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Poor Maternal Nutrition During Gestation Alters Mesenchymal Stem Cell (MSC) Metabolism in Offspring

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ABSTRACT

Poor maternal diet refers to either a nutritional excess or deficiency in the mother during gestation (Reed et al. 2014). It has been well-studied that poor maternal gestational nutrition limits bone and muscle development while increasing adipose development in the offspring (Oreffo et al. 2003; Reed et al. 2014). There is also evidence that suggests poor maternal diet during gestation impairs mesenchymal stem cell (MSC) function (Oreffo et al. 2003). Tissues including bone, muscle, and adipose are derived from this fetal MSC pool (Lawrence et al. 2012). It was hypothesized that poor maternal diet during gestation would negatively alter MSC metabolism in offspring. Eighteen ewes were bred, confirmed pregnant, and individually housed. At d 31 ± 1.3 of gestation, ewes were randomly assigned to one of three diets: 100% (CON), 60% (RES), or 140% (OVER) of NRC requirements for TDN. The diet was maintained until parturition, after which one lamb per ewe was euthanized within 24 h. MSC ($n = 6$ /treatment group) were isolated from the left tibia and femur of the lamb. Cells were plated at 30,000 cells/well and incubated for 48 h at 37°C. Glycolytic and mitochondrial function of the MSC were evaluated using Glycolysis Stress and Cell Mito Stress Test Kits. The assays were performed in the Seahorse XF^c24 Extracellular Flux Analyzer. Data were normalized for total cellular DNA content and statistical analysis was performed using PROC MIXED in SAS. Basal respiration was reduced in RES and OVER, compared with CON (127.4 ± 7.48 , 90.17 ± 9.75 , and 87.51 ± 8.48 $\mu\text{mol O}_2^{-1} \cdot \text{minute}^{-1} \cdot \mu\text{g}$; CON, RES, and OVER, respectively; $P \leq 0.008$). Compared with CON, RES and OVER had reduced ATP production (121.09 ± 6.08 , 84.86 ± 11.05 , and 77.7 ± 6.44 $\mu\text{mol O}_2^{-1} \cdot \text{minute}^{-1} \cdot \mu\text{g}$; CON, RES, and OVER, respectively; $P \leq 0.006$) and reduced maximal respiration (149.29 ± 17.05 , 90.64 ± 23.81 , and 67.93 ± 10.15 $\mu\text{mol O}_2^{-1} \cdot \text{minute}^{-1} \cdot \mu\text{g}$; CON, RES, and OVER, respectively; $P \leq 0.008$).

$^1 \cdot \text{minute}^{-1} \cdot \mu\text{g}$; CON, RES, and OVER, respectively; $P \leq 0.03$). Spare respiratory capacity was reduced in OVER compared with CON ($P = 0.02$) while RES were intermediate (21.9 ± 10.8 , 0.47 ± 12.3 , and $-19.56 \pm 9.24 \text{ pmol O}_2^{-1} \cdot \text{minute}^{-1} \cdot \mu\text{g}$; CON, RES, and OVER, respectively; $P \leq 0.25$). There were no significant differences between groups for proton leak, non-mito-derived OCR, coupling efficiency, glycolysis, glycolytic reserve, non-glucose-derived extracellular acidification rate, and glycolytic reserve capacity ($P \geq 0.18$). In conclusion, poor maternal diet during gestation reduces the basal metabolic state of MSC and restricts the ability to produce ATP during energetic demands. This could possibly contribute to the observed differences in muscle, bone, and adipose development of offspring born to over-fed or under-fed mothers.

INTRODUCTION

It is becoming increasingly difficult to meet the worldwide food demand, with a human population of 7 billion in 2012 and 9 billion people expected by 2050 (Vonnahme et al., 2015). Food production industries must consider not only the supply, but also the efficiency of food animal production. However, several production practices that aim to maximize efficiency inadvertently compromise the quality of fetal growth, often through an inadequate maternal diet (Vonnahme et al., 2015). Poor maternal nutrition refers to either an excess or lack of nutrition during gestation, which can be due to overall nutrients or specific nutrients in the diet (Reed et al., 2014). Maternal over nutrition, such as ‘flushing’, can negatively affect fetal health (Wu et al., 2006). For example, over nutrition in the sow during ovulation increases fetal mortality and over nutrition in both the sow and ewe during ovulation reduces fetal growth (Wu et al., 2006). On the contrary, maternal under nutrition can result from the breeding of immature animals or pasture grazing, and can be detrimental to fetal growth (Wu et al., 2006). This paper will discuss the importance of mesenchymal stem cells (MSC) in the development of bone, muscle, and fat and the effects of poor maternal nutrition on these tissues in the offspring. The effects of poor maternal nutrition on fetal growth are well documented, but the mechanisms are poorly understood. Therefore, the objective of this literature review is to consider several mechanisms connecting poor maternal nutrition to poor fetal growth. Current research focuses on the role of fetal metabolic programming and altered mitochondrial function as possible mechanisms. The metabolism of MSC is an important area of focus because there are several nutrient-sensing pathways that control metabolic status (Agathocleous et al., 2013; Ochocki et al., 2013; Ito et al., 2014). Additionally, the relative ratios of glycolytic and mitochondrial ATP production shift as

MSC differentiate into bone, muscle, and adipose (Shyh-Chang et al., 2013). Fetal metabolism can also be programmed during certain *in utero* conditions, including poor maternal diet (Wu et al., 2006; Fernandez-Twinn et al., 2010). This metabolic programming, specifically of MSC, is a possible mechanism by which poor gestational maternal diet leads to poor fetal growth and development

REVIEW OF LITERATURE

Mesenchymal Stem Cells

Establishment

Mammals are comprised of many different cell types that differ in both morphology and physiology in order to fulfill the specific roles of that tissue. Stem cells are the original, embryonic cells that differentiate into every cell type (Lawrence et al., 2012). Initially, embryonic cells are totipotent, being able to differentiate into any cell type of an adult organism (Lawrence et al., 2012). At the blastocyst stage, the stem cells are considered pluripotent, being able to form only one of two cell types – the epithelium or the mesenchyme (Lawrence et al., 2012). The mesenchymal cells can differentiate into several different cell types; however, the focus of this discussion will be on the formation of muscle, bone, and fat due to the importance of these tissues in food animal production (Lawrence et al., 2012).

Differentiation of MSC into Bone, Muscle, and Adipose Tissues

Endochondrial ossification involves the replacement of a cartilage model with bone (Lawrence et al., 2012). Mesenchymal stem cells first condense, and then differentiate into chondroblasts, followed by differentiation into chondrocytes and extracellular matrix secretion (Lawrence et al., 2012). This primordial cartilage template is replaced with bone through vascularization, calcification, and the replacement of chondroblasts with osteoblasts (Lawrence et al., 2012). Mesenchymal stem cells can also differentiate into an entirely different tissue type, muscle.

Muscle development can be divided into three distinct stages: embryonic, fetal, and postnatal. During embryonic muscle development, MSC differentiate into primary myoblasts (Lawrence et al., 2012). Following proliferation, myoblasts fuse to form primary myotubes and develop the protein scaffolding characteristic of mature myofibers (Lawrence et al., 2012). During fetal muscle development, MSC differentiate into secondary myoblasts, followed by secondary myotubes, and ultimately into secondary myofibers (Lawrence et al., 2012). Postnatal muscle development involves muscle cell replacement in response to injury, muscle fiber hypertrophy, and protein accretion (Lawrence et al., 2012). Muscle cell progenitor cells, known as satellite cells, are quiescent along the periphery of the muscle fiber (Lawrence et al., 2012). Muscle injury signals these satellite cells to differentiate, in a process very similar to primary myogenesis (Lawrence et al., 2012). Mesenchymal stem cells also develop into adipose tissue.

Adipose development can be divided into two phases: prenatal and postnatal. Prior to birth, epigenetic hypomethylation and adipogenic transcription factors signal MSC to differentiate into pre-adipocytes, followed by adipocytes (Lawrence et al., 2012). Unable to proliferate, adipocyte numbers can only increase through the proliferation/differentiation of either MSC or pre-adipocytes (Lawrence et al., 2012). Postnatal adipose development can also occur through lipid droplet accumulation within the adipocyte (Lawrence et al., 2012). Although MSC are predominantly involved in embryogenesis, MSC stores remain in several tissues in the adult organism.

Roles in the Adult

Mesenchymal stem cells can be found in several adult tissues, including bone marrow, adipose, umbilical cord blood, pancreas, and synovial tissue (Ramalingam et al., 2012). However, only bone marrow, and adipose to a lesser extent, contains significant numbers of MSC (Ramalingam et al., 2012). These MSC remain quiescent in the body, but can be signaled to differentiate into new tissue cells during times of cell injury and to proliferate in order to maintain MSC numbers (Ramalingam et al., 2012). However, the function of MSC can be influenced by several factors, including the quality of maternal diet *in utero*.

Poor Maternal Nutrition

Effects on Mesenchymal Stem Cells

The health of fetal MSC is affected by the quality of fetal nourishment provided through the maternal diet (Oreffo et al., 2003). For example, maternal protein restriction was found to delay the normal proliferation and differentiation of MSC (Oreffo et al., 2003). In addition, fetal stem cells from low-protein maternal diets displayed a phenomenon called ‘catch-up growth’, in which a tissue initially of low proliferative ability (due to nutrient restriction in utero) eventually surpasses the proliferative ability of the control (Oreffo et al., 2003). Oreffo et al. (2003) evaluated this phenomenon using total colony forming unit-fibroblastic (CFU-F) colony number, a measure of the ability of MSC to proliferate and form colonies. Total CFU-F numbers of low-protein diet MSC were decreased by 40% at eight weeks, not different at twelve weeks, and increased by 111% at sixteen weeks of fetal age in rats (Oreffo et al., 2003). These differences seen in MSC of poorly nourished mothers can potentially lead to the effects of poor maternal nutrition on bone, muscle, and adipose development.

Effects on Bone, Muscle, and Adipose Development

Bone forms the structural support of the animal, stores excess nutrients, and acts as a reserve of several important cell types, such as red blood cells and stem cells (Lawrence et al., 2012). However, bone development can be affected by the fetus's perinatal environment, including maternal nutrition. Previous studies have demonstrated that poor maternal diet predisposes the offspring to decreased bone density in rats (Oreffo et al., 2003). Similarly, Lanham et al. (2010) found that mice from dams fed a high-fat diet during gestation had both decreased bone mineral content and density, compared with control. Furthermore, rodent studies demonstrate that low-protein maternal diets reduce bone mineral content, bone area, and alter growth plate appearance in offspring (Oreffo et al., 2003; Lanham et al., 2010). Interestingly, offspring from dams fed a high-fat diet during gestation experienced an increase in bone cross-sectional area, yet further investigation attributed this to increased lumen area as opposed to increased wall thickness (Lanham et al., 2010). This suggests that the bone is more prone to fracture (Oreffo et al., 2003). The offspring from dams fed a high-fat gestational diet also experienced reduced femur length and increased bone marrow adiposity (Lanham et al., 2010). Both low-protein and high-fat gestational diets were shown to restrict osteoblast differentiation and arrest the differentiation of recruited marrow stromal cells (Oreffo et al., 2003; Lanham et al., 2010). The development of bone, muscle, and adipose is closely interconnected due to the common MSC origin.

Muscle development can be affected through several ways, such as altered muscle fiber number (determined prenatally) and altered postnatal muscle characteristics, which include fiber type, protein accretion, and fiber size (Lawrence et al., 2012). A study conducted by Reed et al.

(2014) found that maternal over- and under-nutrition during gestation increased cross-sectional area (CSA) of the lamb's semitendinosus muscle at one day of age, but decreased this CSA at three months of age. Additionally, the proportion of muscle fiber types is a major determinate of postnatal growth and is also related to oxidative capacity, insulin sensitivity, and meat quality (Wu et al., 2006; Reed et al., 2014). In pigs, researchers found that lower-weight piglets had more oxidative and fast-oxidative fibers and less glycolytic fibers, as compared with heavier piglets (Wu et al., 2006). Similarly, lambs of over- and under-fed ewes exhibited an increase in oxidative Type IIa and decrease in glycolytic Type I muscle fibers, compared with control (Reed et al., 2014). Oxidative fibers contract slower than glycolytic fibers, and rely more on oxidative mitochondrial function for energy production (Lawrence et al., 2012). Therefore, this increased proportion of oxidative muscle fibers is associated with increased reliance on mitochondrial function and ATP production. These studies demonstrate that (1) fetal muscle fiber type is influenced by maternal diet and (2) the proportion of fiber types predisposes the fetus to a certain type of growth and body type (Wu et al., 2006; Reed et al., 2014). Due to the common MSC origin, it is also important to consider adipose development when considering bone and muscle development.

Adipose is one of the last tissues to develop, given priority behind that of bone and muscle (Lawrence et al., 2012). However, poor maternal diet can alter the priority given to the development of these tissues. When myogenesis is restricted, adipose deposition is given priority over muscle development (Wu et al., 2006). Reed et al. (2014) found that lipid content in semitendinosus muscle of one-day-old lambs was 212.4% and 92.5% greater from overfed and underfed ewes, respectively, compared with control. Yet, at three months of age, lambs from

overfed ewes had 36.1% greater lipid content while those from underfed ewes had 23.6% less lipid content, compared with control (Reed et al., 2014). These changes can alter not only fetal physiology, but are also undesirable for meat quality. Studies show that at slaughter, animals with increased intramuscular fat exhibit increased lipid peroxidation, which oxidizes muscle (Wu et al., 2006). This results in tougher meat and a reduction in the bright color of red meats (Wu et al., 2006). The close relationship between bone, muscle, and adipose tissues is due to the fact that each tissue is originally derived from the mesenchyme stem cell layer (Lawrence et al., 2012). In addition to effects of these specific tissues, poor maternal diet during gestation can alter whole body fetal physiology.

Effects on Whole Body Function

The effects of poor maternal nutrition include reduced survival rates, stunted growth, altered whole body composition, poor meat quality, and permanent changes in health of the offspring (Wu et al., 2006). In sheep, studies have shown poor maternal diet can lead to several fetal metabolic dysfunctions, including insulin resistance, obesity, oxidative stress, and mitochondrial dysfunction (Wu et al., 2006). These offspring showed improved, but not corrected, growth when supplemented with energy, protein, concentrates, or both (Wu et al., 2006). Due to the permanent changes in fetal physiology, fetal programming is one proposed mechanism for the effects exerted by poor gestational diet on fetal growth and development.

Fetal Programming and Maternal Diet

Fetal growth is a complex process that is influenced by many different internal and external factors, including genetics, epigenetics, and the environment (Wu et al., 2006).

However, recent research has emphasized the importance of the offspring's intrauterine and perinatal environments in determining lifelong health status, which were originally not given sufficient consideration (Tamashiro et al., 2010). The term 'fetal programming' refers to permanent changes in the expression of the fetal genome as a result of the fetal environment (Wu et al., 2006; Fernandez-Twinn et al., 2010). Barker and Hales proposed that 'fetal metabolic programming' is a risk factor for the development of several chronic metabolic disorders (Hales et al., 2013). These programming events are believed to be the result of epigenetic modifications to the offspring's genome (Hales et al., 2013). Lanham et al. (2010) notes how organisms experience developmental plasticity, or the ability to alter development based on a certain environment. Developmental plasticity is possible due to epigenetics and the regulation of gene expression (Lanham et al., 2010). Epigenetics refers to posttranslational DNA alterations that regulate gene expression without changing the actual DNA sequence, and includes DNA methylation, genomic imprinting, and chromatin remodeling (Tamashiro et al., 2010). One of the environmental factors that influences fetal programming is maternal/fetal nutrition (Wu et al., 2006; Tamashiro, et al., 2010). The 'developmental origins of health and disease' hypothesis suggests that some chronic diseases can be attributed to the early life environment (Lanham et al., 2010). There are several very similar hypotheses concerning maternal nutrition and fetal programming with a common theme: maternal diet influences fetal health through programming mechanisms.

The 'predictive adaptive response' refers to programmed changes in a fetus's phenotype suited to be advantageous to a certain type of environment (Lanham et al., 2010). Fetal programming is a mechanism that prepares the fetus for birth into this specific environment

(either lack of nutrients or surplus of nutrients); a mismatch between perinatal and postnatal environments results in an altered physiological state, such as fetal metabolic up regulation (Igosheva et al., 2010; Chiaratti et al., 2015). This phenomenon has been referred to as a two-hit process (Tamashiro et al., 2010). The “first hit” is a result of poor *in utero* conditions that promote fetal programming (Tamashiro et al., 2010). However, Tamashiro et al. (2010) suggests that this poor maternal environment is not the *direct* cause of chronic disorders characteristic of the ‘developmental origins of health and disease’ hypothesis. Rather, the “first hit” increases the offspring’s susceptibility to a “second hit”, which is a postnatal challenge that cannot be appropriately met by a “first hit” offspring, resulting in a diseased state (Tamashiro et al., 2010). The postulates of the “thrifty phenotype hypothesis” echo those of the “predictive adaptive response”.

The “thrifty phenotype hypothesis” suggests that offspring of nutrient-restricted mothers develop certain metabolic disorders as a result of programming in utero (Tamashiro et al., 2010). During development, the nutrient-restricted perinatal environment programs the offspring for birth into an equally nutrient-restricted, or thrifty, environment (Tamashiro et al., 2010). For example, malnutrition *in utero* can cause metabolic and physiological changes that prioritize nutrients towards the most vital organs, at the expense of other organs, in order to maximize chances of survival in a nutrient-restricted environment (Fernandez-Twinn et al., 2010). However, this programming negatively affects the offspring when it experiences an energy balance challenge, such as an unexpected abundance of nutrients (Fernandez-Twinn et al., 2010; Tamashiro et al., 2010). Therefore, perinatal conditions are as equally important as postnatal conditions, especially the mismatch between the two. For example, glucose tolerances were

worst in individuals that were born small and developed obesity with age, and therefore experienced a drastic change in perinatal and postnatal environments (Fernandez-Twinn et al., 2010). Similarly, an animal can experience drastic physiological change when they undergo “catch-up growth”, as previously described (Fernandez-Twinn et al., 2010).

Catch-up growth refers to an unexpected increase in a tissue’s proliferative ability following its initial slow growth and development (Oreffo et al., 2003; Fernandez-Twinn et al., 2010). However, catch-up growth is not considered a *corrective* mechanism; rather, it introduces a host of health concerns, as demonstrated by Fernandez-Twinn et al. (2010) study. Offspring from a dam fed an 8% low-protein diet weighed 15% less than control, at birth (Fernandez-Twinn et al., 2010). However, the lower weight rats experienced rapid catch-up growth during lactation, and were referred to subsequently as “recuperated” (Fernandez-Twinn et al., 2010). Despite regaining this initial stunted growth, the life span of the “recuperated” male rats was reduced by 25%, attributable mostly to kidney failure (Fernandez-Twinn et al., 2010). On the contrary, rats that were carried *in utero* by a dam fed a 20% normal protein diet and suckled by a dam fed an 8% low-protein diet dam did not suffer the effects of catch-up growth, specifically reduced longevity (Fernandez-Twinn et al., 2010). Therefore, catch-up growth appears to be a programming mechanism that results from nutrient inadequacies *in utero*. One of common fetal mechanisms susceptible to *in utero* programming is metabolic physiology (Chiaratti et al., 2015).

Changes in fetal metabolic physiology in response to maternal diet can also be referred to as stress-induced metabolic programming (Chiaratti et al., 2015). For example, the least successful mouse litters (defined as low litter fetal weight, low litter placental weight, and low

litter size) had the highest levels of placental mitochondrial DNA (mtDNA); (Chiaratti et al., 2015). The researchers found that mice from protein-restricted mothers contained greater amounts of placental ATP (Chiaratti et al., 2015). These results suggest that fetal metabolism is influenced by maternal diet. Other researchers similarly note links between maternal obesity, increased mitochondrial potential, and increased mtDNA content (Igosheva et al., 2010). These changes in fetal metabolic physiology, possibly due to maternal programming, result in whole body fetal metabolic changes: increase in reactive oxidative species, decrease in glutathione (the principle cellular anti-oxidant), more oxidized redox state, clustered mitochondrial distribution, and increased mtDNA copy number in the oocyte, which all suggest metabolic up-regulation (Igosheva et al., 2010; Ochocki et al., 2013). However, the “quiet embryo hypothesis” suggests that low levels of metabolism promote embryo survival before implantation (Chiaratti et al., 2015). Therefore, altered fetal metabolism could be one of the mechanisms explaining poor fetal growth as a result of poor maternal diet, and will be the focus of the remainder of this literature review.

Metabolism

Normal Metabolic Physiology

Cells can obtain energy through one of two processes: anaerobic glycolysis or oxidative phosphorylation (OXPHOS); (Ochocki et al., 2013). Glycolysis can produce ATP at a much faster rate than OXPHOS, but can only produce a net two ATP molecules per molecule of glucose (Ochocki et al., 2013). On the contrary, OXPHOS metabolizes its substrates more completely in order to produce a net 36 to 38 ATP molecules per molecule of glucose (Ochocki et al., 2013). This is accomplished through the oxygen-demanding electron transport chain,

during which the transfer of electrons between five complexes catalyzes the phosphorylation of ADP to ATP (Bhat et al., 2015). To meet energetic demands, most mature cells rely on OXPHOS over glycolysis (Igosheva et al., 2010; Bhat et al., 2015). However, a cell's metabolic status changes throughout the differentiation process.

Cell Differentiation Status and Metabolic Preference

Metabolism is unique in that it is fairly plastic; a cell's metabolic profile evolves with cellular differentiation (Burgess et al., 2014). This property allows the metabolic physiology to suit the cell's needs at different developmental stages and in different cell types (Agathocleous et al., 2013; Burgess et al., 2014). For example, metabolism is a key determinant of whether a cell proliferates, differentiates, or remains quiescent (Shyh-Chang et al., 2013). In addition, stem cells are influenced by the local microenvironment, known as a stem cell niche, in order to maintain quiescence (Ito et al., 2014). Bone marrow-derived MSC (BMSC) are suited for a low-oxygen, hypoxic niche; this hypoxic niche promotes maintenance of the undifferentiated state, and also favors glycolysis over OXPHOS (Ito et al., 2014). In general, undifferentiated cells prefer anaerobic glycolysis to meet energy demands, while differentiated cells switch to OXPHOS for ATP production (Burgess et al., 2014; Ito et al., 2014). This change represents a cell's physiological evolution as it undergoes differentiation.

The totipotent stem cells of the pre-blastocyst embryo are the least differentiated cells and initially have low glycolysis rates; the glycolytic enzymes hexokinase (HK) and phosphofructokinase 1 (PFK1) are rate limiting and limit glycolysis rates (Shyh-Chang et al., 2013). Energy and carbon sources are derived from analogs of pyruvate, until the embryo

reaches the morula stage and glucose oxidation rivals that of pyruvate (Shyh-Chang et al., 2013). Mitochondrial structure is not yet mature, and therefore oxygen consumption rates are generally low (Shyh-Chang et al., 2013). Prior to blastocyst implantation, the number of mitochondria halves with each cell division, and therefore ATP levels and ATP/ADP ratio decrease with time (Shyh-Chang et al., 2013). ATP levels inhibit PFK1, and therefore this ATP decrease may contribute to the activation of glycolysis at the successive differentiation stage (Shyh-Chang et al., 2013).

Pluripotent stem cells of the blastocyst become more metabolically active. To meet needs for rapid growth, glucose uptake increases due to up-regulated expression of glucose transporters GLUT1 and GLUT3 (Shyh-Chang et al., 2013). Although rates of OXPHOS increase, glycolysis is still the preferred metabolic status of pluripotent stem cells (Shyh-Chang et al., 2013). Interestingly, the generation of induced pluripotent stem cells (iPSCs), which are somatic cells reprogrammed to be pluripotent, initiates a switch from OXPHOS to glycolysis that precedes the presence of pluripotency markers (Shyh-Chang et al., 2013). This suggests that glycolysis is not specific to pluripotency but rather an ideal metabolic state for cells of rapid proliferative ability (Shyh-Chang et al., 2013). Therefore, the ratio of glycolysis to OXPHOS is expected to decrease with increasing differentiation.

Once embryonic stem cells begin differentiation, proliferation slows and cells switch from glycolysis to OXPHOS to meet energetic demands (Shyh-Chang et al., 2013). This switch is partly influenced by the electron transport chain (ETC) re-coupling with ATP synthase, which triggers a sharp increase in oxygen consumption (Shyh-Chang et al., 2013). The coupling

between glycolysis and the Krebs cycle also strengthens (Shyh-Chang et al., 2013). OXPHOS, and specifically the ETC, increase the production of reactive oxidative species (ROS), which promote cellular differentiation under low ROS levels (Shyh-Chang et al., 2013).

Cell types that remain undifferentiated and quiescent in adult tissues, such as MSC, prefer a slow-cycling state (Shyh-Chang et al., 2013). Evidence includes higher expression of glycolytic enzymes and lower expression of OXPHOS proteins (Shyh-Chang et al., 2013). In an undifferentiated bone marrow MSC, glycolysis accounts for about 97% of ATP production while glucose and fatty acid oxidation only account for about 3% (Fillmore et al., 2015). It is believed that MSC prefer this glycolytic state in order to minimize damaging ROS and preserve self-renewal abilities (Shyh-Chang et al., 2013). This is supported by the fact that MSC cultured in normoxia conditions have enhanced proliferative ability yet also experienced increased senescence (Agathocleous et al., 2013; Shyh-Chang et al., 2013; Ito et al., 2014). MSC must maintain their self-renewal capability in order to act as a reserve for adipocytes, osteoblasts, and chondroblasts. In addition, proliferative ability is enhanced by glycolysis, which maintains high rates of macromolecular synthesis (Fillmore et al., 2015). Finally, it appears that cell fate is partially determined by shifts in MSC metabolic status. Differentiation of MSC to pre-adipocytes occurs under conditions of high OXPHOS, high ROS, and high oxygen (Shyh-Chang et al., 2013). However, differentiation towards osteoblast lineage is characterized by conditions of high OXPHOS, low ROS, and high oxygen (Shyh-Chang et al., 2013). On the contrary, chondroblast differentiation prefers low OXPHOS and high glycolysis levels (Shyh-Chang et al., 2013). In addition to differentiation status, several environmental-sensing pathways also influence a cell's metabolic profile.

Regulation of Metabolic State: Role of Oxygen and Nutrient Sensing Pathways

Hypoxia-inducible factors (HIF) are transcription factors that regulate the glycolysis to OXPHOS ratio through sensing of environmental O₂ conditions (Agathocleous et al., 2013; Burgess et al., 2014). HIF plays a critical role in the homeostasis of stem cells (Ochocki et al., 2013). HIF1 is a protein that consists of two different subunits: the HIF-1 α subunit is responsive to hypoxic conditions while the HIF-1 β subunit is constitutively expressed (Ito et al., 2014). During low O₂ conditions (hypoxia), HIF-1 α is critical for the switch from OXPHOS to glycolysis (Agathocleous et al., 2013; Burgess et al., 2014). HIF-1 α promotes the expression of pyruvate dehydrogenase kinase (PDK) isoforms 2 and 4, which are responsible for the phosphorylation of pyruvate dehydrogenase (PDH) and subsequent inhibition of pyruvate entry into the TCA cycle (Ochocki et al., 2013; Ito et al., 2014). HIF-1 α is degraded under normoxic conditions, leading to decreased HIF1 levels (Agathocleous et al., 2013; Ito et al., 2014). HIF-1 α expression is higher in less differentiated cells, which suggests one possible mechanism by which stem cells maintain their preference for glycolysis (Burgess et al., 2014). Other mechanisms for metabolic regulation include nutrient-sensing pathways.

The P13K-AKT-mTOR pathway is a nutrient-sensing pathway that promotes cellular proliferation under the regulation of both growth factors and nutrients, like glucose and amino acids (Ito et al., 2014). Mammalian target of rapamycin (mTOR) is a kinase that senses oxygen, nutrients, and growth factors through the P13K-AKT pathway (Ochocki et al., 2013). There are two distinct complexes: mTORC1 and mTORC2 (Ochocki et al., 2013). mTOR increases the number of mitochondria, and therefore up-regulates metabolism (Shyh-Chang et al., 2013). Upon

stimulation of nutrients or amino acids, mTORC1 is activated and phosphorylates its targets to stimulate mRNA translation, glycolysis, and lipid/nucleotide synthesis (Agathocleous et al., 2013; Ochocki et al., 2013). Overall, the P13K pathway negatively affects stem cell function by initiating stem cell aging, quiescence, and stem cell exhaustion (Ito et al., 2014). Therefore, the P13K pathway may have significant effects in the offspring of over-fed mothers. In addition, mTOR is inhibited by AMPK, which provides a bridge between the P12K-AKT-mTOR and LKB1-AMPK pathways (Agathocleous et al., 2013).

The LKB1-AMPK is another nutrient-sensitive pathway that affects metabolic physiology (Ito et al., 2014). AMP-activated protein kinase (AMPK) is an $\alpha\beta\gamma$ heterotrimeric enzyme that is considered the master regulator of metabolism (Ochocki et al., 2013). The tumor suppressor liver kinase B1 (LKB1) is a serine/threonine kinase that is believed to limit cell growth when poor energetic conditions are sensed (Agathocleous et al., 2013; Ito et al., 2014). Under low energy conditions, the AMP/ATP ratio increases and LKB1 phosphorylates/activates AMPK (Ochocki et al., 2013; Ito et al., 2014). AMPK inhibits mTORC1, resulting in reduced proliferation, inhibition of anabolic pathways such as lipogenesis and protein synthesis, and activation of catabolic pathways such as fatty acid oxidation and glucose uptake (Agathocleous et al., 2013; Ochocki et al., 2013; Ito et al., 2014). Therefore, the P13K and AMPK pathways act in contrast to each other. With restricted cellular proliferation, stem cell longevity is extended through decreased cellular aging, quiescence, and stem cell exhaustion (Ito et al., 2014). This AMPK pathway is of special interest for offspring of under-fed mothers. Metabolic regulation through these pathways is important because sensitive stem cells must compromise the efficiency

of oxygen-derived OXPHOS with the production of potentially harmful reactive oxidative species (Agathocleous et al., 2013; Shyh-Chang et al., 2013; Ito et al., 2014).

Reactive Oxidative Species: Physiological and Pathological Levels

Reactive oxidative species (ROS) are normal byproducts of aerobic energy production. The substrates needed for the ETC, such as NADH, are produced during the citric acid cycle (Igosheva et al., 2010). Nicotinamide adenine dinucleotide (NAD) is one important control mechanism for redox state (Ochocki et al., 2013). NAD is an electron-transferring molecule in redox reactions; it can accept electrons to become the reduced NADH species or donate electrons to become the oxidized NAD^+ state (Ochocki et al., 2013). The NAD^+/NADH ratio is a valuable measurement of a cell's redox status (Ochocki et al., 2013). NADH is oxidized in the ETC, resulting in several changes: (1) an increase in ROS and (2) a decrease in peroxiredoxins, thioredoxin, and oxidized glutathione (Igosheva et al., 2010). In addition, 1 to 5% of the oxygen consumed during oxidative phosphorylation is converted to ROS (Bhat et al., 2015). Normally, complex III of the electron transport chain is the main production center of ROS (Bhat et al., 2015). Although harmful in high concentrations, low concentrations of ROS are needed for several important cellular functions and therefore ROS is considered a physiological molecule (Ochocki et al., 2013).

Low physiological ROS levels have one of two functions: (1) activate DNA repair mechanisms, or (2) promote cellular differentiation (Ochocki et al., 2013). Inhibition of ROS production negatively affects cell function. For example, exogenously added antioxidants (such as hydrogen peroxide) counteract ROS and cause early MSC senescence (Lyublinskaya et al.,

2015). Further, it has been demonstrated that this antioxidant treatment blocks the G₁-S transition of MSC cell cycle (Lyublinskaya et al., 2015). In fact, there is a positive and linear relationship between the number of cells in S phase and the concentration of ROS (Lyublinskaya et al., 2015). In addition, studies have demonstrated a positive correlation between cell proliferation and ROS levels (Lyublinskaya et al., 2015). Therefore, physiologic levels of ROS exert some control over the cell cycle. However, abnormally high levels of ROS pose several health risks, such as oxidative stress (Bhat et al., 2015).

Oxidative stress is defined as the detrimental overproduction of ROS and nitrosative stress as the overproduction of reactive nitrogen species (RNS); (Bhat et al., 2015). Oxidative stress presents a concern during pregnancy because both maternal and fetal metabolisms are performing at their highest rates during gestation (Wu et al., 2006). Therefore, pregnancy is characterized by increasing levels of oxidizing agents, such as the superoxide anion, hydrogen peroxide, lipid peroxide, and hydroxyl radicals (Wu et al., 2006). Both ROS and RNS, in excess concentrations, can injure cells due to their free radical nature (Bhat et al., 2015). A free radical is a molecule with one or more unpaired electrons (Bhat et al., 2015). Free radicals are very reactive and can therefore stimulate inappropriate reactions with cellular components (Bhat et al., 2015). The consensus among current studies is that ROS targets the integrity of DNA, particularly mtDNA (Igosheva et al., 2010; Estrada et al., 2012). While ROS are necessary for normal cell physiology, it must be controlled in order to prevent detrimental oxidative stress. The processes that create a balance between healthy and unhealthy concentrations of ROS comprise the term “Redox Regulation” (Bhat et al., 2015).

Forkhead O (FOXO) transcription factors are one control mechanism for ROS production (Ochocki et al., 2013; Burgess et al., 2014). FOXO transcription factors work by both preventing ROS production, through the increased expression of antioxidant enzymes, and correcting ROS-induced damage, by activating DNA repair enzymes (Ochocki et al., 2013; Burgess et al., 2014; Ito et al., 2014). Of the FOXO family, FOXO3 is particularly important for ROS control because it regulates the expression of manganese superoxide dismutase (Ochocki et al., 2013; Burgess et al., 2014). In addition, FOXO transcription factors regulate the cell cycle and apoptosis, and therefore play a major role in both proliferation and longevity (Ochocki et al., 2013). The FOXO transcription factors are positively regulated by the AMP kinase (AMPK) and negatively regulated by the P13K signaling pathways (Ochocki et al., 2013; Ito et al., 2014). Therefore, a deficiency in FOXO transcription factors can restrict the oxidative stress response, which is crucial to preserving cellular health (Shyh-Chang et al., 2013). Another source of ROS regulation is the ataxia telangiectasia mutated (ATM) pathway (Ito et al., 2014). The ATM protein is a kinase that is required for DNA repair in times of damaging oxidative stress (Ito et al., 2014). When these control mechanisms fail, and ROS levels become pathological, altered metabolic function becomes a concern.

Altered Metabolic Physiology

Research has focused on how maternal diet influences fetal metabolic function (Wu et al., 2006; Igosheva et al., 2010). It was already known that obesity is a form of chronic low-level inflammation characterized by increased levels of oxidative stress (Gallardo et al., 2015). However, researchers discovered that this state is also transmissible to future generations (Gallardo et al., 2015). Obese women had increased levels of ROS in their oocytes/zygotes and

increased levels of oxidative stress during early gestation (Gallardo et al., 2015). In addition, researchers evaluated the presence of two oxidative stress markers, malondialdehyde (MDA) and nitric oxide (NO), in fetal blood. They found that both MDA and NO levels increased with increasing maternal body mass index (BMI); (Gallardo et al., 2015). Gallardo et al. (2015) proposes that since obesity is an inflammatory state, oxidant compounds (such as ROS) can cross the placental barrier and promote oxidative stress in the fetus.

Both maternal over- and under-nutrition can affect the fetal redox regulation system (Wu et al., 2006). For example, children born to obese mothers have an increased risk of developing metabolic alterations, such as increased synthesis of proinflammatory adipocytokines, increased risk of insulin resistance, and increased incidence of oxidative stress (Gallardo et al., 2015). It was found that maternal nutrient restriction weakens the oxidative defense system by reducing the amount of antioxidants (Wu et al., 2006). On the contrary, over nutrition allows for increased oxidation of nutrients, resulting in increased ROS production (Wu et al., 2006). However, both diets result in a more oxidized fetal metabolic state, which is prone to oxidative stress. In fact, carbohydrates and fatty acids are known to be inducers of mitochondrial stress (Igosheva et al., 2010). Therefore, it is important to consider the effects of maternal diet on fetal metabolic physiology, specifically considering mitochondrial function.

Mitochondria are solely maternally inherited, and therefore the fetal metabolic state is maternally influenced (Igosheva et al., 2010). Studies have already demonstrated that poor maternal diet alters normal fetal metabolism (Gallardo et al., 2015; Igosheva et al., 2010; Wu et al., 2006). However, it is unclear how excessive or limited metabolic function affects the whole

fetus. Studies show that increased reliance on the electron transport chain increases the production of ROS (Bhat et al., 2015). This mitochondrial overstimulation is often accompanied with decreased antioxidant presence (Bhat et al., 2015). Combined, these metabolic alterations create a vicious cycle. First, free radicals (such as ROS) attack the mtDNA structure, resulting in a damaged complex I or III of the ETC (Bhat et al., 2015). This results in NADH depletion and oxygen reduction (Bhat et al., 2015). The increased levels of ROS permit the secretion of cytochrome C and Apoptosis Inducing Factor (AIF) from the inner mitochondrial membrane, leading to apoptosis (Bhat et al., 2015). Apoptosis and the resulting oxidative stress lead to cellular injury (Bhat et al., 2015). Therefore, these micro-scale effects of mitochondrial dysfunction could, in part, result in the macro-scale, whole body fetal underdevelopment. In fact, the normal process of aging is very similar to the described abnormal metabolic aging. The “mitochondrial free radical theory of aging” states that aging results from: (1) cumulated mtDNA mutations, (2) abnormal oxidative phosphorylation, (3) imbalance in antioxidant expression, and (4) ROS overproduction (Bhat et al., 2015). The abnormal, environmentally influenced aging of cells presents a challenge because it follows a mechanism of normal aging, yet occurs regardless of animal age. Altered fetal metabolic physiology, and the subsequent premature cellular aging, could be one mechanism by which poor maternal diet restricts fetal growth and development.

Conclusion

The food animal production industry is a vital component of both national employment and the national economy. Therefore, maintaining animal health is essential to both maximizing the efficiency of production and also meeting the ever-increasing food demand. To meet these goals, researchers have considered how the quality of maternal diet affects fetal growth and

development. Several conclusions have already been made: (1) MSC health is vital for proper development of bone, muscle, and fat; (2) maternal diet influences the health of MSC, and therefore growth of the fetus; (3) abnormal ROS levels play negative roles in the health of all cells, especially MSC due to their sensitive stem cell nature; (4) current research suggests correlations between ROS and maternal diet; and (5) altered metabolic regulation can present lifelong complications in health status. Therefore, poor maternal diet and the subsequent poor fetal growth/development could possibly be explained, in part, by diet-induced mitochondrial dysfunction leading to ROS overproduction and the resulting oxidative stress. Research should be continued in order to solidify this mechanism. Efficient growth and production of animals requires normal and healthy MSC, which require proper maternal nutrition. It was hypothesized that poor maternal diet during gestation would negatively alter mesenchymal stem cell metabolism in offspring. Therefore, the objectives of this study included evaluating the glycolytic and mitochondrial functions of MSC obtained from the lambs of ewes fed with a CON, RES, or OVER diet during gestation.

MATERIALS AND METHODS

Animals

All animal experiments were reviewed and approved by the University of Connecticut Institutional Animal Care and Use Committee.

In a previous study, eighteen ewes were estrus synchronized with progesterone controlled intravaginal drug release devices (Pfizer Animal Health, New York, NY) and Lutalyse (Zoetis, Florham Park, New Jersey), followed by live-cover breeding to one of five rams (Reed et al., 2014; Hoffman et al., 2016). On d 31 ± 1.3 of gestation, individually-housed ewes were assigned to one of three diets: 100%, 60%, or 140% of NRC (1992) requirements for TDN of ewes carrying twins (Reed et al., 2014; Hoffman et al., 2016). Rations of complete pelleted feed (Central Connecticut Farmer's Co-Op, Manchester, CT) were calculated based on body weight, weekly (Reed et al., 2014; Hoffman et al., 2016). Treatment diets were maintained until parturition, after which lambs nursed colostrum for up to 24 h (Reed et al., 2014; Hoffman et al., 2016). One lamb per ewe was euthanized through intravenous injection of Beuthanasia-D Special (Merck Animal Health, Summit, NJ) containing 390 mg/mL sodium pentobarbital and 50 mg/mL phentoin based on BW, followed by exsanguination (Reed et al., 2014; Hoffman et al., 2016). Lambs from ewes fed 100, 60, and 140% of NRC will be referred to as CON, RES and OVER, respectively.

Sample Collection

At necropsy, hind legs were removed, followed by removal of wool and muscle. Left femur and tibia were collected, soaked in PBS, and transported to the laboratory for MSC isolation. After 70% ethanol rinse, epiphyses of bones were removed. In the hood, bone marrow was rinsed from bone shaft with α -minimal essential media (MEM; ThermoFisher Scientific, Waltham, MA) into a cell culture dish. This mixture was transferred to a falcon tube and centrifuged for 3 minutes at 2,000 rpm. Red blood cells (RBC) were removed by addition of ammonium chloride (Stem Cell Technologies, Vancouver, Canada), causing RBC lysis, and passage through a 70 μ m filter. The isolated MSC were counted with a hemocytometer and plated at 12 to 15 million cells per cell culture dish in maintenance media. The maintenance media consisted of α -MEM (ThermoFisher Scientific, Waltham, MA), 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, Georgia), 0.5% Penicillin Streptomycin, and 0.25% Fungizone. Media was changed every 2 to 3 d during incubation in 37°C and 5% CO₂. Cells were cultured to reach 70 to 80% adherent confluence. After centrifugation and media removal, MSC were suspended in 1 mL cell guardian (B-Bridge, Cupertino, CA), transferred to cryovials, and stored in liquid nitrogen for future studies.

Cell Culture

Mesenchymal stem cells (n = 6/treatment) in cryovials were removed from liquid nitrogen storage, thawed, and transferred to falcon tubes. The falcon tubes were centrifuged for 3 minutes at 2,000 rpm and supernatant was removed. The MSC were re-suspended in α -MEM maintenance medium previously described and plated on cell culture dishes. The MSC were cultured in α -MEM maintenance medium at 37°C and 5% CO₂. When the MSC reached 70 to 80% confluence, cell adherence to the dish was dissolved by adding a 50% trypsin, 50% PBS

solution to the culture dishes for 3 minutes at 37°C and 5% CO₂. The action of trypsin was stopped with the addition of an equal quantity of α -MEM maintenance medium. The MSC were collected following scraping using a cell scraper and centrifugation for 3 minutes at 2,000 rpm. After removal of supernatant, MSC were re-suspended in α -MEM maintenance medium, counted with a hemocytometer, and plated on Seahorse XF²⁴ cell culture plates at 30,000 cells per well. Six animals were used per treatment group (n = 6/treatment) and there were two technical replicates per animal. These culture plates were incubated for 48 h in 37°C and 5% CO₂.

Glycolysis Stress Test Assay

On the day prior to the assay, the sensor cartridge was hydrated in Seahorse XF Calibrant at 37°C and 0% CO₂ overnight. On the morning of the assay, the assay medium was prepared. Following addition of 2 mM glutamine (Sigma Aldrich, St. Louis, MO), the medium was warmed to 37°C and pH adjusted to 7.4 with 0.1 N NaOH and sterile filtered. From the kit, one vial each of glucose, oligomycin, and 2-DG was opened. The glucose, oligomycin, and 2-DG vials were re-suspended with 3000 μ L, 720 μ L, and 3000 μ L of assay medium, respectively. The solutions were pipetted up and down ten times, and the 2-DG solution was vortexed. The injection ports were loaded using the constant volume method described in the manufacturer's protocol. The XF²⁴ cell culture plate was examined under the microscope to confirm confluence. The α -MEM maintenance medium was removed with a pipette and replaced with prepared 37°C assay medium. The wells were rinsed with assay medium 2 times. The wells were then filled with 525 μ L of assay medium, and the cell culture plate was incubated for one hour in 37°C and 0% CO₂. The cell culture plate and sensor cartridge were loaded into the Seahorse

Bioscience XF²⁴ Extracellular Flux Analyzer. Following a 15 to 30 minute calibration, the lid of the sensor cartridge was removed and the assay was run. The assay was performed using Wave software. The XF²⁴ analyzer automatically injected the wells subsequently with glucose (to stimulate basal glycolysis), oligomycin (to inhibit ATP synthase and maximize glycolysis), and 2-DG (to competitively bind hexokinase and inhibit glycolysis). The analyzer quantifies glycolysis as a measure of extracellular acidification rate (ECAR) of the assay medium in the wells, since protons are extruded during glycolysis.

Cell Mito Stress Test Assay

On the day prior to the assay, the sensor cartridge was hydrated in Seahorse XF Calibrant at 37°C and 0% CO₂ overnight. On the morning of the assay, the assay medium was prepared and sterile filtered. Following addition of 1mM pyruvate (Sigma Aldrich, St. Louis, MO), 2 mM glutamine (Sigma Aldrich, St. Louis, MO), and 10 mM glucose (Sigma Aldrich, St. Louis, MO), the medium was warmed to 37°C and pH adjusted to 7.4 with 0.1 N NaOH. The foil pouch from the assay kit was removed and the reagents were allowed to warm to room temperature in the sealed pouch, for 15 minutes. The oligomycin, FCCP, and rotenone/antimycin A vials were then removed and re-suspended with 630 µL, 720 µL, and 540 µL of prepared assay medium, respectively. The reagents were pipetted up and down ten times to solubilize the compounds. The injection ports were loaded using the constant volume method described in the manufacturer's protocol. The XF²⁴ cell culture plate was examined under the microscope to confirm confluence. The α-MEM maintenance medium was removed with a pipette and replaced with prepared 37°C assay medium. The wells were rinsed with assay medium 2 times. The wells were then filled with 525 µL of assay medium, and the cell culture plate was incubated for one hour in

37°C and 0% CO₂. Following a 15 to 30 minute calibration, the lid of the sensor cartridge was removed and the assay was run. The assay was performed using Wave software. The XF^e24 analyzer automatically injected the wells subsequently with oligomycin (to inhibit ATP synthase and therefore OXPHOS), FCCP (to uncouple and therefore maximize OXPHOS), and rotenone/antimycin A (to inhibit complexes I and III of the ETC and therefore shut down OXPHOS). The analyzer quantifies OXPHOS as a measure of oxygen consumption rate (OCR) of the assay medium in the wells, since oxygen is consumed during OXPHOS.

Data Normalization

Data were normalized for total DNA per well using the Macharey-Nagel NucleoSpin® Tissue kits (Macharey-Nagel Inc., Bethlehem, PA). The procedure was followed according to the manufacturer's instructions.

Calculations

Glycolysis Stress Assay

After the ECAR measurements from the assay were normalized for total DNA, the following calculations were performed in order to evaluate the various parameters associated with glycolysis. These glycolytic parameters can be calculated from the changes in ECAR in response to the various injections of compounds that either stimulated or inhibited glycolysis. Refer to Figure 1 for the designation of measurement number.

Glycolysis: (maximum of ECAR measurements #4 - 6) minus (ECAR measurement #3)

Glycolytic Reserve: (maximum of ECAR measurements #7 - 9) minus (maximum of ECAR measurements #4 - 6)

Glycolytic Reserve Capacity: [(maximum ECAR of measurements #7 - 9) minus (ECAR measurement #3)] divided by GLYCOLYSIS

Non-glucose-derived ECAR: minimum of ECAR measurements #10 - 12

Mito Stress Assay

After the OCR measurements from the assay were normalized for total DNA, the following calculations were performed in order to evaluate the various parameters associated with OXPHOS. These OXPHOS parameters can be calculated from the changes in OCR in response to the various injections of compounds that either stimulated or inhibited the ETC. Refer to Figure 2 for the designation of measurement number.

Non-mitochondria-derived OCR: minimum of OCR measurements #10 - 12

Basal Respiration: (OCR measurement #3) minus NON-MITO OCR

Proton Leak: (minimum of OCR measurements #4 - 6) minus NON-MITO OCR

ATP Production: BASAL RESP minus PROTON LEAK

Maximal Respiration: (maximum of OCR measurements #4 - 9) minus NON-MITO OCR

Spare Respiratory Capacity: MAX RESP minus BASAL RESP

(expressed as fold increase): MAX RESP divided by BASAL RESP

Coupling Efficiency: 100 multiplied by (ATP PROD divided by BASAL RESP)

Statistical Analysis

Statistical analysis was performed using PROC MIXED in Statistical Analysis Software version 9.2 (SAS Inst. Inc, Cary, NC). Least square means were used to make mean comparisons when appropriate. Statistical significance was considered to be $P < 0.05$. Outlier analysis was performed in SAS, and outliers were removed from both the glycolysis stress and cell mito stress data sets. As a result, one OVER animal from the glyco stress test (n = 6, 6, and 5; CON, RES, and OVER, respectively) and one RES animal from the cell mito stress test (n = 6, 5, and 6; CON, RES, and OVER, respectively) were removed from the calculations and data analysis.

RESULTS

Glycolysis Stress Test

ECAR increased in all treatment groups after MSC were injected with glucose, and increased further after the injection of oligomycin (Figure 3). The injection of 2-DG decreased ECAR in all treatment groups (Figure 3). There were no significant differences in basal glycolysis, glycolytic reserve, non-glucose-derived ECAR, and glycolytic reserve capacity between treatment groups ($P \geq 0.3$; Figures 4A and 4B).

Mito Stress Test

In all treatment groups, OCR decreased with the injection of oligomycin, increased with the injection of FCCP, and decreased again with the injection of rotenone and antimycin A (Figure 5). Compared with CON, basal respiration decreased by 29% and 31% in RES and OVER, respectively (127.4 ± 7.5 , 90.2 ± 9.8 , and 87.5 ± 8.5 $\mu\text{mol O}_2^{-1} \cdot \text{minute}^{-1} \cdot \mu\text{g}$; CON, RES, and OVER, respectively; $P \geq 0.004$; Figure 6A). Additionally, ATP production was reduced 30% and 36% in RES and OVER, respectively compared with CON (121.1 ± 6.1 , 84.9 ± 11.1 , and 77.7 ± 6.4 $\mu\text{mol O}_2^{-1} \cdot \text{minute}^{-1} \cdot \mu\text{g}$; CON, RES, and OVER, respectively; $P \geq 0.001$; Figure 6A). Compared with CON, maximal respiration was reduced 39% and 55% in RES and OVER, respectively (149.3 ± 17.1 , 90.6 ± 23.8 , and 67.9 ± 10.1 $\mu\text{mol O}_2^{-1} \cdot \text{minute}^{-1} \cdot \mu\text{g}$; CON, RES, and OVER, respectively; $P \geq 0.004$; Figure 6A). Additionally, spare respiratory capacity was reduced in OVER compared with CON, but was not different between RES and CON (21.9 ± 10.8 , 0.47 ± 15.1 , and -19.6 ± 9.2 $\mu\text{mol O}_2^{-1} \cdot \text{minute}^{-1} \cdot \mu\text{g}$; CON, RES, and OVER, respectively; $P \geq 0.02$; Figure 6A). Proton leak, non-mitochondria-derived OCR, spare respiratory capacity (expressed

as a fold increase), and coupling efficiency were not different among treatment groups ($P \geq 0.07$; Figures 6A, 6B, 6C).

DISCUSSION

The effects of poor maternal diet during gestation on the development of the offspring are well documented (Oreffo et al., 2003; Wu et al., 2006; Lanham et al., 2010; Reed et al., 2014). In general, the development of bone and muscle is restricted while the development of adipose is increased in multiple species (Oreffo et al., 2003; Wu et al., 2006; Lanham et al., 2010; Reed et al., 2014). Specific effects in bone include decreased bone density, reduced bone mineral content, and reduced bone area (Oreffo et al., 2003). In muscle, Reed et al. (2014) found that altered muscle development can be attributed in part to a decrease in muscle cross-sectional area and Wu et al. (2006) noted that the proportion of muscle fiber types varies in relation to maternal diet. These studies also found that carcass adiposity in the offspring is increased in both restricted-fed and over-fed mothers, compared with control (Wu et al., 2006; Reed et al., 2014). Despite this information, the mechanism(s) connecting poor maternal nutrition during gestation to poor fetal growth and development are not established. There are most likely several mechanisms, due to the complexity of growth and development.

Due to the common origin of bone, muscle, and adipose tissues, current research focuses on the effects of poor maternal diet during gestation on MSC function (Oreffo et al., 2003; Pillai et al., 2014). The restricted diet has been found to delay the normal proliferation and differentiation of MSC, which are both required for proper development of bone, muscle, and adipose (Oreffo et al., 2003). Similarly, our laboratory previously demonstrated that the proliferation of the same MSC used in this study was reduced by 51% and 58% in RES and OVER, respectively, compared with CON ($P \geq 0.03$; Pillai et al., 2014). These proliferation data

are evidence of cellular programming. Despite culture in a standard nutrient-rich medium, the function of MSC from RES and OVER are still different from CON. Therefore, poor maternal diet during gestation appears to permanently alter MSC function. The ‘predictive adaptive response’ suggests that the purpose of *in utero* fetal programming is to maximize chances of survival once the fetus is born into a nutrient-rich or nutrient-restricted environment (Lanham et al., 2010). Therefore, the offspring of RES and OVER mothers could possibly be shifting resources away from the development of muscle and bone in order to preserve resources for processes more useful to survival, such as energy storage in adipose.

Fernandez-Twinn et al. (2010) found that malnutrition *in utero* can specifically alter metabolic physiology in order to prioritize nutrients towards more vital organs at the expense of other organs. Several studies have demonstrated that the metabolic state of a cell and its subsequent proliferative ability are correlated (Shyh-Chang et al., 2013; Lyublinskaya et al., 2015). For example, Pillai et al. (2014) found that the proliferation of MSC was reduced in RES and OVER while our current study found that MSC from RES and OVER experienced reduced basal respiration and ATP production. This suggests that poor maternal diet during gestation results in a reduced metabolic state, which is correlated with reduced proliferative ability in MSC. Shyh-Chang et al. (2013) found that metabolism is a key determinant of whether a cell proliferates, differentiates, or remains quiescent. Therefore, the observed alterations in metabolism have the potential to possibly affect the cell cycle of MSC, and either increase or decrease proliferation. Additionally, the literature suggests that stem cell metabolism differs from regular somatic cell metabolism (Shyh-Chang et al., 2013; Lyublinskaya et al., 2015).

These undifferentiated, quiescent stem cells prefer what is known as a “slow-cycling” state (Shyh-Chang et al., 2013). Therefore, MSC tend to rely more on glycolysis as opposed to OXPHOS to meet energy needs (Shyh-Chang et al., 2013). Current studies have found that poor maternal diet during gestation can alter MSC metabolism (Lyublinskaya et al., 2015). In our study, we found that MSC from RES and OVER preferred a reduced metabolic state compared with CON. Both MSC from RES and OVER experienced a reduction in basal respiration and ATP production and no change in glycolysis. Therefore, MSC from RES and OVER are overall less metabolically active than CON. Since stem cell metabolism is correlated with stem cell niche, it is important to consider the role of oxygen.

Bone marrow-derived MSC, such as the ones used in our study, are suited for a low-oxygen niche that promotes the maintenance of the undifferentiated state (Ito et al., 2014). However, the MSC used in the culture and assay conditions are no longer in this hypoxic environment and therefore the increased oxygen availability should increase oxygen utilization (Ito et al., 2014). mTOR is one such signaling pathway that up-regulates metabolism in response to the sensing of high oxygen and nutrient availability (Ito et al., 2014). Therefore, it is expected that cells maintain a greater metabolic state under increased oxygen availability. Under normoxia, the MSC from RES, OVER, and CON all experienced comparable rates of glycolysis, which is likely because oxygen is not involved in glycolysis. However, MSC from RES and OVER experienced reduced reliance on mitochondrial ATP production, compared with CON. This suggests that MSC from RES and OVER do not utilize as much oxygen for energy production via OXPHOS as CON, under normoxic conditions. Considering the relationship

between OXPHOS and cellular injury, this could possibly be a defense mechanism to avoid cellular injury (Shyh-Chang et al., 2013).

Under normoxic conditions, MSC experience increased proliferative ability but at the expense of increased senescence (Shyh-Chang et al., 2013). When proliferation is restricted, stem cell longevity is extended through decreased aging, quiescence, and stem cell exhaustion (Ito et al., 2014). We found that MSC from RES and OVER had both reduced proliferation and reduced OXPHOS, compared with CON (Pillai et al., 2014). Oreffo et al. (2013) similarly found that proliferation of MSC was reduced in protein-restricted ewes. Therefore, it is possible that MSC from RES and OVER are programmed to limit OXPHOS in order to limit ROS and preserve stem cell longevity, because the animal is preparing for birth into an anticipated unfavorable environment. There is an important balance between proliferation (and therefore increased metabolic activity) and stem cell longevity. Increased metabolic activity promotes proliferation but can cause cellular injury, especially in stem cells, through ROS and oxidative stress. However, preserving stem cell longevity through reducing metabolic activity can limit proliferation. Alternatively, the observed differences in MSC metabolism could have been due to changes in an oxygen-sensing pathway.

Hypoxia-inducible factors (HIF) regulate the ratio of glycolysis to OXPHOS in response to the amount of environmental oxygen (Agathocleous et al., 2013; Burgess et al., 2014). HIF-1 α is released during low oxygen conditions, and inhibits the TCA cycle by stimulating pyruvate dehydrogenase kinase (PDK), which phosphorylates and inactivates pyruvate dehydrogenase (PDH), an enzyme critical for the glycolysis to TCA cycle transition (Ochocki et al., 2013; Ito et

al., 2014). Under the normoxic laboratory conditions, it would be expected that HIF is not released, PDK is inactive, PDH is dephosphorylated and active, the TCA cycle continues, and mitochondrial ATP production is increased. However, we found that ATP production was reduced in MSC from RES and OVER, compared with CON. It is possible that the action of HIF, or other oxygen-sensing pathways, is altered in MSC from RES and OVER, and this hypothesis can be pursued further by studying the relative expression of HIF in these MSC.

In addition to this oxygen-sensing pathway, there are nutrient-sensing pathways that regulate the metabolic activity in response to perceived energetic deficits. In our study, we found that MSC from RES and OVER experienced a reduction in maximal respiration while only MSC from OVER experienced a reduction in spare respiratory capacity, compared with CON. This suggests that MSC from RES and OVER have a reduced ability to up-regulate ATP production during energetic deficits. This could possibly suggest an alteration in one of the nutrient-sensing pathways. Liver kinase B1 (LKB1) is a serine/threonine kinase that limits cell growth under low energy conditions, which are sensed as an increased AMP/ATP ratio (Agathocleous et al., 2013; Ito et al., 2014). The pathway works through the phosphorylation and activation of AMPK, which inhibits mTORC1 (Agathocleous et al., 2013; Ito et al., 2014). As previously described, mTOR is a proliferation pathway that is stimulated when high nutrient conditions are sensed. It appears that the metabolisms of MSC from RES and OVER are not responding appropriately to energetic deficits. These studies of maternal-fetal metabolic physiology have demonstrated that *in utero* conditions, such as poor maternal diet, can lead to permanent alterations in metabolism.

The changes in the metabolic profile of MSC observed in our study can potentially lead to the development of whole body metabolic dysfunction, and associated metabolic diseases. Wu et al. (2006) found that poor maternal diet can lead to metabolic diseases, such as insulin resistance, obesity, oxidative stress, and mitochondrial dysfunction in sheep. Some *in utero* conditions, such as poor maternal diet, appear to have permanent, irreversible changes to normal fetal physiology. Barker and Hales proposed an idea that ‘fetal metabolic programming’ is a risk factor for the development of chronic metabolic disorders (Hales et al., 2001). Therefore, continued research in the field of fetal metabolic physiology remains an important aspect of maternal nutritional studies.

In conclusion, data in our study provided some insight into the complex relationship between fetal metabolic physiology, specifically in MSC, and maternal diet during gestation. However, further studies are needed to determine the role of altered MSC metabolism as the mechanism connecting poor maternal diet to poor fetal growth and development. In order to do so, more metabolic parameters than just ECAR and OCR need to be evaluated. Future directions should focus on gene studies (mTOR pathway, LKB1 pathway, FOXO, HIF), ROS levels, and the NAD/NADH ratio. From our study, we can say that maternal over- and under-nutrition during gestation reduce the basal metabolic state of offspring MSC, and the ability of these cells to up-regulate ATP production during energetic deficits. The altered MSC metabolism could possibly contribute to impaired muscle, bone, and adipose growth and maintenance in offspring.

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FIGURES

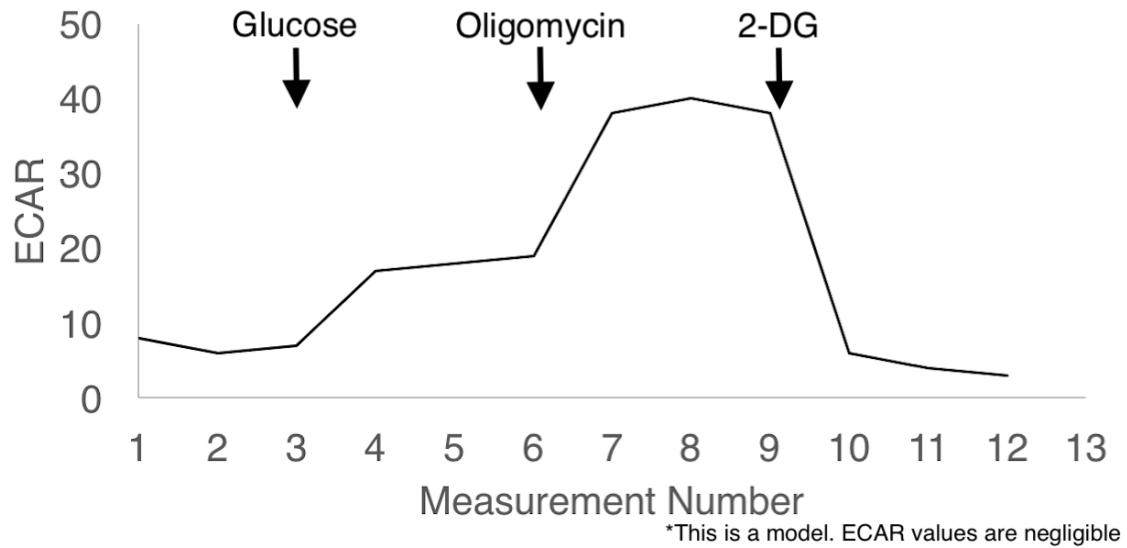


Figure 1. Model Output for Glycolysis Stress Assay. Extracellular acidification rate (ECAR) measured after cells subsequently exposed to glucose, oligomycin, and 2-deoxy-glucose (2-DG). ECAR values are negligible, as this figure is a model for data calculations. Measurements will subsequently be referred to as number 1, 2, 3, etc.

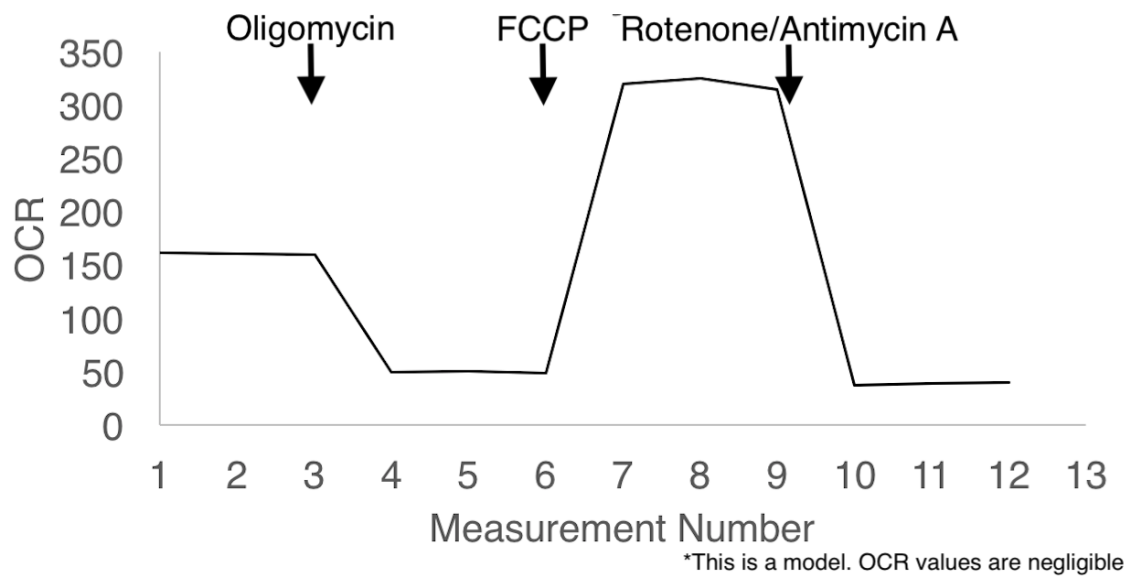


Figure 2. Model Output for Cell Mito Stress Assay. Oxygen consumption rate (OCR) measured after cells subsequently exposed to oligomycin, FCCP, and Antimycin A & Rotenone. OCR values are negligible, as this figure is a model for data calculations. Measurements will subsequently be referred to as number 1, 2, 3, etc.

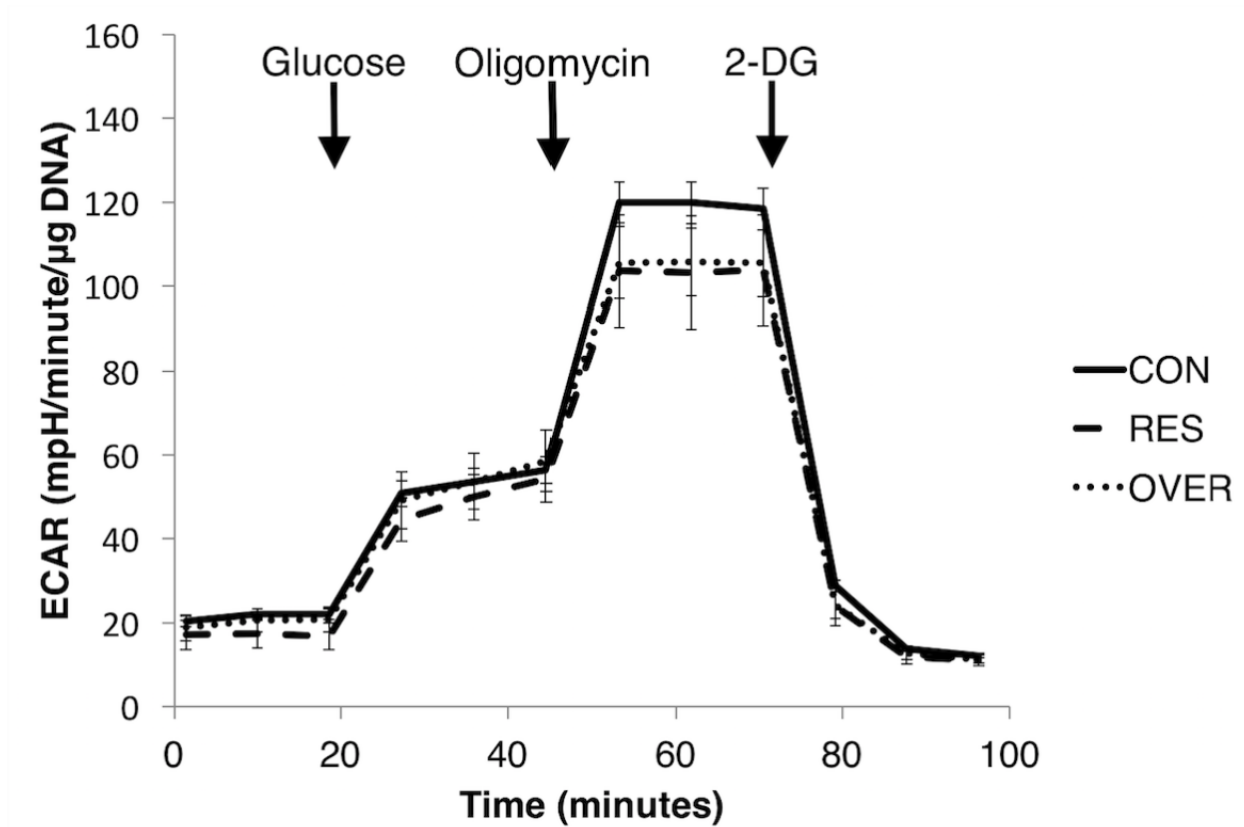


Figure 3. Glycolysis Stress Test. Extracellular acidification rate (ECAR) measured after MSC were subsequently exposed to glucose (to stimulate basal glycolysis), oligomycin (to inhibit ATP synthase and maximize glycolysis), and 2-deoxy-glucose (to competitively bind hexokinase and inhibit glycolysis). n = 6/treatment.

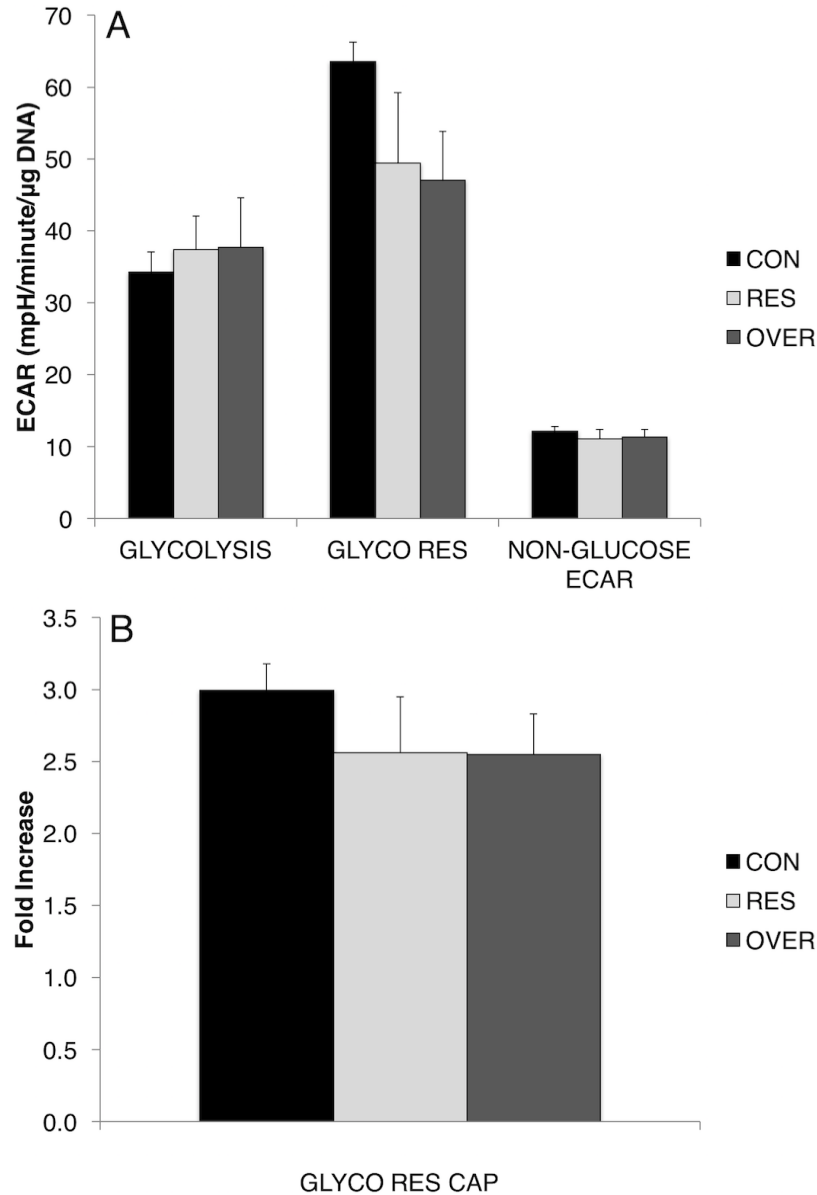


Figure 4. Glycolysis Stress Test Results. (A) Calculations of glycolysis (GLYCOLYSIS), glycolytic reserve (GLYCO RES), and non-glucose-derived ECAR (NON-GLUCOSE ECAR) in MSC. $n = 6/\text{treatment}$. Data are presented as mean \pm SE. $P > 0.05$ among treatment groups. (B) Glycolytic reserve capacity (GLYCO RES CAP) expressed as a function of fold increase. $n = 6/\text{treatment}$. Data are presented as mean \pm SE. $P > 0.05$.

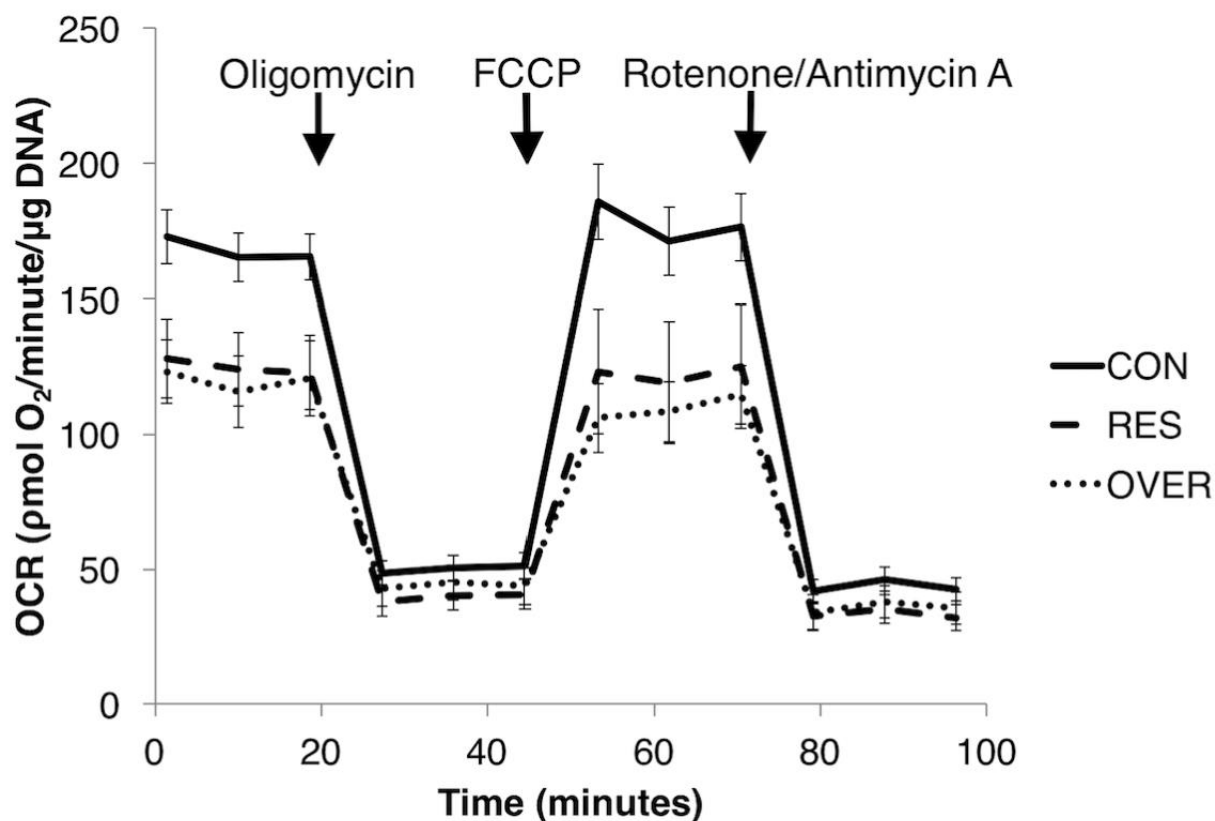
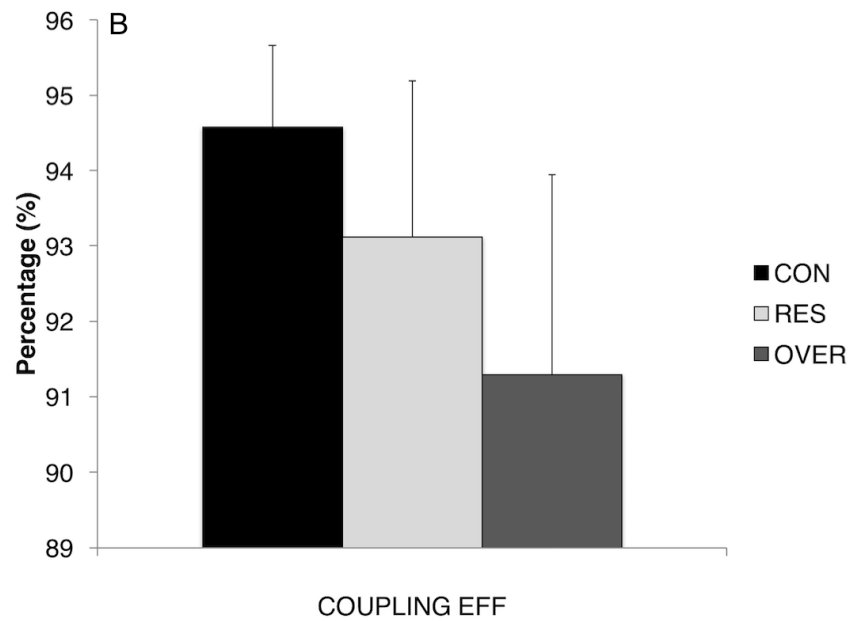
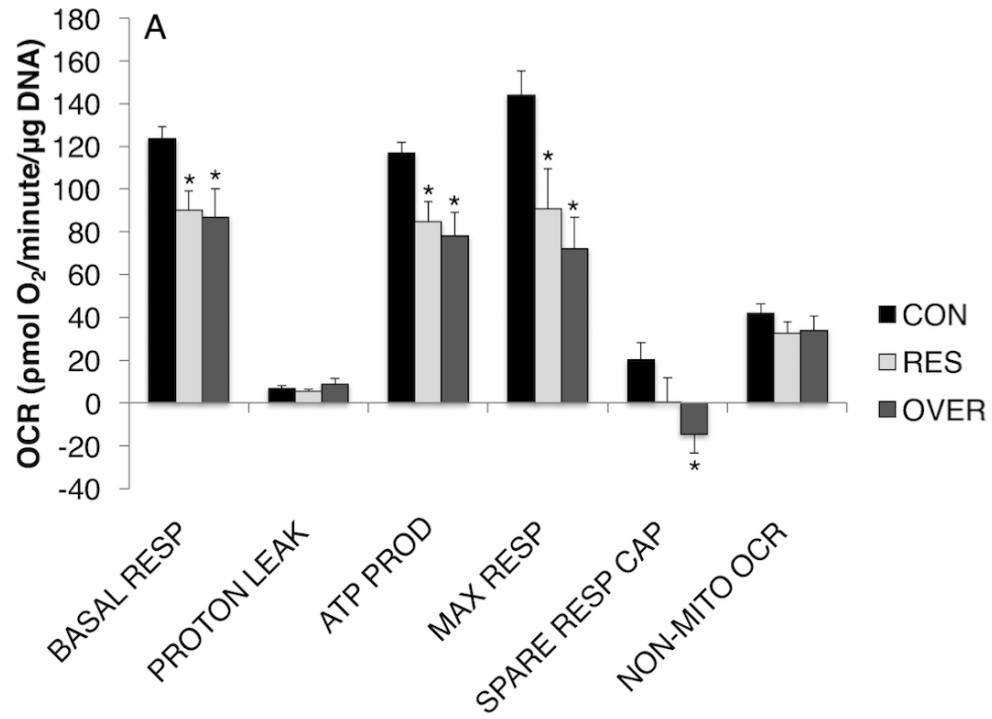


Figure 5. Cell Mito Stress Test. Oxygen consumption rate (OCR) measured after MSC subsequently exposed to oligomycin (to inhibit ATP synthase and therefore oxidative phosphorylation), FCCP (to uncouple and therefore maximize oxidative phosphorylation), and Antimycin A & Rotenone (to inhibit complexes I and III of the electron transport chain and therefore shut down oxidative phosphorylation). n = 6/treatment.



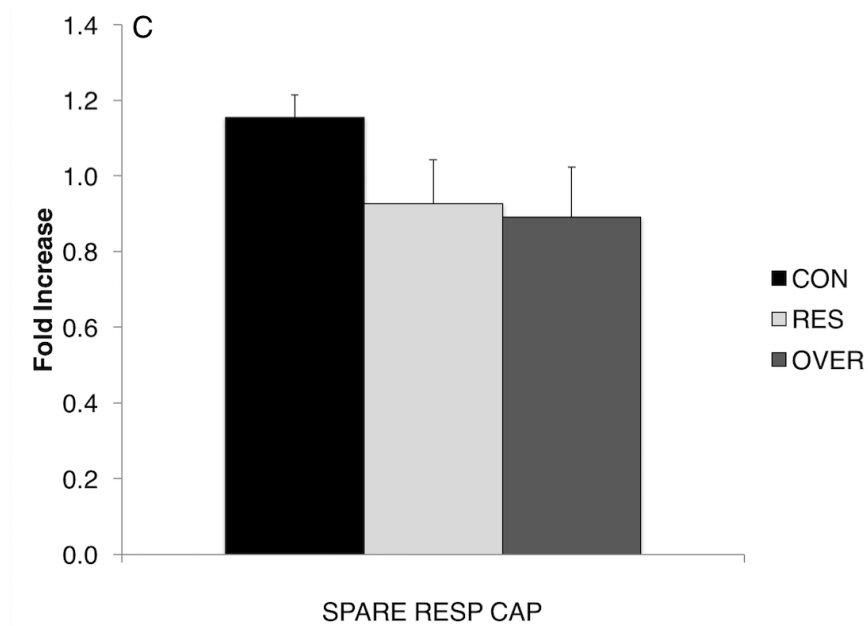


Figure 6. Cell Mito Stress Test Results. (A) Calculations of basal respiration (BASAL RESP), proton leak (PROTON LEAK), ATP production (ATP PROD), maximal respiration (MAX RESP), spare respiratory capacity (SPARE RESP CAP), and non-mitochondria-derived OCR (NON-MITO OCR) in MSC. $n = 6/\text{treatment}$. Data are presented as mean \pm SE. $*P < 0.05$ among treatment groups. (B) Spare respiratory capacity expressed as a function of fold increase. $n = 6/\text{treatment}$. Data are presented as mean \pm SE. $P > 0.05$. (C) Calculation of coupling efficiency in MSC. $n = 6/\text{treatment}$. Data are presented as mean \pm SE. $P > 0.05$.

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