Lymphotoxin-β regulates periderm differentiation during embryonic skin development

Chang-Yi Cui¹, Makoto Kunisada¹, Diana Esibizione¹,², Sergei I. Grivennikov³, Yulan Piao¹, Sergei A. Nedospasov⁴ and David Schlessinger¹,∗

¹Laboratory of Genetics, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA, ²Department of Histology, Embryology and Applied Biology, University of Bologna, Bologna 40126, Italy, ³Basic Research Laboratory, National Cancer Institute, PO Box B, Frederick, MD 21702, USA and ⁴Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow 119991, Russia

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Lymphotoxin-β (LTβ) is a key regulator of immune system development, but also affects late stages in hair development. In addition, high expression of LTβ at an early stage in epidermis hinted at a further function in hair follicle induction or epithelial development. We report that hair follicles were normally induced in LTβ−/− skin, but the periderm detached from the epidermis earlier, accompanied by premature appearance of keratohyalin granules. Expression profiling revealed dramatic down-regulation of a gene cluster encoding periderm-specific keratin-associated protein 13 and four novel paralogs in LTβ−/− skin prior to periderm detachment. Epidermal differentiation markers, including small proline-rich proteins, filaggrins and several keratins, were also affected, but transiently in LTβ−/− skin at the time of abnormal periderm detachment. As expected, Tabby mice, which lack the EDA gene, the putative upstream regulator of LTβ in skin, showed similar though milder periderm histopathology and alterations in gene expression. Overall, LTβ shows a primary early function in periderm differentiation, with later transient effects on epidermal and hair follicle differentiation.

INTRODUCTION

The regulatory circuit for skin development must engineer transitions that start at embryonic day 13.5 (E13.5). At that time mouse skin epithelium consists of a single basal layer of undifferentiated keratinocyte progenitors with an overlying layer called the periderm (1). Committed basal keratinocytes then exit from cell cycle and start to differentiate to form stratified epidermis, and critical reciprocal interactions between epithelium and mesenchyme initiate skin appendage development at sharply defined times from E13.5 to E18.5 (2). During epidermal stratification, keratinizing keratinocytes in the upper epidermis express differentiation markers that include loricrin, involucrin, filaggrin and several keratins (3). They eventually form the characteristic structural features—cornified cell envelopes (CEs), keratohyalin granules and patterned keratins—that mark the terminal differentiation of skin (3).

The first stage in this process involves the little studied periderm, a transitory embryonic skin tissue consisting of a distinct cell population, that is, derived from basal keratinocytes about E8.5 (1). The periderm precursor cells detach from the basement membrane and migrate to the skin surface to form a single layer ‘cell-net’ (1). The periderm cells are connected by tight junctions, which are conspicuously lacking in the underlying epidermis during stratification. The periderm communicates closely with the epidermis, and it is thought to stabilize both differentiating epidermis and epithelium–mesenchyme interactions (1,4). Accordingly, periderm removal during epithelium–mesenchyme interaction for limb development results in ventral polydactyly in rats (5). Once epidermal stratification is complete at E18.5, the periderm is apparently superannuated and is shed from the underlying epidermis. However, the molecular mechanism of periderm differentiation and function in skin development is poorly understood (6).

*To whom correspondence should be addressed at: Laboratory of Genetics, National Institute on Aging/NIH 333 Cassell Dr., Suite 3000, Baltimore, MD 21224, USA. Tel: +1 4105588337; Fax: +1 4105588331; E-mail: schlessingerd@grc.nia.nih.gov

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Here we report that lymphotxin-β (LTβ), a member of the TNF ligand superfamily that is a well known immunomodulatory cytokine, also involved in regulating lipid metabolism (7,8), has a pivotal role in periderm homeostasis. LTβ expression in skin epithelium peaked at E14.5 and was thereafter confined to hair follicles at a lower level (9). We previously showed that its late action in hair follicles regulate hair shaft formation (9). We now report that the earlier expression of LTβ in skin epithelium regulates periderm differentiation, at least in part through periderm-specific keratin-associated proteins (KAPs), and subsequently transiently affects epidermal differentiation.

RESULTS

Normal induction of hair follicles, but abnormal differentiation of periderm and epidermis in LTβ−/− mice

Because LTβ−/−, but not LTα−/− or LTβR−/− mice showed striking hair phenotypes, we looked more extensively for a possible action of LTβ in skin (9). The strong expression of LTβ in early stage embryonic skin suggested two possible roles: (1) LTβ regulates epithelium development at early stages and hair follicle differentiation at late stages or (2) LTβ regulates hair follicle induction as well as hair follicle differentiation from an early stage, with no pronounced effect on epithelium. To distinguish between these alternatives, we collected wild-type and homozygous LTβ−/− embryos at E14.5, 15.5, 16.5 and 18.5, and initially carried out morphological analyses. No gross defects were seen in mutant embryos as compared to wild-type controls at any developmental time point (Supplementary Material, Table S1a). By histological analyses, we observed normal induction of hair germs at E14.5 in LTβ−/− skin (Fig. 1A, solid line at E14.5) and normal down-growth from E15.5 onward (Fig. 1A, arrows at E15.5 and arrowheads in E16.5). These results argue against any early effect of LTβ on hair follicles.

To test the alternative of an effect on early epithelium development, we analyzed skin epithelium more closely and found striking histological abnormalities in the periderm in LTβ−/− mice. The periderm normally covers the epidermis from E8.5 until E17.5 and disappears thereafter (6). Thus, we found that the periderm was intact until E16.5 in wild-type skin (Fig. 1B, depicted by broken lines) and then starts to detach. In contrast, in LTβ−/− mice the periderm was almost completely detached from underlying epidermis at E16.5, one or two days earlier than in wild-type mice (Fig. 1B, arrowheads in E16.5). By immunofluorescent staining with a periderm marker, keratin 6a (10), we further confirmed that periderm was intact until E16.5, but largely detached at E18.5 in wild-type embryos (Fig. 1C; arrows indicate remnants of periderm). In contrast, periderm was lost by E16.5 in LTβ−/− embryos (Fig. 1C).

In addition, at E16.5, epidermal differentiation was also altered in mutant mice. Keratohyalin granules appeared precociously in the upper epidermis, and some embryos even started to form stratum corneum (Fig. 1B, E16.5, arrows in the insert indicate premature appearance of keratohyalin granules and overlying pink horny layer in LTβ−/− mice). By E18.5, keratohyalin granules were also formed in wild-type embryos (Fig. 1B, E18.5).

The apparent anomalies in differentiation of skin epidermis led us to investigate possibly affected differentiation markers in further detail. We performed immunofluorescent staining with antibodies against major epidermal differentiation markers including filaggrin, loricrin, keratin 1 and keratin 14 (Fig. 2 and Supplementary Material, Table S1b). Other
markers were not affected in \(LT^\beta\) skin, but the expression of filaggrin, the major component of keratohyalin granules, was significantly elevated at E16.5, staining strongly from the middle squamous layer to the horny layer (Fig. 2, arrows in upper left panels). Elevation of filaggrin was stage specific: it had not yet begun at E15.5, peaked at E16.5, and was still seen at E18.5 and in newborn mutant mice, though not as abundantly as at E16.5 (Fig. 2). The aberrant differentiation of periderm and underlying epidermis in \(LT^\beta\) embryos was thus in contrast to the apparent normal induction of hair follicles.

Marked downregulation of periderm specific KAP genes in \(LT^\beta\) mice

To look for the spectrum of genes affected in \(LT^\beta\) skin, we carried out whole genome microarray expression analyses with skin samples from wild-type and mutant embryos. Based on the most evident histological abnormalities at E16.5, we have chosen four time points, E15.5, E16.5, E18.5 and postnatal day 5 (p5) for expression profiling (Supplementary Material, Table S1a).

Striking changes in expression were found for several periderm-specific genes. \(KAP13\) and three novel \(KAP13\)-like genes, 2310034C09Rik, LOC546672 and LOC433047 were dramatically down regulated in \(LT^\beta\) skin 10-fold at E15.5 and >100-fold at E16.5, i.e. both before and after premature periderm detachment (Fig. 3A). Another paralogous gene, 2310057N15Rik, was weakly expressed in wild-type and mutant skin at E15.5, but significantly upregulated only in wild-type at E16.5 (Fig. 3A, bottom right panel). Notably, from E18.5 onward, all five genes were expressed at background levels even in wild-type mice, and expression differences between wild-type and \(LT^\beta\) skin were no longer evident (Fig. 3A). This is consistent with the programmed detachment of the periderm, harboring these protein components, during the differentiation process. The time course of expression and loss was almost identical for \(KAP13\), 2310034C09Rik, LOC546672 and LOC433047 in wild-type and mutant skin (Fig. 3A). To verify these findings by an independent method, we carried out Taqman real-time PCR assays for \(KAP13\), 2310034C09Rik, LOC546672 and LOC433047 in wild-type and mutant skin (Fig. 3A). To verify these findings by an independent method, we carried out Taqman real-time PCR assays for \(KAP13\), 2310034C09Rik, LOC546672 and LOC433047 in wild-type and mutant skin (Fig. 3A).

The five genes, especially \(KAP13\), 2310034C09Rik, LOC546672, and 2310057N15Rik, encode highly homologous proteins (Fig. 4A). The periderm genes are tightly clustered within 60 kb of DNA in the ‘KAP complex’ on mouse chromosome 16 C3.3. No additional genes in this region are evident in the current genomic DNA assemblies, where they

![Figure 2. Immunofluorescent staining for epidermal differentiation markers at successive time. Filaggrin was significantly upregulated in \(LT^\beta\) skin at E16.5 (arrows in upper left panels), and slightly at E18.5 and p5. No significant expression differences were found between wild-type and \(LT^\beta\) skin for loricrin or keratin 1.](https://academic.oup.com/hmg/article-abstract/16/21/2583/2356031)

![Figure 3. Expression of \(KAP13\) and its paralogous genes in wild-type and \(LT^\beta\) skin. (A) Expression levels from microarray assays, shown as log-intensities. Expression of \(KAP13\), LOC546672, 2310034C09Rik, LOC433047 was high at E15.5 and E16.5 in wild-type skin but sharply down regulated in \(LT^\beta\) skin. In contrast, expression of another paralog, 2310057N15Rik, was low at E15.5, but sharply upregulated at E16.5 in wild-type skin (right lower panel). At E18.5 and p5, expression of all five genes was at basal levels both in wild-type and \(LT^\beta\) skin. (B) In situ hybridization assays revealed specific localization of \(KAP13\) transcripts in the periderm of wild-type and Tabby skin, but not in \(LT^\beta\) at E15.5 (upper panels). No signals were found from sense probes (lower panels).](https://academic.oup.com/hmg/article-abstract/16/21/2583/2356031)
are transcribed in different directions in a pattern that suggests the presence of multiple promoters (Fig. 4B).

In contrast to KAP13 and its paralogous genes, keratin 6a (Krt6a), another gene specific to periderm (10), was sharply downregulated in LTβ-deficient skin at E16.5, the time of premature periderm detachment, by microarray expression profiling and real-time PCR assays (Supplementary Material, Table S3a), consistent with immunofluorescent staining (Fig. 1C). Whether KAP13 paralogs interact with Krt6a for periderm differentiation is unknown.

**Transiently affected epidermal differentiation markers at E16.5 in LTβ−/− skin**

Terminal differentiation of skin epidermis is marked by formation of three characteristic structures: the cornified CEs, consisting of loricrin, involucrin, small proline-rich proteins (Sprrs), repetin, etc.; the keratohyalin granules mainly comprised of profilaggrin, the precursor of filaggrin and the patterned keratins occurring in bundles (3). These components contribute to the formation of the epidermal barrier and highly insoluble, mechanically tough corneocytes (11). At the time of premature periderm detachment in mutant skin, we found significant expression changes for components of the cornified CEs and keratohyalin granules, encoded by a cluster of genes in the ‘epidermal differentiation complex’ locus on chromosome 3 F1-2.

At E15.5, only two differentiation related genes, the Sprr-like 231002A05Rik, and 230002G24Rik, encoding a novel cysteine-rich protein, were significantly downregulated in mutant skin (Table 1, E15.5). By E16.5, the number of genes affected had grown to 9 (Table 1, E16.5). Sprr1a, 1b and Sprr-like 231002A05Rik were sharply downregulated in mutant skin, and in contrast, Sprr2a, 2d, 2h and repetin were significantly upregulated. Downregulation of Sprr1a protein in mutant skin was confirmed by immunofluorescent staining (Supplementary Material, Table S3b). Sprr proteins are coexpressed with loricrin in cornified CEs and serve as cross-linkers (12); and upregulation of Sprr2d, 2h and repetin was previously reported in loricin-deficient mice (13). Surprisingly, at E18.5 and p5, the expression of these components in mutant skin had reverted to normal level (Table 1, E18.5 and p5). This suggested that a compensatory

**Table 1. Genes from the epidermal differentiation complex affected in LTβ−/− skin**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold-differences (LTβ−/− / WT)</th>
<th>Chr.3 Stranded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E15.5</td>
<td>E16.5</td>
</tr>
<tr>
<td>Sprr2a</td>
<td>0.98</td>
<td>1.89</td>
</tr>
<tr>
<td>Sprr2d</td>
<td>1.05</td>
<td>5.20</td>
</tr>
<tr>
<td>Sprr2h</td>
<td>1.06</td>
<td>1.93</td>
</tr>
<tr>
<td>Rptn</td>
<td>0.94</td>
<td>2.24</td>
</tr>
<tr>
<td>Sprr1b</td>
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<td>0.47</td>
</tr>
<tr>
<td>Sprr1a</td>
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</tr>
<tr>
<td>231002A05Rik</td>
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<td>0.04</td>
</tr>
<tr>
<td>230002G24Rik</td>
<td>0.46</td>
<td>1.21</td>
</tr>
<tr>
<td>Flg2</td>
<td>1.04</td>
<td>3.47</td>
</tr>
<tr>
<td>Flg</td>
<td>0.98</td>
<td>3.70</td>
</tr>
</tbody>
</table>

Bold-face, upregulated; italics, downregulated in LTβ mutant skin.

* Sprr-like, ‘late cornified envelop protein’.

**Figure 4.** Protein homology and clustering of KAP13 paralogous genes on mouse chromosome 16. (A) Protein homology between KAP13, 2310034C09Rik, 2310057N15Rik, and LOC546672. Red, identical; blue, similar amino acids. (B) KAP13 and 4 paralogous genes (red) were tightly clustered within 60 kb distance of the ‘KAP locus’ on chromosome 16, C3.3. Arrows indicate transcription direction for each gene.
mechanism may exist, as already indicated for loricin and involucrin-deficient mice (13,14). Transcription of the major cornified CE components, such as loricin and involucrin (15), however, remained unchanged in mutant mice.

In addition, we found that expression of filaggrin and its paralog filaggrin 2, the major components of keratohyalin granules, was significantly upregulated in mutant skin at E16.5 (Table 1), consistent with protein expression assessed by immunofluorescent staining (Fig. 2). No change in transcription of filaggrin was detected at E18.5 and p5 (Table 1), but immunostaining showed slightly upregulated protein expression in mutant skin at those times (Fig. 2).

Further striking changes were found in the expression of 12 keratin genes, half of them known to be hair follicle related (Table 2). For example, Krt27 is specifically expressed in inner root sheath (IRS) and medulla of hair follicles (16), and Krt71 mutations affect IRS and hair shaft formation (17). Both genes were significantly upregulated in LTβ−/−/skin (Table 2, E16.5). Real-time PCR assays with probe/primers for Krt71 corroborated these micrarray results (not shown). Altered expression of these genes may contribute to the abnormal hair shaft formation observed in LTβ−/−/ mice (9). However, like most of the ‘epidermal differentiation complex’ genes, their transcription was affected only at E16.5. Other major epidermal keratins Krt1, 5, 10 and 14 were not affected in mutant skin.

**Similar but milder periderm phenotypes and delayed expression changes for KAP genes in EDA mutant Tabby mice**

We have reported that LTβ to be a potential target of EDA signaling (9). If LTβ is tightly regulated by EDA in periderm, similar periderm abnormalities can be expected from EDA mutant Tabby mice. We therefore analyzed back skin of Tabby mice before and during periderm detachment. Histology showed that periderm was intact at E15.5, but started to detach at E16.5 in Tabby hemizygous and homozygous mice, when it was still firmly attached in control wild-type and heterozygous Tabby females (Fig. 5A). These findings agree with earlier observations by Vielkind and Hardy (18) and similar to observations in LTβ−/− mice (Fig. 1B). However, the periderm phenotype in Tabby is milder and delayed compared with that in LTβ−/− mice. For instance, at E16.5, only some parts of periderm were detached in Tabby, whereas detachment was almost complete in LTβ−/− mice (Figs 1B and 5A). By E18.5, the periderm was gone in both Tabby and wild-type mice (data not shown).

We carried out additional real-time PCR assays for KAP13, 2310034C09Rik, 2310057N15Rik and Krt6a, the genes most affected in LTβ−/− mice at E16.5, using skin samples from littermate wild-type and Tabby embryos. At E16.5, in Tabby skin, expression of KAP13 and Krt6a was comparable to wild-type controls, whereas Krt6b expression was higher, and 2310034C09Rik was downregulated (Fig. 5B, left panel). However, at E17.5, expression of all four genes was sharply downregulated in Tabby (Fig. 5B, right panel), about 1 day later than LTβ−/− mice but still sooner than in wild-type. The delayed expression changes in Tabby skin were consistent with histological findings (Fig. 5A).

**Expression profiling revealed different downstream targets of LTβ in skin and in immune system**

Based on the phenotypes of adult LTβ−/−, LTα−/− and LTBR−/− mice, we previously suggested that LTβ may initiate a specialized signaling cascade in skin (9). To assess this possibility, we directly compared transcription profiles of LTβ−/− skin and lymph nodes from animals with a lesion of LTβ limited to B-cells. Unlike mice with complete

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**Table 2. Keratin genes affected in LTβ-deficient skin (LTβ−/−/WT)**

<table>
<thead>
<tr>
<th>Genes</th>
<th>E15.5</th>
<th>E16.5</th>
<th>E18.5</th>
<th>p5</th>
<th>Chr.</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krt12</td>
<td>1.04</td>
<td>2.78</td>
<td>1.32</td>
<td>1.00</td>
<td>11D</td>
<td>(−)</td>
</tr>
<tr>
<td>Krt16</td>
<td>0.99</td>
<td>1.56</td>
<td>1.28</td>
<td>1.04</td>
<td>11D</td>
<td>(−)</td>
</tr>
<tr>
<td>Krt27</td>
<td>1.09</td>
<td>2.27</td>
<td>1.18</td>
<td>1.00</td>
<td>11D</td>
<td>(−)</td>
</tr>
<tr>
<td>Krt7</td>
<td>1.03</td>
<td>1.59</td>
<td>1.27</td>
<td>1.04</td>
<td>15F2</td>
<td>(−)</td>
</tr>
<tr>
<td>Krt6a</td>
<td>1.09</td>
<td>0.22</td>
<td>1.12</td>
<td>0.78</td>
<td>15F2</td>
<td>(−)</td>
</tr>
<tr>
<td>Krt19</td>
<td>0.94</td>
<td>0.25</td>
<td>0.90</td>
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<td>11D</td>
<td>(−)</td>
</tr>
<tr>
<td>Krt4</td>
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<td>0.95</td>
<td>1.00</td>
<td>15F3</td>
<td>(−)</td>
</tr>
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<td>Krt7</td>
<td>1.20</td>
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<td>0.81</td>
<td>0.98</td>
<td>15F2</td>
<td>(+)</td>
</tr>
<tr>
<td>Krt8</td>
<td>0.79</td>
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<td>0.88</td>
<td>0.98</td>
<td>15F3</td>
<td>(−)</td>
</tr>
<tr>
<td>Krt13</td>
<td>1.10</td>
<td>0.65</td>
<td>1.08</td>
<td>0.93</td>
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<td>(−)</td>
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<tr>
<td>Krt12</td>
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<td>0.56</td>
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<td>0.93</td>
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<td>0.94</td>
<td>0.93</td>
<td>11D</td>
<td>(−)</td>
</tr>
</tbody>
</table>

Bold-face and italics indicate up and downregulated genes in LTβ-deficient skin, respectively. Genes in upper part are hair follicle related genes.

**Figure 5.** Histology of periderm and expression levels of periderm-specific genes in EDA mutant Tabby skin. (A) Periderm was normal in Tabby (Ta) at E15.5. At E16.5, periderm was still intact in wild-type and heterozygous Tabby (Ta-HT) females, but was detaching from hemizygous (Ta-HE) and homozygous (Ta-HO) Tabby littermates. Broken lines demarcate periderm. (B) Only 2310034C09Rik (C09) was downregulated in Tabby at E16.5, but all four genes were sharply downregulated at E17.5.
LTβ deficiency, B-cell restricted LTβ−/− mice have morphologically ‘normal’ lymph nodes, therefore allowing us to compare expression of mutant and wild-type lymph nodes (19). By same criteria with skin, we found 420 significantly affected genes in lymph nodes of B-cell specific LTβ−/− mice (work in progress). However, only six genes were similarly affected in skin and lymph nodes, and none of these are identified (Fig. 6). Reciprocally, expression of differentiation markers Sprrs, repetin, filaggrins and several keratins—including hair follicle-specific keratins—were unaffected in mutant mice, suggesting a distinct transcriptional regulation of KAP13 genes during periderm differentiation. The apparent coregulation of five genes, three transcribed from one DNA strand and two from the other, suggests that LTβ may affect the higher order structure of chromatin to change the accessibility of genes for transcription in the delimited region of 60 kb.

We infer that the sharp downregulation of KAP genes disturbs the normal differentiation process of periderm and likely contributes to its early detachment in LTβ-deficient mice. However, in part because a putative skin-specific LTβ receptor or interacting protein is not yet identified, the molecular mechanism of LTβ action on KAP genes and periderm differentiation remains to be uncovered.

As seen in amniotic periderm (4), skin periderm also interacts with and protects underlying epidermis during epidermal differentiation and formation of the skin barrier (22). Therefore, abnormal periderm probably secondarily affects the epidermal differentiation process in LTβ-deficient mice. Accordingly, abnormal expression of differentiation markers Sprrs, repetin, filaggrins and several keratins—including hair follicle-specific keratins—was notable in LTβ mutant skin at E16.5, the time at which periderm was prematurely detaching. However, most of the affected genes were normally expressed at E18.5 and p5, strongly suggesting the existence of an unknown compensatory mechanism. A similar ‘backup’ compensatory action was inferred for effects observed in loricin and involucrin-deficient mice (13,14).

**DISCUSSION**

**LTβ function extends to early stage embryonic skin as well as later hair shaft formation**

Significant molecular and histological changes in periderm and underlying epidermis in LTβ-deficient mice imply an important function of LTβ beyond its well-characterized immune system effects. Here we discuss the role of LTβ in skin development and the possible relation of LTβ to EDA during development.

**Primary function of LTβ in skin development**

The most striking and clear abnormalities were sharp downregulation of periderm marker KAP13 and four novel paralogous genes, and precocious detachment of periderm from epidermis in LTβ-deficient skin. The differentiation process of periderm itself and the mechanism of its physiological detachment from underlying epidermis at the time of completion of epidermal stratification are only grossly described, and LTβ-deficient mice, along with EDA mutant Tabby mice (see below), may provide a model for studies of pathophysiological compared with normal periderm differentiation.

KAP proteins generally interact with keratin intermediate filaments to form keratin fibers in hair shaft and epidermis during the final differentiation process of skin (20,21). The KAP family consists of more than 28 small, simply structured genes that cluster in a 0.82 Mb region on mouse chromosome 16 C3.3 and human chromosome 21 q22.11. KAP13 is cens-formic, followed by KAP14, 15 and 16. (20,21). So far, KAP13 is the only member reported to be specifically expressed in mouse periderm (6). That observation is confirmed here, and we found four additional novel KAP13-like genes tightly clustered with KAP13 that behaved similarly in LTβ-deficient mice. Interestingly, immediately neighboring KAP genes in the same genomic region, including KAP13-1, 14, 15 and 16, were unaffected in mutant mice, suggesting a distinct transcriptional regulation of KAP13 genes during periderm differentiation. The apparent coregulation of five genes, three transcribed from one DNA strand and two from the other, suggests that LTβ may affect the higher order structure of chromatin to change the accessibility of genes for transcription in the delimited region of 60 kb.

**Interaction of EDA and LTβ for periderm differentiation**

EDA signaling has a pivotal role in skin appendage development, and LTβ is located downstream of EDA signaling.
Unlike wide-spread EDA expression in skin epidermis and hair follicles, its receptor, EDAR, and receptor adaptor protein, EDARADD, are restricted to hair follicles from E14.5 onward. EDA signaling is therefore often thought to be inactive in skin epidermis and periderm (26–28). However, the periderm abnormalities in Tabby skin suggest that ectodysplasin functions there (Fig. 5A, reviewed in 18). We infer that either EDAR is weakly expressed or ectodysplasin has an indirect partner in skin epithelium.

Compared with LTβ-deficient mice, the periderm phenotype in Tabby mice was histologically similar, though milder and delayed. Expression changes of KAP genes in Tabby skin were also delayed and less extreme than in LTβ-deficient mice. In our previous study, the hair phenotype in LTβ-deficient mice was also milder than in Tabby; and sweat glands were normally developed in LTβ-deficient mice, but absent in Tabby. Furthermore, among a dozen epidermal differentiation markers affected in LTβ-deficient mice, only a minority, including Spry1a, Krt12 and Krt7, was transiently affected in Tabby (9). These data are consistent with findings that EDA signaling operates through multiple pathways (9), and there may be synergistic or compensating interactions of target pathways.

Also, unlike the case in the immune system, LTα and LTβR showed actions distinct from LTβ in skin (9). This reflects the involvement of a special LTβ receptor or interacting protein in skin or the presence of different downstream effectors of signaling pathways in different tissues. Thus animal models have uncovered LTβ function in skin and lymphoid tissues, but with lesions in LTβ have not been reported (31). Nevertheless, LTβ has been shown to be involved in chronic inflammatory disease, and could conceivably affect periderm and hair follicles in humans as well (31).

**MATERIALS AND METHODS**

**Timed-mating with mutant mouse strains**

Two mutant mouse strains in C57BL6 background were used, LTβ knockout and EDA mutant Tabby mice. Two timed matings were set up separately to yield homozygous LTβ knockout, and Tabby hemizygous or homozygous embryos. Male and female LTβ<sup>−/−</sup> mice were crossed to get LTβ<sup>−/−</sup> embryos; and Tabby hemizygous mice were crossed with Tabby heterozygous to obtain wild-type and Ta hemizygous embryos; and Tabby hemizygous mice were crossed with Ta hemizygous/homozygous embryos (C57BL/6J-AW-J-Ta6J, Jackson Laboratory). The morning after mating was designated as E0.5. Embryos were harvested at E14.5–E17.5. Back skin and livers were collected under a dissection microscope, frozen on dry ice and stored at −80°C until use. Genotyping was done as previously reported (9).

**Embryo collection, RNA isolation, microarray assay and real-time PCR**

RNAs from back skin samples of wild-type and LTβ<sup>−/−</sup> embryos at E15.5, E16.5, E18.5 and p5 were profiled on microarrays. RNAs were isolated with Trizol (Invitrogen), LiCl precipitated, and their quality checked by electrophoresis, as previously reported (29). RNAs from three biological replicates (from three different embryos) were hybridized to 44,000 feature 60-mer-oligo microarrays (30). Triplicate data were analyzed, with FDR set to ≤0.1, and genes with <1.5-fold difference were excluded from significant gene lists. We selected 10 genes, including KAP13, 2310034C09, 2310057N15, Zfp318, Entpd4, Wdfy1, BC020077, Krt6a, Krt71 and CCL19 for one-step real-time PCR confirmation with Taqman ‘Assays on-Demand’ probe/primers (Applied Biosystems).

In addition, we isolated RNAs from skin samples of Tabby and wild-type littermates at E16.5 and E17.5 for real-time PCR analyses by the methods described above.

**Histology, immunofluorescent staining and in situ hybridization**

For histological analyses, skin samples from back skin of strains each time point were fixed in 4% paraformaldehyde, embedded in paraffin, and 5 μm sections stained with hematoxylin/eosin. For immunofluorescent staining, anti-mouse keratin 6a (1:50 dilution, Covance), filaggrin (1:750, Covance), loricrin (1:250, Covance), Krt14 (1:50, Santa Cruz Biotechnology) and Krt1 (1:500, Abcam) antibodies were incubated with 10 μm frozen back skin sections, followed by Alexa-fluor secondary antibodies (Invitrogen), and were analyzed under a DeltaVision confocal microscope. For in situ hybridization, 14 μm frozen back skin sections were fixed in 4% paraformaldehyde and hybridized with a KAP13-specific cRNA probe overnight at 60°C. A full-length (902 bp) KAP13 cDNA clone was purchased from Invitrogen and its sequence fully verified before synthesis of a cRNA probe. Digoxigenin (DIG)-labeled sense and antisense probes were prepared using a DIG RNA labeling kit (Roche). After washing with 2x SSC (0.3 M NaCl and 0.03 M sodium citrate, pH 7.0) and 0.1x SSC at 60°C, sections were incubated with anti-DIG antibody for 2 h and signals were visualized with NBT/BCIP stain (Biochain, 1:1000 dilution).

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Conflict of Interest statement. None declared.

**REFERENCES**

5. Peters, T., Sedlmeier, R., Bussow, H., Runkel, F., Luers, G.H., Korthaus, D.,
6. Langbein, L., Rogers, M.A., Praetzel-Wunder, S., Helmke, B.,
8. Lo, J.C., Wang, Y., Tumanov, A.V., Bamji, M., Yao, Z., Reardon, C.A.,
duplication of the autopod: chemical induction by methoxyacetic acid in
tissues.
control of lipid homeostasis.
13. Tumanov, A.V., Bamji, M., Yao, Z., Reardon, C.A.,
14. Nature
15. Fuchs, H., Hrabe de Angelis, M., Stumm, G., Russ, A.P.
17. Peters, T., Sedlmeier, R., Bussow, H., Runkel, F., Luers, G.H., Korthaus, D.,
molecules during mouse pelage hair follicle development. 2. Follicle
envelopes of stratified squamous epithelia.
19. Ware, C.F. (2005) Network communications: lymphotoxins, LIGHT, and
formation in the human fetus is patterned. J. Invest. Dermatol., 113,
30. Carter, M.G., Hamatani, T., Sharov, A.A., Carmack, C.E., Qian, Y., Alba,
31. Ware, C.F. (2005) Network communications: lymphotoxins, LIGHT, and