Characterization of Depot Development and the Role of the Novel Adipokine Relaxin in Regulating Lipid Metabolism, Endocrine Function and the Extracellular Matrix in Porcine Adipose Tissue from Lard and Meat-type Pigs

by

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Abstract

The lard-type, Mangalica pig is a genetic model of excessive adiposity and a breed prized for its excellent pork quality. Therefore, this European heritage breed may serve as a model to improve carcass merit in modern commercial lean breeds and as a unique model to study factors regulating adipose tissue development in pigs. Currently this breed is gaining popularity within the United States swine industry. However, at this time, it is not possible to make science-based recommendations on the ideal harvest weight of Mangalica pigs due to poorly characterized growth and carcass parameters across the breeds growth curve. To resolve this issue, a growth trial was conducted to evaluate 1) growth performance, 2) adipose tissue development, 3) carcass parameters and primal cut measurements, and 4) measures of carcass merit. Previous research involving this breed revealed that porcine adipose tissue synthesizes and secretes the hormone relaxin (RLN), cells of adipose tissue express the RLN receptor, and exposing adipose tissue to exogenous RLN significantly alters the adipocyte transcriptome. To extend these observations, adipose tissue from Mangalica pigs was utilized to further study the effect of RLN on adipose tissue biology. This research demonstrates RLN's ability to 1) decrease preadipocyte number, 2) enhance adipogenesis, 3) alter the mRNA expression of adipokine, fatty acid metabolism and extracellular matrix genes, 4) stimulate lipolysis, and 5) signal through the cAMP pathway. Overall, these data provide novel information on optimal harvest weights of the Mangalica breed and establishes a new function for RLN by supporting the hypothesis that relaxin is a novel regulator of porcine adipose tissue development.

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ACC	Acetyl-CoA carboxylase
Acetyl-CoA	Acetyl Coenzyme A
ACRP	Adiponectin
AMP	Adenosine monophosphate
ATP	Adenosine tri-phosphate
AI	Artificial insemination
ADG	Average daily gain
BAAs	Beta adrenergic agonists
βARs	Beta adrenergic receptors
BAT	Brown adipose tissue
BGN	Biglycan
BPA	Bisphenol A
cAMP	Adenylyl cyclase-cyclic adenosine monophosphate
C/EBP	CCAT-enhancer binding protein
C/EBPα	CCAT-enhancer binding protein alpha
CL	Corpus luteum
CNS	Central nervous system
CO_2	Carbon dioxide
COL1A	Collagen type 1 alpha 1
COL2A	Collagen type 2 alpha 1
COL3A	Collagen type 3 alpha 1
COL4A	Collagen type 4 alpha 1
COL6A	Collagen type 6 alpha 1
CRE	cAMP response element
CREB	cAMP response element binding (protein)
DFD	Dark, firm, dry
DHAP	Dihydroxyacetone phosphate
DNL	de novo lipogenesis
ECM	Extracellular matrix
ERK	Extracellular receptor kinase
ERS1	Estrogen receptor alpha
FAacyl-CoA	Fatty acid acyl-CoA
FAS	Fatty acid synthase
FE	Feed efficiency
FASN	Fatty acid synthase
FN1	Fibronectin

FSH	Follicle-stimulating hormone
GH	Growth hormone
GHIH	Growth hormone inhibiting hormone
GHRH	Growth hormone releasing hormone
GI	Gastrointestinal
GLUT-4	Glucose transporter type 4
GnRH	Gonadotropin releasing hormone
GPCR	Guanine-nucleotide protein coupled receptor
HEK-293T	Human embryonic kidney cells
HEK293T-LGR7	Human embryonic kidney cells transfected with human relaxin receptor
HFD	High-fat diet
HPG	Hypothalamic-pituitary-gonadal
HSL	Hormone sensitive lipase
IGF-1	Insulin-like growth factor-1
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-15	Interleukin-15
In vitro	Within an artificial environment that is outside the body
In vivo	Within the body
LAMA	Laminin
LEA	Loin eye area
LGR7	leucine-rich repeat-containing G-protein-coupled receptor 7
LGR8	leucine-rich repeat-containing G-protein-coupled receptor 8
LH	Luteinizing hormone
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MbFs	Milk-born bioactive factors
MMPs	Matrix metalloproteinases
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
NFκB	Nuclear factor kappa beta
NID1	Nidogen 1
OB	Leptin
PKA	cAMP dependent protein kinase
PI3K	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
PPARs	Peroxisome proliferator-activated receptors
PPARγ	Peroxisome proliferator-activated receptor gamma
PSE	Pale, soft, exudative
RLN	Relaxin
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
RXFPR1	Relaxin family peptide receptor 1
SCD1	Stearoyl-CoA desaturase 1

SOP	Standard operating procedure
SREBP	Sterol regulatory element binding protein
SVC	Stromal vascular cells
TG	Triglyceride
TCA	Tricarboxylic acid
TIMPs	Tissue inhibitors of metalloproteinases
TNF_{α}	Tumor necrosis factor alpha
TLR4	Toll-like receptor 4
UCP-1	Uncoupling protein 1
U.S.	United States
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
7-TM receptor	Seven-transmembrane domain receptor

Introduction

The world population is predicted to exceed nine billion people by the year 2050, with the demand for agricultural products growing 1.5% annually (Food and Agriculture Organization of United Nations, 2003). The demands of such a rapidly growing population and the resultant decreasing farmland acreage have made it increasingly hard to supply enough food and are thus threatening our nutritional security. Threat of future climate change further highlights the need to improve current production practices. The effects of seasonality due to the negative effects of heat stress on animal health and production alone account for approximately $1.69-$2.36 billion worth of economic losses annually to the United States (U.S.) livestock industry (St. Pierre et al. 2003). These losses will surely increase if current climate change models prove correct. To combat these threats to the food supply chain and to keep animal agriculture sustainable, significant innovations in food animal agriculture are imperative.

An important part of this goal involves improving feed efficiency in growing animals. Being able to finely control the development of adipose tissue in an anatomically precise manner would be one way to achieve this goal while maintaining the highest quality food product. Currently consumers demand pork quality standard-lean meat cuts; nonetheless, having significant intramuscular fat accumulation (marbling fat) which is impossible to provide efficiently given our limited understanding of what regulates the development of fat within different anatomical locations on the pig. Presently, the only effective strategies to increase marbling are to either genetically select for marbling which in turn increases overall carcass fat as well and leads to

poorer feed efficiency, or to feed an animal to a certain body fatness which allows adequate marbling and then to trim away and then dispose of the unwanted fat depots during post-harvest fabrication.

Although an immense amount of information has been learned about adipose tissue development during the last twenty years, most of this information has been determined in human and rodent models with potential species-specific differences largely left uncharacterized, such as differences in the site of de novo lipogenesis. For instance, in humans this occurs in the liver, whereas this occurs in adipose tissue in pigs (Bergen and Hausman, 2005). Work done in food animals during the last half century has failed to yield an effective strategy to alter marbling fat accumulation without also altering overall adiposity in the same direction. Making animals leaner also decreases marbling which leads to consumer rejection of the sub-primal cut at the meat counter. Better understanding of the cellular mechanisms that regulate adipose tissue will allow producers to make food animals more efficient by regulating where and how much adipose tissue develops on the animal. The goal of this work is to generate new knowledge that will ultimately allow producers to improve production efficiency and optimize their profitability, all while strengthening the food supply chain and allowing more hungry people to be fed.

In the studies herein, the extreme lard-type Mangalica pig is utilized to study adipose tissue growth on the pig. Adipose tissue accretion is characterized in different anatomical locations with aging in the live animal (Chapter 2) and adipose tissue harvested from this breed is used to facilitate *in vitro* studies using primary cultures of pig preadipocyte cells and adipose tissue explant cultures (Chapter 3). My overall research aims to better define, mechanistically, a new function for the traditional hormone RLN. To achieve this, I will examine the effects of RLN in porcine adipose tissue *in vitro* by determining the effect of exogenous RLN on 1) preadipocyte cell number and

differentiation, 2) the mRNA expression of adipokine, extracellular matrix protein, and fatty acid metabolism genes, 3) lipolysis in the presence and absence of cAMP modulators known to potently regulate lipid metabolism in the pig, and 4) the putative signaling pathway(s) of RLN in porcine adipose tissue. If successful, this work could generate new insights into how to better regulate adipose tissue development.

Chapter 1:

Literature Review

Interestingly, recent studies have revealed that adipose tissue synthesizes and secretes a novel adipokine, bioactive Relaxin (RLN), which has the potential to regulate adipose tissue (Hausman et al. 2006; Roberts et al. 2015). Importantly, adipose tissue exhibits RLN receptor expression, and treating adipocytes with exogenous RLN induces profound changes in the adipocyte transcriptome (Roberts et al. 2015). Identification of RLN as a novel adipokine that can target adipose tissue suggests that the window in which developing adipose tissue is exposed to RLN could span from the emergence of the fetal adipose primordia *in utero* to the harvest of the adult animal; however, RLN receptor expression and RLN secretion from developing adipose tissue has not been characterized *in utero.*

In order to better illustrate why RLN represents a viable candidate regulator of adipose tissue development, I will first elaborate on porcine production and physiology, followed by the biology associated with adipose tissue development and then conclude this literature review with a discussion of the traditional and novel functions of RLN.

1.1 General life cycle of the porcine in swine production and resultant management practices

Throughout the world, more pork is produced and consumed than any other type of meat (McGlone and Pond, 2003). Pork production in the United States is a vital sector of American

agriculture and has been increasing at an average rate of 1.5% annually for the last 70 years (Plain et al., 2003).

1.1.1 Taxonomy and terminology

Common porcine taxonomy and production terminology is used to describe swine given the specific ways this species is used in production systems as a food animal and research model. An understanding of this terminology is useful for understanding how carcass composition develops in growing pigs and how their use in various production cycles can impact this developmental trajectory. Swine belong to the genus S*us* and the species *scrofa*. In the United States, the term "swine" refers to more than one animal or the species as a whole and can be used interchangeable with the word "pig" or "hog." The term "porcine" is an adjective used to describe anything relating to or suggesting swine. An intact, male pig is called a "boar" while a castrated male pig is called a "barrow." A "gilt" is an immature female pig that has not yet been mated, while a "sow" is a mature female. The act of parturition is termed "farrowing" and a "piglet" is a young pig of either sex (McGlone and Pond, 2003). Pigs are monogastrics, meaning they have simple stomachs and are typically fed a standard swine diet consisting of corn and soybean meal with ad libitum access to water (Cheeke, 1999; Lewis and Southern, 2001).

These terms are useful because the body composition and physiological nutritional needs of the pig varies significantly across age, gender classes, and reproductive status. For instance, boars are the leanest, most-muscled, fastest growing class. Barrows are the fattest and grow slower. Gilts fall between these extremes. Sows typically are females that have reached mature frame size and thus grow little. Their metabolic requirements are primarily driven by the need to manage

body condition to maintain optimal fertility or to provide enough nutrition to support the demands of gestation and lactation.

1.1.2 Production phases and management

The U.S. production cycle can be divided into four phases including: gestation, farrowing, nursery and the growing finishing stage (for animals not destined to be retained for the reproductive herd). Each phase has different management obligations that need to be overseen properly to optimize herd health, animal well-being and to achieve an efficient and quality product. Typically, these production classes are separated into dedicated barns to make class-specific management easier and to allow rations to be formulated to meet the unique needs of each class.

Swine are a polyestrous and polytocous species, meaning they have multiple estrous cycles throughout the year, with an average cycle of approximately 21 days, and can farrow many offspring in one birth. Yorkshire gilts may reach puberty and start cycling as early as five months of age and are first bred at approximately six months of age. Mangalica pigs exhibit a delayed onset of puberty and may take as many as 12 months before estrous is first initiated.

Prenatal development, in the porcine, can be divided into three general phases: ovum phase, embryonic phase and fetal stage. The ovum phase is relatively short, roughly 6 days, which extends from the time of fertilization of the ovum to the events leading to implantation of the hatched embryo onto the uterine wall. The embryonic phase extends from the time of implantation to the point of species recognition. The fetal stage extends from the point of species recognition to parturition. Gestation length is roughly 111-115 days (3 months, 3 weeks, 3 days) and depending upon the breed, litter size ranges from 5-18 pigs. For example, Yorkshire sows average 14 piglets with litters ranging between 12-18 depending upon parity, while Mangalica sows average between

5-8 piglets. During gestation, a limited feeding system is implemented on a balanced diet which meets the nutritional requirements of the sow while limiting the consumption of excess energy, as excess energy can increase embryonic mortality (Field and Taylor, 2012).

Neonates are born with less than 1-2% body fat and the inability to regulate internal body temperature making them susceptible to cold stress. They require an external source of heat of about 95˚F until two weeks of age, once they have accumulated approximately 15% body fat (Mersmann et al., 1975). Additionally, neonates are born with very low energy reserves and acquired disease resistance making it vital to receive colostrum for the first twenty-four hours post parturition, to provide passive immunity. Colostrum is the first milk secreted after birth, in mammals, and contains numerous milk-born factors such as hormones, immunoglobulins, peptides and other signaling molecules that get ingested and transported in the circulation of the neonate. These milk-born bioactive factors (MbFs) then bind to specific receptors, in target tissues of the neonate, to alter growth and development and to ensure maximum survival rate and welfare of the animal (George et. al 2018).

Neonates are typically weaned at approximately 3-4 weeks of age, with weanlings weighing approximately twenty pounds. Pigs are transferred from the farrowing facility to the nursery where they are transitioned from, largely, an all milk diet to a corn and soybean diet with whey. During this time, it can be very stressful for the neonates, causing them to be more susceptible to infection and disease and a decrease in feed intake may be observed. After spending roughly 32 days in the nursery and weighing approximately 55-65 pounds, all non-replacement gilts and barrows intended for harvest will transition to the growing-finishing facility.

Most easily appreciated changes in body composition occur between weaning and market weight. Figures 1-1 and 1-2 depict changes in growth and development across the life cycle and

highlight these changes. Young animals grow rapidly during the growing phase (60-120 lbs.) as more muscle protein than fat is deposited. However, as the animal matures and enters the finishing phase (120-260 lbs.), a greater portion of the gain is increasingly due to fat. Growth is measured by weight of the animal and production efficiency. The weight of the animal is measured by the animal's average daily gain (ADG) and is a direct reflection of the animal's ability to grow. Production efficiency or "feed efficiency" (FE) is measured by the pounds of feed required per pound of liveweight gain. Feed accounts for two thirds of the cost of producing swine, so optimizing feed efficiency is crucial for profitability (Lewis and Southern, 2000). The higher the feed efficiency, the more efficient. In the growing-finishing facility, swine are penned by farrowing group and separated by size and sex. Throughout this phase, producers grow the pigs until they reach market weight, roughly 240-280 pounds at approximately 6 months of age for the Yorkshire pig. At this point, growth starts to decline as the maximum amount of lean has been attained while excess energy is converted to fat (Roberts, 2015). Continuing to feed after this period would only lead to increased fat deposition, increased production costs and decreased feed efficiency.

Swine production systems implement a biosecurity protocol to optimize animal health and minimize the spread of infectious agents between and within the herd. Such protocols typically require all workers and guests to shower in and out, have Standard Operating Procedures (SOP) that manage farm transportation trucks, pest management, etc. As part of this SOP, production systems generally operate on an all-in-all-out basis, meaning when pigs transfer to the next production phase, the facility they were housed in is completely cleared and properly disinfected to control pathogen spread amongst the herd before the next group of pigs transition into the barn (McGlone and Pond, 2003; Kyriazakis and Whittemore, 2006; Field and Taylor, 2012).

Furthermore, production systems must meet a list of legal requirements, such as waste treatment rules and regulations, groundwater protection, air quality protection, etc. (McGlone and Pond, 2003). Important elements of these requirements are proper management practices, but also improved feed efficiency of the herd which would allow reduced waste and decrease the environmental impact of swine production. Thus, improving feed efficiency in swine production is an important goal not only because it allows the most feed to be produced per unit of ration fed, but also because it dramatically decreases the impact of swine production upon the environment. Auburn University has its very own biosecure Swine Research and Educational Center which houses naïve herds of both the purebred Mangalica and Yorkshire breeds and works conjointly with Auburn University's Lambert-Powell Meats Laboratory.

1.1.3 Comparison of the Yorkshire and Mangalica breeds

Differences in the growth performance and carcass development between Mangalica and Yorkshire pigs are illustrated in Figure 1-3 and Table 1-1.

The Yorkshire breed originated in England and is widespread, one of United States most popular market hog breeds, due to its superiority of desirable traits such as growth rate, maternal traits and decreased time to market (McGlone and Pond, 2003; Kyriazakis and Whittemore, 2006; Field and Taylor, 2012). Yorkshires are characterized by erect ears and white hair color. This breed exhibits a robust average daily gain (ADG), daily feed intake, and superior feed efficiency (FE) compared to the Mangalica breed, deriving from its lean genetics and resulting in improved carcass characteristics, characterized by a 65-70% carcass lean with minimum backfat (Roberts, 2015).

The Mangalica breed was created in 1833 by the Hungarian Royal Archduke Jozsef and is essentially an unimproved, extreme lard type hog known for its flavorful, marbled meat and high-

quality lard. Mangalica lard contains more unsaturated fat than improved breeds, contributing to the palatability of the meat product by providing a lower melting temperature. This breed is characterized by large, drooping ears and commonly referred to as the "woolly pig" due to its thick woolly coat which comes in one of the three primary hair colors: swallow belly (black), red or blonde. The Mangalica breed lost favor in the early 20th century as genetically improved, efficient breeds, which allowed much more meat to be produced, became popular and prevalent. Mangalica pigs exhibit poor growth parameters with carcasses characteristically exhibiting smaller loin eye area (LEA) and excessive backfat relative to modern breeds such as the Yorkshire and these differences in carcass parameters result in increased time to market and significantly higher production costs for Mangalica (Roberts, 2015). This breed is now preserved as a heritage breed by individuals, as well as gaining attention among niche markets, upscale restaurants and food conscious consumers looking for a more flavorful product, as despite the breed's poorer growth performance, it has maintained its reputation for excellent pork quality.

1.2 Overview of adipose tissue development in the pig

Adipose tissue contains primarily lipid, water, and collagen and is found ubiquitously throughout the animal carcass to varying degrees, but is generally concentrated in predictable anatomical locations of the body termed depots. Furthermore, adipose tissue is comprised of a mosaic of many cell types including preadipocytes, immature pre-adipocytes, mesenchymal stem cells, macrophages, T-regulatory cells, endothelial precursor cells, etc. all of which contribute to the metabolic and endocrine profile of adipose tissue (Poulos et al. 2010; Lafontan, 2011).

As depicted in Figure 1-4, fat accretion in vertebrates is the synergistic result of the processes of hyperplasia (increase in adipocyte number) and hypertrophy (increase in adipocyte

size) and is thus regulated by a complex interaction of genetic, endocrine, and nutritional factors. Hyperplasia functions as a major factor determining the propensity of the adult animal to fatten by determining the total number of fat cells. Adipocyte differentiation gives rise to adipocytes and provides the animal with the necessary metabolic machinery for fat metabolism and consequently, fat accretion. While it is currently thought that fat cell number can increase throughout the lifespan of most domestic animals-especially when the animal is in a positive energy balance- the extent to which the adult animal fattens is ultimately dictated by the hypertrophy of existing fat cells (associated with energy being consumed in excess of the animal's requirements for growth and maintenance) during the "fattening" stage of growth.

Adipose tissue functions as an important energy reserve. Excess energy consumed by the animal is stored in the form of triglyceride (TG) in the mature adipocyte. Thus, the extent of hypertrophy in adipose tissue is largely a function of the relative rates of TG synthesis and degradation. This equilibrium is in turn controlled by the relative contributions of de novo lipogenesis, lipolysis, absorption of dietary fat and fatty acid oxidation (Allen et al., 1976).

1.2.1 Adipose tissue depots and the priority of development model

As depicted in Figure 1-2, there is a priority of development between tissues during normal growth and development such that tissues that are physiologically more important will begin to develop and reach mature size earlier. For instance, the brain will begin to develop first followed by bone, muscle and adipose tissue. Not only is there a priority of development between tissues, but such a priority also exists within tissues and this dictates that adipose tissue develops in a unique pattern as the pig grows and approaches maturity. While whole body adipose is increasingly described as the "adipose organ," adipose tissue develops on vertebrates in defined anatomical

locations. Importantly, each depot has its own unique metabolic and functional profile and develops in the animal at different times while impacting carcass merit differently. In the porcine, these depots include visceral fat, subcutaneous fat, intermuscular fat and intramuscular fat (Wood, 1990).

Visceral fat is located within the body cavity, develops first among depots and is considered "early maturing" while the intramuscular fat or marbling depot is located within the perimysium of whole muscle and fills last being termed "late maturing." Backfat (i.e. subcutaneous fat located between the skin and underlying muscle) and intramuscular fat represent the two most economically important depots in the pork carcass; since backfat is the largest depot but is also highly undesirable and often trimmed and discarded, while marbling is considered a primary determinant of pork quality by consumers at the point of sale (Hammarstedt et al. 2018). Given this, the swine industry is faced with the problem of adopting a strategy to limit subcutaneous fat without negatively affecting the amount of marbling that is present. Currently it is not understood how the temporal development of adipose tissue is regulated in growing animals, but differences in the development of fat depots suggests that the growth of adipose tissue is regulated in a depot specific manner.

Although pigs represent a relatively low fetal load to the dam, newborn pigs are born with extremely low lipid stores (Reed et al., 1993). At birth, the amount of intramuscular fat is greater than the subcutaneous depot and represents greater than 50% of the extractable lipid of the carcass (Hausman and Kauffman, 1986). However, piglets ingest a high fat diet during suckling and there is a resultant rapid increase in fat stores during the immediate postnatal period of growth. At birth, all adipocytes are multilocular (contain multiple fat droplets) but by as early as day 3 postpartum, many have become unilocular (containing one central lipid droplet). Marked increase in adipocytes

size is accompanied by an increase in the size of the central droplet with age (Mersmann et al., 1975).

Adipose tissue accretion becomes the largest contributor to growth as the pig approaches sexual maturity, a period that corresponds to the maturation of muscle growth on the carcass, which allows nutrients to then be repartitioned in support of adipose tissue accretion (Reeds et al., 1993). Presumably as lipid storage is maximized within an adipose tissue depot as it matures and adipocyte size limits are realized, excess energy is then repartitioned toward storage in less developed depots until finally late maturing depots, such as intramuscular fat begin increasing in earnest (Carr et al., 1978; Gu et al., 1992). At market weight, the subcutaneous depot represents 75% of the total extractable lipid in the pork carcass and contains roughly 45% of the adipose cells found in the porcine body (Lee et al., 1973 a, b).

Interestingly, adipose tissue depots display unique metabolic, endocrine, and cellularity profiles as summarized in Table 1-2. Moody and Zobrisky (1966) first characterized three distinct layers within backfat and these individual layers are measurable both in the live animal (via real-time ultrasound) and the carcass, and it appears that these layers also display a developmental priority such that middle > inner > outer layer. Furthermore, each layer is metabolically distinct as the middle subcutaneous layer develops more rapidly than the outer layer with the rate of development of the inner subcutaneous fat layer being intermediate (Allen et al., 1976; Fortin, 1986). Compared to subcutaneous fat, adipocytes within the inter- and intramuscular depots are physiologically less mature and exhibit a delayed growth pattern characteristic of late maturing tissues (Lee and Kauffman, 1974). It appears that intramuscular fat behaves differently from subcutaneous fat, both in terms of cellularity and metabolic activity. Given these inherent depot-specific differences in adipose tissue behavior, it is possible that technologies may be developed

which could effectively target adipose tissue development in a depot specific manner, allowing producers to finely manage the carcass composition of their pigs.

1.2.2 Adipose tissue consequences for production

From a production standpoint, adipose tissue is of great economic importance because it impacts profitability. The degree of adiposity impacts carcass value by influencing cutability or lean yield, marbling, and feed efficiency. Excessive fat accretion in livestock is undesirable from a production standpoint because a great deal of the non-marbling fat that accumulates during growth is then subsequently trimmed off the carcass and disposed of during post-harvest fabrication. Since fat is an undesired commodity and feed represents a considerable productive cost, adipose tissue accretion represents an energy sink which decreases the producer's return on investment.

To illustrate the impact of adipose tissue growth on feed efficiency and growth rate, it is useful to compare the components of lean vs fat tissue. Skeletal muscle is composed of approximately 80% water and 20% protein, whereas adipose tissue is comprised of roughly 90% high-energy lipid and only 10% water. Thus, it takes considerably more feed to support the growth of 1 lb of body fat compared to 1 lb of muscle tissue. Given that feed intake is an asymptotic function of body weight, meaning voluntary feed consumption has an upper limit at any body weight, it is apparent that since adipose tissue requires more feed to produce, the growth of this tissue will be limited by feed intake and thus be correspondingly slower than less energy dense tissues such as muscle.

As illustrated in Figure 1-5, pork quality is measured largely based upon subjective measures, such as color and degree of visual marbling in the retail cut (Lewis and Southern 2001).

Excess accumulation of subcutaneous and intermuscular fat does not contribute to meat quality but rather decreases overall carcass merit, as majority of this fat is removed from wholesale and retail cuts, during carcass fabrication, due to consumers demand for a leaner product (Hood and Allen, 1977). Fatter carcasses bring lower prices. On the contrary, intramuscular fat contributes to the meat's flavor, juiciness and tenderness which greatly increases carcass merit (Lewis and Southern, 2001). Consumers not only consistently select higher marbled products at the point of sale, but have shown a willingness to pay a premium for well marbled retail cuts.

Although the fat content of pork has declined dramatically since the 1980s, regulation of the timing of adipose tissue development is still poorly understood (Lewis and Southern, 2001). Through the years, growth biologists have worked to make animal production more sustainable by decreasing the amount of feed it takes to reach a given unit of mass, increase the rate of growth of the animal and decrease the amount of time it takes to produce the animal; thus, increasing overall production efficiency. However, these improvements have also decreased flavor and quality of the product due to leaner, more muscled animals. Ideally, body composition could be finely manipulated such that intramuscular fat was maximized while economically unwanted depots of subcutaneous and visceral fat were limited. Currently such a result is not yet possible. A better understanding of the basic mechanisms which regulate adipose tissue development is needed to allow development of exciting and novel technologies aimed at preventing the excessive accretion of fat in livestock species.

1.2.3 Critical window aspect of adipose tissue depots

The critical window concept indicates that the confluence of the appearance of a 1) developmental niche, 2) the proper timing of this emergence, and 3) the presence of appropriate

developmental signals, forms a narrow window facilitating key developmental events the interrupting of which results in developmental deficiencies and significantly alters the developmental trajectory of the organ, tissue or organism. Anything that impacts normal growth and development during this window will disrupt the normal developmental trajectory of the animal. Presumably, there is a critical developmental window driving the depot-specific differences and temporal pattern of depot development that are seen in adipose tissue, however, this is currently unclear. During the last four decades, much effort has been devoted to better understanding the regulation of fat accretion in the growing animal based upon the assumption that this critical window occurs postnatally. While this is a reasonable hypothesis given that visually, little fat cover exists on the newborn and differences in depot filling are readily apparent in the growing animal, attempts to regulate adipose tissue accretion in a depot-specific manner postnatally have been failures.

Emerging recent data, driven largely by rodent models, has begun to support a different hypothesis, however. It appears that the critical window driving the ontogeny of depot development occurs much earlier during development, likely manifesting during early fetal development (Figure 1-6). Using fate-mapping strategies during embryonic and fetal development, it now appears that fat cells from different anatomical adipose tissue stores are derived from different progenitor cell populations and their specification may be driven by different transcription factors. This model explains the developmental priority within adipose as observed by the temporal pattern of staggered filling between adipose depots on the growing animal by indicating depots arise from different developmental origins with the timing of progenitor recruitment *in utero*, such that visceral fat primordia appears before subcutaneous fat primordia etc. with the intramuscular fat depot being the last depot to emerge *in utero*. Thus, the pattern of

filling observed on the growing animal is set *in utero* and once set, this temporal pattern of filling is largely intractable to attempts to selectively alter specific depot development after birth. Such emerging models reconfirms the assertion that researchers are prodding at the wrong time point for manipulation of adipose tissue depots and suggests that the animal's critical window is during *in utero* development. Manipulation of adipose tissue depots *in utero* could direct development toward enhanced marbling with limited subcutaneous and visceral fat.

1.3 Adipose tissue biology

Adipose tissue is histologically classified as a specialized form of connective tissue that is highly vascularized with a mosaic of metabolically active cell types, held together by an extracellular matrix composed of collagen and reticular fibers. During normal growth and development, preadipocytes are recruited from stem cells and then differentiate into mature fat cells, which express all the enzymes necessary for lipid metabolism. Adipose tissue also expresses numerous receptors that allow it to communicate with other hormone systems, in addition to the central nervous system (CNS) (Kershaw et al. 2004).

1.3.1 Functions of adipose tissue

Fat cover is important in terms of production efficiency and carcass merit, but adipose tissue is also very important in regulating energy balance, growth and reproduction in the live animal. Traditionally, adipose tissue has been regarded as having a passive role in body homeostasis, but research has proven otherwise. The plasticity of adipose tissue plays a critical role in maintaining metabolic homeostasis of the animal in times of negative or positive energy balance (Crewe et al., 2017). Remodeling of adipose tissue, i.e. the expansion or contraction, is

achieved by the collaborative response of various cell types including adipocytes, immune cells, endothelial cells and fibroblasts (Crewe et al., 2017).

Importantly, human and rodent studies have implicated adipose tissue as an essential, endocrine organ because this tissue can secrete a wide variety of factors, termed adipokines, into the systemic circulation (Zhang et al., 1994; Kershaw et al., 2004). These adipokines can then communicate with other cell types in an autocrine, paracrine and/or endocrine fashion to regulate multiple cellular processes and, ultimately, in regulating the health and disease state of the animal. Too much or too little adipose tissue accretion can have severe metabolic consequences, both in terms of adipose tissue function but also in terms of the resultant alterations in adipokine secretion. For instance, an excess accumulation of adipose tissue, or obesity, is associated with secretion of proinflammatory cytokines from adipose tissue resulting in a low grade, chronic inflammation that can lead to insulin resistance, metabolic syndrome and chronic degenerative disease (Kershaw et al. 2004; Spurlock et al. 2008). Too little adipose tissue accumulation, or lipodystrophy, is also associated with insulin resistance and can lead to other metabolic abnormalities as other soft tissues and organ systems must adjust their metabolic profiles in order to compensate for the lost function of adipose tissue (Leow et al. 2003).

1.3.2 Biochemistry of adipose tissue

Energy balance and the developmental trajectory of an animal are tightly coordinated, and this is especially true of energy balance and the development of adipose tissue within an animal. After birth, hypertrophy is modulated by energy balance and cAMP modulators. Fat cell size is regulated by the processes of lipogenesis and lipolysis and these pathways are exquisitely regulated

by homeorhetic hormones, which orchestrate changes in whole body energy metabolism in response to the transition between negative and positive energy balance within the animal.

1.3.2.1 Lipogenesis

Lipid is stored within adipocytes as neutral triglyceride when energy intake is greater than energy expenditure, i.e. during periods of positive energy balance. The adipocyte synthesizes lipid de novo either through taking up dietary lipid or by synthesizing lipid de novo from glucose carbon and condensing the intracellular free fatty acids with glycerol to form neutral TG. The site of lipogenesis and the primary carbon source varies by species dependency. Research has confirmed that adipose tissue is the primary lipogenic tissue in the pig while in humans, poultry and rodents, lipogenesis primarily occurs within the liver and mammary gland (Bergen and Mersmann, 2005). Glucose is the principal carbon source for humans, pigs, rodents and poultry, while acetate is the principal carbon source for cattle, sheep and goats (Bergen and Mersmann, 2005). This is an important consideration to be aware of when comparing regulation of lipid metabolism in different species. Humans and pigs are generally more alike than any other species, but the site of lipogenesis represents a major departure from this shared biology.

For the purpose of this literature review, I will be focusing on lipid biochemistry in the pig. In a positive energy balance, there is an abundance of glucose in the blood which causes the pancreas to produce insulin. Insulin stimulates adipocytes to translocate glucose transporter type 4 (GLUT-4) to the plasma membrane to absorb glucose and stimulate glycolysis. During glycolysis, glucose is transformed into three-carbon dihydroxyacetone phosphate (DHAP) and further processed into two-carbon acetyl coenzyme A (acetyl-CoA). Acetyl-CoA will then enter the tricarboxylic acid (TCA) cycle, in the mitochondria, where it will condense with oxaloacetate

to become citrate. Citrate can leave the mitochondria and enter back into the cytoplasm of the cell, through a process called the citrate shuttle, where it is converted back into acetyl-CoA by Acetyl-CoA carboxylase (ACC). ACC starts conjoining acetyl-CoAs to form malonyl-CoA which then permits fatty acid synthase (FAS) to produce the hydrocarbon, palmitate. Palmitate can then combine with glycerol, in the adipocyte, to form a triglyceride where it will be stored as a future energy reserve.

Pigs being monogastric can essentially deposit dietary fat in their adipose tissue unchanged; thus, adipose tissue from a pig fed a high fat diet will begin to have a fatty acid composition that reflects the fatty acid composition of their diet. Triglycerides from the diet are broken down in the digestive tract and absorbed through the portal blood supply as glycerol and free fatty acids. These larger substrates will navigate to the liver where they will be reformed into triglycerides and packaged into chylomicrons, transporter lipoproteins. Chylomicrons are then released into the systemic circulation to deliver lipids to adipose tissue for storage. Alternatively, smaller fatty acids will travel through the lymph, bypassing the liver into the systemic circulation, where they will be repackaged by lipoproteins and delivered to adipose tissue. As dietary triglycerides reach the adipocyte, they will be cleaved by lipoprotein lipase (LPL), an enzyme in the fat cell capillary wall that is upregulated by insulin signaling and allows glycerol and free fatty acids to enter the cell. Once inside the adipocyte, free fatty acids are converted to fatty acid acyl-CoA (FAacyl-CoA) and the DHAP from glycolysis can be transported and converted to α–glycerol phosphate. The combining of these two substrates leads to the formation of triglyceride and hypertrophy of the adipocyte.

1.3.2.2 Lipolysis

Lipid, in the form of free fatty acids, is mobilized from adipocytes as neutral triglyceride and is hydrolyzed and broken down when energy intake is less than energy expenditure, i.e. during periods of negative energy balance. The adipocyte catabolizes neutral TG in a straightforward hydrolysis of the bond joining glycerol and long chain fatty acids to yield glycerol and three free fatty acids, which will then be secreted from the fat cell into circulation to support the energy needs of other organs and tissues. Catecholamines, such as epinephrine and norepinephrine, can stimulate lipolysis by binding to specific G protein-coupled receptors that then activate adenylate cyclase. Adenylate cyclase activates the cAMP pathway, leading to activation of cAMP-dependent protein kinase (PKA) and stimulation of hormone sensitive lipase (HSL). HSL targets triglycerides inside adipose tissue and begins breaking them down into free fatty acids and glycerol which will exit the cell to fuel energy needs. Thus, lipolysis is under potent control of cAMP modulators and any hormone that can either signal via or inhibit the cAMP pathway has the potential to regulate lipolysis and hence, adipocyte hypertrophy in the pig.

1.3.3 Adipokines and endocrine function of adipose tissue

Relatively recently within the field of adipose tissue biology, a role for adipose tissue as an important endocrine organ has been established. While several factors were known to be secreted from adipose tissue and it had long been hypothesized that adipose tissue secreted a factor that functioned as a feedback signal to the brain which regulated satiety, the nature of that signal was not determined and the physiological role of known secretory products were not established. In the mid-90's, leptin was established as the adipose-derived satiety factor and interest in adipose tissue as an endocrine organ intensified. Soon after, the tern "adipokine" was coined.

An adipokine is any secreted factor, from adipose tissue, that can bind to a receptor and elicit a response in a target cell regardless of endocrine, autocrine or paracrine mode of action. Thus, the term adipokine can include hormones, cytokines, etc. with the primary character being the secretion from adipose tissue. Some well-known secreted adipokines that perform essential roles in regulating normal growth are leptin, a hormone which regulates satiety, energy balance, and the onset of puberty and adiponectin, a hormone with anti-inflammatory action which also enhances insulin sensitivity in target tissues.

Leptin is produced by white adipose tissue and circulates in the blood at concentrations proportional to the amount of fat deposition and as such represents the prototypical adipokine (Ahima and Flier, 2000). Leptin regulates energy balance by decreasing feeding behavior by decreasing appetite through acting directly on neurons within the arcuate nucleus comprising the feeding center of the hypothalamus. A total lack of leptin or leptin signaling in rodents and humans causes morbid obesity. Leptin is also important in regulating the onset of puberty. As more adipose tissue accumulates, the circulating concentration of leptin increases. Once leptin reaches a threshold level, gonadotropin releasing hormone (GnRH) neurons of the hypothalamus become activated resulting in a spike in GnRH, communicating where the animal is on the growth curve. A sharp rise in GnRH stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary, which then act on the ovaries and testes to initiate sex steroid synthesis. Thus, leptin secretion from adipose tissue is a stark example of a mechanism which tightly couples energy balance with the developmental trajectory of the animal as leptin serves as a proxy that informs the brain that the animal has sufficiently progressed through its growth curve to allow successful gestation. While there are some adipokines that perform essential roles in regulating growth, there are other adipokines such as the pro-inflammatory cytokines that

really do not have a well-studied role. Pro-inflammatory cytokines such as tumor necrosis factor alpha and interleukin-6 have been recognized to increase muscle protein degradation and decrease muscle protein synthesis; however, research still faces an open-ended discussion concerning cytokines and their function in adipose tissue. It is certainly unclear why adipose tissue expresses a functional innate immunity pathway. There are few instances where an inflammatory state within adipose tissue has been associated with a positive health outcome or a permissive effect on optimal growth. In fact, as discussed below, a case can be made for labeling adipose-derived cytokines as endogenous endocrine disruptors.

1.3.4 Adipose tissue recruitment and development

In utero, hyperplasia is initiated by the recruitment of progenitor cells from different stem cell pools through unique developmental fate pathways. The subsequent action of niche-specific transcription factor expression causes depot-specific changes in the transcriptome facilitating preadipocyte specification and subsequent adipogenesis. Adipogenesis is essentially regulated by altering progenitor commitment to preadipocytes and terminal differentiation of preadipocytes (Ruiz-Ojeda et al. 2016). In the first stage, a pluripotent, mesenchymal stem cell commits and develops into a preadipocyte. In the second stage of terminal differentiation, the pre-adipocyte now displays characteristics of a mature adipocyte (Rosen et al. 2006). That progenitor cells originate from different developmental lineages helps explain the characteristic differences seen in adipose tissue depots.

1.3.4.1 Recruitment of progenitor cells

The process of adipose tissue primordia formation begins with the recruitment of mesenchymal stem cells or progenitor cells. During prenatal development, this process largely involves hyperplasia coupled with the formation of new blood vessels, which work to help supply the cells with oxygen and nutrients (Hammarstedt et al. 2018).

Developmentally, this process occurs after embryo implantation, gastrulation and the emergence of mesenchymal stem cells from the mesodermal germ layer as depicted in Figure 1-6. Adipose tissue originates from mesenchyme, a type of loose connective tissue located within the embryonic mesoderm. Mesenchyme consists of a loose aggregate of unspecialized cells, capable of various developmental fates such as bone, connective tissue, cartilage, the lymphatic system and the circulatory system. As mesodermal cells proliferate, they migrate and form ridges along the primitive groove of the developing embryo, becoming the lateral plate mesoderm and paraxial plate mesoderm.

The paraxial plate mesoderm segments into somites containing specialized populations of stem cells including the sclerotome, myotome and dermatome. The sclerotome gives rise to cartilage and bone, the myotome gives rise exclusively to muscle and the dermatome gives rise to muscle, fat and connective tissue. The dermatome consists of a subset of Myf5+ stem cells which can undergo different cell lineages and specialize into different tissue subtypes, stemming from transcription factor expression and regulation. Transcription factors will sit on the promoters of genes of interest, changing the transcriptome and thus the developmental fate of the stem cell. If the Myf5+ stem cell expresses the transcription factor MyoD, the Myf5+ stem cell's transcriptome changes, committing the stem cell into becoming a muscle cell (Bonnet et al., 2010; Du et al., 2015). If the Myf5+ stem cell expresses the transcription factor PRDM16, the Myf5+ stem cell

commits into becoming brown adipose tissue (Bonnet et al., 2010; Du et al., 2015). If the Myf5+ stem cell expresses the transcription factor TGFβ1, the Myf5+ stem cell commits into becoming a fibroblast which secretes collagen fibers or connective tissue (Bonnet et al., 2010; Du et al., 2015). If the Myf5+ stem cell expresses the transcription factor Zfp423, the Myf5+ stem cell can commit into becoming intramuscular fat or marbling (Bonnet et al., 2010; Du et al., 2015).

Emergence of adipocytes from the lateral plate mesoderm has been hypothesized based on fate mapping studies in mice, but this issue is less studied. The lateral plate mesoderm consists of a subset of Myf5- stem cells which will ultimately give rise to endothelial precursor cells, blood vessels and pre-adipocytes. The preadipocytes recruiting from the common progenitor of these cells will commit into becoming visceral or subcutaneous adipose tissue.

1.3.4.2 Regulation of adipogenesis

Once progenitor cells become preadipocytes, adipogenesis is controlled by a regulatory cascade of transcription factors comprised of factors regulated by nutrients, such as fatty acids and factors regulated by homeorhetic hormones such as insulin.

Adipogenesis is initiated as preadipocytes exit the cell cycle and undergo differentiation which results in an increased expression of adipocyte proteins, originating from transcription factor activation, and leading to upregulation of adipocyte specific genes and quiescence of preadipocyte specific genes (Lafontan, 2012). Adipocyte differentiation is a multi-step process catalyzed by transcription factors, predominantly by the CCAAT-enhancer binding protein family (C/EBP), peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element binding protein (SREBP) (Poulos et al. 2010) (Ruiz-Ojeda et al. 2016). Activation of C/EBPβ leads to activation of the PPARγ and C/EBPα genes. PPARγ is the master regulator of adipogenesis and drives the

expression of a host of adipocyte-specific genes. C/EBPα works to maintain expression of PPARγ, but also coordinates with PPARγ to drive expression of the adipocyte specific genes. SREBP-1 assists PPARγ by delivering endogenous ligands which contributes to maximal activation of the master regulator of adipogenesis. Preadipocytes that differentiate through transcriptional activation become mature fat cells containing all the genes and enzymes necessary for lipid biochemistry and for performing the endocrine function of adipose, such as expression and secretion of a host of adipokines which regulate multiple processes such as energy balance, growth and reproduction.

1.3.4.3 Regulation of adipocyte biology via cellular signaling pathways

In general, regardless of tissue or cell type, cellular signaling is a receptor mediated process. Ligand binding generally results in two types of responses: changes in metabolic flux and/or changes in gene expression. Changes in metabolic flux are generally accomplished via orchestrating reversible phosphorylation events that regulate existing proteins such as enzymes, leading to biochemical pathways either being stimulated or shut off. Changes in gene expression occur when secondary messengers act on transcriptional regulators in the nucleus causing genes to either be transcribed or repressed. Changes in metabolic flux are usually orchestrated rapidly while changes in the transcriptome are much slower requiring hours to days for effect. The duration of response is then determined by the affinity between the ligand and its receptor. The stronger the affinity, the greater the strength and duration of the hormone signal.

Adipose tissue displays several well-established paradigms of cellular signaling. Some of the primary pathways which are well documented to either alter metabolic flux or gene expression

patterns include: G-protein coupled receptor (GPCRs), adenylate cyclase-cyclic adenosine monophosphate (cAMP) signaling and the Jak-Stat signaling pathways.

Guanine-nucleotide protein coupled receptors (GPCRs or G-protein coupled receptors) are seven-transmembrane domain receptors (7-TM receptor) that span the membrane seven times, are coupled to trimeric G protein complexes and make up the largest class of cell surface receptors (Tao, 2006). The cAMP and phospholipase C (PLC) pathways are the two major signaling pathways involved with GPCRs in adipose tissue. Depending on the type of protein present, G_i (inhibitory) or G_s (stimulatory), will have opposing effects on intracellular signaling.

When a ligand binds to its receptor in the (cAMP) pathway, it will activate a G_s alpha-subunit protein. The G_s protein becomes activated and in turn will activate the enzyme adenylate cyclase. Adenylate cyclase then converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). When cAMP levels go up, it then activates the cAMP-dependent protein kinase (PKA), which can then work to phosphorylate proteins inside the cell such as the cAMP response element binding protein (CREB). CREB will then bind to a cAMP response element (CRE) on the promoter region of the gene and allow gene expression to occur. This signaling pathway can ultimately lead to upregulation or downregulation of genes which will cause a response in seconds. The regulation of lipolysis by this pathway was already described above.

Jak-Stat signaling, on the other hand, does not require a G protein. Growth hormone or cytokines will bind to their receptors and this binding initiates the transphosphorylation of Jak proteins and attached tyrosine residues. The phosphorylated Jak proteins direct the phosphorylation of two stat proteins. After phosphorylation is complete, the stat proteins will peel off and form a stat dimer which will make its way to the nucleus to regulate gene expression.

1.3.4.4 Endocrine regulation of adipose tissue biology

In general, adipose tissues are regulated by numerous, diverse hormones which play a role in maintaining whole body homeostasis. Specific adipose tissue regulatory hormones consist of growth hormone, cytokines and cAMP modulators such as beta-adrenergic agonists and insulin.

1.3.4.4.1 Growth hormone

Somatotropin, commonly referred to as growth hormone (GH), is a protein hormone secreted in a pulsatile fashion by somatotroph cells of the anterior pituitary. Its release is controlled by growth hormone releasing hormone (GHRH) which signals release from the hypothalamus, while negative regulation of GH is controlled by growth hormone inhibiting hormone (GHIH), also referred to as somatostatin which inhibits the release from the hypothalamus. Once released, GH promotes tissue growth of bone and muscle while having catabolic actions on adipose tissue by inhibiting differentiation of human and porcine primary adipocytes (Armani et al. 2010).

The daily administration of recombinant porcine GH to growing pigs is a technology which favorably alters carcass composition by increasing lean growth while decreasing adipose tissue accretion and feed intake. Consequently, GH can significantly increase animal performance. As a result, GH administration represents a strategy to efficiently produce leaner pork. Growth hormone is clearly a homeorhetic hormone that has effects on multiple tissues resulting in an overall anabolic, diabetogenic state in growing pigs. This is affected through coordinate changes in nutrient metabolism in target tissues so that absorbed nutrients are partitioned away from adipose tissue in support of increased lean growth (Etherton et al., 1989, Etherton et al., 1992, NRC, 1994, Etherton and Bauman, 1998). Growth Hormone alters the metabolism of all major nutrient classes

as evidenced by changes in the concentration of circulating metabolites in response to chronic treatment. The is reflected in significantly altered carcass composition.

Growth hormone can either act directly by binding to growth hormone receptors on other target tissues to alter nutrient metabolism or indirectly by binding to growth hormone receptors on the liver, which stimulates the release of insulin-like growth factor-1 (IGF-1). IGF-1 then functions to coordinate growth of other tissues by stimulating cell proliferation. This is due to changes in nutrient metabolism of target tissues where nutrients are partitioned away from adipose tissue and instead towards lean growth (Etherton et al 1989). The differences in dose-response curves for different parameters of growth performance suggest that GH affects these processes by different mechanisms (Evock et al., 1988). The somatomedin or "dual effector" theory of GH action fits these data well. Current evidence suggests that GH can act either indirectly through the regulation of hepatic IGF-1 to stimulate cell proliferation or directly on target tissues via GH receptors to alter nutrient metabolism (Green et al., 1985, Etherton and Bauman, 1998). The net result of GH action on the adipocyte is to suppress lipogenesis through antagonism of insulin signaling and the downregulation of lipogenic gene expression concomitant with stimulation of lipolysis and lipolytic gene expression.

1.3.4.4.2 Cytokines

Cytokines can also play a role in regulating adipose tissue. Cytokines are a diverse group of hormones involved in several immunological processes that can communicate with other cell types in an autocrine, paracrine and/or endocrine manner often being secreted locally by nonimmune cell types. Adipocytes themselves have a functional innate immunity pathway and

secrete cytokines that in turn activate different responses in adipose tissue or other surrounding tissues (Jacobi et al. 2006).

Generally, there are two different classes of cytokines: anti-inflammatory or pro-inflammatory. Anti-inflammatory cytokines, such as Interleukin-10 (IL-10) and Interleukin-13 (IL-13) have been demonstrated to initiate anabolic processes, antagonistically functioning to counteract adipose tissue inflammation and insulin resistance associated with pro-inflammatory cytokines (Wojdasiewicz et al. 2014), such as tumor necrosis factor alpha (TNFα) and Interleukin-6 (IL-6), which have been demonstrated to initiate catabolic processes (Jacobi et. al, 2006). When endotoxin or lipopolysaccharides (LPS) from bacteria are introduced into circulation, this activates the innate immune pathway. The endotoxin will bind to toll-like receptor 4 (TLR4) that is expressed on adipose tissue (Lin et al. 2000), which will then activate nuclear factor kappa-B (NFκB) transcription factor. NFκB causes the upregulation of pro-inflammatory cytokines. These pro-inflammatory cytokines attract immune cells such as macrophages into adipose tissue which stimulate catabolic processes in target cells. For example, the breakdown of triglycerides and protein into fatty acids and amino acids respectively. Saturated fatty acids have been identified to stimulate pro-inflammatory effects by activating TLR-4 in adipocytes while polyunsaturated fatty acids are typically anti-inflammatory and promote insulin sensitivity (Faris et al. 2012). Pro-inflammatory cytokines promote insulin resistance by downregulating insulin stimulated glucose transporter type 4 (GLUT-4) (Lumeng et al., 2007; Crewe et al., 2017). Under controlled inflammation, pro-inflammatory cytokines such as TNFα and IL-6 have also been identified to have angiogenic effects and may contribute to the healthy expansion of adipose tissue during adipogenesis though this is a controversial assertion (Wagner et al., 2012; Kwon et al., 2013; Crewe et al. 2017). More in-depth research is still needed to characterize this diverse group of

cytokines and the role they perform in adipogenesis because consistent effects have not been fully demonstrated.

1.3.4.4.3 cAMP modulators

cAMP modulators can impact adipose tissue development either by stimulating or inhibiting the cAMP pathway, leading to homeostatic or metabolic changes which largely involve switching the balance between lipogenesis and lipolysis.

Insulin, an established anti-lipolytic hormone, is released from the pancreas in a state of positive energy balance. Upon binding to its receptor on adipocytes, it inhibits the cAMP pathway by activating phosphodiesterase. Phosphodiesterase activation leads to the inhibition of the cAMP pathway and reduction of cAMP levels by converting cAMP to adenosine monophosphate (AMP). Insulin also stimulates adipocytes to translocate glucose transporter type 4 (GLUT-4) to the plasma membrane to absorb glucose. A lot of this glucose will be converted to glycerol and combined with free fatty acids to form triglycerides, which can then be stored in adipose tissue as an energy reserve. Thus, insulin simultaneously inhibits lipolysis and stimulates lipogenesis by activating lipogenic genes and increasing flux through the glycolytic pathway.

Beta adrenergic agonists (BAAs), or catecholamines, are referred to as the “fight or flight” hormones which bind to beta adrenergic receptors (β-AR) and stimulate the cAMP pathway. Physiological, naturally occurring (B-AR) agonists are norepinephrine and epinephrine (Mersmann, 1998). Other studied synthetic agonists include isoproterenol, clenbuterol, cimaterol, ractopamine, etc. There are three primary subtypes of the (β-AR) comprising of β_1-AR, β_2-AR and β_3-AR. These receptors are present on almost all mammalian cells; with β_1-AR and β_2-AR being the predominant receptor isoforms in pig adipose tissue (Mersmann, 1998). The homeostatic

effects of an activated β-AR include increased heart rate, blood flow and force of contraction; while the metabolic effects being an increased protein synthesis in skeletal muscle and increased lipid degradation in adipose tissue.

Catecholamines are potent regulators of lipid metabolism, as the putative way fat cell hypertrophy is regulated is due to β-AR activation or insulin stimulation. Activation of β-ARs in adipose tissue leads to an increased production of intracellular cAMP and the phosphorylation of PKA, which activates HSL and results in increased lipid breakdown or lipolysis (Mills and Mersmann, 1995). β–AR activation also leads to the inhibition of ACC and FAS, blocking glucose and free fatty acid uptake. Oral administration of synthetic β-AR agonists alters growth composition in pigs, cattle, poultry and sheep by promoting protein synthesis and accretion of skeletal muscle while increasing lipid breakdown, reducing backfat depth (Mersmann, 1998). Increased rates of protein synthesis have been observed in pigs fed ractopamine (Bergen et al. 1989; Helferich et al. 1990), whereas the effects of decreased backfat depth have been observed to a greater degree in ruminants, where backfat depth is typically only reduced by 10-15% in swine treated with the same agonist (Anderson et al. 1992; Mills and Mersmann, 1995). These differences are thought to be the result of species differences in receptor subtype variation and receptor desensitization.

1.3.5 Evolutionary relationships between relaxin, insulin and insulin-like growth factor

Relaxin, Insulin and IGF (insulin-like growth factor) all share an evolutionary relationship with one another and contain the basic structural signature of the insulin peptide with six cysteine residues in conserved positions (Wilkinson and Bathgate, 2007). Consequently, it was proposed that relaxin, insulin and IGF were derived from a common ancestral gene and therefore classified

as the insulin superfamily (Wilkinson and Bathgate, 2007). Although each receptor is different, the common hormone morphology allows relaxin, insulin and IGF to cross communicate, or "crosstalk" with one another, binding to each other's receptors, not just their own. Such cross-binding permits overlapping signaling and shared effects on cells and tissues.

1.3.6 Endocrine disruption and a new way to view adipokines

Traditionally, endocrinologists have defined endocrine disrupters as chemicals that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological and immune effects. An example of an endocrine disrupter would be bisphenol A (BPA) coming from the environment. BPA is structurally similar to estrogen allowing it to bind to estrogen receptors and trigger estrogen signaling in a wrong context. Given this definition, it may be appropriate to view cytokines as endocrine disrupters when they are secreted from a non-traditional source such as adipose tissue which is not thought to function during a normal immune challenge. In such a state, they are expressed in the wrong context i.e. a healthy, non-infected or injury state of the animal. In that context, cytokines can then interfere with the normal function of the HPG axis and with the action of homeorhetic hormones and modulate growth and energy homeostasis through their effect on peripheral target tissues.

1.3.6.1 Endogenous endocrine disrupters in disease states

As mentioned previously, adipose tissue expresses a functional innate immune pathway and scientists are not completely sure why. Endotoxin and other ligands for the TL4 receptor stimulate the upregulation of pro-inflammatory cytokines (TNFα) and (IL-6) from macrophages and adipocytes. Normally this stimulation serves to recruit more macrophages and initiate an

inflammatory reaction, but it is unclear what advantage is gained by recruiting macrophages into adipose tissue and stimulating an inflamed state in this important metabolic tissue.

Furthermore, the literature suggests that activation of inflammatory pathways can stunt growth. Holck et al. (1998) demonstrated the striking magnitude of growth depression that occurs in normal swine operations that are relatively free of overt disease presence. These authors grew pigs either in a clean, disease-free or a dirty environment (naïve/biosecure environment vs typical production environment) where the animals were exposed to pathogens. The pigs in the dirty environment exhibited a 30% depression of growth and a significant decrease in fat free lean suggesting that immune challenge is a significant drag on performance. Researchers have always assumed that this drag on performance was mainly due to the energetic cost of paying for an activated immune system and the impacts on growth are a secondary effect. However, other literature such as Defalque et al. (1999) suggests that there are direct links between the immune system and the growth axis. These authors subjected rats to LPS infusion to increase circulating levels of pro-inflammatory cytokines and then measured growth hormone receptor gene expression in the liver. LPS infusion caused a dose-dependent decrease in growth hormone receptor gene expression on the liver, which was associated with the development of growth hormone insensitivity in this important GH-target tissue in part measured by significantly lower circulating IGF-1 levels. These data suggest that pro-inflammatory cytokines can suppress both modes of action of growth hormone-direct through downregulating the GH receptor and indirect through diminishing GH-stimulated IGF1 secretion. Given GH is the major pituitary signal directing progression of the animal through its growth curve due to its pleiotropic action on tissues of the carcass, such direct modulation of GH action via cytokines would be expected to significantly

impact growth and development. In this context, cytokines released from adipose tissue absent of infection, fits the definition of an endocrine disrupter that disrupts normal signaling.

1.3.6.2 Adipokines as endogenous, endocrine disrupters in a healthy state?

Can adipose tissue secrete endogenous, endocrine disrupters that alter feed efficiency and carcass composition in a healthy food animal, i.e. one that does not have an inflammatory signal triggering the innate immunity pathway per se? It has become clear during the last decade that the immune system modulates homeostatic regulation of growth through crosstalk between inflammatory and somatotrophic pathways and the presence of locally derived factors from adipose tissue suggests there could be unappreciated peripheral endocrine axis operating between adipose tissue and skeletal muscle (Carroll, 2008). Research by Hausman et al. (2006) has shown that porcine adipose tissue secretes a wide variety of adipokines including pro-inflammatory cytokines, which have the potential to communicate with and direct other cell types whose regulation by the HPG axis is important for normal regulation of growth**.** Ramsey et al. (2009) revealed that adipokine expression varies among different fat depots and changes throughout normal, neonatal development independent of altered immune contexts suggesting that the secretion of adipokines themselves are under developmental regulation**.** Ramsey et al. (2013) demonstrated that the pro-inflammatory cytokine, TNFα, suppresses lipogenesis, suppresses insulin-stimulated regulation of adipose tissue, and altered the gene expression of other adipokines**.** These data collectively suggest that pro-inflammatory cytokines can act as endogenous, endocrine disrupters and disrupt normal growth and development of the animal calling into focus a great need to better understand the regulation of these factors from developing adipose tissue. Likewise, these

data indicate that more research needs to be directed toward the study of novel adipokines and their actions on adipose tissue growth and development.

1.3.7 Extracellular matrix of adipose tissue

The extracellular matrix (ECM) is the structural component of adipose tissue and acts as the foundation facilitating other important cellular processes such as angiogenesis and adipogenesis while also functioning to anchor cells, isolate tissues from one another and regulate intercellular communications (Hausman, 2012). Fibrogenesis, angiogenesis and adipogenesis appear to be a codependent triad as they appear to be coordinated processes during the development of the adipose tissue primordia *in utero* and during expansion of adipose tissue during the remaining life cycle of the pig. Fibroblasts secrete components such as collagen subunits which determines the structural integrity of the ECM. After fibrogenesis, angiogenesis can begin in earnest. Angiogenesis is a critical component to the subsequent onset of adipogenesis because the vasculature supplies the adipocytes with oxygen, nutrients, hormones and growth factors (Crewe et al., 2017). Fetal adipose tissue development in the pig has been well studied by Hausman's group. Their work supports the model proposed in Figure 3-7 in which fibrogenesis must occur first to provide a supporting network and foundation to allow anchorage for blood vessel formation. By day 50 collagen fiber networks, progenitor cells, endothelial cells, blood vessels and fibroblasts are all visible. By day 60 progenitor cells can begin the process of adipogenesis by differentiating into preadipocytes. By day 80 mature fat cells are visible.

Cell shape, migration, proliferation, differentiation and tissue development are all controlled by the composition of the ECM through interaction with various cell surface receptors and signaling molecules (Nakajima et al. 1998; Toole et al. 1991). Collagen fiber formation plays

a role in altering the ECM (Poulos et al. 2015). Collagen proteins, which consist of three polypeptide chains, called α chains, are the main structural components of connective tissue and the ECM which function to strengthen and support many surrounding cell types and tissues of the body (Hynes and Yamada, 2012). There are more than 14 different types of collagens and each one is given a numerical number in the order in which it was discovered (Hay, 1991). These collagens are further sub-grouped into different classes: fibrillar (COL1, COL2, COL3, COL4, COL11), fibril-associated (COL9, COL12), network-forming (COL4), filamentous (COL6), short chain (COL8, COL10), and long chain (COL7) (Hay, 1991). COL1 is the most abundant collagen in the human body (Karsenty et al., 1995; Di Lullo et al., 2002;) and has also been found to be ubiquitously expressed in porcine somatic cells (Park et al., 2016). Other components of the ECM include elastin, fibronectin, glycosaminoglycans, glycoconjugates, hyaluronic acid, laminins, proteoglycans and vitronectin (Hausman, 2012). Alteration of the collagen fiber network can ultimately alter progenitor recruitment, resulting in diminished adipogenesis.

1.3.7.1 Extracellular matrix components play a role in adipose tissue development and obesity-related fibrosis and inflammation.

The extracellular matrix (ECM) of adipose tissue is immensely populated with collagen fibers, made up of many different subunits which determines the strength of these fibers. Increases in adiposity are accompanied by extensive alterations in the ECM of adipose tissue that, if excessive, will lead to an inflammatory state (Khan et al., 2009). This remodeling is accomplished in part through changes in levels of matrix degrading proteins such as matrix metalloproteinase (MMP) 2, 3 and 12 and extracellular proteins such as COL1, COL3, COL6, as well as biglycan. These changes are accomplished, in part, by altered adipocyte secretory behavior.

The expression of ECM constituents is determined by a balance between their synthesis and release from stromal cells and their degradation under the control of metalloproteases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs). During ECM remodeling, the balance between these processes can shift to accommodate tissue expansion while dysregulation allowing excessive ECM deposition can lead to fibrosis or a hardening of the tissue. Accumulating evidence indicates that the ECM of adipose tissue plays an important role in adipose tissue development and that remodeling of the ECM to a fibrotic phenotype during the onset of obesity may have important consequences for the metabolism and endocrine function of adipocytes (Khan et al., 2009, Nakajima et al., 1998). Studies using murine preadipocyte cell lines have established that adipocytes affect their environment by secreting type 1 and 3 collagens and basement membrane proteins such as type 4 collagen and laminin (Aranti and Kitagawa, 1988; Weiner et al., 1989). Importantly, the expression of ECM components is highly regulated during fat cell differentiation with types 1 and 3 collagens, fibronectin, and beta-1-integrins being down-regulated and type 4 collagen and entactin being up-regulated during adipogenesis (Gregoire et al., 1998). Khan et al., (2009) suggests that collagens are highly upregulated in the adipose tissue of mice during metabolic changes with COL1, COL4 and COL6 demonstrating to be highly expressed in normal adipocytes, whereas COL2 and COL3 exhibited lower expression levels. COL6 has been demonstrated to be an important component of the ECM of adipose tissue as it is highly expressed in various fat depots, clearly demonstrating an important role in adipose tissue physiology (Khan et al., 2009). Lack of COL6 in mice results in larger adipocyte size and a less restrictive ECM environment (Khan et al., 2009).

As would be expected, MMPs also play a role in ECM formation during adipose tissue development. For instance, MMP-2 and -9 are expressed by both preadipocytes and adipocytes

and required for fat cell differentiation, while MMP-3-deficient mice display enhanced rates of adipogenesis, suggesting an inhibitory role for MMP-3 (Bouloumie et al., 2001; Christensen et al., 2016). Inhibition of MMP-9 dramatically reduces adipocyte development by 50% in human stromal vascular (SV) cells, suggesting that MMPs may indirectly influence adipogenesis by regulating turnover of adipocyte ECM proteins (Hausman, 2012). The MMP14 gene involved in collagen breakdown has also been shown to be significantly upregulated in mice fed a high-fat diet (HFD) for one week (Chun et al., 2010; Crewe et al., 2017). Importantly, adipose tissue undergoes extensive remodeling during abnormal development, such as during the onset of obesity, with significant alterations in the ECM. This is characterized by altered levels of MMP 2, 3 and 12 and extracellular proteins such as collagen COL1, COL3, COL6, and biglycan. Khan et al., (2009) showed that connective fiber content of adipose tissue increases dramatically during the onset of obesity due to upregulation of several collagens. This led to a fibrotic state in adipose tissue that was associated with increased proinflammatory cytokine release that was reversed through knockdown of COL6 independent of weight loss. However, mechanisms governing regulation of obesity-related matrix remodeling are poorly understood. Elucidation of these mechanisms will facilitate significant advances in the treatment of obesity related metabolic diseases and diabetes.

1.4 Relaxin biology and new role as a novel adipokine

Relaxin (RLN), a pleiotropic 6 KDa peptide hormone, is generally considered a "pregnancy hormone" due to its production by the ovaries, placenta and breast and its several functions relating to female reproductive tract physiology, including the ability to remodel the ECM (Sherwood, 2004). RLN was first discovered in 1926 by Frederick L. Hisaw when he collected serum from pregnant guinea pigs and injected that serum into non-pregnant guinea pigs and noticed that this

stimulated the relaxation of the pelvic ligaments (Hisaw, 1926; Sherwood, 2004). Depending on the species, different tissues will secrete RLN during different periods of the pregnancy. In humans, the corpus luteum is known to be the primary tissue secreting relaxin. Relaxin is necessary in females to inhibit uterine contractions, relax the pelvic ligament, broaden the birthing canal, and chew up connective tissue in preparation for parturition.

1.4.1 Traditional role of relaxin

The presence of RLN can be detected in both female (i.e., uterus, ovary, corpus luteum, placenta) and male (i.e., prostate, testes, seminal vesicles) reproductive tissues (Sherwood, 2004). A majority of studies have been aimed at the effect of RLN in the female counterpart as the effect of RLN in the male is somewhat controversial. The source of RLN production varies between species with the corpus luteum and placenta being the major sources during gestation (Sherwood et al., 1975a, 1976, 1978, 1979, 1981; Frankshun et al., 2009).

RLN concentrations in the maternal circulation are undetectable in non-pregnant dams and only begin to significantly rise during the final two weeks of gestation peaking at levels of 50 to 300 ng/ml approximately 2 days prior to parturition and rapidly declining to approximately 1 ng/ml by day 7 post-parturition, reflecting the well characterized pattern of upregulated expression and secretion from uterine tissues which wanes upon farrowing (Sherwood et al., 1975a, 1976, 1978, 1979, 1981; Frankshun et al., 2009). Importantly, however, adipose tissue primordia are histologically apparent by day 80 of gestation and adipose tissue development is occurring in earnest during the gestational window in which RLN levels increase within the dam, suggesting developing adipose tissue is exposed to significant levels of RLN during an early critical development window occurring within late fetal development (Hausman and Kauffman, 1986;

Hausman et al., 2009; Hausman et al., 2014). In this regard, several studies suggest that circulating maternal RLN appears to cross the placenta to influence fetal development throughout the second half of rat pregnancy (Hwang and Sherwood, 1988; Burger and Sherwood, 1998; Zhao et al., 1995; Zhao et al., 1996; Zhao and Sherwood, 1998; Sherwood 2004). In these studies, knocking down endogenous RLN through either the immunoneutralization of R1 relaxin or via ovariectomy resulted in fetal weights that were significantly greater than controls. However, species differences in placental structure are of important consideration, as the rodent expresses a discoid placenta, whereas the pig expresses a diffused placenta which might impact RLN uptake into the fetal circulation. The actual concentration of RLN in the porcine fetal circulation during gestation is unknown.

In non-pregnant females, the corpus luteum has been shown to secrete RLN during the estrus cycle with luteal concentrations being estimated at less than 3 μg/g and circulating levels being low (Anderson et al., 1982; Bagnell et al., 1993). In males, RLN has been suggested to play a role in sperm motility (Sasaki et al., 2001). In boars, recent data suggests that the testes are the major source of RLN; although the RLN produced in the male reproductive tract appears to be primarily released into the seminal fluid, with the mean ± SD level of immunoreactive RLN in seminal plasma being 2.61 ± 0.62 ng/mL and essentially undetectable within the systemic circulation (Sasaki et al., 2001; Sherwood, 2004; Kato et al., 2010). Thus, while RLN is produced and secreted by tissues in both genders, the traditional reproductive literature suggests that RLN exposure may not be systemic during much of the developmental trajectory of both genders.

Very little is known about factors that regulate relaxin gene expression. Relaxin upregulates its own expression in HeLa and THP-1 cells through a glucocorticoid-receptor mediated mechanism (Dschietzig et al., 2009). Consistent with this observation, estrogens and

glucocorticoids have also been shown to upregulate relaxin gene expression in reproductive tissues (Crish et al. 1986 a,b; Garibay-Tupas et al., 2004). It is unclear how this relates to the pig.

1.4.2 Relaxin family peptide receptor 1 and RLN signaling

The predominantly known receptor for RLN is a member of the guanine-nucleotide protein coupled receptor family (GPCRs). As such, this receptor is a seven-transmembrane domain receptor (7-TM receptor). The receptor for relaxin is referred to as relaxin family peptide receptor 1, or more commonly referred to as RXFPR1.

That RLN signals through the RXFP1 receptor in the pig is established. However, the intracellular signaling events triggered by RXFP1 are not clearly elucidated, likely because of the multitude of cell type-specific signaling pathways shown to be activated by relaxin to date. Its putative signaling mechanism appears to be the cAMP pathway as RXFP1 is a G-protein coupled receptor capable of activating adenylate cyclase and subsequently PKA (Sherwood, 2004). In female reproduction, this response is directed towards the upregulation of matrix metalloproteinases (MMPs) for connective tissue breakdown and the downregulation of tissue inhibitors of metalloproteinases (TIMPs) which inhibit the matrix metalloproteinases from breaking down this connective tissue (Parry and Vodstrcil, 2007). These matrix metalloproteinases are the main players responsible for the tissue remodeling in the cervix of the female which facilitates parturition.

RLN has also been shown to activate signaling through the MAPK (mitogen-activated protein kinase)/ERK (extracellular receptor kinase) pathway, demonstrating increased phosphorylation of ERK ½; and through the PI3K (phosphatidylinositol 3-kinase) pathway, demonstrating increased cAMP accumulation (Ivell et al., 2007; Halls et al., 2009). Likewise, RLN

has also been shown to stimulate a tyrosine kinase signaling pathway in THP-1 cells (Bartsch et al., 2001). Importantly RLN also activates NFκB, given RLN upregulates IL-6 expression in extraplacental chorionic cytotrophoblasts (Bryant Greenwood et al., 2009) and activates MMP-9 expression in an NFκB-dependent fashion in THP-1 cells (Ho et al., 2007). Consistent with an ability to signal through NFκB, relaxin activates tumor-associated macrophages to a proinflammatory phenotype (Figueiredo et al., 2009). Relaxin also utilizes the nitric oxide pathway in myofibroblasts (Mookerjee et al., 2009) and is known to crosstalk with estrogen signaling pathways. Little is known about relaxin signaling in pig adipose tissue.

1.4.3 Novel role of relaxin

While relaxin is important in reproduction, its action is not limited to cervical ripening. Recent work has indicated that RLN, acting as a milk-born factor, can regulate neonatal development at least until the onset of gut closure, occurring by day 2 post-parturition (Yan et al., 2006; Frankshun et al., 2009). Relaxin can also be transported as a milk-born factor after parturition, in colostrum, to cause important tissue development in the neonate. The neonate is exposed to relaxin and other milk-born factors for approximately two days after birth, corresponding with the timing of gut closure. Once these milk-born factors are in circulation, they can bind to specific receptors of target tissues and impact tissue development in the neonate. For instance, during gestation of the sow, relaxin initiates porcine, uterine development, stimulates MMP-2 and MMP-9 (matrix metalloproteinase) production for connective tissue remodeling and is necessary for stromal and epithelial development of the neonatal uterus (Lenhart et al. 2002; Chen et al. 2011). If the signaling hormone relaxin is not present within the first twenty-four hours of life, uterine development is impaired. The presence of relaxin causes the luminal epithelium to

express estrogen receptor alpha (ERS1) which is necessary in development to allow specific ligands to bind. Estrogen can then bind to estrogen receptor alpha which initiates angiogenesis, the pinching and migration of the glandular epithelium. This pinching and migration of the glandular epithelium initiates glandular development (Chen et al. 2011).

Based upon bioassay measurements using expressed milk from nursing, multiparous Yorkshire sows, porcine RLN concentrations in milk are highest in colostrum, peaking at 9-19 ng/ml and rapidly decline to less than 2 ng/ml by day 7 and essentially becoming undetectable by day 14 post-parturition (Yan et al., 2006; Frankshun et al., 2009). During this timeframe, a period also correlating with a rapid expansion of adipose tissue in the neonate, circulating RLN concentrations rise to 200 pg/ml in nursing piglets reflecting a similar pattern as described for expressed milk (Yan et al., 2006). Thus, a narrow albeit potentially significant window for RLN exposure exists during a critical period of extensive adipose tissue expansion *in utero* and milk-borne transmission extends this period to key early stages of rapid, hypertrophic adipose tissue expansion in the neonate.

Intriguingly, relaxin has recently gained attention as a potential connective tissue modifying agent in non-reproductive tissues; more specifically, the heart and kidney (Conrad and Novak, 2004). Adipose tissue is a specialized form of connective tissue, characterized by mature, lipid-filled adipoctyes held together in a matrix of collagen and reticular fibers. As adipocytes become larger, RLN has the potential to remodel the connective tissue matrix surrounding these adipocytes, making adipose tissue an ideal candidate for regulation by relaxin. Recent studies have discovered that adipose tissue can synthesize and secrete relaxin, cells of adipose tissue express relaxin receptors and exposing adipose tissue to exogenous relaxin encourages fat cell differentiation and alters the transcriptome of adipose tissue (Roberts et al. 2015). Little is known

about the role relaxin may play in regulating adipose tissue development; however, this limited data suggests that relaxin could play a role in adipose tissue development and establish a new role for a traditional, reproductive hormone.

1.4.4 Relaxin as a novel adipokine that regulates adipose tissue development – motive, means and opportunity

Relaxin has the "motive, means and opportunity" to regulate adipose tissue development. Foremost in this argument, RLN is a well-known anti-fibrotic, ECM modifying agent that potently alters connective tissue while adipose tissue is histologically classified as a specialized form of connective tissue. Secondly, upon binding to its receptor (which is expressed by adipose tissue) RLN can activate multiple signaling pathways that are known to be evoked by other hormones that are potent regulators of adipose tissue such as insulin, growth hormone, catecholamines, insulin-like growth factor. Lastly, RLN has multiple opportunities to regulate adipose tissue throughout normal growth and development as recent literature suggests that adipocytes may be exposed to RLN as early as the emergence of the adipose tissue primordia *in utero* and continuously throughout the pig's life cycle thereafter.

This argument is based upon several recent studies and efforts to develop RLN as a therapeutic agent. For instance, RLN has recently gained much attention as a potential anti-fibrotic agent in non-reproductive tissues with the goal of using exogenous RLN therapeutically to reverse scarring in damaged organs such the heart and kidney (Conrad and Novak, 2004; Sasser, 2013; Raleigh et al., 2015). This is in part due to the observation that the relaxin receptor is ubiquitously expressed throughout the body, an observation that had traditionally been discounted as physiologically irrelevant. Interestingly, there is emerging evidence indicating that adipose tissue

might be exposed to significant quantities of endogenous RLN independent of a reproductive context. While RLN is undetectable in the adult circulation outside of the few weeks spanning parturition, recent studies have revealed a lactocrine mode of transmission of RLN from mother to offspring creating a previously unappreciated window during early lactation in which neonatal adipose tissue is exposed to RLN (Frankshun et al., 2009). Importantly, in this context, RLN orchestrates the development of the neonatal uterus by upregulating estrogen receptors and facilitating glandular epithelial maturation, a function that suggests RLN is important for normal development of tissues independent of its action during parturition. In fact, lactocrine transmission extends the potential window of RLN action beyond parturition till at least gut closure in the neonate, whereby transmission from mother's milk can no longer occur. Finally, recent studies have revealed that adipose tissue itself can synthesize and secrete bioactive RLN, adipose tissue exhibits RLN receptor expression, and treating adipocytes with exogenous RLN induces profound changes in the adipocyte transcriptome (Hausman et al. 2006; Roberts et al. 2015). Hausman et al. (2006) characterized the secretome of preadipocytes and adipocytes using 2D Gel Electrophoresis, which separates proteins by size and isoelectric point, and Mass Spectrometry which identifies the protein spots. A list of secreted factors were identified including RLN. This was the first report that RLN may be secreted from adipose tissue, suggesting that relaxin has the opportunity to influence adipose tissue from emergence of fat until the death of the animal. Roberts et al., (2015) followed up these observations to prove that adipocytes secrete bioactive RLN, this secretion is upregulated during fat cell differentiation and in obese versus lean pigs, adipose tissue expresses the RLN receptor, and importantly, treating adipocytes with exogenous RLN profoundly changes the adipocyte transcriptome as measured by RNAseq. Collectively, these data indicate that the

window in which developing adipose tissue is exposed to RLN span from the emergence of the fetal adipose primordia *in utero* to the harvest of the adult animal.

Thus, RLN has the "motive, means and opportunity" to regulate adipose tissue development and could be a novel regulator of adipose tissue development. RLN is no longer your mother's hormone-new data suggest relaxin is involved in much more than just its traditionally understood role in cervical ripening. Armed with this new insight, my overall research aims to better define, mechanistically, a new function for the traditional hormone RLN.

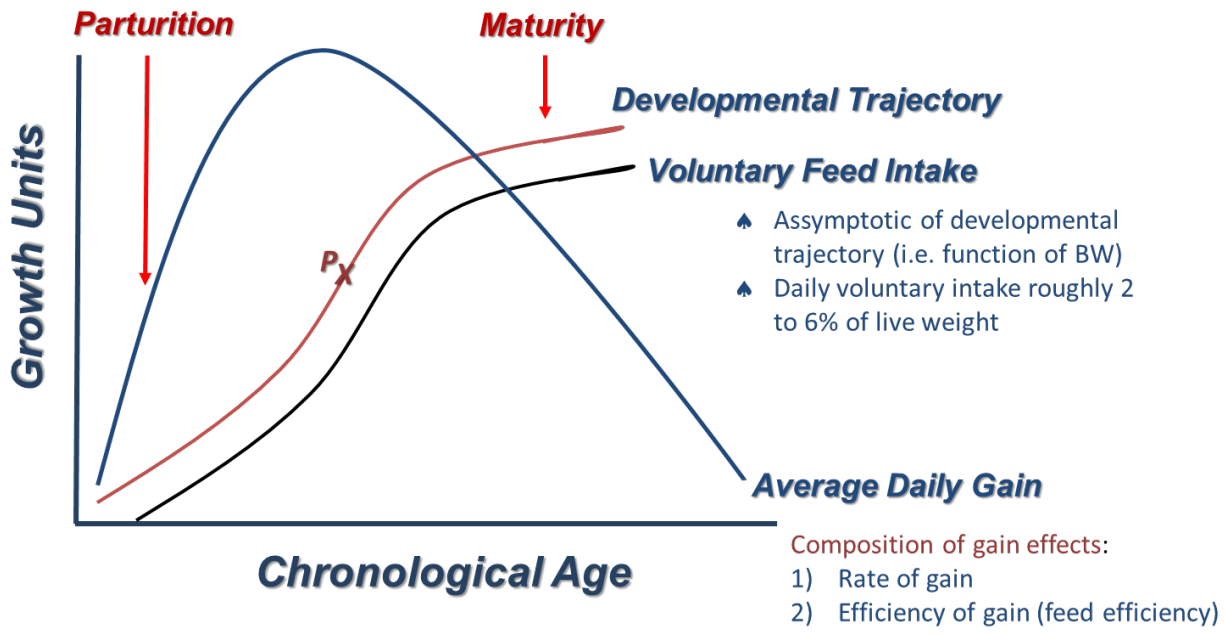

Figure 1-1. The relationship between the developmental trajectory of the porcine and voluntary feed intake and average daily gain. All curves are idealized and the typical timing of onset of puberty is demarcated "P" as it correlates with a break in growth rate.

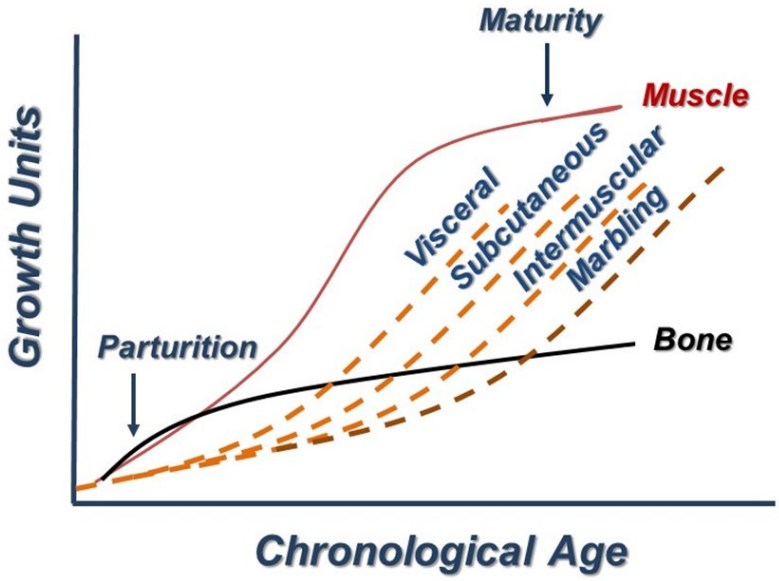

Figure 1-2. Idealized growth curves depicting the temporal patterns of development for the three primary carcass components. Notice the developmental priority between tissues (bone >> muscle >>fat) with bone considered early maturing and fat considered late maturing. Also depicted is the developmental priority within adipose tissue with the visceral depot being the earliest anatomical location to mature and intramuscular fat (marbling) the last depot to mature. Therefore, currently animals must be fattened in order to achieve a desired level of marbling. Undesirable depots must be matured in order to increase marbling and then trimmed and discarded during carcass fabrication post-harvest.

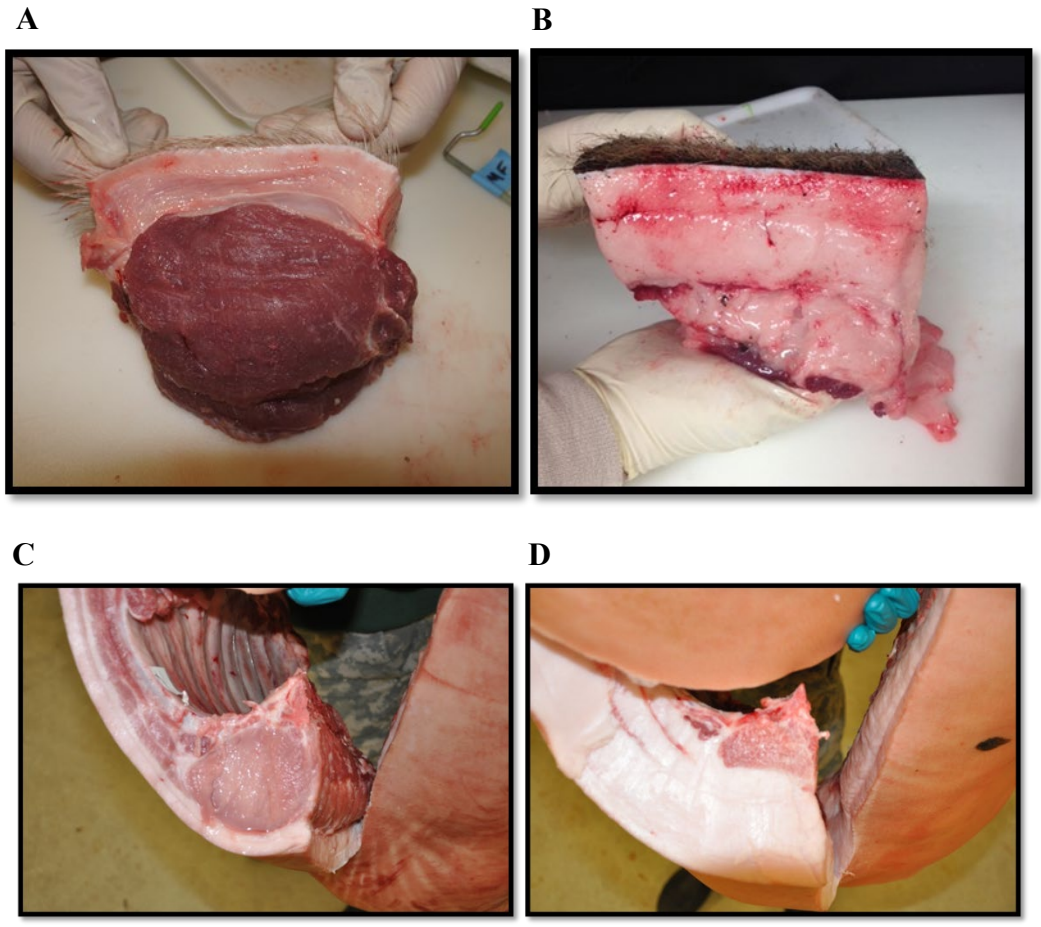

Figure 1-3. Depiction of differences in subcutaneous fat development and thickness and loin eye area at 240 lbs slaughter weight between Yorkshire (***Panels A & C***) and Blonde Mangalica (***Panels B & D***) pigs. For *Panels A & B*, tissue was harvested immediately following exsanguination by dissecting a plug of tissue over the tenth rib from the skin to the underlying *longissimus* muscle. Three distinct adipose tissue layers are visible on the Mangalica sample while the much thinner Yorkshire sample is devoid of the inner layer of subcutaneous fat and exhibits much thinner existing layers compared to the Mangalica. For *Panels C & D*, carcasses were split at the tenth rib to measure backfat thickness and loin eye area following storage at 4 °C for 24 h. Adapted from Roberts (2015) and used by permission.

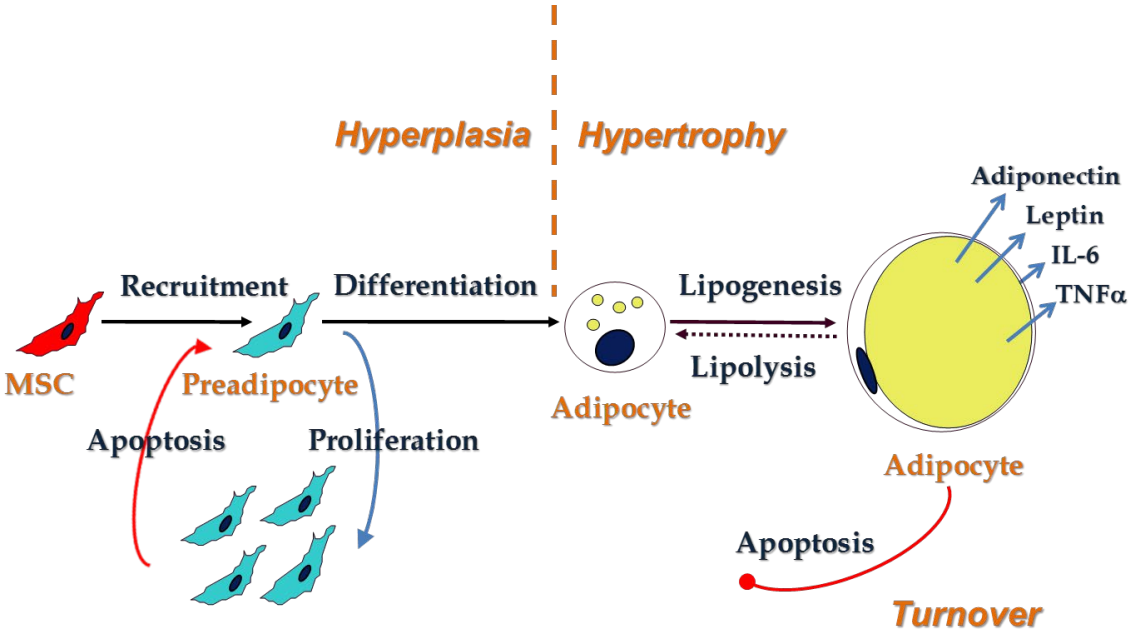

Figure 1-4. Current model of adipose tissue development in mammals. Adipose tissue development is the synergistic function of hyperplasia (increase in cell number) and hypertrophy (increase in cell size). The extent of hyperplasia sets the ultimate potential of the animal to fatten. The degree to which this potential is reached is determined by hypertrophy post-parturition. Cell number is a function of the degree to which adipocyte progenitors are recruited from mesenchymal stem cells *in utero*, the rate of their subsequent commitment to preadipocytes, the balance between the rate of preadipocyte proliferation and apoptosis, and the rate of their terminal differentiation into immature adipocytes (adipogenesis). Fat cell number is generally thought to be set at birth in the pig with only slight increases measurable thereafter in some studies. Cell size is determined by the amount of neutral lipid stored by the adipocyte which is dictated by the balance between triglyceride synthesis (lipogenesis) from lipid uptake from the diet and de novo lipid synthesis from glucose carbon and triglyceride breakdown (lipolysis) resulting in the release of free fatty acids into the circulation. The extent to which cell size is maximized is largely under the influence of energy balance and the endocrine/health status of the animal.

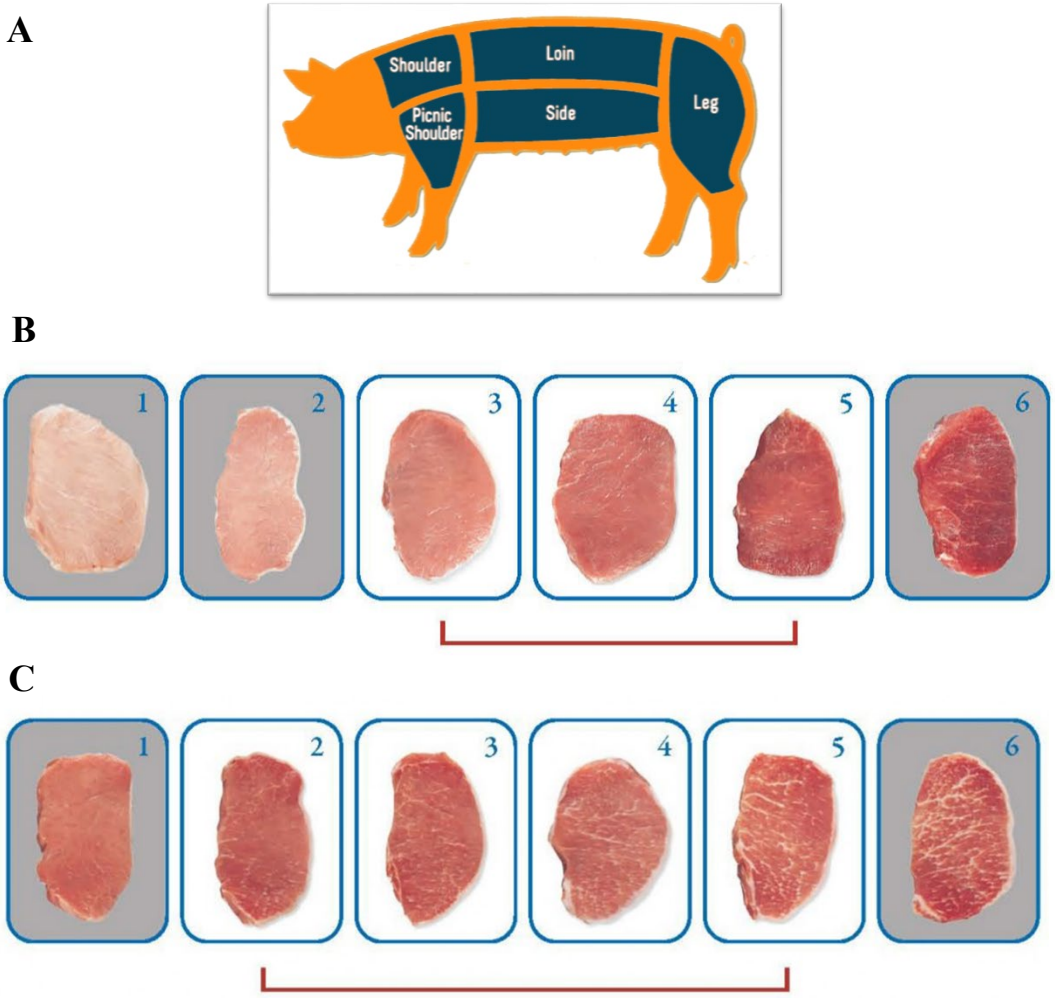

Figure 1-5. Primal cuts from the pork carcass and pork quality standards used to subjectively evaluate the sub-primal cut and assign a visual color and marbling score. ***Panel A***: Depiction of the primal cuts derived from the pork carcass during fabrication post-harvest. ***Panel B***: The six-point scale ranges from 1 (very light) to 6 (dark red) with consumer panel testing consistently indicating a consumer preference for products having a color score between an ideal range of 3 to 5 as color relates to tenderness. Pictured are chops representing a sub-primal cut from the loin. ***Panel C***: The six-point scale ranges from 1 (slight) to 6 (abundant) with consumer panel testing consistently favoring more marbling as it is an indicator of flavor. Pictured are chops representing a sub-primal cut from the loin.

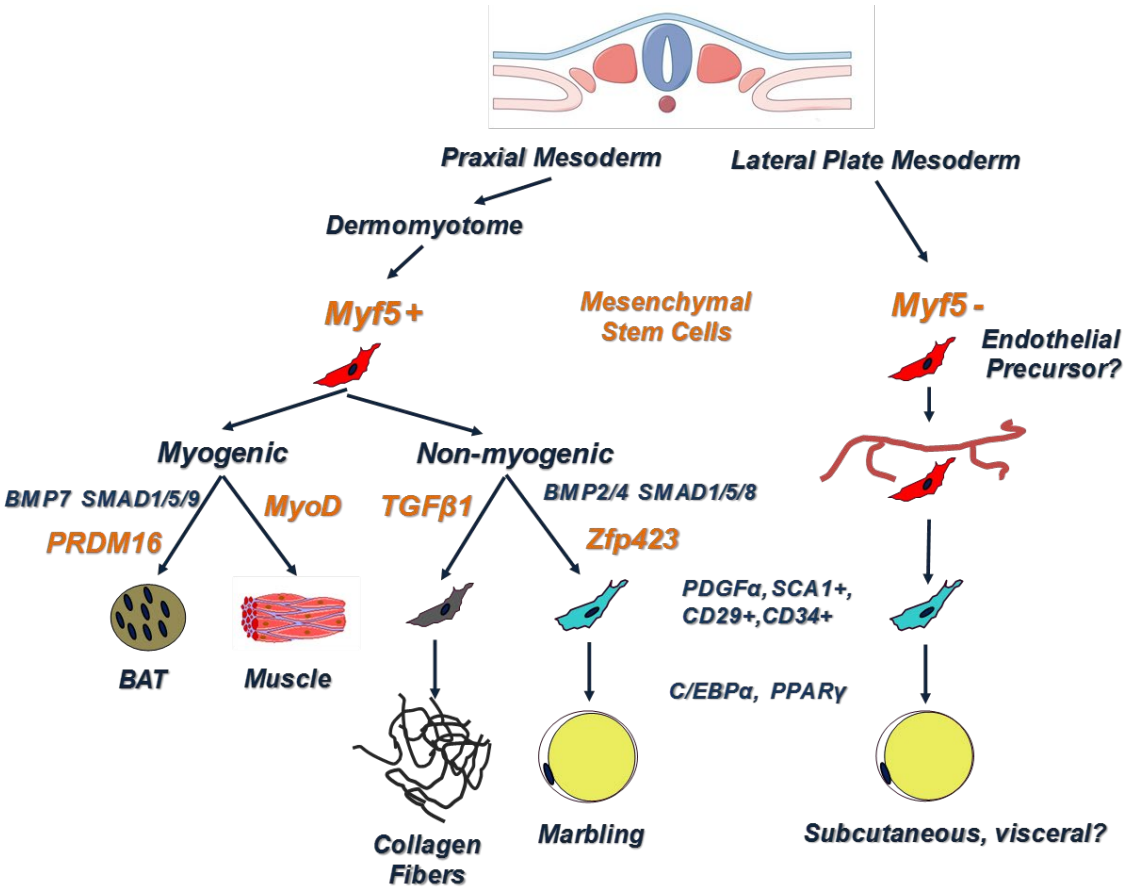

Figure 1-6. The current model explaining the developmental origins of different adipose tissue depots in mammals (Bonnet et al., 2010; Du et al., 2015). Fat cells from different anatomical adipose tissue stores are derived from different progenitor cell populations. This model explains the developmental priority within adipose as observed by the temporal pattern of staggered filling between adipose depots on the growing animal by indicating depots arise from different developmental origins with the timing of progenitor recruitment *in utero* such that visceral fat primordia appears before subcutaneous fat primordia etc. with the intramuscular fat depot being the last depot to emerge *in utero*. Thus, the pattern of filling observed on the growing animal is set *in utero* and once set, this temporal pattern of filling is largely intractable to attempts to selectively alter specific depot development after birth.

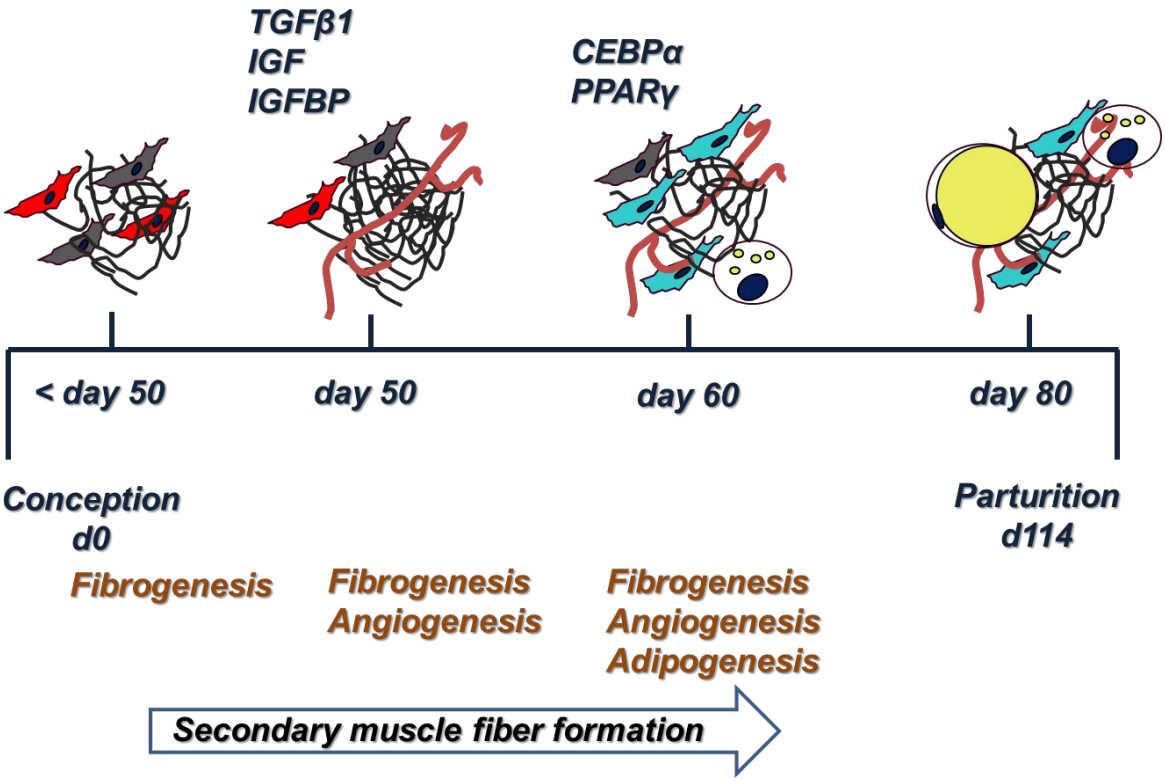

Figure 1-7. Model depicting adipose tissue development *in utero* based upon the collective work from the Hausman laboratory utilizing a fetal model in which subcutaneous fat development was characterized by immunohistochemical approaches. This model indicated that the processes of fibrogenesis, angiogenesis and adipogenesis are coupled processes whereby connective tissue fibers are first laid down allowing an avenue for subsequent blood vessel formation and preadipocyte progenitor cell recruitment. Hormones and nutrients then transported from the blood coupled with the action of autocrine and paracrine acting factors presumably stimulate the preadipocytes to proliferate and differentiate as the adipose tissue primordia emerges. Fibrogenesis is first histochemically observable at approximately day 50 of gestation while the first observable adipocytes emerge at day 60. Uncoupling fibrogenesis, angiogenesis, and adipogenesis by impairing any one process, dramatically decreases the development of the adipose tissue primordia. The timing of these events is concurrent with the fetal wave of muscle fiber formation.

Table 1.1 Breed comparison of typical body composition and growth parameters[1]

Measurement[2]	Unit	Yorkshire	Ossabaw	Mangalica
Live Body Weight[3]	lbs	250	130	375
% Carcass Lean	%	65-70	N.R.[4]	30-35
Backfat at 10th Rib	in	0.9	2.1	3.2
Rib Loin Eye Area	in^2	6.2	5.3	3.80
Average Daily Gain	lb/d	1.70	.75	.55
Daily Feed Intake	lb/d	5.5	3.5	5.0
Feed Efficiency	G/F	.31	.214	.11
Time to Market[5]	d	147	333	454

[1]Table adapted from Roberts, 2015 and used by permission.

[2]Values for Yorkshire adapted from McGlone and Pond, 2003; Stender, 2012. Values for Ossabaw adapted from Martin et al., 1973; Buhlinger et al., 1978; Wangness et al., 1980; Dyson et al., 2006. Values for Mangalica adapted from Egerszegi et al., 2003; Brussow et al., 2005; Ratky et al., 2005.

[3] Live body weight is represented as market weight for Yorkshire and mature body weight for Ossabaw and Mangalica breeds.

[4] N.R. = Not Reported as no citation was available in literature to address this parameter.

[5] Time to market is calculated as market weight set at 250lbs/ADG. Thus, it is normalized for Yorkshire market weight. Ossabaw pigs do not reach 250 lbs mature live weight.

Table 1.2 Differences in cellularity, metabolic activity, endocrine function, and growth and physiology between visceral and subcutaneous adipose tissue based upon the human, rodent, and food animal literature.

Variable	Visceral	Subcutaneous
Cellularity		
Adipogenic Potential	Lower	Higher
Macrophage Infiltration	Higher	Lower
Metabolic Activity		
Overall	Higher	Lower
Lipolytic Rate	Higher	Lower
Glucose Uptake	Higher	Lower
Endocrine Function		
Insulin Sensitivity	Lower	Higher
βAA-stimulation	Higher	Lower
TZD sensitivity	Lower	Higher
Adipokine release		
IL-6, TNFα	Higher	Lower
Leptin	Lower	Higher
Adiponectin	Lower	Higher
Postnatal Growth	Hyperplasia & Hypertrophy	Hypertrophy
Venous Drainage	Portal blood	Systemic circulation

Chapter 2:

Serial Harvest across Progressively Heavier Live Weights in Growing Mangalica Pigs Indicates Optimal Harvest Should Occur at Much Lighter Live Weights than is Currently Practiced by Producers

Hypothesis:

If the current heavy harvest weights of the Mangalica are inappropriate, then serially harvesting at progressively heavier live weights will reveal plateaus in carcass merit measures that occur at live weights lower than the currently adopted harvest weight

Aims:

Aim1: Characterize ideal harvest weight based upon optimal growth and meat quality standards.

Specific objectives:

Objective1: To determine the growth performance of Mangalica pigs across progressively heavier weight classes

Objective2: To determine the carcass composition of Mangalica pigs across progressively heavier weight classes

Objective3: To determine carcass parameters and primal cut measurements of Mangalica pigs across progressively heavier weight classes

Objective4: To determine loin 24h post-harvest pH and color of Mangalica pigs across progressively heavier weight classes

3.1 Abstract

The Mangalica pig is a European heritage breed that was domesticated for its high-quality lard. Recently this breed has begun gaining popularity in the US as a niche breed given its reputation for yielding superior pork quality including high quality fat, darker color and higher degree of marbling. This trend is despite the breed's poor growth performance relative to modern meat breeds which necessitates a higher price point for resultant pork products. However, the Mangalica phenotype is poorly described in the scientific literature and needs to be better characterized in order to assess the breed's true viability as a marketable food animal. Recent work from our group has confirmed that Mangalica pork exhibits superior meat quality attributes supporting the notion that higher price points for Mangalica pork in niche markets may be justified. However, currently, Mangalica producers market their animals at a live weight associated with extreme carcass fat accumulation assuming this maximizes carcass merit. Unfortunately, it is not possible to make science-based recommendations on the proper harvest weight for Mangalica pigs given a lack of information regarding the growth and carcass characteristics across this breed's developmental trajectory. In order to resolve this, a growth trial was conducted utilizing a serial slaughter approach whereby harvest weights were stratified to determine how growth performance, carcass composition, carcass parameters and primal cut measurements, and indices of pork quality such as loin 24hr post-harvest pH and color change in the Mangalica with increasing harvest weight. As expected, live weight significantly increased across weight class. Average daily gain (ADG) and feed efficiency (FE) were similar across classes up to 180 lb live

weight and then they steadily declined with increasing weight class. Loin eye area (LEA), a measure of lean product, was significantly increased up to 180 lb and then plateaued as harvest weight increased. Primal cuts, fat back and fat all significantly increased with increasing live weight. These trends suggest that improvements in pork quality and muscle growth are modest beyond 180 lb live weight while adipose tissue accumulation increases dramatically concomitant with decreased productive efficiency. Collectively, these data indicate an optimal harvest weight occurs between 180 to 225 lb depending upon the premium a producer can receive for marbling. These data offer little objective justification for harvesting Mangalica pigs at heavier weights.

3.2 Introduction

Pork producers have responded to growing consumer demand for leaner, less fatty products by genetically selecting for pigs that grow faster, have little carcass fat, and yield more muscle at heavier harvest weights. Unfortunately, this strategy has also adversely affected important pork quality traits such as flavor, juiciness, tenderness, color, and water-holding capacity which in turn has decreased the palatability of pork and ultimately consumer demand for pork products (Carr et al., 1997; Brewer et al., 2001; NPPC, 2010). In response to lower quality pork, niche markets have developed whereby certain consumers are willing to pay a premium price for a high-quality pork product (Honeyman et al., 2006). This has renewed interest in heritage, lard-type breeds such as the Mangalica pig in order to meet these new market interests.

The Mangalica breed was first developed in 1833 by the Hungarian Royal Archduke Jozsef and is essentially a genetically unimproved, extreme lard type hog known for its flavorful, marbled meat cuts and especially for its high-quality lard, which can be used as an emulsifier to make excellent sausage. Mangalica lard contains more unsaturated fat than modern, improved breeds,

contributing to the palatability of the meat product by providing a lower melting temperature Egerszegi et al., 2003). Previous research from Roberts et al., (2015) confirms that the Mangalica produces higher quality pork due to the redder color and higher degree of marbling at 250 lbs harvest weight compared to modern breeds slaughtered at similar weights. Unfortunately, the higher propensity to fatten is associated with slower, less efficient growth, less muscle, and excessive accretion of trim or waste fat (Roberts, 2015).

Currently Mangalica producers grow pigs to very heavy harvest weights, approaching the mature size for the breed. This is designed to maximize fat accumulation due to the common assumption that this strategy greatly improves carcass merit. However, this assumption is not based upon empirical data and it is possible that such harvest weights are too heavy and allow carcass development to surpass a point where desirable carcass traits continue to increase significantly compared to the rapidly increasing rate of fat accumulation. Unfortunately, it is not currently possible to make science-based recommendations concerning the proper harvest weight for Mangalica pigs because carcass parameters for this breed are largely uncharacterized across its life cycle. Thus, in order to better allow empirical justification for harvest weight for the breed, a growth trial was conducted in which Mangalica pigs were serially slaughtered across seven weight classes and growth performance, carcass composition and meat quality parameters were measured for each harvest weight class.

3.3. Materials and methods

Animals and design

All experimental procedures were approved by the Auburn University Institutional Animal Care and Use Committee. The Auburn University College of Agriculture is accredited by the

Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC) and this study was conducted in accordance with the Federation of Animal Science Societies' Guide for the Care and Use of Agricultural Animals in Research and Teaching**.** A total of fifty-six growing Mangalica pigs were obtained from the Auburn University research herd housed at the Auburn University Swine Research and Education Center. Pigs were individually housed in pens 12.2m^2 in size and were provided *ad libitum* access to food and water. Harvest weights were stratified into 7 weight classes (110, 125, 150, 180, 205, 225, and 280 lb live weight) spanning the traditional grow-finish stages of the porcine growth curve with the heaviest weight class corresponding to the current industry standard harvest weight. Pigs were randomly assigned to weight classes (n=8) in a randomized complete block design. Animals within each weight class were on test for a period of 35 days prior to harvest. All pigs were fed a typical finisher ration (15% crude protein) from 110 to 280 lb live weight. Daily feed intakes and weekly body weights were recorded for all animals on test to facilitate measurement of average daily gain (ADG), feed efficiency (lbs gained/ lbs feed), dressing percentage (hot carcass weight/live weight) and total feed intake.

Carcass fabrication, composition, and merit determination

Mangalica pigs were harvested at the Auburn University Lambert-Powell Meats Lab under USDA-FSIS inspection. Hot carcass weight was recorded after exsanguination and carcasses were chilled at 2 degrees C for 24 hours, at which point, cold carcass weight was recorded. At 24h postmortem, carcasses were split vertically to determine cold side weight and carcass length. Carcasses were then separated between the 12th and 13th rib interface where back fat (BF) depth was measured at the 1st rib, 10th rib, last rib and last lumbar along with LEA. Primal cuts consisting

of the ham, loin, Boston butt, picnic shoulder, and belly were fabricated and individually weighed and recorded along with leftover trim and fat after fabrication. Fat back was separated from the loin and weighed individually. Evaluation of subjective scores for marbling, wetness, firmness, and muscle score were determined by a trained observer using published visual standards (NPPC 2000). Additionally, the longissimus muscle at the 10^{th} rib was evaluated for objective color measurements with a Hunter Miniscan XE Plus (Hunter Lab, Reston, VA) to determine Hunter L*, a*, and b* values. The Miniscan was calibrated according to the manufacturer's recommendations and utilized a D65 light source, a 10° viewing angle, and a 35mm viewing area. Carcass pH was recorded in the loin muscle using a pH Spear probe (Oakton Instruments, Vernon Hills, Il).

Statistical analysis

Growth performance, carcass parameters, fabricated primal cut measurements, and 24h post-harvest pH and color scores were analyzed as a completely randomized block design using a mixed linear model of SAS v9.2 with individual animal serving as the experimental unit, i.e. individual block (SAS Institute, Inc., Cary, NC).

3.4 Results

Growth performance

In order to better allow empirical justification of harvest weight for the Mangalica, live weight, daily feed intake, ADG and FE were evaluated for animals on-test across the 7 weight classes (Table 2.1). As expected, live weight significantly increased as weight class increased ($p < 0.0001$). Daily feed intake was significantly different across weight classes ($p < 0.04$); however,

no apparent trend or pattern was observed. ADG ($p < 0.0025$) and FE ($p < 0.0029$) were similar across classes up to approximately 180 lb and then each steadily declined with increasing weight class. Dressing percentage significantly increased with harvest weight up to 180 lbs. upon which this measure of carcass cutability began to plateau ($p < 0.0001$).

Longissimus dorsi (loin eye) color and pH

In order to better allow empirical justification of harvest weight for the Mangalica, visual color, objective color (L*, a*, b*) and pH were evaluated for animals on-test across the 7 weight classes (Table 2-2). The 24h pH was not significantly different across weight classes ($p < 0.75$) suggesting no beneficial effect on this parameter was associated with harvest weight. On the other hand, visual color score progressively increased with weight class suggesting that heavier harvest weight was associated with a more desirable redder or darker loin ($p < 0.0032$). Consistent with visual color score, the objective color measure, L* (lightness), significantly decreased as weight class increased ($p < 0.024$), while a* (redness) significantly increased as weight class increased ($p < 0.0011$); however, b* (yellowness) did not change with weight ($p < 0.15$). Thus, objective color scores also suggested that heavier harvest weights were associated with more desirable pork color.

Carcass parameters and primal cut measurements

In order to better allow empirical justification of harvest weight for the Mangalica, hot carcass weight, cold carcass weight, cold side weight, carcass length, and primal cuts (ham, loin, Boston butt, picnic shoulder, belly) were evaluated for animals on-test across the 7 weight classes (Table 2-3). As expected, hot carcass weight, cold carcass weight, and cold side weight were all significantly increased in weight as weight class increased ($p < 0.0001$). Likewise, carcass length

was significantly increased with increasing weight class ($p < 0.0001$). During fabrication, the carcass is broken into characteristic primal cuts which then give rise to the sub-primal cuts recognizable by the consumer at the meat counter. When considering these primal cuts, the ham, loin, Boston butt, picnic (shoulder) and belly all significantly increased in weight with increasing weight class ($p < 0.0001$).

Carcass composition

In order to better allow empirical justification of harvest weight for the Mangalica, loin eye area, muscle score, muscle firmness, fat, fat depth along the vertebrate, marbling score, fat back (subcutaneous fat between the skin and longissimus dorsi muscle), and trim were evaluated for animals on-test across the 7 weight classes (Table 2-4). LEA significantly increased up to 180 lb live weight and then began to plateau ($p < 0.003$). Likewise, muscle score, a subjective measure of overall leanness, increased up to 180 lb then was inconsistent without a trend ($p < 0.0001$). There were no differences in muscle firmness across weight classes ($p < 0.08$). Fat depth at the 1st, 10th, last rib, and last lumbar all significantly increased with increasing weight class ($p < 0.0001$). However, Marbling score progressively increased with increasing weight class up to 225lb live weight where Marbling score than plateaued ($p < 0.04$). Fat back and fat both dramatically increased with increasing weight class ($p < 0.0001$). Trim was not significantly different across weight class ($p < 0.13$).

3.5 Discussion

Currently Mangalica producers rear their pigs to very heavy harvest weights that often approach the mature frame size for the breed. This practice is based upon a common assumption

that maximizing fat accumulation on the carcass greatly improves carcass merit in this breed. The study herein provides the first data where carcass composition and merit were systematically examined at increasing harvest weights across the growth curve of the Mangalica pig. These data suggest that improvements in pork quality and muscle growth are modest beyond 180 lb live weight; meanwhile, adipose tissue accumulation increases dramatically concomitant with decreased productive efficiency at heavier live weights. As such, these data should allow producers to make better informed, more profitable decisions concerning the marketing of their pigs.

Growth performance in the Mangalica pig is poorly characterized with few refereed manuscripts existing in the literature addressing this issue. Furthermore, the existing studies largely characterize Mangalica herds that were reared in what would be considered primitive conditions compared to modern production facilities in the U.S, often involving pasture-based systems characteristic of rural Eastern European subsistence farming. Nonetheless, a survey of such studies indicates that Mangalica pigs exhibit an ADG of .55 lbs/day, a daily feed intake of roughly 5 lbs/day and a feed efficiency of .11 (Egerszegi et al., 2003; Brussow et al., 2005; Ratky et al., 2005). In a recent study conducted at Auburn University by Roberts et al., (2015) using Mangalica pigs obtained from a disease-free herd and reared in confinement while fed a concentrated ration, growth performance significantly exceeded the above performance standards. Likewise, performance by animals on-test in the current study also significantly exceeded those standards while being in general agreement with Roberts et al., (2015). Red Mangalica in the Roberts study exhibited higher marbling scores than observed in the current study (4.45 ± 0.39 at 245 lbs. vs. 3.42 at 225 lbs live weight herein). However, the contribution of several Blonde Mangalica pigs in the current trial likely lowered the overall marbling score across weight classes as Red Mangalica pigs exhibit a greater degree of marbling than Blonde Mangalica, and the

Roberts study did not mix Red and Blonde pigs (Roberts, 2015). That Mangalica growth performance exceeded levels reported in the few published studies to date is expected. Mangalica pigs on this trial and those from Roberts et al., (2015) were given ad libitum access to concentrated, balanced rations formulated to match their stage of growth with the express goal of maximizing their growth rate and carcass development. This is in sharp contrast with the nutrition of Mangalica in traditional growing systems in which a significant portion of their diet is met through foraging on lower quality pasture and in woodlots. Furthermore, pigs reared within the SREC are naïve, i.e. disease and parasite free, which is almost certainly not the case for pigs reared on pasture.

Pork quality continues to be a serious issue in the pork industry. The emphasis on selecting pigs for leanness has resulted in a reduction in pork quality due to a loss of color and poorer marbling (NPB, 1998). Color is the most important appearance quality trait affecting the visible appeal of pork to consumers (Faustman and Cassens, 1991; Cheftel and Culioli, 1997; Risvik, 1994). Marbling is an important sensory trait that contributes to the juiciness and flavor of the product and is another key criterion impacting consumer choice at the meat counter (NPB, 1998). Unfortunately, selection for leaner pigs has generally reduced the marbling content, contributing to a less satisfying eating experience by the consumer (NPB. 1998). This has led to the creation of niche markets whereby consumers are willing to pay a premium for high quality pork products, especially at high end restaurants (Honeyman et al., 2006). Mangalica pork is currently being marketed by targeting such niche markets given the breed's reputation for exhibiting superior pork quality and due to the higher price point necessitated by the breed's poor growth performance. However, this reputation for superior quality is largely inferred due to the breed's derivation, place in Eastern European cultural history, and the word of mouth of modern-day chefs. Little data exists in the literature to verify these claims. However, one recent study does support the argument that

Mangalica produce higher quality pork. In that study, Roberts et al., (2015) directly compared Yorkshire and Red Mangalica and observed that Mangalica pork exhibited significantly higher marbling, firmness, and color scores while exhibiting lower cook loss consistent with the perception of juicier chops. Quality parameters measured in the current study were not directly compared to Yorkshire counterparts, but carcass composition and color parameters data reported herein were consistent with values observed by Roberts et al., (2015). Although the Yorkshire breed is one of the most popular breeds for pork production in the United States due to its 3-fold greater ADG and FE compared to heritage breeds such as the lard type Mangalica pig, Yorkshire pork is often pale and devoid of marbling. This study in conjunction with Roberts et al., (2015) indicate that Red Mangalica exhibit darker pork with 2- to 3-fold greater marbling scores. Clearly, regardless of rearing environment, Mangalica pigs exhibit poorer growth performance than their Yorkshire counterparts. However, these data indicate Mangalica pork displays superior meat quality attributes and suggest that the higher price points for Mangalica pork in niche markets that are needed in order to compensate for the poorer productive performance of this breed are indeed justified.

The current study directly addresses the wisdom of the current practice of harvesting Mangalica at heavy live weights associated with excessive accumulation of carcass fat. For instance, LEA was significantly increased up to 180 lbs. and then began to plateau in the current study. Primal cuts were significantly bigger as weight class increased; however, this is not a direct measure of fat free lean as this did not account for leftover subcutaneous fat along with intermuscular and intramuscular fat of the primal cut itself. Thus, given LEA is an excellent proxy for overall lean growth, it is very likely that a substantial proportion of primal cut weight increases across heavier weight classes due to the deposition of fat rather than lean. Unfortunately, marbling

score did not continue to increase past 225lb liveweight despite the rapid accumulation of carcass fat elsewhere. This contradicts the assumption that maximizing fat deposition on the Mangalica frame correlates to maximal marbling score and calls into question the wisdom of utilizing harvest weights greater than 225lb live weight. Excess adipose tissue is energetically wasteful (Hausman et al. 2018) as it requires more feed to grow a pound of fat than it does to grow a pound of muscle, largely due to energy dense lipids comprising roughly 90% of the mass of adipose tissue; whereby, skeletal muscle is composed of approximately 80% water and 20% protein by weight. Thus, it is expensive to fatten animals and after 225 lb, there was no benefit to doing so when considering either lean growth or marbling score.

Collectively, these data further support the claim that Mangalica exhibit high pork quality while indicating an optimal harvest weight occurs between 180 to 225 lb depending upon the premium a producer can receive for marbling. For instance, if the producer prioritizes an optimal compromise between muscle growth and marbling, he or she would harvest at 180lb while a producer who is paid a premium for marbling might harvest at 225 lb live weight as increases in marbling score were minimal beyond this weight class. These data offer little objective justification for harvesting Mangalica pigs at heavier weights unless a market is available to the producer which provides an accessible outlet for excessive lard production.

Table 2-1. Growth performance of Red Mangalica pigs at different weight classes following 35 days on-test when fed *ad libitum*[1]

Variable	110lb	125lb	150lb	180lb	205lb	225lb	280lb	SEM	*P*-Value
Final body weight, lb	112.3^{a}	125.6^{b}	152.0^{c}	181.4^{d}	202.8^{e}	225.1^{f}	286.3^{g}	3.32	0.0001
Daily feed intake, lb	4.09^{b}	4.20^{a}	3.69^{ab}	3.41^{b}	3.25^{b}	4.04^{a}	4.06^{a}	0.25	0.04
Average daily gain, lb	0.944^{a}	1.103^{a}	0.978^{a}	0.865^{a}	0.676^{b}	0.694^{b}	0.642^{b}	0.075	0.0025
Feed efficiency	0.198^{a}	0.220^{a}	0.235^{a}	0.237^{b}	0.180^{b}	0.164^{b}	0.155^{b}	0.017	0.0029
Dressing percentage	66.6^{a}	65.5^{a}	68.5^{b}	71.7^{c}	71.8^{c}	72.8^{c}	73.0^{c}	0.74	0.0001

[1]Values are group mean ± SEM, n=8, differing superscripts within a variable denote differences between weigh classes, $P < 0.05$

Table 2-2. Loin 24h post-harvest pH and color of Red Mangalica pigs at different weight classes following 35 days on-test when fed *ad libitum*[1]

Variable	110lb	125lb	150lb	180lb	205lb	225lb	280lb	SEM	*P*-Value
Loin Ultimate pH[2]	5.51	5.60	5.58	5.61	5.54	5.70	5.64	0.082	0.75
Color[3]	3.42^{a}	3.50^{a}	3.79^{ab}	4.19^{b}	3.83^{a}	4.15^{b}	4.33^{b}	0.15	0.0032
L*, lightness	56.21^{a}	57.43^{a}	53.11^{b}	51.58^{a}	53.67^{b}	52.94^{b}	50.13^{c}	1.34	0.024
a*, redness	9.89^{a}	10.60^{a}	10.73^{a}	12.99^{b}	11.47^{b}	11.31^{b}	12.79^{b}	0.53	0.0011
b*, yellowness	14.89	16.36	14.49	16.52	15.03	14.76	14.56	0.72	0.15

[1]Values are group mean ± SEM, n=8, differing superscripts within a variable denote differences between weigh classes, $P < 0.05$
[2]Ulimate pH: measured 24 h post-harvest on chilled carcasses
[3] Visual (subjective) color score: five-point scale where 1= very light and pale; 5= dark red etc.

Table 2-3. Carcass parameters and primal cut measurements of Red Mangalica pigs at different weight classes following 35 days on-test when fed *ad libitum*[1]

Variable	110lb	125lb	150lb	180lb	205lb	225lb	280lb	SEM	*P*-Value
Hot carcass weight, lb	74.8^{a}	82.3^{b}	104.2^{c}	130.1^{d}	145.6^{e}	163.8^{f}	209.4^{g}	3.3	0.0001
Cold carcass weight, lb	72.2^{a}	81.0^{b}	100.2^{c}	127.3^{d}	141.0^{e}	159.7^{f}	206.5^{g}	3.1	0.0001
Cold side weight, lb	35.8^{a}	40.5^{b}	49.5^{c}	62.5^{d}	68.6^{e}	78.2^{f}	103.1^{g}	1.71	0.0001
Carcass length, in.	24.7^{a}	24.7^{a}	25.8^{b}	27.6^{c}	28.2^{d}	28.8^{e}	30.7^{f}	0.3	0.0001
Ham, lb	6.84^{a}	7.74^{b}	9.49^{c}	11.14^{d}	10.67^{d}	12.92^{e}	16.14^{f}	0.37	0.0001
Loin, lb	5.72^{a}	6.65^{ab}	7.42^{b}	9.24^{c}	9.98^{c}	12.60^{d}	15.40^{d}	0.47	0.0001
Butt, lb	2.74^{a}	2.70^{a}	3.52^{b}	4.90^{c}	5.21^{cd}	5.85^{d}	7.98^{e}	0.29	0.0001
Picnic, lb	2.96^{a}	3.37^{a}	4.34^{b}	5.26^{c}	5.44^{c}	5.98^{d}	8.13^{e}	0.18	0.0001
Belly, lb	4.38^{a}	5.09^{a}	6.44^{b}	7.80^{c}	8.82^{c}	9.90^{d}	11.60^{e}	0.51	0.0001

[1]Values are group mean ± SEM, n=8, differing superscripts within a variable denote differences between weigh classes, $P < 0.05$

Table 2-4. Carcass composition of Red Mangalica pigs at different weight classes following 35 days on-test when fed *ad libitum*[1]

Variable	110lb	125lb	150lb	180lb	205lb	225lb	280lb	SEM	*P*-Value
Loin eye area, in^2	2.52^{a}	2.73^{ab}	2.92^{b}	3.50^{c}	3.26^{c}	3.47^{c}	3.44^{c}	0.17	0.003
Muscle score	1.12^{a}	1.09^{a}	1.42^{b}	1.63^{c}	1.48^{b}	1.85^{d}	1.26^{a}	0.09	0.0001
Muscle Firmness	2.46	2.23	2.67	2.58	2.63	2.58	2.43	0.11	0.08
Fat	2.40^{a}	2.93^{a}	4.26^{b}	5.34^{c}	7.29^{d}	8.01^{e}	10.38^{f}	0.38	0.0001
Fat depth, in									
1^{st} rib	2.12^{a}	2.00^{a}	2.24^{a}	2.63^{b}	2.63^{b}	2.92^{c}	3.54^{d}	0.12	0.0001
10^{th} rib	1.24^{a}	1.35^{ab}	1.49^{b}	1.84^{c}	2.16^{d}	2.17^{d}	2.78^{e}	0.09	0.0001
Last rib	1.20^{a}	1.24^{a}	1.37^{a}	1.64^{b}	1.59^{b}	1.73^{b}	2.36^{c}	0.13	0.0001
Last lumbar	1.44^{a}	1.35^{a}	1.57^{a}	1.94^{b}	2.00^{b}	2.25^{c}	2.63^{d}	0.10	0.0001
Marbling score	1.60^{a}	1.43^{a}	1.85^{a}	2.04^{a}	2.30^{ab}	3.42^{c}	3.41^{c}	0.27	0.04
Fat back, lb	4.08^{a}	4.14^{a}	5.51^{b}	8.46^{c}	8.83^{c}	11.13^{d}	15.83^{e}	0.75	0.0001
Trim	1.32	1.03	1.50	1.44	1.60	1.77	1.86	0.19	0.13

[1]Values are group mean ± SEM, n=8, differing superscripts within a variable denote differences between weigh classes, $P < 0.05$

Chapter 3:

Relaxin Regulates Porcine Adipose Tissue Development by Inhibiting Preadipocyte Number, Stimulating Lipolysis, and Upregulating mRNA Expression of Adipokine, Fatty Acid Metabolism and Extracellular Matrix Genes

Hypothesis:

If Relaxin (RLN) acts as an adipose tissue remodeling agent in the pig, then treating *in vitro* models of porcine adipose tissue with RLN will alter indices of cellularity, lipid biochemistry, and the secretory function of adipose tissue.

Aims:

Aim1: To investigate the ability of RLN to alter porcine preadipocyte and adipocyte cell number.

Aim2: To investigate the ability of RLN to regulate lipolysis in porcine adipose tissue.

Aim3: To investigate the ability of RLN to alter expression of extracellular matrix components of porcine adipose tissue.

Aim4: To characterize the signaling pathway (s) initiated by RLN receptor activation.

Specific objectives:

Objective1: To determine the effect of exogenous RLN on porcine preadipocyte proliferation and differentiation in primary cultures of porcine stromal-vascular cells.

Objective2: To determine the effect of exogenous RLN on mRNA expression of adipokines, extracellular matrix proteins and fatty acid metabolism genes during porcine adipogenesis.

Objective3: To determine the effect of exogenous RLN on glycerol release into the conditioned medium of cultured porcine visceral adipose tissue explants.

Objective4: To determine the putative signaling pathway of RLN in porcine adipose tissue through co-incubation with inhibitors and activators of the cAMP and MAPK pathways.

3.1 Abstract

Relaxin (RLN) has traditionally been considered a reproductive hormone given the important role it plays in orchestrating the remodeling of cervical connective tissue in preparation for parturition. As such, the potential that adipose tissue, a specialized form of connective tissue, could also be a RLN target tissue has been unaddressed. However, recent studies have revealed that porcine adipose tissue can in fact secrete and respond to RLN. These observations suggest the possibility that locally derived RLN could serve to modulate adipose tissue development and function. Therefore, to study the effect of RLN on the regulation of adipose tissue *in vitro*, primary cultures of porcine subcutaneous adipose tissue stromal-vascular cells and visceral adipose tissue explants were utilized as model systems to determine the effect of exogenous RLN on 1) preadipocyte cell number and differentiation, 2) the mRNA expression of adipokine, extracellular matrix protein and fatty acid metabolism genes, 3) lipolysis in the presence and absence of cAMP modulators known to potently regulate lipid metabolism in the pig, and 4) the putative signaling pathway(s) of RLN in porcine adipose tissue. In these studies, RLN decreased preadipocyte number under serum-free conditions, stimulated glycerol release via the cAMP pathway, enhanced

adipogenesis, and increased the mRNA expression of multiple metabolic, adipokine, and extracellular matrix component genes. Collectively, these data support the hypothesis that RLN regulates adipose tissue development in the pig. Overall, these data suggest a working model whereby RLN could potentially function to enhance the ability of the pig to adjust to changes in energy balance through its actions on adipose tissue development and metabolism.

3.2 Introduction

Production efficiency, and hence the sustainability of pork production and our nutritional security, would be significantly enhanced through the discovery of new methods that would allow body composition to be precisely manipulated. Ideally such strategies would simultaneously allow greater accretion of desirable "marbling" or intramuscular fat while limiting the undesirable and energetically wasteful accretion of subcutaneous adipose tissue in important food animals such as the pig. Unfortunately, significant gaps in our understanding of the mechanisms that regulate cellularity and the endocrine/secretory functions of adipose tissue in growing pigs currently prevent achievement of this goal.

Adipose tissue is histologically classified as a specialized form of highly vascularized connective tissue. It appears in distinct anatomical locations termed depots and is comprised of a mosaic of metabolically active and immunoregulatory cell types, all held loosely together by a plastic extracellular matrix composed of collagen and reticular fibers. These depots first emerge within the fetal pig at approximately day 55 of gestation with the coordinate onset of fibrogenesis and angiogenesis as the emergence of the extracellular matrix and capillary bed of the adipose primordia coincides with the recruitment of adipose progenitor cells from the mesenchyme (Hausman and Kauffman1986; Hausman and Richardson, 2004; Hausman et al., 2009; Hausman,

2012; Hausman et al., 2014). These depots then remodel and enlarge when adipogenesis, adipocyte hypertrophy, and extracellular matrix expansion are driven by a positive energy balance. Importantly for food animal production, adipose tissue accretion can directly impact carcass merit as fat distribution on the body influences pork quality, lean yield, and feed efficiency.

Traditionally, adipose tissue has been regarded as having a passive role in whole body homeostasis, serving simply as a reservoir for excess energy in the form of lipid during periods of positive energy balance. However, it is now clear that adipose tissue also acts as an essential endocrine organ capable of secreting a wide variety of factors termed adipokines (Zhang et al., 1994; Kershaw and Flier, 2004). Adipokine secretion has been linked to changes in adipocyte size and adipokines have been implicated in modulating energy balance, immune status, reproductive efficiency, peripheral tissue responsiveness to pituitary and homeorhetic hormones, and the growth and metabolism of neighboring tissues such as skeletal muscle (Zhang et al., 1994; Barb et al., 2001; Kershaw et al., 2004, Hausman, 2012). Given such interactions, better understanding the development and remodeling of adipose tissue in the pig has implications beyond simply the impact of manipulating fat distribution on the growing animal. Discovering novel regulators of adipose biology could not only have the potential to greatly advance our understanding of this essential tissue, but also our understanding of whole-body growth and development.

One such novel regulator of adipose tissue is the reproductive hormone, RLN. Secreted by reproductive tissues during late gestation, RLN is best known for its well-established ability to remodel connective tissue of the birth canal during cervical ripening in preparation for parturition during a narrow time frame in which RLN levels are measurably high in the circulation (Sherwood, 2004; Parry et al., 2005; Parry et al., 2007; Kong et al., 2010). However, RLN has recently gained much attention as a potential anti-fibrotic agent in non-reproductive tissues with the goal of using

exogenous RLN therapeutically to reverse scarring in damaged organs such the heart and kidney (Conrad and Novak, 2004; Sasser, 2013; Raleigh et al., 2015). Interestingly, there is emerging evidence indicating that adipose tissue might be exposed to significant quantities of endogenous RLN independent of a reproductive context. While RLN is undetectable in the adult circulation outside of the few weeks spanning parturition, recent studies have revealed a lactocrine mode of transmission of RLN from mother to offspring creating a previously unappreciated window during early lactation in which neonatal adipose tissue is exposed to RLN (Frankshun et al., 2009). Furthermore, recent studies have revealed that adipose tissue itself can synthesize and secrete bioactive RLN, adipose tissue exhibits RLN receptor expression, and treating adipocytes with exogenous RLN induces profound changes in the adipocyte transcriptome (Hausman et al. 2006; Roberts et al. 2015). Collectively, these data suggest that the window in which developing adipose tissue is exposed to RLN could span from the emergence of the fetal adipose primordia *in utero* to the harvest of the adult animal.

Therefore, my research aims to better define, mechanistically, a new function for the traditional hormone, RLN, as a novel adipose tissue regulatory factor. To achieve this, I: 1) examined the effects of RLN on porcine adipose tissue cellularity, 2) investigated the potential for RLN to regulate adipocyte hypertrophy through acting as a lipolytic agent, 3) characterized the effect of RLN on the mRNA expression of adipokine, fatty acid metabolism and extracellular matrix genes, and finally, 4) determined the specific signaling pathway(s) initiated by RLN receptor activation in porcine adipose tissue.

3.3 Materials and methods

Animals and cell culture

All experimental procedures were approved by the Auburn University Institutional Animal Care and Use Committee. The Auburn University College of Agriculture is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC) and this study was conducted in accordance with the Federation of Animal Science Societies' Guide for the Care and Use of Agricultural Animals in Research and Teaching. Primary stromal-vascular cells (SVCs) were harvested from neonatal pig adipose tissue according to Brandebourg et al. (2005a,b). Six neonatal pigs, two crossbred (Mangalica X Yorkshire) and four purebred Mangalica, were obtained from the Auburn University Swine and Research Education Center. Neonates were euthanized via CO_2 asphyxiation and corneal reflexes were monitored to confirm euthanasia. Subcutaneous back fat located between the shoulder blades was harvested from the neonate to allow isolation of preadipocytes within the stromal-vascular fraction of the tissue. Dissected tissue was minced in 10mL Hank's balanced salt solution containing 2000 Units of type 1 collagenase/gram of tissue and tissue was digested to completion in a shaking incubator at 37°C. The digesta was then strained through a 250 μM mesh filter to remove any undigested tissue and cells were mechanically mixed to avoid clumping. The filtered digesta contains a variety of different cell types including adipocytes, pre-adipocytes, immature fat cells, fibroblasts, etc. Centrifugation was performed to separate mature adipocytes from the SVC fraction containing preadipocytes. The SVC fraction was subsequently washed thrice in Hanks balanced salt solution before being seeded in flasks containing plating medium. These preadipocytes were then used for subsequent cell number and differentiation experiments.

Adipogenesis experiments

Aliquots of SVCs were counted using a hemacytometer and seeded in culture dishes at a density of 5×10^4 cells/cm^2 and incubated at 39°C in 5% CO_2 in air (designated d-1). Plating medium consisted of Dulbecco's Modified Eagles Medium (DME)/Ham's F12 (1:1, vol/vol; Hyclone Laboratories Inc., South Logan, Utah) containing 15 mmol/L of $NaHCO_3$, 15 mmol/L of HEPES buffer, and 50 mg/L of gentamicin sulfate supplemented with 10% FBS (ATTC, Manassas, VA). Plating media for undifferentiated cells consisted of DME/F12 (1:1, vol/vol) containing 15 mmol/L of $NaHCO_3$, 15 mmol/L of HEPES buffer, 50 mg/L of gentamicin sulfate and 10% FBS. Differentiation media consisted of plating media supplemented with 100nM insulin, 5 µM dexamethasone, 0.5 µM rosiglitazone and 10% FBS. Cells were cultured at 39˚C with 5% CO_2 and were subjected to three treatments: undifferentiated (day 0), differentiated with 0 ng RLN (day 4 and 8), and those differentiated with 100 ng/ml RLN (day 4 and 8). In all cultures induced to differentiate, culture media was changed every two days. On day 0, 4 and 8 post-induction, differentiating cultures were lysed by the addition of RNAzol to each well. Lysates were immediately collected and stored at -80˚C until RNA extraction.

Proliferation assay

Aliquots of SVCs were counted using a hemacytometer and seeded in culture dishes at a density of 1×10^3 cells/cm^2 and incubated at 39°C in 5% CO_2 in air (designated d-1). Plating medium consisted of DME/F12 (1:1, vol/vol) containing 15 mmol/L of $NaHCO_3$, 15 mmol/L of HEPES buffer, and 50 mg/L of gentamicin sulfate supplemented with 10% FBS. Cells were plated on 96 well plates to facilitate proliferation studies in the presence and absence of serum and RLN (David Sherwood, Champaign-Urbana, IL). For the first 24 hr, all cells were cultured in the

presence of serum to facilitate robust cell attachment. Wells were then washed twice with Hank's buffered salt solution (HIMEDIA, West Chester, Pennsylvania). The following treatments were applied: DME/F12 w/o serum/no RLN, DME/F12 w/o serum /100 ng/ml RLN, DME/F12 + 5 % serum/no RLN or DME/F12 + 5 % serum/100ng/ml RLN. Treatment media was changed every two days until cell number determination. Cell number was assessed on days 0, 2, 4, and 6 following treatments for cells derived from 6 individual pigs (n=6 with pig as experimental unit) and each treatment mean within replicate representing the average of 8 individual wells. Cell number was estimated by incubation of cultures with [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, Saint Louis, MO) at a final concentration of 0.5 mg/ml for 3 h or by incubation with resazurin (Sigma-Aldrich, Saint Louis, MO) at a final concentration of 50 ug/ml for 3 hr. This was followed by aspiration of the medium and solubilization of the formazan product with 100 µl dimethylsulfoxide. After 30 minutes, absorbance was read at 570 nm (MTT) while fluorescence (resazurin) was determined at 530 nm excitation and 590 nm emission using a 96-well multimode plate reader (Bio-Tek, Winooski, VT).

Gene expression analysis

Total RNA was extracted from lysates obtained from primary cultures of differentiating pig preadipocytes using the RNAzol® RT procedure according to manufacturer directions (MRC, Inc., Cincinnati, OH). RNA was quantified using a BioTek Synergy 4 plate reader utilizing the Take3 system (BioTek US, Winooski, VT) with all samples exhibiting an OD 260/280 between 1.8 and 2.0 and an OD 260/230 value between 1.8 and 2.2. Total RNA integrity was accessed visually by resolving 2 µg of RNA on a denaturing formaldehyde gel containing ethidium bromide. All samples demonstrated sharp ribosomal bands with a 28S to 18S ratio greater than one. 1 µg of

total RNA was then reverse transcribed using Superscript II reverse transcriptase (Promega Inc., Madison, WI) and oligo-dT primers. Real-time PCR was performed on 50 ng of the resultant cDNA using a Roche Lightcycler® 480 Real-time PCR machine and LightCycler® 480 SYBR Green I Master Mix (Roche Applied Science, Indianapolis, IN) according to manufacturer's recommendations. PCR consists of 45 cycles with one cycle defined as: melting at 95˚C, annealing at 54˚C and elongation at 75˚C. All PCR reactions were performed using intron-spanning primers under optimized conditions with primer efficiencies ranging between 72–105% (Tables 3-1 & 3-2) as verified with standard curves. Product purity was assessed by melting curve analysis.

Relaxin lipolysis experiment

Approximately 300 g of visceral adipose tissue was removed from adult Yorkshire pigs at the time of harvest at the Lambert Powell Meats Laboratory, transported back to the laboratory and minced into pieces approximately 5 mm in diameter in Hanks balanced salt solution. These explants (totaling about 200 mg) were then transferred to treatment plates and incubated directly in treatment medium containing phenol-free DME/F12 with 1% FBS supplemented with the experimental treatments (either control or hormone treated medium). Following 2 hr incubation, conditioned medium was collected for determination of glycerol release as a measure of lipolysis. Experiments consisted of first conducting dose-response curves with increasing amounts of RLN (0,1,100 ng/ml). The optimal dose of RLN (100 ng/ml) was then utilized for all subsequent studies, as indicated by previous studies (Roberts et al., 2015). Next, the lipolytic action of RLN was assessed in the absence of or in combination with several cAMP modulators (100 nM insulin, 250 μM IBMX, and 1μM isoproterenol). Finally, RLN signaling was investigated by determining the effect of signaling pathway inhibitors (10μM H89, 10μM UO126) on the lipolytic activity of RLN.

For inhibitor experiments, explants were incubated in the culture medium containing the appropriate inhibitor one hr prior to and continuing throughout the 2 hr RLN treatment. Following collection of conditioned medium, glycerol release was measured by the Trinder colorimetric assay (Sigma-Aldrich, Saint Louis, MO). Glycerol concentration within each well was normalized by explant tissue weight by weighing total explant weight per well and expressed as nmol glycerol/mg tissue/2 hrs. Each experiment was replicated 6 times with each treatment mean within a replicate representing the average of four individual wells.

Statistical analysis

Changes in gene expression were calculated from the cycle threshold, after correction using S15 expression and analyzed using the Pair Wise Fixed Reallocation Randomization Test of REST-MCS v2.0 (http://rest.gene-quantification.info/). Lipolysis and cell number experiments were analyzed as a completely randomized block design using proc glm of SAS v9.2 with individual animal serving as the experimental unit, i.e. individual block (SAS Institute, Inc., Cary, NC).

3.4 Results

Relaxin inhibits preadipocyte proliferation

In order to test the hypothesis that RLN can regulate adipose tissue cellularity, porcine preadipocytes were plated at a density of 1000 cells/cm^2 in either the continuous presence or absence of exogenous RLN (100 ng/ml) for 0, 2, 4 or 6 days upon which cell number was estimated using basic cell proliferation assays (MTT, resazurin). As expected under serum-free conditions, cell number remained unchanged in the control treatment group across days given the absence of

mitogenic signals in the culture (Figure 3-1). However, treatment of cultures with RLN decreased MTT signal by 14 ± 2, 17 ± 2, and 22 ± 2% on days 2, 4, and 6 respectively ($p < 0.05$). Likewise, RLN decreased the resazurin signal by 27 ± 5, 25 ± 5, and 32 ± 5% on days 2, 4, and 6 respectively relative to controls ($p < 0.05$). These data indicate that RLN can decrease preadipocyte number. To test if RLN specifically impairs preadipocyte proliferation, the experiments were duplicated in the presence of 5% serum. Under serum-containing conditions, cell number as estimated by both MTT and resazurin signal increased linearly by day in control cultures indicative of robust preadipocyte proliferation rates ($p < 0.001$). However, under these conditions, RLN failed to statistically alter cell number based upon either MTT ($p < 0.68$) or resazurin ($p < 0.55$) signal compared to controls which may be due to other growth factors and hormones present in the serum (Figure 3-2). These results indicate that RLN can decrease preadipocyte number but not through inhibiting proliferation.

Relaxin enhances adipogenesis.

In order to further test the hypothesis that RLN can alter adipose tissue cellularity, primary cultures of porcine preadipocytes were differentiated in the presence or absence of RLN (100 ng/ml) for 8 days and then morphological, biochemical and gene markers of adipogenesis were measured. Cultures robustly differentiated in the continuous presence of induction medium as depicted by Oil-Red-O (ORO)-stained photomicrographs (Figure 3-3; Panel A) and quantification of extracted ORO dye ($p < 0.05$; Figure 3-3; Panel B) from cultures harvested on day 0 and day 8 post-induction. Apparent adipogenesis was even greater when cultures were induced in the presence of RLN as evidenced by a 2-fold increase in ORO accumulation compared to non-RLN induced cultures ($p < 0.01$). This stimulatory effect was also confirmed biochemically by

measuring glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity (Figure 3-3; Panel C). Preadipocytes express little GAPDH protein while its expression is rapidly upregulated during adipogenesis. GAPDH activity was induced 31-fold in day 8 control cultures relative to day 0 ($p < 0.01$). Meanwhile, consistent with ORO staining, RLN treatment induced GAPDH activity even greater (67-fold; $p < 0.001$). Finally, gene markers for fat cell differentiation, *peroxisome proliferator-activated receptor gamma* (*PPARγ*) and *CCAAT/enhancer-binding protein alpha* (*C/EBPα*) were measured on days 0 and 8 of induction via real-time PCR (Figure 3-3; Panels D & E). Confirming a robust differentiation of cultures that was augmented by the addition of RLN, *PPARγ* was upregulated 9.8-fold in non-RLN treated cultures and 14.3-fold in RLN-treated cultures on day 8 versus day 0 ($p < 0.01$) while *C/EBPα* was likewise upregulated 46.1- and 191-fold respectively ($p < 0.001$).

Relaxin is a lipolytic agent in porcine adipose tissue

In order to test the hypothesis that RLN, a putative cAMP modulator, is a lipolytic agent in pig adipose tissue and to establish optimal treatment conditions using visceral adipose tissue, an initial experiment was performed to evaluate glycerol release from visceral adipose tissue explants in response to increasing doses of RLN (0, 1, 10, 100 ng/ml) and duration of RLN treatment (2, 6 and 24 hr). Relaxin significantly stimulated glycerol release across all durations of treatment, however the response was greatest during acute treatment (2 hr) with 1 ng/ml RLN stimulating glycerol release 2.45-fold ($P < 0.05$) and 100 ng/ml RLN stimulating glycerol release 3.28-fold ($P < 0.01$) compared to untreated controls and data for this time point is shown (Figure 3-4; Panel A). The magnitude of the stimulatory action of RLN decreased with length of RLN treatment and dose-dependence likewise became less consistent. These results indicate that acute incubation (2hr

is the optimal treatment duration). Overall, these experiments support the hypothesis that RLN is a lipolytic agent in porcine adipose tissue. This action was further investigated in all subsequent experiments using 100 ng/ml RLN incubated for 2 hr under basal conditions.

Classic adipose tissue cAMP modulators alter the lipolytic action of RLN.

It is well known that lipolysis is regulated by cAMP modulators *in vivo.* In order to examine the impact of cAMP modulation on the lipolytic action of RLN, experiments were conducted with either insulin, a homeorhetic hormone which inhibits lipolysis in part through activation of phosphodiesterase activity; isoproterenol, a powerful β-adrenergic agonist known to stimulate lipolysis and IBMX, a potent phosphodiesterase inhibitor known to increase cAMP levels in adipocytes.

To test the impact of insulin, RLN was co-incubated with insulin (100 nM) for 2 hr and then glycerol release into the conditioned medium was determined (Figure 3-4; Panel B). Treatment with RLN alone stimulated glycerol release 1.37-fold compared to untreated controls ($p < 0.05$). Insulin treatment alone did not alter glycerol release compared to control cultures ($P < 0.79$), however, co-incubation of insulin with RLN completely blocked the lipolytic action of RLN.

Next, RLN was co-incubated with 1μM isoproterenol for 2 hr and then glycerol release into the conditioned medium was determined (Figure 3-4; Panel C). RLN treatment alone significantly increased glycerol release 1.29-fold compared to control ($p < 0.05$). Isoproterenol treatment alone significantly increased glycerol release 1.56-fold compared to control ($p < 0.01$). Co-incubation of RLN with isoproterenol produced an additive effect, significantly increasing glycerol release 1.70-fold compared to control ($p < 0.001$).

Lastly, RLN was co-incubated with a low dose of IBMX (250 μM) for 2 hr and then glycerol release into the conditioned medium was determined (Figure 3-4; Panel D). Treatment with RLN stimulated glycerol release 1.39-fold compared to control (p <0.05). Treatment with 250 μM IBMX alone stimulated glycerol release 1.51-fold ($p < 0.05$). Co-incubation of IBMX with RLN resulted in a 1.74-fold increase in glycerol release compared to control ($p < 0.05$) but did not produce a significant additive effect compared to RLN or IBMX alone.

Relaxin signals via the cAMP but not MAPK pathway.

To determine the specific signaling pathways initiated by RLN receptor activation in porcine adipose tissue, explants were treated with inhibitors of the cAMP and MAPK pathways.

To evaluate RLN signaling through the cAMP pathway, visceral adipose tissue explants were treated with 10 μM H89, a potent inhibitor of PKA activation, for 2 hr in the presence of RLN and then glycerol release into the conditioned medium was determined (Figure 3-5; Panel A). Treatment with RLN stimulated glycerol release 1.68-fold ($p < 0.05$) and this was completely inhibited by co-incubation of RLN with H89 ($p < 0.05$). The same pattern was seen with the co-incubation of 50 μM H-89 with RLN (data not shown). These data support the hypothesis that RLN signals through the cAMP pathway to stimulate lipolysis in porcine adipose tissue.

To evaluate RLN signaling through the MAPK pathway, visceral adipose tissue explants were treated with 10 μM UO126, a potent global inhibitor of MEK and MAP kinases, for 2 hr in the presence of RLN and then glycerol release into the conditioned medium was determined (Figure 3-5; Panel B). RLN alone modestly stimulated glycerol release 1.23-fold ($p < 0.05$) in this experiment and despite a modest RLN response, UO126 failed to block this action when co-incubated with RLN ($p < 0.05$). Similar results were observed when these experiments were

repeated using 50 μM UO126 (data not shown). These data indicate that the lipolytic action of RLN is not dependent upon activation of the MAPK pathway.

Relaxin alters the mRNA expression of adipokine, metabolic and extracellular matrix component genes.

In order to test the hypothesis that RLN can alter the secretory and metabolic functions of adipose tissue, mRNA expression of adipokines, fatty acid metabolism, and extracellular matrix component genes were measured in primary cultures of porcine preadipocytes that were differentiated in the presence or absence of RLN (100 ng/ml) for 0, 4 and 8 days.

The effect of RLN on the endocrine function of adipose tissue was examined by measuring the mRNA expression of *adiponectin*, *leptin*, *interleukin-6, tumor necrosis factor α, interleukin-15, and VEGF* (Table 3-3, Figure 3-6). A*diponectin* mRNA was upregulated from d0 to d8 by 11.6-fold ($p < 0.05$) on day 8 versus day 0 in the absence of RLN; whereas, RLN treatment upregulated, *adiponectin* mRNA 97.2-fold ($p < 0.01$). Likewise, *leptin* mRNA expression was upregulated from 9.5-fold in day 8 cultures versus day 0 ($p < 0.05$) and addition of RLN resulted in a very robust, additive stimulation of 152-fold versus day 0 ($p < 0.01$). Interestingly, *interleukin-6* mRNA was upregulated 2.83-fold during differentiation in the absence of RLN ($p < 0.05$) but the presence of RLN not only blocked this upregulation but resulted in a striking 80% decrease in mRNA expression compared to day 0 ($p < 0.01$). The mRNA abundance of *interleukin-15* was not altered by either induction of differentiation ($p < 0.76$) or the addition of RLN ($p < 0.99$). On the other hand, *tumor necrosis factor α* mRNA was downregulated during differentiation and this effect was not altered by the addition of RLN ($p < 0.05$). Finally, expression of the RLN target gene, *VEGF*, was upregulated from d0 to d8 by 29.3-fold ($p < 0.05$) in the absence of RLN, and

as expected, the addition of RLN further upregulated its mRNA expression by 77.7-fold ($p < 0.01$). Overall, these data support the hypothesis that RLN can modulate the endocrine/secretory function of adipose tissue.

The ability of RLN to alter adipocyte metabolism was examined be measuring the mRNA expression of the metabolic genes *FASN, HSL, SCD-1*, and *GLUT-4* (Table 3-5, Figure 3-7). As expected, the mRNA abundances of all four metabolic genes measured was significantly induced by differentiation based upon comparison of day 8 values to day 0 values. However, the addition of RLN during differentiation only further induced expression of *FASN* mRNA while having no effect upon the mRNA abundances for *HSL, SCD-1* or *GLUT4*.

Finally, the ability of RLN to alter adipose tissue extracellular matrix components and regulators of extracellular matrix remodeling was examined via assessing gene expression for the *COL1A, COL2A, COL3A, COL4A*, *COL6A, biglycan, fibronectin*, *laminin, NID1, MMP2, MMP9, TIMP3 genes* (Table 3-6, Figure 3.8). The mRNA abundance of several extracellular matrix related genes including COL1A, COL3A and MMP2 were significantly down regulated during differentiation in the absence of RLN but the addition of RLN not only reversed expression, it resulted in a dramatic upregulation of mRNA for these genes compared to day 0 expression levels. Meanwhile the mRNA abundance for the COL2A, COL4A, COL6A, biglycan, fibronectin, laminin, NID1 MMP9 and TIMP3 genes were all significantly upregulated during differentiation and the addition of RLN had a very consistent and robustly additive effect upon the upregulation of these genes. Collectively, these data support the hypothesis that RLN can act as an extracellular remodeling agent in adipose tissue given the consistent and striking modulation of extracellular matrix component gene expression.

3.5 Discussion

Relaxin is a connective tissue remodeling agent in reproductive tissues that prepares the birth canal for parturition and has recently gained attention as an extracellular matrix remodeling agent in non-reproductive tissue such as the heart and kidney (Conrad and Novak, 2004; Sasser, 2013; Raleigh et al., 2015). Adipose tissue is histologically classified as specialized connective tissue, and therefore likewise represents an attractive potential RLN target tissue (Young and Heath, 2000). These data newly generated herein support the hypothesis that RLN acts as a novel adipose tissue remodeling agent in porcine adipose tissue by demonstrating that: 1) RLN can alter preadipocyte number, 2) RLN can enhance adipogenesis, 3) RLN regulates lipolysis through activating the cAMP signaling pathway and, 4) RLN alters the mRNA expression of genes encoding adipokines, extracellular matrix (ECM) components, and ECM regulatory proteins *in vitro*. Collectively, these data point to a much greater role for RLN in regulating growth and development than the traditionally understood role of RLN as primarily a classic reproductive hormone involved in cervical ripening at parturition. Importantly, our data reveal a previously unappreciated role for RLN in modulating adipose tissue biology in the pig.

The presence of RLN can be detected in both female (i.e., uterus, ovary, corpus luteum, placenta) and male (i.e., prostate, testes, seminal vesicles) reproductive tissues (Sherwood, 2004). A majority of studies have been aimed at the effect of RLN in the female counterpart as the effect of RLN in the male is somewhat controversial. The source of RLN production varies between species with the corpus luteum and placenta being the major sources during gestation (Sherwood et al., 1975a, 1976, 1978, 1979, 1981; Frankshun et al., 2009). In non-pregnant females, the corpus luteum has been shown to secrete RLN during the estrus cycle with luteal concentrations being estimated at less than 3 μg/g and circulating levels being low (Anderson et al., 1982; Bagnell et

al., 1993). In males, RLN has been suggested to play a role in sperm motility (Sasaki et al., 2001). In boars, recent data suggests that the testes are the major source of RLN; although the RLN produced in the male reproductive tract appears to be primarily released into the seminal fluid, with the mean ± SD level of immunoreactive RLN in seminal plasma being 2.61 ± 0.62 ng/mL and essentially undetectable within the systemic circulation (Sasaki et al., 2001; Sherwood, 2004; Kato et al., 2010). Thus, while RLN is produced and secreted by tissues in both genders, the traditional reproductive literature suggests that RLN exposure may not be systemic during much of the developmental trajectory of both genders.

It is this relatively narrow window spanning late gestation to early lactation in which peripheral tissues are exposed to high circulating RLN concentrations that has led to RLN largely been ignored as a potential endogenous connective tissue remodeling agent in non-reproductive tissues (Sherwood, 2004). For instance, RLN concentrations in the maternal circulation are undetectable in non-pregnant dams and only begin to significantly rise during the final two weeks of gestation, peaking at levels of 50 to 300 ng/ml approximately 2 days prior to parturition and rapidly declining to approximately 1 ng/ml by day 7 post-parturition; reflecting the well characterized pattern of upregulated expression and secretion from uterine tissues which wanes upon farrowing (Sherwood et al., 1975a, 1976, 1978, 1979, 1981; Frankshun et al., 2009). Importantly, however, adipose tissue primordia are histologically apparent by day 80 of gestation and adipose tissue development is occurring in earnest during the gestational window in which RLN levels increase within the dam; suggesting developing adipose tissue is exposed to significant levels of RLN during an early critical development window occurring within late fetal development (Hausman and Kauffman, 1986; Hausman et al., 2009; Hausman et al., 2014). In this regard, several studies suggest that circulating maternal RLN appears to cross the placenta to

influence fetal development throughout the second half of rat pregnancy (Hwang and Sherwood, 1988; Burger and Sherwood, 1998; Zhao et al., 1995; Zhao et al., 1996; Zhao and Sherwood, 1998; Sherwood 2004). In these studies, knocking down endogenous RLN through either the immunoneutralization of R1 relaxin or via ovariectomy resulted in fetal weights that were significantly greater than controls. However, the actual concentration of RLN in the fetal circulation during gestation is unknown. Interestingly, and consistent with a role for RLN in regulating adipose tissue development, recent work has indicated that RLN, acting as a milk-born factor, can regulate neonatal development at least until the onset of gut closure, occurring by day 2 post-parturition (Yan et al., 2006; Frankshun et al., 2009). Based upon bioassay measurements using expressed milk from nursing, multiparous Yorkshire sows, porcine RLN concentrations in milk are highest in colostrum, peaking at 9-19 ng/ml and rapidly declining to less than 2 ng/ml by day 7 and essentially becoming undetectable by day 14 post-parturition (Yan et al., 2006; Frankshun et al., 2009). During this timeframe, a period also correlating with a rapid expansion of adipose tissue in the neonate, circulating RLN concentrations rise to 200 pg/ml in nursing piglets reflecting a similar pattern as described for expressed milk (Yan et al., 2006). Thus, a narrow albeit potentially significant window for RLN exposure exists during a critical period of extensive adipose tissue expansion *in utero* and milk-borne transmission extends this period to key early stages of rapid, hypertrophic adipose tissue expansion in the neonate.

Given the dogma that relaxin is primarily derived from reproductive tissues, local concentrations of RLN within adipose tissue have not been characterized. However, there is reason to believe that cells within porcine adipose tissue might be exposed to significant amounts of RLN acting in an autocrine or paracrine fashion. Hausman et al., (2006) used 2-D gel electrophoresis and mass spectrometry to investigate the porcine adipose tissue secretome during adipogenesis. In

these studies, RLN was identified as a protein secreted by adipocytes though the authors argued that their observation had little physiological relevance. However, Roberts et al., (2015) demonstrated that porcine adipocytes in both differentiated cultures and explant cultures secrete bioactive RLN, its secretion is upregulated during adipogenesis, RLN expression is higher in subcutaneous adipose tissue of obese versus lean pigs, and that treatment of porcine adipocytes with exogenous RLN causes significant changes in the adipocyte transcriptome. These recent observations demonstrate that porcine adipose tissue can act as a RLN target tissue and importantly, these data essentially extend the window of adipose tissue exposure to RLN from d 80 of gestation to harvest of the mature pig, a timeframe encompassing the entire life cycle of the pig.

Absent micro dialysis studies to quantify *in vivo* adipose tissue concentrations of RLN make it difficult to estimate what the true physiological level of RLN exposure is for pig adipocytes. In the current study, the dose for *in vitro* experiments was determined by initially conducting dose-response curves and then utilizing the maximally effective dose of 100 ng/ml. This dose was consistent with previous *in vitro* studies where exogenous RLN was administered to fat cells though it represents a 1000-fold greater dose compared to quantification of bioactive RLN secretion into adipocyte conditioned medium using the RLN bioassay following 24 incubation (Roberts et al., 2015). However, the dose used in current experiments was well within the physiological range for RLN measured in the dam's circulation at parturition and represents a level only five-fold higher than the concentration of RLN found in colostrum (Sherwood et al., 1975a, 1976, 1978, 1979, 1981; Yan et al., 2006; Frankshun et al., 2009). Given locally secreted concentrations of autocrine and paracrine acting factors can often reach significantly higher levels

locally than are measured in the systemic circulation, the dose used for the studies herein is not likely supraphysiological in magnitude.

While activation of the relaxin receptor can result in significant crosstalk with insulin and the IGF receptors through activation of the MAPK and PKC pathways, with ligands for each of these related receptor systems even being able to somewhat weakly bind to and activate one another's receptors, RLN putatively signals through the cAMP pathway (Ivell et al., 2007; Halls et al., 2009). Importantly, it is well known that mammalian adipocyte hypertrophy is regulated by cAMP-modulators and this is especially so for the pig. For instance, cAMP pathway activators such as beta-adrenergic agonists (βAA) potently stimulate lipolysis and decreases adipocyte size while signals such as insulin, which blunt the pathway, potently inhibit lipolysis and favor increases in cell size (Mills and Mersmann, 1995; Dimitriadis et al., 2011). Thus, RLN, as a putative cAMP activator, is well positioned to regulate lipid biochemistry in adipose tissue.

Indeed, data from our lab confirms that RLN stimulates lipolysis in porcine adipose tissue and does so by signaling through the cAMP pathway. Relaxin alone consistently stimulated lipolysis in the current study between 30-300%. These are the first data to implicate RLN as a metabolic modifier that can directly regulate lipid biochemistry and energy storage in adipose tissue. Further verifying this observation, the co-incubation of RLN with insulin, a phosphodiesterase inhibitor, completely blocked the lipolytic action of RLN. This would be expected as evoking insulin signaling in multiple adipose tissue depots simultaneously stimulates lipogenesis and potently inhibits lipolysis in part through strong inhibition of cAMP signaling (Baumgard et al., 2015; Huttala et al., 2016). Further, as expected, co-incubation of RLN with isoproterenol (ISO), a potent βAA, potentiated the lipolytic action of RLN. It is well documented that β-AR activation in porcine adipose tissue leads to an increased production of intracellular

cAMP and the phosphorylation of PKA, which activates HSL and results in increased lipid breakdown (Mills and Mersmann, 1995). Porcine subcutaneous adipose tissue expresses β1AR, β2AR and β3AR with transcript concentrations of 1.3, 0.4 and 0.14 mol/μg of total RNA respectively (McNeel and Mersmann, 1999). Isoproterenol acts as a pure β-adrenergic compound as it does not appear to bind to other receptors, suggesting that it is directly cross-talking with RLN through the cAMP signaling pathway (Mills and Mersmann, 1995). These observations suggest tempting speculation that locally secreted RLN might serve to potentiate the effect of naturally occurring βAA's such as epinephrine and norepinephrine, the "fight or flight" hormones, and thus serve as a modulatory signal that enhances the transition to a negative energy balance. Importantly, synthetic βAA's such as ractopamine have been approved for use as growth promotants in growing pigs; however, their adoption has been limited by the onset of βAA resistance upon prolonged exposure. The ability of RLN to potentiate the isoproterenol response in the current study may point to potential strategies to overcome such limitations.

In agreement with previous research whereby IBMX increased lipolysis in both retroperitoneal (RWAT) and epididymal (EAT) white adipose tissue of rats, IBMX treatment alone was lipolytic (Feres et al., 2013). Interestingly, however, co-incubation of RLN with IBMX unexpectedly did not potentiate the stimulatory effect of RLN in the current study. It is possible that pig variation may have obscured an additive effect of co-incubation. Explant cultures from different pigs exhibited a highly variable responsiveness to IBMX in the current studies. Further verifying that RLN exerts its lipolytic effect through modulating cAMP in adipose tissue, co-incubation of RLN with H89, a PKA inhibitor, completely inhibited the ability of RLN to stimulate lipolysis. Co-incubation of RLN with U0126, a potent global inhibitor of the MAPK pathway, did not inhibit RLN's lipolytic response, demonstrating that the lipolytic action of RLN is not

dependent upon the MAPK pathway. Taken together, the co-incubation studies with both cAMP modulating agents and specific signaling pathway inhibitors strongly indicate that RLN signals through the cAMP pathway, at least when exerting its lipolytic action on porcine adipose tissue.

Exogenous RLN appears to alter adipose tissue cellularity by decreasing porcine preadipocyte number while stimulating adipogenesis. Treatment of primary cultures of porcine stromal-vascular cells with exogenous RLN decreased cell number in serum free conditions but presented no significant effect on cell proliferation in serum conditions; demonstrating that RLN can decrease pre-adipocyte number but not through inhibiting proliferation in the current study. Interestingly, two studies exist in which porcine RLN was shown to prevent clonal expansion of murine 3T3-L1 preadipocytes upon contact inhibition, supporting a role for RLN as an inhibitor of preadipocyte number (Pawlina et al., 1989; Pawlina et al., 1990). In serum free conditions, the decreased cell number could be explained through either RLN-enhanced necrosis or via RLN stimulation of apoptosis. Based upon morphological observation, it did not appear that treating preadipocytes with 100 ng/ml RLN was detrimental to the cells in the current studies, as all cultures appeared healthy with optimal attachment. Furthermore, there is no data in the literature that suggests RLN is toxic to mammalian cells. However, the cancer literature suggests that RLN has anti-mitogenic properties-consistent with the Pawlina manuscripts-while also indicating that RLN may induce apoptosis in multiple tumor cell lines (Sacchi et al., 1994; Bani et al., 1995). However, other literature suggests that RLN can also be anti-apoptotic in the myocardial tissue, cervix and vagina of rats (Zhao et al., 2001; Moore et al., 2007; Wang et al., 2016; Zhang et al., 2017). Unfortunately, our lab did not assess the effect of RLN treatment on apoptosis in our porcine primary preadipocyte cultures in the studies herein. Therefore, clarifying the potential role for RLN in regulating apoptosis will require further study.

The current study indicates that RLN can stimulate adipogenesis based upon morphological, biochemical, and gene marker studies. Peroxisome proliferator-activated receptor gamma (PPARγ) is recognized as the master regulator of adipogenesis and is also required for the maintenance of adipogenesis (Farmer, 2006; Rosen and MacDougald, 2006). Interestingly, recent research has demonstrated the ability of RLN to increase transcriptional activity of PPARγ in non-adipose tissue cells (Singh and Bennett, 2009; Singh and Bennett, 2010; Singh et al., 2015). Both results from the current studies and from Singh and colleagues are consistent with previous work in our lab in which RLN treatment resulted in both an upregulation of PPARγ and C/EBPα mRNA expression, but also the altered expression of over 800 genes in pig adipocytes as measured via RNAseq experiments (Roberts et al., 2015). Taken together, it is clear that adipose tissue is a RLN target tissue and that RLN enhances adipogenesis likely through stimulatory effects on key adipogenic transcription factors.

Our data supports the hypothesis that RLN regulates the endocrine function of adipose tissue in the pig as the expression of multiple adipokine genes was altered in response to RLN treatment. Adiponectin is an anti-inflammatory hormone associated with increased insulin sensitivity (Ruan and Dong, 2016). Leptin's primary role is associated with the regulation of energy homeostasis and the onset of puberty (Kershaw and Flier, 2004). IL-15 is an anti-inflammatory cytokine and participates in immunological processes such as the stimulation of lymphocytes. On the contrary, TNFα and IL-6 are pro-inflammatory cytokines also involved in several immunological processes and have been demonstrated to have actions on metabolism and adipokine expression (Cawthorn and Sethi, 2008; Galic et al., 2010; Ramsey et al., 2013) Interestingly, in the current study, while TNFα and IL15 mRNA abundance were unaffected, RLN upregulated adiponectin gene expression while downregulating IL6 expression which suggests a

potential anti-inflammatory gene response. While future studies are required to establish a link between adipose tissue derived RLN and inflammatory status, these data indicate that RLN can alter adipokine gene expression and suggests that RLN may play a role in regulating adipose tissue inflammation.

In the current study, RLN upregulated VEGF mRNA expression and this observation is consistent with a role for RLN in adipose tissue remodeling. VEGF is important in tissue and organ development because of its ability to drive the formation of immature vessels through vasculogenesis or angiogenic sprouting (Papetti and Herman, 2002; Liekens et al., 2001; Yanocopoulos et al., 2000; Ribatto et al., 2003). During fetal development of porcine adipose tissue, fibrogenesis, angiogenesis, and adipogenesis are coupled processes and the emergence of fat cells from adipocyte progenitor cells does not occur without extracellular matrix and capillary bed formation (Hausman and Richardson, 2004; Hausman et al., 2014). Likewise, the expansion of adipose tissue requires remodeling of the extracellular matrix and new blood vessel formation to support the increased metabolic and structural needs of enlarging adipocytes. Thus, increased VEGF expression supports the hypothesis that RLN can serve as an adipose tissue remodeling agent.

That RLN consistently and dramatically altered the expression of extracellular matrix component genes in the current study supports a role for RLN in regulating adipose tissue expansion given the essential coordination between fibrogenesis and adipogenesis. This study is the first to assess extracellular matrix gene expression in pig adipose tissue during differentiation. Since connective tissue is an important aspect of adipose tissue biology and RLN is a connective tissue modifying agent, it is reasonable to expect that RLN plays a role in the regulation of collagen proteins within the ECM of adipose tissue itself. Khan et al., 2009 suggests that collagens are

highly upregulated in the adipose tissue of mice during changes in whole body metabolic status with COL1, COL4 and COL6 being highly expressed in adipocytes, whereas COL2 and COL3 exhibited lower expression levels. Our data is the first to demonstrate the ability of RLN to coordinately upregulate COL1A, COL2A, COL3A, COL4A and COL6A mRNA expression in porcine adipocytes *in vitro*. The dramatic changes in expression of these genes demonstrates that collagen plasticity is an important aspect of adipogenesis in the pig. That RLN appears to coordinately regulate collagen gene expression suggests that locally derived RLN may be an important signal driving the coordination of adipogenesis and fibrogenesis in developing adipose tissue.

Importantly, RLN appears to also regulate expression of factors which directly influence collagen protein content in the extracellular matrix. In all tissues, the composition of the ECM is associated with the balance between protein synthesis and degradation (Khan et al., 2009). Matrix metalloproteinases (MMPs) are a family of proteins which function to degrade components of the ECM during normal growth and tissue turnover, playing significant roles in morphogenesis, wound healing, tissue repair and remodeling (Nagase et al., 2006). Tissue inhibitors of matrix metalloproteinases (TIMPs) function to inhibit MMPs and the degradation of the ECM. Research by Lenhart et al., 2002 demonstrate that RLN stimulates the upregulation of MMP2, MMP9, TIMP1 and TIMP2 expression during uterine and cervical growth and remodeling in the pig. Our lab is the first to investigate the effect of RLN on MMP2, MMP9 and TIMP3 expression in pig adipose tissue *in vitro*. Importantly, RLN significantly altered the mRNA expression profiles of *TIMP3*, *MMP2* and *MMP9*. When coupled with the observation that RLN also significantly altered expression of collagen genes, RLN appears to coordinately regulate both the synthesis of new collagens and the turnover of existing collagen proteins. These observations strongly support a

role for RLN as an adipose tissue remodeling agent that can direct changes in the extracellular matrix.

Biglycan (BGN), Fibronectin (FN1), laminin (LAMA), and Nidogen (NID1) are important markers for adipose tissue development and fibrogenesis, but little is known about their precise functions in pig adipose tissue. BGN is best characterized for its role in regulating the mineralization of bone. FN1 is a glycoprotein of the ECM and plays a critical role in cell adhesion, growth, migration and differentiation (George et al., 1993; Pankov and Yamada, 2002; Maurer et al., 2016). LAMA is a major component of basement membranes and is important in cell adhesion, migration and differentiation (Martin et al., 1988; Timpl, 1989; Beck et al., 1990; Yurchenco et al.,1990; Kleinman and Weeks, 1991; Hay, 1991.) NID1 is ubiquitously expressed in basement membranes and binds strongly to laminin (Hay, 1991). Our lab is the first to investigate the effect of RLN on BGN, FN1, LAMA and NID1 expression in pig adipose tissue *in vitro*. The mRNA abundances of *BGN, FN1, LAMA* and *NID1* were significantly upregulated during adipogenesis and treatment with RLN significantly potentiated this upregulation pointing both to an important role for these genes during adipose tissue expansion and for RLN in regulating this process further suggesting RLN plays an important part in the mechanisms that couples fibrogenesis and adipogenesis.

Interestingly, data from the studies herein appear to reveal a paradox concerning the physiological role of RLN in adipose tissue. Clearly, RLN is a locally derived factor that can alter the function of adipose tissue (Hausman, 2007; Roberts et al., 2015) and the present data verifies that pig adipose tissue is a sensitive RLN target tissue. However, the ability of relaxin to promote adipogenesis/fibrogenesis and hence potentiate adipose tissue expansion while also stimulating lipolysis seems at odds. Perhaps the demonstration that insulin effectively blunts the lipolytic

action of RLN may shed some insight into the potential biological activity of RLN. It is tempting to speculate that RLN might serve as a locally derived factor which can modulate the transition between positive and negative energy balance. In this regard, it may be beneficial to visualize RLN signaling as a switch on a railroad track. In the absence of insulin, a state consistent with a negative energy balance, RLN putatively signals through the cAMP pathway, stimulating the breakdown of triglycerides, the mobilization of free fatty acids and the shrinking of adipocytes both directly and perhaps through potentiating other lipolytic signals. However, in the presence of insulin, a state consistent with a positive energy balance, the railroad switch is activated and the train (RLN) switches tracks. Now, perhaps RLN signals through secondary pathways such as the MAPK pathway due to insulin's inhibition of the cAMP pathway. In this context, RLN is no longer lipolytic but instead promotes adipogenesis and directs the coordination of fat cell differentiation and extracellular matrix remodeling, promoting the construction of more warehouse space to facilitate the anabolic action of insulin upon adipocytes. That said, adipocytes do not have an unlimited capacity for expansion and as they become larger, the collagen and reticular fibers of the extracellular matrix begin to constrict the adipocyte (Lafontan, 2011). This is believed to induce cellular stress and the generation of reactive oxygen species (ROS) promoting a mild state of hypoxia (Crewe et al., 2017). Hypoxia and ROS generation promotes adipose tissue inflammation and increased oxidative stress which progressively promotes a state of insulin resistance. In this context, locally derived RLN could serve as a protective signal through stimulating extracellular matrix remodeling which may reduce the constrictive forces of collagen fibers on mature adipocytes and by promoting insulin sensitivity locally. Furthermore, the increased adiponectin and leptin release in response to RLN is consistent with increasing insulin sensitization and would be expected to promote an apparent improvement in the regulation of

satiety, which also may lessen the drive for adipose tissue expansion. Likewise, an anti-inflammatory action of RLN would be consistent with promoting a healthy expansion of adipose tissue; seemingly uncoupling adipose tissue inflammation from adipocyte hypertrophy, at the same time the increased fibrogenesis might reduce the tensile stress on adipocytes. While further research is needed in order to substantiate such a model, our data is consistent with a potential biological role for RLN as a modulating factor facilitating changes in energy balance through its direct action on adipose tissue. Regardless, these data support a role for RLN in regulating the development of pig adipose tissue and point to a novel function for an old hormone which may provide new avenues for the regulation of body composition in the pig.

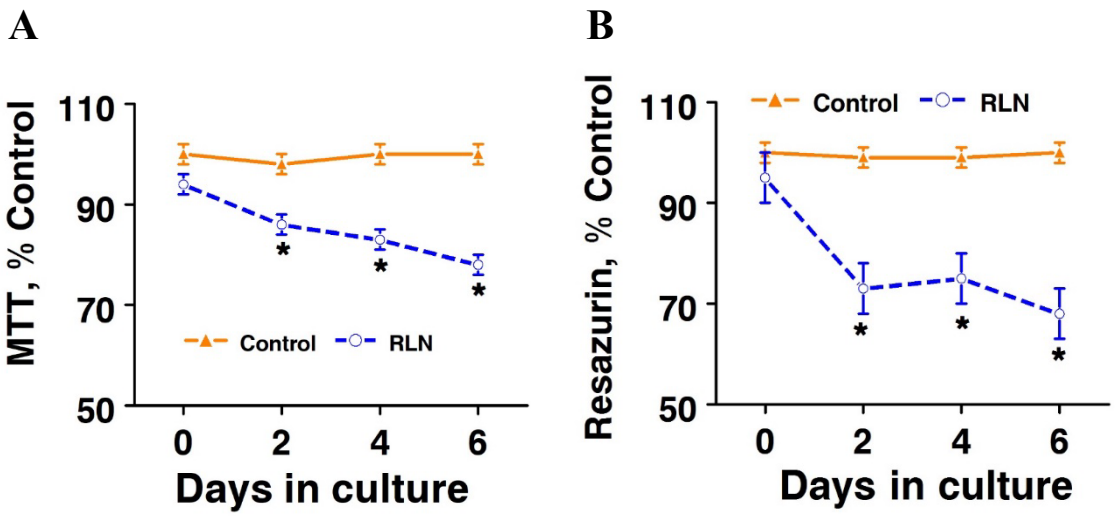

Figure 3-1. Relaxin decreases porcine preadipocyte cell number in serum-free medium based upon both lower cleavage of the tetrazolium salt, MTT and the decreased reduction of resazurin in primary cultures of pig stromal-vascular cells following treatment with RLN for 2, 4 or 6 days. Primary pig pre-adipocytes were plated at a density of 1000 cells/cm^2 in either the continuous presence or absence of exogenous RLN (100 ng/ml) for 0, 2, 4 or 6 days. ***Panel A***: Cell number was estimated using hydrolyzation of the tetrazolum salt, MTT, following 3 hr incubation on the indicated day. Data are expressed as percent control of relative absorbance units (540 nm) and represent the mean +/- SEM (n=6) with * denoting significance at $P < 0.05$ and ** denoting significance at $P < 0.01$. ***Panel B:*** Cell number was estimated using the mitochondrial reduction of resazurin following 3 hr incubation on the indicated day. Data are expressed as percent control of relative fluorescence units and represent the mean +/- SEM (n=6) with * denoting significance at $P < 0.05$ and ** denoting significance at $P < 0.01$.

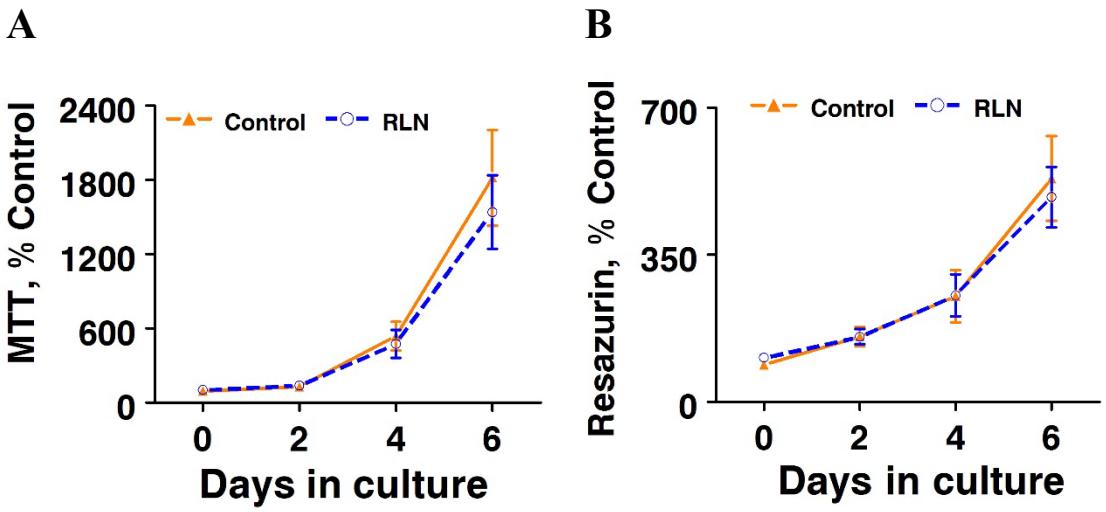

Figure 3-2. Relaxin does not alter porcine preadipocyte cell number in the presence of 5% serum based upon both cleavage of the tetrazolium salt, MTT and the decreased reduction of resazurin in primary cultures of pig stromal-vascular cells following treatment with RLN for 2, 4 or 6 days. Primary pig pre-adipocytes were plated at a density of 1000 cells/cm^2 in either the continuous presence or absence of exogenous RLN (100 ng/ml) for 0, 2, 4 or 6 days. ***Panel A***: Cell number was estimated using hydrolyzation of the tetrazolum salt, MTT, following 3 hr incubation on the indicated day. Data are expressed as percent control of relative absorbance units (540 nm) and represent the mean +/- SEM (n=6) with * denoting significance at $P < 0.05$ and ** denoting significance at $P < 0.01$. ***Panel B:*** Cell number was estimated using the mitochondrial reduction of resazurin following 3 hr incubation on the indicated day. Data are expressed as percent control of relative fluorescence units and represent the mean +/- SEM (n=6) with * denoting significance at $P < 0.05$ and ** denoting significance at $P < 0.01$.

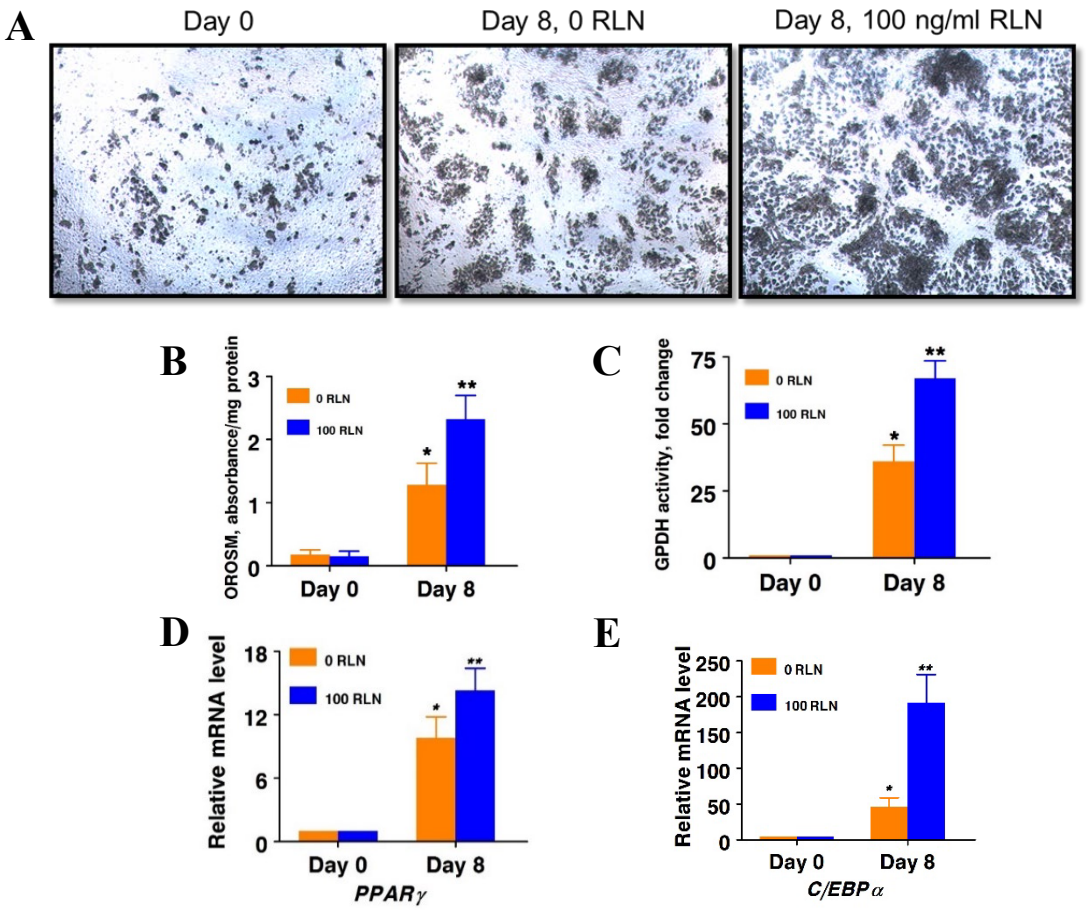

Figure 3-3. Relaxin enhances adipogenesis relative to control based upon elevated morphological, biochemical and gene markers in cultures of differentiating porcine preadipocytes. Pig preadipocytes were plated and continuously induced to differentiate beginning on day 0 in either the presence or absence of 100 ng/ml RLN for 8 days. ***Panel A***: Accumulation of Oil Red O-stained material (OROSM) in primary cultures of differentiating porcine preadipocytes on d 8 after induction of differentiation. Cultures were fixed on the days indicated and stained with ORO and counterstained with hematoxylin. The microscopic magnification was 10× for all images. Photomicrographs are representative of 6 replications each performed with cells isolated from a separate pig. ***Panel B***: Quantification of OROSM in primary cultures of differentiating porcine preadipocytes on d 8 after induction of differentiation. On d 8, plates were stained with Oil Red O, and the extracted stain was quantified spectrophotometrically. Data are means ± SEM from 6 experiments, each performed with cells harvested from a different pig. ***Panel C***: Determination of glycerol-3-phosphate dehydrogenase (GPDH) activity, in primary cultures of differentiating porcine preadipocytes on d 8. Cell lysates were harvested after 8 d of treatment and immediately assayed for GPDH activity. Data are means ± SEM from 6 experiments, each performed with cells harvested from a different pig. Three replicate wells were assayed within treatment for each pig. ***Panels D & E***: Quantification of the mRNA expression of the adipogenic marker genes, *PPARγ and C/EBPα*, using real-time PCR. Expression levels were normalized to the porcine *S15* gene and are presented as fold-change relative to expression in undifferentiated cultures.

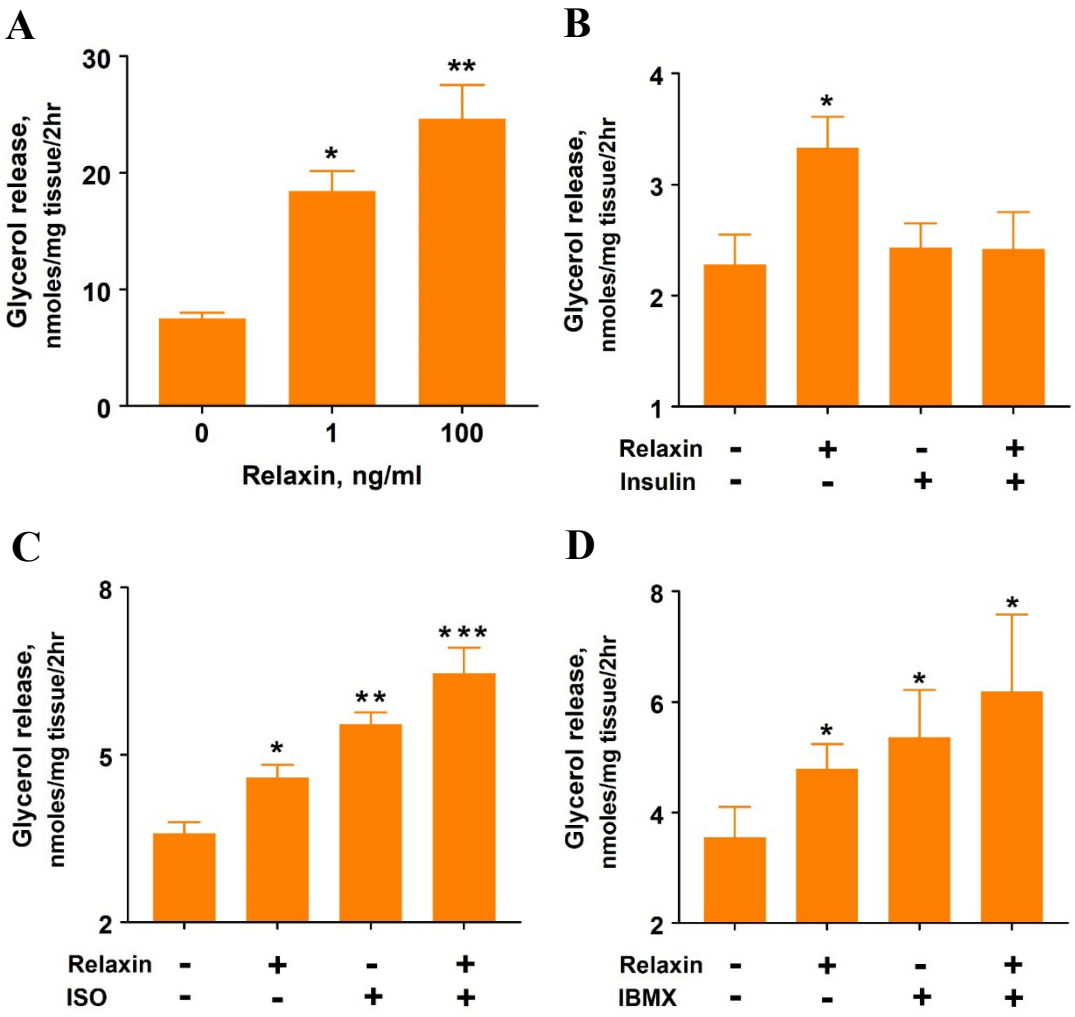

Figure 3-4. Relaxin stimulates lipolysis in porcine adipose tissue and this lipolytic action is altered by cAMP modulators. Porcine visceral adipose tissue explants were incubated in phenol-free DMEM containing 1% FBS containing the indicated doses of RLN. Following a 2 hr incubation, condition medium was collected, and glycerol release measured using the Trinder assay. ***Panel A***: Relaxin stimulates lipolysis in dose-dependent manner. Data are means ± SEM from 6 replicates with significant differences indicated $p < 0.05$ (*) and $P < 0.01$ (**). ***Panel B***: Co-incubation of RLN with 100 nM insulin blocks the stimulatory effect of RLN on glycerol release into the conditioned medium. Data are means ± SEM from 6 replicates with significant differences indicated $p < 0.05$ (*). ***Panel C***: Co-incubation of RLN with 1 μM of the potent βAA agonist, isoproterenol (ISO), enhances the stimulatory effect of RLN on glycerol release into the conditioned medium. Data are means ± SEM from 6 replicates with significant differences indicated $p < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). ***Panel D***: Co-incubation of RLN with 250 μM of the phosphodiesterase activator, IBMX, enhances glycerol release alone but does not alter the stimulatory effect of RLN on glycerol release into the conditioned medium. Data are means ± SEM from 6 replicates with significant differences indicated $p < 0.05$ (*).

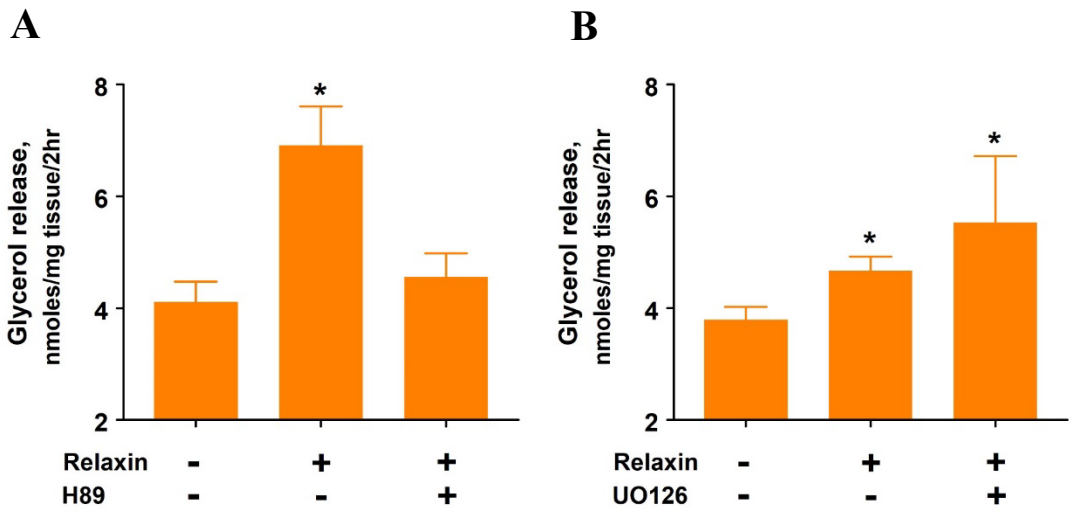

Figure 3-5. Relaxin stimulates lipolysis in porcine adipose tissue by signaling via the cAMP pathway. Porcine visceral adipose tissue explants were incubated in phenol-free DMEM containing 1% FBS containing either 100 ng/ml RLN or inhibitors in combination as indicated. Following a 2 hr incubation, condition medium was collected, and glycerol release measured using the Trinder assay. ***Panel A***: Co-incubation of RLN with 10 µM of the potent PKA inhibitor, H89, effectively blocks the stimulatory effect of RLN on glycerol release into the conditioned medium. Data are means ± SEM from 6 replicates with significant differences indicated $p < 0.05$ (*). ***Panel B***: Co-incubation of RLN with 10 µM of the potent MAP kinase pathway inhibitor, UO126, did not alter the stimulatory effect of RLN on glycerol release into the conditioned medium. Data are means ± SEM from 6 replicates with significant differences indicated $p < 0.05$ (*).

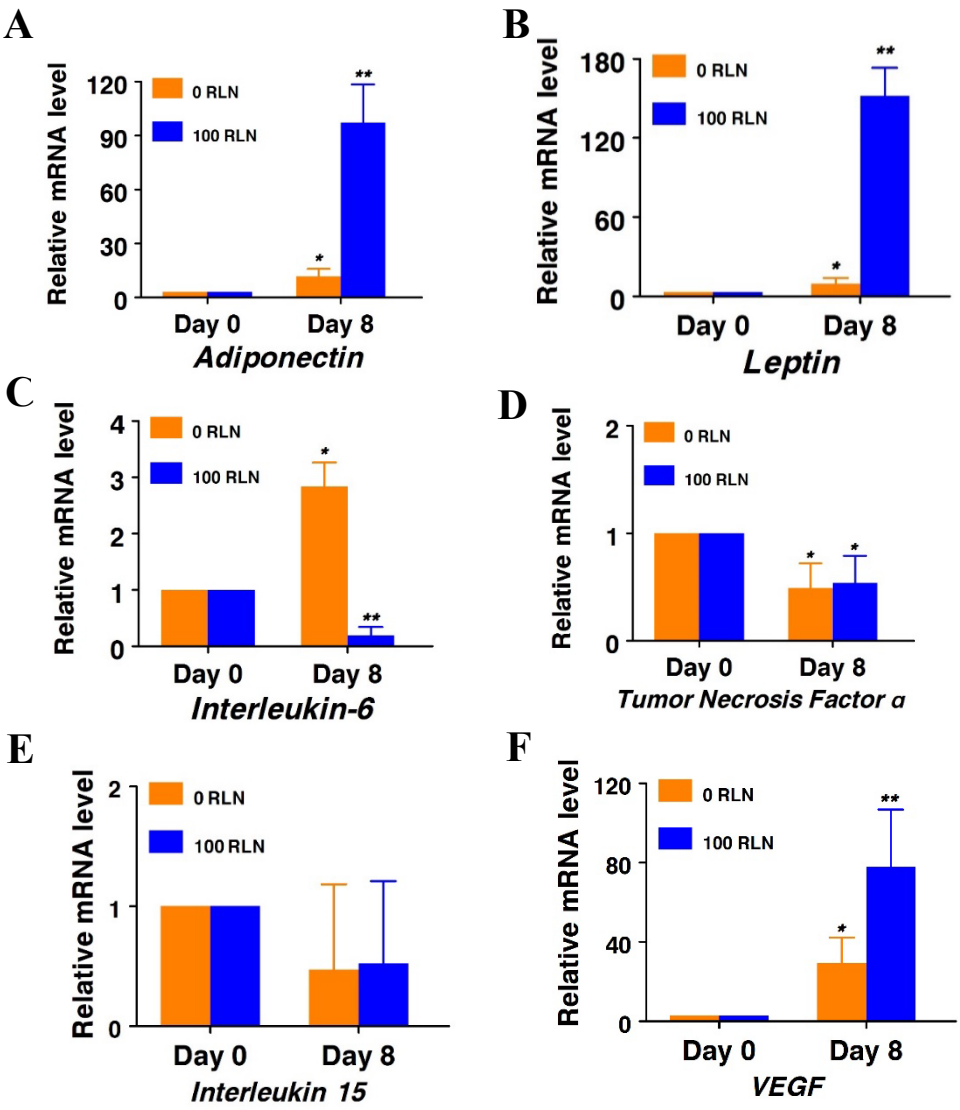

Figure 3-6. Relaxin alters mRNA expression of adipokine genes in cultures of differentiating porcine preadipocytes. Pig preadipocytes were plated and continuously induced to differentiate beginning on day 0 in either the presence or absence of 100 ng/ml RLN for 8 days. Quantification of the mRNA expression using real-time PCR is depicted for *Adiponectin* (***Panel A),*** *Leptin* ***(Panel B),*** *Interleukin-6* ***(Panel C),*** *Tumor necrosis factor-alpha* ***(Panel D),*** *Interleukin-15* ***(Panel E)*** *and VEGF* ***(Panel F).*** Expression levels were normalized to the porcine *S15* gene and are presented as fold-change relative to expression in undifferentiated cultures. Data are means ± SEM from 6 replicates with significant differences indicated $p < 0.05$ (*) and $p < 0.01$ (**).

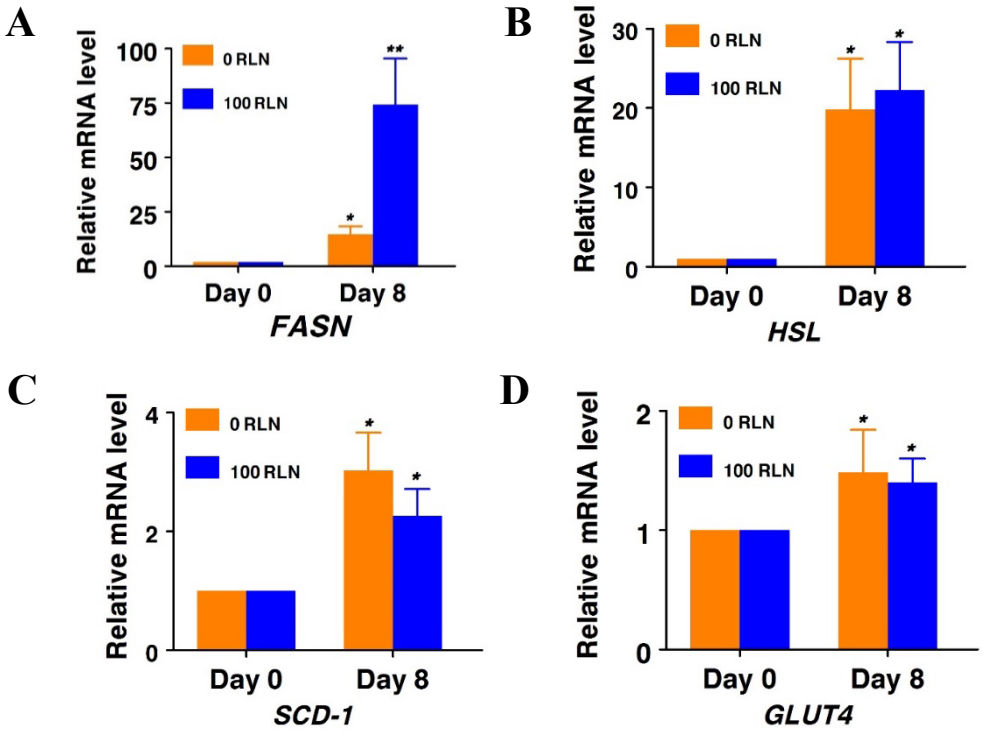

Figure 3-7. Relaxin alters mRNA expression of fatty acid synthetase but not the expression of other metabolic genes in cultures of differentiating porcine preadipocytes. Pig preadipocytes were plated and continuously induced to differentiate beginning on day 0 in either the presence or absence of 100 ng/ml RLN for 8 days. Quantification of the mRNA expression using real-time PCR is depicted for *FASN* (***Panel A),*** *HSL* ***(Panel B),*** *SCD-1* ***(Panel C),*** and *GLUT4* ***(Panel D).*** Expression levels were normalized to the porcine *S15* gene and are presented as fold-change relative to expression in undifferentiated cultures. Data are means ± SEM from 6 replicates with significant differences indicated $p < 0.05$ (*) and $p < 0.01$ (**).

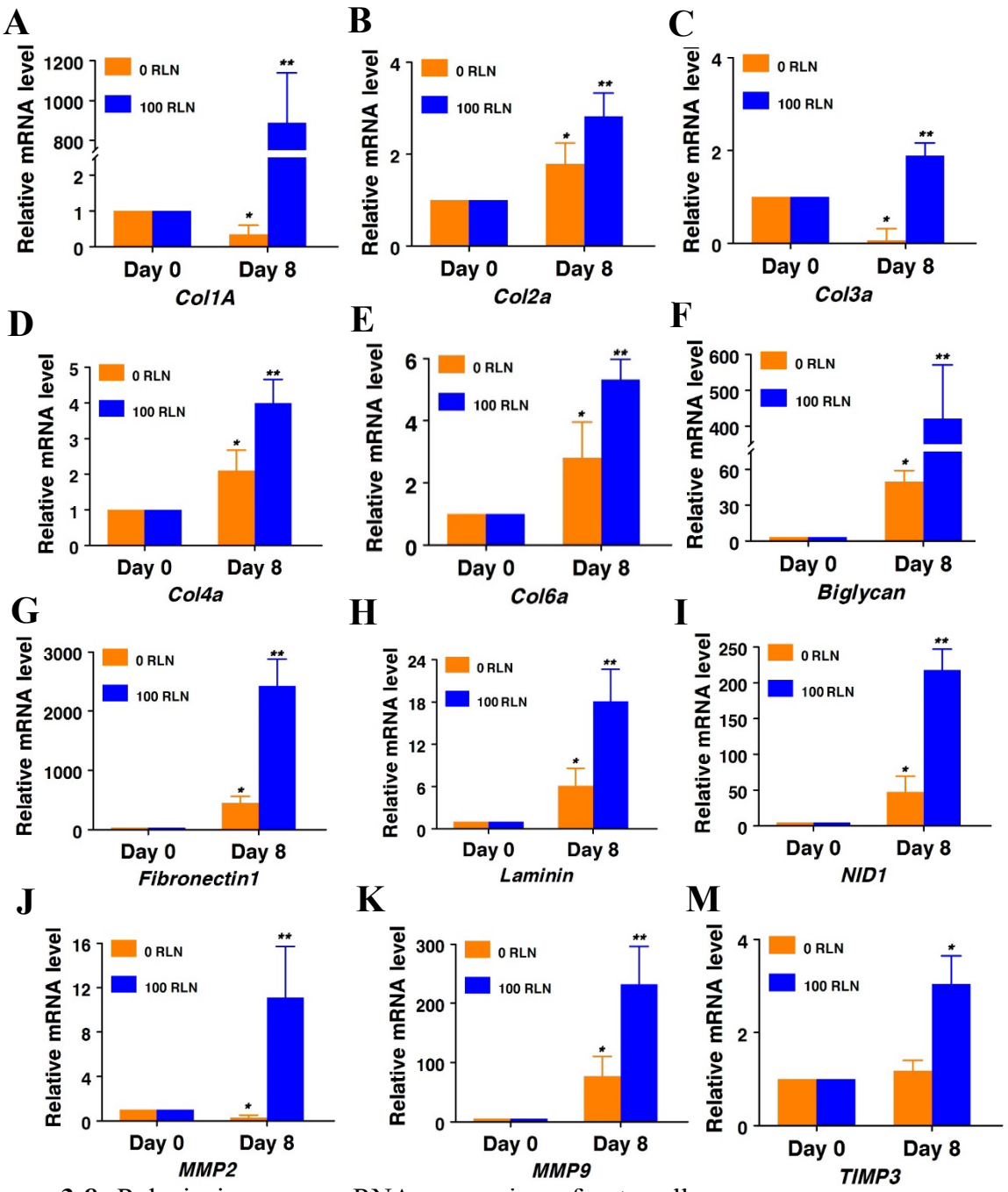

Figure 3-8. Relaxin increases mRNA expression of extracellular matrix component genes in cultures of differentiating porcine preadipocytes. Pig preadipocytes were plated and continuously induced to differentiate beginning on day 0 in either the presence or absence of 100 ng/ml RLN for 8 days. Quantification of the mRNA expression using real-time PCR is depicted for *Col1a* (***Panel A),*** *Col2A* ***(Panel B),*** *Col3A* ***(Panel C),*** *Col4A* ***(Panel D),*** *Col6A* ***(Panel E),*** *biglycan* ***(Panel F),*** *fibronectin1* ***(Panel G),*** *laminin* ***(Panel H),*** *NID1* ***(Panel I),*** *MMP2* ***(Panel J),*** *MMP9* ***(Panel K)*** *and TIMP3* ***(Panel M).*** Expression levels were normalized to the porcine *S15* gene and are presented as fold-change relative to expression in undifferentiated cultures. Data are means ± SEM from 6 replicates with significant differences indicated $p < 0.05$ (*) and $p < 0.01$ (**).

Table 3-1. Oligonucleotide polymerase chain reaction primers for adipogenic and metabolic genes

Gene	Accession No.	Primer sequence (5'→3')	Orientation	Efficiency
S15	NM_214334.1	GGTAGGTGTCTACAATGGCAAGG	Forward	100%
		GGCCGGCCATGCTTC	Reverse	
C/EBPα	XM_003127015.2	TGGACAAGAACAGCAACGAGTACC	Forward	100%
		CACCTTCTGTTGAGTCTCCACGTT	Reverse	
PPARγ	NM_214379.1	AATTAGATGACAGCGACCTGGCGA	Forward	96%
		TGTCTTGAATGTCCTCGATGGGCT	Reverse	
Adiponectin	NM_214370.1	TCTCGGCCAGGAAACCACCGA	Forward	100%
		CGGCCTGGGGTACCGTTGTG	Reverse	
Leptin	NM_213840.1	ACGATTGTCACCAGGATCAG	Forward	92%
		ACAAACTCAGGACAGGATGG	Reverse	
IL-6	NM_214399.1	CACCACAAATGCCGGCCTGCT	Forward	97%
		CAGAGGAGGGAATGCCCGTGG	Reverse	
TNF-α	NM_214022.1	GTAGCCAATGTCAAAGCCGA	Forward	92%
		TTGTCTTTCAGCTTCACGCC	Reverse	
IL-15	XM_021100480.1	TATACTGAAAGTGATGCTCATCCC	Forward	105%
		TACTCAATGGACGATAAACTGCTG	Reverse	
ERS1	AF035775	AGGGAGAGGAGTTTGTGTG	Forward	105%
		TCTCCAGCAGCAGGTCATAG	Reverse	
VEGF	AF041084.1	AGAAATCCCGGTATAAACCCTG	Forward	80%
		GTCGTTCTGTGTCAGTCTTTCC	Reverse	
HSL	NM_214315.3	CCTTTGAAATGCCACTGACTG	Forward	98%
		CTCACTGTCCTGTCCTTCAC	Reverse	
FASN	NM_001099930.1	CTTGTCCTGGGAAGAGTGTAA	Forward	94%
		AGATGGTCACCGTGTCTTTG	Reverse	
SCD-1	NM_213781.1	GTGACACTCACTCTTCCACTT	Forward	95%
		CCTGTTGCTGTAGCCAAATTC	Reverse	
GLUT4	NM_001128433.1	ATCCTGATGACTGTGGCTCTGCTT	Forward	96%
		AGAAGGCCACAAAGCCAAAGATGG	Reverse	

Table 3-2. Oligonucleotide polymerase chain reaction primers for extracellular matrix and regulatory genes

Gene	Accession No.	Primer sequence (5'→3')	Orientation	Efficiency
COL1A	XM_021067155.1	GAAGGGACACAGAGGTTTCAG	Forward	88%
		TAGCACCATCATTTCCACGA	Reverse	
COL2A	XM_021092611.1	GACCAAAGGGACAGAAAGGA	Forward	94%
		TTTGTCACCACGATCACCTC	Reverse	
COL3A	NM_001243297.1	TCCAGGTCTCAAAGGTCCAG	Forward	97%
		CCATCAAAGCCTCTATGCCC	Reverse	
COL4A	XM_021065910.1	TACTCCTTCCCTTCCCTTCTT	Forward	97%
		GCACCTTTATGGTTTCCCTACT	Reverse	
COL6A	XM_005669265.2	GAATTTCACCACCAAGTTCCA	Forward	98%
		AAGATGAGTAGCACCTGTCCT	Reverse	
Biglycan	XM_003135475.5	CATCCATGACAACCGCATCC	Forward	72%
		CGCAGGTAGTTGAGCTTCAG	Reverse	
FN1	XM_003133642.5	ATCCATTGAACTGACCAACTTCC	Forward	95%
		CCAGGCAGCAGATTTGTGAG	Reverse	
LAMA	XM_021096069.1	AGCATCTTCTGACCTCAATCGT	Forward	80%
		AAACACCTTTCCTCACCACTC	Reverse	
NID1	XM_021073759.1	CCTTCTGCTACAACACTCCA	Forward	109%
		ATCGGGTCTTCTCCACTTCTC	Reverse	
MMP2	AF295805.1	CCCACTTTGACGACGATGAG	Forward	93%
		GTTTCCATACTTCACACGCAC	Reverse	
MMP9	NM_001038004.1	CGACAAGGACAAGAAGTGGG	Forward	93%
		TTAGGGCGAGAACCATACAG	Reverse	
TIMP3	XM_003126073.6	TCAAGCAGATGAAGATGTACCGA	Forward	104%
		TCCACAAAGTTGCACAATCCT	Reverse	

Table 3-3. Relative fold changes in adipokine gene expression during adipogenesis in the presence and absence of relaxin[1]

	Day 4			Day 8		
Variable	**Control**	**Relaxin**	***P*-Value**	**Control**	**Relaxin**	***P*-Value**
Adiponectin	1.2 ± 0.27	6.4 ± 1.52[a]	0.05	11.6 ± 4.1a	97.2 ± 21.2[b]	0.01
Leptin	4.7 ± 1.26[a]	19.4 ± 5.22[b]	0.05	9.5 ± 2.09[c]	152 ± 31.4[d]	0.001
Tumor Necrosis Factor-α	0.29 ± 0.26[a]	0.45 ± 0.23[a]	0.05	0.49 ± 0.23[a]	0.54 ± 0.25[a]	0.05
Interleukin-6	0.16 ± 0.30[a]	0.57 ± 0.23[a]	0.05	2.83 ± 0.43[b]	0.19 ± 0.15[a]	0.01
Interleukin-15	0.44 ± 0.52	0.48 ± 0.59	0.76	0.47 ± 0.71	0.52 ± 0.69	0.99

[1]Values are group mean ± SEM, n=6, differing superscripts within a variable denote differences between treatment within days with indicated P-values as determined by Pair Wise Fixed Reallocation Randomization Test of REST-MCS

Table 3-4. Relative fold changes in adipogenic marker and relaxin target gene expression during adipogenesis in the presence and absence of relaxin[1]

	Day 4			Day 8		
Variable	**Control**	**Relaxin**	***P*-Value**	**Control**	**Relaxin**	***P*-Value**
PPARγ	4.3 ± 1.27[a]	7.1 ± 1.44[a]	0.05	9.8 ± 1.1[c]	14.3 ± 3.2[d]	0.01
C/EBPα	7.07 ± 2.08[a]	6.34 ± 1.22[a]	0.05	46.1 ± 12.7[b]	191 ± 40.0[c]	0.001
ERS1	1.40 ± 0.36[a]	1.73 ± 0.33[a]	0.05	2.81 ± 0.39[b]	3.60 ± 0.35[c]	0.05
VEGF	3.26 ± 0.66[a]	5.10 ± 1.02[b]	0.05	29.3 ± 12.8[c]	77.7 ± 29.0[d]	0.01

[1]Values are group mean ± SEM, n=6, differing superscripts within a variable denote differences between treatment within days with indicated P-values as determined by Pair Wise Fixed Reallocation Randomization Test of REST-MCS

Table 3-5. Relative fold changes in metabolic gene expression during adipogenesis in the presence and absence of relaxin[1]

	Day 4			Day 8		
Variable	**Control**	**Relaxin**	***P*-Value**	**Control**	**Relaxin**	***P*-Value**
GLUT4	1.10 ± 0.36	1.30 ± 0.20	0.83	1.48 ± 0.23[a]	1.40 ± 0.32[a]	0.05
Fatty acid synthetase	2.88 ± 0.72[a]	10.4 ± 1.5[b]	0.01	14.4 ± 3.7[b]	74.2 ± 14.5[c]	0.001
Stearoyl-CoA Desaturase	1.95 ± 0.54[a]	1.83 ± 0.43[a]	0.05	3.02 ± 0.64[a]	2.26 ± 0.45[a]	0.01
Hormone Sensitive Lipase	10.8 ± 4.40[a]	10.5 ± 3.07[a]	0.01	19.8 ± 5.4b[b]	22.2 ± 8.1[b]	0.001

[1]Values are group mean ± SEM, n=6, differing superscripts within a variable denote differences between treatment within days with indicated P-values as determined by Pair Wise Fixed Reallocation Randomization Test of REST-MCS

Table 3-6. Relative fold changes in extracellular matrix component and regulatory gene expression during adipogenesis in the presence and absence of relaxin[1]

	Day 4			Day 8		
Variable	**Control**	**Relaxin**	***P*-Value**	**Control**	**Relaxin**	***P*-Value**
Col1A	0.19 ± 0.14^{a}	666 ± 395^{b}	0.05	0.35 ± 0.25^{a}	888 ± 251^{b}	0.001
Col2A	1.20 ± 0.28	1.76 ± 0.35^{a}	0.05	1.79 ± 0.35^{a}	2.82 ± 0.56^{b}	0.05
Col3A	0.02 ± 0.39^{a}	2.70 ± 0.39^{b}	0.01	0.06 ± 0.15^{a}	1.89 ± 0.27^{b}	0.01
Col4A	0.97 ± 0.50^{a}	4.61 ± 1.17^{c}	0.05	2.10 ± 0.57^{b}	3.99 ± 0.67^{c}	0.01
Col6A	1.04 ± 0.63^{a}	2.60 ± 1.30^{a}	0.33	2.80 ± 1.16^{a}	5.42 ± 0.65^{b}	0.01
Biglycan	2.14 ± 0.38^{a}	4.20 ± 0.99^{b}	0.05	49.6 ± 9.57^{c}	421 ± 151^{d}	0.001
Fibronectin	5.10 ± 2.14^{a}	17.5 ± 2.03^{b}	0.01	452 ± 111^{c}	2434 ± 451^{d}	0.001
Laminin	2.30 ± 0.54^{a}	3.59 ± 1.37^{a}	0.05	6.10 ± 2.50^{b}	18.1 ± 4.50^{c}	0.01
NID1	3.68 ± 1.64^{a}	3.17 ± 0.74^{a}	0.05	47.4 ± 22.0^{b}	218 ± 29.0^{c}	0.001
MMP2	0.19 ± 0.10^{a}	11.2 ± 5.10^{b}	0.001	0.29 ± 0.14^{a}	11.1 ± 4.26^{b}	0.001
MMP9	2.14 ± 0.54^{a}	2.03 ± 0.43^{a}	0.05	76.8 ± 34.0^{b}	232 ± 64.0^{c}	0.001
TIMP3	0.82 ± 0.16^{a}	5.50 ± 1.10^{b}	0.01	1.17 ± 0.23^{a}	3.04 ± 0.66^{d}	0.01

[1]Values are group mean ± SEM, n=6, differing superscripts within a variable denote differences between treatment within days with indicated P-values as determined by Pair Wise Fixed Reallocation Randomization Test of REST-MCS

Appendix 1

Pig Primary Stromal Vascular Cells *In Vitro* Cell Model

A.1 History of development

Cell culture involves harvesting tissue or cells, from the live animal, and incubating in a flask used to mimic the *in vivo* (within the body) environment. It is critical to create an *in vitro* (within an artificial environment that is outside the body) environment where the cells or tissue maintain the behavior that they would exhibit inside the live animal. To achieve this, these cells require several pivotal conditions to be met including appropriate cell temperature, provision of a buffer system to maintain physiological pH, aseptic conditions and an appropriate growth medium containing all the metabolites and hormones needed to encourage cell growth and proliferation. Primary culture of fat cells was first described by Rodbell in 1964 and primary culture utilizing tissue explants was first described by Slavin and Elias in 1969. Cell culture allows the study of cell differentiation under controlled substrate concentrations with the concurrent absence of neural and hormonal input, other than conditions induced upon by the researcher (Suryawan et al., 1995). Cell culture allows growth biologists to ask and answer questions that would be difficult to study *in vivo*; however, one must be cognizant that *in vitro* studies cannot fully represent a complete in vivo environment, due to physiological functions that are present in the live organism (Ghorbani and Abedinzade, 2013). For instance, the systemic blood supply of the live organism contains

numerous hormones, chemical messengers, signaling molecules, etc. that could potentially interact with the cell culture cells of interest. This interaction, whether it be substantial or minuscule, could play a role in the cell's overall development and regulation. At this time, researchers cannot fully predict every cell to cell or cell to environment interaction that occurs in the live organism, which presents a notable limitation.

A.2 Immortal vs. primary cells

Immortal cell lines are continuous cultures that can divide indefinitely or are "immortalized." These cells are often derived from tumors such as SW872 cells that were isolated from a human adipose tissue sarcoma or stem cells such as 3T3-L1 cells that were isolated and cloned from Swiss 3T3 mouse embryos by Green and Kehinde, 1975. In theory, these immortalized cell lines can divide forever; however multiple reports have suggested that differentiation capacity decreases with increasing number of passages. During proliferation, pre-adipocyte cell lines display a fibroblast-like morphology and begin to display growth arrest upon reaching confluence (Armani et al. 2010).

Primary cultures are derived directly from harvested neonatal tissue and cultured, either as fragments of tissue called "explants" or cell suspensions following dissociation by collagenase digestion. These cells generally mimic the characteristics of the cells or tissue *in vivo*, but can only be maintained for a limited amount of passages, as these cells will eventually exit the cell cycle after reaching their Hayflick limit or the number of cell divisions a cell can undergo in culture before the cell stops dividing. Primary cells can grow on top of one another and will begin to differentiate with the addition of a precise induction media. For primary cultures to differentiate, the cells need to see an appropriate hormone cocktail of induction signals such as insulin,

dexamethasone and rosiglitazone. These signals drive transcription factor expression which further alters functional gene expression and thus drives cell differentiation. As mentioned previously, insulin stimulates adipocytes to store glucose as glycogen to utilize glucose to make fatty acids. Dexamethasone is a synthetic glucocorticoid and anti-inflammatory which can bind to glucocorticoid receptors in adipocytes resulting in increased insulin sensitivity. Rosiglitazone is a synthetic ligand for $PPAR_{\gamma}$, the master regulator known to drive adipogenesis.

A.3 Types of *in vitro* systems

There are several different varieties of *in vitro* systems used in cell culture including cultures of preadipocytes, mature adipocytes, and primary tissue explants. Subcutaneous back fat located between the shoulder blades is harvested from the neonate to collect tissue or cells of interest. Tissue is then digested via enzymatic, collagenase digestion and transferred to a large conical tube which now contains a variety of different cell types including adipocytes, pre-adipocytes, immature fat cells, fibroblasts, etc. collectively known as stromal vascular cells (SVC). Centrifugation is performed to separate mature adipocytes from preadipocytes. Mature adipocytes are lighter because they are filled with lipid, causing them to float to the top of the conical tube. Pre-adipocytes, on the other hand, are heavier causing a pellet to form at the bottom of the conical tube.

To perform an *in vitro* system studying mature adipocytes, simply collect the cells from the top of the conical tube after centrifugation and incubate in treatment media. The primary advantage of this system is the ability to analyze lipolysis of fully differentiated adipocytes (Ghorbani and Abedinzade, 2013). A disadvantage to this system is that mature fat cells are very fragile and tend to easily lyse in culture, making mature adipocytes very difficult to study.

To perform an *in vitro* system studying preadipocytes, the cell suspension harvested from excised adipose tissue needs to be seeded in a culture flask and incubated under optimal conditions. A primary advantage of this system is the adherent nature of preadipocytes and the ability to supply an abundance of homogeneous cells which can be used to study numerous procedures such as proliferation assays, molecular signaling mechanisms, gene expression assays, etc. (Ghorbani and Abedinzade, 2013). A disadvantage of this system is the difficulty to isolate preadipocytes from fibroblast-like cells of the stromal vascular fraction (SVF) (Armani et al., 2010).

To perform an *in vitro* system studying primary tissue explants, tissue is harvested from the animal, cut into smaller pieces and distributed onto a culture plate containing growth media. An advantage of this system is the ability to study lipolysis from the mature tissue by treating the tissue with activators and inhibitors of the cAMP pathway. A disadvantage of this system is the inability to keep the explants sustainable for a prolonged period without causing profound changes to gene expression.

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