Bioresearch Communications
Volume 01, Issue 02. July 2015.
Journal Homepage: www.bioresearchcommunications.com

Original Article

Fatty Acid Binding Protein 3 (FABP3) Deficiency Does Not Impact on Feto-Placental Morphometry and Fatty Acid Transporters in Mice

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ABSTRACT: In most mammals, proper placentation along with its morphology is critical for fetal development and pregnancy success. Fatty acid binding protein 3 (FABP3) has been shown to regulate the transportation of polyunsaturated fatty acid (PUFA) to the fetus, but its impact on placental morphology and genetic alteration of fatty acid transporters in trophoblast cells is still unknown. Using mice model we found *Fabp3* deficiency does not affect the morphology of placenta along with the gene of fatty acid transporters. *FABP3* deficiency was also found to have no impact on the morphology of human trophoblast cells (BeWo cells) in *in vitro* study. Fatty acid transporter genes were also remains unaltered after *FABP3* knockdown. Our study suggests that placenta may conserve its morphology and genetics although it has PUFAs deficiency.

KEYWORDS: FABP3, PUFA, placenta, trophoblast, morphometry.

CITATION: Islam, A. and Owada, Y. 2015. Fatty Acid Binding Protein 3 (FABP3) Deficiency Does Not Impact on Feto-Placental Morphometry and Fatty Acid Transporters in Mice. *Biores Comm.* **1**(2), 57-61.

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INTRODUCTION

The placenta represents a key organ for fetal growth as it acts as an interface between mother and fetus regulating the maternal-fetal 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 direct regulation of 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 may occur[1].

The essential fatty acids or long-chain polyunsaturated fatty acids (LC-PUFAs) or PUFAs play a fundamental role during pregnancy for both the health of the pregnant woman and for the growth and development of the fetus. Apart from serving as an energy substrate, PUFAs are essential components of membrane lipids that maintain the structure and function of cellular and subcellular membranes and act as regulators of gene expression via nuclear receptors[2]. Among the PUFAs; docosahexanic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) are essential for the development of the fetal neurovisual system, being incorporated in high concentrations in the brain and retina and synthesis of eicosanoids (prostaglandins, thromboxanes and leukotrienes), which are important for the development of fetal nervous, visual, immune and vascular systems [3, 4].

Because of their poor solubility in the cytosol, PUFAs are bound to small (14- to 15-kDa) cytosolic fatty acid-binding proteins (FABPs), which belong to a family of ≥13 different intracellular lipid binding proteins[5,6]. Among these the heart (H)-type FABP (H-FABP) or FABP3 is abundantly present in heart, skeletal muscle and placenta[7-9]. We have previously reported that FABP3 regulates the transportation of n-3 and n-6 PUFAs in rodent trophoblast cells[9]. But their impacts on feto-placental unit or trophoblast morphology and genetics have not been studied yet.

Several studies have suggested that abnormal placental morphology may increase the risk of complications, including low birth weight, malformations[10,11] and possibly involved in alterations of some metabolic parameters[12]. Insulin like growth factor II (*Igf2*) knockout mouse model shows reduced placental growth and fetal growth restriction[13]. Murine studies revealed that plasminogen activator inhibitor–I (*PAI-1)* deficiency may cause aberrant placental morphometry along with altered angiogenesis and genetics[14]. Although basal PPARγ expression is required for proper placental development[15], enhanced activation of placental PPARγ might lead to premature differentiation of trophoblast stem cells and

depletion of the precursor pool, resulting in decreased placental size and abnormal labyrinthine development along with its genetic alteration[16].

In the present study, we evaluated the effect of *Fabp3* deletion on placental morphology and weight variation of feto-placental unit at embryonic day (E) 15.5, and E18.5, by histological comparison and weight measurement. The morphology of trophoblast cells (BeWo cells) and proliferation assay was also checked *in vitro* after knocking down of *FABP3* in BeWo cells. FA transporter genes were also checked *in vitro*.

MATERIALS AND METHODS

Animals. *Fabp3*-knockout (*Fabp3*−/−) mice[17] 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. The presence of a vaginal plug was designated as embryonic day 1 (E1). For our experiment E15.5, and E18.5, dams were used. All experimental protocols were reviewed by the Ethics Committee for Animal Experimentation of Yamaguchi University School of Medicine, Japan.

Cells. BeWo cells (CCL-98; American Type Culture Collection) were cultured at 37°C in a 5% CO_2 atmosphere in Ham's F12 K medium (Wako, Osaka, Japan) with 10% FBS (Hyclone, Logan, UT, USA) as described previously[9].

Morphometric analysis of placenta. Placentas and fetuses from either wild-type or *Fabp3*-KO mice (for E15.5, litter size 6 and for E18.5, litter size 4), were obtained, then placental and fetal weight were compared. For microscopic evaluation, paraffin embedded placental section from either wild-type or *Fabp3*-KO mice at E18.5 were stained with hematoxylin and eosin.

Stealth RNAi-mediated knockdown. *FABP3*-knockdown experiment was performed according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Stealth RNAi directed against the human *FABP3* mRNA [Oligo ID: HSS103512 (Invitrogen)] was used for *FABP3* knockdown. Nontargetting low GC content stealth RNAi (ID: 46:2002) was used as a control (scramble control). After 48-h incubation, FABP3 knockdown was confirmed by RT-qPCR and western blotting.

Real time-PCR (RT-qPCR). Total RNA was extracted from cells (after 48hrs of knockdown of BeWo cells), cDNA was prepared as described previously[9] and reactions were performed by using Step One Plus Real Time PCR system (Applied Biosystem). Among target genes (supplemental table), the house keeping gene glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control.

Morphology. The morphological characteristics and apoptosis of BeWo cells were checked after *FABP3* knockdown at 48 hours by visual assessment from the phase contrast images.

Immunocytochemistry. To identify the localization of FABP3 in BeWo cells, the cells were stained with anti-human FABP3[9] while anti-E-cadherin (Spring bioscience, CA, USA) was used as epithelial cell (trophoblast cell) marker followed by incubation with goat anti-mouse IgG Alexa 568 (Invitrogen, Carlsbad, CA, USA) and goat anti-rabbit IgG Alexa 488 (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).

Proliferation assay of FABP3-knockdown BeWo cells. For proliferation assay BrdU staining was performed as described previosuly[18] with slight modifications. 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 hr and then counterstained with DAPI and cover slipped.

Calculation and Statistics. Values were expressed as means±SEMs. For comparison of the fetoplacental weight variation and RT-qPCR, 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

Fabp3 deficiency did not affect the fetal appearance and weight.

The fetus collected from wild type and *Fabp3*-KO mice at E18.5 stage did not display any variation in their gross appearance (Figure 1A and B). Furthermore placental and/or fetal weights did not show any significant differences between the genotypes (Figure 1C and D).

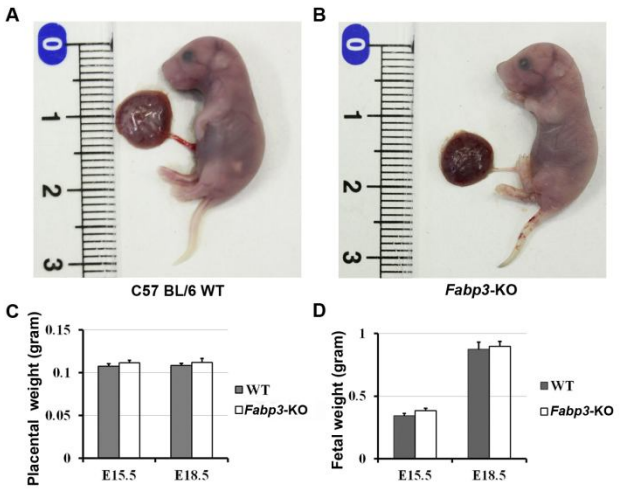

Figure 1. Comparison of feto-placental unit in appearance at E18.5 **(A, B)**. Placental and embryonic weight of both the wild-type and *Fabp3*-KO mice, measured from six litters (E15.5 stage) and four litters (E18.5 stage) **(C, D).**

Fabp3 deficiency did not affect the morphology of mouse placenta.

We examined if there were any morphometric abnormalities in placenta due to *Fabp3* gene ablation. Both of these groups did not present any macroscopic or microscopic abnormalities (Figure 2A–D). Comparison of trophoblast count, maternal space and fetal space in the labyrinthine compartment was checked (unpublished data) and did not display any significant difference between the experimental groups.

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[19,20]. Localization of FABP3 was detected in BeWo cells. This is consistent with our previous findings regarding the localization of FABP family in mouse trophoblast cells[9].

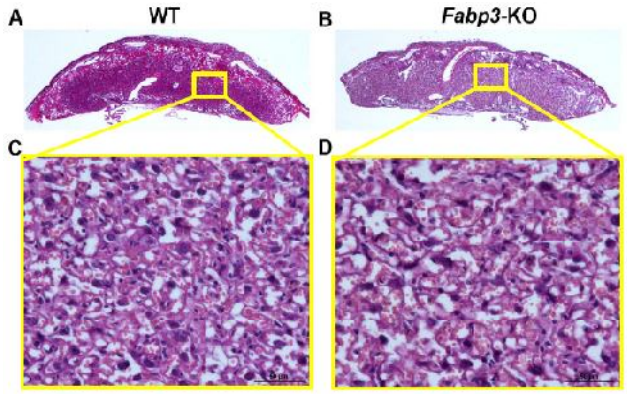

Figure 2. Histological comparison of wild-type and *Fabp3*-KO placenta. Cross-sections of the entire placenta at E18.5 (**A, B**) stained with hematoxylin and eosin. **C, D** shows high magnification image (scale bar, 50 µm).

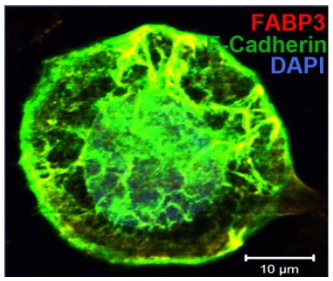

Figure 3. FABP3 immunopositivities were revealed to be overlapped (yellow) with E-Cadherin expression, confirming the localization of FABP3 in human trophoblast cell line (BeWo cells).

FABP3 siRNA transfection did not affect morphology and proliferation of BeWo cells.

Then we checked for any morphological alteration due to *FABP3* siRNA knockdown. Phase contrast images did not show any significant difference in morphology of *FABP3*-KD BeWo cells compared to scramble control (Fig. 4 A, B). Proliferation study using BrdU uptake assay also revealed that *FABP3* siRNA knockdown did not affect proliferation of BeWo cells (Figure 4 C, D).

FABP3-KD *did not affect the gene expression of placental other FA transporters in BeWo cells.*

To assess the influence of *Fabp3* knockdown technique on expression of placental other reported fatty acid transporters, we interrogated the expression of *FABP* and *FATP* isoforms in the wild type and *FABP3*-KD BeWo cells after 48 hrs of transfection. Changes in expression of genes involved in fatty acid transporters were assessed by RT-qPCR[21]. Samples were normalized to the house keeping gene *GAPDH*, which exhibited a stable expression in control or *FABP3*-KD BeWo cells. Importantly *FABP3* knockdown system did not influence the expression level of *FABP4*, *FABP5*, *FATP1*, *FATP4*[22,23], p-*FABP*$_{pm}$[19], *CD36*[24] (fatty acid translocase, FAT) in *FABP3*-KD BeWo cells which is also consistent to our previous *in vivo* findings in mouse placenta[9]. This finding is also supportive to the previous findings[17] where no compensatory expression of related fatty acid transporters in heart and skeletal muscle of *Fabp3*-KO mouse was observed.

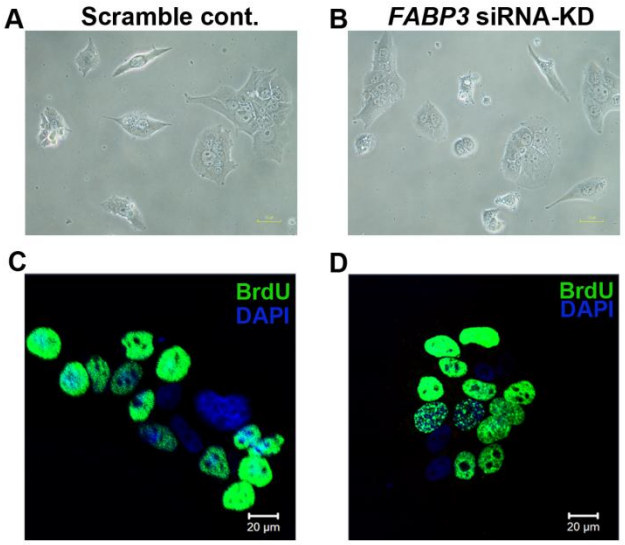

Figure 4. The effect of *FABP3*-knockdown on the morphology (A, B) and proliferation of BeWo cells (C and D).

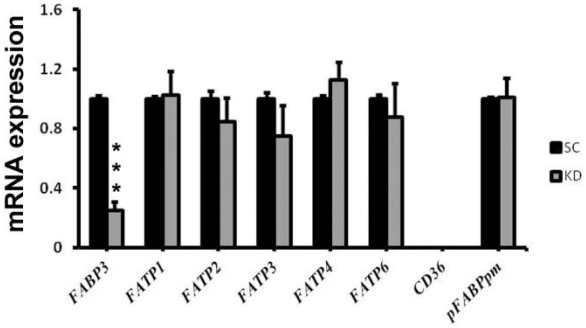

Figure 5. Effect of *FABP3*-knockdown on the gene expression of major FA transporters in BeWo cells after transfection. *FABP*, fatty acid binding protein; *FATP*, fatty acid transport protein; *FAT* (*CD36*), Fatty acid translocase; *p-FABP*$_{pm}$, placental-plasma membrane FABP; *FABP3*-KD, *Fabp3*-knockdown; 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).

DISCUSSION

Proper fetal development is dependent on sufficient nutrient supply to the fetus through the placenta. Because substrate transport, including that of FAs, is directed from the maternal to the fetal circulation under physiologic conditions, the placenta's ability to facilitate this transport is of critical importance for the development of a healthy

fetus[25]. In mice, FABP3 is abundantly expressed by the trophoblast cells of the labyrinthine compartment which has been shown as a regulator of n-3 and n-6 fatty acid transportation through placenta[9]. *In vitro* studies using human trophoblast cell line (BeWo) also revealed that *FABP3* gene knockdown may down regulate fatty acid uptake in trophoblast cells[9]. Although FABP3 was revealed as a regulator of PUFAs transportation but its significance on feto-placental unit is still remain to elucidate. Here we have given morphological and genetic evidences to conserve the integrity of the structure, morphology and fatty acid transporter genes of placental unit inspite of their PUFAs deficiency.

The placenta is the main interface between the fetus and mother. Placental insufficiency results in a failure of the placenta to deliver an adequate supply of substrates to the fetus and IUGR develops. A range of human and animal studies in IUGR pregnancies have suggested that placental restriction and insufficiency may result in a series of placental changes such as altered placental growth and placental substrate transport capacity, increased apoptosis and autophagy. Thus we checked several parameters of the mouse placenta in their gross appearance, weight variation, placental structure and morphology. Our results clearly showed that FABP3 deficiency did not affect the gross appearance, weight variation, placental structure and morphology. Number of trophoblast cells, maternal space and fetal space did not show any significant change due to *Fabp3* deficiency in the placenta. Our *in vitro* results are also supportive to *in vivo* findings. We could not detect any significant difference in the trophoblast cell proliferation due to *FABP3* deficiency.

In FABP4 gene-ablated mice, adipocyte FABP5 expression is enhanced, thus partially compensating for the reduction in the level of FABP4[26]. A similar compensation could occur in the placenta of *FABP3* gene-knockdown trophoblast cells. However, because FABPs are temporally and spatially expressed in different cell types in the placenta[9], the existence of a similar compensatory mechanism is less likely. It is not surprising to note that placental other fatty acid transporters remained virtually unaffected in *FABP3*-KD trophoblast cells. This finding mimic with our previous *in vivo* findings where other FABP family members were unaltered in placenta[9] and heart[8]. Our previous results also demonstrated that there is no compensatory increase in the level of FABP expression in brains from *Fabp3* gene ablated mice[27]. Our current result also coincides with observations in skeletal muscle of *Fabp3*-null mice, where the expression level of *Fat*/*Cd36* and p-$Fabp_{pm}$ remains unaltered[28].

Because the *Fabp3* deficient placenta and fetuses achieved the similar weight, appearance, structure, morphology, proliferation and gene expression, despite a marked impairment in PUFAs transport, we hypothesize that FABP3 deficient conceptuses must modify their metabolism to accommodate a lower availability of long chain fatty acids for its cell integrity, development and energy sources. This "metabolic reprogramming strategy" appeared to allow upregulated transport of glucose to the placentae and fetuses in late gestation[9].

In summary, we provided the direct evidence that FABP3 deficiency or decreased PUFAs transportation through the placenta to fetus does not impact on feto-placental development. Despite there being no compensatory expression of FA transporters *in vitro* and impaired placental PUFA transport[9], *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 were 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 suggested.

ACKNOWLEDGEMENTS

We thank Mr. Masakatsu Tamechika for technical assistance for preparation of paraffin section. This project was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

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