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Protein Kinase C δ Increases Kruppel-like Factor 4 Protein, which Drives Involucrin Gene Transcription in Differentiating Keratinocytes*

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Background: KLF4 is an important regulator of terminal differentiation and barrier formation in epidermis, but its mechanism of action is not well understood.
Results: PKCδ increases KLF4, which increases hINV expression by interaction at specific elements in the hINV promoter.
Conclusion: KLF4 drives expression of involucrin in response to differentiation stimuli.
Significance: This study provides detailed information regarding the mechanism of KLF4 regulation of keratinocyte differentiation.

KLF4 is a member of the Kruppel-like factor family of transcriptional regulators. KLF4 has been shown to be required for normal terminal differentiation of keratinocytes, but the molecular mechanism whereby KLF4 regulates genes associated with the differentiation process has not been studied. In the present study, we explore the impact of KLF4 on expression of involucrin, a gene that is specifically expressed in differentiated keratinocytes. KLF4 overexpression and knockdown studies show that involucrin mRNA and protein level correlates directly with KLF4 level. Moreover, studies of mutant KLF4 proteins indicate that transcriptionally inactive forms do not increase involucrin expression. PKCδ is a regulator of keratinocyte differentiation that increases expression of differentiation-associated target genes, including involucrin. Overexpression of KLF4 augments the PKCδ-dependent increase in involucrin expression, whereas KLF4 knockdown attenuates this response. The KLF4 induction of human involucrin (hINV) promoter activity is mediated via KLF4 binding to a GC-rich element located in the hINV promoter distal regulatory region, a region of the promoter required for *in vivo* involucrin expression. Mutation of the GC-rich element, an adjacent AP1 factor binding site, or both sites severely attenuates the response. Moreover, loss of KLF4 in an epidermal equivalent model of differentiation results in loss of hINV expression. These studies suggest that KLF4 is part of a multiprotein complex that interacts that the hINV promoter distal regulatory region to drive differentiation-dependent hINV gene expression in epidermis.

KLF4 [2] is a member of the Kruppel-like factor transcription factor family that recognizes GC-rich DNA enhancer elements (1). KLF4, like other members of the Kruppel-like transcription factor family, encodes a highly conserved C-terminal zinc finger DNA binding domain that shares 65% sequence identity with other Kruppel-like proteins (1). In contrast, the N-terminal region is variable and contains a nuclear localization signal and transcriptional activator and repressor domains (1). KLF4 is expressed in differentiated, nonproliferating cells, where it serves to suppress proliferation. Gene expression profiling experiments show that KLF4 suppresses expression of proliferation-associated genes and activates expression of differentiation-associated genes (2–5). KLF4 has an important regulatory role in epithelia. It is expressed in suprabasal layers in the epidermis, tongue, palate, esophagus, stomach, cornea, and colon (6–8), and onset of expression is associated with differentiation (6, 7). The impact of KLF4 overexpression and knockdown has been studied in mouse epidermis. Keratin 5 promoter-targeted overexpression of KLF4 in mouse epidermis stimulates premature barrier formation (9–11), whereas KLF4 knock-out mice display impaired barrier function (12). This is consistent with a role for this protein in suppressing proliferation and driving differentiation. Indeed, these findings are consistent with our recent study indicating that KLF4 increases $p21^{Cip1}$ expression in normal keratinocytes (13).

KLF4 has also been studied at the molecular level. KLF4 activates expression of cyclin-dependent kinase inhibitors (2, 13, 14) and cooperates with p53 to increase $p21^{Cip1}$ gene expression (15). Moreover, KLF4 binds to GC-rich elements in the p53 promoter to suppress transcription (16). KLF4 also binds to the cyclin B2 promoter and represses the cyclin B2 expression (17). We have recently shown that a key controller of keratinocyte differentiation, PKCδ, increases $p21^{Cip1}$ mRNA and protein level via a KLF4-dependent mechanism (13). PKCδ stimulates increased KLF4 binding to the six GC-rich elements in the proximal $p21^{Cip1}$ promoter, and mutation of these sites results in loss of the response (13). Thus, KLF4 participates as a regulator to inhibit keratinocyte proliferation.

However, less is known about the molecular details governing KLF4 regulation of genes associated with epidermal differ-

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[2] The abbreviations used are: KLF4, Kruppel-like factor 4; hKLF4, human KLF4; hINV, human involucrin; DRR, distal regulatory region; PRR, proximal regulatory region; KSFM, keratinocyte serum-free medium; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MOI, multiplicity of infection.

entiation. The epidermis is a stratifying epithelium in which the basal layers contain stem cells that undergo intermittent proliferation. These cells then give rise to cells that differentiate to form the suprabasal differentiated layers and that express genes associated with terminal differentiation (18–20). Information available in the literature suggests that genes associated with differentiation should be increased by KLF4 (3, 12, 21); however, this has not been studied at the molecular level. Involucrin is a model for the study of gene expression during keratinocyte differentiation (22–26). It is expressed in the suprabasal epidermal layers, but is absent in basal cells (22, 27, 28). Activation of hINV transcription relies on a variety of mechanisms, but a prominent mechanism is PKCδ activation (13, 29–32). We have demonstrated that PKCδ activity drives hINV expression via activation of a MEKK1, MEK3/MEK6, and p38δ/ERK signaling cascade that elevates Sp1 and AP1 transcription factor level and binding to the hINV promoter to activate transcription (22, 24–26, 29, 33–38). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), a diacylglycerol analog that activates PKC isoforms, is a pharmacologic agent that activates keratinocyte differentiation via this pathway (23, 24, 39), as do some other naturally occurring agents (40).

Our previous study showed that PKCδ activation increases KLF4 mRNA and protein level in normal human keratinocytes (13). In the present study, we extend these studies and examine the effect of the elevation of KLF4 on expression of hINV, an important marker of keratinocyte differentiation. Our results show that KLF4 interacts via a GC-rich element in the hINV promoter distal regulatory region (DRR) to drive transcription. Thus, these findings present the first detailed evidence that KLF4 interacts with response elements on a keratinocyte differentiation-expressed gene to drive transcription.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Keratinocyte serum-free medium (KSFM) and trypsin were purchased from Invitrogen. Sodium butyrate was from Calbiochem, and actinomycin D was purchased from EMD Chemicals (Gibbstown, NJ). Dimethyl sulfoxide and phorbol ester (TPA) were purchased from Sigma-Aldrich. Rabbit polyclonal antibody for human involucrin (hINV) was produced in our laboratory (41), and rabbit polyclonal antibodies for KLF4 (sc-20691) and PKCδ (sc-937) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody for β-actin (A5441) was purchased from Sigma-Aldrich. Normal rabbit IgG (sc-2027) was purchased from Santa Cruz Biotechnology. Peroxidase-conjugated anti-mouse IgG (NXA931) and peroxidase-conjugated anti-rabbit IgG (NA934V) were obtained from GE Healthcare (Buckinghamshire, UK). We present results obtained using the Santa Cruz siRNA (control "scrambled," sc-37007; KLF4, sc-35480), but identical results were observed with KLF4 siRNA directed to other sites in the KLF4 mRNA (Dharmacon, Lafayette, CO, J-005089-10 and J-005089-09). Thus, the observed responses are not due off-site actions of the siRNA.

Plasmids, Viruses, and Cell Culture—The hINV gene expression reporter plasmids were previously described (24, 42). pEGFP-N1 was from Clontech, and pEGFP-N1-PKCδ was described in our previous study (43). pMXs-hKLF4, a gift from Dr. Toshio Kitamura (44), was used as a template to produce pcDNA3-hKLF4(1–470) (wild type), pcDNA3-hKLF4(1–388) (lacks zinc finger domain), and pcDNA3-hKLF4(335–470) (encodes only the zinc finger domain). PKCδ-encoding adenovirus was obtained from Dr. T Kuroki (25, 45). Human KLF4 (hKLF4)-encoding adenovirus was produced in our laboratory (13). Expression of hKLF4 from this virus requires co-infection with Ad5-TA, which produces the tetracycline transactivator (46). For experiments, keratinocytes were incubated with 15 MOI of tAd5-EV or tAd5-hKLF4 in the presence of 2.5 MOI of Ad5-TA virus in keratinocyte serum-free medium containing 6 μg/ml of Polybrene. Primary cultures of human epidermal keratinocytes were prepared from human foreskins and cultured in KSFM supplemented with 5 μg of EGF and 50 μg of pituitary extract/ml (25).

Promoter Activity—For hINV promoter activity analysis, 2 μg of hINV promoter reporter plasmid was mixed with 4 μl of FuGENE 6 reagent diluted with 96 μl of KSFM. The mixture was incubated at 25 °C for 15 min and then added to a 50% confluent culture of primary human epidermal keratinocyte maintained in 2 ml of KSFM in a 9.6-cm^2 dish. For co-transfection experiments, 2 μg of $p21^{Cip1}$ promoter reporter plasmid and 1 μg of hKLF4 expression plasmid were mixed, treated with FuGENE 6, and added to cells as indicated above. After 24 h, the cells were harvested, and extracts were prepared for assay of luciferase activity.

Electroporation and siRNA-mediated Knockdown—Keratinocytes were electroporated with siRNA or plasmids using the Amaxa electroporator and the VPD-1002 nucleofection kit. For electroporation, keratinocytes were harvested with trypsin and replated 1 day prior to the day of electroporation. On the day of electroporation, 1×10^6 of the replated cells were harvested with trypsin and resuspended in KSFM. The cells are collected at 2000 rpm, washed with 1 ml of sterile phosphate-buffered saline (pH 7.5), and suspended in 100 μl of keratinocyte nucleofection solution. The cell suspension, which included 3 μg of gene-specific siRNA, was mixed by gentle pipetting and electroporated using the T-018 settings. Warm KSFM (500 μl) was added, and the suspension was transferred to a 21.3-cm^2 cell culture dish containing 3.5 ml of KSFM. When required, cells were electroporated a second time with luciferase reporter or expression plasmid. This was accomplished by harvesting the cells with trypsin and resuspending in KSFM. The cells were collected, washed with PBS, and resuspended in nucleofection solution as above. The nucleofection suspension, which included 2 μg of plasmid, was electroporated using the T-018 settings. The cells were plated and maintained for various times before extracts were prepared for assay. Our electroporation method delivers nucleic acid reagents with greater than 90% efficiency.

Immunological Analysis—Equivalent amounts of protein were electrophoresed on a 4–15% denaturing polyacrylamide gradient gel and transferred to nitrocellulose. The membranes were blocked, incubated with a specific primary antibody, washed, and exposed to an appropriate horseradish peroxidase-conjugated secondary antibody. Chemiluminescent detection was used to visualize secondary antibody binding.

Real-time PCR—Total RNA was isolated (illustra RNAspin mini kit, GE Healthcare) and reverse-transcribed. Quantification was performed by using the LightCycler 480 system (Roche Applied Science). PCR primers were designed to quantify PKCδ, KLF4, and hINV transcripts using LightCycler 480 SYBR Green I, and signals were normalized using cyclophilin A control primers. Primers for detection of mRNA levels are cyclophilin A (forward, 5′-CATCTGCACTGCCAAGACTGA-3′, reverse, 5′-TTCATGCCTTCTTTCACTTTGC-3′), hINV (forward, 5′-CCTCAGCCTTACTGTGAG-3′, reverse, 5′-GGGAGGCAGTGGAGTTGG-3′), PKCδ (forward, 5′-GGCCACATCAAGATTGCCGACTTT-3′, reverse, 5′-ACTGGCCAATGAGCATCTCGTACA-3′), and KLF4 (forward, 5′-GAAATTCGCCCGCTCAGATGAACT-3′, reverse, 5′-TTCTCTTCTGGCAGTGTGGGTCAT-3′).

KLF4 mRNA Half-life—To analyze KLF4 mRNA decay kinetics, keratinocytes were infected with 15 MOI of Ad5-EV or Ad5-PKCδ for 24 h prior to the addition of 5 μg/ml actinomycin D. At 0, 0.5, 1, 2, 3, and 4 h after actinomycin D addition, RNA was isolated (illustra RNAspin mini kit, GE Healthcare) and analyzed for KLF4 and cyclophilin A mRNA content by quantitative real-time PCR using primers described in the previous section. Values for each mRNA level at each time point are presented as the mean ± S.D. derived from triplicate quantitative RT-PCR reactions of independent samples. First-order decay constants (*k*) were determined by nonlinear regression analysis (Prism version 3.03, GraphPad) of plots measuring the percentage of KLF4 mRNA remaining *versus* time of actinomycin D treatment. KLF4 mRNA decay constants are presented as the mean ± S.D. of three independent time-course experiments, permitting pairwise statistical assessment using the Student's *t* test. Differences were considered significant if $p < 0.005$.

Chromatin immunoprecipitation assay (ChIP)—ChIP assays were conducted as described (13). Enrichment of KLF4-associated DNA sequences in immunoprecipitated samples and input samples was detected by quantitative RT-PCR using sequence-specific primers and LightCycler 480 SYBR Green I master mix. ChIP primers were as follows: hINV AP1-5/Sp1 binding site located at nucleotides −2218/−2055 (forward, 5′-TCAGCTGTATCCACTGCCCTCTTT-3′, reverse, 5′-TCACACCGGTCTTATGGGTTAGCA-3′), and hINV promoter control located at nucleotides −1040/−919 (forward, 5′-CCTCTCAGGGAGAGATTGACATGA-3′, reverse, 5′-CAACAGTGCACCAGCACACTTGAA-3′).

Epidermal Equivalents—Normal human keratinocytes (1.5×10^6), growing in KSFM (25), were harvested with trypsin and electroporated with 3 μg of siRNA. The cells were replated and expanded in KSFM for 72 h, and 2×10^6 cells were re-electroporated with 3 μg of siRNA and then allowed to settle overnight onto Millicell-PCF chambers (diameter = 12 mm, 0.4-μm pore size) in KSFM (Millipore, Billerica, MA). The cells were then shifted to EpiLife medium containing 1.4 mM calcium chloride and 5 μg/ml of vitamin C and cultured at the air-liquid interface. Fresh medium was added every 2 days, and after 4 days, the epidermal skin equivalents were harvested for preparation of histological sections and isolation of RNA for quantitative RT-PCR analysis.

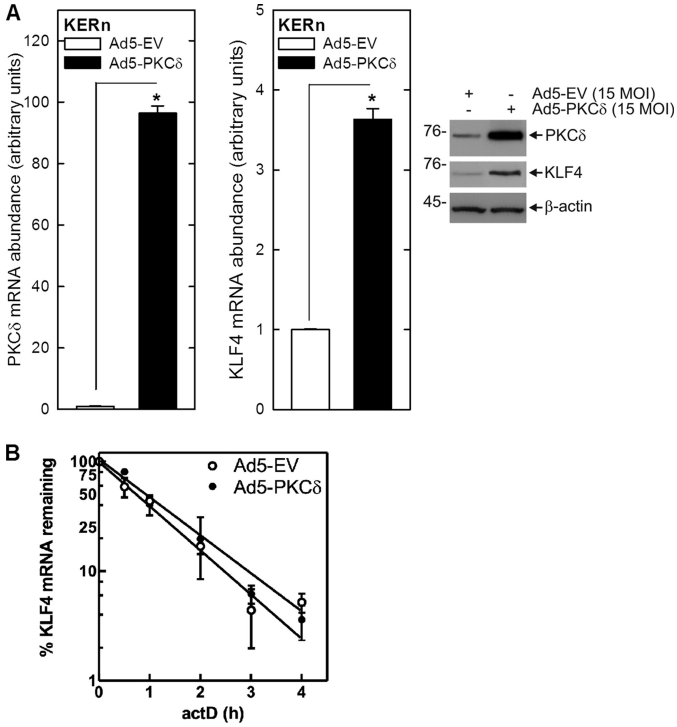

FIGURE 1. **PKCδ controls KLF4 mRNA and protein level.** *A*, keratinocytes (*KERn*) were infected with 15 MOI of Ad5-EV or Ad5-PKCδ, and after 24 h, extracts were prepared for detection of PKCδ and KLF4 mRNA and protein. The mRNA abundance values are mean ± S.D., $n = 3$. The *asterisk* indicates a significant increase over control as determined by Student's *t* test, $p < 0.005$. For immunoblot, cells were treated with adenovirus as above, and after 24 h, extracts were prepared for detection of the indicated epitopes. *B*, PKCδ does not alter KLF4 mRNA half-life. Keratinocytes were infected with 15 MOI of Ad5-EV or Ad5-PKCδ and maintained for 24 h. Actinomycin D was then added to the cultures, and mRNA was harvested at 0–4 h after the addition of 5 μg/ml actinomycin. KLF4 mRNA was detected by quantitative RT-PCR. The RNA decay data are presented as a log-linear plot to determine first-order decay constant.

RESULTS

PKCδ Increases KLF4 Expression—PKCδ is an important regulator of keratinocyte differentiation (22–26, 47), and we recently reported that PKCδ expression increases KLF4 level in keratinocytes (13). We initiated the present studies by confirming the PKCδ-dependent increase in KLF4. Keratinocytes were infected with empty or PKCδ-expressing adenovirus, and after 24 h, cells were harvested for preparation of mRNA. Fig. 1*A* confirms that increasing PKCδ level increases KLF4 mRNA and protein. The human KLF4 gene promoter is presently not available, and so to assess the mechanism responsible for this increase, we monitored the impact of increased PKCδ on KLF4 mRNA turnover. As shown in Fig. 1*B*, KLF4 mRNA half-life is not significantly altered by increased PKCδ level ($t_{1/2} = 0.75$ h in control *versus* 0.87 h in PKCδ overexpressing cells). The lack of change in turnover rate, coupled with the increase in mRNA level, suggests that PKCδ increases KLF4 mRNA level by a transcriptional mechanism.

KLF4 Increases hINV Expression—The Kruppel-like transcription factor, KLF4, is an Sp1-related transcription factor that interacts with the GC-rich DNA elements. KLF4 is an important transcription factor that is required for epidermal barrier formation, suggesting that it has a role in differentiation (12, 21). However, the role of KLF4 in regulating specific differ-

entiation-responsive epidermal genes has not been examined. Involucrin is an extensively studied model of differentiation-associated gene expression in epidermis and in epidermal keratinocytes (22, 49–53). Fig. 2 (*A* and *B*) shows that KLF4 expression increases hINV protein and mRNA level, suggesting that KLF4 may regulate hINV gene transcription. To explore this further, we monitored the impact of KLF4 expression and knockdown on hINV promoter activity. Cells were transfected with the hINV full-length promoter construct, pINV-2473 and hKLF4(1–470) (wild type); an hKLF4 mutant lacking the zinc finger domain, hKLF(1–388); and a mutant encoding only the zinc finger domain, hKLF4(335–470) (13). Treatment with wild-type KLF4 increases transcription, but treatment with the inactive mutants, hKLF4(1–388) and hKLF4(335–470), does not (Fig. 2*C*). hKLF4(1–470) and hKLF4(1–388) were confirmed to be expressed at similar levels by immunoblot, thereby confirming that the difference in hINV promoter activity is not due to a difference in expression of these proteins (Fig. 2*C*). Because anti-KLF4 binds to KLF4 within amino acids 1–180, expression of hKLF4(335–470) could not be monitored. We also assessed the impact of knockdown of endogenous KLF4 on promoter activity. As shown in Fig. 2*D*, reduction in KLF4 level is associated with reduced hINV promoter activity.

KLF4 Is Required for Activation of hINV Gene Expression—To examine the interplay between PKCδ and KLF4, we examined the impact of KLF4 knockdown on PKCδ- and TPA-dependent activation of hINV gene expression. TPA is a strong inducer of keratinocyte differentiation and hINV expression (39). TPA is a diacylglycerol analog that is known to activate calcium- and phospholipid-dependent PKC isoforms (54). Fig. 3 (*A* and *B*), shows that KLF4 siRNA reduces the ability of PKCδ and TPA to increase hINV mRNA level, and Fig. 3*C* shows that KLF4 siRNA reduces TPA-stimulated hINV promoter activity. Consistent with these findings, KLF4 overexpression enhances the PKCδ- (Fig. 3*D*) and TPA- (Fig. 3*E*) dependent increase in hINV promoter activity.

KLF4 Activation of hINV Expression Requires the DRR—Our next goal was to identify hINV promoter regulatory sites responsible for the KLF4-dependent increase in transcription. Previously identified functional domains, including the DRR and the proximal regulatory region (PRR), are indicated in Fig. 4*A*. We tested the response of a series of truncated hINV promoter-luciferase reporter constructs to challenge with KLF4 expression plasmid (37). Activity of the full-length promoter construct, pINV-2473, was increased by KLF4, but the truncated constructs did not respond (Fig. 4*B*). These studies suggest that a KLF4-responsive element is present in the segment spanning nucleotides −2473 to −2136. This region encodes the hINV promoter DRR (39). pINV-41 encodes the hINV minimal promoter (39) and, as expected, has low activity and does not respond to KLF4 stimulation.

The DRR encodes previously characterized AP1 and GC-rich response elements located at nucleotides −2122/−2107 (38, 39). Because the KLF4-associated increase in hINV promoter activity is abolished in the absence of this segment (Fig. 4*B*), we determined whether the AP1-5 or the GC-rich response elements are required for the KLF4-dependent increase. Fig. 5*A*

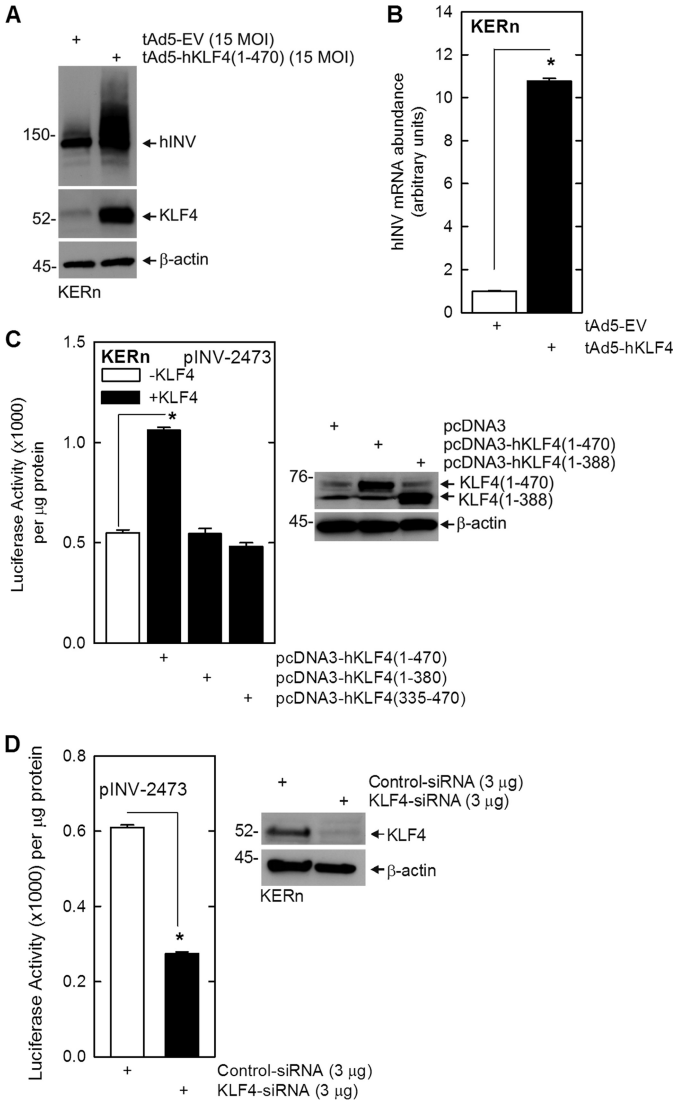

FIGURE 2. **KLF4 expression increases hINV expression.** *A*, keratinocytes (*KERn*) were infected with 15 MOI of tAd5-EV or tAd5-hKLF4. After 48 h, extracts were prepared for detection of KLF4 and hINV protein. *B*, KLF4 increases hINV mRNA level. Keratinocytes were infected with the indicated virus, and after 24 h, RNA was harvested for detection of KLF4 mRNA by quantitative RT-PCR. The values are the mean ± S.D., $n = 3$. The *asterisk* indicates a significant increase over control as determined by Student's *t* test, $p < 0.005$. Similar results were observed in each of three experiments. *C*, keratinocytes were transfected with 2 μg of pINV-2473 luciferase reporter plasmid in the presence of 1 μg of empty vector or the indicated KLF4 expression vector. At 24 h, the cells were harvested, and extracts were assayed for luciferase activity. The *asterisk* indicates a significant increase ($n = 3$) as determined by Student's *t* test, $p < 0.005$. Wild-type and mutant KLF4 expression was monitored by immunoblot and normalized to the level of β-actin. hKLF4(335–470) could not be monitored because the antibody epitope is deleted from this mutant. *D*, keratinocytes were electroporated with 3 μg of the indicated siRNA, and after 48 h, cells were transfected with 4 μg of luciferase reporter plasmid. After an additional 24 h, extracts were prepared for luciferase activity assay and immunoblot. The values are mean ± S.D., $n = 3$. The *asterisk* indicates a significant reduction in luciferase activity, $p < 0.005$. Similar results were observed in each of three experiments. Identical results were observed using several KLF4 siRNA, indicating that the observed responses are not due to off-target effects.

shows a schematic of the hINV luciferase promoter reporter plasmids used in this experiment. Fig. 5*B* shows that mutation of either the AP1-5 or the GC-rich response elements, or both elements, reduces KLF4-stimulated activity of the full-length promoter. This reduction is also observed (Fig. 5*C*) using a con-

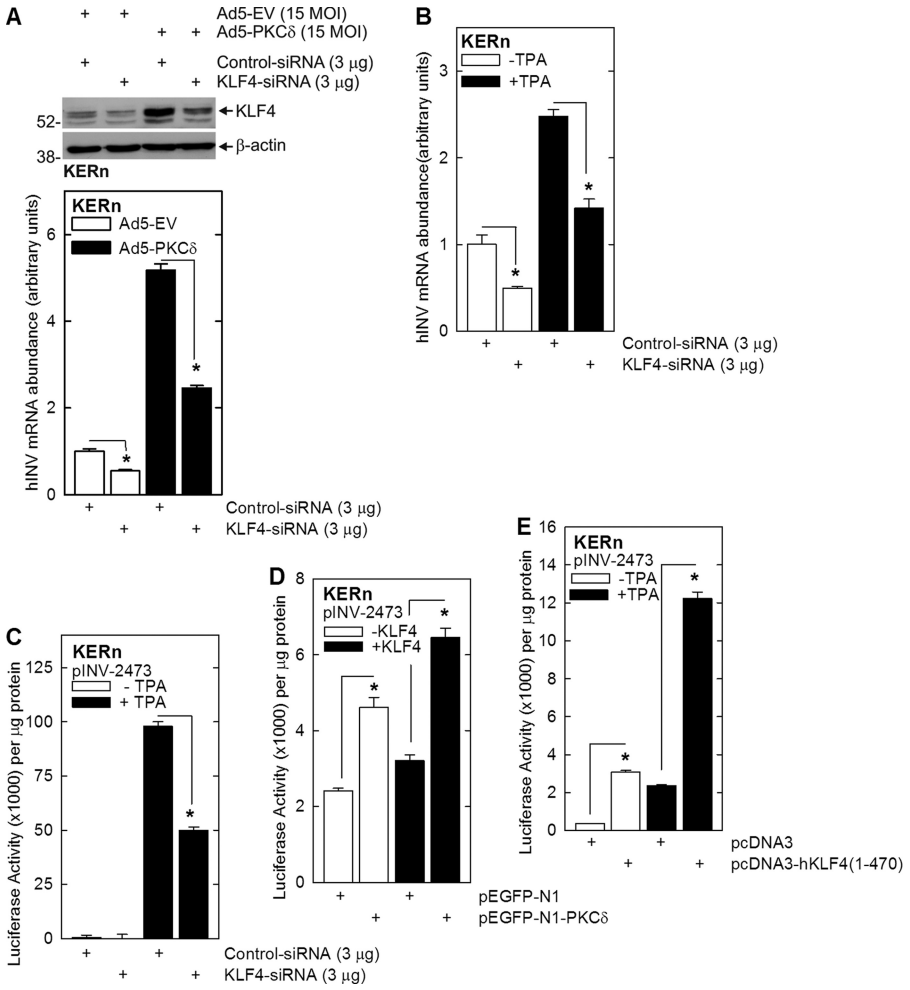

FIGURE 3. **KLF4 is required for PKCδ-induced hINV expression.** *A*, the PKCδ-dependent increase in hINV mRNA level requires KLF4. Keratinocytes (*KERn*) were electroporated with 3 μg of the indicated siRNA, and at 36 h, cells were infected with 15 MOI of Ad5-EV or Ad5-PKCδ. After an additional 36 h, the cells were harvested for assay of hINV mRNA and protein. Similar results were observed in each of three experiments. Moreover, identical results were observed using several KLF4-specific siRNA, indicating that the observed responses are specific. *B* and *C*, the TPA-dependent increase in hINV mRNA level requires KLF4. Keratinocytes were electroporated with 3 μg of the indicated siRNA. After 36 h, the cells were treated in the presence or absence of TPA (50 ng/ml) for an additional 36 h. Extracts were then prepared for detection hINV mRNA and luciferase activity. Similar results were observed in each of three experiments. Identical results were observed using several KLF4 siRNA, indicating that the observed responses are not due to off-target effects. *D*, KLF4 augments the PKCδ-dependent increase in hINV promoter activity. Keratinocytes were transfected with 2 μg of hINV luciferase reporter plasmid, 1 μg of KLF4 expression vector, and 1 μg of PKCδ expression vector. After 24 h, the cells were harvested, and extracts were assayed for luciferase activity. *E*, KLF4 augments the TPA-dependent increase in hINV promoter activity. Keratinocytes were transfected with 2 μg of involucrin luciferase reporter plasmid in the presence or absence of 1 μg of KLF4 expression vector. After 24 h, the cells were treated with or without TPA (50 ng/ml) for 24 h prior to assay for luciferase activity. In all panels, the values are the mean ± S.D. ($n = 3$), and the *asterisk*s indicate a significant increase or decrease as determined using the Student's *t* test, $p < 0.005$. Similar results were obtained in each of three independent experiments.

struct, pINV(−2473/−2088), that encodes a smaller segment of the promoter containing only the DRR region (nucleotides −2473/−2088) linked to the minimal promoter (Fig. 5*A*). This finding indicates that the DRR is necessary and sufficient for the response and rules out a requirement for the PRR AP1-1 site (39). This eliminates the possibility that the PRR has a role in this regulation. pINV-41 encodes the hINV basal promoter (39) and, as expected, does not respond.

We next examined KLF4 interaction at the DRR using chromatin immunoprecipitation. Cells were infected with empty or KLF4-encoding virus, and after 48 h, extracts were prepared for chromatin immunoprecipitation. Fig. 6*A* shows increased KLF4 interaction at the DRR (nucleotides −2218/−2055) in KLF4-expressing cells. In contrast, as a control, we examined KLF4 interaction at a DNA region (nucleotides −1040/−919) that does not encode AP1 or GC-rich elements. As shown in Fig. 6*B*, KLF4 does not bind to this segment.

KLF4 Knockdown Suppresses Keratinocyte Differentiation and Reduces hINV Expression—To assess the impact of KLF4 knockdown on hINV expression during differentiation, we used an epidermal equivalent model that mimics *in vivo* epidermal differentiation (55). Keratinocytes were electroporated with control or KLF4 siRNA and then plated into Millicell chambers to test for the ability to form a stratified epidermal equivalent. Fig. 7*A* confirms that KLF4 siRNA reduces KLF4 mRNA and protein level. Fig. 7*B* shows that that cells treated with control siRNA produce a well differentiated epidermal equivalent that includes a multiple stratified layer and a cornified layer. In contrast, KLF4 siRNA-treated cells form a disordered stratified structure in which suprabasal cells retain their

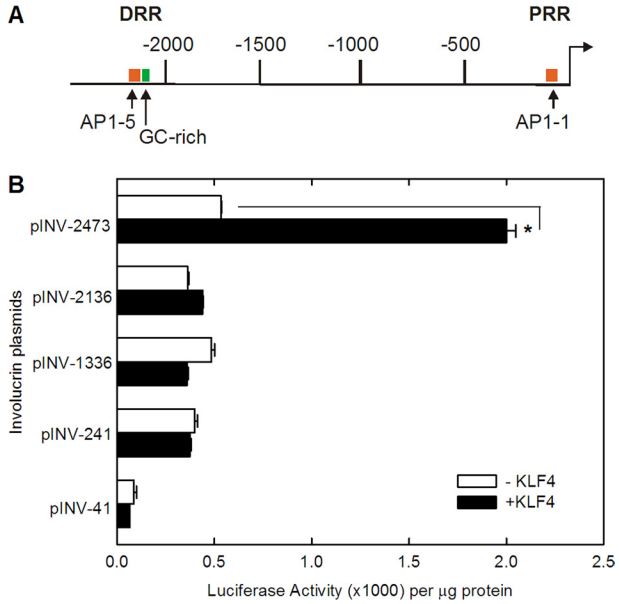

FIGURE 4. **KLF4 activation of hINV promoter requires the DRR.** *A*, the hINV promoter upstream regulatory region showing functionally important (37–39, 50, 52, 53) AP1 (AP1-1 and AP1-5) and GC-rich (Sp1 binding) (22, 37, 38) response elements. The two biologically important AP1 sites present within the upstream regulatory region are indicated (AP1-5 and AP1-1), as is the Sp1 site. The distances are in nucleotides relative to the transcription start site. *B*, KLF4 regulation of hINV promoter activity requires the DRR. Keratinocytes were transfected with 2 μg of the indicated hINV reporter plasmid and 1 μg of empty expression vector or KLF4 expression vector for 24 h prior to harvest and assay for luciferase activity. In all cases, the values are the mean $\pm$ S.D. ($n = 3$), and the *asterisk* indicates a significant increase or decrease, $p < 0.005$. Similar results were observed in three independent experiments.

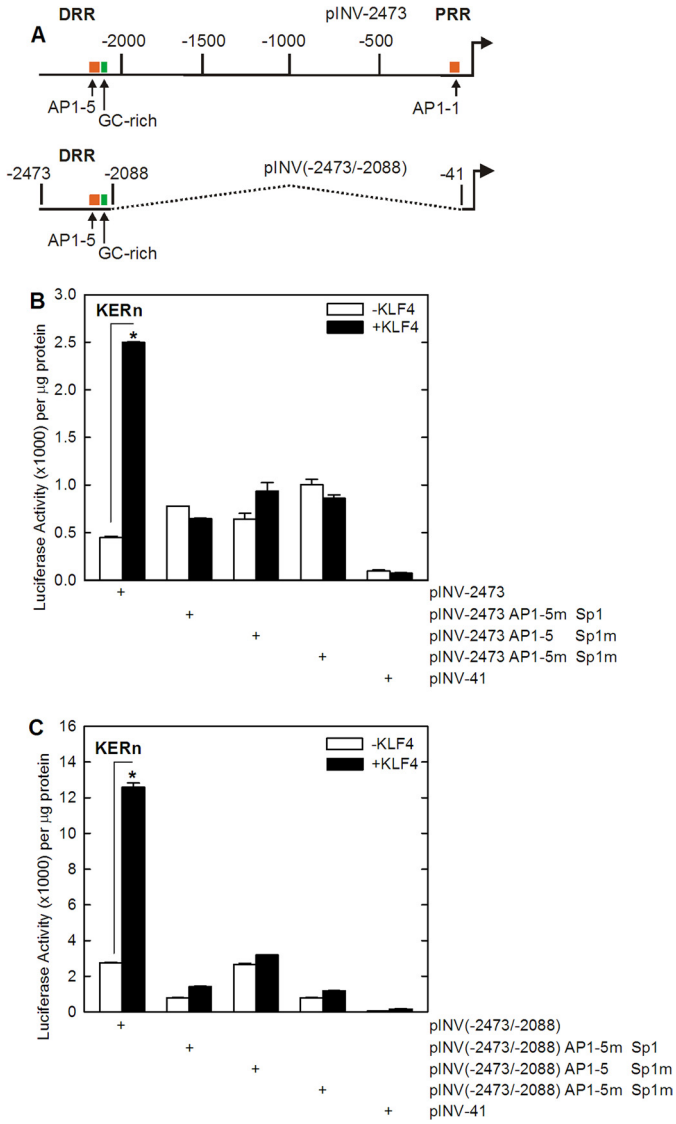

FIGURE 5. **KLF4 activation of hINV promoter activity requires the DRR AP1 and GC-rich response elements.** *A*, schematic showing key regulatory elements in the hINV promoter. pINV-2473 is the full-length promoter. pINV(−2473/−2088) is a construct in which the DRR region (nucleotides −2473/−2088) is linked to the hINV minimal promoter (−41/−1) The *dashed line* indicates the fusion. The functionally important AP1 (AP1-1 and AP1-5) and GC-rich element are indicated. The distances are in nucleotides relative to the transcription start site. *B* and *C*, keratinocytes (*KERn*) were transfected with 2 μg of the indicated reporter plasmid and 1 μg of empty vector or hKLF4-expression vector, and after 24 h, the cells were harvested, and extracts were prepared for luciferase activity assay. The values are mean $\pm$ S.D., $n = 3$. In all cases, the *asterisk* indicates a significant reduction in luciferase activity as determined using the Student's *t* test, $p < 0.005$.

nuclei (*arrows*), showing that KLF4 knockdown impedes the differentiation process. Counting of nucleated suprabasal cells reveals 8 $\pm$ 2 suprabasal nucleated cells in control siRNA-treated cultures, *versus* 41 $\pm$ 3 in KLF4 siRNA-treated cells ($n = 5$, $p < 0.05$). This is evidence of reduced differentiation. Fig. 7*C* shows that hINV mRNA level is reduced in these cultures, thus confirming that KLF4 is required for hINV expression under *in vivo*-like differentiation conditions.

DISCUSSION

Keratinocyte differentiation is a complex process that requires the coordinated activation of a variety of genes (22). Previous studies show that PKCδ is an important driver of cell differentiation (13) and that PKCδ stimulates a MEKK1, MEK3/MEK6, p38δ cascade that triggers differentiation by increasing Sp1 and AP1 transcription factor level (22). In particular, the expression of involucrin, a marker of differentiation, is increased via AP1 and Sp1 factor interaction with DNA elements in the hINV promoter DRR (23–26). However, it is unlikely that these transcription factors are the only one involved in regulating involucrin gene expression.

KLF4 is an important candidate regulator that has not been extensively studied at the mechanistic level in epidermis. Previous studies in mouse transgenic mouse models indicate that KLF4 regulates keratinocyte differentiation (3, 10, 12, 21). Specifically, KLF4 impacts the terminal stages in differentiation. KLF4 knock-out mice die shortly after birth due to loss of skin barrier function, which is accompanied by loss of integrity of late stage differentiation structures such as the cornified envelope (12). These defects are retained when KLF4 knock-out mouse skin is grafted onto nude mice, suggesting that the absence of KLF4 creates an intrinsic defect in the keratinocytes (12). Consistent with these findings, overexpression of KLF4 in mouse epidermis, using the keratin 5 promoter, accelerates barrier formation, which is associated with increase epidermal stratification and increased expression of cornified envelope markers (10). Although global profiling has identified potential KLF4 tar-

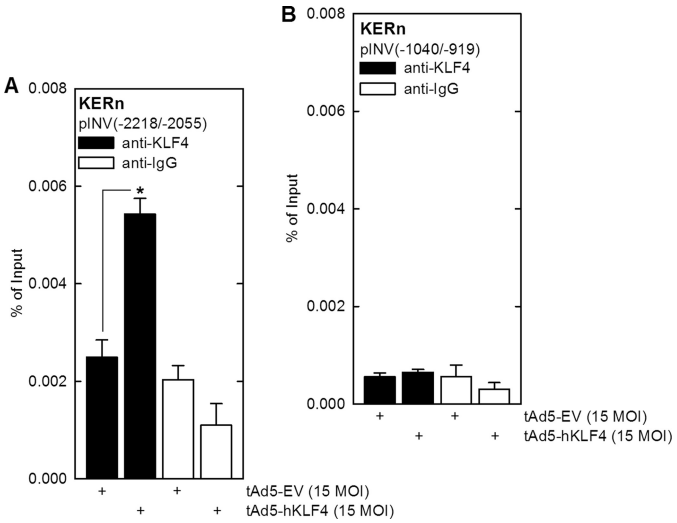

FIGURE 6. **KLF4 interacts with AP1-5/GC-rich response element in the hINV promoter DRR.** *A* and *B*, keratinocytes (*KERn*) were infected with 15 MOI of tAd5-EV or tAd5-hKLF4. After 48 h, cells were prepared for ChIP assay. ChIP was performed as described under "Experimental Procedures" using hINV promoter-derived PCR primers encoding the indicated range of nucleotides. In all panels, the values are the mean ± S.D. ($n = 3$), and the *asterisk* indicates a significant increase or decrease as determined using the Student's *t* test, $p < 0.005$.

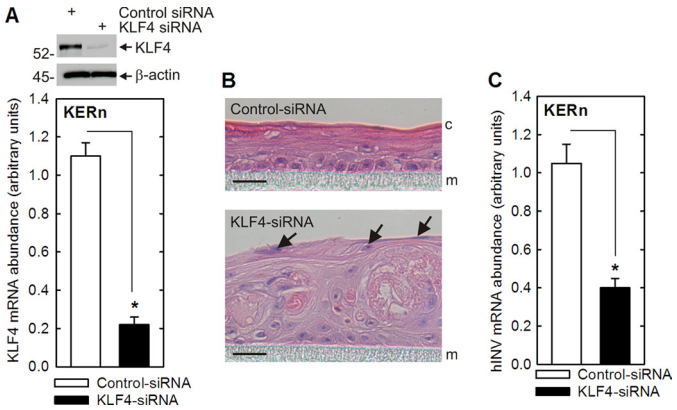

FIGURE 7. **KLF4 is required for hINV expression during keratinocyte differentiation.** *A* and *C*, keratinocytes (*KERn*) were electroporated with control or KLF4 siRNA and seeded into Millicell wells to form epidermal equivalents. After 4 days, the epidermal equivalents were harvested, and extracts were prepared for assay of KLF4 and hINV level. *B*, epidermal equivalents were sectioned and stained with hematoxylin/eosin to assess differentiation status. *Bar* = 100 μm, *m* indicates the membrane, and *c* indicates the cornified layer. The *arrows* indicate nuclei that are retained in cells in the suprabasal layers of the KLF4 siRNA-treated epidermal equivalent. Identical results were obtained with three independent sets of control and KLF4 siRNA. In all cases, the values are the mean ± S.D. ($n = 3$), and the *asterisks* indicate a significant increase or decrease, $p < 0.005$.

get genes in mouse epidermis (3), detailed molecular studies examining KLF4 target gene expression have not been performed. Involucrin is a structural protein that is specifically expressed in differentiated cells of the suprabasal epidermis and is a key precursor protein involved in keratinocyte cornified envelope formation (41, 56–58). Given that KLF4 regulates envelope formation and that involucrin is required for appropriate envelope formation, we surmised that KLF4 may increase involucrin gene expression and that the involucrin gene may be a model for KLF4 action in epidermis.

Mechanism of KLF4 Regulation of Involucrin Expression—We previously showed that hINV expression occurs specifically in differentiated cells and involves interaction of AP1 and Sp1 transcription factors at specific sites in the hINV promoter distal regulatory region (22). This is observed in cultured cells (36, 40, 59), and the DRR is required to drive suprabasal expression *in vivo* when the human gene is placed in transgenic mice (49, 50, 52, 53, 58). Expression is activated by agents and kinases that stimulate keratinocyte differentiation including calcium, phorbol ester, and protein kinase C activity (22). The role of KLF4 in driving expression of differentiation-associated gene in epidermis has not been examined at the molecular level. In the present study, we explore the idea that KLF4 may drive expression of involucrin. We first show that KLF4 expression increases hINV mRNA and protein level, but that this increase is not observed with a transcriptionally inactive KLF4 mutant. The fact that vector-mediated KLF4 increases expression of involucrin, a marker of differentiation, is consistent with the proposed role of KLF4 in driving keratinocyte terminal differentiation (12). Moreover, knockdown of endogenous KLF4 reduces basal hINV mRNA level.

Promoter deletion experiments reveal that the KLF4 regulation requires the promoter DRR, which encodes closely juxtaposed AP1 and GC-rich (Sp1) binding sites. These sites have been implicated in regulation of hINV expression in cultured cells (38, 39, 60) and in transgenic mice *in vivo* (22, 49–53). KLF4 increases activity of the full-length promoter and a promoter segment that encodes only the DRR and flanking sequences. The fact that KLF4 activates the latter construct indicates that the hINV promoter proximal regulatory region is not required for this regulation. This is important because the PRR does have activity in culture models (24, 39). Chromatin immunoprecipitation analysis reveals that KLF4 is enriched at the DRR. It is known that KLF4 interacts at GC-rich elements to drive gene expression and that this interaction requires the KLF4 zinc finger DNA binding domain (1, 61). The Sp1 site of the hINV promoter DRR is a GC-rich sequence (37, 38) that would be predicted to bind KLF4 (1, 61). Indeed mutation of this DNA element results in reduced KLF4-dependent hINV promoter activity. In addition to the GC-rich element, the DRR encodes an AP1 site located immediately upstream of the GC-rich element (50). This site binds AP1 transcription factors including junB, junD, and Fra-1 (39). It is interesting that mutation of this site also reduces the ability of KLF4 to drive transcription. However, this is not unexpected. The AP1 and GC-rich sites are separated by a single nucleotide, and we have previously shown that a large transcriptional complex forms over this region (37, 38, 62) and that mutation of either site disrupts this interaction (38, 60). Thus, we propose that KLF1 forms a complex on this site with AP1 transcription factors and additional adaptor proteins to drive gene expression. These additional proteins are likely to include the p300 histone deacetylase (62). It is interesting that p300 has been reported as a co-regulator with KLF4 (1). In this context, it is plausible that mutation of either the AP1 site or the GC-rich element would reduce KLF4-dependent transcription.

PKCδ Increases KLF4 Level—As mentioned above, PKCδ is an important regulator of keratinocyte differentiation. Vector-mediated overexpression of PKCδ or treatment with phorbol

ester activates this kinase (25, 31, 47, 63, 64). Our present studies show that PKCδ overexpression or treatment with TPA increases KLF4 level in keratinocytes. This is an interesting finding as very little is known about pathways that regulate KLF4 expression. It has been shown that ΔN-p63α suppresses expression in normal epidermal keratinocytes via a mechanism that involves ΔN-p63α direct interaction on the KLF4 promoter (48). ΔN-p63α also regulates KLF4 gene transcription in HaCaT keratinocytes, as determined using a KLF4 promoter-luciferase reporter construct (48). Our actinomycin D inhibitor studies indicate that mRNA stability is not altered in control *versus* PKCδ-expressing keratinocytes, suggesting that the increase in KLF4 expression is mediated via transcriptional mechanisms. Thus, our results are consistent with previously described transcriptional mechanisms for regulation of KLF4 expression (48).

Moreover, these findings are biologically cohesive as ΔN-p63α expression in basal keratinocytes is designed to maintain cell viability in this layer, and so it is expected that ΔNp63α would suppress expression of the pro-differentiation KLF4 protein (ΔNp63α is absent in the upper epidermis, and this is associated with KLF4 expression) (48). Our results show that PKCδ, a pro-differentiation regulator, increases KLF4 expression in keratinocytes to drive differentiation. Moreover, it is interesting that KLF4 is now implicated in two processes in keratinocytes: suppression of proliferation and enhancement of differentiation. Our recent study shows that PKCδ increases $p21^{Cip1}$ expression via a mechanism that requires KLF4 (13).

KLF4 Is Required for hINV Expression in Differentiated Keratinocytes—To confirm that KLF4 is required for hINV expression, we used an epidermal equivalent system that mimics *in vivo* differentiation. In this system, keratinocytes are plated onto a semipermeable membrane and grown at the air-liquid interface. Under these conditions, keratinocytes assemble a highly differentiated multilayered epidermal equivalent that includes a basal proliferative layer, intermediate layers, and a cornified terminal layer (55). Our studies show that knockdown of KLF4 in these cultures severely compromises the differentiation process, resulting in formation of a disordered morphology and the absence of a cornified layer. We further show that hINV expression is markedly reduced. These findings strongly suggest that KLF4 has an *in vivo* role in controlling hINV gene expression.

Based on these studies, we propose that PKCδ induction of KLF4 expression is a key event in the suprabasal epidermis that drives hINV gene expression and keratinocyte terminal differentiation.

REFERENCES

1. Kaczynski, J., Cook, T., and Urrutia, R. (2003) Sp1- and Krüppel-like transcription factors. *Genome Biol.* **4,** 206
2. Swamynathan, S. K., Davis, J., and Piatigorsky, J. (2008) Identification of candidate KLF4 target genes reveals the molecular basis of the diverse regulatory roles of KLF4 in the mouse cornea. *Invest. Ophthalmol. Vis. Sci.* **49,** 3360–3370
3. Patel, S., Xi, Z. F., Seo, E. Y., McGaughey, D., and Segre, J. A. (2006) Klf4 and corticosteroids activate an overlapping set of transcriptional targets to accelerate *in utero* epidermal barrier acquisition. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 18668–18673
4. Chen, X., Whitney, E. M., Gao, S. Y., and Yang, V. W. (2003) Transcriptional profiling of Krüppel-like factor 4 reveals a function in cell cycle regulation and epithelial differentiation. *J. Mol. Biol.* **326,** 665–677
5. Whitney, E. M., Ghaleb, A. M., Chen, X., and Yang, V. W. (2006) Transcriptional profiling of the cell cycle checkpoint gene Krüppel-like factor 4 reveals a global inhibitory function in macromolecular biosynthesis. *Gene Expr.* **13,** 85–96
6. Garrett-Sinha, L. A., Eberspaecher, H., Seldin, M. F., and de Crombrugghe, B. (1996) A gene for a novel zinc-finger protein expressed in differentiated epithelial cells and transiently in certain mesenchymal cells. *J. Biol. Chem.* **271,** 31384–31390
7. Foster, K. W., Ren, S., Louro, I. D., Lobo-Ruppert, S. M., McKie-Bell, P., Grizzle, W., Hayes, M. R., Broker, T. R., Chow, L. T., and Ruppert, J. M. (1999) Oncogene expression cloning by retroviral transduction of adenovirus E1A-immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLF. *Cell Growth Differ.* **10,** 423–434
8. Swamynathan, S. K., Katz, J. P., Kaestner, K. H., Ashery-Padan, R., Crawford, M. A., and Piatigorsky, J. (2007) Conditional deletion of the mouse *Klf4* gene results in corneal epithelial fragility, stromal edema, and loss of conjunctival goblet cells. *Mol. Cell Biol.* **27,** 182–194
9. Jaubert, J., Patel, S., Cheng, J., and Segre, J. A. (2004) Tetracycline-regulated transactivators driven by the involucrin promoter to achieve epidermal conditional gene expression. *J. Invest. Dermatol.* **123,** 313–318
10. Jaubert, J., Cheng, J., and Segre, J. A. (2003) Ectopic expression of Kruppel like factor 4 (*Klf4*) accelerates formation of the epidermal permeability barrier. *Development* **130,** 2767–2777
11. Dai, X., and Segre, J. A. (2004) Transcriptional control of epidermal specification and differentiation. *Curr. Opin. Genet. Dev.* **14,** 485–491
12. Segre, J. A., Bauer, C., and Fuchs, E. (1999) Klf4 is a transcription factor required for establishing the barrier function of the skin. *Nat. Genet.* **22,** 356–360
13. Chew, Y. C., Adhikary, G., Wilson, G. M., Reece, E. A., and Eckert, R. L. (2011) Protein kinase C (PKC) δ suppresses keratinocyte proliferation by increasing $p21^{Cip1}$ level by a KLF4 transcription factor-dependent mechanism. *J. Biol. Chem.* **286,** 28772–28782
14. Wei, D., Kanai, M., Huang, S., and Xie, K. (2006) Emerging role of KLF4 in human gastrointestinal cancer. *Carcinogenesis* **27,** 23–31
15. Zhang, W., Geiman, D. E., Shields, J. M., Dang, D. T., Mahatan, C. S., Kaestner, K. H., Biggs, J. R., Kraft, A. S., and Yang, V. W. (2000) The gut-enriched Kruppel-like factor (Kruppel-like factor 4) mediates the transactivating effect of p53 on the $p21^{WAF1/Cip1}$ promoter. *J. Biol. Chem.* **275,** 18391–18398
16. Rowland, B. D., Bernards, R., and Peeper, D. S. (2005) The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. *Nat. Cell Biol.* **7,** 1074–1082
17. Yoon, H. S., and Yang, V. W. (2004) Requirement of Krüppel-like factor 4 in preventing entry into mitosis following DNA damage. *J. Biol. Chem.* **279,** 5035–5041
18. Pincelli, C., and Marconi, A. (2010) Keratinocyte stem cells: friends and foes. *J. Cell Physiol.* **225,** 310–315
19. Alonso, L., and Fuchs, E. (2003) Stem cells of the skin epithelium. *Proc. Natl. Acad. Sci. U.S.A.* **100,** Suppl. 1, 11830–11835
20. Byrne, C., Tainsky, M., and Fuchs, E. (1994) Programming gene expression in developing epidermis. *Development* **120,** 2369–2383
21. Segre, J. (2003) Complex redundancy to build a simple epidermal permeability barrier. *Curr. Opin. Cell Biol.* **15,** 776–782
22. Eckert, R. L., Crish, J. F., Efimova, T., Dashti, S. R., Deucher, A., Bone, F., Adhikary, G., Huang, G., Gopalakrishnan, R., and Balasubramanian, S. (2004) Regulation of involucrin gene expression. *J. Invest Dermatol.* **123,** 13–22
23. Efimova, T., and Eckert, R. L. (2000) Regulation of human involucrin promoter activity by novel protein kinase C isoforms. *J. Biol. Chem.* **275,** 1601–1607
24. Efimova, T., LaCelle, P., Welter, J. F., and Eckert, R. L. (1998) Regulation of human involucrin promoter activity by a protein kinase C, Ras, MEKK1, MEK3, p38/RK, AP1 signal transduction pathway. *J. Biol. Chem.* **273,** 24387–24395
25. Efimova, T., Deucher, A., Kuroki, T., Ohba, M., and Eckert, R. L. (2002)

Novel protein kinase C isoforms regulate human keratinocyte differentiation by activating a p38δ mitogen-activated protein kinase cascade that targets CCAAT/enhancer-binding protein α. *J. Biol. Chem.* **277,** 31753–31760
26. Efimova, T., Broome, A. M., and Eckert, R. L. (2003) A regulatory role for p38δ MAPK in keratinocyte differentiation: evidence for p38δ-ERK1/2 complex formation. *J. Biol. Chem.* **278,** 34277–34285
27. Rice, R. H., and Green, H. (1979) Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell* **18,** 681–694
28. Rice, R. H., and Green, H. (1977) The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. *Cell* **11,** 417–422
29. Adhikary, G., Chew, Y. C., Reece, E. A., and Eckert, R. L. (2010) PKC-δ and -η, MEKK-1, MEK-6, MEK-3, and p38-δ are essential mediators of the response of normal human epidermal keratinocytes to differentiating agents. *J. Invest Dermatol.* **130,** 2017–2030
30. Deucher, A., Efimova, T., and Eckert, R. L. (2002) Calcium-dependent involucrin expression is inversely regulated by protein kinase C (PKC)α and PKCδ. *J. Biol. Chem.* **277,** 17032–17040
31. Kraft, C. A., Efimova, T., and Eckert, R. L. (2007) Activation of PKCδ and p38δ MAPK during okadaic acid dependent keratinocyte apoptosis. *Arch. Dermatol. Res.* **299,** 71–83
32. Zhu, L., Brodie, C., Balasubramanian, S., and Eckert, R. L. (2008) Multiple PKCδ tyrosine residues are required for PKCδ-dependent activation of involucrin expression–a key role of PKCδ-Y311. *J. Invest Dermatol.* **128,** 833–845
33. Adhikary, G., Crish, J., Lass, J., and Eckert, R. L. (2004) Regulation of involucrin expression in normal human corneal epithelial cells: a role for activator protein one. *Invest Ophthalmol. Vis. Sci.* **45,** 1080–1087
34. Adhikary, G., Crish, J. F., Gopalakrishnan, R., Bone, F., and Eckert, R. L. (2005) Involucrin expression in the corneal epithelium: an essential role for Sp1 transcription factors. *Invest Ophthalmol. Vis. Sci.* **46,** 3109–3120
35. Adhikary, G., Crish, J. F., Bone, F., Gopalakrishnan, R., Lass, J., and Eckert, R. L. (2005) An involucrin promoter AP1 transcription factor binding site is required for expression of involucrin in the corneal epithelium *in vivo*. *Invest Ophthalmol. Vis. Sci.* **46,** 1219–1227
36. Balasubramanian, S., Efimova, T., and Eckert, R. L. (2002) Green tea polyphenol stimulates a Ras, MEKK1, MEK3, and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes. *J. Biol. Chem.* **277,** 1828–1836
37. Banks, E. B., Crish, J. F., and Eckert, R. L. (1999) Transcription factor Sp1 activates involucrin promoter activity in non-epithelial cell types. *Biochem. J.* **337,** 507–512
38. Banks, E. B., Crish, J. F., Welter, J. F., and Eckert, R. L. (1998) Characterization of human involucrin promoter distal regulatory region transcriptional activator elements-a role for Sp1 and AP1 binding sites. *Biochem. J.* **331,** 61–68
39. Welter, J. F., Crish, J. F., Agarwal, C., and Eckert, R. L. (1995) Fos-related antigen (*Fra-1*), *junB*, and *junD* activate human involucrin promoter transcription by binding to proximal and distal AP1 sites to mediate phorbol ester effects on promoter activity. *J. Biol. Chem.* **270,** 12614–12622
40. Balasubramanian, S., and Eckert, R. L. (2007) Keratinocyte proliferation, differentiation, and apoptosis–differential mechanisms of regulation by curcumin, EGCG, and apigenin. *Toxicol. Appl. Pharmacol.* **224,** 214–219
41. Robinson, N. A., LaCelle, P. T., and Eckert, R. L. (1996) Involucrin is a covalently crosslinked constituent of highly purified epidermal corneocytes: evidence for a common pattern of involucrin crosslinking *in vivo* and *in vitro*. *J. Invest Dermatol.* **107,** 101–107
42. Welter, J. F., Gali, H., Crish, J. F., and Eckert, R. L. (1996) Regulation of human involucrin promoter activity by POU domain proteins. *J. Biol. Chem.* **271,** 14727–14733
43. Balasubramanian, S., Zhu, L., and Eckert, R. L. (2006) Apigenin inhibition of involucrin gene expression is associated with a specific reduction in phosphorylation of protein kinase Cδ Tyr^{311}. *J. Biol. Chem.* **281,** 36162–36172
44. Kitamura, T., Koshino, Y., Shibata, F., Oki, T., Nakajima, H., Nosaka, T., and Kumagai, H. (2003) Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp. Hematol.* **31,** 1007–1014
45. Ohba, M., Ishino, K., Kashiwagi, M., Kawabe, S., Chida, K., Huh, N. H., and Kuroki, T. (1998) Induction of differentiation in normal human keratinocytes by adenovirus-mediated introduction of the η and δ isoforms of protein kinase C. *Mol. Cell Biol.* **18,** 5199–5207
46. Sturniolo, M. T., Chandraratna, R. A., and Eckert, R. L. (2005) A novel transglutaminase activator forms a complex with type 1 transglutaminase. *Oncogene.* **24,** 2963–2972
47. Efimova, T., Broome, A. M., and Eckert, R. L. (2004) Protein kinase Cδ regulates keratinocyte death and survival by regulating activity and subcellular localization of a p38δ-extracellular signal-regulated kinase 1/2 complex. *Mol. Cell Biol.* **24,** 8167–8183
48. Cordani, N., Pozzi, S., Martynova, E., Fanoni, D., Borrelli, S., Alotto, D., Castagnoli, C., Berti, E., Viganò, M. A., and Mantovani, R. (2011) Mutant p53 subverts p63 control over KLF4 expression in keratinocytes. *Oncogene* **30,** 922–932
49. Crish, J. F., Howard, J. M., Zaim, T. M., Murthy, S., and Eckert, R. L. (1993) Tissue-specific and differentiation-appropriate expression of the human involucrin gene in transgenic mice: an abnormal epidermal phenotype. *Differentiation* **53,** 191–200
50. Crish, J. F., Zaim, T. M., and Eckert, R. L. (1998) The distal regulatory region of the human involucrin promoter is required for expression in epidermis. *J. Biol. Chem.* **273,** 30460–30465
51. Crish, J. F., Bone, F., Balasubramanian, S., Zaim, T. M., Wagner, T., Yun, J., Rorke, E. A., and Eckert, R. L. (2000) Suprabasal expression of the human papillomavirus type 16 oncoproteins in mouse epidermis alters expression of cell cycle regulatory proteins. *Carcinogenesis* **21,** 1031–1037
52. Crish, J. F., Bone, F., Banks, E. B., and Eckert, R. L. (2002) The human involucrin gene contains spatially distinct regulatory elements that regulate expression during early versus late epidermal differentiation. *Oncogene* **21,** 738–747
53. Crish, J. F., Gopalakrishnan, R., Bone, F., Gilliam, A. C., and Eckert, R. L. (2006) The distal and proximal regulatory regions of the involucrin gene promoter have distinct functions and are required for *in vivo* involucrin expression. *J. Invest Dermatol.* **126,** 305–314
54. Kikkawa, U., Kaibuchi, K., Castagna, M., Yamanishi, J., Sano, K., Tanaka, Y., Miyake, R., Takai, Y., and Nishizuka, Y. (1984) Protein phosphorylation and mechanism of action of tumor-promoting phorbol esters. *Adv. Cyclic. Nucleotide. Protein Phosphorylation. Res.* **17,** 437–442
55. Poumay, Y., Dupont, F., Marcoux, S., Leclercq-Smekens, M., Hérin, M., and Coquette, A. (2004) A simple reconstructed human epidermis: preparation of the culture model and utilization in *in vitro* studies. *Arch. Dermatol. Res.* **296,** 203–211
56. Steinert, P. M., and Marekov, L. N. (1997) Direct evidence that involucrin is a major early isopeptide cross-linked component of the keratinocyte cornified cell envelope. *J. Biol. Chem.* **272,** 2021–2030
57. Robinson, N. A., Lapic, S., Welter, J. F., and Eckert, R. L. (1997) S100A11, S100A10, annexin I, desmosomal proteins, small proline-rich proteins, plasminogen activator inhibitor-2, and involucrin are components of the cornified envelope of cultured human epidermal keratinocytes. *J. Biol. Chem.* **272,** 12035–12046
58. Eckert, R. L., Yaffe, M. B., Crish, J. F., Murthy, S., Rorke, E. A., and Welter, J. F. (1993) Involucrin–structure and role in envelope assembly. *J. Invest Dermatol.* **100,** 613–617
59. Balasubramanian, S., and Eckert, R. L. (2004) Green tea polyphenol and curcumin inversely regulate human involucrin promoter activity via opposing effects on CCAAT/enhancer-binding protein function. *J. Biol. Chem.* **279,** 24007–24014
60. Apfel, C., Bauer, F., Crettaz, M., Forni, L., Kamber, M., Kaufmann, F., LeMotte, P., Pirson, W., and Klaus, M. (1992) A retinoic acid receptor α antagonist selectively counteracts retinoic acid effects. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7129–7133
61. Black, A. R., Black, J. D., and Azizkhan-Clifford, J. (2001) Sp1 and Krüppel-like factor family of transcription factors in cell growth regulation and cancer. *J. Cell Physiol.* **188,** 143–160

62. Crish, J. F., and Eckert, R. L. (2008) Synergistic activation of human involucrin gene expression by Fra-1 and p300–evidence for the presence of a multiprotein complex. *J. Invest Dermatol.* **128,** 530–541
63. Denning, M. F., Dlugosz, A. A., Cheng, C., Dempsey, P. J., Coffey, R. J., Jr., Threadgill, D. W., Magnuson, T., and Yuspa, S. H. (2000) Cross-talk between epidermal growth factor receptor and protein kinase C during calcium-induced differentiation of keratinocytes. *Exp. Dermatol.* **9,** 192–199
64. Denning, M. F., Dlugosz, A. A., Williams, E. K., Szallasi, Z., Blumberg, P. M., and Yuspa, S. H. (1995) Specific protein kinase C isozymes mediate the induction of keratinocyte differentiation markers by calcium. *Cell Growth Differ.* **6,** 149–157