PKCα/ERK/C7ORF41 axis regulates epidermal keratinocyte differentiation through the IKKα nuclear translocation

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Abstract

Aberrant differentiation of keratinocytes disrupts the skin barrier and causes a series of skin diseases. However, the molecular basis of keratinocyte differentiation is still poorly understood. In the present study, we examined the expression of C7ORF41 using tissue microarrays by immunohistochemistry and found that C7ORF41 is specifically expressed in the basal layers of skin epithelium and its expression is gradually decreased during keratinocytes differentiation. Importantly, we corroborated the pivotal role of C7ORF41 during keratinocyte differentiation by C7ORF41 knockdown or overexpression in TPA-induced Hacat keratinocytes. Mechanistically, we first demonstrated that C7ORF41 inhibited keratinocyte differentiation mainly through formatting a complex with IKKα in the cytoplasm, which thus blocked the nuclear translocation of IKKα. Furthermore, we also demonstrated that inhibiting the PKCα/ERK signaling pathway reversed the reduction of C7ORF41 in TPA-induced keratinocytes, indicating that C7ORF41 expression could be regulated by upstream PKCα/ERK signaling pathway during keratinocyte differentiation. Collectively, our study uncovers a novel regulatory network PKCα/ERK/C7ORF41/IKKα during keratinocyte differentiation, which provides potential therapeutic targets for skin diseases.

Key words: keratinocyte differentiation; PKCα; C7ORF41; IKKα; nuclear translocation

1. Introduction

Epidermis, the outermost part of the skin, is a stratified squamous epithelium mainly contains a mitotically active basal layer of keratinocytes, which forms a protective skin barrier physically separating the host from the outside environment [1]. Basal keratinocytes retain undifferentiated state and have continuous self-renewal potential [2, 3]. Upon terminal differentiation, keratinocytes cease proliferation and subsequently migrate into the suprabasal layers and give rise to differentiated cells. Generally, keratinocytes undergo three distinct stages including the spinous, granular and stratum corneum layers in the process of differentiation[4], and express specific differentiation markers at each layer[5]. As cells differentiation into the spinous layer, they begin to express keratin 1 (KRT1) and KRT10 and simultaneously suppress the expression of basal layer marker KRT5 and KRT14 [6, 7]. At a more advanced stage, granular layer cells initiate the expression of Filaggrin (FLG), which aggregates the keratin filaments into tight bundles, forming the flattened terminally differentiated cells [8]. Furthermore, keratinocytes express terminal differentiation markers such as loricrin and involucrin in the cornified layer [9-11], which plays a vital role in the differentiated keratinocytes because of its protective function against the environment [7]. A finely-tuned balance between proliferation and differentiation must be tightly controlled to maintain epidermal homeostasis. However, several evidences showed that the dysregulation of keratinocytes differentiation is associated with skin diseases, such as psoriasis, atopic dermatitis and squamous cell carcinomas [12-14].
Keratinocyte differentiation is a highly coordinated multistep process regulated by intercellular and external signal stimuli. In the epidermis, an extracellular Ca\(^{2+}\) gradient is present from the basal layer to the cornified layer[15], and Ca\(^{2+}\) signaling is indeed involved in the keratinocytes terminal differentiation[16]. Studies indicated that keratinocytes continued to proliferate when cultured in medium containing less than 0.05 mM Ca\(^{2+}\) and were induced to differentiate by elevating the extracellular Ca\(^{2+}\) concentration [15, 17]. Ca\(^{2+}\) induced PKC activation is implicated in the keratinocyte proliferation and differentiation [2, 18, 19]. In addition, the activation of PKC by TPA also stimulates keratinocytes differentiation [20, 21]. Contrarily, inhibition of the PKC activity with specific inhibitor GF109203X attenuated the keratinocytes differentiation and stimulated proliferation [21, 22]. The PKC family contains more than 11 isozyms [23], of which only five different PKC isforms (\(\alpha, \delta, \epsilon, \eta, \xi\)) are expressed in epidermal keratinocytes [24, 25]. (Classical) cPKCs is Ca\(^{2+}\) and phorbol ester dependent, while the (novel) nPKCs (PKC\(\delta, \epsilon, \eta\)) is Ca\(^{2+}\) independent; activation of the atypical PKC\(\xi\) requires neither Ca\(^{2+}\) nor phorbol ester [18, 26]. These specific PKC isoforms showed a characteristic expression pattern and differential regulatory roles in the keratinocytes differentiation [2, 21, 25, 27].

How the PKC signaling pathway controls the balance between proliferation and differentiation in keratinocytes is largely unknown. Several studies indicated that PKC isoforms regulate keratinocytes differentiation by activation MAPK signaling, which leads to the increase of AP1, SP1 and KLF4 transcription factors in the nucleus [27-29]. Interestingly, IKK\(\alpha\), one of the catalytic subunits of the IKK complex, is also a pivotal molecule in balancing growth and differentiation in the epidermis, which does not depend on its kinase activity but requires IKK\(\alpha\) nuclear translocation [30-32]. Numerous studies indicated that the ablation of IKK\(\alpha\) prevented the differentiation of epidermal keratinocytes and enhanced their proliferative potential as well as skin carcinogenesis [32, 33]. IKK\(\alpha\) expression in the skin has been reported to be elevated after TPA or Ca\(^{2+}\) treatment [34, 35], and higher levels of nuclear IKK\(\alpha\) was present in the differentiated keratinocytes [36]. Nuclear localization sequence (NLS) within the kinase domain of IKK\(\alpha\) is essential for its nuclear translocation and its disruption prevents the keratinocytes differentiation [30, 36]. Thereby, it is necessary to illustrate how IKK\(\alpha\) is precisely controlled to translocate into the nucleus.

C7ORF41 namely MTURN, one conserved gene in evolution, is involved in the regulation of diverse development and cellular processes, such as hematopoietic cells differentiation[37], neurogenesis[38]. However, the abnormal expression of C7ORF41 gene is also related to some human diseases like leukemia and nonalcoholic fatty liver disease [37, 39]. Moreover, we also found C7ORF41 is an important molecule that plays a vital role in the innate immune inflammation response through interacting with IKK\(\alpha/\beta\) [40]. Since skin is the first line of defense against infection in the innate immune system, we wonder whether C7ORF41 is involved in the skin function via IKK\(\alpha\).
In the present study, we studied the expression and function of C7ORF41 in epidermal keratinocytes. Our results indicate that C7ORF41 is mainly present in basal layer of epidermis, and its expression is gradually decreased during keratinocytes differentiation. We also investigated the underlying mechanisms of C7ORF41 in keratinocytes differentiation, and found that C7ORF41 could regulate the nuclear translocation of IKKα. Moreover, we demonstrated the downregulation of C7ORF41 was mediated by PKCa/ERK signaling pathway. Taken together, our study identified a novel PKCa/ERK/C7ORF41/IKKα regulatory network to mediate the differentiation of epidermis.

2. Materials and methods

2.1. Cell culture and treatments

Human immortalized Hacat keratinocytes were purchased from China Center for Type Culture Collection (Wuhan University, China) and cultured in DMEM (Gbico, USA) supplemented with 10% fetal bovine serum (Gbico, USA), 1% penicillin and streptomycin at 37°C in a 5% CO₂ atmosphere. To induce keratinocyte differentiation, 2.8 mM CaCl₂ or TPA (Sigma, St. Louis, USA) was added directly to culture media for indicated time, and 0.03 mM CaCl₂ to maintain a basal cell-like population of undifferentiated cells.

All inhibitors were dissolved in DMSO and the final concentrations of DMSO added to cells were less than 0.1%. Cells were pretreated with indicated inhibitors for 0.5 h, followed by stimulated with TPA for 24 h. The final concentration of each inhibitor used is: the c/n PKC inhibitor GF109203X, 10 μM; the cPKC inhibitor GÖ6976, 10 μM; then PKC inhibitor GÖ6983, 10 μM; ERK inhibitor PD98059, 20μM; IKKβ inhibitor SC-514, 100 μM ; JNK inhibitor Sp600125, 50 μM.

2.2. Lentivirus mediated C7ORF41-overexpression or knockdown in Hacat cells

Stable cell lines that overexpressed or knockdown C7ORF41 in Hacat keratinocytes was achieved through lentiviral were performed as previously described [39, 41, 42]. All vectors carried puromycin-resistant gene and the infected cells were selected with puromycin (1 μg/mL) for one week to obtain stable cell lines. C7ORF41 was fused with a flag tag in its C-terminal and the sequence of shRNA for knockdown human C7ORF41 gene was listed in the supplementary Table S1. The corresponding empty vectors were used as control.

2.3. Immunohistochemistry

A tissue microarrays (TMA) containing skin sample (n=4) were purchased from Xi’an Alena Biological Technology Co., and were subjected to immunohistochemical staining. Anti-C7ORF41 antibody was used to detect C7ORF41 expression as previously described [43]. Briefly, immunohistochemistry of tissue microarrays was performed as follows: 4 μm-thick sections were deparaffinized, rehydrated and washed twice with PBS for 10, 5 and 5 min, respectively. After incubating with
hydrogen peroxide for 10 min and antigen retrieval at 98°C for 25 min, the slides were held in blocking solution for 30 min at room temperature, and then incubated with anti-C7ORF41 antibody at 4°C overnight. Subsequently, after washed with PBS, the slides were incubated with HRP-conjugated secondary antibody. The immunohistochemical reaction was visualized with 3,3’-diaminobenzidine (DAB). Sections were removed from DAB solution upon confirmation of color development, rinsed with PBS, and then counterstained with haematoxylin.

2.4. Transfection of Hacat cells with siRNA

Lipofectamine 3000 (Invitrogen Co., USA) was used to transfect cells with siRNA according to the manufacturer’s protocol. The sequences of siRNA for human PKCα and IKKα are listed in the supplementary Table S1. After transfected with siRNA for 24 h, Cells were treated with other stimulations.

2.5. Cell proliferation assay

The proliferation of C7ORF41 overexpression or lower expression in Hacat cells were determined using the cell counting kit (CCK-8) assay (Dojindo, Kumamoto,Japan). Briefly, cells (8000 cells/well) were plated into a 96-well plate in a final volume of 100 μL and were stimulated with 100 nM TPA after 24 h. Proliferation was evaluated at 0, 24, 48, 72, 96 h after TPA stimulation according to the CCK-8 kit manufacturer’s protocol. The number of viable cells was calculated based on absorbance at 450 nm.

2.6. Flow cytometry analysis of cell cycle

Hacat cells that stably overexpressed or knockdown C7ORF41 were harvested after 48 h with TPA treatment. The resultant cells were washed twice with PBS, and fixed with 4% PFA at 4°C overnight and then washed again with PBS. After treatment with 1% saponin and staining with 10 μg/mL DAPI, these cells were examined with flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and data were analyzed by a FlowJo software.

2.7. Quantitative Real-time PCR (q-RT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions. RNA concentrations were determined by absorbance at OD260 and the quality of total RNA was detected at an A260/A280 ratio (NanoDrop Technol). RNA was reverse transcribed using a reverse transcription kit with gDNA Eraser (Takara, Japan) according to the manufacturer’s protocol. The amount of gene expression was measured with SYBR Green PCR Master Mix (DBI-2044, Germany) with triplicates on the ABI step one plus real-time PCR system. For each primer set, the Ct value was normalized to that of GAPDH as inner control, which was further normalized to that of control sample. The results of qRT-PCR was measured using the 2-ΔΔCt method and presented as relative mRNA level.

2.8. Western blot analysis
To prepare total cell lysates, cells were washed with ice-cold PBS for twice and lysed with RIPA buffer containing a complete protease and phosphatase inhibitor cocktail (Roche Diagnostics, Germany) for 30 min on ice. The lysate was then centrifuged at 12000 rpm for 15 min at 4°C and the supernatant was harvested for analysis. Protein concentration was determined using BCA kit (Sigma) according to the manufacturer’s instructions. Equal amounts of sample protein (50μg/well) were loaded and separated by SDS-PAGE gels and transferred onto PVDF membrane (Millipore, USA). After blocking, blots were incubated overnight at 4°C with various primary antibody. Membranes were washed three times for 30 min in TBST followed by incubation with the corresponding HRP-conjugated IgG secondary antibodies for 1 h at room temperature. Membranes were washed and visualized with ECL (Millipore).

2.9. Co-immunoprecipitation assay

To detect the protein-protein interaction, co-immunoprecipitation assay were performed as described in Hacat or cotransfected with reconstructs. Briefly, the cells were lysed and then centrifuged. The cell lysates were incubated with 30 μL protein A/G-agarose beads (GE Healthcare, USA) and primary antibody. The immune complexes was washed with cold immunoprecipitation buffer and eluted by 2×SDS loading buffer. The immunoprecipitates were analyzed through western blot using the indicated primary antibodies and corresponding HRP affinipure IgG light chain or heavy chain as secondary antibodies.

2.10. Subcellular fractionation

Cells were washed with PBS and incubated in cytoplasm lysis buffer (1.5 mM MgCl2, 10 mM HEPES at pH7.4, 10 mM KCl, 1 mM EDTA, 0.2% NP-40, 10% glycerol, 1× protease cocktail) for 10 min on ice. Cell lysates were centrifuged at 12000 rpm for 10 min, and the supernatants were collected as cytoplasm extractions. The pellets were washed twice with cytoplasm lysis buffer and then lysed with nuclear lysis buffer (20 mM HEPES at pH7.4, 10 mM KCl, 350 mM NaCl, 1 mM EDTA, 0.2% NP-40, 20% glycerol, 1×protease cocktail) for 30 min on ice. The lysates were centrifuged for 15 min at 12000 rpm at 4°C to collect nuclear lysates.

2.11. Plasmid construction

The vectors that contains C7ORF41 gene or shRNA targets to C7ORF41 were constructed as described [37, 39]. Human IKKα cDNA was cloned into pCAggs-HA plasmid and the deletion mutation were generated by over-lap PCR method to amplify pCAggs-HA-IKKα. All the constructed vectors were identified by DNA sequencing.

2.12. Statistical analysis

All the experiments were performed three or more times. Statistical analyses were performed using SPSS software and all data are presented as the Means ±STDEV. Student’s t-test was performed to analyse the differences between two groups. Statistical differences among more than 2 groups were examined via one-way analysis of variance (ANOVA) with post hoc Dunnett test. P < 0.05 was considered
statistically significant.

3. Results

3.1. C7ORF41 is downregulated during epidermal differentiation

To understand the physiological role of C7ORF41 during keratinocyte differentiation, we first applied TMA technology and immunohistochemistry to assess C7ORF41 protein expression in the normal skin tissue using the C7ORF41 antibody. As the result shown in Fig. 1A, C7ORF41 staining was not uniformly in the epidermis, but it is primarily localized in the basal cell layer and dramatically reduced in the more differentiated suprabasal layers. To further examine the expression of C7ORF41 in terms of differentiation states, we measured its expression in the cultured Hacat keratinocytes following the high concentrations of calcium for indicated times. We found that under calcium-induced keratinocyte differentiation, the expression of C7ORF41 is gradually decreased in a time-dependent manner both at the mRNA and protein levels, in parallel with an increase of keratin 10 (KRT10) and filaggrin (FLG) expression, markers for differentiation (Fig. 1B-E). In addition, C7ORF41 expression is also reduced in TPA-induced keratinocytes differentiation at both mRNA and protein levels in a dose and time dependent manner (Fig. 1F-M). These results showed that the downregulation of C7ORF41 expression in differentiating keratinocytes is not specific for calcium induced differentiation but can also be achieved by other inducer of keratinocyte differentiation. Altogether, we concluded that the expression pattern of C7ORF41 might be regulated in a differentiation-dependent manner and also involved in keratinocytes differentiation.

3.2. C7ORF41 negatively regulates keratinocytes differentiation

To elucidate the role of C7ORF41 in keratinocyte differentiation, we constructed a C7ORF41 overexpression stable cell line in Hacat cells (designated as Hacat-C7ORF41) using lentiviruses infection, and the overexpression efficiency of C7ORF41 was verified at mRNA and protein levels (Fig. 2A-B). We found that C7ORF41 overexpression significantly retarded keratinocyte differentiation as evidenced by markedly ameliorated the differentiation marker KRT10 and FLG in TPA-treated Hacat C7ORF41 cells (Fig. 2C-E). Besides, we generated C7ORF41 knockdown cells in Hacat using small hairpin RNA (shRNA-C7ORF41)-encoding lentiviruses to further validate the function of C7ORF41 in keratinocytes. The silencing efficiency of C7ORF41 was confirmed by real-time PCR and western blot (Fig. 2F-G). Since the shRNA-C7ORF41-1# cell line showed more profoundly silencing efficiency, we selected this cell line for the following experiments. Our results showed that C7ORF41 silencing increased the expressions of KRT10 and FLG in TPA-treated cells (Fig. 2H-J). Collectively, these data suggest that C7ORF41 negatively regulates the differentiation of keratinocytes under the differentiating conditions.

3.3. C7ORF41 positively regulates keratinocyte growth
Keratinocyte differentiation is often associated with cessation of proliferation. In order to explore the role of C7ORF41 in keratinocytes proliferation, we examined the potential of cell proliferation using CCK-8 in TPA-treated Hacat-C7ORF41 or shC7ORF41-1# cells. As shown in Fig.3A-B, ectopic C7ORF41 expression significantly promoted Hacat cells proliferation after TPA treatment; while C7ORF41 silencing dramatically inhibited cell growth when treated with TPA. In addition, to determine the effect of C7ORF41 on the cell cycle, flow cytometric analysis was performed. The results showed that the cells in the G0/G1 phase were dramatically increased, while there was a significant decrease in the G2/M phase in shC7ORF41-1# cells after TPA treatment for 48h, as compared with control group. On the contrary, C7ORF41 overexpression increased the cells in the S phase (Fig.3C-D). Taken together, all these data suggest that C7ORF41 regulates the cell cycle, and its downregulation could inhibit the proliferation of Hacat keratinocytes and arrest the cell cycle at the G1/S phase. Since cyclin D1 is the major cell-cycle regulator of G1/S phase [44, 45], we examined the effect of C7ORF41 on the expression of the gene CCND1, which encodes for cyclin D1. As the results shown in Fig.3E-H, overexpression of C7ORF41 increased cyclin D1 at both mRNA and protein levels, while knockdown of C7ORF41 inhibited cyclin D1. In sum, all these data suggest that C7ORF41 promotes keratinocytes proliferation through regulating cyclin D1 expression.

3.4. C7ORF41 regulates the keratinocytes differentiation through IKKα nuclear translocation

We next focused on the underlying molecular mechanism of C7ORF41 regulating keratinocytes differentiation. Consistent with our previous studies that IKKα associated with C7ORF41 through its kinase domain [39, 40], we confirmed that C7ORF41 could also interact with IKKα in Hacat cells (Fig.4A-B). Several studies indicated that nuclear translocation of IKKα is critical for its role in keratinocytes differentiation [30, 36]. To examine whether nuclear translocation of IKKα is influenced by C7ORF41, we first measured the subcellular localization of IKKα in Hacat cells with or without TPA treatment. We found that TPA treatment significantly promote IKKα nuclear translocation (Fig.4C). As expected, overexpression of C7ORF41 dramatically reduced the nuclear IKKα (Fig.4D). To further examine whether C7ORF41 blocked the nuclear translocation of IKKα through their interaction with the nuclear localization sequence (NLS) within the kinase domain of IKKα, we performed a co-IP experiments with indicated IKKα truncations. We observed that C7ORF41 protein is immunoprecipitated by the full length IKKa and its kinase domain, whereas the interaction is abolished when the NLS region of IKKα was deleted (Fig.4E). Altogether, all these data suggested that C7ORF41 could form a complex with IKKα in the cytoplasm to block the IKKα entering into the nucleus through masking its NLS.

In order to test whether IKKα is involved in C7ORF41-mediated keratinocytes differentiation, we silenced IKKα using siRNA in TPA-induced shC7ORF41-1# cells. As shown in Fig.4F, silencing IKKα markedly reduced the keratinocytes
differentiation, as evidenced by the expression of differentiation markers. Taken together, these data indicated that C7ORF41 regulates the keratinocytes differentiation through regulating IKKα nuclear translocation.

3.5. C7ORF41 downregulation is mediated by PKC/ERK signaling pathway in keratinocytes

Several studies showed that both Ca^{2+} and TPA could induce keratinocytes differentiation through a PKC-dependent mechanism [15, 46, 47]. To investigate whether PKC signaling is involved in C7ORF41 downregulation during keratinocyte differentiation, we first measured the expression of C7ORF41 with PKC inhibitor GF109203X, GÖ6976, GÖ6983 before TPA stimulation. As the results shown in Fig.5A-D, C7ORF41 expression is markedly reduced by TPA treatment at mRNA and protein levels. However, both general (GF109203X) and classical (GÖ6976), but not novel (GÖ6983) PKC isoform inhibitor reversed TPA-induced C7ORF41 downregulation. Interestingly, all these inhibitors significantly reduced TPA-induced KRT10 and FLG expression (Fig.5B-D). These data suggested that only classical PKC isoform is involved in the regulation of C7ORF41 expression. To further identify which PKC isoform is involved in C7ORF41 mediated keratinocyte differentiation, we first knocked down classic isoform PKCa using siRNA to evaluate its effect on the expression of C7ORF41 and differentiation markers. We found that silencing PKCa could reverse C7ORF41 expression and inhibit keratinocyte differentiation in TPA-induced Hacat cells (Fig.5E-I).

It is of great importance to clarify the underlying downstream pathway of PKC that mediates C7ORF41 reduction in the differentiation conditions. Because MAPK and NF-κB are well known downstream signaling pathways of PKC, which plays key roles in keratinocyte differentiation [15, 17], we next examined their effects on C7ORF41 expression in Hacat cells. Several inhibitors including PD98059 (MEK1/2 inhibitor), Sp600125 (JNK inhibitor) and SC-514 (IKK inhibitor) were added at 30 min before TPA treatment. We found that PD98059 could effectively reverse the TPA-induced downregulation of C7ORF41 (Fig.5J, 5M), which suggested that MEK/ERK signaling pathway may be involved in the regulation of C7ORF41. Interestingly, the expression of differentiation markers was dramatically decreased by PD98059 and SC-514; whereas SP600125 markedly increased KRT10/FLG expression (Fig.5L-N), which is consistent with previously studies [48, 49]. In order to further explore whether the MEK/ERK pathway is also involved in regulating the nuclear translocation of IKKα via C7ORF41, we silenced C7ORF41 using siRNA in PD98059-treated Hacat cells and measured the subcellular distribution of IKKα. As the results shown in Fig.5N, the silencing of C7ORF41 could rescue the inhibitory effect of PD98059 on IKKα nuclear translocation. Collectively, all these data suggested that the activation of PKCa/ERK pathway regulated the downregulation of C7ORF41 under the keratinocytes differentiation conditions.

4. Discussion
Skin is the largest organ of the body and composed of the dermis and epidermis. Keratinocytes constitute the stratified epidermis, and these cells gradually differentiate when they moved to the suprabasal layers and give rise to the cornified layer at the surface of the skin. Skin, as the first line to defense the outside pathogen, plays an important barrier role in protecting against harmful microorganisms, chemical insults and retaining body fluids. Therefore, maintaining a balance between keratinocyte differentiation and proliferation is necessary for epidermal homeostasis. However, aberrant keratinocyte differentiation would disrupt the skin barrier function and cause a series of skin diseases, such as ichthyosis, psoriasis and squamous skin cancer [50]. Although there exist some available therapeutics against these diseases, they are often ineffective and cause some side effects. Therefore, better knowledge of the molecular mechanisms of differentiation in keratinocytes would be useful in the treatment of poorly differentiated skin diseases.

In the present study, we investigated the role of C7ORF41 in the regulation of keratinocyte differentiation. We found C7ORF41 expression mainly localized in the basal layer and its expression gradually decreased in the supabasal layer (Fig.1A). Besides, we also found the expression of C7ORF41 significantly increased in hyperproliferative skin squamous cell carcinoma (SCC) (supplementary 1E). Our results showed that C7ORF41 overexpression inhibited the expression of differentiation markers and promoted the proliferation of keratinocytes, while C7ORF41 knockdown showed the opposite effects (Fig.2, Fig.3). These results suggest that C7ORF41 plays an essential role for basal keratinocytes to keep in the undifferentiated state and its reduction evokes differentiation to suprabasal layers. However, the precise mechanisms of C7ORF41 on keratinocyte function in the epidermis have not been fully elucidated. Numerous studies reported that IKKα is a critical regulator for keratinocyte differentiation as well as proliferation, which requires IKKα nuclear translocation but does not depend on its kinase activity [30, 31]. Our previous studies confirmed that C7ORF41 could interact with IKKα/β in the cytoplasm and regulate the activity of NF-κB [39, 40]. In this study, we found that the expression of IKKα but not IKKβ and IKKε increased in TPA-induced Hacat keratinocyte differentiation (supplementary Fig.1G-H), and demonstrated that C7ORF41 regulates the keratinocyte differentiation by influencing the nuclear translocation of IKKα (Fig.4). Studies showed IKKα could also accelerate the degradation of cyclin D1 during cell cycle regulation [51, 52], which to some extent explain the alterations of cyclin D1 in TPA-treated C7ORF41 overexpression or knockdown cells. Several studies also indicated that EGFR was important in regulating keratinocytes transitioning from proliferation to differentiation and the EGFR cascade activity could be inactivated by IKKα via repressing the expression of its ligands at the transcription level, which further promoted keratinocyte differentiation [53, 54]. Besides, NF-κB activation contributes to the growth arrest and terminal differentiation in stratified epithelium [55]. In this study, our results showed that the phosphorylation levels of NF-κB dramatically increased in TPA-treated Hacat cells (supplementary Fig.1H). We think the downregulation of C7ORF41 in keratinocytes reduced the interaction with IKKα/β complex in the
cytoplasm and the released IKKα/β complex exposed the kinase domain, which caused the nuclear translocation of IKKα and the activation of IKKβ. Altogether, all these factors promoted the keratinocyte differentiation.

PKC isoforms are also involved in keratinocyte proliferation and differentiation, and individual isoforms have differential effects, even opposite in action to others [21, 25]. In this study, we found that silencing PKCα partially rescued the downregulation of C7ORF41 in TPA-treated Hacat cells, which indicated that PKCα had an important role in the regulation of C7ORF41 expression in the keratinocyte differentiation. It has been reported that PKC stimulates keratinocyte differentiation via MAPK signaling pathway [7, 27, 56]. The activation of ERK or p38 MAPK increases AP1 (Jun and Fos), CEBP and SP1 transcription factors activity, which ultimately leads to keratinocyte differentiation. Besides, inhibition of JNK activity promotes differentiation of epidermal keratinocytes via increasing the expression of AP1 [7, 48].

Our previously study showed that C7ORF41 promoter contains two conserved AP1 binding sites and c-Jun could inhibit C7ORF41 gene transcription in NAFLD [39]. However, JNK pathway is not involved in Ca2+ -induced keratinocytes differentiation [57]. Our present study demonstrated that the downregulation of C7ORF41 during TPA-induced keratinocyte differentiation may be controlled by PKCα/ERK pathway, which acts via certain transcription factors to directly regulate the transcription of C7ORF41. However, more detailed situations are still needed to be further investigated.

In conclusion, we examined the expression profile and function of C7ORF41 in keratinocytes and hypothesized its function mechanism as shown in Fig.6. Ca+/TPA induced activation of PKCα/ERK signaling pathway decreased C7ORF41 expression and thus reduced the formation of C7ORF41/IKKα/β complex in the cytoplasm. The free IKKα hence translocated into the nuclear to regulate some differentiation or proliferation related genes expression, which finally induced the keratinocyte differentiation. Altogether, this study indicates the importance of PKCα/ERK/C7ORF41/IKKα axis in regulating keratinocyte differentiation, which provides novel theory evidence and potential therapeutic targets for skin diseases related to keratinocytes differentiation dysfunction.

Data Availability Statement
All supporting data are included within the main article and its supplementary files.

Conflict of interest
All authors declare no conflict of interest.

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**Figure legends**

**Fig.1.** C7ORF41 is downregulated during epidermal differentiation (A)

Immunohistochemical staining of normal human skin was performed to visualize C7ORF41 expression. Original magnification (×400), scale bars: 50 μM (B-D) qPCR analysis C7ORF41 (B), keratin10 (K10) (C) and filaggrin (FLG) (D) gene expression at mRNA level in Hacat cells that treated with 2.8 mM CaCl2 for indicated time. (E)

Protein level analysis C7ORF41, K10, FLG gene expression in above treated cells.

(F-I) Hacat cells were induced to differentiate by different dose of TPA for 24 h, C7ORF41 and differentiation marker expression at mRNA (F-H) and protein level were analyzed. β-actin was used as control. (J-M) C7ORF41, KRT10, FLG mRNA (J-L) and protein (M) levels in Hacat cells that were treated with 100 nM TPA at the indicated time points were assessed by real-time PCR and western blot, respectively. All data (mean ± SD) represent three independent experiments.*P<0.05, **P<0.01, ***P<0.001 vs. 0 day or 0 h group.

**Fig.2.** C7ORF41 negatively controls keratinocytes differentiation (A-B) RT-PCR (A) and western blot (B) were performed to analyze the expression of C7ORF41 in Hacat cells stably transfected with an empty vector (Ctrl) or plasmid encoding C7ORF41 fusing flag tag. ***P<0.001 versus control group. (C-E) Differentiation marker KRT10 and FLG expression were determined by Q-PCR (C-D) and western blot (E) in TPA-treated Hacat-C7ORF41 or control cells at the indicated time points. *P<0.05, **P<0.01 versus Ctrl group. (F-G) The knockdown efficiency of C7ORF41 was assessed at mRNA (F) and protein (G) levels in stably transfected with shRNA control vector (scramble) or targeting C7ORF41 (shC7ORF41-1# or shC7ORF41-2#).

**Fig.3.** C7ORF41 regulates cell proliferation by CyclinD1 (A-B) Cell proliferation of stable C7ORF41 overexpression or downregulation in Hacat cells were tested at TPA-treated time points using CCK-8 assay. *P<0.05, **P<0.01 versus Ctr or scramble group. (C-D) Cell cycle distributions were analyzed after TPA-treated for 48 h in Hacat cells that overexpressed or knocked down C7ORF41 using flow cytometry. The percentage of cell cycle in G1, S and G2/M phases were assessed using flowJo software. (E-H) Cyclin D1, C7ORF41 gene expression at mRNA levels (E, G) and protein levels (F, H) were analyzed in indicated cells. The expression of GAPDH gene was used as inner control. **P<0.01, ***P<0.001 versus control/scramble group.

**Fig.4.** C7ORF41 regulates the keratinocytes differentiation through IKKα nuclear translocation (A) Co-Immunoprecipitation assay was performed with antibody C7ORF41 and normal mouse IgG in Hacat cell lysates and ten percent of cell lysates was used as Input. The immunoprecipitates and the input samples were measured by western blot with anti-C7ORF41 and IKKα antibodies, respectively. (B) Plasmid encoding HA-IKKα was transfected into stable overexpressed C7ORF41.
Hacat cells, then treated with TPA for 24 h after 24 h of transfection. The treated cells were harvested for co-immunoprecipitation with HA antibody and the expression of C7ORF41 and IKKα were detected by blotting with flag and HA antibody. (C) Cytoplasm (C) or nuclear (N) extracts were separated from TPA-treated Hacat cells and the presence of C7ORF41 and IKKα were detected using western blot. (D) C7ORF41 and IKKα protein were measured in Cytoplasm or nucleus extracts from the indicated cells. LaminA/C and GAPDH served as control for nuclear protein or cytoplasm protein. (E) The interaction domains of IKKα with C7ORF41 were explored using truncated IKKα expression constructs and co-immunoprecipitation assays followed by western blotting. (F) si-IKKα or control siRNA were transfected into C7ORF41 knockdown Hacat cells and scramble cells as indicated for 24 h, and then TPA was add into these treated cells for additional 24 h. IKKα, C7ORF41, KRT10 and FLG protein expression were analyzed by western blot.

Fig. 5. C7ORF41 downregulation is mediated by PKC-ERK signaling pathway in keratinocytes (A-D) Hacat cells were pre-incubated for 1 h with GF109203X, G60976 and G60983 before stimulation with TPA. The expression of C7ORF41, KRT10 and FLG were determined at mRNA level (A-C) and protein level (D) by Q-PCR and western blot, respectively. (E-I) Hacat cells were transfected with si-PKCα or control siRNA for 24 h, and then treated with TPA for additional 24 h as indicated. The expression of PKCα, C7ORF41, KRT10 and FLG were analyzed by real time PCR (E-H) and western blot (I). **P<0.01 versus none-treated group; #P<0.05 versus TPA only treated group. (J) Hacat cells were treated with TPA for times as indicated, and the whole cell lysates were used for immunoblot analysis of phosphorylated and total ERK, MEK, JNK, p38, NF-κB, IKKα, IKKβ, IKKe. Equal protein loading was confirmed by GAPDH expression level. (K-N) Hacat cells were pre-treated with PD98059, SC514, SP600125 for 1 h followed by TPA as indicated for additional 24 h. The treated cells were harvested to analyze the expression of C7ORF41, KRT10 and FLG at mRNA and protein levels. Immunoblot analyzed the phosphorylation levels of ERK, JNK, NF-κB to assay the inhibition effect of these three inhibitors. GAPDH serves as loading controls. **p<0.01, ***P<0.001 versus none-treated group; #P<0.05, ##P<0.01 versus TPA/DMSO group.

Fig. 6. A proposed model for the regulation and function of C7ORF41 in epidermal homeostasis Schematic representation of epidermal development stages and the expression of C7ORF41 throughout keratinocyte differentiation (upper panel); schematic diagram illustrates a novel regulatory network that mediates keratinocyte differentiation: differentiation-inducing stimuli (calcium and TPA) activate ERK in a PKCα-dependent fashion, and lead to the transcriptional inhibition of C7ORF41. The downregulation of C7ORF41 enhanced the nuclear translocation of IKKα, which regulates the expression of some keratinocyte differentiation or proliferation related genes.
Figure 2

A

B

E

C

D

F

G

J

H

I

Relative C7ORF41 mRNA expression

Flag
C7ORF41
GAPDH

TPA

0 12 24 48 h

TPA

0 12 24 48 h

 Relative FLG mRNA expression

Ctrl
C7ORF41

Relative KRT10 mRNA expression

Relative C7ORF41 mRNA expression

scramble
shC7ORF41-1#
shC7ORF41-2#

C7ORF41
GAPDH

FLG
KRT10
GAPDH

0 12 24 48 h

scramble
shC7ORF41

FLG
KRT10
GAPDH

0 12 24 48 h

Relative FLG mRNA expression

Relative KRT10 mRNA expression

0 12 24 48 h

0 12 24 48 h

0 12 24 48 h

0 12 24 48 h

0 12 24 48 h

0 12 24 48 h

0 12 24 48 h

0 12 24 48 h
Figure 3

A

Cell counts (10^4)

0 24 48 72 96 h

C7ORF41-TPA

Ctrl-TPA

C7ORF41+TPA

Ctrl+TPA

B

Cell counts (10^4)

0 24 48 72 96 h

shC7ORF41-TPA

Ctrl-TPA

shC7ORF41+TPA

Ctrl+TPA

E

Relative CCND1 mRNA expression

C7ORF41-TPA

Ctrl-TPA

C7ORF41+TPA

Ctrl+TPA

F

Cyclin D1

C7ORF41

GAPDH

34KD

18KD

36KD

G

Relative CCND1 mRNA expression

shC7ORF41-TPA

scramble-TPA

shC7ORF41+TPA

scramble+TPA

34KD

18KD

36KD

H

Cyclin D1

C7ORF41

GAPDH

34KD

18KD

36KD
Figure 5

A

B

C

D

E

F

G
