Fatty Acid Binding Protein 3 (FABP3) Regulates n-3 and n-6 Polyunsaturated Fatty acids (PUFAs) Transportation in Mouse Trophoblast

（脂肪酸結合タンパク質はマウス胎盤栄養膜細胞の多価不飽和脂肪酸輸送を制御する）

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by

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ABSTRACT

Deficiency of placental fatty acid transport during the embryonic period has been suggested to result in fetal developmental disorders and in various adult metabolic diseases. In this study, we examined the localization and functional significance of fatty acid binding protein (FABP), a cellular chaperone of fatty acids, in mouse placenta. Four FABPs (FABP3, FABP4, FABP5 and FABP7) were expressed with spatial heterogeneity in placenta, and FABP3 was dominantly localized to the trophoblast cells. In placenta from *Fabp3* gene-ablated mice, the transportation coefficient for n-6 polyunsaturated fatty acids (PUFA) [n refers to polyunsaturated fatty acid] was reduced by 25 % ($P < 0.01$) and 44% ($P < 0.001$) at embryonic days 15.5 (E15.5) and E18.5 respectively, while that for n-3 PUFAs was reduced by 19 % ($P < 0.05$) and 17 % ($P < 0.05$) respectively. Accumulation of both n-3 and n-6 PUFAs in *Fabp3* gene-ablated fetus was also reduced on days E15.5 and E18.5. In contrast, saturated fatty acid transportation and accumulation was unaffected in gene-ablated mice. An *in vitro* study using a trophoblast cell line (BeWo) also demonstrated that incorporation of n-6 and n-3 PUFAs, but not saturated FAs, was significantly ($P < 0.01$) down-regulated in *FABP3* knock-down BeWo cells. Glucose transportation and utilization was significantly up-regulated in both the *in vivo* and *in vitro* studies. These findings suggest that FABP3 controls PUFA trafficking in trophoblast, strongly indicating that FABP3 is associated with pathophysiology of fetal developmental disorders and adult metabolic/psychiatric diseases.

Dedication

To my parents, parents-in-law and my garments Ayrin Sultana for all of their love, support, and encouragement.

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List of Abbreviations

AA	Arachidonic acid
ALA	α-linolenic acid
ADHD	Attention deficit hyperactivity disorder
BeWo	human choriocarcinoma cell line
CAG-EGFP	CAG promoter driven-enhanced green fluorescence mouse
DAB	3, 3'-diaminobenzidine tetrahydrochloride
DAPI	Diamidino-2-phenylindole
DHA	Docosahexaenoic acid
E	Embryonic day
FABP	Fatty acid binding protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLU	Glucose
kDa	Kilodalton
LCM	Laser capture microdissection
LA	Linoleic acid
MCT1	Monocarboxylated transporter 1
PA	Palmitic Acid
PBS	Phosphate buffer saline

1. INTRODUCTION

1.1 Placenta: the vital temporary organ of mammals

The placenta represents a key organ for fetal growth as it acts as an interface between mother and fetus regulating the fetal-maternal exchange of nutrients, gases, water, ions, and waste products; moreover, it is capable of metabolic, immunologic, and endocrine functions to support successful pregnancy. It plays a key role in fetal programming by directly regulating nutrients (including fatty acids) supply and fetal normal growth. It is essential for sustaining the growth of the fetus during gestation, and defects in its function result in fetal growth restriction or, if more severe, fetal death. In addition, it produces hormones that alter maternal physiology during pregnancy and forms a barrier against the maternal immune system (Cross *et al.,* 2003).

In humans and rodents, the fully developed placenta is composed of three major layers: the outer maternal layer, which includes decidual cells of the uterus as well as the maternal vasculature that brings blood to/from the implantation site; a middle "junctional" region, which attaches the fetal placenta to the uterus and contains fetoplacental (trophoblast) cells that invade the uterine wall and maternal vessels; and an inner layer, composed of highly branched villi that are designed for efficient nutrient exchange (Rossant *et al.,* 2001). The villi are bathed by maternal blood and are

composed of outer epithelial layers that are derived from the trophoblast cell lineage and an inner core of stromal cells and blood vessels.

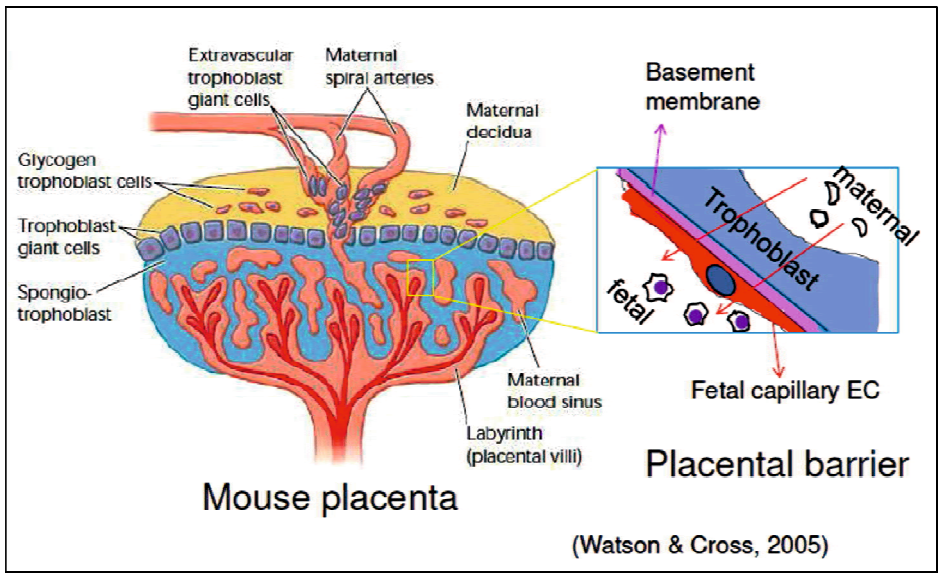


Figure 1: Structure of mouse placenta with it's different compartment (zone)

Placental biology is now being focused by many researchers in relation to the impact of nutrients including fatty acids, amino acids, glucose, growth factors etc on fetal tissue/organ development and their ultimate impact on childhood as well as adult diseases (metabolic or psychiatric). Fetal growth is closely related to the capacity of the placenta to transport nutrients, which is dependent on the expression and activity of specific transporter proteins on the syncytiotrophoblast (Jansson *et al.,* 2009; Sibley 2009).

Abnormal embryonic or fetal nutrition can lead to deleterious consequences in adult life (Barker 2006; Barker *et al.,* 2006; Fowden *et al.,* 2006) and it has been suggested that there is a strong association between low birth weight and the risk of developing type 2 diabetes mellitus(Hales *et al.,* 1991; Ravelli *et al.,* 1998) and cardiovascular disease(Barker *et al.,* 1986; Barker *et al.,* 1989) in adulthood.

1.2 Polyunsaturated fatty acids (PUFAs) and cell biology

Mammalian cells utilize three main types of fatty acids (FAs): (1) saturated FAs, which do not contain any double bonds; monounsaturated FAs (MUFAs), which contain a single double bond; and polyunsaturated FAs (PUFAs), which contain multiple double bonds. Among them PUFAs are important biological constituents having metabolic, structural, and signaling roles. The developing fetus requires substantial amounts of fatty acids to support rapid cellular growth and activity and among them; the n-3 (also termed as ω-3) and n-6 (ω-6) PUFAs are crucial (Haggarty 2010). The most biologically important n-3 and n-6 PUFAs are eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3), dihomo gamma linolenic acid (20:3n-6), and arachidonic acid (AA; 20:4n-6) (Haggarty 2010). While these PUFAs are metabolic derivatives of the essential fatty acids, α-linolenic acid (ALA), and linoleic acid (LA),

their formation from these precursors is energetically demanding and so they are most readily obtained from the diet.

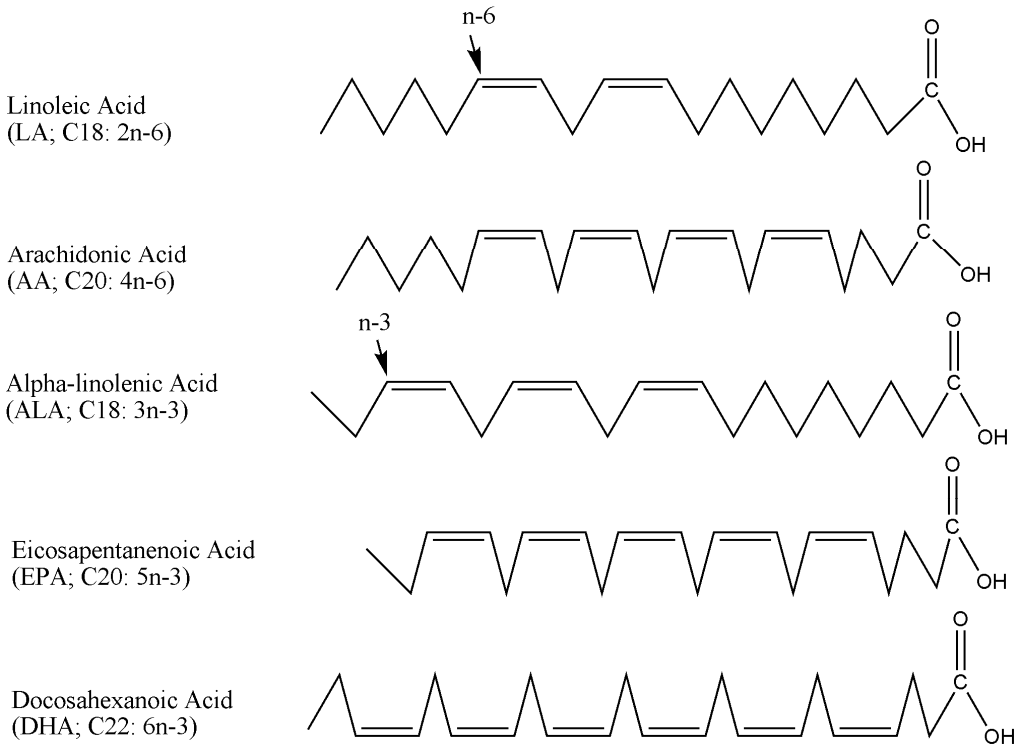


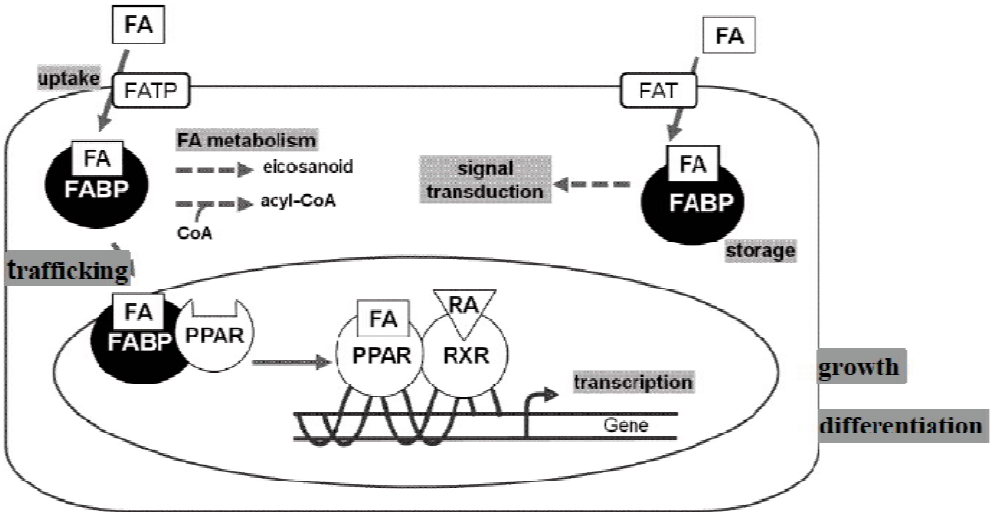
Figure 2: Structure of n-3 and n-6 PUFAs

Unlike plants, mammals are not able to synthesize the parent compounds of both fatty acid families, the n-6 fatty acid LA and the corresponding n-3 fatty acid ALA. Therefore, they must be obtained through the consumption of food. In contrast, mammals are capable of synthesizing the long-chain derivatives of these fatty acids, in particular AA, EPA and DHA, based on LA and ALA in a multistage conversion process, which primarily takes place in the endoplasmic reticulum of liver cells

(Clandinin *et al.,* 1981; Schuchardt *et al.,* 2010). Because the placental desaturase activity/fetal enzyme activity is limited in utero, the fetus depends on placental PUFAs transfer (Hanebutt *et al.,* 2008; Cetin *et al.,* 2009). High amounts of DHA are incorporated into brain/retinal membranes and modulate membrane fluidity and permeability, improve photoreceptor differentiation and may impact on enzyme activity, respectively (Larque *et al.,* 2002).

1.3 Fatty acid binding proteins (FABPs): chaperone of fatty acids

Fatty acid-binding proteins (FABPs) constitute a multi-gene family of intracellular FA carrier molecules with low molecular masses of approximately 14–15 kDa. Multiple FABP isoforms (FABP1–12) have been isolated from distinct tissues. FABPs are generally believed to promote cellular FA uptake and transport toward specific metabolic pathways, and regulate gene transcription by delivering FAs as the ligands for specific nuclear receptors (Owada 2008).



Owada, Y. 2008

Figure 3: Schematic presentation of the cellular FABP function**.** The mechanism of uptake of fatty acids is facilated by membrane associated proteins, i.e. FATP and FAT (CD36). Intracellularly, fatty acids are bound by FABP, and FABP modulates various cellular processes including synthesis of acyl-CoA or eicosanoids. FA-mediated signal transduction and nuclear transcript by PPAR.

FA, fatty acid; FAT, fatty acid translocase (CD36); FATP, fatty acid transport protein; PPAR, peroxisome proliferator activated receptor; RA, retinoic acid; RXR, retinoid X receptor. Produced from (Owada, Y. 2008).

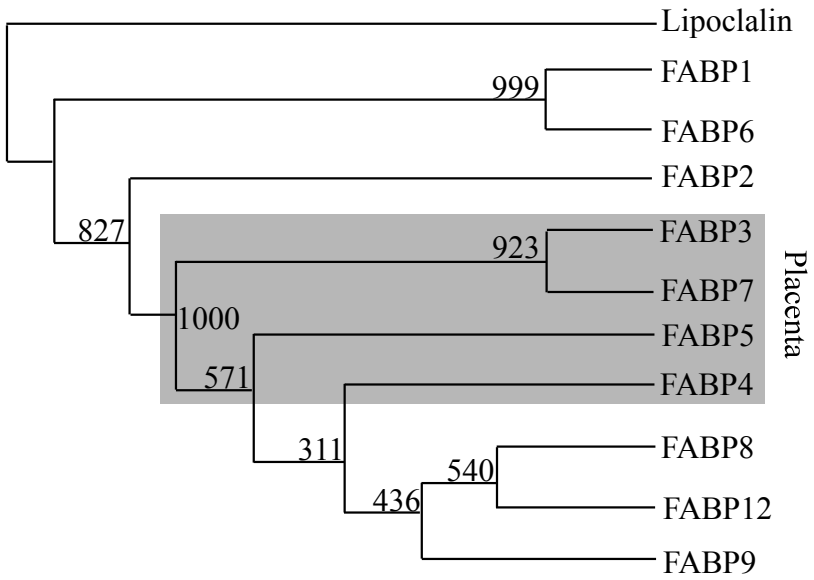


Figure 4: Phylogenetic tree of human fatty acid binding proteins. The bootstrap neighbor-joining phylogenetic tree was constructed using CLUSTAX. The human lipoclalin1 protein sequence (LCN1, GenBank accession number NP_002288) was used as an outgroup. The bootstrap values (based on number per 1000 replicates) are indicated on each node. The four phylogenetically-related FABPs with placenta expression was highlighted. Amino acid sequences of human FABPs were modified and reproduced from *Godbout group (Liu et al., 2010).*

Thus far, among the FABP family, FABP1, FABP3, FABP4, FABP5, and FABP7 have been observed to occur in both the human and rodent placenta (Watanabe *et al.,* 1991; Das *et al.,* 1993; Masouye *et al.,* 1997; Knipp *et al.,* 2000; Daoud *et al.,* 2005; Larque *et al.,* 2006; Biron-Shental *et al.,* 2007). However, the precise localization of each FABP in the placental units and their specific roles in feto-placental PUFA transport remains unknown.

Structurally, AA and DHA are key components of neuronal membranes, making up 15–20% of the brain's dry mass and more than 30% of the retina (Richardson 2004). In early life, both n-3 and n-6 PUFA are critical for supporting brain growth and maturation. AA is crucial for brain growth, and mild deficiencies are associated with low birth weight and reduced head circumference (van de Lagemaat *et al.,* 2011). It also plays a key role in the cellular processes underlying learning and memory (Das *et al.,* 2003). DHA is particularly concentrated in highly active membranes such as synapses and photoreceptors, and adequate supplies are essential for normal visual and cognitive development (Neuringer *et al.,* 1994; Uauy *et al.,* 2001). Pre-formed PUFAs are found naturally in breast milk, and although some controlled studies have shown advantages to both visual and cognitive development from their addition to infant formula (Willatts *et al.,* 2000).

1.4. Aim of this research

The aim of this research was to study the localization of FABP family in rodent placenta and functional role of dominant FABP family member, FABP3 with special consideration to the properties that are unique within the FABP family i.e. intracellular transportation of PUFAs in trophoblast cells.

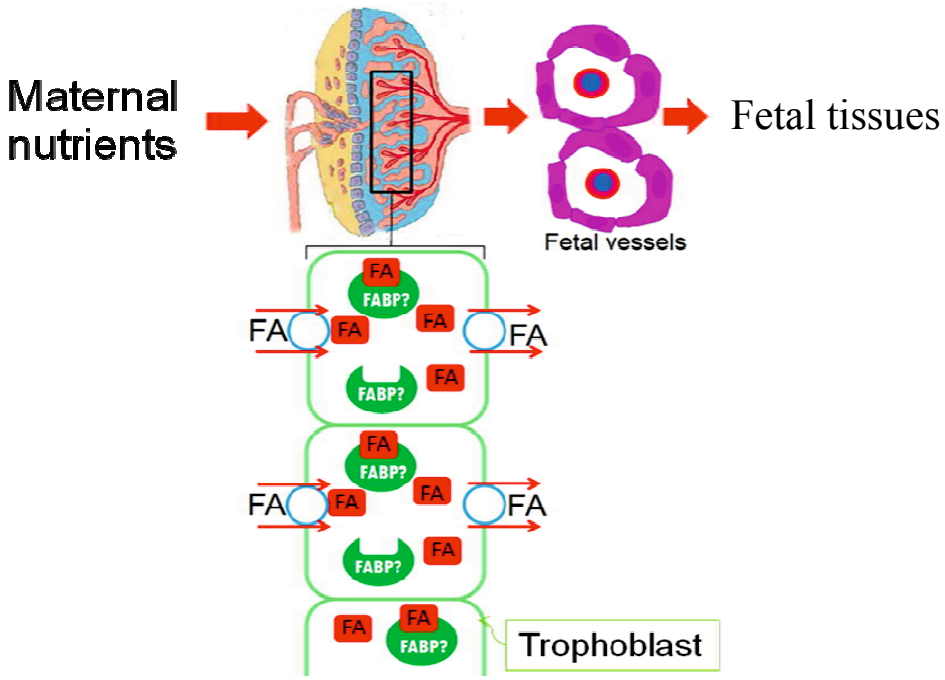


Figure 5: Possible molecular mechanism of FA transport with the help of FABPs in trophoblast cells.

Through this work, we hoped to reveal the molecular mechanism of PUFAs transportation in prenatal stage to explain the phenomena of fetal/adult developmental disorders pertaining to the mechanism of action and biological significance of FABP3 in the rodent placenta. We also hoped to be able to apply this knowledge to better our understanding of the roles of the other members of FABP family in general, as well as their potential FABP3-like functions in the placentas of non-rodent mammals.

MATERIALS AND METHODS

2.1 Animals. *Fabp3*-knockout (*Fabp3*−/−) mice (Binas *et al.,* 1999) and wild-type (*Fabp3*+/+) C57BL/6J mice were housed in a 12-h light/12-h dark cycle with *ad libitum* access to a standard rodent chow and water. At 8 weeks of age, both the *Fabp3*-KO and wild-type female mice were mated with male counterparts. For the preparation of chimeric placenta, a CAG-EGFP male mouse was mated with a wild-type female mouse **(Fig. 6)**. C57BL/6-Tg [CAG-EGFP] mice were kindly supplied by Dr. Masaru Okabe (Osaka University, Osaka, Japan). The presence of a vaginal plug was designated as embryonic day 1 (E1). At E11.5, E15.5, and E18.5, dams were killed and placentas were collected. All experimental protocols were reviewed by the Ethics Committee for Animal Experimentation of Yamaguchi University School of Medicine, Japan.

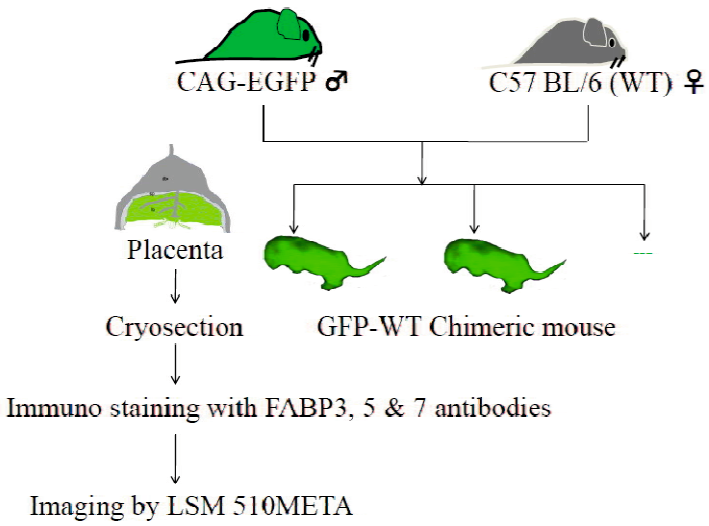


Figure 6: Chimeric placenta preparation paradigm

2.2 Cells. BeWo cells (ATCC, CCL-98) were cultured in Ham's F12 K medium (Wako, Osaka, Japan) with 10% fetal bovine serum (Hyclone, Logan, UT, USA) supplemented with 100 IU/mL penicillin-streptomycin (Wako, Osaka, Japan). The cells were routinely maintained at 37 °C in a 5% CO_2 atmosphere. At confluency, the cells were sub-cultured using a 0.25% trypsin-EDTA (Life Technologies, Carlsbad, CA, USA) solution to suspend the cells.

2.3 Polymerase chain reaction (PCR)

Total RNAs from the frozen placenta of wild-type mice were obtained using the trizol method for reverse transcription-PCR (RT-PCR). The cDNA was synthesized using *ExTaq* polymerase (Takara, Shiga, Japan). Expression levels of *Fabps* 1, 2, 3, 4, 5, 7 (Murphy *et al.,* 2005), 8 (Thumser *et al.,* 2001), and 9 and *β-actin* as the internal control (Iyoda *et al.,* 2002) were examined by RT-PCR with the primers listed in **Table 1**. PCR amplification was performed using a programmed temperature control system (PCR thermal cycler TP600/650, Takara, Shiga, Japan). PCR products separated on 2% agarose gel were visualized by ethidium bromide staining and photographed under ultraviolet (UV) illumination.

Name	Sequence
Fabp1	F(CTCATTGCCACCATGAACTTCTC) R (AGCCTTGTCTAAATTCTCTTGCTGACT)
Fabp2	F (TCTCTCATCTGTCCATATGAGCAACAAATT) R (CAAACATCATTTGGAGGATCCAATGGTTTT)
Fabp3	F(CATGAAGTCACTCGGTGTGG) R (TGCCATGAGTGAGAGTCAGG)
Fabp4	F (TCAACCTGGAAGACAGCTCCT) R (TCGACTTTCCATCCCACTTC)
Fabp5	F (CAAACCGAGAGCACAGTGA) R (TTTGACCGCTCACTGAATTG)
Fabp7	F (GGGTAAGACCCGAGTTCCTC) R (GAGCTTGTCTCCATCCAACC)
Fabp8	F (CAACAGAAAAGCCAAGCGCA) R (CCAGAACAAAGCTCTTACCTTC)
Fabp9	F (GGTTTTCGGTTGTGAATGCC) R (GCTACACCCTTTCGTAGATCC)
β-actin	F (CAGGAGATGGCCACTGCCGCA) R (CTCCTTCTGCATCCTGTCAGCA)

Table 1. Primer sequences used for standard PCR expression analysis.

2.4 Real time-PCR (RT-qPCR). For quantitative PCR (qPCR) analysis, total RNA was extracted from the E15.5 and E18.5 placenta (of wild-type and *Fabp3*-KO mice) and cells using an RNeasy Protect Mini kit and Micro kit (Qiagen, Valencia, CA, USA) then treated with ribonuclease-free deoxyribonuclease according to the instructions of the manufacturer (Qiagen). The quantity of the total RNA was assessed by UV spectrophotometer (DU 640; Beckman Coulter, Boulevard, CA, USA). RT was accomplished using a high fidelity reverse transcriptase cDNA synthesis kit (Roche, GmbH, Mannheim, Germany) following the manufacturer's protocol. Real-time PCR

was performed using a Taqman Fast Universal PCR Master Mix kit (Applied Biosystems, Branchburg, NJ, USA) and reactions were performed in triplicate using 96-well optical plates on a Step One Plus Real-Time PCR System (Applied Biosystems, Foster city, CA, USA). Among the target genes (Table 2 and 3), the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an internal control. Data were analyzed within the log linear phase of the amplification curve obtained for each probe or primer using the comparative threshold cycle method (Applied Biosystems). The thermal cycle conditions used were as follows: 45 cycles of 95 °C for 15 s, 60 °C for 1 min using 100 ng/well of cDNA in a final reaction volume of 20 μL.

Gene symbol	**Taqman gene expression ID (Invitrogen)**
Fabp3	Mm02342495_m1
Fabp4	Mm00445878_m1
Fabp5	Mm00783731_s1
Fatp1	Mm00449511_m1
Fatp4	Mm01327405_m1
p-*Fabp*pm (*got2*)	Mm00494703_m1
Fat (Cd36)	Mm01135198_m1
Gapdh	Mm03302249_g1

Table 2. Primer sequences used for RT-qPCR expression *invivo* analysis.

Gene symbol	Taqman gene expression ID (Invitrogen)
FABP3	Hs00269758_m1
FATP1	Hs01587917_m1
FATP2	Hs00186324_m1
FATP3	Hs00954616_s1
FATP4	Hs00192700_m1
FATP6	Hs01089796_m1
p*FABP*pm (*GOT2*)	Hs00905827_g1
FAT (CD36)	Hs01567185_m1
GAPDH	Hs99999905_g1

Table 3. Primer sequences used for RT-qPCR expression *invitro* analysis.

2.5 Western blotting. The expression of FABP3, FABP4, and FABP5 proteins was analyzed in placental homogenates. Placenta were homogenized and suspended in cold 2xSDS-PAGE sample buffer containing protease inhibitors (Roche Diagnostics, Mannheim, Germany). Twenty micrograms of protein were resolved by SDS PAGE (15% gel) and immunoblotted onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked by soaking in blocking solution [5% nonfat dried milk in Tris-buffered saline with 0.1% Tween 20 (TBST)] for 1 h and incubated at 4° C overnight on a rocking platform with specific antibody: anti-FABP3 [0.5 μg/mL dilution, (Guthmann *et al.,* 1998)], anti-FABP4 [0.5 μg/mL dilution, (Abdelwahab *et al.,* 2007)], anti-FABP5 [0.5 μg/mL dilution, (Owada *et al.,* 2002)], or anti-GAPDH (1:500; Santa Cruz Biotech, Santa Cruz, CA, USA) as the internal control.

The membrane was then washed three times with TBST, and incubated with 1:1000 horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon, Temecula, CA, USA) for 1 h at room temperature, and then the signals were visualized by the ECL-Western Blotting Detection System.

2.6 Laser capture microdissection (LCM) of the labyrinthine compartment. The localization of *Fabp3*, *Fabp4*, and *Fabp5* mRNA was also examined by RT-PCR in the placental labyrinthine compartment using LCM. Whole mouse placenta was collected at E18.5 using sterile apparatus. All specimens were frozen in cryomold in an isopentene-dry ice bath and stored at −80 °C until slicing. Following cryosectioning (CM 1850; Leica, Wetzlar, Germany), sections were captured on RNase-free slides (PEN-membrane; Leica) and placed immediately on dry ice. Staining and dehydration were performed using LCM-certified solutions provided in the histogene frozen section staining kit (Arcturus, Mountain View, CA, USA). Briefly, slides were fixed and dehydrated with solutions of 75% ethanol, 95% ethanol, 100% ethanol, and xylene and stained with RNase-free staining solution. The labyrinthine compartment was cut and collected using the Laser Microdissection (LMD) system (LMD 6500; Leica) under the following conditions: laser output power 35 mV; laser beam size 15 μm; laser power

exposure duration 2–4 s; and the sample was collected in a 0.5-μL single tube. Total RNA was extracted from the tissue using an RNeasy Micro kit (Qiagen, Valencia, CA, USA) and cDNA was amplified using a Transcriptor High Fidelity cDNA synthesis Kit (Roche, GmbH). *Fabp3*, *Fabp4*, *Fabp5*, and *Fabp7* mRNA expression levels were analyzed using primer sets (Table 1).

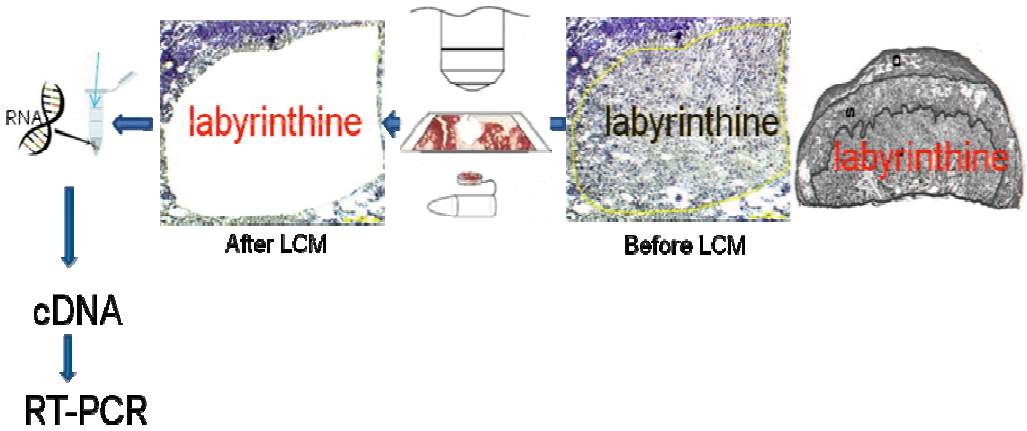


Figure 7: Schematic diagram of LCM system for collection of labyrinthine compartment of mouse placenta.

2.7 Immunohistochemistry. Placenta were removed after transcardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), cryoprotected with 30% (w/v) sucrose in 0.1 M phosphate buffer and then embedded into the freezing medium (OCT compound; Sakura Finetek, Torrance, CA, USA). Cryosections (20 μm thickness) were cut with a cryostat (CM 1850; Leica). The sections were then incubated with rabbit polyclonal primary antibodies for FABP3, FABP4, FABP5, or FABP7 (dilution 0.5 μg/mL) overnight at 4 °C, and subsequently with the biotinylated anti-rabbit

secondary antibody (Vector Laboratories, Burlingame, CA, USA). The immunoreaction sites were visualized using the avidin-biotinylated peroxidase complex (ABC) system (Vector Laboratories) with 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) as a substrate. For double-immunofluorescence staining, sections (12 μm) were incubated with FABP3, FABP5, and chicken anti-rat monocarboxylated transporter 1 [MCT1, 1:200, (Chemicon, Temecula, CA, USA)] antisera followed by incubation with goat anti-rabbit IgG Alexa 488 (1:1000; Invitrogen, Carlsbad, CA, USA) and goat anti-chicken IgG Alexa 594 (1:250; Invitrogen). After counterstaining of the nuclei with diamidino-2- phenylindole (DAPI), the cells were covered with Gel Mount (Biomeda, Foster City, CA, USA) and observed using a confocal laser microscope (LSM510 META; Carl Zeiss, Oberkochen, Germany). For immunofluorescence staining of the chimeric placenta, the placenta was collected using the same procedure as above. Sections (12 μm) were incubated with FABP3, FABP5, and FABP7 antisera, followed by incubation with goat anti-rabbit IgG Alexa 568 (1:1000; Invitrogen). For the immunohistochemistry of human placenta, human full-term placenta (obtained by Cesarean section, Yamaguchi University Hospital, Yamaguchi, Japan) formalin-fixed, paraffin-embedded sections (6 μm) were prepared. The sections were then incubated with a mouse monoclonal anti-human FABP3

antibody (0.5 μg/mL, DS Pharma Biomedical, Osaka, Japan) overnight at 4 °C, washed, and the bound antibodies were detected with a biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlinegame, CA, USA). The immunoreaction sites were visualized using the ABC system with DAB as a substrate.

Antibody table

Peptide/protein target	Name of antibody	Manufacturer catalog/name of providing individual	Species raised in; monoclonal or polyclonal	Dilution used
Mouse fatty acid binding protein3(FABP3)	Anti-mouse FABP3	Yuji Owada, (Yamaguchi University)	Rabbit, polyclonal	0.5 μg/ml
Mouse fatty acid binding protein4(FABP4)	Anti-mouse FABP4	Yuji Owada	Rabbit, polyclonal	0.5 μg/ml
Mouse fatty acid binding protein5(FABP5)	Anti-mouse FABP5	Yuji Owada	Rabbit, polyclonal	0.5 μg/ml
Mouse fatty acid binding protein7(FABP7)	Anti-mouse FABP7	Yuji Owada	Rabbit, polyclonal	0.5 μg/ml
Mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH)	Anti-mouse GAPDH	Santa Cruz Biotech, sc-47724	Human, monoclonal	0.38 μg/ml
Mouse monocarboxylate transporter1(MCT1)	Anti-rat MCT1	Chemicon, AB1286	Chicken; polyclonal	0.18 μg/ml
Human fatty acid binding protein 3(FABP3)	Anti-human FABP3	DS Pharma, Osaka, Japan (clone: 8E3)	Mouse monoclonal	0.5 μg/ml
Human fatty acid binding protein 5(FABP5)	Anti-human FABP5	Yuji Owada	Mouse monoclonal	0.5 μg/ml
Mouse BrdU	Anti-human BrdU	BD Pharmingen Catalog:555627	Mouse monoclonal	0.5 μg/ml

Table 4: List of antibodies used in immunohistochemistry and western blotting.

2.8 Morphometric analysis of placenta. Placentas were sampled from either wild-type or *Fabp3*-KO mice and then morphologically compared to investigate any abnormalities between these groups. First, placentas and fetuses from either wild-type or *Fabp3*-KO mice (for E15.5, litter size six and for E18.5, litter size four), were obtained, then placental and fetal weight were measured. For microscopic observations, placentas from either wild-type or *Fabp3*-KO mice at E18.5 were fixed overnight at 4 °C in fresh 4% paraformaldehyde/PBS, dehydrated, and embedded in paraffin. Sections (6 μm) were prepared by microtome and stained with hematoxylin and eosin.

2.9 Analysis of placental transport of radiolabeled FAs and glucose. Placental transport of radiolabeled FAs and glucose was examined as described previously by Bloise et al. (Bloise *et al.,* 2012) with slight modifications. Experiments were conducted at E15.5 and E18.5, when rapid fetal growth and active nutritional transport are observed in the mouse placenta (Coan *et al.,* 2010). Briefly, *Fabp3*+/− mice were intercrossed (Figure 5), and anesthetized (Isoflurane; Abbott, Chicago, IL, USA) using a small animal anesthetizer (Muromachi Co., Tokyo, Japan) at E15.5 and E18.5. Then, the maternal jugular vein was exposed and total of 100 μL of PBS containing 3.5 μCi of [1-^{14}C]-linoleic acid (LA,18:2n-6), (NEC-501; specific activity 58.2 mCi/mmol, Perkin

Elmer, Waltham, MA, USA), 9,12,15-[1-^{14}C]-linolenic acid (ALA,18:3n-3), (NEC-779; specific activity 51.7 mCi/mmol, Perkin Elmer), [1-^{14}C]-palmitic acid (PA,16:0), (NEC-075H; specific activity 60 mCi/mmol, Perkin Elmer), or D-[^{14}C(U)]-glucose (GLU), (NEC-042X; specific activity 289 mCi/mmol, Perkin Elmer) was injected in the jugular vein via a short piece of tubing (15 cm) attached to a 27-gauge needle.

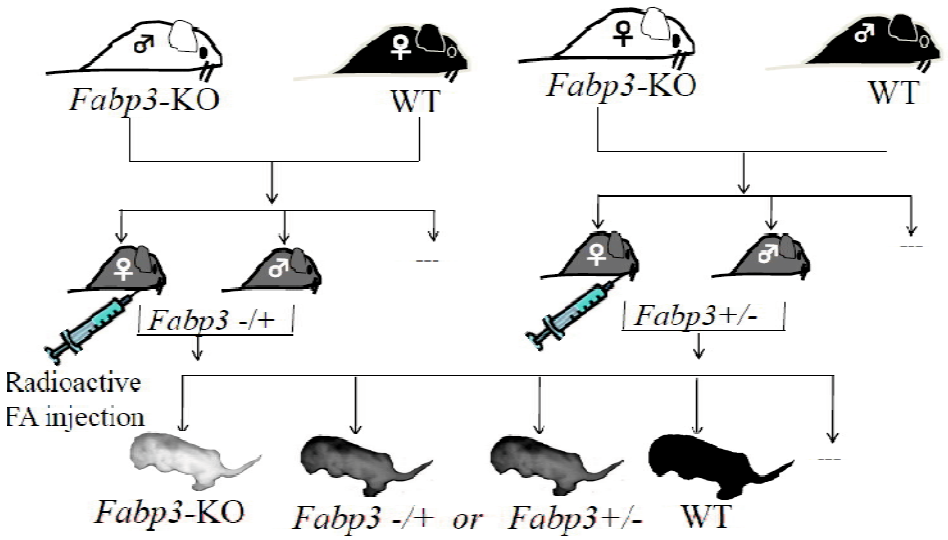


Figure 8: *Fabp3*+/- mouse preparation paradigm

Maternal blood samples (0.3–0.7 mL) were collected from the heart after 4 min of radioisotope injection (Constancia *et al.,* 2002) to measure the maternal plasma radioactivity after cervical dislocation; studies have shown there is minimal tracer backflux at this time (Sibley *et al.,* 2004). Fetuses and placentas were then dissected out and weighed. Fetuses were minced and lysed for 5 h at 55 °C in Solvable (Perkin Elmer). A small section of tail was collected from each fetus for genotyping. The liquid

scintillation cocktail (LSC), Ultima Gold (Perkin Elmer) was then added to aliquots of maternal plasma and fetal fractions in scintillation tubes (Perkin Elmer) for β-counting (liquid scintillation counter 5100; Aloka, Tokyo, Japan). The transfer of radioactive material from mother to fetus was evaluated following the method previously described by Constancia et al. (Constancia *et al.,* 2002). Briefly, radioactive counts detected in each fetus were used to calculate the amount of radioactive material transferred per gram of placenta or per whole embryo. Average values for wild-type and mutant fetuses within a litter were then calculated and expressed as a ratio of mutant to wild type.

2.10 Stealth RNAi-mediated knockdown. BeWo cells were plated in 12-well plates and maintained overnight in Ham's F-12 K medium containing 10% fetal bovine serum (Hyclone). After incubation for 24 h, stealth RNAi directed against the human FABP3 mRNA [Oligo ID: HSS103512 (Invitrogen, Carlsbad, CA, USA)] was used for FABP3 knockdown. Non-targeting low GC content stealth RNAi (ID:46-2002; Invitrogen, Carlsbad, CA, USA) was used as a control (scramble control). Transfection experiments were performed according to the manufacturer's instructions. Stealth RNAi in OptiMEM (Gibco, Carlsbad, CA, USA) was mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at room temperature for 15 min. After incubation for

48 h, FABP3 knockdown was confirmed by quantitative real-time PCR and western immunoblotting.

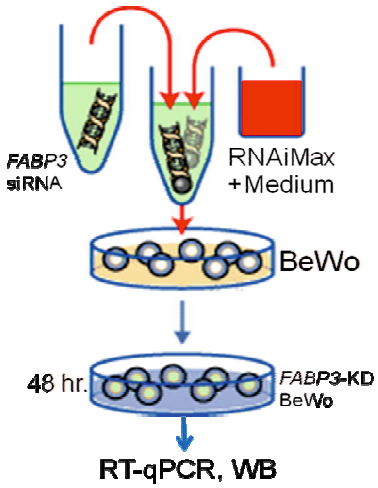


Figure 9: Schematic diagram of *FABP3*-knockdown BeWo cell preparation

2.11 Morphology and proliferation assay of FABP3-knockdown BeWo cells: The morphological characteristics and apoptosis of BeWo cells were checked after *FABP3* knockdown at 24 hours and 48 hours by visual assessement. For proliferation assay BrdU staining was performed. BrdU (10 μM) was added to culture medium at 2 days after seeding when the cells reached a subconfluent state, and was incubated for 5 h. $BrdU^+$ cells were visualized immunocytochemically using an anti-BrdU antibody as described previously with slight modifications (Sharifi *et al.,* 2011). Briefly, cells were incubated with 2 M HCl at room temperature for 30 min followed by washing twice with 0.1 M sodium borate (pH 8.5) over 10 min. After blocking with 5% goat serum at

room temperature for 30 min, they were incubated with mouse anti-BrdU antibody (1:100) at 4°C overnight, and incubated with anti-mouse IgG-Alexa488 (1:1000) at room temperature for 1 h and then counterstained with DAPI. After several washes with PBS, they were mounted and cover slipped.

2.12 FA uptake assay. Cellular uptake of FAs and glucose was examined in BeWo cells, as described previously (Sharifi *et al.,* 2011). Briefly, after cells became confluent in 12-well plates, ^{14}C-labeled LA, ALA, PA, or GLU (0.1 μCi/mL) were added to *FABP3* knockdown (48 h) BeWo cells and incubated for 60 min. Cells were thoroughly washed with cold PBS to remove any surface-bound FAs. The cells were then lysed and harvested in Solvable (Perkin Elmer) and used for scintillation counting. The results were expressed as DPM/μg protein. Incubation with stealth RNAi and scramble control was performed 48 h before the FA uptake study.

2.13 Calculation and Statistics. Values for all data are expressed as means ± SEMs. For comparison of the base pair strength within the embryonic stage, one-way ANOVAs followed by Tukey comparison tests were performed, *Fabp3* expression among the embryonic stages was also compared by same analysis. For compact and comprehensive

presentation of placental *in vivo* transport studies, the one-sample *t-test* (Constancia *et al.,* 2002) was used; for the *in vitro* FA study and qRT-PCR, data were analyzed using Student's two-tailed unpaired *t-test* (SPSS software version 16.0). *P* values < 0.05 were considered statistically significant.

RESULTS

3.1 Expression of FABPs in the mouse placenta. We first examined the gene expression of the *Fabp* family from mid to late embryonic stages (E11.5, E15.5, and E18.5), because FA demand increases in the developing fetus with the progression of gestation. By semi-quantitative RT-PCR, *Fabp3*, *Fabp4*, and *Fabp5* were found to be expressed in whole placentas at E15.5 and E18.5, with *Fabp3* expression of the highest intensity **(Fig. 10A)**. Other *Fabp* mRNA (*Fabp1*, *Fabp2*, *Fabp7*, *Fabp8*, and *Fabp9*) were not detected.

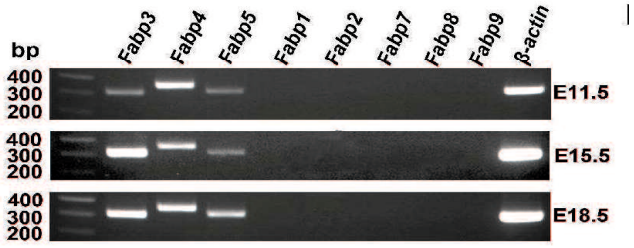


Figure 10A: Identification of the *Fabp* family in the wild-type mouse placenta at mid to late embryonic stages; whole placenta were used to collect the mRNA, which was analyzed by semi-quantitative RT-PCR. β-actin was used as the internal control. bp, base pair; *Fabp,* fatty acid binding protein.

Levels of *Fabp3* expression were significantly higher at E15.5 and E18.5, compared with E11.5

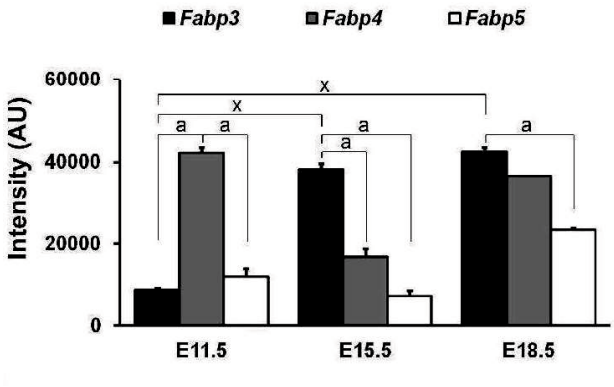


Figure 10 B: Comparison of (means ± SEMs, n = 3 mouse dams) expression intensities of *Fabp* species from base pair intensity (image J software), a and x, $P<0.001$. AU, arbitrary unit; *Fabp,* fatty acid binding protein.

To identify the *Fabp* molecule expressed in the labyrinthine compartment, which is the active compartment for nutrient transport (Watson *et al.,* 2005), we dissected the labyrinthine compartment of mouse placenta at E18.5, using LCM and the mRNA was extracted. In RT-PCR analysis of the dissected samples, *Fabp3* mRNA showed to be highly enriched in the labyrinthine compartment, while only faint to weak expression of *Fabp4* and *Fabp5* was detected **(Fig. 10 C)**.

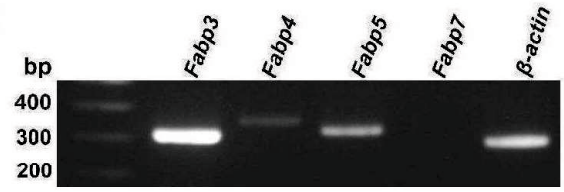


Figure 10 C: Expression of *Fabp3, Fabp4*, *Fabp5* and *Fabp7* in the fetal labyrinthine layer. bp, base pair; *Fabp,* fatty acid binding protein.

In western blot analysis, FABP3, FABP4, and FABP5 were confirmed to be expressed in mouse placenta at mid, mid to late and late stages of pregnancy **(Fig. 10D)**

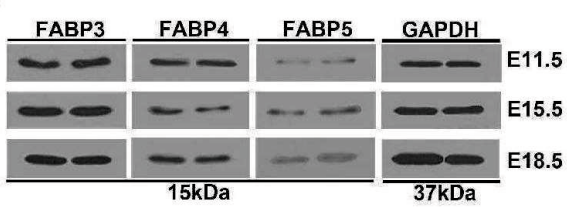


Figure 10 D: Expression of FABP3, FABP4, and FABP5 in WT placenta. kDa, kilodalton.

3.2 Spatially different distribution of FABPs in the mouse placenta. Immunostaining revealed that FABP3, FABP4, FABP5, and FABP7 expression is spatially different in the mouse placenta. Consistent with the results of mRNA expression analysis, FABP3 was strongly localized in the labyrinthine compartment at E15.5 and E18.5 (**Fig. 11, a and b**). Conversely, FABP4 was dominantly localized in the spongiotrophoblast compartment, which is the supportive layer of the labyrinthine compartment (Rossant *et al.,* 2001) (**Fig. 11, d and e**) and FABP5 was weakly, but evenly distributed over the whole placenta (**Fig. 11, g and h**). Unexpectedly, FABP7 was localized to some maternal vessels (sinusoids) of the labyrinthine compartment **(Fig. 11, j and k**). Similar results were obtained from the analysis of E11.5 placenta (data not shown). The placenta obtained from the corresponding gene knockout mice for FABP3, FABP5, and

FABP7 did not show any immunoreaction **(Fig. 11, c, f, and l)** and the placenta stained with primary antibody omission for FABP4 **(Fig. 11, i)** also did not present any immunoreaction, suggesting the authenticity of the immunoreaction of these anti-FABP antibodies.

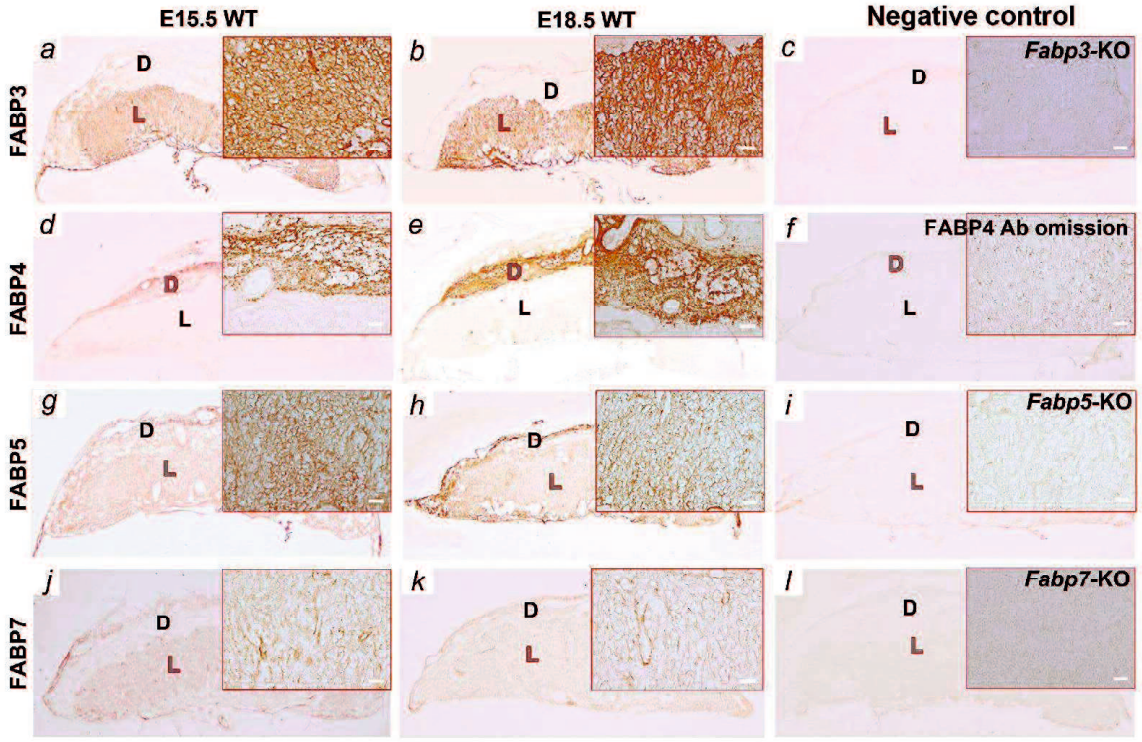


Figure 11: Immunohistochemical localization of FABP3 in the fetal labyrinthine layer **(a, b)**, FABP4 in the maternal decidua layer **(d, e)**, FABP5 in the whole placenta (both fetal and maternal) **(g, h),** and FABP7 in the maternal sinusoids of the fetal labyrinthine layer **(j, k)** at E15.5 and E18.5 stages. *Fabp3*-KO **(c)**, *Fabp5*-KO **(i)**, and *Fabp7*-KO **(l)** mouse placental sections were used as negative control. A primary antibody-omitted section **(f)** was used as the negative control to confirm the FABP4 expression in the mouse placenta. All antibodies were applied under the same conditions and concentrations. Red-colored insets are a higher-magnification image of the labyrinthine or decidua layer. *Fabp,* fatty acid binding protein; WT, wild-type; *Fabp*-KO, *Fabp*-knockout; L, labyrinthine layer; D, decidua layer, scale bar, 100 µm.

3.3 FABP3 is expressed in labyrinthine trophoblast cells. The placenta functions as a hybrid organ between the mother and fetus. The labyrinthine compartment is regarded as the transport compartment where the nutrient exchange mainly occurs. The labyrinthine compartment consists of maternal cells, labyrinthine trophoblasts, as well as fetal cells (Cross *et al.,* 2002). To identify the FABPs that are expressed in labyrinthine trophoblasts, we first performed double immunofluorescence staining using the trophoblast marker, monocarboxylase transporter 1 (MCT1) (Nagai *et al.,* 2010; Bonnin *et al.,* 2011). FABP3 was abundantly localized in $MCT1^+$ trophoblasts **(Fig. 12A and B)** and its subcellular localization pattern was strikingly similar to that of MCT1, suggesting that FABP3 is associated with the apical membrane of trophoblasts. FABP5 was also weakly present in $MCT1^+$ cells, while its distribution was confined to the cytoplasm.

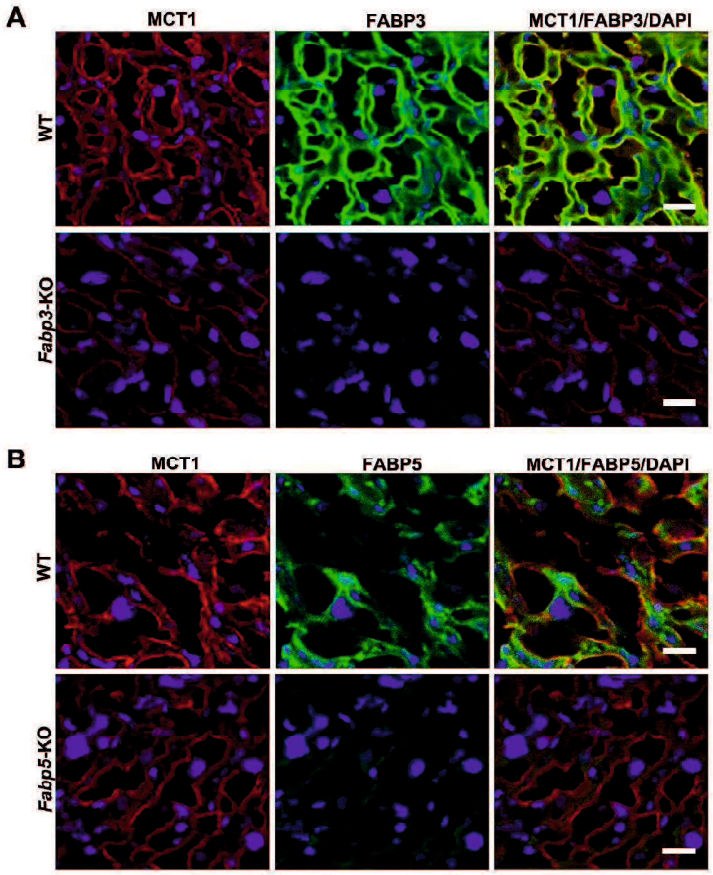


Figure 12 (A, B) Localization of FABP3 and FABP5 in the fetal labyrinthine trophoblast of the mouse placenta. Colocalization of FABP3 **(A)** and FABP5 **(B)** with $MCT1^{+}$ (trophoblast marker) cells. As a negative control, *Fabp3*-KO and *Fabp5*-KO mouse placenta were used under the same conditions. WT, wild-type; *Fabp*-KO, *Fabp*-knockout; FABP, fatty acid binding protein; MCT1, monocarboxylase transporter 1; DAPI, diamidino-2- phenylindole. Scale bar, 20 μm.

3.4* FABP3*and* FABP5 *is expressed in human trophoblast cells. In addition to the localization of FABP3 and FABP5 in mouse placenta, we detected FABP3 and FABP5 expression in the trophoblasts of human full-term placenta.

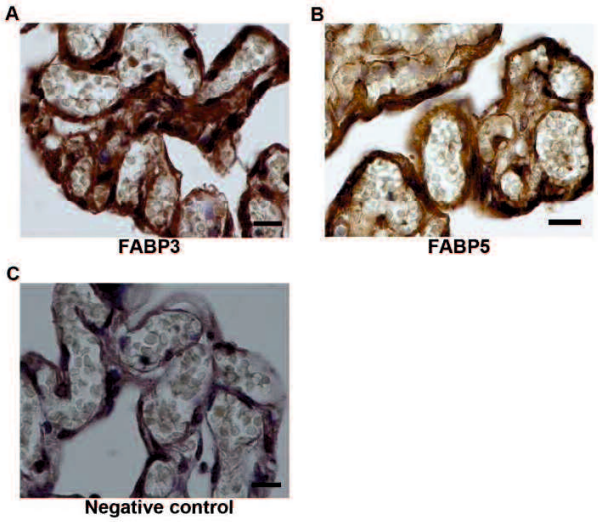


Figure 13. Immunolocalization of FABP3 and FABP5 in human term placental villous **(A** and **B)**. For negative control, the primary antibody was omitted under same conditions (**C**). Scale bar 10μm.

3.5* FABP3*and* FABP5 *is expressed in fetal derived trophoblast cells. Next, to further confirm the localization of FABPs in fetal cells, we stained chimeric placenta obtained by mating a CAG-EGFP male mouse with C57BL/6 wild-type female mouse **(Fig. 6)**. FABP3 and FABP5 were found to be co-localized with fetal-derived GFP^+ cells. In contrast, FABP7 was localized in GFP^- maternal vessels.

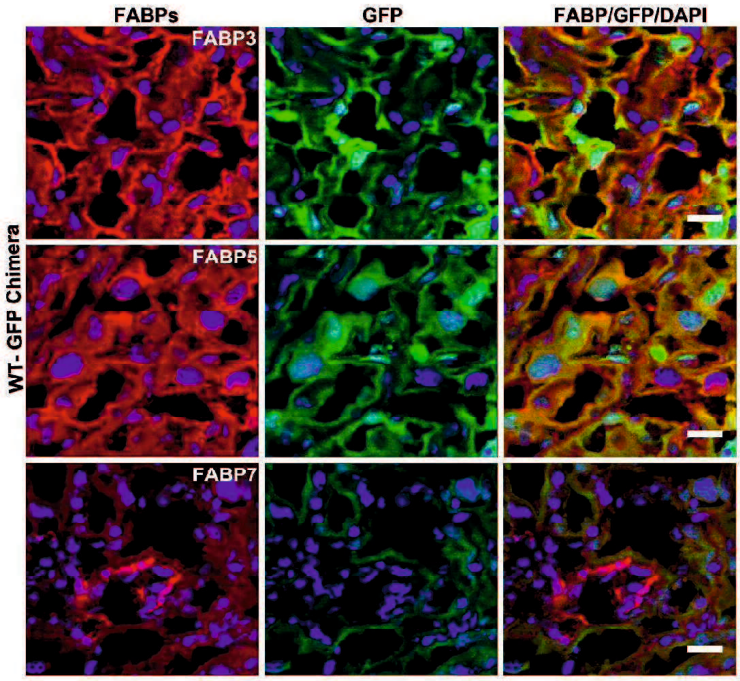


Figure 14: Localization of FABP3, FABP5 and FABP7 in WT-GFP chimera placenta. FABP3 and FABP5 immunopositivities were revealed to be overlapped (yellow) with GFP expression, confirming that both FABPs localize in fetal trophoblast cells, while FABP7 does not. WT-GFP chimera, chimeric placenta; FABP, fatty acid binding protein; DAPI,diamidino-2- phenylindole. Scale bar, 20 μm.

FABPs expression table

FABP	**Localization in placenta**
FABP3	trophoblast in labyrinthine compartment (fetal cells)
FABP5	trophoblast in labyrinthine compartment (fetal cells). spongiotrophoblast (maternal cells), decidua compartment (maternal cells)
FABP4	decidua compartment (maternal cells)
FABP7	sinusoids of labyrinthine (maternal cells)

Table 5. Summary of FABPs expression in mouse placenta

Overall, FABP3 was the major FABP localized in the trophoblasts of mouse placenta, suggesting that it is likely to be involved in PUFA transport through the placenta.

3.6 Fabp3 deficiency does affect the fetal gross appearance and weight. The fetus collected from wild type and *Fabp3*-KO at E18.5 stage did not display any variation in their gross appearance. Furthermore placental and/or fetal weights did not show any significant differences between these two groups.

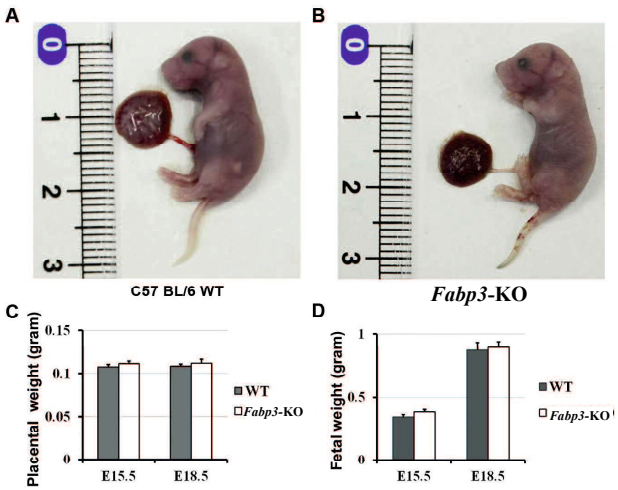


Figure 15: Comparison of feto-placental unit in visual appearance and weight variation at E18.5 **(A, B)**. Placental and embryonic weight of both the wild-type and *Fabp3*-KO mice were measured from six litters (E15.5 stage) and four litters (E18.5 stage) **(C, D).**

3.7 Fabp3 deficiency does affect the histology of mouse placenta. We examined whether there were any morphometric differences between wild-type and *Fabp3*-KO placentas. Both of these groups did not present any macroscopic or microscopic abnormalities **(Fig. 16A–D)**. Comparison of trophoblast count, maternal space and fetal space in the labyrinthine compartment was checked and did not display any significant difference between the experimental groups.

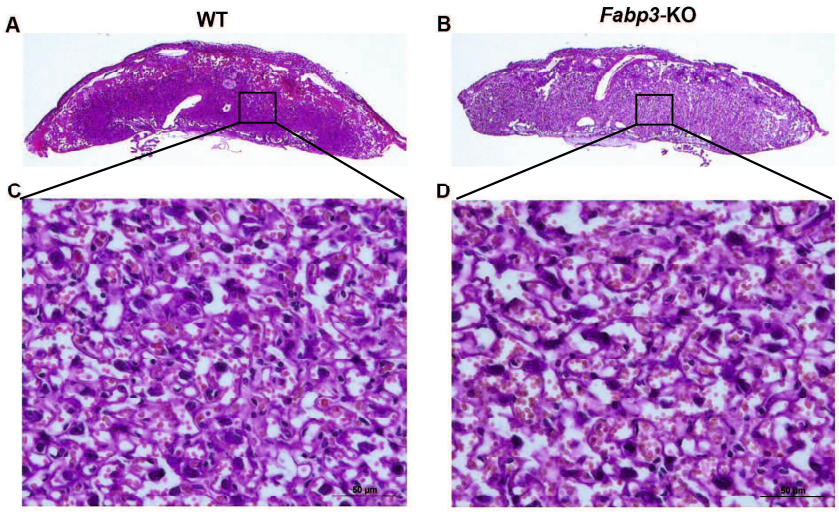


Figure 16: Histological comparison of wild-type placenta with *Fabp3*-KO placenta. Cross-sections of the entire placenta at E18.5 (**A, B**) stained with hematoxylin and eosin**. C, D** shows high magnification image (scale bar, 50 μm).

3.8 ***No compensatory FA transporter gene is observed in Fabp3-KO placenta.*** To assess the influence of *Fabp3* deficiency on placental expression of other reported fatty acid transporters, we initially interrogated the expression of *Fabp* and *Fatp* isoforms in the wild type and *Fabp3*-KO mouse placenta at E15.5 and E18.5. Changes in expression of genes involved in fatty acid transporters were assessed by RT-qPCR (Livak *et al.,* 2001). Samples were normalized to the house keeping gene *Gapdh*, which exhibited a stable expression in control or *Fabp3*-KO placenta. Importantly *Fabp3* deficiency did not influence the expression level of *Fabp4*, *Fabp5*, *Fatp1*, *Fatp4* (Garcia-Martinez *et al.,* 2005; Larque *et al.,* 2006), p-*Fabp*$_{pm}$ (Campbell *et al.,* 1997), *Cd36* (fatty acid translocase, FAT) (Campbell *et al.,* 1998) in mouse placenta (Fig. 16).

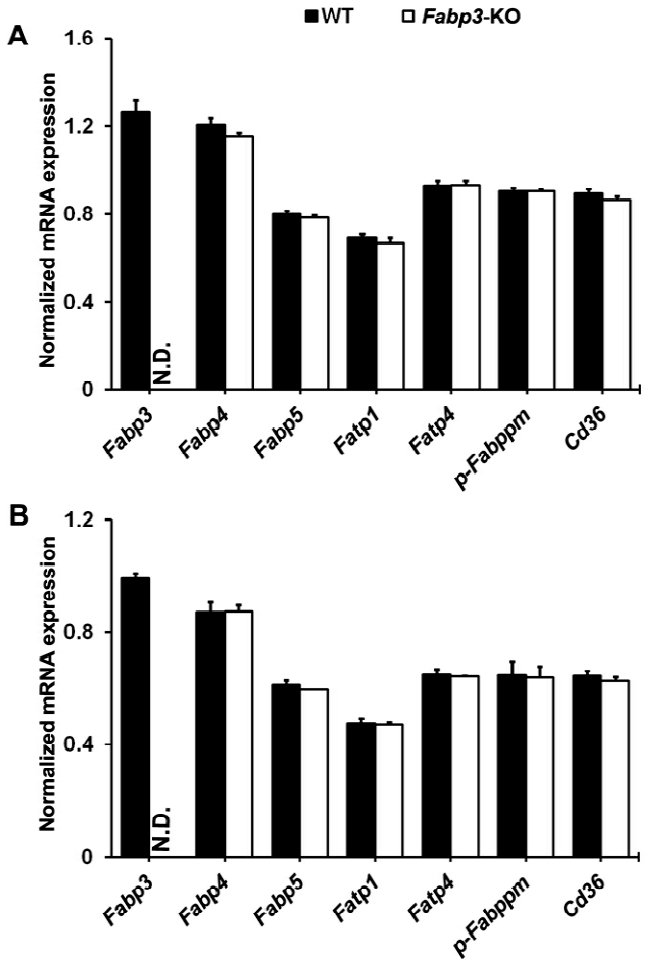


Figure 17: Effect of *Fabp3*-ablation on the gene expression of major FA transporters in the mouse placenta at E15.5 **(A)** and E18.5 **(B)**. *Fabp3* deficiency did not affect the gene expression of other placental FA transporters. Gene expression was normalized by the amount of housekeeping gene (*Gapdh*). *Fabp,* fatty acid binding protein; *Fatp,* fatty acid transport protein; *Fat* (*Cd36*), Fatty acid translocase; *p-Fabp*$_{pm}$, placental-plasma membrane Fabp; *Fabp3*-KO, *Fabp3*-knockout; N.D., not detected (significant value was not detected in *Fabp3*-KO placenta by 40 cycles of PCR amplification). The values are the means ± SEMs (n = 3 mouse dams).

This findings is supportive to the previous findings (Binas *et al.,* 1999) where no compensatory expression of related fatty acid transporters in heart and skeletal muscle of *Fabp3*-KO mouse was observed.

3.9 PUFA traffic is impaired in the Fabp3-KO mouse placenta. We then sought to examine placental transport and fetal accumulation of long chain FAs in *Fabp3*-KO mice to explore the function of FABP3 in placental FA traffic. Interestingly, the placental transport of (n-6) and (n-3) PUFAs was significantly impaired in *Fabp3*-KO mice (both sexes): at E15.5 and E18.5, LA decreased by 25% ($p < 0.01$) and 44 % ($p < 0.001$) and ALA decreased by 19% ($p < 0.05$) and 17% ($p < 0.05$), respectively, compared with wild-type mice **(Fig. 18A)**. Furthermore, fetal accumulation of n-6 and n-3 PUFA was also significantly impaired in *Fabp3*-KO compared with wild-type mice: LA decreased by 18% ($p < 0.05$) and 27% ($p < 0.01$) at E15.5 and E18.5, respectively **(Fig. 18B)** and ALA decreased by 16% ($p < 0.05$) at E15.5 **(Fig. 18B)**. *Fabp3* deficiency did not affect transport and accumulation of a saturated FA (palmitic acid) **(Fig. 18A and B)**. In contrast to the reduced PUFA transport and accumulation, glucose transport significantly increased by 23% ($p < 0.01$) at E18.5 in *Fabp3*-KO placenta **(Fig. 18A)**. These results are generally consistent with the reported phenotypes of *Fabp3*-deficient cardiac myocytes, including the impaired use of FAs and the increased reliance on carbohydrates to meet the cardiac energy demand (Schaa*p et al.,* 1999).

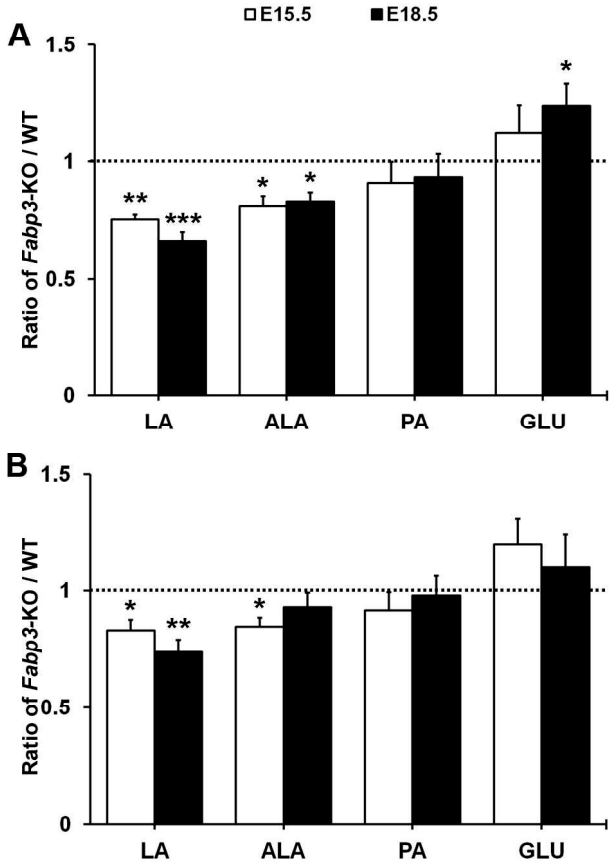


Figure 18: Effect of *Fabp3* ablation on placental transport and fetal accumulation of n-3 and n-6 PUFAs. The placental transport **(A)** and fetal accumulation **(B)** of radiolabeled LA, ALA, PA, and GLU were expressed relative to placental or fetal weight and plotted as a ratio of *Fabp3*-KO to wild-type at two gestational ages (E15.5 and E18.5). Horizontal dashed line indicates standard value for which each bar is being compared to. Ratio < 1 and > 1 indicate 'decreased' and 'increased' transportation or accumulation in *Fabp3*-KO placenta or fetus, respectively. The data are presented here as means ± SEMs for each independent radioisotope, collected from six and four litters at E15.5 and E18.5, respectively. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. WT, wild-type; *Fabp3*-KO, *Fabp3*-knockout; LA, linoleic acid; ALA, α-linolenic acid; PA, palmitic acid;, GLU, glucose.

3.10 Localization of FABP3 in BeWo cells. To further examine the role of FABP3 in trophoblast cells, we used human trophoblast cell line BeWo cells; which are extensively used as a human placental trophoblast cell model (Campbell *et al.,* 1997; Tobin *et al.,* 2009).

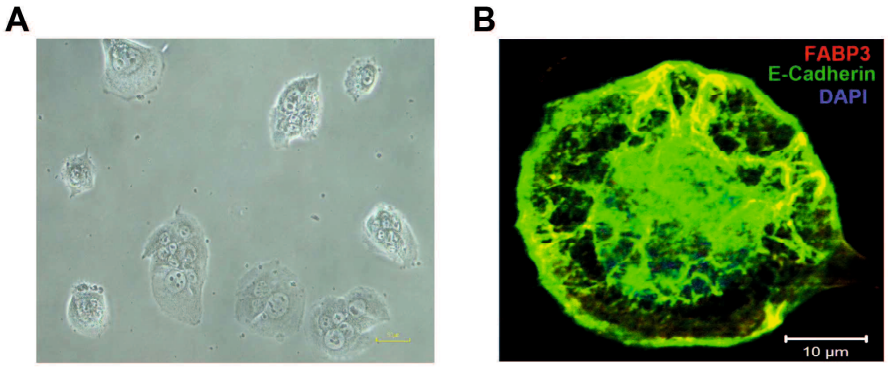


Figure 19: Localization of FABP3 in BeWo cells (**B**). Phase contrast image of BeWo cells (**A**).. E-Cadherin: marker of epithelial cells widely used to mark BeWo cells. FABP3 immunopositivities were revealed to be overlapped (yellow) with E-Cadherin expression, confirming that FABP3 localize in human trophoblast cell line (BeWo cells).

3.11 FABP3 siRNA transfection successfully knockdown FABP3 mRNA and protein expression. To further examine the role of FABP3 in trophoblast cells, we transfected *FABP3* siRNA into BeWo cells (**Fig. 9**). 48 h after siRNA transfection in BeWo cells successfully suppressed gene and protein expression of FABP3

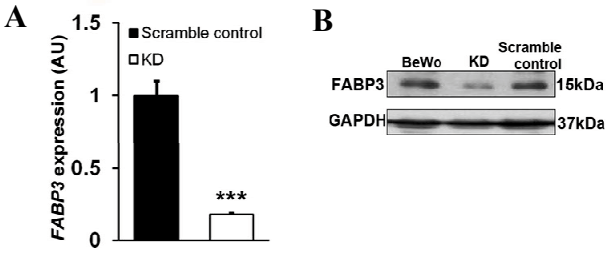


Figure 20: The effect of *FABP3* knockdown in BeWo cells by RT-qPCR **(A)** and Western blot analysis **(B)**, GAPDH was used as the internal control.

3.12 FABP3 siRNA transfection does not affect morphology and proliferation of BeWo cells. Then we checked if there any morphological alteration due to *FABP3* siRNA knockdown following the protocol cited above (Fig. 9). Phase contrast images do not show any significant difference in morphology of *FABP3*-KD BeWo cells compared to scramble control (Fig. 21 A, B). Proliferation checking using BrdU uptake assay also revealed that *FABP3* siRNA knockdown does not affect proliferation of BeWo cells.

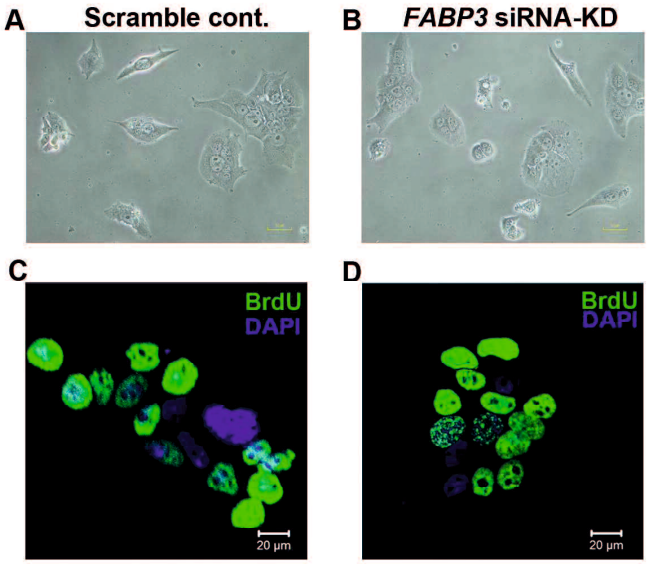


Figure 21: The effect of *FABP3*-knockdown on the morphology and proliferation of BeWo cells (A, B). BrdU uptake properties of *FABP3*-KD BeWo cells compared to scramble control (C, D).

3.13 FABP3-KD does not affect the gene expression of placental other FA transporters in BeWo cells. Similar to *invivo* FA transporters in mouse placenta, the gene expression of other FA transporters (Table 3) in *FABP3*-KD BeWo cell does not significantly changes compared to scramble control.

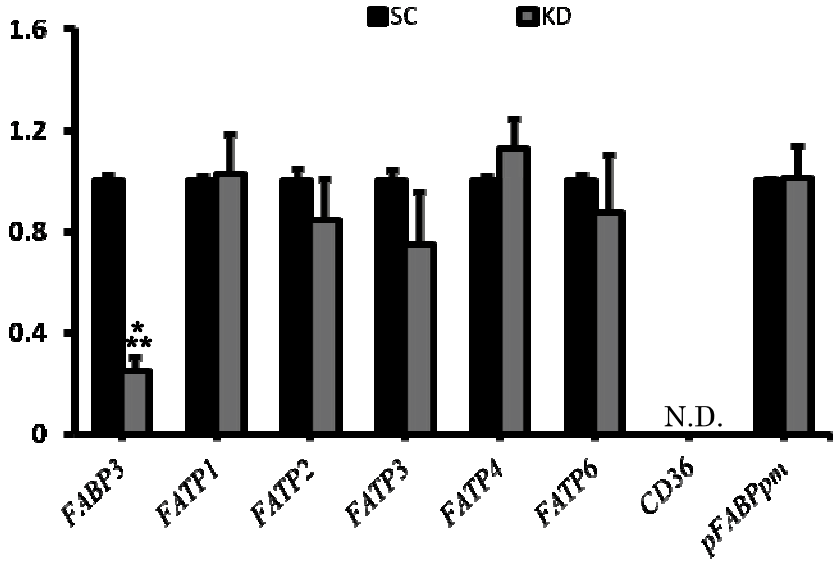


Figure 22: Effect of *FABP3*-knockdown on the gene expression of major FA transporters in BeWo cells after 48 hours transfection. *FABP3*-KD did not affect the gene expression of other placental FA transporters. Gene expression was normalized by the amount of housekeeping gene (*GAPDH*). *FABP,* fatty acid binding protein; *FATP,* fatty acid transport protein; *FAT* (*CD36*), Fatty acid translocase; *p-FABP*$_{pm}$, placental-plasma membrane FABP; *FABP3*-KD, *Fabp3*-knockdown; N.D., not detected (significant value was not detected by 40 cycles of PCR amplification). The values are the means ± SEMs (n = 3 independent trials).

3.14 PUFA uptake is impaired in FABP3 knockdown BeWo cells. To further examine the role of FABP3 in PUFA uptake *invitro*, we investigated the n-3 and n-6 PUFA uptake in *FABP3*-KD BeWo cells. As expected, uptake of LA and ALA was decreased in *FABP3* knockdown BeWo cells, compared with the control **(Fig. 23 A, B)**: LA uptake was decreased by 51% ($P < 0.05$) and 52% ($P < 0.01$) after 1 and 60 min of incubation, respectively. ALA uptake was also decreased by 23% ($P < 0.05$) after 60 min of incubation. Similar to the *in vivo* analysis, GLU uptake was increased by 51% ($P < 0.01$), 50% ($P < 0.05$), 31% ($P < 0.05$), and 33% ($P < 0.05$) at 1, 20, 40, and 60 min after incubation respectively **(Fig. 23D)**. *FABP3* knockdown in BeWo cells did not affect PA uptake **(Fig. 23 C)**.

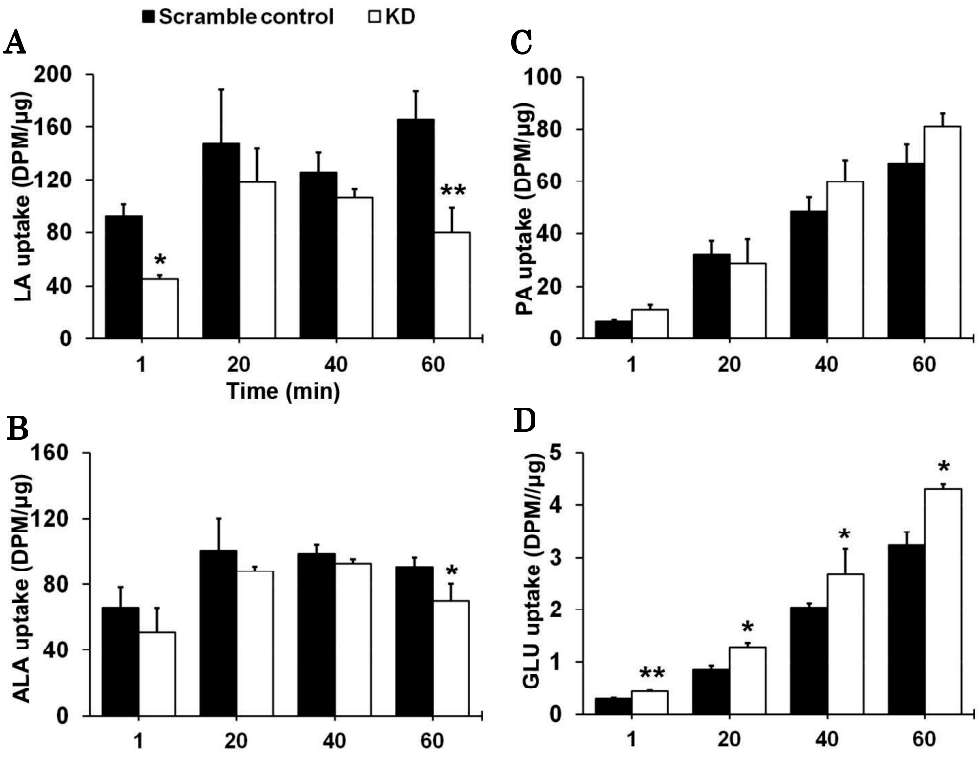


Figure 23. Effect of *FABP3*-knockdown on n-3 and n-6 PUFAs uptake in BeWo cells. Bar graphs **(A–D)** show the incorporation of LA **(A)**, ALA **(B)**, PA **(C)**, and GLU **(D)** in *FABP3*-Knockdown BeWo cells, compared with the scramble control. The amount of FA or GLU uptake was measured in DPM/µg protein of lysates up to 60 min. The graphs shown in **A–D** were representative ones from three independent trials performed in triplicate. Values are means ± SEMs, n = 3. Student's t-test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. FABP3, fatty acid binding protein; AU, arbitrary unit; LA, linoleic acid; ALA, α-linolenic acid; PA, palmitic acid; GLU, glucose; KD, fatty acid binding protein 3 knockdown; BeWo, human choriocarcinoma cell line; kDa, kilodalton; DPM/µg, Disintegration per minute per microgram.

DISCUSSION:

Proper fetal development is dependent on sufficient nutrient supply to the fetus. Because substrate transport, including FAs, is directed from the maternal to the fetal circulation under physiological conditions, the placenta's ability to facilitate this transport is of critical importance for the development of a healthy fetus (Knipp *et al.,* 1999). In this study, we identified FABP3 to be the major FABP expressed in mouse trophoblast cells and evaluated whether FABP3 deficiency may affect transplacental FA transport and placental/fetal development during late gestation.

The mouse placenta is subdivided into three major compartments: the decidua, spongiotrophoblast, and labyrinthine compartments (Malassine *et al.,* 2003; Watson *et al.,* 2005). The labyrinthine compartment, which is bathed in maternal blood, is the main nutrient exchange layer between the mother and fetus (Rinkenberger *et al.,* 2000) and includes both maternal and fetal cells. We have clearly shown the expression of FABP3 and FABP5 in trophoblast cells in the labyrinthine compartment, which are the epithelial cells responsible for the exchange of various nutrients (Jones *et al.,* 2007).

Regarding the molecular mechanism underlying FA transport from mother to fetus, triglycerides in lipoproteins are hydrolyzed by placental lipases in particular lipoprotein lipase, localized in the MVM (Lindegaard *et al.,* 2005). As a result, free fatty acids are released, which either diffuse across the plasma membrane (Herrera *et al.,*

2006) or uptake occurs through fatty acid transport proteins (Dutta-Roy 2000). FFA transported into the syncytial cytoplasm bind to cytoplasmic FABP and are directed to different sites for esterification, beta-oxidation or for transport over to the fetus across the BM. Dutta-Roy group has previously identified p-$FABP_{pm}$ in BeWo cells and proposed the possible involvement of molecules including several membrane associated transporters (FATPs, CD36, and caveolin) and cytoplasmic FABPs (FABP3, and FABP1), all of which are associated with cellular FA trafficking process (Campbell *et al.,* 1998; Dutta-Roy 2000). Furthermore, Schaiff et al. reported the elevation of FATP1, FATP4, CD36 and p-$FABP_{pm}$ expression in placenta after PPARγ agonist administration, in which long-chain FA uptake was markedly induced (Schaiff *et al.,* 2007). However, the direct evidence regarding the function of these molecules in placental FA transport has not so far been available. This study is the first investigation to validate the involvement of FABP3 in n-3 and n-6 PUFA transport in trophoblast cells using gene-ablated mice and gene knockdown techniques at cellular levels (**Fig 24**). Although the functional importance of FABP5 in placental FA transport should be investigated in further studies, it is interesting to propose that saturated FA transport, which was not affected in this study, may be mediated by FABP5 because among the FABP family, FABP5 has been reported to bind preferentially with saturated fatty acids

(Hanhoff *et al.,* 2002).

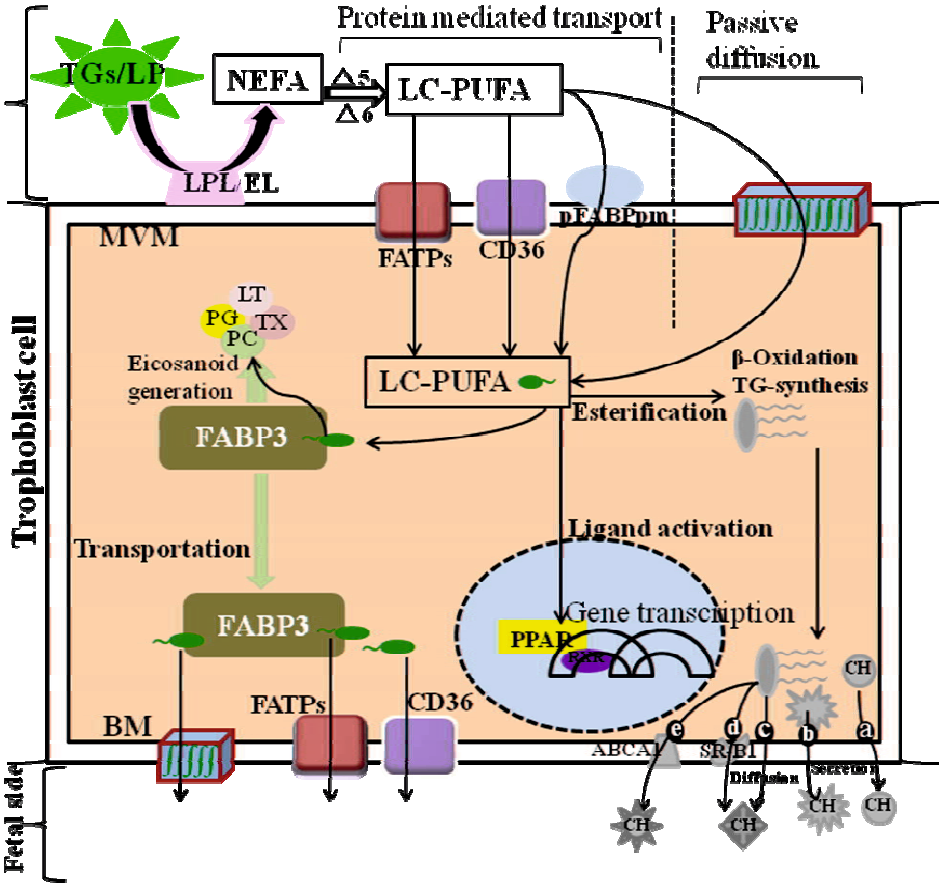


Figure 24: Mechanism of fatty acid Transport across the syncytiotrophoblast cells. Model of placental fatty acid transport through trophoblast cells. A complex interplay of different fatty acid transport proteins orchestrates fatty acid uptake mechanism. Within the cells NEFA are bound by different FABPs and have multiple functions like ferrying function, energy generation, TG and eicosanoid synthesis and activation of nuclear transcription factors like PPAR/RXR. Secretion and efflux of cholesterol out of trophoblasts. Cholesterol can exit cells as lipoproteins, with apolipoproteins, or by being effluxed to acceptors. Efflux can occur by aquous diffusion, scavenger receptor class B type 1 (SR-B1) or ATP-binding cassette transporter A1 (ABCA1), LP; lipoprotein, LPL; lipoprotein lipase, NEFA; non-esterified fatty acid, FATP, fatty acid transport protein, FAT; fatty acid translocase, p-FABPpm; placental plasma membrane FABP, PPAR; peroxisome proliferator activated receptor, RXR; retinoid X receptor. [Figure was modified from Hanebutt *et al*., 2008 and Woollett, 2004].

Most importantly, the *Fabp3*-KO placenta showed significantly impaired PUFA transport, to the extent that the *Fabp3*-KO fetuses had a lower accumulation of PUFAs than the wild-type. PUFAs are used not only as key components of biomembranes, but also play important roles in cell integrity, development, and maintenance. PUFAs are the precursors of important molecules, such as prostaglandins, leukotrienes, and thromboxanes, and they also represent a source of energy. The fetus mostly needs essential FAs and their derivatives, arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3). Intrauterine requirements for essential FAs (derivatives of n-6 and n-3) during the last trimester of pregnancy through to the early weeks of life have been estimated to be 400 and 50 mg/(kg · d) for n-6 and n-3 FAs, respectively (Clandinin *et al.,* 1981). Nevertheless, *Fabp3*-deficient placentas and fetuses achieved a similar weight to those of the wild-type mouse (data not shown), despite a marked impairment in FA transport. In this study, *Fabp3*-deficient placenta became more efficient in glucose transportation. Thus, it is likely that increased placental transport of glucose compensates for the decrease in PUFA transport. In other words, *Fabp*3-deficient conceptuses modified their metabolism to accommodate the lower availability of PUFAs for cell integrity and development, and as an energy source, as observed previously in *Fabp*3-deficient cardiac myocytes (Binas *et al.,* 1999; Schaap

et al., 1999; Binas *et al.,* 2007).

During prenatal development, adequate supplies of FAs are so essential that the placenta doubles the levels circulating in the maternal plasma (Crawford 2000) and severe deficits may have permanent effects if they occur during critical periods of early development. AA is crucial to brain growth and mild deficiencies are associated with low birth weight and reduced head circumference (van de Lagemaat *et al.,* 2011). AA also plays a key role in the cellular processes underlying learning and memory (Auestad *et al.,* 2003; Das *et al.,* 2003). DHA is particularly concentrated in highly active membranes, such as synapses and photoreceptors, and adequate supplies are essential for normal visual and cognitive development (Uauy *et al.,* 2001). Increasing evidence indicates that PUFA deficiencies or imbalances are associated with childhood developmental and psychiatric disorders, including attention deficit hyperactivity disorder (ADHD), dyslexia, dyspraxia, and autistic spectrum disorders(Richardson 2004). These conditions show a high clinical overlap and run in the same families, as well as showing associations with various adult psychiatric disorders in which FA abnormalities are already implicated, such as depression, other mood disorders, and schizophrenia. Recently, we have revealed that the *Fabp3*-KO mouse shows a lower responsiveness to methamphetamine-induced sensitization and enhanced

haloperidol-induced catalepsy, compared with the wild-type mouse (Shioda *et al.,* 2010). It is thus interesting to note the possibility that such altered behavioral responses detected in the *Fabp3*-KO mouse may be partly attributed to a deficiency of *Fabp3* in the placenta during the gestation period.

CONCLUSION

In summary, we have provided the first direct evidence of the detailed distribution of the FABP family in the mouse placenta. Despite there being no compensatory expression of FA transporters *in vivo* and impaired placental PUFA transport, *Fabp3*-deficient placentas are able to achieve litters of normal birth weight. However, the decreased FA nutrient transport and increased glucose transport suggest that *Fabp3*-null conceptuses have been growing with a modified nutrient mixture and may have undergone metabolic reprogramming. To understand the *in vivo* consequences of altered PUFA transport through the *Fabp3*-deficient placenta, detailed long-term health follow-up studies are warranted, as suggested by the evidence for the developmental origins of health and adult disease.

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