

# Modulation of Tight Junctions and the Permeability Barrier in Betaine-Treated Keratinocyte Cultures

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## Research article

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# Abstract

## Background

Tight junctions (TJs) between keratinocytes are important for regulating the inside-out and outside-in permeability barriers. Betaine is a natural intracellular osmolyte utilized for instance in cosmetics. We determined by liquid chromatography that betaine content in human skin samples was higher in the epidermis than in the dermis. This led us to further investigate the effect of betaine on TJs by utilizing different *in vitro* models: topical application of betaine on normal human epidermal keratinocytes (NHEKs) differentiated for 5-days, freshly isolated NHEKs differentiated for 3 days and betaine applied during the differentiation process of organotypic rat epidermal keratinocytes (REKs) differentiated for 12-15 days.

## Results

Topical betaine in amount range of 100 to 500  $\mu\text{M}$  increased transepithelial electrical resistance (TEER) in 5-day-differentiated NHEKs, and downregulated *occludin* mRNA in concentrations 100-250  $\mu\text{M}$ , but not *claudin-4* (*CLDN4*) or *zonula occludens-1* (*ZO-1*). In REKs, betaine applied during differentiation process had no effect on mannitol permeation or the expression of *occludin*, *ZO-1*, *transforming growth factor beta 2* (*TGF- $\beta$ 2*), or *cluster of differentiation* (*CD147*). However, in freshly isolated differentiating human epidermal keratinocytes, betaine treatment increased TJ protein ZO-1 and the differentiation marker involucrin at protein level. By indirect immunofluorescence, prominent staining and co-localization for TJ proteins ZO-1 and claudin-1 was detected in freshly isolated differentiating keratinocytes treated with betaine.

## Conclusions

Taken together, the results provide evidence that betaine modulates TJ proteins in differentiating epidermal keratinocytes and promotes formation of TJs. These results suggest a role for betaine in the regulation of the epidermal barrier and as a natural ingredient in topical cosmetics.

## Background

The epidermis, the squamous stratified epithelium of the skin, consists of multiple sublayers and is one of the most important barriers between the body and the outside world. The *stratum corneum* is the outermost layer of the epidermis and is generated by the final anucleated step in keratinocyte differentiation from cells in nucleated epidermal layers. In the mammalian epidermis, continuous TJs are found in the *stratum granulosum*, or the granular layer of the epithelium, located under the *stratum corneum* [1–3]. TJs are dynamic cell-cell junctions that connect neighboring cells, control the paracellular pathway of molecules acting as barrier, and separate the apical from basolateral cell membrane [4]. In the human epidermis, various TJ proteins have been identified, including occludin (OCLN); CLDN 1, 4, and 7; junctional adhesion molecule-1 (JAM-1); TJ plaque proteins, such as ZO-1 and multi-PDZ protein-1

(MUPP-1); and the aPKC/Par3/Par6 cell polarity complex [4]. The importance of TJs in the epidermal barrier function of the skin has been demonstrated by the finding that *cldn-1* deficiency results in fatal epidermal water loss in neonate mice [2]. ZO-1, OCLN, and claudins 1 and 4 are critical for the formation of functional TJs in keratinocytes—their knockdown increases the permeability to ions and larger molecules [5]. In various skin diseases with perturbed barrier function, such as in psoriasis, ZO-1 and OCLN relocate to the lower epidermal layers [3, 4], and TJs have been implicated in ichthyosis, atopic dermatitis, photoaging, and infections [6, 7]. TJ proteins have other functions and are involved in such processes as proliferation, differentiation, and vesicle transport [8]. They are also target for bacterial and viral insults, and certain viruses and bacteria use them as receptors [9]. For instance, pathogenic *Staphylococcus aureus* downregulates TJ proteins in HaCaT cell model, with a concomitant loss in transepithelial electrical resistance (TEER), and depletes TJ proteins in a porcine skin infection model [10]. Moreover, allergens can disrupt TJs and promote asthma [11]. Ultraviolet (UV) radiation-induced disruptions in the epidermal barrier are associated with TJ barrier impairment [12]. Thus, TJs are important for the inside-out and outside-in barriers in the epidermis.

Betaine (N, N, N-trimethylglycine, or glycine betaine) is a naturally occurring quaternary amine. It is found throughout nature, from microbes to plants and animals, and is used to regulate water balance in response to environmental stresses, such as drought, salinity, extreme temperatures, and UV radiation [13, 14]. In the personal care industry, it is included as a moisturizer in skin and oral care applications; for instance, betaine reduces the mucosal irritation by detergents and relieves dry mouth [15–18]. Betaine is a small molecule (117 daltons (da)), and due to its unique transient interaction with water molecules, it plays an important role in water regulation in various cell types [19]. Physiologically, betaine assists in cell osmoregulation after hyperosmotic stress and UV radiation in normal human keratinocytes [20, 21] and human fibroblasts [22], and when administered orally, it suppresses UVB radiation-induced wrinkle formation in hairless mice [23] and lowers the melanin content in melanocytes [24]. In addition, betaine acts as a molecular chaperone and stabilizes protein structure under denaturing conditions and serves as a methyl donor in various biochemical processes, e.g., in the remethylation of homocysteine, a marker of cardiovascular disease, to methionine [25, 26]. Upon donation of methyl groups by betaine, it is metabolized to glycine through dimethylglycine and sarcosine intermediates, which are also potential osmolytes [21]. Physiologically, betaine can be biosynthesized from choline in two-step enzymatic process by methylation reactions [25].

Permeation of solutes through the intact TJ occurs through two distinct mechanisms: a transport system that is based on charge-selective claudin pores, as quantified by TEER [27], and a lower-capacity leak pathway that allows the passage of larger solutes with no selectivity for ionic charge or size. The latter can be measured using tracer molecules [28]. In cultured monolayer keratinocytes,  $\text{Ca}^{2+}$  ions can be used to induce their differentiation, and the concomitant increase in TEER is associated with lower permeability through the paracellular pathway, with a minor contribution by the TJ-independent transcellular pathway [5].

In this study, the effects of betaine on skin barrier characteristics were studied in human epidermal keratinocytes and multilayered, differentiated REK models both after and during differentiation, respectively. We were interested in determining the effects of betaine on epidermal TJs and permeability characteristics, and for this we investigated betaine content in the skin, and how betaine affects TEER, permeation, and tight junction protein expression both in gene and protein level as well as tight junction protein localization.

## Results

### Epidermis contains more betaine than dermis

The amount of betaine was measured in human skin epidermis and dermis samples. Epidermis contained more betaine ( $74.0 \pm 19.4$  nmol/g wet weight) compared with the dermis ( $45.3 \pm 8.3$  nmol/g wet weight) although the difference did not reach statistical significance due to low  $n$  value ( $p = 0.078$ ) (Fig. 1). Of note, the use of moisturizers by the volunteers was not recorded.

#### **Betaine increases TJ integrity and downregulates OCLN mRNA in cultured normal human epidermal keratinocytes (NHEKs).**

The topical application effect of betaine on the TEER of NHEKs differentiated for 5 days was examined by incubating for 1, 2, 3, 6, 12, and 24 h with 0, 10, 50, 100, 250, and 500  $\mu$ M betaine (Fig. 2a). In the control cells (0  $\mu$ M betaine), TEER decreased to negative values at 2 h timepoint after changing the cell culture medium, but at 3 h to 6 h timepoint the TEER recovered back to the basal level, or to the values in the control cells prior the experiment was initiated. Interestingly, in the samples treated with betaine, the TEER was already much higher after 1 h. Furthermore, the TEER in the betaine treated cells did not decrease at 1 h or 2 h to negative values as was observed with control cells (0  $\mu$ M betaine), even though a decrease in TEER was observed with 250 and 500  $\mu$ M betaine treatment. This suggests that betaine counteracted the stressful situation occurring in cells due to the medium change. At 1 h TEER was at higher level with 100  $\mu$ M ( $8.7 \pm 3.1\%$ ), 250  $\mu$ M ( $19.7 \pm 4.5\%$ ) and 500  $\mu$ M betaine ( $18.8 \pm 4.6\%$ ) being significantly different to untreated control ( $-3.1 \pm 5.0\%$ ,  $p < 0.05$ ,  $p < 0.0001$  and  $p < 0.0001$ , respectively). At 2 h, TEER in 50  $\mu$ M ( $5.8 \pm 6.4\%$ ), 100  $\mu$ M ( $1.9 \pm 5\%$ ), 250  $\mu$ M ( $10.7 \pm 4.1\%$ ), and 500  $\mu$ M ( $13.8 \pm 6.7\%$ ) betaine was significantly different than in untreated control cells ( $-9.4 \pm 3.6\%$ ,  $p < 0.005$ ,  $p < 0.05$ ,  $p < 0.0001$  and  $p < 0.0001$ , respectively).

At 3 and 6 h TEER in betaine-treated samples returned to basal levels observed in control cells, but at later measurements, 12 h and 24 h, TEER in betaine-treated cells started to increase. At 12 h 100  $\mu$ M ( $12.4 \pm 6.5\%$ ), 250  $\mu$ M betaine ( $13.9 \pm 3\%$ ) and 500  $\mu$ M ( $22.7 \pm 5.8\%$ ) betaine induced a significant increase in comparison to untreated control cells ( $-1.1 \pm 4.1\%$ ,  $p < 0.005$ ,  $p < 0.005$  and  $p < 0.0001$ , respectively). At 24 h 50  $\mu$ M ( $39.3 \pm 7.6\%$ ), 100  $\mu$ M ( $41.8 \pm 5.5\%$ ), 250  $\mu$ M ( $39.5 \pm 3.2\%$ ) and 500  $\mu$ M ( $47.8 \pm 8.4\%$ ) betaine had significantly higher TEER when compared to untreated control ( $26.9 \pm 7.3\%$ ,  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.05$  and  $p < 0.0001$ , respectively). However, there was no clear dose-dependency.

The gene expression of *CLDN-4*, *OCLN*, and *ZO-1* at mRNA level was measured in differentiated NHEKs that had been incubated for 24 h from the topical side with 0, 10, 50, 100, 250, and 500  $\mu\text{M}$  betaine (Fig. 2b-d). *CLDN-4* and *ZO-1* mRNA remained at basal levels in betaine-treated samples, whereas *OCLN* was significantly downregulated at 100  $\mu\text{M}$  ( $p < 0.05$ ), and 250  $\mu\text{M}$  betaine ( $p < 0.05$ ) compared to untreated control. However, statistically significant differences were not observed with 10  $\mu\text{M}$  or 500  $\mu\text{M}$  betaine in comparison to control.

### **Betaine does not affect mannitol permeation or TJ component mRNA expression in multilayered organotypic REK cultures during differentiation**

To investigate mannitol permeation in the presence of betaine, REK cultures were differentiated for 12 days. Mannitol permeation was followed for 3 days, but no statistically significant changes in the permeation or differences between the treatments were observed. (Fig. 3a). A numerical increase was observed in the UVB exposed cells that was also observed in UVB exposed cells treated with 10 mM betaine, but it was not statistically significant. Likewise, with acetone treatment, no changes in acetone treated control or acetone and 10 mM betaine treatment was observed (Fig. 3a). Similarly, no changes in the TEER was noted (Fig. 3b).

In REK cultures that were treated with 10 mM betaine and/or UVB, no statistically significant changes in *OCLN*, *ZO-1*, *TGF- $\beta$ 2*, or *CD147* mRNA expression were noted in 15-day old cultures compared with the untreated control (Fig. 4).

## **Increased expression and co-localization of cell junction proteins by betaine in human epidermal keratinocytes**

To examine the effect of betaine treatment on the formation of cell-cell junctions *in vitro*, high  $\text{Ca}^{2+}$  medium (1.4 mM  $\text{Ca}^{2+}$ ) was used to induce differentiation of normal human primary epidermal keratinocytes [3] in the absence or presence of betaine (1-1000  $\mu\text{M}$ ) for 72 h. Thereafter, the keratinocytes were harvested for western blot analysis or used for indirect immunofluorescence IIF staining.

Western blot analysis showed clear upregulation of involucrin and occludin by high  $\text{Ca}^{2+}$  treatment compared to low  $\text{Ca}^{2+}$  (Fig. 5a). High  $\text{Ca}^{2+}$  treatment also induced TJ formation, and localization of ZO-1, desmoplakin, E-cadherin as well as  $\beta$ -catenin to the cell-cell contacts as shown by IIF staining of the same cells (Fig. 5b), as well as colocalization of ZO-1 and claudin-1 on cell-cell contacts (Fig. 5c).

Interestingly, increase in the levels of ZO-1 was detected in cultures treated with betaine in concentrations 1 to 100  $\mu\text{M}$ , and to lesser extent with higher betaine concentrations (200 to 1000  $\mu\text{M}$ ) as determined by western blot (Fig. 5a). In addition, the expression of involucrin was increased by treatment of cultures with all betaine concentrations ( $\geq 1$   $\mu\text{M}$ ). Western blot analysis showed no clear concentration-dependent effects of betaine on the expression of other cell junction proteins examined (Fig. 5a).

In cultures treated with high  $\text{Ca}^{2+}$ , localization of ZO-1, desmoplakin, E-cadherin, and  $\beta$ -catenin was noted in cell-cell junctions in the presence of 50 or 200  $\mu\text{M}$  betaine, as examined with IIF (Fig. 5b). In the merged figure, colocalization of ZO-1 and claudin-1 with higher visual staining intensity was noted in cultures treated especially with high  $\text{Ca}^{2+}$  and 100  $\mu\text{M}$  betaine (Fig. 5c). Taken together, these results indicate that betaine treatment does not disrupt TJs in differentiating epidermal keratinocytes, but in contrast may promote formation of TJs by increasing the protein levels of TJ components.

## Discussion

Betaine regulates cellular volume and acts as an intracellular osmoprotectant in the adaptation of cells to stress. Hyperosmotic stress and ultraviolet B radiation inhibit cell proliferation and induce apoptosis in human keratinocytes [21, 29]. Osmotic stress signals keratinocytes to differentiate [30]. Betaine prevents hyperosmotically induced and UV radiation-induced cell shrinkage, a hallmark of apoptotic cell death [31] in normal human keratinocytes [20] and human HaCaT cells [21, 32]. Dietary and endogenous betaine exists primarily in the liver and kidney but also significantly in other tissues, including skin [19, 33]. Our findings show that betaine is a natural component in the skin, and we are the first to our knowledge to describe that the epidermis contains by number more betaine than dermis. Balanced hydration in the epidermis is needed, for instance, for the optimal differentiation of keratinocytes [30]. Keratinocytes and fibroblasts also express the betaine/ $\gamma$ -amino-n-butyric acid (GABA) transporter BGT-1, the expression of which rises when they are exposed to hyperosmotic conditions or UV irradiation with concomitant increase in the betaine uptake [20, 22, 32]. However, the volunteers in the study were not selected based on any specific exclusion/inclusion criteria, but were women having a mammoplasty. As betaine is used in cosmetic products, a wash out period would have needed to exclude the effect of utilization of betaine containing products in the results. Furthermore, controlling the dietary betaine would have been important in evaluating the effect of diet in the betaine content of the skin. With the scope of this publication, this was not, however, possible, but a simpler approach was selected instead to obtain preliminary results. Therefore, additional studies with more control and increased sample number are warranted.

The increase in TEER at 12 h and 24 h indicates that betaine improves TJ integrity and contributes to the inside-out barrier, and this function is yet another addition to moisturizing function of betaine: maintenance of hydration of the skin through the regulation of TJs by improving water retention [2]. Notably, at 1 h and 2 h, betaine protected cells from the decrease in TEER that was observed with untreated NHEKs. TEER is sensitive to temperature, and the decline in TEER after replacement of the medium might have been due variations in temperature [34]. Osmolytes can be metabolic and cytoprotective under many perturbing conditions, and extreme temperature is a stressor against which osmolytes stabilize cellular function [14]. No consistent changes in TJ protein expression was observed in differentiated NHEKs explaining the effect in TEER; *OCLN* alone was decreased significantly, but not steadily with all tested betaine concentrations. However, betaine was able to increase the ZO-1 protein and also the differentiation marker involucrin when analyzed with betaine-treated freshly isolated human keratinocytes during the 72-h differentiation. We have previously shown, that the expression of ZO-1 in

keratinocytes is regulated by p38 signaling pathway [35]. In experiments with diabetic male mice in relation to protection of blood-testis barrier, betaine has been shown to inhibit p38 phosphorylation and upregulate the protein expressions of ZO-1, OCLN, CLDN-11, N-cadherin, and connexin-43 [36] so it might be a plausible mechanism of action for betaine. However, the increase in ZO-1 in combination with the TEER-increasing effect obtained with the topical modelling of betaine in differentiated NHEKs shown in this study indicate jointly that betaine might strengthen the skin barrier through its capacity to modulate TJ proteins.

The effects of betaine on barrier function in a rat organotypic model of human epidermis in a similar setting has been investigated previously, and no evident changes in histology, epidermal thickness, or proliferation were observed [37]. Likewise, in REK model, no changes in the mRNA expression of *OCLN*, and *ZO-1*, could be observed, which indicates that betaine does not have an impact on the studied TJ protein gene regulation in long term exposure of high amount of betaine, but rather affects to TJs are in a protein level, and our finding is in accordance with previous study showing that organic osmolytes affect TJs through protecting the native protein protein conformation [38]. Betaine has been shown to stabilize proteins from abiotic stress through its high water binding capacity and ability to retain inorganic ions around itself [39].

In a model in which human skin is explanted to a SCID mouse and with cultured human skin equivalent (HSE), the intercellular diffusion of subcutaneous, or basolateral, tracer stops at the level of TJs—specifically, at the level of ZO-1 or OCLN [40]. When the human skin explant and the HSE model are irradiated with UVB, the tracer passes through ZO-1- and OCLN-rich sites, highlighting the importance of TJs in the intercellular permeability barrier to small molecules and the function of UVB as cause of TJ dysfunction [40]. We also examined the permeation of topical mannitol after UVB exposure (30 mJ/cm<sup>2</sup>) in the REK model but failed to observe any differences between the control, UVB-treated, and UVB-plus-betaine-treated samples. In a previous study with REK model, betaine was able to rescue UVB induced permeability increase [38], but the difference is that we utilized cells with mature TJs instead of developing TJs, and developing TJs might be more sensitive to the effects of UVB [38]. The stratum corneum, just above the TJs, is the barrier to water movement across the skin [41], and it has been challenging to demonstrate the function of TJs in the outside-in permeability barrier due to difficulties in accessing the TJs below the stratum corneum [42]. Cornified envelope lipids are key determinants of the types of substances that can be absorbed from the skin surface through diffusion, and these substances are typically nonpolar and less than 500 Da [43]. Mannitol is a 182 Da highly polar sugar alcohol that requires an enhancer for its penetration [44], and it is typically used as a low permeation standard in percutaneous absorption models. We suspect that this property explains in part the lack of an effect; thus, different tracers, such as caffeine with known permeability characteristics, could be used in these types of assays instead [45, 46]. The differences in the permeability of human versus rat skin should be, however, taken into account, as caffeine is more readily absorbed through rat skin than human skin [47].

TJ proteins OCLN, CLDN-1, and CLDN-4 both at mRNA and protein level are not downregulated on exposure of keratinocytes to UVB irradiation [38, 40]. Similarly, we observed no change in the mRNA

expression of the TJ components *CLDN-4*, *ZO-1*, and *OCLN* when REK cultures were exposed to UVB. Although *OCLN* was downregulated at certain concentrations of topical betaine in the NHEK model, the changes were not consistent. TJs and adherens junctions below them, are highly dynamic and regulated by an intricate network of phosphorylation and dephosphorylation reactions by specific protein kinases and phosphatases [48, 49], by translocation of the TJ proteins in and out of these structures [50], and by modulation of the actin cytoskeleton, which is linked to TJs [51]. Betaine did not affect the protein levels of adherens junction components E-cadherin or  $\beta$ -catenin in freshly isolated keratinocytes, nor *CD147* or *TGF- $\beta$ 2* mRNA levels in the REK model. *CD147*, also known as basigin and EMMPRIN, associates with monocarboxylate transporters, interacts with integrins, and forms a complex with CD44, the major hyaluronan receptor [52]. At least 3 TGF- $\beta$  isoforms have been identified: TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3. TGF- $\beta$ 2 is a growth factor that functions in highly malignant and invasive phenotypes of skin cancers [53]. It is also enhanced by UV radiation [54], but we did not see any changes in the expression of these genes—not even in the control treatment. Betaine has anti-inflammatory effects [55, 56], but whether betaine alleviates the inflammatory responses that arise from extensive UV irradiation [57], is still unknown. Nevertheless, it has been shown that in keratinocytes UV irradiation induces osmolyte transporters and that osmolytes are taken up inside the cells after UV irradiation, and that is part of the mechanisms by which cells act against harmful effects of UV radiation [20, 32].

In this study, there was no clinical intervention with topical or oral betaine, and there is no knowledge whether the donors utilized any topical creams containing betaine. It is also possible that the betaine in the epidermis originates from the biosynthetic route from choline, but most likely it originates from the uptake from the extracellular sources, which has been suggested to be the primary source of betaine [25]. Thus, without topical application, the betaine in the epidermis most likely originates from the diet via systemic circulation in the blood [19, 33].

## Conclusions

In conclusion, betaine is naturally present in epidermis at higher amounts than in the dermis. When applied topically after NHEK differentiation, betaine improves TJ integrity at 1 h and 2 h and after 12 h and 24 h. Furthermore, increase in protein amounts of ZO-1 and involucrin in Western blotting, and prominent co-localization of TJ proteins ZO-1 and claudin-1 in the presence of betaine was demonstrated in IIF. The expression of claudin-1 by normal epidermal keratinocytes is high [58] and its expression is downregulated in atopic dermatitis associated with impaired epidermal barrier [59]. These results in combination indicate that betaine may improve skin barrier through modulation of TJs in the epidermis, and mainly at protein level. Strengthening the skin barrier might be particularly important in diseases in which barrier function is impaired, such as atopic dermatitis and dry skin, and in aging. Future research should determine the molecular mechanisms of betaine function in TJ protection as well as whether the effects of betaine on TEER and TJ proteins have significance in various skin conditions.

## Methods

# Dermo-epidermal separation and measurement of betaine content

Human skin biopsies (n = 3) were isolated from women who were undergoing reduction mammoplasty, with the appropriate approval of the ethical committee of the Southwest Finland Hospital District and written informed consent by the patients (Dnro 138/2007). To separate the epidermis and dermis in the biopsies, the specimens were placed directly in 3.8% ammonium thiocyanate (Sigma Aldrich, Darmstadt, Germany) in phosphate buffered saline (PBS), pH 7.4 (Thermo Fisher Scientific, Waltham, MA, US). [60]. After a 30-min incubation at room temperature (RT), the biopsy was split into dermal and epidermal parts with tweezers and rinsed thoroughly with PBS. The samples were stored at -20°C until the betaine content was measured.

Betaine levels were determined as 4-bromophenacyl derivatives by HPLC per published methods [61, 62]. Samples were homogenized and mixed with ice-cold 0.1 M sodium acetate buffer, pH 6 (sodium acetate anhydrous, Merck KGaA, Darmstadt, Germany) and acetic acid 99–100% (J.T.Baker, Center Valley, PA, USA). Proteins were precipitated with 40% (w/w) trichloroacetic acid (TCA) (Riedel de-Haën, Seelze, Germany) in an ice bath, and the precipitate was removed by centrifugation. Betaine was extracted twice from the supernatant with ice-cold diethyl ether (Riedel de-Haën,) by centrifugation. Milli-Q water (Merck KGaA) was added to a final volume of 1.5 ml, and the sample was centrifuged and passed through an Acrodisc 13 mm GHP membrane 0.2-µm filter (Pall, Port Washington, NY, USA). Then, 50 µl of 100 mM KH<sub>2</sub>PO<sub>4</sub> (Merck KGaA) was added to 50 µl of the sample. The betaine in the sample was derivatized to 4-bromophenacyl derivatives by incubation with 66 mg 18-crown-6 (Sigma-Aldrich) and 1390 mg 4-bromophenacyl bromide (Fluka, Seelze, Germany) in 100 ml acetonitrile (VWR International, LLC, Radnor, PA, USA) for 60 min at 70°C. After incubation, the sample was cooled in an ice bath and centrifuged.

Samples were analyzed by liquid chromatography on a Supelcosil™ LC-SCX, 5 µm, 250 x 4.6 mm column (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) with 22 mM choline chloride (Sigma-Aldrich) in 10% H<sub>2</sub>O/90% acetonitrile (v/v) as that eluent at a flow rate of 1.0 ml/min, a column temperature of 25°C, and UV detector wavelength of 254 nm. The run time was 25 minutes, and betaine eluted at 7.6 minutes. The results were calculated using an external standard method with multipoint calibration using peak heights. Betaine monohydrate (Fluka) was used as the reference material.

## Isolation, differentiation and betaine treatments of freshly isolated NHEKs

To isolate NHEKs for western blotting and for IIF staining, skin samples were obtained from breast reduction operations of 2 healthy women (29 and 48 years old) at the Department of Surgery, Turku University Hospital, Turku, Finland with the appropriate approval of the Ethical Committee of the Southwest Finland Hospital District and written consent of the patients (Dnro 138/2007). NHEK cultures were established from skin samples by a modification of the method of described by Boyce and Ham (1985) [63] and cells of 2nd to 4th passages were used for experimentation. Keratinocytes were cultured

in Keratinocyte Growth Medium (KGM-2) (PromoCell GmbH, Heidelberg, Germany) supplemented with SupplementMix and 0.06 mM CaCl<sub>2</sub> (both from PromoCell), 2 mM L-glutamine and penicillin-streptomycin mixture (all from Gibco).

Elevation of the extracellular Ca<sup>2+</sup> level is a well-known physiological stimulus for keratinocyte differentiation. To examine the effects of betaine in TJ protein expression during keratinocyte differentiation *in vitro*, freshly isolated NHEKs were cultured either in low (0.06 mM CaCl<sub>2</sub>) or in high (1.4 mM CaCl<sub>2</sub>) Ca<sup>2+</sup> conditions in KGM-2 containing supplement mix for 72 h, in the presence of betaine (Genencare OSMS BA, Danisco Sweeteners Oy, Naantali, Finland, final concentration 0-1000 µM). The cells were then either harvested for western blotting or fixed for IIF staining.

## **Permeabilization and mRNA expression studies using organotypic REK cultures**

For organotypic cultures, REKs were grown at the air-liquid interface on type I collagen support and treated with betaine and UVB light as previously described [37]. Two independent experiments were performed to assess the permeability of mannitol across these stratified REK cultures. To analyze the effects of betaine on gene expression, 4 independent experiments were performed, from which the 3 most representative ones were used. Small alterations to the published methods [37, 64], were applied during the permeability experiments, as detailed below.

Betaine treatment (10 mM) was initiated on day 4 or 5 after the cells were plated (depending on the growth rate and confluency of the cells), and the cells were differentiated for 6 days (permeability experiments) or 10 days (gene expression experiments) in the presence of betaine, except for the control wells without betaine, before starting the UVB and/or acetone exposures to compromise the permeability barrier. During this time, the cells stratify and differentiate to form an epidermal equivalent that is structurally and functionally like the epidermis *in vivo*, resembling human skin in many respects, including its permeability [64].

For the gene expression experiments (after differentiation, at day 14 after starting the cultures), the cell inserts were placed on a 6-well plate with fresh Dulbecco's Phosphate-Buffered Saline (D-PBS; Euroclone, Pavia, Italy) beneath the insert only. After removing this wash buffer and adding fresh D-PBS, each culture was stressed with UVB (30 mJ/cm<sup>2</sup>) using a portable UV lamp (UVM-57; UVP, Upland, CA) that emitted mid-range UV at 302 nm. After the UVB or sham treatment (UV light off), the PBS was replaced with fresh medium with or without betaine.

For the permeability experiments (after differentiation, at day 11 after starting the cultures), the inserts were similarly placed on D-PBS, and treated for 60 s with 470 µl of acetone (Sigma-Aldrich), pipetting the liquid carefully on the whole area of the epidermis in the insert. After the acetone was removed by careful pipetting, the inserts were placed on a culture plate with fresh medium with or without betaine, and the epidermal surfaces were air-dried briefly from any residual solvent. The UVB exposures were performed

as indicated above, but the initial D-PBS wash step was omitted: both the acetone and the UVB exposures took place with the inserts on well plates containing fresh D-PBS, keeping the living cell layers moist throughout. On the following day, 24 h after the UVB or acetone treatment, TEER was measured as described below and mannitol permeation was examined (in 12-day old cultures; see below), and total RNA was isolated for the real-time quantitative PCR (RT-qPCR) (in 15-day old cultures; see below).

## Permeability of mannitol across the organotypic REK cultures

Permeability of mannitol across REK cultures after different treatments was measured in an apical-to-basolateral direction at 37°C in well-stirred side-by-side diffusion chambers (PermeGear, Bethlehem, PA, USA) similarly as previously described [64]. Briefly, the paracellular low permeability marker [<sup>3</sup>H]mannitol (20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, USA) was diluted (6 µCi/ml) in Hank's balanced salt solution (HBSS, Biowhittaker®, Lonza, Walkersville, MD, USA) buffered with 25 mM Hepes (Sigma-Aldrich) (pH 7.4) and introduced into the donor chamber (3 ml). HBSS buffer solution was added to the receiver chamber (3 ml). The samples from the receiver (200 µl) and donor (10 µl) chambers were withdrawn at 2, 4, 6, 24, 26, 28, 48, 50, 52, 72, 74, and 76 h and fresh HBSS buffer was used to replace the fluid loss from the receiver chamber. Radioactivity of the samples was analyzed by liquid scintillation counting (1450 MicroBeta Trilux Liquid Scintillation and Luminescence Counter, Wallac, Finland) after the addition of 500 µl scintillation cocktail (Optiphase, Wallac, Milton Keynes, UK). The apparent permeability coefficient ( $P_{app}$ , cm/h) was calculated according to equation:  $P_{app} = (\Delta Q_r / \Delta t) (1 / A \times C_d)$ , where  $\Delta Q_r / \Delta t$  is the steady state flux of mannitol (cpm/h), *i.e.*, the slope of the linear region of the cumulative amount of mannitol in receiver chamber versus time (h) plot;  $C_d$  is the average drug concentration in the donor (apical) chamber; and A is the surface area of the insert (cm<sup>2</sup>).

## TEER measurement of differentiated NHEK cultures and organotypic REK cultures

For TEER analysis, adult primary NHEKs (Thermo Fisher Scientific) were maintained in EpiLife® medium with 60 µM calcium (Thermo Fisher Scientific) that was supplemented with Human Keratinocyte Growth Supplement (HKGS) (Thermo Fisher Scientific) (basal medium) in a 5% CO<sub>2</sub> atmosphere at 37°C. The NHEKs were differentiated on cell culture inserts as published [65] with differentiation medium (DM) that consisted of EpiLife® medium, 1.45 mM CaCl<sub>2</sub>, and HKGS and incubated for 4 days in a 5% CO<sub>2</sub> atmosphere at 37°C. The DM was changed from the apical, or topical, and basolateral sides daily. Test substances were given on day 4 of the differentiation. Betaine (Betafin BP20, Finnfeeds Finland Oy, Naantali) was dissolved in DM and applied to the apical side of differentiated NHEKs at 0, 10, 50, 100, 250, and 500 µM. TEER was measured at 1, 2, 3, 6, 12, and 24 hours after the incubation was started with betaine (see below). At the 24-h time point, the NHEKs were lysed for RNA isolation (see below). Two independent replicates were used to calculate the results.

The integrity of the NHEK monolayer was verified by measuring the TEER before and after each time point using EVOM2 (World Precision Instruments, Sarasota, FL, USA) with STX2-electrodes. The background TEER of an empty insert TEER was subtracted from that of the monolayer and insert to yield the resistance of the monolayer, which was multiplied by the area of the insert to obtain the result, expressed as  $\text{ohm} \cdot \text{cm}^2$ . The results were expressed as the percentage change in TEER (%Change in TEER), which reflects the change in resistance at the given time point compared with a time point in the same cell monolayer before betaine was applied (time point 0 h). Per this calculation, the data from time point 0 h was 0.

The integrity of the REK cultures was measured after the treatments explained in paragraph entitled: Permeabilization and mRNA expression studies using organotypic REK cultures. Only Ohm-values are reported from these as the manufacturer recommends utilization of these values in the question of very large, 6-well inserts.

## RNA extraction and cDNA synthesis

NHEK cultures were lysed with buffer RA1 (Macherey Nagel GmbH & Co. KG, Düren, Germany), supplemented with 1%  $\beta$ -mercaptoethanol (Sigma-Aldrich). Total RNA was isolated using the NucleoSpin® 96 RNA kit (Macherey Nagel GmbH & Co. KG, Düren, Germany). Reverse-transcription was performed using SuperScript III (Thermo Fisher Scientific) and random primers (Thermo Fisher Scientific).

Total RNA was isolated from organotypic REK cultures as previously described [37]. The cultures were washed twice with ice-cold D-PBS, and the epidermal layer was separated with fine tweezers and frozen immediately in liquid nitrogen. Samples were homogenized in Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA, USA) on a FastPrep® homogenizer (Savant, Thermo Fisher Scientific Inc.). Total RNA was extracted using the High Pure RNA Tissue Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Reverse-transcription was performed using the Verso™ cDNA synthesis kit (Thermo Fisher Scientific) with anchored oligo-dT primers.

## Real time quantitative PCR (RT-qPCR)

For the NHEK cultures, RT-qPCR of *CLDN4*, *OCLN*, and *ZO-1* were performed using TaqMan gene expression assays (Thermo Fisher Scientific) that were designed for each gene in a 20- $\mu$ l reaction per the manufacturer's instructions on a 7500 Fast Real-time PCR System (Thermo Fisher Scientific) with manufacturer-recommended cycling parameters. As an endogenous control, ribosomal protein large P0 (*RPLP0*) was quantified in parallel in each experiment [66]. The expression of *RPLP0* was first tested with an extensive set of differentially treated samples, and its expression was found to be constant in the various treatments (data not shown).

For the organotypic REK cultures, RT-qPCR was performed on a Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA, USA) using FastStart Universal SYBR Green Master with ROX (Roche) and gene-specific primer pairs for *OCLN*, *ZO-1*, *CD147*, and *TGF- $\beta$ 2*, with *RPLP0* as an endogenous control. The cycling conditions comprised an initial preincubation for 15 min at 95°C, 45 cycles of denaturation at

95°C for 20 s, annealing at a primer-specific temperature for 20 s, and elongation at 72°C for 20 s. Gene-specific amplification was confirmed by melting curve analysis.

In both cases, the data were analyzed per the  $2^{-\Delta\Delta Ct}$  method [67], and the results were expressed as fold-change in mRNA levels of the unknown samples, relative to untreated control samples.

## **Indirect immunofluorescence (IIF) analysis of TJ proteins**

Freshly isolated differentiating human keratinocytes were fixed in ice-cold methanol for 5 min, blocked with PBS that contained 3% bovine serum albumin (BSA) for 30 min at room temperature, and treated with primary antibodies that were diluted in 3% BSA in PBS. Highly PreCross-absorbed goat secondary antibodies against rabbit and mouse antibodies (Thermo Fisher Scientific) were used, and Hoechst stain (Thermo Fisher Scientific) was applied to visualize nuclei. The cells were mounted in Fluorescent Mounting Medium (Agilent) and examined under an Olympus BX60 fluorescence microscope (Olympus Optical Co., Tokyo, Japan) or Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany). Confocal imaging was performed at the Cell Imaging and Cytometry Core (Turku Bioscience Centre, University of Turku, Finland).

## **Western blot analysis**

Equal aliquots of cell lysate harvested from freshly isolated differentiating keratinocytes in Laemmli buffer were separated in 6 % or 10 % SDS-polyacrylamide gel and electroblotted to nitrocellulose membrane (Amersham Biosciences; Piscataway, NJ), as previously described [68]. Horseradish peroxidase (HRP) conjugated secondary antibodies (sheep anti-mouse IgG HRP from Amersham Biosciences and swine anti-rabbit IgG from DakoCytomation) were used and visualized using enhanced chemiluminescence (ECL; Amersham Biosciences).

## **Primary antibodies**

The following antibodies were used in western blotting and/or IIF: Mouse monoclonal antibodies against ZO-1 (33-9100) and E-cadherin (33-4000), both from Invitrogen, Carlsbad, CA;  $\beta$ -catenin (M3539, Dako, Glostrup, Denmark), desmoplakin (ab16434-25, Abcam, Cambridge, UK) and  $\beta$ -actin (A-1978) (from Sigma-Aldrich, St. Louis, MO). The following rabbit polyclonal antibodies were used: involucrin (ab53112) from Abcam, and claudin-1 (51-9000) and occludin (71-1500), both from Invitrogen.

## **Statistical analysis**

For the assays with NHEKs or the REK model, the statistical significance of differences between treatments was determined by one-way analysis of variance (ANOVA) or two-way ANOVA. P-values of 0.05 or less were considered to be significant. All comparisons were made against the respective medium-only-treated controls unless stated otherwise. The statistical analysis of betaine content between epidermis and dermis was performed by unpaired t-test. All analyses were completed using GraphPad Prism version 7.03. Mean + SD values are shown for all results.

## Abbreviations

ANOVA: analysis of variance; BGT-1: betaine/ $\gamma$ -amino-n-butyric acid (GABA) transporter; BSA: bovine serum albumin; CLDN: claudin; CD147: cluster of differentiation 147; Da: Dalton; DM: differentiation medium; D-PBS: Dulbecco's Phosphate-Buffered Saline; ECL: enhanced chemiluminescence; GABA: betaine/ $\gamma$ -amino-n-butyric acid; HKGS: Human Keratinocyte Growth Supplement; HRP: Horseradish peroxidase; HSE: human skin equivalent; IIF: indirect immunofluorescence; KGM-2: Keratinocyte Growth Medium; mRNA: messenger ribonucleic acid; NHEKs: normal human epidermal keratinocytes; Papp: apparent permeability coefficient; PBS: phosphate buffered saline; REK: rat epidermal keratinocytes; RPLP0: ribosomal protein large P0; RT: room temperature; RT-qPCR: Real-time quantitative polymerase chain reaction; SCID: severe combined immunodeficiency; SD: standard deviation; TCA: trichloroacetic acid TCA; TGF- $\beta$ 1: transforming growth factor type beta 1; TGF- $\beta$ 2: transforming growth factor type beta 2; TGF- $\beta$ 3: transforming growth factor type beta 3; TEER: transepithelial electrical resistance; TJ: Tight junctions; UV: ultraviolet; ZO-1: zonula occludens 1;

## Declarations

### Ethics Approval and consent to participate

Appropriate approval for human skin biopsies was obtained from the ethical committee of the Southwest Finland Hospital District and written informed consent by the participants (Dnro 138/2007).

### Consent for publication

not applicable

### Availability of data and materials

Betaine utilized in this study can be purchased under trade name Genencare OSMS BA from Finnfeeds Oy, Naantali, Finland. Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

### Competing interests

HA and KT were employed by IFF Health & Biosciences during the execution of this study, which manufactures and markets the betaine under trade name Genencare OSMS BA that was utilized in this study.

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## Author's contribution's

HA performed the measurement of the betaine content in the skin, as well as investigated the effect of betaine on NHEK TJ protein gene expression and TEER. MT and ES conducted experiments with betaine effect on freshly isolated NHEKs, IIF and Western transfer analysis, LR and JJH investigated effect of betaine on organotypic REK cultures in mannitol permeation and TJ protein gene expression. HA, MT, ES and JJH participated in data visualization and formal analysis. HA writing and original draft preparation, SPS, SP, JP, RT, KT and VMK resourcing, funding, supervision and project administration. All authors participated in reviewing and editing of the manuscript. All authors have read and approved the manuscript.

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## Figures

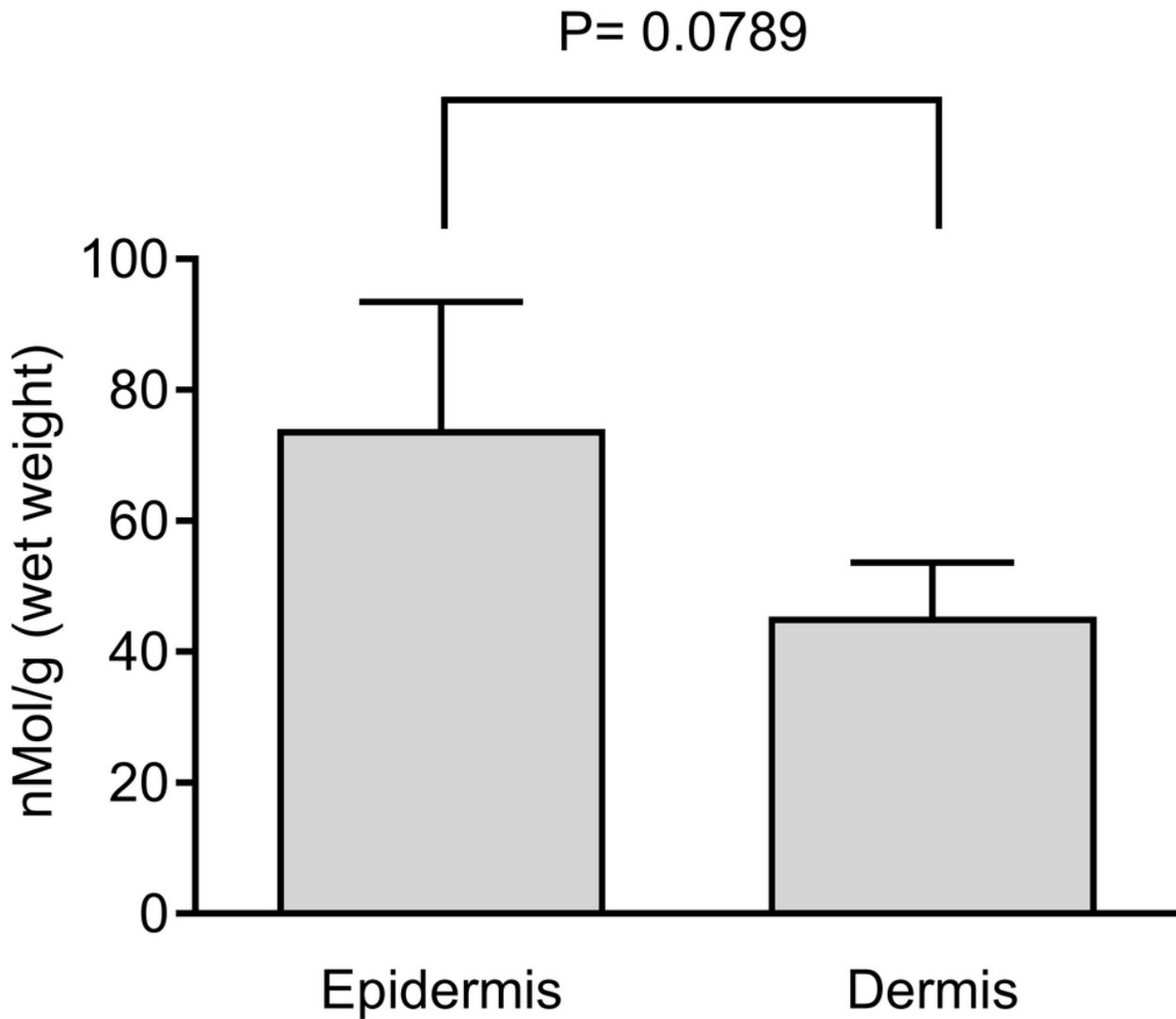
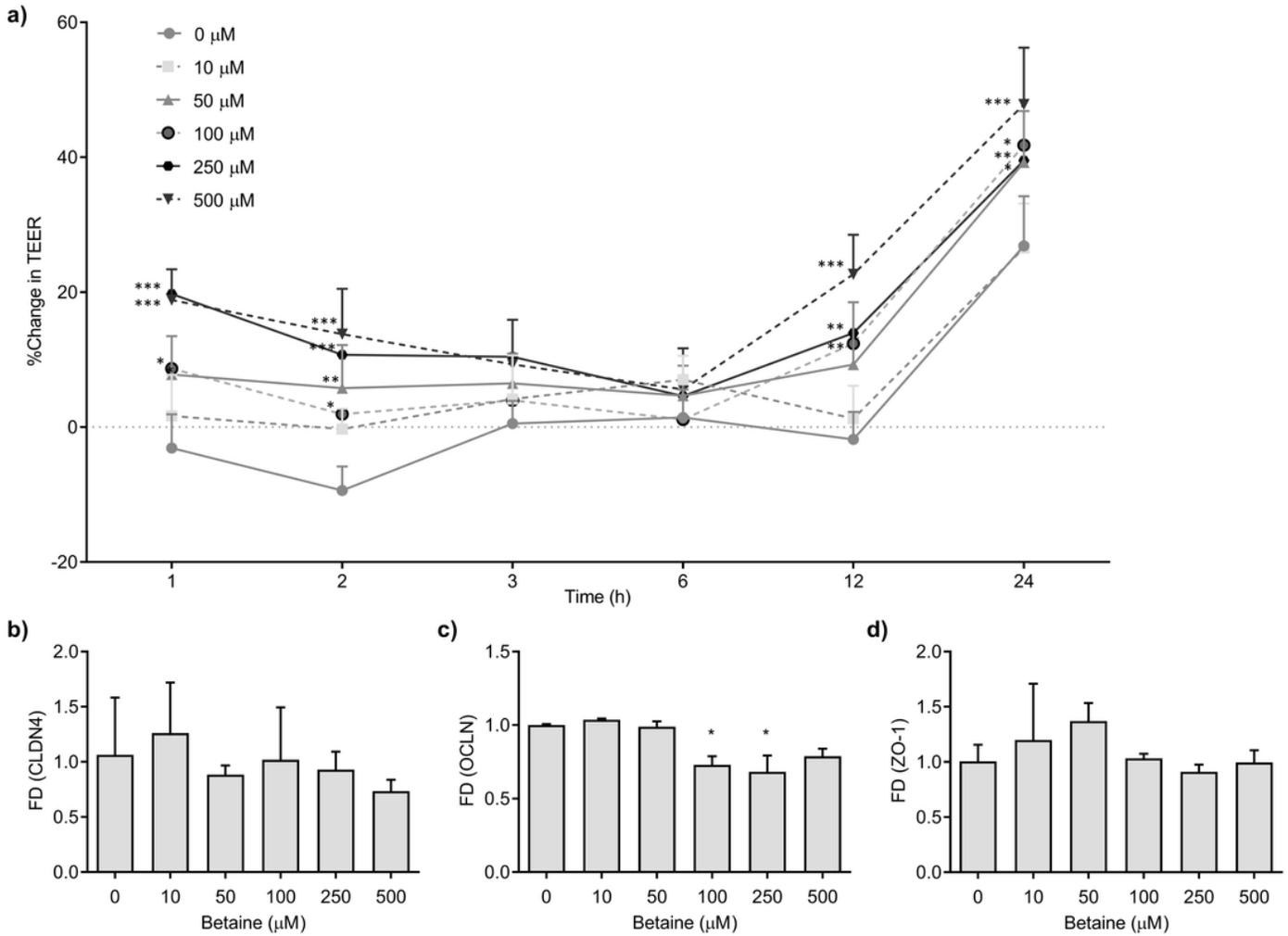


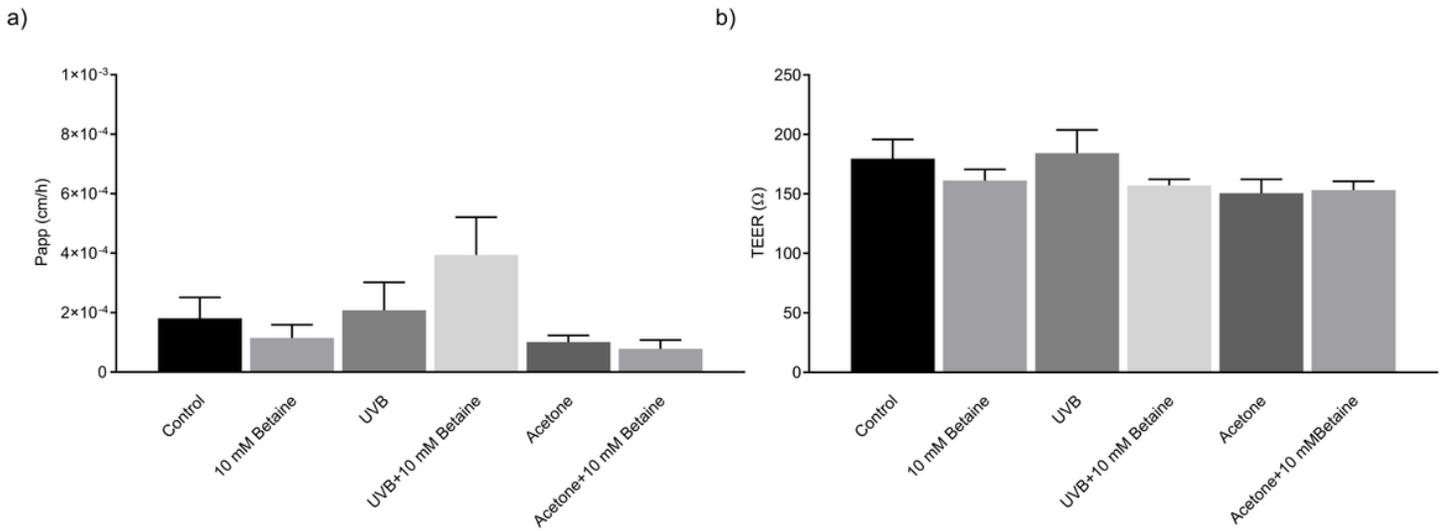
Figure 1

Levels of betaine in human epidermis and dermis. Epidermis and dermis were separated by ammonium isothiocyanate, and the betaine content of the tissue fractions was analyzed by HPLC (t-test, n=3).



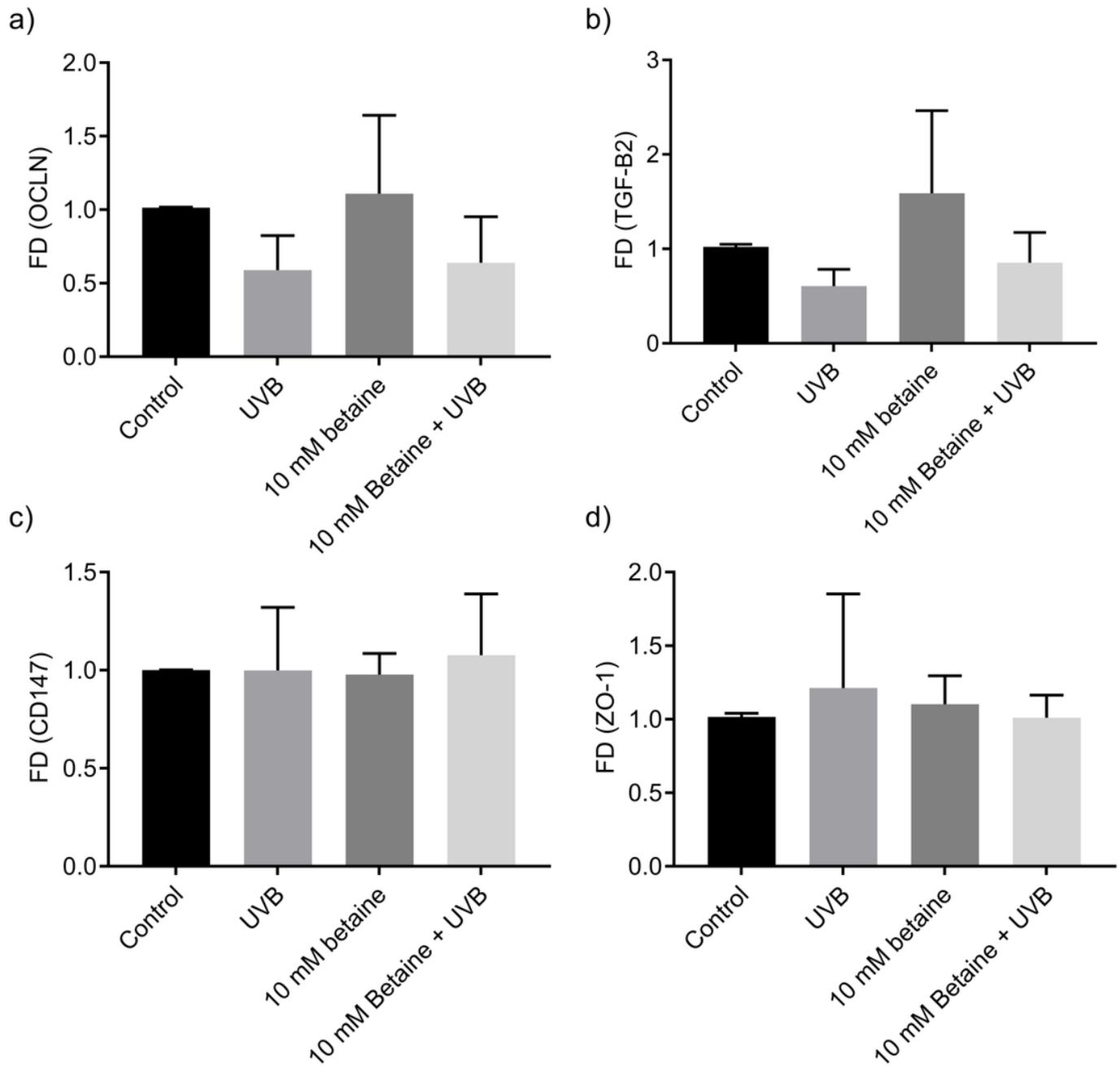
**Figure 2**

a. Measurement of TEER in differentiated NHEK cultures after betaine treatment. Significant differences between betaine-treated versus control cells are indicated by asterisks. mRNA expression of TJ components CLDN4 (b), OCLN (c), and ZO-1 (d). NHEK cultures differentiated for 5 days were treated with apical 0, 10, 50, 100, 250, and 500  $\mu$ M betaine for 24 h, and the mRNA expression of TJ proteins was measured by real-time quantitative PCR. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$  by one-way ANOVA,  $n = 4$  in each.



**Figure 3**

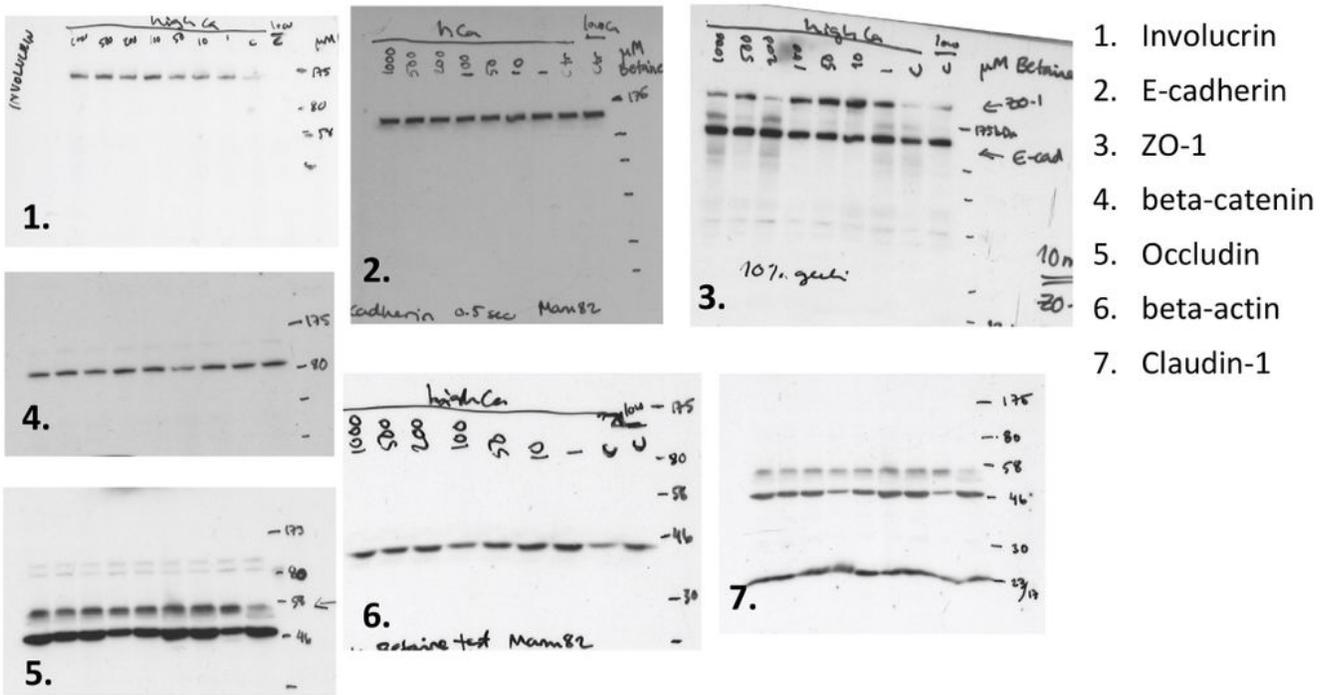
Mannitol permeation assay. REK cells were differentiated for 2 weeks in air-lifted organotypic cultures, and 10 mM betaine was included on differentiation on Day 4 or 5. After differentiation, the cultures were exposed to UVB (30 mJ/cm<sup>2</sup>) or topical acetone, and mannitol permeation was measured after 3 days (a) and TEER 24 hours after the exposure (b). No statistically significant results were observed (One-way ANOVA, n=2)



**Figure 4**

Expression of OCLN (a), TGF-β2 (b), CD147 (c), and ZO-1 (d) in multilayered, organotypic REK cultures as analyzed using RT-qPCR. REK cells were differentiated for 2 weeks in air-lifted organotypic cultures with 10 mM betaine or without betaine, after which the cultures were exposed to UVB radiation (30 mJ/cm<sup>2</sup>) or left unexposed (Sham-treated). After the UVB or sham-exposure the REK cultures continued to be incubated with 10 mM betaine for an additional day. No statistically significant results were observed (One-way ANOVA, n=3).

A



B

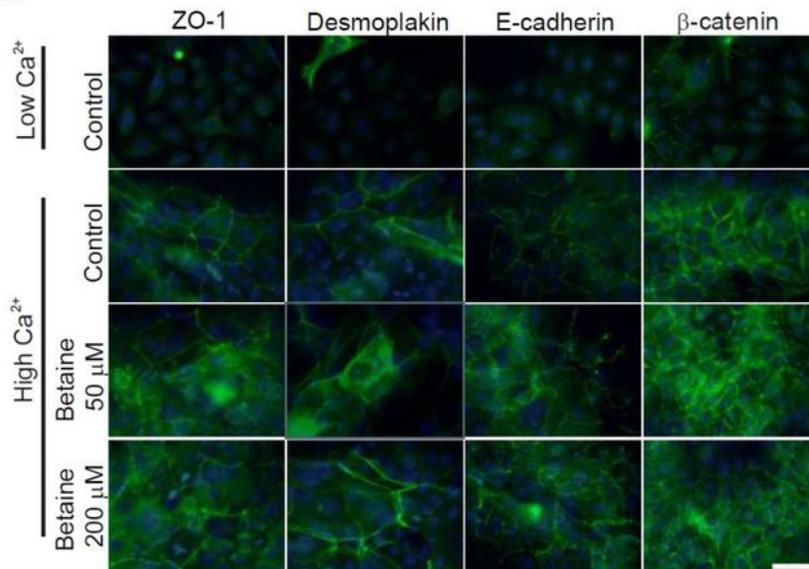


Figure 5

Protein expression using Western transfer analysis and localization of TJ, adherence junction or desmosomal components in betaine treated human epidermal keratinocytes. (a) Human epidermal keratinocytes were cultured either in low  $Ca^{2+}$  concentration without betaine, or in high  $Ca^{2+}$  condition containing different concentrations of betaine (0 - 1000  $\mu$ M) for 72 hours. Samples were then analyzed by western blotting with antibodies against involucrin (depicted in the analysis 1), E-cadherin (depicted in

analysis 2), ZO-1 (depicted in the analysis 3),  $\beta$ -catenin (depicted in analysis 4), occludin (depicted in analysis 5) and claudin-1 (depicted in analysis 7).  $\beta$ -actin was used as a loading control (depicted in analysis 6). (b) Keratinocytes were cultured either in low  $\text{Ca}^{2+}$  concentration without betaine, or in high  $\text{Ca}^{2+}$  concentration in the presence of betaine (0, 50 or 200  $\mu\text{M}$ ) for 72 hours, and followed by IIF for ZO-1, desmoplakin, E-cadherin and  $\beta$ -catenin. Representative wide-field fluorescence images are shown. Bar = 50  $\mu\text{m}$ . (c) Keratinocytes were cultured in high  $\text{Ca}^{2+}$  concentration with 0, 1, 50 or 100  $\mu\text{M}$  betaine, followed by IIF for ZO-1 and claudin-1. Hoechst staining was used to detect the cell nuclei. The confocal images represent single optical sections selected from z-stacks. Bars = 10  $\mu\text{m}$ .