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High Commitment of Embryonic Keratinocytes to Terminal Differentiation through a Notch1-caspase 3 Regulatory Mechanism

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Summary

Embryonic cells are expected to possess high growth/differentiation potential, required for organ morphogenesis and expansion during development. However, little is known about the intrinsic properties of embryonic epithelial cells due to difficulties in their isolation and cultivation. We report here that pure keratinocyte populations from E15.5 mouse embryos commit irreversibly to differentiation much earlier than newborn cells. Notch signaling, which promotes keratinocyte differentiation, is upregulated in embryonic keratinocyte and epidermis, and elevated caspase 3 expression, which we identify as a transcriptional Notch1 target, accounts in part for the high commitment of embryonic keratinocytes to terminal differentiation. In vivo, lack of caspase 3 results in increased proliferation and decreased differentiation of interfollicular embryonic keratinocytes, together with decreased activation of PKC-δ, a caspase 3 substrate which functions as a positive regulator of keratinocyte differentiation. Thus, a Notch1-caspase 3 regulatory mechanism underlies the intrinsically high commitment of embryonic keratinocytes to terminal differentiation.

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Introduction

Mouse skin provides an ideal model for studying the coordinate control of epithelial cell growth and differentiation, and for understanding how this balance is achieved during development. During embryogenesis, complex and rapid developmental changes occur in the skin that are required for the maturation of this organ and for adnexa formation (Hardy, 1992). Skin morphogenesis is determined by a complex set of developmental cues that act in a highly localized fashion to induce skin adnexa formation, while development and maturation of the interfollicular epidermis is thought to occur as a default pathway (Kopan and Fuchs, 1989). The most critical inductive signals leading to skin adnexa formation are acting before day E13.5/E14.5 (McGowan and Coulombe, 1998). At later times of embryonal life, the skin, like other organs, undergoes a complex program of morphogenesis and massive expansion, suggesting that cells at this stage are endowed with a high growth/differentiation potential that progressively decreases after birth. However, while the high growth potential of mesenchymal cells of embryonal origin has been demonstrated, it has not been assessed whether this extends to epithelial cells, and to keratinocytes in particular.

The *Notch* gene family encodes evolutionarily conserved transmembrane receptors with a key role in cell-fate determination and differentiation (Artavanis-Tsakonas et al., 1999). Notch activation is triggered by interactions with ligands of the Delta and Serrate/Jagged families, and results in release of the intracellular region of the receptor by proteolytic cleavage and nuclear translocation. The activated Notch intracellular region binds to a ubiquitous DNA binding protein of the CSL family [*Drosophila* Suppressor of hairless (Su(H)) or its mammalian homolog RBP-Jκ/CBF1], thereby converting it from a repressor into an activator of transcription (Lai, 2002). This results in the induction of gene expression that affects differentiation in a cell type- and context-dependent manner. Notch signaling is likely to play a complex role in skin adnexa formation (Lin et al., 2000; Pourquie, 2000; Yamamoto et al., 2003, and references therein). In the interfollicular epidermis, Notch1 activation is essential for promoting the exit of keratinocytes from the cell cycle and entry into differentiation (Lowell et al., 2000; Rangarajan et al., 2001; Nickoloff et al., 2002).

Recently, great interest has emerged for keratinocyte stem cells, operationally defined as cells with high growth potential that are the likely targets for carcinogenesis (Perez-Losada and Balmain, 2003). An attractive hypothesis was that, like stem cells, embryonic keratinocytes possess a high growth potential required for organ morphogenesis and expansion during development. However, little was known about the intrinsic growth/differentiation properties of these cells due to difficulties in their isolation and cultivation. Contrary to expectations, we report here that pure keratinocyte populations from E15.5 mouse embryos have an intrinsically high

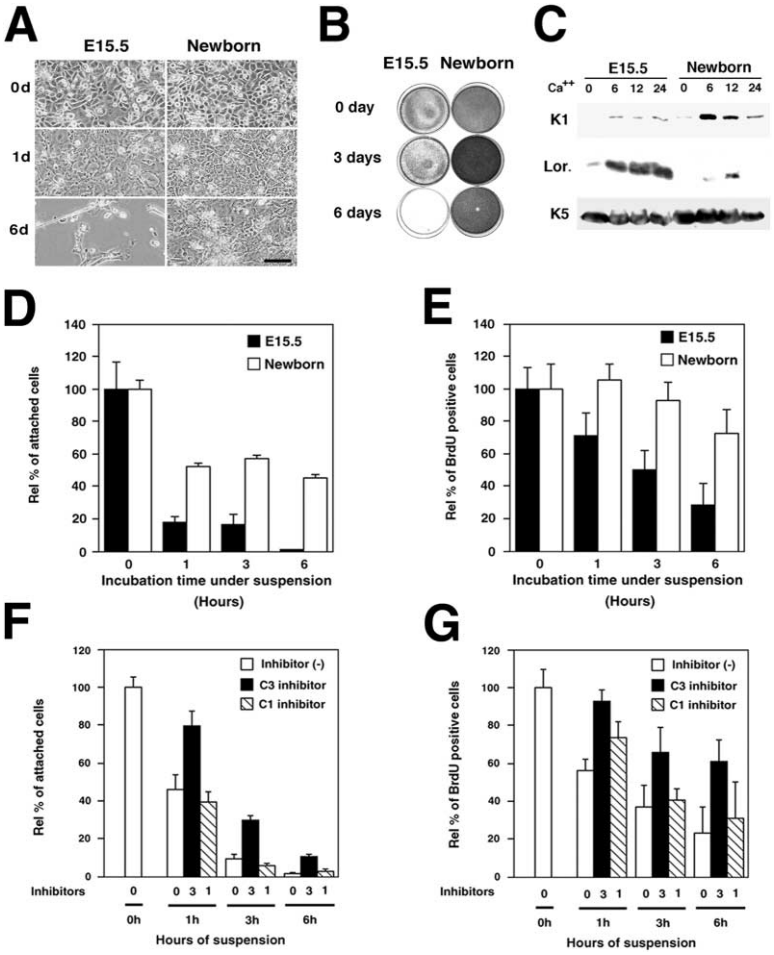

Figure 1. Irreversible Commitment of Embryonic Keratinocytes to Differentiation

(A) Phase contrast photographs of primary keratinocytes derived from E15.5 and newborn mice which were cultured and maintained under low calcium conditions or switched to high calcium medium for 1 or 6 days as indicated. Bar, 100 μm.

(B) Crystal-violet-stained dishes of the same cultures at the indicated times after calcium-induced differentiation.

(C) Terminal differentiation marker expression in embryonic versus newborn mouse keratinocytes as assessed by immunoblotting with the corresponding antibodies. Total cell extracts were prepared from keratinocytes under growing conditions and at various times (hr) of calcium-induced differentiation and analyzed for expression of Keratin 1 (K1), loricrin (Lor), and Keratin 5 (K5). The latter is constitutively expressed in cultured keratinocytes irrespective of their growing versus differentiating conditions and was used as equal loading control.

(D) Attachment efficiency of E15.5 (solid bars) versus newborn (white bars) keratinocytes which were freshly prepared and plated directly or kept in suspension for various amounts of time (hr) prior to plating. Keratinocytes were plated onto collagen type IV-coated dishes, and after 1 hr adherent cells were fixed and stained with 0.1% crystal violet. Staining intensity was determined with an ELISA reader at 570 nm. Values are expressed as percentages relative to attached cells that were plated immediately without culture in suspension. Each point represents the average of six different wells, and similar results were obtained in a second independent experiment.

(E) BrdU labeling index of E15.5 (solid bars) versus newborn (white bars) keratinocytes which attached to the dish after being kept into suspension for the indicated amounts of time (hr). In all cases, cells were pulse labeled with BrdU for 3 hr at 36 hr after plating. The percentage of BrdU-positive nuclei was determined in a minimum of six independent fields (>100 cells/field), and the standard deviation among values from the various fields was calculated. Values are expressed as percentages relative to BrdU labeling of cells that were plated immediately without culture in suspension. Similar results were obtained in another independent experiment.

(F and G) Similar experiments as in (D) and (E), except that cells were kept in suspension either in the absence or presence of specific peptide inhibitors for caspase 3 (DEVD-CHO) or caspase 1 (YVAD-CHO) activity.

commitment to terminal differentiation that can be explained, in part, by elevated Notch signaling and increased caspase 3 expression, which we identify as a Notch transcriptional target.

Results

High Commitment of Embryonic Keratinocytes to Terminal Differentiation

To assess the intrinsic growth/differentiation potential of embryonic keratinocytes, we set up conditions for isolation and cultivation of these cells from E15.5 embryos. Immunofluorescence analysis revealed that these cultures contained >98% keratin-positive cells, with similar morphology and proliferating fraction as keratinocytes from newborn mice (Figure 1A and data not shown). Extracellular calcium concentration is an important determinant of keratinocyte growth and differentiation (Hennings et al., 1980). At 1 day of high calcium exposure, embryonic keratinocytes underwent morphological changes like those of newborn cells (Figure 1A). However, by 3–6 days, many of the embryonic cells maintained in high calcium conditions sloughed off the dish, while newborn cells remained attached and metabolically active (Figures 1A and 1B). This was accompanied by higher induction of loricrin, a terminal differentiation marker of the outermost layers, in differentiating embryonic versus newborn keratinocytes, while expression of keratin 1, an "early" differentiation marker of the intermediate layers, was lower in the embryonic cells (Figure 1C). The increased susceptibility of embryonic keratinocytes to terminal differentiation under these conditions is not associated with enhanced apoptosis as "classically" defined, as the number of cells positive by TUNEL or Annexin V binding assays remained negligible (Supplemental Figures S1A and S1B [http://www.developmentalcell.com/cgi/content/full/6/4/551/DC1]).

To test whether the high propensity to terminally differentiate is an intrinsic property of embryonic keratinocytes independent of their response to calcium, we exploited the fact that maintaining keratinocytes in suspension provides an alternative means to induce late

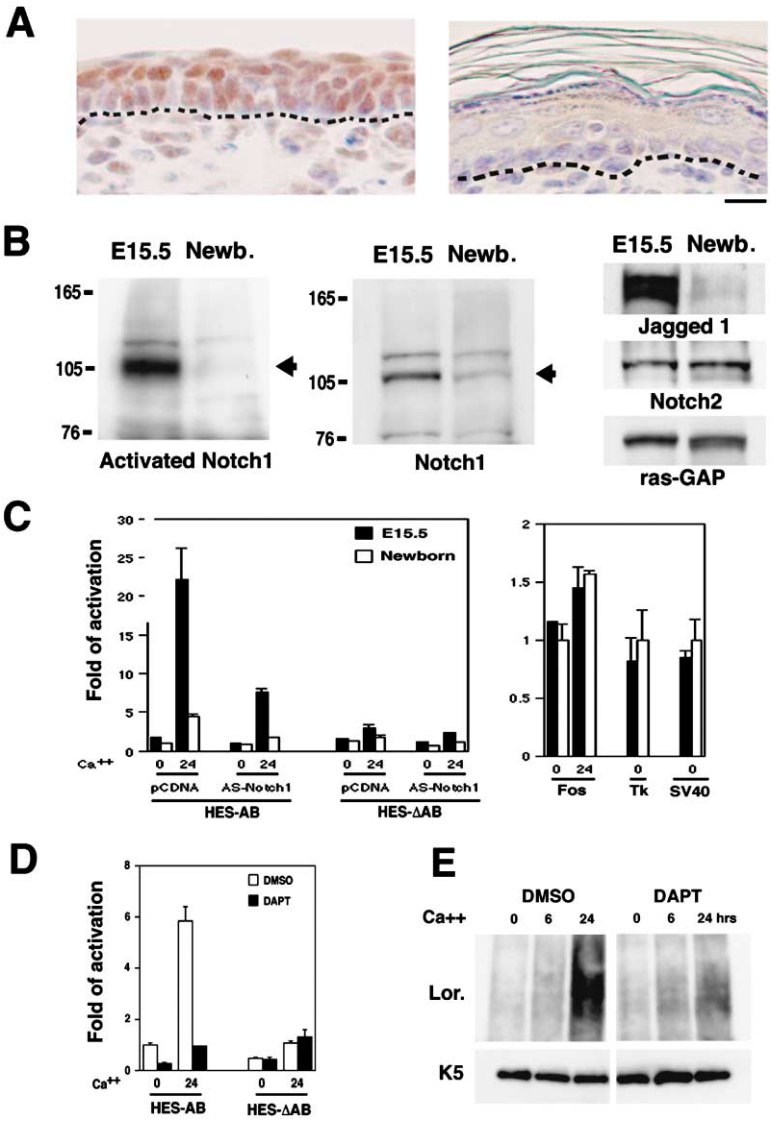

Figure 2. Elevated Notch Expression and Activity in Embryonic versus Newborn Keratinocytes

(A) Immunostaining of embryonic and newborn skin sections with antibodies against the γ-secretase cleaved activated form of Notch1. Bar: 15 μm.

(B) Immunoblot analysis of freshly separated epidermis from E15.5 and newborn mice with antibodies against the indicated proteins. One of these blots was stripped and reprobed with antibodies against Ras-Gap as control for equal loading conditions. The position for molecular weight markers is indicated. Densitometric quantification of the autoradiographs coupled with normalization for levels of ras-GAP expression indicated that relative levels of total and activated Notch1 protein were 3.3- and 13-fold higher, respectively, in embryonic than newborn epidermis.

(C) Left panel: Measurements of endogenous Notch activity by transient transfection of embryonic (black bars) and newborn (white bars) keratinocytes with a reporter for a Notch-responsive promoter (HES-AB) or the promoter with a small internal deletion which abrogates Notch responsiveness (HES-ΔAB). Cells were transfected with the reporter promoters together with pCDNA empty vector or a vector for Notch1 antisense cDNA (AS-Notch1). Cells were either kept under growing low calcium conditions (0) or induced to differentiate by calcium for the last 24 hr (24) of the duration of the experiment (72 hr after transfection). Right panel: similar assays of E15.5 versus newborn keratinocytes transiently transfected with reporter for minimal c-Fos, TK, and SV40 promoters. Values are expressed as folds of activation relative to promoter activity in newborn keratinocytes under low calcium conditions.

(D) Measurements of endogenous Notch activity in embryonic keratinocytes as affected by treatment with the γ-secretase inhibitor DAPT. Cells were transiently transfected with reporters for the Notch-responsive versus unresponsive promoters (HES-AB and HES-ΔAB, respectively) and either treated (black bars) or untreated (white bars) with 1 μM DAPT for 30 hr. Cells were maintained under growing conditions or induced to differentiate by elevated calcium concentrations for the last 24 hr of the experiment.

(E) Immunoblot analysis of embryonic keratinocytes at the indicated times (hr) of high calcium exposure plus/minus DAPT treatment, with antibodies against the terminal differentiation marker loricrin (Lor.) and keratin 5 (K5), which was used as equal loading control.

aspects of differentiation (Watt et al., 1988). Thus, uncultured keratinocyte preparations freshly derived from embryonic and newborn mice were kept in suspension for various times prior to plating. There was a drop in plating efficiency of both embryonic and newborn keratinocytes when these cells were kept in suspension for 1 hr rather than being plated immediately. However, plating efficiency of newborn keratinocytes kept in suspension for longer periods of time did not further decrease, while that of embryonic cells was drastically reduced (Figure 1D). The drop in plating efficiency of embryonic keratinocytes was not accompanied by increased apoptosis, as a similarly low fraction of TUNEL and Annexin V positive cells (<3%–5%) was found with both newborn and embryonic keratinocytes at all times of culture in suspension (Supplemental Figures S1C and S1D). In parallel with these findings, the DNA synthesis capability of embryonic keratinocytes that attached to the dish was progressively reduced when these cells were kept into suspension for various times prior to plating, while newborn keratinocytes showed little or no reduction (Figure 1E).

To confirm conclusions obtained with cultured cells in vivo, we examined the turnover of keratinocytes in the intact skin. Females pregnant for 15.5 and 18.5 days and 2-day-old newborn mice were labeled by intraperitoneal injections with BrdU, and the BrdU labeling index of epidermal cells in embryos and newborn mice was evaluated 3 hr after injection, or 3 and 7 days later. Previous reports indicated that proliferation occurs in both basal and suprabasal epidermal layers of E15.5 embryos, while by day E18 it becomes limited to the basal layer (see, for instance, Byrne et al., 1994). After 3 hr of labeling, the fraction of BrdU-positive cells in

Table 1. Genes Concomitantly Upregulated in Embryonic Keratinocytes and in Response to Increased Notch1 Activity

Gene ID	Gene Name	Fold Upregulation E15.5	Fold Upregulation Notch1	Function
Transcription control				
L12703	Engrailed protein (En-1)	3.88	2.96	Homolog of *Drosophila* engrailed, homeobox gene.
U80078	Transcription factor BFCOL1	3.65	2.45	Kruppel family of zinc finger protein. Repression of the transcription of gastrin, stromelysin, and enolase.
U81604	Eya3 homolog (Eya3)	3.64	2.35	Homolog of *Drosophila* eyes absent. Gene networks with Pax, Six, and Dach.
Y10026	TEF4	2.89	7.66	Transcription factor expressed in preimplantation embryos.
M18401	Homeobox 2.2 protein	2.86	2.08	Homeobox gene in specifying positional identity along the anterior-posterior axis.
Y12783	Ring1B	2.83	2.05	Transcriptional repressor through interaction with Polycomb proteins which suppress homeobox gene.
AF017806	Zn-15 transcription factor (Zfp-15)	2.78	2.18	Zinc finger factor activating transcription of growth hormone.
X98207	Histone deacetylase	2.52	2.54	Controling transcriptional regulation and cell cycle progression through acetylation state of histones.
X07439	Hox-3.1	2.50	2.63	Homeobox gene in specifying positional identity along the anterior-posterior axis.
AI844532	Subunit of the splicing factor SF3B	2.49	2.56	Required for stable binding of U2 snRNP to breakpoint sequence in pre-mRNA.
X13945	L-myc	2.18	3.18	A member of myc oncoprotein family binding to E-box element of transcriptional targets.
D49654	Musashi-1	2.04	2.24	RNA-binding protein affecting cell-fate determination and differentiation through translational repression.
Translation control				
AJ243533	eIF2α kinase/GCN2	3.36	2.17	Regulating protein synthesis by phosphorylating the α subunit of eukaryotic initiation factor (eIF2α).
AA600468	Translational initiation factor 2 β subunit	2.02	2.50	Initiation of protein synthesis.
Intracellular signaling				
U52193	Phosphoinositide 3-kinase	12.35	3.08	Phosphatidyl inositol (PtdIns) 3-kinase distinct from p110/p85 and Vps34. Phosphorylating PtdIns.
U20159	76 kDa tyrosine phosphoprotein SLP-76	14.21	5.41	One substrate of T cell receptor activated tyrosine kinase pathway. Interacting with adaptor protein Grb2.
M80739	Protein tyrosine phosphatase, non-receptor type 2	2.27	2.21	Intracellular phosphotyrosine phosphatase expressed in various tissue.
Apoptosis				
U54803	Caspase 3	2.84	3.46	Cysteine protease responsible for apoptosis execution.
Keratins				
M22832	Keratin 18	4.56	26.12	Type I keratin intermediate filament protein expressed in simple epithelia.
D89902	High-glycine tyrosine keratin type II.4	2.34	2.51	Type II high-glycine/tyrosine keratin chiefly expressed in hair cortical cells.
Transport/sorting				
X75927	ABC transporter (ABC2 gene)	3.18	2.38	One of ATP binding cassette transporter family members responsible for transport across cell membrane.
AF037312	Sulfonylurea receptor-1 (SUR-1)	2.56	2.61	Component of ATP-sensitive potassium channel.
AB026808	Synaptotagmin XI	2.22	2.48	Calcium sensor in the process of vesicular trafficking and exocytosis.
Biosynthesis/metabolism				
D26123	Carbonyl reductase	4.05	2.43	Enzyme to metabolize endogenous carbonyl compounds such as aliphatic aldehydes and ketones.
AF031486	Spermidine aminopropyltransferase	3.26	3.09	Biosynthesis of spermine from spermidine.
AB019541	β-1,4-galactosyltransferase II	2.54	2.87	Synthesis of complex-type N-linked oligosaccharides in glycoproteins and glyolipids.
L06047	Glutathione-S-transferase α 1	6.94	2.85	Conjugation of reduced glutathione to hydrophobic electrophiles.
U07159	Acetyl coenzyme A dehydrogenase	4.29	2.34	Dehydrogenase in mitochondrial fatty acid β-oxidation system.
Membrane proteins				
U49185	Occludin	2.13	5.19	Integral membrane protein localizing at tight junction regulating permeability barrier.

(*continued*)

Table 1. Continued

Gene ID	Gene Name	Fold Upregulation E15.5	Fold Upregulation Notch1	Function
Extracellular matrix				
M15832	Procollagen, type IV, alpha 1	4.44	2.95	Major structural component of basement membrane together with laminins, proteoglycans, and nidogen.
X13986	Minopontin	3.75	19.31	Binding to hydroxyapatite to form the mineralized matrix. Ligand for integrin $\alpha_v\beta_3$.
X70853	MB-90/fibulin C	2.86	3.16	Binding to laminin, nidogen, and collagenIV in calcium-dependent manner.

PolyA+ RNA was prepared from uncultured keratinocytes freshly derived from E15.5 embryos and newborn (day 0) mice, and from primary newborn keratinocyte cultures infected with an adenovirus-expressing GFP together with activated Notch1 (Ad-NIC) or only GFP (Ad-GFP). cRNA probes from each condition were tested in duplicate by hybridization to Affymetrix U74A gene chips according to the manufacturer's recommendations. Only positive flurorescence intensity values above an arbitrary threshold of 300 and changes in gene expression of >2 folds in both duplicates of E15.5 versus newborn keratinocytes, and Notch1-expressing versus control cells, were considered as significant. Shown are genes that could be assigned to a known or likely function, that were concomitantly upregulated in embryonic and Notch1-expressing keratinocytes. Within each functional cluster, genes were ordered on the basis of their average folds of upregulation in embryonic versus newborn cells. Besides caspase 3, increased expression of the En-1, Hox3.1, and Hox2.2 genes was also independently confirmed by real-time PCR.

the basal layer of the E15.5 embryos was significantly greater than in the E18.5 or newborn mice, as expected for the elevated proliferative activity in the rapidly expanding embryonic epidermis. Proliferation was also readily detected in the suprabasal layer of the E15.5 but not E18.5 or newborn epidermis (Supplemental Figure S2). At 3 days after labeling, little or no BrdU-positive cells were found in the basal layer of embryos labeled at day E15.5, while an increased fraction of BrdU-positive cells was found in the suprabasal layers of these mice. By contrast, a substantial number of BrdU-positive cells remained in the basal layer of mice labeled at the E18.5 and newborn stage, with BrdU-labeled cells becoming now also present in the suprabasal layers (Supplemental Figure S2). By 1 week after labeling, little or no labeled keratinocytes were found in the epidermis of mice derived from the E15.5 BrdU-injected embryos in either basal or suprabasal layers, while a substantial fraction of labeled cells was still found in the epidermis of mice derived from similarly labeled E18.5 embryos or newborn (Supplemental Figure S2). Thus, while functional turnover assays provide only an indirect indication of the rate of differentiation, our findings are consistent with E15.5 keratinocytes "transiting" at a faster rate toward terminal differentiation even in the intact skin in vivo.

Elevated Notch1 Expression and Signaling in Embryonic Keratinocytes

Notch signaling plays a key role in promoting keratinocyte differentiation (Lowell et al., 2000; Nickoloff et al., 2002; Rangarajan et al., 2001). We hypothesized that the intrinsically high commitment of embryonic keratinocytes to differentiation is linked to elevated Notch activity. Antibodies have been developed that are specific for the γ-secretase cleaved activated form of Notch1 (Phiel et al., 2003). By immunohistochemistry, many cells stained positive for these antibodies already in the basal layer of the embryonic epidermis, and more pronounced staining was found in the suprabasal layers (Figure 2A). By contrast, little or no signal was seen in newborn epidermis. Interestingly, weak staining for activated Notch1 could also be detected in some cells of the embryonic dermis but not the newborn.

For biochemical determinations, the epidermis of embryonic and newborn skins was separated from the underlying dermis. Immunoblotting showed much higher levels of activated Notch1 in embryonic versus newborn epidermis, which, by densitometric quantification of the autoradiographs, could be explained only in part by higher total levels of the Notch1 protein in the embryonic epidermis (Figure 2B). Jagged1, a Notch ligand whose expression is positively regulated by activated Notch (Luo et al., 1997), was also found at higher levels in the embryonic epidermis (Figure 2B). Amounts of the Notch2 protein, unlike Notch1, were similar in embryonic versus newborn epidermis (Figure 2B).

In parallel with the in vivo results, transient transfection of cultured cells with a reporter plasmid carrying a Notch/RBP-Jκ-responsive promoter (HES-AB) (Jarriault et al., 1995) showed several folds higher promoter activity in embryonic than newborn keratinocytes, especially under differentiating conditions (Figure 2C). The elevated activity of this promoter in embryonic cells was substantially reduced by cotransfection of a Notch1 antisense cDNA, capable of suppressing to a significant extent endogenous Notch signaling (Rangarajan et al., 2001). In contrast to the HES-AB promoter, activities of the Notch-unresponsive HES-ΔAB mutant promoter (Jarriault et al., 1995) or unrelated promoters were similar in embryonic and newborn cells (Figure 2C, right panel). Proteolytic cleavage by γ-secretase is required for activation of endogenous Notch receptors (Kopan and Goate, 2000), and specific γ-secretase inhibitors such as DAPT (N-(N-(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycine t-butyl ester) have been used to suppress Notch activation (Yeh and Crews, 2003). Treatment of embryonic keratinocytes with DAPT at IC_{50} concentrations (1 μM) (Micchelli et al., 2003) was sufficient to suppress activity of the Notch-responsive HES promoter both under basal conditions and after induction of differentiation, while activity of the mutated Notch-unresponsive HES-ΔAB promoter was unaffected (Figure 2D). In parallel with this effect, DAPT treatment suppressed the

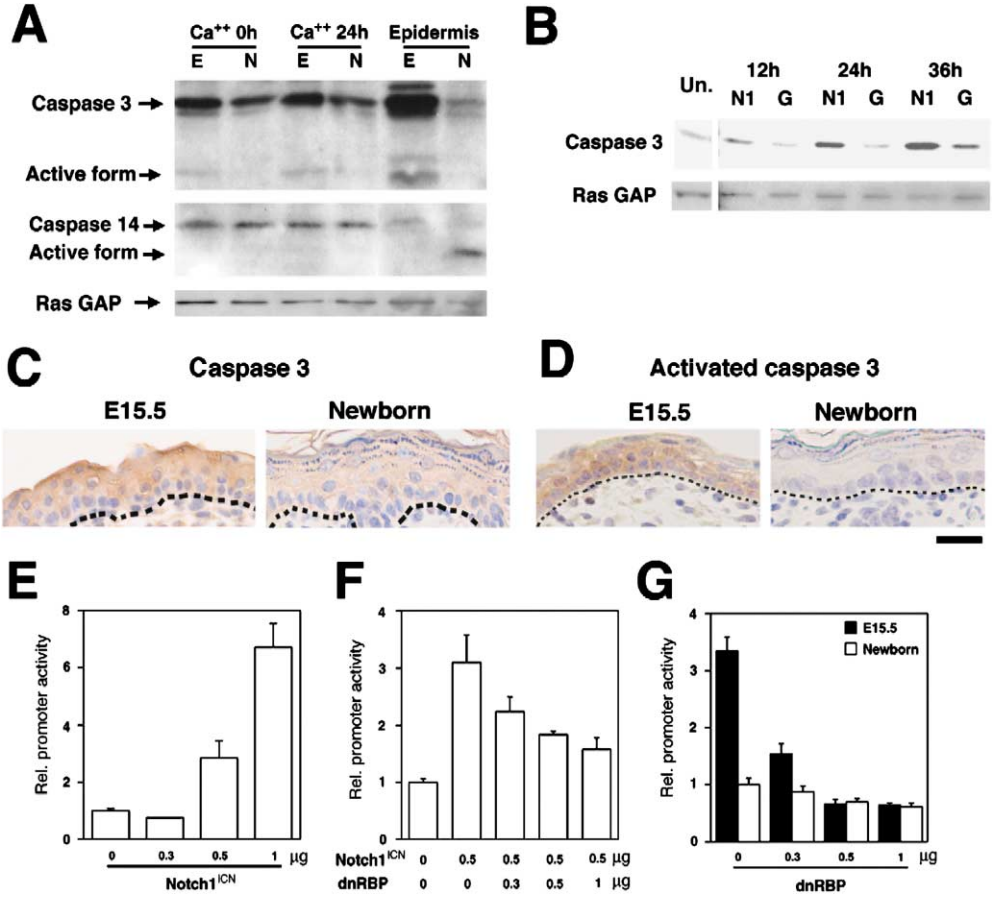

Figure 3. Elevated caspase 3 Expression in Embryonic Keratinocytes and Skin and in Response to Notch1 Activation

(A) Immunoblot analysis with anti-caspase 3 antibodies of total cell extracts derived from embryonic (E) and newborn (N) keratinocytes under low calcium conditions or treated with calcium for 24 hr, and of extracts of freshly separated embryonic and newborn epidermis. Arrows point to the expected positions for caspase 3 in its precursor (32 kDa) and activated (17 kDa) forms. The same extracts were analyzed for levels of caspase 14 expression by immunoblotting with the corresponding antibodies. Reprobing of the immunoblots with antibodies against Ras GAP was used as control for equal loading conditions.

(B) Immunoblot analysis with anti-caspase 3 antibodies of total cell extracts derived from newborn keratinocytes infected with a recombinant adenovirus expressing activated Notch1 (N1) or with an Ad-GFP control (G) at the indicated times after infection (hr). Immunoblotting with anti-Ras GAP antibodies was used as control for equal loading conditions. Un., uninfected control cells.

(C and D) Immunohistochemical analysis of the epidermis of E15.5 embryos and newborn mice with antibodies specific for caspase 3 or its activated form. Bars: 30 μm.

(E) Induction of caspase 3 promoter activity by activated Notch1. Newborn keratinocytes under basal growing conditions were transfected with a luciferase reporter plasmid for the mouse caspase 3 promoter plus/minus an expression vector of the activated form of Notch1 in increasing amounts.

(F) Induction of caspase 3 promoter activity by Notch1 through a RBP-Jκ-dependent mechanism. Newborn keratinocytes were transfected with the caspase 3 promoter reporter together with a vector for activated Notch1^{ICN} plus/minus increasing amounts of a vector for dominant-negative RBP-Jκ (dnRBP).

(G) Increased Notch/RBP-dependent activity of the endogenous caspase 3 promoter activity in embryonic keratinocytes. Parallel cultures of E15.5 (solid bars) and newborn (white bars) keratinocytes under basal growing conditions were transiently transfected with the caspase 3 promoter luc reporter together with a TK-renilla reporter for internal normalization, plus/minus the addition of expression vector for dominant-negative RBP-Jκ.

induction of the loricrin marker in differentiating embryonic keratinocytes (Figure 2E).

Upregulation of caspase 3, a Notch1 Transcriptional Target, in Embryonic Keratinocytes

Terminal differentiation of keratinocytes can be viewed as a specialized form of programmed cell death, and may rely on some of the same mediators as classical apoptosis (Maruoka et al., 1997; Polakowska et al., 1994). Expression of some of these mediators may be selectively elevated in embryonic keratinocytes, as a reflection of increased Notch activity. We investigated this possibility by global analysis of gene expression, by oligonucleotide array hybridization. We found that ~15% of genes with >2-fold higher expression in uncultured embryonic versus newborn keratinocytes (obtained from freshly separated epidermis) were also induced in newborn keratinocytes in response to activated Notch1 expression (Table 1). Among the genes that

could be assigned to known functions, there were several involved in transcription and translation control, signal transduction, intermediate metabolic pathways, cell structure, and adhesion. Only one gene involved in programmed cell death was found: *caspase 3*.

Immunoblot analysis confirmed that caspase 3 is expressed at higher levels in embryonic than newborn keratinocytes, both in culture and the intact epidermis (Figures 3A and 3C), and that its expression is induced in newborn keratinocytes by activated Notch1 (Figure 3B). Moreover, embryonic but not newborn keratinocytes contained also detectable amounts of the 17 kDa caspase 3 activated form (Figure 3A). Consistent with these results, immunohistochemical analysis with antibodies specific for the active form of caspase 3 produced a much stronger positive staining in the embryonic than newborn epidermis (Figure 3D).

Caspase 14 has been recently connected with keratinocyte differentiation control in the mature skin (Lippens et al., 2000). Microarray analysis indicated that expression of this gene, like those for other components of the apoptotic apparatus, is not increased in the embryonic keratinocytes. Direct immunoblot analysis confirmed that caspase 14, unlike caspase 3, is expressed at similar levels in embryonic and newborn keratinocytes, and a cleaved activated form of caspase 14 was found in the intact epidermis of newborn mice but not of embryos (Figure 3A).

To further verify that caspase 3 expression is under Notch control, we cloned the mouse caspase 3 promoter region into a luciferase reporter plasmid. Caspase 3 promoter activity was substantially induced in newborn keratinocytes by coexpression of either activated Notch1 or Notch2, such induction being counteracted by the concomitant expression of a dominant-negative RBP-Jκ mutant (Figures 3E and 3F and data not shown). In embryonic keratinocytes, basal activity of the caspase 3 promoter was significantly higher than in the newborn cells, using a minimal TK promoter as internal normalization control, and such elevated activity in embryonic keratinocytes was suppressed in a dose-dependent manner by cotransfection with a vector for a dominant-negative RBP-Jκ mutant (Figure 3G).

Conventional disruption of the *Notch1* gene results in embryonic lethality at E9.5 days (Conlon et al., 1995; Swiatek et al., 1994). The *Notch1* gene flanked by loxP sites can be efficiently deleted by induction of Cre recombinase activity in the skin of mice only after birth (Rangarajan et al., 2001). As an alternative approach to suppress Notch function during embryonic development, we resorted to the γ-secretase inhibitor DAPT (Dovey et al., 2001). Loss of γ-secretase activity by gene ablation experiments results in a phenotype strikingly similar to that of Notch deletion in both *Drosophila* and mice (Kopan and Goate, 2000), and inhibition of enzymatic activity by DAPT causes a similar phenotype (Micchelli et al., 2003). Skin organ cultures were established from E13.5 embryos and incubated in the presence or absence of DAPT at IC_{50} concentration for 48 hr. Immunohistochemical analysis with antibodies against total versus activated Notch1 indicated that the DAPT treatment caused the expected suppression of Notch1 activation in cultured embryonic skins (Figure 4A). Staining with anti-caspase 3 antibodies showed that expression of this protein and its activated form was strongly decreased in the DAPT-treated skin (Figure 4B), in parallel with an increase in epidermal thickness (Figure 4C), which is similar to that observed with the skins of caspase $3^{-/-}$ embryos (as described further below).

Involvement of caspase 3 in Embryonic Keratinocyte Growth/Differentiation Control

To assess whether elevated caspase 3 expression and activity contribute to the increased commitment of embryonic keratinocytes to terminal differentiation, fresh keratinocyte preparations from embryonic mice were placed in suspension in the presence or absence of a caspase 3 peptide inhibitor (DEVD-CHO) or a similar inhibitor specific for caspase 1 (YVAD-CHO) (Nicholson et al., 1995; Schlegel et al., 1996). As shown in Figure 1F, treatment with the caspase 3 inhibitor could partially circumvent the drop in plating efficiency of embryonic keratinocytes, especially at early times of suspension culture, while treatment with the caspase 1 inhibitor had no such effect. Concomitantly, the caspase 3 inhibitor restored to a substantial degree the DNA synthesis capability of the embryonic cells that attached to the dish after being kept into suspension for various times, while this did not occur with the caspase 1 inhibitor (Figure 1G). Protective effects similar to those of the caspase 3-specific inhibitor were exerted by broader-specificity caspase inhibitors such as Z-VAD-FMK and Boc-D-FMK (Supplemental Figure S3). Parallel experiments with newborn keratinocytes showed no effects on plating efficiency or DNA synthesis by caspase 3 or caspase 1 inhibitors (data not shown).

To assess the role of caspase 3 in the balance between keratinocyte growth and differentiation in vivo, we examined the skin of mice with a null mutation of the *caspase 3* gene (Woo et al., 1998). These mice have an abnormal brain development due to defective apoptosis and a fraction of them dies around birth (Kuida et al., 1996; Woo et al., 1998). Histological analysis revealed a consistent increase ($\sim$30%; $n = 9$) in epidermal thickness of *caspase* $3^{-/-}$ embryos (E15.5) relative to heterozygous and wild-type controls. The fraction of proliferating keratinocytes was 2-fold higher in the knockout skin than controls (Figure 5A). Conversely, expression of terminal differentiation markers loricrin and filaggrin was strongly reduced in the *caspase* $3^{-/-}$ epidermis, while expression of keratin 1, an "early" marker of the spinous layer, was comparable to controls (Figure 5B). At birth, the fraction of proliferating keratinocytes was also 2-fold higher in the epidermis of caspase $3^{-/-}$ versus wild-type or heterozygous mice, while expression of terminal differentiation markers was by this time normalized (data not shown).

Caspase 3 has many possible substrates (as reviewed in Fischer et al., 2003). Among these, the protein kinase C δ isoform is an important inducer of keratinocyte differentiation (Ohba et al., 1998; Tibudan et al., 2002) and is subject to direct activation by caspase 3 cleavage (Emoto et al., 1995; Ghayur et al., 1996). Immunoblotting with antibodies against the activated phosphorylated form of PKC-δ (Emoto et al., 1995) revealed that activated PKC-δ, intact as well as cleaved product retaining the recognized auto-phosphorylation site, is present at

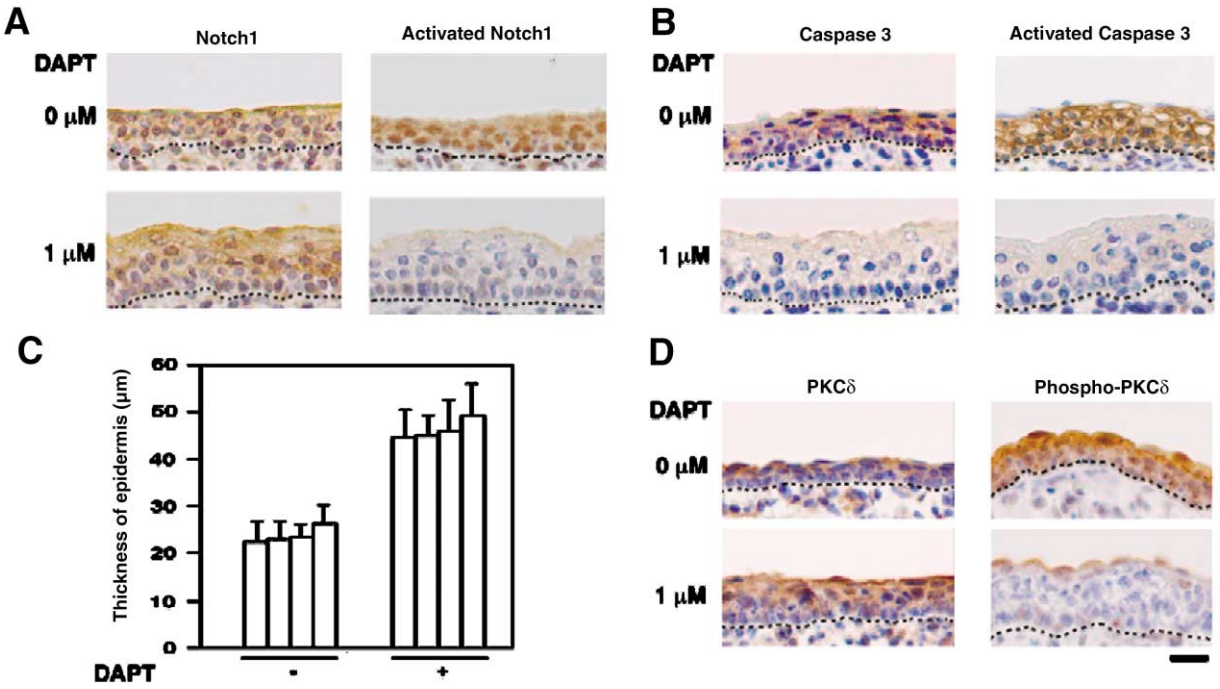

Figure 4. Suppression of Endogenous Notch Activity in Embryonic Skin Organ Cultures by γ-Secretase Inhibition

Dorsal back skins from E13.5 mouse embryos were cultured for 48 hr in medium with the γ-secretase inhibitor DAPT (1 μM) or DMSO vehicle alone. (A, B, and D) Skins were then processed for immunohistochemical analysis with antibodies against the indicated proteins. Similar immunostaining results were obtained with four independent skin cultures treated with DAPT versus four controls. (C) The epidermal thickness of the cultured skins was quantified by a computer-assisted analysis of digitally acquired images of a minimum of ten independent areas per section. Standard deviation of measurements is indicated.

higher levels in embryonic than newborn keratinocytes, especially upon induction of differentiation (Figure 6A). Total PKC-δ amounts were similar in all cases. Poly (ADP-ribose) polymerase (PARP) is another main cleavage target of caspase 3, as well as other caspases, during classical apoptosis (Fischer et al., 2003). Immunoblot analysis showed that there was no detectable PARP cleavage in differentiating embryonic or newborn keratinocytes, while total levels of this protein decreased with differentiation to a similar extent in both types of cells (Figure 6B).

Consistent with the immmunoblotting results, immunohistochemistry with the phospho-specific PKC-δ antibodies produced a much stronger signal in keratinocytes of embryonic than newborn skin (Figure 6C), and the staining was much higher in the skin of wild-type versus *caspase 3* knockout embryos (Figure 6D). Finally, staining with the phospho-specific PKC-δ antibodies was also substantially reduced in cultured embryonic skins treated with the DAPT γ-secretase inhibitor (Figure 4D), consistent with PKC-δ activation, like elevated caspase 3 expression, being dependent on endogenous Notch activity.

Discussion

Genetic analysis of mouse models as well as direct in vivo manipulation experiments have provided significant insights into signaling pathways involved in organ development and morphogenesis. However, with regard to embryonic epithelial cells, little or no information is available on their intrinsic growth/differentiation properties. Contrary to what was expected for cells of an organ undergoing rapid morphogenesis and expansion, we have found that keratinocytes from E15.5 mouse embryos have an intrinsically high propensity to terminally differentiate. This property can be explained, in part, by elevated Notch signaling and increased caspase 3 expression, which we have identified as a transcriptional target of Notch activation.

A global analysis of gene expression indicated that a substantial fraction of genes that are upregulated in embryonic versus newborn keratinocytes are also positively controlled by Notch1 activation. Among these genes there were transcription factors with a known role in development and cell fate determination, such as *En-1*, *Hox3.1*, and *Hox2.2* (Cillo et al., 2001). The functional significance of upregulation of these genes is being explored. Here we focused on *caspase 3*, the only gene involved in programmed cell death that was found to be concomitantly upregulated in embryonic keratinocytes and in response to Notch1.

Caspase 3 is a main effector of both death receptor- and mitochondria-dependent apoptosis (Earnshaw et al., 1999). Most work has focused on regulation of caspase 3 activity at the posttranscriptional level. However, the *caspase 3* gene has also been reported to be differentially expressed in various tissues and developmental times as well as during neuronal apoptosis triggered by various stimuli (Earnshaw et al., 1999). The basis for

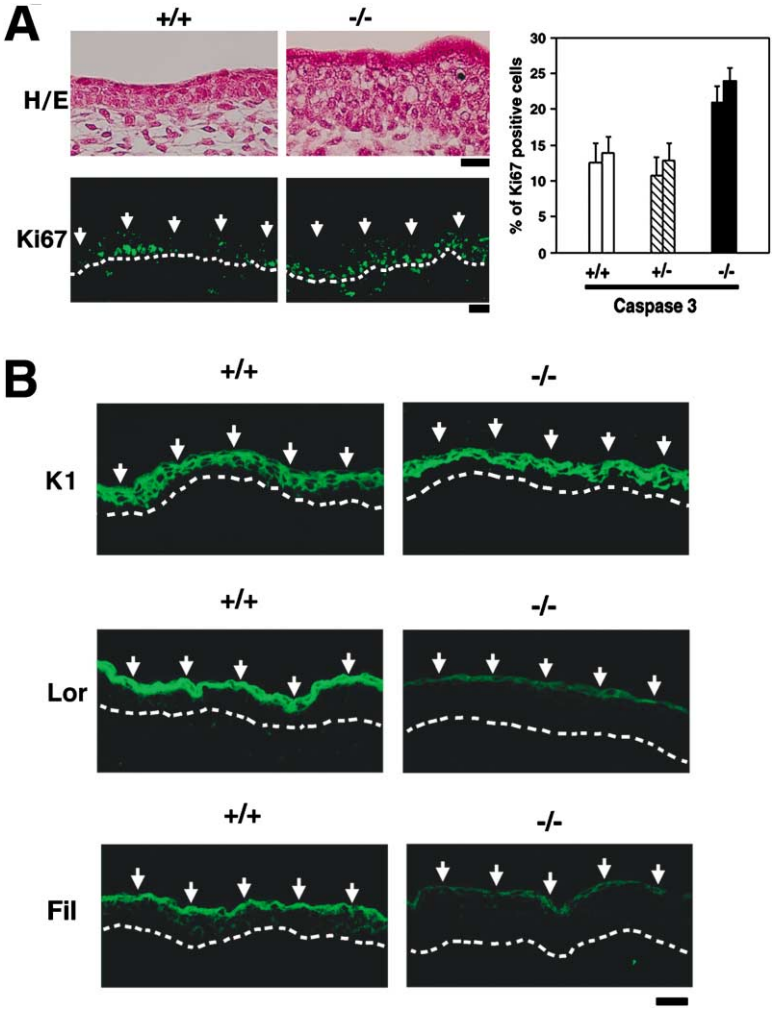

Figure 5. Perturbed Growth/Differentiation Balance of Keratinocytes in Embryonic Skin by Disruption of the *caspase 3* Gene

(A) E15.5 embryos carrying a homozygous disruption of the *caspase 3* gene (Woo et al., 1998) were analyzed in parallel with heterozygous littermates and wild-type control, by H/E staining or immunofluorescence analysis of their epidermis with antibodies against Ki67, a maker of cell proliferation. Bar: 20 μm. Same regions of mouse back skin were analyzed in each case. The percentage of Ki67-positive cells was determined in two animals for each genotype, counting, in each case, at least eight independent fields. The standard deviation from the various fields is indicated. (B) Frozen skin sections from E15.5 embryos with a deletion of the *caspase 3* gene and wild-type controls (+/+) were analyzed by immunofluorescence with polyclonal antibodies against the keratin 1 (K1), loricrin (Lor), and filaggrin (Fil) differentiation markers. Identical exposure and image capture conditions were used for comparison of the *caspase 3*$^{+/+}$ and *caspase 3*$^{-/-}$ skins. Bar: 30 μm.

transcriptional regulation of this gene remains to be elucidated. Our findings indicate that expression of the endogenous caspase 3 gene, as well as transcriptional activity of its promoter, is enhanced in embryonic keratinocytes in a Notch-dependent manner and that the *caspase 3* gene is an upregulated target of Notch1 and Notch2 activation. The elevated levels of endogenous Notch activity in embryonic keratinocytes were not affected by caspase 3 inhibition, indicating that, while controlled by Notch, caspase 3 in turn does not regulate Notch activation. The rat and human *caspase 3* promoter regions have been recently characterized and found to contain several responsive elements for ubiquitous transcription factors such as Sp1 and Ets-1 (Liu et al., 2002), which are fully conserved in the mouse promoter (our unpublished data). This region of the *caspase 3* promoter contains also potential binding sites for RBP-Jκ that do not have, however, a fully conserved consensus sequence. Further biochemical approaches will be required to establish whether, while RBP-Jκ-dependent, upregulation of *caspase 3* by Notch1/2 activation involves direct binding of RBP-Jκ to the *caspase 3* promoter or more indirect mechanisms.

Gene ablation experiments have revealed that caspase 3 plays a critical role during embryonic development in apoptosis of neurons in the brain, with a more restricted requirement for apoptosis of other cell types, such thymocytes or hepatocytes (Kuida et al., 1996; Woo et al., 1998; Zheng et al., 1998). A possible role of caspase 3 beyond classical apoptosis in differentiation of several cell types is currently emerging (De Maria et al., 1999; Fernando et al., 2002; Ishizaki et al., 1998; Sordet et al., 2002; Zermati et al., 2001). An involvement of caspase 3 in keratinocyte differentiation control was previously suggested by pharmacological studies of human keratinocytes in organotypic cultures (Weil et al., 1999). This possibility was later questioned by the fact that no caspase 3 activation was found in differentiating mouse or human epidermal cells derived from newborn or adult skin (Lippens et al., 2000). This is consistent with the fact that, in parallel with increased expression, we could detect caspase 3 activation only in embryonal keratinocytes and skin but not in the newborn.

Disruption of the *caspase 3* gene causes no evident defects in hair follicle development and hair cycle distribution, which depend, to a large extent, on classical apoptosis (Hardy, 1992). Rather, our analysis shows that caspase 3 plays an important role in the balance between growth and differentiation of interfollicular keratinocytes during late stages of embryonic development. In the embryonic epidermis, in spite of the widespread expression and activation of caspase 3, the number of

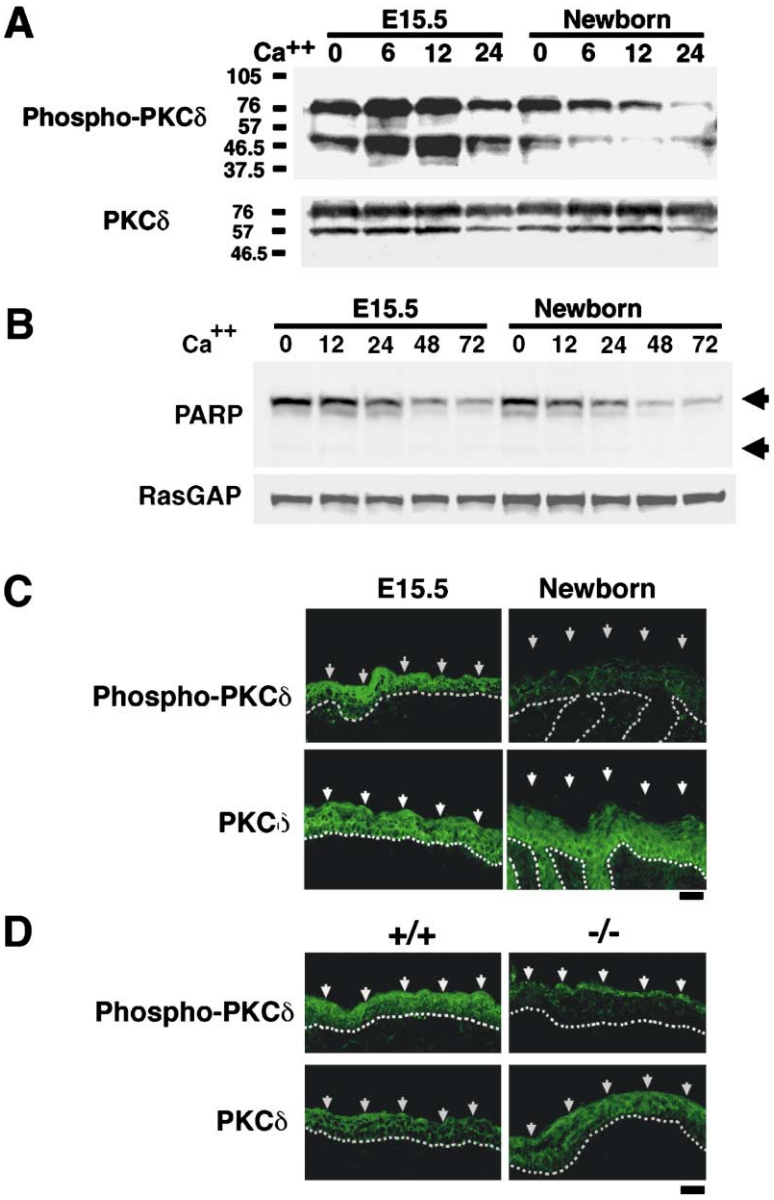

Figure 6. Elevated Levels of Activated PKC-δ in Embryonic Keratinocytes and Skin, Dependent on caspase 3 Function

(A) Total cell extracts from embryonic and newborn keratinocytes under growing conditions (0) and at various times (hr) of calcium-induced differentiation were analyzed by immunoblotting with antibodies against the activated phosphorylated form of PKC-δ (upper panel) or antibodies recognizing the total protein (lower panel).

(B) Total cell extracts from embryonic and newborn keratinocytes under growing conditions (0) and at various times (hr) of calcium-induced differentiation were analyzed by immunoblotting with antibodies against poly (ADP-ribose) polymerase (PARP). PARP is a 116 kDa nuclear protein that is converted by caspase cleavage during apoptosis into a 89 kDa C-terminal catalytic domain and a 24 kDa N-terminal DNA binding domain. Arrows point to the 116 kDa full-length protein and the expected position for the 89 kDa cleavage product.

(C) Frozen skin sections from E15.5 embryos and newborn mice were analyzed by immunostaining with antibodies against the activated phosphorylated form of PKC-δ (upper panel) or antibodies recognizing the total protein (lower panel).

(D) Frozen skin sections from wild-type (+/+) and *caspase 3* negative (−/−) embryos (E15.5) were analyzed by immunostaining with antibodies against the activated phosphorylated form of PKC-δ (upper panel) or antibodies recognizing the total protein (lower panel). Bar: 30 μm.

cells undergoing classical apoptosis is as low as after birth. This suggests that elevated caspase 3 levels in these cells are not sufficient to trigger apoptosis but result in the more selective targeting of substrates that are involved in the keratinocyte differentiation process, including, as we have shown, activation of PKC-δ. In fact, caspase 3 is selectively required in the embryonic epidermis for elevated expression of the "late" terminal differentiation markers of the outermost layers, but not for expression of the "early" markers of the intermediate layers. This specificity of function is in agreement with the substantially higher levels of "late" but not "early" markers in wild-type embryonic versus newborn keratinocytes, and the fact that only the "late" markers are induced by PKC activation while the "early" ones are reduced (Dlugosz and Yuspa, 1993).

While in newborn keratinocytes expression of caspase 3 is usually low, its induction in response to activated Notch1 suggests that caspase 3 could also contribute to the response of these cells to elevated Notch1 activity. Consistent with this possibility, the observed increase in caspase 3 expression is accompanied by production of the caspase 3 activated form as well as phospho-PKC-δ. In addition, levels of phospho-PKC-δ and induction of differentiation marker expression in newborn keratinocytes expressing activated Notch1 are significantly suppressed by inhibition of caspase 3 activity (Supplemental Figure S4). Further analysis of the involvement of caspase 3 in the response of mature keratinocytes to Notch activation will be the topic of future studies.

The surprisingly high commitment to terminal differentiation of embryonic keratinocytes coincides with a crucial time in embryonic skin development when maturation of this organ is being established (Hardman et al., 1998). The somewhat counterintuitive conclusion that embryonic epidermal cells have a higher commitment to differentiation than cells at more mature stages may apply to other epithelial tissues as well. This has important implications for the possible utilization of embryonic

versus newborn or adult tissues as enriched sources of stem cell populations for cell replacement purposes. The intrinsically high commitment to differentiation is also likely to have important consequences for susceptibility to environmental and/or toxic agents and cancer development. Cell populations that terminally differentiate at a fast rate likely provide a smaller target size than cells turning over at a slower rate. Conversely, more deleterious and uncontrolled effects could result from loss of normal growth/differentiation mechanisms in cells that would be otherwise programmed to differentiate at an accelerated rate.

Experimental Procedures

Cell and Organ Cultures

Primary keratinocytes were isolated from mouse embryos at 15.5 days of gestation (E15.5) or from 2-day-old mice of the same strain (Sencar). Epidermis was separated from dermis with 0.8 U/ml dispase (Roche Molecular Biochemicals) overnight at 4°C. Keratinocytes were dissociated by trypsin/EDTA for 5 min at 34°C and plated onto dishes precoated with collagen type IV, and cultured in MEM medium with 4% chelex-treated fetal calf serum, EGF (10 ng/ml), and 0.05 mM $CaCl_2$. For suspension cultures, freshly isolated keratinocytes were incubated on polyhydroxyethylmethacrylate (poly-HEMA)-coated dishes (Sigma, 10 mg/ml) as previously described (Watt et al., 1988). Caspase 1 and 3 activities were blocked by treatment with 5 μM YVAD-CHO or DEVD-CHO peptide inhibitors (Calbiochem). After incubation, cells were washed with medium to remove the inhibitors and plated on collagen type IV-coated dishes.

Organ cultures of back skin E13.5 embryos were prepared as previously described (Foitzik et al., 1999) and incubated at 37°C for 2 days, plus/minus the γ-secretase inhibitor DAPT (N-(N-(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycine t-butyl ester, Calbiochem) dissolved in DMSO.

Antibodies

Affinity-purified antisera against keratin 1, keratin 6, loricrin, and filaggrin antibodies were against the published sequence (Roop et al., 1984). Other antibodies included: goat antibodies against Notch1 and Jagged1; rabbit antibodies against Notch2 and PKC-δ (Santa Cruz Biotechnology); mouse anti-BrdU monoclonals and rabbit-anti caspase 3 antibodies (BD Biosciences); rabbit antibodies against activated caspase 3, the activated form of Notch1, and phosphorylated PKC-δ (#9374) (Cell Signaling Technology); rabbit anti-Ki67 antibody (Novocastra Laboratories); and mouse anti-RasGAP monoclonals (B4F8, Upstate Biotechnology).

Immunostaining of Skin Tissues

Immunofluorescence of frozen skin sections was as previously described (Rangarajan et al., 2001). For immunohistochemical staining, dorsal skin samples were fixed in Bouin's fixative (for cleaved Notch1 antibody) or 4% paraformaldehyde (for other antibodies). Deparaffinized sections were boiled for 20 min in 10 mM sodium citrate (for cleaved Notch1 antibody) for antigen retrieval and incubated in 0.3% H_2O_2 for 30 min to block endogenous peroxidase.

Reporter Assays

The mouse caspase 3 promoter was amplified from total genomic DNA by PCR reaction, generating a fragment extending from the −6617 to the +1 nucleotide position of the caspase 3 gene. The amplified fragment was cloned at the MluI-SmaI site of the pGL3-Luciferase vector. Primary keratinocytes were transiently transfected with various combinations of plasmid DNA as described (Rangarajan et al., 2001). Luciferase activities were normalized for either *Renilla* luciferase activity or total protein concentrations as indicated. All conditions were tested in triplicate wells, and experiments were repeated a minimum of three times.

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