

Spatial confinement induces reciprocating migration of epidermal keratinocytes and forms triphasic epithelia

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In this manuscript the authors established a novel three-dimensional culture system for stratified epithelia that allows epithelial cells to undergo epithelial-to-mesenchymal transition (EMT) and subsequent mesenchymal-to-epithelial transition (MET) while migrating through a membrane with 3.0- μ m micropores, and, thus, provides a **valuable** tool to study EMT and possibly wound regeneration or metastasis. Furthermore, a set of experiments provides **solid** data suggesting that TGF beta signaling and actin polymerization promote movement of epithelial cells into the pores, while Piezo1 and Keratin 6 prevent keratinocyte migration and EMT.

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Abstract

Epithelial cells undergo epithelial–mesenchymal transition (EMT) during migration and regain their epithelial phenotype in the post-migration phase (mesenchymal–epithelial transition; MET). We established an experimental system that reproduced three-dimensional triphasic epithelia, i.e., the original epithelium, its EMT, and MET. Keratinocytes (KCs), skin epithelial cells, placed on a microporous membrane migrated through 3.0- μ m or larger micropores. The 3.0- μ m-pored membrane induced an epithelial structure with three states: stratified KCs above the membrane, KCs showing EMT within the micropores, and a new stratified epithelium under the membrane. The membrane with larger micropores failed to maintain the triphasic epithelia. Live imaging revealed that KCs moved in a reciprocating manner, with actin-rich filopodia-like KC structures extending into and out of the 3.0- μ m micropores, while the cells migrated unidirectionally into larger micropores. Piezo1 and keratin 6 were identified as negative modulators of KC entry to and exit from the 3.0- μ m

micropores. These results demonstrate that non-cancerous epithelial cells migrate through confined spaces in a reciprocating manner, which might help form triphasic epithelia, recapitulating wound healing processes.

Introduction

Organ development and regeneration require cell migration to areas where other cells are absent, a phenomenon known as collective cell migration (Ilina & Friedl, 2009). Epithelial cells typically undergo epithelial–mesenchymal transition (EMT) to facilitate this migration. Once the cells are in the appropriate region, they regain their epithelial phenotype through mesenchymal–epithelial transition (MET; (Dongre & Weinberg, 2019; Kalluri & Weinberg, 2009; Leggett et al., 2021). The epidermis, a stratified epithelium of the skin, serves as a barrier for living organisms (Natsuga, 2014), and its major constituents are keratinocytes (KCs). KC stem cells located at the dermo-epidermal junction provide proliferating and differentiating cells to maintain epidermal integrity (Donati & Watt, 2015). Upon skin wounding, KCs, mainly supplied from hair follicle stem cells, migrate to fill the skin gap (Brownell et al., 2011; Donati et al., 2017; Fujimura et al., 2021; Ito et al., 2005; Joost et al., 2018; Levrà Levron et al., 2023; Page et al., 2013). They do so by transforming themselves into EMT phenotypes and stratifying to re-epithelialize through MET when KCs cover the whole wound area (Cheng et al., 2016; Dongre & Weinberg, 2019; Stone et al., 2016).

Cell migration into interstitial tissues, namely the extracellular matrix (ECM), is a key feature of wound healing and cancer invasion (Wolf et al., 2013). In this setting, the cells must overcome physical constraints and deform themselves to pass through small gaps, possibly through EMT. These characteristics of cell migration upon spatial confinement have been largely explored using cancer cells in the context of cancer invasion (Denais et al., 2016; Mistriotis et al., 2019; Tong et al., 2012; Wolf et al., 2013; Zulueta-Coarasa et al., 2022). However, if and how non-cancerous epithelial cells migrate into confined spaces is unclear.

Cultured epidermal KCs stratify upon air exposure. This three-dimensional (3D)-reconstructed epidermis (hereafter referred to as 3D epidermis) has been used for disease models and drug screening (Enjalbert et al., 2020; Niehues et al., 2018). The 3D epidermis is typically maintained on a microporous membrane with a pore size of 0.4 μm , which allows medium supply while exposing the cells to air (Kumamoto et al., 2018, 2021; Sun et al., 2015). Although this model represents steady-state epidermis, it is not useful for wound healing assays per se. Instead, cell migration that recapitulates wound healing has been examined by other experiments, including Boyden Chamber assay, scratch-wounding assay (Grada et al., 2017; Guy et al., 2017), and microfluidic migration assay (Sala et al., 2022). However, because these assays do not reflect the 3D nature of epithelial tissues, especially stratified epithelia such as the epidermis, a better experimental system that reproduces skin wounds and their repair is warranted.

We introduce here a novel 3D triphasic epithelial tissue by employing KCs cultured on a 3.0- μm -pored membrane. The tissue model comprises three epithelial stages: a primary stratified epithelium, KCs undergoing EMT, and a newly formed stratified epithelium through MET. Our model recaptures KC migration in physiological wound repair. Through this model, we show that KCs move towards such micropores in a reciprocating manner, which is suppressed by Piezo1 and keratin 6.

Results

3.0- μm micropores induced the formation of triphasic epithelia

We began with 3D epidermis and questioned whether the size of the membrane pores affects the integrity of the epidermis. To answer this, we cultured primary human epidermal KCs on membranes with different pore sizes smaller than the diameter of the cells (typically 10–20 μm ; (Barrandon & Green, 1985 [\[1\]](#)) and generated 3D epidermis (**Fig. 1a, b** [\[2\]](#)). KCs passed through pores with a diameter of 3.0 μm , which is much smaller than the cells (**Fig. 1b, c** [\[2\]](#)), in agreement with a previous report using other cells (Wolf et al., 2013 [\[3\]](#)). Intriguingly, while stratified KCs were maintained above the 3.0- μm -pored membrane, a new stratified epithelium formed under the membrane (**Fig. 1b, c** [\[2\]](#)). Membranes with pores larger than 3.0 μm also allowed KCs to pass through, but the stratified epithelium above the membrane was not well-formed (**Fig. 1b** [\[2\]](#)), contrasting with KCs on the 3.0- μm -pored membrane (**Fig. 1b, c** [\[2\]](#)). KCs within and adjacent to 3.0- μm or larger pores expressed vimentin (Vim), a mesenchymal marker, suggesting that the cells undergo EMT (**Fig. 1b, d, e** [\[2\]](#)). We focused on the 3D epidermis kept on the 3.0- μm -pored membrane because of its distinct phenotype.

The stratified epithelium above the membrane expressed E-cadherin (E-cad), an epithelial marker, whereas E-cad was reduced in the cells adjacent to the pores, where N-cadherin (N-cad) expression was present (**Fig. 1d** [\[2\]](#)). Instead, Vim and Slug-positive cells, suggestive of EMT, were observed near the pores (**Fig. 1d, e** [\[2\]](#)). The stratified epithelium under the membrane was positive for E-cad again (**Fig. 1d** [\[2\]](#)), implying that the cells retained an epithelial phenotype (MET). Protrusion of KCs within 3.0- μm micropores was also observed (**Fig. 1e** [\[2\]](#)). Consistent with the morphological data, gene expression of mesenchymal markers, including *FN1*, *VIM*, *SNAI1*, *SNAI2*, and *CDH2*, was upregulated in the tissue maintained on the 3.0 μm -pored membrane compared with that kept on the 0.4- μm -pored membrane, whereas that of *CDH1*, encoding E-cad, was downregulated in the 3.0- μm -pored group (**Fig. 1f, g** [\[2\]](#)). These data indicate that the 3.0- μm -pored membrane allowed KCs to form triphasic epithelia comprising a primary stratified epithelium above the membrane, KCs undergoing EMT within micropores, and another stratified epithelium under the membrane following MET (**Fig. 1h** [\[2\]](#)).

KCs exhibited reciprocating movement through 3.0- μm micropores

We then asked how the triphasic epithelia developed. To answer this, we established HaCaT KCs stably expressing GFP-LifeAct, which labels F-actin in living cells (hereafter referred to as LifeAct-KCs; Supplementary Fig. 1a). Aligning with the results of primary KCs (**Fig. 1** [\[2\]](#)), LifeAct-KCs protruded into 3.0- μm micropores with filopodia-like structures, but not into 0.4- or 1.0- μm micropores (Supplementary Fig. 1b). Electron microscopy also showed that LifeAct-KCs extended their cytoplasm into the micropores (Supplementary Fig. 1c). To further investigate the dynamics of the cells, we conducted live imaging using LifeAct-KCs. Just below the 3.0- μm -pored membrane, cells that had exited the pores and expanded on the bottom of the membrane were observed (**Fig. 2a, b** [\[2\]](#); arrows, Supplementary Movie 1), but at the same time, some cells re-entered the pores (**Fig. 2a, b** [\[2\]](#); arrowheads, Supplementary Movie 1). This reciprocating movement of filopodia-like protrusions was more visible when the focus was set at the level of the micropores, and these protrusions were extended into and out of the pores (**Fig. 2c, d** [\[2\]](#), Supplementary Movie 2). We wondered whether this reciprocating movement of filopodia-like protrusions was specific to the cells kept on 3.0- μm micropores or universal in migrating cells on microporous membranes. Whereas LifeAct-KCs kept on the 3.0- μm -pored membrane showed reciprocating movement of filopodia-like protrusions, the cells kept on 8.0- μm micropores were prone to unidirectional movement (**Fig. 2e, f** [\[2\]](#), Supplementary Movie 3, 4), which was confirmed by quantitative analysis (**Fig. 2g, h** [\[2\]](#), see Materials and Methods section). In line with the data on the triphasic epithelia (**Fig. 1c** [\[2\]](#)), LifeAct-KCs were positive for E-cad but not Vim above the membrane (**Fig. 2i** [\[2\]](#)). The

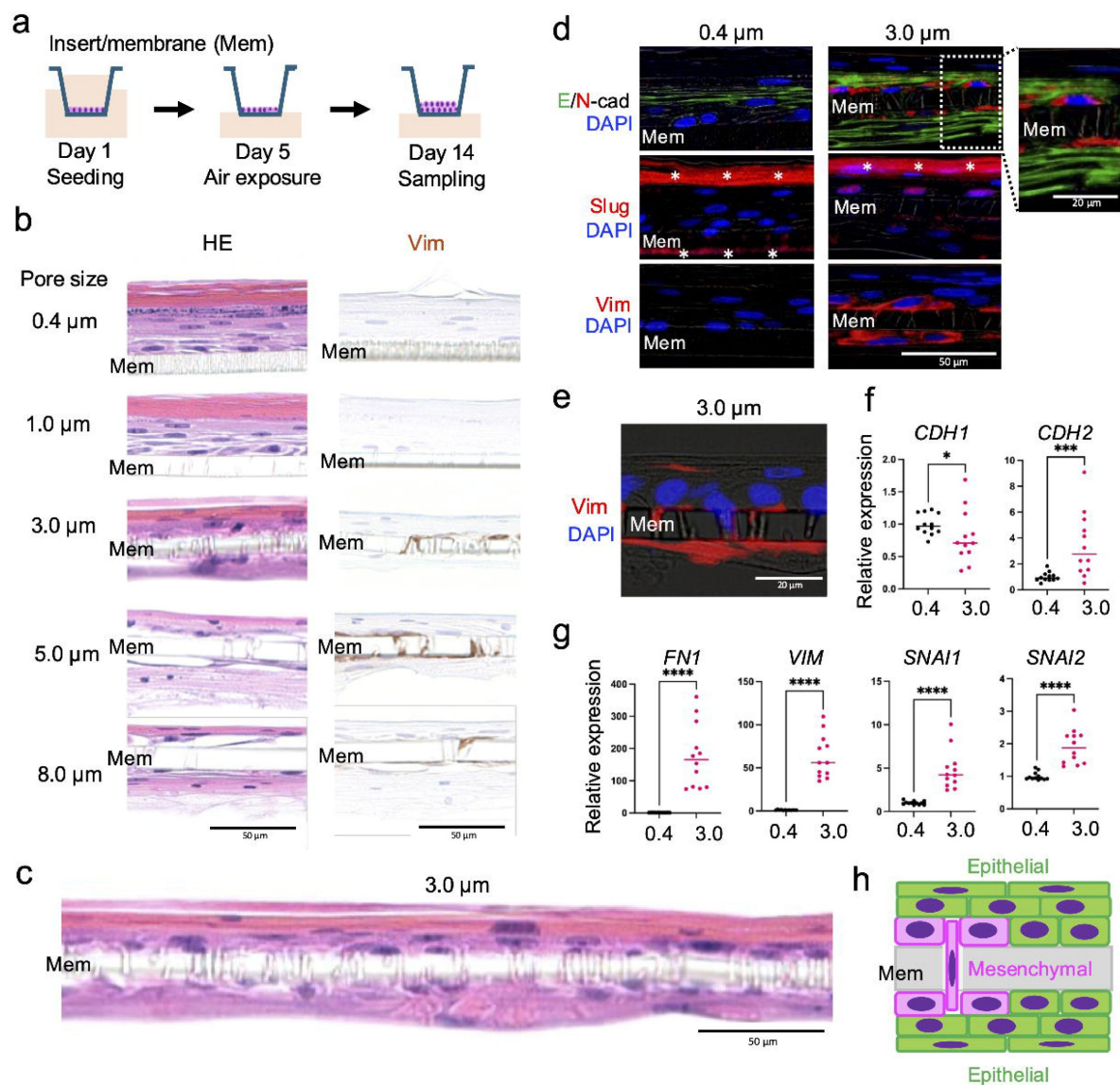


Figure 1.

Triphasic epithelia formed by a 3.0-μm-pored membrane

(a) Schematic diagram of experiments using normal human epidermal keratinocytes cultured on a microporous membrane and exposed to air for 14 days to produce 3D epidermis. (b) Hematoxylin and eosin (HE) staining and vimentin (Vim; brown) labeling of 3D tissue derived from each microporous membrane (Mem). Scale bar: 50 μm. (c) HE staining of 3D tissue formed by a 3.0-μm-pored membrane. Scale bar: 50 μm. (d) E-cadherin (E-cad)/N-cadherin (N-cad) (upper images), Slug (middle images), and Vim (lower images) staining of 3D tissue formed by a 0.4-μm- (left) or 3.0-μm-pored (right) membrane. Scale bar: 50 μm (low-magnified images) and 20 μm (high-magnified image). (e) Vim staining of 3D tissue formed by a 3.0-μm-pored membrane. Scale bar: 20 μm. (f) Quantitative RT-PCR (qRT-PCR) of *CDH1* and *CDH2*. (g) qRT-PCR of *FN1*, *VIM*, *SNAI1*, and *SNAI2*. Data were analyzed with two-tailed Mann–Whitney U tests. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$. (h) Schematic diagram of the triphasic epithelia.

cells in the micropores expressed both E-cad and Vim, showing at least a partial EMT phenotype (Fig. 2j). These data indicate that the KCs kept on 3.0- μ m micropores initially move in a reciprocating manner (Fig. 2e, g, h, Supplementary Movie 3) but can move out of the pores to form a stratified epithelium under the membrane (Fig. 2b), possibly accounting for the formation of the triphasic epithelia (Fig. 1b, c). In contrast, the unidirectional movement of KCs through 8.0- μ m micropores (Fig. 2f-h, Supplementary Movie 4) might explain the improperly formed epithelium above the 8.0 μ m-pored membrane (Fig. 1b).

Actin polymerization is essential for KC migration through 3.0- μ m micropores

We next investigated the mechanisms underlying KC migration through 3.0- μ m micropores, which involves two major steps: cell entry into the gaps and cell exit from the pores. We first examined the TGF- β pathway because KCs on the micropores showed EMT phenotypes (Fig. 1b-d), and the activation of the TGF- β pathway is associated with EMT (Kalluri & Weinberg, 2009). TGF- β ligand promoted LifeAct-KC entry into 3.0- μ m micropores (Fig. 3a, Supplementary Fig. 2a), but blocking TGF- β receptors did not affect this process (Fig. 3b, Supplementary Fig. 2b). The cell exit from the micropores showed similar results across treatments, except that one TGF- β receptor inhibitor slightly promoted cell exit from the pores (Fig. 3c, d, Supplementary Fig. 2c, d).

We then looked into actin because it was enriched in the actin-rich filopodia-like protrusions extending into 3.0- μ m micropores (Fig. 2b). As expected, the inhibition of actin polymerization with cytochalasin D prevented KC entry into 3.0- μ m micropores (Fig. 3e, Supplementary Fig. 2e). In contrast, blebbistatin, an inhibitor of myosin II ATPase, did not affect KC entry into the micropores (Fig. 3f, Supplementary Fig. 2f). Both cytochalasin D and blebbistatin slowed the cell exit from the micropores (Fig. 3g, h, Supplementary Fig. 2g, h). The discrepancy of blebbistatin experiments indicates that cell entry into the small gaps is regulated differently from cell exit from the micropores. Overall, these data suggest that KC migration through confined spaces is promoted by, but does not require, TGF- β and that this migration depends on actin polymerization.

Piezo1 and keratin 6 hinder KC migration through 3.0- μ m micropores and 3D cell invasion

We then hypothesized that mechanosensors might affect KC migration through 3.0- μ m micropores. Intriguingly, ruthenium red, a universal ion-channel blocker, promoted LifeAct-KC entry to and exit from 3.0- μ m micropores (Fig. 4a, b, Supplementary Fig. 2i). Piezo1 is a major mechanosensitive ion channel in KCs, the inhibition of which was implicated in speeding KC migration in a scratch-wounding assay (Holt et al., 2021). Piezo1 knockout (KO) HaCaT KCs showed accelerated LifeAct-KC entry to and exit from 3.0- μ m micropores (Fig. 4c, d, Supplementary Fig. 3a, b, e), whereas Piezo1 agonist Yoda1 slowed these processes (Fig. 4e, f, Supplementary Fig. 2j).

To further delineate the mechanisms underlying this phenomenon, we performed bulk RNA-seq for LifeAct-KCs above 0.4- and 3.0- μ m micropores (Fig. 4g-i). The gene ontology biological process terms enriched among the differentially expressed genes (DEGs) through DESeq2 (Love et al., 2014) included tissue development and cell differentiation (Fig. 4i). Keratins are major cytoskeletal proteins, and differential keratin expression accounts for KC development and differentiation. Among keratin genes, *KRT6A*, *KRT6B*, and *KRT6C*, all encoding keratin 6 (a stress-induced keratin), were upregulated in the KCs kept on 3.0- μ m micropores (Fig. 4j). Keratin 6 was also pronounced in the triphasic epithelia derived from normal human epidermal KCs (Fig. 4k). To uncover the role of *KRT6A*, *KRT6B*, and *KRT6C*, we generated HaCaT KCs with KO for these

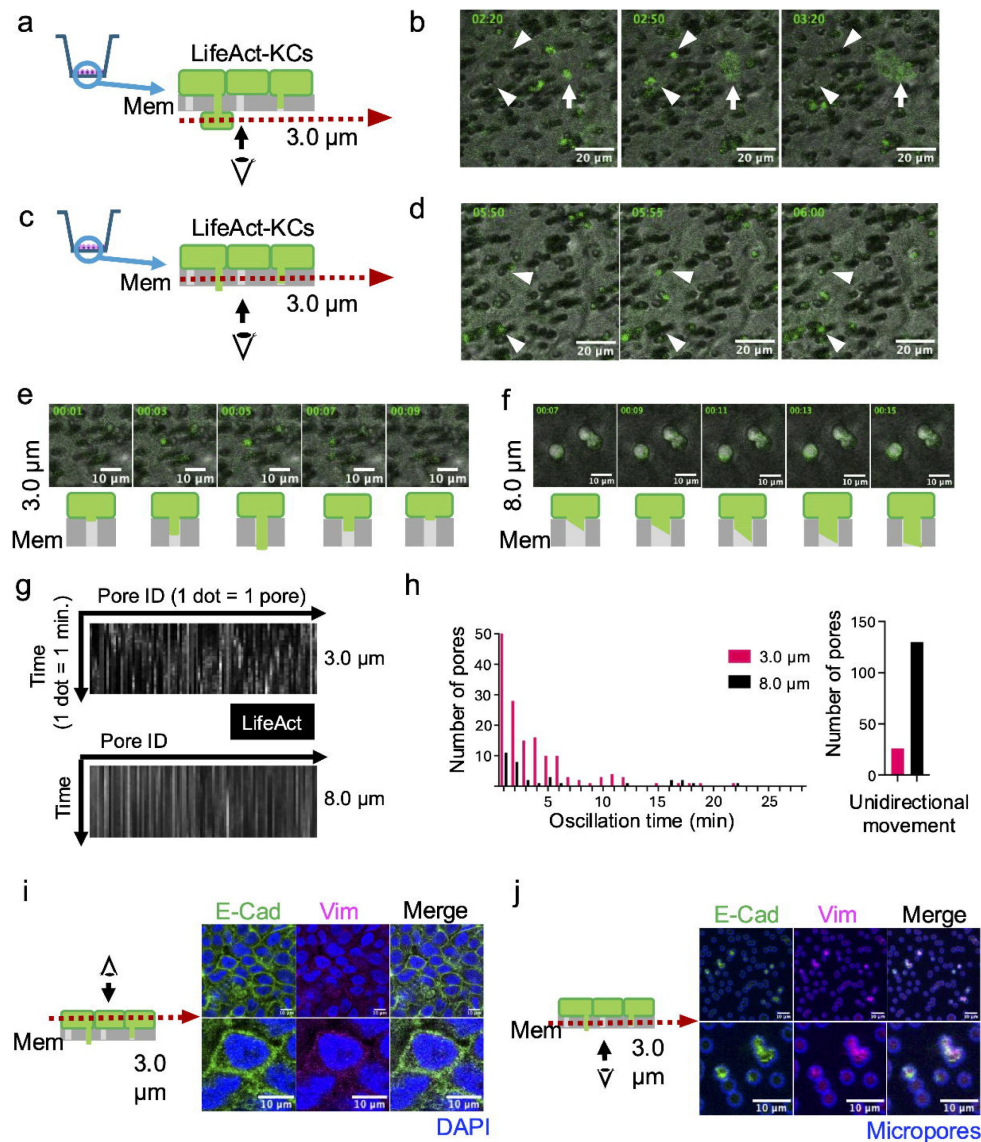


Figure 2.

Reciprocating movement of keratinocytes on a 3.0-μm-pored membrane

(a) Schematic diagram of LifeAct-KCs cultured on a microporous membrane. Observation at the level of cell exit from the micropores. **(b)** Time-lapse images of LifeAct-KCs at the level of cell exit from 3.0-μm micropores. Representative images are shown at 30-minute time intervals. Time stamp shows hour:minute. Arrows indicate the cells that exit from the pores and expand under the membrane. Arrowheads indicate the cells that show reciprocating movement. Scale bar: 20 μm. **(c)** Schematic diagram of LifeAct-KCs cultured on a microporous membrane. Observation at the level of cell entry into the micropores. **(d)** Time-lapse images of LifeAct-KCs at the level of cell entry into 3.0-μm micropores. Representative images are shown at 5-minute intervals. Time stamp shows hour:minute. Arrowheads indicate the cells that show reciprocating movement. Scale bar: 20 μm. **(e, f)** Time-lapse images and schematic diagrams of LifeAct-KCs at the level of cell entry into 3.0-μm **(e)** or 8.0-μm **(f)** micropores. Representative images are shown at 2-minute intervals. Time stamp shows hour:minute. Scale bar: 10 μm. **(g–i)** Quantification of reciprocal movement of LifeAct-KCs on 3.0-μm or 8.0-μm micropores. **(g)** LifeAct signals (white) are shown as a spectrogram, where the X-axis represents each pore, and the Y-axis represents time. **(h)** Graphs comparing the number of pores presenting the LifeAct signals within 3.0-μm or 8.0-μm micropores per time. The left panel shows oscillation time as the X-axis and the number of pores as the Y-axis. The right panel shows unidirectional movement of the cells (no oscillation within 30 minutes) within each micropore. **(i, j)** E-cad and Vim labeling of LifeAct-KCs at the level of cells above **(i)** or within **(j)** 3.0-μm or 8.0-μm micropores. Autofluorescence of micropores is shown in blue **(j)**. Scale bar: 10 μm.

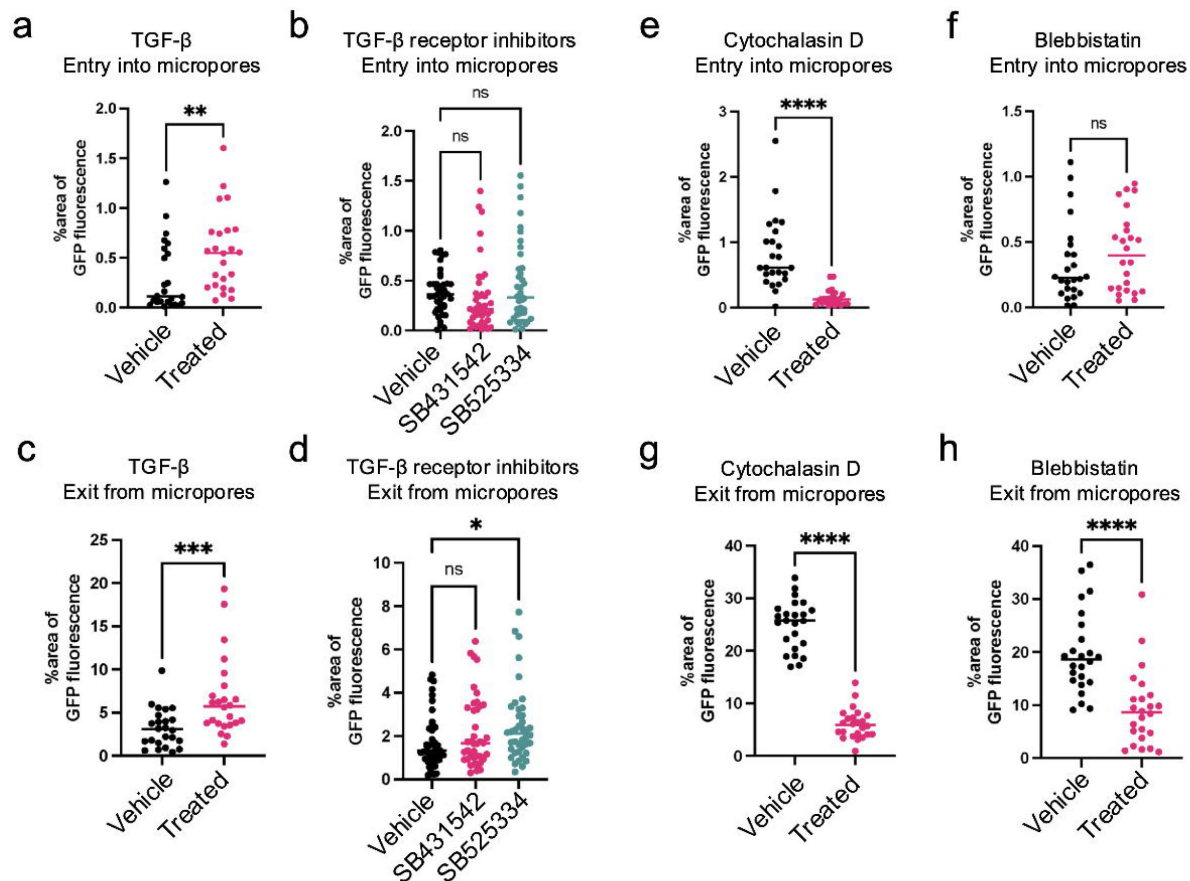


Figure 3.

Chemical treatment on KC entry into and exit from 3.0-μm micropores

(a,b) Quantification of LifeAct-KCs present within 3.0-μm micropores 6 hours after cell seeding. Cells were treated with TGF-β ligand (a) or TGF-β receptor inhibitors, SB431542, and SB525334 (b). (c, d) Quantification of LifeAct-KCs present below 3.0-μm micropores 24 hours after cell seeding. Cells were treated with TGF-β ligand (c) or TGF-β receptor inhibitors (d). (e, f) Quantification of LifeAct-KCs present within 3.0-μm micropores 6 hours after cell seeding. Cells were treated with cytochalasin D (e) or blebbistatin (f). (g, h) Quantification of LifeAct-KCs present below 3.0-μm micropores 24 hours after cell seeding. Cells were treated with cytochalasin D (g) or blebbistatin (h). Two-tailed Mann-Whitney U tests (a, c, e-h) and Kruskal-Wallis tests followed by Dunn's multiple comparison test (b, d) were performed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

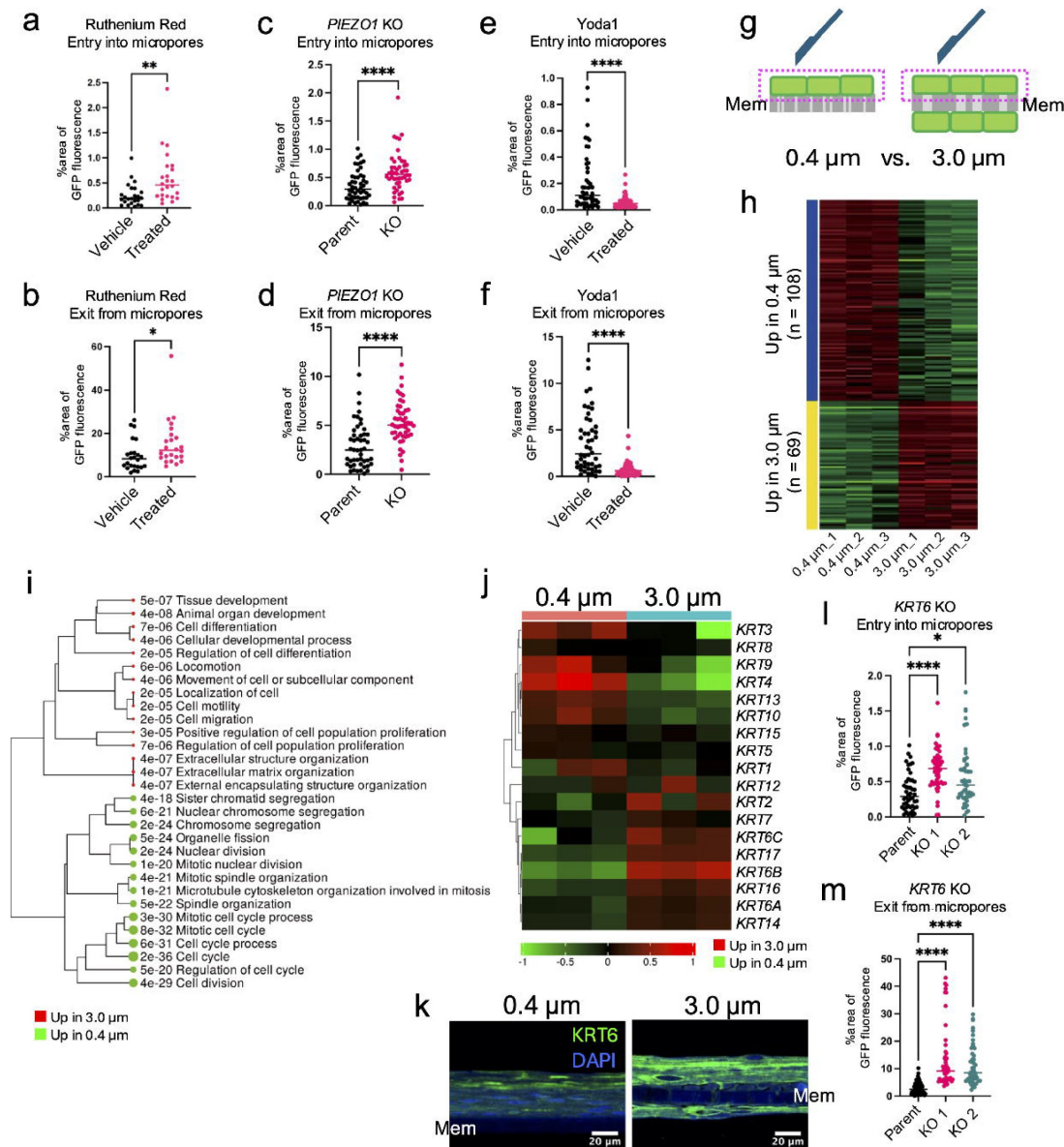


Figure 4.

Regulatory role of Piezo1 and KRT6 on KC entry to and exit from 3.0- µm micropores

(a,b) Quantification of ruthenium red-treated LifeAct-KCs present within 3.0 µm micropores at 6 hours (a) and the cells present below 3.0-µm micropores 24 hours after cell seeding (b). (c, d) Quantification of Piezo1 KO LifeAct-KCs present within 3.0 µm micropores at 6 hours (c) and the cells present below 3.0-µm micropores 24 hours after cell seeding (d). (e, f) Quantification of Yoda1-treated LifeAct-KCs present within 3.0-µm micropores at 6 hours (e) and the cells present below 3.0-µm micropores 24 hours after cell seeding (f). (g) Schematic diagram of bulk RNA-seq comparing HaCaT KCs cultured on a 0.4-µm- or 3.0-µm-pored membrane. The magenta dashed rectangles indicate the cells compared in the bulk RNA-seq. (h) Heatmap of differentially expressed genes identified by the bulk RNA-seq. (i) Gene ontology biological process terms enriched in upregulated and downregulated genes. (j) Heatmap of differentially expressed keratin genes in HaCaT KCs cultured on a 0.4-µm- or 3.0 µm-pored membrane. (k) Keratin 6 (KRT6) labeling of normal human epidermal KCs cultured on a 0.4-µm- or 3.0-µm-pored membrane for 14 days. Scale bar: 20 µm. (l, m) Quantification of pan-KRT6 KO LifeAct-KCs present within 3.0-µm micropores at 6 hours (l) and the cells present below 3.0-µm micropores 24 hours after cell seeding (m). Two-tailed Mann-Whitney U tests (a-f) and Kruskal-Wallis tests followed by Dunn's multiple comparison test (l, m) were performed. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

three keratins (pan-KRT6 KO KCs; Supplementary Fig. 3c, d). Pan-KRT6 KO KCs showed pronounced cell entry and exit from 3.0- μ m micropores (**Fig. 4l, m**, Supplementary Fig. 3e). These data indicate that Piezo1 and keratin 6 negatively modulate KC migration through confined spaces.

Discussion

Our study presents triphasic epithelia in which the original epithelium above 3.0- μ m micropores and a new epithelium under the micropores were interconnected with non-cancerous KCs exhibiting EMT. The 3.0- μ m micropores specifically induced the unique epithelial tissue because larger micropores failed to maintain the epithelium above the membrane and smaller pores did not allow the cells to pass through. KCs entered the 3.0- μ m micropores but showed a reciprocating movement until they finally exited the pores. This reciprocating movement (micropore-induced reciprocating movement of epithelial cells; MIRMET) might underlie the formation of the triphasic epithelia because, otherwise, the unidirectional migration of KCs toward the exit from the pores could deplete the epithelium above the micropores.

Although the TGF- β ligand accelerated the KC entry into 3.0- μ m micropores, the TGF- β pathway is dispensable because the blockade of TGF- β receptors did not slow this process. This is probably because the pivotal step of KC entry into 3.0- μ m micropores involves the extension of actin-rich filopodia-like structures into the micropores, which might not be regulated by the TGF- β pathway. As expected, inhibiting actin polymerization effectively prevented KC entry into 3.0- μ m micropores. The lack of effect of blebbistatin on KC entry implies that this process does not require myosin II-mediated contractile activity. In contrast, our data indicate that blebbistatin slowed KC exit from the micropores, which may rely on actomyosin contractile activity, similar to observations in HeLa cells (Liu et al., 2015).

Reduced Piezo1 is known to accelerate wound healing in mice and KC migration in 2D cultures (Holt et al., 2021). This is explained by the role of Piezo1 in regulating cell shape, causing cell retraction (Holt et al., 2021) and suppressing leader cell formation (Chen et al., 2024). Our data concerning the negative impact of Piezo1 on cell entry into 3.0- μ m micropores might be associated with the less polarized shape of Piezo1-null KCs, as described previously (Holt et al., 2021). Since ruthenium red is a universal ion-channel blocker, mechanosensors other than Piezo1 may be involved in cell entry into micropores. Further research is required to address this issue.

Keratin 6 is a stress-induced keratin that interacts with keratin 16 and 17 (Cohen et al., 2024). Monoallelic variants in *KRT6A*, *KRT6B*, and *KRT6C*, as well as *KRT16* and *KRT17*, are causal for pachyonychia congenita, a disease in which patients develop nail dystrophy, steatocystoma multiplex, and calluses (O'Toole et al., 2024). *Krt6a*- and *Krt6b*-null KCs have shown faster collective cell migration by regulating myosin ATPase activity (Wang et al., 2018), which is possibly compatible with the accelerated cell exit from 3.0- μ m micropores of pan-KRT6 KO KCs in our study. However, the pronounced cell entry into the micropores of pan-KRT6 KO KCs might not be explained by this mechanism because blebbistatin did not prevent KCs from entering the micropores. Instead, as exemplified by the increase of stress fibers in *Krt6a*- and *Krt6b*-null KCs (Wong & Coulombe, 2003), more direct effects on actin organization in pan-KRT6 KO KCs may be involved in the cell entry into the micropores.

Wound healing requires several components and steps: an intact surrounding epithelium that supplies migrating cells to heal the wound, KC migration to the wound area, and re-epithelialization once the wound is fully covered. The triphasic epithelia in our study reproduce these components in a culture system, and the advantage of this model is that each step of the wound healing process is simultaneously visible. Another idea is that the micropores in our system may simulate holes in the ECM or basement membrane (BM), which epithelial cells

protrude into during migration. The intact BM pores are estimated to be around 10 nm (Yurchenco, 2011), much smaller than the micropores in our study. However, in special circumstances such as wound healing, BM can be microbreached due to physical insults or proteases released from KCs or other cells. Similar cell migration occurs in salivary gland development, in which epithelial cells protrude into BM holes with a size of μm (Harunaga et al., 2014). Aligning with our study, salivary epithelial cells move in and out of BM during development (Harunaga et al., 2014). In conclusion, our study presents triphasic epithelia in a culture system, which is possibly based on the unique phenomenon of MIRMET. Our model can be exploited to reproduce wound healing and epithelial development and help develop therapeutics for skin wounds.

Methods

Cells

Normal human epidermal KCs collected from neonatal foreskin were purchased from Kurabo. HaCaT KCs, originally derived from the back skin of a 62-yr-old man (Boukamp et al., 1988), were provided by Dr. Norbert Fussenig's lab (German Cancer Research Center), and the cell identity was confirmed with the Cell Culture STR profile (Biologica). Single-cell-cloned HaCaT KCs with stable Cas9 expression were established previously to enable later KO experiments (S. Mai et al., 2023; Y. Mai et al., 2024). The GFP-LifeAct-expressing vector was generated by inserting the LifeAct sequence (a gift from Dr. Kurisu, Tokushima University) into the pRetroQ- AcGFP1-C1 vector (Takara Bio). The GFP-LifeAct-expressing vector was transfected into Amphi-Pack 293 cells (Clontech) using Avalanche Everyday Transfection Reagent (EZ Biosystems) according to the manufacturer's instructions. GFP-LifeAct-expressing retrovirus was collected from the culture medium of transfected Amphi-Pack 293 and infected into Cas9-expressing HaCaT KC with 8 $\mu\text{g}/\text{mL}$ polybrene (Sigma-Aldrich). After the infection, GFP-LifeAct-expressing cells were sorted by BD FACSaria III (BD Biosciences) and single-cell cloned to generate LifeAct-KCs. Piezo1 KO KCs and pan-KRT6 KO KCs were established from LifeAct-KCs as described previously (Y. Mai et al., 2024). CRISPR RNA (crRNA) was acquired from predesigned Alt-R CRISPR-Cas9 guide RNA (Integrated DNA Technologies), and the crRNA contained the CACGCGCTGGTCTCAACACCGG sequence, with the CGG PAM sequence targeting exon 8 of the *PIEZO1* genomic sequence (NG_042229.1). The crRNA to generate pan-KRT6 KO KCs contained the CGACCTGCGCAACCAAGCAGG sequence to target a shared region among *KRT6A* (NG_008298.1), *KRT6B* (NG_008299.1), and *KRT6C* (NG_012416.1). The KO of each gene was confirmed by quantitative RT-PCR (qRT-PCR) and Western blotting. We routinely tested all cells for mycoplasma contamination and confirmed negative results.

Cell culture

Normal human epidermal KCs were thawed and cultured in EpiLife Medium with 60 μM calcium (ThermoFisher) using a supplement kit containing insulin, human epidermal growth factor, hydrocortisone, antibiotics, and bovine pituitary extract (Kurabo), then stocked in CELLBANKER 2 (ZNQ) after two passages. For NHEK 3D modeling, NHEKs were thawed from stocks into EpiLife, treated with trypsin at subconfluent, and suspended in CnT-Prime (CELLnTEC) at a concentration of 2.5×10^5 cells/mL for seeding. The day before seeding, Millicell Hanging Cell Culture Insert, PET 0.4, 1.0, 3.0, 5.0, or 8.0 μm , 12-well (Merck), was placed in 12-well plates (Corning) and coated with CELLstart solution (ThermoFisher) diluted 50-fold in PBS with MgCl_2 and CaCl_2 (Sigma-Aldrich) and incubated overnight at 4 °C. After removal of the coating solution, 1 mL of CnT-Prime was added in 12 well plates, and 1 mL of cell suspension was seeded inside the inserts. Three days after seeding, the medium was replaced with CnT-Prime 3D Barrier Culture Medium (CELLnTEC) supplemented with L(+)-ascorbic acid at a concentration of 50 $\mu\text{g}/\text{mL}$. The medium inside the insert was removed, and the cell surface was exposed to air on the following day. After the air exposure, Extra Thick Blot Filter Paper (Bio-Rad), cut into 28 mm squares and with two 12-mm holes, was placed on Falcon 6-well deep well plates (Corning), and each well was filled with 11.5

mL of CnT-Prime-3D (Y. Mai et al., 2024 [DOI](#)). The inserts were placed on the holes of the filter paper, followed by medium changes every 3 days. After 14 days from seeding, the membrane was cut from the insert and used for histology, immunofluorescence, and RNA extraction. Cas9-expressing HaCaT KCs and LifeAct-KCs were cultured in Dulbecco's modified eagle medium (DMEM) with 4.5g/L glucose and L-glutamine (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; HyClone) and 1x antibiotic–antimycotic mixed stock solution (Nacalai Tesque). The procedure for 3D modeling of HaCaT KCs using inserts was similar to that used for normal human epidermal KCs, except that the DMEM, FBS, and antibiotics were used after seeding and air exposure.

Live cell imaging

Live cell imaging on LifeAct-KCs cultured on a microporous membrane was carried out with LSM710 (Carl Zeiss) or FV1000 (Olympus) confocal microscopes equipped with an incubation chamber maintained at 37 °C and 5% CO₂. During live cell imaging, LifeAct-KCs were cultured in DMEM (4.5 g/L glucose) without phenol red (Nacalai Tesque) supplemented with L-Glutamine (ThermoFisher). LifeAct-KCs were seeded at 7.0×10^5 cells/mL per pre-coated insert. The inserts were placed on the glass-bottomed dishes containing 1 mL of medium. The lids were fixed by wrapping their sides with Parafilm with air holes. Live cell imaging was performed after 6 hours of incubation from seeding.

Quantification of reciprocating movement of KCs

The time sequence of the image files taken with the LSM710 and FV1000 was used for the quantification of the cell movement. Cellular locations were estimated by the region with high GFP intensity. We first smoothed the images by taking the average intensity of the nine neighboring pixels. Then, the pixels with top X percent intensity were detected. If more than Y pixels with top X percent high-intensity pixels were connected, the region should correspond to the pore where a cell entered and exited. The pore region was defined as a circular region with a radius of Z pixels, where the center corresponds to the center of mass of the connected region. We calculated the average intensity in each pore region as a function of time. For each pore region, the minimum of the maximum values in each pore region of one experiment was used as the threshold, and the number of consecutive images exceeding the threshold was counted. When the first or last image exceeded the threshold, the consecutive counts were excluded if it was 5 or less. We set the parameters $X = 1$, $Y = 10$, and $Z = 5$ for the case with 3- μ m pores and $X = 3$, $Y = 50$, and $Z = 20$ for the case with 8- μ m pores.

Quantification of cell entry to and cell exit from micropores

Image files taken with the LSM710 and FV1000 were uniformly processed by an ImageJ macro that binarized the images after Gaussian blurring. For each image, the %Area was calculated by dividing the number of GFP pixels by the total number of pixels in each image.

Ligand and chemical treatments

Recombinant human TGF- β 1 (Peprotech), TGF- β receptor 1 inhibitor, SB431542 (Selleck) and SB525334 (Selleck), cytochalasin D (Cayman Chemical), blebbistatin (Cayman Chemical), ruthenium red (Wako), and Yoda1 (Cayman Chemical) were used at concentrations of 5 ng/mL, 10 μ M, 10 μ M, 0.25 μ M, 50 μ M, 100 μ M, and 25 μ M, respectively. These reagents were added at seeding, except cytochalasin D, blebbistatin, and Yoda1, which were added 5.5 hours after seeding because they prevent cell attachment to the membrane. The cells were evaluated 6 hours after seeding for cell entry into the micropores and at 24 hours for cell exit from the pores after seeding. The cytoskeletal blockers cytochalasin D and blebbistatin were washed out with fresh medium after the imaging, 6 hours after seeding.

RNA extraction and quantitative RT-PCR

RNA was extracted from normal human epidermal KCs or cultured HaCaT cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was prepared using the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific). qRT-PCR was performed using the designated primers and fast SYBR Green (Thermo Fisher Scientific) in a STEP-One plus sequence detection system (Applied Biosystems, Waltham, MA, USA). All primers used in this study are listed in Supplementary Table 1. Pan-*KRT6* primers were designed based on the sequences common to *KRT6A*, *KRT6B*, and *KRT6C*. *GAPDH* and *YWHAZ* were used as internal controls in the qRT-PCR of normal human epidermal KCs and HaCaT KCs, respectively.

Western blotting

Cell lysates were collected using a lysis buffer containing 1% Nonidet P-40 (Nacalai Tesque), 25 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid, and a 1:100 dilution protease inhibitor cocktail (P8340; Sigma-Aldrich) on ice for 30 minutes with shaking. The lysates were centrifuged at 15,300 g at 4 °C for 20 minutes. Cell lysate supernatants were denatured with a 5× loading buffer (0.25 M Tris-HCl; 8% sodium dodecyl sulfate; 30% glycerol; 0.02% bromophenol blue; 0.3 M β-mercaptoethanol; pH 6.8). Samples were subjected to electrophoresis using NuPAGE 4% to 12%, Bis-Tris, 1.0–1.5 mm, Mini Protein Gels (Thermo Fisher Scientific). Proteins separated in the gels were transferred onto PVDF transfer membranes (Bio-Rad). The membranes were blocked with 2% skim milk and incubated with rabbit anti-PIEZO1 antibody (Cat# NBP1-78537, RRID:AB_11003149, 1:500 dilution; Novus), rabbit anti-KRT6A antibody (Cat# PRB-169P, RRID:AB_10063923, 1:1,000 dilution; Biolegend), or mouse anti-GAPDH antibody (Cat# G8795, RRID:AB_1078991, 1:5,000 dilution; Sigma) in 2% skim milk at 4 °C overnight. After washing with Tris-buffered saline, the membranes were incubated with peroxidase-conjugated anti-rabbit IgG antibody (Cat# 711-035-152, RRID:AB_10015282, 1:5,000 dilution; Jackson ImmunoResearch) or anti-mouse IgM antibody (Cat# 315-035-020, RRID:AB_2340064, 1:5,000 dilution; Jackson ImmunoResearch) in 2% skim milk at RT for 1 hour. Signals were visualized by Clarity Western ECL Substrate (Bio-Rad) and detected with an ImageQuant LAS 4000 mini camera system (Fujifilm).

Electron microscopy

Ultrastructural observation of HaCaT KCs was performed as previously described (Kosumi et al., 2022 [\[1\]](#)). Briefly, HaCaT KCs were cultured for 4 hours after seeding in DMEM supplemented with 10% FBS (HyClone) and 1× antibiotic–antimycotic mixed stock solution (Nacalai Tesque) on 3.0-μm-pored cell culture inserts (Merck KGaA, Darmstadt, Germany). The inserts were pre-coated with CELLstart (Thermo Fisher Scientific, Waltham, MA, USA). HaCaT KCs on the inserts were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The samples were dehydrated and embedded in Epon 812. Thin sections for electron microscopy were cut to 70 nm in thickness, stained with uranyl acetate and lead citrate, and examined using a JEM1400 (Japan Electron Optics Laboratory, Tokyo, Japan).

RNA-seq

HaCaT KCs were cultured on a 0.4-μm- or 3.0-μm-pored micromembrane for 48 hours. KCs above the membrane were then collected using a scraper. RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). Library preparation, sequencing, and further analysis were performed as described previously (Y. Mai et al., 2024 [\[2\]](#)).

Histology

KCs cultured on a microporous membrane were fixed with formalin and embedded in paraffin after dehydration. Sectioned paraffin samples were deparaffinized and stained with hematoxylin and eosin (HE). For immunohistochemistry, KCs were fixed with 4% paraformaldehyde in PBS, followed by dehydration and embedding in paraffin. Anti-Vim antibody (Cat# EPR3776, RRID:AB_10562134, 1:500 dilution; Abcam), biotin-conjugated anti-rabbit IgG antibody (Cat# E0432, RRID:AB_2313609, 1:200 dilution; Agilent), VECTASTAIN ABC Standard Kit (Vector Laboratories), and DAB substrate (Roche Diagnostics Inc.) were used for staining. Images were taken with a BZ-9000 microscope (Keyence, Osaka, Japan).

Immunofluorescence

Cultured KCs were fixed with 4% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100 in PBS for 20 minutes at room temperature. Formalin-fixed paraffin-embedded samples were sectioned and deparaffinized. The cells or sectioned deparaffinized samples were then incubated with primary antibodies overnight at 4°C. After washing in PBS, the cells were incubated with secondary antibodies conjugated with Alexa488, Alexa568, or Alexa647 for 1 hour at room temperature. The nuclei were stained with 4',6-diamidino-2-phenylindole. All stained immunofluorescent samples were observed using a confocal laser scanning microscope (FV1000, Olympus). The following antibodies were used: anti-Vim (Cat# EPR3776, RRID:AB_10562134, 1:500 dilution; Abcam or Cat# sc-373717, RRID:AB_10917747, 1:200 dilution; Santa Cruz), anti-E-cad (Cat# AF648, RRID:AB_355504, 1:1,000 dilution; R and D system or Cat# 3195, RRID:AB_2291471, 1:200 dilution; Cell Signaling), anti-N-cad (Cat# ab18203, RRID:AB_444317, 1:100 dilution; Abcam), anti-SLUG (Cat# 9585, RRID:AB_2239535, 1/100 dilution; Cell Signaling), and anti-KRT6A (Cat# PRB-169P, RRID:AB_10063923, 1:500 dilution; Biolegend).

Statistics

Statistical analyses were performed with GraphPad Prism 10 (GraphPad Software). P-values were determined with two-tailed Mann–Whitney U tests or Kruskal–Wallis tests followed by Dunn's multiple comparison test. P-values of <0.05 were considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

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Supplementary material

Gene	Forward (5'→3')	Reverse (5'→3')
<i>CDH1</i>	GCTGGACCGAGAGAGT TTCCCTACGTATAC	CTAACGTCGTAATCACC ACACTGAAAGTGA
<i>CDH2</i>	TCTGACAATGGAATTCC TCCTATGAGTGGA	CCTCAAGTGTTACCTCA AGAGGCAGAGACT
<i>FN1</i>	GTGTCAGATACCAGTG CTAC	CAGACCTATCCAAGCT CAAG
<i>VIM</i>	CTTGACATTGAGATTGC CACCTACAGGAAG	GATGGACAGGTTATCA ACGAAACTTCTCAG
<i>SNAI1</i>	CGCTCTTTCCTCGTCA GG	TCTAATCCAGAGTTTAC CTTCCA
<i>SNAI2</i>	CTTCCTGGTCAAGAAG CATT	TGGACACACATACAGT GATTAT
<i>GAPDH</i>	GAAGGTGAAGGTCGGA GTC	GAAGATTGGTGATGGG ATTTTC
<i>PIEZO1</i>	CAAATGCAGCCGAGAG ACAG	CAGCGACATGAAGAGC AGTG
<i>KRT6</i>	GGGTTTCAGTGCCAAC TCAG	CCAGGCCATACAGACT GCGG
<i>YWHAZ</i>	ACTTTTGGTACATTGTG GCTTCAA	CCGCCAGGACAAACCA GTAT

Supplementary Table 1.

Primers used in qRT-PCR

Additional files

Supplementary Figure 1. Characterization of HaCaT KCs and LifeAct-KCs. **(a)** LifeAct-KCs kept on a dish. Scale bar: 20 μm . **(b)** HE staining of parental HaCaT KCs cultured on a 1.0- or 3.0- μm -pored membrane for 14 days. Scale bar: 20 μm . **(c)** Ultrastructural findings of HaCaT KCs cultured on a 3.0- μm -pored membrane. Scale bar: 5.0 μm . [🔗](#)

Supplementary Figure 2. LifeAct-KCs treated with chemicals. **(a, b)** Representative images of LifeAct-KCs present within 3.0- μm micropores 6 hours after cell seeding. Cells were treated with TGF- β ligand **(a)** or TGF- β receptor inhibitors, SB431542, and SB525334 **(b)**. **(c, d)** Representative images of LifeAct-KCs present below 3.0- μm micropores 24 hours after cell seeding. Cells were treated with TGF- β ligand **(c)** or TGF- β receptor inhibitors **(d)**. **(e, f)** Representative images of LifeAct-KCs present within 3.0- μm micropores 6 hours after cell seeding. Cells were treated with cytochalasin D **(e)** or blebbistatin **(f)**. **(g, h)** Representative images of LifeAct-KCs present below 3.0- μm micropores 24 hours after cell seeding. Cells were treated with cytochalasin D **(g)** or blebbistatin **(h)**. **(i)** Representative images of ruthenium red-treated LifeAct-KCs present within 3.0- μm micropores 6 hours after cell seeding (left images) and below 3.0- μm micropores 24 hours after cell seeding (right images). **(j)** Representative images of LifeAct-KCs treated with Yoda1 present within 3.0- μm micropores 6 hours after cell seeding (left images) and below 3.0- μm micropores 24 hours after cell seeding (right images). Scale bar: 30 μm . [🔗](#)

Supplementary Figure 3. Supplementary Figure 3. PIEZO1 and pan-KRT6 knockout keratinocytes. **(a)** qRT-PCR results of PIEZO1 on wild-type (WT) and PIEZO1 knockout (KO) keratinocytes (KCs). **(b)** Western blot analysis using WT and PIEZO1 KO KCs. Immunoblotted by anti-PIEZO1 antibody and anti-GAPDH antibody. **(c)** qRT-PCR results of pan-KRT6 on WT and pan-KRT6 KO KCs. **(d)** Western blot analysis using WT and pan-KRT6 KO KCs. Immunoblotted by anti-KRT6 antibody and anti-GAPDH antibody. **(e)** Representative images of LifeAct-KCs present within 3.0- μm micropores 6 hours after cell seeding (upper images) and below 3.0- μm micropores 24 hours after cell seeding (lower images). Scale bar: 30 μm . [🔗](#)

Supplementary Movie 1. A time-lapse movie of LifeAct-KCs that exited the 3.0- μm -pored membrane (related to Figure 2b). Images are each separated by 5 minutes. [🔗](#)

Supplementary Movie 2. A time-lapse movie of LifeAct-KCs that entered the 3.0- μm micropores (related to Figure 2d). Images are each separated by 5 minutes. [🔗](#)

Supplementary Movie 3. A time-lapse movie of LifeAct-KCs that entered the 3.0- μm micropores (high-magnified view; related to Figure 2e). Images are each separated by 1 minute. [🔗](#)

Supplementary Movie 4. A time-lapse movie of LifeAct-KCs that entered the 8.0- μm micropores (high-magnified view; related to Figure 2f). Images are each separated by 1 minute. [🔗](#)

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Reviewer #1 (Public review):

Summary:

The study describes the migration of epidermal keratinocytes through porous membranes and observes a unique size selection whereby only on 3-micron membrane are keratinocytes able to migrate and reform an intact epidermis. The authors propose that the model replicates three cell states of the intact epidermis, EMT, and MET. They also show that this response depends on the actin cytoskeleton and Piezo1, and the migration could be stimulated with TGFbeta ligands.

Strengths:

Strengths of the study include the establishment of a simple yet robust in vitro model that captures all three cell states, which could be useful for future investigation of wound healing or metastasis. There is also good characterisation of the pore size effects, providing some interesting observations about the physical regulation of keratinocyte migration. The images and presentation are clear.

Weaknesses:

(1) Some of the terminology would benefit from better definition or refinement. Triphasic suggests different physical behaviours (e.g. liquid-liquid phase separation) rather than cellular properties. Perhaps it would be better to refer to these as cell states or to describe the model more specifically as an invasion or EMT model. Likewise, the term 'reciprocating' implies two-way communication, but it is used to describe two-way migration or oscillating migration. Here, perhaps oscillatory would be clearer.

(2) The quantification and statistical analysis of key results could be improved. Notably, quantification of immunostaining in Figures 1 and 2 would strengthen core findings, and greater detail is needed on the sample sizes and number of experiments used for statistical analysis. These details are missing or only appear to N=1 in some places.

(3) There is an attempt to analyse the underlying molecular mechanisms, but these studies lack depth and detail. For example, it is not clear how actin, keratins, and piezo1 communicate to regulate cell migration. Are they acting directly on EMT genes such as SNAI1 or through changes in cell mechanics and cell-cell adhesions? Likewise, is TGF-beta signalling active in the system (e.g. nuclear pSMAD during cell migration)? As a result, the new biological insight is somewhat limited and confirms much of what is known about these pathways in keratinocyte migration.

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Reviewer #2 (Public review):

Summary:

The manuscript by Nohara et al. presents a novel 3D assay that allows for stratification of epithelia, active EMT through small pores, and active MET. They show that 3µm pores allow for keratinocytes to sample the pore through filopodia and up-regulate EMT genes to transverse the pores to the other side of the membrane where EMT genes are downregulated as the cells re-establish stratified epithelia. The TGFbeta pathway and actin polymerization promote the movement of cells into the pores and Piezo1 and KRT6 actively block this movement. This work provides a novel 3D assay that is likely to become a benchmark to analyze these processes using a more complex system than other current culture-specific EMT and MET assays.

Strengths:

The strengths of the manuscript include the foundational analysis of the pathways involved in establishing the tri-phasic epithelium. The authors have incorporated live imaging, drug studies, KO analysis, and RNA sequencing to show the relevant pathways involved.

Weaknesses:

While the authors provide strong evidence that the tri-phasic epithelium represents the EMT process, the MET process is largely relegated to the absence of EMT genes. It would be interesting to know how the stratified MET epithelia submerged in the media is similar or different from the stratified epithelia at the air-liquid interface.

Reviewer #3 (Public review):**Summary:**

The authors established an experimental system that reproduced three-dimensional triphasic epithelia, i.e., the original epithelium, its EMT, and MET. Keratinocytes (KCs), skin epithelial cells, placed on a microporous membrane migrated through 3.0-um or larger micropores. The 3.0-um-pored membrane induced an epithelial structure with three states: stratified KCs above the membrane, KCs showing EMT within the micropores, and a new stratified epithelium under the membrane. The membrane with larger micropores failed to maintain this triphasic epithelium. Live imaging revealed that KCs moved in a reciprocating manner, with actin-rich filopodia-like KC structures extending into and out of the 3.0-um micropores, while the cells migrated unidirectionally into larger micropores. KO of Piezo1 and keratin 6 increased KC entry to and exit from the 3.0-um micropores. Their results demonstrate that benign keratinocytes migrate through confined spaces in a reciprocating manner, which might help form triphasic epithelia, recapitulating wound healing processes.

Strengths:

Careful observation of the behaviour of keratinocytes on the different-sized pores. CrispR-Cas9 gene editing to KO Piezo 1 and keratin 6 isoforms in HaCaT keratinocytes.

Weaknesses:

There is no analysis of the matrix produced by the keratinocytes on the different pore sizes as this may influence migration.

HaCaT cells are quite different from normal keratinocytes in terms of migration. Pilcher et al. PMID: 9182674

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