



Claudin-3 Loss Causes Leakage of Sweat from the Sweat Gland to Contribute to the Pathogenesis of Atopic Dermatitis

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The transfer of sweat to the skin surface without leakage is important for the homeostatic regulation of skin and is impaired in atopic dermatitis. Although the precise composition of the leakage barrier remains obscure, there is a large contribution from claudins, the major components of tight junctions. In humans, claudin-1, -3, and -15 are expressed on sweat ducts, and claudin-3 and -10 are expressed on secretory coils. Although only two claudins are expressed in murine sweat glands, we found that the expression of claudin-3 is conserved. Atopic dermatitis lesional skin had decreased claudin-3 expression in sweat glands, which was accompanied by sweat leakage. This critical role in water barrier function was confirmed in *Cldn3*^{-/-} and *Cldn3*^{+/-} mice and those with experimentally decreased claudin-3. Our results show the crucial role of claudin-3 in preventing sweat gland leakage and suggest that the pathogenesis of dermatoses accompanied by hypohidrosis involves abnormally decreased claudin-3.

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INTRODUCTION

Sweat is defined as a diluted electrolyte for regulating homeostasis and is indispensable for human activity (Murota et al., 2015; Sato et al., 1989). Sweat is delivered to the skin surface by the sweat gland apparatus. To avoid leakage, the sweat apparatus must include a water barrier that remains to be identified. Epithelial cells have tight junctions (TJs) that form a barrier between the paracellular space and the ectoplasmic membrane leaflet and regulate the selective transport of critical substances (Tamura and Tsukita, 2014; Zihni et al., 2016). Electron microscopic analysis showed the existence of a TJ structure in the paracellular space of luminal cells of the sweat gland apparatus, forming a barrier to prevent body fluid leakage (Hashimoto, 1971a, 1971b). TJs are primarily formed by the polymerization of claudins, which are associated with other transmembrane proteins (e.g., occludin and tricellulin) and scaffold proteins (e.g., zonula occludens [ZO] proteins, cingulin, and membrane-associated guanylate kinase inverted proteins) (Tsukita et al., 2001; Zihni et al., 2016). Of the 27 known claudins (Tamura and Tsukita, 2014; Tsukita et al.,

2001), skin epithelial cells express claudin-1 and claudin-4, which serve mainly as the paracellular water barrier (Tamura and Tsukita, 2014; Tokumasu et al., 2016). Although their expression has also been confirmed in the sweat gland apparatus, their association with TJs remains obscure (Wilke et al., 2006).

The pathophysiology of atopic dermatitis (AD) may provide a clue to understanding the mechanism for preventing sweat leakage. Historically, AD has been considered as a sweat retention syndrome, characterized by environmental temperature-evoked attacks of pruritus and reduced sweating (Sulzberger et al., 1947). Immunohistochemical identification of dermcidin (i.e., DCD), a sweat-specific antimicrobial peptide, in tissue surrounding sweat glands indicated possible leakage, which was proposed as a cause of decreased sweating in AD (Eishi et al., 2002; Kijima et al., 2012; Murota et al., 2015; Shiohara et al., 2011; Sulzberger et al., 1947; Takahashi et al., 2013). To identify claudins regulating the permeability of the sweat gland apparatus, we compared tissue samples from subjects with and without AD. The results of these studies show a crucial role for the TJ protein claudin-3 in regulating leakage from the sweat gland apparatus.

RESULTS

Leakage of sweat in AD

After applying sudomotor stimulation (acetylcholine [ACh]) to the skin surface, we found that the sweat volume was decreased in AD subjects (n = 20) relative to that in control individuals (n = 14) (Figure 1a). Immunohistochemistry for DCD showed its ectopic distribution throughout the dermis surrounding the sweat gland apparatus in samples from AD subjects (n = 8), whereas it was restricted to the lumens of sweat gland apparatuses in healthy skin (n = 5) and psoriasis samples (n = 3) (Figure 1b, and see Supplementary Figure S1

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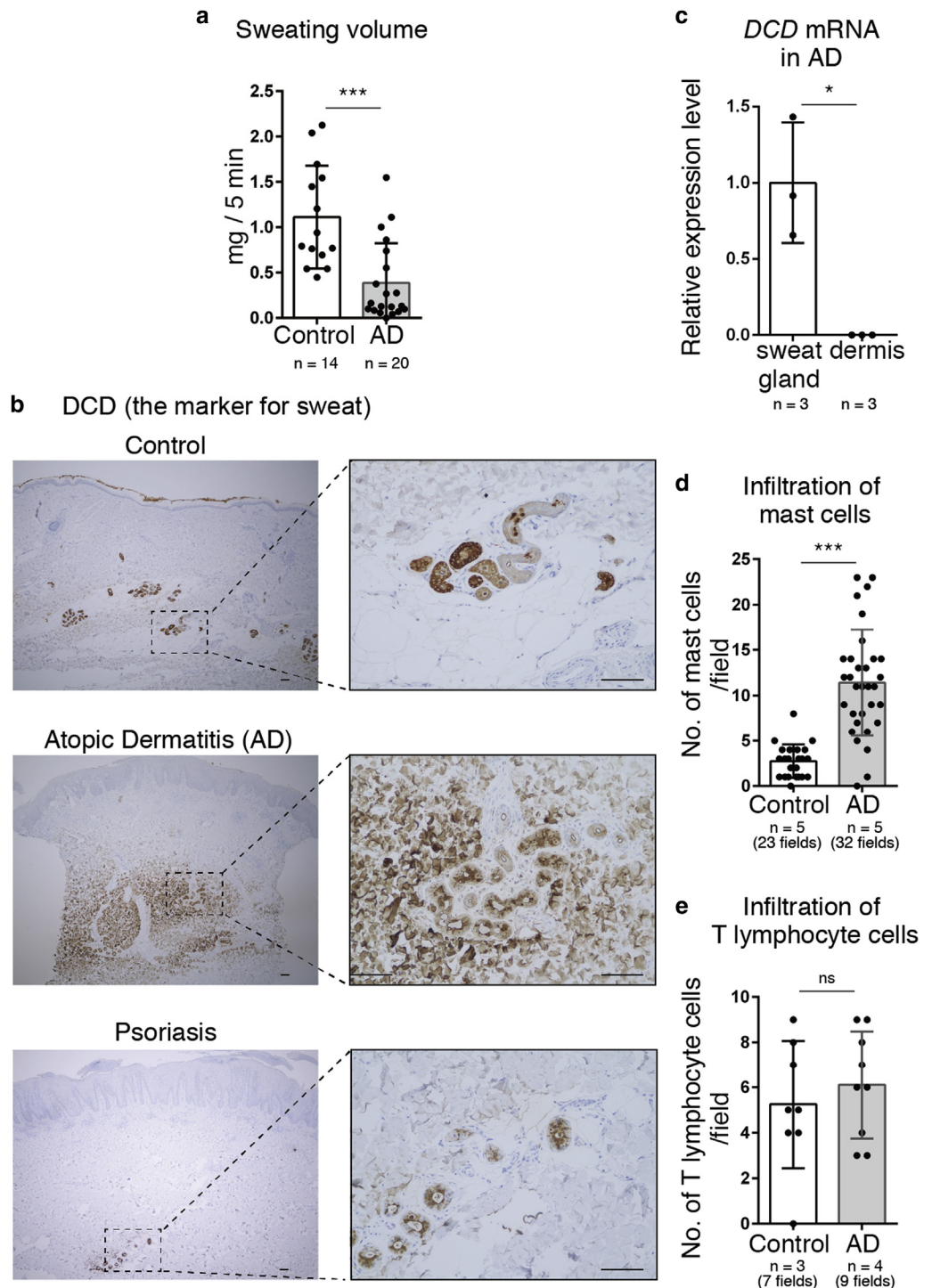
Abbreviations: ACh, acetylcholine; AD, atopic dermatitis; AIGA, acquired idiopathic generalized anhidrosis; QSART, quantitative sudomotor axon reflex test; TJ, tight junction; ZO, zonula occludens

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Figure 1. Patients with AD showed impaired sweating and ectopic localization of DCD. (a) Axon reflex-mediated sweating volume in healthy control individuals (n = 14) and AD patients (n = 20).

(b) Localization of DCD. Representative findings from healthy control individuals (n = 5) and AD (n = 8) and psoriasis patients (n = 3). Scale bars = 100 μ m. (c) *DCD* mRNA expression in the sweat glands and the dermis around sweat glands of AD patients (n = 3 each). This suggests that DCD is produced only in the sweat glands. Graphs indicate average numbers of (d) mast cells and (e) T lymphocyte cells around sweat glands in healthy control individuals and AD patients (see Supplementary Figure S2).

* $P < 0.05$; *** $P < 0.001$; ns, not significant by unpaired Student *t* test. Error bars indicate standard error of the mean. AD, atopic dermatitis; DCD, dermcidin.



online). Because DCD mRNA was detected in sweat glands but not the surrounding dermal tissue (n = 3) (Figure 1c), it is unlikely that the protein was from DCD-producing cells around the sweat glands in the AD samples. Another histopathological finding from AD lesions was a slight increase in the number of mast cells around sweat glands, whereas there was no apparent infiltration of inflammatory cells around the sweat glands in AD lesions (n = 4–5 from 9–32 microscopic fields) or in healthy skin (n = 3–5 from 7–23 microscopic fields) (Figure 1d and e, and see Supplementary Figure S2 online).

Expression of TJs in the human sweat gland apparatus

To investigate the role of TJs of the human sweat gland apparatus, we performed immunohistochemistry to assess the expression of TJ components in healthy skin (Figure 2) and in AD lesions (Figure 3a). The sweat duct and secretory coil are composed of a double-decker structure of epithelial cells and a single secretory cell layer surrounded by smooth muscle actin-positive myoepithelial cells, respectively. Claudin-1 localized to basal cells of the sweat duct but was not expressed on secretory coils (Figure 2). Claudin-4

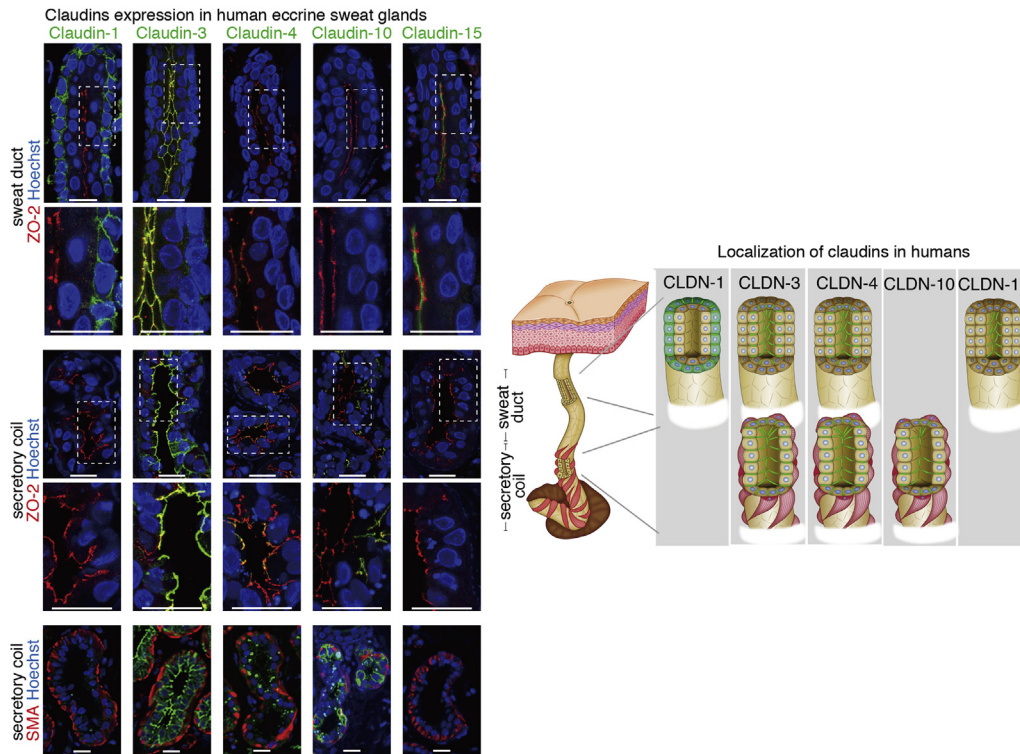


Figure 2. Distribution of claudins in human sweat glands. Immunolocalization of claudin-1, -3, -4, -10, and -15 (green) in the sweat ducts (upper panels; red, ZO-2) and the secretory coils (middle and lower panels; red, ZO-2 or SMA) from healthy control individuals. Scale bars = 20 μ m. Far right panel shows a schematic diagram of claudin expression (green). SMA, smooth muscle actin.

co-localized with ZO-2 and claudin-3 localized to the paracellular space around luminal cells of the sweat duct and the secretory coil (Figure 2). The co-localization of ZO-2 with claudin-3 indicates that TJs throughout the sweat gland apparatus comprise claudin-3. Contrary to our expectations, claudin-1 did not co-localize with occludin, ZO-1, or ZO-2 and thus may not be a component of these TJs (Figure 2). Meanwhile, claudin-10 and claudin-15, which regulate cation transport across TJs (Tamura and Tsukita, 2014), were expressed distinctively in the paracellular space of the secretory coil and in the lumen of the sweat duct, respectively, and colocalized with ZO-2 (Figure 2). A proportion of secretory coil cells, but not all, expressed claudin-10 (Figure 2). In AD skin lesions ($n = 5-10$), the expressions of claudin-1, -3, and -15 were apparently decreased compared with those in control skin ($n = 4-6$) (Figure 3a and b). By contrast, in psoriasis skin lesions, claudin-3 was normally expressed in both the sweat duct and the secretory coil (see Supplementary Figure S3 online). On the basis of these findings, we focused on claudin-3, which is expressed by luminal cells throughout the sweat gland apparatus, and hypothesized that its decreased expression leads to sweat leakage in AD skin lesions.

Claudin-3 prevents leakage of sweat in murine sweat gland apparatus

To test the hypothesis that claudin-3 prevents sweat leakage, we turned to an animal model. We first characterized the expression of claudins in the murine sweat gland apparatus. Claudin-1, -2 and -15 were not expressed in the murine sweat gland apparatus (see Supplementary Figure S4 online). Although claudin-3 and claudin-4 localized to the sweat apparatus as in humans, claudin-10 was found to localize to

the paracellular space around sweat duct basal cells. The expression of claudin-3 was found throughout the sweat gland apparatus (Figure 4a and b). To investigate the function of claudin-3 in preventing sweat leakage, we visualized the counterflow of a tracer (biotin solution) from the skin surface to the lumen of the sweat gland apparatus of wild-type (*Cldn3*^{+/+}) and claudin-3-knockout (*Cldn3*^{-/-}) mice (Figure 5a). In *Cldn3*^{+/+} mice, the tracer was retained inside the lumen, whereas the tracer extended beyond the TJ in the paracellular space around luminal cells of the sweat duct in *Cldn3*^{-/-} mice despite the stable expression of claudin-4 (Figure 4c and 5b, and see Supplementary Movies S1 and S2 online). Additionally, the tracer accumulated in the paracellular spaces of corneocytes and epidermal granular cells in *Cldn3*^{-/-} mice (Figure 5c).

These results suggest that TJs expressing claudin-3 are responsible for maintaining the water barrier in the sweat gland apparatus. Furthermore, a starch-iodine reaction showed that sweat volumes on the skin surface after sudomotor stimulation were decreased in *Cldn3*^{-/-} mice ($n = 6$ /genotype) (Figure 5d). This finding was confirmed by the dynamic observation of sweat flow using optical coherent tomography (Figure 5e), and sweat spouts were more abundant in *Cldn3*^{+/+} mice after ACh administration than in *Cldn3*^{-/-} mice ($n = 7$ /genotype) (Figure 5e).

Increased sweat leakage with dose-dependent decrease in claudin-3

The decrease in claudin-3 expression in AD skin lesions likely increases the permeability of sweat glands, resulting in sweat leakage. In support of this idea, claudin-3-heterozygous (*Cldn3*^{+/-}) mice showed a moderate influx of the tracer into the paracellular space around luminal cells

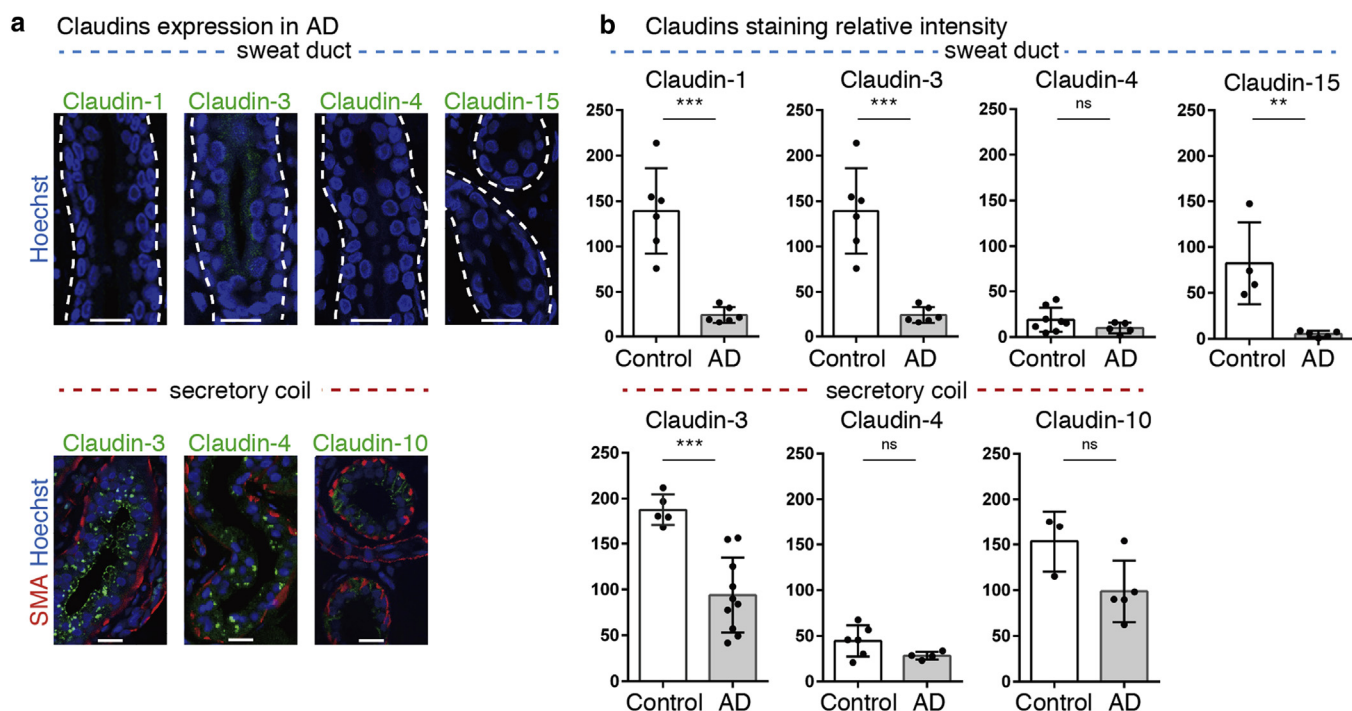


Figure 3. Patients with AD showed decreased expression of claudins in sweat glands. (a) Immunofluorescent images of claudin-1, -3, -4, and -15 (green) in the sweat ducts (upper panels) and claudin-3, -4, and -10 in the secretory coils (lower panels) from AD patients. (b) Relative intensities of claudin-1, -3, -4, and -15 immunoreactivity in the sweat duct and of claudin-3, -4, and -10 in the secretory coil. The intensities of positive signals for claudins were analyzed semiquantitatively in healthy control individuals (white bars; $n = 4-6$) and in patients with AD (gray bars; $n = 5-10$). Scale bars = 20 μ m. Error bars indicate standard error of the mean. ** $P < 0.01$, *** $P < 0.001$; ns, not significant by unpaired Student t test. SMA, smooth muscle actin.

(Figure 6a, and see Supplementary Figure S5 online for relative expression levels). We next immersed the footpads of *Cldn3*^{+/+} mice in a solution containing histamine, which is known to down-regulate the expression of claudins (Gschwandtner et al., 2013). As expected, the expressions of *Cldn3* and *Cldn7* mRNAs in the skin were reduced by histamine ($n = 5$) (Figure 6b). This treatment also resulted in leakage of the tracer into the paracellular space (Figure 6c).

As with histamine-mediated dermatoses, urticaria also occurs in patients with acquired idiopathic generalized anhidrosis (AIGA). Sweat glands of patients with this type of cholinergic urticaria, as well as those with AD, exhibit an ectopic distribution of DCD and decreased claudin-3 expression (see Supplementary Figure S6 online).

DISCUSSION

The results of this study show a crucial role of claudin-3 in preventing the leakage of sweat from the sweat gland apparatus. Thus, claudin-3 likely contributes to the pathogenesis of AD (Figure 6d).

Although the genetic background is an important etiology of atopic diathesis, past genome-wide association studies did not identify claudin-3 (7q11.23) as an atopy locus (Esparza-Gordillo et al., 2009; Hirota et al., 2012; Paternoster et al., 2011; Sun et al., 2011; Weidinger et al., 2013). Thus, we assumed that conditions that transiently reduced claudin-3 expression in the sweat gland apparatus in AD skin might cause sweat leakage. We found that when the luminal face of the sweat gland was exposed to histamine, a decrease in claudin-3 expression and sweat leakage

was observed. However, other conditions known to decrease the expression of claudin, such as intradermally administered histamine (Gschwandtner et al., 2013), topically applied mite antigen (Wan et al., 1999), and hapten-induced chronic dermatitis (Yokouchi et al., 2015), did not alter the permeability of the sweat gland apparatus in *Cldn3*^{+/+} mice (see Supplementary Figure S7 online). These results suggest that histamine exposure to the exterior of the skin but not from the interior contributes to the decrease in claudin-3 in the AD sweat gland apparatus. One candidate for this source of histamine external to the sweat gland in AD pathogenesis might be keratinocytes, because they synthesize histamine de novo via the enzyme histidine decarboxylase, which is up-regulated in AD (Gutowska-Owsiak et al., 2014; Shimizu et al., 2015). Furthermore, AD keratinocytes exposed to lipopolysaccharides, house dust mites, TNF- α , and thymic stromal lymphopoietin produce large amounts of histamine (Gutowska-Owsiak et al., 2014). Thus, we speculate that the up-regulated histamine in AD keratinocytes might reach the skin surface and be exposed to the lumen of the sweat gland apparatus to decrease the expression of claudin-3.

Another impact of histamine is that it decreases sweating by impairing sweat expulsion from the sweat gland (Matsui et al., 2014) and induces an urticarial reaction (Tokura, 2016). The pathogenetic relationship between cholinergic urticaria and AIGA has been explained by the responsiveness to ACh (Tokura, 2016) or to histamine (Suma et al., 2014). Our findings reported here suggest that the leakage of sweat via decreased claudin-3 expression may partly explain the

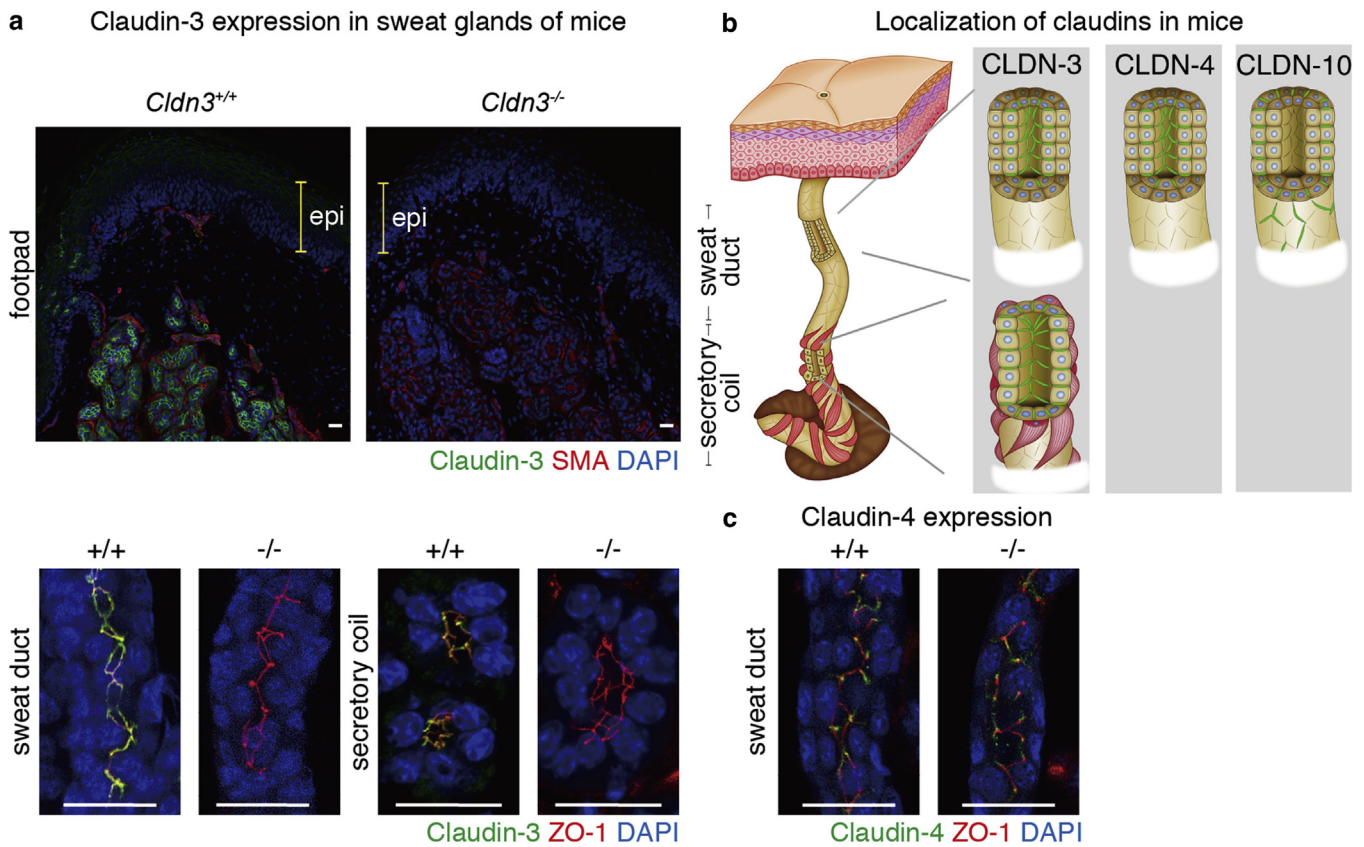


Figure 4. Claudin-3 expression in sweat glands of mice. (a) Top panels: immunofluorescent images for claudin-3 (green), SMA (red), and DAPI (blue) in sweat glands of footpads of wild-type (*Cldn3*^{+/+}) and claudin-3-knockout (*Cldn3*^{-/-}) mice. Bottom panels: high-magnification images of sweat gland apparatuses. Scale bars = 20 μ m. (b) Schematic diagram of claudin expression (green) in sweat glands of mice. (c) Representative immunofluorescent images for claudin-4 (green), along with ZO-1 (red) and DAPI (blue) in the sweat ducts of *Cldn3*^{+/+} and *Cldn3*^{-/-} mice. Scale bars = 20 μ m. epi, epidermis; SMA, smooth muscle actin.

tingling sensation upon sweating that is common among AD, cholinergic urticaria, and AIGA.

Another important finding from this study is that claudins are differentially localized and may have distinct roles in the human sweat gland apparatus. The localization of claudin-10 and claudin-15 suggests that these proteins may be involved in excreting and reabsorbing sodium through the sweat gland apparatus. However, further studies are needed to clarify the distinctive functions of claudins, especially of claudin-1, -10, and -15, which are differentially localized between human and murine sweat gland apparatuses. These differences may be related to the functions that sweat glands play in the two species. It is assumed that human sweat glands evolved to address the pleiotropic functional requirements of sweat, whereas murine sweat glands are needed simply to meet a limited function, such as providing slip resistance for the feet.

MATERIALS AND METHODS

Quantitative sudomotor axon reflex test (QSART)

To test the ACh-induced sweating volume, a QSART was conducted as described previously (Kijima et al., 2012; Takahashi et al., 2013). Twenty patients with AD and 14 healthy control individuals participated in this study. In short, the subjects were asked to rest for 20 minutes before the QSART. QSART was performed at a temperature of 20°C and at a humidity of 60%. The sweat capsule of the QSART

consisted of the outer and central compartments. ACh (100 mg/ml) was iontophoretically applied to the skin from the outer compartment, which directly stimulates the underlying sympathetic nerves. Sweat glands of the skin under the central compartment were activated via axon reflux. Sweating volume was measured at the central compartment during the first 5 minutes of the iontophoresis. The QSART was performed on the forearm.

Antibodies

The following antibodies were used: claudin-3 (rabbit, 34-1700; Invitrogen, Waltham, MA), claudin-1 (rabbit, 51-9000; Invitrogen), claudin-2 (rabbit; S. Tsukita Laboratory, Osaka, Japan), claudin-4 (rabbit; Invitrogen), claudin-10 (rabbit, 41-5100; Invitrogen), claudin-15 (rabbit; S. Tsukita Laboratory), DCD (mouse, sc-33656; Santa Cruz Biotechnology, Dallas, TX), smooth muscle actin (mouse, 1A4; Dako, Santa Clara, CA), ZO-1 (mouse, T8-754; S. Tsukita Laboratory), ZO-2 (goat, sc-8148; Santa Cruz Biotechnology), occludin (rat, MOC37; S. Tsukita Laboratory), and CD3 (mouse, M7254; DAKO). Rabbit-, goat-, rat-, and mouse-specific secondary antibodies labeled with Alexa Fluor 488, 568, or 647 (Invitrogen) were used. Alexa Fluor 568-conjugated streptavidin (Invitrogen) was used to detect biotinylated proteins. Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA) or DAPI was used to detect cell nuclei.

Histological analysis of human skin samples

Skin samples from 10 patients with AD, 4 patients with psoriasis, 3 patients with AIGA, and 2 patients with AIGA accompanied by

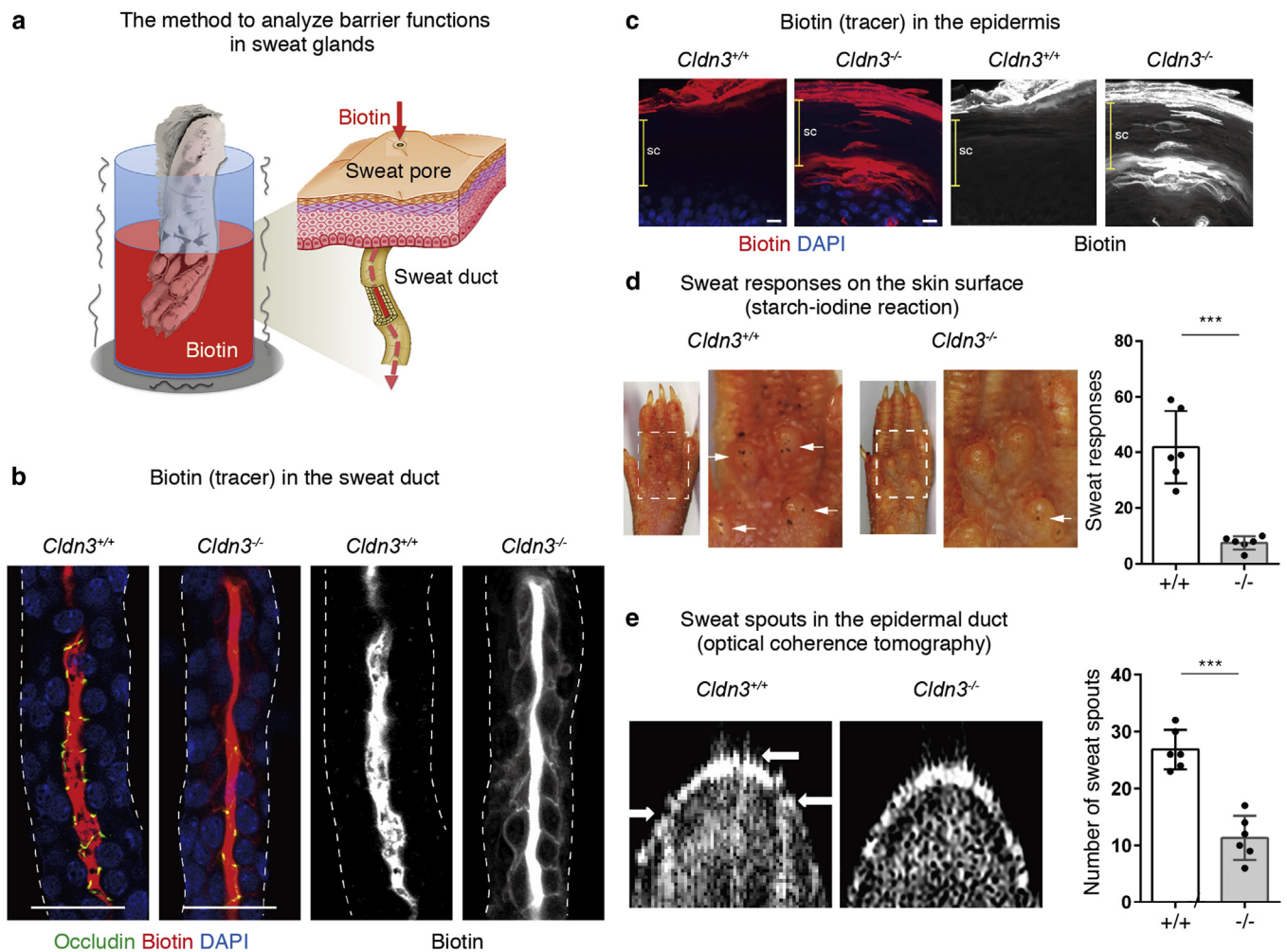


Figure 5. *Cldn3*^{-/-} mice developed leakage of sweat and decreased sweating on the skin surface. (a) Schematic of the method to visualize the lumen of the sweat gland. (b) Localization of biotin (red) in sweat ducts. Green indicates occludin; blue indicates DAPI. Scale bars = 20 μ m. (c) Localization of tracer in the epidermis. Scale bars = 10 μ m. (d) Evaluation of skin surface sweat by starch-iodine reaction (arrows). Graph indicates average numbers of sweat responses (n = 6/genotype). (e) Evaluation of sweating on the epidermal sweat duct (arrows) visualized by optical coherence tomography. Graph indicates average numbers of sweat spouts in each footpad (n = 7/genotype). Error bars indicate standard error of the mean. ***P < 0.001 by unpaired Student t test. sc, stratum corneum.

cholinergic urticaria were used in this study. Six preserved and trimmed skin samples obtained by the excision of benign skin tumors (e.g., epidermal cyst, lipoma, etc.) were used as the normal control skin. All skin samples used in this study had been stored after written informed consent was obtained. AD skin samples were obtained from the eczematous lesions of the trunk or the extremities. The other samples were also obtained from the trunk or the extremities. Paraffin-embedded skin samples (4- μ m thick) were processed as described previously (Tokumasu et al., 2016) (see Supplementary Materials and Methods online).

The numbers of mast cells around sweat glands (Figure 1d, and see Supplementary Figure S2a) were counted from healthy control samples (n = 5 from 23 microscopic fields) and AD patients (n = 5 from 32 microscopic fields). The numbers of T lymphocyte cells around sweat glands (Figure 1e, and see Supplementary Figure S2b) were also counted from healthy control samples (n = 3 from seven microscopic fields) and AD patients (n = 4 from nine microscopic fields).

For the semiquantitative analysis, 10 regions were defined per raw image. By using ImageJ software (National Institutes of Health,

Bethesda, MD), the mean fluorescence intensity for each image was measured after subtracting the background.

Mice

Cldn3-deficient mice were generated by Tsukita et al. (M Imasato, personal communication). Mice were maintained on the background of C57BL/6J and were used between 6 and 10 weeks of age.

Sweat response

According to the method described in a previous report (Matsui et al., 2014), a 3.5% (weight/volume) iodine (Sigma-Aldrich, St. Louis, MO)/ethanol solution and then a 10% (weight/volume) starch (Wako, Osaka, Japan)/mineral oil (Nacalai Tesque, Kyoto, Japan) solution were first applied to the footpads of anesthetized mice. Subsequently, 50 μ g ACh (pilocarpine hydrochloride; Junsei Chemical, Tokyo, Japan) in 20 μ l phosphate buffered saline was injected subcutaneously. Five minutes after the injection, the footpads of mice were photographed. Finally, the numbers of black dots induced by the starch-iodine reaction were counted.

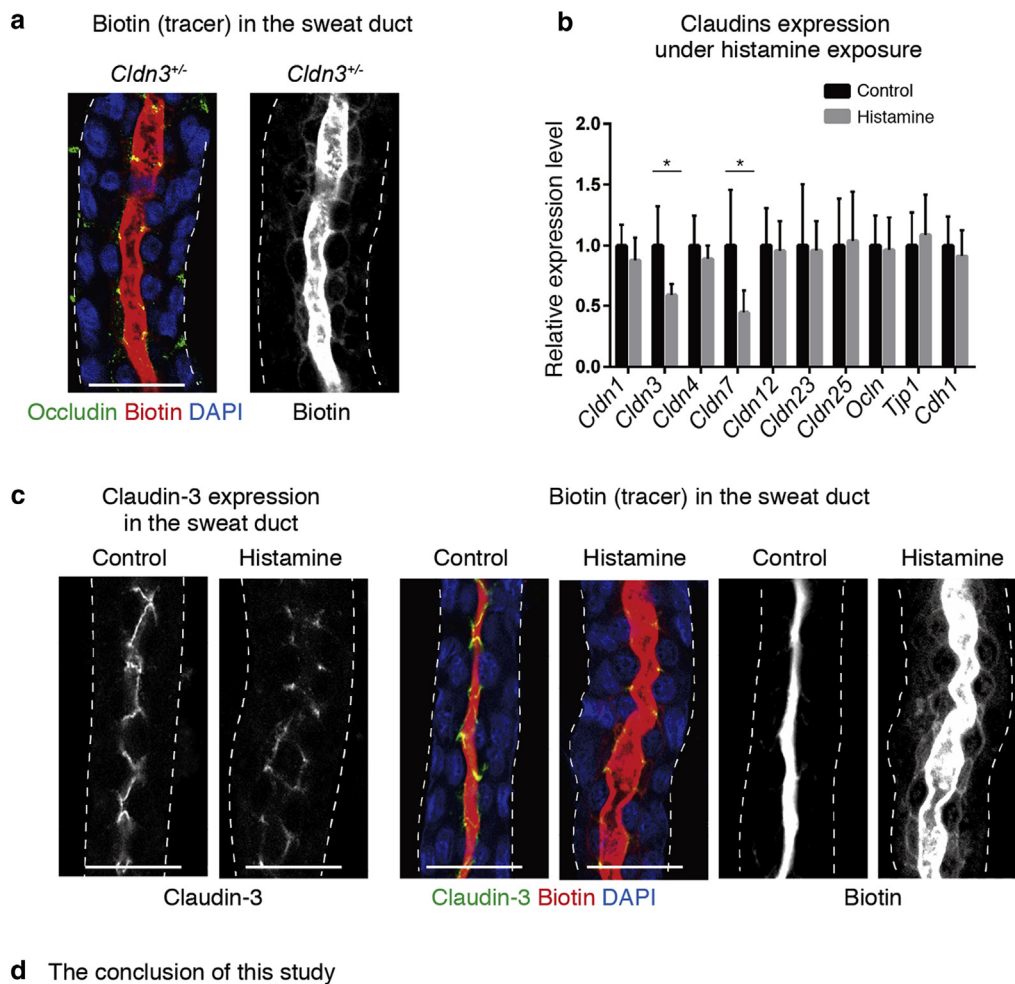
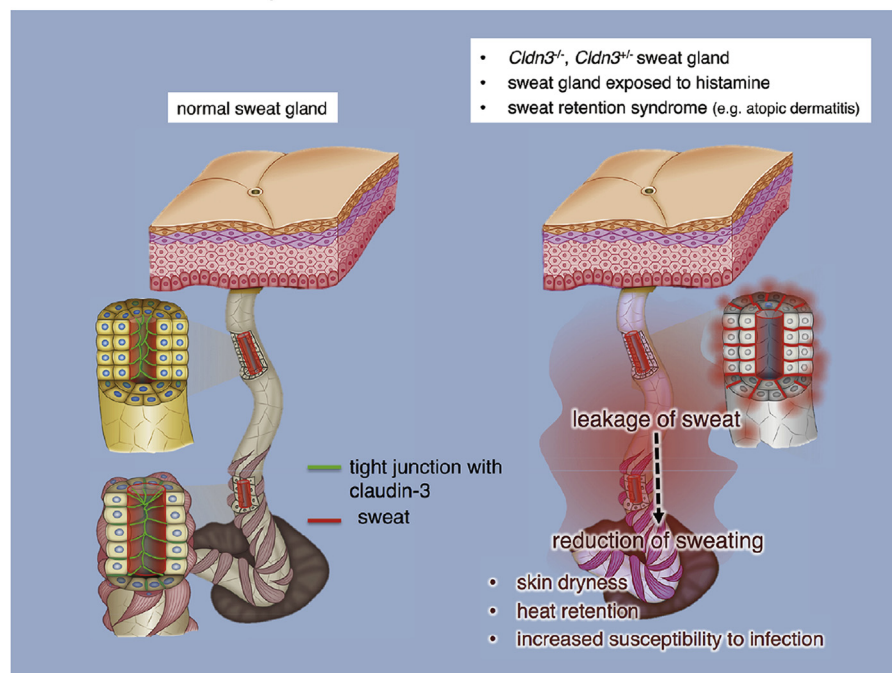


Figure 6. Decreased claudin-3 induced by histamine leads to sweat leakage. (a) Immunofluorescence image of the barrier assay in the sweat duct of a claudin-3 heterozygous (*Cldn3^{+/-}*) mouse. Scale bars = 20 μ m. (b) mRNA expression levels of several claudins in phosphate buffered saline-treated (control, black bars) and histamine-treated (gray bars) hind paws of *Cldn3^{+/-}* mice (n = 5/each). Error bars indicate standard error of the mean. **P* < 0.05 by unpaired Student *t* test. (c) Images of claudin-3 in the sweat ducts (left) and in the biotin assay (right) of phosphate buffered saline-treated (control) and histamine-treated hind paws. Scale bars = 20 μ m. (d) Schematic diagram of the conclusions of this study.



Optical coherence tomography

According to a previous report, the movement of sweat in the epidermal sweat ducts was observed by optical coherence tomography,

which can create images by detecting a large difference in the refractive indices between sweat and the surrounding keratinocytes. The dynamic images of ACh-induced sweating in the *x/z*-plane of

mouse feet were taken using B-mode optical coherence tomography. The administrations were conducted in the same way as for the starch-iodine reaction. The numbers of sweat spouts in epidermal sweat ducts of one footpad were counted 2 minutes after a subcutaneous injection of ACh (Matsui et al., 2014).

Barrier function assays in sweat glands

To investigate the water barrier function of TJs in sweat gland lumens, the localization of biotin solution (as a contrast dye) inside the lumen was observed, as illustrated in Figure 5a. The hind limbs of mice were amputated, and the cut sections were covered with Parafilm (Bemis, Neena, WI). The amputated limbs were immersed in the biotin solution (10 mg/ml of EZ-Link Sulfo-NHS-LC-Biotin [Pierce Chemical Co., Dallas, TX] in phosphate buffered saline containing 1 mmol/L CaCl₂) and incubated at 4°C for 2 days with vibration by an MT-360 microtube mixer (Tomy, Katsushika, Japan). In this way, the biotin solution flowed from the skin surface into the lumens of the sweat ducts. After the incubation, the hind paws were dissected and incubated in 5 mmol/L EDTA in phosphate buffered saline at 37°C for 4 hours to peel the epidermis from the dermis. When the epidermis was peeled off, the attached sweat gland apparatuses could be pulled from the dermis. As a result, most of the sweat gland apparatuses broke off, but the sweat ducts, which connect to the epidermis, were recovered successfully. Therefore, we could observe the lumens of sweat ducts but not the lumens of sweat glands with this method. The epidermal sheets were fixed in 95% methanol at -20°C for 10 minutes and incubated with the blocking buffer described. Then, the epidermal sheets were incubated with primary antibodies at 37°C for 2 hours and then with secondary antibodies (Alexa Fluor 568-conjugated streptavidin and DAPI) at 37°C for 2 hours. The staining procedure was conducted with stirring at 100 r.p.m. The epidermal sheets were mounted in a whole-mount manner. Images were obtained by a Zeiss LSM 710 confocal laser-scanning microscope (Carl Zeiss Micro-Imaging, Jena, Germany).

In Supplementary Movies S1 and S2, these whole-mounted tissues were observed by inverted two-photon excitation microscopy. The imaging system was composed of a two-photon inverted microscope (A1R-MP; Nikon, Tokyo, Japan) equipped with a water multi-immersion objective lens (Plan Fluor; 0.75 numerical aperture; Nikon) and a laser (Chameleon Vision II Ti:Sapphire; Coherent, Santa Clara, CA) tuned to 840 nm. Fluorescent signals were detected through band-pass filters at 492/SP nm (for DAPI), 525/50 nm (for Alexa Fluor 488), and 575/25 nm (for Alexa Fluor 568). Raw imaging data were processed with NIS-Elements (Nikon).

For the histamine assay, mouse hind limbs were immersed in a 6 µg/µl histamine diphosphate and biotin solution. For the hapten-induced dermatitis model, 20 µl of 2% oxazolone (Sigma-Aldrich) in acetone/olive oil (4:1) was applied (to sensitize mice) to shaved abdomens 5 days before the first elicitation. Then, the application (20 µl of 2% oxazolone) was repeated on the paws 4 times per week for 2 weeks. For mite antigen application, 30 mg of *Dermatophagoides farinae* body extract ointment (Biostir AD, Biostir, Kobe, Japan) was applied to the paws, and then the paws were occluded by Parafilm for 1 day. For the histamine-injection model, 6-µg/µl histamine diphosphate diluted in phosphate buffered saline was subcutaneously applied to hind paws for 4 consecutive days.

Isolation of human sweat glands and dermis

Human sweat glands and dermis were isolated from skin samples (10-µm sections) using a laser microdissection system (LMD7000, Leica Microsystems, Wetzlar, Germany).

Real-time PCR

The footpad skins of mice were dissected and collected under a stereomicroscope. Total RNA of murine footpad skins was isolated with an RNeasy kit (Qiagen, Dusseldorf, Germany), and that of dissected human sweat glands and dermis was isolated with an RNeasy FFPE kit (Qiagen). The RNA was reverse transcribed to cDNA using SuperScript II (Invitrogen) reverse transcriptase and was measured using SYBR green. The gene expression was normalized to the GAPDH expression level and calculated using the 2^{-ΔCT} method (Tokumasu et al., 2016).

Statistics

All data are presented as mean ± standard error of the mean. *P*-values were determined by two-tailed Student *t* tests or by one-way analysis of variance. *P* less than 0.05 was considered to be statistically significant. The statistical analyses were conducted with using GraphPad Prism 6 software (GraphPad, La Jolla, CA).

Study approval

Clinical investigations, including skin biopsies, were performed after obtaining written informed consent. The study protocol was approved by the ethics committee of Osaka University Graduate School of Medicine (institutional approval no. 13429). This study was performed in adherence with the Declaration of Helsinki. All animal experiments were performed in accordance with protocols approved by the animal studies committee of Osaka University School of Medicine.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

KY, H. Murota, IK, and ST designed the experiments. KY, H. Murota., ST, and AT conducted the experiments and analyzed the data. H. Miyata and MO performed the optical coherent tomography. JK and MI performed the two-photon microscopy. KY and H. Murota wrote the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2017.11.040>.

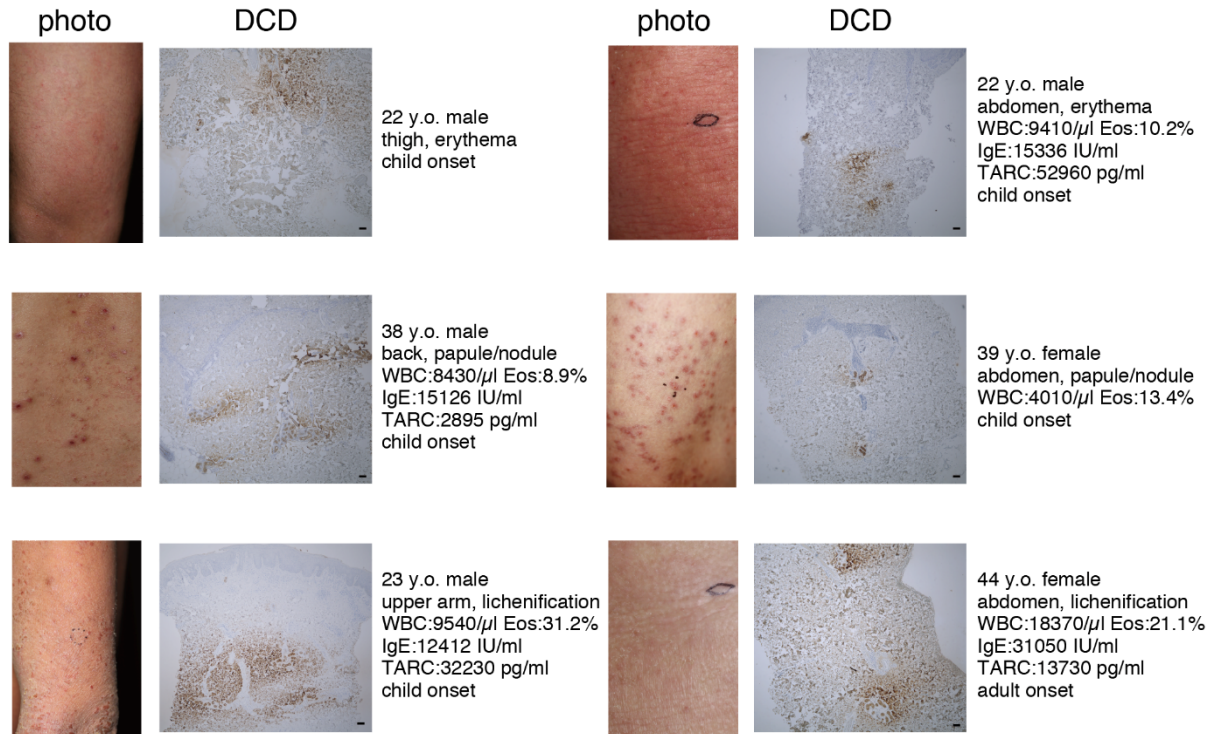
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SUPPLEMENTARY MATERIAL

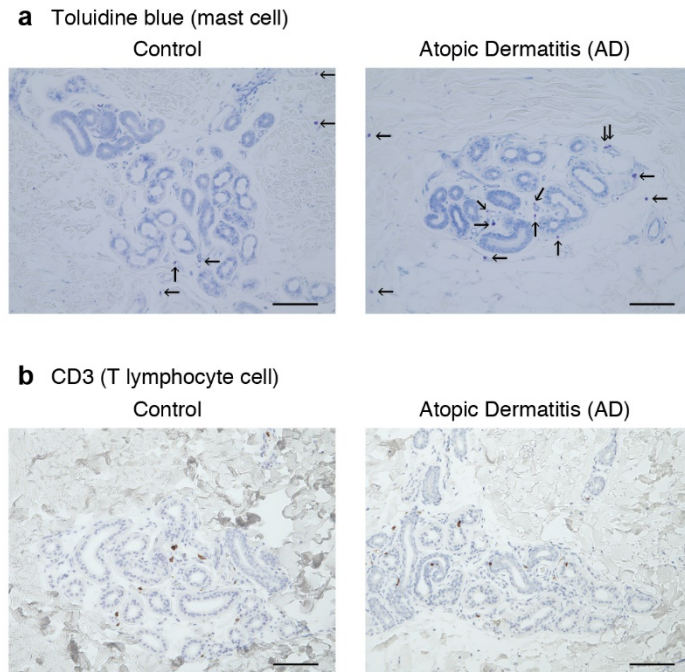
Supplementary Figure S1



Supplementary Figure S1. Diverse skin manifestations in AD patients showing ectopic localization of DCD.

The skin manifestation (left), localization of DCD (middle), and clinical information (right) of AD patients. Note that ectopic localization of DCD was observed in diverse skin manifestations of AD patients, including those in erythema, papules, nodules, and lichenification. Scale bars: 100 μ m. AD, atopic dermatitis; DCD, dermcidin; y.o., years old; WBC, white blood cells; Eos, eosinophils; IgE, Immunoglobulin E, TARC, thymus, and activation-regulated chemokine.

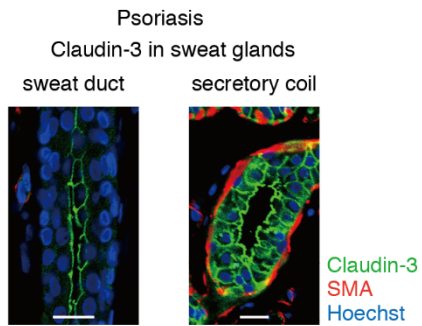
Supplementary Figure S2



Supplementary Figure S2. Increase in mast cells around sweat glands in patients with AD.

Representative images of the infiltration of mast cells (a) and T lymphocyte cells (b) around sweat glands of healthy controls (left) and AD patients (middle). Mast cells were stained with toluidine blue, and the numbers of cells with metachromasia (purple) were counted (see Figure 1d). Scale bars: 100 μ m. AD, atopic dermatitis.

Supplementary Figure S3

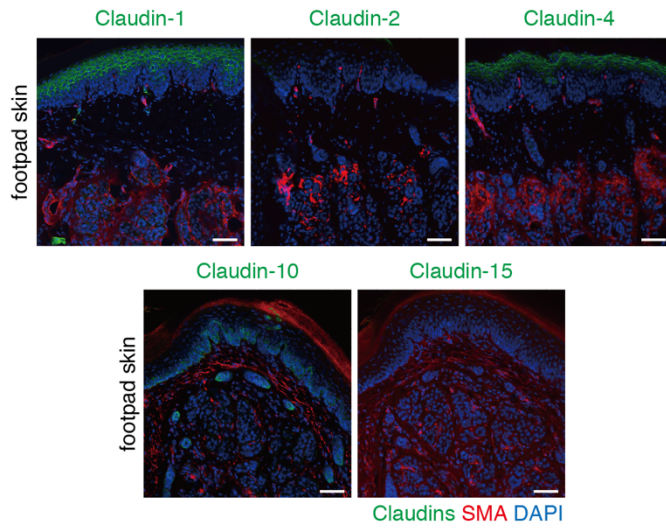


Supplementary Figure S3. Expression of Claudin-3 is normal in patients with psoriasis.

Immunofluorescence images of claudin-3 in the sweat duct (left) and the secretory coil (right).

Scale bars: 20 μ m. SMA, smooth muscle actin.

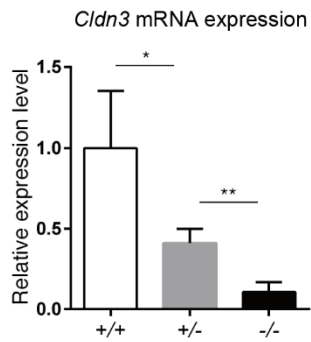
Supplementary Figure S4



Supplementary Figure S4. Claudin expression in mouse footpads (including sweat glands).

Immunofluorescence images of claudin-1, -2, -4, -10, and -15 (green), SMA (red), and DAPI (blue) in sweat glands of mouse footpads. Scale bars: 100 μ m. SMA, smooth muscle actin; DAPI, 4'6'-diamidino-2-phenylindole.

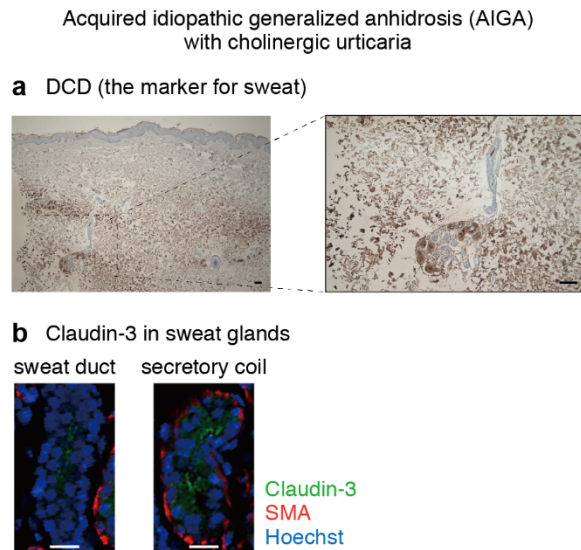
Supplementary Figure S5



Supplementary Figure S5. *Cldn3* mRNA expression in each genotype.

Relative *Cldn3* mRNA expression in footpads of wild-type (*Cldn3*^{+/+}), claudin-3-heterozygous (*Cldn3*^{+/-}), and claudin-3-knockout (*Cldn3*^{-/-}) mice ($n = 3/\text{genotype}$). Here, the mean value of *Cldn3*^{+/+} was set as 1.0. Error bars, SEM. * $P < 0.05$ and ** $P < 0.01$ by one-way ANOVA.

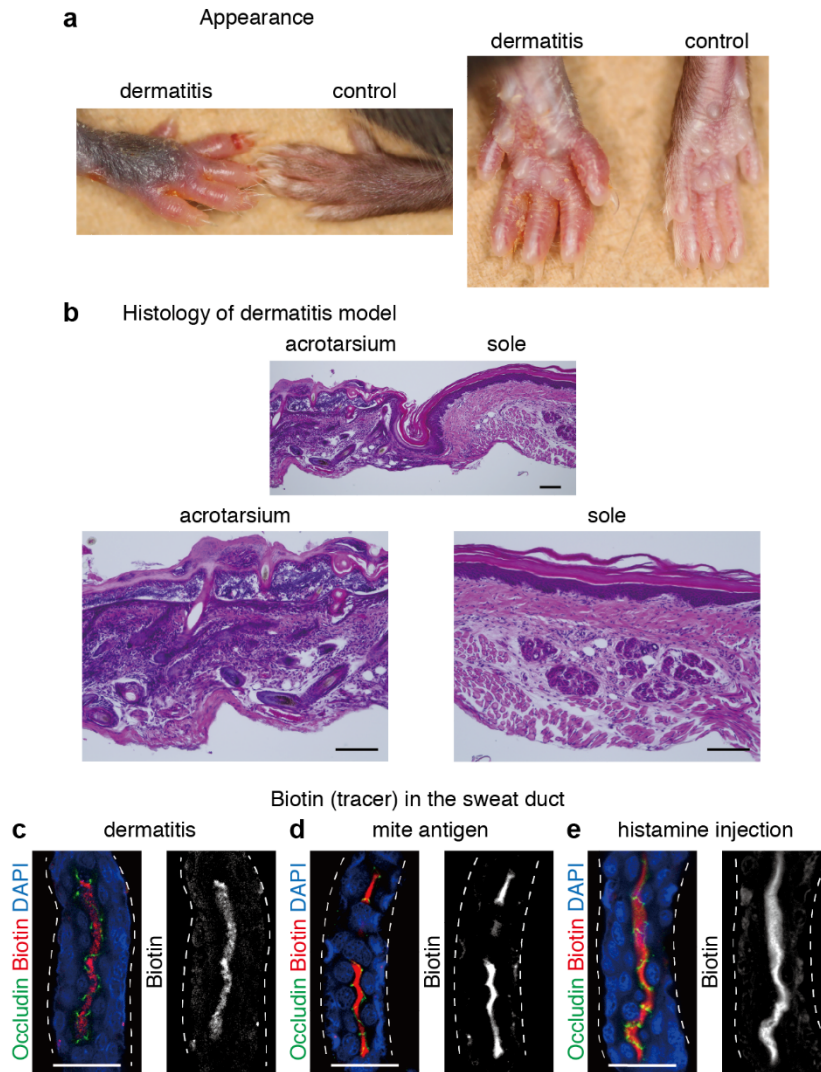
Supplementary Figure S6



Supplementary Figure S6. Patients with AIGA with cholinergic urticaria showed abnormal distribution of DCD and decreased expression of claudin-3 in sweat glands.

(a) Representative image of localization of DCD. Scale bars: 100 μ m. (b) Immunofluorescence images of claudin-3 in the sweat duct (left) and the secretory coil (right) of a patient with AIGA. Scale bars: 20 μ m. AIGA, acquired idiopathic generalized anhidrosis; DCD, dermcidin; SMA, smooth muscle actin.

Supplementary Figure S7



Supplementary Figure S7. Hapten-induced dermatitis, mite antigen, and injected histamine did not alter the paracellular barrier function in the sweat duct of *Cldn3*^{+/+} mice.

(a) Appearance of the oxazolone-induced dermatitis model and vehicle-treated control hind paws. Note that paws of the dermatitis model are reddish and swollen with desquamation on the border of the sole and the acrotarsium. (b) Histological analysis of dermatitis of the acrotarsium and the sole. Note that many inflammatory cells infiltrated into the dermis and epidermis of the acrotarsium, while there was almost no inflammatory infiltration in the planter skin (containing

sweat glands). This indicates that dermatitis cannot be induced in the sole but can in the acrotarsium. Scale bars: 100 μm . Localization of biotin (tracer, red) in the sweat ducts of the oxazolone-induced dermatitis model (c), the mite antigen applied model (d), and the histamine injected model (e). Green: occludin (TJs); blue: DAPI. Scale bars: 20 μm .

LEGENDS FOR SUPPLEMENTARY MOVIES

Supplementary Movie 1. 3D-image observations by inverted two-photon excitation microscopy in barrier function assays (see Figure 5a) show the localization of the fluorescent tracer (red) in the sweat duct of a *Cldn3*^{+/+} mouse.

Green: occludin (TJs). Blue: DAPI. Scale bars: 20 μm.

Supplementary Movie 2. 3D-image observations by inverted two-photon excitation microscopy in barrier function assays (see Figure 5a) show the localization of the fluorescent tracer (red) in the sweat duct of a *Cldn3*^{-/-} mouse.

Green: occludin (TJs). Blue: DAPI. Scale bars: 20 μm.

SUPPLEMENTARY MATERIALS AND METHODS

Histological analysis of human skin samples

All skin samples were fixed in a 4% formaldehyde solution at 4°C overnight. For staining, paraffin-embedded skin samples were sectioned (4 µm), deparaffinized with xylene, and dehydrated by a graded ethanol series. Some sections were stained with hematoxylin and eosin (HE). The other sections underwent antigen retrieval with 10 mM Tris-1 mM EDTA (pH 9.0) at 120°C for 16 min or with 10 mM sodium citrate buffer (pH 6.0) at 120°C for 11 min. The samples were blocked with Protein Block Serum-Free solution (Dako) for 15 min, incubated with primary antibodies at room temperature (RT) for 1 h, and then washed three times with phosphate-buffered saline (PBS). For 3,3'-diaminobenzidine (DAB) staining, the samples were incubated with Dako LSAP + System-AP (DakoCytomation) at RT for 25 min and were washed with PBS, treated with Dako ChemMate Envision kit HRP (DAB), washed with distilled water, counterstained with hematoxylin, washed again with distilled water, and mounted with Clear Plus (Falma). For immunofluorescence staining, the samples were incubated with secondary antibodies at RT for 30 min and washed with PBS, and then were counterstained with Hoechst 33342 at RT for 10 min, washed with PBS, and mounted in Prolong Gold (Invitrogen). The mounted specimens were observed under a fluorescence microscope (BZ-9000; Keyence) equipped with 4×, 10×, and 20× objectives or a Zeiss LSM710 laser-scanning confocal microscope (Carl Zeiss MicroImaging) equipped with 20× and 63× objectives.

Histological analysis of murine skin samples

Footpad tissue specimens were embedded in OCT (Tissue Tek) and frozen, and were then cryosectioned (10 µm), dried for 30 min, and fixed in 95% methanol at -20°C for 10 min. Sections

were washed with PBS and then blocked at RT for 1–2 h with PBS containing 1% fetal bovine serum and 1% NaCl. For labeling with mouse antibodies, we used M.O.M basic kits (Vector laboratories). Primary antibodies in blocking buffer were incubated at 4°C overnight. After rinsing with PBS, secondary antibodies (conjugated with Alexa Fluor 488, 568, or 647) and DAPI were incubated at RT for 1 h. Slides were rinsed with PBS and then mounted with Prolong Gold (Invitrogen). Fluorescent images were obtained using a Zeiss LSM 710 confocal laser-scanning microscope (Carl Zeiss MicroImaging).