Keratinocyte-specific Expression of Fatty Acid Transport Protein 4 Rescues the Wrinkle-free Phenotype in Slc27a4/Fatp4 Mutant Mice*

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Fatp4 (fatty acid transport protein 4; also known as SLC27A4) is the most widely expressed member of a family of six long chain fatty acid transporters. FATP4 is highly expressed in enterocytes and has therefore been proposed to be a major importer of dietary fatty acids. Two independent mutations in Fatp4 cause mice to be born with thick, tight, shiny, “wrinkle-free” skin and a defective skin barrier; they die within hours of birth from dehydration and restricted movements. In contrast, induced keratinocyte-specific deficiency of FATP4 in adult mice causes only mild skin abnormalities. Therefore, whether the loss of FATP4 from skin or a systemic gestational metabolic defect causes the severe skin defects and neonatal lethality remain important unanswered questions. To investigate the basis for the phenotype, we first generated wild-type tetraploid/mutant diploid aggregates that should lead to rescue of any abnormalities caused by loss of FATP4 from the placenta. However, the skin phenotype was not ameliorated. We then generated transgenic mice expressing exogenous FATP4 either widely or specifically in suprabasal keratinocytes, and we bred the transgenes onto the Fatp4−/− background. Both modes of FATP4 expression led to rescue of the neonatally lethal skin defects, and the resulting mice were viable and fertile. Keratinocyte expression of an FATP4 variant with mutations in the acyl-CoA synthetase domain did not provide any degree of rescue. We conclude that expression of FATP4 with an intact acyl-CoA synthetase domain in suprabasal keratinocytes is necessary for normal skin development and that FATP4 functions in establishing the cornified envelope.

Fatty acid transport proteins (FATPs)2 compose a family of transmembrane proteins that facilitate cellular long chain fatty acid uptake (1–3). The most widely expressed member of this family is FATP4 (4, 5), which is encoded by Slc27a4 (solute carrier family 27 member 4 gene). The most robust expression of FATP4 is in intestinal epithelial cells (5), but its broad expression pattern is suggestive of functions in many organs. Surprisingly, the “wrinkle-free” phenotype we discovered in mice with a spontaneous mutation in Slc27a4 (Slc27a4wrfr) and the identical phenotype of mice with a targeted Slc27a4 mutation (Slc27a4Δexons2&3) indicate critical roles for FATP4 in skin and hair development (6, 7). For these mutations, which both affect exon 3, homozygotes are born with tight, thick, shiny skin and a defective skin barrier; they die shortly after birth because of restricted movements and dehydration. These defects are reminiscent of human restrictive dermopathy, but this disease has recently been linked to mutations in zinc metalloprotease STE24 (8–10). Interestingly, deletion of Slc27a4 exons 2 and 3 (the first two coding exons; Slc27a4Δexons2&3) results in embryonic lethality prior to embryonic day 9.5 (11). The reason for the striking difference in phenotypes remains unknown, but it may involve a partially functional truncated protein in the first two mutants or early embryonic death unrelated to the interruption of the Slc27a4 gene in the third mutant. For simplicity, the Slc27a4 gene will henceforth be referred to as Fatp4.

Several hypotheses can explain the etiology of the skin phenotype of Fatp4−/− mice. First, the skin defects could result from essential fatty acid deficiency because of loss of FATP4 from the placenta and insufficient maternal to fetal fatty acid transport (7). Second, the lack of FATP4 expression in other fetal tissues, such as liver or adipose tissue, could cause systemic defects that lead to the skin phenotype. Third, FATP4 could be required in the skin; it could directly impact development of the epidermis via effects on fatty acids that serve as signaling molecules (7), and/or it could be important for formation of the epidermal barrier by directly regulating the availability and nature of fatty acid derivatives that are incorporated into the lipid-rich barrier of the skin (6). Regarding an intrinsic role for FATP4 in skin, FATP4 has been conditionally deleted in the skin of adult mice. By gross appearance the mice appear normal, but several mild histological abnormalities are present in the epidermis (12), supporting a direct role for FATP4 in the skin.

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2 The abbreviations used are: FATP, fatty acid transport protein; IV, involucrin; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SSC, standard saline citrate; ACS, acyl-CoA synthetase; WT, wild type; E, embryonic day.
However, the mildness of these defects compared with the severity of skin defects in Fatp4−/− animals suggests that expression in other tissues could also be relevant.

We have now tested these hypotheses directly, using tetraploid aggregations to rescue potential placental abnormalities and transgenes to restore FATP4 expression either widely or specifically in keratinocytes. We found that FATP4 expressed in suprabasal keratinocytes, under the control of the involucrin promoter, is sufficient to rescue the lethality and most of the skin defects of Fatp4−/− mice. These results support the notion that the only absolute requirement for FATP4 expression is in the upper layers of the skin, consistent with a crucial role in the initial formation of the cornified envelope and the barrier of the skin during late fetal development.

**EXPERIMENTAL PROCEDURES**

Tetraploid Aggregations—Tetraploid aggregations were performed as described (13). Briefly, wild-type ICR two-celled embryos were electrosouffed to form tetraploids and cultured for 24 h. Embryonic day (E) 2.5 embryos from Fatp4−/− females mated to Fatp4−/− males were then isolated, and each diploid embryo, 25% of which should be Fatp4−/−, was sandwiched between two wild-type tetraploid embryos. After 24 h, aggregates that had developed to single blastocysts were transferred to pseudopregnant recipient females. Fetuses and the attached placentas were dissected at E18.5 or E19.5 for analysis.

**Generation of Fatp4 Transgenic Mice**—The Miw-Fatp4 transgene was produced by PCR amplification of the Fatp4 cDNA with Pfu polymerase (Stratagene, La Jolla, CA) using primers that added HindIII sites to both ends of the cDNA. The PCR product was purified over a Wizard PCR clean up column (Promega, Madison, WI), cut with HindIII, and ligated directly into the HindIII site separating the promoter and polyadenylation signal sequences of the Miw plasmid. Miw contains a Rous sarcoma virus long terminal repeat inserted into the chicken β-actin promoter (14, 15). The transgene was liberated from the vector by digestion with NotI and purified from a gel using the Geneclean purification system (MP Biicals, Solon, OH).

The Ivl-Fatp4 transgene was produced by PCR amplification of Fatp4 cDNA with Pfu polymerase using primers that added NotI sites to both ends of the Fatp4 cDNA. The PCR product was purified, cut with NotI, and ligated into the NotI site of pH3700-pL2 containing the human involucrin promoter (16). The transgene was liberated from the vector by digestion with Sall and purified as above.

The Ivl-ΔFatp4 transgene was similarly produced from the Fatp4 cDNA with the S247G and T249G mutations introduced using primers containing the mutations and a QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions.

Transgenic mice were created by microinjection of DNA into the pronuclei of 1-cell stage B6CBAF2 mouse embryos. Potential founder mice were screened for incorporation of the transgene by PCR using primers from two different exons of Fatp4. Founders were confirmed by Southern blotting.

**Immunohistochemistry**—Anti-FATP4 antibody was generated as described (17). Whole mouse embryos or individual tissues were frozen by immersion in optimal cutting temperature solution and quick-freezing in 2-methylbutane cooled in a dry ice/ethanol bath. Sections were cut at 7 μm on a cryostat, air-dried, and fixed in cold 100% ethanol for 10 min. For anti-FATP4 staining, ethanol-fixed sections were denatured for 1 h in cold 6 M urea, 0.1 M glycine, pH 3.5, blocked in 1% normal goat serum and 1% bovine serum albumin in PBS, and incubated with the primary antibody. All antibody incubations were performed in PBS containing 1% bovine serum albumin, and all washes were performed in PBS. Secondary antibodies were conjugated to fluorescein isothiocyanate (ICN, Costa Mesa, CA) or Cy3 (Chemicon, Temecula, CA). After several washes, sections were mounted in 90% glycerol containing 0.1× PBS and 1 mg/ml propidium iodide. Images were examined using a Nikon Eclipse E800 microscope. Images were captured with a Spot 2 cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI) using Spot software version 3.5.9. Images were imported into Adobe Photoshop and Adobe Illustrator for processing and layout.

**RNA Isolation and Northern Blotting**—Tissues from newborn mice were frozen on dry ice and stored at −80 °C. Total RNA was extracted from frozen tissues using TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s protocol. Following resuspension of RNA in water and determination of concentration, mRNA was isolated using Poly(A) Purist™ MAG magnetic mRNA purification kit (Ambion, Austin, TX). Poly(A) RNA (1 or 1.5 μg) Northern blotting was performed as described (7). Hybridized blots were exposed to a phosphor screen and scanned with a Storm 840 scanner (GE Healthcare). The GAPDH probe was a 700-bp reverse transcription-PCR product, and the FATP4 probe was an ~1200-bp BstXI cDNA fragment.

**In Situ Hybridization and Riboprobes**—In situ hybridization was performed as described (18) with some modifications. Hairs were removed using a beard clipper, and the dorsal skin was dissected. Samples were fixed at 4 °C overnight in 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned at 5 μm. Sections were deparaffinized in X-3 xylene substitute (StatLab Medical Product, Lewisville, TX), rehydrated in graded ethanols, fixed again in 4% paraformaldehyde, and digested in 5 μg/ml proteinase K in PBS for 10 min. All washes following rehydration were performed in PBS with 0.1% Tween 20. Samples were post-fixed for 10 min in 4% paraformaldehyde, acidified in 0.1 M triethanolamine, pH 8, with 0.25% acetic anhydride for 10 min, and prehybridized in hybridization solution (50% formamide, 10 mM Tris, pH 7.5, 600 mM NaCl, 1 mM EDTA, pH 8.0, 1× Denhardt’s, 200 μg/ml yeast tRNA, 0.25% SDS) without probe at room temperature for 1 h. Hybridization was performed with 0.3 ng/μl heat-denatured riboprobes at 60 °C overnight. Sections were washed in 5× SSC (1× SSC: 0.15 M NaCl, 15 mM sodium citrate, pH 7) at room temperature, 1× SSC and 50% formamide at 55 °C for 30 min, and TNE (10 mM Tris, pH 7.5, 500 mM NaCl, and 1 mM EDTA, pH 8.0) at room temperature. Sections were digested in RNase A (20 μg/ml in TNE) at 37 °C for 30 min, washed in TNE at 37 °C, in 2× SSC at 55 °C for 20 min, and twice in 0.2× SSC at 55 °C for 20 min. Sections were washed in MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5), blocked in blocking...
solution I (20% heat-inactivated normal sheep serum and 2% blocking reagent in MABT) (Roche Applied Science) at room temperature for 1 h, and incubated in 1:3000 anti-digoxigenin-alkaline phosphatase (Roche Applied Science) in blocking solution II (5% heat-inactivated normal sheep serum and 2% blocking Reagent in MABT) at 4 °C overnight. Sections were washed in MABT, in NTMT (10 mM NaCl, 50 mM MgCl₂, 100 mM Tris, pH 9.5, 0.1% Tween 20), then in NTMT with 2 mM levamisol, and color-developed in BM Purple (Roche Applied Science) with 2 mM levamisol and 0.1% Tween 20 for 1 h to 3 days in the dark. Sections were finally washed in NTMT and in PBS, mounted in Crystal/Mount (Biomeda Corp., Foster City, CA), and postmounted in VectaMount (Vector Laboratories, Burlingame, CA). Some sections were counterstained with Nuclear Fast Red (Sigma) before mounting. Adjacent sections were stained with hematoxylin and eosin by standard methods.

Riboprobes were synthesized from a linearized pcDNA3-based plasmid containing the full-length FATP4 coding region using digoxigenin RNA labeling mix (Roche Applied Science) and RNA Polymerase Plus (Ambion, Austin, TX). Riboprobes were purified by NucAway spin columns (Ambion) following the manufacturer’s instructions. To synthesize antisense and sense riboprobes, the plasmid was linearized by EcoRI or BstEII and transcribed with SP6 or T7 RNA polymerase, respectively.

Hair Counts—Hairs were plucked in bunches from the back at various ages using forceps. Fur was pulled apart to individual hairs, spread on microscope slides, and mounted. Hair types were scored according to appearance as described (19) and expressed as percentage of total counted hairs. For each sample, 80–200 hairs were counted.

Lipid Analysis of Epidermis—Newborn epidermis was separated from the dermis by overnight digestion at 4 °C in 5 mg/ml dispase in Hanks’ buffered saline solution. Epidermal pieces were then rinsed several times in 50 mM lithium chloride and homogenized in water. Lipids were extracted with chloroform and methanol (20) and characterized by electrospray ionization mass spectrometry as described (21, 22).

RESULTS

Restoration of Fatp4 Expression in the Placenta—Fatp4 is highly expressed in both mouse and human placenta (7, 23). The placenta is composed of extraembryonic cells that originate from the blastocyst; thus it is derived from and genetically identical to the embryo (24). In mouse placenta, expression of Fatp4 is most prominent in trophoblasts (Fig. 1G), the cells that transport all substances between mother and fetus. Because embryos obtain lipids from the maternal circulation only in the form of nonesterified fatty acids (25, 26), we hypothesized that embryos might experience essential fatty acid deficiency in the absence of placental FATP4 (7). Symptoms of essential fatty acid deficiency show many similarities to the Fatp4 mutant phenotype, including loss of barrier function, thickened epidermis, loss of skin elasticity, and loss of hair (27–30).

To test this hypothesis, we performed tetraploid/diploid embryo aggregations. Tetraploid cells are severely limited in their ability to contribute to embryonic tissues (31, 32) and therefore contribute primarily to the placenta, whereas diploid cells in aggregates contribute primarily to the embryo proper (31, 33). Here, tetraploid embryos were derived from matings between wild-type mice, and these were aggregated with diploid embryos derived from matings between Fatp4<sup>−/−</sup> mice, of which ¼ are expected to be Fatp4<sup>−/−</sup> embryos. The aggregates were reimplanted into pseudopregnant foster mothers and allowed to develop to near term. With this strategy, Fatp4<sup>−/−</sup> embryos develop with wild-type placentas, and any abnormalities resulting from loss of FATP4 in trophoblasts should be rescued.

From two litters of reimplanted aggregates, no fetuses with a normal phenotype had the Fatp4<sup>−/−</sup> genotype. However, we did identify two phenotypically wrinkle-free mice that were confirmed as Fatp4<sup>−/−</sup> mutants by genotyping. Compared with control, the mutant skin appeared histologically abnormal, with a thickened epidermis and a flat dermal-epidermal junction (Fig. 1, A and B); this appeared identical to mutant skin from a conventional pregnancy (Fig. 1C). Absence of FATP4 in the skin was confirmed by immunofluorescence (Fig. 1, D–F). As expected, the tetraploid-derived placenta stained positive for FATP4 (Fig. 1, G and H), whereas a mutant placenta from a conventional pregnancy did not (Fig. 1I). These results indicate that even when nourished by a placenta containing wild-type trophoblasts expressing Fatp4, Fatp4<sup>−/−</sup> fetuses exhibit the wrinkle-free phenotype; therefore, this phenotype does not result from loss of FATP4 in the placenta.

Widespread Restoration of FATP4 Expression—With the original goal of proving that the wrinkle-free phenotype was caused by the absence of FATP4, we generated mice with a ubiquitously expressed Fatp4 transgene for breeding onto the Fatp4<sup>−/−</sup> background. Transgene expression was driven by the Miw promoter, which is a hybrid chicken β-actin promoter/
Fatp4 mRNA is widely expressed throughout mouse development (14). Widespread expression of FATP4 from the Miw-Fatp4 transgene rescues the skin phenotype. A. Northern analysis of Fatp4 expression in newborn Fatp4−/−;Miw-Fatp4+/− mice. Transgene-derived Fatp4 mRNA runs faster because of a lack of the 3′-untranslated region. GAPDH was used as a control. B and C, localization of FATP4 in the skin of an E18.5 control mouse (B), and a Miw-Fatp4−/− rescued mutant (C) shows suprabasal accumulation in the control but diffuse expression in the mutant epidermis. The epidermal basement membrane is indicated with a dashed line. D, rescued mutants appear normal. E and F, hematoxylin and eosin staining of adult control (E) and Fatp4−/−;Miw-Fatp4+/− skin (F) reveals normal architecture. Bar is 20 μm.

Histological analysis of Miw-Fatp4−/− rescued mutant skin by hematoxylin and eosin staining at 5 months of age indicated that the epidermis was, for the most part, no different from the control (Fig. 2, E and F). We did observe some segmental, slight thickening of the epidermis at 5 months of age, and this was more frequently observed at 9 months (data not shown). However, we have never observed any gross abnormalities in the skin or hair of these mice, even at 16 months of age. From these studies we conclude the following: 1) the absence of FATP4 causes the wrinkle-free phenotype; and 2) the Miw-Fatp4 transgene is expressed in a spatial and temporal fashion that is sufficient to replace endogenous FATP4 wherever it is necessary for normal skin development.

Keratinocyte-specific Restoration of Fatp4 Expression—We next sought to determine whether the Fatp4−/− skin phenotype results from loss of FATP4 specifically in the epidermis, where it is normally concentrated in the upper spinous and the granular layers (Fig. 2B) (6). We therefore used the well characterized human involucrin (Ivl) promoter to drive expression of FATP4 in transgenic mice. Involucrin is a scaffold protein of the cornified envelope (34) expressed in suprabasal keratinocytes (16, 35, 36) and hair follicles (37). Two independent lines of transgenic mice carrying the Ivl-Fatp4 transgene were mated to Fatp4−/− mice for two generations to obtain Fatp4−/−;Ivl-Fatp4+/− mice. Most Fatp4−/−;Ivl-Fatp4+/− mice were indistinguishable from control mice at birth. Northern analysis of mRNA from several tissues from Fatp4−/−;Ivl-Fatp4+/− newborn mice confirmed that the transgene was expressed specifically in the skin, in contrast to the widespread expression of endogenous FATP4 (Fig. 3A) and Miw-Fatp4 (Fig. 2A). (Endogenous FATP4 expression appears higher in the Ivl-Fatp4 mice (Fig. 3A) than in the Miw-Fatp4 mice (Fig. 2A) because of different exposure times.)

We next assayed the expression of transgene-derived FATP4 protein in E18.5 Fatp4−/−;Ivl-Fatp4+/− skin by immunohistochemistry. The results varied somewhat
Histologically, there was a thickened epidermis (Fig. 4, sheath of hair follicles (Fig. 4, easily detected in the granular layer and in the inner root genic epidermis (Fig. 4). FATP4 RNA could not be detected in the control, nontransgenic samples prepared from the same mice as above. Keratin 6 expression was only activated in the scaly epidermis of the mutant phenotype in these mice; in a partial penetrance of the mutant phenotype in these mice; in a variability in terms of level and/or timing of transgene expression in type (7). In contrast, there was no epidermal keratin 6 expression of FATP4 (Fig. 3). Ally appeared to be fully rescued by keratinocyte-specific expression of keratin 6 in inner root sheath (arrowhead), with much stronger signals in the more thickened epidermis (left margin, small bracket, right margin, large bracket).

Note the normal expression of keratin 6 in inner root sheath (arrowhead in M–P) of hair follicles in all samples. Comparison between the two lines. FATP4 was localized to the granular layer of the epidermis in both; however, in line 1 the expression pattern was patchy and not continuous as it was in control (Fig. 3, B and C), and in line 2 the expression was continuous (Fig. 3D). By in situ hybridization endogenous FATP4 RNA could not be detected in the control, nontransgenic epidermis (Fig. 4E), but in transgenic skin the RNA was easily detected in the granular layer and in the inner root sheath of hair follicles (Fig. 4, F and G).

Skin and Hair Defects in a Subset of Fatp4−/−;Ivl-Fatp4tg+/+ Mice—Although the skin abnormalities of Fatp4−/− mice usually appeared to be fully rescued by keratinocyte-specific expression of FATP4 (Fig. 3E and Fig. 4, C and K), we occasionally observed dry, scaly skin beginning at an early age (Fig. 5A). Histologically, there was a thickened epidermis (Fig. 4, D and L) and patchy expression of keratin 6 (Fig. 4P) in mice with this phenotype, characteristics reminiscent of the Fatp4−/− phenotype (7). In contrast, there was no epidermal keratin 6 expression in Fatp4−/−;Ivl-Fatp4tg+/+ mice exhibiting a normal appearance (Fig. 4O). This likely indicates mouse-to-mouse variability in terms of level and/or timing of transgene expression and the fraction of granular cells that express. Paradoxically, transgene-derived FATP4 RNA levels appeared to be increased in the thickened epidermis of scaly rescued mice (Fig. 4H). We believe this is because of up-regulation of involucrin promoter activity (and thus the Ivl-Fatp4 transgene) caused by partial penetrance of the mutant phenotype in these mice; in a preliminary gene expression profiling study comparing embryonic Fatp4−/− to control skin, involucrin expression was increased ~20-fold in Fatp4−/− skin.

Beginning at 2–6 weeks of age, the fur of some Fatp4−/−;Ivl-Fatp4tg+/+ mice began to look matted and unngroomed (Fig. 5B). At 8–12 weeks of age, some began to show thinning fur and/or small patchy areas of hair loss, particularly between the ears and on the back (Fig. 5, C and D). Thinning fur and alopecia occurred in ~50% of the Fatp4−/−;Ivl-Fatp4tg+/+ mice and varied greatly in terms of age of onset and severity of hair loss (Fig. 5, C and D).

Another variable phenotype observed in Ivl-Fatp4 rescued mutant mice relates to hair types. The unngroomed appearance
of some young rescued mutants (Fig. 5B) suggested a possible alteration in hair types. Mouse pelage is normally composed of four types of hairs that can be differentiated by numbers of rows of air cells, characteristics of bending, and hair length (19). Microscopic analysis of fur from mice with an unguarded or “ruffled” appearance indicated shorter zigzag hairs than normal. Furthermore, hair counts obtained from a number of Iv-Ivl-Fatp4 rescued mutants showed significant increases in guard and awl hair percentages, a decreased percentage of zigzag hairs, and an increased percentage of unidentifiable hairs (Table 1). The increased percentage of unidentifiable hairs may result from hair breakage or hair fragility. Mice with ruffled fur and hair count abnormalities were the same ones that later developed thinning fur and alopecia.

X-linkage of the Line 1 Iv-Ivl-Fatp4 Transgene—The most severe skin and fur phenotypes were observed in rescued female mice, but not in rescued male mice, from line 1. Analysis of transmission of this transgene in 18 litters revealed that male transgenic mice only transmitted the transgene to female offspring; male to male transmission was never observed. These results indicate that the transgene is X-linked, which likely causes the stronger, variable skin phenotypes in females because of random X chromosome inactivation and the lack of transgene expression in a subset of keratinocytes. This is consistent with the patchy transgene expression observed in skin from females (Fig. 3C).

Molecular Analysis of Ceramide Fatty Acid Composition—Herrmann et al. (6) demonstrated previously an increased molar content of ceramides in Fatp4 mutant epidermis compared with control littermates; detailed analysis of the fatty acid species of epidermal ceramides indicated a lower proportion of very long chain fatty acids (≥26 carbons) and an increased proportion of <26 carbon fatty acids. Significant differences in any lipids were not found in other tissues. Because of the importance of ceramides to skin barrier formation, we sought to determine whether the phenotypically rescued mice were also biochemically rescued. Electrospray ionization mass spectrometry was performed to analyze the fatty acid composition of epidermal ceramides from WT mice, Fatp4−/− mice, and rescued Fatp4−/−;Ivl-Fatp4G/A+ mice (Table 2). A significantly lower proportion of very long chain fatty acids (≥26 carbons) was observed in the mutants (15.6% in Fatp4−/− versus 33.8% in WT), but these proportions were restored to near WT values in the rescued mice (32.2% in Fatp4−/−;Ivl-Fatp4G/A+ mice). In comparisons of individual ceramide fatty acid species, Fatp4−/−;Ivl-Fatp4G/A+ ratios were not significantly different from WT ratios for most fatty acids, with the exception of 20:0 and 24:1, which are very minor components of normal skin but were increased in the mutant and rescued mutant epidermis. The significant increase in 24:1 resulted in a significant decrease in the profile of saturated fatty acid species.

Expression of a Mutated FATP4 Does Not Rescue the Skin Phenotype—FATP4 has a conserved ACS domain, and the ACS activity of FATP4 is thought to drive fatty acid uptake (4, 38–41), as demonstrated most recently by transfections of WT and ACS mutant FATP4 into cells (42). To investigate the role of ACS activity in FATP4 in vivo, and to determine whether FATP4 might exhibit any function in the absence of ACS activity, we generated a Fatp4 CDNA (ΔFatp4) carrying two amino acid substitutions, S247G and T249G. Altering these critical residues inhibits ACS activity (43, 44) and blocks uptake of Bodipy B12-FA (Ref. 42 and personal observations). The ΔFatp4 CDNA was placed under the control of the involucrin promoter and microinjected to make several lines of transgenic mice, three of which were shown to express the transgene well by Northern analysis (Fig. 6A). We then determined whether FATP4 lacking ACS activity was capable of rescuing the skin phenotype by generating Fatp4−/−;Ivl-Fatp4G/A+ mice for all three lines. In all cases, newborn Fatp4−/−;Ivl-ΔFatp4G/A+ mice showed the typical wrinkle-free phenotype and died shortly after birth (data not shown). Hematoxylin and eosin staining of E18.5 skin showed the thickened epidermis (Fig. 6, B–D) and epidermal keratin 6 expression (Fig. 6, H–J) typical of Fatp4−/− mice (7). Expression of the mutant FATP4 protein in suprabasal keratinocytes was confirmed by immunofluorescence (Fig. 6F).

These results confirm that FATP4 carrying a mutated ACS domain is functionally inactive in vivo and support the hypothesis that the fatty acid transport activity of FATP4 depends upon its ACS activity.

**DISCUSSION**

Fatp4−/− mice die shortly after birth, apparently because of skin defects; the tight skin prevents the movements necessary for breathing and suckling, and the lack of an effective barrier causes rapid dehydration (6, 7). Given the broad expression pattern of FATP4, a phenotype restricted to skin was therefore somewhat surprising. We have investigated the root cause of this phenotype through both placental complementation and transgenic rescue experiments. We initially hypothesized that the skin defects in Fatp4−/− mice may be secondary to the loss of Fatp4 from the placenta and defective fatty acid transport to the embryo, which could then cause essential fatty acid deficiency-like symptoms. However, the tetraploid aggregation experiments reported here proved that a normal placenta was unable to rescue the skin phenotype. Only the transgenes were able to rescue the

### TABLE 1
Hair counts from rescued mutant and control mice

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Guard</th>
<th>Awl</th>
<th>Auchene</th>
<th>Zigzag</th>
<th>Unidentified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>1.30 ± 0.21</td>
<td>15.00 ± 1.45</td>
<td>4.10 ± 0.87</td>
<td>76.90 ± 2.06</td>
<td>2.50 ± 0.62</td>
</tr>
<tr>
<td>Rescue (n = 8)</td>
<td>2.63 ± 0.26</td>
<td>23.63 ± 4.04</td>
<td>3.00 ± 0.78</td>
<td>58.63 ± 5.80</td>
<td>12.50 ± 4.94</td>
</tr>
<tr>
<td>% change in rescue</td>
<td>+103%</td>
<td>+57.5%</td>
<td>-26.8%</td>
<td>-23.8%</td>
<td>+400%</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.0007</td>
<td>&lt;0.0381</td>
<td>&lt;0.1809</td>
<td>&lt;0.0081</td>
<td>&lt;0.0417</td>
</tr>
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requirement for FATP4 in skin

Table 2

<table>
<thead>
<tr>
<th>Fat chain</th>
<th>Sat.</th>
<th>Unsat.</th>
</tr>
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<tbody>
<tr>
<td>16:0</td>
<td>4.3</td>
<td>0.0</td>
</tr>
<tr>
<td>18:0</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>20:0</td>
<td>0.2</td>
<td>2.6</td>
</tr>
<tr>
<td>22:0</td>
<td>9.2</td>
<td>22.8</td>
</tr>
<tr>
<td>24:0</td>
<td>0.6</td>
<td>4.6</td>
</tr>
<tr>
<td>26:0</td>
<td>0.5</td>
<td>30.9</td>
</tr>
</tbody>
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For significantly different values compared with Fatp4 knock-out mice, p < 0.05.

Figure 6. The Ivl-ΔFatp4 transgene with two amino substitutions in the acyl-CoA synthetase domain is unable to rescue the Fatp4−/− phenotype. A, expression of the ΔFatp4 transgene is higher than endogenous Fatp4 in several mutant transgenic lines, as shown by Northern blotting. B–D, hematoxylin and eosin staining of sections from control (B), Fatp4−/−;Jvl-ΔFatp4/w (C), and Fatp4−/−;Jvl-ΔFatp4/w (D) E18.5 embryos shows a thickened epidermis and typical mutant epidermal morphology in Fatp4−/−;Jvl-ΔFatp4/w mice. E–G, appropriate expression of ΔFatp4 was confirmed by immunofluorescence analysis of control (E), Fatp4−/−;Jvl-ΔFatp4/w (F), and Fatp4−/−;Jvl-ΔFatp4/w (G) skin. Keratin 6 expression was activated in the epidermis of Fatp4−/−;Jvl-ΔFatp4/w mice (I), as in Fatp4−/− epidermis (J), but not in control (H). The epidermal basement membrane is indicated with a dashed line. Bar is 100 μm.

It is interesting that FATP4 expression restricted to suprabasal cells of stratified squamous epithelia and hair follicles was sufficient to rescue the neonatal lethality of Fatp4−/− mice. However, there was not complete rescue of the skin phenotype in all mice, and the gross abnormalities seemed to be restricted to the skin and hair (Fig. 5). We realized while characterizing the skin and hair phenotypes that the most severe phenotypes were observed only in female mice of line 1 Fatp4−/−;Jvl-Fatp4−/− mice, whereas males showed only mild hair loss, indicating an influence of gender on the skin phenotype. Insertion of the transgene on the X chromosome was confirmed in this line by analysis of transmission of the transgene by gender. Therefore, we conclude that transgene-rescued females of line 1 have severe skin phenotypes because of lack of expression of FATP4 in some regions of the skin where the X chromosome carrying the transgene was inactivated. For the second transgenic line that rescues the Fatp4−/− phenotype, both males and females exhibit some mild alopecia after several months of age.

Herrmann et al. (12) generated a conditional Fatp4 knock-out allele and demonstrated a role for FATP4 in the mainte-
nance of normal epidermal structure. In that study, mice carrying a tamoxifen-inducible keratinocyte-specific Cre and the floxed Fatp4 allele were treated topically with tamoxifen at 10 weeks for 5 days and again 2, 4, and 6 weeks later. No gross abnormalities were observed in the mice. Epidermal abnormalities were revealed only by histological analysis, which showed a thickened stratum corneum, increased cell layers in the stratum spinosum, hyperproliferation in the basal layer, and compromised barrier function. Interestingly, FATP4 should be missing throughout the epidermis, but the phenotype is much milder than that of newborn mice with complete loss of FATP4.

Here we sought to determine whether epidermal FATP4 expression is sufficient to rescue the wrinkle-free phenotype and neonatal lethality, rather than whether it is important for maintenance of the epidermis in adults. We not only showed that FATP4 expression in the epidermis during development can rescue the mutation, but we further showed that FATP4 is only critical in the granular layer, and therefore it is likely to be crucial for initial barrier formation. But similar to the findings of Herrmann et al. (12), we did observe areas of thickened epidermis in older Miw-Fatp4 rescued mutants by histological analysis, although the mice appeared overtly normal. This is likely because of decreased expression of the transgene in non-muscle lineages (including skin) postnatally, which we have consistently observed in transgenic mouse studies that utilize the Miw regulatory element.

FATP4 has previously been hypothesized to be necessary for development of the skin barrier (6). Here, we demonstrate that FATP4 expressed in the same pattern as involucrin, a major protein of the cornified envelope (45–47), can rescue the skin phenotype. The lipids of the cornified envelope that form the skin barrier consist primarily of ceramides, cholesterol, and nonesterified fatty acids derived from lamellar granules in the granular layer (48–50). We speculate that FATP4 may help ensure the supply of fatty acids necessary for formation of the cornified envelope; without the proper balance of free fatty acids in the cornified envelope, the skin barrier is defective (48, 49, 51). This function appears to require the very long chain ACS activity that has been attributed to FATP4 (4) and that we have demonstrated herein to be necessary for the rescue of the Fatp4-/- phenotype. Hall et al. (41) showed a virtual absence of very long chain ACS activity in the skin of Fatp4-/- mice, despite the fact that FATP1, FATP3, and FATP6 should still be present (52). These results point to a lack of FATP functional redundancy in suprabasal keratinocytes, as suggested previously by yeast complementation assays (39), and underscore the importance of FATP4. We also demonstrate an abnormal fatty acid composition of ceramides in the epidermis, consistent with data from Herrmann et al. (6), indicating a decreased proportion of longer chain fatty acids and an increased proportion of shorter chain fatty acids; this fatty acid chain length composition is restored to that of control animals in the transgene-rescued mutants, suggesting that the fatty acid chain lengths of skin lipids are determined in part by FATP4 activity and are essential to normal skin function. The fatty acid composition of ceramides has also been shown to be critical for the skin barrier and proper skin function in Elav4-/- mutant mice that die several hours after birth from a defective skin barrier (53–55); the defective skin permeability barrier was attributed to a reduced very long chain fatty acid composition of ceramides in those mice (55).

Schmuth et al. (52) reported FATP4 to be highly expressed in sebaceous glands and subcutaneous fat, but they also detected lower levels of expression in the dermis, hair follicles, and basal as well as suprabasal keratinocytes. In contrast, Herrmann et al. (6) found FATP4 expression only in the granular layer of newborn mice, when the hair follicles are beginning to develop and sebocytes are not yet differentiated. Our data are in closer agreement with those of Herrmann et al. (12), as we observed FATP4 expression primarily in suprabasal keratinocytes. We have detected FATP4 in the dermis, although it is only barely detectable, and the staining is inconsistent. We also found very high expression of FATP4 in sebaceous glands and hair follicles when analyzing skin at an age when these structures are mature. The reasons for the discrepancies in staining patterns may reflect different antibody preparations used in different laboratories or different staining techniques with varying degrees of tissue penetration or epitope retrieval. Although there is conflicting data on where FATP4 is actually expressed in the skin, our transgenetic rescue data indicate that its expression only in suprabasal keratinocytes and hair follicles is sufficient for survival of the animal. Thus, FATP4 is dispensable in all other tissues for normal development, suckling, growth, and fertility.

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Requirement for FATP4 in Skin


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