The crosstalk between human fatty acid transport protein 1 and fatty acid transport protein 4

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THE CROSSTALK BETWEEN HUMAN FATTY ACID TRANSPORT PROTEIN 1 AND FATTY ACID TRANSPORT PROTEIN 4

By

Zhe Yuan

A THESIS

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Fatty acid transport proteins (FATPs) provide pivotal roles in fatty acid transport and activation and thus are crucial for overall fatty acid homeostasis. Peroxisome Proliferators Activated Receptors (PPARs) are important transcription factors, which control many genes that govern lipid metabolism.

Using 293 T-REx cell lines that stably express FATP1 or FATP4 from a tetracycline-inducible (Tet) promoter, this work evaluated gene expression of key genes involved in fatty acid metabolism using quantitative polymerase chain reaction (Q-PCR), protein expression of FATP1 and FATP4 using Western blots, and fatty acid transport to address the roles of these two FATP isoforms in fatty acid homeostasis. This work found that the expression of FATP1 from the heterologous Tet promoter resulted in
increased FATP4 expression from its native promoter; the expression of FATP4 from the Tet promoter did not, however, increase the expression of FATP1. These results identified an unexpected regulatory loop between FATP1 and FATP4, which is hypothesized to occur at the transcriptional or post transcriptional level. Western blots confirmed these relationships by evaluating the protein expression of FATP1 and FATP4. Studies monitoring the transport of fatty acids using the fluorescent long chain fatty acid C_{12}-BODIPY-C_{12} demonstrated the expression of FATP1 resulted in higher levels of transport relative to FATP4 overexpression. To address whether this regulatory loop proceeds through PPARα or PPARγ, the expression of these two genes along with PPARs target genes were monitored. The overexpression of FATP1 and FATP4 from the Tet promoter did not increase PPARs gene expression; the expression of FATP1 from the Tet promoter did result in an increased expression of PPARα target genes. Collectively these results supported the conclusion that the enzymatic product of FATP1 resulted in increased expression of FATP4 through a PPARα-mediated process. To further address this question, fatty acid and fatty acyl CoA profiles were measured in 293 T-REx cells expressing FATP1 or FATP4. The acyl CoA profiles showed both C18:2-CoA and C20:4-CoA were
elevated in FATP1 expressing cell lines, perhaps indicating a relationship in n-3 fatty acid activation including further downstream metabolism. Likely effectors in this regulatory loop are arachidonic acid metabolites, including prostaglandins.
Acknowledgments

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List of Abbreviations

FATPs: Fatty acid transport proteins
ACSL: Long chain acyl-CoA synthetase
CoA: Coenzyme A
FA: Fatty acid
FFA: Free fatty acid
PPARs: Peroxisome proliferator-activated receptors
BODIPY: Boron-dipyrromethene
RXR: Retinoic X receptor
TLR: Toll-like receptor
LCFAs: Long chain fatty acids
GPCR: G-protein-coupled receptor
FABP: Fatty acid binding protein
PPREs: Peroxisome Proliferator Response Elements
OE: Over expression
Chapter 1. Introduction

I. Significance

Long chain fatty acids (LCFAs) are important molecules for our life. They are important components in the storage and generation of energy, and also contribute to many essential cellular processes (1).

Different research studies indicate that LCFAs function as hormone-like components (2, 3, and 4). For example, they can affect gene expression and function as effectors by activating nuclear receptors including PPAR family members and can initiate the release of insulin through G-protein-coupled receptor (GPCR) (5, 6). LCFAs are also involved in the innate immune response by modulating toll-like receptor (TLR) signaling (7, 8 and 9). The chronic imbalances in lipid metabolism can give rise to many different kinds of health disorders including nonalcoholic fatty liver disease, obesity, cancer, Type II diabetes, heart disease, and hyperlipidemia. From a fundamental standpoint, LCFAs have a profound physiological significance. (10, 11, 12, 13)
To assert their diverse effects, LCFAs must first be transported across the plasma membrane. Evidence shows that LCFAs traverse the membrane using several distinct mechanisms. The uptake of LCFAs is likely protein-mediated via fatty acid transport proteins (FATP), fatty acid translocase (FAT/CD36) and/or fatty acid binding proteins (FABP) (14, 15, 16, 17 and 18).

There is a significant body of work that has been done establishing these proteins in the process of fatty acid uptake. An additional level of complexity comes from studies showing the expressions of FATP, FAT/CD6, and FABP proteins are controlled at multiple levels including transcription and post-transcription (19, 20). Because of these controls, the lipid balance inside of the cells is very tightly regulated (21).
II. Fatty acid transport proteins (FATPs)

Of the proteins implicated in fatty acid uptake, the fatty acid transport proteins (FATPs) represent an important group. In humans, there are six FATP members: hsFATP1 to hsFATP6. Each of these proteins is expressed in the tissues or cells which utilize fatty acids and/or have high levels of lipid metabolism. FATP1 and 4 are the most predominant FATPs in mammalian cells (22, 23). Fatty acid transport proteins function in fatty acid uptake by working independently or coordinately with a long chain acyl-CoA synthetase; in both cases fatty acid transport and activation represent a coupled process referred to vectorial acylation.

FATP1 is the predominant FATP in adipose tissue and muscle. It is quite important for energy homeostasis, thermogenesis and insulin resistance (24). When FATP1 is knocked out in mice, they do not show any signs of insulin resistance when they are fed a high fat diet. Under pathological conditions, FATP1-mediated fatty acid uptake may result in the accumulation of neutral lipid intramuscularly, which has been linked to insulin resistance (25).
FATP4 knockout mice are lethal; evidence to date suggests they cannot form a normal embryo. To date, there is no evidence linking FATP4 to insulin resistance (26). FATP4 is over expressed in human adipose tissue in obese individuals. Tissue specific deletion of FATP4 in the skin results in a phenotype of skin homeostasis failure, which is likely linked to the formation of skin waxes (27). Studies of different FATPs show their unique and crucial roles in the epidermis for maintaining skin barrier and keratinocyte homeostasis. Animal models of FATP4 deficiency, as well as human phenotype in IPS, have demonstrated a vital role for FATP4 in mammalian skin development and formation of epithelial barrier, which may be due to its very long chain acyl-coenzyme A synthetase activity. Pathomechanism of FATP4 deficiency in skin is still poorly understood and clarifying of specific pathways affected by such deficiency may yield new insights for the role of fatty acid metabolism in epidermal biology.

FATP5 knockout mice have abnormal hepatic fatty acid uptake and bile acid reconjugation. These data suggest that FATP5 is a bifunctional protein both at the level of fatty acid uptake, but also in bile acid activation and reconjugation (28).
Indeed most fatty acid transport proteins are increasingly being recognized as multifunctional proteins that mediate the uptake of fatty acids and catalyze the formation of coenzyme A derivatives using long-chain fatty acids, very-long chain fatty acids, and bile acids. The enzymatic product for most FATPs is likely to be a fatty acyl CoA that may function as an effector of different metabolic pathways. The interrelationships between the different isoforms of FATPs in the context of fatty acid metabolism have not been studied (29, 30).

Modulation of fatty acid transport protein function can result in altered energy homeostasis and insulin sensitivity, defective skin homeostasis, and altered bile acid metabolism. Both fatty acid uptake and enzymatic activity of fatty acid transport proteins likely contribute to these phenotypes. Future studies are needed to better understand the molecular mechanism of fatty acid transport protein function and the physiological role of FATP2, FATP3, and FATP6.
III. Peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor superfamily, of which there are three subtypes: PPAR alpha, PPAR beta (or delta) and PPAR gamma. (31)

Fibrates and fatty acids are peroxisome proliferators which can activate the transcriptional activity of these receptors. Prostaglandin derivatives are the only natural ligands of PPAR gamma. PPAR have been identified as an antidiabetic molecule by binding thiazolidinedione with high affinity (32). After binding with the activating ligands, PPARs form heterodimers with retinoic X receptor (RXR) and translocate into the nucleus where they alter the transcription level of its target genes through binding Peroxisome Proliferator Response Elements (PPREs). PPRE is a simple repeat of the hexameric DNA recognition motif (XGGTCA) spaced by 1 nucleotide (33). Natural fatty acids and drugs are often used to activate PPARs and affect lipid metabolism. Each PPAR isotype is preferentially activated by specific ligands. For example, leukotriene B4 activates all isotypes, but it activates
PPARα preferentially. (42) PPARα is also activated by arachidonic acid (AA) and fibrates, which links this nuclear factor to atherosclerosis. (43)

PPARs regulate the expression of target genes, which are implicated in cellular lipid metabolism. Our current understanding of PPARs show they can be considered as multiple function factors because their activation can trigger adipocyte differentiation and other genes crucial to adipogenesis (34). This suggests the PPARs are involved in the regulation of the immune system, maintain nutrient balance and lipid metabolism, and regulate the transcription of key genes involved in lipid metabolism. At present, there are reports showing the enzymatic products of one or more of the FATPs activate the different PPAR subtypes.

PPARα are expressed mainly in the liver, heart, skeletal muscle, kidney and small intestine. They are present in important mechanisms, such as the capture and oxidation of fatty acids; synthesis of ketone bodies; metabolism of apolipoproteins (apoAI and apoAII); expression of genes involved in
gluconeogenesis; inhibition of transamination and deamination of amino acids, as well as the blocking of urea synthesis. (39)

PPARβ/γ is widely distributed in the tissues and is expressed in the placenta and small intestine. The mechanisms involving the gene expression regulation of this receptor are unknown, but it is an important agonist in the treatment of dyslipidemia and cancer and acts in the differentiation of central nervous system cells. (38, 40)

PPARγ is abundant in adipose tissue and is present in low concentrations in the skeletal muscle of humans. As a receptor of antidiabetic drugs (troglitazone and rosiglitazone), it leads to increased sensitivity to insulin in the adipose and muscle tissues, by improving glucose metabolism; reduces inflammation and promotes the differentiation of pre-adipocytes in adipocytes. (41)
IV. Previous Work

For defining the roles of the different isoforms of fatty acid transporter proteins in fatty acid trafficking precisely, all six murine isoforms were expressed and characterized in a genetically modified yeast strain. This strain cannot transport long-chain fatty acids and has deficient long-chain acyl-CoA synthetase activity. Murine FATP1, FATP2 and FATP4 rescued the mutation in fatty acid transport and very long-chain fatty acid esterification associated with a remove of the yeast FAT1 gene; but three of these 6 isoforms: FATP3, FATP5 and FATP6 did not complement the transporter function even though all of them were localized in the yeast plasma membrane (35).

FATP1 was the predominant isoform located in adipocytes consistent with the function in the transportation and activation of exogenetic fatty acids destined for storage of triglycerides. In intestinal-like Caco-2cells and liver-like HepG2 cells, in which there were lots of fatty acid processing, FATP2 was the predominant isoform in these two cell lines(36).
Dr. Melton did some important research work on FATP2 in our lab. Two splice variants of human FATP2: FATP2a and FATP2b were identified and shown to have distinguishing metabolic characteristic suggesting differential functions in fatty acid homeostasis. Dr. Melton overexpressed FATP2a and FATP2b in yeast or 293 T-REx cells. Both of them were sufficient for long chain fatty acid import. However, only the full length variant FATP2a performed very long chain acyl-CoA synthetase activity (37).

Our lab is also investigating main fatty acid transport proteins in human. And the coding sequences for FATP1 and FATP4 were amplified by PCR using suitable generating double restriction enzyme cut sites respectively and cloned into pcDNA4/TO/myc-His (Invitrogen) and propagated in an Escherichia coli strain; the sequences of both expression clones were verified. Stable cell lines expressing human FATP1 and FATP4 were generated by transfection of the constructs into stable 293T-REx cell lines (InVitrogen; stably expressing the tetracycline repressor protein) by Dr. Elsa Arias-Barrau. Western blot has been done by her using multiclonal antibody of human FATP1 and FATP4. And the band for FATP1 only exist in FATP1
over expression cell line sample, but the band for FATP4 exist in both FATP1 and FATP4 over expression cell lines samples.
V. Hypothesis

The expression of human FATP1 increases the expression of human FTAP4 through a mechanism involving PPARα and not PPARγ. This regulation of FATP4 by FATP1 may be mediated by the enzymatic product of FATP1, which may activate PPARα post-translationally.
Chapter 2. Materials and Methods

I. Cell Culture

293T-REx stable cell lines allowing the controlled expression of FATP1 and FATP4 were established by Elsa Arias-Barraua with the vector pcDNA4/TO/myc-His (Invitrogen) and were seeded at $5 \times 10^3$ cells/cm$^2$ in T75 flasks in DMEM medium containing 10% FBS, 5µg/ml blasticidin and 125µg/ml of zeocin to keep and expand the cell lines. The selective medium was replaced every 3-4 days. When cells were more than 90% confluent they were split 1:3 into new T25, T75, and T150 flasks. Induction of expression of FATP1 or FATP4 was accomplished by the addition of tetracycline to a final concentration of 2µg/ml for 48h; the cells were harvested and then subjected to analytical studies as detailed below.
II. Time-course fatty acid uptake assay

The ability of fatty acid transport was monitored using the live-cell, real-time method and the fluorescently labeled fatty acid, 4, 4-difluoro-5-methyl-4-bora-3a, 4adiaza-s-indacene-3-dodecanoic acid, C1-BODIPY-C12, in combination with the quenching agent trypan blue. To monitor fatty acid transport, the stable 293 T-REx cell lines grown under standard conditions in the presence of tetracycline (2µg/ml) (see above) in optically transparent 96 well plates. Cells were seeded at 50,000 cells per 96 well in standard media containing 5µg/ml Blasticidin and 125 µg/ml Zeocin. 24h after plating 2µg/ml of tetracycline was added to the cells for induction. 48h post induction cells were serum-starved for 1h in MEM prior to performing the C1-BODIPY-C12 transport assay. Subsequently 50µL of the C1- BODIPY-C12 mixture in different final concentration of BODIPY: 1 µM, 5 µM, 10 µM, 20 µM and 40 µM (For 5 µM final concentration of BODIPY: 10µM C1-BODIPY-C12, 10µM BSA, 4mM trypan blue) was added to each well. Transport was determined immediately by measuring the cell associated fluorescence, which was measured using a Bio-Tek Synergy HT multidetection microplate reader (Bio-Tek Instruments, Inc. Winooski, VT)
using filter sets of 485 nm ± 20 excitation and 528 nm ± 20 emission. Data were collected as arbitrary fluorescence units (AFU)/3 x 10^6 cells/30sec. The cell number in each well was determined using a standard Hoechst assay used to quantify DNA. The fluorescence was read at 350nm/450nm (excitation/emission). The cell number was calculated from a standard curve generated for cell number verses fluorescence units. Data analysis is done with PRISM software. (n=3)
III. Western Blot

The expression of FATP1 and FATP4 were monitored by western blot analysis using FATP1 and FATP4 monoclonal antibodies (Invitrogen) coupled with the appropriate secondary antibodies. After 48 h Tet induction, the 293 T-Rex cells were harvested. The cell pellets of FATP1 overexpression, FATP4 overexpression and empty cell lines were resuspended. Cells were sonicated for 150 sec (3 sec on, 5 sec off), 20% amp. Then, cells were centrifuged at 13200 rpm for 15 min, 4 °C. Supernatants were transferred to a new tube. The protein concentrations were tested using a plate reader: 200ul 1:4 diluted protein assay reagent (from Bio-Rad) + 1 ul my sample or + 10 ul standard BSA. Then a 12% Poly Acrylamide Gel Electrophoresis was performed. Gel was run at 80V (constant) through the stacker and 120V (constant) through the running gel. Then, the semi-dry transfer was performed using 0.4 amps constant and run for 30 minutes. The membrane was wet in PBS for several minutes. Then, the first antibody is used in 1:3000 dilution and 1:15000 in Li-Cor western buffer for second antibody for the blot. The membrane is scanned by membrane scanner.
IV. LC- MS/MS Analysis of Fatty Acyl CoA Pool

Cells were grown to confluence in T75 flask and harvested in PBS. Cell pellet was gently washed with 5 ml PBS and placed on ice immediately. Cells were resuspend in 0.5 ml water and mixed up and down several times with pellet tip to get uniform suspension. 50ul of all was used for cell counting and protein concentration determination. 200ul aliquot of this suspension was pipetted out into a screw cap tube on ice. 500ul of methanol containing 1mM EDTA was added to the aliquot. Then, the mixture was sonicated for 30 seconds. 10ul of internal standard (C17:0 CoA, C19:0 CoA, C23:0 CoA) was added. 250ul chloroform was added at last. The mixture was sonicated for 30 seconds and incubated at 50 ºC for 30 minutes in a heating block or water bath. After this, the mixture was cooled to room temperature. 250ul water was added to it, and the mixture was vortexed and mixed. The mix was centrifuged to get pellet at 3000 rpm, 5 min, 25 ºC. The upper phase was removed with pipette to a new screw cap tube. The remainder was re-extracted twice each time with 500ul (H2O/CH3OH/CHCl3-45/50/5) followed by centrifuge (3000 rpm, 5 min, 25 ºC). The upper phase was collected and combined to the first extract. 180ul
methanol/butanol/chloroform (50/25/25) was added to keep the very long chain acyl CoAs stable. The samples were run on LC-MS by Dr. Padmamalini Srinivasan. Analyze monitoring and quantification was conducted with Analyst 1.5 Software from AB Sciex.
V. GC-MS Analysis of Fatty Acid Profile

Cells were resuspended in 1ml chloroform with 0.05% BHT and 2 ml methanol with 0.05% BHT. 10 ug C17 standard was added to it and incubated at room temperature with 30 min shaking. Cells were centrifuged at 3000 rpm, 5 min at room temperature. Supernatant was transferred to glass tubes and 1ml chloroform and 1.8ml water were added and the mixture was vortexed. Cells were centrifuged at 3000 rpm for 5min at room temperature. The bottom phase was transferred to a small black capped glass tube. It was dried under nitrogen stream and resuspended in 1ml chloroform. This 1ml was split to 500ul for FAME and 500ul for GC-MS total lipid. Both of them were dried under N2. The total lipid sample was kept in -20ºC. The 500ul for FAME was resuspended in 250ul toluene and 500ul 1% sulfuric acid in methanol. The sample was incubated at 50ºC overnight. The 1.25ml 5% NaCl was added and vortexed. The sample was extracted twice using 1.25ml hexane. 1ml 2% potassium was added to the upper phase combo and vortexed. The upper hexane phase was passed over 0.5g sodium sulfate in glass packed pasture pipet and collected in a new tube. The sample
was dried under N₂ and resuspended in 1ml methyl acetate and transferred to GC-MS vial for GC-MS running.
VI. Quantitative Real-Time PCR

After 48 h of Tet induction, $1 \times 10^7$ cells were harvested using Qiagen total RNA extraction kit. Then Bio-Rad cDNA synthesis kit was used for reverse transcription step to obtain cDNA, which is used for the template in QPCR. 1 µg RNA/20 µl RT system was used. 1/10 dilution is used as the template of PCR. For the standard curve preparation, genomic DNA is used as template and the interested gene’s primers to do PCR. After the amplification, an agarose gel was run to analyze the samples. The bands which are clear and at right sizes were cut. Gel extraction kit was used to extract the PCR products. Nanodrop was used to see the concentration of the gene fragments. Then the absolute number of each fragment according to the concentration and the base pair numbers were calculated. Molecular copy numbers/ µl=$6.022 \times 10^{23} \times (\text{Concentration of DNA (ng/µl)} \times 10^{-9})/ (\text{base pair X 650}) 10^{11}/ µl$ is usually kept for storage concentration. Then a series of dilutions from $10^{10}$ to $10^3$ will be done with PCR H$_2$O.

For the QPCR, β-actin was used as the reference gene. Because the R2 is the straightness of every gene’s standard curve, the R2s should be bigger than
We used 10μl PCR reaction mix including 5μl of Syber Green mix, 1μl cDNA template, 0.25μl each primer (10mM primer), 3.5μl ddH2O in 96 wells plate. Each sample was vortexed and spun down prior to PCR. The PCR program was 95°C, 15 min; (95°C, 30s; 55°C, 30s; 68°C, 30s ) X 35 Cycles; melting curve 20min, 0.5°C increase from 55°C to 95°C per minute; end 4°C. We keep the standard deviation lower than 0.2, R2 of each gene higher than 0.95. The samples are triplicated.

The primers used are the following:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin (reference gene)</td>
<td>5’ CTGGGACGACATGGAGAAAA 3’</td>
<td>5’ AAGGAAGGCTGGAAGAGTGC 3’</td>
</tr>
<tr>
<td>hsFATP1 (target gene)</td>
<td>5’ CTGCCCTTAAATGAGGCAGTCT 3’</td>
<td>5’ AACAGCTTCAGAGGGCGAAG 3’</td>
</tr>
<tr>
<td>hsFATP4 (target gene)</td>
<td>5’ TTCTGTGAAAGTCTCATGTCCAAGT 3’</td>
<td>5’ TCTCAGCCTGGGAACCAGAG 3’</td>
</tr>
<tr>
<td>hsPPAR-α (target gene)</td>
<td>5- GCAAAACTTGGACCTGAAC-3</td>
<td>5- CCCATTTCCATACGCTAC-3</td>
</tr>
<tr>
<td>hsPPAR-γ (target gene)</td>
<td>5- CCAAGCTGCTCCAGAAAA-3</td>
<td>5- AGCGGGGTGAAGACTCATGT-3</td>
</tr>
<tr>
<td>hsPLTP (target gene of PPARα)</td>
<td>5- CATGCGGGGTGAGGACTCATGT-3</td>
<td>5- GAGGGGGCACTACAGGCTAT-3</td>
</tr>
<tr>
<td>hsPEPCK (target gene of PPARγ)</td>
<td>5- GAGGGGGCACTACAGGCTAT-3</td>
<td>5- GAGGGGGCACTACAGGCTAT-3</td>
</tr>
<tr>
<td>Forward: 5-TGCTGGCTGCCCCGCACAGACCCC-3</td>
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<td></td>
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<tr>
<td>--------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse: 5-GAGAAGGAGTTACAATCACCGTCT-3</td>
<td></td>
<td></td>
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<tr>
<td>hsHMGCS2: (target gene of PPARα)</td>
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<td>Forward: 5-CAGATCCATGGAAAAGCTTCCCTGGG-3</td>
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<td>Reverse: 5-ATTTACCAGCTAAGAGTGGGATCT-3</td>
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<td>hsCD36: (target gene of PPARγ)</td>
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</tr>
<tr>
<td>Reverse: 5-TTGACCTGCAATATCAGAAGAAA-3</td>
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</table>

For the QPCR data analysis, we use software Excel to process the data we collected from the thermo cycler to a bar figure (n=3). The copy number of each gene / the copy number of β-actin = Y, the Y of empty vector was set as 1 to compare the expression profile of these genes mentioned above.
Chapter 3. Results and Discussion

I. The expression of human FATP1 can increase the expression of human FATP4 at the protein level.

From our previous data, we used multiclonal antibodies to develop the western blot of FATP1 and FATP4 and found put that FTAP4 can be seen in both FATP1 overexpression and FATP4 overexpression cell lines. This can be explained by two possibilities. One is the epitope of FATP1 and FATP4 in the multiclonal antibodies have some cross over. So, the FATP1 multiclonal antibody can bind with FATP4, but FATP4 antibody cannot bind with FATP1. This epitope cross over can cause this western blot result although these two genes don’t have any crosstalk between each other. Another possibility is the human FATP1 really turn on the expression of FATP4 on protein level.

For addressing this problem, we use the monoclonal antibody of each protein. And the antigens sequences for each of them are checked to make sure that they have no overlapping epitope from the vendor’s website. And
for the better quantitative result comparable to QPCR assay for mRNA level, we use Li-Cor system to execute our western blot.

From the western result, we can see there are still two bands in both FATP1 over expression and FATP4 over expression wells on the membrane anti-hsFATP4 (Figure 1) and only one band in FATP1 over expression well on the membrane anti-hsFATP1 (Figure 2). This is consistent with our previous preliminary data. We also find that the FATP4 band in FATP1 over expression well is heavier than the band in FATP4 over expression itself.

This suggests that the expression of human FATP1 can increase the expression of human FATP4 in protein level not because of the epitope cross over. And the turned on FATP4 has a stronger expression level than the construct overexpression. This gives us a clue that there are some mechanisms involving in the crosstalk of these two proteins, at least on protein expression level, the reason why FATP4 is turned on is unclear.
II. FATP1 can turn on FATP4 not only at protein level but also at mRNA level.

To check the crosstalk on mRNA level between these two genes, quantitative PCR was used. We checked the human FATP1, human FATP4 mRNA level in empty vector (as control), FATP1 over expression cell line and FATP4 over expression cell line and used β-actin as a reference gene. The QPCR result is consistent with the western data (Figure 3, n=3). hsFATP1 is over expressed in FATP1 over expression cell line and no change in vector and FATP4 over expression cell line. hsFATP4 is over expressed in FATP4 over expression cell line and almost 4 folds than vector in FATP1 over expression cell line. This means hsFATP1 overexpression really can turn on the mRNA level of hsFATP4. And this turn on effect is robust, even higher than the construct overexpression itself.

For the expression of FATPs, they are regulated by the transcriptional factors PPARs. If the FATP4 expression is up regulated, there should be some reason to turn on the PPARs regulation of FATP4 specifically. Two
possible approaches can achieve this up regulation. One is the expression of PPAR is turned on. There are more PPARs to start the transcription. Another potential way is the PPARs are more active than control because there is more ligand binding to them.
III. The effect of increasing FATP4 expression caused by FATP1 overexpression is through PPARα not through PPARγ, and it is intermediated on post-transcription level not the transcription level of PPAR γ.

PPARα and PPARγ mRNA levels and their target genes were evaluated by QPCR. PEPCK is the specific target gene of PPARγ, HMGCS2 and PLTP are the specific target genes of PPARα. Our idea is if the crosstalk is mediated on transcriptional level, PPAR itself is over expressed. So, there are more transcriptional factors to turn on the target genes expression. And if this process is through post-transcription level, the expression of PPAR itself will not change obviously, but the target genes are turned on.

From the QPCR result (n=3) (Figure 4.), neither of the PPAR expression is turned on. This is the evidence that the effect is not caused by transcriptional level. For the PPARα, target genes PLTP (phospholipid transfer protein) and HMGCS2 (3-hydroxy-3-methylglutaryl-CoA synthase 2) are highly over expressed than empty vector. But for the PPARγ, we find both FATP1 over
expression and FATP4 over expression can turn on PEPCK (phosphoenolpyruvate carboxykinase), this cannot explain the one-way turn on effect.

All fold changes of the genes we checked can be seen in Table I. FATP1 is overexpressed in FATP1 OE cell line. FATP4 is 2 folds overexpressed in FATP4 OE cell line but 4 folds in FATP1 OE cell line. This means FATP4 expression is elevated by the FTAP1 overexpression. Expressions of PPARα and PPARγ keep the same in all of three cell lines. The target gene of PPARγ, PEPCK is elevated by 2 times in both of FATP1 and FATP4 overexpression cell line. This may not the causal factor of FATP4 expression elevation. PLTP is 4 times of control group; HMGCS2 is more than 40 times of control group. They are the target gene of PPARα. This shows us that the elevation effect may cause the activation of PPARα and the consequence of that is the expressions of these target genes are elevated. CD36 keeps the same in each cell line. So, we can conclude that the increase expression of FATP4 effect is through PPARα activation elevation not the transcriptional level of it.
IV. Time-course fatty acid uptake assay of empty vector, FATP1 over expression and FATP4 over expression.

To measure the initial uptake efficiency of different overexpression cell lines, kinetic C1-BODIPY-C12 uptake assay is used. The patterns of fatty acid transport were monitored using the live-cell, real-time method and the fluorescently labeled fatty acid, 4, 4-difluoro-5-methyl-4-bora-3a, 4adiaza-s-indacene-3-dodecanolic acid, C1-BODIPY-C12, in combination with the quenching agent trypan blue (Figure 5).

From the C1-BODIPY-C12 initial uptake data (n=3, Figure 6 and 7), we know that the over expression of FATP1 can uptake the C1-BODIPY-C12 more efficiently than FATP4 OE than empty vector. There should be some reason that FATP1 over expression is effective than FATP4 over expression.

Our conclusion which is consistent with western and QPCR data is that FATP1 can turn on FATP4.
V. LC-MS/MS Analysis of Fatty Acyl CoA Pool and GC-MS analysis of the total fatty acids change of different 293 T-Rex over expression cell lines.

After the 48 hours of Tet induction, we used LC-MS to see the CoA pool change of different 293 T-Rex over expression cell lines. From the LC-MS data, the acyl CoA profiles showed both C18:2-CoA and C20:4-CoA were elevated in FATP1 expressing cell lines, perhaps indicating a relationship in n-3 fatty acid activation including further downstream metabolism (n=3, Figure 8). Likely effectors in this regulatory loop are arachidonic acid metabolites, including prostaglandins.

And the GC-MS of lipids extraction of 293 T-Rex overexpression cell lines after Tet induction also has been done to see the lipidome change of different 293 T-Rex over expression cell lines. From the GC-MS data (n=3, Figure 9), we cannot see the overexpression of these two transporters will affect the fatty acids pool.
But this can be explained by the method of our lipids sample extraction. FAME was used to extract the total lipids inside of the cells. So, most portion of this pool is at steady state, which can mask our data as minor change. That’s why there is no significant change of fatty acids profile.
Chapter 4. Summary

From the PQCR, Western and C1-BODIPY-C12 data collected from this work, we found a phenomenon that FATP1 can turn up FATP4 expression, but FATP4 cannot control FATP1.

And this regulation may be through a certain mechanism of post-transcriptional activation of PPARα because the up regulation of PPARα target genes uniquely when the FATP1 is over expressed. The over expression of FATP1 can uptake the C1-BODIPY-C12 more efficiently than FATP4 than empty.

The LC/MS/MS for CoA pool and GC/MS for total fatty acids profile results of FATP1 and FATP4 over expression cell lines also show some differences. C18:2-CoA can be utilized to produce C20:4-CoA. So, increased C18:2-CoA would cause C20:4-CoA increased. Prostaglandins were derived from C20:4. There are some references also showing that arachidonic acid and its
derivatives, exp. prostaglandin, can be the activators of PPAR. From this, we may conclude our finding as increased expression of FATP1 elevated the arachidonic acid CoA. But some references show that Acyl-CoA cannot be the effector of PPARα. So, probably the downstream of C20:4-CoA can be the effector causing the elevation of PPARα activation. C20:4-CoA can form phospholipids, then C24:0 can be derived from phospholipids. And the derivative of C24:0, prostaglandin, is likely to be the effector which activates PPARα to upregulate the expression of FATP4.

For the future work, we should make sure the relationship between the elevation of FATP expression and the activation of PPARα using a luciferase PPAR reporter gene. Then we need to try to specify the group which can activate this PPAR subtype, if it is arachidonic acid CoA or prostaglandin.

From this work, we suggest a novel regulatory mechanism in fatty acid metabolism that involves an enzymatic product, directly or downstream, of FATP1, which functions as a PPARα effector. The eventual identification
of this ligand may result in a target for eventual therapeutics directed to control lipids homeostasis through PPARα.
Figure 1. Western blot using anti-hsFATP1. Lane empty means the empty vector as control. FATP1 OE is the cell line over expressing FATP1. Lane FATP4 OE is the cell line over expressing FATP4. M represents the molecular weight markers. There is only one band in the lane of FATP1 overexpression indicating that FATP1 is overexpressed.
Figure 2. Western blot using anti-hsFATP4. Lane empty means the empty vector as control. FATP1 OE is the cell line over expressing FATP1. Lane FATP4 OE is the cell line over expressing FATP4. M represents the molecular weight markers. Both lanes for FATP1OE and FATP4 OE are showing the band for FATP4 overexpression.
Figure 3. mRNA levels of hsFATP1 and hsFATP4 in 3 cell lines. (n=3)

This is the QPCR data showing the fold change of interesting genes in mRNA level relative to empty vector. The copy number of each gene / the copy number of b-actin = Y, the Y of empty vector was set as one. Blue bars present the FATP1 gene. Red bars present the FATP4 gene. The error bars show the standard deviation. This data is consistent with western blot result.
Figure 4. mRNA level of PPARα, PPARγ and their target genes in 3 cell lines. (n=3) This is the QPCR data showing the fold change of interesting genes in mRNA level relative to empty vector. The copy number of each gene / the copy number of b-actin = Y, the Y of empty vector was set as 1. Blue bars present the empty vector control cell line. Red bars present the FATP1 over expression cell line. Green bars present the FATP4 over expression cell line. The error bars show the standard deviation.
Figure 5. The illustration of C1-BODIPY-C12 assay. The chemical structure shows the molecular structure of C1-BODIPY-C12. Yellow zone presents the cell membrane. Blue parts are the FATP as transporter of lipids. Green dots are C1-BODIPY-C12 molecules. Trypan blue is used as the quencher of C1-BODIPY-C12 outside of the cells. And the C1-BODIPY-C12 gives out the signal to plate reader.
Figure 6. C1-BODIPY-C12 assay of three interested cell lines. (n=3) This figure shows the initial uptake rate of BODIPY by different cell lines at different concentrations of BODIPY in medium. The data are present in AFU/min/50,000 cells. Blue bars present the empty vector control cell line. Red bars present the FATP1 over expression cell line. Green bars present the FATP4 over expression cell line. The error bars show the standard deviation.
Figure 7. Kinetic analysis of initial uptake rate using software PRISM.

Analysis of the initial uptake rate of C1-BODIPY-C12 mimics the lipids uptake by FATP as the enzyme process substrates. Round dots presents the empty vector cell line. Triangle presents the FATP1 over expression cell line. Square presents the FATP4 over expression cell line. X-axis is the concentration of C1-BODIPY-C12. Y-axis is the AFU data per minute per 50,000 cells. For uptake rate, FATP1 OE > FATP4 OE > empty vector.
Figure 8. LC-MS of FA-CoAs extraction of 293 T-Rex Overexpression cell lines after Tet induction. (n=3) X-axis is the different CoA forms. Y-axis is the Mol percentage of different CoAs. Blue bars present the empty vector control cell line. Red bars present the FATP1 over expression cell line. Green bars present the FATP4 over expression cell line. The square is the amplification of some part of data. The error bars show the standard deviation.
Figure 9. GC-MS of FAMEs extraction of 293 T-Rex Overexpression cell lines after Tet induction. (n=3)

X-axis is the different fatty acids. Y-axis is the Mol percentage of different FAs. Blue bars present the empty vector control cell line. Red bars present the FATP1 over expression cell line. Green bars present the FATP4 over expression cell line. The error bars show the standard deviation. The error bars show the standard deviation.
Table 1. The QPCR data of all of interested genes (n=3) This is the overall QPCR data showing the fold change of interesting genes in mRNA level relative to empty vector. The copy number of each gene/the copy number of b-actin=Y. The Y of empty vector is set to one. ± shows the deviation of data. The target genes of PPARα are overexpressed only in FATP1 OE cell line, which gives a clue that the activation of PPARα may be elevated.

<table>
<thead>
<tr>
<th>Genes tested</th>
<th>Empty vector (Control set as 1.0)</th>
<th>FATP1 OE</th>
<th>FATP4 OE</th>
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<tbody>
<tr>
<td>FATP1</td>
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<td>2.2±0.3</td>
<td>0.95±0.1</td>
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<td>FATP4</td>
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### Table 2. The QPCR data of other tested genes (n=2, HPRT as the reference gene)

This is the overall QPCR data showing the fold change of interesting genes in mRNA level relative to empty vector. The copy number of each gene/the copy number of β-actin=Y. The Y of empty vector is set to one. ± shows the deviation of data.

<table>
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<tr>
<th>Genes tested</th>
<th>Cell Lines</th>
<th>Empty vector (Control set as 1.0)</th>
<th>FATP1 OE</th>
<th>FATP4 OE</th>
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<td>FATP6</td>
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References


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