UTEROPLACENTAL INSUFFICIENCY CAUSES SEX-DIVERGENT CHANGES IN THE PLACENTAL PPARγ-SETD8 AXIS IN THE RAT

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Fetal outcomes after uteroplacental insufficiency (UPI) include growth restriction and sex-divergent increase in neonatal and adult-onset disease. Fetal acquisition of long-chain polyunsaturated fatty acids (LCPUFA) is 1) linked to disease outcomes, 2) mediated by the placenta, and 3) impaired in pregnancies complicated by UPI. Peroxisome proliferator activated receptor gamma (PPARγ) regulates placental LCPUFA transport via direct control of gene expression and by initiating chromatin modifications through Setd8. We previously showed in our rat model that UPI results in fetal growth restriction and development of sex-divergent neonatal and adult-onset disease. We hypothesize that UPI in the rat results in sex-divergent changes in the placental PPARγ-Setd8 axis and sex-divergent changes in fetal serum LCPUFA profiles.

UPI was induced by bilateral uterine artery ligation at embryonic day 19 in pregnant Sprague Dawley rats. Male and female fetal rat serum and corresponding placenta were surgically collected at term (embryonic day 21). Serum LCPUFA profiles were measured using gas chromatography-mass spectrometry. mRNA was measured using real-time RT-PCR, protein was measured using western blotting, and PPARγ occupancy at the Setd8 promoter was measured using ChIP.

Data are expressed as mean ± standard deviation (SD), *$p<0.05$. UPI did not affect PPARγ mRNA levels and protein abundance in male placenta or female placenta compared to sex-matched control. UPI increased Setd8 mRNA levels, protein abundance,

and total PPARγ at the promoter of the Setd8 compared to sex-matched control in male placenta but not in female placenta. In male placenta, UPI increased global H4K20me compared to sex-matched control. No differences were observed in female placenta. In male fetuses, UPI increased the LCPUFA palmitic acid (PAL), linoleic acid (LA), and arachidonic acid (AA), while decreasing palmitoleic acid (PA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). In female fetuses, UPI increased AA with no change in other LCPUFA.

In conclusion, UPI in the rat results in sex-divergent changes in the PPARγ-Setd8 axis in association with sex-divergent changes in fetal serum LCPUFA profiles. Given the role of Setd8 in chromatin maintenance, sex-divergent changes in PPARγ-driven Setd8 expression may influence UPI-induced sex-divergent changes in the placental transcriptome and subsequent placental transfer of LCPUFA.

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INTRODUCTION

Developmental Origins of Health and Disease

The developmental origins of health and disease (DOHaD) were first described by Barker et al., with the recognition that perinatal events were important determinants of health later in life.[1] DOHaD begins *in utero*. Adverse prenatal events, including maternal diet, obesity, toxin exposure, and pathologic conditions of pregnancy, lead to sex-divergent organ-specific structural and functional changes that contribute to disease onset in the neonate and adult periods. Such insults can establish heritable patterns of epigenetic marks, such as chromatin modifications, that "program" states of gene expression in offspring.[2]

One pathological condition of pregnancy is uteroplacental insufficiency (UPI). In developed countries, a major cause of UPI is preeclampsia. UPI is characterized by impaired placental blood flow to the fetus, resulting in compromised transport of oxygen and nutrients to the fetus. Organ-specific structural and functional changes resulting from UPI may allow the fetus to survive *in utero* but may predispose the offspring to disease onset in the long run.[3,4] Disease outcomes involve many organ systems and include metabolic diseases such as type 2 diabetes, cardiovascular disease, obesity, and impaired neurodevelopment. An important caveat is that disease outcomes are sex-divergent with males often having worse outcomes than females.

The placenta is the key interface between the maternal environment and the

developing fetus and is appreciated to contribute to developmentally programmed sex-divergent disease outcomes.[5] The placenta is genetically aligned with the sex of the fetus and responds to stressors (like UPI) in a sex-divergent manner. Male and female placenta display unique UPI-induced changes in placental structure, efficiency, and molecular characteristics. Differences in structure, function, and adaptation between male and female placenta are now being appreciated.[3]

Long-Chain Polyunsaturated Fatty Acids

In pregnancies complicated by UPI, the placenta impairs transport of oxygen and nutrients to the fetus. This impairment is especially the case for long-chain polyunsaturated fatty acids (LCPUFA) which are selectively transferred to the fetus during late gestation. The placenta regulates selective transport of LCPUFA from maternal to fetal circulation and also storage of LCPUFA in the placenta for membrane construction and energy production.[6,7]

Fetal acquisition of LCPUFA is critical for normal development. Specifically, impaired fetal LCPUFA acquisition during development is associated with neonatal and adult disease.[8,9] Impaired fetal LCPUFA acquisition occurs in pregnancies complicated by UPI. Little is known about the mechanisms regulating LCPUFA metabolism in the placenta in normal pregnancies and pregnancies complicated by UPI. Furthermore, the current literature rarely considers the sex of the placenta as a variable even though programming of the placental transcriptome is sex-divergent.

PPARγ and Setd8

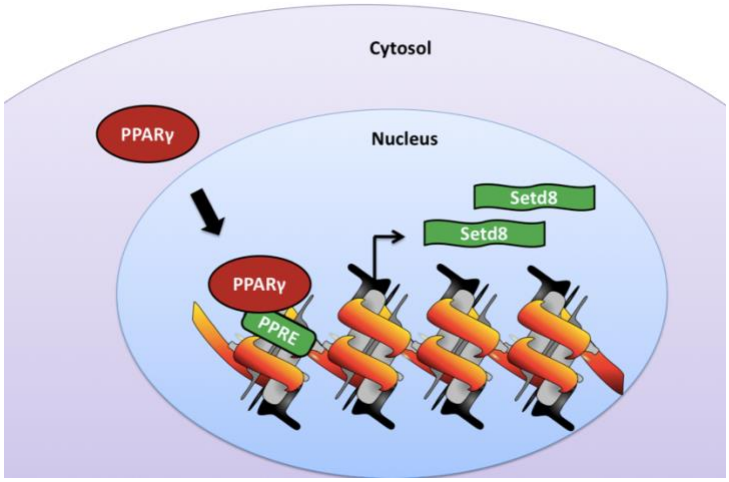
Peroxisome proliferator activated receptor gamma (PPARγ) is a transcription factor activated by LCPUFA and their metabolites. PPARγ is a key regulator of signaling pathways governing LCPUFA metabolism in the placenta. In many tissues, including placenta, PPARγ regulates LCPUFA metabolism by directly promoting the transcription of lipid metabolizing genes. In nonplacental tissue, PPARγ regulates LCPUFA metabolism by indirectly influencing the transcription of suites of fatty acid metabolizing genes via chromatin modifications. However, whether indirect regulation of LCPUFA metabolism occurs in the placenta via PPARγ and its downstream target gene Setd8 (Figure 1) is unknown. The aim of this study is to investigate the sex-divergent effects of UPI on the placental PPARγ-Setd8 axis and quantify LCPUFA profiles of offspring in the rat.

Hypothesis

DOHaD begins *in utero*. UPI results in structural and functional changes that leads to impaired fetal acquisition of LCPUFA and sex-divergent programming of metabolic disease. Placental LCPUFA metabolism is governed by PPARγ, and in non-placental tissue, PPARγ targets the epigenetic modifier Setd8.

We hypothesized that UPI induces sex-divergent changes in the PPARγ-Setd8 axis in rat placenta. We further hypothesized that this occurs in association with sex-divergent changes in serum LCPUFA in the rat fetus.

A.



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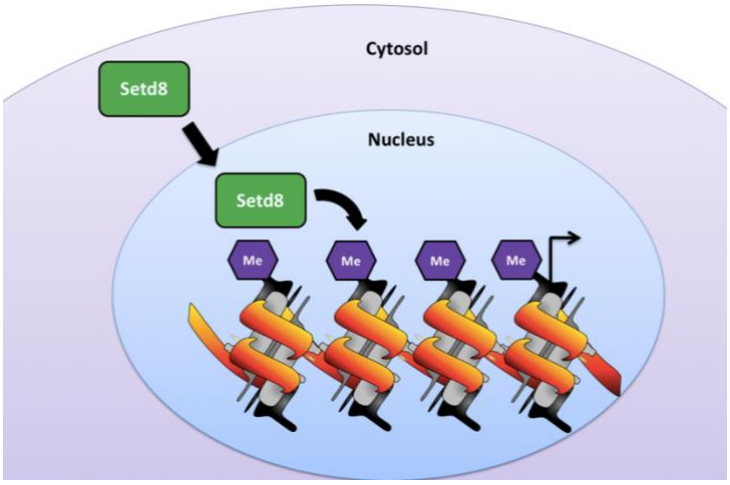

Figure 1. The PPARγ-Setd8 axis. A) Ligand activated PPARγ protein, a transcription factor, binds to a PPAR response element (PPRE) in the promoter of the Setd8 gene. The Setd8 gene is transcribed and translated to the Setd8 protein. B) The Setd8 protein, a histone lysine methyltransferase, enzymatically places a monomethyl group on Histone 4 Lysine 20 on suites of various LCPUFA metabolizing target genes. The presence of the epigenetic H4K20me mark has the effect of increasing gene transcription.

METHODS

Rat Model of UPI

UPI was induced in pregnant, Sprague-Dawley rat dams by bilateral uterine artery ligation on embryonic day 19 (E19) as previously described.[10,11] Control rat dams underwent comparable anesthesia but did not undergo surgery. At embryonic day 19 (E19), rat pups were delivered by C-section and separated into male or female groups. At embryonic day 21 (E21), rat offspring underwent euthanasia, and placenta and serum from male and female rat pups was collected and immediately flash frozen in liquid nitrogen. Samples were stored at -80°C. In this study, we examined 4 groups: male control, male UPI, female control, and female UPI. Each group consisted of n=8 rat pups derived from different litters. Our lab has previously demonstrated in our rat model that UPI results in offspring that are asymmetrically growth restricted, have sex-divergent programming of adult metabolic disease onset in various tissues (adipose, lung, liver, and brain), and have metabolic profiles comparable to those expected in a human UPI pregnancy.[10,11]

Real-Time RT-PCR

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to measure mRNA transcript levels of PPARγ and Setd8 in whole placenta homogenate of control and UPI rat pups. RNA was extracted from placenta tissue samples using an

RNeasy Mini Kit (Qiagen). cDNA was generated using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The following Assay-on-Demand primer/probe sets were used: PPARγ Rn01492274_m1; Setd8 Rn01477383_g1; GAPDH-Mm99999915_g1. GAPDH was used as an internal control. GAPDH primer and probe sequences were as follows: Forward: CAAGATGGTGAAGGTCGGTGT; Reverse: CAAGAGAAGGCAGCCCTGGT; Probe: GCGTCCGATACGGCCAAATCCG. Data and RT-PCR amplification were analyzed by the QuantiStudio 12K Flex Real Time PCR system at the University of Utah Core Facility.

Western Blot

Western blotting was used to measure protein abundance of PPARγ, Setd8, and global acid extracted H4K20me in whole placenta homogenate of control and UPI rat pups. Rat placenta tissue was crushed using a mortar and pestle with liquid nitrogen. Total protein was isolated from rat pup whole placenta homogenate using RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% Na-deoxy-cholate, 1% NP- 40 (Igepal), and 0.1% SDS) and protease inhibitor cocktail (Roche-Complete Mini). Pierce BCA protein assay kit (ThermoScientific) was used to quantify protein abundance. Protein samples were stored at -80°C until use. A total of 30 μg of protein homogenate was loaded on Bis-Tris Gels (Novex by Life Technologies) to analyze protein abundance. GAPDH was used as an internal control for all experiments. The following antibodies were used: PPARγ sc-271392 (Santa Cruz Biotechnology); Setd8 ab177488 (abcam); H4k20me ab9051 (abcam); H4 2592 (Cell Signaling Technology); GAPDH 14C10 (Cell Signaling Technology). Antibodies were detected with Western Lightning enhanced

chemiluminescence and quantified using an Image Station 2000R (Eastman Kodak).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was used to measure PPARγ occupancy at the promoter regions of the Setd8 output gene. Rat placenta tissue was crushed using a mortar and pestle with liquid nitrogen. Crosslinking and chromatin isolation was performed as previously described in rat pup whole placenta homogenate.[10,11] ChIP was conducted using the EpiQuik Chromatin Immunoprecipitation (ChIP) Kit (Epigentek Group Inc.) according to manufacturer's directions. We used antibody PPARγ ab41928 (abcam). The following primer and probe sequences were used: (Setd8 primer and probe sequences: Forward: AGCCCAGCCCTTCGAATC; Reverse: CAGCAGGGAGGTCAGAATGAC; Probe: AGAGTCAGTTTTCCC. Samples were transferred to the University of Utah Core Facility where qPCR was used to quantify PPARγ occupancy at the Setd8 promoter.

Serum Fatty Acids

Serum fatty acid profiles were quantified using gas chromatography-mass spectrometry (GC-MS) at the University of Utah Core Facility. Frozen serum was transferred to the University of Utah Core Facility to quantify relative amounts of fatty acids. Supelco's FAMES 37 component mixture was used as a reference standard.

Statistical Analysis

UPI rat pups were compared to sex-matched control pups. Male and female rats were considered separate groups. Data are expressed as mean ± standard deviation (SD) for a sample size of n=8 per group. Mann Whitney U test was used to detect statistically significant differences between UPI groups and sex-matched controls. Two-way ANOVA was used to determine significant interaction effects between UPI and sex. Statistical significance was defined as $p<0.05$ using GraphPad Prism 7 software (GraphPad Software).

RESULTS

PPARγ

First, we measured PPARγ mRNA transcript levels and protein abundance relative to GAPDH in rat placenta tissue. We detected no basal sex differences in PPARγ mRNA transcript levels between male control and female control. UPI did not alter levels of PPARγ mRNA transcript levels compared to sex-matched control in male or female placenta (Figure 2). Similarly, we detected no basal sex differences in PPARγ protein abundance between male control and female control. UPI did not alter levels of PPARγ protein abundance compared to sex-matched control in male or female placenta (Figure 3).

Setd8

In order to assess downstream PPARγ signaling, we measured Setd8 mRNA transcript levels and protein abundance relative to GAPDH rat placenta tissue. We detected no basal sex differences in Setd8 mRNA transcript levels between male control and female control. UPI significantly increased Setd8 mRNA transcript levels compared to sex-matched control in male placenta ($p<0.05$) but not female placenta (Figure 4). A significant interaction effect between UPI and sex was observed for Setd8 mRNA ($p<0.05$). We detected no basal sex differences in Setd8 protein abundance between male control and female control. Similarly, UPI increased Setd8 protein abundance compared

to sex-matched control in male ($p<0.05$) but not female placenta (Figure 5). Again, a significant interaction effect was observed between UPI and sex for Setd8 protein ($p<0.05$). We also measured PPARγ occupancy at the Setd8 promoter region expressed as % input. Compared to sex-matched control, UPI increased PPARγ occupancy at the Setd8 promoter in male placenta ($p<0.05$) but did not affect occupancy in female placenta (Figure 6).

H4K20me

To assess the activity of Setd8, we quantified global H4K20me relative to total H4 protein. Global H4K20me was significantly increased in control male placenta compared to control female placenta ($p<0.05$). Furthermore, UPI increased global H4K20me in male placenta compared to sex-matched control ($p<0.05$), but no differences were observed between female placenta and sex-matched control (Figure 7).

Serum Fatty Acids

Finally, in order to understand the effects of UPI on serum fatty acid profiles, we measured saturated fatty acids, monounsaturated fatty acids, omega-6 fatty acids, and omega-3 fatty acids in rat pup serum. Fatty acid profiles were compared to weight% of total fatty acids. We detected no basal sex differences in fatty acids between male control and female control. UPI increased palmitic acid (PAL), linoleic acid (LA), and arachidonic acid (AA) and decreased palmitoleic acid (PA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) compared to sex-match control ($p<0.05$). UPI increased AA only in female placenta compared to sex-matched control ($p<0.05$). We

observed a significant interaction effect between UPI and sex for PAL, PA, and EPA in female placenta only.

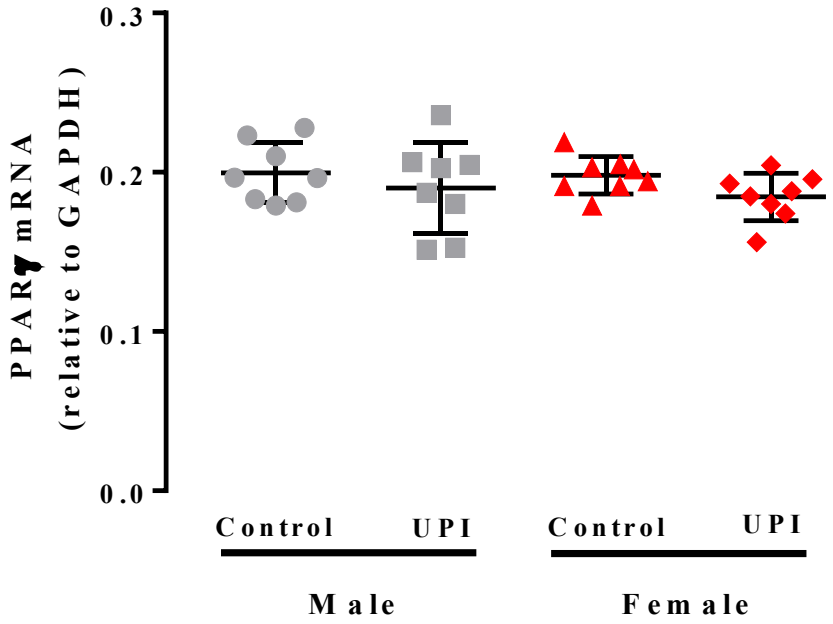

Figure 2. PPARγ mRNA transcript levels relative to GADPH (n=8/group). *p<0.05 compared to sex-matched control.

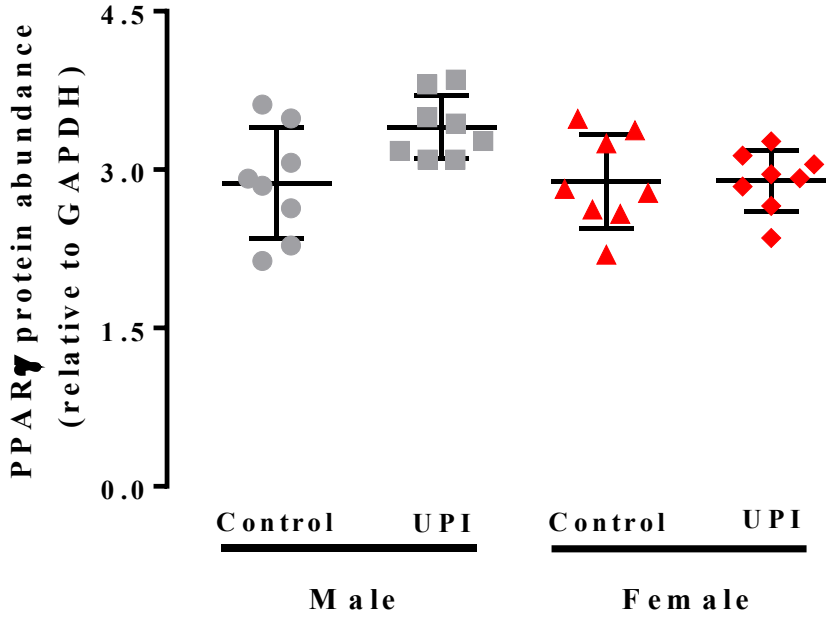

Figure 3. PPARγ protein abundance relative to GAPDH (n=8/group). *p<0.05 compared to sex-matched control.

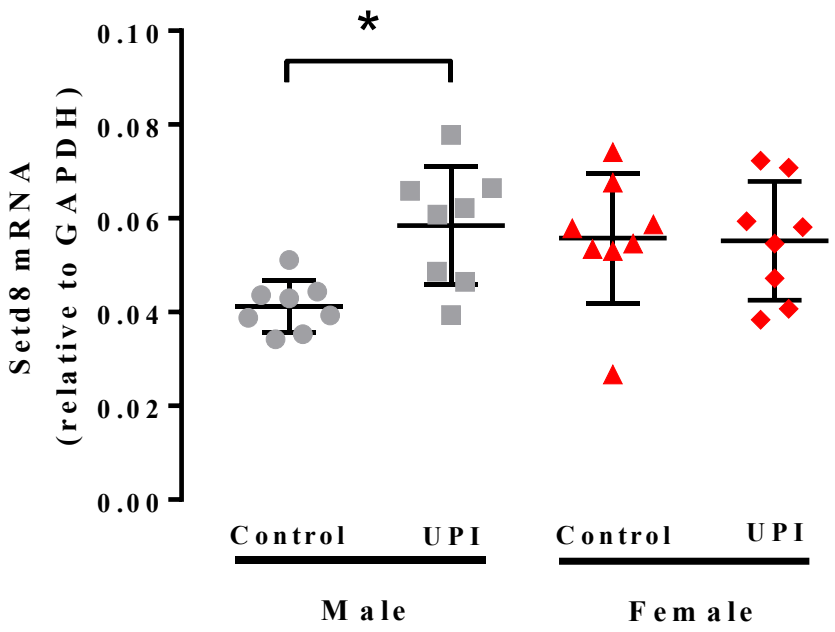

Figure 4. Setd8 mRNA transcript levels relative to GAPDH (n=8/group). *p<0.05 compared to sex-matched control, significant interaction effect UPI x sex.

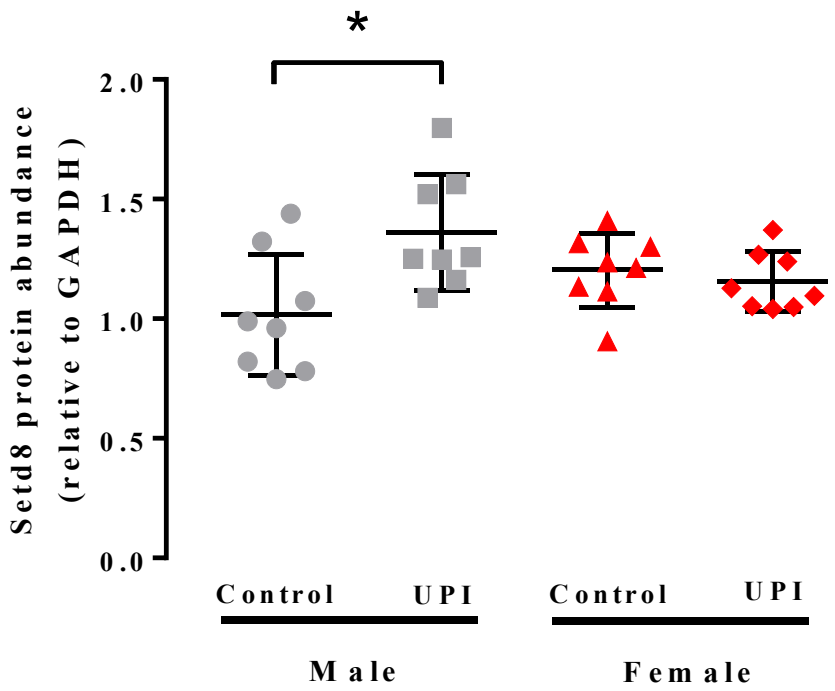

Figure 5. Setd8 protein abundance relative to GAPDH (n=8/group). *p<0.05 compared to sex-matched control, significant interaction effect UPI x sex.

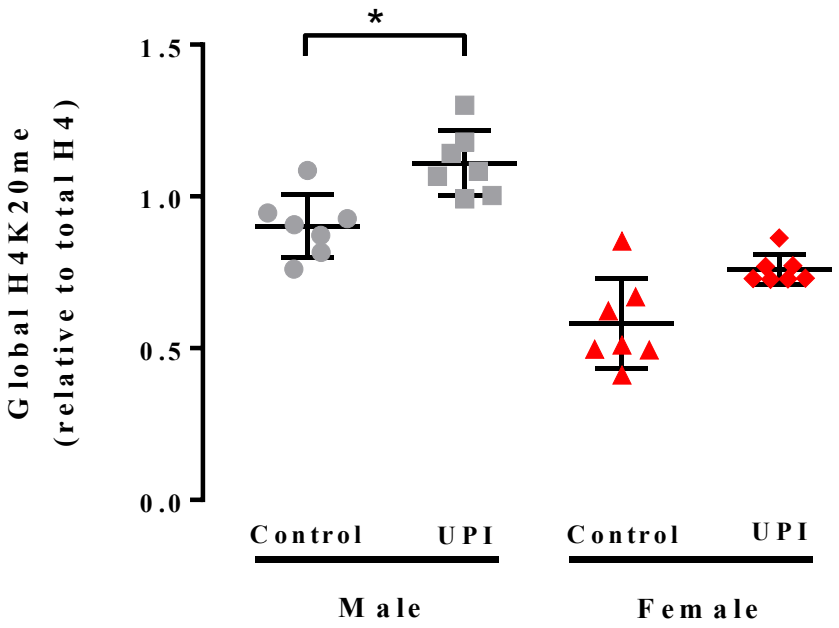

Figure 6. PPARγ occupancy at the Setd8 promoter expressed as % input (n=8/group). *p<0.05 compared to sex-matched control.

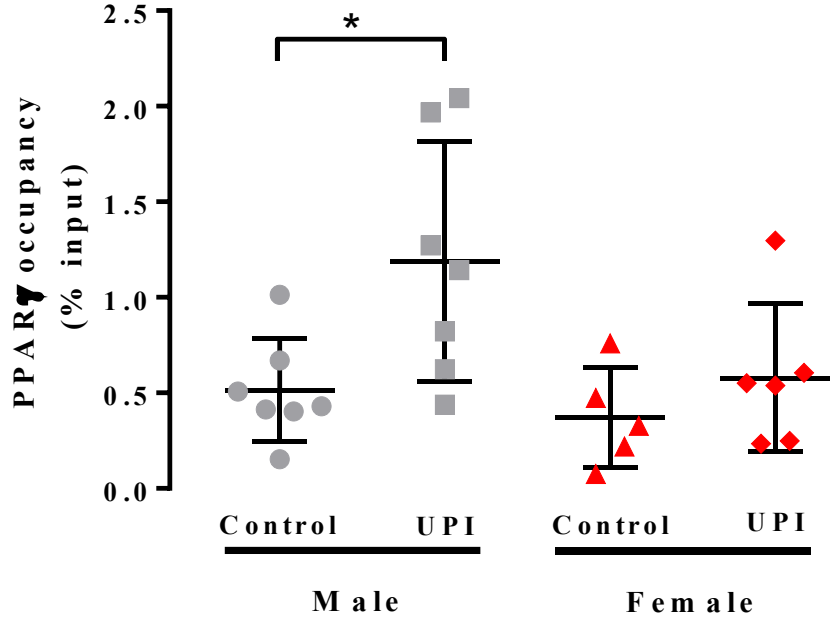

Figure 7. Global H4k20me relative to total H4 protein (n=8/group). *p<0.05 compared to sex-matched control.

Table 1. Serum Fatty Acids in Male and Female Term Rat Fetuses

	Male		Female	
Fatty Acid (wt:wt% of FA)	**Control**	**UPI**	**Control**	**UPI**
Palmitic (16:0)	21.6 ± 1.2	**23.7 ± 1.1***	21.4 ± 1.1	20.6 ± 1.7†
Stearic (18:0)	9.7 ± 0.5	10.5 ± 1.0	9.4 ± 0.7	10.0 ± 2.2
Palmitoleic (16:1)	4.6 ± 0.7	**2.3 ± 1.0***	4.8 ± 0.6	4.0 ± 07†
Oleic (18:1)	18.8 ± 1.8	19.7 ± 3.7	18.7 ± 2.6	18.3 ± 4.0
Linoleic (18:2, ω-6)	9.9 ± 0.5	**12.6 ± 1.7***	9.4 ± 1.3	10.6 ± 0.9
Arachidonic (20:4, ω-6)	7.9 ± 0.2	**11.8 ± 1.9***	7.0 ± 0.9	**9.0 ± 1.2***
A-Linolenic (18:3, ω-3)	2.2 ± 0.4	2.7 ± 0.4	2.2 ± 0.7	2.1 ± 0.2
Eicosapentaenoic (20:5, ω-3)	3.0 ± 0.4	**0.8 ± 0.4***	2.9 ± 1.2	2.6 ± 1.0†
Docosahexaenoic (22:6, ω-3)	3.9 ± 0.6	**2.9 ± 0.5***	3.4 ± 1.2	3.6 ± 0.7

*$p<0.05$ compared to sex-matched control. †$p<0.05$ significant interaction effect UPI x sex

DISCUSSION

The purpose of this study was to investigate the sex-divergent effects of UPI on the placental PPARγ-Setd8 axis and quantify serum LCPUFA profiles of offspring in the rat. We demonstrated that UPI causes sex-divergent changes in placental PPARγ-Setd8 signaling in association with altered serum LCPUFA profiles. These data emphasize the importance of sex of the placenta and how it may impact LCPUFA metabolism and molecular signaling in UPI pregnancies. Knowledge gained from this research adds to the understanding of mechanisms involved in placental LCPUFA metabolism. These data will inform studies aimed at determining the mechanisms by which the placenta contributes to the developmental programming of disease.

Our research is consistent with previous work conducted in adipose tissue. Wakabayashi et al. demonstrated the presence of the PPARγ-Setd8 axis in adipose tissue and that PPARγ upregulates the expression of Setd8.[12] To our knowledge, our study is the first to report the PPARγ-Setd8 axis in placenta tissue. PPARγ mRNA levels and protein abundance were not different between groups. However, Setd8 mRNA levels, Setd8 protein abundance, and PPARγ binding at the Setd8 promoter were increased in male placenta compared to sex-matched control, demonstrating that PPARγ upregulates the expression of Setd8. We also demonstrated a significant interaction effect between UPI and sex for Setd8 mRNA levels and protein abundance. This significant interaction effect suggests that changes in Setd8 expression are sex-divergent.

Studies clearly show that the placental transcriptome and DNA methylation profile is different between male and female placenta.[13-15] It has been suggested that female placenta develop slowly, investing more nutrients into the placenta itself which allows females to adapt to insults late in gestation. Conversely, male placentas may develop more quickly and earlier in gestation. Quick development allows more nutrients to be invested into the fetus itself but may result in males being unable to adapt to late gestation insults.[3] Our results showed that LCPUFA profiles of offspring are altered in a sex-divergent manner in UPI pregnancies. In males, UPI increased PAL, LA, AA and decreased PA, EPA, and DHA. In females, UPI increased AA. A significant interaction effect between UPI and sex was observed in female placenta for PAL, PA, and EPA. These data suggest that female placenta may be able to adapt to a late gestational insult to restore LCPUFA profiles to normal levels. We speculate that a late gestational insult may result in compensatory molecular signaling to increase transcription of specific target genes in male placenta.

Our study has strengths and limitations. The UPI rat model is a strength of the study because it is well-characterized. Furthermore, since UPI is induced late in gestation and rat pups are delivered two days after surgery, physiological maternal adaptations are excluded. Bilateral uterine artery ligation compromises blood, oxygen, and nutrient transfer from maternal circulation to the fetus. Thus, observed changes in placental PPARγ signaling and LCPUFA metabolism are likely due to feedback from the fetus. Another strength of the study is that we investigate molecular changes with phenotypic outcomes. Our study examines molecular measures of PPARγ and its downstream targets in addition to phenotypic serum LCPUFA in term rat fetuses.

A limitation to the study is that molecular measurements were done in whole placenta homogenate and, therefore, were from multiple cell types of the placenta. Ongoing studies are mastering techniques to isolate specific cell types in the placenta. While our study is investigating PPARγ, we recognize that we have not considered all molecules involved in placental LCPUFA metabolism. We also recognize compensatory PPARγ signaling may have occurred following UPI. Our study is descriptive, and we will not establish a cause and effect relationship between PPARγ signaling and LCPUFA metabolism. Ongoing studies in the lab are supplementing rat dams with diets containing DHA in the context of UPI to determine if serum lipid profiles can be restored in term rat fetuses. Lastly, our study did not examine specific target genes affected by increased H4K20me. Ongoing studies are investigating specific targets of Setd8 and the effect of increased H4K20me along the length of the genome. We speculate that the H4K20me increases transcription of fatty acid transport protein (FATP) and fatty acid binding protein (FABP) genes. Specifically, we speculate FATP2 may be a target of the PPARγ-Setd8 axis since PPARγ is a regulator of FATP2. Additionally, Lager et al. demonstrated that FATP2 is upregulated in placenta tissue in overweight/obese pregnancies and speculated that increased FATP2 expression could contribute to increased LCPUFA transfer to the fetus.[16]

Future studies should aim to examine the sex-divergent effects of UPI on the PPARγ-Setd8 axis in specific cell types and layers in the placenta. Human and animal studies have examined the mRNA and less often the protein levels of PPARγ in the placenta of normal and pathologic pregnancies. In preeclampsia, human and animal studies show altered placental PPARγ mRNA in association with disturbed placental lipid

metabolism and fetal outcomes.[17-20] Furthermore, many studies yield conflicting results in the effects of therapeutic maternal LCPUFA supplementation during pregnancy. Some studies suggest a positive effect while other studies are inconclusive or yield negative results.[21-29] We speculate a sex-based mechanism by which placental LCPUFA handling is altered in UPI pregnancies and, therefore, future studies should investigate how the placenta responds to sex-specific therapeutic supplementation of LCPUFA in the context of UPI pregnancies. Since the placental transcriptome and methylation patterns are different between male and female placenta, future studies should consider sex as a variable.

In conclusion, in male rat placenta, UPI alters the placental PPARγ-Setd8 axis in a sex-divergent manner. UPI increased Setd8 mRNA, protein levels of Setd8, PPARγ occupancy at the Setd8 promoter, and global H4K20me in male rat placenta. These changes occurred in association with sex-divergent changes in LCPUFA profiles in term gestation male rat pups. In female rats, UPI does not affect the placental PPARγ-Setd8 axis. We speculate our results represent a sex-based mechanism by which placental LCPUFA handling is altered in UPI pregnancies.

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