3. 360° animated graphical reconstruction of a fibroblast-repopulated d3D-LTC.

This movie displays a 360° horizontal animation of a 3D surface rendered confocal z-stack of a d3D-LTC that was repopulated with mouse lung fibroblasts (MLg lung fibroblasts, stained for Phalloidin and depicted in red) and immunolabeled for Collagen 1 (yellow). The graphical reconstruction clearly demonstrates that fibroblasts occupy also niches deep within the d3D-LTCs. Scale bar = 70 µm.

Movie 4. Anchorage of fibroblasts to 3D-LTCs by focal adhesion contacts (FAs).

4D confocal live-cell imaging of an MLg lung fibroblast. The fibroblast ectopically expressed EGFP-αActinin1 and was found migrating on a decellularized 3D-LTC. The red circles point out areas of streak-like structures reminiscent of focal-adhesion contacts (FAs) usually found in fibroblasts and other cells cultured in conventional 2D-PDs. The confocal z-stack is shown as a maximum intensity projection. Scale bar = 10 μm.

Movie 5. Morphological plasticity of fibroblasts as a function of their surrounding microenvironment.
3D confocal live-cell imaging of MLg lung fibroblasts which ectopically express EGFP. Note that the fibroblasts adopt their morphology according to their surrounding microenvironment. Two fibroblasts, which engrafted into an alveolar region, with distinct morphologies (flat and dome-like; elongated and spindle-shaped) are shown as isosurface rendered structures (red). Nuclei are rendered in blue and the autofluorescence of the decellularized 3D-LTCs' ECM is depicted in green. Scale bar = 30 μm.

Movie 6. Engraftment of fibroblasts to the ECM of d3D-LTCs.
4D confocal live-cell imaging of an MLg lung fibroblast, which ectopically expresses EGFP, demonstrates that this cell does not migrate, though creates protrusions and largely keeps up its dome-like morphology over time, thus lining the inner side of an alveolus. Scale bar = 20 μm.

Movie 7. 3D-migration of lung fibroblasts in decellularized 3D-LTCs differs between distinct ECM niches and 2D plastic dishes.
4D confocal live-cell imaging of HOECHST stained MLg lung fibroblasts that engrafted on decellularized 3D-LTCs for 72 hours. Different ECM niches (2D plastic dish, alveolar, fibrotic, emphysematous, airway/vessel, mesothelium) were chosen for imaging. Fibrotic areas, airway/vessel and part of the mesothelium are indicated by an encircled, red-dashed line.