Targeted Multiplex Gene Expression Profiling to Measure High-Fat Diet and Metformin Effects on Fetal Gene Expression in a Mouse Model

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Abstract

Background: Maternal obesity and excessive gestational weight gain (GWG) are associated with delivery of a large-for-gestational-age infant. We used a high-fat diet (HFD) mouse model to separate the effect of maternal obesity from excessive GWG on fetal growth. Our objective was to identify fetal gene expression changes in an HFD and control diet (CD) mouse model with and without metformin exposure. **Study Design:** Normal weight timed-pregnant (Female Friend virus B) strain mice were allocated on day e0.5 to receive HFD or CD and either plain water or metformin (2.5 mg/mL in drinking water). Dams were euthanized on day e17.5 and fetal livers harvested and frozen at -80°C . RNA was extracted and hybridized to a customized 96-gene Nanostring panel focused on angiogenesis, inflammation, and growth gene expression. Fetal liver gene expression was compared between metformin and plain water groups using analysis of variance. Significant differences in gene expression, defined by a false discovery controlled q value <0.01 , were then analyzed using Ingenuity pathway analysis (IPA). **Results:** In HFD-fed dams, compared to controls, the metformin-treated group had significantly lower fetal weight and 39 differentially expressed liver genes; 15 (38%) were in the growth/angiogenesis gene expression network. IPA predicted that fetal liver gene upregulation associated with metformin exposure is a result of metformin inhibition of the common upstream regulator, phosphatase and tensin homolog (*PTEN*). **Conclusions:** Metformin-exposed fetuses from dams fed HFD and CD have significant gene expression differences in genes specific to growth and angiogenesis pathways in the fetal liver. Diet alone did not alter fetal liver gene expression.

Keywords

angiogenesis, fetal gene expression, growth, high-fat diet, metformin, *PTEN*

Introduction

Maternal obesity and excessive gestational weight gain (GWG) are known risk factors for infant macrosomia, birth trauma, and adverse metabolic consequences. Obese pregnant women are more likely to experience excessive GWG,¹ making it difficult to determine the relative importance of baseline maternal weight versus GWG on infant birth weight. A recent randomized clinical trial of nondiabetic, obese pregnant women found that compared to placebo, antepartum metformin treatment reduced maternal GWG but not infant birth weight.² The maternal and fetal effects of antepartum metformin exposure in nondiabetic, nonobese pregnant women are unknown. This gap in knowledge is potentially important because metformin acts in the liver to decrease lipogenic enzymes,³ which could affect the metabolic phenotype in exposed offspring. Because metformin crosses the placenta and is commonly prescribed during

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Table 1. Dam, Fetus, and Placental Weight Characteristics by Treatment Group.

	Group 1 (CD + CT), n = 5 Dams, n = 45 Fetuses/Placentas	Group 2 (CD + MT), n = 5 Dams, n = 49 Fetuses/Placentas	Group 3 (HFD + CT), n = 5 Dams, n = 45 Fetuses/Placentas	Group 4 (HFD + MT), n = 5 Dams, n = 45 Fetuses/Placentas
Dam starting weight, g, mean (SEM)	23.2 (0.34)	23.0 (0.39)	22.2 (0.58)	24.4 (0.93)
Fetus weight, g, mean (SEM)	0.84 (0.01)	0.85 (0.01)	0.97 (0.01)	0.90 (0.02)
Litter size, n (median-IQR)	11 (11-11)	9 (9-9)	10 (9-10)	10 (9-11)
Mean placenta weight, g, mean (SEM)	0.088 (0.01)	0.072 (0.003)	0.10 (0.004)	0.092 (0.005)

Abbreviations: CD, control diet; CT, control; HFD, high-fat diet; IQR, interquartile range; MT, metformin; SEM, standard error of the mean.

pregnancy,⁴ it is critical to study the *fetal* effects of metformin exposure to further understand the metabolic effects diet and metformin play with regard to fetal programming.

Our objective was to measure dam GWG and fetal weight and identify fetal gene expression changes in a high-fat diet (HFD) and control diet (CD) mouse model with and without metformin exposure. We hypothesized that in utero metformin exposure alters fetal liver gene expression to support normal rather than excessive fetal growth. To test this hypothesis, we measured fetal liver gene expression in control and HFD dams with and without metformin exposure.

Materials and Methods

The Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina approved this protocol (15-163.0), and all experimental animals were maintained in accordance with the IACUC and the National Institutes of Health guidelines for the Care and Use of Laboratory Animals.

Female Friend virus B (FVB)-strain mice (n = 20) were weighed and then bred with control FVB males. On embryonic day (e) 0.5 (defined upon the detection of a vaginal plug), half of the pregnant dams were fed a CD (10% calories from fat) and half an HFD (60% calories from fat). Each group was further randomized to control treatment (water) versus metformin in water (2.5 mg/mL).^{5,6} All groups were fed ad libitum within their diet and treatment groups until euthanasia. On e17.5, dams were anesthetized with ketamine and euthanized by cervical dislocation. Fetuses were immediately removed from the uterine horns and weighed. Livers were then removed and frozen at -80°C. Dam weight gain was defined as the net gain in weight (g) from the day of mating to the day of sacrifice and reported as mean (standard error of the mean [SEM]) by group. Fetal weight was reported as mean (SEM) by group.

For this analysis, we selected 12 frozen fetal livers from each group (total sample size of 48). The livers were randomly sampled from the 45 to 49 fetuses from each group. The sample size of 12 livers across all groups and 96 genes per liver is adequately powered (>0.80) to show statistically significant differential gene expression (*q* < 0.01) between control and treated animals. Statistical tests developed for Nanostring data will identify genes that are differentially expressed relative to a fold-change threshold. We based our sample size on previously published work, demonstrating that a sample size of 12 across

groups allows for detection of a 1.5-fold difference in gene expression.⁷

RNA was extracted using the AllPrep DNA/RNA/miRNA universal kit (Qiagen, Germantown, Maryland), according to manufacturer’s specifications. RNA quantity was determined by Nanodrop (Thermo Scientific, Wilmington, Delaware), and quality was determined using the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, California). The RNA was hybridized to a customized 96-gene Nanostring (NanoString Technologies, Inc, Seattle, Washington) panel focused on 80 genes specific to angiogenesis, inflammation, and growth and 16 housekeeping genes (Supplemental table 1). Genes were chosen based on literature review of their relevance to glucose/energy/metformin pathway and based on use of string-db.org, a publicly available website that predicts functional interactions of genes. The Nanostring analysis was performed in the core preclinical genomic pathology laboratory at the University of North Carolina at Chapel Hill Lineberger Comprehensive Cancer Center. Briefly, the nCounter Analysis System probe library contains 2 sequence-specific probes, the capture probe, and the reporter probe for each gene of interest. Probe pairs are mixed with total RNA in one hybridization reaction and then imaged with the use of fluorescent microscopy.^{8,9} Expression is measured by counting the number of unique color tags within the gene-probe tripartite structures and is reported as “counts.” This platform permits quantifying the expression of up to 800 targets, without the need to use enzymes (reverse transcriptase or Taq for polymerase chain reaction), which could introduce bias. Raw expression data were extracted using the nSolver software (Nanostring Technologies) and normalized both to positive controls and to housekeeping genes.

We created analysis of variance (ANOVA) models to examine the fetal gene expression differences in liver of 80 genes based on diet and metformin treatment. Gene expression data were normalized using both housekeeping genes and positive controls. Four ANOVA models were tested: Model 1 examined fetal liver gene expression differences between metformin and placebo among fetuses in the CD group. Model 2 examined fetal liver gene expression differences between metformin and placebo among fetuses in the HFD group. Model 3 examined fetal liver gene expression differences in an HFD compared to CD groups receiving placebo treatment. Finally, model 4 examined fetal liver gene expression between the CD group on placebo compared to HFD group on metformin. Statistical

significance was defined as (1) a false discovery (type II error) controlled q value ≤ 0.01 and (2) a fold-change ≥ 1.5 or ≤ -1.5 . The ANOVA models were run using Partek Genomics Suite software (version 6.6; Partek, Inc, St Louis, Missouri).

Genes identified with statistically significant differential expression between metformin and placebo in both diet groups were analyzed for enrichment of biological functions, canonical pathways, upstream regulatory molecules, and interacting molecular networks, where P values were determined using Fisher exact test, a measure of the probability that the association between the statistically significant gene identified within the data set is due to random chance. All functional analyses of genes were run using Ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, California). IPA utilizes a manually curated database containing biological interactions and functional annotations to identify differentially represented biological functions and/or diseases in a data set. IPA utilizes a right-tailed Fisher exact test to generate a significance score for each association between genes in the experimental data set and a biological function.¹⁰

The upstream regulator analysis feature of IPA was utilized to predict the activation or inhibition of transcriptional regulators based on the direction of gene expression changes in our data set which can provide information about the biological activities occurring in the tissue (liver) being studied.¹¹ We defined upstream regulators as significantly activated or inhibited if the bias-corrected Z-score was ≥ 2.0 or ≤ -2.0 , respectively, in accordance with recommended threshold.¹¹

Results

Descriptive Data for Dams and Fetuses

Descriptive data for the dams and fetuses by diet group and metformin exposure are reported in Table 1. As shown in Figure 1, compared to CD-fed dams, HFD-fed dams gained significantly more weight during pregnancy (21.9 g [1.55] vs 16.2 g [1.66], $P = .04$). Among HFD-fed dams, there were no significant differences in dam weight gain between control- and metformin-treated dams (21.9 g [1.55] vs 20.8 g [1.22], $P = .59$).

The 20 dams had a total of 192 fetuses and placentas. Compared to CD-fed dams (group 1), HFD-fed dams (group 3) had significantly heavier fetuses (0.97 g [0.009] vs 0.84 g [0.01], $P < .001$; Figure 1). In HFD-fed dams, compared to control-treated dams (group 3), the metformin-treated dams (group 4) had significantly lower fetal weight (0.97 g [0.009] vs 0.90 g [0.02], $P = .0003$; Figure 2).

Fetal Gene Expression Data

The housekeeping genes had stable expression across all the experimental groups. In ANOVA model 1, dams fed CDs $\pm$ metformin exposure showed differential fetal liver gene expression with decreased expression of 6 genes-specific growth and angiogenesis (FKBP1A, FOS, IRS1, KDM5D,

Figure 1. Mean dam weight gain by treatment group. * $P < .05$.

Figure 2. Mean fetal weight by treatment group. ** $P < .001$. SEM and P values were calculated using a generalized estimating equation model to control for clustering by dam. SEM indicates standard error of the mean.

NFKB1, NRP2; Table 2). In ANOVA model 2, dams fed HFDs $\pm$ metformin showed significantly increased fetal liver gene expression in 39 genes; 15 (38%) of 39 genes were in the growth/angiogenesis gene expression network and were significantly increased (range 3.5- to 9.4-fold) in metformin-exposed compared to plain water-exposed fetuses (Table 3). Of note, 5 (80%) of the 6 genes found to have decreased expression in the fetal liver of CD-fed mice with in utero metformin exposure showed increased expression in the fetal liver of HFD-fed mice with in utero exposure to metformin. Model 3 examined fetal liver gene expression differences in an HFD compared to CD. Using our targeted panel, no significant differences in gene expression were found between the groups based on diet alone. Model 4 examined fetal liver gene expression differences in a CD on placebo compared to HFD on metformin; no significant differences in gene expression were found between these groups.

Table 2. Genes (n = 6) With Decreased Expression in Fetal Liver of CD-Fed Mice After in Utero Metformin Exposure.

Gene Name	Full Gene Name	P Value	q Value	Fold-Change
<i>Fos</i>	Fos proto-oncogene, AP-1 transcription factor subunit	.00	≤0.01	−5.60
<i>Irs1</i>	Insulin receptor substrate 1	.01	≤0.01	−8.17
<i>Nfkb1</i>	Nuclear factor κB subunit 1	.04	≤0.01	−6.90
<i>Nrp2</i>	Neuropilin 2 (Nrp2)	.04	≤0.01	−7.81
<i>Fkbp1a</i>	FK506-binding protein 1a	.05	≤0.01	−5.82
<i>Kdm5d</i>	Lysine demethylase 5D	.01	≤0.01	−11.31

Abbreviation: CD, control diet.

Table 3. Genes (n = 39) With Increased Expression in Fetal Liver of HFD-Fed Mice After in Utero Metformin Exposure.

Gene Name	Full Gene Name	P Value	q Value	Fold-Change
<i>Fos</i>	Fos proto-oncogene, AP-1 transcription factor subunit	.019	≤0.01	2.68
<i>Irs1</i>	Insulin receptor substrate 1	.05	≤0.01	3.15
<i>Jun</i>	Jun proto-oncogene, AP-1 transcription factor subunit	.034	≤0.01	5.77
<i>Igf1bp5</i>	Insulin-like growth factor-binding protein 5	.003	≤0.01	5.64
<i>Kit</i>	KIT proto-oncogene receptor tyrosine kinase	.034	≤0.01	3.72
<i>Flt4</i>	Fms-related tyrosine kinase 4	.03	≤0.01	3.53
<i>Epas1</i>	Endothelial PAS domain protein 1	.034	≤0.01	4.73
<i>Ghrl</i>	Ghrelin and obestatin prepropeptide	.009	≤0.01	3.04
<i>Nfkb1</i>	Nuclear factor κB subunit 1	.03	≤0.01	3.92
<i>Rptor</i>	Regulatory-associated protein of mTOR complex 1	.01	≤0.01	9.39
<i>Stat3</i>	Signal transducer and activator of transcription 3	.049	≤0.01	3.75
<i>Nrp2</i>	Neuropilin 2 (Nrp2)	.032	≤0.01	4.62
<i>Hif1a</i>	Hypoxia inducible factor 1, α subunit	.049	≤0.01	4.04
<i>Fkbp1a</i>	FK506-binding protein 1a	.038	≤0.01	3.52
<i>Igfals</i>	Insulin-like growth factor-binding protein, acid labile subunit	.04	≤0.01	4.17
<i>Vegfb</i>	Vascular endothelial growth factor B	.033	≤0.01	4.05
<i>Nrp1</i>	Neuropilin 1	.027	≤0.01	5.84
<i>Tgfb1</i>	Transforming growth factor β1	.037	≤0.01	4.04
<i>Hnf4a</i>	Hepatic nuclear factor 4α	.043	≤0.01	4.69
<i>Jak2</i>	Janus kinase 2	.05	≤0.01	3.65
<i>Igf2</i>	Insulin-like growth factor 2	.017	≤0.01	11.22
<i>Rps6kb1</i>	Ribosomal protein S6 kinase, polypeptide 1	.015	≤0.01	7.23
<i>Rictor</i>	RPTOR-independent companion of mTOR, complex 2	.047	≤0.01	4.23
<i>Akt1</i>	Thymoma viral proto-oncogene 1	.036	≤0.01	4.8
<i>Mapkap1</i>	Mitogen-activated protein kinase-associated protein 1	.044	≤0.01	3.58
<i>Igf1r</i>	Insulin-like growth factor 1 receptor	.049	≤0.01	3.03
<i>Ep300</i>	E1A-binding protein p300	.044	≤0.01	3.79
<i>Ripk1</i>	Receptor (TNFRSF)-interacting serine-threonine kinase 1	.027	≤0.01	5.73
<i>Grb10</i>	Growth factor receptor-bound protein 10	.048	≤0.01	4.45
<i>Tnfrsf1a</i>	Tumor necrosis factor receptor superfamily, member 1a	.046	≤0.01	3.17
<i>Shbg</i>	Sex hormone-binding globulin	.039	≤0.01	5.08
<i>Ppargc1a</i>	Pparg coactivator 1α	.032	≤0.01	4.57
<i>Trp53</i>	Transformation-related protein 53	.049	≤0.01	3.84
<i>Vegfc</i>	Vascular endothelial growth factor C	.033	≤0.01	4.87
<i>Tek</i>	Endothelial-specific receptor tyrosine kinase	.042	≤0.01	4.19
<i>Foxa2</i>	Forkhead box A2	.033	≤0.01	4.96
<i>Pparg</i>	Peroxisome proliferator-activated receptor γ	.042	≤0.01	2.65
<i>Mlst8</i>	mTOR-associated protein, LST8 homolog (<i>Saccharomyces cerevisiae</i>)	.046	≤0.01	4.42
<i>Figf</i>	Vascular endothelial growth factor D	.034	≤0.01	6.24

Abbreviations: HFD, high-fat diet; mTOR, mechanistic target of rapamycin.

Ingenuity Pathway Analysis

The working files for the IPA analyses are in Supplementary files. Affected biological processes and pathways in fetal livers of dams fed HFDs ± metformin are depicted in Supplementary

figure 4. IPA predicted that the fetal liver gene upregulation seen with metformin exposure is the result of metformin inhibition of the common upstream regulator, phosphatase and tensin homolog (*PTEN*; Figure 3). IPA upstream regulation

Figure 3. Regulatory network of metformin-associated genes. An upstream network predicted to regulate many (44%) of the 39 metformin-associated genes within the livers of the high-fat diet group. Genes are displayed as predicted to be activated (orange) or inhibited (blue) with metformin treatment. PTEN indicates phosphatase and tensin homolog.

analysis was unable to be done for model 1 (CD $\pm$ metformin) because the gene set ($n = 6$) did not allow adequate power to predict upstream regulation.

Discussion

Using a normal weight pregnant mouse model, we found that compared to a CD, a HFD resulted in higher dam GWG and heavier fetuses. We also found that in utero metformin exposure attenuated fetal weight without significantly affecting dam GWG. In this mouse model, we also found that metformin altered gene expression involved in growth and angiogenesis in livers of fetuses exposed to a maternal HFD. Using IPA, we were able to predict that fetal liver gene expression alterations in in utero metformin-exposed fetuses were a result of metformin inhibition of the common upstream regulator PTEN. In mice fed a CD, in utero exposure to metformin decreased 5 of 6 genes that were upregulated in the livers of in utero exposed mice fed an HFD. Thus, the fetal effects of metformin appeared to be dependent on type of diet during pregnancy. In our model of normal weight mice, diet alone (HFD vs CD) did not alter fetal liver gene expression of 80 focused genes on our custom-made panel.

In utero metformin exposure in mice has previously been shown to alter the metabolic phenotype in adulthood of offspring of dams fed an HFD. Salomaki et al showed beneficial effects of prenatal metformin exposure by improved glucose tolerance and fat mass accumulation in offspring of dams fed an HFD.¹² Transcriptome data obtained from livers and subcutaneous adipose tissue on a 4-day-old male offspring showed significant gene expression effects on genes involved in adipocyte differentiation and mitochondrial adenosine triphosphate production.¹² Another study showed, similar to our findings, that metformin-exposed fetuses fed an HFD were lighter at e18.5 compared to CD-fed mice and that there was no effect

of metformin on maternal body weight composition.¹³ Our study confirms the findings on attenuation of fetal weight with metformin exposure in an HFD and adds to the literature by showing direct fetal gene expression differences in fetal mouse livers with exposure to metformin.

Our data help to fill a knowledge gap. Little is known regarding in utero metformin exposure on fetal gene expression. The use of a mouse model permitted direct examination of the fetal liver in an HFD model during pregnancy. This provided us with the following advantages: (1) the use of a mouse model allowed us to definitively address possible in utero origins of the effects of metformin in pregnancy¹⁴ due to altered fetal gene expression; (2) hepatocytes are derived primarily from preexisting hepatocytes rather than stem cells; thus, the epigenetic effects of metformin could have long-term outcomes if chromatin modifications are passed onto daughter cells^{12,13,15,16}; and (3) metformin has previously been shown to decrease the expression of many lipogenic enzymes in liver,³ which may have an effect on metabolic phenotype of offspring. Metformin inhibition of the PTEN pathway found via interrogation of fetal liver gene expression may play a role in altering metabolic offspring phenotypes, given that it is a key signaling pathway that regulates growth, survival, and proliferation.

Our IPA showed that metformin has an effect on the fetus via alteration of fetal gene expression in the liver with evidence of inhibition of the PTEN/Akt/mechanistic target of rapamycin (mTOR) pathway. PTEN negatively regulates the insulin signal, and PTEN downregulation is likely to increase the cellular response to insulin, which may explain the lower fetal weights in the metformin-exposed group on an HFD compared to control group (no metformin) on an HFD.¹⁷

Metformin regulates and inhibits the Akt/mTOR pathway,¹⁸⁻²² culminating in increased AMPK signaling. AMPK is a central regulator of multiple signaling pathways that control cellular proliferation and metabolism, including mTOR

and complex 1 (mTORC1) inhibition. This is relevant in the context of our finding because the mTORC1 pathway has a role in nutrient and growth signaling. The mTOR is present in human and animal placentas, and placental mTOR dysregulation in animal models is associated with fetal growth restriction and overgrowth.^{23,24} Human placental mTOR-nutrient signaling links maternal nutrient availability to fetal growth by modulating delivery of amino acids across the placenta and is downregulated in growth-restricted compared to normally grown fetuses.²⁵ However, it is unknown whether changes in mTOR expression in the placenta are an adaptive response by the fetoplacental unit or a defect in the nutrient sensing system.⁴ Our findings raise the question whether or not there is a link between metformin's effect on the PTEN/Akt/mTOR pathway and fetal liver gene expression and growth, leading us to postulate that there is an adaptive response by the fetoplacental unit when metformin crosses the placenta. The PTEN plays a role in the regulation of the PI3K/Akt/mTOR pathway by inhibiting the downstream phosphorylation of Akt. An Akt is a serine/threonine kinase that acts downstream of growth factors such as insulin and upstream of mTORC1. Loss of functional PTEN results in increased Akt/mTOR signaling and promotes cellular proliferation in cancer.²⁶ The link between metformin's effect on the PTEN pathway and fetal growth via mTOR should further be examined by mechanistic studies in animal models and prospective human studies.

Our study has several strengths. We were able to measure the effects of an HFD and metformin in well-timed and characterized pregnancies. The diets that the mice received were standardized, which cannot be easily accomplished in humans. Stages of embryonic development have different gene expression patterns. To mitigate variation by fetal age, we harvested fetuses at the same time point. In human pregnant women, it is difficult to tease out effects of obesity from excessive GWG. By studying normal weight mice, we were able to limit gene expression effects due to maternal obesity. Although the effects of metformin in this model were different from those seen in humans (antepartum metformin reduced maternal GWG but did not change fetal/neonatal weight),² we did not use an obese prepregnancy model as used in the human studies. The next step is to study the effects of different GWG on lean and obese mice and to use PTEN inhibitors to further understand the metabolic effects of metformin on mothers and offspring.

The limitations of our study include that we were restricted to studying the effects of an HFD as a surrogate for conditions that produce excessive GWG, such as obesity and diabetes. Weight gain from excessive calories due to a diet high in sugar, protein, or total calories may affect different genes than an HFD. Also, it is unclear whether fetal findings are comparable to offspring gene expression findings. Finally, the number of dams and livers from each group may have limited our ability to detect differences in gene expression as a result of metformin exposure. We cannot make conclusions about mouse maternal weight effects due to metformin exposure because we were underpowered to detect a difference. We chose preselected genes to study rather than using an agnostic whole

transcriptome approach; thus, there may be other fetal gene expression alterations that we did not detect. However, by using a custom-made gene panel, we were able to take a hypothesis-driven approach based on genes that would be important in the metformin pathway.

Our findings show that in mice, metformin impacts embryonic liver gene expression. Pathway analysis shows metformin inhibition of PTEN in fetal liver, which may play a role in altering fetal growth. It is important to consider the effects of diet and metformin exposure on long-term offspring phenotypes. Further studies are needed to confirm these associations and to understand the long-term metabolic impact of diet and metformin use on the developing fetus.

Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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