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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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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

<u>Incidence of Maternal Obesity and its Correlation to Childhood Obesity</u>

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).
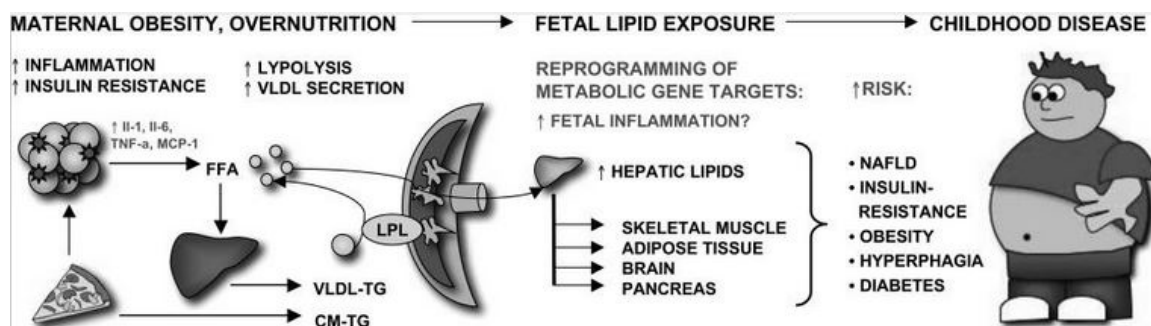
Figure 1.1. The Effect of Maternal Lipids on Fetal Development and its Consequences in Adulthood. Maternal obesity results in increased inflammation, insulin resistance, lipolysis and hepatic very-low-density lipoprotein (VLDL) accumulation in the adipose tissue. These changes in adipose tissue phenotype lead to an increase in lipid exposure by a developing fetus, impacting organ development and resulting in an increase in the risk for metabolic disease in childhood. Monocyte chemotractant 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
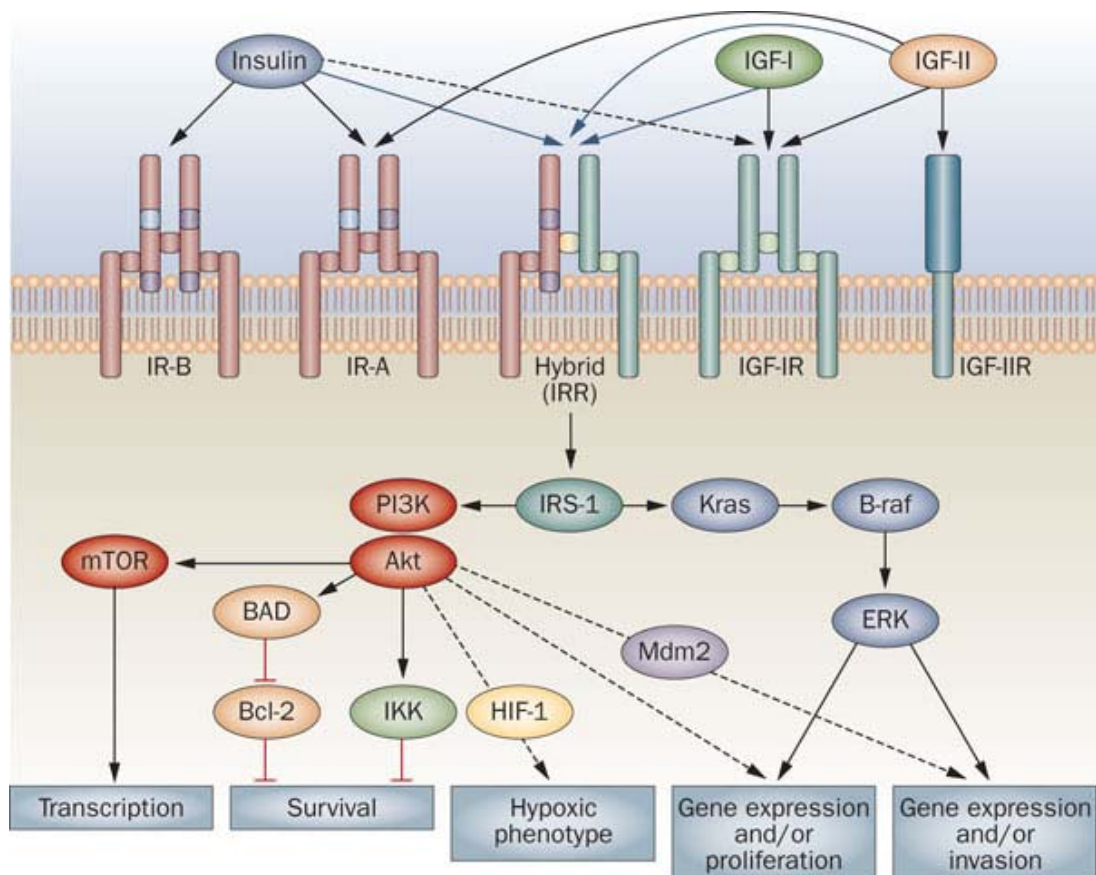
Figure 1.2. Insulin and IGF Signaling Pathways. Both Insulin and IGFs share similar signal
transduction pathways and are able to bind the different receptors with varying affinities. Ligand
binding results in MAPK and PI3K signaling pathway activation resulting in transcription,
proliferation and gene 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).
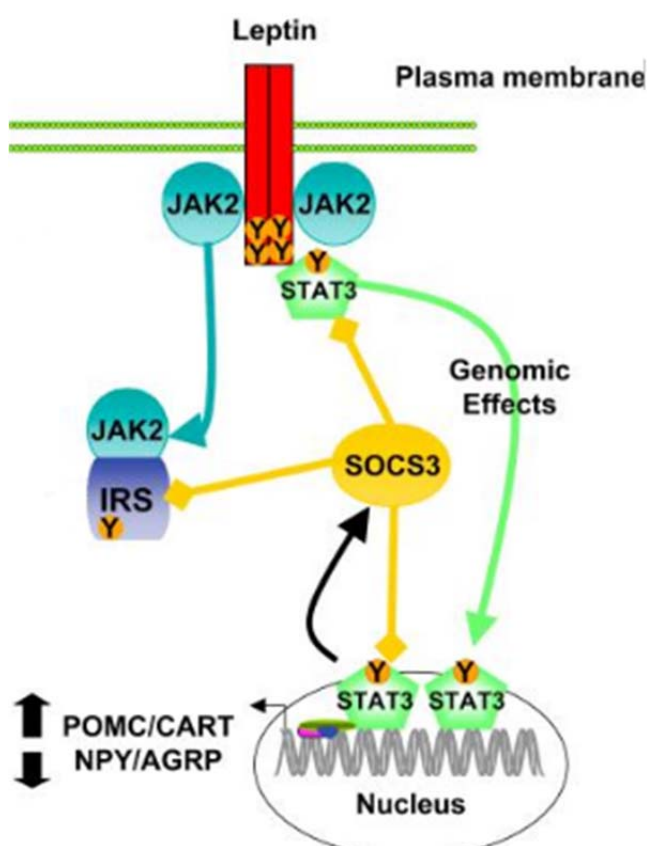
Figure 1.3. Leptin Signal Transduction Pathway. Leptin binds to its receptor where it activates JAK2 which then phosphorylates STAT3. STAT3 then results in gene transcription to induce POMC/CART and inhibit NPY/AGRP (60).

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).
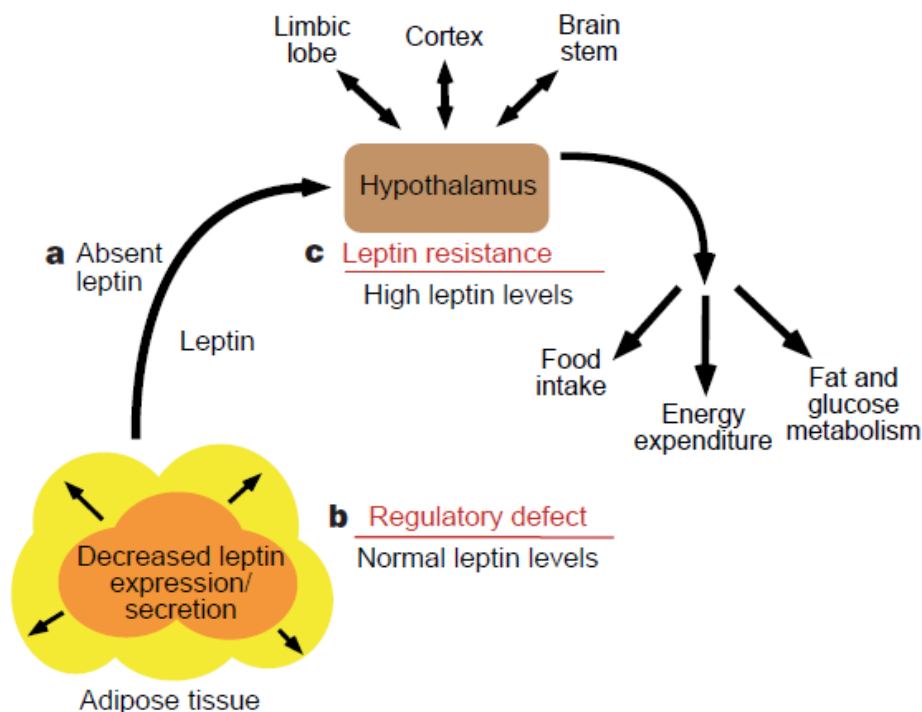
Figure 1.4. Obesity can Result from Three Different Alterations in Leptin Signaling. Obesity is generated in three ways in regard to the leptin regulatory loop. a) Failure to produce leptin leads to a drastic increase in fat mass, b) inappropriately low leptin secretion for a given fat mass leads to 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 NH_2-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).
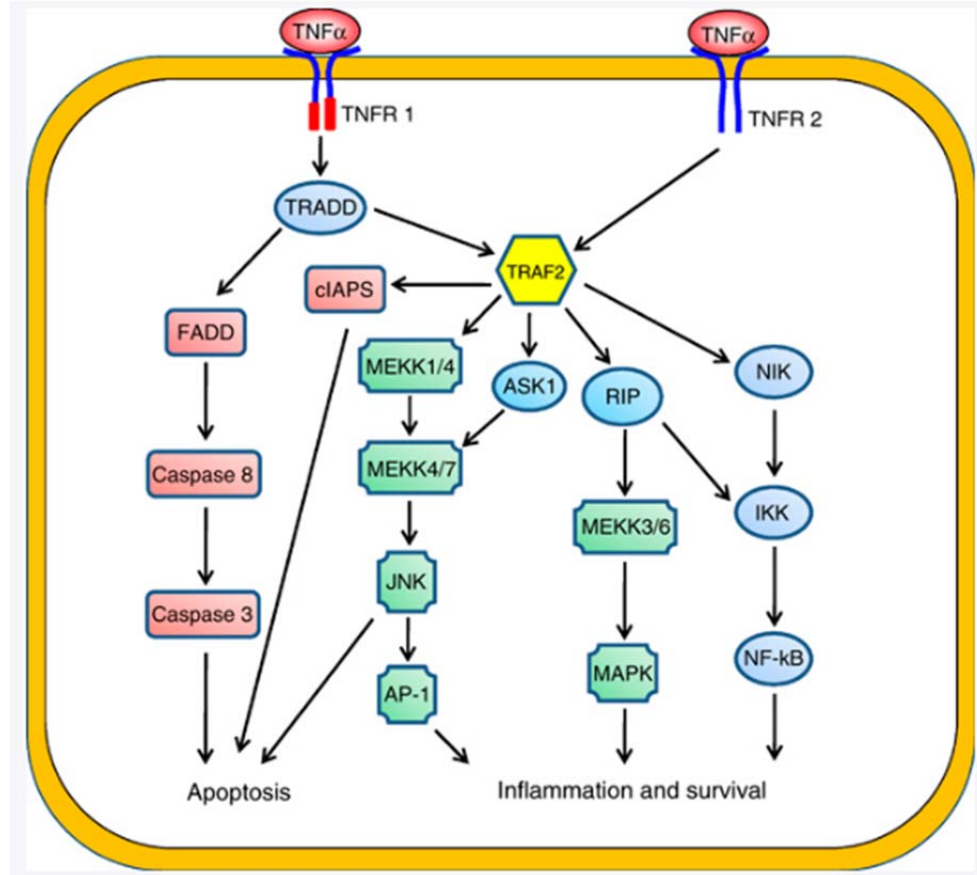
Figure 1.5. TNF-α Signal Transduction Pathway. TNF-α binds to either TNF-R1 or TNF-R2 which activate signaling pathways to induce apoptosis, cell survival or inflammation (81).

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).
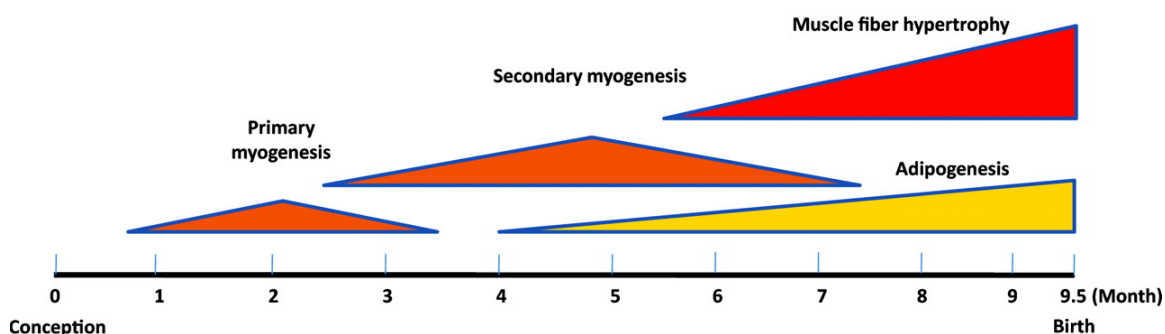
Figure 1.6. Stages of Bovine Fetal Skeletal Muscle Development. The progression through the various bovine fetal skeletal muscle developmental stages during gestation may be impacted by nutrient restriction or excess. The stage in which the nutrient deficiency or excess occurs will determine what organs 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 O_2 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
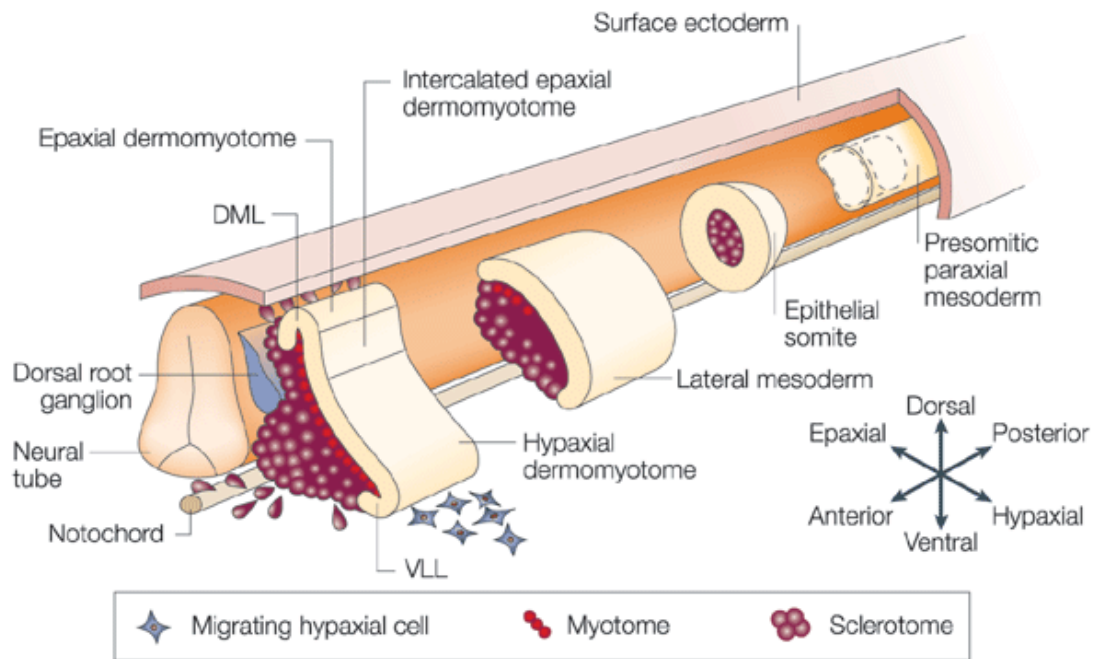
Figure 1.7. Spatial Representation of Somite Development. Segmentation of the presomitic paraxial mesoderm into ball-like structures, known as somites, occurs along the dorsal–ventral axis and in an anterior to posterior direction. In response to signals from the notochord and the neural tube, the somites 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).
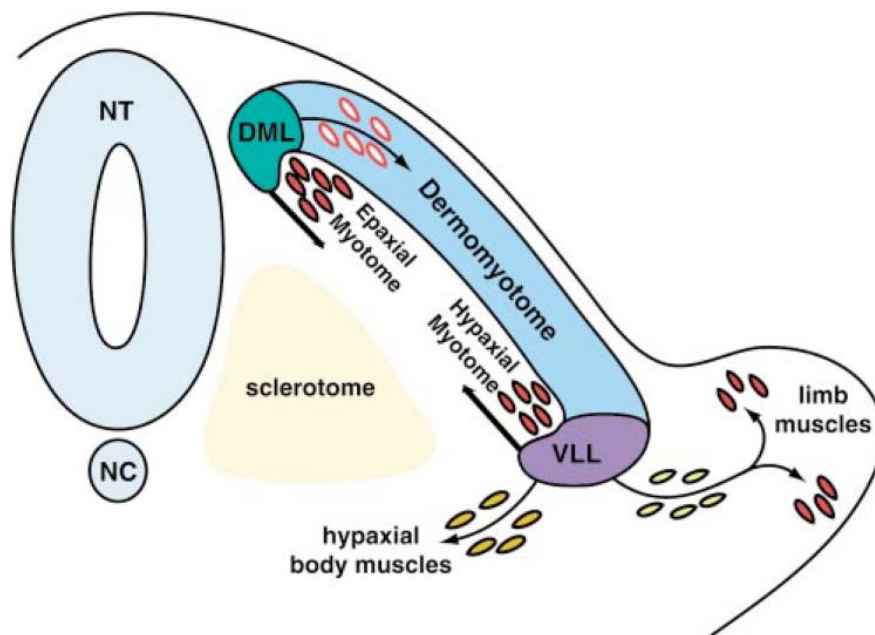
Figure 1.8. Somite Origin of Myogenic Progenitor Cells. Myogenic progenitors originate in
the dorsal-medial lip (DML) and ventral-lateral lip (VLL) of the dermomyotome. Cells of the
dorsal medial lip (DML) migrate under the dermomyotome to form the epaxial myotome. A
similar event occurs at the ventral 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).
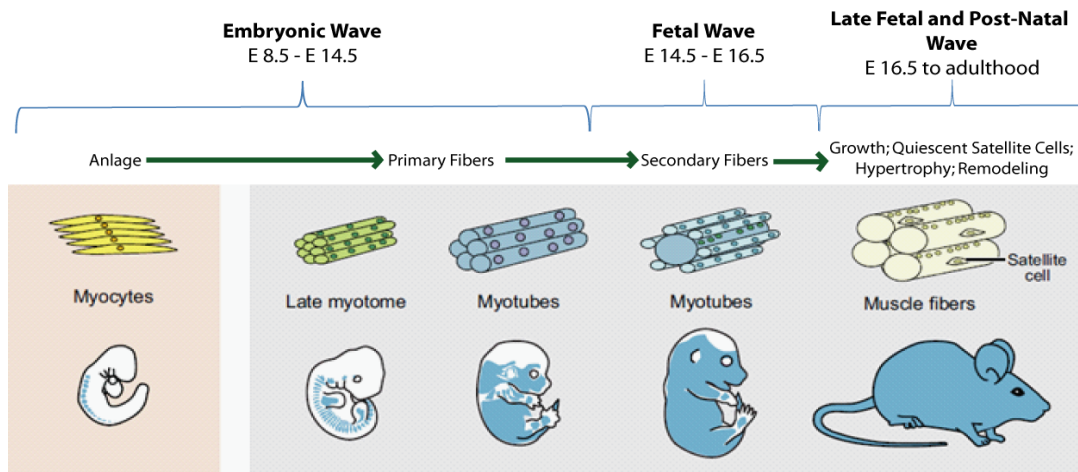
Figure 1.9. Waves of Developmental Myogenesis in the Mouse. The early embryonic wave begins around embryonic day (E) 8.5 and results in the formation of primary fibers. The fetal wave, between E 14.5 and E 16.5, consists of the scaffolding of secondary fibers. Around E 16.5, proliferating myotubes form 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
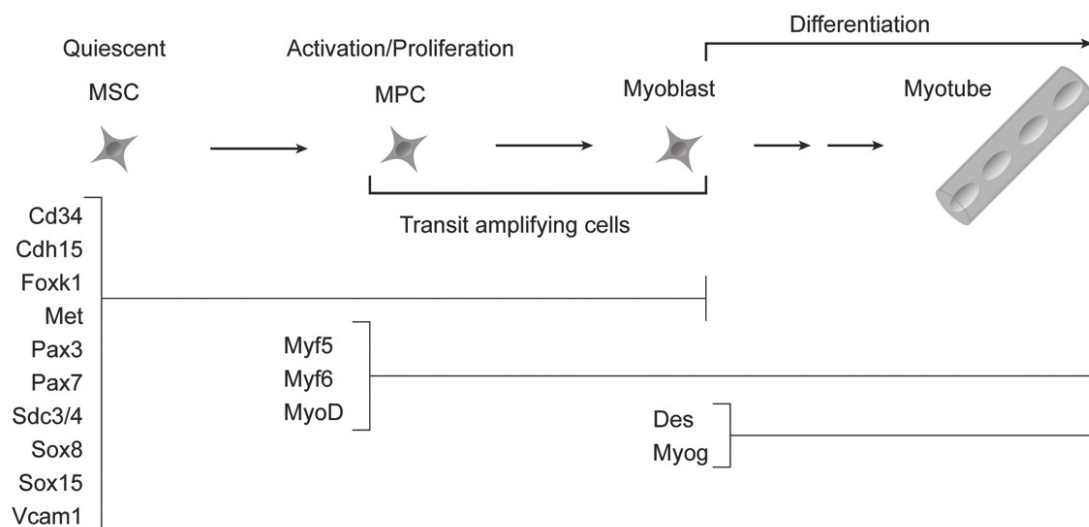
Figure 1.10. Gene Expression Profiles during Myogenesis. Myogenic stem cells (MSC), myogenic progenitor cells (MPC), myoblasts, and the multinucleated myotube express different genes during myogenesis (148).

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).
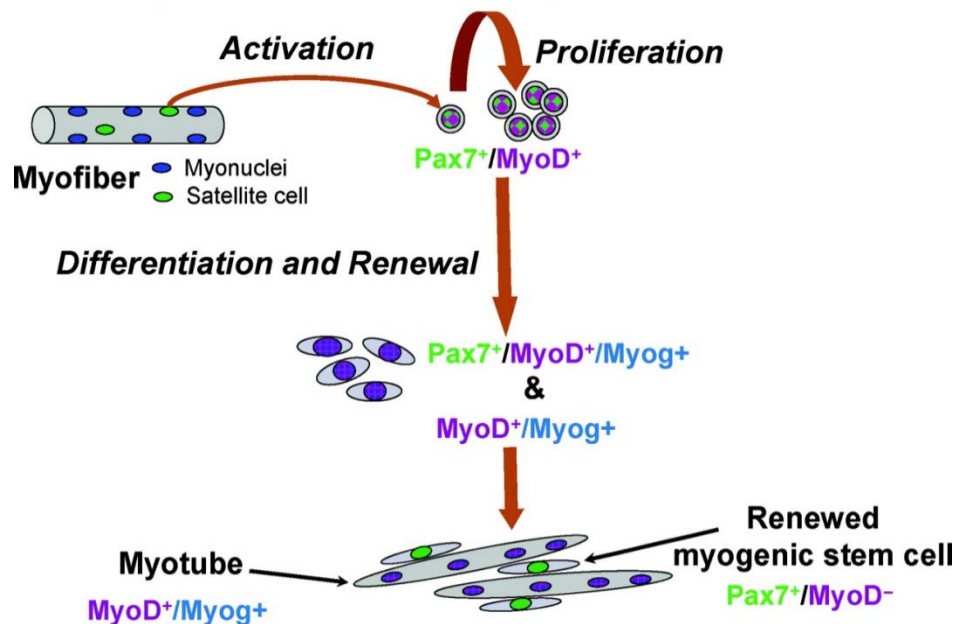
Figure 1.11. Expression Profile of Satellite Cells. The proliferating myoblast population is represented by the *Pax7+/MyoD+* mononuclear cells. Nuclei that are *MyoD+/Myog+* (and no longer express *Pax7*) are found within differentiated mononuclear cells and myotubes, whereas a minor population of *Pax7+/MyoD+/Myog+* 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).
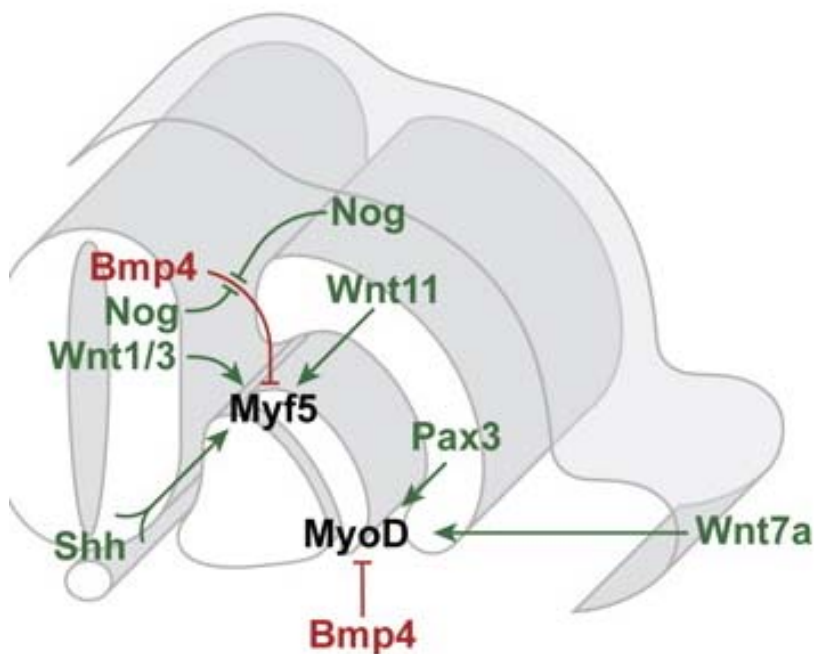
Figure 1.12.   Signals from Surrounding Tissues Impact Myogenic Markers. Signals from adjacent lineages, notochord, neural tube, and surface ectoderm, impact the expression of genes in the dermomyotome, myotome, and sclerotome (148).

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).
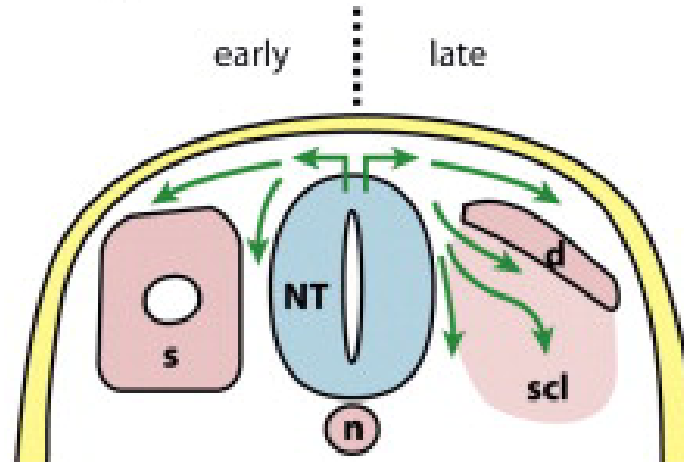
Figure 1.13. Migration Pattern of Neural Crest Cells. Trunk neural crest cells in the mouse migrate from the neural tube (NT) through the dermomyotome (d) and sclerotome (scl) and impact myogenic differentiation. Somite (s); and notochord (n) (200).

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).
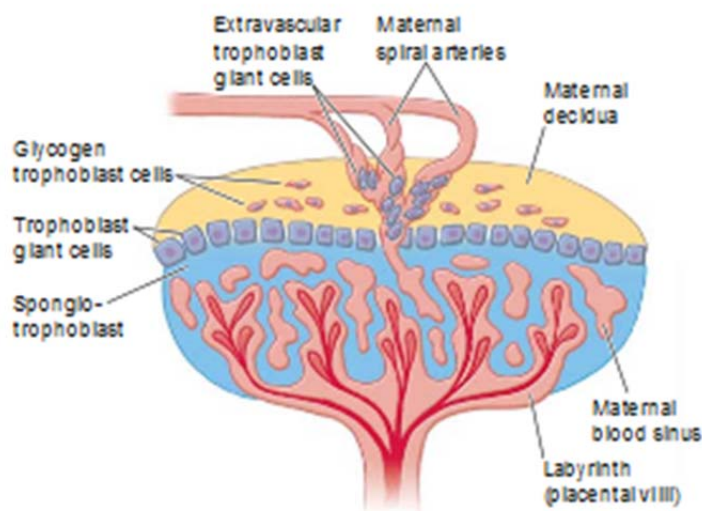
Figure 1.14. Structure of the Mouse Placenta. The mouse placenta is composed of a labyrinth, spongiotrophoblast, trophoblast giant cell layer and maternal decidua. The labyrinth layer consists of fetal capillaries and maternal blood and is the site of nutrient and gas exchange between the fetal and maternal 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,

223). 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 ($O^{\bullet-}_2$), hydrogen peroxide (H_2O_2) and hydroxyl

($OH^{\bullet}$) (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 $O^{\bullet-}_2$ 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 $O^{\bullet-}_2$ leads to a cascade of other ROS development. For example,

dismutation of $O^{\bullet-}_2$ produces H_2O_2 which can be reduced to H_2O and O_2 by various

antioxidants. Likewise, in the presence of iron, $O^{\bullet-}_2$ is converted to $OH^{\bullet}$. 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 in

utero 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 $O^{\bullet-}_2$ and H_2O_2

as byproducts of cellular energy production (236). All of these factors increase the

generation of $O^{\bullet-}_2$ 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 $O^{\bullet-}_2$ with the first being

conversion of $O^{\bullet-}_2$ to H_2O_2 by superoxide dismutases (SOD) (Figure 1.15). There are

three different SODs: SOD1 which contains Cooper 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_2O_2 to O_2 and H_2O and therefore limit cell exposure to H_2O_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).
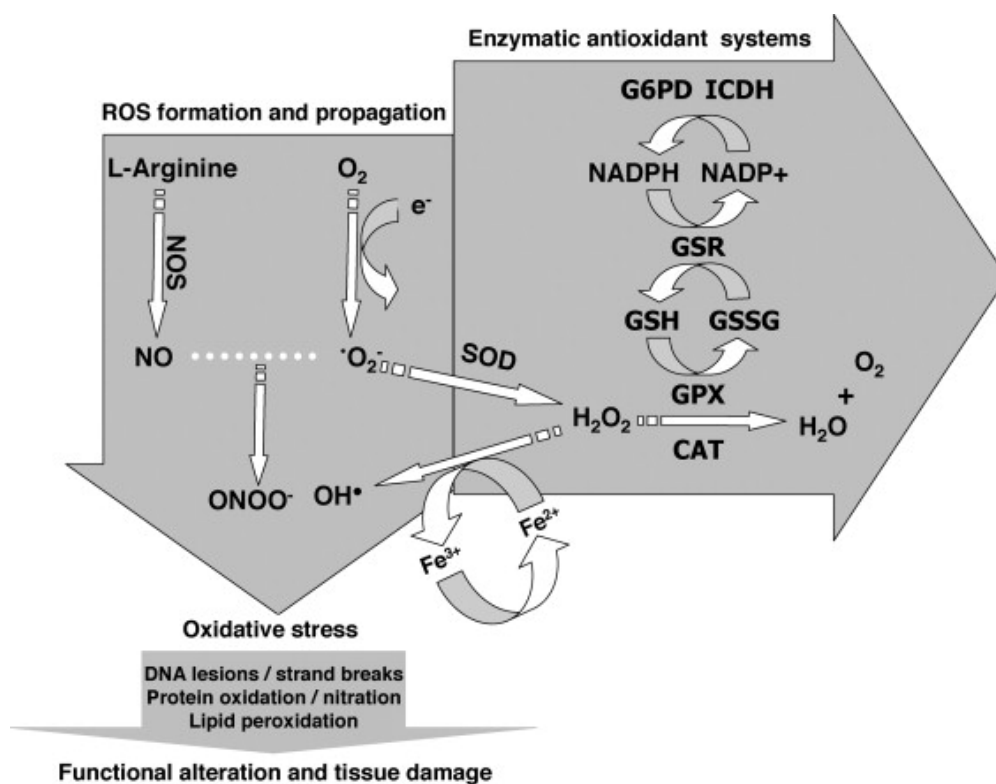
Figure 1.15. Oxidative Stress Results from the Production of Reactive Oxygen Species.
Reactive oxygen species (ROS) are produced from molecular oxygen (O_2) being converted to hydroxyl radicals (·OH). There are multiple key cellular antioxidant enzymatic pathways that try to convert ROS back into O_2 and water (H_2O). Superoxide anion (·O_2^-); hydrogen peroxide (H_2O_2); copper–zinc containing SOD (Cu,Zn-SOD or SOD1); manganese containing SOD (Mn-SOD or SOD2); catalase (CAT); glutathione peroxidase (GPX) (230).
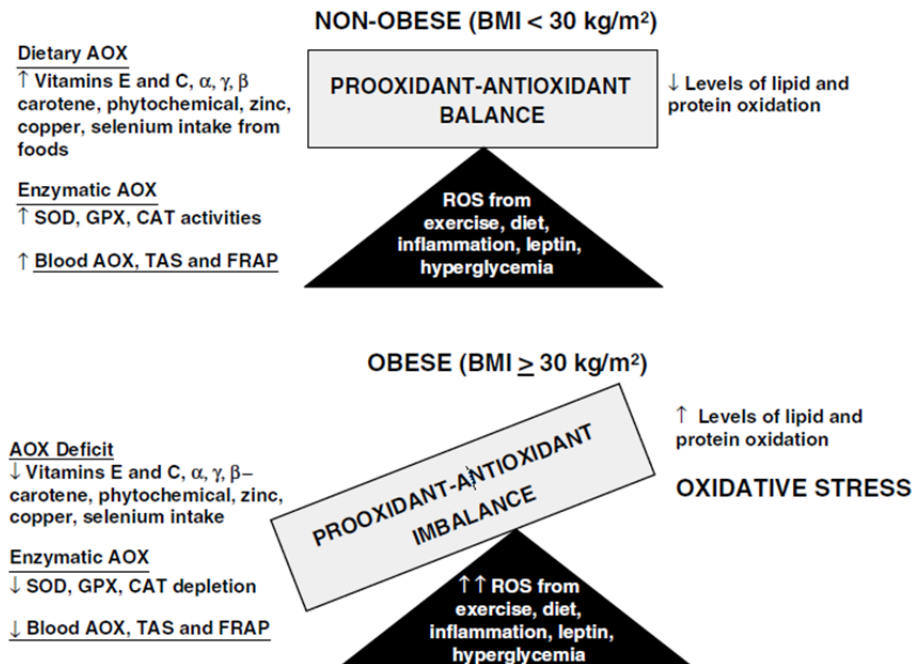
Figure 1.16. Prooxidant-Antioxidant Imbalance Results in Oxidative Stress. A balance is needed between the amount of antioxidants and ROS present in order to not create an oxidative stress environment. Reactive oxygen species (ROS); antioxidant (AOX); superoxide dismutase (SOD); glutathione peroxidase (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).
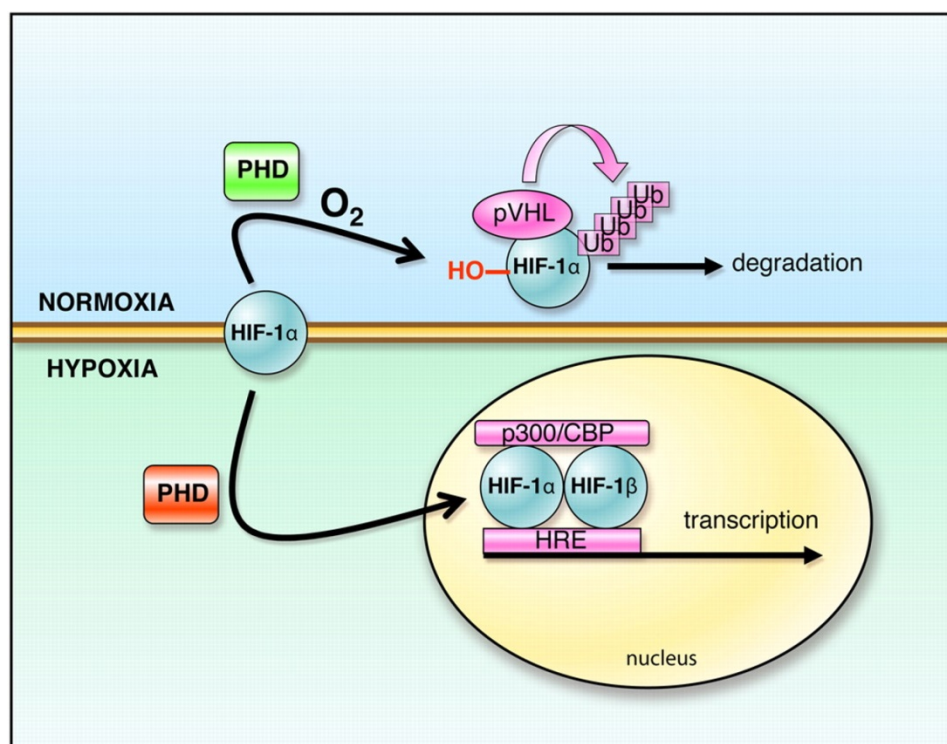
Figure 1.17. Hypoxia/Normoxia Signaling Cascade. Hypoxia inducible factor (HIF)-1α is
degraded in a normoxia environment, while in a hypoxic environment it binds to a hypoxia
responsive element (HRE) and promotes transcription of genes. Prolyl hydroxylase (PHD); von
Hippel Lindau protein (pVHL); and ubiquitination (Ub) (262).

Under normal oxygen conditions, PHDs in the presence of O_2 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

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).

Glucocorticoids:

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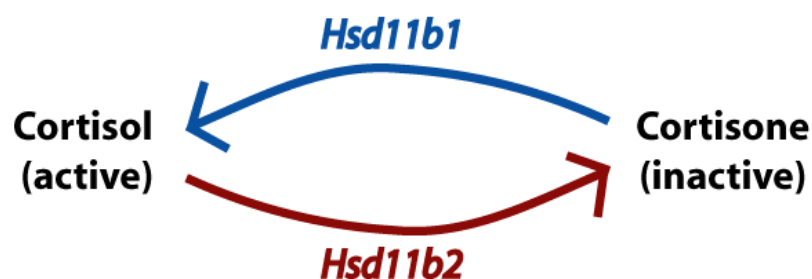
Figure 1.18. Enzymatic Regulation of Glucocorticoids. *Hsd11b1* is responsible for the conversion of 'inactive' cortisone to 'active' cortisol while *Hsd11b2* converts 'active' cortisol to 'inactive' cortisone.

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
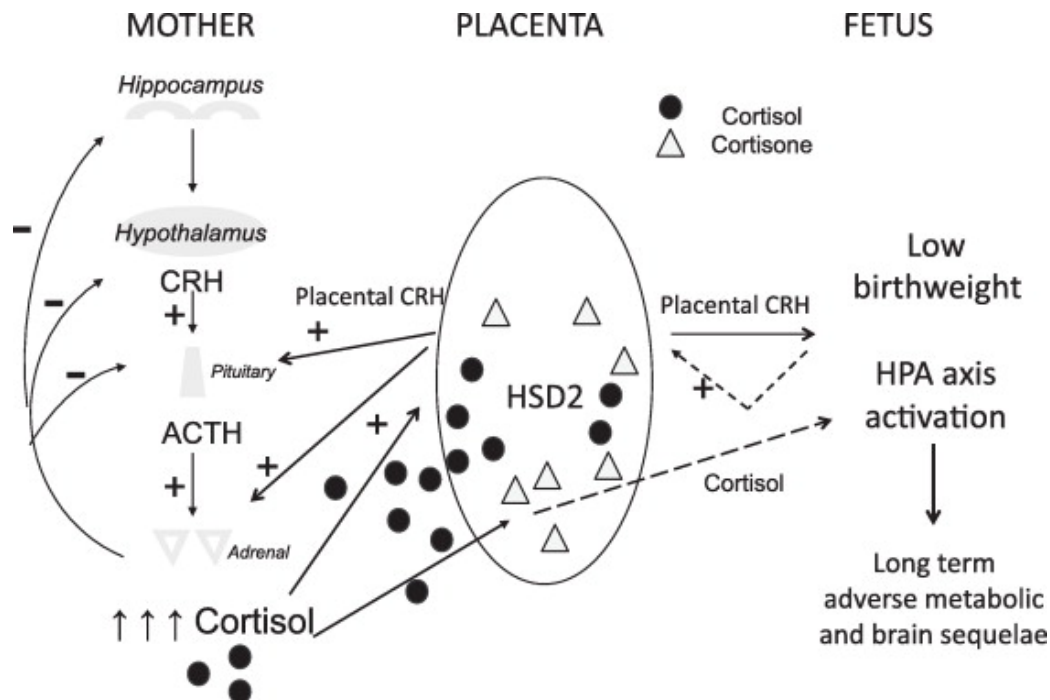
Figure 1.19. Glucocorticoid Signaling between the Mother, Placenta and Fetus. Activation of the maternal hypothalamic–pituitary–adrenal (HPA) axis during pregnancy leads to increased circulating levels of cortisol (filled circles). Maternal cortisol is transported to the placenta where it is either broken 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).

<u>Conclusion</u>

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.
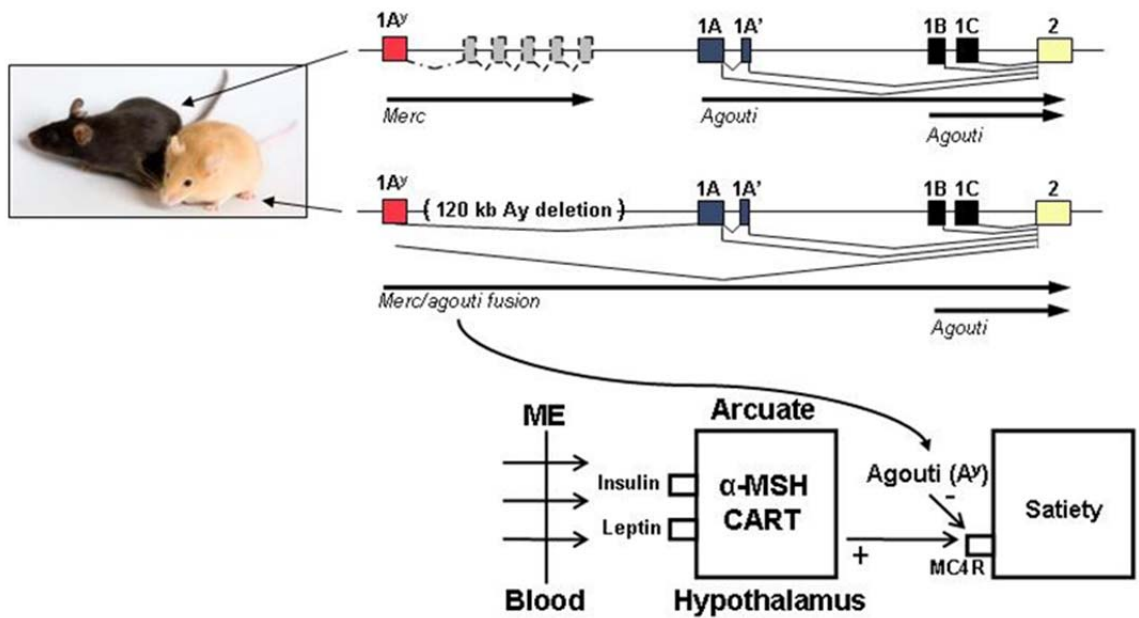
Figure 1.20. Lethal Yellow Mouse Model of Obesity. Schematic representation of the deletion mutation on the C57 BL/6 background which produces the Lethal Yellow phenotype.

Literature Cited

1. **Guo SS, Roche AF, Chumlea WC, Gardner JD, Siervogel RM** 1994 The predictive value of childhood body mass index values for overweight at age 35 y. Am J Clin Nutr 59:810-819

2. **Siega-Riz AM, Laraia B** 2006 The implications of maternal overweight and obesity on the course of pregnancy and birth outcomes. Matern Child Health J 10:S153-156

3. 2011 Prevalence and Trends Data - Nationalwide 2011 Overweight and Obesity (BMI). In: CDC, Office of Surveillance, Epidemiology, and Labratory Services

4. **Trayhurn P, Bing C, Wood IS** 2006 Adipose tissue and adipokines--energy regulation from the human perspective. J Nutr 136:1935S-1939S

5. **Bays HE, Gonzalez-Campoy JM, Bray GA, Kitabchi AE, Bergman DA, Schorr AB, Rodbard HW, Henry RR** 2008 Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity. Expert Rev Cardiovasc Ther 6:343-368

6. **Wu G, Bazer FW, Cudd TA, Meininger CJ, Spencer TE** 2004 Maternal nutrition and fetal development. J Nutr 134:2169-2172

7. **Heslehurst N, Ells LJ, Simpson H, Batterham A, Wilkinson J, Summerbell CD** 2007 Trends in maternal obesity incidence rates, demographic predictors, and health inequalities in 36,821 women over a 15-year period. BJOG 114:187-194

8. **O'Reilly JR, Reynolds RM** 2013 The risk of maternal obesity to the long-term health of the offspring. Clin Endocrinol (Oxf) 78:9-16

9. **Heerwagen MJ, Miller MR, Barbour LA, Friedman JE** 2010 Maternal obesity and fetal metabolic programming: a fertile epigenetic soil. Am J Physiol Regul Integr Comp Physiol 299:R711-722

10. **Fleming TP, Kwong WY, Porter R, Ursell E, Fesenko I, Wilkins A, Miller DJ, Watkins AJ, Eckert JJ** 2004 The embryo and its future. Biology of reproduction 71:1046-1054

11. **Denison FC, Roberts KA, Barr SM, Norman JE** 2010 Obesity, pregnancy, inflammation, and vascular function. Reproduction 140:373-385

12. **Boney CM, Verma A, Tucker R, Vohr BR** 2005 Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics 115:e290-296

13. **Bispham J, Gopalakrishnan GS, Dandrea J, Wilson V, Budge H, Keisler DH, Broughton Pipkin F, Stephenson T, Symonds ME** 2003 Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. Endocrinology 144:3575-3585

14. **Symonds ME, Sebert SP, Budge H** 2009 The impact of diet during early life and its contribution to later disease: critical checkpoints in development and their long-term consequences for metabolic health. Proc Nutr Soc 68:416-421

15. **Power ML, Schulkin J** 2012 Maternal obesity, metabolic disease, and allostatic load. Physiol Behav 106:22-28

16. **Briana DD, Malamitsi-Puchner A** 2010 The role of adipocytokines in fetal growth. Ann N Y Acad Sci 1205:82-87

17. **O'Connor JC, McCusker RH, Strle K, Johnson RW, Dantzer R, Kelley KW** 2008 Regulation of IGF-I function by proinflammatory cytokines: at the interface of immunology and endocrinology. Cell Immunol 252:91-110

18. **Poulos SP, Hausman DB, Hausman GJ** 2010 The development and endocrine functions of adipose tissue. Mol Cell Endocrinol 323:20-34

19. **Lupu F, Terwilliger JD, Lee K, Segre GV, Efstratiadis A** 2001 Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. Dev Biol 229:141-162

20. **Agha A, Monson JP** 2007 Modulation of glucocorticoid metabolism by the growth hormone - IGF-1 axis. Clin Endocrinol (Oxf) 66:459-465

21. **Moller N, Jorgensen JO** 2009 Effects of growth hormone on glucose, lipid, and protein metabolism in human subjects. Endocr Rev 30:152-177

22. **Garten A, Schuster S, Kiess W** 2012 The insulin-like growth factors in adipogenesis and obesity. Endocrinol Metab Clin North Am 41:283-295, v-vi

23. **Ruan W, Lai M** 2010 Insulin-like growth factor binding protein: a possible marker for the metabolic syndrome? Acta Diabetol 47:5-14

24. **Rinderknecht E, Humbel RE** 1978 Primary structure of human insulin-like growth factor II. FEBS Lett 89:283-286

25. **Bellows CG, Jia D, Jia Y, Hassanloo A, Heersche JN** 2006 Different effects of insulin and insulin-like growth factors I and II on osteoprogenitors and adipocyte progenitors in fetal rat bone cell populations. Calcif Tissue Int 79:57-65

26. **DeChiara TM, Efstratiadis A, Robertson EJ** 1990 A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature 345:78-80

27. **Han VK, Lund PK, Lee DC, D'Ercole AJ** 1988 Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization, and tissue distribution. J Clin Endocrinol Metab 66:422-429

28. **Jones JI, Clemmons DR** 1995 Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3-34

29. **Yakar S, Leroith D, Brodt P** 2005 The role of the growth hormone/insulin-like growth factor axis in tumor growth and progression: Lessons from animal models. Cytokine Growth Factor Rev 16:407-420

30. **Baxter RC** 2000 Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. Am J Physiol Endocrinol Metab 278:E967-976

31. **Kloting N, Koch L, Wunderlich T, Kern M, Ruschke K, Krone W, Bruning JC, Bluher M** 2008 Autocrine IGF-1 action in adipocytes controls systemic IGF-1 concentrations and growth. Diabetes 57:2074-2082

32. **Mavalli MD, DiGirolamo DJ, Fan Y, Riddle RC, Campbell KS, van Groen T, Frank SJ, Sperling MA, Esser KA, Bamman MM, Clemens TL** 2010 Distinct growth hormone receptor signaling modes regulate skeletal muscle development and insulin sensitivity in mice. J Clin Invest 120:4007-4020

33. **Back K, Arnqvist HJ** 2009 Changes in insulin and IGF-I receptor expression during differentiation of human preadipocytes. Growth Horm IGF Res 19:101-111

34. **Adams TE, Epa VC, Garrett TP, Ward CW** 2000 Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci 57:1050-1093

35. **LeRoith D** 2000 Insulin-like growth factor I receptor signaling--overlapping or redundant pathways? Endocrinology 141:1287-1288

36. **Isaksson OG, Lindahl A, Nilsson A, Isgaard J** 1987 Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth. Endocr Rev 8:426-438

37. **Clayton PE, Banerjee I, Murray PG, Renehan AG** 2011 Growth hormone, the insulin-like growth factor axis, insulin and cancer risk. Nat Rev Endocrinol 7:11-24

38. **Baxter RC, Twigg SM** 2009 Actions of IGF binding proteins and related proteins in adipose tissue. Trends Endocrinol Metab 20:499-505

39. **Rechler MM, Zapf J, Nissley SP, Froesch ER, Moses AC, Podskalny JM, Schilling EE, Humbel RE** 1980 Interactions of insulin-like growth factors I and II and multiplication-stimulating activity with receptors and serum carrier proteins. Endocrinology 107:1451-1459

40. **Kulik G, Klippel A, Weber MJ** 1997 Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol 17:1595-1606

41. **Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME** 1997 Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91:231-241

42. **Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME** 1997 Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661-665

43. **Sekimoto H, Boney CM** 2003 C-terminal Src kinase (CSK) modulates insulin-like growth factor-I signaling through Src in 3T3-L1 differentiation. Endocrinology 144:2546-2552

44. **Shen WH, Zhou JH, Broussard SR, Freund GG, Dantzer R, Kelley KW** 2002 Proinflammatory cytokines block growth of breast cancer cells by impairing signals from a growth factor receptor. Cancer Res 62:4746-4756

45. **Villafuerte BC, Fine JB, Bai Y, Zhao W, Fleming S, DiGirolamo M** 2000 Expressions of leptin and insulin-like growth factor-I are highly correlated and region-specific in adipose tissue of growing rats. Obes Res 8:646-655

46. **Ozanne SE, Jensen CB, Tingey KJ, Martin-Gronert MS, Grunnet L, Brons C, Storgaard H, Vaag AA** 2006 Decreased protein levels of key insulin signalling molecules in adipose tissue from young men with a low birthweight: potential link to increased risk of diabetes? Diabetologia 49:2993-2999

47. **Iniguez G, Ormazabal P, Lopez T, Maldonado D, Avila A, Roman R, Cassorla F** 2009 IGF-IR/ERK content and response to IGF-I and insulin in adipocytes from small for gestational age children. Growth Horm IGF Res 19:256-261

48. **Reul BA, Ongemba LN, Pottier AM, Henquin JC, Brichard SM** 1997 Insulin and insulin-like growth factor 1 antagonize the stimulation of ob gene expression by dexamethasone in cultured rat adipose tissue. Biochem J 324 (Pt 2):605-610

49. **Friedman JM, Halaas JL** 1998 Leptin and the regulation of body weight in mammals. Nature 395:763-770

50. **Houseknecht KL, Baile CA, Matteri RL, Spurlock ME** 1998 The biology of leptin: a review. J Anim Sci 76:1405-1420

51. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM** 1994 Positional cloning of the mouse obese gene and its human homologue. Nature 372:425-432

52. **Long YC, Zierath JR** 2006 AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest 116:1776-1783

53. **Unger RH, Zhou YT, Orci L** 1999 Regulation of fatty acid homeostasis in cells: novel role of leptin. Proc Natl Acad Sci U S A 96:2327-2332

54. **Muoio DM, Dohm GL, Fiedorek FT, Jr., Tapscott EB, Coleman RA** 1997 Leptin directly alters lipid partitioning in skeletal muscle. Diabetes 46:1360-1363

55. **Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ** 1997 Acute stimulation of glucose metabolism in mice by leptin treatment. Nature 389:374-377

56. **Haque MS, Minokoshi Y, Hamai M, Iwai M, Horiuchi M, Shimazu T** 1999 Role of the sympathetic nervous system and insulin in enhancing glucose uptake in peripheral tissues after intrahypothalamic injection of leptin in rats. Diabetes 48:1706-1712

57. **Unger RH** 2002 Lipotoxic diseases. Annu Rev Med 53:319-336

58. **M.E. Hadley LJE** ed. 2007 Endocrinology. 6th ed. Upper Saddle River: Pearson Prentice Hall

59. **Chehab FF, Mounzih K, Lu R, Lim ME** 1997 Early onset of reproductive function in normal female mice treated with leptin. Science 275:88-90

60. **Ahima RS, Antwi DA** 2008 Brain regulation of appetite and satiety. Endocrinol Metab Clin North Am 37:811-823

61. **Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, Tepper RI** 1995 Identification and expression cloning of a leptin receptor, OB-R. Cell 83:1263-1271

62. **Li C, Ioffe E, Fidahusein N, Connolly E, Friedman JM** 1998 Absence of soluble leptin receptor in plasma from dbPas/dbPas and other db/db mice. J Biol Chem 273:10078-10082

63. **Devos R, Guisez Y, Van der Heyden J, White DW, Kalai M, Fountoulakis M, Plaetinck G** 1997 Ligand-independent dimerization of the extracellular domain of the leptin receptor and determination of the stoichiometry of leptin binding. J Biol Chem 272:18304-18310

64. **Carpenter LR, Farruggella TJ, Symes A, Karow ML, Yancopoulos GD, Stahl N** 1998 Enhancing leptin response by preventing SH2-containing

phosphatase 2 interaction with Ob receptor. Proc Natl Acad Sci U S A 95:6061-6066

65. **Vaisse C, Halaas JL, Horvath CM, Darnell JE, Jr., Stoffel M, Friedman JM** 1996 Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. Nat Genet 14:95-97

66. **Kahn BB, Alquier T, Carling D, Hardie DG** 2005 AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 1:15-25

67. **Spiegelman BM, Flier JS** 2001 Obesity and the regulation of energy balance. Cell 104:531-543

68. **Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD, Low MJ** 2001 Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 411:480-484

69. **Balthasar N, Coppari R, McMinn J, Liu SM, Lee CE, Tang V, Kenny CD, McGovern RA, Chua SC, Jr., Elmquist JK, Lowell BB** 2004 Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron 42:983-991

70. **Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB, O'Rahilly S** 1997 Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 387:903-908

71. **Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS** 1995 Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med 1:1311-1314

72. **Yeo GS, Heisler LK** 2012 Unraveling the brain regulation of appetite: lessons from genetics. Nat Neurosci 15:1343-1349

73. **Kriegler M, Perez C, DeFay K, Albert I, Lu SD** 1988 A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell 53:45-53

74. **Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL, et al.** 1994 Processing of tumour necrosis factor-alpha precursor by metalloproteinases. Nature 370:555-557

75. **Olszewski MB, Groot AJ, Dastych J, Knol EF** 2007 TNF trafficking to human mast cell granules: mature chain-dependent endocytosis. J Immunol 178:5701-5709

76. **Galic S, Oakhill JS, Steinberg GR** 2010 Adipose tissue as an endocrine organ. Mol Cell Endocrinol 316:129-139

77. **Sullivan KE, Reddy AB, Dietzmann K, Suriano AR, Kocieda VP, Stewart M, Bhatia M** 2007 Epigenetic regulation of tumor necrosis factor alpha. Mol Cell Biol 27:5147-5160

78. **Coppack SW** 2001 Pro-inflammatory cytokines and adipose tissue. Proc Nutr Soc 60:349-356

79. **Valsamakis G, Kumar S, Creatsas G, Mastorakos G** 2010 The effects of adipose tissue and adipocytokines in human pregnancy. Ann N Y Acad Sci 1205:76-81

80. **Hube F, Birgel M, Lee YM, Hauner H** 1999 Expression pattern of tumour necrosis factor receptors in subcutaneous and omental human adipose tissue: role of obesity and non-insulin-dependent diabetes mellitus. Eur J Clin Invest 29:672-678

81. **Wu Y, Zhou BP** 2010 TNF-alpha/NF-kappaB/Snail pathway in cancer cell migration and invasion. Br J Cancer 102:639-644

82. **Sewter CP, Digby JE, Blows F, Prins J, O'Rahilly S** 1999 Regulation of tumour necrosis factor-alpha release from human adipose tissue in vitro. J Endocrinol 163:33-38

83. **Hunt JS, Chen HL, Miller L** 1996 Tumor necrosis factors: pivotal components of pregnancy? Biol Reprod 54:554-562

84. **Challier JC, Basu S, Bintein T, Minium J, Hotmire K, Catalano PM, Hauguel-de Mouzon S** 2008 Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta. Placenta 29:274-281

85. **Drake AJ, Reynolds RM** 2010 Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. Reproduction 140:387-398

86. **Taylor PD, Poston L** 2007 Developmental programming of obesity in mammals. Exp Physiol 92:287-298

87. **Parsons TJ, Power C, Manor O** 2001 Fetal and early life growth and body mass index from birth to early adulthood in 1958 British cohort: longitudinal study. BMJ 323:1331-1335

88. **Jensen DM, Damm P, Sorensen B, Molsted-Pedersen L, Westergaard JG, Ovesen P, Beck-Nielsen H** 2003 Pregnancy outcome and prepregnancy body mass index in 2459 glucose-tolerant Danish women. Am J Obstet Gynecol 189:239-244

89. **Sebire NJ, Jolly M, Harris JP, Wadsworth J, Joffe M, Beard RW, Regan L, Robinson S** 2001 Maternal obesity and pregnancy outcome: a study of 287,213 pregnancies in London. Int J Obes Relat Metab Disord 25:1175-1182

90. **Lowe A, Braback L, Ekeus C, Hjern A, Forsberg B** 2011 Maternal obesity during pregnancy as a risk for early-life asthma. J Allergy Clin Immunol 128:1107-1109 e1101-1102

91. **Catalano PM, Thomas A, Huston-Presley L, Amini SB** 2003 Increased fetal adiposity: a very sensitive marker of abnormal in utero development. Am J Obstet Gynecol 189:1698-1704

92. **Luzzo KM, Wang Q, Purcell SH, Chi M, Jimenez PT, Grindler N, Schedl T, Moley KH** 2012 High fat diet induced developmental defects in the mouse: oocyte meiotic aneuploidy and fetal growth retardation/brain defects. PLoS One 7:e49217

93. **Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, Moley KH** 2010 Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. Endocrinology 151:4039-4046

94. **Wei JN, Sung FC, Li CY, Chang CH, Lin RS, Lin CC, Chiang CC, Chuang LM** 2003 Low birth weight and high birth weight infants are both at an increased risk to have type 2 diabetes among schoolchildren in taiwan. Diabetes Care 26:343-348

95. **McMillen IC, Muhlhausler BS, Duffield JA, Yuen BS** 2004 Prenatal programming of postnatal obesity: fetal nutrition and the regulation of leptin synthesis and secretion before birth. Proc Nutr Soc 63:405-412

96. **Catalano PM, Presley L, Minium J, Hauguel-de Mouzon S** 2009 Fetuses of obese mothers develop insulin resistance in utero. Diabetes Care 32:1076-1080

97. **Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH, Piersma AH, Ozanne SE, Twinn DF, Remacle C, Rowlerson A, Poston L, Taylor PD** 2008 Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. Hypertension 51:383-392

98. **Luo ZC, Xiao L, Nuyt AM** 2010 Mechanisms of developmental programming of the metabolic syndrome and related disorders. World J Diabetes 1:89-98

99. **Plagemann A, Harder T, Rake A, Voits M, Fink H, Rohde W, Dorner G** 1999 Perinatal elevation of hypothalamic insulin, acquired malformation of hypothalamic galaninergic neurons, and syndrome x-like alterations in adulthood of neonatally overfed rats. Brain Res 836:146-155

100. **George LA, Uthlaut AB, Long NM, Zhang L, Ma Y, Smith DT, Nathanielsz PW, Ford SP** 2010 Different levels of overnutrition and weight gain during pregnancy have differential effects on fetal growth and organ development. Reprod Biol Endocrinol 8:75

101. **Levin BE** 2006 Metabolic imprinting: critical impact of the perinatal environment on the regulation of energy homeostasis. Philos Trans R Soc Lond B Biol Sci 361:1107-1121

102. **Armitage JA, Taylor PD, Poston L** 2005 Experimental models of developmental programming: consequences of exposure to an energy rich diet during development. J Physiol 565:3-8

103. **Langley-Evans SC** 1996 Intrauterine programming of hypertension in the rat: nutrient interactions. Comp Biochem Physiol A Physiol 114:327-333

104. **Muhlhausler BS, Adam CL, Findlay PA, Duffield JA, McMillen IC** 2006 Increased maternal nutrition alters development of the appetite-regulating network in the brain. FASEB J 20:1257-1259

105. **Nivoit P, Morens C, Van Assche FA, Jansen E, Poston L, Remacle C, Reusens B** 2009 Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. Diabetologia 52:1133-1142

106. **McMillen IC, Adam CL, Muhlhausler BS** 2005 Early origins of obesity: programming the appetite regulatory system. J Physiol 565:9-17

107. **Ford SP, Zhang L, Zhu M, Miller MM, Smith DT, Hess BW, Moss GE, Nathanielsz PW, Nijland MJ** 2009 Maternal obesity accelerates fetal pancreatic beta-cell but not alpha-cell development in sheep: prenatal consequences. Am J Physiol Regul Integr Comp Physiol 297:R835-843

108. **Aagaard-Tillery KM, Grove K, Bishop J, Ke X, Fu Q, McKnight R, Lane RH** 2008 Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. J Mol Endocrinol 41:91-102

109. **van der Poorten D, Milner KL, Hui J, Hodge A, Trenell MI, Kench JG, London R, Peduto T, Chisholm DJ, George J** 2008 Visceral fat: a key mediator of steatohepatitis in metabolic liver disease. Hepatology 48:449-457

110. **Oben JA, Patel T, Mouralidarane A, Samuelsson AM, Matthews P, Pombo J, Morgan M, McKee C, Soeda J, Novelli M, Poston L, Taylor P** 2010 Maternal obesity programmes offspring development of non-alcoholic fatty pancreas disease. Biochem Biophys Res Commun 394:24-28

111. **Fan L, Lindsley SR, Comstock SM, Takahashi DL, Evans AE, He GW, Thornburg KL, Grove KL** 2013 Maternal high-fat diet impacts endothelial function in nonhuman primate offspring. Int J Obes (Lond) 37:254-262

112. **Khan IY, Dekou V, Douglas G, Jensen R, Hanson MA, Poston L, Taylor PD** 2005 A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. Am J Physiol Regul Integr Comp Physiol 288:R127-133

113. **Elahi MM, Cagampang FR, Mukhtar D, Anthony FW, Ohri SK, Hanson MA** 2009 Long-term maternal high-fat feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice. Br J Nutr 102:514-519

114. **Moraeus L, Lissner L, Yngve A, Poortvliet E, Al-Ansari U, Sjoberg A** 2012 Multi-level influences on childhood obesity in Sweden: societal factors, parental determinants and child's lifestyle. Int J Obes (Lond) 36:969-976

115. **Zhu MJ, Ford SP, Means WJ, Hess BW, Nathanielsz PW, Du M** 2006 Maternal nutrient restriction affects properties of skeletal muscle in offspring. The Journal of Physiology 575:241-250

116. **Larciprete G, Valensise H, Di Pierro G, Vasapollo B, Casalino B, Arduini D, Jarvis S, Cirese E** 2005 Intrauterine growth restriction and fetal body composition. Ultrasound Obstet Gynecol 26:258-262

117. **Zhu MJ, Ford SP, Nathanielsz PW, Du M** 2004 Effect of maternal nutrient restriction in sheep on the development of fetal skeletal muscle. Biol Reprod 71:1968-1973

118. **Yates DT, Macko AR, Nearing M, Chen X, Rhoads RP, Limesand SW** 2012 Developmental programming in response to intrauterine growth restriction impairs myoblast function and skeletal muscle metabolism. J Pregnancy 2012:631038

119. **Zhu MJ, Han B, Tong J, Ma C, Kimzey JM, Underwood KR, Xiao Y, Hess BW, Ford SP, Nathanielsz PW, Du M** 2008 AMP-activated protein kinase signalling pathways are down regulated and skeletal muscle development impaired in fetuses of obese, over-nourished sheep. The Journal of physiology 586:2651-2664

120. **Winick M, Noble A** 1965 Quantitative changes in DNA, RNA, and protein during prenatal and postnatal growth in the rat. Dev Biol 12:451-466

121. **Allen RE, Merkel RA, Young RB** 1979 Cellular aspects of muscle growth: myogenic cell proliferation. J Anim Sci 49:115-127

122. **Greenwood PL, Hunt AS, Hermanson JW, Bell AW** 2000 Effects of birth weight and postnatal nutrition on neonatal sheep: II. Skeletal muscle growth and development. J Anim Sci 78:50-61

123. **Huang Y, Zhao JX, Yan X, Zhu MJ, Long NM, McCormick RJ, Ford SP, Nathanielsz PW, Du M** 2012 Maternal obesity enhances collagen accumulation and cross-linking in skeletal muscle of ovine offspring. PLoS One 7:e31691

124. **Yan X, Huang Y, Zhao JX, Long NM, Uthlaut AB, Zhu MJ, Ford SP, Nathanielsz PW, Du M** 2011 Maternal obesity-impaired insulin signaling in sheep and induced lipid accumulation and fibrosis in skeletal muscle of offspring. Biology of reproduction 85:172-178

125. **Colomiere M, Permezel M, Lappas M** 2010 Diabetes and obesity during pregnancy alter insulin signalling and glucose transporter expression in maternal skeletal muscle and subcutaneous adipose tissue. J Mol Endocrinol 44:213-223

126. **Tong JF, Yan X, Zhu MJ, Ford SP, Nathanielsz PW, Du M** 2009 Maternal obesity downregulates myogenesis and beta-catenin signaling in fetal skeletal muscle. American journal of physiology 296:E917-924

127. **Yan X, Zhu MJ, Xu W, Tong JF, Ford SP, Nathanielsz PW, Du M** 2010 Up-regulation of Toll-like receptor 4/nuclear factor-kappaB signaling is associated with enhanced adipogenesis and insulin resistance in fetal skeletal muscle of obese sheep at late gestation. Endocrinology 151:380-387

128. **Roth SM, Metter EJ, Ling S, Ferrucci L** 2006 Inflammatory factors in age-related muscle wasting. Curr Opin Rheumatol 18:625-630

129. **Spate U, Schulze PC** 2004 Proinflammatory cytokines and skeletal muscle. Curr Opin Clin Nutr Metab Care 7:265-269

130. **Frost RA, Nystrom GJ, Lang CH** 2003 Tumor necrosis factor-alpha decreases insulin-like growth factor-I messenger ribonucleic acid expression in C2C12 myoblasts via a Jun N-terminal kinase pathway. Endocrinology 144:1770-1779

131. **Gokulakrishnan G, Estrada IJ, Sosa HA, Fiorotto ML** 2012 In utero glucocorticoid exposure reduces fetal skeletal muscle mass in rats independent of effects on maternal nutrition. Am J Physiol Regul Integr Comp Physiol 302:R1143-1152

132. **Odedra BR, Bates PC, Millward DJ** 1983 Time course of the effect of catabolic doses of corticosterone on protein turnover in rat skeletal muscle and liver. Biochem J 214:617-627

133. **Relaix F, Rocancourt D, Mansouri A, Buckingham M** 2005 A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature 435:948-953

134. **Liu W, Wen Y, Bi P, Lai X, Liu XS, Liu X, Kuang S** 2012 Hypoxia promotes satellite cell self-renewal and enhances the efficiency of myoblast transplantation. Development 139:2857-2865

135. **Nissen PM, Danielsen VO, Jorgensen PF, Oksbjerg N** 2003 Increased maternal nutrition of sows has no beneficial effects on muscle fiber number or postnatal growth and has no impact on the meat quality of the offspring. J Anim Sci 81:3018-3027

136. **Lee RC, Wang ZM, Heymsfield SB** 2001 Skeletal muscle mass and aging: regional and whole-body measurement methods. Can J Appl Physiol 26:102-122

137. **Mok GF, Sweetman D** 2011 Many routes to the same destination: lessons from skeletal muscle development. Reproduction 141:301-312

138. **Feve B** 2005 Adipogenesis: cellular and molecular aspects. Best Pract Res Clin Endocrinol Metab 19:483-499

139. **Buckingham M, Bajard L, Chang T, Daubas P, Hadchouel J, Meilhac S, Montarras D, Rocancourt D, Relaix F** 2003 The formation of skeletal muscle: from somite to limb. J Anat 202:59-68

140. **Du M, Tong J, Zhao J, Underwood KR, Zhu M, Ford SP, Nathanielsz PW** 2010 Fetal programming of skeletal muscle development in ruminant animals. J Anim Sci 88:E51-60

141. **Sambasivan R, Tajbakhsh S** 2007 Skeletal muscle stem cell birth and properties. Semin Cell Dev Biol 18:870-882

142. **Tajbakhsh S, Buckingham M** 2000 The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. Curr Top Dev Biol 48:225-268

143. **Parker MH, Seale P, Rudnicki MA** 2003 Looking back to the embryo: defining transcriptional networks in adult myogenesis. Nat Rev Genet 4:497-507

144. **Kalcheim C, Ben-Yair R** 2005 Cell rearrangements during development of the somite and its derivatives. Curr Opin Genet Dev 15:371-380

145. **Pownall ME, Gustafsson MK, Emerson CP, Jr.** 2002 Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu Rev Cell Dev Biol 18:747-783

146. **Denetclaw WF, Ordahl CP** 2000 The growth of the dermomyotome and formation of early myotome lineages in thoracolumbar somites of chicken embryos. Development 127:893-905

147. **Christ B, Huang R, Scaal M** 2007 Amniote somite derivatives. Dev Dyn 236:2382-2396

148. **Shi X, Garry DJ** 2006 Muscle stem cells in development, regeneration, and disease. Genes Dev 20:1692-1708

149. **Buckingham M, Relaix F** 2007 The role of Pax genes in the development of tissues and organs: Pax3 and Pax7 regulate muscle progenitor cell functions. Annu Rev Cell Dev Biol 23:645-673

150. **Tajbakhsh S** 2009 Skeletal muscle stem cells in developmental versus regenerative myogenesis. J Intern Med 266:372-389

151. **Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell TK, Turner D, Rupp R, Hollenberg S, et al.** 1991 The myoD gene family: nodal point during specification of the muscle cell lineage. Science 251:761-766

152. **Ludolph DC, Konieczny SF** 1995 Transcription factor families: muscling in on the myogenic program. FASEB J 9:1595-1604

153. **Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, Lassar AB, Miller AD** 1989 Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci U S A 86:5434-5438

154. **Lassar AB, Davis RL, Wright WE, Kadesch T, Murre C, Voronova A, Baltimore D, Weintraub H** 1991 Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell 66:305-315

155. **Brack AS, Rando TA** 2012 Tissue-specific stem cells: lessons from the skeletal muscle satellite cell. Cell Stem Cell 10:504-514

156. **Bergstrom DA, Tapscott SJ** 2001 Molecular distinction between specification and differentiation in the myogenic basic helix-loop-helix transcription factor family. Mol Cell Biol 21:2404-2412

157. **Kitzmann M, Carnac G, Vandromme M, Primig M, Lamb NJ, Fernandez A** 1998 The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. J Cell Biol 142:1447-1459

158. **Haldar M, Karan G, Tvrdik P, Capecchi MR** 2008 Two cell lineages, myf5 and myf5-independent, participate in mouse skeletal myogenesis. Dev Cell 14:437-445

159. **Gensch N, Borchardt T, Schneider A, Riethmacher D, Braun T** 2008 Different autonomous myogenic cell populations revealed by ablation of Myf5-expressing cells during mouse embryogenesis. Development 135:1597-1604

160. **Gustafsson MK, Pan H, Pinney DF, Liu Y, Lewandowski A, Epstein DJ, Emerson CP, Jr.** 2002 Myf5 is a direct target of long-range Shh signaling and Gli regulation for muscle specification. Genes Dev 16:114-126

161. **Yablonka-Reuveni Z, Rudnicki MA, Rivera AJ, Primig M, Anderson JE, Natanson P** 1999 The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. Dev Biol 210:440-455

162. **Halevy O, Piestun Y, Allouh MZ, Rosser BW, Rinkevich Y, Reshef R, Rozenboim I, Wleklinski-Lee M, Yablonka-Reuveni Z** 2004 Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal. Dev Dyn 231:489-502

163. **Megeney LA, Rudnicki MA** 1995 Determination versus differentiation and the MyoD family of transcription factors. Biochem Cell Biol 73:723-732

164. **Rhodes SJ, Konieczny SF** 1989 Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev 3:2050-2061

165. **Yablonka-Reuveni Z** 2011 The skeletal muscle satellite cell: still young and fascinating at 50. J Histochem Cytochem 59:1041-1059

166. **Hinterberger TJ, Sassoon DA, Rhodes SJ, Konieczny SF** 1991 Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. Dev Biol 147:144-156

167. **Mauro A** 1961 Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 9:493-495

168. **Kassar-Duchossoy L, Giacone E, Gayraud-Morel B, Jory A, Gomes D, Tajbakhsh S** 2005 Pax3/Pax7 mark a novel population of primitive myogenic cells during development. Genes Dev 19:1426-1431

169. **Bajard L, Relaix F, Lagha M, Rocancourt D, Daubas P, Buckingham ME** 2006 A novel genetic hierarchy functions during hypaxial myogenesis: Pax3

directly activates Myf5 in muscle progenitor cells in the limb. Genes Dev 20:2450-2464

170. **Schultz E** 1996 Satellite cell proliferative compartments in growing skeletal muscles. Dev Biol 175:84-94
171. **Shefer G, Van de Mark DP, Richardson JB, Yablonka-Reuveni Z** 2006 Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. Dev Biol 294:50-66
172. **Pannerec A, Marazzi G, Sassoon D** 2012 Stem cells in the hood: the skeletal muscle niche. Trends Mol Med 18:599-606
173. **Charge SB, Rudnicki MA** 2004 Cellular and molecular regulation of muscle regeneration. Physiol Rev 84:209-238
174. **Kastner S, Elias MC, Rivera AJ, Yablonka-Reuveni Z** 2000 Gene expression patterns of the fibroblast growth factors and their receptors during myogenesis of rat satellite cells. J Histochem Cytochem 48:1079-1096
175. **Woo M, Isganaitis E, Cerletti M, Fitzpatrick C, Wagers AJ, Jimenez-Chillaron J, Patti ME** 2011 Early life nutrition modulates muscle stem cell number: implications for muscle mass and repair. Stem Cells Dev 20:1763-1769
176. **Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR** 2004 Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J Cell Biol 166:347-357
177. **Yablonka-Reuveni Z, Day K, Vine A, Shefer G** 2008 Defining the transcriptional signature of skeletal muscle stem cells. J Anim Sci 86:E207-216
178. **Pownall ME, Strunk KE, Emerson CP, Jr.** 1996 Notochord signals control the transcriptional cascade of myogenic bHLH genes in somites of quail embryos. Development 122:1475-1488
179. **Rong PM, Teillet MA, Ziller C, Le Douarin NM** 1992 The neural tube/notochord complex is necessary for vertebral but not limb and body wall striated muscle differentiation. Development 115:657-672
180. **Kuang S, Gillespie MA, Rudnicki MA** 2008 Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell 2:22-31
181. **Rios AC, Serralbo O, Salgado D, Marcelle C** 2011 Neural crest regulates myogenesis through the transient activation of NOTCH. Nature 473:532-535
182. **Bjornson CR, Cheung TH, Liu L, Tripathi PV, Steeper KM, Rando TA** 2012 Notch signaling is necessary to maintain quiescence in adult muscle stem cells. Stem Cells 30:232-242
183. **Schuster-Gossler K, Cordes R, Gossler A** 2007 Premature myogenic differentiation and depletion of progenitor cells cause severe muscle hypotrophy in Delta1 mutants. Proc Natl Acad Sci U S A 104:537-542
184. **Conboy IM, Rando TA** 2002 The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell 3:397-409
185. **Kuang S, Kuroda K, Le Grand F, Rudnicki MA** 2007 Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 129:999-1010
186. **Borycki AG, Emerson CP, Jr.** 2000 Multiple tissue interactions and signal transduction pathways control somite myogenesis. Curr Top Dev Biol 48:165-224

187. **Brack AS, Conboy IM, Conboy MJ, Shen J, Rando TA** 2008 A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. Cell Stem Cell 2:50-59

188. **Fan CM, Lee CS, Tessier-Lavigne M** 1997 A role for WNT proteins in induction of dermomyotome. Dev Biol 191:160-165

189. **Wiggan O, Hamel PA** 2002 Pax3 regulates morphogenetic cell behavior in vitro coincident with activation of a PCP/non-canonical Wnt-signaling cascade. J Cell Sci 115:531-541

190. **Borycki AG, Mendham L, Emerson CP, Jr.** 1998 Control of somite patterning by Sonic hedgehog and its downstream signal response genes. Development 125:777-790

191. **Kruger M, Mennerich D, Fees S, Schafer R, Mundlos S, Braun T** 2001 Sonic hedgehog is a survival factor for hypaxial muscles during mouse development. Development 128:743-752

192. **Teillet M, Watanabe Y, Jeffs P, Duprez D, Lapointe F, Le Douarin NM** 1998 Sonic hedgehog is required for survival of both myogenic and chondrogenic somitic lineages. Development 125:2019-2030

193. **Hirsinger E, Duprez D, Jouve C, Malapert P, Cooke J, Pourquie O** 1997 Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning. Development 124:4605-4614

194. **Borello U, Berarducci B, Murphy P, Bajard L, Buffa V, Piccolo S, Buckingham M, Cossu G** 2006 The Wnt/beta-catenin pathway regulates Gli-mediated Myf5 expression during somitogenesis. Development 133:3723-3732

195. **Hay ED** 1995 An overview of epithelio-mesenchymal transformation. Acta Anat (Basel) 154:8-20

196. **Duband JL, Monier F, Delannet M, Newgreen D** 1995 Epithelium-mesenchyme transition during neural crest development. Acta Anat (Basel) 154:63-78

197. **Nieto MA** 2001 The early steps of neural crest development. Mech Dev 105:27-35

198. **Billon N, Iannarelli P, Monteiro MC, Glavieux-Pardanaud C, Richardson WD, Kessaris N, Dani C, Dupin E** 2007 The generation of adipocytes by the neural crest. Development 134:2283-2292

199. **Gammill LS, Roffers-Agarwal J** 2010 Division of labor during trunk neural crest development. Dev Biol 344:555-565

200. **Theveneau E, Mayor R** 2012 Neural crest delamination and migration: from epithelium-to-mesenchyme transition to collective cell migration. Dev Biol 366:34-54

201. **Hall BK** 2008 The neural crest and neural crest cells: discovery and significance for theories of embryonic organization. J Biosci 33:781-793

202. **Kuriyama S, Mayor R** 2008 Molecular analysis of neural crest migration. Philos Trans R Soc Lond B Biol Sci 363:1349-1362

203. **Knecht AK, Bronner-Fraser M** 2002 Induction of the neural crest: a multigene process. Nat Rev Genet 3:453-461

204. **Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA** 2000 The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2:76-83

205. **Van Ho AT, Hayashi S, Brohl D, Aurade F, Rattenbach R, Relaix F** 2011 Neural crest cell lineage restricts skeletal muscle progenitor cell differentiation through Neuregulin1-ErbB3 signaling. Developmental cell 21:273-287

206. **Murphy VE, Smith R, Giles WB, Clifton VL** 2006 Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. Endocr Rev 27:141-169

207. **Lesage J, Del-Favero F, Leonhardt M, Louvart H, Maccari S, Vieau D, Darnaudery M** 2004 Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat. J Endocrinol 181:291-296

208. **Gluckman PD, Hanson MA** 2004 The developmental origins of the metabolic syndrome. Trends Endocrinol Metab 15:183-187

209. **Nathanielsz PW, Poston L, Taylor PD** 2007 In utero exposure to maternal obesity and diabetes: animal models that identify and characterize implications for future health. Clin Perinatol 34:515-526, v

210. **Sebert S, Sharkey D, Budge H, Symonds ME** 2011 The early programming of metabolic health: is epigenetic setting the missing link? Am J Clin Nutr 94:1953S-1958S

211. **Funston RN, Larson DM, Vonnahme KA** 2010 Effects of maternal nutrition on conceptus growth and offspring performance: implications for beef cattle production. J Anim Sci 88:E205-215

212. **Rossant J, Cross JC** 2001 Placental development: lessons from mouse mutants. Nat Rev Genet 2:538-548

213. **Watson ED, Cross JC** 2005 Development of structures and transport functions in the mouse placenta. Physiology (Bethesda) 20:180-193

214. **Adamson SL, Lu Y, Whiteley KJ, Holmyard D, Hemberger M, Pfarrer C, Cross JC** 2002 Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. Dev Biol 250:358-373

215. **Reynolds LP, Millaway DS, Kirsch JD, Infeld JE, Redmer DA** 1990 Growth and in-vitro metabolism of placental tissues of cows from day 100 to day 250 of gestation. J Reprod Fertil 89:213-222

216. **Reynolds LP, Redmer DA** 1995 Utero-placental vascular development and placental function. J Anim Sci 73:1839-1851

217. **Reynolds LP, Redmer DA** 2001 Angiogenesis in the placenta. Biol Reprod 64:1033-1040

218. **Caluwaerts S, Vercruysse L, Luyten C, Pijnenborg R** 2005 Endovascular trophoblast invasion and associated structural changes in uterine spiral arteries of the pregnant rat. Placenta 26:574-584

219. **Hayes EK, Lechowicz A, Petrik JJ, Storozhuk Y, Paez-Parent S, Dai Q, Samjoo IA, Mansell M, Gruslin A, Holloway AC, Raha S** 2012 Adverse fetal

and neonatal outcomes associated with a life-long high fat diet: role of altered development of the placental vasculature. PLoS One 7:e33370

220. **Godfrey KM, Barker DJ** 2001 Fetal programming and adult health. Public Health Nutr 4:611-624

221. **Godfrey KM** 2002 The role of the placenta in fetal programming-a review. Placenta 23 Suppl A:S20-27

222. **Eckstein P, Mc KT, Record RG** 1955 Variation in placental weight according to litter size in the guinea-pig. J Endocrinol 12:108-114

223. **Anthony RV, Bellows RA, Short RE, Staigmiller RB, Kaltenbach CC, Dunn TG** 1986 Fetal growth of beef calves. II. Effect of sire on prenatal development of the calf and related placental characteristics. J Anim Sci 62:1375-1387

224. **Walsh SW** 1998 Maternal-placental interactions of oxidative stress and antioxidants in preeclampsia. Semin Reprod Endocrinol 16:93-104

225. **King JC** 2006 Maternal obesity, metabolism, and pregnancy outcomes. Annu Rev Nutr 26:271-291

226. **Jauniaux E, Poston L, Burton GJ** 2006 Placental-related diseases of pregnancy: Involvement of oxidative stress and implications in human evolution. Hum Reprod Update 12:747-755

227. **Luo ZC, Fraser WD, Julien P, Deal CL, Audibert F, Smith GN, Xiong X, Walker M** 2006 Tracing the origins of "fetal origins" of adult diseases: programming by oxidative stress? Med Hypotheses 66:38-44

228. **Agarwal A, Gupta S, Sharma RK** 2005 Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 3:28

229. **Genestra M** 2007 Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Cell Signal 19:1807-1819

230. **Al-Gubory KH, Fowler PA, Garrel C** 2010 The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol 42:1634-1650

231. **Hancock JT, Desikan R, Neill SJ** 2001 Role of reactive oxygen species in cell signalling pathways. Biochem Soc Trans 29:345-350

232. **Ornoy A** 2007 Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. Reprod Toxicol 24:31-41

233. **Vincent HK, Taylor AG** 2006 Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. Int J Obes (Lond) 30:400-418

234. **Rani N, Dhingra R, Arya DS, Kalaivani M, Bhatla N, Kumar R** 2010 Role of oxidative stress markers and antioxidants in the placenta of preeclamptic patients. J Obstet Gynaecol Res 36:1189-1194

235. **Myatt L** 2006 Placental adaptive responses and fetal programming. J Physiol 572:25-30

236. **Storz P** 2007 Mitochondrial ROS--radical detoxification, mediated by protein kinase D. Trends Cell Biol 17:13-18

237. **Storz P** 2011 Forkhead homeobox type O transcription factors in the responses to oxidative stress. Antioxid Redox Signal 14:593-605

238. **Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruiter ND, Bos JL, Burgering BM** 2004 FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J 23:4802-4812

239. **Machlin LJ, Bendich A** 1987 Free radical tissue damage: protective role of antioxidant nutrients. FASEB J 1:441-445

240. **Ponugoti B, Dong G, Graves DT** 2012 Role of forkhead transcription factors in diabetes-induced oxidative stress. Exp Diabetes Res 2012:939751

241. **Manolagas SC, Almeida M** 2007 Gone with the Wnts: beta-catenin, T-cell factor, forkhead box O, and oxidative stress in age-dependent diseases of bone, lipid, and glucose metabolism. Molecular endocrinology (Baltimore, Md 21:2605-2614

242. **Wang MC, Bohmann D, Jasper H** 2005 JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. Cell 121:115-125

243. **Almeida M, Han L, Ambrogini E, Weinstein RS, Manolagas SC** 2011 Glucocorticoids and tumor necrosis factor alpha increase oxidative stress and suppress Wnt protein signaling in osteoblasts. The Journal of biological chemistry 286:44326-44335

244. **Shen HM, Liu ZG** 2006 JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. Free Radic Biol Med 40:928-939

245. **Kyaw M, Yoshizumi M, Tsuchiya K, Izawa Y, Kanematsu Y, Tamaki T** 2004 Atheroprotective effects of antioxidants through inhibition of mitogen-activated protein kinases. Acta Pharmacol Sin 25:977-985

246. **Brasier AR** 2006 The NF-kappaB regulatory network. Cardiovasc Toxicol 6:111-130

247. **Ornoy A, Rand SB, Bischitz N** 2010 Hyperglycemia and hypoxia are interrelated in their teratogenic mechanism: studies on cultured rat embryos. Birth Defects Res B Dev Reprod Toxicol 89:106-115

248. **Hung TH, Skepper JN, Burton GJ** 2001 In vitro ischemia-reperfusion injury in term human placenta as a model for oxidative stress in pathological pregnancies. Am J Pathol 159:1031-1043

249. **Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, Furukawa S, Tochino Y, Komuro R, Matsuda M, Shimomura I** 2007 Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. Diabetes 56:901-911

250. **Trayhurn P, Wood IS** 2004 Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr 92:347-355

251. **Ye J, Gao Z, Yin J, He Q** 2007 Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. Am J Physiol Endocrinol Metab 293:E1118-1128

252. **Iiyori N, Alonso LC, Li J, Sanders MH, Garcia-Ocana A, O'Doherty RM, Polotsky VY, O'Donnell CP** 2007 Intermittent hypoxia causes insulin resistance in lean mice independent of autonomic activity. Am J Respir Crit Care Med 175:851-857

253. **Lolmede K, Durand de Saint Front V, Galitzky J, Lafontan M, Bouloumie A** 2003 Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. Int J Obes Relat Metab Disord 27:1187-1195

254. **Semenza GL** 2002 HIF-1 and tumor progression: pathophysiology and therapeutics. Trends Mol Med 8:S62-67

255. **Ye J** 2009 Emerging role of adipose tissue hypoxia in obesity and insulin resistance. Int J Obes (Lond) 33:54-66

256. **Benizri E, Ginouves A, Berra E** 2008 The magic of the hypoxia-signaling cascade. Cell Mol Life Sci 65:1133-1149

257. **Yun Z, Lin Q, Giaccia AJ** 2005 Adaptive myogenesis under hypoxia. Mol Cell Biol 25:3040-3055

258. **Ornoy A, Livshitz A, Ergaz Z, Stodgell CJ, Miller RK** 2011 Hyperglycemia, hypoxia and their combination exert oxidative stress and changes in antioxidant gene expression: studies on cultured rat embryos. Birth Defects Res B Dev Reprod Toxicol 92:231-239

259. **Semenza GL** 2000 HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 88:1474-1480

260. **Lee KA, Lynd JD, O'Reilly S, Kiupel M, McCormick JJ, LaPres JJ** 2008 The biphasic role of the hypoxia-inducible factor prolyl-4-hydroxylase, PHD2, in modulating tumor-forming potential. Mol Cancer Res 6:829-842

261. **Berra E, Benizri E, Ginouves A, Volmat V, Roux D, Pouyssegur J** 2003 HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J 22:4082-4090

262. **Balligand JL, Feron O, Dessy C** 2009 eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues. Physiol Rev 89:481-534

263. **Semenza G** 2002 Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol 64:993-998

264. **Huang LE, Gu J, Schau M, Bunn HF** 1998 Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A 95:7987-7992

265. **Zhou G, Golden T, Aragon IV, Honkanen RE** 2004 Ser/Thr protein phosphatase 5 inactivates hypoxia-induced activation of an apoptosis signal-regulating kinase 1/MKK-4/JNK signaling cascade. J Biol Chem 279:46595-46605

266. **Seta KA, Millhorn DE** 2004 Functional genomics approach to hypoxia signaling. J Appl Physiol 96:765-773

267. **Cooper MS, Bujalska I, Rabbitt E, Walker EA, Bland R, Sheppard MC, Hewison M, Stewart PM** 2001 Modulation of 11beta-hydroxysteroid dehydrogenase isozymes by proinflammatory cytokines in osteoblasts: an autocrine switch from glucocorticoid inactivation to activation. J Bone Miner Res 16:1037-1044

268. **Alfaidy N, Gupta S, DeMarco C, Caniggia I, Challis JR** 2002 Oxygen regulation of placental 11 beta-hydroxysteroid dehydrogenase 2: physiological and pathological implications. J Clin Endocrinol Metab 87:4797-4805

269. **Semenza GL, Wang GL** 1992 A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 12:5447-5454

270. **Constancia M, Angiolini E, Sandovici I, Smith P, Smith R, Kelsey G, Dean W, Ferguson-Smith A, Sibley CP, Reik W, Fowden A** 2005 Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems. Proc Natl Acad Sci U S A 102:19219-19224

271. **Livingstone DE, Jones GC, Smith K, Jamieson PM, Andrew R, Kenyon CJ, Walker BR** 2000 Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. Endocrinology 141:560-563

272. **Seckl JR, Cleasby M, Nyirenda MJ** 2000 Glucocorticoids, 11beta-hydroxysteroid dehydrogenase, and fetal programming. Kidney Int 57:1412-1417

273. **Speirs HJ, Seckl JR, Brown RW** 2004 Ontogeny of glucocorticoid receptor and 11beta-hydroxysteroid dehydrogenase type-1 gene expression identifies potential critical periods of glucocorticoid susceptibility during development. J Endocrinol 181:105-116

274. **Sun K, Yang K, Challis JR** 1997 Differential expression of 11 beta-hydroxysteroid dehydrogenase types 1 and 2 in human placenta and fetal membranes. J Clin Endocrinol Metab 82:300-305

275. **Gunberg DL** 1957 Some effects of exogenous hydrocortisone on pregnancy in the rat. Anat Rec 129:133-153

276. **Osinski PA** 1960 Steroid 11beta-ol dehydrogenase in human placenta. Nature 187:777

277. **Krozowski Z** 1992 11 beta-hydroxysteroid dehydrogenase and the short-chain alcohol dehydrogenase (SCAD) superfamily. Mol Cell Endocrinol 84:C25-31

278. **Tannin GM, Agarwal AK, Monder C, New MI, White PC** 1991 The human gene for 11 beta-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization. J Biol Chem 266:16653-16658

279. **Agarwal AK, Rogerson FM, Mune T, White PC** 1995 Gene structure and chromosomal localization of the human HSD11K gene encoding the kidney (type 2) isozyme of 11 beta-hydroxysteroid dehydrogenase. Genomics 29:195-199

280. **Bujalska I, Shimojo M, Howie A, Stewart PM** 1997 Human 11 beta-hydroxysteroid dehydrogenase: studies on the stably transfected isoforms and localization of the type 2 isozyme within renal tissue. Steroids 62:77-82

281. **White PC, Mune T, Agarwal AK** 1997 11 beta-Hydroxysteroid dehydrogenase and the syndrome of apparent mineralocorticoid excess. Endocr Rev 18:135-156

282. **Mericq V, Medina P, Kakarieka E, Marquez L, Johnson MC, Iniguez G** 2009 Differences in expression and activity of 11beta-hydroxysteroid dehydrogenase type 1 and 2 in human placentas of term pregnancies according to birth weight and gender. Eur J Endocrinol 161:419-425

283. **Seckl JR, Meaney MJ** 2004 Glucocorticoid programming. Ann N Y Acad Sci 1032:63-84

284. **Fowden AL, Li J, Forhead AJ** 1998 Glucocorticoids and the preparation for life after birth: are there long-term consequences of the life insurance? Proc Nutr Soc 57:113-122

285. **Reinisch JM, Simon NG, Karow WG, Gandelman R** 1978 Prenatal exposure to prednisone in humans and animals retards intrauterine growth. Science 202:436-438

286. **Liggins GC** 1976 Adrenocortical-related maturational events in the fetus. Am J Obstet Gynecol 126:931-941

287. **Liggins GC** 1994 The role of cortisol in preparing the fetus for birth. Reprod Fertil Dev 6:141-150

288. **Campbell AL, Murphy BE** 1977 The maternal-fetal cortisol gradient during pregnancy and at delivery. J Clin Endocrinol Metab 45:435-440

289. **Benediktsson R, Calder AA, Edwards CR, Seckl JR** 1997 Placental 11 beta-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. Clin Endocrinol (Oxf) 46:161-166

290. **Seckl JR** 2004 Prenatal glucocorticoids and long-term programming. Eur J Endocrinol 151 Suppl 3:U49-62

291. **Brown RW, Chapman KE, Kotelevtsev Y, Yau JL, Lindsay RS, Brett L, Leckie C, Murad P, Lyons V, Mullins JJ, Edwards CR, Seckl JR** 1996 Cloning and production of antisera to human placental 11 beta-hydroxysteroid dehydrogenase type 2. Biochem J 313 (Pt 3):1007-1017

292. **Murphy VE, Clifton VL** 2003 Alterations in human placental 11beta-hydroxysteroid dehydrogenase type 1 and 2 with gestational age and labour. Placenta 24:739-744

293. **Homan A, Guan H, Hardy DB, Gratton RJ, Yang K** 2006 Hypoxia blocks 11beta-hydroxysteroid dehydrogenase type 2 induction in human trophoblast cells during differentiation by a time-dependent mechanism that involves both translation and transcription. Placenta 27:832-840

294. **Shams M, Kilby MD, Somerset DA, Howie AJ, Gupta A, Wood PJ, Afnan M, Stewart PM** 1998 11Beta-hydroxysteroid dehydrogenase type 2 in human pregnancy and reduced expression in intrauterine growth restriction. Hum Reprod 13:799-804

295. **Sugden MC, Langdown ML, Munns MJ, Holness MJ** 2001 Maternal glucocorticoid treatment modulates placental leptin and leptin receptor expression and materno-fetal leptin physiology during late pregnancy, and elicits hypertension associated with hyperleptinaemia in the early-growth-retarded adult offspring. Eur J Endocrinol 145:529-539

296. **Edwards LJ, Coulter CL, Symonds ME, McMillen IC** 2001 Prenatal undernutrition, glucocorticoids and the programming of adult hypertension. Clin Exp Pharmacol Physiol 28:938-941

297. **Reynolds RM** 2013 Glucocorticoid excess and the developmental origins of disease: two decades of testing the hypothesis--2012 Curt Richter Award Winner. Psychoneuroendocrinology 38:1-11

298. **Clifton VL, Murphy VE** 2004 Maternal asthma as a model for examining fetal sex-specific effects on maternal physiology and placental mechanisms that regulate human fetal growth. Placenta 25 Suppl A:S45-52

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.

Gene	Accession #	Forward Sequence	Reverse Sequence
Actb	NM_007393	5'-agatgacccagatcatgtttgaga	5'-cacagcctggatggctacgt
Angpt1	NM_009640	5'-ggctgcaaaaacttgagaattacatt	5'-tggccgtgtggtttttgaac
Arnt	NM_009709	5'-gcatgggctcacgaaggt	5'-aaacagggtccacggagcta
Hif1a	NM_010431	5'-gcgggcaccgattcg	5'-ttcagaactcatcttttcttctcgtt
Hsd11b1	NM_008288	5'-ggcactatggaagacatgacattt	5'-actccgcccatgagcttc
Hsd11b2	NM_008289	5'-gcactgcttatggacacattcg	5'-cattagtcactgcatctgtcttgaag
Pecam1	NM_008816	5'-tgcacagtgatgctgaacaaca	5'-tggatacgccatgcacctt
Snai1	NM_011427.2	5'-gcctgtgcccgaacctt	5'-gccagactcttggtgcttgtg
Snai2	NM_011415.2	5'-gcctgggtgccctgaag	5'-ttgcagacacaaggcaatgtg
Twist1	NM_011658.2	5'-ccggagacctagatgtcattgtt	5'-agttatccagctccagagtctctagac
Vcam1	NM_011693	5'-acaagtctacatctctcccaggaatac	5'-cacagcaccaccctcttgaa
Vegfa_164	NM_031836	5'-gaaaaattcactgtgagccttgttc	5'-cttggcttgtcacatctgcaa
Zeb2	NM_015753	5'-ggtgcaagaggcgcaaac	5'-gcctgcgtccactacgttgt

Table 2.1. Primer Sequences for QPCR Analysis

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 (Hitschfel 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, Bethseda, 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 *Mc1r* and *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).
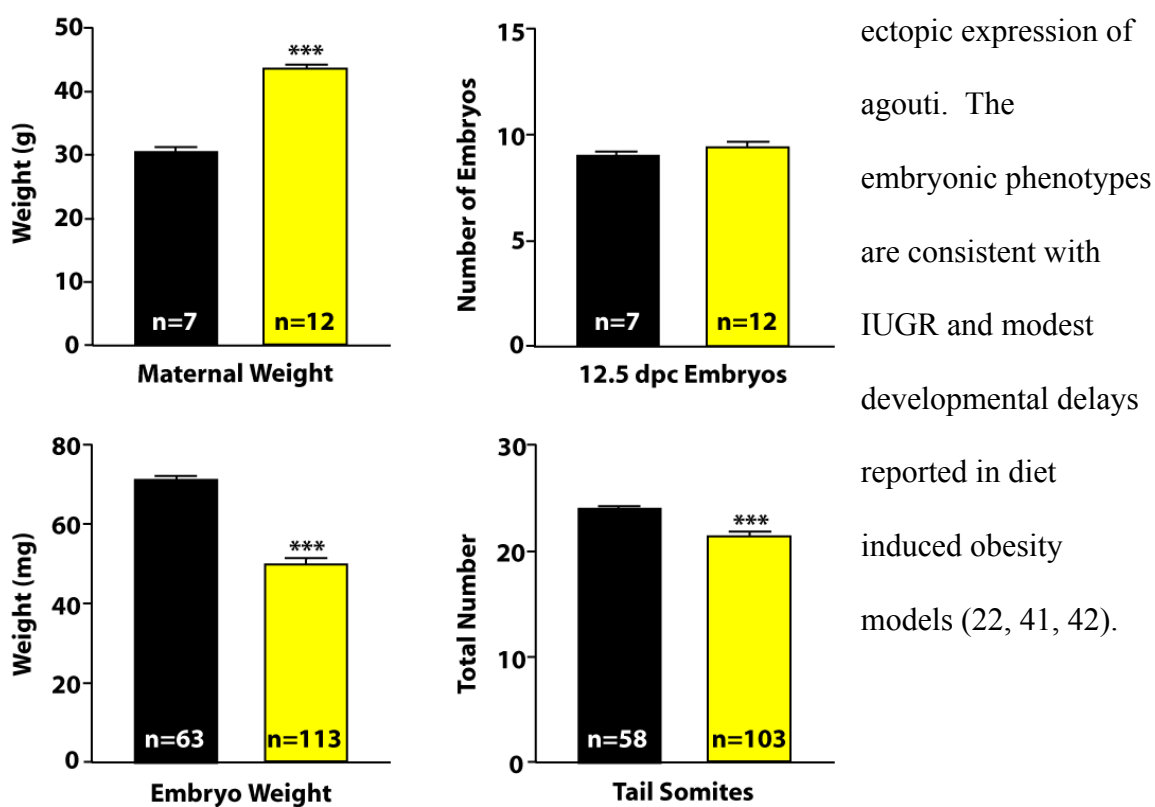
Figure 2.1. LY Mice Exhibit an Obese Phenotype and Altered Growth of their Embryos.
Maternal body weight (A) and the number of embryos at 12.5 dpc (B) were determined for B6 (black bar, n=7) and LY (yellow bar, n=12) dams. The wet weights (C) were recorded for embryos (B6-derived, black bar, n=63; LY-derived, 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).
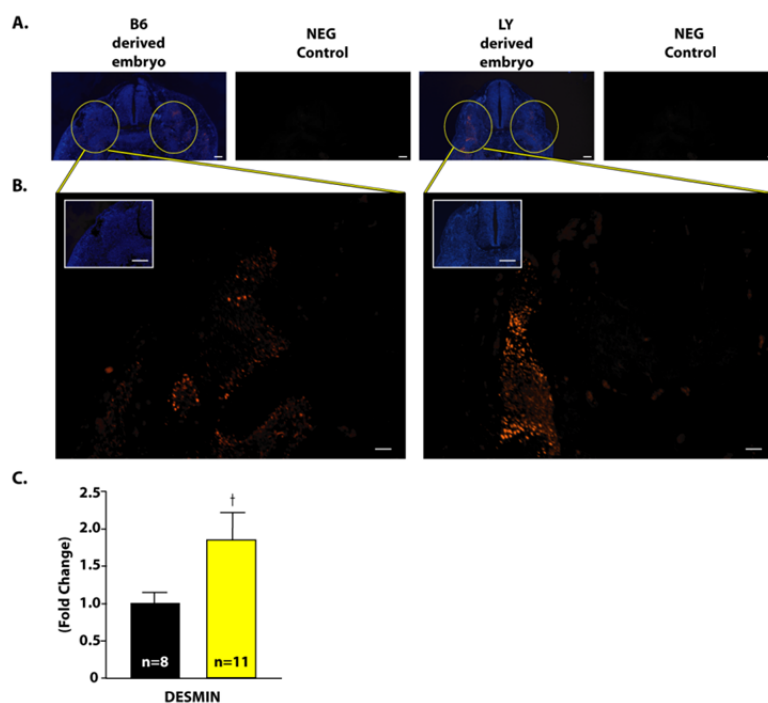
Figure 2.2. DES Expression is Increased in the Somite in LY-Derived Embryos. (A, B) Immunofluorescent detection of DES was carried out using transverse sections of 12.5 dpc embryos derived from B6 and LY dams. Representative images (A=40X, B=100X) are shown for DES (orange) and DAPI (blue) expression. 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 ($P < 0.09$, †). 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 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).
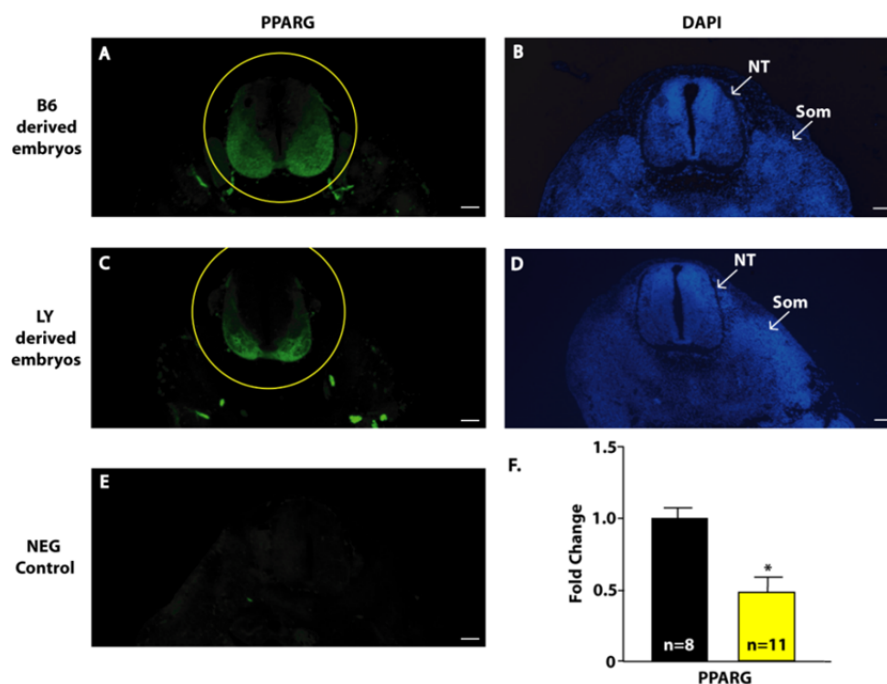
Figure 2.3. PPARG Expression is Decreased in the Neural Tube of LY-Derived 12.5 dpc Embryos. Representative embryos (40X) from B6 and LY dams were stained for immunofluorescent detection of PPARG (green) and DAPI (blue). A representative section processed without primary antibody served as a negative 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.
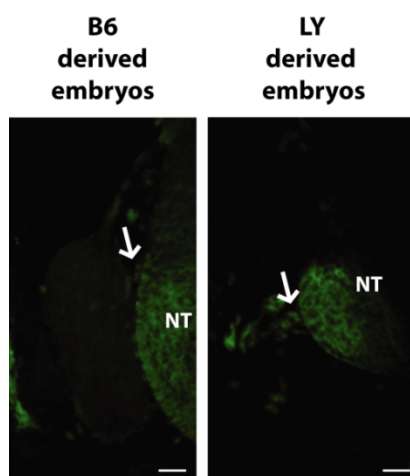
Figure 2.4. PPARG is Expressed in Cells Adjacent to the Neural Tube in 12.5 dpc Embryos. Representative images (100X) of B6 (n=8) and LY-derived embryos (n=11) from Figure 3 showing expression of PPARG (green). Arrows indicate PPARG-positive cells adjacent to the neural tube. Scale bars=50 μm. NT=neural 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).
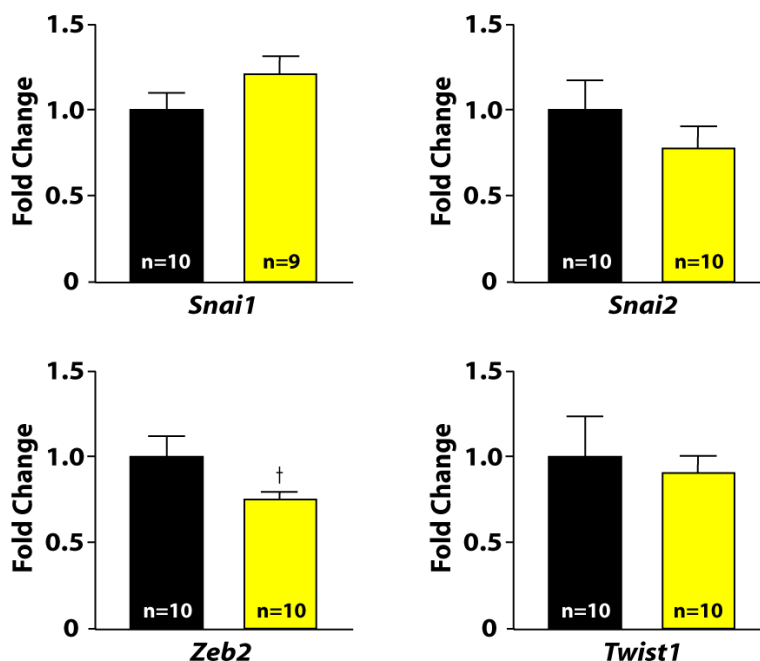
Figure 2.5. Abundance of EMT mRNAs in Whole Embryos from B6 and LY Mothers. QPCR analysis of *Snai1*, *Snai2*, *Twist1* and *Zeb2* was carried out. The normalized, relative abundance of each gene from embryos derived in LY dams (yellow bar, n=10) was compared to the mean, normalized relative abundance 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-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
(*Hsd11b2*) and active (*Hsd11b1*) isoforms. There was no difference in *Hsd11b1* mRNA
abundance in placentas derived from B6 and LY dams. However, *Hsd11b2* mRNA
levels were decreased 1.59-fold ($P < 0.04$) (Figure 2.6D).
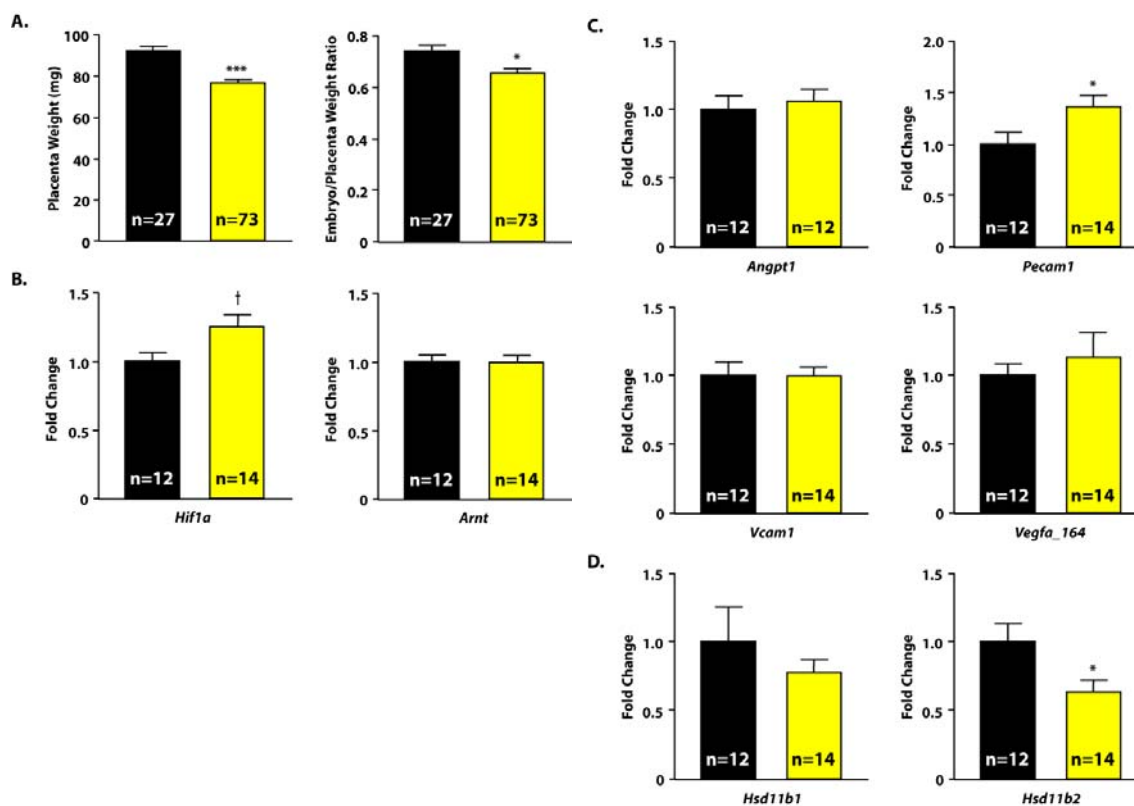
Figure 2.6. Placental Morphology and Function is Altered in LY Dams. Wet weights (A) were recorded for B6-derived (black bar, n=27) and LY-derived (yellow bar, n=73) placenta as well as the ratio of embryo to placenta weight was determined. (C, D) QPCR analysis of *Hif1a*, *Arnt*, *Angpt1*, *Pecam1*, *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

B6 from B6 dam ☐ **B6 from LY dam** ☐ **LY from LY dam**
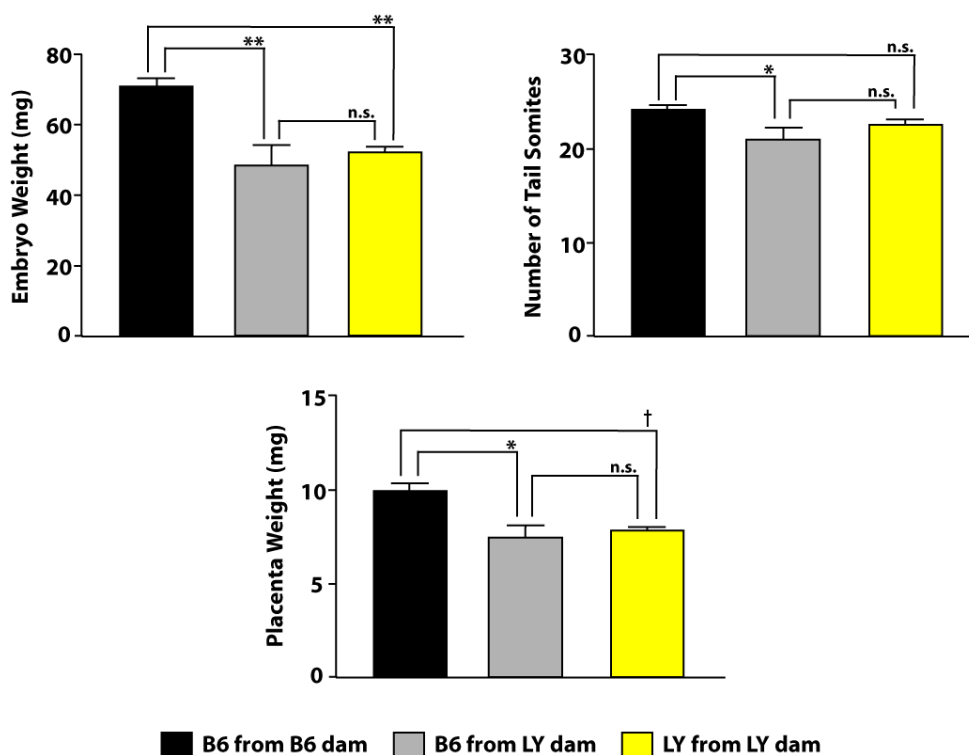
Supplemental Figure 2.7. Embryos from LY Mice Do Not Differ in Growth Regardless of Agouti Expression. Embryos and their corresponding placenta were isolated from B6 dams (B6 embryo, black bar, n=10) or LY dams (B6 embryo, grey bar, n=7; LY embryo, yellow bar, n=3). Wet weights for each embryo and placenta 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.

LITERATURE CITED

1. **Trayhurn P, Bing C, Wood IS** 2006 Adipose tissue and adipokines--energy regulation from the human perspective. J Nutr 136:1935S-1939S
2. **Osmond C, Barker DJ** 2000 Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women. Environ Health Perspect 108 Suppl 3:545-553
3. **Siega-Riz AM, Laraia B** 2006 The implications of maternal overweight and obesity on the course of pregnancy and birth outcomes. Matern Child Health J 10:S153-156
4. **Nelson SM, Matthews P, Poston L** 2010 Maternal metabolism and obesity: modifiable determinants of pregnancy outcome. Hum Reprod Update 16:255-275
5. **Martin-Gronert MS, Ozanne SE** 2010 Mechanisms linking suboptimal early nutrition and increased risk of type 2 diabetes and obesity. J Nutr 140:662-666
6. **Sewell MF, Huston-Presley L, Super DM, Catalano P** 2006 Increased neonatal fat mass, not lean body mass, is associated with maternal obesity. Am J Obstet Gynecol 195:1100-1103
7. **Denison FC, Roberts KA, Barr SM, Norman JE** 2010 Obesity, pregnancy, inflammation, and vascular function. Reproduction 140:373-385
8. **Huang Y, Yan X, Zhu MJ, McCormick RJ, Ford SP, Nathanielsz PW, Du M** 2010 Enhanced transforming growth factor-beta signaling and fibrogenesis in ovine fetal skeletal muscle of obese dams at late gestation. Am J Physiol Endocrinol Metab 298:E1254-1260
9. **Yan X, Zhu MJ, Xu W, Tong JF, Ford SP, Nathanielsz PW, Du M** 2010 Up-regulation of Toll-like receptor 4/nuclear factor-kappaB signaling is associated with enhanced adipogenesis and insulin resistance in fetal skeletal muscle of obese sheep at late gestation. Endocrinology 151:380-387
10. **Huang Y, Zhao JX, Yan X, Zhu MJ, Long NM, McCormick RJ, Ford SP, Nathanielsz PW, Du M** 2012 Maternal obesity enhances collagen accumulation and cross-linking in skeletal muscle of ovine offspring. PLoS One 7:e31691
11. **Tong JF, Yan X, Zhu MJ, Ford SP, Nathanielsz PW, Du M** 2009 Maternal obesity downregulates myogenesis and beta-catenin signaling in fetal skeletal muscle. American journal of physiology 296:E917-924
12. **Yan X, Huang Y, Zhao JX, Long NM, Uthlaut AB, Zhu MJ, Ford SP, Nathanielsz PW, Du M** 2011 Maternal obesity-impaired insulin signaling in sheep and induced lipid accumulation and fibrosis in skeletal muscle of offspring. Biology of reproduction 85:172-178
13. **Du M, Yan X, Tong JF, Zhao J, Zhu MJ** 2010 Maternal obesity, inflammation, and fetal skeletal muscle development. Biology of reproduction 82:4-12
14. **Kassar-Duchossoy L, Giacone E, Gayraud-Morel B, Jory A, Gomes D, Tajbakhsh S** 2005 Pax3/Pax7 mark a novel population of primitive myogenic cells during development. Genes Dev 19:1426-1431
15. **Maroto M, Reshef R, Munsterberg AE, Koester S, Goulding M, Lassar AB** 1997 Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell 89:139-148

16. **Sato T, Rocancourt D, Marques L, Thorsteinsdottir S, Buckingham M** 2010 A Pax3/Dmrt2/Myf5 regulatory cascade functions at the onset of myogenesis. PLoS Genet 6:e1000897

17. **Mok GF, Sweetman D** 2011 Many routes to the same destination: lessons from skeletal muscle development. Reproduction 141:301-312

18. **Francis-West PH, Antoni L, Anakwe K** 2003 Regulation of myogenic differentiation in the developing limb bud. J Anat 202:69-81

19. **Pownall ME, Gustafsson MK, Emerson CP, Jr.** 2002 Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu Rev Cell Dev Biol 18:747-783

20. **Munsterberg AE, Lassar AB** 1995 Combinatorial signals from the neural tube, floor plate and notochord induce myogenic bHLH gene expression in the somite. Development 121:651-660

21. **Van Ho AT, Hayashi S, Brohl D, Aurade F, Rattenbach R, Relaix F** 2011 Neural crest cell lineage restricts skeletal muscle progenitor cell differentiation through Neuregulin1-ErbB3 signaling. Developmental cell 21:273-287

22. **Kappen C, Kruger C, MacGowan J, Salbaum JM** 2012 Maternal diet modulates placenta growth and gene expression in a mouse model of diabetic pregnancy. PloS one 7:e38445

23. **Hayes EK, Lechowicz A, Petrik JJ, Storozhuk Y, Paez-Parent S, Dai Q, Samjoo IA, Mansell M, Gruslin A, Holloway AC, Raha S** 2012 Adverse fetal and neonatal outcomes associated with a life-long high fat diet: role of altered development of the placental vasculature. PLoS One 7:e33370

24. **Khankin EV, Royle C, Karumanchi SA** 2010 Placental vasculature in health and disease. Semin Thromb Hemost 36:309-320

25. **Jansson T, Powell TL** 2007 Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. Clin Sci (Lond) 113:1-13

26. **Krebs C, Macara LM, Leiser R, Bowman AW, Greer IA, Kingdom JC** 1996 Intrauterine growth restriction with absent end-diastolic flow velocity in the umbilical artery is associated with maldevelopment of the placental terminal villous tree. Am J Obstet Gynecol 175:1534-1542

27. **Miyata T, Takizawa S, van Ypersele de Strihou C** 2011 Hypoxia. 1. Intracellular sensors for oxygen and oxidative stress: novel therapeutic targets. Am J Physiol Cell Physiol 300:C226-231

28. **Murphy VE, Smith R, Giles WB, Clifton VL** 2006 Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. Endocr Rev 27:141-169

29. **Myatt L** 2006 Placental adaptive responses and fetal programming. J Physiol 572:25-30

30. **Benediktsson R, Calder AA, Edwards CR, Seckl JR** 1997 Placental 11 beta-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. Clin Endocrinol (Oxf) 46:161-166

31. **Seckl JR, Cleasby M, Nyirenda MJ** 2000 Glucocorticoids, 11beta-hydroxysteroid dehydrogenase, and fetal programming. Kidney Int 57:1412-1417

32. **Klebig ML, Wilkinson JE, Geisler JG, Woychik RP** 1995 Ectopic expression of the agouti gene in transgenic mice causes obesity, features of type II diabetes, and yellow fur. Proc Natl Acad Sci U S A 92:4728-4732

33. **Michaud EJ, Bultman SJ, Klebig ML, van Vugt MJ, Stubbs LJ, Russell LB, Woychik RP** 1994 A molecular model for the genetic and phenotypic characteristics of the mouse lethal yellow (Ay) mutation. Proc Natl Acad Sci U S A 91:2562-2566

34. **Duhl DM, Stevens ME, Vrieling H, Saxon PJ, Miller MW, Epstein CJ, Barsh GS** 1994 Pleiotropic effects of the mouse lethal yellow (Ay) mutation explained by deletion of a maternally expressed gene and the simultaneous production of agouti fusion RNAs. Development 120:1695-1708

35. **Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO, et al.** 1994 Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature 371:799-802

36. **Yang Z, Norwood KA, Smith JE, Kerl JG, Wood JR** 2012 Genes involved in the immediate early response and epithelial-mesenchymal transition are regulated by adipocytokines in the female reproductive tract. Mol Reprod Dev 79:128-137

37. **Mack EM, Smith JE, Kurz SG, Wood JR** 2012 Cyclic AMP dependent regulation of ovulatory response genes is amplified by IGF1 due to synergistic effects on Akt phosphorylation and NFkappaB transcription factors. Reproduction

38. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F** 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.0031-RESEARCH0034.0011

39. **McFee RM, Artac RA, McFee RM, Clopton DT, Smith RA, Rozell TG, Cupp AS** 2009 Inhibition of vascular endothelial growth factor receptor signal transduction blocks follicle progression but does not necessarily disrupt vascular development in perinatal rat ovaries. Biology of reproduction 81:966-977

40. **Bott RC, Clopton DT, Fuller AM, McFee RM, Lu N, Cupp AS** 2010 KDR-LacZ-expressing cells are involved in ovarian and testis-specific vascular development, suggesting a role for VEGFA in the regulation of this vasculature. Cell Tissue Res 342:117-130

41. **Luzzo KM, Wang Q, Purcell SH, Chi M, Jimenez PT, Grindler N, Schedl T, Moley KH** 2012 High fat diet induced developmental defects in the mouse: oocyte meiotic aneuploidy and fetal growth retardation/brain defects. PLoS One 7:e49217

42. **Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, Moley KH** 2010 Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. Endocrinology 151:4039-4046

43. **Davis LA, Zur Nieden NI** 2008 Mesodermal fate decisions of a stem cell: the Wnt switch. Cell Mol Life Sci 65:2658-2674

44. **Chen L, Necela BM, Su W, Yanagisawa M, Anastasiadis PZ, Fields AP, Thompson EA** 2006 Peroxisome proliferator-activated receptor gamma promotes epithelial to mesenchymal transformation by Rho GTPase-dependent activation of ERK1/2. The Journal of biological chemistry 281:24575-24587

45. **Reka AK, Kurapati H, Narala VR, Bommer G, Chen J, Standiford TJ, Keshamouni VG** 2010 Peroxisome proliferator-activated receptor-gamma activation inhibits tumor metastasis by antagonizing Smad3-mediated epithelial-mesenchymal transition. Mol Cancer Ther 9:3221-3232

46. **Zhu MJ, Han B, Tong J, Ma C, Kimzey JM, Underwood KR, Xiao Y, Hess BW, Ford SP, Nathanielsz PW, Du M** 2008 AMP-activated protein kinase signalling pathways are down regulated and skeletal muscle development impaired in fetuses of obese, over-nourished sheep. The Journal of physiology 586:2651-2664

47. **Huang Y, Yan X, Zhao JX, Zhu MJ, McCormick RJ, Ford SP, Nathanielsz PW, Ren J, Du M** 2010 Maternal obesity induces fibrosis in fetal myocardium of sheep. American journal of physiology 299:E968-975

48. **Bayol SA, Macharia R, Farrington SJ, Simbi BH, Stickland NC** 2009 Evidence that a maternal "junk food" diet during pregnancy and lactation can reduce muscle force in offspring. Eur J Nutr 48:62-65

49. **Simar D, Chen H, Lambert K, Mercier J, Morris MJ** 2012 Interaction between maternal obesity and post-natal over-nutrition on skeletal muscle metabolism. Nutr Metab Cardiovasc Dis 22:269-276

50. **Sambasivan R, Tajbakhsh S** 2007 Skeletal muscle stem cell birth and properties. Semin Cell Dev Biol 18:870-882

51. **Rose O, Rohwedel J, Reinhardt S, Bachmann M, Cramer M, Rotter M, Wobus A, Starzinski-Powitz A** 1994 Expression of M-cadherin protein in myogenic cells during prenatal mouse development and differentiation of embryonic stem cells in culture. Dev Dyn 201:245-259

52. **Creuzet S, Lescaudron L, Li Z, Fontaine-Perus J** 1998 MyoD, myogenin, and desmin-nls-lacZ transgene emphasize the distinct patterns of satellite cell activation in growth and regeneration. Exp Cell Res 243:241-253

53. **Capetanaki Y, Milner DJ, Weitzer G** 1997 Desmin in muscle formation and maintenance: knockouts and consequences. Cell Struct Funct 22:103-116

54. **Li H, Choudhary SK, Milner DJ, Munir MI, Kuisk IR, Capetanaki Y** 1994 Inhibition of desmin expression blocks myoblast fusion and interferes with the myogenic regulators MyoD and myogenin. The Journal of cell biology 124:827-841

55. **Cawthorn WP, Scheller EL, Macdougald OA** 2012 Adipose tissue stem cells meet preadipocyte commitment: going back to the future. J Lipid Res 53:227-246

56. **Michalik L, Desvergne B, Dreyer C, Gavillet M, Laurini RN, Wahli W** 2002 PPAR expression and function during vertebrate development. Int J Dev Biol 46:105-114

57. **Wada K, Nakajima A, Katayama K, Kudo C, Shibuya A, Kubota N, Terauchi Y, Tachibana M, Miyoshi H, Kamisaki Y, Mayumi T, Kadowaki T, Blumberg RS** 2006 Peroxisome proliferator-activated receptor gamma-mediated regulation of neural stem cell proliferation and differentiation. J Biol Chem 281:12673-12681

58. **Gesta S, Tseng YH, Kahn CR** 2007 Developmental origin of fat: tracking obesity to its source. Cell 131:242-256

59.	**Tajbakhsh S** 2009 Skeletal muscle stem cells in developmental versus regenerative myogenesis. J Intern Med 266:372-389

60.	**Kang JS, Krauss RS** 2010 Muscle stem cells in developmental and regenerative myogenesis. Current opinion in clinical nutrition and metabolic care 13:243-248

61.	**Billon N, Iannarelli P, Monteiro MC, Glavieux-Pardanaud C, Richardson WD, Kessaris N, Dani C, Dupin E** 2007 The generation of adipocytes by the neural crest. Development 134:2283-2292

62.	**Yu CK, Teoh TG, Robinson S** 2006 Obesity in pregnancy. Bjog 113:1117-1125

63.	**Ramachenderan J, Bradford J, McLean M** 2008 Maternal obesity and pregnancy complications: a review. Aust N Z J Obstet Gynaecol 48:228-235

64.	**Cnattingius S, Bergstrom R, Lipworth L, Kramer MS** 1998 Prepregnancy weight and the risk of adverse pregnancy outcomes. N Engl J Med 338:147-152

65.	**Jones HN, Woollett LA, Barbour N, Prasad PD, Powell TL, Jansson T** 2009 High-fat diet before and during pregnancy causes marked up-regulation of placental nutrient transport and fetal overgrowth in C57/BL6 mice. FASEB J 23:271-278

66.	**Roberts KA, Riley SC, Reynolds RM, Barr S, Evans M, Statham A, Hor K, Jabbour HN, Norman JE, Denison FC** 2011 Placental structure and inflammation in pregnancies associated with obesity. Placenta 32:247-254

67.	**Challier JC, Basu S, Bintein T, Minium J, Hotmire K, Catalano PM, Hauguel-de Mouzon S** 2008 Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta. Placenta 29:274-281

68.	**Gokulakrishnan G, Estrada IJ, Sosa HA, Fiorotto ML** 2012 In utero glucocorticoid exposure reduces fetal skeletal muscle mass in rats independent of effects on maternal nutrition. Am J Physiol Regul Integr Comp Physiol 302:R1143-1152

69.	**Nesan D, Kamkar M, Burrows J, Scott IC, Marsden M, Vijayan MM** 2012 Glucocorticoid receptor signaling is essential for mesoderm formation and muscle development in zebrafish. Endocrinology 153:1288-1300

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 hydroxlyated 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 *Hsd11b2* is regulated by oxygen (12, 36) and Mericq et al. (37) showed that *Hsd11b2* enzyme activity in the placenta to be seven- to eightfold higher compared with the activity of *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

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.

Gene	Accession #	Forward Sequence	Reverse Sequence
Actb	NM_007393	5'-agatgacccagatcatgtttgaga	5'-cacagcctggatggctacgt
Arnt	NM_009709	5'-gcatgggctcacgaaggt	5'-aaacagggtccacggagcta
Gpx1	NM_008160	5'-cgacatgaacgatctgcagaag	5'-attctcctggtgtccgaactga
Hif1a	NM_010431	5'-gcgggcaccgattcg	5'-ttcagaactcatcttttcttctcgtt
Hsd11b1	NM_008288	5'-ggcactatggaagacatgacattt	5'-actccgcccatgagctttc
Hsd11b2	NM_008289	5'-gcactgcttatggacacattcg	5'-cattagtcactgcatctgtcttgaag
Pecam1	NM_008816	5'-tgcacagtgatgctgaacaaca	5'-tggatacgccatgcacctt
Sod1	NM_011434	5'-gtgcagggaaccatccactt	5'-gtcctgacaacacaactggttca
Sod2	NM_013671	5'-gctctggccaagggagatg	5'-tgattaatatgtcccccaccatt
Vcam1	NM_011693	5'-acaagtctacatctctcccaggaatac	5'-cacagcaccaccctcttgaa
Vegfa_164	NM_031836	5'-gaaaaattcactgtgagccttgttc	5'-cttggcttgtcacatctgcaa
Vegfa_165b	NM_031836	5'-cagaaaatcactgtgagccttgtt	5'-ggtgagaggtctgcaagtacgtt

Table 3.1. Primer sequences for QPCR analysis.

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_2O_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).
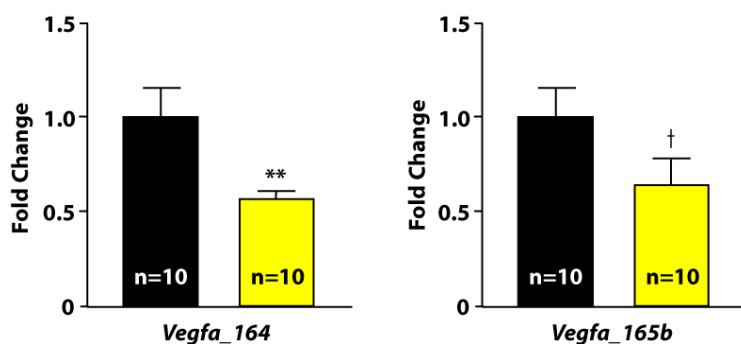
Figure 3.1. Vasculature Markers in Whole Embryos from LY Dams are Decreased. QPCR
analysis of the vasculature markers *Vegfa_164* and *Vegfa_165b* was carried out. The normalized,
relative abundance of each gene from embryos from LY dams (yellow bars, n=10) was compared
to the mean, normalized relative 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 ($P < 0.1$, † 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-

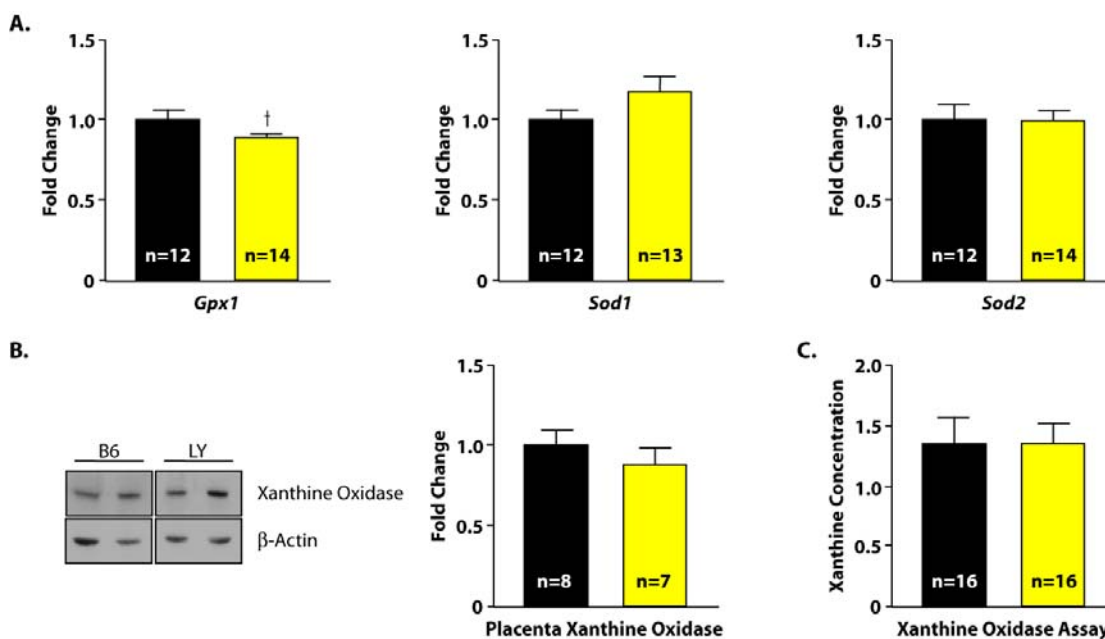fold, $P < 0.09$) in placenta from LY mothers (Figure 3.2A). Next, we looked at the

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 ($P < 0.1$, †).

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

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).
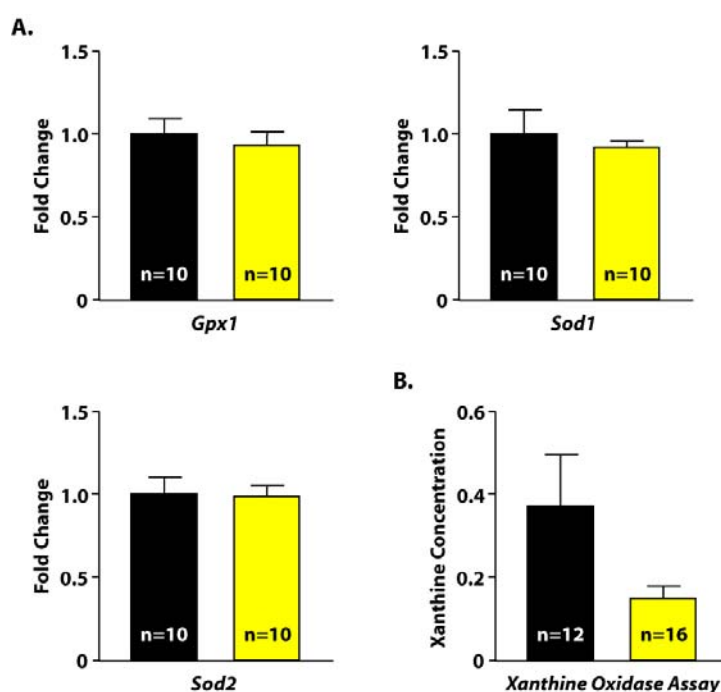
Figure 3.3. LY-Derived Embryos Show No Signs of Oxidative Stress. QPCR analysis of the oxidative stress primers (A) *Gpx1*, *Sod1* and *Sod2* was carried out. The normalized, relative abundance of each gene from embryos from LY dams (yellow bars, n=10) was compared to the mean, normalized relative abundance 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 promotor 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 H_2O_2 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 glucorticoids 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.

LITERATURE CITED

1. 2011 Prevalence and Trends Data - Nationalwide 2011 Overweight and Obesity (BMI). In: CDC, Office of Surveillance, Epidemiology, and Labratory Services

2. **Heslehurst N, Ells LJ, Simpson H, Batterham A, Wilkinson J, Summerbell CD** 2007 Trends in maternal obesity incidence rates, demographic predictors, and health inequalities in 36,821 women over a 15-year period. BJOG 114:187-194

3. **O'Reilly JR, Reynolds RM** 2013 The risk of maternal obesity to the long-term health of the offspring. Clin Endocrinol (Oxf) 78:9-16

4. **Fleming TP, Kwong WY, Porter R, Ursell E, Fesenko I, Wilkins A, Miller DJ, Watkins AJ, Eckert JJ** 2004 The embryo and its future. Biology of reproduction 71:1046-1054

5. **Reynolds LP, Redmer DA** 1995 Utero-placental vascular development and placental function. J Anim Sci 73:1839-1851

6. **Reynolds LP, Redmer DA** 2001 Angiogenesis in the placenta. Biol Reprod 64:1033-1040

7. **Funston RN, Larson DM, Vonnahme KA** 2010 Effects of maternal nutrition on conceptus growth and offspring performance: implications for beef cattle production. J Anim Sci 88:E205-215

8. **Caluwaerts S, Vercruysse L, Luyten C, Pijnenborg R** 2005 Endovascular trophoblast invasion and associated structural changes in uterine spiral arteries of the pregnant rat. Placenta 26:574-584

9. **Hayes EK, Lechowicz A, Petrik JJ, Storozhuk Y, Paez-Parent S, Dai Q, Samjoo IA, Mansell M, Gruslin A, Holloway AC, Raha S** 2012 Adverse fetal and neonatal outcomes associated with a life-long high fat diet: role of altered development of the placental vasculature. PLoS One 7:e33370

10. **Rossant J, Cross JC** 2001 Placental development: lessons from mouse mutants. Nat Rev Genet 2:538-548

11. **Reynolds LP, Borowicz PP, Caton JS, Vonnahme KA, Luther JS, Buchanan DS, Hafez SA, Grazul-Bilska AT, Redmer DA** 2010 Uteroplacental vascular development and placental function: an update. Int J Dev Biol 54:355-366

12. **Alfaidy N, Gupta S, DeMarco C, Caniggia I, Challis JR** 2002 Oxygen regulation of placental 11 beta-hydroxysteroid dehydrogenase 2: physiological and pathological implications. J Clin Endocrinol Metab 87:4797-4805

13. **Cowden Dahl KD, Fryer BH, Mack FA, Compernolle V, Maltepe E, Adelman DM, Carmeliet P, Simon MC** 2005 Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. Mol Cell Biol 25:10479-10491

14. **Wang YP, Walsh SW, Guo JD, Zhang JY** 1991 Maternal levels of prostacyclin, thromboxane, vitamin E, and lipid peroxides throughout normal pregnancy. Am J Obstet Gynecol 165:1690-1694

15. **Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S** 2012 The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol 10:49

16. **Denison FC, Roberts KA, Barr SM, Norman JE** 2010 Obesity, pregnancy, inflammation, and vascular function. Reproduction 140:373-385

17. **Oliva K, Barker G, Riley C, Bailey MJ, Permezel M, Rice GE, Lappas M** 2012 The effect of pre-existing maternal obesity on the placental proteome: two-dimensional difference gel electrophoresis coupled with mass spectrometry. J Mol Endocrinol 48:139-149

18. **Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, Furukawa S, Tochino Y, Komuro R, Matsuda M, Shimomura I** 2007 Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. Diabetes 56:901-911

19. **Benizri E, Ginouves A, Berra E** 2008 The magic of the hypoxia-signaling cascade. Cell Mol Life Sci 65:1133-1149

20. **Semenza GL** 2000 HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 88:1474-1480

21. **Balligand JL, Feron O, Dessy C** 2009 eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues. Physiol Rev 89:481-534

22. **Semenza G** 2002 Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol 64:993-998

23. **Semenza GL, Wang GL** 1992 A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 12:5447-5454

24. **Ornoy A, Rand SB, Bischitz N** 2010 Hyperglycemia and hypoxia are interrelated in their teratogenic mechanism: studies on cultured rat embryos. Birth Defects Res B Dev Reprod Toxicol 89:106-115

25. **Hung TH, Skepper JN, Burton GJ** 2001 In vitro ischemia-reperfusion injury in term human placenta as a model for oxidative stress in pathological pregnancies. Am J Pathol 159:1031-1043

26. **Agarwal A, Gupta S, Sharma RK** 2005 Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 3:28

27. **Genestra M** 2007 Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Cell Signal 19:1807-1819

28. **Vincent HK, Taylor AG** 2006 Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. Int J Obes (Lond) 30:400-418

29. **Seckl JR, Meaney MJ** 2004 Glucocorticoid programming. Ann N Y Acad Sci 1032:63-84

30. **Fowden AL, Li J, Forhead AJ** 1998 Glucocorticoids and the preparation for life after birth: are there long-term consequences of the life insurance? Proc Nutr Soc 57:113-122

31. **Reinisch JM, Simon NG, Karow WG, Gandelman R** 1978 Prenatal exposure to prednisone in humans and animals retards intrauterine growth. Science 202:436-438

32. **Liggins GC** 1976 Adrenocortical-related maturational events in the fetus. Am J Obstet Gynecol 126:931-941

33. **Liggins GC** 1994 The role of cortisol in preparing the fetus for birth. Reprod Fertil Dev 6:141-150

34. **Seckl JR, Cleasby M, Nyirenda MJ** 2000 Glucocorticoids, 11beta-hydroxysteroid dehydrogenase, and fetal programming. Kidney Int 57:1412-1417

35. **Bujalska I, Shimojo M, Howie A, Stewart PM** 1997 Human 11 beta-hydroxysteroid dehydrogenase: studies on the stably transfected isoforms and localization of the type 2 isozyme within renal tissue. Steroids 62:77-82

36. **White PC, Mune T, Agarwal AK** 1997 11 beta-Hydroxysteroid dehydrogenase and the syndrome of apparent mineralocorticoid excess. Endocr Rev 18:135-156

37. **Mericq V, Medina P, Kakarieka E, Marquez L, Johnson MC, Iniguez G** 2009 Differences in expression and activity of 11beta-hydroxysteroid dehydrogenase type 1 and 2 in human placentas of term pregnancies according to birth weight and gender. Eur J Endocrinol 161:419-425

38. **O'Reilly JR, Drake AJ, Jones R, Critchley HO, Riley SC, Seckl J, Reynolds R** 2012 Maternal Obesity in Human Pregnancy is Associated with Altered Placental Expression of Key Genes in Fetal Growth During the First Trimester, but not at Term. Endocrine Abstracts 28:P191

39. **Norwood KA, Brandt A, Wood JR** 2013 Maternal Obesity Alters Embryonic Expression of Desmin and PPARγ, Reduces Placental Function and Alters Placental Expression of Stress-Related Genes Endocrinology Submited

40. **Mack EM, Smith JE, Kurz SG, Wood JR** 2012 Cyclic AMP dependent regulation of ovulatory response genes is amplified by IGF1 due to synergistic effects on Akt phosphorylation and NFkappaB transcription factors. Reproduction

41. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F** 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.0031-RESEARCH0034.0011

42. **Wang GL, Semenza GL** 1993 General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci U S A 90:4304-4308

43. **Richter HG, Camm EJ, Modi BN, Naeem F, Cross CM, Cindrova-Davies T, Spasic-Boskovic O, Dunster C, Mudway IS, Kelly FJ, Burton GJ, Poston L, Giussani DA** 2012 Ascorbate prevents placental oxidative stress and enhances birth weight in hypoxic pregnancy in rats. J Physiol 590:1377-1387

44. **Homan A, Guan H, Hardy DB, Gratton RJ, Yang K** 2006 Hypoxia blocks 11beta-hydroxysteroid dehydrogenase type 2 induction in human trophoblast cells during differentiation by a time-dependent mechanism that involves both translation and transcription. Placenta 27:832-840

45. **Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, Moley KH** 2010 Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. Endocrinology 151:4039-4046

46. **Luzzo KM, Wang Q, Purcell SH, Chi M, Jimenez PT, Grindler N, Schedl T, Moley KH** 2012 High fat diet induced developmental defects in the mouse: oocyte meiotic aneuploidy and fetal growth retardation/brain defects. PLoS One 7:e49217

47. **Kappen C, Kruger C, MacGowan J, Salbaum JM** 2012 Maternal diet modulates placenta growth and gene expression in a mouse model of diabetic pregnancy. PloS one 7:e38445

48. **Luo ZC, Xiao L, Nuyt AM** 2010 Mechanisms of developmental programming of the metabolic syndrome and related disorders. World J Diabetes 1:89-98

49. **Carver TD, Quick AA, Teng CC, Pike AW, Fennessey PV, Hay WW, Jr.** 1997 Leucine metabolism in chronically hypoglycemic hypoinsulinemic growth-restricted fetal sheep. Am J Physiol 272:E107-117

50. **Myatt L** 2006 Placental adaptive responses and fetal programming. J Physiol 572:25-30

51. **Ornoy A** 2007 Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. Reprod Toxicol 24:31-41

52. **Barrington JW, Lindsay P, James D, Smith S, Roberts A** 1996 Selenium deficiency and miscarriage: a possible link? Br J Obstet Gynaecol 103:130-132

53. **Lake BG** 1995 Mechanisms of hepatocarcinogenicity of peroxisome-proliferating drugs and chemicals. Annu Rev Pharmacol Toxicol 35:483-507

54. **Jones DP** 2008 Radical-free biology of oxidative stress. Am J Physiol Cell Physiol 295:C849-868

55. **Murphy VE, Smith R, Giles WB, Clifton VL** 2006 Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. Endocr Rev 27:141-169

56. **Gokulakrishnan G, Estrada IJ, Sosa HA, Fiorotto ML** 2012 In utero glucocorticoid exposure reduces fetal skeletal muscle mass in rats independent of effects on maternal nutrition. Am J Physiol Regul Integr Comp Physiol 302:R1143-1152

57. **Nesan D, Kamkar M, Burrows J, Scott IC, Marsden M, Vijayan MM** 2012 Glucocorticoid receptor signaling is essential for mesoderm formation and muscle development in zebrafish. Endocrinology 153:1288-1300

58. **Shams M, Kilby MD, Somerset DA, Howie AJ, Gupta A, Wood PJ, Afnan M, Stewart PM** 1998 11Beta-hydroxysteroid dehydrogenase type 2 in human pregnancy and reduced expression in intrauterine growth restriction. Hum Reprod 13:799-804

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 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.

Gene	Accession #	Forward Sequence	Reverse Sequence
Actb	NM_007393	5'-agatgacccagatcatgtttgaga	5'-cacagcctggatggctacgt
Des	NM_010043	5'-tgagaccatcgcggctaag	5'-gtctgaaaccttggacttgtacca
Igf2	NM_010514	5'-ccgtacttccggacgacttc	5'-cgtcccgcggactgtct
Myf5	NM_008656.5	5'-tgtctggtcccgaaagaacag	5'-cagcacatgcatttgatacatca
Myod1	NM_010866.2	5'-gccggtgtgcattccaa	5'-cactccggaaccccaacag
Myog	NM_031189.2	5'-cccatggtgcccagtgaa	5'-gcagattgtgggcgtctgta
Pax7	NM_011039.2	5'-ggccaaactgctgttgattacc	5'-cggagtgttccccaagctt
Pparg	NM_011146	5'-cacaagagctgacccaatggt	5'-gatcgcactttggtattcttgga
Slc2a4	NM_009204	5'-cagcctacgccaccatagga	5'-ctaagagcaccgagaccaacgt

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.
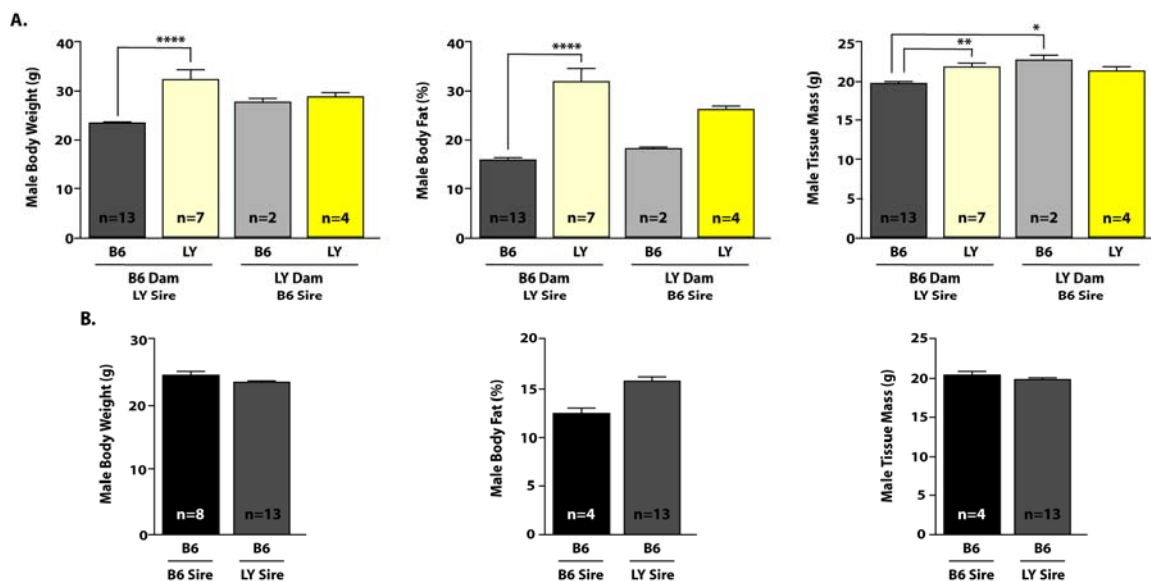
Figure A.1. Male LY Offspring from B6 Dams Display Increased Body, Fat and Total Lean Tissue Mass. (A) Male offspring from B6 dams and LY sires (B6, dark grey bar, n=13; LY, light yellow bar, n=7) and from LY dams and B6 sires (B6, light grey bar, n=2; LY, dark yellow bar, n=4) were collected at 12 weeks 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).
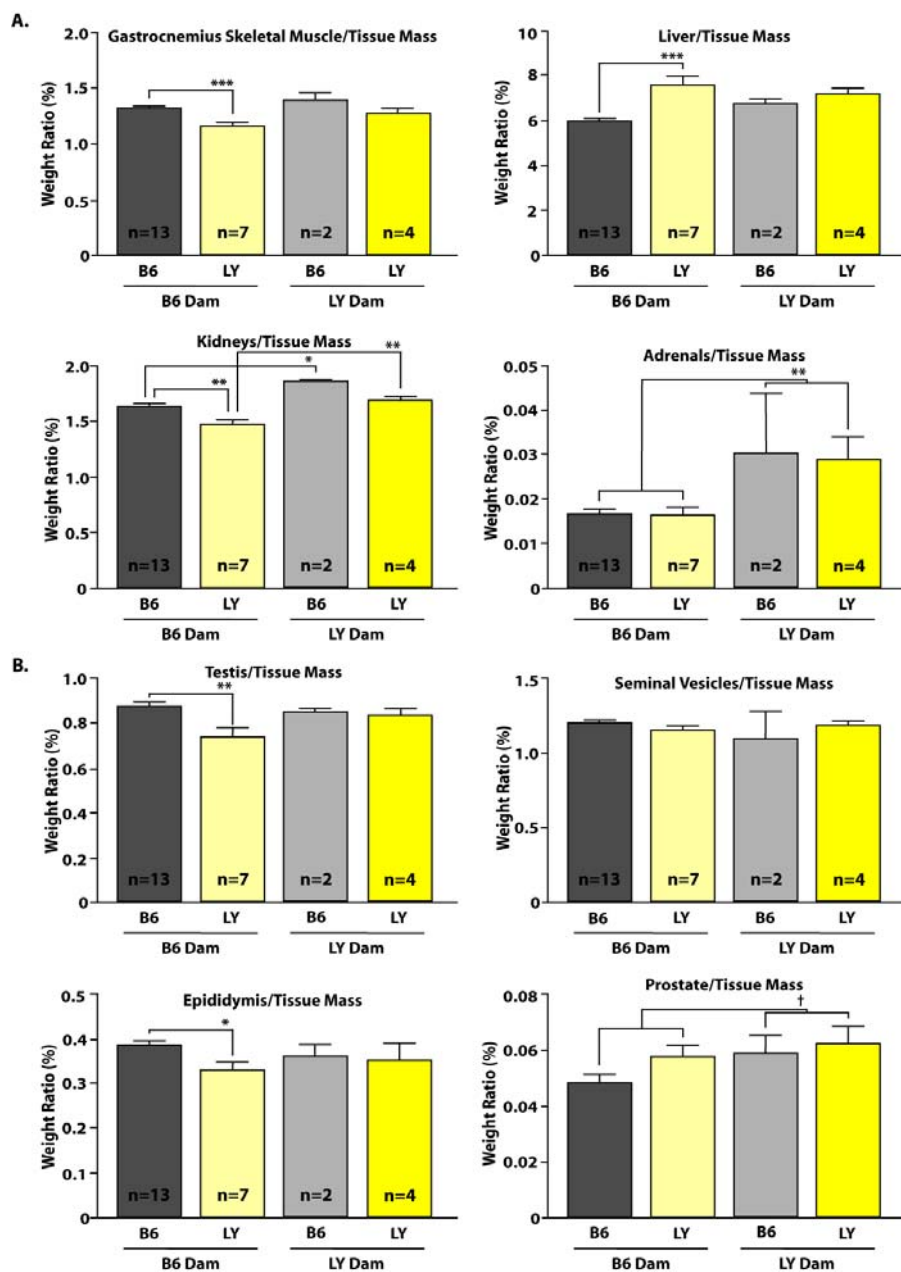
Figure A.2. Organ Growth Differs in Male Offspring from B6 Dams and LY Dams. (A, B) Male offspring from B6 dams (B6, dark grey bar, n=13; LY, light yellow bar, n=7) and LY dams (B6, light grey bar, n=2; LY, dark yellow bar, n=4) were euthanized and organs were removed. All organ weights were normalized 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

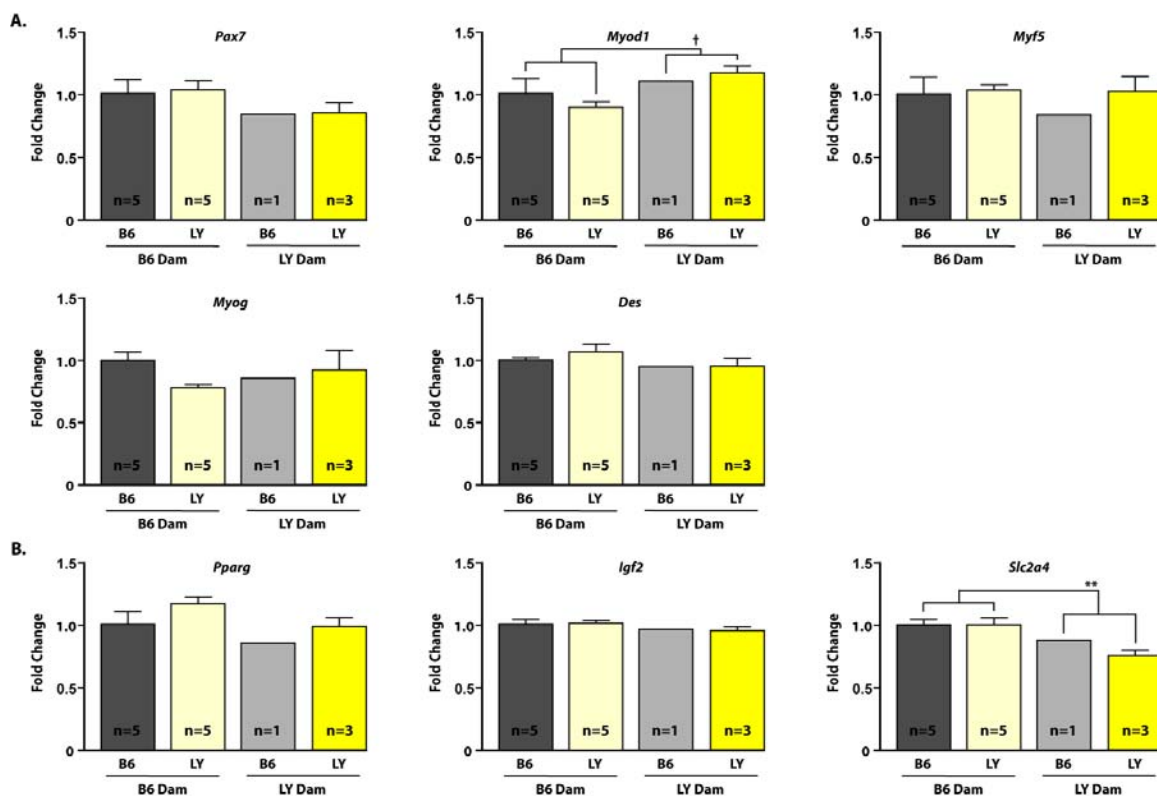sample size, we grouped all muscle samples from offspring developed from a B6-LY

Figure A.3. Muscle Function in Male Offspring is Altered. (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 *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 (*Pparg*, *Igf2* and *Slc2a4*) and found *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).

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.
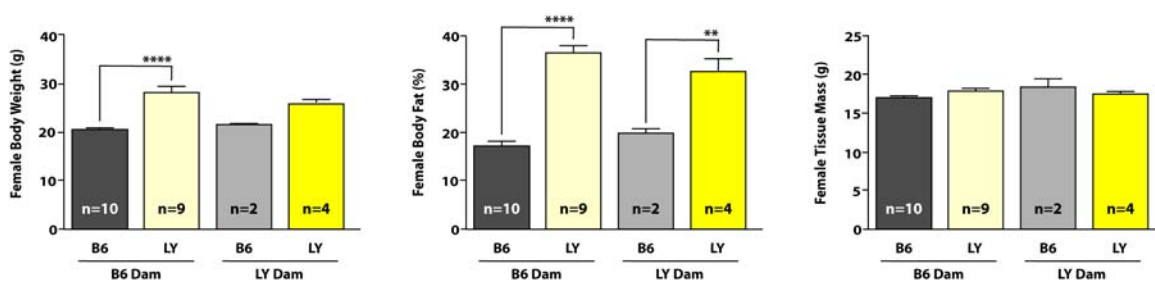

Figure A.4. Female Offspring from B6 and LY Dams Exhibit Differing Body Growth Depending on their Phenotype. Female offspring from B6 dams and LY sires (B6, dark grey bar, n=10; LY, light yellow bar, n=9) and from LY dams and B6 sires (B6, light grey bar, n=2; LY, dark yellow bar, n=4) were collected 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.

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).
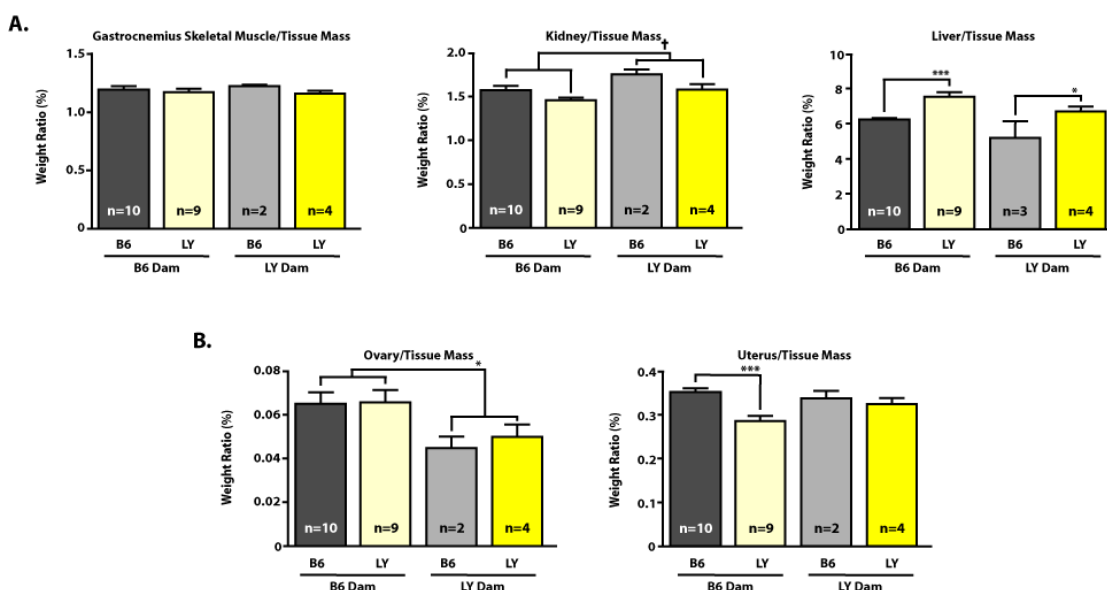

Figure A.5. Female Offspring Display Altered Organ Growth When From a B6 Compared to LY Dam. (A, B) Female offspring from B6 dams (B6, dark grey bar, n=10; LY, light yellow bar, n=9) and LY dams (B6, light grey bar, n=2; LY, dark yellow bar, n=4) were euthanized and the gastrocnemius skeletal muscle, 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, †; *P* < 0.05, *; *P* < 0.001, ***) 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
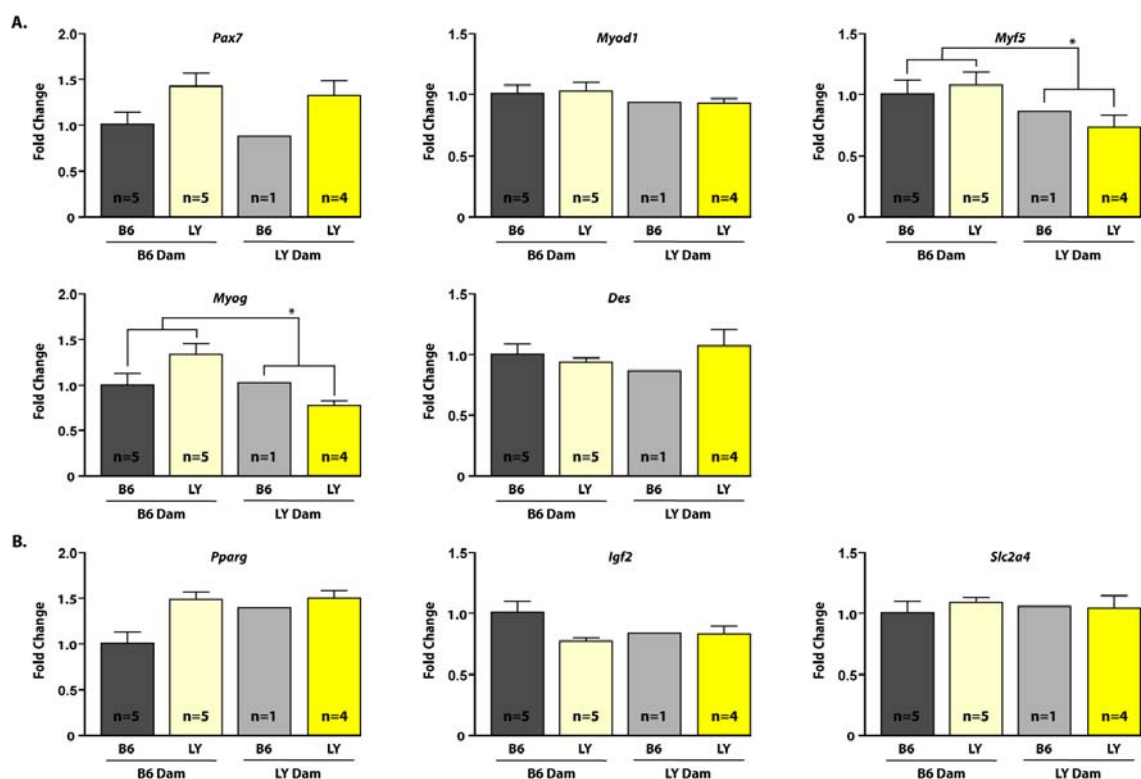

Figure A.6. Muscle mRNA Expression Differs in Female Offspring Skeletal Muscle. (A, B) QPCR analysis of *Pax7*, *Myod1*, *Myf5*, *Myog*, *Des*, *Pparg*, *Igf2* and *Slc2a4* was carried out. The normalized, relative abundance of each gene from female B6 offspring derived in B6 dam (dark grey bar, n=5) 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.

LITERATURE CITED

1. **Siega-Riz AM, Laraia B** 2006 The implications of maternal overweight and obesity on the course of pregnancy and birth outcomes. Matern Child Health J 10:S153-156

2. **Bays HE, Gonzalez-Campoy JM, Bray GA, Kitabchi AE, Bergman DA, Schorr AB, Rodbard HW, Henry RR** 2008 Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity. Expert Rev Cardiovasc Ther 6:343-368

3. **Symonds ME, Sebert SP, Budge H** 2009 The impact of diet during early life and its contribution to later disease: critical checkpoints in development and their long-term consequences for metabolic health. Proc Nutr Soc 68:416-421

4. **Denison FC, Roberts KA, Barr SM, Norman JE** 2010 Obesity, pregnancy, inflammation, and vascular function. Reproduction 140:373-385

5. **Boney CM, Verma A, Tucker R, Vohr BR** 2005 Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics 115:e290-296

6. **Fleming TP, Kwong WY, Porter R, Ursell E, Fesenko I, Wilkins A, Miller DJ, Watkins AJ, Eckert JJ** 2004 The embryo and its future. Biology of reproduction 71:1046-1054

7. **Drake AJ, Reynolds RM** 2010 Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. Reproduction 140:387-398

8. **Taylor PD, Poston L** 2007 Developmental programming of obesity in mammals. Exp Physiol 92:287-298

9. **O'Reilly JR, Reynolds RM** 2013 The risk of maternal obesity to the long-term health of the offspring. Clin Endocrinol (Oxf) 78:9-16

10. **Armitage JA, Taylor PD, Poston L** 2005 Experimental models of developmental programming: consequences of exposure to an energy rich diet during development. J Physiol 565:3-8

11. **George LA, Uthlaut AB, Long NM, Zhang L, Ma Y, Smith DT, Nathanielsz PW, Ford SP** 2010 Different levels of overnutrition and weight gain during pregnancy have differential effects on fetal growth and organ development. Reprod Biol Endocrinol 8:75

12. **Ford SP, Zhang L, Zhu M, Miller MM, Smith DT, Hess BW, Moss GE, Nathanielsz PW, Nijland MJ** 2009 Maternal obesity accelerates fetal pancreatic beta-cell but not alpha-cell development in sheep: prenatal consequences. Am J Physiol Regul Integr Comp Physiol 297:R835-843

13. **Sebert SP, Hyatt MA, Chan LL, Yiallourides M, Fainberg HP, Patel N, Sharkey D, Stephenson T, Rhind SM, Bell RC, Budge H, Gardner DS, Symonds ME** 2010 Influence of prenatal nutrition and obesity on tissue specific fat mass and obesity-associated (FTO) gene expression. Reproduction 139:265-274

14. **Aagaard-Tillery KM, Grove K, Bishop J, Ke X, Fu Q, McKnight R, Lane RH** 2008 Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. J Mol Endocrinol 41:91-102

15. **Oben JA, Patel T, Mouralidarane A, Samuelsson AM, Matthews P, Pombo J, Morgan M, McKee C, Soeda J, Novelli M, Poston L, Taylor P** 2010 Maternal obesity programmes offspring development of non-alcoholic fatty pancreas disease. Biochem Biophys Res Commun 394:24-28

16. **Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH, Piersma AH, Ozanne SE, Twinn DF, Remacle C, Rowlerson A, Poston L, Taylor PD** 2008 Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. Hypertension 51:383-392

17. **Long NM, George LA, Uthlaut AB, Smith DT, Nijland MJ, Nathanielsz PW, Ford SP** 2010 Maternal obesity and increased nutrient intake before and during gestation in the ewe results in altered growth, adiposity, and glucose tolerance in adult offspring. J Anim Sci 88:3546-3553

18. **Larciprete G, Valensise H, Di Pierro G, Vasapollo B, Casalino B, Arduini D, Jarvis S, Cirese E** 2005 Intrauterine growth restriction and fetal body composition. Ultrasound Obstet Gynecol 26:258-262

19. **Yates DT, Macko AR, Nearing M, Chen X, Rhoads RP, Limesand SW** 2012 Developmental programming in response to intrauterine growth restriction impairs myoblast function and skeletal muscle metabolism. J Pregnancy 2012:631038

20. **Zhu MJ, Han B, Tong J, Ma C, Kimzey JM, Underwood KR, Xiao Y, Hess BW, Ford SP, Nathanielsz PW, Du M** 2008 AMP-activated protein kinase signalling pathways are down regulated and skeletal muscle development impaired in fetuses of obese, over-nourished sheep. The Journal of physiology 586:2651-2664

21. **Yan X, Huang Y, Zhao JX, Long NM, Uthlaut AB, Zhu MJ, Ford SP, Nathanielsz PW, Du M** 2011 Maternal obesity-impaired insulin signaling in sheep and induced lipid accumulation and fibrosis in skeletal muscle of offspring. Biology of reproduction 85:172-178

22. **Maroto M, Reshef R, Munsterberg AE, Koester S, Goulding M, Lassar AB** 1997 Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell 89:139-148

23. **Sato T, Rocancourt D, Marques L, Thorsteinsdottir S, Buckingham M** 2010 A Pax3/Dmrt2/Myf5 regulatory cascade functions at the onset of myogenesis. PLoS Genet 6:e1000897

24. **Mok GF, Sweetman D** 2011 Many routes to the same destination: lessons from skeletal muscle development. Reproduction 141:301-312

25. **Francis-West PH, Antoni L, Anakwe K** 2003 Regulation of myogenic differentiation in the developing limb bud. J Anat 202:69-81

26. **Pownall ME, Gustafsson MK, Emerson CP, Jr.** 2002 Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu Rev Cell Dev Biol 18:747-783

27. **Muhlhausler BS, Adam CL, Findlay PA, Duffield JA, McMillen IC** 2006 Increased maternal nutrition alters development of the appetite-regulating network in the brain. FASEB J 20:1257-1259

28. **Nivoit P, Morens C, Van Assche FA, Jansen E, Poston L, Remacle C, Reusens B** 2009 Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. Diabetologia 52:1133-1142

29. **Mack EM, Smith JE, Kurz SG, Wood JR** 2012 Cyclic AMP dependent regulation of ovulatory response genes is amplified by IGF1 due to synergistic effects on Akt phosphorylation and NFkappaB transcription factors. Reproduction

30. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F** 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.0031-RESEARCH0034.0011

APPENDIX B

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

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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 *Snai1* 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.
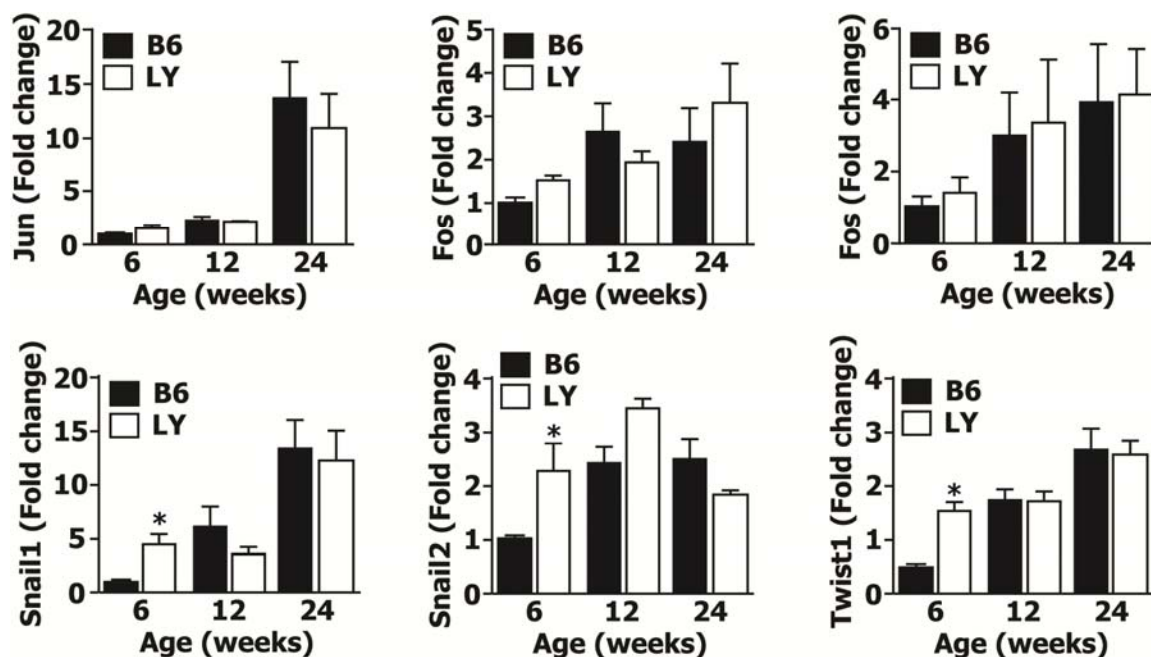

Figure B.4. Abundance of IE, EMT, and IGF family mRNAs in Uterus of Obese and Normal-Weight Females. Total RNA was isolated from the whole uterus of 6, 12, or 24 week-old B6 (black bars) or LY (white bars) females 44 h after eCG stimulation. Quantitative, real-time PCR was carried out using primers specific 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

1.   **Maekawa T, Takeuchi S, Kanayama M, Takahashi S** 2009 Estradiol, progesterone, and transforming growth factor alpha regulate insulin-like growth factor binding protein-3 (IGFBP3) expression in mouse endometrial cells. Zoological science 26:131-138

2.   **Hewitt SC, Li Y, Li L, Korach KS** 2010 Estrogen-mediated regulation of Igf1 transcription and uterine growth involves direct binding of estrogen receptor alpha to estrogen-responsive elements. The Journal of biological chemistry 285:2676-2685