Notch down-regulation in regenerated epidermis contributes to enhanced expression of interleukin-36α and suppression of keratinocyte differentiation during wound healing

Yuko Takazawa[a], Eisaku Ogawa[a], Rumiko Saito[b], Ryuhei Uchiyama[a], Shuntaro Ikawa[c], Hisashi Uhara[a], Ryuhei Okuyama[a]

[a]Department of Dermatology, Shinshu University School of Medicine, Matsumoto, Japan

[b]Tohoku Medical Megabank Organization, Tohoku University, Sendai, Japan

[c]Department of Project Programs, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan.

Running title: Notch down-regulation in keratinocytes during wound healing

Corresponding author: Ryuhei Okuyama, Department of Dermatology, Shinshu

University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. Tel: +81-263-37-2643. Fax: +81-263-37-2646. E-mail: rokuyama@shinshu-u.ac.jp

Keywords: Notch pathway; wound healing; keratinocyte; cytokine; keratin.

Disclosure Statement

The authors declare no financial support or relationships that may pose a conflict of interest.

Word count = 3892

Number of references = 35

Number of figures = 5

Number of tables = 0

Abstract

Background: Notch signaling controls a number of cellular processes, including cell fate decisions, proliferation, differentiation, and survival/apoptosis, in multiple tissues. In the epidermis, Notch1 functions as a molecular switch that controls the transition of cells from an undifferentiated state into a differentiated state.

Objective: To clarify the functions of Notch in the regenerated epidermis during wound healing.

Methods: Wounds on mouse skin were immunostained. To investigate the functions of Notch, Notch was inhibited in primary keratinocytes by treatment with a γ-secretase inhibitor and by small interfering RNA-mediated knockdown, and was activated by a recombinant adenovirus approach.

Results: Notch1 and Notch2 were down-regulated in the regenerated epidermis during wound healing. To clarify the significance of this down-regulation, we examined its effect on expression of the interleukin (IL)-1 family of proinflammatory cytokines because wounds are exposed to pathogens from the outside world. Among the IL-1 family, IL-36α expression was induced by Notch

inhibition. This was consistent with the decreased IL-36α expression in Notch-overexpressing keratinocytes. Notch down-regulation in the regenerated epidermis may reinforce defense against stress from the outside world by inducing IL-36α expression. Next, we examined the effects of Notch down-regulation on keratinocyte growth and differentiation. Notch down-regulation did not alter keratinocyte proliferation. On the other hand, Notch1 down-regulation suppressed induction of spinous layer-specific keratins (keratin1 and keratin10) in keratinocytes, which was consistent with the decreased expression of these keratins in the regenerated epidermis. The reduced levels of these keratins would increase cellular flexibility.

Conclusion: Notch down-regulation in the epidermis appears to contribute to tissue regeneration during wound healing.

1. Introduction

Notch (Notch1–4 in mice and humans) is a transmembrane receptor that regulates cell fate decisions during development and contributes to the maintenance of cell proliferation, differentiation, and survival/apoptosis. Notch signaling is initiated by ligand-receptor interactions between neighboring cells. In response to ligand engagement, Notch undergoes proteolysis by γ-secretase. This releases the Notch intracellular domain, resulting in translocation of the intracellular domain into the nucleus, where it binds to the DNA-binding protein CSL. While CSL functions as a transcriptional repressor by binding to the promoter regions of particular genes, it is converted into a transcriptional activator by association with Notch, resulting in activation of downstream target genes, such as Hes and Hey [1-3].

Notch activation is generally thought to maintain stem cell potential and inhibit differentiation. However, in some cell types, such as keratinocytes, increased Notch activity causes cell-cycle exit and commitment to differentiation, whereas down-modulation or loss of Notch1 function promotes carcinogenesis

[4,5]. Keratinocytes form a multilayered stratified epithelium, the epidermis, which comprises basal, spinous, granular, and cornified layers. Each of these layers is characterized by specific expression of a repertoire of keratins and other proteins [6]. Notch1–3 are expressed in the epidermis, and their activation is considered to regulate skin homeostasis [7,8]. Postnatal knockout of Notch1 leads to a hyperproliferative epidermis due to interference with the commitment of basal cells to differentiate into spinous cells [4,5]. This suggests that Notch1 functions as a molecular switch that controls the transition of basal cells into spinous cells.

The epidermis is a barrier-forming tissue that protects the internal milieu from the outside environment; therefore, keratinocytes are the first to respond to injury. After injury, keratinocytes are activated and secrete various cytokines, including interleukin (IL)-1, which alert surrounding cells to barrier damage and attract inflammatory cells to the wound site to remove contaminating bacteria. Simultaneously, re-epithelization is a crucial step, which involves the migration and proliferation of keratinocytes at the periphery of the wound to cover the denuded dermal surface. If this process is dysregulated, patients will require

long-term care of their wounds and are at increased risk of infection and fluid loss. In this instance, migrating keratinocytes undergo various modifications beyond cytokine secretion. In healthy epidermis, keratinocytes exhibit a columnar phenotype and are tightly connected with neighboring keratinocytes. However, as the cells start to migrate across the wound bed, keratinocytes become flatter and more elongated, which requires dramatic reorganization of their cytoskeleton [9]. Basal keratinocytes usually express keratin 5 (K5) and K14, whereas spinous, differentiating keratinocytes express K1 and K10. However, in the regenerated epidermis, K1 and K10 expression is attenuated and replaced by K6, K16, and K17 expression. While abundant expression of K1 and K10 in the context of normal epidermal differentiation promotes increased mechanical resilience, the abundance of these keratins disturbs the pliability of keratinocytes, leading to retardation of their migration. The transition from K1/K10 expression to K6/K16/K17 expression likely reflects a compromise between conflicting needs i.e., retaining sufficient cell pliability for migration, while acquiring sufficient resilience to survive in the wound environment [9].

Cutaneous wound healing is a highly coordinated physiological process that rapidly and efficiently restores skin integrity. We found that Notch expression was decreased in the regenerated epidermis. Notch may play a role in epidermal regeneration. To better understand the role of Notch in wound healing, we analyzed the effects of Notch down-regulation on keratinocytes.

2. Materials and Methods

2.1. Cell Culture and Reagents

Primary keratinocytes were prepared from the epidermis of newborn ICR (Institute for Cancer Research) mice, as described previously [10]. In brief, the epidermis was separated from the dermis with 0.25% trypsin (Gibco BRL, Tokyo, Japan), plated in dishes precoated with collagen type I (Nitta Gelatin, Osaka, Japan), and cultured in minimum essential medium supplemented with 4% Chelex-treated fetal calf serum, epidermal growth factor (10 ng/ml; Gibco BRL), and 0.05 mM $CaCl_2$. Under these conditions, keratinocytes are maintained in an immature state, and differentiation was induced by the addition of $CaCl_2$ to a final

concentration of 2 mM. For migration assays using the scratch method, keratinocytes were grown to confluency in 60-mm culture dishes and a scratch was made through the monolayer with a 200-μl pipette tip. Cells were further incubated, and the scratch areas were filled by migrating cells. Images of more than six fields were captured, and migration into the scratch area was analyzed using ImageJ software. Recombinant adenoviruses expressing either LacZ, Notch1 intracytoplasmic domain (a constitutively active form of Notch1), or Notch2 intracytoplasmic domain (a constitutively active form of Notch2) were generated, as described previously, and were used at a multiplicity of infection (MOI) of 50 [10]. The adenovirus expressing LacZ was used as a control. The following antibodies were purchased and used as recommended by their suppliers: goat anti-Notch1 and goat anti-Notch2 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal antibodies against K1, K5, K6, and K10 (Covance, Emeryville, CA); mouse anti-tubulin-α monoclonal antibody (Sigma, Tokyo, Japan); mouse anti-bromodeoxyuridine (BrdU) monoclonal antibody (BD Biosciences, Tokyo, Japan); rabbit anti-Ki67 polyclonal

antibody (Novocastra, Wetzlar, Germany); rabbit anti-phospho-epidermal growth factor receptor (EGFR) monoclonal antibody and rabbit anti-cleaved Notch1 polyclonal antibody (Cell Signaling Technology, Tokyo, Japan); goat anti-IL-36α polyclonal antibody (R&D Systems, Tokyo, Japan); and fluorescein isothiocyanate (FITC)-coupled and horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG antibodies (Amersham, Tokyo, Japan). N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a selective inhibitor of γ-secretase, was purchased from Cayman (Ann Arbor, MI).

2.2. Wound Formation and Immunostaining

Female mice (8–10 weeks old) of a C57BL/6 background were anesthetized by intraperitoneal injection of 300 μl of 2.5% avertin. Full-thickness skin wounds were created on the back of each mouse using a 4-mm biopsy punch. To obtain regenerated skin samples from the biopsied areas, mice were euthanized by an overdose of sodium pentobarbital and the tissues were subsequently removed. As regenerated skin, recently re-epithelialized areas were obtained before the

skin defect closed. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Research Committee of Shinshu University (permit number: 240030). All efforts were made to minimize animal suffering. Formalin-fixed samples were sectioned at a thickness of 4 μm. Frozen skin sections (6 μm thick) were fixed with 2% paraformaldehyde or cold acetone. Non-specific binding was blocked using 5% serum, and the skin sections were incubated with primary antibodies and subsequently with isotype-specific secondary antibodies. After immunostaining, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Tokyo, Japan). Stained preparations were observed with a LSM 5 exciter (Zeiss, Jena, Germany).

In addition, DAPT was topically administered to the skin on the backs of mice. Mice were shaved under anesthesia 2 days prior to treatment with DAPT. On day 0, Scotchguard (3M) tape was firmly applied to the hairless area and removed in a sudden motion three times to eliminate the horny layer. Thereafter,

DAPT (100 µl of a 100 µM solution prepared in DMSO) was topically applied three times every other day. As a control, DMSO was topically applied.

2.3. Immunoblotting

Proteins were extracted from cultured keratinocytes and subjected to immunoblotting, as described previously [10]. In brief, electroblotted membranes were blocked with TBST (50 mM Tris [pH 7.5] and 0.5% Tween 20) containing 5% non-fat dried milk. The membranes were then incubated with primary antibodies, rinsed with TBST, and incubated with peroxidase-conjugated secondary antibodies. After additional rinses, the blots were exposed to LumiGLO reagent (Cell Signaling Technology). Equal loading was achieved by normalization of the protein concentration using a bicinchoninic acid protein assay (Pierce, Rockford, IL).

2.4. BrdU Incorporation

Cells labeled with BrdU for the last 5 hours of cultivation were permeabilized

in 0.1% NP-40 and denatured in 50 mM NaOH. BrdU incorporation was detected using an anti-BrdU antibody. The total number of nuclei was determined by fluorescence microscopic observation of nuclei stained with DAPI. At least six fields were assayed per sample (> 100 cells/field).

2.5. Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), as recommended by the manufacturer. cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and 3 μg of total RNA as the template. qRT-PCR analyses were performed using a StepOne™ RT-PCR System (Applied Biosystem, Tokyo, Japan), a LightCycler System, and a Universal Probe Library Set (Roche Applied Science, Indianapolis, IN). Primers and probes were purchased for Notch1, Notch2, Hes1, K1, K10, and hypoxanthine phosphoribosyl transferase (HPRT) from Applied Biosystems. Furthermore, specific primers were designed to sequence one exon-exon region by the Universal ProbeLibrary Assay Design Center (Roche

Applied Science) as follows: for IL-1α, 5'-TTG GTT AAA TGA CCT GCA ACA-3' and 5'-GAG CGC TCA CGA ACA GTT G-3'; for IL-1β, 5'-TGT AAT GAA AGA CGG CAC ACC-3' and 5'-TCT TCT TTG GGT ATT GCT TGG-3'; for IL-18, 5'-CAA ACC TTC CAA ATC ACT TCC T-3' and 5'-TCC TTG AAG TTG ACG CAA GA-3'; for IL-33, 5'-GGT GAA CAT GAG TCC CAT CA-3' and 5'-CGT CAC CCC TTT GAA GCT C-3'; for IL-36α, 5'-CAC AAA GGA TGG GGA GCA-3' and 5'-GGT TCC TTT TTG TTG TAC ATT TCC-3'; for IL-36β, 5'-CTT CGA TCC CAG AGA CAA GAC T-3' and 5'-ATT CGG TTC CCA CAT TTG AA-3'; for IL-36γ, 5'-GGA CAC CCT ACT TTG CTG CTA-3' and 5'-AAC AGG AAT GGC TTC ATT GG-3' ; for K6, 5'- ATC CAG CGG GTC AGG ACT-3' and 5'-GTC CAG GAC CTT GTT CTG CT-3'; for K16, 5'-TGA GCT GAC CCT GTC CAG A-3' and 5'-CTC AAG GCA AGC ATC TCC TC-3'; and for K17, 5'-AGG AGC TGG CCT ACC TGA A-3' and 5'-ACC TGG CCT CTC AGA GCA T-3'. Universal ProbeLibrary Set #52, #78, #46, #71, #60, #69, #77, #32, #85, and #63 (Roche Applied Science) were used as specific probes of IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, K6, K16, and K17, respectively. Expression was given as the mRNA level of the

protein of interest divided by that of HPRT.

2.6. RNA Interference

Small interfering RNAs (siRNAs) targeting Notch1 or Notch2 were purchased from Invitrogen. siRNAs were transfected using Lipofectamine 2000 (Invitrogen). Block-iT Fluorescent Oligo (Invitrogen) showed that siRNAs were introduced into more than 80% of keratinocytes.

2.7. Statistical Analyses

The data were analyzed using unpaired Student's *t*-tests. Differences were considered significant for $P < 0.05$.

3. Results

3.1. Decreased Expression of Notch1 and Notch2 in the Regenerated Epidermis during Wound Healing

To clarify the roles of Notch in wound healing, we examined Notch

expression in the regenerated epidermis. In intact mouse skin, Notch1 was expressed in the basal and spinous layers, while labeling was absent in the upper layers. Notch2 was observed throughout all epithelial cell layers, with weak expression in the basal layer. The intensities of Notch1 and Notch2 labeling were decreased in the regenerated epidermis during wound healing (Fig. 1A). Next, we examined the expression of Hes1, a downstream molecule of the Notch signaling pathway. Similar to Notch expression, the intensity of Hes1 staining was decreased in the regenerated epidermis (Fig. 1B), showing that Notch function was reduced during epidermal regeneration. Furthermore, we examined activation of EGFR because this receptor is reportedly a key negative regulator of Notch1 expression [11]. The intensity of phospho-EGFR staining was increased in the regenerated epidermis inversely to the intensities of Notch1 and Notch2 staining (Fig. 1C). In addition, expression of Notch1 and Notch2 was restored after closure of the skin defect (Fig. 1D); thus, Notch expression was reduced in the regenerated epidermis.

3.2. Notch Down-Regulation Induces IL-36α Expression in Keratinocytes

During wound healing, regenerated tissues are exposed to the outside world and are therefore vulnerable to pathogen attack. Keratinocytes should alert surrounding cells of this risk to prevent pathogen invasion. We examined whether Notch regulated the production of IL-1 family cytokines, which are induced in response to tissue damage and promote inflammation [12,13]. First, we used a γ-secretase inhibitor, DAPT, to inhibit the Notch signaling pathway by blocking the proteolytic cleavage of Notch. As expected, Hes1 expression was reduced by DAPT treatment (Fig. 2A). Among IL-1 family cytokines (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ), DAPT treatment only increased the mRNA expression of IL-36α (Fig. 2B); expression of the other members was not induced by DAPT treatment. The increase in IL-36α expression was confirmed at the protein level (Fig. 2C). To verify that IL-36α expression is increased by Notch down-regulation, we knocked down Notch using sequence-specific siRNA (Fig. 2D). IL-36α expression was increased by Notch1 silencing, as well as Notch2 silencing (Fig. 2E, 2F). Notch knockdown had the same effects as

treatment with a γ-secretase inhibitor. In contrast to Notch down-regulation, we used an adenovirus to overexpress the intracytoplasmic domain of Notch1 or Notch2. Overexpression of the intracytoplasmic domain of Notch1 or Notch2 decreased expression of IL-36α, at both the mRNA and protein levels (Fig. 2G, 2H); thus Notch negatively regulates IL-36α expression in keratinocytes.

3.3. Notch Down-Regulation Results in Induction of IL-36α Expression in the Epidermis

Next, we examined IL-36α expression in the regenerated epidermis. In immunohistological analyses, the intact epidermis did not show clear expression of IL-36α, whereas IL-36α was clearly expressed in the regenerated epidermis, chiefly in the upper and middle layers (Fig. 3A), and this was inversely correlated with Notch down-regulation. After closure of the skin defect, the induced IL-36α expression disappeared in association with restoration of Notch expression (Fig. 3B). To define the contribution of reduced Notch expression to induction of IL-36α expression, we examined IL-36α expression *in vivo* after topical

administration of DAPT, which inhibits the Notch signaling pathway by blocking the proteolytic cleavage of Notch. DAPT was applied to the skin surface after the stratum corneum had been removed to increase its penetration. While DAPT did not affect the expression of Notch1 or Notch2, it decreased the level of cleaved Notch1, the active form of Notch1 (Fig. 3C, Supplemental Fig. 1). Application of DAPT induced IL-36α expression (Fig. 3D). Therefore, Notch down-regulation seems to help to alert surrounding cells of barrier damage by inducing expression of IL-36α in the regenerated epidermis, which is related to induction of inflammation around the wound.

3.4. Notch Down-Regulation Does Not Affect Keratinocyte Proliferation in the Regenerated Epidermis

Notch functions in the transition of cells from an undifferentiated proliferative state into a differentiated state in which growth is arrested [4,5]. We examined whether Notch down-regulation alters keratinocyte proliferation in the regenerated epidermis. We overexpressed Notch intracytoplasmic domain (a

constitutively active form) in primary keratinocytes. Notch1 induces the proliferation arrest of keratinocytes [5,14]. Similar to Notch1 intracytoplasmic domain overexpression, Notch2 intracytoplasmic domain overexpression suppressed keratinocyte proliferation (Fig. 4A). Considering the growth suppressive function of Notch together with the endogenous expression of Notch, keratinocyte proliferation was expected to increase upon DAPT treatment. However, unexpectedly, DAPT did not alter keratinocyte proliferation (Fig. 4B), in contrast to keratinocyte-specific deletion of *Notch1*, which accelerates keratinocyte proliferation [5]. Notch activity may be weak in undifferentiated cells, thereby leading to no alteration in cell proliferation upon partial suppression of Notch signaling by DAPT treatment. Next, we examined keratinocyte proliferation in the regenerated epidermis. The epidermis at wound edges is the source of keratinocytes for wound closure because of the removal of skin appendages in full-thickness skin wounds. Immunostaining of Ki67, a proliferation marker, did not show an increased number of positive cells in the regenerated epidermis (Fig. 4C). This is consistent with a previous report that

keratinocytes proliferate many cell diameters away from the proximal wound edge during regeneration [15,16]. Therefore, Notch1 and Notch2 suppress keratinocyte proliferation, but it is unlikely that Notch down-regulation contributes to growth regulation in the regenerated epidermis.

3.5. Notch Down-Regulation Alters Keratinocyte Differentiation

Finally, we asked whether Notch down-regulation alters keratinocyte differentiation in the regenerated epidermis. DAPT treatment suppressed expression of spinous layer-specific keratins (K1 and K10) (Fig. 5A, 5B). Similarly, Notch1 knockdown decreased K1 and K10 expression (Fig. 5C). Notch2 knockdown weakly decreased K1 and K10 expression (Fig. 5D), suggesting that Notch1 contributes greatly to the expression of spinous layer-specific keratins. In contrast to Notch down-regulation, Notch intracytoplasmic domain overexpression increased K1 and K10 expression (Fig. 5E, 5F). Notch1 and Notch2 overexpression had similar effects on K1 and K10 expression *in vitro*, although the effects of Notch2 intracytoplasmic domain

overexpression were slightly weaker than those of Notch1 intracytoplasmic domain overexpression. Furthermore, we examined expression of K1 and K10 in the regenerated epidermis. In parallel with Notch down-regulation, the intensity of K1/K10 staining was obviously lower in the regenerated epidermis than in the intact epidermis (Fig. 5G). To further corroborate the involvement of Notch in K1/K10 expression, we topically administered DAPT to mouse skin. In contrast to control DMSO-treated skin, inhibition of Notch signaling decreased K1 expression in the epidermis (Fig. 5H), although K10 expression was not markedly altered. Notch down-regulation likely leads to alteration of spinous layer-specific keratin expression in the regenerated epidermis, which may help to increase cellular pliability necessary for epidermal reconstitution.

In addition, we investigated whether expression of K6, K16, and K17 was altered by inhibition of Notch signaling because these keratins are induced in regenerated epidermis [9]. In contrast to K1 and K10, the expression of K6, K16, and K17 was not altered by DAPT treatment (Fig. 5I), suggesting that the Notch signaling pathway does not directly regulate K6/K16/K17 expression.

Furthermore, we examined the effects of IL-36α on the expression of K6, K16, and K17. However, IL-36α did not alter the expression of these keratins (Fig. 5J). Finally, we investigated whether IL-36α altered keratinocyte migration because of the need for cellular migration in the regenerated epidermis. IL-36α treatment did not alter keratinocyte migration in the scratch assay (Fig. 5K), suggesting that IL-36α induction by Notch down-regulation does not play a role in regulation of keratinocyte migration in the regenerated epidermis.

4. Discussion

Notch signaling controls a number of cellular processes in multiple tissues. We found that expression of Notch1 and Notch2 was decreased in the regenerated epidermis of wounds, where the features of keratinocytes are altered. In our analysis, Notch down-regulation contributed to the induction of IL-36α expression, which would help to alerting surrounding cells to barrier damage. Furthermore, Notch down-regulation suppressed K1/K10 expression,

which would lead to changes in the viscoelastic properties of keratinocytes during regeneration.

Cutaneous wound healing is a coordinated physiological process. While rebuilding an effective epidermal barrier over the wound site, the avoidance of infections is a high priority. In response to epidermal injury, keratinocytes release a variety of factors, such as IL-1α and other proinflammatory cytokines, around the wound [12,13]. Proinflammatory cytokines serve as a paracrine signal to fibroblasts, endothelial cells, and lymphocytes [17]. Furthermore, proinflammatory cytokines serve as an autocrine signal to keratinocytes, stimulating them to produce other cytokines and growth factors [18]. Here, we found that among the IL-1 family members examined, IL-36α was up-regulated upon Notch down-regulation. IL-36 is expressed in keratinocytes and other epithelial cells and refers to three molecules, namely, IL-36α, IL-36β, and IL-36γ, which have similar biological activities [19]. IL-36 exerts stimulatory effects on dendritic cells and T helper 1 cells [20]. Therefore, induction of IL-36α expression would be beneficial to prevent infection during wound healing. The

Notch-IL-36α axis likely acts as an early danger signal to stimulate host responses. There are very few reports concerning the mechanism underlying induction of IL-36α, and it is unclear how Notch regulates IL-36α expression. Based on the inconsistent expression pattern of IL-36α with those of Notch1 and Notch2, we hypothesize that Notch may regulate IL-36α expression in an indirect manner.

The cellular features of regenerated skin resemble those in psoriasis, a chronic inflammatory skin disease [21]. This resemblance may be related to the Notch signaling pathway because IL-36 plays a role in the pathomechanism of psoriasis: IL-36 is overexpressed in psoriatic lesional skin [22,23]. Furthermore, mutations of the natural antagonist of IL-36, IL-36Ra, lead to the development of pustular psoriasis, a subtype of psoriasis [24,25]. In addition, overexpression of IL-36α can lead to psoriatic-like skin inflammation in mice [26,27]. Intriguingly, trauma frequently induces new eruptions on non-lesional skin in patients with psoriasis (Koebner phenomenon). Similar to regenerated skin, trauma of the epidermis may enhance IL-36α expression due to Notch down-regulation in

these patients, leading to the development of new eruptions. Wound healing is different from psoriasis in that IL-36α expression is decreased until wound closure. Restoration of Notch expression after wound closure is important to prevent continued overproduction of IL-36α and to terminate the inflammatory reaction. In addition, impaired Notch signaling results in hidradenitis suppurativa, a chronic recurrent inflammatory skin disease, which further supports the close relationship between decreased Notch signaling and skin inflammation [28].

The viscoelastic properties of keratinocytes are important for rebuilding an effective epidermal barrier. The features of keratinocytes, such as their keratin composition, can be altered. Differentiating keratinocytes express K1 and K10, while the levels of these keratins are decreased concurrent with the induction of K6, K16, and K17 expression in regenerated epidermis [9]. Notch down-regulation results in K1/K10 down-regulation. This affects cellular resilience and pliability because keratins act as a flexible scaffold that enables cells to resist physical stress. While K5/K14 expression renders basal keratinocytes pliable and able to undergo directed cell migration, K1/K10

expression in differentiated keratinocytes promotes strong mechanical resilience at the expense of cellular pliability [29]. Cellular plasticity is important for tissue reconstitution; therefore, decreased K1/K10 expression upon Notch down-regulation enables keratinocytes to adjust their viscoelastic properties. K6, K16, and K17 assist regeneration because K6/K16/K17-expressing keratinocytes are an intermediate between K5/K14-expressing basal cells and K1/K10-expressing differentiated cells [29]. In addition, K6/K16/K17 expression seems to be independent of the Notch signaling pathway because expression of these keratins was not altered by treatment with DAPT or IL-36α. Damaged keratinocytes release prestored IL-1α/β, which serves as an autocrine signal to the surrounding undamaged keratinocytes [18]. IL-1 may lead to induction of K6, K16, and K17 expression [30].

IL-36α expression was induced not only by Notch1 knockdown but also by Notch2 knockdown, whereas Notch2 knockdown had a limited effect on K1/K10 expression. A recent report revealed that Notch1, but not Notch2, triggers keratinocyte differentiation through Rho-associated coiled-coil-containing protein

kinase 1 activation [31], which suggests that downstream signaling varies among Notch family members. Notch1-ablated epidermis is susceptible to chemical-induced carcinogenesis [4]. Although the functions of Notch2 in keratinocytes remain to be fully elucidated, the removal of Notch2 expression in Notch1-deficient skin increases susceptibility to tumorigenesis [32,33]. In addition, loss of CSL or presenilin (the catalytic component of γ-secretase and the core component in signaling of all Notch receptors) results in more severe developmental epidermal phenotypes than loss of Notch1 alone [7,34]. Notch2 likely also contributes to epidermal homeostasis, including wound healing.

In the present study, we elucidated the role of Notch in regenerated keratinocytes. A reduction in Notch signaling alters IL-36α and keratin expression, leading to the regeneration of skin tissue. On the other hand, Notch signaling regulates many other cells aside from keratinocytes in the wound healing process. Notch signaling activation is required for vascular endothelial cell proliferation and migration, and topical application of DAPT leads to delayed wound closure due to effects on endothelial cells [35]. The role of Notch

signaling varies in a cell type-specific manner [2]. However, our results suggest that Notch signaling plays an important role in keratinocyte regulation during wound healing, and that regulation of Notch signaling could provide a novel strategy for the treatment of wounds in pathological conditions.

Acknowledgements

This study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government (to RO, grant number 26461686).

Footnote

The abbreviations used are: BrdU, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; EGFR, epidermal growth factor receptor; FITC, fluorescein isothiocyanate; HPRT, hypoxanthine phosphoribosyl transferase; IL, interleukin; K, keratin; MOI,

multiplicity of infection; qRT-PCR, quantitative reverse transcription-PCR; siRNA, small interfering RNA.

References

[1] Dotto GP. Notch tumor suppressor function. Oncogene 2008;27:5115-23.

[2] Okuyama R, Tagami H, Aiba S. Notch signaling: its role in epidermal homeostasis and in the pathogenesis of skin diseases. J Dermatol Sci 2008;49:187-94.

[3] Watt FM, Estrach S, Ambler CA. Epidermal Notch signalling: differentiation, cancer and adhesion. Curr Opin Cell Biol 2008;20:171-9.

[4] Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, et al. Notch1 functions as a tumor suppressor in mouse skin. Nat Genet 2003;33:416-21.

[5] Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, et al. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J 2001;20:3427-36.

[6] Koster MI, Roop DR. Mechanisms regulating epithelial stratification. Annu Rev Cell Dev Biol 2007;23:93-113.

[7] Blanpain C, Lowry WE, Pasolli HA, Fuchs E. Canonical notch signaling functions as a commitment switch in the epidermal lineage. Genes Dev

2006;20:3022-35.

[8] Ezratty EJ, Stokes N, Chai S, Shah AS, Williams SE, Fuchs E. A role for the primary cilium in Notch signaling and epidermal differentiation during skin development. Cell 2011;145:1129-41.

[9] Coulombe PA. Wound epithelialization: accelerating the pace of discovery. J Invest Dermatol 2003;121:219-30.

[10] Okuyama R, Ogawa E, Nagoshi H, Yabuki M, Kurihara A, Terui T, et al. p53 homologue, p51/p63, maintains the immaturity of keratinocyte stem cells by inhibiting Notch1 activity. Oncogene 2007;26:4478-88.

[11] Kolev V, Mandinova A, Guinea-Viniegra J, Hu B, Lefort K, Lambertini C, et al. EGFR signalling as a negative regulator of Notch1 gene transcription and function in proliferating keratinocytes and cancer. Nat Cell Biol 2008;10:902-11.

[12] Groves RW, Rauschmayr T, Nakamura K, Sarkar S, Williams IR, Kupper TS. Inflammatory and hyperproliferative skin disease in mice that express elevated levels of the IL-1 receptor (type I) on epidermal keratinocytes.

Evidence that IL-1-inducible secondary cytokines produced by keratinocytes in vivo can cause skin disease. J Clin Invest 1996;98:336-44.

[13] Tomic-Canic M, Komine M, Freedberg IM, Blumenberg M. Epidermal signal transduction and transcription factor activation in activated keratinocytes. J Dermatol Sci 1998;17:167-81.

[14] Okuyama R, Nguyen BC, Talora C, Ogawa E, Tommasi di Vignano A, Lioumi M, et al. High commitment of embryonic keratinocytes to terminal differentiation through a Notch1-caspase 3 regulatory mechanism. Dev Cell 2004;6:551-62.

[15] Odland G, Ross R. Human wound repair. I. Epidermal regeneration. J Cell Biol 1968;39:135-51.

[16] Santoro MM, Gaudino G. Cellular and molecular facets of keratinocyte reepithelization during wound healing. Exp Cell Res 2005;304:274-86.

[17] Dinarello CA, Wolff SM. The role of interleukin-1 in disease. N Engl J Med 1993;328:106-13.

[18] Kupper TS, Groves RW. The interleukin-1 axis and cutaneous inflammation.

J Invest Dermatol 1995;105(1 Suppl):62S-6S.

[19] Sims JE, Smith DE. The IL-1 family: regulators of immunity. Nat Rev Immunol 2010;10:89-102.

[20] Vigne S, Palmer G, Martin P, Lamacchia C, Strebel D, Rodriguez E, et al. IL-36 signaling amplifies Th1 responses by enhancing proliferation and Th1 polarization of naive CD4+ T cells. Blood 2012;120:3478-87.

[21] Nickoloff BJ, Bonish BK, Marble DJ, Schriedel KA, DiPietro LA, Gordon KB, et al. Lessons learned from psoriatic plaques concerning mechanisms of tissue repair, remodeling, and inflammation. J Investig Dermatol Symp Proc 2006;11:16-29.

[22] Johnston A, Xing X, Guzman AM, Riblett M, Loyd CM, Ward NL, et al. IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. J Immunol 2011;186:2613-22.

[23] Carrier Y, Ma HL, Ramon HE, Napierata L, Small C, O'Toole M, et al. Inter-regulation of Th17 cytokines and the IL-36 cytokines in vitro and in

vivo: implications in psoriasis pathogenesis. J Invest Dermatol 2011;131:2428-37.

[24] Marrakchi S, Guigue P, Renshaw BR, Puel A, Pei XY, Fraitag S, et al. Interleukin-36-receptor antagonist deficiency and generalized pustular psoriasis. N Engl J Med 2011;365:620-8.

[25] Onoufriadis A, Simpson MA, Pink AE, Di Meglio P, Smith CH, Pullabhatla V, et al. Mutations in IL36RN/IL1F5 are associated with the severe episodic inflammatory skin disease known as generalized pustular psoriasis. Am J Hum Genet 2011;89:432-7.

[26] Blumberg H, Dinh H, Trueblood ES, Pretorius J, Kugler D, Weng N, et al. Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. J Exp Med 2007;204:2603-14.

[27] Blumberg H, Dinh H, Dean C, Jr., Trueblood ES, Bailey K, Shows D, et al. IL-1RL2 and its ligands contribute to the cytokine network in psoriasis. J Immunol 2010;185:4354-62.

[28] Pink AE, Simpson MA, Desai N, Dafou D, Hills A, Mortimer P, et al.

Mutations in the gamma-secretase genes NCSTN, PSENEN, and PSEN1 underlie rare forms of hidradenitis suppurativa (acne inversa). J Invest Dermatol 2012;132:2459-61.

[29] Wong P, Coulombe PA. Loss of keratin 6 (K6) proteins reveals a function for intermediate filaments during wound repair. J Cell Biol 2003;163:327-37.

[30] Freedberg IM, Tomic-Canic M, Komine M, Blumenberg M. Keratins and the keratinocyte activation cycle. J Invest Dermatol 2001;116:633-40.

[31] Yugawa T, Nishino K, Ohno S, Nakahara T, Fujita M, Goshima N, et al. Noncanonical NOTCH Signaling Limits Self-Renewal of Human Epithelial and Induced Pluripotent Stem Cells through ROCK Activation. Mol Cell Biol 2013;33:4434-47.

[32] Demehri S, Turkoz A, Kopan R. Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment. Cancer Cell 2009;16:55-66.

[33] Mazur PK, Gruner BM, Nakhai H, Sipos B, Zimber-Strobl U, Strobl LJ, et al. Identification of epidermal Pdx1 expression discloses different roles of

Notch1 and Notch2 in murine Kras(G12D)-induced skin carcinogenesis in vivo. PLoS One 2010;5:e13578.

[34] Pan Y, Lin MH, Tian X, Cheng HT, Gridley T, Shen J, et al. gamma-secretase functions through Notch signaling to maintain skin appendages but is not required for their patterning or initial morphogenesis. Dev Cell 2004;7:731-43.

[35] Chigurupati S, Arumugam TV, Son TG, Lathia JD, Jameel S, Mughal MR, et al. Involvement of notch signaling in wound healing. PLoS One 2007;2:e1167.

Figure Legends

Figure 1. **Notch expression in the regenerated epidermis during wound healing.** (A) Notch1 and Notch2 expression in regenerated skin. The expression of Notch1 and Notch2 was decreased in the recently re-epithelialized area before closure of the skin defect. Skin sections of regenerated skin were obtained from wounds 3 days after injury. (B) Expression of Hes1 in regenerated skin. (C) Phospho-EGFR (p-EGFR) expression in regenerated skin. (D) Notch1 and Notch2 expression in skin wounds after closure of the defect. The expression of Notch1 and Notch2 was restored 6 weeks after closure. Skin sections were immunostained with antibodies against the indicated proteins and FITC-conjugated secondary antibodies. Nuclei were counterstained with DAPI (blue). Arrows: skin surface, dashed lines: dermal-epidermal junctions. Scale bars: 20 μm. These findings are representative of three independent experiments.

Figure 2. **Notch down-regulation induces IL-36α expression.** (A)

Suppression of Notch activity by DAPT. Primary mouse keratinocytes were treated with DAPT (10 μM, +) or vehicle control (-) for 48 h. Suppression of Hes1 expression, a Notch downstream target, was verified by real-time RT-PCR, using HPRT for internal normalization. (B) Effect of Notch suppression on IL-1 family cytokines. Primary keratinocytes were treated with DAPT (10 μM, +) or vehicle control (-) for 48 h, after which the mRNA levels of IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ were analyzed by real-time RT-PCR. Expression is shown relative to that of HPRT. (C) Effect of Notch suppression on IL-36α expression. IL-36α protein levels were quantified by immunoblotting. Keratinocytes were incubated with $CaCl_2$ (Ca^{2+}) to induce differentiation and/or with DAPT. The same blot was stripped and reprobed for tubulin-α as an internal control. (D) Knockdown of Notch by specific siRNAs. Primary keratinocytes were transfected with siRNA against Notch1 (N1i-1 and N1i-2) or Notch2 (N2i-1 and N2i-2) or with the scrambled siRNA control (Ctrl). Notch silencing was confirmed by real-time RT-PCR 48 hours after transfection. Expression is shown relative to that of HPRT. (E, F) Effect of Notch knockdown on IL-36α expression.

Keratinocytes were transfected with siRNA against Notch1 (N1i-1 and N1i-2) or Notch2 (N2i-1 and N2i-2) or with the scrambled siRNA control (Ctrl). Total RNA was analyzed by real-time PCR with primers designed to amplify IL-36α. Results were normalized against the level of HPRT (E). Keratinocytes were incubated with or without $CaCl_2$ (Ca^{2+}) and then total cell extracts were analyzed by immunoblotting with antibodies against IL-36α and tubulin-α as an equal loading control (F). (G, H) Suppression of IL-36α expression by Notch1 and Notch2 overexpression. Keratinocytes were infected with a Notch1 or Notch2 intracytoplasmic domain-expressing adenovirus (N1 and N2, respectively) or with a LacZ-expressing control adenovirus (Ctrl) at a MOI of 50 for 48 h. Thereafter, IL-36α was analyzed by real-time RT-PCR (G) or immunoblotting (H) as described in the previous panels. In (H), cells were treated with $CaCl_2$ for various amounts of time. Data are presented as the mean ± standard deviation. $^{**}P < 0.01$ (Student's *t*-test). The results are representative of three independent experiments.

Figure 3. ***In vivo*** **Notch suppression induces IL-36α expression in the epidermis.** (A, B) IL-36α expression in the regenerated epidermis. IL-36α expression was increased in the recently re-epithelialized area before closure of the skin defect (A), and reduced after closure (B). Frozen sections of skin wounds 3 days after injury and 6 weeks after closure as well as of intact skin were immunostained with an anti-IL-36α antibody and a FITC-conjugated secondary antibody. Nuclei were counterstained with DAPI (blue). (C, D) Expression of cleaved Notch1 and IL-36α in DAPT-treated skin. DAPT (100 μM) or vehicle control (DMSO) was applied to the skin, and immunostaining was then performed for cleaved Notch1 (C) and IL-36α (D). Arrows: skin surface, dashed lines: dermal-epidermal junctions. Scale bars: 20 μm. These findings are representative of three independent experiments.

Figure 4. **Effect of Notch down-regulation on keratinocyte proliferation in the regenerated epidermis.** (A) Overexpression of Notch1 or Notch2 induces keratinocyte growth arrest. Primary keratinocytes were infected with a Notch1-

or Notch2 intracytoplasmic domain-expressing adenovirus (N1 and N2, respectively) or a LacZ-expressing control adenovirus (Ctrl) at a MOI of 50 for 48 hours. The mean percentage of BrdU-positive keratinocytes is shown. The error bars indicate standard deviations. *P < 0.05; **P < 0.01 (Student's t-test). (B) Effect of suppression of Notch activity on keratinocyte proliferation. Primary keratinocytes were treated with various concentrations of DAPT. The percentage of BrdU-positive cells is shown. The bars and error bars indicate the mean values and standard deviations, respectively. (C) Immunohistochemical analysis with an anti-Ki67 antibody in a healing wound. The low magnification image shows hematoxylin-eosin staining of a whole-wound section. The high magnification images show (i) intact skin, (ii) skin immediately proximal to the wound edge, and (iii) regenerated skin. The number of Ki67-positive keratinocytes was not higher in the regenerated epidermis than in intact skin. Sections were immunostained with an anti-Ki67 antibody and a FITC-conjugated secondary antibody. Nuclei were counterstained with DAPI (blue). Arrows: skin surface, dashed lines: dermal-epidermal junctions, arrowheads: Ki67-positive

cells. Scale bar: 400 μm (low magnification) and 20 μm (high magnification). These findings are representative of three independent experiments.

Figure 5. **Effect of Notch down-regulation on keratinocyte differentiation.** (A) Effect of Notch suppression on the spinous layer-specific keratins, K1 and K10. Primary keratinocytes were treated with DAPT (10 μM, +) or vehicle control (-) for 48 h, and then the mRNA levels of K1 and K10 were analyzed by real-time RT-PCR. The expression is shown relative to that of HPRT. (B) Effect of Notch suppression on K1 and K10 expression. $CaCl_2$ (Ca^{2+}) was used to induce keratinocyte differentiation and cells were treated with various concentrations of DAPT. Total cell extracts were analyzed by immunoblotting with antibodies against K1 and K10, as well as tubulin-α as the equal loading control. (C) Effect of Notch1 knockdown on K1 and K10 expression. Primary keratinocytes were transfected with siRNA against Notch1 (N1i-1 and N1i-2) or with the scrambled siRNA control (Ctrl). K1 and K10 were analyzed by real-time RT-PCR, using HPRT for internal normalization. (D) Effect of Notch2 knockdown on K1 and K10

expression. Primary keratinocytes were transfected with siRNA against Notch2 (N2i-1 and N2i-2) or with the scrambled siRNA control (Ctrl). K1 and K10 were analyzed by real-time RT-PCR, using HPRT for internal normalization. (E, F) Induction of K1 and K10 expression by Notch1 and Notch2 overexpression. Keratinocytes were infected with a Notch1- or a Notch2 intracytoplasmic domain-expressing adenovirus (N1 and N2, respectively) or a LacZ-expressing control adenovirus (Ctrl) at a MOI of 50 for 48 h. Total RNA was analyzed by real-time PCR with primers designed to amplify K1 or K10. Results were normalized against the level of HPRT (E). Total cell extracts were analyzed by immunoblotting with antibodies against K1, K10, Notch1, and Notch2 (F). $CaCl_2$ (Ca^{2+}) was used to induce keratinocyte differentiation. The same blot was stripped and reprobed for K5 as the internal control. (G) K1 and K10 expression in regenerated epidermis. Expression of K1 and K10 was decreased in the recently re-epithelialized area of the skin wound 3 days after injury. Frozen sections of regenerated skin and intact skin were immunostained with an anti-K1 antibody or an anti-K10 antibody and a FITC-conjugated secondary antibody.

(H) Expression of K1 in DAPT-treated skin. DAPT or the vehicle control (DMSO) was applied to the skin, and then immunostaining was performed for K1 and K10. Arrows: skin surface, dashed lines: dermal-epidermal junctions. Scale bars: 20 μm. (I) Effect of Notch suppression on the expression of K6, K16, and K17. Primary keratinocytes were treated with DAPT (10 μM, +) or vehicle control (-) for 48 h, and then the mRNA levels of K6, K16, and K17 were analyzed by real-time RT-PCR. The expression is shown relative to that of HPRT. (J) Effect of IL-36α on the expression of K6, K16 and K17. Primary keratinocytes were treated with IL-36α or vehicle control for 24 h, and then the mRNA levels of K6, K16, and K17 were analyzed by real-time RT-PCR. (K) Effect of IL-36α on keratinocyte cell migration in a scratch assay. IL-36α did not alter keratinocyte migration. Scale bars: 200 μm. Data are presented as the mean ± standard deviation. *P < 0.05; **P < 0.01 (Student's t-test). These findings are representative of three independent experiments.

Figure 1
Click here to download high resolution image

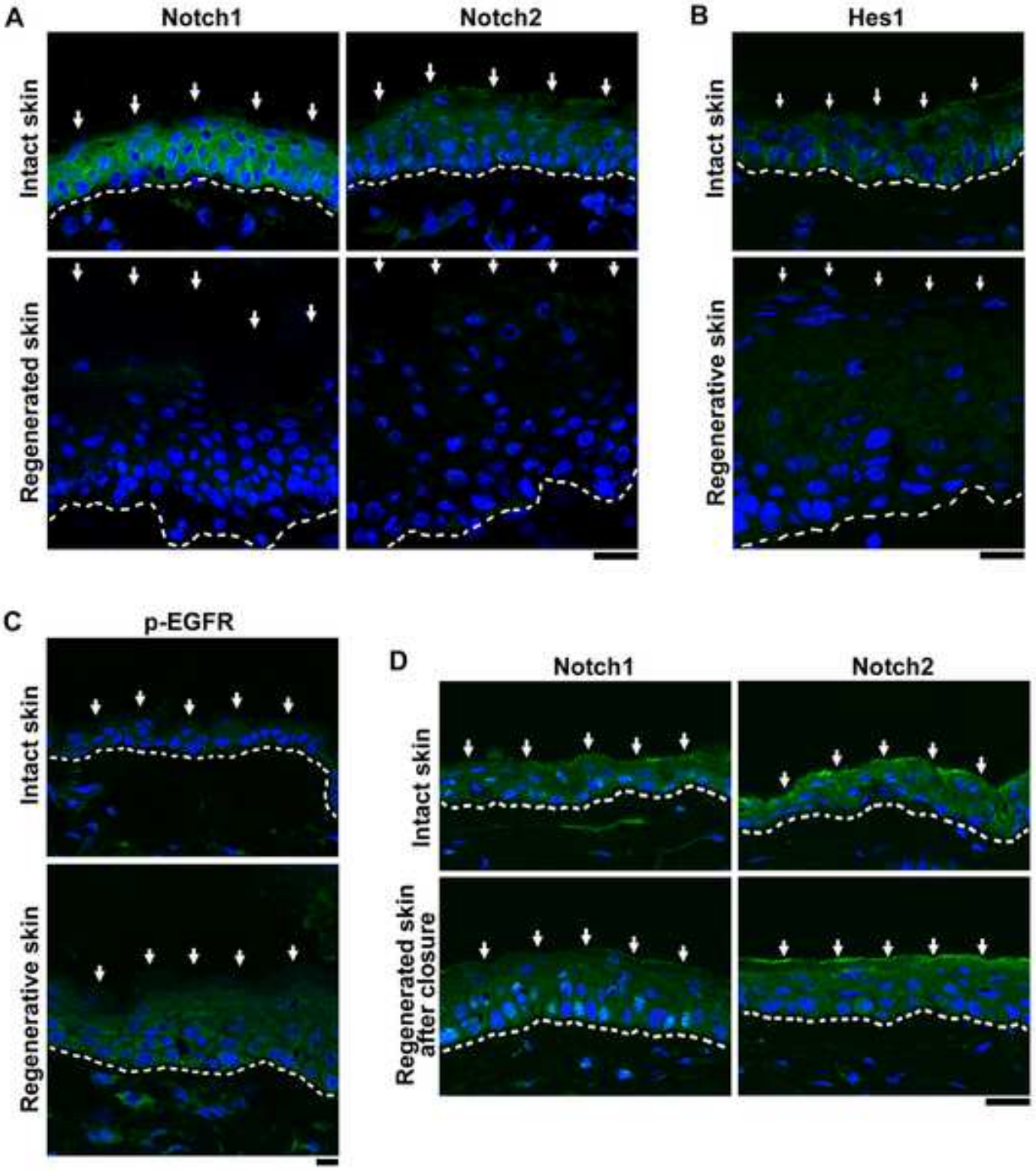

Figure 2

Click here to download high resolution image

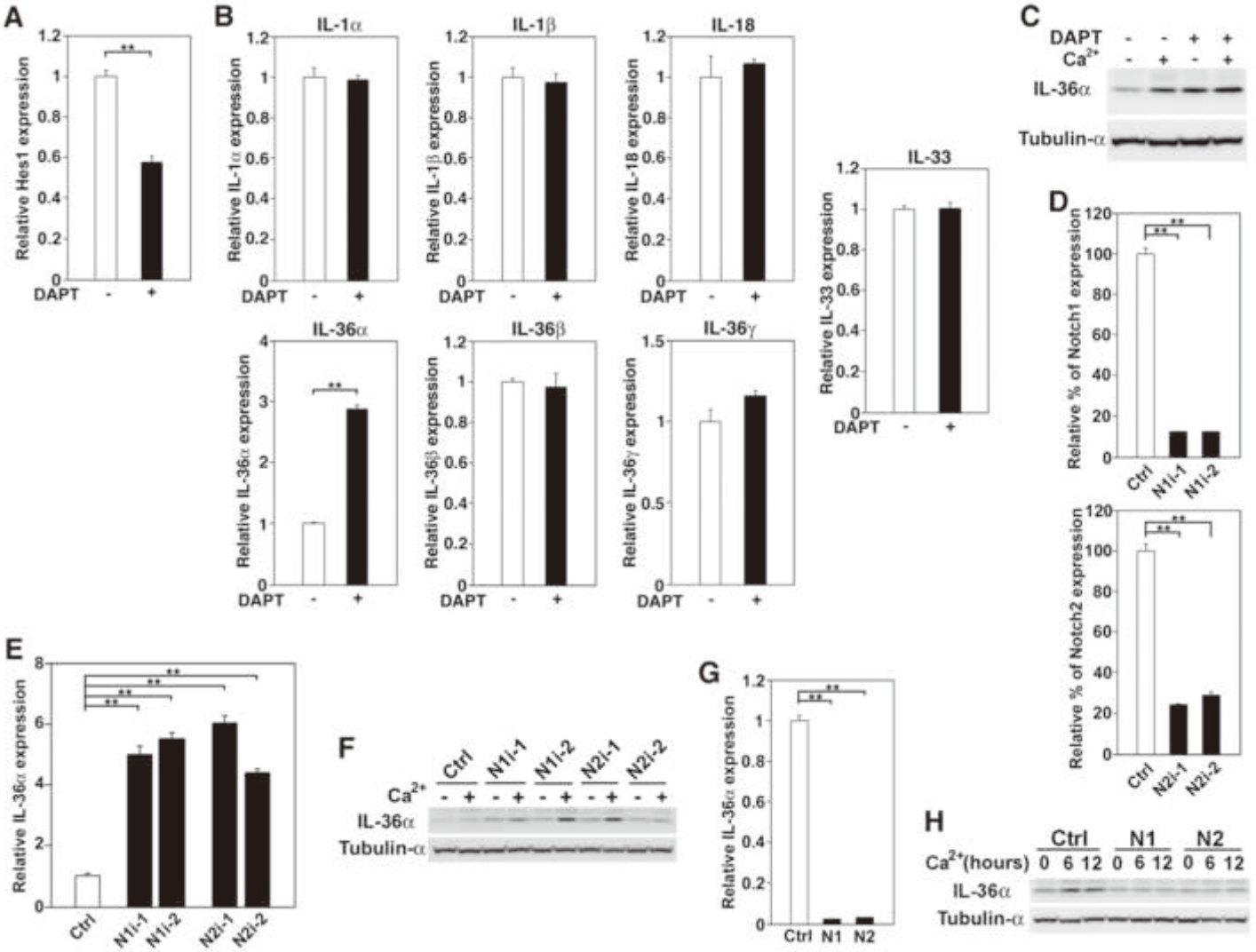

Figure 3
Click here to download high resolution image

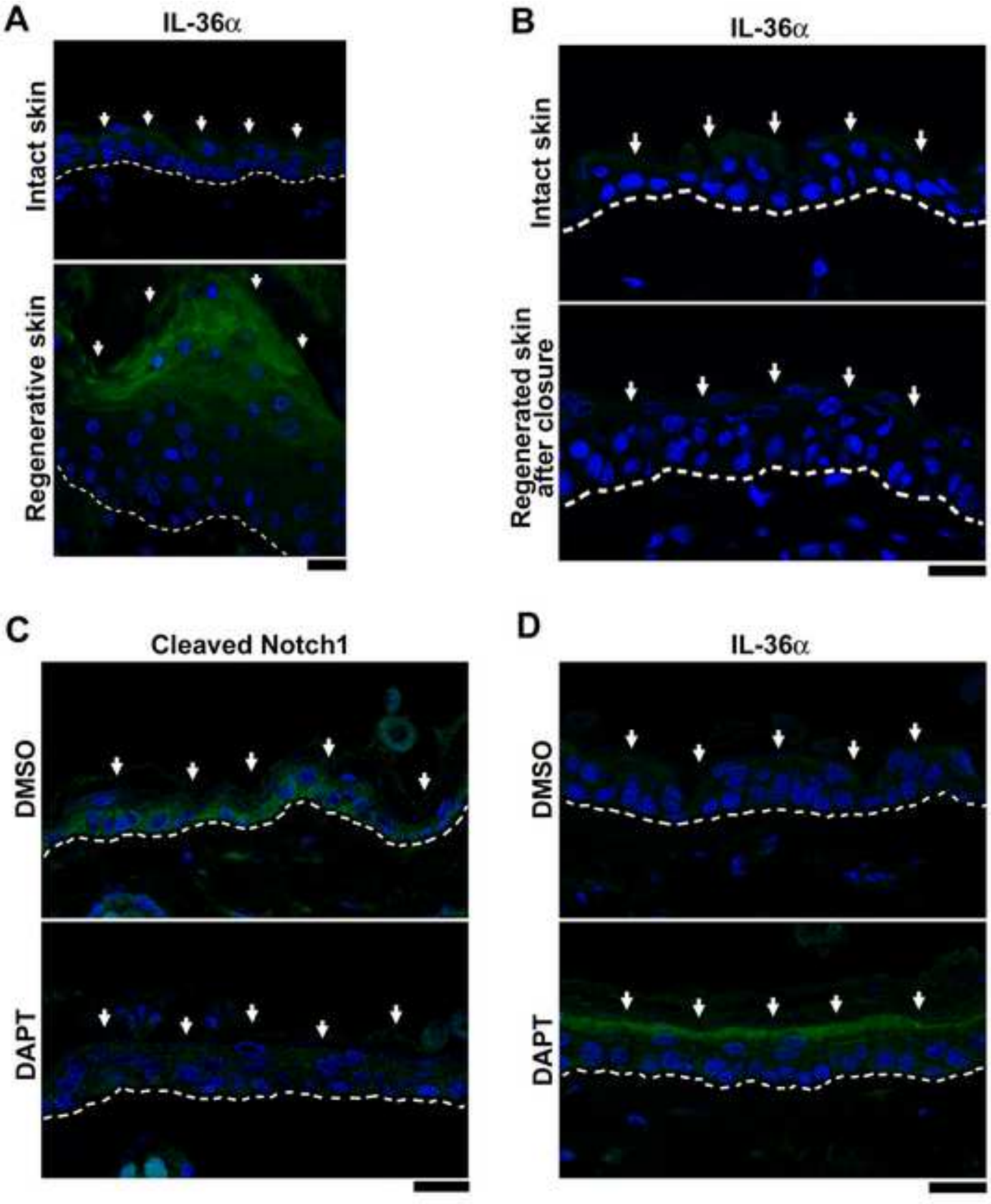

Figure 4
Click here to download high resolution image

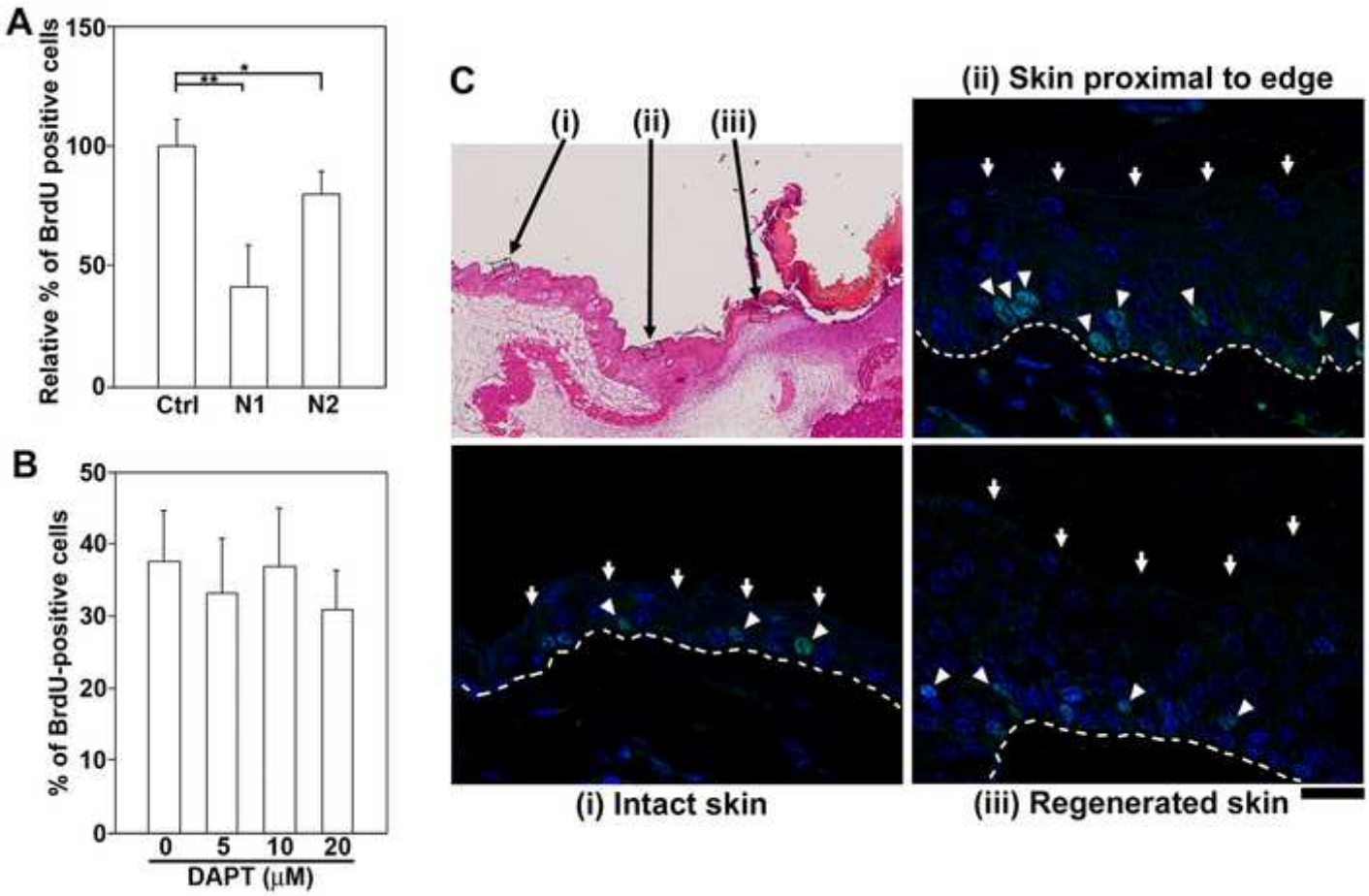

Figure 5

Click here to download high resolution image