Targeted disruption of LIG-1 gene results in psoriasiform epidermal hyperplasia[1]

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Abstract The gene encoding a transmembrane glycoprotein LIG-1, of which the extracellular region was organized with the leucine-rich repeats and immunoglobulin-like domains, was disrupted in mice by gene targeting. LIG-1-deficient mice developed a skin change on the tail and facial area after birth. The affected skin was histologically reminiscent of the epidermis in human common skin disease 'psoriasis'. LIG-1 was expressed in basal cells of the epidermis and outer root sheath cells of hair follicles in mice. Interestingly, the LIG-1 expression was apparently down-regulated in the psoriatic lesions, suggesting that LIG-1 inversely correlates with proliferative ability of epidermal keratinocytes.

Key words: LIG-1; Knockout mouse; Psoriasis; Keratinocyte

1. Introduction

Psoriasis is one of the most common yet enigmatic human skin diseases that affect about 2% of the world's population. Psoriatic lesion is histologically characterized by epidermal hyperplasia, neutrophil influx, and also accumulation of T lymphocytes [1]. There has been long-standing debate about whether psoriasis is primarily a disorder of the immune system or of keratinocytes. Current thinking regarding the pathogenetic mechanism of psoriasis favors an immunological process; human epidermal keratinocytes are responsive to growth stimulatory signals released by activated T lymphocytes in the lesion [2,3]. However, there is a great deal of evidence suggesting that the keratinocytes in psoriasis have a genetic susceptibility to external stimuli [4,5].

The LIG-1 gene was originally found in a screen for genes up-regulated during neural differentiation of mouse embryonal carcinoma cell line P19 [6]. Our previous study showed that the mouse LIG-1 gene is expressed predominantly in the brain, and is restricted to a small subset of glial cells in the cerebellum and the olfactory bulb. Nucleotide sequence analysis of LIG-1 cDNA revealed that it encoded a type I transmembrane glycoprotein of which the extracellular region was entirely composed of 15 leucine-rich repeats (LRRs) and three immunoglobulin (Ig) domains. Although each kind of motif is found in a wide range of proteins involved in cell adhesion and specific ligand binding [7], the combination of LRR and Ig domains within a molecule is a fairly unique structural feature; a similar domain organization is shared only by a few membrane glycoproteins such as the vertebrate Trk family of neurotrophin receptors [8] and *Drosophila* Kek1 [9], both of which are thought to have neural-related functions. Therefore, we inferred that LIG-1 might have roles mainly in neural-related functions as a cell adhesion molecule and/or receptor, and that the lack of LIG-1 might result in crucial defects in the nervous system.

To clarify the physiological roles of LIG-1 in vivo, we generated LIG-1-deficient mice and analyzed their phenotype. These analyses revealed novel findings: (1) LIG-1 is expressed in the skin, and (2) LIG-1 inversely correlated with proliferative ability of epidermal keratinocytes.

2. Materials and methods

2.1. Generation of LIG-1-deficient mice

An approximately 20 kb genomic fragment containing six exons of the LIG-1 gene was isolated from a mouse 129/Sv genomic library (Stratagene). Since the complete exon/intron structure has not been fully established, we refer to these exons as E1–E6 in the order of 5′–3′ orientation. Exon E1 was the 'first coding exon' containing the initiation codon for the first methionine of the LIG-1. In the targeting vector pTVLIG1 (Fig. 1A), the short and long arms used for homologous recombination were the 0.75 kb *Pst*I fragment containing the upstream half of exon E1 and 6 kb *Eco*RI fragment located downstream, respectively. The neomycin-resistance (*neo*) and a herpes simplex virus thymidine kinase (*tk*) gene cassettes were also placed. Linearized pTVLIG1 was electroporated into R1 embryonic stem (ES) cells [10], selected with G418 (200 μg/ml), and the homologous recombinant clones were screened for by Southern blot genotyping. Of 525 clones, two (A4 and C9) were correctly targeted and showed normal karyotype ($2n = 40$) (data not shown). These clones were used

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[1] Data deposition: human and mouse LIG-1 cDNA nucleotide sequences have been submitted to the GenBank database with the accession numbers AB050468 and D78572, respectively.
[2] These authors contributed equally to this work.

Abbreviations: ES, embryonic stem

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to produce chimeras by the modified aggregation method [11]. The male chimeras were mated with C57BL/6 females (Nippon SLC Inc.), and transmitted the mutation through the germ line. The generated F1 progeny heterozygous for LIG-1 mutation (LIG-$1^{+/-}$) were normal, and were intercrossed to obtain homozygous mutant offspring (LIG-$1^{-/-}$). The F2 littermates (wild-type, LIG-$1^{+/-}$ and LIG-$1^{-/-}$ mice) were used for all experiments. Since the results derived from the A4 mutant ES cell clone were the same as from C9, all results described in this report were from the A4 clone. All animals were maintained under specific pathogen-free conditions, and experiments were carried out with strict adherence to our institutional guidelines to minimize distress in experimental animals.

2.2. Southern blot genotyping

Genomic DNA (10 μg) was digested with *Eco*RI, separated through 0.7% agarose gels, transferred onto Hybond-N^+ nylon membranes (Amersham), and hybridized with ^{32}P-labeled DNA probes 1–4 (Fig. 1A).

2.3. Cloning and nucleotide sequencing of human LIG-1 cDNA

In the present study, human LIG-1 cDNA clones were isolated from a human heart cDNA library (Stratagene) and subjected to nucleotide sequence analyses. Human and mouse LIG-1 cDNA sequences have been submitted to the GenBank database with the accession numbers AB050468 and D78572, respectively.

2.4. In situ hybridization

Cryostat or paraffin-embedded sections were hybridized with ^{35}S-labeled antisense cRNA probes corresponding to the mouse LIG-1 cDNA (0.96 kb *Eco*RI-XhoI fragment) subcloned into pBluescript SK(−) (Stratagene). Hybridization was performed according to the standard protocol. After hybridization, hematoxylin–eosin staining was performed to facilitate visualization of the histological structure of the sections. Sense probes were always used as controls.

2.5. Immunochemical analysis

For preparation of anti-LIG-1 antibody, an oligo-peptide sequence corresponding to amino acid residues 203–222 of mouse LIG-1 (TQLPVKAFKLPRLTQLDLNR; 20 amino acids) was chemically synthesized, purified, and inoculated into rabbits. Although this peptide sequence differed from human LIG-1 at position 208 (K to R in human), we confirmed that the anti-LIG-1 antibody was cross-reactive with human LIG-1 (data not shown). The following antibodies were also used: rabbit anti-Ki67, mouse anti-keratin 14 (K14) (Novocastra), rabbit anti-mouse keratin 6 (K6), keratin 10 (K10) (BAbCO), mouse anti-filaggrin (Biomedical Technologies) and rat anti-mouse β_1-, α_6-integrins (Chemicon International Inc.). Skin specimens were fixed with cold acetone. After fixation, cryostat sections (5 μm thick) of skin were incubated with primary antibodies for 1 h at room temperature, washed in phosphate-buffered saline, and incubated with fluorescein-conjugated donkey anti-rat IgG antibody, rhodamine-conjugated anti-rabbit IgG antibody or with fluorescein-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories). Cells isolated from the skin of tails and ears of newborn mice were air-dried and incubated as described above. Slides were then analyzed by fluorescence microscopy. Human specimens were obtained from involved skin of psoriatic patients ($n = 5$) and normal volunteers ($n = 5$) with their informed consent.

2.6. In vitro keratinocyte studies

Full-thickness skin of newborn mice was treated with 250 U/ml of dispase overnight at 4°C and the epidermis was peeled off from the dermis and trypsinized to prepare single cells. These cells were suspended in KGM medium (Kyokuto) and plated on dishes for 10 min (rapidly adherent cells: RACs), and then non-attached cells (late adherent cells: LACs) were transferred to new dishes by washing. On reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, the primers used for amplification of LIG-1 mRNA were as follows: 5′-TGAGGACTTGACGAATCTGC-3′ (upstream) and 5′-ATGGAGTTGTTGCTGAGGTG-3′ (downstream) to amplify a fragment corresponding to nucleotides 723–1343 of the mouse LIG-1 cDNA that includes exons 2–6. Each fraction of the cells was also immunocytochemically analyzed using anti-LIG-1 antibody and anti-β_1 or α_6-integrin antibody.

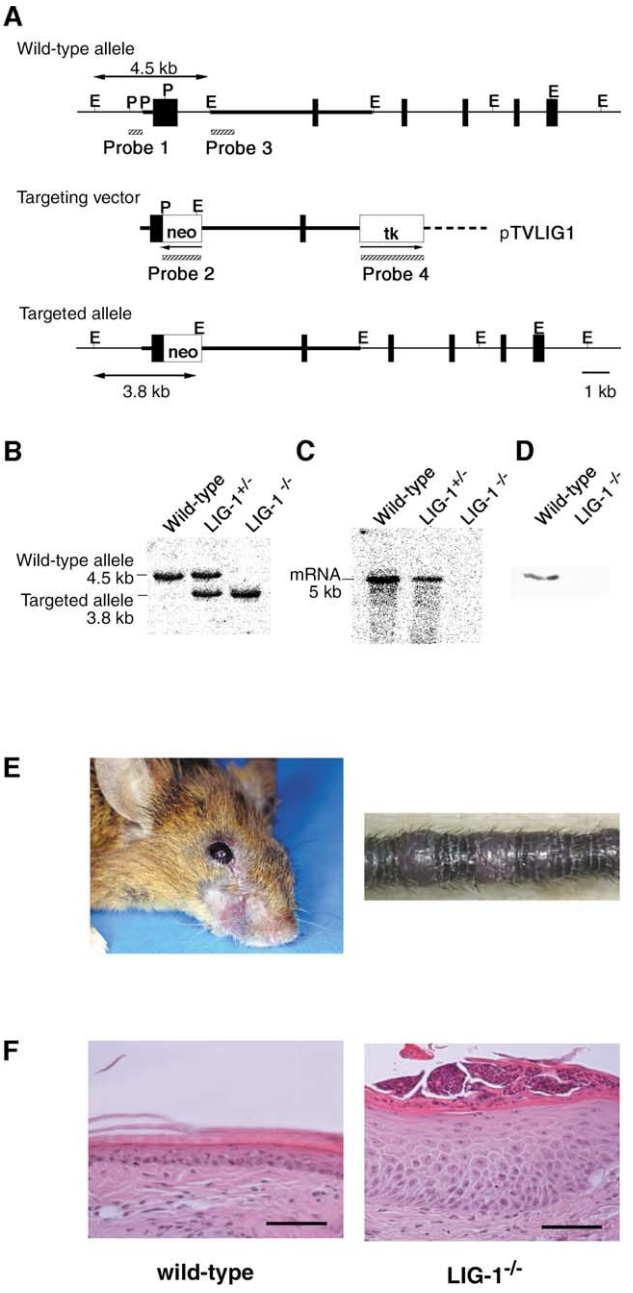

Fig. 1. Targeted disruption of LIG-1 gene results in skin abnormalities. A: Structures of the wild-type allele, the targeting vector pTVLIG1 and the targeted allele. Vertical closed boxes represent exons E1–E6. Thick horizontal lines represent genomic sequences used for homologous recombination. The neomycin-resistance (*neo*) and the thymidine kinase (*tk*) gene cassettes, and probes 1–4 used for Southern blot genotyping are also indicated. Restriction enzyme sites; E, *Eco*RI; P, *Pst*I. B: Southern blot genotyping of the wild-type, LIG-$1^{+/-}$ and LIG-$1^{-/-}$ mice. The result was obtained with probe 1. The 4.5 kb and 3.8 kb bands represent the wild-type allele and the targeted allele, respectively. C: Northern blotting analysis to determine LIG-1 gene expression in mice brain. D: Western blotting analysis to determine LIG-1 protein expression in mice brain with anti-LIG-1 antibody. E: Macroscopic phenotype of face and tail. F: Histopathology of the affected tail skin. Sections (4 μm thick) were stained with hematoxylin and eosin. Scale bar 100 μm.

3. Results and discussion

We generated LIG-1-deficient (LIG-1$^{-/-}$) mice by gene targeting (Fig. 1A,B). A targeting vector was constructed to terminate intrinsic translation at codon 55 by insertion of the neomycin-resistance gene cassette. Northern and Western blotting analyses confirmed that the targeted mutation completely abolished the expression of LIG-1 mRNA and protein and that the truncated forms of LIG-1 were absent (Figs. 1C,D and 4C). Since the LIG-1 gene was expressed predominantly in neural tissue and encoded a protein similar to other neural-related cell adhesion- and/or receptor-like molecules, we inferred that LIG-1 might have roles mainly in neural-related functions and that the lack of LIG-1 might result in crucial defects in the nervous system. Although Northern and Western blotting analyses confirmed that the targeted mutation completely abolished the expression of LIG-1 mRNA and protein (Fig. 1C,D), LIG-1$^{-/-}$ mice were born at Mendelian ratios, and were normal with respect to growth, behavior and fertility. Morpho-histological screening revealed no apparent defects in neural tissue (data not shown). It was therefore assumed that LIG-1 might be either of minor importance for overall development or other molecules in the nervous system might compensate its deficiency.

Unexpectedly, at the postnatal age of 3 weeks to 4 months, only the LIG-1$^{-/-}$ mice developed skin abnormalities on their tail, facial area and ear, even though they were housed under pathogen-free conditions. Grossly, tail skin of the LIG-1$^{-/-}$ mice showed a knot-like thickening with scales and the facial area exhibited alopecia with scales and erosion (Fig. 1E). Histological examination of the affected tail skin showed epidermal hyperplasia, hyperkeratosis with parakeratosis, neutrophil influx and subcorneal pustules similar to 'Munro's microabscesses' (Fig. 1F). All of these features were reminiscent of those of the epidermis in psoriasis, while they lack infiltrations of T lymphocytes and increased dermal capillarization [1]. In the mildly affected mice, they showed only the thickened epidermis with no inflammatory cell infiltration (data not shown), suggesting that the lack of LIG-1 initially altered the keratinocyte growth and differentiation, and then subsequently recruited neutrophils into the dermis and epidermis. No other area such as trunk and feet manifested such skin change. The affected areas of the LIG-1$^{-/-}$ mice were sites that were not covered with thick hair and were exposed more readily to non-specific stimuli except for the feet where development of the lesion is less common in psoriasis [1]. This finding was consistent with the Kobner phenomenon [12], in which inflammation or wounding of psoriatic uninvolved skin can result in development of psoriatic lesions, whereas similar perturbation of normal skin does not result in such changes. The psoriasiform epidermal changes were reversed by the treatment of common anti-psoriatic drugs such as steroid and vitamin D3 analogue ointments (Fig. 2).

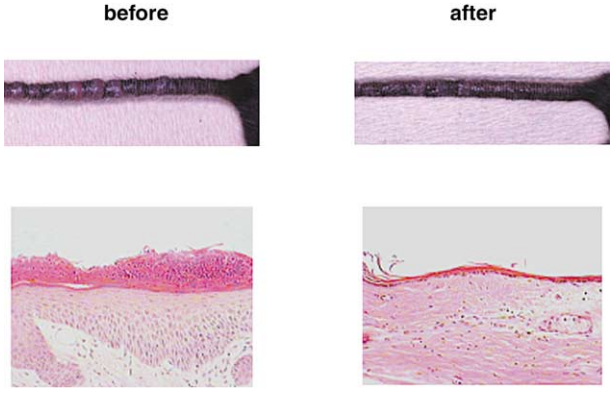

Fig. 2. Reversal of psoriasiform epidermal change in LIG-1$^{-/-}$ mice by anti-psoriatic drugs. Tail skin of LIG-1$^{+/-}$ mice was treated by topical application of corticosteroid or vitamin D3 analogue for 2 weeks. These treatments gave the same results.

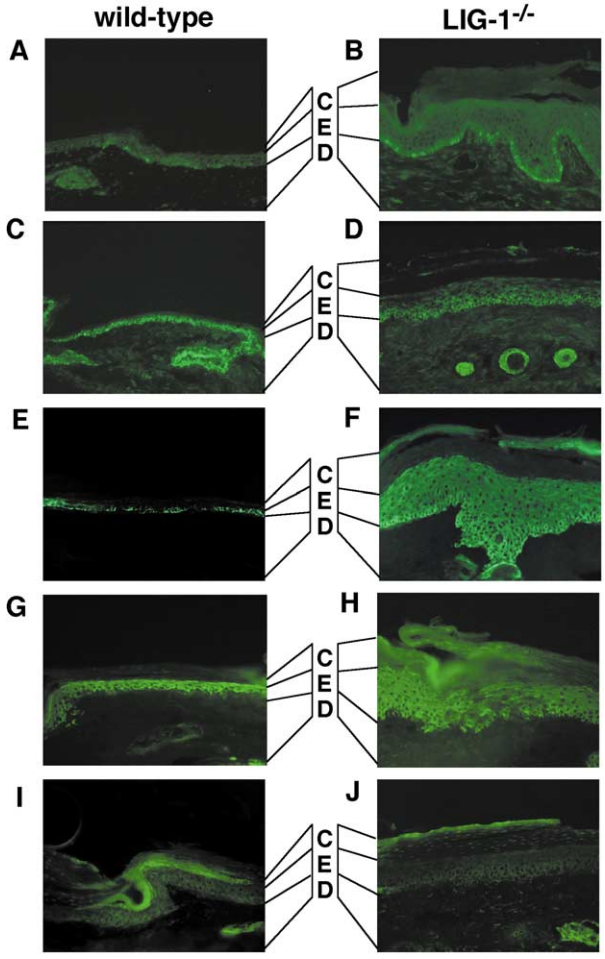

Fig. 3. Immunohistochemical analyses for keratinocyte proliferation and differentiation. Tail skin specimens were immunostained with anti-Ki67 (A, B), anti-K14 (C, D), anti-K6 (E, F), anti-K10 (G, H), and anti-filaggrin antibodies (I, J). C, stratum corneum; E, epidermis; D, dermis. Scale bar 100 μm.

To examine whether the LIG-1$^{-/-}$ epidermal keratinocytes were altered in their proliferation and/or maturation, we immunohistochemically analyzed the affected tail skin of LIG-1$^{-/-}$ mice. Relatively few Ki67-positive cells were observed in the epidermal basal cell layer of wild-type mice (Fig. 3A). In contrast, almost all of the basal cells and a few suprabasal cells were positive for Ki67 in the LIG-1$^{-/-}$ mouse lesion (Fig. 3B), indicating that LIG-1$^{-/-}$ keratinocytes were highly proliferative. K14 was expressed in basal cells of wild-type mice, whereas it was also expressed in the suprabasal cells in LIG-1$^{-/-}$ mouse lesions (Fig. 3C,D). K6, which is normally expressed in the epidermal basal cells in the tail skin, was highly induced in the whole epidermis of LIG-1$^{-/-}$ mice (Fig. 3E,F). K10, which was expressed in differentiating ke-

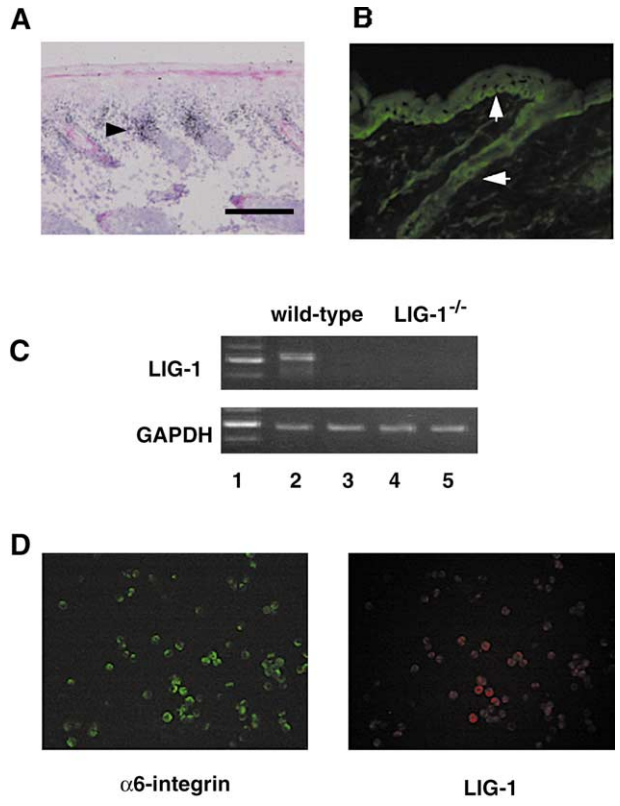

Fig. 4. In vivo and in vitro LIG-1 expression in mouse skin. A: LIG-1 mRNA expression in the mouse skin (arrowhead). Scale bar 100 μm. B: LIG-1 protein expression (arrowhead). C: RT-PCR detection of LIG-1 gene expression in newborn mice keratinocytes from wild-type and LIG-1$^{-/-}$ mice. Lane 1, molecular weight marker; lanes 2 and 4, RACs; lanes 3 and 5, LACs. D: Immunocytochemical detection of LIG-1 and α_6-integrin on wild-type mouse keratinocytes.

ratinocytes, was focally down-regulated in the LIG-1$^{-/-}$ epidermis (Fig. 3G,H). Filaggrin, a late differentiation marker of keratinocytes, was found in the stratum corneum and granulosum in the wild-type skin, while it was reduced and limited to the outer side of the thickened corneal layer in LIG-1$^{-/-}$ skin (Fig. 3I,J). These observations indicated that the lesional LIG-1$^{-/-}$ keratinocytes were activated and in a hyperproliferative state with perturbed terminal differentiation, as reported in psoriasiform epidermis [13].

Since the phenotype of LIG-1$^{-/-}$ mice suggested that LIG-1 is also expressed in the skin, we examined cutaneous LIG-1 expression. In situ hybridization analyses indicated that LIG-1 mRNA was abundantly expressed in the upper part of hair follicles and in the basal cells of the epidermis in a lesser extent (Fig. 4A). Immunohistochemical analysis revealed that LIG-1 protein was found on the epidermal basal cells as well as the hair follicle cells in the mouse-tail skin (Fig. 4B). The LIG-1-positive basal cells were clustered in patches. The LIG-1 expression in the tail and facial skin was not different as compared to the trunk skin, although trunk skin never showed psoriasiform skin change (data not shown). We tried to induce the similar phenotype by creating minor injuries on the trunk skin of LIG-1$^{-/-}$ mice, but the mice did not develop any psoriasiform skin changes. More continuous stimulation might be necessary to induce such skin change in the areas covered with thick hair.

Since undifferentiated basal keratinocytes strongly express α_6- and β_1-integrins on their cell surface, these cells adhere rapidly to the extracellular matrix [14,15]. Therefore, we divided the mouse keratinocytes into two fractions of RACs and LACs, and analyzed LIG-1 expression in the two fractions (see Section 2). RT-PCR analysis showed that LIG-1 mRNA was abundantly expressed in RACs (Fig. 4C) but was absent in LACs. LIG-1 mRNA was not detected in either fraction in LIG-1$^{-/-}$ mouse. Immunocytochemical examination confirmed that all of the RACs expressed α_6-integrin, and a subpopulation of the α_6-integrin-positive RACs showed strong LIG-1 expression on their cell surface (Fig. 4D). This cell population also expressed β_1-integrins strongly (data not shown). These results and the strong expression of LIG-1 mRNA in the bulge area of the hair follicles may suggest the strong expression of LIG-1 in the undifferentiated keratinocyte stem cells [16]. Further studies are required to define the characteristics of LIG-1 strong positive keratinocytes.

The LIG-1$^{-/-}$ mouse phenotype suggested that the loss of LIG-1 might trigger and/or influence the development of psoriasiform epidermal hyperplasia. Therefore, we compared the LIG-1 expression level between the psoriatic skin and the normal skin by immunohistochemistry. LIG-1 expression was significantly down-regulated in the psoriatic epidermis as compared with the normal skin (Fig. 5). Basal cells in non-lesional skin of psoriatic patients showed no difference of LIG-1 expression with normal skin (data not shown). These results indicate that LIG-1 may inversely correlate with the proliferative ability of epidermal keratinocytes in psoriasis.

While the mechanism directing the down-regulation of LIG-1 in psoriasis remains undefined, we hypothesized that LIG-1 might negatively regulate the epidermal growth factor (EGF) receptor-signaling pathway in the keratinocytes. Activation of the EGF receptor–ligand system in epidermal keratinocytes is well characterized in psoriasis, and mice transgenic for EGF receptor ligands such as transforming growth factor-α and amphiregulin in epidermal keratinocytes show psoriatic phenotypes similar to that in LIG-1$^{-/-}$ mice [17,18]. Recently, the *Drosophila* Kek1, a transmembrane protein with domain organization similar to LIG-1, has been reported to act in a feedback loop to negatively regulate the activity of EGF receptors through the extracellular domain [19]. We inferred that LIG-1 may function as a vertebrate Kek1-like molecule to suppress the growth of keratinocyte and that the cells would be released from the inhibitory effect of LIG-1 in the psoriasiform epidermis.

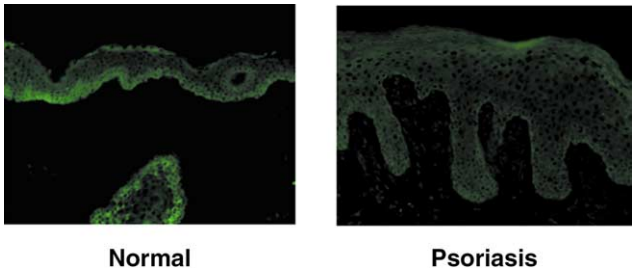

Fig. 5. LIG-1 expression in psoriatic skin. LIG-1 was strongly expressed in the epidermal basal cells and outer root sheath cells of hair follicles in normal human skin, but was apparently decreased in the psoriatic skin.

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