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Maternal obesity alters brain derived neurotrophic factor (BDNF) signaling in the placenta in a sexually dimorphic manner

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Abstract

Introduction—Obesity is a major clinical problem in obstetrics being associated with adverse pregnancy outcomes and fetal programming. Brain derived neurotrophic factor (BDNF), a validated miR-210 target, is necessary for placental development, fetal growth, glucose metabolism, and energy homeostasis. Plasma BDNF levels are reduced in obese individuals; however, placental BDNF has yet to be studied in the context of maternal obesity. In this study, we investigated the effect of maternal obesity and sexual dimorphism on placental BDNF signaling.

Methods—BDNF signaling was measured in placentas from lean (pre-pregnancy BMI < 25) and obese (pre-pregnancy BMI > 30) women at term without medical complications that delivered via cesarean section without labor. MiRNA-210, *BDNF* mRNA, proBDNF, and mature BDNF were measured by RT – PCR, ELISA, and Western blot. Downstream signaling via TRKB (BDNF receptor) was measured using Western blot.

Results—Maternal obesity was associated with increased miRNA-210 and decreased *BDNF* mRNA in placentas from female fetuses, and decreased proBDNF in placentas from male fetuses. We also identified decreased mature BDNF in placentas from male fetuses when compared to female fetuses. Mir-210 expression was negatively correlated with mature BDNF protein. TRKB phosphorylated at tyrosine 817, not tyrosine 515, was increased in placentas from obese women. Maternal obesity was associated with increased phosphorylation of MAPK p38 in placentas from male fetuses, but not phosphorylation of ERK p42/44.

Discussion—BDNF regulation is complex and highly regulated. Pre-pregnancy/early maternal obesity adversely affects BDNF/TRKB signaling in the placenta in a sexually dimorphic manner.

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These data collectively suggest that induction of placental TRKB signaling could ameliorate the placental OB phenotype, thus improving perinatal outcome.

Keywords

placenta; obesity; microRNA; neurotrophin; sexual dimorphism

1. Introduction

The placenta regulates maternal metabolism, mediates substrate supply to the developing fetus, and facilitates fetal development [1]. The increasing prevalence of obesity [2] in women of reproductive age presents a major challenge to placental and fetal development as it increases the risk for placental hypoxia [3], increases placental inflammation [4, 5], decreases placental cellular respiration [6], and dysregulates the placental transcriptome [6-8]. The fetal and neonatal consequences of maternal obesity include increased risk for congenital abnormalities [9, 10], large for gestational age or intrauterine growth restriction [11], and stillbirth [12, 13]. Recent work has highlighted the fact that to examine the influence of an adverse intrauterine environment on placenta function, it is imperative to consider fetal sex. Differences in gene expression have been reported between male and female placentas [14] and maternal asthma significantly increased pro-inflammatory cytokine gene expression in female placentas [15, 16]. Increased expression of NF κ B1 (p50) in female placentas from obese women [8] resulted in upregulation of miR-210. The *sexually dimorphic* increase in miR-210 expression consequently diminished mitochondrial function in primary trophoblast cells by targeting subunits of the mitochondrial electron transport chain. The interplay between maternal obesity, placental inflammation, miR-210 regulation, and fetal sex is evident; however, we are just beginning to understand the physiologic consequences of miR-210 expression on placental and mitochondrial function in maternal obesity.

An *in vitro* validated miR-210 target gene is brain derived neurotrophic factor (*BDNF*) [17], a small, secreted member of the neurotrophin family of growth factors [18]. BDNF was first identified in the mammalian brain [19] and has since been associated with several cellular functions in peripheral tissue via the high affinity cognate receptor, tropomyosin receptor kinase B (TRKB), and the low affinity pan neurotrophin receptor, p75NTR. Phosphorylation of TRKB results in induction of MAPK, PI3K, and PLC γ signaling cascades while binding of the BDNF precursor (proBDNF) to p75NTR results in apoptosis via caspase dependent signaling. Surprisingly, maternal obesity is associated with decreased placental apoptosis [20], suggesting contextually restrictive proBDNF/p75NTR signaling. However, BDNF and TRKB are expressed in the placenta [21], reported to play critical roles in implantation [22], placental development [22, 23], and fetal growth [24]. In addition to its involvement in placental development, BDNF/TRKB signaling regulates energy homeostasis and mediates mitochondrial function [25]. Interestingly, BDNF expression is attenuated in the plasma of obese adults [26], but has yet to be evaluated in placentas from pregnancies complicated by maternal obesity. Based on previous evidence, we tested the hypothesis that maternal obesity would alter BDNF/TRKB signaling in the placenta.

2. Methods

2.1 Placenta collection and tissue processing

The research protocol was approved by the Institutional Review Board of the University of Texas Health Science Center San Antonio. Exclusion criteria for the placenta study were: abnormal oral glucose tolerance test, concurrent diseases (diabetes, preeclampsia, hypertension, infections), tobacco or drug/medication use, excessive weight gain/loss prior to pregnancy, and labor with regular contractions. Placentas were collected following informed consent from patients in the Labor and Delivery Unit of University Hospital San Antonio. Placentas from uncomplicated, term pregnancies of lean (LN, pre/early pregnancy BMI: 18.5-24.9) and obese (OB, pre/early pregnancy BMI ≥ 30) women were immediately collected following elective cesarean section delivery at term in the absence of labor (**Table 1**). Five random tissue samples were taken from an area approximately 2.5 cm from the periphery. Villous tissue were dissected away from the basal and chorionic plates, flash frozen in liquid nitrogen, and stored at -80°C .

2.2 RNA extraction, cDNA synthesis, and semi-quantitative RT-PCR

Total RNA was extracted from villous tissue using the Qiagen miRNeasy kit. RNA concentration was determined by the Nanodrop spectrophotometer. MiR-210 expression was determined as described [8]. To determine the expression of placenta specific BDNFmRNA [27], 2 μg of total RNA were reverse transcribed using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. One third of the first strand synthesis reaction was amplified and detected using the Invitrogen Platinum SYBR Green qPCR SuperMix-UDG kit. A melting curve analysis was completed following RT-PCR. The cycling parameters were: 95°C for 15 sec, 60°C for 1 min, and 0.6°C incremental increase. PrimeTime qPCR Primers (IDT,) were used for the specific detection of the internal control, RNA18S5 [28, 29]. After amplification, the samples were separated on a 2% agarose gel and visualized by ethidium bromide.

2.3 Placental proBDNF immunoassay

ProBDNF [30] was measured in placental homogenates using the BDNF E_{max} ImmunoAssay acid treatment protocol (Promega) to facilitate dissociation of BDNF from TRKB, thus improving detection. BDNF concentrations were normalized to total protein amounts.

2.4 Western blot

20 μg of protein were separated by SDS-PAGE as described [8]. Mature BDNF (1:200 rabbit anti-BDNF; Santa Cruz), TRKB (1:500 rabbit anti-TrkB; Santa Cruz), phosphorylated TRKB at Y515 and Y817 (1:400 rabbit anti-TrkB phospho Y515; 1:1000 rabbit anti-TrkB phospho Y817; Abcam), p38 MAPK (1:1000 rabbit anti-p38; Cell Signaling), p42/44 MAPK (1:1000 rabbit anti-p42/44 (ERK1/2); Cell Signaling), phosphorylated p38 MAPK (1:1000 rabbit anti-Pp38; Cell Signaling), phosphorylated p42/44 MAPK (1:1000 rabbit anti-Pp42/44 (ERK1/2; Cell Signaling), and β actin (loading control; 1:1000 mouse anti- β -

actin; Sigma) were measured by Western blotting. The membranes were then washed, incubated with the appropriate peroxidase conjugated secondary antibody, and visualized.

2.5 Statistical analysis

Kolmogorov-Smirnov/Lilliefors Test for Normality was performed. Normally distributed data are reported as mean $\pm$ SEM. Parametric statistical analysis was performed using Student's t test, one or two way ANOVA. The post-hoc analysis was completed using Student-Neuman-Keuls. P values < 0.05 were considered statistically significant. Non-parametric data are reported as median with minimum and maximum values. Non-parametric statistical analyses comparing two groups or more were performed using either Mann-Whitney U or Kruskal Wallis, respectively. Post-hoc analysis was completed using Dunn's multiple comparison test. Multiplicity adjusted p values for Dunn's multiple comparison test were calculated and are reported with rejection of the null hypothesis at $\alpha = 0.05$ [31, 32]. MiR-210 expression was analyzed by linear regression and correlation analysis against BDNF Exon IVS, Exon VS, proBDNF, and mature BDNF. Analyses were performed using StatPlus:mac Pro Software (AnalystSoft Inc., Walnut, CA, USA) and GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Demographic and clinical characteristics

Maternal and fetal characteristics are presented in **Table 1**. By design, pre-pregnancy/first trimester BMI was significantly greater in the obese group vs the lean group ($p < 0.001$). Weight gain in the obese group was significantly lower than the lean group ($p < 0.001$). There were no differences across groups in gestational age, maternal age at delivery, parity, or birth weight ($p > 0.05$). Each group contained equal numbers of male and female fetuses. The majority of the participants were Hispanic.

3.2 Placental mir-210 and BDNF mRNA expression

MiR-210 expression is induced by inflammation and hypoxia, two states that are associated with pregnancy and exacerbated with maternal obesity. We confirmed our previous findings of no differences in miR-210 expression in placentas from obese women when compared to lean women (**Fig. 1A**) or in male vs female placentas (**Fig. 1A**) and that there was an increase of miR-210 expression in female placentas from obese women vs lean women [8] (**Fig. 1A**). Five placenta specific BDNF transcripts have been reported in a population of Estonian participants [27]; however in our Hispanic population, only two of the five transcripts were apparently expressed, Exon IVS and Exon VS. BDNF-IVS mRNA was significantly decreased in placentas from obese women compared to placentas from lean women (**Fig. 1B**). However, there were no differences in BDNF-IVS mRNA in male or female placentas (**Fig. 1B**). When analyzing for sexual dimorphism in the context of maternal BMI, BDNF-IVS mRNA was significantly decreased in female placentas from obese women vs lean women (**Fig. 1B**). In order to determine if miR-210 was associated with the decrease in BDNF-IVS mRNA, we performed a linear regression and correlation analysis and found no relationship between miR-210 and BDNF-IVS expression (**Fig. 1C**).

BDNF-VS mRNA expression did not differ across groups (**Fig. 1D**) nor was there a relationship between miR-210 and BDNF-VS mRNA expression (**Fig. 1E**).

3.3 Placental miR-210 expression and proBDNF levels

ProBDNF protein levels were significantly decreased in placentas from obese women compared to lean women (**Fig. 2A**) but there were no differences in proBDNF levels in male vs female placentas. When analyzing for sexual dimorphism in the context of maternal BMI, proBDNF levels were significantly decreased in male placentas from obese women vs lean women carrying a male fetus (**Fig. 2A**). We found no relationship between placental miR-210 expression and proBDNF levels in the setting of obesity or fetal sex (**Fig. 2B, C, D**).

3.4 Placental miR-210 expression and mature BDNF levels

ProBDNF is proteolytically processed into a mature isoform [18, 33]. Although maternal BMI was not associated with placental mature BDNF levels (**Fig. 3A and B**), mature BDNF was found to be significantly decreased in male vs female placentas (**Fig. 3A and B**). When examining sexual dimorphism in the context of maternal BMI, mature BDNF was significantly decreased in male placentas from lean women vs lean women with a female fetus (**Fig. 3A and B**). Placental miR-210 was found to negatively correlate with mature BDNF in placentas when all patients were included (lean and obese women carrying either a male or female fetus; **Fig. 3C**) and in lean women alone (with either a male or female fetus; **Fig. 3D**), but not in placentas from obese women alone (with either a male or female fetus; **Fig. 3E**).

3.5 Placental TRKB levels and tyrosine phosphorylation

TRKB is a membrane bound, tyrosine receptor kinase that was found to be significantly decreased in placentas from obese women when compared to lean women (**Fig. 4G**). Although there were no differences in TRKB levels in placentas from male vs female fetuses (**Fig. 4G**), TRKB was significantly decreased in female placentas from obese women vs lean women (**Fig. 4G**). Following the binding of BDNF, TRKB undergoes autophosphorylation at several tyrosine residues. We examined phosphorylation at tyrosine 515 (Y515) and tyrosine 817 (Y817). Pre/early maternal BMI and fetal sex did not affect the ratio of pY515/TRKB in placental homogenates (**Fig. 4A and B**), however, the ratio of pY515/ β actin was decreased in placentas from obese women (**Fig. 4C**). Conversely, pY817/TRKB ratios were significantly increased in placentas from obese women when compared to lean women (**Fig. 4D and E**), but, pre/early pregnancy BMI and fetal sex did not influence placental pY817/ β actin ratios (**4F**).

3.6 Placental MAPK signaling

Autophosphorylation at Y515 is associated with induction of ERK signaling. Autophosphorylation at Y817 is associated with induction of PLC γ , PKC, and MAPK signaling [34-36]. We examined phosphorylation at threonine 180/tyrosine 182 of p38 MAPK (pp38 MAPK) and phosphorylation at threonine 202/tyrosine 204 of p42/44 MAPK (pp42/44). pp38 was significantly increased in placentas from obese women when compared

to placentas from lean women (**Fig. 5A and B**). There were no differences in pp38 in male vs female placentas (**Fig. 5A and B**); however, pp38 was significantly increased in male placentas from obese women vs lean women (**Fig. 5A and B**). We then measured pp42/44 in placentas and determined that pre/early maternal BMI and fetal sex did not affect pp42/44 (**Fig. 5C and D**).

4. Discussion

We have previously described a sexually dimorphic interaction between maternal obesity, placental inflammation and miR-210, which regulates trophoblast respiration. In this current study, we confirmed the results of Muralimanoharan et al. [8] utilizing villous tissue from a large, independent group of participants. One miR-210 target is BDNF [17]. Since purification, BDNF has been identified in several peripheral tissues and is necessary for energy homeostasis and mitochondrial respiration [25], improving oxygen consumption rates [37], preventing oxidative stress [38], and decreasing apoptosis [39]. BDNF dependent signal transduction is mediated via the low affinity pan-neurotrophin receptor, p75NTR, and TRKB. The precursors of NGF, NT-3, NT-4/5, and BDNF bind to p75NTR, a member of the TNF superfamily of receptors [40, 41], and induce apoptosis via caspase dependent signaling. Although p75NTR is expressed in the placenta, *NGRF* was not detected in isolated trophoblasts [22], suggesting that p75NTR expression is cell specific. Cytokeratin m30, a marker for caspase activation, was found to be decreased in placentas from obese women in the presence and absence of labor (vaginal or elective cesarean delivery) [20], however, p53 and caspase 3 in placentas from elective cesarean deliveries, were not affected by maternal obesity (unpublished; A. Maloyan, L. Myatt). Many of the studies thus far have examined BDNF in the context of neurodegenerative, psychiatric, and metabolic disorders. In several cortical regions of individuals with schizophrenia and major depressive disorder, there is a significant decrease in BDNF and TRKB mRNA expression [42]. BDNF is significantly decreased in the hippocampus of individuals with Alzheimer's disease [43], and BDNF heterozygous mice display depressive traits [44]. This present study is the first to examine placental BDNF/TRKB signaling in the context of maternal obesity and fetal gender. Strengths of the study are the well-defined patient groups, consideration of the role of sexual dimorphism, inclusion of 13 subjects in each group, the predominance of one ethnic group (Hispanic) and collection of tissue at term in the absence of labor.

In our population, we identified variant dependent *BDNF* mRNA expression. Our findings appear to contradict those of Pruunsild et al. [27] where in an Estonian population, *BDNF* transcripts containing Exon IVS, VhS, VS, VIS, or IXS were identified in the placenta [27]. In addition to the difference in ethnicities between studies, the gestational age of sampling in the Pruunsild et al. study was not stated. BDNF decreases as gestation progresses [21], which suggests that placentas at term will have differing expression profiles than placentas acquired in the first and second trimesters. Maternal obesity decreased the expression of *BDNF-IVS* in female placentas from obese women; however, *BDNF-VS* expression was neither affected by maternal obesity nor fetal sex. The linear regression and correlation analysis indicate that the decrease in *BDNF-IVS* is independent of miR-210 expression, suggesting that decreased *BDNF* is not due to miR-dependent mRNA degradation. Although our population is predominantly Hispanic, the placental *BDNF* mRNA expression profile is

similar to obese individuals that are carriers of the minor C allele (rs12291063) within an intronic region of *BDNF* [45]. Binding of the transcriptional regulator, hnRNPDOB, was decreased as a consequence of the minor C allele, decreasing *BDNF* mRNA in the ventromedial hypothalamus. This suggests decreased *BDNF* mRNA in placentas from female fetuses of obese women is related to possible alterations in *BDNF* gene structure that mechanistically influence expression.

We hypothesized that proBDNF and mature BDNF protein levels would be decreased in female placentas from obese women. ProBDNF levels were, in fact, decreased with obesity; however, the changes were only significant in male placentas from obese women and were not related to miR-210 expression. Mature BDNF levels were also decreased in placentas from male vs female fetuses; however, the sexual dimorphic reduction of BDNF was independent of maternal obesity. Interestingly, miR-210 expression was negatively correlated with mature BDNF levels in placentas from lean but not obese women, suggesting different mechanisms for regulating BDNF in placentas of lean and obese women. The increase in mature BDNF levels in placentas from female fetuses suggested that TRKB would be upregulated when compared to placentas from male fetuses. On the contrary, TRKB protein was significantly decreased in placentas from female fetuses of obese women. The decrease in pY515/ β actin ratios suggested that diminished receptor levels were responsible for the attenuation of phosphorylation. When normalizing with total TRKB, the phosphorylation patterns indicated that maternal obesity did not influence the induction of pY515. Maternal obesity and fetal sex did not change Y817/ β actin ratios, however, normalizing with TRKB suggested that maternal obesity induces Y817 phosphorylation. These data collectively suggest maternal obesity adversely influences BDNF binding and induction of TRKB phosphorylation in placentas from female fetuses. In an animal model of traumatic brain injury, female rats that underwent experimental traumatic brain injury showed increased BDNF in the hippocampus. However, spatial memory did not recover when compared to male rats suggesting possible differences in response to injury [46]. The mechanism underlying sexual dimorphism, at this point in time, is not clear. However, BDNF/TRKB signaling in the placenta as a result of maternal obesity appears to mirror that in the central nervous system of an animal model of stress.

We next examined downstream signaling pathways that are associated with phosphorylation of TRKB at Y515 and Y817. p42/44 MAPK (ERK) and p38 MAPK are associated with cellular growth/differentiation and mitochondrial function, respectively. As we saw maternal obesity associated phosphorylation of TRKB at Y817, but not Y515, we hypothesized that there would be increased phosphorylation of p38 with no changes in phosphorylation of p42/44 in placentas from female fetuses. Maternal BMI and fetal sex had no effect on phosphorylation of p42/44. Interestingly, induction of p38 MAPK occurred in placentas from obese women and in placentas from obese women with a male fetus. This suggests, collectively with the BDNF data, that phosphorylation of MAPK is independent of the BDNF/TRKB pathway. Alternatively, p38 has been shown to negatively regulate p42/44 [47], which suggests that maternal obesity could adversely influence the crosstalk between p38 and p42/44 in the placenta. Identifying clinical relevance at this stage of investigation is a limitation of our study, however, these data contribute to our understanding of neuropeptide signaling in the placenta.

One out of three women of childbearing age in the United States are obese (BMI ≥ 30) [2, 48], which increases adverse pregnancy outcomes and is associated with irreversible, long-term effects on the health of the offspring (fetal programming) [49, 50]. These studies suggest a link between maternal obesity and fetal development perhaps involving the placenta, which has implications for understanding fetal programming. Maternal obesity presents a challenge to the intrauterine environment that, in turn, influences placental function. In this study, we have demonstrated that maternal obesity influences placental BDNF/TRKB signaling in a sexually dimorphic manner. The findings are the first to identify aberrant BDNF/TRKB signaling in female placentas from obese women. Further investigation of BDNF/TRKB signaling may provide insight into how the obese phenotype can be ameliorated in placentas and potentially, influence fetal programming.

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Abbreviations

BDNF	brain derived neurotrophic factor
TRKB	tropomyosin receptor kinase

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- Validation of increased placental miR-210 from obese women carrying a female fetus.
- Decreased placental *BDNF* in obese women carrying a female fetus.
- Decreased placental proBDNF in obese women carrying a male fetus.
- Decreased placental mature BDNF from male fetuses, independent of maternal BMI.
- Dysregulated placental TRKB and MAPK signaling with maternal obesity.

Figure 1. miR-210 and BDNF mRNA expression in placentas from lean and obese women
Quantification of miR-210 (A), BDNF Exon IVS (B), and Exon VS (D) by RT-PCR. U18 was used as an internal control for miR-210 and ribosomal 18S was used as an internal control for BDNF mRNA. Data were fitted using linear regression analysis. Correlation coefficient (R) and p values are shown. Correlation analysis between miR-210 expression, BDNF Exon IVS expression (C), and BDNF Exon VS expression (E) in placentas from lean and obese women carrying either a male or a female fetus. Values from miR-210 RT-PCR are mean $\pm$ SEM; a: $p = 0.01$ vs. LN female; $n = 13$ per group/sex. Values from BDNF RT-

PCR and mean $\pm$ SEM; *: $p = 0.0018$ vs LN; a: $p = 0.012$ vs LN female; $n=13$ per group/sex. Dotted regression line: no correlation ($p>0.05$).

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Figure 2. ProBDNF levels and correlation with miR-210 expression in placentas from lean and obese women

ProBDNF protein in placental homogenates from lean or obese women carrying either a male or female fetus (A). ProBDNF was normalized to total protein. Data were fitted using linear regression analysis. Correlation coefficient (R) and p values are shown. Correlation between miR-210 expression and proBDNF levels in placentas from lean and obese women with male or female fetuses (B), placentas from lean and obese women carrying a male fetus (C), and placentas from lean and obese women carrying a female fetus (D). Values from the BDNF ELISA are median with minimum and maximum values, *: $p=0.02$ vs LN; a: $p=0.03$ vs. LN male; $n = 10$ per group/sex. Dotted regression line: no correlation ($p>0.05$).

Figure 3. BDNF protein levels and correlation with miR-210 in placentas from lean and obese women

Representative Western blot (A) and quantification (B) of mature BDNF in placentas from lean and obese women carrying either a male or female fetus. β -Actin was used as the loading control. Data were fitted using linear regression analysis. Correlation coefficient (R) and p values are shown. Correlation between miR-210 expression and BDNF levels in placentas from lean and obese women carrying either a male or female fetus (C). Correlation between miR-210 expression and BDNF levels in placentas from lean women carrying either a male or female fetus (D). Correlation between miR-210 expression and BDNF levels in placentas from obese women carrying either a male or female fetus (E). Values from

Western blot are mean $\pm$ SEM; *:p = 0.02 vs female; a: p = 0.04 vs LN female; n=10 per group/sex. Dotted regression line: no correlation (p>0.05); solid regression line: correlation (p = 0.04).

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Figure 4. TRKB levels and TRKB phosphorylation at tyrosine 515 and 817 in placentas from lean and obese women with male or female fetuses

Representative Western blots (A, D) and quantification of pY515 TRKB (B, C), pY817 TRKB (E, F), and TRKB (G). β actin was used as the loading control. Values are mean $\pm$ SEM; *: p < 0.05 vs. LN; n = 10 per group/sex.

Figure 5. p38 MAPK and p42/44 MAPK phosphorylation in placentas from lean and obese women with male or female fetuses

Representative Western blots and quantification of pp38 MAPK (A, B) and pp42/44 MAPK (C, D). Samples were normalized to p38 and p42/44 respectively. β actin was used as the loading control. Values are mean $\pm$ SEM; *: $p = 0.01$ vs. LN; a: $p = 0.01$ vs LN male; $n = 6$ per group/sex (pp38); $n = 13$ per group/sex (pp42/44).

Table 1

Maternal and Fetal Characteristics

	Lean (n = 26)	Obese (n = 26)
Pre/Early Pregnancy BMI (kg/m ²)	22.0±0.3	36.7±0.7 *
Gestational Age (weeks)	39.0±0.2	39.2±0.2
Maternal Age at Delivery (years)	29.0±1.1	27.4±1.0
Maternal Weight Gain (lbs)	29.9±2.5	16.0±1.9 *
Parity	1.2±0.2	1.5±0.2
Birth Weight (g)	3326±77	3532±93
Fetal Sex (M/F)	13/13	13/13
Ethnicity (Hispanic/Non-Hispanic)	23/3	22/4

Data are presented as mean ± SEM.

*
p<0.001 vs. lean.