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PKCη promotes a proliferation to differentiation switch in keratinocytes via upregulation of p27^{Kip1} mRNA through suppression of JNK/c-Jun signaling under stress conditions

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To maintain epidermal homeostasis, the balance between keratinocyte proliferation and differentiation is tightly controlled. However, the molecular mechanisms underlying this balance remain unclear. In 3D organotypic coculture with mouse keratinocytes and fibroblasts, the thickness of stratified cell layers was prolonged, and growth arrest and terminal differentiation were delayed when *PKCη*-null keratinocytes were used. Re-expression of PKCη in *PKCη*-null keratinocytes restored stratified cell layer thickness, growth arrest and terminal differentiation. We show that in 3D cocultured *PKCη*-null keratinocytes, p27^{Kip1} mRNA was downregulated, whereas JNK/c-Jun signaling was enhanced. Furthermore, inhibition of JNK/c-Jun signaling in *PKCη*-null keratinocytes led to upregulation of p27^{Kip1} mRNA, and to thinner stratified cell layers. Collectively, our findings indicate that PKCη upregulates p27^{Kip1} mRNA through suppression of JNK/c-Jun signaling. This results in promoting a proliferation to differentiation switch in keratinocytes.

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The epidermis is a stratified squamous epithelium consisting of a basal layer, a suprabasal layer, a granular layer and a stratum corneum.[1] The keratinocytes in the basal layer have proliferative potential and are undifferentiated. On terminal differentiation, basal keratinocytes exit from the cell cycle and subsequently move into the suprabasal layers. Exit from the cell cycle and terminal differentiation are closely linked and the balance between keratinocyte proliferation and differentiation must be tightly controlled to maintain epidermal homeostasis.[1]

Changes in many processes, such as gene expression, control keratinocyte proliferation and differentiation.[2] Of these changes, upregulation of CDK inhibitors (CKI) p21$^{Cip1/WAF1}$ and p27^{Kip1} is associated with keratinocyte growth arrest and onset of terminal differentiation.[3,4] Emerging evidence reveals that several signaling pathways, including those of Notch and calcineurin, and transcription factors, such as Sp1/Sp3 and NFAT, regulate the induction of p21$^{Cip1/WAF1}$ mRNA.[4–6] In contrast, the molecular mechanisms underlying the induction of p27^{Kip1} during keratinocyte growth arrest and onset of terminal differentiation are poorly understood.

PKC is also involved in keratinocyte proliferation and differentiation.[7,8] PKCη was originally cloned from a mouse skin cDNA library.[9] Overexpression of PKCη induces differentiation of human and mouse keratinocytes.[10,11] Previously, we have reported that *PKCη*-null mice show normal skin development, but *PKCη*-null mice display prolonged epidermal hyperplasia after topical 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment and during re-epithelialization after skin injury.[12] Furthermore, *PKCη*-null mice are susceptible to tumor formation via a two-stage skin carcinogenesis protocol; a single application of 7,12-dimethylbenz(*a*)anthracene for tumor initiation followed by TPA treatment for tumor promotion.[12] However, the regulation of keratinocyte proliferation, differentiation and tumor formation by PKCη remains unclear and requires detailed study.

Because PKCη is mainly expressed in the suprabasal and granular layers of the epidermis,[13,14] the use of monolayer-cultured keratinocytes is insufficient to elucidate a function of PKCη. Furthermore, although *in vivo* studies provide valuable insight into keratinocyte proliferation and differentiation, the complex regulation of this phenomenon is difficult to analyze, because the interactions between keratinocytes and other cells, such as inflammatory and immune cells, need to be considered.[15,16] To elucidate a function of PKCη in keratinocytes, it is, therefore, necessary to induce keratinocyte stratification in culture.

The 3D organotypic coculture of keratinocytes with dermal fibroblasts can be used to induce stratification and to elucidate the molecular mechanisms underlying keratinocyte proliferation and differentiation, and to understand the interactions of keratinocytes with fibroblasts.[17] The 3D organotypic coculture is also useful to understand re-epithelialization during wound healing.[18] Although many studies using human keratinocytes in 3D coculture have been reported, studies using genetically defined primary mouse keratinocytes and fibroblasts are

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rare.[19,20] It is important to use primary mouse keratinocytes and fibroblasts in 3D coculture to enable the use of transgenic and knockout mice.

In this study, we used a 3D organotypic coculture system with primary mouse keratinocytes and fibroblasts that we have recently developed,[21] and we investigated the role of PKCη on epidermal keratinocyte proliferation and differentiation. We provide evidence that PKCη promotes keratinocytes to switch from proliferation to differentiation via the induction of $p27^{Kip1}$ mRNA through the suppression of JNK/c-Jun signaling.

Results

The thickness of stratified cell layers produced using *PKCη*-null keratinocytes was prolonged in 3D organotypic coculture. To elucidate a possible role of PKCη in keratinocytes, we performed 3D organotypic coculture. Because PKCη is not expressed in the dermis,[13] we have used dermal fibroblasts from wild-type (WT) newborn mice. Dermal fibroblasts were embedded in a gel of type-I collagen within a hanging-drop insert. Keratinocytes isolated from WT and *PKCη*-null (KO) mice were overlaid onto the surface of the gel. To induce keratinocyte stratification, the gel surface was raised to the air–liquid interface by lowering the medium.

After air exposure, WT keratinocytes gradually stratified, and by day 3 had formed multiple nucleated cell layers, resembling the mouse epidermis under hyperplasia (Figure 1a). The thickness of the upper WT cell layer gradually decreased to $28 \pm 1\,\mu$m (mean ± S.E.M.) by day 5 and to $22 \pm 2\,\mu$m by day 7 (Figure 1b). KO keratinocytes also stratified, and by day 3 had formed multiple nucleated cell layers, which were very similar to those of WT cells (Figures 1a and b). However, on day 5, the thickness of the upper KO cell layer was $41 \pm 2\,\mu$m, which was significantly more than that of WT cells ($P < 0.01$). On day 7, the thickness of the KO cells was $27 \pm 4\,\mu$m, which was not significantly different from that of the WT cells. In addition, on day 4 and 6, and later at day 7, no difference was observed between WT and KO cells (data not shown). KO keratinocytes produced prolonged thickness of stratified cell layers in 3D coculture, which resembled the prolonged epidermal hyperplasia seen in KO mice.[12]

We next re-expressed PKCη in KO keratinocytes in 3D coculture using a recombinant adenoviral vector (Ad-PKCη). On day 2, PKCη re-expression in KO cells produced comparable or slightly greater levels of PKCη expression relative to WT (Figures 1c and 5d). The thickness on day 5 of the upper keratinocyte layer of WT and KO cells infected with empty vector was 28 ± 3 and $40 \pm 3\,\mu$m, respectively (Figures 1d and e). In contrast, the thickness of the upper keratinocyte layer of KO cells infected with Ad-PKCη was $31 \pm 2\,\mu$m on day 5, which was almost the same as that of WT cells infected with empty vector. These results indicate that prolonged thickness of stratified cell layers produced using KO keratinocytes is caused by the loss of PKCη.

PKCη promotes keratinocyte growth arrest. We next examined the effects of PKCη loss on keratinocyte DNA synthesis using the BrdU labeling assay. In WT cells, on days 1 and 2, the BrdU-labeling index in the lowest layer was about 50–60%, which was comparable with that in KO cells (Figures 2a and b). On day 3, the labeling index in WT cells decreased to 22%, indicating that keratinocytes underwent growth arrest from day 2 to day 3. In contrast, in KO cells, the labeling index was 39%, which was more than that in WT cells. On day 5, the labeling index in KO cells was not significantly different from that in WT cells. When PKCη was re-expressed in KO keratinocytes by Ad-PKCη, the labeling index on day 3 was 25%, which was almost the same as that in WT cells infected with empty vector (Figures 2c and d).

In addition, we examined DNA synthesis in cultured monolayer keratinocytes after changing the medium to one containing a high Ca^{2+} concentration ($[Ca^{2+}]$), which induces keratinocyte growth arrest and differentiation. In low $[Ca^{2+}]$ medium, the labeling index was similar between WT and KO cells (Figure 3a). After keratinocytes were exposed to high $[Ca^{2+}]$ medium, the labeling index in WT cells decreased. In contrast, the labeling index in KO cells remained higher (Figures 3a and b). Collectively, these results indicate that PKCη promotes keratinocyte growth arrest.

PKCη triggers keratinocyte terminal differentiation. Because KO keratinocytes showed growth arrest delay, PKCη may trigger keratinocyte terminal differentiation. To address this question, we examined expression of keratinocyte differentiation markers in 3D coculture. In the epidermis, keratin 1 (K1) is an early differentiation marker expressed in the suprabasal layer. Upregulation of K1 is associated with the onset of keratinocyte differentiation. Loricrin is a late differentiation maker expressed in the granular layer. Keratin 5 (K5) is a marker of the basal layer. Levels of K1 and loricrin in KO cells in 3D coculture were decreased (Figure 4a). K5 was expressed throughout the upper keratinocyte layer, which is similar to that seen in re-epithelialization during wound healing.[22] K5 was not different between WT and KO cells. Immunoblot analysis also showed that K1 and loricrin, but not K5, were decreased in KO cells on day 3 (Figure 4b). On day 2, K1 and loricrin in KO cells were at comparable levels with those in WT cells (Figure 4c). These results suggest that in KO keratinocytes, terminal differentiation is delayed, but not blocked. When PKCη was re-expressed in KO keratinocytes by Ad-PKCη, K1 and loricrin on day 3 were at similar levels compared with those in WT cells infected with empty vector (Figure 4d).

We also examined the induced level of K1 in cultured monolayer keratinocytes in high $[Ca^{2+}]$ medium. The expression level of K1 was low and similar between WT and KO cells in low $[Ca^{2+}]$ medium (Figure 4e). K1 was markedly induced in WT cells after exposure to high $[Ca^{2+}]$ medium (Figure 4e). In contrast, K1 was weakly induced in KO cells. PKCη was also induced in keratinocytes exposed to high $[Ca^{2+}]$ medium, as previously reported.[7] When PKCη was re-expressed in KO cells by Ad-PKCη, the induction of K1 was at similar levels compared with that in WT cells infected with empty vector (Figure 4f). Collectively, these results indicate that PKCη triggers keratinocyte terminal differentiation.

PKCη upregulates $p27^{Kip1}$ mRNA in keratinocytes. To explore the molecular mechanisms of PKCη's role in growth

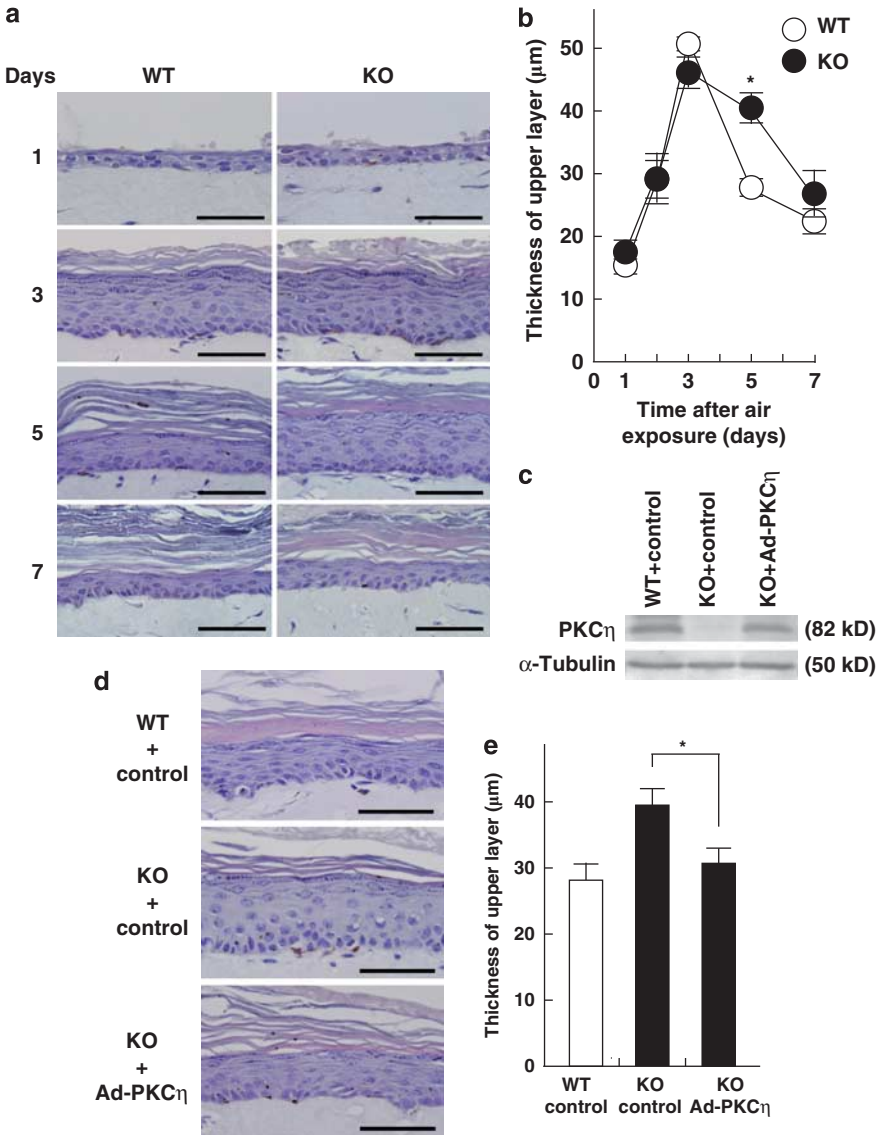

Figure 1 The thickness of stratified cell layers was prolonged using KO keratinocytes in 3D organotypic coculture. (**a**) Hematoxylin and eosin-stained sections of 3D coculture after air exposure. Bar, 50 μm. (**b**) Time-dependent change in thickness of upper layer. Five independent experiments were performed ($n=5$); $^*P<0.01$. (**c**) Immunoblot analysis of PKCη levels in keratinocytes of day 2 3D coculture infected with empty vector or Ad-PKCη. (**d**) Hematoxylin and eosin-stained sections of day 5 3D coculture infected with Ad-PKCη or empty vector. Bar, 50 μm. (**e**) Thickness of upper layer of day 5 3D coculture infected with Ad-PKCη or empty vector. Five independent experiments were performed ($n=5$); $^*P<0.01$

arrest and differentiation, we first focused on expression of CKI genes, which are important for cell growth arrest. Because keratinocytes underwent growth arrest from day 2 to day 3, we examined mRNA levels in 3D coculture of keratinocytes on day 2. We found that $p27^{Kip1}$ mRNA levels in KO cells were reduced (Figure 5a). Expression levels in KO cells of other CKI genes, including *p21$^{Cip1/WAF1}$*, were comparable with those in WT cells (Figure 5a and Supplementary Figure S1a). We also examined the mRNA levels of genes that are important for keratinocyte proliferation and differentiation (Supplementary Figure S1b). The expression levels of these genes were similar between WT and KO cells.

We examined time-dependent changes in $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ mRNA levels after air exposure. $p27^{Kip1}$ mRNA in WT keratinocytes was upregulated from day 1 to day 2 and downregulated from day 2 to day 3 (Figure 5b). In contrast, $p27^{Kip1}$ mRNA in KO cells was not induced on day 2. On day 3, $p27^{Kip1}$ mRNA in KO cells was slightly reduced, but was not significantly different compared with that in WT cells ($P>0.06$). Levels of $p21^{Cip1/WAF1}$ mRNA in WT and KO cells were similarly decreased from day 1 to day 3.

Immunoblot analysis showed that the level of $p27^{Kip1}$, but not $p21^{Cip1/WAF1}$, was reduced in KO keratinocytes (Figure 5c). When PKCη was re-expressed in KO keratinocytes by Ad-PKCη, the level of $p27^{Kip1}$ protein was restored to

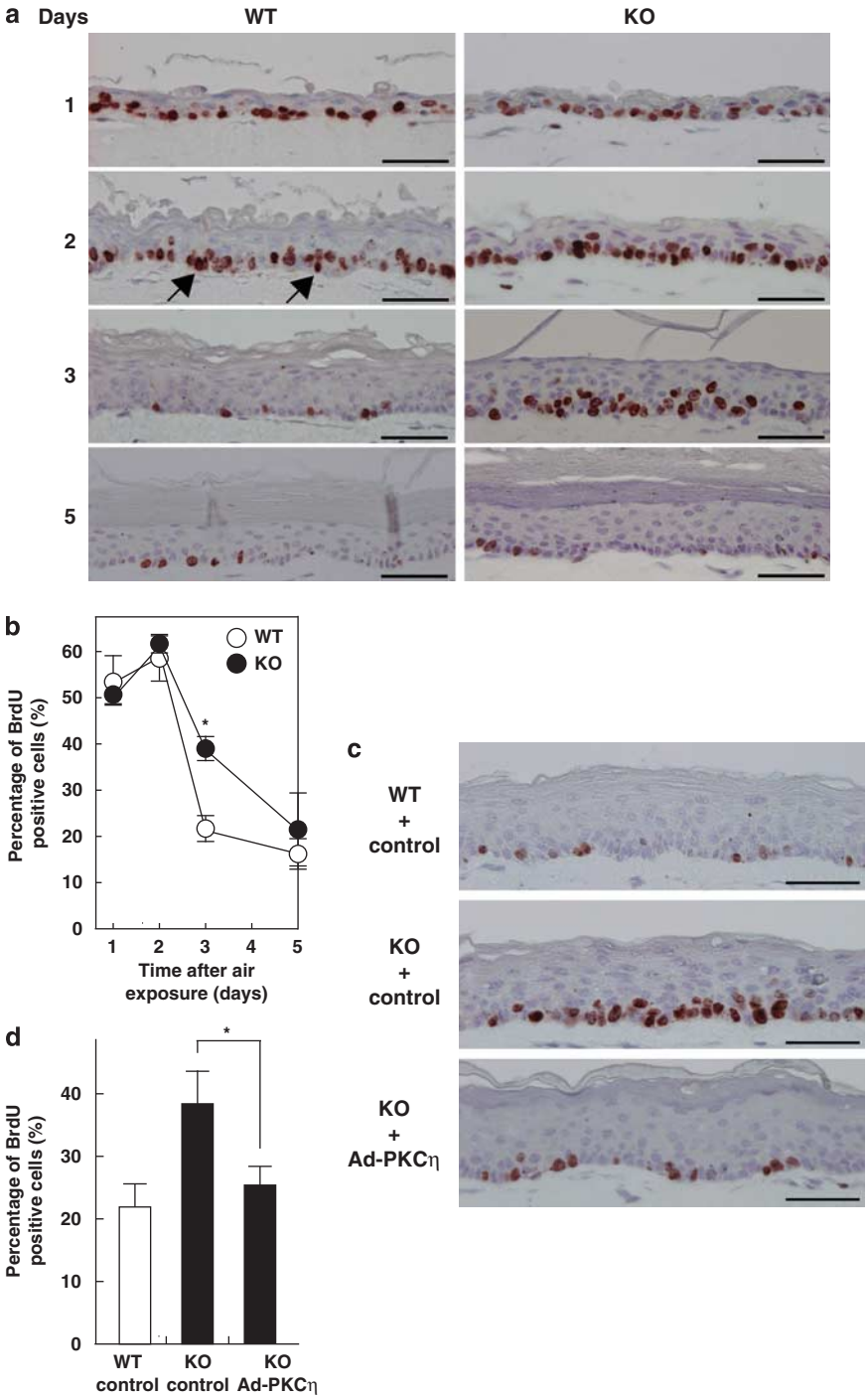

Figure 2 PKCη promotes keratinocyte growth arrest in 3D coculture. (**a**) Immunostaining of BrdU in sections of 3D coculture. Arrows indicate BrdU-labeled cells. Bar, 50 μm. (**b**) BrdU-labeling index in the lowest layer. Five independent experiments were performed ($n=5$); *$P<0.01$. (**c**) Immunostaining of BrdU in sections of day 3 3D coculture infected with Ad-PKCη or empty vector. Bar, 50 μm. (**d**) BrdU-labeling index in the lowest layer. Five independent experiments were performed ($n=5$); *$P<0.01$

almost the same level as that in WT cells infected with empty vector (Figure 5d). CDK2, CDK4 and cyclin D1, which are also required for keratinocyte proliferation,[23–25] were at similar levels in WT and KO cells (Supplementary Figure S2a). These results indicate that PKCη upregulates p27^{Kip1} mRNA in keratinocytes.

We also examined induction of p27^{Kip1} in cultured monolayer keratinocytes after changing in the medium to one containing a high [Ca^{2+}]. The level of p27^{Kip1} protein was markedly increased in WT cells, but weakly induced in KO cells (Figure 5e). When PKCη was re-expressed in KO cells by Ad-PKCη, the induction of p27^{Kip1} was at similar levels

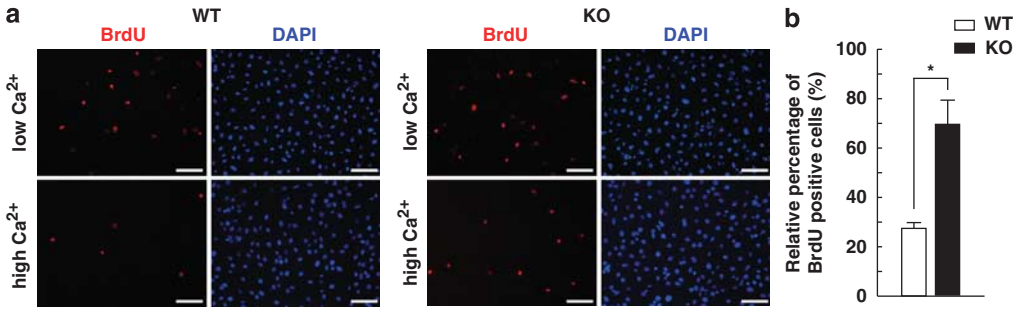

Figure 3 PKCη promotes keratinocyte growth arrest in cultured monolayer keratinocytes in high [Ca^{2+}] medium. (**a**) BrdU immunostaining of monolayer-cultured keratinocytes after the medium was changed to one containing high [Ca^{2+}] and culture continued for 18 h. Bar, 50 μm. (**b**) Relative percentages of BrdU-labeled cells in keratinocytes exposed to high [Ca^{2+}] medium. Relative % denotes percentages of BrdU-labeled cells in high [Ca^{2+}]/low [Ca^{2+}] conditions. At least, 1500 cells per sample were counted. Three independent experiments were performed ($n=3$); *$P<0.01$

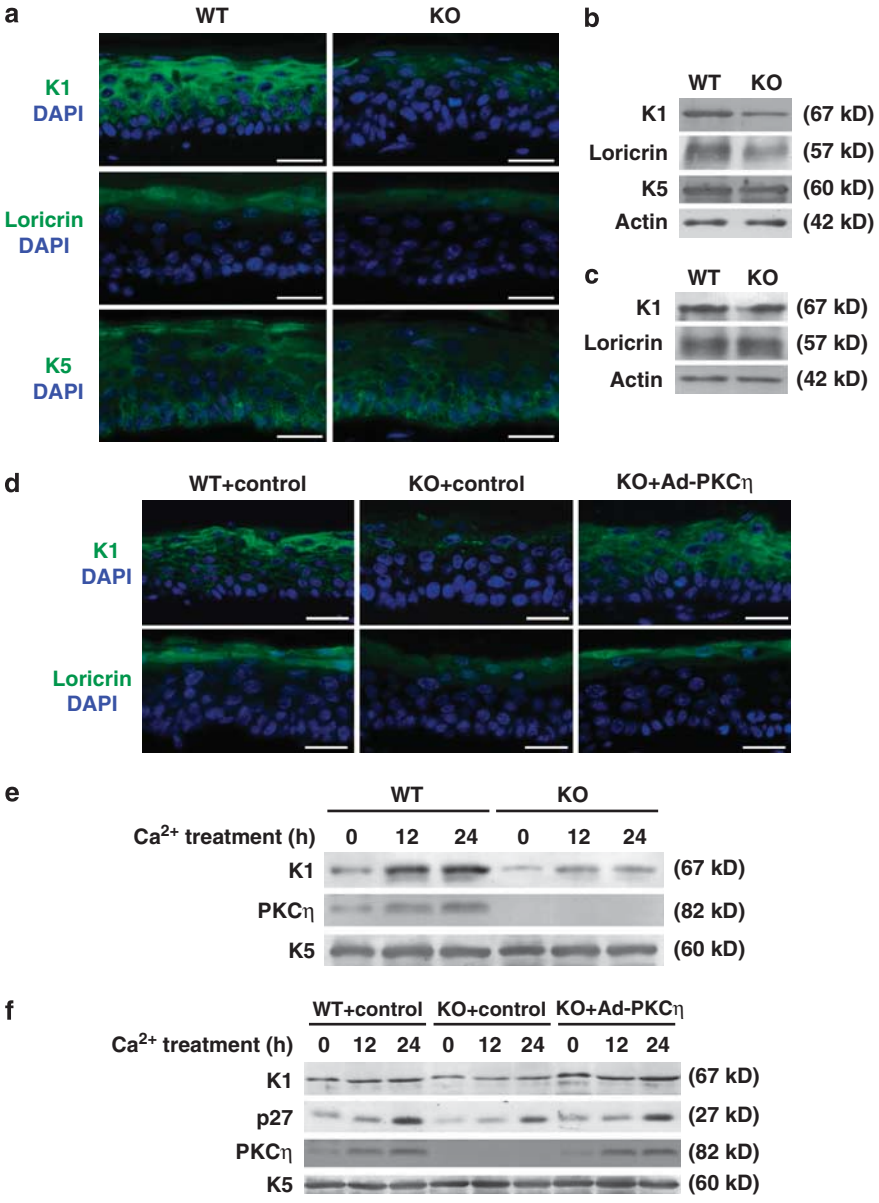

Figure 4 PKCη triggers keratinocyte terminal differentiation. (**a**) Immunostaining of K5, K1 and loricrin in sections of 3D coculture on day 3. Bar, 25 μm. (**b**) Immunoblot analysis of K5, K1 and loricrin levels on day 3. Representative results are shown. (**c**) Levels of K1 and loricrin on day 2. (**d**) Immunostaining of K1 and loricrin in sections of 3D coculture infected with Ad-PKCη or empty vector. Bar, 25 μm. (**e**) Levels of K1 and PKCη in cultured monolayer keratinocytes exposed to high [Ca^{2+}] medium. K5 was used as equal loading control. (**f**) Levels of K1, $p27^{Kip1}$ and PKCη in cultured monolayer keratinocytes infected with Ad-PKCη or empty vector in high [Ca^{2+}] medium

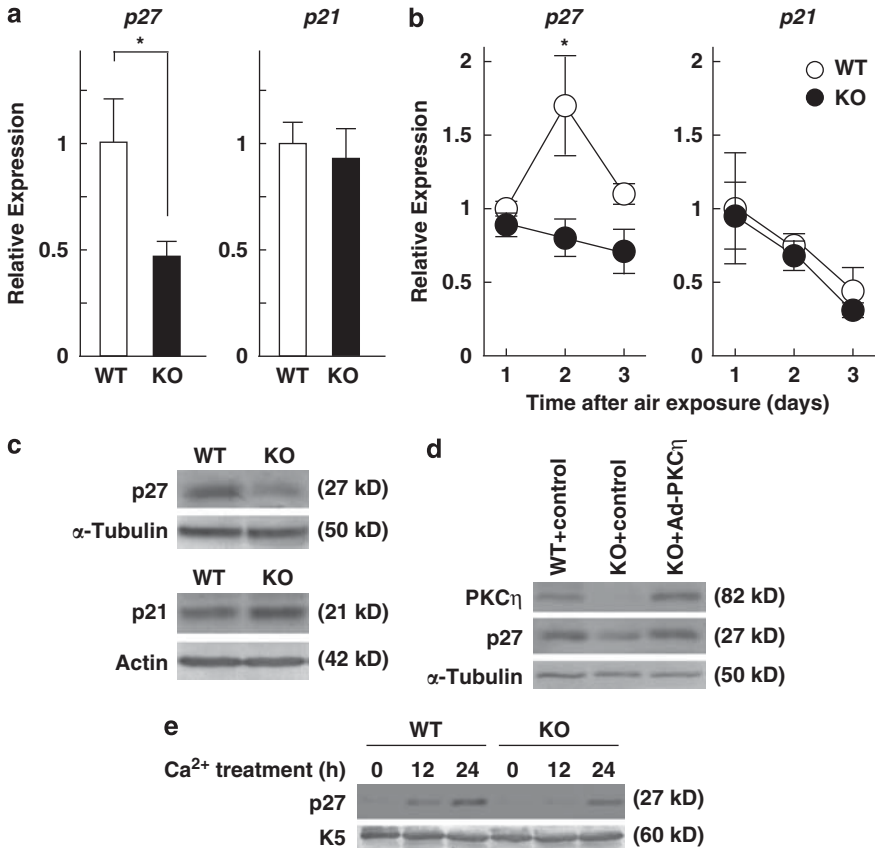

Figure 5 PKCη regulates the induction of $p27^{Kip1}$ mRNA in keratinocytes. (**a**) Real-time PCR analysis of $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ mRNA levels in keratinocytes on day 2. KO levels are expressed as fold changes *versus* WT (set to 1). Five independent experiments were performed ($n=5$); $^*P<0.04$. (**b**) Time-dependent changes of $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ mRNA levels on days 1–3. Results are shown as mean ± S.E.M. relative to WT mRNA levels on day 1 (set to 1). Five independent experiments were performed ($n=5$). (**c**) Immunoblot analysis of $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ levels on day 2. Representative results are shown. (**d**) Levels of $p27^{Kip1}$ in day 2 keratinocytes infected with Ad-PKCη or empty vector. (**e**) Levels of $p27^{Kip1}$ in cultured monolayer keratinocytes exposed to high [Ca^{2+}] medium

compared with that in WT cells infected with empty vector (Figure 4f).

PKCη suppresses JNK/c-Jun signaling in keratinocytes. To determine how PKCη upregulates $p27^{Kip1}$ expression on day 2, we examined MAPK signaling,[1,24,26,27] EGFR signaling[1,26] and NF-κB signaling,[1,2,24] which are important for keratinocyte proliferation and differentiation (Figure 6a and Supplementary Figure S2b). We found that phosphorylated (activated) JNK was elevated in KO keratinocytes. Furthermore, phosphorylated c-Jun, which is downstream of JNK, was also elevated in KO cells. Total JNK and c-Jun in KO cells were at similar levels to those in WT cells. When PKCη was re-expressed in KO keratinocytes by Ad-PKCη, phosphorylated JNK and c-Jun were restored to similar levels compared with those in WT cells infected with empty vector (Figure 6b). These results indicate that PKCη suppresses JNK/c-Jun signaling in keratinocytes.

JNK/c-Jun signaling upregulates $p27^{Kip1}$ mRNA in keratinocytes. We next determined whether upregulation of JNK/c-Jun signaling in KO keratinocytes leads to downregulation of $p27^{Kip1}$ expression on day 2. We examined the expression of $p27^{Kip1}$ in KO cells treated with SP600125, a JNK-specific protein kinase inhibitor. When the level of phosphorylated c-Jun in KO cells treated with SP600125 was comparable with that in WT cells, the levels of $p27^{Kip1}$ protein and mRNA were restored to levels comparable with those in WT cells (Figures 6c and d). Furthermore, on day 5, the thickness of the upper keratinocyte layer in KO cells treated with SP600125 was not different from that in WT cells (Figures 6e and f). Collectively, these results indicate that PKCη suppresses JNK/c-Jun signaling, which leads to the induction of $p27^{Kip1}$ mRNA.

Discussion

In this study, using 3D organotypic coculture with mouse primary keratinocytes and fibroblasts, we found that in *PKCη*-null (KO) keratinocytes, the thickness of stratified cell-layers was prolonged, and growth arrest and terminal differentiation were delayed. Furthermore, in KO keratinocytes exposed to high [Ca^{2+}], growth arrest and terminal differentiation were delayed. In KO keratinocytes, $p27^{Kip1}$ mRNA was downregulated, whereas JNK/c-Jun signaling was enhanced. Inhibition of JNK/c-Jun signaling leads to upregulation of $p27^{Kip1}$ mRNA and to thinner stratified cell layers in KO

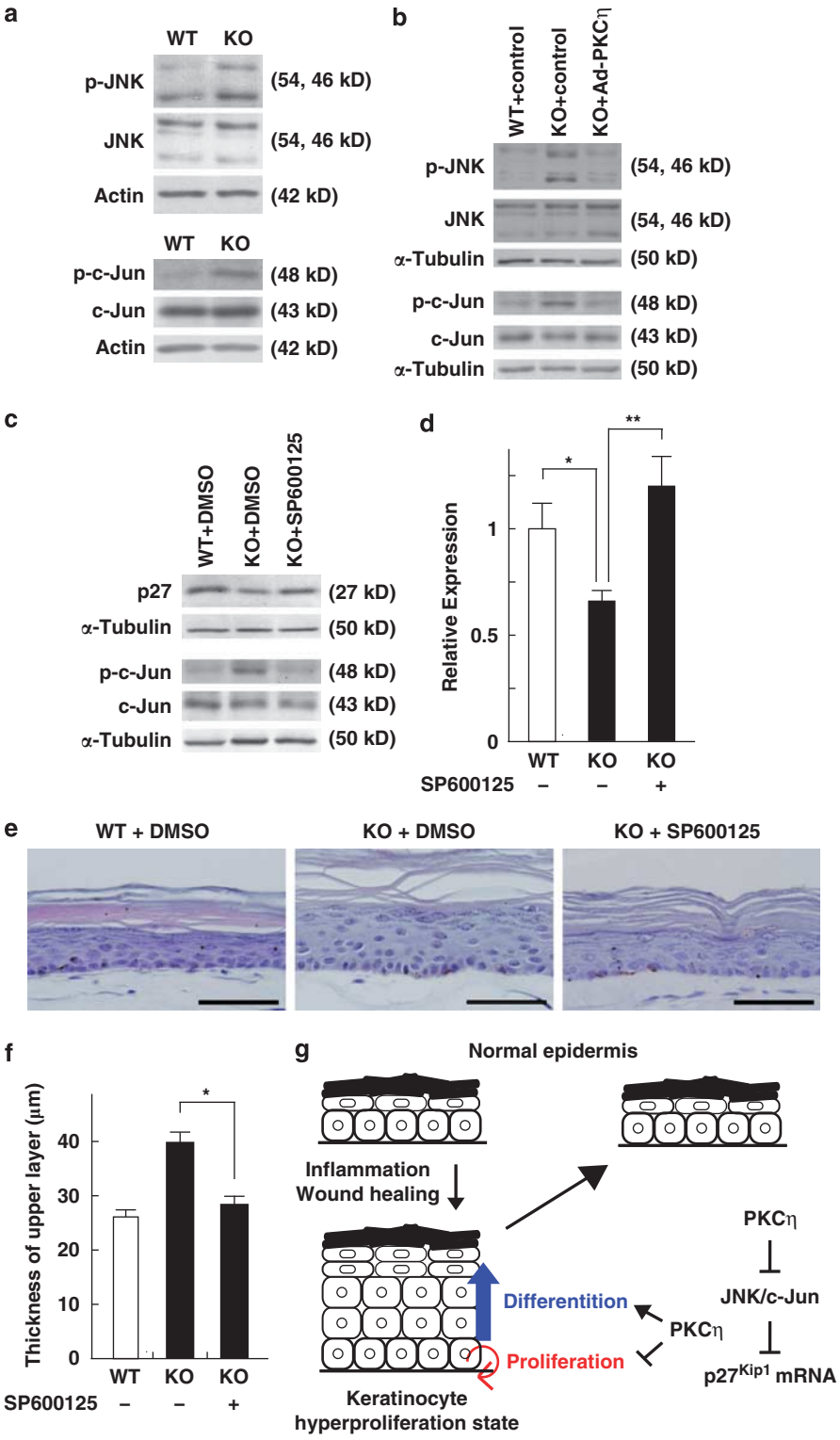

Figure 6 PKCη suppresses JNK/c-Jun signaling, which leads to the induction of $p27^{KIP1}$ mRNA in keratinocytes. (**a**) Total and phosphorylated JNK and c-Jun in keratinocytes on day 2. (**b**) Total and phosphorylated JNK and c-Jun in day 2 cells infected with Ad-PKCη or empty vector. (**c**) Expression on day 2 of $p27^{Kip1}$, c-Jun and phosphorylated c-Jun in KO cells treated with SP600125. (**d**) Real-time PCR analysis of $p27^{Kip1}$ mRNA levels in day 2 KO cells treated with SP600125. Results are shown as mean ± S.E.M. of relative mRNA levels, WT = 1. Five independent experiments were performed ($n = 5$); $^*P < 0.03$, $^{**}P < 0.01$. (**e**) Hematoxylin and eosin-stained sections of day 5 3D coculture treated with SP600125. Bar, 50 μm. (**f**) Thickness of upper layer. Four independent experiments were performed ($n = 4$); $^*P < 0.01$. (**g**) A working hypothesis of PKCη function in the transition from proliferation to differentiation in keratinocytes. PKCη inhibits JNK/c-Jun signaling that leads to the induced expression of $p27^{Kip1}$ mRNA

keratinocytes. Our findings provide molecular links among PKCη, upregulation of $p27^{Kip1}$ mRNA and JNK/c-Jun signaling in keratinocytes.

Overexpression of PKCη in human keratinocytes partially inhibits DNA synthesis and induces epidermal transglutaminase, which is involved in the crosslinking of proteins in the outermost epidermal layers, but does not change the expression of K1 and loricrin.[10] Similarly, overexpression of PKCη in mouse keratinocytes partially inhibits DNA synthesis and induces epidermal transglutaminase,[11] suggesting that PKCη functions during late events of keratinocyte terminal differentiation. On the basis of our findings, PKCη promotes keratinocyte growth arrest and triggers terminal differentiation. Taken together, PKCη has dual functions in keratinocytes; a trigger for transition from the proliferation state to the differentiation state, and a late function in terminal differentiation.

Overexpression of a constitutively active form of PKCη stimulates JNK activation in HEK293 cells.[28] In contrast, inducible PKCη expression in MCF-7 cells inhibits JNK activity and apoptosis in response to DNA damage.[29] Molecular mechanisms underlying the regulation of JNK/c-Jun signaling by PKCη may be dependent on physiological conditions, or be cell-type specific. Further studies are required to elucidate the molecular mechanisms underlying the negative regulation of JNK activity by PKCη during the switch from proliferation to differentiation in keratinocytes.

What factors are upstream of PKCη during keratinocyte growth arrest and differentiation? Because TGFβ signaling induces keratinocyte growth arrest,[30] we examined the effects of loss of PKCη on TGFβ1-induced growth arrest in cultured monolayer keratinocytes. However, the growth arrest in WT and KO keratinocytes was similarly induced after TGFβ1 treatment (data not shown). Furthermore, expression of *p15^{Ink4b}*, *Mad1* and *c-Myc*, target genes of TGFβ signaling,[30,31] was not different between WT and KO keratinocytes in 3D coculture on day 2 (Supplementary Figure S1a). Our findings suggest that TGFβ does not act upstream of PKCη to induce keratinocyte growth arrest. Alternatively, a high extracellular [Ca^{2+}] would at least act upstream of PKCη, because KO cultured monolayer keratinocytes exposed to a high [Ca^{2+}] had delayed growth arrest and terminal differentiation. Increased extracellular [Ca^{2+}] induces increased intracellular [Ca^{2+}] and phosphatidylinositol turnover in keratinocytes.[5,32] PKCη is activated by diacylglycerol, but not Ca^{2+} and diacylglycerol is produced by phosphatidylinositol turnover.[32]

$p27^{Kip1}$ is an important regulator of cell growth arrest and terminal differentiation in other cell types, such as oligodendrocytes and erythroid cells.[33,34] Furthermore, $p27^{Kip1}$ and Mad1 cooperate to control self-renewal and differentiation of hematopoietic stem cells.[35] It would be interesting to determine whether PKCη has a function for cell growth arrest and terminal differentiation in other cell types, such as oligodendrocytes or erythroid cells.

Regulation of $p27^{Kip1}$ has been shown to be primarily controlled at the protein level instead of the mRNA level.[36] However, accumulating evidence indicates that $p27^{Kip1}$ is also regulated at the mRNA level.[37] On the basis of our findings, PKCη upregulates $p27^{Kip1}$ mRNA in keratinocytes. What kind of transcriptional factor functions in keratinocytes? c-Jun is a member of transcriptional factor AP-1 family, which consists of various dimmers of the Fos, Jun and CREB/ATF families.[38] AP-1 has an important role in regulating keratinocyte proliferation and differentiation.[38] For example, AP-1 regulates numerous genes, including *EGFR*, several keratins, involucrin and loricrin.[26,38] In addition, AP-1 inactivation in the suprabasal epidermis causes increased epidermal hyperproliferation, delayed differentiation and hyperkeratosis.[39] c-Jun in combination with binding partners has a function in regulating the promoter activity positively or negatively. c-Jun binds directly to the AP-1 element at -469 in the human $p27^{Kip1}$ promoter and represses $p27^{Kip1}$ transcription in HEK293 and Huh7 cell lines.[37] It is necessary to determine whether c-Jun directly represses the expression of $p27^{Kip1}$ in keratinocytes. However, using a chromatin immunoprecipitation assay with samples from 3D coculture, we did not detect binding of c-Jun to TPA- or cAMP-response elements, which are located within 3 kb upstream from the transcription initiation site, in exon 1 and intron 1 of *p27^{Kip1}* gene (data not shown). c-Jun may indirectly repress the expression of $p27^{Kip1}$ and may regulate the expression of the other transcription factors such as C/EBP, AP2, Id3, Sp1 and NFAT in keratinocytes.[1,2,5,6] These transcriptional factors may repress the expression of $p27^{Kip1}$. Further studies are required to elucidate the molecular mechanisms underlying the repression of $p27^{Kip1}$.

Keratinocytes cultured in high [Ca^{2+}] medium do not show JNK activation.[27] On the basis of our findings, phosphorylated JNK was not induced in WT- and KO-monolayer-cultured keratinocytes exposed to high [Ca^{2+}] medium (data not shown). However, when keratinocytes were induced to stratify in culture, phosphorylated JNK was elevated in KO cells. This discrepancy may account for the high levels of phosphorylated JNK seen in WT- and KO-monolayer-cultured keratinocytes in low [Ca^{2+}] medium (data not shown). Thus, 3D coculture is necessary to elucidate the molecular mechanisms underlying keratinocyte proliferation and differentiation.

Inflammatory and immune cells influence keratinocyte proliferation and differentiation under stress conditions, such as the inflammation response and wound healing.[15,16] These cells may modulate keratinocyte proliferation in *PKCη*-null mice, which display prolonged epidermal hyperplasia. However, *in vivo* studies alone could not determine a function of PKCη in keratinocyte proliferation and differentiation in a keratinocyte-autonomous manner, because inflammatory and immune cells had to be considered. On the basis of our findings, that KO keratinocytes produced prolonged thickness of stratified cell layers in 3D coculture, we suggest that PKCη regulates keratinocyte proliferation and differentiation in a keratinocyte-autonomous manner.

We hypothesize that PKCη functions in the epidermis as shown in Figure 6g. *PKCη*-null mice show normal skin development; therefore, under normal conditions in the epidermis, PKCη is not essential for proliferation and differentiation.[12] Under stress conditions, such as the inflammation response and wound healing, keratinocytes are induced into a hyperproliferative state. In the transition from the hyperproliferative state to the normal state, PKCη functions in the transition from proliferation to differentiation.

During this process, PKCη upregulates p27^{Kip1} expression through the suppression of JNK/c-Jun signaling. However, further studies are required to understand the role of p27^{Kip1} in epidermis. We propose that PKCη is an important regulator of the proliferation–differentiation switch in keratinocytes during the inflammatory response and wound healing, but not in normal skin development, through the regulation of JNK/c-Jun signaling and p27^{Kip1}. Furthermore, we also propose that PKCη suppresses skin tumor formation through regulation of the keratinocyte proliferation–differentiation switch.

In conclusion, we provide evidence that PKCη promotes transition of keratinocyte proliferation to differentiation via the upregulation of p27^{Kip1} mRNA through suppression of JNK/c-Jun signaling.

Materials and Methods

Mice. *PKCη*-null (*Prkch*$-/-$) mice were generated as reported previously.[12] They were backcrossed to C57BL/6J through 13 generations. All animal experiments were performed in accordance with the policies of the Animal Ethics Committee of The University of Tokyo.

Antibodies. Primary antibodies were as follows: K5, K1 and loricrin (Covance, Berkeley, CA, USA); cyclin D1 (72-13G), phospho-EGFR (Tyr 1173; sc-12351), PKCη (sc-215), c-Jun (sc-45), p21 (sc-397) and p27 (sc-528; Santa Cruz, Santa Cruz, CA, USA); phospho-Akt (Ser473), Akt, EGFR, phospho-Erk (Thr202/Tyr204), Erk, phospho-IKKα (Ser180)/IKKβ (Ser181), IKKα, IKKβ, IκBα, phospho-JNK (Thr183/Tyr185), JNK, phospho-c-Jun (Ser63), phospho-p38 (Thr180/Tyr182), p38, phospho-p65 (Ser536) and p65 (Cell Signaling Technology, Beverly, MA, USA); CDK2 and CDK4 (Transduction Laboratories, San Diego, CA, USA); β-actin (AC-15) and α-tubulin (B-5-1-2; Sigma, Saint Louis, MO, USA); BrdU (BMG 6H8, Roche, Mannheim, Germany). Secondary antibodies conjugated with alkaline phosphatase for immunoblot analysis were purchased from Chemicon (Temecula, CA, USA). Secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 was purchased from Invitrogen (Carlsbad, CA, USA).

Construction of recombinant adenoviral vectors. Recombinant adenoviral vectors were constructed as previously described.[40] Briefly, the mouse *Prkch* cDNA was subcloned into the pKSCX-EGFP vector. The *Prkch* expression cassette was inserted into the *Swa*I site of the pALC3 cosmid to make pALC3-PKCη. The recombinant adenoviral vector encoding *Prkch* was produced by transfecting HEK293T cells with pALC3-PKCη and pMC1-Cre. The empty vector (for use as a control) was produced by infecting HEK293T cells with pALC3 and pMC1-Cre. The titer of virus stocks was determined with the Adeno-X Rapid Titer Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

Cell isolation and culture. Epidermal keratinocytes and dermal fibroblasts were isolated from the dorsal skin of 1- to 3-day-old mice, and cultured as described previously.[21] Briefly, the epidermis was incubated in a 0.1% collagenase solution (Worthington, Lakewood, NJ, USA) in MCDB153 medium (Sigma) supplemented with 10% dialyzed FCS (dFCS) overnight at 4°C. After removal of the dermis, keratinocytes were isolated and used as primary keratinocytes. Almost all the cells isolated from the epidermis expressed cytokeratin but not vimentin.[21] In monolayer cultures, isolated keratinocytes were plated at 1.5×10^6 cells/35 mm dish or at 5×10^5 cells/well in collagen I (BD Biosciences, San Jose, CA, USA) -coated 12-well plastic plates, and cultured in MCDB153 supplemented with 10% dFCS. To induce keratinocyte differentiation, the $[Ca^{2+}]$ of the media was raised from 0.03 to 0.5 mM by adding $CaCl_2$ on day 3 after plating. Adenovirus infection of keratinocytes was carried out on day 1 after plating for 3 h in MCDB153 supplemented with 10% dFCS. Adenoviral infections were performed at an MOI of five.

Fibroblasts were isolated from the remaining dermis, cultured in DMEM (Nissui, Tokyo, Japan) supplemented with 10% FCS, passaged two times to achieve a homogenous population of spindle cells and used for organotypic coculture.

Organotypic coculture. Primary keratinocytes and dermal fibroblasts, isolated as described above, were used to reconstruct the dermal equivalent, as described previously with slight modifications.[21] Briefly, primary keratinocytes were seeded (8×10^5 cells/insert in a 12-well plate) onto collagen type I gels (Nitta Gelatin, Osaka, Japan) containing 9.1×10^4 per ml primary fibroblasts and cultured in MCDB 153 supplemented with 10% dFCS. After 24 h, the culture medium was replaced with a mixture of conditioned MCDB153 supplemented with 10% dFCS and FAD medium (1 : 1 volume) and cultures incubated for 24 h. The gel surface was raised to the air–liquid interface by lowering the level of the FAD (to 0.8 ml in each well of a 12-well plate). FAD was replaced every 2 days. Adenovirus infection (2.6×10^7 p.f.u./insert in a 12-well plate) was carried out on day 1 after seeding onto collagen type I gels in a mixture of conditioned MCDB153 supplemented with 10% dFCS and FAD (1 : 1 volume) for 24 h. Treatment with SP600125 (100 μM final concentration; Wako, Osaka, Japan) was carried out for 24 h after air exposure on day 1.

Histological analysis. Organotypic coculture samples were fixed in 4% formaldehyde/PBS at room temperature overnight and embedded in paraffin. The samples (4 μm thick) were deparaffinized with xylene and ethanol, and then stained with hematoxylin and eosin according to standard procedures. Malinol (Muto Pure Chemicals, Tokyo, Japan) was used as mounting medium. The epithelial thickness without the cornified layer was measured in at least 12 independent regions.

Immunofluorescence histochemistry. Deparaffinized sections (4 μm thick) were washed three times with PBS. For K5, after deparaffinization, antigen retrieval was performed by incubation in 10 mM citrate buffer (pH 6.0) at 98°C for 10 min. Sections were blocked with 5% normal goat serum for 30 min at room temperature and then incubated with antibodies at 4°C. After washing three times with PBS containing 0.05% Tween-20, the sections were incubated with secondary antibody conjugated with Alexa Fluor 488 (Invitrogen) at 37°C for 1 h. After washing three times with PBS containing 0.05% Tween 20, the sections were incubated with DAPI (Wako) for 10 min, washed two times with PBS and mounted with PermaFluor Mountant Medium (Thermo Shandon, Pittsburgh, PA, USA).

BrdU incorporation assay. For 3D organotypic coculture, BrdU (Sigma) at 65 μM final concentration was added to the FAD medium 4 h before fixation. Deparaffinized sections (4 μm thick) were washed with PBS three times, treated with methanol/H_2O_2 (80/20% of a 3% H_2O_2 solution) for 20 min, followed by washing with PBS three times, and then incubated with 0.25% trypsin at 37°C for 5–15 min. After washing with PBS three times, sections were treated with 2 N HCl for 10 min at room temperature, followed by three washes with PBS, and incubated with anti-BrdU antibody conjugated with peroxidase at 37°C for 2 h. After washing with PBS three times, DAB (Dako Japan, Kyoto, Japan) was used as chromogen. Hematoxylin was used as a counterstain. Sections were mounted with PermaFluor Mountant Medium (Thermo Shandon). At least 10 independent regions were measured.

For monolayer-cultured keratinocytes, $CaCl_2$ (0.5 mM final concentration) was added to medium on day 3 after plating. At 18 h after addition, cells were fixed with 70% ethanol/15 mM glycine solution (pH 2.0) for 20 min at -20°C. BrdU at 65 μM final concentration was added to the medium 6 h before fixation. The assay was performed with a 5-bromo-2′-deoxyuridine Labeling and Detection Kit II (Roche) according to the manufacturer's instructions, except for the secondary antibody used; antibody conjugated with Alexa Fluor 594 (Invitrogen) was used as the secondary antibody. Nuclei were stained with DAPI. At least 10 independent regions were measured.

Immunoblot analysis. For 3D organotypic coculture, the upper keratinocyte layer was physically separated from the collagen gels with forceps, treated with standard SDS sample buffer and boiled at 98°C for 5 min. After centrifugation, supernatants were subjected to SDS-PAGE. For monolayer-cultured keratinocytes induced to differentiate by Ca^{2+}, floating and adhered keratinocytes were treated with SDS sample buffer and boiled at 98°C for 5 min. After centrifugation, supernatants were subjected to SDS-PAGE. The separated proteins were electrically blotted onto a nitrocellulose membrane. At least three independent experiments were performed. Detection was performed using BCIP/NBT (Sigma) substrates.

Real-time PCR analysis. Total RNAs were isolated from the upper keratinocyte layer, physically separated from the collagen gels, or from monolayer-cultured keratinocytes using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using a first strand synthesis kit (Roche) and random primers. The resulting cDNA (20 ng)

was amplified in triplicate by PCR using 2 × SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) in the ABI Prism 7900 Sequence Detection System. The expression levels of all samples were normalized to an endogenous control, *Gapdh*. Quantification was carried out using the $2^{-\Delta\Delta Ct}$ method. Primer Sequences are shown in Supplementary Table S1.

Statistical analysis. All results are expressed as mean ± S.E.M. The two-tailed unpaired *t*-test was used for all experiments. *P*-values <0.05 were considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)