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Protein kinase D1 deficiency promotes differentiation in epidermal keratinocytes

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SUMMARY

Background: Protein kinase D (PKD or PKD1) is a serine/threonine protein kinase that has been shown to play a role in a variety of cellular processes; however, the function of PKD1 in the skin has not been fully investigated. The balance between proliferation and differentiation processes in the predominant cells of the epidermis, the keratinocytes, is essential for normal skin function.
Objective: To investigate the effect of PKD1 deficiency on proliferation and differentiation of epidermal keratinocytes.
Methods: We utilized a floxed PKD1 mouse model such that infecting epidermal keratinocytes derived from these mice with an adenovirus expressing Cre-recombinase allowed us to determine the effect of PKD1 gene loss *in vitro*. Proliferation and differentiation were monitored using qRT-PCR, Western blot, transglutaminase activity assays, [^{3}H]thymidine incorporation into DNA and cell cycle analysis.
Results: A significant decrease in PKD1 mRNA and protein levels was achieved in adenoviral Cre-recombinase-infected cells. Deficiency of PKD1 resulted in significant increases in the mRNA and protein expression of various differentiation markers such as loricrin, involucrin, and keratin 10 either basally and/or upon stimulation of differentiation. PKD1-deficient keratinocytes also showed an increase in transglutaminase expression and activity, indicating an anti-differentiative role of PKD1. Furthermore, the PKD1-deficient keratinocytes exhibited decreased proliferation. However, PKD1 loss had no effect on stem cell marker expression.
Conclusions: Cre-recombinase-mediated knockdown represents an additional approach demonstrating that PKD1 is an anti-differentiative, pro-proliferative signal in mouse keratinocytes.
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1. Introduction

Keratinocytes are the predominant cell type present in the outermost layer of the skin, the epidermis. Keratinocytes undergo a distinct pattern of proliferation and differentiation. The proliferating keratinocytes in the basal layer move upward upon growth arrest and initially express mature keratins 1 and 10 in the first differentiated epidermal layer, the spinous layer, followed by intermediate markers such as involucrin. The upper differentiated granular layer is marked by expression of late (*e.g.*, loricrin) differentiation markers. Late differentiation is also accompanied by expression/activation of proteins, like transglutaminase, that are essential for the formation of the cornified envelope and corneocytes at the outer layer of the epidermis, the stratum corneum. Although the epidermis is similar in mice and humans, differences exist. Thus, in humans, the stratum spinosum is composed of multiple layers of keratinocytes, whereas in mouse epidermis the stratum spinosum is usually a single cell layer [1]. Also, average epidermal thickness (30 μM) in mouse skin is less than that of human epidermis (50 μM) [2]. In addition, human hair follicles cycle asynchronously through growth (anagen), involution (catagen) and resting (telogen) phases, while those of mice are synchronized [3].

The equilibrium between keratinocyte proliferation and differentiation is essential for maintaining the protective barrier

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and to prevent skin diseases (reviewed in Refs. [4–7]). For example, in squamous and basal cell carcinoma, the malignant cells fail to complete the keratinocyte differentiation program. Abnormal keratinocyte differentiation also characterizes another human skin disease, psoriasis, treatment costs of which are estimated at over $1 billion annually (www.aad.org). Therefore, to develop better treatments, it is important to understand the regulation of keratinocyte proliferation and differentiation.

Protein kinase D or protein kinase D1 (PKD or PKD1) is a serine/threonine protein kinase that has been shown to play a role in a variety of cell processes (reviewed in Refs. [8–10]). PKD has been classified as the first member, PKD1, of a new family that includes also PKD2 and PKD3, with sequence homology to calcium/calmodulin-dependent kinases in the catalytic domain. PKD can be activated by transphosphorylation mediated by protein kinase C and a Src/Abl tyrosine kinase cascade in response to a variety of growth factors, hormones and other extracellular signals (reviewed in Refs. [10,11]). PKD is also activated by oxidative stress [12–16] and DNA damage [17]. Importantly, we have recently shown activation of PKD in keratinocytes exposed to ultraviolet B (UVB) irradiation, the primary risk factor for the development of non-melanoma skin cancer, providing a link between these cancers, sun exposure (*i.e.*, UVB), and PKD [18].

Ours and others' results suggest that PKD plays a role in regulating keratinocyte proliferation and differentiation. Increased PKD levels in mouse epidermal carcinomas [19], decreased DNA synthesis [19,20] and increased differentiation upon inhibition of its activity [20], and increased DNA synthesis [18] and promoter activity of keratin 5, a basal layer marker [21], upon PKD overexpression all suggest the pro-proliferative role of PKD in keratinocytes. Reversal of calcium-differentiated keratinocytes to a proliferative basal-like phenotype upon switching of the cells to a low calcium medium has also been described [22], and this low calcium switch-induced mitogenic response is dependent upon PKD signaling. Recently, *in vivo* studies involving targeted deletion of PKD1 in epidermal keratinocytes suggested a pro-proliferative role of PKD1 during wounding and upon challenge with tumor-promoting phorbol esters [23]. These authors also suggested that PKD1 is dispensable, under normal conditions, for skin development and homeostasis [23]. However, this report did not show cumulative quantified results of multiple animals for proliferative or differentiative markers under basal conditions nor was epidermal PKD1 deletion verified in the skin *in situ*. In addition, possible compensatory effects related to the loss of PKD1 *in utero* could not be excluded. On the other hand, Ivanova and coworkers have shown that siRNA-mediated down-regulation of PKD1 decreases human keratinocyte proliferation, although these authors failed to demonstrate PKD1 knockdown at the protein level [24]. Thus, all of the strategies used to date have both benefits and the potential for artifacts, and the results from multiple approaches can only strengthen the evidence for PKD1's role in keratinocytes.

To determine PKD's role in keratinocyte function, we have used a recently generated transgenic mouse model [25] in which the PKD1 gene is flanked by loxP sites (*i.e.*, a floxed PKD1 mouse model). Infecting epidermal keratinocytes derived from these mice with an adenovirus possessing Cre-recombinase allowed us to determine the effect of PKD1 loss *in vitro*. Our hypothesis was that PKD1 exerts anti-differentiative effects on epidermal keratinocytes such that its loss will result in increased differentiation.

2. Materials and methods

2.1. Materials

Antibodies were purchased as described in Table 1 of the supplementary materials. PVDF membrane was from Millipore (Billerica, MA), and iScript cDNA synthesis kits were purchased from Bio-Rad (Hercules, CA). The keratinocyte serum-free media (K-SFM) kit was purchased from Life Technologies (Carlsbad, CA). [^{3}H]Thymidine and [^{3}H]putrescine were purchased from Dupont/NEN (Boston, MA).

2.2. Adenoviral constructs amplification

The GFP-tagged adenovirus construct for Cre-recombinase was purchased from Vector Biolabs (Philadelphia, PA). Adenoviruses containing wild type-PKD (WT-PKD) and tyrosine-463-to-phenylalanine PKD mutant (mutant-PKD) constructs were made previously in our laboratory using the AdEasy adenoviral system as described elsewhere [18,26]. The amplification and purification of viruses were performed as described earlier [18,27].

2.3. Cell culture and experimental design

Primary epidermal mouse keratinocytes were prepared from 1 to 3 day old neonatal floxed-PKD1 [25] or outbred ICR CD-1 mice (Harlan Laboratories, Indianapolis, IN) and cultured in K-SFM containing 50 μM $CaCl_2$. Isolated keratinocytes were seeded on 6-well plates using dialyzed fetal bovine serum-containing plating media as described earlier [28]. For PKD1 depletion experiments, the day after plating, when the floxed-PKD1 keratinocytes were approximately 40% confluent, the keratinocytes were infected with Cre-recombinase (also expressing GFP) adenovirus or GFP adenovirus [at a multiplicity of infection (MOI) of 0.5 or 5] in K-SFM. The virus-containing media was removed 24 h post-infection and replaced with K-SFM and every 24 h thereafter. 48 h after infection, the cells were stimulated to differentiate with K-SFM containing a moderately elevated calcium concentration (125 μM) [29,30]. After 24 h of calcium treatment (for a total of 72 h after viral infection), the cells were harvested either for quantitative RT-PCR (qRT-PCR) or Western analysis. For time course experiments, 125 μM calcium-containing medium was added 24 h prior to termination of the experiments and harvesting of the cells. For over-expression studies, CD-1 mouse keratinocytes were incubated with K-SFM and allowed to grow for 1–2 days. Sub-confluent cells were infected with adenoviral constructs over-expressing wild-type PKD (WT-PKD) or tyrosine-463-to-phenylalanine PKD (mutant-PKD) or with GFP alone (Vector) using an MOI of 50. After 24 h of adenoviral infection, the cells were harvested for qRT-PCR or Western analysis.

2.4. RNA extraction, cDNA synthesis, and quantitative RT-PCR

After treatment the keratinocytes were processed for total RNA extraction using PerfectPure RNA tissue kits (5 PRIME, Inc., Gaithersburg, MD, USA) as per the manufacturer's protocol. Spectroscopic quality testing and quantitation of total RNA was performed using a Nanodrop instrument (NanoDrop Technologies, Wilmington, DE). Equal quantities of total RNA (1 μg) were reverse transcribed using iScript cDNA synthesis kits (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. Equal volumes of 5-times diluted cDNA were used in qRT-PCR reactions with Taqman probes purchased from Applied Biosystems (Applied Biosystems, Grand Island, NY), as indicated in Supplementary Table 2. The qRT-PCR reaction was performed using the Fast Reagent PCR Master Mix (Applied Biosystems) and the StepOnePlus Real-Time PCR System (Applied Biosystems) as per the manufacturer's protocol. The relative gene expression was calculated by the delta-delta Ct method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein, large, P0 (Rplp0) as endogenous control genes (average value of the two genes) and normalized to the GFP (Vector)-infected group.