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1 **Peptidylarginine deiminase is involved in maintaining the cornified oral mucosa**
2 **of rats**

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20

1 Abstract

2 *Background and Objective:* Epithelial cells derived from different regions exhibit
3 marked differences in their differentiation capacity, allowing them to provide a suitable
4 protective barrier. We aimed to clarify the role of peptidylarginine deiminase (PAD) in
5 modifying the key epidermal proteins filaggrin (FLG) and keratin 1 (K1) during
6 stratification of the rat palate and buccal mucosa.

7 *Materials and Methods:* We performed immunofluorescence, immunoblotting, PAD
8 activity assays, and two-dimensional electrophoresis, and developed an organotypic
9 culture model.

10 *Results:* PAD1 expression was highest in the palate, whereas PAD2, PAD3, and PAD4
11 expression was highest in the skin, suggesting the tissue-specific expression of PAD
12 isozymes that leads to differences in calcium dependency. Immunoblotting showed that
13 the FLG monomer, as well as its degradation products and precursor (proFLG), were
14 most abundantly expressed in the skin but had low expression in the palate, whereas
15 only faint proFLG expression was detected in the buccal mucosa. FLG and K1 were
16 co-localized with PAD1 and were likely to be citrullinated in the cornified layers of the
17 skin; this co-localization was not detected on the palatal surface, and dot-like presence
18 of proFLG that might be citrullinated and that of PAD1 were found in the granules of
19 the palate. Organotypic models derived from the rat palate revealed that PAD inhibition
20 reduced the breakdown of FLG, increased its association with K1 together with
21 epithelial compaction, and decreased permeability in a dye permeability assay.
22 Conversely, PAD stimulation had the opposite effects.

23 *Conclusions:* Citrullination is likely a protein modification that plays an important role
24 in maintaining the structure and function of oral cornified mucosa in a way that is
25 distinctly different from that of the skin.

1 **Introduction**

2 Citrullination or deimination is one of the post-translational modifications of
3 proteins. In this process, peptidylarginine deiminase (PAD) changes the structure of key
4 epidermal proteins, such as filaggrin (FLG) and keratins, by converting arginine
5 residues into neutral citrulline residues in the stratifying epithelium (1). Profilaggrin
6 (ProFLG) is a large molecule that accumulates in keratohyalin granules. During
7 cornification of the skin, it is proteolyzed into FLG repeats (monomers) and other
8 domains (2). FLG monomers bind to keratin intermediate filaments (KIFs), causing
9 tight aggregation into macrofibrils (3). The tightly packed KIF leads to cellular
10 compaction aligned parallel to the outer surface of the epidermis, which generates a
11 barrier against the environment (4). However, loss of positive charge per citrulline
12 residue induced by PAD changes the intramolecular and intermolecular interactions
13 with the keratin matrix, making the less ordered FLG structure more susceptible to
14 proteolytic degradation (1).

15 The PAD family is composed of five calcium-dependent isozymes. PAD1 has
16 mainly been detected in the human epidermis and uterus (1), and rat epidermis and
17 stomach (5). Rat PAD1 cDNA was cloned from a retinoic acid-treated culture of a
18 newborn keratinocyte cell line (6). PAD1 plays a major role in the citrullination of
19 keratin 1 (K1) in the epidermis (7, 8). K1 citrullination was shown to be decreased in
20 the hyperproliferative plaques in psoriasis and bullous congenital ichthyosiform
21 erythroderma (9, 10). PAD2 is expressed in a variety of rat tissues such as the epidermis,
22 brain, lung, spleen, stomach, and uterus (5). PAD3 is expressed in the inner root sheath
23 and medulla of hair follicles where it crosslinks the structural protein trichohyalin (1,
24 11). PAD4 is found in hematopoietic cells (1). All of these PAD subtypes are reportedly
25 expressed in the rat epidermis (5). Among them, PAD1 and PAD3 proteins are

1 colocalized with FLG, suggesting that they are both involved in FLG citrullination in
2 the epidermis (8, 11).

3 Epithelial cells derived from different regions including the oral mucosa exhibit
4 marked differences in their differentiation capacity, allowing them to provide a suitable
5 protective barrier (12). Dale *et al.* (13) demonstrated site and age differences in the
6 distribution of FLG and its precursor protein in rat oral mucosa; as in the epidermis, the
7 protein was observed in cornified layers and in keratohyalin granules throughout the
8 mouth of newborn (0–2 days old) rats, whereas it was mainly present in keratohyalin
9 granules in adult rats. Our previous immunohistochemical and real-time PCR studies
10 showed that the relative levels of FLG differed among the palate, buccal mucosa,
11 esophagus, and skin in 7-day-old rats (14). FLG is expressed in the human oral mucosa
12 as well as in the epidermis (15, 16). ProFLG is detectable in human nonkeratinized,
13 parakeratinized, and orthokeratinized oral epithelium, although FLG monomer is
14 expressed in only the orthokeratinized palate (15). Expression of FLG proteins in the
15 human oral mucosa was shown to markedly increase under pathological conditions
16 involving hyperkeratosis such as lichen planus and leucoplakia (17), which occur
17 independently of defects in the FLG gene (18). These data suggest that the enzymatic
18 breakdown of proFLG and the resulting pattern of stratification are likely to be distinct
19 among stratified tissues such as the skin and oral mucosa. Thus, it is likely that the post-
20 translational modification of proFLG and FLG by PAD influences epithelial
21 differentiation and cornification of oral mucosa (15).

22 In this study, we aimed to clarify the role of protein citrullination in the
23 stratification and cornification of oral mucosa. First, we compared the expression of
24 FLG, proFLG, and PAD isozymes, and evaluated PAD activity and protein
25 citrullination in the epithelial cell layers of the skin, palate, and buccal mucosa of 7-

1 day-old rats. Second, we developed a 3-dimensional (3D) mucosal model derived from
2 epithelial cells and fibroblasts isolated from the rat palate. Finally, we examined
3 whether an inhibitor and activator of PAD could alter FLG expression, as well as the
4 structure and function of the epithelial layers.

5

6 **Materials and Methods**

7 **Antibodies and reagents**

8 The following primary antibodies were used: mouse monoclonal K1 (NB100-
9 2756; Novus Biologicals, Littleton, CO), β -actin (sc-47778; Santa Cruz Biotechnology,
10 Santa Cruz, CA), rabbit polyclonal anti-cyclic citrullinated peptide (CCP, bs-1053R;
11 Bioss Inc., Woburn, MA), anti-PAD1 (NBP2-37761; Novus Biologicals), human anti-
12 modified citrulline (MC, clone C4; EMD Millipore Corp., Temecula, CA), and rabbit
13 anti-FLG antiserum (generously gifted by Dr. Ishigami, Tokyo Metropolitan Institute
14 of Gerontology, Tokyo, Japan). Cl-Amidine trifluoroacetate salt was purchased from
15 Cayman Chemical (Ann Arbor, MI) and theophylline-7-acetic acid (acefylline) was
16 purchased from Toronto Research Chemicals, Inc. (North York, Canada). The other
17 compounds were obtained from Sigma-Aldrich (St. Louis, MO).

18

19 **Histological and immunohistochemical examination**

20 Neonatal Wistar rats (7 days old) were anesthetized with isoflurane and sacrificed.
21 Permission for the procedures was granted by the Animal Research Committee of
22 Fukuoka Dental College (Fukuoka, Japan). The palate, buccal mucosa, and abdominal
23 skin were dissected as previously described (14). The *in vivo* rat tissues or reconstructed
24 model were fixed in 10% formalin solution, routinely processed, and embedded in
25 paraffin. Sections 3 μ m thick were stained with hematoxylin and eosin (H&E). To

1 obtain frozen sections 10 μm thick, the samples were fixed briefly in 4%
2 paraformaldehyde (PFA) solution, gradually dehydrated with 10%, 15%, and 20%
3 sucrose solution, and embedded in Super Cryoembedding Medium (Section-Lab Co.,
4 Hiroshima, Japan). To detect MC immunoreactivity in the tissue sections, citrulline
5 residues on the sections were chemically modified with 2,3-butanedione monoxime and
6 antipyrine under acidic conditions (19) prior to applying the antibodies. For
7 immunofluorescence double staining, the tissue sections with or without chemical
8 modification were blocked in 10% normal goat serum and then incubated with a
9 combination of two antibodies listed below for 2 h; anti-FLG serum (1:40,000), anti-
10 MC (1:100), CCP (1:200), PAD1 (1:100), and K1 (1:100) antibodies, followed by a 1 h
11 incubation at room temperature with goat anti-rabbit, anti-mouse, or anti-human
12 immunoglobulin G (IgG) antibodies conjugated with Alexa 488 or Alexa 594 (1:800).
13 The sections were counterstained with DAPI (ProLong Gold; Thermo Fisher Scientific,
14 Waltham, MA). Fluorescence was observed using a fluorescence microscope (BZ-
15 9000; Keyence, Osaka, Japan) and a confocal laser scanning microscope (LSM710;
16 Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Quantification of the relative
17 area and width of the layers in the images and calculation of Pearson's colocalization
18 coefficient were performed with ImageJ software (National Institutes of Health [NIH],
19 Bethesda, MD).

20

21 **Evaluation of mRNA expression levels by real-time PCR**

22 Total RNA was isolated from the rat tissues and the reconstructed model
23 (NucleoSpin RNA Kit; Macherey-Nagel GmbH & Co. KG, Düren, Germany), after
24 which the cDNA was reverse transcribed (PrimeScript RT Reagent Kit; Takara Bio Inc.,
25 Tokyo, Japan). Quantitative PCR (qPCR) was performed with the Applied Biosystems

1 7500 Real-Time PCR System with the thermal cycling conditions recommended by the
2 manufacturer, and with the SYBR^R Green method using a pair of specific primers to
3 target rat proFLG (Forward: 5'-ATGCTAGATGTGGACCACGATGAC-3', Reverse:
4 5'-TGTTCCCTCTTCCTCTTGGGCTT-3'; GenBank accession number:
5 XM_003753631), rat PAD1 (Forward: 5'-CACAGGCAAGGTGAAGAAAGG-3',
6 Reverse: 5'-TGTTTGTAGTTGGAGAGGGAGG-3'; GenBank accession number:
7 NM_019332.1), rat PAD2 (Forward: 5'-TCCTGAAAGAGGTGAAGAACCTG-3',
8 Reverse: 5'-TACTGGGAAGCCTTTGTGAGG-3'; GenBank accession number:
9 NM_017226.1), rat PAD3 (Forward: 5'-TTGGCTTGTGCTTCCTATGGT-3', Reverse:
10 5'-GTAGTGAGGGTGTGAAATAGTCTG-3'; GenBank accession number:
11 NM_017230.2) and rat PAD4 (Forward: 5'-GCTGGGAAGGATCAGAGCAC-3',
12 Reverse: 5'-GGGAGTCTTCGTGCTTAGGG-3'; GenBank accession number:
13 NM_017227.1). The qPCR reactions were performed in duplicate. A standard curve
14 was used to calculate expression of the PADs and proFLG normalized to rat GAPDH,
15 which served as the endogenous control (Forward: 5'-
16 GACATGCCGCCTGGAGAAAC-3', Reverse: 5'-AGCCCAGGATGCCCTTTAGT-
17 3'; GenBank accession number: NM_017008), and the relative quantity was compared.

18

19 **Gel electrophoresis and western blot analysis**

20 The rat oral mucosa was minced and homogenized in lysis buffer containing 8 M
21 urea, 10 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM Tris-HCl (pH 8.0), and 1%
22 protease inhibitor cocktail (Sigma). After extraction, the lysates (15 µg) were
23 centrifuged for 5 min at 1000 × g, and the proteins were resolved on gradient sodium
24 dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (4-20%). For
25 two-dimensional (2D) gel electrophoresis, the proteins (50–80 µg) were extracted with

1 lysis buffer containing 8 M urea, 2 M thiourea, 2% CHAPS, 1% Triton X-100, 2%
2 Biolyte (pH 4–10; BioRad, Hercules, CA), 10 mg/mL DTT, 1% protease inhibitor
3 cocktail, and 10 mM Tris-Cl (pH 8.0). Proteins were loaded on agarose gels with a pH
4 range of 3–10 (A-M310, agarGEL; ATTO Corp., Tokyo, Japan), separated in the first
5 dimension using non-equilibrium pH gradient electrophoresis (NEpHGE) (20), and
6 separated on SDS-PAGE gradient gels (4–20%). Then the samples were
7 electrophoretically transferred to a PVDF membrane (Hybond-P; GE Healthcare,
8 Wauwatosa, WI). Total protein staining was performed using colloidal gold solution
9 (ProtoGold; Agar Scientific, Essex, UK). To detect MC proteins, citrulline residues on
10 the membrane were modified with 2,3-butanedion monoxime and antipyrine by
11 overnight incubation under acidic conditions (Anti-Citrulline [Modified] Detection Kit,
12 17-347B; EMD Millipore). For protein detection, membranes were incubated with anti-
13 FLG serum (1:100,000), and anti-MC (1:1000), CCP (1:500), β -actin (1:1000), and K1
14 (1:100), antibodies followed by incubation with horseradish peroxidase (HRP)-
15 conjugated goat anti-rabbit and anti-mouse IgG antibodies (1:400,000; Jackson
16 ImmunoResearch, West Grove, PA), and goat anti-human IgG (1:2000, EMD
17 Millipore). For stripping of immunoblots, membrane was incubated with 62.5 mM Tris
18 buffer (pH=6.7) containing 100 mM 2-mercaptoethanol and 2% SDS at 37 °C for 15
19 min. Proteins were visualized using enhanced chemiluminescence (ImmunoStar LD;
20 Wako Pure Chemical, Osaka, Japan).

21

22 **PAD activity**

23 An antibody-based assay for PAD activity (ABAP assay) was performed to measure
24 PAD activity (21) (MQ17.101-96, ModiQuest Research, Nijmegen, The Netherlands).
25 Briefly, tissue samples were homogenized in lysis buffer (100 mM NaCl, 10 mM β -

1 mercaptoethanol, 10% glycerol, 1% protease inhibitor cocktail, 20 mM Tris-HCl pH
2 7.4). After centrifugation at 4°C, the tissue supernatant (1 µg total protein) or blank
3 supernatant diluted in citrullination buffer containing different concentrations of
4 calcium was incubated at 37°C for 75 min in the wells in which arginine-containing
5 peptides were immobilized. Immobilized peptides containing citrulline were used as a
6 positive control. PAD activity was assayed using a specific monoclonal mouse antibody
7 for citrulline, HRP-labeled anti-mouse IgG antibody, and tetramethylbenzidine
8 substrate, followed by measurement of optical density at 450 nm (OD₄₅₀) in a
9 microplate reader (1420 ARVO MX; Perkin Elmer Inc., Waltham, MA). Specific PAD
10 activity in several tissue types was calculated using a regression line of the relationship
11 between the measured OD₄₅₀ values and the known activities (0.012–3 mU) of human
12 recombinant PAD4 (hPAD4) on a semi-log scale.

13

14 **Reconstruction of a rat oral mucosal model using collagen gel matrix culture**

15 Oral mucosa was obtained from the palatal and buccal regions of 7-day-old Wistar
16 rats. All procedures were conducted based on previous studies on oral mucosa (14, 22,
17 23). Epithelium and lamina propria were separated from the mucosa after incubation
18 with dispase. The latter was minced into pieces and cultured in Dulbecco's Modified
19 Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). After fibroblasts
20 were sprouted from the tissue, the cells were cultured in new medium for 5–6 days,
21 followed by one round of subculturing. A total of 3 mL type I collagen gel was mixed
22 with 2×10^6 fibroblast cells, and poured onto a cell culture insert (2.5 cm diameter,
23 Millicell CM; Millipore) as previously described (14). After digestion of the epithelial
24 tissue with trypsin, the cells were suspended in MCDB-153 media (Kinousei Peptide
25 Institute, Yamagata, Japan) and then overlaid on the collagen gel. The culture insert was

1 placed in an outer dish containing MCDB-153 + DMEM medium (1:1) supplemented
2 with additives as previously reported (14), and incubated at 37°C, 5% CO₂. After the
3 epithelial cells grew to confluency, the cells on the gel surface were cultured at an air-
4 liquid interface (airlift) by removing the medium from the cell culture insert and
5 replacing the medium in the outer dish with DMEM supplemented with 10% FBS and
6 growth factors in the presence of Cl-amidine, acefylline or vehicle (0.1% dimethyl
7 sulfoxide) as a control.

8 To analyze permeability of the oral mucosal model, 200 µL of 1 M Lucifer yellow
9 CH dilithium salt solution (Sigma) was added to the surface of the model. After
10 incubation at 37°C for 10 h, the model was fixed in 4% PFA and embedded in paraffin.
11 The sections were examined under a fluorescence microscope, and the fluorescence
12 intensity within the non-cornified epithelial area was quantified using ImageJ software
13 (NIH). The permeability index was calculated by dividing the intensity by the non-
14 cornified area.

15

16 **Statistics**

17 All values are presented as the mean ± standard error of the mean. Statistical
18 analysis was performed using one-way analysis of variance followed by a *post hoc* test.
19 P values less than 0.05 were considered statistically significant.

20

21 **Results**

22 **Expression and degradation of FLG and proFLG in rat stratified epithelial tissues**

23 We performed histological examination of the skin, palate, and buccal mucosa from
24 7-day-old rats by H&E staining (Fig. 1A). In the skin, interfollicular keratinocytes were
25 well flattened within the granular layers, and dot-like staining of the keratohyalin

1 granules was observed. In the palate, squamous epithelial cells and fewer granules were
2 observed. The buccal mucosa possessed thick layers with poor cornification where the
3 cells exhibited minimal flattening and contained granules.

4 Initially, we confirmed that positive staining was not observed without using the
5 primary antibodies and antiserum employed in the present study (data not shown).
6 Previously, Senshu *et al.* (19) demonstrated that the anti-FLG antiserum they raised was
7 capable of detecting rat FLG precursors and breakdown products as well as FLG
8 monomers. Fluorescence microscopy showed that the same antiserum exhibited intense
9 immunofluorescence in the lower cornified layer of 7-day-old rat skin (Fig. 1A). The
10 immunoreactivity faded towards the superficial part of the cornified layer. The presence
11 of many stained nodules with obscured outlines in the granular layer indicated the
12 notable presence of proFLG in the keratohyalin granules. In the palate, continuous
13 staining was barely detected in the mucosal layers, although punctuate staining was
14 visible throughout the granular layer, suggesting the presence of proFLG. Faint granular
15 immunoreactivity was observed in the buccal mucosa. Figure 1B shows the quantitative
16 analysis of FLG and proFLG immunofluorescence from four preparations. The relative
17 values of the immunoreactive areas were statistically different among the three tissues
18 in the descending order of skin > palate \geq buccal mucosa.

19 Figure 1C shows the immunoblot profiles obtained after probing with anti-FLG
20 antiserum. The major band located at 48 kDa from skin samples represented the FLG
21 monomer, as previously identified (19). Additional bands were also noted below 40
22 kDa, representing the degradation products of FLG. Diffuse signals in the upper part of
23 the lane probably represent high molecular weight precursors of FLG, such as proFLG
24 (~400 kDa) and its processing intermediates including FLG dimers. In contrast, the
25 FLG monomer and its high molecular weight precursor were less intensely stained in

1 palate samples, and only a faint proFLG signal was detected in the upper part of the
2 lane of the buccal mucosa samples.

3

4 **Expression and activity of PAD in stratified rat epithelial tissues**

5 To investigate the expression of PAD isozymes in rat skin and oral mucosal tissues,
6 we performed qPCR and immunoblot experiments. First, representative amplification
7 of cDNA with specific primers showed that PAD1, 2, 3, and 4 and proFLG were
8 expressed in the three types of epithelial tissues or whole skin as a control (Fig. 2A).
9 PCR analysis demonstrated that the mRNA levels of proFLG, a major substrate of the
10 PADs, were expressed in descending order of skin >> palate > buccal mucosa (Fig. 2B),
11 consistent with the expression of FLG protein. We used qPCR to examine the mRNA
12 levels of PAD1, PAD2, PAD3, and PAD4 in the epithelial layers (Fig. 2C). Both PAD2
13 and PAD4 mRNA was expressed in descending order of skin >> palate = buccal mucosa,
14 whereas PAD1 mRNA expression was highest in the palate (Fig. 2C). PAD3 mRNA
15 was present at very low levels in oral tissues relative to the skin.

16 The ABAP assay was performed in 5 mM calcium-containing deimination buffer.
17 The specific activity of PAD in the epithelial layers was highest in the skin, next highest
18 in the palate, and lowest in the buccal mucosa (Fig. 2D). We assayed PAD activity in
19 different calcium conditions as each PAD isozyme reportedly has distinct calcium
20 dependency. Interestingly, in the presence of 10 μ M calcium, PAD activity was highest
21 in the palatal mucosa of the three tissues, and mirrored PAD1 mRNA expression. In the
22 presence of 100 μ M calcium, PAD activity was the same in the skin and palate. The
23 calcium dependency index suggested that PAD activity was considerably dependent on
24 calcium in the skin, but had small dependence in the oral mucosa, and activity was
25 likely to be retained in the palate even in the presence of calcium concentrations as low

1 as 10 μ M.

2

3 **Detection of citrullinated proteins in stratified rat epithelial tissues**

4 Figure 3A shows the immunoblot profiles upon probing with the anti-CPP antibody,
5 which recognizes synthetic CCP originating from the primary structure of FLG. The
6 major signal had an apparent molecular weight of 50 kDa. Immunoblots probed with
7 anti-MC antibody, which recognizes citrulline residues after chemical modification,
8 revealed a major band at 50 kDa and a strong smear signal in the upper region of the
9 lane. Without the chemical modification, this antibody would not show reactivity. The
10 intensity of these signals was in the descending order of skin > palate > buccal mucosa.
11 Although citrullinated FLG and proFLG signals were likely present, other citrullinated
12 proteins in the upper part of the lane and those at about 50–60 kDa, such as K1, should
13 also be taken into account.

14 To discriminate between citrullinated keratins and FLG by protein charge, we
15 performed 2D gel electrophoresis using NEpHGE in the first dimension and SDS-
16 PAGE in the second dimension (Fig. 3B). Type I and type II keratins were acidic
17 (isoelectric point [pI] = 4–5) and relatively basic (pI = 6–8), respectively, whereas FLG
18 was a much more basic protein (pI = 10). The twin protein spots located around 50 kDa
19 on the most cathodic side of the membrane were immunoreactive to anti-FLG antiserum.
20 After the blot was stripped, we confirmed that there was no immunodetection of spots
21 after incubation with the secondary antibody alone (data not shown). This suggests that
22 no residual traces of bound FLG antibody immunoreacted with the spots. Re-probing
23 of the blot with anti-CCP antibody revealed that FLG-immunoreactive spots included
24 anti-CCP reactivity, suggesting that the FLG monomer was partly citrullinated in the
25 skin (Fig. 3B). Total protein staining revealed multiple spots in the middle and anodic

1 side of the membrane, which likely included K1 and K10. Some of the spots (50 and
2 60 kDa) reacted positively with the anti-CCP antibody. In the palate, a small spot
3 located around 50 kDa on the most cathodic side of the membrane also reacted with
4 anti-FLG antiserum, although the signal was rather weak, so CCP immunoreactivity
5 was not evident (Fig. 3B).

6

7 **Co-existence of structural proteins and PAD in the cornified and granular layers** 8 **of the skin and palatal mucosa**

9 To determine the tissues where the structural proteins (FLG and K1) and PAD1
10 associated physically or functionally, we performed immunofluorescence experiments
11 and viewed the images by confocal microscopy (Fig. 4). Signals for both FLG and K1
12 overlapped in the lower cornified layer of the skin. Pearson's colocalization coefficient
13 was 0.96 in the cornified layer and 0.62 in the granular layer (squares in Fig. 4),
14 suggesting the probable physical association of these proteins in KIFs. Merged
15 localization was not observed in the palate or buccal mucosa (coefficients; 0.54 in the
16 palate and 0.34 in the buccal mucosa). In both the skin and palatal mucosa, we observed
17 the continuous immunofluorescence of PAD1 in the cornified layer, and a number of
18 dot-like stains in the granular layer. Intense co-localization of PAD1 and K1 was
19 detected in the cornified layer of the skin, suggesting that the chemical modification of
20 K1 and probably FLG occurs in this tissue. However, such co-localization was not
21 found in the superficial layers of the palatal mucosa. In the buccal mucosa, PAD
22 localization was not observed.

23 Next, we immunostained the citrullinated proteins using anti-CCP antibody (Fig.
24 4) and observed an intense signal in the cornified layer of the skin, but a faint signal in
25 the palatal and buccal mucosal layers. A considerable part of the K1 signal was merged

1 with the CCP signal, indicating that K1 and probably FLG are citrullinated in the
2 cornified layer of the skin. The continuous MC staining in the cornified layer of skin
3 was also merged with FLG staining. Scattered staining of MC and proFLG was found
4 in the granular layer of the palate, some of which overlapped, indicating that proFLG
5 may be citrullinated in keratohyalin granules (Fig. 4).

6

7 **Organotypic culture models of rat palatal mucosa: Involvement of PAD in** 8 **modification of FLG**

9 Next, we analyzed the structure of the stratified layers during keratinization using
10 reconstruction models (8 days after airlifting) derived from epithelial cells and
11 fibroblasts isolated from the palatal mucosa of 7-day-old rats. As previously reported
12 (14), the culture models retained their multi-layered epithelial structure with a cornified
13 layer (Fig. 5A, control). The dot-like distribution of proFLG and diffusible localization
14 of K1 were found in the granular layers. Similar to the *in vivo* palate preparation (see
15 Fig. 4), the scattered staining of proFLG was merged with MC staining in the granular
16 layer of the organotypic culture model (Fig. 5B, control).

17 Cl-amidine is a pan-inhibitor of PAD (24). We confirmed that this compound (10
18 μM) effectively reduced citrullination of histone H3 in the presence of hPAD4 using
19 anti-MC antibody in immunoblot study (Fig. 5C). Cl-amidine significantly decreased
20 PAD activity in rat palatal epithelial lysates and recombinant hPAD4 in the ABAP assay.
21 In the presence of Cl-amidine, the reconstruction model appeared to be tightly overlaid
22 with the cornified layer and the cells were flattened in the granular layer (Fig. 5A).
23 Acefylline (100 μM), an activator of PAD1 and PAD3 (25), increased PAD activity of
24 the rat palatal epithelium in the ABAP assay. The surface structure appeared to be looser
25 and the cells exhibited minimal flattening (Fig. 5A).

1 We examined the effects of Cl-amidine and acefylline on the expression of FLG,
2 K1, and citrullinated proteins in the reconstruction models. Neither Cl-amidine nor
3 acefylline changed K1 immunostaining in the granular layer, but nonspecific staining
4 was occasionally observed in some basal cells. Treatment with Cl-amidine resulted in
5 continuous FLG staining in the cornified layers as well as granular proFLG staining
6 (Fig. 5A, B). Interspersed co-localization was found in the confocal images of surface
7 FLG and K1 immunoreactivity (Fig. 5B). Both continuous and granular FLG staining
8 was negative to the anti-MC antibody. Acefylline did not cause apparent changes in
9 FLG immunoreactivity (Fig. 5A). Some fine dots showed co-immunoreactivity with
10 proFLG and MC (Fig. 5B). Immunoblot analysis showed that Cl-amidine significantly
11 increased the FLG monomer signal (48 kDa) 2.5-fold, whereas acefylline tended to
12 decrease the signal (Fig. 6A).

13 To examine whether PAD activity is involved in the epithelial structure, epithelial
14 thickness was quantified in the presence or absence of Cl-amidine and acefylline. Cl-
15 amidine significantly decreased epithelial thickness and the relative value of the
16 cornified layer relative to the entire epithelial layer (Fig. 6B). Acefylline significantly
17 increased epithelial thickness although relative thickness remained unchanged. Then
18 we further investigated whether the change in PAD activity altered mucosal
19 permeability. The fluorescence dye Lucifer yellow was applied to the surface of the
20 models in the presence or absence of these compounds. The dye was allowed to
21 penetrate the cornified layer and left to stain below the layer for 10 h. Cl-amidine
22 significantly inhibited dye permeability whereas acefylline enhanced it (Fig. 6C).

23

24 **Discussion**

25 Processing of proFLG has several crucial roles in maintaining epidermal integrity

1 such as aligning KIFs, and controlling significant changes in cell shape and epidermal
2 texture (4). Knockdown of FLG in the reconstructed epidermis or organotypic skin
3 models by single hairpin RNA and small interference RNA technology was shown to
4 disrupt the diffusion barrier in a dye penetration assay (26, 27). In flaky tail mice, which
5 lack normal proFLG, barrier function was impaired (28). FLG-null (*Flg*^{-/-}) mice
6 exhibited loss of keratin patterns and deficiency of barrier integrity (29). Transient
7 overexpression of FLG resulted in collapse of the intermediate filament network (30,
8 31). Thus, the proFLG system should be distinct among epithelia types to provide
9 suitable rigidity and permeability to the environment. This study demonstrated the
10 diffuse localization of FLG in the cornified layer of the skin, but not in the palate or
11 buccal mucosa of rats, whereas granular localization of proFLG was detected in the rat
12 skin and palate. The distinct expression of FLG and proFLG in different epithelial types
13 has also been previously reported in humans. Immunofluorescence with the antibody
14 against (pro)FLG showed diffuse staining in the cornified layer of the human palate and
15 scattered dot-like staining in the granular layer of the palate and buccal mucosa,
16 suggestive of proFLG deposition in keratohyalin granules (15, 16). Immunoblot
17 analysis showed that both the FLG monomer and proFLG were present in the palate,
18 whereas only proFLG was found in the buccal mucosa (15). These results suggest some
19 species-specific differences in FLG and proFLG expression in different parts of the oral
20 mucosa.

21 PAD1 is distributed in all living layers of the human epidermis and in granular and
22 weakly cornified layers of the mouse epidermis (11). Other PAD isozymes may also be
23 involved in the granular and lower cornified layers, with some redundancy (5, 11). In
24 this study, the relative expression level of PAD1 was highest in the rat palate, whereas
25 that of PAD2, PAD3, and PAD4 was highest in the skin, indicative of some tissue-

1 specific PAD expression. Interestingly, the ABAP assay revealed that maximal PAD
2 activity in the presence of 5 mM calcium was lower in the palate than in the skin, but
3 the rank order of PAD activity reversed with decreasing concentrations of calcium,
4 suggesting that calcium dependency is completely different between the two epithelial
5 types. Notably, hPAD1 and hPAD3 were fully effective in catalyzing FLG citrullination
6 at calcium concentrations less than 1.0 mM and 1.5 mM, respectively (8). In contrast,
7 hPAD2 required more than 2.5 mM calcium to produce maximal activity in
8 citrullinating FLG (8). Therefore, the high level of PAD1 in the palate allowed FLG
9 citrullination even under low calcium concentrations. Because a gradient of calcium
10 concentration has been reported in the epidermis (32), a relationship between
11 intracellular calcium levels and the specific expression of the PAD isozymes with
12 distinct calcium dependency in oral mucosa should be clarified in future studies.

13 FLG and K1 are preferentially citrullinated during the terminal differentiation of
14 human epidermis (33). PAD1, but not other isozymes, is present in the uppermost
15 cornified layer in the skin (8) and is responsible for K1 citrullination at this level (7,
16 10). FLG has been reported to be co-localized with PAD3 as well as PAD1 in the keratin
17 matrix of lower cornified layer. In the human granular layer, proFLG has been shown
18 to be co-localized with PAD1 and PAD3 on the granules; only PAD1 but not proFLG
19 nor PAD3 were detected on the KIFs (8). The present study showed entirely distinct
20 patterns of co-localization among the epithelial types in rats; FLG and K1 were co-
21 localized with PAD1 in the cornified layers of skin, suggesting that FLG monomer and
22 PAD may associate with KIFs and create tight bundles. However, neither FLG
23 monomer nor K1 was co-expressed on the surface of the palate even though PAD1 was
24 present (Fig. 7). Because of the granular presence of proFLG and PAD1, keratohyalin
25 granules may be another place for citrullination by PAD1 in the palate, although other

1 protein substrates of PAD1, as yet uncharacterized, might be involved.

2 To determine where citrullinated proteins are localized in these epithelia, we took
3 two approaches to detect the citrullination of FLG and keratins. It was reported that
4 synthetic CCP originating from FLG recognized autoantibodies directed toward
5 citrullinated protein generated in rheumatoid arthritis patients (34). This study used an
6 anti-CCP antibody raised against cyclic synthetic peptide including citrulline residues
7 within human FLG. This antibody likely recognized citrullinated FLG in our
8 immunoblot and immunohistochemistry experiments. Furthermore, detection of the
9 MC residues revealed the presence of citrullinated (pro)FLG (19). In our western blot
10 analyses combined with 2D electrophoresis, the anti-CCP antibody was capable of
11 detecting FLG monomer, whereas the anti-MC antibody likely detected proFLG, its
12 processing intermediates, and the FLG monomer as well as all citrullinated proteins.
13 The more intense FLG immunoreactivity in the three epithelial types and additional
14 immunoreactive spots observed by 2D gel electrophoresis indicated the involvement of
15 other citrullinated proteins such as K1, K10, and hornerin (35).

16 Our immunofluorescence studies revealed the overlapping surface distribution of
17 K1, FLG, and CCP staining, suggesting that chemical modification of K1/FLG likely
18 take places in the cornified layers of the skin. In the palate and its culture model, clear
19 dot-like staining was observed in the immunofluorescent images for proFLG and MC,
20 suggesting that chemical modification of proFLG is likely to be predominantly in
21 keratohyalin granules, consistent with the previous notion of proFLG citrullination in
22 keratohyalin granules (36–38). Loss of positive charges in proFLG could affect the
23 integrity of the oral mucosa, for example, by altering the alignment of KIFs and
24 changing the cell shape and mucosal texture (Fig. 7).

25 As mentioned above, the amount and type of PAD isozymes, their substrates, and

1 conditions of citrullination reactions were clearly distinct among the three epithelial
2 types. To understand the precise role of PAD in the structure and function of oral
3 mucosa, we attempted to make experimental conditions other than PAD activity the
4 same using a rat 3D mucosal model and two types of compounds that are known to
5 modify PAD. Cl-amidine inhibits the calcium-bound form of PADs and is the most
6 widely used pan-PAD inhibitor in numerous disease models (24). As a PAD activator,
7 xanthine derivative acefylline is useful for the citrullination of FLG by PAD1 and PAD3
8 (25). The present study revealed that PAD inhibition decreased the epithelium thickness
9 and Lucifer yellow permeability concomitantly with an increase in expression of the
10 FLG monomer. The fact that citrullination was lost in granular proFLG staining and
11 that K1 and FLG monomer were co-expressed suggests that the persistent presence of
12 FLG monomer may cause keratins to bundle tightly to make the epithelial compact in
13 the PAD-deficient palate. On the other hand, further activation of PAD by acefylline in
14 the palatal epithelium decreased expression of the FLG monomer, and increased the
15 epithelium thickness and dye permeability. These data suggest that FLG citrullination
16 contributes to the breakdown of (pro)FLG and the intrinsic structure of the oral mucosa
17 to affect the barrier function. The present study strongly supports the notion that
18 citrullinated filaggrin is involved in the function and morphology of stratified epithelia
19 (10).

20 In conclusion, citrullination is likely to play an important role in maintaining
21 structure and function of oral mucosa. The results of this study provide new insights
22 into the complex mechanism underlying the cornification and stratification of
23 epithelium in the oral mucosa, and paves the way for new pharmacological approaches
24 directed toward the maintenance of sustainable oral health.

25

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- 14

1 **Figure Legends**

2 Fig. 1 Histological and immunofluorescence examination of the skin, palate, and buccal
3 mucosa in 7-day-old rats. A, Hematoxylin and eosin staining (upper) and profilaggrin
4 (proFLG) immunofluorescence using anti-filaggrin (FLG) antiserum (middle and
5 lower). The arrowheads indicate keratohyalin granules (upper) and dot-like
6 immunoreactivity for proFLG (lower). The squares a, b, and c are enlarged in the lower
7 panels. Purple: DAPI staining. Broken line indicates the basement membrane. Dotted
8 line indicates the surface of the epithelium. B, Quantitative analysis of relative area of
9 the proFLG immunofluorescence. **P < 0.01; one-way analysis of variance followed
10 by the *post hoc* Dunnett's test (n = 4). C, A representative image of immunoblot for
11 proFLG protein. P, I, F, and D denote proFLG (~400 kDa), the processing intermediates
12 of proFLG, FLG monomers (~48 kDa), and the degradation products of the monomers,
13 respectively. Expression of β -actin is shown as a loading control.

14

15 Fig. 2 Expression of peptidylarginine deiminase (PAD) and filaggrin in the skin, palate,
16 and buccal mucosa of rats. A, Typical real-time PCR results obtained using cDNA
17 derived from three types of epithelial tissues and whole skin with specific primer pairs
18 for PAD1–4 and GAPDH. B, Typical real-time PCR results and quantitative PCR data
19 obtained using primer pairs for profilaggrin (proFLG) (n = 4 animals per group). C,
20 Quantitative real-time PCR measurements of PAD1, PAD2, PAD3, and PAD4 mRNA
21 are shown (n = 4 animals per group). Expression values were calculated by the relative
22 standard curve method. *P < 0.05, **P < 0.01, ***P < 0.001; one-way analysis of
23 variance followed by the *post hoc* Dunnett's *t*-test. D, PAD specific activity was
24 measured in the presence of 10 μ M, 100 μ M, and 5 mM Ca²⁺ (antibody-based assay for
25 PAD activity assay) using lysate (1 μ g) obtained from 3 epithelial types (n = 3–4) (left).

1 The ratio of PAD activity in 5 mM Ca²⁺ to that in 10 μM Ca²⁺ is shown to clarify the
2 distinct calcium dependency among the three. *P < 0.05, **P < 0.01, ***P < 0.001;
3 one-way analysis of variance followed by the *post hoc* Bonferroni's *t*-test.

4

5 Fig. 3 Separation of citrullinated protein in the skin, palate, and buccal mucosa of rats.
6 A, immunoblot following 4–20% gradient sodium dodecyl sulfate-polyacrylamide gel
7 electrophoresis (SDS-PAGE) using anti-cyclic citrullinated peptide (CCP) and anti-
8 modified citrulline (MC) protein antibodies. Immunoreactivity of anti-MC antibody
9 with or without citrulline modification of the skin sample is also shown. Expression of
10 β-actin is shown as a loading control. B, Two-dimensional profiles of immunoblot using
11 anti-filaggrin (FLG) antiserum and anti-CPP antibody. The samples were loaded on
12 agarose gels, separated in the first dimension using NEpHGE, and separated in the
13 second dimension by SDS-PAGE. FLG: immunodetection of (pro)FLG. CCP:
14 immunodetection of citrullinated protein. Merged skin and palate: images obtained by
15 superimposing total protein (colloidal gold) with (pro)FLG (green) and the citrullinated
16 proteins (red).

17

18 Fig. 4 Immunofluorescence images for expression of (pro)filaggrin [(pro)FLG],
19 peptidylarginine deiminase (PAD)1, and citrullinated proteins in the skin, palate, and
20 buccal mucosa of rats under a confocal microscope. For detection of the citrullinated
21 proteins, anti-cyclic citrullinated peptide (CCP) antibody and anti-modified citrulline
22 (MC) antibody were used. (G): green. (R): red. Yellow staining denotes the overlapping
23 signals. Squares are enlarged in the lower panels. Arrowheads indicate the dot-like
24 immunoreactivity for proFLG and MC. Purple: DAPI staining. Broken line indicates
25 the basement membrane. Dotted line indicates the surface of the epithelium.

1

2 Fig. 5 Organotypic reconstruction models using epithelial cells and fibroblasts
3 dissociated from rat palatal mucosa. The models were cultured in the presence or
4 absence of a pan- peptidylarginine deiminase (PAD) inhibitor Cl-amidine (10 μ M) or
5 acefylline (100 μ M) for 5 days. A, Hematoxylin and eosin (H&E) staining and
6 immunofluorescence using anti-filaggrin (FLG) antiserum in the reconstruction models.
7 The squares are enlarged. Arrowheads denote granular presence of profilaggrin
8 (proFLG). Broken line indicates the basement membrane. B, Confocal images using
9 anti-FLG antiserum, anti-keratin 1 (K1), and anti-modified citrulline (MC) antibodies
10 in the reconstruction models. (G): green. (R): red. Yellow staining (arrows) denotes the
11 overlapping granular signals. Dotted line indicates the surface of the epithelium. C,
12 Validity of Cl-amidine and acefylline in modulating PAD activity was tested.
13 Immunoblotting using anti-MC antibody was performed in the presence of recombinant
14 human PAD4 (hPAD4) and histone H3 as a substrate with or without Cl-amidine. An
15 antibody-based assay for PAD activity assay to check the validity of Cl-amidine and
16 acefylline was also performed using hPAD4 and rat palatal epithelial lysate (n = 3).
17 PAD specific activity was measured in the presence of 5 mM Ca^{2+} using lysate (1 μ g)
18 obtained from rat palatal epithelium.

19

20 Fig. 6 Quantitative analysis of epithelial structure and expression pattern of filaggrin
21 (FLG) in the reconstruction models treated for 5 days with or without Cl-amidine (10
22 μ M) or acefylline (100 μ M). A, Immunoblot using anti-FLG antiserum (upper).
23 Quantitative measurement of FLG monomer (48 kDa, closed triangle) is shown (n = 4)
24 (lower). Expression of β -actin is shown as a loading control. B, Thickness of all
25 epithelial layers (upper) and relative thickness of the cornified layer with respect to all

1 epithelial layers (lower) (n = 6). C, Permeability to lower epithelium with Lucifer
2 yellow applied onto the surface of the palate models. After 10 h, the models were fixed.
3 Permeability index was calculated using fluorescence intensity within the noncornified
4 area (surrounded by broken lines) (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001; one-
5 way analysis of variance followed by *post hoc* Bonferroni's *t*-test.

6

7 Fig. 7 Schematic models of enzymatic breakdown of profilaggrin (proFLG) in rat skin
8 and palate. ProFLG is stored in keratohyalin granules of the granular layer. ProFLG is
9 proteolyzed to FLG monomers which associate with keratin intermediate filaments
10 (KIF) in the cornified layer of the skin. Peptidylarginine deiminase (PAD) including
11 PAD1 is co-localized in KIF and/or keratohyalin granules, and is likely to citrullinate
12 FLG or proFLG (denoted as open marks) and to decrease the intermolecular interaction
13 resulting in proteolytic degradation. PAD3 may be involved in protein citrullination in
14 skin (8). The pattern of proFLG breakdown in the palate is likely to be distinct from
15 that in the skin.

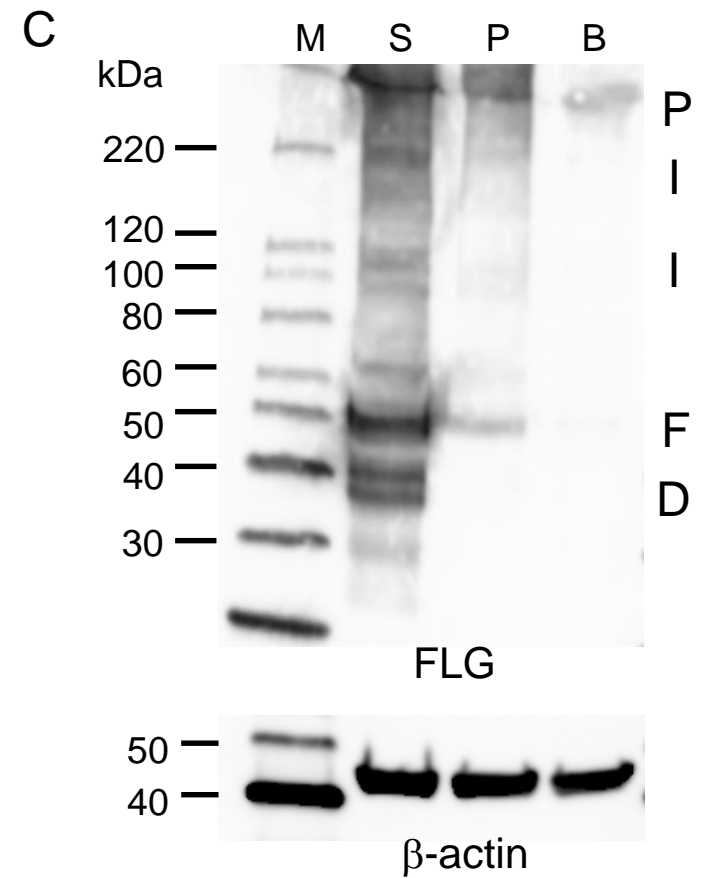
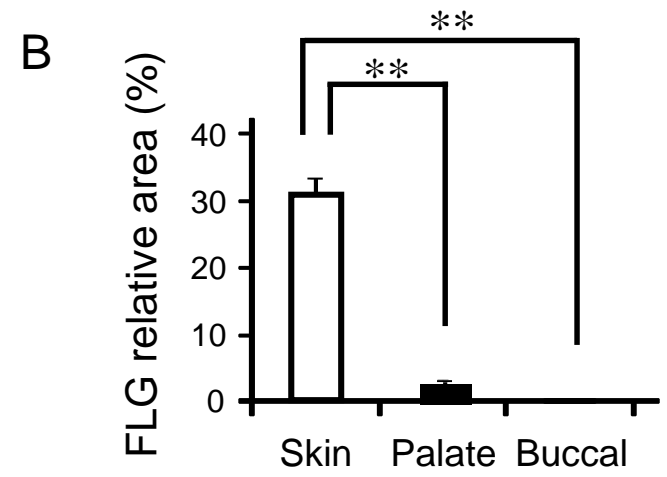
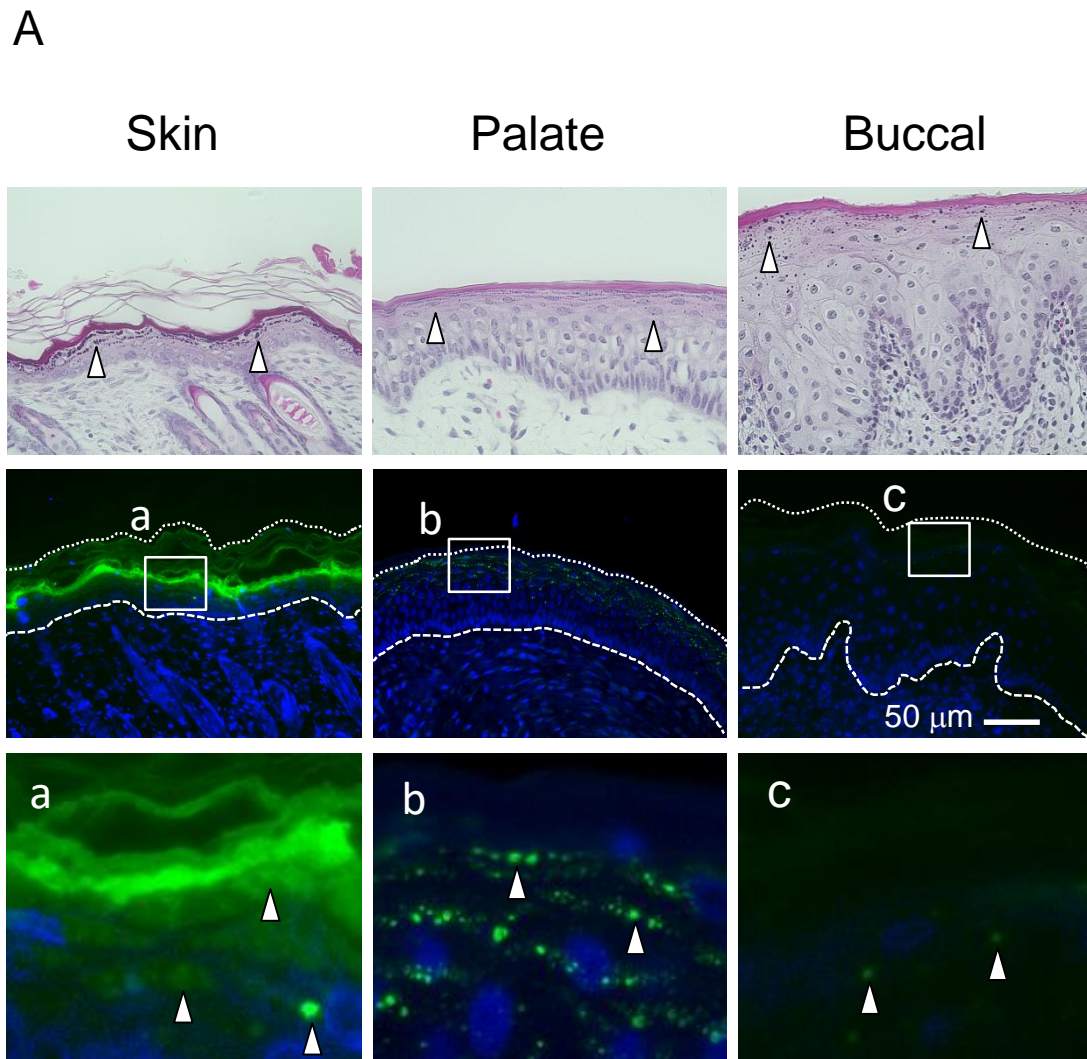


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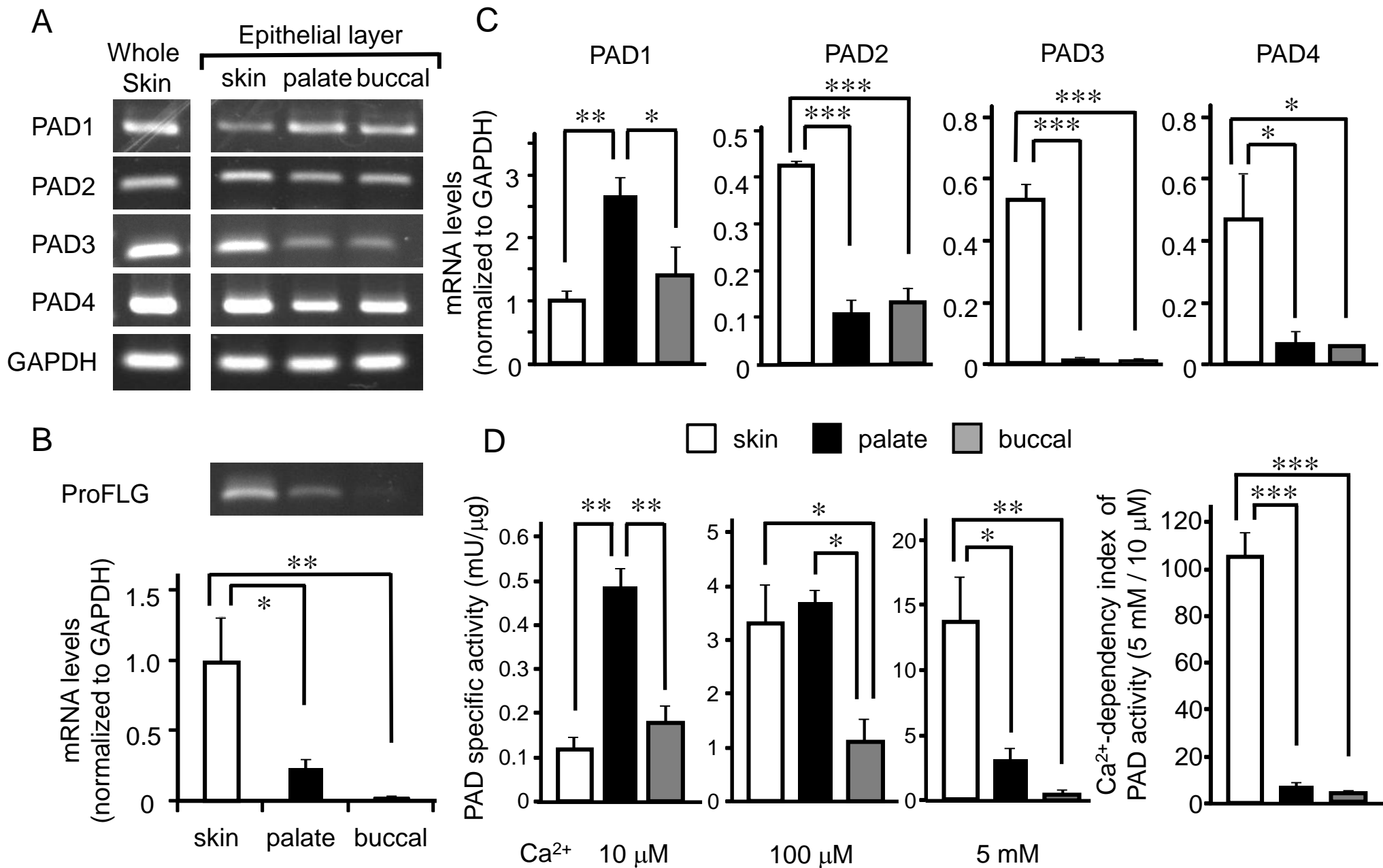


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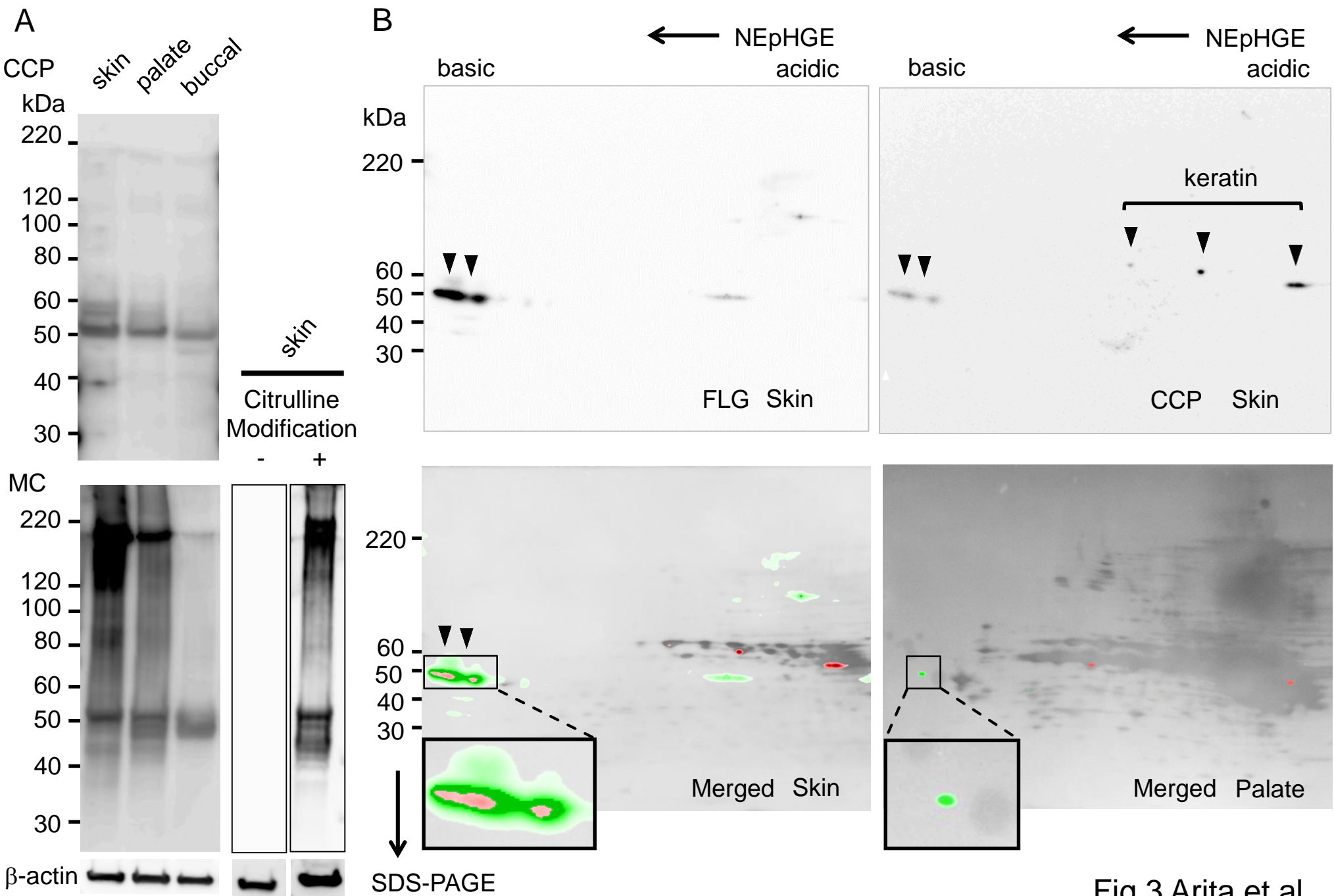


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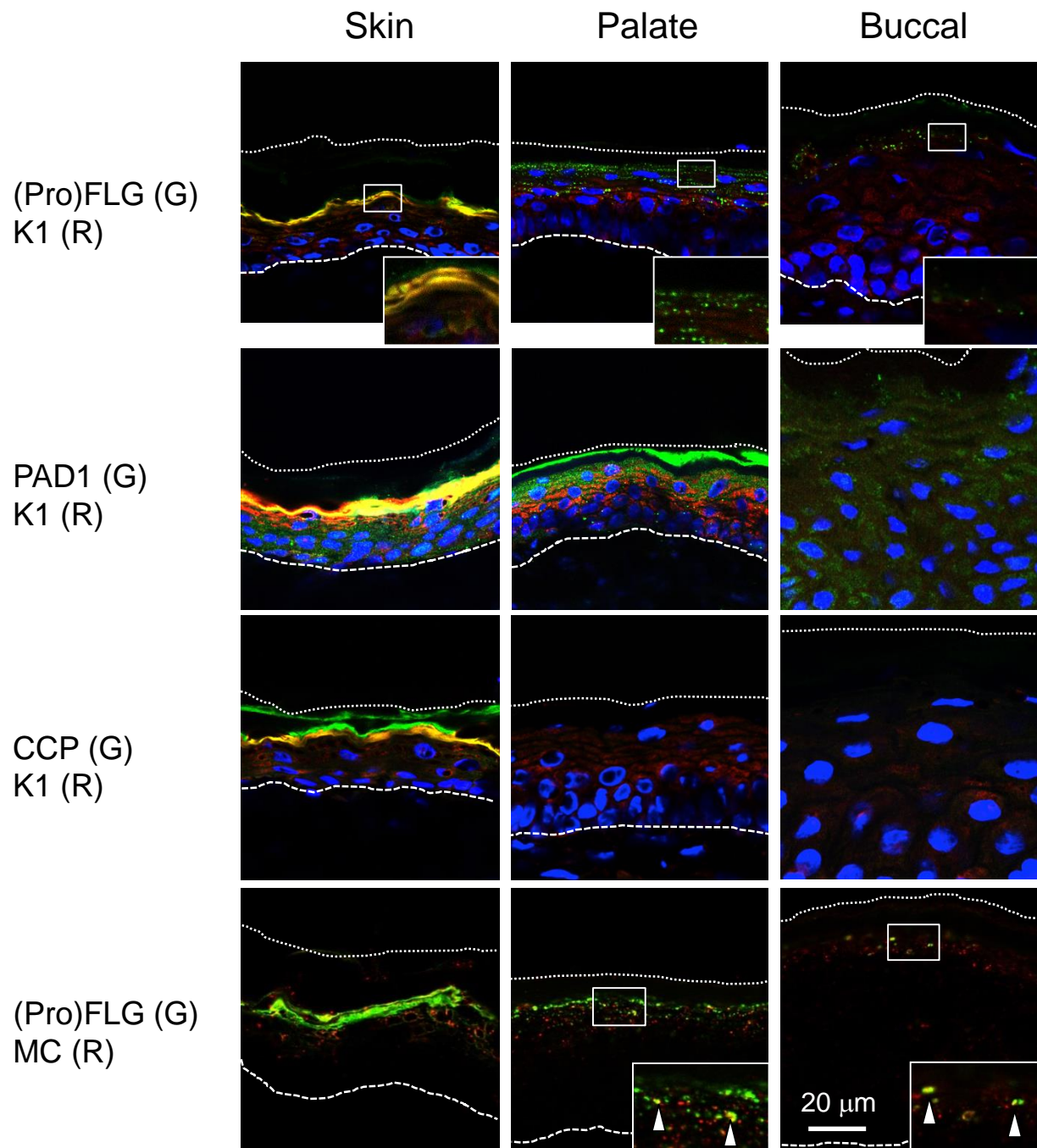


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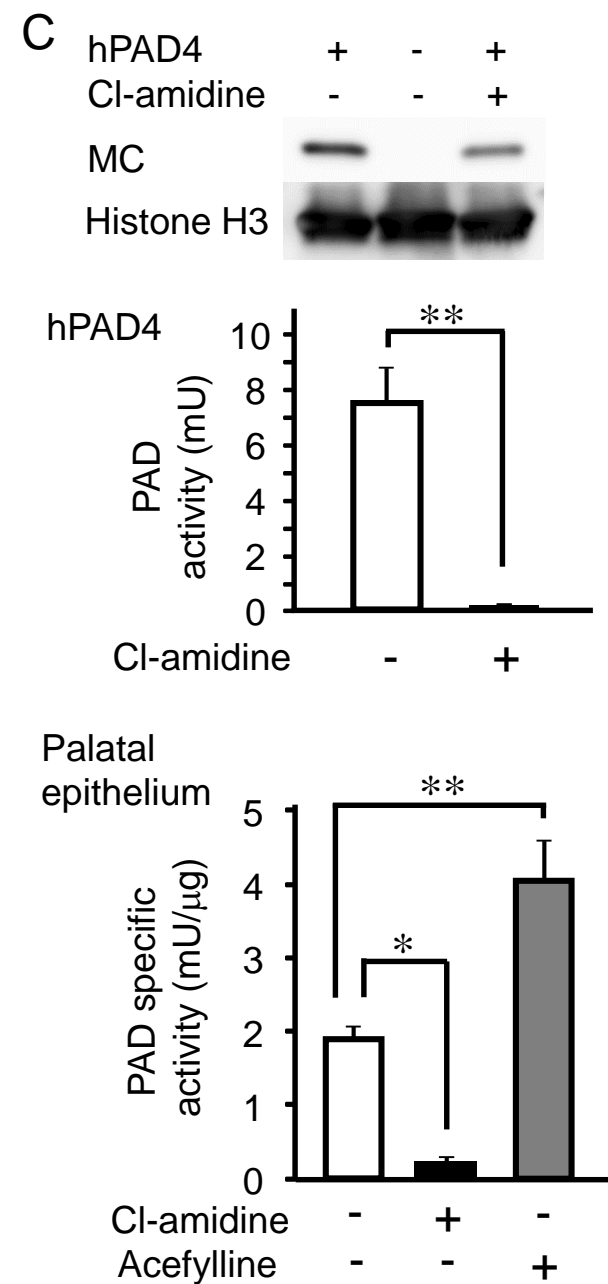
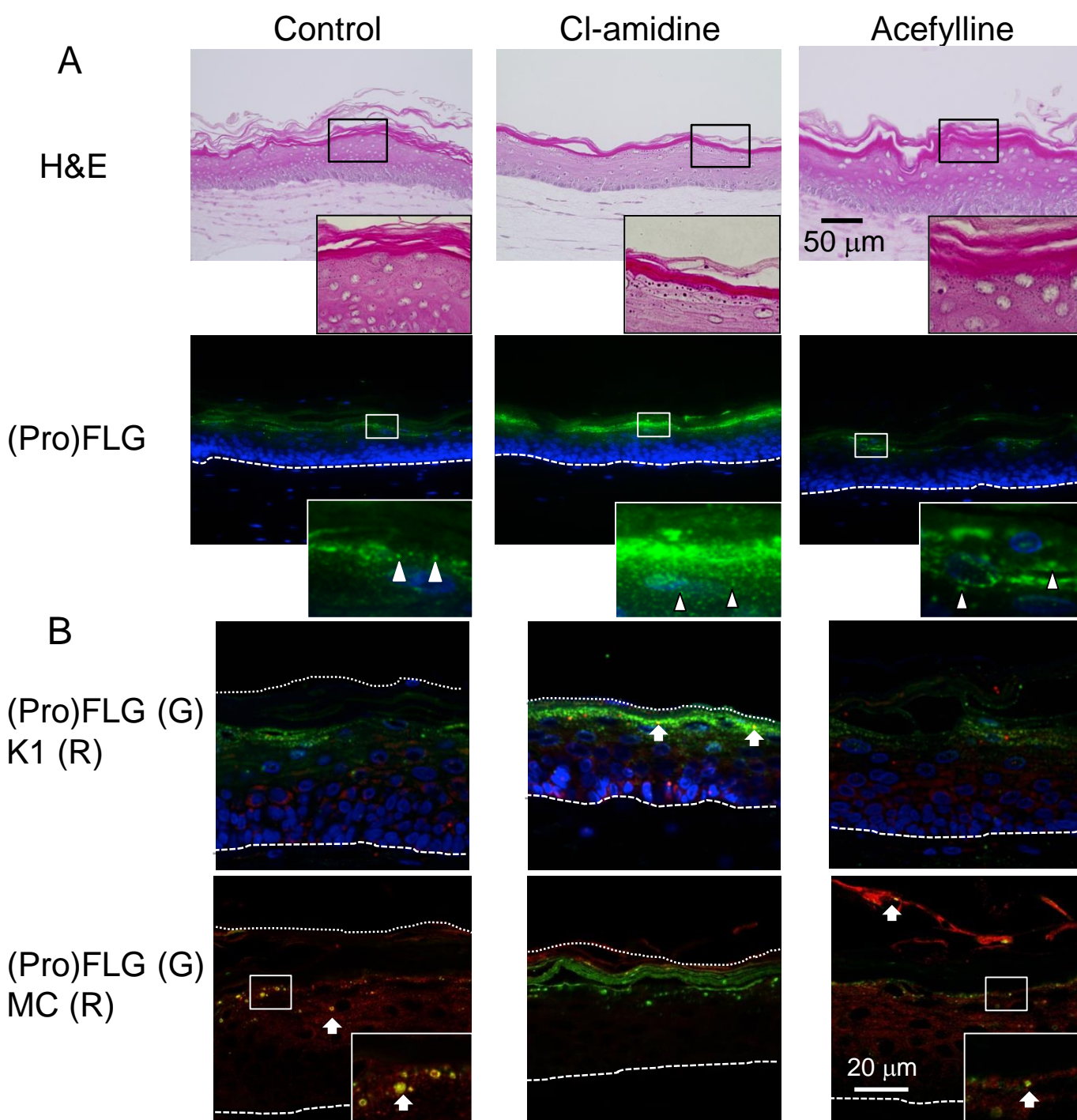


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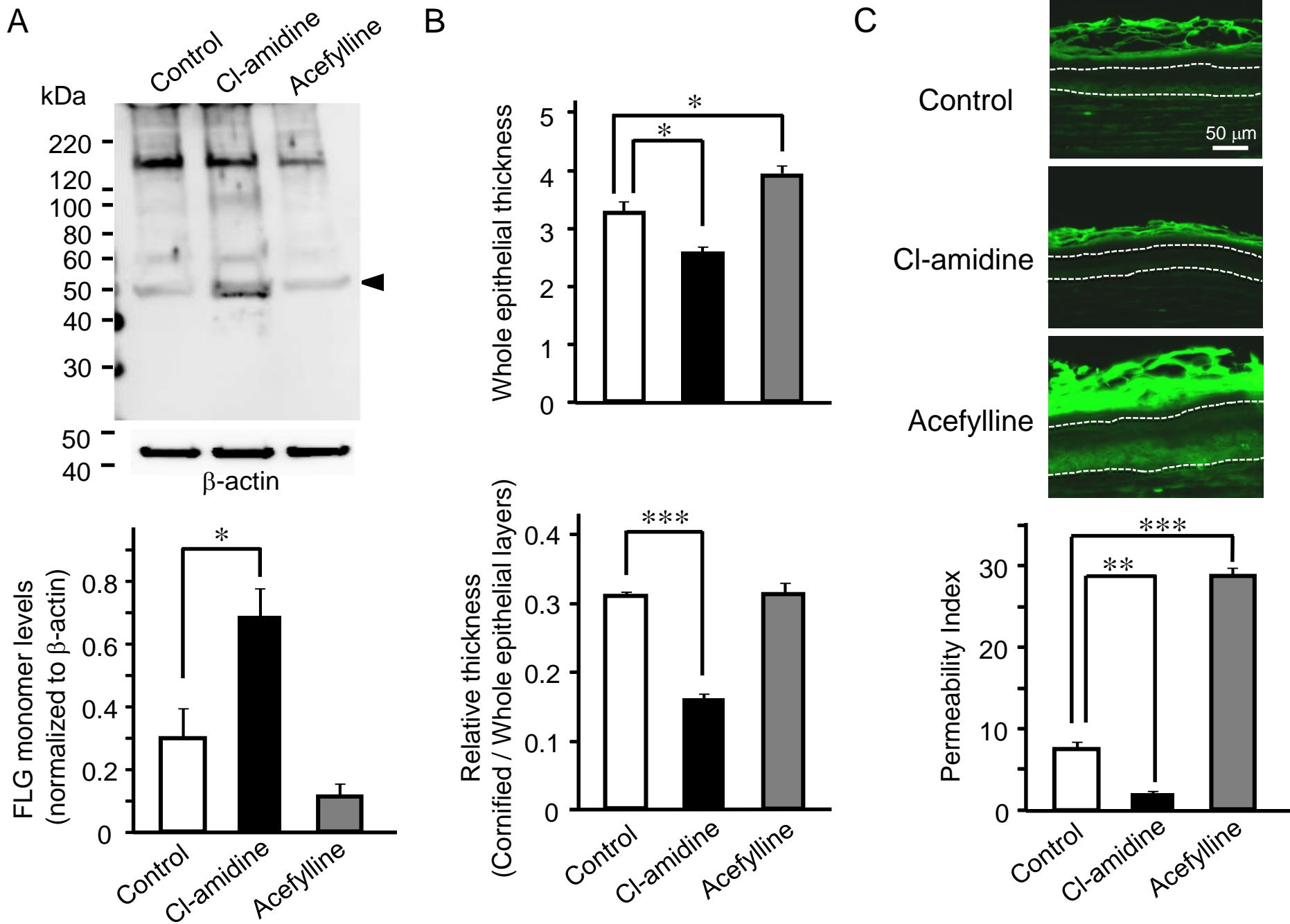


Fig. 6 Arita et al.

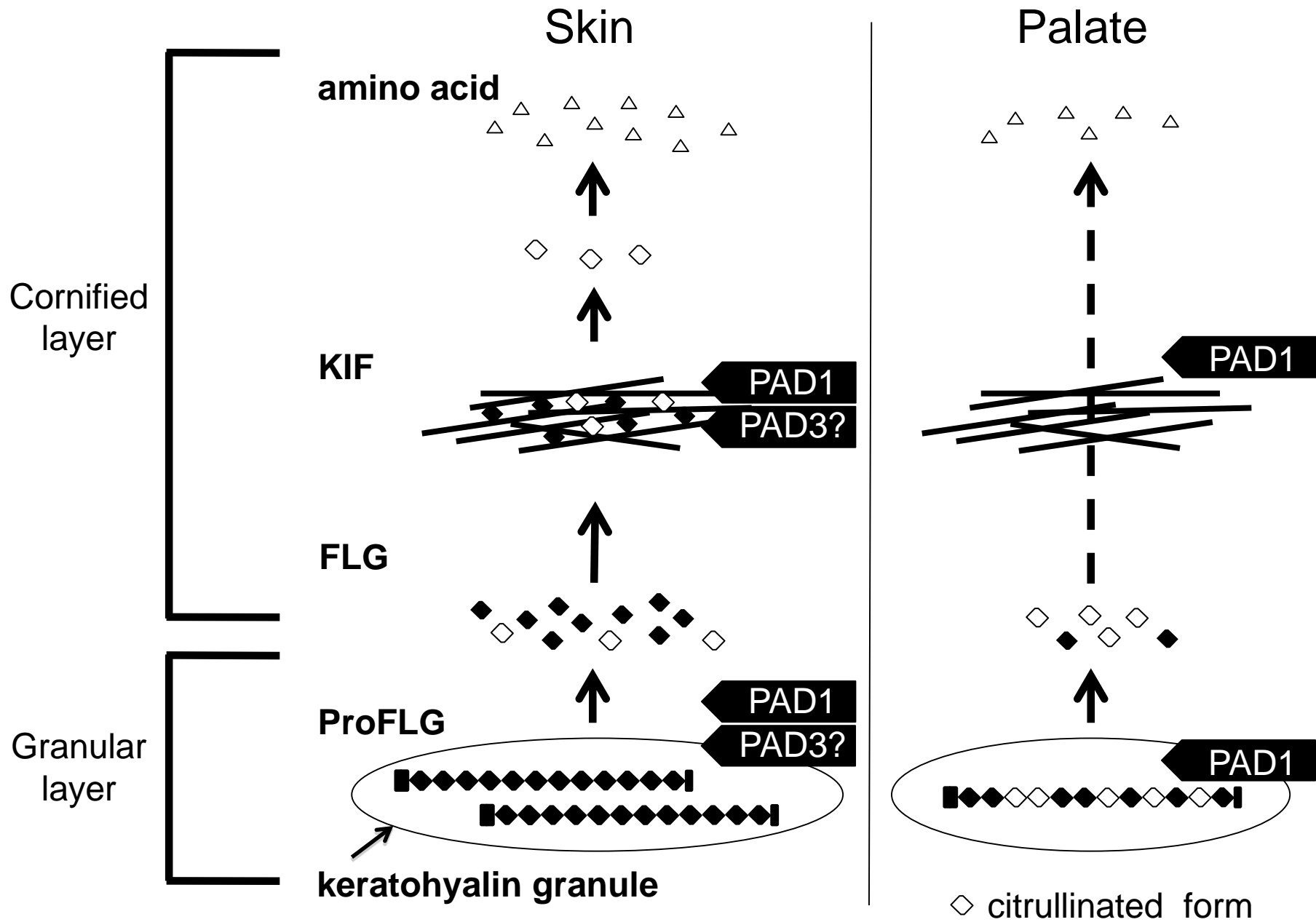


Fig. 7 Arita et al.