The roles of ABCA12 in keratinocyte differentiation and lipid barrier formation in the epidermis

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ABCA12 is a member of the large superfamily of ATP-binding cassette (ABC) transporters, which bind and hydrolyze ATP to transport various molecules across limiting membranes or into vesicles. The ABCA subfamily members are thought to be lipid transporters. ABCA12 is a keratinocyte transmembrane lipid transporter protein associated with the transport of lipids in lamellar granules to the apical surface of granular layer keratinocytes. Extracellular lipids, including ceramide, are thought to be essential for skin barrier function. ABCA12 mutations are known to underlie the three main types of autosomal recessive congenital ichthyoses: harlequin ichthyosis, lamellar ichthyosis and congenital ichthyosiform erythroderma. ABCA12 mutations lead to defective lipid transport via lamellar granules in the keratinocytes, resulting in malformation of the epidermal lipid barrier and ichthyosis phenotypes. Studies of ABCA12-deficient model mice indicate that lipid transport by ABCA12 is also indispensable for intact differentiation of keratinocytes.

Introduction

ABCA12 is a member of the large superfamily of ATP-binding cassette (ABC) transporters,[1] which bind and hydrolyze ATP to transport various molecules across limiting membranes or into vesicles.[2] The ABCA subfamily members are thought to be lipid transporters.[3] The ABC transporter A12 (ABCA12) is known to be a key molecule in keratinocyte lipid transport (**Fig. 1**).[4-6] ABCA12 is a keratinocyte transmembrane lipid transporter protein associated with the transport of lipids in lamellar granules to the apical surface of granular layer keratinocytes.[4] This article reviews the importance of *ABCA12* as a keratinocyte lipid transporter in the context of keratinocyte differentiation and skin lipid barrier formation.

ABCA12 and Other ABCA Transporters

Several genetic diseases have been shown to be caused by mutations in ABCA subfamily genes. The ABCA subfamily, of which the *ABCA12* gene is a member, comprises 12 full transporters and one pseudogene (ABCA11) that are essential for lipid transport and secretion.[7] Three ABCA genes of the same subfamily as ABCA12 have been also implicated in the development of genetic diseases affecting cellular lipid transport. In the phylogenetic tree of ABCA subfamily proteins, ABCA3 is very close ABC12.[1] ABCA3 is known to aid lipid secretion from alveolar type II cells via lamellar granules,[8] and an ABCA3 deficiency recently was reported to underlie a fatal lung surfactant deficiency in newborns,[9] a condition that often leads to death shortly after birth.

Another important member of the ABCA subfamily is ABCA1. Mutations in the human ABCA1 gene underlie familial alpha-lipoprotein deficiency syndrome (Tangier disease), which suggests that ABCA1 is a major regulator of high-density lipoprotein metabolism.[10-12]

ABCA2, ABCA3 and ABCA7 mRNA levels were reported to be upregulated after sustained cholesterol influx,[13,14] suggesting that ABCA transporters are involved in the transmembrane transport of endogenous lipids.[15] From these facts, transporters in the ABCA subfamily are thought to be involved in the transmembrane transport of cholesterol.[16-18] Interestingly, ABCA3, a member of the same protein superfamily as ABCA12, functions in pulmonary surfactant lipid secretion through the production of similar lamellar-type granules within lung alveolar type II cells.[8,9]

The Role of ABCA12 in the Transport of Lipids into Lamellar Granules

Extracellular lipids, including ceramide, are thought to be essential for skin barrier function.[19] Mutations in the ABCA12 gene (*ABCA12*) were reported to underlie the devastating phenotype seen in harlequin ichthyosis (HI) patients,[4,20] the most severe keratinization disorder. ABCA12 mutations underlying HI are thought to have major disruptive defects on ABCA12 lipid
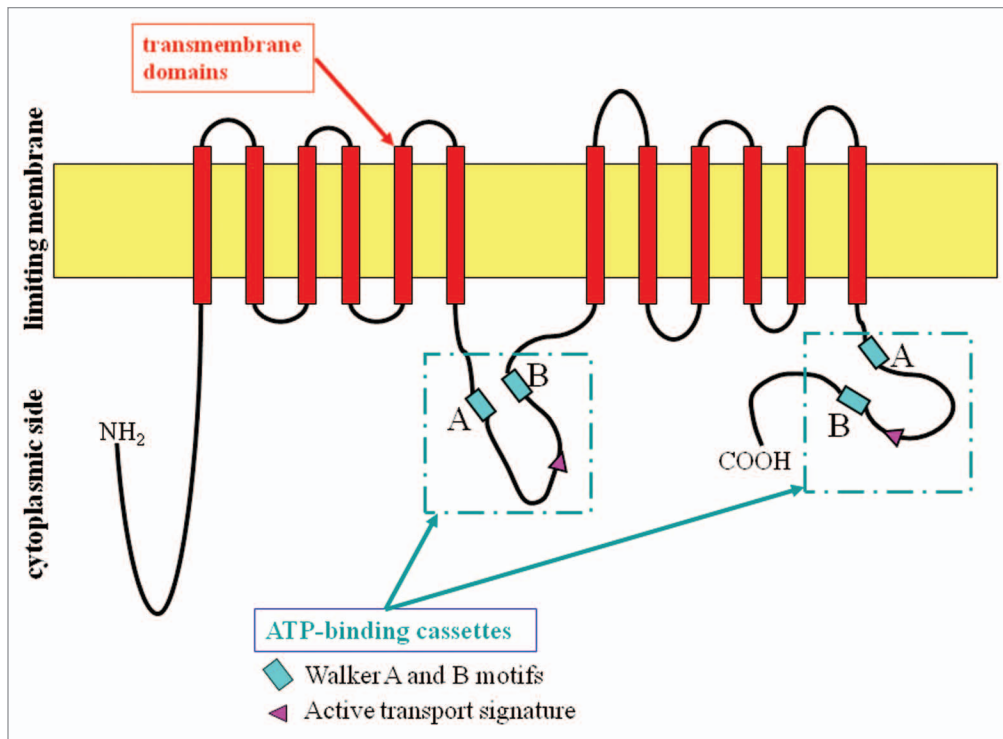
Figure 1. ABCA12 protein structure and domains. Analysis of the predicted structure of the ABCA12 protein reveals features typical of ABCA transporters.[1]

transporter function, resulting in the HI phenotype.[4] We reported that ABCA12 is localized in lamellar granules (LGs) in the granular layer keratinocytes and might work in lipid transport through LGs to form the intercellular lipid layers in the stratum corneum.[4] We have analyzed the epidermal localization of ABCA12 in comparison with the localization of Golgi apparatus markers and LG-associated proteins together with transglutaminase 1, because LGs are thought to be a part of the continuous tubular network that originates from the Golgi apparatus and extends to the cell membrane.[5] We employed antibodies to well-established marker molecules of each part of the Golgi apparatus-LG-cell membrane network, i.e., the GM130, anti-TGN-46 and anti-transglutaminase 1 antibodies (B.C1), as markers for cis-Golgi, trans-Golgi and cell membrane, respectively. Our results show that ABCA12 localizes throughout the entire Golgi apparatus to LGs at the cell periphery, mainly in the granular layer keratinocytes. These results suggest that ABCA12 works in the transport of lipids from the Golgi apparatus to LGs in the granular layer cells.[5] Double-labeling immunofluorescence staining of cultured keratinocytes clearly indicates that ABCA12 is localized from the Golgi apparatus (colocalized with cis-Golgi marker GM130 and trans-Golgi marker TGN-46) to the cell periphery (close to the plasma membrane stained with transglutaminase 1). ABCA12 fails to colocalize with TGase1, a cell membrane-bounding protein, both in vivo and in cultured keratinocytes and ABCA12 is thought to distribute only very sparsely on the cell membrane.[5]

In normal human epidermis, ABCA12 is expressed throughout, but mainly in the upper spinous and granular layers.[5] Immunofluorecent double labeling reveals that the majority of ABCA12 colocalizes with glucosylceramide in the cytoplasm within

the upper spinous and granular cells (**Fig. 2**).[5] Immunofluorescence labeling on ultrathin cryosections clearly reveals localization of ABCA12 and glucosylceramide. In immunofluorescence labeling under light microscopy, ABCA12 and glucosylceramide staining almost completely overlap within the granular layer keratinocytes.[5] Post-embedding immunoelectron microscopy reveals both ABCA12 and glucosylceramide in the LGs of the uppermost granular layer keratinocytes.[5] Under immunoelectron microscopy using ultrathin cryosections, glucosylceramide labeling is seen with the lamellar structures in the LGs. ABCA12 immunogold labeling is observed on or close to the membrane surrounding LGs in the uppermost granular layer cells.[5]

We can hypothesize that ABCA12 is likely to be a membrane lipid transporter that functions in the transport of lipids from the trans-Golgi network to LGs at the keratinocyte periphery (**Fig. 3**).[4,5] Recently, it was confirmed biochemically that ABCA12 deficiency impairs glucosylceramide accumulation in lamellar granules and that ABCA12 transports glucosylceramide to the inner side of lamellar granules.[6] In addition, ceramide was reported to upregulate ABCA12 expression via PPAR delta-mediated signaling pathway, providing a substrate-driven, feed-forward mechanism for regulation of this key lipid transporter.[21] More recently, studies using *Abca12-/-* mice suggested that ABCA12 plays an important role in the normal differentiation of epidermal keratinocytes.[22]

ABCA12 Mutations and Ichthyoses

ABCA12 mutations are known to underlie the three main types of autosomal recessive congenital ichthyoses: harlequin ichthyosis

(HI), lamellar ichthyosis (LI) and congenital ichthyosiform erythroderma (CIE). Harlequin ichthyosis is the most severe ichthyosis subtype. Affected patients show plate-like scales over the whole body, severe eclabium and ectropion.

In 2010, a review of the literature was performed to identify all known *ABCA12* mutations in patients with ARCI and 56 *ABCA12* mutations were described (online database: www.derm-hokudai.jp/ABCA12/) in 66 unrelated families, including 48 HI, 10 LI and 8 CIE families.[23] Mutations have been reported among autosomal recessive congenital ichthyosis patients with African, European, Pakistani/Indian and Japanese backgrounds in most parts of the world. Of the 56 mutations, 36% (20) are nonsense, 25% (14) are missense, 20% (11) comprise small deletions, 11% (6) are splice site, 5% (3) are large deletions and 4% (2) are insertion mutations. At least 62.5% (35) of all the reported mutations are predicted to result in truncated proteins. There is no apparent mutation hot spot in *ABCA12*, although mutations underlying the LI phenotype are clustered in the region of the first ATP-binding cassette.[24]

In HI-affected epidermis, several morphologic abnormalities have been reported, including abnormal lamellar granules in the keratinocyte granular layer and a lack of extracellular lipid lamellae within the stratum corneum.[25-28] Lack of ABCA12 function subsequently leads to disruption of lamellar granule lipid transport in the upper keratinizing epidermal cells, resulting in malformation of the intercellular lipid layers of the stratum corneum in HI.[4] Cultured epidermal keratinocytes from an HI patient carrying *ABCA12* mutations demonstrate defective glucosylceramide transport, and this phenotype is recoverable by in vitro *ABCA12* corrective gene transfer.[4] Intracytoplasmic glucosylceramide transport has been studied using cultured keratinocytes from a total of three patients harboring *ABCA12* mutations. One patient was homozygous for the splice site mutation c.3295-2A>G[4] and another was compound heterozygous for p.Ser387Asn and p.Thr1387del.[29] Only one heterozygous mutation, p.Ile1494Thr, was identified in the other patient.[30] Cultured keratinocytes from all three patients showed apparently disturbed glucosylceramide transport, although this assay is not quantitative.

In addition, defective lamellar granule formation was observed in the skin of two CIE patients with *ABCA12* mutations.[30] Electron microscopy revealed that, in the cytoplasm of granular layer keratinocytes, abnormal, defective lamellar granules are assembled with some normal-appearing lamellar granules.[30]

Formation of the intercellular lipid layers is essential for epidermal barrier function. In ichthyotic skin with ABCA12 deficiency, defective formation of the lipid layers is thought to result in a serious loss of barrier function and a likely extensive compensatory hyperkeratosis.[31]

One hypothetical pathomechanism for ABCA12 deficiency in autosomal recessive congenital ichthyosis is explained by the "differentiation defect theory," which is derived from the clinical features of HI patients. Fetuses affected with HI start developing the ichthyotic phenotype while they are in the amniotic fluid, where
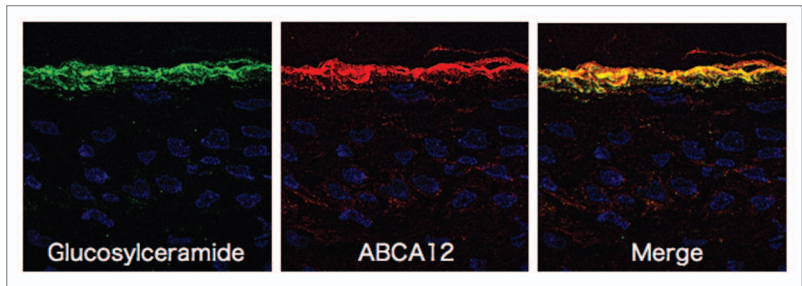

Figure 2. Immunofluorescence labeling using ultrathin cryosections as substrates reveal that glucosylceramide (green) and ABCA12 (red) overlap in the granular layers (derived from ref. 5).

stratum corneum barrier function is not required. According to this theory, barrier defects cannot be involved directly in the pathogenesis of the HI phenotype, at least during the in utero fetal period. In light of this, disturbed keratinocyte differentiation is speculated to play an important role in the pathogenesis of the HI phenotype. In fact, three-dimensional culture studies reveal that HI keratinocytes differentiate poorly according to morphologic criteria and show reduced expression of keratin 1 and defective conversion from profilaggrin to filaggrin.[32]

In an *ABCA12*-ablated organotypic co-culture system, which is an in vitro model of HI skin, the expression of keratinocyte late differentiation-specific molecules is dysregulated.[33] The expression of specific proteases associated with desquamation (kallikrein 5 and cathepsin D) is dramatically reduced in the ABCA12-ablated organotypic co-culture system.[33] In this model system, *ABCA12* ablation results in a premature terminal differentiation phenotype.[33] Furthermore, in mutant mice carrying a homozygous spontaneous missense mutation, loss of Abca12 function leads to the premature differentiation of basal keratinocytes.[34] In contrast, in our *Abca12-/-* HI model mice, immunofluorescence and immunoblotting of *Abca12-/-* neonatal epidermis revealed defective profilaggrin/filaggrin conversion and reduced expression of the differentiation-specific molecules (loricrin, kallikrein 5 and transglutaminase 1), although their mRNA expression is upregulated.[22] These data suggest that ABCA12 deficiency may lead to disturbances in keratinocyte differentiation during fetal development, resulting in an ichthyotic phenotype at birth. These observations suggest that ABCA12 deficiency might have global effects on keratinocyte differentiation, resulting in both impaired terminal differentiation and premature differentiation of the epidermis.

HI patients often die in the first week or two of life. However, those that survive beyond the neonatal period phenotypically improve within several weeks after birth. To clarify the mechanisms of phenotypic recovery, we studied grafted skin and keratinocytes from *Abca12*-disrupted (*Abca12-/-*) mice.[22] *Abca12-/-* skin grafts kept in a dry environment exhibited dramatic improvements in all the abnormalities seen in the model mice. Increased transepidermal water loss, a parameter of barrier defect, is remarkably decreased in grafted *Abca12-/-* skin. Ten-passage sub-cultured *Abca12-/-* keratinocytes show restoration of intact ceramide distribution, differentiation-specific protein
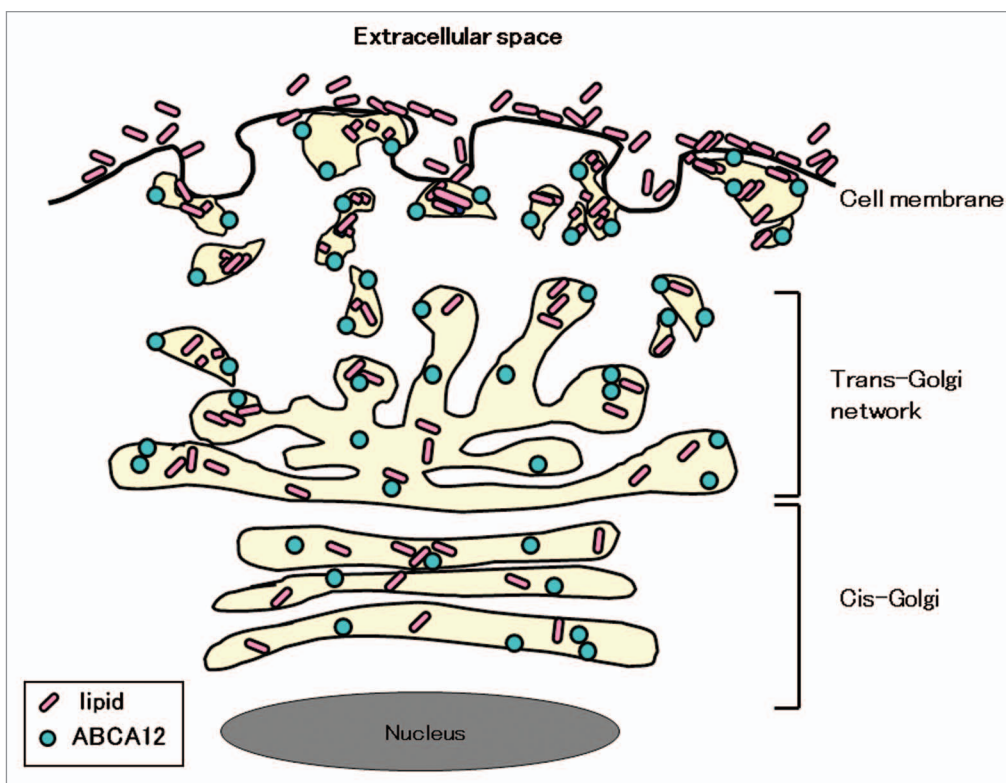
Figure 3. Scheme of ABCA12 distribution from the cis-Golgi, trans-Golgi network to lamellar granules in the upper spinous and granular layer keratinocytes (derived from ref. 5).

expression and profilaggrin/filaggrin conversion, which are defective in the primary-culture.[22] These observations suggest that, during maturation, *Abca12*^{-/-} epidermal keratinocytes regain normal differentiation processes, although the exact mechanisms of this restoration remain unknown.[22]

ABCA12-deficient Animal Models

Recently, bioengineered disease models were established to investigate the ichthyotic pathomechanisms that result from defective ABCA12 function and to aid the development of innovative treatments for ichthyosis with ABCA12 deficiency.

We transplanted cultured keratinocytes from patients with HI and succeeded in reproducing HI skin lesions in immunodeficient mice.[35] These reconstituted HI lesions show similar changes to those observed in HI patients' skin. In addition, we generated *Abca12*-disrupted (*Abca12*^{-/-}) mice that closely reproduced the human HI phenotype, showing marked hyperkeratosis with eclabium and skin fissures.[36] Lamellar granule abnormalities and defective ceramide distribution were remarkable in the epidermis. Skin permeability assays of *Abca12*^{-/-} mouse 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 from *Abca12*^{-/-} mice lack normal lamellar structures.[36] The level of surfactant protein B, an essential component of alveolar surfactant, is reduced in the *Abca12*^{-/-} mice.[36]

Another group independently developed *Abca12*^{-/-} mice and these also had the clinical features of HI.[37]

A study in one *Abca12*-disrupted HI model mouse indicates that a lack of desquamation of skin cells, rather than enhanced proliferation of basal-layer keratinocytes accounts for the five-fold thickening of the *Abca12*^{-/-} stratum corneum determined by in vivo skin proliferation measurements.[37] It was suggested that this lack of desquamation is associated with a profound reduction in skin linoleic esters of long-chain omega-hydroxyceramides and a corresponding increase in their glucosylceramide precursors. Omega-hydroxyceramides are required for correct skin barrier function, and these results from HI model mice establish that ABCA12 activity is required for the generation of the long-chain ceramide esters that are essential for the development of normal skin structure and function.[37]

In addition, a mouse strain carrying a homozygous spontaneous missense mutation was reported to show skin manifestations similar to ichthyosis.[34] Lipid analysis of *Abca12* mutant epidermis revealed defects in lipid homeostasis, suggesting that *Abca12* plays a crucial role in maintaining lipid balance in the skin.[34] The cells from the *Abca12* mutant mouse have severely impaired lipid efflux and intracellular accumulation of neutral lipids.[34] Abca12 was also demonstrated as a mediator of Abca1-regulated cellular cholesterol efflux.[34] Injection of a morpholino designed to target a splice site at the exon 4/intron 4 junction to block *Abca12* pre-mRNA processing induced altered skin surface contours, disorganization of the melanophore distribution, pericardial edema
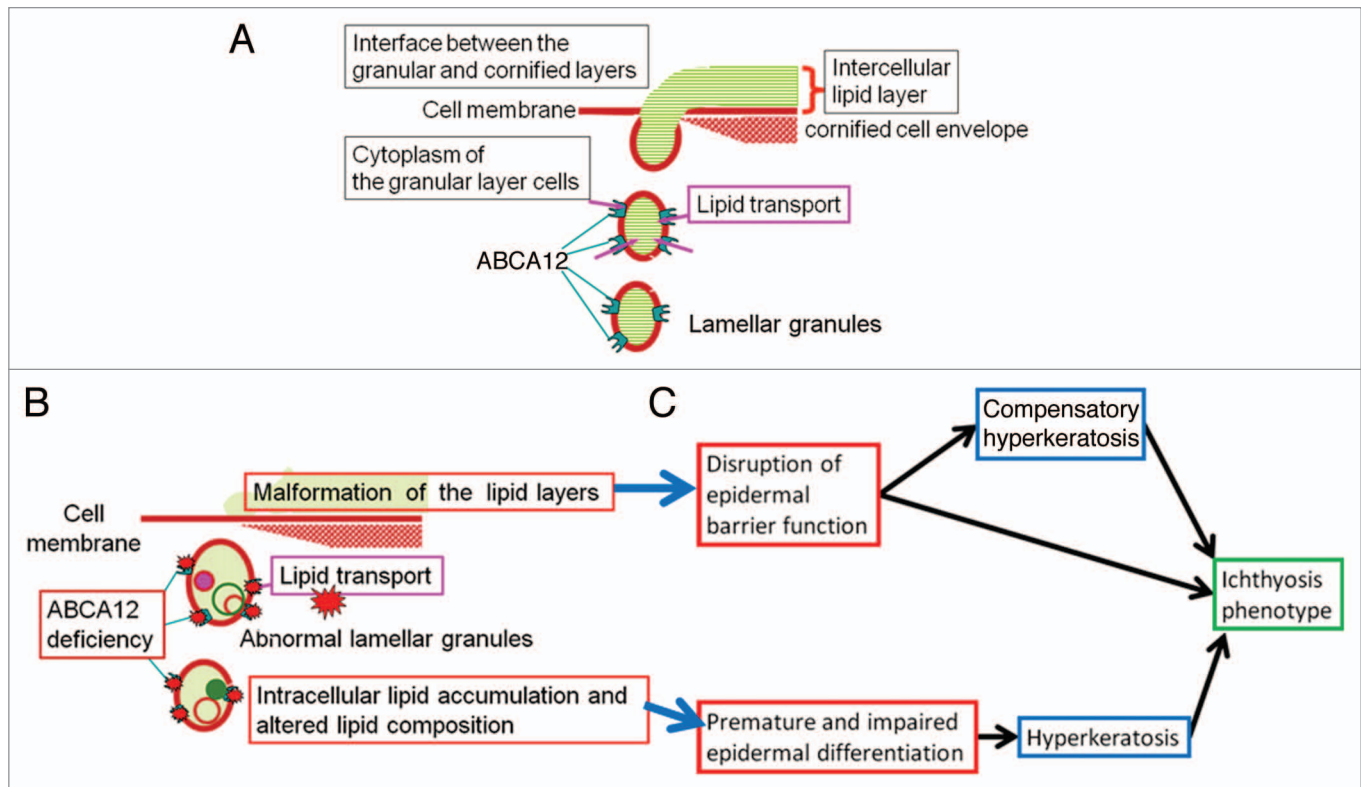
Figure 4. Physiological role(s) of ABCA12 in lipid trafficking of epidermal keratinocytes and the model of pathogenetic mechanisms in ichthyosis phenotypes caused by ABCA12 deficiency. (A) Model of how ABCA12 transports lipids in epidermal keratinocytes. (B) Model of how loss of ABCA12 function leads to lipid abnormality and lipid barrier malformation in the upper epidermis. (C) It is hypothesized that the combination of lipid barrier defects and disturbed keratinocyte differentiation cause hyperkerasosis and the ichthyosis phenotype (derived from ref. 20).

and enlargement of the yolk sac at 3 days post-fertilization in the larvae of zebrafish. It was also associated with premature death at around 6 days post-fertilization. These results suggest that *Abca12* is an essential gene for normal zebrafish skin development and provide novel insight into the function of ABCA12 (reported at the Annual Meeting of the Society for Investigative Dermatology 2010; Abstract, Frank et al. J Invest Dermatol 2010; 130:86).

Using our Abca12^{-/-} HI model mice, we tried fetal therapy with systemic administration to the pregnant mother mice of retinoid or dexamethasone, which are effective treatments for neonatal HI and neonatal respiratory distress, respectively. However, neither of these improved the skin phenotype nor extended the survival period.[36] Retinoids were also ineffective in in vivo studies using cultured keratinocytes from the model mice.[22]

Conclusion

ABCA12 is apparently localized in the membrane of the trans-Golgi network and lamellar granules in the upper epidermis, mainly in the uppermost spinous and granular layer cells. Our own studies and a review of the literature suggest that ABCA12 works in the transport of lipids into the trans-Golgi network and lamellar granules, to accumulate lipids that are essential to skin barrier formation. Consequently, the lipids packed in lamellar granules are secreted to the extracellular space to form intercellular lipid layers in the stratum cornuem, which is important for skin barrier function (**Fig. 4**). In addition, model mouse studies indicate that lipid transport by ABCA12 is indispensable for intact differentiation of keratinocytes. To elucidate the mechanisms of ABCA12 in keratinocyte differentiation/proliferation, further accumulation of data is needed.

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