Effects of Intrauterine Growth Restriction on Wharton's Jelly Cells and Preweaning Traits in Pigs

by

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B.S., University of Nebraska-Lincoln, 2010
M.S., Kansas State University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
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Abstract

Intrauterine growth restriction (IUGR) affects all mammals. In the swine industry IUGR pigs result from intrauterine crowding. Prenatal programming in IUGR pigs has substantial effects on myogenesis and adipogenesis. Prenatal programming due to IUGR is also a problem in humans and long-term effects on adipogenesis are well established for small for gestational age (SGA) babies. Mesenchymal stem cells (MSCs) are the precursors for adipocytes. The umbilical cord contains a population of MSCs in Wharton's jelly (WJ) and they can be harvested postnatally without ethical issues. Therefore, WJMSCs are proposed as models for studying prenatal programming of adipogenesis. We selected genes from studies of adipogenesis in humans and other species and examined their expression in pig WJ. We assigned pigs within litter as High, Medium, or Low birth weight and evaluated these categories for expression of *Cox1*, *Cox2*, *EGR1*, *PPARɣ1*, *PPARɣ2*, and *Pref1*. Differences due to size classification within litter were limited but there were correlations between weaning weight and delta cycle threshold (ΔCt) for *EGR1* ($r = 0.28$; $P < 0.009$), *PPARɣ1* ($r = 0.29$; $P < 0.007$), and *PPARɣ2* ($r = 0.30$; $P < 0.005$). This may be consistent with the reports for SGA babies where *EGR1* is upregulated by prenatal growth restriction. To gain insight into when during pregnancy IUGR affects WJ cells we collected umbilical cords at d 60 and d 95. In d 60 umbilical cords, small fetuses had increased ($P = 0.06$) *Cox1* gene expression. We tested the ability of d 60 WJ cells to undergo adipogenic differentiation using standard protocols and a cycling protocol that exposed the cells to adipogenic differentiation conditions interposed with a rest phase with high insulin. It has been reported that the cycling protocol revealed increased glucose uptake in WJ cells from human SGA babies. We found that d 60 WJ cells did not show adipogenic differentiation in any of the protocols tested however glucose uptake correlated negatively with birth weight at Cycle 0

($P < 0.02$; $r = 0.61$). In summary, pig WJ cells reveal some effects of IUGR but they appear to differ from the relationship demonstrated reported for human SGA babies. A new finding was that at midgestation pig WJ cells do not appear to be competent to complete adipogenesis.

We also studied nursing managements to improve outcomes for IUGR pigs. Colostrum intake may be a problem, particularly for light weight pigs and those born later during farrowing. Split suckling is the removal of some pigs to allow others unrestricted nursing access. We temporarily removed the six heaviest pigs and this treatment increased gain and weight by d 7 of age. Colostrum intake was highest for the high birth weight pigs. When we temporarily removed the first half of the litter, colostrum intake was increased for the second half of litter born and the difference in immunocrit was reduced between the two litter halves.

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Major Professor
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Dedication

To my family; my parents, grandparents, and siblings, for your love, support, and guidance along the way. To my nephew and nieces, Charlie, Isabella, Dream, and Vatalia Rainbow, may you follow your dreams and never give up on what you want in life.

Chapter 1 - General Review of the Literature

The developmental origin of health and disease (DOHaD) describes the effects of the *in utero* environment on long term health and physiological characteristics of placental mammals (Langley-Evans 2006; Rinaudo and Wang 2012). An understanding of DOHaD can help reduce the global epidemic of obesity and metabolic diseases in humans (Mraz and Haluzik 2014). Developmental origin of health and disease also has important implications for production of food animals, particularly in pigs where the large litter sizes increase the incidence of light birth weights (Foxcroft et al., 2006).

In humans, family income, diet, and lifestyle affect DOHaD. Low birth weight offspring can result from environmental and maternal factors. After birth, affected offspring have relatively greater postnatal growth when compared to normal sized offspring. This compensatory growth consists mostly of adipose tissue (Symonds et al., 2009; Okada et al., 2015). Understanding the adipogenesis pathway and its alterations during development and obesity could be key to finding ways to fight obesity (Zhu et al., 2016).

In livestock, low birth weight offspring are more prevalent in polytocous species, but also occur in single-offspring species (Gonzalez-Bulnes et al., 2016). The smallest pigs at birth often don't reach market weight. However, they take resources that could be used by more profitable pigs.

The increase in number of pigs/litter has brought with it an increase in within litter birth weight variation. This is due to limited uterine capacity and placental insufficiencies for individual pigs (Foxcroft et al., 2006; Louveau et al., 2016). Understanding how the *in utero* environment affects intrauterine growth restricted (IUGR) pigs could lead to methods to reduce the effects of growth restriction (Gondret et al., 2011; Gondret et al., 2013). For pork production,

this could mean increased survivability and more full value pigs reaching market. This review is first directed at DOHaD in humans to consider the large volume of information available on the mechanisms and effects in babies.

Developmental Origin of Health and Disease (DOHaD)

The First 25 Years

The understanding that the *in utero* environment affects a person after birth and throughout one's adult life originated in the mid-1980s. David Barker evaluated epidemiological evidence to determine the risk of adult diseases in England and Wales based on socioeconomic status and location (Langley-Evans 2006). Using country wide data, he found a regional correlation between infant mortality in 1921-1925 and ischemic heart disease in 1968-1978 (Wadhwa et al., 2009). A fetus adapts to its environment and if the fetus is restricted in its supply of nutrients, its body will adjust for a similar environment after birth. When fed a nutrient rich diet after birth, the mismatch results in the inability to completely adjust to the postnatal environment and the risk for metabolic diseases increases (Langley-Evans 2006; Rinaudo and Wang 2012). In humans, low birth weight alone can be an identifier for problems in adult life. Low birth weight humans have a greater risk for coronary heart disease, non-insulin dependent diabetes, high blood pressure, obesity, and risk for metabolic syndrome (Langley-Evans, 2006). However, birth weight is a simplistic measurement and does not consider other identifiers of adulthood diseases. Thinness, decreased abdominal circumference, and altered head to body ratio are also indicators of restricted prenatal nutrition (Langley-Evans, 2006).

For humans, cohort studies provide most of the data on DOHaD because designed experiments that harm or reduce caloric intake to negatively affect unborn children would be unethical. The Dutch Famine is the best known cohort study due to a limited food supply in a

normally nourished society. The Famine lasted from December 1944 to April 1945 (Langley-Evans, 2006; Rinaudo and Wang, 2012). There was severe caloric reduction, but pregnancies were still occurring. Food rations for adults dropped to 400 calories per day and pregnant women had less than 1000 calories per day (Painter et al., 2008). Hospital and midwife training schools kept thorough records of the babies that were gestated during the famine and provided data for cohort studies.

Nutrient restriction at different times during gestation had different impacts on the offspring. If the mother had a reduced nutrient intake during her first or second trimester, birth weight did not decrease. However, restriction in late gestation resulted in babies that weighed 300g less (Lumey, 1992; Lumey et al., 2007). Intelligence was not altered, but obesity rate doubled for babies affected in early or midgestation (Lumey et al., 2007).

The mechanisms of birthweight and risk of adult diseases have not been completely determined. Proposed mechanisms are altered cell numbers, cell type, alteration of the hypothalamic-pituitary-adrenal axis, increased glucocorticoid and endocrine sensitivity, changes in epigenetic regulation, and reduction in mitochondrial function and oxidative activity (Symonds et al., 2009).

Heijmans et al. (2008) looked at epigenetic differences in offspring that were gestating *in utero* during or after the Dutch Famine occurred. Insulin-like growth factor II (*IGF2*) differentially methylated region (DMR) methylation was altered in offspring exposed to the famine early in gestation, while late gestation restriction caused no detected epigenetic changes (Heijmans et al., 2008). Reduced nutrient intake altered DNA methylation of leptin, guanine nucleotide binding protein, alpha stimulating subunit (GNAS) in blood cells along with *IGF2* (Ratnasingham et al., 2017).

Critical Windows of Development

There are important periods during gestation when the offspring are vulnerable to intrinsic and environmental factors. At these times, nutrient restriction or over nutrition can have a detrimental effect on the offspring's development. The embryo stage in large mammals is when organ buds and the placenta are developing (Symonds et al., 2009). Offspring resulting from early nutrient restriction have a greater incidence of atherogenic lipid profiles, obesity, metabolic diseases and a threefold increase in cardiovascular disease although their body size is not affected (Symonds et al., 2009). In midgestation there is rapid growth of the placenta, tissue remodeling, and fetal hyperplasia (Symonds et al., 2009). During the Dutch Famine, reduced calories during midgestation did not affect birth weight or body conformation, but offspring were more likely to have moderately increased albuminuria, kidney disease and obstructive airway disease (Symonds et al., 2009). Late gestation is when most of the fetal growth occurs. Offspring with nutrient restriction during late gestation have decreased adipocytes, altered IGF1 profiles, hyperinsulinemia, and glucose intolerance. These babies were thin, short and had smaller head circumferences (Symonds et al., 2009).

Epigenetic changes usually happen during critical periods of embryo development when cells are rapidly dividing. These are the times that cellular injury, alterations of the epigenome, reduced cellular fitness, function or capacity can occur. Stem cells reside in niches of some if not all tissues. Reducing the cellular fitness of stem cell populations could lead to early senescence and reduction of cellular division (Einstein et al., 2010). Reducing the stem cell niche, and affecting its role in cellular maintenance, could lead to postnatal age-related problems at an earlier time or a higher prevalence in growth restricted offspring.

2010 to Present

Preventative methods to reduce DOHaD in offspring focus on nutrition, physical activity, and tobacco and alcohol use as adults (Heindel et al., 2015). Current understanding is that an overall healthy lifestyle needs to start at a young age. When mothers are obese, it can lead to increased birth weights. There is a U shaped effect for birth weight, with both high and low birth weight children having increased risk for adult diseases (Ratnasingham et al., 2017). Mothers who are overweight, obese, have altered glucose tolerance, or gestational diabetes are more likely to have a large for gestational age (LGA) baby. Large for gestational age babies have a higher prevalence for childhood obesity and for developing metabolic syndrome by age 11 (Nicholas et al., 2016).

In modern societies the diet often is not balanced for nutrient intake and there is a high caloric intake on many days. Most research has focused on prenatal programming due to nutrient restrictions. However, in developed countries, maternal over nutrition also affects the offspring. Animal models of macronutrient intake have shown that the types of food the mother eats while pregnant affects her offspring (Kereliuk et al., 2017). Maternal diets that restrict protein cause growth restriction of the cranium and organs with brain and heart sparing. There is increased blood pressure, insulin sensitivity, fasting insulin, lipid levels and reduced number of muscle fibers, altered cardiac function, altered gene expression of the glucocorticoid receptor and peroxisome proliferator-activated receptor α (*PPARα*) in the liver (Vickers, 2014; Kereliuk et al., 2017). Diets with increased simple sugars can alter glucose sensitivity and insulin tolerance (Kereliuk et al., 2017).

A low carbohydrate diet has become popular, but it can also be detrimental to offspring. Low carbohydrate diets during early gestation increase methylation of the retinoid X receptor in umbilical cord tissue. Children with increased retinoid X receptor expression have increased

adiposity. The retinoid X receptor is found in adipocytes and is stimulated by the peroxisome proliferator-activated receptor ɣ (*PPARɣ*). The result can be increased rate of adipogenesis in early development due to increase the total number of adipocytes (Ratnasingham et al. 2017).

High fat in maternal diets can lead to problems for both the mother and the offspring. Increased percentage of body fat, a decline in cardiovascular health, insulin resistance, type-2 diabetes, and altered methylation and gene expression of dopamine and opioid related genes were seen in offspring of mothers consuming high fat diets (Vickers, 2014; Kereliuk et al., 2017). The increase in adiposity is expressed predominately as visceral fat in the offspring (Nicholas et al., 2016). Having dopamine and opioid related genes altered by a high fat diet can lead to changes in appetite and increased preferences for unhealthy foods (Vickers, 2014). When ewes were obese during gestation and their lambs were fed *ad libitum* after weaning, the lambs had increased appetite, adiposity, and growth rates. The changes in body composition were associated with changes in insulin sensitivity and glucose tolerance (Nicholas et al., 2016).

Nutritional status of both parents can affect the offspring. The developmental origins of health and disease is transgenerational and both germline and somatic inheritance of epigenetic modifications leads to phenotypic changes (Vickers, 2014). In mice, paternal obesity was shown to alter metabolic profiles of offspring for up to two generations (Vickers, 2014). Maternal transgenerational effects have been seen resulting from high fat diets and obesity of the mother and maternal grandmother. High fat diets increased the rate of obesity and epigenetic modifications in adipose tissue and the livers of mice (Nicholas et al., 2016).

Offspring usually (but not always) have diets comparable to their parents. In the Dutch famine, adult offspring of fathers exposed to the famine had greater weights and body mass indexes (BMI) (Veenendaal et al., 2013). This could result from learning about diet and exercise

from their parents and following that pattern into adulthood. However, the study did not find a transgenerational effect on the health of these children as adults (Veenendaal et al., 2013).

Catch-Up Growth

In humans, the terms small for gestational age (SGA), low birth weight (LBW), and fetal growth restricted (FGR) are often used interchangeably. Small for gestational age and LBW are defined as two standard deviation below the mean birth weight, or in the 10th percentile of birth weight, based on gestational age (Okada et al., 2015). Fetal growth restriction can be symmetrical or asymmetrical. Symmetrical FGR occurs when the whole animal and organ weights are restricted uniformly. This is usually due to disease or genetics (Gonzalez-Bulnes et al., 2016). Asymmetrical FGR occurs when some of the animal's organs, or its overall growth, are affected more severely and usually there is a sparing on the brain. This is also known intrauterine growth restricted (IUGR) (Gonzalez-Bulnes et al., 2016). Not all LBW offspring are IUGR and not all IUGR have LBW. Low birth weight refers to size at birth, not the pattern of growth *in utero* (Verkauskiene et al., 2007). Intrauterine growth restricted offspring can occur naturally or due to maternal nutrient restriction. Infants diagnosed as IUGR are predicted to have a higher incidence of metabolic disorders in their adult lives (Tosh et al., 2010). Small for gestation age infants are shorter, lighter, thinner, have smaller head circumferences, and have lower muscle mass, fat mass, and bone mineral density (Verkauskiene et al., 2007).

The SGA or IUGR offspring seems to be prepared for a postnatal environment that is similar to the *in utero* environment. When there is adequate nutrition postnatally, offspring even though born with a lower weight, have increased rate of growth particularly in adipose tissue. Thin babies lack muscle mass and the catch-up growth disproportionally consists of adipose tissue (Eriksson et al., 1999). Subcutaneous fat is mainly affected in catch-up growth, while no

changes are seen in the intra-abdominal adipose tissue (Symonds et al., 2009; Okada et al., 2015). Children that experience catch-up growth have a higher body mass index, insulin-like growth factor I levels, and fasting glucose levels, while adiponectin values are lower compared to normal birth weight children (Okada et al., 2015).

Catch-up growth is rapid and usually linked to metabolic syndromes, type-2 diabetes and cardiovascular diseases (Symonds et al., 2009, Tosh et al., 2010; Okada et al., 2015). There are some thoughts that the rapidity of weight gained is more important to adverse effects than the timing of catch-up growth. Leunissen et al. (2009) found that if catch-up growth happens in the first three months of life, children have higher risks for metabolic syndromes at early adulthood than those with slower growth rate. Having slow growth, followed by rapid growth at age 2, can cause cardiovascular disease and type 2 diabetes (Leunissen et al., 2009). In sheep, the rapid catch-up caused inflammation, endoplasmic reticulum stress, and altered insulin signaling in perirenal adipose tissue (Symonds et al., 2009).

The activating mechanism that links catch-up growth and adult diseases is unclear. Small for gestation age babies seem to have a delay in adipose cell hyperplasia in subcutaneous tissue, while the adipose cells ofnormal weight infants undergo hypertrophy in the first few months of life (Okada et al., 2015). A possible explanation is that FGR leads to limited cell numbers while *in utero*, then after birth the catch-up growth is due to extensive cell hypertrophy in adipose tissue (Eriksson et al., 1999).

Alleviating the effects of postnatal catch-up growth for SGA and IUGR offspring has been attempted. Small for gestational age babies benefit from breastmilk by having lower adiposity, decreased development of type 2 diabetes, and a lower incidence of cardiovascular disease compared to formula fed babies (Leunissen et al., 2009; Okada et al., 2015). In rats,

Tosh et al. (2010) compared the offspring of ad libitum and restricted diet fed mothers. Postnatally, IUGR pups were allowed to nurse on *ad libitum* or restricted diet fed mothers. Pups that were IUGR and nursed on *ad libitum* fed mothers had catch-up growth, while IUGR pups nursed on restricted fed mothers showed delayed catch-up growth (Tosh et al., 2010).

Adipose Tissue

Adipose tissue is a metabolic organ for energy storage, homeostasis and insulin sensitivity (Iñiguez et al., 2009; Lefterova and Lazar, 2009). There are two main types of adipose tissue, white and brown. White adipose tissue stores energy and releases lipids and fatty acids, while brown adipose tissue has a role in thermoregulation and is usually found in newborns (Lefterova and Lazar 2009; Smith and Kahn 2016). White adipose tissue has multiple depots. These different anatomical locations have differences in gene expression and physiological characteristics (Lefterova et al., 2014). Adipose tissue is composed of multiple cell types: white and brown adipocytes, fibroblasts, inflammatory cells, vascular cells and mesenchymal stem cells (Gustafson et al., 2007).

Adipokines are secreted from adipose tissue and help regulate physiology including appetite, glucose metabolism, reproduction, inflammatory responses, blood pressure, and angiogenesis (Lefterova and Lazar, 2009). Adipokines affect immune cells in adipose tissue, and throughout the body they affect metabolic organs to regulate lipid and glucose homeostasis (Cao, 2014). The adipokines are leptin, adiponectin, resistin, tumor necrosis factor alpha (*TNFα*), aP2, and IL6 (Cao, 2014).

Adiponectin facilitates fatty acid oxidation. During obesity and diabetes type II, it helps decrease insulin resistance (Gustafson et al., 2007; Liu et al., 2012). Plasma concentrations of adiponectin are inversely related to the mass of adipose tissue and are correlated with insulin

sensitivity (Tontonoz and Spiegelman, 2008). Leptin regulates body weight by signaling nutritional status to the hypothalamus and other organs. The hypothalamus secretes neuropeptides and neurotransmitters that control food intake and energy expenditure. Tumor necrosis factor alpha stimulates lipolysis which increases release of free fatty acids, and in turn increases *TNFα* production in macrophages in adipose tissue. Tumor necrosis factor alpha increases leptin receptor expression, which may increase circulating leptin. Resistin seems to be a suitable marker for the identification of metabolic diseases. IL6 is needed for glucose metabolism and metabolic homeostasis for the whole body, increased IL6 positively correlates to insulin resistance and obesity. Circulating aP2, a lipid activated adipocytokine of the fatty acid-binding protein family, may factor into human metabolic syndrome, plasma levels have been associated with obesity and metabolic syndrome for multiple ethnicities (Cao, 2014). Adipokines are involved in adipogenesis and could influence obesity and health related issues related to obesity.

Adipogenesis

The commitment of pre-adipocytes to become mature adipocytes is the process of adipogenesis. Pre-adipocytes undergo a morphological change from fusiform to round, lipid filled cells. Differentiation into adipocytes includes morphological transformations, lipid accumulation, cell arrest, and attainment of insulin sensitivity and expression of adipokines (Gustafson et al., 2007). Research using cell cultures has identified the cell types starting from mesenchymal stem cells, that can differentiate into multiple lineages. Stem cells proliferate and then become committed to pre-adipocytes that can proliferate or differentiate into the adipogenic lineage. Preadipocytes will stop proliferation due to contact inhibition and are growth-arrested preadipocytes. With hormonal stimulation the growth-arrested cells re-enter the cell cycle and

undergo cell division again. Through upregulated gene expression the cells again will stop dividing and go into terminal differentiation. When the cells are differentiated into adipocytes they are round and have a large lipid droplet (Lefterova and Lazar 2009). The lipid droplet is important in energy homeostasis. It takes up most of the cell volume and is near the endoplasmic reticulum and mitochondria which are the locations of triglyceride esterification and hydrolyzation (Rutkowski et al., 2015).

A cascade of gene expression is needed to convert pre-adipocytes to adipocytes by activating the early transcription factors CCAAT/enhancing binding protein beta (*C/EBPβ*), *C/EBPδ,* and *PPARɣ* (Gustafson et al., 2007). Peroxisome proliferator-activated receptor gamma is the key regulator in adipogenesis and no other element is able to promote adipocyte differentiation (Tontonoz and Spielgelman 2008; Lefterova and Lazar 2009). Peroxisome proliferator-activated receptor gamma promotes fatty acid absorption, regulates lipid deposition and during adipogenesis activates the metabolic program (Liu et al., 2011; Lefterova et al., 2014). Peroxisome proliferator-activated receptor gamma expression varies among the different adipose tissue depots and this gives each depot its own individual physiological properties (Lefterova et al., 2014). There are two isoforms of *PPARɣ*, *PPARɣ1* and *PPARɣ2*. Peroxisome proliferator-activated receptor gamma 2 has an additional 30 amino acids compared to *PPARɣ1* and is more potent in starting differentiation. Peroxisome proliferator-activated receptor gamma 2 is almost exclusively limited to adipocytes (Tontonoz and Spiegelman 2008; Lefterova and Lazar 2009).

Expression of PPARɣ has to be maintained in adult adipocytes or apoptosis occurs with insulin resistance and lipodystrophy, along with a repopulation of pre-adipocytes (Tontonoz and Spielgelman 2008; Lefterova and Lazar 2009). In fetal pigs, *PPARɣ* and *C/EBPα* are already

found in adipose tissue prior to adipogenesis and both genes need to be expressed for differentiation of pre-adipocytes (Hausman, 2003; Lefterova et al., 2014). CCAAT/enhancing binding protein alpha is induced later in adipogenesis and of the C/EBPs, it is more abundant in mature adipocytes, so the cells can utilize insulin-dependent glucose uptake (Lefterova and Lazar 2009). Studies have shown that *PPARɣ* expression in *C/EBPα* null cells stimulates normal levels of adipogenesis, but the cells have low insulin sensitivity (Tontonoz and Spiegelman 2008).

Adipocyte numbers in humans increases during childhood and adolescence, but in adulthood of both lean and obese individuals, the adipocyte numbers are level. Obese individuals that undergo weight loss have reduced volume but not number of adipocytes. Adipocyte turnover is similar in lean and obese adults with half of all adipocytes replaced every 8.3 years (Spalding et al., 2008).

Adipocytes and Obesity

Obesity is a global epidemic. The World Health Organization estimated in 2011 that 500 million people around the world were considered obese. By 2030, that number could double. Obesity is an important disease to understand because it is linked to metabolic syndrome and type 2 diabetes, arterial hypertension, dyslipidemia, and with cardiovascular and cerebrovascular mortalities (Mraz and Haluzik 2014). In obese individuals, insulin resistance reduces the inhibition of lipolysis by insulin and increases fatty acid and glycerol release (Smith and Kahn, 2016). Excess energy from the diet or de novo lipogenesis is stored in the lipid droplets of adipocytes as triglycerides (Ruthowski et al., 2015). Adipokine expression and white adipose tissue mediated disposal of dietary glucose and lipids are unregulated and increase hyperglycemia, hyperlipidemia, and chronic inflammation (Lefterova and Lazar 2009). This can be intensified by lipid deposition in non-adipogenic tissues, in particular the liver (Ruthowski et

al., 2015). In obese individuals, enlarged adipocytes are surrounded by more macrophages compared to healthy weight individuals (Gustafson et al., 2007). The influx of macrophages brings increased production of inflammatory mediators that influence insulin resistance (Tontonoz and Spiegelman 2008). Normal weight mice have 5-10% of their macrophages in their adipose tissue, but obese mice can have up to 50% of their total macrophages in adipose tissue (Mraz and Haluzik 2014).

Adipose tissue can make up to half the weight of obese individuals (Zhu et al., 2016). The site of adipose tissue depots can greatly influence overall health status of an individual. Subcutaneous fat is the largest fat depot, and the least harmful storage site for lipids (Smith and Kahn 2016). Subcutaneous adipose tissue from obese donors has impaired cell proliferation, reduced numbers of cells positive for mesenchymal stem cell surface markers CD54 and CD90, and reduced ability for chondrogenic differentiation, while osteogenic and adipogenic potential for differentiation is still present. (De Girolamo et al., 2013; Zhu et al., 2016). Ectopic fat depots in the liver, heart, and muscle are linked to insulin resistance and obesity. Pericardial and visceral fat also detrimental to human health and correlate positively with the amount of ectopic fat in the body (Smith and Kahn 2016). Measuring abdominal circumference is used as an indicator for both visceral and ectopic fat depots.

Intrauterine Growth Restricted and Low Birth Weight Pigs

Development *in utero*

Polytocous mammals are more likely to have IUGR offspring and pigs have one of the highest rates of LBW and IUGR offspring (Foxcroft et al., 2006; Amdi et al., 2013). This appears to be due to genetic selection for increased total number of pigs born. Economically, more pigs per litter is important, but pigs need to make it to market to be of value and the

efficiency of their growth and the value of the carcasses they produce must be considered. Factors that increase litter size are increased ovulation rate, embryonic survival, and uterine capacity. Uterine capacity is the number of fully formed fetuses a gravid uterus is able to support to term. The production of IUGR pigs detracts from the value of increased litter size in pork production (Wang et al., 2017). Pigs per litter has increased, but uterine capacity has not increased to the same extent (Foxcroft et al., 2006). With more pigs born, more are low birth weight and an increase in within litter variation of birth weights is observed (Foxcroft et al., 2006).

The uterine capacity limitation is apparent as early as gestation d 25 and was reported for any litter that has 14 or more embryos (reviewed by Foxcroft et al., 2006). Gestation d 30-40 is the critical window of uterine capacity limitation, and by d 44, size of fetuses will vary due to intrauterine crowding. Current maternal line sows and gilts often ovulate 20 or more oocytes and this leads to between 40 and 60 percent prenatal loss during the total gestation. There is a competition for space in the uterus to develop placentas for fetal/maternal exchange of nutrition and oxygen (Foxcroft et al., 2006; Gonzalez-Bulnes et al., 2016). Location in the uterus can exacerbate growth retardation by d 70 of gestation and fetal weight seems to decrease linearly from the utero-tubal junction to the cervix (Wang et al., 2017).

Intrauterine growth restricted pigs usually exhibit brain sparing. Brain sparing is the limiting of nutrients supplied to non-brain tissues such as liver and kidneys, so that the brain is less restricted in its nutrient supply. An important mechanism is alteration of blood flow (Bauer et al., 2003; Gonzalez-Bulnes et al., 2016). The result of brain sparing is smaller organs in IUGR pigs and even a decrease in the number of nephrons in the kidneys (Bauer et al., 2003).

Muscle fiber development starts around d 35 gestation. This is when the first effects of intrauterine crowding are observed (Foxcroft et al., 2006). Secondary myogenesis begins around d 55 of gestation. Secondary muscle fibers development is limited in IUGR pigs (Foxcroft et al., 2006). At gestation d 110, LBW pigs had increased expression of preadipocyte factor 1 (*Pref1*) in their *longissimus dorsi* muscle, which could be attributable to the delay of muscle fiber development in these pigs (Perruchot et al., 2015). Limiting muscle fiber development and delaying myogenesis may cause cells to default to the adipogenic pathway or become connective tissue. In late gestation, LBW pigs have increased amounts of collagen I and adipose tissue in their muscle (Karunaratne et al., 2005).

Pigs are born with one to two percent of total body weight as adipose tissue (Louveau et al., 2016). Adipogenesis starts in subcutaneous tissue during gestation d 50-75, while perirenal adipose tissue begins to develop around d 75 of gestation (Louveau et al., 2016). By d 60, lipid deposits are found in adipose cells. Adipose tissue of low birth weight pigs at d 75 of gestation has higher expression of *Pref1* than adipose tissue in their heaviest littermates, while LBW pigs' gene expression of *PPARɣ* is lower. This suggests that adipose tissue in LBW pigs is less differentiated compared to normal birth weight pigs (Gondret et al., 2011).

Gestation diets for sows and gilts may be inadequate for the dam and fetuses. The nutrient supply could be limited by dietary amino acids or the total amount of feed (Wang et al., 2017). Restricted nutrition in early development alters organ development, while late gestation restriction causes an overall reduction for pig birth weight (Ji et al., 2017). Maternal nutrition has a larger effect on secondary muscle fibers than on primary muscle fibers (Foxcroft et al., 2006). Supplementation with L-arginine has been shown to negate the effects of intrauterine crowding (Gondret et al., 2011). The larger pigs *in utero* take resources from other pigs, increasing within

birth weight variation (Gonzalez-Bulnes et al., 2016). Excessive feed and energy levels in the maternal diet does not improve muscle weight (Perruchot et al., 2015). Maternal diets with protein levels outside recommended values (> 70% or > 115%) result in no effect on muscle weight and reduced adipose tissue at birth (Gondret et al., 2013; Perruchot et al., 2015).

Birth to weaning

The percentage of newborn pigs that are considered LBW (< 1.11kg) range from 15-25% (Feldpausch et al., 2016; Wang et al., 2017). Mortality is increased for LBW/IUGR pigs. Stillborn rate is 35% for pigs if they weigh < 0.8 kg (Wu et al., 2006). Preweaning mortality of 76% is reported for pigs weighing < 1.10 kg at birth (Ji et al., 2017). Intrauterine growth restricted pigs can have respiratory, neurological, and circulatory problems, compromised immunity, gastrointestinal tracts, and metabolic functions (Wu et al., 2006; Gonzalez-Bulnes et al., 2016). Altered gastrointestinal tracts in small pigs may affect absorption of immunoglobulins from colostrum (Gonzalez-Bulnes et al., 2016). In general, the increase in total pigs born alive makes it difficult for all pigs in the litter to receive an adequate amount of colostrum for immunity. Sows that are over or underfed during gestation can have altered colostrum quality and quantity (Wu et al., 2006).

Colostrum is the source of immunoglobulin G (IgG) for immunity, energy, development of the gastrointestinal and reproductive tracts and blood glucose regulation. Colostrum is needed by newborn pigs. Adequate intake of colostrum is especially a problem for LBW pigs, those late in the birth order, and low viability pigs (Devillers et al., 2004; Campbell et al., 2012; Vallet et al., 2016). De Vos et al. (2016) concluded that an adequate amount of colostrum for piglet survival is 150g/kg of body weight. Intrauterine growth restricted pigs ingest on average 100g of colostrum/kg of body weight (Amdi et al., 2016). Estimation of colostrum intake can be

impractical for farms because of the need for excess handling, time, invasive procedures and expense (Devillers et al., 2004). The immunocrit method has been used as a quick, inexpensive way to determine if a pig ingested a sufficient amount of colostrum. This is done by collection of < 1ml of blood and evaluating IgG precipitated in serum. Low immunocrit values are correlated with limited stomach fill and poor survival in pigs (Vallet et al., 2016). The correlation of colostrum IgG concentration and pig serum immunocrit is done at 14-h after birth of the first pig (Vallet and Miles, 2017).

Pigs do not have brown adipose tissue which is found in offspring of other mammals and regulates thermogenesis (Louveau et al., 2016). Instead, pigs use energy reserves in the form of glycogen in the liver and skeletal muscles (Gondret et al., 2011). Pigs are born with limited white adipose tissue and LBW pigs have even less adipose tissue compared to normal size pigs. After birth, LBW pigs often experience catch-up growth which is largely adipose tissue. The catch-up growth is thought to reflect delayed development of adipose tissue (Louveau et al., 2016, Chen et al., 2017). Low birth weight pigs have increased levels of *Pref1* 2 d postnatally in their dorsal subcutaneous adipose tissue (Gondret et al., 2013). At d 7, the subcutaneous adipose tissue of LBW pigs has decreased gene expression of fatty acid binding protein 4 (*FABP4*) and downregulation of *PPARγ* compared to normal sized pigs (Gondret et al., 2011). This downregulation suggests that there is a delay in adipogenesis for these pigs.

Secondary myofiber development is affected by the delayed development (Perruchot et al., 2015). Low birth weight pigs at birth have a lower number of total muscle fibers and lower ratio of secondary to primary muscle fibers compared to normal sized littermates. The muscle fibers also have increased expression of embryonic myosin heavy chain (*MyHC*) compared to adult fast *MyHC* indicating that muscle development is delayed in newborn LBW pigs.

Intrauterine growth restricted pigs have low birth weight. Pigs can be LBW, but gain weight by weaning comparable to normal sized pigs (Amdi et al., 2013; Douglas et al., 2016). Intrauterine growth restricted pigs can also be identified by physical characteristics. Using head morphology IUGR identification is considered to be at least 2 of 3 of the following: a dolphin-like shaped head, bulging eyes, and nose wrinkles perpendicular to the mouth (Amdi et al., 2013). A combination of birth weight and physical characteristics would be a simple and fast way to identify IUGR pigs (Douglas et al., 2016).

Nutritional intervention for IUGR pigs has met with limited success. High nutrient concentrations can increase the risk of lipid metabolic disorders in IUGR pigs (Chen et al., 2017). When fed a high protein formula, IUGR pigs have poor growth rates, increased mortality from elevated ammonia and blood urea concentrations (Ji et al., 2017). Supplementing milk replacer during nursing is an additional expense. It is bovine derived and there is limited voluntary ingestion by nursing pigs (Wu et al., 2006; De Vos et al., 2014). In general, creep feeding has limited benefits due to high variability in pig intake. However, consumption of creep feed can help adapt the gastrointestinal tract for a complete solid feed at weaning. (De Vos et al., 2014). Creep feed is usually provided when the pigs are older and at a time after most pre-weaning mortalities have occurred.

Another approach to benefit smaller pigs later in lactation is split weaning. This involves removing part of the litter late in lactation. The rest are allowed to nurse to continue gaining weight before entering the nursery (De Vos et al., 2014).

Split suckling and cross fostering are other management practices used to help LBW pigs. Split suckling is applied soon after birth by removing the heaviest pigs to allow LBW pigs access to colostrum (De Vos et al., 2014). This is done in the first 24-h of life to ensure colostrum

ingestion and absorption by the gastrointestinal tract (Donovan and Dritz 2000). Cross fostering is commonly practiced equalizing number and/or by size of nursing pigs and should be done in the first two days of life to allow establishment a teat nursing order. Research results for cross fostering for LBW pigs has produced mixed results.

Weaning to market

Intrauterine growth restricted pigs often don't have the growth potential to become a 'full market value pig'. Liu et al. (2015) reported that IUGR pigs needed 26 more days to reach slaughter weight. German Landrace LBW pigs had more subcutaneous fat at 11 weeks of age and more abdominal fat at 19 weeks of age compared to pigs of normal birth weight (Metges et al., 2015). LBW pigs have reduced feed intake, increased feed to gain ratio, lower daily weight gain, and lower carcass quality with less muscle and more adipose tissue and collagen. Pork processors prefer want market hogs that are uniform in size and have similar carcass values (Karunaratne et al., 2005; Foxcroft et al., 2006; Gonzalez-Bulnes et al., 2016).

Feed restriction has similar effects on LBW and normal birth weight pigs for changes in body composition, plasma lipids and stress hormone concentrations, and refeeding response during the finishing period (Metges et al., 2015). Lipogenesis and fat deposition increased when high fat/ high energy diets were fed to IUGR pigs during the finishing phase (Liu et al., 2015).

Reproduction

Breeding herd replacement animals should not be IUGR or LBW pigs. Even coming from litters that had increased with-in litter birth variation could lead to potential problems such as: delayed puberty, reduced number of oocytes ovulated, increased number of LBW offspring, and decreased sperm counts (Foxcroft et al., 2006). Low birth weight gilts have smaller uteri, less uterine secretions, delayed follicular development on their ovaries and take more days to reach

puberty compared to larger birth weight females. Low birth weight boars have lower sperm concentrations and fewer sperm per ejaculate (Ji et al., 2017).

Litter size nursed can also have an effect on puberty and reproductive development for gilts and boars. When the nursing litter was 6 or less compared to 14 pigs per litter, gilts raised in the smaller litters ovulated more oocytes and had more viable embryos at d 25 of gestation (Nelson and Robison, 1976). Over 3 parities sows raised from litters of 7 or less pigs, had increased farrowing rates and litter numbers compared to sows raised in litters of 10 or more pigs (Flowers, 2008). When boars were raised in litters of 6 or less, they reached puberty sooner and had more sperm per ejaculate compared to boars raised in litters of 9 or more (Flowers, 2008).

Immunocrit levels at d-1 of age are predictive for reproductive success in gilts. Gilts with higher immunocrit values reached puberty up to 8 d sooner. These gilts had more pigs born alive and better preweaning growth performance of offspring (Vallet et al., 2016). The combination of birth weight and IgG levels would be beneficial in identifying replacement gilts and boars for maximum reproductive success.

Chapter 2 - Relationships Between Pig Weights at Birth and Weaning and Expression of *EGR1*, *Pref1*, *Cox1*, *Cox2*, *PPARɣ1*, and *PPARɣ2* in Wharton's Jelly and the Epididymal Fat Pad

Introduction

Pigs that are small at birth are a major problem in swine production. Low birth weight (< 1.11 kg; LBW) pigs have decreased survival to weaning (Feldpausch et al., 2016) and various researchers have identified reduced growth to weaning and market as a problem associated with the smallest pigs at birth (Bergstrom et al., 2009). Wu et al. (2006) reviewed multiple reports that found an increased backfat at market weight was problematic and pigs with small birth weights are generally considered as unlikely to reach 'full market value'. These findings lead to the conclusion that pigs below a certain birth weight have damaged physiology due to IUGR and that has programmed their genetic potential for postnatal growth and development (Foxcroft et al., 2006). Adipogenesis is programmed by IUGR and appears to be delayed for the smallest pigs in the litter (Gondret et al., 2011). Low birth weight pigs are generally considered to be analogous to small for gestational age (SGA) human babies. Therefore, the extensive literature on SGA babies contains valuable information for understanding the situation in LBW pigs.

Adipocytes are one of the mesodermal lineages that develop from MSCs. Sukarieh et al. (2014) proposed that the MSCs in Wharton's jelly (WJ) in the umbilical cord may be programmed in a manner similar to other MSCs in the fetus and thus provide an *in vitro* model to understand IUGR effects in SGA babies. They reported that expression of the immediate early gene, early growth response 1 (*EGR1*) and cyclooxygenase 2 (*Cox2*) in the umbilical cord WJ cells (WJC) is affected in SGA babies resulting in decreased insulin sensitivity and increased

adipogenic development (Sukarieh et al., 2014). Low birth weight pigs are generally considered to be analogous to SGA human babies. Small for gestational age babies are known to develop slower than normal sized babies and to be programmed for obesity later in life and these traits parallel reduced growth rates and increased fatness in LBW pigs (Louveau et al., 2016).

Our objective was to determine whether LBW pigs have increased *EGR1* and *Cox2* gene expression when compared to normal birth weight pigs in their WJ. We also examined epididymal fat pads (EFPs) of male pigs and measured expression of *Cox1*, preadipocyte factor (*Pref1*), and the peroxisome proliferator activated receptor genes *PPARɣ1* and *PPARɣ2*.

Materials and Methods

Umbilical Cord and Epididymal Fat Pad Collection

Umbilical cords (n = 104; PIC 326 × 1050 and DNA 241 × 610) were collected at birth from 9 litters at the Swine Teaching and Research Unit at Kansas State University. Paired testes (n = 55) with attached fat pads were collected at d 7 of age after routine castration. Care was taken to minimize contamination with the environment. Individual cords and testes pairs were placed in polypropylene tubes containing phosphate buffered saline, pH 7.2 (PBS, Invitrogen, Carlsbad, CA) and antibiotic/antimycotic solution (penicillin, 400 u/ml, streptomycin, 400 mg/ml, amphotericin B, 1 µg/ml: Gibco, Gaithersburg, MD).

Umbilical cords and testes were kept at 4˚C and processed within 24 h. For isolation of WJ, the cords were placed in sterile 150 mm^2 petri dishes (TPP Cultureware, MidSci St. Louis, MO) and opened longitudinally with two hemostats. The blood vessels were removed from the cord and WJ was scraped out with a surgical blade. For isolation of EFP, each testis with the epididymis and fat was placed in a sterile 100 cm^2 petri dish (MidSci) and the visceral vaginal tunic was removed with two hemostats. The EFP was separated from the testis using surgical

scissors. After mincing, the WJ and EFP were placed separately into cryovials (Nalgene, 2ml), snap frozen in liquid nitrogen, and stored at -80˚C until RNA extraction.

RNA extraction

RNA extraction was performed using the Trizol method. Tissue samples (< 100 mg) were each placed in 15 ml conical tubes with 1ml RiboZol^{Tm} (Amresco; Solon, OH) and homogenized twice. Chloroform was added (200µl/1 ml RiboZol^{Tm}) and the samples were centrifuged (4500 × *g*) for 30 min at 4˚C. The supernatant was pipetted into another 15 ml conical tube with chloroform and centrifuged. After the second centrifugation, the supernatant was pipetted into a Qiagen spin column. For RNA purification, a Qiagen RNeasy Mini (Qiagen, Venlo, Netherlands) kit was used according to the manufacturer's instructions. A DNase digest (RNase-free DNAase Set, Qiagen) was performed to remove genomic DNA. Columns were eluted with 30 µl of 50˚C nuclease-free water. RNA quantity was determined with the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and RNA quality was evaluated with the Agilent 2100 Bioanalyzer using the 6000 Nano kit (Agilent, Santa Clara, CA) at the Kansas State University's Center for Biomedical Research Excellence (COBRE). RNA samples were considered usable with a RNA integrity number (RIN) of 4 or greater based on the 28s to 18s ratio. This value was set because tissues have lower RIN scores than cells and higher connective tissue or fat in samples result in lower RIN scores (Shabihkani et al, 2014). In addition, PCR amplicons < 400 base pairs (bp) can tolerate lower RIN scores with little effect on results (Fleige et al., 2006). Total RNA was stored at -80˚C.

Quantitative Real Time PCR (rtPCR)

Ribonucleic acid was reverse transcribed using an Applied Biosystems High Capacity cDNA Reverse Transcription kit. In a 20 µl reaction, 200 ng (10 ng/µl) of RNA was used

following manufacturer's instructions. Complementary DNA (cDNA) was diluted to 5 ng/µl and was stored at -20˚C until further analysis.

The WJ and EFP cDNA was subjected to quantitative RT-PCR using *Cox1*, *Cox2*, *EGR1*, *Pref1*, *PPARɣ1*, and *PPARɣ2* and *18s* ribosomal subunit primers. Primer sequences (Invitrogen) for all genes are in Table 1. *18s* was used at the housekeeping gene for all experiments.

The reactions were assembled using 2µl of cDNA, 18µl of power SYBR green master mix (Applied Biosystems, Rostercity, CA) with 100nM each of forward and reverse primers in a total reaction volume of 20µl in 96 well plates. Relative expression for genes of interest were normalized for 18s. For *Cox1*, *Cox2*, *EGR1*, and *Pref1*, swine testes cDNA was included on all plates to remove between plate variation. For *PPARɣ1*, and *PPARɣ2* subcutaneous adipose tissue cDNA from a market weight pig was used to remove between plate variation. Gene expression is expressed as change in threshold cycle Ct(gene of interest)- Ct(18s) (ΔCt).

Quantitative real time PCR was conducted using a 7500 fast Applied Biosystems PCR system with the following conditions, a heat step of 50˚C for 2 min, a denaturing step of 95˚C for 10 min, 40 cycles of 95˚C for 15 s, 60˚C for 1 min, 95˚C for 30s, and 60˚C for 15s. Threshold values were normalized for all plates. Samples with a standard deviation higher than 0.3 between triplicates were repeated.

Growth Data

Pigs were weighed at birth and 21-d of age weights (nearest 0.01 kg, Scale Model ES6R, OHAUS, Parsippany, NJ). Birth weight categories were: low birth weight < 1.11 kg, mid birth weight ≥ 1.11 kg and ≤ 1.45 kg, and high birth weight > 1.45 kg. Average daily gain (ADG) was calculated by subtracting birth weight from weaning weight and dividing by age for each pig.

Statistical Analysis

Data were analyzed using Proc GLIMMIX of SAS (Version 9.4, SAS Institute Inc., Cary, NC). For this study, we collected tissue from 9 randomly selected litters. Pigs were assigned to a birth weight category based on their initial body weight. The model is a split plot which compared expression ΔCt of each gene. Sow was the experimental unit with individual pigs placed into a birth weight category. Correlations for each gene and birth weight, weaning weight, and ADG were determined using Proc Corr of SAS. Because gene expression is reported as ΔCt, larger numbers indicate less gene expression. Similarly, a positive correlation shows a negative relationship biologically. Statistical significance was set at $P \leq 0.05$ and a tendency at $P \leq 0.10$.

Results

Expression of *Cox1* tended (Table 2, $P = 0.08$) to be affected by birth weight category with the expression highest in high birth weight pigs. For the 5 other genes, there were no ($P > 0.10$) differences due to birth weight category. There was a positive correlation ($r = 0.65$; $P < 0.01$) between birth weight and weaning weight.

However, there was considerable overlap for gain between birth weight categories (Fig. 1). Therefore, we evaluated the relationship between weaning weight, ADG, and gene expression (Table 3). This analysis revealed that *EGR1* ($r = 0.28$), *PPARɣ1* ($r = 0.29$), and *PPARɣ2* ($r = 0.30$) ΔCts were positively correlated ($P < 0.01$) with wean weights and *Cox1* ΔCt tended ($r = -0.20$; $P = 0.07$) to be negatively correlated with wean weight. Average daily gain to weaning was similarly related to expression of these genes. However, *Cox2* and *Pref1* were not ($P > 0.20$) related to gain or weaning weights. Therefore, as weaning weight increased the expression of *EGR1*, *PPARɣ1*, and *PPARɣ2* was decreased, but *Cox1* tended to increase.

For EFP there were no ($P > 0.10$) effects of birth weight category for expression of any of the 6 genes evaluated (Table 4). Litter affected ($P = 0.05$) expression of *PPARɣ1* and tended ($P < 0.06$) to affect *PPARɣ2* expression. For male pigs, birth and weaning weights were ($r = 0.65$; $P < 0.01$) correlated and expression of *PPARɣ1* tended to be correlated ($r = 0.24$, $P = 0.08$) with ADG in EFP, but expression of the other genes were not correlated with birth weight, weaning weight, or ADG ($P > 0.10$, Table 5).

Discussion

Naturally occurring IUGR in pig litters has been used as a model for physiological studies of human SGA babies (Radlowski et al., 2014). Therefore, observations for SGA babies may also provide information valuable for understanding IUGR in pigs. Here we followed up human studies by Sukarieh et al. (2014) who found that prenatal programming in IUGR humans was detectable in MSCs in WJ and the effects were consistent with programming for increased adipogenesis. Wharton's jelly in the umbilical cord contains MSCs that can differentiate into a wide array of cell types including adipose, muscle, bone and cartilage. Sukarieh et al. (2014) observed increased expression of *EGR1* and *Cox2* in the Wharton's jelly of SGA babies compared to normal size babies. Therefore, we studied the gene expression in pig umbilical cords.

At postnatal d 7, LBW pigs still have increased expression of *Pref1* and decreased levels of *PPARɣ* in subcutaneous adipose tissue (Gondret et al., 2011; Louveau et al., 2016). High levels of *Pref1* would indicate that there are still pre-adipocytes in the tissue depot that have not differentiated into adipocytes (Hudak and Sul, 2013).

A standard experimental design for studies of nursing pigs is to use litter as a block recognizing that there are similarities within litters. Therefore, our experiment was designed to

evaluate small, medium, and large birth weight pigs within litters. In our data, the weaning weight and ADG of pigs in the different birth weight categories overlapped substantially. Therefore, we also evaluated correlations between gene expression and ADG, weaning weight, and birth weight. These correlations revealed relationships between *EGR1* and gain to weaning. Expression of *EGR1* is anti-adipogenic and can induce expression of *Pref1* in murine 3T3-L1 cells. When murine 3T3-L1 cells expressed *EGR1*, *PPARɣ2* expression is reduced (Boyle et al., 2009). Our results indicate similar relationships between weaning weight, gain, and *EGR1*, *PPARɣ1,* and *PPARɣ2*, however there is no indication that changes in *Pref1* expression is associated with these correlations. Expression of *EGR1*, *PPARɣ1,* and *PPARɣ2* appear to be linked by pathways that do not include *Pref1* and we do not know if the associations we observed are related to adipogenesis.

The expression of specific *PPARɣ* isoforms in porcine preadipocytes has received limited attention (Nakajima, 2015). Expression of *PPARɣ* in subcutaneous tissue of pigs was greater at postnatal d 28 compared to pigs at birth. Tissue from littermates was pooled in that study and did not account for pig size variation (Ding et al., 1999).

For WJ, *Cox1* tended to be affected by birth weight category. Cyclooxygenase 1 is the constitutive form of cyclooxygenase. Most adipogenic research has focused on the inducible form of cyclooxygenase, *Cox2* (Rahmen et al., 2013; Fujimori et al., 2014). Differences we found in *Cox1* expression could indicate differences in species regulation and the cyclooxygenase could affect the types or amounts of prostanoids synthesized (Rahmen et al., 2013). Further evaluation of the role of *Cox1* in adipogenesis for IUGR pigs should be conducted.

Expression of *EGR1* in WJ of human SGA babies appears to induce expression of *Cox2* (Sukarieh et al., 2014). In humans and mice, uncoupling protein 1 (UCP1) is important for thermogenesis. In white adipose tissue, *Cox2* is involved in upregulating *UCP1*, but pigs lack a functioning *UCP1* gene (Madsen et al., 2010; Sukarieh et al., 2014; Louveau et al., 2016; Hou et al., 2017; Milet et al., 2017). Therefore, the effect of IUGR on *Cox2* may not be needed in pigs. However, *Cox2* has other effects on adipogenesis including inflammation in adipose tissue in humans and other species (Ghoshal et al., 2011). The different relationships observed in human and pig WJ require further study.

Wharton's jelly cells could be an important model for prenatal effects on postnatal traits such as preweaning gain. Wharton's jelly could be beneficial for studies that examine WJ at birth followed by collection of growth data to market and carcass data. Identification of gene expression effects at birth that correlate with postnatal growth may help separate prenatal and postnatal effects. Associations between gene expression at birth and growth to weaning may reveal pathways leading to these responses and could be important clues for deciphering the associations between prenatal and postnatal physiology.

Table 1. List of primer sequences by gene

Gene	Primer Sequence	T_M	Amplicon Length, bp	Efficiency, %
Pref 1 Forward	AGGACGGCTGGGATGGA	82.6	90	82%
Pref 1 Reverse	CGAGGTTCGCGCAGGTT			
Cox1 Forward	GGGAGTCCTTCTCCAATGTG	84.4	288	95%
Cox1 Reverse	CATAAATGTGGCCGAGGTCT			
Cox2 Forward	ATGATCTACCCGCCTCACAC	83.1	284	105%
Cox2 Reverse	AAAAGCAGCTCTGGGTCAAA			
EGR1 Forward	ACCGCAGAGTCTTTTCCCGA	77.7	77	83%
EGR1 Reverse	GTGGTTTGGCTGGGGTAACT			
PPARγ1 Forward	ATTTGGTGGAAGCCAACTCTA	75.1	86	95%
PPARγ1 Reverse	CCATGGTCACCTCGCTAAAAG			
PPARγ2 Forward	TCATGACAAGGGAGTTTCTAAGGA	76.7	73	94%
PPARγ2 Reverse	GCAAACTCGAACTTGGGCTC			
18s Forward	GAGGTTCGAAGACGATCAGA	84.0	317	92%
18s Reverse	TCGCTCCACCAACTAAGAAC			

Table 2. Gene expression in Wharton's jelly as affected by birth weight category

	Birth Weight Category				*P*-value	
Gene	High[a]	Mid[b]	Low[c]	SEM	Litter	BWC[d]
Cox1	14.19[e]	15.05	14.79	0.48	0.13	0.08
Cox2	14.91	15.18	14.57	0.89	0.12	0.82
EGR1	10.40	10.82	11.00	0.55	< 0.01	0.55
Pref1	8.96	9.52	9.80	0.43	< 0.01	0.13
PPARɣ1	21.97	22.00	22.58	0.48	< 0.01	0.54
PPARɣ2	20.03	19.89	20.32	0.62	0.08	0.83

[a] High Birth Weight Category: > 1.45 kg at birth
[b] Mid Birth Weight Category: ≥ 1.11 kg and ≤ 1.45 kg at birth
[c] Low Birth Weight Category: < 1.11 kg at birth
[d] Birth weight category
[e] ΔCt

Table 3. Correlations between Wharton's jelly gene expression and weights at birth and weaning, and average daily gain

	Genes					
Item	Cox1	Cox2	EGR1	Pref1	PPARɣ1	PPARɣ2
Birth Weight	-0.02	0.02	0.04	0.05	-0.12	0.01
P-value	0.87	0.87	0.72	0.67	0.25	0.95
Wean Weight	-0.20	0.13	0.28	-0.09	0.29	0.30
P-value	0.07	0.24	0.01	0.41	0.01	0.01
Average Daily Gain	-0.21	0.12	0.28	-0.07	0.29	0.29
P-value	0.06	0.27	0.01	0.51	0.01	0.01

Table 4. Gene expression in epididymal fat as affected by birth weight category

	Birth Weight Category				*P*-value	
Gene	High[a]	Mid[b]	Low[c]	SEM	Litter	BWC[d]
Cox1	18.57[e]	19.04	19.08	0.44	0.15	0.40
Cox2	19.73	19.42	19.10	0.55	0.16	0.56
EGR1	9.71	9.54	9.40	0.55	0.66	0.94
Pref1	18.12	18.35	16.49	0.95	0.76	0.30
PPARɣ1	20.54	20.92	20.55	0.24	0.05	0.13
PPARɣ2	18.95	19.20	18.89	0.22	0.06	0.27

[a] High Birth Weight Category: > 1.45 kg at birth

[b] Mid Birth Weight Category: ≥ 1.11 kg and ≤ 1.45 kg at birth

[c] Low Birth Weight Category: < 1.11 kg at birth

[d] Birth weight category

[e] ΔCt

Table 5. Correlations between gene expression in epididymal fat and weights at birth and weaning, and average daily gain

Item	Genes					
	Cox1	Cox2	EGR1	Pref1	PPARɣ1	PPARɣ2
Birth Weight	-0.12	0.16	0.14	0.12	0.04	-0.009
P-value	0.38	0.24	0.32	0.38	0.75	0.95
Wean Weight	-0.10	0.14	0.07	0.21	0.22	0.03
P-value	0.46	0.32	0.60	0.14	0.12	0.81
Average Daily Gain	-0.11	0.14	0.06	0.21	0.24	0.06
P-value	0.43	0.31	0.69	0.13	0.08	0.67

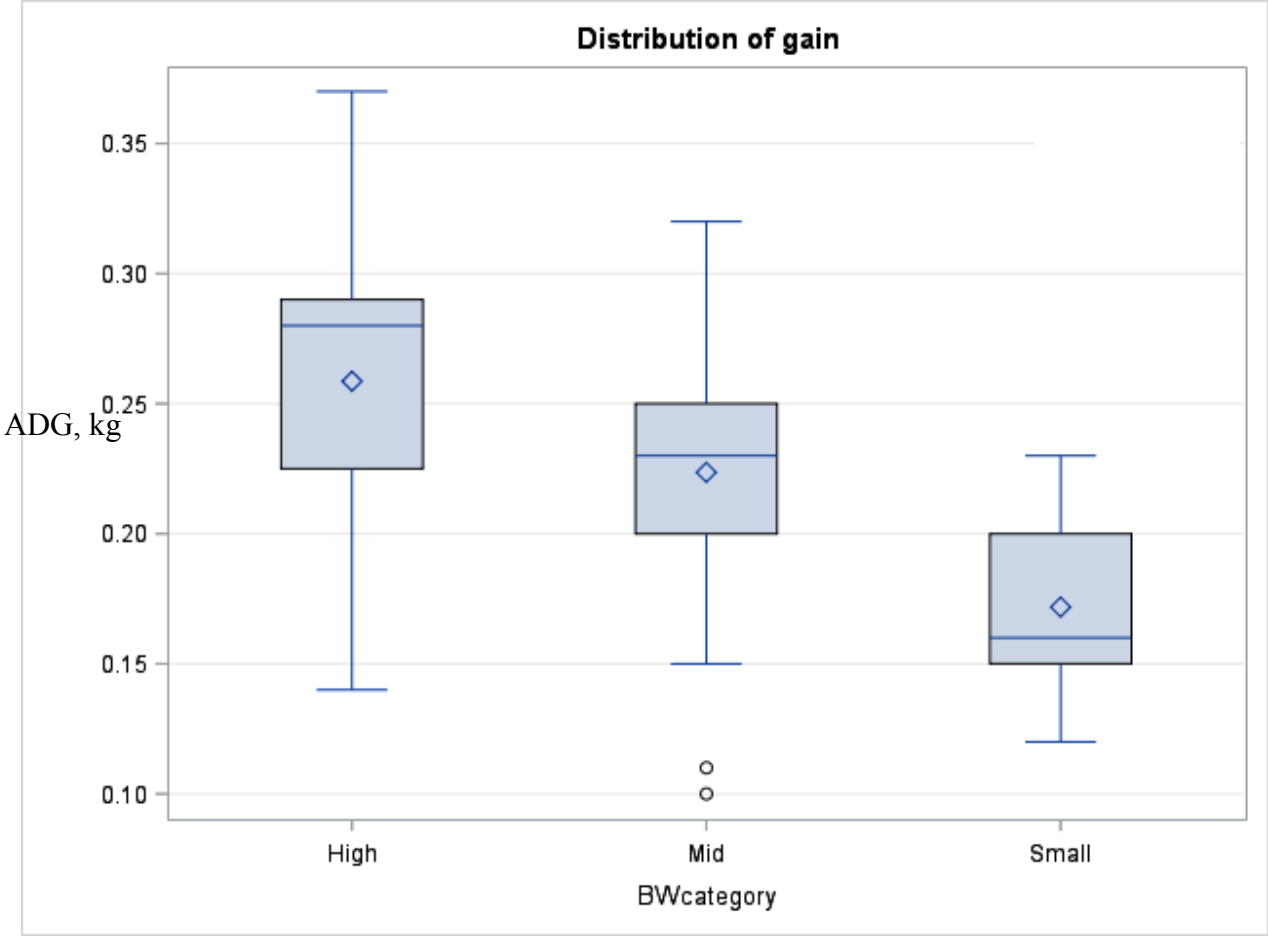

Figure 1. Box plots showing the distribution of average daily gain from birth to weaning for pigs in different birth weight categories.

Chapter 3 - Effects of Fetus Size on Gene Expression in Tissues and Cells

Introduction

Low birth weight (LBW) pigs exhibit a delay in adipogenic development until after birth which then comes in the form of catch-up growth (Gondret et al., 2011). In addition to low survival rates these pigs lack the growth potential to achieve market weight on the same schedule as their larger birth weight littermates. Mesenchymal stem cells (MSCs) are precursors to cells in the adipogenic pathway. Sukarieh et al. (2014) used MSCs in Wharton's jelly of human umbilical cords (WJ cells) to study fetal programming effects on adipogenesis. Sukarieh et al. (2014) suggest that WJ cells provide a model for the effects of IUGR.

Placental insufficiency is a major cause of IUGR and intrauterine crowding causes placental insufficiency in pigs. Crowding begins at least by d 30 in pigs and by midgestation programming of myogenesis is detected in pigs (Foxcroft et al., 2006). In this study, we examined two points in gestation: d 60 and d 95. Day 60 of gestation is earlier than most previous research on adipogenesis which focuses around d 71 when lipid deposition starts to occur in pigs (Gondret et al., 2011; Gondret et al., 2013). Evaluation of d 60 MSCs may provide insights into programming occurring in the first half of gestation that could affect adipogenesis.

The objective of this study was to identify gene expression changes in d 60 and 95 fetal umbilical cord WJ cells that may give insights to critical developmental regulation and lead to a better understanding of IUGR and adipogenic development in pigs.

Materials and Methods

The protocol for this experiment was approved by the Kansas State University Institutional Animal Care and Use Committee (protocol number 3405).

Animal Management and Housing

Sexually mature gilts (d 60 *n* =12 and d 95 *n* =5; PIC 327 × 1050; 168 kg initial BW and age 7 mo) across three different breeding groups. When gilts were approximately 185 d of age, group-penned gilts were exposed to a mature boar daily for estrous detection. After detecting estrus, gilts were moved into individual gestation stalls (2.88 m^2). Gilts received Matrix (6.8mL/d; 15 mg altrenogest; Intervet/Schering-Plough Animal Health, Millsboro, DE) top-dressed on their daily feed allowance for 14 consecutive d for estrus synchronization. At d 3 after end of Matrix addition, gilts were heat checked twice daily with sexually mature boars. When gilts were found in standing estrus, they were artificially inseminated (PIC 337) 12 h later and up to 3 times following the 12 h intervals if in estrus for 48 h or longer. Pregnancy was confirmed at gestation d 24 by transcutaneous ultrasound. All gilts were limit fed (2.2 kg/d) a standard non-medicated diet daily that met or exceeded their nutrient requirements (NRC, 2012).

Tissue Collection

On gestation d 59 ± 2 and d 95 ± 1 gilts were transported to Kansas State University Abattoir. Gilts were euthanized by exsanguination after electrical stunning. The gravid uteri were collected. Fetuses were weighed, and size categories established for each sex. At d 60 the smallest, median, and largest males were identified and the whole umbilical cords was collected in a cryovial (Nalgene, 2ml) containing Recovery™ Cell Culture Freezing Medium (Gibco™), frozen (-80˚C) and transferred to LN_2 vapor for storage. For the smallest, median, and largest females, whole umbilical cords were placed in cryovials and snap frozen in LN_2 for RNA

isolation. Day 95 whole umbilical cords were collected from smallest, median, and largest male fetuses in each litter were collected and stored in the same way.

Explant and Cell Culturing

Cords frozen in Recovery™ Cell Culture Freezing Medium were quick-thawed in a bead bath (37˚C) for 2 min and poured into 50 ml conical tubes. Phosphate Buffered Saline (PBS) with 5% Fetal Bovine Serum (FBS; Gibco) was added drop wise (10 ml) into the tube and incubated for 5 minutes at room temperature. Phosphate Buffered Saline was removed by pipetting, the cords were rinsed in the same manner twice more. Cords were then placed in a sterile 100 mm^2 petri dish (TPP Cultureware, MidSci St. Louis, MO) and opened longitudinally by blunt dissection with two hemostats. The blood vessels were removed from the cords and the rest of the cord which included serosa and WJ was minced into explants (approx. 1 mm^3) with scissors and cultured with growth medium (8ml) consisting of high glucose Dulbecco's Minimum Essential Medium (DMEM, Invitrogen) with 20% FBS, and antibiotic/antimycotic (penicillin, 1000U/ml, streptomycin, 100mg/ml, amphotericin B, 0.25µg/ml, Gibco), Normocin-O (1000µg/ml, Invitrogen), Gentamicin (25µg/ml, Invitrogen), and β-mercaptoethanol (55µmol/ml, Sigma).

The petri dishes were placed were cultured (38.5˚C, 5% CO_2, 95% air atmosphere) and after 3 d, 4 ml of growth medium was added. After 5 d in culture half the medium was removed, and 4 ml of growth medium added. At d 7, complete medium was replaced without disturbing the explants. On d 10, all medium was removed, and fresh medium added. Cells were passaged when dishes were 80% confluent or at d 14 after explant plating.

Cells were passaged using 0.05% Trypsin-EDTA (1ml/25cm^2, Gibco) and explants were separated from the medium by vacuum filtration (Steriflip 60µm Millipore). The viable cells

were counted using a microcapillary flow cytometer (Millipore Guava Viacount EasyCyte Plus software and reagent). Cells were replated at 10,000 viable cells/cm^2. When cells reached 80-85% confluence they were lifted with trypsin-EDTA and replated. At passage 3, cells were lifted, counted, and resuspended in Recovery™ Cell Culture Freezing Medium (Gibco) and aliquoted into cryovials (1 x 10^6 to 2 x 10^6 cells/vial; Nalgene, 2ml). Cells were frozen -80˚C before transfer to LN_2 vapor.

For immunocytochemistry and glucose uptake assays the frozen aliquots of cells were quick-thawed in a bead bath (37˚C) and transferred to a 15 ml conical tube. Warm growth medium was added drop wise. Cells were pelleted by centrifugation (500 × *g* for 10min). Supernatant was removed, and the pellet resuspended in fresh medium and cells replated. When cells reached 80-85% confluence, they were lifted, counted, and aliquoted to wells for glucose uptake assay, replated for immunocytochemistry for Cluster of Differentiation (CD) markers, or lysed for RNA extraction.

Cluster of Differentiation Markers

Day 60 WJ cells were analyzed to determine surface phenotype cluster of differentiation (CD) markers. Mouse monoclonal antibodies for porcine CD31 (LCI-4): IgG1-RPE, CD45 (K252-1E4): IgG1-FITC, and SLA class II DR (2E9/13): IgG2b-FITC (AbD Serotec, Bio-Rad, Hercules, CA), porcine CD 105 (MEM-263): IgG1-FITC and CD90 (5E10): IgG1-FITC (Abcam, Cambridge, MA) and porcine CD44 (MEM-263): IgG-FITC (Thermo Scientific, Middletown, VA) were used. All isotype control antibodies were derived from mice (IgG1-FITC, IgG1-RPE, IgG2a-FITC, and IgG2b-FITC, Invitrogen, Carlsbad, CA). Cultures were washed twice with PBS, and cells detached with trypsin (0.05% trypsin/EDTA in PBS). Culture

medium was added to inactivate trypsin and cells were washed twice with PBS and volume adjusted to 1 x 10^6 cells/ml of PBS.

Cells (7 x 10^6) were washed with PBS, resuspended in 7ml PBS with 5% Normal Goat Serum, then divided into 1ml aliquots and incubated for 10 min at room temperature to block non-specific binding sights. Cells were centrifuged (1000 × g, 5 min) and resuspended in either negative control (1ml PBS) or 100µl with 10µl isotype control or labeled antibody. Cells were then incubated in the dark for 45 min at 4˚C, washed twice with PBS and resuspended in 500µl of PBS. Fluorescence emission of cells was analyzed using microcapillary cytometer (Guava EasyCyte Plus, Millipore). Nonspecific background was evaluated by parallel staining with isotype-matched IgG1-FITC, IgG1-RPE, IgG2a-FITC, and IgG2b-FITC. The cell population was gated separately from the cell debris using a side scatter/forward scatter dot plot and then was applied to the histograms for green or red fluorescence. The number and percent positive cells was generated by Cytosoft^{TM} software, Guava ExpressPro assay (Millipore).

Glucose Uptake

The glucose uptake assay was adapted from procedures of Joseph et al. (2015). Isolates for glucose uptake were selected from donor gilts for which all three fetal size categories grew after frozen storage (n = 5/fetal size). Each cycle consisted of 3 d adipocyte induction medium then 1 d insulin medium.

Cells were aliquoted into 2 wells (1 x 10^5 viable cells/ well) of a 6 well plate. Four plates were prepared for each cord for harvest after cycle 0, 1, 3, or 6. For each cycle cells were grown 3 d in adipocyte differentiation medium and 1 d in insulin medium. The Cycle 0 plates were cultured in insulin medium for 1 d for comparison to cells exposed to differentiation medium. Insulin medium consisted of growth medium with the addition of insulin (10µm) and

rosiglitazone (1µm) (Joseph et al., 2015). Cycles 1-6 consisted of 3 d of adipocyte induction medium followed by insulin medium. Adipocyte induction medium consisted of growth medium with the addition of isobutylmethylxanthine (0.5mM), dexamethasone (1µm), insulin (10µm), indomethacin (200µm) and rosiglitazone (1µm) (Joseph et al., 2015).

Medium (500µl) for glucose assay was collected after 24 h incubation in insulin medium and stored (-80˚C) for later analysis. After the collection of medium the cells were lifted with trypsin, resuspended in 500µl of PBS and an aliquot counted by Guava microcapillary flow cytometer. The remaining cells were pelleted by centrifugation and lysed with Buffer RLT (Qiagen) for RNA extraction.

Glucose concentration was determined using the QuantiChrom Glucose Assay Kit (BioAssay Systems, Thermo Fisher Scientific) after diluting (1:2). Samples were analyzed in duplicate on a 96 well plate and concentration was read on a BioTek microplate reader (Winooski,VT).

RNA Extraction

RNA extraction was performed using the Trizol method. Tissue samples (< 100 mg) were each placed into 15 ml conical tubes with 1ml RiboZol^{Tm} (Amresco; Solon, OH) and homogenized twice. Chloroform was added (200µl) and samples were centrifuged (4500 × *g* for 30 min at 4˚C). Supernatant was pipetted into another 15 ml conical tube with chloroform and the centrifugation repeated. After the second centrifugation, supernatant was pipetted into a Qiagen spin column. For RNA purification, a Qiagen RNeasy Mini kit was used according to the manufacturer's instructions. A DNase digest (RNase-free DNAase Set, Qiagen) was performed to remove genomic DNA. Columns were eluted with 30 µl of 50˚C nuclease-free water. RNA was extracted from d 60 Wharton's jelly cells using Qiagen RNeasy Mini kit with a Qiashredder

and following manufacturer's instructions. A DNase digest (RNase-free DNAase Set, Qiagen) was performed to remove genomic DNA. Columns were eluted with 30 µl of 50˚C nuclease-free water. RNA quantity was analyzed with the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific), and RNA quality was determined with the Agilent 2100 Bioanalyzer with the 6000 Nano kit (Agilent) at the Kansas State University's Center for Biomedical Research Excellence (COBRE). RNA samples were considered usable with a RIN of 4 or greater for tissue and RIN of 8 or greater for cells, based on 28s to 18s ratio. Total RNA was stored at -80˚C.

Quantitative Real Time PCR

RNA was reverse transcribed using an Applied Biosystems High Capacity cDNA Reverse Transcription kit. In a 20 µl reaction, 200 ng (10 ng/µl) of RNA was used following manufacturer's instructions. cDNA was diluted to 5 ng/µl and was stored at -20˚C until further analysis.

The cDNA from d 60 fetal WJ cell, d 60 cords, and d 95 cords were subjected to quantitative RT-PCR using *Cox1*, *Cox2*, *EGR1*, *Pref1*, *PPARɣ1*, and *PPARɣ2* and *18s* ribosomal subunit primers. Primer sequences (Invitrogen) are located in Table 1 for all genes. *18s* was used at the housekeeping gene for all experiments.

The reactions were assembled using 2µl of cDNA, 18µl of power SYBR green master mix (Applied Biosystems, Rostercity, CA), 100nM each of Forward and Reverse primers for a total reaction volume of 20µl reaction per well in a 96 well plate. Relative expression for genes of interest were normalized by *18s*. To remove between plate variation, subcutaneous adipose tissue cDNA from a market weight pig was used for all genes for d 60 fetal WJ cells, and for *PPARɣ1* and *PPARɣ2* genes for the d 60 and d 90 umbilical cords. For *Cox1*, *Cox2*, *EGR1*, and *Pref1* in the fetal tissue, swine testes cDNA was included on all plates to remove between plate

variation. Gene expression is expressed as change in threshold cycle, Ct(gene of interest)-Ct(18s) ΔCt for whole cords. Gene expression for d 60 WJ cells used fold difference, $-2^{\Delta\Delta CT}$. For fold difference, ΔΔCt calculation used was ΔCt(cycle 6) – ΔCt(control cells).

Quantitative real time PCR was conducted using a 7500 fast Applied Biosystems PCR system with the following conditions, a heat step of 50˚C for 2 min, a denaturing step of 95˚C for 10 min, 40 cycles of 95˚C for 15 s, 60˚C for 1 min, 95˚C for 30s, and 60˚C for 15s. Threshold values were normalized for all plates. Samples with a standard deviation higher than 0.3 between triplicates were repeated.

Statistics

Data were analyzed using Proc GLIMMIX of SAS (Version 9.4, SAS Institute Inc., Cary, NC). For d 95 and 60 umbilical cords, fetuses were assigned to a weight category (High, Mid, Low). Our split plot model compared expression (ΔCt) of each gene by gilt and fetal weight category. Gilt was the experimental unit with fetal weight category nested within gilt as the random effect. The LS means were determined for gilt and fetal weight category. Correlations were evaluated using Proc Corr of SAS.

Day 60 cells for the glucose uptake assay compared uptake for size × cycle and gilt when normalized as milligrams of glucose per 1 x 10^5 cells. Gilt was the experimental unit with fetal weight category nested within gilt as the random statement. The LS means were determined for gilt and fetal weight category. Proc Corr of SAS was used to evaluate the correlation between fetal body weight and gene expression for cycle 0 or 6.

Gene expression by d 60 WJ cells was evaluated using ΔCt. Our model compared expression by gilt and fetal weight category. Gilt was the experimental unit with fetal weight category nested within gilt as the random effect. The LS means were determined for gilt and fetal

weight category. Proc Corr of SAS was used to evaluate the correlation between fetal body weight with each gene cycle 6 cells.

Gene expression for d 60 and 95 umbilical cords is reported as ΔCt. Therefore, when comparting means, larger numbers indicate less gene expression. Similarly, a positive correlation actually shows a negative relationship biologically. Data were considered significant at $P < 0.05$ and a tendency at $P \leq 0.10$.

Results

Day 60 Cells

At d 60 cells the mean fetal body weight for each category was: High, 125.4 g; Mid, 117.7 g; and Low, 110.9 g (± 31.5 g). The d-60 cells had a mesenchymal morphology and readily established and grew *in vitro* (Fig. 2) however adipogenic induction medium did not induce lipid droplets as evaluated by Oil Red O staining. We also tested continuous exposure to other adipose differentiation media and procedures with similar results. However, pig umbilical cord MSCs established from cords collected at birth readily differentiated in all the induction media during a pilot study (unpublished). Therefore, it appears that mid gestation umbilical cord mesenchymal stem cells are not competent for adipogenic differentiation. We also examined the CD markers used to identify MSCs (Table 6). Few cells in each isolate positively expressed the hematopoietic markers CD33, CD45, and SLA-DR as expected. The majority of the cells in the isolates tested were positive for CD44 but although substantial numbers of the cells were positive for CD90 and CD105, the numbers were less than expected for MSCs and less than our observations with MSCs for term cords (Packthongsuk et al., 2018). Therefore, midgestation pig umbilical cord MSCs may be at an earlier stage of development of cell surface markers than cells harvested at term.

Glucose uptake (Table 7) was not affected ($P > 0.10$) by fetal size category, cycle or the size × cycle interaction. However fetal weight was negatively correlated ($r = -0.61$, $P < 0.02$) with glucose uptake at cycle 0.

During 6 cycles of adipogenic induction, there was an increased in *Cox1*, *Cox2*, and *Pref1*. During this time, there was also a decrease in expression of *EGR1*, *PPARɣ1,* and *PPARɣ2* (Table 8). Before induction, *Cox1* expression was positively ($r = -0.59$; $P = 0.02$) with fetal weight (Table 9).

Day 60 Tissue

Umbilical cord tissue frozen immediately after harvest revealed no effects of fetal size for *Cox2*, *EGR1*, *Pref1*, *PPARɣ1*, and *PPARɣ2* ($P > 0.10$; Table 10), however small fetuses tended ($P < 0.06$) to express more *Cox1*. There were no ($P > 0.10$) correlations detected between gene expression and fetal weight (Table 11).

Day 95 Tissue

Umbilical cord tissue revealed no ($P > 0.10$) gene expression differences due to fetal size (Table 12). However, *EGR1* expression was negatively ($r = 0.54$, $P < 0.04$) correlated with fetal weight (Table 13) and there was a tendency ($r = -0.48$, $P < 0.07$) for a positive correlation between *EGR1* and the head circumference/fetal weight ratio.

Discussion

We do not know of others who have investigated differentiation capabilities of porcine midgestion umbilical cord cells, but our inability to achieve their adipogenic differentiation may indicate they have not achieved full competence as MSCs. Term umbilical cord cells can undergo adipocyte differentiation, using a 21-d protocol, term cells changed morphologically from fibroblast-like to spherical cells containing lipid droplets. Lipid droplets were confirmed by

LipidTOX™ Red stain (Packthongsuk et al., 2018). The lack of adipogenic potential in d 60 WJ cells may be consistent with our observations on their expression of CD markers which are only somewhat consistent with what is expected for MSCs and with our previous observations with term pig umbilical cord cells. Phenotype characterization for term Wharton's jelly cells indicated that they were over 90% positive for mesenchymal stem cell markers CD 44, CD 90, and CD 105. These term cell isolates lacked expression for CD 31, CD 45, and SLA-DR, which are hematopoietic surface markers (Packthongsuk et al., 2018). Our d-60 cells, also lacked expression for the hematopoietic surface markers and were positive for CD44. However, they exhibited lower than expected expression of CD 90 and 105.

Wharton's jelly MSCs collected at midgestation (2-3 mo) from caprines were able to differentiate into adipocytes by 14 d with identification of lipid droplets by oil red o stain. The percentage of cells positive for CD 90 (23%) and 105 (3.66%) was low for the goat midgestation cells (Somal et al., 2016). Bovine WJ MSCs from each trimester of gestation were able to differentiate into adipocytes. Differences were seen in percentage of positive cells for surface CD markers 90 and 105 between gestational age groups and were lower than expected for MSCs (Cardoso et al., 2017).

Penolazzi et al. (2009) compared the ability for term and preterm human babies WJ MSCs to differentiate into osteoblasts. Some of the premature birth babies WJ MSCs were still undifferentiated after the 14-d protocol and did not deposit mineral matrix compared to all the term (> 37 weeks) cells. Penolazzi et al. (2009) hypothesized this was due to the babies being treated with betamethasone to develop their lungs, which could also favor adipocyte differentiation due to exposure to the glucocorticoid. Wharton's jelly MSCs from midgestation

canines did not produce calcium-phosphate mineralization during differentiation into osteoblast, but the cultures did express osteogenic genes (Filioli Uranio et al., 2014).

Even though adipose tissue begins to develop d 45 of gestation, protein and fat accretion accelerate after d 69 of gestation in pig fetuses (McPherson et al., 2004; Gondret et al., 2011). At gestation d 75, adipose development delays are seen in subcutaneous adipose tissue of smaller fetuses (Gondret et al., 2011).

Our data reveal interesting effects of adipogenic medium on porcine d-60 umbilical cord cells. First, the absence of adipogenesis is consistent with elevated *Pref1* in response to our adipogenic induction conditions. Expression of *Pref1* is expected to decrease to allow adipogenesis, but it is also reported to increase in pre-adipocytes (Hudak and Sul, 2013). Therefore, elevated *Pref1* may indicate the cells are blocked as pre-adipocytes and unable to progress. In term human cord blood MSCs there was high expression of *Pref1* and the cells did not differentiate even after 60 d in differentiation medium. Expression of *Pref1* increased throughout the protocol. Expression of *PPARɣ* increased in the cord blood MSCs cultures, but since the *Pref1* expression did not downregulate, no differentiation or lipid droplets were found (Karagianni et al., 2013).

The increase in *Cox2* gene expression in SGA fetuses reported by Sukarieh et al. (2014) may be consistent with our observations for *Cox2*. *Cyclooxygenase 1* is a central regulator of the prostaglandin pathway and is usually expressed constitutively. *Cyclooxygenase 2* is inducible and activated during inflammation (Rahmen et al., 2013). Both *Cox1* and *Cox2* play a role in delaying adipocyte differentiation (Rahmen et al., 2013; Fjimori et al., 2014).

Expression of PPARɣ2 in postnatal d 7 pigs is related to birth weight. The “normal” pigs had higher expression compared to small and large birth weight pigs (Williams et al., 2009).

Gondret et al. (2011) found the same gene expression down regulation for the small gestational d 75 fetuses in subcutaneous adipose tissue. We did not see difference by birth weight category at either fetal age. Therefore, prenatal umbilical cord cells do not duplicate the gene expression seen in adipose tissue.

The hypothesis put forward by Sukarieh et al. (2014) is that *EGR1* and *Cox2* are upregulated after insulin stimulation in term SGA derived umbilical cord cells. In our study, *EGR1* is associated with fetal size. In d 95 umbilical cords, the expression of *EGR1* was negatively correlated to fetal weight. This is consistent with the negative relationship between *EGR1* and the head circumference/fetal weight, a measure of brain sparing. However, this relationship was not apparent in the brain/liver weight ratio, a classical measure of brain sparing.

Our glucose uptake data indicates a greater uptake by smaller fetuses during the first exposure to high insulin medium. This affect was not statistically significant in cycles 3 and 6 although the numerical trend was similar. Sukarieh et al. (2014) observed SGA fetuses took up more glucose, an effect they interpreted as indicating an increased insulin sensitivity. An increase in glucose uptake by the undifferentiated cells of smaller pig fetuses at cycle 0 suggests the effects of IUGR are present by midgestation.

In summary, we observed changes in gene expression and glucose uptake associated with fetal size. These observations indicate that the WJ cells are affected by growth restriction in the smaller fetuses. In addition to the effects on glucose uptake, the expression of *EGR1* and perhaps *Cox1* may be fruitful areas of study to understand the effects of IUGR in pig fetuses. Further work is needed to understand these relationships and explore their role in fetal development. Other work in this thesis (Ch 2) also points to the prenatal effects of these genes in term umbilical cords. The consistent relationship of *EGR1* upregulation in small fetuses in our work

and the report of Sukarieh et al. (2014) in human SGA babies is strong evidence for a conserved role for the transcription factor in the response to IUGR.

Table 6. Cluster of Differentiation markers as percentage of positive cells per isolate

		% Positive					
Size	Gilt	CD31	CD44	CD45	CD90	CD105	SLA II-DR
Large	B28	0.27	82.29	4.25	61.80	18.74	1.04
	B29	0.07	71.68	2.09	27.74	27.88	1.22
	B30	0.03	84.88	8.80	57.01	49.55	1.13
Mid	B29	0.23	70.98	1.66	43.96	21.82	0.40
	B30	0.14	72.71	2.73	87.73	49.61	0.55
	O54	0.22	86.41	2.16	63.36	31.92	0.57
Low	B28	0.16	76.69	4.00	47.90	24.44	1.10
	B29	0.16	67.15	2.05	38.49	8.35	2.32
	O54	0.23	70.98	1.66	43.96	21.82	1.92

Table 7. Glucose uptake following 24 h in insulin medium

	Size Category				Correlation with fetal weight		*P*-value			
	High	Mid	Low	SEM	R	*P*-value	Size	Cycle	Cycle×Size	Litter
Cycle 0	1.46	1.69	2.42	0.50	-0.61	0.02	0.25	0.22	0.95	0.61
Cycle 3	1.58	1.92	2.48		-0.31	0.27				
Cycle 6	1.66	2.18	2.97		-0.43	0.11				

Table 8. Gene expression in Wharton's jelly cells before and after 6 cycles of adipogenic differentiation induction

	Control			Cycle 6			*P*-value			
Gene	High	Mid	Low	High	Mid	Low	SEM	Size[a]	Cycle[b]	Size×Cycle
Cox1	15.56[c]	15.93	16.62	13.96	14.01	14.46	0.42	0.35	0.01	0.64
Cox2	11.68	12.14	11.87	9.18	9.34	8.89	0.32	0.58	0.01	0.68
EGR1	10.58	10.71	11.57	11.89	11.60	12.09	0.32	0.19	0.02	0.40
Pref1	26.49	26.02	26.39	19.01	19.71	19.00	0.89	0.98	0.01	0.77
PPARγ1	22.53	22.52	22.30	24.05	24.06	24.12	0.67	0.99	0.01	0.91
PPARγ2	20.93	21.47	19.98	22.67	22.36	22.07	0.69	0.56	0.01	0.41

[a] Differences by fetal weight category

[b] Control vs. cycle 6

[c] *Δ*CT

Table 9. Correlation between fetal weight and gene expression in Wharton's jelly cells

	Genes					
	Cox1	Cox2	EGR1	Pref1	PPARɣ1	PPARɣ2
Control Cycle	-0.59	-0.04	-0.38	-0.06	0.32	0.58
P-value	0.02	0.90	0.16	0.84	0.24	0.03
Cycle 6	-0.54	0.12	-0.34	0.16	0.24	0.39
P-value	0.04	0.67	0.21	0.56	0.39	0.15

Table 10. Gene expression of d 60 umbilical cord tissue

	Size Category				*P*-value	
Gene	High	Mid	Low	SEM	Size[a]	Litter
Cox1	14.61[b]	15.74	10.58	1.50	0.06	0.41
Cox2	6.78	7.60	7.31	1.05	0.85	0.02
EGR1	4.93	5.86	5.32	0.46	0.38	0.45
Pref1	7.08	7.09	7.18	0.86	1.00	0.03
PPARɣ1	22.15	21.80	21.77	0.35	0.68	0.53
PPARɣ2	18.07	18.04	18.32	1.57	0.99	0.71

[a] Differences by body weight category
[b] ΔCt

Table 11. Correlation between d 60 umbilical cord tissue gene expression with fetal weight

	Genes					
	Cox1	Cox2	EGR1	Pref1	PPARɣ1	PPARɣ2
Fetal weight	0.28	0.09	-0.08	0.09	0.12	0.02
P-value	0.12	0.64	0.64	0.61	0.50	0.92

Table 12. Gene expression of d 95 umbilical cord tissue

	Size Category				*P*-value	
Gene	High	Mid	Low	SEM	Size[a]	Litter
Cox1	15.17[b]	16.58	14.85	2.37	0.86	0.52
Cox2	8.64	11.58	10.50	2.4	0.69	0.78
EGR1	7.32	6.55	6.33	0.61	0.52	0.50
Pref1	7.95	11.03	9.79	1.44	0.31	0.63
PPARɣ1	22.89	21.97	20.03	2.79	0.77	0.35
PPARɣ2	20.57	15.98	20.83	2.63	0.39	0.60

[a] Differences by fetal weight category

[b] ΔCt

Table 13. Correlation between d 95 umbilical cord tissue gene expression with fetal weight, brain to liver ratio, and head circumference to fetal weight ratio

	Genes					
	Cox1	Cox2	EGR1	Pref1	PPARɣ1	PPARɣ2
Fetal weight	0.23	-0.28	0.54	-0.15	-0.09	-0.23
P-value	0.41	0.32	0.04	0.61	0.74	0.41
Brain/liver ratio[a]	0.13	-0.09	-0.06	-0.17	0.27	-0.09
P-value	0.65	0.74	0.84	0.57	0.33	0.76
HC/FW ratio[b]	-0.19	0.32	-0.48	0.10	0.16	0.19
P-value	0.50	0.24	0.07	0.74	0.57	0.49

[a] Fetal weight (0.02, $P = 0.95$) with brain to liver ratio

[b] Fetal weight (-0.98, $P < 0.01$) with head circumference to fetal weight ratio (HC/FW)

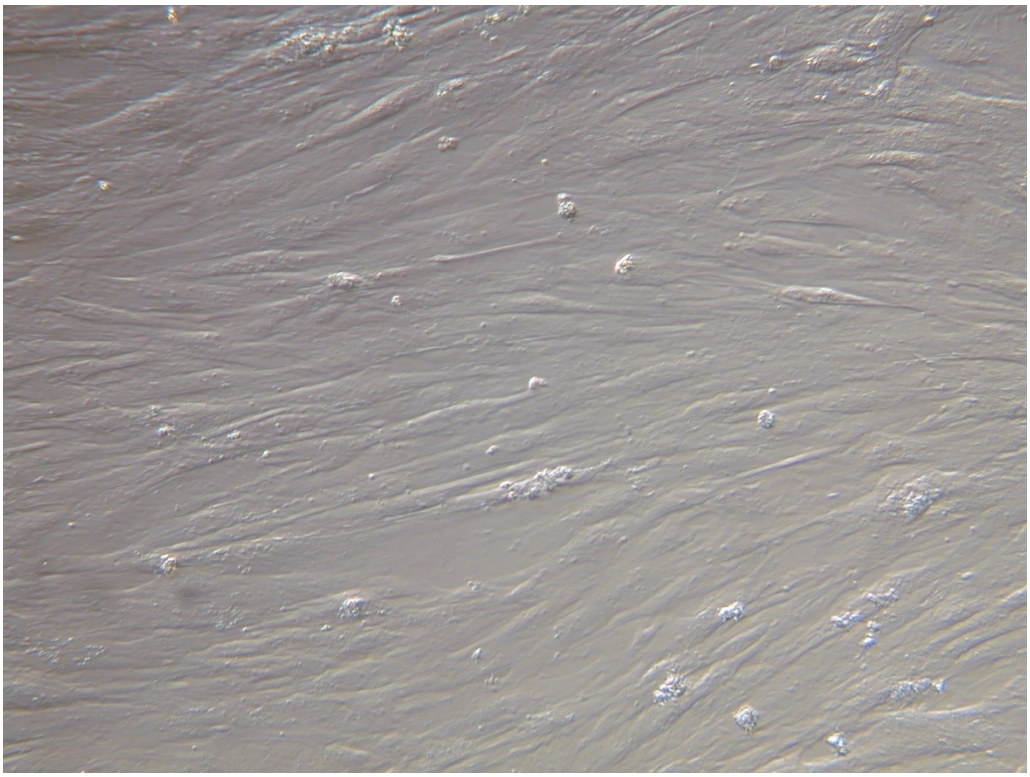

Figure 2. Day 60 Porcine umbilical cord stem cells in culture at 200x

Chapter 4 - Split Suckling, Birth Order, and Birth Weight Affects Colostrum Intake and Pre-Weaning Weight Gain

Introduction

Colostrum is an important source of nutrients and immunoglobulins for newborn pigs. As litter size increases, the access to adequate amounts of colostrum may be limited for low birth weight (< 1.11 kg; LBW) pigs and pigs born later in the birth order (Le Dividich et al., 2005; De Vos et al., 2014; Feldpausch et al., 2016). Temporary removal of part of the litter (split suckling) allows disadvantaged pigs access to the teats to increase colostrum consumption with minimum competition for 1 or 2 suckling events (Donovan and Dritz, 2000). However, there is no management standard for a split suckling protocol that is beneficial to all disadvantaged pigs in a litter.

Immunoglobulin immunocrit was developed as a cheap and simple alternative to identifying pigs that ingested inadequate amounts of colostrum (Vallet et al., 2015). Colostrum intake calculations require weighing pigs twice, the first weight is taken before nursing and the second is after the first 24 h of life (Amdi et al., 2013). This can lead to excess handling of the pigs and take up too much time for workers in the farrowing house. Being able to identify which pigs need extra colostrum in the first 24 h could reduce preweaning mortality through added energy and immunity.

Our objectives were to evaluate two methods of split suckling, one that is based on birth weight and one that considers the birth sequence. To evaluate these methods, we determined immunocrits one day after farrowing and estimated colostrum intake using published protocols.

Materials and Methods

Split Suckling

Thirty sows (DNA Line 241, n = 10/treatment, balanced by parity) were observed over three farrowing groups at the Kansas State University STRC when they neared farrowing. Time of the first pig born was recorded. Each pig (n = 412, sired by DNA line 610) was dried with a towel, and weighed after removal of the umbilical cord. Umbilical cords were tied with a plastic zip tie to stem blood loss and cords trimmed to half an inch in length. Pigs were then tagged and returned to the farrowing crate directly behind the sow. Sows that had less than 9 live pigs or farrowing duration extended for more than 7.5 h were not included in this study.

Six hours after the birth of the first pig, the litters were randomly assigned to one of three split suckling treatments: 1) control, all pigs allowed to suckle ad libitum; 2) weight based, 6 of the heaviest pigs removed for 1.5 h; 3) birth order based, first half of the litter removed for 1.5 h. During separation from the sow the pigs were placed in plastic storage totes behind the crate with a heat lamp to prevent chilling. The pigs that remained with the sows were observed to confirm at least one successful suckle in the 1.5 h time period. After 1.5 h, all pigs were returned to the sow and were allowed to nurse ad libitum.

Pigs were weighed 24 h after birth of the first pig and at d 7 and 20 post farrowing. At 24 h, blood was collected from the cephalic or mammary vein (0.5ml) of each pig. Blood was allowed to clot for more than 30 min. Then serum was separated by centrifugation (18,000 × g, 4˚C). Serum was pipetted into 1.7 ml tubes and frozen at -80˚C until analysis.

Immunocrit and Colostrum Intake

Serum was combined with 40% (wt/vol) ammonium sulfate in distilled water (50µl each). Precipitated samples were loaded into a hematocrit centrifuge tube and centrifuged (12,000 × *g*) for 10 min at room temperature. Immunocrit was determined by the ratio of the precipitate length divided by the total length of serum in the column (0-0.3).

An enzyme-linked immunosorbent assay (ELISA) for porcine IgG (Bethyl Laboratories Inc., Montgomery, TX) was used to validate immunocrit ratios from a subsample of pigs (*n* = 30). Samples were diluted (1:5 x 10^5) and analyzed following the manufacturer's directions. IgG concentrations were read on microplate plate reader (BioTek, Winooski, VT).

A simplified colostrum intake calculation developed by Amdi et al. (2013) was used. Colostrum/24h = ((1.55 × 0.994 × body weight gain) + 115). Body weight gain was 24-h weight – birth weight before nursing.

Statistics

The assignment of pigs to the experiment is presented in Table 14. Data were analyzed using Proc GLIMMIX of SAS (Version 9.4, SAS Institute Inc., Cary, NC) as a split-plot design. This model included treatment × birth weight category, treatment × birth order, and birth weight category × birth order interactions. Sow was the experimental unit with treatment nested within sows as the random statement. The LS means were determined for treatment × birth weight category, treatment × birth order, and birth weight category × birth order interactions. Simple correlations were evaluated using Proc Corr of SAS between weight, gain, immunocrit, and colostrum intake with number of pigs nursed. Data were considered significant at $P < 0.05$ and a tendency at $P \leq 0.10$.

Results

Nursing treatments did not differ for birth weight, 24-h gain, d-1 weights, overall gain, d-20 weights, colostrum intake, or immunocrit ($P > 0.10$; Table 15). Treatment 2 pigs had greater ($P < 0.05$) weight gain to d-7 and d-7 weights compared to control pigs. No treatment × birth weight category or treatment × birth order interactions were observed ($P > 0.10$; Table 16).

Pigs in the HBW group had the greatest colostrum intake, weight gain, and weights at birth, d-1, d-7, and weaning compared to medium and low birth weight pigs ($P < 0.01$; Table 17). Immunocrit was not affected by birth weight category ($P > 0.10$; Table 18). No birth weight category × treatment or birth order interactions were seen ($P > 0.10$). Immunocrit and colostrum intake were positively correlated ($r = 0.49$; $P < 0.01$).

Pigs born in the first half of the litter tended to be heavier than pigs born in the second half ($P < 0.09$; Table 19) and had greater ($P < 0.01$; Table 20) immunocrits. The first half pigs also tended ($P = 0.10$) to have increased weight gain to d 7 compared to the second half. By chance, the control and weight-based treatments both had half the number of LBW pigs compared to the litter order based split suckling treatment. This could be a reason for the treatment's overall weaning weight being numerically lower than the control and split suckling treatment.

Increasing the number of pigs born alive/litter affected ($P < 0.01$) birth weight ($r = -0.37$), d 1 weight ($r = -0.41$), colostrum intake ($r = -0.41$), and immunocrit ($r = -0.21$).

Discussion

Birth weight and order can affect pigs in the preweaning period. When pigs ingest > 200 grams of colostrum the mortality rate was < than 10% while pigs that ingest < 100 grams of colostrum had a mortality rate of about 63% (Theil et al., 2014). In our experiment, high birth

weight pigs had the highest colostrum intake and gain throughout the preweaning period when compared to the other birth weight categories.

Weight-based split suckling improved gain to d 7 and pigs had the numerically greatest weaning weight among treatments. Weight-based split suckling is a commonly applied method for split suckling because most farrowing workers do not keep track of birth order (Donovan and Dritz, 2000). One recommendation is to repeatedly rotate light and heavy pigs at 1 h intervals throughout the working day (Baxter et al., 2013). This requires considerable labor for continually rotating pigs and monitoring pigs while they are away from the sow. Therefore, the single period of separation in our experiment would be easier to adapt to commercial settings.

Pigs need colostrum for energy and immunity. Colostrum quality changes as it transitions to milk, so beginning spilt suckling early as applied in our experiment may be beneficial. Split suckling applied early postpartum may also be beneficial by providing more energy to challenged pigs before they use up their glycogen reserves for energy and thermogenesis (Gondret et al., 2011). Glycogen reserves in pigs deplete by 16 h after birth if colostrum ingestion does not occur (Theil et al., 2011). Therefore, it is important to maximize ingestion of colostrum in the first 12 h of a pig's life (Baxter et al., 2013; Theil et al., 2014). De Vos et al. (2014) recommend pigs receive at least 150g of colostrum/kg of birth weight. Allowing low birth weight pigs and the late born pigs unrestricted nursing access early could allow them to have up to two milk letdown periods in 1.5 h and potentially ensure enough colostrum for energy to improve survivability.

Immunocrit is a relatively cheap and effective way to identify pigs that have not ingested enough colostrum and to identify sows not producing enough colostrum (Vallet et al., 2013). Le Dividich et al. (2017) found there were no differences in colostrum intake between the first two

and last two pigs born in litters, but the pigs born last had lower IgG levels at d 2 of age. Cabrera et al. (2012) also saw a decline in serum IgG levels related to birth order but found no differences in serum IgG levels related to birth weight of pigs. The lower immunocrit, but similar colostrum intake could be due to changes in colostrum IgG levels, as lactation proceeds. These observations are consistent with our results which show that birth order, but not birth weight affects immunocrit.

Immunocrits for most of the pigs in our study are considered normal to high, with only 45 pigs having lower than adequate immunocrit levels (Peters et al., 2016). The immunocrit values attained from our pigs were similar to those reported by Vallet et al. (2013). By attending each farrowing and drying pigs as they were born, we may have improved early nursing compared to management that does not achieve these steps for all pigs.

The colostrum intake calculation we used was a simplified version of Devillers et al. (2004) as suggested by Amdi et al. (2013). In a pilot study, we found that the Amdi et al. (2013) calculation gave results similar to the complete equation suggested by Devillers et al. (2004) which included the interval to first suckle in the calculation. Another method to estimate colostrum intake is deuterium oxide dilution, which may be even more accurate, but is considerably more expensive (Theil et al., 2014).

We found that number of pigs born alive correlates negatively to weight, weight gain, colostrum intake, and immunocrit. These relationships have welfare implications for the pigs which includes the risk of mortality. In the larger litters there is more competition at the udder, and LBW pigs are more likely to be chilled, die of starvation, or be crushed (Rutherford et al., 2013). Colostrum production is independent of litter size and for each additional pig born, colostrum intake drops by 16 g (Le Dividich et al., 2017). In rats, the pups from larger litters

received less colostrum, had lower IgG serum levels, and decreased immunocompetence compared to pups from small litters (Prager et al., 2010).

As the litter size increases in swine production, more management will be needed for low birth weight and late born pigs. Although it would be difficult to implement with current technology, a split suckling strategy that included both weight and birth order might be most effective. For example, the small pigs in the second half of the birth order may benefit most from split suckling and temporarily removing the heaviest pigs in the first half of the birth order might produce the most benefit.

Table 14. Experimental design

Nursing treatment	Birth weight category			
	High	Mid	Low	Total/litter
Control	57	53	22	132/10
Weight based	60	48	29	137/10
Litter-order based	41	52	50	143/10
Total	158	153	101	

Table 15. Weight and gain of pigs by treatment

	Treatment				*P*-value	
Weight, kg	Control[a]	Weight based[b]	Litter order[c]	SEM	Treatment	Trt×BWC[d]
Birth weight	1.27	1.28	1.27	0.02	0.92	0.21
24-h gain	0.11	0.12	0.11	0.016	0.85	0.82
d-1 weight	1.38	1.40	1.38	0.027	0.79	0.31
d-7 gain	1.14	1.33	1.20	0.051	0.04	0.47
d-7 weight	2.44	2.65	2.50	0.058	0.05	0.38
d-20 gain	4.30	4.58	4.20	0.17	0.47	0.91
d-20 weight	5.61	5.89	5.48	0.18	0.24	0.89

[a] Treatment 1: control pigs, n = 132
[b] Treatment 2: weight based split suckling, n = 137
[c] Treatment 3: litter order based split suckling n = 143
[d] Treatment × birth weight category

Table 16. Colostrum intake, immunocrit and survival to d 20 by treatment

	Treatment				*P*-value	
	Control[a]	Weight based[b]	Litter order[c]	SEM	Treatment	Trt×BWC[d]
Colostrum intake, g	246.34	273.94	267.16	21.61	0.64	0.81
Immunocrit	0.145	0.152	0.148	0.009	0.87	0.13
Survival to d 20, %	86.4	88.3	85.3			

[a] Treatment 1: control pigs, n = 132

[b] Treatment 2: weight based split suckling, n = 137

[c] Treatment 3: litter order based split suckling n = 143

[d] Treatment × birth weight category

Table 17. Weight and gain of pigs by birth weight category

	Birth Weight Category				*P*-value		
Weight, kg	High[a]	Mid[b]	Low[c]	SEM	BWC[d]	BWC×Trt[e]	BWC×BO[f]
Birth weight	1.63	1.30	0.90	0.017	<0.01	0.21	0.39
24-h gain	0.14	0.12	0.07	0.01	<0.01	0.82	0.38
d-1 weight	1.76	1.40	0.99	0.022	<0.01	0.31	0.70
d-7 gain	1.43	1.30	0.94	0.044	<0.01	0.47	0.54
d-7 weight	3.07	2.61	1.90	0.052	<0.01	0.38	0.81
d-20 gain	4.78	4.51	3.79	0.141	<0.01	0.91	0.93
d-20 weight	6.40	5.82	4.76	0.15	<0.01	0.89	0.94

[a] High birth weight category: > 1.45kg at birth, n = 158

[b] Mid birth weight category: ≥1.11kg and ≤ 1.45 kg at birth, n = 153

[c] Low birth weight category: < 1.11 kg at birth, n = 101

[d] Birth weight category

[e] Treatment × birth weight category

[f] Treatment × birth order

Table 18. Colostrum intake and immunocrit by birth weight category

	Birth Weight Category				*P*-value	
	High[a]	Mid[b]	Low[c]	SEM	BWC[d]	BWC×Trt[e]
Colostrum intake, g	311.32	266.02	210.09	15.52	<0.01	0.81
Immunocrit	0.147	0.152	0.146	0.017	0.42	0.13

[a] High birth weight category: > 1.45kg at birth, n = 158

[b] Mid birth weight category: ≥1.11kg and ≤ 1.45 kg at birth, n = 153

[c] Low birth weight category: < 1.11 kg at birth, n = 101

[d] Birth weight category

[e] Birth weight category × treatment

Table 19. Weight and gain of pigs by birth order

	Birth Order			*P*-value		
Weight, kg	First half	Second half	SEM	Birth order	BO×Trt[a]	BO×BWC[b]
Birth weight	1.29	1.26	0.013	0.09	0.14	0.39
24-h gain	0.11	0.11	0.009	0.33	0.13	0.38
d-1 weight	1.39	1.37	0.017	0.21	0.19	0.70
d-7 gain	1.25	1.20	0.033	0.10	0.18	0.54
d-7 weight	2.55	2.50	0.038	0.25	0.21	0.81
d-20 gain	4.40	4.32	0.107	0.42	0.84	0.93
d-20 weight	5.70	5.62	0.110	0.39	0.81	0.94

[a] Birth order × treatment

[b] Birth order × birth weight category

Table 20. Colostrum intake and immunocrit by birth order

	Birth Order			*P*-value		
	First half	Second half	SEM	Birth order	BO×Trt[a]	BO×BW[b]
Colostrum intake, g	267.15	257.81	13.20	0.31	0.13	0.42
Immunocrit	0.155	0.142	0.006	0.01	0.46	0.98

[a] Birth order × treatment

[b] Birth order × birth weight category

Chapter 5 - References

Amdi, C., U. Krogh, C. Flummer, N. Oksbjerg, C. F. Hansen, and P. K. Theil. 2013. Intrauterine growth restricted piglets defined by their head shape ingest insufficient amounts of colostrum. J. Anim. Sci. doi:10.2527/jas2013-6824.

Bauer, R., B. Walter, P. Brust, F. Füchtner, and U. Zwiener. 2003. Impact of asymmetric intrauterine growth restriction on organ function in newborn piglets. European Journal of Obstetrics Gynecology and Reproductive Biology. 110:S40-S49.

Baxter E. M., K. M. D. Rutherford, R. B. D'Eath, G. Arnott, S. P. Turner, P. Sandoe, V. A. Moustsen, F. Thorup, S. A. Edwards, and A. B. Lawrence. 2013. The welfare implications of large litter size in the domestic pig II: management factors. Animal Welfare. 22: 219-238.

Boyle, K. B., D. Hadaschik, S. Virtue, W. P. Cawthorn, S. H Ridley, S. O'Rahilly, and K. Siddle. 2009. The trasciption factors Egr1 and Egr2 have opposing influences on adipocyte differentiation. Cell Death Differ.16(5): 782-789.

Cabrera R. A., X. Lin, J. M. Campbell, A. J. Moeser, and J. Odle. 2012. Influence of birth order, birth weight, colostrum and serum immunoglobulin G on neonatal piglet survival. Journal of Animal Science and Biotechnology. 3:42.

Campbell, J., S. Jacobi, Y. Liu, K. H. Robertson, J. Drayton, I. Medina, J. Polo, J. Crenshaw, and J. Odle. 2012. Evaluation of immunoglobulin G absorption from colostrum supplements gavaged to newborn piglets. J. Anim. Sci. 90:299–301. doi:10.2527/jas51544.

Cao, H. 2014. Adipocytokines in obesity and metabolic disease. J. Endocrinol. 220:47–59. doi:10.1530/JOE-13-0339.

Cardoso T. C., L. H. Okamura, J. C. Baptistella, R. Gameiro, H. L. Ferreira, M. Marinho, E. F. Flores. 2017. Isolation, characterization and immunomodulatory-associated gene transcription of Wharton's jell-derived multipotent mesenchymal stromal cells at different trimesters of cow pregnancy. Cell Tissue Res. 367:243-256.

Chen, L., X. Mao, F. Han, B. Yu, J. He, P. Zheng, J. Yu, J. Luo, and D. Chen. 2017. The effect of high nutrient on the growth performance, adipose deposition and gene expression of lipid metabolism in the neonatal intrauterine growth-retarded piglets. J. Appl. Anim. Res. 45:39–44. doi:10.1080/09712119.2015.1091330. l

De Girolamo, L., D. Stanco, L. Salvatori, G. Coroniti, E. Arrigoni, G. Silecchia, M. A. Russo, S. Niada, E. Petrangeli, and A. T. Brini. 2013. Stemness and osteogenic and adipogenic potential are differently impaired in subcutaneous and visceral adipose derived stem cells (ASCs) isolated from obese donors. Int. J. Immunopathol. Pharmacol. 26:11–21. doi:10.1177/03946320130260S103.l

Devillers, N., J. van Milgen, A. Prunier, and J. Le Dividich. 2004. Estimation of colostrum intake in the neonatal pig. Anim. Sci. 78:305–313.

De Vos, M., L. Che, V. Huygelen, S. Willemen, J. Michiels, S. Van Cruchten, and C. Van Ginneken. 2014. Nutritional interventions to prevent and rear low-birthweight piglets. J. Anim. Physiol. Anim. Nutr. (Berl). 98:609-619. doi:10.1111/jpn.12133.

Ding S.T., R.L. McNeel, and H.J. Mersmann. 1999. Expression of porcine adipocyte transcripts: tissue distribution and differentiation in virto and in vivo. Comparative Biochemistry and Physiology Part B. 123:307-318.

Donovan TS and Dritz SS. 2000. Effect of split nursing on variation in pig growth from birth to weaning. J Am Vet Med Assoc. 217:79-81.

Douglas, S. L., S. A. Edwards, and I. Kyriazakis. 2016. Are all piglets born lightweight alike? Morphological measurements as predictors of postnatal performance. J. Anim. Sci. 94:3510-3518. doi:10.2527/jas2015-0142.

Einstein, F., R. F. Thompson, T. D. Bhagat, M. J. Fazzari, A. Verma, N. Barzilai, and J. M. Greally. 2010. Cytosine methylation dysregulation in neonates following intrauterine growth restriction. PLoS One. doi:10.1371/journal.pone.0008887.

Eriksson, J. G., T. Forsén, J. Tuomilehto, P. D. Winter, C. Osmond, D. J. P Barker, and T. Fors. 1999. Catch-Up Growth in Childhood and Death from Coronary Heart Disease: Longitudinal Catch-up growth in childhood and death from coronary heart disease: longitudinal study. Source BMJ Br. Med. J. 318:427–431.

Filioli Uranio, M., M. E. Dell'aquila, M. Caira, A. C. Guaricci, M. Ventura, C. R. Catacchio, N. A. Martino, and L. Valentini. 2014. Characterization and in vitro differentiation potency of early-passage canine amnion- and umbilical cord-derived mesenchymal stem cells as related to gestational age. Mol. Reprod. Dev. 81:539-551.

Fleige S., V. Walf, S. Huch, C. Prgomet, J. Sehm, and M.W. Pfaffl. 2006. Comparison of relative mRNA quantification models and the impact of RNA integrity in quantiative real-time RT-PCR. Biotechnol Lett. 28:1601-13.

Flowers, W. L. 2008. New opportunities for reproductive management. Available from: https://www.cabi.org/cabdirect/FullTextPDF/2013/20133208893.pdf

Foxcroft, G. R., W. T. Dixon, S. Novak, C. T. Putman, S. C. Town, and M. D. Vinsky. 2006. The biological basis for prenatal programming of postnatal performance in pigs. J. Anim. Sci. doi:84/13_suppl/E105 [pii].

Fujimori, K., M. Yano, H. Miyake, and H. Kimura. 2014. Termination mechanism of CREB-dependent activation of COX-2 expression in early phase of adipogenesis. Molecular and Cellular Endocrinology. 384:12-22.

Ghoshal, S., D.B. Trivedi, G.A. Graf, C.D. Loftin. 2011. Cyclooxygenase-2 deficiency attenuates

adipose tissue differentiation and inflammation in mice. J. Biol. Chem. 286(1):889-98.

Gondret, F., M. H. Perruchot, S. Tacher, J. Bérard, and G. Bee. 2011. Differential gene expressions in subcutaneous adipose tissue pointed to a delayed adipocytic differentiation in small pig fetuses compared to their heavier siblings. Differentiation. doi:10.1016/j.diff.2011.02.002.

Gondret, F., MC. Pere, S. Tacher, S. Dare, C. Trefeu, I. Le Huerou-luron, I Louveau. 2013. Spontaneous intra-uterine growth restriction modulates the endocrine status and the developmental expression of genes in porcine fetal and neonatal adipose tissue. General and Comparative Endocrinology. 194:208-216.

Gonzalez-Bulnes, A., S. Astiz, C. Ovilo, C. J. Lopez-Bote, L. Torres-Rovira, A. Barbero, M. Ayuso, C. Garcia-Contreras, and M. Vazquez-Gomez. 2016. Developmental Origins of Health and Disease in swine: Implications for animal production and biomedical research. Theriogenology. doi:10.1016/j.theriogenology.2016.03.024.

Gustafson, B., A. Hammarstedt, C. X. Andersson, and U. Smith. 2007. Inflamed adipose tissue: A culprit underlying the metabolic syndrome and atherosclerosis. Arterioscler. Thromb. Vasc. Biol. doi:10.1161/ATVBAHA.107.147835.

Hausman, G. J. 2003. Dexamethasone induced preadipocyte recruitment and expression of CCAAT/enhancing binding protein α and peroxisome proliferator activated receptor-γ proteins in porcine stromal-vascular (S-V) cell cultures obtained before and after the onset of fetal adipogenesis. Gen. Comp. Endocrinol. doi:10.1016/S0016-6480(03)00149-7.

Heijmans, B. T., E. W. Tobi, A. D. Stein, H. Putter, G. J. Blauw, E. S. Susser, P. E. Slagboom, and L. H. Lumey. 2008. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc. Natl. Acad. Sci. doi:10.1073/pnas.0806560105.

Heindel, J. J., J. Balbus, L. Birnbaum, M. N. Brune-Drisse, P. Grandjean, K. Gray, P. J. Landrigan, P. D. Sly, W. Suk, D. C. Slechta, C. Thompson, and M. Hanson. 2015. Developmental origins of health and disease: Integrating environmental influences. Endocrinology. doi:10.1210/EN.2015-1394.

Hou L, J. Shi, L. Cao, G. Xu, C. Hu, and C. Wang. 2017. Pig has no uncoupling protein 1. Bioch Bioplys Res Commun. 487:795-800.

Hudak, C.S and H.S. Sul. 2013. Pref-1, a gatekeeper of adipogenesis. Front Endocrinol (Lausanne). 4:79. doi: 10.3389/fendo.2013.00079.

Iñiguez, G., P. Ormazabal, T. López, D. Maldonado, A. Avila, R. Román, and F. Cassorla. 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. doi:10.1016/j.ghir.2008.12.005. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1096637408001445

Ji, Y., Z. Wu, Z. Dai, X. Wang, J. Li, B. Wang, and G. Wu. 2017. Fetal and neonatal programming of postnatal growth and feed efficiency in swine. J. Anim. Sci. Biotechnol. doi:10.1186/s40104-017-0173-5.

Joseph, R., J. Poschmann, R. Sukarieh, P. G. Too, S. G. Julien, F. Xu, A. L. Teh, J. D. Holbrook, K. L. Ng, Y. S. Chong, P. D. Gluckman, S. Prabhakar, and W. Stünkel. 2015. ACSL1 Is Associated With Fetal Programming of Insulin Sensitivity and Cellular Lipid Content. Mol. Endocrinol. doi:10.1210/me.2015-1020.

Karagianni M., I. Binkmann, S. Kinzebach, M. Grassl, C. Weiss, P. Bugert, and K. Bieback. 2013. A comparative analysis of the adipogenic potential in human mesenchymal stromal cells from cord blood and other sources. Cytotherapy. 15:76-88.

Karunaratne, J. F., C. J. Ashton, and N. C. Stickland. 2005. Fetal programming of fat and collagen in porcine skeletal muscles. J. Anat. doi:10.1111/j.1469-7580.2005.00494.x.

Kereliuk, S. M., G. M. Brawerman, and V. W. Dolinsky. 2017. Maternal macronutrient consumption and the developmental origins of metabolic disease in the offspring. Int. J. Mol. Sci. doi:10.3390/ijms18071451.

Langley-Evans, S. C. 2006. Developmental programming of health and disease. Proc Nutr Soc. 65(1):97-105.

Le Dividich J., R. Charneca, and F. Thomas. 2017. Relationship between birth order, birth weight, colostrum intake, acquistion of passive immunit and pre-weaning mortality of piglets. Spanish Journal of Agricultural Research. doi.org/10.5424/sjar/2017152-9921.

Lefterova, M. I., A. K. Haakonsson, M. A. Lazar, and S. Mandrup. 2014. PPARg and the global map of adipogenesis and beyond. Trends Endocrinol. Metab. 25:293–302. doi:10.1016/j.tem.2014.04.001.

Lefterova, M. I., and M. A. Lazar. 2009. New developments in adipogenesis. Trends Endocrinol. Metab. doi:10.1016/j.tem.2008.11.005.

Leunissen, R. W. J., G. F. Kerkhof, T. Stijnen, and A. Hokken-Koelega. 2009. Timing and Tempo of First-Year Rapid Growth in Relation to Cardiovascular and Metabolic Risk Profile in Early Adulthood.JAMA. 301(21):2234-2242.

Liu, H. F., M. X. Gui, H. Dong, X. Wang, and X. W. Li. 2012. Differential expression of AdipoR1, IGFBP3, PPAR?? and correlative genes during porcine preadipocyte differentiation. Vitr. Cell. Dev. Biol. - Anim. doi:10.1007/s11626-011-9468-6.

Louveau, I., M.-H. Perruchot, M. Bonnet, and F. Gondret. 2016. Invited review: Pre- and postnatal adipose tissue development in farm animals: from stem cells to adipocyte physiology. animal. doi:10.1017/S1751731116000872.

Lumey, L. H. 1992. Decreased birthweights in infants after maternal in utero exposure to the Dutch famine of 19441945. Paediatr. Perinat. Epidemiol. 6:24–253.

Lumey, L. H., A. D. Stein, H. S. Kahn, K. M. Van der Pal-de Bruin, G. J. Blauw, P. A. Zybert, and E. S. Susser. 2007. Cohort profile: The Dutch Hunger Winter families study. Int. J. Epidemiol. doi:10.1093/ije/dym126.

Madsen L., L.M. Pedersen, H.H. Lillefosse, E.Fjaere, I. Bronstad, Q. Hao, R.K. Petersen, P. Hallenborg, T. Ma, R. De Matteis, P. Araujo, J. Mercader. M. L. Bonet, B. Cannon, J. Nedergaard, J. Wang, S. Cinti, P. Voshol, S.O. Doskeland, and K. Kristiansen. 2010. UCP1 induction during recruitement of brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. PLoS ONE. 5(6): e11391.

Metges, C. C., S. Görs, K. Martens, R. Krueger, B. U. Metzler-Zebeli, C. Nebendahl, W. Otten, E. Kanitz, A. Zeyner, H. M. Hammon, R. Pfuhl, and G. Nürnberg. 2015. Body composition and plasma lipid and stress hormone levels during 3 weeks of feed restriction and refeeding in low birth weight female pigs. J. Anim. Sci. 93:999. doi:10.2527/jas.2014-8616.

Milet C., M. Bleher, K. Allbright, M. Orgeur, F. Coulpier, D. Duprez, and E. Havis. 2017. Egr1 deficiency induces browning of inguinal subcutaneous white adipose tisuee in mice. Nature. 7:6153. doi:10.1038/s41598-017-1654307.

Mraz, M., and M. Haluzik. 2014. The role of adipose tissue immune cells in obesity and low-grade inflammation. J. Endocrinol. doi:10.1530/JOE-14-0283.

Nakajima I. 2015. Characterization of PSPA cell differentiation, an established preadipocyte cell line as an in vitro model for pig fat development. JARQ. 49(2):85-90.

Nelson, R. E., and O. W. Robison. 1976. Effects of postnatal maternal environment on reproduction of gilts. JAS. 43:71-77.

Nicholas LM, JL. Morrison, L. Rattanatray, S Zhang, SE Ozanne, and IC McMillen. 2016. The early origins of obesity and insulin resistance: timing, programming and mechanisms. International Journal of Obesity. 40:229-238.

NRC. 2012. Nutrient requirements of swine. 11th rev. ed. Natl. Acad. Press, Washington, DC.

Okada, T., S. Takahashi, N. Nagano, K. Yoshikawa, Y. Usukura, and S. Hosono. 2015. Early postnatal alteration of body composition in preterm and small-for-gestational-age infants: implications of catch-up fat. Pediatr. Res. doi:10.1038/pr.2014.164

Packthongsuk K., T. Rathbun, D. Troyer, and D. L. Davis. 2018. Poricine Wharton's jelly cells distribute throughout the body after intraperitoneal injection. Stem Cell Res. Ther. 9:38. doi: 10.1186/s13287-018-0775-7.

Painter, R. C., C. Osmond, P. Gluckman, M. Hanson, D. I. W. Phillips, and T. J. Roseboom. 2008. Transgenerational effects of prenatal exposure to the Dutch famine on neonatal adiposity and health in later life. BJOG An Int. J. Obstet. Gynaecol. doi:10.1111/j.1471-0528.2008.01822.x.

Penolazzi L., R. Vecchiatini, S. Bignardi, E. Lambertini, E. Torreggiani, A. Canella, T. Franceschetti, G. Calura, F. Vesce, and R. Piva. 2009. Influence of obstetric factors on osteogenic potential of umbilical cord-derived mesenchymal stem cells. Reproductive Biology and Endocrinology. 7:106. doi:10:1186/1477-7827-7-106.

Perruchot, M. H., L. Lefaucheur, I. Louveau, L. Mobuchon, M. F. Palin, C. Farmer, and F. Gondret. 2015. Delayed muscle development in small pig fetuses around birth cannot be rectified by maternal early feed restriction and subsequent overfeeding during gestation. animal. doi:10.1017/S1751731115001202.

Peters, B. M., K. Sa, and H. Dj. 2016. Reference values for immunocrit ratios to assess maternal antibody uptake in 1-day-old piglets. J. Swine Heal. Prod. — January Febr. 24:36–41.

Prager G., V. Stefanski, R. Hudson, and H. G. Rodel. 2010. Family matters: maternal and litter-size effects on immune parameters in young laboratory rats. Brain, Beahavior, and Immunity. 24: 1371-1378.

Pu Y. and A. Veiga-Lopez. 2017. PPARɣ agonist through the terminal differentiation phase is essentail for adipogenic differentiation of ovine preadipocytes. Cellular & Molecular Biology Letters. 22:6. DOI 10.1186/s11658-017-0037-1.

Radlowski E.C., M.S. Conrad, S. Lezmi, R.N. Dilger, B. Sutton, R. Larsen, and R.W. Johnson. 2014 A neonatal piglet model for investigating brain and cognitive development in small for gesation age human infants.PLoS ONE. 9(3): e91951. doi.org/10.1371/journal.pone.0091951.

Rahman M.S., P.K. Syeda, F. Khan, K. Nishimura, M. Jisaka, T. Nagaya, F. Shono, and K. Yokota. 2013. Cultured preadipocytes undergoing stable transfection with cyclooxygenase-1 in the antisense direction accelerate adipogenesis during the maturation phase. Appl Biochm Biotechnol. 171:128-144.

Ratnasingham, A., Y. A. Eiby, M. Dekker Nitert, T. Donovan, and B. E. Lingwood. 2017. Review: Is rapid fat accumulation in early life associated with adverse later health outcomes? Placenta. doi:10.1016/j.placenta.2017.01.101.

Rinaudo, P., and E. Wang. 2012. Fetal Programming and Metabolic Syndrome. Annu. Rev. Physiol. doi:10.1146/annurev-physiol-020911-153245.

Rutherford K. M. D., E. M. Baxter, R. B. D'Eath, S. P. Turner, G. Arnott, R. Roehe, B. Ask, P. Sandoe, V. A. Moustsen, F. Thorup, S. A. Edwards, P. Berg, and A. B. Lawrence. 2013 The welfare implications of large litter size in the domestic pig I: biological factors. Animal Welfare. 22:199-218.

Rutkowski, J. M., J. H. Stern, and P. E. Scherer. 2015. The cell biology of fat expansion. J. Cell Biol. doi:10.1083/jcb.201409063.

Shabihkhani M., G.M. Lucey, B. Wei, S. Mareninov, J.J. Lou, H.V. Vinters, E.J. Singer, T.F. Cloughesy, and W.H. Yong. 2014. The procurement, storage, and quality assurance of

frozen blood and tissue biospecimens in pathology, biorepository, and biobank settings. Clin Biochem. 47 (4-5):258-66.

Somal A., I.A. Bhat, I. B., S. Pandey, B.S.K. Panda, N. Thakur, M. Sarkar, V. Chandra, G. Saikumar, G. T. Sharma. 2016. A comparative study of growth kinetics, in vitro differentiation potential and molecular characterization of fetal adnexa derived caprine mesenchymal stem cells. PLoS ONE. 11(6):e0156821. doi:10.1371/journal.pone.0156821.

Smith, U., and B. B. Kahn. 2016. Adipose tissue regulates insulin sensitivity: role of adipogenesis, de novo lipogenesis and novel lipids. J. Intern. Med. doi:10.1111/joim.12540.

Spalding, K. L., E. Arner, P. O. Westermark, S. Bernard, B. A. Buchholz, O. Bergmann, L. Blomqvist, J. Hoffstedt, E. Näslund, T. Britton, H. Concha, M. Hassan, M. Rydén, J. Frisén, and P. Arner. 2008. Dynamics of fat cell turnover in humans. Nature. doi:10.1038/nature06902.

Sukarieh, R., R. Joseph, S. Chi Leow, Y. Li, M. Lö ffler, I. M. Aris, J. Hao Tan, A. Ling Teh, L. Chen, J. D. Holbrook, K. Lyn Ng, Y. Seng Lee, Y. Seng Chong, S. A. Summers, P. D. Gluckman, and W. Stü nkel. 2014. Molecular pathways reflecting poor intrauterine growth are found in Wharton's jelly-derived mesenchymal stem cells. Hum. Reprod. Adv. Access Publ. August. 29:2287–230110. doi:10.1093/humrep/deu209.

Symonds, M. E., S. P. Sebert, M. A. Hyatt, and H. Budge. 2009. Nutritional programming of the metabolic syndrome. Nat. Rev. Endocrinol. 5:604–610. doi:10.1038/nrendo.2009.195. Available from: http://www.nature.com/doifinder/10.1038/nrendo.2009.195

Theil P. K., G. Cordero, P. Henckel, L. Puggaard, N. Oksbjerg, and M. T. Sorensen. 2011. Effects of gestation and transition diets, piglet birth weight, and fasting time on depletion of glycogen pools in livers and 3 muscles of newborn piglets. JAS. 89: 1805-1816.

Theil P. K., C. Flummer, W. L. Hurley, N. B. Kristensen, R. L. Labouriau, and M. T. Sorensen. 2014. Mechanistic model to predict colostrum intake based on deuterium oxide dilution technique data and impact of gesation and prefarrowing diets on piglet intake and sow yield of colostrum. JAS.92:5507-5519.

Theil P. K., C. Lauridsen, and H. Quesnel. 2014. Neonatal piglet survival: impact of sow nutrtion around partuition on fetal glycogen deposition and production and composition of colostrum and transient milk. Animal. 8:7 1021-1030.

Tontonoz, P., and B. M. Spiegelman. 2008. Fat and Beyond: The Diverse Biology of PPARγ. Annu. Rev. Biochem. doi:10.1146/annurev.biochem.77.061307.091829.

Tosh, D. N., Q. Fu, C. W. Callaway, R. A. Mcknight, I. C. Mcmillen, M. G. Ross, R. H. Lane, and M. Desai. 2010. Epigenetics of programmed obesity: alteration in IUGR rat hepatic IGF1 mRNA expression and histone structure in rapid vs. delayed postnatal catch-up growth. Am J Physiol Gastrointest Liver Physiol. 299:G1023-G1029.

Vallet, J. L., J. A. Calderón-Díaz, K. J. Stalder, C. Phillips, R. A. Cushman, J. R. Miles, L. A. Rempel, G. A. Rohrer, C. A. Lents, B. A. Freking, and D. J. Nonneman. 2016. Litter-of-origin trait effects on gilt development. J. Anim. Sci. doi:10.2527/jas.2015-9644.

Vallet, J. L., and J. R. Miles. 2017. The effect of farrowing induction on colostrums and piglet serum immunocrits is dependent on parity. J. Anim. Sci. 95:688–696. doi:10.2527/jas2016.0993.

Vallet, J. L., J. R. Miles, L. A. Rempel, D. J. Nonneman, C. A. Lents, A. McNeel, M. Judy, S. Watts, S. Corman, W. Peshek, and M. Anderson. 2015. Relationships between day one piglet serum immunoglobulin immunocrit and subsequent growth, puberty attainment, litter size, and lactation performance 1. J. Anim. Sci. 93:2722–2729. doi:10.2527/jas2014-8535.

Vallet J. L., J. R. Miles, and L. A. Rempel. 2013. A simple novel measure of passive transfer of maternal immunoglobulin is predictive of preweaning mortality in piglets. The Veterinary Journal. 195: 91-97.

Veenendaal, M. V. E., R. C. Painter, S. R. De Rooij, P. M. M. Bossuyt, J. A. M. Van Der Post, P. D. Gluckman, M. A. Hanson, and T. J. Roseboom. 2013. Transgenerational effects of prenatal exposure to the 1944-45 Dutch famine. BJOG An Int. J. Obstet. Gynaecol. doi:10.1111/1471-0528.12136.

Verkauskiene, R., J. Beltrand, O. Claris, D. Chevenne, S. Deghmoun, S. Dorgeret, M. Alison, P. Gaucherand, O. Sibony, and C. Lévy-Marchal. 2007. Impact of fetal growth restriction on body composition and hormonal status at birth in infants of small and appropriate weight for gestational age. Eur. J. Endocrinol. doi:10.1530/EJE-07-0286.

Vickers MH. 2014. Early life nutrition, epigenetics and programming of later in life disease. Nutrients. 6:2165-2178.

Wadhwa, P. D., C. Buss, S. Entringer, and J. M. Swanson. 2009. Developmental origins of health and disease: Brief history of the approach and current focus on epigenetic mechanisms. Semin. Reprod. Med. doi:10.1055/s-0029-1237424.

Wang, J., C. Feng, T. Liu, M. Shi, G. Wu, F. W. Bazer, and C. Junjun Wang. 2017. Physiological alterations associated with intrauterine growth restriction in fetal pigs: Causes and insights for nutritional optimization; Physiological alterations associated with intrauterine growth restriction in fetal pigs: Causes and insights for nut. doi:10.1002/mrd.22842.

Wu, G., F. W. Bazer, J. M. Wallace, and T. E. Spencer. 2006. Board-invited review: Intrauterine growth retardation: Implications for the animal sciences. J. Anim. Sci. doi:10.2527/jas.2006-156.

Zhu, X.-Y., S. Ma, A. Eirin, J. R. Woollard, L. J. Hickson, D. Sun, A. Lerman, and L. O. Lerman. 2016.Tissue-Specific Progenitor and Stem Cells Functional Plasticity of Adipose-Derived Stromal Cells During Development of Obesity. doi:10.5966/sctm.2015-

0240.

Appendix A - Experimental Design

Table 21. Individual sow information for term litter umbilical cord and epididymal fat collection

	Sow		Litter	
	Genetics[a]	Parity	Total number born	Number born alive
B92	PIC	5	13	12
B110	PIC	5	15	13
B192	PIC	3	13	13
B199	PIC	3	16	14
R14	PIC	2	14	12
B64	DNA	1	16	16
B145	DNA	1	10	9
B146	DNA	1	12	12
B147	DNA	1	13	13

[a]Sow and pig genetics

Table 22. Gilt and litter information for d 60

	Block	Viable fetuses	Total fetuses/empty spaces	Average fetal weight, g
B8	1	11	11	126.7
B12	1	11	11	125.3
B14	1	15	17	133.6
B15	1	14	15	123.7
B25	1	12	14	122.5
B28	1	13	14	126.6
B29	1	15	17	109.9
B30	1	13	15	114.8
O51	2	10	17	155.7
O54	2	15	16	150.3
O55	2	7	9	143.9
O62	2	11	12	159.2

Table 23. Gilt and litter data for d 95

	Viable fetuses	Total fetuses	Average fetal weight, g
Y3	12	13	814.7
Y10	14	14	836.9
Y16	12	12	940.4
Y19	15	15	719.6
Y23	15	15	793.7