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Targeted Deletion of Fatty Acid Transport Protein-4 Results in Early Embryonic Lethality*

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Ruth E. Gimeno‡, David J. Hirsch§, Sandhya Punreddy‡, Ying Sun‡, Angelica M. Ortegon¶, Hui Wu‡, Tom Daniels‡, Alain Stricker-Krongrad‡, Harvey F. Lodish§, and Andreas Stahl¶‖

From the ¶Palo Alto Medical Foundation and Stanford University School of Medicine, Palo Alto, California 94301, §The Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142, and ‡Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts 02139

Fatty acid transport protein-4 (FATP4) is the major FATP in the small intestine. We previously demonstrated, using *in vitro* antisense experiments, that FATP4 is required for fatty acid uptake into intestinal epithelial cells. To further examine the physiological role of FATP4, mice carrying a targeted deletion of *FATP4* were generated. Deletion of one allele of *FATP4* resulted in 48% reduction of FATP4 protein levels and a 40% reduction of fatty acid uptake by isolated enterocytes. However, loss of one *FATP4* allele did not cause any detectable effects on fat absorption on either a normal or a high fat diet. Deletion of both *FATP4* alleles resulted in embryonic lethality as crosses between heterozygous *FATP4* parents resulted in no homozygous offspring; furthermore, no homozygous embryos were detected as early as day 9.5 of gestation. Early embryonic lethality has been observed with deletion of other genes involved in lipid absorption in the small intestine, namely microsomal triglyceride transfer protein and apolipoprotein B, and has been attributed to a requirement for fat absorption early in embryonic development across the visceral endoderm. In mice, the extraembryonic endoderm supplies nutrients to the embryo prior to development of a chorioallantoic placenta. In wild-type mice we found that FATP4 protein is highly expressed by the epithelial cells of the visceral endoderm and localized to the brush-border membrane of extraembryonic endodermal cells. This localization is consistent with a role for FATP4 in fat absorption in early embryogenesis and suggests a novel requirement for FATP4 function during development.

Uptake of unesterified long-chain fatty acids (LCFAs)[1] into mammalian cells occurs through both a passive flip-flop as well as a saturable, protein-mediated mechanism. A protein-mediated uptake of LCFAs has been demonstrated for the intestine (1, 2), liver (3), adipose tissue (4), and heart (5, 6). Several membrane proteins that increase the uptake of LCFAs when overexpressed in cultured mammalian cells have been identified. The most prominent and best characterized of these are FAT/CD36 (7), long-chain fatty acyl-CoA synthetases (8, 9), and fatty acid transport proteins 1 through 6 (FATP1–6, solute carrier family 27). FATP1 is a 71-kDa transmembrane protein and is the major FATP in adipose tissue, and is also found in heart and skeletal muscle (9–11). FATP2 is found almost exclusively in liver and kidney cortex (10), whereas FATP3 is broadly expressed with notably high mRNA levels in the lung (10, 12). FATP5 expression is liver specific (10), and FATP6 is principally expressed in the heart, where it is the predominant FATP family member (13, 14).

FATP4 is the only FATP expressed in the small intestine, and is localized to the apical brush border of the epithelial cells, where it is thought to be responsible for absorption of dietary lipids. Studies with FATP4 overexpressing cell lines and with isolated enterocytes demonstrated that FATP4 is both necessary and sufficient for efficient uptake of long-chain and very long-chain fatty acids (15). FATP4 and FATP1 are also the predominant FATPs in the brain (16).

An AMP-binding sequence is found in all known FATPs located at the beginning of the 300-amino acid long FATP signature sequence (10). The AMP-binding motive in FATP1 mediates ATP binding and is required for LCFA uptake, since mutations within this motif abolish uptake activity (17, 18). Oligomerization also seems to be required for transport function, because co-expression of non-functional FATP1 mutants with wild-type FATP1 resulted in dominant negative inhibition of transport (19). Based on the finding that both long-chain and very long-chain acyl-CoA synthetase activities are increased in lysates from FATP overexpressing cells (20) and in partially purified FATP1 and -4 precipitates (21, 22), it has been suggested that FATPs are acyl-CoA ligases that directly couple LCFA uptake with CoA activation, thereby trapping fatty acids inside the cell.

Dietary fatty acids contribute ~30–40% of the calories in a normal Western diet (23). Inhibition of fat absorption, by blocking generation of fatty acid from lipids with pancreatic lipase inhibitors (25, 26), has been shown to be an effective treatment for obesity and to improve obesity-associated disorders, such as cardiovascular disease, dyslipidemia, and insulin resistance (24). An alternative or additional treatment, which may result in an improved side effect profile, could be direct inhibition of fatty acid uptake, which could be theoretically achieved by blocking FATP4 function.

In this study, we attempted to better understand the function of FATP4 *in vivo* by engineering a *FATP4* null mutation through deletion of the first two exons in the murine gene. We found that homozygous deletion of the *FATP4* gene resulted in early embryonic lethality and that FATP4 protein was expressed during early post-implantation development in the yolk sac, suggesting a critical role for FATP4 in the maternal-fetal transport of fatty acids.

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‖ To whom correspondence should be addressed. Tel.: 650-614-3293; Fax: 650-329-9114; E-mail: AStahl@Stanford.edu.

[1] The abbreviations used are: LCFA, long-chain fatty acids; FATP, fatty acid transport protein.

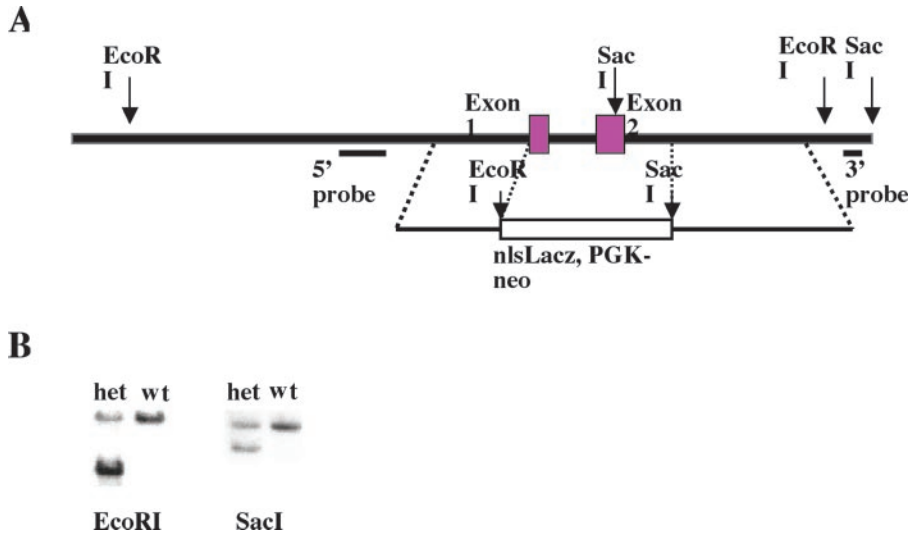

FIG. 1. **Targeted disruption of *FATP4*.** *A*, structure of the wild-type and the mutated *FATP4* alleles. The first two coding exons of *FATP4* were replaced with a cassette containing a nuclear localized *lacZ* gene and a selectable marker. The mutated sequence is flanked by an EcoRI and a SacI site. The 5′ and 3′ arms of the construct as well as the positions of the 5′ and 3′ probes are indicated. *B*, Southern blot analysis of genomic DNA from mice derived from a *FATP4* +/− cross. Genomic DNA was digested with EcoRI or SacI and hybridized with the 5′ and 3′ probes, respectively. *het*, heterozygote; *wt*, wild-type.

MATERIALS AND METHODS

Generation of the FATP4 Deletion Allele—129/Sv genomic library was obtained from Research Genetics and the targeting vector was based on pGEM from Promega. A 577-nucleotide fragment ending 37 base pairs upstream of the 5′ arm and a 599-nucleotide fragment starting 444 bp downstream of the 3′ arm were generated by PCR amplification, subcloned into a shuttle vector, and used as probes for Southern blots.

129SvEv ES cells were cultured on SNL76/7 mitotically inactive feeder cells as described by Robertson (27). Electroporation of the cells was performed as described by Huszar *et al.* (28). G418-resistant clones were screened by Southern blot hybridization using 5′ and 3′ flanking probes to confirm recombination. ES cell clones that had undergone homologous recombination were injected into blastocysts, which were then transferred to pseudopregnant female mice to generate chimeric offspring. Male chimeras were mated with C57Bl/6J females to obtain germline transmission of the disrupted *FATP4* gene. The resulting heterozygotes were interbred to generate additional offspring. Genotyping of offspring was performed using a PCR genotyping assay.

Western Blotting and Immunofluorescence—FATP4 protein levels were determined in enterocytes of five male wild-type and heterozygous litter pairs. Enterocyte lysates were prepared from *ad lib* fed mice as previously described (15). Western blotting was performed using polyclonal anti-FATP4 antibody (15) and quantitated using densitometry.

Immunofluorescence was performed using embryos from timed-pregnant C57Bl/6J mice. 8 days after conception mice were anesthetized with Isoflurane and sacrificed. Embryos were removed from the uterus, fixed in 4% paraformaldehyde for 1 h, and cryoprotected by overnight incubation in Hanks' buffered salt solution containing 30% sucrose and 10% OCT (optimal cutting temperature, Tissue-Tek, Sakura). Fresh-frozen sections of embryos were stained and imaged on a Zeiss LSM510 confocal microscope as previously described (14).

Animal Experiments—Embryos at different stages of development were obtained by dissecting pregnant females. The appearance of a vaginal plug was counted as day 0.5 of pregnancy. Phenotyping experiments were performed using male animals at the indicated ages. Animals were fed standard lab chow or a high fat diet containing 58 kcal % fat from hydrogenated coconut oil (D12330, Research Diets, New Brunswick, NJ). To examine the acute effects of high fat feeding, 200 μl of corn oil (Sigma) was administered to animals twice a day by oral gavage while allowing free access to chow diet and water. Fat and lean mass was determined by dual-energy x-ray absorptiometry using a PIXImus mouse densitometer (Lunar Corp., Madison, WI). Fecal fat was measured by extracting dried feces with 1:3.3:4 volumes of H_2O: chloroform:methanol, and determining the weight of the dried organic phase. Data were expressed as mean ± S.E. The Millennium and PAMFRI IACUC committees approved all animals and procedures.

Enterocyte Isolation and Bodipy-Fatty Acid Uptake Assays—Enterocytes from female 6-month-old C57Bl/6J mice were isolated as previously described (15). In brief, small intestines (duodenum, ileum, and jejunum) were removed, rinsed with Hanks' buffered salt solution (Invitrogen), cut into ~1-cm long sections, and incubated in 50 ml of Hanks' buffered salt solution containing 0.1 M sucrose (Bio-Rad) and 20 mM EDTA. Intestinal sections were gently stirred for 10 min. The detached enterocytes were filtered through sterile cheesecloth (VWR) and pelleted by centrifugation. The cells were then gently re-suspended in Hanks' buffered salt solution. Bodipy-fatty acid (C1-Bodipy-C12, Molecular Probes) uptake assays were performed at 37 °C with 2 μM Bodipy-fatty acid bound to 0.1% bovine serum albumin and quantitated as previously described (15).

TABLE I
Genotype of offspring from FATP4 heterozygote matings

Observed and, for embryonic lethality, expected distribution of F1 offspring from FATP4 heterozygote matings.

Genotype	+/+	Total number genotyped: 332	
		Male, 190 +/−	Female, 142 −/−
Observed	105	227	0
Expected	111	221	0

RESULTS

Targeted Deletion of FATP4 Causes Embryonic Lethality—Genomic DNA containing the mouse *FATP4* locus was obtained by screening a 129/Sv genomic bacterial artificial chromosome library with a fragment containing the 5′ end of the mouse *FATP4* coding sequence and subcloning *FATP4*-containing fragments into a shuttle vector. 11 kb of genomic DNA covering the first two coding exons of *FATP4* were sequenced. A 5′ and a 3′ arm for homologous recombination were then generated by PCR amplification of 3.05 kb of genomic DNA just upstream of the initiation codon (5′ arm) and 5.48 kb of genomic DNA 1,285 nucleotides downstream of the 2nd coding exon (3′ arm). The sequence of the 5′ and 3′ arms as well as the presence of the restriction sites was confirmed by sequencing. Arms were subcloned into the targeting vector pGEMneo-lacz, a pGEM-based vector containing a PGK-neo expression cassette as well as a promoter-less *lacZ* gene containing a nuclear localization sequence (29). The PCR primers were designed to introduce an EcoRI site at the 3′ end of the 5′ arm and a SacI site at the 5′ end of the 3′ arm (Fig. 1*A*) allowing for detection of recombination events by Southern blot (Fig. 1*B*). The correct recombination event results in the removal of exons 1 and 2 deleting the first 185 amino acids of the FATP4 protein.

Whereas heterozygous mice were born at the expected frequencies, we were unable to generate homozygous knockout mice (Table I). From 15 different heterozygous mating pairs we obtained a total of 332 offspring, none of which were homozygous for the *FATP4* deletion allele. Heterozygous mating pairs bred well and produced an average litter size of 8 pups; as expected by Mendelian distribution, heterozygous and wild-

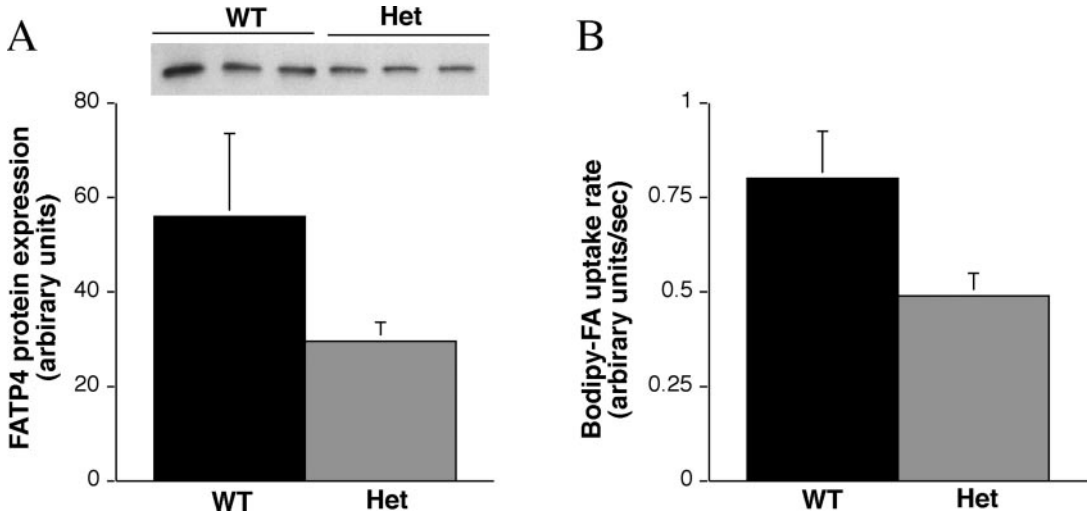

FIG. 2. **Quantitation of FATP4 protein expression and LCFA uptake by isolated enterocytes.** *A*, enterocytes from three age- and sex-matched wild-type and FATP4 heterozygote (*Het*) mice were isolated, lysed, and FATP4 protein content assessed by Western blot/densitometry. *B*, enterocytes from three age- and sex-matched wild-type (*WT*) and FATP4 heterozygote mice were isolated and immediately used for Bodipy-fatty acid uptake assays. Uptake rates were calculated by linear regression and expressed as arbitrary fluorescent units per second.

TABLE II
High fat challenge of FATP4 wild-type and heterozygote mice

Age-matched littermates were weighed at 7 weeks of age and then placed on a high-fat diet. After 10 weeks on a high-fat diet, mice were weighed again, fat and lean mass were determined by DEXA scanning, and fecal fat content was determined using gravimetry.

	Body weight	Body weight	Fat mass	Lean mass	Fecal fat
		g			%
Treatment	Chow	High fat	High fat	High fat	High fat
+/+ (n = 10)	27.7 ± 1.3	40.6 ± 2.5	13.7 ± 1.9	26.6 ± 0.99	6.35 ± 0.37
+/− (n = 10)	28.0 ± 1.1	40.6 ± 2.3	13.8 ± 1.4	26.4 ± 0.8	6.24 ± 0.36

type mice were present at a ratio of 2:1. To address the possibility of neonatal lethality, the cages of pregnant animals were inspected daily and any dead pups were removed, visually examined, and genotyped. We found only a small number of non-viable offspring, which appeared phenotypically normal; none of these pups were homozygous for the FATP4 deletion allele. We therefore conclude that targeted deletion of *FATP4* results in embryonic lethality.

FATP4 Heterozygosity Causes Reduced Enterocyte LCFA Uptake but Does Not Affect Lipid Absorption in Vivo—Mice carrying one copy of the *FATP4* deletion allele had an average 48% decrease in FATP4 protein levels in their intestinal epithelial cells (Fig. 2*A*). All other FATP family members remained undetectable by Western blot (data not shown). Isolated primary enterocytes from *FATP4* heterozygote animals also showed a significant 40% reduction in the average rate of Bodipy-FA uptake (Fig. 2*B*). To examine the *in vivo* consequences of this decrease in FATP4 protein levels and LCFA uptake rates, we performed two sets of experiments. In the first experiment, we placed 7-week-old male wild-type and FATP4 heterozygous littermates on a high fat diet for 10 weeks and recorded body weight and food intake on a weekly basis. At the end of the experiment, we collected feces, and then measured the body composition using dual-energy x-ray absorptiometry. We found no differences between wild-type and *FATP4* heterozygous mice in any of the parameters measured (Table II). To examine the effects of an acute challenge with high fat, 200 μl of corn oil was administered to chow-fed wild-type and *FATP4* heterozygous animals twice a day for 2 days, and the effect on fecal fat content was determined. As expected, administration of corn oil resulted in a significant increase in fecal fat during the first 24 h. Importantly, wild-type and *FATP4* heterozygous mice showed a similar increase (Table III). Thus, 52% of the wild-type FATP4 protein levels appear to be sufficient for normal fat absorption in the small intestine.

Deletion of FATP4 Causes Lethality Early in Embryonic Development—To further examine the mechanism by which *FATP4* deletion causes lethality we dissected embryos at different stages of gestation and obtained their genotypes (Table IV). We were unable to obtain any homozygous *FATP4* knockout mice as early as day 9.5 of gestation. The absence of homozygous knockout mice is significant for embryos younger than day 11.5 of gestation ($p < 0.05$ using Chi square analysis) and highly significant for embryos younger than day 15.5 of gestation ($p < 0.01$). Our attempts to genotype embryos earlier than day 9.5 of gestation were unsuccessful because of contamination of dissected early stage embryos with maternal tissues that compromised the PCR-based genotyping assay.

Staining of neutral lipids with Oil Red O in sections of day 8 wild-type embryos demonstrated that lipids at this stage predominantly accumulate in trophoblasts directly surrounding the yolk sac cavity and by the yolk sac itself (Fig. 3*A*). Immunofluorescent staining of similar sections through day 8 embryos with FATP4-specific antibodies showed that FATP4 was also expressed by both tissues (Fig. 3*B*). More detailed confocal fluorescent microscopy examinations revealed that embryonic FATP4 expression was restricted to the extraembryonic visceral endoderm, a layer of columnar/cuboidal cells with characteristic actin-rich brush-border membranes (Figs. 3*C* and 4) and apical vacuoles (Fig. 4*B*). FATP4 protein in the extraembryonic endodermal cells was restricted in its subcellular localization to the apical brush-border membrane (Figs. 3*C* and 4) reminiscent of the FATP4 localization to apical brush-border membranes in the adult intestine (15).

TABLE III
Corn oil challenge of FATP4 wild-type and heterozygote mice

12-week-old male mice fed a chow diet were weighed and then 200 ul of corn oil was administered twice a day by oral gavage. Feces was collected every 24 h and fecal fat was determined using gravimetry. Fecal fat content was significantly higher after 1 day of corn oil challenge ($p < 0.05$); however, no significant differences were detected between FATP4 +/+ and +/− mice.

	Body weight	Fecal fat	Fecal fat	Fecal fat
	g		*%*	
Treatment	None	None	Corn oil 1 day	Corn oil 2 days
+/+ (n = 9)	30.2 ± 0.7	5.45 ± 0.93	7.51 ± 0.61	6.08 ± 0.77
+/− (n = 9)	32.5 ± 1.1	4.86 ± 1.1	8.11 ± 0.65	5.94 ± 0.49

TABLE IV
Genotyping of embryos from FATP4 heterozygote matings

Timed-pregnant mice were sacrificed at the indicated number of days post-conception and embryos were dissected away from maternal tissues. DNA was extracted from embryonic tissues and used for PCR based genotyping. p value (χ-square) for total numbers < 0.0001.

Days after conception	Number of embryos	+/+	+/−	−/−
9.5	8	3	5	0
11.5	10	3	7	0
13.5	8	2	5	0
15.5	7	0	7	0
18.5	7	1	6	0
19.5	9	3	6	0
Total	49	12	36	0

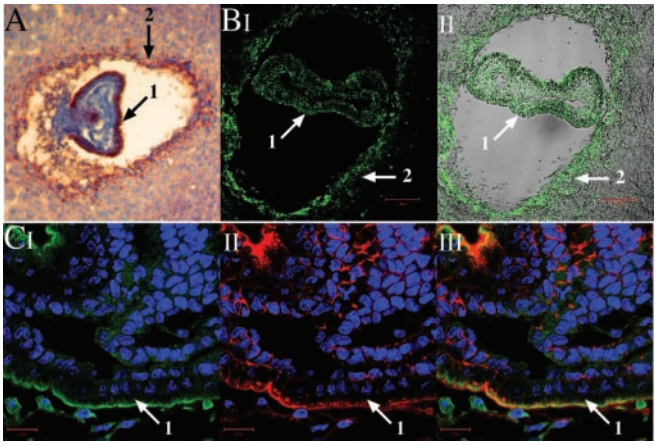

FIG. 3. **Lipid and FATP4 localization in day 8 mouse embryos.** *A*, Oil red/hematoxylin staining of a fixed, fresh frozen embryo demonstrating neutral lipid accumulation by cells of the yolk sac (*1*) and trophoblasts (*2*) surrounding the yolk sac. *B*, localization of FATP4 expression by maternal and embryonic tissues using confocal immunofluorescent microscopy (*I*) and an overlay of *I* with a bright field image (*II*). *Size bar*, 100 μM. *C*, FATP4 (*I*) and actin (*II*) expression in day 8 yolk sac were detected using anti-FATP4 antibodies and rhodamine phalloidin, respectively. Nuclei are shown in *blue*. Colocalization of FATP4 and actin is demonstrated in the overlay by *yellow areas* (*III*). *Size bar*, 20 μM.

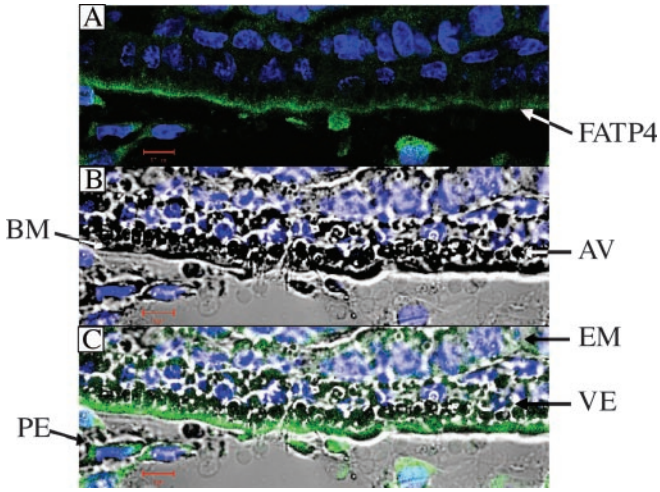

FIG. 4. **Subcellular localization of FATP4.** FATP4 immunofluorescent image (*A*), bright field image (*B*), and composite of both (*C*) showing a section of day 8 embryonic yolk sac membrane. *Arrows* highlight FATP4, apical vacuoles (*AV*), brush-border membrane (*BM*), extraembryonic mesodermal (*EM*) component of yolk sac, visceral endoderm (*VE*), and parietal endoderm (*PE*). *Size bar*, 10 μM.

DISCUSSION

Cellular uptake of LCFAs seems to involve two components, passive diffusion and protein-mediated transport. The family of FATPs has been implicated in the later process (see Ref. 12 for a more detailed review). FATP4 is expressed by several tissues including small intestine, brain, liver, kidney, skin, and heart (10, 22, 30). We have previously shown that FATP4 enhances fatty acid uptake when overexpressed in cell lines and is the major FATP in the small intestine (15). FATP4 has also been demonstrated to possess long-chain and very long-chain acyl-CoA synthetase activity (22) and may therefore mediate uptake through metabolic activation of fatty acids. In our attempt to further investigate the role of FATP4 in the absorption of dietary lipids and fatty acid transport by generating targeted deletions of the *FATP4* gene we uncovered a novel function for FATP4 during early embryonic development in the mouse. Not one, of 332 genotyped pups from *FATP4* heterozygote mating pairs, showed a homozygote deletion of the FATP4 allele. We also were unable to detect any *FATP4* null embryos between developmental days 9.5 to 19.5, indicating a severe early embryonic defect caused by the complete loss of FATP4 function. Partial loss of FATP4 function during development, however, did not affect viability, because the frequency of heterozygote offspring was as predicted.

We have previously shown that FATP4 is the principal FATP in the small intestine (15), and that reduction of FATP4 protein levels via antisense oligonucleotide treatment resulted in reduced LCFA uptake by isolated enterocytes (15). Consistent with this observation, we have shown here that isolated enterocytes from *FATP4* heterozygote mice have both reduced FATP4 protein levels and LCFA uptake rates. The 48% reduction in FATP4 protein levels observed in heterozygote mice did not, however, translate into an observable phenotype, as body composition, weight changes, and fecal fat content in FATP4 heterozygote mice were not significantly different from wild-type littermates. The 40% reduction in enterocyte LCFA uptake may not have been sufficient to impact overall absorption because of the large capacity of the small bowel for lipid uptake. Similarly, the gastric and pancreatic lipase inhibitor, Orlistat, only affects lipid absorption and weight gain after inhibiting more than 90% of lipase activity (31).

Maternal to fetal transport of essential and non-essential fatty acids plays an important role during embryonic development (32, 33). In mice and rats, the yolk sac is the primary interface for nutrient exchange before the development of a

functional chorioallantoic placenta (days 9 to 10) (34). The inner layer of the yolk sac is comprised of the visceral endoderm, a columnar layer of cells with an apical brush-border membrane that is responsible for the absorption of nutrients coming from maternal blood sinuses, and trophoblasts surrounding the Reichert's membrane (34). Interestingly, we found that FATP4 is highly expressed by this cell layer by embryonic day 8 and targeted predominantly to the brush-border membrane. Thus, a likely cause for the observed embryonic lethality of *FATP4* null animals is an insufficient embryonic absorption and/or activation of maternal saturated and unsaturated fatty acids. This hypothesis is indirectly supported by the observation that deletions of two other proteins that are expressed by the yolk sac and are involved in lipid metabolism, microsomal triglyceride transfer protein and apolipoprotein B, both lead to early embryonic lethality (35, 36). Whereas *MTP* and *apoB* deletions impair the formation and basolateral secretion of nascent very low density lipoprotein particles from the visceral endodermal cells, loss of FATP4 function could result in a reduced absorption of fatty acids from hydrolyzed maternal lipids on the apical site of the cells. All three mutations would result in severely reduced levels of lipids and essential fatty acids reaching the embryo.

During the preparation of this manuscript, two publications have also reported mouse strains with FATP4 loss of function due either to a spontaneous transposon insertion in exon 3 (30), or by targeted disruption of exon 3 via homologous recombination (37). Both groups report that *FATP4* null mice are viable at birth but die within days, because of a phenotype reminiscent of restrictive dermopathy with tight, wrinkle-free skin and disrupted skin barrier function (30, 37). The exact reasons behind the observed discrepancies in the *FATP4* null phenotype are unclear. Because our *FATP4* null mice were based on a single ES clone, it is a formal possibility that an unrelated mutation in this clone occurred and is causal for the embryonic lethality. However, chances of this occurring seem remote because the hypothetical mutation would have to be in close linkage to the *FATP4* locus given that not a single FATP4 homozygote mouse of 400 genotyped mice was observed. Furthermore, independent commercial efforts to create targeted deletions of the *FATP4* gene also resulted in embryonic lethality.[2] Other possible explanations include genetic strain differences, and the fact that our targeting construct deleted exons 1 and 2, whereas in both non-lethal *FATP4* mutations, the first two exons were left intact, leaving the possibility that abbreviated FATP4 fusion proteins are produced. Whereas it is highly speculative that the first 185 amino acids encoded by exons 1 and 2 would present a peptide with biological activity, it is worthwhile noting that this region is homologous to fragments of FATP1 that would include the entire extracellular N terminus, the transmembrane region, and cytoplasmic membrane-associated sequences (38). Because FATPs may be multifunctional proteins with separable residues involved in LCFA transport and activation (39), the hypothetical fusion proteins could retain some aspects of FATP4 function, such as LCFA transport, or interaction with other proteins, for instance long-chain acyl-CoA synthetases (40) or other FATPs (19). Although the causes of neonatal mortality, described by Moulson *et al.* (30) and Herrmann *et al.* (22), may differ from the embryonic death described in this article, both lethalities ultimately underscore the importance of FATP4-mediated LCFA uptake/activation for normal physiology.

[2] R. Phillips, personal communication.

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