The Expression and Function of Fatty Acid Transport Protein-2 and -4 in the Murine Placenta

Takuya Mishima¹, Jeffrey H. Miner², Mayumi Morizane¹, Andreas Stahl³, Yoel Sadovsky¹,⁴*

¹ Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, ² Departments of Internal Medicine, Cell Biology and Physiology, Washington University, St. Louis, Missouri, United States of America, ³ Department of Nutritional Sciences and Toxicology, University of California, Berkeley, California, United States of America, ⁴ Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America

Abstract

**Background:** The uptake and trans-placental trafficking of fatty acids from the maternal blood into the fetal circulation are essential for embryonic development, and involve several families of proteins. Fatty acid transport proteins (FATPs) uniquely transport fatty acids into cells. We surmised that placental FATPs are germane for fetal growth, and are regulated during hypoxic stress, which is associated with reduced fat supply to the fetus.

**Methodology/Principal Findings:** Using cultured primary term human trophoblasts we found that FATP2, FATP4 and FATP6 were highly expressed in trophoblasts. Hypoxia enhanced the expression of trophoblastic FATP2 and reduced the expression of FATP4, with no change in FATP6. We also found that Fatp2 and Fatp4 are expressed in the mouse amnion and placenta, respectively. Mice deficient in Fatp2 or Fatp4 did not deviate from normal Mendelian distribution, with both embryos and placentas exhibiting normal weight and morphology, triglyceride content, and expression of genes related to fatty acid mobilization.

**Conclusions/Significance:** We conclude that even though hypoxia regulates the expression of FATP2 and FATP4 in human trophoblasts, mouse Fatp2 and Fatp4 are not essential for intrauterine fetal growth.

Introduction

Both the human and mouse placenta are hemochorial, with fetal-derived trophoblasts bathed in maternal blood, and are thus well-positioned to regulate placental transport functions, including transport of oxygen, nutrients, and waste products between the maternal and fetal blood. Among transported nutrients, the uptake and trafficking of fatty acids is critical for embryonic development and growth in all eutherians, particularly during the second half of pregnancy, when the fetal/placental growth ratio is markedly increased, corresponding to increasing fetal caloric demands [1–3]. Transported essential fatty acids (linoleic acid, and α-linolenic acid) are metabolized into long chain poly-unsaturated fatty acids (LCPUFAs), and are necessary for development of vital organs such as the heart and lung. A particularly high amount of arachidonic acid and docosahexaenoic acid is needed for development of the brain and retina [3–8]. Fatty acids are also essential for biosynthesis of membrane phospholipids, myelin, gangliosides, glycolipids and sphingolipids, and for production of signaling eicosanoids [9–12]. Albumin-bound free fatty acids (FFA), VLDL, and chylomicrons in the maternal circulation are the major source of fatty acids to the placenta, and require the action of trophoblastic triglyceride hydrolase for liberation of FFA and transport across the trophoblastic microvillus membrane [13–15]. The mechanisms underlying trophoblast fatty acid uptake and trafficking are largely unknown. Membrane-bound and cytoplasmic fatty acid binding proteins (FABPs) are expressed in trophoblasts, but their function in intracellular trafficking of fatty acids in trophoblasts is unknown [16–18].

Cytoplasmic FFAs bound to fatty acid binding proteins (FABPs) are targeted for metabolism or storage in lipid droplets, which are dynamic organelles that actively store neutral lipids (such as triglycerides, cholesteryl esters and retinol esters) [19–21]. In addition to their neutral fats, lipid droplets are encased within a layer of amphipathic lipids, and coated by lipid droplet-associated (PLIN) proteins that regulate the assembly, maintenance, and composition of lipid droplets, as well as lipolysis and lipid efflux [22–25].

The family of fatty acid transport proteins (FATPs, solute carrier family 27, SLC27) is an evolutionarily conserved group of integral trans-membrane proteins which, along with fatty acid translocase (FAT/CD36), mediate cellular uptake of long-chain and very long chain fatty acids. This prevalent, saturable, carrier-regulated process is distinct from the less common, passive (“flip-flop”) membrane diffusion [26–28]. FATPs comprise a family of six highly homologous proteins, which are expressed primarily in...
fatty acid-utilizing tissues [28–30]. Interestingly, FATP4 is also highly expressed by epithelial cells of the visceral endoderm and localizes to the brush-border of extraembryonic endodermal cells [31]. It is hypothesized that FATP1, FATP2, and FATP4 are bifunctional, exhibiting both transport and acyl-CoA synthase activities, which facilitate fatty acid influx across biological membranes [32–34].

The expression of placental FATPs and their regulation in this tissue is largely unknown. We recently showed that ligand-stimulated PPARG enhances the expression of FATP1 and FATP4 as well as PLIN2 in primary human trophoblast (PHT) [35], and that hypoxic trophoblasts retain neutral lipids in the form of lipid droplets [35, and manuscript in preparation]. In this study we sought to identify key FATPs that are expressed in the human placenta and regulated during hypoxic stress, and use Fatp mutant mice to decipher the function of relevant FATPs in vivo.

Results

We initially examined the expression of FATP transcripts in the human placenta and in isolated primary term trophoblasts (PHTs), and compared the level of FATP expression to that of other human tissues, serving as controls. Because hypoxia increases the accumulation of lipid droplets in trophoblasts [35], we also assessed the expression of the lipid droplet–associated (PLIN) transcripts, and detected the expression of PLIN2 and PLIN3, but not the other members of this family. Among the highly expressed FATPs, we found that hypoxia enhanced the expression of FATP2, but diminished the expression of FATP4, with no change in FATP6 (Fig. 1B). Notably, the weakly expressed FATP1 was also increased in hypoxia. The increase in PLIN2 in hypoxic PHTs (Fig. 1B) was expected [36], and suggests lipid droplet accumulation in hypoxic PHTs. Together, these data indicate that placental FATP2 and FATP4 are relatively highly expressed in primary human trophoblasts, and that hypoxia has an opposite influence on their expression in human trophoblasts. Therefore, our subsequent analysis centered on FATP2 and FATP4.

To gain insight into the role of placental FATP2 and FATP4 in vivo, we initially sought to examine the expression of Fatp in placentas of wild type C57Bl/6 mice. We found several differences in Fatp expression between human and murine placentas. The near term mouse placenta expresses primarily Fatp1, Fatp3, Fatp4, and Fatp6 (Fig. 2A). Interestingly, Fatp2 is expressed mainly in the mouse amnion, but not in the placenta (Fig. 2A). We confirmed the expression pattern of FATP2 and FATP4 proteins in the placenta and amnion (Fig. 2B–C).

The expression of Fatp in other mouse organs was similar, but not identical, to that in humans (compare Fig. 1A–2A). The expression pattern of murine Plin was similar to that of human tissues, with weak expression of Plin4 in the mouse placenta. Hypoxia had a weak and statistically insignificant effect on the expression of relevant murine Fatp and Plin in both the placenta and the amnion (Fig. 2D–E). As a control, we showed reduced expression of placental transferrin receptor (Tfrc), a marker of hypoxic mouse placenta [37,38].

We used ISH to localize the expression of Fatp2, Fatp4, and Fatp6 at E7.5 (prior to establishment of maternal-fetal trafficking), E12.5 (after maternal-fetal trafficking is initiated), and E17.5 (near-term placenta, Fig. 3). None of the three Fatps were definitively detected at E7.5. Using renal cortical expression of Fatp2 as positive control [39], we found that Fatp2 was expressed in the murine amnion but not in the placenta proper at E12.5 and E17.5 (Fig. 3, A–E). Similarly, Fatp4 was expressed in amnion at E12.5, and exhibited weak, relatively diffuse placental expression at E12.5 and E17.5. This was confirmed using Fatp4 expression in placenta derived from Fatp4 transgenic mice (Fig. 3I), and using Fatp4 expression in the intestinal villous epithelium as positive control (Fig. 3J). Fatp6 was primarily detected in the spongiosotrophoblast at E12.5 and E17.5, but not at E7.3 clearly (Fig. 3, L–O).

We next sought to assess the impact of Fatp2 or Fatp4 deficiency on the murine placenta. Crossing heterozygous Fatp2 or Fatp4 males and females, we found that at E17.5 Fatp2 or Fatp4 deficient fetuses were indistinguishable from their wild type littermates in either standard or hypoxic conditions with respect to litter size, genotype distribution, placental and embryo weight (Fig. 4). In
addition, placental histology was unchanged among the genotypes in either normal or hypoxic placenta (data not shown). Because FATPs play a role in cellular fatty acid uptake, we examined the accumulation of neutral fat, as well as triglyceride levels, in the placentas of wild type and Fatp2 or Fatp4 deficient mice. Oil Red O staining (Fig. 5A–C) and Sudan Black B staining (Fig. 5D–F) of placentas from all genotypes showed a similar pattern of diffuse fat staining, with more abundant fat in the decidua. This expression pattern was unchanged in hypoxic placentas (data not shown). Similarly, the concentration of placental triglycerides was also

Figure 3. ISH for detection of Fatp2, Fatp4 and Fatp6 expression in the mouse placenta. Fatp2 expression in the mouse embryo at E7.5 (A), placenta at E12.5 (B) and at E17.5 (C), and kidney (D). Arrowheads point to the amnion. Fatp4 expression in mouse embryo at E7.5 (F), placenta at E12.5 (G) and at E17.5 (H), in the Fatp4 transgenic placenta (I) and in the small intestine (J). Fatp6 expression in mouse embryo at E7.5 (L), placenta at E12.5 (M) and at E17.5 (N). Negative controls using a sense probe are shown in E, K, O. Scale bars = 0.5 mm.

doi:10.1371/journal.pone.0025865.g003
similar among the genotypes, with hypoxia causing a small yet significant increase in triglyceride concentration in the hypoxic Fatp2 KO placenta (Fig. 5G). Lastly, we used quantitative RT-PCR to rule out the possibility that the expression of other Fatps might compensate for the reduced levels of Fatp2 or Fatp4. As shown in Fig. 6, the expression of Fatp2 or Fatp4 was appropriately reduced in the respective placentas and amnion tissues, with reduced expression in placental Fatp6 or Fatp3 in the Fatp2 and

Figure 4. The feto-placental phenotype of Fatp2 and Fatp4 deficient mice. The litter size, genotype distribution, placental and embryo weight at E17.5 in standard or hypoxic conditions after cross-breeding of heterozygous pregnant mice. (A) Fatp2 analysis, (B) Fatp4 analysis. The graphs depict placenta and embryo weight. * denotes p<0.05.
doi:10.1371/journal.pone.0025865.g004

Figure 5. The effect of Fatp2 or Fatp4 deficiency on the levels of neutral lipids in the mouse placenta. Frozen sections stained with oil Red O (A–C) or with Sudan Black B (D–F) of wild type, Fatp2 KO, or Fatp4 KO at E17.5. Scale bars = 1 mm and 100 μm in larger panels and insets, respectively. (G) Triglyceride concentration of Fatp2 wild type or KO (left panel), or Fatp4 wild type or KO (right panel) in standard or hypoxic condition (n = 5 for each strain and each condition). * denotes p<0.05.
doi:10.1371/journal.pone.0025865.g005
Fatp4 KO placentas, respectively. Importantly, none of the Fatps, Plin2 or Plin3 exhibited a compensatory increase in expression.

Discussion

The uptake, mobilization and efflux of fatty acids are critical for fetal growth, with fetuses of malnourished pregnant women being at risk for intrauterine growth restriction [10,14,40,41]. Several observations regarding FATPs led us to interrogate the expression and function of placental FATPs: (a) hypoxic human trophoblasts accumulate neutral lipids with lipid droplets [35], and manuscript in preparation, (b) PPARg/RXR increase trophoblastic FATP1 and FATP4 expression, reduce FATP2 expression, and enhance trophoblast uptake of fatty acids and lipid droplet accumulation [42], and (c) inhibition of p38 (which mimics key aspects of PPARg deficiency) also up-regulates FATP2 and down-regulates FATP4 expression [42]. We found that among FATPs, FATP2, FATP4 and FATP6 are expressed in human trophoblasts, and that hypoxia enhanced FATP2 and reduced FATP4 expression levels. These data, which are opposite of the effect of PPARg/RXR signaling on FATP2 and FATP4, suggest that FATP2 and FATP4 play a role in trophoblast fatty acid trafficking. We therefore used pregnant mice to assess the function of FATP2 and FATP4 in the placenta in vivo. Although the human and mouse placenta share many structural, functional, and gene expression patterns [43,44], there are marked morphological and functional differences between placentas of the two species, including differences in transport functions [45–47]. Whereas FATP4 was expressed in both the human and murine placenta, the expression of murine Fatp2 was restricted to the amnion. Unlike the expression changes in human trophoblastic FATP2 and FATP4 when cultured in hypoxic atmosphere, we found no difference in the expression of murine placental Fatp2 and Fatp4 between standard and hypoxic conditions in vivo.

We produced hypoxia during the latter part of mouse pregnancy using an O2 concentration of 12% for 6 days, which is similar to the level of hypoxia used by others and us [48,49]. The most suitable degree of hypoxia for cultured PHT cells remains controversial. Low oxygen tension (pO2 of 15–20 mmHg) characterizes the early human placenta, before maternal blood begins to perfuse the intervillous space, with a rise to ~55 mmHg after 12 weeks of pregnancy [50,51]. Placental hypoxia is abnormal after that gestational age [52,53]. Exposure of cultured third trimester trophoblasts to pO2<1%, as we chose in our experiments, is commonly used to model hypoperfusion-induced villous injury [54–56]. Notably, the differences between our in vitro analysis using PHT cells and intact mouse placentas likely reflect inter-species differences in expression patterns and functions of FATPs. Moreover, there is a clear dissimilarity between exposure to hypoxia in vitro and in vivo, where the response of purified cultured cells to extreme hypoxia might be different from that of intact tissue, which is exposed to marked, yet life-sustaining hypoxia.

Genetic ablation of murine Fatp2 and Fatp4 expression did not lead to any functional consequences with respect to feto-placental growth, and specifically, lipid accumulation. These data are consistent with those of Heinzer et al [57], which did not specifically focus on embryonic development, yet reported normal growth, behavior and activity of Fatp2 KO mice. Moubon et al [58] reported that Fatp4 KO mice died soon after birth, with shiny, tight, thick skin. This phenotype was reproduced by Herrmann et al [59,60], who showed that epidermal-specific conditional Fatp4 KO mice exhibited similar morphological abnormalities as embryos, indicating that the skin-related abnormalities of Fatp4 KO fetuses reflect fetal maldevelopment and not placental dysfunction. In addition, Gimeno et al [61] demonstrated early embryonic lethality in Fatp4 KO mice, possibly related to the expression of FATP4 in the extraembryonic endoderm. Interestingly, two other membrane-related fatty acid transporters, FABPpm and CD36 (FAT), are also expressed in the murine placenta, primarily in the labyrinth and junctional zone [17]. While a knockout mouse model for FABPpm has not been
published, CD36 deficient mice do not exhibit a pregnancy or placenta-related phenotype [62].

Although we ruled out significant compensatory changes in placental Fatp expression in Fatp2- or Fatp4-deficient mice, we cannot rule out functional redundancy among fatty acid transporters. Such redundancy is unlikely, though, because the expression of the different Fatps in the placenta is dissimilar. Furthermore, as noted above, the phenotype of Fatp2- and Fatp4-deficient mice in non-placental tissues does not overlap. Our data are the first to show the expression of Fatp6, hitherto known to be expressed in the human heart and bovine mammary tissue [12,63], in human trophoblasts and mouse placentas, as well as in the female and male gonads. It is possible that Fatp6 plays a role on fat trafficking in the placenta as well as in other organs, which may be redundant with the function of other FATPs.

While critical experiments that might have uncovered the role of FATPs in the human placenta cannot be ethically performed, our results underscore limitations in the use of animal models to inform human biology. Mutations in human FATP2 are currently unknown. Several mutations in human FATP4 are associated with ichthyosis prematurity syndrome, where preterm delivery is related to polyhydramnios, not a placental function [64], or with congenital verruciform hyperkeratosis [65]. New animal models as well as manipulation of FATP expression using ex vivo human samples might be necessary in order to fully analyze the role of FATPs in placental fat uptake and trafficking.

Materials and Methods

Primary human trophoblast (PHT) isolation and culture

Placental tissue samples were collected by the Obstetrical Specimen Procurement Unit at Magee-Womens Hospital of the University of Pittsburgh Medical Center. Collection was conducted under an approved exempt protocol by the Institutional Review Board of the University of Pittsburgh. Patients provided written consent for the use of de-identified, discarded tissues for research upon admittance to the Hospital. PHT cells were isolated from term human placentas \( n = 5 \) and cultured as we previously described [55]. Cells were cultured in an atmosphere of 20% O\(_2\) with 5% CO\(_2\) at 37C as standard condition, or in O\(_2\)-deficient mice, we

Mouse breeding, genotyping and exposure to hypoxia

Our experiments were conducted under protocol number 0806669-B4, which was approved by the Institutional Animal Care and Use Committee of University of Pittsburgh. Fatp2 (Slc27a2tm1Kds) heterozygous C57Bl/6 mice harboring a targeted mutation in the Fatp2 gene were generously provided by Dr. Kirby Smith (Johns Hopkins). Fatp4 heterozygous (Slc27a4tm1verf) C57Bl/6 mice, which harbor a spontaneous transposon insertion in Fatp4 gene, as well as the Fatp4 overexpressing mouse, were previously described [66]. Timed matings were carried out by pairing heterozygous males and females for one night, with the morning after mating designated as embryonic day 0.5 (E0.5). Pregnancy was assumed based the presence of vaginal plug and a 10% weight gain on E10.5. Mice were kept under constant conditions until E11.5, which were assumed a standard rodent chow and water ad libitum, and kept on a 12:12 h light-dark cycle in room air. Delivery typically occurs on E19.5. Exposure to hypoxia, where relevant, was initiated on E11.5, when the mice were either exposed to FiO\(_2\) = 12% between E11.5 and E17.5 (hypoxia group) or normoxia at standard atmospheric conditions. For exposure to normobaric hypoxia we used a Polymer Hypoxic Glove Box with a Purge Airlock system with CO\(_2\) and O\(_2\) control indicators (Coy Laboratory Products, Grass Lake, MI), which is specifically designed for experiments in live rodents and regulates ambient temperature, humidity, and gas composition. Dams were euthanized by CO\(_2\) asphyxiation. Embryos and placentas were weighed, and immediately processed for further analysis (see below). Each set of analyses included ten pregnant mice, each carrying 6–10 embryos. Fetuses from uterine horns containing only one embryo were excluded from the final analysis. Genomic DNA was extracted from embryo tails by the alkaline lysis and boiling method [67] and genotyped using standard PCR, as previously described [34,58].

Histological Analysis

For oil Red O staining, 4% PFA-fixed samples were immersed in 10%, then 20% sucrose in PBS, followed by OCT embedding. Sections were cut using a cryostat (Cryotome FSE, Thermo Scientific, Wilmington, DE) at 7 µm thickness, then stained with oil Red O (Sigma) [68] and counter-stained with hematoxylin or stained with Sudan Black B (Sigma) and counter-stained with nuclear fast red.

For detection of Fatp2, Fatp4 and Fatp6 by ISH we used digoxigenin-labeled cRNA probes, synthesized using digoxigenin RNA labeling kit (Roche, Basel, Switzerland). Cryosections (10 µm) of the OCT-embedded placentas were rehydrated in PBS, digested with protease K (10 µg/ml, 5 min at 37C), treated with 0.2 N HCl for 10 min at RT, acetylated (0.25% acetic anhydride in TEA for 10 min at RT), and then hybridized with cRNA probes overnight at 60°C. Slides were washed four times with 4×SSC, digested with RNaseA (5 µg/ml) for 15 min at 37C, and washed twice with 0.5×SSC for 15 min at 30C. Slides were blocked using 1% blocking reagent (Roche) in maleic acid buffer (MAB), followed by incubation with anti-DIG-AP antibody (0.5 U/ml) for 2 h at RT, washed with MABT (MAB with 0.2% Tween20), and then reacted with BM purple (Roche) with 1 mM levamisole overnight. The sections were examined using Nikon 90i microscope (Nikon, Tokyo, Japan) equipped with DS-Ri1 CCD camera (Nikon).

Standard and quantitative RT-PCR

Total RNA was extracted from PHT cells or from diverse tissues of eight weeks old mice using TriReagent (MRC, Cincinnati, OH) according to the manufacturer’s instructions. Some of the analyses were also performed using Human Total RNA Survey Panel (Ambion, Austin, CA). RNA samples were treated with DNaseI using a Turbo DNA-free Kit (Ambion). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA in 20 µl of reaction mixture using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA). Synthesized cDNA samples were diluted 1:5 in DEPC-treated H\(_2\)O. RNA quality was assessed by 260/280 and 260/230 absorbance ratio using NanoDrop (Thermo). Standard RT-PCR was performed using KOD Extreme DNA polymerase (EMD, Gibbstown, NJ) with 2 µl cDNA per 20 µl reaction volume, with amplification in a Veriti thermal cycler (Applied Biosystems) using the following conditions: 94°C for 2 min; 35 cycles at 98°C for 10 sec, 60°C for 30 sec, and 68°C at 30 sec. PCR products were electrophoresed on a 2% TAE agarose gel and DNA detected using ethidium bromide. Quantitative RT-PCR was carried out in duplicates using 384 well plates with 2 µl of cDNA per 10 µl of reaction mixture using SYBR Green PCR master mix (Applied Biosystems). A total of 8–12 cDNA from mouse samples and five cDNAs from human samples were used for each analysis. PCR was carried out in
Table S1 Primers used in standard and RT-qPCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession</th>
<th>Description</th>
</tr>
</thead>
</table>

**Western blot analysis**

Proteins for western blot were prepared from cells using lysis buffer, and by homogenization in lysis buffer for tissues, as we previously described [35]. Protein lysates were electrophoresed using 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel at 180 V for 1 h, then transferred to polyvinylidene difluoride membranes (Biorad, Hercules, CA) at 23 V for overnight. After blocking with 5% non-fat dried milk in TBST, the membranes were incubated overnight with rabbit anti-FATP2, FATP4 antibodies as previously described [30] or mouse anti-actin, Millipore, Bedford, MA) antibodies at 4 C. After washing with TBST, the membranes were incubated with goat anti-rabbit IgG peroxidase conjugated (Santa Cruz Biotech, Santa Cruz, CA) and TBST, the membranes were incubated with goat anti-rabbit IgG peroxidase conjugated (Santa Cruz Biotech, Santa Cruz, CA) and donkey anti-mouse IgG peroxidase conjugated (Santa Cruz Biotech, Santa Cruz, CA) for 2 h at RT. Detection was performed with Western Lightning ECL kit (Perkin Elmer).

**Triglyceride assay**

Lipids were extracted from each murine placenta by the Folch method [72], and triglyceride concentration determined using a triglyceride spectrophotometer assay kit (Cayman Chemical, Ann Arbor, MI), detected using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). Five placental fragments, each 10–20 μg, were used for analysis, and normalized by weight.

**Statistics**

Statistical analysis was performed using analysis of variance (ANOVA) with Bonferroni post hoc test for multiple comparisons of placental and embryo weight, and by Mann-Whitney test for RT-qPCR and triglyceride assay using Dr SPSS II for Windows (SPSS, Chicago, IL). Significance was determined at p<0.05.

**Supporting information**

**Table S1 Primers used in standard and RT-qPCR.**

**Acknowledgments**

We thank Kirby Smith (Johns Hopkins) for the VLCS (FATP2) heterozygous mice. We also thank Tonia Tse and Karen Peters for technical assistance, and Lori Rideout for assistance in manuscript preparation.

**Author Contributions**

Conceived and designed the experiments: TM JHM AS YS. Performed the experiments: TM MM. Analyzed the data: TM YS. Wrote the paper: TM JHM AS YS. Generated FATP4 transgenic mice: JHM.

**References**


54. Bionaz M, Loor JJ (2008) ACSL1, AGPAT6, FABP3, LPIN1, and SLC27A6 are the most abundant isoforms in bovine mammary tissue and their expression is initially described as affected by self-healing congenital verruciform hyperkeratosis. Am J Med Genet A 152A: 2664–2665.

