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# Expression of N-terminal truncated desmoglein 3 (ΔNDg3) in epidermis and its role in keratinocyte differentiation

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Abbreviations: Dg3, desmoglein 3; ΔNDg3, N-terminal truncated intracellular domain of Dg3

## Abstract

**During a search for keratinocyte differentiation-related genes, we obtained a cDNA fragment from the 5'-untranslated region of a previously identified splicing variant of desmoglein 3 (Dg3). This transcript encodes a protein of 282 amino acids, which corresponds to the N-terminal truncated intracellular domain of Dg3 (ΔNDg3). Northern blot analysis detected a 4.6-kb transcript matching the predicted size of ΔNDg3 mRNA, and Western blot analysis with an antibody raised against the Dg3 C-terminus (H-145) detected a 31-kDa protein. Increased ΔNDg3 expression was observed in differentiating keratinocytes by RT-PCR and Western blot analysis, suggesting that ΔNDg3 is indeed a differentiation-related gene product. In immunohistochemical studies of normal and pathologic tissues, H-145 antibody detected the protein in the cytoplasm of suprabasal layer cells, whereas an antibody directed against the N-terminal region of Dg3 (AF1720) reacted with a membrane protein in the basal layer. In addition, ΔNDg3 transcript and protein were upregulated in psoriatic epidermis, and protein expression appeared to increase in epidermal tumors including Bowen's disease and squamous cell carcinoma. Moreover, overexpression of ΔNDg3 led to increased migration and weakening of cell adhesion. These results suggest that ΔNDg3 have a role in keratinocyte differentiation, and that may be related with tumorigenesis of epithelial origin.**

**Keywords:** cell adhesion; cell differentiation; cell movement; desmoglein 3; epidermis; keratinocytes; skin neoplasms

## Introduction

Cell-cell adhesion mediated by membrane structures such as adherens junctions and desmosomes plays an important role in development and in tissue integrity. Adhesion structures are composed of large proteins, including cadherins, catenins, and plakins, which are ultimately linked to the cytoskeleton (Kowalczyk *et al*., 1999). With the cooperation of these proteins, cytoskeletal elements such as actin and keratin filaments anchor to the plasma membrane and add strength to cell-cell adhesions, resulting in adherens junctions and desmosomes, respectively (Angst *et al*., 2001). At desmosomes, desmogleins (Dgs) and desmocollins (Dscs) are cadherin family proteins that form a bridge between two cells. The cytoplasmic portions of desmosomes contain the intracellular domains of Dgs and Dscs, as well as the linking proteins plakoglobin, plakophilins, and desmoplakins (Kowalczyk *et al*., 1999). Desmogleins and classical cadherins are homologous in amino acid sequence both in their extracellular domains and in their intracellular cadherin-specific regions. Their C-terminal subdomain contains a desmoglein-specific repeating amino acid motif, which is termed the intracellular repeat region. Whereas the classical cadherins localize to adherens junctions, the desmogleins are found in desmosomes (Angst *et al*., 2001). Two of the desmogleins, Dg1 and Dg3, are autoantigens in the autoimmune blistering skin diseases pemphigus foliaceus and pemphigus vulgaris, respectively (Stanley *et al*., 1982; Payne *et al*., 2004; Baroni *et al*., 2007).

Terminal differentiation of skin keratinocyte is a vertically directed multi-step process that is tightly

controlled by the sequential expression of a variety of genes. To gain further insight into the molecular events involved in this process, we previously attempted to identify the differentially expressed genes using the techniques of suppression subtraction hybridization and cDNA microarray (Seo *et al*., 2004, 2005). Among the many candidates, we chose one transcript that has now been identified as a splicing variant of Dg3 (Genbank accession, BX538327). This transcript is identified originally from the human esophagus tumor cDNA, and thought to encode a hypothetical protein of 282 amino acids. However, the expression has not yet been determined in epidermis of human skin. Because it is predicted to encode a N-terminal truncated intracellular domain of Dg3, we named it as ΔNDg3, and investigated the expression and putative role in keratinocyte differentiation.

## Results

### Isolation of ΔNDg3, a novel gene associated with keratinocyte differentiation

We previously identified many of differentiation-related genes from primary skin keratinocytes cultured *in vitro* after treatment with calcium (Seo *et al*., 2004). We selected one differentially expressed clone that matched to the 5'-untranslated region of the cDNA BX538327, annotated as 'similar to desmoglein 3, differentially spliced'. This transcript is predicted to encode a hypothetical protein of 282 amino acids, which corresponds to the N-terminal truncated intracellular domain of Dg3. At this point, we designated it as ΔNDg3 (Figure 1A).

To investigate the relationship of this gene to Dg3, its mRNA was sized by Northern blot analysis. We made two different probes, one complementary to bases 1360-1619 of the Dg3 mRNA sequence (Genbank accession, NM_001944) and another recognizing a non-homologous sequence of ΔNDg3 (bases 866-1082 of the BX538327 mRNA). Using the model system in which immortalized keratinocytes HaCaT were induced to differentiate by calcium and ionophore A23187 (Fuchs, 1990; Zhao *et al*., 1992; Lee *et al*., 2005), we detected differential expression in RNAs. The sizes of the new gene product (4.6 kb) and of Dg3 (5.6 kb) corresponded to predictions from database sequences, and the clone's mRNA expression was upregulated in keratinocyte differentiation (Figure 1B).

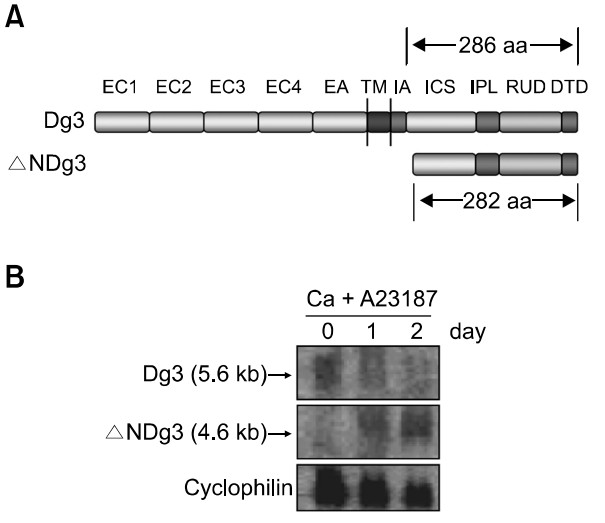


**Figure 1.** (A) Overall structure of Dg3 and ΔNDg3. EC1-EC4, four extracellular cadherin-typical repeats; EA, extracellular anchor domain; TM, transmembrane domain; IA, intracellular anchor domain; ICS, intracellular cadherin-specific domain; IPL, proline-rich linker domain; RUD, repeating unit domain; DTD, desmoglein-specific terminal domain. ΔNDg3 contains slightly shorter ICS, IPL, RUD and DTD domains. (B) Northern blot analysis. HaCaT cells were treated with 1 μM A23187 and 0.3 mM calcium for the indicated time points. About 5.6 kb Dg3 mRNA and 4.6 kb ΔNDg3 mRNA were shown. Cyclophilin was detected as a loading control.

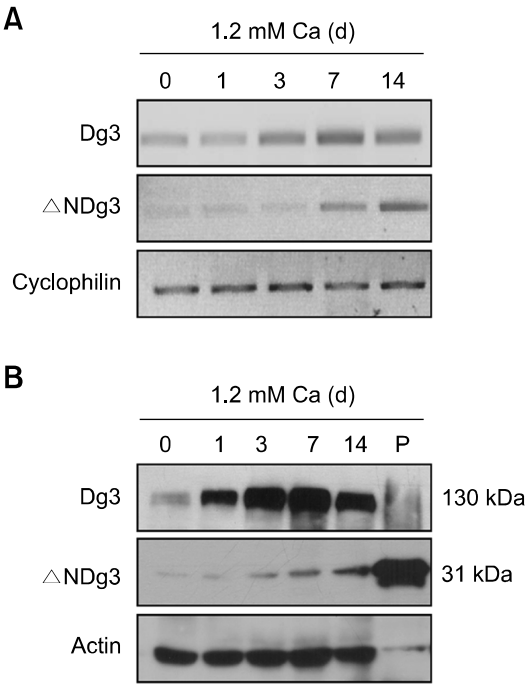


**Figure 2.** (A) RT-PCR analysis. Primary normal human epidermal keratinocytes were treated with high calcium at the indicated time points. Two μg of total RNAs were reverse transcribed with M-MLV reverse transcriptase and used for PCR amplification. (B) Western blot analysis. Cellular proteins were extracted from primary cultured keratinocytes, then separated on polyacrylamide gels. Blot was probed with C-term anti-Dg3 antibody. The positive control (P) for 31 kDa protein was prepared by transfection of pcDNA3.1-ΔNDg3 to HEK293 cells.

### Expression of ΔNDg3 in cultured keratinocytes and the epidermis

To further confirm the expression of ΔNDg3, we adopted another experimental model in which primary cultured human epidermal keratinocytes were differentiated by high calcium treatment. Consistent with previous data, RT-PCR showed that ΔNDg3 expression was markedly increased by calcium, in a time-dependent manner (Figure 2A). To determine the expression of ΔNDg3 at the protein level, we used two antibodies; one raised against the intracellular domain (residues 855-999, C-term) of Dg3 and one against the N-terminus (N-term) of Dg3. The N-term antibody could detect only Dg3, but the C-term antibody could bind to both Dg3 and ΔNDg3. Western blot analysis with C-term antibody showed the bands of Dg3 and ΔNDg3, with expected sizes (130 kDa and 31 kDa) respectively (Figure 2B).

Immunostaining with the C-term antibody identified the presence of ΔNDg3 protein at the spinous layer in normal epidermis, increasing in accordance with differentiation to the granular layer (Figure 3). In contrast, experiments with the Dg3 N-term antibody showed Dg3 expression primarily at the basal layer in normal epidermis, indicating different localization of the two homologs. Additionally, the N-term antibody stained along cell mem-

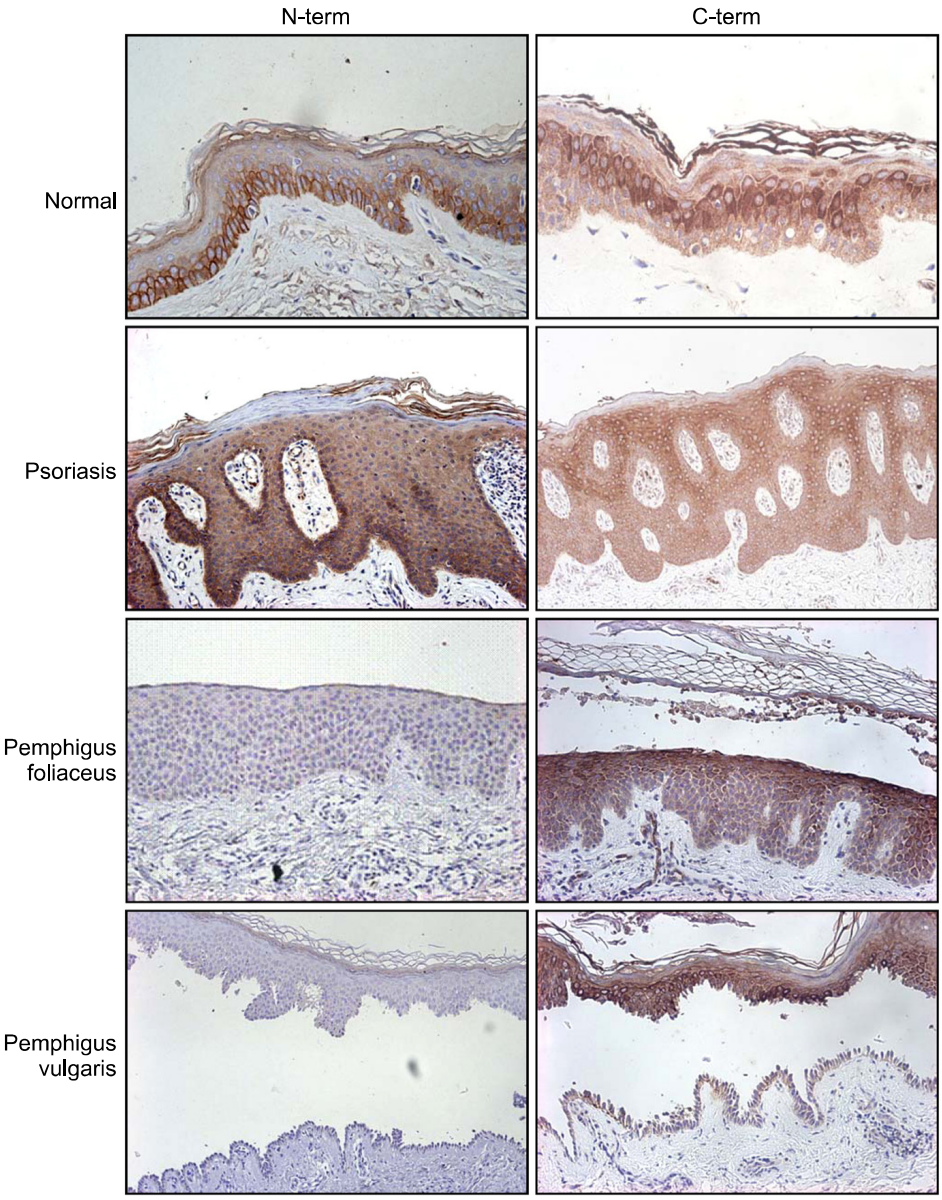


**Figure 3.** Immunohistochemical staining. Paraffin-embedded tissue sections of skin specimens were stained with N-term and/or C-term anti-Dg3 antibodies. The N-term antibody stains primarily along the cell membrane of basal layer, while the C-term antibody stains the cytoplasm of suprabasal layer.

branes, while the C-term antibody reacted more strongly in cytoplasmic regions. In psoriasis, staining with the C-term antibody showed the same pattern of expression as seen in *in situ* hybridization. We also examined immunostaining in two bullous diseases, pemphigus foliaceus, in which separation occurs in the subcorneal layer, and in pemphigus vulgaris, separating at the suprabasal layer. We did not see Dg3 expression in these samples, but confirmed ΔNDg3 expression from the suprabasal layer to subcorneal layer in the epidermis (Figure 3).

### ΔNDg3 supports epithelial cell migration and weakens keratinocyte intercellular adhesion in epithelial cells

Its expression from the suprabasal to the subcorneal layer in the epidermis suggested that ΔNDg3 could play a specialized role in the migration of keratinocytes. If adhesion between keratinocytes is too strong, they may not be able to move upward in the epidermis. To determine whether ΔNDg3 expression affected cell migration, we transfected HaCaT cells with ΔNDg3 expression vector, then performed *in vitro* wound-healing assay. Compared to vector control, HaCaT overexpressing ΔNDg3 showed markedly enhanced migratory behavior (Figure 4A).

To confirm the role of ΔNDg3 in weakening keratinocyte intercellular adhesion, we examined the adhesive strength of cells overexpressing ΔNDg3, using an enzymatic and mechanical dissociation assay. HaCaT cell monolayers were lifted from culture dishes using dispase, a recombinant protease that cleaves cell-matrix, but not cell-cell adhesion molecules (Calautti *et al*., 1998; Huen *et al*., 2002). Under shear stress, both vector control and ΔNDg3-transfected cell sheets dissociated into fragments. However, ΔNDg3-transfected cell sheet fragmented more than control, supporting the idea that ΔNDg3 has impact for weakening intercellular adhesion (Figure 4B).

### ΔNDg3 is associated with plakoglobin

As a mechanism for weakening intercellular junction,

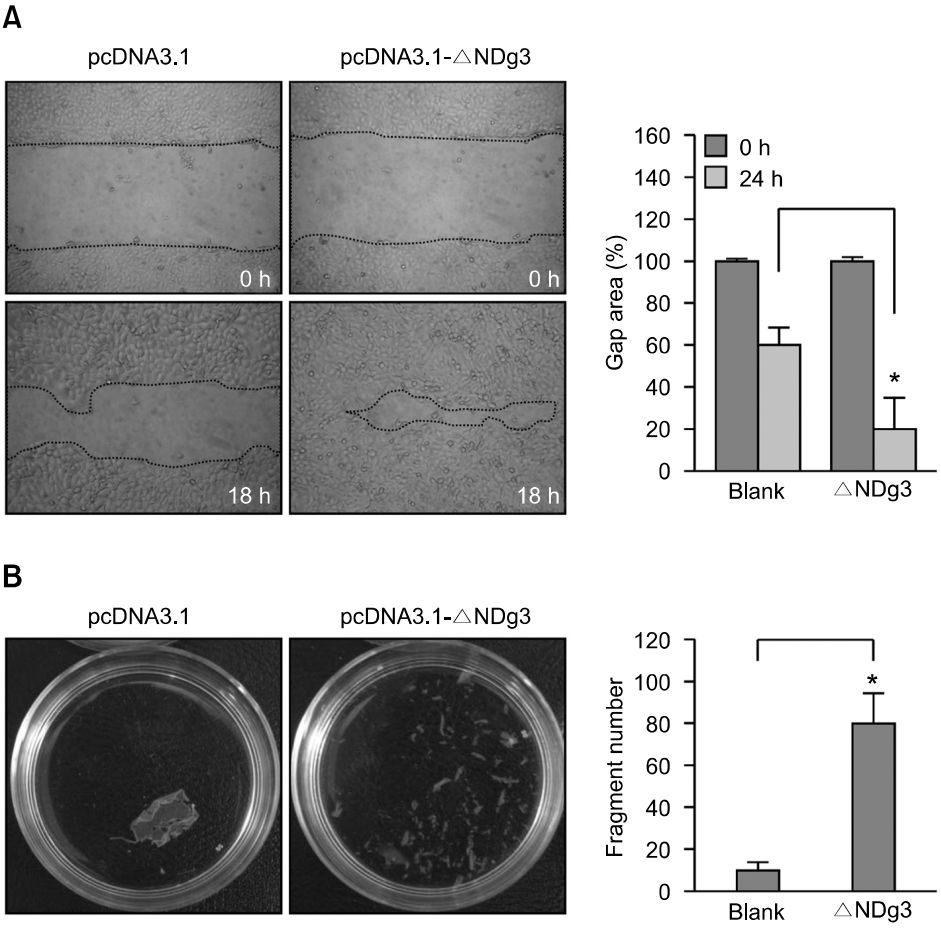


**Figure 4.** ΔNDg3 regulates migration of epithelial cells. (A) HaCaT cells were transfected with control vector (pcDNA3.1) or ΔNDg3 expressing construct (pcDNA3.1-ΔNDg3). Scratch wounds were made using a pipette tip, then cells were incubated for 18 h. A cell-free area was photographed and measured by image analyzer. Data are represented as percent control and SEM, measured from three independent experiments (*$P < 0.05$). (B) Control and ΔNDg3 transfectants were grown to confluence. Monolayers were separated from culture dishes via incubation with dispase. Dissociation assay results were quantified by counting the number of total particles. Data represents the average and SEM from three independent experiments (*$P < 0.05$).

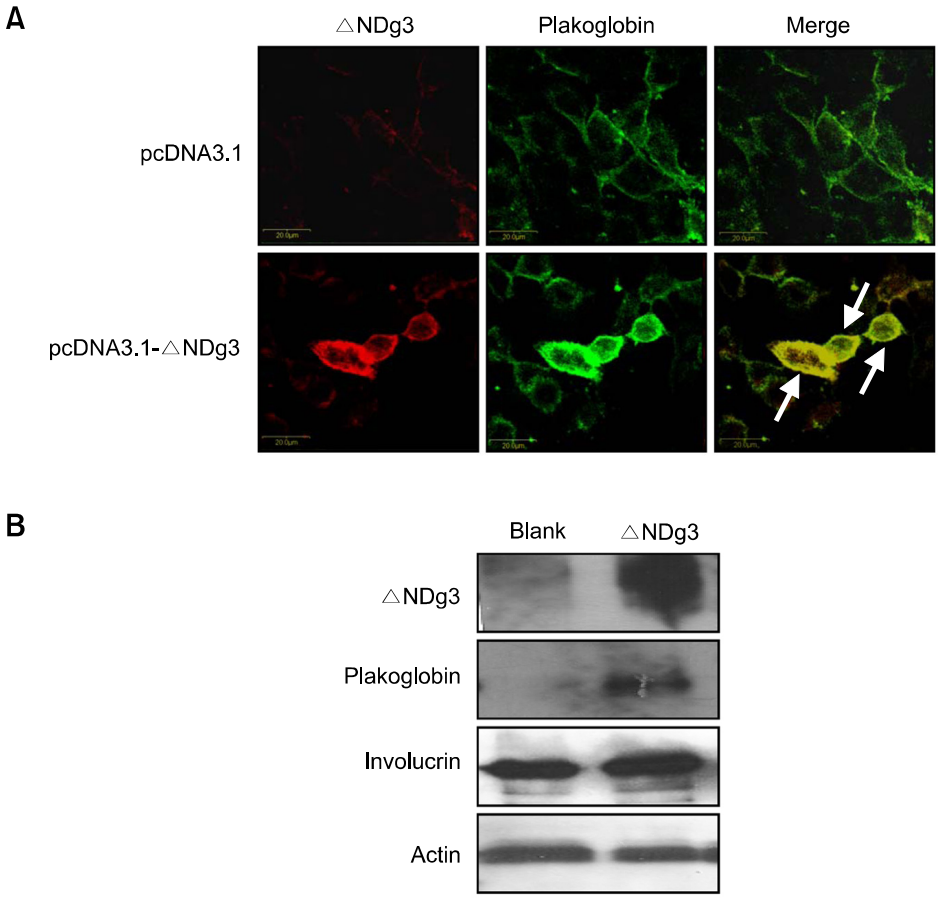


**Figure 5.** Plakoglobin and ΔNDg3 expression in HaCaT cells. (A) Cells were transfected with control vector or ΔNDg3 expressing vector. Cells were double-stained with C-term anti-Dg3 antibody and anti-plakoglobin antibody. Plakoglobin colocalizes with ΔNDg3 in merged cells (arrows). (B) Cells were transfected with ΔNDg3 overexpressing vector, then protein levels for ΔNDg3, plakoglobin and involucrin were detected by Western blot. Actin was used as a loading control.

we hypothesized that ΔNDg3 might recruit and compete for plakoglobin, which is necessary for Dg3 to connect to keratin intermediate filaments. Using ΔNDg3 overexpressing keratinocytes, we demonstrated that plakoglobin could co-localize with ΔNDg3. Interestingly, plakoglobin increased its expression in those cells, as evidenced by immunocytochemistry and immunoblotting, and its localization changed from membranes to cytoplasm with ΔNDg3 overexpression (Figure 5). These results suggest that ΔNDg3 could be involved in the regulation of plakoglobin, although such an interaction remains uncharacterized.

### Expression of ΔNDg3 in several skin diseases and cancer tissues

Next, we examined ΔNDg3 expression in diseases known to have alterations in keratinocyte migration using immunohistochemistry. Psoriasis is known to show abnormal differentiation of keratinocytes, with enhanced migration. In contrast, lichen simplex chronicus (LSC) is a disease characterized by a thickened epidermis due to delayed migration of individual keratinocytes from repeated scratching (Bernardin *et al*., 2006; Lotti *et al*., 2008). As expected, LSC tissue showed positive staining in the basal cells and in a few suprabasal layers (Figure 6A). In psoriasis, however, strong expression was observed in the cytoplasm of keratinocytes through almost the entire thickness of the epidermis (Figure 3).

A fundamental characteristic of cancer cells is altered adhesion, both to other cells and to the extracellular matrix. The possibility of a role for ΔNDg3 in keratinocyte migration in the epidermis led us to wonder whether its expression might be altered in cancers originating from keratinocytes. We examined ΔNDg3 expression in epidermal cancers, including Bowen's disease, basal cell carcinoma (BCC), and squamous cell carcinoma (SCC) by tissue immunohistochemistry. The expression of ΔNDg3 was upregulated in cancer cells of Bowen's disease and SCC, while the N-term anti-Dg3 antibody stained primarily the basal layer keratinocytes and did not stain the cancer mass (Figure 6B-G). This results support the view that upregulation of ΔNDg3 may have impact for skin cancer

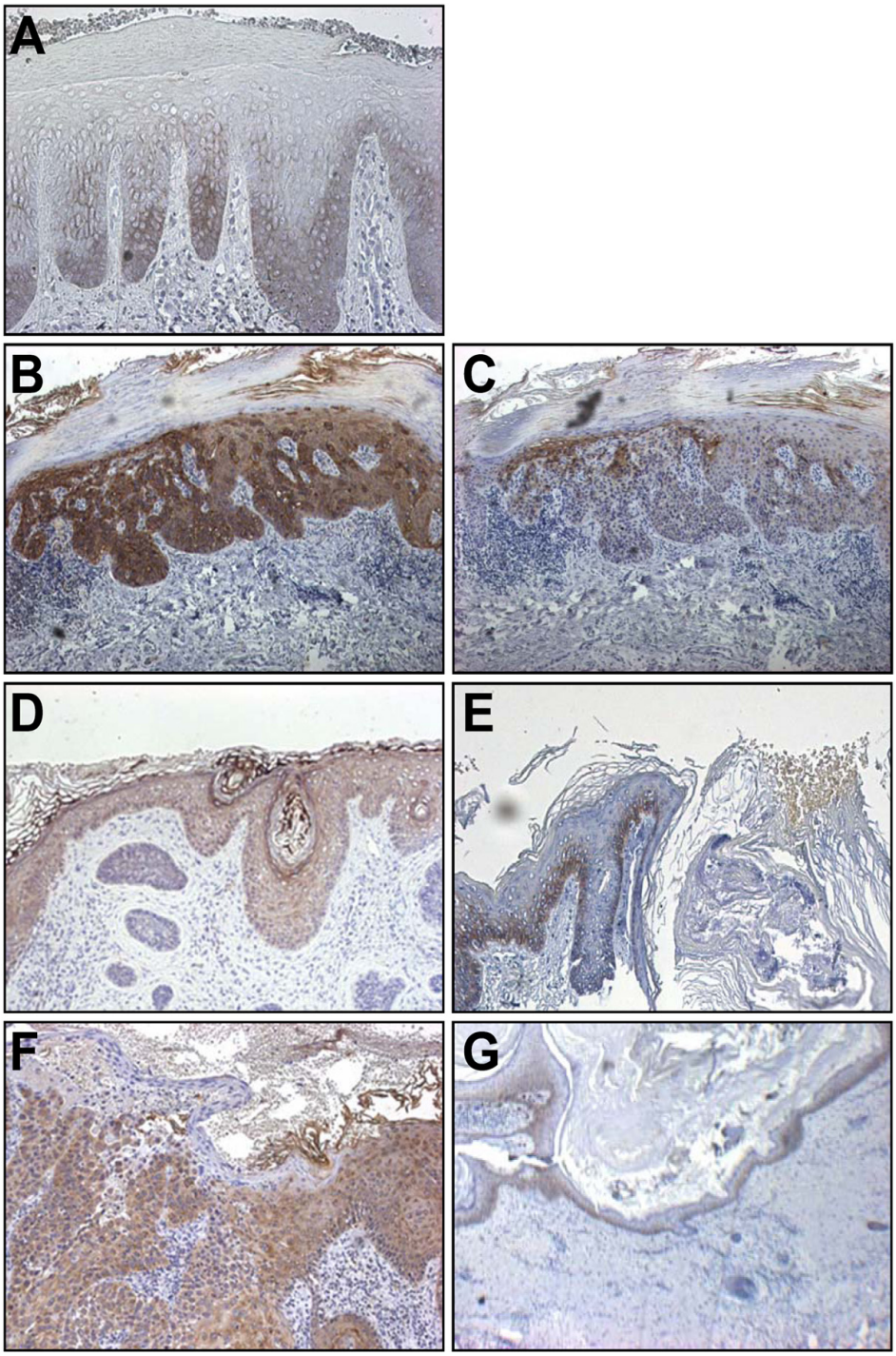


**Figure 6.** (A) ΔNDg3 expression in lichen simplex chronicus (LSC), a skin disease related with delayed migration of keratinocytes. Skin specimens were stained with C-term anti-Dg3 antibody. (B-G) ΔNDg3 expression in skin cancers. Specimens were stained with C-term anti-Dg3 antibody (B, D, F) and N-term anti-Dg3 antibody (C, E, G). (B, C) Bowen's disease, (D, E) basal cell carcinoma (BCC), (F, G) squamous cell carcinoma (SCC).

development, and that it might be a new target for cancer therapy.

## Discussion

Although many genes required for keratinocyte differentiation have been discovered, it is not sufficient to understand the sophisticated molecular mechanism underlying this process. In this study, we identified a differentially expressed human gene product in a calcium-induced keratinocyte differentiation model. We designated this transcript, which was previously cloned and identified

as the product of alternative splicing of the desmoglein 3 gene, as ΔNDg3. The results presented here highlight a previously unrecognized role of ΔNDg3 in support of epithelial cell migration in the keratinocyte differentiation process. Specifically, we found that ΔNDg3 is expressed at comparatively high levels in differentiated epidermal keratinocytes; forced ΔNDg3 expression enhances migration of keratinocytes, but decreases intercellular adhesion in epidermal sheets; ΔNDg3 expression in cancer tissues is upregulated relative to normal tissues; and ΔNDg3 may act in concert with plakoglobin to influence cell adhesion and migration during differentiation.

The identification of ΔNDg3 and its pattern of expression may help clarify why Dg3 staining was observed in all epidermal layers, using antibodies raised against multiple epitopes of Dg3 rather than against a specific epitope. These antibodies could detect both Dg3 and ΔNDg3, although earlier explanations proposed cross-reactivity between Dg3 and other desmogleins or desmocollins (Suhrbier and Garrod, 1986; Holton *et al*., 1990). In efforts to elucidate the processing of Dg3, the presence of ΔNDg3 could obfuscate our understanding of events in the cytoplasm, provided antibodies reacting with ΔNDg3 are not carefully excluded. For example, antibodies raised against the IA domain of Dg3, not present in ΔNDg3, could be chosen for studies of Dg3 processing inside cells.

The deduced amino acid sequence from the human ΔNDg3 cDNA predicted a protein of 282 amino acids with a calculated molecular mass of 31 kDa. The protein contained plakoglobin-binding sites like other desmoglein family members, but no E-cadherin binding domain. In contrast to Dg3 expression in membrane of the basal layer, ΔNDg3 was found to be cytoplasmically expressed in the suprabasal layer of the epidermis. Because of its expression pattern, we hypothesized that ΔNDg3 play a role in the migration of keratinocytes during differentiation. In our experiments, keratinocytes showed markedly enhanced migratory behavior and weakened intercellular adhesion after transfection with ΔNDg3. We speculate that this effect may be, in part, due to the competitive binding of ΔNDg3 to the Dg3 binding partners. Based on the previous data and its domain structure, we postulated that one possible binding partner for ΔNDg3 is plakoglobin. Plakoglobin may contribute to desmosome assembly through its interaction with the intracellular region of desmocollins and desmogleins (Mathur *et al*., 1994; Yin and Green, 2004; Stokes, 2007). In our experiments, overexpression of ΔNDg3 resulted in broken focal cell adhesions and the movement of plakoglobin from the membrane to the cytoplasm, despite increasing plakoglobin protein levels. These results suggest that ΔNDg3 may disturb desmosome assembly by depleting the free intracellular desmosome components, thereby contribute to the weakening of cell-cell interaction. The precise mechanism remains to be investigated further.

Two disorders showing significant increases in epidermal thickness (acanthosis) from different mechanisms revealed different patterns of ΔNDg3 expression. Lichen simplex chronicus (LSC) from delayed keratinocyte migration was associated with downregulation of ΔNDg3, but psoriasis, with increased keratinocyte migration, showed upregulation of ΔNDg3. These results further hint a role for ΔNDg3 in keratinocyte migration.

As previously recognized, alterations in epithelial cell interactions have impact on carcinogenesis, tumor invasion, and metastasis. For example, reduced synthesis of epithelial junctional proteins was frequently observed in dedifferentiation, tumorigenesis, and metastasis. Thus, it is generally accepted that loss of adhesive molecules and of adhesion structures is implicated in the development of an invasive phenotype, and predicts a poor prognosis for patients (Trosko *et al*., 2004; Kartenbeck *et al*., 2005). In this study, the expression of ΔNDg3 is highly increased in SCC, but not in BCC, suggesting that ΔNDg3 may have a relation with tumor cell invasion. A better understanding of invasion patterns in many skin diseases are needed to explain the effects of ΔNDg3 on cancer development.

In conclusion, we identified a new gene, ΔNDg3, and characterized its structure and expression in cultured human keratinocytes, normal epidermis, and various skin disorders, including psoriasis and several epithelial cancers. Our results suggest that ΔNDg3 is involved in the differentiation and migration of cells in epithelia. It may have a novel function in epidermal differentiation and in related processes, and thus serve as a potential target in skin cancer and other disease states.

## Methods

### Cell culture and transfection

All skin samples were obtained under the written informed consent of donors, in accordance with the ethical committee approval process of Chungnam National University Hospital. Skin specimens were briefly sterilized in 70% ethanol, minced, and then treated with dispase for overnight at 4°C. The epidermis was separated and placed in a solution containing 0.05% trypsin and 0.02% EDTA (Gibco BRL, Rockville, MD) for 15 min at 37°C. After vigorous pipetting, cells were pelleted and resuspended in keratino-

cyte-serum free medium (K-SFM) supplemented with bovine pituitary extract and recombinant human EGF (Gibco BRL). Primary keratinocytes were differentiated by addition of 1.2 mM calcium. Immortalized human keratinocytes HaCaT and HEK293 cells were cultured in DMEM supplemented with 10% FBS (Gibco BRL). For differentiation of HaCaT, cells were cultured with 1 μM A23187 and 0.3 mM calcium for the indicated time points. For transfection, cells were incubated with FuGENE6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

### Northern blot analysis

Total RNAs were prepared using an Easy blue RNA isolation kit (Intron, Daejeon, Korea). For Northern blot analysis, 20 μg of total RNAs were electrophoresed in 1% agarose gels containing formaldehyde and transferred onto Hybond-$N^+$ membranes (Amersham, Buckinghamshire, UK). Blots were prehybridized for 2 h at 42°C, then hybridized overnight at 42°C in the same solution containing $^{32}$P-labeled probe (specific activity is ~$1 \times 10^9$ cpm/μg). Probes were prepared by PCR amplification of cDNA with following primer sets: Dg3, 5'-CAAAGCTGCCTCAAATGTCA and 5'-TGCAAACTGCATCTTTTTCG; ΔNDg3, 5'-TGTTTCCTCTTTGGTTTCCA and 5'-GAGGGCATATAAACGGGTGA.

### Western blot analysis

Cell extracts were prepared using Pro-prep protein extraction solution (Intron). Protein samples were run on 7-12% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane and incubated with appropriate antibodies. To detect ΔNDg3, the antibody raised against C-term (855-999 amino acids) of human Dg3 (H-145, Santa Cruz Biotechnologies, Santa Cruz, CA) was used. Blots were then incubated with peroxidase-conjugated secondary antibody and developed by enhanced chemiluminescence (Amersham). Anti-plakoglobin and anti-involucrin antibodies were purchased from Santa Cruz Biotechnologies.

### Cell migration assay

Cell migration assay was performed according to the previously reported method with slight modifications (Kim *et al*., 2007). HaCaT cells were seeded in six-well plates and transfected with pcDNA3.1-ΔNDg3 or empty vector using FuGENE6. To rule out the proliferative effect, cells were pretreated with 10 μg/ml mitomycin C (Sigma, St. Louis, MO) for 2 h. A cell-free area was introduced by scraping the monolayer with a pipette tip. After incubation, cells were photographed by using an inverted phase- contrast microscope, and gap areas were measured using Image-Pro Plus software (MediaCybernetics, Silver Spring, MD).

### Dispase-based dissociation assay

The dispase-based dissociation assay was modified from a published protocol (Calkins *et al*., 2006). HaCaT cells were seeded in triplicate onto 60-mm dishes. At about 40～50% confluency, cells were transfected with pcDNA3.1-ΔNDg3 and mock vector. After reaching confluency, culture dish were washed twice in PBS and then incubated in 4.8 Unit of dispase (Roche Applied Science) for 30 min. Released monolayers were carefully washed with PBS and transferred to 15 ml tubes. Enough PBS was added for a final volume of 5 ml. Tubes were subjected to 50 inversions. Fragments were counted using a dissecting microscope. Statistical analysis was performed on the average of three separate experiments. Statistical significance (*t* test) was defined as $P < 0.05$.

### Immunofluorescence

HaCaT cells were grown on coverslips. After transfection, cells were fixed and permeabilized with 4% paraformaldehyde for 15 min at room temperature, and 0.5% tritonX-100 for 1 min. Cell were then incubated with appropriate antibodies for overnight at room temperature, then incubated with FITC- and TRITC-conjugated secondary antibodies, and visualized under the fluorescent microscope.

### Acknowledgements

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