The serine proteases dipeptidyl-peptidase 4 and urokinase are key molecules in human and mouse scar formation

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Despite recent advances in understanding skin scarring, mechanisms triggering hypertrophic scar formation are still poorly understood. In the present study, we investigate mature human hypertrophic scars and developing scars in mice at single cell resolution. Compared to normal skin, we find significant differences in gene expression in most cell types present in scar tissue. Fibroblasts show the most prominent alterations in gene expression, displaying a distinct fibrotic signature. By comparing genes upregulated in murine fibroblasts during scar development with genes highly expressed in mature human hypertrophic scars, we identify a group of serine proteases, tentatively involved in scar formation. Two of them, dipeptidyl-peptidase 4 (DPP4) and urokinase (PLAU), are further analyzed in functional assays, revealing a role in TGFβ1-mediated myofibroblast differentiation and over-production of components of the extracellular matrix in vitro. Topical treatment with inhibitors of DPP4 and PLAU during scar formation in vivo shows anti-fibrotic activity and improvement of scar quality, most prominently after application of the PLAU inhibitor BC-11. In this study, we delineate the genetic landscape of hypertrophic scars and present insights into mechanisms involved in hypertrophic scar formation. Our data suggest the use of serine protease inhibitors for the treatment of skin fibrosis.
Hypertrophic scars are a complex medical problem and a significant global disease burden. In the western world, an estimated number of 100 million people develop scars every year, ~11 million of which bear keloid scars and 4 million suffer from burn scars. In the USA, an estimated amount of 12 billion dollars is spent annually on the treatment of skin scarring. For affected persons, a pathological hypertrophic scar can cause significant functional impairment, pain, pruritus, and a reduction in quality of life.

Wound healing is a tightly coordinated, three-step process, characterized by an acute inflammatory phase, a proliferative phase, and a remodeling phase. Prolonged inflammation results in increased fibroblast (FB) activity, with enhanced secretion of transforming growth factor beta 1 (TGFβ1), TGFβ2, insulin-like growth factor (IGF1), and other cytokines. TGFβ1 drives differentiation of FBs into myofibroblasts, which have a contractile phenotype, are characterized by excessive secretion of ECM-components, and are the major contributors to the formation of hypertrophic scars. Mature hypertrophic scars display strong tissue contraction, and dense, parallel, or whorl-like ECM. Topical silicone application, compression or massage therapy, intralesional injection of triamcinolone (TAC), corticosteroids, or 5-Fluorouracil (5-FU), laser ablation, and surgery are the most commonly used options for prevention or treatment of hypertrophic scars. However, many of these therapies lack evidence of efficacy and safety, show high recurrence rates, and mechanisms of actions are still unclear.

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. Serine proteases/peptidases constitute a large, diverse group of proteases, divided into 13 clans and 40 families. The group of trypsins comprises proteases contributing to vital processes such as blood coagulation, fibrinolysis, apoptosis, and immunity. Members of this family include urokinase, HTRA1/3 (high-temperture requirement A1/3 peptidase), several coagulation factors and complement components, PRSS-like serine proteases, granzymes, and cathepsin G. Inhibitors of PLAU have been shown to counteract fibrotic processes in cardiac and pulmonary fibrosis in human in vitro studies and in mouse experiments. Recently, the serine protease DPP4 became the center of attention, since DPP4 inhibitors (gliptins) have been clinically used for the treatment of diabetes mellitus. DPP4 was also implicated in a variety of fibrotic pathologies, including cardiac, hepatic, renal, and dermal fibrosis, and inhibition of DPP4 activity mitigated fibrotic processes in animal models. However, the contribution of serine proteases to human scar formation and the underlying anti-fibrotic mechanisms are so far not known. Even though scRNAseq was previously performed to identify factors important for embryonic and postnatal skin development as well as for tissue regeneration by investigating murine wound healing, scar tissue on single-cell level has not been investigated yet.

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

**Results**

The single-cell landscape of hypertrophic scars. To elucidate the complex biological processes of scar formation, we performed droplet-based single-cell transcriptome analysis of human hypertrophic scar tissue and healthy skin. In both samples, Unsupervised Uniform Manifold Approximation and Projection (UMAP)-clustering revealed 21 cell clusters, which were further classified as specific cell types by well-established marker genes (Figure S2A), expression patterns of all clusters (Figure S2B), and transcriptional cluster proximity via a phylogenetic cluster tree (Figure 1B). We identified seven FB clusters, smooth muscle cells and pericytes (SMC/Peri), three clusters of endothelial cells (EC), and lymphatic endothelial cells (LECs), two clusters of T cells and of dendritic cells (DC), macrophages (Mac), three keratinocyte (KC) clusters, and melanocytes (Mel).

All cells of specific subsets were clustered together, and skin and scar samples displayed comparable cellular cluster composition (Fig. 1C, D). Only cluster FB1 was mainly present in scar tissue. The clusters of skin and scars showed different relative cell number ratios (Fig. 1E, F). Whereas FBs represented 40% of all cells in healthy skin, a significant increase (53%) was observed in scar tissue. Similarly, we detected more ECs (16.31%) in scar tissue as compared to normal skin (8.1%). Contrary, the relative numbers of epithelial cells (6.37%) and immune cells (12.47%) in mature hypertrophic scars were significantly reduced compared to skin (22.47% and 19.97%, respectively).

When comparing scar to skin, we identified considerably more up- (Fig. 1G) than downregulated genes (Fig. 1H), and the most abundant differential gene expression (number of differentially expressed genes, nDEG) was found in FBs, SMC/PCs, macrophages, DC1 and KC1 (Fig. 1G, H). The top 50 up- and downregulated genes for FBs, SMC/PCs, ECs, T cells, DCs, and KCs are listed in Figure S3. Genes related to ECM production (e.g., COL1A1/2, COL5A1/2, FN1, BGN, LOX, LUM, OGN, PCOLCE) were mainly overrepresented in FBs, but notably also in PCs and ECs (Figure S3A-C). Several significantly regulated genes with so far undescribed roles in fibrosis and scar formation (e.g., ARL4C, COP22, CRABP2, HSPA1A/B, MDK, OGN, among others) were found in all cell types (Figure S3A-F). These distinctly regulated genes might provide valuable new candidates to understand and modulate skin scarring.

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

After subssetting and reclustering of all FBs, we identified 11 separate clusters (Fig. 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 Fig. 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 Fig. 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, e.g., BGN, COL1A1, COL1A2, COL3A1, COL5A1/2, FN1, MMP23B, OGN, PCOLCE (Figure S3). 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 (associated genes: ASPN, COL1A1, COL1A2, FBNI, HSPAI1A, HTRA1, INHBA, JUN, LOX, POSTN, red circles) and ECM-formation (associated genes: AEIIP1, BGN, CCN2, COL1A1, COL1A2, COL1A2, COL3A1, COL5A2, COL6A1, COL6A2, COMP, CREB3L1, DPP4, EGFLL6, FBNI, FN1, HTRA1, LOX, LUM, MFAP2, MMP11, PHLD2, PHLD3, PHLD5, RAG2, RAN3, RTN4L, RTN6, RTN6R1, RTN6R2, THBS1, THBS3, THRB, and TLE4).

**Projection (UMAP)-clustering revealed 21 cell clusters, which were further classified as specific cell types by well-established marker genes (Figure S2A), expression patterns of all clusters (Figure S2B), and transcriptional cluster proximity via a phylogenetic cluster tree (Figure 1B). We identified seven FB clusters, smooth muscle cells and pericytes (SMC/Peri), three clusters of endothelial cells (EC), and lymphatic endothelial cells (LECs), two clusters of T cells and of dendritic cells (DC), macrophages (Mac), three keratinocyte (KC) clusters, and melanocytes (Mel). All cells of specific subsets were clustered together, and skin and scar samples displayed comparable cellular cluster composition (Fig. 1C, D). Only cluster FB1 was mainly present in scar tissue. The clusters of skin and scars showed different relative cell number ratios (Fig. 1E, F). Whereas FBs represented 40% of all cells in healthy skin, a significant increase (53%) was observed in scar tissue. Similarly, we detected more ECs (16.31%) in scar tissue as compared to normal skin (8.1%). Contrary, the relative numbers of epithelial cells (6.37%) and immune cells (12.47%) in mature hypertrophic scars were significantly reduced compared to skin (22.47% and 19.97%, respectively).

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POSTN, SERPINE1, SFRP2, SPARC, TGFBI, THBS1, TNC, VCAN (purple circles) (Fig. 2F), further corroborating its role in skin fibrosis. In addition, our analysis indicated a role of FB1 in processes important for several other cell types, including platelets, smooth muscle cells (associated genes: CCN3, CHN1, IGF1, IGFBP3, PLAT, PLAU, POSTN, SERPINE1, green circles), and cells of the skeletal system (associated genes: CCN2, CCN3, COL12A1, COL14A1, COL1A1, COL1A2, COL3A1, COL5A2, COL6A1, COL6A2, COMP, ECM1, FBN1, FRZB, HYAL2, IGF1, INHBA, LOX, LUM, PAPSS2, POSTN, SFRP2, SFRP4, SOX4, SPARC, TGFBI, VCAN, yellow circles), suggesting paracrine actions of FB1.

Pseudotime calculation and trajectory construction effectively identified possible cell fates and time-regulated genes, even when analyzing cells of only one timepoint35,36. Thus, we next sorted human skin and scar FBs along a pseudotime axis and constructed trajectories (Fig. 3A, B). The trajectories revealed a division at a certain timepoint where FBs divided into two

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

A Workflow of scRNAseq in human skin (n = 3) and scar (n = 3) samples. B Phylogenetic clustertree calculated 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.
branches (Fig. 3C). Whereas the majority of FBs preferentially aligned with branch 1 in normal skin (Fig. 3D), we observed a significantly longer branch 2 with FBs of hypertrophic scar tissue (Fig. 3E). Branched expression analysis identified genes most regulated in a pseudotime-dependent manner in normal skin and hypertrophic scars (Fig. 3F). Interestingly, the collagens COL1A1, COL1A2, and COL3A1, known to contribute to all fibrotic processes, are most upregulated at the end of Branch 2, but are not among the most pseudotime-regulated genes in scar (Fig. 3F). In contrast, other collagens, including COL5A1/2, COL8A1, COL11A1, and COL12A1, dominated the late pseudotime-dependent gene expression in branch 2. The role of these collagens in (hypertrophic) scar is scarcely investigated, and merits further exploration. Together, our trajectory analysis
models the temporal dynamics of gene expression in scars and might provide a basis to target respective genes at different stages of scar development. Interestingly, no genes were strongly regulated directly at the branching point, suggesting cell-fate is already determined at the beginning of pseudotime.

**scRNAseq of murine scars identifies genes involved in scar maturation.** As our approach so far only gave information on the current state of mature scars, we further investigated mechanisms leading to scar formation and maturation, using a murine full-thickness skin wound model (Figure S4A). Whereas scar formation and maturation in humans is a long-lasting process, it only takes up to 80 days in rodents. Although the here used murine scar model does not completely reflect hypertrophic scar formation in humans, the analysis of genes that are regulated in both, human hypertrophic scars and during normal scar formation in mice, might identify the most evolutionarily conserved and most interesting targets for therapeutic interventions.

In order to detect dynamic differences in gene expression related to scar formation rather than wound healing, we compared samples from normal mouse skin, and from mice 6 and 8 weeks after wounding (Fig. 4A). Analogously to the human dataset, the murine scRNAseq dataset was clustered, and cell types were identified using established marker genes (Figure S4B), expression patterns of all clusters (Figure S4C), and transcriptome proximity of clusters via a phylogenetic clustertree (Figure 4B). All clusters aligned homogeneously, and all major skin cell types were represented in normal skin and at both time points after scar development (Fig. 4C, Figure S4). In accordance with human scar tissue, 8-week-old mouse scars contained a higher proportion of murine FBs (mFBs) (32.6%) compared to 6-week-old scars (17.39%), and more immune cells (9.6 versus 6.3%). In contrast, less of the endothelium (2.8 versus 1.5%) and less keratinocytes (63.3 vs 45%) were present (Fig. 4D). We next calculated up- and downregulated genes for FBs, PCs, ECs, T cells, DCs, and KCs, comparing 8 weeks to 6-week-old scars (top 50 are shown in Figure S5A-F). In contrast to human scars, the highest number of differentially expressed genes was found in mFBs and mKCs (Fig. 4E, F), which was most likely due to ongoing epidermal tissue regeneration. Expression of Acta2 and collagen genes showed only minor regulation between 6 and 8 weeks in mFBs (Fig. 4G). In addition, expression of several other matricellular and ECM-modulating proteins, e.g., Fbln1 (Fibulin1), Ogn (osteoglycin), Lum (Lumican), and Pcolce (Procollagen C-Endopeptidase Enhancer), and Tgfb1 (transforming growth factor, beta-induced) increased in mFBs during scar maturation (Fig. 4H). Together, our scRNAseq identified a gene profile specific for scar maturation in mice.

**Serine proteases are strongly upregulated during scar maturation.** To identify genes that are crucial for scar maturation, we next compared our human scar dataset with genes upregulated in mouse scars 6 weeks after wounding (Fig. 5A). While in both datasets only one gene (LEPR) was downregulated, 16 genes were mutually upregulated (Fig. 5B–D). Stuningly, 5 of these genes (AEBP1, DPP4, HTRA1, PLAU, and PRSS23) were members of the superfamily of serine proteases (Fig. 5C, E). All five serine proteases were upregulated in scRNAseq in human scar tissue, particularly in FBs, but also in other cell types (Fig. 5E–J). AEBP1 and PRSS23 expression also increased in ECs and melanocytes, HTRA1 in ECs and KCs, 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 AEBP1 and HTRA1 significantly enriched at the end of branch 2 (Figure S7). Together, these data suggest a major role of serine proteases in scar formation and/or maturation.

The serine proteases DPP4 and urokinase regulate TGFβ1-mediated myofibroblasts differentiation and ECM overproduction. 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 on 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 S8), and immunofluorescence staining of human (Fig. 6A–C) and murine (Figure S8E-G) skin and scars. Immunofluorescence staining revealed expression of urokinase in the dermis and epidermis of healthy skin. In contrast, DPP4 was only present in the dermal compartment of healthy skin. Whereas the expression of DPP4 was significantly increased in the epidermis and dermis of hypertrophic scars in both species, immunofluorescence staining revealed only a slight, not significant upregulation of PLAU in the dermal compartment of hypertrophic scars. Since detection of released proteins by immunofluorescence often shows difficulties, we further quantified urokinase and DPP4 in human tissue biopsies using ELISA. Interestingly, both urokinase and DPP4 were significantly increased in human scar tissue compared to normal skin (Fig. 6F, G).

As TGFβ1 is one of the key inducers of scarring and tissue fibrosis, causing differentiation of FBs to profibrotic myofibroblasts, we hypothesized that the serine proteases interact with TGFβ-signaling. To test this, we performed siRNA-mediated gene knockdown of DPP4 and PLAU in primary FBs from healthy human skin. The knockdown significantly downregulated DPP4 and PLAU mRNA expression levels (Figure S9A, B) and almost completely abolished the production of the respective proteins (Fig. 7A). Knockdown of both genes strongly reduced TGFβ1-mediated expression of alpha-smooth muscle actin (αSMA), a marker for myofibroblasts (Fig. 7B). The reduced αSMA expression was accompanied by a reduced ability to
Fig. 3 Pseudotime analysis of human scar FBs identifies cell fates and pseudotime-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. 
(C) Color code represents pseudotime progression. 
(D, E) Cell trajectories were calculated based on pseudotime values, split by tissue. 
(F) Branched expression analysis modeling (BEAM) of skin and scar fibroblasts. Colors represent q-value, the expression of the respective gene in relation to pseudotime.
contract a matrix of collagen in vitro (Fig. 7C). We also analyzed components of the ECM and found significantly lower expression of different collagens and fibronectin (FN1) in knockdown FBs (Figure S9C-F). While FN1 protein release was strongly reduced (Fig. 7E), protein levels of COL1α1 were not reduced 48 h after gene silencing (Fig. 7D). Of note, transfection of cells led to a strong increase of baseline levels of FN1 and COL1α1, which might be the reason for the weak response to TGFβ1 stimulation.

Next, we assessed these effects using the specific inhibitors for DPP4 (Sitagliptin) and PLAU (BC-11). Both inhibitors were able to abolish TGFβ1-induced αSMA production to a comparable degree as the specific gene knockdown (Fig. 7F, G). Surprisingly, collagen contraction was not inhibited with the inhibitors...
The serine protease inhibitors Sitagliptin and BC-11 improve scar formation by interfering with production and organization of the ECM. We next attempted to assess the effects of Sitagliptin and BC-11 in vivo scar formation in mice (Fig. 8A). Application of the inhibitors did not interfere with wound healing (Fig. 8B), and even showed a slight, non-significant trend toward faster wound closure after application of BC-11 (Fig. 8C). scRNAseq of scars (Fig. 8D–I) showed a lower number of the main matrix producing FB clusters mFB1 and mFB2 in BC-11 stimulated scars after 8 weeks (Fig. 8F, I). The top 50 regulated genes are shown in Figure S11. Treatment of mice scars with BC-11 and Sitagliptin resulted in a slightly higher expression of Colla1, but significantly lower expression of Col3a1, Col5a1, and Fbn1. Interestingly, both inhibitors reduced the expression of their target proteases. Of note, BC-11 treatment do not interfere with canonical or known non-canonical TGFß1 signaling. Figure 7H, indicating off-target or unspecific inhibitor effects. Moreover, Sitagliptin and BC-11 attenuated TGFß1-induced overproduction of the ECM-proteins Colla1 (Fig. 7I), and fibronectin (Fig. 7J) by FBs. These results demonstrate that serine proteases are involved in TGFß1-induced myofibroblast differentiation. Of note, not all observed effects found in FBs deficient for PLAU or DPP4, were mirrored with pharmacological inhibitors. To investigate whether the serine protease inhibitors interfere with TGFß1 signaling, we analyzed the TGFß1-induced SMAD and ERK signaling pathways. Neither knockdown of DPP4 or PLAU nor addition of the inhibitors led to a significant inhibition of the SMAD and ERK1/2 signaling pathway (Figure S10A). To further identify other signaling pathways that might be involved in the action of the serine protease inhibitors, we used a signaling proteome profiler, showing that none of the signaling molecules were blocked by the inhibitors (Figure S10B). Interestingly, the GSKα/β-pathway, known to attenuate fibrotic processes in the heart was significantly activated by BC-11 (Figure S10B-D), indicating a counter-regulatory action. Together, these data suggest that sitagliptin and BC-11 do not interfere with canonical or known non-canonical TGFß1 signaling.

Discussion

Although skin fibrosis has been extensively studied, key mechanisms leading to the development of hypertrophic scars are still not well understood. In addition, treatment options to prevent or treat (hypertrophic) scars are still scarce and not exceptionally effective. In the present study, we used scRNAseq to elucidate the genetic landscape of hypertrophic scar tissue at a hitherto unmet single-cell resolution.

As expected, our scRNAseq analysis confirmed a plethora of previous studies, but also identified numerous genes, which have so far not been described in the context of skin scarring or tissue fibrosis. For example, the cytokines MDK (midkine) and PTN (pleiotrophin), both involved in cell growth, migration, and angiogenesis, were strongly upregulated in scar FBs. In contrast, SOD2/3 (superoxide dismutase 2/3), an enzyme controlling the release of reactive oxygen species (ROS), hence acting as important antioxidant, was strongly downregulated in scar FBs. Intriguingly, failure of ROS-scavenging has already been shown to contribute to hypertrophic scar formation. Another interesting and significantly downregulated gene in scars was SFN (stratifin). As stratfin has been identified as potent collagenase-stimulating factor in FBs, its downregulation in scars suggests a contribution to the maintenance and/or progression of the fibrotic phenotype by preventing matrix degradation. However, we also identified interesting, so far undescribed differences in other cell types. In human SMC/Pericytes for example, we found a strong upregulation of a group of metallothionins (MT1G, MT1E, MT2A, MT1A), which were previously found to be increased in keloid FBs and concomitantly regulated with collagens upon treatment with TGFß, however, their role in hypertrophic scars has yet to be determined. We also identified a rearrangement of T-cell subsets in mouse scar tissue (Figure S12). In the light of a previous publication by Kalekar et al., demonstrating that GATA3-expressing regulatory T cells contribute to FB activation in murine dermal fibrosis, and our finding, that Gata3 is strongly upregulated in mouse scars, it is likely that T-cell subsets contribute significantly to scar formation. However, we were not able
to identify comparable variations in T-cell subsets in human mature hypertrophic scars (Figure S13). It is therefore conceivable that these T-cell subsets play a role in initial scar formation rather than in established mature hypertrophic scars. In that the T-cell subsets but also in several other cell clusters in human scars and mouse scars (Figure S14). Together, these and many other novel factors identified in our study could be important, decisive molecules for the development and/or maturation of hypertrophic scars. Thus, our study has built a basis for future studies.
Fig. 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 remaining scar FBs significantly (adj. p-value <0.05) regulated genes were compared with mouse scar FBs 6 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 Violin plots and feature plots of serine proteases in mouse skin and scars. 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. J Feature plots and violin plots of serine proteases in human skin and scar. AEBP1 (adipocyte enhancer-binding protein 1) (p = 2.22e−16, p = 2.22e−16), DPP4 (dipeptidyl-peptidase 4) (p = 6.8e−9, p = 1.1e−15), HTRA1 (high-temperature requirement A serine peptidase 1) (p = 2.22e−16, p = 2.22e−16), PLAU (urokinase) (p = 2.22e−16, p = 2.22e−16), PRSS23 (serine protease 23) (p = 2.22e−16, p = 4e−14). In violin plots, dots represent individual cells, y-axis represents log 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. A two-sided Wilcoxon-ranked sign test was used in R. NS p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001.

In this study, we analyzed human hypertrophic scars and mouse scar formation on a single-cell level. However, several limitations should be considered. Due to the high costs and the fact that scRNAseq yields large datasets of tens of thousands of cells, thereby smoothing donor and technical variances, low donor numbers are usually justifiable. Nevertheless, the relatively small sample size in our study should be considered a limitation of our study. Differences in body sites between scar tissue and healthy skin, and the fact that healthy skin and scars were not taken from the same donors could affect comparability of the data. However, a recent study by Ascension et al. comparing different single-cell datasets of skin samples from different body regions showed that the major FB populations were consistently present in all donors and body sites, suggesting high comparability.

Furthermore, there are certainly considerable differences between human and murine wound healing; while mouse wounds heal predominantly via contraction promoted by the subcutaneous *panniculus carnosus*, de novo formation and deposition of ECM and subsequent re-epithelialization prevails in human wound healing. However, a study assessing contribution of epithelialization and contraction in mice found that each accounted for 40–60%, and that mouse wound models can thus be considered a valid model also for human wound healing. Moreover, our mouse scarring model does not fully reflect the pathological fibrotic state of human hypertrophic scars. Although mouse models for hypertrophic scars, e.g., subcutaneous bleomycin injection, or tight-skin mice have been described, their transcriptome comparability with human hypertrophic scars is not well investigated. We therefore suggest that future studies testing the efficacy of serine protease inhibitors should be performed in large animal models, e.g., pigs, which better reflect the pathology of human hypertrophic scars. In our experimental model, creams containing protease inhibitors were topically applied on wounds and scar tissue after complete wound closure.
Fig. 6 Immunofluorescence staining confirms elevated expression of PLAU and DPP4 in human and mouse skin and scar. A H&E staining of human skin and scar; immunofluorescent staining of B Urokinase and C DPP4 in human skin and scar. Quantification of staining intensity separate for epidermis and dermis for D urokinase \( (p = 0.329 \text{ dermis}, \ p = 0.815 \text{ epidermis}) \) and E DPP4 \( (p < 0.0001 \text{ dermis}, \ p < 0.0001 \text{ epidermis}) \). For all stainings, \( n = 2-3 \) normal skin samples were analyzed, and \( n = 4 \) scars. From each sample, five regions of interest per sample were quantified. ELISA from human whole skin \( (n = 6) \) and scar \( (n = 6) \) lysate for F urokinase \( (p < 0.0001) \) and G DPP4 \( (p = 0.0037) \) is shown. Statistical significance was tested using two-tailed unpaired Student t-test. Lines and error bars indicate mean and standard deviation. NS \( p > 0.05 \), * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \). Source data are provided as a Source data file.
Whether the actives are able to penetrate wound scabs and/or scar tissue, or the initial treatment on open wounds is already enough to improve scar formation is currently not known. A recent study investigating transdermal resorption of Sitagliptin\(^6\), however, indicates sufficient skin penetration. As literature for BC-11 is scarce, future studies are needed to evaluate its pharmacodynamics and pharmacokinetics properties.

Since we have demonstrated significant differences between specific knockdown of \(PLAU\) and \(DPP4\) and the inhibitors with regard to collagen contraction, it is conceivable that both inhibitors show side effects which have to be fully elucidated in further studies. Finally, histological analysis revealed up to 10\% normal adjacent skin in the mouse scar samples, which slightly impacts our single-cell analysis.

Together, our study provides a genetic landscape of hypertrophic scars which is the basis for further investigations on genes and fibrotic processes hitherto not studied in the context of skin scarring. Our in vitro and in vivo approaches suggest the use of serine protease inhibitors as treatment option for the prevention or improvement of hypertrophic scar development.

**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-66.009/0075-V/3b/2018) and performed in accordance with the Austrian guidelines for the use and care of laboratory animals. Mouse experiments were performed once, repetition of the experiment was not permitted by the ethics committee.

**Scar and skin samples.** Resected scar tissue \((n = 3)\) was obtained from patients who underwent elective scar resection surgery (donor information is provided in...
Fig. 7 Knockdown and pharmacological inhibition of DPP4 or urokinase prevents TGFβ-induced myofibroblast differentiation. A, B Western blot of primary FBs after knockdown of PLAU or DPP4. B Western blot of primary FBs after knockdown of DPP4 or PLAU stimulated with active TGFβ1 for 24 h to differentiate FBs into alpha-smooth muscle actin-expressing (αSMA) myofibroblasts, and western blot quantification (n = 3). scr vs scr-IGFβ1, p = 0.0006; scr-IGFβ1 vs PLAUsi-IGFβ1, p = 0.0010; scr-IGFβ1 vs DPP4si-IGFβ1, p = 0.0017. C Collagen contractility with FBs after knockdown of PLAU or DPP4 and stimulation with or without active TGFβ1. scr vs PLAUunsi p = 0.0194; scr vs scr-IGFβ1 p = 0.0005; scr-IGFβ1 vs PLAUsi-IGFβ1 = 0.0018. D Collagen I (p = 0.005) and E fibronectin (p < 0.001) expression were calculated by pixel density measurement in ImageJ, adjusted to GAPDH and normalized to respective Ctrl values. Experiments were performed in duplicates of n = 5 each. 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 two-way ANOVA with Tukey post-test. N S p < 0.05, * p < 0.01, ** p < 0.001. Source data are provided as a Source data file.

Table S1). Scars were classified as hypertrophic, pathological scars according to POSAS70 by a plastic surgeon. Only mature scars, which had not been treated before and persisted for more than 2 years were used for all experiments. All donors had no known chronic diseases and received no chronic medication. The quality of scar tissue was assessed by histological analysis. No adjacent normal skin was observed in any of the scar samples. Healthy skin (n = 3) was obtained from female donors between 25 and 45 years from surplus abdominal skin removed during elective abdominoplasty.

Mouse full skin wounding and scar maturation. Female Balb/c mice bred at the animal facility of the Medical University of Vienna (Himberg, Austria) were housed under specific-pathogen-free conditions at 22 ± 2 °C room temperature and 55 ± 10% humidity, with 12 h/12 h light/dark cycles and food and water access ad libidum. Female mice were used due to easier handling and better experimental compliance, which was necessary to enable frequent handling and application of wound dressings. Mice were randomly assigned to the different treatment groups. Immediately after wounding, mice were treated with control or inhibitors by applying ointments or protein inhibitors, Sitagliptin (Ultrasicc/Ultrabas and 1 part water) were mixed and used as control treatment. For wound healing, mice were treated with control or inhibitors by applying ointments or protein inhibitors, Sitagliptin (Ultrasicc/Ultrabas 1:2; Hecht-Pharma, Bremervörde, Germany) was used as carrier substance for all treatments. Four parts concentration 5 mM) were dissolved in water and mixed with the ointment. Immediately after sorting, viable cells were loaded on a 10X-chromium instrument (single-cell gene expression 3’v2/3, 10X Genomics, Pleasanton, CA, USA) to generate GEMs. GEM-generation, library preparation, RNA-sequencing, demultiplexing, and counting were done by the Biomedical Sequencing Core Facility of the Center for Molecular Medicine (CeMM, Vienna, Austria). Sequencing was performed on an Illumina HiSeq 3000/4000 (Illumina, San Diego, CA, USA) with 3 samples per lane, 2 × 75 bp, and paired-end sequencing.

Cell-genome matrix preparation and downstream analysis. Raw sequencing files were demultiplexed, aligned to the human or mouse reference genome (GrCh38/mm10) and counted using the Cellranger pipelines (Cellranger v3, 10X Genomics). The resulting cell-genome matrices were processed using the ‘Seurat’ package (Seurat v3.1.0, Satija Lab, New York, NY, USA) to generate GEMs. GEM-generation, library preparation, RNA-sequencing, demultiplexing, and counting were done by the Biomedical Sequencing Core Facility of the Center for Molecular Medicine (CeMM, Vienna, Austria). From each sample, unwanted variations and low-quality cells were filtered by removing cells with high and low (>3000 and <200) unique molecular identifier (UMI)-counts. First, healthy skin and scar samples were integrated separately to avoid clustering according to donors, and for batch correction. Subsequently, skin and scar data were integrated again into one dataset. Data integration was performed according to the recommended workflow by Butler et al. and Stuart et al.36,37. After quality control comparing all donors, we obtained transcriptome data from a total of 25,083 human skin and scar cells, with a median of 24,943 reads and 851 detected genes per cell. In mice, we obtained data from 6561 cells 6 weeks after wounding, and 9393 cells 8 weeks after wounding. The samples displayed a median of 24,774 reads per cell, and median of 1969 detected genes per cell. After quality control, all mouse samples were integrated together in one integration step. In both datasets, normalized count numbers were used for differential expression analyses with Seurat v3.1.0. More than 2000 genes were identified as differentially expressed genes in skin and scar samples. After quality control, all mouse samples were integrated together in one integration step. In both datasets, normalized count numbers were used for differential expression analyses with Seurat v3.1.0. More than 2000 genes were identified as differentially expressed genes in skin and scar samples. 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 cause technical background noise and batch effect in mouse scRNAseq, as described before34, and were thus excluded from the dataset.

Pseudotime analyses. Pseudotime analyses, trajectory construction, and calculation of pseudotime-dependent gene expression were performed in Monocle2 (Monocle2, v2.14.0, Trapnell Lab, University of Washington, Seattle, WA, USA). From the integrated FB subset Seurat-obtained data were integrated 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 performed9,35,85,75. As input for pseudotime ordering, differentially expressed genes between skin and scar were used, and trajectories were constructed with DDTree (package ‘DDTree’ v0.1.5, 2015)36.

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 Cytoscape v3.7.2 with medium GO-sensitivity, with

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GO-term fusion, and only significant (P value < 0.05) GO terms are depicted as circles, whereby circle size correlates with P value, and lines represent functional connection of respective GO terms.

**Immunofluorescence staining.** Immunofluorescence staining on formalin-fixed, paraffin-embedded (FFPE) sections of skin and scar tissue were performed according to the protocol provided by the respective antibody manufacturer as described previously. In brief, sections were deparaffinized in xylene and alcohol, antigen retrieval was performed with pH6 citric acid buffer, sections were washed in phosphate-buffered saline, and incubated with antibodies overnight at 4 °C. Sections were washed again, and incubated with secondary antibodies, blocking sera of secondary antibodies species, and Hoechst, for 1 h at room temperature. Antibodies were used as indicated in Table S2. After the last wash, sections were mounted in aqueous mounting medium. Stainings were photographed using an Olympus BX63 microscope (Olympus, Tokyo, Japan) with Olympus CellSens Dimension v2.3 (Olympus) software with standardized exposure time for all samples. Staining intensity was quantified separately in dermis and epidermis using ImageJ v1.53c. For dermal quantification, regions of interest (ROIs) of 70 × 70 pixels were directly adjacent and parallel to the epidermis, contained no hair follicles or blood vessels, and were obtained from all regions of the specimen. For epidermal quantification, ROIs of 30 × 30 pixels located within the epidermis from all regions of each specimen. The total corrected fluorescence was measured by subtracting background values from area integrated density in the respective ROI.
Picrosirius red staining. Picrosirius Red staining was performed according to the manufacturer’s protocol of the staining kit (ab150681, Abcam, Cambridge, UK).

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

TGFβ1-induced myofibroblast differentiation. After the first passage, isolated primary FBs were plated in 6-well plates, supplied with DMEM + 10% fetal bovine serum (FBS, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific) and grown until 100% confluency. FBs were then stimulated with 10 ng/ml TGFβ1 (HEK-293-derived, Peprotech, Rocky Hill, NJ, USA), and with or without DPP4 inhibitor Sitagliptin (10 µM) (Thermo Fisher Scientific) or urokinase-inhibitor BC-11 hydroiodide (10 µM) (Tocris by Bio-Technne, Bristol, UK) for 24 h. Supernatants were removed and medium and inhibitors were resupplied for another 24 h. Supernatants were collected and stored at −80 °C and cells were lyzed in 1x Laemmli Buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for further analysis. To analyze signaling pathways, FBs were stimulated with TGFβ1 and inhibitors for 1 h, and then harvested in 1x Laemmli Buffer with protease inhibitor (Complete, MiniProtease Inhibitor Cocktail Tablets, Roche, Basel, Switzerland) and phosphatase inhibitor (Pierce Phosphatase Inhibitor Mini Tablets, Thermo Scientific).

siRNA-mediated gene silencing. Small interfering RNA (siRNA) transfection was conducted according to the previously published protocol88 with minor modifications. siRNAs targeting PLAU (HS1108076, Thermo Fisher Scientific) and DPP4 (HS1102872, Thermo Fisher Scientific) were used. Briefly, primary human FBs of 3rd passage were transfected using Lipofectamine 2000 (Thermo Fisher Scientific). A total of 5 µl of Opti-MEM medium (+-L-Glutamine, 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, Phenol Red; Gibco by Life Technologies) were mixed with 50 µl of Lipofectamine 2000 and 65 µl of a 20 µM small interfering RNA, or scrambled control RNA (Low GC Duplex: Thermo Fisher Scientific). After 15 min incubation, the solution was added to 20 ml DMEM medium and FBs were further incubated with DMEM and gels with normal fibroblast were either treated with sitagliptin (10 µM) and BC-11 (10 µM) or left untreated. Collagen gels were further maintained in the absence or presence of TGFβ1 (10 ng/ml). After 48 h, gels were photographed, and gel areas were calculated using ImageJ software.

Western blotting. Primary FBs were lysed in 1x Laemmli Buffer (Bio-Rad Laboratories, Inc.) and loaded on 4-15% SDS-PAGE gels (Bio-Rad Laboratories, Inc.). Proteins were transferred on a nitrocellulose membrane (Bio-Rad Laboratories, Inc.), membranes were blocked in non-fat milk with 0.1% Tween 20 (Sigma-Aldrich for h, and incubated with antibodies as indicated in Table S2 at 4 °C overnight. After washing, membranes were incubated with horseradish-peroxidase conjugated secondary antibodies as indicated in Table S2 for 1 h at room temperature. Signals were developed with SuperSignal West Dura substrate (Thermo Fisher Scientific) and imaged with a Gel Doc XR + device (Bio-Rad Laboratories, Inc.). Quantification analysis was performed with the Volume tool in ImageLab 6.0.1 (Bio-Rad), adjusted to GAPDH expression, and normalized to respective Ctrl samples to calculate fold change to Ctrl.

Proteome profiling of signaling pathways. To analyze signaling pathways, we used a proteome profiler for human phospho-kinases (ARY003C, R&D Systems, Biotechnie, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Enzyme-linked immunosorbent assay (ELISA). Supernatants of TGFβ1-stimulated FBs after gene knockdown and treatment with protease inhibition were collected, centrifuged, and stored at −20 °C for further use. Protein levels of human procollagen Iα1 ELISA (R&D Systems) and human fibronectin ELISA (R&D Systems) were measured according to the manufacturer’s manual. Absorbance was detected by FluoroStar Optima microplate reader (BMG Labtech, Ortenberg, Germany).

Scar planimetry. Collagen bundle alignment has been calculated with Curvealign V4.0 Beta, a curvelet transform-based, open-source MATLAB software. Images of H&E-stained tissues were edited by Adobe Photoshop CS6 (Adobe Inc, San Jose, CA, USA) to adapt the collagen color, contrast, brightness, and in some cases alignment as comparable value for the relative fiber alignment for each region was calculated. In total, 14 regions of interest calculated from 5 images taken from 4 to 5 animals for each condition.

RNAseq in situ hybridization. FFPE-sections of human skin and scar tissue were prepared according to RNAscope (ACDBio, Bio-Technie, Bristol, UK) pre-treatment protocol, hybridized with probes targeting human DPP4 (RNAscope® Probe-Hs-DPP4) and PLAU (RNAscope® Probe-Hs-PLAU), and visualized with
RNAscope 2.5 HD Assay—RED as suggested by the manufacturer. Images were acquired by AX70 microscope (Olympus, Tokyo, Japan) using the imaging software MetaMorph (Olympus).

**Statistical analyses.** Two groups with normally distributed data were compared by student’s t test. Data of three and more groups were compared by one-way ANOVA with Tukey post hoc test. All statistical analyses were performed in GraphPad Prism v8.0.1 (GraphPad Software, San Diego, USA). P-values were marked in figure using asterisks indicating *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**Data availability**  The scRNAseq data generated in this study have been deposited in the NCBI GEO database under accession "GSE156326". The raw sequencing data are protected and are not available due to data privacy laws. If raw sequencing data are absolutely necessary for replication or extension of our research, they will be made available upon request to the corresponding author within a 2-week timeframe. All other relevant data supporting the key findings of this study are available within the article and its Supplementary.
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**Acknowledgements**

This research project was financed in part by the FFG Grant “APOSEC” (852748 and 862068; 2015-2019), by the Vienna Business Agency “APOSEC to clinic,” (ID 2343727, 2018-2020), and by the ApoScience AG under group leader HIA. MM was funded by the Sparkling Science Program of the Austrian Federal Ministry of Education, Science and Research (SPA06/055). We thank the HPH Haselsteiner and the CRISCAR Familien-stiftung for their belief in this private public partnership to augment basic and translational clinical research. We thank Stefan Spalt for his support. The authors acknowledge the core facilities of the Medical University of Vienna, a member of Vienna Life Science Instruments.

**Author contributions**

M.M., H.J.A., E.T. and V.V. provided study conception and design; W.H. and C.R. provided patient sample material; H.J.A. and M.M. acquired funding; V.V., D.C., K.K., M.D., Y.C. and B.G. conducted experiments and prepared samples; V.V., M.L. and M.M. performed data analysis, visualization and figure design; V.V., M.L., K.K., E.T., K.H. and M.M. participated in data interpretation; V.V., M.L. and M.M. drafted the manuscript. All authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information**

The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-26495-2.

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**Peer review information**

*Nature Communications* thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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