Single cell sequencing identifies serine proteases as regulators of myofibroblast differentiation

Vera Vorstandlechner

Medical University of Vienna

Maria Laggner

Medical University of Vienna

Dragan Copic

Medical University of Vienna

Yiyang Chen

Medical University of Vienna

Bahar Golabi

Medical University of Vienna

Werner Haslik

Medical University of Vienna

Christine Radtke

Medical University of Vienna

Erwin Tschachler

Medical University of Vienna

Hendrik Jan Ankersmit

Medical University of Vienna

Michael Mildner (✉ michael.mildner@meduniwien.ac.at)

Medical University of Vienna <https://orcid.org/0000-0002-6892-925X>

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1 **Single cell sequencing identifies serine proteases as regulators of myofibroblast differentiation**

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3

4 Abstract

5 Despite recent advances in understanding skin scarring, mechanisms triggering hypertrophic scar
6 formation are still poorly understood. In the present study we performed single-cell sequencing of
7 mature human hypertrophic scars and developing scars in mice.

8 Compared to normal skin, we found significant differences in gene expression in most cell types
9 present in scar tissue. Fibroblasts (FBs) showed the most prominent alterations in gene expression,
10 displaying a distinct fibrotic signature. By comparing genes upregulated in murine FBs during scar
11 development with genes highly expressed in mature human hypertrophic scars, we identified a group
12 of serine proteases, tentatively involved in scar formation. Two of them, dipeptidyl-peptidase 4
13 (DPP4) and urokinase (PLAU), were further analyzed in functional assays, revealing a role in TGF β 1-
14 mediated myofibroblast differentiation and over-production of components of the extracellular matrix
15 (ECM) without interfering with the canonical TGF β 1-signaling pathway.

16 In this study, we delineate the genetic landscape of hypertrophic scars and present new insights into
17 mechanisms involved in hypertrophic scar formation. Our data suggest the use of serine protease
18 inhibitors for the treatment of skin fibrosis.

19 **Significance statement**

20 Our study deepens the understanding of skin scarring, and provides unique biological insights in
21 tissue fibrosis. We provide data on the transcriptomic landscape of human hypertrophic scars on a
22 single-cell level and investigated scar maturation in vivo. Our study identified numerous genes
23 potentially contributing to scarring in various cell types. Especially a group of serine proteases have
24 been shown to play a key role in scar formation by interfering with non-canonical TGF β -signaling
25 and prevention of myofibroblast-differentiation. Targeting these proteases with inhibitors might
26 improve scar formation.

27

28

29 **Introduction**

30 Hypertrophic scars are a complex medical problem and a significant global disease burden ^{1,2}. In the
31 western world, an estimated number of 100 million people develop scars every year, approximately
32 11 million of which bear keloid scars and 4 million suffer from burn scars ¹. In the USA, an estimated
33 amount of 12 Billion Dollars is spent annually on the treatment of skin scarring ³. For affected
34 persons, a pathological hypertrophic scar causes significant functional impairment, pain, pruritus, and
35 a reduction in quality of life ^{4,5}.

36 Wound healing is a tightly coordinated, three-step process, characterized by an acute inflammatory
37 phase, a proliferative phase, and a remodeling phase. Prolonged inflammation results in increased
38 fibroblast (FB) activity, with enhanced secretion of transforming growth factor beta 1 (TGF β 1), TGF β
39 2, insulin-like growth factor (IGF1), and other cytokines ⁶. TGF β 1 drives differentiation of FBs into
40 myofibroblasts, which have a contractile phenotype, are characterized by excessive secretion of
41 ECM-components ⁷, and are the major contributors to the formation of hypertrophic scars ⁸. Mature
42 hypertrophic scars display strong tissue contraction ⁶, and dense, parallel or whorl-like ECM ⁸.

43 Topical silicone application, compression or massage therapy, laser ablation, surgery, and
44 intralesional injection of triamcinolone (TAC), corticosteroids, or 5-Fluorouracil (5-FU) are the most

commonly used options for prevention or treatment of hypertrophic scars^{6, 9, 10, 11}. However, many of these therapies lack evidence of efficacy and safety, and mechanisms of actions are still unclear^{10, 12}. Recently, several proteases became the focus of drug development in fibrotic diseases, as they have been shown to be involved in ECM-breakdown and the activation of growth factors in tissue remodeling^{13, 14}. Serine proteases/peptidases constitute a large, diverse group of proteases, divided into 13 clans and 40 families¹⁵. The group of trypsins comprises proteases contributing to vital processes such as blood coagulation, fibrinolysis, apoptosis and immunity¹⁶. Members of this family include urokinase, HTRA1/3 (high temperature requirement A1/3 peptidase), several coagulation factors and complement components, PRSS-like serine proteases, granzymes, and cathepsin G^{16, 17}. Inhibitors of PLA₂ have been shown to counteract fibrotic processes in cardiac and pulmonary fibrosis in human *in vitro* studies and in mouse experiments^{18, 19}. Recently, the serine protease DPP4 became the center of attention, since DPP4 inhibitors (gliptins) have been clinically used for the treatment of diabetes mellitus²⁰. DPP4 was also implicated in a variety of fibrotic pathologies, including cardiac, hepatic, renal, and dermal fibrosis^{21, 22, 23, 24, 25} and inhibition of DPP4 activity mitigated fibrotic processes in animal models^{18, 19, 26, 27, 28, 29}. However, the contribution to human scar formation and the underlying anti-fibrotic mechanisms are so far not known.

Here, we used the new approach of scRNAseq to thoroughly study gene expression and mechanisms involved in hypertrophic scar formation. We aimed to identify new genes regulated in scar tissue, and to uncover potential new targets for drug development towards scar-free wound healing or full reversion of a present scar.

Methods

Ethical statement

The Vienna Medical University ethics committee approved the use of healthy abdominal skin (Vote Nr. 217/2010) and of scar tissue (Vote Nr. 1533/2017) and all donors provided written informed consent. Animal experiments were approved by the Medical University of Vienna ethics committee and by the Austrian Federal Ministry of Education, Science and Research (Vote Nr. BMBWF-

71 66.009/0075-V/3b/2018) and performed in accordance with the Austrian guidelines for the use and
72 care of laboratory animals.

73 **Scar and skin samples**

74 Resected scar tissue was obtained from patients who underwent elective scar resection surgery (donor
75 information is provided in Table S1). Scars were classified as hypertrophic, pathological scars
76 according to POSAS ³⁰ by a plastic surgeon. Only mature scars, which had not been treated before
77 and persisted for more than two years were used for all experiments. All donors had no known chronic
78 diseases and received no chronic medication. Healthy skin was obtained from female donors between
79 25-45 years from surplus abdominal skin removed during elective abdominoplasty.

80 **Mouse full skin wounding and scar maturation**

81 Female Balb/c mice bred at the animal facility of the Medical University of Vienna (Himberg, Austria)
82 were housed under specific-pathogen-free conditions with 12h/12h light/dark cycles and food and
83 water access *ad libidum*. For full thickness skin wounds, mice were anesthetized with 100 mg/kg
84 Xylazin and 5 mg/kg ketamin (both Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally.
85 Postoperative analgesia was provided with 0.1 mg/kg Buprenorphin (Temgesic®, Indivior Inc., North
86 Chesterfield, VA, USA) subcutaneously and 0.125 mg/ml Piritramid (Janssen-Cilag Pharma, Vienna,
87 Austria) in drinking water *ad libidum*. A 9x9 mm² area was marked on shaved backs and excised with
88 sharp scissors. The wounds were left to heal uncovered without any further intervention. Mice were
89 sacrificed 6 or 8 weeks after wounding, and scar tissues were isolated.

90 **Single cell isolation and fluorescence-activated cell sorting (FACS)**

91 Four mm biopsies were taken from human skin, human scar, and from mouse scar tissue, and
92 enzymatically digested with MACS Miltenyi Whole Skin Dissociation Kit (Miltenyi Biotec,
93 Bergisch-Gladbach, Germany) for 2.5h according to the manufacturer's protocol. After processing on
94 a GentleMACS OctoDissociator (Miltenyi), cell suspensions were passed through a 70 µm and a 40
95 µm filter and stained with DAPI nuclear dye. Cells were sorted on a MoFlo Astrios high speed cell

96 sorting device (Beckman-Coulter, Brea, CA, USA), and only DAPI-negative cells, representing
97 viable cells, were used for single cell RNAseq.

98 **Generation of single-cell gel-bead in emulsions (GEMs) and library preparation**

99 Immediately after sorting, viable cells were loaded on a 10X-chromium instrument (Single cell gene
100 expression 3'v2/3, 10X Genomics, Pleasanton, CA, USA) to generate GEMs. GEM-generation,
101 library preparation, RNA-sequencing, demultiplexing and counting were done by the Biomedical
102 Sequencing Core Facility of the Center for Molecular Medicine (CeMM, Vienna, Austria).
103 Sequencing was performed on an Illumina HiSeq 3000/4000 (Illumina, San Diego, CA, USA) with 3
104 samples per lane, 2x75bp, and paired-end sequencing.

105 **Cell-gene matrix preparation and downstream analysis**

106 Raw sequencing files were demultiplexed, aligned to the human or mouse reference genome
107 (GrCh38/ mm10) and counted using the Cellranger pipelines (Cellranger v3, 10X Genomics). The
108 resulting cell-gene matrices were processed using the 'Seurat'-package (Seurat v3.1.0, Satija Lab,
109 New York, NY, USA) in R-studio in R (R v3.6.2, The R Foundation, Vienna, Austria). From each
110 sample, unwanted variations and low-quality cells were filtered by removing cells with high and low
111 (>3000 and <200) Unique Molecular Identifier (UMI)-counts. First, healthy skin and scar samples
112 were integrated separately to avoid clustering according to donors, and for batch correction.
113 Subsequently, skin and scar data were integrated again into one dataset. Data integration was
114 performed according to the recommended workflow by Butler *et al.* and Stuart *et al.* ^{31, 32}. After
115 quality control comparing all donors, we obtained transcriptome data from a total of 25.083 human
116 skin and scar cells, with a median of 24943 reads and 851 detected genes per cell. In mice, we obtained
117 data from 6561 cells 6 weeks after wounding, and 9393 cells 8 weeks after wounding. The samples
118 displayed a median of 24774 reads per cell, and median of 1969 detected genes per cell. After quality
119 control, all mouse samples were integrated together in one integration step. In both datasets,
120 normalized count numbers were used for differential gene expression analysis, for visualization in
121 violin plots, feature plots, dotplots and heatmaps, when displaying features that vary across

conditions, as recommended by current guidelines ³³. In both datasets, cell types were identified by well-established marker gene expression (Figure S1A, Figure S4A). For identification of differentially expressed genes (DEGs), normalized count numbers were used, including genes present in the integrated dataset to avoid calculation of batch effects. As keratin and collagen genes were previously found to contaminate skin biopsy datasets and potentially provide a false-positive signal ³⁴, these genes (*COL1A1*, *COL1A2*, *COL3A1* and *KRT1 KRT5*, *KRT10*, *KRT14*, *KRTDAP*) were excluded from DEG calculation in non-fibroblast clusters (collagens) or non-keratinocyte clusters (keratins), respectively. Moreover, genes *Gm42418*, *Gm17056* and *Gm26917* caused technical background noise and batch effect in mouse scRNAseq, as described before ³⁵, and were thus excluded from the dataset.

Pseudotime analyses

Pseudotime analyses, trajectory-construction and calculation of pseudotime-dependent gene expression were performed in Monocle 2 (Monocle2, v2.14.0, Trapnell Lab, University of Washington, Seattle, WA, USA) ^{36, 37}. From the integrated FB subset Seurat-object, data were converted into a monocle-compatible CellDataSet. Analysis was then performed according to the recommended pipeline. Cells with mRNA counts two standard deviations above or below the mean were excluded. Size factors and dispersions were estimated, tSNE-reduction and clustering were performed ^{36, 37, 38}. As input for pseudotime ordering, differentially expressed genes between skin and scar were used, and trajectories were constructed with DDRTree (R-package ‘DDRTree’ v0.1.5, by Qi Mao, Li Wang *et al.*, 2015)³⁸.

STRING functional protein interaction networks

Protein interaction networks were generated by imputing a list of genes (e.g. upregulated genes in FBs scar vs skin) into the String v11.0 online tool ^{39, 40} (<https://string-db.org/>, String consortium, Swiss Institute of Bioinformatics/EMBL Heidelberg/University of Zurich/Novo Nordisk Foundation CPR).

Gene ontology (GO)-networks

148 Gene lists of significantly regulated genes (adjusted p-value <0.05, average log fold change
149 [avg_logFC] >0.1) were imputed in ClueGO v2.5.5⁴¹ plug-in in Cytoscape v3.7.2⁴² with medium
150 GO-specificity, with GO-term fusion, and only significant (*P* value <.05) GO-terms are depicted as
151 circles, whereby circle size correlates with *P* value, and lines represent functional connection of
152 respective GO-terms.

153 **Immunofluorescence stainings**

154 Immunofluorescence staining on formalin-fixed, paraffin-embedded (FFPE) sections of skin and
155 scar tissue were performed as described previously⁴³. Antibodies were used as indicated in Table S2.

156 **RNAScope in situ hybridization**

157 FFPE-sections of human skin and scar tissue were prepared according to RNAScope (ACDBio, Bio-
158 Techne, Bristol, UK) pre-treatment protocol, hybridized with probes targeting human DPP4
159 (RNAScope® Probe-Hs-DPP4) and PLAU (RNAScope® Probe-Hs-PLAU), and visualized with
160 RNAScope 2.5 HD Assay – RED as suggested by the manufacturer. Images were acquired by AX70
161 microscope (Olympus, Tokyo, Japan) using the imaging software MetaMorph (Olympus).

162 **Isolation of primary skin fibroblasts**

163 Five mm biopsies were taken from fresh abdominal skin, washed in phosphate-buffered saline (PBS),
164 and incubated in 2.4 U/ml Dispase II (Roche, Basel, Switzerland) overnight at 4°C. The next day,
165 epidermis was separated from dermis, and dermis was incubated with Liberase TM (Merck Millipore,
166 Burlington, MA, USA) in Dulbeccos modified eagle medium (DMEM, Thermo Fisher Scientific,
167 Waltham, MA, USA) without supplements at 37°C for 2h. Next, the dermis was passed through 100
168 µm and 40 µm filters, rinsed with PBS, and cells were plated in a T175 cell culture flask. Medium
169 was changed the next day, and then every other day until FBs reached 90% confluency. First passage
170 FBs were used for TGFβ1-stimulation experiments.

171 **TGFβ 1-induced myofibroblast differentiation**

172 After the first passage, isolated primary FBs were plated in 6-well plates, supplied with DMEM +
173 10% fetal bovine serum (FBS, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo
174 Fisher Scientific) and grown until 100% confluency. FBs were then stimulated with 10ng/ml TGFβ1
175 (HEK-293-derived, Peprotech, Rocky Hill, NJ, USA), and with or without DPP4-inhibitor Sitagliptin
176 (10μM) (Thermo Fisher Scientific) or urokinase-inhibitor BC-11 hydrobromide (10μM) (Tocris by
177 Bio-Techne, Bristol, UK) for 24h. Supernatants were removed and medium and inhibitors were
178 resupplied for another 24h. Supernatants were collected and stored at -80°C and cells were lysed in
179 1x Laemmli Buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for further analysis.

180 To analyse signaling pathways, FBs were stimulated with TGFβ1 and inhibitors for 1h, and then
181 harvested in 1x Laemmli Buffer with protease inhibitor (cOmplete, MiniProtease Inhibitor Cocktail
182 Tablets, Roche, Basel, Switzerland) and phosphatase inhibitor (Pierce™Phosphatase Inhibitor Mini
183 Tablets, Thermo Scientific, Waltham, MA, USA).

184 **Western blotting**

185 Primary FBs were lysed in 1x Laemmli Buffer (Bio-Rad Laboratories, Inc.) and loaded on 4-15%
186 SDS-PAGE gels (Bio-Rad Laboratories, Inc.). Proteins were transferred on a nitrocellulose membrane
187 (Bio-Rad Laboratories, Inc.), membranes were blocked in non-fat milk with 0,1% Tween 20 for 1h
188 (Sigma-Aldrich), and incubated with antibodies as indicated in Table S2 at 4°C overnight. After
189 washing, membranes were incubated with horseradish-peroxidase conjugated secondary antibodies
190 as indicated in Table S2 for 1h at room temperature. Signals were developed with SuperSignal West
191 Dura substrate (Thermo Fisher Scientific) and imaged with a Gel Doc XR+ device (Bio-Rad
192 Laboratories, Inc.).

193 **Proteome profiling of signaling pathways**

194 To analyse signaling pathways, we used a proteome profiler for human phospho-kinases (ARY003C,
195 R&D Systems, Biotechne, Minneapolis, MN, USA) according to the manufacturer's instructions.

196

197 **Enzyme-linked Immunosorbent assay (ELISA)**

198 Human pro-collagen Ia1 ELISA (R&D Systems) and human fibronectin ELISA (R&D Systems) were
199 performed with supernatants of TGF β 1-stimulated FBs according to the manufacturer's manual.
200 Absorbance was detected by FluoStar Optima microplate reader (BMG Labtech, Ortenberg,
201 Germany).

202

203 Results

204 The single-cell landscape of hypertrophic scars

205 To elucidate the complex biological processes of scar formation, we performed droplet-based single-
206 cell transcriptome analysis of human hypertrophic scar tissue and healthy skin⁴⁴ (Figure 1A). In both
207 samples, Unsupervised Uniform Manifold Approximation and Projection (*UMAP*)-clustering
208 revealed 21 cell clusters, which were further classified as specific cell types by well-established
209 marker genes (Figure S1A), expression patterns of all clusters (Figure S1B), and transcriptional
210 cluster proximity *via* a phylogenetic cluster tree (Figure 1B). We found seven FB clusters, smooth
211 muscle cells and pericytes (SMC/Peri), three clusters of endothelial cells (EC), and lymphatic
212 endothelial cells (LECs), two clusters of T-cells and of dendritic cells (DC), macrophages (Mac),
213 three keratinocyte (KC) clusters, and melanocytes. All cells of specific subsets were clustered
214 together, and skin and scar samples displayed comparable cellular cluster composition (Figure 1C,
215 D). Only cluster FB1 was mainly present in scar tissue. The clusters of skin and scars showed different
216 relative cell number ratios (Figure 1E, F). Whereas FBs represented 40% of all cells in healthy skin,
217 a significant increase (53%) was observed in scar tissue. Similarly, we detected more ECs (16,31%)
218 in scar tissue as compared to normal skin (8,1%). Contrary, the relative numbers of KCs (5,97%) and
219 macrophages (4,42%) in mature hypertrophic scars were significantly reduced compared to skin
220 (18,97% and 7,11%).

221 To identify the strongest differences in gene expression between normal skin and scars, we calculated
222 all up- and downregulated genes for FBs, PCs, ECs, T-cells, DCs and KCs (Figure S2). In general,
223 we identified considerably more up- (Figure 1G) than downregulated genes (Figure 1H), and the most
224 abundant differential gene expression of scar compared to skin (number of differentially expressed
225 genes, nDEG) was found in FBs, SMC/pericytes, macrophages, DC1 and KC1 (Figure 1G, H). Genes
226 related to ECM production were mainly overrepresented in FBs, but notably also in PCs and ECs
227 (Figure S2A-C). Several significantly regulated genes with so far undescribed roles in fibrosis and

228 scar formation were found in all cell types (Figure S2A-F). These distinctly regulated genes might
229 provide valuable new candidates to understand and modulate skin scarring.

230

231 **The fibrotic gene expression pattern of fibroblasts in hypertrophic scars.**

232 Since FBs showed strongest gene regulation in our scRNAseq dataset, and have been considered as
233 the major drivers of skin scarring and an important source for myofibroblasts⁷, we focused our further
234 analysis on differences between FBs of healthy skin and hypertrophic scars (Figure 2).

235 After subsetting and reclustering of all FBs, we identified 11 separate clusters (Figure 2A-C) showing
236 110 significantly up- and 85 downregulated genes in FBs derived from scar tissue compared to healthy
237 skin. The top 50 differentially up- and downregulated genes are shown in Figure 2D. Interestingly,
238 one FB cluster (FB1) was almost exclusively present in hypertrophic scars, suggesting a specific role
239 in tissue fibrosis. Comparison of FB cluster 1 to all other scar FBs revealed 141 significantly up- and
240 179 downregulated genes. The top 50 differentially up- and downregulated genes are shown in Figure
241 2E. Most of the upregulated genes in scar-derived FBs are well-studied in the context of skin scarring
242 and are functionally related to collagens and ECM-modifying genes (Figure S3A, B). However, the
243 interaction on protein level of several genes found here (Figure S3A, B, bold) was hitherto not studied
244 with regard to scar formation.

245 Analysis of the biological processes associated with differentially regulated genes between FB1 and
246 other FB clusters by gene ontology network analysis revealed a strong association of FB1 with TGFβ-
247 signaling (red circles) and ECM-formation (purple circles) (Figure 2F), further corroborating its role
248 in skin fibrosis. In addition, our analysis indicates a role of FB1 in processes important for several
249 other cell types, including platelets, smooth muscle cells and cells of the skeletal system, suggesting
250 paracrine actions of FB1.

251 Pseudotime calculation and trajectory construction effectively identifies possible cell fates and time-
252 regulated genes, even when analyzing cells of only one timepoint^{36, 38}. Thus, we next sorted human

253 skin and scar FBs along a pseudotime axis and constructed trajectories (Figure 3A, B). The
254 trajectories revealed a division at a certain time point where FBs divided into two branches (Figure
255 3C). Whereas the majority of FBs preferentially aligned with branch 1 in normal skin (Figure 3D),
256 we observed a significantly longer branch 2 with FBs of hypertrophic scar tissue (Figure 3E). Genes
257 most regulated in a pseudotime-dependent manner in normal skin and hypertrophic scars are shown
258 in Figure 3F and 3G. Interestingly, the collagens *COL1A1*, *COL1A2* and *COL3A1*, known to
259 contribute to all fibrotic processes, are most upregulated at the end of skin pseudotime, but are not
260 among the most pseudotime-regulated genes in scar (Figure 3F,G). In contrast, *COL5A1/2*, *COL8A1*,
261 *COL11A1* and *COL12A1*, dominated the late pseudotime-dependent gene expression in scars. The
262 role of these collagens in (hypertrophic) scar is scarcely investigated, and merits further exploration.
263 Together, our trajectory analysis models the temporal dynamics of gene expression in scars and might
264 provide a basis to target respective genes at different stages of scar development.

265 **scRNAseq of murine scars identifies genes involved in scar maturation**

266 As our approach so far only gave information on the current state of mature scars, we further
267 investigated mechanisms leading to scar formation and maturation, using a murine full thickness skin
268 wound model. Whereas scar formation and maturation in humans is a long lasting process ⁴⁵, it only
269 takes up to 80 days in rodents ⁴⁶. Hence, we compared scar formation in mice 6 and 8 weeks after
270 wounding (Figure 4A). Analogously to the human dataset, all cells were clustered, and cell types were
271 identified using established marker genes (Figure S4A), expression patterns of all clusters (Figure
272 S4B) and transcriptome proximity of clusters *via* a phylogenetic cluster tree (Figure 4B). All clusters
273 aligned homogeneously, and all major skin cell types were represented at both time points (Figure
274 4C, D).

275 In accordance with human scar tissue, 8 weeks old mouse scars contained a higher proportion of
276 murine FBs (mFBs) (32,6%) compared to 6 weeks old scars (17,39%), and more DCs (9,6 versus
277 6,3%). In contrast, less pericytes and endothelial cells (2,8 versus 1,5%) and less keratinocytes (63,3
278 vs 45%) were present (Figure 4E, F). We next calculated up- and downregulated genes for FBs, PCs,

279 ECs, T-cells, DCs and KCs, comparing 8 weeks to 6-week-old scars (Figure S5A-F). In contrast to
280 human scars, the highest number of differentially expressed genes was found in mFBs and mKCs
281 (Figure 4G,H), which was most likely due to ongoing epidermal tissue regeneration. Surprisingly, the
282 expression of collagens did not change significantly between 6 and 8 weeks in mFBs, and only mECs
283 showed a slightly increased expression of collagens (Figure S5A-C). However, expression of several
284 matricellular proteins and other ECM-components, e.g. *Fbln1* (Fibulin1), *Ogn* (osteoglycin), *Lum*
285 (Lumican), and *Pcolce* (Procollagen C-Endopeptidase Enhancer) increased in mFBs during scar
286 maturation (Figure S5A). Together, our scRNAseq identified a gene profile specific for scar
287 maturation in mice.

288

289 **Serine proteases are strongly upregulated during scar maturation**

290 To identify genes that are crucial for scar maturation, we next compared our human scar data set with
291 genes upregulated in mouse scars 8 weeks after wounding in comparison to mouse scars 6 weeks after
292 wounding (Figure 5A). While in both data sets only one gene (*LEPR*) was downregulated, 16 genes
293 were mutually upregulated (Figure 5B-D). Stunningly, 5 of these genes (*AEBP1*, *DPP4*, *HTRA1*,
294 *PLAU* and *PRSS23*) were members of the superfamily of serine proteases (Figure 5B, C). All five
295 serine proteases were upregulated in scRNAseq in human scar tissue, particularly in FBs, but also in
296 other cell types (Figure 5E-I). *AEBP1* and *PRSS23* expression also increased in ECs and melanocytes,
297 *HTRA1* in ECs and KC3, and *PLAU* in DCs. Several additional serine proteases, *HTRA3* (High-
298 Temperature Requirement A Serine Peptidase 3), *DPP7* (Dipeptidyl-Peptidase 7), *FAP* (Fibroblast
299 activation protein alpha), were upregulated in human scars (Figure S6), and also showed a trend in
300 mouse scars.

301 Analysis of these serine proteases by pseudotime trajectories in human FBs revealed that their
302 expression mainly increased over time and *AEBP1* and *HTRA1* significantly enriched at the end of
303 branch 2 (Figure 6A,C). Together, these data suggest a major role of serine proteases in scar formation
304 and/or maturation.

305 **The serine proteases DPP4 and urokinase regulate TGFβ1-mediated myofibroblasts** 306 **differentiation and ECM over-production**

307 We next wanted to investigate the contribution of the identified serine proteases to scar formation.
308 Since specific inhibitors are commercially available only for DPP4 and urokinase, we focused our
309 further functional studies on these two serine proteases. First, we corroborated our scRNAseq data by
310 analyzing RNA and protein expression of DPP4 and urokinase (*PLAU*) using in-situ hybridization
311 (Figure 7A, B), and immunofluorescence stainings (Figure 7C, D), respectively. Both methods
312 showed significantly higher expression of DPP4 and PLAU in hypertrophic scars as compared to
313 healthy skin. In addition to dermal FBs, *PLAU* was also induced in the epidermis of scar tissue. As
314 we have previously identified a strong upregulation of *PLAU* in scars in DC1 (Figure 5H), the positive
315 cells in the epidermis most likely represent Langerhans cells.

316 As TGFβ1 is one of the key inducers of scarring and tissue fibrosis, causing differentiation of FBs to
317 pro-fibrotic myofibroblasts^{19, 47, 48, 49}, we hypothesized that the serine proteases interact with TGFβ-
318 signaling. To test this, we stimulated primary FBs from healthy abdominal human skin with active
319 TGFβ1, which induces expression of alpha-smooth muscle actin (αSMA), a marker for
320 myofibroblasts. Addition of the DPP4 inhibitor Sitagliptin and the urokinase inhibitor BC11 almost
321 completely abolished the TGFβ1-mediated myofibroblast-differentiation as shown by αSMA Western
322 blot (Figure 8A, B). Moreover, Sitagliptin and to an even greater extent BC-11 attenuated TGFβ1-
323 induced overproduction of the ECM-proteins Colla1 (Figure 8C, D), and fibronectin (Figure 8E, F)
324 by FBs. These results suggest that serine proteases are involved in the TGFβ1-induced myofibroblast
325 differentiation.

326

327 **The serine proteases DPP4 and urokinase do not interfere with canonical TGFβ1-signaling**
328 **pathways**

329 To investigate whether the serine protease inhibitors interfere with TGFβ1 signaling, we investigated
330 the TGFβ1-induced signaling pathways SMAD2 and ERK1/2⁵⁰. While SMAD2 and ERK1/2
331 phosphorylation was readily induced upon stimulation with TGFβ1, addition of the inhibitors showed
332 no effects (Figure 9A). In contrast, SMAD1, 5 and 9 showed no activation after TGFβ1 stimulation
333 (Figure 9A). To further identify other signaling pathways that might be involved in the action of the
334 serine protease inhibitors, we used a signaling proteome profiler, showing that none of the signaling
335 molecules were inhibited by the inhibitors (Figure 9B). Interestingly, the GSKα/β-pathway, known to
336 attenuate fibrotic processes in the heart⁵¹ was significantly activated by BC-11 (Figure 9B, Figure
337 S7), suggesting a counter-regulatory action. Together, these data suggest that sitagliptin and BC-11
338 do not interfere with canonical or known non-canonical TGFβ1 signaling.

339

340 Discussion

341 Although skin fibrosis has been extensively studied, key mechanisms leading to the development of
342 hypertrophic scars are still not well understood. In addition, treatment options to prevent or treat
343 (hypertrophic) scars are still scarce ⁵² and not exceptionally effective. In the present study, we used
344 scRNAseq to elucidate the genetic landscape of hypertrophic scar tissue at a hitherto unmet single
345 cell resolution. We found numerous regulated genes not yet investigated in scar formation and
346 identified serine proteases as regulators of TGF β -induced myofibroblast differentiation.

347 As expected, our scRNAseq analysis confirmed a plethora of previous studies, but also identified
348 numerous genes, which have so far not been described in the context of skin scarring or tissue fibrosis.
349 For example, the cytokines *MDK* (midkine) and *PTN* (pleiotrophin), both involved in cell growth,
350 migration, and angiogenesis ⁵³, were strongly upregulated in scar FBs. In contrast, *SOD2/3*
351 (superoxide dismutase 2/3), an enzyme controlling the release of reactive oxygen species (ROS),
352 hence acting as important antioxidant ⁵⁴, was strongly downregulated in scar FBs. Interestingly,
353 failure of ROS-scavenging has already been shown to contribute to hypertrophic scar formation ⁵⁵.
354 Another interesting and significantly down-regulated gene in scars was *SFN* (stratifin). As stratifin
355 has been identified as potent collagenase-stimulating factor in FBs, its down-regulation in scars
356 suggests a contribution to the maintenance and/or progression of the fibrotic phenotype by preventing
357 matrix degradation. These and many other novel factors identified in our study could be important,
358 decisive molecules for the development and/or maturation of hypertrophic scars. Thus, our study has
359 built a basis for future studies describing the role of these molecules in skin scarring and tissue
360 fibrosis.

361 Our combined study of human mature hypertrophic scars and scar maturation in mice identified a
362 group of serine proteases as key player for scar development and maturation. Although, DPP4-
363 positive FBs have previously been identified as the main source of ECM production in the skin ⁵⁶,
364 and urokinase has been shown to be involved in lung fibrosis ¹⁹, their roles in myofibroblast
365 differentiation and production of ECM are still unclear. Our finding that addition of specific DPP4

366 and urokinase inhibitors to TGFβ1-stimulated FBs almost completely inhibited myofibroblast
367 differentiation and up-regulation of matrix proteins was striking. Sitagliptin, the here used DPP4
368 inhibitor, is an effective drug widely used for the treatment of diabetes mellitus⁵⁷. Based on our study,
369 it might be very interesting to systemically evaluate differences in scar formation and scar quality of
370 diabetic patients treated with either gliptins or other drugs. Furthermore, topical application of
371 gliptins, during the phase of tissue remodeling after full wound closure, might prevent (hypertrophic)
372 skin scarring. As gliptins are already approved for clinical use, an off-label topical application would
373 be a promising step forward to test its efficacy on skin scarring *in vivo*. Interestingly, the urokinase
374 inhibitor BC-11 showed an even stronger effect on inhibiting TGFβ1-induced myofibroblast
375 differentiation and matrix production. However, so far it has only been used *in vitro*, and further *in*
376 *vivo* testing for efficacy and safety is still required. Inhibition of urokinase to attenuate tissue fibrosis
377 *per se* might appear counterintuitive, as urokinase facilitates fibrinolysis and regulates ECM-turnover,
378 eliciting anti-fibrotic action⁵⁸. However, literature on urokinase inhibitors and fibrosis is
379 contradictory. The best investigated endogenous urokinase-inhibitor, plasminogen activator inhibitor-
380 1 (PAI-1, *SERPINE1*), was found to cause excessive matrix deposition after injury⁵⁹. By contrast,
381 and in line with our results, inhibition of urokinase by PAI-1 suppressed profibrotic response in FBs
382 from fibrotic lungs and prevented cardiac fibrosis in mice¹⁸. Therefore, our study suggests the use of
383 urokinase inhibitor BC-11 as a possible new therapeutic strategy for the treatment of skin scars.
384 Further studies are necessary to fully elucidate its efficacy *in vivo*.

385 Strikingly, our analyses revealed no influence of the inhibitors on the canonical TGFβ1 signaling
386 pathway. As to the anti-fibrotic action of sitagliptin, we have no mechanism to offer yet. Although
387 DPP4 inhibition has previously been demonstrated to directly inhibit canonical TGFβ signaling *via*
388 Smad2 in renal fibrosis⁶⁰ and TGFβ-mediated myoFB-differentiation by interfering with ERK-
389 signaling⁶¹, we were not able to confirm these mechanisms in skin FBs. Possible explanations for
390 alternative the anti-fibrotic action of sitagliptin could be interactions with CXCL12/SDF1 (Stromal
391 derived factor 1), a substrate of DPP4⁶² which was found to promote scar formation⁶³, or with

392 components of the ECM, such as fibronectin ⁶², which have been shown to bind latent TGFβ ¹⁹, or
393 DPP4 induced cleavage of growth factors or receptors ⁶². Deciphering these interactions will be the
394 scope of further mechanistic studies. Regarding BC-11, we found a significant activation of GSK3α/β
395 in TGFβ1-stimulated FBs. Since GSK3β was previously found to interact with WNT/β-catenin
396 signaling ^{64, 65}, and deletion of GSK-3β induced a pro-fibrotic myofibroblast phenotype in isolated
397 cardiac FBs in mice ⁵¹, the activation of GSK3α/β suggests a counter-regulation of TGFβ1-signaling.
398 It is therefore conceivable that BC-11, at least partially, exerts its anti-fibrotic action *via* activation of
399 GSK3α/β.

400 Together, our study provides a genetic landscape of hypertrophic scars which is the basis for further
401 investigations on novel genes and fibrotic processes hitherto not studied in the context of skin
402 scarring. In addition, we identified a group of serine proteases involved in scar formation and/or
403 maturation and further *in vivo* studies will demonstrate the efficacy of serine protease inhibitors on
404 the prevention of scar development and on the improvement of already existing scars.

405

406

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408 **Secondary Sources**

409 **Uncategorized References**

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637 **Conflict of interest**

638 The authors declare no conflict of interest.

639

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649

650 **Author contributions**

651 MM, HJA, ET and VV provided study conception and design; WH and CR provided patient sample
652 material; HJA and MM acquired funding; VV, DC, YC and BG conducted experiments and prepared
653 samples; VV performed data analysis, visualization and figure design; VV, ML, ET, and MM
654 participated in data interpretation; VV, ML and MM drafted the manuscript. All authors reviewed the
655 manuscript.

656

Figure 1

658

659 **Figure 1: Characterization of human skin and scar samples with scRNAseq identifies specific**
660 **cell clusters and a distinct fibrotic gene signature.**

661 A) Illustration of workflow of scRNAseq in human skin and scar samples. B) Phylogenetic clustertree
662 calculated unsupervised based on UMAP-clustering. C, D) UMAP-plots of human skin and scar
663 samples, split by tissue, after integration of all samples, identifying seven fibroblast clusters (FB1-7),
664 smooth muscle cells and pericytes (SMC/Peri), endothelial cells (EC1+2), lymphatic endothelial cells
665 (LEC), T-cells, macrophages (Mac), dendritic cells (DC1+2), three keratinocyte clusters (KC1-3),
666 and melanocytes (Mel). E, F) Pie charts showing ratios of cell clusters in skin and scars. Feature plots
667 and bar graphs of number of differentially expressed genes (nDEG) per cluster of G) up- and H)
668 downregulated genes. DEGs were calculated per cluster comparing scar versus skin using Wilcoxon

669 rank sum test, including genes with average logarithmic fold change (avglogFC) of > 0.1 or < -0.1
670 and Bonferroni-adjusted p-value < 0.05 . Feature plots show projection of nDEG onto the UMAP-plot,
671 color intensity represents nDEG. Bar graphs show absolute numbers of nDEG per cluster, y-axis
672 represents nDEG. UMAP, uniform manifold approximation and projection.
673

Figure 2

674

675 Figure 2: Subset analysis of fibroblasts

676 A) Phylogenetic clustertree calculated unsupervised based on UMAP-clustering of subsetted
677 fibroblasts only. B, C) UMAP-plots of re-clustered skin and scar fibroblasts, split by tissue, re-
678 clustering identified 11 fibroblast clusters (subFB1-11). D) Feature plots illustrating computational
679 basis for dotplots, dotplots of top 50 regulated genes (according to lowest adjusted p-value)
680 comparing scar FBs versus skin FBs. E) Dotplot of top 50 regulated genes (according to lowest
681 adjusted p-value) cluster subFB1 compared to all other scar FBs. F) Gene ontology-term network was
682 calculated based on significantly upregulated (adj. p-val <0.05, avg.logFC >0.1) genes comparing
683 subFB1 to all other scar FBs. Gene list was imputed in ClueGO plug-in in Cytoscape with medium
684 GO-specificity, with GO-term fusion, only significant (*P* value <.05) GO terms are shown. Circle size
685 correlates with *P* value, lines (“edges”) represent functional connection of respective GO terms. Red
686 circles represent association of GO-term with TGFβ-signaling, purple, with extracellular matrix,
687 green, with smooth muscle differentiation, blue, with signaling factors, and yellow with bone
688 formation and -development. UMAP, uniform manifold approximation and projection.
689

Figure 3

690

691 **Figure 3: Pseudotime analysis of human scar FBs identifies cell fates and time-regulated genes**
 692 A, B) Ordering skin and scar FBs along a pseudotime axis. X-axis, pseudotime. y axis, % of cells in
 693 respective pseudotime-bin. Cell trajectory with pre-branch and branches is shown. Color code
 694 represents pseudotime progression. C, D) Cell trajectories were calculated based on pseudotime
 695 values, split by tissue. E, F) Heatmaps of pseudotime-dependent gene expression in skin and scar.
 696 Colors represent q-value, the expression of the respective gene in relation to pseudotime.

697

698

A) Illustration of workflow of mouse scar model and two-timepoint scRNAseq. Two mice were analyzed per timepoint. B) Phylogenetic clustertree calculated unsupervised based on unsupervised UMAP-clustering. C, D) UMAP-plots of mouse scar tissue, split by timepoint, after integration of all samples, identifying four fibroblast clusters (mFB1-4), smooth muscle cells and pericytes (mPC/SMC), endothelial cells and lymphatic endothelial cells (mEC/LEC), T-cells, dendritic cells (mDC), Langerhans cells (mLC), nine keratinocyte clusters (KC1-9), adipocytes (mAdipo) and melanocytes (Mel). E, F) Pie charts show relative numbers of cells in clusters, split by timepoint. Feature plots and bar graphs of number of differentially expressed genes (nDEG) per cluster of G) up- and H) downregulated genes per cluster. DEGs were calculated per cluster comparing 8 weeks vs

710 6 weeks old scars using Wilcoxon rank sum test, including genes with average logarithmic fold
711 change (avg_logFC) of > 0.1 or < -0.1 and Bonferroni-adjusted p-value < 0.05 . Feature plots show
712 projection of nDEG onto the UMAP-plot, color intensity represents nDEG. Bar graphs show absolute
713 number of nDEG per cluster, y-axis represents nDEG. UMAP, uniform manifold approximation and
714 projection.
715

Figure 5

716

717 **Figure 5: Comparing human scar gene expression and mouse scar maturation identifies mutual**
718 **drivers of skin fibrosis**

719 A) Illustration of computational basis for comparison human and mouse. Human cluster subFB1 vs
720 rest scar FBs significantly (adj. p-value<0,05) regulated genes were compared with mouse scar FBs
721 8 weeks vs 6 weeks significantly regulated genes. B) Venn diagrams of human and mouse up- (upper
722 panel) and down- (lower panel) regulated genes. C) Table of mouse and human mutually up and D)
723 downregulated genes. E-I) Feature plots and violin plots of serine proteases in human skin and scar.
724 *AEBP1* (adipocyte enhancer binding protein 1), *DPP4* (dipeptidyl-peptidase 4), *HTRA1* (High-
725 Temperature Requirement A Serine Peptidase 1), *PLAU* (urokinase), *PRSS23* (Serine protease 23). In
726 violin plots, dots represent individual cells, y-axis represents log2 fold change of the normalized genes
727 and log-transformed single-cell expression. Vertical lines in violin plots represent maximum

728 expression, shape of each violin represents all results, and width of each violin represents frequency
729 of cells at the respective expression level. In feature plots, normalized log expression of the respective
730 gene is mapped onto the UMAP-plot. Color intensity indicates level of gene expressions. UMAP,
731 uniform manifold approximation and projection.
732

Figure 6

Figure 6: Pseudotime analysis corroborates the putative role of serine proteases as drivers of scar maturation

A-E) Trajectory plots and pseudotime plots of Serine proteases in skin and scar FBs. AEBP1 (adipocyte enhancer binding protein 1), DPP4 (dipeptidyl-peptidase 4), HTRA1 (High-Temperature Requirement A Serine Peptidase 1), PLAU (urokinase), PRSS23 (Serine protease 23). In trajectory plots, normalized log expressions are plotted on the trajectories, split by skin and scar. In pseudotime plots, normalized log expressions are plotted against the pseudotime axis, and a spline curve represents expression dynamics over pseudotime. Y-axis, normalized log expression of respective gene, x-axis, pseudotime.

744

745 **Figure 7: *In-situ* hybridization and immunofluorescence staining confirm elevated expression**
 746 **of *PLAU* and *DPP4* in human skin and scar**

747 A, B) RNAScope in-hybridization of skin and scar tissue with *DPP4*- and *PLAU*-probes. Red dots
 748 indicate single mRNA molecules. Inserts show high magnification micrographs. C, D)
 749 Immunofluorescent staining of *DPP4* and *PLAU* in human skin and scar tissues.

Figure 8

750

751 **Figure 8: Pharmacological inhibition of DPP4 or urokinase prevents TGFβ-induced**
752 **myofibroblast-differentiation**

753 A,B) Western blot of primary FBs stimulated with active TGFβ1 for 24h to differentiate FBs into
754 alpha-smooth muscle actin-expressing (αSMA) myofibroblasts. Myofibroblast differentiation
755 inhibited with A) DPP4-inhibitor Sitagliptin or B) urokinase-inhibitor BC-11. C, D) Collagen I or E,
756 F) fibronectin in supernatants of stimulated primary skin FBs, detected by Enzyme-linked
757 Immunosorbent Assay (ELISA). Whiskers represent range maximum and minimum values with < 1.5
758 interquartile range, boxes represent 25th -75th quartiles, line represents mean. Statistical significance
759 was tested using one-way ANOVA with Tukey post-test. NS p>0.05, *p<0.05, **p<0.01, ***p<0.001.
760 Experiments were performed in duplicates of five donors each.

761

Figure 9

Figure 9: Signaling pathway activation after TGFβ-stimulation and DPP4/PLAU inhibition

E) Western blot of primary FB lysate stimulated with active TGFβ1 for 1h and analysis of canonical TGFβ1 signaling pathways. B) Analysis of non-canonical TGFβ1 signaling pathways and kinase pathways detected by proteome profiler from lysates of primary FBs after 1h stimulation with TGFβ1 alone, TGFβ1 with sitagliptin, and TGFβ1 with BC-11. Bars represent fold change compared to stimulation with TGFβ1 only, marked by dotted line.

Figures

Figure 1

Characterization of human skin and scar samples with scRNAseq identifies specific cell clusters and a distinct fibrotic gene signature. A) Illustration of workflow of scRNAseq in human skin and scar samples. B) Phylogenetic clustertree calculated unsupervised based on UMAP-clustering. C, D) UMAP-plots of

human skin and scar samples, split by tissue, after integration of all samples, identifying seven fibroblast clusters (FB1-7), smooth muscle cells and pericytes (SMC/Peri), endothelial cells (EC1+2), lymphatic endothelial cells (LEC), T-cells, macrophages (Mac), dendritic cells (DC1+2), three keratinocyte clusters (KC1-3), and melanocytes (Mel). E, F) Pie charts showing ratios of cell clusters in skin and scars. Feature plots and bar graphs of number of differentially expressed genes (nDEG) per cluster of G) up- and H) downregulated genes. DEGs were calculated per cluster comparing scar versus skin using Wilcoxon rank sum test, including genes with average logarithmic fold change (avglogFC) of > 0.1 or < -0.1 and Bonferroni-adjusted p-value < 0.05 . Feature plots show projection of nDEG onto the UMAP-plot, color intensity represents nDEG. Bar graphs show absolute numbers of nDEG per cluster, y-axis represents nDEG. UMAP, uniform manifold approximation and projection.

Figure 2

Subset analysis of fibroblasts

Figure 3

Pseudotime analysis of human scar FBs identifies cell fates and time-regulated genes A, B) Ordering skin and scar FBs along a pseudotime axis. X-axis, pseudotime. y axis, % of cells in respective pseudotime-bin. Cell trajectory with pre-branch and branches is shown. Color code represents pseudotime progression. C, D) Cell trajectories were calculated based on pseudotime values, split by tissue. E, F) Heatmaps of pseudotime-dependent gene expression in skin and scar. Colors represent q-value, the expression of the respective gene in relation to pseudotime.

Figure 4

Two-timepoint mouse scar model identifies genes regulated in scar maturation A) Illustration of workflow of mouse scar model and two-timepoint scRNAseq. Two mice were analyzed per timepoint. B) Phylogenetic cluster tree calculated unsupervised based on unsupervised UMAP-clustering. C, D) UMAP-plots of mouse scar tissue, split by timepoint, after integration of all samples, identifying four fibroblast clusters (mFB1-4), smooth muscle cells and pericytes (mPC/SMC), endothelial cells and lymphatic

endothelial cells (mEC/LEC), T-cells, dendritic cells (mDC), Langerhans cells (mLC), nine keratinocyte clusters (KC1-9), adipocytes (mAdipo) and melanocytes (Mel). E, F) Pie charts show relative numbers of cells in clusters, split by timepoint. Feature plots and bar graphs of number of differentially expressed genes (nDEG) per cluster of G) up- and H) downregulated genes per cluster. DEGs were calculated per cluster comparing 8 weeks vs 6 weeks old scars using Wilcoxon rank sum test, including genes with average logarithmic fold change (avg_logFC) of > 0.1 or < -0.1 and Bonferroni-adjusted p-value < 0.05 . Feature plots show projection of nDEG onto the UMAP-plot, color intensity represents nDEG. Bar graphs show absolute number of nDEG per cluster, y-axis represents nDEG. UMAP, uniform manifold approximation and projection.

Figure 5

Comparing human scar gene expression and mouse scar maturation identifies mutual drivers of skin fibrosis A) Illustration of computational basis for comparison human and mouse. Human cluster subFB1 vs rest scar FBs significantly (adj. p-value<0,05) regulated genes were compared with mouse scar FBs 8 weeks vs 6 weeks significantly regulated genes. B) Venn diagrams of human and mouse up- (upper panel) and down- (lower panel) regulated genes. C) Table of mouse and human mutually up and D)

downregulated genes. E-I) Feature plots and violin plots of serine proteases in human skin and scar. AEBP1 (adipocyte enhancer binding protein 1), DPP4 (dipeptidyl-peptidase 4), HTRA1 (High-Temperature Requirement A Serine Peptidase 1), PLA1 (urokinase), PRSS23 (Serine protease 23). In violin plots, dots represent individual cells, y-axis represents log2 fold change of the normalized genes and log transformed single-cell expression. Vertical lines in violin plots represent maximum expression, shape of each violin represents all results, and width of each violin represents frequency of cells at the respective expression level. In feature plots, normalized log expression of the respective gene is mapped onto the UMAP plot. Color intensity indicates level of gene expressions. UMAP, uniform manifold approximation and projection.

Figure 6

Pseudotime analysis corroborates the putative role of serine proteases as drivers of scar maturation A-E) Trajectory plots and pseudotime plots of Serine proteases in skin and scar FBs. AEBP1 (adipocyte enhancer binding protein 1), DPP4 (dipeptidyl-peptidase 4), HTRA1 (High-Temperature Requirement A Serine Peptidase 1), PLAU (urokinase), PRSS23 (Serine protease 23). In trajectory plots, normalized log expressions are plotted on the trajectories, split by skin and scar. In pseudotime plots, normalized log

expressions are plotted against the pseudotime axis, and a spline curve represents expression dynamics over pseudotime. Y-axis, normalized log expression of respective gene, x-axis, pseudotime.

Figure 7

In-situ hybridization and immunofluorescence staining confirm elevated expression of PLAU and DPP4 in human skin and scar A, B) RNAScope in-hybridization of skin and scar tissue with DPP4- and PLAU-

probes. Red dots indicate single mRNA molecules. Inserts show high magnification micrographs. C, D) Immunofluorescent staining of DPP4 and PLAU in human skin and scar tissues.

Figure 8

Pharmacological inhibition of DPP4 or urokinase prevents TGFβ-induced myofibroblast-differentiation A,B) Western blot of primary FBs stimulated with active TGFβ1 for 24h to differentiate FBs into alpha-smooth muscle actin-expressing (αSMA) myofibroblasts. Myofibroblast differentiation inhibited with A) DPP4-inhibitor Sitagliptin or B) urokinase-inhibitor BC-11. C, D) Collagen I or E, F) fibronectin in supernatants of stimulated primary skin FBs, detected by Enzyme-linked Immunosorbent Assay (ELISA). Whiskers represent range maximum and minimum values with < 1.5 interquartile range, boxes represent 25th -75th quartiles, line represents mean. Statistical significance was tested using one-way ANOVA with Tukey post-test. NS p>0.05, *p<0.05, **p<0.01, ***p<0.001. Experiments were performed in duplicates of five donors each.

Figure 9

Signaling pathway activation after TGFβ-stimulation and DPP4/PLAU inhibition E) Western blot of primary FB lysate stimulated with active TGFβ1 for 1h and analysis of canonical TGFβ1 signaling pathways. B) Analysis of non-canonical TGFβ1 signaling pathways and kinase pathways detected by proteome profiler from lysates of primary FBs after 1h stimulation with TGFβ1 alone, TGFβ1 with sitagliptin, and TGFβ1 with BC-11. Bars represent fold change compared to stimulation with TGFβ1 only, marked by dotted line.

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