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Review

The role of sphingolipid metabolism in cutaneous permeability barrier formation☆

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ABSTRACT

The epidermal permeability barrier of mammalian skin is localized in the stratum corneum. Corneocytes are embedded in an extracellular, highly ordered lipid matrix of hydrophobic lipids consisting of about 50% ceramides, 25% cholesterol and 15% long and very long chain fatty acids. The most important lipids for the epidermal barrier are ceramides. The scaffold of the lipid matrix is built of acylceramides, containing ω-hydroxylated very long chain fatty acids, acylated at the ω-position with linoleic acid. After glucosylation of the acylceramides at Golgi membranes and secretion, the linoleic acid residues are replaced by glutamate residues originating from proteins exposed on the surface of corneocytes. Removal of their glucosyl residues generates a hydrophobic surface on the corneocytes used as a template for the formation of extracellular lipid layers of the water permeability barrier. Misregulation or defects in the formation of extracellular ceramide structures disturb barrier function. Important anabolic steps are the synthesis of ultra long chain fatty acids, their ω-hydroxylation, and formation of ultra long chain ceramides and glucosylceramides. The main probarrier precursor lipids, glucosylceramides and sphingomyelins, are packed in lamellar bodies together with hydrolytic enzymes such as glucosylceramide-β-glucosidase and acid sphingomyelinase and secreted into the intercelullar space between the stratum corneum and stratum granulosum. Inherited defects in the extracellular hydrolytic processing of the probarrier acylglucosylceramides impair epidermal barrier formation and cause fatal diseases: such as prosaposin deficiency resulting in lack of lysosomal lipid binding and transfer proteins, or the symptomatic clinical picture of the "collodion baby" in the absence of glucocerebrosidase. This article is part of a Special Issue entitled The Important Role of Lipids in the Epidermis and their Role in the Formation and Maintenance of the Cutaneous Barrier. Guest Editors: Kenneth R. Feingold and Peter Elias.

1. Introduction

All land dwelling animals and plants are protected by a functional barrier between the organisms and their environment. The permeability barrier of mammals is localized in the stratum corneum (SC), the outermost cellular layer of the epidermis. This barrier protects the organisms against desiccation by transepidermal water loss and against the infiltration of pathogens and toxins.

The epidermis has a unique structure built of several layers mainly composed of differentiating keratinocytes. Keratinocytes of the stratum basale contain intact organelles and proliferate. During their migration through the adjacent outer epidermal layers, the stratum spinosum and the stratum granulosum (SG), they experience an increasing Ca^{2+} gradient and differentiate. The outer cell layer, the SC, is composed of terminally differentiated keratinocytes (corneocytes), which are dead flattened cells devoid of organelles but packed with keratin filaments. Corneocyte are embedded in a matrix of extracellular lipid lamellae, consisting mainly of ceramides, free fatty acids, and cholesterol [1,2]. These lipids form two lamellar phases with short and long periodicity of approximately 6 nm and 13 nm, respectively [3].

Human SC contains 12 free extractable ceramide fractions with different hydroxylation patterns in the sphingoid base and in the fatty acid (FA) moiety [4–8]. Two nomenclature systems are used for the description of SC ceramides. One is a numbering system based on the chromatographic migration (ceramide 1 to ceramide 8) and chronological arrangement of their publication (from ceramide 9 on). The other system is based on the molecular structure of the ceramides. It was proposed by Motta et al. [9] and modified by Robson et al. [5]. The last letter designates the sphingoid base: S (sphingosine), DS (sphinganine), P (phytosphingosine), and H (6-hydroxy sphingosine) and the front

Abbreviations: ABCA12, ATP binding cassette subfamily A member 12; ASM, acid sphingomyelinase; CE, cornified envelope; Cer, ceramide; CerS, ceramide synthase; ELOVL, fatty acid elongase; FA, fatty acid; GlcCer, glucosylceramide; LB, lamellar bodies; PPAR, peroxisome proliferator-activated receptor; Sap, saposin; SC, stratum corneum; SG, stratum granulosum; SM, sphingomyelin; SPT, serine palmitoyltransferase; VLC, very long chain; VLC-FA, very long chain FA (>C20); ULC-FA, ultra long chain FAs (≥C26)

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letter(s) designate the acyl chain: N (non-hydroxy FA), A (α-hydroxy FA), and EO (esterification of the ω-hydroxy FA with linoleic acid). Their structures and distribution in human SC are depicted in Fig. 1. In human SC, 342 different ceramide species belonging to eleven ceramide classes were identified by Masukawa et al. [10]. The analysis of sphingoid bases and fatty acids demonstrated carbon chain length in the range of 14–28 and 16–36, respectively [7,10]. Recently a new class of ceramides with four hydroxyl (OH) groups in the sphingoid base (T) has been identified in human epidermis by mass spectrometry [7], however, the position of OH-groups remains unknown. This new ceramide would be abbreviated as ceramide (NT).

2. The permeability barrier in the epidermis

Cellular membranes and lipid bilayers are permeable for water [11]. To avoid desiccation by excessive water loss, nature developed multilayered arrays of rather hydrophobic very long chain (VLC) and ultra long chain (ULC) nanostructured waxes (esters of ULC alcohols and ULC fatty acids (ULC-FAs)) on surfaces of plants (e.g. cacti) and insects, and extracellular lipid layers composed of ULC-FA, ceramides with ULC-FAs, and cholesterol on surfaces of the skin of land dwelling animals. The permeability barrier of mammalian epidermis is localized in the SC. The most important sphingolipids of keratinocytes are glucosylceramides (GlcCers) and ceramides containing a broad range of FA species. FAs are classified due to carbon chain length: FAs from C12 to C20 are long chain FAs, FAs >C20 are called VLC-FAs, and FAs ≥C26 are ULC-FAs [12].

Alterations in lipid composition of SC and their organization impair epidermal function resulting in disease. The scaffold for the lipid organization in the extracellular lipid matrix is made of ULC-sphingolipids, mainly ULC-ceramides, which are covalently attached to a three-dimensional network of cross-linked proteins (e.g. envoplakin and involucrin) of the cornified envelope (CE) [13,14]. They form a template on the surface of corneocytes to organize the extracellular lipid layers as a barrier in the interstices of the SC. Ceramides with ULC-FAs are major

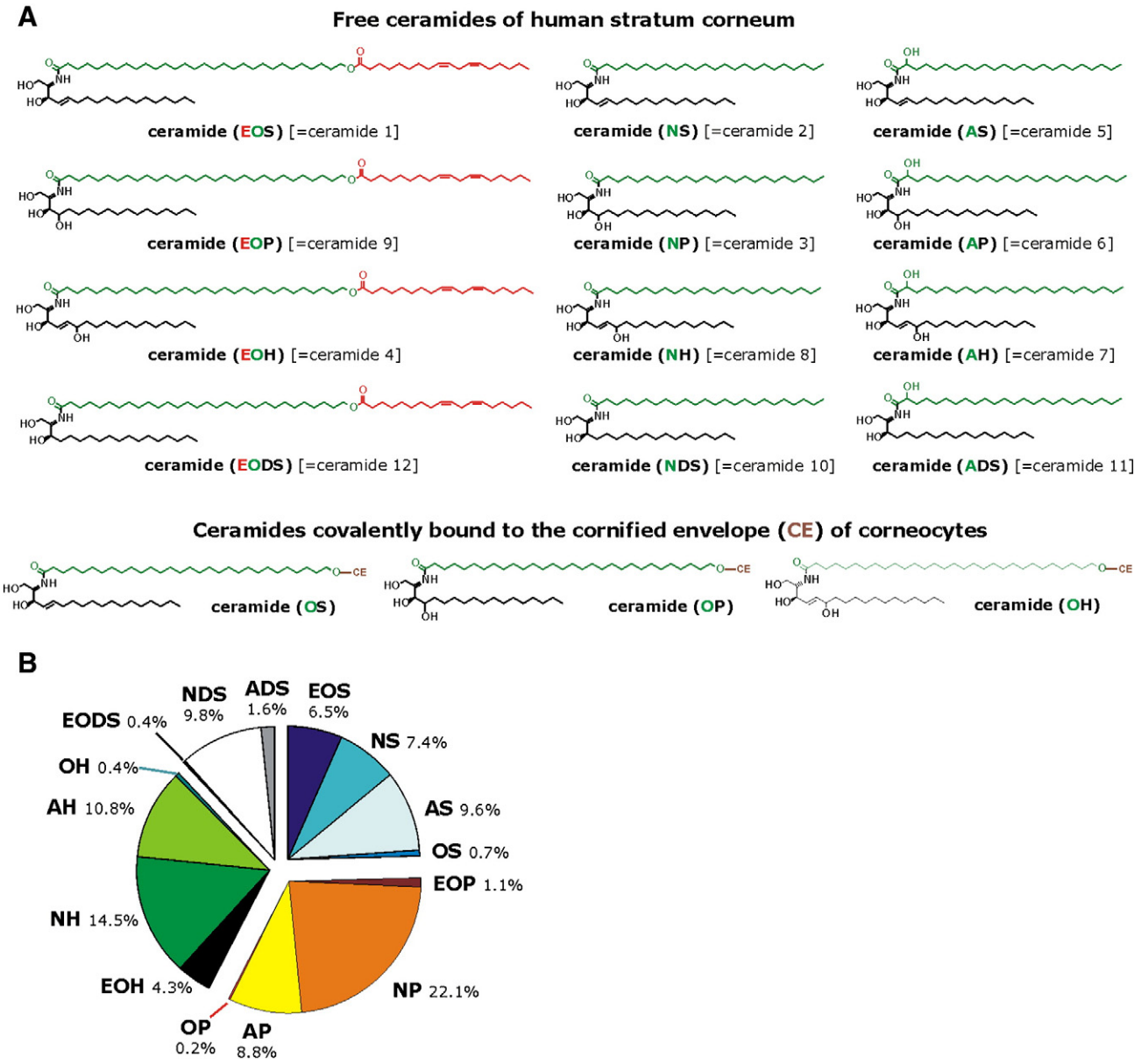

Fig. 1. Molecular structures and distribution of free and covalently-bound ceramides of the human SC. Structures (A) are shown in accordance with the terminology proposed by Motta et al. and modified by Robson et al. [5,9]. The structures are classified according to the sphingoid base (black, S: sphingosine, P: phytosphingosine, H: 6-hydroxysphingosine, DS: sphinganine) and the N-acyl residue (green, A: α-hydroxy-FA, O: ω-hydroxy-FA, N: no hydroxy-FA). E: acylated with linoleic acid (red) in ω-OH position. Panel B presents the distribution of different ceramide species in human SC modified from t'Kindt et al. [7].

components of the barrier, the extracellular multilamellar lipid layers of the SC, but not of cellular membranes. They are synthesized together with ULC-FA-containing GlcCers by differentiating keratinocytes and stored in lamellar bodies (LB, also called Odland bodies), forming disk-like structures (Fig. 2). LBs release their content (probarrier lipids including GlcCer, sphingomyelin (SM), and phospholipids) into the extracellular space between SG and SC before the cells transform into corneocytes [15–18]. Lipid loading of LBs (especially with linoleoyl-ω-esterified ceramides) is severely reduced in patients with harlequin ichthyosis and in a mouse model for the disease, both of which are

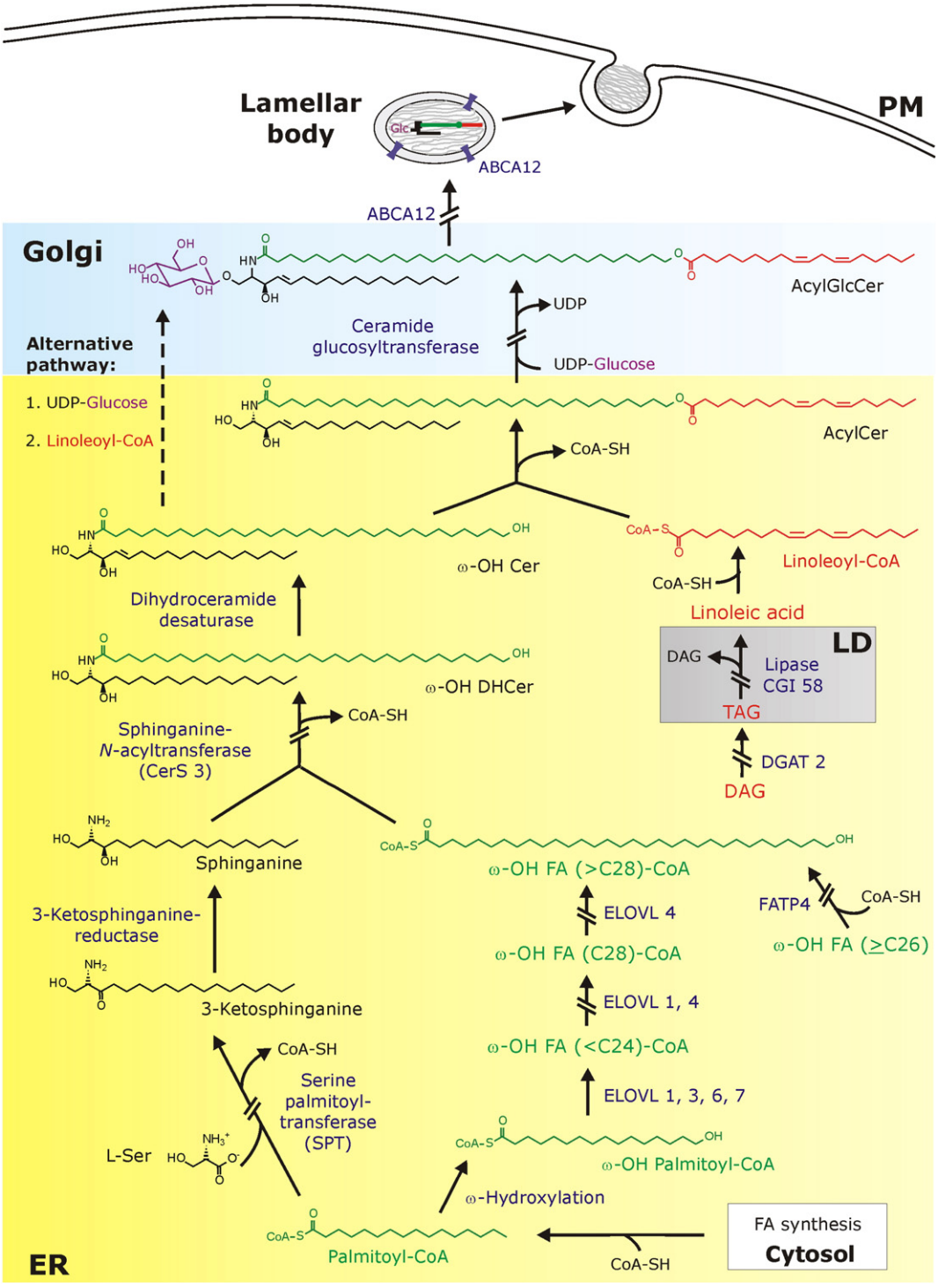

Fig. 2. Biosynthesis of acylglucosylceramide. Scaffold of the extracellular permeability barrier are ultra long chain ω-OH ceramides. The intracellular precursors of these ceramide species are acylGlcCers. Biosynthesis of their sphingoid bases, VLC- and ULC-FAs with their ω-hydroxylation and the acylation of the bases to ceramides by CerS 3 occur at the ER. We suggest that the elongation of ω-OH-FAs follows a mechanism similar to that of FAs. We also assume that each FA ≥C16 could be ω-hydroxylated. The pathway of the ω-hydroxylation of VLC- and ULC-FAs has not been elucidated yet. Subsequently ceramides are probably first esterified at the ω-position with linoleic acid and then glucosylated at the cytosolic side of the Golgi membranes. An alternative pathway (1. glucosylation, 2. acylation) cannot be excluded at present. The precursor lipids are packed in lamellar bodies (LB) and secreted into the intercellular space. Anabolic blocks in SPT, ELOVL 1, ELOVL 4, FATP4, CerS 3, ABCA12, DGAT2, and cofactor CGI-58 are depicted by arrows with slashes through them. LD: lipid droplets, Cer: ceramide, GlcCer: glucosylceramide, FA: fatty acid.

deficient in the ATP binding cassette transporter ABCA12. ABCA12 deficiency causes a loss of the lamellar sheets in LBs [19–21] and impairs barrier function resulting in early neonatal death.

Secreted probarrier lipids are extracellularly processed mainly by lysosomal hydrolases and lipid binding proteins (saposins (Saps)) in a slightly acidic environment.

3. Biosynthesis of barrier sphingolipids with ULC-FAs

Mammalian sphingolipids are synthesized in a cell type and differentiation specific manner. The major ULC-sphingolipids of keratinocytes are GlcCers and ceramides. The most unique and essential ones contain ω-hydroxylated fatty acids (ω-OH-ULC-FAs with a chain length of 28–36 carbons) esterified in ω-position with linoleic acid [22–26].

3.1. Biosynthesis of VLC- and ULC-FAs

Starting from acetyl-CoA and malonyl-CoA, fatty acid synthase complex (FAS)-I in the cytosol and the enzymes of FAS-II in mitochondria generate mainly palmitoyl-CoA [27], which can be ω-hydroxylated and also elongated.

As suggested by inhibitor studies, FAs and possibly also ceramides containing ULC-FAs can undergo ω-hydroxylation most likely catalyzed by a P-450-type 4 isoform (CYP4F) [28], a prerequisite for the formation of ω-OH-ULC-FA containing ceramides by ceramide synthase (CerS) 3 [12]. The mechanism of ω-hydroxylation has not been clarified yet.

Another disorder affecting the ω-hydroxylation of the FA metabolism is Sjögren–Larsson syndrome which is due to a deficiency of fatty aldehyde dehydrogenase. The disease is characterized by ichthyosis with abnormal LBs [29,30] and reduced levels of acylceramides [31]. Fatty aldehyde dehydrogenase is a part of the fatty alcohol:NAD oxidoreductase enzyme complex which catalyzes the oxidation of long chain aliphatic aldehyde to FA. It is also involved in the ω-oxidation of ULC-FA [32]. The specific function of fatty aldehyde dehydrogenase in barrier formation is not fully elucidated.

The elongation of FAs ≥16 carbon atoms is performed by a 4-step reaction cycle (1. condensation by 3-ketoacyl-CoA synthase (elongation-of-very-long-chain-fatty acids (ELOVL)-protein), 2. reduction by 3-keto-acyl-CoA reductase, 3. dehydration by 3-hydroxyacyl-CoA dehydratase, and 4. reduction by 2,3 enoyl-CoA reductase), which results in a chain extension of two carbons per cycle [33]. Seven distinct but homologous fatty acid elongases, ELOVLs 1–7, were discovered and described as membrane-bound enzymes of the ER [34]. They differ in cellular expression profile and substrate specificities [35,36] (Fig. 2). ELOVLs have an ER retention signal and contain five transmembrane spans [37]. ELOVLs 1, 3 and 4 have been detected in skin [34]. ELOVL 1 and ELOVL 4 seem to be the crucial elongases to generate ULC-FAs with ≥26 carbon atoms (Fig. 2). In vitro ELOVL 1 accepts acyl-CoAs with a chain length of C18 to C26 [35] and ELOVL 4 acyl-CoAs with carbon atoms ≥24 [38,39]. Mice deficient in one of these elongases have a disturbed barrier function and die neonatally from an increased transepidermal water loss [38,40]. The epidermis of ELOV 1 deficient mice has a decreased level of ceramides with acyl chains ≥C26 and an increased level of ceramides with FA residues ≤C24 [40].

Model mice with a functional deletion of ELOV 4 activity are deficient in ULC-FAs with more than 28 carbon atoms and corresponding ω-OH-ceramides, whereas their precursors with 26 carbon atoms accumulate [38,39,41–43]. Patients with a heterozygous mutation of *ELOVL 4* suffer from juvenile dominant macular degeneration (Stargardt disease 3) [44]. Patients with recessive *ELOVL 4* mutations have a congenital ichthyosis comparable to the skin phenotype of ELOV 4 deficient mice [45].

Fatty acid transport protein 4 (FATP4) activates ULC-FA with CoA [46]. A mutation in the *FATP4* gene was found in patients with the ichthyosis prematurity syndrome [47,48]. FATP4 deficient mice die shortly after birth due to increased transepidermal water loss [49,50]. The exact role or function of FATP4 for the formation of the epidermal barrier has not been elucidated.

3.2. Biosynthesis of ceramides

The sphinganine base is generated by a de novo pathway at the ER, whereas sphingosine is formed in a salvage pathway [51,52]. The first step of the de novo synthesis is the condensation of palmitoyl-CoA and L-serine by serine palmitoyltransferase (SPT) (Fig. 2). SPT, a key enzyme in regulating sphingolipid synthesis, is a heterodimer of subunits, SPTLC 1 either with SPTLC 2 or with SPTLC 3 [53]. Significant reductions of the SPT expression and of the ceramide levels have been reported in psoriatic lesions compared with non-lesional skin [54]. The conditional SPT knock-out mice (loss of SPTLC 2) show inflammatory skin like psoriasis, with a reduced ceramide level [55]. Similar results were observed in mice treated with ISP-I (myriocin or thermozymocidin), a potent inhibitor of the SPT [56].

Sphinganine containing sphingolipids, mainly SM and GlcCer, are degraded to dihydroceramide, presumably mostly by lysosomal hydrolases, and slowly desaturated by a dihydroceramide desaturase [51,52,57] to form ceramides at the ER. Hydroxylation of dihydroceramide by desaturase 2 (DES2) forms 4-OH dihydroceramide (phytoceramide) [57] and hydroxylation of ceramide by an unknown enzyme generates 6-OH ceramide [58]. Six different ceramide synthases (CerS 1–6) utilize acyl-CoAs with selected carbon chain lengths as substrates to N-acylate sphingoid bases [59,60].

CerS 3 seems to be the only ceramide synthase being able to generate the key components of the water permeability barrier, ceramides containing ULC-FAs (≥C26) including ω-OH-ULC-FAs (Fig. 2) [12]. Its genetically engineered deficiency in model mice results in a complete loss of all ceramides with ULC-FAs and a lack of extracellular lipid matrix. Mutant mice die shortly after birth from transepidermal water loss and are prone to infection [12]. Recently, a homozygous missense mutation in *CERS3* has been identified in patients with autosomal recessive congenital ichthyosis [61,62].

Acylation of the ω-OH-ceramides with linoleate (or in a small amount with C17:2, C19:2, and C20:2 [63]) residues leads to the formation of rather hydrophobic ULC-acylceramides (acylCer) (Fig. 2) [64,65]. The linoleate required for acylation is released from triacylglycerides by a triacylglyceride lipase activated by CGI-58 [64,65]. Loss-of-function mutations in CGI-58 were found in a patient with Dorfman–Chanarin syndrome, a neutral lipid storage disorder with ichthyosis and abnormal skin barrier function. A CGI-58 deficient mouse model showed a neonatal lethal skin barrier defect and a deficiency of both, ω-OH-ceramides and CE-bound-ω-OH-ceramides [65].

The final step of triacylglyceride biosynthesis is the acylation of diacylglyceride by acyl-CoA: diacylglycerol acyltransferase 2 (DGAT 2). This enzyme is decreased in human psoriatic skin [66]. *Dgat 2* deficient mice have a disturbed barrier function with increased transepidermal water loss due to drastically reduced content of total linoleic acid (>90%) and acylceramides (>60%) [67]. A deficiency in the peroxisomal phytanoyl-CoA-hydroxylase or its peroxisomal import receptor peroxin 7 leads to a loss of the lipid-bound envelope in mutant murine models of Refsum disease. Patients with Refsum disease either cannot use branched fatty acids to form acylceramides, or they can make acylceramides containing branched fatty acids instead of linoleate, but the acylceramides containing branched fatty acids cannot be converted to covalently bound omega-hydroxyceramides [68].

4. Glucosylation of acyl ceramides

Glucosylation of ceramides containing acyl residues of up to 26 carbon atoms normally occurs at the cytosolic surface of Golgi membranes by a membrane bound glucosyltransferase [69,70]. The newly formed GlcCer can penetrate and flip the Golgi and/or ER membranes to reach the luminal surfaces and serve as educt for the synthesis of lactosylceramide and

complex glycosphingolipids [71–73]. So far it is unclear if processing of (acyl-) probarrier ceramides containing ULC-FAs follows a similar topological pathway. Glucosylation of acylceramides, however, is an essential step in the processing of these probarrier lipids (Fig. 2). During terminal differentiation of cultured human keratinocytes, the expression of GlcCer synthase mRNA increases 40 fold [74]. A keratinocyte specific knock-out of the glucosyltransferase causes the loss of probarrier GlcCers and a 4 fold increase of the precursor ω-OH-ceramides in 4 day old mutant mice [75]. They show increased desquamation of the SC causing desiccation resulting in early death [75]. Mutant mice with an inducible and conditional loss of glucosyltransferase have been published recently [76]. They show an ichthyosis like phenotype with hyperproliferation, an increased transepidermal water loss, and a decreased level of free extractable GlcCers, protein-bound ceramides and GlcCers, while free extractable ceramides were increased. In addition, the levels of ω-OH-SM which are very low in wild type epidermis increase dramatically [75,76]. Obviously, probarrier acyl-ULC-ceramides which cannot be glucosylated in the mutant mice are channeled into the synthesis of (acyl-)ULC-SMs.

Currently it is unclear if ω-OH-ceramides are glucosylated first to ω-OH-GlcCer and then acylated to acylGlcCer (Fig. 2). The pathway has not been elucidated yet.

Probarrier lipids synthesized and processed during their voyage through the secretory pathway, the ER, Golgi and TGN membranes (acylGlcCers, acyl-ceramides, ULC-FAs etc.), are packed in LBs, forming disk-like lipid membrane structures and are secreted into the extracellular space at the interphase of the SG and SC shortly before the keratinocytes transform into corneocytes.

Packing of probarrier lipids in LBs is impaired by functional loss of ATP binding cassette transporter 12 (ABCA12) activity in harlequin ichthyosis, characterized by impaired skin barrier functions and early death [19–21].

5. Extracellular processing of probarrier lipids

Proper formation, packing and secretion of LBs into the interstices between SG and SC are vital for the generation of the skin barrier (Figs. 3, 4). Still little is known about their biochemistry and molecular biology. They contain the main probarrier lipids mentioned above and several lysosomal hydrolases essential for the processing of the probarrier lipids such as β-glucocerebrosidase [18,77,78] and acid lipase [18,79,80]. There is some evidence that they are formed from the TGN in a similar way as lysosomes [81]. How the secretion of their disk-like lipid membranes and hydrolases into the intercellular space at the interstices of SG and SC is facilitated and regulated at the molecular level, however, is mostly unknown. Extracellular processing of the secreted probarrier lipids requires mainly lysosomal hydrolases, some of which are secreted from the LBs, and Saps, which are predominantly found in the lysosomal compartment [82].

Transcription rates of mRNAs of the key lysosomal proteins, β-glucocerebrosidase (30 fold), prosaposin (15 fold) and acid sphingomyelinase (ASM) (5 fold), increase dramatically during differentiation of cultured human keratinocytes [74]. Secretion of these proteins (ASM, prosaposin and/or individual Saps) has not been experimentally investigated, but may well occur by exocytosis during degradation of nuclei and other intracellular organelles and the replacement

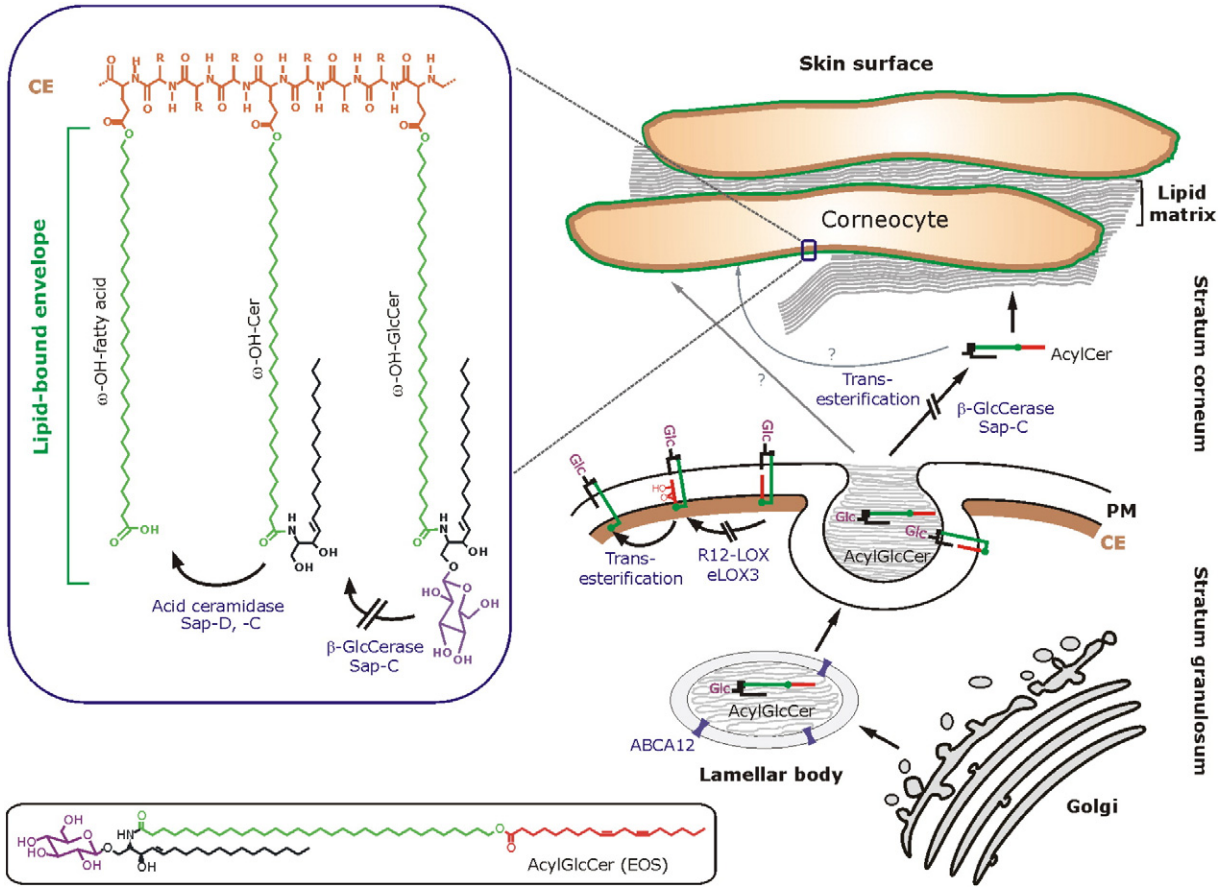

Fig. 3. Formation of the lipid bound envelope. The permeability barrier of the skin is localized in the stratum corneum. A lipid-bound envelope is generated on the cornified envelope (CE, brown) by transesterification of acylGlcCers (consisting of sphingosine (black), glucose (violet), and a long chain ω-OH-fatty acid (green), which is acylated in ω-position with linoleic acid (red)) to γ-glutamate residues of CE proteins. Linoleinyl residues are oxidized and then replaced by γ-glutamate. Transesterification leads to protein bound GlcCers that are metabolized to protein bound ceramides and particularly to protein bound fatty acids by secreted lysosomal hydrolases and cofactors. Catabolic blocks in ß-glucocerebrosidase and Sap-C deficiency are indicated by arrows with slashes through them. PM: plasma membrane.

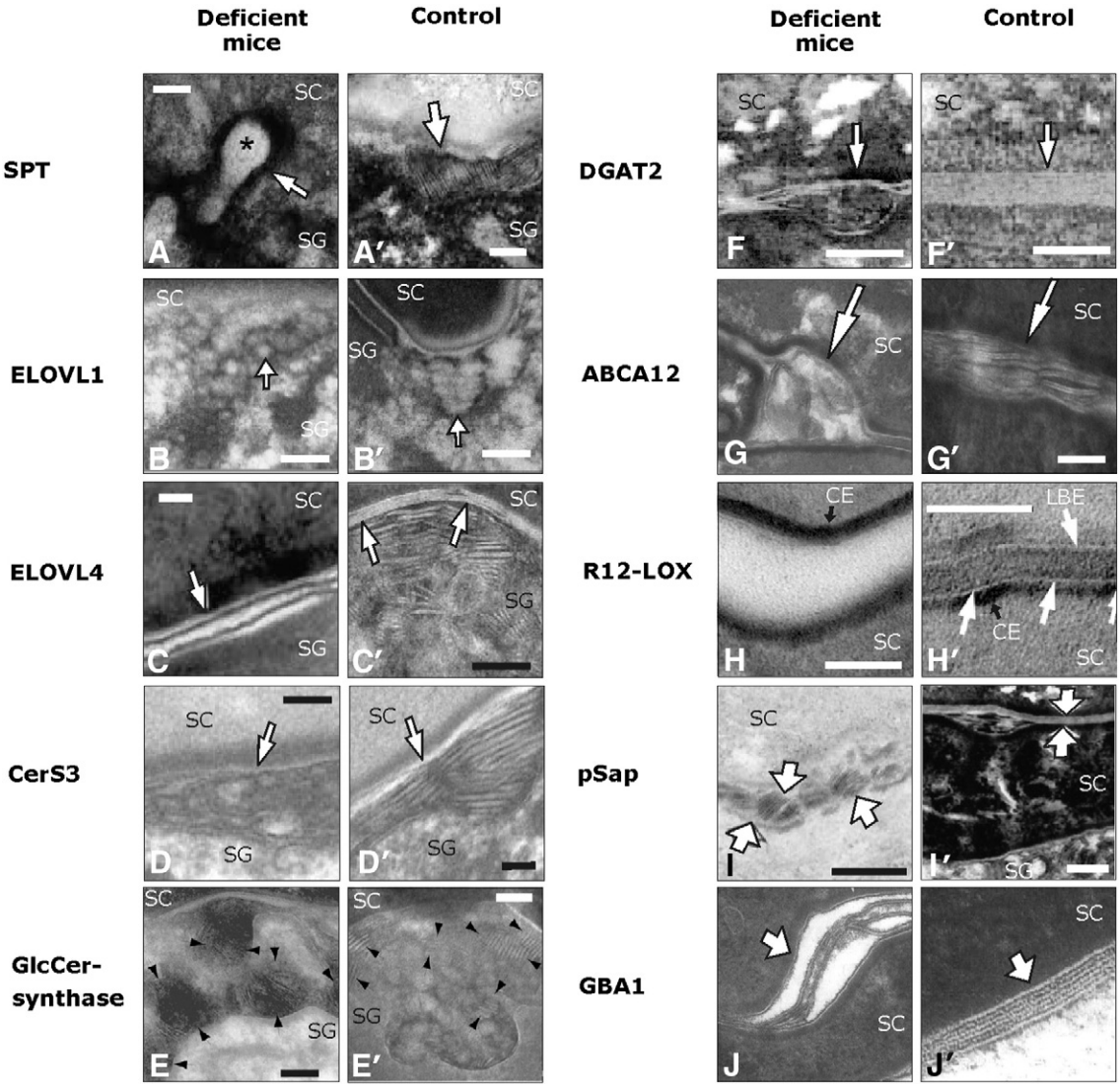

Fig. 4. Ultrastructure of murine epidermis deficient in proteins involved in the formation of acylceramides and the lipid bound envelope. Misregulation or defects in the formation of acylceramides disturb barrier function. Defects in the anabolic pathway in SPT (A), ELOVL 1 (B), ELOVL 4 (C), CerS 3 (D), GlcCer-synthase (E), DGAT2 (F), ABCA12 (G) lead to irregular lamellar bodies (white arrows in A–D, black arrows in E) and abnormal lamellar structure in the interstitial space of the SC (white arrows in F, G). R12-LOX deficient mice show no lipid bound envelope surrounding the corneocytes (H) as compared to the wild type littermates (H′) (white arrows: lipid-bound envelope, black arrows: CE). Defects in the deglucosylation of acylGlcCer and covalently-bound GlcCer to ceramide by loss of prosaposin (pSap) (I) and β-glucocerebrosidase (GBA1) (J), respectively, reveal immature (unprocessed) lamellar structures (white arrows) in the upper to outer SC interstices. EM-pictures are taken from following publications: A: [55], B: [40], C: [39], D: [12], E: [75], F: [67], G: [19], H: [89] I: [25], J: [83]. CE: cornified envelope, LBE: lipid-bound envelope, SC: stratum corneum, SG: stratum granulosum. Bars: 100 nm.

of plasma membranes by the CE, during terminal differentiation of keratinocytes.

5.1. Processing of glucosylceramides

Deglucosylation of GlcCers is assumed to be a major pathway for the generation of extracellular ceramides [83] requiring both, β-glucocerebrosidase (also called GBA1) and Sap-C [84]. Indeed, deficiencies of β-glucocerebrosidase in severely affected Gaucher patients cause a defective skin phenotype with increased transepidermal water loss and early death [85,86] similarly observed in β-glucocerebrosidase-deficient mice [26,83,87].

An almost complete deficiency of β-glucocerebrosidase activity in genetically engineered mice caused the accumulation of all epidermal GlcCer species and was accompanied by a decrease in the related ceramide levels (Fig. 2). Especially protein-bound ω-OH-GlcCer of the epidermal CE accumulated up to 35 fold in the mutant mice, whereas the related protein-bound ceramides and ω-OH ULC-FAs were completely missing [26], causing a defective epidermal barrier with excessive water loss and neonatal death as observed in collodion babies (Gaucher disease type 2) [83,85].

Essential functions of Saps for the maturation of the epidermal barrier have been identified in a mouse model with complete prosaposin deficiency (pSap-deficiency) [88]. The mutant mice suffer from increased water loss and show an ichthyotic skin. Furthermore, morphological alterations were observed, such as thickening of the stratum lucidum and the SC. Probarrier GlcCers accumulated in the epidermis of the prosaposin deficient mice, and the respective ceramides were decreased, indicating a precursor–product relationship [25]. Moreover, ω-OH-GlcCers covalently bound to proteins of the CE were discovered in the mutant mice as key intermediates for the formation of protein-bound ceramides on the surface of corneocytes. Their level was strongly increased and the contents of covalently bound ω-OH-ceramides and ω-OH-FAs were correspondingly decreased. These protein-bound GlcCers were also detected in healthy human epidermis [25]. Lipid analysis of prosaposin deficient and wild type mice indicated a pathway for the generation of hydrophobic surfaces on the CE, serving as a template for the generation of the intercellular multilamellar lipid layers in the

SC. Oxidation [89,90] and removal of the oxidized linoleic acid residue allow its replacement by γ-glutamate residues of proteins (mostly involocrin) of the CE and the formation of a lipid layer with hydrophilic glucosyl head groups on the surface of the CE (Fig. 3). Before acylGlcCers (and possibly also acylceramides) are transacylated to the CE, the linoleic moiety is stepwise oxidized by 12R lipoxygenase (12R-LOX) and epidermal lipogenase 3 (eLOX3) [89,90]. Mice deficient in one of these enzymes show neonatal lethality, a disturbed barrier function and loss of covalently bound ceramide [90–92]. A loss of 12R lipoxygenase or epidermal lipogenase 3 was found in patients with an autosomal recessive congenital ichthyosis [93].

The subsequent release of the hydrophilic β-glucosyl headgroups from the protein bound GlcCers at the CE by the concerted action of β-glucocerebrosidase and Saps, mostly Sap-C, allows the generation of a hydrophobic surface on the corneocytes, which can serve as a base for the organization of the extracellular multilamellar lipid layers in the SC. Sap-C is not only needed as an essential cofactor for the hydrolysis of GlcCers by β-glucocerebrosidase [84], but together with the other Saps for the generation of linearized and continuous multilayered lipid arrays in the extracellular space surrounding the corneocytes in the SC (Fig. 4). In prosaposin deficient epidermis (Fig. 4), the extruded lamellar body content appears to be highly disorganized and retains a characteristic pattern of discontinuously secreted piles in the interstices [25].

Saps have been identified as lipid-binding and transfer proteins, which can also fuse lipid layers [94–96]. Apparently, their absence in prosaposin deficient mice impairs the generation of continuous, lamellar lipid arrays from the secreted spherical and disconnected lamellar body piles as observed in wild type epidermis (Fig. 4). In affected SC of patients with psoriasis vulgaris reduced levels of ω-OH ceramide (Breiden & Sandhoff, unpublished data), prosaposin and β-glucocerebrosidase [97,98] have been observed.

Epidermal protein-bound GlcCers are detectable in mice three days before birth, reach a maximum and decrease with gestational age. They appear on the apical side of the outermost granular keratinocyte layer and disappear concurrently with the formation of the CE and the development of a functional epidermal barrier [99].

5.2. Sphingomyelin derived ceramides

SM also serves as a precursor of barrier ceramides. It is synthesized by the transfer of phosphorylcholine from phosphatidylcholine to ceramide. The reaction is catalyzed by sphingomyelin synthase 1 at the lumenal side of trans-Golgi membranes and by sphingomyelin synthases 2 in the plasma membrane [100]. Barrier ceramides can originate from two sources: a) from LBs [18,77,101] and b) possibly also from plasma membranes, which have a high SM content, especially in the outer leaflet, and disappear during terminal differentiation. It has been demonstrated that the epidermis contains three major SM fractions with distinct fatty acid residues (1. long chain fatty acids (C22–26), 2. short chain fatty acids (primarily C16), and 3. short chain α-OH-FAs (C16–18)) [102]. In contrast to GlcCer, which is a precursor for all ceramide species, only two ceramide species (Cer NS and Cer AS) are derived from SM.

Two isoenzymes can convert SM to ceramide, lysosomal ASM with a pH optimum at pH 5.0 and a non-lysosomal, Mg^{2+} dependent neutral sphingomyelinase. Both enzymes play an important role in permeability barrier repair processes [103,104].

Due to the acidic environment in the SC [105,106] we assume that primarily ASM is engaged in SM digestion. ASM activity has been localized in LBs and SC interface [18,79,107] and ASM deficient Niemann–Pick patients have a reduced barrier recovery [108].

5.3. Can membrane lipids regulate the extracellular processing of probarrier lipids?

Processing of probarrier lipids occurs at the SC–SG interface and the lowermost (up to three layers) SC layers demonstrated by immunoelectro microscopy for GlcCer [109] and SM [101], and degrading hydrolases [110]. Hydrolytic reactions mediated by lysosomal hydrolases take place at slightly acidic pH values [106]. Despite the fact that the SC is a mostly lipophilic environment with low water content, it should contain enough water to allow these hydrolytic processes. At least the lipophilic phase of lipid bilayers contains a water concentration of around 13 mM [11]. The water content in the lipophilic portion of the rather hydrophobic lipid layers in the interstices of the SC, however, should be even lower.

mRNA levels of key hydrolases for processing of probarrier lipids are upregulated in terminally differentiated keratinocytes, glucocerebrosidase and ASM as well as prosaposin which is processed to four different lipid binding, transfer and membrane-fusion proteins, the Saps-A, -B, -C and -D [74,94]. At least in vitro experiments established a strong stimulation of β-glucosidase catalyzed hydrolysis of GlcCers by Sap-C and anionic lipids such as bis(monoacylglycero)phosphate, phosphatidylserine, phosphatidylinositol, phosphatidic acid, and dolicholphosphates [84] and a strong inhibition by cationic bacteriocidic lipids such as glucosylsphinganine and sphingosine [84,94,111]. The extent of their actual impact on the lipid processing in the SC, however, remains unclear, since data on the exact pH, water and lipid concentrations in the SC are not available.

Sap functions are also modulated by membrane lipids. Lipid solubilizing and transfer activities of Saps-A and -B are activated by anionic lipids like bis(monoacylglycero)phosphate and inhibited by cholesterol in the lipid carrying membranes [112,113]. Furthermore, membrane fusogenic activities of Saps-C and -D are enhanced by bis(monoacylglycero)phosphate [96] as well as the ceramide cleaving activity of acid ceramidase in the presence of bis(monoacylglycero)phosphate, phosphatidylinositol and Sap-D [114].

Saps are not only required for the enzymatic hydrolysis of various GlcCers (see above), but also to establish the water permeability barrier by forming continuous extracellular multilamellar lipid layers from the secreted unconnected lipid heaps of the LBs (see above and Fig. 4). In vitro experiments established a strong regulatory influence of membrane lipids on the lipid transfer and bilayer fusion activity of Saps [94,112,113]. Anionic lipids such as bis(monoacylglycero)phosphate, phosphatidylinositol, phosphatidylserine stimulate whereas cholesterol and others are inhibitory. Their actual impact, however, remains undetermined since no relevant data are available for the SC.

Lipid hydrolyzing activities of ASM, acid ceramidase and phospholipases A2 are modulated by membrane lipids with a strong stimulatory impact of anionic lipids [94,114–117]. The influence of ASM on the extracellular lipid processing is probably larger than expected, since the enzyme cleaves not only SM but also other membrane phospholipids depending on the pH, lipid and Sap concentrations in its environment [94,116,118]. Hydrolysis of liposomal phospholipids by ASM is strongly stimulated by several anionic phospholipids in the liposomal membranes such as phosphatidylglycerol, phosphatidic acid, bis(monoacylglycero) phosphate, phosphatidylinositol, phosphatidylserine, dolicholphosphate and many others as well as by Sap-C [114]. Obviously, the biochemistry of the extracellular multilamellar lipid arrays in the SC and its enzymatic processing are much more complex than previously thought.

6. Regulation of keratinocyte differentiation and ceramide metabolism

Many proteins and factors are essential for keratinocyte differentiation and the formation of the permeability barrier. Ca^{2+}, cytokines and growth factors as well as the activation of nuclear hormone receptors have stimulating effects on the barrier formation. In contrast, testosterone, NO, and PAR2 activation inhibit the development and repair of the epidermal barrier.

In the epidermis, a Ca^{2+}-gradient has been observed with concentrations increasing from the stratum basale to the SG [119]. The Ca^{2+}-concentration is a regulator for the secretion of LBs [120]. Ca^{2+}-receptors are predominantly localized in the suprabasal layers of

Table 1
Proteins involved in the formation of the lipid bound envelope of corneocytes.

Protein	Gene	Function in the epidermis	Human disease	Knock-out mice
Serine palmitoyltransferase (heterodimer between SPTLC1 and SPTLC2 or SPTLC3)	SPTLC1–3	Generate sphinganine	Psoriasis (upregulation of SPT)	TEWL, decreased ceramide levels
Fatty acid elongase 1	*ELOVL1*	Elongates fatty acids (to C20–C28 FA)	–	TEWL, loss of acylceramides
Fatty acid elongase 4	*ELOVL4*	Elongates fatty acids (≥C26 FA)	• Heterozygous mutation: Stargardt disease 3 (juvenile dominant macular degeneration, no skin defect) • Recessive mutation: congenital ichthyosis	TEWL, loss of acylceramides
Fatty acid transport protein 4	*FATP4*	Transport of FA and synthesis of acyl-CoA	Ichthyosis prematurity syndrome	TEWL, reduction of VLCF
Cytochrome P450, family 4, subfamily F, polypeptide 22	*CYP4F22*	ω-hydroxylation of ULC-FA	Autosomal recessive congenital ichthyosis	
Ceramide synthase 3	*CERS3* (*LASS3*)	Generates ceramides	Autosomal recessive congenital ichthyosis	TEWL, loss of lipid bound envelope, accumulation of ω-OH-Cer
Ceramide glucosyltransferase	*UGCG*	Generates GlcCers	–	TEWL, loss of GlcCer, generation of acyl-SM
Acyl-CoA: diacylglycerol acyltransferase	*DAGT2*	Generates triacylglycerides	Psoriasis (upregulation of *DGAT2*)	TEWL and neonatal lethal, deficiency of ω-OH-Cer & lipid bound envelope
CGI-58 (also called abhydrolase domain containing 5)	*ABHD5*	Cofactor for hydrolysis of triacylglyceride	Dorfman–Chanarin syndrome	TEWL and neonatal lethal, deficiency of ω-OH-Cer & lipid bound envelope
ATP binding cassette sub-family A member 12	*ABCA12*	Lipid transporter of GlcCer into LB	Harlequin ichthyosis	TEWL and neonatal lethal
12R-LOX	*ALOXE12B*	Oxygenates linoleic acid residue in acylCer	Autosomal recessive congenital ichthyosis	TEWL and neonatal lethal, loss of lipid bound envelope
eLOX 3	*ALOXE3*	Oxygenates linoleic acid residue in acylCer	Autosomal recessive congenital ichthyosis	Neonatal lethal, loss of lipid bound envelope
ß-Glucocerebrosidase	*GBA1*	Hydrolysis of GlcCer to the barrier lipid ceramide in the extracellular matrix	Type 2 Gaucher disease (collodion babies)	TEWL and neonatal lethal, accumulation of GlcCer, decrease of Cer
Prosaposin	*PSAP*	Generation of saposins as lipid binding and transfer proteins, necessary for the hydrolysis of glucosphingolipids	Prosaposin deficiency	TEWL and neonatal lethal, accumulation of GlcCer, decrease of Cer
Sphingosine-1-phosphate lyase	*SGPL1*	Dephosphorylation of sphingosine-1-phosphate	Atopic dermatitis (upregulation of *SGPL1*)	No skin defect
Phytanoyl-CoA-hydroxylase	*PHYHIP*	Oxidates plant-derived branched FA	Refsum syndrome	Loss of acylceramides and of lipid bound envelope
Peroxin 7	*PEX7*	Oxidates plant-derived branched FA	Refsum syndrome	Loss of acylceramides and of lipid bound envelope
Fatty aldehyde dehydrogenase	*ALDH3A2*	Oxidation of aldehyde to FA, involved in ω-hydroxylation of ULC-FA	Sjögren–Larsson syndrome	Reduced level of acylceramide

Cer: ceramide, TEWL: transepidermal water loss.

the epidermis and sense the extracellular Ca^{2+} levels. Keratinocyte-specific Ca^{2+} sensing receptor knock-out mice have a strong barrier dysfunction due to a decreased formation and secretion of LBs and downregulation of sphingolipid transport and processing proteins (glucosyltransferase, ABCA12, β-glucocerebrosidase, ASM) [121].

Peroxisome proliferator-activated receptor (PPAR; isoforms PPARα, PPARβ/δ, PPARγ) and liver X receptor (LXR; isoforms: LXRα and LXRβ) belong to the nuclear hormone receptors which heterodimerize with retinoid X receptor (RXR). All these receptors are expressed in the epidermis and their activation stimulates keratinocyte differentiation, epidermal lipid synthesis, LB formation, and secretion of degrading enzymes in the extracellular lipid matrix. Lipids, which are required for the LB formation and barrier function, activate PPARs: FAs (e.g. linoleic acid) stimulate PPARα, PPARβ/δ, PPARγ and ceramide stimulates PPARβ/δ [122]. Expression of SPT and long chain acyl-CoA synthase is stimulated by PPARα [123] and that of ABCA12 by PPARβ/δ, PPARγ, and LXR [124]. Treatment of mice epidermis and organotypic cultures of human keratinocytes with PPAR and LXR triggers the expression of β-glucocerebrosidase [123,125].

Vitamin D receptor, another nuclear hormone receptor, controls transcription of target genes and regulates proliferation and differentiation in keratinocytes [126]. Vitamin D receptor requires the binding of cofactors to stimulate the transcription of its target genes: vitamin D receptor-interacting protein (DRIP) in basal keratinocytes and steroid receptor coactivator in differentiated cells [127,128]. Both, vitamin D receptor and steroid receptor coactivator, are important for the epidermis-specific synthesis of sphingolipids and barrier formation. Blocking vitamin D receptor and steroid receptor coactivator downregulates ELOVL3, ELOVL4, ceramide glucosyltransferase, and ABCA12 [129]. These results are comparable to vitamin D receptor knock-out mice [130].

Many proteins essential for keratinocyte differentiation are encoded by genes of the epidermal differentiation complex (EDC) clustered in region 1q21 of the human chromosome 1 [131]. Their expression is regulated by a pool of transcription factors: Krüppel-like factor 4 (Klf4), grainyhead-like 3 (Grhl3), and aryl hydrocarbon receptor nuclear translocator (Arnt). Mutations in the EDC genes are the strongest risk factors for atopic dermatitis (fillagrin genes) and faulty formation of the CE (transglutaminase 1 deficiency) causing lamellar ichthyosis [131].

Ctip2 (chicken ovalbumin upstream promoter transcription factor-interacting protein 2) also regulates genes of sphingolipid biosynthesis in the epidermis and is important for the formation of the epidermal permeability barrier [132,133]. Adult mice deficient in Ctip2 selectively in keratinocytes develop an atopic like skin with increased transepidermal water loss [134].

Atopic patients show a general deficiency in biosynthetic rates and reduced levels of barrier lipids with ULC-FA moieties, including

acylceramides. Reduced levels of epidermal protein-bound ω-OH ceramides down to 25% of control skin were observed in non-lesional areas and down to 15% of control skin in affected areas [135].

Sphingosine-1-phosphate lyase catalyzes the final step of sphingolipid catabolism, the cleavage of the sphingoid base. Sphingosine-1-phosphate is a metabolic product of sphingomyelin degradation and has an anti-proliferate effect in keratinocytes [136]. Increased expression of sphingosine-1-phosphate lyase and a resulting decrease of sphingosine-1-phosphate were observed in patients with psoriasis or atopic dermatitis [137].

Differentiation of keratinocytes and the synthesis of barrier lipids are regulated by small molecules added to the culture medium. Stimulators are Ca^{2+} (≥1 mM) [138], linoleic acid, vitamin C [139], EGF, and a slightly acidic culture medium [140]. Vitamin A, however, a component of serum usually added to the culture medium, inhibits differentiation and decreases ceramide and GlcCer content [140].

Differentiation of cultured keratinocytes is triggered by low concentrations of NO [141], up-regulating genes for structural proteins such as keratin 10 and profillagrin [74], and genes for the production of barrier acylceramides such as ceramide glucosyltransferase, glucocerebrosidase, prosaposin, SPT, and ASM [74].

Though there is no complete and coherent picture so far on the regulation of skin barrier formation, several transcription factors critically involved have been identified. Altered signaling of epidermal TCF (T cell factor) affects expression of ELOVL 3 and stearoyl-CoA desaturases causing major changes of lipid composition, compromising the water permeability barrier [142]. Formation of a functional epidermal barrier with a normal lipid composition is also dependent on the membrane-anchored serine protease CAP1/Prss8 [143,144]. Its downstream effector, the proteinase-activated receptor 2 (PAR2) [144], co-regulates keratinocyte proliferation and differentiation [145], the pH of the SC [146,147], LB secretion, and repair of the epidermal barrier.

Maintenance of the epidermal barrier function also requires a functional IκB kinase 1 (IKK1) [148] and an epithelial sodium channel (ENaC). Its deficiency causes in mice a progressive dehydration due to a decrease of the barrier lipids, ceramides and cholesterol, an increase in probarrier lipids and a strong reduction in lipids covalently bound to proteins, essential as a template on the surfaces of corneocytes for the organization of lipid layers in the interstices of the SC [149].

A functional permeability barrier also protects from microbial invasion and oxidative stress [150]. Though activation of the Nrf2 (nuclear factor erythroid 2-related factor 2) transcription factor provides skin protection under stress, mice with enhanced Nrf2 activity in keratinocytes, however, develop hyperkeratosis and inflammation which also alters the epidermal lipid barrier.

Prolonged activation of Nrf2 in keratinocytes causes acne-like skin disease in mice with sebaceous gland hyperplasia and loss of hair [151].

7. Conclusion

The permeability barrier is essential for terrestrial life. A large number of enzymes, proteins and other factors are involved in its formation and regulation. Defects of proteins involved result in an abnormal skin morphology (Fig. 4) and a disturbed barrier function. Table 1 gives an overview of human disorders and knock-out mice caused by a defect in sphingolipid metabolizing proteins.

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