p63/p51-induced Onset of Keratinocyte Differentiation via the c-Jun N-terminal Kinase Pathway Is Counteracted by Keratinocyte Growth Factor

Eisaku Ogawa,1,2 Ryuhei Okuyama,1,2 Teie Egawa, Hirokazu Nagoshi,1 Masuo Obinata,1 Hachiro Tagami,1,3 Shuntaro Ikawa,1,4,5 and Setsuya Aiba1

From the 1Department of Dermatology, Tohoku University Graduate School of Medicine, the 2Department of Cell Biology, Institute of Development, Aging, and Cancer, and the 3Ikawa Group, Center for Interdisciplinary Research, Tohoku University, Sendai 980-8574, Japan

p63/p51, a homolog of the tumor suppressor protein p53, is chiefly expressed in epithelial tissues, including the epidermis. p63 affects cell death similar to p53, and also plays important roles in the development of epithelial tissues and the maintenance of epithelial stem cells. Because it remains unclear how p63 regulates epithelial cell differentiation, we examined the function(s) of p63 in keratinocyte differentiation through the use of a keratinocyte culture system. ΔNp63α (ΔNp51B), a p63 isoform specifically expressed in basal keratinocytes, suppressed the differentiation of specific late-stage proteins, such as filaggrin and loricrin. In contrast, ΔNp63α induced keratin 1 (K1), which is expressed at the start of differentiation, via c-Jun N-terminal kinase (JNK)/AP-1 activation. However, p63 did not induce K1 expression in the basal layer in vivo, although basal keratinocytes had high levels of p63. This discrepancy was explained by the suppression of K1 expression by dermis-secreted keratinocyte growth factor. This suppression occurred via extracellular signal-related kinase (ERK) signaling, and counteracted the p63-mediated induction of K1. Thus, a precise balance between p63 and keratinocyte growth factor mediates the onset of epithelial cell differentiation, through JNK and ERK signaling. These data may provide mechanistic explanations for the pathological features of skin diseases, including psoriasis.

1 The abbreviations used are: TA, transactivation; Lor, loricrin; Fil, filaggrin; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; KGF, keratinocyte growth factor; FGF, fibroblast growth factor; GFR2-IIIβ, GFR receptor 2 IIIβ; CM, conditioned medium; K1, keratin 1; RT, reverse transcriptase; siRNA, small interfering RNA; GM-CSF, granulocyte macrophage-colony-stimulating factor.
p63-induced Keratinocyte Differentiation by JNK Activation

**FIGURE 1. Induction of K1 and suppression of late differentiation markers by △Np63α.** A, △Np63α modulates the expression of differentiation markers in keratinocytes. Primary keratinocytes were infected with the LacZ (control) or △Np63α adenoviruses (Ad-LacZ or Ad-△Np63α), and were cultivated for the time period indicated (in hours) after replacement into a high calcium medium (2 mM). Total cell extracts were collected, and the proteins were analyzed by 7.5% SDS-PAGE and immunoblotting with antibodies against the various differentiation markers. Filaggrin was synthesized as a high molecular weight precursor, profilaggrin, which was subsequently processed. The diffuse bands correspond to the multiple products of this processing. The same blots were stripped and reprobed with an anti-KS antibody, as a control for equal loading conditions. B, △Np63α expression dose-dependently increases K1 expression. Keratinocytes were infected with increasing multiplicity of infections of Ad-△Np63α. Virus was compensated for a total of 50 multiplicity of infection (MOI) by LacZ virus. K5 was used as a loading control. C, △Np63α expression dose-dependently inhibits Fil expression. Keratinocytes, infected similarly to those in B, were cultivated in low (−) or high (+) calcium concentrations. D, K1 transcription is increased by △Np63α. K1 mRNA levels were determined by real time RT-PCR with primers specific for the K1 gene. Values were normalized for glyceraldehyde-3-phosphate dehydrogenase mRNA levels and expressed as arbitrary units. E, immunoblots of keratinocytes transfected with △Np63α-specific siRNAs. K1 levels decreased with △Np63 down-regulation by △Np63 sequence-specific (P-1 and -2), but not with green fluorescent protein sequence-specific (C), siRNAs. Arrow indicates △Np63α. K5 and tubulin-α were used as loading controls. The above results are representatives of at least three independent experiments.

The following antibodies were purchased and used as recommended by their suppliers: mouse anti-p63 monoclonal (4A4, Neomarkers, Fremont, CA), rabbit antibodies against phosphorylated c-Jun N-terminal kinase (JNK) (Thr183/Tyr185), JNK, phosphorylated extracellular signal-regulated kinase (ERK) (Thr202/Tyr204), ERK, phosphorylated mitogen-activated protein kinase/ERK kinase (MEK) (Ser217/Ser221), and MEK (Cell Signaling Technology, Tokyo); rabbit antibodies against K1, K5, K10, Fil, and Lor (Covance, Emeryville, CA); mouse antibubulin-α monoclonal (Sigma, Tokyo); and goat anti-KGF antibody (R&D Systems, Minneapolis, MN). A fluorescein isothiocyanate-coupled goat anti-rabbit antibody and horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG antibodies (Amersham Biosciences, Tokyo) were used as secondary antibodies.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Antibodies**—The plasmid Rc/CMV-△Np63α was constructed as described previously (21). The plasmid with the AP-1 response element, which was the kind gift of Dr. K. Yokoyama (Riken BioResource Center, Tsukuba, Japan), contains 3 copies of 5′-TGA CTA A-3′ upstream of the thymidine kinase promoter in phRL-TK. The reporter plasmid for the K1 regulatory region was obtained by PCR amplification, with the oligonucleotide 5′-CAC AAG AGA TGA AGG ATG TGT CAA CC-3′ as the forward primer and 5′-TCT CTA GAC CAA ACC-3′ as the reverse primer. The amplified fragment spanning +7795 to +8395 of the human K1 gene was cloned into the luciferase reporter pGL3-promoter vector (Promega, Tokyo). This region includes the AP-1 consensus sequence 5′-TGA TTC A-3′, which is activated in response to increased calcium concentration (22, 23).

The following antibodies were purchased and used as recommended by their suppliers: mouse anti-p63 monoclonal (4A4, Neomarkers, Fremont, CA), rabbit antibodies against phosphorylated c-Jun N-terminal kinase (JNK) (Thr183/Tyr185), JNK, phosphorylated extracellular signal-regulated kinase (ERK) (Thr202/Tyr204), ERK, phosphorylated mitogen-activated protein kinase/ERK kinase (MEK) (Ser217/Ser221), and MEK (Cell Signaling Technology, Tokyo); rabbit antibodies against K1, K5, K10, Fil, and Lor (Covance, Emeryville, CA); mouse antibubulin-α monoclonal (Sigma, Tokyo); and goat anti-KGF antibody (R&D Systems, Minneapolis, MN). A fluorescein isothiocyanate-coupled goat anti-rabbit antibody and horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG antibodies (Amersham Biosciences, Tokyo) were used as secondary antibodies.

**Cell Cultures, Transfection, and Adenoviral Infection**—Primary keratinocytes were prepared from the newborn ICR mouse epidermis as described (24). In brief, the epidermis was separated from the dermis with 0.25% trypsin (Invitrogen, Tokyo) overnight at 4 °C, plated in dishes precoated with colla-
p63-induced Keratinocyte Differentiation by JNK Activation

LacZ were generated as described and used at a multiplicity of infection of 50 or as indicated (19, 20). Adenovirus with LacZ was used as a control.

Immunoblotting and Immunostaining—Proteins were extracted from cultured keratinocytes and subjected to immunoblotting as described previously (24). In brief, electrophlated membranes were blocked with TBST (50 mM Tris, pH 7.5, 0.5% Tween 20) containing 5% nonfat dried milk. The membranes were then incubated with primary antibodies, rinsed with TBST, and incubated with peroxidase-conjugated secondary antibodies. After additional rinses, the blots were exposed to LumiGLO reagent (Cell Signaling Technology) and then to x-ray film. Equal loading was achieved by normalization of the protein concentration using a BCA protein assay (Pierce). Frozen skin sections (6 μm thick) were fixed with 2% paraformaldehyde. Non-specific binding was blocked by using 5% serum, and the skin sections were incubated with primary antibodies followed by incubation with isotype-specific secondary antibodies. Nuclei were stained with propidium iodide after the immunostaining. Stained preparations were photographed with a TCS 4D scanner (Leica, Heerbrugg, Switzerland) connected to an inverted LEITZ DM IRB microscope (Oberkochen, Germany).

Real-time PCR—The total RNA was prepared from primary keratinocytes using the RNeasy mini kit (Qiagen, Tokyo) according to the manufacturer’s instructions. The cDNAs were synthesized by SuperScript II reverse transcriptase (Invitrogen), as recommended by the manufacturer, using 3 μg of the total RNA as a template. Real-time PCR analyses were performed with a Quantitect SYBR Green PCR kit (Qiagen) and an iCycler (Bio-Rad, Tokyo), using the following primers: for K1, 5’-GAC CAG TCA CGG ATG GAT TC-3’ and 5’-CGA ACT CAT TCT CTG CGT TG-3’; for FGF receptor 2 III, reduction of ΔNp63α expression increases the activity of the AP-1 response element (19, 20). Adenovirus with LacZ was used as a control.

RESULTS

ΔNp63α Suppresses Late Differentiation Proteins but Increases K1 Expression—We first examined the effects of p63 on the induction of differentiation markers in keratinocytes.

To examine promoter activity, the transfected keratinocytes were subjected to a dual luciferase reporter assay (Promega) 72 h after transfection. Small interfering RNAs (siRNAs) targeting nucleotides 376–396 (P-1) and 1728–1748 (P-2) of ΔNp63α, where nucleotide 1 corresponds to the A base of the ΔNp63α cDNA start codon, were synthesized commercially (Thermo, Ulm, Germany). P-1 and P-2 siRNAs recognize the DNA-binding domains of all isoforms and the carboxyl-terminal domains specific to ΔNp63α and TAp63α, respectively. An siRNA targeting green fluorescent protein was used as a control. Recombinant adenosviruses expressing either ΔNp63α or

The above results are representatives of at least three independent experiments.

To examine promoter activity, the transfected keratinocytes were subjected to a dual luciferase reporter assay (Promega) 72 h after transfection. Small interfering RNAs (siRNAs) targeting nucleotides 376–396 (P-1) and 1728–1748 (P-2) of ΔNp63α, where nucleotide 1 corresponds to the A base of the ΔNp63α cDNA start codon, were synthesized commercially (Thermo, Ulm, Germany). P-1 and P-2 siRNAs recognize the DNA-binding domains of all isoforms and the carboxyl-terminal domains specific to ΔNp63α and TAp63α, respectively. An siRNA targeting green fluorescent protein was used as a control. Recombinant adenosviruses expressing either ΔNp63α or

gen type I (Nitta Gelatin, Osaka, Japan), and cultured in minimum essential medium supplemented with 4% Chelex-treated fetal calf serum, epidermal growth factor (10 ng/ml; Invitrogen), and 0.05 mM CaCl2. Under these conditions, keratinocytes were maintained in an immature state, and differentiation was used as a loading control. The above results are representatives of at least three independent experiments.
p63-induced Keratinocyte Differentiation by JNK Activation

A

\[
\begin{array}{c|ccc|ccc}
 & \text{LacZ} & + & \Delta \text{Np63}_a & + \\
\hline
\text{CM} & - & 0 & 6 & 24 & 0 & 6 & 24 \\
\text{Ca}^{++} & 0 & 6 & 24 & 0 & 6 & 24 & 0 & 6 & 24 \\
\end{array}
\]

K1

K5

B

\[
\begin{array}{c|ccc|ccc}
 & \text{LacZ} & \Delta \text{Np63}_a \\
\hline
\text{G} & - & K & G & K & G & K \\
\text{KGF (ng/ml)} & 0 & 1 & 5 & 20 & 0 & 1 & 5 & 20 \\
\end{array}
\]

K1

K5

C

\[
\begin{array}{c|ccc|ccc}
 & \text{LacZ} & + & \Delta \text{Np63}_a & + \\
\hline
\text{KGF (ng/ml)} & 0 & 1 & 5 & 20 & 0 & 1 & 5 & 20 \\
\end{array}
\]

K1

K5

D

\[
\begin{array}{c|ccc|ccc}
 & \text{Control} & \Delta \text{Np63}_a \\
\hline
\text{KGF (ng/ml)} & 0 & 1 & 5 & 20 & 0 & 1 & 5 & 20 \\
\end{array}
\]

E

\[
\begin{array}{c|ccc|ccc}
 & \text{LacZ} & \Delta \text{Np63}_a \\
\hline
\text{IgG \alpha-K} & - & \text{IgG \alpha-K} & - & \text{IgG \alpha-K} \\
\end{array}
\]

K1

K5

F

\[
\begin{array}{c|ccc|ccc}
 & \text{LacZ} & \Delta \text{Np63}_a \\
\hline
\text{K} & 0 & 1 & 5 & 20 & 0 & 1 & 5 & 20 \\
\end{array}
\]

G

\[
\begin{array}{c|ccc|ccc}
 & \text{LacZ} & + & \Delta \text{Np63}_a & + \\
\hline
\text{FGF10} & - & 0 & 6 & 24 & 0 & 6 & 24 \\
\text{Ca}^{++} & 0 & 6 & 24 & 0 & 6 & 24 & 0 & 6 & 24 \\
\end{array}
\]

K1

K5

H

\[
\begin{array}{c|ccc|ccc}
 & \text{LacZ} & \Delta \text{Np63}_a \\
\hline
\text{Ca}^{++} & - & KGF & - & KGF \\
\end{array}
\]

Lor

K10

Fil

K5
The keratinocyte is an excellent tool for analyzing epithelial tissues, because its features can be modulated in vitro through changes in extracellular calcium concentration. Cells cultivated in a low calcium environment mimic undifferentiated, proliferating keratinocytes. Upon replacement with a high calcium medium, the cells mimic in vivo differentiation processes, which include induction of differentiation-specific proteins such as K1, K10, Fil, and Lor. These proteins can be categorized as early markers (K1 and K10), which are initially expressed in the spinous layer, and late markers (Fil and Lor), whose expression is initially in the granular layer and beyond (11).

ΔNp63α is the predominant p63 isoform expressed in keratinocytes; it is down-regulated at the start of differentiation (25). We overexpressed ΔNp63α in keratinocytes using an adenovirus transduction system. We observed that ΔNp63α overexpression decreased levels of the late differentiation markers Fil and Lor, but did not alter K10 levels (Fig. 1A). However, ΔNp63α increased K1 expression (Fig. 1A). This was unexpected, because ΔNp63α is abundantly expressed in the basal layer where K1 is not expressed. K1 expression in the spinous layers was initiated during the differentiation process at time points when ΔNp63α levels were gradually decreasing.

To confirm that ΔNp63α was responsible for these changes in differentiation marker levels, we added various amounts of the ΔNp63α vector to our culture system. K1 levels increased with increasing levels of ΔNp63α, in a dose-dependent manner. In contrast, Fil levels decreased dose-dependently (Fig. 1, B and C). K1 up-regulation was also verified by real time RT-PCR (Fig. 1D). Conversely, ΔNp63α reduction by two independent p63 sequence-specific siRNAs decreased K1 expression (Fig. 1E). Taken together, these results indicate that ΔNp63α induces the expression of K1, which suggests that p63 induces the initiation of keratinocyte differentiation.

ΔNp63α Induction of K1 Is Mediated by JNK Signaling Activation—To investigate the mechanism of the ΔNp63α-dependent induction of K1, we examined the effects of p63 on K1 transcription. Human K1 induction is controlled by a regulatory region located in the 3′ flanking area that includes the AP-1 response element (22, 23). We isolated the K1 regulatory region and monitored luciferase reporter activity to examine the effects of p63 on K1 gene expression. A shift from low to high calcium concentration transactivated the K1 regulatory region (Fig. 2A), which mimicked the endogenous response of the K1 gene. Transfection of ΔNp63α transactivated the K1 regulatory region (Fig. 2B) and the AP-1 response element (Fig. 2C). Conversely, the activity of the AP-1 response element was decreased following siRNA reduction of p63 (Fig. 2D).

AP-1 activity is activated by JNK via Jun phosphorylation. Therefore, we used Western blot analysis to examine the effects of p63 on JNK activity by detecting JNK phosphorylation at thr-o-nine 183 and tyrosine 185. ΔNp63α induced JNK phosphorylation in a dose-dependent manner (Fig. 2E). Furthermore, the JNK inhibitor SP600125 abrogated the ΔNp63α-dependent expression of K1 (Fig. 2E). These results indicate that ΔNp63α induces K1 expression by activating the JNK/AP-1 pathway.

KGF Blocks the p63 Induction of K1 Expression—Despite K1 induction by ΔNp63α in pure keratinocyte cultures in vitro, K1 is not induced in basal keratinocytes in vivo, even with high amounts of ΔNp63α. To investigate the reasons for this discrepancy, we sought to determine whether K1 induction was suppressed by the paracrine field effect that is mediated by skin cells except keratinocytes. We focused on dermal fibroblasts, which are thought to be responsible for preserving keratinocyte homeostasis. We collected conditioned medium (CM) from a culture of dermal fibroblasts, and added this CM to a keratinocyte culture (Fig. 3A). Cultured medium partially suppressed the ΔNp63α-induced expression of K1, suggesting that K1 was suppressed by some soluble factor(s) secreted from dermal fibroblasts.

We examined the roles of KGF (also known as FGF-7) and granulocyte macrophage-colony stimulating factor (GM-CSF) in the suppression of K1, because both KGF and GM-CSF regulate keratinocyte homeostasis in a paracrine manner (26). ΔNp63α-dependent K1 induction was blocked by KGF, but not by GM-CSF (Fig. 3B). KGF down-regulated the expression of the K1 protein and its mRNA in a dose-dependent manner (Fig. 3, C and D). Furthermore, a KGF neutralization antibody clearly enhanced K1 expression, albeit weakly, in LacZ-expressing or ΔNp63α-overexpressing keratinocytes (Fig. 3E). Because our cultured dermal fibroblasts produced KGF mRNA (Fig. 3F), these results indicated that KGF secreted from dermal fibroblasts was responsible for K1 down-regulation. However, epidermal homeostasis is controlled by several soluble factors in addition to KGF, including interleukin-1, interleukin-6, tumor growth factor-β, and platelet-derived growth factor (27). The ΔNp63α induction of K1 was inhibited by FGFI0, an FGFI family member that closely resembles KGF (Fig. 3G). Thus, the
induction of K1 expression by ΔNp63α was inhibited by dermal fibroblast-expressed KGF. In addition, KGF blocked the expression of K10 and Lor, but did not alter the expression of Fil (Fig. 3H).

The KGF-activating ERK Signal Competes with p63 in Inducing K1 Expression—To assess the mechanism of KGF-dependent K1 induction, we first tested the possibility that KGF inhibits the activity of the transcriptional regulatory element. ΔNp63α increased K1 mRNA levels (Fig. 1D) and the activity of the K1 regulatory element (Fig. 2B), but KGF inhibited the ΔNp63α-dependent increase of K1 mRNA (Fig. 3D). However, KGF did not inhibit luciferase activity in the K1 regulatory element or in the AP-1 response element (Fig. 2A and B). These results suggest that KGF controls K1 expression through a mechanism other than the AP-1 response element.

We next analyzed ERK signaling, which is thought to mediate not only cell proliferation but also cell differentiation. KGF induced MEK-ERK phosphorylation, and hence MEK-ERK activation (Fig. 4C). Use of the MEK inhibitor PD98059 revealed that K1 expression was up-regulated by the reduction of ERK activity (Fig. 4D). These results suggested the possibility that KGF inhibits K1 expression via ERK activation. However, despite its role in inducing K1 expression, ΔNp63α activated the ERK pathway, similar to KGF (Fig. 4E). To clarify the role of ERK signaling in p63/KGF-mediated K1 induction, we inhibited KGF signaling in ΔNp63α-overexpressing keratinocytes enhanced K1 induction (Fig. 4F), indicating that ΔNp63α-mediated KGF activation inhibits K1 induction. Furthermore, ERK signaling inhibition counteracted the KGF-mediated inhibition of p63-induced K1 expression (Fig. 4G). Therefore, we concluded that ΔNp63α suppresses K1 induction by ERK signaling, but that this suppression can be overwhelmed by JNK/AP-1-mediated K1 induction.

KGF Inhibits K1 Induction in Vivo—We examined the expression of the KGF receptor, FGFR2-IIIβ, by real time RT-PCR, which revealed an abundance of FGFR2-IIIβ in undifferentiated keratinocytes under low calcium conditions (Fig. 5A). FGFR2-IIIβ expression was minimal in differentiated keratinocytes and in dermal fibroblasts, consistent with previous data showing that FGFR2-IIIβ localizes to the basal layer in vivo (28). Thus, KGF chiefly affects undifferentiated keratinocytes, such as basal keratinocytes. We also examined the effect of p63 on FGFR2-IIIβ expression, and found that FGFR2-IIIβ transcription was increased by ΔNp63α expression, but not by TAp63α expression (Fig. 5B).

To assess the effects of KGF in vivo, we injected a KGF neutralizing antibody into the subcutaneous space of mouse skin.
p63-induced Keratinocyte Differentiation by JNK Activation

Although K1 is usually absent from basal keratinocytes, the blockade of KGF-induced K1 expression (Fig. 5, C and D). This suggests that KGF competes with p63 for K1 expression in undifferentiated keratinocytes, both in vitro and in vivo.

**DISCUSSION**

Previous studies have established specific requirements for p63 in epithelial homeostasis. Here, we show that p63 initiates keratinocyte differentiation via JNK activation, but suppresses keratinocyte progression to the late stages of differentiation. By signaling through ERK, dermis-secreted KGF can counteract K1 expression. Although King et al. (29) have reported that ∆Np63α suppresses K1 expression, which is contrary to our findings, Nguyen et al. (18) have reported K1 induction in primary mouse keratinocytes, similar to our results. The reason for the discrepancy between our study and that of King et al. (29) remains to be elucidated.

Recently, analyses using skin organotypic culture have suggested that p63 plays an important role in initiating keratinocyte differentiation, as well as in maintaining cell growth in developmentally mature keratinocytes (17). In the organotypic epidermis, ∆Np63α knockdown leads to the impaired expression of squamous epithelium-specific proteins, including K1, K10, and Lor. ∆Np63α knockdown also leads to the induction of the simple epithelium-specific proteins K8 and K18. In the absence of ∆Np63α, this differentiation should be blocked at an early point and forbidden to proceed further. Therefore, p63 supports the continuation of cell differentiation, rather than the maintenance of cells in an undifferentiated state.

We showed here that p63 activates JNK, a classic stress-activated protein kinase. A number of stress stimuli, including UV radiation and inflammatory cytokines, induce JNK activation. Activated JNK can phosphorylate Jun family transcription factors, such as c-Jun that participates in the activation/formation of the AP-1 complex. The AP-1 complex is a heterogenous set of dimeric proteins, consisting of members of the Jun, Fos, and ATF families. In keratinocytes, AP-1 activation induces K1 transcription (22, 23). However, many other genes have AP-1 binding sites in their promoters, including the late differentiation markers Fil and Lor (30). A detailed analysis of whether p63 controls late differentiation markers through the JNK/AP-1 pathway has yet to be performed. However, based on our data that ∆Np63α differentially regulates the expressions of K1 and

![FIGURE 5. KGF blockade induces K1 expression in basal keratinocytes in vivo. A, FGFR2-IIIβ expression in keratinocytes and dermal fibroblasts. Keratinocytes were cultivated in low (–) or high (+) calcium concentrations. FGFR2-IIIβ mRNA levels were determined by real time RT-PCR with primers specific for the FGFR2-IIIβ gene. Values were normalized for glyceraldehyde-3-phosphate dehydrogenase mRNA levels and are expressed as arbitrary units. B, ∆Np63α increases FGFR2-IIIβ expression. Keratinocytes were transfected with an empty vector control, or with the expression plasmids of ∆Np63α or TAp63α, and mRNA levels were determined as in A. C, K1 expression in basal keratinocytes occurs in skin injected with a KGF-specific antibody. K1 staining was performed 48 h after a KGF antibody (α-K) was injected into the subcutaneous tissue. Normal mouse IgG (IgG) was used as a control. The number of K1-positive basal keratinocytes was calculated by counting a minimum of six fields (≥50 basal cells/field). Values are expressed as the percentage of K1-positive cells. Standard deviations are shown by the error bars, *p < 0.05. D, representative images of K1 staining in the KGF-blocked skin. Green, K1; red, nuclei. Arrow, skin surface; arrowhead, K1-positive basal keratinocytes; broken lines, dermal/epidermal junction. Scale bar, 20 μm.

p63-induced Keratinocyte Differentiation Onset via JNK Activation—Defects in the squamous epithelia of p63−/− mice indicate that p63 is necessary for epithelial development. However, p63 is expressed abundantly in the squamous epithelia even after birth and also in some types of cancers, suggesting that p63 can still play an important role in the post-developmental stage. Previously, we reported that p63 supports cell proliferation and integrin expression by inhibiting Notch activity, causing growth-capable keratinocytes to remain in the basal layer (19). In the present study, we showed that ∆Np63α-induced K1, the earliest marker that is normally observed in the suprabasal layer, and blocked the induction of the late markers Fil and Lor. Although King et al. (29) have reported that ∆Np63α suppresses K1 expression, which is contrary to our findings, Nguyen et al. (18) have reported K1 induction in primary mouse keratinocytes, similar to our results. The reason for the discrepancy between our study and that of King et al. (29) remains to be elucidated.
Fil/Lor, ΔNp63α would be required for the initial commitment to the differentiated phenotype (K1 induction). Furthermore, the subsequent down-regulation of ΔNp63α would be required for differentiation progression (Fil/Lor induction). In addition, because AP-1 binding sites are also located in the epidermal growth factor receptor, JNK activation in keratinocytes results in hyperproliferation and tumor formation via epidermal growth factor signaling (31, 32). Therefore, p63 induces JNK activation, which can modulate not only differentiation but also proliferation, cell motility, and apoptosis.

We primarily analyzed the predominant ΔNp63α isoform of p63, and not the TAp63 isoforms. The ΔNp63 isoforms were originally proposed to act as repressor molecules against TAp63 and p53, but recent studies have indicated that ΔNp63 proteins have unique and independent biological functions. Similar to ΔNp63α, TAp63α could induce K1 expression, but its effect was weaker than that of ΔNp63α (data not shown). Given that the ΔNp63 isoform shares a number of downstream genes with TAp63 (17), the weak induction by TAp63 may be caused by unstable characteristics of the isoform. Furthermore, the skin phenotype of p63-null mice can be partially rescued by the expression of ΔNp63, but not by the expression of TAp63 (33). Thus, differentiation can be chiefly governed by ΔNp63α, although we cannot neglect the contribution of the TAp63 isoforms.

KGF Counteracts p63-induced K1 Expression—In contrast with the predominantly held view of p63, our data reveal that this protein promotes the expression of K1, a factor expressed at the start of keratinocyte differentiation. Despite its role in inducing K1 expression in vitro, p63 is highly expressed in basal keratinocytes, which do not induce K1 expression in vivo. This discrepancy suggests that the processes regulating the start of differentiation must involve other factors presumably derived from non-keratinocytes, because our cell culture consisted of almost pure keratinocytes. KGF meets this criterion.

KGF is a paracrine-acting growth factor produced by mesenchymal cells to stimulate epithelial cell proliferation via FGFR2-IIIβ (34). It stimulates keratinocyte proliferation, consistent with its rapid induction in the healing wound (35). However, the effects of KGF on differentiation have not been well examined. In organotypic culture, KGF induces a delay in keratinocyte differentiation (36), suggesting that it inhibits the differentiation process. We show here that KGF counteracts differentiation progression via ERK phosphorylation, consistent with the function of ERK in delaying the differentiation process (37). In addition, the effect of KGF is chiefly localized to the basal layer, because FGFR2-IIIβ is restricted to this layer (28). Therefore, basal keratinocytes can maintain their undifferentiated state through p63 and KGF signaling, whereas suprabasal keratinocytes undergo differentiation due to the function of p63 and the loss of FGFR2-IIIβ. Although not thought to be the sole dermis signaling molecule, KGF regulates keratinocyte homeostasis cooperatively with p63.

p63 can induce FGFR2 splicing toward the IIIβ type by binding with ABBP1, a member of the RNA processing machinery (38). The p63 mutations found in Hay-Wells syndrome abolish p63/ABBP1 complex formation, which disturbs splicing to FGFR2-IIIβ. The altered skin phenotype observed in Hay-Wells syndrome is attributed to abnormalities in FGFR2 splicing. We observed an increase in FGFR2-IIIβ expression when ΔNp63α was overexpressed by keratinocytes, but not when TAp63 was overexpressed. This result suggests the possibility that p63 suppresses differentiation via KGF receptor expression while inducing differentiation via the JNK/AP-1 pathway. Furthermore, p63 dysregulation is also observed in psoriasis, a common skin disease marked by the hyperproliferation and delayed differentiation of epidermal cells (39). p63 is expressed throughout almost the whole epidermis, except for a few layers located at or near the skin surface. Interestingly, and at the same time as p63 expressed, FGFR2-IIIβ is expressed basally, and in psoriasis is also expressed by differentiated keratinocytes (28). In addition, KGF production is increased in the mesenchymal cells subjacent to the epidermis (28). Whereas p63 induces the initiation of keratinocyte differentiation through JNK activation, the cooperative increase in KGF signaling may enhance cell proliferation and delay differentiation by activating both the JNK and ERK signaling pathways. This model is consistent with the activation of both signals in psoriasis (37, 40, 41). Our data on the role of p63 in keratinocyte differentiation may serve as a basis for the dissection of disease mechanisms, and may shed new light on the molecular mechanisms underlying skin physiology and pathology.

REFERENCES
1. Mills, A. A. (2006) Curr. Opin. Genet. Dev. 16, 38–44
2. Glickman, J. N., Yang, A., Shahsafaei, A., McKeon, F., and Odze, R. D. (2001) Hum. Pathol. 32, 1157–1165
3. Hu, H., Xia, S. H., Li, A. D., Xu, X., Cai, Y., Han, Y. L., Wei, F., Chen, B. S., Huang, X. P., Han, Y. S., Zhang, J. W., Zhang, X., Wu, M., and Wang, M. R. (2002) Int. J. Cancer 102, 580–583
4. Massion, P. P., Taflan, P. M., Jamsheed Rahman, S. M., Yildiz, P., Szy, Y., Edgerton, M. E., Westfall, M. D., Roberts, J. R., Pietenpol, J. A., Carbone, D. P., and Gonzalez, A. L. (2003) Cancer Res. 63, 7113–7121
5. van Bokhoven, H. (2002) Am. J. Hum. Genet. 71, 1–13
6. Mills, A. A., Zheng, B., Wang, X. J., Vogel, H., Roop, D. R., and Bradley, A. (1999) Nature 398, 708–713
7. Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C., and McKeon, F. (1999) Nature 398, 714–718
8. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992) Nature 356, 215–221
9. Dotto, G. P. (1999) Crit. Rev. Oral Biol. Med. 10, 442–457
10. Fuchs, E. (2008) J. Cell Biol. 180, 273–284
11. Dlugosz, A. A., and Yuspa, S. H. (1993) J. Cell Biol. 120, 217–225
12. Honeycutt, K. A., Koster, M. I., and Roop, D. R. (2004) J. Investig. Dermatol. Symp. Proc. 9, 261–268
13. Okuyama, R., Tagami, H., and Aiba, S. (2008) J. Dermatol. Sci. 47, 189–194
14. Ridky, T. W., and Khavari, P. A. (2004) Cell Cycle 3, 621–624
15. Koster, M. I., Kim, S., and Roop, D. R. (2005) J. Investig. Dermatol. Symp. Proc. 10, 118–123
16. Truong, A. B., and Khavari, P. A. (2007) Cell Cycle 6, 295–299
17. Truong, A. B., Kretz, M., Ridky, T. W., Kimmel, R., and Khavari, P. A. (2006) Genes Dev. 20, 3185–3197
18. Nguyen, B. C., Lefort, K., Mandinova, A., Antonini, D., Devgan, V., Della Gatta, G., Koster, M. I., Zhang, Z., Wang, J., Tommasi di Vignano, A., Kitajewski, J., Chiorino, G., Roop, D. R., Missiero, C., and Dotto, G. P. (2006) Genes Dev. 20, 1028–1042
19. Okuyama, R., Ogawa, E., Nagoshi, H., Yabuki, M., Kurihara, A., Terui, T., Aiba, S., Obinata, M., Tagami, H., and Ikawa, S. (2007) Oncogene 26, 4478–4488
20. Ogawa, E., Okuyama, R., Ikawa, S., Nagoshi, H., Egawa, T., Kurihara, A., Yabuki, M., Tagami, H., Obinata, M., and Aiba, S. (2008) Oncogene 27,
p63-induced Keratinocyte Differentiation by JNK Activation

21. Osada, M., Ohba, M., Kawahara, C., Ishioka, C., Kanamaru, R., Katoh, I., Ikawa, Y., Nimura, Y., Nakagawara, A., Obinata, M., and Ikawa, S. (1998) Nat. Med. 4, 839–843
22. Huff, C. A., Yuspa, S. H., and Rosenthal, D. (1993) J. Biol. Chem. 268, 377–384
23. Lu, B., Rothnagel, J. A., Longley, M. A., Tsai, S. Y., and Roop, D. R. (1994) J. Biol. Chem. 269, 7443–7449
24. Okuyama, R., Nguyen, B. C., Talora, C., Ogawa, E., Tommasi di Vignano, A., Lioumi, M., Chiorino, G., Tagami, H., Woo, M., and Dotto, G. P. (2004) Dev. Cell 6, 551–562
25. Laurikkala, J., Mikkola, M. L., James, M., Tummers, M., Mills, A. A., and Thesleff, I. (2006) Development 133, 1553–1563
26. Szabowski, A., Maas-Szabowski, N., Andrecht, S., Kolbus, A., Schorpp-Kistner, M., Fusenig, N. E., and Angel, P. (2000) Cell 103, 745–755
27. Schroder, J. M. (1995) J. Investig. Dermatol. 105, Suppl. 1, 20S–24S
28. Finch, P. W., Murphy, F., Cardinale, I., and Krueger, J. G. (1997) Am. J. Pathol. 151, 1619–1628
29. King, K. E., Ponnamperuma, R. M., Yamashita, T., Tokino, T., Lee, L. A., Young, M. F., and Weinberg, W. C. (2003) Oncogene 22, 3635–3644
30. Angel, P., Szabowski, A., and Schorpp-Kistner, M. (2001) Oncogene 20, 2413–2423
31. Li, G., Gustafson-Brown, C., Hanks, S. K., Nason, K., Arbeit, J. M., Pogliono, K., Wisdom, R. M., and Johnson, R. S. (2003) Dev. Cell 4, 865–877
32. Zenz, R., Scheuch, H., Martin, P., Frank, C., Eferl, R., Kenner, L., Sibilia, M., and Wagner, E. F. (2003) Dev. Cell 4, 879–889
33. Candi, E., Rufini, A., Terrinoni, A., Dinsdale, D., Ranalli, M., Paradisi, A., De Laurenzi, V., Spagnoli, L. G., Catani, M. V., Ramadan, S., Knight, R. A., and Melino, G. (2006) Cell Death Differ. 13, 1037–1047
34. Werner, S., Smola, H., Liao, X., Longaker, M. T., Krieg, T., Hofschneider, P. H., and Williams, L. T. (1994) Science 266, 819–822
35. Finch, P. W., Rubin, J. S., Miki, T., Ron, D., and Aaronson, S. A. (1989) Science 245, 752–755
36. Andreadis, S. T., Hamoen, K. E., Yarmush, M. L., and Morgan, J. R. (2001) FASEB J. 15, 898–906
37. Haase, I., Hobbs, R. M., Romero, M. R., Broad, S., and Watt, F. M. (2001) J. Clin. Investig. 108, 527–536
38. Fomenkov, A., Huang, Y. P., Topaloglu, O., Brechman, A., Osada, M., Fomenkova, T., Yuriditsky, E., Trink, B., Sidransky, D., and Ratovitski, E. (2003) J. Biol. Chem. 278, 23906–23914
39. Shen, C. S., Tsuda, T., Fushiki, S., Mizutani, H., and Yamanishi, K. (2005) J. Investig. Dermatol. 32, 236–242
40. Takahashi, H., Ibe, M., Nakamura, S., Ishida-Yamamoto, A., Hashimoto, Y., and Izuka, H. (2002) J. Investig. Dermatol. Sci. 30, 94–99
41. Zenz, R., and Wagner, E. F. (2006) Int. J. Biochem. Cell Biol. 38, 1043–1049