Epidermal ablation of Dlx3 is linked to IL-17–associated skin inflammation

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In an effort to understand the role of Distal-less 3 (Dlx3) in cutaneous biology and pathophysiology, we generated and characterized a mouse model with epidermal ablation of Dlx3. K14cre;Dlx3$^{Kin/f}$ mice exhibited epidermal hyperproliferation and abnormal differentiation of keratinocytes. Results from subsequent analyses revealed cutaneous inflammation that featured accumulation of IL-17–producing CD4$^+$ T, CD8$^+$ T, and γδ T cells in the skin and lymph nodes of K14cre;Dlx3$^{Kin/f}$ mice. The gene expression signature of K14cre;Dlx3$^{Kin/f}$ skin shared features with lesional psoriatic skin, and Dlx3 expression was markedly and selectively decreased in psoriatic skin. Interestingly, cultured Dlx3 null keratinocytes triggered cytokine production that is potentially linked to inflammatory responses in K14cre;Dlx3$^{Kin/f}$ mice. Thus, Dlx3 ablation in epidermis is linked to altered epidermal differentiation, barrier development, and IL-17–associated skin inflammation. This model provides a platform that will allow the systematic exploration of the contributions of keratinocytes to cutaneous inflammation.

barrier function | psoriasis | inflammatory diseases | homeobox transcription factor | mouse model

Skin functions as a barrier that protects the body from the external environment and excessive water loss. Barrier function is established through multistage differentiation of keratinocytes in the epidermis. During this process, keratinocytes differentiate from proliferative cells in the basal layer into flattened cornified cells in the stratum corneum, associated with expression of differentiation-specific proteins, many of which are encoded by genes clustered in the epidermal differentiation complex (EDC). Leukocytes in skin play a key role as sentinels, and crosstalk between keratinocytes and leukocytes is crucial for maintenance of the skin homeostasis (1). When the epidermal barrier is disrupted, keratinocytes produce cytokines and chemokines to recruit leukocytes from blood into skin (2, 3).

Recent studies indicate that genetic mutations, particularly in genes in the EDC, are linked to barrier dysfunction in three common skin disorders: ichthyosis vulgaris, atopic dermatitis (AD), and psoriasis (PS). The EDC encodes proteins including filaggrin (Flg), loricrin (Lor), S100 proteins, late cornified envelope (LCE) proteins, and the small proline-rich (Sprr) proteins (4). Loss-of-function mutations in the Flg gene have been identified as causative in ichthyosis vulgaris and AD (5, 6). Genome-wide association studies also identified susceptibility variants within the LCE gene cluster in PS (7, 8).

Although inflammatory skin disorders are often accompanied by decreased barrier function, the role of keratinocyte dysfunction in disease causality is often unclear. Most notably, inflamed skin generally exhibits abnormal keratinocyte differentiation, disrupted barrier function, and/or immunocyte abnormalities (9–11). Studying key genes that are active in immunologic functions as well as epidermal homeostasis will provide insight into the intricate regulatory networks involved in complex inflammatory skin diseases.

Distal-less 3 (Dlx3) is a homeobox transcription factor involved in terminal differentiation of keratinocytes (12). Misexpression of Dlx3 in the basal layer results in decreased keratinocyte proliferation and premature terminal differentiation (13). To study the role of Dlx3 in the skin homeostasis, we generated conditional epidermis-specific knockout K14cre;Dlx3$^{Kin/f}$ mice (14). In the present study, we characterized the abnormal differentiation and hyperproliferation of keratinocytes in the skin of K14cre;Dlx3$^{Kin/f}$ mice, along with the development of IL-17–associated skin inflammation distinguished by cytokine and chemokine up-regulation and leukocyte infiltration that resembles a human skin inflammatory disease.

Results

Epidermal-Specific Ablation of Dlx3 Results in Altered Barrier Formation. K14cre;Dlx3$^{Kin/f}$ mice presented a gross phenotype distinguished by persistent scaly skin (Fig. 1*A*). To assess barrier function, we performed dye exclusion assays (15) at E17.5 and found that WT embryos excluded dye, whereas K14cre;Dlx3$^{Kin/f}$ embryos uniformly exhibited dye penetration into skin (Fig. 1*B*). By E18.5, the K14cre;Dlx3$^{Kin/f}$ fetuses did not show obvious percutaneous dye penetration, demonstrating delayed establishment of the water barrier in developing skin. To further assess barrier function-related features of K14cre;Dlx3$^{Kin/f}$ skin, we analyzed E18.5 cornified envelopes (CEs) using phase-contrast microscopy. Compared with the WT CEs and consistent with abnormal barrier function, mutant mice exhibited fragile CEs (Fig. 1*C*, arrowheads) with a rigidly polygonal shape.

Mice Lacking Dlx3 in Epidermis Exhibit Abnormalities in Epidermal Differentiation and Hyperplasia. To determine whether the K14cre;Dlx3$^{Kin/f}$ mice exhibited impaired epidermal differentiation, we compared the expression of Lor and Flg proteins in epidermis via Western blot analysis. The K14cre;Dlx3$^{Kin/f}$ mice exhibited diminished expression of Lor and Flg at P3 (Fig. 1*D*). Claudins, which are key components of tight junctions (16), especially Claudin 7, also were decreased in P3 K14cre;Dlx3$^{Kin/f}$ skin (Fig. 1*D*).

K6 and K16 are hyperproliferation-associated keratins that are up-regulated in uninvolved and lesional psoriatic epidermis (2).

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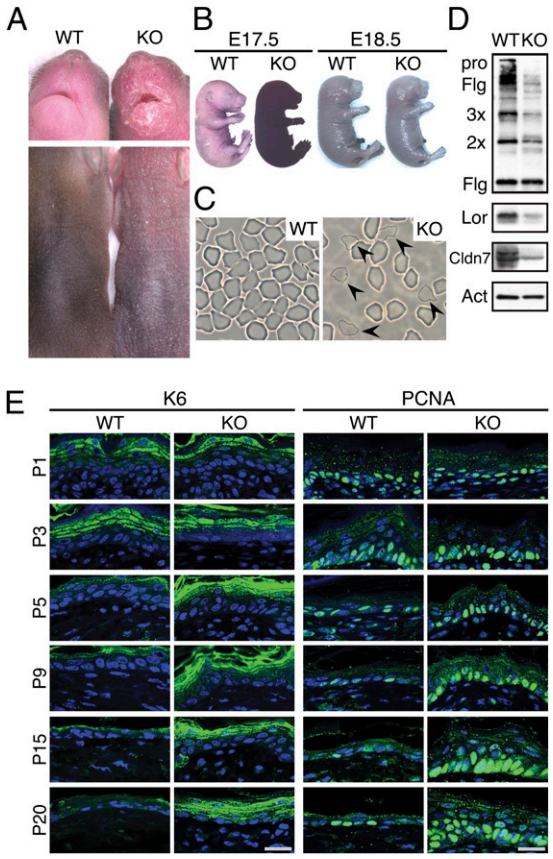


Fig. 1. Mice lacking Dlx3 in the epidermis exhibit epidermal barrier defects. (*A*) K14cre;Dlx3$^{Kin/f}$ mice at P5 exhibited dry skin near the mouth and across the dorsal skin. (*B*) Dye-exclusion assays showed barrier dysfunction in E17.5 K14cre;Dlx3$^{Kin/f}$ fetuses. Obvious permeability differences were not detected at E18.5. (*C*) Cell envelope preparations from skin of E18.5 fetuses analyzed by phase contrast microscopy exhibited fragile and sparse cell envelopes in the K14cre;Dlx3$^{Kin/f}$ skin. (*D*) Western blot analysis was performed using skin protein extracts. (*E*) Expression of Flg, Lor, and claudin 7 was decreased in K14cre;Dlx3$^{Kin/f}$ mice at P3. Skin samples were obtained from WT and K14Cre;Dlx3$^{Kin/f}$ mice (KO) at P1, P3, P5, P9, P15, and P20, and sections were stained with anti-K6 and anti-PCNA antibodies (green) and DAPI (blue). (Scale bar: 20 μm.)

Elevated proliferation was detected in the skin of K14cre;Dlx3$^{Kin/f}$ mice by immunohistochemistry using anti-K6 and anti-proliferating cell nuclear antigen (PCNA) antibodies (Fig. 1*E*) and confirmed by microarray analysis (Fig. 2*A*). The status of epidermal differentiation in K14cre;Dlx3$^{Kin/f}$ mice also was assessed via gene expression analysis of whole skin (Fig. 2*A*). Most prominently, Lce(s) genes, which are frequently up-regulated during barrier dysfunction, were induced in K14cre;Dlx3$^{Kin/f}$ skin (Fig. 2*A*). The significant up-regulation of Sprr2h and Sprr2d in the K14cre;Dlx3$^{Kin/f}$ mice suggested stimulation of a compensatory mechanism similar to that reported previously (17). Expression of the differentiation-specific markers Lor and Flg was assessed by qPCR analysis and immunohistochemistry (for Flg), showing that although expression was lower in mutant skin than in WT at P1, P3, and P5, expression of these differentiation markers was significantly up-regulated beginning on P9 (Fig. 2*B* and Fig. S1). Altered expression of differentiation and cell envelope proteins that are frequently up-regulated during barrier dysfunction (Fig. 2*A*) are consistent with sustained abnormalities in epidermal differentiation.

Other genes involved in barrier formation and function, including peroxisome proliferator-activated receptor (PPAR) and transglutaminase (Tgm), were down-regulated in K14cre;Dlx3$^{Kin/f}$ mice (Fig. 2*A*). qPCR performed to validate Dlx3 ablation in the K14cre;Dlx3$^{Kin/f}$ samples showed decreased expression of Dlx3 and hair-specific keratin 32 (K32), a Dlx3 target (14), in K14cre; Dlx3$^{Kin/f}$ mice (Fig. S2).

Development of an Inflammatory Response in K14cre;Dlx3$^{Kin/f}$ Skin. The skin of K14cre;Dlx3$^{Kin/f}$ mice demonstrated up-regulation of genes encoding inflammatory mediators and antimicrobial peptides (Fig. 2*A*). Progressively increased expression of S100a8 and S100a9 was observed in K14cre;Dlx3$^{Kin/f}$ skin. Defensins, particularly β-defensin 3 (Defb3), also were significantly up-regulated at P20, and up-regulation of lipocalin 2 (Lcn2) was detected at P9 and P20. In addition to its antimicrobial activity, Lcn2 acts as a marker for abnormal keratinocyte differentiation (18).

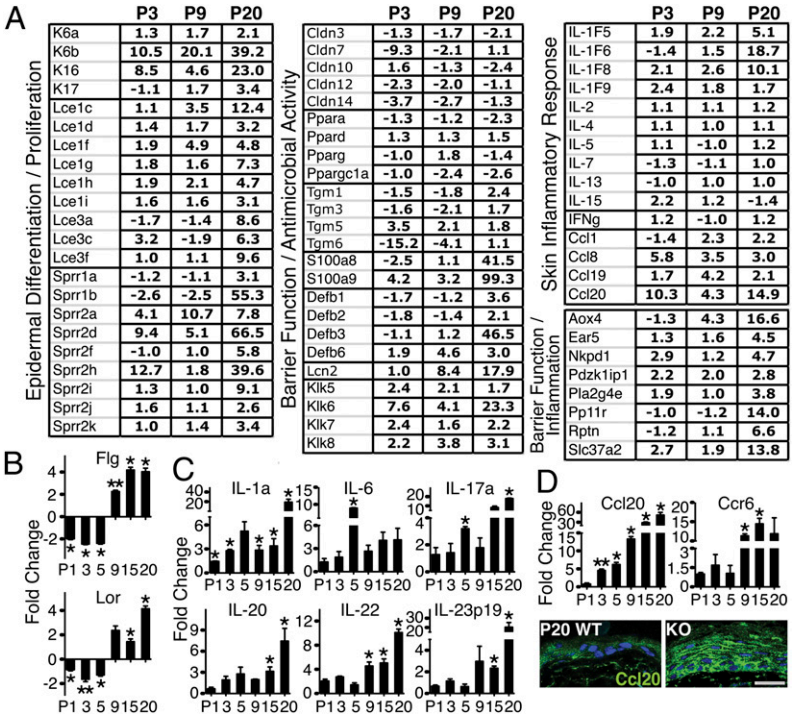
K14cre;Dlx3$^{Kin/f}$ skin also exhibited up-regulation of several members of the kallikrein chymotrypsin-like secreted serine protease family (Klk5, Klk6, Klk7, and Klk8), which are involved in skin barrier maintenance (19). Up-regulation of Klk family members in K14cre;Dlx3$^{Kin/f}$ skin may indicate activation of pathways involved in barrier function and innate immunity. Interestingly, Klk5 and Klk6 expression was up-regulated in PS skin compared with normal and AD skin (9).

Systems biology analysis identified a linkage network of correlated expression of genes related to barrier function and inflammation (20). Many of the genes in this linkage network (Lce3a, Sprr2i, Sprr2d, K6b, S100a8, Defb3, IL-1F5, IL-1F6, IL-1F8, and IL-1F9) were differentially expressed in K14cre; Dlx3$^{Kin/f}$ (Fig. 2*A*). IL-1F5, IL-1F6, IL-1F8, and IL-1F9 are recently identified IL-1 family cytokines (21, 22).

Elevated expression of IL-1α, IL-6, and IL-17a was found in K14cre;Dlx3$^{Kin/f}$ skin (Fig. 2*C*). IL-1α is a key proinflammatory cytokine secreted by keratinocytes, and both IL-1α and IL-6 play crucial roles in cutaneous inflammation (23, 24). Up-regulation of IL-17a expression is particularly interesting because IL-17a is produced by T helper 17 (Th17) cells, a recently identified subset of T helper cells that can induce antimicrobial activity and recruit dendritic cells and neutrophils (25, 26). It is well known that IL-23, a cytokine composed of a unique p19 subunit and the p40 subunit shared with IL-12, promotes the expansion of Th17 cells. Thus, we examined the expression level of the p19 subunit and confirmed increased expression (Fig. 2*C*). Recent studies have shown that IL-23 or IL-6 can trigger IL-22 production from T cells, and that IL-22 can mediate the crosstalk between keratinocytes and leukocytes by inducing IL-20 production in keratinocytes (27, 28). Both IL-20 and IL-22 expression were up-regulated in K14cre;Dlx3$^{Kin/f}$ skin (Fig. 2*C*). The expression level of other cytokines, including IL-2, IL-4, IL-5, IL-7, IL-13, IL-15, and IFN-γ, was not significantly changed in K14cre;Dlx3$^{Kin/f}$ skin (Fig. 2*A*), suggesting that up-regulation of IL-17 production in skin may be particularly relevant.

Several chemokines were increased in the skin of K14cre; Dlx3$^{Kin/f}$ mice (Fig. 2*A*). Ccl20 and its receptor Ccr6 were strongly up-regulated (Fig. 2 *A* and *D*). A recent study found that Ccl20 is secreted by keratinocytes, providing the capacity to recruit Ccr6-positive Th17 cells (29). The up-regulation of Ccl20 was corroborated in the results of our microarray analysis (Fig. 2*A*), qPCR, and immunohistochemistry (Fig. 2*D*).

IL-17–Expressing T Cells and Leukocytes Are Increased in the Skin of K14cre;Dlx3$^{Kin/f}$ Mice. To investigate the inflammatory response in K14cre;Dlx3$^{Kin/f}$ mice, we performed immunohistochemistry with antibodies reactive with CD45 and F4/80 (Fig. 3*A*). CD45 is a pan-leukocyte cell surface marker (30), and F4/80 is a marker for macrophages and epidermal Langerhans cells (21). Cells expressing these markers were markedly increased from P3 onward in K14cre;Dlx3$^{Kin/f}$ skin (Fig. 3*A*). The numbers of CD45$^+$ cells and infiltrating F4/80$^+$ cells were significantly higher in K14cre;Dlx3$^{Kin/f}$ skin by P3 and remained at increased levels through P20 (Fig. S3).



	P3	P9	P20
K6a	1.3	1.7	2.1
K6b	10.5	20.1	39.2
K16	8.5	4.6	23.0
K17	-1.1	1.7	3.4
Lce1c	1.1	3.5	12.4
Lce1d	1.4	1.7	3.2
Lce1f	1.9	4.9	4.8
Lce1g	1.8	1.6	7.3
Lce1h	1.9	2.1	4.7
Lce1i	1.6	1.6	3.1
Lce3a	-1.7	-1.4	8.6
Lce3c	3.2	-1.9	6.3
Lce3f	1.0	1.1	9.6
Sprr1a	-1.2	-1.1	3.1
Sprr1b	-2.6	-2.5	55.3
Sprr2a	4.1	10.7	7.8
Sprr2d	9.4	5.1	66.5
Sprr2f	-1.0	1.0	5.8
Sprr2h	12.7	1.8	39.6
Sprr2i	1.3	1.0	9.1
Sprr2j	1.6	1.1	2.6
Sprr2k	1.0	1.4	3.4

	P3	P9	P20
Cldn3	-1.3	-1.7	-2.1
Cldn7	-9.3	-2.1	1.1
Cldn10	1.6	-1.3	-2.4
Cldn12	-2.3	-2.0	-1.1
Cldn14	-3.7	-2.7	-1.3
Ppara	-1.3	-1.2	-2.3
Ppard	1.3	1.3	1.5
Pparg	-1.0	1.8	-1.4
Ppargc1a	-1.0	-2.4	-2.6
Tgm1	-1.5	-1.8	2.4
Tgm3	-1.6	-2.1	1.7
Tgm5	3.5	2.1	1.8
Tgm6	-15.2	-4.1	1.1
S100a8	-2.5	1.1	41.5
S100a9	4.2	3.2	99.3
Defb1	-1.7	-1.2	3.6
Defb2	-1.8	-1.4	2.1
Defb3	-1.1	1.2	46.5
Defb6	1.9	4.6	3.0
Lcn2	1.0	8.4	17.9
Klk5	2.4	2.1	1.7
Klk6	7.6	4.1	23.3
Klk7	2.4	1.6	2.2
Klk8	2.2	3.8	3.1

	P3	P9	P20
IL-1F5	1.9	2.2	5.1
IL-1F6	-1.4	1.5	18.7
IL-1F8	2.1	2.6	10.1
IL-1F9	2.4	1.8	1.7
IL-2	1.1	1.1	1.2
IL-4	1.1	1.0	1.1
IL-5	1.1	-1.0	1.2
IL-7	-1.3	-1.1	1.0
IL-13	-1.0	1.0	1.0
IL-15	2.2	1.2	-1.4
IFNg	1.2	-1.0	1.2
Ccl1	-1.4	2.3	2.2
Ccl8	5.8	3.5	3.0
Ccl19	1.7	4.2	2.1
Ccl20	10.3	4.3	14.9

	P3	P9	P20
Aox4	-1.3	4.3	16.6
Ear5	1.3	1.6	4.5
Nkpd1	2.9	1.2	4.7
Pdzk1ip1	2.2	2.0	2.8
Pla2g4e	1.9	1.0	3.8
Pp11r	-1.0	-1.2	14.0
Rptn	-1.2	1.1	6.6
Slc37a2	2.7	1.9	13.8

Fig. 2. Gene expression analysis of WT and K14Cre;Dlx3$^{Kin/f}$ skin by microarray and qPCR. (*A*) Microarray analysis of total RNA isolated from skin indicated abnormal terminal differentiation and inflammation in K14cre;Dlx3$^{Kin/f}$ mice at P3, P9, and P20. Numbers indicate fold changes in K14cre; Dlx3$^{Kin/f}$ skin (KO) vs. WT controls. Aox4, aldehyde oxidase 4; Ear5, eosinophil-associated, ribonuclease A family member 5; Nkpd1, NTPase, KAP family P-loop domain–containing 1; Pdzk1ip1, PDZK1-interacting protein 1; Pla2g4e, phospholipase A2, group IVE; Pp11r, placental protein 11-related; Rptn, Repetin; Slc37a2, solute carrier family 37 (glycerol-3-phosphate transporter) member 2. (*B*) Fold changes of KO vs. WT in the expression of Flg and Lor analyzed by qPCR in P1–P20 skin. (*C*) Fold changes of KO vs. WT in the expression of IL-1a, IL-6, IL-17a, IL-20, IL-22, and IL-23p19 were analyzed by qPCR in P1–P20 skin, showing up-regulated expression of cytokines in K14Cre;Dlx3$^{Kin/f}$ skin. (*D*) qPCR analysis and immunohistochemistry for Ccl20 (green) expression in K14cre; Dlx3$^{Kin/f}$ skin. Sections were counterstained with DAPI (blue). Ccr6, a receptor for Ccl20, was up-regulated in K14cre; Dlx3$^{Kin/f}$ skin. n = 3–5 mice per time point. $^{*}P < 0.05$; $^{**}P < 0.005$. (Scale bar: 20 μm.)

The frequencies of CD45$^+$ cells and infiltrating F4/80$^+$ cells remained significantly higher in K14cre;Dlx3$^{Kin/f}$ skin at 9 wk (Fig. S4). In addition, systemic inflammation was demonstrated by gross inspection of spleens and lymph nodes at different stages of postnatal development (Fig. S4). The K14cre;Dlx3$^{Kin/f}$ mice exhibited progressively enlarged spleens and lymph nodes compared with those of their WT littermates.

Total leukocytes (CD45$^+$) were isolated from P20 WT and K14cre;Dlx3$^{Kin/f}$ skin, and these cells were then gated for antigen-presenting cells (APCs), T cells, B cells, Langerhans cells, and neutrophils (Fig. 3*B* and Fig. S5*A*). The fold changes and total numbers of each of these cell types were increased in K14cre;Dlx3$^{Kin/f}$ skin, with significant increases in neutrophils and Langerhans cells.

Results from qPCR showed markedly increased IL-17 and IL-23 expression in the skin of K14cre;Dlx3$^{Kin/f}$ mice (Fig. 2*C*). In addition to Th17 cells, other sources of IL-17, including CD8$^+$ T cells and γδ T cells, have been reported in skin inflammatory responses (31, 32). To evaluate IL-17a–expressing cells in skin and draining lymph nodes (DLNs) of WT and K14cre;Dlx3$^{Kin/f}$ mice, we performed analytical flow cytometry for CD4$^+$ T cells, CD8$^+$ T cells, and γδ T cells and then analyzed intracellular IL-17a expression in each of these gated cell populations (Fig. 3*C* and Fig. S5). The results indicate that IL-17a–producing T cells were increased in the skin and DLNs of K14cre;Dlx3$^{Kin/f}$ mice. Interestingly, in addition to Th17 cells, increases in IL-17$^+$ CD8$^+$ T cells and IL-17$^+$ γδ T cells also were detected in both skin and DLNs of K14cre; Dlx3$^{Kin/f}$ mice. These results are consistent with recent reports demonstrating that IL-17 can be produced by IL-17$^+$ CD8$^+$ T cells and γδ T cells, as well as by Th 17 cells (31, 33). The absolute numbers for each subpopulation are presented in Fig. S5*C*.

K14cre;Dlx3$^{Kin/f}$ Skin Presents a Gene Expression Signature with Similarities to Human Inflammatory Skin Diseases. To identify possible similarities between human inflammatory skin diseases and the skin inflammation in K14cre;Dlx3$^{Kin/f}$ mice, qPCR analysis was performed for genes composing signatures for PS and AD (9, 10). These results showed sustained up-regulation of Sprr2d, Defb3, and S100a9 expression in the K14cre;Dlx3$^{Kin/f}$ mice (Fig. 4*A*), in agreement with the microarray results (Fig. 2*A*). Expression of the Corneodesmosin (Cdsn) gene, located in the PS susceptibility locus 1 and up-regulated in PS (34), was up-regulated in K14cre;Dlx3$^{Kin/f}$ skin as well. Thus, K14cre;Dlx3$^{Kin/f}$ mice have a gene expression signature resembling that seen in PS.

To assess the expression of Dlx3 in human inflammatory skin diseases, we performed immunohistochemistry using Dlx3 antibody on sections of PS and AD skin. Dlx3 expression was remarkably diminished in the PS skin, with no detectable nuclear localization (Fig. 4*B*, high-magnification insets). In contrast, Dlx3 was readily detectable in most nuclei of the upper differentiated layers of normal and AD skin (Fig. 4*B* and Fig. S6).

Specific Contributions of Dlx3-Null Keratinocytes to Inflammatory Mediator Production. We reasoned that Dlx3 deletion in keratinocytes might be linked directly to inflammatory mediator production. To examine this, we isolated primary keratinocytes from Dlx3$^{f/f}$ mice and transduced them with adenovirus expressing Cre recombinase. qPCR analyses revealed increased expression of IL-1α, IL-1F8, S100a9, and Sprr2d in Dlx3-null keratinocytes (Fig. 5*A*). These results indicate that ablation of Dlx3 expression in keratinocytes directly causes altered differentiation and triggers cytokine production that could potentially initiate the inflammatory responses observed in K14cre;Dlx3$^{Kin/f}$ mice, including the infiltration of leukocytes into skin.

It is well recognized that PPAR expression in the epidermis is related to epidermal differentiation and inflammation (35). We found decreased expression of PPARα and PPARγ in Dlx3-null keratinocytes (Fig. 5*A*). Moreover, both lesional psoriatic and K14cre;Dlx3$^{Kin/f}$ skin demonstrated increased PPARβ/δ expression but decreased PPARα and PPARγ expression (36) (Fig. 2*A*).

To further analyze the effects of PPARγ on Dlx3-null keratinocytes, we transduced Adeno-PPARγ viruses into Adeno-cre; Dlx3$^{f/f}$ keratinocytes. qPCR analyses showed that the expression levels of IL-1Iα, IL-1F8, S100a9, and Sprr2d in Dlx3-null keratinocytes reverted to the levels seen in WT control keratinocytes (Fig. 5*B*). These results suggest that increased cytokine/chemokine production by Dlx3-null keratinocytes is due, at least in part, to decreased PPARγ production.

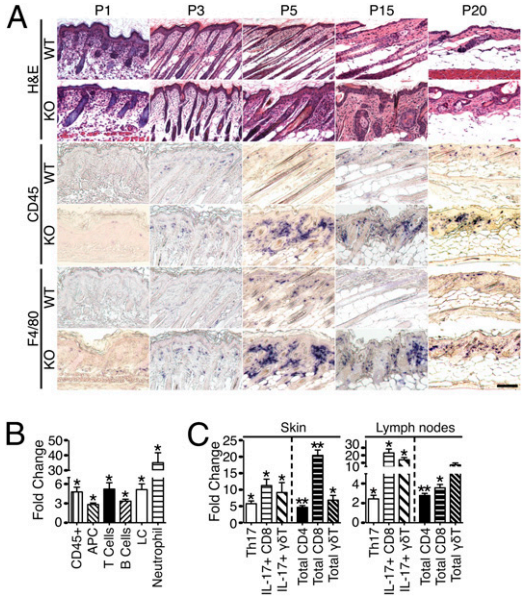


Fig. 3. Epidermal deletion of Dlx3 triggers infiltration of IL-17–producing and other inflammatory cells into the skin of K14cre;Dlx3$^{Kin/f}$ mice. (*A*) H&E staining revealed epidermal hyperplasia in K14cre;Dlx3$^{Kin/f}$ skin from P1–P20. Immunostaining using anti-CD45 and anti-F4/80 antibodies showed infiltration of leukocytes, macrophages, and Langerhans cells into K14cre;Dlx3$^{Kin/f}$ skin. (Scale bar: 50 μm.) (*B*) FACS analyses were performed to determine fold changes of total CD45$^+$ leukocytes, CD45$^+$CD3ε$^-$MHCII$^+$ APCs, CD45$^+$CD3ε$^+$MHCII$^-$ T cells, CD45$^+$CD3ε$^-$CD19$^+$MHCII$^+$ B cells, CD45$^+$CD3ε$^-$MHCII$^+$EPCAM^{high} Langerhans cells (LC), and CD45$^+$CD3ε$^-$CD11b$^+$Ly-6g$^+$F4/80$^-$ neutrophils in the skin of P20 mice. Error bars indicate SEMs for each group of four or five mice. Data are representative of three independent experiments. Fold changes were calculated by dividing the absolute cell numbers from each K14cre;Dlx3$^{Kin/f}$ mouse by the average of absolute cell numbers from WT mice. (*C*) Fold changes of IL-17a–expressing lymphocytes in skin and DLNs. Single cell suspensions of dorsal skin were gated on live CD4$^+$ T cells (TCRβ$^+$TCRγδ$^-$CD4$^+$CD8$^-$), CD8$^+$ T cells (TCRβ$^+$TCRγδ$^-$CD4$^-$CD8$^+$), and γδT cells (TCRβ$^-$TCRγδ$^+$). Numbers of IL-17a–expressing T cells from P20 K14cre;Dlx3$^{Kin/f}$ mice are expressed as relative fold changes compared with average cell numbers from WT mice. Error bars indicate SEMs for each group of four or five mice. Data are representative of three independent experiments. $*P < 0.05$; $**P < 0.005$.

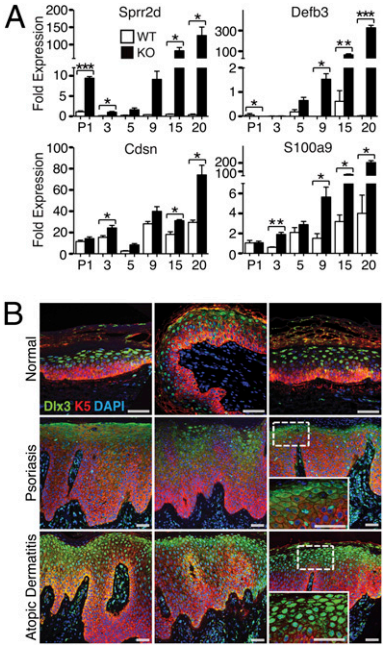


Fig. 4. Gene expression similarity of K14cre;Dlx3$^{Kin/f}$ skin and PS, and histological analysis of Dlx3 expression in patient samples and normal counterparts. (*A*) Significant up-regulation of Sprr2d, DefB3, Cdsn, and S100a9 was observed by qPCR analysis in K14cre;Dlx3$^{Kin/f}$ skin from P1–P20. (*B*) Dlx3 expression was detected using an anti-Dlx3 antibody (green) and keratin 5 (K5, red) as a marker for basal keratinocytes. Images include DAPI (blue) counterstaining to denote nuclear staining (*Insets*, high magnification). (Scale bar: 50 μm.)

Discussion

Dlx3 Is an Essential Regulator of Epidermal Differentiation. Delayed acquisition of barrier function was observed in K14cre;Dlx3$^{Kin/f}$ fetuses. In postnatal mice, gene expression profiles of K14cre;Dlx3$^{Kin/f}$ skin suggest that differentiation defects persist and are linked to epidermal hyperplasia, accompanied by down-regulation of claudins and transglutaminases and up-regulation of K6 and K16. Altered expression of S100As, β-defensins, and PPAR genes suggest that abnormal differentiation of epidermis may lead to inflammatory responses associated with the production of IL-1Fs and IL-17 cytokines. Our findings of down-regulation of Pparα and Pparγ in K14cre;Dlx3$^{Kin/f}$ mice and Dlx3-null keratinocytes suggests a possible link between the intrinsic role of Dlx3 as a regulator of epidermal differentiation and inflammatory responses in the skin (36). Based on these findings, we propose that Dlx3 is a central and critical regulator of epidermal homeostasis, barrier function, and skin inflammation (Fig. 5*C*).

PS and AD are chronic inflammatory skin diseases characterized by epidermal hyperproliferation and abnormal differentiation of keratinocytes associated with a defective skin barrier. These diseases are prevalent, affecting ~7–10% of the total population, but their etiology remains incompletely understood. Characterization of altered gene expression patterns has provided insights into the pathophysiology of each disorder (9). In this study, we demonstrated up-regulation of Sprr2d, Defb3, Cdsn, and S100a9 expression in K14cre;Dlx3$^{Kin/f}$ skin, indicating overlap in gene expression between this mouse model and the PS gene expression signature (9, 22). Moreover, marked down-regulation of Dlx3 expression was detected in human psoriatic lesions, suggesting

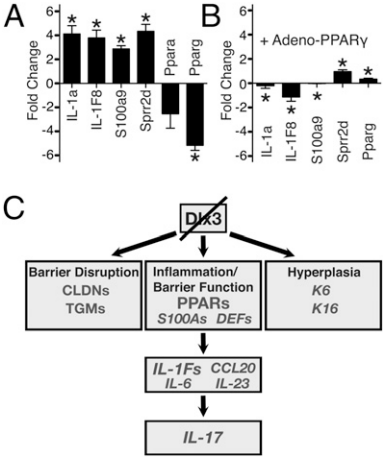


Fig. 5. Inflammatory cytokine production triggered in Dlx3-null keratinocytes. (*A*) Total RNA was isolated from Adeno-cre;Dlx3$^{f/f}$ keratinocytes (KO) and Adenocre;Dlx3$^{wt/wt}$ (control keratinocytes, WT). Levels of gene expression were evaluated by qPCR, and fold changes (KO vs. WT) were calculated for each gene. (*B*) Adeno-PPARγ viruses were transduced into WT control and Dlx3-null keratinocytes. qPCR analyses were performed to assess levels of expression of each gene. (*C*) Schematic diagram showing the proposed role of Dlx3 in epidermal homeostasis, barrier function, and skin inflammation. Up-regulated genes are shown in italic type, and major genes related to barrier function/inflammation are shown in larger type. ($*P < 0.05$).

that the lack of Dlx3 transcriptional function is potentially involved in the pathophysiology of human inflammatory diseases.

IL-17–Mediated Inflammatory Responses in K14cre;Dlx$^{Kin/f}$ Skin. Expression of multiple IL-1 family members was significantly elevated in the skin of K14cre;Dlx$^{Kin/f}$ mice by P20 (Fig. 2). Previous studies have demonstrated the critical role of IL-1α, an epidermal cytokine, in the maintenance of skin barrier function (37). The addition of IL-1α to cultured human tumor cell lines was shown to induce IL-6 and G-CSF production (38). Up-regulation of IL-6 also was detected in the skin of K14cre;Dlx3$^{Kin/f}$ mice (Fig. 2). Increased IL-6 secretion might be crucial to understanding the IL-17/Th17-dependent inflammatory response in K14cre;Dlx3$^{Kin/f}$ mice, given that IL-6 also promotes the generation of Th17 cells from naïve T cells (39).

Recent studies indicate that PS is strongly associated with a Th17-mediated immune response, and infiltration of CD8$^+$ T cells into psoriatic epidermis has been reported (10). The accumulation of IL-17–expressing T cells (including Th17 and CD8$^+$ T cells) was corroborated in both skin and lymph nodes of K14cre;Dlx3$^{Kin/f}$ mice, in which increased IL-17a expression was seen as well. A contribution of γδ T cells to skin inflammation in K14cre;Dlx3$^{Kin/f}$ mice was also observed. The possible relevance of this finding is supported by the fact that intraepithelial lymphocytes bearing γδ TCRs have been identified as immune modulators in skin, and that crosstalk between skin γδ T cells and keratinocytes is known to play an essential role in the maintenance of skin homeostasis (40).

The survival and proliferation of Th17 cells are dependent on the production of IL-23 (41), and up-regulation of IL-23 was observed in K14cre;Dlx3$^{Kin/f}$ skin (Fig. 2*B*). The events and factors that trigger IL-23 production by either keratinocytes and/or dendritic cells in the absence of Dlx3 function remain to be elucidated. In addition, strongly increased Ccl20 expression was detected in K14cre;Dlx3$^{Kin/f}$ skin (Fig. 2). The chemokine Ccl20 is produced primarily by keratinocytes and acts as a chemoattractant to stimulate migration of chemokine-receptor Ccr6$^+$ cells from blood into inflamed cutaneous tissue (10, 29). Ccl20 is particularly relevant for Th17-mediated immune responses, because Ccr6 is expressed by IL-17–producing cells.

In characterizing our mouse model, we determined that infiltration of T cells producing IL-17 was prominent, and that an intrinsic defect in K14cre;Dlx3$^{Kin/f}$ keratinocytes was associated with the development of an IL-17–mediated inflammatory response. The degree to which perturbation of barrier function contributes to the subsequent inflammatory response remains to be determined.

K14cre;Dlx3$^{Kin/f}$ Mice as a Mouse Model for Human Inflammatory Skin Disease. Although inflammatory skin disorders are often accompanied by decreased barrier function, the role of this barrier dysfunction in disease causality is often unclear. Most notably, inflamed skin generally exhibits abnormal keratinocyte differentiation, disrupted barrier function, and/or immunocyte abnormalities (9–11). Mouse models have been critical tools in examining some of these complex interactions (42, 43). Targeted epidermal deletion of such genes as JunD, Klf4, Gata3, and Notch in mice results in defects in barrier formation due to abnormal skin differentiation (44–47), and many barrier dysfunction models exhibit aspects of AD and PS (42, 43). Numerous mouse models have been generated in the hope of elucidating the pathogenesis of human PS (42). However, human skin differs from mouse skin in terms of thickness, width of interfollicular regions, and epidermal turnover, and a complete psoriasiform phenotype is not readily observed in mice.

In this study, we characterized gene expression profiles and cutaneous inflammation in K14cre;Dlx3$^{Kin/f}$ mice and in Dlx3-null keratinocytes cultured in vitro. These profiles revealed altered keratinocyte differentiation and up-regulated cytokine and down-regulated PPAR expression. These findings suggest that certain aspects of the inflammatory responses observed in K14cre;Dlx3$^{Kin/f}$ mice, including the infiltration of leukocytes into skin, are keratinocyte-initiated and are mediated in part by a PPAR-regulated mechanism.

In summary, we have described a mouse model with epidermal deletion of Dlx3 that can be used to define the contributing role of keratinocytes to the pathogenesis of cutaneous inflammation that may be relevant to one or more human skin diseases. This model should allow detailed and systematic exploration of important relationships between keratinocyte and recruited immunocytes in inflammatory responses.

Materials and Methods

Mice. Mouse strains Dlx3$^{Kin/+}$ (LacZ knock-in heterozygote), Dlx3$^{f/f}$ (floxed homozygote), and K14cre;Dlx3$^{Kin/f}$were generated and genotyped as reported previously (14). All animal care and experimental protocols were approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases Animal Care and Use Committee.

Human Skin Samples. Skin biopsy specimens were obtained from patients and normal adults after provision of written informed consent. All procedures received previous approval from Yonsei University College of Medicine's Institutional Review Board in accordance with the Declaration of Helsinki.

Skin Barrier Function Analysis and Preparation of CEs. Dye permeability assays were performed with 1% hematoxylin (EMD Chemicals) for 1 h at 30 °C (15). CEs were prepared from skin samples as described previously (13). Isolated CEs were analyzed by phase-contrast microscopy (Axiophot; Zeiss).

Western Blot Analysis. Protein samples from dorsal skin of mice were subjected to Western blot analysis. The following antibodies and dilutions were used: anti-Flg (1:1,000; Covance), anti-Lor (1:1,000; Covance), anti-Claudin 7 (1:500; Invitrogen), anti-actin (1:1,000; Sigma-Aldrich), and horseradish peroxidase-conjugated secondary antibody (Vector Laboratories).

Histology, Immunofluorescence, and Confocal Microscopy. Skin sections (10 μm thick) were stained with H&E or used for immunohistochemistry after incubation with primary antibodies (14): anti-Dlx3 (1:250; Morasso Laboratory), anti-K6 (1:100; Covance), anti-PCNA (1:100; Calbiochem), anti-CD45 (1:50; BD Bioscience), anti-F4/80 (1:50; AbD Serotec), anti-K5 (1:50; Lifespan Biosciences), and anti-Ccl20 (1:50; R&D Systems) and secondary antibodies Alexa Fluor 488 or Alexa Fluor 546 goat IgG (1:250; Molecular Probes).

RNA Isolation, Microarray Analysis, and qPCR. Total RNA was prepared from the dorsal skin of mice or cultured keratinocytes using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, and quantity and quality were evaluated by RNA capillary electrophoresis (Agilent). RNA was reverse-transcribed into cDNA using the ImProm-II Reverse-Transcription System (Promega). Microarray analysis and data processing were performed at the National Institute of Diabetes and Digestive and Kidney Diseases Genomics Core Facility using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) (48). qPCR analysis was done in triplicate using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). The following primers for qPCR were purchased from Qiagen or synthesized according to previous reports: Ccl20 (QT02326394), Ccr6 (QT 02256002), Cdsn (QT00287217), DefB3 (QT00265517), GAPDH (QT01658692), IL-1a (QT00113505), IL-1F8 (QT01067045), IL-6 (QT00098875), IL-17a (QT00103278), K10 (QT01748397), Pparα (QT00137984), Pparγ (QT00100296), S100a9 (QT00105252), Sprr2d (QT00133931), IL-23p19 (49), and RPLP0 (50). The primers for Dlx3 as follows: forward, ATTACAGCGCTCCTCAGCAT; reverse, CTTCCGGCTCCTCTTTCAC. Relative expression was normalized against the housekeeping genes RPLP0 and GAPDH. Fold changes were calculated by the Ct method (51).

Microarray and qPCR analyses were performed for each group with at least three WT and three mutant mice at each developmental stage: P1, P3, P5, P9, P15, and P20 in this study.

Flow Cytometry. Dorsal skin (2 cm × 3 cm) was incubated for 1 h, 45 min at 37 °C with 5 mL of RPMI medium containing 500 μg/mL of Liberase CI (Roche) and then minced with sharp scissors. Single-cell suspensions were prepared after an additional 15 min incubation with 0.05% DNase I (Sigma-Aldrich),

followed by sequential filtering through 100-, 70-, and 40-μm nylon mesh. Lymph node (inguinal, axillary, and brachial) capsules were torn with sharp forceps, and tissues were incubated for 30 min at 37 °C in 2 mL of RPMI medium containing 100 μg/mL of Liberase CI (Roche). Lymph node cell suspensions were prepared by vigorous trituration and filtered through 40-μm nylon mesh. For T cell intracellular cytokine staining, cells were stimulated with 50 ng/mL of phorbol myristate acetate and 500 ng/mL of ionomycin (Sigma-Aldrich) in the presence of brefeldin A and monensin (eBioscience) for 4 h before surface staining with antibodies. Isolated cells were stained in PBS, 2% FCS, 1 mM EDTA, and 0.01% NaN_3 with the following antibodies in the presence of Fc blocking antibodies: anti–CD4-Pacific Blue (RM4-5), anti–CD8-APC-eFluor780 (53-6.7), anti–TCRβ-PE (H57-597), anti–TCRγδ-PerCP-eFluor710 (GL3), anti–CD45-eFluor450 (30-F11), anti–CD11b-eFluor605NC (M1/70), anti–Ly-6G-PE-Cy5 (RB6-8C5), anti–CD3e PerCP-eFluor710 (17A2), anti–EPCAM-PE-Cy7 (G8.8), anti–IL-17a-AF488 (eBio17B7), and anti–IFN-γ-APC (XMG1.2). All antibodies were purchased from eBioscience except anti–F4/80-AF700 (CI:A3-1, Abd Serotec). Lower limit thresholds were set using isotype control mAb. Data were acquired on a LSR II flow cytometer (BD), and analysis was performed with Flowjo Software 7.5 (Treestar).

Four or five WT and mutant mice were used for each experiment, and results presented are representative of three independent experiments.

Dlx3 Deletion in Cultured Keratinocytes by adenoCre Transduction. Primary keratinocytes were isolated from $Dlx3^{f/f}$ mice, and CD1 cells were transduced with adenovirus expressing Cre-recombinase and/or PPARγ (Vector Biolabs) at 5 MOI in infection media (S-MEM with 4 μg/mL of polybrene and 0.05 mM Ca^{2+}) for 30 min. The cells were subsequently cultured in low-calcium medium (10% chelated FCS, 0.05 mM Ca^{2+}) for 72 h and lysed in TRIzol (Invitrogen).

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