Harlequin ichthyosis model mouse reveals alveolar collapse and severe fetal skin barrier defects

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Harlequin ichthyosis (HI), which is the most severe genodermatosis, is caused by loss-of-function mutations in ABCA12, a member of the ATP-binding cassette transporter family. To investigate the pathomechanism of HI and the function of the ABCA12 protein, we generated ABCA12-deficient mice (Abca12−/−) by targeting Abca12. Abca12−/− mice closely reproduce the human HI phenotype, showing marked hyperkeratosis with eclabium and skin fissure. Lamellar granule abnormalities and defective ceramide distribution were remarkable in the epidermis. Skin permeability assay of Abca12−/− fetuses revealed severe skin barrier dysfunction after the initiation of keratinization. Surprisingly, the Abca12−/− mice also demonstrated lung alveolar collapse immediately after birth. Lamellar bodies in alveolar type II cells of the Abca12−/− mice lacked normal lamellar structures. The level of surfactant protein B, an essential component of alveolar surfactant, was reduced in the Abca12−/− mice. Fetal therapeutic trials with systemic administration of retinoid or dexamethasone, which are effective for HI and respiratory distress, respectively, to the pregnant mother mice neither improved the skin phenotype nor extended the survival period. Our HI model mice reproduce the human HI skin phenotype soon after the initiation of fetal skin keratinization and provide evidence that ABCA12 plays pivotal roles in lung and skin barrier functions.

INTRODUCTION

Harlequin ichthyosis (HI) (OMIM 242500) is the most severe ichthyosis, and its clinical features at birth include severe ectropion, eclabium, flattening of the ears and large thick plate-like scales over the entire body. Infants affected with HI frequently die within the first days of life. In 2005, we and other independent research groups identified mutations in the ATP-binding cassette transporter A12 (ABCA12) gene as the cause of HI (1,2). We previously demonstrated that a severe ABCA12 deficiency causes defective lipid transport within lamellar granules (LGs) in the granular layer keratinocytes, resulting in malformation of intercellular lipid layers in the stratum corneum and severe thickening of the stratum corneum of the HI phenotype. We also reported that LG lipid secretion could be recovered in cultured keratinocytes by corrective gene transfer of functional ABCA12 (1).

Formation of the intercellular lipid layer is essential for proper epidermal barrier function. Defective formation of the lipid layers is thought to result in the loss of such function and abnormal hyperkeratosis (3). Defective formation of the intercellular lipid layers in the stratum corneum due to abnormal keratinocyte lipid metabolism, transport and/or secretion is the major pathogenic mechanism in congenital ichthyoses (3). ABCA12 belongs to a large superfamily of ATP-binding cassette (ABC) transporters that aid in the transport of various biomolecules across the cell membrane (4–6). The ABCA subfamily consists of 12 full transporters and one pseudogene (ABCA11), which are essential for lipid transport and secretion (7). Several genetic diseases are caused by mutations in ABCA subfamily genes. ABCA3, a molecule that is close to ABCA12 in the phylogenic tree of ABCA subfamily proteins (8), aids lipid secretion from alveolar type II cells via lamellar bodies (9). The ABCA3 deficiency underlies a fatal surfactant deficiency and associated lung collapse in newborns (10). Recently, several groups have reported on ABCA3-deficient mice (11–14). These mice showed neonatal lethality due to respiratory failure and that ABCA3 is necessary for lamellar body biogenesis and proper surfactant protein processing.

†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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In the present study, to clarify the function of ABCA12 and investigate the pathomechanisms of the most severe ichthyosis, HI, we generated ABCA12-deficient mice by homologous recombination.

RESULTS

Generation of mice with targeted disruption of Abca12

To inactivate Abca12, we constructed a targeting vector to delete exon 30 of the Abca12 gene by homologous recombination, replacing that exon with a neomycin-resistance cassette (Fig. 1A). We intercrossed the heterozygous mice, which were phenotypically indistinguishable from their wild-type (WT) littermates, to generate Abca12−/− mice. PCR analysis confirmed correct targeting of Abca12 (Fig. 1B). Reverse transcription–PCR (RT–PCR) and western blot analysis using rabbit anti-mouse Abca12 antibody showed that expression of Abca12 mRNA and protein was abolished in the Abca12−/− mice (Fig. 1C and D). The anti-mouse Abca12 antibody used in the present study recognizes a 14-amino acid sequence in the C-terminal region of mouse Abca12. This antibody is unable to detect the truncated Abca12 proteins lacking the C-terminal region. Thus, we could not detect a protein with different size in the western blotting.

The Abca12−/− mice were born with smaller bodies than the WT mice (WT neonate 1.34 ± 0.17 g, Abca12−/− neonate 0.91 ± 0.27 g; mean ± 2SD, n = 5) and erythematous skin covered the entire body of the Abca12−/− mice, making their skin less flexible than that of the WT mice (Fig. 2A). The entire skin surface was covered with thick scales and some of the Abca12−/− mice developed skin fissures and eversions of the lips (eclabium) (Fig. 2B, D and F), which are characteristic of HI in humans (Fig. 2C, E and G). The Abca12−/− mice died within a few hours, with none surviving the postnatal period (n = 20). We examined the development of fetal skin phenotype of the Abca12−/−. There was no significant difference between Abca12−/− and WT fetuses until E15.5, before the skin keratinization started. At E18.5, Abca12−/− fetuses developed taut and shiny skin without normal skin folds and showed contractures of the limbs (Fig. 2A). At E18.5, the size of Abca12−/− fetuses seemed to be smaller than WT littermates (E18.5 WT fetus 1.07 ± 0.14 g, E18.5 Abca12−/− fetus 0.96 ± 0.14 g; mean ± 2SD, n = 5).

Morphological observation of the skin showed that the Abca12−/− mice exhibited features similar to those of human HI patients. Light microscopy showed strikingly thick, compact stratum corneum without the normal basket-weave appearance (Fig. 3A). Electron microscopy demonstrated numerous defective LGs and lipid droplets in the upper epidermis (Fig. 3B). By immunostaining with goat anti-ABCA12 antibody, Abca12 was detected mainly in the granular layer of the WT skin; the skin of the Abca12−/− mice lacked ABCA12 (Fig. 3C). Immunofluorescent staining showed that ceramide, a major lipid component of LGs (15,16) and an essential component of the epidermal permeability barrier (17), was sparsely distributed in the Abca12−/− mice stratum corneum, in marked contrast to the restricted, intense distribution in the stratum corneum of the WT skin (Fig. 3D). Glucosyleramidase immunocytochemistry showed congested patterns in differentiated keratinocytes of the Abca12−/− mice, in contrast to the peripheral patterns that were shown in the differentiated keratinocytes of the WT mice, when both cell types were cultured under high (1.2 mM) Ca2+ conditions (Fig. 3E).

Skin barrier function of the Abca12−/− mice

We first examined the development and patterning of the skin barrier in Abca12−/− mice using an in situ skin permeability assay. In WT E15.5 fetuses, the entire skin stained with Toluidine blue. In fetuses after E18.5 and in neonates, the skin did not stain. In contrast, the skin of the Abca12−/− fetuses from E15.5 to E18.5, and even neonates, stained completely blue (Fig. 4A).

The development of the skin barrier in the late gestational period was also assessed by measuring trans-epidermal water loss (TEWL) (Fig. 4B). No significant difference in TEWL was found between the WT and Abca12−/− E15.5 fetuses.
However, the Abca12<sup>−/−</sup> E18.5 fetuses showed much higher levels of TEWL than the WT E18.5 fetuses.

To confirm the ‘inward out’ defect in the skin in the Abca12<sup>−/−</sup> mice, we evaluated the rate of fluid loss through evaporation in the Abca12<sup>−/−</sup> and WT mice by monitoring body weight as a function of time. The Abca12<sup>−/−</sup> E15.5, E18.5 fetuses and neonates lost 14.5, 14 and 13.5% of their body weight in 3 h, respectively (Fig. 4C). This weight loss was attributed to trans-epidermal fluid evaporation due to compromised skin barrier function in fetuses and neonates, in the absence of either feeding or urinating during the evaluation period.

To evaluate the correlation between developing skin barrier function and epidermal structural changes, we performed light microscopy on the E15.5 and E18.5 fetuses and electron microscopy on the E18.5 fetuses. There was no significant difference between unkeratinized Abca12<sup>−/−</sup> and WT mice skin at E15.5; however, at E18.5, Abca12<sup>−/−</sup> fetuses started to show hyperkeratosis by light microscopy and cytoplasmic lipid droplets in the upper epidermis including the stratum corneum by electron microscopy (Fig. 4D and E). Thus, keratinization was disturbed and the development of the skin barrier was clearly defective even during fetal development in Abca12<sup>−/−</sup> mice.

**Alveolar surfactant deficiency in the Abca12<sup>−/−</sup> lungs**

We confirmed Abca12 mRNA expression in WT lung tissue through RT–PCR (Fig. 5A). Histological examination of the Abca12<sup>−/−</sup> mice lungs revealed alveolar collapse and pulmonary congestion. The Abca12<sup>−/−</sup> mice lungs had thicker interalveolar septa and smaller alveolar spaces than the WT mice (Fig. 5B and C). Ultrastructural analysis of alveolar type II cells in Abca12<sup>−/−</sup> mice showed that lamellar body like organelles in Abca12<sup>−/−</sup> mice lungs lacked the typically clear lamellar structure compared with that of WT mice (Fig. 5D and E).

We performed double staining with goat anti-ABCA12 antibody and rabbit anti-surfactant protein-B (SP-B) antibody (Fig. 6A). In the neonatal lung cryosections, Abca12 was expressed in the lung epithelial cell cytosol of the WT mice but not in the lungs of the Abca12<sup>−/−</sup> mice. By immunostaining and western blotting, SP-B expression was found to be lower in the Abca12<sup>−/−</sup> mice than in the WT mice (Fig. 6B). No significant differences in mRNA levels of SP-B, ABCA3 and GAPDH were found between the WT and Abca12<sup>−/−</sup> lungs (Fig. 6C). These data suggested that Abca12 protein is involved in lipid metabolism of certain pulmonary components including surfactant and plays a significant role in surfactant biogenesis.

**Fetal therapy with oral or intrauterine administration of retinoid or subcutaneous administration of corticosteroid to pregnant mice**

Protocol 1: Systemic administration of etretinate. Oral administration of any dose of etretinate (1, 10 or 100 mg/kg daily, 5 consecutive days) or intrauterine administration (intra-amniotic...
fluid administration) of etretinate (one 1 mg shot) to the pregnant mice did not change the skin phenotype at birth in any way. All the Abca12⁻/⁻ newborns who had received the fetal therapy died within a few hours after birth. Congenital abnormalities (limb defects and anencephaly) were observed in several of the Abca12⁺/⁺ or Abca12⁺/⁻ littermates who had received fetal therapy of oral etretinate (100 mg/kg daily, 5 consecutive days) via the pregnant mother mice.

**DISCUSSION**

Previously, we identified mutations in ABCA12 gene as the cause of HI and demonstrated that severe ABCA12 deficiency causes defective lipid transport of LGs in the upper epidermal keratinocytes, resulting in the abnormal thickening of the stratum corneum and HI phenotype (1). Subsequently, our group showed that ABCA12 is highly expressed in the fetal skin, and we developed an HI skin model in immunodeficient mice (18). More recently, we demonstrated that ABCA12 protein localizes from Golgi apparatuses to LGs with glucosylceramide (19). Furthermore, we conducted DNA-based prenatal diagnosis and exclusion from identification of the gene whose abnormality causes HI, ABCA12 (20,21). Based on this research, to clarify the function of ABCA12 and investigate the pathomechanisms of the most severe ichthyosis, HI, we generated ABCA12-deficient mice by homologous recombination.

Here we have succeeded in generating Abca12⁻/⁻ mice as a human disease model of HI. The model mice showed severe skin barrier defect and skin histopathology similar to that of HI patients. Interestingly, the lungs of the present model mice showed signs of alveolar collapse. Western blotting and immunostaining of the Abca12⁻/⁻ mice lung showed the SP-B expression to be decreased. These findings suggest that ABCA12 is involved in not only lipid transport in the epidermis, but also in surfactant biogenesis and/or transport in the lung.

The Abca12⁻/⁻ mice were born with smaller bodies and with erythematous, less flexible skin covering the entire body. The entire skin surface was covered with thick scales, and

**Figure 3.** Morphological observations of the skin show that the Abca12⁻/⁻ mice have similar features to those in HI. (A) Light microscopy histology showed strikingly thick, compact stratum corneum without the normal basket-weave appearance in the Abca12⁻/⁻ mouse skin (right), compared with normal WT neonatal skin (left). (B) Electron micrograph of Abca12⁻/⁻ skin (right column) and WT neonatal skin (left column). There were numerous lipid droplets in the upper epidermis (top and middle) and defective lamellar granules (arrows) (bottom). In the WT skin, no lipid droplets are seen (top and middle), although intact intercellular lipid layers (middle, arrows) and normal lamellar granules (bottom, arrowheads) were observed. (C) By immunofluorescence staining, the Abca12 protein (FITC, green) was detected in the granular layer of the WT mice skin (left) but not in the Abca12⁻/⁻ mice skin (right). (D) Immunofluorescence staining shows the ceramide (FITC, green), a major lipid component of lamellar granules and an essential component of the epidermal permeability barrier, to be distributed remarkably sparse in the Abca12⁻/⁻ mice stratum corneum (right). This contrasts with the intense restricted distribution in the stratum corneum of the WT skin (left) (dotted lines are the skin surface.). (E) Glucosylceramide immunocytochemistry demonstrated congested patterns of glucosylceramide (Alexa488, green) in differentiated Abca12⁻/⁻ mice keratinocytes (right), in contrast with peripheral patterns of glucosylceramide distribution in the differentiated keratinocytes of the WT mice (left) (nuclear stain; Propidium iodide, red).
Figure 4. Skin barrier defect in the Abca12−/− mice. (A) In situ dye permeability assay. The entire skin of the WT E15.5 fetus is permeable to Toluidine blue. At E18.5 and thereafter, the WT skin is resistant to the stain (lower). In contrast, the skin of the Abca12−/− fetus and neonates is totally permeable to the dye throughout their development (upper). (B) TEWL assay. There is no significant difference in TEWL between the WT (blue) and Abca12−/− E15.5 fetuses (red). However, the Abca12−/− E18.5 fetuses (red) show significantly greater TEWL than the WT fetuses (blue) show. The embryonic development of the skin barrier is defective in the Abca12−/− mice. (P, 0.01) (C) Dehydration assay. Loss of fluid at room temperature from the E15.5 fetuses, E18.5 fetuses and neonates of the Abca12−/− and WT mice (removed before feeding) was recorded by monitoring body weight loss. No significant difference is found in percent weight loss between WT (blue) and Abca12−/− (red) E15.5 fetus. The Abca12−/− E18.5 fetuses and neonates (blue) show significantly greater weight loss than that in the WT E18.5 fetuses and neonates (red). (P < 0.01) The data are expressed as percentages of initial body weights. (D) Histopathological features of fetuses. There is no significant difference between Abca12−/− fetal skin (top, right) and WT fetal skin (top, left) at E15.5. After keratinization at E18.5, Abca12−/− fetuses showed marked hyperkeratosis (bottom, right) compared with normal keratinization of E18.5 WT fetus (bottom, left). (E) Ultrastructural features of the Abca12−/− E18.5 fetus. Cytoplasmic lipid droplets in the upper epidermis including the stratum corneum are seen by electron microscopy in the Abca12−/− E18.5 fetus (right), but not in the WT E18.5 fetus (left).

Figure 5. The Abca12−/− mice show alveolar collapse in the lung. (A) RT semi-nested PCR of lung tissue homogenates shows Abca12 mRNA to exist only in the lung of the WT mice (left) and not in that of the Abca12−/− mice (right). (B and C) Histological examination of the lung from the Abca12−/− mice (right column) reveals collapsed alveoli and pulmonary congestion. The Abca12−/− mice lung has thicker interalveolar septa and smaller alveolar spaces than the WT mice lung has (left column) (B: hematoxylin–eosin stain, C: Elastica-Masson stain). (D) Electron microscopy reveals collapse of an alveoli in Abca12−/− lung (right), but a normal alveoli in the WT lung (left). (E) Ultrastructural analysis of alveolar type II cells showed that lamellar body-like organelles (asterisks) in Abca12−/− mice lung (right) lacked lamellar structure compared with those of WT mice (left, arrows).
of human HI. From analysis of the skin and keratinocytes, we conclude that lamellar-body-like organelles lacked a normal lamellar structure in Abca12−/− mice compared with that of WT mice.

Interestingly, the Abca12−/− mice in the present study showed that ABCA12 plays a crucial role in the lungs. High-dose systemic retinoid treatment is applied to the neonates recently and effective in many HI cases. However, the mortality rate of HI is high even now and more than half of the affected neonates die within a few weeks of life. Until now, the cause of HI death has not been elucidated completely, and it has been thought that respiratory distress, bacterial infection, feeding problems and dehydration are associated with HI death (22–24).

ABCA3, a molecule closely related to ABCA12 (8), aids lipid secretion from alveolar type II cells via lamellar bodies (9). ABCA3 deficiency underlies a fatal surfactant deficiency in newborns (10), and ABCA3-deficient mice show neonatal lethality from respiratory failure (11–14). Apart from the skin, it has been reported that ABCA12 is expressed in the lungs, testis and placenta (25); however, the precise function of ABCA12 in the lungs had not been investigated in detail. In the present study, we investigated the effect of Abca12 disruption in our Abca12−/− mice lungs. Histological examination of lungs from the Abca12−/− mice revealed alveolar collapse and pulmonary congestion. Double staining with anti-ABCA12 antibody and anti-SP-B antibody revealed that Abca12 is co-expressed with SP-B in the cytoplasm of type II alveolar epithelial cells in WT mice. Immunostaining and western blotting revealed SP-B expression to be lower in the Abca12−/− mice than in the WT mice. Ultrastructural analysis of alveolar type II cells in the Abca12−/− mice showed that lamellar-body-like organelles lacked a normal lamellar structure in Abca12−/− mice compared with that of WT mice.

It is well known that expression of surfactant proteins in the peripheral lung normally increases just prior to birth (26). In particular, hydrophobic SP-B plays crucial roles in surfactant function and homeostasis; the proteins enhance the spreading, absorption and stability of required surfactant lipid (27). We were unable to clarify the precise details of ABCA12’s role in lipid transport in the lung tissue, but the present data suggest that the ABCA12 protein is involved in the metabolism or transport of lipid components in pulmonary surfactant and that this protein plays a role in surfactant biogenesis and/or secretion. Our results support the hypothesis that respiratory distress may contribute to the observed high mortality rate in HI neonates to a significant extent.

The development of treatments for this condition is crucial to improve patient survival, and the high-dose systemic retinoid therapy that has been applied to HI newborns in neonatal intensive care unit has produced several long time survivors (28–31). Based on these clinical reports, we conducted systemic etretinate administration to the pregnant mice to clarify the efficacy of retinoid therapy as an HI treatment. We gave high-dose retinoid to the pregnant mother mice orally and directly into the uterus (amniotic fluid) by intrauterine (intra-amniotic fluid) injection. Unfortunately, neither improvement of the skin manifestations nor extension of the survival period was obtained by systemic retinoid administration. We do not know why the...
systemic retinoid had no effect, but from the results of our retinoid fetal therapy, we conjecture that retinoid therapy is effective against hyperkeratosis only after birth and not against the development of skin lesions during the fetal period. Systemic steroids have been used to treat respiratory distress syndrome. Steroids have been shown, in vitro and in vivo, to enhance fetal lung maturation including differentiation of alveolar type II cells and production of both lipid and protein components of surfactant. Prenatal steroid treatment decreases the incidence of respiratory distress syndrome in premature infants by promoting the maturation of fetal lungs, including the surfactant system (32). That is why we conducted the systemic steroid therapy on the pregnant mice by subcutaneous injection. However, we obtained positive effects on neither the skin symptoms nor the lung manifestations. Probably, a combination of alveolar surfactant compensation therapy and intra-amniotic gene therapy might be the next logical treatment approach.

In conclusion, our findings suggest that ABCA12 is involved not only in lipid transport and assembly of a proper skin barrier, but also in lung surfactant biogenesis. Both these tissues heavily depend on ABCA12 function and are essential for survival of organisms in a dry environment. Our novel findings together demonstrate that ABCA12 is a crucially important protein for life in dry environment.

MATERIALS AND METHODS

Animals

All the studies involving animals were reviewed and approved by the Animal Use and Care Committee of the Hokkaido University Graduate School of Medicine. All animals used for this study were maintained under pathogen-free conditions.

Generation of Abca12−/− mice

We cloned mouse genomic DNA Abca12 fragments from the mouse 129Sv/Ev genomic library (Bacpac Resources Center, Children’s Hospital Oakland Research Institute, Oakland, CA, USA). We subcloned a 10.6 kb fragment to make the targeting vector. We inserted the PGK/Neo cassette between 47 bp upstream of the exon 30 and 203 bp downstream of exon 30. We transfected the targeting vector by electroporation into 129Sv/Ev embryonic stem cells, then microinjected the correctly targeted embryonic stem cell line into blastocysts obtained from C57BL/6J mice (Jackson Laboratories) to generate chimeric mice, which we then mated with C57BL/6J females. We crossed the heterozygotes with C57BL/6J over at least five generations, and then intercrossed them to generate the Abca12−/− mice.

PCR screening of the Abca12−/− mice

Genotypes of fetuses and neonates were determined by PCR analysis of tail tissue DNA using the following primers: P1 (5′-CACCTCAGTGACATCTA-3′), P2 (5′-TCGAGTTATGTCAGTTGCTGAA-3′), and P4 (5′-TGCCAGGGCCAGGGCGTATTTGC-3′). Primer sets P1–P2 and P3–P4 correspond to WT allele 310 bp and knockout allele 2.2 kb, respectively. As controls for these experiments, we used Abca12+/− or Abca12+/+ of the Abca12−/− mice.

Extraction of total RNA and RT–PCR

We isolated total RNA from skin and lung tissue of the WT and Abca12−/− mice pups using the QuickGene RNA Tissue Kit SII (Fujifilm Corp.). We reverse-transcribed RNA using superscript II following the manufacturer’s instructions (Invitrogen Corp.). Specific Abca12 primers for cDNA to PCR amplification were as follows: forward 5′-CATCGTGGCTCAGGGTAG-3′ and reverse 5′-CTTGTGAAGTGGAGGTGGTAAC-3′. These primers were used for amplification of a 256 bp fragment around exons 30–31 boundary of normal cDNA. For semi-nested RT–PCR of lung mRNA, forward primer was changed to 5′-TGATTTGGAACATCACCAC-3′. Specific primers of Abca3 cDNA for PCR amplification were as follows: forward 5′-AAGGTCTAGTGACGTCTG-3′ and reverse 5′-GAGGAGTGTGGTCTACAGGC-3′. Specific primers of SP-B cDNA to PCR amplification were as follows: forward 5′-CTTAGCAACAGCTCCTCCCCATT-3′ and reverse 5′-TGCCATAGCCTGTCATGCG-3′. As a control, we amplified GAPDH cDNA as a housekeeping gene.

Western blotting

For western blotting, we used lung tissue protein homogenates and primary cultured keratinocyte proteins prepared from a lysis buffer of 50 mM Tris–HCl, pH7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS and Roche protease cocktail 1 tablet. The lysates were separated by 5–10% gradient gel SDS–PAGE and transferred to polyvinylidene difluoride membranes. Membrane blocking and incubation with antibodies were carried out in Tris-buffered saline with 2% skim milk. Signals were revealed with chemiluminescence reagents.

Antibodies

Rabbit polyclonal affinity purified anti-mouse Abca12 antibody was raised in rabbits using a 14-amino acid sequence synthetic peptide (residues 2581–2594) derived from the mouse Abca12 sequence (XM001002308) as the immunogen. The other primary antibodies were mouse monoclonal anti-ceramide antibody (Alexis Biochemicals), rabbit anti-glucosylceramide antibody (GlycobioTech), rabbit anti-mouse surfactant protein-B antibody (SP-B, AB3780; Chemicon), mouse monoclonal anti-beta actin antibody (Sigma) and goat anti-ABCA12 antibody (N-20; Santa Cruz Biotechnology). We used rabbit anti-mouse Abca12 antibody for western blotting and goat anti-ABCA12 antibody for immunostaining. As the secondary antibodies, Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Molecular Probes), TRITC-conjugated goat anti-rabbit IgG (Southern Biotech), FITC conjugated donkey anti-goat IgG (Jackson Immuno Research), horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse F(ab′)2 (Biosource) were used.
Establishment of Abca12−/− mice keratinocyte culture
Skin samples from WT and Abca12−/− mice were processed for primary keratinocyte culture, and cells were grown according to standard procedures in CnT-57 medium (Cellntec Advanced Cell Systems). For differentiation, cultures were grown for several passages in CnT-57 medium and then switched to CnT-02 medium (Cellntec Advanced Cell Systems), and 24 h later, the calcium concentration was changed to 1.2 mM. Forty-eight hours later, we performed immunocytochemistry or protein extraction.

Light microscopy and immunofluorescence analysis
We killed the newborn pups, fixed them for 24 h in 10% neutral buffered formalin, dehydrated them for 24 h in 70% ethanol and embedded them in paraffin. We cut 4 μm sagittal sections and stained them with hematoxylin–eosin and Elastica–Masson. Immunofluorescent labeling was performed on frozen tissue sections and keratinocyte cultures as previously described (33). Fluorescent labeling was performed with secondary antibodies, followed by 10 μg/ml of propidium iodide (Sigma) to counter-stain the nuclei. The stained samples were observed under a confocal laser-scanning microscope.

Electron microscopy
Skin and lung samples were fixed in 5% glutaraldehyde solution, post-fixed in 1% OsO4, dehydrated and embedded in Epon 812. The samples were sectioned for electron microscopy. The thin sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy.

Skin permeability assay
In situ skin permeability assay using Toluidine blue was performed as described by Hardman et al. (34). Briefly, we subjected fatal and neonatal mice to methanol dehydration and subsequent rehydration as described. We then washed them in phosphate-buffered saline (PBS) for 1 min, stained them for 5 min at room temperature in 0.1% Toluidine blue. They were briefly washed in PBS for 15 min in PBS at room temperature and immediately photographed.

TEWL was measured on live mouse pups by evaporimeter (AS-VT100RS: Asahibiomed Corp.). The AS-VT100RS utilizes the ventilated-chamber method of measuring TEWL. Its hygrometer measures the humidity of incoming air and of outgoing air that has passed over the test area of skin, and TEWL is calculated from the difference. TEWL measurements were done on the back of the embryos and neonates.

Therapeutic trials for the fetuses
Protocol 1: systemic administration of etretinate. We dissolved several doses of etretinate (a sample drug from Chugai Pharmaceuticals, Tokyo, Japan) in water, and single oral doses (1, 10, 100 mg/kg) of etretinate were administered to the pregnant mice every day from E15 to delivery. We also tried 1 μg etretinate injection to the intra-amniotic fluid of every yolk sac at E15.

Protocol 2: systemic administration of corticosteroid. We dissolved the several doses of dexamethasone (Sigma) in the water, and single doses (0.1 mg or 1 mg) of dexamethasone were administrated by subcutaneous injection to the pregnant mice every day from E15 to delivery.

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Conflict of Interest statement. None declared.

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