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Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-κB and PPARγ

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Abstract

Establishing an effective epidermal barrier requires a series of coordinated molecular events involving keratinocytes (KCs) within a stratified epithelium. Epidermal maturation depends on convergence of pathways involving components of NF-κB and peroxisome proliferator activated receptor (PPAR) signaling systems that promote terminal differentiation and production of a stratum corneum. The Notch-1 receptor and its ligand Delta-1 have been proposed by others to participate in early events in KC differentiation. Here, we establish differential expression patterns for several Notch receptors and ligands in normal human skin. These immunolocalization findings, together with functional studies demonstrating increased levels of Notch ligand/receptors occurring during the onset of differentiation, prompted use of a soluble Notch ligand, a peptide derived from the most conspicuously expressed ligand in skin, Jagged-1. Exposing submerged KC monolayers to this peptide (JAG-1) in co-presence of elevated calcium ion concentration, produced stratification with loricrin expression. Using a living human epidermal equivalent (EE) model system, when submerged cultures were raised to an air/liquid interface to generate a fully mature epidermis, activation of Notch signaling was detected. Addition of JAG-1 peptide to submerged EEs was sufficient to induce epidermal maturation. Moreover, a soluble decoy Notch inhibitor prevented such differentiation and corneogenesis in human EEs exposed to either an air/liquid interface or to the JAG-1 peptide. In KC monolayers, addition of JAG-1 peptide induced IKKα mediated NF-κB activation, as well as increased PPARγ expression. Immunoprecipitation/Western blot analysis revealed a physical association between the p65 subunit of NF-κB and PPARγ. These results indicate that activation of Notch signaling is necessary for maturation of human epidermis, and activation by a soluble Notch ligand is sufficient to trigger complete KC differentiation including cornification.

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Abbreviations: EE, epidermal equivalent; KC, keratinocyte; JAG, jagged; PBS, phosphate buffered saline; PPAR, peroxisome proliferator activated receptor

Introduction

The most important function of mammalian skin is to generate a barrier preventing desiccation, infection, and damage by toxic agents.[1] This protective shield is created in the outermost layers of epidermis when keratinocytes (KCs) undergo terminal differentiation with formation of stratum corneum.[2] The precise molecular pathways responsible for triggering growth arrest, terminal differentiation and corneogenesis in epidermis remain to be determined.[3,4] Elucidation of this differentiation program is also important from an oncological perspective, as a block in KC differentiation predisposes to development of skin cancer.[5]

The most widely studied inducer of murine and human KC differentiation is the calcium ion. An epidermal calcium ion gradient is present with a low concentration in the proliferative basal cell layer, progressively increasing toward the skin surface.[6] Exactly how this ion gradient is generated or maintained is unknown, but investigators take advantage of this to promote KC proliferation in-vitro using low calcium (0.07 mM), serum-free medium. In general, while mouse-derived KCs are highly responsive to elevated extracellular calcium ion levels to trigger differentiation, human KCs are much less responsive to calcium levels.[7,8]

Notch signaling is a well-recognized, highly evolutionarily conserved complex network that orchestrates cell fate decisions involving proliferation, migration, differentiation and cell death in organisms ranging from insects to humans.[9–11] Notch genes encode transmembrane receptors involved in cell-to-cell developmental interactions. These interactions are mediated by signals exchanged between neighboring cells expressing Notch ligands that bind and activate adjacent Notch receptors (ibid). Drosophila has a single Notch receptor and two Notch ligands, Delta and Serrate.[11] Humans and mice have four Notch homologues (Notch 1–4) and a still undetermined number of Notch ligands divided into Delta-like and Jagged (Serrate homologues). Notch 1 has been implicated in the regulation of differentiation programs in several vertebrate cell types including adipocytes,[12] T-cells (reviewed in [13–16]) retinal neurons,[17,18] erythroid cells,[19] myeloid cells,[20] hair,[21] and

several other cell types. Recent evidence suggests that Notch 1 and Delta-1 participate in the regulation of the early stages of epidermopoiesis. Lowell *et al*.[22] have shown that basal keratinocytes (KC) expressing high levels of Delta-1 tend to be unresponsive to Notch activation and remain within the stem cell compartment, possibly due to Delta-1/Delta-1 homotypic interactions. Conversely, activation of Notch 1 by Delta-1 induces KC contiguous to stem cells to initiate differentiation. More recently, Rangarajan *et al*.[23] have shown that in murine KC Notch 1 is necessary for expression of $p21^{cip1/waf1}$ and exit from the cell cycle. These events occur as KCs begin their differentiation program after a transient amplification. Moreover, constitutively active Notch-1 induces expression of early differentiation markers and prevents expression of late differentiation markers. Taken together, these observations suggest that Delta-1/Notch 1 interaction may promote exit from the stem cell compartment and initial KC differentiation. However, a role for Notch signaling in KC terminal differentiation, or creation of stratum corneum, has not been previously described.

Two transcriptional regulators that are known to play key roles in KC differentiation and maturation, NF-κB and peroxisome-proliferator activated receptor (PPAR)γ, have been suggested to interact with the Notch signaling network. Constitutively active forms of Notch 1 have been reported to inhibit NF-κB through a direct physical interaction[24,25] and to transactivate the p105/p52 NF-κB2 promoter.[26] Very recently, Cheng *et al*.[27] have shown that Notch 1 is necessary for physiologic expression and activity of several subunits of NF-κB in murine hematopoietic precursors. Similarly, Notch 1 is necessary for expression of PPARγ in murine preadipocytes.[12]

NF-κB signaling pathway and the NF-κB-activating kinase IKK are necessary for murine epidermopoiesis, as demonstrated by transgenic mouse models.[28–34] Knocking-out the IKKα subunit revealed a critically important function in epidermopoiesis and corneogenesis. However, when NF-κB activity was reduced by either overexpressing a dominant negative form of IKBα, or disrupting the gene encoding the IKKδ subunit (i.e. NEMO); increased proliferation but no alteration in terminal differentiation or cornification were observed.[31,33] These results strongly indicate that NF-κB activation plays a role in the early stages of KC differentiation, while IKKα is necessary for both early and terminal differentiation. This suggests that IKKα may participate in NF-κB-dependent and independent signaling during epidermopoiesis.[33,34]

Nuclear hormone receptors belonging to the PPAR family are necessary for physiologic epidermopoiesis.[35–37] PPARα and PPARγ heterodimerize with retinoid X receptor, thereby regulating transcription of genes influencing KC differentiation in normal and diseased skin.[35–38] Using a reconstructed epidermis resembling the EE model, addition of PPAR agonists triggered transcriptional events leading to cornification.[36] This suggests that PPAR activation participates in late differentiation events in KC.

Taken together, these observations prompted us to conduct a comprehensive investigation of Notch signaling in human epidermopoieis and its possible functional relationships with NF-κB, IKKα and PPARγ. In this report, we demonstrate the following: (1) biochemical identity, state of activation and specific localization of distinct Notch ligands and receptors in normal human epidermis, confirmed and extended by immunoblot analysis of KCs fractionated by their maturational state; (2) ability of peptides derived from a Notch ligand, Jagged-1,[39] to induce KC differentiation and activate NF-κB signaling in cultured KCs, including IKKα activity when calcium ion concentration was increased from 0.07 mM to 2.0 mM; (3) upregulation of Jagged-1 and specific Notch receptors during induction of differentiation when submerged EEs were raised to the air/liquid interface; (4) ability of a specific inhibitor of Notch signaling to block Notch activation and induction of cornification, following raising of EEs to air/liquid interface; (5) ability of Jagged-1 peptide to trigger terminal differentiation, and corneogenesis in submerged EEs; (6) discovery of a biochemical link between Notch signaling, NF-κB and PPAR activation including a feedback loop whereby PPARγ could bind the p65 subunit of NF-κB. Our data indicate that Notch signaling orchestrates the entire process of epidermopoiesis including cornification, through the sequential activity of different Notch ligands, receptors and downstream pathways.

Results

Localization and characterization of Notch ligand/receptors in normal human skin

Immunostaining cryostat sections of skin with a panel of antibodies directed against various members of the Notch receptor/ligand family revealed consistent results for all skin specimens examined (Figure 1A). Jagged-1 was strongly and diffusely expressed predominantly on the plasma membrane of KCs beginning in supra-basal cell layer (with focal staining of basal layers), and involving multiple cell layers up to, but not always including, the granular cell layer, producing a chicken-wire appearance (upper left panel). There was only rare to absent Jagged-1 staining of either granular cell or the stratum corneum layers. Jagged-2 expression was weak to undetectable (data not shown). Notch 1 immunoreactivity was primarily confined to KCs in lower and mid level epidermal layers, with only faint and focal staining of granular cell layer and stratum corneum (Figure 1A, upper middle panel). Notch 1 was detected in these KCs with a more stippled staining pattern of the plasma membrane, and while there was minimal cytoplasmic staining, occasional positive nuclear staining was observed (white arrows). Notch 2 was detectable as well, with primarily cytoplasmic staining and distribution clearly limited to KCs in the basal layer (Figure 1A, upper right panel).

Immunostaining to detect Notch 3 revealed primarily localization to KCs in mid and upper layers of epidermis. Plasma membrane staining was more prevalent than cytoplasmic staining, and no consistent nuclear patterns were observed (Figure 1A, lower left panel). In contrast to Jagged-1 and Notch 1 staining, Notch 3 staining included more diffuse and consistent plasma membrane reactivity by KCs near the granular cell layer. In addition, Langerhans

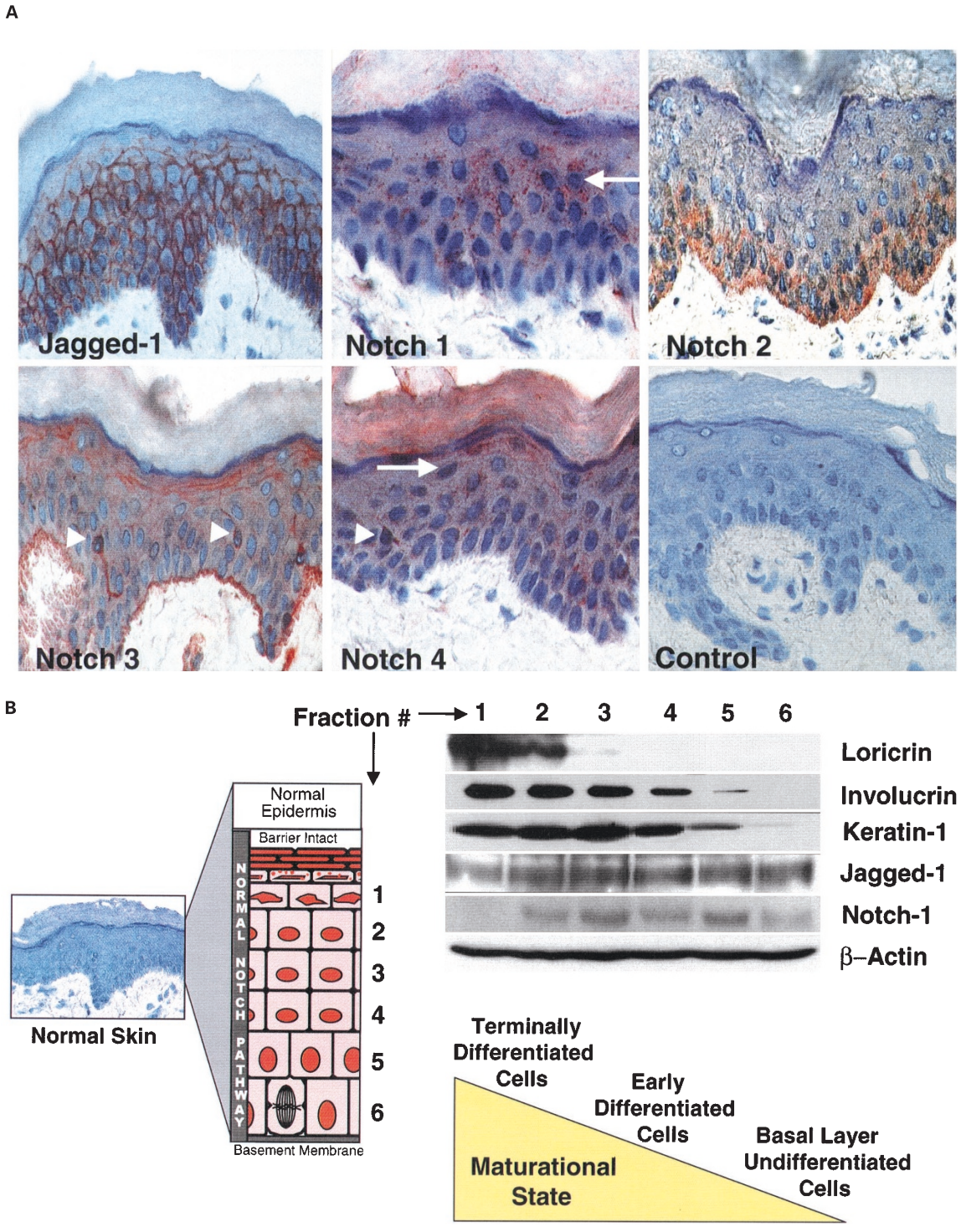

Figure 1 (**A**) Immunohistochemical analysis of normal human skin for Jagged-1 (upper left panel), Notch 1 (upper middle panel), Notch 2 (upper right panel), Notch 3 (lower left panel), and Notch 4 (lower middle panel). Control goat anti-serum was used to identify non-specific staining which was insignificant (lower right panel). All magnifications ×100. (**B**) Immunoblot analysis for KCs located at different levels of epidermis – (fractions 1 and 2) including most superficial layers representing highly differentiated KCs (stratum corneum, granular cell layer), as well as mid-epidermal layers (stratum spinosum cells – fractions 3 and 4), and basal cell layers (fractions 5 and 6). KCs were fractionated according to their maturational state, proteins extracted followed by Western blot analysis to detect loricrin, involucrin, keratin-1, Jagged-1, and Notch 1. β-actin is included as a loading control

cells were also positive for Notch 3 (arrowheads). Intense signal in the basement membrane was also observed. Notch 4 signal was primarily localized in the suprabasal layers and included plasma membrane staining of KCs near the

granular cell layer (Figure 1A, lower middle panel). The Notch 4 staining also included peri-nuclear staining of the cytoplasm, and occasional nuclear staining (white arrows), as well as Langerhans cells (arrowheads). In contrast to Jagged-1 expression, antibodies to Delta-like 1 and Jagged-2 revealed weak to negative staining in suprabasal layers (data not shown), with the isotype control antibody revealing no significant staining (Figure 1A, lower right panel).

Besides these epidermal-based staining profiles for Notch ligand/receptors, we also examined adnexal structures such as hair follicles, sweat glands, and underlying dermis, including dendritic cells and endothelial cells lining blood vessels. Table 1 summarizes these immunolocalization results for Notch ligand/receptors.

To confirm and extend these immunohistochemical-based studies, each cell layer of epidermis was characterized by Western blot analysis. Six different bands from Percoll gradients were initially analyzed for loricrin levels. Intact human skin contains a band of loricrin positive cells in outermost layers of epidermis representing terminally differentiated KCs (Figure 1B). Presence of loricrin in the most buoyant bands (fractions 1 and 2) of Percoll gradients represents KCs from the granular cell layer and stratum corneum; whereas KCs from mid-level stratum spinosum (fractions 3, 4) and basal cell layers (fractions 5, 6) were devoid of loricrin (Figure 1B). Involucrin was detected in suprabasal layer fractions being most prominent in fractions 1 to 3 (Figure 1B). All cell layers except basal cells (fraction 6) expressed the suprabasal marker – keratin-1. When identical protein fractions were analyzed for Jagged-1 content, it was detected in all cell fractions, in agreement with immunostaining. In these fractions, Jagged-1 appeared as a doubled, possibly related to post-translational modification. Antigen pre-absorption experiments showed both bands to be specific (data not shown). Notch 1 was detectable in its mature, transmembrane form ($\approx$120 kd) and was most abundant in the more immature fractions. We could not detect Notch 2 by Western blotting with the only antibody that gave us positive immunostaining. Notch 3 and 4 could not be reproducibly extracted in soluble form from these fractions. Thus, using immunostaining and immunoblot analysis of KCs fractionated by maturational state, it appeared that Jagged-1 could potentially participate in both early and late stages of KC differentiation, possibly by activating different combinations of Notch receptors in different layers.

Induction of terminal differentiation/cornification in epidermal equivalents is accompanied by increased expression of Notch ligands and receptors

We explored the relationship between induction of differentiation and Notch pathway in an experimental model closely resembling the *in vivo* state. For these experiments a living EE model system was used, where KCs are maintained in a multilayered or stratified configuration that closely resembles normal human skin. EEs consist of KCs grown to produce a multi-layered structure that are initially prepared using submerged KCs. Under these conditions, approximately 3–5 layers are produced with a progressive flattening of top level KCs, which may include occasional cells with keratohyalin granules, and only focal appearance of loricrin positive cells without corneocytes (Figure 2, upper panels). When cultures reach this stage, they can either, continue to be maintained as submerged cultures, or lifted-up and maintained on a support to create an air/liquid interface. After lifting of cultures, multilayered KCs are exposed to air, with the subjacent membrane being moistened and feeding occurring from underlying medium. As previously reported,[40,41] lifting of previously submerged cultures, triggers the terminal differentiation process. Examination of day 4 air/liquid interface cultures reveals a greater flattening and stratification of all KC layers, with appearance of a granular cell layer, diffuse loricrin positivity creating a continuous band-like effect at the top layer of viable KCs, accompanied by a thick stratum corneum including stacked-layers of corneocytes (Figure 2, lower panels).

To determine if Notch ligand / receptors were being modulated by air/liquid interface-induced triggering of terminal differentiation, immunostaining of these EEs was performed. Formalin-fixed paraffin embedded sections of EEs before being lifted revealed focal and faint plasma membrane staining to detect Jagged-1 in mid-level KCs (Figure 3A). Similarly, staining to detect Notch- 1, Notch 3, and Notch 4 revealed only faint, localized signal, primarily confined to plasma membranes of KCs in submerged EEs. However, 5 days after being lifted, cultures at air/liquid interface revealed enhanced expression of Jagged-1, as well as Notch receptors. Staining profiles in these lifted cultures included an increase predominantly in the cytoplasmic compartment with some plasma membrane staining, but no detectable nuclear

Table 1 Summary of non-epidermal localization of Notch receptors and Jagged-1 in normal skin

	Notch 1	Notch 2	Notch 3	Notch 4	Jagged-1
Hair Follicle:					
Outer Root Sheath	Positive	Positive	Positive	Positive	Positive
Inner Root Sheath	Positive	Positive	Positive	Positive	Positive
Matrix	Negative	Negative	Focally Positive	Focally Positive	Positive
Dermal Papillae	Negative	Negative	Negative	Negative	Negative
Sweat Gland:					
Ductal Epithelial	Positive	Positive	Positive	Positive	Positive
Secretory Glands	Negative	Positive	Negative	Negative	Positive
Dermal Dendritic Cells	Focally Positive	Negative	Positive	Positive	Negative
Endothelial Cells	Negative	Positive	Positive	Focally Positive	Negative

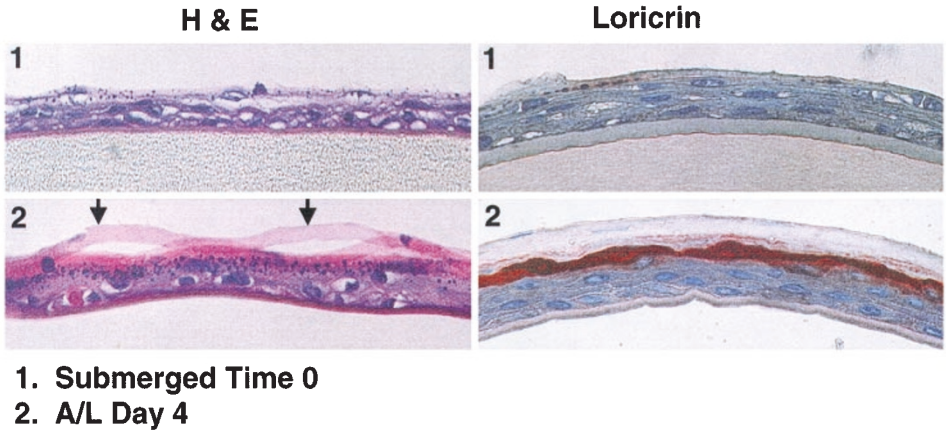

Figure 2 Routine light microscopic appearance (H & E; left side panels) and immunostained appearance (loricrin, right side panels) of EEs before (panels 1) and after being raised to an air/liquid interface (panels 2). All magnifications $\times 250$

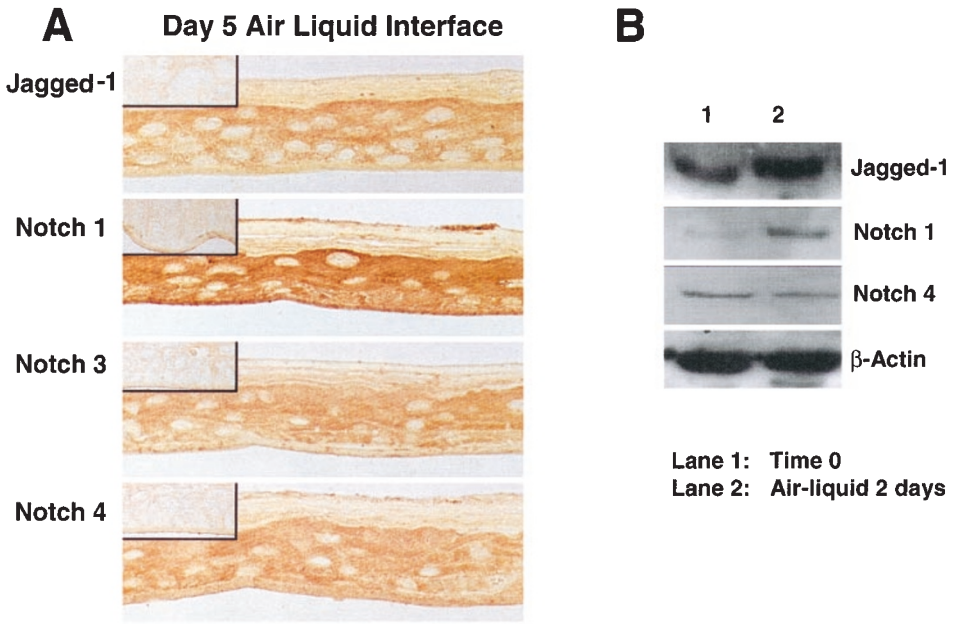

Figure 3 (**A**) Immunohistochemical analysis of EEs before (inserts) and 5 days after being raised to air/liquid interface. Note the triggering of terminal differentiation in the lifted cultures is associated with increased levels of expression for Jagged-1, as well as Notch 1, 3, and 4 compared to the submerged cultures. (**B**) Immunoblot analysis of EEs before (lane 1), and 2 days after (lane 2), being raised to the liquid/air interface. Note the triggering of terminal differentiation in the lifted cultures is associated with increased levels of expression for Jagged-1 and Notch 1, but not Notch 4. β-actin is included as a loading control

localization. In addition to these immunolocalization studies, immunoblot assays were also performed using cell extracts derived from KCs in EEs before and after being lifted to an air/liquid interface. Figure 3B reveals that levels of Jagged-1 and Notch 1 (but not Notch 4) were increased after induction of terminal differentiation by exposure to an air/liquid interface. Given the enhanced expression of Jagged-1 and Notch receptors during EE differentiation and the strong expression of Jagged-1 in normal human epidermis, we sought to determine if JAG-1 peptide could simulate air/liquid interface induced triggering of terminal differentiation and cornification as described in the following sections.

JAG-1 peptide triggers terminal differentiation in cultured KCs

To determine if activation of Notch pathway triggers terminal differentiation, KC monolayers were treated with two different active peptides, i.e. JAG-1 or R-JAG, as well as a scrambled control peptide (SC-JAG). Unlike mouse KCs in-vitro that undergo terminal differentiation in response to elevated extracellular calcium ion concentration,[7] human KCs behave differently. Raising extracellular calcium ion concentration to 2 mM by itself does not induce stratification of monolayers, or induce significant levels of proteins involved in terminal differentiation - loricrin or involucrin (Figure 4, left side panels). As early as 3 days following addition of JAG-1 or R-JAG

peptide to KC cultures maintained in 2 mM calcium, KCs began piling-up to form 3-dimensional stratified clusters including appearance of keratohyalin granules (Figure 4, left side and middle panels). Moreover, immunostaining demonstrated strong induction of both loricrin and involucrin with a gradient of intensity in staining, beginning with mild to moderate staining of KCs at the bottom layer, and increasing in intensity by KCs that had moved-up to the top layers within those stratified clusters (Figure 4, right side and middle panels). Semiquantitative assessment revealed between 25 to 35% of all KCs were induced to express loricrin or involucrin after exposure to JAG-1 or R-JAG peptides. Western blot analysis confirmed the induction of loricrin and involucrin by cultured KCs exposed to JAG-1 peptide (40 μM; 72 h) in the co-presence of elevated calcium (2 mM), but not by the SC-JAG peptide (Figure 4, upper right panel).

When Notch signaling components were stained in cultured KCs, Notch 1 was also predominantly localized to plasma membranes in untreated KC cultures resembling KCs in mid-layers of epidermis in normal skin (data not shown). Twenty-four hours after addition of JAG-1 peptide, Notch 1 was redistributed to the cytoplasm with occasional nuclear positivity (data not shown). Similar results were obtained with Notch 3 and 4. JAG-1 peptide treatment also was accompanied by loss of plasma membrane staining for Jagged-1, with focal peri-nuclear cytoplasmic re-distribution. This data indicates JAG-1 treatment can induce cellular redistribution of Notch 1, 3, and 4, suggesting this ligand can activate all these Notch receptors in KCs.

JAG-1 peptide triggers terminal differentiation/ corneogenesis in living EE

To confirm and extend the ability of JAG-1 peptide to induce terminal differentiations of cultured KC monolayers, the living EE model system was employed. In this system, submerged multi-layered KC cultures need to be raised to air/liquid interface to produce a stratum corneum. Strikingly, the addition of JAG-1 peptide alone, to submerged cultures, was sufficient to induce a stratum corneum in the absence of air/liquid interface (Figures 5A, B, D). The addition of a scrambled control (SC-JAG) failed to induce a stratum corneum. To further establish that Notch signaling was involved in complete maturation of epidermis, a specific soluble Notch inhibitor was employed. A soluble human Notch 1 decoy, rh11-12, that mimics the ligand binding region of human Notch 1[12,23] abolished terminal differentiation and cornification of EE induced by exposure to an air/liquid interface (Figure 5C) and blocked JAG-1 mediated induction of KC maturation under submerged EE culture conditions

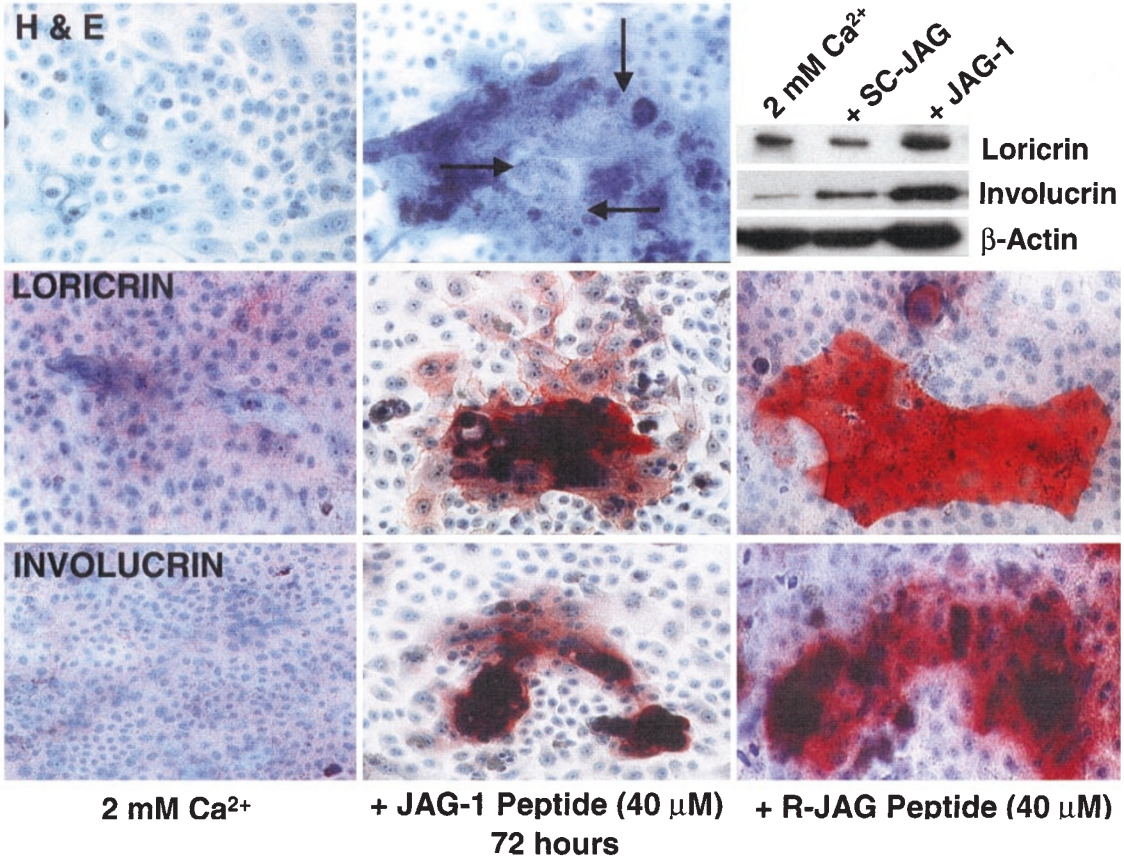

Figure 4 Light microscopic and immunohistochemical analysis of cultured KCs. KC monolayers were triggered to stratify and express keratohyalin granules (upper panel – H & E; arrows), and markers of terminal differentiation – loricrin (middle panels), involucrin (lower panels), after addition for 72 h of JAG-1 peptide or R-JAG peptide with elevated Ca^{2+} concentration (2 mM). Western blot analysis (upper right panel) of KC extracts reveal enhanced loricrin and involucrin levels after exposure for 72 h to JAG-1 peptide, but not by SC-JAG peptide. β-actin is included as a loading control

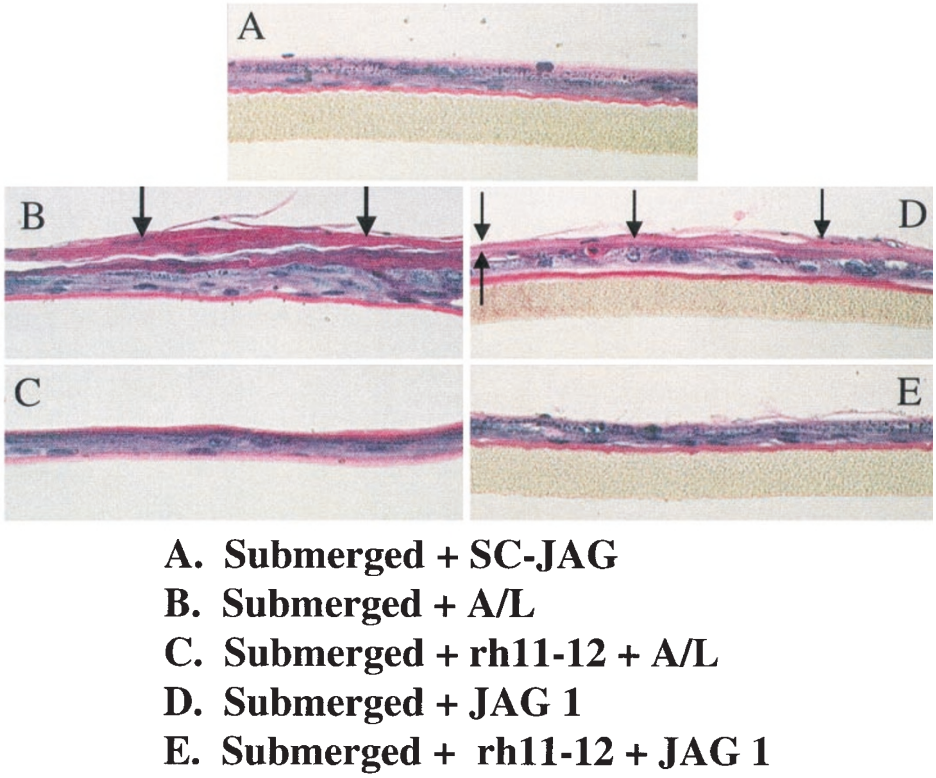

Figure 5 Microscopic appearance of living EEs (day 5) either submerged with SC-JAG (**A**) or after being raised to the air/liquid interface (**B**), and after being raised to air/liquid interface in the presence of soluble inhibitor rh11-2 (**C**). Treating submerged cultures with JAG-1 peptide in the absence (**D**) or presence (**E**) of soluble Notch inhibitor rh11-12 (4 μM). Arrows denote production of (stratum corneum) in panels B and D

(Figure 5D, E). These results indicate that Notch signaling is necessary for correct differentiation of EEs exposed to an air/liquid interface.

Ability of JAG-1 peptide to activate NF-κB signaling

Since NF-κB primarily regulates KC differentiation,[28–34] we investigated whether JAG-1 peptide could influence NF-κB activity. First, KCs were transfected to allow use of a luciferase-based NF-κB reporter assay, and NF-κB activity determined using KCs maintained in either a low calcium (0.07 mM) or high calcium (2 mM) medium. Figure 6A reveals that exposure of KCs for 18 h to elevated calcium ion concentrations did not, by itself, induce NF-κB activity. Addition of JAG-1 peptide at the indicated concentrations above 1 μM (i.e. 10, 40 and 100 μM) did trigger NF-κB activity after 6 h in high calcium, but not low calcium conditions. Treatment of KCs with TNF-α served as a positive control for NF-κB activation under both low and high calcium conditions. To verify the specificity of JAG-1 induced NF-κB activation, a retroviral vector with a dominant negative mutant (i.e. IKBα DN) for NF-κB activity was utilized. Compared to KCs infected with an empty retroviral construct (i.e. linker-control), co-infection with IκBα DN retrovirus demonstrated that blocking NF-κB activity inhibited the ability of either TNF-α or JAG-1 to activate NF-κB dependent transcription. Thus, these results indicate that the calcium ion can be regarded as a co-factor, important for induction of NF-κB activity triggered by JAG-1 peptide.

Activation of NF-κB in KCs may be mediated by p50 and/or p65 subunits[42] that become liberated from their cytoplasmic location and rapidly translocated to the nucleus. To assess the molecular composition of the NF-κB dimers mediating the transcriptional activation detected by luciferase-based assays, nuclei from cultured KCs were isolated before and after JAG-1 exposure followed by Western blot analysis. Eighteen hours following an increase in calcium ion concentration only a low intranuclear amount of p50 was detected, accompanied by a barely detectable level of p65 (Figure 6B). However, addition of JAG-1 peptide induced markedly enhanced intranuclear levels of both p50 and p65 subunits at 30 min and 1 h. Rapid induction of these heterodimers was followed by a subsequent decline in their levels at 2, 6, and 24 hours; being greater for p50 compared to the p65 subunit. In some experiments, levels of p50 and p65 peaked at 1 h rather than 30 min after exposure to JAG-1 peptide.

To confirm and extend the luciferase-based assay of NF-κB activity, and the intranuclear immunoblot analysis, we measured NF-κB DNA binding activity, using EMSA. Nuclear extracts were prepared and incubated with a specific probe containing NF-κB binding sites (Figure 7A). These results indicated that JAG-1 peptide could activate NF-κB DNA-binding complexes including p50/p65 heterodimers as well as p50/p50 homodimers.

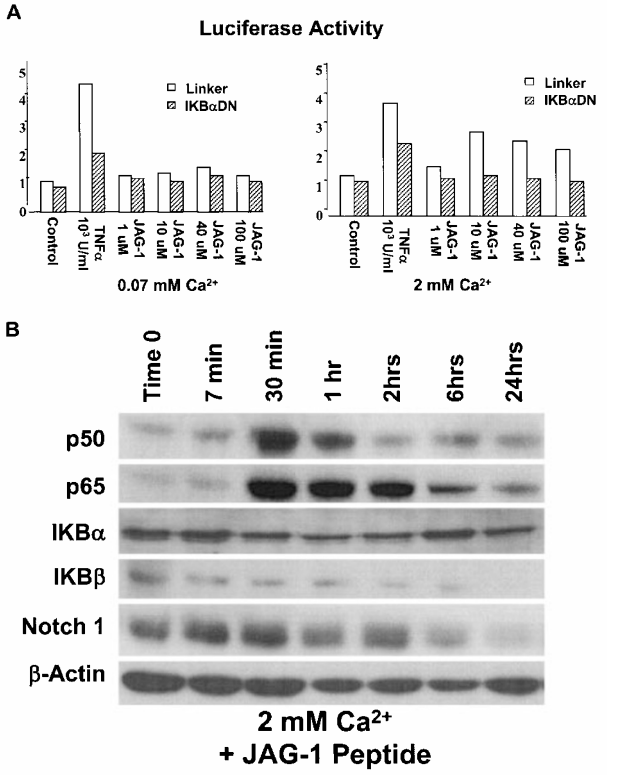

Figure 6 (**A**) NF-κB luciferase-based construct reveals prominent induction of NF-κB activity by JAG-1 peptide (at the indicated concentrations) in co-presence of high calcium (2 mM) ion levels after 6 h, but not in medium containing a low calcium ion concentration (0.07 mM). (**B**) Western blot analysis of nuclear (NF-κB subunits-p50, p65) and cytoplasmic extracts (IκB proteins, Notch 1) in KCs maintained in 2 mM calcium ion containing medium before, and after, addition of JAG-1 peptide. Upper panels reveal markedly elevated intranuclear levels for p50 and p65 after 30 min and 60 min of stimulation, which diminished at subsequent time points. Middle panel reveals degradation of IκBα, which began 30 min following treatment with JAG-1 peptide. IκBβ was only present at relatively low levels compared to IκBα, and was degraded beginning at the 7 min time point. Lower panel reveals the degradation of Notch 1 by the JAG-1 peptide after 7 min and 30 min treatment, followed by the degradation of Notch 1 at the 6 h time point with progressively lower levels at the 24 h time point. β-Actin levels used to confirm equivalent protein loading

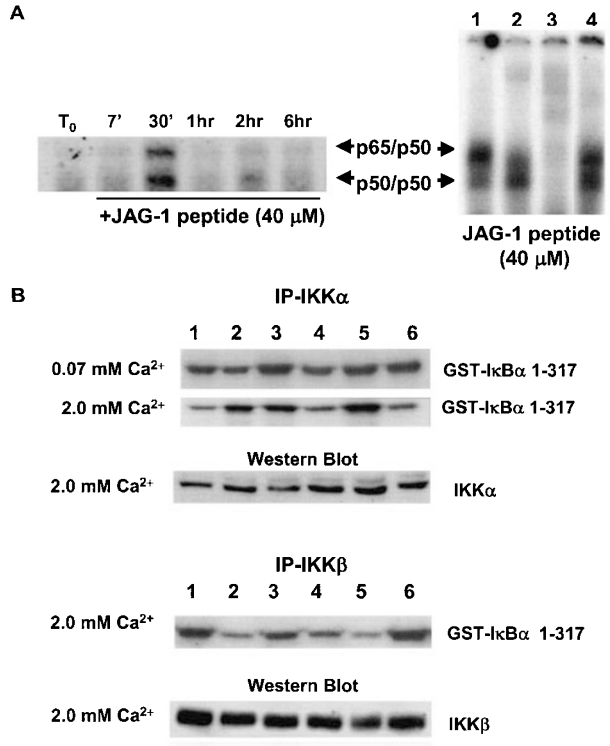

Figure 7 (**A**) EMSA analysis demonstrating the presence of both homodimers (p50/p50) and heterodimers (p65/p50) for NF-κB subunits induced by JAG-1 peptide after 30 min using 2 mM calcium ion conditions (left side). Supershift analysis performed by pre-incubating nuclear extracts obtained after JAG-1 peptide exposure with either IgG isotype control Ab (lane 1) and mAbs against p50 (lane 2), p65 (lane 3), and Rel-B (lane 4) right side. Note the loss of the upper band in lane 2 using the p65 Ab, and loss of both upper and lower bands by the p50 Ab; whereas no change in either band was induced by the Rel-B Ab. (**B**) IKKα immunoprecipitation followed by kinase activity assay using KCs in either low (0.07 mM) or high (2.0 mM) calcium ion concentration, before or after indicated stimuli. Lane 1: no stimulus; Lanes 2, 3: JAG-1 (40 μM) 30 min to 60 min respectively; Lane 4: SC-peptide (40 μM) 60 min; Lane 5: R-Jag (40 μM) 60 min; Lane 6: TNF-α (10^3 U/ml) 60 min. Note the higher constitutive levels of activity for KCs maintained in low calcium medium but without significant induction following the stimuli. However, in the high calcium medium, addition of JAG-1 peptide and R-JAG peptide stimulated the kinase activity, whereas the SC-peptide and TNF-α, did not induce IKKα kinase activity. Western blot analysis for IKKα and IKKβ revealed only slight changes in these protein levels after exposure to either JAG-1 or R-JAG peptides (lower panels)

In mouse hematopoietic precursors, Notch-1 is required for the expression of several NF-κB subunits and thus for NF-κB activity.[27] However, the time course of NF-κB activation induced by JAG-1 in KC suggests that this is a non-transcriptional event. Thus, we investigated whether JAG-1 affects IκB proteins. The most prominent inhibitory protein in KC extracts was IκBα (Figure 6B). Addition of JAG-1 peptide triggers IκBα degradation that initially becomes apparent after 30 min of treatment (Figure 6B). The relatively low constitutive level of IκBα was reduced after 7 min and continued to reduce between 30 min and 6 h levels and became undetectable after 24 h. Notch-1 itself has been previously identified as being capable of binding NF-κB.[25] There was no significant change in the levels of the $\approx$120 Kd form of Notch 1 during the initial 2 h of JAG-1 peptide exposure, but 6 and 24 h after JAG-1 addition, Notch 1 decreased to barely detectable levels. This suggests that the signaling cascade activated by JAG-1 is limited by feedback mechanisms that control the amount of Notch 1 protein. Taken together, these data indicate that JAG-1 activates NF-κB through a mechanism that may involve phosphorylation of IκBα and possibly IκBβ, rather than through the dissociation of a Notch-NF-κB cytoplasmic complex.[43]

Characterization of IKK complex following JAG-1 peptide activation

The protein kinase complex that phosphorylates IκBs contain two catalytic subunits (i.e. IKKα and IKKβ) as previously described.[29–33,44] Since IKKα appears to be critically important for terminal differentiation and corneogenesis of

epidermis through both NF-κB-dependent and independent mechanisms, the ability of JAG-1 peptide to activate the kinase activity of IKKα was investigated. To measure activation kinetics, KC extracts before and after various time points following addition of JAG-1 peptide, or a scrambled control (SC-JAG) peptide, were immunoprecipitated with an anti-IKK ab, and incubated with a glutathione S-transferase – IκBα (1 – 54) as a substrate. Figure 7 reveals that while TNF-α or the SC-peptide had little effect on the activation of IKKα, addition of either JAG-1 peptide or R-JAG peptide did activate the kinase activity of IKKα after 30 – 60 mins. This increase in kinase activity was not accompanied by any significant change in the amount of IKKα present immediately following the stimulation by JAG-1 peptide (see Western blot analysis, Figure 7), suggesting that soluble Notch ligands can directly trigger IKKα activation through non-transcriptional mechanisms. In contrast to the IKKα results, neither JAG-1 nor R-JAG peptides could induce kinase activity immunoprecipitable with IKKβ antibodies compared to no treatment (compare lanes 1 *versus* 2, 3 and 5). While the scrambled control peptide (lane 4) also failed to activate IKKβ, addition of the cytokine TNF-α did increase the IKKβ associated kinase activity (lane 6). No consistent increase in the protein levels of IKKβ were observed immediately after exposure to JAG-1 by Western blot analysis (Figure 7).

PPARγ is induced by JAG-1 and forms a complex with NF-κB

Besides a role for NF-κB in regulating epidermal differentiation, it has been established by others that PPAR family members may also influence KC differentiation.[35–38] PPARγ has been shown to be induced by Notch-1 in 3T3 L1 preadipocytes.[12] Figure 8A reveals that addition of JAG-1 peptide induces PPARγ levels as detected by Western blot analysis. These increases were rapid (being evident at 30 min). Levels of PPARγ continued to be elevated for 6 h before returning to baseline levels at 24 h, whereas PPARα appeared to decrease beginning after 2 h exposure and then also returning to baseline by 24 h. Exposure of EE to an air/liquid interface induces PPARγ and this effect is completely abolished by rh11-12 (Figure 8B). Since induction of NF-κB DNA binding was also transient (Figures 6 and 7), and because PPARγ has been observed in other systems to inhibit NF-κB activity,[45] we determined if there was a physical association between PPARγ and p65. Figure 8C reveals Western blot, and immunoprecipitation followed by Western blot analysis, for p65 and PPARγ under 2 mM calcium ion conditions. After 24 h of exposure to 2 mM calcium ion levels, there is low constitutive levels of p65 by Western blot (Figure 8C, upper panel, lane A), but after exposure to JAG-1 for 60 min (Figure 8C, lane B), and 120 min (Figure 8C, lane C), but not SC peptide for 60 min (Figure 8C, lane D) or 120 min (Figure 8C, lane E), intranuclear p65 levels were increased. When nuclear proteins were initially immunoprecipitated using an anti-PPARγ ab, the subsequent immunoblot using anti-p65 ab revealed a physical association between p65 and PPARγ when KCs were treated with JAG-1 peptide at the 60 min (Figure 8C, lane B) and 120 min (Figure 8C, lane C) time points. Either no or minimal binding between p65 and PPARγ was detected in KCs not exposed to JAG-1 peptide (Figure 8C, lane A) or after exposure to SC-peptide (Figure 8C, lanes D,E).

Finally, a reciprocal analysis was also performed. In these cultures, nuclear extracts were initially probed by Western blot analysis to detect PPARγ which revealed (Figure 8C, lower panels) low, but detectable constitutive levels for PPARγ in the 2 mM calcium ion containing cultures (lane A), which was further increased after 60 min (lane B), and 120 min (lane C) addition of JAG-1 peptide, but not after SC-peptide (60 min, lane D; 120 min, lane E). When nuclear extracts were initially immunoprecipitated with an anti-PPARγ ab, and then immunoblotted to detect p65, no constitutive co-binding was identified (lane A). Upon addition of JAG-1 peptide, increased physical association between PPARγ and p65 was detected at 60 min (lane B) and 120 min (lane C), but not after addition of SC-peptide (lanes D,E). Taken together both sets of results portrayed in Figure 8C demonstrate not only that JAG-1 induces p65 and PPARγ, but that these proteins physically interact within the nucleus of KCs exposed to JAG-1 peptide.

Discussion

Various Notch receptors and ligands are expressed during the development of rodent hair follicles[21,46] and bird feathers.[47] A role for Notch 1 and Delta-like-1 in regulating cell fate in the basal layer of human epidermis has been recently suggested.[22,23] However, a pivotal role for Notch signaling in orchestrating the entire KC differentiation program, including terminal differentiation and creation of stratum corneum, has not been previously described using human KCs. In this report we provide a detailed molecular analysis of location, characterization, and function for Notch ligands and multiple Notch receptors amongst KCs present in various cell layers of normal human skin. The results demonstrate that Jagged-1 may be able to influence both early and late stages of differentiation within the epidermal compartment, because KCs bearing Jagged-1 on their plasma membrane were in physical contact with basal layer undifferentiated KCs below, and partially differentiated KCs above. Not only were Jagged-1 and Notch receptor levels increased during the differentiation process, but triggering of KC terminal differentiation and cornification was found to be dependent on activation of the Notch pathway.

Addition of JAG-1 peptide could influence the cellular distribution of multiple Notch receptors, and activated NF-κB signaling including the kinase activity of IKKα. We selected JAG-1 peptide (and related R-JAG peptide) because of the prominent expression of Jagged-1 *in vivo*, and its enhancement during differentiation. The spatial distribution of Notch receptors and ligands in normal skin suggested that sequential activation of different Notch receptors may coordinate the KC differentiation program. Delta-1, which is expressed exclusively in basal layers, has been reported to maintain human epidermal stem cells in an undifferentiated state through homotypic interactions, and to trigger initial differentiation through Notch receptor activation in basal KC that exit the stem compartment.[22]

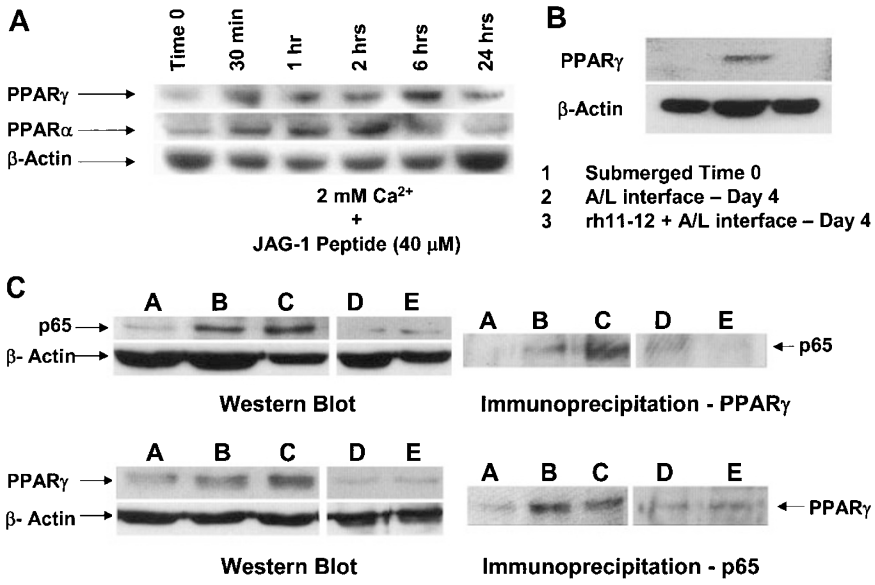

Figure 8 (**A**) Immunoblot analysis of nuclear extracts of KCs maintained in 2 mM calcium ion containing medium before, and after, the addition of JAG-1 peptide to detect time course for induction of PPARγ and PPARα. Note that intranuclear levels of both PPARγ (upper panel) and PPARα (mid panel) are rapidly induced by addition of JAG-1 peptide with the highest level detected at 6 h for PPARγ, compared to the highest levels detected for PPARα at 2 h. β-actin levels (lower panel) used to confirm equivalent protein loading. (**B**) Western blot and immunoprecipitation/Western blot analysis demonstrating physical association between p65 and PPARγ. Nuclear extracts were either directly assessed by Western blots for p65 (upper panels) or PPARγ (lower panels); or also immunoprecipitated with an anti-PPARγ mAb, followed by Western blot to detect p65 using KCs maintained in 2 mM calcium ion concentration before and after exposure to JAG-1 peptide or SC peptide; or were immunoprecipitated with an anti-p65 mAb followed by Western blotting to detect PPARγ as indicated. Lane A: no stimulus. Lanes B,C: JAG-1 peptide (40 μM) treatment for 60 min and 120 min, respectively. Lanes D, E: SC-peptide (40 μm) treatment for 60 and 120 min, respectively. Note the Western blot in upper panel reveals that JAG-1 peptide induces p65 intranuclear levels of p65 after exposure to JAG-1 peptide, but not SC-peptide. Immunoprecipitation with anti-PPARγ ab followed by Western blot analysis reveals physical association between PPARγ and p65 after exposure to JAG-1 peptide but not SC-peptide. Conversely, lower panel reveals that low constitutive PPARγ levels are increased by exposure to JAG-1 peptide but not SC-peptide; and the immunoprecipitation using anti-p65 ab followed by Western blot analysis confirms physical association between PPARγ and p65. Equal loading of lanes for the Western blots was confirmed by β-actin levels

Our immunohistochemical data suggest that Notch 1 and 2 may be involved in this process, possibly in cooperation. Rangarajan *et al*.[23] have shown that constitutively active Notch 1 can trigger cell cycle arrest in KCs and induce early differentiation markers while preventing the expression of late differentiation markers. However, expression of constitutively active Notch 1 may not be subject to physiologic feedback mechanisms that normally limit the levels and activity of wild type Notch 1, and cannot reproduce the sequential activation of several Notch receptors. Since all four known Notch receptors are expressed in anatomically distinct layers of human skin, rather than overexpressing a single Notch receptor, we sought to use a complementary approach with a more physiological stimulus that could engage multiple Notch receptors. Jagged-1 derived peptides, but not the scrambled control peptide, were clearly capable of activating Notch signaling, including cellular redistribution of multiple Notch receptors.

Interestingly, prolonged exposure to JAG-1 peptide was followed by Notch 1 downregulation, suggesting that wild-type Notch 1 may be subject to feedback mechanisms that limit the duration of its activity, such as ubiquitination.[48] JAG-1 peptide and structurally related R-JAG peptide induced a differentiation switch in submerged living EEs, mimicking the unknown signal induced by exposure to air/liquid interface. These effects were clearly dependent upon Notch activation, since they could be abolished by a soluble decoy Notch receptor consisting of EGF repeats 11 and 12 derived from human Notch 1.[39] Furthermore, the same Notch 1 decoy abrogated the differentiation program in living EEs raised to an air/liquid interface. This indicates that endogenous activation of Notch signaling is required not just for differentiation induced by Jagged-derived peptides, but also for spontaneous differentiation in the absence of soluble Notch ligands. Further studies are warranted to elucidate specific distal molecular events that mediate terminal differentiation in response to soluble Notch ligands, and relative roles of various Notch receptors. Based on the literature and the anatomical distribution of Notch receptors and ligands in human skin, our data support a model in which the earliest steps of differentiation are mediated by Delta-1 interactions with Notch 1 and possibly Notch 2. Subsequent steps, up to and including cornification, are mediated instead by Jagged-1 interactions with Notch 1, 2, 3 and 4.

The role of calcium ion levels in this experimental system deserves comment. In cultured human KCs, unlike murine KCs, elevating calcium ion levels does not, by itself, trigger terminal differentiation.[7] As noted in several figures, the ability of JAG-1 peptide to influence KC differentiation was enhanced by high calcium (i.e. 2 mM) concentration.

Experiments are underway to determine if high calcium promotes interaction between soluble Jagged-1 derived peptides with the extracellular domain of Notch receptors, as shown in other cell systems.[49]

Notch signaling is a complex and incompletely understood network.[9,11] Upon receptor engagement, cleavage and nuclear redistribution of the intracellular domain of Notch receptors is thought to convert the ubiquitous transcription factor CBF-1/RBP-Jk from a repressor to an activator, through dissociation of nuclear co-repressors and histone deacetylase 1[50] and recruitment of co-activators[51] and histone acetyltransferases.[52] This triggers a cascade of transcriptional regulation that ultimately affects cell fate. Additionally, CBF-1 independent signaling through various candidate mediators is thought to occur.[9,11] The best characterized CBF-1 independent pathway involves the cytoplasmic Notch-binding protein Deltex, which somehow inhibits JNK-mediated activation of the transcription factor E47[53,54] and has transcriptional effects distinct from CBF-1.[55] The rapid kinetics of IKKα and NF-κB activation following JAG-1 peptide treatment suggest these effects are not mediated by the transcriptional regulatory functions of Notch receptors and that we have identified a novel cytoplasmic pathway of Notch signaling triggered within minutes of ligand engagement. For example, it is unknown whether known cytoplasmic Notch-associated proteins such as Deltex participate in this effect. It is also unclear which of the Notch receptors that JAG-1 can activate in KCs is responsible for IKKα and NF-κB activation. It is likely that in vivo simultaneous and/or sequential activation of various Notch components are required to induce terminal differentiation and cornification.

Our data suggest for the first time that ligand binding to one or more Notch receptors triggers almost immediate activation of IKKα and NF-κB-mediated transcription. This novel, 'early stage' of Notch signaling begins within minutes of ligand binding and appears completed by 2 h after receptor engagement. Thus, it is very unlikely to represent a transcriptional effect of Notch activation. Our data indicate that IκBα and possibly IκBβ are phosphorylated as a result of JAG-1 mediated IKKα activation. Constitutively active, oncogenic forms of Notch 3 activate IKK-mediated IκBα phosphorylation in mouse T-cells, causing sustained NF-κB activation.[56] Our data show that a soluble Notch ligand can trigger rapid but transient activation of NF-κB. This suggests that wild-type Notch signaling is controlled by feedback mechanisms that limit the duration of NF-κB activation. One such mechanism consistent with our data may be the degradation of active Notch receptors after activation. The diverse effects on NF-κB of overexpressed nuclear forms of Notch receptors[25,43] may represent an aberrant consequence of physiologic binding between Notch and NF-κB or a distinct, nuclear stage of Notch signaling.

Our data indicate Notch-1 induces PPARγ expression in KC, similar to its effect in 3T3-L1 preadipocytes.[12] PPARγ plays a pivotal role in orchestrating KC differentiation through its interaction with the RXR retinoid receptors.[35–38] In addition, PPARγ has been reported to inhibit NF-κB activity.[45] Our data indicates that one mechanism by which PPARγ inhibits NF-κB activity involves physical association between the p65 subunit of NF-κB and PPARγ. This may be responsible for transient nature of the NF-κB activation in response to H-JAG-1 peptide treatment. Taken together, our observations suggest a hypothesis in KCs involving cross-talk between Notch, NF-κB, and PPARγ signaling. Rapid activation of NF-κB by Notch ligands would result in a simultaneous transactivation of NF-κB target genes and, through the 'conventional' pathway, CBF-1 target genes. PPARγ would then be upregulated by NF-κB and in turn quench NF-κB activity by forming a complex with it, thus closing a negative feedback loop. Whether a similar negative feedback exists for CBF-1 effects it is still unclear.

In conclusion, we demonstrated that activation of Notch signaling by soluble ligands in high calcium is sufficient to trigger terminal differentiation and cornification of human KCs, with rapid activation of IKKα and NF-κB as well as induction of PPARγ. These molecules known to play crucial roles in KC differentiation, are triggered by soluble Notch ligands through a novel signaling mechanism. Relative roles of this pathway and subsequent Notch mediated regulation in KC differentiation deserve further investigation, as does possible cooperation amongst different Notch receptors in coordinating epidermal development. Our findings have potential clinical implications in that soluble Notch ligands may be used therapeutically to correct disorders of epidermal differentiation such as those occurring in psoriasis,[57] and/or to generate well-differentiated artificial skin surfaces to be used in both *in vitro* as well as *in vivo* settings.

Material and Methods

Clinical tissue samples

Three mm punch biopsies of normal human adult skin were obtained (n=5) after informed consent and approval of the Institutional Review Board. Portions of skin biopsies were fixed in formalin and paraffin embedded, or were cryopreserved by mounting on gum tragacanth and snap-frozen in liquid nitrogen-chilled isopentane, and stored at -80°C.

Cell culture and treatments

KC cultures were initiated from discarded neonatal foreskins, in which epidermis was separated from dermis using dispase as previously described.[42] KCs were induced to proliferate using a low calcium (0.07 mM), serum-free medium (KGM, Clonetics Corp., San Diego, CA, USA). KCs were grown in 10 cm plastic Petri dishes (Corning, New York), in a humidified incubator at 37°C with 5% CO_2 and passaged at 60–70% confluency, KCs were also grown in 8-well Lab Tek Chambers as previously described.[42] KCs were used between 2 and 4 passages. In some experiments, KCs were pre-treated with KGM containing an elevated calcium ion concentration (i.e. 2 mM) for 24 h prior to addition of JAG-1 peptide, or other stimuli as indicated. To perform protein analysis on KCs from different layers of the skin, thin keratome samples (1 mm thick) of normal human skin were treated with trypsin (0.05%, 1 h at 37°C) and the single cell suspension was transferred to a discontinuous Percoll gradient as previously described.[58] Briefly, six fractions were collected representing KCs

with a specific buoyant density and maturational state. By cytological examination, each fraction contains a distinct layer of KCs with the small, highest-density basal layer KCs in the first fraction and the most superficial layer with highly differentiated, lowest-density squames in the last fraction.

Jagged-related peptides and Notch inhibitor

Several different peptides were synthesized and utilized in these studies. A synthetic peptide (designated JAG-1) with Notch agonist activity *in vitro* corresponding to Jagged-1 residues 188–204 (CDDYYYGFGCNKFCRPR) is part of the DSL region, and highly conserved between human Jagged-1 and Jagged-2.[10,39] Other peptides included a scrambled control peptide without agonist activity designated SC-JAG (RCGPDCFDNYGRYKYCF), and a second peptide with agonist activity designated R-JAG (CDDYYYGFGCNK-FGRPRDD) derived from same regions of Jagged-1, but with specific amino acid changes. These peptides were designed based on the assumption that all Cys residues in mature Jagged-1 DSL are involved in disulfide bonds and on structure predictions for that region of Jagged-1. All peptides were purchased from GeneMed Synthesis, San Francisco, CA, USA, and their activities confirmed using identical sequences provided by Peptide Technologies Inc. (Gaithersburg, MD, USA). Peptide stock solutions (10 mM) were prepared in sterile distilled water, aliquoted and snap-frozen. Appropriate volumes of stock solution were dissolved in medium immediately before use. Thawed peptide aliquots were not reused. A specific Notch inhibitor was produced as a soluble Notch decoy protein (designated rh11-12) consisting of EGF repeats 11 and 12 of human Notch 1 with a 6-histidine tag at the N-terminus expressed in *E.coli* using pLD101,[59] and purified as previously described.[60] Purified rh11-12 was stored in sterile PBS at -80°C and diluted in medium before use.

Retroviral vectors and transduction of normal KCs

Dominant negative IκBα cDNA (IκBαDN) was kindly provided by Dr. Tom Ellis (Loyola University Medical Center) and subcloned into *Bam*HI and *Not*I of LZRS and MGF-based retroviral expression vector as previously described.[42] LZRS vector containing enhanced green fluorescent protein was kindly provided by Dr. Paul A Khavari (Stanford University School of Medicine, Stanford CA, USA). Phoenix-Ampho retroviral packaging cells were obtained from American Type Culture Collection (Manassas, VA, USA) with permission from Dr. Gary P Nolan (Stanford University Medical Center, Stanford, CA, USA). Packaging cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY, USA), and transfected with LZRS-IκBαDN vector by using $CaC1_2$ and 2$\times$ Hanks Balanced Salt Solution. After overnight incubation, cells were fed with fresh medium and incubated at 32°C for 24–48 h. The supernatants were collected for cell infection. Normal KCs were seeded into 6-well plates and infected with 300 μl of viral supernatant in presence of 4 μg/ml hexadimethrine bromide (Polybrene; Sigma H-9268) for 1 h at 32°C, then supernatant was removed and replaced with fresh medium, incubated at 37°C in 5% CO_2 overnight.

NF-κB luciferase assay

To perform luciferase-based NF-κB reporter assays, normal KCs were seeded in 6-well plates as previously described.[42] At approximately 50–60% confluence, cells were co-transfected with 0.8 μg of pNF-κB-LUC vector (CLONTECH Laboratories, Palo Alto, CA, USA) containing a firefly luciferase gene with κ enhancer and 0.2 μg of PRL-TK plasmid DNA which contains Renilla Luciferase gene to normalize transfection efficiency. The same amount of control vector in which κ enhancer was removed from pNF-κB-LUC was also used to co-transfect cells.

DNA was transfected into KCs using Fu-Gene 6 Transfection Reagent (Roche Molecular Biochemicals) according to manufacturer's protocol. Thirty-six hours after transfection, cells were treated with either TNF-α (10^3 U/ml; R&D Systems, Minn, MN, USA) or indicated peptides. Preparation of cell lysate and luciferase activity measurements were made with Dual Luciferase^{TM} Reporter Assay System (Promega, Madison WI, USA) according to manufacturer's instructions. Samples were placed in a TD-20/20 luminometer (CLONTECH Laboratories) for detection of chemiluminescence intensity.

Antibodies

Anti-Notch 1, 2, 3, 4 receptor antibodies; Notch 1 (SC-6014), Notch 2 (SC-5545), Notch 3 (SC-7424), Notch 4 (SC-8644) abs and anti-Jagged-1 (SC-6011) ab, and anti-p50 (SC-7178) anti-p65 (SC-109), and anti IκBα (SC-371) and anti-PPARγ (SC-7273) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-loricrin and anti-involucrin abs were obtained from (BabCO); Richmond, CA, USA). Anti-keratin 1 ab was purchased from Upstate Biotechnologies Inc (Lake Placid, NY, USA).

IKKα immunocomplex kinase assay

For immunoprecipitation 2 μg of anti-IKKα ab was added to whole cell lysate for 2 h on ice. 30 μl of protein A-G Sepharose beads were added and incubated at 40°C for 90 min. Beads were washed three times in wash buffer, and were boiled with 2$\times$ SDS sample buffer. Kinase activity was determined as previously described using GST-IκBα as substrate.[30]

Electromobility shift assay (EMSA)

EMSA were performed as described previously.[42] Five μg of nuclear proteins were incubated on ice with 1 μg of poly (dI-dC) (Amersham Pharmacia Biotech) in a buffer containing 10 mM HEPES, 60 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 4% Ficoll for 10 min. ^{32}P-labeled double strand NF-κB binding oligonucleotide (105 c.p.m.) was added to reaction mix for an additional 20 min on ice. NF-κB oligonucleotide had the following sequence: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. For experiments involving supershift analysis, 2–4 μg of polyclonal antibodies against different subunits of NF-κB (Santa Cruz Biotech) were incubated with nuclear proteins for 30 min on ice prior to addition of ^{32}P-labeled NF-κB probe. All reaction mixtures were resolved on 4% polyacrylamide gels.

Western blot analysis

Whole cell lysates, or nuclear and cytoplasmic extracts, were prepared to detect different proteins as previously described.[61] Briefly, KCs were washed with ice-cold phosphate buffered saline (PBS), harvested by scraping monolayers into 1 ml PBS, and pelleted in a microcentrifuge tube. The cell pellet was suspended in 400 μl of buffer A (10 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethlysulfonyl fluoride, and 5 μg/ml leupeptin). After 20 min incubation on ice, 25 μl of 10% Nonidet P-40 was added and samples were centrifuged briefly. Supernatants represented cytoplasmic extracts. Nuclear pellets were resuspended in 60–80 μl of buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl

fluoride, and 5 μg/ml leupeptin) and incubated at 4°C with shaking for 15 min. Nuclear debris was removed by centrifugation at 4°C.

For whole cell lysates, cells were washed with cold PBS and incubated in CHAPS buffer on ice for 15 min. After centrifugation at 4°C, supernatants were saved as whole cell lysates. Protein concentrations were determined using Bio-Rad protein assay reagent. Thirty μg of protein were loaded onto 8–12.5% SDS-polyacrylamide gels, transferred to Immobilon-P (polyvinylidene difluoride) membrane and blocked in 5% powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween 20). The membrane was incubated with primary antibody overnight at 4°C in 2.5% powdered milk in TBST and washed extensively with TBST; incubated with 1 : 1500 anti-rabbit or mouse-HRP (Amersham Pharmacia Biotech). Proteins were visualized with ECL detection kit (Amersham Pharmacia Biotech). Equivalent loading of proteins in each well was confirmed by Ponceau staining.

For immunoprecipitation/Western blot analysis, protein lysates were first immunoprecipitated using an anti-p65 ab, and Western blotting was subsequently performed using an anti-PPARγ ab, or vice versa, as previously described.[62]

Living epidermal equivalent (EE) systems

Reconstituted epidermis, known as EEs were purchased from MatTek Corp., Ashland, MA. EEs consist of several layers of relatively undifferentiated KCs submerged in standard medium (DMEM plus EGF, insulin, hydrocortisone-free) as previously described.[40] In this system, exposure of EEs to an air/liquid interface is a potent stimulus for differentiation with creation of a stratum corneum comparable to normal human skin *in vivo*.[40,41]

Histological analysis and immunohistochemistry

Portions of EEs were fixed in 10% neutral buffered formalin, embedded in paraffin and routinely stained with 1% hematoxylin and eosin. For immunohistochemical analysis, some sections underwent antigen retrieval using a standard microwave procedure (to detect Notch 1, 3, 4, Jagged-1, while other staining (to detect loricrin) did not require such retrieval. When using Lab Tek cultures, slides were rinsed in PBS and fixed with ice-chilled acetone for 10 mins prior to addition of primary abs. In all cases, a highly sensitive avidin biotin peroxidase procedure (Vectastain Kit, Vector Labs, Burlingame, CA, USA) was used with a positive red reaction product obtained using 3-amino-4-ethylcarbazole as chromogen.[61]

References

1. Landmann L (1988) The epidermal permeability barrier. Anat. Embryol. 178: 1–10
2. Elias PM (1996) The stratum corneum revisited. J. Dermatol. 23: 756–768
3. Nemes Z and Steinert PM (1999) Bricks and mortar of the epidermal barrier. Exp. Mol. Med. 31: 5–19
4. Roop D Z (1995) Defects in the barrier. Science 267: 474–475
5. Yuspa SH (1994) The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. Cancer Res. 54: 1178–1189
6. Menon GK, Elias PM, Lee SH and Feingold KR (1992) Localization of calcium in murine epidermis following disruption and repair of the permeability barrier. Cell Tissue Res. 270: 503–512
7. Green H (1977) Terminal differentiation of cultured human epidermal cells. Cell 11: 405–416
8. Yuspa SH, Kilkenny AE, Steinert PM and Roop DR (1989) Expression of murine epidermal differentiation markers is highly regulated by restricted extracellular calcium concentrations in vitro. J. Cell. Biol. 109: 1207–1217
9. Artavanis-Tsakonas S, Rand MD and Lake RJ (1999) Notch signaling: Cell fate control and signal integration in development. Science 284: 770–776
10. Li L, Milner LA, Deng Y, Iwata M, Banta A, Graf L, Marcovina S, Friedman C, Trask BJ, Hood L and Torok-Storb, B (1998) The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch 1. Immunity 8: 43–55
11. Miele L and Osborne B (1999) Arbiter of differentiation and death: Notch signaling meets apoptosis. J. Cell. Physiol. 181: 393–409
12. Garces C, Ruiz-Hidalgo MJ, Font de Mora J, Park C, Miele L, Goldstein J, Bonvini E, Porras A and Laborda J (1997) Notch-1 controls the expression of fatty acid-activated transcription factors and is required for adipogenesis. J. Biol. Chem. 272: 29729–29734
13. Osborne B and Miele L (1999) Notch and the immune system. Immunity. 11: 653–663
14. Robey E (1999) Regulation of T cell fate by Notch. Annu. Rev. Immunol. 17: 283–295
15. Pui JC, Altman D, Xu L, DeRocco S, Kamell FG, Bakkour S, Lee JY, Kadesch T, Hardy RR, Aster JC and Pear WS (1999) Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity 11: 299–308
16. Yasutomo K, Doyle C, Miele L and Germain RN (2000) The duration of antigen receptor signalling determines CD4+ versus CD8+ T-cell lineage fate. Nature 404: 506–510
17. Ahmad I, Zaqouras P and Artavanis-Tsakonas S (1995) Involvement of Notch-1 in mammalian retinal neurogenesis: association of Notch-1 activity with both immature and terminally differentiated cells. Mech. Dev. 53: 73–85
18. Austin CP, Feldman DE, Ida Jr JA and Cepko CL (1995) Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. Development 121: 3637–3650
19. Shelly LL, Fuchs C and Miele L (1999) Notch-1 prevents apoptosis in murine erythroleukemia cells and is necessary for differentiation induced by hybrid polar drugs. J.Cell Biochem. 73: 164–175
20. Milner LA and Bigas A (1999) Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. Blood 93: 2431–2448
21. Kopan R, and Weintraub H (1993) Mouse Notch: expression in hair follicles correlates with cell fate determination. J. Cell. Biol. I 121: 631–641
22. Lowell S, Jones P, Le RI, Dunne J and Watt FM (2000) Stimulation of human epidermal differentiation by delta-notch signaling at the boundaries of stem-cell clusters. Curr. Biol. 10: 491–500
23. Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, Aster JC, Krishna S, Metzger D, Chambon P, Miele L, Aguet M, Radtke F and Dotto GP (2001) Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J. 20: 3427–3436
24. Guan E, Wang J, Laborda J, Norcross M, Baeuerle PA and Hoffman T (1996) T cell leukemia-associated human Notch/translocation-associated Notch homologue has I kappa B-like activity and physically interacts with nuclear factor-kappa B proteins in T cells. J.Exp.Med. 183: 2025–2032
25. Wang J, Shelly L, Miele L, Boykins R, Norcross MA and Guan E (2001) Human Notch-1 inhibits NF-kappa B activity in the nucleus through a direct interaction involving a novel domain. J. Immunol. 167: 289–295
26. Oswald F, Liptay S, Adler G and Schmid RM (1998) NF-kappaB2 is a putative target gene of activated Notch-1 via RBP-J kappa. Mol.Cell Biol. 18: 2077–2088
27. Cheng P, Zlobin A, Volgina V, Gottipati S, Osborne B, Simel EJ, Miele L and Gabrilovich DI (2001) Notch-1 regulates NF-kappaB activity in hemopoietic progenitor cells. J. Immunol. 167: 4458–4467
28. Seitz CS, Lin Q, Deng, H and Khavari PA (1998) Alterations in NF-κB function in transgenic epithelial tissue demonstrates a growth inhibitory role for NF-κB. Proc. Natl. Acad. Sci. USA. 95: 2307–2312
29. Takeda K, Takeuchi O, Tsujimura T, Itami S, Adachi O, Kawai T, Sanjo H, Yoshikawa K, Terada N and Akira S (1999) Limb and skin abnormalities in mice lacking IKKα. Science 284: 313–316
30. Hu Y, Baud V, Delhase M, Zhang P, Derrinck T, Ellisman M, Johnson, R and Karin M (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKKα subunit of IκB kinase. Science 284: 316–320
31. Tanaka M, Fuentes ME, Yamaguchi K, Durnin MH, Dalrymple SA, Hardy KL and Goeddel DV (1999) Embryonic lethality, liver degeneration, and impaired NF-κB activation in IKK-β-deficient mice. Immunity 10: 421–429

32. Schnidt-Supprian M, Bloch W, Courtois G, Addicks K, Israel A, Rajewsky K and Pasparakis M (2000) NEMO/IKKγ-deficient mice model incontinentia pigmenti. Mol. Cell 5: 981 – 992
33. Makris C, Godfrey VL, Krahn-Senftleben, G, Takahashi T, Roberts JL, Schwarz T, Feng L, Johnson RS and Karin M (2000) Female mice heterozygous for IKK-γ/NEMO deficiencies develop a dermatopathy similar to the human X-linked disorder incontinentia pigmenti. Mol. Cell. 5: 969 – 979
34. Kaufman CK and Fuchs E (2000) It's got you covered; NF-κB in the epidermis. J. Cell. Biol. 149: 999 – 1004
35. Rivier M, Castiel I, Ailhaud G and Michel S (1998) Differentiated expression of peroxisome proliferator-activated receptor subtypes during the differentiation of human keratinocytes. J. Invest. Dermatol. 111: 1116 – 1121
36. Rivier M, Castiel I, Irina Safonova I, Ailhaud G and Michel S (2000) Peroxisome proliferator-activated receptor-α enhances lipid metabolism in a skin equivalent model. J. Invest. Dermatol. 114: 681 – 687
37. Komuves LG, Hanley K, Lefebvre AM, Man MQ, Ng DC, Bikle DD, Williams ML, Elias PM, Auwerx J and Feingold KR (2000) Stimulation of PPARα promotes epidermal keratinocyte differentiation in vivo. J. Invest. Dermatol. 115: 353 – 360
38. Ellis CN, Varani J, Fisher GJ, Ziegler ME, Pershadsingh HA, Benson, SC, Chi Y and Kurtz TW (2000) Troglitazone improves psoriasis and normalizes models of proliferative skin disease: ligands for peroxisome proliferator-activated receptor-gamma inhibit keratinocyte proliferation. Arch. Dermatol. 136: 609 – 616
39. Zlobin A, Jang M and Miele L (2000) Toward the rationale design of cell fate modifiers: Notch signaling as a target for novel biopharmaceuticals. Curr. Phar. Biotech. 1: 83 – 106
40. Kubilus J, Christopher C, Pat N, Holly S and Klausner M (1996) Response of the EpiDerm skin model to topically applied irritants and allergens. In Vitro Toxicol. 9: 157 – 166
41. Asselineau D, Bernhard B, Bailly C and Darmon M (1985) Epidermal morphogenesis and induction of the 67 KD keratin polypeptide by culture of human keratinocytes at the liquid-air interface. Exp. Cell. Res. 159: 536 – 539
42. Qin, J-Z, Chaturvedi V, Denning M.F, Choubey D, Diaz, MO and Nickoloff BJ (1999) Role of NF-κB in the apoptotic-resistant phenotype of KCs. J. Biol. Chem. 274: 37957 – 37964
43. Guan E, Wang J, Laborda J, Norcross M, Baeuerle PA and Hoffman T (1996) T cell leukemia associated human Notch/translocation-associated Notch homologues has I kappa B-like activity and physically interacts with nuclear factor-kappa B proteins in T cells. J. Exp. Med. 183: 2025 – 2032
44. Delhase M, Hayakawa M, Chen Y and Karin M (1999) Positive and negative regulation of IκB activity through IKKβ subunit phosphorylation. Science 284: 309 – 316
45. Ricote M, Li AC, Wilsons TM, Kelly CJ and Glass CK (1998) The peroxisome proliferator-activated receptors - γ is a negative regulator of macrophage activation. Nature 391: 79 – 82
46. Powell BC, Passmore EA, Nesci A and Dunn SM (1998) The Notch signaling pathway in hair growth. Mech. Dev. 78: 189 – 192
47. Crowe R, Henrique D, Ish-Horowicz D and Niswander L (1998) A new role for Notch and Delta in cell fate decisions: patterning the feather array. Development 125: 767 – 775
48. Qiu L, Joazeiro C, Fang N, Wang HY, Elly C, Altman Y, Fang D, Hunter T and Liu YC (2000) Recognition and ubiquitination of Notch by Itch, a Hect-type E3 ubiquitin ligase. J.Biol.Chem. 275: 35734 – 35737
49. Rand MD, Grimm LM, Artavanis-Tsakonas S, Patriasb V, Blacklow SC, Sklar J and Aster JD (2000) Calcium depletion disassociates and activates heterodimeric Notch receptors. Mol. Cell. Biol. 20: 1825 – 1835
50. Kao HY, Ordentlich P, Koyano-Nakagawa N, Tang Z, Downes M, Kintner CR, Evans RM and Kadesch T (1998) A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes. Dev. 12: 2269 – 2277
51. Zhou S, Fujimuro M, Hsieh JC, Chen L, Miyamoto A, Weinmaster G and Hayward SD (2000) SKIP, a CBF-1 associated protein, interacts with the ankyrin repeat domain of NotchIC to facilitate NotchIC function. Mol. Cell. Biol. 20: 2400 – 2410
52. Kurooka H and Honjo T (2000) Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5. J. Biol. Chem. 275: 17211 – 17220
53. Ordentlich P, Lin A, Shen CP, Blaumueller C, Matsuno K, Artavanis-Tsakonas S and Kadesch T (1998) Notch inhibition of E47 supports the existence of a novel signaling pathway. Mol. Cell. Biol. 18: 2230 – 2239
54. Matsuno K, Eastman D, Mitsiades T, Quinn AM, Carcanciu ML, Ordentlich P, Kadesch T and Artavanis-Tsakonas S (1998) Human deltex is a conserved regulator of Notch signalling. Nat. Genet. 19: 74 – 78
55. Yamamoto N, Yamamoto SS, Inagaki F, Kawaichi M, Fukamizu A, Kishi N, Matsuno K, Nakamura K, Weinmaster G, Okano H and Nakafuku M (2001) Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. J. Biol. Chem. 276: 45031 – 45040
56. Bellavia D, Campese AF, Alesse E, Vacca A, Felli MP, Balestri A, Stoppacciaro A, Tiveron C, Tatangelo L, Giovarelli M, Gaetano C, Ruco L, Hoffman ES, Hayday AC, Lendahl U, Frati L, Gulinio A and Screpanti I (2000) Constitutive activation of NF-κB and T-cell leukemia/lymphoma in Notch 3 transgenic mice. EMBO J. 19: 33337 – 3348
57. Miele L, Bacon PE, Chaturvedi V, Qin J-Z and Nickoloff BJ (2000) Discordant expression of Notch-1 and -2 compared to Notch ligands (Jagged, Delta) in psoriatic epidermis. J Invest Dermatol 114: 758 (abstract)
58. Rennecke J, Rehberger PA, Furstenberger G, Johannes FJ, Stohr M, Marks F and Richter KH (1999) Protein kinase-cμ expression correlates with enhanced keratinocyte proliferation in normal and neoplastic mouse epidermis and in cell culture. Int. J. Cancer 80: 98 – 103
59. Miele L, Cordella-Miele E and Mukherjee AB (1990) High level bacterial expression of uteroglobin, a dimeric eukaryotic protein with interchain disulfide bridges, in its natural quaternary structure. J. Biol. Chem. 265: 6427 – 6435
60. Mantile G, Fuchs C, Cordella-Meile E, Peri A, Mukherjee AB and Miele L (2000) Stable, long-term bacterial production of soluble, dimeric, disulfide-bonded protein pharmaceuticals without antibiotic selection. Biotechnol. Prog 16: 17 – 25
61. Chaturvedi V, Qin J-Z, Denning, MF, Choubey D, Diaz MO and Nickoloff BJ (1999) Apoptosis in proliferating, senescent, and immortalized keratinocytes. J. Biol. Chem. 274: 23358 – 23367
62. Chaturvedi V, Qin J-Z and Nickoloff BJ (2001) Abnormal NF-κB signaling pathway with enhanced susceptibility to apoptosis in immortalized keratinocytes. J. Dermatol. Sci. 26: 67 – 78