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Protein kinase C δ and η differently regulate the expression of loricrin and Jun family proteins in human keratinocytes

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ABSTRACT

Barrier function of the epidermis is maintained by precise expression of keratinocyte-specific structural proteins to form the cornified cell envelope (CE). Loricrin, a major component of the CE, is expressed at the late stage of keratinocyte differentiation. In this study, we reveal the isoform-specific function of protein kinase C (PKC) in the regulation of loricrin expression. Both PKCδ and PKCη have been recognized as differentiation-promoting isoforms. However, loricrin expression was inversely controlled by PKCδ and PKCη in cultured keratinocytes and 3D skin culture; i.e. loricrin expression was decreased by PKCδ and increased by PKCη. To clarify the mechanisms that PKCδ and PKCη oppositely regulate the loricrin expression, we examined the expression of activator protein-1 (AP-1) family proteins, which modulate the transcription of loricrin and are downstream molecules of PKC. PKCδ decreased c-Jun expression, whereas PKCη increased JunD, which are positive regulators of loricrin transcription. These findings suggest that inverse effects of PKCδ and PKCη on loricrin expression attributes to the expression of c-Jun and JunD.

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1. Introduction

The epidermis is the major component to defend the organism against irritants in its external surroundings. The multilayer structure of the epidermis is maintained by a finely tuned balance of growth, differentiation and cell death. Once the basal cells detach from the basement membrane, they proceed to terminal differentiation. They exit the cell cycle and sequentially induce a set of differentiation-associated genes including keratin1/10 (K1/K10), SPRs, involucrin and loricrin [1,2]. Eventually, activation of transglutaminase 1 (TGase 1) leads to the formation of the cornified envelope by cross-linking its substrate proteins such as loricrin.

Protein kinase C (PKC) is a serine/threonine kinase which regulates a wide variety of cellular functions including keratinocyte differentiation [3–5]. PKC consists of 10 isoforms which are classified into conventional (α, β, γ), novel (δ, ε, η, θ, μ) and atypical (ζ, λ/ι) subfamilies based upon their molecular structure and co-factor requirements [6]. Among them, five PKC isoforms are expressed in keratinocytes [7], i.e. PKCα, δ, ε, η and ζ. In particular, PKCη has a unique feature in the skin; it is specifically expressed in the differentiated epithelial tissues including the upper layer of epidermis. PKCη is associated with Fyn tyrosine kinase in keratinocytes, leading to the inactivation of CDK2 [8]. Moreover, in our previous study, PKCη induces the cell cycle arrest at G1 phase and the terminal differentiation by the activation of TGase 1 [4].

Some studies have shown that PKCη shares the common features with PKCδ. PKCδ also inhibits the cell proliferation and activates TGase1 [4]. Involucrin mRNA and proteins are increased by PKCδ and PKCη via the MEKK1/MEK3/p38δ MAPK pathway [9,10]. Schindler et al. reported that PKCδ and PKCη elicit proline-rich protein tyrosine kinase (Pyk2) activation, resulting in the increase of promoter activity and protein level of involucrin [11]. However, PKCδ and PKCη possess discrete properties in the activation and down-regulation, subcellular localization and its associated proteins [12–15]. PKCδ is located in whole layers of the epidermis, while PKCη is restricted to the differentiated layer [16]. PKCδ promoted the caspase-3 activity during UV-induced apoptosis, whereas PKCη reduced its activity [17]. These findings suggest the possibility that PKCδ and PKCη have specific function in keratinocytes.

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In the current study, we evaluated the roles of PKCδ and PKCη in keratinocyte differentiation by suspension culture. Furthermore, we used a three-dimensional (3D) skin culture to examine the functions of PKCδ and PKCη in the epidermis. We describe here that PKCδ and PKCη have opposite abilities to modulate the expression of loricrin and diversely affect the expression of Jun family transcriptional factors, which are known as downstream molecules of PKCs.

2. Materials and methods

2.1. Antibodies

Anti-human keratin 1, keratin14, anti-involucrin, and loricrin antibodies were from Covance (Richmond, CA). Anti-PKCδ (C-20), c-fos (H-125), c-Jun (H-79), JunB (C-11) and JunD (329) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-PKCη antibody (GTX24134) was from GeneTex Inc.

2.2. Cell culture and 3D skin culture

Primary normal human keratinocytes (NHK) were purchased from Lonza (Walkersville, MD) and cultured in serum-free keratinocyte growth medium2 (KGM2, Lonza) including human keratinocyte growth supplement as described previously [4] For suspension-induced differentiation, NHK were detached with 0.025% trypsin and 0.02% EDTA and suspended in 1.45% methylcellulose-containing KGM2 on polyhydroxyethylmethacrylate (poly-HEMA)-coated dishes [18]. *In vitro* skin reconstitution (3D culture) was prepared as described previously [19]. Briefly, the dermal equivalent was made with 2.5×10^5 normal human dermal fibroblasts (Lonza) per collagen (Collagen Type A, 2.4 mg/ml, Nitta Gratin, Osaka, Japan) in cell culture inserts (1.0 mm pore size, PET track-etched membrane, Becton Dickinson Lab ware, Franklin lakes, NJ). NHK were seeded on the collagen matrix at the density of 1.2×10^6 cells per gel. The medium was replaced with KGM2: FAD = 1:1 after 24 h of seeding and the culture was further continued for 24 h. The gel surface was raised to the air–liquid interface. After 9 days, the gel was embedded in Tissue-Tek OTC compound (Sakura Finetek) and placed into liquid nitrogen for frozen section or fixed with formalin for paraffin-embedded section.

2.3. Adenovirus-mediated gene transfer

Replicative-deficient Ad5 type adenovirus vectors of wild and the kinase negative mutants of mouse PKCδ and mouse PKCη were constructed as described previously [4]. Kinase negative mutants of PKC were generated by substitution of arginine or alanine for lysine at the ATP binding site of PKC [17]. Purification and titration of adenovirus was performed with Adeno-X adenovirus purification kit and Adeno-X rapid titer kit (Clontech) according to the manufacturer's instructions. For introducing the adenovirus vectors into 3D cultures, adenovirus (1.0×10^8 pfu/gel) was added both onto the keratinocytes and into the medium of the culture 1 day before air-exposure of the gel.

2.4. Real-time PCR

Total RNA was extracted from the cells by using Illustra RNA spin kit (GE Healthcare) according to the manufacture's protocol. One microgram of total RNA was reverse-transcribed to cDNA by using Superscript III reverse transcriptase (Invitorgen Inc.) and random primers (Promega). Gene expression was measured by quantitative RT-PCR by using ABI PRISM 7000 or StepOne Real-Time PCR System (Applied Biosystems) and SYBR Green method (SYBR GreenER qPCR SuperMix, Invitrogen). Expression levels of mRNA were normalized to corresponding glyceraldehyde-3-phosphate de-hydrogenase (GAPDH) house keeping gene. The sequence of the primer pairs, 5′ and 3′, were as fellows: K1, AGGGGGCTTC AGCTCTGGCT and TGGTGGTCCTGCGCTGGTAGT; involucrin, TCCAC TGCCTCCCCCATGCC and GCAGCTCCTGCTCCTGTGGC; loricrin, GG AGTTGGAGGTGTTTTCCA and CAAACCTCGGGTAGCATCAT; SPR2a, TGGTACCTGAGCACTGATCTGCC and CCAAATATCCTTATCCTTTCTT GG; c-Jun, GTACCTGATGAACCTGATGC and GGTCACAGCACATGCCA CTT; JunB, GTCACCGAGGAGCAGGAGG and TCTTGTGCAGATCGTCCA GG; JunD, TGACGCTGAGCCTGA GTGAG and TCGGGAGAGGCGAGCA.

2.5. RNA interference

For transient RNA interference, PKCδ siRNA (sc-36353) from SantaCruz Biotech was transfected into NHK with Lipofectamine RNAiMAX (invitrogen). Twenty-four hours after transfection, the cells were trypsinized from the dishes and cultured in the suspension medium as described above. Control siRNA-A (sc-37007, SantaCruz Biotech.), which consists of a scrambled sequence, was used as a negative control.

2.6. Immunoblotting

Whole cell lysates were prepared by lysing the cells in 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, 20 mM NaF, 50 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF. Lysates were centrifuged for 10 min at 4 °C and an equal amount of protein from the supernatants was used for SDS–PAGE and immunoblotting.

2.7. Immunohistochemistry

3D cultures were fixed with 3.7% formaldehyde and embedded in paraffin. Sections were de-waxed and boiled in 10 mM citrate buffer (pH 6.0) in a microwave oven at 500 W for 10 min. For staining of Jun/Fos family genes, the specimen were blocked with 2% goat serum and incubated with the primary antibody. Proteins were detected with Histofine Max-PO kit (Nichirei, Tokyo). Frozen cryostat sections were fixed with ethanol: acetone = 1:1 at −20 °C for 10 min. After washing with phosphate-buffered saline (PBS), the specimen was blocked with 2% goat serum and incubated with the appropriate primary antibodies against the keratinocyte differentiation markers or PKC isoforms. After additional three washes with PBS, the sections were visualized with Alexa488- or Alexa546-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (Invitrogen). Nuclear staining was performed with DAPI.

3. Results

3.1. Opposite effects of PKCδ and PKCη on loricrin induction during suspension-induced differentiation of keratinocytes

Throughout the present study, we used the adenovirus vectors expressing dominant negative mutants of PKCδ (D/Nδ) and PKCη (D/Nη) to inhibit each kinase activity in keratinocytes. First, we examined the expression of various differentiation markers in suspension-induced differentiation of NHK introduced D/Nδ and D/Nη. Real-time PCR analysis revealed that K1 mRNA expression, an early marker of differentiation, was significantly increased by D/Nδ and D/Nη. Induction of SPR2a and involucrin genes, upper spinous and granular layer markers, were decreased in D/Nδ- and D/Nη-expressing keratinocytes (Fig. 1A). These results suggest that both PKC isoforms function as positive regulators in the mid/late stage

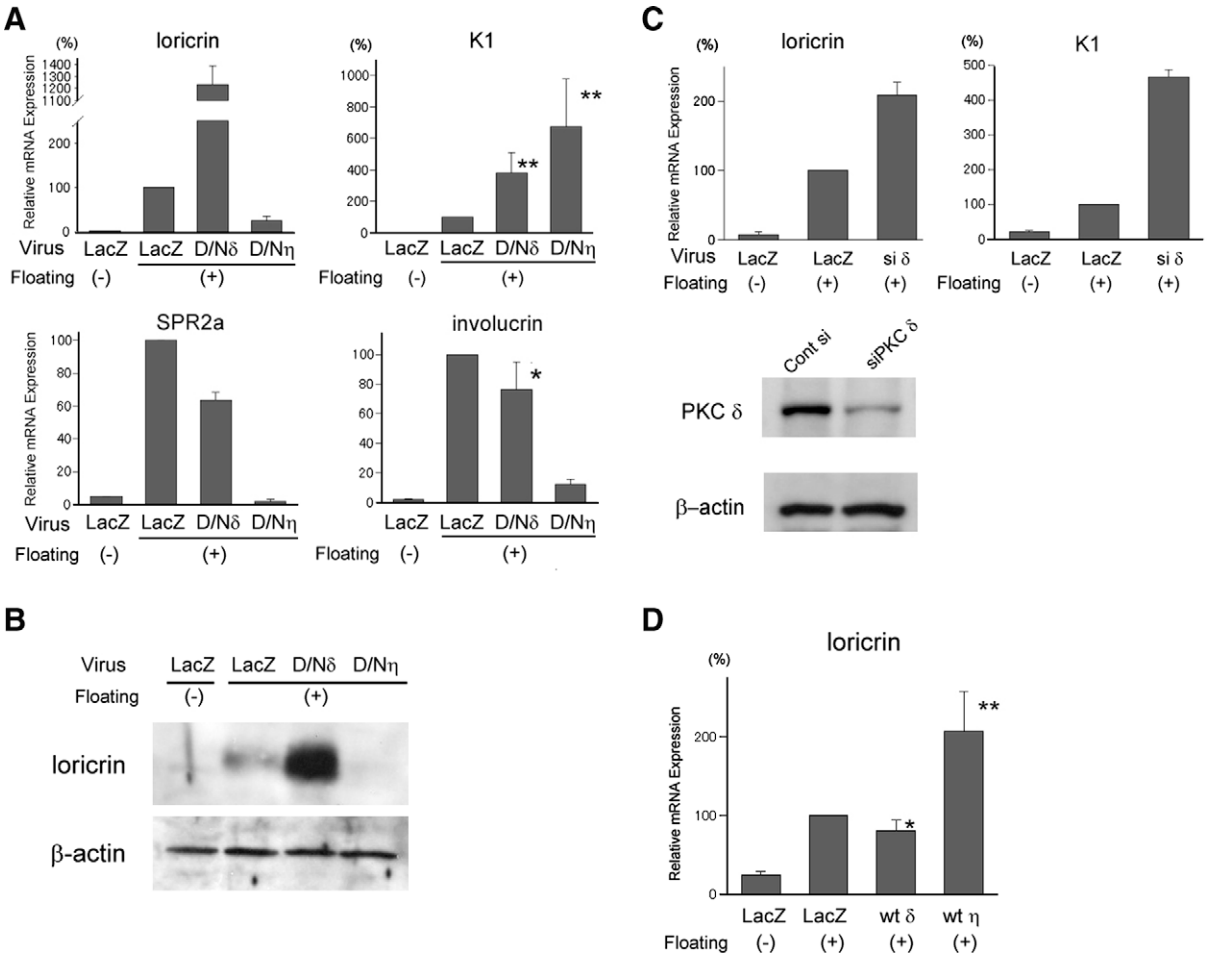

Fig. 1. PKCδ and PKCη exhibit opposite effects on the expression of loricrin in human keratinocytes. (A) Real-time PCR analysis of the differentiation marker in human keratinocytes differentiated by suspension culture (Floating). Twelve hours after infection with the adenovirus vector of β-galactosidase (LacZ), or the dominant negative PKCδ (D/Nδ) or PKCη (D/N η), normal human keratinocytes (NHK) were induced to differentiation by the suspension for 48 h. Values represent means ± standard deviations of triplicate determinations and are shown as a relative ratio to the value for the suspended lacZ-infected cells. (B) Western blot analysis of loricrin in dominant negative PKC–expressing NHK. Differentiation was induced by suspension for 60 h. (C) Effects of PKCδ siRNA on the induction of loricrin and K1 mRNA. (D) Effects of wild type PKCδ and PKCη on the induction of loricrin by the suspension culture. Cells were subjected to the suspension culture for 36 h. MOI of the adenovirus vectors were at 15 in all experiments. Asterisks indicate the significant difference from the suspended LacZ-infected cells ($^{*}P < 0.1$, $^{**}P < 0.05$).

of keratinocyte differentiation. However, only the loricrin expression, a late marker of differentiation, was inversely affected by D/Nδ and D/Nη (Fig. 1A, upper left). D/Nη inhibited the induction of loricrin significantly, whereas D/Nδ increased strikingly. These contrastive effects between PKCδ and PKCη were verified by the experiment of immunoblot shown in Fig. 1B. The above results were unexpected, because 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) increases loricrin in keratinocytes [3]. Therefore, we confirmed the effects of PKCδ on suspension-induced differentiation by using PKCδ siRNA. Compared to scramble siRNA-transfected NHK, the loricrin mRNA level was elevated in the siPKCδ-transfected cells (Fig. 1C). K1 expression was also increased by PKCδ siRNA. Furthermore, we examined the effects of wild type PKCδ (wtδ) and PKCη (wtη). The gain of function analysis revealed the inverse effects on the loricrin expression to the loss of function analysis shown above (Fig. 1A–C). Overexpression of wtδ slightly reduced the expression of loricrin, while wtη increased it (Fig. 1D). These results indicate that PKCδ represses the loricrin expression and PKCη increases it during the suspension-induced differentiation.

3.2. Inhibition of PKCδ increases the loricrin expression in 3D culture

To elucidate the role of PKCδ and PKCη in the epidermal differentiation in more physiological condition, we explored the influences of the dominant negative PKCδ or PKCη in *in vitro* reconstituted skin equivalent (3D culture). The D/Nδ or D/Nη adenovirus vector was transduced into 3D culture before forming the cornified layer and lifting to the air-exposure. Elevated expression of either D/Nδ or D/Nη was confirmed in the 3D culture even after 9 days of infection (Fig. 2A). HE staining showed the moderately thicker epidermis in both D/Nδ and D/Nη-introduced 3D cultures than LacZ-transduced control culture (Fig 2B, top panel). In accord with the results seen in Fig. 1, loricrin expression was inversely affected by PKCδ and PKCη (Fig. 2B). In the D/Nδ-introduced 3D culture, the expression of loricrin apparently increased in the granular layer, and its weak expression can be seen even in some cells of the spinous layer, suggesting that PKCδ suppresses the expression of loricrin in the epidermis. In contrast, loricrin expression decreased in the D/Nη-introduced 3D culture (Fig. 2). Expression of involucrin was attenuated by both D/Nδ and D/Nη, and K1 was increased (Fig. 2B).

3.3. Inhibition of PKCδ and PKCη results in the different expression pattern of AP-1 family genes

Loricrin gene harbors the AP-1 binding sites in the promoter, and Jun family proteins mainly regulate the transcriptional activity of loricrin [20]; c-Jun and JunD increase the transcription activity,

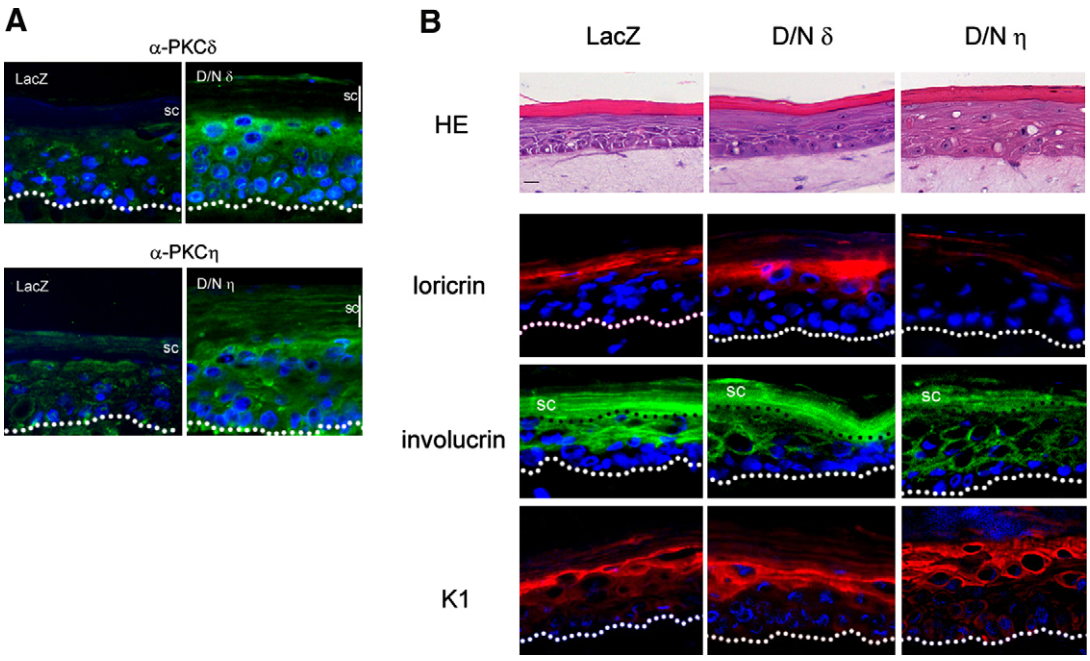

Fig. 2. Expression of differentiation marker proteins in 3D skin culture introduced the dominant negative PKC adenoviruses. (A) Expression of PKC isoforms in the adenovirus-infected 3D skin culture. Keratinocytes in the 3D culture were infected with adenovirus vectors of lacZ, D/Nδ or D/Nη as described in the Material and methods. Expression of each PKC (upper panel: PKCδ, lower panel: PKCη) was detected at 9 days after air-exposure. (B) Sections from the 3D cultures were stained with hematoxilin–eosin (HE) or immunostained for the differentiation markers:loricrin, involucrin and K1. Nuclei were stained with DAPI. The white dots and the black dots represent the boundaries between the epidermis and the dermis, the granular and cornified layers, respectively. sc: stratified cornium, Scale bar; 20 μm.

while JunB decreases it. Furthermore, AP-1 transcription factors are known to be a nuclear target of the PKC signaling pathway [21]. Therefore, we examined the expression of AP-1 proteins in D/Nδ or D/Nη-introduced 3D culture to clarify the mechanisms that both PKC isoforms play opposite roles in the loricrin expression. Immunohistochemical analysis showed the apparent different expression pattern of Jun/Fos families between D/Nδ or D/Nη-introduced 3D culture. D/Nδ increased c-Jun and JunD expression, especially in the differentiating layer (Fig. 3A). Little alteration of JunB in D/Nδ-introduced 3D culture was observed. In contrast, D/Nη significantly decreased JunD expression and moderately JunB. In addition, c-Jun was localized in both the cytoplasm and nuclei in D/Nη-expressing 3D culture, while it was present in the nuclei in LacZ- and D/Nδ-expressing 3D culture (Fig. 3A). Slight increase of c-Fos expression was observed in both D/Nδ- and D/Nη-expressing 3D culture (Fig. 3A). Finally, we examined the mRNA expression of Jun family genes in suspension culture of keratinocytes. In a similar way, D/Nδ significantly increased c-jun and modestly JunD, while D/Nη decreased them (Fig. 3B). These results suggest that PKCδ down-regulates the loricrin expression by the decrease of c-Jun, while PKCη up-regulates it by the increase of JunB and JunD in the epidermis.

4. Discussion

The structure and function of the epidermis are maintained by precise execution of the complex differentiation program, including the induction of the cornified envelope precursor proteins and the activation of a variety of protein kinases. PKC fulfills critical roles in the differentiation process of keratinocytes [5,8]. In the present study, we propose a novel function of PKCδ and PKCη in the expression of loricrin and AP-1 transcription factors in keratinocytes.

We showed that the inhibition of PKCδ increased loricrin expression, indicating the negative function of PKCδ in the late stage of differentiation (Figs. 1 and 2). However, several lines of evidence have shown that PKCδ is a differentiation inducer. The expression level of PKCδ is apparently elevated in differentiating keratinocytes [7]. Overexpression of PKCδ gives rise to the activation of TGase1 and the induction of desmoglein 1, an adhesion molecule in the differentiated keratinocytes [4,22] In current study, we also display that PKCδ is involved in the induction of involucrin and SPR2a genes (Figs. 1 and 2). Therefore, PKCδ acts as a negative regulator of differentiation only for the loricrin expression. Our data regarding loricrin is the first evidence to show the inhibitory effect of PKCδ in keratinocyte differentiation.

In contrast, the suppression of PKCη prevented loricrin expression as well as involucrin and SPR2a. Considering together the previous reports [4,8,23] and present results, PKCη appear to possess only the ability to induce the terminal differentiation of keratinocytes. PKCη is mainly expressed in the granular layers [16], while PKCδ is localized throughout the epidermis. These findings suggest that the limited distribution of loricrin in the granular layer results from the balance between the positive effect by PKCη and the negative effect by PKCδ on loricrin expression.

In some previous works, the gene function was analyzed in 3D culture by using lentivirus vectors or stable transformants of HacaT cells, an immortalized human keratinocytes [5,24]. In this study, we utilized the combination of 3D skin culture with adenovirus-mediated gene transfer to elucidate PKC function. The adenovirus vector is more useful to introduce desired genes into 3D culture. It is not necessary to establish the stable transformants and is possible to introduce one or more genes at the same time only by adding the adenoviruses to 3D culture. Dose-dependent effects of introduced gene can be easily examined by increase of the concentration of adenovirus.

The experiments using 3D cultures revealed that PKCη positively regulated JunB and JunD expression, whereas PKCδ negatively modulated c-Jun expression. JunB suppresses proliferation of keratinocytes and promotes stratification [24]. JunD and JunB, but not c-Jun, increase the involucrin transcriptional activity [25,26]. Moreover, JunD activates the transcription

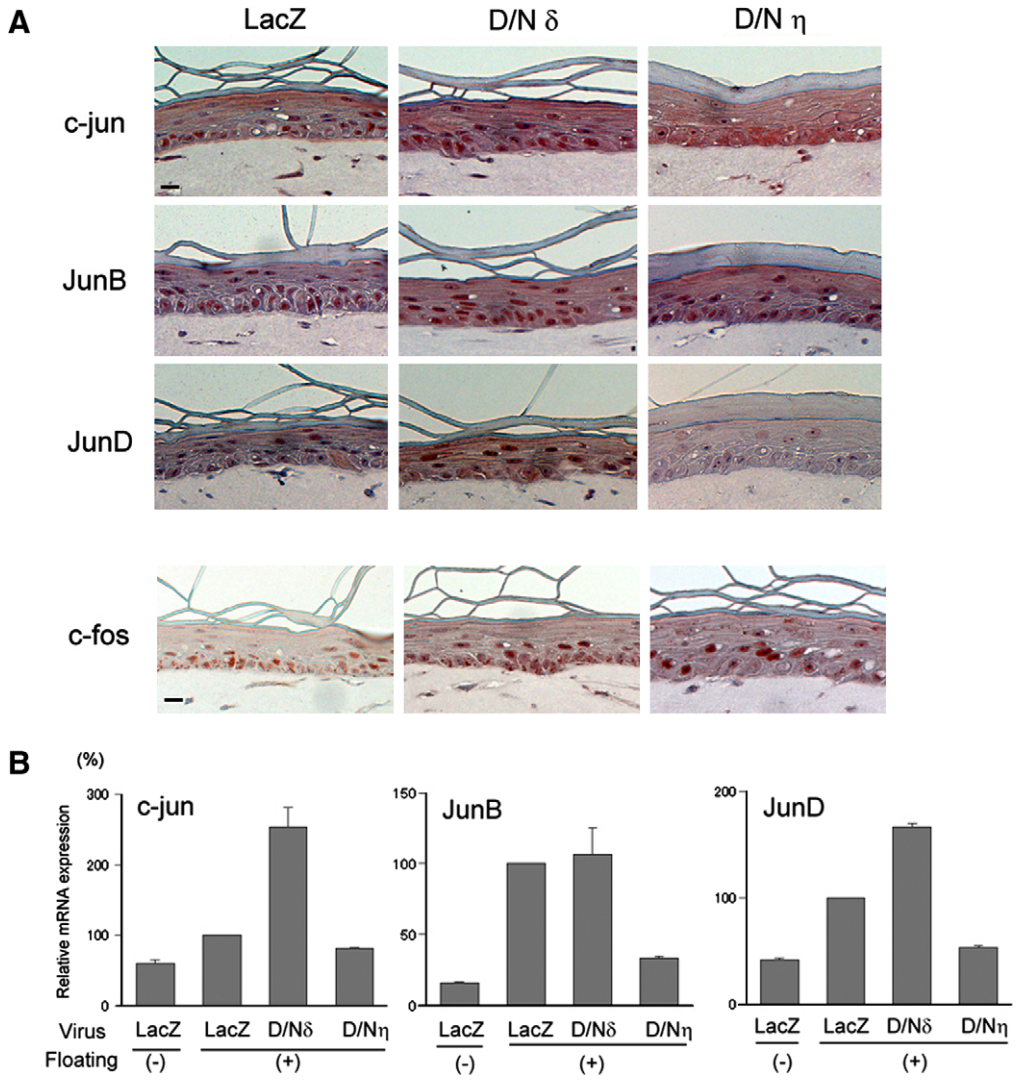

Fig. 3. (A) Dominant negative PKCη decreases the expression of JunB and JunD proteins in 3D skin culture. (A) LacZ, D/Nδ or D/Nη adenovirus vector was infected into 3D culture for 9 days. The sections were immunoreacted with c-Jun, JunB, JunD and c-fos specific antibodies and visualized with AEC dye. The sections were counterstained with hematoxilin. Scale bar; 20 μm. (B) Effects of dominant negative PKC on the mRNA expression of Jun family genes in NHK induced to differentiation by suspension culture. Values represent means ± standard deviations of triplicate determinations and are shown as a relative ratio to the value for the suspended lacZ-infected cells.

activity of fillaggrin, a late marker of differentiation [27]. On the other hand, c-Jun enhances proliferation and tumor formation of the skin [28]. c-Jun-deficient keratinocytes poorly proliferate and show increased differentiation, accompanied by decreased expression of EGFR [29]. These findings suggest that keratinocyte differentiation (except for the loricrin expression) is promoted by PKCη through the up-regulation of JunB/JunD and by PKCδ through the down-regulation of c-Jun. The distinct regulation of loricin expression from other differentiation-associated genes by PKCδ might attribute to the dependence on c-Jun for its transcriptional activation [20,30]. The expression of loricrin is also controlled by some other factors such as Sp-1, AP-2 and p300/CBP [30]. Further exploration is needed to clarify the precise regulation mechanisms of the expression differentiation marker by PKC isoforms.

In conclusion, we present the evidence that PKCδ and PKCη inversely regulate the loricrin expression, although both isoforms similarly control the expression of other differentiation markers. Moreover, the expression of AP-1 family genes is also diversely modulated by PKCδ and PKCη. These findings help to understand the complicated signaling pathway of human keratinocyte differentiation.

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