



Studies on the Pathophysiological Role of TRPV3 in Itchy Dermatitis

Kasai, Erika

(Degree)

博士 (理学)

(Date of Degree)

2014-03-25

(Date of Publication)

2016-03-25

(Resource Type)

doctoral thesis

(Report Number)

甲第6125号

(URL)

<https://hdl.handle.net/20.500.14094/D1006125>

※ 当コンテンツは神戸大学の学術成果です。無断複製・不正使用等を禁じます。著作権法で認められている範囲内で、適切にご利用ください。



Doctoral Dissertation

Studies on the Pathophysiological Role of
TRPV3 in Itchy Dermatitis

(そう痒性皮膚疾患における TRPV3 の病態生理学的役割に関する研究)

January 2014

Department of Biology, Graduate School of Science

Kobe University

Erika KASAI

Table of Contents

List of abbreviations	- 1 -
Introduction	- 2 -
1. Atopic dermatitis	- 2 -
2. DS- <i>Nh</i> mouse.....	- 3 -
3. TRPV3	- 4 -
Chapter 1 Impact of TRPV3 on spontaneous itch in a mouse model of dry skin- 7 -	
1. Materials and Methods	- 8 -
2. Results and Discussion	- 12 -
3. Summary.....	- 17 -
Chapter 2 Impact of TRPV3 on the development of allergic dermatitis as a dendritic cell modulator	- 18 -
1. Materials and Methods	- 20 -
2. Results and Discussion	- 23 -
3. Summary.....	- 32 -
Chapter 3 Impact of TRPV3 on acute skin inflammation and pruritogen induced itch-related response in mice.	- 34 -
1. Materials and Methods	- 35 -
2. Results and Discussion	- 37 -
3. Summary.....	- 43 -
Overview	- 45 -
Lists of Papers.....	- 47 -
Acnkowlegdements	- 48 -
References.....	- 49 -

List of abbreviations

AA; Arachidonic acid
AD; Atopic dermatitis
AEW; Acetone/diethylether and water
CD; Cluster of differentiation
CHS; Contact hypersensitivity
COX; Cyclooxygenase
DC; Dendritic cell
DNFB; 2,4-dinitrofluorobenzene
DRG; Dorsal root ganglia
FITC; Fluorescein isothiocyanate
G-CSF; Granulocyte-colony stimulating factor
GM-CSF; Granulocyte/macrophage-colony stimulating factor
IFN; Interferon
IL; Interleukin
KC; Keratinocyte chemoattractant (keratinocyte-derived chemokine)
LC; Langerhans cell
LT; Leukotrien
LO; Lipoxygenase
MCP; Monocyte chemoattractant protein
MIP; Macrophage inflammatory protein
MPO; Myeloperoxidase
NF- κ B; Nuclear factor-kappa B
NGF; Nerve growth factor
NOS; Nitric oxide synthase
PG; Prostaglandin
RANTES; Regulated on activation normal T cell expressed and secreted
SC; Stratum corneum
Sema3A; Semaphorin 3A
SP; Substance P
TEWL; Transepidermal water loss
TNF; Tumor Necrosis Factor
TPA; 12-O-tetradecanoylphorbol-13-acetate
TS; Tape stripping
TSLP; Thymic stromal lymphopoietin
TRPV3; Transient receptor potential vanilloid subfamily 3

Introduction

1. Atopic dermatitis

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease accompanied by severe itching, can lead to serious impairment of quality of life in patients. It is a major public health problem that affects at least 15% of children and is characterized by cutaneous hyperreactivity to environmental triggers (Geha, 2003; Leung and Bieber, 2003; Novak et al., 2003). Pathological studies revealed spongiosis, hyperkeratosis and parakeratosis in acute lesions and marked epidermal hyperplasia and perivascular accumulation of inflammatory cells in chronic lesions. AD has a complex etiology that involves abnormal immunological and inflammatory pathways that include defective skin barrier, dry skin, exposure to environmental agents, and neuropsychological factors (Fartasch, 1997; Pastore et al., 1997; Trautmann et al., 2000; Geha, 2003; Leung and Bieber, 2003; Novak et al., 2003; Howell et al., 2004). Prevalence of atopic dermatitis has increased in recent years, but the pathophysiology of this condition is only partly understood and available treatments for this disorder are only palliative.

Pruritus, regularly defined as an unpleasant sensation provoking the desire to scratch (Rothman, 1941), is an essential feature of AD. Itch-elicited scratching aggravates lesions of the skin, and the itch is intensified. Therefore, reduction of itching would be one of the most effective strategies for management of AD (Kimura and Miyazawa, 1989; Wahlgren, 1999; Ikoma et al., 2006). However H1 receptor (histamine H1R) antagonists are used widely in the treatment of AD, they do not have sufficient inhibitory effect against pruritus (Wahlgren et al., 1990). Various studies have clearly shown that the number of cutaneous nerve fibers is altered in atopic skin lesions (Tobin et al., 1992; Sugiura et al., 1997; Urashima and Mihara, 1998) and this is probably caused by nerve growth factor (NGF), which is released from keratinocytes (Albers et al., 1994; Pincelli et al., 1994). Histamine does not therefore play a major role in pruritus treatment of patients with human AD, but its neuronal mechanism

is not fully understood.

2. DS-*Nh* mouse

In 1976, DS-*Nh* mice were artificially selected on the basis of hairless phenotype from a colony of an inbred DS strain, which was developed from out-bred ddN stock obtained in 1954 from the Central Institute for Experimental Animals, Tokyo, Japan. DS-*Nh* mice develop spontaneous dermatitis when they are maintained in conventional conditions and are considered to be a model of human AD (Fig. 1), with the following features: (i) superantigen-producing *Staphylococcus aureus* can be isolated from skin lesions; (ii) serum levels of IgE, interleukin (IL)-4 and IL-13 are significantly increased; (iii) numbers of whole mast cells and cluster of differentiation (CD) 4 positive T cells are significantly increased; (iv) serum and tissue levels of NGF are significantly increased; and (v) itch-associated behavior becomes significantly severe (Hikita et al., 2002; Yoshioka et al., 2003 and 2006).

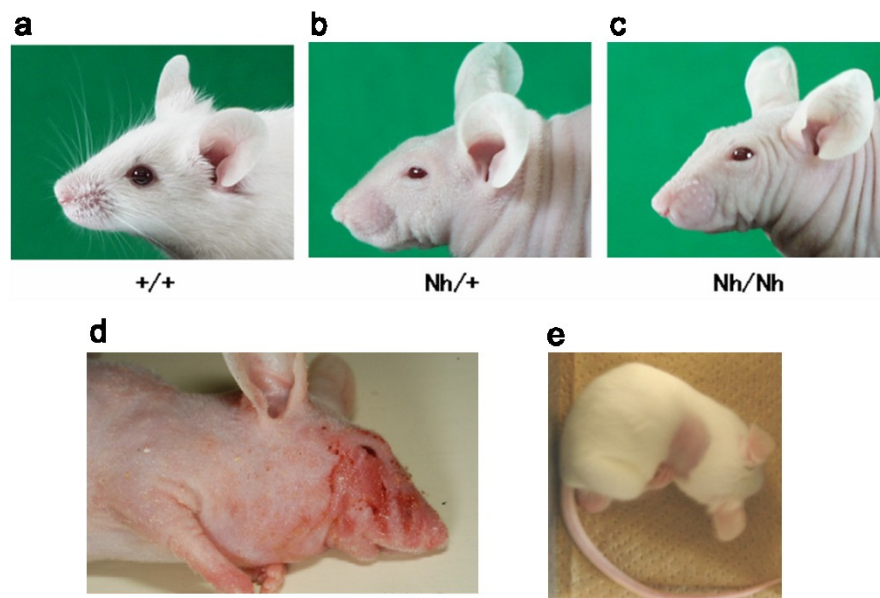


Fig. 1 Clinical features of DS and DS-*Nh* mice. (a) DS (+/+) mice kept under conventional conditions (b, c) and DS-*Nh* (Nh/+, Nh/Nh) mice kept under specific pathogen-free condition. (d) Spontaneous dermatitis of DS-*Nh* mice kept under conventional conditions at 20 weeks. (e) A typical example of itch-associated behavior (scratching behavior) in mice (e).

We have reported previously that DS-*Nh* mice have a one amino-acid substitution (Gly573 to Ser) in the transient receptor potential (TRP) cation channel subfamily V member 3 (TRPV3) and Gly573Ser substitution of TRPV3 is a gain-of-function mutation, as shown by the results of Ca^{2+} influx stimulated by temperature (Asakawa et al., 2006; Imura et al., 2007). Xiao et al. have speculated recently that Gly573Ser-substituted TRPV3 is active constitutively *in vivo* under normal physiological conditions (Xiao et al., 2008). Therefore, this mouse is thought to be good tool for investigating the association between activation of TRPV3 and development of dermatitis.

3. TRPV3

The TRP channel superfamily contains 28 mammalian members (27 in human) subdivided in 6 subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin) and TRPA (ankyrin) (Fig. 2). TRP ion channels are widely expressed in many different tissues and cell types, where they are involved in diverse physiological processes, such as sensation of different stimuli or ion homeostasis (Nilius et al., 2007). Most TRPs are non-selective cation channels, only few are highly Ca^{2+} selective, some are even permeable for highly hydrated Mg^{2+} ions (Nilius and Owsianik, 2011). Members of the TRPV family comprise 6 ion channels based on homology. Four groups can be identified: TRPV1/TRPV2, TRPV3, TRPV4, and TRPV5/6. Among the TRPVs channels, TRPV1, 2, 3, and 4 have striking temperature sensitivities with activation threshold at 43 °C, 52°C, 33°C and 30 °C, respectively (Voets, 2012). TRPV3, a channel that has high sequence homology and located in the same chromosome as TRPV1 was identified using expressed sequence tag (EST) clones by three different groups simultaneously (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002). After its identification, it appeared as a surprise that this channel was not highly expressed in the sensory dorsal root (DRG) or trigeminal (TG) ganglia (at least in rodents). Instead, TRPV3 was found to be most abundantly expressed in the skin, especially in epidermal and hair follicle keratinocytes (Peier et al.,

2002). *In vivo* study, it was shown that TRPV3 deficient mice exhibit loss of behavioral responses to innocuous and noxious heat but not in other sensory modalities (Moqrich et al., 2005) and TRPV3^{gly573ser} mice spontaneously develop hairless phenotype. It is also reported that TRPV3 is involved in normal hair development and epidermal barrier formation in mice (Cheng et al., 2010). With respect to skin physiology and pathophysiology, probably TRPV3 is the most important TRPV channel (Nilius and Biró, 2013). However, detailed functions of TRPV3 in various diseases and its therapeutic potential have not been fully understood yet.

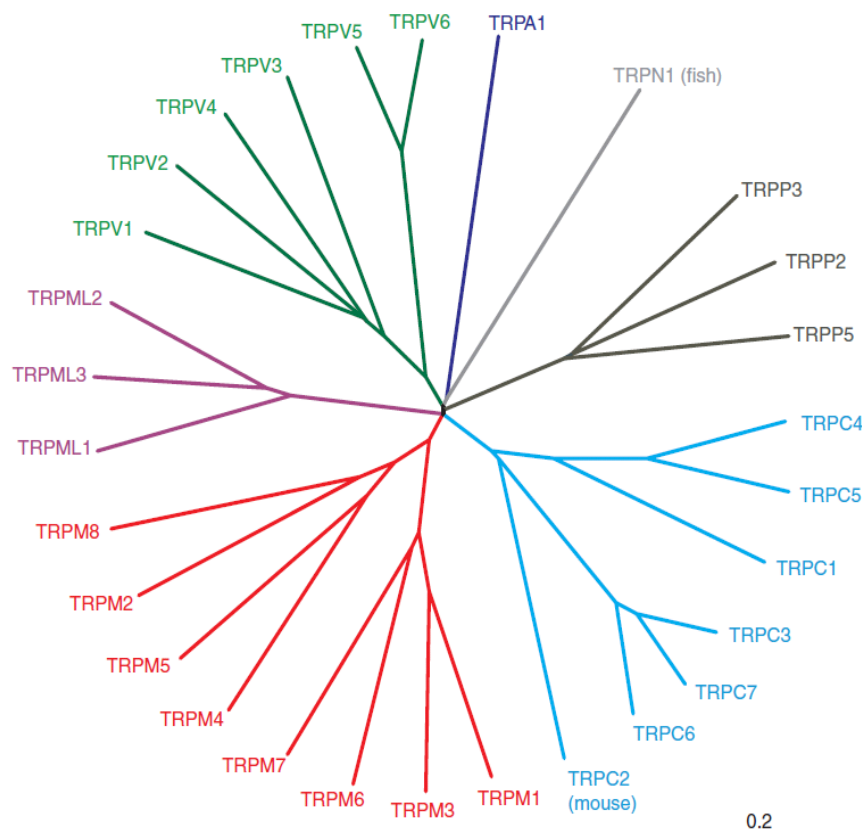


Fig. 2 A phylogenetic tree of human TRP channels. (Nilius and Owsianik, 2011)

Therefore, these studies are conducted with the aim of investigation of pathophysiological role of TRPV3 in the skin by analyzing phenotype in *DS-Nh* and TRPV3 knockout mice. Chapter 1 attempts to illustrate that TRPV3 is involved in a certain type of itch. Chapter 2 describes that TRPV3 regulates dendritic cell migration and release of thymic stromal lymphopoietin (TSLP) from keratinocytes. Chapter 3 shows that physiological response of TRPV3 to various stimulations by pruritogen and irritant to the skin.

Chapter 1 Impact of TRPV3 on spontaneous itch in a mouse model of dry skin

Itch is a common symptom in various forms of dermatitis characterized by dry skin, such as senile xerosis and atopic dermatitis. It is defined as an unpleasant sensation that provokes a desire to scratch and, since itch-elicited scratching aggravates lesions of the skin, the itch is intensified (Ikoma et al., 2006). Therefore, reduction of itching and scratching is an effective strategy for preventing aggravation of skin lesions and improving quality of life. Histamine is the best-known pruritogen and has been regarded as a main target for antipruritic therapies, but H1 receptor (histamine H1R) antagonists are often ineffective against certain kinds of pruritus including dry skin pruritus (Wahlgren et al., 1990). It is suggested that mediators other than histamine also play a key role in itch; for example, physiological temperature, inflammatory mediators and nitric oxide. These putative pruritogens are also recognized as activators of TRPV3 (Xu et al., 2002; Hu et al., 2006; Yoshida et al., 2006), which is a warm-sensitive Ca^{2+} -permeable cation channel highly expressed in epidermal keratinocytes (Peier et al., 2002). Interestingly, we indicated previously that a gain-of-function mutation in TRPV3 caused itchy dermatitis in rodents (Asakawa et al., 2005 and 2006; Yoshioka et al., 2009). However, we could not sufficiently suggest a possibility of TRPV3 as a therapeutic target of pruritus. Hence, I focused on TRPV3 as a new therapeutic target for histamine H1R antagonist-resistant pruritus and carried out experiments using ICR^{TRPV3-/-} mice to determine the maximum therapeutic impact of TRPV3 for a certain type of itch.

1. Materials and Methods

1-1. Animals

ICR mice and C57BL/6 mice were used. C57BL^{TRPV3^{-/-}} mice were constructed according to previous report (Moqrich et al., 2005). C57BL^{TRPV3^{-/-}} mice were crossed with ICR, since ICR mouse is a good responder for scratching behavior against various pruritogens (Inagaki et al., 2001). These F1 progenies without TRPV3 backcrossed with ICR for several generations to produce ICR^{TRPV3^{-/-}} mice. All mice used in this study were maintained in cages, exposed to a 12 hours light/12 hours dark cycle and provided with standard food and water ad libitum. This study was conducted according to the Guidelines for Animal Experimentation at Shionogi & Co., Ltd.

1-2. Drugs

Naltrexone hydrochloride (Sigma-Aldrich, St. Louis, MO) were dissolved in physiological saline in a concentration of 0.1 mg/mL and injected subcutaneously in a volume of 0.1 mL per body weight of 10 g (10 mg/kg) into the caudal part of the back 15 minutes before the start of videotaping. Physiological saline was injected as the control.

1-3. Treatment of cutaneous barrier disruption

Recently, Miyamoto et al. developed a murine model of dry skin pruritus, which was constructed by daily treatment of an acetone/ether (1:1) mixture and water (AEW) applied to the rostral back and the resulting spontaneous scratching of these models were thought to be histamine-independent (Miyamoto et al., 2002). The hair of mice was shaved over the rostral part of the back at least 4 days before the start of the experiment. The skin was treated by applying a piece of cotton soaked with a mixture of acetone and diethylether (1:1(v/v)) directly onto the shaved area for 15 seconds, followed immediately by application of a piece of cotton soaked with distilled water to the

exact same skin area for 30 seconds. AEW treatments were performed twice daily for 4 consecutive days.

1-4. Measurement of TEWL and SC hydration

Skin dryness and barrier disruption after AEW treatment were determined by measuring transepidermal water loss (TEWL) for 30 seconds using a Tewameter[®] TM300 (Courage & Khazawa, Cologne, Germany) or stratum corneum (SC) hydration expressed as relative capacitance using Moisture checker MY-808S (Scalar, Tokyo, Japan). Each parameter was measured on the day before the first treatment for cutaneous barrier disruption.

1-5. Behavioral observation

After the 4th treatment day, the mice were put individually into an acrylic box composed of six cells. They were acclimated to the experimental environment for at least 1 hour, and then behaviors were videotaped for 2 hours with experimenters kept out of the observation room. Playing back of the video served for counting scratching behavior. The mouse generally scratches several times for about 1 second and a series of these movements was counted as one bout of scratching. Locomotor activity were also measured over a 6 hour period as the amounts of spontaneous motor activity in both mice strains without AEW treatment using Activity Sensor Model NS-AS01 (NeuroScience, inc., Tokyo, Japan).

1-6. Histopathological analysis

The dorsal skin was excised and fixed with 10% neutral formalin, embedded in paraffin and sectioned at 4 μ m. The sections were stained with hematoxylin and eosin for histopathological analysis by light microscopy. Paraffin sections were also prepared for immunohistochemical analyses. These sections were pretreated with 2.5 mg/mL of purified mouse IgG (BD Pharmingen, San Diego, CA) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and Dako Protein Block (Dako,

Glostrup, Denmark). Next, the sections were immunostained with a rabbit anti-mouse protein gene product (PGP) 9.5 (Biomol, Plymouth Meeting, PA). After washing in Tris buffered saline-0.05% Tween 20 (TBS-T), the sections were reacted with biotin-conjugated secondary antibodies (BD Pharmingen). After washing with PBS, the sections were treated with UltraAvidin-Horseradish Peroxidase (Leinco Technology Inc., St. Louis, MO). Finally, antibody-positive cells in the sections were visualized by enzyme-based color staining after washing with PBS.

1-7. RT-PCR

Total RNA from back skin was extracted using TRIzol reagent (Invitrogen/GIBCO Life Technologies, Carlsbad, CA). Approximately 1 µg of total RNA was reverse transcribed with PrimeScript[®] RT reagent Kit (Takara Bio, Shiga, Japan) and realtime PCR was performed with SYBR[®] Premix Ex Taq[™] II (Takara Bio) using 1 µL of cDNAs from each sample as template in a DNA thermal cycler (ABI7700; Applied Biosystems, Foster City, CA). The primer pairs for β-actin and Semaphorin 3A were also purchased from Takara Bio.

1-8. Western blotting

Skin tissue samples were collected from ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice, homogenized in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) and stored at -80°C until analysis. The concentration of protein in homogenate was determined using a BCA reagent (Pierce). Samples were diluted with Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and boiled for 5 min. Ten micrograms of proteins loaded onto a 4-12% NuPAGE[®] Novex Bis-Tris Gel (Invitrogen). After electrophoresis, the proteins were electrotransferred onto polyvinylidene difluoride membranes (Invitrogen) overnight at 4°C. The membranes were incubated in blocking buffer (TBS-T with 4% w/v fat-free dry milk) for 2 hours at room temperature. The membranes were then incubated with the primary antibodies for 1.5 hours at room temperature. Primary antibodies used were rabbit anti-Sema

3A (1:100; Santa Cruz Bioechnology, Santa Cruz, CA) and rabbit anti-actin (1:1000; Santa Cruz Biotechnology). After incubation, the membranes were washed with TBS-T (TBS and 0.05% Tween 20) four times for 10 minutes and incubated with anti-rabbit HRP-conjugated secondary antibody (1: 2000; Santa Cruz Biotechnology) for 1 hour at room temperature. The membrane was then washed with TBS-T four times for 15 minutes. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL kit; Amersham Biosciences, Arlington Heights, IL). The bands were quantified a lumminoimage analyzer (LAS 1000-Mini; Fuji Film, Tokyo, Japan).

1-9. Statistics

All data are shown as the values of means and SEM. Statistical significance was tested by a Student's *t*-test for non-paired samples or a paired *t*-test for paired samples. For multiple comparisons, analysis of variance (ANOVA) was performed followed by a post hoc test (Dunnett's test).

2. Results and Discussion

C57BL^{TRPV3-/-} mice were constructed according to previous report (Moqrich et al., 2005). Fig. 3 shows simple schematic drawing of the TRPV3 wild-type and TRPV3 mutant allele and the results of genotyping. I indicated the minimal separation of both alleles and position and size for PCR primers to perform the genetic testing. C57BL^{TRPV3-/-} mice were crossed with ICR and these F1 progenies without TRPV3 backcrossed with ICR for several generations to produce ICR^{TRPV3-/-} mice. Mice lacking the TRPV3 gene also exhibit wavy whiskers (Fig. 3c). I can distinguish between wild type (ICR^{TRPV3+/+}) and ICR^{TRPV3-/-} mice based on these results.

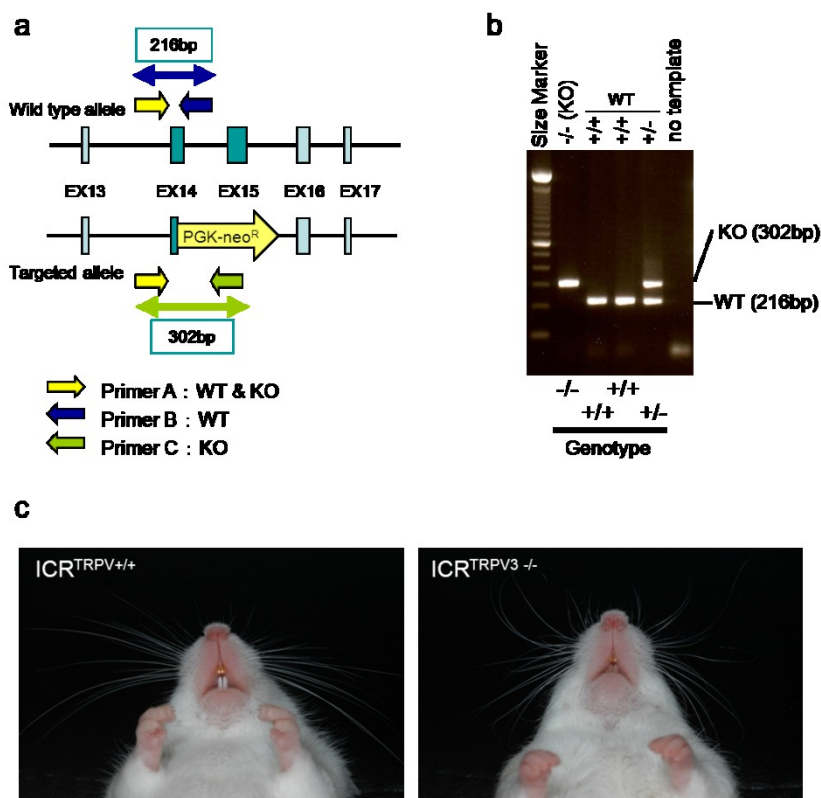


Fig. 3 Information regarding TRPV3 knockout mice and phenotypes associated with lack of TRPV3. (a) Simple schematic drawing of the TRPV3 wild-type and TRPV3 mutant allele. I indicated the minimal separation of both alleles and position and size for PCR primers to perform the genetic testing. (b) The results of genotyping. (c) Mice lacking the TRPV3 gene also exhibit wavy whiskers and dorsal coat.

To develop skin dryness and barrier disruption, the dorsal back of ICR^{TRPV3^{-/-}} mice and ICR^{TRPV3^{+/+}} mice were treated with AEW for 4 days. Daily treatment with AEW increased transepidermal water loss (TEWL) and decreased SC hydration in both ICR^{TRPV3^{+/+}} and ICR^{TRPV3^{-/-}} mice (Fig. 4a). Hematoxylin-eosin (HE) staining revealed remarkable epidermal hyperplasia without infiltration of inflammatory cells in the dermis in both ICR^{TRPV3^{+/+}} and ICR^{TRPV3^{-/-}} mice (Fig. 4b). It has been reported that TRPV3 is required for the formation of the stratum corneum layer, which is thought to be a key player in maintaining skin barrier functions (Cheng et al., 2010). Contrary to my expectations, there was no difference in the manner of the development of dry skin caused by AEW treatment between mice with and without TRPV3 in this study. This may be because the mouse strain used in each study was different or effects of AEW-treatment are too strong to detect the effects of TRPV3 on the development of dry skin.

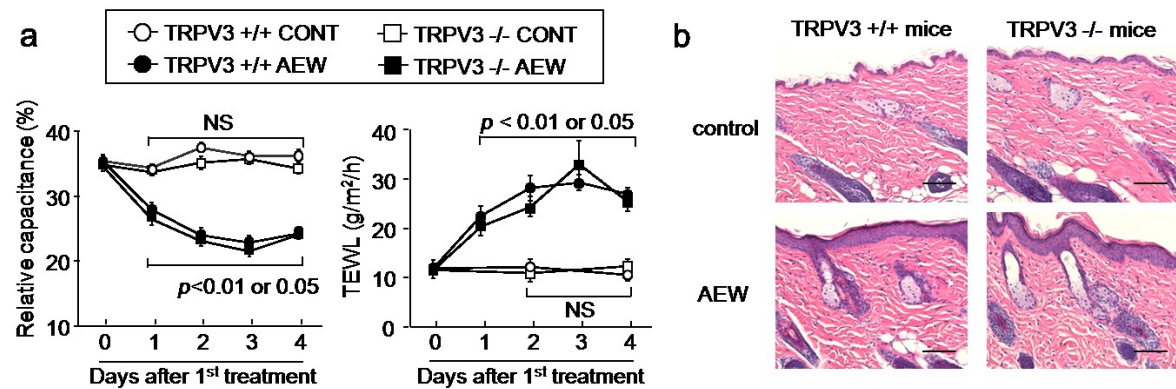


Fig. 4 Physiological and histological features of experimental dry skin induced by AEW in ICR^{TRPV3^{+/+}} and ICR^{TRPV3^{-/-}} mice. (a) Changes of TEWL and SC hydration of daily treatment with AEW. Each value represents mean $\pm$ SEM for 8 mice. NS, not significant. (Student's *t*-test) (b) The dorsal skin was excised and fixed with 10% neutral formalin, embedded in paraffin and sectioned at 4 μ m. Sections were stained with hematoxylin-eosin (HE). Scale bar = 100 μ m.

To examine the impact of TRPV3-deletion in the development of spontaneous scratching behavior, I performed behavioral tests. Repeated treatment with AEW resulted in a significant increase in spontaneous scratching directed toward the AEW-treated area; which was suppressed by subcutaneous injections of naltrexone, a μ -opioid receptor antagonist (Fig. 5b). Given that μ -opioid receptor antagonists reduce experimentally-induced itch in humans (Heyer et al., 1997), spontaneous scratching behavior observed in the AEW-treated mice might reflect similar itch sensation in humans. In the AEW-treated ICR^{TRPV3-/-} mice, there was a smaller increase in numbers of scratching behaviors compared to those of wild-type mice (Fig. 5c). To rule out the possibility of a sedative effect, I also measured the spontaneous motor activity and could not find any locomotor effects evoked by TRPV3 deletion in these mice (Fig. 5a). These results indicated that TRPV3 plays a pivotal role in the development of itch in mice with dry skin.

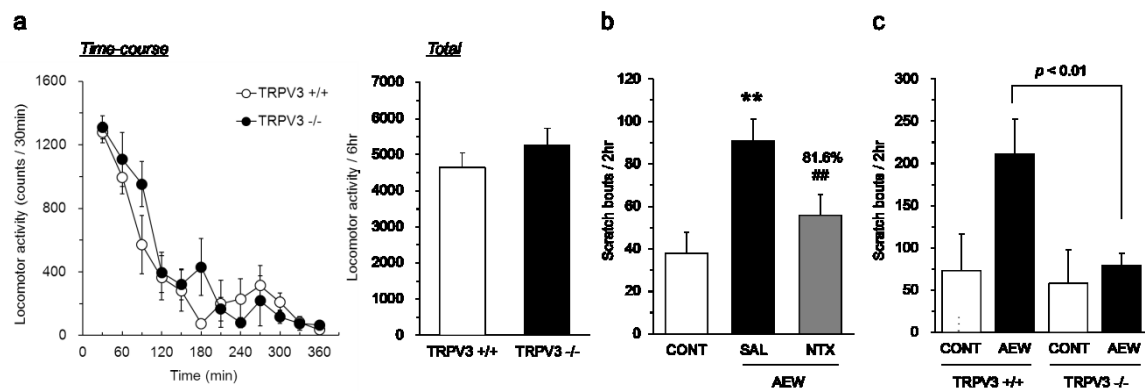


Fig. 5 Spontaneous scratching induced by AEW and locomotor activity in ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice. (a) Behavior was measured over a 6-hour period as the amount of spontaneous motor activity in both mice strains. Each value represents mean $\pm$ SEM for 8-16 mice. (b) Scratching behavior of ICR mice with and without AEW treatments was evaluated during a 2-hour observation period. Naltrexone (NTX, 10 mg/kg) was dissolved in saline and injected subcutaneously 15 minutes before behavioral observation. Each value represents mean $\pm$ SEM for 6 or 7 mice. ** $p < 0.01$ when compared with control (CONT), ## $p < 0.01$ when compared with saline (SAL). (c) Scratching behavior of ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice with and without AEW treatments was evaluated during a 2-hour observation period. Each value represents mean $\pm$ SEM for 8-16 mice

It is well known that increased nerve density is involved in producing pruritus. In AD patients, increased sprouting of epidermal C-fibers is seen, inducing hypersensitivity to itching, which aggravates the disease (Paus et al., 2006). In the present study, I also found that the density of the invasive nerve fibers in the skin was increased in AEW-treated mice by immunohistochemical examination of the sensory neurons using anti-PGP9.5 antibody (Fig. 6). PGP9.5-positive neurites in the dermis were more prominent in the AEW-treated skin of wild-type mice compared to that of ICR^{TRPV3-/-} mice, and some fibers expanded into the epidermal layer. These results suggested that TRPV3 affected nerve sprouting in dry skin.

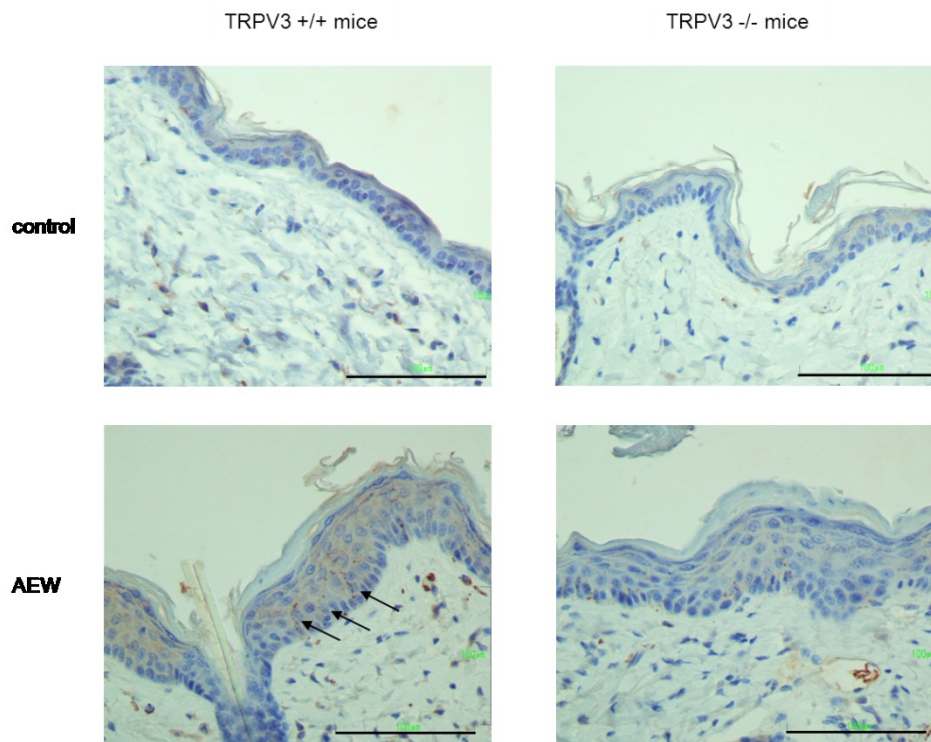


Fig. 6 Lower nerve response to AEW treatment in the skin of ICR^{TRPV3-/-} mice. Frozen sections were prepared from dorsal skin samples obtained from ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice and immunostained for mouse protein gene product 9.5 (PGP9.5). Arrows indicate PGP9.5-expressing neurites. Bar = 100 μ m.

Semaphorin3A (Sema3A), an axon guidance molecule, potently induces retraction of NGF-sensitive neurons among dorsal root ganglia neurons (Dontchev and Letourneau, 2002). It is reported that Sema3A expressed in keratinocyte may play a crucial inhibitory role for C-fiber elongation/sprouting in the upper layers of the epidermis in human and alleviates skin lesions and scratching behavior in NC/Nga mice (Fukamachi et al., 2011; Yamaguchi et al., 2008). The protein and mRNA expression levels of Sema3A in the skin of wild-type mice were significantly decreased by AEW-treatment, but no change is found in ICR^{TRPV3-/-} mice treated with AEW (Fig. 7). In addition, previous studies indicate that TRPV3 affects the production of epidermal NGF (Gopinath et al., 2005; Yoshioka et al., 2009). These results suggest that TRPV3 causes pruritus in lesional skin through nerve fiber expansion. In AD patients with pruritus, higher expressions of TRPV3 mRNA are observed compared to those without pruritus (data not shown). This suggests that TRPV3 is involved in human and murine pruritus in a similar manner.

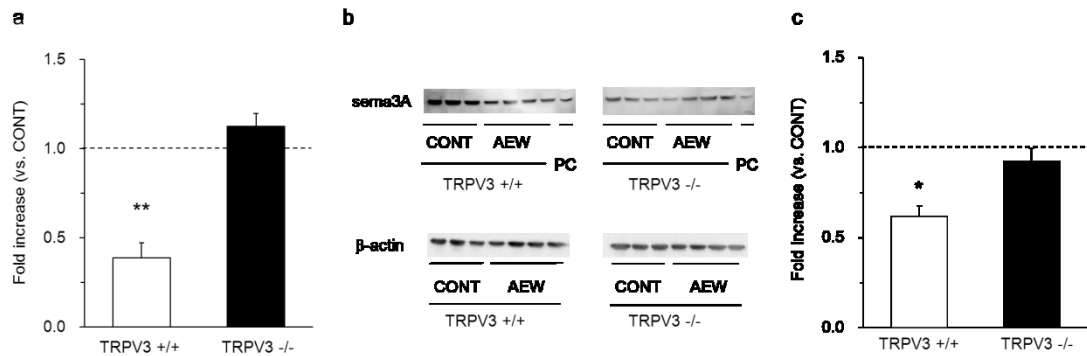


Fig. 7 Effects of AEW-treatment on Sema3A production in the skin of ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice. AEW treatment was given to the hair-clipped skin of murine back twice a day for 4 days. (a) The expression levels of Sema3A mRNA were examined by RT-PCR and normalized with β-actin. Dotted lines indicate the expression levels of Sema3A in non-treated murine skin. Each value represents mean ± SEM for 8 mice. (b) Typical examples and (c) the expression level of Sema3A were examined by western blotting and normalized with β-actin. Dotted lines indicate the expression levels of Sema3A in non-treated murine skin. Each value represents mean ± SEM for 3 or 4 mice. **p*<0.05, Student's *t*-test, when compared with control (CONT).

3. Summary

1. Daily treatment with AEW increased TEWL and decreased SC hydration in both ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice. Remarkable epidermal hyperplasia without infiltration of inflammatory cells in the dermis was observed in both ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice treated with AEW.
2. Repeated treatment with AEW resulted in a significant increase in spontaneous scratching directed toward the AEW-treated area in ICR^{TRPV3+/+} mice, however there was a smaller increase in AEW-treated ICR^{TRPV3-/-} mice compared to those of ICR^{TRPV3+/+} mice.
3. The density of the invasive nerve fibers in the skin was increased in AEW-treated mice and neurites in the dermis were more prominent in the AEW-treated skin of ICR^{TRPV3+/+} mice compared to that of ICR^{TRPV3-/-} mice.
4. The expression levels of Sema3A in the skin was significantly decreased by AEW-treatment in ICR^{TRPV3+/+} mice, but no change is found in ICR^{TRPV3-/-} mice treated with AEW

These results suggest that TRPV3 causes pruritus in lesional skin through nerve fiber expansion. Although I could not carry out pharmacological studies using TRPV3-antagonists to elucidate the therapeutic role of TRPV3 for a certain type of pruritus, I believe that this is the first report referring their role in itch sensation by genetic studies using ICR^{TRPV3-/-} mice. TRPV3 may play an important role in dry skin itch and TRPV3 antagonists could be a therapeutic option in managing AD and dry skin patients with histamine H1R antagonist-resistant pruritus.

Chapter 2 Impact of TRPV3 on the development of allergic dermatitis as a dendritic cell modulator

Inflammatory responses are one of the body's defense systems against harmful agents or injury and the classical signs of acute inflammation are pain, heat, redness and swelling. Although the thermal element of inflammation and/or fever might regulate the activation and migration of dendritic cells (DCs) in the skin (Ostberg et al., 2000), a detailed mechanism for this has not been reported. Cutaneous DCs, such as epidermal Langerhans cells (LCs) and dermal DCs play a major role in regulating skin immune responses. As professional antigen presenting cells, they detect exogenous substances that have penetrated the epithelial barrier and traffic to the draining lymph nodes (LNs) where they interact with T and B cells to initiate and shape adaptive immune responses.

DS-*Nh* mice were selected on the basis of a hairless phenotype from an inbred DS strain mouse colony, developed from an out-bred ddN stock. DS-*Nh* mice develop spontaneous dermatitis and are considered a model of human atopic dermatitis (AD) (Hikita et al., 2002; Yoshioka et al., 2003, 2006 and 2007). We previously reported on a one amino-acid substitution (Gly573 to Ser) in the TRPV3 (TRPV3^{gly573ser}) of DS-*Nh* mice and showed that this is a gain-of-function mutation, as Ca²⁺ influx was stimulated by temperature (Asakawa et al., 2006). TRP channels are expressed in almost all organs of the body and act as cellular sensors for a variety of environmental and endogenous stimuli such as temperature, touch, pain, osmolarity, pheromones and taste (Clapham, 2003). TRPV3 is a Ca²⁺-permeable cation channel that is highly expressed in keratinocytes and activated by moderate thermal heating ($\geq 32^{\circ}\text{C}$) in the range where warmth is sensed (Xu et al., 2002; Peier et al., 2002; Smith et al., 2002). Studies using TRPV3-deficient mice revealed that the TRPV3 channel is necessary for normal hair development, epidermal barrier formation, and thermosensation (Moqrich et al., 2005; Cheng et al., 2010). However, there have been few reports on specific ligands for TRPV3.

Results of DNA microarray (Table S1) and cytokine array (Fig. S1) analysis using

keratinocytes from DS, DS-*Nh*, TRPV3 knockout mice (TRPV3^{-/-}) and their wild-type littermates (TRPV3^{+/+}) as well as a previous study regarding DC migration (Ostberg et al., 2000) motivated me to investigate whether TRPV3 might be a key player in the development of dermatitis, acting as a DC modulator. I was also interested in determining which pathological conditions would still maintain expression of TRPV3 in lesional areas using skin samples from patients with AD as one of the DC-mediated skin disorders, although little is known about its direct and indirect involvement in human dermatitis via dermal DC. Taking this into account, I investigated the dermal DC-mediated phenotype using DS-*Nh* mice as a potentially useful model in which to validate the pathological or therapeutic role of TRPV3.

1. Materials and Methods

1-1. Animals

Male DS, DS-*Nh*, TRPV3 knockout (TRPV3^{-/-}) mice in an ICR genetic background and their wild-type (TRPV3^{+/+}) littermates (Yamamoto-Kasai et al., 2012) were used in this study. All mice were maintained in cages exposed to a 12 hours light/12 hours dark cycle and provided with standard food and water *ad libitum*. The animals were housed in rooms under specific pathogen-free or conventional conditions. This study was conducted according to the Guidelines for Animal Experimentation at Shionogi & Co., Ltd.

1-2. Contact hypersensitivity

Mice were sensitized by painting 60 μ L of 0.5% 2,4-dinitrofluorobenzene (DNFB, Sigma-Aldrich, St Louis, MO) in 4:1 acetone:olive oil (AOO) on the shaved abdomen of each animal. Seven days after sensitization, each mouse was challenged with 20 μ L of 0.2% DNFB in AOO applied to the side of the ear. The control animals received AOO without DNFB. At 0 and 24 hours post-DNFB challenge, ear swelling was evaluated by measuring the ear thickness using a dial thickness gauge (Teclock, Nagano, Japan).

1-3. Hematoxylin and eosin staining

Lesional and nonlesional skins from three female patients with AD were purchased from Asterand (Detroit, MI), and fixed in 10% neutral formalin. Ears were excised from DS mice and DS-*Nh* mice 24 hours after DNFB or vehicle challenge, fixed in 10% neutral formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) for histopathological analysis using light microscopy. This study was conducted according to the Declaration of Helsinki principles and approved by the local Research Ethics Committee.

1-4. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from human skin (Asterand) using an RNeasy kit (Qiagen, Valencia, CA) with DNaseI (Qiagen) treatment, and 1 µg of total RNA was used to synthesize cDNA using PrimeScript® 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). The cDNA was then used for quantitative RT-PCR analysis performed using respective pairs of gene-specific primers for TSLP, TRPV3 and Homo sapiens hypoxanthine phosphoribosyltransferase 1 (HPRT1) genes. The primer pairs for TSLP, TRPV3, HPRT1 and SYBR® Premix Ex Taq™ II were purchased from Takara Bio.

1-5. FITC-induced cutaneous DC migration and fluorescence-activated cell sorting analysis of lymph node single-cell suspensions

Inguinal LNs were isolated from mice 16 hours after abdominal application of 400 µL of 0.5% fluorescein isothiocyanate (FITC; Sigma-Aldrich) in 1:1 acetone:dibutyl phthalate and cut on ice. Tissue pieces were then suspended in RPMI 1640 medium including Liberase DL (Roche Applied Science, Indianapolis, IN) and DNase I (Sigma-Aldrich) and incubated at 37°C for 20 minutes. The tissue was completely dissociated using a 70-µm cell strainer (BD Biosciences, Stockholm, Sweden) to obtain a single cell suspension. After Fc receptor blocking with the CD16/32 antibody (Ab) (BD Biosciences), cells were stained with anti-CD11c-PE (eBioscience, San Diego, CA), anti-CD49d-PE (BD Biosciences) and anti-Langerin-PE (Biolegend, San Diego, CA). Cells were resuspended in fluorescence-activated cell sorting (FACS) staining buffer (BD Biosciences) before acquisition on a FACS Aria (BD Biosciences)

1-6. Primary culture of keratinocytes

Trunk skins from mice with the hair removed were incubated in 0.5% collagenase (Wako, Osaka, Japan) solution for 1 hour at 37°C, and then incubated with 1000 U/mL Dispase I (Godo Shusei, Tokyo, Japan) solution for 1 hour at 37°C. After washing with PBS, skin fragments were pressed

through a 100- μ m cell strainer (BD Biosciences). The cell suspension was centrifuged and cultured in keratinocyte growth medium-2 (KGM-2 BulletKit; Cambrex, Walkersville, MD) at 33°C and 5% CO₂. The culture supernatant was collected 24 hours after the last medium change and used for cytokine analysis using the Bio-Plex suspension array system (Bio-Rad, Hercules, CA) their TSLP levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Gene expression in keratinocytes from DS and DS-*Nh* mice strains was analyzed using Agilent microarray hybridization. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and purified using RNeasy kits (Qiagen). Equal amounts of total RNA samples were pooled from six wells for each strain and cDNA was prepared using the Agilent kit (Agilent, Santa Clara, CA). cDNAs were then hybridized to immobilized mouse oligo microarrays and analyzed according to the manufacturer's instructions (Agilent).

1-7. Measurement of TSLP

Skin tissue samples were collected from DS and DS-*Nh* mice, homogenized in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) and stored at -80°C until analysis. After the homogenates were thawed and centrifuged, supernatants were collected and their TSLP levels were measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions.

1-8. Statistics

Statistical analyses were performed using the Student's *t*-test for paired comparisons, unless stated otherwise.

2. Results and Discussion

In Fig. S1, because the same degree of G-CSF, KC, MCP-1, and TNF- α responses were observed in keratinocytes from TRPV3^{-/-} mice compared with TRPV3^{+/+} mice, keratinocytes from TRPV3^{-/-} mice were thought to be equally physiologically active. On the other hand, greater TSLP responses were observed in keratinocytes from TRPV3^{+/+} mice compared with TRPV3^{-/-} mice. These results indicate that TRPV3 is a key player in regulating TSLP levels in keratinocytes. Detailed results of the TSLP response in keratinocytes are shown in Fig. 11b. I also analyzed the gene profiles of primary keratinocytes from DS and DS-*Nh* mice using DNA microarrays. In Table S1, the value of “DNA microarray fold (*Nh* / DS)” is not lower than 1.90 or higher than 0.60, and the value of “Raw (DS-*Nh* or DS)” is not less than 5000. To set these criterion values, gene expression data reflect the most important difference in function of these keratinocytes. Hence, the genes listed in Table S1 were thought to be closely related to the gain-of-function TRPV3 mutant. According to the references shown in table S1, underlined genes are thought to be associated with DC or DC-mediated dermatitis directly or indirectly. Hence I hypothesized that TRPV3 regulates immune cell migration in the skin.

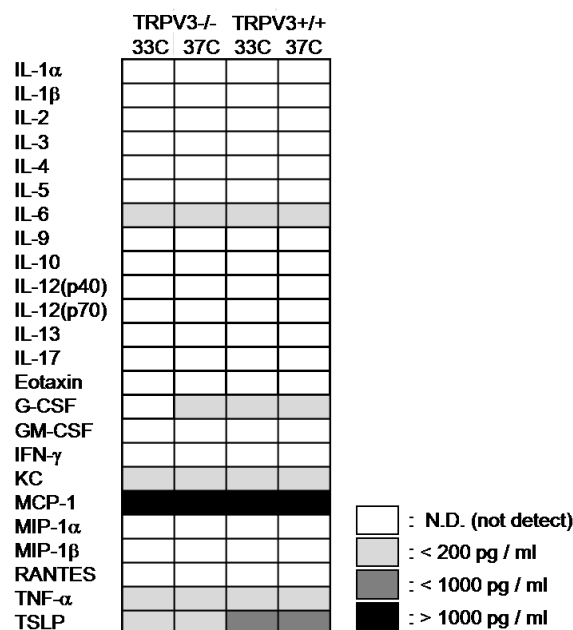


Fig. S1 Cytokine array analysis using keratinocytes from TRPV3^{-/-} and TRPV3^{+/+} mice. Release of cytokines from keratinocytes of TRPV3^{-/-} mice and TRPV3^{+/+} mice into culture medium was measured using cytokine analysis using

the Bio-Plex suspension array system and ELISA kits

Table S1 Genetic analysis of the primary keratinocytes from DS-*Nh* and DS mice

DNA micro array fold (<i>Nh</i> /DS)	Raw		GenBank	Description	Ref.
	DS- <i>Nh</i>	DS			
0.51	13050	25773	NM_025984	small proline rich-like 3	
0.53	8522	16173		tropomyosin 1, alpha	
0.58	4854	8430	NM_026822	small proline rich-like 5	
3.22	8237	2555	NM_010809	matrix metalloproteinase 3	
3.11	13080	4202	NM_009263	secreted phosphoprotein 1	Renkl et al., 2005
2.34	23565	10078	NM_011311	S100 calcium binding protein A4	
2.11	8197	3893	NM_133903	spondin 2, extracellular matrix protein	
2.01	6531	3254	NM_011118	proliferin 2	Hyun-Ok et al., 2009
1.95	8010	4100	NM_181852	mitogen regulated protein, proliferin 4	
1.94	12230	6288	NM_009114	S100 calcium binding protein A9 (calgranulin B)	Cheng et al., 2008

In Fig. 8a, histological analysis of the clinical features of three human AD patients used for RT-PCR analysis and the characteristics of these patients are shown. The level of TRPV3 mRNA in lesional skins from patients with AD (Fig. 8c) suggests that TRPV3 plays a role in the development of human AD. With respect to skin physiology and pathophysiology, TRPV3 might be the most important TRPV channel (Nilius and Bíró, 2013). I observed an up-regulation of TSLP and TRPV3 mRNA in lesional skin samples compared with nonlesional skin samples from patients with AD (Fig. 8d and 8e). TSLP is an epithelial cell-derived cytokine that regulates T cell differentiation by conditioning DC maturation (Soumelis et al., 2002). It was also reported that TSLP expression in patients with AD was associated with LC migration and activation (Soumelis et al., 2002; Ito et al., 2012). In this study, I tested whether TRPV3 acted as a DC modulator by controlling the TSLP response in DS-*Nh* mice.

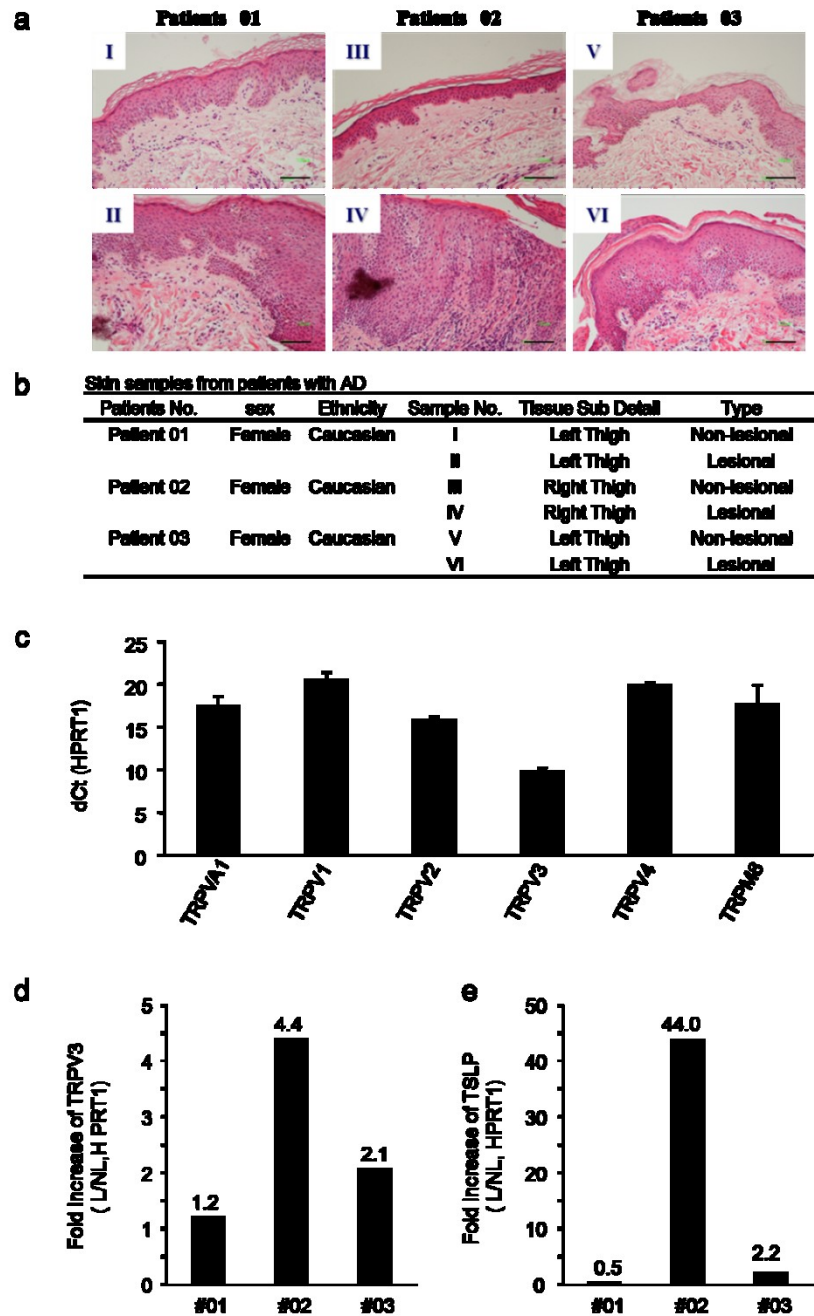


Fig. 8 Higher expression of TRPV3 and TSLP mRNA in the skin of patients with AD. (a) Skin sections from nonlesional skin (I, III, V) and lesional skin (II, IV, VI) of three patients with AD were stained with HE. Pairs of nonlesional and lesional skin (I-II, III-IV, V-VI) from the same patients are shown in the order corresponding to #01-03. Scale bar = 100 μ m. (b) Skin samples were obtained from three female patients with AD and #01-03 indicates patient number. (c) Expression levels of thermo-TRP mRNA in lesional and skin from patients with AD were examined by RT-PCR. Expression of HPRT1 was used as a normalization control. Each value represents the mean $\pm$ SEM. The fold difference in expression of (d) TRPV3 and (e) TSLP mRNA between lesional (L) and nonlesional (NL) skin showed that there was a tendency for increased expression of these two mRNAs in lesional skin. Expression of HPRT1 was used as a normalization control.

Since DCs are known to play an important role in the development of contact hypersensitivity (CHS), I compared DNFB-induced CHS responses in DS-*Nh* and DS mice. To induce CHS, DS and DS-*Nh* mice were sensitized with DNFB on day 0 and challenged with DNFB on day 7 as shown in Fig. 9a. DNFB challenge in DS mice failed to provoke a CHS response, whereas in DNFB challenged DS-*Nh* mice induced marked ear redness and swelling (Fig. 9b). The ear thickness in DNFB-challenged DS-*Nh* mice was markedly increased compared with DNFB-challenged DS mice, and HE stained sections showed edema and inflammatory cell infiltration in DS-*Nh* mice (Fig. 9c and 9d). These results suggested that TRPV3 activation is involved in the enhancement of CHS responses in DS-*Nh* mice.

CHS is a DC-dependent T cell-mediated skin inflammation that develops in two distinct phases: the sensitization and elicitation phase. During the sensitization phase, haptens penetrating the skin are captured by resident LCs and dermal DCs that migrate to regional LNs and induce the activation of specific T cell precursors. In the elicitation phase, reexposure of the skin to the hapten activates specific T cells in the dermis and triggers the inflammatory process responsible for cutaneous lesions, which peaks at 24-48 hours after reexposure. Therefore, the difference in CHS responses between DS and DS-*Nh* mice may be partly related to DC function regulated by TRPV3.

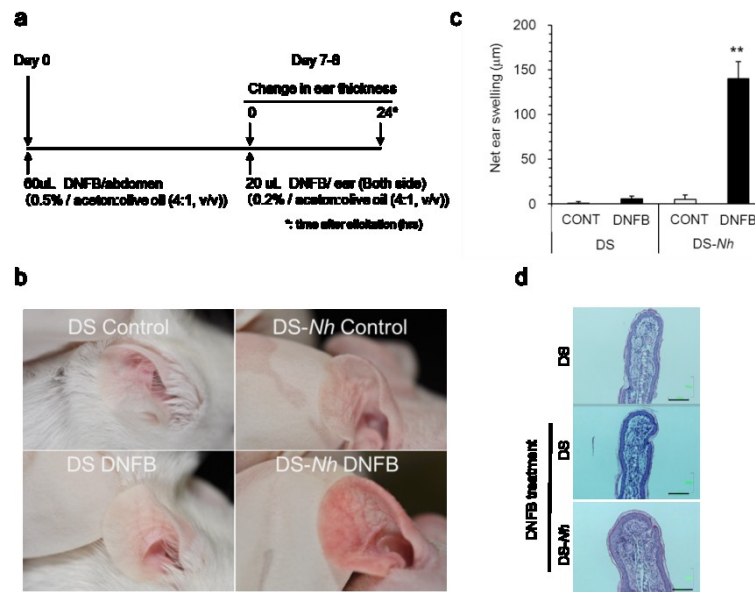


Fig. 9 TRPV3^{gly573ser} enhanced DNFB-induced CHS. (a) CHS induction protocol. Mice were sensitized with DNFB by applying 60 μ L of 0.5% DNFB in acetone:olive oil (4:1) to the shaved abdomen of mice on day 0. On day 7, sensitized mice were challenged on the side of the ears with 0.2% DNFB and ear thickness was measured 24 hours later. (b) Ear appearance was compared between DS and DS-*Nh* mice 24 hours after challenge with 0.2% DNFB or vehicle (control). (c) Ear thickness was increased in DS-*Nh* mice challenged with DNFB, but not in DS mice challenged with DNFB. Each value represents the mean $\pm$ SEM of 4-10 mice. ** $p < 0.01$, Student's *t*-test, compared with vehicle-challenged control groups (control). (d) DNFB-sensitized DS and DS-*Nh* mice were challenged with 0.2% DNFB and 24 hours later the ears were excised and fixed in formalin, and prepared sections were stained with HE. Representative images were captured using light microscopy. Scale bar = 100 μ m.

Activated DCs bearing antigen migrate into the draining LNs to initiate T cell responses. T cells memorize the characteristics of the antigen and upon a second exposure induce CHS responses. I hypothesized that DC activation contributes to the enhancement of CHS responses in DS-*Nh* mice and evaluated the migration of FITC-bearing DCs to the draining LNs after FITC application to abdominal skin using FACS (Fig. 10). FITC, acting as a hapten, can induce DC maturation and mobilization, therefore FITC-induced DC migration assays have been used for *in vivo* studies (Macatonia et al., 1987; Kripke et al., 1990). The uptake and transport of FITC by DCs can be easily traced by its fluorescence and this system allows for the investigation of the entire pathway of DC migration, from the skin to the LN. FITC was painted on the shaved abdomen and inguinal LNs cells were isolated 16 hours later. As

shown in Fig. 10, the frequency of FITC-bearing cells among CD11c⁺ (DC marker) cells in the draining LNs was higher in DS-*Nh* mice (30.52%) than in DS mice (8.77%). Similar results were obtained among Langerin⁺ cells or CD49⁺ cells (42.81% vs. 11.44% and 49.83% vs. 14.21%, respectively). Langerin is a highly selective marker for LCs, a specialized subset of DCs that populate the epidermal layer of the skin and CD49 expression is indicative of DC maturation (Valladeau et al., 2000; Puig-Kröger et al., 2000). These data indicated that hapten-induced DC activation was enhanced in DS-*Nh* mice, suggesting that TRPV3 was involved in DC activation in the skin.

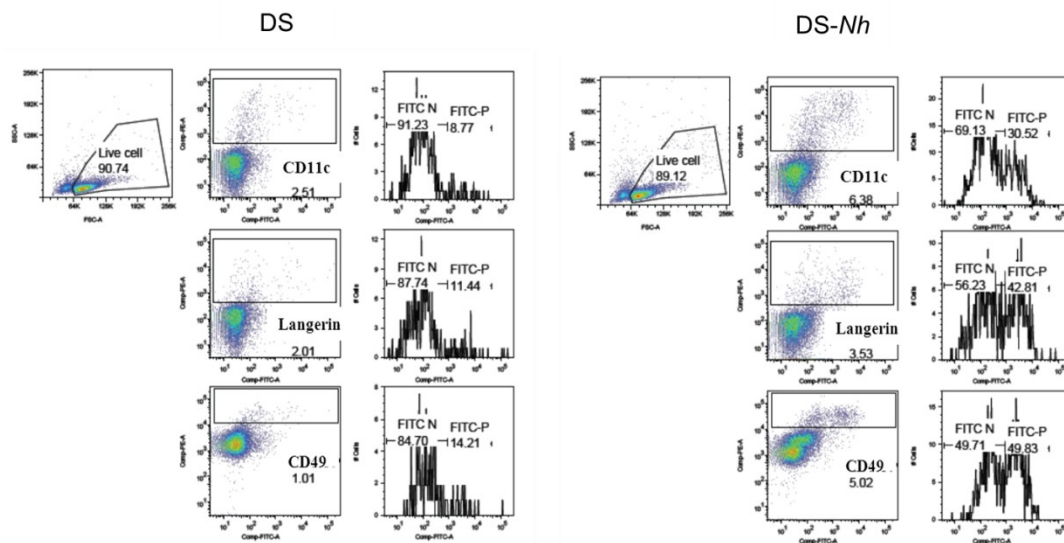


Fig. 10 TRPV3^{gly573ser} potentiates DC migration from skin to draining lymph nodes. DS and DS-*Nh* mice were administered 400 μ L of 0.5% FITC in acetone/dibutyl phalate (1:1) to the shaved abdomen and 16 hours later inguinal LNs were collected. Single-cell suspensions were prepared and stained with anti-CD11c/PE, anti-Langerin/PE, and anti-CD49/PE to identify infiltrating DCs, LCs and activated DCs. A higher frequency of FITC-bearing cells was detected from the draining lymph node of DS-*Nh* mice compared with controls.

TSLP is an epithelial cell-derived cytokine that regulates T cell differentiation by conditioning DC maturation. I observed the up-regulation of TSLP mRNA in the lesional skin of patients with AD (Fig. 8e). Furthermore, it was previously reported that patients with AD expressed elevated TSLP levels in the epidermis (Sano et al., 2013; Soumelis et al., 2002). Thus, I measured the expression levels of TSLP in the skin of DS and DS-*Nh* mice using an ELISA. TSLP levels were significantly higher in DS-*Nh* mice compared with DS mice (Fig. 11a). To confirm whether TRPV3 was involved in the production/release of TSLP from keratinocytes, TSLP levels in supernatants from primary cultures of keratinocytes taken from TRPV3^{-/-} mice and DS-*Nh* mice were measured. Fig. 11b shows that TSLP release from TRPV3^{-/-} keratinocytes was significantly decreased compared with TRPV3^{+/+} keratinocytes and only TRPV3^{+/+} keratinocytes responded to the higher temperature (37°C) without any difference in cell activity (data not shown). Moreover, higher levels of TSLP released from keratinocytes of DS-*Nh* mice compared with DS mice were observed (Fig. 11b). These findings suggested that TRPV3 is an important player in the regulation of TSLP production by keratinocytes. Keratinocytes are activated upon encountering haptens and produce various chemical mediators, such as tumor necrosis factor α , interleukin (IL)-1 β , and prostaglandin (PG) E₂ that promote the migration and maturation of skin DCs (Cumberbatch et al., 1995 and 1997; Kabashima et al., 2003). Cytokines that are found at high levels in lesional skin in patients with DC-mediated allergic skin inflammation (i.e. IL-1 β , TNF- α , IL-4 and IL-13) can also synergize to induce TSLP expression by keratinocytes (Bogiatzi et al., 2007). Thus, keratinocytes may play a pivotal role in the development of CHS.

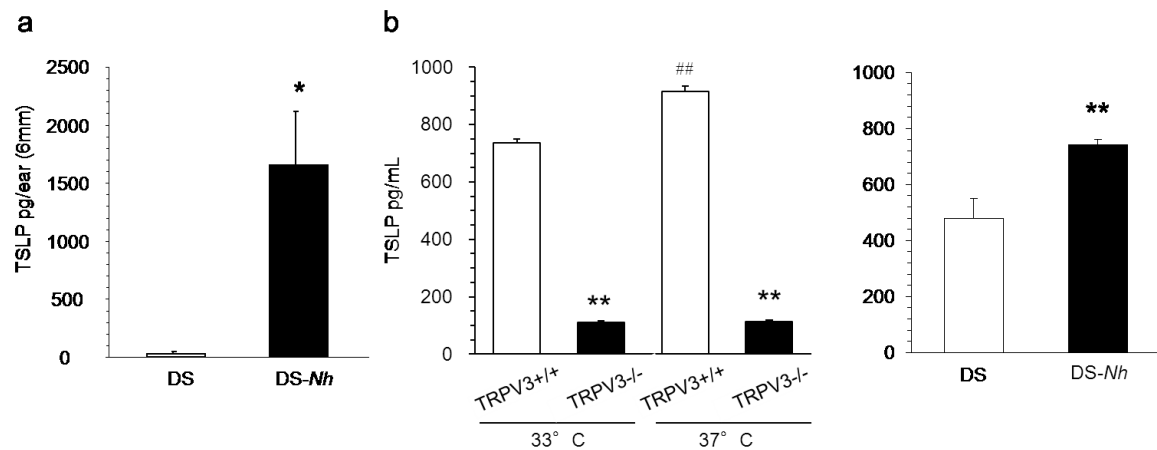


Fig. 11 Essential role of TRPV3 in TSLP release from keratinocytes. (a) Ear samples collected from DS and DS-*Nh* mice were homogenized, and supernatant TSLP levels were measured using ELISA. Each value represents the mean $\pm$ SEM of 4 mice. * $p < 0.05$, Student's *t*-test, compared with DS mice. (b) Release of TSLP from keratinocytes in TRPV3^{-/-} and TRPV3^{+/+} mice (left) or DS and DS-*Nh* mice (right) into culture medium was measured using ELISA. Each value represents the mean $\pm$ SEM of 6-12 mice. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test, compared with TRPV3^{+/+} mice or DS mice. ## $p < 0.01$, Student's *t*-test, compared with TRPV3^{+/+} cultured at 33°C.

In this study, FITC-bearing cells were rarely observed in the LNs and CHS responses induced by DNFB were not observed in DS mice. This suggested that DNFB was insufficient to trigger a primary immune response in DS mice under these conditions. However, enhanced skin responses to DNFB were observed in DS-*Nh* mice, and skin TSLP levels were higher than in DS mice. TSLP expression in patients with AD was associated with LC migration and activation *in situ*, suggesting that TSLP may contribute directly to the activation of DCs, which may then migrate into the draining LNs and prime allergen-specific T cell responses (Soumelis et al., 2002). Taken together, high level constitutive expression of TSLP may have an important role in DC activation and result in the aggravation of allergic skin inflammation. TSLP was also shown to participate in innate immune reactions in AD-like inflammation and early control of innate immune responses before activation of adaptive immune responses by conventional T and B cells that perpetuate chronic skin inflammation. Therefore, TSLP may adequately alleviate acute exacerbations of AD (Park et al., 2013).

Camphor is a well-known TRPV3 agonist and commonly used as a topical antipruritic in

medicine. From this perspective, TRPV3 activation may be expected to reduce an inflammatory response, but the results of the present study indicated an opposite effect of TRPV3. More recently, mutations in TRPV3 (Gly573Ser) have been linked with a rare genodermatosis known as the Olmsted syndrome (Lin et al., 2012). Moreover, Sherkheli *et al.* reported that prolonged exposure to monoterpenoids resulted in agonist-specific desensitization of TRPV3, whereas the *Nh* mutation in TRPV3 is a “gain-of-function” one (Sherkheli et al., 2009). While the bicyclic monoterpenoids (camphor or 1,8-cineol) induced both acute desensitization and tachyphylaxis, monocyclic monoterpenoids (dihydrocarveol or menthol) preferentially induced only tachyphylaxis. However, while the molecular basis for these differences remain unknown, the finding itself points to important biophysical and pharmacological aspects of TRPV3 activity that is modulated by different desensitization profiles of ligands. Taking this into consideration, it is difficult to validate an orphan receptor such as TRPV3 by pharmacological methods using artificial ligands. Given this information, I decided to employ DS-*Nh* mice with genetically active TRPV3 as a good tool to study this orphan ion-channel.

3. Summary

1. Analysis of data obtained from the gene profiles of primary keratinocytes from DS and DS-*Nh* mice using DNA microarrays revealed that immune cell trafficking genes were altered in DS-*Nh* compared with DS mice.
2. TSLP and TRPV3 mRNA in the lesional skin from the patients with AD were up-regulated compared with those in non-lesional skin.
3. Ear thickness in DNFB-challenged DS-*Nh* mice was markedly increased compared with DNFB-challenged DS mice.
4. The frequency of FITC-bearing cells among CD11c/Langerin/CD49 positive cells in the draining LN was higher in DS-*Nh* mice than in DS mice.
5. TSLP release from TRPV3^{-/-} keratinocytes was significantly decreased compared with TRPV3^{+/+} keratinocytes and only TRPV3^{+/+} keratinocyte responded to higher temperature (37°C). The higher levels of TSLP released from keratinocytes of DS-*Nh* mice were also observed compared with DS mice. And the expression level of TSLP in the skin of DS-*Nh* mice was significantly higher compared with DS mice.

These results demonstrated that the augmentation of CHS in DS-*Nh* mice might be due to the enhanced migration capability of DCs and that TRPV3-activation was partly involved in DC migration by playing a role in the constitutive release of TSLP from keratinocytes. TSLP was also shown to participate in innate immune reactions in AD-like inflammation and early control of innate immune

responses before activation of adaptive immune responses by conventional T and B cells that perpetuate chronic skin inflammation. Therefore, TSLP may adequately alleviate acute exacerbations of AD (Park et al., 2013). Similar to DS-*Nh* mice, TRPV3 may play a part in the aggravation of dermatitis through the regulation of DC functions in human AD. In addition, we previously reported that a gain-of-function mutation of TRPV3 (TRPV3^{gly573ser}) caused itchy dermatitis in rodents (Asakawa et al., 2006; Yoshioka et al., 2009) and may be a therapeutic target for pruritus (Yamamoto-Kasai et al., 2012). In conclusion, I believe that TRPV3 is a potential therapeutic target for certain types of dermatitis, via the management of inflammation and pruritus.

Chapter 3 Impact of TRPV3 on acute skin inflammation and pruritogen induced itch-related response in mice.

Skin is well-known for its functional role as a protective physical barrier. It is now considered that skin is far more than a mere container but rather is a dynamic organ that has other recognised functions, such as endogenous homeostasis, metabolism and sensory input. In addition, skin actively participates in immunological regulatory processes and inflammatory responses. However, some inflammatory or immunological reactions lead to chronic inflammation processes, such as psoriasis or to intolerable skin inflammation conditions, such as contact dermatitis, which requires medication (Robert and Kupper, 1999).

TRPV3 is highly expressed in epidermal keratinocytes and required for the formation of the skin barrier by regulating the activities of transglutaminases, a family of Ca^{2+} -dependent crosslinking enzymes essential for keratinocyte cornification (Peier et al., 2002; Cheng et al., 2010). Moreover, we reported previously that the gain-of-function mutation of the *trpv3* gene ($\text{TRPV3}^{\text{Gly573Ser}}$) in keratinocytes is responsible for the development of a spontaneous hairless phenotype and pruritic, AD-like skin alterations in rodents (Asakawa et al., 2005 and 2006; Yoshioka et al., 2009). However, pathophysiological role of TRPV3 in various skin conditions have not been fully understood yet. Here, I elucidated the roles of TRPV3 in the acute itch and inflammation in the skin using *DS-Nh* mice and TRPV3 knockout mice.

1. Materials and Methods

1-1. Animals

ICR mice, ICR^{TRPV3^{-/-}} mice, C57BL mice, C57BL^{TRPV3^{-/-}} mice, DS mice and DS-*Nh* were used (Yamamoto-Kasai et al., 2012). All mice used in this study were maintained in cages, exposed to a 12 hours light/12 hours dark cycle and provided with standard food and water ad libitum. This study was conducted according to the Guidelines for Animal Experimentation at Shionogi & Co., Ltd.

1-2. Recovery of skin barrier function

Skin barrier function was evaluated by measurement of Transepidermal water loss (TEWL) using a Tewameter[®] TM300 (Courage & Khazawa, Cologne, Germany). The hair of mice was shaved over the rostral part of the back before the start of the experiment. Mice were anaesthetized with pentobarbital (50 mg/kg) and the dorsal surface of the back was treated by tape stripping (TS) with cellophane tape (Scotch; Sumitomo 3M, Tokyo, Japan) until TEWL reached 40-50 g/m²/h. TEWL was measured just before, immediately after and at 1, 3 and 6 hours after tape stripping. The percentage of barrier recovery was calculated using the following formula: (TEWL immediately after tape stripping - TEWL at indicated time point)/(TEWL immediately after tape stripping - TEWL before tape stripping) × 100%.

1-3. TPA-induced dermatitis in mice

The hair of mice was shaved over the rostral part of the back before the start of the experiment. Acute irritant dermatitis was induced by application of 100 µL of 0.006% (w/v) 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich, St. Louis, MO) dissolved in acetone. The dorsal skin was excised 72 hours after TPA application and fixed with 10% neutral formalin, embedded in paraffin and sectioned at 4 µm. The sections were stained with hematoxylin and eosin for

histopathological analysis by light microscopy. The epidermal thickness was analyzed using Image-Pro Plus image analysis software (Media Cybernetics, Bethesda, MD).

1-4. AA-induced mouse ear edema

The inflammatory response was induced by application of 20 μ L of 1% or 3% (w/v) arachidonic acid (AA; Sigma-Aldrich) dissolved in acetone on the surface of ears. The ear thickness was measured with a dial thickness gauge (Teclock, Nagano, Japan) before and 15, 30, 60, 90, 120 minutes after application of AA or acetone.

1-5. Behavioral observation

Substance P (SP; Peptide Institute, Osaka, Japan) was dissolved in physiological saline at the dose of 50 and 100 nmol/site and Compound 48/80 (Sigma-Aldrich) was also dissolved in physiological saline at the dose of 1 and 3 μ g/site and injected intradermally to the rostral back. Immediately after an intradermal (i.d.) injection, the mice were put individually into an acrylic box composed of six cells. They were acclimated to the experimental environment for at least 1 hour, and then behaviors were videotaped for 30 minutes with experimenters kept out of the observation room. Playing back of the video served for counting scratching behavior. The mouse generally scratches several times for about 1 second and a series of these movements was counted as one bout of scratching.

1-6. Statistics

All data are shown as the values of means and SEM. Statistical significance was tested by a Student's *t*-test for non-paired samples or a paired *t*-test for paired samples. For multiple comparisons, analysis of variance (ANOVA) was performed followed by a post hoc test (Dunnett's test).

2. Results and Discussion

Tape stripping (TS) of the stratum corneum, a long-standing technique used in dermatology research to induce epidermal damage, disrupts the cutaneous epithelial barrier and induces various biological responses in the skin as part of the homeostatic repair response (De Koning et al., 2012; Man et al., 1999). To develop skin barrier disruption, the dorsal back of C57BL^{TRPV3^{-/-}} mice and their age-matched littermates (C57BL^{TRPV3^{+/+}}) were treated with TS. Multiple strips immediately increased TEWL in both C57BL^{TRPV3^{+/+}} and C57BL^{TRPV3^{-/-}} mice and recovered 6 hours after TS (Fig. 12). Although it has been reported that TRPV3 is required for the formation of the stratum corneum layer, which is thought to be a key player in maintaining skin barrier functions (Cheng et al., 2010), there were no remarkable difference in skin repair response between C57BL^{TRPV3^{+/+}} and C57BL^{TRPV3^{-/-}} mice. This may be because the effects of TS were too strong to detect the effects of TRPV3 on the skin barrier recovery.

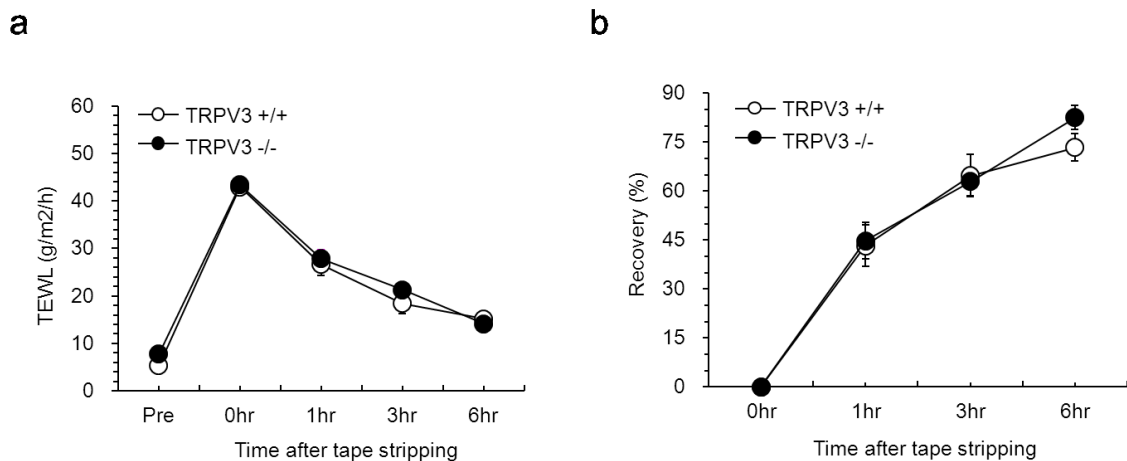


Fig. 12 Tape stripping-induced acute barrier disruption in C56BL^{TRPV3^{+/+}} and C57BL^{TRPV3^{-/-}} mice. (a) TEWL recovery curve after tape stripping mouse skin. TEWL was measured just before, immediately after and at 1, 3 and 6 hours after tape stripping. (b) Recovery rates of TEWL after TS. The percentage of barrier recovery was calculated using the following formula: (TEWL immediately after tape stripping - TEWL at indicated time point)/(TEWL immediately after tape stripping - TEWL before tape stripping) $\times$ 100%. Each value represents the mean $\pm$ SEM of 10 mice.

Next, I examined the impact of TRPV3 on acute irritant dermatitis induced by TPA and AA. Mouse skin inflammation induced by TPA and AA is commonly used as an animal model for evaluation of anti-inflammatory activity (Murakawa et al., 2006; Young et al., 1984; Puignero V and Queralt J, 1997). The application of TPA to mouse skin stimulates inflammatory responses that are mediated by leucocyte infiltration. An early hallmark of skin irritation and local inflammation is thickening within 1–4 hour due to increased vascular permeability, edema and swelling within the dermis. Secondly, polymorphonuclear leukocytes migrate to the dermis within about 24 hours, and produce MPO, a marker enzyme of neutrophil granules, which can be quantified as a measure of the magnitude of neutrophil activation (De Vry et al., 2005). TPA applied to the rostral back skin of mice induced significant increase in the epidermal thickness and infiltration of inflammatory cells in both ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice, but there is no apparent difference between ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice (Fig. 13).

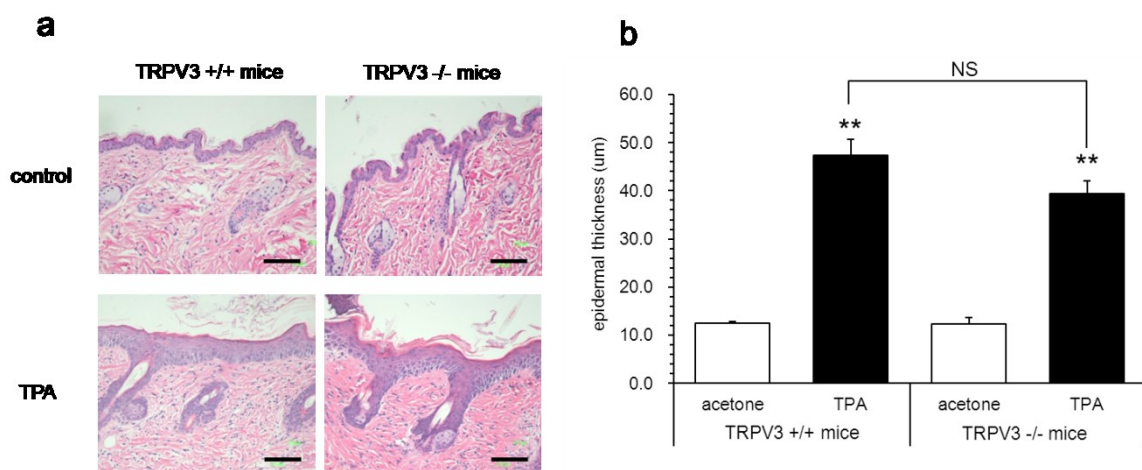


Fig. 13 Acute irritant dermatitis induced by TPA in ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice. (a) TPA were applied to the rostral back skin and 72 hours later the skins were excised and fixed in formalin, and prepared sections were stained with HE. Representative images were captured by light microscopy. Scale bar = 100 μm. (b) Epidermal thickness was increased in both ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice treated with TPA. Each value represents means ± SEM of 4 mice. ***p* < 0.01, Student's *t*-test, compared with acetone-treated control groups (acetone). NS, not significant. (Student's *t*-test)

A single topical application of AA to mice ears caused marked swelling within 15 min of AA application and was maximal at 30 min in a dose dependent manner (Fig. 14). Ear swelling of ICR^{TRPV3^{-/-}} mice treated with AA was milder than that of ICR^{TRPV3^{+/+}} mice (Fig. 14a and 14b), whereas the ear thickness in AA-challenged DS-*Nh* mice was markedly increased compared with DS mice (Fig. 14c and 14d).

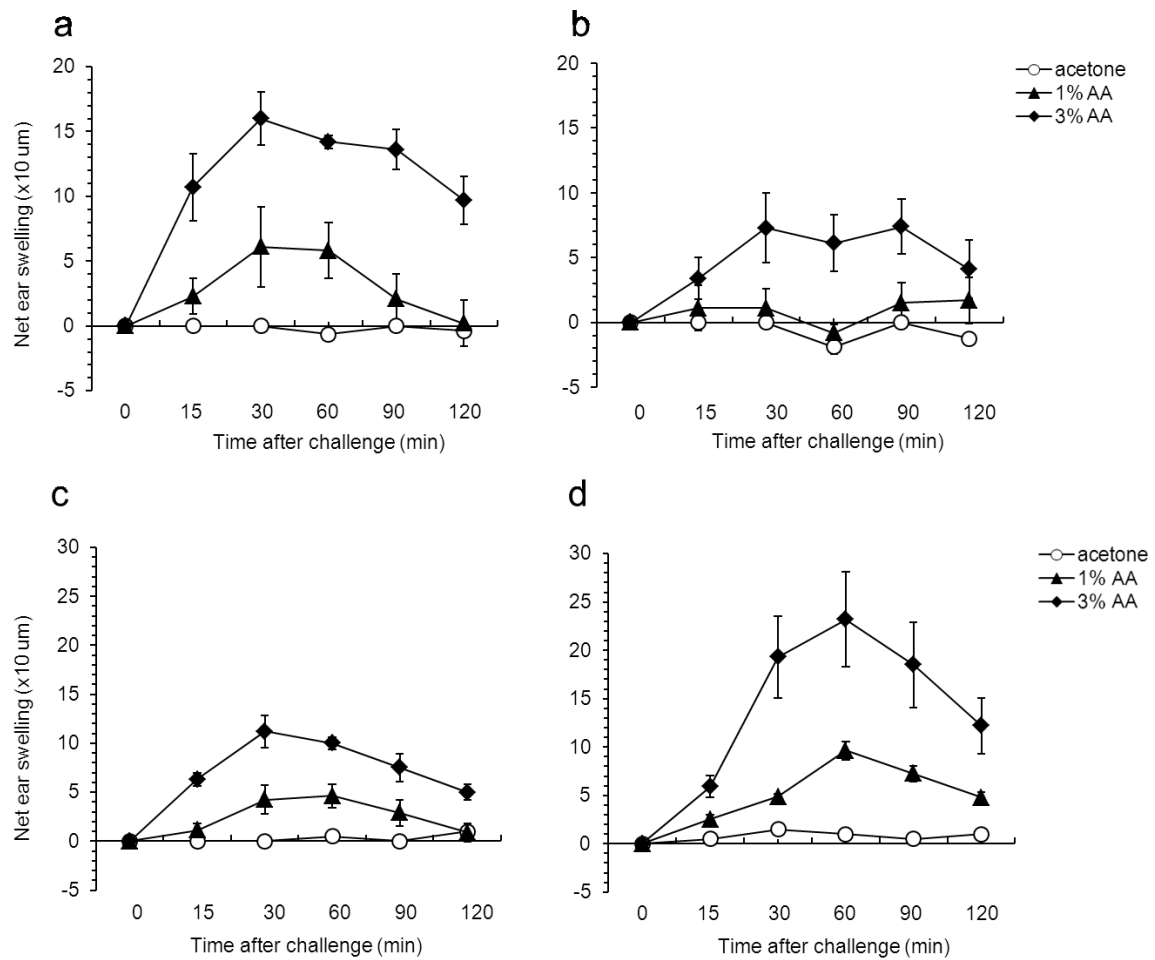


Fig. 14 Effects of TRPV3 on ear swelling induced by AA in mice. Time-course of ear thickness after AA application in ICR^{TRPV3^{+/+}} mice (a), ICR^{TRPV3^{-/-}} mice (b), DS mice (c) and DS-*Nh* mice (d). Ear thickness was measured before and 15, 30, 60, 90, 120 minutes after application of AA or vehicle. Each value represents mean $\pm$ SEM for 4 or 5 mice.

As previously described, topical application of TPA to the skin of mice promotes acute irritant dermatitis and inflammatory responses, including development of edema, initiation of oxidative stress and induction of pro-inflammatory cytokines, cyclooxygenase (COX) 2 and nitric oxide synthase (NOS) 2 (Ho et al., 2007; Song et al., 2008). Previous studies have reported that TPA-induced cellular functions, such as NF- κ B activation and protein kinase C, are responsible for the expression of proinflammatory cytokines and inflammatory enzymes (Cataisson et al., 2005; Ho et al., 2007; Auphan et al., 1995). On the other hand, some pharmacological studies and/or clinical experiences have demonstrated that biologically active metabolites of AA, which is a member of free fatty acids, such as prostaglandins (PGs) and leukotriens (LTs) play an important role in the development of a certain type of inflammatory skin disorders. Inhibitors of COX and lipoxygenase (LO) suppress acute inflammatory responses induced by AA, suggesting that metabolites of AA has important role in acute inflammation induced by AA, most probably related to LTC₄ and LTB₄ release (Inoue et al., 1988; Griswold et al., 1991). Topical application of AA to the ear was caused acute swelling in ICR^{TRPV3+/+} mice, whereas that in ICR^{TRPV3-/-} mice was relatively mild. Interestingly, higher response was observed in DS-*Nh* mice compared with DS mice. However, unlike AA, there was no difference of the skin inflammation induced by TPA between ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice. Therefore there is a possibility that TRPV3 may less affect the expression of pro-inflammatory cytokines and inflammatory enzymes such as COX2 and NOS2 in the irritant skin inflammation, but control the activation or inactivation of enzymes which could metabolize AA such as 5-LO and this contribute to development of the acute edema induced by AA.

To confirm that TRPV3 is involved in transmission of itch sensation itself, substance P (SP) and compound 48/80 known as potent pruritogen were injected intradermally to the rostral back and counted scratching behavior in ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice. An intradermal injection of compound 48/80 (1 or 3 $\mu\text{g}/\text{site}$) and SP (50 and 100 nmol/site), but not saline, elicited scratching behavior to the injected skin by the hind paws; the effect peaked in the initial 10 minutes and almost subsided by 30 minutes (Fig. 15b and 15d). The number of scratching induced by both SP and compound 48/80 was similar between ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice (Fig. 15a and 15c).

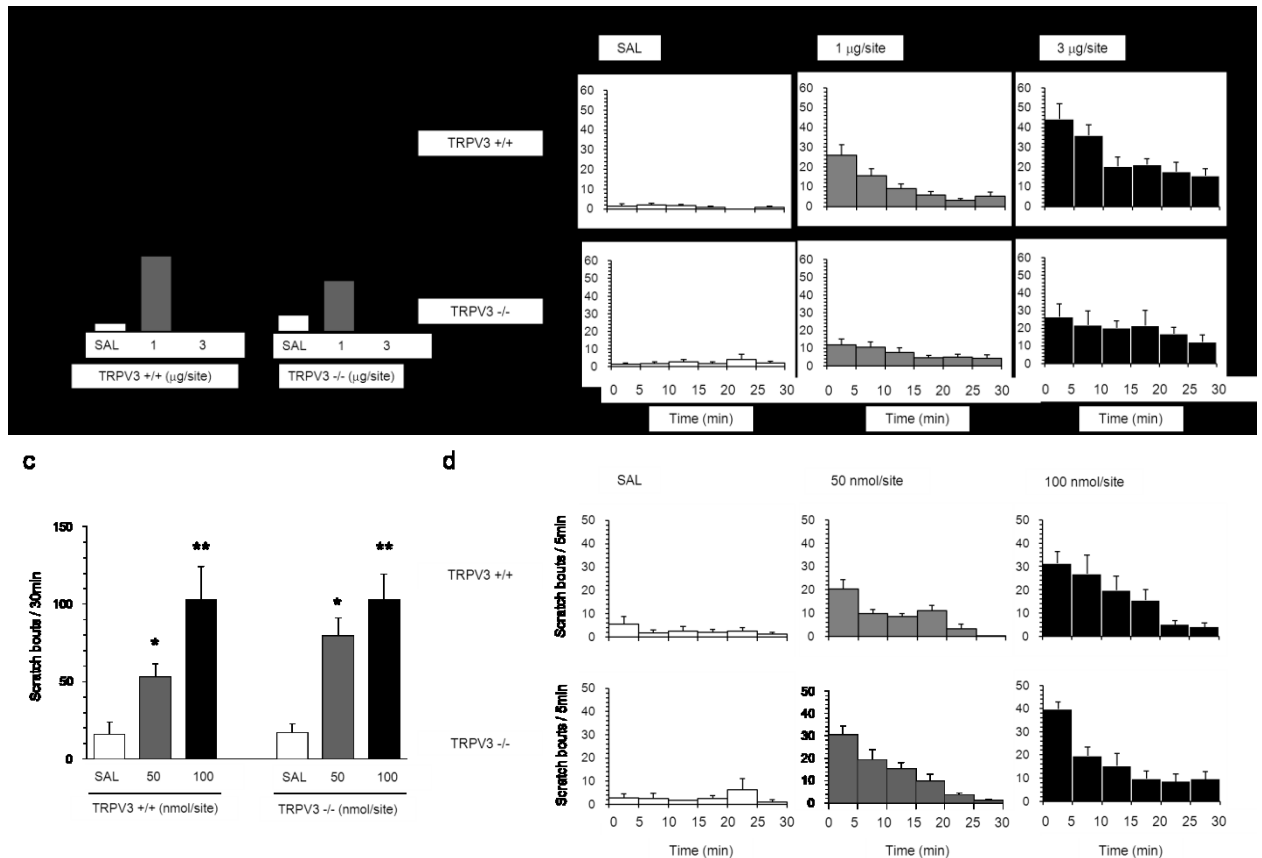


Fig. 15 Spontaneous scratching induced by pruritogen in ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice. The number of scratch bouts for 30 min induced by intradermal injection of compound 48/80 (a) and SP (c). The time course of scratching following compound 48/80 injection (b) and substance P(d). Each value represents mean $\pm$ SEM for 4-8 animals. * $p < 0.05$, ** $p < 0.01$ vs SAL. (Dunnett's test)

There are several possible sites of action of SP (the location of NK1 tachykinin receptors) in the skin. SP acts on mast cells to release histamine (Ebertz et al., 1987; Lowman et al., 1988) and the other cells such as macrophages (Lucey et al., 1994), keratinocytes (Koizumi et al., 1994) and endothelial cells (Bowden et al., 1994) are also potential site of action of intradermal SP to release itch-related mediator. SP also acts directly on the peripheral terminals of primary sensory neurons through NK1 receptors (Andoh et al., 1996). Compound 48/80, a condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde, is well known as a peripheral pruritogen. It produces the itch sensation and causes degranulation of mast cells and results in the release of pruritogens such as histamine and leukotriene B4 (Andoh and Kuraishi, 1998). In this study, ICR^{TRPV3-/-} mice exhibited scratching behavior following injection of SP and compound 48/80 as well as ICR^{TRPV3+/+} mice. In chapter 1, I indicated that TRPV3 is involved in dry skin pruritus associated with nerve fiber expansion in the epidermis via at least partly modulating expression of the nerve extension related molecules such as NGF and Sema3A. Taken together, it is suggested that TRPV3 has little influence on transmission of acute itch sensation induced by pruritogenic agents.

3. Summary

1. Tape stripping immediately increased TEWL in both C57BL^{TRPV3+/+} and C57BL^{TRPV3-/-} mice and recovered 6 hours after TS. However, there were no remarkable difference in skin repair response between C57BL^{TRPV3+/+} and C57BL^{TRPV3-/-} mice.
2. TPA applied to the rostral back skin of mice induced significant increase in the epidermal thickness and inflammatory cells in both ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice and there is no apparent difference between ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice.
3. Ear swelling of ICR^{TRPV3-/-} mice treated with AA was milder than that of ICR^{TRPV3+/+} mice, whereas the ear thickness in AA-treated DS-*N/h* mice was markedly increased compared with DS mice.
4. An intradermal injection of SP and compound 48/80 elicited scratching behavior to the injected skin by the hind paws, however the number of scratching induced by both SP and compound 48/80 were similar between ICR^{TRPV3+/+} and ICR^{TRPV3-/-} mice.

These results demonstrated that TRPV3 seems to have little influence on skin barrier recovery and transmission of acute itch sensation induced by pruritogenic agents. Moreover, TRPV3 may less affect the expression of pro-inflammatory cytokines and inflammatory enzymes such as COX and iNOS in the irritant skin inflammation, but modulate the activity of enzymes which could metabolize AA such as 5-LO and this contribute to maintenance of the skin physiology. In the inflamed tissues, AA is either released from the infiltrating lymphocytes or produced within the sensory fibers or skin cells following the activation of receptors by other inflammatory mediators. Although I could not

carry out analysis of skin levels of AA metabolite profiles or assessment of enzymes activity, TRPV3 might play a part in the aggravation of dermatitis through control of its metabolism and TRPV3 antagonists could be a therapeutic option in managing skin inflammation associated with AA.

Overview

DS-*Nh* mice have a one amino-acid substitution (Gly573 to Ser) in the TRPV3 and Gly573Ser substitution is a gain-of-function mutation. DS-*Nh* mice develop spontaneous dermatitis and are considered to be a model of human AD therefore this mouse is thought to be good tool for investigating the association between activation of TRPV3 and development of dermatitis. With these findings, TRPV3 might have important role in skin physiology and pathophysiology. However, detailed functions of TRPV3 in various skin diseases and a possibility of TRPV3 as a therapeutic target of pruritus have not been fully understood yet. Thus, in this study, I carried out the experiments using ICR^{TRPV3^{-/-}} mice to determine the maximum therapeutic impact of TRPV3 for a certain type of itch and using DS-*Nh* mice to elucidate the role of TRPV3 in the development of DC-mediated skin inflammation. Moreover, To investigate pathophysiological role of TRPV3 in various skin conditions, I elucidated the roles of TRPV3 in the acute itch and inflammation in the skin using DS-*Nh* mice and TRPV3^{-/-} mice.

In chapter 1, it was revealed that TRPV3 might be involved in dry skin pruritus through the nerve fiber expansion and a therapeutic potential of TRPV3 for a certain type of pruritus was suggested by genetic studies using ICR^{TRPV3^{-/-}} mice. TRPV3 may play an important role in histamine independent itch and TRPV3 antagonists could be a therapeutic option in managing AD and dry skin patients with histamine H1R antagonist-resistant pruritus.

In chapter 2, I indicated that TRPV3 might regulate the function of dermal dendritic cells via release of TSLP from keratinocyte at least partly and be involved in exacerbation of allergic skin inflammation. There is a possibility that the increasing skin temperature seems to be strategically used by dermal DC through TRPV3 but not just a warning of some disease.

In chapter 3, I examined the physiological role of TRPV3 in the skin. TRPV3 is unlikely to have a significant impact on skin barrier recovery and transmission of acute itch sensation induced by pruritogenic agents, however might control the activity of enzymes which metabolize AA such as 5-LO

in the irritant skin inflammation. This function of TRPV3 might contribute to maintenance of the skin physiology and play a part in the aggravation of dermatitis. I therefore think that TRPV3 has a therapeutic potential for dermatitis involving AA and its metabolites.

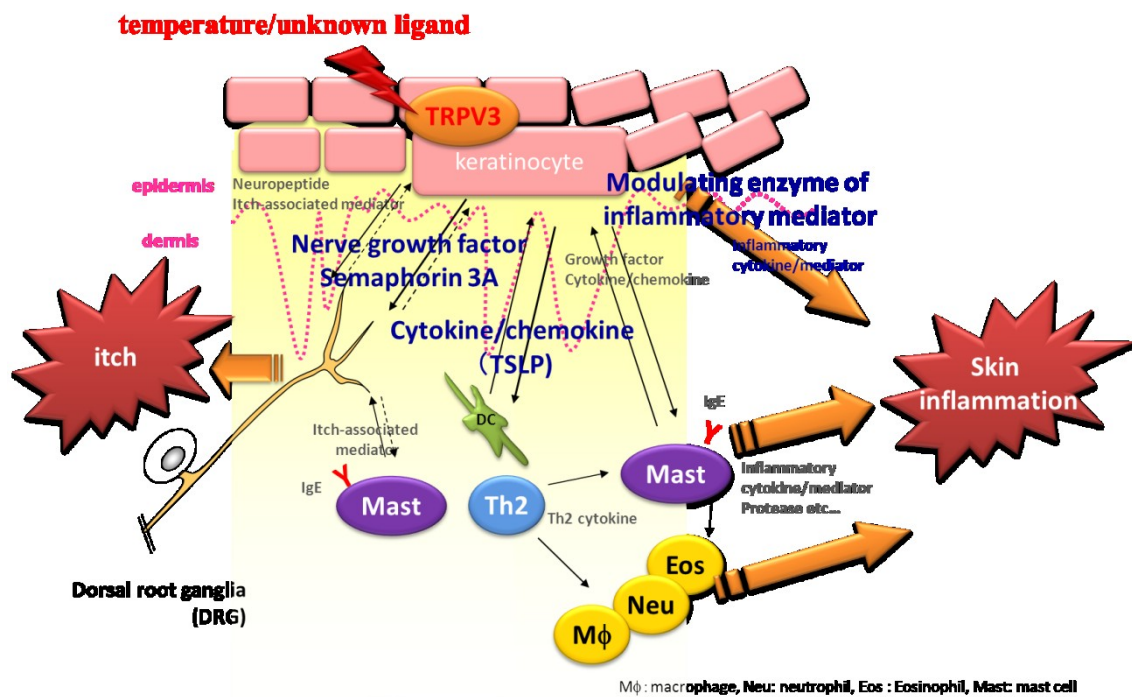


Fig. 16 The pathophysiological mechanism related to TRPV3 in itchy dermatitis.

In conclusion, the role of TRPV3 in a certain type of itch and dermal DC activation were firstly defined, and the physiological role of TRPV3 which may control the activity of lipid-metabolizing enzyme was revealed. These observations provide new insight into mechanisms responsible for the development of skin inflammation in allergic dermatitis and suggested therapeutic potential for pruritus. I believe that TRPV3-targeted drugs become a useful and effective therapy for AD, via the management of inflammation and pruritus.

Lists of Papers

1. Yamamoto-Kasai E, Imura K, Yasui K, Shichijo M, Oshima I, Hirasawa T, Sakata T, Yoshioka T. TRPV3 as a therapeutic target for itch. *J Invest Dermatol* (2012) 132 (8) :2109-2112
2. Yamamoto-Kasai E, Yasui K, Shichijo M, Sakata T, Yoshioka T. Impact of TRPV3 on the Development of Allergic Dermatitis as a Dendritic cell Modulator *Exp Dermatol* (2013) 22 (12) : 820-824
3. 葛西 えりか・吉岡 健 アトピー性皮膚炎の動物モデル アレルギーの臨床 (2012) 430: 527-532
4. 葛西 えりか・吉岡 健 アトピー性皮膚炎の動物モデル アレルギーの臨床 (2013) 451: 1230-1236

Acnkowlegdements

I am very thankful to Dr. Tsuneaki Sakata for his support and encouragement on the planning of experiments. I thank Dr. Michitaka Shichijo, Dr. Gaku Sakaguchi, Dr. Kiyoshi Yasui, and Dr. Shunji Shinohara for giving the opportunity to do the present study and Dr. Takeshi Yoshioka for grateful suggestions, supports and discussions to do the present study. And I would like to thank Dr. Kinichi Imura for valuable discussions on the progress of the experiments. I also wish to thank my colleagues for their help during the research.

References

- Albers KM, Wright DE, Davis BM, *et al* (1994). Overexpression of nerve growth factor in epidermis of transgenic mice causes hypertrophy of peripheral nerve system. *J Neurosci* 14: 1422-1432
- Andoh T and Kuraishi Y, (1998). Intradermal leukotriene B₄, but not prostaglandin E₂, induces itch-associated responses in mice. *Eur J Pharmacol* 353: 93-96
- Andoh T, Nagasawa T and Kuraishi Y, *et al* (1996). Expression of tachykinin NK1 receptor mRNA in dorsal root ganglia of the mouse. *Mol Brain Res* 35:329-332
- Asakawa M, Yoshioka T, Hikita I, *et al* (2005). WBN/Kob-Ht Rats Spontaneously Develop Dermatitis under Conventional Conditions: Another Possible Model for Atopic Dermatitis. *Exp Anim* 54: 461-465
- Asakawa M, Yoshioka T, Matsutani T, *et al* (2006). Association of a mutation in TRPV3 with defective hair growth in rodents. *J Invest Dermatol* 126: 2664-2672
- Auphan N, DiDonato JA, Rosette C, *et al* (1995). Immunosuppression by glucocorticoids: Inhibition of Nf-kappa B activity through induction of I kappa B synthesis. *Science* 270: 286-290.
- Bogiatzi SI, Fernandez I, Bichet JC *et al* (2007). Cutting edge: Proinflammatory and Th2 cytokines synergize to induce thymic stromal lymphopoietin production by human skin keratinocytes. *J Immunol* 178: 3373-3377
- Bowden JJ, Garland AM, Baluk P, *et al* (1994). Direct observation of substance P-induced internalization of neurokinin 1 (NK1) receptors at sites of inflammation. *Proc Natl Acad Sci USA* 91:8964-8968.
- Cataisson C, Pearson AJ, Torgerson S, Nedospasov SA, Yuspa SH, *et al* (2005). Protein kinase C alpha-mediated chemotaxis of neutrophils requires NF-kappa B activity but is independent of TNF alpha signaling in mouse skin in vivo. *J Immunol* 174: 1686-1692
- Cheng P, Corzo CA, Luetke N, *et al* (2008). Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *J Exp Med* 205: 2235-2249
- Cheng X, Jin J, Hu L, *et al* (2010). TRP channel regulates EGFR signaling in hair morphogenesis and skin barrier formation. *Cell* 141: 331-343
- Clapham DE (2003). TRP channels as cellular sensors. *Nature* 426: 517-524
- Cumberbatch M and Kimber I (1995). Tumour necrosis factor-alpha is required for accumulation of dendritic cells in draining lymph nodes and for optimal contact sensitization. *Immunology* 94: 31-35
- Cumberbatch M, Dearman RJ, Kimber I, *et al* (1997). Langerhans cells require signals from both tumour necrosis factor-alpha and interleukin-1 beta for migration. *Immunology* 92: 388-395

- De Koning HD, van den Bogaard EH, Bergboer JG, *et al* (2012). Expression profile of cornified envelope structural proteins and keratinocyte differentiation-regulating proteins during skin barrier repair. *Br J Dermatol* 166: 1245-1254
- De Vry CG, Valdez M, Lazarov M, *et al* (2005). Topical application of a novel immunomodulatory peptide, RDP58, reduces skin inflammation in the phorbol ester-induced dermatitis model. *J Invest Dermatol* 125: 473-481
- Dontchev VD and Letourneau PC (2002). Nerve growth factor and semaphorin 3A signaling pathways interact in regulating sensory neuronal growth cone motility. *Neuroscience* 22: 6659-6669
- Ebertz JM, Hirshman CA, Kettelkamp NS, *et al* (1987). Substance P-induced histamine release in human cutaneous mast cells. *J Invest Dermatol* 88:682-685
- Fartasch M (1997). Epidermal barrier in disorders of the skin. *Microsc Res Tech* 38: 361-372
- Fukamachi S, Bito T, Shiraishi N, *et al* (2011). Modulation of semaphorin 3A expression by calcium concentration and histamine in human keratinocytes and fibroblasts. *J Dermatol Sci* 61: 118-123
- Geha RS (2003). Allergy and hypersensitivity nature versus nurture in allergy and hypersensitivity editorial overview. *Curr Opin Immunol* 15: 603-608
- Gopinath P, Wan E, Holdcroft A, *et al* (2005). Increased capsaicin receptor TRPV1 in skin nerve fibres and related vanilloid receptors TRPV3 and TRPV4 in keratinocytes in human breast pain. *BMC Womens Health*. 8: 2
- Griswold DE, Marshall PJ, Lee JC, *et al* (1991). Pharmacology of the pyrroloimidazole, SK&F 105809--II. Antiinflammatory activity and inhibition of mediator production in vivo. *Biochem Pharmacol* 25: 42: 825-831
- Heyer G, Dotzer M, Diepgen TL, *et al* (1997). Opiate and H1 antagonist effects on histamine induced pruritus and alopecia, *Pain* 73: 239-243
- Hikita I, Yoshioka T, Mizoguchi T, *et al* (2002). Characterization of dermatitis arising spontaneously in DS-Nh mice maintained under conventional conditions: another possible model for atopic dermatitis. *J Dermatol Sci* 30: 142-153
- Ho YS, Lai CS, Liu HI, *et al* (2007). Dihydrolipoic acid inhibits skin tumor promotion through anti-inflammation and anti-oxidation. *Biochem Pharmacol* 73: 1786-1795
- Howell MD, Jones JF, Kisich KO, *et al* (2004). Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. *J Immunol* 172: 1763-1767
- Hu HZ, Xiao R, Wang C, *et al* (2006). Potentiation of TRPV3 channel function by unsaturated fatty acids. *J Cell Physiol* 208: 201-212
- Hyun-Ok Kua HO, Jeonga SH, Kanga HG, *et al* (2009). Gene expression profiles and pathways in skin inflammation induced by three different sensitizers and an irritant. *Toxicol Lett* 190: 231-237
- Ikoma A, Steinhoff M, Ständer S, *et al* (2006). The neurobiology of itch. *Nat Rev Neurosci* 7: 535-547

- Imura K, Yoshioka T, Hikita I, *et al* (2007). Influence of TRPV3 mutation on hair growth cycle in mice. *Biochem Biophys Res Commun* 363: 479-483
- Inagaki N, Nagao M, Igeta K, *et al* (2001). Scratching behavior in various strains of mice. *Skin Pharmacol Appl Skin Physiol* 14: 87-96
- Inoue H, Mori T, Koshihara Y, *et al* (1988). Sulfidopeptide-leukotrienes are major mediators of arachidonic acid-induced mouse ear edema. *Prostaglandins* 36: 5: 731-739
- Ito T, Liu YJ, Arima K, *et al* (2012). Cellular and molecular mechanisms of TSLP function in human allergic disorders--TSLP programs the "Th2 code" in dendritic cells. *Allergol Int* 61: 35-43.
- Kabashima K, Sakata D, Nagamachi M, *et al* (2003). Prostaglandin E2-EP4 signaling initiates skin immune responses by promoting migration and maturation of Langerhans cells. *Nat Med* 9: 744-749
- Kimura T and Miyazawa H, (1989). The 'butterfly' sign in patients with atopic dermatitis. evidence for the role of scratching in the development of skin manifestations. *J Am Acad Dermatol* 21: 579-580
- Koizumi H, Yasui C, Fukaya T, *et al* (1994). Substance P induces inositol 1,4,5-trisphosphate and intracellular free calcium increase in cultured normal human epidermal keratinocytes. *Exp Dermatol* 3:40-44
- Kripke ML, Munn CG, Jeevan A, *et al* (1990). Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. *J Immunol* 145: 2833-2838
- Leung DYM and Bieber T (2003). Atopic dermatitis. *Lancet* 361: 151-160
- Lin Z, Chen Q, Lee M *et al* (2012). Exome sequencing reveals mutations in TRPV3 as a cause of Olmsted syndrome. *Am J Hum Genet* 90: 558-564
- Lowman MA, Benyon RC and Chruch MK, *et al* (1988). Characterization of neuropeptide-induced histamine release from human dispersed skin mast cells. *Br J Pharmacol* 95: 121-130
- Lucey DR, Novak JM, Polonis VR, *et al* (1994). Characterization of substance P binding to human monocytes/macrophages. *Clin Diagn Lab Immunol* 1:330-335
- Macatonia SE, Knight SC, Edwards AJ, *et al* (1987). Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. *J Exp Med* 166: 1654-1667
- Man MQ, Wood L, Elias PM, *et al* (1999). Cutaneous barrier repair and pathophysiology following barrier disruption in IL-1 and TNF type I receptor deficient mice. *Exp Dermatol* 8:261-266
- Miyamoto T, Nojima H, Shinkado T, *et al* (2002). Itch-associated response induced by experimental dry skin in mice. *Jpn J Pharmacol* 88: 285-292
- Moqrich A, Hwang SW, Earley TJ, *et al* (2005). Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. *Science* 307: 1468-1472

- Murakawa M, Yamaoka K, Tanaka Y, *et al* (2006). Involvement of tumor necrosis factor (TNF)- α in phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin edema in mice. *Biochem Pharmacol* 71: 1331-1336
- Nilius B, Owsianik G, Voets T, *et al* (2007). Transient Receptor Potential Channels in Disease. *Physiol Rev* 87: 165-217
- Nilius B and Owsianik G (2011). The transient receptor potential family of ion channels. *Genome Biol* 12: 3: 218-229
- Nilius B and Bíró T (2013). TRPV3: a “more than skinny” channel. *Exp Dermatol* 22: 447-452
- Novak N, Bieber T, Leung DY, *et al* (2003). Immune mechanisms leading to atopic dermatitis. *J Allergy Clin Immunol* 112: S128-139
- Ostberg JR, Patel R, Repasky EA, *et al* (2000). Regulation of immune activity by mild (fever-range) whole body hyperthermia: effects on epidermal Langerhans cells. *Cell Stress Chaperones* 5: 458-461
- Park CO, Noh S, Jin S *et al* (2013). Insight into newly discovered innate immune modulation in atopic dermatitis. *Exp Dermatol* 22: 6-9
- Pastore S, FanalesBelasio E, Albanesi C, *et al* (1997). Granulocyte macrophage colony-stimulating factor is overproduced by keratinocytes in atopic dermatitis: implications for sustained dendritic cell activation in the skin. *J Clin Invest* 99: 3009-3017
- Paus R, Schmelz M, Bíró T, *et al* (2006). Frontiers in pruritus research: scratching the brain for more effective itch therapy. *J Clin Invest* 116: 1174-1186
- Peier AM, Reeve AJ, Andersson DA, *et al* (2002). A heat-sensitive TRP channel expressed in keratinocytes. *Science* 296: 2046-2049
- Pincelli C, Seignani C, Manfredini R, *et al* (1994). Expression and function of nerve growth factor and nerve growth factor receptor on cultured keratinocytes. *J Invest Dermatol* 103: 13-18
- Puig-Kröger A, Sanz-Rodríguez F, Longo N, *et al* (2000). Maturation-dependent expression and function of the CD49d integrin on monocyte-derived human dendritic cells. *J Immunol* 165:4338-4345
- Puigneró V and Queralt J (1997). Effect of topically applied cyclosporin A on arachidonic acid (AA)-and tetradecanoylphorbol acetate (TPA)-induced dermal inflammation in mouse ear. *Inflammation* 21:357-369
- Renkl AC, Wussler J, Ahrens T, *et al* (2005). Osteopontin functionally activates dendritic cells and induces their differentiation toward a Th1-polarizing phenotype. *Blood* 106: 946-955
- Robert C and Kupper T.S, (1999). Inflammatory skin diseases, T cells and immune surveillance. *N Engl J Med.* 341: 1817-1828
- Rothman S (1941). Physiology of itching. *Physiol Rev* 21: 357-381

- Sano Y, Masuda K, Tamagawa-Mineoka R, *et al* (2013). Thymic stromal lymphopoietin expression is increased in the horny layer of patients with atopic dermatitis. *Clin Exp Immunol* 171: 330-337
- Sherkheli MA, Benecke H, Doerner JF, *et al* (2009). Monoterpenoids induce agonist-specific desensitization of transient receptor potential vanilloid-3 (TRPV3) ion channels. *J Pharm Sci* 12:116-128
- Smith GD, Gunthorpe MJ, Kelsell RE, *et al* (2002). TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature* 418: 186-190
- Song HY, Lee JA, Ju SM, *et al* (2008). Topical transduction of superoxide dismutase mediated by HIV-1 Tat protein transduction domain ameliorates 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice. *Biochem Pharmacol* 75: 1348-1357
- Soumelis V, Reche PA, Kanzler H, *et al* (2002). Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 3: 673-680
- Sugiura H, Omoto M, Hirota Y, Danno K, Uehara M, *et al* (1997). Density and fine structure of peripheral nerves in various skin lesions at atopic dermatitis. *Arch Derm Res* 289: 125-131
- Trautmann A, Akdis M, Kleemann D, *et al* (2000). T cellmediate Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. *J Clin Invest* 106: 25-35
- Tobin D, Nabarro G, Baart de la Faille H, van Vloten WA, van der Putte SC, Schuurman HJ, *et al* (1992). Increased number of immunoreactive nerve fibers in atopic dermatitis. *J Allergy Clin Immunol* 90: 613-622
- Urashima R and Mihara M, (1998). Cutaneous nerves in atopic dermatitis. A histological, immunohistochemical and electron microscopic study. *Virchows Arch* 432: 363-370
- Valladeau J, Ravel O, Dezutter-Dambuyant C, *et al* (2000). Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* 12: 71-81
- Voets T (2012). Quantifying and Modeling the Temperature-Dependent Gating of TRP Channels. *Rev Physiol Biochem Pharmacol* 162: 91-119
- Wahlgren CF (1999). Itch and atopic dermatitis: an overview. *J Dermatol* 26: 770-779
- Wahlgren CF, Hägermark O, Bergström R, *et al* (1990). The antipruritic effect of a sedative and a non-sedative antihistamine in atopic dermatitis. *Br J Dermatol* 122: 545-551
- Xiao R, Tian J, Tang J, *et al* (2008). The TRPV3 mutation associated with the hairless phenotype in rodents is constitutively active. *Cell Calcium* 43: 334-343
- Xu H, Ramsey IS, Kotecha SA, *et al* (2002). TRPV3 is a calcium-permeable temperature- sensitive cation channel. *Nature* 418: 181-186
- Yamaguchi J, Nakamura F, Aihara M, *et al* (2008). Semaphorin3A alleviates skin lesions and scratching behavior in NC/Nga mice, an atopic dermatitis model. *J Invest Dermatol* 128: 2842-2849

- Yamamoto-Kasai E, Imura K, Yasui K, *et al* (2012). TRPV3 as a therapeutic target for itch. *J Invest Dermatol* 132: 2109-2112
- Yoshioka T, Hikita I, Matsutani T, *et al* (2003). DS-*Nh* as an experimental model of atopic dermatitis induced by *Staphylococcus aureus* producing staphylococcal enterotoxin C. *Immunology* 108: 562-569
- Yoshioka T, Hikita I, Asakawa M, *et al* (2006). Spontaneous scratching behaviour in DS-*Nh* mice as a possible model for pruritus in atopic dermatitis. *Immunology* 118: 293-301
- Yoshioka T, Imura K, Hikita I, *et al* (2007). Impact of T-cell receptor V-beta haplotypes on the development of dermatitis in DS-*Nh* mice: synergistic production of interleukin-13 caused by staphylococcal enterotoxin C and peptide glycans from *Staphylococcus aureus*. *Immunology* 121: 51-61
- Yoshioka T, Imura K, Asakawa M, *et al* (2009). Impact of the Gly573Ser Substitution in TRPV3 on the Development of Allergic and Pruritic Dermatitis in Mice. *J Invest Dermatol* 129: 714-722
- Yoshida T, Inoue R, Morii T, *et al* (2006). Nitric oxide activates TRP channels by cysteine S-nitrosylation. *Nat Chem Biol* 2: 596-607.
- Young JM, Spires DA, Bedord CJ, *et al* (1984). The mouse ear inflammatory response to topical arachidonic acid. *J Invest Dermatol* 82: 367-371