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Tight junction protein claudin-4 is modulated via ΔNp63 in human keratinocytes

Terufumi Kubo [a,*], Kotaro Sugimoto [a,*,1], Takashi Kojima [b], Norimasa Sawada [a], Noriyuki Sato [a], Shingo Ichimiya [c]

[a] Department of Pathology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan
[b] Department of Cell Science, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan
[c] Department of Human Immunology, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan

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ABSTRACT

In the epidermis, tight junction (TJ) structure is specifically located in the stratum granulosum, where the expression of ΔNp63, a p53 family transcription factor, is attenuated. Since the relationship between ΔNp63 and barrier function has not been fully uncovered, we assessed expression profiles of TJ proteins in skin tissues and cultured keratinocytes. The results showed that expression of ΔNp63 and that of claudin-4 were inversely correlated in healthy human epidermis. *In vitro* studies using HaCaT keratinocytes revealed functional relevance of ΔNp63 and claudin-4. Curiously, Toll-like receptor (TLR)-3 ligand, which is known to be liberated from damaged cells, suppressed ΔNp63 expression and concomitantly upregulated claudin-4 expression in primary keratinocytes. More interestingly, a broad expression pattern of claudin-4 was found in the epidermis of atopic dermatitis (AD), a barrier defect disorder, which contains ΔNp63-lacking keratinocytes as we reported previously. Therefore, upregulation of claudin-4 expression regulated by ΔNp63 might be associated with complementary or repair responses of damaged keratinocytes with AD.

1. Introduction

The skin is the first site of exposure to the external environment and plays a crucial role in prevention of chemical, physical or biological invasion as well as maintenance of moisture and temperature of the body. Dysfunction of this barrier in cases such as an extensive burn is often lethal. Keratinocytes, the main components of the epidermis, are stratified epithelia composed of several distinct characteristic layers by their differentiation stages: stratum corneum, granulosum, spinosum, and basale. The stratum corneum is composed of fully keratinized cell layers and works as a forefront of the physical barrier. In the stratum granulosum, just beneath the stratum corneum, tight junctions work as the secondary barrier preventing the entry of small molecules and water loss [1,2].

Tight junctions are the apical-most constituents of the intercellular junctional complex in mammalian epithelia and act as a semipermeable barrier to the paracellular transport of restricted small molecules. Claudins are essential components of tight junctions and consist of 27 or more members of a gene family. In addition, distinct sets of claudins are generally expressed in a cell- and tissue-specific manner. For instance, several leaktight claudins such as claudin-1 and -4 are expressed in the skin for protection against invasion of outer pathogens and prevention of water loss, whereas leaky, paracellular channel-forming claudins such as claudin-2 and -15 are expressed in the intestine for nutritional absorption [3]. Therefore, in the stratum granulosum, claudin expression and distribution should be strictly regulated to maintain the skin barrier, but the mechanisms of regulation remain largely unknown.

A p53 family transcription factor p63 is expressed in stratified epithelia and is known to rule epithelial cellular fate and function. In epidermal keratinocytes, ΔNp63, a p63 variant lacking the N-terminal transactivation domain, is dominantly expressed and plays a crucial role in regulating the differentiation of keratinocytes [4]. Experiments using inducible ΔNp63 knockout mice experiments revealed impaired keratinocyte differentiation and wound healing, suggesting ΔNp63 plays a fundamental role in

* Corresponding authors at: Department of Pathology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan. Fax: +81 11 643 2310.
E-mail addresses: kuboteru@sapmed.ac.jp (T. Kubo), k.sugimoto@victorchang.edu.au (K. Sugimoto).
[1] Present address: Developmental and Stem Cell Biology Division, Victor Chang Cardiac Research Institute, Darlinghurst, New South Wales 2010, Australia.

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epidermal integrity [5]. While we previously reported that p53 family transcription factors regulate some claudin proteins in thymic epithelial cells and a knockout mouse study has shown that ΔNp63 regulates claudin-1 expression, the relationships between human ΔNp63 and other tight junction proteins in keratinocytes have not been fully investigated [6,7].

In this study, we found that the expression of ΔNp63 was inversely correlated with claudin-4 in the human epidermis. *In vitro* study using cultured keratinocytes and the RNA interfering method revealed that ΔNp63 negatively regulated claudin-4, consistent with their distribution pattern in the epidermis. Treatment with TLR3 ligand, potentially released from damaged epithelial cells [8], suppressed ΔNp63 expression and concomitantly increased claudin-4 expression. Interestingly, claudin-4 protein was expressed more widely in the epidermis of the affected region of atopic dermatitis (AD), which is considered to be a barrier defect disorder, than in healthy skin. Since claudins are considered to have non-barrier forming activities, increased claudin-4 in AD would contribute to an unknown repair response of damaged keratinocytes as well as complementary responses of the impaired barrier in AD [9].

2. Materials and methods

2.1. Tissues

Skin tissues were obtained from patients with AD and patients presenting without pathological findings who underwent skin biopsies at Sapporo Medical University Hospital. The diagnosis of AD was established by both dermatologists and pathologists. Human tissue specimens were obtained with written informed consent and the study was approved by the institutional review board of Sapporo Medical University Hospital.

2.2. Cell cultures

Commercially available human primary keratinocytes were obtained from DS Pharma Biomedical (Tokyo, Japan) and cultured in a serum-free medium for human keratinocytes (DS Pharma Biomedical). Human HaCaT epidermal cells (RIKEN, Tsukuba, Japan) were maintained in DMEM (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin and 5% fetal bovine serum. For stimulation of cells, culture media were supplemented with the following reagents: 10 μg/ml zymozan (InvivoGen, San Diego, CA), 25 μg/ml polyinosine-polycytidylic acid (poly I:C, InvivoGen), 100 ng/ml lipopolysaccharide (LPS, InvivoGen), 5 μM Type B CpG oligonucleotide (ODN2006, InvivoGen) and 10 ng/ml TNFα (R&D Systems, Minneapolis, ME). All cells were cultured at 37 °C in a humidified atmosphere in 5% CO_2.

2.3. RT-PCR and real-time PCR analyses

Total RNA was extracted and purified using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). For RT-PCR, 2 μg of total RNA was reverse-transcribed into cDNA using a reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time PCR was performed as described in the manufacturer's protocol for Assays-on-Demand Gene Expression products (Applied Biosystems). The amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in each sample was used to standardize the quantities of p63 mRNA (Hs00978344), claudin-1 mRNA (Hs00221623) and claudin-4 mRNA (Hs00976831). To calculate the relative mRNA expression of triplicate specimens, the ΔΔCT method was employed according to the manufacturer's instructions.

2.4. siRNA preparation and transfection

A cocktail of three siRNAs for human p63 (siTrio full set) was purchased from B-Bridge International (Sunnyvale, CA). The sense sequences of the siRNAs for human p63 were as follows: 5′-CAGAAGAUGGUGCGACAAATT-3′, 5′-GUGAAUUCAACGAGGGACATT-3′, and 5′-GCAAAAAAGAGUUGGGUGUTT-3′. Negative control siRNA was obtained from Invitrogen (Carlsbad, CA). Cells were cultured at a density of 2×10^5 cells/well in a 6-well plate in 2.0 ml culture medium. After 24 h, the culture medium was replaced by a medium containing a complex of the siRNAs specific for p63 and Lipofectamine RNAi MAX (Invitrogen), giving a final concentration of siRNA of 40 nM following the manufacturer's instructions. After 72 h, cells were harvested to be subjected to quantitative PCR, Immunoblot or immunofluorescence staining analysis.

2.5. Antibodies

The primary antibodies used were a rabbit polyclonal antibody (pAb) anti ΔNp63 (p40; Merck KGaA, Darmstadt, Germany), a rabbit anti-claudin-1 pAb (JAY.8; Invitrogen), mouse anti-claudin-4 monoclonal antibody (Clone 3E2C1; Invitrogen) and a rabbit anti-cytokeratin pAb (Biogenesis, Poole, England). Alexa 488 (green)- and Alexa 594 (red)-conjugated anti-mouse and anti-rabbit IgGs were purchased from Invitrogen. Peroxidase-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from KPL (Gaithersburg, MD).

2.6. Immunostaining analysis

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. The cultured cells were fixed with cold acetone–ethanol (1:1 volume mixture). After rinsing these specimens with PBS, they were incubated with optimally diluted primary antibodies at room temperature for 1 h and secondary antibodies under the same conditions for another 1 h. Then in the immunofluorescence, 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich) was employed for counterstaining of the cell nuclei. Specimens were examined under a fluorescence microscope (IX81, Olympus, Tokyo, Japan).

2.7. Immunoblot analysis

Cell lysis was performed with 0.5% NP-40, 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and protease inhibitors (Roche, Basel, Switzerland) for 30 min at 4 °C. Aliquots of the supernatants were applied to SDS-10% polyacrylamide gels under reducing conditions and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were incubated with blocking buffer and then incubated with optimally diluted antibodies for 1 h at room temperature. After washing with wash buffer (0.1% Tween 20 in PBS), the membranes were reacted with a peroxidase-labeled secondary antibody for 1 h. After washing again with wash buffer three times, signals were visualized using an enhanced chemiluminescence detection system (Amersham Life Science, Arlington Height, IL). The levels of intensity of signals detected in immunoblots were quantified using NIH Image-J software. The intensity levels were normalized to the corresponding levels of β-actin, and their relative levels were shown in histograms.

2.8. Statistical analysis

Statistical significance was examined using the unpaired *t* test or ANOVA with Tukey's post hoc tests. Graph bars in the figures present means ± SD.

3. Results

3.1. Distribution of ΔNp63 and claudin-4 in healthy epidermis

A previous study showed the expression of several types of claudin in the human epidermis and cultured keratinocytes [10]. Among them, claudin-1 and -4 have been extensively studied [11], and they seem to be the chief contributors to maintenance of the barrier function in skin. First, we performed immunohistochemical staining of paraffin- embedded specimens of healthy skin to investigate the distribution of ΔNp63, claudin-1, and -4 in the epidermis. Using the ΔNp63-specific antibody p40, ΔNp63 was detected in nuclei of keratinocytes through basal to spinous layers and was significantly decreased in the granular layer, where a functional tight junction structure is assembled (Fig. 1A). Claudin-1 was distributed in the cell boundary of keratinocytes of para-basal to granular layers, whereas claudin-4 was expressed specifically in the granular layer (dotted line; Fig. 1A and B). Because ΔNp63 determines epithelial differentiation and its distribution was inversely correlated with claudin-4 in the epidermis, we hypothesized that ΔNp63 negatively regulates claudin-4 [12].

3.2. Expression of claudin-4 is negatively modulated by ΔNp63

To verify this hypothesis, we established ΔNp63 knockdown HaCaT keratinocytes by the RNA interfering method. Quantitative PCR and immunoblot analysis revealed that ΔNp63 was suppressed at both transcription ($P < 0.001$) and protein levels in the groups of HaCaT keratinocytes into which p63-specific siRNA had been introduced (Fig. 2A–C). In contrast, ΔNp63 knockdown keratinocytes

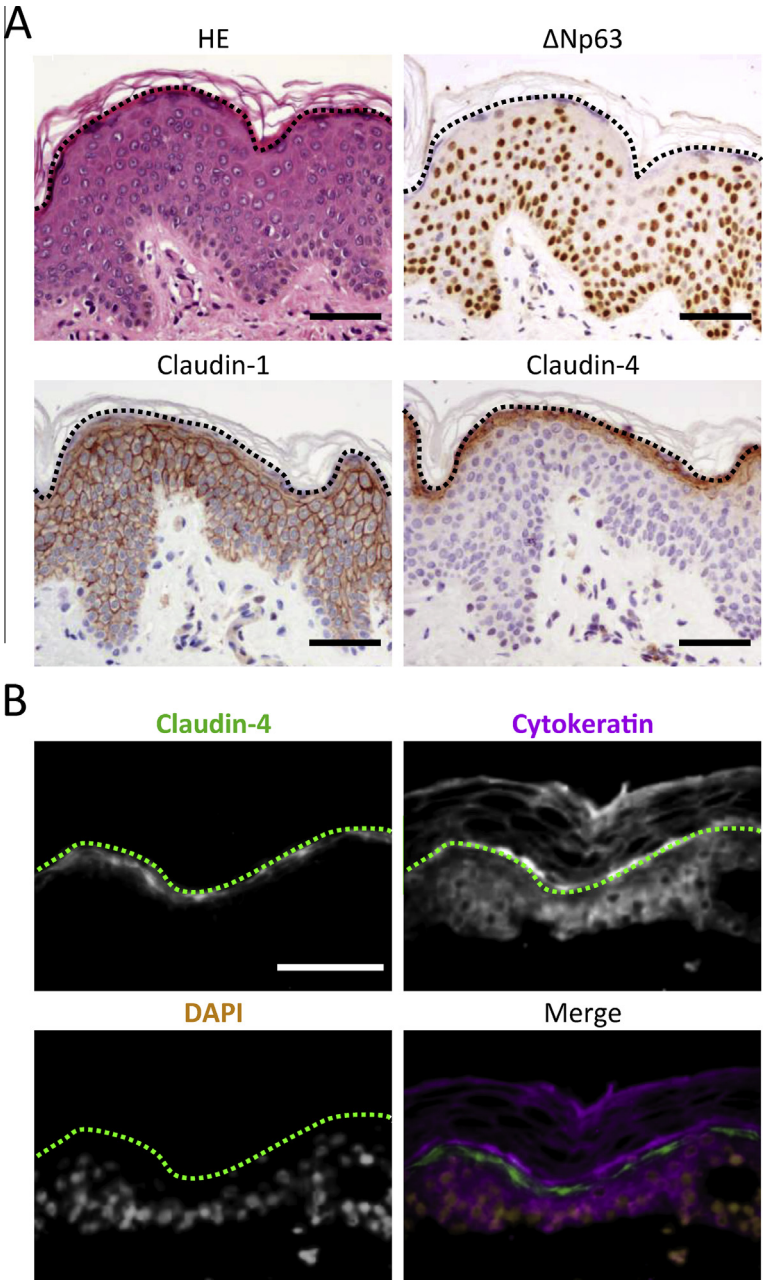

Fig. 1. Distribution of ΔNp63 and claudin-4 was inversely correlated in the epidermis. (A, B) Immunohistochemical analysis to determine the localization of ΔNp63, claudin-1 and claudin-4 in paraffin-embedded human epidermis. ΔNp63-specific antibody detects nuclei of keratinocytes in the basal to spinous layers (A). Claudin-1 is expressed in the para-basal to granulous layers (A). Claudin-4 is expressed in the sternum granulosm (A and B). DAPI, 4′,6-diamidino-2-phenylindole. Dotted lines show the granulous layer. Bar = 50 μm.

showed a several-fold increase of claudin-4 in quantitative PCR and immunoblot analysis, whereas claudin-1 showed no significant change (Fig. 2B and C). Collectively, ΔNp63, a coordinator of epithelial integrity, negatively regulates claudin-4 expression in keratinocytes.

3.3. TLR3 ligand suppressed ΔNp63 expression and increased claudin-4 expression in cultured keratinocytes

Accumulating evidence has indicated that damaged cells liberate their nucleic acids or proteins as danger signals, which are sensed via various innate immune receptors of infiltrating inflammatory cells or surrounding tissue cells [13]. Keratinocytes express functional TLRs, and we previously found that TLR3 ligand, potentially originating from damaged epithelial cells, constitutively suppresses ΔNp63 expression in primary keratinocytes [14]. To investigate whether TLR3 ligands regulate claudin-4 expression, the expression of claudin-4 in keratinocytes stimulated with various ligands of TLRs was analyzed by quantitative PCR. When primary cultured keratinocytes were treated with poly I:C, a synthetic TLR3 ligand, for 24 h, the shape of keratinocytes was altered from a polygonal to spindle pattern, resembling differentiated keratinocytes lacking ΔNp63 (Fig. 3A) [14]. Primary keratinocytes treated with the TLR3 ligand expressed a decreased level of

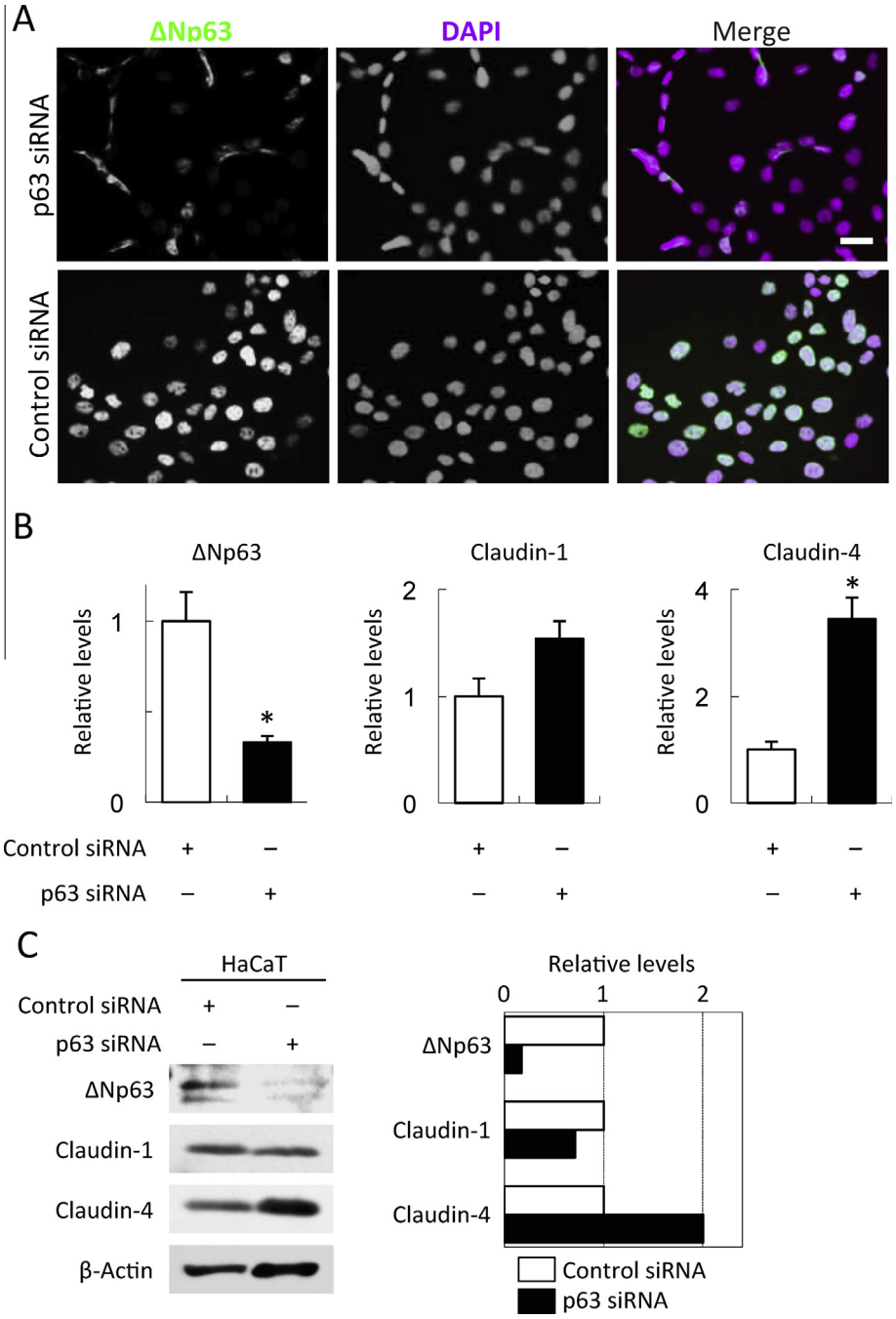

Fig. 2. Regulation of tight junction proteins by ΔNp63 in HaCaT keratinocytes. (A) Immunofluorescence labeling microscopy to detect ΔNp63 in HaCaT keratinocytes transfected with p63 siRNA or control siRNA. (B) Quantitative PCR for ΔNp63 and claudin-4 in HaCaT cells in which p63-specific siRNA or scramble siRNA for a control had been introduced. Transcript of claudin-4 is increased in ΔNp63 knockdown keratinocytes. Statistical analyses were performed using Student's *t* test. $^{*}P < 0.001$. (C) Immunoblot analyses also show that the level of claudin-4 is upregulated in ΔNp63 knockdown keratinocytes (left panel). The histogram shows the relative expression of tight junction proteins normalized to β-actin, which was used as a loading control (right panel).

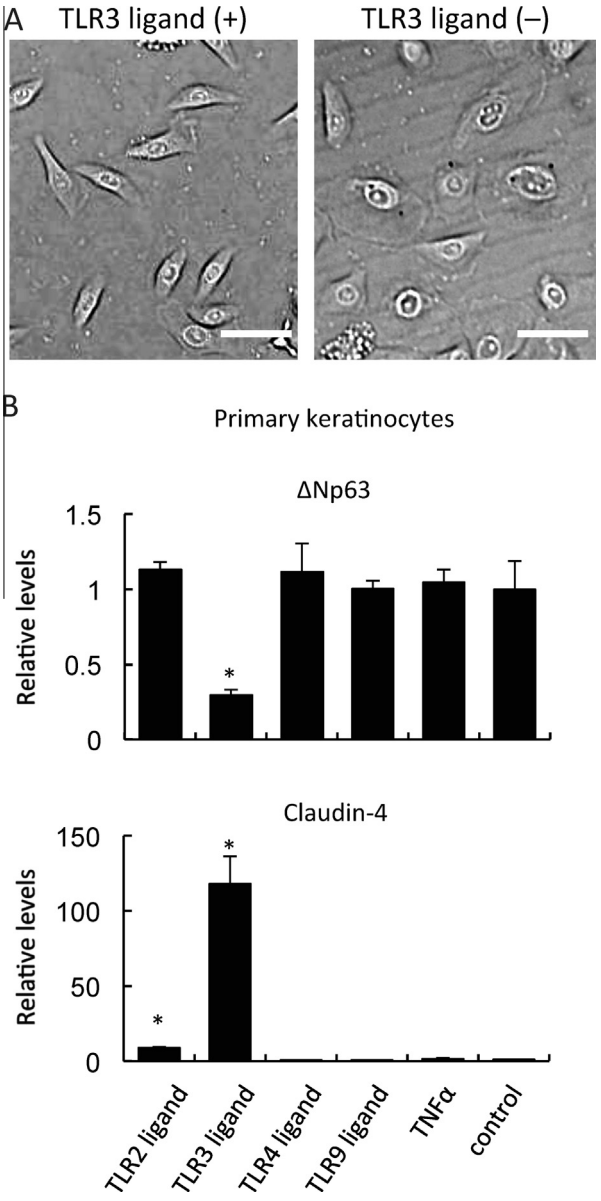

Fig. 3. Stimulation of keratinocytes by TLR3 ligand induced claudin-4 expression. (A) Treatment of primary cultured keratinocytes with poly I:C, an artificial TLR3 ligand, for 24 h induced morphological change from a polygonal to spindle shape similar to differentiated keratinocytes in the spinous layer, where ΔNp63 expression is lacking. (B) Treatment of primary cultured keratinocytes with the TLR3 ligand for 24 h decreased ΔNp63 expression and concomitantly increased claudin-4 expression. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison test. $^{*}P < 0.001$, versus control.

ΔNp63 and increased level of claudin-4 (Fig. 3B). Taken together with earlier results, our results suggest that the TLR3 signal upregulates claudin-4 via ΔNp63 suppression.

3.4. Aberrant claudin-4 expression in the epidermis of atopic dermatitis

In the pathogenesis of atopic dermatitis (AD), both acute and chronic dermatitis, aberrant expression of immune-related and barrier-related proteins in keratinocytes has reported [15,16]. These alterations of keratinocytes status are assumed to be induced by microorganisms or self-derived TLR ligand milieu. We recently reported that the expression of ΔNp63 is decreased in the epidermis of AD (Fig. 4A) [14]. These findings and our results shown above aroused our interest in whether the expression pattern of claudin-4 is altered in AD epidermis, and we performed immunofluorescent staining of AD skin specimen. As expected, claudin-4 was expressed more broadly at the cell boundary of keratinocytes in AD skin than in control skin (Fig. 4B). Collectively, the results indicate that claudin-4 is expressed aberrantly and that its expression is inversely correlated with ΔNp63 expression in the epidermis of AD.

4. Discussion

The present study demonstrated that claudin-4 is physiologically expressed distinctively in the stratum granulosum among the four layers of the epidermis and that its expression is modulated by ΔNp63, which plays a crucial role in epithelial differentiation. Various types of claudins and other tight junction-associated molecules are expressed in all of the layers of the epidermis; however, the functional tight junction structure is found only in the stratum granulosum, where claudin-4 is specifically expressed [1,2]. Although there has been no report about whether claudin-4 knockout mice present an epidermal barrier defect like that in claudin-1 knockout mice, which die shortly after birth with dehydration, an *in vitro* study using cultured keratinocytes revealed that claudin-4 knockdown resulted in a barrier defect [17,18]. Therefore, claudin-4 would play an important role in shaping an efficient barrier of the tight junction in healthy epidermis.

Tight junctions are not a static barrier structure but dynamically regulated in response to innate and adaptive immunity [11]. Our observations showed that claudin-4 in primary keratinocytes is induced by treatment with a TLR3 ligand probably via suppression of ΔNp63. Furthermore, claudin-4 was increased in keratinocytes of atopic dermatitis (AD). These findings are consistent with the results of our previous study showing decreased expression of ΔNp63 in AD, which is postulated to be induced by double-strand (ds) RNA, a natural TLR3 ligand potentially derived from damaged cells [14]. On the other hand, a number of studies have shown that AD is a disease caused, at least partly, by a barrier defect of epidermal keratinocytes [19]. In particular, claudin-1 expression in the affected area of AD skin is decreased [16]. Nevertheless, until the current study, expression profiles of claudin-4 in AD skin had not been investigated in detail. Considering that dsRNA is liberated from damaged epithelial cells [8,20] and that ΔNp63 is a master coordinator altering cellular status in response to an extrinsic insult [21], increase of claudin-4 expression in AD skin, which is regulated by the TLR3-ΔNp63 axis, might be associated with complementary responses to barrier dysfunction.

It is still unclear whether increased claudin-4 is correctly assembled into a functional tight junction structure and contributes to the constitution of an efficient barrier in AD skin. However, even if that is not the case, some studies have shown that claudin proteins not only act as a module for barrier function. It has been reported that claudin-4 is increased in human nasal epithelia by cytokines without significant change of barrier function [22]. In addition, a recent study showed that the second extracellular loop of claudin-4 excluded from the tight junction is able to interact with the extracellular environment to promote cell motility in normal mammary epithelial cells as well as in breast and ovarian cancer cell lines [23]. Although the function of non-junctional claudins has not been completely uncovered, they seem to have many important roles in cell functions such as proliferation, migration and apoptosis [9]. Therefore, it is possible that increased claudin-4 in AD skin contribute some repair reactions of keratinocytes that have been damaged due to barrier impairment and/or inflammation. Alternatively, since claudin-1 on epithelial cells and dendritic

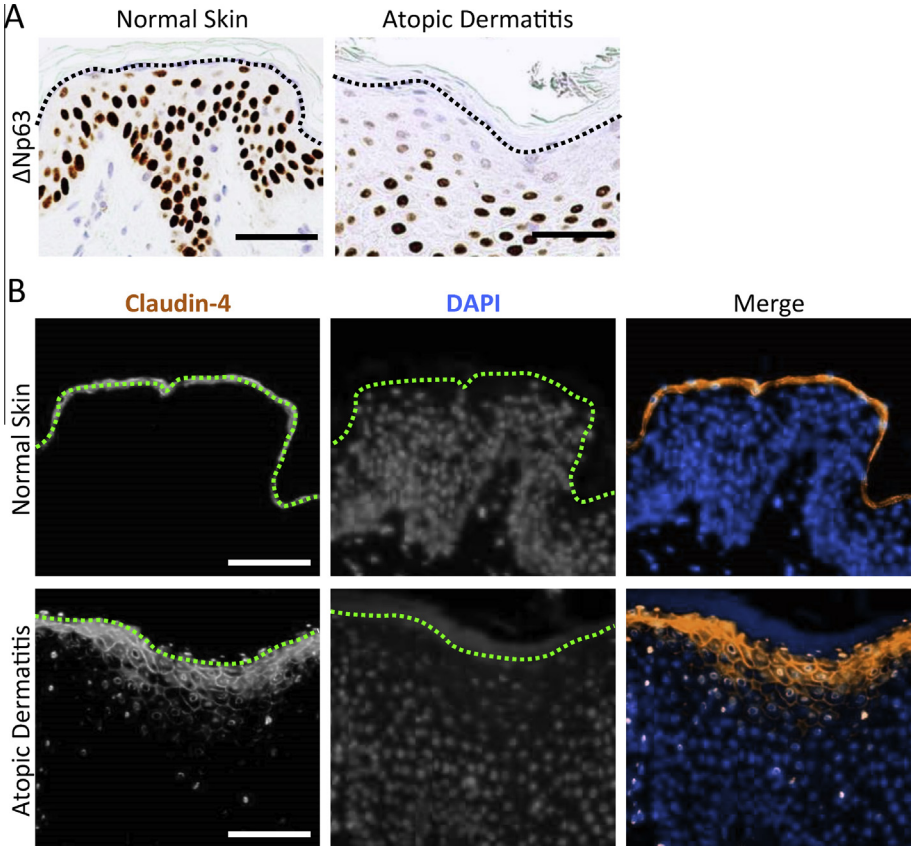

Fig. 4. Aberrant expression of claudin-4 in the epidermis of atopic dermatitis. (A) Paraffin-embedded sections of the normal or atopic dermatitis were stained for ΔNp63. In the epidermis of atopic dermatitis, the ratio of ΔNp63-negative nuclei is increased. Bar = 50 μm. (B) Immunofluorescence labeling of claudin-4 in the corresponding region of serial sections. Claudin-4 is distributed in the deeper spinous layer, where ΔNp63 is predominantly lacking. Dotted lines show the granulous layer. Bar = 50 μm.

cells (DC) interact with each other for penetrating dendrites of DC to access antigens, claudin-4 of keratinocytes might participate in such an interaction between epithelial cells and DC in response to an inflammatory environment [24,25].

In conclusion, the present study has shown that downregulation of ΔNp63 during the keratinocyte differentiation process or by TLR3 stimulation induces claudin-4 expression. Elucidation of the functional significance of this finding in preserving the barrier or other roles of claudin-4 may provide further insights into epidermal physiology and pathology.

Declaration of financial disclosure

The authors have no financial conflict of interest.

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