1 **Single cell landscape of hypertrophic scars identifies serine proteases as key regulators of** 2 **myofibroblast differentiation**

3 Vera Vorstandlechner^{1,2,3}, Maria Laggner^{1,2}, Dragan Copic^{1,2}, Yiyan Chen⁴, Bahar Golabi⁴, Werner
4 Haslik³, Christine Radtke³, Erwin Tschachler⁴, Hendrik Jan Ankersmit^{1,2,*}, Michael Mildner^{4,*}

5
6 ¹Laboratory for Cardiac and Thoracic Diagnosis, Regeneration and Applied Immunology, Division
7 of Thoracic Surgery, Medical University of Vienna

8 ²Aposcience AG, Vienna, Austria.

9 ³Division of Plastic and Reconstructive Surgery, Department of Surgery, Medical University of
10 Vienna, Vienna, Austria

11 ⁴Department of Dermatology, Medical University of Vienna, Vienna, Austria

12 * contributed equally to this work

13

14 **corresponding authors:**

15 Michael Mildner, PhD

16 Department of Dermatology, Medical University of Vienna

17 Lazarettgasse 14, 1090 Vienna, Austria

18 e-mail: michael.mildner@meduniwien.ac.at

19 phone: +43-1-40400-73507

20 fax: +43-1-40400-73590

21

22 Hendrik Jan Ankersmit, MD

23 Laboratory for Cardiac and Thoracic Diagnosis, Regeneration and Applied Immunology, Division of

24 Thoracic Surgery, Medical University of Vienna

25 Waehringer Guertel 18-20, 1090 Vienna, Austria

26 e-mail: hendrik.ankersmit@meduniwien.ac.at

27 phone: +43-1-40400-69770

28 fax: +40-1-40400-67820

29 **Abstract**

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

33 Compared to normal skin, we found significant differences in gene expression in most cell types
34 present in scar tissue. Fibroblasts (FBs) showed the most prominent alterations in gene expression,
35 displaying a distinct fibrotic signature. By comparing genes upregulated in murine FBs during scar
36 development with genes highly expressed in mature human hypertrophic scars, we identified a group
37 of serine proteases, tentatively involved in scar formation. Two of them, dipeptidyl-peptidase 4
38 (DPP4) and urokinase (PLAU), were further analyzed in functional assays, revealing a role in TGFβ1-
39 mediated myofibroblast differentiation and over-production of components of the extracellular matrix
40 (ECM) without interfering with the canonical TGFβ1-signaling pathway.

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

44 **Introduction**

45 Hypertrophic scars are a complex medical problem and a significant global disease burden (Bayat et
46 al, 2003; Leavitt et al, 2016). In the western world, an estimated number of 100 million people
47 develop scars every year, approximately 11 million of which bear keloid scars and 4 million suffer
48 from burn scars (Bayat et al, 2003). In the USA, an estimated amount of 12 Billion Dollars is spent
49 annually on the treatment of skin scarring (Sen et al, 2009). For affected persons, a pathological
50 hypertrophic scar causes significant functional impairment, pain, pruritus, and a reduction in quality
51 of life (Bock et al, 2006; Van Loey et al, 2008).

52 Wound healing is a tightly coordinated, three-step process, characterized by an acute inflammatory
53 phase, a proliferative phase, and a remodeling phase. Prolonged inflammation results in increased
54 fibroblast (FB) activity, with enhanced secretion of transforming growth factor beta 1 (TGFβ1), TGFβ
55 2, insulin-like growth factor (IGF1), and other cytokines (Lee & Jang, 2018). TGFβ1 drives
56 differentiation of FBs into myofibroblasts, which have a contractile phenotype, are characterized by
57 excessive secretion of ECM-components (Hinz, 2016), and are the major contributors to the formation
58 of hypertrophic scars (Nabai et al, 2020). Mature hypertrophic scars display strong tissue contraction
59 (Lee & Jang, 2018), and dense, parallel or whorl-like ECM (Nabai et al, 2020).

60 Topical silicone application, compression or massage therapy, laser ablation, surgery, and
61 intralesional injection of triamcinolone (TAC), corticosteroids, or 5-Fluorouracil (5-FU) are the most
62 commonly used options for prevention or treatment of hypertrophic scars (Anthonissen et al, 2016;
63 Kafka et al, 2017; Lee & Jang, 2018; Tredget et al, 2017). However, many of these therapies lack
64 evidence of efficacy and safety, and mechanisms of actions are still unclear (Bao et al, 2019; Kafka
65 et al, 2017).

66 Recently, several proteases became the focus of drug development in fibrotic diseases, as they have
67 been shown to be involved in ECM-breakdown and the activation of growth factors in tissue

remodeling (Kanno, 2019; Menou et al, 2018). Serine proteases/peptidases constitute a large, diverse group of proteases, divided into 13 clans and 40 families (Page & Di Cera, 2008). The group of trypsins comprises proteases contributing to vital processes such as blood coagulation, fibrinolysis, apoptosis and immunity (Di Cera, 2009). 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 (Di Cera, 2009; Rawlings & Barrett, 1999). Inhibitors of PLA₂ have been shown to counteract fibrotic processes in cardiac and pulmonary fibrosis in human *in vitro* studies and in mouse experiments (Gupta & Donahue, 2017; Schuliga et al, 2017). Recently, the serine protease DPP4 became the center of attention, since DPP4 inhibitors (gliptins) have been clinically used for the treatment of diabetes mellitus (Makrilakis, 2019). DPP4 was also implicated in a variety of fibrotic pathologies, including cardiac, hepatic, renal, and dermal fibrosis (Aroor et al, 2017; Hong et al, 2017; Kaji et al, 2014; Suzuki et al, 2017; Uchida et al, 2017) and inhibition of DPP4 activity mitigated fibrotic processes in animal models (Gupta & Donahue, 2017; Hu & Longaker, 2016; Lay et al, 2019; Schuliga et al, 2018; Schuliga et al, 2017; Shi et al, 2016). 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.

88

89 **Methods**

90 **Ethical statement**

91 The Vienna Medical University ethics committee approved the use of healthy abdominal skin (Vote
92 Nr. 217/2010) and of scar tissue (Vote Nr. 1533/2017) and all donors provided written informed
93 consent. Animal experiments were approved by the Medical University of Vienna ethics committee
94 and by the Austrian Federal Ministry of Education, Science and Research (Vote Nr. BMBWF-
95 66.009/0075-V/3b/2018) and performed in accordance with the Austrian guidelines for the use and
96 care of laboratory animals.

97 **Scar and skin samples**

98 Resected scar tissue was obtained from patients who underwent elective scar resection surgery (donor
99 information is provided in Table S1). Scars were classified as hypertrophic, pathological scars
100 according to POSAS (Fearmonti et al, 2011) by a plastic surgeon. Only mature scars, which had not
101 been treated before and persisted for more than two years were used for all experiments. All donors
102 had no known chronic diseases and received no chronic medication. Healthy skin was obtained from
103 female donors between 25-45 years from surplus abdominal skin removed during elective
104 abdominoplasty.

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

106 Female Balb/c mice bred at the animal facility of the Medical University of Vienna (Himberg, Austria)
107 were housed under specific-pathogen-free conditions with 12h/12h light/dark cycles and food and
108 water access *ad libidum*. For full thickness skin wounds, mice were anesthetized with 100 mg/kg
109 Xylazin and 5 mg/kg ketamin (both Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally.
110 Postoperative analgesia was provided with 0.1 mg/kg Buprenorphin (Temgesic®, Indivior Inc., North
111 Chesterfield, VA, USA) subcutaneously and 0.125 mg/ml Piritramid (Janssen-Cilag Pharma, Vienna,
112 Austria) in drinking water *ad libidum*. A 9x9 mm² area was marked on shaved backs and excised with

113 sharp scissors. The wounds were left to heal uncovered without any further intervention. Mice were
114 sacrificed 6 or 8 weeks after wounding, and scar tissues were isolated.

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

116 Four mm biopsies were taken from human skin, human scar, and from mouse scar tissue, and
117 enzymatically digested with MACS Miltenyi Whole Skin Dissociation Kit (Miltenyi Biotec,
118 Bergisch-Gladbach, Germany) for 2.5h according to the manufacturer's protocol. After processing on
119 a GentleMACS OctoDissociator (Miltenyi), cell suspensions were passed through a 70 µm and a 40
120 µm filter and stained with DAPI nuclear dye. Cells were sorted on a MoFlo Astrios high speed cell
121 sorting device (Beckman-Coulter, Brea, CA, USA), and only DAPI-negative cells, representing
122 viable cells, were used for single cell RNAseq.

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

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

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

131 Raw sequencing files were demultiplexed, aligned to the human or mouse reference genome
132 (GrCh38/ mm10) and counted using the Cellranger pipelines (Cellranger v3, 10X Genomics). The
133 resulting cell-gene matrices were processed using the 'Seurat'-package (Seurat v3.1.0, Satija Lab,
134 New York, NY, USA) in R-studio in R (R v3.6.2, The R Foundation, Vienna, Austria). From each
135 sample, unwanted variations and low-quality cells were filtered by removing cells with high and low
136 (>3000 and <200) Unique Molecular Identifier (UMI)-counts. First, healthy skin and scar samples

137 were integrated separately to avoid clustering according to donors, and for batch correction.
 138 Subsequently, skin and scar data were integrated again into one dataset. Data integration was
 139 performed according to the recommended workflow by Butler *et al.* and Stuart *et al.* (Butler et al,
 140 2018; Stuart et al, 2018). After quality control comparing all donors, we obtained transcriptome data
 141 from a total of 25,083 human skin and scar cells, with a median of 24,943 reads and 851 detected
 142 genes per cell. In mice, we obtained data from 6,561 cells 6 weeks after wounding, and 9,393 cells 8
 143 weeks after wounding. The samples displayed a median of 24,774 reads per cell, and median of 1,969
 144 detected genes per cell. After quality control, all mouse samples were integrated together in one
 145 integration step. In both datasets, normalized count numbers were used for differential gene
 146 expression analysis, for visualization in violin plots, feature plots, dotplots and heatmaps, when
 147 displaying features that vary across conditions, as recommended by current guidelines (Luecken &
 148 Theis, 2019). In both datasets, cell types were identified by well-established marker gene expression
 149 (Figure S1A, Figure S4A). For identification of differentially expressed genes (DEGs), normalized
 150 count numbers were used, including genes present in the integrated dataset to avoid calculation of
 151 batch effects. As keratin and collagen genes were previously found to contaminate skin biopsy
 152 datasets and potentially provide a false-positive signal (Rojahn et al, 2020), these genes (*COL1A1*,
 153 *COL1A2*, *COL3A1* and *KRT1* *KRT5*, *KRT10*, *KRT14*, *KRTDAP*) were excluded from DEG
 154 calculation in non-fibroblast clusters (collagens) or non-keratinocyte clusters (keratins), respectively.
 155 Moreover, genes *Gm42418*, *Gm17056* and *Gm26917* caused technical background noise and batch
 156 effect in mouse scRNAseq, as described before (Hammond et al, 2019), and were thus excluded from
 157 the dataset.

158 **Pseudotime analyses**

159 Pseudotime analyses, trajectory-construction and calculation of pseudotime-dependent gene
 160 expression were performed in Monocle 2 (Monocle2, v2.14.0, Trapnell Lab, University of
 161 Washington, Seattle, WA, USA) (Qiu et al, 2017a; Trapnell et al, 2014). 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 (Qiu et al, 2017a; Qiu et al, 2017b; Trapnell et al, 2014). 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)(Qiu et al, 2017b).

169 **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 (Snel et al, 2000; Szklarczyk et al, 2019) (<https://string-db.org/>, String consortium, Swiss Institute of Bioinformatics/EMBL Heidelberg/University of Zurich/Novo Nordisk Foundation CPR).

174 **Gene ontology (GO)-networks**

Gene lists of significantly regulated genes (adjusted p-value <0.05, average log fold change [avg_logFC] >0.1) were imputed in ClueGO v2.5.5 (Bindea et al, 2009) plug-in in Cytoscape v3.7.2 (Lotia et al, 2013) with medium GO-specificity, with GO-term fusion, and only significant (*P* value <.05) GO-terms are depicted as circles, whereby circle size correlates with *P* value, and lines represent functional connection of respective GO-terms.

180 **Immunofluorescence stainings**

Immunofluorescence staining on formalin-fixed, paraffin-embedded (FFPE) sections of skin and scar tissue were performed as described previously (Gschwandtner et al, 2013). Antibodies were used as indicated in Table S2.

184 **RNAScope in situ hybridization**

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

190 **Isolation of primary skin fibroblasts**

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

199 **TGFβ 1-induced myofibroblast differentiation**

200 After the first passage, isolated primary FBs were plated in 6-well plates, supplied with DMEM +
201 10% fetal bovine serum (FBS, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo
202 Fisher Scientific) and grown until 100% confluency. FBs were then stimulated with 10ng/ml TGFβ1
203 (HEK-293-derived, Peprotech, Rocky Hill, NJ, USA), and with or without DPP4-inhibitor Sitagliptin
204 (10µM) (Thermo Fisher Scientific) or urokinase-inhibitor BC-11 hydrobromide (10µM) (Tocris by
205 Bio-Techne, Bristol, UK) for 24h. Supernatants were removed and medium and inhibitors were
206 resupplied for another 24h. Supernatants were collected and stored at -80°C and cells were lysed in
207 1x Laemmli Buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for further analysis.

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

212 **Western blotting**

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

221 **Proteome profiling of signaling pathways**

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

224

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

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

230

231 Results

232 The single-cell landscape of hypertrophic scars

233 To elucidate the complex biological processes of scar formation, we performed droplet-based single-
 234 cell transcriptome analysis of human hypertrophic scar tissue and healthy skin (Figure 1A). In both
 235 samples, Unsupervised Uniform Manifold Approximation and Projection (*UMAP*)-clustering
 236 revealed 21 cell clusters, which were further classified as specific cell types by well-established
 237 marker genes (Figure S1A), expression patterns of all clusters (Figure S1B), and transcriptional
 238 cluster proximity *via* a phylogenetic cluster tree (Figure 1B). We found seven FB clusters, smooth
 239 muscle cells and pericytes (SMC/Peri), three clusters of endothelial cells (EC), and lymphatic
 240 endothelial cells (LECs), two clusters of T-cells and of dendritic cells (DC), macrophages (Mac),
 241 three keratinocyte (KC) clusters, and melanocytes. All cells of specific subsets were clustered
 242 together, and skin and scar samples displayed comparable cellular cluster composition (Figure 1C,
 243 D). Only cluster FB1 was mainly present in scar tissue. The clusters of skin and scars showed different
 244 relative cell number ratios (Figure 1E, F). Whereas FBs represented 40% of all cells in healthy skin,
 245 a significant increase (53%) was observed in scar tissue. Similarly, we detected more ECs (16,31%)
 246 in scar tissue as compared to normal skin (8,1%). Contrary, the relative numbers of KCs (5,97%) and
 247 macrophages (4,42%) in mature hypertrophic scars were significantly reduced compared to skin
 248 (18,97% and 7,11%).

249 To identify the strongest differences in gene expression between normal skin and scars, we calculated
 250 all up- and downregulated genes for FBs, PCs, ECs, T-cells, DCs and KCs (Figure S2). In general,
 251 we identified considerably more up- (Figure 1G) than downregulated genes (Figure 1H), and the most
 252 abundant differential gene expression of scar compared to skin (number of differentially expressed
 253 genes, nDEG) was found in FBs, SMC/pericytes, macrophages, DC1 and KC1 (Figure 1G, H). Genes
 254 related to ECM production were mainly overrepresented in FBs, but notably also in PCs and ECs

(Figure S2A-C). Several significantly regulated genes with so far undescribed roles in fibrosis and scar formation were found in all cell types (Figure S2A-F). These distinctly regulated genes might provide valuable new candidates to understand and modulate skin scarring.

258

The fibrotic gene expression pattern of fibroblasts in hypertrophic scars.

Since FBs showed strongest gene regulation in our scRNAseq dataset, and have been considered as the major drivers of skin scarring and an important source for myofibroblasts (Hinz, 2016), we focused our further analysis on differences between FBs of healthy skin and hypertrophic scars (Figure 2).

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

Analysis of the biological processes associated with differentially regulated genes between FB1 and other FB clusters by gene ontology network analysis revealed a strong association of FB1 with TGFβ-signaling (red circles) and ECM-formation (purple circles) (Figure 2F), further corroborating its role in skin fibrosis. In addition, our analysis indicates a role of FB1 in processes important for several

278 other cell types, including platelets, smooth muscle cells and cells of the skeletal system, suggesting
279 paracrine actions of FB1.

280 Pseudotime calculation and trajectory construction effectively identifies possible cell fates and time-
281 regulated genes, even when analyzing cells of only one timepoint (Qiu et al, 2017b; Trapnell et al,
282 2014). Thus, we next sorted human skin and scar FBs along a pseudotime axis and constructed
283 trajectories (Figure 3A, B). The trajectories revealed a division at a certain time point where FBs
284 divided into two branches (Figure 3C). Whereas the majority of FBs preferentially aligned with
285 branch 1 in normal skin (Figure 3D), we observed a significantly longer branch 2 with FBs of
286 hypertrophic scar tissue (Figure 3E). Genes most regulated in a pseudotime-dependent manner in
287 normal skin and hypertrophic scars are shown in Figure 3F and 3G. Interestingly, the collagens
288 *COL1A1*, *COL1A2* and *COL3A1*, known to contribute to all fibrotic processes, are most upregulated
289 at the end of skin pseudotime, but are not among the most pseudotime-regulated genes in scar (Figure
290 3F,G). In contrast, *COL5A1/2*, *COL8A1*, *COL11A1* and *COL12A1*, dominated the late pseudotime-
291 dependent gene expression in scars. The role of these collagens in (hypertrophic) scar is scarcely
292 investigated, and merits further exploration. Together, our trajectory analysis models the temporal
293 dynamics of gene expression in scars and might provide a basis to target respective genes at different
294 stages of scar development.

295 296 **scRNAseq of murine scars identifies genes involved in scar maturation**

297 As our approach so far only gave information on the current state of mature scars, we further
298 investigated mechanisms leading to scar formation and maturation, using a murine full thickness skin
299 wound model. Whereas scar formation and maturation in humans is a long lasting process (Kant et
300 al, 2019), it only takes up to 80 days in rodents (Ferguson & O'Kane, 2004). Hence, we compared
301 scar formation in mice 6 and 8 weeks after wounding (Figure 4A). Analogously to the human dataset,

all cells were clustered, and cell types were identified using established marker genes (Figure S4A), expression patterns of all clusters (Figure S4B) and transcriptome proximity of clusters *via* a phylogenetic cluster tree (Figure 4B). All clusters aligned homogeneously, and all major skin cell types were represented at both time points (Figure 4C, D).

In accordance with human scar tissue, 8 weeks old mouse scars contained a higher proportion of murine FBs (mFBs) (32,6%) compared to 6 weeks old scars (17,39%), and more DCs (9,6 versus 6,3%). In contrast, less pericytes and endothelial cells (2,8 versus 1,5%) and less keratinocytes (63,3 vs 45%) were present (Figure 4E, F.). We next calculated up- and downregulated genes for FBs, PCs, ECs, T-cells, DCs and KCs, comparing 8 weeks to 6-week-old scars (Figure S5A-F). In contrast to human scars, the highest number of differentially expressed genes was found in mFBs and mKCs (Figure 4G,H), which was most likely due to ongoing epidermal tissue regeneration. Surprisingly, the expression of collagens did not change significantly between 6 and 8 weeks in mFBs, and only mECs showed a slightly increased expression of collagens (Figure S5A-C). However, expression of several matricellular proteins and other ECM-components, e.g. *Fbln1* (Fibulin1), *Ogn* (osteoglycin), *Lum* (Lumican), and *Pcolce* (Procollagen C-Endopeptidase Enhancer) increased in mFBs during scar maturation (Figure S5A). Together, our scRNAseq identified a gene profile specific for scar maturation in mice.

319

320 Serine proteases are strongly upregulated during scar maturation

To identify genes that are crucial for scar maturation, we next compared our human scar data set with genes upregulated in mouse scars 8 weeks after wounding in comparison to mouse scars 6 weeks after wounding (Figure 5A). While in both data sets only one gene (*LEPR*) was downregulated, 16 genes were mutually upregulated (Figure 5B-D). Stunningly, 5 of these genes (*AEBP1*, *DPP4*, *HTRA1*, *PLAU* and *PRSS23*) were members of the superfamily of serine proteases (Figure 5B, C). All five

serine proteases were upregulated in scRNAseq in human scar tissue, particularly in FBs, but also in other cell types (Figure 5E-I). *AEBPI* and *PRSS23* expression also increased in ECs and melanocytes, *HTRA1* in ECs and KC3, and *PLAU* in DCs. Several additional serine proteases, *HTRA3* (High-Temperature Requirement A Serine Peptidase 3), *DPP7* (Dipeptidyl-Peptidase 7), *FAP* (Fibroblast activation protein alpha), were upregulated in human scars (Figure S6), and also showed a trend in mouse scars. Analysis of these serine proteases by pseudotime trajectories in human FBs revealed that their expression mainly increased over time and *AEBPI* and *HTRA1* significantly enriched at the end of branch 2 (Figure 6A). Together, these data suggest a major role of serine proteases in scar formation and/or maturation.

335

336 **The serine proteases DPP4 and urokinase regulate TGFβ1-mediated myofibroblasts** 337 **differentiation and ECM over-production**

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

As TGFβ1 is one of the key inducers of scarring and tissue fibrosis, causing differentiation of FBs to pro-fibrotic myofibroblasts (Carthy, 2018; Lodyga & Hinz, 2019; Roberts et al, 1986; Schuliga et al, 2017), we hypothesized that the serine proteases interact with TGFβ-signaling. To test this, we

350 stimulated primary FBs from healthy abdominal human skin with active TGFβ1, which induces
 351 expression of alpha-smooth muscle actin (αSMA), a marker for myofibroblasts. Addition of the DPP4
 352 inhibitor Sitagliptin and the urokinase inhibitor BC11 almost completely abolished the TGFβ1-
 353 mediated myofibroblast-differentiation as shown by αSMA Western blot (Figure 8A, B). Moreover,
 354 Sitagliptin and to an even greater extent BC-11 attenuated TGFβ1-induced overproduction of the
 355 ECM-proteins Colla1 (Figure 8C, D), and fibronectin (Figure 8E, F) by FBs. These results suggest
 356 that serine proteases are involved in the TGFβ1-induced myofibroblast differentiation.

357

358 **The serine proteases DPP4 and urokinase do not interfere with canonical TGFβ1-signaling** 359 **pathways**

360 To investigate whether the serine protease inhibitors interfere with TGFβ1 signaling, we investigated
 361 the TGFβ1-induced signaling pathways SMAD2 and ERK1/2 (Hata & Chen, 2016). While SMAD2
 362 and ERK1/2 phosphorylation was readily induced upon stimulation with TGFβ1, addition of the
 363 inhibitors showed no effects (Figure 9A). In contrast, SMAD1, 5 and 9 showed no activation after
 364 TGFβ1 stimulation (Figure 9A). To further identify other signaling pathways that might be involved
 365 in the action of the serine protease inhibitors, we used a signaling proteome profiler, showing that
 366 none of the signaling molecules were inhibited by the inhibitors (Figure 9B). Interestingly, the
 367 GSKα/β-pathway, known to attenuate fibrotic processes in the heart (Lal et al, 2014) was significantly
 368 activated by BC-11 (Figure 9B, Figure S7), suggesting a counter-regulatory action. Together, these
 369 data suggest that sitagliptin and BC-11 do not interfere with canonical or known non-canonical
 370 TGFβ1 signaling.

371 Discussion

372 Although skin fibrosis has been extensively studied, key mechanisms leading to the development of
373 hypertrophic scars are still not well understood. In addition, treatment options to prevent or treat
374 (hypertrophic) scars are still scarce (Sidgwick et al, 2015) and not exceptionally effective. In the
375 present study, we used scRNAseq to elucidate the genetic landscape of hypertrophic scar tissue at a
376 hitherto unmet single cell resolution. We found numerous regulated genes not yet investigated in scar
377 formation, and identified serine proteases as regulators of TGF β -induced myofibroblast
378 differentiation.

379 As expected, our scRNAseq analysis confirmed a plethora of previous studies, but also identified
380 numerous genes, which have so far not been described in the context of skin scarring or tissue fibrosis.
381 For example, the cytokines *MDK* (midkine) and *PTN* (pleiotrophin), both involved in cell growth,
382 migration, and angiogenesis (Muramatsu, 2010), were strongly upregulated in scar FBs. In contrast,
383 *SOD2/3* (superoxide dismutase 2/3), an enzyme controlling the release of reactive oxygen species
384 (ROS), hence acting as important antioxidant (Miao & St Clair, 2009), was strongly downregulated
385 in scar FBs. Interestingly, failure of ROS-scavenging has already been shown to contribute to
386 hypertrophic scar formation (Carney et al, 2019). Another interesting and significantly down-
387 regulated gene in scars was *SFN* (stratifin). As stratifin has been identified as potent collagenase-
388 stimulating factor in FBs, its down-regulation in scars suggests a contribution to the maintenance
389 and/or progression of the fibrotic phenotype by preventing matrix degradation. These and many other
390 novel factors identified in our study could be important, decisive molecules for the development
391 and/or maturation of hypertrophic scars. Thus, our study has built a basis for future studies describing
392 the role of these molecules in skin scarring and tissue fibrosis.

393

Our combined study of human mature hypertrophic scars and scar maturation in mice identified a group of serine proteases as key player for scar development and maturation. Although, DPP4-positive FBs have previously been identified as the main source of ECM production in the skin (Vorstandlechner et al, 2020), and urokinase has been shown to be involved in lung fibrosis (Schuliga et al, 2017), their roles in myofibroblast differentiation and production of ECM are still unclear. Our finding that addition of specific DPP4 and urokinase inhibitors to TGFβ1-stimulated FBs almost completely inhibited myofibroblast differentiation and up-regulation of matrix proteins was striking. Sitagliptin, the here used DPP4 inhibitor, is an effective drug widely used for the treatment of diabetes mellitus (Juillerat-Jeanneret, 2014). Based on our study, it might be very interesting to systemically evaluate differences in scar formation and scar quality of diabetic patients treated with either gliptins or other drugs. Furthermore, topical application of gliptins, during the phase of tissue remodeling after full wound closure, might prevent (hypertrophic) skin scarring. As gliptins are already approved for clinical use, an off-label topical application would be a promising step forward to test its efficacy on skin scarring *in vivo*. Interestingly, the urokinase inhibitor BC-11 showed an even stronger effect on inhibiting TGFβ1-induced myofibroblast differentiation and matrix production. However, so far it has only been used *in vitro*, and further *in vivo* testing for efficacy and safety is still required. Inhibition of urokinase to attenuate tissue fibrosis *per se* might appear counterintuitive, as urokinase facilitates fibrinolysis and regulates ECM-turnover, eliciting anti-fibrotic action (Behrendt, 2004). However, literature on urokinase inhibitors and fibrosis is contradictory. The best investigated endogenous urokinase-inhibitor, plasminogen activator inhibitor-1 (PAI-1, *SERPINE1*), was found to cause excessive matrix deposition after injury (Rabieian et al, 2018). By contrast, and in line with our results, inhibition of urokinase by PAI-1 suppressed profibrotic response in FBs from fibrotic lungs and prevented cardiac fibrosis in mice (Gupta & Donahue, 2017). Therefore, our study suggests the use of urokinase inhibitor BC-11 as a possible new therapeutic strategy for the treatment of skin scars. Further studies are necessary to fully elucidate its efficacy *in vivo*.

419

420 Strikingly, our analyses revealed no influence of the inhibitors on the canonical TGF β 1 signaling
 421 pathway. As to the anti-fibrotic action of sitagliptin, we have no mechanism to offer yet. Although
 422 DPP4 inhibition has previously been demonstrated to directly inhibit canonical TGF β signaling *via*
 423 Smad2 in renal fibrosis (Wang et al, 2018) and TGF β -mediated myoFB-differentiation by interfering
 424 with ERK-signaling (Thielitz et al, 2008), we were not able to confirm these mechanisms in skin FBs.
 425 Possible explanations for alternative the anti-fibrotic action of sitagliptin could be interactions with
 426 CXCL12/SDF1 (Stromal derived factor 1), a substrate of DPP4 (Elmansi et al, 2019) which was
 427 found to promote scar formation (Nishiguchi et al, 2018), or with components of the ECM, such as
 428 fibronectin (Elmansi et al, 2019), which have been shown to bind latent TGF β (Schuliga et al, 2017),
 429 or DPP4 induced cleavage of growth factors or receptors (Elmansi et al, 2019). Deciphering these
 430 interactions will be the scope of further mechanistic studies. Regarding BC-11, we found a significant
 431 activation of GSK3 α/β in TGF β 1-stimulated FBs. Since GSK3 β was previously found to interact
 432 with WNT/ β -catenin signaling (Guo et al, 2017; Vallée et al, 2017), and deletion of GSK-3 β induced
 433 a pro-fibrotic myofibroblast phenotype in isolated cardiac FBs in mice (Lal et al, 2014), the activation
 434 of GSK3 α/β suggests a counter-regulation of TGF β 1-signaling. It is therefore conceivable that BC-
 435 11, at least partially, exerts its anti-fibrotic action *via* activation of GSK3 α/β .

436

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

442

444 LiteraturePrimary Sources

445 Secondary Sources

446 Uncategorized References

447 Anthonissen M, Daly D, Janssens T, Van den Kerckhove E (2016) The effects of conservative
448 treatments on burn scars: A systematic review. *Burns : journal of the International Society for Burn*
449 *Injuries* **42**: 508-518

450

451 Aroor AR, Habibi J, Kandikattu HK, Garro-Kacher M, Barron B, Chen D, Hayden MR, Whaley-
452 Connell A, Bender SB, Klein T, Padilla J, Sowers JR, Chandrasekar B, DeMarco VG (2017)
453 Dipeptidyl peptidase-4 (DPP-4) inhibition with linagliptin reduces western diet-induced myocardial
454 TRAF3IP2 expression, inflammation and fibrosis in female mice. *Cardiovascular diabetology* **16**: 61

455

456 Bao Y, Xu S, Pan Z, Deng J, Li X, Pan F (2019) Comparative Efficacy and Safety of Common
457 Therapies in Keloids and Hypertrophic Scars: A Systematic Review and Meta-analysis. *Aesthetic*
458 *plastic surgery*

459

460 Bayat A, McGrouther DA, Ferguson MWJ (2003) Skin scarring. *BMJ : British Medical Journal* **326**:
461 88-92

462

463 Behrendt N (2004) The urokinase receptor (uPAR) and the uPAR-associated protein
464 (uPARAP/Endo180): membrane proteins engaged in matrix turnover during tissue remodeling.
465 *Biological chemistry* **385**: 103-136

466

467 Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, Fridman WH, Pages F,
468 Trajanoski Z, Galon J (2009) ClueGO: a Cytoscape plug-in to decipher functionally grouped gene
469 ontology and pathway annotation networks. *Bioinformatics (Oxford, England)* **25**: 1091-1093

470

471 Bock O, Schmid-Ott G, Malewski P, Mrowietz U (2006) Quality of life of patients with keloid and
472 hypertrophic scarring. *Archives of dermatological research* **297**: 433

473

474 Butler A, Hoffman P, Smibert P, Papalexi E, Satija R (2018) Integrating single-cell transcriptomic
475 data across different conditions, technologies, and species. *Nature Biotechnology* **36**: 411

476

477 Carney BC, Chen JH, Kent RA, Rummani M, Alkhalil A, Moffatt LT, Rosenthal DS, Shupp JW
478 (2019) Reactive Oxygen Species Scavenging Potential Contributes to Hypertrophic Scar Formation.
479 *The Journal of surgical research* **244**: 312-323

480

481 Carthy JM (2018) TGFbeta signaling and the control of myofibroblast differentiation: Implications
482 for chronic inflammatory disorders. *Journal of cellular physiology* **233**: 98-106
483
484 Di Cera E (2009) Serine proteases. *IUBMB life* **61**: 510-515
485
486 Elmanshi AM, Awad ME, Eisa NH, Kondrikov D, Hussein KA, Aguilar-Pérez A, Herberg S,
487 Periyasamy-Thandavan S, Fulzele S, Hamrick MW, McGee-Lawrence ME, Isales CM, Volkman BF,
488 Hill WD (2019) What doesn't kill you makes you stranger: Dipeptidyl peptidase-4 (CD26) proteolysis
489 differentially modulates the activity of many peptide hormones and cytokines generating novel
490 cryptic bioactive ligands. *Pharmacology & therapeutics* **198**: 90-108
491
492 Fearmonti RM, Bond JE, Erdmann D, Levin LS, Pizzo SV, Levinson H (2011) The modified Patient
493 and Observer Scar Assessment Scale: a novel approach to defining pathologic and nonpathologic
494 scarring. *Plastic and reconstructive surgery* **127**: 242-247
495
496 Ferguson MW, O'Kane S (2004) Scar-free healing: from embryonic mechanisms to adult therapeutic
497 intervention. *Philosophical transactions of the Royal Society of London Series B, Biological sciences*
498 **359**: 839-850
499
500 Gschwandtner M, Mildner M, Mlitz V, Gruber F, Eckhart L, Werfel T, Gutzmer R, Elias PM,
501 Tschachler E (2013) Histamine suppresses epidermal keratinocyte differentiation and impairs skin
502 barrier function in a human skin model. *Allergy* **68**: 37-47
503
504 Guo Y, Gupte M, Umbarkar P, Singh AP, Sui JY, Force T, Lal H (2017) Entanglement of GSK-3β, β-
505 catenin and TGF-β1 signaling network to regulate myocardial fibrosis. *Journal of molecular and*
506 *cellular cardiology* **110**: 109-120
507
508 Gupta KK, Donahue DL (2017) Plasminogen Activator Inhibitor-1 Protects Mice Against Cardiac
509 Fibrosis by Inhibiting Urokinase-type Plasminogen Activator-mediated Plasminogen Activation. **7**:
510 365
511
512 Hammond TR, Dufort C, Dissing-Olesen L, Giera S, Young A, Wysoker A, Walker AJ, Gergits F,
513 Segel M, Nemesh J, Marsh SE, Saunders A, Macosko E, Ginhoux F, Chen J, Franklin RJM, Piao X,
514 McCarroll SA, Stevens B (2019) Single-Cell RNA Sequencing of Microglia throughout the Mouse
515 Lifespan and in the Injured Brain Reveals Complex Cell-State Changes. *Immunity* **50**: 253-271.e256
516
517 Hata A, Chen YG (2016) TGF-β Signaling from Receptors to Smads. *Cold Spring Harbor*
518 *perspectives in biology* **8**
519
520 Hinz B (2016) Myofibroblasts. *Experimental eye research* **142**: 56-70
521

522 Hong SK, Choo EH, Ihm SH, Chang K, Seung KB (2017) Dipeptidyl peptidase 4 inhibitor attenuates
523 obesity-induced myocardial fibrosis by inhibiting transforming growth factor-beta1 and Smad2/3
524 pathways in high-fat diet-induced obesity rat model. *Metabolism: clinical and experimental* **76**: 42-
525 55

526

527 Hu MS, Longaker MT (2016) Dipeptidyl Peptidase-4, Wound Healing, Scarring, and Fibrosis. *Plastic*
528 *and reconstructive surgery* **138**: 1026-1031

529

530 Juillerat-Jeanneret L (2014) Dipeptidyl peptidase IV and its inhibitors: therapeutics for type 2 diabetes
531 and what else? *Journal of medicinal chemistry* **57**: 2197-2212

532

533 Kafka M, Collins V, Kamolz LP, Rappl T, Branski LK, Wurzner P (2017) Evidence of invasive and
534 noninvasive treatment modalities for hypertrophic scars: A systematic review. *Wound repair and*
535 *regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair*
536 *Society* **25**: 139-144

537

538 Kaji K, Yoshiji H, Ikenaka Y, Noguchi R, Aihara Y, Douhara A, Moriya K, Kawaratani H, Shirai Y,
539 Yoshii J, Yanase K, Kitade M, Namisaki T, Fukui H (2014) Dipeptidyl peptidase-4 inhibitor attenuates
540 hepatic fibrosis via suppression of activated hepatic stellate cell in rats. *Journal of gastroenterology*
541 **49**: 481-491

542

543 Kanno Y (2019) The Role of Fibrinolytic Regulators in Vascular Dysfunction of Systemic Sclerosis.
544 *International journal of molecular sciences* **20**

545

546 Kant S, van den Kerckhove E, Colla C, van der Hulst R, Piatkowski de Grzymala A (2019) Duration
547 of Scar Maturation: Retrospective Analyses of 361 Hypertrophic Scars Over 5 Years. *Advances in*
548 *skin & wound care* **32**: 26-34

549

550 Lal H, Ahmad F, Zhou J, Yu JE, Vagnozzi RJ, Guo Y, Yu D, Tsai EJ, Woodgett J, Gao E, Force T
551 (2014) Cardiac fibroblast glycogen synthase kinase-3 β regulates ventricular remodeling and
552 dysfunction in ischemic heart. *Circulation* **130**: 419-430

553

554 Lay AJ, Zhang HE, McCaughan GW, Gorrell MD (2019) Fibroblast activation protein in liver
555 fibrosis. *Frontiers in bioscience (Landmark edition)* **24**: 1-17

556

557 Leavitt T, Hu MS, Marshall CD, Barnes LA, Lorenz HP, Longaker MT (2016) Scarless wound
558 healing: finding the right cells and signals. *Cell and tissue research* **365**: 483-493

559

560 Lee HJ, Jang YJ (2018) Recent Understandings of Biology, Prophylaxis and Treatment Strategies for
561 Hypertrophic Scars and Keloids. *International journal of molecular sciences* **19**

562

563 Lodyga M, Hinz B (2019) TGF-beta1 - A truly transforming growth factor in fibrosis and immunity.
564 *Seminars in cell & developmental biology*
565
566 Lotia S, Montojo J, Dong Y, Bader GD, Pico AR (2013) Cytoscape app store. *Bioinformatics (Oxford, England)* **29**: 1350-1351
567
568
569 Luecken MD, Theis FJ (2019) Current best practices in single-cell RNA-seq analysis: a tutorial.
570 *Molecular Systems Biology* **15**: e8746
571
572 Makrilakis K (2019) The Role of DPP-4 Inhibitors in the Treatment Algorithm of Type 2 Diabetes
573 Mellitus: When to Select, What to Expect. *International journal of environmental research and public health* **16**
574
575
576 Menou A, Duitman J, Crestani B (2018) The impaired proteases and anti-proteases balance in
577 Idiopathic Pulmonary Fibrosis. *Matrix biology : journal of the International Society for Matrix Biology* **68-69**: 382-403
578
579
580 Miao L, St Clair DK (2009) Regulation of superoxide dismutase genes: implications in disease. *Free radical biology & medicine* **47**: 344-356
581
582
583 Muramatsu T (2010) Midkine, a heparin-binding cytokine with multiple roles in development, repair
584 and diseases. *Proceedings of the Japan Academy Series B, Physical and biological sciences* **86**: 410-
585 425
586
587 Nabai L, Pourghadiri A, Ghahary A (2020) Hypertrophic Scarring: Current Knowledge of
588 Predisposing Factors, Cellular and Molecular Mechanisms. *Journal of Burn Care & Research* **41**: 48-
589 56
590
591 Nishiguchi MA, Spencer CA, Leung DH, Leung TH (2018) Aging Suppresses Skin-Derived
592 Circulating SDF1 to Promote Full-Thickness Tissue Regeneration. *Cell reports* **24**: 3383-3392.e3385
593
594 Page MJ, Di Cera E (2008) Serine peptidases: classification, structure and function. *Cellular and molecular life sciences : CMLS* **65**: 1220-1236
595
596
597 Qiu X, Hill A, Packer J, Lin D, Ma YA, Trapnell C (2017a) Single-cell mRNA quantification and
598 differential analysis with Census. *Nature methods* **14**: 309-315
599
600 Qiu X, Mao Q, Tang Y, Wang L, Chawla R, Pliner H, Trapnell C (2017b) Reversed graph embedding
601 resolves complex single-cell developmental trajectories. *bioRxiv*: 110668
602

603 Rabieian R, Boshtam M, Zareei M, Kouhpayeh S, Masoudifar A, Mirzaei H (2018) Plasminogen
604 Activator Inhibitor Type-1 as a Regulator of Fibrosis. **119**: 17-27
605

606 Rawlings ND, Barrett AJ (1999) MEROPS: the peptidase database. *Nucleic Acids Res* **27**: 325-331
607

608 Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA,
609 Falanga V, Kehrl JH, et al. (1986) Transforming growth factor type beta: rapid induction of fibrosis
610 and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proceedings of the National*
611 *Academy of Sciences of the United States of America* **83**: 4167-4171
612

613 Rojahn TB, Vorstandlechner V, Krausgruber T, Bauer WM, Alkon N, Bangert C, Thaler FM,
614 Sadeghyar F, Fortelny N, Gernedl V, Rindler K, Elbe-Bürger A, Bock C, Mildner M, Brunner PM
615 (2020) Single-cell transcriptomics combined with interstitial fluid proteomics defines cell-type-
616 specific immune regulation in atopic dermatitis. *Journal of Allergy and Clinical Immunology*
617

618 Schuliga M, Grainge C, Westall G, Knight D (2018) The fibrogenic actions of the coagulant and
619 plasminogen activation systems in pulmonary fibrosis. *Int J Biochem Cell Biol* **97**: 108-117
620

621 Schuliga M, Jaffar J, Harris T, Knight DA, Westall G, Stewart AG (2017) The fibrogenic actions of
622 lung fibroblast-derived urokinase: a potential drug target in IPF. *Scientific reports* **7**: 41770
623

624 Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT
625 (2009) Human skin wounds: a major and snowballing threat to public health and the economy. *Wound*
626 *repair and regeneration : official publication of the Wound Healing Society [and] the European*
627 *Tissue Repair Society* **17**: 763-771
628

629 Shi S, Koya D, Kanasaki K (2016) Dipeptidyl peptidase-4 and kidney fibrosis in diabetes.
630 *Fibrogenesis & tissue repair* **9**: 1
631

632 Sidgwick GP, McGeorge D, Bayat A (2015) A comprehensive evidence-based review on the role of
633 topicals and dressings in the management of skin scarring. *Archives of dermatological research* **307**:
634 461-477
635

636 Snel B, Lehmann G, Bork P, Huynen MA (2000) STRING: a web-server to retrieve and display the
637 repeatedly occurring neighbourhood of a gene. *Nucleic Acids Res* **28**: 3442-3444
638

639 Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM, Stoeckius M, Smibert P, Satija
640 R (2018) Comprehensive integration of single cell data. *bioRxiv*: 460147
641

642 Suzuki T, Tada Y, Gladson S, Nishimura R, Shimomura I, Karasawa S, Tatsumi K, West J (2017)
643 Vildagliptin ameliorates pulmonary fibrosis in lipopolysaccharide-induced lung injury by inhibiting
644 endothelial-to-mesenchymal transition. *Respiratory research* **18**: 177
645
646 Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT,
647 Morris JH, Bork P, Jensen LJ, Mering CV (2019) STRING v11: protein-protein association networks
648 with increased coverage, supporting functional discovery in genome-wide experimental datasets.
649 *Nucleic Acids Res* **47**: D607-d613
650
651 Thielitz A, Vetter RW, Schultze B, Wrenger S, Simeoni L, Ansorge S, Neubert K, Faust J, Lindenlaub
652 P, Gollnick HP, Reinhold D (2008) Inhibitors of dipeptidyl peptidase IV-like activity mediate
653 antifibrotic effects in normal and keloid-derived skin fibroblasts. *The Journal of investigative*
654 *dermatology* **128**: 855-866
655
656 Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, Lennon NJ, Livak KJ, Mikkelsen
657 TS, Rinn JL (2014) The dynamics and regulators of cell fate decisions are revealed by pseudotemporal
658 ordering of single cells. *Nat Biotechnol* **32**: 381-386
659
660 Tredget EE, Shupp JW, Schneider JC (2017) Scar Management Following Burn Injury. *Journal of*
661 *burn care & research : official publication of the American Burn Association* **38**: 146-147
662
663 Uchida T, Oda T, Matsubara H, Watanabe A, Takechi H, Oshima N, Sakurai Y, Kumagai H (2017)
664 Renoprotective effects of a dipeptidyl peptidase 4 inhibitor in a mouse model of progressive renal
665 fibrosis. *Renal failure* **39**: 340-349
666
667 Vallée A, Lecarpentier Y, Guillemin R, Vallée JN (2017) Interactions between TGF- β 1, canonical
668 WNT/ β -catenin pathway and PPAR γ in radiation-induced fibrosis. *Oncotarget* **8**: 90579-90604
669
670 Van Loey NE, Bremer M, Faber AW, Middelkoop E, Nieuwenhuis MK (2008) Itching following
671 burns: epidemiology and predictors. *The British journal of dermatology* **158**: 95-100
672
673 Vorstandlechner V, Laggner M, Kalinina P, Haslik W, Radtke C, Shaw L, Lichtenberger BM,
674 Tschachler E, Ankersmit HJ, Mildner M (2020) Deciphering the functional heterogeneity of skin
675 fibroblasts using single-cell RNA sequencing. *FASEB journal : official publication of the Federation*
676 *of American Societies for Experimental Biology* **34**: 3677-3692
677
678 Wang D, Zhang G, Chen X, Wei T, Liu C, Chen C, Gong Y, Wei Q (2018) Sitagliptin ameliorates
679 diabetic nephropathy by blocking TGF- β 1/Smad signaling pathway. *International journal of*
680 *molecular medicine* **41**: 2784-2792
681
682

684 **Figure Legends**

685 **Figure 1: Characterization of human skin and scar samples with scRNAseq identifies specific** 686 **cell clusters and a distinct fibrotic gene signature.**

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

699 **Figure 2: Subset analysis of fibroblasts**

700 A) Phylogenetic clustertree calculated unsupervised based on UMAP-clustering of subsetted
701 fibroblasts only. B, C) UMAP-plots of re-clustered skin and scar fibroblasts, split by tissue, re-
702 clustering identified 11 fibroblast clusters (subFB1-11). D) Feature plots illustrating computational
703 basis for dotplots, dotplots of top 50 regulated genes (according to lowest adjusted p-value)
704 comparing scar FBs versus skin FBs. E) Dotplot of top 50 regulated genes (according to lowest
705 adjusted p-value) cluster subFB1 compared to all other scar FBs. F) Gene ontology-term network was
706 calculated based on significantly upregulated (adj. p-val < 0.05 , avg.logFC > 0.1) genes comparing
707 subFB1 to all other scar FBs. Gene list was imputed in ClueGO plug-in in Cytoscape with medium

708 GO-specificity, with GO-term fusion, only significant (P value $< .05$) GO terms are shown. Circle size
709 correlates with P value, lines (“edges”) represent functional connection of respective GO terms. Red
710 circles represent association of GO-term with TGF β -signaling, purple, with extracellular matrix,
711 green, with smooth muscle differentiation, blue, with signaling factors, and yellow with bone
712 formation and -development. UMAP, uniform manifold approximation and projection.

713 **Figure 3: Pseudotime analysis of human scar FBs identifies cell fates and time-regulated genes**

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

719 **Figure 4: Two-timepoint mouse scar model identifies genes regulated in scar maturation**

720 A) Illustration of workflow of mouse scar model and two-timepoint scRNAseq. Two mice were
721 analyzed per timepoint. B) Phylogenetic clustertree calculated unsupervised based on unsupervised
722 UMAP-clustering. C, D) UMAP-plots of mouse scar tissue, split by timepoint, after integration of all
723 samples, identifying four fibroblast clusters (mFB1-4), smooth muscle cells and pericytes
724 (mPC/SMC), endothelial cells and lymphatic endothelial cells (mEC/LEC), T-cells, dendritic cells
725 (mDC), Langerhans cells (mLC), nine keratinocyte clusters (KC1-9), adipocytes (mAdipo) and
726 melanocytes (Mel). E, F) Pie charts show relative numbers of cells in clusters, split by timepoint.

727 Feature plots and bar graphs of number of differentially expressed genes (nDEG) per cluster of G)
728 up- and H) downregulated genes per cluster. DEGs were calculated per cluster comparing 8 weeks vs
729 6 weeks old scars using Wilcoxon rank sum test, including genes with average logarithmic fold
730 change (avg_logFC) of > 0.1 or < -0.1 and Bonferroni-adjusted p-value < 0.05 . Feature plots show
731 projection of nDEG onto the UMAP-plot, color intensity represents nDEG. Bar graphs show absolute

732 number of nDEG per cluster, y-axis represents nDEG. UMAP, uniform manifold approximation and
733 projection.

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

736 A) Illustration of computational basis for comparison human and mouse. Human cluster subFB1 vs
737 rest scar FBs significantly (adj. p-value<0,05) regulated genes were compared with mouse scar FBs
738 8 weeks vs 6 weeks significantly regulated genes. B) Venn diagrams of human and mouse up- (upper
739 panel) and down- (lower panel) regulated genes. C) Table of mouse and human mutually up and D)
740 downregulated genes. E-I) Feature plots and violin plots of serine proteases in human skin and scar.
741 *AEBP1* (adipocyte enhancer binding protein 1), *DPP4* (dipeptidyl-peptidase 4), *HTRA1* (High-
742 Temperature Requirement A Serine Peptidase 1), *PLAU* (urokinase), *PRSS23* (Serine protease 23). In
743 violin plots, dots represent individual cells, y-axis represents log2 fold change of the normalized genes
744 and log-transformed single-cell expression. Vertical lines in violin plots represent maximum
745 expression, shape of each violin represents all results, and width of each violin represents frequency
746 of cells at the respective expression level. In feature plots, normalized log expression of the respective
747 gene is mapped onto the UMAP-plot. Color intensity indicates level of gene expressions. UMAP,
748 uniform manifold approximation and projection.

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

751 A-E) Trajectory plots and pseudotime plots of Serine proteases in skin and scar FBs. *AEBP1*
752 (adipocyte enhancer binding protein 1), *DPP4* (dipeptidyl-peptidase 4), *HTRA1* (High-Temperature
753 Requirement A Serine Peptidase 1), *PLAU* (urokinase), *PRSS23* (Serine protease 23). In trajectory
754 plots, normalized log expressions are plotted on the trajectories, split by skin and scar. In pseudotime
755 plots, normalized log expressions are plotted against the pseudotime axis, and a spline curve

756 represents expression dynamics over pseudotime. Y-axis, normalized log expression of respective
757 gene, x-axis, pseudotime.

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

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

763 **Figure 8: Pharmacological inhibition of *DPP4* or urokinase prevents TGF β -induced**
764 **myofibroblast-differentiation**

765 A,B) Western blot of primary FBs stimulated with active TGF β 1 for 24h to differentiate FBs into
766 alpha-smooth muscle actin-expressing (α SMA) myofibroblasts. Myofibroblast differentiation
767 inhibited with A) *DPP4*-inhibitor Sitagliptin or B) urokinase-inhibitor BC-11. C, D) Collagen I or E,
768 F) fibronectin in supernatants of stimulated primary skin FBs, detected by Enzyme-linked
769 Immunosorbent Assay (ELISA). Whiskers represent range maximum and minimum values with < 1.5
770 interquartile range, boxes represent 25th -75th quartiles, line represents mean. Statistical significance
771 was tested using one-way ANOVA with Tukey post-test. NS $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
772 Experiments were performed in duplicates of five donors each.

773 **Figure 9: Signaling pathway activation after TGF β -stimulation and *DPP4*/*PLAU* inhibition**

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

779

780 **Supplementary figure legends**

781 **Figure S1: Identification of cell types by marker genes and marker gene expression patterns in** 782 **human skin and scar**

783 A) Feature Plots of cluster markers *KRT1* (Keratin1) for spinous and granular keratinocytes (KCs),
784 *KRT5* (Keratin 5) for basal KCs, *COL1A1* (collagen I alpha 1) for fibroblasts (FBs), *ACTA2* (smooth
785 muscle actin) for smooth muscle cells and myofibroblasts, *RGS5* (Regulator Of G Protein Signaling
786 5) for pericytes, *SELE* (E-selectin) for endothelial cells, *LYVE1* (Lymphatic Vessel Endothelial
787 Hyaluronan Receptor 1) for lymphatic endothelial cells, *CD3D* (cluster of differentiation 3D) for T-
788 cells, *ITGAX* (Integrin Subunit Alpha X, CD11C) and *CD1A* for dendritic cells, *AIF1* (Allograft
789 Inflammatory Factor 1) for macrophages, and *MLANA* (Melan-A) for melanocytes. In feature plots,
790 normalized log expression of the respective gene is mapped onto the UMAP-plot. B) Heatmap of top
791 10 clustermarker (differentially upregulated genes of each cluster compared to the rest of the dataset).
792 Heatmap shows scaled expression values for genes, rows represent genes, columns represent
793 individual cells. DEGs were calculated per cluster comparing scar versus skin using Wilcoxon rank
794 sum test, including genes with average logarithmic fold change (avglogFC) of > 0.1 or < -0.1 and
795 Bonferroni-adjusted p-value < 0.05 . Feature plot shows projection of nDEG onto the UMAP-plot,
796 color intensity represents nDEG. UMAP, uniform manifold approximation and projection.

797 **Figure S2: Top 50 regulated genes per cell group in scar compared to skin.**

798 In cellgroups, i.e. A) fibroblasts (FBs), B) smooth muscle cells and pericytes (SMC/PCs), C)
799 endothelial cells (ECs), D) T-cells, E) dendritic cells (DCs), and keratinocytes (KCs), differentially
800 expressed genes (DEGs) were calculated comparing scar versus skin using Wilcoxon rank sum test,
801 including genes with average logarithmic fold change (avglogFC) of > 0.1 or < -0.1 and Bonferroni-
802 adjusted p-value < 0.05 . For each cell group, top 50 DEGs according to lowest adjusted p-value are

803 displayed, split by skin and scar. Dot size represents percent of cells expressing the respective gene,
804 color correlates with average expression.

805 **Figure S3: Gene interaction networks of upregulated genes in FBs**

806 STRING networks of gene/protein interactions from A) top 50 regulated genes (according to lowest
807 adjusted p-value) comparing skin FBs versus scar FBs and B) top 50 regulated genes (according to
808 lowest adjusted p-value) cluster subFB1 to all other scar FBs. Lines indicate protein interaction with
809 evidence level according to legend. Bold gene names indicate protein with no hitherto described
810 relation to skin scarring.

811 **Figure S4: Identification of cell types by marker genes and gene expression patterns in mouse** 812 **scars**

813 A) Feature Plots of cluster markers *Krt1* (Keratin1) for spinous and granular keratinocytes (KCs),
814 *Krt5* (Keratin 5) for basal KCs, *Mki67* (Marker Of Proliferation Ki-67) for proliferating cells, *Colla1*
815 (collagen I alpha 1) for fibroblasts; *Pecam* (Platelet And Endothelial Cell Adhesion Molecule 1) for
816 endothelial cells, *Lyve1* (Lymphatic Vessel Endothelial Hyaluronan Receptor 1) for lymphatic
817 endothelial cells, *Acta2* (smooth muscle actin) for smooth muscle cells and myofibroblasts, *Rgs5*
818 (Regulator Of G Protein Signaling 5) for pericytes, *Cd3d* (cluster of differentiation 3D) for T-cells,
819 *Cd14* for dendritic cells, *Cd207* (Langerin) for Langerhans cells, and *Pmel* (Premelanosome Protein)
820 for melanocytes. In feature plots, normalized log expression of the respective gene is mapped onto
821 the UMAP-plot.

822 B) heatmap of top 10 upregulated clustermarker (differentially expressed genes of each cluster
823 compared to the rest of the dataset). Heatmap showing scaled expression values for genes, rows
824 represent genes, columns represent individual cells.

825 **Figure S5: Top 50 regulated genes per cell group in 6 weeks and 8 weeks old murine scars.**

826 In A) fibroblasts (FBs), B) smooth muscle cells and pericytes (SMC/PCs), C) endothelial cells (ECs),
 827 D) T-cells, E) dendritic cells (DCs), and keratinocytes (KCs), differentially expressed genes (DEGs)
 828 were calculated comparing 8 weeks versus 6 weeks old mouse scars using Wilcoxon rank sum test,
 829 including genes with average logarithmic fold change (avglogFC) of > 0.1 or < -0.1 and Bonferroni-
 830 adjusted p-value < 0.05 . For each cellgroup, top 50 DEGs according to lowest adjusted p-value are
 831 displayed, split by timepoint. Dot size represents percent of cells expressing the respective gene, color
 832 correlates with average expression.

833 **Figure S6: Feature Plots and Violin plots of serine proteases**

834 A-C) Feature plots and violin plots of serine proteases in human skin and scar. *DPP7* (dipeptidyl-
 835 peptidase 7), *FAP* (Fibroblast Activation Protein Alpha), *HTRA3* (High-Temperature Requirement A
 836 Serine Peptidase 3). In violin plots, dots represent individual cells, y-axis represents log2 fold change
 837 of the normalized genes and log-transformed single-cell expression. Vertical lines in violin plots
 838 represent maximum expression, shape of each violin represents all results, and width of each violin
 839 represents frequency of respective expression level. In feature plots, normalized log expression of the
 840 respective gene is mapped onto the UMAP-plot. Color intensity indicates level of gene expressions.
 841 UMAP, uniform manifold approximation and projection.

842 **Figure S7: A proteome profiler-assisted identification of signaling pathways after stimulation** 843 **with TGF β and serine protease inhibitors.**

844 Proteome profiler analysis of signaling pathways of primary human skin FBs stimulated with A)
 845 TGF β 1, B) TGF β 1 and DPP4-inhibitor Sitagliptin, and C) TGF β 1 and PLAU-inhibitor BC-11. D)
 846 Legend table for proteome profiler data points.

847

848

849

850 **Conflict of interest**

851 The authors declare no conflict of interest.

852

853 **Acknowledgements**

854 This research project was financed in part by the FFG Grant “APOSEC” (852748 and 862068; 2015-
855 2019), by the Vienna Business Agency “APOSEC to clinic,” (ID 2343727, 2018-2020), and by the
856 Aposcience AG under group leader HJA. MM was funded by the Sparkling Science Program of the
857 Austrian Federal Ministry of Education, Science and Research (SPA06/055). We thank HPH for his
858 belief in this private-public partnership to augment patients' health. We thank the Biomedical
859 Sequencing Facility, Center for Molecular Medicine, Vienna, Austria, for the processing and
860 sequencing of our samples. The authors acknowledge the core facilities of the Medical University of
861 Vienna, a member of Vienna Life Science Instruments.

862

863 **Author contributions**

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

Skin

Scar

(A)

(B)

(C)

(D)