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The Class II Phosphoinositide 3-Kinase C2β Is Not Essential for Epidermal Differentiation

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Phosphoinositide 3-kinases (PI3Ks) regulate an array of cellular processes and are comprised of three classes. Class I PI3Ks include the well-studied agonist-sensitive p110 isoforms; however, the functions of class II and III PI3Ks are less well characterized. Of the three class II PI3Ks, C2α and C2β are widely expressed in many tissues, including the epidermis, while C2γ is confined to the liver. In contrast to the class I PI3K p110α, which is expressed throughout the epidermis, C2β was found to be localized in suprabasal cells, suggesting a potential role for C2β in epidermal differentiation. Overexpressing C2β in epidermal cells in vitro induced differentiation markers. To study a role for C2β in tissue, we generated transgenic mice overexpressing C2β in both suprabasal and basal epidermal layers. These mice lacked epidermal abnormalities. Mice deficient in C2β were then generated by targeted gene deletion. C2β knockout mice were viable and fertile and displayed normal epidermal growth, differentiation, barrier function, and wound healing. To exclude compensation by C2α, RNA interference was then used to knock down both C2α and C2β in epidermal cells simultaneously. Induction of differentiation markers was unaffected in the absence of C2α and C2β. These findings indicate that class II PI3Ks are not essential for epidermal differentiation.

The phosphoinositide 3-kinase (PI3K) family is conserved through evolution and is implicated in a diverse array of biologic processes, including cell survival, proliferation, inflammation, adhesion, glucose metabolism, chemotaxis, and cancer. In mammals, there are eight PI3K family members divided into three classes by sequence homology (8, 14, 18). All proteins share homologous kinase domains and can phosphorylate the 3-hydroxyl position of the inositol head group of phosphoinositides. Class I PI3Ks exist as heterodimers and are divided into two subclasses. The ubiquitously expressed class Ia PI3Ks are activated by receptor tyrosine kinases and their effectors, notably Ras. They are comprised of a 110-kDa catalytic subunit (p110α, p110β, or p110δ) that is constitutively bound via amino-terminal sequences to a regulatory subunit (p85α, p55α, p50α, p85β, or p55γ). A major product of phosphorylation of phosphoinositide substrates by class Ia PI3Ks is phosphatidylinositol 3,4,5-triphosphate [PtdIns(3,4,5)P_3]. PtdIns(3,4,5)P_3 promotes membrane localization and activation of a host of downstream effectors that contain pleckstrin homology domains, including Akt, Sos, PDK-1, and PLCγ. PtdIns(3,4,5)P_3 action is opposed by phosphatases that include PTEN and SHIP (1, 18). Class Ia PI3Ks are implicated in a variety of cellular processes and have recently been found as active mutant forms in a variety of human cancers (23). Class Ib PI3Ks act downstream of G-protein-coupled receptors and are composed of a catalytic subunit (p110γ) bound to a p101 regulatory subunit. In contrast to the ubiquitous class Ia PI3Ks, class Ib PI3Ks are primarily found in hematopoietic cells. Class III PI3K is composed of a catalytic homolog of the Vps34 protein, which is implicated in vesicle sorting, and a p150 regulatory subunit. While diverse effects have been assigned to class I PI3Ks, the functions of class II and class III PI3Ks are still being elucidated.

Class II PI3Ks have been a focus of increasing recent interest. In contrast to class I PI3Ks, these lipid kinases lack regulatory subunits, phosphorylate PIP_2 poorly in vitro, and are constitutively associated with cellular membranes. Class II PI3Ks include the ubiquitously expressed PI3K C2α and C2β proteins as well as the liver-restricted C2γ protein (13). Unlike class I PI3Ks, class II PI3Ks do not appear to be directly activated by Ras. Class II PI3K C2 proteins do appear to associate with and function downstream of a number of transmembrane proteins, including clathrin, integrins, chemokine receptors, and growth factor receptors (2, 4, 10, 14, 15, 21, 25, 31), and C2β can also be activated by other stimuli, such as lysophosphatidic acid, insulin and platelet aggregation (6, 20, 33). Like other class II members, PI3K C2β contains a Ras binding domain, a PI kinase (PIK) domain, a catalytic domain, and a C2 domain (3, 14, 18). Although less well characterized than class I PI3Ks at the functional level, C2β has recently been implicated as being important in cell migration in several epithelial lines (20) and in the differentiation of HL-60 hematopoietic cells by retinoic acid (27). While a number of class I PI3Ks have been disrupted in mice, the phenotypic effects of targeted deletion of C2β have not been reported.

Cutaneous epidermis is a stratified epithelial tissue that undergoes continual, spatially controlled differentiation and self-renewal throughout life. The processes governing the induction of differentiation in developmentally mature mammalian epidermis are not fully understood. Given expression patterns suggestive of involvement in the differentiation of a variety of tissues (13), PI3Ks represent potential candidates for control of epidermal differentiation. Directly contradictory findings for

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a potential role for PI3Ks in this process, however, have recently been presented. Adenoviral overexpression of active PI3K p110α inhibited expression of the keratin 1/10 differentiation markers in cultured keratinocytes while dominant-negative p85 induced these markers, suggesting that PI3K may prevent epidermal differentiation (24). In contrast, a more recent study demonstrated that PI3K inhibitors blocked calcium-induced keratinocyte differentiation, suggesting that intact PI3K function is required for this process (32). A limitation in both cases is that these studies relied only on cultured cells, and the effects of PI3K on epidermal differentiation in tissue have not been reported.

Here we have assessed the role of PI3K C2β in epidermal tissue by generation of both transgenic and knockout mice. C2β was found to be expressed predominantly in suprabasal epidermal layers, suggesting a possible role in epidermal differentiation. While pharmacologic blockade of PI3K inhibited keratinocyte differentiation in vitro and overexpression of wild-type C2β enhanced it, these effects were not seen in tissue engineered for either gain or loss of C2β function. Specifically, both C2β overexpression in both basal and suprabasal epidermal compartments and C2β gene disruption failed to alter epidermal growth, differentiation, barrier function, and wound healing. C2β knockout mice lacking C2β protein expression in cutaneous and visceral tissues were viable and fertile, indicating that C2β is dispensable for normal development, survival, and reproduction. To examine potential redundancy of class II PI3Ks in epidermal differentiation, simultaneous knockdown of both C2α and C2β protein levels was achieved in epidermal cells by RNA interference; however, this also failed to block induction of differentiation. These findings indicate that C2β is dispensable for viability and for epidermal homeostasis.

MATERIALS AND METHODS

Cell culture and gene transfer. Coding sequences for human PI3K C2α (12), PI3K C2β (3), PI3K p110αCAAX (30), and PI3K Δp85 (17) were subcloned into the EcoRI/XhoI, BamHI, and BamHI sites of the LZRS vector (19), respectively. To generate deletions in the PI kinase, catalytic, and C2 domains of C2β, the cDNA encoding the wild-type version of the enzyme was digested with FspI, EagI, and BsrG1/ClaI, respectively, and religated to generate in-frame deletions. All constructs were verified by restriction mapping and sequencing. Primary human keratinocytes were isolated and grown as described previously (9). Retrovirus was prepared in human 293T packaging cells as described previously (11). Primary keratinocytes underwent retroviral transduction at a multiplicity of infection of 15 without drug selection (22).

Targeting vector construction and generation of PI3K C2β-deficient mice. A 3-kb PI3K C2β genomic fragment containing exon 2, a 2.3-kb fragment containing exons 3 to 5, and a 4.4-kb fragment containing exons 6 to 8 were amplified from 129/Sv mouse DNA by PCR. Primers were designed to incorporate EcoRI (3-kb fragment), BamHI (2.3-kb fragment), and NotI (4.4-kb fragment) restriction enzyme cleavage sites for ligation into the targeting vector. After amplification by PCR, all exons and exon-intron borders were sequenced. The 3-kb EcoRI fragment, the 2.3-kb BamHI fragment, and the 4.4-kb NotI fragment were inserted separately as the short arm, *loxP* site-flanked fragment, and long arm, respectively, into a modified pPNT-loxP vector, which contained a neomycin and a thymidine kinase cassette. The targeting vector was linearized with PvuI and electroporated into R1 embryonic stem cells. Resistant cells were selected in the presence of G418 and ganciclovir. DNA was isolated from a total of 397 clones. Homologous recombinants were screened by Southern blotting. Genomic DNA was digested with BamHI, and identified with probe 1, a specific PCR fragment of 540 bases located downstream of the targeted *PIK3C2B* locus. Two clones, A7 and C6, were injected into C57BL/6 blastocysts, which were subsequently transferred into pseudopregnant females to generate chimeric offspring. The chimeras were crossed into C57BL/6 mice for germ line transmission. The null allele was generated by crossing the $C2\beta^{fl/+}$ line into the protamine-Cre deleter line (PrmCre1). For genotyping, the genotypes of mutant mice were determined by PCR and confirmed by Southern blot analysis of genomic DNA from tail biopsies. Tail samples were collected and digested with 40 μg proteinase K in 400 μl buffer (0.5% sodium dodecyl sulfate [SDS], 0.1 M NaCl, 50 mM Tris-HCl [pH 8], 2.5 mM EDTA) at 58°C overnight. Routine genotyping through PCR was performed with genomic DNA with specific primers for the deleted allele (P1, 5′-TGT TAGAACCTGCCGCCTTTAC-3′, and P2, 5′-CCGAATCAGCCTCATTTCCTC TC-3′) and for the wild-type allele (P3, 5′-GGCACACACTAACCACAGCACC-3′, and P4, 5′-TCGATGCACGT CTCTCC GC-3′). The PCR product for the deleted allele was 404 bp, whereas the product for the wild-type allele was 251 bp. Genotyping of animals was confirmed by Southern blotting. Genomic DNA was digested with HindIII and identified by probe 2, a specific PCR fragment of 517 bases located within exon 2.

Total RNA isolation and reverse transcription-PCR. Total cellular RNA was isolated from cultured keratinocytes obtained from postnatal day 0 mice with the RNeasy minikit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed by using Superscript II RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA). RT-PCR was performed with a forward primer (RT1, 5′-ACACCTCTGGGAAACCTGTG-3′) designed in exon 2 and a reverse primer (RT2, 5′-GCCTCTCTTGGAGATGGA TG-3′) designed in exon 7.

Histology and protein expression. Necropsies were performed on 6- to 8-week-old mice. For histological examination, tissue was fixed in 10% buffered formalin and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. For immunostaining, 5-μm skin cryosections were allowed to air dry for 10 min and permeabilized with cold acetone for 10 min. Sections were blocked with 1% bovine serum albumin–phosphate-buffered saline (BSA-PBS) for 1 h and treated with the primary antibody diluted in 10% BSA-PBS overnight at 4°C. Slides were then washed three times with PBS and incubated for 1 h with secondary antibodies with 2 mg/ml Hoechst (Molecular Probes, Eugene, OR). After three washes with PBS, slides were mounted in Vectashield (Vector Laboratories) and examined under a Zeiss 100M Axiovert microscope. The following panel of antibodies was used in immunostaining: anti-mouse K14, anti-mouse K10, antiloricrin, antifilaggrin, and anti-mouse involucrin (Covance, Berkeley, CA); PI3K C2β (BD Biosciences, San Jose, CA); anti-mouse Ki-67 (Dako, Carpinteria, CA); Cy3-conjugated goat anti-rabbit immunoglobulin G (IgG) and Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories). For immunoblotting, human keratinocyte extracts were homogenized in lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 1% NP-40) with protease inhibitors (Complete Mini EDTA free; Roche, Indianapolis, IN) and phosphatase inhibitor mixture II (Sigma, St. Louis, MO). Mouse keratinocytes were lysed in lysis buffer (8 M urea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 10% 2-mercaptoethanol, 40 mM Tris [pH 8.0], 2.5 mM EDTA). Protein extract was loaded at 20 to 100 μg/lane and subjected to 10% SDS-PAGE. The following antibodies were used: PI3K p110α (Cell Signaling, Beverly, MA), PI3K C2α (a gift of Jan Domin), PI3K C2β (BD Biosciences, San Jose, CA), human keratin 1 and filaggrin (Babco), human involucrin (Sigma), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), donkey anti-rabbit IgG–horseradish peroxidase (Amersham Biosciences, Piscataway, NJ), sheep anti-mouse IgG–horseradish peroxidase (Amersham), and donkey anti-goat IgG–horseradish peroxidase (Santa Cruz).

Generation of transgenic mice. The sequence encoding human PI3K C2β (3) was subcloned downstream of a 2,075-bp human keratin 14 (K14) promoter construct, which targets expression of C2β to keratinocytes within the basal epidermal layer (26). In addition, the C2β coding sequence was subcloned downstream of a human keratin 1 (HK1) promoter construct to target gene expression to keratinocytes in the suprabasal epidermal layers (16). Both constructs were used to generate transgenic mice. Transgene integration was confirmed by PCR.

Wound healing and barrier analysis. Six-week old mice ($C2\beta^{-/-}$, $C2\beta^{+/-}$, $C2\beta^{+/+}$) were anesthetized and shaved. Two full-thickness skin biopsy punch wounds were made on either side of the midline of the mouse, and the wound area was measured daily for 10 days. For transepidermal water loss, C2β-deficient-mouse and control mouse skin were analyzed using an evaporimeter (Servomed, Stockholm, Sweden). To further assess the epidermal permeability barrier by a β-galactosidase skin permeability assay, unfixed and freshly isolated embryos were rinsed in PBS and then immersed in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) reaction mix at pH 4.5 [100 mM $NaPO_4$, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$ and 1 mg/ml X-Gal] and incubated at room temperature overnight.

RNA interference. Human PI3K C2β and PI3K C2α small interfering RNA (siRNA) pools were designed and synthesized by Dharmacon (Chicago, IL).

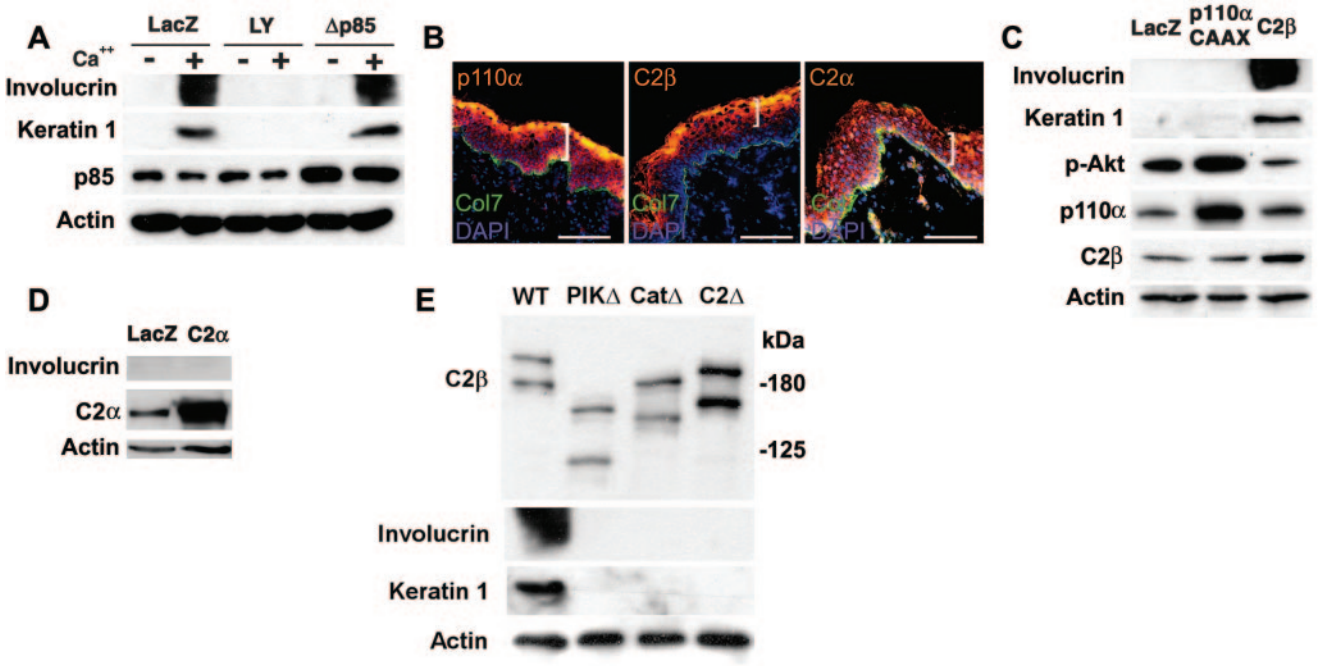


FIG. 1. PI3K C2β induces keratinocyte differentiation. (A) Expression of keratinocyte differentiation proteins keratin 1 and involucrin in the presence of low (0.07 mM [−]) or elevated (0.20 mM [+]) calcium in the medium. Cells were coincubated with the PI3K inhibitor LY294002 or transduced with retroviral vectors expressing either the PI3K Δp85 mutant dominant-negative for class Ia PI3K function or a LacZ marker control. Proteins blotted are shown at left. (B) PI3K protein distribution in human epidermis. Note p110α and C2α throughout the epidermis and the restriction of C2β to suprabasal layers. Brackets delineate expression regions: orange, PI3K; green, collagen VII denoting the epidermal basement membrane; and blue, DAPI (4′,6′-diamidino-2-phenylindole) nuclear stain. Bars = 100 μm. (C) Differentiation protein expression in keratinocytes expressing retrovirally introduced LacZ control, p110α, and C2β. Retroviral expression constructs are noted at the top of each lane, and the blotted proteins are indicated at the left of each panel. (D) Differentiation marker protein expression in keratinocytes expressing retrovirally introduced PI3K C2α and LacZ control. (E) Deletion of the C2β PI kinase (PIKΔ), catalytic (CatΔ), or C-terminal C2 (C2Δ) domain abolishes induction of keratinocyte differentiation. Immunoblot verification of expression of the mutants noted at the top of each lane is shown in the top panel, with molecular mass markers to show the size of each mutant. The proteins studied are shown at the left of each panel; retroviral expression of wild-type and mutant C2β proteins invariably produced a doublet on immunoblotting.

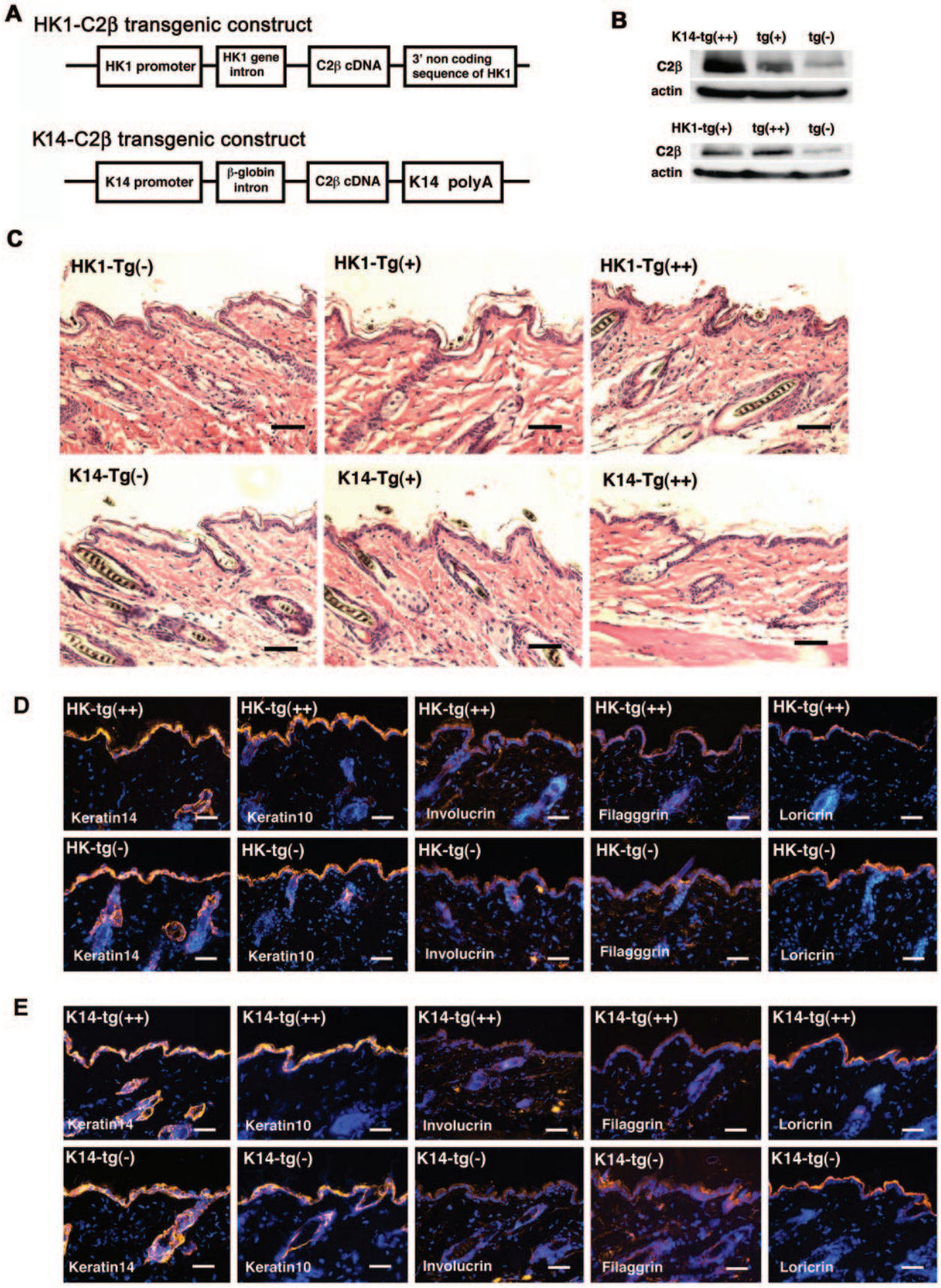
Keratinocytes (10^6) were electroporated with 200 pmol of the of the indicated siRNA oligonucleotide using the Amaxa nucleofection kit (Amaxa, Gaithersburg, MD) according to the manufacturer's recommended protocol. Twenty-four hours after nucleofection, the concentration of calcium in the medium was raised to 1.0 mM to induce differentiation. Cells were harvested and analyzed 48 h later.

RESULTS

Epidermal differentiation is blocked by PI3K inhibition and can be induced by C2β in vitro. Conflicting data have been reported regarding a potential role of PI3Ks in epidermal differentiation (24, 32). We therefore examined the effect of blocking PI3K function on differentiation of epidermal keratinocytes. To inhibit PI3K function, two approaches were used: retroviral expression of a dominant-negative p85 mutant (Δp85) inhibitory for class Ia PI3Ks and addition of the pharmacologic inhibitor LY294002, which inhibits action by multiple classes of PI3Ks (28, 29). While Δp85 failed to alter calcium-induced differentiation protein expression, LY294002 blocked it entirely (Fig. 1A). This suggested that function of non-class Ia PI3Ks may be required for keratinocyte differentiation and raised the possibility that expression of active PI3Ks might induce it.

To begin to address this possibility, we examined PI3K expression in epidermis. While PI3K p110α and C2α protein were detected throughout the epidermis in both undifferentiated and differentiating layers, C2β was confined to differentiating suprabasal layers of human epidermis (Fig. 1B), consistent with a potential C2β role in differentiation. In agreement with the lack of Δp85 effects, expression of active class I PI3K p110α failed to increase differentiation protein expression un-

FIG. 2. K14 and HK1-C2β transgenic mice exhibit normal skin. (A) Transgene cassettes used to generate targeted expression of C2β under control of the basal K14 promoter or suprabasal HK1 promoter. Three independent lines were generated for each construct and displayed similar phenotypes. (B) C2β protein expression in keratinocyte extracts isolated from wild-type [tg(−)], singly transgenic [tg(+)], or doubly transgenic [tg(++)] mice. Actin expression is included as a loading control. (C) Histology of wild-type (−) and transgenic skin. Note normal architecture in all cases. Expression of keratin 14 and the differentiation proteins keratin 10, involucrin, filaggrin, and loricrin in (D) wild-type and HK1-C2β and (E) K14-C2β transgenic skin (orange, differentiation marker; blue, DAPI). Bars = 100 μm.


A
HK1-C2β transgenic construct
HK1 promoter
HK1 gene intron
C2β cDNA
3' non coding sequence of HK1
K14-C2β transgenic construct
K14 promoter
β-globin intron
C2β cDNA
K14 polyA
B
K14-tg(++) tg(+) tg(-)
C2β
actin
HK1-tg(+) tg(++) tg(-)
C2β
actin
C
HK1-Tg(-)
HK1-Tg(+)
HK1-Tg(++)
K14-Tg(-)
K14-Tg(+)
K14-Tg(++)
D
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Keratin14
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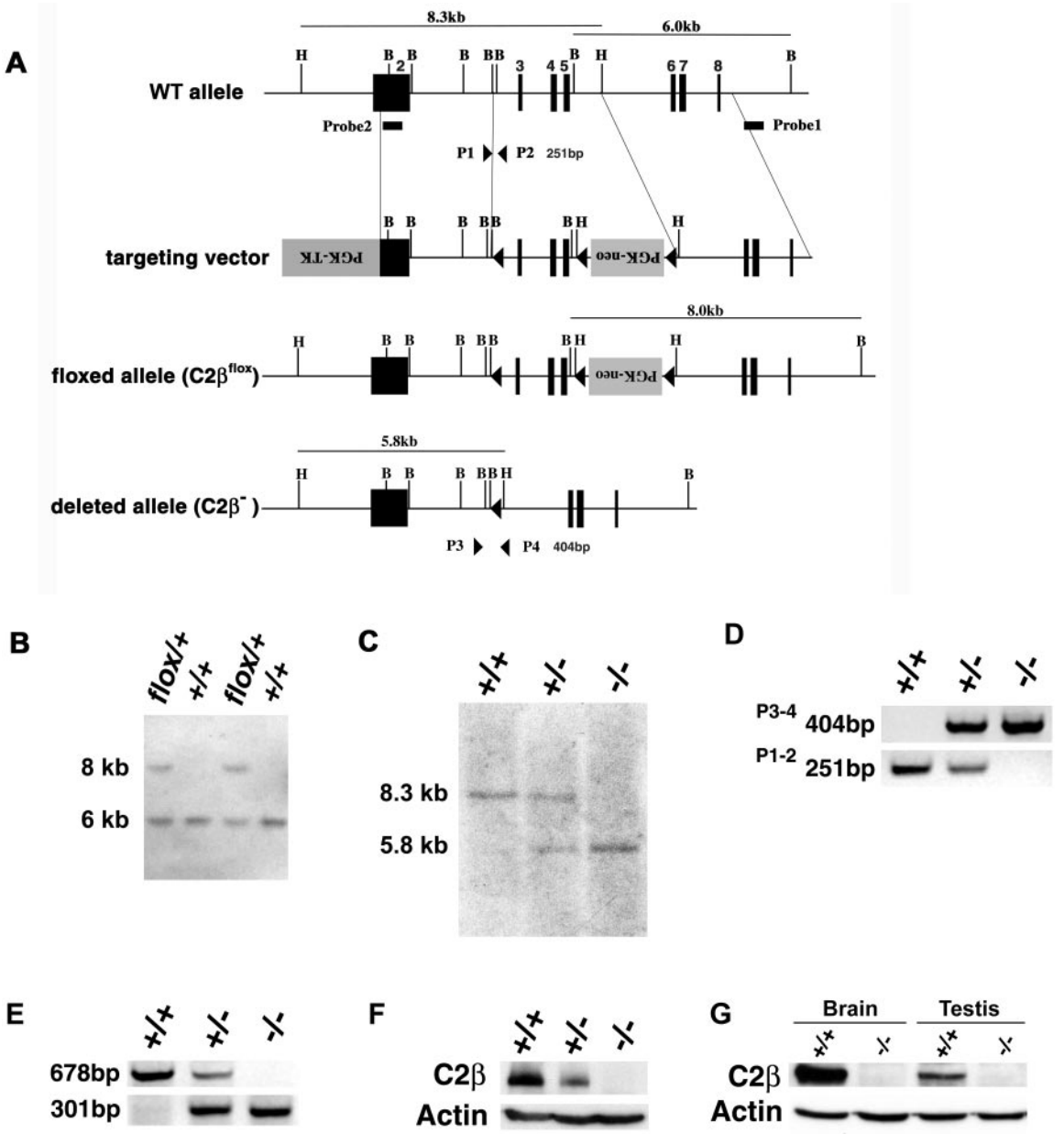


FIG. 3. Targeted disruption of the mouse *C2β* gene. (A) Targeting strategy. Partial restriction enzyme maps and schematic representation of the strategy for ablation of the C2β gene. The *PIK3C2B* gene of 129Sv mice (wild-type [WT] allele), the targeting vector, the recombinant *loxP*-flanked locus (floxed allele), and the C2β exon-deleted locus (deleted allele) are shown. Cleavage sites for BamHI (B) and HindIII (H) are marked. The locations of probes 1 and 2 for Southern analysis are indicated by closed bars, and primers for PCR analysis are shown as arrowheads. Exons are symbolized as numbered rectangles. Exon 1 encodes an untranslated region; the start codon is located in exon 2. (B) Southern blot analysis of BamHI-digested genomic DNA and hybridization to probe 1 reveal a 6-kb and an 8-kb band corresponding to wild-type and floxed alleles, respectively. (C) Southern hybridization for detection of the null allele. The HindIII fragments in the wild-type allele and deleted alleles are 8.3 kb and 5.8 kb, respectively, when detected with probe 2. (D) Mice were genotyped by PCR. The amplicon with primer pair 1 and 2 is 251 bp for the wild-type allele. The amplicon with primer pair 3 and 4 is 404 bp for the null allele. (E) Loss of normal C2β mRNA transcript in $C2\beta^{-/-}$ mice. RNA was isolated from cultured keratinocytes. The forward primer was designed in exon 2, and the reverse primer was designed in exon 7. The wild-type allele yields a 678-bp amplicon, and the deleted allele yields a 301-bp amplicon. (F) Loss of epidermal C2β protein in $C2\beta^{-/-}$ mice. Western blot analysis showing absence of C2β protein expression in the knockout mice. Proteins were isolated from epidermal extracts and detected with an antiserum to C2β. (G) Loss of C2β protein in visceral tissues of $C2\beta^{-/-}$ mice.

der basal medium conditions (Fig. 1C). In contrast, the class II PI3K C2β increased it strongly (Fig. 1C). Expression of the only other class II PI3K expressed in epidermis, C2α, failed to induce differentiation (Fig. 1D), indicating that this effect is specific to C2β. Induction of keratinocyte differentiation by C2β required intact PIK, catalytic, and C2 domains, because expression of mutant C2β proteins lacking these sequences failed to produce this effect (Fig. 1E). C2β is thus expressed

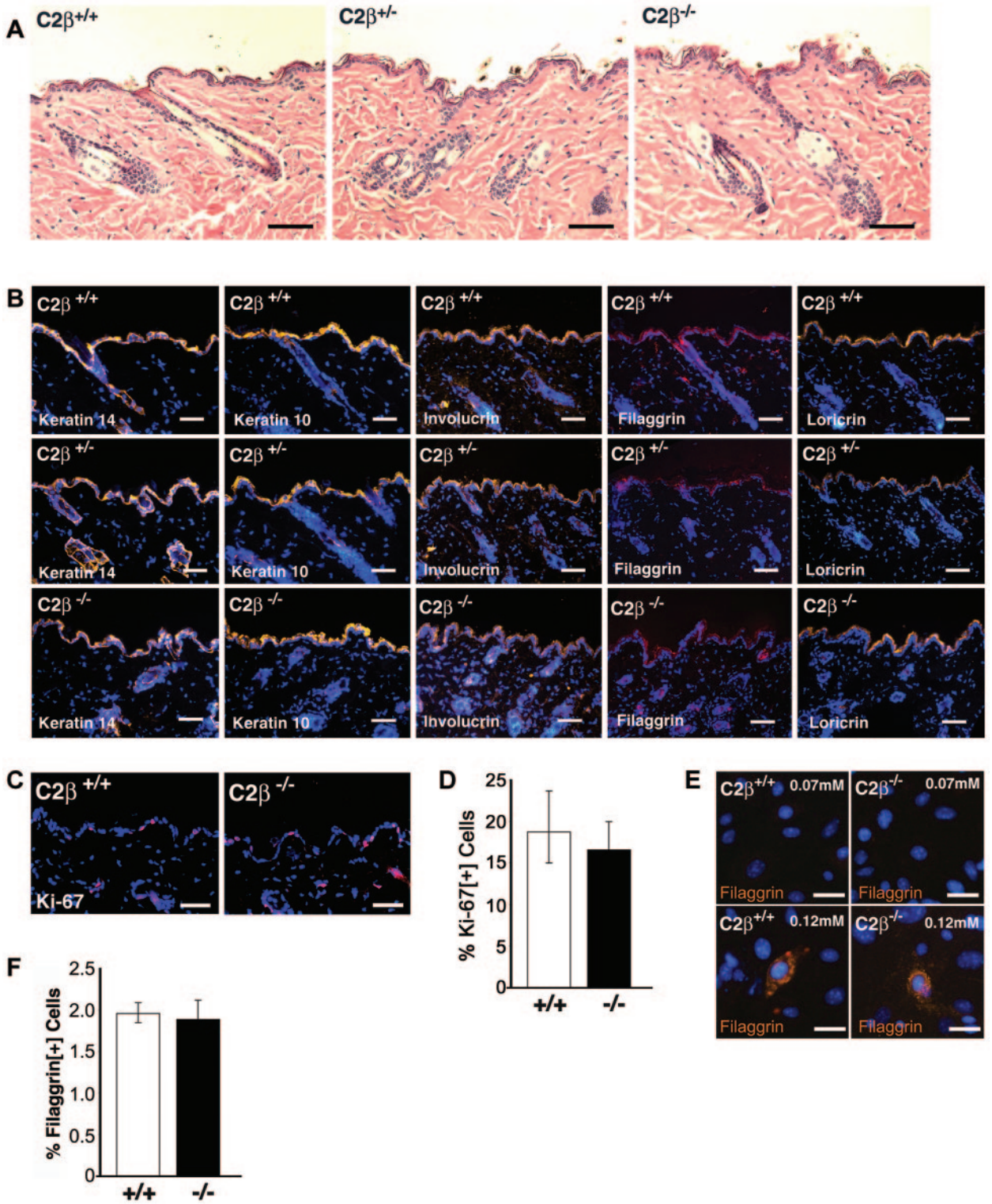
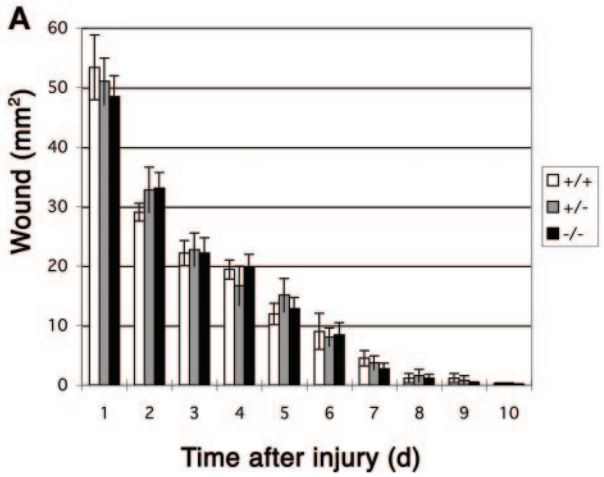
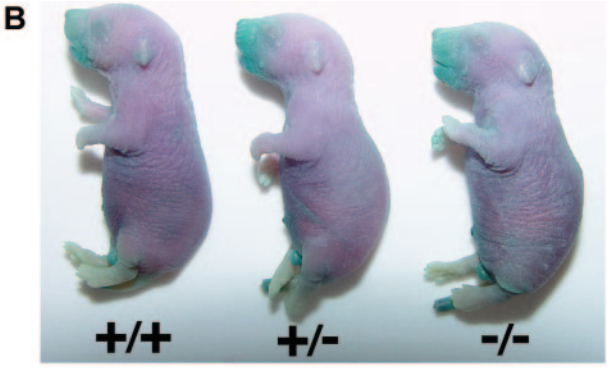


FIG. 4. *C2β*$^{-/-}$ mice display normal epidermal differentiation and proliferation. (A) Histology of back skin from C2β wild-type, heterozygous, and null mice at 8 weeks of age. Note normal tissue architecture in C2β-null skin. (B) Expression of keratin 14 and the differentiation markers keratin 10, involucrin, filaggrin, and loricrin in 8-week-old mice. Bars = 100 μm. (C) Ki-67 (orange) in tissue counterstained with DAPI (blue). Bars = 100 μm. (D) Percentage of Ki-67(+) epidermal cells in skin tissue from C2β wild-type (+/+) and null (−/−) mice (three mice each; values are means ± standard deviations). (E) Filaggrin (orange)-expressing primary keratinocytes in culture from C2β wild-type and null mice when cells were grown in low (0.07 mM)-calcium and higher (0.12 mM)-calcium media. (F) Quantitation of the percent of filaggrin-positive cells in C2β wild-type and null murine keratinocytes after 24 h in 0.12 mM calcium (three independent experiments; values are means ± standard deviations).





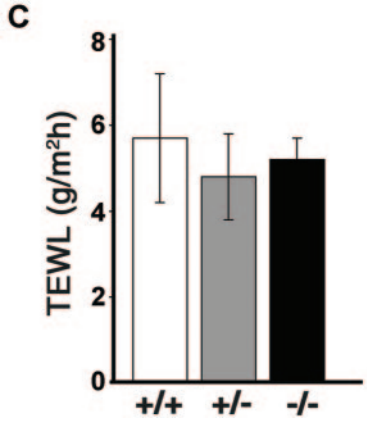


FIG. 5. Normal wound healing and barrier function in C2β-deficient mice. (A) *C2*β$^{-/-}$ mice exhibited normal re-epithelialization. Wound area over a 10-day period following injury (three mice each; values are means ± standard deviations). (B) β-Galactosidase epidermal permeability barrier assay of *C2*β$^{-/-}$ mice compared to the wild type. Note the lack of increased penetration of X-Gal through the epidermal barrier (as detected by blue staining) of the wild-type and *C2*β$^{-/-}$ mice. (C) Transepidermal water loss in newborn mice (three mice each; values are means ± standard deviations).

within the differentiating cell compartment of epidermal tissue in vivo and can induce keratinocyte differentiation in vitro.

Transgenic mice with targeted epidermal C2β expression are normal. To assess the effects of C2β in tissue, transgenic mice that express increased levels of C2β in the epidermis were generated. To examine C2β effects in both undifferentiated and differentiated layers, the keratin 14 (K14) and keratin 1 (HK1) promoters were used, respectively (Fig. 2A). Increased expression of C2β protein was observed in epidermal keratinocytes of both singly and doubly transgenic mice; because available C2β antibodies do not work well for immunohistochemistry in murine tissue, immunoblotting of transgenic and wild-type control littermate keratinocyte extracts was performed (Fig. 2B).

Targeted expression of C2β in both undifferentiated and differentiating epidermal layers failed to alter epidermal morphology from that of normal (Fig. 2C). Additionally, increased C2β expression did not alter epidermal differentiation marker expression patterns in tissue (Fig. 2D and E). These data indicate that increased expression of C2β in vivo does not exert dramatic effects on epidermal differentiation.

Generation of C2β knockout mice. The discrepancy between our in vitro and in vivo overexpression findings did not address a potential requirement for C2β function in epidermal homeostasis. To study this, we undertook targeted deletion of sequences at the murine *PIK3C2B* locus, which encodes C2β. Exons 3 to 5 were flanked with *loxP* sites (Fig. 3A). Deletion of these sequences produces a truncated, nonfunctional protein lacking the PIK, catalytic, and C2 domains that are required for induction of keratinocyte differentiation in vitro. C2β-null mice were generated by crossing *C2*β$^{fl/+}$ animals with protamine-Cre deleter mice (PrmCre1); the progeny were then backcrossed to obtain *C2*β$^{-/-}$ mice that lacked C2β expression (Fig. 3B to E). Loss of targeted exons in C2β-null mice led to a total lack of C2β protein, as detected by immunoblotting to the amino terminus, in both epidermis (Fig. 3F) and visceral tissues (Fig. 3G). Null mice were born and survived to adulthood at a normal Mendelian ratio. C2β knockout mice were viable and fertile and displayed no detectable abnormalities on visual inspection and on histologic survey of visceral tissues.

Characterization of C2β knockout mice. To examine a possible role for C2β in epidermal homeostasis, epidermal tissue architecture, differentiation marker expression and proliferative indices were analyzed. Epidermal thickness and morphology appeared to be normal in C2β knockout mice (Fig. 4A). Differentiation marker expression was also localized in a manner indistinguishable from normal (Fig. 4B). Epidermal proliferation was also normal, as measured by mitotic indices using the proliferation marker Ki-67 (Fig. 4C and D), and C2β-null keratinocytes retained the capacity to induce differentiation marker expression in vitro in response to calcium in a fashion similar to that of normal controls (Fig. 4E and F). C2β knockout mice also healed with normal kinetics after wounding (Fig. 5A). They displayed normal epidermal barrier function, as judged by both X-Gal penetration and transepidermal water loss (Fig. 5B, C). These findings indicate that C2β is not essential for normal epidermal growth, differentiation, wound healing, or barrier formation.

Effects of simultaneous knockdown of C2α and C2β. The lack of epidermal effects with C2β loss could be due to com-

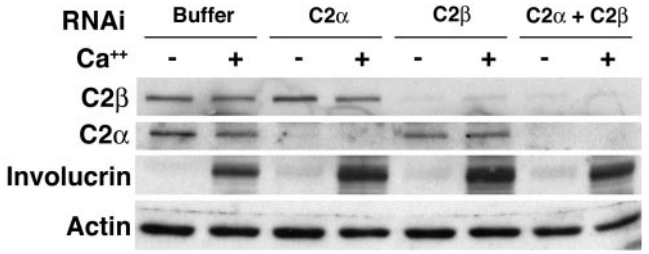


FIG. 6. Simultaneous C2α and C2β knockdown fails to prevent keratinocyte differentiation marker expression. Human keratinocytes treated with duplex RNAs to C2α, C2β, or C2α plus C2β were exposed to high and low extracellular calcium concentrations for 2 days. The proteins blotted are shown at the left of each panel, and the RNAi used is at the top. Note the expression of the involucrin differentiation marker and its induction by calcium in the absence of C2α and C2β proteins.

pensation by C2α. We therefore simultaneously knocked down expression of both proteins using RNA interference (RNAi) and examined the effects on keratinocyte differentiation in vitro, the setting where C2β effects were observed. RNA duplexes effectively diminished expression of both C2α and C2β but did not abolish normal induction of differentiation marker expression by calcium (Fig. 6). This finding suggests that the class II PI3Ks C2α and C2β are not required for epidermal differentiation.

DISCUSSION

Here we have shown that PI3K C2β is not essential for epidermal homeostasis. Because our gene deletion strategy also produced C2β protein loss in other somatic tissues in addition to epidermis, these data indicate that C2β is not required for normal development and postnatal viability in mice. This observation is surprising, given the discrete phenotypes obtained with knockouts of class I PI3Ks (1, 8, 18), and argues for generation of multigene knockouts for class II PI3Ks to look for genetic redundancy in development. Our knockdown data suggest, however, that class II PI3Ks are dispensable for at least epidermal differentiation because simultaneous knockdown of both C2α and C2β, the only class II PI3Ks expressed in epidermis, fails to impair differentiation protein induction.

Two conflicting studies have argued for (32) and against (24) a role for PI3K function in epidermal differentiation. While the two studies used different sets of reagents and differentiation conditions, both were limited by exploring the role of PI3K in this setting using wholly in vitro experiments. In vitro approaches alone also proved misleading in the present study and highlighted the need for in vivo genetic experiments, including loss-of-function efforts via gene deletion, to uncover any requirements for PI3K isoform function in specific tissue settings. It is formally possible that multiple other PI3K isoforms cooperate to regulate this process. Future efforts to study this issue may include multigene knockout animals to explore the impacts of deficiencies in multiple PI3K isoforms on epidermal development and differentiation.

The observation that PI3K pharmacologic inhibition blocks differentiation in vitro suggested a role for PI3Ks in this process. One potential explanation for the discrepancy between pharmacologic and genetic studies is that inhibitors altered the function of another protein independent of the PI3K family, consistent with the known lack of complete specificity of multiple classes of kinase inhibitors. For example, both LY294002 and wortmannin, widely used inhibitors of PI3K function, also effectively inhibit the function of the mTOR kinase when used at identical concentrations (7). Additionally, the observed findings may represent a potential in vitro artifact where inhibitors function in a manner that does not reflect the effects of inhibitor action in tissue. In agreement with the latter possibility, we observed no effect of topical LY294002 application for 8 weeks to normal mouse skin using an approach demonstrated previously to achieve significant topical PI3K inhibition in a cutaneous melanoma model (5). Addressing a potential role for alternative PI3K inhibitor-sensitive processes in epidermal differentiation will require further studies.

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