

Lessons from Loricrin-deficient Mice: Compensatory Mechanisms Maintaining Skin Barrier Function in the Absence of a Major Cornified Envelope Protein

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Abstract. The epidermal cornified cell envelope (CE) is a complex protein–lipid composite that replaces the plasma membrane of terminally differentiated keratinocytes. This lamellar structure is essential for the barrier function of the skin and has the ability to prevent the loss of water and ions and to protect from environmental hazards. The major protein of the epidermal CE is loricrin, contributing ~70% by mass. We have generated mice that are deficient for this protein. These mice showed a delay in the formation of the skin barrier in embryonic development. At birth, homozygous mutant mice weighed less than control littermates and showed skin abnormalities, such as congenital erythroderma with a shiny, translucent skin. Tape stripping experiments suggested that the stratum corneum stability was reduced in newborn *Lor*^{-/-} mice compared with wild-

type controls. Isolated mutant CEs were more easily fragmented by sonication *in vitro*, indicating a greater susceptibility to mechanical stress. Nevertheless, we did not detect impaired epidermal barrier function in these mice. Surprisingly, the skin phenotype disappeared 4–5 d after birth. At least one of the compensatory mechanisms preventing a more severe skin phenotype in newborn *Lor*^{-/-} mice is an increase in the expression of other CE components, such as SPRRP2D and SPRRP2H, members of the family of “small proline rich proteins”, and repetin, a member of the “fused gene” subgroup of the S100 gene family.

Key words: loricrin • knockout mice • cornified envelope • small proline-rich protein • repetin

Introduction

The cornified cell envelope (CE)¹ is a protein–lipid layer that replaces the plasma membrane of corneocytes and is crucial for epidermal barrier function (Roop, 1995). The

CE consists of a complex mixture of covalently cross-linked proteins and a layer of characteristic lipids attached to the extracellular surface of the protein layer (Wertz et al., 1989; Steinert and Marekov, 1995; Ishida-Yamamoto and Iizuka, 1998; Marekov and Steinert, 1998; Steinert et al., 1998a,b). On the cytoplasmic side, keratin filaments and their attachment sites at the plasma membrane, the desmosomes, are embedded in the CE (Koch and Franke, 1994). The CE components are cross-linked mainly via ϵ -(γ -glutamyl)lysine isopeptide bonds, which are catalyzed by calcium-dependent transglutaminases (TGases). This cross-linking leads to a protein complex that is highly insoluble and resistant to conventional biochemical extraction procedures. The significance of the TGase-mediated cross-linking for skin function has been demonstrated by the discovery that mutations in one of these enzymes (TGase 1) can lead to lamellar ichthyosis, an autosomal recessive skin

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¹*Abbreviations used in this paper:* CE, cornified cell envelope; E, embryonic day; ES, embryonic stem; HSV-tk, herpes simplex virus type 1 thymidine-kinase minigene; RPA, RNase protection assay; SPRRP, small proline-rich protein; TEWL, transepidermal water loss; TGase, transglutaminase; VS, Vohwinkel's syndrome.

disease (Russell et al., 1995; Huber et al., 1995, 1997). Patients with this severe disease develop hyperkeratosis (thickening of the stratum corneum) and acanthosis (thickening of the epidermis) due to a failure to form CE (Jeon et al., 1998). Furthermore, mice that are deficient for TGase 1 exhibit a severe defect in epidermal barrier function resulting in neonatal lethality (Matsuki et al., 1998).

Several protein components of the CE have been identified, among them are involucrin, members of a family of small proline-rich proteins (SPRRPs), loricrin as well as cystatin α , desmoplakin, envoplakin, elafin, filaggrin, and several keratins (Ishida-Yamamoto and Iizuka, 1998). Furthermore, repetin has been identified as a putative CE precursor protein (Krieg et al., 1997). Some of these proteins (involucrin, SPRRPs, loricrin, filaggrin, and repetin) are encoded by genes clustered in the so-called "epidermal differentiation complex" on human chromosome 1q21 (Volz et al., 1993; Marenholz et al., 1996; Mischke et al., 1996) and mouse chromosome 3 (Rothnagel et al., 1994; Song et al., 1999), respectively. Involucrin, SPRRPs, repetin, and loricrin also share amino acid sequence homologies in their COOH-terminal domains, indicating a common origin of these genes (Backendorf and Hohl, 1992; Krieg et al., 1997).

Interestingly, the protein composition of the CE varies between different tissues and body sites. Loricrin is the major component of the CE in the interfollicular epidermis, in which as much as 70% of the CE mass is derived from this protein (Hohl et al., 1991; Steven and Steinert, 1994; Jarnik et al., 1996; Steinert et al., 1998a). For example, in human foreskin epidermis, the inner (cytoplasmic) two thirds of the CE consists of >85% of loricrin (Steinert and Marekov, 1995). On the other hand, loricrin is absent in most internal epithelia, though one notable exception is the mouse forestomach (Hohl et al., 1993). Furthermore, the molar ratio of the CE components may vary between different tissues. A comparison of mouse epidermal and forestomach CE showed that the ratio of loricrin to SPRRPs was 100:1 in the newborn epidermis and 3:1 in the forestomach (Jarnik et al., 1996; Steinert et al., 1998a). SPRRP proteins are coexpressed with loricrin and serve as molecular cross-linkers (Steinert et al., 1998a). It has been speculated that the relative molar ratio of loricrin and SPRRP proteins may determine the biomechanical properties of the CE. For example, CE that are subject to mechanical stress may have a higher relative amount of SPRRP proteins (Jarnik et al., 1996; Steinert et al., 1998a).

It has been proposed that the first step in epidermal CE formation in the stratum granulosum is the deposition of involucrin at the inner surface of the plasma membrane (Steinert and Marekov, 1997). Involucrin is thought to serve as a scaffold for the addition of other CE components (Steinert and Marekov, 1997). Loricrin (Mehrel et al., 1990) is expressed in the granular layer of the epidermis where it accumulates in granules (termed L-granules) before it is integrated into the developing CE (Steven et al., 1990; Bickenbach et al., 1995).

Mouse loricrin is a glycine- (55.1% of total amino acids), serine- (22.3%), and cysteine-rich (7.1%) basic protein. It consists of 481 amino acids and has a predicted molecular weight of 37,828 (Mehrel et al., 1990). The protein con-

tains tandem repeats that have been predicted to form "glycine loops" (Hohl et al., 1991; Steinert et al., 1991). It is thought that these structures confer flexibility to the protein and the CE. Interestingly, these biochemical features of loricrin, glycine-rich sequences and high flexibility, are also characteristic for the NH₂-terminal and COOH-terminal domains of keratins 1 and 10 (Jorcano et al., 1984; Zhou et al., 1988). This pair of intermediate filament proteins is coexpressed with loricrin in embryonic development of the epidermis (Bickenbach et al., 1995). The glycine loops of loricrin are flanked by glutamine- or glutamine/lysine-rich sequences, which are targets for TGase-mediated intramolecular and intermolecular cross-linking through isopeptide bonds (Steinert and Marekov, 1995). The abundance of cysteine residues in the loricrin sequence suggests that this protein also forms extensive disulfide bridges (Hohl et al., 1991). This prediction is supported by the observation that loricrin can only be solubilized with high concentrations of reducing agents (Mehrel et al., 1990).

The unique structural features of loricrin and its abundance in orthokeratinizing epithelia, in particular tissues that are subject to considerable mechanical stress, such as the epidermis and forestomach (in mouse), have led to the assumption that expression of this protein is essential for the function of these tissues. To test this hypothesis, we have generated loricrin knockout mice. Unexpectedly, loss of loricrin led to a mild transient phenotype in mice. We present evidence suggesting that Lor^{-/-} mice compensate for the loss of the main epidermal CE component by up-regulating the expression of other CE components, in particular SPRRPs and repetin.

Materials and Methods

Construction of Targeting Vector

We have described previously the isolation and characterization of the loricrin gene of the BALB/c mouse strain (Rothnagel et al., 1994). To achieve a high frequency of recombination with the genome of mouse embryonic stem (ES) cells, gene targeting/replacement vectors were constructed using gene sequences from the 129/Sv mouse strain (Capecchi, 1989; Deng and Capecchi, 1992; Ramirez-Solis et al., 1993; Horie et al., 1994). We have isolated the loricrin gene from this mouse strain. In our targeting vector (Fig. 1 A), a 2.457-kb DNA fragment encompassing the entire loricrin coding sequence is deleted and replaced by a neomycin-resistance minigene (see Fig. 1 A, PGKneobpA) (provided by Allan Bradley, Baylor College of Medicine). The minigene is flanked by DNA sequences derived from the loricrin gene locus (5.8-kb 5' flanking sequence; 1.5-kb 3' flanking sequence). To facilitate the isolation of targeted ES cell clones, we inserted a herpes simplex virus type 1 thymidine-kinase minigene (HSV-tk, Fig. 1 A) (Mansour et al., 1988) into the targeting vector (provided by John Lydon, Baylor College of Medicine). Both minigenes are under the transcriptional control of the phosphoglycerate kinase I promoter.

Targeting of ES Cells and Generation of Loricrin-deficient Mice

The ES cell line AB2.2 (provided by Allan Bradley, Baylor College of Medicine) was cultured as described previously (Ramirez-Solis et al., 1993). Linearized targeting vector was introduced into ES cells by electroporation with a Bio-Rad Gene Pulser II (Bio-Rad Laboratories). Electroporated ES cells were grown on gamma-irradiated neomycin-resistant SNL feeder cells (Hogan et al., 1994), provided by Allan Bradley (Baylor College of Medicine) in selection medium containing G418 (GIBCO BRL) and FIAU. G418/FIAU-resistant ES cell colonies were isolated and screened for successful recombination using a mini-Southern procedure (Ramirez-Solis et al., 1992, 1993). As shown in Fig. 1, we used a SacI-SphI

fragment (5' probe) and a SacI–BamHI fragment (3' probe) as probes in this Southern blot analysis. Recombinant ES cell clones that showed the expected DNA bands in Southern blots, indicating a deletion of the loricrin coding sequence in one allele, were also tested with a probe derived from the neomycin-resistance minigene. This probe indicated the presence of a single copy of the neomycin-resistance minigene in the recombinant ES cell clones tested (data not shown). The recombinant ES cells were injected into C57Bl/6J blastocysts. Chimeric offsprings were crossed with C57Bl/6J mice. DNA from agouti offspring was prepared from the tail (Hogan et al., 1994) and tested for the presence of the targeted allele by Southern blot hybridization using the 5' and/or 3' probes. Mice heterozygous for the targeted mutation were intercrossed to obtain homozygous loricrin-null mutants. To minimize phenotype variations due to the mixed (129/Sv and C57Bl/6) genetic background, we crossed the loricrin-null allele into the FvB mouse strain. The experiments described in this paper were performed using mice from the fifth back cross.

Southern Blot Analysis

Genomic DNA from mouse tails (Hogan et al., 1994) was digested overnight with the appropriate restriction enzymes. DNA fragments were separated by agarose gel electrophoresis, transferred to GeneScreen Plus membranes (NEN Life Science Products), and then UV cross-linked (UV Stratalinker 1800; Stratagene). Radioactive DNA probes were synthesized using the DecaprimeII kit (Ambion) with [α^{32} P]dCTP (NEN Life Science Product). Blots were hybridized for 2 h at 65°C with probes in "rapid hybridization buffer" (Amersham Pharmacia Biotech), washed once for 10 min at room temperature in 2× SSC/0.1% SDS, followed by two washes (20 min each) at 65°C in 0.2× SSC/0.1% SDS. The membranes were then exposed to Kodak BIOMAX MR film (Eastman Kodak).

RNase Protection Assay

The following mouse DNA clones were used to synthesize antisense cRNA probes: loricrin (200-bp 3' coding sequence), SPRRP2D (position 309–640, sequence data available from EMBL/GenBank/DDBJ under accession number AJ005562), SPRRP2H (position 469–642, sequence data available from EMBL/GenBank/DDBJ under accession number AJ005566), repetin (position 8651–8854, sequence data available from EMBL/GenBank/DDBJ under accession number X99251), β -actin (pTRI-actin M, Ambion), GAPDH (pTRI-GAPDH, Ambion), cyclophilin (Ambion), and desmoglein 3 (clone B9; Koch et al., 1997). Mouse epidermis was prepared as described previously (DiSepio et al., 1999). In some experiments, total RNA was isolated from epidermis with the RNazol B reagent (Tel-test). Biotinylated probes were synthesized with a MAXIScript II Kit (Ambion) using biotin-16-UTP (Enzo). The β -actin probe was synthesized with 1/50 of the standard biotin-16-UTP concentration to obtain RPA signals comparable with those obtained with the other probes. For nonradioactive RPA, tissue lysates were prepared using the Direct Protect Kit (Ambion). RPA products were separated in 6% polyacrylamide/TBE/8 M urea gels, blotted onto GeneScreen Plus membranes (NEN Life Science Products) with a Mini-Trans-Blot Cell (Bio-Rad Laboratories), and then UV cross-linked (UV Stratalinker 1800; Stratagene). Biotin-labeled RNA fragments were detected with a chemiluminescent detection system (BrightStar Biotodect; Ambion). RPA with radioactive labeled probes were done with the RPAII kit (Ambion). Expression levels of RNAs were determined by scanning RPA autoradiograms followed by densitometric analysis with the QuantiScan software package (Biosoft).

Antibodies

The following antibodies were used: loricrin (Mehrel et al., 1990), K14 (Roop et al., 1984), repetin (Huber, M., and D. Hohl, unpublished data) filaggrin repeat (AF 111), which was generated against synthetic peptide DSQVHSGVQVEGRRGH (Yuspa et al., 1989), and filaggrin linker (AF133), which was generated against the synthetic peptide GYYEEQHSEESD. Anti-rabbit, anti-guinea pig, and anti-mouse IgG coupled to fluorescent dyes (FITC, Texas red) were purchased from Zymed Laboratories and Dako.

Western Blotting

Skin samples of newborn mice were pulverized in liquid nitrogen and then incubated for 10 min in extraction buffer (0.0625 M Tris-HCl, 5% SDS, 10% glycerol, 20% β -mercaptoethanol, pH 6.8) at 95°C. Equal amounts of protein were separated in 10% SDS-PAGE gels and then transferred onto

nitrocellulose membranes (S&S NC; Schleicher & Schuell) with a Mini-Trans-Blot Cell (Bio-Rad Laboratories). Membranes were blocked for 1 h in blocking buffer (3% nonfat milk/TBS) and then incubated with the primary antibody, which was dissolved in blocking buffer, for 1 h. After three washes (10 min each) in TBST buffer (0.05% Tween 20 in TBS), membranes were incubated with the secondary antibody (alkaline phosphatase-conjugated anti-rabbit or anti-mouse antibodies; Zymed Laboratories) for 30 min and then washed as described above. Antibody binding was visualized with the NBT/BCIP system (Boehringer).

Immunofluorescence Microscopy

Cryosections (5- μ m thick) were fixed in acetone for 10 min at –20°C and then washed in PBS. The tissues samples were incubated with the first antibody at the appropriate dilution in 1% BSA/PBS over night at 4°C. After washing three times for 10 min each in PBS, samples were incubated with a fluorescent dye-coupled antibody diluted in 1% BSA/PBS for 30 min and washed as described above. Stained sections were examined and photographed with a Nikon OPTIPHOT photomicroscope (Nikon).

Preparation of CEs and Sonication Experiments

Epidermis from newborn and 4-d-old mice was isolated essentially as described previously (Jarnik et al., 1996). CEs were prepared by boiling epidermis for 20–40 min in a buffer consisting of 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DTT, and 2% SDS. After centrifugation (5,000 g), CEs were washed twice at room temperature with a buffer consisting of 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DTT, and 0.2% SDS. For sonication, CEs were suspended in a 2% SDS solution. The concentration of CE was determined with a hemacytometer. The CE suspension was sonicated in a Branson 2210 cup sonicator (Branson Ultrasonics) at 4°, 20°, 30°, and 60°C. At various time points, CE aliquots were removed from the sonicator, counted with the hemacytometer, and photographed with a Nikon OPTIPHOT photomicroscope.

Tape Stripping Experiments

Newborn mice were tape stripped 10 times. Backskin from treated and untreated areas were excised, fixed, and processed for electron microscopy as described previously (Bickenbach et al., 1996).

Toluidine Blue Staining of Mouse Embryos

The developmental stage of mouse embryos was determined based on the assumption that fertilization occurred in the middle of the dark cycle the day before plugs were identified. The embryos were stained essentially as described (Hardman et al., 1998). In brief, embryos were dehydrated by incubations (1 min each) in 25%, 50%, and 75% methanol/PBS followed by 1 min in 100% methanol. The embryos were then rehydrated with the same series of methanol solutions (1 min incubations), washed in PBS, and stained for 1 min in 0.0125% toluidine blue O (Fisher Scientific)/PBS. After destaining in PBS, embryos were photographed with a Nikon SMZ-2T microscope.

Measurement of Transepidermal Water Loss

Transepidermal water loss (TEWL) from the ventral skin of newborn mice was determined using a Tewameter TM210 (Courage and Khazaka), as described previously (Matsuki et al., 1998). In addition, we used a simple dye penetration assay to analyze potential barrier defects (Matsuki et al., 1998). In brief, the backs of mice were immersed in 1 mM Lucifer yellow/PBS (Fluka Chemical Co.). Mice were killed after 1 h incubation and frozen sections were prepared from the back skin. Penetration of the dye was analyzed using a fluorescence microscope.

Results

Cloning of the Mouse Loricrin Gene and Generation of Loricrin-deficient Mice

We have described previously the structure and chromosomal localization of the BALB/c loricrin gene (Rothnagel et al., 1994). To construct an efficient gene targeting vec-

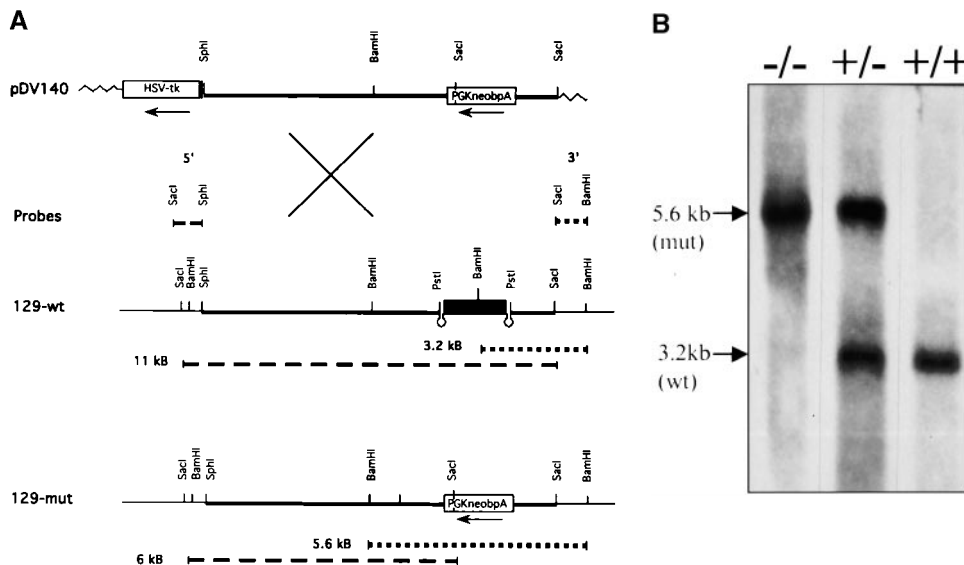


Figure 1. Targeting strategy and confirmation of gene targeting event. (A) Partial restriction enzyme maps and schematic representation of the strategy used to ablate loricrin expression in mice. The targeting vector (pDV140, top, arrows indicate the orientation of transcription units), the loricrin gene of 129Sv mice (129-wt, middle) and the recombinant loricrin locus (129-mut, bottom) are shown. Solid lines represent loricrin sequences included in the targeting vector. Thin lines represent sequences excluded from the targeting vector. Zigzag lines symbolize plasmid sequences. The location of the 5' (SacI-SphI) and 3' (SacI-BamHI) probes used to identify the recombinant allele in Southern blots are indicated above the 129-wt gene. The dotted lines below the middle and bottom schematics indicate DNA fragments that hybridize to the 3' and 5' probe, respectively. In the gene targeting vector, a Pst I fragment containing the entire coding sequence of the loricrin gene (middle, solid box) was deleted and replaced by a neomycin-resistance minigene (PGKneobpA). To facilitate the identification of recombinant ES cell clones, we introduced an HSV-tk into the targeting vector, as well. Note that recombination between the targeting vector and the wild-type loricrin gene locus deleted a BamHI site (middle, solid box) and introduced a new SacI site (in PGKneobpA cassette, bottom). (B) Example of a Southern blot analysis. Tail DNA from the offspring of a $lor^{+/-}$ intercross was cut with BamHI and hybridized to the 3' probe. The genotype of the mice analyzed is indicated above the lanes. Arrows indicate the position of mutant (mut) and wild-type (wt) DNA fragments.

tor, we isolated the loricrin gene from the 129Sv/J mouse strain. It has been shown that the use of isogenic DNA can significantly increase the efficiency of gene targeting vectors in ES cells (Deng and Capecchi, 1992; Zhang et al., 1994; Simpson, 1997).

The loricrin protein sequence is encoded by a single exon (Yoneda et al., 1992; Rothnagel et al., 1994). In the targeting vector pDV140, a 2.4-kb PstI fragment encompassing the entire loricrin coding sequence was deleted and replaced by a neomycin-resistance minigene (Fig. 1 A). To allow homologous recombination between targeting vector and the loricrin gene in ES cells, the neomycin minigene was flanked by 5.8-kb 5' and 1.5-kb 3' loricrin sequences, respectively. Furthermore, a herpes simplex virus thymidine kinase (Fig. 1 A, HSV-tk) expression cassette was introduced into the targeting construct to allow a "positive/negative" selection procedure (Mansour et al., 1988). As shown in Fig. 1 A, successful recombination between the targeting vector and the loricrin gene was predicted to eliminate a BamHI site and introduce a new SacI site into the loricrin gene locus. Neo^r/FIAU^r ES cell clones were tested for the presence/absence of these two restriction sites by Southern blot analysis using probes that were located outside the loricrin region used in the targeting vector (Fig. 1 B). Six independent clones that had undergone homologous recombination at the loricrin gene locus were identified. Southern blots hybridized with a probe derived from the neomycin minigene indicated that these clones had integrated a single copy of the neomycin minigene (data not shown).

All six ES cell clones were injected into C57Bl/6J blastocysts. Three clones (VIL2, VIL4, and VIL5) generated highly chimeric mice as judged by their coat color. Two of these clones (VIL2 and VIL5) showed germline transmis-

sion of the targeted loricrin allele. $Lor^{-/-}$ animals generated from both ES clones were phenotypically identical. The targeted mutation was inherited according to Mendelian law (data not shown), indicating that loricrin ablation did not prevent prenatal development. Furthermore, $Lor^{-/-}$ mice were viable and fertile.

Initially, $Lor^{-/-}$ mice were derived from $Lor^{+/-}$ intercrosses on a segregating C57Bl/6 and 129Sv background. To minimize possible phenotypic variations due to this heterogeneous genetic background, we crossed the loricrin mutation into the FVB mouse inbred strain. The experiments described below were performed using animals from the fifth generation of backcrosses. To confirm that mice homozygous for the targeted mutation did not synthesize loricrin, we performed ribonuclease protection assays (RPA), Western blot analysis, and indirect immunofluorescence microscopy. As shown in Fig. 2, newborn $Lor^{-/-}$ mice did not express loricrin as demonstrated by the absence of loricrin mRNA and protein.

Effects of Loricrin Ablation on Prenatal Development

Hardman and colleagues recently developed a method to follow epidermal barrier formation in mouse embryonic development (Hardman et al., 1998). This method is based on the staining of embryos with a histological dye (see Materials and Methods). Impermeability of the skin for the dye indicates maturation of the skin barrier and is accompanied by the development of the cornified cell layers. At the molecular level, deposition of loricrin at the plasma membrane coincides with dye impermeability.

Focal expression of loricrin in the skin is first detected at embryonic day (E) 16 (Bickenbach et al., 1995; Hardman et al., 1998). We wondered whether the ablation of loricrin affected skin barrier formation at that stage of develop-

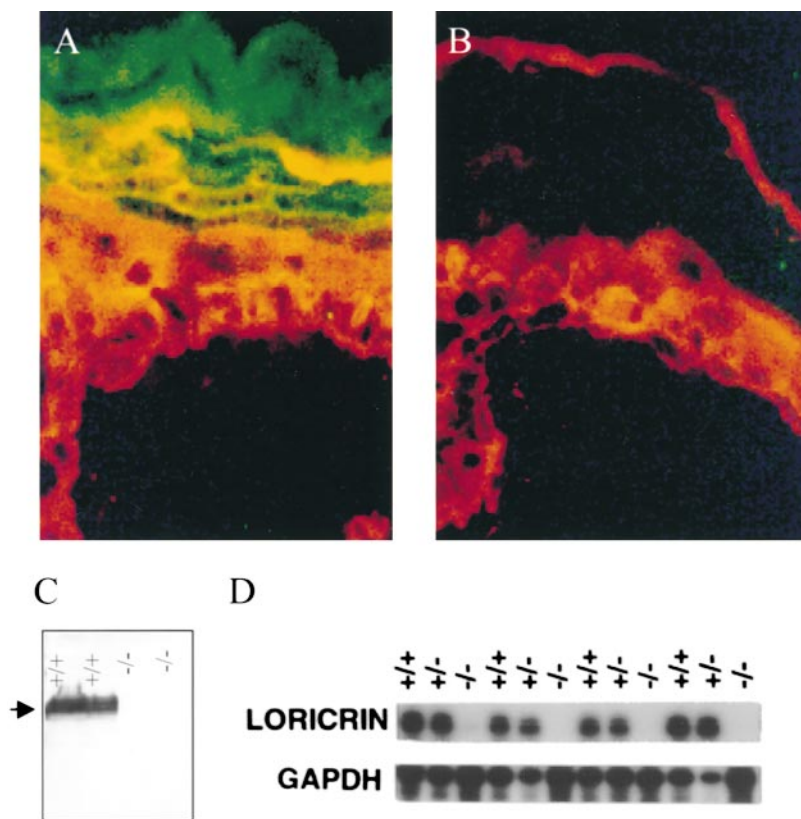


Figure 2. Lack of loricrin expression in newborn $Lor^{-/-}$ mice as confirmed by immunofluorescence microscopy (B), Western blots (C), and RPA (D). (A) Staining of backskin from a newborn wild-type mouse with antibodies against loricrin (green) and K14 (red). Overlapping expression of both proteins results in yellow fluorescence. K14 protein is present in the suprabasal layers of the epidermis although the K14 gene is only transcribed in the basal cell layer. (B) Staining of $Lor^{-/-}$ back skin. Note the lack of loricrin staining. (C) Western blots probed with loricrin antibody. The arrowhead points to the loricrin band in skin extracts from wild-type back skin. (D) RPA analysis of RNA isolated from the epidermis of newborn pups from a $Lor^{+/-}$ intercross. GAPDH was used as an internal loading control. Note the absence of loricrin mRNA in $Lor^{-/-}$ mice.

ment. Therefore, we examined $Lor^{-/-}$ and control embryos at E16.5, E17.5, and E18.5 (Fig. 3). $Lor^{+/-}$ and $+/+$ embryos showed a similar staining pattern at E16.5, with a large unstained area on the backskin indicating barrier formation. $Lor^{-/-}$ embryos showed a different staining pattern. Their back skin was either completely stained or showed smaller areas of unstained skin compared with control animals, clearly indicating impaired barrier formation. However, at E17.5, $Lor^{-/-}$ and control animals did not show any skin staining. These results suggest that the development of skin barrier function, at least as defined by the staining method, is delayed in $Lor^{-/-}$ mice, but reaches wild-type level at E17.5. Nevertheless, given that $Lor^{-/-}$ mice are born with a skin phenotype (see below), it is quite likely that skin barrier defects persist beyond E17.5.

Loricrin Knockout Mice Develop a Transient Congenital Erythroderma with a Shiny, Translucent Skin

Mice heterozygous for the loricrin mutation ($Lor^{+/-}$) were phenotypically indistinguishable from wild-type littermates. However, $Lor^{-/-}$ mice were clearly abnormal at birth. They were runt and had a shiny, translucent skin with signs of erythroderma (Fig. 4). To determine whether runt $Lor^{-/-}$ mice grew more slowly than control littermates, we monitored their body weight. The postpartum growth rate of $Lor^{-/-}$ mice was similar to control animals (data not shown). This suggested that food intake and digestion were normal in these mice, that is the loricrin-expressing tissues of the gastrointestinal tract were functioning properly. Consequently, histological examination did not reveal abnormalities in the tissues that are known

to express loricrin, oral cavity, esophagus, and forestomach, of $Lor^{-/-}$ mice (Hohl et al., 1993). The skin phenotype persisted for ~ 4 –5 d and then gradually disappeared. Histological examination of newborn $Lor^{-/-}$ skin did not reveal abnormalities with respect to thickness and proper development and differentiation (data not shown).

Mechanical Properties of $Lor^{-/-}$ CE

We next prepared CEs from wild-type and $Lor^{-/-}$ newborn epidermis. Microscopic examination revealed structural abnormalities in $Lor^{-/-}$ CE (Fig. 5). It has been proposed that loricrin confers flexibility to the epidermal CE. To test whether $Lor^{-/-}$ CEs were more susceptible to mechanical stress, we exposed purified mutant and wild-type CE to ultrasound (Table I). The percentage of intact CE from $Lor^{-/-}$ epidermis decreased dramatically after 10

Table I. Ultrasound Treatment of Isolated CEs from Newborn $Lor^{-/-}$ (MT) and Wild-Type (WT) Mice

Treatment	WT/MT			
	4°C	20°C	30°C	60°C
None	100/100	100/100	100/100	100/100
5 min	100/100	100/100	100/100	100/100
10 min	100/50	100/100	100/100	100/100
15 min	100/0	95/90	100/95	100/95
20 min	90/0	95/90	95/95	100/95
25 min	50/0	90/50	95/90	95/90
30 min	ND	ND	95/90	95/90

CEs were isolated from four $Lor^{-/-}$ mice and two wild-type controls. Each sample was sonicated as indicated at four different temperatures. Numbers indicate percentage of intact CE. ND, not done.

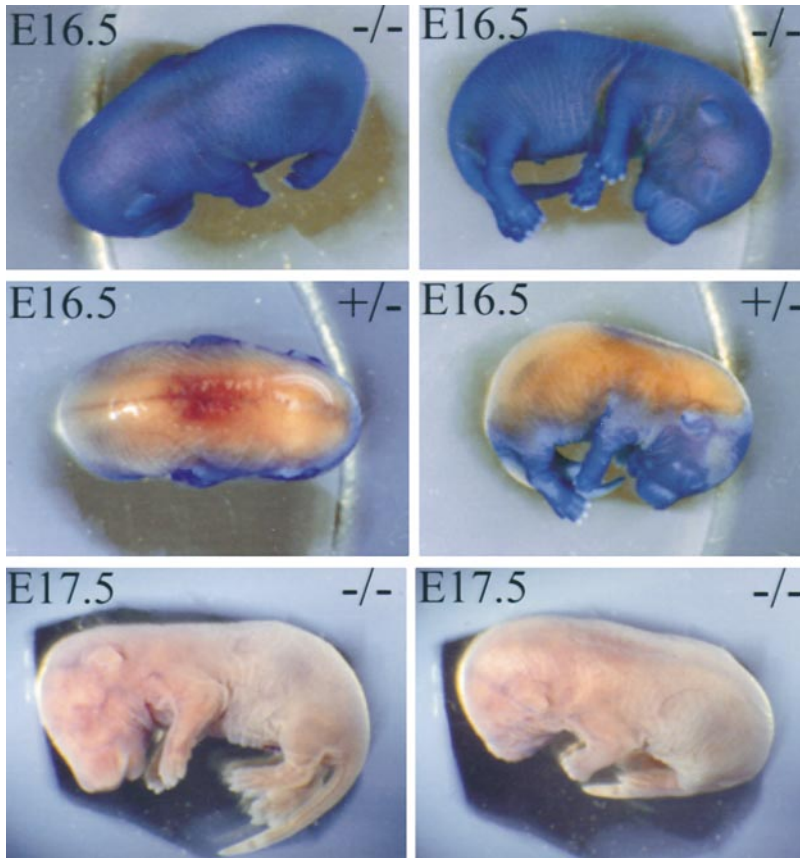


Figure 3. Delay in the development of the skin barrier in $Lor^{-/-}$ embryos. Embryos from $lor^{+/-}$ intercrosses were extracted with methanol, which is thought to partially extract polar lipids, and stained with toluidine blue. Staining indicates permeability for the dye and shows immature barrier function. The approximate age of the embryos and their genotype is indicated. At E16.5, $Lor^{+/-}$ embryos showed large unstained areas on the back skin, whereas $Lor^{-/-}$ embryos were completely stained, indicating a delay in the formation of the skin barrier in these embryos. Wild-type ($Lor^{+/+}$) and heterozygous mutant embryos ($Lor^{+/-}$) showed a similar staining pattern (data not shown). At E17.5, mutant and wild-type (data not shown) embryos were largely unstained.

min of ultrasound treatment (50% mutant CE versus 100% wild-type CE) (Fig. 5 and Table I). After 15 min, essentially all mutant CEs were destroyed. In contrast, no damage was evident in wild-type CE after 15 min of ultrasound treatment. Interestingly, the susceptibility of mutant CE to ultrasound was temperature dependent. Initially, CEs were treated with ultrasound at 4°C. At higher temperature, mutant CE were partially protected from ultrasound damage (Table I). For example, whereas essentially all mutant CEs were destroyed after 15 min treatment at 4°C, only 10% were damaged at 20°C (Table I). Adult $Lor^{-/-}$ CE also showed the same susceptibility to sonication (data not shown).

To determine whether the abnormal CE affected the biomechanical properties of the mutant epidermis, we performed tape stripping experiments and determined the loss of cell layers from the stratum corneum by electron microscopy. As summarized in Fig. 6, the stratum corneum of $Lor^{-/-}$ backskin was less resistant to mechanical stress than wild-type control tissue. Mutant mice lost approximately three times more cell layers than control mice. Nevertheless, the fact that tissues that are naturally exposed to mechanical stress (paws, legs, and axilla) do not show gross or microscopic lesions, suggests that the mechanical stability of $Lor^{-/-}$ epidermis is not significantly impaired.

The $Lor^{-/-}$ Mutation Does Not Increase TEWL

We speculated that the shiny skin phenotype of $Lor^{-/-}$ mice resulted from dehydration, that is an increased TEWL due to a skin barrier defect. To investigate this pos-

sibility, we measured water loss in newborn mice with a Tewameter (Barel and Clarys, 1995). We also determined whether the passive diffusion of the dye Lucifer yellow (Matsuki et al., 1998) through the stratum corneum of newborn $Lor^{-/-}$ mice was increased compared with wild-type controls. We have shown previously that the disruption of the epidermal barrier in transgenic mice expressing a mutant form of loricrin leads to an increase in the permeability of the skin for this dye, as well as an increase in TEWL (Suga et al., 2000). However, neither of these methods revealed an apparent increase in TEWL of $Lor^{-/-}$ epidermis (data not shown).

Upregulation of Other CE Components in $Lor^{-/-}$ Epidermis

The fast regeneration of the $Lor^{-/-}$ epidermis with normal TEWL shortly after birth and the disappearance of the phenotype by day 4 or 5, suggested a compensatory mechanism that was induced either in utero or immediately after birth. The fact that the CE of newborn $Lor^{-/-}$ mice was of normal thickness (Jarnik, M., P.A. de Viragh, D. Bundman, M. Simon, D.R. Roop, and A.C. Stevens, manuscript submitted for publication), but abnormal structure, further supported the idea that another CE component was compensating for the loss of loricrin. Therefore, we compared the expression levels of several known CE components in E16.5 and E17.5 embryos, newborn, and 4-d-old $Lor^{-/-}$ and $Lor^{+/+}$ mice. We found an increase in the expression of repetin and two members of the SPRRP protein family in $Lor^{-/-}$ epidermis at all time points examined (Fig. 7, A and B), except for E16.5. At E16.5, we did not obtain de-

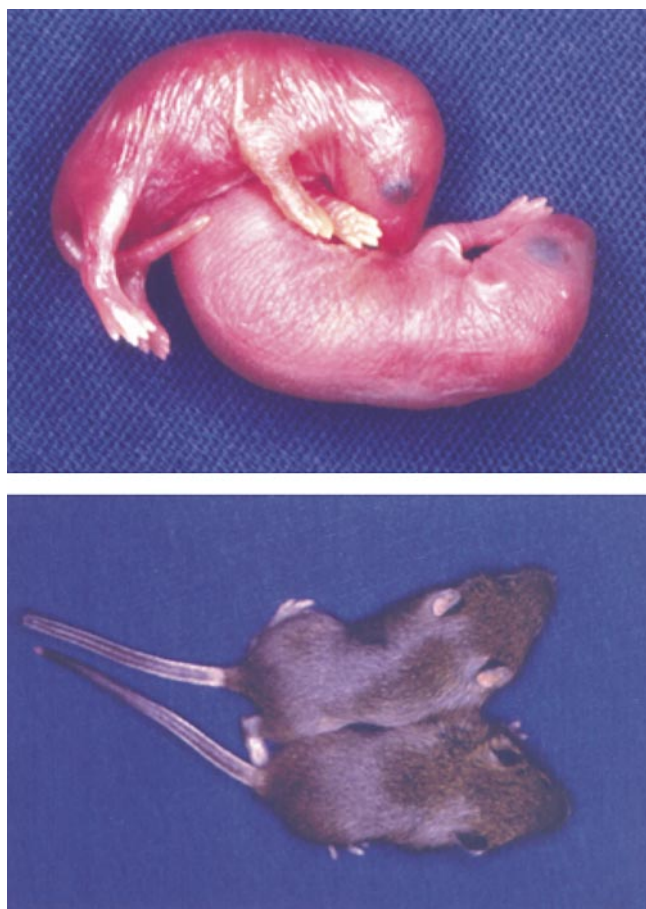


Figure 4. Gross abnormalities in newborn *Lor*^{-/-} mice. (Top) Comparison of a *Lor*^{-/-} (top pup) and wild-type (bottom pup) newborn mouse. *Lor*^{-/-} mice were born with a shiny, translucent skin. (Bottom) This phenotype disappeared in older mice. Adult mutant (top animal) and wild-type (bottom animal) mice were indistinguishable.

tectable signals with the repetin and SPRRP2D probe, respectively. The SPRRP2H probe, however, gave reproducible signals. The expression levels of this gene were identical in *Lor*^{-/-} and wild-type mice at E16.5 (Fig. 7 C).

SPRRPs are components of the innermost cytoplasmic layer of the CE and serve as cross-linkers between loricrin molecules in orthokeratinizing epithelia (see Introduction). Mouse SPRRPs constitute a family of proteins that have been subdivided into three groups based on their amino acid sequences: SPRRP1 (2 genes), SPRRP2 (11 genes), and SPRRP3 (1 gene) (Ishida-Yamamoto and Iizuka, 1998; Song et al., 1999). Preliminary RT-PCR experiments indicated that two members of the SPRRP2 subfamily, SPRRP2D and SPRRP2H, were upregulated in *Lor*^{-/-} epidermis (data not shown). To confirm these results, we used RPAs to determine the mRNA levels of SPRRP2D and SPRRP2H in *Lor*^{-/-} and control (*Lor*^{+/-} and *Lor*^{+/+}) back skin epidermis.

As shown in Fig. 7 A, the two SPRRP2 mRNAs were already upregulated in E17.5 embryos, ~1 d after loricrin expression is initiated in normal mice (see Introduction). The expression of both SPRRP2D and SPRRP2H increased by approximately fivefold at E17.5. The expression in newborn mice increased by fivefold (SPRRP2D)

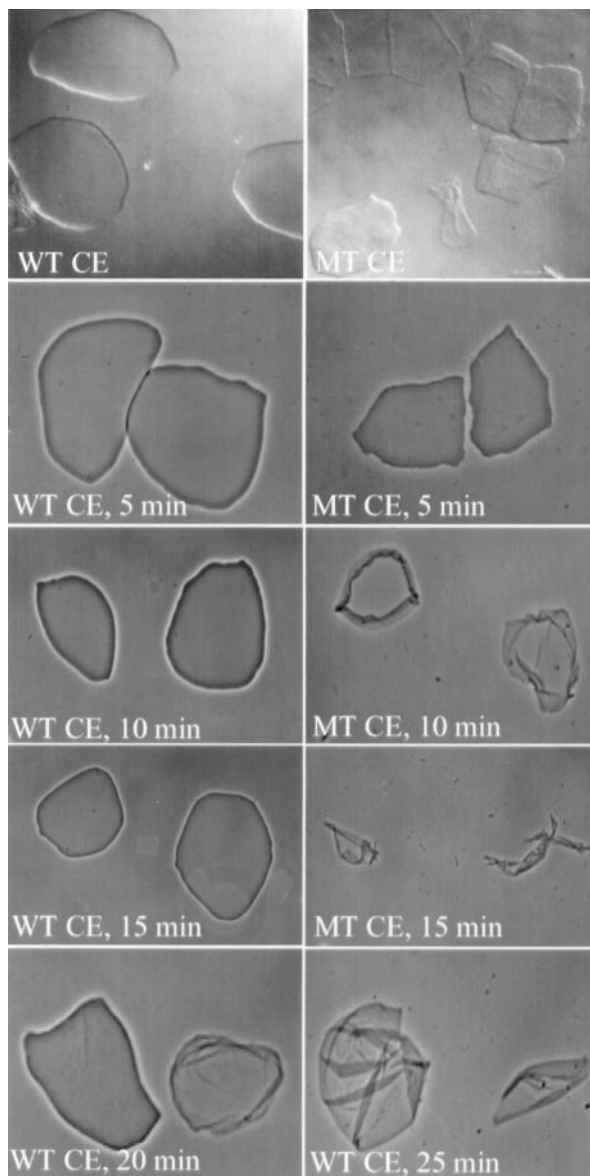


Figure 5. Morphological abnormalities and fragility of mutant CE. Wild-type (WT) and *Lor*^{-/-} (MT) CE were isolated and treated with ultrasound for 5, 10, 15, 20, and 25 min, respectively (for details see Materials and Methods). Note that untreated mutant CE are of irregular shape. Wild-type CE appear to be more symmetrical and have a smooth surface. After 15 min of treatment, mutant CE were completely destroyed, whereas wild-type CE were largely intact.

and twofold (SPRRP2H), respectively. At day 4 of postnatal development, SPRRP2D and SPRRP2H expression decreased, coinciding with the disappearance of the shiny skin phenotype. Nevertheless, whereas both mRNAs were easily detectable in RPA analysis of *Lor*^{-/-} skin, no (SPRRP2D) or only faint signals (SPRRP2H) were obtained using wild-type skin mRNA (Fig. 7 A).

We also examined the expression of repetin by RPA. Repetin is a relatively new member of the “fused gene” S100 subgroup consisting of profilaggrin, trichohyalin, and repetin (Krieg et al., 1997). Repetin also shares homology with CE components and was recently shown to be upreg-

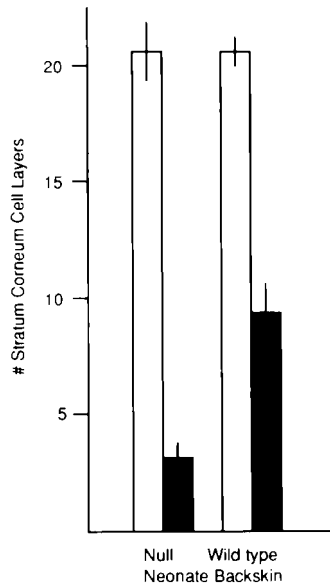


Figure 6. The stratum corneum of $Lor^{-/-}$ mice shows increased susceptibility to mechanical stress. The back skin of newborn $Lor^{-/-}$ (Null) and wild-type pups was tape stripped ten times. Animals were killed and the number of stratum corneum cell layers was determined by electron microscopy. Open bars (untreated skin); closed bars (tape stripped skin). Note that mutant mice lost approximately three times more cell layers.

ulated along with SPRRP2A in another knockout mouse exhibiting a barrier defect (Segre et al., 1999). Repetin transcripts were increased approximately threefold at E17.5, twofold in newborns, and fourfold in 4-d-old $Lor^{-/-}$ back skin when compared with controls (Fig. 7 B). We were also able to demonstrate an increase in repetin in $Lor^{-/-}$ epidermis at the protein level by immunofluores-

cence microscopy. In wild-type epidermis, repetin is expressed focally, and it appears to be localized in granules. There is no obvious accumulation in the stratum corneum (Fig. 8 A). However, in $Lor^{-/-}$ epidermis, repetin is expressed more uniformly, and it appears to localize at the periphery of CE in the stratum corneum (Fig. 8 B).

Other biochemical markers of keratinocyte differentiation, such as profilaggrin, keratin 10, involucrin, sciellin, and trichohyalin (which is a loricrin cross-linker in mouse forestomach) (Steinert et al., 1998a), were expressed at similar levels in newborn $Lor^{-/-}$ and control backskin (data not shown). However, in the case of profilaggrin, we noticed an additional high molecular weight band in Western blots from newborn $Lor^{-/-}$ back skin extracts which was not present in $Lor^{+/+}$ extracts (Fig. 7 D). Profilaggrin is a polyprotein consisting of multiple filaggrin units joined by linker sequences and flanked by unique NH₂- and COOH-terminal domains (Rothnagel et al., 1987). The polyprotein is processed to yield filaggrin in a complex biochemical cascade involving phosphatases and proteases. Filaggrin has a high affinity for keratins and provides a matrix in which keratin filaments are embedded. In the stratum corneum, filaggrin is eventually completely hydrolyzed. Free amino acids derived from this protein are thought to contribute to water retention in the stratum corneum.

Interestingly, one of the filaggrin antibodies used in this study (AF133, Fig. 7 D) indicated a reduction of the amount of mature filaggrin in $Lor^{-/-}$ epidermis extracts

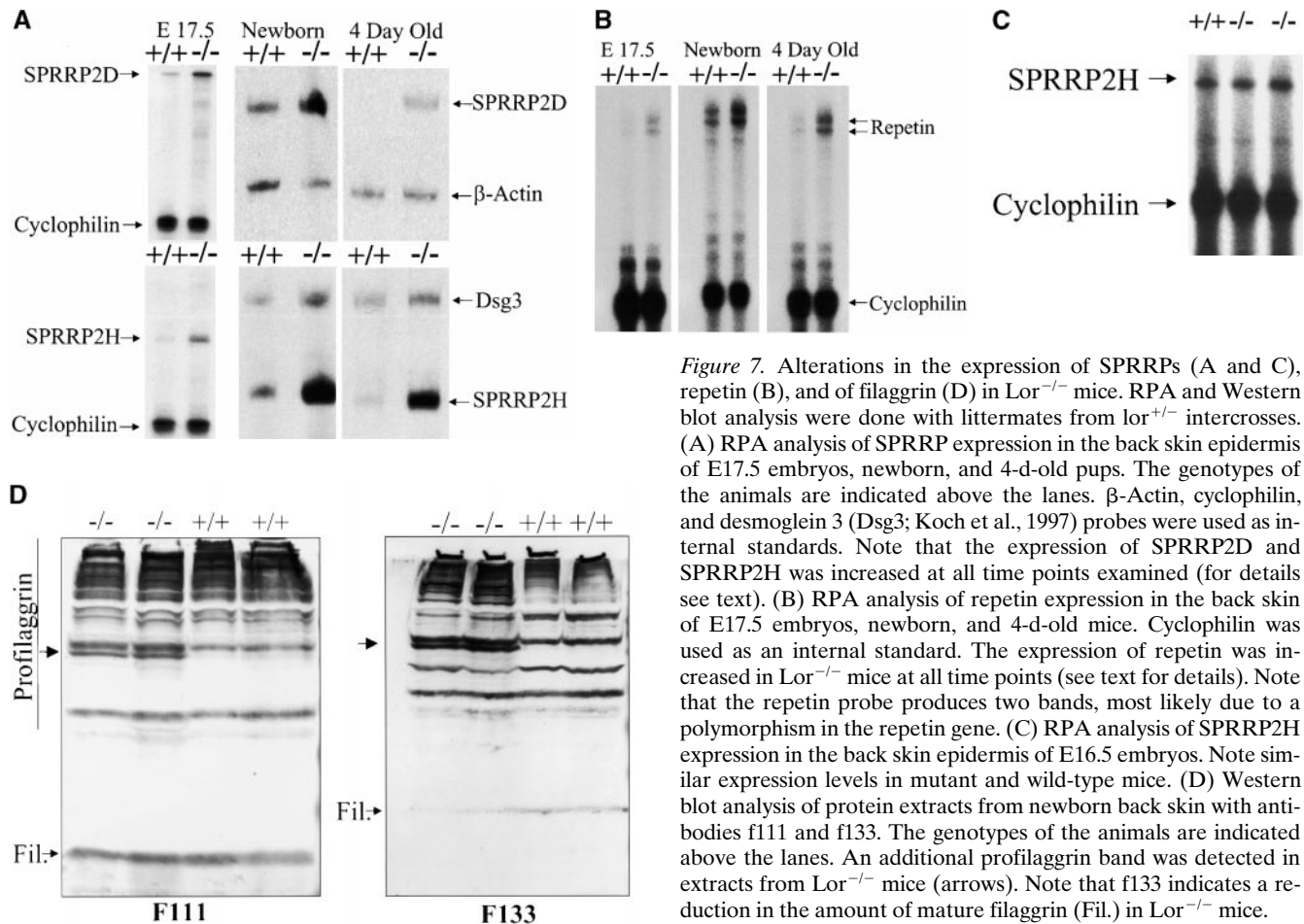


Figure 7. Alterations in the expression of SPRRPs (A and C), repetin (B), and of filaggrin (D) in $Lor^{-/-}$ mice. RPA and Western blot analysis were done with littermates from $lor^{+/-}$ intercrosses. (A) RPA analysis of SPRRP expression in the back skin epidermis of E17.5 embryos, newborn, and 4-d-old pups. The genotypes of the animals are indicated above the lanes. β -Actin, cyclophilin, and desmoglein 3 (Dsg3; Koch et al., 1997) probes were used as internal standards. Note that the expression of SPRRP2D and SPRRP2H was increased at all time points examined (for details see text). (B) RPA analysis of repetin expression in the back skin of E17.5 embryos, newborn, and 4-d-old mice. Cyclophilin was used as an internal standard. The expression of repetin was increased in $Lor^{-/-}$ mice at all time points (see text for details). Note that the repetin probe produces two bands, most likely due to a polymorphism in the repetin gene. (C) RPA analysis of SPRRP2H expression in the back skin epidermis of E16.5 embryos. Note similar expression levels in mutant and wild-type mice. (D) Western blot analysis of protein extracts from newborn back skin with antibodies f111 and f133. The genotypes of the animals are indicated above the lanes. An additional profilaggrin band was detected in extracts from $Lor^{-/-}$ mice (arrows). Note that f133 indicates a reduction in the amount of mature filaggrin (Fil.) in $Lor^{-/-}$ mice.

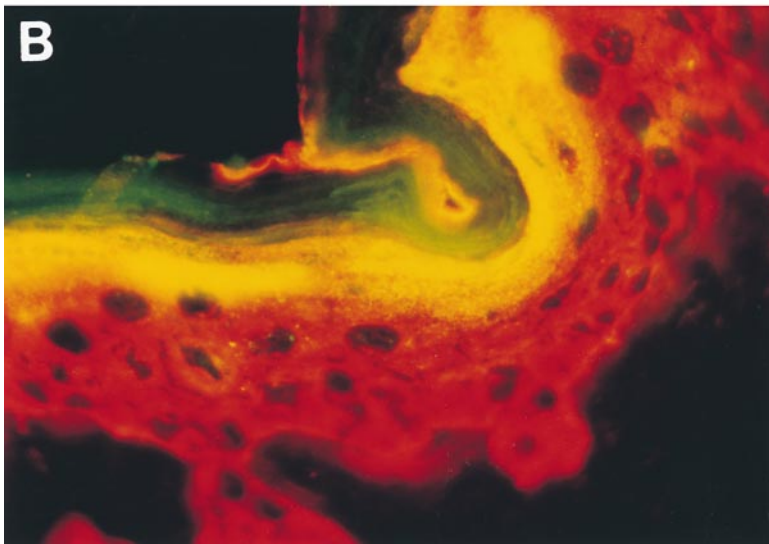
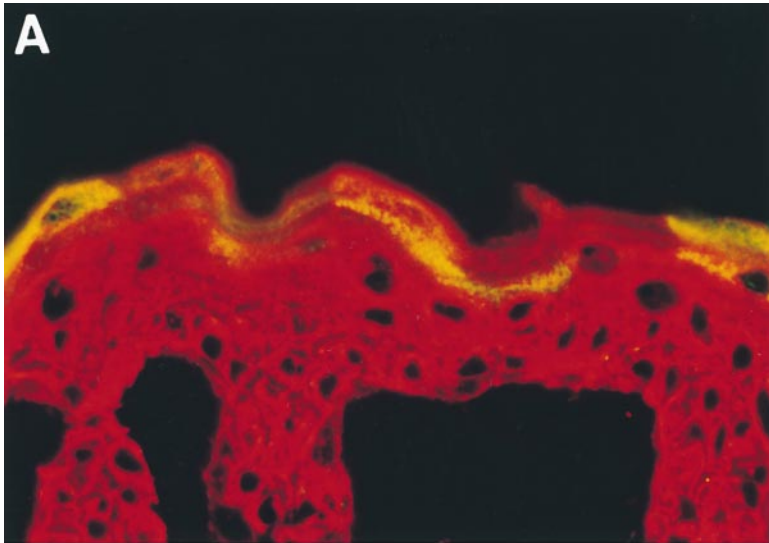


Figure 8. Increased repetin expression in $Lor^{-/-}$ mice. (A) Staining of back skin from a newborn wild-type mouse with antibodies against repetin (green) and K14 (red). Overlapping expression of both proteins results in yellow fluorescence. Note the focal expression pattern and lack of accumulation in the stratum corneum. (B) Staining of $Lor^{-/-}$ neonatal back skin. Note the more uniform expression pattern and the presence of repetin at the periphery of corneocytes in the stratum corneum. Also, note the apparent accumulation of repetin in granules in the granular layer of both wild-type and $Lor^{-/-}$ epidermis.

compared with $Lor^{+/+}$ control extracts. However, the other antibody (AF111, Fig. 7 D) showed equal amounts of filaggrin in $Lor^{-/-}$ and $Lor^{+/+}$ extracts. Nevertheless, both antibodies detected the additional high molecular band. AF133 was raised against a peptide sequence from the linker region that connects the filaggrin units in the profilaggrin precursor polypeptide (GYYYEQEHSEEESD). On the other hand, AF111 was raised against a peptide found in the filaggrin repeat (DSQVHSGVQVEGRRGH).

Therefore, the Western blot results suggest abnormal proteolytic processing of profilaggrin. Immunofluorescence microscopy with AF133 showed a normal distribution of the protein in $Lor^{-/-}$ epidermis (data not shown). Furthermore, electron microscopy on newborn epidermis showed that $Lor^{-/-}$ mice form normal profilaggrin-containing F-granules (Jarnik, M., P.A. de Viragh, D. Bundman, M. Simon, D.R. Roop, and A.C. Stevens, manuscript submitted for publication). It remains to be seen whether the observed aberrant processing of profilaggrin contributes to the phenotype of $Lor^{-/-}$ mice.

Discussion

Delayed Development of Skin Barrier Function in Embryonic $Lor^{-/-}$ Mice

Loricrin expression is first detected at day E16 in mouse embryonic development. The protein initially accumulates in L-granules (Bickenbach et al., 1995) where it is subsequently dispersed and incorporated into the nascent CE. At this stage, a rudimentary barrier function is established. By staining methanol-extracted E16.5 embryos with a histological dye, a process that presumably extracts polar lipids from the epidermis, unstained patches of skin become apparent in normal mouse embryos, indicating the initiation of barrier formation (Hardman et al., 1998). It has been shown that deposition of loricrin into the CE correlates with onset of dye impermeability (Hardman et al., 1998). At E16.5, $Lor^{-/-}$ embryos showed a different staining pattern than control embryos, indicating impaired skin barrier function. Their back skin was either completely stained or

showed only small patches of epidermis that were impermeable to the dye. In contrast, Lor^{+/-} and Lor^{+/+} embryos showed large unstained areas on their backs (Fig. 3), and in most cases, the entire back was unstained. However, E17.5 mutant and control back skins were equally impermeable to the dye. Thus, the development of skin barrier function was delayed, but not abolished, in Lor^{-/-} mutant embryos. Coincident with establishment of the barrier function in Lor^{-/-} embryos, we observed increased expression of certain CE components in utero (see below).

Lor^{-/-} Epidermis Is More Prone To Desquamation but Not To TEWL

Although the mutant epidermis eventually became impermeable to the dye, it was not completely normal. The skin of Lor^{-/-} mice was red and shiny at birth, and the mutants weighed less than +/- and +/+ littermates. Interestingly, E18.5 embryos derived by cesarean section did not show gross skin abnormalities (data not shown), indicating that the skin phenotype developed shortly after birth, most likely within minutes.

Based on the gross appearance of newborn Lor^{-/-} mice, which was very similar to another transgenic mouse model we characterized that exhibited dehydration due to a defect in epidermal barrier function (Imakado et al., 1995), we expected to see an increase in TEWL. However, at the time of measurement (~1 h after birth), we were not able to measure significant differences in TEWL in Lor^{-/-} neonates.

The physiological basis for the red and shiny skin remains unclear. Staining tissue sections of Lor^{-/-} and control mice with antibodies against endothelial markers did not reveal an increase in the number or size of blood vessels in Lor^{-/-} mice (data not shown). Nevertheless, it is tempting to speculate that changes in the molecular composition of mutant CE might affect the optical properties of the skin.

A significant difference between Lor^{-/-} and control epidermis was revealed by tape stripping experiments. Mutant mice lost significantly more layers of back skin stratum corneum cells than control mice.

Lor^{-/-} Cell Envelopes Show Enhanced Susceptibility to Mechanical Stress

The above observation suggested that the loss of loricrin affects the biomechanical properties of the stratum corneum, making it more susceptible to physical stress. This hypothesis is supported by our sonication experiments, which showed that purified Lor^{-/-} CEs were reduced to small fragments under conditions in which wild-type CEs remained largely intact (Table I). Although CEs isolated from Lor^{-/-} mice had less regular shapes than wild-type CEs (Fig. 5), their thickness, as determined by electron microscopy, was essentially normal, and was measured to be ~15 nm (a detailed analysis is reported in Jarnik, M., P.A. de Viragh, D. Bundman, M. Simon, D.R. Roop, and A.C. Stevens, manuscript submitted for publication). Since the procedure used to isolate CEs is harsh, it may be that the altered overall shapes of Lor^{-/-} CEs reflects their enhanced susceptibility to mechanical stress (in situ, the CE of an individual corneocyte should be a continuous closed bag that is ruptured and perhaps fragmented during isolation).

Upregulation of SPRRP2 and Repetin in Lor^{-/-} Epidermis

From the conserved thickness of the mutant CE, we suspected that another CE component(s) substitutes for loricrin in Lor^{-/-} mice. Of several known CE components examined, increased transcript levels were detected only for SPRRP2D, SPRRP2H, and repetin. SPRRPs mainly serve as loricrin cross-linkers in orthokeratinizing epithelia. Interestingly, although the total amount of loricrin and SPRRP proteins is approximately constant at 80–85% of CE protein mass, their relative molar ratio varies markedly between different epithelia and even between different body sites (Jarnik et al., 1996; Steinert et al., 1998a). The loricrin/SPRRP ratio is >100:1 in the newborn epidermis, 4.5–7.5:1 in the footpad, and 3:1 in the forestomach (Steinert et al., 1998a). In general, tissues that have to withstand higher mechanical stress seem to have more SPRRP protein. Although the elevated levels of SPRRP2 in Lor^{-/-} mice would imply a more robust CE, their effect appears to be offset by other components of the mutant cell envelope.

Increased expression in Lor^{-/-} mice was also observed for repetin, a recently identified member of the “fused gene” subgroup of S100 proteins (Krieg et al., 1997). Repetin is expressed in terminally differentiated keratinocytes of mouse epidermis, tongue, and forestomach (Krieg et al., 1997). This protein has a high content of glutamine residues (20.2%) that might serve as a substrate for TGases. Repetin also shares sequence homologies in its COOH terminus with the corresponding domains of loricrin, involucrin, and SPRRP proteins (Krieg et al., 1997). These properties, together with the observation that repetin accumulates at the periphery of corneocytes in Lor^{-/-} mice (Fig. 8 b), suggests that repetin is a component of the CE.

Analysis of the amino acid composition of Lor^{-/-} CEs from newborn back skin indicated an approximately threefold increase in total SPRRP proteins compared with wild-type (Jarnik, M., P.A. de Viragh, D. Bundman, M. Simon, D.R. Roop, and A.C. Stevens, manuscript submitted for publication), which is consistent with the observed increase in SPRRP2 transcripts (this study). According to this analysis, repetin is unlikely to be a major component of either mutant or wild-type CEs, because it is detected at levels less than the estimated detection threshold of a few percent. This method was not sensitive enough to detect the increased repetin content of the CE implied by its elevated transcript level. More generally, mutant CEs have high contents of Gly and Ser, like wild-type CEs, in which these amino acids are contributed mainly by loricrin. The high Gly/Ser contents of the Lor^{-/-} mutant may be contributed in part by keratins 1 and 10, whose end domains are rich in these residues, but other components are likely to be involved (Jarnik, M., P.A. de Viragh, D. Bundman, M. Simon, D.R. Roop, and A.C. Stevens, manuscript submitted for publication). Moreover, our Western blot analysis and RPA of Lor^{-/-} epidermis did not indicate increased expression of the keratins. Further studies will be necessary to determine if a novel loricrin-like protein is indeed integrated into the CE of loricrin-deficient mice.

The expression of other CE components, such as involucrin, scellin, filaggrin, trichohylin, and K10, were similar in Lor^{-/-} and control animals. However, in the case of profil-

aggrin, we noted an additional band in Western blots of total epidermal lysates, indicating abnormal processing of the precursor protein. Both immunofluorescence and immunoelectron microscopy of *Lor*^{-/-} epidermis showed a normal distribution of profilaggrin and F-granules (data not shown). Therefore, it is unclear whether the abnormal processing of profilaggrin contributes to the skin phenotype of *Lor*^{-/-} mice.

Loricrin Mutations in Vohwinkel's Syndrome and Progressive Symmetric Erythrokeratoderma

Although loricrin-null mutations have not been reported in a human skin disease, a subset of patients with Vohwinkel's syndrome (VS) (Maestrini et al., 1996; Korge et al., 1997; Armstrong et al., 1998; Ishida-Yamamoto et al., 1998; Takahashi et al., 1999) and one pedigree with "progressive symmetric erythrokeratoderma" (Ishida-Yamamoto et al., 1997) were shown to have mutations in the loricrin gene. These diseases are rare autosomal dominant disorders that are clinically characterized by diffuse palmoplantar keratoderma (thickening of the stratum corneum of palms and soles) with a honeycomb appearance and the development of constricting bands around digits that can lead to auto-amputation. In pedigrees analyzed at the molecular level, mutations in gene sequences coding for the COOH terminus of loricrin have been found (Maestrini et al., 1996; Ishida-Yamamoto et al., 1997; Korge et al., 1997; Armstrong et al., 1998; Takahashi et al., 1999). All of the mutations reported to date are single base pair insertions that lead to the synthesis of an aberrant protein in which COOH-terminal amino acid sequences are replaced by missense amino acids. These mutations also eliminate several glutamine and lysine residues that normally form isopeptide bonds (compare mutations reported in Maestrini et al., 1996; Ishida-Yamamoto et al., 1997; Korge et al., 1997; Armstrong et al., 1998; Takahashi et al., 1999; with cross-linking sites in Candi et al., 1995; Steinert et al., 1998a).

We have recently generated a transgenic mouse model for this disease that reproduces most of the characteristics of VS (Suga et al., 2000). The severe phenotype resulting from expression of the loricrin-VS mutant, as compared with the mild *Lor*^{-/-} phenotype, suggests that it is not functioning in a classic dominant negative fashion; it does not produce a functional knockout of loricrin. To test this prediction, we mated the loricrin-VS mutation into the *Lor*^{-/-} background. The severe phenotype resulting from this cross confirmed that the VS mutation was interfering with late stages of epidermal differentiation in a more global manner and did not require a direct interaction with wild-type loricrin (Suga et al., 2000).

Implications of the Mild Transient Phenotype Resulting from Loss of Loricrin Expression

In summary, we have shown that in newborn mice, the *Lor*^{-/-} phenotype includes erythroderma and a shiny, translucent skin. The absence of a more severe phenotype is, at least in part, due to a compensatory increase in the expression of SPRRPs and repetin. Although CEs of mutant mice show diminished stability when subjected to extreme mechanical stress *in vitro*, we did not notice skin abnormalities in adult mice. This might be due to the fact

that the dense coat of mice provides protection against mechanical stress, since the CE isolated from adult *Lor*^{-/-} mice exhibited the same susceptibility to sonication as newborn *Lor*^{-/-} CE. This leaves one to speculate about the potential phenotypic consequences of loricrin deficiency in man. Patients born with a self-healing skin disorder similar to lamellar ichthyosis have been reported (Frenk and de Techtermann, 1992), however preliminary screening of a few of these patients did not reveal a deficiency in loricrin (Hohl, D., and P. de Viragh, unpublished data). The longer gestation time and the earlier onset of epidermal differentiation in human embryonic development may result in a subclinical presentation of loricrin deficiency at birth. If human *Lor*^{-/-} CE exhibit the same brittle characteristics as mouse *Lor*^{-/-} CE, would the absence of a dense coat of hair make these individuals more susceptible to harsh environmental conditions such as moisture and cold? Alternatively, are the requirements for fully functional CE in the maintenance of epidermal barrier function so critical that this need was met with the evolution of a series of replacement components that almost completely compensate for each other?

In this respect, it is interesting to note that mice deficient for involucrin do not develop an obvious phenotype (Djian et al., 2000). Regardless of the answers, an understanding of the molecular mechanisms that allow an embryo to compensate for a defect in epidermal barrier function *in utero* could have practical implications for the treatment of premature infants in the future. The *Lor*^{-/-} mouse provides us with an excellent model to discover these mechanisms.

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