

**The antimicrobial protein S100A7/psoriasin enhances expression of keratinocyte differentiation markers and strengthens the skin tight junction barrier**

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**Bulleted statements:**

- **Already known:** S100A7/psoriasin is a component of the epidermal differentiation complex, however its direct role in keratinocyte differentiation and the tight junction barrier is not known.
- **This study adds:** S100A7/psoriasin enhances expression of keratinocyte differentiation markers and tight junction components and strengthens the tight junction barrier. This finding provides novel evidence that S100A7/psoriasin contributes to cutaneous innate immunity by enhancing the skin barrier, besides having antimicrobial properties.

## Abstract

**Background:** S100A7/psoriasin is a member of the S100 protein family and is encoded in the epidermal differentiation complex, which contains genes for markers for epidermal differentiation. S100A7/psoriasin is overexpressed in hyperproliferative skin diseases, where it is believed to not only exhibit antimicrobial functions but also to induce immunomodulatory activities, including chemotaxis and cytokine/chemokine production.

**Objective:** To evaluate the effect of S100A7/psoriasin on keratinocyte differentiation and regulation of the tight junction barrier function.

**Methods:** Expression of differentiation markers and tight junction proteins in human keratinocytes was determined by real-time PCR and Western blot. The changes in tight junction barrier function were assessed by transepithelial electrical resistance and paracellular permeability assays. Glycogen synthase kinase-3 (GSK-3) and MAP kinase activation was analysed by Western blot, whereas  $\beta$ -catenin and E-cadherin activation was evaluated by Western blot and immunofluorescence.

**Results:** S100A7/psoriasin enhanced expression of several differentiation markers and selectively increased expression of tight junction proteins, such as claudins and occludin, that are known to strengthen the tight junction barrier. Furthermore, S100A7/psoriasin increased  $\beta$ -catenin and E-cadherin accumulation at cell-cell contact, and enhanced transepithelial electrical resistance while reducing the paracellular permeability of keratinocyte layers. The data suggested that S100A7/psoriasin-mediated regulation of tight junction barrier was via both the GSK-3 and MAP kinase pathways, as evidenced by the inhibitory effects of inhibitors for GSK-3 and MAPKs.

**Conclusions:** Our finding that S100A7/psoriasin regulates differentiation and strengthens tight junction barrier function provides novel evidence that S100A7/psoriasin is involved in skin innate immunity, in addition to its antimicrobial and immunoregulatory activities.

## Introduction

The human skin forms an effective barrier against invading microorganisms and water loss. This protective function is partially mediated by antimicrobial peptides/proteins, also known as host defence peptides/proteins (HDPs). Several types of HDPs, including the human  $\beta$ -defensins (hBDs), cathelicidin LL-37 and S100A7/psoriasin, have been identified in the human skin<sup>1-3</sup>. Skin-derived HDPs not only act as endogenous antibiotics but also display various immunomodulatory functions, such as promoting chemotaxis, stimulating the production of cytokines/chemokines, regulating cell proliferation and differentiation, and accelerating wound healing<sup>1-4</sup>.

S100A7/psoriasin belongs to the S100A family of  $\text{Ca}^{2+}$ -binding proteins, which regulate cell proliferation, differentiation, invasion and metastasis<sup>5,6</sup>. Many of the genes encoding members of this family of proteins are located in the epidermal differentiation complex (EDC). This region is of particular interest because it encodes genes, including involucrin, filaggrin, keratins (KRTs) and loricrin, that are essential for epidermal differentiation<sup>7,8</sup>. Of the more than 20 S100A proteins that have been identified to date, 13 members are expressed in the epidermis, where they are involved in the pathology of various skin disorders<sup>8</sup>. S100A7/psoriasin is overexpressed in psoriasis, atopic dermatitis, Darier's disease, mycosis fungoides, wound healing, and skin cancer<sup>8</sup>. All of these conditions are characterised by an inflammatory reaction and disturbed keratinocyte differentiation, suggesting an important role for S100A7/psoriasin in inflammation and keratinocyte differentiation. In addition to the skin defence against *Escherichia coli*, S100A7/psoriasin is a chemoattractant for lymphocytes and neutrophils<sup>9</sup> and an inducer of chemokine production by keratinocytes<sup>10</sup>.

In addition to the cutaneous barrier provided by the stratum corneum, there are multiple important components to barrier function in the epidermis. One of these components is the

tight junction (TJ). The TJ functions as a paracellular barrier beneath the stratum corneum by forming a physical barrier that prevents solutes and water from passing through the paracellular space by forming cell-cell junctions<sup>11</sup>. Furthermore, TJs act as a fence that separates the apical and basolateral portions of the cell membrane<sup>12</sup> and play an important role in cell differentiation and proliferation, cell communication, and cell polarity<sup>13,14</sup>. TJs are mainly composed of transmembrane proteins, such as claudins, occludin, and junctional adhesion molecules, that associate with the cytoplasmic plaque proteins zonula occludens<sup>15,16</sup>. Among the TJ proteins, claudins are particularly important in regulating TJ barrier function by tightening the epithelial barrier<sup>17-19</sup> or disrupting the barrier<sup>18,20</sup>. Furthermore, TJ proteins influence the expression of keratinocyte differentiation markers, as shown by the finding that claudin-1-deficient mice exhibit downregulation of involucrin and filaggrin<sup>21</sup> and the finding that overexpression of claudin-6 leads to abnormal expression of various keratinocyte differentiation markers<sup>22</sup>. Conversely, because downregulation of TJ proteins has been reported in patients with filaggrin mutations, it has been suggested that TJ components are also influenced by keratinocyte differentiation<sup>23</sup>.

Although S100A7/psoriasis has been proposed to be involved in cell differentiation<sup>6,8,24</sup>, its effect on keratinocyte differentiation is still unknown. This study aimed to investigate the effects of S100A7/psoriasis on keratinocyte differentiation and regulation of TJ barrier function. Here, we show that S100A7/psoriasis enhances the expression of various keratinisation markers and TJ proteins. Furthermore, S100A7/psoriasis regulates TJ barrier function by increasing transepithelial electrical resistance (TER) and reducing paracellular flux in keratinocyte layers, and this activity is controlled by the glycogen synthase kinase (GSK)-3 and mitogen-activated protein kinase (MAPK) pathways. This finding provides

novel evidence that S100A7/psoriasin contributes to the modulation of cutaneous innate immunity by strengthening TJ barrier function.

## **Materials and methods**

### ***1. Reagents***

Human S100A7/psoriasin (low endotoxin) was purchased from CircuLex, Nagano, Japan. The involucrin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the keratin (KRT)1, KRT10, transglutaminase (TGM)1 and TGM3 antibodies were purchased from Novus Biologicals (Littleton, CO). The claudin-1, claudin-4 and occludin antibodies were purchased from Invitrogen (Carlsbad, CA), and the claudin-14, filaggrin,  $\beta$ -catenin and E-cadherin antibodies were obtained from Abcam (Tokyo, Japan). The phosphorylated and unphosphorylated antibodies against GSK-3 $\alpha/\beta$  (Y279/Y216), ERK, JNK, p38,  $\beta$ -catenin and E-cadherin were purchased from Cell Signaling Technology (Beverly, MA). SB 415286 was obtained from Tocris Bioscience (Bristol, UK), and U0126, JNK inhibitor II and SB203580 were purchased from Calbiochem (La Jolla, CA).

### ***2. Cell culture and stimulation***

Primary human keratinocytes purchased from Kurabo Industries (Osaka, Japan) were cultured in HuMedia-KG2 (Kurabo Industries), as previously described<sup>25</sup>. For total RNA extractions and Western blotting, the keratinocytes were cultured in 12-well plates and incubated with S100A7/psoriasin in HuMedia without supplements. For the TER and paracellular flux assays,  $0.72 \times 10^5$  cells were plated in transwells with 0.4- $\mu$ m pores (Millipore, Billerica, MA), and the medium was replaced with high-Ca<sup>2+</sup> (1.35 mM) medium after the cells reached confluence.

### ***3. Total RNA extraction and real-time PCR***

Following cell stimulation, total RNA was extracted using the RNeasy Plus Micro kit (Qiagen, Hilden, Germany) and first-strand cDNA was synthesised using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Real-time PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ). Amplification and detection of mRNA were performed using the StepOnePlus Real-time PCR System (Applied Biosystems) following the manufacturer's specifications. All primer/probe sets were obtained from Applied Biosystems' Assays-on-Demand.

#### ***4. Western blot analysis***

Keratinocytes were stimulated with S100A7/psoriasin and lysed in RIPA buffer (Cell Signaling Technology), and equal amounts of total protein were subjected to 12.5% SDS-PAGE. Non-specific binding sites were blocked, and the blots were incubated with appropriate antibodies overnight. The membrane was developed with the Luminata Forte Western HRP substrate (Millipore, Billerica, MA) and imaged using Fujifilm LAS-4000 Plus (Tokyo, Japan). A densitometry analysis was performed using the software program Image Gauge (LAS-4000 Plus, Fujifilm) to correct for protein loading discrepancies. In some of the experiments, the cells in high-Ca<sup>2+</sup> medium were pre-treated with various inhibitors and Western blotting was performed as above.

#### ***5. TER and paracellular flux measurements***

TER was measured across keratinocyte layers grown on 0.6-cm<sup>2</sup> transwell filters that were stimulated with S100A7/psoriasin for 0-48 h using a CellZscope (nanoAnalytics, Münster, Germany). TER values were corrected by subtracting the blank value (no cells) and were expressed in ohm × cm<sup>2</sup>. Keratinocytes were also grown on 0.3-cm<sup>2</sup> transwell filters and

stimulated with S100A7/psoriasis for 0-48 h, and then, a paracellular flux assay was performed as described previously using 4 kDa FITC-dextran (Sigma-Aldrich, St Louis, MO) as a tracer<sup>26</sup>. The fluorescence was measured with a fluorimeter (Nihon Molecular Devices, Tokyo, Japan). In some experiments, keratinocyte layers in high-Ca<sup>2+</sup> medium were treated with various inhibitors for 48 h before S100A7/psoriasis addition, and TER and paracellular flux were measured at 36 h.

### ***6. Immunofluorescence microscopy***

Keratinocytes cultured in high-Ca<sup>2+</sup> medium on collagen I-coated chamber slides (BD Biosciences, Bedford, MA) were stimulated with S100A7/psoriasis for 36 h. After fixation with methanol, cells were blocked in Protein Block Serum-Free (DakoCytomation, Carpinteria, CA) and then incubated overnight with primary antibodies diluted in 1% BSA-PBS, followed by incubation with secondary antibodies coupled to Alexa 594 (Invitrogen). Images were captured using confocal laser scanning microscopy (Carl Zeiss, Jena, Germany).

### ***7. Statistical analysis***

Statistical analysis was performed with ANOVA tests using Prism GraphPad software (GraphPad Software, San Diego, CA).  $P < 0.05$  was considered significant. The results are presented as the mean values  $\pm$  SD.

## Results

### *1. S100A7/psoriasin enhances the expression of keratinocyte differentiation markers*

As seen in Fig. 1a, S100A7/psoriasin markedly enhanced the mRNA expression levels of various differentiation markers, such as filaggrin, involucrin, KRT1, KRT10, loricrin, TGM1 and TGM3. Furthermore, Western blotting revealed that S100A7/psoriasin increased the protein expression of above differentiation markers, except loricrin (Fig. 1b). S100A7/psoriasin likely had no effect on the processing of filaggrin because there was no notable change in the high-molecular filaggrin (Fig. S1). The increased differentiation was not due to some toxic effects of the recombinant protein because transfection of S100A7/psoriasin in keratinocytes showed increases in expression of differentiation markers, which increases were comparable to those induced by the addition of recombinant protein (Fig. S2). Furthermore, S100A7/psoriasin did not show any cytotoxicity as evaluated by lactate dehydrogenase activity and CCK-8 assays (Fig. S3).

### *2. S100A7/psoriasin increases the expression of TJ proteins*

Keratinocyte differentiation markers influence the assembly of the TJ barrier as downregulation of TJ proteins has been observed in patients with ichthyosis vulgaris who have filaggrin mutations<sup>27</sup>. As shown in Fig. 2a, S100A7/psoriasin significantly upregulated the mRNA expression levels of claudin-1, claudin-3, claudin-4, claudin-7, claudin-9, claudin-14 and occludin. S100A7/psoriasin did not remarkably influence the expression of other claudins, junctional adhesion molecules or zonula occludens proteins (data not shown). Among the TJ proteins whose mRNA levels were increased by S100A7/psoriasin, claudin-1, claudin-4, claudin-14 and occludin were enhanced at the protein level following

S100A7/psoriasin stimulation (Fig. 2b). This result implies the importance of these TJ components in the S100A7/psoriasin-mediated regulation of TJ barrier function.

### ***3. S100A7/psoriasin increases TER while decreasing the paracellular flux of FITC-dextran***

To confirm whether the changes in the expression of the TJ proteins resulted in altered regulation of TJ barrier function, we assessed the TER and paracellular permeability, two parameters used to evaluate TJ integrity and barrier function<sup>28</sup>. Keratinocyte layers incubated with S100A7/psoriasin displayed significant increases in TER 24 h after stimulation, and this TER reached maximal values at 36 h before decreasing at 48 h (Fig. 3a). S100A7/psoriasin-treated keratinocyte layers also had significantly reduced 4 kDa FITC-dextran flux from the apical compartment to the basolateral compartment 24 h after stimulation, and this permeability further decreased to a minimum level at 36 h before increasing again at 48 h (Fig. 3b). Together, these observations suggest that S100A7/psoriasin is involved in regulating TJ barrier function.

### ***4. S100A7/psoriasin regulates TJ barrier function by activating GSK-3 $\alpha/\beta$***

To clarify the mechanism by which S100A7/psoriasin regulates TJ barrier function, we tested the involvement of GSK-3, which is implicated in the assembly and maintenance of TJ barrier function by regulating TJ protein expression<sup>29,30</sup>. We first examined the phosphorylation of GSK-3 $\alpha/\beta$  at Y279/Y216 as phosphorylation at these residues leads to full GSK-3 activation<sup>29,31</sup>. S100A7/psoriasin induced increases in the level of tyrosine-phosphorylated GSK-3 $\alpha/\beta$  (Fig. 4a). In contrast, S100A7/psoriasin did not change GSK-3 $\alpha/\beta$  phosphorylation at S21/S9 (data not shown). Phosphorylation at these residues inactivates GSK-3<sup>29</sup>, indicating that S100A7/psoriasin enhances GSK-3 activation in

keratinocytes. This activation was necessary for S100A7/psoriasin-mediated regulation of TJ barrier function because the GSK-3-specific inhibitor SB 415286<sup>30</sup> almost completely suppressed TER increases and also enhanced the paracellular permeability of FITC-dextran that was induced by S100A7/psoriasin up to fourfold (Fig. 4b). GSK-3 inhibition also decreased the S100A7/psoriasin-induced expression of claudin-1, claudin-4, claudin-14 and occludin, suggesting that GSK-3 controls the TJ barrier function through regulation of TJ protein expression (Fig. 4c).

### ***5. S100A7/psoriasin increases activation and accumulation of $\beta$ -catenin and E-cadherin***

GSK-3 also regulates cadherin/catenin complex at the adherens junctions to stabilize cell-cell contacts<sup>32</sup>; therefore, we examined the effect of S100A7/psoriasin on  $\beta$ -catenin and E-cadherin activation. S100A7/psoriasin induced phosphorylation of both  $\beta$ -catenin and E-cadherin.  $\beta$ -Catenin phosphorylation was dose-dependent and observed even at doses as low as 63 nM S100A7/psoriasin, whereas E-cadherin phosphorylation was seen at the highest concentrations (Fig. 5a). Similarly, S100A7/psoriasin induced a widespread accumulation of  $\beta$ -catenin and E-cadherin at cell-cell contacts (Fig. 5b). Because the total proteins of  $\beta$ -catenin and E-cadherin were unchanged in response to S100A7/psoriasin, this implies that S100A7/psoriasin regulates the functions of these adherens junctions mainly through induction of their phosphorylation and distribution to cell-cell contacts rather than increasing their total proteins.

### ***6. Activation of MAPK is required for S100A7/psoriasin-induced regulation of TJ barrier function***

We also investigated MAPK activation to determine whether this signalling pathway might be involved in S100A7/psoriasin-induced regulation of TJ barrier function. Following

S100A7/psoriasin stimulation, phosphorylation of ERK enhanced between 2-10 min before the signal faded. In addition, high levels of JNK and p38 phosphorylation were seen at the 30-min time point (Fig. 6a). U0126 (ERK inhibitor), JNK inhibitor II and SB203580 (p38 inhibitor) almost completely reduced the TER increases and reversed the paracellular flux of FITC-dextran mediated by S100A7/psoriasin up to fourfold (Fig. 6b), and also suppressed the S100A7/psoriasin-induced expression of claudin-1, claudin-4, claudin-14 and occludin (Fig. 6c). This implies that MAPK activation is part of a signalling pathway necessary for the S100A7/psoriasin-mediated regulation of TJ barrier function

MAPKs have been also reported to regulate keratinocyte differentiation<sup>33,34</sup>; therefore, the effects of MAPK inhibitors on S100A7/psoriasin-induced increases of differentiation were examined. As observed in Fig. 7, MAPK inhibitors selectively affected the keratinocyte differentiation: all inhibitors markedly suppressed the expression of involucrin, KRT1 and KRT10, but had no effect on the filaggrin, TGM1 and TGM3 expression.

## Discussion

Recently, there has been an interest in the role of TJs in the skin barrier as studies have demonstrated that TJs assist the stratum corneum to form a secondary barrier, serve as a rescue system when the stratum corneum barrier is perturbed, and regulate epidermal differentiation<sup>12,21,35</sup>. Because S100A7/psoriasin is located in the EDC, where genes for many differentiation markers are encoded, we hypothesised that S100A7/psoriasin may influence the expression of keratinisation markers and also regulate TJ barrier function. Here, we showed that S100A7/psoriasin upregulated the expression of differentiation markers and TJ proteins in human keratinocytes, and improved TJ barrier function by increasing TER and decreasing paracellular flux. S100A7/psoriasin-mediated regulation of the TJ barrier function was controlled by the GSK-3 and MAPK pathways.

Although S100A7/psoriasin has been suggested to be involved in epidermal differentiation because of the high concentration of this protein in skin diseases characterised by disturbed keratinocyte differentiation<sup>8</sup>, the mechanisms by which S100A7/psoriasin effects keratinisation markers have been unclear. Here, we demonstrated that S100A7/psoriasin enhanced the expression of various differentiation markers, such as filaggrin, involucrin, KRT1, KRT10, loricrin, TGM1 and TGM3 under MAPK pathway. Thus, our study provides insight into how S100A7/psoriasin contributes to the regulation of epidermal differentiation. Another S100 protein, S100A11, has been reported to modulate filaggrin expression but had no effect on loricrin and involucrin expression<sup>36</sup>, indicating the unique role for S100A7/psoriasin in keratinocyte differentiation.

Alterations of the TJ barrier and keratinocyte differentiation each influence the other<sup>21</sup>, implying that both the TJ barrier and keratinocyte differentiation are closely and complementarily related in the maintenance of the skin barrier. For example, there is a

downregulation of involucrin and filaggrin in keratinocytes from claudin-1-deficient mice <sup>21</sup>, and altered expression of differentiation markers has also been demonstrated in mice overexpressing claudin-6 <sup>22</sup>. Conversely, downregulation of TJ proteins has been observed in ichthyosis vulgaris patients with filaggrin mutations <sup>23</sup>. Because S100A7/psoriasin is overexpressed in skin diseases, such as psoriasis, lichen planus and ichthyosis vulgaris, in which changes in expression of TJ proteins have been reported <sup>12,37,38</sup>, this led us to investigate the regulation of TJ proteins by S100A7/psoriasin. S100A7/psoriasin selectively increased the expression of claudin-1, claudin-3, claudin-4, claudin-7, claudin-9, claudin-14 and occludin, and all of these TJ proteins have been reported to tighten TJ barrier <sup>39-41</sup>. This result indicates that S100A7/psoriasin contributes to the strengthening of the epidermal barrier through enhancing the expression of TJ components. In fact, this phenomenon is demonstrated by the ability of S100A7/psoriasin to increase TER and reduce paracellular permeability.

The molecular mechanism by which S100A7/psoriasin regulates TJ barrier function involved the GSK-3 and MAPK pathways. GSK-3 consists of two isoforms, GSK-3 $\alpha$  and GSK-3 $\beta$ , and is a key regulator of several physiological processes, including the regulation of TJ components <sup>42</sup>. Until recently, it was believed that GSK-3 is a constitutively active enzyme and could be regulated only by inhibition via phosphorylation of serine residue (S21 in GSK- $\alpha$  and S9 in GSK-3 $\beta$ ) <sup>29</sup>. However, some recent reports have indicated that GSK-3 is also activated by tyrosine phosphorylation (Y279 in GSK- $\alpha$  and Y216 in GSK-3 $\beta$ ) in response to diverse stimuli, including lysophosphatidic acid, apoptotic stimuli, ischemia, increases in intracellular Ca<sup>2+</sup>, and insulin <sup>43-46</sup>. We showed that S100A7/psoriasin increased tyrosine phosphorylation of GSK-3 $\alpha/\beta$  and that inhibition of GSK-3 suppressed the TJ protein expression and reversed S100A7/psoriasin-induced TER increases and paracellular flux

reduction. S100A7/psoriasin also increased accumulation of chief components of adherens junctions,  $\beta$ -catenin and E-cadherin, at cell-cell contacts. The phosphorylation of  $\beta$ -catenin and E-cadherin is controlled by GSK-3<sup>32,47,48</sup>, and these proteins play an important role in both adherens junction formation and regulation of epithelial barrier function<sup>32</sup>. Thus, S100A7/psoriasin contributes to both TJ and adherens junction regulation.

To further understand the S100A7/psoriasin activation mechanism, we also investigated the involvement of the MAPK pathway, which regulates various cellular activities, including differentiation and TJ function. MAPKs control the expression of differentiation markers, including involucrin, KRTs and loricrin<sup>33,34</sup>. Furthermore, activation of the MAPK increases TER and decreases paracellular permeability due to increased expression of claudins<sup>49</sup>, and thiols promote TJ sealing by activating MAPKs<sup>50</sup>. Conversely, activation of MAPK is also involved in TJ leaking caused by various stimuli, including cytokines, growth factors and bacteria<sup>51</sup>. Our finding that MAPK-specific inhibitors reversed S100A7/psoriasin-mediated TJ barrier regulation and selectively attenuated differentiation indicates that S100A7/psoriasin regulates TJ function and differentiation through the activation of MAPKs.

HDPs S100A7/psoriasin, hBDs and LL-37 are present within the permeability barrier, stratum corneum<sup>52,53</sup>. It was recently reported that the antimicrobial and permeability barriers of the skin are closely linked: skin barrier disruption leads to increased expression of S100A7/psoriasin, cathelin-related antimicrobial peptide CRAMP (murine homologue of LL-37), hBD-2 and hBD-3, and murine  $\beta$ -defensins mBD-1, mBD-3 and mBD-14 (orthologs of hBD-1, hBD-2 and hBD-3, respectively)<sup>54-57</sup>, and this increase in expression is reduced by permeability barrier restoration<sup>55,57</sup>. Importantly, CRAMP knockout mice display delayed permeability barrier recovery, demonstrating that HDPs are essential for the permeability barrier homeostasis<sup>55</sup>. Because CRAMP knockout mice exhibit defective cutaneous

antimicrobial defense against streptococcal infections <sup>58</sup>, the increased expression of HDPs may reflect a defense response to protect the skin against harmful microorganisms invading skin with impaired permeability barrier.

Although the exact concentrations of S100A7/psoriasin *in vivo* are not known, this protein is estimated to be the principal HDP expressed in normal adult skin <sup>53</sup>. Our findings that S100A7/psoriasin regulates TJ barrier function and keratinocyte differentiation provide novel evidence of the importance of S100A7/psoriasin in cutaneous immunity, in addition to its antimicrobial and immunomodulatory activities.

**Abbreviations:**

**CRAMP:** Cathelin-related antimicrobial peptide; **EDC:** Epidermal differentiation complex;  
**GSK-3:** Glycogen synthase kinase-3; **hBD:** Human  $\beta$ -defensin; **HDP:** Host defence peptide;  
**KRT:** Keratin; **MAPK:** Mitogen-activated protein kinase; **TGM:** Transglutaminase; **TJ:**  
Tight junction; **TER:** Transepithelial electrical resistance

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## Figure legends

### Figure 1. Effect of S100A7/psoriasin on the expression of keratinocyte differentiation markers

(a) Normal human keratinocytes were stimulated with indicated concentrations of S100A7/psoriasin or the diluent (Med) for 24 h. Following this incubation, total RNA was extracted and reverse transcribed into cDNA, and real-time PCR was performed. The values from four separate experiments represent fold-increases in gene expression relative to those of controls stimulated with medium alone (Med). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . (b) Human keratinocytes were stimulated with S100A7/psoriasin or the diluent (Med) for 48 h. After stimulation, cell lysates were obtained and equal amounts of total protein were subjected to 12.5% SDS-PAGE analysis. The blots were incubated with antibodies against filaggrin (FLG, 40 kDa monomer), involucrin (IVL), keratin 1 (KRT1), keratin 10 (KRT10), loricrin (LOR), transglutaminase 1 (TGM1) and transglutaminase 3 (TGM3). The results of one representative of three separate experiments yielding similar results are shown. **Right panels:** Bands were quantified by densitometry to correct for protein loading discrepancies. The data represent the ratio of the intensity of the target protein divided by that of  $\beta$ -actin.

### Figure 2. S100A7/psoriasin increases the expression of TJ proteins in keratinocytes

(a) Human keratinocytes were incubated with S100A7/psoriasin or medium alone for 24 h. After stimulation, total RNA was extracted and reverse transcribed into cDNA, and real-time PCR was performed. The values from five separate experiments represent fold-increases in gene expression relative to those of controls stimulated with medium alone (Med). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . (b) Keratinocytes were stimulated with S100A7/psoriasin or the

diluent alone (Med) for 48 h. The levels of claudin-1 (Cldn-1), claudin-4 (Cldn-4), claudin-14 (Cldn-14) and occludin (Ocdn) in cell lysates were determined by Western blot. The results of one representative of four separate experiments yielding similar results are shown. **Lower panels:** Bands were quantified by densitometry to correct for protein loading discrepancies. The data represent the ratio of the intensity of the target protein divided by that of  $\beta$ -actin.

**Figure 3. S100A7/psoriasis increases transepithelial electrical resistance and decreases paracellular flux in keratinocyte layers**

Keratinocyte layers grown on transwell inserts were stimulated with 125-500 nM S100A7/psoriasis or the diluent (Med) for 0-48 h. **(a)** The transepithelial electrical resistance (TER) values were assessed using a CellZscope and are expressed in  $\text{ohm} \times \text{cm}^2$ . **(b)** Paracellular flux of 4 kDa FITC-dextran was determined by measuring the fluorescence using a fluorimeter. The values obtained from four separate experiments using stimulated and non-stimulated cells (Med) were compared. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ .

**Figure 4. S100A7/psoriasis regulates TJ barrier function through GSK-3 $\alpha$ / $\beta$  activation**

**(a)** Human keratinocytes were incubated with 500 nM S100A7/psoriasis or diluent (Med) for 0.5-8 h, and lysates were separated by 12.5% SDS-PAGE using antibodies directed against phosphorylated (p-) GSK-3 $\alpha$ / $\beta$  (Y279/Y216) and GSK-3 $\alpha$ / $\beta$  proteins. The results of one representative of four separate experiments yielding similar results are shown. **Right panel:** Upper and lower bands were quantified by densitometry to correct for protein loading

discrepancies. The data represent the ratio of the intensity of p-GSK-3 $\alpha$ / $\beta$  divided by that of GSK-3 $\alpha$ / $\beta$  protein. **(b)** Human keratinocyte layers grown on transwell inserts were pre-treated with 25-100 nM SB 415286 or 0.1% DMSO for 48 h and then stimulated with 500 nM S100A7/psoriasis or diluent (Med) for 36 h, and the TER and paracellular flux were determined. The values obtained from four separate experiments using stimulated and non-stimulated cells (Med) or in the presence and absence of an inhibitor were compared. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ . **(c)** Effects of SB 415286 on the S100A7/psoriasis-mediated expression of TJ proteins. Keratinocytes were pre-treated with 50 nM SB 415286 (+SB) or 0.1% DMSO for 48 h and were then stimulated with 500 nM S100A7/psoriasis (Pso) or diluent alone (Med) for 48 h. The levels of claudin-1 (Cldn-1), claudin-4 (Cldn-4), claudin-14 (Cldn-14) and occludin (Ocdn) in cell lysates were determined by Western blot. The results of one representative of three separate experiments yielding similar results are shown. **Lower panels:** Bands were quantified by densitometry to correct for protein loading discrepancies. The data represent the ratio of the intensity of the target protein divided by that of  $\beta$ -actin.

**Figure 5. S100A7/psoriasis increases phosphorylation and accumulation at cell-cell contact of  $\beta$ -catenin and E-cadherin**

**(a)** Keratinocytes were incubated with S100A7/psoriasis or diluent alone (Med) for 36 h and lysed. Equal amounts of proteins were immunoblotted using antibodies directed against phosphorylated or unphosphorylated  $\beta$ -catenin (p- $\beta$ -catenin and  $\beta$ -catenin) and E-cadherin (p-E-cadherin and E-cadherin). The results of one representative experiment of four separate experiments yielding similar results are shown. **Right panels:** Bands were quantified by

densitometry to correct for protein loading discrepancies. The data represent the ratio of the intensity of phosphorylated protein divided by that of unphosphorylated protein. **(b)** Keratinocytes grown to confluence on collagen I-coated chamber slides were stimulated with 500 nM S100A7/psoriasin or diluent alone (Medium) for 36 h. Cells were processed for overnight immunofluorescence staining with antibodies against  $\beta$ -catenin, E-cadherin or isotype control (mouse IgG1), followed by incubation with secondary antibodies coupled to Alexa 594.  $\beta$ -catenin and E-cadherin were visualized in red using confocal laser scanning microscopy. The results of one representative experiment of four separate experiments yielding similar results are shown. Scale bar = 10  $\mu$ m.

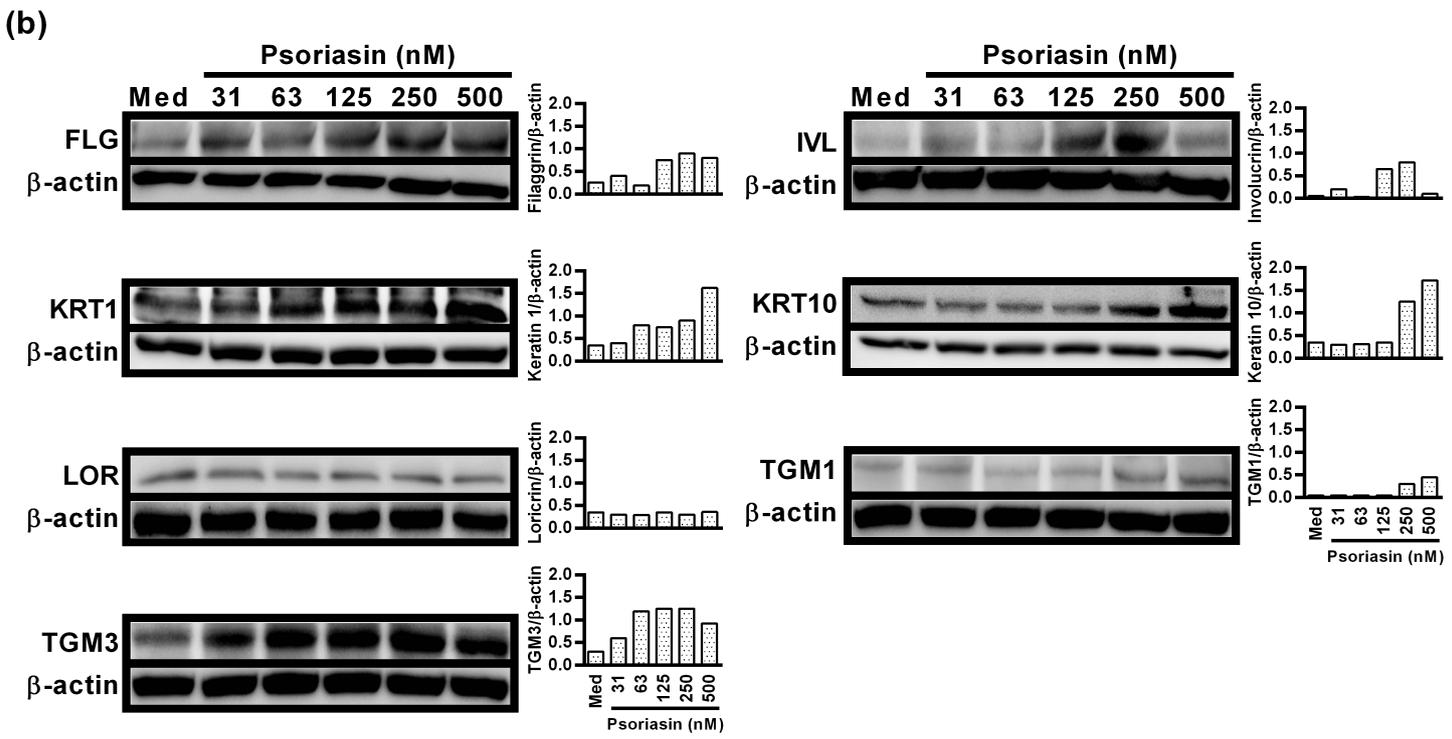
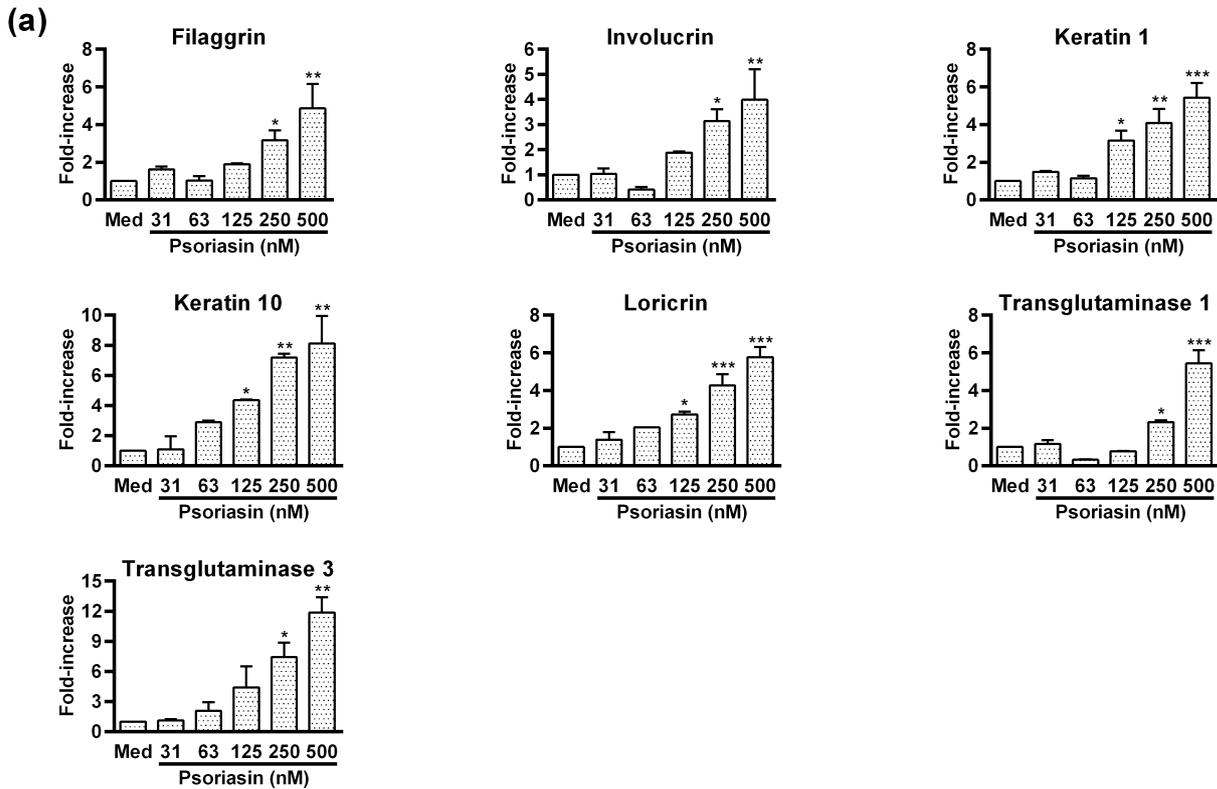
**Figure 6. S100A7/psoriasin regulates TJ barrier function via MAPK activation**

**(a)** Keratinocytes were incubated with 500 nM S100A7/psoriasin or the diluent (Med) for 2-60 min and then lysed, and equal amounts of protein were subjected to 12.5% SDS-PAGE analysis using antibodies directed against phosphorylated or unphosphorylated ERK (p-ERK and ERK), JNK (p-JNK and JNK) and p38 (p-p38 and p38). The results of one representative of three separate experiments yielding similar results are shown. **Right panels:** Bands were quantified by densitometry to correct for protein loading discrepancies. The data represent the ratio of the intensity of phosphorylated protein divided by that of unphosphorylated protein. **(b)** The effects of ERK, JNK and p38 inhibitors. Keratinocytes were pre-treated with 10  $\mu$ M of U0126, JNK inhibitor II (JNK inh II) or SB203580 or with 0.1% DMSO for 24 h, stimulated with 500 nM S100A7/psoriasin or the diluent (Med) for 36 h, and then, the TER and paracellular flux were determined. The values obtained from four separate experiments using stimulated and non-stimulated cells (Med) or in the presence and absence of an inhibitor

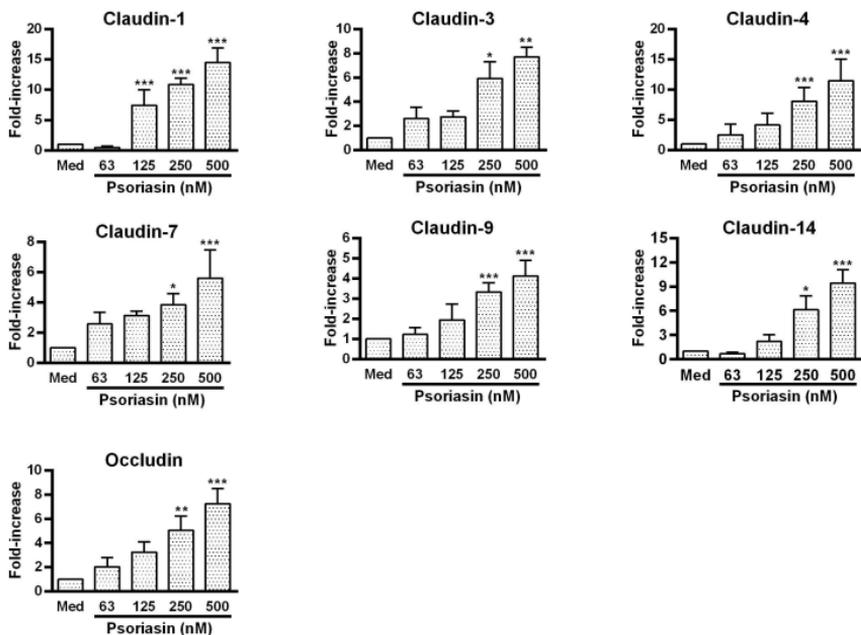
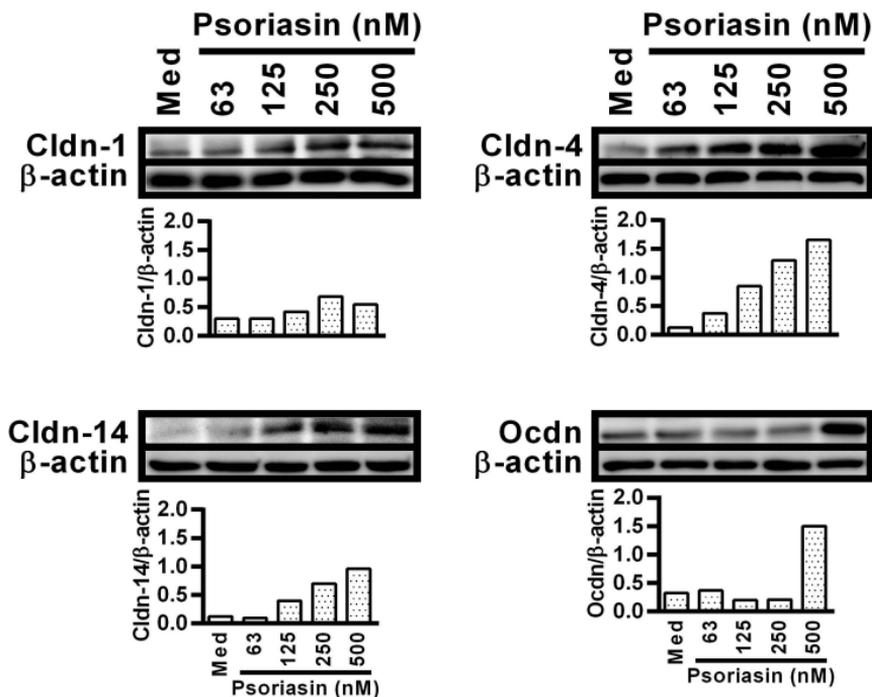
were compared. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ . (c) Keratinocytes were pre-treated with 10  $\mu\text{M}$  of U0126, JNK inhibitor II (+JNK II) or SB203580 (+SB) or with 0.1% DMSO for 24 h, and were then stimulated with 500 nM S100A7/psoriasin (Pso) or diluent alone (Med) for 48 h. The levels of claudin-1 (Cldn-1), claudin-4 (Cldn-4), claudin-14 (Cldn-14) and occludin (Ocdn) in cell lysates were determined by Western blot. The results of one representative of three separate experiments yielding similar results are shown. **Lower panels:** Bands were quantified by densitometry to correct for protein loading discrepancies. The data represent the ratio of the intensity of the target protein divided by that of  $\beta$ -actin.

#### **Figure 7. Effects of MAPK inhibitors on S100A7/psoriasin-mediated differentiation**

Keratinocytes were pre-treated with 10  $\mu\text{M}$  of U0126, JNK inhibitor II (+JNK II) or SB203580 (+SB) or with 0.1% DMSO for 24 h, and were then stimulated with 500 nM S100A7/psoriasin (Pso) or diluent alone (Med) for 48 h. The levels of differentiation markers in cell lysates were determined by Western blot. The results of one representative of four separate experiments yielding similar results are shown. **Lower panels:** Bands were quantified by densitometry to correct for protein loading discrepancies. The data represent the ratio of the intensity of the target protein divided by that of  $\beta$ -actin.

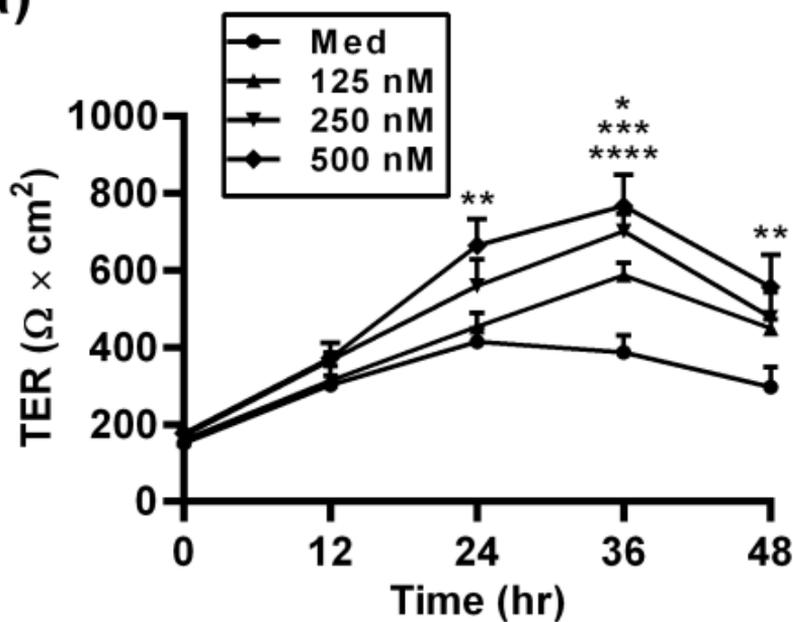


Hattori F. *et al.* Figure 1

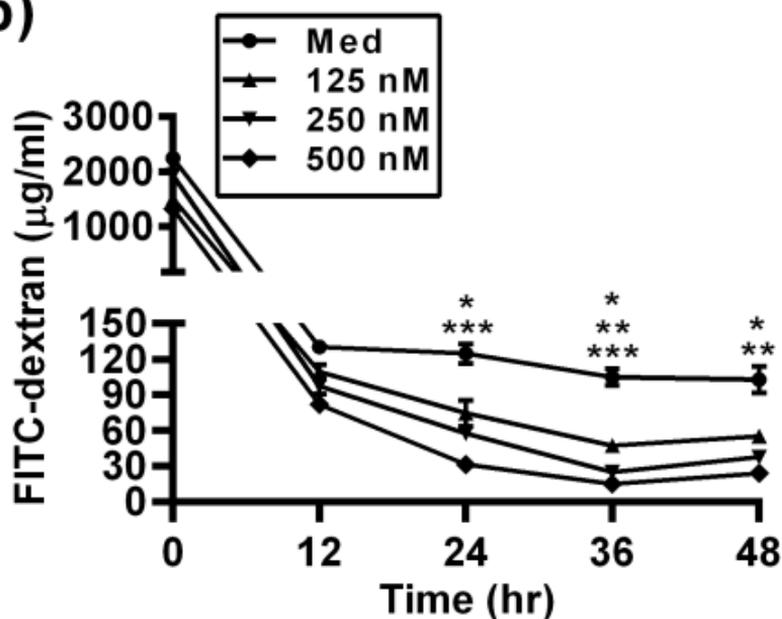
**(a)****(b)**

Hattori F. *et al.* Figure 2

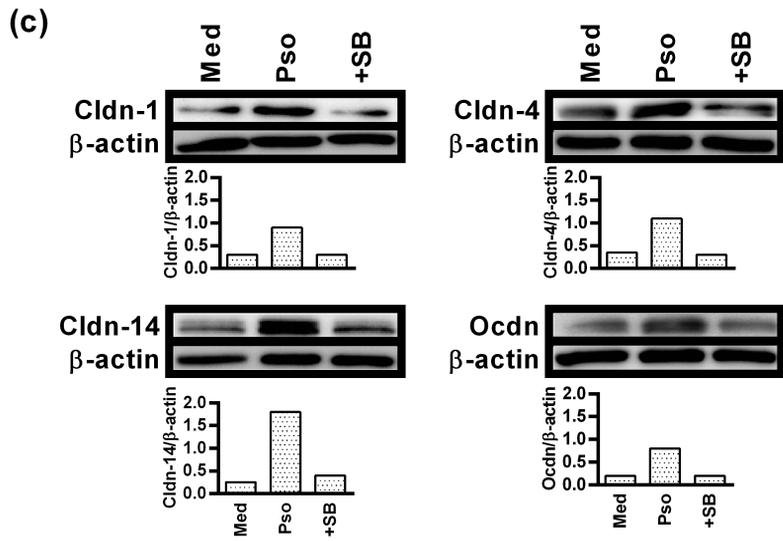
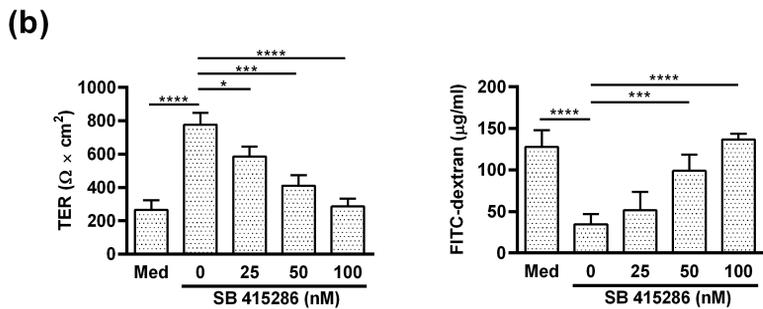
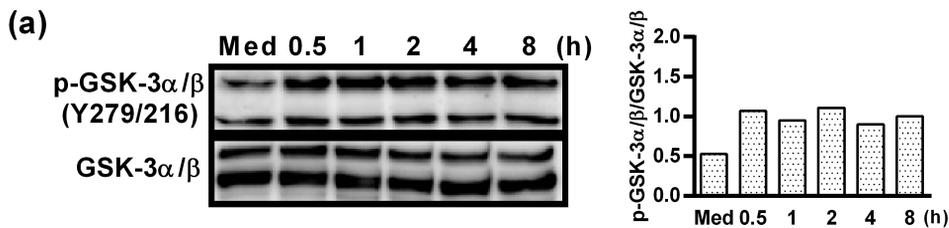
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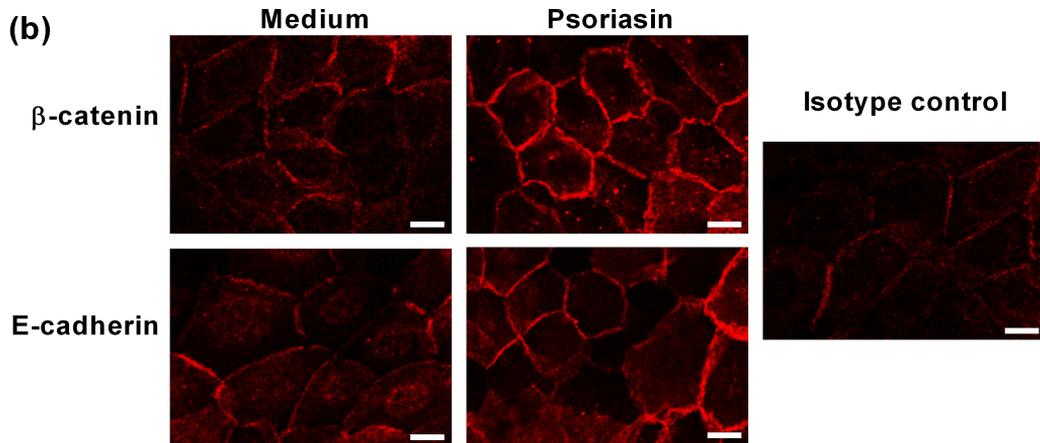
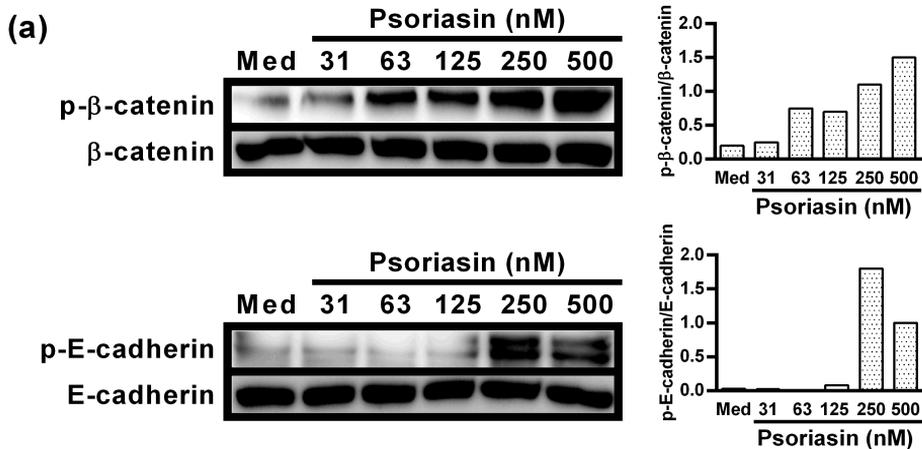


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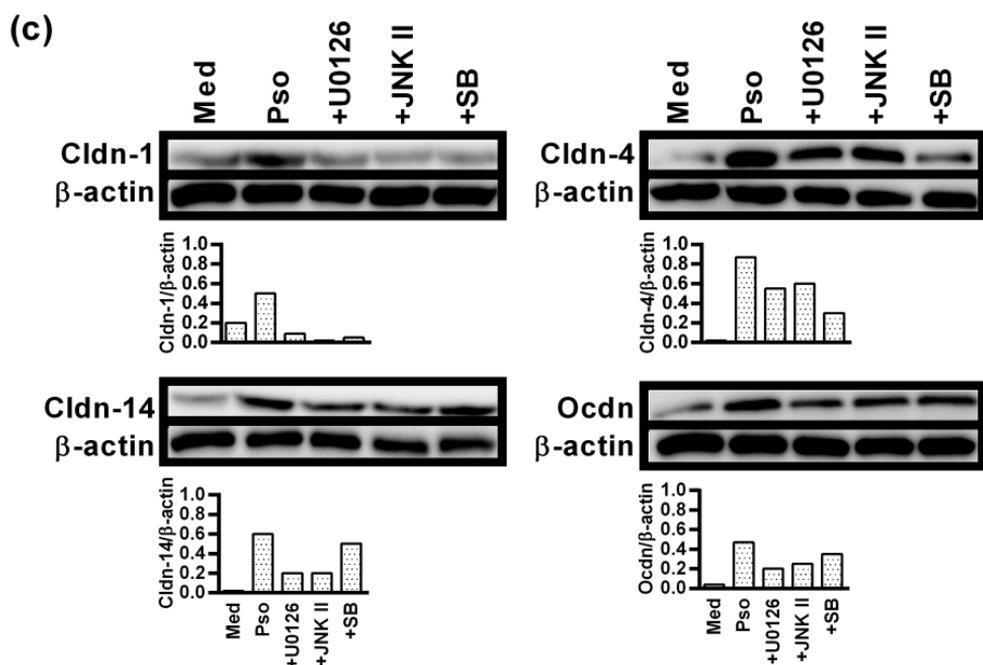
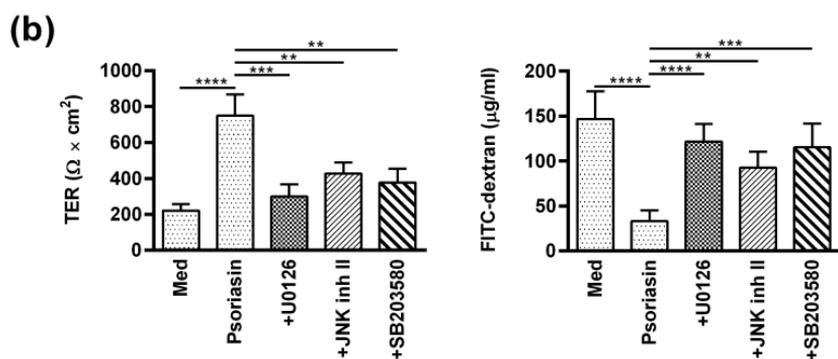
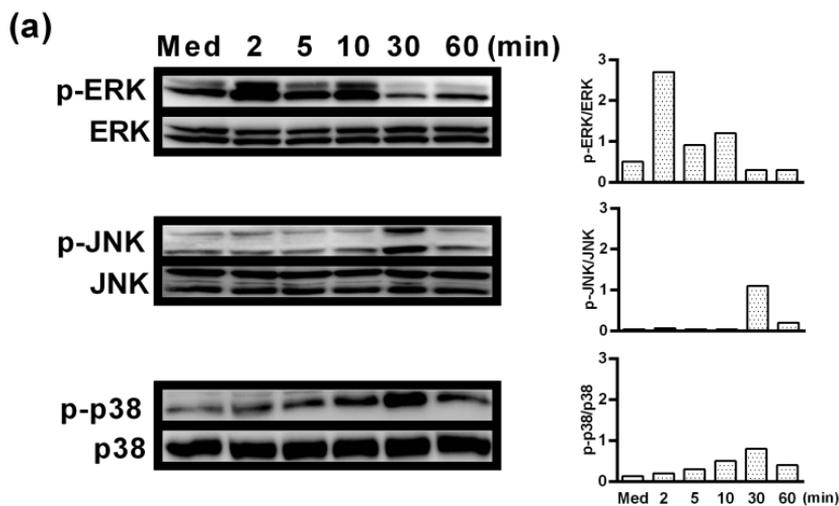


Hattori F. *et al.* Figure 3

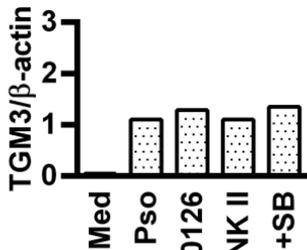
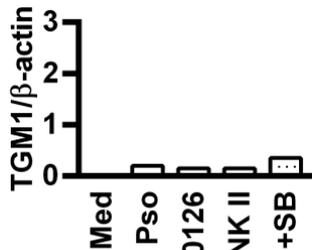
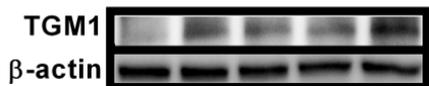
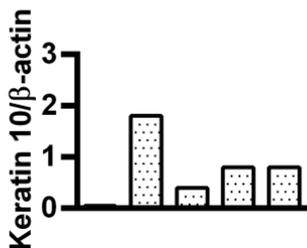
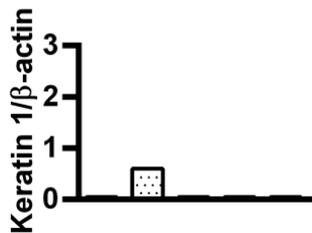
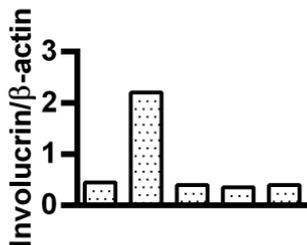
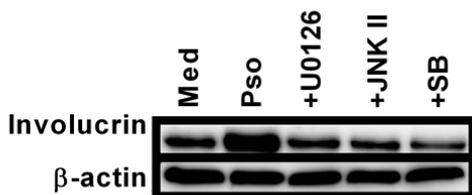
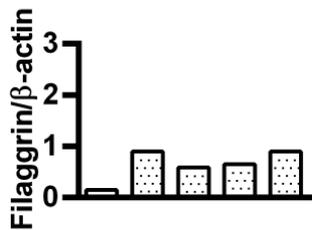
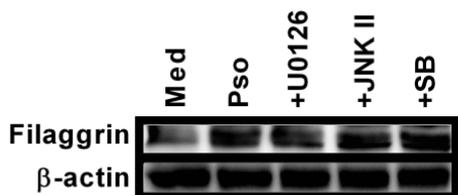




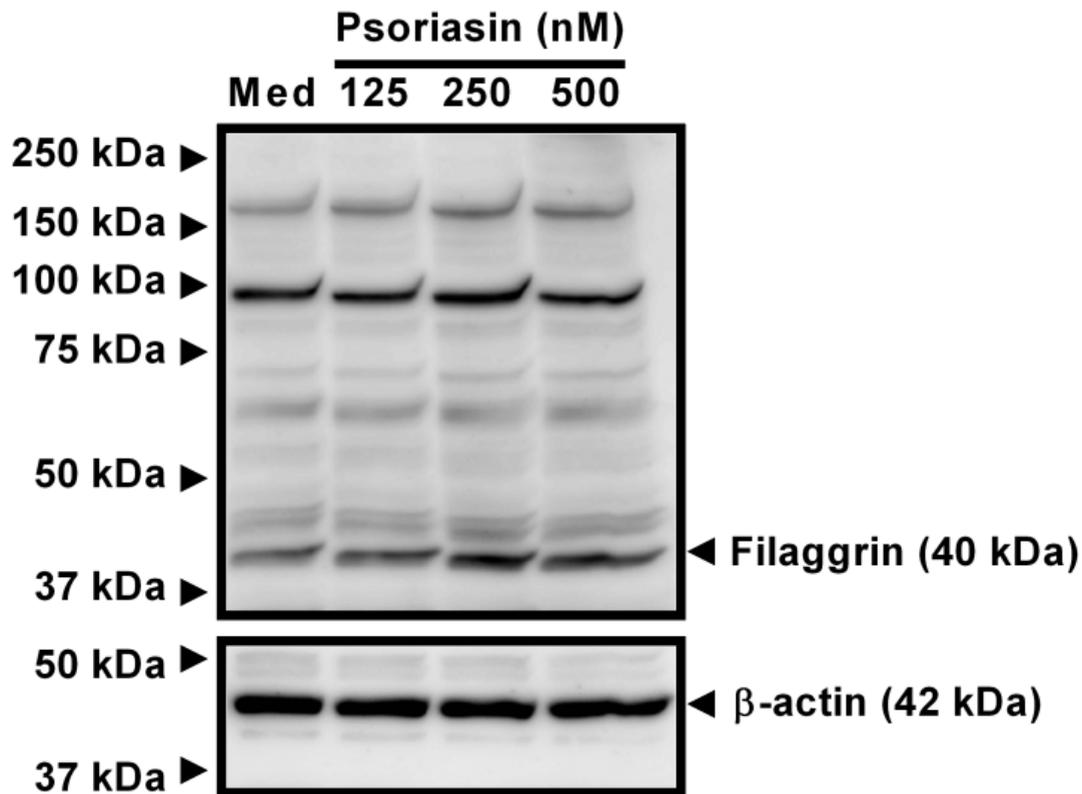
Hattori F. *et al.* Figure 5



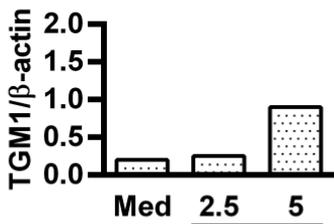
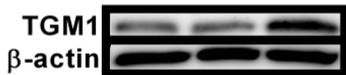
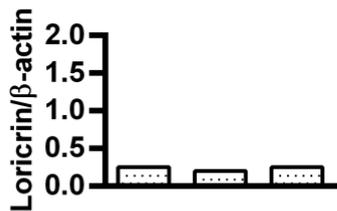
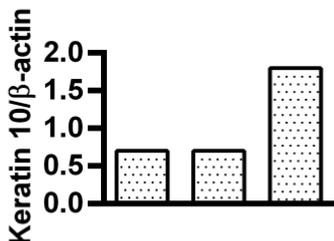
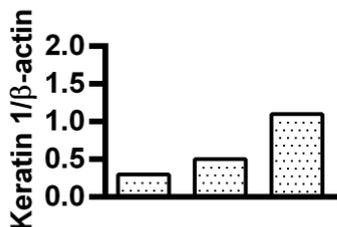
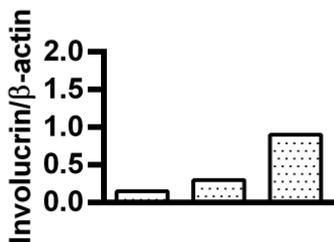
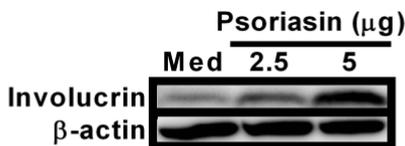
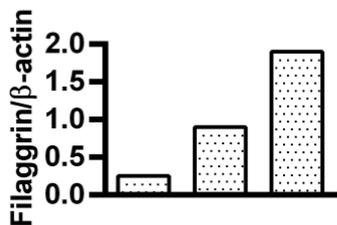
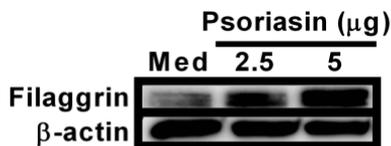
Hattori F. *et al.* Figure 6



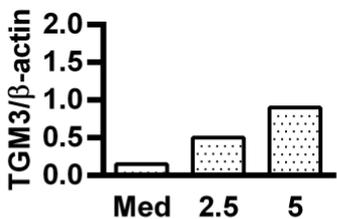
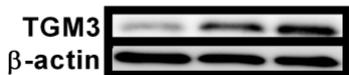
Hattori F. *et al.* Figure 7



Hattori F. *et al.* Figure S1



Med 2.5 5  
Psoriasis ( $\mu\text{g}$ )



Med 2.5 5  
Psoriasis ( $\mu\text{g}$ )

