MATERIALS AND METHODS

Ethical clearance statement

Primary cells were isolated from foreskin biopsies from juvenile donors (1 - 3 years). All donors' legal representative(s) provided full informed consent in writing. For scaffold preparation, porcine jejunal segments were obtained from German Landrace pigs; these animals received humane care in compliance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and the German Animal Welfare Act (last amended by Art. 3 G v. 28.7.2014 I 1308) after approval from the institutional animal protection board.

Cell isolation and culture

Human-derived keratinocytes, dermal fibroblasts and microvascular endothelial cells were isolated from foreskin samples as previously described (1, 2). Keratinocytes were subsequently cultured in EpiLife basal medium supplemented with HKGS (Human Keratinocyte Growth Supplement), and 1% penicillin/streptomycin (Fisher Scientific GmbH, Schwerte, Germany). Fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM, Fisher Scientific GmbH) with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) and 1% penicillin/streptomycin. Microvascular endothelial cells were cultured in VascuLife Basal medium supplemented with VascuLife EnGS LifeFactors (both Lifeline Cell Technology, Troisdorf, Germany) and 1% penicillin/streptomycin. To generate the vascularized human skin equivalents, keratinocytes were employed from first to second passage, fibroblasts and microvascular endothelial cells from third to fifth passage, once they reached 80-90% confluence.

Generation of a vascularized skin equivalent

The scaffold used to seed the human cells was prepared from a segment of porcine jejunum supplied by a single artery-vein pair with intact outer vascular system as previously described (Suppl. Fig. 1C) (3, 4). The jejunum segments were decellularized by perfusing the vasculature with sodium-desoxycholate at 100 mmHg (constant pressure). After sterilization with gamma irradiation, the matrix was stored at 4°C for up to one year. To create a matrix for the vascularized skin equivalent, the segment of jejunum was cut longitudinally on the antimesenteric side and fixed onto a polycarbonate frame. After equilibration in VascuLife EnGS Medium for 24 h in cell culture conditions, vascular channels in the matrix were reseeded with 1.2 x 10^7 human dermal microvascular endothelial cells in 1000 µL: 700 µL of cell suspension were injected in the arterial inlet and 300 µl in the venous inlet. After 48 h of incubation, the matrix was placed in a bioreactor system with its artery and vein connected to a fluidic circuit containing VascuLife EnGS, as previously described (2). With the matrix in the bioreactor, 3 x 10^6 human dermal fibroblasts were seeded on the matrix surface. After further 48 h of incubation, perfusion was started with a pressure of 10 mmHg, increased hourly by 10 mmHg up to 80 mmHg, and then changed to an oscillating pressure profile between 120 and 80 mmHg that resembles physiological human blood pressure. The pressure was measured and controlled at the arterial end of the skin equivalent during the whole culture period. Beside the circuit to the vascular system, two other circuits delivered medium to the surface and lower side of the submerged model, respectively, or later, in air-liquid interface conditions, ensured the air flow on top and medium flow below the skin model. At the following day, the cell culture medium in the fluidic system was replaced with EpiLife basal medium supplemented with HKGS (Thermo Fisher, Massachusetts, USA), 1.5 mM $CaCl_2$, 73 µL/mL 2-Phospho-L-ascorbic acid trisodium salt, 5 ng/mL keratinocyte growth factor and 1% penicillin/streptomycin (Sigma Aldrich, Steinheim, Germany). Four x 10^6 hEK were seeded on the surface and incubated for 24 h. After further 24 h of submerged perfusion, the

bioreactor was switched to air-liquid interface conditions that ensured sterile airflow on top of the skin model, promoting epidermal differentiation (5). Culture was continued until day 28 with medium change every 4-5 days. A tailor-designed incubator with programmable peristaltic pumps ensured the pressure control and standard cell culture conditions at 37°C and 5% CO_2 (Fig. 1F).

Optimization of the bioreactor design

The previously described bioreactor system was optimized to overcome difficulties in general handling and issues regarding sterility and leakproofness during culture (5). The shape of the bioreactor body was changed from a round to a rectangular shape (Fig 1B, C). To allow easy access to the cannulas of arterial and venous vessels of the skin equivalent during culture, the inner dimensions of the bioreactor were adjusted to fit the frame with the matrix and the vessels with approximately 7 cm length, with an additional length of 10 mm of the luer connectors where the vessels are connected for perfusion. The bioreactor body had 100 mm length and 70 mm width. The lid of the bioreactor was cast with polydimethylsiloxane (PDMS), thus being transparent and allowing a direct view inside the bioreactor during culture. At the same time, the PDMS lid provides a tight sealing without the need of extra o-ring seals, which would not have been the optimal choice of sealing for the rectangular shape.

The frame for carrying the matrix was not changed in basic dimensions compared to the old bioreactor setup. However, the frame was not placed horizontally into the bioreactor, but at an angled position of 15° to prevent air bubbles entrapment underneath the matrix (Fig. 1D). The highest point underneath the matrix was thus shifted to the very rim of the frame. Together with a bevel on this side of the lower part of the frame, air bubbles escaped from below the matrix to the open compartment of the bioreactor, or could be cleared away by slightly

swaying the bioreactor. An additional bevel on the lowermost rim of the upper part of the frame ensured proper drain of medium off the matrix, in case of leakages through the gaps between frame and bioreactor during airlift culture. To provide a horizontal matrix for cell seeding, the bioreactor was placed on a ramp that compensates the 15° inclination of the matrix. After incubation for cell attachment, the ramp was removed and the bioreactor was put back in regular orientation. Due to this change during cell seeding, the total height of the bioreactor was set to 56mm (without lid) to prevent overflow of medium when placed on the ramp.

The fluidic circuit to which the bioreactor was connected was slightly modified from the previous setup. As before, it included two glass bottles, one of 100 ml as a medium reservoir, one of 25 ml for pressure balance. A pressure sensor was connected to a pressure dome right before the arterial inlet of the bioreactor. To allow easier switching between submerged and airlift culture, two three-way-cocks were integrated into the circuit. Instead of connecting all tubes to the system from the beginning, the system could be reduced to two pump tubes for submerged culture, with addition of two pump tubes for airlift culture, thus providing easier handling during medium change.

Conversion of the vascularized skin equivalent to fibrotic skin and prevention of fibrosis

The vascularized human skin equivalent models were exposed to TGFβ (PeproTech, Hamburg, Germany) at a concentration of 10 ng/mL to generate fibrotic skin. Recombinant human TGFβ was added with each medium change (every 4-5 days) 1 week after switching to air-liquid interface. Nintedanib was chosen as a prototypical antifibrotic compound based on its antifibrotic effects on SSc fibroblasts, in different mouse models of SSc and in patients with SSc-ILD (6-8). Nintedanib was added at a final concentration of 1 µM (6). The final

DMSO concentration (the solvent of nintedanib) was 1/10000. Skin equivalents without TGFβ or nintedanib, but with DMSO in the same concentration served as control.

Histological analysis

Formalin-fixed, paraffin-embedded (FFPE) sections of human skin and vascularized human skin equivalent models (5 µm) were deparaffinized and stained with haematoxylin and eosin as previously described (9).

Immunohistochemistry and immunofluorescence stainings

For immunohistochemistry, FFPE sections of skin equivalents were used. For immunofluorescence, both FFPE- and cryo-sections from human skin and vascularized human skin equivalent models were employed.

The FFPE-sections were deparaffinized and for immunofluorescence stainings epitopes were subsequently retrieved using a heat-induced method. For epitope retrieval, sections were alternately bathed in boiling sodium citrate buffer (10 mM sodium citrate, pH 6.0) and Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0), each bathing step for 2 min up to a total of 10 min, followed by a cooling step of 20 min and a washing step in distilled water of 5 min (10).

For cryo-sections, tissues were placed in OCT compound (Tissue-Tek) (Newcomer Supply) and then snap-frozen in liquid nitrogen and cut to 7-µm slices. Sections were washed in distilled water after thawing.

Next, sections were blocked for 1 h in 2% BSA in PBS. Primary antibodies were incubated overnight at 4 °C, followed by an intense washing step and 2 hours incubation at room

temperature with HRP- or Alexa Fluor 488/555/647-conjugated secondary antibodies (Thermo Fisher, Massachusetts, USA). The slides were counterstained with haematoxylin (for immunohistochemistry) or DAPI (1:800, sc-3598, Santa Cruz Biotechnology) (for immunofluorescence stainings) (11). Cross-reactivity was minimized by pre-incubation with species-specific immunoglobulins. The following primary antibodies were used:

- for immunohistochemistry: anti-αSMA (A5228, Sigma-Aldrich, Steinheim, Germany)
- for immunofluorescence on cryo-sections: anti-laminin 5 (ab78286, Abcam, Cambridge, UK), anti-VSV (clone P5D4, SAB4200695, Sigma-Aldrich, Steinheim, Germany), anti-collagen 1 (ab138492, Abcam, Cambridge, UK), VSV- and HIS-tagged GD3A12 antibody anti-dermatan sulfate (from periplasmic fractions isolated from bacteria expressing the antibody) (12-14)
- for immunofluorescence on FFPE-sections: anti-CD31 (AF3628, R&D Systems, Minneapolis, USA), anti-pSMAD3 (ab52903, Abcam, Cambridge, UK), anti-prolyl-4-hydoxylase β (P4Hβ) (MA3-019, Thermo Fisher, Massachusetts, USA), anti-collagen 1 (ab138492, Abcam, Cambridge, UK), anti-keratin 10 (ab9025, Abcam, Cambridge, UK), anti-keratin 14 (HPA023040, Sigma Aldrich, Steinheim, Germany), anti-vimentin (ab92547, Abcam, Cambridge, UK) (10)

Stainings were visualized with a Nikon Eclipse Ni-U microscope (Nikon) and integrated fluorescence intensity was analyzed using Fiji (15). Voronoi diagrams were generated as previously described (16).

Quantitative PCR (qPCR)

Gene expression was quantified by SYBR Green real-time PCR using the ABI Prism 7300 Sequence Detection System (Life Technologies, Darmstadt, Germany) (17). Samples without

enzyme in the reverse transcription reaction (non-RT controls) were used as negative controls. Unspecific signals caused by primer-dimers were excluded by non-template controls and by dissociation curve analysis. The comparative threshold cycle (Ct) method was used for relative quantification. β-actin was used to normalize for the amounts of cDNA within each sample. Primer sequences were: *PAI1* fwd: TCA TTG CTG CCC CTT ATG A; *PAI1* rev: GTT GGT GAG GGC AGA GAG AG; *SMAD7* fwd: TAC TCC AGA TAC CCG ATG GAT T; *SMAD7* rev: TCT GGA CAG TCT GCA GTT GG; *ACTA2* fwd: AAG AGG AAT CCT GAC CCT GAA; *ACTA2* rev: TGG TGA TGA TGC CAT GTT CT; *COL1A1* fwd: ACG AAG ACA TCC CAC CAA TC; *COL1A1* rev: ATG GTA CCT GAG GCC GTT C; *COL1A2* fwd: GGT CAG CAC CAC CGA TGT C; *COL1A2* rev: CAC GCC TGC CCT TCC TT; *FIBRONECTIN* fwd: TTC TAA GAT TTG GTT TGG GAT CAA T; *FIBRONECTIN* rev: TCT TGG TTG GCT GCA TAT GC; *ACTB* fwd: AGA AAA TCT GGC ACC ACA CC; *ACTB* rev: TAG CAC AGC CTG GAT AGC AA.

Capillary Western immunoassay

Most reagents and the Wes™ equipment were purchased from ProteinSimple (Wiesbaden, Germany). Cell lysates were incubated at 95 °C for 5 min. Three microliters of each sample were loaded into the top rows of plates preloaded with electrophoresis buffers to separate proteins of 12–230 kDa. Subsequent rows were filled with blocking buffer, primary and secondary antibody solutions, chemiluminescence reagents, and wash buffer. Primary antibodies were anti-collagen type I (ab138492, Abcam, Cambridge, UK) and anti-β-actin (A5441, Sigma Aldrich, Steinheim, Germany). Secondary antibodies were anti-mouse secondary HRP conjugate and anti-rabbit secondary HRP conjugate.

Plates were loaded into the Wes machine, and assays were performed using the standard 12- to 230-kDa separation range. The Compass software (ProteinSimple, Wiesbaden, Germany) reports data as spectra of chemiluminescence signals versus apparent molecular weights; the

later are determined by mapping ladder peaks to capillary positions. Compass was also used to generate artificial lane view images from the spectra.

Data presentation and statistical analysis

Data were analyzed with GraphPad Prism 5 and are presented as mean x-fold value relative to the control group ± SEM. The differences between the groups were tested for statistical significance by the Mann-Whitney U non-parametric test. P values are represented as suggested by GraphPad Prism: $0.05>p>0.01$ as *; $0.01>p>0.001$ as **; $p<0.001$ as *** (values less than 0.05 were considered significant).

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