Maternal Obesity Alters Fetal Development Due to Impaired Placental Function and has Lasting Effects on Adult Offspring

Kristin Ann Norwood

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MATERNAL OBESITY ALTERS FETAL DEVELOPMENT DUE TO IMPAIRED PLACENTAL FUNCTION AND HAS LASTING EFFECTS ON ADULT OFFSPRING

by

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MATERNAL OBESITY ALTERS FETAL DEVELOPMENT DUE TO IMPAIRED PLACENTAL FUNCTION AND HAS LASTING EFFECTS ON ADULT OFFSPRING

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Obesity is an epidemic in many developed nations and maternal obesity can result in developmental alterations in offspring that have long-lasting effects. Two experiments were conducted to determine the impact of maternal obesity on placental development and early embryonic growth and muscle development. Experiment one utilized obese Lethal Yellow (LY) and normal weight C57BL/6 (B6) dams to assess how maternal obesity alters skeletal muscle development in mid-gestational embryos. Embryos from LY dams exhibited decreased embryo and placental weights as well as an increase in the myogenic marker desmin. Furthermore, the adipogenic marker PPARG expression was predominately localized to the neural tube and was significantly decreased in LY-derived embryos. The objective of experiment 2 was to identify how maternal obesity alters placenta development and function and may be associated with altered development of the fetus. The same mid-gestation embryos and corresponding placenta from LY and B6 dams were used. Placenta from LY dams were smaller than when developed in a B6 dam and exhibited a phenotype of reduced function. The placenta also displayed increased hypoxia markers and decreased gene expression of enzymes which regulate the transfer
of active glucocorticoids from the mother to developing embryo. Interestingly, the embryos reared in an obese dam possessed decreased expression of vasculature markers. In summary, these experiments support the following findings: (1) maternal obesity decreases embryonic and placental weight and results in altered temporal regulation of myogenesis; (2) PPARG expression is localized to the neural tube and decreased in LY-derived embryos indicating a function for this transcription factor in neural tube development and suggesting that obesity alters this function; (3) placenta from an obese dam display increased hypoxemia and altered glucocorticoid metabolism resulting in altered embryonic vasculature and potentially differences in the function of various organ systems. These data represent an important shift in understanding how maternal obesity reduces skeletal muscle density during development and its long-term effects on the metabolic health of their children.
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CHAPTER I
LITERATURE REVIEW

Incidence of Maternal Obesity and its Correlation to Childhood Obesity

There has been a rapid rise in the incidence of obesity due to changes in diet and activity levels of individuals. The body mass index (BMI), which is directly correlated with the amount of body fat, is the measure used to determine if one is obese. In order to calculate BMI, an individual’s weight in pounds is divided by their height in inches squared and multiplied by a conversion factor of 703. When an adult has a BMI between 25 and 29.9 they are considered overweight, while an adult with a BMI of 30 or higher is considered obese (1). Obesity worldwide now exists at a prevalence of 15–20% (2). In 2011, 27.8% of the total USA population had a BMI greater than 30 (3) while in the UK, ~25% of the adult population is classified as obese (4). Bays et al. (5) reported that in the USA for the past 20-30 years the rate of overweight or obesity has increased from 15 to 33% in adults, 5 to 14% in children (2-5 years), 7 to 19% in children (6-11 years), and 5 to 17% in adolescents (12-19 years). Due to the increased incidence of obesity, it is now considered a significant public health problem which is associated with a reduction in life expectancy of ~8 years (4) and is attributed to 2-7 % of the total health care costs in the USA (2). Obesity also has been shown to increase the risk of developing metabolic syndrome which comprises several major diseases including type 2 diabetes and coronary artery disease (4).

Overweight and obese women are more likely to gain excessive gestational weight and maintain higher weights after delivery which is one factor that attributes to
the growing obesity epidemic (2, 6). In the USA, approximately 64% of reproductive aged women are overweight and 35% are obese which is translated to more than one in five pregnant women being obese (7, 8). Pregnant women accumulate fat as a result of enhanced lipogenesis and increased adipose tissue lipoprotein lipase activity. From mid-to late gestation, maternal lipid metabolism switches from an anabolic to a catabolic state concomitant with increasing maternal insulin resistance (9). This increase in insulin resistance results in excess weight gain during pregnancy which has detrimental effects on offspring (10). The fetus is not able to regulate their own food intake or growth, so they are under the control of their mothers’ nutrient availability. Figure 1.1 depicts how obesity combined with pregnancy leads to an increase in maternal circulating lipids that increases the lifetime risk of obesity in offspring as well as metabolic syndrome during childhood and adolescence (11, 12).

![Figure 1.1. The Effect of Maternal Lipids on Fetal Development and its Consequences in Adulthood.](image-url)

Monocyte chemotactant protein-1 (MCP-1); chylomicron (CM); triglycerides (TG); lipoprotein lipase (LPL); free fatty acid (FFA); nonalcoholic liver disease (NAFLD) (9).
The nutritional and hormonal environment encountered by the fetus is a strong determinant of not only fetal growth, but also cardiovascular disease risk in later life (13). Changes in maternal diet throughout pregnancy modify the mother’s endocrine status which can have pronounced effects on the growth and development of their conceptus (14).

**Metabolic Stress in Obese Individuals**

Potentially important insights into the mechanisms controlling intrauterine growth are provided by recent studies which demonstrate that white adipose tissue is a highly active endocrine organ. Immature adipose tissue first appears in the fetus between 14 and 16 weeks of gestation in the human and once adulthood is reached, adipose cell numbers remain relatively constant (11). Mature adipose tissue consists of adipocytes, macrophages, and stroma-vascular cells which are composed of vascular endothelial cells, fibroblasts, and cells of the hematopoietic lineage (11, 15). Adipose tissue is known to secrete a range of hormones that are important in modulating metabolism, energy homeostasis and growth, collectively called adipocytokines (16). These adipocytokines can either produce proinflammatory cytokines or metabolic regulators that often act as negative regulatory signals that temper the action of hormones and growth factors (17). In obesity, the expansion of adipose tissue mass (not cell number) is associated with increased inflammation of adipose tissue (11) which results in an imbalance in adipocytokine levels that in the pregnant female can impact the development of the growing fetus.
**IGF-Family:**

The insulin-like growth factor (IGF) system is comprised of IGF-1, IGF-2, the two cell surface receptors, IGF-1R and IGF-2R, as well as six binding proteins, IGFBP 1-6 and proteases which degrade these binding proteins (18). Overall, the IGF system, in combination with growth hormone (GH), accounts for 83% of postnatal body growth (19). The primary mechanism of GH/IGF axis function involves GH-dependent stimulation of IGF-1 which regulates both metabolism and growth (20). IGF-1 is predominately produced in the liver in the presence of sufficient nutrient intake and elevated hepatic portal insulin levels, but is also synthesized in many other tissues and cell types (21, 22). Interestingly, adipose tissue is a major source of circulating IGF-1 as well as IGF-1 being a critical mediator of preadipocyte proliferation, differentiation, and survival (22). IGF-1 concentrations are inversely related to life span, increased cell proliferation, and increased IGF-1 levels have been positively correlated with tumor progression (17). Along with GH and the IGF family, insulin is also a central hormone in regulating metabolism, capable of stimulating glucose uptake, glycogen synthesis, lipogenesis, and prevention of proteolysis (23). IGF-1 and insulin are known to act in a synergistic manner in order to induce adipocyte differentiation.

The structure of IGF-2 is similar, but not identical to IGF-1 (24). IGF-2 is known to regulate fetal growth and, in an *in vitro* model of rat adipocyte progenitor cells, stimulate adipocyte differentiation (22, 25). The phenotype of mice lacking IGF-2 exhibit decreased birth weight compared to control animals, but growth is unaffected after birth suggesting that IGF-2 is only essential for prenatal growth (26). In contrast to
IGF-1 which is GH dependent, IGF-2 is, for the most part, GH independent and is expressed in most tissues during fetal development, predominantly by cells of mesenchymal origin (27, 28). The activities of IGF-1 and IGF-2 are modulated by three distinct mechanisms with the first being the control of ligand expression. Secretion of IGF-1 is sensitive to nutritional and endocrine control and thus is positively related to growth rate (17). Secondly, high affinity binding proteins are responsible for maintaining a large circulating pool of IGF-1 and IGF-2 (29). Specifically, IGFBP6 binds to IGF-2 with higher affinity than IGF-1 while other IGFBPs bind to both IGF-1 and IGF-2 with similar affinities (30). Finally, tissue specific expression of IGF and insulin receptors is an important mechanism that confers specificity of IGF actions.

IGF-1R, IGF-2R, and insulin receptor are ligand-activated transmembrane tyrosine kinases with IGF-1R being widely expressed in many cell types in both fetal and postnatal tissues (31). As depicted in Figure 1.2, the IGF-1R and insulin receptors are heterotetramer glycoproteins consisting of two alpha and two beta subunits which recruit insulin receptor substrate 1 (IRS-1) to induce phosphorylation and initiate a cascade of signal transduction reactions (32). Insulin receptor shares a high degree of homology with IGF-1R, so much so that hybrid receptors naturally occur with full ligand-activated signal transduction capacity (17, 28). The insulin receptor, is comprised of two isoforms (A and B) which differ by only 12 amino acids (22). These receptors are able to bind IGFs with a low affinity (28). Certain cell types are found to contain different receptors. For example, preadipocytes express IGF-1R and insulin receptor isoform A, whereas mature adipocytes express predominantly insulin receptor isoform A and B (33). The
IGF-1R is known to bind IGF-1 with high affinity and initiates a physiological response. It is also able to bind IGF-2, but with a lower affinity, and is responsible for the mitogenic effects of IGF-2 during fetal development (34, 35). The IGF-1R has traditionally been viewed in the context of its impact on somatic growth, in particular the synergistic relationship with GH that is essential for normal postnatal growth (36). IGF resistance due to receptor desensitization plays a role in growth retardation and metabolic

Figure 1.2. Insulin and IGF Signaling Pathways. Both Insulin and IGFs share similar signal transduction pathways and are able to bind the different receptors with varying affinities. Ligand binding results in MAPK and PI3K signaling pathway activation resulting in transcription, proliferation and gene expression (37).
disorders which can be caused by a variety of factors including but not limited to diabetes, obesity and IGF-1R mutations (17). The final receptor, IGF-2R, acts as a decoy receptor due to it not regulating any significant signaling cascades. However, IGF-2R has been implicated in the regulation of intracellular trafficking of mannose-6-phosphate proteins such as lysosomal enzymes (38). IGF-2 competes for binding with IGF-1R but has preferential affinity for IGF-2R over IGF-1 and does not bind insulin receptor (35, 39).

Ligand binding to IGF-1R and insulin receptor results in the phosphorylation of the beta subunits and stimulates tyrosine kinase activity. This phosphorylation results in the binding of IRS proteins to the receptors (28). There are two major pathways that have been identified as playing an important role in IGFs cellular proliferation and the inhibition of apoptosis (35). The mitogen-activated protein kinase (MAPK) and the phosphoinositide-3 kinase (PI3K) pathways both demonstrate a role in mediating the anti-apoptotic and cell proliferative responses of the IGFs (40-42). IGF-1 is implicated in the regulation of adipocyte differentiation and cell cycle by activation of the PI3K pathway (22). Various downstream components of the PI3K pathway are associated with enhancing specific cellular events, including metabolism, transcription, hypertrophy and differentiation (17). IGFs also stimulate MAPK which is mediated through Src family of nonreceptor tyrosine kinases (43). On the other hand, pro-inflammatory cytokines have been found to exhibit properties that induce a state of IGF-1 resistance (17, 44).

Both IGFs and insulin are key molecules in mediating the metabolic-related function of adipocytes. IGF-1, which mediates the clonal expansion of preadipocyte cells
prior to differentiation, is equipotent with insulin in promoting glucose uptake and inhibiting lipolysis in mature adipocytes (23). Villafuerte et al. (45) looked at adipocyte depot-specific expression patterns of IGF-1 (and leptin). They found that IGF-1 mRNAs correlate with adipocyte volume, such that there is increased expression of IGF-1 in retroperitoneal compared to subcutaneous fat where retroperitoneal fat has a much greater adipocyte volume compared to subcutaneous fat. They also determined that both leptin and IGF-1 function at the autocrine level which could modulate region-specific patterns of adipose tissue growth. Similarly, knowing that individuals with low birth weight have an increased risk for metabolic syndrome later in life, a study compared the expression of insulin/IGF-signaling molecules in adipose tissue of low-birth-weight and normal-birth-weight young males. They found a lower expression of genes for PI3K p85 and p110 subunits as well as decreased IRS-1 in the adipose tissue of low-birth-weight compared to normal-birth weight males (46). Likewise, in another study, IGF-1R, insulin receptor, and AKT are decreased in subcutaneous adipocytes from small for gestational age compared to appropriate for gestational age children (47). These studies show that IGFs do indeed play a role in the generation of adipose depots in offspring. Studies have also demonstrated that the IGF system may influence other adipokines such as leptin (48).

Leptin:

Leptin (meaning thin in Greek) is a 16-kDa protein containing 167 amino acids that was discovered in 1994 by Jeffrey Friedman’s group when a mutation in the obese (ob) gene resulted in profound obesity and type II diabetes (49-51). They characterized leptin as an adipocyte-derived hormone which is structurally similar to pro-inflammatory
cytokines. Initially leptin was found to diminish adiposity by reducing food intake while improving insulin sensitivity, at least in part by depleting triglyceride stores in peripheral tissues (52, 53). Normally, in peripheral tissues, leptin induces fatty acid oxidation (54) and glucose uptake (55, 56) as well as stimulates lipolysis by promoting insulin insensitivity in adipose tissue, but not in muscle or liver tissue (57).

Leptin is a molecule intimately linked with fat and feeding behavior, but it also has important reproductive functions, including fetal growth and developmental processes such as brain development, angiogenesis, hematopoiesis, and immune cell function (15, 58). Mice treated with high amounts of leptin show accelerated maturation of the female reproductive tract which leads to an earlier onset of the oestrous cycle and reproductive capacity (59). Leptin has also been found to be synthesized in the placenta of many mammalian species even though its exact function there is unknown (15).

Figure 1.3. Leptin Signal Transduction Pathway. Leptin binds to its receptor where it activates JAK2 which then phosphorylates STAT3. STAT3 then results in gene transcription to induce POMC/CART and inhibit NPY/AGRP (60).
Leptin functions through binding its receptors which belong to the cytokine receptor superfamily. The leptin receptor, in turn, activates the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway (Figure 1.3). There are five alternatively spliced forms of the leptin receptor which all bind leptin with nanomolar affinity (61). Mutations in the receptor cause an obese phenotype identical to leptin deficiency (62). The activation of the leptin receptor homodimer depends on phosphorylation of the kinase Jak2 after ligand binding (63). Phosphorylation of Jak2 results in activation of Stat3 as well as leads to tyrosine phosphorylation of SHP-2, a phosphotyrosine phosphatase, which decreases both the state of Jak2 phosphorylation and transcription of a leptin-inducible reporter gene (49, 64, 65). Even though many of leptin’s effects are mediated through the JAK/STAT pathway, it is also found to selectively stimulate phosphorylation and activation of the α2 catalytic subunit of the 5' adenosine monophosphate-activated protein kinase (AMPK) pathway in skeletal muscle. The activation of AMPK results in the suppression of acetyl CoA carboxylase activity, thereby stimulating fatty acid oxidation in muscle (66).

Leptin’s primary site of action is the brain, where it promotes decreased food intake and increased energy expenditure (67). Specifically, leptin acts at the level of the arcuate nucleus which contains orexigenic neuropeptide Y (NPY)/agouti related protein (AGRP) and anorexigenic cocaine- and amphetamine-regulated transcript (CART)/proopiomelanocortin (POMC) neurons. Leptin modulates energy homeostasis in the melanocortin axis by positively regulating CART/POMC neurons and negatively regulating NPY/AGRP neurons (68). Specifically, POMC neurons have been proposed
as the primary cell type for mediating leptin’s anorexigenic effect (68, 69). POMC is the precursor of melanocortin-stimulating hormone which when it acts on its receptor, melanocortin-4 receptor, in response to an increased plasma leptin concentration, it results in decreased food intake and increased energy expenditure (49). Corticotrophin-releasing hormone (CRH) also mediates some of leptin's effects, as pretreatment with an anti-CRH antibody blunts the anorexigenic effects of leptin. Other studies have indicated NPY as an important component of the biological response to low levels of leptin and possibly starvation (49).

There are three general ways in which alterations in the leptin signaling pathway can result in obesity which are depicted in Figure 1.4. First, a failure to produce leptin can occur. This would result in unchecked expansion of fat cell mass and consequently obesity results. Second, a regulatory defect can result in decreased leptin production for a given fat mass. This leads to a lower plasma leptin concentration secreted then what is expected and results in moderate obesity. Finally, obesity can result from receptor insensitivity to leptin. An increase in circulating leptin would result from this insensitivity and cause increased food intake (49). The complete loss of leptin or leptin receptor is the most severe reported monogenic cause of obesity in mice and humans; however, it is extremely rare (70). In fact, most obese animals have higher leptin levels than controls indicating that these forms of obesity are associated with some form of leptin resistance (71, 72).
Figure 1.4. Obesity can Result from Three Different Alterations in Leptin Signaling.

Obesity is generated in three ways in regard to the leptin regulatory loop. a) Failure to produce leptin leads to a drastic increase in fat mass, b) inappropriately low leptin secretion for a given fat mass leads to fat expanding until ‘normal’ levels of leptin are reached, and c) insensitivity to leptin resulting in high leptin levels (49).

TNF-α:

Tumor necrosis factor alpha (TNF-α) is synthesized as a 26 kDa, 233 amino acid transmembrane protein that after cleavage is released into circulation as a 17 kDa, 157 amino acid soluble TNF-α molecule (73, 74). TNF-α is predominately produced by macrophages (75, 76), but is also produced by a broad range of other cell types including endothelial cells, cardiac myocytes, fibroblasts, neurons and adipose tissue. The stimulation of Toll-like receptors and activation of cytokines and lipid mediators lead to the increased infiltration of adipose tissue with macrophages which increases TNF-α
production. However, no matter the tissue, TNF-α is a rapidly up regulated cytokine whose transcription is initiated within minutes and protein production within a few hours. Alterations in this complex regulatory system including over and under expression of TNF-α has significant pathological consequences (77). In adipose tissue, TNF-α inhibits lipoprotein lipase, stimulate hormone-sensitive lipase and induce uncoupling protein expression. Also, TNF-α functions to down regulate insulin-stimulated glucose uptake (78). All of these effects reduce lipid accumulation within adipose tissue. TNF-α has also been found to be produced by the placenta during pregnancy where it is able to not only induce its own synthesis but also the synthesis of its receptors (78, 79).

Membrane-bound as well as soluble TNF-α interact with TNF receptors (TNF-R) 1 and 2 (80). It is through these receptors that TNF-α can activate cell survival, cell proliferation, and apoptotic pathways (Figure 1.5). These differing effects of TNF-α are dependent on the signaling pathways activated. For example, TNF-α induces apoptosis through activation of FADD which binds to caspase-8. Conversely, TNF-α promotes inflammation and survival through TRAF2 via c-Jun NH2-terminal kinase (JNK)-dependent kinase cascade, MAPK and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways (81). While the separate pathways are well defined, the balance between cell survival and death remains unclear. TNF-α has also been found to impact other signaling pathways by down regulating insulin-stimulated glucose uptake via effects on glucose transporter 4, insulin receptor autophosphorylation and IRS-1 (78).
Figure 1.5. TNF-α Signal Transduction Pathway. TNF-α binds to either TNF-R1 or TNF-R2 which activate signaling pathways to induce apoptosis, cell survival or inflammation (81).

Interestingly, TNF-α was the first discovered adipose-derived factor that indicated a link between obesity, inflammation and diabetes. Upon development of obesity, macrophages migrate into the adipose tissue and appear to be the predominant source of adipose-derived TNF-α (82). Studies using genetic models of TNF-α deficiency confirmed a causal role for this inflammatory cytokine in the development of obesity-induced insulin resistance (76). Furthermore, insulin has been reported to stimulate macrophages in adipose tissue to produce more TNF-α (11, 82). Interestingly, TNF-α is involved in ovarian and uterine cyclicity as well as in the establishment of the dialogue
between the implanting embryo and the receptive uterus (83). Likewise, elevated circulating TNF-α increases placental produced maternal peripheral blood mononuclear cells (84). Altogether, these data have established the now well accepted paradigm that obesity is a chronic condition of low-grade inflammation and activated adipose tissue macrophages resulting in increased TNF-α production (4).

The increase in the production of adipocytokines including IGFs, leptin, and TNF-α upon increased maternal adipose tissue mass may directly impact the development of the growing fetus. The placenta, which plays an important role in the transfer of nutrients from the mother to the fetus, can also be impacted by these adipocytokines. Thus, an indirect effect of increased maternal adipocytokines may be to alter nutrient transfer resulting in abnormalities in the development of multiple fetal organ systems.

**Effect of Maternal Obesity on Fetal Growth**

There is a developmental overnutrition hypothesis which proposes that increased fuel supply to the fetus due to maternal obesity or overnutrition leads to permanent changes in offspring metabolism, behavior, and appetite regulation (8, 85, 86). A study by Parsons et al. (87) in humans found that heavier mothers give birth to heavier babies which go on to have a high BMI in adult life as well as are at an increased risk for metabolic syndrome, asthma and developmental abnormalities (88-90). Even though human studies have found a prevalence for large-for-gestational-age (LGA) fetuses, maternal obesity has also been associated with intrauterine growth restriction (IUGR) in humans as well as in rodents and livestock species (91-93). Interestingly, both LGA and
IUGR result in offspring exhibiting similar developmental abnormalities (94-97). This may be due to catch-up growth in IUGR infants which could put them at a higher risk for metabolic syndrome in adulthood (98) as well as the postnatal accelerated growth adversely affecting glucose tolerance in rats (99).

Based on these data, it is clear that maternal overfeeding and adiposity prior to pregnancy has a significant impact on fetal, neonate, and adult growth. Furthermore, this effect on growth has been correlated to alterations in fetal organ development (100). Animal models (mainly rodents and sheep) have proven to be invaluable tools to define the mechanistic links between maternal diet and body composition as well as the offspring phenotype of metabolic syndrome (101-103). For example, prenatal overfeeding in the rat, mouse, and sheep leads to altered appetite regulation in the postnatal offspring (97, 104, 105) which may be due to programming changes at appetite centers in the hypothalamus (86, 92, 106). This altered appetite regulation is tied to the increased fat deposition resulting in postnatal weight gain and eventually offspring obesity. Prior to birth, embryos from mice fed a high fat diet display significant growth retardation, with live pups being significantly smaller at birth compared to control fetuses (93).

The development of the cardiovascular system, liver, and pancreas may be particularly sensitive to pre-conception and/or early gestational changes in maternal body composition and metabolism beyond what is compensated for by fetal visceral fat deposition (100). Excess energy is normally stored as visceral fat, but if stores are surpassed, circulating lipid levels are increased. Indeed, offspring from obese dams are
found to display not only higher percentage of body fat (97, 107), but also increases in triglyceride levels (108) which together can predispose the animal to postnatal development of metabolic disease (e.g. fatty liver disease) (100, 109).

Along with the impact of increased adiposity on fetal growth and metabolic disturbance, there is evidence that maternal obesity in rodents and ewes can predispose offspring to alterations in organ development. For example, pancreas weight is increased and displays enhanced early β-cell maturation in offspring from obese dams (100, 102, 107). A similar increase is also seen in regards to liver weights which have been shown to lead to the development of nonalcoholic fatty liver disease (100, 108, 110). Animal studies also suggest that the vasculature of the offspring is significantly affected by maternal high-fat diet during pregnancy. A study in non-human primates showed that offspring of mothers fed a high-fat diet had a threefold reduction of dilation capacity in the abdominal aorta, increased intimal wall thickness and an increase in the expression of vascular inflammation markers, suggesting a negative effect of maternal high-fat diet on offspring endothelial function (111). These data represent a potential mechanistic link between maternal obesity and the development of high blood pressure (97, 112-114) seen in animal models. However, despite this animal evidence, there has been no data in humans that explains the association between maternal obesity and blood pressure (85). Interestingly, there are also sexually dimorphic differences in the impact of maternal obesity on organ function, particularly in relation to glucose homeostasis and liver weights. A study by Samuelsson et al. (97) found that liver weights are increased in male offspring but not in female offspring.
Maternal Obesity Alters Muscle Development

The impact of maternal obesity on skeletal muscle development is of particular importance. Skeletal muscle is a major metabolic organ and alterations in muscle development could result in long-lasting effects on the metabolic health of offspring. Skeletal muscle development is known to have a lower priority in nutrient partitioning than the development of the neural system, internal organs, and bone, making it susceptible to nutrient fluctuation (115). Due to this fact, studies have been conducted that look at the effects of an altered maternal diet on embryonic muscle development. One study carried out by Larciprete et al. (116) used ultrasonic measurements of IUGR fetuses to show a reduction in muscle mass in these IUGR fetuses. Likewise, Zhu et al. (117) indicate that maternal nutrient availability is associated with the number of secondary fibers present in prenatal muscles. During early and mid-gestation in the ewe nutrients have been shown to reduce skeletal muscle fiber density suggesting abnormalities in myotube formation (118, 119). The incorporation of additional myoblasts into the myofiber during fetal development continues via fiber hypertrophy and requires additional myoblast incorporation to increase genomic DNA content (120, 121). However, in fetal sheep with placental insufficiency caused by alterations in maternal nutrients, skeletal muscle fibers contain fewer myonuclei than fibers from control fetuses, resulting in less DNA, RNA and protein per fiber (122). Also, Huang et al. showed that maternal obesity enhances collagen content and crosslinking in fetal skeletal muscle, which might be due to reduced collagen remodeling, and leads to fibrosis which impairs muscle function and is a hallmark of muscle aging (123). These
alterations that are occurring in muscle may be impacting the myogenic program and altering the stages of skeletal muscle development (Figure 1.6). The pre-adipocyte marker, peroxisome proliferator-activated receptor γ (PPARγ), has also been found to be expressed in higher concentrations in skeletal muscle of offspring from obese dams indicating increased intramuscular adiposity in offspring from over nourished mothers (119, 124). In addition to abnormalities in muscle mass, the metabolic function of muscle is also impaired in fetuses of obese dams with obesity during pregnancy causing defects in insulin-signal transduction (125).

Figure 1.6. Stages of Bovine Fetal Skeletal Muscle Development. The progression through the various bovine fetal skeletal muscle developmental stages during gestation may be impacted by nutrient restriction or excess. The stage in which the nutrient deficiency or excess occurs will determine what organs are impacted based on their developmental program. Adapted from (140).

Maternal obesity dependent reductions in myogenesis and increased intramuscular fat in the late gestation fetal sheep has been associated with increased expression of inflammatory markers and altered AMP-activated protein kinase signaling (119, 126, 127). Skeletal muscle, which is the largest tissue in the body, synthesizes and responds to many inflammatory mediators. For example, elevated proinflammatory cytokines including TNF-α are implicated in muscle wasting (128, 129). This muscle wasting may
be a result of inflammatory cytokines suppressing IGF-1 signaling in myofibers and myoblasts. Indeed, TNF-α has been shown to decrease IGF-1 mRNA by 80% (17, 130). Along with the impact of proinflammatory cytokines, high maternal glucocorticoid levels have been shown to impair fetal skeletal muscle growth (131). In mature muscle, glucocorticoid exposure decreases muscle protein synthesis and promotes protein degradation (132). Thus, increased glucocorticoid levels during gestation may negatively impact muscle development by disrupting the differentiation process resulting in decreased muscle mass. During normal development, skeletal muscle development is induced by reduced O₂ availability, or hypoxemia. Embryonic somites have been shown to exhibit increased expression of hypoxic markers prior to the formation of local blood vessels and embryonic muscle (133) with hypoxemia increasing the undifferentiated state of muscle stem cells. However, a chronic hypoxic environment diminishes the differentiation of muscle stem cells which promotes self-renewal divisions without affecting the overall proliferation of primary myoblasts (134).

Previous studies have focused on the impact of maternal obesity on skeletal muscle development during late gestation (secondary myogenesis) and the phenotype of skeletal muscle in the adult offspring. However, there is a gap in understanding how maternal obesity affects primary myogenesis and the consequences of potential alterations during primary myogenesis on the later stages of muscle development. In order to understand the alterations that are occurring in muscle development due to maternal obesity, it is imperative to understand how skeletal muscle normally develops.
Normal Skeletal Muscle Development

The fetal period is crucial for skeletal muscle development due to no net increase in the number of muscle fibers after birth (117, 122, 135). Adult skeletal muscle consists of about 40-50% of the total body mass (136) and is a key metabolic organ which is responsible for the oxidation of glucose and fatty acids (126). The myogenic program is a highly conserved process in all species (137). However, the formation of secondary myofibers and adipogenesis begins around mid-gestation in humans, sheep and cattle (138), while rodents are born highly immature with secondary myogenesis occurring late in gestation and continuing throughout the neonatal period (126, 139). Thus, even though there is a difference in the timing of myogenesis, the process and its regulation is still very similar.

The Somite Region Harbors Muscle Progenitor Cells:

All skeletal muscles in the body arise from muscle founder stem cells located in the somites. Somites are transitory structures that form in pairs, on either side of the neural tube, as epithelial spheres from the presomitic paraxial mesoderm (Figure 1.7) (141, 142). Somites begin forming at the anterior (head) end of the embryo and are added posteriorly (tail) as the embryo extends. This means that the anterior somites are more developed than the posterior somites and result in a range of somite stages within a single embryo (137). Since an anterior/posterior developmental gradient occurs in somitogenesis, there is also a difference in limb formation with the forelimbs forming before the hindlimbs (139). Once somitogenesis is complete there are a total of 60 pairs
Figure 1.7. Spatial Representation of Somite Development. Segmentation of the presomitic paraxial mesoderm into ball-like structures, known as somites, occurs along the dorsal–ventral axis and in an anterior to posterior direction. In response to signals from the notochord and the neural tube, the somites differentiate and subdivide to give rise to the dermomyotome and the sclerotome which result in the development of many different tissues and organs throughout the body (143).

of somites in the developing mouse embryo (139). The somites harbor progenitor cells that give rise to not only skeletal muscle, but also cartilage, endothelial cells, tendons, connective tissue and the dermis of the back (144).

As the cells of the somite proliferate, they divide into multiple distinct regions (Figure 1.8); the sclerotome which gives rise to vertebral and rib cartilage, dermal lineages, and blood vessel lineages and the dermomyotome (DM) which harbors skeletal muscle progenitor cells (MPC) of the trunk, limbs and some head muscles as well as dermal progenitors (139, 141, 145). The DM has a region that lies directly below it called the myotome. Further, both the DM and the myotome are divided into two
regions, epaxial and hypaxial. The epaxial region gives rise to the deep muscles of the back, while the hypaxial region produces the rest of the body and limb musculature (137, 142). Even more specifically, the DM has two regions on the ends, the ventral lateral lip (VLL) and the dorsal medial lip (DML). The VLL progenitors delaminate from limb level somites and migrate to limb buds to form limb muscles (145). Medial DM cells as well as the cells at the DML produce the epaxial muscle progenitors that migrate underneath the DM. These cells then differentiate into mononucleated myocytes to form the myotome which is the region where the first skeletal muscles form (133, 141, 146, 147).

Figure 1.8. Somite Origin of Myogenic Progenitor Cells. Myogenic progenitors originate in the dorsal-medial lip (DML) and ventral-lateral lip (VLL) of the dermomyotome. Cells of the dorsal medial lip (DML) migrate under the dermomyotome to form the epaxial myotome. A similar event occurs at the ventral lateral lip (VLL), which results in the formation of the hypaxial myotome. Cells of the VLL also undergo an epithelial to mesenchymal transition, delaminate and migrate to regions of limbs for muscle development (145).
As somites develop, the complex muscle differentiation process is occurring in tandem. Myogenesis has multiple waves in the developing embryo that build the framework for adult musculature. As shown in Figure 1.9, the mouse has its first embryonic wave of muscle development around embryonic day (E) 8.5 which continues until around E 14.5. During this wave, quiescent myogenic stem cells (MSC) are activated and proliferate making them muscle progenitor cells (MPC) (Figure 1.10). During this time MPC further differentiate into myoblasts which are able to begin to differentiate and fuse to form primary fibers or myotubes. Those primary fibers then act as a scaffold for the formation of fetal (secondary) fibers which occurs in the fetal wave of muscle development (E 14.5 - E 16.5). Around E 16.5, the late fetal wave begins which is the final wave where secondary fibers fill in the spaces not already occupied by existing fibers and complete myogenesis (148).

**Figure 1.9. Waves of Developmental Myogenesis in the Mouse.** The early embryonic wave begins around embryonic day (E) 8.5 and results in the formation of primary fibers. The fetal wave, between E 14.5 and E 16.5, consists of the scaffolding of secondary fibers. Around E 16.5, proliferating myotubes form muscle fibers completing myogenesis. Adapted from (141, 149, 150).
**Myogenic Regulatory Factors:**

The process of differentiation from MSC to myotubes is dependent on the temporal expression of several transcription factors which belong to the basic-Helix-Loop-Helix (bHLH) family (Figure 1.10). These myogenic regulatory factors (MRFs) include myogenic determination factor 1 (MyoD), myogenic factor 5 (Myf5), myogenic regulator factor 4 (Mrf4, also known as myogenic factor 6-Myf6 when first discovered) and myogenin (Myog, also known as myogenic factor 4-Myf4 when first discovered) (151, 152). The expression of two paired box (Pax) transcription factors, Pax3 and Pax7, is the first step in differentiation of DM progenitor cells to skeletal muscle (141). *Pax3* and *Pax7* are also members of the bHLH family of transcription factors and are expressed in the central nervous system, neural crest cells and somites. Their expression is necessary for the emergence and survival of MPC and the regulation MRFs (141, 149). In a

![Figure 1.10. Gene Expression Profiles during Myogenesis.](#)

Myogenic stem cells (MSC), myogenic progenitor cells (MPC), myoblasts, and the multinucleated myotube express different genes during myogenesis (148).
coordinated fashion, MRFs form heterodimeric DNA-binding complexes with other bHLH transcription factors such as the E2 gene family (E47 and E12) and bind a canonical DNA sequence (CANNTG, also referred to as an E box) to regulate the expression of an array of genes that regulate the assembly of skeletal muscle (145, 153, 154).

The switch from MSC to MPC marks the start of differentiation (137) with the temporal activation of MRFs regulating the myogenic program (outlined in Figure 1.10). Specifically, in the developing embryo Pax7 plays a large part in the activation of MSCs which begins the differentiation process into mature muscle (155). The activation of Pax7 results in the expression of Myf5 which is the earliest marker of MPC and is closely followed by MyoD expression. Myf5, MyoD and Mrf4 are all co-expressed in MPC as well as myoblasts and play a role in the establishment and maintenance of muscle progenitor lineages by directing the developmental program during embryogenesis (156-159). Due to the Myf5 and MyoD transcripts being relatively unstable, their continued expression requires constant signals from the neural tube and notochord in order for these cells to later initiate myotome differentiation (160). Further differentiation is marked by the onset of Myog expression and the formation of myoblasts (Figure 1.10) (161, 162). Under the proper signals, levels of Myog will increase, followed by up-regulation of Des (Desmin) (163, 164). DES, an intermediate filament protein, is expressed in differentiated myotubes that, along with MYOG expression, is critical for muscle formation during embryogenesis, however is not needed for proper muscle differentiation in adulthood (141, 165). In adult myofibers, the expression profile switches to Mrf4,
whereas MyoD, Myf5 and Myog expression is relatively lower, making Mrf4 a predominant factor in adult muscle (166).

**Satellite Cells in Adult Muscle:**

Towards the end of fetal muscle development (around E16.5) (10), MSC begin to be enveloped under a basal lamina that forms around the muscle fibers (133). In 1961, Alexander Mauro discovered and termed these cells satellite cells (167). Satellite cells arise from a novel population of MSC that express both Pax3 and Pax7 (133, 168). However, while Pax3 is not solely required for satellite cell emergence or maintenance, Pax7 is required otherwise they will fail to repopulate the stem cell niche and eventually die (133, 168, 169). In newborn animals, the proportion of satellite cells in muscle is higher. It is during this time that they participate in proliferation and adding nuclei to the growing muscles. Once growth is achieved, satellite cells become quiescent and represent a very small proportion of nuclei in adult muscles (170). For example, the number of satellite cells in muscle declines after birth from 30% of myonuclei in the juvenile phase (0 - 3 weeks) to 4% in the adult phase (3 weeks to ~18 months), followed by a small decrease to 2% in aged mice (18 months to end of life) (171, 172).

Adequate satellite cell availability is needed for proper muscle maintenance and repair after injury in the adult animal (Figure 1.11). Muscle satellite cells are normally quiescent, expressing both Pax7 and Myf5, but lacking MyoD expression (173, 174). When muscle fiber is damaged, satellite cells become activated. At this time satellite cells can either replicate and differentiate to form new fibers permitting muscle repair or
they will become renewed satellite cells allowing for repopulation of the satellite stem cell niche (173, 175). When a satellite cell is activated, they will express the MYOD protein which is the hallmark of an activated satellite cell. The satellite cell then will either lose MYOD expression and maintain PAX7 expression as they will leave the cell cycle and self-renew (176) or will retain the MYOD expression. If retained, MYOG expression is activated to signal terminal differentiation to a mature myoblast (173). The terminal differentiation results in the fusion of the recently differentiated myoblast to form nascent multinucleate myofibers or fuse with myofiber end-fragments to develop a new muscle fiber (148). So, MYF5 and MYOD commit progenitors to a muscle fate whereas MYOG is required for terminal differentiation of committed progenitors (141).

**Figure 1.11. Expression Profile of Satellite Cells.** The proliferating myoblast population is represented by the Pax7+/MyoD+ mononuclear cells. Nuclei that are MyoD+/Myog+ (and no longer express Pax7) are found within differentiated mononuclear cells and myotubes, whereas a minor population of Pax7+/MyoD+ cells represents a transitional stage within recently differentiated myoblasts; newly formed myotubes occasionally display Pax7+/ MyoD+/Myog+ nuclei as well. Renewed cells Pax7+/MyoD− represent reentry into the satellite cell niche (177).
Signals Impacting Muscle Development

Neural Tube Regulation of Myogenesis:

Secreted factors from the neural tube, notochord and surface ectoderm regulate the myogenic program including regulating the expression of MRFs and Pax transcription factors (Figure 1.7). While the notochord releases signals that are required for the activation of MRFs, the signals from the neural tube aid in the maintenance of MRF expression during somite maturation prior to the initiation of myotome differentiation (178). Both the neural tube and notochord provide essential signals specific for the formation of epaxial muscles, but not for hypaxial or limb muscles (179). These signals from the adjacent notochord, neural tube, and surface ectoderm impinge upon the cells in the DM to regulate the emergence of MPC (141, 148).

Factors secreted by the neural tube including Notch, Wnt, Sonic Hedgehog (Shh), and Bone Morphogenetic Proteins (BMPs) function in the activation of MSCs, their downstream progeny, and their lineage progression (Figure 1.12) (180). Notch signaling is necessary for the initial phases of myogenesis and MSC maintenance in DM cells which is activated by mild hypoxia (134, 181, 182). The Notch signaling pathway is responsible for the reduction in the proliferative capacity of the Pax3 and Pax7 positive MSCs (183). If the Notch signaling pathway is disrupted, the progenitor pool depletion is accelerated due to their commitment to myogenesis thereby compromising muscle development (141). A sustained activation of Notch reverses the myogenic program, resulting in a downregulation of Myf5 and MyoD expression and a return to a quiescent
MSC state (181). There is some evidence that MSC self-renew through asymmetric cell divisions is controlled by not only the Notch but also Wnt signaling pathways (184-186). Canonical Wnt signaling, which follows Notch, promotes differentiation of satellite cells to become muscle fibers (187). Specifically, Wnt6, produced by the surface ectoderm, activates $Pax3$ to initiate further differentiation into MPC (188, 189). Wnt signaling has also been shown to regulate the initiation of Sonic hedgehog (Shh) signaling from the surface ectoderm and dorsal neural tube during somite formation (190). Shh is an essential and sufficient notochord signal for $Myf5$ and $MyoD$ activation (190-192). Noggin, a secreted BMP antagonist, also regulates $Myf5$ and $MyoD$ expression by inhibiting Bmp4 signaling from the neural tube (193). Altogether, the activation of $Myf5$ and $MyoD$ genes in the somite, as well as in the limbs, depends on signaling from Wnts, Bmp4, Noggin, and Shh (139, 193, 194).

**Figure 1.12. Signals from Surrounding Tissues Impact Myogenic Markers.** Signals from adjacent lineages, notochord, neural tube, and surface ectoderm, impact the expression of genes in the dermomyotome, myotome, and sclerotome (148).
While the mechanisms are not clear, it is likely that factors expressed due to maternal obesity alter these signaling pathways and therefore disrupt myogenesis.

**Neural Crest Cell Regulation of Myogenesis:**

In addition to neural tube signals that impact myogenesis, neural crest cells also play a part in the regulation of muscle development. Neural crest cells originate in the neural tube and go through a transformation where they delaminate and migrate away from the neural tube to diverse regions in the developing embryo (Figure 1.13) (195-200). One region they migrate past following somite maturation is the anterior half of the sclerotome and along the basement membrane of the DM (201, 202). It is in this region that they play a part in the regulation of myogenesis (200, 201, 203). In order for neural crest migration to occur these cells must undergo an epithelial-mesenchymal transition (EMT). EMT is a mechanism where epithelial cells are converted to a mesenchymal phenotype. In order for this to occur, the expression of Snail, Twist and Zeb2 are needed to repress the expression of epithelial polarity/adhesion factors (e.g. E-cadherin) (204). Along with neural crest cells providing signals to the surround cells, they are also able to differentiate into neurons, cartilage, melanocytes, the dorsal root ganglia and many other types of cells (149, 200, 203).
Figure 1.13. Migration Pattern of Neural Crest Cells. Trunk neural crest cells in the mouse migrate from the neural tube (NT) through the dermomyotome (d) and sclerotome (scl) and impact myogenic differentiation. Somite (s); and notochord (n) (200).

Functional signaling exists between neural crest cells and MPC in early muscle development where they aid in the survival of the MPC and maintain them in an undifferentiated state (181, 205). Neural crest cell migration is limited to a short time period during early development, and therefore, neural-crest-mediated regulation of muscle growth is limited to the initial phases of myotome formation (181). Specifically, when somites are going through the embryonic wave of myogenic differentiation characterized by expression of MYF5, neural crest cells are migrating from the neural tube and playing a role in regulating this stage of muscle development (181). Hence, the timing of myotome formation to that of neural crest migration may provide a mechanistic link for the concurrence of these two events (181, 199). Moreover, neural crest cells have been found to secrete Neuregulin1 which plays a part in muscle regulation by restraining MPC from precocious differentiation (205). Interestingly, the depletion of migrating neural crest cells has been shown to compromise myogenesis by altering the balance
between Pax7 positive progenitors and differentiating muscle (181, 205). Altogether, MPC rely on the migration of neural crest cells to trigger the transient activation of Notch signaling which guarantees a balanced and progressive differentiation of the MPC pool (181).

**Fetal Programming is Impacted by Maternal Environment**

Fetal growth and development are influenced by genetic as well as environmental factors (206). The overall fetal response to its *in utero* environment is defined as “fetal programming” (207). Fetal programming involves developmental plasticity with disturbances during critical periods of fetal development altering the differentiation of specific cells, organ systems or homeostatic pathways (208, 209) which persist into adult life. Furthermore, the postnatal environment may either mask or magnify the effects of programming on the expression of a phenotype (98). Two of the major environmental factors that have been proposed to influence fetal programming are malnutrition and stress. For example, many have hypothesized that the macro- and micro-nutrient components of the diet, together with caloric content, act to set the cellular control of energy oxidation and storage (210). Likewise, maternal overnutrition can stimulate programming mechanisms which regulate mitochondrial bioactivity, cellular stress and inflammation (210). Altogether, the nutrient status of the mother during pregnancy is responsible for the development of the placenta (211), which is a key organ in respiratory gas, nutrient, and waste exchange between the mother and fetus. If alterations in placental development occur, the growth and future health of the fetus may be impacted (10).
Placenta’s Role in Embryonic Development

The late, eminent reproductive physiologist Samuel Yen referred to the placenta as the ‘third brain’ in pregnancy in recognition of the regulatory nature of placental function (15). The placenta acts as an important source of pregnancy-associated hormones and growth factors, and is involved in immune protection of the fetus (212). The placenta itself is derived from the trophectoderm with the number of cells that differentiate into this lineage determining its developmental potential (213). Specifically, at the time of implantation the trophectoderm cells become trophoblast giant cells (212, 213). The cells immediately adjacent to the inner cell mass are then transformed in a number of ways to differentiate into the remaining placental structures (Figure 1.14). One of these ways is when the trophoblast, with its associated fetal blood vessels, undergoes extensive villous branching to create a densely packed structure called the labyrinth (212). While the labyrinth is developing, it is supported structurally by the spongiotrophoblast, which form a compact layer of non-syncytial cells between the labyrinth and outer giant cells which is in contact with maternal blood (15, 212). The fetal vascular compartment of the placenta arises from the allantoic mesoderm of the embryo while the maternal components are derived from the maternal vasculature and decidual cells (212). Maternal nutrients are able to be exchanged when blood enters the small spaces of the labyrinth where it flows in a countercurrent manner with fetal blood in the trophoblastic villi to maximize nutrient transport (212, 214).
Figure 1.14. **Structure of the Mouse Placenta.** The mouse placenta is composed of a labyrinth, spongiotrophoblast, trophoblast giant cell layer and maternal decidua. The labyrinth layer consists of fetal capillaries and maternal blood and is the site of nutrient and gas exchange between the fetal and maternal blood (213).

During the early phase of fetal development (i.e. the first two-thirds of gestation) maximal placental growth, differentiation, and vascularization are occurring (211, 215). It is during this time that an adequate establishment of the uteroplacental blood flow is needed to allow for exchange of all respiratory gases, nutrients, and waste products between the maternal and fetal systems (211, 216-219). Placental function is a dynamic process which is influenced by maternal diet, body composition and lifestyle and has an important regulatory role in maternal well-being during pregnancy (11). If an altered nutrition and hormone profile occurs, changes in nutrient transfer and partitioning between mother, placenta, and fetus results which modifies placental weights (220). Interestingly, placental weight is used to predict growth and long-term health of the fetus (221) and under normal conditions, placental and fetal weights are highly correlated (222,
Abnormalities in maternal metabolism (e.g. due to obesity) alters the balance between placental and fetal weight (224, 225). In addition, normal processes of placental function like oxidative stress and hypoxia can be altered due to maternal obese environment. Together, these alterations can contribute to differences in the normal program of fetal development. These changes then result in altered differentiation of the developing fetus due to changes in uteroplacental blood flow, placental metabolism of glucocorticoids and the transport of nutrients to the developing fetus.

*Oxidative Stress:*

The syncytiotrophoblast cells come in direct contact with the relatively high oxygen concentration in maternal blood, but have reduced levels of antioxidants making the placenta highly susceptible to oxidative stress (226). Sources of oxidative stress including nutritional and environmental factors play a major role in the susceptibility of programming disease in offspring (227). Oxidative stress is a result of the generation of reactive oxygen species (ROS) in the absence of sufficient antioxidant support (228, 229). There are three ROS: superoxide (O\(^{\cdot-}\)), hydrogen peroxide (H\(_2\)O\(_2\)) and hydroxyl (OH\(^{\cdot}\)) (228, 230, 231), all of which are in a more reactive state than molecular oxygen and are generated within the cell as by-products of aerobic respiration and metabolism. The generation of O\(^{\cdot-}\) by a single electron donation to O\(_2\) is the initial step in the formation and propagation of ROS within and out of the cell (Figure 1.15). The formation of O\(^{\cdot-}\) leads to a cascade of other ROS development. For example, dismutation of O\(^{\cdot-}\) produces H\(_2\)O\(_2\) which can be reduced to H\(_2\)O and O\(_2\) by various antioxidants. Likewise, in the presence of iron, O\(^{\cdot-}\) is converted to OH\(^{\cdot}\). Interestingly,
due to the ROS have a very short half-life, the development of oxidative stress in the placenta does not directly affect signal transduction and cell survival in the developing embryo. However, placental changes as a result of an adverse maternal environment may induce oxidative stress in the embryo (232). If uncontrolled, ROS can damage different biological targets such as lipids, DNA, carbohydrates and proteins and compromise cell function (228, 229, 233).

During a normal pregnancy, there is an important balance between ROS production and antioxidant defenses (234). This balance is altered to favor ROS production in pregnancies complicated by obesity resulting in the development of an intrauterine environment of oxidative stress (Figure 1.16) (235). The production of ROS is facilitated by growth factors and cytokines like insulin, transforming growth factor beta (TGFβ), or TNF-α which are normally increased due to an obese environment. Also, major sources for intracellular ROS are the mitochondria, which generate $O^{•-2}$ and $H_2O_2$ as byproducts of cellular energy production (236). All of these factors increase the generation of $O^{•-2}$ through the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases or via mitochondrial antioxidants (237, 238). There are a number of steps where antioxidants work to control the exposure of cells to $O^{•-2}$ with the first being conversion of $O^{•-2}$ to $H_2O_2$ by superoxide dismutases (SOD) (Figure 1.15). There are three different SODs: SOD1 which contains Copper and Zinc and is located in the cytoplasm, Manganese containing SOD (SOD2) which is located in the mitochondria and extracellular SOD3 which contains Copper and Zinc (230). In addition to SODs, the antioxidants glutathione peroxidase 1 and catalase are reducing enzymes which convert
H$_2$O$_2$ to O$_2$ and H$_2$O and therefore limit cell exposure to H$_2$O$_2$ (Figure 1.15). They also act on lipid hydroperoxides (233). All of these antioxidants, along with non-enzymatic dietary antioxidants (239), work together to defend the body against oxidative stress (Figure 1.16).

**Figure 1.15. Oxidative Stress Results from the Production of Reactive Oxygen Species.** Reactive oxygen species (ROS) are produced from molecular oxygen (O$_2$) being converted to hydroxyl radicals (OH). There are multiple key cellular antioxidant enzymatic pathways that try to convert ROS back into O$_2$ and water (H$_2$O). Superoxide anion (O$_2^-$); hydrogen peroxide (H$_2$O$_2$); copper–zinc containing SOD (Cu,Zn-SOD or SOD1); manganese containing SOD (Mn-SOD or SOD2); catalase (CAT); glutathione peroxidase (GPX) (230).
Figure 1.16. **Prooxidant-Antioxidant Imbalance Results in Oxidative Stress.** A balance is needed between the amount of antioxidants and ROS present in order to not create an oxidative stress environment. Reactive oxygen species (ROS); antioxidant (AOX); superoxide dismutase (SOD); glutathione peroxidase (GPX); catalase (CAT); total antioxidant status (TAS) (233).

Small changes in ROS associated with maternal obesity modulate the expression of genes via activation of multiple signaling pathways (98). For example, ROS activate members of the JNK and p38 stress kinase family, MAPKs, PI3K, and NF-κB (238). The JNK and p38 stress kinase family mediate inflammatory cytokine- and stress-induced apoptosis and stress-responsive gene expression functions through the phosphorylation of the Forkhead Box (FOXO) family of transcription factors (240). ROS are known to promote FOXO1 translocation to the nucleus of β-cells by a mechanism that involves activation of JNK and the phosphorylation of FOXO (241). The JNK-induced phosphorylation by oxidative stress overrides the effects of FOXO phosphorylation,
which then provides a mechanism where oxidative stress results in an increase in insulin sensitivity (242). The activation of FOXO transcription factors due to increased oxidative stress can also induce the expression of a wide range of genes that regulate cellular responses such as cell cycle arrest, apoptosis. Interestingly, FOXO transcription factors also stimulate the expression of SODs and antioxidants in order to alleviate the oxidative stress (237, 243, 244). ROS are also known to activate MAPKs which are protein serine/threonine kinases which play an important role in cell differentiation, growth, apoptosis, via the activation of a variety of transcription factors which regulate target gene expressions (245). Finally, PI3K is activated in response to ROS which induces cell growth, proliferation, differentiation, motility, and survival while NF-κB signaling results in growth control, immune response to infection, and inflammation (246). Altogether, all of these pathways are activated in response to oxidative stress which can have a negative impact on placental nutrient regulation impacting embryonic development.

**Hypoxia:**

Oxidative stress is also induced by hypoxia (247, 248). Hypoxia occurs when oxygen availability does not match the demand of the surrounding tissue, resulting in decreased oxygen tension (249). For example, if oxygen supply is equal to the oxygen demand then a state of normoxia results but, if oxygen supply is not enough to fulfill tissue demands then a hypoxic environment results. Hypoxia was first proposed as a possible cause of inflammation in obesity in 2004 (249-251) when hypoxia was found in only the adipose tissue of obese mice (251). The decreased perfusion as well as
decreased blood flow per blood vessel occurs in adipose tissue of obese mice (249). Systemic hypoxia has also been shown to induce insulin resistance as glucose infusion rate was decreased which occurs in obese individuals (251, 252). Hypoxia has been found to stimulate both leptin and vascular endothelial growth factor (VEGF) from mature adipocytes in vitro as well as being the primary signal for angiogenesis (249, 253, 254). Inflammation in adipose tissue is induced by hypoxia due to the induction of gene expression in adipocytes and macrophages (255).

Low oxygen tension is important in many normal physiological processes in the adult animal including postnatal growth, differentiation and migration. During embryonic development, low oxygen tension promotes the establishment and differentiation of the vascular and hematopoietic systems (256). However, reductions in oxygen tension, to the level of hypoxia, are known to differentially regulate cell differentiation and therefore alter the fetal developmental program (247). For example, hypoxia plays a major part in inhibiting the differentiation of preadipocytes into adipocytes and myoblasts into myocytes (257) which leads to decreased fetal growth and skeletal anomalies (258).

Cells respond to hypoxia by coordinating expression of numerous genes to ensure adaptation (249). The primary pathway that responds to tissue hypoxia is the hypoxia inducible factor (HIF) signaling cascade. The HIF signaling cascade is activated in response to low oxygen availability and is central in maintaining oxygen homeostasis (256, 259). The HIF signaling cascade is composed of a HIF-1β subunit and one of the three HIFα subunits (HIF-1α, HIF-2α or HIF-3α). It also contains prolyl hydroxylase
domain-containing proteins (PHDs) which aid in the regulation of HIF signaling. There are three types of PHDs with each having a distinct tissue distribution, pattern of subcellular localization, and substrate specificity (260). PHD2 is the rate-limiting enzyme that sets the low steady-state levels of HIF-1α in normoxia, whereas PHD1 and PHD3 contribute to HIF-1α regulation only upon chronic hypoxia (261).

Figure 1.17. Hypoxia/Normoxia Signaling Cascade. Hypoxia inducible factor (HIF)-1α is degraded in a normoxia environment, while in a hypoxic environment it binds to a hypoxia responsive element (HRE) and promotes transcription of genes. Prolyl hydroxylase (PHD); von Hippel Lindau protein (pVHL); and ubiquitination (Ub) (262).

Under normal oxygen conditions, PHDs in the presence of O2 hydroxylates HIF-1α, which can then bind to von Hippel Lindau protein (Figure 1.17). This event promotes the polyubiquitination of HIF-1α followed by its degradation (263). In well-oxygenated conditions, HIF-1α is degraded and its levels are maintained at low levels.
cells, HIF-1α is an exceptionally short-lived protein with a half-life of less than 5 minutes (264). On the other hand, under hypoxic conditions, the lack of oxygen prevents the hydroxylation of HIF-1α by PHD, leading to its stabilization (Figure 1.17). The lack of oxygen results in HIF-1α migrating to the nucleus where it dimerizes with HIF-1β which then binds the cofactor p300/CBP. This HIF-1 complex is then able to bind to and induce the transcription of genes containing hypoxia responsive elements in their promoter region (262, 263). Hypoxia is associated with the activation of several stress-induced protein kinases, including JNK, p38, ERK and the NF-κB pathways which trigger cell death (255, 265, 266). NF-κB is a master regulator of inflammation response and controls the transcription of many pro-inflammatory cytokines like TNF-α and IL-1β which are increased in hypoxic tissues (267).

Impaired placental perfusion in early development can lead to hypoxia in the placenta which often causes fetal growth restriction and in severe cases even fetal death (98, 268). Normally, in early pregnancy, placentation occurs in relatively hypoxic conditions which is critical for proper placental and embryonic development (268). Hypoxia is needed to express adequate amounts of a variety of genes, including VEGFA, glycolytic enzymes and inducible nitric oxide synthetase (269). Similarly, placental insufficiency (i.e. reduced placental blood flow) causes a chronic state of fetal hypoxemia and hypoglycemia. As a result, endocrine and metabolic adaptations in the placenta, including the interaction between the Igf2 gene in feto-placental tissues and nutrient transporter genes in placental tissues, conserve fetal nutrients (270).
Maternal obesity not only increases hypoxia and oxidative stress in tissues but also stimulates the production of adrenal glucocorticoids (271). Physiological glucocorticoids (cortisol in humans, corticosterone in rats and mice) are synthesized in the adrenal cortex (272). Cortisol synthesis is regulated by the activity of the hypothalamic–pituitary–adrenal (HPA) axis with adrenocorticotropin hormone (ACTH) stimulating cortisol production by the adrenals. Cortisol, in turn, reduces corticotropin releasing hormone (CRH) and ACTH release by the hypothalamus and pituitary. Cortisol produced by the mother, is transported to the placenta which regulates fetal exposure to glucocorticoids. Glucocorticoid receptors are found in the placenta as well as most fetal tissues and are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (273, 274). In the placenta, glucocorticoid receptors are thought to mediate both metabolic and anti-inflammatory effects (274) and increased levels of glucocorticoids in the placenta directly affect placental size (275).

The ability of the placenta to inter-convert glucocorticoids between their active and inactive forms cortisol was first described by Osinski (276) in 1960. In the placenta, there are two enzymes, 11β hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2), that regulate the amount of circulating glucocorticoids in the body, specifically the active form cortisol (Figure 1.18). Both 11β-HSD1 and 11β-HSD2 are members of the short-chain alcohol dehydrogenase superfamily (277) with the genes encoding them being found on chromosome 1 (278) and chromosome 16 (279), respectively. The enzyme 11β-HSD1 catalyzes the bidirectional conversion of active-
cortisol and inactive-cortisone activities, but operates predominately as an oxoreductase, converting cortisone to cortisol, due to its higher affinity for cortisone (Figure 1.18) (280). In contrast, 11β-HSD2 is a high-affinity unidirectional enzyme that exhibits only oxidase activity, cortisol to cortisone, under physiological conditions and is regulated by oxygen (268, 281). Interestingly, Mericq et al. (282) found 11β-HSD2 enzyme activity in the placenta to be is seven- to eightfold higher compared with the activity of 11β-HSD1.

![Figure 1.18. Enzymatic Regulation of Glucocorticoids.](image)

**Figure 1.18. Enzymatic Regulation of Glucocorticoids.** *Hsd11b1* is responsible for the conversion of ‘inactive’ cortisone to ‘active’ cortisol while *Hsd11b2* converts ‘active’ cortisol to ‘inactive’ cortisone.

Glucocorticoids are essential for the development and maturation of fetal organs (lung, thymus, gastrointestinal tract, heart, liver, and kidneys) at the end of gestation (283-287). However, excessive exposure to glucocorticoids during earlier stages of development leads to altered maturation of organs as well as has a negative impact on fetal growth resulting in IUGR (286). Normally the fetus has much lower levels of physiological glucocorticoids than its mother (288), with the concentrations being very high in maternal blood during pregnancy. The placenta cannot stop lipophilic steroids from crossing to the fetus and therefore limiting embryonic exposure to cortisol is dependent on placental 11β-HSD2 concentration/activity which rapidly inactivates
cortisol to inert cortisone (Figure 1.19) (289, 290). Even though 11β-HSD2 forms a potent barrier to the high levels of maternal glucocorticoids, approximately 10-20% of the maternal glucocorticoids are still able to cross intact to the fetus which are required for the development and maturation of fetal organs (289, 291). Also, the barrier function of 11β-HSD2 is found to decrease in the last part of gestation, along with an increase in 11β-HSD1 gene expression, which may be a mechanism by which cortisol concentrations rise at term to regulate fetal maturation and activate pathways associated with labor (206, 292). Alfaidy et al. (268) showed that oxygen may be an important regulator of placental 11β-HSD2. Indeed, hypoxia has been shown to modulate placental 11β-HSD2 levels by diminishing its promoter activity and down-regulating its protein synthesis (293, 294) while an increase in oxygen levels up-regulates glucocorticoid metabolism through 11β-HSD2 activation. There are also some indirect factors, TNF-α and IL-1β, which have been shown to decrease 11β-HSD2 activity in the placenta (256, 267).

When the placenta exhibits reduced 11β-HSD2 activity there is an increase in fetal exposure to cortisol which is correlated to abnormalities in organ development. For example, reduced 11β-HSD2 activity is associated with increased blood pressure as well as the programming of permanent hyperglycemia in the offspring during adult life (295, 296). Similarly, in humans, mutations in the 11β-HSD2 gene have been associated with low birth weight and reduced 11β-HSD2 activity, and increased fetal cortisol levels have
Figure 1.19. Glucocorticoid Signaling between the Mother, Placenta and Fetus. Activation of the maternal hypothalamic–pituitary–adrenal (HPA) axis during pregnancy leads to increased circulating levels of cortisol (filled circles). Maternal cortisol is transported to the placenta where it is either broken down by the enzyme HSD2 into inactive cortisone (grey triangles) or passes directly through to the fetus. If there is not enough HSD2 in the placenta it can lead to fetal HPA axis activation (associated with low birthweight, metabolic and brain abnormalities). Corticotropin releasing hormone (CRH); adrenocorticotropin hormone (ACTH); and 11β hydroxysteroid dehydrogenase type 2 (HSD2) (297).

been reported in association with IUGR (Figure 1.19) (272). Overall, fetal exposure to high levels of glucocorticoids reduces fetal growth independent of its effects on maternal food intake as well as alters the trajectory of fetal tissue maturation (131). Interestingly, placental glucocorticoid metabolism differs according to sex of the fetus with the male fetus being less sensitive to the effects of glucocorticoids compared to the female fetus (298).
Conclusion

Obesity impacts not only the health of the mother but when combined with pregnancy is associated with an increased risk for their offspring to be obese and develop metabolic syndrome. Offspring from obese dams are more likely to experience IUGR which if accompanied by a catch-up growth in the first few weeks after birth has been correlated to alterations in adult organ function. Interestingly, the extent of the alterations may be sex dependent, but more research is needed to determine the exact mechanisms that are impacting gender-dependent alterations in organ development. Of special interest is the impact of maternal obesity on skeletal muscle development given that it is an important metabolic organ. Currently, there is a gap in understanding how maternal nutrition affects early muscle differentiation; although, a number of studies have documented a negative effect of maternal obesity on secondary and tertiary myogenesis and the metabolic function of this skeletal muscle.

The intrauterine growth of a fetus is largely determined by the capacity of the placenta, which is a key organ in respiratory gas, nutrient, and waste exchange, to supply nutrients from the mother to the fetus. Normal levels of oxygen tension and ROS are needed in the placenta for proper development to occur. However, oxidative stress, hypoxia and altered glucocorticoid metabolism in the placenta inhibit the transport of nutrients which can negatively impact fetal development. The placenta is unique in that it is able to produce an adaptive response if the fetus is not developing well; however, this adaptation can only partly compensate for impaired growth in utero.
While the normal development of the placenta and muscle is well described, the mechanisms by which maternal obesity alters this development are unknown. The long-term goal of our laboratory is to understand how maternal obesity alters placental function and impacts cell differentiation during embryonic/fetal development. The working hypothesis of this project is that maternal obesity induces placental hypoxia and/or oxidative stress resulting in the altered differentiation of somite-associated mesodermal cells and reduced skeletal muscle development. In order to test this hypothesis, the lethal yellow (LY) mouse model of obesity was used. The LY mouse has a deletion mutation on the C57BL/6 (B6) background which results in ectopic expression of agouti (Figure 1.20). Agouti, in turn, inhibits hypothalamic signaling of α-MSH and CART via the melanocortin 4 receptor (MC4R) resulting in inhibition of satiety, excess caloric intake, and progressive obesity. In this study, mid-gestation embryos and placentas that were developed in a B6 (lean) or LY (obese) environment were examined for placental abnormalities and differences in embryonic cell differentiation, specifically alterations in primary myogenesis.
Figure 1.20. Lethal Yellow Mouse Model of Obesity. Schematic representation of the deletion mutation on the C57 BL/6 background which produces the Lethal Yellow phenotype.
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CHAPTER II

Maternal Obesity Alters Embryonic Expression of Desmin and PPARγ, Reduces Placental Function and Alters Placental Expression of Stress-Related Genes

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ABSTRACT

Maternal obesity has been correlated to decreased skeletal muscle mass, increased adipose tissue mass and increased risk for metabolic syndrome in their children. However, mechanistic links between maternal obesity and the development of these phenotypes remain unclear. Thus, the objective of the current study was to determine how maternal obesity alters the temporal program of skeletal muscle differentiation. To achieve this objective, mid gestation embryos (12.5 days post coitus) were collected from age-matched, obese Lethal Yellow (LY) and normal weight C57BL/6 (B6) dams. As expected, the body weight of LY dams was heavier than B6 dams while the average wet weight and tail somite number of LY-derived embryos was decreased. To determine the impact of maternal obesity and decreased embryo weight on mesodermal cell differentiation, the expression of the myogenic marker desmin (DES) and adipogenic marker PPARG were examined. Surprisingly, DES expression tended to be increased ($P < 0.09$) in somite-associated cells of LY-derived embryos. Furthermore, PPARG expression was predominately localized to the neural tube and was significantly decreased ($P < 0.04$) in LY-derived embryos. Placental function in the LY dams was
also altered. Specifically, the ratio of embryo to placenta weight was decreased 12% in LY-derived embryos suggesting abnormalities in placental development. Furthermore, the mRNA abundance of *Hif1a*, *Pecam1*, and *Hsd11b2* was altered in the placenta indicative of chronic hypoxemia and stress. Taken together, these data demonstrate that temporal regulation of myogenesis is altered in embryos derived in an obese environment and may be associated with obesity-induced placental stress.

**Key Words:** Obesity, Cortisol, Muscle Development, Neural Tube

**INTRODUCTION**

Obesity is a considerable public health problem that is associated with an increased risk of several chronic diseases including type 2 diabetes and cardiovascular disease (1, 2). The prevalence of obesity worldwide is 15–20% with at least 1/3 of reproductive age women classified as obese in the United States (3, 4). Maternal obesity negatively affects the infant’s health by increasing fetal adiposity as well as increasing the lifetime risk of obesity and development of metabolic syndrome during childhood or early adult life (5-7). Specifically, maternal obesity increases fetal intramuscular adipocytes and fibrosis (8-10) as well as decreases muscle fiber density (11). Furthermore, Yan *et al.* (12) have shown attenuated insulin signaling in the skeletal muscle of offspring derived from over nourished mothers.

These changes in the structure and function of skeletal muscle (12, 13) suggests that the activation and/or regulation of the skeletal muscle program are altered when the
fetus is exposed to an obese in utero environment. During normal fetal development, skeletal muscle in the trunk and limb develops from stem cells in the dermomyotome layer of the somite which differentiate into myogenic progenitor cells upon stimulation of paired box (Pax) 3 and Pax7 expression (14). PAX3 and PAX7 are transcription factors that regulate the expression of myogenic factor-5 (Myf5), myogenic differentiation 1 (MyoD), myogenin (Myog), and desmin (Des) which, in turn, promote differentiation of proliferating muscle progenitor cells into myocytes and ultimately mature muscle fibers (15-19). Regulation of the myogenic program is dependent on multiple signals from the neural tube located adjacent to each somite and neural crest cells which migrate around and within the somite (20, 21). Together, these signals maintain an important balance between the maintenance and/or migration of stem and progenitor cells and the differentiation and establishment of mature myofibers which are the major components of adult skeletal muscle.

The effect of maternal obesity on fetal development is mediated by the placenta which serves as a dynamic interface between the mother and developing fetus (22). Hayes et al. (23) showed that excessive adiposity during pregnancy results in increased platelet endothelial cell adhesion marker (PECAM1) and decreased smooth muscle actin expression, indicative of poor placental vascularization. Decreased placental vascular density has been tied to reduced tissue oxygenation and the development of a hypoxic environment (24). Poor development of placental vasculature also results in reduced placental blood flow, leading to not only a smaller placenta but also insufficient nutrient transport (25, 26). Hypoxia, in turn, can induce oxidative stress due to decreased
expression of antioxidants (27) as well as decreased expression of 11β-hydroxysteroid dehydrogenase 2 (Hsd11b2) which converts glucocorticoids from an active to inactive state (28). Collectively, these maternal obesity dependent changes to placental function have been linked to intrauterine growth restriction (IUGR). Furthermore, activation of signaling pathways due to hypoxia and oxidative stress as well as changes to DNA methylation and histone modifications by the glucocorticoid, cortisol, result in changes in the fetal program which can directly impact phenotypes expressed by the children of obese mothers (29-31).

Taken together, these data indicate that the increased incidence of maternal obesity may perpetuate the obesity epidemic in the next generation due in part to altered skeletal muscle programming. However, mechanistic links between maternal obesity and the regulation of the skeletal muscle program during embryonic development remain poorly defined. In the current study, the Lethal Yellow (LY) mouse model was used to identify maternal obesity-dependent temporal and/or spatial changes in primary myogenesis. The LY mouse has a deletion mutation on the C57BL/6 (B6) background which results in ectopic expression of agouti (32-34). Agouti, in turn, inhibits hypothalamic signaling of α-MSH and CART via the melanocortin 4 receptor (MC4R) resulting in inhibition of satiety, excess caloric intake, and progressive obesity (35). We have previously demonstrated that the obese phenotype of LY females includes a significant increase in total body weight, visceral fat weight, and circulating leptin and insulin concentrations (36). Using this model, we have identified novel differences in the
expression of myogenic markers and abnormalities in placental function of mid-gestation embryos collected from lean (B6) and obese (LY) dams.

MATERIALS AND METHODS

Animal Model: Founder B6 (C57BL/6 a/a) and LY (C57BL/6 Ay/a) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Fertile, male B6 mice were mated with aged-matched (17-week-old) B6 or LY female littermates. Twelve days after visual confirmation of a vaginal plug (i.e., 12.5 dpc), B6 (n=7) and LY (n=12) dams were euthanized and maternal weights were determined. Viable and degenerative embryos were collected from each dam (degenerative: B6=7.01% and LY=5.53%). Each viable embryo and its placenta were weighed and the number of tail somites per embryo counted from hind limb to the tip of the tail. Half of the embryos from each litter were fixed in Bouin’s solution and embedded in paraffin for morphological and immunohistochemical (IHC) analyses while the other half were flash frozen for RNA extraction. All of the placentas were flash frozen for RNA extraction. Agouti mRNA abundance (see QPCR methods below) was used to distinguish between B6 and LY embryos derived from LY dams. All animal experiments were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

Reverse Transcription: Whole placenta and whole embryo RNA was isolated using Tri-Reagent (Invitrogen, Carlsbad, CA). RNA (5 μg) from each individual sample was treated with DNase I (Promega, Madison, WI) and reverse transcribed using random
hexamer primers (Roche Diagnostics, Indianapolis, IN) and Moloney Murine Leukemia Virus reverse transcriptase (Promega) according to manufacturer’s instructions.

**Quantitative, Real-Time PCR (QPCR):** Forward and reverse primers for candidate genes and the housekeeping gene β-actin (*Actb*) (Table 2.1) were designed (Primer Express, Applied Biosystems, Foster City, CA) and synthesized (Integrated DNA Technologies, Coralville, IA). Primers were tested empirically to determine the maximal concentration that produced specific amplification of the target sequence in the absence of primer dimers. Quantitative PCR (QPCR) reactions were carried out using each primer set and equivalent amounts of cDNA from each sample as previously described (37). QPCR amplification of *Gapdh* and *18s* rRNA was carried out using an endogenous control kit (Applied Biosystems, Foster City, CA). The relative abundance of the candidate mRNA in each sample was normalized using the most stably expressed housekeeping gene(s) (*Gapdh*, *18s*, or *Actb*) which was determined using Normfinder (38). Specifically, embryo candidate genes were normalized using the geometric mean of *18s* rRNA and *Gapdh* mRNA abundance for each embryo sample while *Gapdh* mRNA abundance was used to normalize candidate genes for each placenta sample. The resulting normalized data for each candidate mRNA was then compared to the mean normalized mRNA abundance in B6-derived placenta or embryo and expressed as a fold change.
Table 2.1. Primer Sequences for QPCR Analysis

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</tbody>
</table>

**Immunofluorescence Analyses:** Transverse sections (8 µm) were generated from the Bouin’s-fixed embryos from LY and B6 dams for IHC analyses. Samples were deparaffinized and hydrated followed by boiling in 10 mM sodium citrate for antigen retrieval (39). Samples were blocked in 1X PBS, 5% normal goat serum and 0.3% Triton X-100. After blocking, anti-DES (1:200) (Abcam, Cambridge, MA), anti-PPARG (1:200) (Cell Signaling, Danvers, MA), or no primary antibody diluted in SignalStain Antibody Diluent (Cell Signaling, Danvers, MA) was added to each section and incubated overnight at 4°C. Sections were washed and subsequently incubated with Alexa Fluor 555 anti-rabbit secondary antibody (DES) or Alexa Fluor 488 anti-rabbit secondary antibody (PPARG) (Cell Signaling, Danvers, MA). Sections were washed and mounted with VectaShield Hard Mount containing DAPI (Vector Labs, Burlingame, CA) to stain cell nuclei. Sections exposed to only Alexa Fluor 555 or Alexa Fluor 488 anti-rabbit secondary antibodies were used as a negative control in order to ensure specific detection of signal. Detection of DES or PPARG positive cells was carried out using an IX71 Olympus inverted brightfield and fluorescence microscope (Hischfeld Instruments,
Inc., St. Louis, MO) and images captured using the Slidebook 4.2 Software (Intelligent Imaging Innovations, Inc., Denver, CO) (40). Images were captured using the same exposure length and analyzed using Image J Software (National Institute of Health, Bethesda, MD) for the percent area stained. The mean value for embryos derived in LY dams was compared to the mean value from embryos derived in B6 dams and shown as a fold change.

Statistical Analyses: All statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Statistically significant differences in weights, mRNA abundance or protein expression between B6 and LY dams or B6- and LY-derived embryos or placentas (P < 0.05) were determined using Student t-test.

RESULTS

Maternal Obesity Reduces Fetal Growth and Induces Modest Developmental Delays in Viable Fetuses: As expected based on our previous study (36), maternal body weights of female LY mice were significantly higher (P < 0.0001) than age-matched B6 mice at embryonic day 12.5 (E12.5) (Figure 2.1A). While there was an increase in body weight, no difference in litter size of viable embryos (Figure 2.1B) or the number of degenerating embryos (data not shown) was identified. Individual wet weights of the viable embryos showed a significant 30% reduction (P < 0.0001) in LY-derived compared to B6-derived embryos (Figure 2.1C). Furthermore, embryos derived from LY dams had a modest but significant reduction (P < 0.0001) in tail somite number (Figure 2.1D). To establish that these morphological effects were due to the maternal
obese phenotype and not overexpression of agouti, B6 and LY embryos collected from LY dams were compared. There was no difference in embryo and placental wet weights or tail somite number (Supplemental Figure 2.7) between LY and B6 littermates. All placentas from LY dams, regardless of embryo phenotype, expressed agouti. Agouti can bind to the melanocortin family of receptors including MC1R and MC4R; however, there were negligible levels of \( \text{Mc1r} \) and \( \text{Mc4r} \) mRNA detected in either the placentas or embryos collected from LY dams (data not shown). Together, these data indicated that the embryonic phenotypes are induced by the obese phenotype of the dams and not the ectopic expression of agouti. The embryonic phenotypes are consistent with IUGR and modest developmental delays reported in diet induced obesity models (22, 41, 42).

![Bar chart](image)

**Figure 2.1. LY Mice Exhibit an Obese Phenotype and Altered Growth of their Embryos.** Maternal body weight (A) and the number of embryos at 12.5 dpc (B) were determined for B6 (black bar, \( n=7 \)) and LY (yellow bar, \( n=12 \)) dams. The wet weights (C) were recorded for embryos (B6-derived, black bar, \( n=63 \); LY-derived, yellow bar, \( n=113 \)) and the number of tail somites (D) for each B6-derived (black bar, \( n=58 \)) or LY-derived (yellow bar, \( n=103 \)) embryo were counted from hind limb to tip of the tail. The mean ± SEM normalized values are presented, and asterisks represent a statistically significant difference in means (\( P < 0.0001 \), ***).
Primary Myogenesis is Accelerated in Embryos Developed in an Obese In Utero Environment: To assess differences in myocyte development between LY- and B6-derived embryos, IHC analysis of DES expression was carried out. DES is a marker of differentiating myocytes and, upon its expression, myocytes are destined to complete myogenesis (i.e. this is an irreversible differentiation step). As expected, DES-positive cells were located exclusively in the somite regions adjacent to the neural tube in both B6- and LY-derived embryos (Figure 2.2A and 2.2B). In contrast, there was no signal detected in sections incubated with only secondary antibody (NEG Control, Figure 2.2A).

Figure 2.2. DES Expression is Increased in the Somite in LY-Derived Embryos. (A, B) Immunofluorescent detection of DES was carried out using transverse sections of 12.5 dpc embryos derived from B6 and LY dams. Representative images (A=40X, B=100X) are shown for DES (orange) and DAPI (blue) expression. Sections processed without primary antibody were used to verify specific detection of DES (NEG control). (C) The percent of the indicated area (circled in A) which is positive for DES was determined for each sample (B6, black bar, n=8; LY, yellow bar, n=11), normalized to the average percent area for the B6-derived embryos, and expressed as a fold change (mean ± SEM). The mean ± SEM normalized values are presented and the data was tested for statistical significance ($P < 0.09, \dagger$). Scale bars=100 µm (A) and 50 µm (B).
The percentage of DES-positive cells in LY- compared to B6-derived embryos was also different. However, there tended to be 2-fold more DES-positive cells \( (P < 0.09) \) in LY-derived embryos (Figure 2.2C) which was unexpected given that fetal and adult muscle mass is decreased in other models of maternal obesity (11, 13, 43, 44).

**PPARG is Predominately Expressed in the Neural Tube and Its Expression is Decreased in LY-Derived Embryos:** Myocytes and adipocytes arise from the same stem cells in vitro (43). Therefore, given the difference in DES expression in LY-derived embryos, we also examined the expression of a marker of adipocyte differentiation, peroxisome proliferator-activated receptor gamma (PPARG), in each embryo (Figure 2.3, 2.4). While some PPARG-positive cells were identified in the somites of both LY- and B6-derived embryos, PPARG expression was primarily localized to the neural tube of each embryo (Figure 2.3A-D). Conversely, no signal was detected in sections incubated with only secondary antibody (NEG control, Figure 2.3E). Furthermore, PPARG was expressed in cells that appeared to be delaminating from the neural tube (Figure 2.4). Semi-quantitative analysis of PPARG expression in the somite region revealed no differences in the percentage of PPARG positive cells in B6- or LY-derived embryos \( (1.0 \pm 0.50 \text{ vs. } 0.66 \pm 0.27, P = 0.52) \). However, we did detect a significant 2-fold decrease \( (P < 0.04) \) in PPARG expression in the neural tube region (circled regions; Figure 2.3A and C) of LY-derived embryos (Figure 2.3F).
Figure 2.3. PPARG Expression is Decreased in the Neural Tube of LY-Derived 12.5 dpc Embryos. Representative embryos (40X) from B6 and LY dams were stained for immunofluorescent detection of PPARG (green) and DAPI (blue). A representative section processed without primary antibody served as a negative control (E) to verify specific detection of PPARG. The percent of the indicated area (circled in A, C) which is positive for PPARG was determined for each sample (B6, black bar, n=8; LY, yellow bar, n=11), normalized to the average percent area for the B6-derived embryos, and expressed as a fold change (mean ± SEM). Asterisk represents a statistically significant difference in means ($P < 0.05$, *). Scale bars=100 µm. NT=neural tube and Som=somite.

Figure 2.4. PPARG is Expressed in Cells Adjacent to the Neural Tube in 12.5 dpc Embryos. Representative images (100X) of B6 (n=8) and LY-derived embryos (n=11) from Figure 3 showing expression of PPARG (green). Arrows indicate PPARG-positive cells adjacent to the neural tube. Scale bars=50 µm. NT=neural tube.
**Expression of the Epithelial-Mesenchymal Transition Marker Zeb2 is Decreased in Embryos Developed in an Obese In Utero Environment:**

The conversion of neural tube cells from an epithelial to a mesenchymal lineage is an important process prior to delamination and migration of these cells during embryonic development. Furthermore, there is *in vitro* evidence that PPARG regulates the epithelial to mesenchymal transition (EMT) (44, 45). Therefore, to determine if decreased neural tube expression of PPARG was associated with changes in the mRNA abundance of transcription factors that regulate EMT (SNAI1, SNAI2, TWIST1, ZEB2), QPCR was carried out using RNA collected from the whole embryo. While there were no differences in the mRNA abundance of *Snai1, Snai2, or Twist1*; Zeb2 tended to be decreased 1.35-fold (*P < 0.07*) in LY- compared to B6-derived embryos (Figure 2.5).

**Figure 2.5. Abundance of EMT mRNAs in Whole Embryos from B6 and LY Mothers.**

QPCR analysis of *Snai1, Snai2, Twist1* and *Zeb2* was carried out. The normalized, relative abundance of each gene from embryos derived in LY dams (yellow bar, n=10) was compared to the mean, normalized relative abundance of each gene from embryos derived in B6 dams (black bar, n=10) and the data shown as a fold change (mean ± SEM). The data was tested for statistical significance (*P < 0.1, †*).
Reduced Placental Growth and Altered Gene Expression in LY-derived Placenta:

Given that the placenta serves as a critical interface between the dam and fetus, the effect of maternal obesity on placental function was examined. The placental weight was significantly reduced \((P < 0.0001)\) when collected from LY compared to B6 dams (Figure 2.6A). Furthermore, there was a 12\% decrease \((P < 0.02)\) in the ratio of embryo to placenta weight when embryos were collected from LY dams (Figure 2.6A) suggesting abnormalities in placental development and/or function. To determine if the placentas collected from LY dams exhibited evidence of hypoxemia, the mRNA abundance of hypoxia induced transcription factors Hif1a and Arnt were determined by QPCR. While Hif1a mRNA abundance was modestly increased \((1.24\text{-fold}, P < 0.07)\), there was no difference in Arnt expression (Figure 2.6B). In addition to these transcription factors, markers of angiogenesis which are increased upon hypoxia were also examined. The mRNA abundance of Pecam1 was increased 1.34-fold \((P < 0.05)\) in placenta from LY compared to B6 dams (Figure 2.6C). However, the mRNA abundance of other angiogenesis makers including Angpt1, Vcam1, or Vegfa_164 was not different in LY compared to B6 placentas. Chronic hypoxemia can also impact the expression of enzymes which regulate the conversion of glucocorticoids between their inactive \((\text{Hsd11b2})\) and active \((\text{Hsd11b1})\) isoforms. There was no difference in \(\text{Hsd11b1}\) mRNA abundance in placentas derived from B6 and LY dams. However, \(\text{Hsd11b2}\) mRNA levels were decreased 1.59-fold \((P < 0.04)\) (Figure 2.6D).
Figure 2.6. Placental Morphology and Function is Altered in LY Dams. Wet weights (A) were recorded for B6-derived (black bar, n=27) and LY-derived (yellow bar, n=73) placenta as well as the ratio of embryo to placenta weight was determined. (C, D) QPCR analysis of Hif1a, Arnt, Angpt1, Pecam1, Vcam1, Vegfa_164, Hsd11b1 and Hsd11b2 was carried out. The normalized, relative abundance of each gene in placenta from LY dams (yellow bar, n=12) was compared to the mean, normalized relative abundance of each gene from placenta from B6 dams (black bar, n=11) and the data shown as a fold change (mean ± SEM). The data was tested for statistical significance ($P < 0.1$, †; $P < 0.05$, *; and $P < 0.0001$, ***)

DISCUSSION

Numerous studies have demonstrated a clear correlation between maternal obesity and altered physiological function in adult offspring. Indeed, maternal overnutrition has been linked to decreased density and/or metabolic function of fetal and adult skeletal muscle in sheep (11, 12, 46, 47) and rodents (48, 49). Based on in vitro studies of
mesodermal stem cells and in vivo studies carried out in the last third of gestation, one underlying mechanism of this phenotype could be decreased development of skeletal muscle and increased development of adipose tissue from common stem or progenitor cells (13, 43). However, data in the current study indicates that maternal obesity increased DES expression in the dermomyotome layer of somites in mid-gestation embryos suggesting increased differentiation of skeletal muscle cells. Furthermore, at mid-gestation, PPARG expression was primarily localized to the neural tube with very little expression in the somite indicating temporal uncoupling of myogenesis and adipogenesis. Thus, for the first time, we provide evidence that maternal obesity increases myogenesis early in development and suggest that these events may cause changes in the trajectory of skeletal muscle progenitor cell differentiation. This may subsequently result in reduced skeletal muscle development later in gestation and reduced muscle mass and/or function in adult offspring.

Primary myogenesis is initiated in the early embryo (E8.5-E9.0 in the mouse) and results in the differentiation of stem cells into myoblasts which proliferate, migrate, and ultimately fuse to form primary myotubes (i.e., primitive muscle fibers) (50). The intermediate filament protein, Des, is initially expressed in proliferating myoblasts and its expression is maintained through differentiation into mature myofibers (51-53). DES expression in the somite is detected at E9.0, with cranial somites exhibiting expression first followed by caudal somites. Interestingly, we showed that expression of DES in the dermomyotome layer of the somite is increased in E12.5 embryos developed in an obese compared to a normal weight environment. This difference in expression was not
attributed to differences in the expression profile of cranial versus caudal somites as each embryo was processed to ensure that somites at similar anatomical positions were examined. Li et al. (54) showed that loss of DES expression in the C2C12 myoblast cell line results in poor fusion of myoblasts into myotubes suggesting that one function of DES is to promote myotube formation over myoblast proliferation and/or migration. In sheep, decreases in muscle fiber density were identified in both the semitendinosus (limb) and longissimus dorsi (trunk) muscles (10-12) indicating an overall reduction in myotube formation. Based on these collective data, we hypothesize that maternal obesity favors myoblast exit from the cell cycle and fusion during primary myogenesis resulting in decreased myoblast proliferation.

PPARG is a well described transcriptional regulator of adipocyte differentiation and is expressed in the committed preadipocyte and mature white adipocyte (55). Given this role of PPARG and evidence that adipocytes and myocytes arise from common mesodermal stem cells in vitro (43), we anticipated that PPARG expression would be localized to the somite and be increased in embryos derived from LY dams. In contrast, however, PPARG expression was primarily localized to the ventral neural tube at E12.5 and the percentage of PPARG-positive cells was decreased in the neural tube of embryos developed in an obese environment (Figure 2.3). These data are consistent with PPARG localization studies during development carried out by Michalik et al. (56). Likewise, Wada et al. (57) showed high levels of PPARG expression in the embryonic brain and demonstrated that PPARG is required for neural stem cell proliferation and the formation of neurospheres in vitro. The novel decrease in PPARG upon maternal obesity identified
in the current study suggests that, not only is PPARG functionally important during neural development, but also provides the first evidence that decreased PPARG may contribute to abnormalities in neural development in an obese environment.

Irrespective of the role of PPARG in the neural tube, the lack of PPARG-positive cells in the somite of mid-gestation embryos (Figure 2.3) indicates that temporal regulation of primary myogenesis and adipogenesis are uncoupled. While these findings oppose *in vitro* studies which show that adult mesenchymal stem cells divergently differentiate into myogenic versus adipogenic cells (58), mechanistic differences between embryonic cell differentiation during *in vivo* development and adult stem cell differentiation during tissue regeneration are not unprecedented. For example, regeneration of skeletal muscle, which includes activation of satellite cells, in the adult animal is mechanistically different from the myogenic program during embryonic development (59, 60). Thus, given the metabolic importance of the skeletal muscle to adipose tissue ratio in the adult animal, a better understanding of how adipogenesis is stimulated and regulated during normal embryo and fetal development is needed. Interestingly, Billon et al. demonstrated that neural crest cells have the potential to differentiate into adipocytes (61). Likewise, we detected PPARG expression in cells that may be delaminating from the neural tube and therefore may represent neural crest cells (Figure 2.4). However, while adult adipose tissue in the head and neck have been traced to a neural crest cell lineage, fat pads in the trunk including perigonadal, subcutaneous, and perirenal depots were not derived from the neural crest cell lineage (61). These data indicate that different adipose tissue depots likely develop by unique mechanisms and
arise from different stem and progenitor cell lineages during development adding another layer of complexity to adipose tissue development.

The specific mechanisms by which the maternal environment alters embryo growth and development are uncertain, but include changes in placental function. Indeed, in our model of maternal obesity, embryo weight, placenta weight, and the ratio of embryo to placenta weight were decreased (Figure 2.1 and 2.6) indicating potential abnormalities in placenta development and/or function. The decrease in placenta and embryo weight at mid-gestation is consistent with other rodent models of maternal obesity or gestational diabetes (22, 41, 42). However, an increased risk for macrosomia due to increased growth during gestation (large for gestational age, LGA) is the common phenotype reported upon human maternal obesity (62-64). It is important to note that the rodent studies detected growth restriction at mid-gestation of pregnancy (E12.5-14.5) while most human studies are identifying small and large for gestational age babies at birth. Furthermore, Jones et al. (65) showed that E18.5 fetuses from high-fat fed C57/BL6 dams weighed more than fetuses from control-fed dams due to increased placental transport of glucose and aminoisobutyric acid. In our mouse colony, weaning weights of offspring from obese, LY dams were not different than age-matched offspring from lean, B6 dams (Wood, unpublished data). Thus, restricted growth during the first half of gestation may be followed by compensatory growth during the second half of gestation ultimately resulting in normal or increased birth weights in the murine model of maternal obesity and may represent a mechanism of macrosomia development.
The abnormalities in fetal growth associated with maternal obesity have been correlated to placental inflammation and hypoxemia (7, 66, 67). In the current study, we detected no evidence of inflammation (data not shown) but did detect indications of hypoxemia (Figure 2.6) in the placenta from obese LY dams. Furthermore, we demonstrated that Hsd11b2 mRNA abundance was decreased in placenta from LY dams (Figure 2.6). The 11β-HSD type 2 enzyme converts active glucocorticoids to their inactive form and its expression in the placenta is crucial in order to limit transport of maternal cortisol to the fetus (28). Interestingly, Gokulakrishnan et al. (68) showed that in utero exposure of rat fetuses to dexamethasone, a synthetic glucocorticoid, during the second half of gestation (E12-E22) resulted in reduced placenta and fetal weight, a decreased fetal-to-placenta weight ratio, and decreased protein mass in skeletal muscle of the quadriceps and diaphragm. Furthermore, Nesan et al. (69) demonstrated that knockdown of the glucocorticoid receptor (GR) during early embryogenesis of zebrafish resulted in reduced embryo length but increased expression of Myog. Together, these data indicate an important role of cortisol in regulating the myogenic program.

In summary, we have presented novel evidence that maternal obesity-dependent increases in fetal exposure to hypoxemia and/or cortisol results in increased expression of the skeletal muscle differentiation marker DES during primary myogenesis. Based on these data, we propose that primary myogenesis is precociously activated when the embryo develops in an obese environment resulting in depletion of skeletal muscle progenitor cells required for secondary myogenesis. In addition, we have shown that PPARG expression is localized to the neural tube indicating a function for this
transcription factor independent of adipocyte development. These key findings represent an important shift in our understanding of how maternal obesity reduces skeletal muscle fiber density and increases adipose tissue mass during development which will have long-lasting effects on the metabolic health of their children.

SUPPLEMENTAL FIGURE

**Supplemental Figure 2.7. Embryos from LY Mice Do Not Differ in Growth Regardless of Agouti Expression.** Embryos and their corresponding placenta were isolated from B6 dams (B6 embryo, black bar, n=10) or LY dams (B6 embryo, grey bar, n=7; LY embryo, yellow bar, n=3). Wet weights for each embryo and placenta were recorded as well as the number of tail somites were counted (mean ± SEM). Statistically significant differences (n.s., not significant; \( P < 0.1 \), †; \( P < 0.05 \), *; and \( P < 0.001 \), **) were determined using one-way ANOVA.
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CHAPTER III

Placenta from Obese, Lethal Yellow Dams Exhibit Reduced Weight and Show Evidence of Hypoxia and Abnormal Cortisol Metabolism but Not Oxidative Stress

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ABSTRACT

Proper placental development is critical for adequate gas, nutrient, and waste exchange and ensures appropriate transfer of substrates needed to support normal fetal growth. Previous studies demonstrate that maternal obesity negatively impacts placental function but the mechanisms have not been defined. Thus, the objective of this study was to determine how maternal obesity impacts the overall growth and molecular phenotype of the mid-gestation placenta. To achieve this objective, embryos and their corresponding placenta were collected 12.5 days post coitus from age-matched, obese Lethal Yellow (LY) and normal weight C57BL/6 (B6) dams. Placenta weight and the ratio of embryo to placenta weight were decreased in LY-derived embryos suggesting abnormalities in placental development and/or function. To determine if the reduced placental weight is associated with abnormalities in hypoxia, oxidative stress or glucocorticoid metabolism, whole placenta was collected for quantitative real-time PCR, Western blot, and enzyme activity assays. We identified significant changes in the mRNA abundance of *Hif1a*, *Pecam1*, *Gpx1* and *Hsd11b2* in the placenta of LY compared to B6 dams. However, there were no differences in the expression of *Sod1* or *Sod2* and there was no increase in the expression or activity of xanthine oxidase. The impact of
altered placental gene expression on embryonic gene expression was also examined. Interestingly, embryos from an obese environment displayed decreased mRNA abundance of *Vegfa_164* and *Vegfa_165b*. Taken together, the data provide evidence that maternal obesity increases placenta hypoxemia and reduces glucocorticoid metabolism which may be altering nutrient transport by the placenta and negatively impacting angiogenesis in the embryo.

**Key Words: obesity, placenta, hypoxia, glucocorticoids**

**INTRODUCTION**

Obesity in the USA is a rising epidemic with 27.8% of the total USA population considered obese in 2011 (1). Furthermore, approximately 35% of reproductive aged women are obese which translates to more than one in five pregnant women being obese (2, 3). Excess weight gain during pregnancy has detrimental effects on offspring development as well as their future health (4). During development, nutrient availability and fetal growth are dependent on maternal nutrient availability which is transported via the placenta. Specifically, the placenta’s role is to regulate the respiratory gas, nutrient, and waste exchange between the mother and developing fetus (5-9).

The placenta is the first organ to develop during mammalian embryogenesis and forms from trophoblast cells, which provide the main structural and functional components needed to bring the fetal and maternal blood systems into close contact (10). Normal placental angiogenesis is critically important to ensure adequate blood flow to the
placenta and therefore, to provide the substrates that support normal fetal growth (11). Reduced oxygen levels and the synthesis of reactive oxygen species (ROS) which are associated with abnormal functions in other physiological systems play an important role in normal placenta development. For example, during early pregnancy, placentation occurs under low oxygen tension conditions which promote placental morphogenesis, angiogenesis, and cell fate decisions critical for proper placental development (12, 13). A moderate increase in ROS levels is also a normal occurrence in the placenta during the second trimester of pregnancy which leads to the stimulation of cell growth and proliferation (14, 15). The development of the placenta and its functions during gestation are dynamic processes which are influenced by maternal diet, body composition and lifestyle (7, 16). Increased maternal nutrition or environmental stress results in abnormal placental angiogenesis and altered fetal growth (6, 11). Likewise, hypoxia and oxidative stress associated with maternal obesity inhibits normal placental angiogenesis and can reduce placental blood flow and nutrient transport (9, 17).

Hypoxia occurs when oxygen availability does not match the demand of the surrounding tissue (18). In the presence of a hypoxic environment, cells activate the hypoxia inducible factor (HIF) signaling cascade which coordinates the expression of genes and ensures adaptation (18-20). The primary targets of the HIF signaling cascade are the transcription factors HIF1a and ARNT. Under normoxic conditions, HIF1a is rapidly hydroxylated resulting in its degradation. However, when oxygen levels are decreased, HIF1a hydroxylation is reduced allowing for its translocation to the nucleus where it dimerizes with ARNT. This complex then binds to and induces the transcription
of genes (vascular endothelial growth factor A (VEGFA), glycolytic enzymes and inducible nitric oxide synthetase) which contain hypoxia responsive elements in their promoter region leading to their increased expression (21-23).

When there is an increase in the level of hypoxia, an increase in oxidative stress usually arises as well (24, 25). The generation of oxidative stress occurs due to an increase in reactive oxygen species (ROS) (26, 27); however, antioxidants like glutathione peroxidase (Gpx1) and superoxide dismutase (Sod) work to defend the body against their generation. Conversely, xanthine oxidase, which is stimulated by hypoxia, promotes the production of ROS. ROS can damage different biological targets such as lipids, DNA, carbohydrates and proteins which compromise cell function and can alter the transport of nutrients through the placenta (27, 28).

Another important function of the placenta is regulating glucocorticoid availability to the embryo and fetus. At the end of gestation, glucocorticoids are essential for the development and maturation of fetal organs (e.g. lung, thymus, gastrointestinal tract, heart, liver, and kidneys) (29-33). Physiological glucocorticoids (cortisol in humans, corticosterone in rats and mice) are synthesized throughout pregnancy by the maternal adrenal cortex (34) and once produced, are able to be transported from the mother, through the placenta, to the developing fetus. In the placenta, there are two enzymes, 11β hydroxysteroid dehydrogenase type 1 (Hsd11b1) and type 2 (Hsd11b2) which regulate the amount of maternal glucocorticoids that reach the fetus. The enzyme Hsd11b1 catalyzes the conversion of cortisone (11-dehydrocorticosterone (11-DHC) in mice) to corticosterone/cortisol (35) while Hsd11b2 converts corticosterone/cortisol to
11-DHC/cortisone. The expression of \textit{Hsd11b2} is regulated by oxygen (12, 36) and Mericq et al. (37) showed that \textit{Hsd11b2} enzyme activity in the placenta to be seven- to eightfold higher compared with the activity of \textit{Hsd11b1}. These data suggest that the primary role of the placenta is to convert glucocorticoids to their inactive form and limit fetal exposure of glucocorticoids until the end of the gestation. There is evidence that glucocorticoid metabolism in the placenta may be altered due to a maternal obesity resulting in altered differentiation of the developing fetus (38).

While there is evidence that maternal obesity negatively effects placental development and function, it is unclear if maternal obesity increases hypoxia and oxidative stress or alters glucocorticoid metabolism in the placenta. In the current study, we use a Lethal Yellow (LY) obese mouse model to identify the impact of maternal obesity on markers of placental hypoxia, oxidative stress and glucocorticoid metabolism. Previously, we characterized LY mice and showed that they over eat and have an altered hormone profile similar to that of obese humans (39). Using this LY mouse model of obesity we have discovered differences in the placenta due to a maternal obese environment that may impact placental function and nutrient transport.

**MATERIALS AND METHODS**

\textit{Animal Model}: Founder B6 (C57BL/6 a/a) and LY (C57BL/6 Ay/a) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Fertile, male B6 mice were mated with aged-matched (17 week old) B6 or LY female littermates. Twelve days after visual confirmation of a vaginal plug (i.e., 12.5 dpc), B6 \((n = 7)\) and LY \((n = 12)\) dams were euthanized and maternal weights were determined. Viable embryos and their
corresponding placenta were collected and wet weights were determined. All of the
placentas were flash frozen for RNA and protein extraction whereas half of the embryos
from each litter were flash frozen for RNA and protein extraction. All animal
experiments were approved by the University of Nebraska-Lincoln Institutional Animal
Care and Use Committee.

**Reverse transcription:** Whole placenta and whole embryo RNA was isolated
using Tri-Reagent (Invitrogen, Carlsbad, CA). RNA (5 μg) from each individual sample
was treated with DNase I (Promega, Madison, WI) and reverse transcribed using random
hexamer primers (Roche Diagnostics, Indianapolis, IN) and Moloney Murine Leukemia
Virus reverse transcriptase (Promega) according to manufacturer’s instructions.

**Quantitative, real-time PCR (QPCR):** Forward and reverse primers for candidate
genes and the housekeeping gene beta actin (*Actb*) (Table 3.1) were designed (Primer
Express, Applied Biosystems, Foster City, CA) and synthesized (Integrated DNA
Technologies, Coralville, IA). Primers were tested empirically to determine the maximal
concentration that produced specific amplification of the target sequence in the absence
of primer dimers. Quantitative PCR (QPCR) reactions were carried out using each
primer set and equivalent amounts of cDNA from each sample as previously described
(40). QPCR amplification of *Gapdh* and *18s rRNA* was carried out using an endogenous
control kit (Applied Biosystems, Foster City, CA). The relative abundance of the
candidate mRNA in each sample was normalized using the most stably expressed
housekeeping gene(s) (*Gapdh, 18s, or Actb*) which was determined using Normfinder
(41). Specifically, embryo candidate genes were normalized using the geometric mean of
18S rRNA and Gapdh mRNA abundance for each embryo sample while Gapdh mRNA abundance was used to normalize candidate genes for each placenta sample. The resulting normalized data for each candidate mRNA was then compared to the mean normalized mRNA abundance in B6-derived placenta or embryos and expressed as a fold change.

### Table 3.1. Primer sequences for QPCR analysis.

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<th>Gene</th>
<th>Accession #</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<td>Actb</td>
<td>NM 007393</td>
<td>5'-agatgacccagatcgattgaga</td>
<td>5'-cacacggccttgctgacgctacgt</td>
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**Protein Extraction:** Whole placenta and whole embryo samples were homogenized in RIPA buffer (150 mM NaCl, 1 mM EDTA, 50mM Tris-HCl pH7.4, 1% NP-40, 0.25% Na-deoxycholate) containing phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4) and Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics). Following homogenization, samples were sonicated and then centrifuged to separate cell debris from the supernatant which contained the soluble protein. The protein concentration of each sample was determined using the Pierce BCA Protein Assay Kit.
(Rockford, IL, USA). Light absorbance was measured on a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA) at 562 nm.

**Western Blot Analyses:** Placenta protein samples were resolved by SDS-polyacrylamide gel electrophoresis using a 4% stacking gel and 7% separating gel. Protein samples were loaded together with loading buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS and 5% β-mercaptoethanol) and electrophoresis carried out at constant voltage (100V) for 90 minutes. The separated protein was transferred to Immobilon PVDF (Millipore, Billerica, MA). Following transfer, the membranes were blocked with 5% nonfat dry milk in 1X TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween 20) for 1 hour with gentle shaking in order to block nonspecific proteins. The blots were probed with primary antibody against Xanthine Oxidase (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 5% w/v BSA in 1XTBST overnight at 4°C with gentle shaking. Blots were then washed with 1X TBST and then incubated for 1 hour with HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA) which was diluted with 5% nonfat dry milk in 1X TBST. Blots were incubated with West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min and exposed to autoradiograph film (Fisher Scientific, Fairlawn, IL). After visualization of the protein, blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA) at room temperature for 15 min. Blots were then rinsed with 1X TBST and re-blocked with 5% milk in 1XTBST. Next, they were incubated with primary antibody against β-Actin (Cell Signaling Technology, Danvers, MA) overnight at 4°C with gentle shaking. Total protein was exposed and visualized as described above. The
visualized total protein for β-Actin served as a loading control for each sample. The autoradiograph images films were scanned and the density of the protein band was determined in Adobe Photoshop. The density of the Xanthine Oxidase band was normalized to the density of the β-Actin band in each sample. The normalized abundance of Xanthine Oxidase protein in LY samples was subsequently compared to the B6 control samples to determine the fold-change in the LY samples.

**Xanthine Oxidase Assay:** Placenta and embryo protein samples were analyzed for superoxide production using the Amplex® Red Xanthine Oxidase Assay Kit (Molecular Probes, Eugene, OR) according to manufacturer instructions with hypoxanthine as the substrate. H$_2$O$_2$ was used as a positive control and reaction buffer without sample was used as a negative control. After 30 minutes of incubation at 37°C, absorbance was read on a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA) at 560 nm. Hypoxanthine supplied in the kit was used as a standard and final sample concentrations were calculated based on the standard curve.

**Statistical Analyses:** All statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Statistically significant differences in weights, mRNA abundance, protein expression or protein activity between B6 and LY dams or B6- and LY-derived embryos or placentas ($P < 0.05$) were determined using the Student’s t-test.
RESULTS

Maternal Obesity Reduces Placental Weight and the Ratio of Embryo to Placental Weight: We have previously demonstrated that the obese phenotype of LY females includes a significant increase in total body weight. However, embryos from LY dams exhibit decreased mid-gestational embryonic weight (39). Given that the placenta serves as a critical interface between the dam and fetus (5), the effect of maternal obesity on placental weight was examined. Placenta weight was significantly reduced ($P < 0.0001$) when collected from a LY compared to a B6 dam (Chapter II, Figure 2.6A).

Furthermore, there was a 12% decrease ($P < 0.05$) in the ratio of embryo to placenta weight when collected from LY dams (Chapter II, Figure 2.6A) which is indicative of reduced placental function and may explain the reductions of embryo weight and developmental delay previously identified (39).

LY-Derived Placenta Display Mild Hypoxia and Altered Vasculature: To determine how the molecular phenotype of the placenta was affected by maternal obesity, changes in the mRNA abundance of hypoxia candidate genes Hif1a and Arnt were measured. Interestingly, Hif1a mRNA abundance was increased 20% (1.25-fold, $P < 0.06$) in the placenta from LY mothers compared to B6 mothers (Chapter II, Figure 2.6B) suggesting hypoxemia (mild hypoxia) (42). Since decreased oxygen levels results in the compensatory expression of vascular markers, we examined the mRNA abundance of vasculature markers Pecam1, Vcam1, Vegfa_164 and Vegfa_165b which are essential in the development of placental vasculature (23). Pecam1 was significantly increased (1.34-fold, $P < 0.05$) in LY-derived placenta (Chapter II, Figure 2.6C); although none of
the other mRNAs showed significant changes in abundance (Chapter II, Figure 2.6C; data not shown for VEGFA_165b).

Placental Hypoxemia Negatively Impacts Embryonic Vasculature: Placental vascular development ensures for the proper exchange of nutrients exchange between the mother and fetus. If a hypoxic environment in the placenta is altering its vasculature development, nutrient transfer may be decreased and negatively impact vascular development in the embryo. Thus, we examined the expression of the vascular markers Vegfa_164 and Vegfa_165b in the mid-gestation embryos to discover if embryo vascular development is impaired. We found a significant decrease (1.79-fold, \( P < 0.004 \)) in Vegfa_164 and a tendency (1.59-fold, \( P < 0.10 \)) for Vegfa_165b to be decreased in LY-derived embryos (Figure 3.1).

Figure 3.1. Vasculature Markers in Whole Embryos from LY Dams are Decreased. QPCR analysis of the vasculature markers Vegfa_164 and Vegfa_165b was carried out. The normalized, relative abundance of each gene from embryos from LY dams (yellow bars, \( n=10 \)) was compared to the mean, normalized relative abundance of each gene from embryos from B6 dams (black bars, \( n=10 \)) and the data shown as a fold change (mean ± SEM). The data was tested for statistical significance (\( P < 0.1, \dagger \) and \( P < 0.01, ** \)).

Maternal Obesity does not Induce Oxidative Stress in the Placenta of our LY Mouse Model: It has been documented that hypoxia increases placental oxidative stress (25, 43). Therefore, we examined several markers in B6- and LY-derived placenta
which, when their expression is altered, results in the development of oxidative stress. First, changes in the mRNA abundance of the antioxidant and superoxide dismutase genes *Gpx1*, *Sod1* and *Sod2* were measured. While there was no difference in *Sod1* or *Sod2* expression, there was a tendency for the antioxidant *Gpx1* to be decreased (1.14-fold, $P < 0.09$) in placenta from LY mothers (Figure 3.2A). Next, we looked at the activity of the xanthine oxidase pathway which is one mechanism by which ROS is generated. We found no difference in either the concentration or the activity of xanthine

**Figure 3.2. LY-Derived Placenta Exhibit Mild Oxidative Stress Compared to B6-Derived Placenta.** QPCR analysis of the oxidative stress primers (A) *Gpx1*, *Sod1* and *Sod2* was carried out. The normalized, relative abundance of each gene from placenta from LY dams (yellow bars, $n=14$) was compared to the mean, normalized relative abundance of each gene from placenta from B6 dams (black bars, $n=12$) and the data shown as a fold change (mean ± SEM). (B) Western blot analysis was carried out using antibodies against Xanthine Oxidase. Total β-Actin was subsequently probed and served as a loading control. Semi-quantitative analysis of band density between LY (yellow bar, $n=7$) and B6-derived (black bar, $n=8$) placenta was calculated and shown as a fold change. (C) Xanthine Oxidase activity in the placenta was measured using an Amplex Red Xanthine Oxidase Assay Kit and activity level is displayed between LY dams (yellow bar, $n=16$) and B6 dams (black bar, $n=16$). The data was tested for statistical significance ($P < 0.1$, †).
oxidase in the placenta from B6- and LY-derived placenta (Figure 3.2B, C). In addition to the placenta, we also examined these markers of oxidative stress in the embryos of B6 and LY dams. There were no differences in the mRNA abundance of Gpx1, Sod1 and Sod2 between B6- and LY-derived embryos (Figure 3.3A). Likewise, we found no difference in xanthine oxidase activity in B6- compared to LY-derived embryos (Figure 3.3B).

Figure 3.3. LY-Derived Embryos Show No Signs of Oxidative Stress. QPCR analysis of the oxidative stress primers (A) Gpx1, Sod1 and Sod2 was carried out. The normalized, relative abundance of each gene from embryos from LY dams (yellow bars, n=10) was compared to the mean, normalized relative abundance of each gene from embryos from B6 dams (black bars, n=10) and the data shown as a fold change (mean ± SEM). (B) Xanthine Oxidase activity in the embryos was measured using an Amplex Red Xanthine Oxidase Assay Kit and activity level is displayed between LY dams (yellow bar, n=16) and B6 dams (black bar, n=12).

Maternal Obesity Results in Placenta with Alterations in Glucocorticoid Regulation: In addition to oxidative stress, hypoxia also regulates placental
glucocorticoid metabolism; specifically, the enzymes *Hsd11b1* and *Hsd11b2* (responsible for the conversion of active-corticosterone/cortisol and inactive-11-DHC/cortisone) levels (44). In order to determine if these enzymes were impacted in placenta collected from obese or lean dams, mRNA abundance was determined using primers specific for *Hsd11b1* and *Hsd11b2*. While there was no difference in *Hsd11b1* mRNA levels, we found a 1.59 fold (*P* < 0.03) decrease in *Hsd11b2* mRNA levels in placenta from LY compared to B6 mothers (Chapter II, Figure 2.6D).

**DISCUSSION**

Other studies have demonstrated that maternal obesity causes reduced angiogenesis and nutrient transport but the mechanisms are unclear. In the current study, embryo weight (39), placenta weight and ratio were decreased (Figure 3.1), indicating potential abnormalities in placenta development and/or function. The decrease in placenta and embryo weight (39) at mid-gestation is consistent with other rodent models of maternal obesity or gestational diabetes (45-47). However, the exact role of the placenta in altered nutrient transfer and fetal growth restriction is unknown.

Based on *in vivo* studies in human, sheep and rats, excessive exposure to a hypoxic environment can be detrimental to development by resulting in the inadequate perfusion of the placenta resulting in fetal growth restriction (12, 48). Similarly, decreased placental function and nutrient transport has been found to result in a chronic state of fetal hypoxemia (49). When looking at the impact of maternal obesity as opposed to induced hypoxia on the placenta we found a trend for increased HIF1a (Figure 3.2) in placenta from an obese environment suggesting modest hypoxemia. Hypoxia
causes increased expression of genes which contain the hypoxia response element in their promoter region (21-23). Knowing this, additional evidence of mild hypoxemia in the placenta is the increased expression of Pecam1 (Figure 3.2B).

While we found no change in VEGFA isoform expression in the placenta of LY compared to B6 dams (Figure 3.2), we found decreased or a tendency for decreased expression of the major angiogenic and anti-angiogenic isoforms (Figure 3.3) in LY-derived compared to B6-derived embryos. Mild hypoxemia in the placenta will alter its function and nutrient transport which plays a direct role in fetal programming, specifically modifications in embryonic vascular development which ultimately leads to cardiovascular or metabolic disease later in adult life (50).

Alterations in the placental ROS/antioxidant balance due to maternal obesity can also affect placental function and nutrient transport and result in congenital irregularities or embryonic death (51). Barrington et al. (52) found that women with low concentrations of serum selenium, the transition metal embedded within GPX, display increased placental oxidative stress and experience a higher rate of early pregnancy failure. In our model of maternal obesity, there was no evidence of oxidative stress in the placenta or embryo using the markers selected for this study. However, while Sod1 and Sod2 mRNA abundance was not different and xanthine oxidase protein and enzyme activity levels were not different between LY and B6 dams (Figure 3.4), there are other pathways which play a role in the synthesis of ROS that we did not examine. These pathways that are known to increase H2O2 include NADPH oxidase, lipid peroxides, substrates of monoamine oxidases, isoprostanes and peroxisomal proliferators (53, 54).
Glucocorticoid regulation in the placenta is crucial to limit transport of maternal cortisol in the fetus (55). Excessive exposure to glucocorticoids in utero can lead to altered maturation of organs as well as fetal growth restriction (32). In humans, mutations in the Hsd11b2 gene reduces Hsd11b2 activity which leads to an increase in fetal corticosterone/cortisol levels and fetal growth restriction (34). Likewise, exposure of rat fetuses to dexamethasone, a synthetic glucocorticoid, during the second half of gestation (12-22 dpc) resulted in reduced placenta and fetal weight and a decreased fetal-to-placenta weight ratio (56). The knockdown of the glucocorticoid receptor during early embryogenesis has also been found to reduce embryo length (57). Together, these data indicate that an important function of the placenta is to regulate embryo exposure to active glucocorticoids and the expression of Hsd11b2 is critical for this function. Data in our current study indicates that maternal obesity decreases Hsd11b2 mRNA abundance (Figure 3.6). Interestingly, hypoxia decreases placental Hsd11b2 levels (44, 58) while an increase in oxygen levels up-regulates glucocorticoid metabolism through Hsd11b2 activation. This suggests that the chronic, modest hypoxemia in the placenta of LY dams may result in altered glucocorticoid metabolism and increased exposure of these embryos to corticosterone.

All in all, we have presented evidence that maternal obesity increases placental hypoxemia and decreases placental regulation of glucocorticoid metabolism. These alterations in the molecular function of the placenta may represent plausible mechanisms for altered vascular development and growth of the embryos from obese dams.
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APPENDIX A

Effect of Maternal Obesity on Body Weight, Percent Body Fat, Reproductive Organ Weights, and Skeletal Muscle Molecular Phenotypes of Male and Female Offspring

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INTRODUCTION

Obesity worldwide is a rising epidemic which exists at a prevalence of 15–20% of the total population (1). Over the past 20-30 years, the rate of overweight or obesity in the USA has increased from 15 to 33% in adults and has experienced similar increases in children and adolescents (2). With the rise in the incidence of obesity it is now considered a significant public health problem which contributes 2-7 % of the total health care costs in the USA (1). Obesity combined with pregnancy has been found to lead to an increase in maternal circulating lipids resulting in a pronounced effect on the growth and development of their conceptus (3). Maternal obesity has been shown to increase the lifetime risk of obesity in offspring as well as lead to metabolic syndrome in childhood and adolescence (4-6).

The correlation between maternal obesity and abnormalities in fetal growth/development has led to the establishment of a developmental overnutrition hypothesis which proposes that increased fuel supply to the fetus due to maternal overnutrition leads to permanent changes in offspring metabolism and growth (7-9). Other studies have established a correlation between maternal obesity and abnormalities in organ and whole body development. For example, studies have found increased
pancreas weight with enhanced early β–cell maturation (10-13), increases in triglyceride levels (14), increased liver weight leading to the development of nonalcoholic fatty liver disease (11, 14, 15), increased perirenal body fat weight or percent body fat (11, 12, 16, 17) and only select studies have found an increase in heart weight (11, 13). One study looked at gender differences and found liver weights to be increased in male offspring but not in female offspring while both males and females exhibit decreased muscle and increased fat pad weight when developed in an obese dam (16).

While maternal obesity impacts multiple organ systems, how it is impacting skeletal muscle development is of particular importance given that muscle is a major metabolic organ and directly contributes to the development of obesity. Several studies have looked at the effects of an altered maternal diet on embryonic muscle growth. These studies that have found IUGR fetuses to display a reduction in muscle mass (16, 18), which may be due to maternal overnutrition interfering with myotube formation (19, 20). The pre-adipocyte marker, peroxisome proliferator-activated receptor gamma (PPARG) is expressed in skeletal muscle and its expression is higher in the skeletal muscle of offspring from obese dams indicating increased intramuscular adiposity in these offspring (20, 21). Regulation of adult muscle mass is a complex process that is controlled by the transcription factor, PAX7, which drives the expression of myogenic factors (myogenic factor-5 (Myf5), myogenic differentiation 1 (MyoD), myogenin (Myog), and desmin (Des)) to promote the differentiation of proliferating muscle progenitor cells into myocytes and ultimately mature muscle fibers (22-26). The
regulation of these genes is imperative for proper adult muscle regeneration, but how these genes are altered in the adult offspring of obese mothers has not been examined.

Due to the rising incidence in obesity, many animal models have been used to determine the impact maternal obesity is playing on life-long offspring outcomes. For example, prenatal overfeeding in the rat, mouse, and sheep leads to altered appetite regulation in the postnatal offspring (16, 27, 28) which is tied to increased postnatal weight gain and development of obesity. These studies show that a maternal obese environment alters the development of their offspring resulting in altered organ development and body growth. It is unknown if prenatal exposure to an obese environment coupled with postnatal stimulus for increased food intake has a synergistic effect on the morphology and/or function of specific organs.

**MATERIALS AND METHODS**

**Animal Model:** Founder B6 (C57BL/6, a/a) and LY (C57BL/6, Ay/a) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Fertile, female B6 mice were mated with LY male littermates (B6-LY) or female LY mice were mated with male B6 littermates (LY-B6). Following a natural birth, animals were weaned at 3 weeks, housed with 1 to 4 mice per cage, and provided ad libitum access to water and normal rodent chow. At 12 weeks of age, both male and female offspring were euthanized, weighed and subjected to dual-energy X-ray absorptiometry (DEXA) using the Lunar P PIXImus Densitometer (GE Medical Systems, Fitchburg, WI). Following the DEXA scan, organs were removed from males (gastrocnemius skeletal muscle, liver, kidneys, adrenals, testis, seminal vesicles, epididymis and prostate) and females (gastrocnemius skeletal muscle,
kidneys, liver, ovaries and uterus) and their wet-weights were determined. All organ
weights were normalized to the total lean tissue mass of the animal. The gastrocnemius
skeletal muscle was flash frozen for RNA extraction. In addition, B6 females were mated
with fertile B6 males (B6-B6) and male offspring were collected at twelve weeks of age
for body weight and DEXA analysis. All animal experiments were approved by the
University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

Reverse Transcription: Gastrocnemius skeletal muscle RNA was isolated using
the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). RNA (1 μg) from each
individual sample was treated with DNase I (Promega, Madison, WI) and reverse
transcribed using random hexamer primers (Roche Diagnostics, Indianapolis, IN) and
Moloney Murine Leukemia Virus reverse transcriptase (Promega) according to
manufacturer’s instructions.

Quantitative, Real-Time PCR (QPCR): Forward and reverse primers for
candidate genes and the housekeeping gene β-actin (Actb) (Table A.1) were designed
(Primer Express, Applied Biosystems, Foster City, CA) and synthesized (Integrated DNA
Technologies, Coralville, IA). Primers were tested empirically to determine the maximal
concentration that produced specific amplification of the target sequence in the absence
of primer dimers. Quantitative PCR (QPCR) reactions were carried out using each
primer set and equivalent amounts of cDNA from each sample as previously described
(29). QPCR amplification of Gapdh and 18s rRNA was carried out using an endogenous
control kit (Applied Biosystems, Foster City, CA). The relative abundance of the
candidate mRNA in each sample was normalized using the most stably expressed
housekeeping gene(s) (Gapdh, 18s, or Actb) which was determined using Normfinder (30). Specifically, muscle candidate genes were normalized using the geometric mean of Actb and Gapdh mRNA abundance for each male or female muscle sample. The resulting normalized data for each candidate mRNA was then compared to the mean normalized mRNA abundance in B6 offspring derived in a B6 dam and expressed as a fold change.

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**Table A.1. Primer Sequences for QPCR Analysis**

**Statistical Analyses:** All statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Differences between male and female B6 and LY offspring body weights, percent fat, total tissue mass, organ weights and mRNA abundance from either a B6 dam and LY sire or a LY dam and B6 sire were determined using one-way ANOVA and Bonferroni post-test. If no difference was found between offspring from the same dam, then statistically significant differences between all offspring from a B6 dam and LY sire was compared to all offspring from a LY dam and B6 sire ($P < 0.05$) using Student $t$-test.
RESULTS

*Maternal Obese Environment Impacts Male Offspring Growth and Fat Mass*

*Irrespective of Paternal Phenotype:* In order to compare the adult phenotypes of male offspring developed in a lean versus obese environment, twelve week old male mice were examined. B6 and LY males from B6-LY and LY-B6 crosses were compared. Also, in order to verify that there was no paternal obesity influence, B6 males from a B6-B6 cross were examined. As expected, LY males from B6-LY crosses showed increased body weight (1.39-fold, \( P < 0.0001 \)), a 51% increase (2-fold, \( P < 0.0001 \)) in percent body fat and increased (1.11-fold, \( P < 0.01 \)) in total lean tissue mass compared to B6 male offspring from a B6-LY cross (Figure A.1A). In addition, B6 male offspring from a LY-B6 cross display increased (1.15-fold, \( P < 0.05 \)) total lean tissue mass compared to B6 male offspring from a B6-LY cross. However, we surprisingly found no differences in body weight, percent fat and total lean tissue mass when comparing the B6 and LY male offspring from a LY-B6 cross (Figure A.1A). In order to validate that a sire effect was not occurring with our model, we examined the impact of paternal obesity on body weight, percent body fat, or total lean tissue mass of B6 male offspring from a B6-B6 cross with B6 male offspring from a B6-LY cross. This comparison showed no difference in body weight, percent fat or total lean tissue mass (Figure A.1B) indicating no effect of paternal obesity on offspring phenotypes. Together, this data shows that regardless of the sire phenotype, male B6 offspring from a B6-LY cross are significantly smaller and exhibit decreased body fat and total tissue mass compared to age-matched LY littermates.
Male LY Offspring from B6 Dams Display Increased Body, Fat and Total Lean Tissue Mass.

(A) Male offspring from B6 dams and LY sires (B6, dark grey bar, n=13; LY, light yellow bar, n=7) and from LY dams and B6 sires (B6, light grey bar, n=2; LY, dark yellow bar, n=4) were collected at 12 weeks of age. Body weight was calculated and percent body fat and total lean tissue mass were determined via DEXA scan (mean ± SEM). (B) For each comparison, male B6 offspring from a B6 dam and B6 sire (black bar, n=4 or 8) were compared to B6 offspring from a B6 dam and LY sire (dark grey bar, n=13) to justify our control (dark grey bar). Statistically significant differences ($P < 0.05$, $*$; $P < 0.01$, $**$; $P < 0.0001$, $****$) were determined using one-way ANOVA.

Male Organ Weights are Altered Based on their In Utero Maternal Environment:

To determine if maternal obesity affects the growth of individual organs irrespective of total lean body mass, we next compared the weights of various organs between B6-LY and LY-B6 derived 12-week old offspring. Gastrocnemius skeletal muscle (1.14-fold, $P < 0.001$) and kidney weight ratios (1.11-fold, $P < 0.01$) were decreased in LY compared to B6 offspring from a B6-LY cross (Figure A.2A). The weight ratio for kidneys between B6 male offspring from a LY dam were increased (1.27-fold, $P < 0.05$) compared to B6 males from a B6 dam. Likewise, we found an increase (1.15-fold, $P < 0.01$) in the kidney weight ratio between LY male offspring from a LY dam compared to
a B6 dam. Also, the testis (1.19-fold, \( P < 0.01 \)) and epididymis (1.17-fold, \( P < 0.05 \)) were significantly smaller in LY compared to B6 offspring from a B6-LY cross (Figure A.2B). Due to the fact that there was no difference in adrenal and prostate weights between LY and B6 offspring from B6 dams or from LY dams, we grouped the weights of the adrenals from B6-LY and LY-B6 crosses regardless of the offspring’s phenotype. We found that both the adrenal glands (1.79-fold, \( P < 0.01 \)) as well as the prostate (1.19-fold, \( P < 0.10 \)) from offspring developed from a LY-B6 cross were heavier than offspring from a B6-LY cross (Figure A.2A).
Figure A.2. Organ Growth Differs in Male Offspring from B6 Dams and LY Dams. (A, B) Male offspring from B6 dams (B6, dark grey bar, n=13; LY, light yellow bar, n=7) and LY dams (B6, light grey bar, n=2; LY, dark yellow bar, n=4) were euthanized and organs were removed. All organ weights were normalized to their corresponding total lean tissue mass (mean ± SEM) and statistically significant differences ($P < 0.10$, $^+$; $P < 0.05$, $^*$; $P < 0.01$, $^{**}$; $P < 0.001$, $^{***}$) and tendencies were determined using one-way ANOVA. If there were no differences between male offspring from the same dam a Students $t$-test was used for the combination of male offspring from a B6 dam compared to male offspring from a LY dam.
Dependent on Maternal Weight, Adult Male Offspring Skeletal Muscle Display

Differences in mRNA Expression: The proper differentiation of muscle progenitor cells is needed for muscle to develop normally. Thus, we examined the myogenic markers Pax7, Myod1, Myf5, Myog and Des in whole gastrocnemius skeletal muscle. Due to low sample size, we grouped all muscle samples from offspring developed from a B6-LY

![Figure A.3. Muscle Function in Male Offspring is Altered.](image)

(A, B) QPCR analysis of Pax7, Myod1, Myf5, Myog, Des, Pparg, Igf2 and Slc2a4 was carried out. The normalized, relative abundance of each gene from male B6 offspring derived in B6 dam (dark grey bar, n=5) was compared to the mean, normalized relative abundance of each gene from LY offspring derived in a B6 dam (light yellow bar, n=5), B6 offspring derived in a LY dam (light grey bar, n=1) or LY offspring derived in a LY dam (dark yellow bar, n=3) with the data shown as a fold change (mean ± SEM). Statistically significant differences (P < 0.1, †; P < 0.01, **) were determined using the Students t-test for the combination of male offspring from a B6 dam compared to male offspring from a LY dam.
cross and from a LY-B6 cross. We found a tendency (1.22-fold, \( P < 0.08 \)) for an increase in \textit{Myod1} expression in skeletal muscle from a LY-B6 cross compared to muscle developed from a B6-LY cross (Figure A.3A). We also examined other markers that impact skeletal muscle metabolism (\textit{Pparg}, \textit{Igf2} and \textit{Slc2a4}) and found \textit{Slc2a4} expression to be 21\% decreased (1.27-fold, \( P < 0.001 \)) in muscle developed from a LY-B6 cross compared to a B6-LY cross (Figure A.3B).

\textit{Female Offspring Show a Similar Growth Pattern as Their Male Littermates:} We found the female LY compared to B6 offspring from a B6-LY cross to exhibit a 27\% increase (1.38-fold, \( P < 0.0001 \)) in body weight (Figure A.4). Similarly, there was a significant increase (\( P < 0.0001 \)) in percent body fat (2.13-fold, 53\%) in LY offspring from a B6-LY cross compared to B6 littermates (Figure A.4). In contrast to the male offspring, we found a 40\% increase (1.67-fold, \( P < 0.01 \)) in percent body fat in LY compared to B6 offspring from a LY-B6 cross. However, while we found differences in total lean tissue mass in male offspring, no differences were seen in female offspring.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Images/FigureA4.png}
\caption{Female Offspring from B6 and LY Dams Exhibit Differing Body Growth Depending on their Phenotype.} \textit{Female offspring from B6 dams and LY sires (B6, dark grey bar, \( n=10 \); LY, light yellow bar, \( n=9 \)) and from LY dams and B6 sires (B6, light grey bar, \( n=2 \); LY, dark yellow bar, \( n=4 \)) were collected at 12 weeks of age. Body weight was calculated and percent body fat and total lean tissue mass were determined via DEXA scan (mean ± SEM). Statistically significant differences (\( P < 0.01 \), **; \( P < 0.0001 \), ****) were determined using one-way ANOVA.}
\end{figure}
LY Female Organ Weights are Decreased Compared to B6 Littermates

Regardless of Dam Phenotype: Female kidney weight ratio was increased (1.08-fold, \( P < 0.10 \)) in B6 and LY female offspring from a LY-B6 cross compared to B6 and LY offspring from a B6-LY cross (Figure A.5A, B). The liver showed an increased weight ratio between LY and B6 offspring from a LY dam (1.30-fold, \( P < 0.05 \)) as well as when from a B6 dam (1.21-fold, \( P < 0.001 \)). We also determined the uterus to be significantly smaller (1.23-fold, \( P < 0.001 \)) in LY compared to B6 littermates from a B6-LY cross (Figure A.5B). Interestingly, when the ovaries were grouped according to dam, we found a decrease (1.36-fold, \( P < 0.05 \)) in weight ratio when developed from a LY-B6 cross compared to a B6-LY cross (Figure A.5B).

Figure A.5. Female Offspring Display Altered Organ Growth When From a B6 Compared to LY Dam. (A, B) Female offspring from B6 dams (B6, dark grey bar, \( n=10 \); LY, light yellow bar, \( n=9 \)) and LY dams (B6, light grey bar, \( n=2 \); LY, dark yellow bar, \( n=4 \)) were euthanized and the gastrocnemius skeletal muscle, kidneys, liver, ovaries and uterus were removed. All organ weights were normalized to their corresponding total lean tissue mass (mean ± SEM) and statistically significant differences (\( P < 0.10, \dagger \); \( P < 0.05, \ast \); \( P < 0.001, \ast\ast \)) were determined using one-way ANOVA. If there were no differences between female offspring from the same dam a Students\( t\)-test was used for the combination of female offspring from a B6 dam compared to female offspring from a LY dam.
Female Skeletal Muscle Myogenic Factors are Altered in LY-B6 Crosses: The myogenic factors Pax7, Myod1, Myf5, Myog and Des were examined in female whole gastrocnemius skeletal muscle. While no difference was found for Pax7, Myod1, or Des, we found a 27% reduction (1.37-fold, \( P < 0.05 \)) for Myf5 and a 29% reduction (1.42-fold, \( P < 0.05 \)) for Myog mRNA expression of offspring skeletal muscle from a LY-B6 cross.

Figure A.6. Muscle mRNA Expression Differs in Female Offspring Skeletal Muscle. (A, B) QPCR analysis of Pax7, Myod1, Myf5, Myog, Des, Pparg, Igf2 and Slc2a4 was carried out. The normalized, relative abundance of each gene from female B6 offspring derived in B6 dam (dark grey bar, n=5) was compared to the mean, normalized relative abundance of each gene from LY offspring derived in a B6 dam (light yellow bar, n=5), B6 offspring derived in a LY dam (light grey bar, n=1) or LY offspring derived in a LY dam (dark yellow bar, n=3) with the data shown as a fold change (mean ± SEM). Statistically significant differences (\( P < 0.05 \), *) were determined using the Students t-test for the combination of female offspring from B6 dams compared to female offspring from LY dams.
compared to a B6-LY cross (Figure A.6A). We also examined other muscle markers (Pparg, Igf2 or Slc2a4) that impact muscle metabolism but failed to find any differences in gene expression between offspring from a B6-LY compared to LY-B6 cross (Figure A.6B).

**SUMMARY**

The preliminary data collected in this study suggests that there are genetic alterations that are occurring in offspring reared in an obese compared to a normal weight environment. However, the small sample size from each experimental group made it difficult to ascertain the statistical significance of these data. We did, however, detect an environmental effect due to maternal obesity with the difference in kidney and ovary weights in female offspring where kidneys were increased and ovary were decreased in LY-B6 compared to B6-LY crosses (Figure A.5). Additionally, male adrenal weights were significantly larger when developed from a LY-B6 compared to a B6-LY cross (Figure A.2A) and prostate weight ratio was increased in male offspring from a LY-B6 compared to a B6-LY cross (Figure A.2B. We also experienced some gender dependent differences in gastrocnemius skeletal muscle mRNA expression but it is not clear how these changes are affecting muscle signaling.

We expected to see a synergistic effect when LY offspring were developed in a LY dam; however, we only experienced this effect in male kidneys. The lack of a synergistic effect of prenatal and postnatal obesity on organ growth and development in LY derived from LY-B6 cross may be attributed to our low animal numbers, differences in the number of mice per cage and variations in litter size. Also, our offspring are 12
weeks old which may be too early to see a dramatic phenotype. Later time points may be needed in order to allow for more drastic effects to take form. Altogether, further studies are needed that control for litter size, mice per cage, food intake and take into account the impact of paternal obesity in order to make valid conclusions as to the effect of maternal obesity on offspring development and organ function.
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APPENDIX B

Genes Involved in the Immediate Early Response and Epithelial-Mesenchymal Transition are Regulated by Adipocytokines in the Female Reproductive Tract.

Zhufeng Yang, Kristin A. Norwood, Jacqueline E. Smith, Jill G. Kerl, and Jennifer R. Wood
Department of Animal Science, University of Nebraska-Lincoln, Lincoln, NE
Molecular Reproduction & Development. 2012 Feb; 79(2): 128-137

These results were performed and then combined with work done by Zhufeng Yang and others which was published in Molecular Reproduction & Development in 2012.

Epithelial-Mesenchymal Transition (EMT) mRNA Levels are Increased in the LY Uterus:
The effect of an obese phenotype on the abundance of IE and EMT mRNAs in the whole uterus was subsequently determined by QPCR. Uterus was collected 44 hours after eCG stimulation from 6, 12, or 24 week old LY and B6 females. RNA was isolated from the whole uterus and QPCR was carried out using primers against cJun, cFos, cMyc, Snai1, Snai2, or Twist1 (Figure B.4). There was no difference in cJun, cFos, or cMyc mRNA abundance in the uterus of LY compared to B6 at any age (Figure B.4A). However, both cJun and cMyc mRNA levels were increased in 24 compared to 6 and 12 week-old LY and B6 females. Conversely, Snai1, Snai2, and Twist1 mRNA abundance was increased in the whole uterus of 6 week-old LY compared to age-matched B6 females (Figure B.4B). Similar to cJun and cMyc, the mRNA abundance of Snail was also increased in 24 week-old LY and B6 uterus compared to 6 and 12 week-old uterus.
The differences in uterine Snai1, Snai2, and Twist1 mRNA levels between 6 week-old LY and B6 females were correlated to increased circulating IGF-1 levels at this age (compare Figure B.3B and B.4B). IGF-1 and IGF-1 binding proteins (IGFBPs) are also expressed in the murine uterus (1, 2). Therefore, QPCR was carried out to identify differences in uterine levels of Igf1, Igfbp2, or Igfbp3 mRNAs between age-matched LY and B6 females (Figure B.4C). While Igf1 and Igfbp2 mRNA levels were not different between LY and B6, Igfbp3 mRNA abundance was increased in uterus of 6 week-old LY compared to B6 females. Furthermore, Igfbp3 tended to be increased in 24 week-old LY compared to B6 uterus.

![Figure B.4. Abundance of IE, EMT, and IGF family mRNAs in Uterus of Obese and Normal-Weight Females.](image)

Total RNA was isolated from the whole uterus of 6, 12, or 24 week-old B6 (black bars) or LY (white bars) females 44 h after eCG stimulation. Quantitative, real-time PCR was carried out using primers specific for (A) cJun, cFos, or cMyc; (B) Snai1, Snai2, or Twist1; or (C) Igf1, Igfbp2, or Igfbp3 as described in Figure 1. Analysis of the resulting QPCR data was also carried out as described in Figure 1. Statistically significant differences in fold change (P < 0.05) were determined using one-way ANOVA and Tukey-Kramer post-test and indicated by different letters.
LITERATURE CITED
