Single-cell RNA sequencing identifies a migratory keratinocyte subpopulation expressing THBS1 in epidermal wound healing

Highlights
- Single-cell RNA sequencing reveals a keratinocyte subpopulation expressing THBS1
- LPA and ERK activity are required for the induction of THBS1+ keratinocyte
- Thbs1+ keratinocytes are differentiated from basal keratinocytes upon epidermal wounding
- Thbs1+ keratinocytes are migratory cells and may contribute to epidermal wound healing
Single-cell RNA sequencing identifies a migratory keratinocyte subpopulation expressing THBS1 in epidermal wound healing

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SUMMARY
Keratinocyte differentiation is an intricate process that is regulated by multiple mediators. Using cultured human keratinocytes, we found that lysophosphatidic acid (LPA) induced the differentiation of a previously unsuspected keratinocyte subpopulation expressing the extracellular matrix protein, thrombospondin-1 (THBS1). This action of LPA was mediated by the RHO/ROCK-SRF signaling downstream of LPA1 and LPA5 receptors and required ERK activity. Suppression of THBS1 in vitro suggested a migratory role of THBS1+ keratinocytes. Moreover, we analyzed publicly deposited single-cell RNA sequencing dataset and identified Thbs1-expressing keratinocytes in the mouse wound skin. Immunohistochemistry analysis revealed that Thbs1+ keratinocytes were apparently differentiated from basal keratinocytes upon wounding, subsequently polarized and migrated suprabasally toward the wound front, and eventually underwent terminal differentiation in the neo-epidermis. Importantly, inhibition of Erk activity suppressed Thbs1+ keratinocyte differentiation in wound healing. Based on these findings, we suggest that THBS1+ keratinocyte is a migratory keratinocyte subpopulation that facilitates epidermal wound healing.

INTRODUCTION
The epidermis of human skin functions as a barrier and protects our body against dehydration, physical damage, external antigen exposure, and pathogen invasion (Elias, 2007). As the predominant cell type in the epidermis, keratinocytes represent approximately 95 percent of the total epidermal cells (McGrath et al., 2004). They are conventionally classified into three different subpopulations namely the basal, spinous, and granular keratinocytes that reside in their corresponding layers of the epidermis and are covered by the outermost stratum corneum (McGrath et al., 2004). In homeostasis, basal keratinocytes proliferate and subsequently differentiate into spinous and then granular keratinocytes, and the balance of this differentiation process is regulated by multiple growth factors and bioactive mediators (An et al., 1998; Kim et al., 2004).

Lysophosphatidic acid (LPA) is a lipid mediator of the lysophospholipid family and multiple species of LPA have been detected in vivo (Aoki et al., 2008; Aikawa et al., 2015). It is produced either from lysophospholipids by the action of lysophospholipase D/autotaxin in the serum or from phosphatidic acid by phospholipase A1 or A2 on the cell membrane (Aoki et al., 2008). In the skin, it is known that LPA could be synthesized through the catalytic activity of the enzyme mPA-PLA1α (or LIPH) that is expressed in epidermal keratinocytes (Aoki et al., 2008; Diribarne et al., 2012). As a bioactive mediator, LPA is involved in various cellular processes such as cell proliferation, anti-apoptosis, migration, cytokine and chemokine secretion, platelet aggregation, contraction and transformation of smooth muscles, and neurite retraction (Aikawa et al., 2015). It is known that LPA exerts its physiological functions through six cognate G protein-coupled receptors (GPCRs), LPA1–6 (Aikawa et al., 2015; Yung et al., 2015). In the skin, it was previously reported that LPA regulates hair follicle development through LPA4 (Shimomura et al., 2008; Pastermack et al., 2008; Inoue et al., 2011). Moreover, we have recently reported that LPA facilitates the differentiation of basal keratinocytes to granular keratinocytes through the RHO/ROCK-SRF signaling downstream of LPA1 and LPA5, and this action of LPA enhances skin barrier function in vivo (Sumitomo...
Figure 1. LPA induced THBS1+ keratinocytes in vitro
(A) Overview of the workflow for scRNA-seq analysis of NHEKs.
(B) Two-dimensional visualized with uniform manifold approximation and projection (UMAP) of cells that passed the quality control (n = 119), colored according to unsupervised clustering. Round mark (○) indicates control NHEKs and triangle mark (△) indicates cells treated with 10 µM LPA for 72 h. Bar chart on the right indicates the percentage of cells assigned to each cluster per condition, colored according to unsupervised clustering.
seq data (n = 119) using graph-based clustering in Seurat (Gribov et al., 2010) identified three different treatment group were subjected for downstream analysis. Unsupervised clustering of integrated scRNA-injured skin (Mazereeuw-Hautier et al., 2005) and has been shown to promote wound healing (Demoyer 2019). LPA is one such mediator that was detected at relatively high concentrations in blister fluids of profiles of NHEKs stimulated with either vehicle or 10 μM LPA for 72 h. Scale bars, 20 μm.

Skin wound healing is a highly dynamic process in which different cell types work together in a coordinated fashion under the tight control of various wound-induced mediators (Cañedo-Dorantes and Cañedo-Ayala, 2019). LPA is one such mediator that was detected at relatively high concentrations in blister fluids of injured skin (Mazereeuw-Hautier et al., 2005) and has been shown to promote wound healing (Demoyer et al., 2000; Balazs et al., 2001). Previous works have proposed that LPA facilitates wound healing through various different mechanisms such as debris clearance via macrophage accumulation and wound contraction via increased contractility of fibroblasts and collagen matrix (Mio et al., 2002; Zhao et al., 2005; Abe et al., 2007). However, how LPA directly modulates keratinocyte functions in the wound healing process remains largely unknown and needs further investigation.

Single-cell RNA sequencing (scRNA-seq) is a novel and powerful technology that allows the detection and quantification of gene expression at single-cell resolution (Kolodziejczyk et al., 2015). Since its first introduction in 2009 (Tang et al., 2009), with subsequent technology breakthroughs and reducing cost, scRNA-seq has been rapidly introduced into various fields of biology and medicine. By enabling the transcriptomic profiling of thousands of cells in parallel, scRNA-seq not only uncovers the uniqueness of each cell within a population but also helps researchers to address various biological questions regarding new cell types/phenotypes, cellular state transition, developmental dynamics, gene regulatory networks, and allele-specific gene expression (Gru¨ n et al., 2015; Rizvi et al., 2017; Xue et al., 2013; Benitez et al., 2017). In the field of skin biology, several scRNA-seq studies on mouse and human skin have been recently reported (Joost et al., 2016, 2018, 2020; Cheng et al., 2018; Finnegan et al., 2019; Haensel et al., 2020; Reynolds et al., 2021). These studies have revealed the marked heterogeneity of keratinocytes in skin both in homeostasis and under pathophysiological conditions. In this study, we employed scRNA-seq to further unravel LPA actions on keratinocyte differentiation and its previously unsuspected roles in epidermal wound healing.

RESULTS

LPA induced a novel THBS1+ keratinocyte subpopulation in vitro

We previously reported that LPA facilitates the differentiation of granular keratinocytes using normal human epidermal keratinocytes (NHEKs) isolated from neonatal foreskin (Sumitomo et al., 2019). However, in our previous study, besides FLG+ granular keratinocytes, a fraction of keratinocytes was stained negative for FLG under LPA stimulation. Moreover, the morphology of these keratinocytes was distinct from undifferentiated NHEKs in the control condition. Based on these observations, we hypothesized that LPA might also induce a subpopulation of keratinocytes that is different from the control NHEKs and granular keratinocytes. To test this hypothesis, we employed scRNA-seq to dissect the transcription profiles of NHEKs stimulated with either vehicle or 10 μM LPA for 72 h at single-cell resolution (Figure 1A). After quality control (Figures S1A–S1G), 59 cells from the control group and 60 cells from the LPA treatment group were subjected for downstream analysis. Unsupervised clustering of integrated scRNA-seq data (n = 119) using graph-based clustering in Seurat (Gribov et al., 2010) identified three different subpopulations of keratinocytes (Figure 1B, left). It should also be noted that most of vehicle-treated control cells were classified in cluster 1 keratinocytes, whereas the majority of LPA-treated cells were classified in cluster 2 and 3 keratinocytes (Figure 1B, right). We further conducted differential expression genes (DEGs) analysis on each cluster to identify the specific marker genes for each cluster (Table S1). We found

Figure 1. Continued

(C) Feature plot showing Thrombospondin-1 (THBS1) expression projected on UMAP.

(D) LPA (10 μM) induced THBS1 gene expression as quantitated by qRT-PCR. Normalized fold changes are shown using GAPDH as the reference. Results are represented as means ± SEM, n = 3 for each condition. **p < 0.001 (two-tailed Student’s t test).

(E) Representative stacked confocal images of immunofluorescence staining of nuclei (Hoechst 33342, magenta) and THBS1 (green) in NHEKs treated with vehicle or 10 μM LPA for 72 h. Scale bars, 20 μm.

(F) Quantification of THBS1+ cell number per area of 0.1 mm² induced by different LPA concentrations. Positive cells were counted over 10 representative pictures per condition (duplicates) and results are shown as means ± SEM. **p < 0.001 (Kruskal-Wallis test followed by Dunn’s multiple comparisons test).

(G) Representative stacked confocal images of immunofluorescence staining of nuclei (Hoechst 33342, magenta), THBS1 (green), and the granular keratinocyte marker, FLG (cyan) of NHEKs stimulated with 10 μM LPA for 72 h. Reconstituted image on the x-z axis is shown below. Note the different spatial location of THBS1+ keratinocytes (white arrows) in the bottom cell layer and FLG+ keratinocytes (white arrowheads) in the upper cell layers. Scale bar, 20 μm.

(H) Linescan profiles of the signal intensities of THBS1 and FLG along the dotted white line shown in the corresponding images in (G).
that cluster 1 keratinocytes expressed the highest levels of basal keratinocyte markers, KRT5 and KRT14, among the 3 clusters, and therefore annotated them as basal-like keratinocytes (Figure S2A). On the other hand, given its high expression of early granular keratinocyte markers such as TGM1 and SCEL and partial expression of late granular keratinocyte markers such as IVL and FLG, cluster 2 was annotated as granular-like keratinocytes (Figure S2A). Interestingly, we found that cluster 3 keratinocytes significantly expressed high levels of thrombospondin-1 (THBS1), a matricellular protein involved in tissue repair (Tan and Lawler, 2009; Adams and Lawler, 2011; Sweetwyne and Murphy-Ullrich, 2012; Resovi et al., 2014) and therefore, annotated cluster 3 as THBS1-expressing keratinocytes (Figures 1C and S2B). Gene set enrichment analysis (GSEA) further indicated a number of gene sets significantly enriched in THBS1-expressing keratinocytes compared to the basal keratinocytes, especially those involved in cell migration such as actomyosin, anchoring, and cell-substrate junction (Figure S2C and Table S2). Moreover, we generated violin plots for genes listed in GO term “EMT (epithelial-mesenchymal transition)” and found that some of representative EMT genes such as HMGA2, FLNA, TGFBR1, and NOTCH1 were highly expressed in cluster 3 (Figure S2D). Given these results, we deduced that LPA promotes the differentiation of NHEKs to two cell populations, granular-like keratinocytes and a unique subpopulation of keratinocytes specifically expressing THBS1 with enriched migratory signatures.

To validate the LPA-induced THBS1-expressing keratinocytes as suggested by scRNA-seq analysis, we next examined THBS1 gene expression by quantitative RT-PCR (qRT-PCR) and confirmed that LPA significantly enhanced THBS1 expression (Figure 1D). Moreover, we examined the presence of THBS1-expressing keratinocytes by immunocytochemistry for THBS1. Although THBS1 was rarely detected in vehicle-treated control NHEKs, it was clearly observed in the perinuclear region of a fraction of cells treated with LPA (Figure 1E). Given that the perinuclear staining of THBS1 in ER and Golgi apparatus is consistent with THBS1 staining pattern observed in other cell types (Cunha et al., 2016; Jerónimo et al., 2017), we defined keratinocytes detected with such perinuclear THBS1 signals as THBS1 keratinocytes. We found an increased number of THBS1 keratinocytes in an LPA concentration-dependent manner, whereas they were rarely detected among vehicle-treated control NHEKs (Figure 1F). Notably, THBS1 keratinocytes in LPA-treated NHEKs tended to form small clusters consisting of several cells (Figure 1E). Co-staining with a different anti-THBS1 antibody showed a complete overlap of perinuclear signals, further supporting that these signals specifically represent THBS1 (Figures S3A and S3B).

In addition, co-immunostaining for THBS1 and FLG revealed that LPA-induced THBS1 keratinocytes were distinct from FLG granular keratinocytes (Figure 1G). They were always mutually exclusive. Notably, there was a remarkable difference in the three-dimensional spatial localization between these two subpopulations in differentiated-multilayered NHEKs such that THBS1 keratinocytes were mainly located at the bottom, whereas FLG keratinocytes were localized on the top layers (Figure 1H). Taken together, these results indicate that LPA induced THBS1 keratinocytes in addition to FLG granular keratinocytes in vitro.

**Characterization of THBS1 keratinocyte subpopulation induced by LPA**

We next conducted immunocytochemistry to further examine the characteristics of THBS1 keratinocytes induced by LPA. First, we treated NHEKs with vehicle or 10 μM LPA for 72 h and conducted co-staining of THBS1 and Ki67, a cell proliferation marker. Both THBS1 Ki67 keratinocytes and THBS1 Ki67 keratinocytes could be observed upon LPA treatment (Figure 2A), suggesting that THBS1 keratinocytes were composed of both proliferative cells and cells that exit the cell cycle at this time point. It should be noted that LPA did not trigger apoptosis in NHEKs as TUNEL staining showed no significant difference between vehicle control and LPA-treated conditions (Figures 2B and 2C). Therefore, LPA induced THBS1 keratinocytes without affecting cell apoptosis.

We next conducted small interfering RNAs (siRNAs)-mediated knockdown experiments to investigate the function of THBS1 produced by THBS1 keratinocytes (Figure 2D). Given the involvement of THBS1 in wound healing previously reported in literature (DiPietro et al., 1996; Agah et al., 2002; Nor et al., 2005), we examined the wound-healing capability of keratinocytes upon THBS1 knockdown using an in vitro scratching assay. Time-lapse imaging revealed that knockdown of THBS1 resulted in slower wound healing compared to the control (Figure 2E and Video S1). Quantification of the reepithelialized area versus time further revealed a significant delay in cell migration upon THBS1 knockdown (Figure 2F). These results suggest a critical role of THBS1 produced by THBS1 keratinocytes in epidermal wound healing.
THBS1+ keratinocyte induction was regulated by Rho/ROCK-SRF signaling downstream of LPA1 and LPA5 receptors

We previously reported that LPA promotes NHEK differentiation to FLG+ granular keratinocytes via LPA1 and LPA5 receptors (Sumitomo et al., 2019). Given these findings, we next examined the involvement of LPA1 and LPA5 receptors in THBS1+ keratinocyte differentiation with siRNAs. We selectively knocked down the expression of LPAR1, LPAR5, or both in NHEKs. siRNA for each receptor was confirmed to effectively knockdown the respective receptor (Figures 3A and 3B). Single knockdown of LPAR1 suppressed LPA-induced THBS1 expression by ~60%, single knockdown of LPAR5 by ~15%, and combined

Figure 2. Characterization of in vitro LPA-induced THBS1+ keratinocytes

(A) Representative stacked confocal images of immunofluorescence staining of THBS1 (cyan), Ki67 (green), and nuclei (Hoechst 33342, magenta) in NHEKs stimulated with 10 μM LPA for 72 h. White arrows indicate THBS1+Ki67+ keratinocytes and white arrowheads indicate THBS1+Ki67- keratinocytes. Scale bar, 20 μm.

(B) Representative stacked confocal images of immunofluorescence staining of TUNEL (green), and nuclei (Hoechst 33342, magenta) in NHEKs treated with vehicle or 10 μM LPA for 72 h. White arrows indicate TUNEL+ keratinocytes. Scale bars, 20 μm.

(C) Quantification of the percentage of TUNEL+ keratinocytes per total keratinocytes induced by 10 μM LPA for 72 h. Positive cells were counted over 10 representative pictures per condition (triplicates) and results are shown as means ± SEM. There was no statistical significance between the two conditions (two-tailed Student’s t test).

(D) siRNA-mediated knockdown of THBS1 in NHEKs. Suppression of THBS1 gene expression at the time of stimulation (24 h after siRNA transfection) is shown. Normalized fold changes are shown using GAPDH as the reference. Results are represented as means ± SEM, n = 3 for each condition. **p < 0.01 (two-tailed Student’s t test).

(E) Representative phase contrast images of control and THBS1-knockdown NHEKs after wound scratching every 2 h. Time is relative to wound scratching. Scale bars, 50 μm.

(F) Normalized wound area (wound area at each time point normalized to wound area at 0 h) as imaged in (E) is plotted versus time. Data is shown as means ± SD, n = 3 for each condition. **p < 0.01 (two-tailed Student’s t test at each time point).

THBS1+ keratinocyte induction was regulated by Rho/ROCK-SRF signaling downstream of LPA1 and LPA5 receptors

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Figure 3. LPA induced THBS1+ keratinocytes through Rho/ROCK-SRF signaling downstream of LPA₁/LPA₅ receptors

(A and B) siRNA-mediated single and double knockdown of LPAR1 and LPAR5 in NHEKs. LPAR1 and LPAR5 gene expressions were measured by qRT-PCR at the time of LPA stimulation (24 h after siRNA transfection). Normalized fold changes are shown using GAPDH as the reference. Results are represented as means ± SEM, n = 3 for each condition. *p < 0.05, **p < 0.01 (compared with the control siNT group) (one-way ANOVA followed by Bonferroni’s post-hoc comparisons test).

(C) THBS1 gene expression in LPAR1-knockdown, LPAR5-knockdown, or LPAR1/5-double knockdown NHEKs upon LPA stimulation for 72 h. Normalized fold changes are shown using GAPDH as the reference. Results are represented as means ± SEM, n = 3 for each condition. *p < 0.05 (compared with the vehicle control of the indicated treated group) (Kruskal-Wallis test followed by Dunn’s multiple comparisons test), #p < 0.05, ##p < 0.01 (compared with the siNT control of the corresponding LPA stimulation) (Kruskal-Wallis test followed by Dunn’s multiple comparisons test).

(D) 10 μM LPA-treated NHEKs in the absence or presence of Y-27632 (30 μM). LPA-induced THBS1 gene expression at 72 h post-stimulation is shown.

(E) 10 μM LPA-treated NHEKs in the presence or absence of CCG-1423 (30 μM). LPA-induced THBS1 gene expression at 72 h post-stimulation is shown.
knockdown of LPAR1 and LPAR5 additively suppressed LPA-induced THBS1 expression by ~90% (Figure 3C). In addition, we conducted experiments using LPA1 antagonist (AM095) or LPA5 antagonist (TCLPA54) to further validate and consistently found a significant decrease in the number of THBS1+ cells in the presence of either antagonist (Figures S4A and S4B). Therefore, LPA induces THBS1+ keratinocytes through both LPA1 and LPA5 receptors. Given that we also previously reported that RHO/ROCK-SRF is the critical signaling pathway downstream of LPA1 and LPA5, we next examined the role of this signaling pathway with a ROCK-specific inhibitor, Y-27632 (Uehata et al., 1997), and an SRF-specific inhibitor, CCG-1423 (Hayashi et al., 2014). We found that LPA-dependent THBS1 expression was significantly suppressed in the presence of Y-27632 (Figures 3D and S4C) and CCG-1423 (Figure 3E). Taken together, these data suggest that similar to FLG+ keratinocytes, LPA also induced THBS1+ keratinocytes via LPA1 and LPA5 receptors and their downstream RHO/ROCK-SRF signaling.

ERK activity dictates the different cell fates of NHEKs upon LPA stimulation

Given that LPA induced both THBS1+ keratinocytes and FLG+ granular keratinocytes through the same LPA1/LPA5-RHO/ROCK-SRF signaling, we next questioned what determines the different cell fates of NHEKs. We reasoned that cellular heterogeneity of NHEKs at the time of LPA stimulation might be the underlying critical factor responsible for such cell fate decisions. Given that the heterogeneity of ERK activity in cultured cells including keratinocytes was previously reported (Aoki et al., 2013; Hiratsuka et al., 2015, 2020), we examined ERK activity in our cultured NHEKs by immunocytochemistry for phosphorylated ERK (pERK). We found that there were two different types of staining patterns of pERK in NHEKs: nuclear staining and diffused staining patterns (Figure 4A). Notably, nuclei of NHEKs that showed diffused-pERK staining pattern were located slightly higher from the bottom cell layer (Figure 4A, below) than nuclei of NHEKs with nuclear-pERK staining pattern (Figure 4A, below). Moreover, the staining intensity was varied among NHEKs with nuclear-pERK staining pattern (Figure 4B). Double staining of pERK and Ki67 further revealed a positive correlation between nuclear-pERK staining and Ki67 staining (Figures S5A and S5B). These results together suggest that there is a large heterogeneity of ERK activity in cultured NHEKs before LPA stimulation, and cells with high ERK activity are proliferative cells.

We next examined how ERK activity affects NHEK differentiation upon LPA stimulation. To this end, we used U0126, a MEK-specific inhibitor. MEK is an upstream kinase of ERK and it was previously reported that U0126 binds to the MEK1/2 kinases and inhibits their phosphorylation and subsequent ERK activation (Cargnello and Roux, 2011). First of all, we confirmed that U0126 treatment for 30 min significantly suppressed nuclear ERK staining (Figures S6A and S6B). We then next pre-treated NHEKs with U0126 for 30 min to suppress ERK activity, stimulated with LPA for 72 h, and then examined the number of THBS1+ and FLG+ keratinocytes. We found that the number of FLG+ keratinocytes was increased in the presence of U0126 (Figures 4C and 4D). This result was consistent with our previous report (Sumitomo et al., 2019). In contrast, the number of THBS1+ keratinocytes upon LPA treatment for 72 h was significantly reduced in the presence of U0126 (Figures 4C and 4E). It should be noted that treatment of NHEKs with U0126 without LPA stimulation had no significant effect on the differentiation of both FLG+ keratinocytes and THBS1+ keratinocytes (Figures 4D and 4E). These results suggest that ERK activity at the moment of LPA stimulation is indispensable for the induction of THBS1+ keratinocytes but not FLG+ keratinocytes.

To determine possible sources of basal ERK activity, we further investigated the involvement of EGFR, a receptor for the growth factor EGF, in THBS1+ keratinocyte induction. It is widely known that MEK-ERK signaling cascade is activated downstream of EGFR (Lemmon and Schlessinger, 2010). We used PD168393, an EGFR-specific inhibitor, and found that the number of THBS1+ keratinocytes, but not FLG+ keratinocytes, upon LPA treatment was reduced in the presence of EGFR inhibitor (Figures S7A and S7B) in a similar fashion to MEK inhibitor (Figures 4C–4E). Therefore, it is likely that signaling through...
PI3K plays an inhibitory role in the LPA-induced NHEK differentiation. However, there are some Thbs1+ cells that detached from the basement membrane and were located in the basal layer at approximately the same distance but with their nuclei elongated (Figures 6D and 6I). Notably, that was previously reported by Haensel et al., 2020. From this deposited data, we were able to reproduce Thbs1 wound healing. To examine this possibility, we investigated Col17a1 expression in mouse interfollicular keratinocytes in the interfollicular epidermis (IFE): Col17a1+ keratinocytes are specifically induced in the proliferation zone of epidermal basal keratinocytes upon wounding and subsequently migrated toward the wound front in mice (Figure 5A and 5B) as previously described (Haensel et al., 2020). In this work by Haensel et al., it was demonstrated that GA basal keratinocytes are specifically increased in wounded skin at day 1 PWI (Haensel et al., 2020). Intriguingly, our reanalysis results further revealed that GA basal keratinocytes specifically expressed high Thbs1 levels compared to the other IFE basal keratinocytes (Figure 5C). In addition, GO analysis indicated that GA basal keratinocytes possessed migratory signatures with significant enrichment for cell migration-related gene sets (Figure 5D). Based on these findings, we deduced a possibility that Thbs1+ keratinocytes are differentiated from basal keratinocytes and acquire migratory ability upon epidermal wounding in vivo.

To further validate the presence of Thbs1+ keratinocytes in wound skin, we conducted immunohistochemistry of Thbs1 at different time points in the dorsal skin of mice subjected to excisional dorsal full-thickness skin wounds (Figure 6A). The specificity of Thbs1 staining in the epidermis was confirmed by the use of two different antibodies that show an identical staining pattern (Figures S8A and S8B). In normal skin, Thbs1+ keratinocytes were rarely observed in the IFE and mainly restricted to keratinocytes in hair follicle (HF) bulges (Figure 6B). This finding is consistent with previous in situ hybridization findings on mRNA expression of Thbs1 (Streit et al., 2000). Intriguingly, wounded skin at day 1 PWI showed a large number of Thbs1+ keratinocytes in the basal layer of the epidermis spanning a distance of 0.6–1 mm from the wound edge (Figures 6C and 6I), presumably in the proliferation zone as defined by previous studies (Aragona et al., 2017; Park et al., 2017). Consistently, double staining of Thbs1 together with Ki67 indicated a number of Thbs1+ keratinocytes positive for Ki67 in the proliferation zone (Figure S9A). It should be noted that nuclei of Thbs1+ keratinocytes were relatively round at this stage (Figure 6C). Co-staining of Thbs1 and Krt14 further revealed that Thbs1+ keratinocytes are basal keratinocytes at day 1 PWI, because they are Krt14-positive (Figure S9B). Subsequently, on day 2 PWI, most of Thbs1+ keratinocytes remained in the basal layer at approximately the same distance but with their nuclei elongated (Figures 6D and 6I). Notably, there were also some Thbs1+ cells that detached from the basement membrane and were located in the

EGFR regulates the ERK activity in NHEKs and contributes to THBS1+ keratinocyte differentiation. It should be noted that we also examined the involvement of phosphatidylinositol three-kinase (PI3K) using a specific inhibitor, Wortmannin, and found that it stimulated both growth arrest (GA) and cell proliferation (response), and GA (growth arrest) (Figures 5A and 5B) as previously described (Haensel et al., 2020). In vitro differentiation of NHEKs induced the expression of THBS1 (Figures S7C and S7D), thus suggesting that PI3K plays an inhibitory role in the LPA-induced NHEK differentiation.

Thbs1+ keratinocytes were specifically induced in the proliferation zone of epidermal basal layer upon wounding and subsequently migrated toward the wound front in mice

Given the previously reported elevation of THBS1 expression in NHEKs upon scratching in vitro wound assay (Fitsialos et al., 2007), our aforementioned findings that THBS1 promotes cell migration of NHEKs in vitro, and previous reports suggesting the upregulation of Thbs1 in wounded skin of mice (Joost et al., 2018; Haensel et al., 2020), we speculated the involvement of THBS1+ keratinocytes in epidermal wound healing. To examine this possibility, we investigated Thbs1 expression in mouse interfollicular keratinocytes upon wounding at day 4 post wound induction (PWI) using a public scRNA-seq dataset that was previously reported by Haensel et al., 2020. From this deposited data, we were able to reproduce the result of three basal keratinocyte states in the interfollicular epidermis (IFE): Col17a1+ keratinocytes are specifically induced in the proliferation zone of epidermal basal keratinocytes upon wounding and could be observed in the migrating front of the wound in the IFE (Haensel et al., 2020). Intriguingly, our reanalysis results further revealed that GA basal keratinocytes specifically expressed high Thbs1 levels compared to the other IFE basal keratinocytes (Figure 5C). In addition, GO analysis indicated that GA basal keratinocytes possessed migratory signatures with significant enrichment for cell migration-related gene sets (Figure 5D). Based on these findings, we deduced a possibility that Thbs1+ keratinocytes are differentiated from basal keratinocytes and acquire migratory ability upon epidermal wounding in vivo.
suprabasal layer (Figure 6D). We next observed an increased number of Thbs1+ keratinocytes in the suprabasal layer at the distance closer to the wound edge (0.3–0.6 mm) at day 3 PWI (Figures 6E and 6I). This area is likely the migration zone as defined by previous studies (Aragona et al., 2017; Park et al., 2017). It should be noted that a fraction of Thbs1+ keratinocytes at this stage was polarized, elongated, and apparently moved toward the wound front. These morphological characteristics are the hallmark of migratory cells. Moreover, polarized Thbs1+ keratinocytes observed at day 4 PWI were Ki67-negative (Figure S9C). These results are consistent with the features of Thbs1-enriched GA keratinocytes at day 4 PWI described previously (Haensel et al., 2020), including migratory signatures and cell-cycle arrest.

On day 5 PWI when reepithelialization was completed, Thbs1+ keratinocytes were found in the thick neo-epidermis (Figure 6F). Thbs1+ keratinocytes formed a stream of cells that upwardly extended from the edge to the center of the neo-epidermis (Figure 6F). To examine the identity of these cells, Krt10, a spinous keratinocyte marker, was stained using consecutive skin sections. We found that the neo-epidermis consisted of multiple layers of Krt10+ spinous keratinocytes but only a single layer of Krt10+ basal keratinocytes. Notably, a fraction of Krt10+ keratinocytes in lower suprabasal layers was also positive for Thbs1, suggesting Thbs1+ keratinocytes further differentiated into spinous keratinocytes (Figure 6G). Finally, Thbs1 signals disappeared as the wound closure completed and the neo-epidermis became fully stratified on day 7 PWI (Figure 6H). Altogether, these findings suggest that Thbs1+ keratinocytes differentiated into Krt10+ spinous keratinocytes upon their arrival at the wound and then finally downregulated their Thbs1 expression.

Taken together, Thbs1+ keratinocytes were apparently specifically generated from basal keratinocytes of the proliferation zone upon wounding, migrated toward the wound front, and eventually differentiated into spinous keratinocytes as they arrived at the neo-epidermis.
Figure 6. Thbs1+ keratinocytes were transiently induced upon wound healing in vivo
(A) Scheme represents the experiment design.
(B–F) Representative stacked confocal images showing immunostaining for Thbs1 (green) and nuclei (Hoechst 33342, magenta) in (B) normal skin and wounded skin on day 1, 2, 3, and 5 PWI (C–F). Dotted lines: approximate location of the basement membrane beneath the normal epidermis. Filled lines: approximate location of the basement membrane beneath the leading edge or neo-epidermis. Der, dermis; Epi, normal epidermis; Eschar, eschar; LE, leading edge; WEpi, neo-epidermis. Scale bars, 100 μm (low magnification), and 10 μm (high magnification).
(G) Representative stacked confocal images showing immunostaining for nuclei (Hoechst 33342, magenta), Krt10 (cyan blue), and Thbs1 (green) using consecutive wounded skin sections on day 5 PWI. Dotted lines: approximate location of the basement membrane beneath the neo-epidermis. Scale bars, 50 μm.
(H) Representative stacked confocal images showing immunostaining for Thbs1 (green) and nuclei (Hoechst 33342, magenta) in wounded skin on day 7 PWI. Dotted lines: approximate location of the basement membrane beneath the normal epidermis. Dotted lines: approximate location of the basement membrane beneath the leading edge or...
Erk activity is indispensable for Thbs1+ keratinocytes differentiation and epidermal wound healing in vivo

We next investigated the involvement of LPA in the epidermal wound healing process in vivo. We generated an epithelial debridement wound with a 6-mm punch in the dorsal skin and examined the effects of LPA on the healing of the epithelial wound. However, we could not detect any significant difference between LPA-treated and control groups (Figures S10A–S10C). We then examined the potential role of Erk activity in Thbs1+ keratinocyte differentiation and wound healing using the MEK inhibitor, Trametinib (Yamaguchi et al., 2011), which could be administered orally in vivo. Mice were orally administered with vehicle or Trametinib at the dose of 1 mg/kg/day for 2 days before excisional wound induction on the dorsal skin (Figure 7A). We then collected the wounded skin on day 1 PWI and performed immunohistochemical staining for Thbs1. Consistently with our above results, we found that a large number of Thbs1+ keratinocytes were induced in the basal layer of the epidermis near the wound edge in the control mice treated with vehicle (Figure 7B). On the other hand, only few Thbs1+ keratinocytes were observed in the epidermal basal layer in mice treated with Trametinib (Figure 7C). Quantitative analysis further confirmed that the number of Thbs1+ keratinocytes in the wounded epidermis was significantly reduced upon Trametinib treatment (Figure 7D). These results together suggest that the induction of Thbs1+ keratinocytes upon wounding is dependent on Erk activity. Finally, we examined the effect of Trametinib on wound closure and reepithelialization. To this end, we conducted wound healing assay in vehicle-treated control mice and Trametinib-treated mice and monitored the wound closure rate on day 1, 2, 4, and 6 PWI. Trametinib treatment significantly delayed the wound closure throughout the repairing process (Figures 7E and 7F). Therefore, Erk activity is indispensable for Thbs1+ keratinocyte differentiation and skin-wound healing in vivo.

DISCUSSION

Accumulating evidences have now unveiled a remarkable heterogeneity of keratinocytes in the skin (Joost et al., 2016, 2018, 2020; Cheng et al., 2018; Finnegan et al., 2019; Haensel et al., 2020; Reynolds et al., 2021). Such heterogeneity becomes even larger as skin is challenged by injury or inflammation (Joost et al., 2018; Cheng et al., 2018; Haensel et al., 2020; Reynolds et al., 2021). Our study employed scRNA-seq to investigate the differentiation of NHEKs and revealed a previously unsuspected THBS1+ keratinocyte subpopulation that exhibits migration and wound-response signatures and does not belong to the conventional classification of keratinocyte subtypes in the epidermis. Importantly, we found that THBS1-expressing keratinocytes were rare in the control but LPA strongly promoted their differentiation in vitro. Given that LPA production through the catalytic activity of a keratinocyte-specific phospholipase A, mPA-PLA1α (or LIPH), is known (Aoki et al., 2008; Diribarne et al., 2012), the small number of THBS1+ keratinocytes observed in control conditions might be because of the effect of endogenous LPA produced by keratinocytes themselves in the culture. In this study, we also confirmed our previous report that LPA induces NHEK differentiation into FLG+ granular keratinocytes (Sumitomo et al., 2019). Therefore, LPA has a potential to promote the differentiation of both THBS1+ keratinocytes and FLG+ granular keratinocytes in vitro.

The dual action of LPA on THBS1+ keratinocyte differentiation and FLG+ keratinocyte differentiation was mediated through LPA1 and LPA2 receptors and their downstream Rho/ROCK-SRF signaling. However, with the same LPA1/LPA2-RHO/ROCK-SRF axis responsible for both LPA-induced THBS1+ and granular keratinocytes, then what determines the cell fate of NHEKs? Here, we showed that there was a heterogeneity of ERK activity in NHEKs before LPA stimulation. The heterogeneity of ERK activity in cells including keratinocytes was observed previously (Sampattavanich et al., 2018; Hiratsuka et al., 2015, 2020), and it was reported that changes in basal ERK activity and pulses account for different cell states and could dynamically regulate keratinocyte differentiation (Hiratsuka et al., 2020). Given that MEK inhibition specifically suppressed the differentiation of LPA-treated NHEKs into THBS1+ keratinocytes, it is likely that THBS1+ keratinocytes are derived from NHEK cells with high basal ERK activity. Moreover, as ERK activation can be propagated to neighboring cells via paracrine signaling (Aoki et al., 2013; Hiratsuka et al., 2020), this might also be involved in the cluster appearance of THBS1+ keratinocytes that we observed in vitro.

Figure 6. Continued

neo-epidermis. Der, dermis; Epi, normal epidermis; Eschar, eschar; WEpi, neo-epidermis. Scale bars, 100 μm (low magnification), and 10 μm (high magnification).

(i) Quantification of the distance of Thbs1+ keratinocytes to the neo-epidermis margin. Means of three to seven measurements at each time point are represented and bars represent the distance range.
EGFR-specific inhibitor suppressed THBS1⁺ keratinocyte differentiation in a similar fashion to suppression of ERK by MEK inhibitor, we suggest that a potential candidate for such paracrine signaling molecule is EGF. Notably, high ERK activity was observed in Ki67⁺ proliferating keratinocytes that could be possibly driven by EGF. Therefore, it is likely that LPA acts on proliferating basal keratinocytes with high ERK activity and facilitates their differentiation into THBS1⁺ keratinocytes.

It was previously reported that THBS1 expression is upregulated in the wound skin of mice (Joost et al., 2018; Haensel et al., 2020). Particularly, it was shown that THBS1 expression is upregulated in basal
keratinocytes upon skin wounding in the latter (Haensel et al., 2020). In this work, we have analyzed the publicly deposited scRNA-seq data of mouse normal skin and day 4 PWI wound skin in Haensel et al.’s paper (Haensel et al., 2020). We found that of basal keratinocytes, the GA basal keratinocytes expressed the highest level of Thbs1 at this time point (Figure 5C) and were enriched in genes related to cell migration similarly to the in vitro human THBS1+ keratinocytes, suggesting their migratory roles. Consistently, we found Thbs1+ keratinocytes in keratinocyte migration as knockdown of THBS1 impaired wound healing in vitro scratching assay. It should be noted that not only THBS1-producing keratinocytes move faster but also the surrounding keratinocytes (Video S1). This might be possible because THBS1 is a secretory matrix protein that has a potential to remodel the microenvironment. In vivo, we demonstrated that Thbs1+ keratinocytes were specifically and transiently induced in mouse skin upon wound injury by immunohistochemistry. These cells originate from basal keratinocytes in the proliferation zone distant from the wound edge (Aragona et al., 2017; Park et al., 2017). Thereafter, Thbs1+ keratinocytes apparently detach from the basal layer, polarize, and migrate through the suprabasal layer toward the wound front and exit cell cycle (Figure S11). In wound healing, for the reepithelialization to take place efficiently, we speculate that these Thbs1+ keratinocytes promote keratinocyte migration through the secretion of Thbs1. Consistently, delay of skin wound closure was reported in wounds treated with antisense-Thbs1 (DiPietro et al., 1996) and in Thbs1-deficient mice (Agah et al., 2002; Nor et al., 2005). Eventually, upon Thbs1+ keratinocytes’ arrival at the wound, they differentiate into spinous keratinocytes and subsequently downregulate their Thbs1 expression (Figure S11). Therefore, we speculate that Thbs1+ keratinocytes might also supply the keratinocytes for reepithelialization and stratification of the neo-epidermis in wound healing. However, given the small number of Thbs1+ keratinocytes compared to the total keratinocytes in neo-epidermis after wounding, their contribution might be relatively small. In addition, it should be noted that Thbs1 is also expressed by other cell types such as monocytes, macrophages, fibroblasts, and myofibroblasts. Because these cells could be observed in the injured dermis of wounded skin, it is possible that Thbs1 produced by these cells may also contribute to the wound healing besides Thbs1+ keratinocytes in the epidermis.

Accumulating evidence has suggested different cellular states of IFE keratinocytes in wound healing; however, whether all wound-responsive IFE cells are derived from a single subpopulation is still an open question. Our study indicated that Thbs1+ keratinocytes are differentiated from proliferating basal keratinocytes, then transiently migrate through the suprabasal layers toward the migratory zone before Thbs1 being downregulated as a consequence of terminal differentiation in the neo-epidermis. Given that IFE-derived keratinocytes were reported to make long-term contributions to the regenerative basal layer after migration (Plikus et al., 2012; Vagnozzi et al., 2015), it is possible that in addition to Thbs1+ cells, other subpopulation(s) of IFE keratinocytes are also involved in the wound healing process. In such a scenario, Thbs1+ keratinocytes may account for early and fast reepithelialization actions, whereas other IFE keratinocyte subpopulation(s) are responsible for the subsequent permanent epidermal regeneration.

It is also known that in addition to IFE-derived cells, hair follicle (HF)-derived stem cells from the isthmus, junctional zone, and bulge also contribute to the neo-epidermis formation during the epidermal wound healing process (Plikus et al., 2012). It was reported that although the migration of HF-derived stem cells is relatively slower compared to that of IFE-derived cells, both are able to generate long-lasting clones in the regenerative epidermis together with transient suprabasal cells (Plikus et al., 2012; Vagnozzi et al., 2015). In this study, we also observed Thbs1+ keratinocytes in hair follicles of normal skin. In addition, we were able to detect two subpopulations of HF keratinocytes that expressed high levels of Thbs1 in Haensel et al.’s scRNA-seq data (Figures S12A–S12C). One is the Lgr5+ HF stem cells that have been previously reported to migrate toward the wound front and contribute to the new-forming IFE epidermis (Joost et al., 2018). The other Thbs1+ expressing HF subpopulation was enriched for intermediate early genes such as Ier3. However, it is not clear whether these cells could also migrate out of the bulge. This issue should be further addressed in future studies.

In this study, we also examined the effect of LPA on the differentiation of Thbs1+ keratinocytes and skin wound healing in mice. However, we could not detect any obvious effect. There are several possible interpretations for this result. First, it is possible that Thbs1+ keratinocytes in vivo are different from THBS1+ keratinocytes that we have observed in vitro, and their differentiation is not dependent on LPA. Second, the response of keratinocytes to LPA might have already been saturated by the large amount of LPA released
upon skin wounding (Mazereeuw-Hautier et al., 2005). Third, other bioactive mediators might function redundantly to LPA in vivo. Finally, given that LPA is susceptible to oxidation and unstable in vivo, LPA might rapidly lose its activity in the in vivo experiment setting, thus making it difficult to be used as a therapeutic agent for wound healing. In such case, future development of LPA1 and LPA5 agonists that could be administered in vivo will help us to further clarify this issue.

Notably, in this work, we found that inhibition of Erk activity with a MEK inhibitor suppressed Thbs1+ keratinocyte induction upon wounding in vivo. Therefore, Erk activity is also indispensable for Thbs1+ keratinocytes both in vitro and in vivo. Moreover, we also found that MEK inhibitor suppressed wound healing. However, this result should be interpreted with caution. Given the pleiotropic biological role of MEK-ERK signaling, we could not exclude the possibility that MEK inhibitor may also interfere with other processes such as contraction that also contributes to wound healing in addition to the interference of Thbs1+ keratinocyte differentiation. Nevertheless, our finding that wound healing is impaired upon MEK inhibitor treatment and the fact that various MEK inhibitors are now widely used in cancer therapy have implications for the treatment of these patients. It should also be noted that skin adverse effects are highly common in cancer patients who received MEK inhibitor treatment (Varvaresou et al., 2020).

Limitations of the study
Regarding the limitation of our study, firstly, it should be noted that in vitro maintenance of keratinocytes is not a physiological condition. Therefore, it is difficult to conclude that THBS1+ keratinocytes we observed in vitro are exactly the same to those Thbs1+ keratinocytes in vivo. However, given several similar characteristics between THBS1+ keratinocytes in vitro and Thbs1+ keratinocytes in vivo as discussed above, we suggest that it is possible to extract useful hypothesis from our in vitro studies for further validations. The second limitation of our study was although our suggestion for the migratory features of THBS1+ keratinocytes during wound healing was based on a combination of in vitro assays, scRNA-seq data analysis, and spatiotemporal correlation of Thbs1+ keratinocytes in vivo, the direct evidence is still lacking. The lineage tracing model would be an ideal approach to further confirm the nature of THBS1+ keratinocytes in the future.

STAR METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104130.
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AUTHOR CONTRIBUTIONS

R.S., A.Q.N., S.N., and D.T. designed the experiments. R.S. and D.T. performed single cell isolation, RNA extraction, and library preparation with the Fluidigm C1 system. S.S. conducted sequencing using the Illumina HiSeq2500. R.S., A.Q.N., and D.T. performed scRNA-seq data analysis. A.Q.N. and M.H. performed validation experiments with NHEKs. A.Q.N., D.T., and A.K. performed wound healing experiments with mice. A.Q.N. and D.T. conducted immunohistochemistry. A.Q.N., M.H., and D.T. performed statistical analysis. A.W. supervised scRNA-seq experiment and data analysis. R.S., A.Q.N., M.H., S.N., and D.T. wrote the manuscript. All authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS

S.N. is a scientific advisor to Astellas Pharma Inc and Toray Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

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### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-THBS1 | Invitrogen | Cat# MA5-13398; RRID: AB_10984611 |
| Mouse monoclonal anti-THBS1 | Santa Cruz | Cat# sc-59887; RRID: AB_793045 |
| Rabbit polyclonal anti-THBS1 | Abcam | Cat# ab85762; RRID: AB_10674322 |
| Mouse monoclonal anti-FLG | Santa Cruz | Cat# sc-66192; RRID: AB_1122916 |
| Rabbit polyclonal anti-Krt10 | Biolegend | Cat# PRB-159P; RRID: AB_291580 |
| Mouse monoclonal anti-KRT14 (Alexa Fluor 647-conjugated) | Santa Cruz | Cat# sc53253; RRID: AB_2134820 |
| Mouse monoclonal anti-Ki67 | Leica | Cat# NCL-L-Ki67-MM1; RRID: AB_563841 |
| Rabbit monoclonal anti-Ki67 | Sigma | Cat# ZRB1007 |
| Mouse monoclonal anti-MAP kinase, Activated (phosphorylated) | Sigma | Cat# M8159; RRID: AB_477245 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Recombinant human IL-4 | PeproTech | Cat# 200-04 |
| LPA 18:1 | Avanti Polar Lipids | Cat# 287130 |
| AM095 | Sigma | Cat# BM0029 |
| TCLPA54 | Tocris | Cat# 4708/10 |
| Y-27632 | Wako | Cat# 259-00613 |
| U0126 | Sigma | Cat# 19-147 |
| CCG-1423 | Sigma | Cat# 555558 |
| PD168393 | Sigma | Cat# 194423-15-9 |
| Wortmannin | Tocris | Cat# 1232/1 |
| Trametinib | ChemScene | Cat# CS-0060 |
| Lipofectamine RNAiMAX Transfection Reagent | Life Technologies | Cat# 13778150 |
| **Critical commercial assays** |        |            |
| C1 Single-Cell Open App IFC, 17 - 25 μm | Fluidigm | Cat# 100-8135 |
| Click-iT™ TUNEL Alexa Fluor™ 488 Imaging Assay | Thermo Fisher | Cat# C10245 |
| **Deposited data** |        |            |
| scRNA-seq data | This paper | GEO: GSE167056 |
| Raw quantification data | This paper | Mendeley Data: https://doi.org/10.17632/693s5mbxen.1 |
| **Experimental models: Cell lines** |        |            |
| Normal Human Epidermal Keratinocytes (NHEKs) | KURABO | Cat# KK-4009 |
| **Experimental Models: Organisms/Strains** |        |            |
| CS7BL/6N | SLC | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dean Thumkeo (d.thumkeo@mfour.med.kyoto-u.ac.jp).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The scRNA-seq data were deposited in Gene Expression Omnibus (GEO) repository, NCBI (GEO: GSE167056). All codes were used were from prior reports, as listed in the “Software and Algorithms” section of the key resources table. Raw quantification data have been deposited at Mendeley (Mendeley Data: https://doi.org/10.17632/693s5mbxnn.1) and are public available as the data of publication. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture
Normal Human Epidermal Keratinocytes (NHEKs, KURABO) were used at passages 1-2 and maintained in an undifferentiated state in HuMedia KG-2 media (KURABO). At 24 h after seeding, cells were starved in EpiLife medium (Invitrogen) containing 60 mM Ca\(^{2+}\) (Gibco) in the absence of growth factors for 48 h. For stimulation, cells were cultured in EpiLife medium containing 1.2 mM Ca\(^{2+}\) (Gibco) and supplemented with 30 ng/ml recombinant human IL-4 in the presence or absence of LPA. Depending on the assay, cells were pretreated with either AM095 (10 \(\mu\)M), TCLPA54 (3 \(\mu\)M), Y-27632 (30 \(\mu\)M), CCG-1423 (3 \(\mu\)M), U0126 (5 \(\mu\)M or 10 \(\mu\)M), PD168393 (1 \(\mu\)M), or Wortmannin (1 \(\mu\)M) prior to stimulation.

Animal model
C57BL/6N mice (female, 6-7 week old) were purchased from SLC (Shizuoka, Japan) and kept under pathogen-free conditions. All animal experiments were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

METHOD DETAILS

Single-cell isolation, cDNA library preparation and next generation sequencing
At 72 h post stimulation, single cell suspension of 300 cells/\(\mu\)l from either vehicle control or LPA stimulation was prepared and loaded into a 96-well microfluidic chip (designed for cells from 17 - 25 \(\mu\)m diameter) for single cell capture using the Fluidigm C1 Autoprep system. The capturing efficiency was evaluated on a
microscope and only single cells were subjected to library preparation. The SMART-Seq® v4 (Clontech) was used for cDNA synthesis from the single cells. Library was amplified using the Nextera XT DNA Library Preparation Kit (Illumina) and quality control on multiplexed libraries was performed by Bioanalyzer 2100 (Agilent). Finally, libraries were deep sequenced as single-ended on an Illumina HiSeq 2500 platform.

Analysis of scRNA-seq data
Raw sequencing data in FASTQ format was evaluated using FastQC (v0.11.8) and adapter sequences were removed with Cutadapt (v2.3, Martin, 2011). Then reads were mapped to the reference genome (hg38) together with the GENCODE v38 annotation file using STAR (v2.7.0f, Dobin et al., 2013). A count matrix was generated with featureCounts (v1.6.4, Liao et al., 2014) for downstream analysis in R (v3.6.3).

Cells with total counts of less than 500,000, total number of expressed genes less than 2,000, a percentage of counts in mitochondrial genes more than 30%, and genes expressed in less than 2 cells were excluded. Raw counts were normalized by library size and log-transformed with NormalizeData function in Seurat R package (v3.1.5, Gribov et al., 2010). We performed batch correction by applying Harmony (v1.0, Korsunsky et al., 2019) onto the principle component analysis (PCA) matrix with theta = 0.5. The batch corrected PCA matrix was then used for clustering and UMAP visualization with Seurat. DEG analysis was performed based on two-sided Wilcoxon rank sum test using the default Seurat’s function FindAllMarkers. GO analysis was conducted using TPM values with GSEA tools (Subramanian et al., 2005) on GO gene sets.

scRNA-seq analysis of public data GEO: GSE142471
Haensel et al.’s scRNA-seq data (GEO: GSE142471) was downloaded from the GEO repository. Count matrices were imported and similar quality control parameters were used to filter cells with 200 - 5,000 genes and less than 10% mitochondrial percentage as previously described by Haensel et al. Two unwounded and three wounded skin samples were integrated together and downstream analysis was similarly performed as described above in “analysis for scRNA-seq data” using Seurat and Harmony.

siRNA-mediated knockdown
NHEKs were transfected with Silencer Select siRNAs (at a final concentration of 5 nM) using Lipofectamine RNAiMAX (Life Technologies) at 36 h prior to stimulation. Silencer Select Negative control no. 1 (Life Technologies) was used as a negative control in all experiments. The sequences of Silencer Select siRNAs used were as follows:

LPAR1: GAGACUGACUGUUAGCACAAtt and
UGUGCUAACAGUCAGUCUCcg,

LPAR5: AGUGACUGUGACAAUUUAtt and
UAAAUUGUCACAAGACACUat,

THBS1: GGACUGCGUUGGUGAUGUAtt and
UACAUCAACACGCAUGCtt.

Quantitative RT-PCR
mRNA was extracted from cells using TRIzol® LS Reagent (Life Technologies) and reverse-transcribed to cDNA using PrimeScript RT Master Mix (TaKaRa). cDNA, primers, and TB Green Premix Ex Taq (Tli RNaseH Plus) (TaKaRa) were mixed in a 96-well PCR plate. Subsequently, quantitative PCR (qPCR) was performed using a CFX96 Real-Time System (Bio-Rad). The sequences of primers are as the following:

FLG F: 5’-AGTCACGTGGCAGTCCTCACA-3’,

FLG R: 5’-TCTAAACCAGGATTCCACATTCAATA-3’,

GAPDH F: 5’-ATGACATCAAGAAGGTTG-3’,

GAPDH R: 5’-TCTTCTTACGTCAAGCTTG-3’,
GAPDH R: 5’-CATACCAGGAAATGAGCTTG-3’,
LPAR1 F: 5’-AATCGGGATACCATGATGAGTCTT-3’,
LPAR1 R: 5’-CCAAGGAGTCCAGCAGATGATAAA-3’,
LPAR5 F: 5’-CGCCATCTTCCAGATGAAC-3’,
LPAR5 R: 5’-TAGCGGTCCACGTTGATG-3’,
THBS1 F: 5’-GCTGGAATGTGGGTGCTTGC-3’,
THBS1 R: 5’-CTCCATTGTGGTTGAAGCAGGC-3’.

NHEKs wound scratching assay
The cell scratching was conducted using the P200 pipette tip. NHEKs at the confluency 70-80% were scratched to generate a wound with the width ~300 - 400 μm. Cell stimulation was conducted by the addition of 1.2 mM Ca²⁺ or of 10 μM LPA plus 1.2 mM Ca²⁺ immediately after wound scratching.

Time-lapse imaging
Time-lapse imaging of NHEKs wound healing in vitro was performed using BZ-X700 microscope (Keyence, Japan) equipped with a 10x dry objective lens (Keyence, Japan) and a chamber box to stably control the temperature (37°C) and CO₂ level (5%). Phase-contrast images were acquired every 5 min for 10 h.

Immunocytochemistry
Cultured cells were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) in PBS, permeabilized and blocked in PBS containing 10% normal goat/donkey serum and 0.3% Triton X-100. Cells were incubated with primary antibodies for either THBS1 (1:500 (Abcam, ab85762) or 1:250 (Invitrogen, MA5-13398)), MAP kinase, activated (1:50), or Ki67 (1:500) overnight at 4°C and counterstained with Hoechst 33342 (1:1000). Fluorescence images were acquired with an LSM 710 confocal microscope (Zeiss) using a 40x NA 1.30 oil-immersion objective lens or a FV3000 confocal microscope (Olympus) using a 20x NA 0.8 objective lens, 40x NA 0.95 objective lens or 60x NA 1.42 oil-immersion objective lens. Images were processed by Imaris software (Bitplane) and ImageJ (NIH).

Immunohistochemistry
Normal skin and wounded skin samples at different days after wounding were collected and fixed in 4% PFA overnight, cryoprotected in PBS containing 30% sucrose, frozen in Tissue-Tek OCT compound (Sakura), and cut into sections of 5 μm thickness using a cryostat (Leica). Antigen retrieval was performed in 10 mM citrate buffer (pH 5.8) using a pressure cooker. Sections were incubated with primary antibodies for either THBS1 (1:500 (Abcam, ab85762) or 1:250 (Santa Cruz, sc-59887)), Krt10 (1:2000), KRT14 (1:500) or Ki67 (1:500) overnight at 4°C and counterstained with Hoechst 33342 (1:1000). Fluorescence images were acquired with a TCS SP8 scanning confocal microscope (Leica) using a 40x NA 1.30 oil-immersion objective lens or a FV3000 confocal microscope (Olympus) using a 20x NA objective lens. Images were processed by Imaris software (Bitplane) and ImageJ (NIH).

Wound healing experiments on mice
Mice received oral administration of vehicle or Trametinib prior to wound induction. Trametinib dissolved in 10% PEG400 (Nacalai) and 10% Cremophor EL (Nacalai) in sterile dH₂O was administered as a single dose of 1mg/kg per day. For immunohistochemistry experiments, four 3 mm full thickness wounds were created by ear punch on the dorsal telogen phase skin of each anesthetized mouse. For wound closure monitoring experiment, 8 mm biopsy punch (KAI Medical) was used to generate 2 wounds on the dorsal telogen phase skin of each anesthetized mouse.

Topical application of LPA cream to the wounded skin
Due to the hydrophobic nature of LPA, 1% LPA cream for topical application was prepared by adding 18:1 LPA into the following ingredients (constituent concentration, wt.%): milliQ water 56, propylene glycol (Nacalai) 10, paraffin liquid 10, vaseline (Nacalai) 5, glycerol monostearate (Alfa aesar) 10, 1-hexadecanol 10, and LPA 1% (Sigma).
(Nacalai) 6, polyoxyethylene hexadecyl ether (Nacalai) 2. Control vehicle cream formulation was the same as above except that milliQ water was 57 (constituent concentration, wt.%). Lipid phase and water phase were separately heated to 70°C, then combined and homogenized by T10 basic ULTRA-TURRAX Homogenizer (IKA) and cooled down. LPA was added before cooling down to room temperature. Control vehicle cream (25 mg) or 1% LPA cream (25 mg) was topically applied to the wounded skin for consecutive 4 days, twice for the first 2 days and once thereafter.

QUANTIFICATION AND STATISTICAL ANALYSIS
Prism (GraphPad Software) was used for statistical analyses. Data were presented as means ± standard deviation (SD) or means ± standard error of the mean (SEM). When only two groups were compared, a two-tailed Student’s t-test was used. Alternatively, multiple comparisons were performed using one-way ANOVA followed by Bonferroni’s post-hoc comparisons test for parametric data and the Kruskal-Wallis test followed by Dunn’s multiple comparisons test for non-parametric data.