



Skin permeability barrier formation by the ichthyosis-causative gene *FATP4* through formation of the barrier lipid ω -O-acylceramide

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The epidermis-specific lipid acylceramide plays a pivotal role in the formation of the permeability barrier in the skin; abrogation of its synthesis causes the skin disorder ichthyosis. However, the acylceramide synthetic pathway has not yet been fully elucidated. Namely, the acyl-CoA synthetase (ACS) involved in this pathway remains to be identified. Here, we hypothesized it to be encoded by *FATP4/ACSVL4*, the causative gene of ichthyosis prematurity syndrome (IPS). *In vitro* experiments revealed that *FATP4* exhibits ACS activity toward an ω -hydroxy fatty acid (FA), an intermediate of the acylceramide synthetic pathway. *Fatp4* knockout (KO) mice exhibited severe skin barrier dysfunction and morphological abnormalities in the epidermis. The total amount of acylceramide in *Fatp4* KO mice was reduced to ~10% of wild-type mice. Decreased levels and shortening of chain lengths were observed in the saturated, nonacylated ceramides. FA levels were not decreased in the epidermis of *Fatp4* KO mice. The expression levels of the FA elongase *Elovl1* were reduced in *Fatp4* KO epidermis, partly accounting for the reduction and shortening of saturated, nonacylated ceramides. A decrease in acylceramide levels was also observed in human keratinocytes with *FATP4* knockdown. From these results, we conclude that skin barrier dysfunction observed in IPS patients and *Fatp4* KO mice is caused mainly by reduced acylceramide production. Our findings further elucidate the molecular mechanism governing acylceramide synthesis and IPS pathology.

acylceramide | ceramide | lipid | skin | sphingolipid

Skin possesses a powerful permeability barrier (the skin barrier), which functions to prevent infectious diseases and allergies by blocking the invasion of external substances such as pathogens, allergens, and chemicals. It also prevents water loss from the body, an essential function for terrestrial animals. The skin barrier is so strong that infections through the skin rarely occur under normal circumstances; but if it is damaged, such as by a burn or cut, the risk of infection increases dramatically. Ichthyosis is an inherited disease featuring skin barrier abnormalities and is characterized by dryness, thickening, and scaly skin (1). Similarly, atopic dermatitis patients suffer from reduced skin barrier function, allowing allergens to enter (2).

Ichthyosis prematurity syndrome (IPS) is one of the syndromic forms of ichthyosis (3, 4). IPS is characterized by premature birth, neonatal asphyxia, and ichthyosis. Ichthyosis improves during the first several weeks after birth but persists for life, often accompanying atopic dermatitis and recurrent infections. The epidermis of IPS patients is thickened, and hyperkeratosis and presence of droplets are observed in the stratum corneum (3, 5). Furthermore, curved multilamellar membranes exist in the stratum corneum and granular layer of such patients (3, 5, 6). The causative gene of IPS is *FATP4* (fatty acid transporter, member 4; also known as *ACSVL4* [acyl-CoA synthetase very-long-chain, member 4] or *SLC27A4* [solute carrier family 27, member 4]) (3). As the alternative name of *FATP4*, *ACSVL4*, indicates, *FATP4* is an acyl-CoA synthetase (ACS) that converts fatty acids (FAs) to

acyl-CoAs. FAs are classified by chain length: Long-chain (LC) FAs have a chain length of C11–20 and very-long-chain (VLC) FAs have a length of \geq C21. VLCFAs with \geq C26 are sometimes denominated ultra-long-chain (ULC) FAs, since their functions and tissue distributions differ from shorter VLCFAs (7, 8). *FATP4/ACSVL4* belongs to the very-long (VL) subfamily of ACSs and exhibits higher ACS activity toward VLCFAs than LCFAs (9). Several *Fatp4* knockout (KO) mice have been reported, either generated spontaneously or by gene targeting (10–14). Except for one report indicating prenatal lethality (12), *Fatp4* KO mice are neonatal lethal (10, 11, 13, 14). Newborn *Fatp4* KO mice exhibit severe skin barrier abnormalities, hyperkeratosis, keratinocyte hyperproliferation, acanthosis, and small keratohyalin granules (10, 11, 13, 14). In addition, the rigid skin of *Fatp4* KO mice causes facial dysmorphism, movement restriction, and respiratory failure. However, the mechanism by which mutations in the *FATP4/Fatp4* gene cause ichthyosis in IPS patients and *Fatp4* KO mice remains unclear.

Lipids are suitable for the formation of permeability barriers due to their high hydrophobicity. The stratum corneum contains a multilayer lipid structure (the lipid lamellae) that plays a central role in skin barrier formation (8, 15). The lipid lamellae are mainly composed of ceramides, FAs, and cholesterol. Ceramide forms the

Significance

Acylceramide is essential for skin permeability barrier formation. However, its biosynthesis pathway has not yet been elucidated in its entirety. In the present study, we found that *Fatp4* disruption substantially decreased the amount of acylceramides in mice, as did *FATP4* knockdown in human keratinocytes. In addition, *in vitro* experiments demonstrated that *FATP4* exhibited acyl-CoA synthetase activity toward an ω -hydroxy ultra-long-chain fatty acid, an intermediate of the acylceramide biosynthetic pathway. From these results, we conclude that *FATP4* functions in skin barrier formation through acylceramide synthesis. Our findings not only reveal the pathogenic mechanism of ichthyosis prematurity syndrome, but also help to elucidate the molecular mechanism of the synthesis of the skin barrier lipid acylceramide.

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backbone of sphingolipids (biological membrane lipids) consisting of two hydrophobic chains: a long-chain base and a FA (Fig. 1A). In addition to this normal-type ceramide, acylceramide (ω -O-acylceramide), a ceramide class specialized for skin barrier formation, exists in the epidermis (8, 16, 17). Acylceramide shows a unique three hydrophobic-chain structure, in which linoleic acid is esterified to the ω -position of the FA moiety of ceramide (Fig. 1B). As a C30–36 ULCFA, acylceramide also has a characteristic and much longer FA chain length than normal ceramide (C16–24). The unique structure of acylceramide is important for the formation and maintenance of lipid lamellae and skin barrier formation: Mutations in the human genes involved in its synthesis

cause autosomal recessive congenital ichthyosis, the most severe type in the family of disorders (1, 8, 17, 18). In mice, these mutations lead to neonatal lethality due to skin barrier dysfunction (19–24).

Despite the importance of acylceramide in skin barrier formation, most of the genes involved in acylceramide production have only recently been identified. The identified genes include the FA elongases *ELOVL1* and *ELOVL4* for elongation of FAs up to C30–36 (19, 21, 25), the ceramide synthase *CERS3* for amide bond formation between a long-chain base and a ULCFA (20), the cytochrome P450 member *CYP4F22* for ULCFA ω -hydroxylation (26), and the transacylase *PNPLA1* for ester bond formation between a ULCFA and a linoleic acid (22–24, 27) (*SI Appendix*, Fig. S1).

We previously identified the above-mentioned FA ω -hydroxylase gene *CYP4F22*, which hydroxylates the ω -position of ULCFAs (26). *CYP4F22* is one of the causative genes of autosomal recessive congenital ichthyosis (28–30), and the amount of acylceramide was greatly reduced in the patient examined in that study (26). We also found that the substrates of *CYP4F22* are ULCFAs using biochemical and cell-based assays (26). The FA elongases *ELOVL1* and *ELOVL4* are responsible for the elongation of shorter FAs to ULCFAs (19, 21, 25). Since the elongation reactions proceed in acyl-CoA forms (7, 8), ULC acyl-CoAs produced by the FA elongases must be converted to ULCFAs in order to be catalyzed by *CYP4F22*. After ω -hydroxylation by *CYP4F22*, the resulting ω -hydroxy (ω -OH) ULCFAs need to be reconverted to acyl-CoA forms (Fig. 1B), the substrates of the subsequent reaction catalyzed by the ceramide synthase *CERS3* (20). However, the ACS involved in this reaction has remained unknown.

Given the importance of acylceramide in skin barrier formation, mutations in the unidentified ACS gene in the acylceramide synthesis pathway are expected to cause congenital ichthyosis. Twenty-six ACSs exist in mammals (31). Of these, we speculate that *FATP4* is the unidentified ACS gene that functions in the acylceramide synthesis pathway, based on the severe skin symptoms/phenotypes observed in human IPS patients and *Fatp4* KO mice. Since no hypotheses about the involvement of *FATP4* in acylceramide synthesis have been proposed so far, acylceramide levels have not yet been measured in *Fatp4* KO mice. Shortening of the chain length of nonacylated (normal type) ceramides has been reported in *Fatp4* KO mice, specifically, decreases in \geq C26 ceramides and increases in \leq C24 ceramides (10). In the present study, we performed several analyses of *Fatp4* KO mouse epidermis to examine the function of *Fatp4* in acylceramide synthesis and to reveal the causes of skin barrier dysfunction observed in IPS patients.

Results

Abnormalities in Skin Barrier Formation and Epidermal Morphology in *Fatp4* KO Mice. To date, several *Fatp4* mutant mouse lines have been generated (10–14). To investigate the involvement of *FATP4* in acylceramide synthesis in the present study, we created another *Fatp4* KO mouse line by genome editing using CRISPR-Cas9. Setting the target sequence to exon 3 of the *Fatp4* gene, we obtained *Fatp4* KO mice having a single nucleotide insertion between the 196th thymine and the 197th guanine in the coding sequence (Fig. 1C). This gene product is predicted to contain the N-terminal 66 amino acids of wild-type (WT) protein (643 amino acids) followed by an unrelated 42 amino acids, due to a frameshift and its accompanying early stop codon. Since the translated region is very short, this mutated gene product is expected to be completely nonfunctional. As in the previously reported *Fatp4* mutant mice (10, 11, 14), our *Fatp4* KO mice also had more rigid, less wrinkled skin than WT mice (Fig. 1D) and were neonatal lethal. We then examined the time course of the survival rates of the mice obtained by cesarean section on embryonic day 18.5 (E18.5). All KO mice died within 1 h after

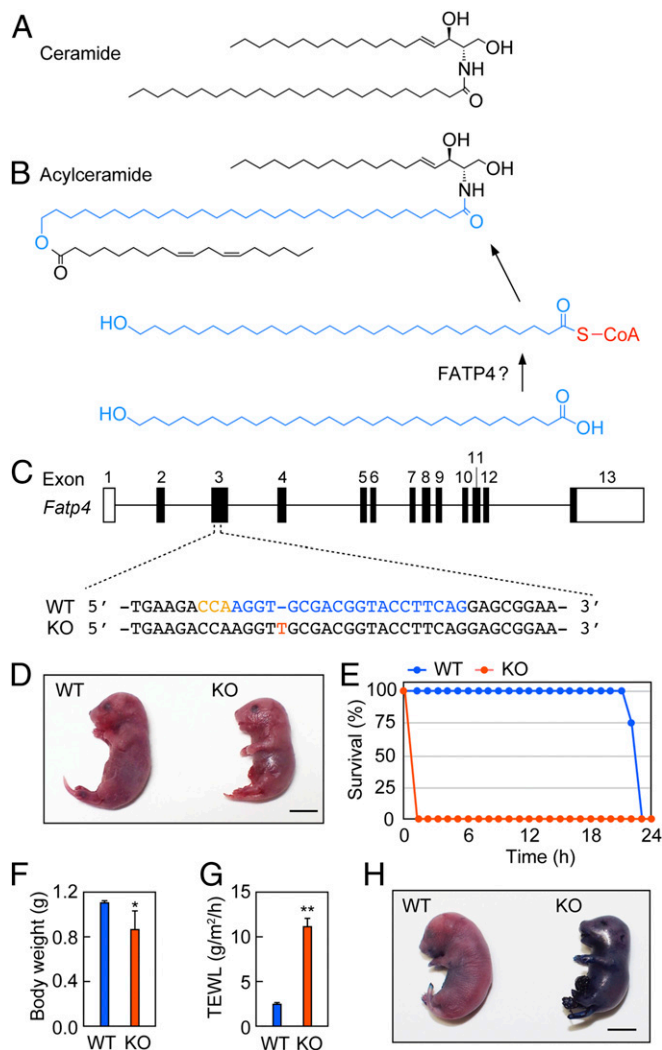


Fig. 1. Skin barrier dysfunction in *Fatp4* KO mice. (A) Structure of ceramide. (B) Structure of acylceramide and reactions that introduce ω -OH ULCFA into acylceramide. (C) Genome structure of mouse *Fatp4* gene and WT and mutant nucleotide sequences around CRISPR-Cas9 target sequence. The black boxes represent coding sequences. Red, inserted nucleotide in *Fatp4* KO mouse; blue, target sequence of the guide RNA; orange, protospacer adjacent motif (PAM) sequence. (D–H) WT and *Fatp4* KO mice were prepared by cesarean section at E18.5. (D) Photograph of specimens. (Scale bar, 1 cm.) (E) Measurement of survival rates (WT, $n = 4$; KO, $n = 3$). (F) Measurement of body weights (WT, $n = 4$; KO, $n = 4$). (G) Measurement of TEWL (WT, $n = 4$; KO, $n = 4$). Values represent the means \pm SD. Statistically significant differences are indicated (two-tailed Student's *t* test; * $P < 0.05$; ** $P < 0.01$). (H) WT and *Fatp4* KO mice were incubated with 0.1% toluidine blue solution for 2 h and photographed. (Scale bar, 1 cm.)

cesarean section (Fig. 1E). Under the same conditions, where newborn mice were taken from their mothers and could not drink milk, even WT mice did not survive beyond 22 h after cesarean section. The body weight of E18.5 KO mice was lower than that of WT mice (Fig. 1F). The value of the trans-epidermal water loss (TEWL), an index of the skin permeability barrier function from inside the body to the outside, was 4.4-fold higher in *Fatp4* KO mice than WT mice (Fig. 1G). The inverse skin permeability barrier function, from the outside to the body, was examined by toluidine blue staining. Little dye penetration was observed in WT mice, while strong penetration was observed in *Fatp4* KO mice (Fig. 1H). These high TEWL values and enhanced dye staining have also been reported in the previous *Fatp4* KO mice (10, 13), confirming the presence of impaired skin barrier function in our newly created *Fatp4* KO mice.

Hematoxylin/eosin staining was conducted to examine morphological changes in the epidermis of *Fatp4* KO mice. In the stratum corneum of WT mice, intercorneocyte gaps, which correspond to lipid lamellae, were observed (Fig. 2A). In *Fatp4* KO mice, the existence of gaps was less obvious, indicating impaired lipid lamellae formation. Acanthosis was observed in the stratum spinosum of *Fatp4* KO mice. A more detailed examination of the structure of the epidermis was carried out by transmission electron microscopy. The number of cell layers in the stratum corneum was increased in *Fatp4* KO mice compared to WT mice (Fig. 2B and C). The existence of keratohyalin granules filled with profilaggrin is a feature of the stratum granulosum. These granules in *Fatp4* KO mice were smaller than those in WT mice (Fig. 2B). Hyperkeratosis, acanthosis, and small keratohyalin granules were also observed in the previously reported *Fatp4* mutant mice (10, 11, 13, 14). We found vacuoles and curved membrane structures, which may represent deposits of lipids/membranes probably caused by impaired lamellar body release, in the stratum corneum of *Fatp4* KO mice (Fig. 2C and D). Similar structures have also been observed in IPS patients (3, 5, 6). These morphological abnormalities suggest that keratinocyte differentiation and lipid lamellae formation are impaired in the *Fatp4* KO mouse epidermis.

Decreased Amount of Acylceramide in *Fatp4* KO Mouse Epidermis. To examine the effect of *Fatp4* disruption on epidermal lipid composition, lipids were extracted from the mouse epidermis, separated by thin-layer chromatography (TLC), and stained with copper sulfate/phosphoric acid solution. We observed decreases in the amounts of acylceramide and acyl-glucosylceramide in *Fatp4* KO mice compared with WT mice, whereas FA and triacylglycerol (triglyceride; TAG) levels were increased (Fig. 3A). No apparent differences between WT and *Fatp4* KO mice were found in the amounts of cholesterol and glycerophospholipids.

Quantitative analysis of acylceramide species was performed using liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS). In *Fatp4* KO mice, the amounts of all of the acylceramide species, regardless of FA chain length and degree of unsaturation (saturated or monounsaturated), were substantially decreased compared with those in WT mice (Fig. 3B and C). The total amount of acylceramides in *Fatp4* KO mice was 9.8% of WT mice, with saturated and monounsaturated acylceramides being similarly reduced (saturated, 7.4%; monounsaturated, 11%). These results indicate that *Fatp4* is indeed involved in the acylceramide synthesis.

We also measured the amounts of acyl-glucosylceramides, which are acylceramide metabolites (SI Appendix, Fig. S1). The total amount of saturated acyl-glucosylceramides was substantially reduced in *Fatp4* KO mice (9.1% of WT mice; Fig. 3D), as was that of saturated acylceramides (Fig. 3C). On the other hand, the decrease in the total amount of monounsaturated acyl-glucosylceramides in KO mice was rather small (46% of WT mice; Fig. 3D), in contrast to

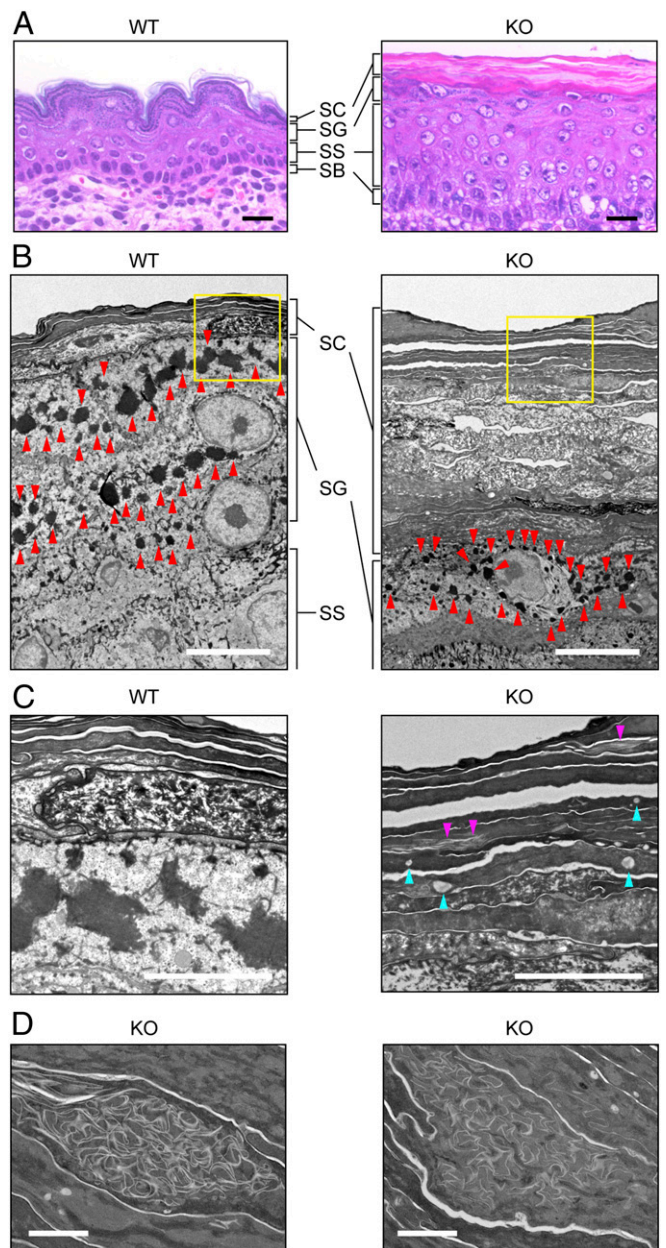


Fig. 2. Hyperkeratosis and impaired formation of lipid lamellae and keratohyalin granules in *Fatp4* KO mice. (A) Paraffin sections of E18.5 WT and *Fatp4* KO mouse skin were prepared and stained with hematoxylin and eosin, and bright field images were photographed. (Scale bars, 20 μm .) (B–D) Ultrathin sections of E18.5 WT and *Fatp4* KO mouse skin were prepared and subjected to transmission electron microscopy. (B) Red arrowheads indicate keratohyalin granules. (Scale bars, 10 μm .) (C) Enlarged views of the yellow boxes in B. Magenta and light blue arrowheads indicate curved membrane structures and vacuoles in corneocytes, respectively. (Scale bars, 5 μm .) (D) Enlarged views of the curved membrane structures observed in *Fatp4* KO mouse skin are shown. (Scale bars, 1 μm .) SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale.

that of monounsaturated acylceramides (Fig. 3C). The total amount of acyl-glucosylceramides (saturated plus monounsaturated) was 37% of that in WT mice (Fig. 3D).

We next measured the amounts of protein-bound ceramides in *Fatp4* KO mice, into which a portion of the acylceramide is converted (SI Appendix, Fig. S1). Protein-bound ceramide is a crucial component of a unique membrane structure to corneocytes, termed the corneocyte lipid envelope, which is thought to function

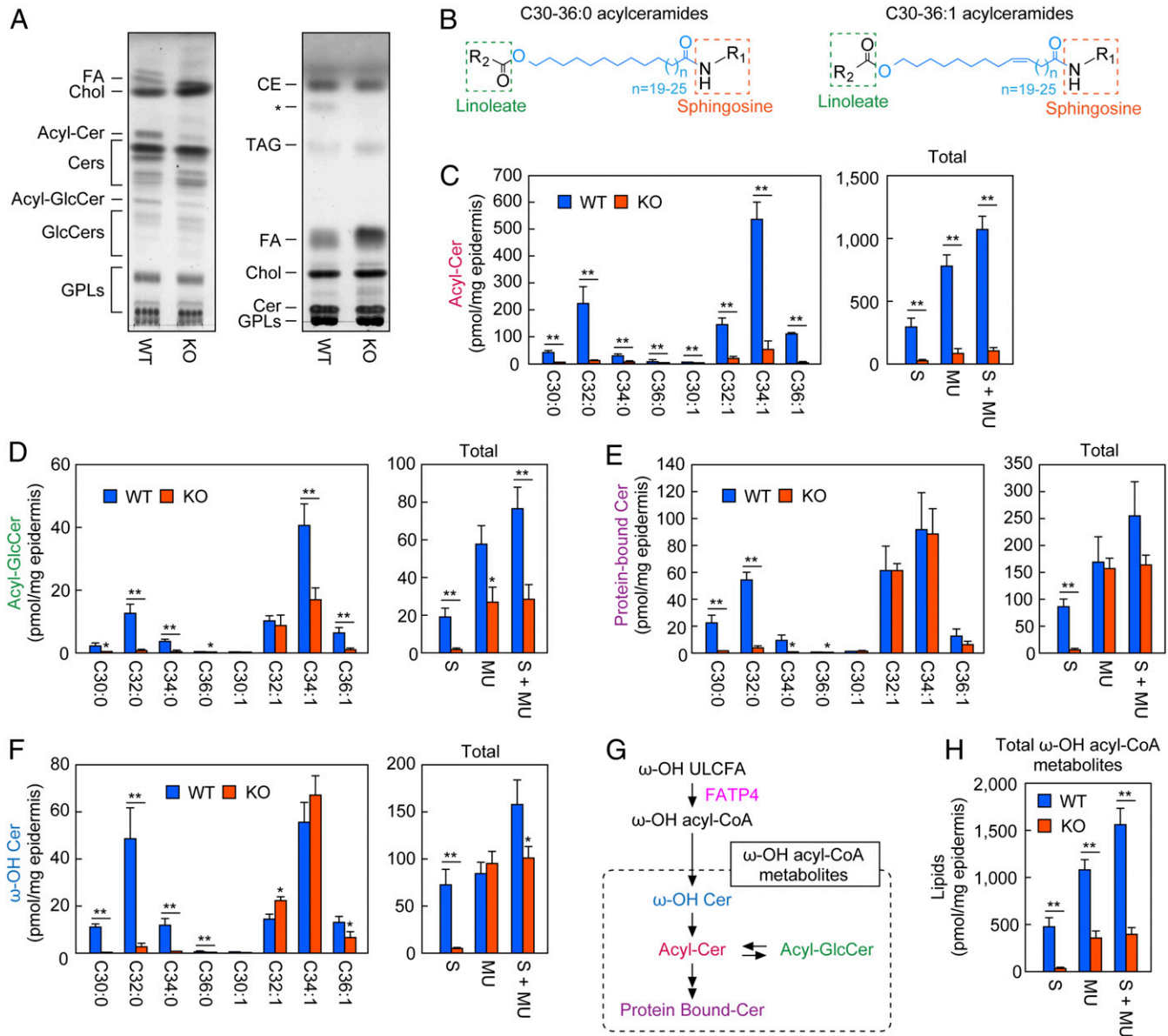


Fig. 3. Decreased amounts of acylceramides in the epidermis of *Fatp4* KO mice. (A) Lipids were extracted from the epidermis of WT and *Fatp4* KO mice at postnatal day 0. Lipids (2.5 mg skin) were separated by TLC using the solvent system for separation of ceramide and glucosylceramide (Left) and that for separation of TAG, FA, and cholesterol (Right) and stained with copper sulfate/phosphoric acid solution. The asterisk represents unidentified lipid band. (B) Structures of saturated (Left) and monounsaturated (Right) acylceramides. The position of the double bond in the monounsaturated FA of acylceramide is n-9. Sphingosine is the most abundant long-chain base among mammalian long-chain bases. (C–F) Lipids were extracted from the epidermis of WT ($n = 3$) and *Fatp4* KO ($n = 3$) mice at postnatal day 0. LC-MS/MS analyses were performed on acylceramides (C), acyl-glucosylceramides (D), protein-bound ceramides (E), and ω -OH ceramides (F). The Left shows the amount of each lipid species, and the Right shows the total of saturated (S) species, total of monounsaturated (MU) species, and total of all species. (G) The metabolic pathway of ω -OH ULCFA, an intermediate of acylceramide. (H) Sum of the amounts of ω -OH acyl-CoA metabolites [acylceramide (C), acyl-glucosylceramide (D), protein-bound ceramide (E), and ω -OH ceramide (F)]. Values represent the means \pm SD. Statistically significant differences are indicated (two-tailed Student's *t* test; * $P < 0.05$; ** $P < 0.01$). Chol, cholesterol; Acyl-Cer, acylceramide; Cer, ceramide; Acyl-GlcCer, acyl-glucosylceramide; GlcCer, glucosylceramides; GPL, glycerophospholipid.

in connecting lipid lamellae and corneocytes (32–34). Again, a difference in the degree of reduction between saturated and monounsaturated types owing to *Fatp4* disruption was observed. The amount of total saturated protein-bound ceramides in *Fatp4* KO mice was reduced to 6.8% of that in WT mice, whereas the total amount of monounsaturated protein-bound ceramides remained comparable (Fig. 3E). In addition, the total amount of protein-bound ceramides (saturated plus monounsaturated) was 65% of that in WT mice.

The final step of acylceramide production is the ester bond formation between ω -OH ceramide and linoleic acid (SI Appendix,

Fig. S1). This reaction is catalyzed by the transacylase PNPLA1, where PNPLA1 transfers the linoleic acid in TAG to ω -OH ceramide (27). We measured the amounts of ω -OH ceramides, which are precursors of acylceramides, and found a large decrease (6.5% of WT mice) in the total amount of saturated ω -OH ceramides in *Fatp4* KO mice (Fig. 3F). In contrast, no difference was observed in the total amounts of monounsaturated ω -OH ceramides. The total amount of ω -OH ceramides (saturated plus monounsaturated) in *Fatp4* KO mice was decreased to 64% of that in WT mice.

In the acylceramide synthetic pathway, ω -OH acyl-CoA, the product of FATP4, is converted to ω -OH ceramide, acylceramide,

acyl-glucosylceramide, and protein-bound ceramide (Fig. 3G and *SI Appendix*, Fig. S1). The sum of all these ω -OH acyl-CoA metabolites in *Fatp4* KO mice was reduced to 25.6% of WT mice (Fig. 3H). The decrease was large in saturated types (7.2%) but smaller in monounsaturated types (33.5%).

***Fatp4* Gene Disruption Does Not Reduce the Uptake of FAs into the Epidermis.** Previous studies measuring ceramides (nonacylated, normal type) reported that \geq C26 ceramides were decreased in *Fatp4* KO epidermis compared with WT mice, whereas \leq C24 ceramides were increased (10). In the present study, we performed more detailed analyses. First, we confirmed the decreases in \geq C26 and increases in \leq C24 for saturated ceramides in the epidermis of the *Fatp4* KO mice (Fig. 4A), consistent with the previous report (10). While that study measured only C18:1 and C24:1 ceramides for monounsaturated ceramides (10), we here expanded the analyses to \geq C24 monounsaturated species. As in the case of saturated ceramides, the amount of monounsaturated ceramide with a chain length of C24 was also increased in *Fatp4* KO mice compared with WT mice (Fig. 4A). On the other hand, unlike the saturated types, the amount of C26 monounsaturated ceramide was unchanged, and those of \geq C28 monounsaturated ceramides were unexpectedly increased. In summary, the total amount of \leq C24 ceramides was increased approximately threefold in *Fatp4* KO mice compared with WT mice, whereas that of \geq C26 saturated ceramides was decreased

to 34%. The total amount of \geq C26 monounsaturated ceramides as well as total amount of ceramides (saturated plus monounsaturated) was almost unchanged. Thus, *Fatp4* disruption had a greater effect on acylceramides than nonacylated ceramides. Similar changes in ceramide composition (increases in \leq C24 and decreases in \geq C26 ceramides, and large effects on saturated ceramides but mild effects on monounsaturated ceramides) were also observed in *Elovl1* (a FA elongase)-KO mice (21), implying a reduced activity of *Elovl1* in *Fatp4* KO mice.

Since the effect of *Fatp4* disruption on the abundance of individual FA species has so far not been investigated using MS, we estimated species composition and abundance using epidermal samples. Similar to ceramides (Fig. 4A), the amounts of most \leq C24 FAs were increased in *Fatp4* KO mice compared with WT mice (Fig. 4B). In contrast, the amounts of \geq C26 saturated FAs in *Fatp4* KO mice were not significantly different from those in WT mice, and \geq C26 monounsaturated FAs were unexpectedly increased. The total amount of FAs in *Fatp4* KO mice was 2.1-fold higher than that of WT mice. The substrates of FA elongases in the FA elongation process are acyl-CoAs rather than FAs (7). Therefore, the VLCFAs and ULCFAs in *Fatp4* KO mice are most likely produced from the corresponding acyl-CoAs via hydrolysis of CoA by thioesterases after FA elongation. The increases in many FAs, especially monounsaturated FAs, in *Fatp4* KO mice suggest that FATP4 has a function of converting the FAs generated by thioesterase back to acyl-CoAs. It is likely that the equilibrium between FAs and acyl-CoA shifts to FAs due to *Fatp4* disruption. Although an FA transporter function of FATP4 has been hypothesized (4, 11), our results (specifically the increases in the amounts of FAs in *Fatp4* KO mice) did not support this hypothesis. Since linoleic acid (C18:2), a component of acylceramide, is an essential FA that cannot be synthesized in mammals, keratinocytes must procure it from outside the cells. However, the amount of linoleic acid was also increased in *Fatp4* KO epidermis (Fig. 4B).

The linoleic acid moiety in acylceramide is derived from TAG; *Pnpla1* transfers linoleic acid in TAG to ω -OH ceramide by transacylation (27) (*SI Appendix*, Fig. S1). We examined the amounts of linoleic acid-containing TAGs in the epidermis of *Fatp4* KO mice and found that all TAG species examined were increased compared with WT mice (Fig. 4C). These results indicate that the skin barrier dysfunction and the decreased acylceramide in *Fatp4* KO mice are not due to reduced uptake of linoleic acid.

Decreased Expression of *Elovl1* and *Pnpla1* due to *Fatp4* Disruption.

Abnormalities in epidermal morphology and changes in lipid metabolism were observed in *Fatp4* KO mice (Figs. 2–4). To investigate the causes behind these changes, the expression levels of keratinocyte differentiation markers and lipid metabolism genes were analyzed by quantitative RT-PCR. Among keratinocyte differentiation markers, the stratum basale marker *Krt14* (keratin 14) showed a tendency to increase in *Fatp4* KO mice compared with WT mice, although this was not statistically significant (Fig. 5A). In contrast, *Krt10* (keratin 10), a marker of the stratum spinosum and stratum granulosum, was reduced in the KO mice (8.3% of WT mice). The stratum granulosum markers *Flg* (filaggrin) and *Ivl* (involucrin) were also decreased (1.2% and 7.8% of WT mice, respectively). We confirmed decreases in the protein levels of filaggrin and keratin 10 by immunoblotting (*SI Appendix*, Fig. S2). These results indicate that differentiation into stratum spinosum and/or stratum granulosum was impaired in the *Fatp4* KO mouse epidermis. Abnormal expression of differentiation markers has been observed in mice with KO of several genes involved in acylceramide synthesis (*Pnpla1*, *Cers3*, and *Cyp4f39* KO mice) (20, 22, 23, 35). Although the exact reason is unknown, it is possible that keratinocytes sense and respond to impaired lamellar body and lipid lamella formation.

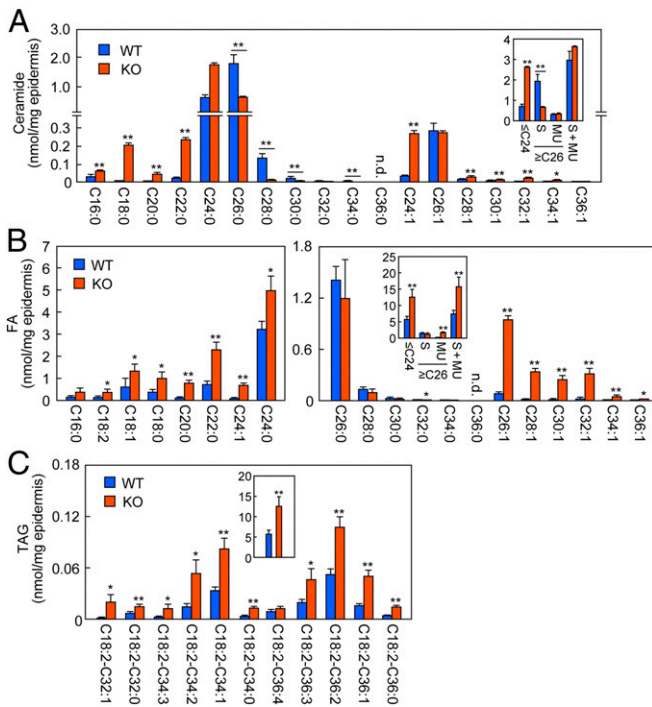


Fig. 4. Effects of *Fatp4* disruption on ceramide, FA, and TAG levels. (A–C) Lipids were extracted from the epidermis of WT ($n = 3$) and *Fatp4* KO ($n = 3$) mice at postnatal day 0. LC-MS/MS analyses were performed on ceramides (A), FAs (B), and TAGs containing linoleic acid (C). (A and B) The graphs show the amount of each lipid species, and the *Insets* show the total of \leq C24 species, total of \geq C26 saturated (S) species, total of \geq C26 monounsaturated (MU) species, and total of all species. (C) The graph shows the amount of each TAG species containing linoleic acid (C18:2), while the *Inset* shows the total TAG quantities. The numbers after “C18:2-” represent the sum of two FAs other than linoleic acid. For example, C32:1 indicates that the summed chain length and degree of unsaturation of the two FA chains are 32 and 1, respectively. Values represent the means \pm SD. Statistically significant differences are indicated (two-tailed Student’s *t* test; * $P < 0.05$; ** $P < 0.01$). n.d., not detected.

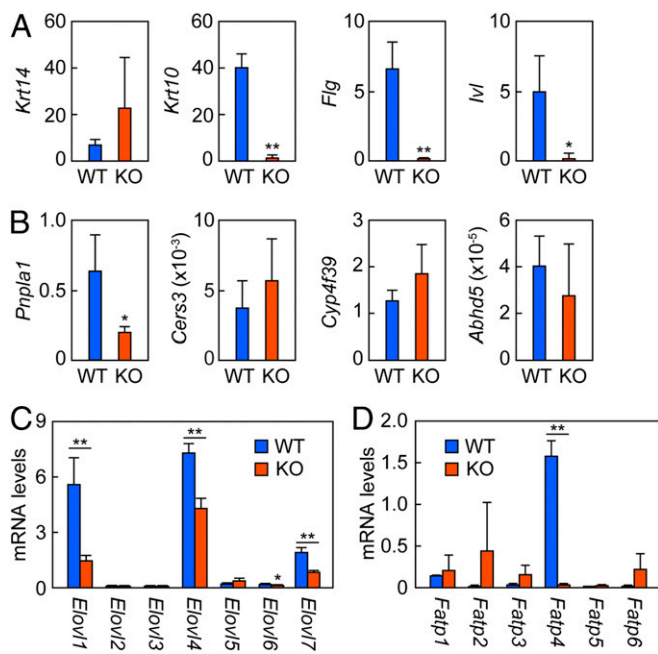


Fig. 5. Effects of *Fatp4* disruption on the expression levels of keratinocyte differentiation markers and acylceramide synthesis-related genes. Total RNA was prepared from the epidermis of WT ($n = 3$) and *Fatp4* KO ($n = 3$) mice at postnatal day 0. Real-time quantitative RT-PCR was performed on *Hprt1*, keratinocyte differentiation markers (*Krt14*, *Krt10*, *Flg*, and *IvI*) (A), acylceramide synthesis-related genes (*Pnpla1*, *Cers3*, *Cyp4f39*, and *Abhd5*) (B), *Elov1* family members (*Elov1*–*7*) (C), and *Fatp* family members (*Fatp1*–*6*) (D). Values represent the means \pm SD and indicate the relative amounts to *Hprt1*. Statistically significant differences are indicated (two-tailed Student's *t* test; * $P < 0.05$; ** $P < 0.01$).

Next, the expression levels of acylceramide synthesis-related genes were examined (Fig. 5B). The expression levels of the transacylase *Pnpla1* were reduced in the *Fatp4* KO mouse epidermis (32% of WT mice), while almost no changes were observed in the expression levels of the ceramide synthase *Cers3*, the FA ω -hydroxylase *Cyp4f39* (a mouse ortholog of human *CYP4F22*), or *Abhd5*, a gene product that functions to promote utilization of TAGs by *Pnpla1* (36).

Since changes in the chain lengths of ceramides and FAs were observed (Fig. 4A and B), the expression levels of the FA elongase *Elov1* family members were examined. In *Fatp4* KO mice, the expression levels of *Elov1* were reduced to 25% of WT mice (Fig. 5C). *Elov1* plays an important role in the elongation of C24:0-CoA to C26:0-CoA in epidermis (21). The expression levels of *Elov4*, which is responsible for elongation of C26-CoA to \geq C28-CoAs (7, 8, 19), were reduced to 59% of WT mice. The expression levels of *Elov6* and *Elov7*, which respectively exhibit activity toward C16:0-CoA and C18-CoAs (7, 8, 25), were reduced to about half the levels of WT mice.

We investigated the possibility of compensatory changes in the expression of other *Fatp/Acsvl* family members due to *Fatp4* disruption. In WT mice, expression levels of *Fatp4* were the highest among *Fatp* family members, as also observed in human keratinocytes (37). The expression levels of *Fatp4* were substantially reduced in *Fatp4* KO mice (Fig. 5D), possibly due to destabilization of *Fatp4* mRNA by nonsense-mediated mRNA decay. In *Fatp4* KO mice, the expression levels of *Fatp1*, *Fatp2*, *Fatp3*, *Fatp5*, and *Fatp6* showed tendencies to increase, although these were not statistically significant. In summary, our results suggest that changes in ceramide/fatty acid chain lengths and decreased acylceramide levels observed in *Fatp4* KO mice are at

least partly attributable to decreased expression levels of *Elov1* and *Pnpla1*.

FATP4 Exhibits ACS Activity toward ω -OH ULCFA. We speculate that FATP4 acts as an ACS that converts ω -OH ULCFAs to ω -OH ULC acyl-CoAs in the acylceramide synthetic pathway. However, it has not been examined whether FATP4 has ACS activity toward ω -OH ULCFAs, although activity toward LC and VLC nonhydroxylated FAs has been reported (9, 38, 39). Therefore, we performed in vitro ACS assays using ω -OH C30:0 FA as a substrate and membrane fractions prepared from HEK 293T cells overexpressing human FATP4 as enzyme source. Overexpression of FATP4 caused a 10.5-fold increase in ACS activity compared with control (Fig. 6). Furthermore, overexpression of FATP4 increased ACS activities toward C24:0 and C24:1 FAs by 6.6- and 3.5-fold, respectively. It is worthwhile to note that substrate preference for FATP4 among FA substrates cannot be evaluated by this method, since the water solubility of the substrate FAs, which affects the enzyme activity, and the ionization efficiency of the product acyl-CoAs are different. Our results indicate that FATP4 can indeed produce ω -OH ULC acyl-CoAs, which are required for acylceramide synthesis. In addition, our findings reveal that FATP4 is active toward a wide range of FAs from LC to ULC, regardless of nonhydroxylated or ω -hydroxylated status.

FATP4 Knockdown in Human Keratinocytes Decreases Acylceramide Production. We next performed knockdown analyses to investigate whether FATP4 is involved in acylceramide production in human keratinocytes. Human keratinocytes were infected with lentiviral plasmids to express an shRNA targeting *FATP4*, and they then differentiated. Two shRNAs targeting different *FATP4* sequences (sh*FATP4*-1 and sh*FATP4*-2) both showed a knockdown efficiency of over 90% (Fig. 7A). These *FATP4* shRNAs reduced the amounts of all C30–36 acylceramide species, and they were decreased overall by \sim 40% compared to the control

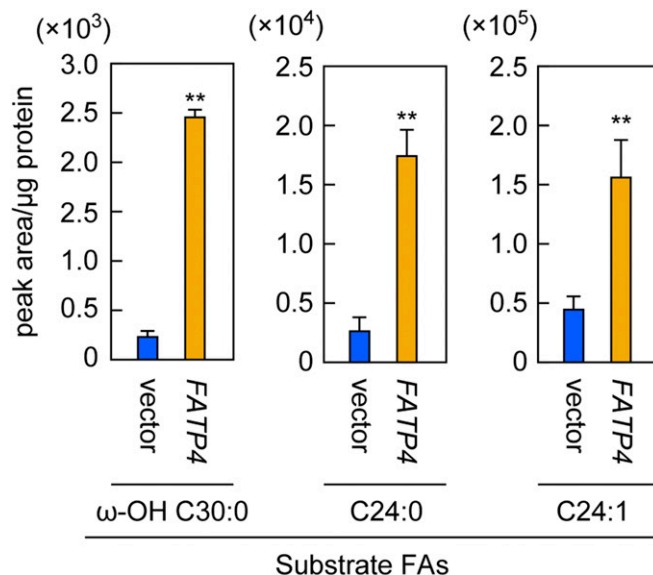


Fig. 6. ACS activity of FATP4 toward ω -OH ULCFA. HEK 293T cells were transfected with vector (pCE-puro 3 \times FLAG-1) or a plasmid encoding 3 \times FLAG-FATP4 (pCE-puro 3 \times FLAG-FATP4), and membrane fractions were prepared 24 h after transfection. Membrane fractions (20 μ g) were incubated with 10 μ M FA (ω -OH C30:0 FA, C24:0 FA, or C24:1 FA) and 0.5 mM CoA, 2.4 mM ATP at 37 $^{\circ}$ C for 1 h. The generated acyl-CoAs were extracted and quantified by LC-MS/MS. Values represent the means \pm SD obtained from three independently prepared samples. Statistically significant differences are indicated (two-tailed Student's *t* test; ** $P < 0.01$).

(Fig. 7B and C). On the other hand, the shRNAs did not affect the amounts of nonacylated ceramides (Fig. 7D). These results indicate that FATP4 is also involved in acylceramide production in human keratinocytes.

Discussion

Acylceramide is a lipid that is essential for skin barrier formation, and the abrogation of its production causes ichthyosis in humans and neonatal lethal barrier defects in mice (1, 8, 17–24). However, not all genes involved in the production of acylceramide have been identified to date. Based on our previous finding that the substrates of the FA ω -hydroxylase CYP4F22 are ULCFAs (26), we speculate that the acylceramide synthesis pathway includes three successive reactions: ULC acyl-CoAs to ULCFAs by a thioesterase, ULCFAs to ω -OH ULCFAs by the FA ω -hydroxylase CYP4F22, and ω -OH ULCFAs to ω -OH ULC acyl-CoAs by an ACS (SI Appendix, Fig. S1). Of these, the thioesterase and ACS remained to be identified. In the present study, we found that acylceramide levels in *Fatp4* KO mice are reduced to ~10% of those in WT mice (Fig. 3C). Given this large decrease in acylceramide levels, it is reasonable to conclude that *Fatp4* is directly involved in acylceramide production. We also confirmed the involvement of FATP4 in acylceramide production using human keratinocytes and knockdown analyses (Fig. 7). Furthermore, these results support the validity of our model, which assumes that ACS is involved in the acylceramide synthesis pathway. While the thioesterase(s) has not yet been identified, members of the ACOT (acyl-CoA thioesterase) family are considered candidates. Mammals have 15 ACOTs (ACOT1–13, THEM4,

and THEM5), and it is possible that some of these redundantly function in acylceramide synthesis.

Several previous studies have generated *Fatp4* mutant mice and reported the presence of severe skin barrier abnormalities (10, 11, 13, 14). However, the mechanism that causes the abnormalities has remained unknown. One hypothesis as to the cause of the skin barrier dysfunction is the loss of FA transporter activity by *Fatp4* (4, 11); another is changes in (nonacylated) ceramide metabolism (10). In recent years, many acylceramide-related genes have been identified, and the respective KO mice were created and analyzed. These mice exhibited severe skin barrier abnormalities that resemble those observed in *Fatp4* mutant mice (19–24). This phenotypic resemblance led to our hypothesis that the impaired skin barrier formation observed in IPS patients and *Fatp4* mutant mice is caused by a defect in acylceramide synthesis. In the present study, we provide proof for this notion (Fig. 3C). With respect to the reduced FA uptake hypothesis, we found that the amounts of linoleic acid and linoleic acid-containing TAGs were not decreased but rather increased in the epidermis of *Fatp4* KO mice (Fig. 4B and C), contradicting the hypothesis. With respect to changes in (nonacylated) ceramide metabolism, the decreases in \geq C26 ceramides and increases in \leq C24 ceramides (10) (Fig. 4A) in *Fatp4* KO mice likely partly contribute to the skin barrier dysfunction. However, the effect of *Fatp4* disruption on the amounts of acylceramide was much greater than on ceramides. Furthermore, considering the severe skin barrier abnormality in *Pnpla1* KO mice in which only the amounts of acylceramides (but not of ceramides) are decreased (23, 24), it is very possible that the decreases in acylceramide amounts are mainly responsible for the observed skin barrier dysfunction.

The FA elongase *Elovl1* plays important roles in the elongation of saturated VLC acyl-CoA, especially of C24:0-CoA to C26:0-CoA, although its contribution to the elongation of monounsaturated VLC acyl-CoAs is lower (21, 40). Expression levels of *Elovl1* were decreased in *Fatp4* KO mouse epidermis (Fig. 5C). Therefore, we speculate that this decrease in *Elovl1* expression causes the changes in ceramide metabolism—i.e., the decreases in \geq C26 saturated ceramides and increases in \leq C24 saturated ceramides—as observed in the epidermis of *Elovl1* KO mice (21). The exact cause for the decreased *Elovl1* expression is unknown. However, it is likely that impaired skin barrier formation due to reduced acylceramide production indirectly leads to expression changes in various genes including *Elovl1* (SI Appendix, Fig. S3A). Decreased expression of *Elovl1* has also been observed in KO mice of *Nipal4* (another ichthyosis-causative gene) (41).

The expression levels of *Pnpla1* were also reduced in *Fatp4* KO mice (Fig. 5B). *Pnpla1* catalyzes the final step of acylceramide production, the conversion of ω -OH ceramide to acylceramide (27). The decreased expression of *Pnpla1* in *Fatp4* KO mice may be partly responsible for reduced acylceramide production. The monounsaturated ω -OH ceramide was unexpectedly not reduced in *Fatp4* KO mice (Fig. 3F). We speculate that two effects—reduced production due to *Fatp4* deficiency and substrate accumulation due to decreased expression of *Pnpla1*—acted in a mutually countervailing manner here.

The reaction products of *Fatp4*, ω -OH ULC acyl-CoAs, are rapidly metabolized to ω -OH ceramides, acylceramides, acylglucosylceramides, and protein-bound ceramides (Fig. 3G and SI Appendix, Fig. S1). Since ω -OH ULC acyl-CoAs are transient metabolic intermediates, the sum of the ω -OH ULC acyl-CoA metabolites, rather than the amounts of ω -OH ULC acyl-CoAs themselves, may represent the actual amounts of ω -OH ULC acyl-CoAs produced by *Fatp4* (and other *Fatp*/*Acsvl* subfamily members having redundant functions). The sum of saturated ω -OH ULC acyl-CoA metabolites was reduced to ~1/14 by *Fatp4* disruption (Fig. 3H). We consider this to be caused by the combined effects of the decrease in the conversion step of ω -OH ULCFAs to ω -OH ULC acyl-CoAs due to *Fatp4* disruption, and

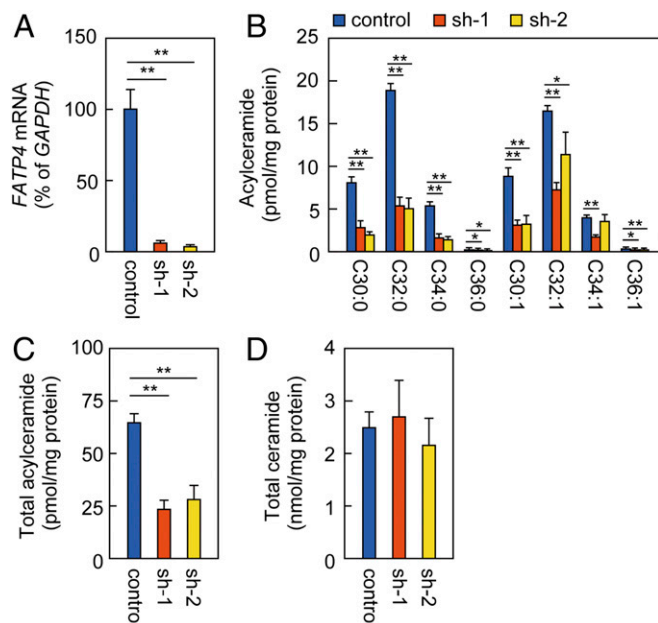


Fig. 7. Reduction in acylceramide levels with *FATP4* knockdown in human keratinocytes. Human keratinocytes were infected with lentiviral particles expressing control shRNA, sh*FATP4*-1, or sh*FATP4*-2, and then differentiation was induced for 7 d. (A) Total RNA was prepared, and real-time quantitative RT-PCR was performed for *GAPDH* and *FATP4*. Values represent the means \pm SD obtained from three independent experiments and indicate the expression levels relative to *GAPDH*. (B and C) Lipids were extracted, and acylceramides (B and C) and (nonacylated) ceramides (D) were quantified by LC-MS/MS. The amount of each acylceramide species (B), total amount of acylceramides (C), and total amount of ceramides (D) are shown. Values represent the means \pm SD obtained from three independent experiments. Statistically significant differences are indicated (Dunnett's test; * $P < 0.05$; ** $P < 0.01$). sh-1, sh*FATP4*-1; sh-2, sh*FATP4*-2.

the decreased supply of ULC acyl-CoAs due to reduced expression levels of *Elovl1* (SI Appendix, Fig. S3B). However, it is difficult to estimate the degree of contribution of the *Fatp4* disruption. On the other hand, since the contribution of *Elovl1* to the elongation of the monounsaturated VLCFAs is small, the decrease in the sum of monounsaturated ω -OH ULC acyl-CoA metabolites as well as total amount of monounsaturated acylceramides can be considered to be simply due to *Fatp4* disruption (SI Appendix, Fig. S3B). Indeed, the amounts of nonacylated ceramides with \geq C30:1 were not reduced in *Fatp4* KO mice compared with WT mice (Fig. 4A), indicating that the amounts of \geq C30:1 acyl-CoAs were not reduced by the *Fatp4* disruption. In *Fatp4* KO mice, the sum of monounsaturated ω -OH ULC acyl-CoA metabolites was reduced to \sim 1/3 compared with WT mice (Fig. 3H). This implies that the contribution of *Fatp4* to conversion of ω -OH VLCFAs to ω -OH ULC acyl-CoAs in the acylceramide production pathway is \sim 2/3 of the total. This may even be an underestimate, considering that the expressions of other *Fatp* family members had tendencies to increase in *Fatp4* KO mice (Fig. 5D). Among *Fatp* subfamily members, *Fatp1* shares the highest amino acid sequence similarity with *Fatp4*, and forced expression of *Fatp1* in the epidermis of *Fatp4* KO mice can complement the skin barrier abnormalities (42).

In the present study, we elucidated one of the missing links in the acylceramide synthesis pathway. Furthermore, we revealed that the cause of IPS pathology is impaired acylceramide production, not reduction in FA uptake as assumed to date. Further research into the treatment of IPS with acylceramide or compounds with similar effects is thus required.

Methods

Extended details for all methods are available in SI Appendix, Materials and Methods.

Production and Breeding of Mice. *Fatp4* KO mice were generated by CRISPR-Cas9. Mice were housed at $23 \pm 1^\circ\text{C}$ ambient temperature and at $50 \pm 5\%$ humidity with a 12-h light/dark cycle with food available ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of Hokkaido University and conducted in accordance with the institutional guidelines.

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