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IUGR DECREASES PPAR γ AND SETD8 EXPRESSION IN NEONATAL RAT LUNG AND THESE EFFECTS ARE AMELIORATED BY MATERNAL DHA SUPPLEMENTATION

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Abstract

Intrauterine growth restriction (IUGR) is associated with altered lung development in human and rat. The transcription factor PPAR γ , is thought to contribute to lung development. PPAR γ is activated by docosahexanoic acid (DHA). One contribution of PPAR γ to lung development may be its direct regulation of chromatin modifying enzymes, such as Setd8. In this study, we hypothesized that IUGR would result in a gender-specific reduction in PPAR γ , Setd8 and associated H4K20Me levels in the neonatal rat lung. Because DHA activates PPAR γ , we also hypothesized that maternal DHA supplementation would normalize PPAR γ , Setd8, and H4K20Me levels in the IUGR rat lung. We found that IUGR decreased PPAR γ levels, with an associated decrease in Setd8 levels in both male and female rat lungs. Levels of the Setd8-dependent histone modification, H4K20Me, were reduced on the PPAR γ gene in both males and females while whole lung H4K20Me was only reduced in male lung. Maternal DHA supplementation ameliorated these effects in offspring. We conclude that IUGR decreases lung PPAR γ , Setd8 and PPAR γ H4K20Me independent of gender, while decreasing whole lung H4K20Me in males only. These outcomes are offset by maternal DHA. We speculate that maintenance of the epigenetic milieu may be one role of PPAR γ in the lung and suggest a novel benefit of maternal DHA supplementation in IUGR.

Introduction

Intrauterine growth restriction (IUGR) increases the risk of postnatal morbidities, with male IUGR infants more severely affected (1–5). In the United States, 5–12% of premature babies born annually are IUGR (6). IUGR, particularly when combined with prematurity, results in pulmonary morbidities, including bronchopulmonary dysplasia (BPD) (7–9). BPD is characterized histologically by impaired lung development (10–12). In a rat model of IUGR, we have demonstrated thickening of lung mesenchyme at birth, consistent with abnormal lung development (13).

Impaired lung development in IUGR is due to disruptions in epithelial-mesenchymal interactions and several lines of evidence suggest that the nuclear receptor transcription

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Conflict of Interest Statement

None of the authors have any conflicts of interest.

factor, peroxisome proliferator activated receptor gamma (PPAR γ) is involved in these interactions. Evidence of PPAR γ 's involvement in epithelial-mesenchymal interactions in the lung is twofold. Firstly, a PPAR γ -targeted lung epithelial knockout mouse has altered postnatal lung development, and changes in the expression of mesenchymal genes, resulting in enlarged airspaces, increased lung volumes, and decreased tissue resistance (14). Secondly, PPAR γ is a critical component of the parathyroid hormone related protein (PTHrP)-driven epithelial-mesenchymal paracrine loop that also contributes to lung development (15).

A further role for PPAR γ in lung development may be the transcriptional regulation of epigenetic modifying enzymes. Epigenetic modifying enzymes affect developmental processes by altering gene expression patterns of target genes. This is accomplished by the dynamic placement of epigenetic marks (such as methylation and acetylation) on histones associated with a particular gene. The marks then dictate interactions of the gene with transcription machinery. A number of chromatin modifying enzymes have PPAR response elements (PPRE) in their promoters and are bona fide transcriptional targets of PPAR γ (16). One of these PPAR γ responsive genes is the set domain containing histone methyltransferase, Setd8, which puts a monomethyl (Me) group on lysine (K) 20 of Histone (H) 4. Regulation of the placement of H4K20Me on the PPAR γ gene may represent a feedback loop by which levels of PPAR γ are regulated (16).

The PPAR γ gene is well conserved between the human and rat gives rise to at least three mRNA variants, PPAR γ 1a, PPAR γ 1b and PPAR γ 2, and two protein isoforms, PPAR γ 1 and PPAR γ 2 (17–19) (detailed in Supplementary Data Figure 1). Expression of PPAR γ isoforms is tissue-specific. PPAR γ 1 is ubiquitously expressed, while PPAR γ 2 is thought to be expressed exclusively in adipose tissue (17, 20–21). Levels and activity of PPAR γ are increased by ligand activation. Ligand binding induces a conformational change affecting transcription of PPAR γ and PPAR γ target genes (reviewed in (22)) and the resulting effects are ligand specific. PPAR γ ligands include the long chain fatty acid (FA) docosahexaenoic acid (DHA) (23–24). The effects of DHA on PPAR γ expression and activity are well documented and include activation of ectopically expressed PPAR γ in myoblasts; in differentiating monocytes; in endometrial tissue, and in tumor cells (25–30). A maternal plasma FA profile low in DHA has also recently been shown to be associated with small for gestational age (SGA) and preterm birth, and maternal supplementation with DHA during gestation is associated with longer gestation duration (31–33).

The effects of IUGR on PPAR γ and Setd8 expression or associated levels of H4K20Me are unknown. The effects of maternal DHA supplementation on neonatal lung PPAR γ are also unknown. In this study, we hypothesized that IUGR would result in a gender-specific reduction in PPAR γ , Setd8 and associated H4K20Me levels in the neonatal rat lung. Because DHA activates PPAR γ , we also hypothesized that maternal DHA supplementation would normalize PPAR γ , Setd8, and H4K20Me levels in the IUGR rat lung. To test these hypotheses, we used a well-characterized model of uteroplacental insufficiency (UPI)-induced IUGR in the rat (34–38) and developed a model of maternal DHA supplementation in the context of the UPI-induced IUGR rat.

Materials and Methods

Animals

The rat uteroplacental insufficiency model of IUGR has been described in detail previously (39–41). All procedures were approved by the University of Utah Animal Care Committee and are in accordance with the American Physiological Society's guiding principles (42). Body weights of IUGR pups are approximately 25% smaller than the control pups (43). The

surgical procedures have been described previously (44–45). Briefly, on day 19 of gestation, pregnant Sprague-Dawley rats were anesthetized with intraperitoneal xylazine (8 mg/kg) and ketamine (40 mg/kg), and both uterine arteries ligated giving rise to IUGR pups. Control dams underwent identical anesthetic procedures. Day 0 (d0) pups were delivered by caesarian section at term, 2.5 days after bilateral uterine artery ligation. Rat pups were killed by decapitation and blood collected for serum separation. Lungs were immediately removed from the same animals, flash-frozen in liquid nitrogen, and stored at -80°C . For each experiment, each group (control, IUGR and DHA-IUGR) had 6 male pups and 6 female pups, unless otherwise noted. Pups within each group were derived from different litters.

Maternal DHA Supplementation

DHA was administered via a custom diet. The diet, based on Harlan Teklad 8640 standard rodent diet (TD.8640, Harlan-Teklad, WI), substitutes 1% of the soybean oil in the standard chow with 1% purified DHA (cis-docosahexaenoic acid, # U-84-A, Nu-Chek Prep, MN). The resulting diet (here called 1% DHA diet) contains the same macronutrient content as standard rodent chow (21.8% protein, 40.8% carbohydrate and 5.4% fat – with a resulting caloric density of 3 Kcal/g). The pregnant rats were pair-fed regular diet or 1% DHA diet from E13 until term. However, the amount of food consumed by the pregnant rats was independent of diet.

Serum DHA quantification

Serum levels of DHA were measured in control and IUGR pups using gas chromatography. Samples were directly transesterified to fatty acid methyl esters (FAMES) [42]. Rat serum (0.25 μl) was mixed with 0.25 ml of methanol:benzene 3:2 (v:v). This was followed by the addition of an equal amount of freshly prepared acetylchloride-methanol 5:100 (v:v) in glass tubes incubated at 100°C for 1 hour. FAME composition was determined by gas chromatography using an HP5890 GC equipped with an Omegawax 250 Capillary Column (Sigma-Aldrich, 30m x 0.25 mm x 0.25 μm film thickness). Gilson Unipoint software was used to measure the area under the curve (AUC) for each peak. Dividing each FAME AUC by the sum AUC of all peaks provided the wt:wt% for the fatty acids of interest.

Real-Time RT PCR

Real-time reverse transcriptase PCR was used to evaluate mRNA abundance of lung PPAR γ variants as well as PPAR γ responsive downstream gene, Setd8 as previously described, with GAPDH as an internal control (43). The following Assay-on-demand primer/probe sets were used: PPAR γ 1a- Rn01492275_m1, PPAR γ 1b - Rn01492273_m1, PPAR γ 2 - Rn00440940_m1, Setd8 - Rn01477383_g1 (Applied Biosystems). GAPDH primer and probe sequences; Forward: CAAGATGGTGAAGGTCGGTGT; Reverse: CAAGAGAAGGCAGCCCTGGT; Probe: GCGTCCGATACGGCCAAATCCG.

Protein Isolation, Immunoprecipitation and Mass Spectrometry

In order to confirm the specificity of the c-terminal PPAR γ antibody for both isoforms of PPAR γ , protein isolated from whole lungs was immunoprecipitated, electrophoresed, commassie stained and bands were then analyzed by mass spectrometry. Immunoprecipitation was performed with antibody to c-terminal PPAR γ (PPAR γ H-100, sc-7196, Santa Cruz Biotechnology). Protein samples were ZiptipTM purified prior to digestion with TPCK-modified trypsin (Promega) or chymotrypsin (Princeton). Mass spectrometry analysis was performed using a LTQ-FT hybrid mass spectrometer (ThermoElectron Corp). Peptide molecular masses were measured by FT-ICR. Proteins were identified from peptides using the Mascot search engine (in-house licensed, ver. 2.2.1,

Matrix Science, Inc.) and NCBI protein sequence files for PPAR γ isoforms (NCBI accession numbers, PPAR γ 1 AAD40118, PPAR γ 2 NP-037256).

Immunoblot

Immunoblotting was used to determine relative abundance of lung PPAR γ and Setd8 protein in IUGR and control rats as previously described (46–48) (antibodies, PPAR γ H-100, sc-7196, Santa Cruz Biotechnology and Setd8 (sc-54998, Santa Cruz Biotechnology). Whole lung H4K20Me immunoblots were performed on acid-extracted histones, prepared as previously described (48). Levels of H4K20Me were quantified relative to total H4 (Histone H4 monomethyl Lys20 pAb, 39175, Active Motif).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was used to investigate the levels of histone modifications along the PPAR γ gene. Positions along the PPAR γ gene that were examined are marked with an * on Supplementary Data Figure 1 and include 5' of P1, at P1, P2, Exon 4 and at the 3' end of the PPAR γ gene. Table 1 contains primers used for real-time PCR of ChIP DNA. ChIP was performed as as previously described (49–50) using anti- H4K20Me antibody (39175, Active Motif).

Statistics

Data are presented as mean $\pm$ SEM. Statistical differences between control, IUGR and DHA-IUGR groups were determined using analysis of variance (ANOVA) followed by Fisher's PLSD nonparametric test. We used Statistix 8 software package (Analytical Software, Tallahassee, FL). We accepted $p \leq 0.05$ for statistical significance.

Results

IUGR decreases serum DHA in male neonatal IUGR pups

IUGR significantly decreased serum DHA from 4 ± 0.2 wt:wt% to 3 ± 0.3 wt:wt% ($p=0.032$) in male pups, with no significant difference in female pups (Figure 1). The addition of DHA to the maternal diet increased serum DHA levels in male and female DHA-IUGR pups to 5.9 ± 0.6 wt:wt% and 6.7 ± 0.3 wt:wt%, respectively.

IUGR decreases PPAR γ variant mRNA levels at birth in neonatal rat lung; maternal DHA supplementation ameliorates this decrease

IUGR decreased PPAR γ 1a mRNA at birth in males ($p=0.007$) and females ($p=0.004$) (Figure 2A). In male rat lungs, maternal DHA supplementation significantly increased PPAR γ 2 mRNA levels in DHA-IUGR compared to IUGR ($p=0.05$). IUGR also decreased PPAR γ 1b mRNA at birth in males ($p=0.01$) and females ($p=0.003$) (Figure 2B). Similarly, IUGR decreased PPAR γ 2 mRNA at birth in males ($p=0.04$) and females ($p=0.02$) (Figure 2C). Maternal DHA supplementation significantly increased PPAR γ 2 mRNA levels in male ($p=0.04$) and female ($p=0.05$) DHA-IUGR compared to IUGR (Figure 2). No significant difference to control was observed for DHA-IUGR PPAR γ 1a, - γ 1b or γ 2 mRNA (Figure 2).

IUGR decreases PPAR γ 2 protein abundance at birth in neonatal rat lung; maternal DHA supplementation ameliorates this decrease

Mass spectrometry used to confirm immunoblot band identity. The two bands resulting from immunoprecipitation with a c-terminal PPAR γ antibody, each contained peptides for the common region of PPAR γ and one band contained peptides for the unique PPAR γ 2 N-terminus (data not shown).

In accordance with mRNA data, IUGR significantly reduced PPAR γ 2 protein at birth in males ($p=0.02$) and females ($p=0.03$) (Figure 3B). Maternal DHA supplementation ameliorated this effect with abundance of PPAR γ 2 protein in DHA-IUGR lung not significantly different from gender-matched controls. In both male and female rat lungs, maternal DHA supplementation significantly increased PPAR γ 2 protein abundance in DHA-IUGR compared to IUGR in males ($p=0.02$) and females ($p=0.03$). (Figure 3B). Abundance of PPAR γ 1 protein was unchanged from control in IUGR and DHA-IUGR lungs at birth (Figure 3A).

IUGR decreases Setd8 levels at birth in neonatal rat lung; maternal DHA supplementation ameliorates this decrease

Because Setd8 is a direct transcriptional target of PPAR γ , the effect of IUGR and maternal DHA supplementation on Setd8 mRNA expression and protein abundance in rat lungs was evaluated. IUGR decreased Setd8 mRNA expression in males ($p=0.01$) and females ($p=0.03$) (Figure 4A). Maternal DHA supplementation ameliorated this decrease, with levels of Setd8 mRNA expression in DHA-IUGR lung not significantly different from gender-matched controls. In male rat lungs, maternal DHA supplementation significantly increased Setd8 mRNA levels in DHA-IUGR compared to IUGR ($p=0.03$) (Figure 4A).

IUGR significantly reduced Setd8 protein abundance in males ($p=0.02$). In females, IUGR did not decrease Setd8 abundance ($p=0.07$). Maternal DHA supplementation ameliorated the decrease in male IUGR pups, with levels of Setd8 protein abundance in DHA-IUGR lung not significantly different from male controls. (Figure 4B).

IUGR decreases whole lung H4K20Me in male neonatal rats; maternal DHA supplementation ameliorates this decrease

In light of reduced Setd8 mRNA expression and protein abundance in the IUGR lung, we examined levels of whole lung H4K20Me relative to total H4 in control, IUGR, and DHA-IUGR at birth. IUGR significantly reduced levels of H4K20Me at birth in lung of male IUGR pups ($p=0.02$) (Figure 5). Maternal DHA supplementation ameliorated this decrease, with levels of whole lung H4K20Me in lung of male DHA-IUGR pups not significantly different from male controls. No significant changes were observed in whole lung H4K20Me in lung of female pups.

Maternal DHA ameliorates the IUGR decrease in H4K20Me at Exon 4 of the PPAR γ gene in neonatal rat lung

Because Setd8 mRNA is known to specifically target the PPAR γ gene, we examined levels of H4K20Me at 5 positions along the PPAR γ gene at birth, in control, IUGR and DHA-IUGR rat lung. The abundance of H4K20Me was determined at the 5' end, P1, P2, Exon 4, and the 5' end of the PPAR γ gene. Levels of H4K20Me were quantified relative to a non-transcribed intergenic region that has previously been shown to contain low levels of all histone modifications (51). Data are presented as IUGR and DHA-IUGR relative to gender-matched normalized controls $\pm$ SEM in order to demonstrate the effect of IUGR and DHA-IUGR on H4K20Me levels along the length of the PPAR γ gene. IUGR significantly reduced H4K20 levels at Exon 4 in both males ($p=0.02$) and females ($p=0.05$) (Figure 6). Maternal DHA supplementation ameliorated this decrease with levels of Exon 4 H4K20Me in DHA-IUGR lung not significantly different from control. In female rat lungs, maternal DHA supplementation significantly increased Exon 4 H4K20Me in DHA-IUGR compared to IUGR ($p=0.05$) (Figure 6).

Discussion

Novel findings of this study are fourfold. First, the neonatal rat lung expresses all PPAR γ mRNAs and protein isoforms. Second, in both male and female rats, IUGR decreases PPAR γ mRNA and protein, with an associated decrease in a PPAR γ downstream target: Setd8. Thirdly, this decrease in Setd8 is associated with a reduction in PPAR γ specific H4K20Me in both males and females and a reduction in whole lung H4K20Me in males only. Finally, maternal DHA supplementation ameliorates these changes. Together, these data suggest both a role for PPAR γ in IUGR induced epigenetic changes in the lung during development and a novel benefit of maternal DHA supplementation in IUGR.

While the importance of total PPAR γ in the lung has been established (14, 52–53), the role of individual isoforms is unknown. In this study, we demonstrated that PPAR γ 2 is expressed in the neonatal rat lung. To the best of our knowledge, this is the first report of PPAR γ 2 expression in the lung. The PPAR γ 2 isoform has previously been associated predominantly with adipose tissue, where it acts as key regulator of adipogenesis (54–55). Only PPAR γ 1 expression has previously been reported in the lung. A possible explanation for the lack of detection of PPAR γ 2 mRNA in rabbit primary alveolar type II (ATII) cells (56) is that the first 7 nucleotides of the PPAR γ 2-specific primer used is not contained within any PPAR γ 2 sequences currently listed on the NCBI database. The additional nucleotides may have hampered detection. In a second study, using embryonic rat whole lung tissue and ATII cells, the primer sequence used to differentiate PPAR γ 1 from PPAR γ 2 was not reported (20). Western Blot analysis of cultured ATII cells was reported but a second band in the gels is not explained. In our study, we showed, by mass spectrometry that the whole rat lung expresses both PPAR γ 1 and PPAR γ 2.

PPAR γ has roles in acute and chronic inflammatory responses in the lung (57–59), pulmonary fibrosis (57, 60), lung cancer (61) and pulmonary vascular disease (62); however, specific roles of PPAR γ in lung development have yet to be determined. The discovery that PPAR γ regulates the expression of chromatin modifying enzymes (16) suggests that PPAR γ 's role in lung development may be via the regulation of epigenetic events. Epigenetics provides a way to selectively express information contained within the genome. This is particularly important in the context of developmentally specific gene expression. Methylation of histones by methyltransferase enzymes contributes to this regulation. The histone lysine methyltransferase, Setd8, places the H4K20Me mark. This mark is enriched in the promoter or coding regions of active genes and strongly correlated with gene activation when it appears in regions downstream of the transcriptional start site (63–64). Considering the potential importance of PPAR γ and Setd8 in lung development, our findings of IUGR-induced reductions in PPAR γ and Setd8 in the neonatal rat lung are consistent with the impaired lung development observed in IUGR rats. Our observation of decreased PPAR γ 2 and Setd8 in IUGR during the saccular stage of lung development, when the lung is primed to begin alveolar formation, is intriguing.

Whole lung H4K20Me and H4K20Me at Exon 4 of the PPAR γ gene at the outset of alveolar formation may be an important determinant of appropriate expression of PPAR γ and other genes governing alveolar formation. We have demonstrated that IUGR reduces levels of H4K20Me at PPAR γ Exon4 in both males and females. This is likely an important determinant of the reduced PPAR γ 2 levels observed in our studies. Several lines of evidence suggest PPAR γ 2 levels are maintained by a feedback loop. Firstly, previous studies have demonstrated that reduced H4K20Me of the PPAR γ gene is associated with reduced PPAR γ transcription. Secondly, PPAR γ regulates transcription of the H4K20Me placement enzyme Setd8. Finally, the PPAR γ 2 promoter binds PPAR γ promoting the transcription of the PPAR γ 2 variant (16).

Our observation of a reduction in whole lung H4K20Me only in males, despite reduced Setd8 protein in both genders, suggests a gender-specific response in global Setd8 targets. This is important in the context of IUGR because gender-specific molecular changes are often observed (65–70). The mechanisms driving the gender-specific molecular responses remain unknown, and formulate an important and unanswered question in the field. Gender-specific responses to PPAR γ polymorphisms have been demonstrated in human obesity (68, 71). These studies suggest that females may utilize other pathways to regulate PPAR γ expression and that of down-stream targets, potentially those related to sex-steroids. Gender-specific vulnerability of Setd8 target genes may provide insight into mechanisms of gender-specific molecular responses and will warrant further investigation.

Maternal transfer of DHA to the fetus occurs primarily at the end of gestation, a process interrupted in the preterm infant, exacerbated by placental dysfunction (72–73). Our finding of reduced serum DHA in IUGR male rat pups is consistent with this observation. Again, it is interesting that, in our model, female rat pups do not appear to be as affected. We have also demonstrated that DHA administered to the rat dam via diet is transferred to the pups *in utero*. Activation of PPAR γ by long chain fatty acids, particularly DHA, has received a great deal of attention over past years. This is particularly true in the context of maternal supplementation of pregnant women and has been the subject of extensive biochemical and clinical investigations (74–76). A number of these studies have addressed the role of DHA in brain development, visual acuity and child behavior (reviewed in (77)). Direct binding affinities for DHA and PPAR γ remain to be determined, but molecular modeling studies suggest that the affinity of PPAR γ for DHA is high and exceeds that of other long chain FA (78–79). In light of the improved levels of PPAR γ , setd8 and H4K20Me observed in DHA-IUGR rat pups, maternal DHA supplementation may improve aspects of neonatal lung development in IUGR and warrants further investigation.

While we have shown that PPAR γ isoforms can be detected in whole lung, we have not described the location of the mRNA variants or protein isoforms. Future studies will need to address the location of PPAR γ 1a, - γ 1b and - γ 2 mRNA expression and subsequent protein accumulation. This will be important in delineating isoform specific epithelial-mesenchymal signaling. A further limitation of this study is the lack of morphometric data describing the structure of the lung of DHA-IUGR rats in the context of our pharmacological dose of DHA. In future experiments, it will be important to include morphometric and functional studies to delineate clinically significant DHA doses that may improve the IUGR lung phenotype.

In conclusion, we have described the presence of all PPAR γ mRNA variants and protein isoforms in the neonatal rat lung. We have also demonstrated that in male and female IUGR lungs, levels of PPAR γ 2 and its target, histone modifying enzyme setd8, are significantly reduced in association with decreased H4K20Me of the PPAR γ gene. We have also demonstrated that maternal DHA supplementation ameliorates these effects. We speculate that in the rat, IUGR impairs lung development via alterations in the PPAR γ regulated epigenetic milieu. We further speculate that the dietary activation of PPAR γ via maternal DHA supplementation may favorably effect this epigenetic environment, with potential improvement of the pulmonary phenotype seen in IUGR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Maternal DHA supplementation increases serum DHA in deficient male neonatal IUGR pups. IUGR decreased serum DHA in male neonatal rat pups compared to control, with no significant difference seen in females. The addition of DHA to the maternal diet increased serum DHA levels in male and female DHA-IUGR relative to control. n=6, * $p \leq 0.05$, ** $p \leq 0.01$.

Figure 2. IUGR decreases PPARγ variant mRNA levels at birth in neonatal rat lung and maternal DHA supplementation ameliorates this decrease. A) PPARγ1a mRNA, B) PPARγ1b mRNA and C) PPARγ2 mRNA. Asterisks denote significant differences n=6, *p≤0.05, **p≤0.01.

Figure 3. IUGR decreases PPAR γ 2 protein levels at birth in neonatal rat lung and maternal DHA supplementation ameliorates this decrease. A) PPAR γ 1 protein, B) PPAR γ 2 protein. Asterisks denote significant differences. n=6, *p<0.05.

Figure 4. IUGR decreased mRNA (A) and protein levels (B) of the PPAR γ target, Setd8, at birth in male and female rat lungs. Maternal DHA supplementation ameliorates the IUGR decreased in Setd8 mRNA and protein in rat lungs at birth. Asterisks denote significant differences. n=6, *p $\leq$ 0.05, #p=0.07.

Figure 5.

IUGR decreases whole lung H4K20Me, relative to total H4, in male rats at birth, with no change in females. Maternal DHA ameliorates this decrease in whole lung H4K20Me in male neonatal rats. Asterisks denote significant differences. n=6, *p≤0.05, **p≤0.01, #p=0.08.

Figure 6. IUGR decreases levels of H4K20Me at Exon 4 of the PPAR γ gene in male and female neonatal rat lung and maternal DHA ameliorates this decrease. Levels of H4K20Me, relative to a non-transcribing intergenic region, were quantified along the PPAR γ gene in male and female lungs. Asterisks denote significant differences. n=5, *p≤0.05, **p≤0.01.

Table 1

Primer/probe sets for ChIP analysis

Transcript position relative to PPAR γ gene	Sequence
5' of P1	For: 5' TCGACGGCTTCTGAATGTG
	Rev: 5' CTTGCCCTCTTTCAGCTCTTTC
	Probe: 5' ATCTTTAGGACAGATCATG
P1	For: 5' AAAAACAACCTTCTGCGTGACAGT
	Rev: 5' GGTCCCACGTTCTCAGACA
	Probe: 5' AGGGCACCAGCCGG
P2	For: 5' CCAAGTCTTGCCAAAGAAGCA
	Rev: 5' GATTGAGAGCCAGCTGTGACAA
	Probe: 5' ACAGCATTATGACACACCAT
Exon 4	For: 5' CCATCAGGTTTGGGCGAAT
	Rev: 5' GATCTCCGCCAACAGCTTCT
	Probe: 5' CCACAGGCCGAGAAG
3' End	For: 5' CGCCAAGGTGCTCCAGAA
	Rev: 5' CTGCACGTGCTCTGTGACAA
	Probe: 5' ATGACAGACCTCAGGCAG