Improving the skin barrier by targeting the TRPV4 ion channel with small-molecule bioactive compounds

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Abstract

Background: The barrier function of the human skin is constantly challenged by detrimental environmental factors. Hence it is of major interest to develop cosmetic treatment strategies that support the formation of a healthy skin barrier. Here we present a targeted approach based on modulating the TRPV4 ion channel by natural small-molecule bioactive compounds to improve human epidermal barrier formation.

Methods: We characterized TRPV4 function by employing CRISPR/Cas9 genome editing, electrophysiological patch clamp analysis and live cell Ca^{2+} imaging in cultured human keratinocytes as well as in a novel spheroid-based 3D epidermal model. The contribution of TRPV4 to epidermal development was determined by analyzing *in vitro* differentiation and 3D barrier function assays using reconstituted epidermal equivalents. Natural bioactives targeting TRPV4 were identified by a cell-based compound screening and validated in 3D barrier function assays.

Results: Expression of *TRPV4* increased during *in vitro* differentiation and TRPV4 activity was localized to the outer, differentiated keratinocyte layers. *TRPV4* gene knockout affected expression of differentiation markers at later stages of keratinocyte maturation. *TRPV4*-KO or treatment with TRPV4 inhibitor RN1734 increased the permeability of the epidermal

barrier (accompanied by reduced claudin-1 levels), whereas benchmark TRPV4 activator GSK101 and one of our natural bioactives improved the barrier function.

Conclusion: Our study suggests that Ca^{2+} signaling by TRPV4 is important for proper keratinocyte maturation and barrier formation, probably by influencing tight junction stability. Moreover, we proved that modulation of TRPV4 by using our natural small-molecule bioactive is a feasible approach to improve epidermal barrier formation *in vitro*.

Keywords: skin barrier; epidermis, TRP, bioactives, skin repair

Introduction

The main function of the human skin is to provide a physical barrier protecting the body from the environment. In addition it serves as an inside-out barrier, which prevents transepidermal water loss (TEWL) and dehydration of the body [1]. Various stress factors, such as temperature and humidity changes, mechanical stress, air pollution or UV radiation are constantly challenging the skin's barrier integrity and hence contribute to barrier disruption, inflammation, dry and fragile skin as well as premature ageing [2]. Barrier disruption is often associated with stressful symptoms such as itching and feeling of tension or can even aggravate inflammatory skin diseases such as psoriasis and atopic dermatitis [2]. Therefore, it is of major interest to develop strategies for cosmetic and dermatological skin care to improve the barrier integrity of the skin.

The skin barrier is created by a continuously renewing epidermis composed of different keratinocyte layers. In the basal layer, keratinocyte stem cells (KSC) divide asymmetrically into KSCs and transient amplifying cells (TA) [3]. TA cells leave the basal layer and successively develop into post-mitotic cells (PM) that further differentiate to eventually form the spinous, granular and the outermost corneal layers, characterized by ordered expression of keratins (KRT) and other characteristic marker proteins such as involucrin (IVL) or filaggrin [4]. At the end of this differentiation process, the outermost *stratum corneum* barrier is formed by non-viable corneocytes, which are surrounded by a rigid cornified envelope (CE), in which the major CE protein is IVL, which binds ceramides to form a corneocyte-bound lipid envelope (CLE). The corneocytes are additionally embedded in an extracellular matrix of lamellar-arranged lipids to seal the epidermis [5].

A second barrier below the *stratum corneum* is created by intercellular junctions, called tight junctions (TJ) and adherens junctions (AJ) [6, 7]. These complex organized structures not only provide structural integrity but also control the paracellular movement of solutes and ions. TJs harbor proteins to form a connection with the intracellular cytoskeleton network but also consist of several different transmembrane structures, such as occludins and claudins, to form the actual cell-to-cell contacts [6, 7]. The importance of AJs and TJs becomes obvious in several skin diseases in which barrier function is severely impaired, for example due to lack or reduced levels of claudin-1 in the TJ complex [8].

Clearly, healthy human skin can cope with detrimental external stress factors. But how the skin is sensing changes in the environment and initiates compensatory repair processes is still puzzling. For a wide variety of different stimuli, members of the transient receptor potential (TRP) ion channel family have been identified as sensory receptors in the skin [9]. In particular, the Ca^{2+} permeable TRPV4 ion channel, which is composed of six transmembrane domains and N-terminal ankyrin-rich repeats, was reported to be sensitive to osmotic [10, 11] and temperature changes [12, 13]. It was also reported that TRPV4 promotes (temperature-dependent) intercellular junction formation in human keratinocytes in 2D cell culture [14, 15], however, direct evidence based on genetic knockout of *TRPV4* in human keratinocytes as well as the relevance of TRPV4 in a 3D organized human epidermal organoid is missing.

Hence the aim of our study was to validate TRPV4 as a new molecular target for improving the barrier function of the human epidermis. For this purpose, we first analyzed the contribution of TRPV4 to human keratinocyte differentiation and epidermal barrier function by using small-molecule compounds as well as genetic knockout keratinocytes in classical and novel spheroid-based epidermal equivalents. Next, we identified natural small-molecule modulators of TRPV4 proving that targeting TRPV4 is a feasible approach to improve epidermal barrier function *in vitro*.

Materials and Methods

Cell culture

General cell culture conditions and cultivation of HaCaT cell lines was described earlier [16]. The NHEK-E6/E7 cell line was generated by immortalization of primary juvenile keratinocytes (Promocell, Heidelberg, Germany) using transduction of lentivirus HPV-E6/E7-Puromycin (Sirion Biotech, Graefelfing, Germany) (MOI 1) in presence of 8 µg/ml polybrene based on [17]. NHEK-E6/E7 cells were routinely cultivated in KGM-2/Keratinocyte Growth Medium 2 (Promocell) in presence of 0.3 µg/ml puromycin.

Genome editing

To generate a clonal *TRPV4*-deficient HaCaT knockout cell line (HaCaT-*TRPV4*$^{-/-}$) the CRISPR-Cas9 genome editing technology was applied. The knockout strategy was based on Cas9/sgRNA-mediated insertion of a mutated homology DNA repair (HDR) template, which contains a mutated *TRPV4* start codon, one frame-shift mutation as well as a hygromycin resistance gene, into the genomic TRPV4 locus. After transfection of the cells, single cell clones were raised by hygromycin selection and correct genomic modifications were determined by Sanger sequencing.

RNA-Sequencing

Isolation of KSCs, TA and PM cells is described in detail in [18]. RNA isolation and subsequent RNA-Seq was performed as described in [16]. Transcript abundancies were displayed in fragments per kilobase of exon per million fragments mapped (FPKM).

Electrophysiology

Voltage-clamp recordings were performed under RT in the whole-cell configuration using an EPC-10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). The internal solution contained (in mM): 140 CsCl, 1.93 $CaCl_2$, 5 EGTA, 10 HEPES, 2 $MgCl_2$, 2 Mg-ATP, 10 D-glucose, adjusted to 315 mOsmol/kg with mannitol and pH 7.2 with CsOH. The external solution contained (in mM): 70 NaCl, 5 KCl, 2 $CaCl_2$, 10 HEPES, 1 $MgCl_2$, 10 D-glucose, adjusted to 325 mOsmol/kg with mannitol and pH 7.4 with NaOH. Currents were recorded with a 10 kHz low-pass filter and sampled with a frequency of 50 kHz. Data were

memorized with Patchmaster (HEKA Elektronik) and analyzed with Fitmaster (HEKA Elektronik). Bath solution exchange was performed using a gravity flow perfusion system. Whole-cell currents were elicited by repetitively applying 500 ms voltage pulses from -120 mV to +80 mV (20 mV increments) from a holding potential of -40 mV and 500 ms voltage ramps from -100 mV to +100 mV from a holding potential of 0 mV. To account for the different sizes of the measured cells, whole-cell currents were normalized to the cell membrane capacity.

Small-molecule compounds

GSK1016790A (hereinafter called GSK101) (Sigma Aldrich and Tocris) and RN1734 (MedChemExpress and Tocris) stock solutions were prepared by dissolving 10 mM in DMSO. Gd^{3+} stock solution was prepared by dissolving 50 mM of $GdCl_3$ in external solution. Screening compounds were prepared by AnalytiCon Discovery GmbH and dissolved in DMSO/H_2O (90%/10%) (10 mM).

Ca^{2+} imaging in 2D and 3D cell culture

Measuring intracellular Ca^{2+} concentration using the Ca^{2+}-sensitive Fluo4 dye in monolayer HaCaT cell culture in a plate-reader settings was performed as described in [16]. 3D spheroids were preparred with 5000 HaCaT cells in U-shaped low attachemnt plates (Greiner Bio-One) as described in [19]. For live-cell Ca^{2+} imaging, cells were loaded with CalRed (Biomol) (30 min at 37 ^{o}C in isotonic solution). The cells were grown as monolayer directly in a perfusion chamber (Ibidi), while the spheroids were located in the Ibidi chamber on the day of the experiment and stabilized with a gelatine-based mash (Scaffolene). The isotonic (control) and the GSK101 solutions were applied with a gravitiy-mediated perfusion system. Images were acquired every 3 sec with a confocal microcope equipped with a 20x objective (Leica) at 500 nm and 600 nm emission bands. The emission ratio (500 nm / 600 nm) at each time point was normalized to the emission ratio in control solution and plotted over time.

For whole-mount immunostaining spheroids were fixed in PFA 4% and processed with IVL (Abcam ab20202, 1:1000) and KRT14 (Thermo Fisher PA5-16722, 1:1000) primary antibodies and AF-488 and AF-555 secondary antibodies (Thermo Fisher) plus glycerol-based clearing, as described in [19]. Upon confocal microscopy, the reconstructed 3D image

of the spheroids were analyzed by „neural network computation“, to segment the three-dimensional nuclei. For each cell, the fluorescence intensity in the cytoplasmic region and distance from the spheroid´s border were calculated.

Differentiation assays

In vitro differentiation protocol described by Deyrieux & Wilson [20] was used to convert HaCaT cells back to a basal cell-like state, followed by induction of differentiation using $CaCl_2$.

Western blot analysis and antibodies

Western blot analysis were performed as described in [21]. The following antibodies were used Anti-Cytokeratin 10 (ab76318, 1:10,000; Abcam, Cambridge, UK) , Anti-Involucrin (Abcam ab20202, 1:10,000), Anti-Claudin-1 (Thermo Fisher 51-9000), Anti-β-Actin (Sigma A1978, 1:10,000; Merck KGaA, Darmstadt, Germany).

3D epidermis equivalents

HaCaT or NHEK-E6/E7 cells were seeded in transwell inserts (ThinCerts, Greiner Bio-One GmbH, Frickenhausen, Germany), submerged in CnT-PR (CELLnTEC, Bern, Switzerland). After 3 days, medium was switched to 3D differentiation medium (3 parts CnT-PR-3D (CELLnTEC) / 2 parts DMEM (Thermo Fisher, Dreieich, Germany)) for 24 h. Cells were then transferred into deep well plates and lifted to the air-liquid interface. 3D Medium was refreshed every other day. The epidermis equivalents were harvested after 10 days.

Lucifer yellow barrier assay

1 mM Lucifer Yellow (LY) (Sigma Aldrich, St. Louis, USA) was applied on top of the epidermal equivalents for 60 min at 37 °C. Subsequently, 1.67 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher) was applied on the bottom of the insert for 60 min at RT. After fixation and paraffin embedding, 4 µm sections were deparaffinised and stained with Alexa Fluor 594 streptavidin (Thermo Fisher) for 30 min at RT. Sections were mounted in VECTAshield, containing DAPI (Biozol, Eching, Germany). Fluorescence signals were detected using the appropriate filter sets (FITC filter for LY, TexasRed filter for

Biotin/Strepavidin) of the Nikon Eclipse Ci microscope. Alternatively, LY fluorescence in the flow through was recorded using a fluorescence plate-reader (FITC filter) and compared to LY fluorescence obtained from a standard LY curve to quantify the concentration of LY that passed through the epidermal equivalent.

Immunohistochemistry

Processing and staining of the epidermis equivalents was performed as described in [18]. Claudin-1 antibody (51-9000, Thermo Fisher) or concentration adjusted isotype control antibody was applied overnight at 4 °C.

Statistical analysis

The applied statistical tests, the number of replicates, mean values and error bars are shown in the figures and figure legends. P-values were defined as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, $p\geq0.05$ not statistically significant (ns).

Results

Expression of *TRPV4* in keratinocytes

To address the function of TRPV4 during differentiation, we first analyzed the expression of *TRPV4* in different epidermal keratinocytes. We therefore isolated KSCs, TA and PM cells from the epidermis of healthy human donors and performed RNA-Seq analysis. We also included the widely used keratinocytes of the HaCaT cell line and of our proprietary NHEK-E6/E7 cell line. We found that *TRPV4* is expressed at similar, rather low mRNA levels in all keratinocyte populations and in the two cell lines (Fig. 1A). In differentiated PM cells there is a tendency towards higher *TRPV4* mRNA levels compared to the undifferentiated KSCs. When we induced differentiation *in vitro*, we observed the expected mRNA increase of differentiation marker genes *IVL* and *KRT10* (Fig. 1B), and also an increase of *TRPV4* mRNA by a factor of approx. three (Fig. 1B), which suggests that the *TRPV4* gene is regulated during epidermal development.

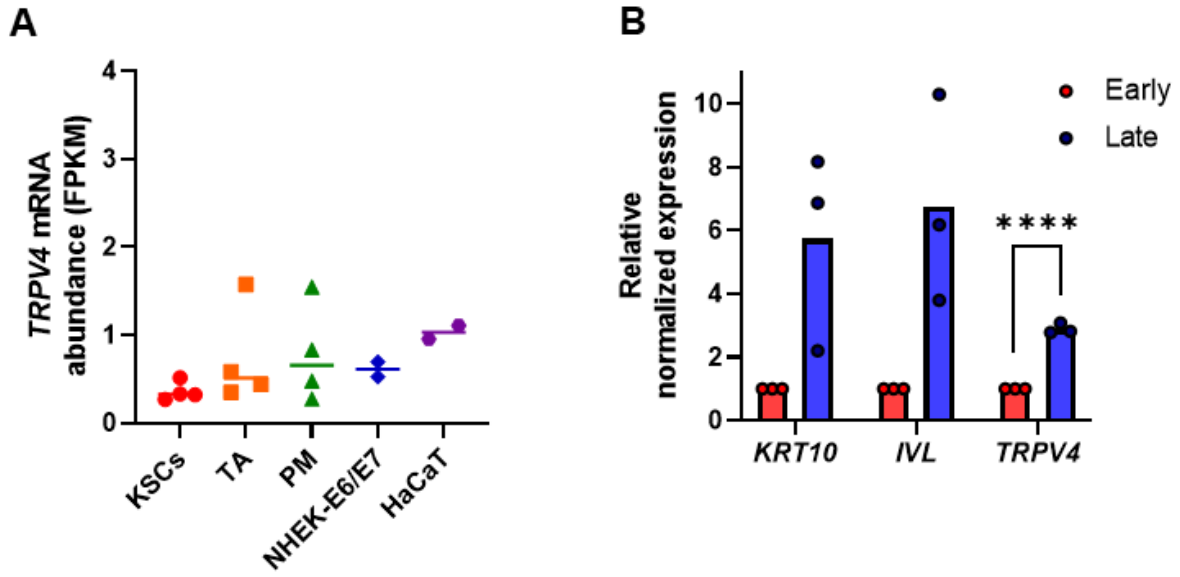

Fig. 1: (A) Expression of *TRPV4* was determined by RNA-Seq either in primary keratinocyte populations, isolated from the human epidermis (KSC: keratinocyte stem cells, TA: transient amplifying cells, PM: post-mitotic cells), or in HaCaT and NHEK-E6/E7 cell lines. mRNA abundancies of *TRPV4* are depicted as FPKM values. (B) Differences in differentiation marker expression in basal (early) vs. *in vitro* differentiated HaCaT cells (late). FPKM values of *TRPV4*, the differentiation markers keratin 10 (KRT10) and involucrin (IVL) were normalized to values of basal HaCaT cells. Individual values from n=2-4 (A) and n=3 (B) are shown. **** $p<0.0001$ (unpaired, 2-sided t-test).

CRISPR/Cas9-based knockout of *TRPV4*

To study the importance of TRPV4 in keratinocytes we created *TRPV4* knockout HaCaT cells (HaCaT-*TRPV4*$^{-/-}$) using the CRISPR-Cas9 genome editing technology. To ensure that the genomic knockout also led to a non-functional TRPV4 ion channel, we performed whole-cell patch clamp experiments. As can be seen from the low whole-cell currents, HaCaT-WT cells show negligible TRPV4 activity in the absence of an activating stimulus (Fig. 2A). Addition of the specific TRPV4 activator GSK101 (200 nM), to the external solution, resulted in strong activation of a TRPV4-typical, slightly outward rectifying current (Fig. 2A). Importantly, these GSK101-activated currents could be inhibited by addition of both the nonspecific TRPV4 blocker Gd^{3+} (100 µM) (Fig. 2D) and the specific TRPV4 inhibitor RN1734 (100 µM) (Fig. 2C, D), clearly indicating that the ionic currents measured after addition of GSK101 are conducted by TRPV4. In stark contrast to HaCaT-WT cells, the addition of GSK101 to the external solution had no effect on the whole-cell currents of HaCaT-*TRPV4*$^{-/-}$ cells (Fig. 2B, D), undoubtedly demonstrating the absence of functional TRPV4 at the plasma membrane.

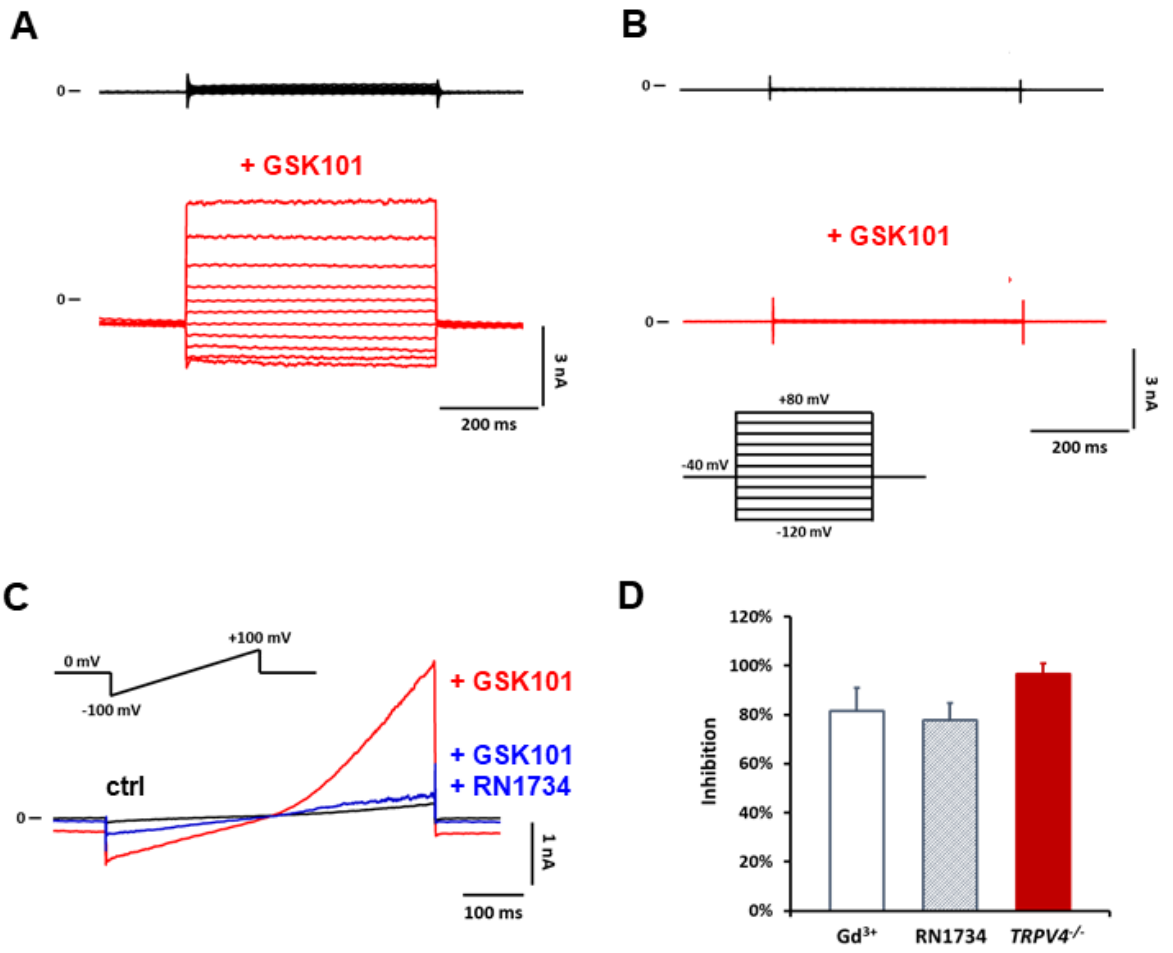

Fig. 2: Genetic knockout of *TRPV4* causes disappearance of GSK101-evoked currents in HaCaT cells. Representative whole-cell current traces from HaCaT-WT (A) and HaCaT-*TRPV4*$^{-/-}$ (B) cells evoked by the voltage-step protocol shown in (B, inset below). Currents were recorded in standard bath solution (black traces) and in the presence of TRPV4 activator GSK101 (200 nM) (red traces). (C, D) GSK101-evoked currents in HaCaT cells can be inhibited by blockers Gd^{3+} and RN1734 (D) on ionic whole-cell currents elicited by the addition of GSK101. Whole-cell currents shown in (C) and (D) were evoked using the voltage-ramp protocol shown in the left upper corner in (C). Black traces: control measurement (ctrl) before addition of GSK101, red traces: currents measured 2 min after addition of 200 nM (C) or 100 nM (D) GSK101 to the external solution, blue traces: currents measured 2 min after addition of 100 µM Gd^{3+} (C) and 100 µM RN1734 (D) to the external solution. (D) Inhibition of GSK101-activated currents at +80 mV by 100 µM Gd^{3+}, 100 µM RN1734 or *TRPV4*$^{-/-}$. Data points in (D) represent arithmetic means +/- standard deviation of 5 to 16 independent experiments.

TRPV4-mediated Ca^{2+} signaling in 2D tissue culture and 3D epidermal spheroids

Next we studied the consequence of the *TRPV4* knockout on Ca^{2+} signaling in keratinocytes. We applied increasing concentrations of GSK101 to HaCaT-WT and HaCaT-*TRPV4*$^{-/-}$ cells and monitored (in a plate-reader setting) intracellular Ca^{2+} changes using the Ca^{2+}-sensitive dye Fluo4. We observed a concentration-dependent increase of Fluo4 fluorescence in

HaCaT-WT cells, whereas none of the applied GSK101 concentrations evoked a change of intracellular Ca^{2+} concentration in HaCaT-*TRPV4*$^{-/-}$ cells (Fig. 3A). Accordingly, blocking TRPV4 by using the specific TRPV4 inhibitor RN1734 led to dose-dependent reduction of GSK101-evoked Ca^{2+} transients (Fig. 3B). Consistently, confocal live-imaging microscopy in HaCaT cells, loaded with the ratiometric dye CalRed, proved that the agonist GSK101 evoked a Ca^{2+} rise in many cells (Fig. 3C). In summary, this shows that the intracellular Ca^{2+} fluxes measured after addition of GSK101 are mediated by TRPV4.

These experiments were performed in classical 2D tissue culture, but the epidermis is organized in a 3D structure allowing extensive cell-to-cell communication. To gain more physiologically relevant, spatio-temporal insights into the TRPV4-mediated Ca^{2+} signaling in a 3D environment, we created 3D spheroids composed of HaCaT cells and performed comparable live cell imaging experiments. After stimulation of the spheroids with GSK101, large Ca^{2+} transients were observed in the cells located in the most outer region, while the cell in the center did not respond (Fig. 3C, D). This suggests a preferential functional expression of TRPV4 in the cells at the spheroid´s rim, which is the region of differentiation.

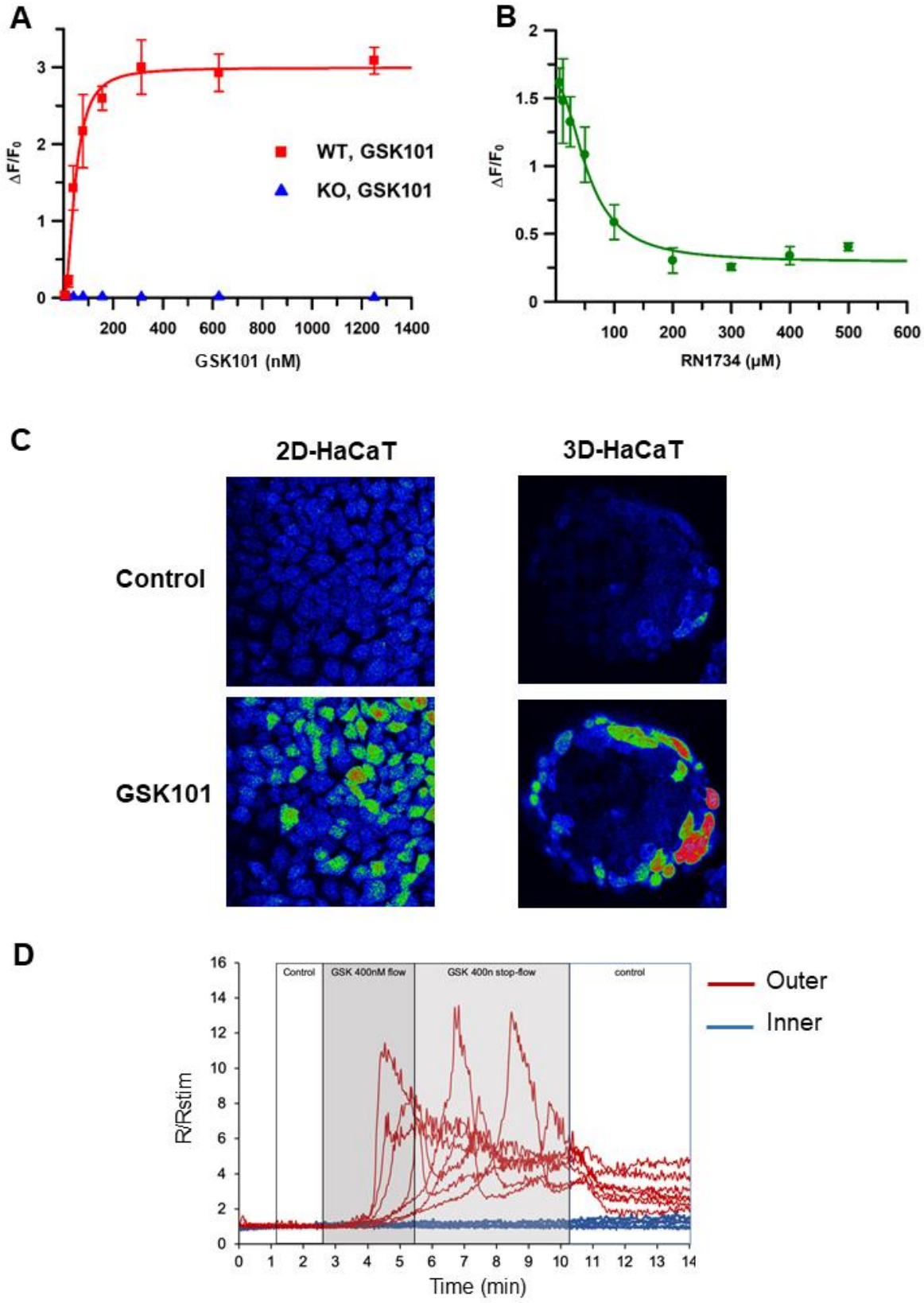

Fig. 3: TRPV4-mediated Ca^{2+} response in 2D and in 3D organized HaCaT cells. Dose-response curves in Fluo4-stained HaCaT-WT and HaCaT-*TRPV4*$^{-/-}$ (KO) cells treated with TRPV4 activator

GSK101 (A) and TRPV4 inhibitor RN1734 (B) (n=4) using a plate-reader. (C) Confocal live Ca^{2+} imaging of monolayer HaCaT cells and spheroids upon loading with CalRed. (C) Representative ratio images are shown (emission 530 nm / 650 nm) in control and upon perfusion with GSK101 (400 nM). (D) The plot shows ratiometric (500 nm / 600 nm emission bands) fluorescence changes over time in representative cells located on the border (red) and in the center (blue) of the spheroid shown in C. Value were normalized to the fluorescence before stimulation.

Function of TRPV4 during keratinocyte differentiation

Given the fact that Ca^{2+} is a crucial trigger for differentiation we now asked the intriguing question whether the Ca^{2+}-permeable TRPV4 ion channel plays a role in epidermal differentiation. For this purpose, we used basal HaCaT-WT and basal HaCaT-*TRPV4*$^{-/-}$ cells, induced differentiation by $CaCl_2$ and monitored the *in vitro* differentiation process by quantitative Western blot analysis using antibodies against the differentiation markers IVL and KRT10. As can be seen, both marker proteins increased over time, but absence of TRPV4 led to elevated levels of IVL and KRT10 in particular at later stages of differentiation (Fig. 4A, B).

To determine whether this effect is present also in a 3D epidermal model, we employed HaCaT spheroids to analyze by whole-mount immunostainings the expression of the differentiation marker IVL and of KRT14, usually present in basal keratinocytes. HaCaT cells self-assembled into 3D spheroids; while KRT14-positive cells were present also in the spheroid center, IVL-positive cells were mainly located in the outer layer (Fig. 4C). Over time, while KRT14 signal decreased, IVL expression increased, but remained localized at the rim (Fig, 4C-E). Interestingly, spheroids with HaCaT-*TRPV4*$^{-/-}$ cells showed higher expression of IVL, which is more evident at the spheroid border (Fig. 4D, E). Altogether, these findings clearly indicate a role of TRPV4 in keratinocyte differentiation.

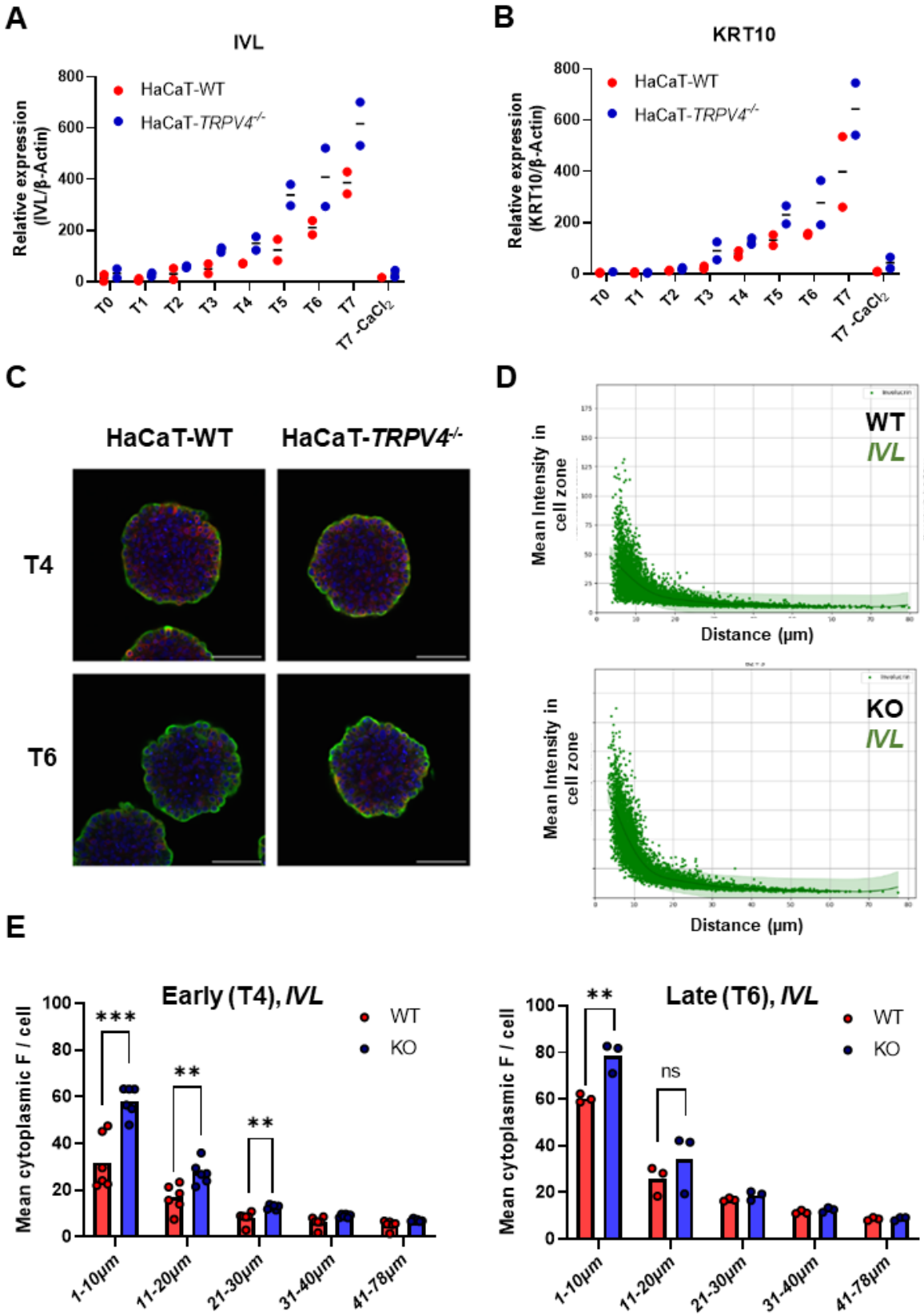

Fig. 4: Knockout of *TRPV4* affects *in vitro* differentiation. Quantitative Western blot analysis of IVL (A) and KRT10 (B) normalized to ß-Actin at different time points (T, in days) from Ca^{2+}-mediated

induction of differentiation in basal HaCaT-WT and HaCaT-*TRPV4*$^{-/-}$ (KO) cells grown as monolayers. Individual data points of n=2 are shown. (C) Representative z-projections of whole-mount immunostainings and 3D analysis of cleared WT and KO spheroids are shown at T4 and T6 using IVL (green) and KRT14 (red) antibodies. Nuclei in blue. (D) 3D analysis of spheroids at T4. Scatter plots of the mean intensity versus the distance from the spheroid´s border. Each dot represents a cell, and in each plot the cells of at at least 3 spheroids plotted together. (E) The mean fluorescence intensities of IVL in the cells, located at defined distance intervals (x-axis) from the spheroid´s border, are plotted for WT and KO cells.

Importance of TRPV4 for barrier function

Since keratinocyte differentiation was affected, we wondered whether also epidermal barrier function is impaired in absence of TRPV4. To test this, we reconstituted *in vitro* 3D epidermal equivalents from HaCaT cells and measured the barrier integrity using Lucifer yellow (LY) penetration assays. By measuring the fluorescence in the flow through, we showed that epidermal equivalents from HaCaT-WT cells had low permeability for LY, which was drastically increased when SDS was used to disrupt the barrier (Fig. 5A, B). In contrast, equivalents reconstituted from HaCaT-*TRPV4*$^{-/-}$ cells showed already in untreated conditions higher LY permeability than WT equivalents. Upon SDS treatment, the LY permeability was even further increased (Fig. 5A, B). Next we tested, whether similar effects can be achieved not only by knocking out the *TRPV4* gene but also by reducing TRPV4 activity with small-molecule compounds. We therefore applied the benchmark blocker RN1734 and measured barrier integrity by the LY assay. Strikingly, treatment with RN1734 greatly enhanced LY permeability of the WT epidermal equivalents already in unchallenged (i.e. non-SDS treated) conditions (Fig. 5B).

To better understand on the molecular level the defect occurring in absence of TRPV4 on the molecular level, we focused on the tight junctions, which form the intercellular barrier in the *s. granulosum*. Immunohistochemistry and Western blot analysis of the epidermal equivalents showed that staining of the tight junction protein claudin-1 was weaker in 3D epidermal equivalents from HaCaT-*TRPV4*$^{-/-}$ cells compared to WT cells (Fig. 5C, D). These findings suggest that the contribution of TRPV4 to barrier function probably involves the formation or stabilization of tight junction complexes.

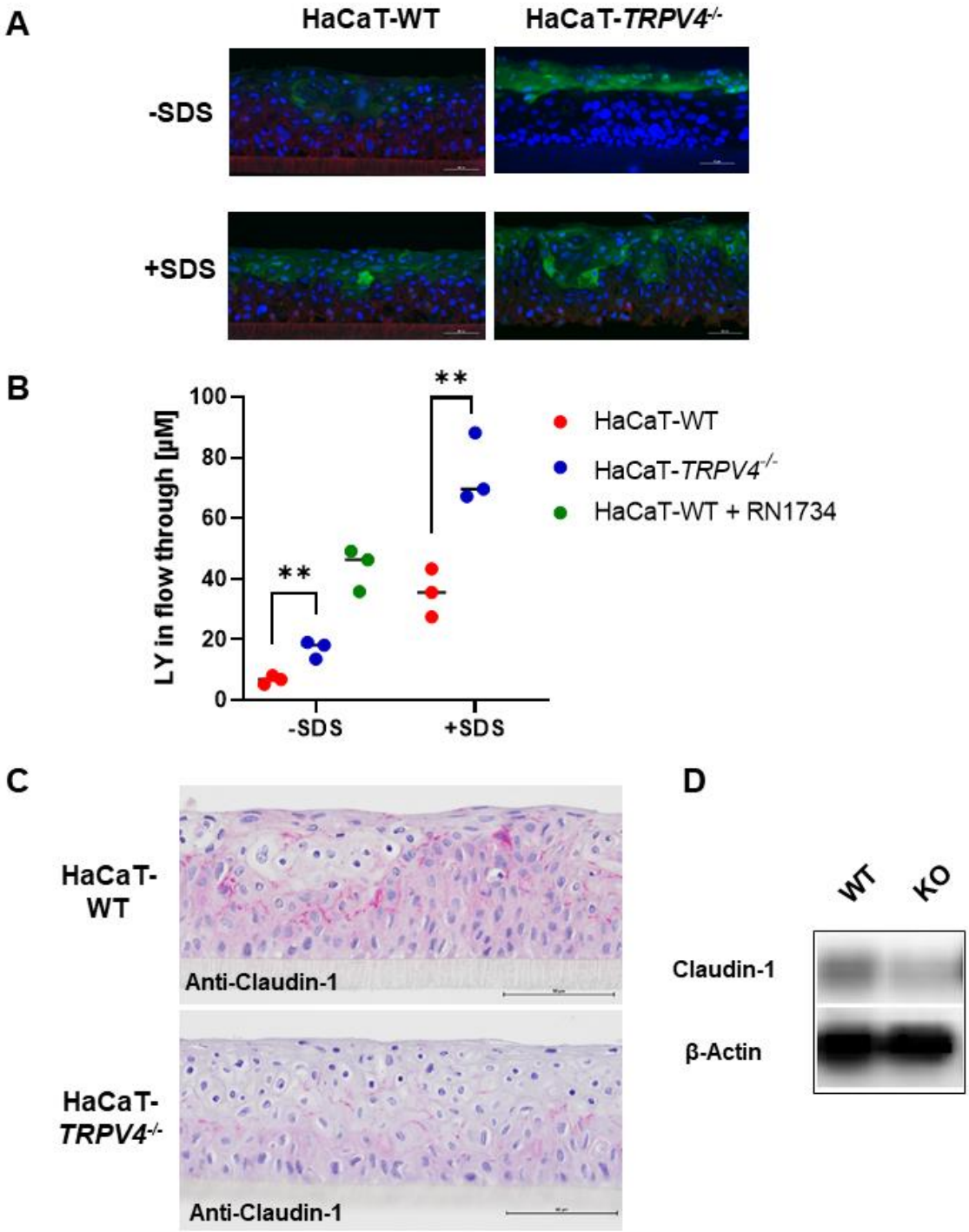

Fig. 5: Fig. 5: Barrier function was measured in reconstituted 3D epidermal models from HaCaT cells by using LY penetration assays. Penetration of LY (in green) and biotin (in red; not stained in -SDS of *TRPV4*$^{-/-}$) was analyzed after 1 h incubation (1 mM LY at the top of the model) by (A) fluorescence immunostainings of epidermal sections. Representative images are shown (A). Scale bar 50 µm. SDS 0.1 % treatment was applied. (B) Quantification of LY concentration in the flow through obtained from the bottom of the transwell. SDS treatment (0.5 %) increased penetration of LY, which was even higher in TRPV4 knockout or when TRPV4 activity was inhibited by RN1734 (200 µM). Mean and individual values (n=3) are shown. ** $p<0.01$ (unpaired, 2-sided t-test). (C) Immunohistochemistry

of 3D equivalents using an antibody against claudin-1 was performed. Claudin-1 staining (in red) is limited to few epidermal layers and appears weaker in 3D models raised from HaCaT-*TRPV4*$^{-/-}$ cells. Scale bar 50 µm. (D) Western blot of 3D equivalents confirms reduced claudin-1 levels in HaCaT-*TRPV4*$^{-/-}$ (KO) (n=1).

Identification of small-molecule modulators of TRPV4

After having shown that TRPV4 is involved in barrier function and differentiation, we next hypothesized that controlling TRPV4 ion channel activity by small-molecule compounds could be a new approach to modulate epidermal barrier function. To identify small-molecule compounds targeting the TRPV4 ion channels we performed a screening using a smart selection of compounds. We have focused on compounds that are purely natural, compliant to the Nagoya protocol, possess low regulatory and toxicological burdens, with easy availability and relatively low costs. To determine the specificity of these compounds, we measured compound-dependent Ca^{2+} fluxes, followed by a counter screening against related ion channels *TRPV1* and *TRPA1*. Next, dose-response experiments were performed to determine compound potency and efficacy. At the end of this screening, several purely natural TRPV4 modulators could be identified.

Effect of TRPV4 modulators on keratinocyte differentiation and barrier function

We finally tested whether it is indeed a feasible approach to use TRPV4 modulators for improving the skin barrier. For this purpose we reconstituted epidermal equivalents from NHEK-E6/E7 cells, which are characterized by complete stratification and formation of all keratinocyte layers similar to the native epidermis (Fig. 6A). As a consequence, the barrier of the NEHK-E6/E7 equivalents almost completely retained the LY dye confirming the presence of an intact barrier, but nonetheless it can be disrupted by treatment with SDS (Fig. 6A, B). We pre-treated the epidermal equivalents with Cmpd S and Cmpd H, two natural TRPV4 modulators identified from our screening, as well as with GSK101 as a reference TRPV4 activator and measured barrier integrity using the LY assay. LY permeability was not altered after Cmpd S treatment. However, GSK101 and Cmpd H reduced the LY permeability, which is indicative for the formation of a barrier even tighter than in untreated WT conditions. This example can be regarded as an *in vitro* proof-of-concept showing that targeting TRPV4 by small-molecule compounds is a feasible strategy for improving the epidermal barrier.

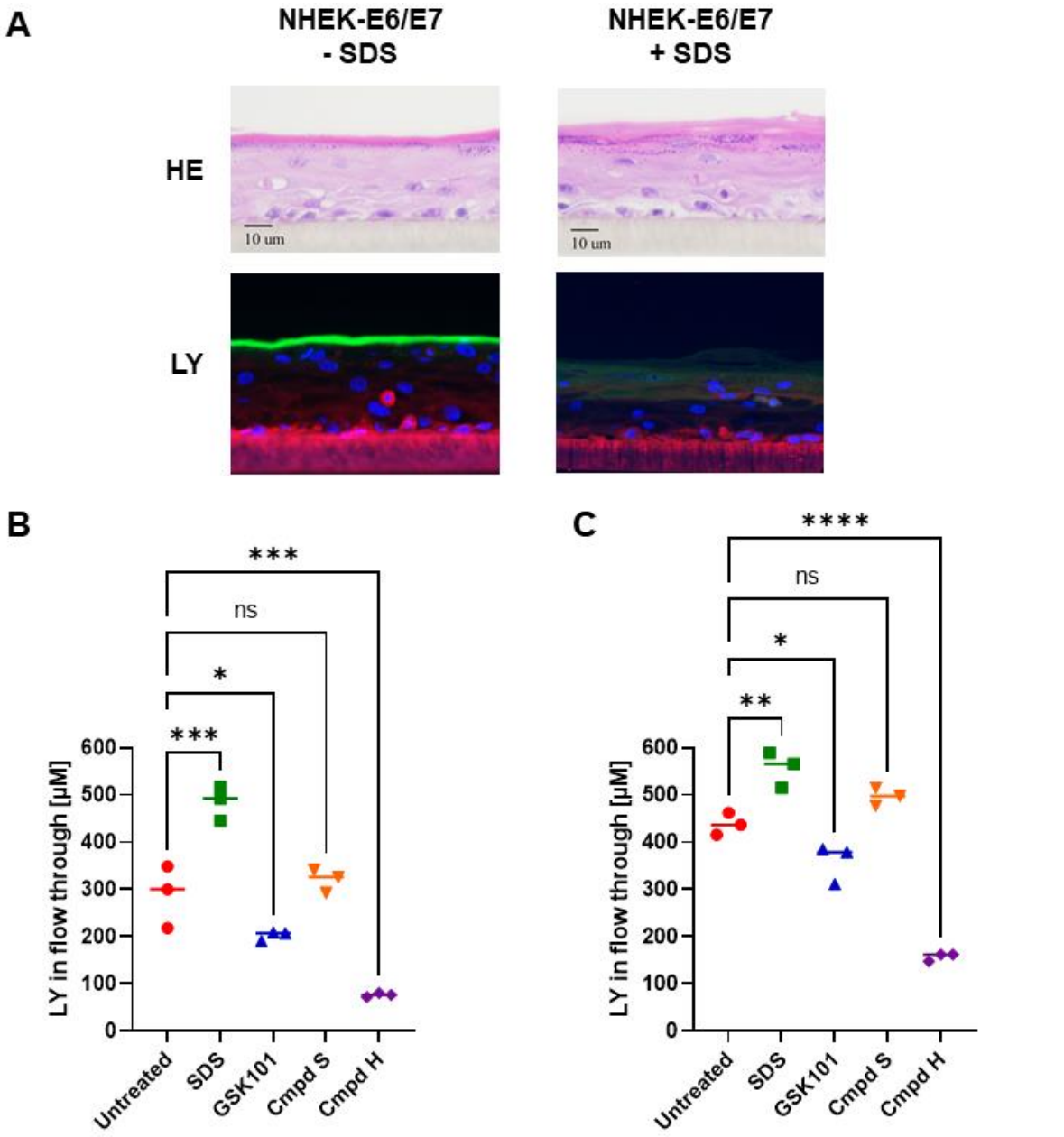

Fig. 6: Effect of small-molecule compounds targeting TRPV4 on barrier function. (A, top) HE staining of reconstituted equivalents from NHEK-E6/E7 cells shows complete stratification with keratinocyte layers similar to the native epidermis. After SDS treatment, the *s. corneum* appears looser and more softened. (A, bottom) Penetration of fluorescent LY (in green) and biotin (in red) was analyzed by fluorescence microscopy imaging of epidermal sections. Scale bar 10 µm. Notice that fully stratified NHEK-E6/E7 strongly retain LY at the border of the *s. corneum* (left panel), whereas barrier disruption by SDS (0.1 %) caused deep LY penetration (right panel). Flow through was quantified by plate reader after 60 min (B) and 120 min (C). Pre-treatment of the equivalents with GSK101 (150 nM) and Cmpd H (100 µM) reduced LY penetration after 60 min (B) and 120 min (C), while Cmpd S (100 µM) shows no effects. Mean and individual values (n=3) are shown (B, C). Stars indicate statistical significance according to one-way ANOVA test with multiple comparison.

Discussion

In our study we directly tested the importance of TRPV4 by applying a genetic CRISPR-based *TRPV4* knockout approach in human keratinocytes, which, to our best knowledge, has so far not been reported. This approach allowed us to uncover a role of TRPV4 in human keratinocyte differentiation *in vitro*. On the contrary, studies in other organisms did explicitly state no effect of *TRPV4* gene knockout on epidermal differentiation [21] indicating organism-specific differences of TRPV4 function.

Bulk expression of *TRPV4* in primary human keratinocytes has been reported previously [14, 15]. Here we went a step further and quantitatively determined *TRPV4* mRNA levels in the different keratinocyte populations (isolated from the epidermis), which represent different stages of epidermal development. We observed a tendency, towards higher expression levels of *TRPV4* mRNA in further differentiated primary keratinocytes. This is in line with our finding that *TRPV4* expression increases roughly three-fold during *in vitro* differentiation of HaCaT cells. Admittedly, the differences between the different keratinocyte populations were statistically not significant, and hence additional experiments are required to conclusively answer this question. It should be kept in mind that TRPV4 may act *in vivo* only in very few epidermal layers (such as the *s. granulosum*, see discussion below); consequently further experiments would require an even more advanced cell sorting methodology or truly specific antibodies against TRPV4 to precisely determine the site of TRPV4 action in the native human epidermis.

In our *in vitro* differentiation experiments (in 2D and in 3D) we have so far focused on few markers such as KRT10, IVL and claudin-1. To get a broader understanding about the gene families and gene clusters affected in absence of TRPV4, we are currently performing transcriptome-wide gene expression analysis using WT vs KO cells at different time points of *in vitro* differentiation. In this way we want to elucidate the potential interaction partners of TRPV4 and the downstream located signaling pathways that control formation of TJ complexes, for example via Rho signaling and β-catenin interaction [21, 22].

For basic as well as applied skin research, *in vitro* skin models of different complexities have become powerful tools [23]. Here we applied classical reconstituted epidermal equivalents not only to study barrier function mediated by TRPV4, but also to qualify new compounds with respect to their capability of strengthening the epidermal barrier. With our novel

spheroid-based model we are aiming at circumventing the inherent limitations of classical, air-lift models. Indeed, by developing our spheroid model we could shorten the time of maturation (5 vs. 14 days), increase the throughput (96- vs. 24-well format), facilitate the downstream staining protocols (whole-mount vs. sectioning the samples) and most importantly, we were able to quantify marker stainings within the different keratinocyte layers with cell precision. Another major advantage that we are presenting here, is that our spheroid model permits the real-time measurements of ion fluxes occurring in the entire epidermal structure. Consequently, we revealed that TRPV4 is functionally active only in the outermost keratinocyte layers, and not in the inner, more proliferative layers; an observation which has so far not been possible to make with bulk Ca^{2+} imaging in planar keratinocyte cell culture [15]. Such a localized, functional expression of TRPV4 only in one epidermal layer, such as the *s. granulosum*, would be in perfect agreement with a function of TRPV4 in the maintenance of TJ or AJ complexes.

Here we describe for the first time the use of 3D epidermal equivalents composed of *TRPV4*-KO human keratinocytes. This allowed us to show that TRPV4 contributes to proper barrier function of the human epidermis. Previous studies have worked with human keratinocytes in 2D culture, which allows very limited transferability to the processes that are ongoing in a 3D organized epidermis. In such a planar setting it could be shown that direct activation of TRPV4 by using pharmacological TRPV4 activators (4α-PDD and GSK101) led to increased transepithelial electrical resistance (TEER) and increased paracellular barrier [15]. The other way round, reducing the level of TRPV4 in cultured keratinocytes using an siRNA approach reduced TEER and the paracellular barrier [15]. Our study is in line with these findings, and additionally proves the importance of TRPV4 in a more physiologically relevant 3D setting. In other organisms, it was also shown that gene knockout of *TRPV4* (and not of the related *TRPV3*), led to increased TEWL *in vivo*, but only when the skin barrier was already damaged [21]. Mechanistically, it was proposed that TRPV4 is binding to ß-catenin leading to local Ca^{2+} changes around AJ complexes, which activates Rho and stabilizes intercellular junctions [21, 22]. Here, we provide first hints that in human keratinocytes, TRPV4 is required for maintenance of TJ complexes, but whether similar interactions with ß-catenin and Rho are also occurring in the human epidermis remains to be determined.

In our study we went a step further since we not only characterized TRPV4 function, but also performed a pilot screening to identify new modulators of TRPV4. In contrast to a typical pharma screening approach we have created a smart selection of compounds based on properties that would later facilitate safety assessment, compound procurement and market entry. Despite having focused on a smaller compound library, we achieved to identify TRPV4 specific modulators, which are not targeting other family members such as TRPV1 and TRPA1, and which are fully natural, in contrast to the already described synthetic TRPV4 modulators [24].

Most importantly, we could show that not only the benchmark agonist GSK101 but also one of our TRPV4 modulators (Cmpd H) can indeed be applied to improve the 3D barrier function *in vitro*. Of course, at that development stage, it cannot be ruled out that this beneficial effect of Cmpd H is not solely due to the modulation of TRPV4 since it may also involve effects on other molecular structures. Interestingly, one study showed that GSK101 is capable of improving TEWL when GSK101 was applied to human skin after tape stripping in an *ex vivo* approach [15]. In this context, it will be interesting to analyze the actual performance of our identified bioactives in *in vivo* situations, for example in the treatment of barrier disrupted, dry or inflammatory skin conditions.

Conclusion

Altogether, we came to conclude that the TRPV4 ion channel contributes to human epidermal development and barrier function and that modulation of TRPV4 by using our natural small-molecule bioactive is a feasible approach to improve epidermal barrier formation *in vitro*.

Acknowledgments

This work was funded by BRAIN Biotech AG funds, and by the German Federal Ministry of Education and Research (BMBF) as part of the Innovation Partnership M^2Aind, projects M^2OGA (03FH8I02IA) and Drugs4Future (13FH8I05IA) within the framework “Starke Fachhochschulen- Impuls für die Region (FH-Impuls)” and NatLifE 2020 UfIB Programme 031B0716.

Conflict of Interest Statement

None.

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