A DECREASE IN DKK1, A WNT INHIBITOR, CONTRIBUTES TO PLACENTAL AND FETAL LIPID ACCUMULATION IN AN OBESITY-PRONE RAT MODEL

BY

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DISSERTATION

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ABSTRACT

Placenta, as the sole transport mechanism between mother and fetus, links the maternal physical state and the immediate and life-long outcomes of the offspring. The present study examined the mechanisms behind the effect of maternal obesity on placental lipid accumulation and metabolism. Pregnant Obese Prone (OP) and Obese Resistant (OR) rat strains were fed a control diet throughout gestation. Placentas were collected on gestational d21 for analysis and frozen placental sections were analyzed for fat accumulation as well as β-Catenin and Dkk1 localization. Additionally, DKK1 was overexpressed in JEG3 trophoblast cells, followed by treatment with NEFA and Oil Red O stain quantification and mRNA analysis to determine the relationship between placental DKK1 and lipid accumulation. Maternal plasma and placental NEFA and TG were elevated in OP dams, and offspring of OP dams were smaller than OR. Placental *Dkk1* mRNA content was 4-fold lower in OP placentas, and there was a significant increase in β-Catenin accumulation as well as mRNA content of fat transport and TG synthesis enzymes, including *Ppar-delta, Fatp1, Fat/Cd36, Lipin1, and Lipin3.* There was significant lipid accumulation within the decidual zones in OP but not OR placentas, and the thickness of the decidual and junctional zones was significantly smaller in OP than OR placentas. Overexpression of DKK1 in JEG3 cells decreased lipid accumulation and the mRNA content of *PPAR-Delta, FATP1, FAT/CD36, LIPIN1,* and *LIPIN3*. Our results indicate that Dkk1 may be regulating placental lipid metabolism through Wnt-mediated mechanisms.

Additionally, recent studies have suggested that maternal obesity may also program early development of non-alcoholic fatty liver disease (NAFLD), rates of which

have correlated with the increase in the obesity epidemic. In the current study, livers of OP offspring had significantly increased TG content ($P<0.05$) and lipid accumulation when compared to offspring of OR dams. Additionally, hepatic *Dkk1* mRNA content was significantly decreased in OP livers when compared to OR ($P<0.05$), and treating H4IIECR rat hepatocyte cells with NEFA showed that *Dkk1* mRNA was also decreased in NEFA-treated cells ($P<0.05$) that also had lipid accumulation. Chromatin Immunoprecipitation (ChIP) analysis of the *Dkk1* promoter in fetal livers showed a pattern of histone modifications associated with decreased gene transcription in OP offspring, which agrees with our gene expression data. These results demonstrate that the hepatic *Dkk1* gene is epigenetically regulated via histone modification in neonatal offspring in the current model of gestational obesity, and future studies will be needed to determine whether these changes contribute to excessive hepatic lipid accumulation in offspring of obese dams.

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LIST OF ABBREVIATIONS

ADA: American Dietetic Association

BMI: Body mass index

cDNA: Complementary deoxyribonucleic acid

ChIP: Chromatin Immunoprecipitation

CHO: Carbohydrate

Ckiα: Casein kinase I isoform alpha

*DGAT: Diacylglycerol acyltransferase

DHA: Docosahexaenoic acid

DIO: Diet-induced obesity

*DKK1: Dickkopf homolog 1

DVL: Dishevelled

Dz: Decidual zone

FABP-pm: Plasma membrane fatty acid binding protein

*FAT/CD36: Fatty acid translocase

*FATP: Fatty acid transport protein

FBS: Fetal bovine serum

FFA: Free fatty acids

FZD: Frizzled

Frat: Frequently rearranged in advanced T-cell lymphomas

Gsk3β: Glycogen synthase kinase 3 beta

HDL: High density lipoprotein

H3Ac: Acetylation of Histone H3

H3K4Me2: Di-methylation of Histone H3 at Lysine residue 4

H3K4Me3: Tri-methylation of Histone H3 at Lysine residue 4

H3K9Me2: Di-methylation of Histone H3 at Lysine residue 9

H3K9Me3: Tri-methylation of Histone H3 at Lysine residue 9

H3K9Ac: Acetylation of Histone H3 at Lysine residue 9

H3K14Ac: Acetylation of Histone H3 at Lysine residue 14

H3K18Ac: Acetylation of Histone H3 at Lysine residue 18

H3K27Me2: Di-methylation of Histone H3 at Lysine residue 27

H3K27Me3: Tri-methylation of Histone H3 at Lysine residue 27

H4Ac: Acetylation of Histone H4

H4K20Me3: Tri-methylation of Histone H4 at Lysine residue 20

IgG: Immunoglobulin G

IOM: Institute Of Medicine

IUGR: Intrauterine growth restriction

Jz: Junctional zone

Lz: Labyrinthine zone

Lrp5/6: Low density lipoprotein receptor-related protein

MDA: Malondialdehyde

MEM: Minimum essential medium

mRNA: Messenger ribonucleic acid

NAFLD: Non-alcoholic fatty liver disease

NEFA: Non-esterified fatty acids,

NFAT: Nuclear factor of activated T cells

NLK: Nemo-like kinase

OP: Obese/obesity-prone

OR: Obese/obesity-resistant

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

*PPAR: Peroxisome proliferator-activated receptors

Pro: Protein

PYGO: Pygopus

RNA: Messenger ribonucleic acid

SGA: Small for gestational age

Sfrp: Secreted frizzled-related protein

TBARS: Thiobarbituric acid reactive substances

TCF/LEF: T-cell factor/lymphoid enhancer factor

TG: Triglyceride/triacylglyceride

Wnt: Wingless-type MMTV integration site family

WT: Wild-type

*Human genes are shown in all uppercase, while rodent genes are shown with the first letter capitalized and the rest in lowercase. Additionally, any reference to the mRNA expression of genes is denoted by italicizing the full gene name, while protein expression is not italicized.

CHAPTER 1: INTRODUCTION

Significance

The rates of gestational obesity have been increasing at the same rapid rate as the obesity epidemic in the general population (1). These statistics are alarming not only because obese pregnancies are accompanied by various birth complications, but also because human and animal studies have shown that being obese during pregnancy programs offspring for a variety of adult-onset diseases. The placenta regulates the transport of all nutrients between maternal and fetal circulation, and is at a marked risk for the accumulation of ectopic fat in instances of obesity during pregnancy. Although the connection between fetal outcomes and maternal obesity has been irrefutably established, the molecular mechanisms remain undefined. As the principal link between mother and child, the placenta has become an important factor in unraveling these mechanisms (2, 3). Poor placentation has been associated with Intrauterine Growth Restriction (IUGR) as well as Small for Gestational Age (SGA) offspring, gestational diabetes, and a risk for the offspring of developing numerous late-onset diseases (4-6).

The current study focused on the regulation of placental lipogenesis in response to gestational obesity in an obesity-prone (OP) rat model. We hypothesized that placentas of OP animals would have an increased lipid accumulation and that this would be accompanied by an increase in oxidative stress. Additionally, because of its involvement in vascularization, development, and recent association with adipogenesis, we hypothesized that the Wnt pathway has a critical role in placental lipid metabolism. As the primary mode of communication between mother and fetus, the placenta has an

integral part in the adaptations that occur in a nutrient-restricted or abundant pregnancy, and we aimed to demonstrate that Wnt signaling may be involved in this adaptation.

Additionally, because the rates of non-alcoholic fatty liver disease have increased at the same rapid rate as the obesity epidemic, we propose that maternal obesity will be associated with substantial lipid accumulation in livers of offspring of obese dams. Because Wnt has also been connected to liver development, we hypothesize that livers of offspring born to obese dams will have altered *Dkk1* expression, and that this may be due to epigenetic modifications of the gene. The current study contributes novel and substantial knowledge to the study of placental lipid metabolism as well as hepatic lipid accumulation in neonatal offspring in response to the growing obesity epidemic, and clearly understanding the consequences of obesity during pregnancy is imperative, as it may lead to significant changes in the current pre-pregnancy weight recommendations.

Specific Aims

The proposed hypotheses were tested by pursuing the following specific aims:

Specific Aim 1:

Determine the consequences of gestational obesity on placental physiology, with a principal focus on placental lipid accumulation and metabolism.

We hypothesized that maternal obesity will result in increased lipid accumulation as well as oxidative stress, and this will be associated with changes in maternal circulating lipids. Additionally, we proposed that placental structure will be altered in OP placentas, and that genes related to placental fatty acid uptake and metabolism will be increased.

Specific Aim 2:

Determine the events occurring within the placental canonical Wnt signaling pathway in response to gestational obesity and investigate the potential role of this pathway in placental lipid metabolism.

We hypothesized that OP placentas would have a disregulation in canonical Wnt signaling, and that these changes would be directly associated with altered placental lipid metabolism.

Specific Aim 3:

Determine the consequences of gestational obesity on hepatic lipid accumulation and *Dkk1* mRNA expression in neonatal offspring.

We hypothesize that offspring of obese dams will have an increase in lipid accumulation, and this will be associated with a change in hepatic *Dkk1* mRNA expression, potentially due to histone modifications within the promoter of the gene.

CHAPTER 2: LITERATURE REVIEW

Common Rodent Models of Obesity and Complications of Maternal Obesity

Common Rodent Models of Obesity

The model of obesity-proneness and resistance has been thoroughly studied and characterized and is a valuable tool for modeling obesity and metabolic syndrome in the human population. The earliest studies of rats that tended to either gain weight or were resistant to weight gain on a high-fat (HF) diet (60% of calories from fat) showed that after 4 weeks on the HF diet, obesity prone (OP) rats ate more than obesity resistant (OR) animals, had lower insulin sensitivity, higher plasma free fatty acids (FFA), as well as a higher respiratory quotient, suggesting that OP animals oxidized a higher proportion of fat than OR (7). Additionally, after 2 or 7 months of either HF or low-fat (LF) feeding, OP rats remained heavier and fatter than the LF diet controls after 2 months, and after food restriction, their body weight and body energy content stabilized at a higher level than the LF controls, suggesting that these animals were defending their adiposity. After refeeding, OP rats regained their obese state (8). When muscle metabolism was compared between OR and OP rats, insulin-stimulated glucose uptake and oxidation was significantly lower in OP rats, as was glycogen storage. Although OP rats gained 60% more body weight than OR, they ate only 10% more energy than OR (9). Further analysis of lipid metabolism differences between OR and OP rats showed that after 5 weeks on the HF diet, fat pad weights were significantly heavier in OP rats, while the FFA rates of appearance and oxidation were not different between OR and OP rats. However, glycerol rates of appearance and intracellular FFA were significantly elevated in OP rats (10). Later studies showed that OP rats can be characterized by an increase in

plasma TG (11) and have reduced capacity for fatty acid oxidation (12), especially in the liver (13). Barry Levin's group was instrumental in selectively breeding the obesity phenotype out of the Sprague-Dawley strain (14), and their interest was primary in the brain differences between OR and OP rats. They found that OP rats had increased glucose-induced norepinephrine release (15), but the turnover of norepinephrine, an index of sympathetic activity, was significantly decreased in the heart, pancrease, and hypothalamus (16). Additionally, they showed that OP rats have decreased leptin sensitivity (17, 18), are more sensitive to stress-induced weight gain (19), have inappropriate insulin signaling (20, 21) and beta-cell mass with age (22), and defend their body weight in response to exercise (23), potentially because of their altered energy expenditure (24). While there are still many unknowns in this model of obesity development, it appears that these rats have many of the characteristics of humans who are obese, which makes this an appropriate model for the current study.

Current Gestational Weight and Weight Gain Recommendations

In a 2009 response to the growing obesity epidemic, especially in women of childbearing age, the American Dietetic Association (ADA) issued a statement focusing on maternal obesity and weight gain (25). The ADA statement asserted that all obese and overweight women of reproductive age should receive nutrition and diet counseling prior to pregnancy, during pregnancy, and after pregnancy to alleviate the adverse outcomes of being obese during pregnancy. This statement from the ADA also reviewed the current weight and weight gain recommendations for all women, focusing on the obese and overweight population. Specifically, it was suggested that pre-pregnancy weight loss is

the best recommendation for obese women. However, weight loss is difficult, and restrictive eating or dieting before and during pregnancy may be detrimental to maternal and fetal health. Therefore, it has been stated that the most realistic interventions should occur during the time of pregnancy, when women are most likely to make changes to guarantee fetal health. The primary intervention is to urge women to gain within the Institute of Medicine (IOM) 1990 guidelines for each pre-pregnancy BMI (15-25 lb for BMI>26-29, 15 lb for BMI>29). Since this statement was published, the IOM updated its recommendations to prescribe a 15-25 lb weight gain for overweight women (BMI=25-29.9), and a gain of 11-20 lb for obese women (BMI≥30.0) (26).

Consequences for the Mother

The World Health Organization (WHO) predicts that in the United States, one-third of women of reproductive age are classified as obese, and the IOM recently reported that since the mid-1990s, about half of women of childbearing age are overweight (27, 28). Historically, maternal and gestational obesity has been discouraged because of its effect on maternal and immediate fetal health, as obese pregnancies are often complicated by gestational diabetes, preeclampsia, macrosomia and cesarean section (29). Normal pregnancy is a time of weight gain, but the weight gained in normal-weight mothers is quite different than in obese women. Because of the metabolic shift that occurs in obese individuals, studies have shown that obese women tend to gain weight more centrally than lean women. This central-visceral adiposity has been associated with the negative outcomes described above, including gestational diabetes, hypertension, pre-eclampsia, and insulin resistance (30-33). An analysis of 151,025 Swedish women found that the

risk of pre-eclampsia, gestational hypertension, gestational diabetes, cesarean delivery, stillbirths, and large-for-gestational age births was increased during the second pregnancies of women who experienced an increase in BMI score of 3 or more between their first and second pregnancies (34). Obese women have a decrease in HDL cholesterol, together with a severe increase in plasma triglycerides and total cholesterol (35). Additionally, the normal pregnancy-associated insulin resistance is exacerbated in women who are obese during pregnancy, increasing the risk for gestational diabetes (36, 37).

Short-Term Consequences for the Fetus

The immediate short-term effects of maternal BMI on the offspring were recently assessed through a metanalysis, where it was shown that as maternal BMI increased, so did fetal compromise, meconium, shoulder dislocation, and NICU use. There was also a decrease in Apgar scores in neonates of obese mothers (38). Other studies have shown that gestational obesity is associated with stillbirth, neonatal death, and other severe fetal complications (39, 40). Data related to the effect of maternal obesity on fetal birth weight are contradictory. Because gestational obesity is associated with an increase in fuel availability to the fetus in the form of glucose, it has been believed that maternal obesity and macrosomia are strongly associated. However, recent studies have shown that maternal obesity in humans and animals can also be accompanied by low fetal birth weights and IUGR (41-44).

Long-Term Consequences for the Offspring

The idea of in-utero programming of adult-onset disease has become of increasing interest in the scientific community. Numerous epidemiological studies have shown that the uterine environment has a predictive effect on the long-term health of offspring (45-48). These studies have focused on the programming of obesity, hypertension, insulin resistance and heart disease, and are supported by numerous animal studies showing the effects of maternal obesity on the weight, adiposity, insulin resistance and health of offspring in adulthood (29, 49). Additionally, recent studies have suggested that maternal obesity may also program early development of non-alcoholic fatty liver disease (NAFLD) (42, 50, 51), rates of which have been correlated with the increase in the obesity epidemic (52, 53). **Table 2.1** provides a review of current data related to maternal obesity and offspring outcomes and **Table 2.2** focuses on obesity development in the offspring of obese mothers (54).

Table 2.1: Consequences of gestational obesity. Adapted from Ruager-Martin, *et al* (54), refer to the original review for a full list of references.

Factor	***Conclusion***
Infertility	Women with a BMI > 35 are 26–49% less likely to conceive compared to women with BMI of 21–29.
Miscarriage and still birth	Women with pre-pregnancy obesity have an odds ratio for late antepartum death ranging from 2.4 to 3.1 when compared to non-obese women.
Gestational diabetes	A meta-analysis of 57,000 subjects from 18 cohort studies showed that the odds-ratio of developing gestational diabetes increases with increasing BMI, from 3.56 [3.05–4.21 95% CI] in obese women to 8.56 [5.07–16.04] in severely obese women, compared to normal weight controls.
Gestational hypertension	For each unit increase in pre-pregnancy BMI the relative risk for gestational hypertension is 1.09. Women with a waist circumference > 80 cm at 16 weeks of gestation had a risk ratio for gestational hypertension of 1.8 [1.1–2.9 95% CI] compared to mothers with a waist circumference < 80 cm.
Pre-eclampsia	Approximately 65,000 subjects showed a risk for pre-eclampsia of 2.47 [1.66 to 3.67 95% CI] for raised pre-pregnancy BMI. A systematic review of 13 cohort studies including 1.4 million women, showed that the risk of pre-eclampsia almost doubles for every 6 unit increase in pre-pregnancy BMI.
Delivery complications	Rates of Caesarean section were 20.7% in normal weight mothers, 33.8% in obese mothers and 47.4% in morbidly obese mothers.
Maternal death	35% of all of pregnancy-related death involved obese women. More than half of all the women who died from direct or indirect causes during (late) pregnancy or labor were either overweight or obese, and more than 15% were morbidly obese.
Fetal	Maternal obesity was associated with a 1.7-fold increased risk of neural tube defects.If the woman was severely obese, the risk was increased more than 3 fold. In a population based study, for every incremental unit increase in BMI the risk of birth defects increased by 7%.
Small for gestational age	A study involving 385 obese nulliparous women found an overall incidence of SGA infants of 18.8% (72/382) compared to 10% in the general population. Additionally, stillborn infants born to obese mothers are smaller than those born to mothers of normal body weight.
Fetal overgrowth	A correlation between neonatal weight and the mother's pre-pregnancy BMI has been shown ($r = 0.20$, $p = 0.044$). A study of 2329 mother–baby pairs in showed an increase in average birth weight with increasing maternal BMI. An adjusted odds ratio for birth weight > 4500 g of 2.0 (1.4–3.0 95% CI) has been reported in obese, mothers (2.4 (1.5–3.8) in morbidly obese) when compared with controls.
Preterm birth and neonatal mortality	A population based study of 300,000 births showed that delivery before 32 weeks gestation was significantly less likely to occur in women with BMI > 30 (OD: 0.73, 95%CI: 0.65–0.82). Studying 284 preterm births, mortality increased from 9% (5/53) in babies born to thin mothers, to 47% (8/17) in offspring of obese mothers.
Breastfeeding	A population based study of 300,000 births suggested that a BMI > 30 was associated with an odds ratio for breast feeding at discharge of 0.86 (95%CI: 0.84–0.88) compared to normal weight mothers.
Body composition	In a study of 216 9-year-old children, for 1 standard deviation increase in maternal prepregnant BMI, there was an increase of 0.26 standard deviations in the fat mass index (total body fat (kg) / height (m)) of the offspring (obtained using dual x-ray absorptiometry).
Metabolic effects	In a population-based screening of 3 million children, a U-shape relationship was found between birth weight and risk of developing type-2 diabetes mellitus in childhood, and mothers of children who developed type-2 diabetes had significantly higher BMI than mothers of children without diabetes.

Table 2.2: Relationship between maternal obesity and the risk of childhood obesity. Consequences of gestational obesity. Adapted from Ruager-Martin, *et al* (54), refer to the original review for a full list of references.

Cohort size	***Follow up age***	***Conclusion***
547	2 years	Maternal BMI associated with offspring weight at birth, 1 year and 2 years. Maternal prepregnancy BMI is correlated with offspring BMI at 2years ($r = 0.18$, $p < 0.001$) during the first two years of life. After 1 year post-birth offspring BMI is also correlated with paternal BMI (at 2 years: $r = 0.23$, $p < 0.001$).
8494	4 years	The risk of childhood obesity at 4 years is associated with maternal obesity (measured during first trimester; BMI > 30) (OR: 2.3; 95% CI: 2.0–2.6).
313	5 years	Children of obese mothers (pre-pregnancy BMI > 30) have 0.54 kg more fat mass than children of non-obese mothers as determined by dual-energy x-ray absorptiometry.
4062	5 years	Pre-pregnancy maternal obesity (≥ 95 percentile) (OR: 4.7; 95% CI: 3.3–6.8) and paternal obesity (≥ 95 percentile) (OR: 2.9; 95% CI: 1.9–4.5) are independent predictors of severe obesity (≥ 95 percentile) at 5 years.
3022	2–7 years	Maternal pre-pregnancy BMI > 30 associated with an increased odds ratio for overweight in the offspring (OR: 1.37; 95% CI: 1.02–1.84).
3306	5–7 years	Children's BMI is significantly correlated with parental BMI, although a closer correlation was observed between maternal ($r = 0.249$; $p < 0.01$) than paternal ($r = 0.159$; $p < 0.01$) BMI. Also there was a closer correlation between the BMI of boys and parental BMI than that for girls.
871	7 years	Maternal obesity (BMI > 30) results in higher offspring body fat measured by bioelectrical impedance analysis (OR: 4.0 (95% CI: 0.4–7.7).
1779	7 years	Maternal index (kg/cm) is associated with offspring obesity at 7 years of age ($p < 0.5$).
8234	7 years	Maternal BMI during pregnancy and Paternal BMI are independent predictors of obesity at 7 years (both parents: adjusted OR: 10.44; 95% CI: 5.11–21.32).
4654	7.5 years	Child BMI is associated equally with maternal ($r = 0.295$ (0.267 to 0.322)) and paternal ($r = 0.250$ (95% CI: 0.218 to 0.274)) BMI.
216	9 years	For 1 standard deviation increase in maternal pre-pregnancy BMI, fat mass index increased by 0.26 standard deviations.
2636	2–14 years	Maternal pre-pregnancy BMI > 30 associated with an increased odds ratio for overweight in the offspring (adjusted OR: 4.1; 95% CI): 2.6–6.4).
3340	14 years	Pre-pregnancy maternal BMI (β 0.353 (95% CI: 0.304 – 0.401) a stronger predictor of offspring BMI than paternal BMI (β 0.251 (95% CI: 0.199 – 0.304) (difference between maternal and paternal: $p = 0.009$).
1103	18 years	Maternal pre-pregnancy BMI is the strongest predictor of offspring obesity (OR: 0.39; 95% CI: 0.28–0.49).
17,414	33 years	Pre-pregnancy maternal BMI explained association between birth weight and adult BMI.

Consequences for the Placenta

As the primary link between the maternal and fetal environments, the placenta has become an important focus of studies aiming to uncover the role of maternal obesity in fetal programming and immediate health. Human placental studies are still limited, but several have shown that maternal obesity has a significant effect on placental inflammation and oxidative stress (55-57). Specifically, they have shown that as maternal BMI increases so do placental nitrative stress, oxidative stress, cytokine production, and macrophage accumulation. Although human research related to placental development during obesity is limited, a variety of animal studies and outcomes are available. A recent study utilizing the baboon as the animal model demonstrated that maternal obesity leads to an increase in placental macrophages, a decrease in System A transporter activity, and that these effects correlated negatively with maternal leptin concentrations (58). Another study, focusing on diet-induced obesity in a pregnant rat model, demonstrated that although the placentas of heavier animals did not differ in size when compared to the lean rats, the ratio of fetal weight to placental weight was significantly decreased. These results indicated that the placentas of the obese dams were less efficient in supporting fetal development than placentas of lean animals (59).

Placental Characteristics

Placental Function

The placenta functions as the sole transport mechanism between mother and fetus for all essential nutrients and substances, as well as, waste products. The placenta is responsible for transporting oxygen, carbon dioxide, water, and any other essential nutrients. Respiratory gases

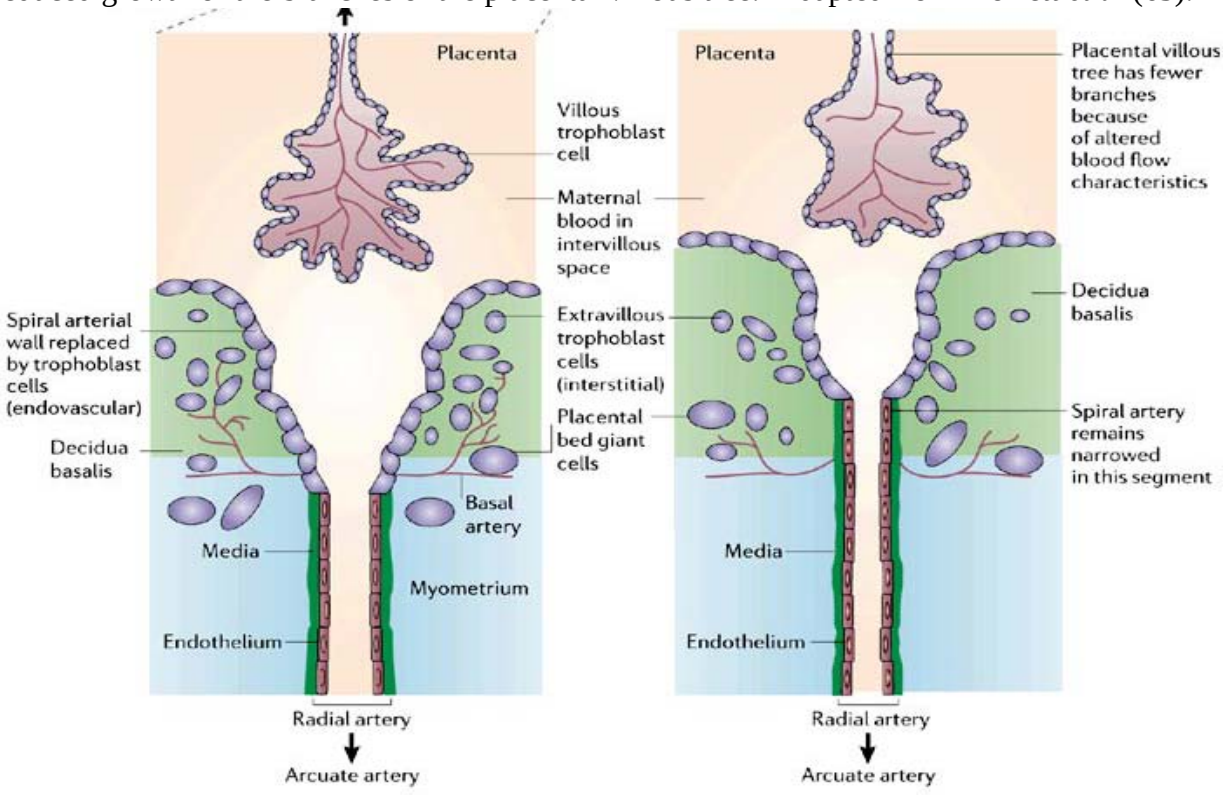
pass freely from maternal to fetal blood, as do other lipophilic substances, and their transport depends primarily on concentration differences of these substances between mother and fetus. Hydrophilic substances, such as ions, amino acids and glucose have poor diffusion capacity across the placenta, and require transport proteins and ion channels in order to become available to the fetus (60). The weight of placenta required for adequate fetal development varies across species. The appropriate fetal-to-placental weight ratio in humans is approximately 6:1, while rats have a ratio of 10:1 (61). The rat's ability to produce more fetus per gram of placenta is thought to be due to the rodents' placental countercurrent blood exchange. In this arrangement, maternal and fetal capillaries are in parallel to each other, and blood flows in opposing directions (62). Humans have the less-efficient multivillous arrangement, resulting in the need for a larger placenta (61).

Placentation and Placental Structure

In humans, placentation requires an extensive invasion of placental trophoblasts into the uterus, and these trophoblastic cells have a wide aray of vital functions, including anchoring the placenta to the uterine wall, transporting nutrients and oxygen to the fetus, removing waste products, secreting hormones and other placental proteins, and acting as a physical barrier between maternal and fetal circulations (63). **Figure 2.1** represents the process of human placentation and trophoblast invasion. The diagram on the left represents normal placentation, with the appropriate conversion of the spiral arteries of the placental bed to uteroplacental arteries by migratory extravillous trophoblast cells. Trophoblasts invade both the arterial media and the endothelium, converting the artery into a wide vessel that can deliver blood to the

intervillous space. During inappropriate placental development (on the right), trophoblast cells do not invade adequately, causing the spiral arteries to remain narrow, thus reducing the amount of blood flowing to the fetus, and leading to poor fetal growth (63).

Figure 2.1: Representative image of normal (left) and inappropriate (right) placentation. In normal placentation, the placental spiral arteries are converted to uteroplacental arteries by extravillous trophoblast cells, which converts the artery into a wide vessel that can deliver blood to the intervillous space. During inappropriate placentation, trophoblast invasion is inadequate, and the spiral arteries are not properly transformed, which disrupts blood flow and causes reduced growth of the branches of the placental villous tree. Adapted from Moffett *et al* (63).



Species differences in placental structure become important when selecting an animal model to study placental development and efficiency. Placentas are most often classified using two major factors:

1) the shape of the placenta as well as the number of contact points between mother and fetus, and
2) the number of tissue layers separating fetal and maternal vascular systems.

In regards to the first classification, primates and rodents develop a single "discoid" placenta, while horses, cats, dogs, pigs and ruminants have alternate placental structures. When classifying placentas according to the number of intermediate layers (also known as the Grosser Classification), primates and rodents once again fall into the same "hemochorial" category. With this architecture, all pregnancy-associated maternal tissue layers are reorganized to become the placenta, eliminating any layers between the placenta and fetus, and exposing the fetal chorionic epithelium to maternal blood (61). Although the metabolisms of primates and rodents are not identical, the similarities between their placental structures make the rat the most appropriate species for modeling human placental function and transport capacity. **Figure 2.2** is a visual representation of different types of placentas, **Figure 2.3** diagrams rodent placentation (top), and a similar diagram of human placentation (bottom).

Figure 2.2: Three main types of placentation, showing the relationship between the fetal trophoblast cells and maternal blood. In haemochorial placentation (panel C), as seen in humans and rodents, maternal uterine blood vessels are infiltrated by trophoblast cells, allowing the release of blood into the intervillous space. Adapted from Moffett *et al* (63)**.**

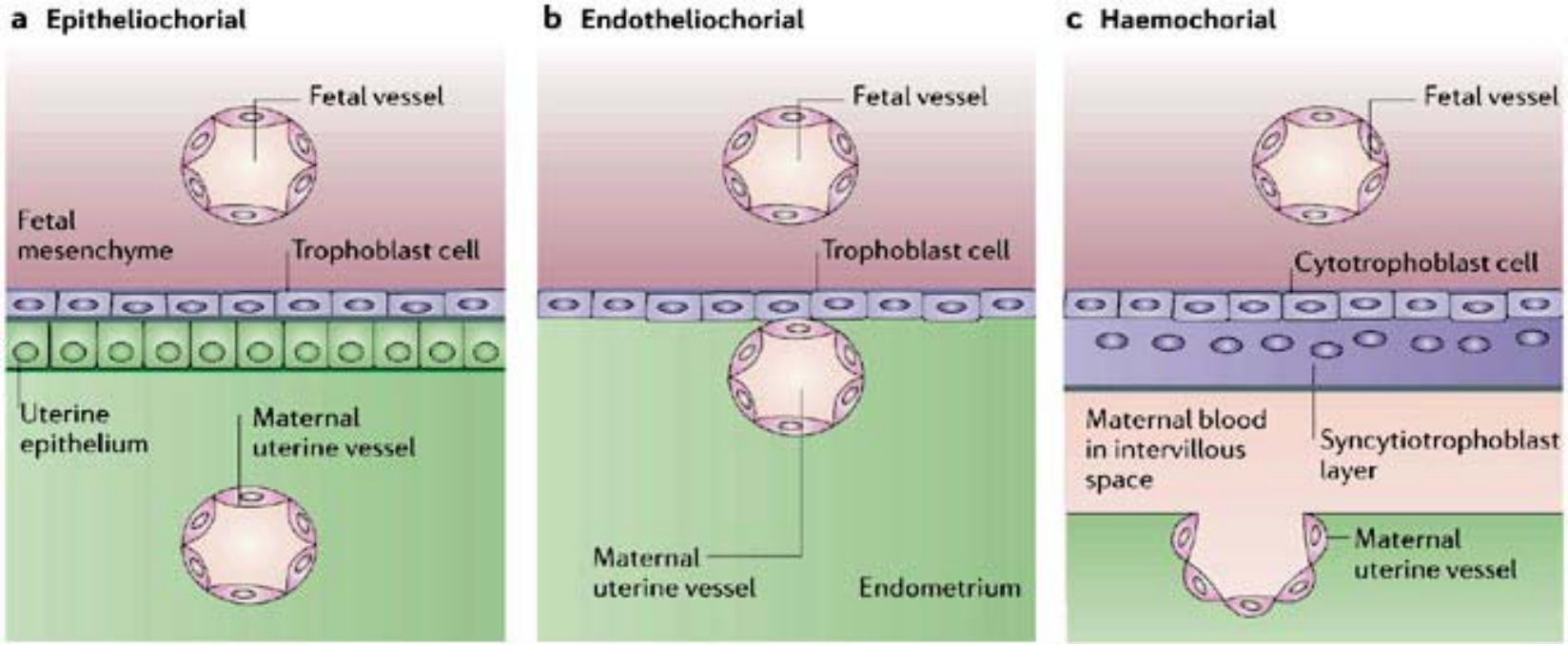
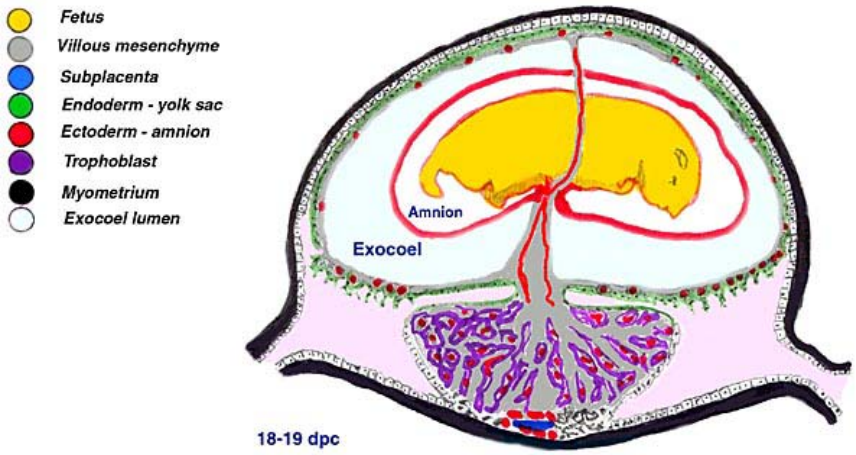
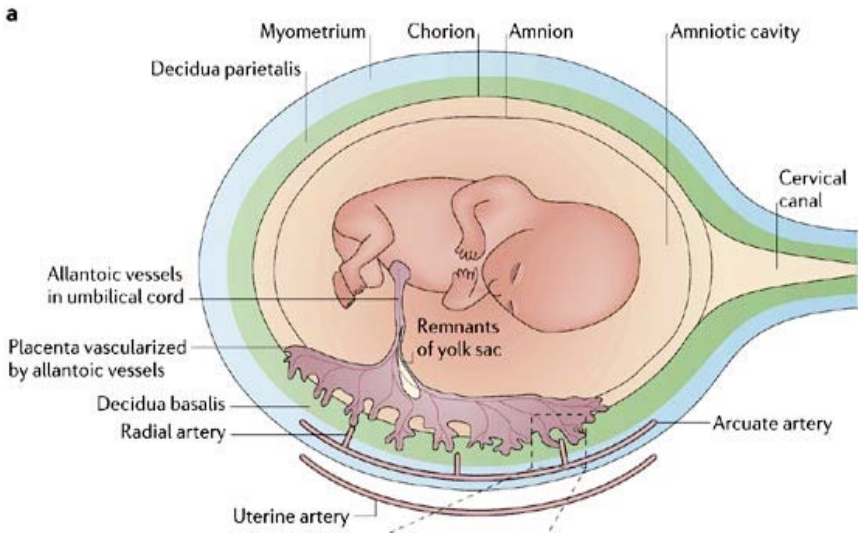


Figure 2.3: Rodent placentation (top, adapted from *Comparative Placentation [internet source]*, UCSD, Kurt Benirschke) and human placentation (bottom, adapted from Moffett *et* al (63).)





Placental Lipid Metabolism

Because triglycerides (TG) are not transported across the placenta, their hydrolysis to free fatty acids (non-esterified fatty acids, NEFA) provides a vital source of essential fatty acids for both the placenta and fetus (64). Additionally, studies have shown that placental uptake of free fatty acids from maternal circulation is not a passive event, and occurs primarily via transport proteins, such as fatty acid transport proteins 1-6 (FATP), the plasma membrane fatty acid binding protein (FABP-pm) as well as a fatty acid translocase (FAT/CD36) (65). Once these free fatty acids are taken up, they may be oxidized as an energy source for the placenta, transferred directly to the fetus or esterified to TG before being delivered to fetal circulation. Although mechanisms behind placental fatty acid metabolism are not thoroughly understood, it has been suggested that nuclear receptors, especially the PPARs, that regulate lipid metabolism in other tissues, also participate in placental fat metabolism (66). Because these genes have been shown to be key regulators of placental lipid transport, *Fatp1* and *Fat/CD36* were analyzed in the current study. Additionally, *Dgat1, Lipin 1, 2* and *3* were measured as markers of triglyceride synthesis, and *Ppar-delta* as a potential regulator of these events.

Programming of Non-Alcoholic Fatty Liver Disease

NAFLD has numerous etiologies, but the primary characteristic is the presence of fat in the liver, with potential inflammation and fibrosis (67). Rates of childhood NAFLD and the accompanying steatohepatitis are increasing (68), and recent studies have shown that maternal obesity may program early development of NAFLD (42, 50, 51), rates of which have correlated with the increase in the obesity epidemic (52). An

excess of hepatocyte triglycerides (TG) has often been considered as the first and hallmark sign of NAFLD development (69), often called the “first hit”, as it is accompanied by oxidative stress. Additional inflammation and fibrosis follow as the “second hit” and inevitably leads to excessive cellular liver damage. Because NAFLD is difficult to treat in adulthood and often leads to irreparable liver damage if left untreated, the potential programming of this disease during the *in utero* period confirms the importance of maternal health and diet.

Programming Gene Expression Through Histone Modifications

In utero histone modification in response to changes in maternal physiology or nutrition has been demonstrated in a number of studies. Gestational food restriction in rats was shown to affect *Igf1* (Insulin-like growth factor) mRNA expression and the levels of dimethylation of histone H3 at lysine residue 4 (H3K4Me2) within its promoter (70), and gestational protein restriction in mice resulted in a decrease in the mRNA expression of *Igf2*, with a concurrent decrease of H3K4Me3 and H4K20Me3, and an increase of H3K9Me3 and H3K27Me3 (71). In another study, gestational choline supplementation led to an increase in H3K9Me2 and H3K27Me2, while choline-deficiency increased the levels of H3K4Me2 (72) in offspring of rat dams. The consequences of gestational obesity on the histone code have not been thoroughly studied. A recent study in primates, Japanese macaques, showed that chronic consumption of a maternal high-fat diet with accompanying obesity development in the mother led to an increase in fetal liver TG and NAFLD. In fetal hepatic tissues, there was an increase in H3K14Ac, and a trend of increase in H3K9Ac and H3K18Ac. There

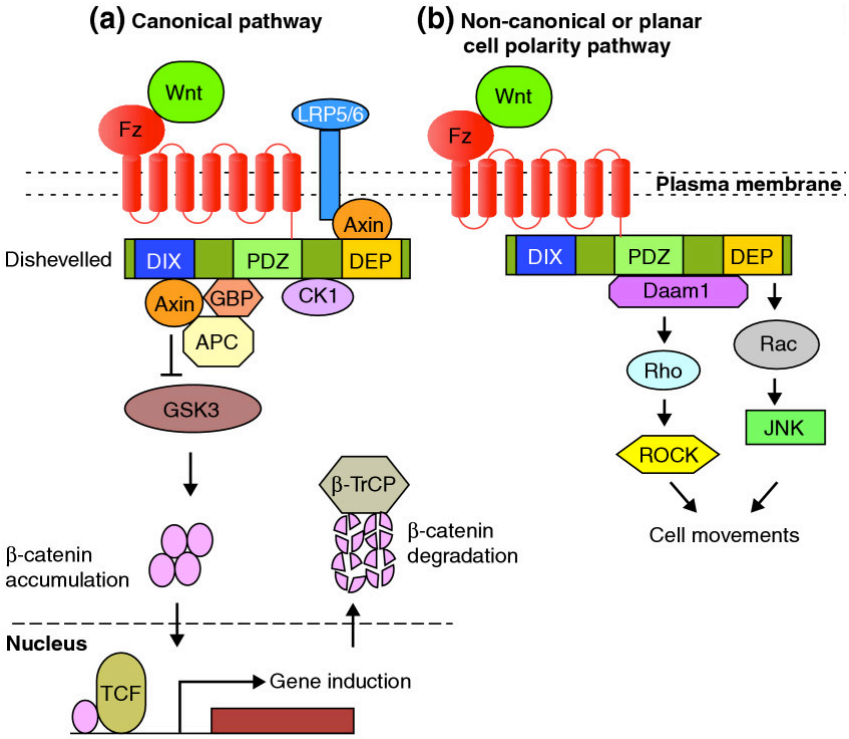
was also a trend of increase in H3K9Me2, H3K9Me3 and H3K27Me3 (73). These results suggest that maternal diet and physiology have the ability to alter fetal chromatin structure in primates via covalent histone modifications.

Wnt Signaling

Function of Wnt Signaling

While the complexity of the Wnt signaling pathway makes it difficult to assign it a singular function, the most common approach to classifying Wnt signaling and function has been to assign Wnt components to either the canonical or noncanonical pathways (**Figure 2.4**). The canonical Wnt Pathway is linked to cell fate determination, while the noncanonical Wnt pathway is associated with the control of cell movement and cell polarity (74). Because of its extensive involvement in the control of cell growth, a strong connection has been made between canonical Wnt signaling and the progression of colorectal cancer (75), as well as bone and eye development (76). A connection has also been made between TCF4 (a Wnt component) and the development of Type 2 Diabetes (77), but this relationship is not well-understood.

Figure 2.4: Wnt components in the canonical (panel a) or noncanonical (panel b) pathways, adapted from Habas, *et al* (78).



Components of Wnt Signaling

Canonical Wnt signaling is transduced through Frizzled (FZD) receptors and acts through the complexing of nuclear β-catenin with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, Legless family docking proteins, and PYGO family coactivators to enhance the transcription of target genes (79-84). Canonical Wnt signaling occurs following the binding of a Wnt molecule and the downstream phosphorylation of Dishevelled (DVL) by Ckiα and its binding to Frat. The subsequent assembly of the FZD-DVL and Lrp5/6-Axin-Frat complexes releases β-Catenin from Ckiα and Gsk3β phosphorylation and allows it to travel into and accumulate in the nucleus (85, 86).

Noncanonical Wnt signals are also transduced through FZD receptors, but do not involve the translocation of β-catenin into the nucleus to induce gene expression. Instead, the pathway acts through DVL-dependent small G proteins and c-jun NH2-terminal kinase (87, 88), as well as Calcium-dependent Nemo-like kinase (NLK) and nuclear factor of activated T cells (NFAT) (89, 90).

Wnt antagonists are either secreted molecules or function at the intracellular level, acting through various routes. Secreted-type inhibitors include Sfrp1, Sfrp2, Sfrp3, Sfrp4, Sfrp5, WIF1, Dkk1, Dkk2, Dkk3, and Dkk4 (91). Members of the Sfrp and WIF family members inhibit the binding of Wnt to the FZD receptors, while the Dkk family of molecules interact with and causethe endocytosis of LRP5/LRP6 coreceptor to prevent the formation of the Wnt-FZD-LRP5/LRP6 complex.

The complicated nature of Wnt-signaling is confirmed by various reports of Wnt's self-regulation. Wnt signaling has been shown to feedback-activate Axin2, Dkk1

and Nkd, and feedback-inhibit FZD and Lrp6, thereby dampening Wnt-signaling (79, 92, 93). Wnt's feedforward signaling has also been shown to activate Rspo and TCF/LEF to activate or reinforce Wnt-signaling (94-96).

Placental Wnt signaling

Because of its rapid growth and vascularization, it has been proposed that Wnt signaling has a vital role in the development of the placenta. In fact, out of the 19 known Wnt ligands, 14 were found in the placenta, and 8 out of the 10 FZD receptors were detectable in placental tissue (97). Numerous studies have explored Wnt-signaling in placental tissues and cell culture experiments. β-Catenin, the key mediator of canonical Wnt-signaling, was implicated in trophoblast adhesion, survival and differentiation (98-100). Other studies have shown that Wnt7a and TCF/LEF1 are required for chorioallantoic fusion (101, 102), and that Wnt2 (103), Wnt3a (104), Wnt5a, Wnt10b, Fzd5 (105), Sfrp4 (106, 107), Rspo (108, 109) may be essential for proper placental vascularization and growth. Additionally, Dkk1, a potent inhibitor of Wnt-signaling, has been shown to be involved in trophoblast invasion and migration (110). Therefore, it appears that many components of the Wnt signaling pathway may have a strong link to the poor fetal outcomes associated with inadequate placental development. While numerous studies have shown that Wnt signaling has a vital role in regulating adipogenesis and lipid accumulation (111), little is known about the adipogenic action of the Wnt signaling pathway in the placenta. A study in JEG-3 trophoblasts showed that the activation of β-Catenin resulted in the increased expression of the StarD7 gene, which has been associated with lipid transport (112). The authors hypothesized that because β-

Catenin is often associated with proper placental development and vascularization, an increase in StarD7 likely results in the appropriate uptake of phospholipids. However, they also noted that excessive StarD7 expression is observed in placental pathologies, and that this could be due to the activation of the gene through aberrant β-Catenin signaling.

Hepatic Wnt Signaling

The Wnt signaling pathway is essential for hepatic cellular proliferation, differentiation, survival and metabolism, and thus regulates liver development, stem cell–assisted regeneration, and zonation (113). A knockout (KO) of β-Catenin in early development resulted in little or no hepatic tissues, and a KO of Wnt2b, a Wnt ligand, resulted in delayed liver development (114). Additionally, in mice, a hepatoblast-specific β-Catenin KO was lethal, and was accompanied by undersized livers with little cell proliferation and increased proliferation. These livers also had decreased α-fetoprotein, albumin, cyclin-D1, the adherens junction protein E-cadherin, the tight junction protein ZO-2, as well as β-catenin targets glutamine synthetase, regucalcin, *Egfr*, cytochrome p450 oxidases *Cyp2e1* and *Cyp1a2*, Glutathione-S-transferase *Gsta3*, *Gsto1*, and *Gstm1*. The immature cells also showed elevated oxidative stress, potentially due to a decrease in the expression of glutathione-S-transferases (115). Other than acting as an inhibitor of Wnt signaling, the role of hepatic Dkk1 is unclear. Dkk1 is overexpressed in cases of hepatocellular carcinoma (116) with a concurrent decrease in β-catenin, but its expression has not been characterized in NAFLD. In a liver-specific β-catenin KO mouse model similar to the one discussed above, mice were fed a control or steatogenic diet, and KO mice on both diets had higher hepatic cholesterol levels, and KO mice on the steatogenic

diet had significantly higher steatohepatitis and fibrosis when compared with wild-type mice . Therefore, while Wnt appears to be imperative for hepatic development and may play a role in hepatic liver metabolism, its activity and the mechanism behind its control in fatty livers remain unknown.

Summary

While the dangers of being obese during pregnancy have been confirmed, the mechanisms behind these outcomes remain undefined. A strong link has been demonstrated between placental efficiency, maternal physiology and pregnancy outcomes. Additionally, because of its role in cell determination and cycling, the Wnt signaling pathway has been designated as a possible determinant of appropriate placental development. The precise action of Wnt signaling in placenta has not been elucidated, especially in response to maternal obesity. and despite there existing substantial evidence for the role of Wnt in adipose tissue expansion, the role of Wnt in placental lipogenesis has not been clearly categorized. By utilizing a rat model of obesity-prone pregnancy as well as a trophoblasic cell culture model, the current study will not only provide vital data related to the physiological events occurring in women who are obese during pregnancy, but will contribute knowledge to the mechanistic regulation of lipogenesis occurring within the placenta in response to gestational obesity. Moreover, analysis of livers of neonates born to obese animals will provide further confirmation of the importance of appropriate maternal weight during pregnancy.

CHAPTER 3: MATERIALS AND METHODS

Animal Study

Animal Model and Dietary Treatment

Timed-pregnant rats were obtained from Charles River Laboratories (Wilmington, MA) on gestational day 2. These rats originated from a line of Crl:CD (Sprague Dawley (SD)) rats (Charles River Laboratories strain CD(SD) and substrain Crl) with two lines being developed from the outbred colony: the OP(CD) (Obese Prone) become obese when fed high-fat diets, and OR(CD) (Obese Resistant) do not become obese when fed high-fat diets. The Charles River company was not helpful in providing information regarding the diet fed to the animals before they arrived at our facility, however, after reviewing the growth charts available from the company, we concluded that the 25% difference in body weight observed upon arrival between the OR and OP animals, they were most likely maintained on a standard rodent chow, and not a high-fat diet. Five pregnant rats from each strain group were fed a control diet (Research Diets, Inc, New Brunswick, NJ) with the following caloric distribution: 64%CHO, 20%Pro, 16%Fat **(Table 1)** *ad libitum* until gestational day 20, when they were fasted overnight and underwent cesarean delivery to collect placentas. All placental as well as neonatal liver samples were immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Fetal and placental weights were also recorded. Additionally, trunk blood from each dam was collected and immediately spun down to isolate serum and plasma. Body weights were recorded once a week, and food intake data was recorded every 3 days. Because these two values were not recorded at the same time and because both food intake and body weight stayed consistent throughout pregnancy, the ratio of food intake

per gram of body weight was calculated my taking the average daily food intake and dividing it by the body weight of dams on d 21 of gestation. The value for products of conception was calculated by adding the weights of all offspring and placentas for each dam and then calculating the mean of each group.

Maternal Plasma and Serum Analysis

To determine plasma TG levels, plasma samples were thawed on ice and analyzed via the Thermo Infinity Triglycerides Liquid Stable Reagent (Thermo Fisher Scientific, Rockford, IL) following company protocol and using a commercially available standard reference kit (Verichem Laboratories, Providence, RI). Maternal serum NEFA was analyzed utilizing a commercially available kit (HR-2 Series, Wako Diagnostics, Richmond, VA).

Placental Lipid and Lipid Peroxidation Analysis

In order to determine whether an increase of circulating TG and NEFA corresponds to their increased accumulation within the placenta, placental samples (50 mg) were ground using a mortar and pestle with liquid nitrogen and mixed with 0.15 mL saline (0.9% w/v NaCl) as previously reported (117). Homogenized samples were quickly frozen in liquid nitrogen and kept in -70°C until analysis. The samples were quickly thawed at 37°C and diluted 5 times in saline to 0.75 mL. Twenty microliters of the diluted samples were incubated with 20 μL 1% deoxycholate at 37°C for 5 min, and 10 μL of the samples were used to analyze either TG or NEFA. TG content was analyzed via the Thermo Infinity Triglycerides Liquid Stable Reagent (Thermo Fisher Scientific) following company protocol and using a commercially available standard

reference kit (Verichem Laboratories). Placental NEFA concentration was determined using a commercially available kit (HR-2 Series, Wako Diagnostics). The Lowry assay was performed to determine the protein concentration of each sample, which was then used to normalize the TG and NEFA concentrations.

Because lipid accumulation has been shown to be accompanied by an increase in oxidative stress, the TBARS assay (Catalog # 10009055, Cayman Chemical, Ann Arbor, MI) was performed per company protocol to determine the concentration of MDA, a product of lipid peroxidation, within placental tissues. Lowry assay was performed to normalize the data; however protein content did not differ between treatment groups, therefore raw data from this experiment are presented.

Placental Oil Red O Staining

Because OP placentas had increased accumulation of TG and NEFA when compared to OR, the localization of the lipid within the tissues was characterized. In order to do this, frozen placentas from 5 dams (2 from each dam, for n=10) in each strain group were embedded in Tissue-Tek OCT compound (VWR, Radnor, PA) on dry-ice and 2 sections were cut from each sample at a thickness of 7 μm and mounted on glass slides. Slides were then briefly washed in water, 60% Isopropanol and stained for 10 min in a 60% working Oil Red O solution (Catalog #1277, Newcomer Supply, Middleton, NJ). Slides were then washed well in water and 60% Isopropanol, counterstained for 20 sec in hematoxylin, washed again in running water and coverslipped using an aqueous mounting media. Images were obtained using a NanoZoomer Slide Scanner and NDP View software (Hamamatsu, Bridgewater, NJ).

Placental RNA Isolation and RT-PCR Analysis

Because OP placentas had visible lipid accumulation within all regions, the mRNA expression of lipogenic genes was also analyzed in these placentas. Additionally, because it was hypothesized that Dkk1 may be affected in placentas of obese animals, frozen placentas from 5 dams (2 from each dam, for n=10) from each strain were randomly chosen for mRNA analysis and matched to the placentas used for NEFA, TG, and TBARS analyses. Total RNA was isolated using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and quantified using Nano Drop Spectophotometer ND-1000. Two micrograms of cDNA was synthesized in a 20 μl reaction volume using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random primers and a thermal cycler (Applied Biosystems 2700), with the following program: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds, and a 4°C hold. Real-time PCR was performed using 25 ng cDNA as the template, SYBR Green PCR Master Mix (Applied Biosystems), and 5 μmol/L of each forward and reverse primer **(Table 7.2)** in the 7300 Real-Time PCR System (Applied Biosystem), with the following program: 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds, 55°C for 1 minute, 95°C for 15 seconds, with 40 cycles of steps 2 and 3. Primers were designed using the Vector NTI software (Invitrogen) and the Ensembl database and obtained from IDT (Integrated DNA Technologies, Coralville, IA). Selection criteria for primers included the following: primer length of 18-25, a product length of 60-80 base pairs, a product that spans an exon and intron (whenever possible), few or no hairpin loops, and a melting temperature difference of < 0.3°C. For each reaction, a serial dilution was created from a sample that originated from each study, and was used to

create a standard curve for quantification and a dissociation curve was analyzed following each reaction. The reaction was deemed appropriate if the standard curve had a slope of -3.3±0.2 and $R^2 > 0.98$. **Appendix B** shows the dissociation/melting curves for all primers utilized in the current study. All mRNA data are expressed as a value following normalization to the L7a gene, the mean of which did not differ between treatment groups.

Placental Immunofluorescence

Because Dkk1 mRNA was significantly decreased in OP placentas, its protein content was also determined, as was the protein content of β-Catenin, the indicator of aberrant Wnt-signaling. Frozen placentas (1 from each dam for n=5) in each strain group were embedded in Tissue-Tek OCT compound (VWR) on dry-ice and 2 sections were cut from each sample at a thickness of 10 μm and mounted on glass slides. Samples were allowed to air dry and then fixed in ice-cold acetone for 10 minutes, followed by 10 minutes of washing in PBS. Samples were then blocked in Image-iT FX (Catalog # I36933, Invitrogen, Carlsbad, CA) for 45 minutes, washed 3 X 5 minutes with PBS, incubated in primary antibody for 1.5 hrs (1:150 for β-Catenin, Santa Cruz Catalog # sc-1496 and 1:50 for Dkk1, AbCam Catalog # ab61034), counterstained with Alexa Fluor 647(Catalog #A-21245, Invitrogen) secondary antibody at a 1:200 dilution for 1 hr at room temperature, washed 3 X 5 minutes with PBS. Nuclei were stained with Hoechst (Catalog #33342, Invitrogen) for 15 minutes, followed by washing 2 X 5 minutes with PBS. Slides were then coverslipped using ProLong Gold (Catalog# P36934, Invitrogen) and stored at room temperature overnight and viewed using the Zeiss Axiovert 200M

with the Apotome Structured Illumination Optical Sectioning System (Thornwood, NY). Results were determined by a blinded analysis of at least 6 areas within each sample.

Hepatic Lipid Analysis and Oil Red O Staining in Neonatal Offspring

The effect of maternal obesity on liver lipid accumulation in offspring was analyzed, and the procedure for this analysis was identical to that utilized for the placental study.

Hepatic RNA Isolation and RT-PCR Analysis in Neonatal Offspring

In order to determine whether lipid accumulation in livers of OP offspring was also associated with a decrease in *Dkk1* (as seen in placenta), the mRNA expression of this gene was determined, and the procedure for this analysis was identical to that utilized for the placental study.

Hepatic Chromatin Immunoprecipitation (ChIP) Analysis of the Dkk1 Gene in Neonatal Offspring

In order to determine whether there are any epigenetic modifications occurring within the hepatic *Dkk1* gene that lead to its decreased transcription in livers of OP offspring, ChIP analysis of histone modifications was performed using a modified protocol (118). Livers from 10 offspring (5 male and 5 female) from each strain group were randomly chosen for sampling to assure that offspring from all dams were represented in the analysis. Briefly, 200 mg of frozen liver samples were ground using a mortar and pestle with liquid nitrogen and washed with PBS. The samples were

resuspended in PBS and cross-linked in 1% formaldehyde. The chromatin was sonicated (Fisher Scientific model 100 Sonic Dismembrator) on ice with 6 bursts for 40 s at power setting 5 with 2 min cooling intervals between each burst. Sheared chromatin was diluted in ChIP Dilution Buffer and incubated overnight on a hematology mixer (Model 346, Fisher Scientific) with 2 μg of each primary rabbit antibody at 4°C (H3Ac: Millipore catalog # 06-599, H4Ac: Upstate catalog # 06-866, H3K4Me2: Millipore catalog # 07-030, H3K9Me3: Upstate catalog # 07-442, H3K27Me3: Upstate catalog # 07-449). Pre-blocked salmon sperm DNA/protein G agarose beads (60 μL, 50% slurry; Upstate Biotechnology, Lake Placid, NY) were then added to the chromatin samples, followed by 2 hrs of incubation at 4°C. Supernatant of normal rabbit IgG (Santa Cruz catalog # sc-2027) was saved as input control. Pellets containing the immunoprecipitated complexes were washed andd eluted from Protein G beads by adding elution buffer. The supernatants were incubated at 65°C for 5 hr to reverse the formaldehyde cross-linking and release the DNA fragments. Samples were then treated with proteinase K (Sigma-Aldrich) at 37°C for 1 hr to remove any protein. DNA was purified with a DNA miniprep system (Qiagen). 5μL of immunoprecipitated DNA was used for the real-time PCR reaction to analyze different genomic regions on the *Dkk1* gene. The Real Time PCR primers for the promoter covered the region that was between 143 base pairs (-143 Forward, CCCAGCTTCCGATACACAAACACT) and 76 base pairs (-76 Reverse, AGCAGGGATGGGATTTCAAAGC) upstream of the transcription start site.

Cell Culture Studies

Cell Culture and Treatment

To determine whether the decreased Dkk1 expression was associated with elevated plasma NEFA observed in our model of gestational obesity, human JEG3 trophoblast cells were obtained from ATCC (Manassas, VA, USA). Minimum essential medium (MEM) was purchased from SCS Cell Media Facility at the University of Illinois at Urbana-Champaign (Urbana, IL, USA). Fetal bovine serum (FBS) and other cell culture media supplements were purchased from Mediatech (Herndon, VA, USA). Cells were maintained at 37°C in a 5% CO_2/95% air incubator, and were cultured and maintained in MEM media containing 10% (v/v) FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Sigma-Aldrich).

Passage 3 cells were seeded in a 6-well plate at 0.2 million cells per well on top of 22X22 mm cover slips for Oil Red O staining and immunofluorescence, at 8 million cells per dish in 100 mm dishes for TBARS assay, at 1 million cells per dish in 60 mm dishes for RNA isolation in triplicate, and at 0.05 million cells per well in 24-well plates for Oil Red O quantification. For the initial experiment using wild-type cells, cells were treated with either 0 or 400 μmol/L of Fatty Acid Supplement containing 2 moles of linoleic and 1 mole oleic acid per mole of Bovine Serum Albumin (BSA) (Catalog #F7175, Sigma-Aldrich) in triplicate. This particular mixture of free fatty acids was selected because it has been suggested that the placenta has a preferential uptake of the fatty acids (and other PUFAs). Please see the Discussion section for further details. The 400 μmol/L concentration was chosen because a concentration gradient using 0, 25, 50, 100, 200, and 400 μmol/L NEFA showed that DKK1 was significantly decreased following the 25, 50,

100, and 200 μmol/L NEFA treatment when compared to the 0 μmol/L treatment, but was maximally decreased following the 400 μmol/L NEFA treatment (data not shown). Additionally, 400 μmol/L has been shown to be a physiologically attainable NEFA content in the plasma of pregnant women, and corresponds to a level of plasma NEFA that is between OR and OP rats in the current study. Treatment media made for the 0 μmol/L treatments was supplemented with additional BSA to mimic the 400 μmol/L treatment.

As discussed below, cells were incubated with NEFA for a total of 24 hours. Initial time course experiments showed that by 12 hours, the expression of DKK1 was not consistent among the triplicates, and that the 24 and 48 hour treatments showed a similar pattern of expression (data not shown). Because the overexpression protocol requires that the transfection not exceed 72 hours, we chose the 24 hour NEFA treatment for the current experiment (24 hours of NEFA treatment+48 hours of transfection).

Overexpression of Dkk1 in JEG3 Cells

Because Dkk1 mRNA was downregulated and fat accumulation increased in NEFA-treated JEG3 cells, the ability of Dkk1 overexpression to prevent the accumulation fat in these cells following NEFA treatment was also determined. Transfection-ready Dickkopf homolog 1 (Dkk1) DNA inserted into a pCMV6-XL5 vector was purchased from OriGene (Catalog #SC303946, mRNA NCBI Reference Sequence NM_012242.2, Rockville, MD). The empty pCMV vector was isolated and used as a transfection control. JEG3 Cells were grown as described above and transfected with either the Dkk1-containing vector or the empty pCMV. The transfection mixture was prepared

with each vector (1.5 μg per well for 6-well plates and 0.5 μg per well for 24-well plates), serum-free media and Superfect Transfection Reagent (Catalog #301307, Qiagen, Valencia, CA). After being transfected with the resulting mixture for 3 hours at 37°C in a 5% CO_2/95% air incubator, cells were replenished with fresh complete MEM (containing 10% (v/v) FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B) for 48 hours to allow for the expression of both Dkk1 mRNA and protein. Cells were then treated with either 0 or 400 μmol/L of Fatty Acid Supplement containing 2 moles of linoleic and 1 mole oleic acid per mole of BSA.

Analysis of Wnt Components and Fat Accumulation in Normal and Dkk1-Overexpressed JEG3 Cells Following NEFA Treatment

After 24 hours of NEFA treatment, cells were collected into Tri Reagent (Catalog # T9424, Sigma-Aldrich) to isolate RNA per manufacturer's protocol. Methodology for RNA quantification and mRNA analysis in JEG3 cells is identical to that described for placental tissues.

After 24 hours of NEFA treatment, cells were fixed in 4% Paraformaldehyde (Catalog #P6148, Sigma-Aldrich) in PBS for 1.5 hours for Oil Red O staining. Each well was then washed with PBS, and the same protocol was followed as for tissue Oil Red O staining. Following staining, coverslips containing the stained cells were mounted on glass slides and visualized as with tissue samples. For quantification of the Oil Red O staining, cells grown in 24-well plates were also fixed in 4% Paraformaldehyde as discussed above, and then were washed 3 times with water, stained with Oil Red O stain for 20 minutes and washed 3 times very well with water. The dye was extruded with

100% Isopropanol for 10 minutes, and the resulting supernatant was transferred to 96-well plates and analyzed specrophotometrically at an OD of 490 nm.

For the initial experiment in JEG3 cells, data were presented using a modified y-axis to account for the background from the visible oil red O stain still remaining on the walls of all wells. For the transfection experiment, data were presented as the % of change between the pCMV and Dkk1-transfected cells following 400 μmol/L of NEFA treatment. The lack of a transfection effect on cell number was confirmed by performing Lowry Protein analysis on adjacent cells exposed to the identical treatment.

The protocol for immunofluorescence staining of β-Catenin protein in JEG3 cells was similar to that used for tissue samples, except that cells grown on coverslips were first mounted on glass slides before proceeding with the staining protocol.

TBARS assay (Catalog # 10009055, Cayman Chemical) was performed per company protocol to determine the concentration of MDA, a product of lipid peroxidation, within JEG3 cells following NEFA treatment.. Lowry assay was performed to normalize the data; however protein content did not differ between treatment groups, therefore raw data from this experiment are presented.

Analysis of Dkk1 mRNA Expression and Fat Accumulation in H4IIEC3 Cells Following NEFA Treatment

In order to determine the potential effect of elevated circulating or hepatic lipids on *Dkk1* mRNA content and lipid accumulation, rat H4IIEC3 hepatocyte cells were obtained from ATCC (Manassas, VA, USA) and incubated with NEFA. All lipid

accumulation and mRNA analyses were identical to that used for the JEG3 study, except that H4IIEC3 cells were analyzed following 48 hours of NEFA treatment.

Statistical Analysis

Results are reported as means ± SEM. The n=5 for maternal food intake, weight gain, and plasma analysis. Fetal and placental weight analysis was performed by taking the average of weights by litter, with a resulting n=5. The fetal:placental ratio was obtained for every individual offspring and placenta, and the average was taken for all litters, with a resulting n=5. For placental and hepatic mRNA analysis, ChIP analysis, Oil Red O staining, TBARS, lipid content, and placental thickness measurements, n=10. For determination of decidual/junctional thickness, 5-7 measurements were taken per placenta using NDP View software (Hamamatsu, Bridgewater, NJ) from samples previously stained and scanned for Oil Red O content. For placental immunofluorescence, n=5. All cell culture experiments we run in triplicate.

Maternal weight and food intake were analyzed using the repeated measures ANOVA (SAS software, Cary, NC). For all remaining data, Student's *t*-test was used to test differences between means, and means were considered significantly different when $P<0.05$.

CHAPTER 4: RESULTS

Animal Study: Maternal and Placental Data

Maternal Gestational Characteristics

Gestational food intake **(Figure 8.1)** was not different between OR and OP dams throughout gestation, however, OP dams were significantly heavier ($P<0.01$) than OR throughout gestation **(Figure 8.2),** and the ratio of food intake per gram of body weight was significantly lower ($P<0.05$) in OP dams when compared to OR (**Figure 8.3**). Total gestational weight gain was not different between OR and OP dams, and neither was mass gain (calculated by subtracting fetal and placental weights from total gestational weight gain) **(Table 7.3).** At the time of cesarean delivery on gestational d 21, OP dams had significantly higher ($P<0.01$) levels of fasting plasma TG **(Figure 8.4)** as well as serum NEFA **(Figure 8.5)** ($P<0.01$).

Offspring Observations And Placental Lipid Analysis

At the time of Cesarean delivery on gestational day 21, offspring of OP dams were significantly lighter ($P<0.01$), when compared to offspring of OR dams **(Table 7.3),** the size of OP litters was significantly larger ($P<0.05$) than that of OR **(Table 7.3)**, but the products of conception did not differ between the two groups **(Table 7.3).** Placental weight was not different between the two strains, but the fetal:placental weight ratio was significantly lower in OP dams when compared to OR **(Table 7.3).** OP dams had significantly ($P<0.05$) higher placental TG **(Figure 8.6)** and NEFA **(Figure 8.7)** content when compared to OR placentas. Oil Red O staining of OR and OP placentas suggested

that OP placentas had visibly more lipid associated with the labyrinthine, decidual and junctional zones, while OR placentas had little lipid accumulation within all zones **(Figure 8.8).** Additionally, the combined thickness of the decidual and junctional zones was significantly narrower ($P<0.01$) in OP placentas when compared to OR **(Figure 8.9).**

The mRNA content of fatty transporters *Fatp1* and *Fat/Cd36* were significantly higher ($P<0.01$) in OP dams, as was the mRNA content of genes associated with TG synthesis, including *Ppar-delta, Dgat, Lipin1*, and *Lipin3*, without a significant change in *Lipin2* **(Figure 8.10).** Despite the increase in lipid content within the OP placentas, there was no significant difference in the MDA concentration between OR and OP placentas **(Figure 8.11).**

Placental Dkk1 And β-Catenin Content

Dkk1 mRNA content was significantly ($P<0.01$) lower (>4 fold) in OP placentas when compared to OR **(Figure 8.12)**, as was the amount of Dkk1 protein within the decidial/junctional and labyrinthine zones **(Figure 8.13)**. OP placentas also had a visible increase of β-Catenin protein within all regions when compared to OR **(Figure 8.14)**.

Cell Culture Study: Trophoblast Analysis

Fat Accumulation And Dkk1 mRNA Content in NEFA-treated JEG3 Cells

Treating JEG3 cells with 400 μmol/L of NEFA resulted in a significant decrease ($P<0.01$) in the content of *DKK1* mRNA when compared to the 0 μmol/L treatment **(Figure 8.15)**. Additionally there was obvious fat accumulation in cells treated with 400 μmol/L of NEFA when compared to the 0 μmol/L treatment as observed by Oil Red O

staining **(Figure 8.16)** and quantified by extruding the Oil Red O dye ($P<0.01$) **(Figure 8.16)**, without a significant difference in the MDA concentration between the two treatments **(Figure 8.17).**

Fat Accumulation and Wnt Signaling in NEFA-treated JEG3 Cells Overexpressing DKK1

The overexpression of DKK1 was confirmed by testing mRNA content of the *DKK1* gene following transfection and NEFA treatment **(Figure 8.18).** Additionally, the overexpression of DKK1 (+Dkk1) and NEFA treatment resulted in a decrease of β-Catenin protein when compared to the WT (wild-type) transfection **(Figure 8.19).** Although we did not observe any obvious differences in the number of lipid droplets between WT-transfected and +Dkk1 cells, there did appear to be fewer bright red droplets and more pink drops in the +Dkk1 samples **(Figure 8.20 top).** Additionally, quantification of the Oil Red O staining showed that there was a significant ($P<0.01$) decrease of 10% in fat accumulation in +Dkk1 cells following NEFA treatment when compared to WT-transfected cells **(Figure 8.20 bottom)**.

When compared to WT-transfected cells, +Dkk1 cells also had a significant decrease in the mRNA content of *PPAR-Delta* ($P<0.01$), *FATP* ($P<0.05$), *DGAT* ($P<0.01$), *LIPIN2* ($P<0.05$), and *LIPIN3* ($P<0.05$) without a significant change in *FAT/CD36* and only a trend of decrease of *LIPIN1* ($P=0.059$) **(Figure 8.21)**.

Animal Study: Liver Analysis in Neonatal Offspring

Hepatic Lipid Content and Accumulation and Dkk1mRNA Expression in Neonatal Offspring

OP offspring had significantly higher ($P<0.05$) liver TG content when compared to OR **(Figure 8.22)**, while there was no significant difference in hepatic NEFA content between OR and OP offspring **(Figure 8.23)**. Oil Red O staining in livers of OR and OP offspring suggested that OP neonatal livers had visibly more lipid content than livers of OR neonates **(Figure 8.24).** *Dkk1* mRNA content was significantly lower ($P<0.05$) in livers of OP offspring when compared to OR (**Figure 8.25)**.

Hepatic Histone Modifications by ChIP Analysis of the Dkk1 Gene in Neonatal Offspring

Promoter analysis of the hepatic *Dkk1* gene demonstrated that maternal obesity led to a significant decrease ($P<0.05$) in the amount of H3Ac, H4Ac, as well as H3K4Me2 associated with the Dkk1 promoter in livers of OP offspring when compared to OR (**Figure 8.26)**. However, there was no difference in the amount of either H3K9Me3 or H3K27Me3 associated with the hepatic *Dkk1* promoter between OR and OP offspring (**Figure 8.26)**. There was also no effect by maternal obesity in the amount of IgG binding within the region tested, which was used as a control (**Figure 8.26)**.

Cell Culture Study: Hepatocyte Analysis

Lipid Accumulation and Dkk1 Expression in NEFA-Treated H4IIEC3 Cells

At the highest NEFA concentration of 400 μmol/L, there was a significant decrease ($P<0.05$) of *Dkk1* mRNA content **(Figure 8.27).** There was also an increase in lipid accumulation with increasing NEFA concentration **(Figure 8.28 bottom)**, with no observable red droplets in the 0 μmol/L NEFA-treated group and many visibly red droplets in the 400 μmol/L NEFA-treated cells **(Figure 8.28 top).**

CHAPTER 5: DISCUSSION

To our knowledge, the present study is the first to present evidence that placental lipid storage and metabolism may be regulated in part through the placental Wnt pathway. These novel findings demonstrate that Wnt signaling may be directly involved in the accumulation of fat within the placenta, a pathophysiology that may have dire consequences for placental efficiency and fetal development.

Although data related to the current obesity prevalence in pregnancy is limited, several cohort studies predict that the figures match the increase in obesity in the population as a whole (28). Current recommendations for pregnant women focus primarily on controlling weight gain during pregnancy. However, there is increased evidence that pre-pregnancy weight may be as important to gestational and fetal health as the total amount of weight gained. Overall, studies have shown that maternal obesity, which has been associated with central subcutaneous adiposity, correlates strongly to gestational diabetes, hypertension and pre-eclampsia (30-34).

The current study utilized an obesity-prone rat model, which allowed us to observe the consequence of excessive body weight on the placenta without the potentially confounding effect of high-fat intake often observed in other models of diet-induced obesity. This approach is a unique and critical feature of the current study, as women are more likely to follow appropriate dietary recommendations during pregnancy than they may be before or after becoming pregnant. Therefore, a HF-diet-induced model of obesity was not appropriate for to accomplish our study aims. The current model of obesity has been well-characterized and is thought to exhibit many of the characteristics

of humans who are obese and have other symptoms of metabolic syndrome. **Appendix A** contains a thorough review of studies utilizing the model of Diet-Induced Obesity (DIO) as well as Obesity Proneness (OP) and Obesity Resistance (OR).

Fasting triglycerides were higher in OP than in OR dams, suggesting that the obese animals have a decreased ability to clear triglycerides, even after an overnight fast. This observation has been shown to be characteristic of the current model during the non-pregnant state, and has been likened to severely obese individuals who also have a disregulation in TG metabolism (11). In our study, OP dams had significantly higher NEFA levels than OR, and that OP dams also gave birth to growth-restricted offspring. Elevated NEFA levels are often seen in obese individuals (119), and it has been observed that pregnancies complicated with IUGR are also associated with elevated NEFA (120, 121), which agrees with our results.

We observed a significant decrease in birth weights of offspring from OP dams when compared to OR. Low birth weight, or IUGR, is associated with increased risk of numerous adult-onset diseases (122). Maternal obesity has most often been associated with fetal overgrowth, but there is evidence that obesity can also result in small-for-gestational-age offspring (41-44). It was previously believed that conditions that lead to decreased placental size (and therefore decreased area for nutrient transport and waste export) lead to IUGR (2, 3, 59). However, recent reports suggest that the ratio of fetal-to-placental weight is a better marker of placental efficiency, as it represents the amount of fetus produced for each gram of placenta. The ratio has been evaluated by several studies, which have concluded that a large placenta, when accompanied by a smaller fetus, is associated with IUGR as well as SGA offspring, gestational diabetes, and

possible risks of late-onset diseases (4-6, 59). In this study, we found that placental weight was not affected by maternal weight, but that the fetal:placental ratio was decreased in obese dams, indicating that despite being the same size as those of lean dams, the placentas of these dams were not able to support proper fetal development. Additionally, in the current study, placentas of OP dams had a significant decrease in the thickness of the decidual and junctional zones, with a concurrent increase in lipid accumulation. Appropriate vascularization and angiogenesis within the decidual zone is imperative for placental and fetal development (123), and any disruptions within its development could be potentially detrimental. We observed extensive decidual lipid accumulation, and although there is little data available regarding the consequence of this observation on the placenta, studies in other organ systems have shown that excessive fat accumulation results in substantial damage to cellular components and tissue structure (124).

Offspring born small for gestational (SGA) age are at risk for developing metabolic syndrome in adulthood (122), and it has recently been reported that SGA children may also be at risk for NAFLD (125). This is consistent with our results, since although OP offspring were smaller at birth, they had higher hepatic TG than OR and appeared to have an increase in lipid accumulation as measured by Oil Red O staining than OR, indicating their tendency to store lipid within the liver instead of utilizing the available energy substrates for growth. While no other studies have looked at the effects of obesity on pregnancy outcomes in the current model, a study of OR and OP mice showed that although OP dams gave birth to smaller pups, after being weaned to a standard diet for 13 weeks, offspring of OP dams were significantly heavier than

offspring of OR dams (126). Although we did not keep our pups past neonatal d 1, this observation is likely to also be true for animals in the current study, and likely accounts for reports that IUGR offspring tend to weigh more in adulthood than those babies born normal for gestational size.

Canonical Wnt signaling is activated by the binding of a Wnt molecule, which allows β-Catenin to accumulate in the nucleus and act as a transcription factor (85, 86, 127). Dkk1, a secreted-type inhibitor, interacts with and cause the endocytosis of LRP5/LRP6 coreceptor to prevent the formation of the Wnt-FZD-LRP5/LRP6 complex. In a pregnant mouse model, Dkk1 was highly expressed in the maternal decidual compartment, and culturing decidual cells in the presence of antisense Dkk1 decreased trophoblast invasiveness, while treatment with antisense β-Catenin increased trophoblast attachment and invasiveness, suggesting that an increase in Wnt signaling may prevent decidualization and trophoblast cell invasion (128). The inhibitory role of Wnt signaling on vascularization, in association with decreased Dkk1, has been shown in cases of diabetic retinopathy (129), in human umbilical vein endothelial cells (130), as well as in human breast tumors (131). In the current study, Dkk1 was significantly decreased and β-Catenin significantly increased in OP placentas, which corresponds to an inappropriate activation of Wnt signaling in "fatty" placentas that had a significant decrease in decidual/junctional thickness. Dkk1 mRNA expression was also decreased in trophoblast cells treated with NEFA, and the accumulation of fat was decreased when Dkk1 was overexpressed in these cells followed by NEFA treatment, suggesting that fat accumulation within trophoblasts is regulated in part by Wnt signaling.

The mRNA content of *Ppar-delta* was significantly increased in placentas of OP dams, and significantly decreased when JEG3 cells that overexpress DKK1 were treated with NEFA. Because PPAR-Delta has been shown to contain a β-Catenin responsive element within its promoter (132), we propose that the aberrant activation of Wnt signaling in OP placentas results in the activation of *Ppar-delta*, which was been shown to be a potent inducer of adipogenesis and lipogenesis (133). Although all three PPAR isoforms have been shown to be expressed in the placenta (134), their exact actions have not been thoroughly classified.

In the current study, we utilized the JEG3 trophoblast cell line in order to study the relationship between fat accumulation and the Wnt signaling pathway. JEG3 choriocarcinoma cells originated from a line of human extravillous trophoblasts, which makes them an ideal model for studying invasion, vascularization, and the transport of molecules that occurs at the primary interface between maternal and fetal tissues (135). Extravillous trophoblasts function as anchors connecting maternal and fetal interfaces, which is the reason for the frequent use of JEG3 cells in placental functional studies (136-138). The cell line has also been utilized in numerous studies to model syncytiotrophoblasts, or the direct layer of villous chorionic villi exposed to the maternal blood supply (139).

Additionally, we treated cells with a NEFA mixture composed of oleic and linoleic acids, an approach similar to that which has been utilized by previous studies of trophoblast fat accumulation (140-142). Additionally, studies have shown that the placenta may have a preference for transporting these acids (as well as DHA), presumably to guarantee an ample supply of these essential fatty acids to the fetus (143,

144). It has also been suggested that because glucose is the primary energy source for the fetus, the polyunsaturated fatty acids in particular function to provide the fetus with structural and metabolic components and do not serve as energy substrates (145).

The rates of childhood non-alcoholic fatty liver disease are increasing, and children with NAFLD are at risk for end-stage liver disease as well as insulin resistance and other symptoms of metabolic syndrome in adulthood (68, 146). The present study is the first to demonstrate that gestational obesity programs lipid accumulation in fetal liver with a concurrent decrease in the hepatic mRNA content of a potent inhibitor of Wnt, *Dkk1*, through histone modifications. We further showed that NEFA alone might lead to a decrease of *Dkk1* mRNA content *in vitro*. The role of Dkk1 in hepatic lipid metabolism is poorly understood. Although *Dkk1* expression is elevated during hepatocellular carcinoma, its function in cancer and hepatic lipid accumulation is likely to be different, as Dkk1 has been shown to be both upstream as well as downstream of β-Catenin (79), so the full Wnt pathway will need to be evaluated to fully characterize *Dkk1*'s mechanism of action.

Although the role of *Dkk1* in NAFLD remains unclear, it is certain that Wnt plays an important role in liver development as well as lipid metabolism, as shown by our placenta study. Therefore, it is notable that out ChIP analysis of the *Dkk1* promoter in neonatal livers demonstrated that the gene is regulated epigenetically in response to maternal obesity. The modification of histone tails through H3Ac, H4Ac, as well as H3K4Me2 are all known to be increased in actively transcribed genes (147-149), and we propose that the decreased expression of *Dkk1* in livers of OP offspring is directly related to the decrease in these histone marks. If these marks persist into adulthood, this histone

code will likely result in the perpetual decrease in *Dkk1*'s transcription, which will potentially lead to the uncontrolled activity of Wnt signaling, the consequence of which may be detrimental.

In conclusion, this study demonstrates, for the first time, a coordinated regulation of placental lipid metabolism by a β-Catenin inhibitor, *Dkk1*. While both the placenta and fetus require an extensive supply of essential fatty acids during gestation, we show that an excessive availability of NEFA in circulation leads to placental fat accumulation, potentially in response to aberrant Wnt activation. As the primary mode of communication between mother and fetus, the placenta plays an integral part in the adaptations that occur in a nutrient-restricted or abundant pregnancy, and any disregulations within placental structure may be deleterious for both placental and fetal development. Additionally, we showed that maternal obesity was associated with lipid accumulation in livers of neonatal offspring, which is a classic marker of NAFLD, and that *Dkk1* in these livers was epigenetically regulated. We propose that determining the mechanisms behind the *in utero* development of the disease may help decrease the rates of childhood NAFLD and prevent its progression into adulthood.

CHAPTER 6: FUTURE DIRECTIONS

Alternate Models of Gestational Obesity

Although the OP rats should become obese on a HF diet, our OP rats maintained their obesity status despite being on a control diet throughout gestation. This phenomenon has been observed in previous studies of obesity-prone rats (150), and allowed us to effectively demonstrate the consequences of being obese during pregnancy without the confounding effect of diet. As demonstrated in **Appendix A**, diet-induced, as well as obesity models developed by selective breeding in rats are commonly utilized approaches towards modeling metabolic syndrome in the human population. However, fewer studies have focused on the consequences of being obese during pregnancy, so it still remains unclear which models are most advantageous for this analysis. Feeding OP rats a Control and HF diet would add valuable information on this strain, and would potentially model human cases in which obese women did or did not follow dietary recommendations during pregnancy. Because obesity is often familial as well as lifestyle-induced, we consider that our strain of breeding-induced obesity is optimal for the current analysis. However, an additional approach would be to utilize standard Sprague-Dawley rats fed a HF diet for an extended period of time in order to obtain a true diet-induced model of obesity. Although this would eliminate any breeding-induced obesity characteristics, it would also introduce diet as a confounding factor. Additionally, Sprague-Dawley rats are notoriously inconsistent in their weight gain response to high-fat or high-energy feeding, making them similar to humans, so it is

probable that the animal number for such a study would be fairly large and would depend on whether the animals could gain enough weight on the diet.

Analysis of Placental Size and Dimensions as an Additional Indicator of Placental Efficiency

In the current study, placental weight remained unchanged while lipid accumulation increased in OP placentas when compared to OR. Because lipid likely weighs less than other placental components, it is probable that while placental weights did not change, there was a difference in placental size and dimension between OR and OP placentas, and these factors were not measured in the current study. Although studies are limited, it has been suggested that placental surface area and other dimensions, and not weight per-se, may be associated with poor fetal outcomes (151-153), so performing such an analysis in any future studies of obesity during pregnancy may be a valuable marker of fetal as well as placental development.

Placental Primary Cultures with NEFA Incubation, or Incubation of JEG3 Cells With Sera From Obese Versus Lean Women

Primary cultures of specialized cells are difficult to obtain and maintain, so JEG3 was selected for the current analysis because it originated from a line of cells vital for placental structure and transport. Our cell culture analysis showed a direct relationship between NEFA, Wnt, and placental lipid accumulation. However, the JEG3 cell line represents just one of several placental cell-types, so a primary culture of a variety of human placental cell-types would be an additional approach to further study the

consequences of being obese during pregnancy on placental molecular pathways. Additionally, while we isolated the effect of elevated NEFA in obese women on Wnt signaling, other plasma factors differ between lean and obese women, so utilizing complete sera from women with varying degrees of obesity or other markers of metabolic syndrome would add important knowledge to the study of obesity during pregnancy and placental development.

Alternate Measures of Oxidative Stress or Determination of the Mechanisms Behind Peroxidation Prevention

While numerous studies have shown that excess lipid accumulation leads to the oxidation of lipids, we did not observe an increase of MDA, a marker of lipid peroxidation, in OP placentas when compared to OR. Although the TBARS assay is a valid and commonly utilized approach to measuring lipid-induced oxidation, it has been suggested to be somewhat unspecific and dependent on the content of specific fatty acids (154-156), so other approaches may be more appropriate. Additionally, if our TBARS results are in fact correct, and there is no increase in lipid peroxidaton in OP placentas when compared to OR, it would be valuable to elucidate the molecular mechanisms behind this antioxidant defense system.

Binding Analysis within the Ppar-Delta PromoteraAs well as Within the Promoters of Target Genes to Determine the Precise Role of β-Catenin and the Mechanism Behind its Activation

We observed that the overexpression of DKK1 in NEFA-treated cells results in decreased lipid accumulation and expression of lipid metabolism-associated genes. Specifically, we observed that an increase in β-Catenin was also accompanied by a decrease in PPPAR-Delta, a potent activator of lipid metabolism. We also observed that OP placentas had increased Ppar-Delta mRNA content and β-Catenin protein. It has been suggested that PPAR-Delta is a downstream target of β-Catenin, but this needs to be confirmed in the current study in order to determine whether the decrease in lipid accumulation we observed was due to the decrease in PPAR-Delta-activated genes in response to β-Catenin inhibition. ChIP analysis within the PPAR-Delta promoter, both within our cell culture model and in placental tissue samples, would add valuable information related to its control by β-Catenin. Additionally, binding studies of PPAR-Delta itself within the promoters of lipid transporters and TG synthesis-related genes would confirm the role of this transcription factor in placental lipid accumulation in response to gestational obesity.

Role of Wnt in other Tissues that have Potential Ectopic Lipid Accumulation, Including Liver, Pancreas, and Muscle

We have demonstrated a potentially novel role of Dkk1 and Wnt signaling in placental lipid metabolism. However, the role of Wnt in other tissues is not yet well-characterized. Studies have suggested that the inhibition of Wnt is necessary for the

maturation of pre-adipocytes in adipose tissues. However, the additional accumulation of mature adipocytes is associated with increased levels of β-Catenin, suggesting that in adipose tissue, the role and action of Wnt fluctuates with adipogenesis progression (157). In liver, β-Catenin has been shown to be necessary for the prevention of lipid accumulation, since mice with liver-specific β-Catenin knockouts developed non-alcoholic fatty liver disease in response to a steatogenic diet (158). Therefore, it appears that Wnt may be a relatively novel and promising pathway for the study of adipogenesis, and particularly in metabolically-active tissues at risk for ectopic lipid accumulation in response to obesity, such as liver, pancreas, and muscle.

CHAPTER 7: TABLES

Table 7.1: Animal Diets. Obesity-Resistant (OR) and Obesity-Prone (OP) dams were fed a control diet *ad libitum* beginning on gestational day 2 until they were fasted on gestational day 20.

Diet Composition (Per 12.5 kg)			
		Control (C)	
		Gram %	**kcal %**
Protein		20	20
Carbohydrate		64	64
Fat		7	16
	Total	*91.0*	*100*
	kcal/g	*3.9*	
Ingredients		**Gram**	**kcal**
Casein		200	800
L-Cystine		3	12
Corn Starch		437.2	1749
Maltodextrin		100	400
Sucrose		102	408
Cellulose		50	0
Soybean Oil		25	225
Lard		47	423
Mineral Mix		10	0
DiCalcium Phosphate		13	0
Calcium Carbonate		5.5	0
Potassium Citrate		16.5	0
Vitamin Mix		10	40
Choline Bitartrate		2	0
	Total	*1021.25*	*4057*

Table 7.2: Primer Information.

Gene	Species	Forward Sequence and Location	Reverse Sequence and Location	Ensembl ID
Dkk1	Rat	ATGCCCTCTGACCACAGCCATT (+439)	CACCGTGGTCATTGCCAAGGT (+517)	ENSRNOT00000015771
	Human	GATCATAGCACCTTGGATGGG (+635)	GGCACAGTCTGATGACCGG (+742)	ENST00000373970
Ppar-Delta	Rat	GCTCACCGAGTTCGCCAAGAAC (+1024)	CCTCATGCACGCCGTACTTGAG (+1112)	ENSRNOT00000042539
	Human	CCCCACGTCTGTCCTCCTTTCTTAT (+1954)	TGTGCAAAAGCAGAGGTCCTGTTC (+2039)	ENST00000360694
Fatp1	Rat	CCACCATTCCTACAGCAT (+996)	TGCTGAGTGGTAGAGAGGTA (+1062)	ENSRNOT00000024659
	Human	AACCTCAGAGGAACCCGTGCCT (+2137)	TGAAAAGCAGGGAGAGGAGGC (+2206)	ENST00000252595
Fat/CD36	Rat	AGTGCTCTCCCTTGATTCTGC (+151)	GAGCCCACAGTTCAGATCACA (+213)	ENSRNOT00000061687
	Human	CAAGAAAAATGGGCTGTGACCG (+247)	AACACAGCCAGGACAGCACCAAT (+319)	ENST00000447544
Dgat1	Rat	TCAATCTGTGGTGCCGCCAG (+708)	CCCACTGACCTTCTTCCCTGCA (+775)	ENSRNOT00000039795
	Human	GTGGCTTCAGCAACTACCGT (+507)	CGGGCATTGCTCAAGATC (+573)	ENST00000332324
Lipin1	Rat	AGCCTGGTAGATTGTCAGAG (+738)	GAGGACAAGAGCTAGAGAGAAC (+804)	ENSRNOT00000005863
	Human	GCGTAAAATGTCCCAAGCAGCC (+2761)	CGGGGAGACCTATCCTTTAATGGG (+2836)	ENST00000256720
Lipin2	Rat	CCAGTTACCCACAGACAGTGTGCC (+792)	CTCTCGGATGGCTTCACCTCCA (+855)	ENSRNOT00000020476
	Human	ACCACCTATCCCCAGACAGCGT (+936)	CAGGCTCTCCGCAGGTTTCA (+1004)	ENST00000261596
Lipin3	Rat	CCCTGAAGAGAAGCCAGCACCT (+1107)	TCAGAGTCCAGGGAGGGCAGAT (+1176)	ENSRNOT00000067531
	Human	CGGCACCATCACCAAGTCAGAT (+2029)	TGGTGTGTCCAGTCTTTCCCCA (+2097)	ENST00000373257
L7a	Rat	GAGGCCAAAAAGGTGGTCAATCC (+64)	CCTGCCCAATGCCGAAGTTCT (+127)	ENSRNOT00000006754
	Human	TTTGGCATTGGACAGGACATCC (+145)	AGCGGGGCCATTTCACAAAG (+208)	ENST00000323345

Table 7.3: Maternal and Fetal Observations. Obesity-Resistant (OR) and Obesity-Prone (OP) dams underwent cesarean delivery on gestational day 21. n=5 litters. Results are reported as means ± SEM. *P<0.05 when compared to OR. #P<0.01 when compared to OR.

Maternal And Fetal Observations		
Variable	**Strain**	
	OR	OP
Total Gestational Weight Gain (g)	124.20 ± 2.67	128.78 ± 6.36
Total Mass Gain (g)	74.98 ± 4.74	78.63 ± 6.93
Birth Weight (g)	4.14 ± 0.24	3.44 ± 0.08#
Placental Weight (g)	0.50 ± 0.01	0.49 ± 0.01
Fetal:Placental Ratio	8.26 ± 0.41	7.11 ± 0.16#
Products of Conception (g)	49.22 ± 3.17	50.15 ± 1.26
Litter Size	10.60 ± 0.68	12.80 ± 0.58*

*P<0.05 When Compared to OR

#P<0.001 When Compared to OR

CHAPTER 8: FIGURES

Figure 8.1, 8.2, 8.3: Food Intake and Body Weight Information. Gestational food intake **(Figure 8.1)**, body weight **(Figure 8.2)** curves, and amount of food (g) per gram of body weight **(Figure 8.3)** in Obesity-Resistant (OR) and Obesity-Prone (OP) dams throughout pregnancy. Results are reported as means ± SEM. n=5 litters. *P<0.01 when compared to OR.

Figure 8.1

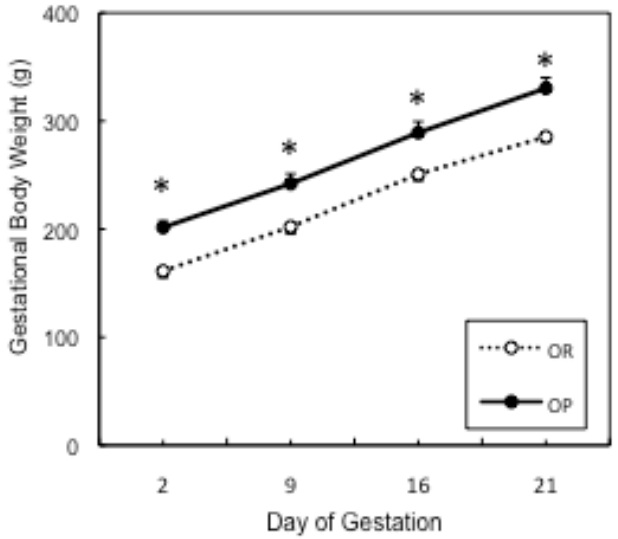


Figure 8.2

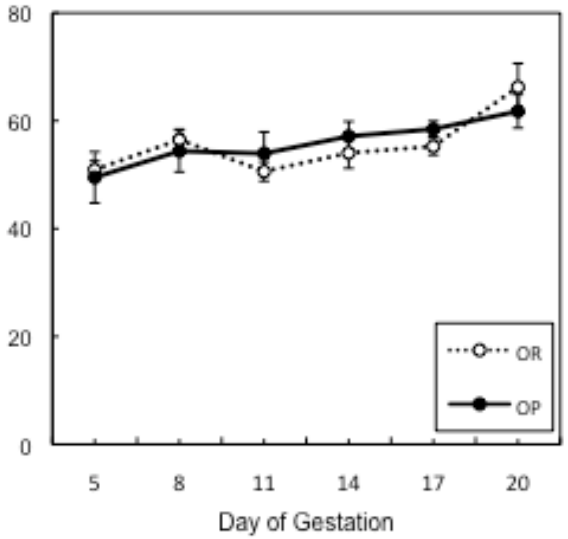


Figure 8.3

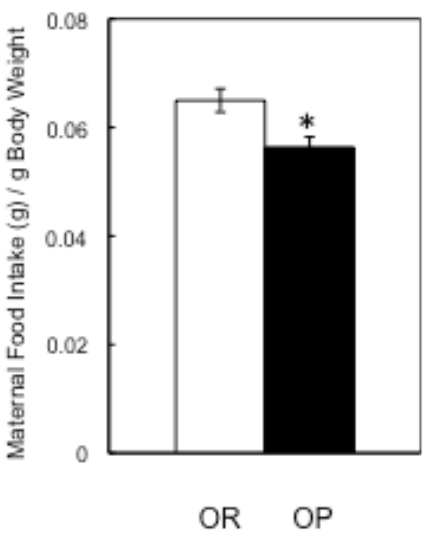


Figure 8.4, 8.5, 8.6, 8.7: Triglyceride and Non-Esterified Fatty Acid Measurements. Fasting plasma Triglycerides (TAG) **(Figure 8.4)** and Non-Esterified Fatty Acids (NEFA) **(Figure 8.5),** as well as placental TG **(Figure 8.6)** and NEFA **(Figure 8.7)** in Obesity-Resistant (OR) and Obesity-Prone (OP) dams at the time of cesarean delivery on gestational d21. Results are reported as means ± SEM. n=5 litters. *P <0.05 when compared to OR. #P <0.01 when compared to OR.

Figure 8.4

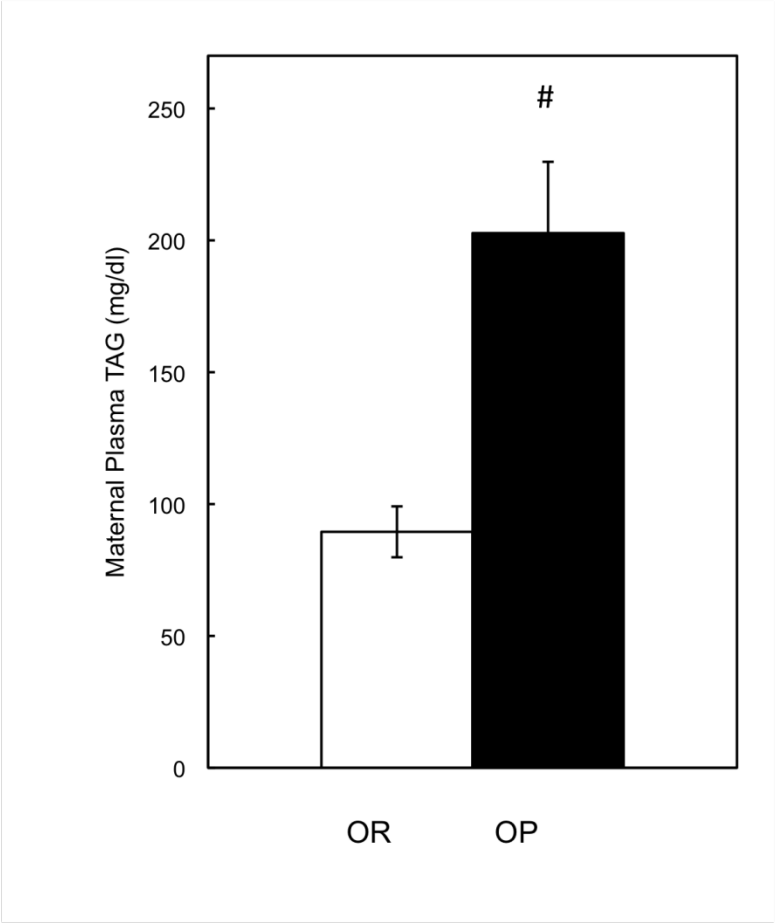
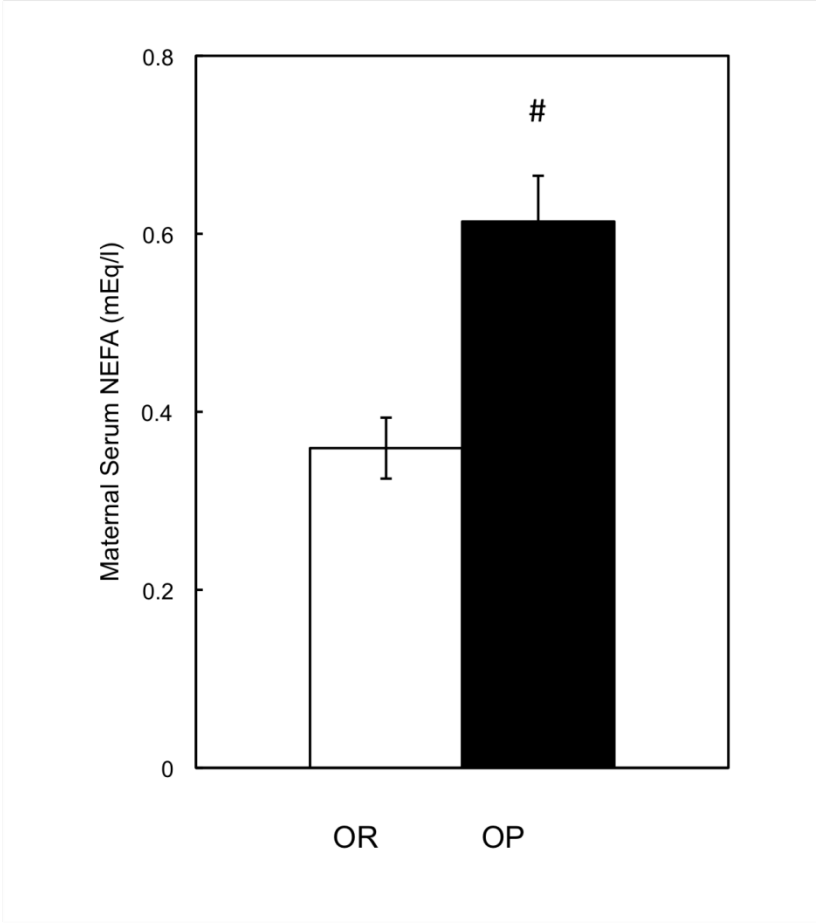


Figure 8.5



(Figure 8.4, 8.5, 8.6, 8.7 Continued)

Figure 8.6

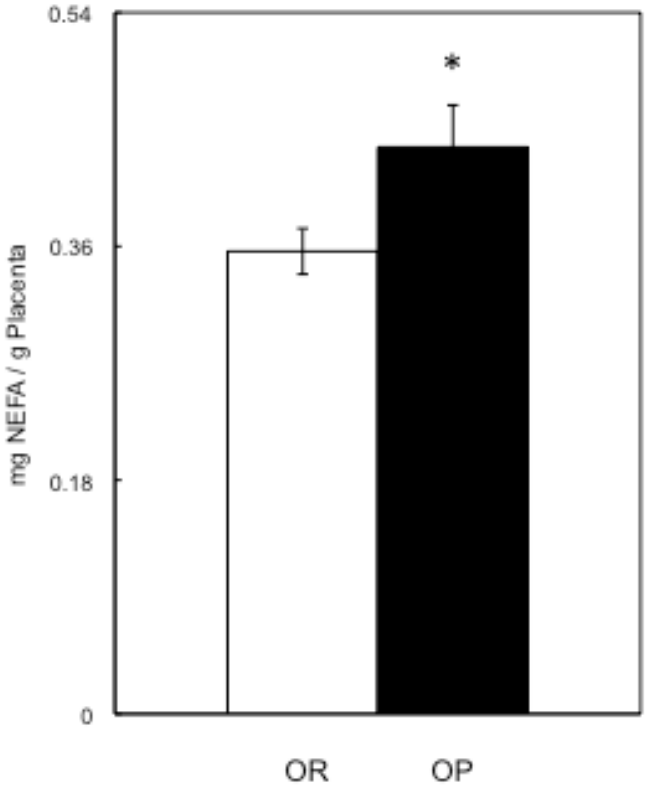


Figure 8.7

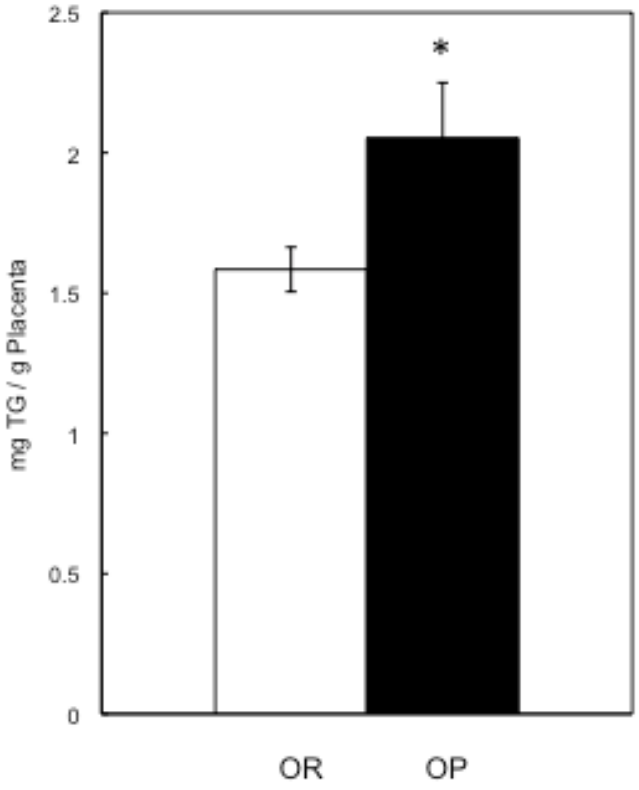


Figure 8.8, 8.9, 8.10, and 8.11: Placental Characteristics. Representative image of Oil Red O Staining of placental fat accumulation in placentas of Obesity-Resistant (OR) **(Figure 8.8 left)** and Obesity-Prone (OP) **(Figure 8.8 right)** dams with magnified images for clearer viewing. "Lz": Labyrinthine Zone, "Jz": Junctional Zone, "Dz": Decidual Zone". Arrows point to areas of significant red staining. Measurement of the decidual/junctional thickness **(Figure 8.9)**, mRNA analysis **(Figure 8.10)**, and Malondialdehyde MDA concentration analysis **(Figure 8.11)** was performed in placentas of 5 dams (2 from each dam, for n=10) from each strain, with 5-7 measurements per placenta for thickness measurement. Results are reported as means ± SEM. *P<0.01 when compared to OR.

Figure 8.8

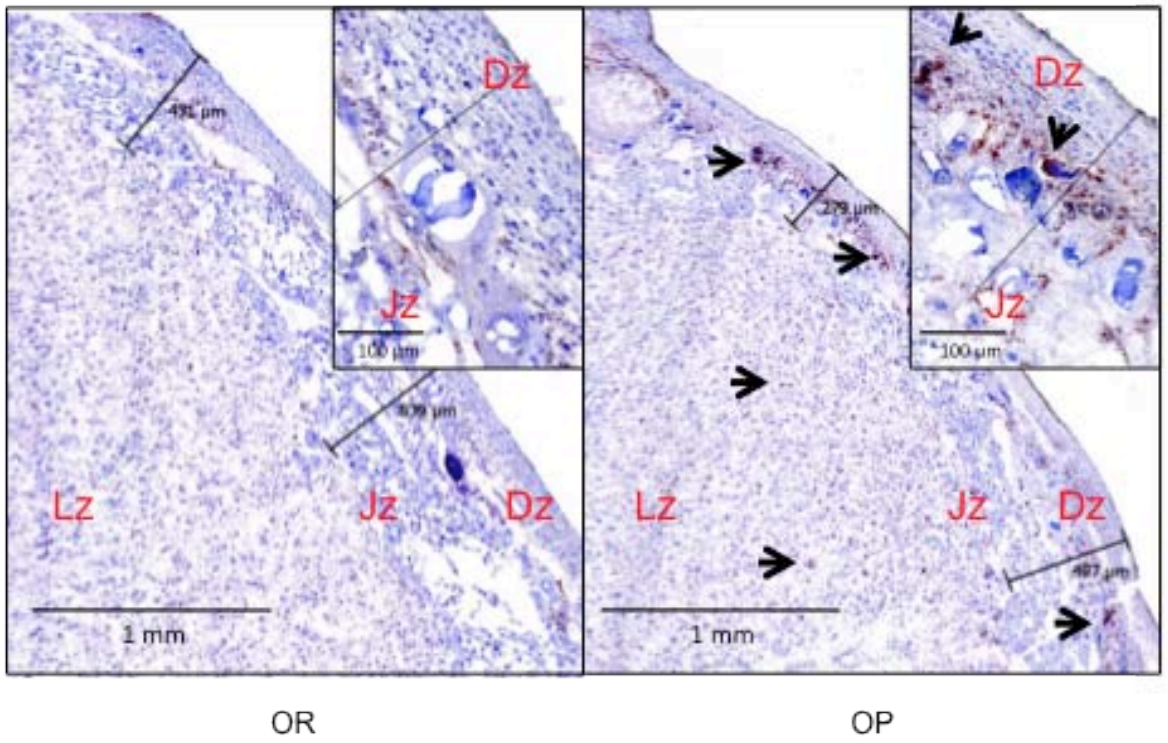


Figure 8.9

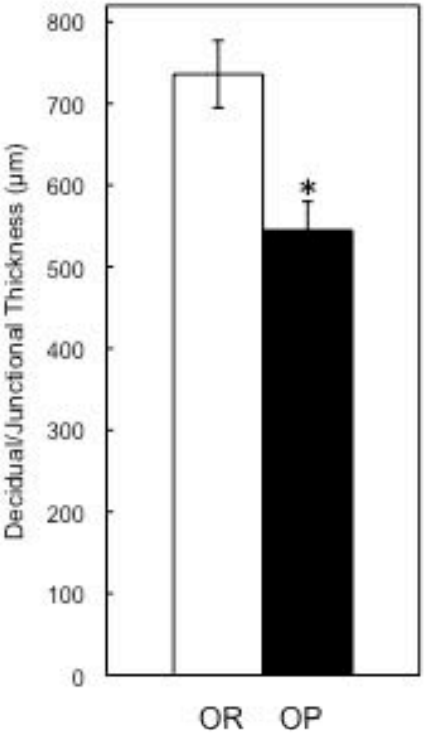


Figure 8.8, 8.9, 8.10, and 8.11 (Continued)

Figure 8.10

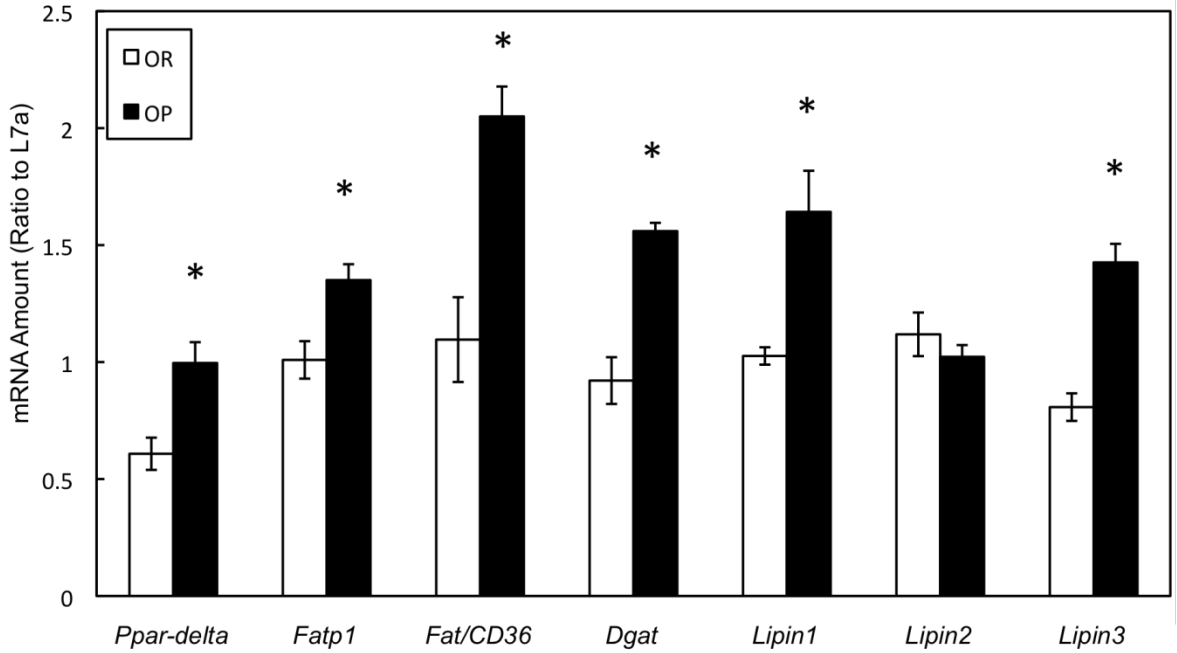


Figure 8.11

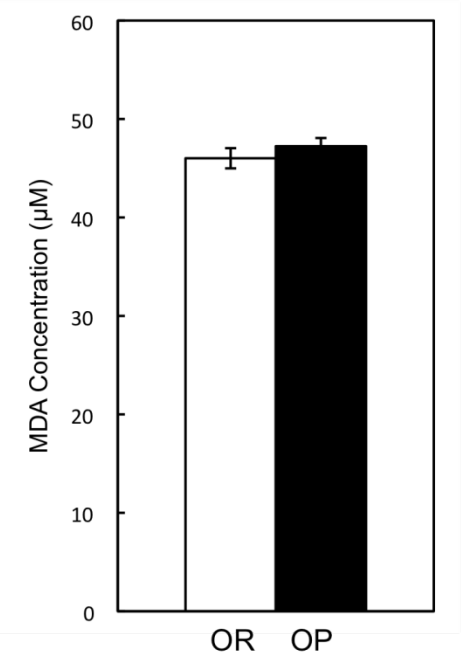


Figure 8.12, 8.13, 8.14: Placental *Dkk1* and β-Catenin Analysis. Whole-tissue Dkk1 mRNA content **(Figure 8.12)** in placentas of Obesity-Resistant (OR) and Obesity-Prone (OP) dams. Representative image of Dkk1 protein localization in the decidual/junctional zones in placentas of OR and OP dams as well as in the labyrinthine zone of placentas of OR and OP dams **(Figure 8.13).** Representative image of β-Catenin protein localization in the decidual/junctional zones in placentas of OR and OP dams as well as in the labyrinthine zone of placentas of OR and OP dams **(Figure 8.14)**. Red color represents β -Catenin or Dkk1 protein, and blue color represents nuclear staining. n=10 for mRNA analysis, and n=5 for staining. Results are reported as means ± SEM. *P<0.01 when compared to OR.

Figure 8.12 **Figure 8.13**

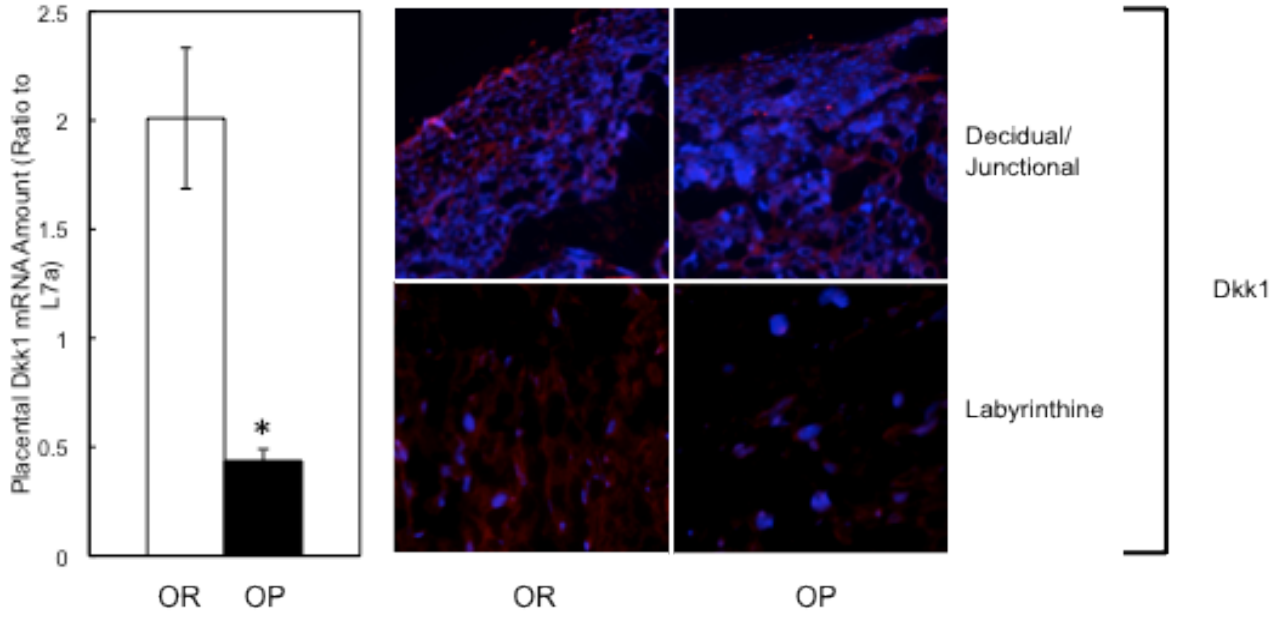


Figure 8.12, 8.13, 8.14 (Continued)

Figure 8.14

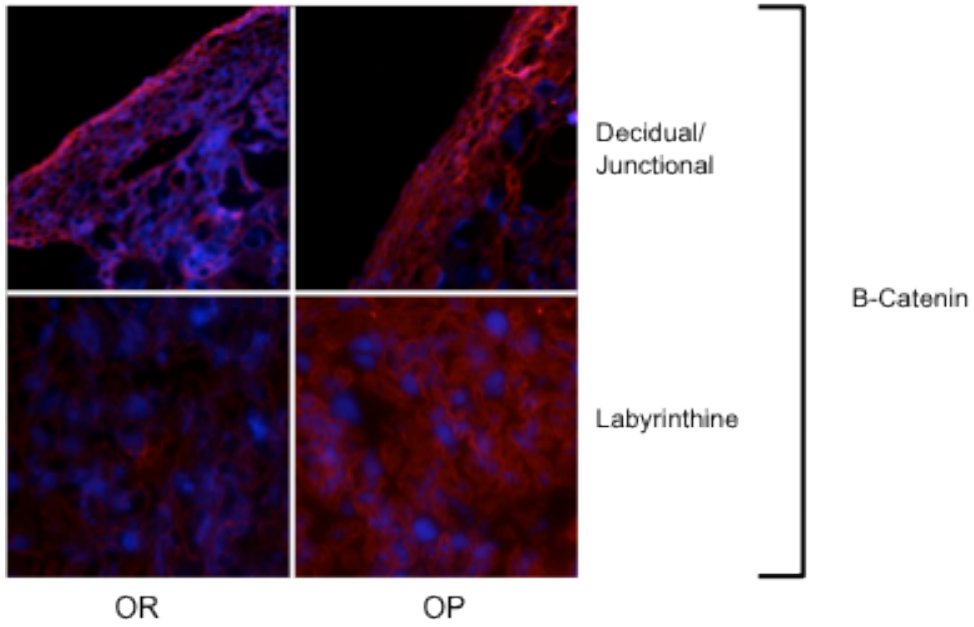


Figure 8.15, 8.16, 8.17: *DKK1* mRNA and Lipid Content in NEFA-Treated JEG3 Cells.
DKK1 mRNA content in JEG3 cells following treatment with 400 µ mol/L Non-Esterified Fatty Acids (NEFA) **(Figure 8.15)**, with a representative images of Oil Red O staining **(Figure 8.16 top)** in cells following 0 and 400 µ mol/L NEFA treatment and quantification of the Oil Red O staining **(Figure 8.16 bottom),** as well as the MDA concentration from TBARS assay from the same experiment **(Figure 8.17).** n=3 for mRNA and staining analyses. Results are reported as means ± SEM. *P<0.01 when compared to 0 µ mol/L treatment.

Figure 8.15

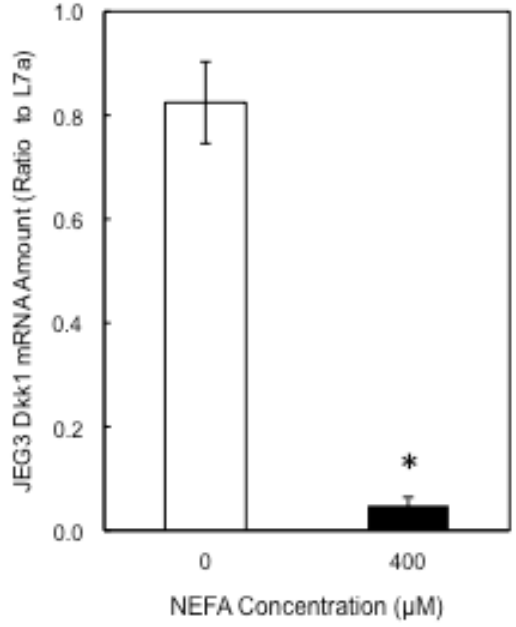


Figure 8.16

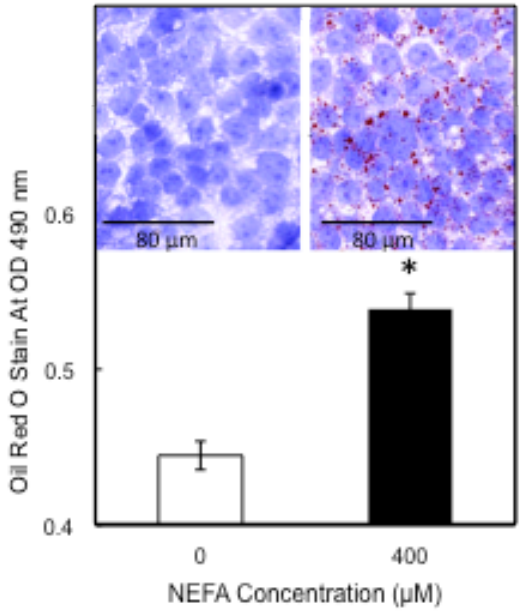


Figure 8.17

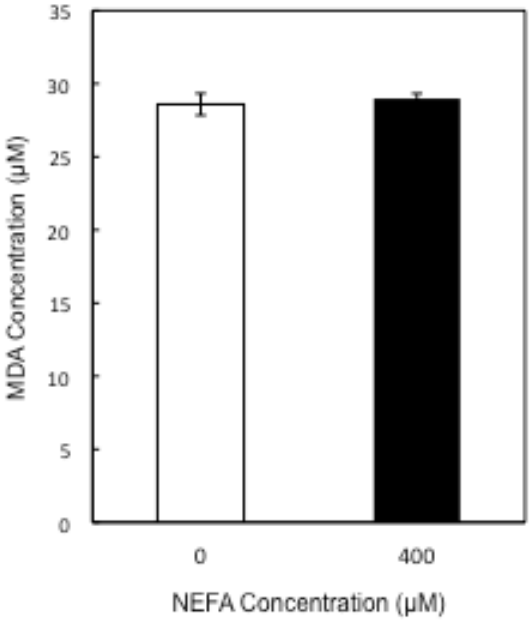


Figure 8.18, 8.19, 8.20, 8.21: DKK1, β -Catenin and Lipid Analysis in NEFA-Treated JEG3 Cells Following Dkk1 Overexpression. *DKK1* mRNA content following DKK1 overexpression in JEG3 cells treated with 400 µ mol/L Non-Esterified Fatty Acids (NEFA) **(Figure 8.18),** as well as a representative image of β -Catenin protein localization in WT (wild-type, following transfection of empty pCMV) and +Dkk1 (Dkk1-overexpressed) JEG3 cells following treatment with 400 µ mol/L NEFA **(Figure 8.19)**. Red color represents β -Catenin protein, and blue color represents nuclear staining. Representative image of Oil Red O staining in WT and +Dkk1 JEG3 cells following treatment with 400 µ mol/L NEFA **(Figure 8.20, top)** and quantification of the Oil Red O staining **(Figure 8.20 bottom).** mRNA analysis of +Dkk1 JEG3 cells following NEFA treatment **(Figure 8.21)**. n=3 for mRNA analysis, staining, and Oil Red O quantification. Results are reported as means ± SEM. *P<0.05 when compared to WT cells. #P<0.01 when compared to WT cells.

Figure 8.18

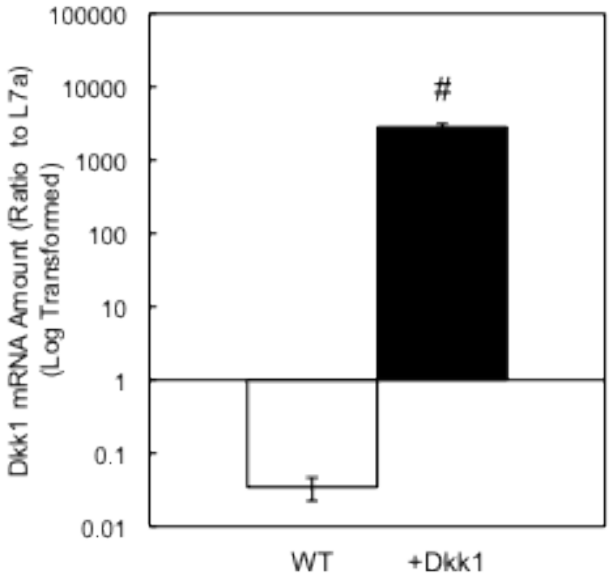


Figure 8.19

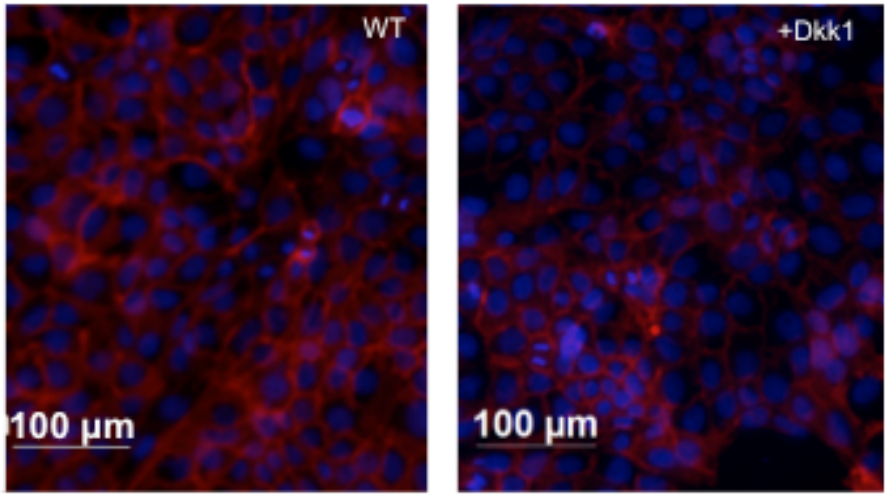


Figure 8.18, 8.19, 8.20, 8.21 (Continued)

Figure 8.20

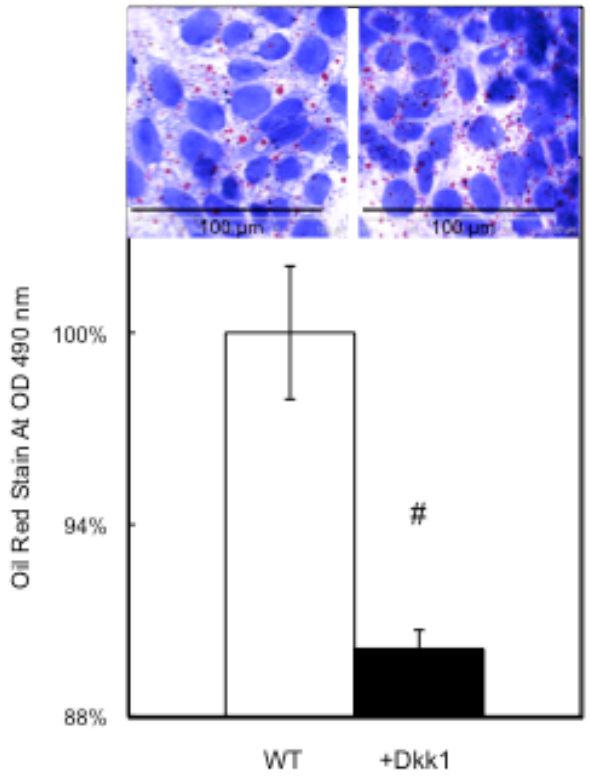


Figure 8.21

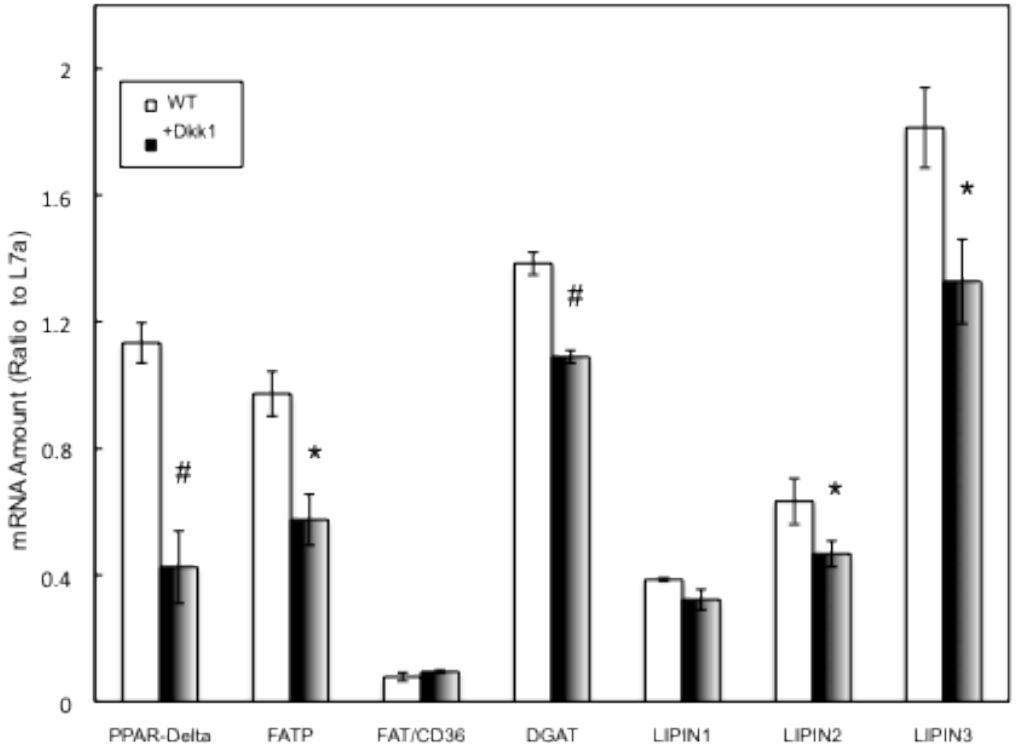


Figure 8.22, 8.23, 8.24: Hepatic Triglyceride (TG), Non-Esterified Fatty Acid (NEFA) Measurements and Oil Red O Staining in Neonatal Offspring. Hepatic TG **(Figure 8.22)**, NEFA **(Figure 8.23),** and Oil Red O staining **(Figure 8.24)** in neonatal offspring of Obesity-Resistant (OR) and Obesity-Prone (OP) dams at the time of cesarean delivery on gestational d21. Results are reported as means ± SEM. n=10 livers. *P <0.05 when compared to OR.

Figure 8.22

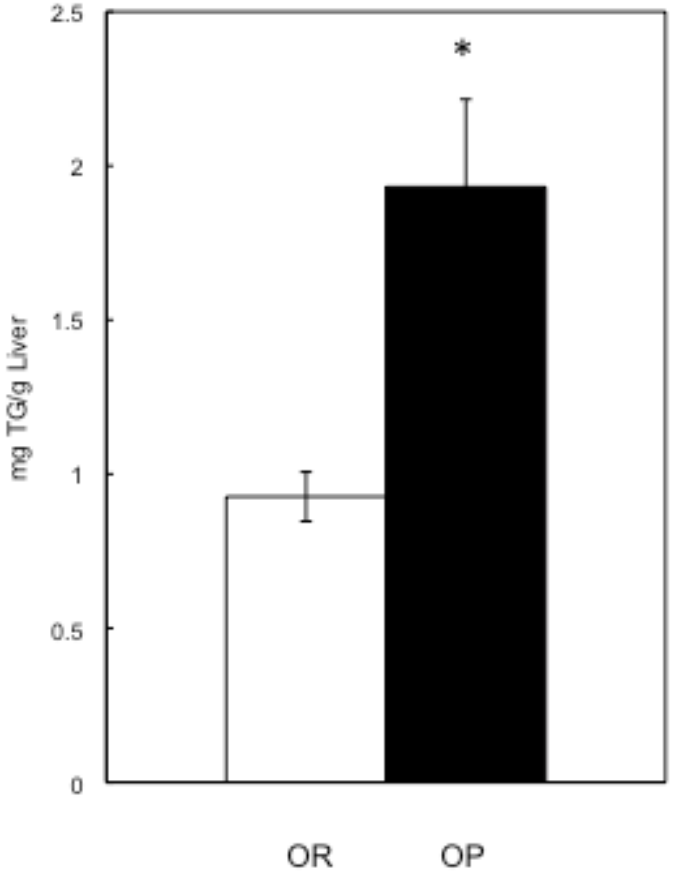


Figure 8.23

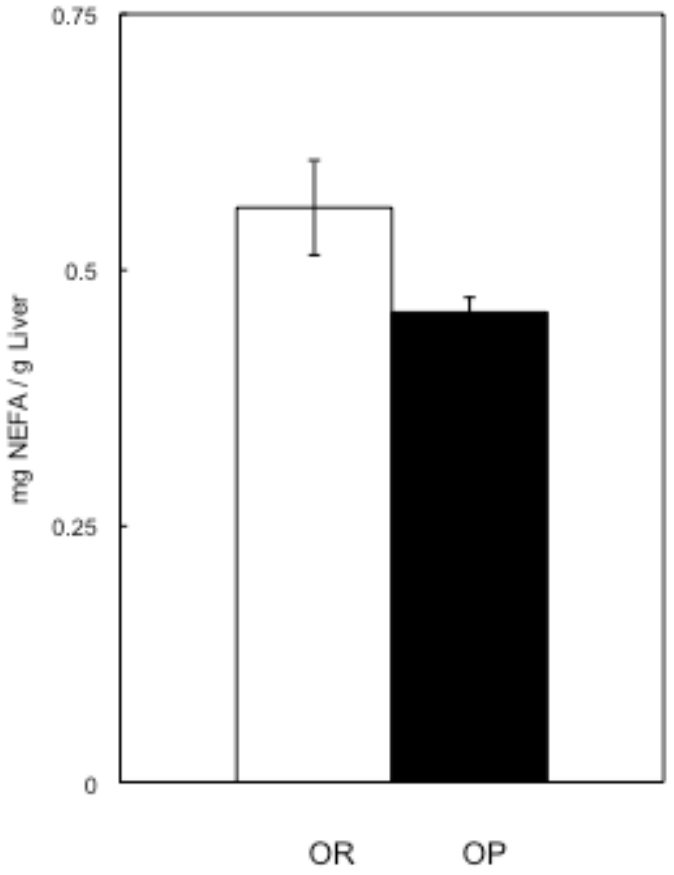
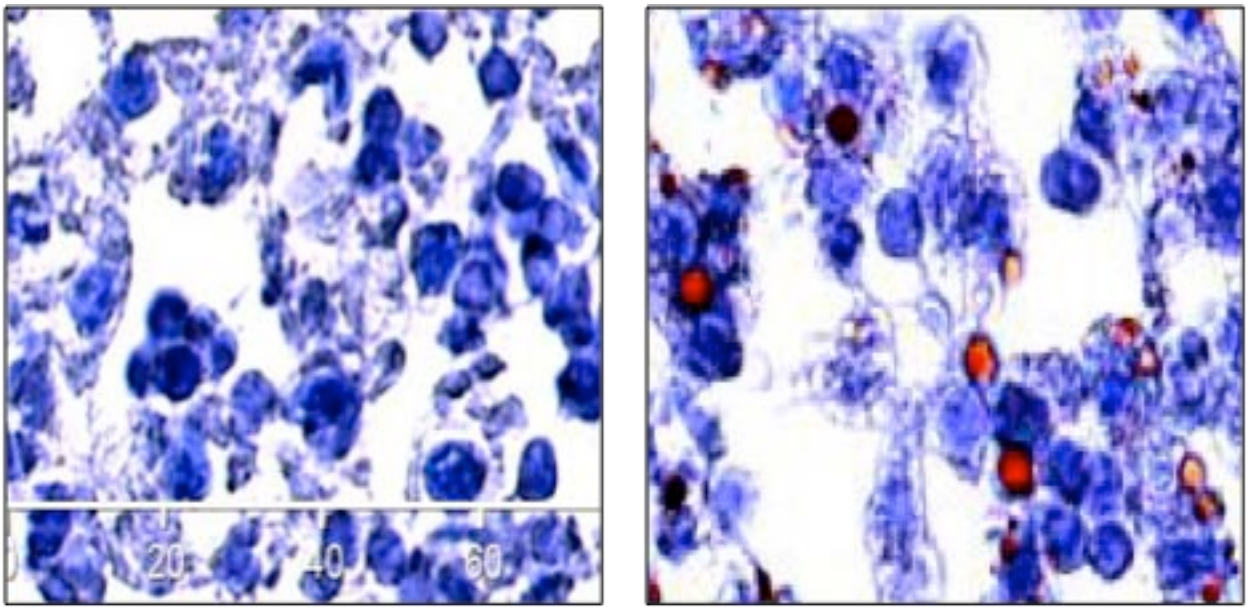


Figure 8.22, 8.23, 8.24 (Continued)

Figure 8.24



OR OP

Figure 8.25, 8.26: *Dkk1* Expression and Histone Modifications in Livers of OR and OP Neonatal Offspring. Whole-tissue Dkk1 mRNA content **(Figure 8.25)** and promoter Chromatin Immunoprecipitation (ChIP) analysis **(Figure 8.26)** in livers of neonatal offspring of Obesity-Resistant (OR) and Obesity-Prone (OP) dams. n=10 livers. Results are reported as means ± SEM. *P<0.05 when compared to OR.

Figure 8.25

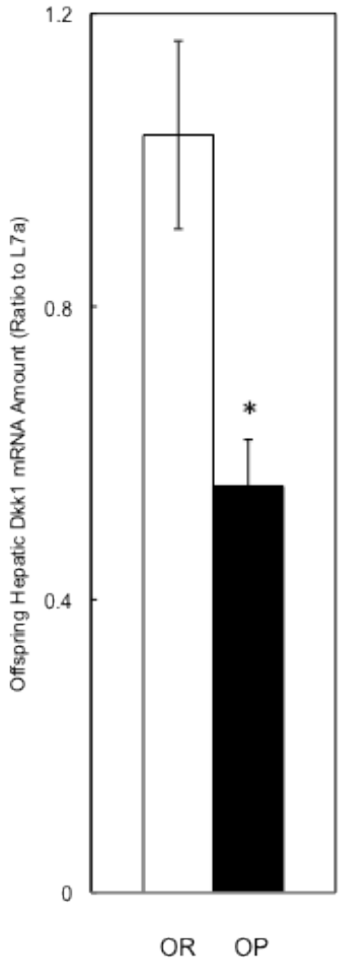


Figure 8.26

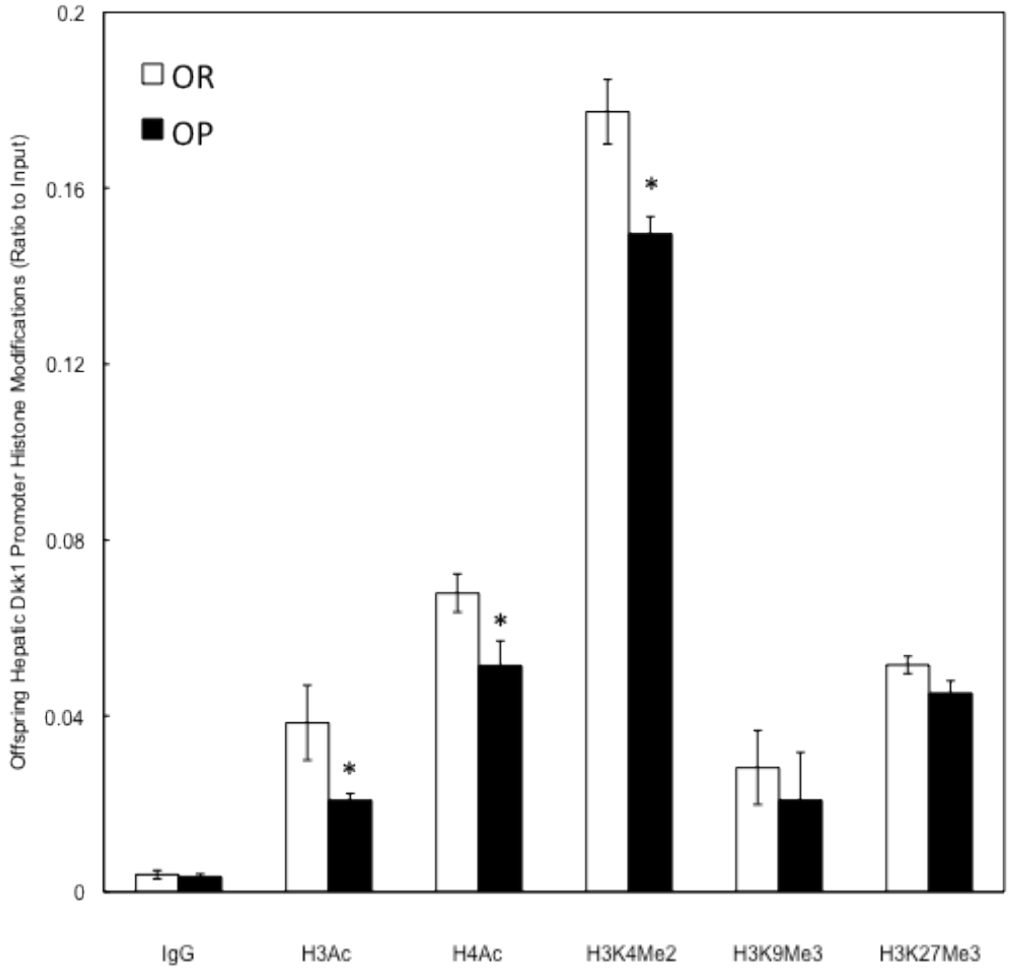


Figure 8.27, 8.28: *Dkk1* mRNA and Lipid Content in NEFA-Treated H4IIEC3 Cells.
Dkk1 mRNA content in H4IIEC3 rat hepatocyte cells following treatment with 400 µ mol/L Non-Esterified Fatty Acids (NEFA) **(Figure 8.27)**, with a representative images of Oil Red O staining **(Figure 8.28 top)** in cells following 0 and 400 µ mol/L NEFA treatment and quantification of the Oil Red O staining **(Figure 8.28 bottom).** n=3 for mRNA and staining analyses. Results are reported as means ± SEM. *P<0.01 when compared to 0 µ mol/L treatment.

Figure 8.27

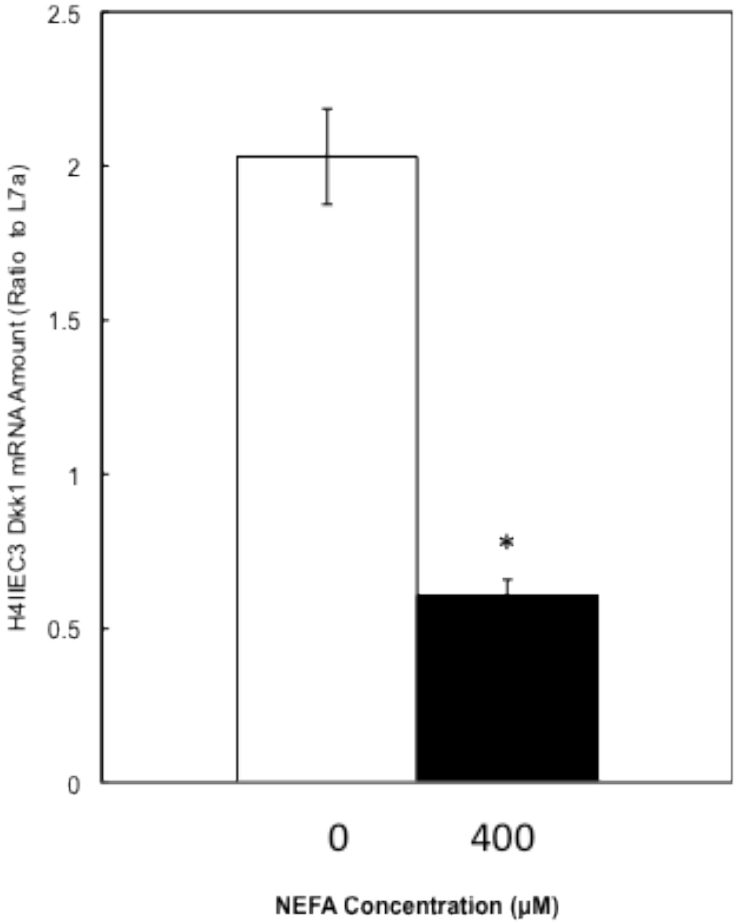
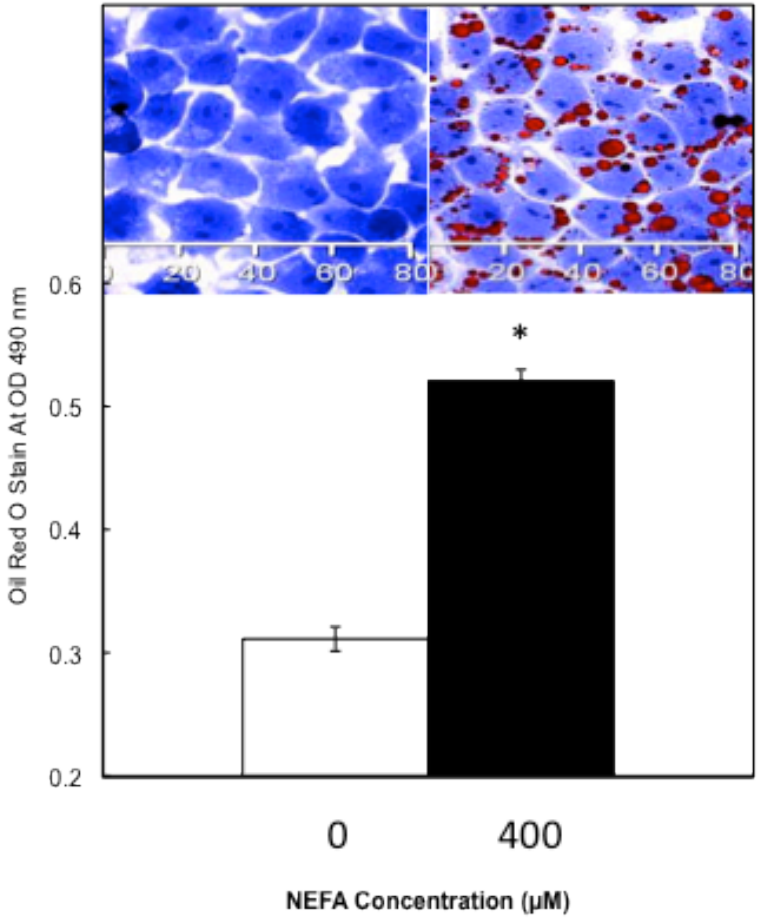


Figure 8.28



APPENDIX A: COMMON RODENT MODELS OF OBESITY

A thorough review of rodent models of diet-induced obesity (DIO), dietary resistance (DR), obesity proneness (OP) and obesity resistance (OR) has been made available as a supplemental file named **Appendix A-Rodent Obesity Models.pdf.**

APPENDIX B: QUANTITATIVE REAL-TIME PCR DISSOCIATION CURVES

The dissociation (melting) curves for all primers used in the current studies have been made available as a supplemental file named **Appendix B-qRT-PCR Curves.pdf**

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160. Strakovsky RS, Zhang X, Zhou D, Pan YX. Gestational High Fat Diet Programs Hepatic Phosphoenolpyruvate Carboxykinase (Pck) Expression and Histone Modification in Neonatal Offspring Rats. J Physiol. 2011 Mar 28.

CURRICULUM VITAE

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EDUCATION

PhD, Nutritional Sciences
University of Illinois Champaign - Urbana, August 2006 - May 2011.

Dissertation Title: *A Decrease in Dkk1, a Wnt Inhibitor, Contributes to Placental and Fetal Lipid Accumulation in an Obesity-Prone Rat Model.*

Director of Research and Dissertation Advisor: Yuan-Xiang Pan, PhD.

Dietetic Internship
University Of Illinois Champaign - Urbana, August 2009 - May 2010.

B.S Molecular and Cellular Biology
University of Illinois Champaign - Urbana, August 2001 - May 2005.

EMPLOYMENT

University of Illinois, Center For Teaching Excellence
Training Session Facilitator, August 2010 & January 2011

- Trained teaching assistance for the campus-wide Graduate Academy for College Teaching.
- Trained graders and office hours teaching assistants for the campus-wide Graduate Symposium on Grading and Office Hours.

University of Illinois, Department of Food Science and Human Nutrition and Kraft Company
Research Apprentice Program (RAP1) Team Leader, May-August 2010

- Organized learning opportunities for High School students on campus in collaboration with Oscar Meyer/Kraft.

University of Illinois, Division of Nutritional Sciences
Research Assistant, August 2006-Present

- Perform animal and molecular research in the area of genetics of fetal and maternal nutrition in the laboratory of Y-X Pan, PhD and Hong Chen, PhD.

University of Illinois, Department of Food Science and Human Nutrition
Teaching Assistant, August 2007-May 2009

- Organized and provided reviews, held discussions, graded exams/quizzes and held office hours for Nutritional Aspects of Disease, Nutritional Biochemistry I/II and Principles of Nutrition. Earned "Excellent" ranking from students during each semester.

Krannert Center For the Performing Arts
Ticket Office Supervisor, January 2005-August 2008

- Trained students, provided customer service by phone and in person, had responsibility for nightly cash counts and all weekend sales

Camp Ben Frankel
Unit Head and Assistant Program Director, June 2003-June 2006

- Trained future and current staff, planned programs and organized events for over 100 campers at an overnight camp.

VOLUNTEERING AND PROFESSIONAL SERVICE

American Society for Nutrition
Student Representative, Nutrient-Gene Interactions Research Interest Section, September 2010 - June 2011.

- Assist leaders in the field in planning ASN events for the 2011 Experimental Biology Meeting in Washington, DC.

Sprouts At The Market
Volunteer

- Provided nutritional programming for kids under the age of seven at the Urbana Farmer's Market, Summer 2010.

Nutritional Sciences Grad Student Association
Various Event Planning Committees, Fall 2008, Spring 2009.

Nutritional Sciences Grad Student Association
Fundraising Committee, Summer 2008.

Volunteer Illini Projects, Board of Directors
Tutoring Director 2003-2005.

- Headed and organized over ten tutoring opportunities for University of Illinois students within the Champaign - Urbana community.

Volunteer Illini Projects, Tutor
Matthew House and Thomas Payne Elementary, Champaign, IL, 2001-2005.

Volunteer Illini Projects
Champaign Park District, 2004-2005.

Volunteer Illini Projects
Volunteer, Blood Drives, 2001-2002.

PROFESSIONAL PRESENTATIONS

Oral Presentations
Experimental Biology 2011, Washington, DC

- *Gestational High Fat Diet Programs Hepatic Gluconeogenic Gene Expression and Histone Modification in Offspring Rats.*

Nutritional Sciences Oral Competition, University of Illinois, Champaign - Urbana, April 2011

- *Gestational High Fat Diet Programs Hepatic Gluconeogenic Gene Expression and Histone Modification in Offspring Rats.*

Experimental Biology 2010, Anaheim, CA

- *Obese Rat Pregnancy Impaired Fetal Development Independent of Diet and Involved The Placental Wnt Pathway.*

Nutritional Sciences Oral Competition, University of Illinois, Champaign - Urbana, April 2010

- *Obese Rat Pregnancy Impaired Fetal Development Independent of Diet and Involved The Placental Wnt Pathway.*

Staff Presentation, Omega-3 Fatty Acids
McKinley Health Center, University of Illinois, Champaign - Urbana, December 2009.

Poster Presentations

Experimental Biology 2009, New Orleans, LA

- *Prenatal Low Protein Diet Influences Placental Gene Expression in Rats.*

Nutritional Sciences Symposium Poster Competition, University of Illinois, Champaign - Urbana, April 2009.

Experimental Biology 2008, San Diego, CA

- *Maternal Protein and Folate Intake Affects Gene Expression and DNA Methylation in Rat Placenta.*

Experimental Biology 2007, Washington, DC

- *Expression of TRB3 mRNA Correlates to the Temporal Phosphorylation of eIF2α.*

PUBLICATIONS

Abstracts

- **R. Strakovsky**, Y-X. Pan. Gestational High Fat Diet Programs Hepatic Gluconeogenic Gene Expression And Histone Modification In Offspring Rats. *FASEB J* March 17, 2011 25:351.5

- **R. Strakovsky**, Y-X. Pan. Obese Rat Pregnancy Impaired Fetal Development Independent of Diet and Involved the Placental Wnt Pathway. *FASEB J*. 2010; 24:212.3
-
- **R. Strakovsky**, Y-X. Pan. Prenatal Low Protein Diet Influences Placental Gene Expression In Rats. *FASEB J*. 2009; 23:724.6

- **R. Strakovsky**, Y-X. Pan. Maternal Protein and Folate Intake Affects Gene Expression and DNA Methylation in Rat Placenta. *FASEB J.* 2008; 22:727.

- **R. Strakovsky**, Y-X. Pan. Expression of TRB3 mRNA Correlates to the Temporal Phosphorylation of eIF2α. *FASEB J.* 2007; 21:lb203

Book Chapters

- Y-X. Pan, **R. Strakovsky**, S. Zheng. 2010. Maternal Nutrition, Intrauterine Development and Disease Risks in the Offspring through Epigenetic Regulation of Gene Expression. In "Nutrition, Epigenetic Mechanisms and Human Disease", (N. Maulik, eds), CRC Press, Taylor & Francis Group, Baca Raton, FL, USA.

Peer-Reviewed Journal Articles

- **R. Strakovsky**, D. Zhou, Y-X. Pan, 2010. A Low-Protein Diet during Gestation in Rats Activates the Placental Mammalian Amino Acid Response Pathway and Programs the Growth Capacity of Offspring. *J Nutr*. 2010 Dec; 140(12):2116-20. Epub 2010 Oct 27.

- **R. Strakovsky**, X. Zhang, D. Zhou, Y-X. Pan. 2011. Gestational High Fat Diet Programs Hepatic Gluconeogenic Gene Expression And Histone Modification In Offspring Rats. *J Physiol.* 2011 Mar 28. [Epub ahead of print].

- X. Zhang, **R. Strakovsky**, D. Zhou, Y-X. Pan. 2011. Maternal High Fat Diet Programs Cellular Senescence Markers and Antioxidant Defense Genes in the Liver of Offspring Rats. *J.Nutr*, accepted.

- **R. Strakovsky,** Y-X. Pan, 2011. A Decrease In Dkk1, A Wnt Inhibitor, Contributes to Placental Lipogenesis In An Obesity-Prone Rat Model, *Journal of Biological Chemistry*, Submitted.

- X. Zhang, D. Zhou, **R. Strakovsky**, Y-X. Pan. 2011. A High Fat Diet Causes Increased Expression of Genes in Cellular Senescence Pathway in the Liver of Obesity Prone Rats through Histone Modifications. *Hepatology*, submitted.

TEACHING EXPERIENCE

Center for Teaching Excellence Graduate Teaching Certificate

Teaching Assistant
Department of Food Science and Human Nutrition, University of Illinois, Urbana - Champaign

- **"Nutritional Biochemistry II"**, Spring 2009.
- **"Nutritional Biochemistry I"**, Fall 2008.
- **"Principles of Nutrition" Discussion Leader**, Spring 2008.
- **"Nutritional Aspects of Disease" Teaching Assistant**, Fall 2007.

ACES Teaching College Course, Fall 2007.

CURRENT PROFESSIONAL MEMBERSHIPS

NACTA: North American Colleges and Teachers of Agriculture
ASN: American Society for Nutrition

ADA: American Dietetic Association
Gamma Sigma Delta: Honor Society of Agriculture, May 2010.

AWARDS AND HONORS

Oral Presentation Competition
Nutritional Sciences Oral Presentation Competition, Universtity Of Illinois, Urbana - Champaign, April 2011, 1st place.

Margin of Excellence Travel Award
Division of Nutritional Sciences, February 2011.
Division of Nutritional Sciences, November 2009.

2011 Frank W. Kari Memorial Award
Division of Nutritional Sciences, January 2011.

Graduate College Travel Award
October 2010.

2010 William Rose Endowed Award
Division of Nutritional Sciences**,** January 2010.

Margin of Excellence Research Grant
Division of Nutritional Sciences, 2009.

Department of Food Science and Human Nutrition 2009 Outstanding Teaching Assistant Award, May 2009.

Poster Competition
Nutritional Sciences Poster Competition, Universtity of Illinois, Urbana - Champaign, April 2009, 2nd place.

List of Teachers Ranked as Excellent:

- **"Nutritional Biochemistry II" Teaching Assistant (Outstanding Ranking)**, Spring 2009.
- **"Principles of Nutrition" Discussion Leader,** Spring 2008
- **"Nutritional Biochemistry I" Teaching Assistant,** Fall 2008
- **"Nutritional Aspects of Disease" Teaching Assistant,** Fall 2007