Lipin-1 expression is critical for keratinocyte differentiation

Short title: Function of lipin-1 in keratinocyte differentiation

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**Abbreviations:** BCC, basal cell carcinoma; CDK, cyclin dependent kinase; DAB, 3,3’-Diaminobenzidine; DAG, diacylglycerol; GFP, green fluorescent protein; KC, keratinocyte; KD, knockdown; MARCKS, myristoylated alanine rich C kinase substrate; NHEK, normal human epidermal keratinocyte; PA, phosphatidic acid; PAP, phosphatidate phosphatase; PI, propidium iodide; PKC, protein kinase C; Rb, retinoblastoma; TAG, triacylglycerol; TPA, 12-O-tetradecanoylphorbol-13-acetate
Abstract

Lipin-1 is an Mg$^{2+}$-dependent phosphatidate phosphatase that facilitates the dephosphorylation of phosphatidic acid to generate diacylglycerol. Little is known about the expression and function of lipin-1 in normal human epidermal keratinocytes (NHEKs).

Here, we demonstrate that lipin-1 is present in basal and spinous layers of the normal human epidermis, and lipin-1 expression is gradually down-regulated during NHEK differentiation. Interestingly, lipin-1 knockdown inhibited keratinocyte differentiation and caused G1 arrest by up-regulating p21 expression. Cell cycle arrest by p21 is required for commitment of keratinocytes to differentiation, but must be down-regulated for the progress of keratinocyte differentiation. Therefore, reduced keratinocyte differentiation result from sustained up-regulation of p21 by lipin-1 knockdown. Lipin-1 knockdown also decreased the phosphorylation/activation of protein kinase C alpha (PKC$\alpha$), whereas lipin-1 overexpression increased PKC$\alpha$ phosphorylation. Treatment with PKC$\alpha$ inhibitors, like lipin-1 knockdown, stimulated p21 expression, while lipin-1 overexpression reduced p21 expression, implicating PKC$\alpha$ in lipin-1-induced regulation of p21 expression. Taken together, these results suggest that lipin-1-mediated down-regulation of p21 is critical for the progress of keratinocyte differentiation after the initial commitment of keratinocytes to differentiation induced by p21, and that PKC$\alpha$ is involved in p21 expression regulation by lipin-1.
Keywords
Lipin-1, PKCa, G1 arrest, p21, keratinocyte differentiation, keratin 1, keratin 10
**Introduction**

Lipin-1 was first described as the gene responsible for lipodystrophy in the fatty liver dystrophy mouse (1). The function of lipin-1 as a mammalian Mg\(^{2+}\)-dependent phosphatidate phosphatase (PAP) was revealed by the identification of Pah1p, a yeast homolog, as an Mg\(^{2+}\)-dependent phosphatidate phosphatase (2). Mechanistic target of rapamycin complex-1 (mTORC-1) phosphorylates lipin-1 to regulate its subcellular localization and function (3-5). In the cytoplasm, lipin-1 is involved in the biosynthesis of triacylglycerol (TAG) and phospholipids by catalyzing the dephosphorylation of phosphatidic acid (PA) to produce diacylglycerol (DAG) at the endoplasmic reticulum (ER) membrane (6). In the nucleus, lipin-1 acts as a transcriptional co-activator to enhance fatty acid oxidation by increasing the expression of peroxisome proliferator activated receptor alpha (PPAR\(\alpha\)) in cooperation with PPAR-\(\gamma\) coactivator 1 alpha (PGC-1\(\alpha\)) (6, 7). Through these functions, lipin-1 and its orthologs are involved in various cellular events such as adipogenesis, lipid metabolism, mitochondrial morphology, vacuole fusion, and autophagy in diverse species (8-10). Although much is known about the role of lipin-1 in various cell types, little is known about lipin-1 in keratinocytes.

DAG functions as a lipid second messenger and activates a signaling cascade involving protein kinase C (PKC). The PKC family consists of at least 11 members, of which only 5 (\(\alpha\), \(\delta\), \(\epsilon\), \(\eta\), \(\zeta\)) are found in human keratinocytes (11-13). Classical PKC\(\alpha\) is calcium- and DAG-dependent, while the novel PKCs \(\delta\), \(\epsilon\), and \(\eta\) only respond to DAG; activation of the atypical PKC\(\zeta\) requires neither DAG nor calcium. PKC levels and intracellular distribution change during keratinocyte differentiation (14). PKC\(\mu\)/PKD is classified in a distinct kinase subfamily due to lack of significant homology to the PKC catalytic domains and also expressed in human keratinocytes (15). PKC activity is required for growth and differentiation of normal keratinocytes (16-19).
Accordingly, alterations in PKC signaling are fundamental to the pathogenesis of benign skin diseases such as hyperproliferative, inflammatory, and neoplastic lesions (20-22).

The cyclin-dependent kinase (CDK) inhibitor, p21, suppresses the cyclin-dependent kinases, cyclinE/CDK2, and cyclinD/CDK4, thus inhibiting phosphorylation of retinoblastoma (Rb) protein to control cell cycle arrest, cell differentiation, or senescence (23-27). p21 controls the transition of cells from G1 to S phase of the cell cycle by inhibiting cyclin activity at the G1 checkpoint (28). Induction of p21 in early differentiation is necessary to initiate keratinocyte differentiation (29, 30). However, because expression of terminal differentiation markers is attenuated by sustained overexpression of p21 independent of the cell cycle, p21 expression must be down-regulated at later stages of differentiation (31). Therefore, tight regulation of p21 expression is critical for the process of keratinocyte differentiation (32, 33).

In this study, we investigated the expression and function of lipin-1 in normal human epidermal keratinocytes (NHEKs). We show that lipin-1 is present in basal and spinous layers of normal human epidermis, and lipin-1 expression is gradually down-regulated during NHEK differentiation. We also demonstrate that lipin-1 knockdown (KD) causes G1 arrest associated with p21 up-regulation, which is known to be necessary for initial commitment of keratinocytes to differentiation. However, interestingly, lipin-1 KD suppressed keratinocyte differentiation. We demonstrate that the reduced keratinocyte differentiation was due to the sustained expression of p21 driven by lipin-1 KD, mediated by inhibition of PKCα activation.
Results

Lipin-1 is down-regulated during epidermal differentiation

To understand the physiological role of lipin-1 in keratinocytes, lipin-1 protein level was measured during proliferation and differentiation. NHEKs were cultured in low calcium KGM-Gold medium up to 90% confluence (day 0). To study the regulation of lipin-1 protein expression by keratinocyte differentiation, NHEKs were cultured for an additional 7 days (day 7) under either low calcium (50 μM) or high calcium (1.2 mM) conditions. Western blot analysis showed that lipin-1 was strongly down-regulated regardless of calcium concentration (Fig. 1A-B) and b), corresponding to an increase in expression of involucrin, a marker for differentiation (29, 31). A previous report demonstrated that PKC activation mediates human keratinocyte differentiation at high cell densities, independent of changes in extracellular calcium (34), which may be associated with similar patterns of lipin-1 expression in low and high calcium conditions. The data indicate that lipin-1 expression is gradually decreased during keratinocyte differentiation. To assess the expression pattern and localization of lipin-1 in human epidermis, immunostaining was performed for lipin-1 in human foreskin sections. Lipin-1 was largely detected in both the basal layer and the spinous layer (Fig. 1C). Lipin-1 expression appeared to be purplish brown as a result of brown DAB chromogen with blue hematoxylin. These data suggest that lipin-1 may play a role in keratinocyte proliferation or early differentiation.

Lipin-1 regulates cell cycle progression by regulating p53/p21

Because lipin-1 is predominantly expressed in proliferating keratinocytes, its effect on proliferation was examined by cell cycle analysis using propidium iodide (PI) staining. After transfection with control siRNA or Lipin-1 siRNA, NHEKs were subjected to serum deprivation
for 16 h and DNA content was analyzed by FACS. The DNA histogram revealed that the proportion of G1 phase was increased in a serum free medium compared with control (43.03 % ± 0.34 vs 56.07 % ± 0.24) (see supplementary Fig. I). FACS analysis revealed that lipin-1 siRNA induced G1 cell-cycle arrest (56.00 % ± 0.38) 24 h after transfection compared with control siRNA (50.77 % ± 0.57; Fig. 2A). Analysis of proteins regulating the progression through the G1 phase of the cell cycle showed a strong increase in the levels of p21 and p53, but not p16 (Fig. 2C). Activation of p21 signaling is an important mechanism to prevent G1 cells from entering the S phase. p21 is activated by p53-dependent or p53-independent mechanisms (35). Thus, we predict that lipin-1 KD results in increased p53 level, which in turn induces its downstream target p21 expression and G1 arrest in NHEKs. Taken together, lipin-1 may be required for the progression of G1 phase in the cell cycle through regulating p53/p21 expression.

**DAG content and PKCα activity are regulated by lipin-1 in NHEKs**

To confirm DAG formation via dephosphorylation of PA by lipin-1 in keratinocytes, like in hepatocytes and adipocytes (1, 8, 9), PA and DAG contents were assayed in NHEKs transfected with lipin-1 or control siRNA. As expected, lipin-1 KD resulted in elevated PA and reduced DAG levels compared with control (Fig. 3A). The data indicated that lipin-1 is involved in DAG formation in NHEKs (supplementary Fig. 2). PKC activation by DAG controls the proliferation of keratinocytes (36, 37). To assess whether altered DAG contents led to impaired PKC activation, we assessed the phosphorylation of PKC substrates. Phosphorylation of PKC substrates and myristoylated alanine-rich C kinase substrate (MARCKS), a major PKC substrate, was inhibited by lipin-1 KD (Fig. 3B-E). Phosphorylation of five PKC isoforms expressed in
keratinocytes (11) was also analyzed by western blot. Most PKC isoforms exhibited no significant change in phosphorylation levels in lipin-1 KD cells compared with control, but PKCα phosphorylation at Ser657 was inhibited by lipin-1 KD (Fig. 4A). To confirm the inhibition of PKCα activation, we demonstrated that lipin-1 KD decreased phosphorylation at another PKCα autophosphorylation site (Thr638) (Fig. 4C). We transfected vectors containing lipin-1 cDNA or pCMV-AC empty vector as a negative control to assess the effect of exogenous expression of lipin-1. Western blot analysis indicated that overexpression of lipin-1 enhances PKCα phosphorylation (Fig. 4E). These results indicate that lipin-1-catalyzed DAG formation is associated with the activation of PKC, specifically PKCα, in NHEKs.

**Lipin-1-induced PKCα activation inhibits p21 expression**

It has been reported that PKC down-regulation mediated growth arrest is associated with p21 induction in fibroblasts (38). Thus, to confirm that inhibition of PKCα is associated with up-regulation of p21 expression in NHEKs, cells transfected with lipin-1 or control siRNA were treated with Go6976 (PKCα/β inhibitor) or safingol (PKCα inhibitor/sphingosine kinase 1 inhibitor). Go6976 treatment stimulated p21 expression in both lipin-1 siRNA and control siRNA transfected cells (Fig. 5A). Although safingol lack specificity to PKCα due to its activity of sphingosine kinase 1 inhibitor (39), safingol treatment also increased p21 expression in both lipin-1 siRNA and control siRNA transfected cells but the increase was less pronounced compared to Go6976 treatment. These data suggest that PKCα inhibition is associated with the stimulation of p21 expression in NHEKs. Therefore, PKCα inactivation by lipin-1 siRNA may be associated with elevated p21 in NHEKs. To further confirm the effect of lipin-1 on p21 expression, NHEKs transfected with lipin-1 vector or pCMV-AC empty vector were analyzed by
western blot. Lipin-1 overexpression induced PKCα phosphorylation/activation (Fig. 4E) and reduced p21 expression in both Go6976-treated and untreated cells (Fig. 5C). Collectively, these results suggest that lipin-1-induced PKCα activation inhibits p21 expression in NHEKs.

**Lipin-1 down-regulation inhibits keratinocyte differentiation by sustaining p21 induction.**

We investigated whether the cell cycle arrest linked to up-regulation of p21 by lipin-1 KD affects keratinocyte differentiation. NHEKs were transfected with lipin-1 or control siRNA for 24 h (day 1) and differentiation marker expression was assessed 4 days post-transfection by western blot. Inhibition of lipin-1 expression was observed in lipin-1 KD cells on day 4, indicating that lipin-1 siRNA was effective at least 4 days after transfection. Surprisingly, the differentiation markers, keratin 1 and keratin 10, were reduced in lipin-1 KD cells compared with control (Fig. 6A). Previous studies demonstrated that elevated expression of p21 is observed at the induction stage of differentiation, while sustained up-regulation of p21 expression inhibits keratinocyte differentiation. Therefore, elevated p21 expression must be down-regulated at a later differentiation stage (31). Thus, we examined whether lipin-1 KD affects tight regulation of p21 during early differentiation (0, 1, 2, 3, and 4 days). On the transfection day (0 day), cell confluency was approximately 60% to conduct siRNA transfection. Lipin-1 expression was increased (day 2) before reaching confluence, but suppressed when the cells began to differentiate (Fig. 6C). The data reflect lipin-1 expression peaks when cell reaches full confluence and is gradually decreased by cell differentiation as observed in Fig. 1A. Our data showed that p21 expression was induced on day 1, maintained until day 3, and then down-regulated on day 4 in both control and lipin-1 KD cells. Our data also showed that the expression of p21 was sustained at a higher level in lipin-1 KD cells compared with control. In addition,
keratin 1 and keratin 10 began to express later in lipin-1 KD cells compared to control. These results imply that sustained p21 up-regulation resulted from lipin-1 KD may lead to the inhibition of the differentiation markers, keratin 1 and keratin 10.

To verify the effect of lipin-1 KD-induced p21 expression on keratinocyte differentiation, NHEKs were knocked down for p21, lipin-1 or both genes and incubated for 4 days. p21 KD showed more pronounced inhibition of keratin 1 and keratin 10 expression compared to lipin-1 KD (Fig. 6E). Previous study demonstrated that p21 null keratinocytes show lower expression level of keratin 1 compared to wild-type keratinocytes (29). While lipin-1 KD inhibited the expression level of keratin 1 and keratin 10 as shown in previous data (Fig. 6A-D), simultaneous KD of both p21 and lipin-1 restored not only the levels of p21, but also the levels of keratin 1 and keratin 10 to almost the same level as control, indicating that lipin-1 KD-induced p21 contributes to the inhibition of keratin 1 and keratin 10 expression.
Discussion

Lipin-1 catalyzes the formation of DAG by dephosphorylating PA. The DAG generated from the lipin-1-mediated reaction is utilized for the formation of TAG, as well as the phospholipids phosphatidyl-choline (PC), phosphatidyl-ethanolamine (PE) and phosphatidyl-serine (PS) (9, 40). In addition, both substrates and products have functions in lipid signaling. PA is involved in transcription, activation of cell growth, membrane proliferation, secretion, and vesicular trafficking, while DAG is known to be involved in the activation of PKC (9). In keratinocytes, TAG is a minor lipid species in lamellar bodies (41, 42) and thus, epidermal TAG metabolism has not been focused in the past (43). However, diacylglycerol acyltransferase-2 (DGAT-2)-deficient mice lacking the enzyme catalyzing the final reaction in TAG synthesis die shortly after birth due to abnormal energy metabolism and impaired skin permeability barrier function (44). Mice deficient in comparative gene identification-58 (CGI-58), a lipid droplet-associated protein that controls intracellular TAG levels by activating adipose triglyceride lipase (ATGL), develop a severe permeability barrier defect (41). These reports demonstrate that efficient inter-conversion between TAG and DAG is required for permeability barrier homeostasis in epidermis. Given the critical role of lipin-1 in formation of DAG and subsequent synthesis of TAG and signaling lipids, in this study, we investigated the role of lipin-1 in epidermis. We found that lipin-1 expression gradually decreases during differentiation of NHEKs (Fig. 1A-B). Immunostaining of skin tissue showed that lipin-localizes in basal and spinous layers of human epidermis (Fig. 1C). The localization of lipin-1 was somewhat surprising because TAG synthesis reportedly increases during keratinocyte differentiation (45). These results suggest that lipin-1 functions in proliferating NHEKs rather than increasing TAG synthesis during differentiation. In this study, we show that PKCα activation is regulated by
lipin-1 in NHEKs. PKCα is expressed and activated in the first basal or lower spinous layers in human epidermis (37). These results suggest that lipin-1 may be involved in PKCα activation in the basal and spinous layers to control proliferation or induce early differentiation of NHEKs.

Because lipin-1 KD caused G1 arrest linked to p21 induction, the relationship between lipin-1 and p21 was investigated by analyzing DAG content and PKC activation. Several studies demonstrated that activation of PKC isoforms is regulated by DAG converted by lipin-1 in various cell types. For example, activation of the PKCμ-Vps34 signaling cascade is dependent on lipin-1 in skeletal muscle (10). In addition, PKCε activity requires lipin-1 in liver (46). We observed that DAG formation and PKCα activation were inhibited by lipin-1 KD in NHEKs (Fig. 3). PKC activation with TPA, a PKC agonist, triggers an irreversible growth arrest, which is accompanied by the induction of p21 in normal keratinocytes (47, 48). Consistent with TPA treatment, overexpression of PKCδ and -α in keratinocytes prevents proliferation by a mechanism associated with elevated p21 expression (36, 37). On the contrary, we found that lipin-1-KD-induced suppression and lipin-1-induced activation of PKCα are associated with induction and inhibition of p21 expression, respectively, in NHEKs (Fig. 5). The effect of PKCα activation on cell cycle progression is somewhat controversial, and depends on cell type and strength/duration of stimuli. In intestinal epithelial cells, PKCα overexpression and TPA treatment-induced G1 arrest are linked to activation of the extracellular signaling cascade, Rb hypophosphorylation, induction of CDK inhibitors p21/p27, and inhibition of cyclin D1 (49, 50). In addition, sustained extracellular signal-regulated kinase activation by phorbol 12-myristate 13-acetate (PMA)-induced PKC/PKCα stimulation results in growth arrest and differentiation, while transient activation of extracellular signal-regulated kinase cascades with serum leads to cell cycle progression in these cells (51). On the other hand, similar to our results, PKCα and
PKCθ suppression induces p21 expression and growth arrest in Swiss3T3 fibroblast cells (38). In our study, inhibition of lipin-1 expression by targeting lipin-1 siRNA and consequent up-regulation of p21 lasted for at least 4 days after transfection (Fig. 6) while PKCα overexpression transiently induced PKCα and p21 expression (37). Therefore, the discrepancy of the effect of PKCα activation/inhibition on p21 may be attributed to differences in duration/magnitude of stimuli. Also, difference in phosphorylation status of PKCα might affect the level of p21 expression.

Earlier work has demonstrated the complexity of p21 function in keratinocyte differentiation. Induction of p21 expression is one of the earliest cell cycle regulatory events contributing to differentiation-associated growth arrest (29, 30). In addition, increased p21 expression must be down-regulated by proteasome-mediated degradation for the later stages of differentiation; otherwise, sustained p21 expression blocks the expression of differentiation markers including keratin 1, loricrin, involucrin, and filaggrin (31). In our study, p21 induction caused by lipin-1 KD was sustained during keratinocyte differentiation stages, resulting in delayed and insufficient down-regulation of p21 and consequently reduced expression of early differentiation markers compared with control. A study of atopic eczema (AE) accompanied with impaired epidermal differentiation indicates lipin-1 down-regulation in AE skin, suggesting lipin-1 inhibition may play a role in pathogenesis of AE (52). Relationship of lipin-1 down-regulation on abnormal differentiation in AE skin should be further investigated.

In summary, lipin-1 is required for production of DAG and activation of PKCα signaling, which is associated with down-regulation of p21 expression, in NHEKs (Fig. 7). Lipin-1 KD caused G1 arrest associated with p21 induction, which is necessary to initiate keratinocyte differentiation. Lipin-1 KD also suppressed the expression of keratin 1 and keratin 10 due to
delayed down-regulation of p21, which is critical for proper progress of keratinocyte differentiation after the initial commitment of keratinocytes to differentiation induced by p21. While further studies are required to verify involvement of lipin-1 in various PKCα-downstream signaling pathways in keratinocytes, our study provides fundamental insights into the function of lipin-1 and the importance of lipin-1-mediated down-regulation of p21 in differentiation of NHEKs.
Conflict of Interest

The authors state no conflicts of interest.
Materials & Methods

Cell culture

NHEKs from neonatal foreskin were purchased from Lonza (Basel, Switzerland) and cultured in KBM-GOLD medium with KGM-GOLD growth supplements containing insulin, human epidermal growth factor, bovine pituitary extract, hydrocortisone, epinephrine, transferrin, and gentamicin/amphotericin B. The cells were serially passaged at 70-80% confluence no more than 3 times.

Immunostaining of tissue section

Normal human skin purchased from Biochain (Hayward, CA, USA) was incubated with polyclonal rabbit anti-lipin-1 antibody (Sigma, St Louis, MO, USA) for 40 min at room temperature. Anti-rabbit HRP conjugated antibody (ab6802. Abcam Inc., Cambridge, MA, USA) was then applied as the secondary antibody for 20 min. The secondary antibody was detected with using chromogenic substrate 3,3'-Diaminobenzidine (DAB). Immunoreactivity was evaluated under a light microscope (BX-53, Olympus, Tokyo, Japan) and photomicrographs were taken using a digital camera (DP72, Olympus, Tokyo, Japan).

Plasmid construction

The human full-length lipin-1 cDNA (isoform 1α, accession number NM_145963) cloned into the pCMV6-AC-GFP vector was obtained from Origene (Rockville, MD, USA). To generate lipin-1β isoform (accession number NM_001261428), lipin-1 fragment 1 (F1) was amplified by PCR from the genomic DNA template (Forward, 5’-GAGGCAGACAGCACCATACA-3’; Reverse, 5’-TCAATGGGGCTGGACTCTTT-3’). The
DNA template was extended by 4 successive PCR reactions with 1st primers (Forward, 5’-TGGATTCCAATGTATGGTCTTCAATTTAACGTGCAGCACCATCGAATTACGTGGGCGAG-3’; Reverse, 5’-CTGCAAGATAGGAGGGCAGTCTTTTGCAATCTACCAGGCTACTGGAGGTGGGTGACCACCTC-3’), 2nd primers (Forward, 5’-GACTCGGCTTGGTATGGGACCAGATTCCCAATAATGAGAGACCTGGGATGGTGGAATACGTGGGAGCGAG-3’; Reverse, 5’-GTGGAGGGCAAGAATGACCCTCTCCCGGGCAGCAACTGCAAGATGAGGGGCGAG-3’), 3rd primers (Forward, 5’-CGCAGCTCCAGCTGGAGACCTCCAGGGCAAGAGCTCCCAGACTGGGCTTGGTATGGGACCAGATTCCCAATAATGAGAGACCTGGGATGGTGGAATACGTGGGAGCGAG-3’; Reverse, 5’-GAACCGGAAGGACTTTTCCGAAGGATGGAACAGGGAAGACTGACGGCAAGAAGAAGACTGTGGGAGGGCAAGAACTAG-3’), and 4th primers (Forward, 5’-AGATCTCGCCGCCGATCGCCATGGGGGAACAGGACGGCATTCGCAGCTCCCAGCTCAGGTGGGAGG-3’; Reverse, 5’-GAACCGGAAGGACTTTTCCGAAGGATGGAACAGGGAAGACTGACGGCAAGAAGAAGACTGTGGGAGGGCAAGAACTAG-3’). The extended PCR product was amplified using primers with added restriction sites (Forward, 5’-AGATCTGCGCCGCCGATCGCCATGGGGGAACAGGACGGCATTCGCAGCTCCCAGCTCAGGTGGGAGG-3’; Reverse, 5’-GGGCGCGCGTACGGTGTACAGGGAAGACTGTGGGAGGGCAAGAACTAG-3’), and the resultant lipin-1 fragment (F2) was cloned into the pCMV6-AC-GFP vector at Sgf I/Mlu I sites using the In-Fusion HD cloning kit (Clontech, Mountain view, CA, USA). Lipin-1 fragment 3 (F3) was amplified by PCR from the genomic DNA template (Forward, 5’-GAGGCAGACAGCACCATA-3’; Reverse, 5’-TCAATGGGCTGGACTCCTTTC-3’) and the resultant fragment was cloned into the pCMV6-AC vector by inserting F2 at Hpa I/Mlu I sites using the In-Fusion HD cloning kit.

**Western blot analysis**

To prepare total cell lysate, NHEKs were washed with ice-cold PBS and lysed in RIPA
buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) in the presence of protease and phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA). The lysate was then centrifuged at 15,000×g for 20 min, and the supernatant was used for analysis. Protein concentration was determined using a BCA kit (Sigma) with bovine serum albumin (BSA) as the standard. Equal protein (40 μg/well) from cell lysates was loaded and separated by 8-12% gradient SDS-PAGE and transferred onto PVDF membrane. Membranes were blocked in 3% BSA in TBST (20 mM Tris-HCl, pH 8.5, 150 mM NaCl, 0.5% Tween) at room temperature for 30 min. Blots were incubated at 4°C with anti-lipin-1 antibody (R&D Systems, Minneapolis, MN, USA), and anti-cytokeratin 5, -cytokeratin 14, -cytokeratin 1, -cytokeratin 10 (Covance, Princeton, NJ, USA) and anti-β actin, -GAPDH, -p21, -phospho MARCKS (Santa Cruz, Santa Cruz, CA, USA) and anti-phospho PKC-α (Ser657), -phospho PKC-α (Thr638), -phospho PKC-ε (Ser729), -aquaporin 3, -involucrin, (Abcam, Cambridge, UK), and anti-phospho PKC-δ (Thr505), -phospho PKC-η (Ser674), -phospho PKC-ζ (Thr410), -phospho-serine PKC substrate, -MARCKS (Cell Signaling, Boston, MA, USA) antibodies overnight in 3% BSA in TBST. Membranes were washed three times for 15 min in TBST followed by incubation with the appropriate horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-goat IgG secondary antibodies (Bio-Rad, Hercules, CA, USA) for 1 h at room temperature. Membranes were washed and visualized with enhanced chemiluminescent reagent (ECL) immunofluorescence staining (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Image analysis of immunoblots was performed using ImageQuant TL software (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

**Knockdown or overexpression**
Predesigned ON-TARGET plus human siRNA against lipin-1(#L01742701), p21(#L00347100) and non-targeting pool siRNA (#D-001810-10) were purchased from Dharmacon (Lafayette, CO, USA). NHEKs were plated 24 h before transfection and then transfected by lipofection using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) and OPTI-MEM (Invitrogen, Carlsbad, CA, USA) with siRNA at a final concentration of 25 nM for 6 h. The medium was then changed to KGM-Gold containing all appropriate supplements. For lipin-1 overexpression, NHEKs were seeded 24 h before transfection and then transfected with 1 μg/ml of plasmid using the X-tremeGENE HP DNA transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. We used a 1.5:1 ratio of μl of X-tremeGENE HP DNA transfection reagent to μg of pCMV6-AC-GFP vector as a transfection control.

Cell cycle analysis

After cells were transfected with siRNAs, complete medium was replaced with serum free medium for 16 h. The cells were centrifuged, washed in PBS, and fixed in 70% ethanol overnight at −20°C. After washing twice in PBS, the cells were stained in 0.5 ml of propidium iodide (PI)/RNase solution (BD BioScience, San Jose, CA, USA) for 15 min. Cell cycle distribution was analyzed using FACS Calibur (BD Bioscience). Ten-thousand events were counted during data collection. The percentage of cells in G1, S, G2/M phase was determined using ModFit LT curve fitting software (Verity software, Topsham, ME, USA).

Lipid analysis

Lipids were extracted by a modification of the Bligh and Dyer method (53). Briefly,
keratinocyte cells were collected by centrifugation at 1,500 × g for 5 min and suspended in 0.5 ml of cold PBS buffer, pH 7.4 followed by sonication. Pellet was extracted with 1.5 ml of methanol, 2.25 ml of 1 M of sodium chloride and 2.5 ml of chloroform and the phase was separated by centrifugation at 1,500 × g for 5 min. The lower phase was dried and re-dissolved in 500 μl of 1% Triton X-100 for PA analysis or 100 μl of chloroform for DAG analysis. PA was measured with the Total Phosphatidic Acid Fluorometric Assay Kit (Cayman, Ann Arbor, MI, USA)(10). For the DAG quantification, extracted lipids in chloroform were spotted on silica 60 TLC plate (Sigma, St Louis, MO, USA)(54). DAG was migrated with diethylether/heptane/acetic acid (75:25:1 v:v:v) mixture, the plate were dried and stained with 0.003% Comassie brilliant blue in 30% methanol and 10 mM sodium chloride for 30 min. The plates were de-stained for 5 min in dyefree solution and the band density was calculated using ImageQuant TL software (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The amount of DAG was calculated using a standard curve of 1,2-dioleyl-sn-diacylglycerol (Avanti, Alabaster, AL, USA)

**Statistical Analysis**

Statistical comparisons were performed using Student’s t-test between two groups or one-way ANOVA test within multiple groups, followed by Turkey’s post-hoc test. All measurements were obtained from at least three independent experiments, and values are expressed as the means ± SD.
REFERENCES


34. Lee, Y. S., S. H. Yuspa, and A. A. Dlugosz. 1998. Differentiation of cultured human epidermal keratinocytes at high cell densities is mediated by endogenous activation of the protein


Figure Legends

Fig. 1. Lipin-1 expression is repressed during keratinocyte differentiation.
Cell lysates of normal human epidermal keratinocytes (NHEKs) at each differentiation day (1, 3, 5 and 7 days) under low calcium concentration (0.5 μM) (A) or high calcium concentration (1.2 mM) (B) were subjected to immunoblot analysis to assess lipin-1 expression levels. Involucrin was also analyzed as a differentiation marker. C: Immunoperoxidase staining of normal human foreskin was performed to visualize lipin-1 localization. Negative control was done by using normal rabbit IgG. Scale bar is 40μm. These data are representative of three independent experiments.

Fig. 2. Lipin-1 knockdown induces G1 arrest via p53/ p21 induction.
A-B: NHEKs were transfected with lipin-1 siRNA or control siRNA, and cell cycle distributions were analyzed at 24 h post-transfection following serum starvation for 16 h using flow cytometry. Curve fitting analysis was performed to compare the distribution of lipin-1-KD or control NHEKs in G1, S, and G2/M phases. C-D: NHEK cell lysates prepared after lipin-1 siRNA or control siRNA transfection for 24 h were subjected to western blot analysis for p53, p21, and p16. Protein levels of p53, p21 and p16 were normalized to GAPDH. All data (mean ± SD) represent three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

Fig. 3. Lipin-1 knockdown inhibits DAG production and phosphorylation of PKC substrates.
A: After transfection of lipin-1 siRNA or control siRNA in NHEKs for 24 h, total lipids were extracted from the cells and analyzed biochemically to quantify PA and DAG. B-C: Cell lysates
after transfection of lipin-1 siRNA or control siRNA in NHEKs for 24 h were immunoblotted to assess phosphorylation of PKC substrates using anti-phospho-serine PKC substrate antibody and the level of phosphorylation of PKC substrates normalized to GAPDH was expressed as mean ± SD for three independent experiments. D-E: MARCKS was also detected using anti-phospho MARCKS and anti-MARCKs antibodies and the level of phospho-MARCKS normalized to MARCKS was expressed as mean ± SD for three independent experiments. *p < 0.05, **p < 0.001 versus control.

Fig. 4. Lipin-1 regulates PKCα activity.
A-B: Cell lysates after transfection of lipin-1 siRNA or control siRNA in NHEKs for 24 h were immunoblotted to assess phosphorylation levels of protein kinase C (PKC) isoforms with indicated antibodies to PKC isoforms. Protein levels of PKC isoforms normalized to GAPDH were expressed as mean ± SD for three independent experiments. C-D: The cell lysates were also detected by phospho-PKCα (Thr638) or PKCα antibodies. The level of phospho-PKCα (Thr638) normalized to PKCα were expressed as mean ± SD for three independent experiments. E-F: NHEK cell lysates transfected overnight with vector containing lipin-1 cDNA or pCMV-AC empty vector were subjected to western blot analysis for phospho-PKCα (Ser657) and total PKCα. The level of phospho-PKCα (Ser657) normalized to PKCα were expressed as mean ± SD for three independent experiments. *p < 0.05, **p < 0.01 versus control.

Fig. 5. Lipin-1-induced PKCα activation inhibits p21 expression.
A-B: NHEKs transfected with lipin-1 siRNA or control siRNA for 24 h were treated with Go6976 (500 nM) or safingol (10 μM) overnight. Cells were lysed and analyzed for p21
expression. The p21 levels were normalized to GAPDH. C-D: NHEKs transfected overnight with vector containing lipin-1 cDNA or pCMV-AC empty vector were treated with Go6976 (500 nM) for 4 h. Cells lysates were immunoblotted to assess p21 expression. The p21 levels were normalized to GAPDH. All data (mean ± SD) represent three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

Fig. 6. Lipin-1 down-regulation inhibits keratinocyte differentiation by sustaining p21 induction.

NHEKs transfected with lipin-1 siRNA or control siRNA for 24 h were incubated in KGM-Gold media. A-B: Cells were harvested after 4 days and cell extracts were analyzed by immunoblotting with antibodies specific for the indicated differentiation markers. Protein levels of keratin5, keratin14, keratin1, keratin10, aquaporin 3 (glycosylated (upper band) and unglycosylated (lower band) forms) and involucrin were normalized to GAPDH. C-D: Cells were harvested at the indicated time points (0, 1, 2, 3, and 4 day). Cell lysates were subjected to immunoblot analysis for lipin-1, keratin 1, keratin 10, and p21. The p21 level was normalized to GAPDH. E-F: NHEKs transfected with either control siRNA, p21 siRNA, lipin-1 siRNA or both siRNAs were incubated in KGM-Gold media for 4 days. Cells were lysed and analyzed for p21, keratin1 and keratin 10. Protein levels of p21, keratin1 and keratin10 were normalized to GAPDH. All data (mean ± SD) represent three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

Fig. 7. Proposed role of lipin-1 in regulating the expression of differentiation markers in keratinocytes. Lipin-1 KD down-regulates DAG production in NHEKs. This inhibition
deactivates PKCa, leading to promote p21 expression. When lipin-1 KD-induced p21 upregulation is sustained, the expression of keratin 1 and keratin 10 is suppressed, resulting in delayed keratinocyte differentiation.
Fig. 1

A

Day of culture (50 μM calcium)

B

Day of culture (1.2 mM calcium)

C

Control IgG

Lipin-1

stratum corneum
stratum granulosum
stratum spinosum
stratum basale
Fig. 2

A  siControl

B

C

D

Normalized protein levels to GAPDH

siControl  siLipin-1

*** ** * ** ***

G1 G1

G2/M G2/M

S S

Lipin-1 p53 p21 p16

G1 S G2/M
Fig. 3

A

![Graph](image1)

B

![Image](image2)

C

![Graph](image3)

D

![Image](image4)

E

![Graph](image5)
Fig. 4

A

\[
\begin{align*}
\text{siControl} & \quad \text{siLipin-1} \\
\text{Lipin-1} & \\
P-\text{PKC} \alpha \text{ (Ser657)} & \\
P-\text{PKC} \delta \text{ (Thr505)} & \\
P-\text{PKC} \eta \text{ (Ser674)} & \\
P-\text{PKC} \epsilon \text{ (Ser729)} & \\
P-\text{PKC} \zeta \text{ (Thr410)} & \\
P-\text{PKC} \mu \text{ (Ser744/748)} & \\
\text{PKC} \alpha & \\
\text{GAPDH} & 
\end{align*}
\]

B

Normalized protein levels to GAPDH

C

\[
\begin{align*}
\text{siControl} & \quad \text{siLipin-1} \\
\text{Lipin-1} & \\
P-\text{PKC} \alpha \text{ (Thr638)} & \\
\text{PKC} \alpha & 
\end{align*}
\]

D

Normalized P-PKC\alpha (Thr638) level to PKC\alpha

E

\[
\begin{align*}
\text{Empty vector} & \quad \text{Lipin-1 vector} \\
\text{Lipin-1} & \\
P-\text{PKC} \alpha \text{ (Ser657)} & \\
\text{PKC} \alpha & 
\end{align*}
\]

F

Normalized P-PKC\alpha level to PKC\alpha
Fig. 5

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lipin-1 siRNA</th>
<th>Go6976</th>
<th>Safingol</th>
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<tbody>
<tr>
<td>Control siRNA</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lipin-1 siRNA</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control siRNA + Go6976</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control siRNA + Safingol</td>
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<td>-</td>
<td>+</td>
</tr>
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</table>

B

![Normalized p21 level to GAPDH](image)

C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lipin-1 vector</th>
<th>Go6976</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vector</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lipin-1 vector</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

D

![Normalized protein levels to GAPDH](image)
Fig. 6

A

D0 D1 D2 D3 D4 D0 D1 D2 D3 D4
siControl siLipin-1

B

Normalized protein level to GAPDH

siControl siLipin-1

C

siControl siLipin-1

D

**
***

E

F

siControl siLipin-1 siLipin-1+sип21

GAPDH
Fig. 7

PA $\uparrow$

Lipin-1 $\downarrow$ $\downarrow$

DAG $\downarrow$

$\downarrow$

PKC$\alpha$ $\downarrow$

$\downarrow$

p21 $\uparrow$

$\downarrow$

$\downarrow$

Keratin1, Keratin10 $\downarrow$